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DOI
10.1016/j.wroa.2025.100324

Publication date
2025

Document Version
Final published version

Published in
Water Research X

Water Research X 28 (2025) 100324

Contents lists available at ScienceDirect

Water Research X

journal homepage: www.sciencedirect.com/journal/water-research-x

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ARTICLE INFO

Keywords:
Sponge-based trickling filter
Comammox
Nitrous oxide, Nitrification, Wastewater treatment

ABSTRACT

Nitrification/denitrification mitigates excess nitrogen in wastewater and reduces nutrient pollution in recipient surface waters but emits substantial amounts of nitrous oxide (N_2O). Complete ammonia-oxidizing (comammox) bacteria provide novel opportunities to mitigate N_2O emissions from wastewater treatment systems. In this study, a down-flow hanging sponge (DHS) reactor with low-strength ammonia-based synthetic wastewater was used to culture comammox bacteria, to study the microbial community structure, and to assess the nitrogen removal performance. The results showed a high NH_4^+-N removal efficiency of 98 ± 4 % and complete nitrification during the entire experimental period. 16S rRNA gene sequencing and metagenomic analysis showed that comammox-like *Nitrospira* dominated the DHS-retained sludge, and that comammox-like *Nitrospira* and ammonia-oxidizing archaea may have coexisted symbiotically. The dissolved N_2O emissions per NH_4^+-N removed from the DHS reactor were much lower than those from conventional activated sludge processes, indicating that the DHS reactor could be effective in reducing N_2O emissions during wastewater treatment.

Introduction

Nitrification, a biochemical process that converts ammonia (NH_4^+-N) to nitrate (NO_3^--N) through intermediate stages, is essential in the global nitrogen cycle. It plays a vital role in transforming nitrogen compounds in the environment, thereby supporting plant growth and maintaining ecological balance. In engineered systems such as biological wastewater treatment plants, nitrification/denitrification is used in the removal process of excess nitrogen from wastewater, which helps to reduce nutrient pollution in recipient surface waters. However, despite these benefits, emission of nitrous oxide (N_2O), a potent greenhouse gas and ozone-depleting substance, remains a significant environmental challenge posed by wastewater treatment facilities. Current estimates suggest that N_2O emissions from wastewater treatment processes contribute to approximately 3.4 % of global anthropogenic N_2O emissions (Kits et al., 2019), and these emissions are expected to increase with increasing wastewater production worldwide. The need to control and mitigate N_2O emissions from wastewater treatment has spurred research on the microbial communities responsible for nitrification. Traditional nitrification is typically carried out by two bacteria types: ammonia-oxidizing bacteria (AOB), which convert NH_4^+-N to nitrite (NO_2^--N), and nitrite-oxidizing bacteria (NOB), which convert NO_2^--N to NO_3^--N. However, in 2015, a groundbreaking discovery was made with the identification of complete ammonia-oxidizing (comammox) bacteria that could perform the entire nitrification process from NH_4^+-N to NO_3^--N within a single organism (van Kessel et al., 2015). This unique capability distinguishes comammox bacteria from conventional two-step nitrifying organisms, and offers new avenues for controlling N_2O emissions. Studies have indicated that wastewater treatment systems dominated by comammox bacteria tend to emit less N_2O than those dominated by traditional AOB, making comammox a promising target for developing

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https://doi.org/10.1016/j.wroa.2025.100324
Received 30 December 2024; Received in revised form 15 February 2025; Accepted 24 February 2025
Available online 25 February 2025

Nomenclature

AOA	Ammonia-oxidizing archaea
AOB	Ammonia-oxidizing bacteria
DHS	Down-flow hanging sponge
DO	Dissolved oxygen
NOB	Nitrite-oxidizing bacteria
PCR	Polymerase chain reaction
TN	Total nitrogen

more sustainable nitrification processes (Kits et al., 2019).

One of the technological innovations explored to harness comammox bacteria in wastewater treatment is the down-flow hanging sponge (DHS) reactor (Watari et al., 2024). A DHS reactor is a type of biological trickling filter system designed to promote high biomass retention using sponge materials as carriers for microbial growth (Tyagi et al., 2021). The sponge material provides a large surface area and protective environment that fosters microbial communities, allowing for efficient retention and cultivation of nitrifying bacteria, including comammox. Several factors render the DHS reactor particularly suitable for enhancing nitrification, while potentially reducing N_2O emissions. First, sponge carriers in the DHS reactor support a high biomass density, which contributes to rapid nitrification rates (Watari et al., 2021). Second, the sponge structure creates an oxygen gradient with varying dissolved oxygen (DO) levels within different sections. It supports diverse microbial activities, effectively mitigating nitrite accumulation through rapid nitrification (Tyagi et al., 2021; Watari et al., 2021). Finally, the DHS reactor enables a long sludge retention time, allowing slow-growing bacteria such as comammox to establish stable populations within the reactor (Watari et al., 2024).

In this study, a laboratory-scale DHS reactor was used to cultivate comammox bacteria under controlled conditions, while feeding simulated domestic sewage to investigate their nitrification efficiency and N_2O emission characteristics. We assessed the process performance, microbial community structure, and N_2O emissions under various conditions. Understanding these interactions can help to develop a nitrification process that minimizes N_2O emissions and reduces the environmental impact of wastewater treatment. This study offers insights into the design of next-generation treatment systems that support global efforts to reduce greenhouse gas emissions and promote sustainable water management.

Results and discussion

Process performance of DHS reactor and nitrifying microorganism growth

Fig. 1 shows the abundance of nitrifying microorganisms in the seed sludge and DHS retained sludge over the height of the reactor. The seed-activated sludge contained 0.5 % of *Nitrosomonas* and 0.4 % of *Nitrospira*. *Nitrosomonas* is an NOB commonly detected in DHS reactors that treat domestic and industrial wastewater (Kirishima et al., 2022). *Nitrospira* are commonly detected in DHS reactors that treat wastewater and municipal sewage (Kubota et al., 2014; Watari et al., 2024).

The DHS operational period of 415 days was divided into three phases based on the influent conditions in terms of DO and temperature (Table 1). The DHS reactor showed partial nitrification activity in week 1 and accumulated 21.8 mg NO_2^--N·L^{-1} (Fig. 2); however, after 9 days of operation, 99 % complete nitrification to NO_3^- was observed. This observation was in agreement with our previous study applying specific DHS carrier material that enhanced the growth and retention of nitrifying microorganisms (Watari et al., 2022). However, the nitrifying microorganism abundance remained low (0.4 %) until day 21. The minimal doubling times of AOB and NOB are approximately 7–8 h and 10–13 h, respectively, which are longer than those of other bacteria, such as *Escherichia coli* (Peng and Zhu, 2006; Tuttle et al., 2021). Additionally, the doubling time of nitrifying bacteria significantly increases in low-temperature environments, requiring 45 days of cultivation in reactors operating at 5–10 °C (Nikolaev et al., 2015). In the activated sludge process for treating domestic wastewater, low-temperature wastewater below 15 °C flows during winter, leading

