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Diversity of bacterial communities related to the nitrogen cycle in a coastal tropical bay

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Abstract A culture-independent molecular phylogenetic analysis was carried out to study for the first time the diversity of bacterial ammonia monooxygenase subunit A (*amoA)* and nitrogenase reductase subunit H (*nifH*) genes from Urca inlet at Guanabara Bay in Rio de Janeiro, Brazil. Most bacterial *amoA* and *nifH* sequences exhibited identities of less than 95% to those in the GenBank database revealing that novel ammonia-oxidizing bacteria and nitrogen-fixing microorganisms may exist in this tropical marine environment. The observation of a large number of clones related to uncultured bacteria also indicates the necessity to describe these microorganisms and to develop new cultivation methodologies.

Keywords Marine ecology · Marine microorganisms · Nitrogen cycle · Guanabara bay

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Introduction

Culture independent molecular studies based on 16S rRNA sequences have contributed to increase the knowledge of prokaryotic diversity and biogeography. Bacterial communities have a high degree of endemism at global level, presenting marked differences in community composition that vary with the location and latitudinal gradient [1]. Genes that encode key metabolic enzymes such as the *amoA* and *nifH* have also been used as alternative targets for the determination of ammonia-oxidizing bacteria (AOB) and nitrogen-fixing (N-fixing) microorganisms, contributing to a better understanding of the distribution of prokaryotes and the nitrogen cycle [2]. As they are directly involved in metabolic processes, they provide new information about the physiology and ecology of these groups. Albeit being abundant and productive components of the plankton, little is known about microbial estuarine diversity and metabolic processes in tropical regions [3, 4].

Nitrogen is one of the most important elements in aquatic ecosystems and is used in high abundance by all living organisms. This nutrient is introduced into aquatic environments by continental inputs, rainfall, diffusion from the sediments, and by microbiological fixation. Low concentrations of inorganic nitrogen affect aquatic primary productivity and hence, the whole trophic web [5].

Several steps, such as N_2-fixation and nitrification, constitute the nitrogen cycle. In nature, a small group of

diazotrophic microorganisms have the capacity to reduce atmospheric nitrogen to ammonia. These microbes have a nitrogenase protein complex, encoded by the *nifHDK* genes, which is relatively conserved in structure and function [6]. Nitrification is mainly mediated by two distinct groups of chemolithoautotrophic microorganisms: the AOB and the nitrite-oxidizing bacteria (NOB). Two enzymes are involved in the ammonia oxidation process: ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) The AMO enzyme is constituted by 3 subunits encoded by the functional operon *amo*. The *amoA* gene encodes the active site of the enzyme [7].

Nitrifying activity has been reported for several estuarine ecosystems [8] while N_2-fixation is rarely detected [9]. In spite of that, distinct *nifH* genotypes have been found in other bay systems [10] pointing out to the relevance of diazotrophic activity in estuaries. We have previously provided details about the environmental chemistry, phylogeny, and distribution of the bacterial [11] and archaeal [12] communities present in Guanabara Bay, where few sequences related to nitrifying and N-fixing organisms were obtained probably because they were below the detection limit of our 16S rRNA gene analyses.

In the present study, we reveal the AOB and N-fixing bacteria diversity in an oceanic site near the entrance of Guanabara bay using marker genes (*amoA* and *nifH*). Urca inlet (UR) is situated at the vicinity of the bay's entrance, and is supplied by relatively pristine coastal seawater. This site is characterized by low levels of ammonia and nitrite and by high concentrations of nitrate promoted by an elevated tidal influence and by low inputs from rivers, clandestine effluents and drainage-basin runoff discharges [15]. Knowledge about how these microbial groups may be linked to environmental conditions in Guanabara Bay is important to determine changes in environmental processes and to better understand microbial loops and the role of local nitrogen transformation.

Materials and methods

Sampling

Surface water samples (0.5 m) from UR were collected with a 3 l Van Dorn bottle, transferred to sterile polypropylene flasks, and kept on ice in the dark until taken to the laboratory.

amoA and *nifH* genes clones libraries construction

DNAs were prepared after water filtration (1 l) through a 3.0 μm cellulose Millipore membrane and subsequent concentration on a 0.22 μm Millipore Sterivex-filter as previously described [12]. PCR was performed in 50 μl reaction mixtures (2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 50 pmol of each primer, 2.5 U of *Taq* DNA polymerase [Promega Corporation, USA], 1× PCR buffer and 200 ng of environmental DNA sample, using the *amoA*-1f (5′-GGGGTTTCTACTGGTGGT-3′) and *amoA*-2r (5′-CCCCTCKGSAAAGCCTTCTTC-3′) [7] primers for AOB of the beta-Proteobacteria subclass, and *nifH*f (5′-GGHAARGGHGGHATHGGNAARTC-3′) and *nifH*r (5′–GGCATNGCRAANCCVCCRCANAC-3′) [29] for N-fixing bacteria.

