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Competition between Ammonia-Oxidizing Archaea and Bacteria from Freshwater Environments

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ABSTRACT In the environment, nutrients are rarely available in a constant supply. Therefore, microorganisms require strategies to compete for limiting nutrients. In freshwater systems, ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) compete with heterotrophic bacteria, photosynthetic microorganisms, and each other for ammonium, which AOA and AOB utilize as their sole source of energy and nitrogen. We investigated the competition between highly enriched cultures of AOA (AOA-AC1) and AOB (AOB-G5-7) for ammonium. Based on the *amoA* gene, the newly enriched archaeal ammonia oxidizer in AOA-AC1 was closely related to *Nitrosotenuis* spp., and the bacterial ammonia oxidizer in AOB-G5-7, *Nitrosomonas* sp. strain Is79, belonged to the *Nitrosomonas oligotropha* group (*Nitrosomonas* cluster 6a). Growth experiments in batch cultures showed that AOB-G5-7 had higher growth rates than AOA-AC1 at higher ammonium concentrations. During chemostat competition experiments under ammonium-limiting conditions, AOA-AC1 dominated the cultures, while AOB-G5-7 decreased in abundance. In batch cultures, the outcome of the competition between AOA and AOB was determined by the initial ammonium concentrations. AOA-AC1 was the dominant ammonia oxidizer at an initial ammonium concentration of 50 μM, and AOB-G5-7 was dominant at 500 μM. These findings indicate that during direct competition, AOA-AC1 was able to use ammonium that was unavailable to AOB-G5-7, while AOB-G5-7 dominated at higher ammonium concentrations. The results are in strong accordance with environmental survey data suggesting that AOA are mainly responsible for ammonia oxidation under more oligotrophic conditions, whereas AOB dominate under eutrophic conditions.

IMPORTANCE Nitrification is an important process in the global nitrogen cycle. The first step, ammonia oxidation to nitrite, can be carried out by ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB). In many natural environments, these ammonia oxidizers coexist. Therefore, it is important to understand the population dynamics in response to increasing ammonium concentrations. Here, we study the competition between AOA and AOB enriched from freshwater systems. The results demonstrate that AOA are more abundant in systems with low ammonium availabilities and that AOB are more abundant when the ammonium availability increases. These results will help to predict potential shifts in the community composition of ammonia oxidizers in the environment due to changes in ammonium availability.

KEYWORDS ammonia oxidation, ammonia-oxidizing archaea, ammonia-oxidizing bacteria, competition, freshwater, nitrification

Nitrification, the oxidation of ammonia to nitrate via nitrite, is an important and central process in the global nitrogen cycle (1). The first step of nitrification, the oxidation of ammonia to nitrite, is performed by ammonia-oxidizing bacteria (AOB), which have been grown in laboratory cultures for many decades (2–4), and ammonia-oxidizing archaea (AOA), which were discovered in 2005 (5, 6). Both groups use chemolithotrophic

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ammonia oxidation as the sole energy-generating process and fix carbon dioxide autotrophically via either the Calvin cycle (AOB) or a modified version of the 3-hydroxypropionate/4-hydroxybutyrate cycle (AOA) (3, 4, 6–8). Nitrite oxidation, the second step of nitrification, is attributed to nitrite-oxidizing bacteria (NOB), a group of bacteria belonging to diverse phyla (9, 10). In natural systems, ammonia oxidizers and NOB often exist in physical proximity, converting ammonia directly to nitrate (11), with ammonia oxidation usually being the rate-limiting step. A few years ago, the ability for the complete oxidation of ammonia to nitrate (Comammox) was discovered in members of the phylum *Nitrospira* (12, 13).

Ammonium availability is a major factor controlling activity and community composition (6, 14–16). Ammonium concentrations in aquatic environments can range from 0.03 to 0.5 μM in oligotrophic environments such as Lake Superior and the open ocean and can be up to 100 μM in eutrophic environments such as Lake Erie and Lake Taihu (17–20). Raw wastewater coming into wastewater treatment plants can even have 1 to 3 mM ammonium (21).