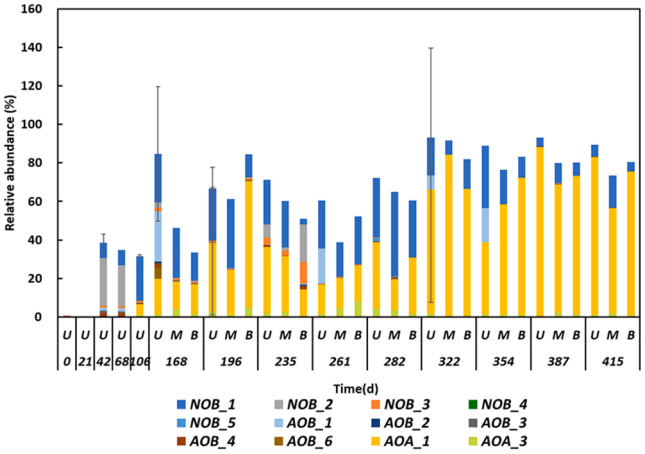

Fig. 1. Abundance of nitrifying microorganisms during the experimental period (U: upper part of the reactor, M; middle part of the reactor, B: bottom of the reactor).
AOB_1: Nitrospmonas. sp, AOB_2: Nitrosomonas bacterium enrichment, AOB_3: Nitrosomonas uncultured, AOB_4: Nitrosomonas_ASV1, AOB_6: Nitrosomonas communis, AOA_1: Nitrosotenuis uncultured, AOA_3, Nitrocosmicus_ASV1, NOB_1: Nitrospira_ASV1, NOB_2: Nitrospira defluvii, NOB_3: Nitrospira uncultured, NOB_4: Nitrospira unidentified, NOB_5: Nitrospira japonica

Table 1
Temperature, dissolved oxygen (DO), and pH changes during phases 1–3.

	Temperature (°C)		DO (mg·L^{-1})		pH	
	Influent	Effluent	Influent	Effluent	Influent	Effluent
Phase 1 (Days 0–161)	14.3 ± 3.5	26.7 ± 4.7	7.8 ± 1.3	5.7 ± 0.8	8.0 ± 0.3	7.1 ± 1.3
Phase 2 (Days 162–313)	23.5 ± 4.3	30.1 ± 1.9	2.4 ± 2.3	4.9 ± 1.6	7.9 ± 1.0	7.2 ± 0.4
Phase 3 (Days 313–415)	19.8 ± 6.6	28.4 ± 5.6	4.3 ± 3.3	5.0 ± 1.6	7.7 ± 1.4	7.1 ± 1.1

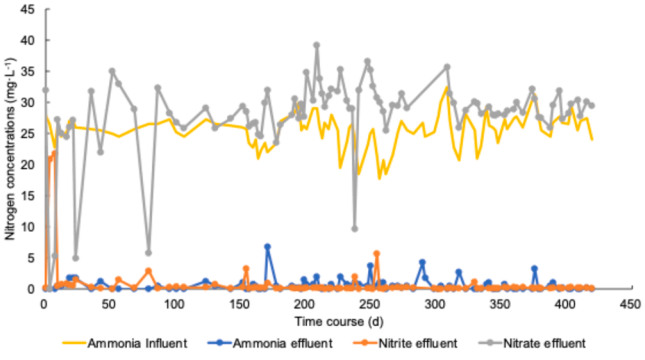

Fig. 2. Time course of influent NH_4^+-N and effluent NH_4^+-N, NO_2^--N, and NO_3^--N concentrations during the experimental period.

to a decline in nitrifying bacterial activity and overall performance (Coskuner et al., 2008). A multiple regression model indicated that maintaining a high MLSS concentration is necessary to sustain a high treatment performance under low-temperature conditions (Coskuner et al., 2008). In the DHS reactor, the low temperature of the influent substrate likely led to an extended doubling time for AOB and NOB, resulting in low initial abundance of nitrifying bacteria. Furthermore, the low relative abundance of nitrifying bacteria is considered the primary cause of the incomplete nitrification observed up to day 9. During phase 1, the temperature and DO concentration of the influent were 14.3 ± 3.5 °C and 7.8 ± 1.3 mg·L^{-1}, respectively, and the NH_4^+-N removal efficiency was 92.3 ± 17.9 %. Nitrification reactions are affected by DO concentrations ranging 0.3–4.0 mg·L^{-1}, with concentrations > 4.0 mg·L^{-1} needed for optimal nitrification rates (Zhao et al., 2022). In addition, nitrification by comammox bacteria has been reported to remove high NH_4^+-N concentrations in environments where the DO concentrations are > 1–3 mg·L^{-1} (Zhao et al., 2022). Moreover, in a batch test with varying DO concentrations (0.5–17.4 mg·L^{-1}), *Nitrospira nitrosa* was dominant under all oxygen conditions (Zheng et al., 2022). In the present study, on day 42 of operation, *Nitrospira* spp. abundance increased, with 33.7 % detected in the upper part of the reactor; 24.7 % were *Nitrospira defluvii*, 8.9 % were uncultivated *Nitrospira* species (NOB_1, 3, 4), and 4.6 % were *Nitrosomonas* spp.. *N. defluvii* was the most dominant species (21.3–24.7 %) after 68 days of operation. On day 106 of operation, *Nitrospira* spp. accounted for 24.3 %, *N. defluvii* accounted for 0.1 %, and *Nitrosomonas* spp. were detected in 0.7 % of samples. From day 106 of operation, the most preferential bacteria switched from *N. defluvii* to NOB_1, which is one of uncultivated *Nitrospira* spp.. Moreover, the ammonia-oxidizing archaea (AOA) *Nitrosotenuis* and *Nitrocosmicus* were detected at 5.6 % and 1.0 %, respectively. *N. defluvii* is a major nitrite-oxidizing bacteria in wastewater treatment plants and exhibits excellent biofilm formation; however, no literature exists on its role as a catalyst for ammonia oxidation (Nowka et al., 2014). Additionally, the solid retention time of the treatment system is a key parameter associated with an increase in the abundance of comammox bacteria (Cotto et al., 2020). Therefore, it can be inferred that NOB_1 (presumably comammox bacteria) and AOA_1 contribute to ammonia oxidation, leading to a decrease in the relative abundance of NOB_2, which lacks the ability to oxidize ammonia.

On day 161, the influent temperature increased to 23.5 °C, causing the DO concentration to decrease to 2.4 ± 2.3 mg·L^{-1}. Despite the lower DO concentration of influent, the NH_4^+-N removal efficiency reached 95.6 ± 9.8 %. The DO concentration of effluent of 4.9 ± 1.6 mg·L^{-1} indicated that the DHS reactor exhibited high oxygen transfer (T. Watari et al., 2021) and could supply sufficient DO for complete nitrification. On day 196 of operation, the AOA abundance began to increase, with 36.1 % of *Nitrosotenuis* spp. and 2.2 % of *Nitrocosmicus* spp. detected. These AOAs are autotrophic and grow at an optimal temperature of 30 °C (Nakagawa et al., 2021). The nitrifying bacteria abundance was 66.7 %, 61.2 %, and 84.5 % in the upper, middle, and lower parts of the reactor, respectively.

