



## Nitrification characteristics and microbial community changes during conversion of freshwater to seawater in down-flow hanging sponge reactor

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### ABSTRACT

In recirculating aquaculture systems (RAS), maintaining water quality in aquaculture tanks is a paramount factor for effective fish production. A down-flow hanging sponge (DHS) reactor, a trickling filter system used for water treatment of RAS that employs sponges to retain biomass, has high nitrification activity. However, nitrification in seawater RAS requires a long start-up time owing to the high salinity stress. Therefore, this study aimed to evaluate the nitrification characteristics and changes in the microbial community during the conversion of freshwater to seawater in a DHS reactor fed with ammonia-based artificial seawater. The total ammonia nitrogen concentration reached  $1.0 \text{ mg-N-L}^{-1}$  (initial concentration  $10 \text{ mg-N-L}^{-1}$ ) within 11 days of operation, and nitrate production was observed. The 16 S rRNA gene sequence of the DHS-retained sludge indicated that the detection rate of the ammonia-oxidizing archaeon *Candidatus Nitrosocosmicus* decreased from 23.9 % to 14.0 % and 25.8–17.6 % in the upper and lower parts of the DHS reactor, respectively, after the introduction of seawater. In contrast, the nitrite-oxidizing bacteria *Nitrospira* spp. increased from 0.1 % to 9.5 % and from 0.5 % to 10.5 %, respectively. The ammonia oxidation rates of  $0.12 \pm 0.064$  and  $0.051 \pm 0.0043 \text{ mg-N-g-MLVSS}^{-1}\cdot\text{h}^{-1}$  on the 37th day in the upper and bottom layers, respectively. Thus, nitrification in the DHS reactor performed well, even under high-salinity conditions with short operational days. This finding makes the transition from freshwater to saltwater fish in the RAS system simple and economical, and has the potential for early start-up of the RAS.

### 1. Introduction

Food shortages have recently become a grave concern due to population growth, especially in developing countries. The world population has been estimated to reach 9.7 billion by 2050, making the establishment of sustainable food production methods an urgent necessity. In addition, the demand for fish and shellfish is increasing as they comprise essential proteins in the human diet. However, catches of fish and shellfish may decline owing to global warming, and the supply of marine products from capture fisheries in the ocean is limited. Therefore, aquaculture is becoming increasingly important for seafood production. Global aquaculture production accounts for approximately 50 % of the total fish production and is growing steadily (FAO 2020). However,

marine aquaculture may lead to environmental issues. For example, nutrient runoff from feed can result in eutrophication, and mangrove growth along rivers may need to be removed to secure land for aquaculture. The closed recirculating aquaculture system (RAS) is a sustainable food production method, particularly in areas with challenging geographical features such as mountainous and desert regions (Ahmed and Turchini, 2021). This method can substantially reduce environmental risks by recirculating 90–99 % of the aquaculture water used (Badiola et al., 2012). However, one shortcoming of RAS is the risk of fish mortality due to the accumulation of total ammonia nitrogen (TAN) and nitrite ( $\text{NO}_2^-$ -N) in enclosed spaces. Ammonia ( $\text{NH}_3$ ) and  $\text{NO}_2^-$ -N are highly toxic even at low concentrations (Losordo et al., 2000; Neori et al., 2004).  $\text{NH}_4^+$  levels should be below  $1.0 \text{ mg N-L}^{-1}$ , and  $\text{NH}_3$  levels

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should range from 0.01 to 0.5 mg N-L<sup>-1</sup>, as per Losordo et al. (1998). Therefore, biological nitrification processes in which ammonia-oxidizing bacteria (AOBs), ammonia-oxidizing archaea (AOAs), and nitrite-oxidizing bacteria (NOB) oxidize TAN and NO<sub>2</sub>-N to low toxic nitrate (NO<sub>3</sub>-N) are commonly used as a system (He et al., 2020; Lu et al., 2020; Ruiz et al., 2019).

Various water treatment devices are used in RAS, including physical filtration devices such as solid-liquid separators and sand filters, sterilization devices that use ozone or ultraviolet light to sterilize water, fluidized sand biofilters, moving bed biofilm reactors (MBBRs), sprinkler filter bed biofilm reactors, fixed bed biofilm reactors, and biological filtration devices such as rotating biological contactors (Xiao et al., 2019). Biological filtration devices are frequently used to control water quality because they are responsible for nitrification and denitrification reactions in RAS (Xiao et al., 2019). In addition, biological filtration has gained widespread adoption within RAS in recent years owing to its advantages, such as low cost and lack of chemicals (Tadda et al., 2021).