AOA and AOB differ in their affinities for ammonium. The affinity of AOA ranges from as low as 0.003 μM NH_3 ($\sim$0.1 μM NH_3 plus NH_4^+) for *Nitrosopumilus maritimus* and *Nitrosoarchaeum koreensis* MY1 to up to 5 μM NH_3 ($\sim$40 μM NH_3 plus NH_4^+) in "*Candidatus* Nitrosotenuis uzonensis" (6, 22, 23). The affinity of AOB for ammonia spans from 3 μM ($\sim$150 μM NH_3 plus NH_4^+) in *Nitrosomonas* sp. strain Is79 and *Nitrosospira briensis* to more than 60 μM ammonia ($\sim$3 mM NH_3 plus NH_4^+) in members of the *Nitrosomonas eutropha* cluster (24–26). Microorganisms with a high affinity for their energy substrate have the ability to successfully compete with other microorganisms for the limiting substrate (27). The competition for ammonium among AOB, heterotrophs, and plants has been investigated in detail (28–33). *Nitrosomonas europaea* is a poor competitor for ammonium in comparison to the heterotrophic bacteria *Arthrobacter globiformis* and *Thiosphaera pantotropha* and the AOB *Nitrosomonas* sp. Is79 from the enrichment culture AOB-G5-7 (28, 32, 33).

The differences in affinity for ammonium within and between AOA and AOB indicate that ammonium is an important factor in the niche differentiation of ammonia oxidizers. However, direct competition of AOA and AOB for ammonium has not been investigated in detail. Here, we present a study investigating the competition between AOA and AOB enriched from freshwater systems for ammonium. Competition was tested under ammonium-limiting conditions in continuous cultures and at elevated ammonium concentrations in batch cultures. The experiments were conducted with the AOB *Nitrosomonas* sp. Is79 in the enrichment culture AOB-G5-7 and the newly enriched AOA culture AOA-AC1 with an AOA belonging to the *Nitrosotenuis* group. AOA-AC1 was enriched from freshwater sediment and is briefly described here.

RESULTS

Enrichment of freshwater AOA and phylogenetic affiliation of the enrichment culture. The enrichment culture AOA-AC1 was obtained from Lake Acton under autotrophic conditions with ammonia as the electron donor in the medium. The culture oxidized ammonium to nitrate stoichiometrically, indicating the presence of a nitrite oxidizer. Based on the *amoA* sequence, the enrichment culture belongs to *Thaumarchaeota-Nitrosopumilales* group I.1.a and within the group to clade NP-η, genus *Nitrosotenuis*, a genus dominated by freshwater and hot spring sequences (34–37) (Fig. 1). The *amoA* gene sequence of the AOA in the enrichment culture was between 86.4% and 98.6% identical to those of the other AOA enrichment cultures from *Nitrosopumilus* cluster 5 (see Table S2 in the supplemental material). The culture AOA-AC1 was 79% enriched based on catalyzed reporter deposition fluorescence *in situ* hybridization (CARD FISH) counts using AOA-specific 16S rRNA probes versus 4′,6-diamidino-2-phenylindole (DAPI) counts of all microbes in the sample (Table S3). AOB were not detected in the enrichment culture AOA-AC1 as tested by the amplification of the DNA with AOB-specific 16S rRNA and *amoA* primers (results not shown) and by FISH using AOB-specific 16S rRNA probes (Table S3).

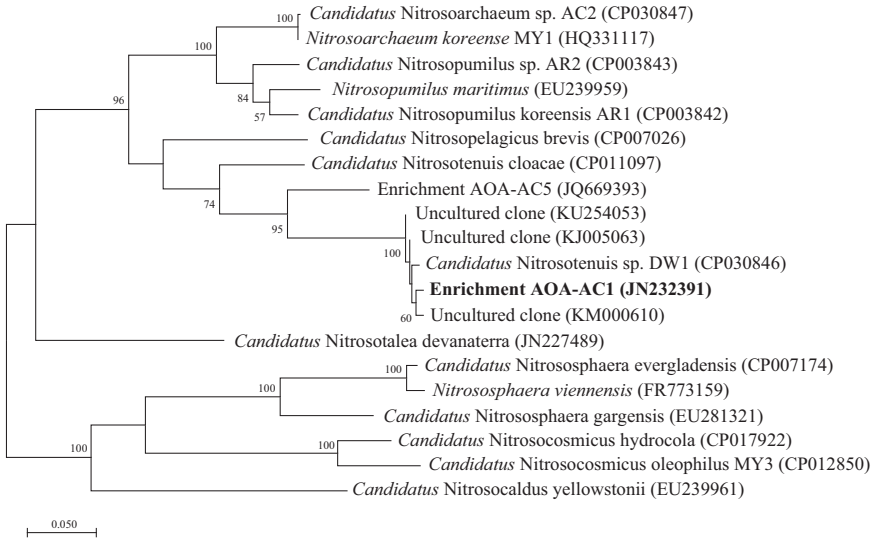

FIG 1 Neighbor-joining phylogenetic tree of the AOA enrichment culture AOA-AC1 based on *amoA* gene sequences (595 bp). Bootstrap values of >50 for 100 replicates are shown at the nodes. GenBank accession numbers are in parentheses.