Amplification of the *amoA* target sequences was as follows: one cycle at 94°C for 3 min, 32 cycles consisting of 94°C for 1 min, 55°C for 1 min, and 72°C for 50 s, and a final cycle at 72°C for 10 min. A positive control containing DNA from *Nitrosomonas europaea* was included in the amplification set. The template DNA (200 ng) for *nifH* amplification was denatured at 94°C for 2 min, followed by 35 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. A final extension step was carried out at 72°C for 10 min. Positive controls were obtained with *Rhizobium meliloti*, *Agrobacterium thumefaciens*, *Klebsiella pneumonia,* and *Bradrhizobium japonicum* DNAs. Negative controls for *amoA* and *nifH* PCR amplifications were made using DNA from an *Escherichia coli* strain.

PCR products were concentrated and purified with a GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, UK) after electrophoresis on a 1% (w/v) agarose gel. PCR products were cloned into the pGEM-T cloning vector (Promega Corporation, USA) and used to transform *Escherichia coli* DH10B competent cells. Positive colonies were picked and frozen at −70°C. Two representative clone libraries were constructed for *amoA* and *nifH* genes from the UR sample. We sequenced 96 *amoA* and 96 *nifH* clones and, for that, DNA from each clone (400 ng) was prepared and PCR cycle-sequencing reactions were carried out with *amoA*-1f and *nifH*f primers for AOB and N-fixing bacteria, respectively. Partial *nifH* and *amoA* sequences were obtained by capillary electrophoresis on a MegaBace1000 platform using the DYEnamic ET terminator cycle-sequencing kit (GE Healthcare, UK).

Valid sequences, with Phred score ≥20, were compared with sequences in GenBank by BLAST searches. The AOB and N-fixing bacterial sequences were deposited under the accession numbers EF468353 to EF468421 and EF468422 to EF468459, respectively. Bacterial sequences from each clone library were aligned and clustered as Operational Taxonomic Units (OTUs) at an overlap identity cutoff of 99, 97, 95 or 80% stringency by using DOTUR software [30]. Phylogenetic trees were constructed by the Maximum-likelihood method based on distance estimates calculated by Kimura-2 algorithm. Tree construction was

performed with MEGA2 program version 2.1, and bootstrap analysis was performed with 1000 replications [12].

Results and discussion

Ammonia monooxygenase gene diversity

Phylogenetic analysis of *amoA* gene showed that the Guanabara Bay sequences are not related to any cultured AOB species (Fig. 1). All sequences were most closely related (bootstrap support 85) to medium and deep, October samples from Monterey Bay, belonging to clades referred to as A and C in analysis by Mullan and Ward [16]. It has been hypothesized that Monterey Bay *amoA* clades A, B and C might correspond to the *Nitrosospira*-like 16S group I. Our tree suggests that whereas *amoA* clades A and C might indeed be related, the relationship to *Nitrosospira*, or any other cultivated AOB species, is unresolved. These sequences might represent a novel AOB species, not present among cultivated representatives. More sequences and phylogenies are necessary to resolve its placement. Neighbor-joining trees suggested one well-supported clade (96 bootstrap) but the same sequences did not cluster in the maximum likelihood tree and their long branches might suggest their grouping is an artifact. Despite the fact that some members of AOB have low survival in saline waters and are predominantly found in freshwater ecosystems they have been observed in several marine and estuarine samples [17–19]. Interestingly, all UR *amoA* sequences were strongly related to the uncultured environmental clones obtained in Monterey Bay, a semi-protected bay located at the central Californian coast of the Pacific Ocean [16]. This bay is influenced by the cold California current while the Monterey Submarine Canyon provides upwelling nutrient-rich water during some seasons of the year [20].

