RESEARCH ARTICLE

Deep-sea methane seep sediments in the Okhotsk Sea sustain diverse and abundant anammox bacteria

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Abstract

Marginal sea methane seep sediments sustain highly productive chemosynthetic ecosystems and are hotspots of intense biogeochemical cycling. Rich methane supply stimulates rapid microbial consumption of oxygen; these systems are thus usually hypoxic to anoxic. This and reported evidence for resident nitrogen fixation suggest the presence of an anaerobic ammonium-oxidizing (anammox) bacterial community in methane seep sediments. To test this hypothesis, we employed detection of genes encoding 16S rRNA gene and hydrazine dehydrogenase (*hzo*) to investigate the structure, abundance and distribution of the anammox bacterial community in the methane seep sediments of the Okhotsk Sea. Diverse complements of *Candidatus* Scalindua-related 16S rRNA and *hzo* gene sequences were obtained. Most of the deep-sea sites harbored abundant *hzo* genes with copy numbers as high as 10^7 g^{-1} sediment. In general, anammox bacterial signatures were significantly more abundant in the deep-water sediments. Sediment porewater NO_3^-, NO_x^- (i.e. NO_3^- + NO_2^-), NO_x^-/NH_4^+ and sediment silt content correlated with *in situ* distribution patterns of anammox bacterial marker genes, likely because they determine anammox substrate availability and sediment geochemistry, respectively. The abundance and distribution of anammox bacterial gene markers indicate a potentially significant contribution of anammox bacteria to the marine N cycle in the deep-sea methane seep sediments.

Introduction

The anaerobic ammonium oxidation (anammox, $NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$) process removes fixed nitrogen and produces N_2 in anoxic environments (Strous & Jetten, 2004). Anammox bacteria and their activity have been detected in diverse terrestrial, marine and engineered ecosystems, where they were discovered less than one and half decades ago (Strous *et al.*, 1999; Hu *et al.*, 2011; and references therein). In some regions of the world's oceans, more than 50% of N_2 production in marine sediment and oxygen-depleted seawater zones may be attributed to anammox, making it an important process in the marine biogeochemical nitrogen cycle (Jetten *et al.*, 2009, and references therein).

The obligate chemolithoautotrophic anammox bacteria form a distinct clade of deeply branched *Planctomycetes* (Strous *et al.*, 1999). Five genera of anammox bacteria including *Candidatus* Anammoxoglobus, *Candidatus* Brocadia, *Candidatus* Jettenia, *Candidatus* Kuenenia and *Candidatus* Scalindua have been identified (Jetten *et al.*, 2009). Because anammox bacteria have not yet been isolated into pure culture, one of the earlier approaches for studying them in the environment was the molecular

detection of their 16S rRNA genes. The broad molecular diversity of anammox bacterial 16S rRNA genes, however, rendered them an inefficient tool for capturing the diversity and distribution of anammox bacteria in environment. Genome-informed reconstruction of anammox catabolic pathways (Strous *et al.*, 2006) and an increased sequence database provided for the development of primer sets for molecular detection that target the functional genes encoding hydrazine dehydrogenase [formerly called hydrazine oxidoreductase; *hzo* (Schmid *et al.*, 2008)] and hydrazine synthase [formerly called hydrazine hydrolase; *hzsA* (Harhangi *et al.*, 2012)] and their successful application provided evidence of their broader coverage and higher resolution (Dang *et al.*, 2010a, 2013; Li *et al.*, 2010; Hirsch *et al.*, 2011; Harhangi *et al.*, 2012).

Methane seeps (also called cold seeps), mainly formed in gas-hydrate-bearing sediments, sustain highly productive deep-sea chemosynthetic ecosystems (Vanreusel *et al.*, 2009). Coupled C and S cycling catalyzed by microbial processes appears to form the ecophysiological foundation of these unique systems (Jørgensen & Boetius, 2007; Reeburgh, 2007; Pernthaler *et al.*, 2008), which concomitantly requires a high supply of nitrogenous nutrients (Dang *et al.*, 2009, 2010b). The rapid oxygen consumption of many of these C, S and N transformation processes maintains hypoxic to anoxic methane seep sediments and provides a suitable environment for the anammox process. The removal of nitrogen from the methane seep sediments via the anammox pathway likely constitutes ecophysiological pressures on the methane seep ecosystem. Although methane seeps are hotspots of intense biogeochemical processes in the ocean (Dang *et al.*, 2009; Dekas *et al.*, 2009), little is known about the biology of the anammox bacteria in these unique systems, including the environmental factors that control their distribution and abundance.

The Okhotsk Sea, a marginal sea located on the continental slope offshore Sakhalin Island in the northwestern Pacific Ocean, is one of the largest reservoirs of methane hydrates in the world (Ginsburg *et al.*, 1993). The Okhotsk Sea methane seep sediments harbor diverse and novel *nifH*- and *amoA*-carrying bacteria and archaea (Dang *et al.*, 2009, 2010b), providing the biological potential for active *in situ* production of ammonia and nitrite as substrates for the anammox process. In addition, the methane seep sediments are O_2-limited due to *in situ* O_2 consumption by intense methane oxidation (Sahling *et al.*, 2003), ecologically favorable to anaerobic microbial processes such as anammox and denitrification. Without a direct implication of the anammox bacteria, indeed, a recent study in the Gulf of Mexico indicated high rates of denitrification and nitrate removal in deep-sea methane seep sediments (Bowles & Joye, 2011).

In this study, we used 16S rRNA and *hzo* genes as molecular targets to determine anammox bacterial diversity, abundance and community structure, and to identify pertinent environmental factors that shape their spatial distribution in the methane seep sediments of the Okhotsk Sea.

Materials and methods

Sample collection and environmental factor measurement

Sediment cores were collected from eight methane seep sites of the Okhotsk Sea (Supporting Information, Fig. S1) as described previously (Luan *et al.*, 2008; Dang *et al.*, 2009, 2010b). In addition to the measured environmental factors reported previously (Dang *et al.*, 2010b), concentrations of sediment porewater nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+), phosphate (PO_4^{3-}) and silicate (SiO_3^{2-}) were measured using a nutrient QUAATRO AutoAnalyzer (Bran+Luebbe, Germany) in this study (Table S1).

DNA extraction and anammox bacterial 16S rRNA and *hzo* gene analyses

Sediment DNA was extracted as described previously (Dang *et al.*, 2008b, 2010b). The 16S rRNA gene PCR primers Brod541F and Brod1260R, targeting specifically the *C.* Scalindua group (Penton *et al.*, 2006), and the *hzo* gene PCR primers *hzo*F1 and *hzo*R1, targeting all known anammox bacterial groups (Li *et al.*, 2010), were used respectively for 16S rRNA and *hzo* gene amplifications following previous protocols (Dang *et al.*, 2010a, 2013). PCR products from 10 reactions were pooled to minimize PCR bias, gel purified, ligated into pMD19-T Simple vectors (Takara, Tokyo, Japan), and transformed into competent *Escherichia coli* TOP10 cells (Dang *et al.*, 2008a, 2010a). Plasmid insert-positive recombinants were selected using Luria–Bertani medium agar plates amended with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), IPTG (isopropyl-β-D-thio-galactopyranoside) and 100 μg mL^{-1} ampicillin. A Miniprep method was used to isolate plasmids (Dang & Lovell, 2000) and the cloning vector primers RV-M and M13-D were used to reamplify the cloned DNA fragments (Dang *et al.*, 2008a). The resulting PCR products were screened for correct size and purity by electrophoresis using 1% agarose gels.