Our research group has been attempting to develop a water quality treatment system for RAS using a down-flow hanging sponge (DHS) reactor with a nitrification function in aquaculture tanks (Kotcharoen et al., 2021; Watari et al., 2021b). The DHS reactor was designed to serve as an affordable solution for sewage treatment in developing countries (Tyagi et al., 2021) and is used for treating domestic and industrial wastewater. In a DHS reactor, as water flows down the surface of the sponge carrier, it absorbs oxygen, facilitating nitrifying bacteria to conduct nitrification reactions inside the sponge (Onodera et al., 2016). In addition, the DHS reactor can be operated at low cost because it does not require aeration in the tank owing to its ability to supply dissolved oxygen (DO) through the sponge carrier (Uemura et al., 2016; Watari et al., 2021c). Recently, our research group successfully utilized denitrification and DHS reactors to operate both freshwater and marine aquaria and aquaculture systems (Adlin et al., 2022; Furukawa et al., 2016; Kotcharoen et al., 2021; Watari et al., 2021b). Watari et al. (2021b) reported that the DHS reactor could maintain a low TAN of 0.33 ± 0.12 mg-N-L<sup>-1</sup> in closed recirculating aquaculture tanks housing *Epinephelus bruneus*. Kotcharoen et al. (2021) used a combined system of DHS and up-flow sludge blanket (USB) reactor to rear *Epinephelus bruneus* and found that the DHS reactor could maintain a TAN value below 1.0 mg-N-L<sup>-1</sup> in the aquaculture tank at various salinity levels. However, initial water quality control of the seawater RAS is difficult because seawater has a longer start-up period for nitrification than freshwater (Navada et al., 2021; Zhang et al., 2023). Additionally, Zhang et al. (2023) found that moving bed systems treating freshwater RAS are more stable than seawater RAS, and partial nitrification was observed in seawater RAS. Navada et al. (2021) investigated nitrification performance in freshwater MBBRs with increasing salinity and reported that at any rate of salinity increase, nitrification performance decreased when the salinity of seawater (31‰–32‰) was reached, but recovered as acclimation time to seawater increased. Liu et al. (2019) reported that seawater-infused trickling filters have been reported to have a faster nitrification rate than MBBRs, and that inoculating MBBRs with their biocarriers causes a faster nitrification ramp-up of MBBRs (Liu et al., 2019). DHS reactors have been used for sewage treatment in various countries because of their start-up characteristics, and a study by Watari et al. (2022) reported that nitrification reactions started in approximately two weeks in a sewage-treating DHS reactor (Watari et al., 2022). High nitrification activity in the DHS reactor was observed after 13 days with the use of sponge biomass-retained carrier start-up (Kirishima et al., 2024). Rapid preparation of nitrifying bacteria is important in RAS, where ammonia accumulation is a problem. The rapid start-up of nitrification equipment is essential for an RAS, which is expected to expand its business in the future. The application of nitrifying bacteria from freshwater nitrification systems to seawater is a useful method (Zhang et al., 2023); however, to the best of our knowledge, the nitrification performance and microbial community change in the DHS reactor for marine RAS using freshwater-inoculated sponge carrier and

artificial seawater have not been reported. Artificial seawater is used for RAS to prevent pathogens bring from outside

To bridge these gaps in the literature, this study aimed to present the findings of a restart study that investigated the transection of DHS biomass to seawater conditions. The primary objective of this study was to develop start-up methods for marine RAS systems that employ freshwater DHS sponges coupled with NH<sub>4</sub>Cl addition to artificial seawater tanks to enhance nitrification. The start-up characteristics were determined by evaluating the nitrification performance of the DHS reactor for 37 days. Furthermore, this study aimed to characterize the nitrification rate of the microbial community present in the DHS reactor to better understand the nitrifying microorganisms.

## 2. Material and methods

### 2.1. DHS reactor and operating conditions

Fig. 1 shows a schematic of the DHS reactor used in this study, which consists of two layers, each with a volume of 20 L (total 40 L) and filled with 700 sponge carriers (sponge volume: 20 L; 10 L each in the upper and bottom layers). The aquarium tank and DHS reactor were placed in a large experimental building at Nagaoka University of Technology and operated at ambient temperature. The sponge carriers used were sourced from a freshwater RAS that reared Nile tilapia (*Oreochromis niloticus*) with stable nitrification performance at ambient temperature. A 33 mm × 33 mm × 33 mm cubic sponge, encased in a plastic net ring 33 mm in diameter and 33 mm in length, served as the biomass-retaining carrier. The sponge carrier had a pore size of 0.46 mm, a void ratio of 0.98, and a specific surface area of 1.87 cm<sup>2</sup>·cm<sup>-3</sup>. The aquarium tank had a total capacity of 5000 L. Artificial seawater was prepared using 2000 L of water and syntenic seawater salt (Marine Art, Tomita Pharmaceutical Co., Ltd.) with the salinity adjusted to 23‰–27‰ to prevent the introduction of pathogens into the RAS system. The syntenic seawater contained 1.01 w/v% sodium, 0.058 w/v% potassium, 0.119 w/v% magnesium, 0.033 w/v% calcium, 1.97 w/v% chlorine and 0.28 w/v% sulfuric acid. Simulated aquaculture water was fed to the top of the DHS reactor using a submerged pump (1260, Ehieim) at a flow