The sample used for library constructions was collected in October and, at Rio de Janeiro, the upwelling phenomenon is normally observed from September to March [21]. This phenomenon occurs at the Cabo Frio coastal region and is induced by Northeast winds that predominate in the summer season. The Cabo Frio upwelling source is the South Atlantic Central Water (SACW), found at 200 m depth and characterized by low temperatures and high nutrient levels [22]. Guanabara Bay is strongly influenced by the Cabo Frio upwelling [23]. The occurrence of similar sequences in geographically distinct areas such as Monterey and Guanabara Bays suggests that a similar environmental factor related to upwelling could be regulating the AOB community in these ecosystems. The presence of similar clones in both ecosystems also suggests that they could have originated in a dominant pool of oceanic ammonia-oxidizing microorganisms.

The *amoA* library from Monterey Bay was dominated by clones belonging to the *Nitrosospira* group [16] which represents one of the most important planktonic AOB clades in estuarine and marine environments. Sequences related to *Nitrosospira*, for instance, dominated the 16S rRNA and *amoA* clone libraries from the oxygen minimum zone of the Eastern South Pacific off the Northern Chilean coastline [24]. Uncultivated AOB of the *Nitrosospira* group also represented 50% of the total *amoA* diversity found in Chesapeake Bay [25].

Nitrogenase reductase gene diversity

The *nifH* gene library showed a clear structure with the sequences belonging to three noticeably distinct clusters (Fig. 2). Cluster B comprised 59.46% of the sequences, which were related to uncultured clones obtained at Lake Michigan [26]. Cluster C, represented by one sequence was most closely related to a clone from Monterey Bay. Cluster A was composed by 37.84% of the sequences. There is a well-supported (100 bootstrap) clade of 7 clones which had some evidence for clustering with an uncultured bacteria found in oligotrophic oceanic samples. There is weak evidence (59 bootstrap support) that three clones might be related to *Arcobacter nitrofigilis* and also some evidence (81 bootstrap) that another 4 are related to *Sulfurospirillum multivoran*, both of the cultured species affiliated to the epsilon-Proteobacteria group. The presence of putative N-fixing microorganisms in Guanabara Bay 16S rRNA clone libraries was, therefore, supported by our *nifH* gene analysis. Several sequences related to uncultured clones were also observed in *nifH* gene library. As described in Vieira et al. [11], sequences related to *Arcobacter nitrofigilis*, a free-living N-fixing epsilon-Proteobacteria, were found. Although normally found in nitrogen-rich ecosystems [10] there is little information on the spatial distribution of *A. nitrofigilis*. Its role in nutrient cycling is still not well defined, but actually it is known that N-fixation is not always inhibited by nitrate [27], the predominant nitrogen form at UR site. In addition, the occurrence of microorganisms with N-fixing capacity in nitrogen rich environments does not necessarily mean that they are performing the process [28]. The presence of the *nifH* gene was detected in Chesapeake Bay even though N-fixation activity was not, suggesting that its expression may be regulated by some environmental factor, such as, for example, high nitrogen availability [27]. Like Monterey Bay [16], Guanabara Bay presents different levels of complexity regarding AOB and N-fixing bacterial communities. In spite of that, differences were observed when