Influence of ammonium concentrations on the growth of AOA-AC1 and AOB-G5-7. The growth of the AOA enrichment culture AOA-AC1 was characterized at different ammonium concentrations (Fig. 2). The growth rates were compared to the growth rates of the AOB enrichment culture AOB-G5-7 (Fig. 2) (data for G5-7 were retrieved from reference 34). The growth rate of AOA-AC1 was not affected by increasing ammonium concentrations, while the growth rate of AOB-G5-7 increased with increasing ammonium concentrations (Fig. 2).

Effect of spent medium on the growth of AOA-AC1 and AOB-G5-7. Growth experiments with AOA-AC1 and AOB-G5-7 were conducted in the spent medium of the same culture or the other culture (Fig. 3; Fig. S1). This experiment serves as an important control experiment to exclude chemical inhibition of one ammonia oxidizer by the other or by other bacteria present in the enrichment cultures. The ammonium consumption rates of AOA-AC1 and AOB-G5-7 in standard mineral salts medium and in AOA-AC1 or AOB-G5-7 spent medium were similar (Fig. 3). This indicates that the outcome of the competition experiments is not impacted by the production of inhibitory substances by members of the enrichment cultures.

Short-lived reactive oxygen species (ROS) were very likely not playing a role in the interaction between AOA and AOB because both cultures are enrichment cultures. Heterotrophic bacteria in the ammonia-oxidizing enrichment cultures reduce the oxidative stress for the ammonia oxidizers (26, 38).

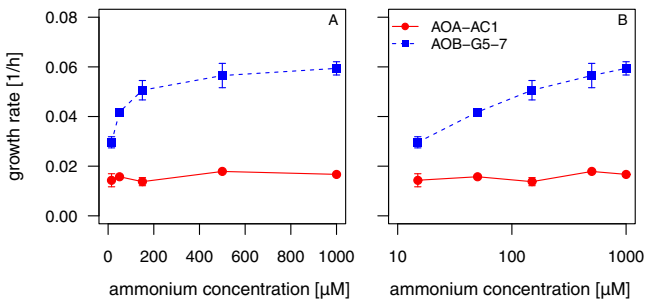

FIG 2 Influence of the ammonium concentration on the growth rates of the enrichment cultures AOA-AC1 and AOB-G5-7 (means ± standard deviations [SD] [n = 3]). Ammonium concentrations are shown on a linear scale (A) and a logarithmic scale (B). All data for the culture AOB-G5-7 were presented previously by French et al. (34) and are included here for better comparability of the data.

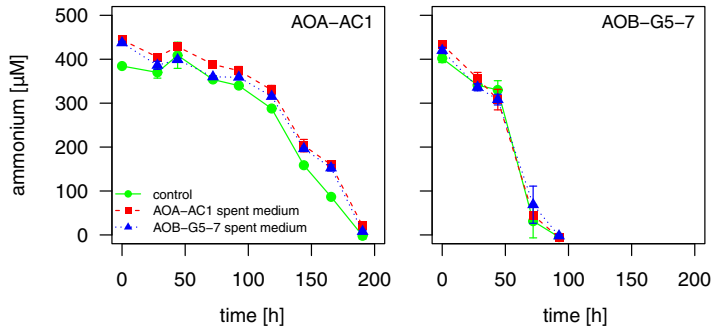

FIG 3 Ammonium consumption of AOA-AC1 and AOB-G5-7 in mineral salts medium and spent medium from AOA-AC1 and AOB-G5-7 (means ± SD [*n* = 3]).

Competition between AOA-AC1 and AOB-G5-7 for ammonium under ammonium-limiting conditions in continuous cultures. AOA-AC1 and AOB-G5-7 were grown in continuous cultures with ammonium as the growth-limiting substrate (Fig. 4A and B; Fig. S2). After inoculation, the ammonia oxidizers started to consume ammonium and to produce nitrite and subsequently also nitrate due to the presence of nitrite oxidizers in the enrichment cultures (Fig. S2). When the ammonium was consumed after 6 days in chemostat L (inoculated with AOB-G5-7) and after 20 days in chemostat R (inoculated with AOA-AC1), the pumps were started to add fresh mineral salts medium with ammonium. The ammonium and nitrite concentrations stayed approximately at the detection limit (<5 μM for ammonium and <1 μM for nitrite) for the remainder of the experiment, while nitrate increased to 500 μM, indicating that ammonium was converted to nitrate in both chemostats (Fig. 4A and B; Fig. S2). No differences between the ammonium concentrations in the chemostats could be detected because the ammonium concentration was below or