In phase 3, the DO concentration of the influent increased due to a decrease in the temperature to 19.8 ± 6.6 °C. On day 322 of operation, AOA abundance surged, with 66.1 %, 84.2 %, and 66.6 % in the upper, middle, and lower parts, respectively. Nitrifying microorganisms accounted for 93.3 %, 91.6 %, and 81.9 % of the microorganisms in the upper, middle, and lower reactor sections, respectively. From day 322 of operation, nitrifying bacteria were the predominant bacteria in the reactor. AOB abundance did not exceed 1.0 % from days 322–415, and only uncultivated *Nitrospira* species were detected during this period. The uncultivated *Nitrospira* abundance increased on day 354 of operation, reaching a maximum of 32.4 %. However, the uncultivated *Nitrospira* species abundance decreased after 354 days of operation, with 6.0 %, 16.8 %, and 4.7 % detected in the upper, middle, and lower parts, respectively, on day 415. A comparison of AOA_1 and NOB_1, 3, 4 abundance after day 322 of operation confirmed that uncultivated *Nitrospira* abundance decreased with an increased AOA_1 abundance, and uncultivated *Nitrospira* abundance increased with a decreased AOA_1 abundance. This indicated a possible competitive relationship between AOA and uncultivated *Nitrospira* for NH_4^+-N oxidation. As uncultivated *Nitrospira* species are likely to be comammox bacteria, the possibility of comammox bacterial growth in DHS reactors has been reported (Watari et al., 2024). Considering the nitrification performance, it is suggested that complete oxidation of ammonia and nitrite from day 9 to the final day was achieved because of the dominance of uncultivated *Nitrospira* and AOA. Compared to conventional plastic carriers, sponge carriers demonstrate superior biomass retention (Deng et al., 2016). Moreover, cultivation of comammox bacteria in reactors employing sponge carriers has been documented (Huang et al., 2022). Consequently, these findings suggest that DHS is advantageous for facilitating biofilm formation by the comammox bacteria.

In previous studies and pilot tests, the DHS reactor has been shown to exhibit high performance not only in the removal of nitrogen from sewage but also in the removal of other substances. The DHS reactor, employed as a post-treatment unit after the anaerobic baffled reactor (ABR) used for treating synthetic molasses wastewater simulating industrial wastewater, was found to facilitate the oxidation of hydrogen sulfide in biogas, achieve 57.7 % removal of ammonia, and obtain an 88.3 % COD removal efficiency (Tanikawa et al., 2020). A pilot DHS reactor at a wastewater treatment plant in Khon Kaen, Thailand, achieved 63.8 % COD and 96.9 % ammonia removal with a 1-hour hydraulic retension time (HRT) for low-strength combined sewer wastewater (Watari et al., 2022); however, reports on phosphorus recovery using a single-chamber DHS reactor are limited, and techniques combining the DHS reactor with a duckweed pond for phosphorus recovery have been documented (Kubota et al., 2024). Furthermore, beyond its application in wastewater treatment, the DHS reactor is considered applicable to water treatment technologies for both marine and freshwater closed recirculating aquaculture systems (RAS), owing to its robust treatment performance and rapid startup phase (Akamine et al., 2024; Watari et al., 2021b). Complete nitrification facilitated by comammox bacteria expands the potential for developing integrated water treatment systems in conjunction with the technologies mentioned above to mitigate greenhouse gas emissions. The DHS process enhances economic efficiency not only because it eliminates the need for external aeration, but also because of its negligible excess sludge production, which accounts for only 2 % of the total COD removed (Tyagi et al., 2021). Therefore, given its ability to achieve organic matter removal and complete nitrification at a pilot scale in a highly sustainable manner, along with its capacity to minimize excess sludge production, the DHS process is considered economically feasible.

Molecular phylogenetic analysis of nitrifying microorganisms

The results of molecular phylogenetic analysis targeting the 16 s rRNA gene are shown in Fig. 3. Six AOB species, three AOA species, and four NOB species were classified by Silva 138 99 % OTUs from 515F/806R. Among the AOB, AOB_4 and AOB_6 exhibited abundances > 1.0 %; AOB_4 and AOB_6 reached abundances of 2.2 % and 5.8 %, respectively, at 168 days of operation. AOB_4 (*Nitrosomonas*_ASV1) and AOB_6 (*Nitrosomonas communis*) are closely related to *Nitrosomonas communis* growth in terrestrial and freshwater environments, respectively (Pommerening-Röser et al., 1996). A previous study reported that AOB switch from *N. communis* to other bacteria in a reactor inoculated with sludge containing *N. communis,* when the pH changes to 7.5–8.0 and the temperature changes to 25–30 °C (Egli et al., 2003), indicating that *N. communis* grows in environments with pH ≤ 7.5 and temperatures ≤ 25 °C. Therefore, it would be difficult for *N. communis* to grow in the DHS of our present study, which treats water with pH of 6.9 ± 0.3 at a temperature of 30 ± 1.3 °C (Table 1).

Among the AOA, AOA_1 and AOA_3 were present at ratios ≥ 1 %. AOA_1 was the most predominant species in the reactor, increasing to a

