Transcriptional Activity of Aerobic and Anaerobic Ammonia-Oxidizing community in the Intertidal Sponge *Cinachyrella Australiensis*, Ambient Seawater, and Sediment

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Abstract

Microbial ammonia oxidation plays a central role in nitrogen cycling. Hitherto, four types of autotrophic ammonia-oxidizing microorganisms are identified, including aerobic ammonia-oxidizing archaea (AOA), aerobic partial-nitrification ammonia-oxidizing bacteria (parAOB), aerobic complete-nitrification AOB (comAOB), and anaerobic AOB (AnAOB). However, revelation and comparison of the active ammonia-oxidizing community in the marine sponges and their ambient environments is scarce. Here, transcribed ammonia oxidation phylomarker gene *amoA* of AOA, parAOB, and comAOB and *hzsB* of AnAOB were amplified to investigate the active ammonia-oxidizing populations in a representative marine sponge *Cinachyrella australiensis*, ambient seawater, and sediment niches. Ammonia-oxidizing population in *C. australiensis* consists of AOA, parAOB, and AnAOB, significantly different from that in seawaters comprising of AOA and in sediments containing AOA, parAOB, comAOB, and AnAOB. The quantitative assay demonstrates that AOA *amoA* transcripts are exclusively detectable or higher in abundance than parAOB *amoA*, comAOB *amoA*, or AnAOB *hzsB* transcripts by orders of magnitude in *C. australiensis*, seawater, and sediment niches. This transcript-based analysis clarifies the remarkable niche differentiation of putatively active ammonia-oxidizing microbiota in *C. australiensis* and the ambient environments. Such a work further contributes to the understanding of *in situ* active ecological functions of sponge microsymbionts in nitrogen cycling.

Introduction

Ammonia oxidizing prokaryotes (AOP) play a fundamental role in the global nitrogen cycling [1]. Hitherto, four autotrophic AOP types are uncovered in nature. Among them, three aerobic AOP types, i.e., ammonia-oxidizing archaea (AOA) of the Thaumarchaeota phylum, partial-nitrification ammonia-oxidizing bacteria (parAOB) of the Proteobacteria phylum, and comammox bacteria (comAOB) of the Nitrospirae phylum, can drive the oxidation of ammonia to nitrite during the nitrification step [2–4]. While the remaining one anaerobic AOP type, i.e., anaerobic AOB (AnAOB) of the Planctomycetes phylum, can functionates the oxidation of ammonia to nitrogen gas via the anammox procedure [5].

Sponges are widely distributed ecological participators in marine ecosystems [6]. Sponges often host abundant diverse microbiota [7–9] and excrete ammonia as a waste product which is toxic with its accumulation in sponge tissues [10]. The accumulated ammonia in sponges can be scavenged by AOP, since nitrification and anammox strategies have been confirmed in sponges [11–13].

The *amoA* gene, encoding a subunit of ammonia monooxygenase in AOA and parAOB, is commonly used as a phylogenetic marker to verify the residence of AOA and parAOB in environments [14–16]. For example, *amoA* gene or transcript-based analyses have revealed diverse Thaumarchaeota AOA or *Nitrosospira* parAOB in sponges [17–19]. Besides, *amoA* gene and transcript co-detection surveys demonstrated that parAOB *amoA* genes could be detected in the sponges *Antho dichotoma* and *Phakellia ventilabrum* whereas their transcript counterparts were only revealed from *A. dichotoma* [20], suggesting that some sponge-associated AOP may be not metabolically active at the time of sampling [20].

Metagenomic and metatranscriptomic analyses have also disclosed abundant *amo* homologues of Thaumarchaeota AOA and Proteobacteria parAOB in different sponges [21–26]. Besides, metagenome-assembled genomes have further revealed various AOA species, e.g., *Nitrosopumilus* sp. LS_AOA, Ca. *Nitrosospongia ianthellae*, Ca. *Nitrosopumilus hexadellus*, Ca. *Nitrosopumilus detritiferus*, and Ca. *Cenporiarchaeum stylissum* in sponges [27–29]. These studies demonstrate the remarkable function of AOA or parAOB in aerobic ammonia oxidation in sponges.

The *amoA* gene in comAOB, distinctive from its homologues in AOA and parAOB, is also a useful biomarker to screen the comAOB in ecosystems [30]. *amoA* gene-based studies have revealed diverse comAOB in marine environments, such as estuary sediments [31], mangrove sediments [32], and estuarine tidal flat wetlands [33]. As a comparison, comAOB has not been reported inhabiting sponges yet. Co-existence of *Nitrosopumilus* and *Nitrospira* was found in the sponges *Theonella swinhoei* [34] and *Cymbastela concentrica* [35]. However, whether these sponge-associated *Nitrospira* belonged to comAOB were not verified. Further comAOB *amoA* gene-based assessment [30] would be helpful to verify the inhabitancy of comAOB in sponges.