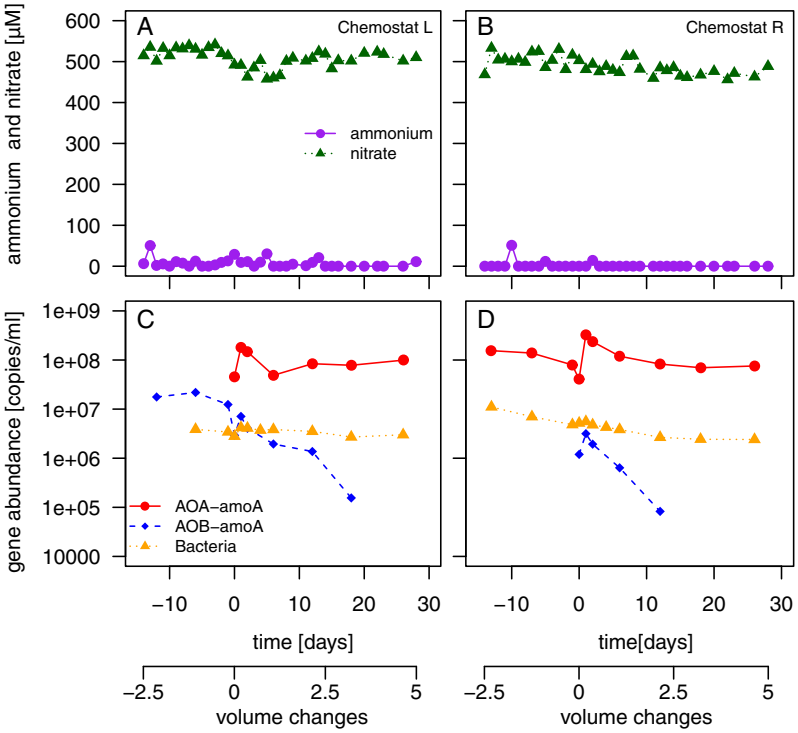

FIG 4 Ammonium and nitrate concentrations (A and B) and gene abundances (AOA-AC1 *amoA*, AOB-G5-7 *amoA*, and total bacteria) (C and D) in the chemostats during the competition experiment between AOA-AC1 and AOB-G5-7. Chemostat L was inoculated with AOB-G5-7, and chemostat R was inoculated with AOA-AC1. At day 0, the cultures were mixed.

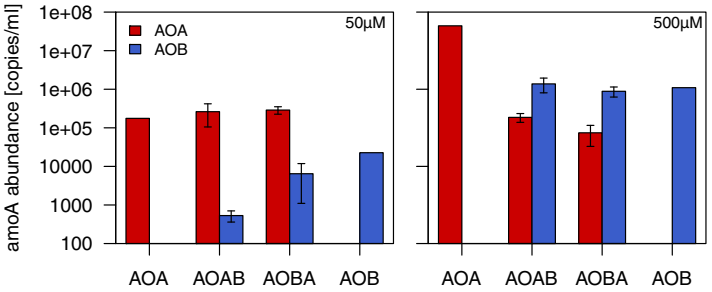

FIG 5 *amoA* gene abundances in the batch culture competition experiments at initial ammonium concentrations of 50 μM and 500 μM. The batch cultures were inoculated with cultures of AOA-AC1 and AOB-G5-7 mixed at 9:1 (vol/vol) (AOAB) and 1:9 (vol/vol) (AOBA) ratios and with AOA-AC1 (AOA) and AOB-G5-7 (AOB) alone as controls (means $\pm$ SD [n = 3] for AOAB and AOBA; means [n = 2] for AOA and AOB). The cultures were transferred twice to fresh medium (2 ml of culture into 48 ml of fresh medium) when the ammonium was used up. The *amoA* gene abundance was determined at the end of the third growth cycle.

around the detection limit of the method used. When the ammonium, nitrite, and nitrate concentrations in the chemostats were constant for 2 to 3 volume changes, the cultures were mixed (time point, 0 days). The concentrations of ammonium, nitrite, and nitrate were stable after mixing, indicating that ammonia and nitrite oxidation went on without disturbance. Molecular methods were used to determine the abundances of AOA-AC1 and AOB-G5-7 in the chemostats. In chemostat L (inoculated with AOB-G5-7), approximately 10^7 bacterial *amoA* copies/ml culture were detected (Fig. 4C), which would correspond to 3×10^6 AOB cells/ml culture because *Nitrosomonas* sp. Is79, the AOB in the enrichment culture AOB-G5-7, has 3 copies of the *amoA* gene in the genome (39). The abundance of AOA-AC1 was above 10^8 *amoA* copies/ml culture (Fig. 4D), corresponding to the same number for AOA in the cultures because all sequenced AOA have only 1 copy of the *amoA* gene in their genome (7, 35, 40).