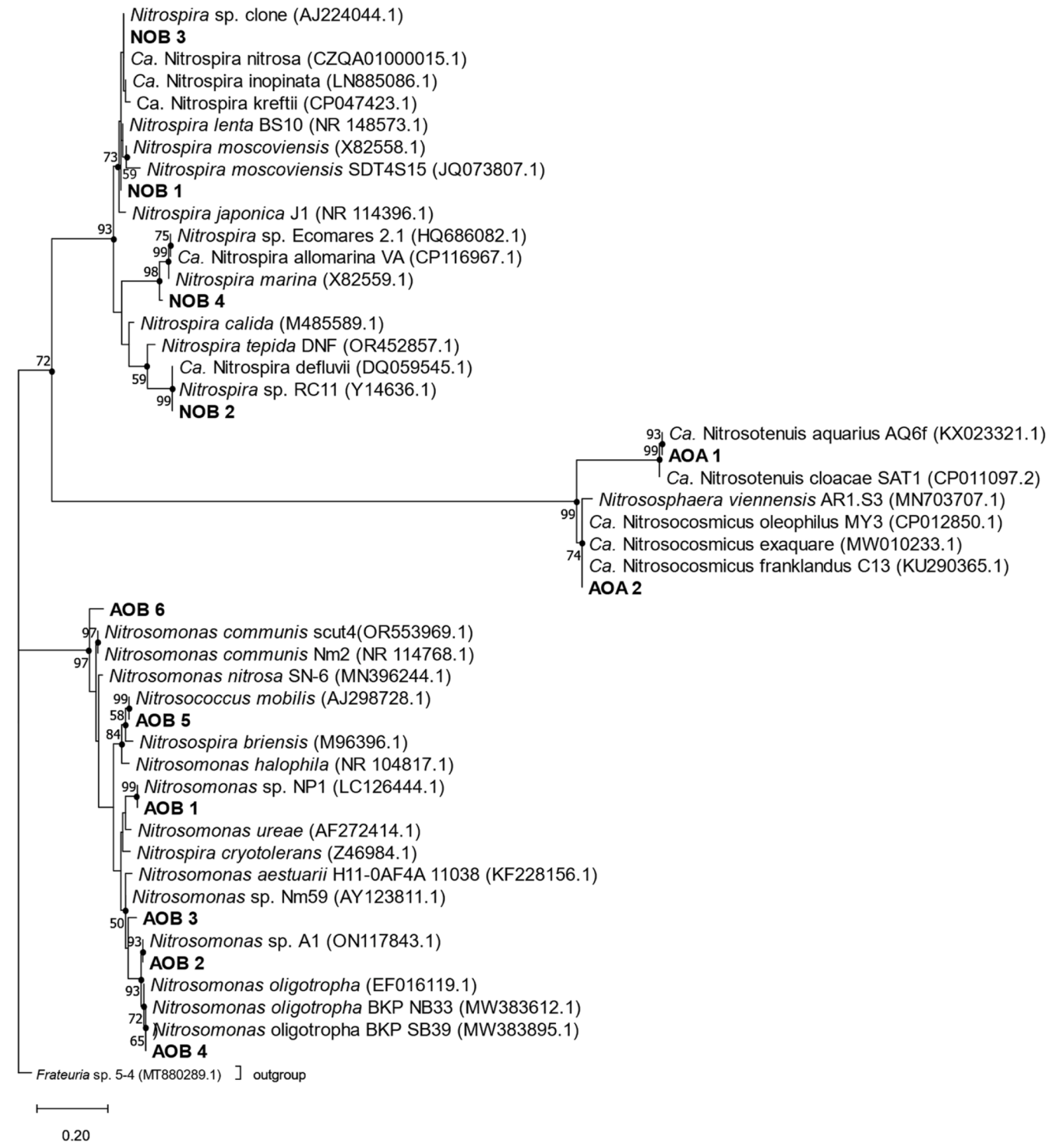

Fig. 3. Molecular phylogenetic analysis based on 16S rRNA gene sequences of nitrifying microorganisms. Bootstrap values higher than 50 % are indicated with black circles.
AOB_1: Nitrospmonas. sp, AOB_2: Nitrosomonas bacterium enrichment, AOB_3: Nitrosomonas uncultured, AOB_4: Nitrosomonas_ASV1, AOB_5: Nitrosomonas mobilis, AOB_6: Nitrosomonas communis, AOA_1: Nitrosotenuis uncultured, AOA_3, Nitrocosmicus_ASV1, NOB_1: Nitrospira_ASV1, NOB_2: Nitrospira defluvii, NOB_3: Nitrospira uncultured, NOB_4: Nitrospira unidentified, NOB_5: Nitrospira japonica

maximum of 87.6 % (at 387 days of operation). AOA_1 (*Nitrosotenuis*_uncultured) is closely related to *Candidatus* Nitrosotenuis aquarius found in freshwater, which has been reported to grow in an environment with pH of 6.8–7.3 and at an optimal temperature of 33 °C (Sauder et al., 2018). AOA_3 (*Nitrosocosmicus*_ASV1) was present at a maximum rate of 8.3 % (261 days of operation), and was confirmed to be closely related to *Candidatus* Nitrosocosmicus arcticus, a bacterium that inhabits Arctic soil and is reported to perform optimal NH_4^+-N oxidation at temperatures ranging 20–28 °C (Alves et al., 2019). During Phase 3, the influent water had a temperature of 19.8 ± 6.6 °C and pH of 7.7 ± 1.4. The reactor was operated in a 32 °C incubator, which facilitated nitrification, resulting in an effluent temperature of 28.4 ± 5.6 °C

and a pH of 7.1 ± 1.1. Additionally, circulating water with a pH of 6.9 ± 0.3 and a temperature of 30 ± 1.3 °C contributed to the optimization of the cultivation environment for AOA_1 and AOA_3. Therefore, the two spaces of AOA, particularly *Candidatus* Nitrosotenuis aquarius, were able to accumulate in the DHS reactor.

Among the NOB, NOB_1, NOB_2, and NOB_3 were present at ≥ 1.0 % abundance. NOB_2 (*N. defluvii*) is closely related to *N. defluvii*, a common NOB that grows in activated sludge and is reported to be dominant in environments with a pH of 6.4, while the population switches to other NOB in environments with a pH of 7.4 (Wegen et al., 2019). In the present study, *N. defluvii* was present in the seed sludge of the DHS reactor, with a maximum of 24.7 % on day 45 of operation; however, its presence was not confirmed on day 282 of operation. The latter could be caused by a population shift, since the inlet pH of this reactor was 7.6 ± 0.8, leading to a replacement of *N. defluvii* by other NOB.

All comammox bacteria were confirmed to be *Nitrospira* belonging to lineage II (Sakoula et al., 2021) and two species (NOB_1 and NOB_3) observed in this study belonged to lineage II. NOB_1 (*Nitrospira*_ASV1) and NOB_3 (*Nitrospira*_uncultured), which belong to the genus *Nitrospira*, were considered comammox bacteria because they are closely related. The NOB_1 presence rate increased to a maximum of 44.1 % (282 days of operation), confirming proliferation of comammox bacteria during operation of the DHS reactor. It has been reported that reactors in which comammox bacteria predominated were cultivated in an environment with a pH of 7.0–7.5 and water temperatures of 21–25 °C (Koike et al., 2022; Zheng et al., 2022). The influent pH of this reactor was 7.6 ± 0.8 and the treated water temperature was 30 ± 3.3 °C, which were considered suitable conditions for culturing comammox bacteria. However, the water temperature in this study was > 5 °C higher than the optimal temperature; hence, it was considered necessary to maintain the water temperature at 20–25 °C to further accumulate comammox bacteria.

Metagenomic analysis

Metagenomic analysis yielded 60 bins, of which one was identified as *Candidatus* Nitrospira nitrosa. The bin of comammox *Nitrospira* exhibited a high degree of reliability, with 97.25 % completeness and 9.43 % contamination, and accounted for a relative abundance of 1.84 %. Therefore, the uncultivated species of *Nitrospira* was considered to be a Comammox bacterium closely related to "*Ca N. nitrosa*.". *Ca N. nitrosa* is widely distributed in wastewater treatment plants (Zheng et al., 2023) and is the most important Comammox bacterium in wastewater treatment. The relative abundance of bin in "*Candidatus* Nitrosotenuis aquarius" with a completeness of 99.97 % and contamination of 1.28 % accounted for 16.57 %. These results suggest that Comammox bacteria were detected by nitrogen removal in the DHS reactor and coexist with AOA and contribute to nitrification in the reactor.