The *hzsA* or *hzsB* gene encoding a subunit of hydrazine synthase in AnAOB is used as a phylomarker to uncover the AnAOB in niches [36]. An attempt to detect AnAOB *hzsA* fragments from sponges was failed [19]. However, co-detection of *amoA* and AnAOB-specific 16SrRNA genes have verified the *Nitrosarchaeum* AOA and *Scalindua* AnAOB in the sponge *Geodia barretti* [13], and *Nitrosospira* AOB and *Brocadia* AnAOB in the sponges *Ircinia strobilina* and *Mycale laxissima* [20]. Therefore, the function of AnAOB in anaerobic ammonia oxidation should not be neglected in sponges.

Notably, the presence of the functional genes (e.g., *amoA* or *hzsB*) in ecosystems is not the direct evidence of AOP metabolic activity. Rather, the corresponding transcripts of genes are more suitable to represent the functional activity, and are more sensitive when facing environmental variables [37]. Thus, analysis of the ammonia-oxidation transcripts would provide valuable hints on the active ammonia-oxidizing microorganisms in environments [38], such as in sponges [20].

Since the ubiquity of comAOB in various environments [39], it is necessary to reappraise and expand the understanding of the AOP community in environments, such as sponges and the ambient niches. *Cinachyrella australiensis* (Demospongiae Class, Tetractinellida Order, Tetillidae Family) is an ecologically dominant and representative sponge species of the coastal community [40]. This sponge distributes in a broad range of Indo-Pacific [41]. A previous study has discovered both aerobic bacteria (e.g., *Nitrospira* and Methylococcales) and anaerobic bacteria (e.g., Anaerolineae and Chromatiales) in *C. australiensis* [42], implying the presence of oxic-anoxic microenvironments in this sponge. Here, *C. australiensis* was chosen to access the presence, diversity, and abundance of the active AOP community in sponge and the ambient environments. To this aim, transcripts of phylomarkers involved in aerobic and anaerobic ammonia oxidation procedures were analyzed to uncover and compare the active ammonia-oxidizers in *C. australiensis*, ambient seawater, and sediment niches.

Materials And Methods

Study Site and Sampling

The sponge individuals were collected at the low tide sites S1 (109°29'33" E 18°15'37" N), S2 (109°29'11" E 18°15'37" N), and S3 (109°26'18" E 18°15'37" N) from the intertidal zone of Hainan Island in the South China Sea at 8:00 – 10:00 am in July 6th, 2019. The seawater temperature was 27.2 – 27.4°C. The sponge is solitary individual of globular size in appearance, typically up to 4 – 6 cm diameter. In each site, triplicate samples of sponge tissue slice, ambient seawater (two liters each), and ambient sediment (0 – 2 cm, ~ 5 g) were separately collected (Fig. S1 A and B). Collections and pretreatments of sponge, seawater, and sediment samples referred to the reported strategy [19, 20, 43] before being transferred into the RNeasy Protect Bacteria Reagent (Qiagen, Hilden, Germany). Identification of the sponge species based on gross morphology and PCR confirmation of the mitochondrial *coxI* (cytochrome c oxidase subunit I) gene using the primer pair ClnaF2/dgHCO2198 [44]. The amplified *coxI* clone gene showed 99% nucleotide sequence identity to the reported *C. australiensis* voucher LB_815 mitochondrial *cox1* gene (JX177880) and was submitted to GenBank with the accession number MT913441. The time between sample acquisition and fixation was no longer than 20 min to minimize RNA degradation [45]. All the RNA protector-fixed samples were stored at -80°C before total RNA and DNA extraction within two weeks.

RNA, DNA Extraction and cDNA Synthesis

RNA protector-fixed *C. australiensis*, seawater, and sediment samples were ground in liquid nitrogen with a sterilized mortar and pestle. Both RNA and DNA were extracted from ground powders using the PrepRNA/DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. RNA and DNA were separately extracted from each sample of *C. australiensis*, seawater, and sediment. RNase-free DNase I (Fermentas, Hanover, USA) was used to digest the residual genomic DNA at 37°C for 60 min. RNA quality and integrity were checked by gel electrophoresis and by examining the A260/A280 ratio (ranging from 1.97 to 2.02) using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, USA). The final RNA concentration and purity were quantified using the Qubit system (Invitrogen, Darmstadt, Germany). First-strand cDNA synthesis was performed using the SuperScript FirstStrand Synthesis System (Invitrogen, Carlsbad, USA). Each reaction volume was 10 µl containing 100 ng RNA, 0.5 µl random hexamer primer (50 ng µl⁻¹), 5 µl cDNA Synthesis Mix, and proper RNase-free water. This reaction system was incubated at 25°C for 10 min and then 50°C for 50 min and terminated at 85°C for 5 min. All cDNA aliquots were stored at -80°C before PCR amplification.