The cultures were mixed based on volume at 1:2 and 2:1 ratios. After mixing, we detected around 10 to 100 times more AOA than AOB *amoA* copies. Over the course of 2 to 3 volume changes (up to 20 days), the abundance of AOB-G5-7 decreased to $<10^5$ copies/ml culture (Fig. 4C and D). Twenty days after mixing, AOB-G5-7 was not detectable anymore in the mixed cultures. The washout rate of the AOB was calculated (41) (Fig. S3). The theoretical washout rate of the AOB was comparable to the decrease of the measured abundance of the AOB in the chemostats, indicating that the AOB were not actively growing during the competition experiment. The abundance of AOA-AC1 stayed constant for most of the experiment, at approximately 10^8 *amoA* copies/ml culture (Fig. 4C and D).

Both cultures contained nitrite-oxidizing bacteria. Based on the amplicon sequencing data, the NOB in AOA-AC1 belonged to the genus *Nitrospira*, and the NOB from AOB-G5-7 belonged to the genus *Nitrobacter*. After mixing, *Nitrospira* sp. became the dominant NOB in both chemostats, while the relative abundance of *Nitrobacter* decreased, indicating that *Nitrobacter* was washed out (Fig. S4C and D).

The heterotrophic community was analyzed using weighted UniFrac after removing all operational taxonomic units (OTUs) assigned to ammonia and nitrite oxidizers. Principal-coordinate analysis (PCoA) showed that the heterotrophic community in the mixed cultures was more closely related to the heterotrophic community in AOA-AC1 than to the heterotrophic community in AOB-G5-7 (Fig. S5).

Competition for ammonium between AOA-AC1 and AOB-G5-7 under various, non-ammonium-limiting conditions in batch cultures. Batch competition experiments were conducted with AOA-AC1 and AOB-G5-7 in media with different initial ammonium concentrations of between 50 and 500 μM (Fig. 5; Fig. S6). AOA-AC1 and the mixed cultures used the ammonium faster than AOB-G5-7 when incubated in the presence of 50 μM ammonium (Fig. S6). Conversely, AOB-G5-7 and the mixed cultures with

a higher proportion of AOB-G5-7 consumed ammonium faster than AOA-AC1 when cultures were incubated with an initial ammonium concentration of 500 μM (Fig. S6).

We determined the abundances of AOA-AC1 and AOB-G5-7 at the end of the experiment. The abundance of AOA-AC1 in the mixed cultures at 50 μM ammonium was at the same level as that in the AOA control (AOA-AC1 alone), while the abundance of AOB-G5-7 decreased in the mixed cultures compared to the AOB control (AOB-G5-7 alone) (Fig. 5). At 500 μM ammonium, the opposite effect was observed: the abundance of AOA-AC1 was lower in the mixed cultures than in the AOA control, and the abundance of AOB-G5-7 was at the same level as that in the AOB control, indicating that AOB-G5-7 became dominant (Fig. 5). The results indicate that AOA-AC1 became dominant in the mixed cultures at 50 μM ammonium and that AOB-G5-7 became dominant at 500 μM ammonium.

The nitrite oxidizer *Nitrospira* sp. became dominant at 50 μM ammonium together with the archaeal ammonia oxidizer (Fig. S7). At 500 μM ammonium, *Nitrobacter* sp. originating from the AOB enrichment culture became dominant at an AOA/AOB mixing ratio of 1:9. At an AOA/AOB mixing ratio of 9:1, *Nitrospira* sp. from the AOA enrichment culture was the dominant nitrite oxidizer.

The heterotrophic communities in the batch competition treatments represented a mixture of the heterotrophic communities of the AOA and AOB control cultures (Fig. S5).

DISCUSSION

Competition between AOA-AC1 and AOB-G5-7. AOA-AC1 outcompeted AOB-G5-7 under ammonium-limiting conditions in chemostats and at 50 μM ammonium in batch cultures (Fig. 4 and 5). However, AOB-G5-7 outcompeted AOA-AC1 at 500 μM ammonium and grew faster in batch cultures with ammonium concentrations of between 50 and 1,000 μM (Fig. 2 and 5).