Redundancy analysis

Fig. 4 shows the Redundancy Analysis (RDA) results of the reactor inflow characteristics (DO, pH, Temp., total nitrogen [TN], and NH_4^+-N) and the abundance ratio of the uncultivated *Nitrospira* species, *N. defluvii*, AOB, and AOA in the microbial community of the DHS. The axes of RDA1 and RDA2 showed contribution rates of 44.9 % and 10.2 %, respectively

N. defluvii corresponded to NH_4^+-N and DO; whereas no correlation was observed between NH_4^+-N and DO in the uncultivated *Nitrospira* (possibly comammox) and AOA. Other environmental variables (e.g., pH and TN) did not contribute to the abundance ratios of *Nitrospira, N. defluvii*, AOB, or AOA. The microbial species that proliferated differed depending on the environmental conditions within the RDA range used. In contrast, uncultivated *Nitrospira* and AOA plotted close to each other. Therefore, a correlation existed between the increase and decrease in the abundance ratio of uncultivated *Nitrospira* and AOA. Comparison of the

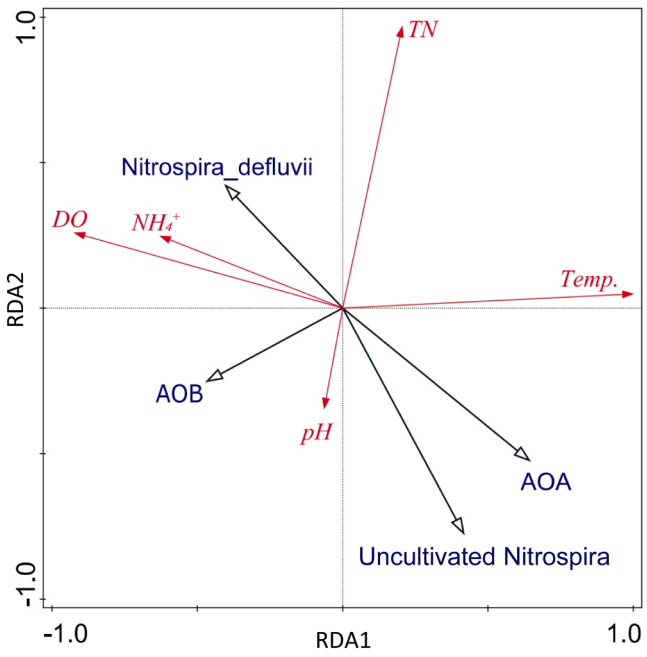

Fig. 4. Redundancy Analysis (RDA) results for nitrifying microorganisms and environmental factors. DO – dissolved oxygen; AOB - ammonia-oxidizing bacteria; AOA – ammonia-oxidizing archaea, Uncultivated Nitrospira: NOB_1, 3, 4.

abundance ratios of AOA and NOB after 322 days of operation in Fig. 1 confirmed that the abundance ratio of NOB decreased in samples in which the abundance ratio of AOA increased, and that of NOB increased in samples in which that of AOA decreased. Uncultivated *Nitrospira* are possibly comammox bacterium, and therefore they are thought to compete with AOA for NH_4^+-N oxidation. However, although AOA accounted for the majority of NH_4^+-N oxidation, uncultivated *Nitrospira* was present in > 4.7 % of all sampled areas. These findings suggest that uncultivated *Nitrospira* spp. and AOA may have coexisted symbiotically..

N_2O production from DHS reactor

Fig. 5 shows the dissolved N_2O production from the DHS reactor determined during days 380 to 382. Dissolved N_2O (0.6 ± 0.3 mg-N·L^{-1}) was detected over a period of approximately 3 days. The amount of dissolved N_2O emitted per unit of NH_4^+-N removed by this reactor was 0.02 ± 0.01 kg N_2O—N·kg NH_4^+-N^{-1}. The 2006 IPCC Guidelines for Greenhouse Gas Inventory report that the N_2O conversion rate associated with natural wastewater decomposition is 0.5–250 kg N_2O—N·kg NH_4^+-N^{-1} (Eggleston et al., 2006). In addition, in a wastewaster treatment plant that removes nitrogen using conventional activated sludge process, the nitrous oxide generation coefficient around the removed nitrogen was a maximum of 0.253 kg N_2O—N kg-N^{-1} and an average of 0.035 ± 0.027 kg N_2O—N kg-N^{-1} (Foley et al., 2010). In a full-scale A^2O

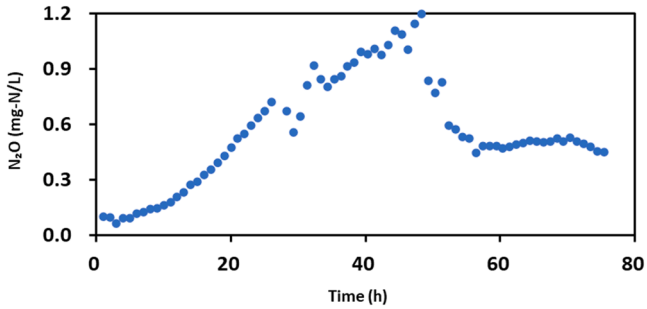

Fig. 5. Total emission of dissolved nitrous oxide on day 380 to day 382.

reactor for nitrogen treatment in Sydney, dissolved nitrous oxide emissions ranged from 0.010 to 0.018 kg N_2O—N per kg-N, depending on the process stage (Foley et al., 2010). In the typical partial nitrification process that forms part of the anammox process, half of the ammonia is converted into nitrite, which accumulates and serves as a precursor for the formation of nitrous oxide in both the gaseous and liquid phases. In the typical partial nitrification process, which serves as the pre-treatment stage for the anammox process, approximately half of the ammonia is oxidized to nitrite. This nitrite accumulation triggers the generation of nitrous oxide in both the gaseous and liquid phases. Kong et al. reported that during partial nitrification, a maximum dissolved nitrous oxide concentration of 21.44 μmol L^{-1}—equivalent to approximately 0.944 mg L^{-1}—was detected during aeration (Kong et al., 2013). The reactor exhibited a significantly lower amount of dissolved N_2O emitted per unit of NH_4^+-N removed than both the N_2O conversion rate specified in the IPCC guidelines and the emissions observed in conventional activated sludge and partial nitrification processes, yet it was higher than that of the A^2O process, which achieved complete nitrification and denitrification. Therefore, nitrogen removal by operating the DHS reactor could contribute to reducing N_2O emissions. Increasing the aeration volume has been cited as a method for reducing N_2O generation in the nitrification process (Wu et al., 2014). However, owing to problems of increased carbon dioxide production and operating costs associated with increased aeration volume, aeration measures are not considered a useful means by which to reduce greenhouse gases. In this regard, this study confirmed that DHS reactors are easy to operate and emit less N_2O per unit of NH_4^+-N removed than the conventional activated sludge process without any aeration. Therefore, introduction of a DHS reactor in the nitrification process is considered highly effective in suppressing N_2O emissions.

On the day when dissolved N_2O measurements began (380 days of operation), the abundance ratios of the AOA genus *Nitrosotenuis* (88 % on day 387 of operation) and the uncultured comammox bacteria genus *Nitrospira* (11 % on day 387 of operation) increased, which is thought to have contributed to N_2O production. The main N_2O production pathway during aerobic NH_4^+-N oxidation is aerobic denitrification of NO_2^--N by AOB, and AOA is thought to produce less N_2O than AOB (Hink et al., 2018). The amount of N_2O produced by comammox bacteria is approximately 3–15 % of that produced by AOA (Li et al., 2019). The results of the present study confirm that the DHS reactor suppressed N_2O generation. Nevertheless, dissolved and gaseous N_2O emissions have been confirmed not only from the nitrification process but also from denitrification processes characterized by low COD/N ratios and low DO concentrations, as well as from phosphorus removal systems in which glycogen-accumulating organisms (GAOs) proliferate (Kampschreur et al., 2009). The DHS reactor operates as an open system; additional research is necessary to elucidate N_2O generation including environmental conditions and types of microorganisms enriched in greater detail.