Amplification and Sequencing

The AOA *amoA*, AAOB *amoA*, CoAOB *amoA*, and AnAOB *hzsB* gene fragments were amplified, respectively, using the cDNA and DNA templates with the primers listed in Table 1. PCR amplifications were performed in a total volume of 40 µl containing 2 µl cDNA or 2 ng DNA, 0.1 µM of each primer, and 20 µl TaqMasterMix (CoWin Biotech, Beijing, China) on a Thermocycler (Eppendorf, Hamburg, Germany) according to the following procedures: 95°C for 5 min; followed by 30 cycles at 95°C for 40 s, annealing

(temperature referring to Table 1) for 30 s and 72°C for 30 s, and finally 72°C for 10 min. For negative control, a similar procedure was carried out using purified RNA to ensure that there was no genomic DNA contamination. PCR products originated from the triplicate samples of *C. australiensis*, seawater, or sediment from each sampling site were pooled to reduce potential amplification bias and maximize the transcript richness referring to the previous strategies [19]. The presence and sizes of these amplification products were estimated by gel electrophoresis (1.5% agarose gel). Since this study focused on the transcriptional activity of the ammonia-oxidizing community, the performance of DNA-based PCR amplification was to only test the presence of the targeted genes in the investigated biotopes, whose PCR products were not sequenced [19]. cDNA-based PCR products were gel-purified with MinElute Gel Extraction Kit (Qiagen), cloned with pUCm-T Vector Rapid Cloning Kit (Sangon Biotech, Shanghai), and transformed to the DH5α competent cells (Sangon Biotech) according to the standardized instructions. The positive clones were screened by ampicillin resistance and identified by PCR screening with vector-specific M13 primers. A variable number of clones (13 – 69) from each clone library were sequenced (Table S1) on an ABI 3100 capillary sequencer (Sangon Corp., Shanghai, China).

Sequence Analysis

All the obtained nucleotide sequences were trimmed manually by using ClustalW implemented in MEGA [46] with default settings [46]. The trimmed sequences were performed BLAST searches against the NCBI Nucleotide database. A phylotype was defined by 3% dissimilarity threshold [47, 48] for *amoA*, or by 5% for *hzsB* [36], using the Mothur Version 1.44.1 package [49]. Phylotype representative sequences were taxonomically classified using BLASTn against NCBI Nucleotide database. Rarefaction curves and Good's coverage estimators were determined using the Mothur package [49] to estimate whether the sequencing depth is enough to cover most of the transcribed genes in each clone library.

One representative sequence from each phylotype and its closest sequence retrieved from the NCBI Nucleotide database were aligned using ClustalW implemented in the MEGA [46]. Maximum-likelihood (ML) tree was constructed by using the MEGA [46] with the Kimura-2 parameter model according to a published guideline [50]. Bootstrap analysis was used to estimate the reliability of phylogenetic reconstructions (1000 replicates).

RT-qPCR Assays

RT-qPCR assays were performed using an ABI 7500 Fast Real-time qPCR platform (Applied Biosystems, Foster, USA), following the reported strategy on sponges [19]. Gene expression was tested using technical triplicates for each sample of *C. australiensis*, seawater, and sediment. PCR was performed in a total volume of 25 µl containing 12.5 µl of SYBR Premix Ex Taq™ II (Takara, Dalian, China), 1 µl of cDNA template (tenfold serial dilution), and 0.1 µM of each primer (Table 1). PCR thermocycling steps were set as follows: 95°C for 5 min and 40 cycles at 95°C for 45 s, annealing (temperature setting showed in Table 1) for 45 s, and 72°C for 45 s. For quantification, standard curves (log-linear $R^2 > 0.99$, $E = 92\% - 110\%$) were generated using purified and quantified plasmids containing AOA *amoA* (sequence of the clone