Amplicons of correct size obtained from 16S rRNA and *hzo* gene amplifications were digested separately with the MspI, HhaI and TaqI endonucleases (Fermentas, Glen Burnie, MD). Restriction fragments were resolved

by electrophoresis on 3% agarose gels in 0.5× Tris-borate-EDTA (TBE) buffer. Band patterns digitally photographed with an AlphaImager HP imaging system (Alpha Innotech, Santa Clara, CA) were compared for restriction fragment length polymorphisms (RFLP) to identify identical clones.

Cloning vector primers M13-D and RV-M were used for sequencing plasmid inserts with an ABI 3770 automatic sequencer (Applied Biosystems, Foster City, CA). DNA sequences were checked for possible chimera with programs CHIMERA_CHECK (Cole *et al.*, 2003), BELLEROPHON (Huber *et al.*, 2004) and PINTAIL (Ashelford *et al.*, 2005). The BLASTN and BLASTX algorithms were used for retrieving the top-hit 16S rRNA gene and deduced Hzo protein sequences, respectively, from GenBank (Altschul *et al.*, 1997). The 16S rRNA gene sequences were grouped into operational taxonomic units (OTUs) with 0.5% distance cutoff and the Hzo protein sequences were grouped with 1% distance cutoff using the DOTUR program (Schloss & Handelsman, 2005). The small distance cutoffs were used, because the 16S rRNA gene sequence identities between some described anammox bacterial species so far, reach 99% (van Niftrik & Jetten, 2012) and microdiversity among environmental anammox bacteria has been reported (Woebken *et al.*, 2008). The choice of these sequence distance cutoffs also took into consideration the known sequence error rates due to PCR and sequencing, which are usually < 0.3% (Tindall & Kunkel, 1988; Eckert & Kunkel, 1991; Kwiatowski *et al.*, 1991; Clark & Whittam, 1992; Lee *et al.*, 2010). Phylogenetic analyses followed previously reported procedures (Dang *et al.*, 2010a, 2013) including sequence alignments using CLUSTAL X (version 2.0; Larkin *et al.*, 2007) and phylogenetic tree construction using PHYLIP (version 3.69; Felsenstein, 1989).

Quantification of sediment 16S rRNA and *hzo* genes

Plasmids carrying *C.* Scalindua 16S rRNA or *hzo* gene fragments constructed previously (Dang *et al.*, 2010a) were extracted from *E. coli* hosts using a plasmid mini kit (Qiagen, Valencia, CA) and linearized with an endonuclease specific to the vector region. Concentrations of plasmid DNAs and sediment genomic DNAs were measured using PicoGreen (Molecular Probes, Eugene, OR) and a Modulus single-tube multimode-reader fluorometer (Turner Biosystems, Sunnyvale, CA). All real-time fluorescence quantitative PCR (qPCR) assays targeting the 16S rRNA or *hzo* genes were carried out in triplicate with an ABI PRISM 7500 sequence detection system (Applied Biosystems) using previously established SYBR green qPCR methods (Dang *et al.*, 2010a, 2013). Agarose gel electrophoresis and melting-curve analysis were routinely employed to confirm specificities of the qPCRs. Standard curves were obtained with serial dilution of standard plasmids containing target 16S rRNA or *hzo* gene fragments as the insert. The abundance of standard plasmid inserts ranged from 1.27×10^1 to 1.27×10^6 for the *C.* Scalindua 16S rRNA gene and 1.22×10^1 to 1.22×10^7 for the *hzo* gene. In all experiments, negative controls containing no template DNA were subjected to the same qPCR procedure to detect and exclude any possible contamination or carryover.

Sediment *C.* Scalindua 16S rRNA gene copies were quantified using primers Brod541F and Brod1260R (Penton *et al.*, 2006; Dang *et al.*, 2010a, 2013; Jaeschke *et al.*, 2010) and anammox bacterial *hzo* gene copies quantified using primers *hzo*F1 and *hzo*R1 (Dang *et al.*, 2010a, 2013; Li *et al.*, 2010). The qPCR reaction conditions, thermocycling parameters and data collection procedures were presented in previous publications (Dang *et al.*, 2010a, 2013). Sediment total bacterial and archaeal 16S rRNA gene copies were determined previously (Dang *et al.*, 2010b).

Statistical analyses

The coverage of each clone library was calculated as $C = [1-(n_1/N)] \times 100$, where n_1 is the number of unique OTUs and N the total number of clones in a library (Mullins *et al.*, 1995). Indices of gene diversity (Shannon–Weiner H and Simpson D) and evenness (J) were calculated using the OTU data for each library. Rarefaction analysis and two nonparametric richness estimators, the abundance-based coverage estimator (S_{ACE}) and the bias-corrected Chao1 (S_{Chao1}), were calculated using program DOTUR (Schloss & Handelsman, 2005).

Community classification of the sediment anammox bacterial assemblages was performed with Jackknife environment clustering and principal coordinates analysis (PCoA) using the FAST UNIFRAC program (Hamady *et al.*, 2010) following previously established procedures (Dang *et al.*, 2010a, 2013). Correlations of the anammox bacterial assemblages with environment factors were explored using canonical correspondence analysis (CCA) with statistics software CANOCO (version 4.5, Microcomputer Power, Ithaca, NY, ter Braak & Šmilauer, 2002) following previously described procedures (Dang *et al.*, 2010a, 2013).

Data normal distribution (normality) was examined using the statistics software MINITAB (release 13.32; Minitab Inc., State College, PA). Pearson correlation analyses (significance level $\alpha = 0.05$) of the abundance of sediment *C.* Scalindua 16S rRNA or anammox bacterial *hzo* genes with environmental factors were also performed using MINITAB following previous procedures (Dang *et al.*, 2010a, 2013).

Nucleic acid sequence accession numbers

The sequences of 16S rRNA and *hzo* gene amplicons have been deposited in GenBank under accession numbers JQ901713–JQ901759 and JQ901760–JQ901837, respectively.

Results

Diversity of deep-sea sediment anammox bacterial 16S rRNA and *hzo* gene sequences

Of the eight *C.* Scalindua 16S rRNA gene clone libraries constructed from the Okhotsk Sea sediment samples, 746 clones were identified to contain a valid gene fragment, resulting in 47 unique RFLP sequences and 36 OTUs. The values of library coverage (*C*) ranged from 88.3% to 95.7% (Table 1), which together with rarefaction analysis (Fig. S2a) indicated that the *C.* Scalindua bacteria were sufficiently represented in these clone libraries. Station 39H had the highest OTU diversity and the gas hydrate-bearing station 25H the lowest, based on the majority of the diversity indices (*H*, 1/*D* and *J*). However, the S_{ACE} and S_{Chao1} richness estimators indicated that the actual *C.* Scalindua bacterial diversity might have been higher than what was obtained at these eight stations in the current study (Table 1). The calculated S_{ACE} and S_{Chao1} richness estimators also indicated that station 40H might potentially have the highest *C.* Scalindua bacterial diversity (Table 1).