Conclusions

In this study, a DHS reactor utilizing low-strength ammonia-based synthetic wastewater was employed to cultivate comammox bacteria and assess the nitrogen removal efficiency and microbial community composition. The findings demonstrated a high NH_4^+-N removal efficiency (98 ± 4 %) and complete nitrification throughout the experimental period. 16S rRNA gene sequencing and metagenomic analysis revealed that comammox-like *Nitrospira* predominated in the sludge retained in the DHS and that comammox-like *Nitrospira* and AOA potentially coexisted symbiotically. N_2O emissions per NH_4^+-N removed from the DHS reactor were substantially lower than those of conventional nitrification processes, suggesting that the DHS reactor could be effective in mitigating N_2O emissions.

Materials and methods

Laboratory-scale DHS reactor

The DHS reactor was a 21-L column installed in an incubator at 32 °C. DHS sponges (33 mm on the side) were used as microorganism carriers (Oshiki et al., 2022). The 250 sponge carriers were randomly filled with a sponge volume of 7.0 L (Figure. S-1). Carriers designed to retain biomass were created using polyurethane sponge, which was shaped into a cylindrical form (33 mm in both diameter and length) and encased in a polypropylene mesh for support (Okubo et al., 2016). The sponge carriers employed in the DHS reactor exhibited a void ratio > 90 %, suggesting that they contained a considerable amount of unoccupied space, and had a surface area of 1.87 $cm^2 \cdot cm^{-3}$ (Okubo et al., 2016; Watari et al., 2022). The pH of the substrate was adjusted to 7.5–8.5. The substrate composition was based on a previous study (van de Graaf et al., 1996). The NH_4^+-N concentration and pH were adjusted to 30 mg $N \cdot L^{-1}$ and 7.5−8.5, respectively, by adding $(NH_4)_2SO_4$ and KH_2PO_4 and $NaHCO_3$, respectively. $NaHCO_3$ supplied carbon dioxide as an inorganic carbon source for nitrifying bacteria while simultaneously mitigating the decline in pH. The substrate tank was stored indoors and fed using a pump (Masterflex L/S, USA). The treated water was circulated to enhance water distribution and nitrification (Watari et al., 2017). The HRT was calculated based on the sponge volume and set to 7.5 h. To prevent drying of the DHS in the reactor, the treated water was circulated at a flow rate of 0.84 L/min. The nitrogen-loading rate of this study was 0.1 kg $N \cdot L^{-1} \cdot day^{-1}$. Activated sludge (300 mL; 3.12 g-MLSS·L^{-1}) collected from the Nagaoka Central Purification Center, Japan, was used for seeding.

Water quality analysis

Water quality analyses were performed on the reactor influent and effluent. pH and DO were measured using a portable pH meter (MM-42DP, TOA DKK) and DO meter (HQ30d, HACH), respectively. NH_4^+-N was analyzed using the Nessler method (DR3900, HACH) after filtration through a 0.22 μm filter. NO_2^--N and NO_3^--N were measured using a continuous-flow analyzer (Auto Analyzer QuAAtro19; BL TEC.) after filtering through a 0.22-μm membrane filter.

Microbial community analysis

Microbial community analysis was conducted on sponge carriers sampled from various heights in the reactor. These samples were preserved at −20 °C until examination. Prior to DNA extraction, an ultrasonic homogenizer (VCX 130 PB; Sonics & Materials Inc., Newtown, CT, USA) was used to disaggregate the sludge up for 1–2 s. Samples were then washed with phosphate-buffered saline. DNA was extracted from the sludge using a Fast DNA SPIN Kit for Soil (MP Biomedicals, Irvine, CA, USA). The extracted DNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). To gather genetic material from all bacteria and archaea in the samples, the universal forward primer Univ515F and universal reverse primer Univ806R were used for targeted amplification of the 16S rRNA gene. A QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) was used to purify the PCR products and eliminate residual primers and undesired components. The 16S rRNA gene was subjected to massive parallel sequencing using an Illumina MiSeq System (San Diego, CA, USA) following the protocol outlined in a previous study (Caporaso et al., 2012). The sequence reads obtained from the MiSeq System were analyzed using Quantitative Insights into Microbial Ecology (version 2022.8) (QIIME 2), a software package designed specifically for microbiome analysis (Caporaso et al., 2010). Operational taxonomic units (OTUs) were established by clustering similar sequences based on a 97 % sequence identity threshold, which enabled identification of distinct microbial taxa in samples. The Silva 138 99 % OTUs from 515F/806R

was used for taxonomic classification to provide information on the detected microbial species or groups.

The 16S rRNA gene amplicon sequences ware subjected to an NCBI BLAST search to identify closely related species. Maximum likelihood phylogenetic tree of 16S rRNA sequences obtained from samples and NCBI using Mega 11 software. The General Time reversible model was used as the base substitution model. The Phylogeny Test was repeated 500 times using the Bootstrap method. The numbers in the nodes indicate the Bootstrap value of each branch.

Metagenomic analysis was performed on DHS-retained sludge samples obtained on day 196. Sequences were obtained using DNBSEQ-G400 (MGI Tech). Low quality sequences were trimmed using Fastp (v0.20.1) (-W 6 -M 30). Filtered reads were assembled using SPAdes assembler (v3.15.5). Metabat (ver2.15) was applied to the metagenomic assembly and generated sets of bins. Completeness and contamination of the final bins were assessed using CheckM (v1.0.12). Taxonomic classification of the final bins was performed using GTDB-Tk (v2.1.1) with the GTDB_r207 database as a reference.

RDA was performed using CANOCO (version 4), in which correlations between the inflow characteristics of the reactor (DO, pH, Temp., TN, and NH_4^+-N) and the abundance of uncultivated *Nitrospira, N. defluvii*, AOB, and AOA in the microbial community of the DHS were investigated.

N_2O emission measurements

To determine the amount of dissolved N_2O emitted from the reactor, a microsensor (Unisense, Aarhus, Denmark) was placed at the reactor outlet, and the amount of dissolved N_2O produced was continuously monitored for 3 days from Day 380 to Day 382. Dissolved N_2O was automatically recorded at 1-s intervals, and the concentration was converted using a calibration curve.

Funding

This research was financially supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science [Grant numbers 22K14349, 24K01011].

CRediT authorship contribution statement

Sota Kabasawa: Writing – original draft, Methodology, Investigation, Conceptualization. **Takahiro Watari:** Writing – original draft, Supervision, Investigation, Funding acquisition, Conceptualization. **Yuki Sato:** Software, Investigation. **Yuga Hirakata:** Writing – review & editing, Software, Methodology, Formal analysis. **Masashi Hatamoto:** Writing – review & editing. **Tsutomu Okubo:** Writing – review & editing. **Carols Lopez Vazquez:** Writing – review & editing, Conceptualization. **Jules B. van Lier:** Writing – review & editing, Conceptualization. **Takashi Yamaguchi:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Takahiro Watari reports financial support was provided by Japan Society for the Promotion of Science. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.wroa.2025.100324.

Data availability

Data will be made available on request

References

Akamine, T., Nagai, M., Watari, T., Netsu, H., Adlin, N., Satanwat, P., Riquelme, C., Hatamoto, M., Yamaguchi, T., 2024. Nitrification characteristics and microbial community changes during conversion of freshwater to seawater in down-flow hanging sponge reactor. Ecoto. And Environ. Saf. 283, 116839. https://doi.org/10.1016/j.ecoenv.2024.116839.