AOA-spg-1, GenBank ID MT925791), parAOB *amoA* (sequence of the clone parAOB-spg-1, GenBank ID MT925730), comAOB *amoA* (sequence of the clone comAOB-sed-1, GenBank ID MT925742), or AnAOB *hzsB* (sequence of the clone AnAOB-spg-1, GenBank ID MT925769) fragment in a dilution series that spanned from 10^1 to 10^7 gene copies per reaction. All standard dilutions were prepared in $10 \text{ ng } \mu\text{l}^{-1}$ aqueous tRNA solution (Sigma-Aldrich, Steinheim, Germany). Plasmid DNA was extracted using the PurePlasmid 96 Kit (CoWin Biotech), and the plasmid concentration was measured using the Qubit system (Invitrogen). Since the sequences of the vector and PCR insert are known, copy numbers of transcribed *amoA* or *hzsB* genes were directly calculated according to the reported formula: copy numbers $\mu\text{l}^{-1} = (A \times 6.022 \times 10^{23}) \times (660 \times B)^{-1}$, where A is the plasmid concentration ($\text{g } \mu\text{l}^{-1}$), B is the recombinant plasmid length (bp) containing the *amoA* or *hzsB* fragment, 6.022×10^{23} is the Avogadro's number, and 660 is the average molecular weight of 1 bp [51]. For negative control, a similar procedure was performed using purified RNA to ensure that there was no genomic DNA contamination. After the qPCR assay, the specificity of amplification was verified by the generation of melting curves (in steps of 0.5°C for 5 s, with temperatures ranging from 60 to 95°C) and the qPCR product size and specificity were checked by 2% agarose gel electrophoresis.

Statistical Analysis

Data acquisition of the qPCR assay was performed using the 7500 System SDS Software Version 1.2 (Applied Biosystems). One-way analysis of variance (ANOVA) was performed to evaluate the abundance variations of the *amoA* or *hzsB* transcripts between *C. australiensis*, seawater, and sediment niches using the commands in SPSS 19.0. The thetaYC matrix distances based on the *amoA* or *hzsB* transcript sequences were calculated via Mothur commands and were visualized by the principal co-ordinate analysis (PCoA) in Canoco 5.0. Comparison between the *amoA* or *hzsB*-harboring communities from *C. australiensis*, seawater, and sediment niches were analyzed using the analysis of similarity statistics (ANOSIM) on thetaYC indices through Mothur. Statistical differences were determined at the level of $\alpha = 0.05$.

Results

Rarefaction Curves and Good's Coverage Values

PCR and sequencing result showed that AOA *amoA* was detected from *C. australiensis*, seawater, and sediment niches; parAOB *amoA* and AnAOB *hzsB* were revealed from *C. australiensis* and sediment niches, whereas comAOB *amoA* was only uncovered from sediment niches (Fig. S2). Rarefaction curve analysis showed that most of the *amoA* and *hzsB* transcript curves reach an asymptote based on 3% or 5% nucleotide sequence dissimilarity (Fig. S3), consistent with the Good's Coverage values ranging from 76.9% – 100% (Table S1). Therefore, enough *amoA* and *hzsB* transcript clones were sequenced to represent their diversity in corresponding clone libraries.

The phylotype sequences determined in this study were submitted to GenBank under accession numbers MT925778 - MT925797 (for the AOA *amoA* sequences), MT925728 - MT925741 (for the parAOB *amoA* sequences), MT925742 - MT925755 (for the comAOB *amoA* sequences), and MT925756-MT925777 (for the AnAOB *hzsB* sequences).

Community Compositions Of Active Aop

Most of the AOA *amoA* transcripts were taxonomically classified into the *Nitrosopumilus*, *Nitrosotenuis*, *Nitrososphaera*, *Nitrosopelagicus*, and *Cenarchaeum* taxa, and the remaining ones (7.3% of total) cannot gather into a definite taxon (Unclassified). As shown in Fig. 1A, AOA population in *C. australiensis* was composed of *Nitrosopumilus*, *Nitrosopelagicus*, and *Cenarchaeum*. Such a composition differed from that in sediment niches (consisting of *Nitrosopumilus*, *Cenarchaeum*, and Unclassified) and in seawater niches (consisting of *Nitrosopumilus*, *Nitrosotenuis*, *Nitrosopelagicus*, *Cenarchaeum*, and Unclassified). Similarly, parAOB or AnAOB compositions were different between *C. australiensis* and sediment niches. Thus, the parAOB population consisted of *Nitrosospira* in *C. australiensis* and *Nitrosospira* and *Nitrosomonas* in sediment niches (Fig. 1B); while the AnAOB population comprised *Kuenenia* and *Scalindua* in *C. australiensis* and *Brocadia*, *Scalindua*, and *Jettenia* in sediment niches (Fig. 1D). Moreover, *Nitrospira* Clade A and Clade B composed the comAOB population in sediment niches with Clade A taking a dominant proportion (95.6% of total) (Fig. 1C).