Of the eight anammox bacterial *hzo* gene clone libraries constructed from the Okhotsk Sea sediment samples, a total of 775 insert-positive clones were identified to represent 61 unique RFLP sequences and 32 OTUs. The values of library coverage (*C*) ranged from 90.4% to 96.9% (Table 1), which together with rarefaction analysis (Fig. S2b) indicated that the anammox bacteria were sufficiently represented in these clone libraries. Station 37H had the highest diversity of OTUs and the gas hydrate-bearing station 25H the lowest, based on the majority of diversity indices (*H*, 1/*D* and *J*). Station 40H had the highest richness of OTUs based on the S_{ACE} and S_{Chao1} estimators (Table 1).

Phylogenetic analysis of *C.* Scalindua 16S rRNA gene sequences

The 47 distinct *C.* Scalindua 16S rRNA gene sequences obtained were 91.6–99.9% identical with one other, and 98.3–100.0% identical to the closest GenBank sequence matches. Most of the top-hit GenBank sequences (76.6%) were originally retrieved from marine sediments or anoxic seawater (Penton *et al.*, 2006; Woebken *et al.*, 2007, 2008; Shu & Jiao, 2008; Dang *et al.*, 2010a).

The constructed 16S rRNA gene phylogenetic tree revealed that the methane seep sediments harbored diverse anammox bacteria affiliated with the *C.* Scalindua lineage (Fig. 1). Six putative *C.* Scalindua clades were identified, including *C.* Scalindua wagneri (Schmid *et al.*, 2003), *C.* Scalindua pacifica (Dang *et al.*, 2013), *C.* Scalindua marina, *C.* Scalindua sorokinii/brodae (Schmid *et al.*, 2003), *C.* Scalindua arabica (Woebken *et al.*, 2008), and a previously identified 'novel *C.* Scalindua clade' (Dang *et al.*, 2010a) (Fig. 1). Based on DOTUR analysis, the sequences of any two of these clades were < 97% identical. More than 72.0% of the 16S rRNA gene sequences were affiliated with the *C.* Scalindua sorokinii/brodae clade, which included sequences from all the eight sampling sites, indicating that anammox bacteria from this clade are prevalent in the deep-sea

Table 1. Biodiversity and predicted richness of the sediment *Candidatus* Scalindua 16S rRNA and anammox *hzo* gene sequences recovered from the sampling stations of the Okhotsk Sea

Station	No. of clones	No. of unique gene sequences*	No. of OTUs†	*C* (%)	*H*	1/*D*	*J*	S_{ACE}	S_{Chao1}
17H	93/92‡	8/20	8/11	94.6/94.6	1.50/1.85	2.36/2.28	0.50/0.53	23.00/22.45	13.00/13.50
25H	94/97	9/18	7/8	95.7/96.9	1.42/1.23	2.31/1.57	0.51/0.41	17.00/11.37	10.00/11.00
30H	87/91	11/17	10/9	92.0/96.7	1.68/1.92	2.51/2.52	0.50/0.61	38.00/11.57	20.50/12.00
37H	96/114	10/26	8/14	94.8/95.6	1.50/2.39	2.31/3.20	0.50/0.63	27.52/19.16	18.00/16.50
38H	91/103	14/26	12/13	91.2/95.1	1.90/2.39	2.74/3.13	0.53/0.65	34.93/18.86	26.00/18.00
39H	94/94	16/23	15/11	88.3/94.7	2.04/1.72	2.81/1.99	0.52/0.50	50.75/18.07	33.33/16.00
40H	100/94	13/22	11/13	92.0/90.4	1.75/1.69	2.59/2.00	0.51/0.46	63.91/41.72	39.00/31.00
48H	91/90	11/19	10/9	94.5/95.6	1.90/1.52	2.85/1.97	0.57/0.48	21.07/13.00	13.33/11.00

*Unique *C.* Scalindua 16S rRNA and anammox *hzo* gene sequences were determined via RFLP analyses.

†OTUs of the *C.* Scalindua 16S rRNA gene sequences were determined at 0.5% distance cutoff and OTUs of the anammox Hzo protein sequences were determined at 1% distance cutoff using the DOTUR program. The coverage (*C*), Shannon–Weiner (*H*), Simpson (*D*) and evenness (*J*) indices, and S_{ACE} and S_{Chao1} richness estimators were calculated using the OTU data.

‡Index and estimator data are presented for the '*C.* Scalindua 16S rRNA gene/anammox *hzo* gene or Hzo protein' sequences.