The observation that AOA-AC1 outcompeted AOB-G5-7 under ammonium-limiting and low-ammonium conditions further confirmed the ability of AOA to grow and persist under conditions of substrate limitation better than AOB, while the higher growth rates of AOB-G5-7 in batch cultures point to a competitive advantage of the AOB over the AOA under conditions with higher ammonium availability. This observation is well supported by studies using cultivation and molecular approaches. *Nitrosopumilus maritimus* and several AOA enrichment cultures have very high affinities for ammonium, with K_m values ranging from 0.003 μM to 4.4 μM ammonia (0.1 to 40 μM NH_3 plus NH_4^+) (5, 6, 22, 23, 36, 42). AOA also dominate many aquatic environments with very low ammonium availability, such as the open ocean, oligotrophic lakes, and drinking water treatment plants (16, 43–49). AOB, on the other hand, have a lower affinity for ammonium, with K_m values of 3 μM ($\sim$150 μM NH_3 plus NH_4^+) in *Nitrosomonas* sp. Is79 and *N. briensis* and up to 60 μM ($\sim$3 mM NH_3 plus NH_4^+) in *N. eutropha* (24–26). Additionally, they are found in high abundances in more eutrophic aquatic environments such as Lake Erie and Lake Taihu and in agricultural/fertilized soils, indicating their adaptation to higher ammonium availabilities than AOA (16, 50–54). These results indicate that AOA-AC1 is the better competitor for low-concentration ammonium than AOB-G5-7 and therefore will likely perform better in more oligotrophic environments.

One of the most successful ways of separating freshwater AOA from AOB after initial enrichment is sequential filtration through 0.45-μm filters, indicating that the enriched AOA are much smaller than the AOB (34, 55, 56). The theoretical volume of AOB ranges between 0.5 and 3.3 μm^3, and the surface area ranges between 3.5 and 12 μm^2, while both parameters are more variable in AOA (see Table S4 in the supplemental material). Comparison of the volumes and surface areas of *Nitrosomonas oligotropha* (as a representative of *Nitrosomonas* sp. Is79 in AOB-G5-7) and "*Ca*. Nitrosotenuis uzonensis" (as a representative of the AOA in AOA-AC1) (Table S4) shows that the AOB have a 50-times-larger volume and a 10-times-larger surface area than the AOA. The abundance of AOA-AC1 in the chemostats and batch cultures was approximately 100 times higher than the abundance of AOB-G5-7, while both cultures grew under the

same conditions and on the same amount of ammonium as the energy source. Combining our results on abundance of AOA and AOB with the theoretical size of the cells (Table S4) indicates that cell size has an important impact on the ammonia-oxidizing activity in general. A more in-depth study was just published in the *ISME Journal* (57). Smaller cells must be present in higher abundances than larger cells to oxidize the same amount of ammonium. If we apply this observation with the same assumptions to environmental samples, this would indicate that freshwater AOA from the *Nitrosopumilus* and *Nitrosotenuis* groups and AOB from the *Nitrosomonas oligotropha* group would contribute equally to ammonia oxidation in an environment only if the AOA abundance is 10 to 100 times higher than the AOB abundance. If the abundances of these AOA and AOB are of the same order of magnitude, it would be very likely that AOB are responsible for the majority of the ammonia oxidation. Therefore, it would be important to consider the cell sizes of the dominant AOA and AOB when determining which ammonia oxidizers are most likely the metabolically dominant microorganisms in an environment. This observation has an important impact on the interpretation of data presenting the abundances of AOA and AOB in the environment since many studies conduct direct comparisons between the abundances of AOA and AOB to evaluate their contributions to ammonia oxidation (58–61) and even use the ratio between AOA and AOB as a measure of the trophic status of an environment (62).

Ecological implications. From an ecological perspective, AOA and AOB might represent *K*- and *r*-strategists (63) or, as more directly described for microbes, oligotrophs and copiotrophs (64). Among other characteristics, oligo- and copiotrophs differ in their maximum growth rate, affinity for the substrate, responsiveness to substrate addition (starvation response), and ease of cultivation (64). Differences in all these characteristics were determined for AOA-AC1 and AOB-G5-7 as well as AOA and AOB in general. AOB-G5-7 grows faster than AOA-AC1 and other freshwater AOA (Fig. 2) (22, 34), AOA have higher affinities for ammonium than AOB (6, 22, 23, 36, 42, 65), AOA-AC1 responds more slowly after starvation than AOB-G5-7 does (66), and AOA are more challenging to cultivate than AOB (55). Based on these observations, AOA can be described as oligotrophs and AOB can be described as copiotrophs in this experimental setup.