Community Dissimilarity Of Aop Communities Between Different Niches

PCoA based on the thetaYC matrix distance showed that AOA populations within *C. australiensis*, seawater, and sediment niches were clearly separated by axes PC1 (explaining 71.2% of the variation) and PC2 (explaining 18.3% of the variation) (Fig. 2A). Similarly, the parAOB or AnAOB populations from *C. australiensis* and sediment niches were clearly separated by axes PC1 (explaining 52.6% or 68.5% of the variation) and PC2 (explaining 16.8% or 20.4% of the variation) (Fig. 2B and C). ANOSIM results based on thetaYC indices showed that the AOA, parAOB, or AnAOB populations within *C. australiensis*, seawater, and sediment niches was significantly different from each other ($r = 0.812-0.639$, $p = 0.002-0.028 < 0.05$).

Phylogeny of *amoA* and *hzsB* Transcript Phylotypes

All the AOA *amoA* transcript phylotypes were most similar to the uncultured environmental sequences from sponges, seawaters, and sediments (Table S3) and phylogenetically fell into six clusters (Fig. 3). Basically, five phylotypes fell into the *Nitrosopumilus* cluster; these five phylotypes related to *Nitrosopumilus* spp. and the uncultured sequences from aquarium biofilter, marine sediments, and seawaters. One phylotype gathered into the *Nitrosotenuis* cluster and was similar to Ca. *Nitrosotenuis* spp. and the uncultured sequences from the Black Sea. Another phylotype falling into the *Nitrososphaera*

cluster was related to Ca. *Nitrososphaera* spp. and the uncultured sequence from marine sediment. Four phylotypes gathered into the *Nitrosopelagicus* cluster; these phylotypes were closely related to Ca. *Nitrosopelagicus brevis*, Crenarchaeote SCGC AAA288-J14, and the uncultured sequences from seawater and sponge. Another four phylotypes were ascribed into the *Cenarchaeum* cluster which were closely related to *Cenarchaeum symbiosum* and the uncultured sequences from seawater and sponge. Besides, the remaining four phylotypes gathered into the Unclassified cluster; these phylotypes were similar to the uncultured sequences from marine sediments and seawaters. Thus, a complex active AOA population was uncovered from *C. australiensis*, seawater, and sediment niches. Analysis of the Proportion of AOA amoA phylotypes in each niche showed that, The phylotypes accounting for the highest proportion in turn fell into the *Cenarchaeum*, *Nitrosopumilus*, and *Nitrosopelagicus* cluster in *C. australiensis*, sediment, and seawater niches, respectively.

The parAOB *amoA* transcript phylotypes which were most similar to the uncultured environmental sequences (Table S3) fell into the *Nitrospira* and *Nitrosomonas* clusters (Fig. 4A). Eleven phylotypes fell into the *Nitrospira* cluster and were mainly related to *Nitrospira* spp. and the uncultured sequences from various sponges and marine sediments; while the remaining three sediment-derived phylotypes gathered into the *Nitrosomonas* cluster and were related to *Nitrosomonas* spp. and the uncultured sequences from marine sediments and nitrifying granules (Fig. 4A). The phylotype accounting for the highest proportion in turn gathered into the *Nitrospira* and *Nitrosomonas* cluster in *C. australiensis* and sediment niches, respectively.

Specially, comAOB *amoA* transcripts were most similar to the uncultured environmental sequences from marine sediments and other niches (Table S3) and gathered into the *Nitrospira* Clade A and B clusters (Fig. 4B), according to their distinct divergence [4]. Clade A included 12 phylotypes which were closely related to the sequences from various types of sediments, wastewater treatment plant, biofilter sand, and the known comAOB species, i.e., Ca. *Nitrospira nitrificans*, Ca. *Nitrospira nitrosa*, Ca. *Nitrospira inopinata*, and *Nitrospira moscoviensis*. The Clade B containing two phylotypes were similar to the wetland derived sequences and the Ca. *Nitrospira* sp. comreactor17. Thus, a complex comAOB population with transcriptional activity inhabits the surface sediment adjacent to the sponge *C. australiensis*. The phylotype accounting for the highest proportion gathered into the *Nitrospira* Clade A cluster in sediment niches.