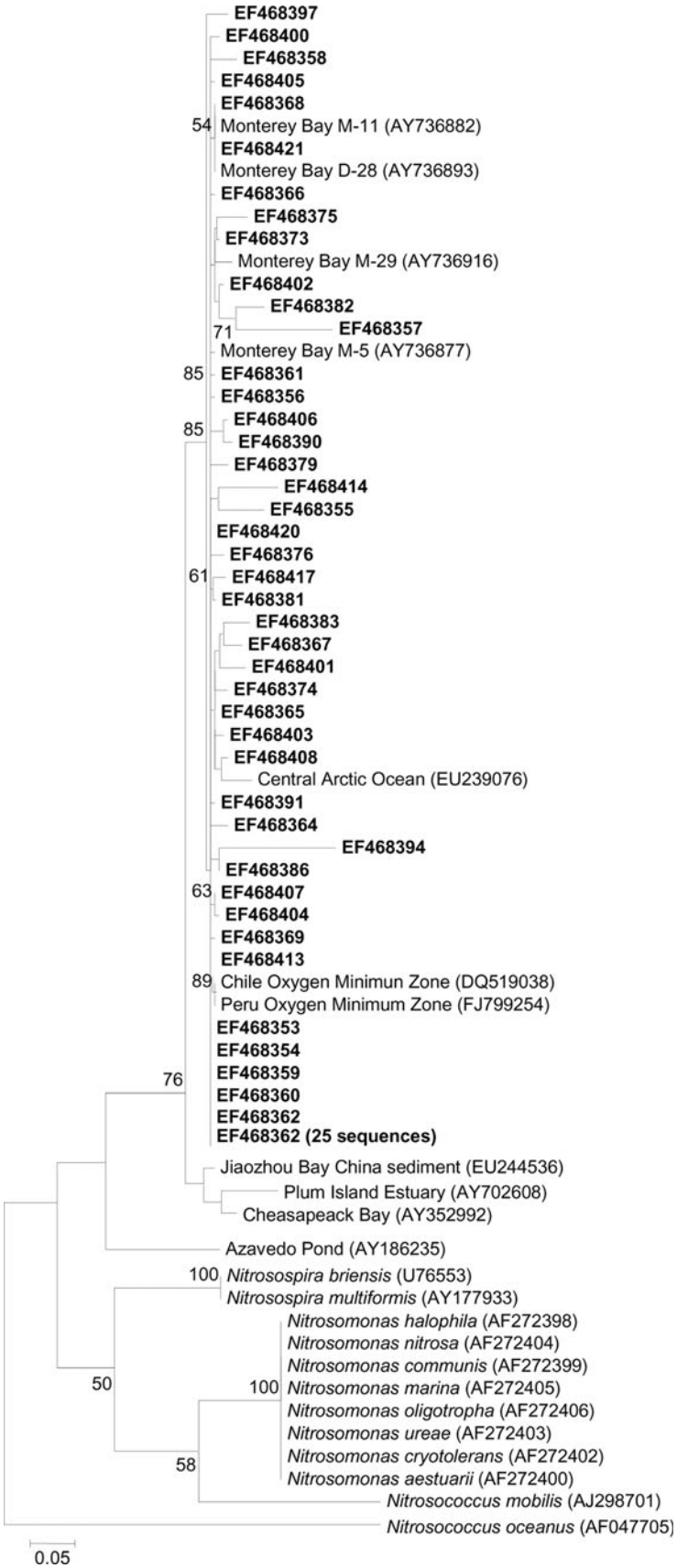

Fig. 1 Maximum-likelihood phylogenetic tree of *amoA* gene clones obtained at Guanabara Bay. Genbank accession numbers are shown for sequences from other studies. Sequences from this study are in bold. The tree is outgroup rooted with *Nitrosococcus oceanus* (AF047705). Bootstrap values lower than 50% are omitted