Alves, R.J.E., Kerou, M., Zappe, A., Bittner, R., Abby, S.S., Schmidt, H.A., Pfeifer, K., Schleper, C., 2019. Ammonia oxidation by the Arctic terrestrial thaumarchaeote *Candidatus* Nitrosocosmicus arcticus is stimulated by increasing temperatures. Front. Microbiol. 10, 1571. https://doi.org/10.3389/fmicb.2019.01571.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods. 7, 335–336. https://doi.org/10.1038/nmeth.f.303.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G., Knight, R., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J 6, 1621–1624. https://doi.org/10.1038/ismej.2012.8.

Coskuner, G., Jassim, M.S., 2008. Development of a correlation to study parameters affecting nitrification in a domestic wastewater treatment plant. J. Chem. Technol. Biotechnol. 83, 299–308.

Cotto, I., Dai, Z., Huo, L., Anderson, C.L., Vilardi, K.J., 2020. Long solids retention times and attached growth phase favor prevalence of comammox bacteria in nitrogen removal systems. Water Res 169, 115268. https://doi.org/10.1016/j.watres.2019.115268.

Deng, L., Guo, W., Ngo, H.H., Zhang, X., Wang, X.C., Zhang, Q., Chen, R., 2016. New functional biocarriers for enhancing the performance of a hybrid moving bed biofilm reactor–membrane bioreactor system. Bioresour. Technol. 208, 87–93. https://doi.org/10.1016/j.biortech.2016.02.057.

Eggleston, H.S., Buendia, L., Miwa, K., Ngara, T., Tanabe, K. 2006. 2006 IPCC guidelines for national greenhouse gas inventories. http://www.ipcc-nggip.iges.or.jp/public/2006gl/index.htm.

Egli, K., Langer, C., Siegrist, H.R., Zehnder, A.J.B., Wagner, M., van der Meer, J.R., 2003. Community analysis of ammonia and nitrite oxidizers during start-up of nitritation reactors. Appl. Environ. Microbiol. 69, 3213–3222. https://doi.org/10.1128/AEM.69.6.3213-3222.2003.

Foley, J., Haas, D., Yuan, Z., Lant, P., 2010. Nitrous oxide generation in full-scale biological nutrient removal wastewater treatment plants. Water Res 44, 831–844. https://doi.org/10.1016/j.watres.2009.10.033.

Huang, T., Xia, J., Liu, T., Su, T., Guan, Y., 2022. Comammox nitrospira bacteria are dominant ammonia oxidizers in mainstream nitrification bioreactors emended with sponge carriers. Environ. Sci. Technol. 56 (17), 12584–12591. https://doi.org/10.1021/acs.est.2c03641.

Hink, L., Gubry-Rangin, C., Nicol, G.W., Prosser, J.I., 2018. The consequences of niche and physiological differentiation of archaeal and bacterial ammonia oxidisers for nitrous oxide emissions. ISME J 12 (4), 1084–1093. https://doi.org/10.1038/s41396-017-0025-5.

Kampschreur, M.J., Temmink, H., Kleerebezem, R., Jetten, M.S.M., van Loosdrecht, M.C. M., 2009. Nitrous oxide emission during wastewater treatment. Water Res 43, 4093–4103. https://doi.org/10.1016/j.watres.2009.03.001.

Kirishima, Y., Choeisai, P., Khotwieng, W., Hatamoto, M., Watari, T., Choeisai, K., Panchaban, P., Wong-Asa, T., Yamaguchi, T., 2022. Efficiency of high rate treatment of low-strength municipality sewage by a pilot-scale combination system of a sedimentation tank and a down-flow hanging sponge reactor. Environ. Technol. 43, 2457–2466. https://doi.org/10.1080/09593330.2021.1882584.

Kits, K.D., Jung, M.Y., Vierheilig, J., Pjevac, P., Sedlacek, C.J., Liu, S., Herbold, C., Stein, L.Y., Richter, A., Wissel, H., Brüggemann, N., Wagner, M., Daims, H., 2019. Low yield and abiotic origin of N_2O formed by the complete nitrifier Nitrospira inopinata. Nat. Commun. 10, 1836. https://doi.org/10.1038/s41467-019-09790-x.

Koike, K., Smith, G.J., Yamamoto-Ikemoto, R., Lücker, S., Matsuura, N., 2022. Distinct comammox Nitrospira catalyze ammonia oxidation in a full-scale groundwater treatment bioreactor under copper limited conditions. Water Res 210, 117986. https://doi.org/10.1016/j.watres.2021.117986.

Kubota, K., Hayashi, M., Matsunaga, K., Iguchi, A., Ohashi, A., Li, Y.Y., Yamaguchi, T., Harada, H., 2014. Microbial community composition of a down-flow hanging sponge (DHS) reactor combined with an up-flow anaerobic sludge blanket (UASB) reactor for the treatment of municipal sewage. Bioresour. Technol. 151, 144–150. https://doi.org/10.1016/j.biortech.2013.10.058.

Kubota, K., Otani, T., Hariu, T., Jin, T., Tagawa, M., Morikawa, M., Li, Y., 2024. Evaluation of sewage treatment performance and duckweed biomass production efficiency in a primary sedimentation basin + duckweed pond + downflow hanging sponge (PSB + DWP + DHS) system. J. Water Process Eng. 65, 105818. https://doi.org/10.1016/j.jwpe.2024.105818.

Li, C., Hu, H.W., Chen, Q.L., Chen, D., He, J.Z., 2019. Comammox Nitrospira play an active role in nitrification of agricultural soils amended with nitrogen fertilizers. Soil Biol. Biochem. 138. https://doi.org/10.1016/j.soilbio.2019.107609.

Nakagawa, T., Koji, M., Hosoyama, A., Yamazoe, A., Tsuchiya, Y., Ueda, S., Takahashi, R., Stahl, D.A., 2021. Nitrosopumilus zosterae sp. nov., an autotrophic ammonia-oxidizing archaeon of phylum Thaumarchaeota isolated from coastal eelgrass sediments of Japan. Int. J. Syst. Evol. Microbiol. 71. https://doi.org/10.1099/ijsem.0.004961.

Nikolaev, Yu.A., Kozlov, M.N., Kevbrina, M.V., Dorofeev, A.G., Aseeva, V.G., Pimenov, N.V., Zharkov, A.V., 2015. Study of the low temperature anoxic ammonia oxidation feasibility. Water Prac. Technol. 10, 172–177. https://doi.org/10.2166/wpt.2015.019.

Nowka, B., Off, S., Daims, H., Spieck, E., 2014. Improved isolation strategies allowed the phenotypic differentiation of two Nitrospira strains from widespread phylogenetic lineages. FEMS Microbiol. Ecol. 91, fiu031. https://doi.org/10.1093/femsec/fiu031.

Okubo, T., Kubota, K., Yamaguchi, T., Uemura, S., Harada, H., 2016. Development of a new non-aeration-based sewage treatment technology: performance evaluation of a full scale down-flow hanging sponge reactor employing third-generation sponge carriers. Water Res 102, 138–146. https://doi.org/10.1016/j.watres.2016.06.035.