All the AnAOB *hzsB* transcript phylotypes were most similar to the uncultured environmental sequences from the South China Sea and other soil or sediment environments (Table S3) and fell into four clusters (Fig. 5). Briefly, the *Brocadia* cluster including seven phylotypes were related to the sequences from granular sludge and different types of wetlands. The *Jettenia* cluster included one phylotype related to the sequence from paddy soil. Two phylotype fell into the *Kuenenia* cluster and were similar to the sequence from South China Sea sediment, wastewater treatment plant, and Ca. *Kuenenia stuttgartiensis*; while the *Scalindua* cluster included 12 phylotypes which were closely related to Ca. *Scalindua rubra* and the South China Sea sediment-derived sequences. The phylotypes accounting for the highest proportion in turn gathered into the *Scalindua* and *Brocadia* cluster in *C. australiensis* and sediment niches,

respectively. Taken together, the *amoA* and *hzsB* transcript phylotypes fell into different clusters and were clustered with the uncultured environmental sequences from marine sponges, seawaters, sediments, and other aquatic niches.

Quantification of *amoA* and *hzsB* Transcripts

Quantitative surveys showed that (Fig. 6 and Table S3), transcript abundances of AOA *amoA* > parAOB *amoA* > AnAOB *hzsB* in *C. australiensis* niches ($r = 0.894-0.625$, $p = 0.001-0.035$) while, transcript abundances of AOA *amoA* > parAOB *amoA* > comAOB *amoA* and AnAOB *hzsB* in sediment niches ($r = 0.792-0.584$, $p = 0.008-0.042$). In seawater, copy of AOA *amoA* transcripts was $(8.73 \pm 0.22) \times 10^7$ copies ng^{-1} seawater RNA, whereas other AOP type were below the limit of PCR detection. Thus, transcript abundance of AOA *amoA*, ParAOB *amoA*, ComAOB *amoA*, and AnAOB *hzsB* varied in *C. australiensis*, ambient seawater, or sediment niches.

Discussion

An active AOP community composing of AOA, parAOB, and AnAOB was revealed in the sponge *C. australiensis*. Such a AOP community was significantly different from that in ambient seawater (consisting of AOA) and sediment niches (consisting of AOA, parAOB, comAOB, and AnAOB). Such a finding was consistent with previous studies that microorganism compositions were significantly varied between sponges, environmental seawaters, and sediments [43, 52]. Therefore, marine sponges may have eco-physiological preferences for specific microbes distinctive from ambient seawater and sediment niches [53].

AOA are ubiquitous and significant contributors to nitrogen cycling in marine environments [54]. *Nitrosopumilus*, *Nitrosopelagicus*, *Nitrosotenuis*, *Nitrososphaera*, and *Cenarchaeum* AOA were detected (Fig. 1). The active AOA population in *C. australiensis* mainly consisting of *Nitrosopumilus*, *Nitrosopelagicus*, and *Cenarchaeum* differed from that in sediment niches (consisting of *Nitrosopumilus*, *Cenarchaeum*) and in seawater niches (consisting of *Nitrosopumilus*, *Nitrosotenuis*, *Nitrosopelagicus*, *Cenarchaeum*) (Figs. 1A and 2A). Generally, there is an obvious niche differentiation of AOA population between *C. australiensis*, and their ambient seawater and sediment niches. Similar condition has been found in another case that the South China Sea sponges harbored an AOA population (*Nitrosopelagicus* and *Cenarchaeum*) differed from that in ambient seawaters (*Nitrosopumilus*) [19]. Among the detected AOA lineages, *Nitrosopumilus* was detected from *C. australiensis*, seawater, and sediment niches (Fig. 1A), in agreement with previous findings that *Nitrosopumilus* is prevalent in ammonia oxidation in marine sponges (Fig. S4), seawaters, and sediments [19, 55, 56]. *Nitrosopelagicus* and *Cenarchaeum* were detected from *C. australiensis* and seawater niches (Fig. 1A), consonant with former reports that these two AOA lineages are commonly found in various sponges (Fig. S4) and seawaters [24, 57]. *Nitrosotenuis* and *Nitrososphaera* were detected from seawater and sediment niches, respectively (Fig. 1A), consistent with previous revelations that *Nitrosotenuis* can be found widely distributed in seawaters [58] while *Nitrososphaera* represents an AOA genus frequently found in marine sediments [59].

Generally, there is an obvious niche differentiation of putative active AOA population among *C. australiensis*, ambient seawater, and sediment niches.

The parAOB also contribute to ammonia oxidation in marine environments [60]. The parAOB population in *C. australiensis* comprised of *Nitrosospira* distinctive from that in sediment niches consisting of *Nitrosospira* and *Nitrosomonas* (Figs. 1B and 2B). Conclusion of the reported sponge-associated parAOB *amoA* sequences showed that, *Nitrosospira amoA* sequences were reported in diverse sponges (Fig. S5), while *Nitrosomonas amoA* homologues were only uncovered from few sponges, e.g., *Neamphius huxleyi* and *Placospongia* sp. (Fig. S5), indicating that *Nitrosospira* rather than *Nitrosomonas* may the dominate the parAOB population in sponges. Many studies also demonstrated that *Nitrosomonas* and *Nitrosospira* parAOB have also been uncovered from marine sediments [61–63], in agreement with our finding (Fig. 1). Whereas the negative detection of parAOB in seawaters different from previous findings that parAOB residence in seawaters [17, 64, 65]. Therefore, the presence of parAOB in marine environments may be varied in different geographic areas and a specific niche, e.g., sponge, seawater, or sediment niche may harbor a specific parAOB population.