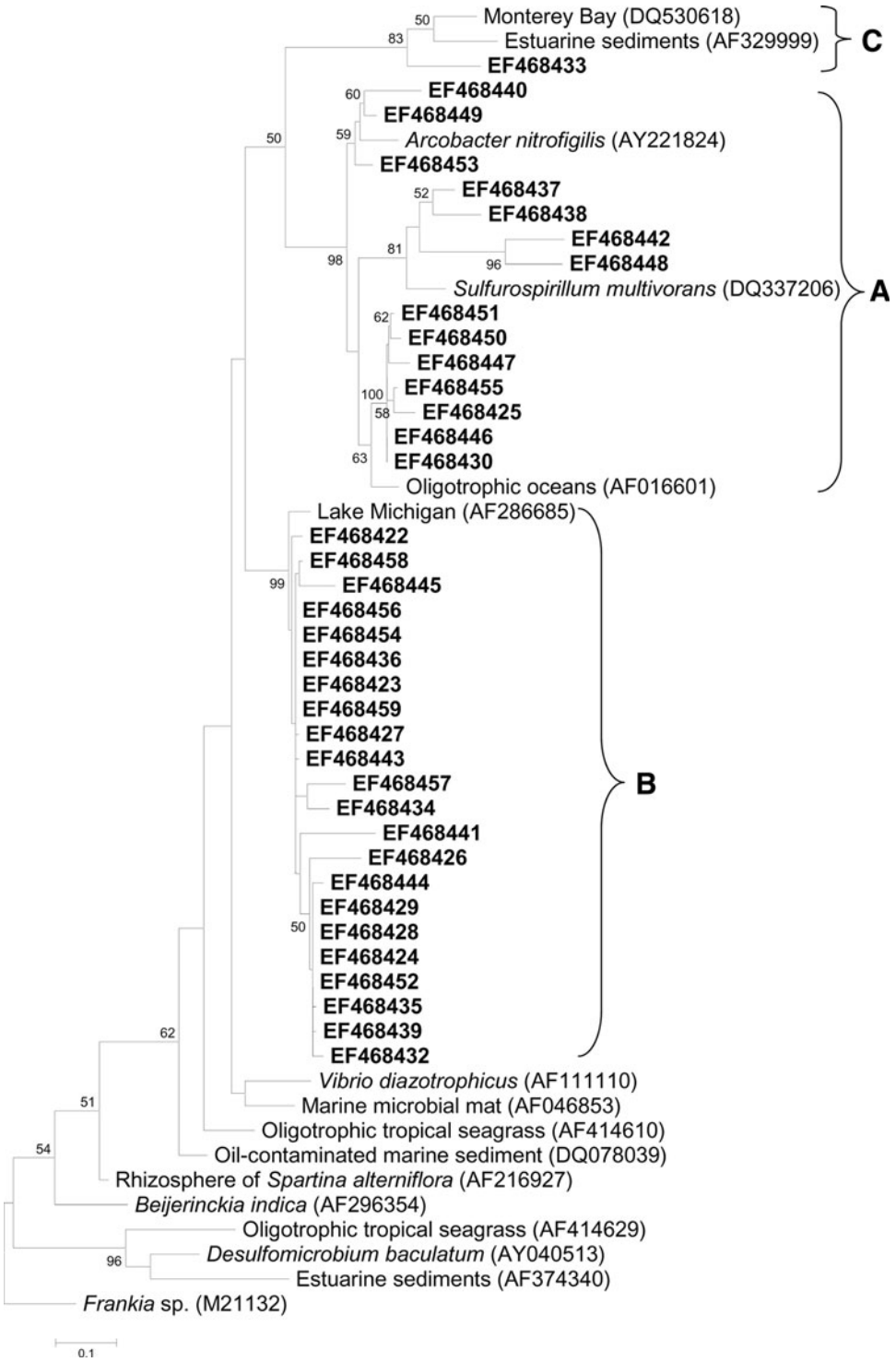

Fig. 2 Maximum-likelihood phylogenetic tree of *nifH* gene clones obtained at Guanabara Bay. Genbank accession numbers are shown for sequences from other studies. Sequences from this study are in bold. The tree is outgroup rooted with *Frankia* sp. (M21132). Bootstrap values lower than 50% are omitted

we compared the bacterial community diversities of both environments.

Future studies

Nitrogen biogeochemical cycles are mediated by several microbiological processes involving many genes and metabolic routes distributed in different microbial species. In this paper, we provide baseline information about AOB and N-fixing bacterial diversity in UR, an unexplored tropical environmental area in Guanabara Bay. Our analysis, however, did not include *amoA* and *nifH* gene expression and the presence of these genes does not necessarily indicate N-fixing or nitrifying activity. Thus, a

more detailed evaluation is necessary for a better comprehension of Guanabara Bay's nitrification potential and N-fixing rates as well as its microbial diversity. Biogeochemical activity measurements related to gene expression and abundance analyses should be used in future works to investigate the distribution of nitrifiers and N-fixing microorganisms on Guanabara Bay and to determine their environmental control factors. Studies on nutrient dynamics, mainly the nitrogenous compounds, are of great importance to determine the pollution impact on the Guanabara Bay ecosystem as well as to verify its self-cleaning potential. The molecular biology approach utilized in this work and the sequences obtained can be applied in future studies on the seasonal distribution of AOB and N-fixing microorganisms in this tropical area. The observation of a large number of clones related to uncultured bacteria also indicates the necessity to develop new cultivation methodologies to describe these novel microorganisms. Recently, a marine Crenarchaeota capable of nitrification was isolated [31]. The number of Crenarchaeota species that are functional ammonia oxidizers is unknown, but their high abundance in the oceans underpins their importance in global biogeochemical cycles. Upcoming studies about *amoA* marker genes in Guanabara Bay should also include investigation of Archaea species related to ammonia oxidation to evaluate their contribution for the nitrifying community diversity of this environment. In summary, as this urban aquatic estuary is submitted to accelerated degradation processes and presents different levels of water quality [13] and complex microbial metabolism [14], it can be used as an environmental model for nitrogen cycle studies in tropical regions.

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