Conclusions. Environmentally relevant factors such as ammonium concentrations determined the ability of AOA-AC1 or AOB-G5-7 (chemostat versus batch culture) to grow faster and therefore outcompete the other. These results reflect the distribution of AOA and AOB in freshwater systems, with AOA being more abundant in more oligotrophic–nutrient-poor systems (low ammonium) and AOB being more abundant in more meso/eutrophic–nutrient-rich systems (high ammonium). The number of AOA-AC1 cells was 10 to 100 times higher than the number of AOB-G5-7 cells when grown under autotrophic conditions on the same amount of ammonium (Fig. 4 and 5). Based on this observation, one could conclude that these AOA and AOB contribute equally to ammonia oxidation in the freshwater environment, where the abundance of AOA is found to be 10 to 100 times higher than the abundance of AOB.

MATERIALS AND METHODS

Medium. Mineral salts medium used in all experiments contained 10 mM NaCl, 1 mM KCl, 1 mM $CaCl_2 \cdot 2H_2O$, 0.2 mM $MgSO_4 \cdot 7H_2O$, and 1 ml liter^{-1} trace elements solution (55). For batch culture experiments, HEPES buffer was added in a 4:1 molar ratio to the ammonium concentration, and the pH was adjusted with 1 M NaOH to 7.5 before autoclaving. After autoclaving, a sterile KH_2PO_4 solution was added to obtain a final concentration of 0.4 mM (55). For the continuous cultures, unbuffered mineral salts medium was prepared and autoclaved in the chemostats. The sterile KH_2PO_4 solution was added after autoclaving, and the pH was adjusted to 7.5 with a 2% (wt/vol) Na_2CO_3 solution.

Enrichment of AOA-AC1. Sediment samples were taken near the shoreline of Lake Acton (39°57′N, 84°74′W) in the fall of 2008 and inoculated into fresh mineral salts medium containing 250 μM ammonium. The AOA culture was further enriched in mineral salts medium with 250 μM ammonium as previously described (34, 55). During enrichment, the cultures were passed through 0.45-μm filters for the first six transfers to exclude AOB. The phylogenetic affiliation of the AOA was determined as described previously (34).

AOB-G5-7. We used the previously described AOB freshwater enrichment culture G5-7 (AOB-G5-7) as a representative AOB culture. AOB-G5-7, a well-characterized AOB enrichment culture that was

obtained from freshwater sediment in the Netherlands, contains the AOB *Nitrosomonas* sp. Is79 and is adapted to low ammonium concentrations (26, 33, 39, 67).

Batch growth experiments. All batch growth experiments were conducted in 125-ml Erlenmeyer flasks with a 50-ml culture in mineral salts medium and cotton plugs to allow for gas exchange. The cultures were incubated at 27°C in the dark. Samples for nitrite/nitrate analysis were taken daily.

Batch culture growth experiment at different ammonium concentrations. The growth experiments were conducted in mineral salts medium with different ammonium concentrations (34, 55). The growth rates were calculated from the linear increase of the log-transformed nitrite/nitrate concentrations over time, assuming that the production in the cultures is correlated with the growth of AOA and AOB (5, 34, 55, 68).

Spent medium growth experiments. AOA-AC1 and AOB-G5-7 were grown in 500-ml mineral salts medium in a 1-liter Erlenmeyer flask with 500 μM ammonium. Once the ammonium was consumed, the cultures were spun down (20 min at 22,000 $\times$ *g*), and the supernatant was sterile filtered through 0.1-μm filters. Ammonium was added to the spent medium to reach a final ammonium concentration of 500 μM, and the pH was readjusted to 7.5. Spent media of AOA-AC1 and AOB-G5-7 (45 ml) were inoculated with late-logarithmic-phase cultures of AOA-AC1 and AOB-G5-7 (5 ml), respectively. In addition, AOA-AC1 and AOB-G5-7 were inoculated into mineral salts medium with ammonium as controls. The cultures were incubated at 27°C in the dark, and samples for ammonium, nitrite, and nitrate analyses were taken daily.