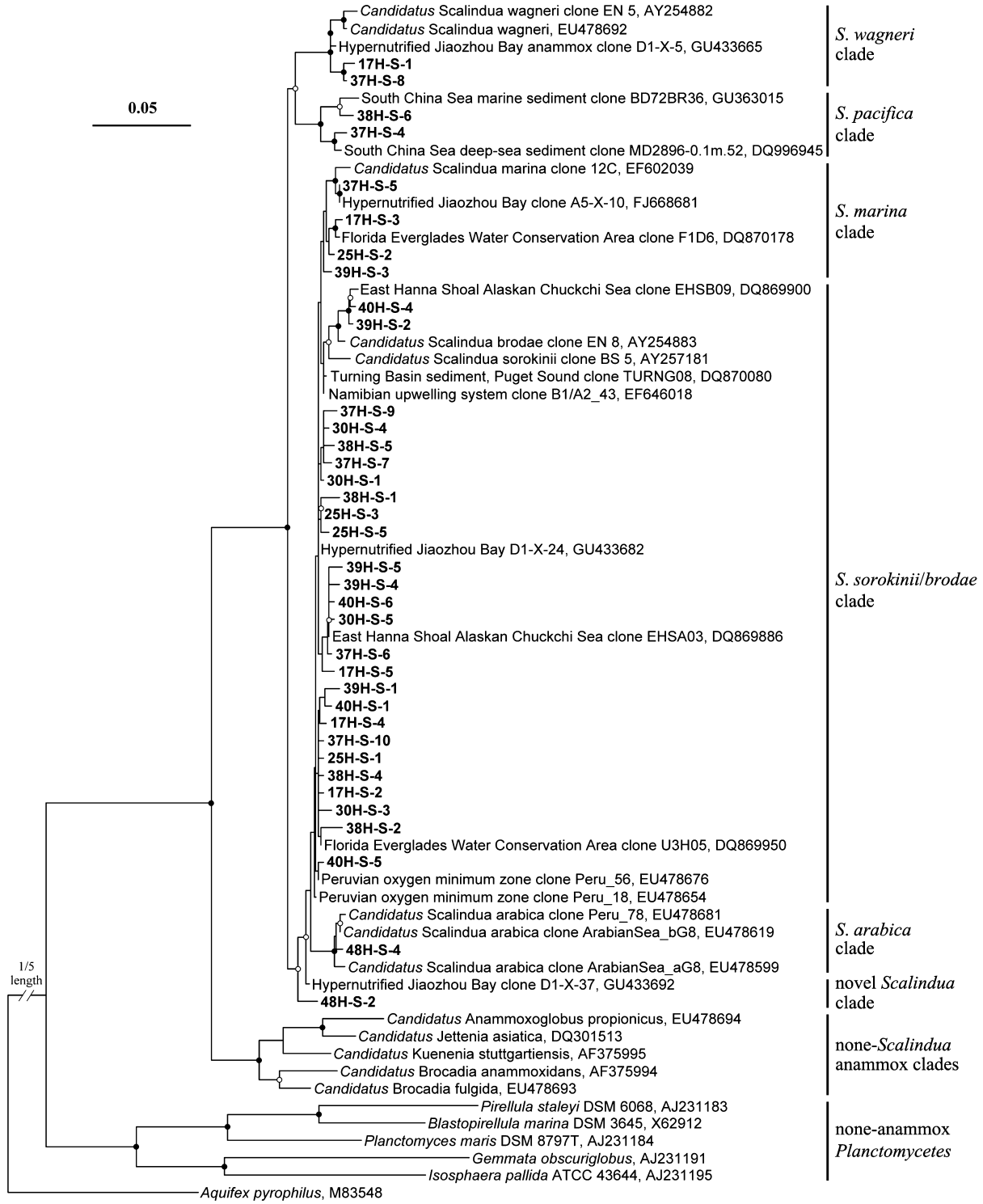

Fig. 1. Phylogenetic analysis of the *Candidatus* Scalindua 16S rRNA gene sequences obtained from the deep-sea methane seep surface sediments of the Okhotsk Sea. An alignment of experimental and publicly available 16S rRNA gene sequences was subjected to a distance neighbor-joining inference of phylogeny. The 16S rRNA gene sequence from *Aquifex pyrophilus* (GenBank accession number M83548) was used as outgroup. The tree branch distances represent nucleotide substitution rates, and the scale bar represents the expected number of changes per homologous position. Bootstrap values (100 resamplings) higher than 70% are shown with solid circle symbols, and those < 70% but ≥ 50% are shown with open circle symbols on the corresponding nodes. The *C.* Scalindua 16S rRNA gene sequences obtained in this study are shown in bold.

methane seep sediments of the Okhotsk Sea. Two OTUs, 25H-S-3 and 37H-S-10 affiliated with the *C.* Scalindua sorokinii/brodae clade, occurred in all eight 16S rRNA gene clone libraries. One other OTU, 37H-S-5 affiliated with the *C.* Scalindua marina clade, occurred in six of the eight clone libraries. Although these three OTUs accounted for only 8.3% of all the OTUs, they accounted for 89.8% of all the clones in our 16S rRNA gene clone libraries, likely representing the most abundant and prevalent *C.* Scalindua bacteria in the deep-sea methane seep sediments of the Okhotsk Sea.

Phylogenetic analysis of anammox bacterial Hzo protein sequences

The 61 unique *hzo* sequences obtained were 75.1–99.7% identical with one other and 94.5–99.7% identical to the closest-match GenBank sequences. The deduced partial Hzo protein sequences (331 amino acid residues) were 97.3–100% identical to the closest-match GenBank sequences. Most of the top-hit GenBank sequences were originally retrieved from coastal or marine sediments from, for example, the hypernutrified Jiaozhou Bay (Dang *et al.*, 2010a), the Hong Kong Mai Po nature reserve (Li *et al.*, 2011) and the South China Sea deep sea (Hong *et al.*, 2011a).

The constructed Hzo phylogenetic tree revealed that the sequences obtained from the Okhotsk Sea deep-sea methane seep sediments are all closely related to the *C.* Scalindua lineage, in which four distinct Hzo sequence clades were identified (Fig. 2), including the previously reported *C.* Scalindua clade, *C.* Scalindua-like clade I, *C.* Scalindua-like clade II, and *C.* Scalindua-like clade III (Dang *et al.*, 2010a). Based on DOTUR analysis, the sequences of any two of these clades were < 90% identical. The top-hit GenBank sequences affiliated with the *C.* Scalindua clade and the *C.* Scalindua-like clade I and clade II were originally obtained mainly from the hypernutrified Jiaozhou Bay and the South China Sea deep-sea sediments (Dang *et al.*, 2010a; Hong *et al.*, 2011a), and those affiliated with the *C.* Scalindua-like clade III mainly from the hypernutrified Jiaozhou Bay and the Hong Kong Mai Po nature reserve sediments (Dang *et al.*, 2010a; Li *et al.*, 2011).

Two Hzo OTUs, 25H-H-10 and 25H-H-1, occurred in all eight *hzo* clone libraries, and three OTUs, 30H-H-6, 25H-H-8 and 48H-H-9, occurred in seven and six, respectively, of the eight *hzo* clone libraries. Although these five OTUs accounted for only 15.6% of all the OTUs, they accounted for 86.7% of all the clones in our *hzo* gene clone libraries, likely representing the most abundant and prevalent anammox bacteria in the deep-sea methane seep sediments of the Okhotsk Sea.

Anammox bacterial community classification and spatial distribution

FAST UNIFRAC environment clustering analysis using the obtained 16S rRNA gene sequences indicated that the *C.* Scalindua bacterial assemblages of stations 38H, 39H and 48H were different from all the other stations (Fig. 3a). PCoA analysis further confirmed this difference (Fig. S3a). The stations 17H, 25H, 30H, 37H and 40H could be separated from the above three stations along the first PCoA principal coordinate (P1), which explained 55.89% of the total variability of the *C.* Scalindua bacterial assemblages among all the sampling stations (Fig. S3a).

In the CCA analysis using the *C.* Scalindua 16S rRNA gene sequences (Fig. 4a), the first two CCA axes (CCA1 and CCA2) explained 40.2% of the total variance in the *C.* Scalindua composition and 44.8% of the cumulative variance of the *C.* Scalindua–environment relationship. This analysis also showed that the *C.* Scalindua assemblages at the sampling stations 38H and 39H were different from those at all the other sampling stations, including 48H (Fig. 4a). In contrast to the results of the FAST UNIFRAC environment clustering and PCoA analyses (Figs 3a and S3a), the *C.* Scalindua assemblage at sampling station 48H was similar to those at the sampling stations 37H and 40H (Fig. 4a). Of all the environment factors analyzed, the ratio of sediment porewater total nitrogen oxides ($NO_3^- + NO_2^-$, i.e. NO_x^-) to NH_4^+ (i.e. NO_x^-/NH_4^+) was identified as the most important environment factor, although only with a marginal significance ($P = 0.068$, 1000 Monte Carlo permutations), in shaping the *C.* Scalindua bacterial community structure and spatial distribution, providing 16.7% of the total CCA explanatory power. Although none of the other major environment factors identified by the CCA analysis (as shown in Fig. 4a) contributed significantly ($P > 0.300$) to the *C.* Scalindua bacteria–environment relationship, the combination of these environment factors provided an additional 72.2% of the total CCA explanatory power.