Oshiki, M., Netsu, H., Kuroda, K., Narihiro, T., Fujii, N., Kindaichi, T., Suzuki, Y., Watari, T., Hatamoto, M., Yamaguchi, T., Araki, N., Okabe, S., 2022. Growth of nitrite-oxidizing Nitrospira and ammonia-oxidizing Nitrosomonas in marine recirculating trickling biofilter reactors. Environ. Microbiol. 24, 3735–3750. https://doi.org/10.1111/1462-2920.16085.

Peng, Y.Z., Zhu, G.B., 2006. Biological nitrogen removal with nitrification and denitrification via nitrite pathway. Appl. Environ. Microbiol. 73, 15–26. https://doi.org/10.1007/s00253-006-0534-z.

Pommerening-Röser, A., Rath, G., Koops, H.P., 1996. Phylogenetic diversity within the genus Nitrosomonas. Syst. Appl. Microbiol. 19, 344–351. https://doi.org/10.1016/S0723-2020(96)80061-0.

Kong, Q., Liang, S., Zhang, J., Xie, H., Miao, M., Tian, L., 2013. N_2O emission in a partial nitrification system: dynamic emission characteristics and the ammonium-oxidizing bacteria community. Bioresour. Technol. 127, 400–406. https://doi.org/10.1016/j.biortech.2012.10.011.

Sauder, L.A., Engel, K., Lo, C.C., Chain, P., Neufeld, J.D., 2018. Candidatus Nitrosotenuis aquarius,' an Ammonia-Oxidizing Archaeon from a Freshwater Aquarium biofilter. Appl. Environ. Microbiol. 84, e01430. https://doi.org/10.1128/AEM.01430-18. -18.

Sakoula, D., Koch, H., Frank, J., Jetten, M.S., van Kessel, M.A., Lücker, S., 2021. Enrichment and physiological characterization of a novel comammox Nitrospira indicates ammonium inhibition of complete nitrification. ISME J 15 (4), 1010–1024. https://doi.org/10.1038/s41396-020-00827-4.

Tanikawa, D., Seo, S., Motogawa, D., 2020. Development of a molasses wastewater treatment system equipped with a biological desulfurization process. Environ. Sci. Pollut. Res. 27, 24738–24748. https://doi.org/10.1007/s11356-019-07077-8.

Tuttle, A.R., Trahan, N.D., Son, M.S., 2021. Growth and maintenance of Escherichia coli laboratory strains. Curr. Protoc. 1 e20. https://doi.org/10.1002/cpz1.20.

Tyagi, V.K., Ali, M., Tawfik, A., Maharjan, N., Kazmi, A.A., Okubo, T., Harada, H., 2021. Future perspectives of energy saving down-flow hanging sponge (DHS) technology for wastewater valorization—a review. Rev. Environ. Sci. Bio Technol. 20, 389–418. https://doi.org/10.1007/s11157-021-09573-1.

van de Graaf, A.A., de Bruijn, P., Robertson, L.A., Jetten, M.S.M., Kuenen, J.G., 1996. Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor. Microbiology 142, 2187–2196. https://doi.org/10.1099/13500872-142-8-2187.

van Kessel, M.A.H.J., Speth, D.R., Albertsen, M., Nielsen, P.H., op den Camp, H.J.M., Kartal, B., Jetten, M.S.M., Lücker, S., 2015. Complete nitrification by a single microorganism. Nature 528, 555–559. https://doi.org/10.1038/nature16459.

Watari, T., Kirishima, Y., Choeisai, P., Harada, H., Kotcharon, W., Matsueda, T., Tanaka, N., Kawakami, S., Hatamoto, M., Yamaguchi, T., 2022. Performance evaluation of quick and compact package-type down-flow hanging sponge system for domestic sewage treatment. J. Water Process Eng. 47. https://doi.org/10.1016/j.jwpe.2022.102798.

Watari, T., Kirishima, Y., Choeisai, P., Thepubon, T., Hirakata, Y., Matsueda, T., Nagano, A., Yamaguchi, T., Okubo, T., Hatamoto, M., Yamaguchi, T., 2024. Distinct comammox Nitrospira in high rate down-flow hanging sponge reactor treating municipal wastewater. Bioresour. Technol. 408, 131160. https://doi.org/10.1016/j.biortech.2024.131160.

Watari, T., Mai, T.C., Tanikawa, D., Hirakata, Y., Hatamoto, M., Syutsubo, K., Fukuda, M., Nguyen, N.B., Yamaguchi, T., 2017. Performance evaluation of the pilot scale upflow anaerobic sludge blanket – Downflow hanging sponge system for natural rubber processing wastewater treatment in South Vietnam. Bioresour. Technol. 237, 204–212. https://doi.org/10.1016/j.biortech.2017.02.058.

Watari, T., Vazquez, C.L., Hatamoto, M., Yamaguchi, T., van Lier, J.B., 2021a. Development of a single-stage mainstream anammox process using a sponge-bed trickling filter. Environ. Technol. 42, 3036–3047. https://doi.org/10.1080/09593330.2020.1720309.

Watari, T., Nakamura, Y., Kotcharoen, W., Hirakata, Y., Satanwat, P., Pungrasmi, W., Powtongsook, S., Takeuchi, Y., Hatamoto, M., Yamaguchi, T., 2021b. Application of down-flow hanging sponge – Upflow sludge blanket system for nitrogen removal in *Epinephelus bruneus* closed recirculating aquaculture system. Aquaculture 532, 735997. https://doi.org/10.1016/j.aquaculture.2020.735997.

Wegen, S., Nowka, B., Spieck, E., 2019. Low temperature and neutral pH define "Candidatus Nitrotoga sp." as a competitive nitrite oxidizer in coculture with *Nitrospira defluvii*. Appl. Environ. Microbiol. 85. https://doi.org/10.1128/AEM.02569-18.

Wu, G., Zheng, D., Xing, L., 2014. Nitritation and N2O emission in a denitrification and nitrification two-sludge system treating high ammonium containing wastewater. Water (Basel) 6 (10), 2978–2992. https://doi.org/10.3390/w6102978.

Zhao, J., Zheng, M., Su, Z., Liu, T., Li, J., Guo, J., Yuan, Z., Hu, S., 2022. Selective enrichment of comammox Nitrospira in a moving bed biofilm reactor with sufficient oxygen supply. Environ. Sci. Technol. 56, 13338–13346. https://doi.org/10.1021/acs.est.2c03299.

Zheng, M., Mu, G., Zhang, A., Wang, J., Chang, F., Niu, J., Wang, X., Gao, T., Zhao, Z., 2022. Predominance of comammox bacteria among ammonia oxidizers under low dissolved oxygen condition. Chemosphere 308, 136436. https://doi.org/10.1016/j.chemosphere.2022.136436.

Zheng, M., Tian, Z., Chai, Z., Zhang, A., Gu, A., Mu, G., Wu, D., Guo, J., 2023. Ubiquitous occurrence and functional dominance of comammox Nitrospira in full-scale wastewater treatment plants. Water Res. 236, 119931. https://doi.org/10.1016/j.watres.2023.119931.