A comAOB population composed of *Nitrospira* Clade A and B lineages (Fig. 3) was detected in sediments (Fig. 1C and Fig. S2). Such a comAOB *amoA* population composition were also found in marine coastal waters and sediments [31–33, 66, 67] and river waters and sediments [68]. Such a comAOB population differs from that in eutrophic lake sediments [69] and river tidal flat sediments [31] where only *Nitrospira* clade A were detected. The comAOB *amoA* was undetectable in seawaters, in agreement with the findings in former studies [4, 70]. Besides, no comAOB *amoA* fragment was detected from *C. australiensis*, and no reports have confirmed the presence of comAOB in sponges so far. Therefore, whether the comAOB resident in sponges needs further investigation. These findings indicate the niche differentiation of comAOB in different environments.

AnAOB greatly contributes to marine nitrogen removal from the oceans [71]. The AnAOB population in *C. australiensis* niches comprised of *Kuenenia* and *Scalindua* (Fig. 1) which was different from the *Brocadia* AnAOB population in the sponge *Mycale laxissima* [20]. Moreover, AnAOB population in sediment niches were divergent from that in *C. australiensis* niches (Fig. 2C) and consisted of *Brocadia*, *Jettenia*, *Kuenenia*, and *Scalindua* (Fig. 1D), consistent with that in China's coastal wetlands [72], but different from that in surface sediments of the Bohai Sea [73] and north marginal seas of China [74] which was composed of *Scalindua*, *Jettenia*, and *Anammoxoglobus*. Conclusively, the ecological niche segregation of AnAOB was prevalent in marine environments, such as in sponges, seawaters, and sediments.

Quantitative surveys of *amoA* or *hzsB* transcripts demonstrated that AOA *amoA* transcripts were either exclusively detectable or were higher in abundance than parAOB *amoA*, comAOB *amoA* and AnAOB *hzsB* transcripts by orders of magnitude in *C. australiensis*, seawater, and sediment niches (Fig. 6). Such a finding indicated the dominant transcriptional expression of AOA in these niches. Similar condition has been revealed from the cold-water sponges where higher *amoA* transcripts abundance of AOA than that of parAOB were detected [12]. Based on the nitrification kinetics of pure cultures, ammonia affinity of

ammonia-oxidizing species ranking from high to low is marine AOA, comAOB, terrestrial AOA, and parAOB [75]. A higher ammonia affinity of AOA would contribute to their survival in the oligotrophic ocean. Meanwhile, although comAOB showed a higher ammonia affinity than parAOB, both parAOB and comAOB were uncovered in sediment niches, while parAOB rather than comAOB were uncovered in *C. australiensis*. Therefore, besides ammonia contents, other environmental factors may also influence the residence of comAOB in sponges, which need further investigation.

Generally, microbially mediated ammonia oxidation process by phylogenetically divergent AOP lineages in the sponge *C. australiensis*, ambient seawater, and sediment niches. Certainly, some aspects need further study. For example, the comAOB *amoA* sequence was not detected from *C. australiensis*. Therefore, more sponge species from distant geographic scales over different sampling timepoints can be investigated to verify the residence of comAOB in sponges. Additionally, the investigated *C. australiensis*, ambient seawater, and sediment niches were only collected at one timepoint rather than serial timepoints. Temporal change in gene expression is possible, such as the AOA and parAOB *amoA* transcripts in coastal sediments [76]. Therefore, more data from different sponges in time series will give us a better insight into the ammonia oxidation and other nitrogen metabolic processes.

Conclusion

In this study, the community structure and abundance of active ammonia-oxidizing microbiota in the sponge *C. australiensis*, ambient seawaters, and sediments were investigated using transcript-based strategies. Active AOP community selectively consisting of AOA, parAOB, comAOB, or AnAOB was uncovered inhabiting *C. australiensis*, seawater, and sediment niches. The AOP community in *C. australiensis* was significantly different from that in ambient seawater and sediment niches. AOA *amoA* transcripts were exclusive or higher in abundance than parAOB *amoA*, comAOB *amoA*, or AnAOB *hzsB* transcripts by orders of magnitude in each niche type. This study indicates the obvious divergence of AOP population structures and significant variations of AOP transcriptional expressions among *C. australiensis*, ambient seawater, and sediment niches. This research would extend our understanding of the putative metabolic activity and ecological functions of the ammonia-oxidizing microbiota within marine sponges and the ambient environments.