FAST UNIFRAC environment clustering analysis using the obtained Hzo protein sequences indicated the existence of distinct clusters of the anammox bacterial assemblages (Fig. 3b). The stations 37H and 38H anammox bacterial assemblages represented one cluster and the remaining stations comprised another. PCoA analysis further confirmed this classification (Fig. S3b). The anammox bacterial assemblages of stations 37H and 38H were separated from those of the other stations along the first PCoA principal coordinate (P1), which explained 82.63% of the total variability of the anammox bacterial assemblages among all the sampling stations (Fig. S3b).

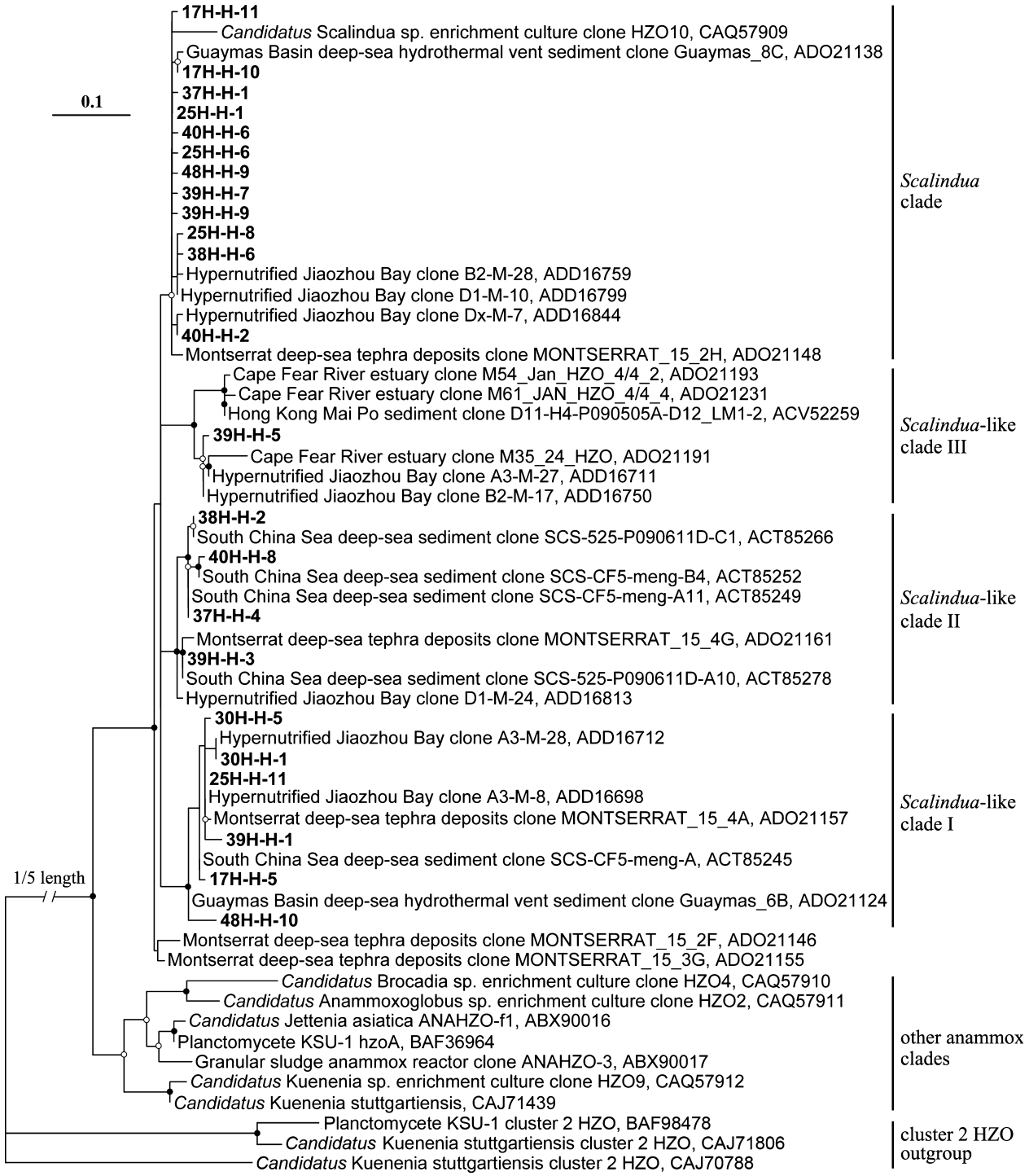

Fig. 2. Phylogenetic consensus tree constructed after neighbor-joining distance analysis of an alignment of publicly available anammox bacterial Hzo protein sequences and those deduced from experimental sequences of *hzo* genes recovered from the deep-sea methane seep surface sediments of the Okhotsk Sea. The tree branch distances represent amino acid substitution rates, and the scale bar represents the expected number of changes per homologous position. The three Hzo sequences in 'cluster 2' (BAF98478, CAJ71806 and CAJ70788) were used as outgroup. Bootstrap values higher than 70% of 100 resamplings in support of the presented tree are shown with solid circle symbols, and those < 70% but ≥ 50% are shown with open circle symbols on the corresponding nodes. The anammox bacterial Hzo sequences obtained in this study are shown in bold.

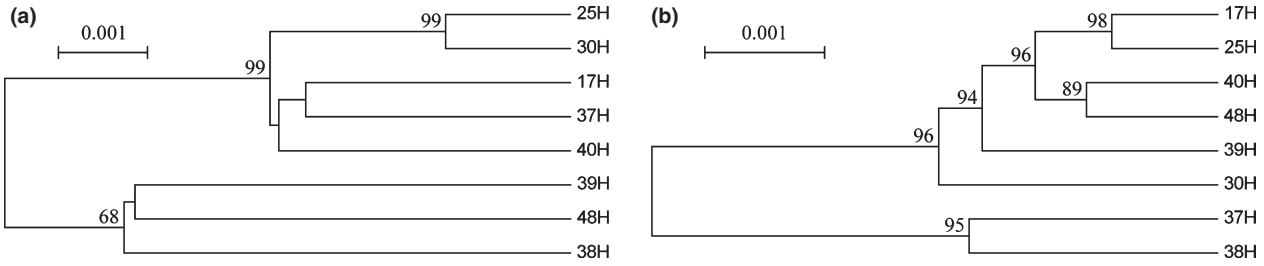

Fig. 3. Dendrogram of the hierarchical clustering analysis of the methane seep surface sediments anammox bacterial assemblages as revealed by the *Candidatus* Scalindua 16S rRNA gene sequences (a) or the anammox Hzo protein sequences (b), constructed using the FAST UNIFRAC normalized and weighted Jackknife environment clusters statistical method. The scale bars stand for the unit of the UNIFRAC distance and the percentage supports of the classification tested with jackknifing resamplings are shown near the corresponding nodes.