Continuous-culture competition experiment. Two chemostats with a vessel volume of 5 liters were assembled, filled with 2 liters of unbuffered mineral salts medium with 500 μM ammonium, and autoclaved. The temperature was then adjusted to 27°C, stirring was set to 50 rpm, bubbling with sterile filtered air was set to a rate of 500 ml min^{-1}, a sterile KH_2PO_4 solution was added to a final concentration of 0.4 mM, and the pH value was adjusted to 7.5 using a 2% (wt/vol) Na_2CO_3 solution. The chemostats were inoculated with 1 liter of late-logarithmic-phase cultures of AOA-AC1 or AOB-G5-7 as determined by the consumption of ammonium. When the cultures started to consume ammonium and produce nitrite/nitrate, the stirrer speed was increased stepwise from 50 rpm to 300 rpm and stayed at 300 rpm for the remainder of the experiment. When the cultures had consumed around 80% of the initial ammonium concentration, unbuffered mineral salts medium with 500 μM ammonium was added to each chemostat with a dilution rate of 535 ml day^{-1}, equal to 1 volume change in 5.6 days and a growth rate of 0.0074 h^{-1}. After the cultures reached steady state, the competition experiment was started by mixing the cultures. One liter of each culture was removed from the original vessel and transferred to the other, to create 1:2 and 2:1 (vol/vol) mixtures of the cultures. The cultures were mixed in different ratios to ensure that the initial mixing ratio did not affect the outcome of the competition experiment. Samples were taken daily throughout the course of the experiment (around 2 months) to determine the ammonium, nitrite, and nitrate concentrations. The samples were filtered (0.22-μm polycarbonate filters) and stored at $-$20°C until analysis. After every volume change and before and after mixing the cultures, two 50-ml samples from each chemostat were taken and filtered on 0.1-μm polycarbonate membranes, which were stored at $-$80°C for molecular analysis.

Batch culture competition experiment. AOA-AC1 and AOB-G5-7 were grown in mineral salts medium with 500 μM ammonium to the late logarithmic phase as determined by the consumption of ammonium. These cultures were mixed in volumetric ratios of 1:9 and 9:1. The mixed cultures as well as both single cultures (2 ml) were used to inoculate mineral salts medium (48 ml) with 50 μM and 500 μM ammonium. The cultures were incubated in the dark at 27°C, and the ammonium concentration was determined every second day. When the ammonium was consumed, the cultures were transferred to fresh medium with the same ammonium concentration (2 ml into 48 ml). At the end of the third growth cycle, the cultures were filtered onto 0.1-μm polycarbonate membranes, and the filters were stored at $-$80°C for molecular analysis.

Chemical analysis. Ammonium, nitrite, and nitrate concentrations were determined using colorimetric methods (55, 69–71).

Molecular analysis. (i) DNA isolation (competition experiment). DNA was isolated from filters using the Fast DNA spin kit for soil (MP Biomedicals, Solon, OH, USA).

(ii) Quantitative PCR (qPCR). The copy numbers of the 16S rRNA and *amoA* genes (DNA) were quantified using the Bioline SensiFAST SYBR NoROX kit (Bioline USA, Taunton, MA, USA) in 5-μl reaction mixtures with 0.5- or 1-μl samples in an Illumina Eco real-time PCR system (Illumina, San Diego, CA, USA) with the primers and conditions presented in Table 1 and Table S1 in the supplemental material. Standard curves were constructed using plasmids containing the gene sequence of interest. The efficiency of the reactions ranged from 87% to 118%, and the R^2 value in all experiments was $>$0.99.

TABLE 1 Primers used for quantification of *amoA* genes in the DNA samples from the competition experiment

Target (reference)	Primer name, sequence
AOA *amoA* (73)	Arch amoA F, 5′-STA ATG GTC TGG CTT AGA CG-3′
	Arch amoA R, 5′-GCG GCC ATC CAT CTG TAT GT-3′
AOB *amoA* (74)	amoA-1F, 5′-GGG GTT TCT ACT GGT GGT-3′
	amoA-2R KS, 5′-CCC CTC KGS AAA GCC TTC TTC-3′
Eubacteria (75)	357F, 5′-CCT ACG GGA GGC AGC AG-3′
	518R, 5′-ATT ACC GCG GCT GCT GG-3′

(iii) 16S rRNA gene amplicon sequencing. DNA from selected samples from the chemostats and batch cultures was amplified in triplicate with Illumina-compatible barcoded primers designed for the V4 region of the 16S rRNA gene (515F-806R) (76, 77). The PCR products were mixed in equal ratios and sequenced on the Illumina MiSeq system at the Center for Bioinformatics and Functional Genomics (CBFG) at Miami University. The sequences were processed by the software package MiSeq Reporter into files containing the sequences and quality information and a file containing the barcodes assigned to each sequence. The software package QIIME 2 was used for quality control and analysis of the sequences (see the supplemental material) (72).

Data availability. The *amoA* sequence of the AOA enrichment culture AOA-AC1 was deposited in GenBank under the accession number JN232391. The sequences of the microbial communities were deposited in the NCBI SRA database under the accession number PRJNA259942.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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