Declarations

Author contribution

Study conception and design: FG, SL, LZ, and HT; acquisition of data: FG, SL, LZ, and HT; bioinformatic analysis: FG and SL; analysis and interpretation of data: FG; drafting of manuscript: FG, SL, LZ, and HT; critical revision: FG, SL, LZ, and HT.

Conflict of interest The authors declare no competing interests.

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Tables

Table 1 Primers for PCR amplification of *amoA* and *hzsB* gene fragments

Primer	Sequence (5'-3')	Length (bp)	Anneal. Temp. (°C)	Operation	Reference
AOA <i>amoA</i>					
ArchamoA 23F	ATGGTCTGGYTWAGACG	624	58	PCR	[14]
ArchamoA 616R	GCCATCCABCKRTANGTCCA				
amo196F	GGWGTKCCRGGRACWGCMAC	103	55	qPCR	[16]
amo277R	CRATGAAGTCRTAHGGRTADCC				
ParAOB <i>amoA</i>					
BacamoA 1F	GGGGTTTCTACTGGTGGT	491	60	PCR, qPCR	[15]
BacamoA 2R	CCCCTCKGSAAAGCCTTCTTC				
ComAOB <i>amoA</i>					
comamoA F	AGGNGAYTGGGAYTTCTGG	436	62	PCR, qPCR	[30]
comamoA R	CGGACAWABRTGAABCCCAT				
AnAOB <i>hzsB</i>					
hzsB364f-	AGCTGGAAGAAYRYRAAGTAYAT	450	55	PCR, qPCR	[36]
hzsB790r	CCSGTYCCHACRTCATGVGTCTG				

Figures

Figure 1

Population composition of AOA, parAOB, comAOB, and AnAOB in *C. australiensis*, seawater and sediment niches based on the taxonomic classification of *amoA* and *hzsB* transcript sequences.

A

B

C

Figure 2

PCoA of the AOA (A), parAOB (B), and AnAOB (C) populations based on the amoA or hzsB transcript phylotypes retrieved from *C. australiensis*, seawater and sediment niches from three sampling sites.

Figure 3

Phylogenetic maximum-likelihood tree of AOA amoA transcript phylotypes retrieved from the sponge *C. australiensis*, ambient seawater, and sediment niches. Scale bar represents 20% sequence divergence per homologous position. Bootstrap values more than 50% of 1000 replicates are shown. Phylotypes retrieved from *C. australiensis*, seawater and sediment niches were marked with purple, blue, red, and yellow, respectively. The outgroup symbolized by an arrow represented the amoA sequence of *Nitrosomonas europaea* ATCC 19178 (JN099309). AOA, ammonia-oxidizing archaea

Figure 4

Phylogenetic maximum-likelihood tree of parAOB amoA (A) and comAOB amoA (B) transcript phylotypes retrieved from the sponge *C. australiensis*, ambient seawater, and sediment niches. Scale bar represents 10% sequence divergence per homologous position. Bootstrap values more than 50% of 1000 replicates are shown. Phylotypes retrieved from *C. australiensis*, and sediment niches were marked with blue and yellow, respectively. The outgroup symbolized by an arrow represented the amoA sequence of *Ca*.

Nitrosocosmicus arcticus (MK978767) (A) or by an arrow represented the pmoA sequence of Crenothrix polyspora (DQ295902) (B). parAOB, partial-nitrification ammonia-oxidizing bacteria; comAOB, complete-nitrification ammonia-oxidizing bacteria

Figure 5

Phylogenetic maximum-likelihood tree of AnAOB hzsB transcript phylotypes retrieved from the sponge *C. australiensis*, ambient seawater, and sediment niches. Scale bar represents 5% sequence divergence per homologous position. Bootstrap values more than 50% of 1000 replicates are shown. Phylotypes retrieved from *C. australiensis*, seawater and sediment niches were marked with blue and yellow, respectively. The outgroup symbolized by an arrow represented the hzsB sequence of Planctomycetes bacterium (A3D13_04020). AnAOB, anaerobic ammonia-oxidizing bacteria

Figure 6

Abundance of transcribed *amoA* and *hzsB* genes estimated by qPCR for the sponge *C. australiensis*, seawater and sediment niches. The depicted values are the means of sample triplicates and technical triplicates in each site, and the error bars indicate the standard error. The * symbol indicated the significant difference of transcript abundance ($p < 0.05$)

Supplementary Files

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