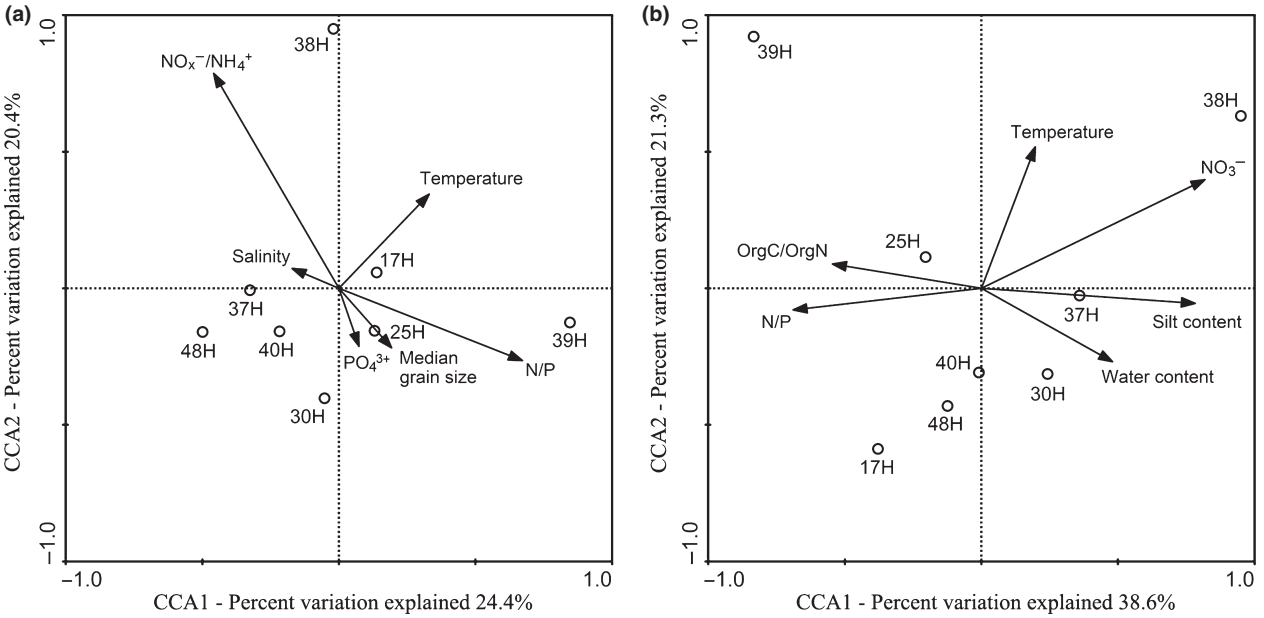

Fig. 4. CCA ordination plots for the first two dimensions showing the relationship between the methane seep surface sediment *Candidatus* Scalindua 16S rRNA gene (a) and the anammox Hzo gene (b) assemblages from the Okhotsk Sea with environmental parameters analyzed using the weighted OTU data. Correlations between environmental variables and CCA axes are represented by the length and angle of the arrows (environmental factor vectors). Kurtosis, sediment kurtosis; Median, sediment median grain size; Skewness, sediment skewness; Sorting, sediment sorting coefficient.

For the CCA analysis using the Hzo protein sequences (Fig. 4b), the first two CCA axes (CCA1 and CCA2) explained 55.9% of the total variance in the anammox bacterial composition and 59.9% of the cumulative variance of the anammox bacteria-environment relationship. Similar to the CCA result employing the *C.* Scalindua 16S rRNA gene OTUs (Fig. 4a), the *hzo*-harboring anammox bacterial assemblages at the sampling stations 38H and 39H were different from those at all the other sampling stations (Fig. 4b). However, unlike the results of the FAST UNIFRAC environment clustering and PCoA analyses (Figs 3b and S3b), the *hzo*-harboring anammox bacterial assemblage at sampling station 37H was not grouped closely with that at 38H (Fig. 4b). Of all the environment factors analyzed, sediment porewater NO_3^- ($P = 0.019$) and sediment silt content ($P = 0.043$) contributed significantly to the anammox bacteria–environment relationship, providing 28.9% and 17.8% of the total CCA explanatory power, respectively. Although none of the other major environment factors identified by the CCA analysis (as shown in Fig. 4b) contributed significantly ($P > 0.250$) to the anammox bacteria–environment

relationship, the combination of these environment factors provided an additional 46.7% of the total CCA explanatory power, indicating that the sediment anammox bacterial community structure and spatial distribution were influenced by a combination of environmental parameters with varying individual contributions.

Quantification of sediment anammox bacterial assemblages

Melting curve analyses of qPCR reactions targeting the *C.* Scalindua 16S rRNA and anammox *hzo* genes confirmed the qPCR specificity. Standard curves generated using plasmids containing 16S rRNA or *hzo* gene fragments to relate the threshold cycle (C_t) to gene copy number revealed a linearity ($r^2 \geq 0.990$) of over six orders of magnitude of the standard plasmid DNA concentration (Table S2). The obtained high correlation coefficients and similar slopes indicated high primer hybridization and extension efficiencies, making comparison of gene abundances reasonable. The absence of detectable influence of sediment PCR-inhibitory substances was confirmed by obtaining similar amplification efficiencies with 10-fold-diluted environmental DNAs extracted from the sediments of station 25H (data not shown).

The qPCR results reflect a heterogeneous distribution of the *C.* Scalindua 16S rRNA gene abundance across all sampling sites of the Okhotsk Sea (Table 2), where station 38H had the highest (4.74×10^6 g^{-1} sediment) and station 48H the lowest gene copy numbers (5.91×10^3 g^{-1} sediment). Similarly, the *hzo* gene abundance also reflected a heterogeneous distribution (Table 2), station 38H having the highest (1.03×10^7 g^{-1} sediment) and station 39H the lowest gene copy numbers (1.13×10^4 g^{-1} sediment). The ratio of anammox bacterial *hzo* genes to *C.* Scalindua 16S rRNA genes ranged from 1.18 : 1 to 5.13 : 1, with an average of 2.32 ± 1.27, station 48H having the highest ratio (5.13 : 1) and station 39H the lowest (1.18 : 1; Table 2).

Pearson correlation analyses (Table S3) indicated that the abundances of the sediment *C.* Scalindua 16S rRNA genes and anammox bacterial *hzo* genes both correlated positively ($P < 0.05$) with sediment silt content, skewness and water depth, and negatively with sediment sorting coefficient. The relative abundance of sediment anammox bacteria, revealed as the ratio of *C.* Scalindua 16S rRNA gene abundance or the total anammox bacterial *hzo* gene abundance to the total bacterial 16S rRNA gene abundance (as reported in Dang *et al.*, 2010b), correlated positively with sediment porewater NO_3^-, porewater NO_x^-, sediment OrgN, sediment silt content, sediment skewness and water depth, and negatively with sediment sorting coefficient in the deep-sea methane seep sediments of the Okhotsk Sea (Table S3).

Table 2. Copy numbers of the bacterial, archaeal and *Candidatus* Scalindua 16S rRNA genes and the anammox *hzo* genes in sediments of the eight sampling stations of the Okhotsk Sea

Sampling station	Target gene copy number g^{-1} sediment*				Ratio		
	Bacterial 16S rRNA†	Archaeal 16S rRNA†	*Scalindua* 16S rRNA	Anammox *hzo*	*Scalindua* 16S rRNA/ Bacterial 16S rRNA ($\times 10^{-6}$)	Anammox *hzo*/ Bacterial 16S rRNA ($\times 10^{-6}$)	Anammox *hzo*/ *Scalindua* 16S rRNA
17H	7.74×10^{11} (3.30×10^{10})	2.49×10^{10} (2.54×10^{9})	1.68×10^{6} (1.24×10^{5})	2.53×10^{6} (7.20×10^{3})	2.17	3.27	1.51
25H	3.26×10^{11} (2.40×10^{10})	7.77×10^{9} (8.80×10^{8})	9.85×10^{4} (8.43×10^{3})	3.04×10^{5} (3.02×10^{4})	0.30	0.93	3.09
30H	6.33×10^{11} (2.41×10^{10})	1.19×10^{10} (1.89×10^{9})	3.75×10^{6} (2.22×10^{5})	6.74×10^{6} (3.26×10^{5})	5.93	10.65	1.80
37H	4.67×10^{11} (2.68×10^{10})	1.70×10^{10} (2.09×10^{9})	4.05×10^{6} (3.54×10^{5})	8.61×10^{6} (3.26×10^{5})	8.68	18.46	2.13
38H	4.13×10^{11} (1.14×10^{10})	1.63×10^{10} (1.95×10^{9})	4.74×10^{6} (1.72×10^{5})	1.03×10^{7} (6.35×10^{5})	11.49	24.96	2.17
39H	1.55×10^{11} (4.59×10^{9})	5.93×10^{9} (8.64×10^{8})	9.59×10^{3} (8.91×10^{2})	1.13×10^{4} (1.05×10^{3})	0.06	0.07	1.18
40H	4.60×10^{11} (3.68×10^{10})	1.85×10^{10} (3.38×10^{9})	2.32×10^{5} (7.61×10^{3})	3.59×10^{5} (1.85×10^{4})	0.50	0.78	1.55
48H	3.28×10^{11} (2.86×10^{9})	1.14×10^{10} (6.09×10^{8})	5.91×10^{3} (5.51×10^{2})	3.03×10^{4} (5.71×10^{2})	0.02	0.09	5.13

*Standard errors indicated in parentheses.
†The abundances of bacterial and archaeal 16S rRNA genes were determined in a previous study (Dang *et al.*, 2010b).

Discussion

Methane seep sediments support diverse and abundant anammox bacteria

In agreement with our hypothesis, we identified an abundant and diverse complement of anammox bacterial 16S rRNA and *hzo* gene sequences including several new *C.* Scalindua-related clades in the deep-sea methane seep sediments of the Okhotsk Sea (Figs 1 and 2). Both 16S rRNA and *hzo* gene clone libraries included gene sequences exclusively related to the *C.* Scalindua lineage (Figs 1 and 2). The average ratio of the sediment *hzo* and *C.* Scalindua 16S rRNA gene abundances in the Okhotsk Sea was 2.3 : 1 (Table 2), which is close to the 2 : 1 ratio reported for the genomes of *C.* Kuenenia stuttgartiensis, *C.* Scalindua profunda and anammox bacterium strain KSU-1 (Strous *et al.*, 2006; Shimamura *et al.*, 2007; van de Vossenberg *et al.*, 2013). This also suggests that the anammox bacterial assemblages in the Okhotsk Sea methane seep sediments consist exclusively of bacteria in the *C.* Scalindua lineage.

The fact that anammox bacterial assemblages in the deep-sea appear to be less diverse than those in estuarine and coastal environments (Amano *et al.*, 2007, 2011; Dale *et al.*, 2009; Dang *et al.*, 2010a, 2013; Li *et al.*, 2010, 2011; Hirsch *et al.*, 2011) does not necessarily mean that anammox bacteria are of less importance to ecosystem functions in deep-sea sediments. Our qPCR results of the *C.* Scalindua 16S rRNA and anammox bacterial *hzo* genes indicate that the abundance of the anammox bacteria ranged from 10^3 to 10^7 g^{-1} sediment in the Okhotsk Sea, which is equivalent to or even higher than the abundance of sediment anammox bacteria found in the marginal seas at other geographic locations of the Pacific Ocean such as the Jiaozhou Bay (10^5–10^6 g^{-1} sediment; Dang *et al.*, 2010a), Bohai Sea (10^5–10^7 g^{-1} sediment; Dang *et al.*, 2013), Mai Po Nature Reserve estuary (10^4–10^5 g^{-1} sediment; Li *et al.*, 2011) and South China Sea and equatorial Pacific (10^3–10^4 g^{-1} sediment; Hong *et al.*, 2011a, b). Anammox bacteria are chemolithoautotrophic and recent studies provided evidence that the deep-sea sediment anammox bacteria are alive and active in N_2 production (Engström *et al.*, 2009; Glud *et al.*, 2009; Trimmer & Nicholls, 2009; Sokoll *et al.*, 2012). The significant positive correlation of the relative abundance of anammox bacteria with sediment porewater NO_3^- and NO_x^- suggests that the sediment anammox bacteria actively facilitate the anaerobic ammonium oxidation process in deep-sea methane seep sediments of the Okhotsk Sea (Table S3).

Methane seeps are widespread in the world's continental margins and represent a unique type of highly productive ecosystem in the ocean (Vanreusel *et al.*, 2009). Methane seep sediments are generally hypoxic (Sahling *et al.*, 2003; Vanreusel *et al.*, 2009), thereby providing the necessary habitat for the anammox process. Anammox bacteria were recently found to be active over a range of O_2 concentrations, suggesting that their distribution might be broader than previously thought (Kalvelage *et al.*, 2011). However, the prevailing hypoxia in deep-sea methane seep sediments may contribute to the enrichment and resulting higher activity of anammox bacteria. In comparison with other deep-sea sites of the Pacific Ocean, our data suggest that anammox bacteria may be more important in methane seep sediments than in non-seep sediments.

Anammox bacterial community structure is shaped by sediment environments

Both clustering and PCoA analyses revealed an uneven spatial distribution of the anammox bacterial assemblages in the deep-sea sediments of the Okhotsk Sea (Figs 3 and S3). Several environmental factors, including the availability of anammox substrates (inorganic N nutrients), OrgN, water depth and several sedimentological factors, correlated significantly with anammox bacterial abundance and spatial distribution patterns (Table S3, Fig. 4). On the other hand, our data indicate that the 16S rRNA gene-based and Hzo-based statistical results were not totally consistent (Figs 3, 4 and S3). The reason for this inconsistency is not fully understood at present. We speculate that it is caused by differences in molecular diversity of 16S rRNA and *hzo* gene sequences and that the employed primers target slightly different subsets of the anammox bacterial assemblages. This difference in molecular diversity of 16S rRNA and *hzo* genes may have evolved in the context of the difference in gene copy number per cell, whereby the sequence rectification of multiple gene copies (*hzo* genes) introduces biases not encountered in a single gene copy (16S rRNA gene) scenario (Klotz & Stein, 2011).

The NO_2^- concentration is usually very low and likely limiting for anammox bacterial population size and activity in marine environment (Meyer *et al.*, 2005; Trimmer *et al.*, 2005; Dang *et al.*, 2010a). NO_2^- is mainly generated by reduction of NO_3^-, indicating that NO_3^- or the total level of nitrogen oxides (NO_x^-) constitutes the limiting factor for the anammox process (Rysgaard *et al.*, 2004; Nicholls & Trimmer, 2009; Dang *et al.*, 2013). In oligotrophic marine environments, NH_4^+ may be limiting as well; however, some anammox bacteria can utilize organic reductants to reduce nitrite to ammonium (van de Vossenberg *et al.*, 2013; and references therein). Our current study identified sediment porewater NO_3^-, NO_x^- and

NO_x^-/NH_4^+ as key environmental factors influencing the abundance, community structure and spatial distribution of the sediment anammox bacteria (Fig. 4, Table S3). Sediment porewater NO_2^- and NO_3^- concentrations were generally low, whereas the NH_4^+ concentrations were highly variable (ranging from 11.39 to 245.81 μM) across the different sampling sites in the Okhotsk Sea (Table S1). The detected correlation of anammox bacterial gene marker distribution patterns and *in situ* inorganic nitrogenous nutrients suggests that the resident bacteria were active in catalyzing the anammox process in the deep-sea methane seep sediments of the Okhotsk Sea.

In this study, sediment OrgN was found to have a significant influence on the relative abundance of anammox bacterial gene markers (Table S3), suggesting that organic nitrogen remineralization is an important source of NH_4^+ for anammox in certain deep-sea methane seep sediments of the Okhotsk Sea. In our study, sediment OrgN was found to correlate negatively with sediment NH_4^+ ($r = -0.687$, $P = 0.088$) and DIN ($r = -0.682$, $P = 0.092$), suggesting the *in situ* compensative effect of OrgN to the localized deficiency of ammonium or total inorganic nitrogenous nutrients for the sediment anammox process in certain Okhotsk Sea deep-sea sites. A recent study showed the potential of marine *C.* Scalindua bacteria to take up amino acids, oligopeptides and other small organic compounds for an alternative source of NH_4^+ and/or energy production (van de Vossenberg *et al.*, 2013). Our data support this newly identified ecophysiology of marine *C.* Scalindua bacteria (van de Vossenberg *et al.*, 2013).

Our CCA analyses identified sediment silt content as another significant environmental factor influencing the abundance and spatial distribution of anammox bacterial gene markers in the deep-sea methane seep sediments of the Okhotsk Sea (Fig. 4b, Table S3). Anammox activity requires the simultaneous presence of ammonium and nitrite, which may be found at or near the oxic–anoxic interface of sediments (Kuenen, 2008). Deep-sea sediment ammonium supply is likely provided via several *in situ* microbial processes, including nitrogen fixation (Pernthaler *et al.*, 2008; Dang *et al.*, 2009; Dekas *et al.*, 2009; Miyazaki *et al.*, 2009), organic nitrogen remineralization and dissimilatory nitrate reduction to ammonium (Glud *et al.*, 2009; Lam *et al.*, 2009; Jensen *et al.*, 2011). Nitrite may be produced *in situ* by anaerobic nitrate reduction or by aerobic ammonia oxidation (Lam *et al.*, 2009; Dang *et al.*, 2010b). Our previous study also identified sediment silt content as the most important environmental factor influencing the composition, structure and distribution of the *amoA*-encoding archaea assemblages in the deep-sea methane seep sediments of the Okhotsk Sea (Dang *et al.*, 2010b). The likely reliance of anammox bacteria on ammonia-oxidizing microorganisms to acquire an *in situ* nitrite source provides a reasonable explanation for the phenomenon that both microbial groups correlate with the same environmental factor: sediment silt content. Compared with oxygen-rich sand-dominant or oxygen-scarce clay- dominant sediments, silt-dominant sediments may sustain a more stable oxic–anoxic interface for suitable sediment porosity (constituting the diffusional constraints), geohydrological flow of interstitial water and dissolved O_2 and thus for simultaneous microbial production of ammonium and nitrite. Silt-dominant sediments are more favorable to the anammox process and therefore are a key environmental factor in shaping the community structure, abundance and spatial distribution of the anammox bacteria via its influence on the geochemistry and mutualistic cooperation between anammox bacteria and other N-transforming microorganisms in the sediments (Hulth *et al.*, 2005; Glud *et al.*, 2009; Lam *et al.*, 2009; Yan *et al.*, 2010; Jensen *et al.*, 2011).

Two other sedimentological parameters, sediment sorting coefficient and skewness, were also found to correlate with anammox bacterial gene marker abundance in the Okhotsk Sea sediments (Table S3). The importance of sedimentological parameters for abundance and spatial distribution of sediment microbiota has been reported recently (Dang *et al.*, 2010a, b, 2013, and references therein). Our current study presents another ecologically meaningful example of this rather intriguing finding. Further exploration using additional approaches is indicated.

Water depth was found to be another significant environmental factor positively correlated with the sediment anammox bacteria abundance in the Okhotsk Sea (Table S3). A similar trend has also been discovered in other marginal seas (Jaeschke *et al.*, 2010; Sokoll *et al.*, 2012; Trimmer *et al.*, 2013). Lower *in situ* carbon mineralization rate at deeper sites was proposed as an explanation for this phenomenon (Jaeschke *et al.*, 2010). Furthermore, the temperature optimum of anammox bacteria is lower than that of psychrotrophic denitrifying bacteria. Thus, colder environments favor the anammox process (Rysgaard *et al.*, 2004). This may be one of the reasons why the relative contribution of anammox vs. denitrification increases with water depth in marine environments (Dalsgaard *et al.*, 2005; Trimmer & Nicholls, 2009; Jaeschke *et al.*, 2010; Sokoll *et al.*, 2012) and why cold deep-sea sediments are a favorable habitat for anammox bacteria. Methane seep sediments are usually colder than the ambient cold deep-sea environments due to the endothermic nature of methane hydrate decomposition (Luan *et al.*, 2008). Thus the colder methane seep environments may favor anammox biology. This ecological context may provide an the impetus for future molecular, physiological and biochemical anammox research.

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Authors' contributions

S.S. and X.L. contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Maps show (a) the Okhotsk Sea and (b) the sampling stations in the methane seep areas.

Fig. S2. Rarefaction of anammox bacterial communities based on *Candidatus* Scalindua 16S rRNA gene (a) and deduced Hzo protein sequences (b) from eight stations of the deep-sea methane seep sediments in the Okhotsk Sea.

Fig. S3. Ordination diagrams of the FAST UNIFRAC weighted and normalized PCoA analyses of the methane seep surface sediment anammox bacterial assemblages as revealed by the *Candidatus* Scalindua 16S rRNA gene sequences (a) or the anammox Hzo protein sequences (b). The plots of the first two principal coordinate axes (P1 and P2) for PCoA and the distribution of the *C*. Scalindua 16S rRNA gene-typic assemblages (a) and the anammox bacterial Hzo protein-typic assemblages (designated with the sampling station names) (b) in response to these axes.

Table S1. Sampling stations and environmental parameters of the top 5-cm layer deep-sea methane seep sediments collected from the Okhotsk Sea.

Table S2. Efficiency and sensitivity of individual qPCR standard curve determined via plasmid DNA.

Table S3. Pearson correlation analyses of the key microbial abundance with measured environmental factors.