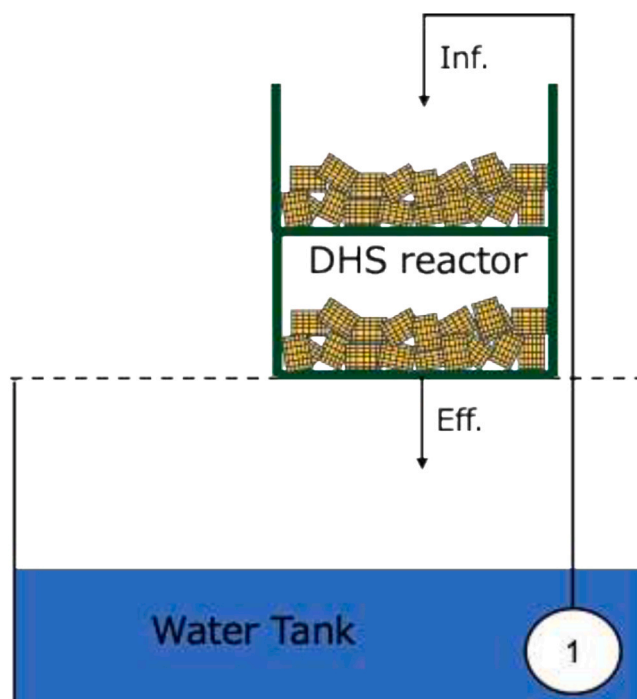


Fig. 1. Schematic of water tank and downflow hanging sponge (DHS) reactor. (1) Submerged pumps.

rate of 600 L·day<sup>-1</sup> and 300 L·day<sup>-1</sup>, respectively, at phase 1 and phase 2. The hydraulic retention time (HRT) was calculated based on the sponge volume. The HRT of the DHS reactor was set at 1.8 min for the first 20 days of operation (Phase 1) and changed to 3.75 min after the 21st day (Phase 2). When the TAN concentration was decreased to 1.0 mg·N·L<sup>-1</sup>, NH<sub>4</sub>Cl was added to achieve a concentration of 10 mg·N·L<sup>-1</sup>. The pH was maintained above 7.0 by adding 100 mg·L<sup>-1</sup> of NaHCO<sub>3</sub>.

## 2.2. Chemical analysis

The samples were collected daily from the seawater tank, inflow (inf), and outflow (eff) of the DHS reactor. DO and water temperature were measured using a DO meter (HQ30d; HACH). The salinity was determined using a handheld salinity meter (MASTER-S28α; Atago). Water samples for TAN, NO<sub>2</sub><sup>-</sup>-N, and NO<sub>3</sub><sup>-</sup>-N measurements were filtered through a 0.22 μm membrane filter immediately after collection and stored at 4°C until measurements. TAN was measured using the Nessler method (DR3900; HACH), and NO<sub>2</sub><sup>-</sup>-N and NO<sub>3</sub><sup>-</sup>-N were measured using an autoanalyzer (QuAAatro39; BLTEC).

## 2.3. Nitrification rate test

On days 30 and 37 of operation, sponge carriers were collected from the center of the upper and bottom layers of the DHS reactor, suspended in 20 mL of milli-q (MQ) water, centrifuged, washed with phosphate-buffered saline (PBS), and used as the seeding solution for nitrification rate tests. The tests were conducted using 190 mL of artificial seawater and 10 mL of seedling solution in a 300-mL triangular flask. NH<sub>4</sub>Cl was added as the ammonia source at an initial concentration of 10 mg·L<sup>-1</sup>. The nitrification rate was calculated using the following equation:

$$\text{Nitrification rate} = (\text{NO}_2\text{-N effluent (mg-N}\cdot\text{L}^{-1}) + \text{NO}_3\text{-N effluent (mg-N}\cdot\text{L}^{-1}) / \text{Time course (hours)} / \text{MLSS of the retained sludge (g-MLVSS}^{-1}) \quad (1)$$

## 2.4. Microbial community structure analysis based on 16 S rRNA genes

For the microbial community structure analysis, sponges in the DHS reactor were collected from the center of the upper and bottom layers, and the microorganisms attached to the sponge carriers were suspended and squeezed in 20 mL of ultrapure water and used as samples. The samples were centrifuged, and the supernatant was discarded. Subsequently, the samples were washed with PBS and stored at -20°C until DNA extraction. DNA extraction was performed according to DNeasy PowerSoil Pro-Soil - IRT using QIAcube Connect. The extracted DNA was adjusted to the appropriate Tris-EDTA buffer (Nippon Gene). The concentration of extracted DNA was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific). The obtained DNA was analyzed by 16 S rRNA PCR amplification using the 515 F-806R primer pair for the 16 S rRNA gene using the QIAcube Connect (Watari et al., 2021b). The PCR conditions were 95°C for 3 min and 25 cycles of 95°C for 45 s, 50°C for 1 min, 72°C for 1.5 min; and 72°C for 10 min. PCR products were purified according to the Cleanup - QIAquick PCR - Amplification reactions - Standard using QIAcube Connect and DNA extraction. The purified products were analyzed using a next-generation sequencer (iSeq 100, Illumina), and the obtained gene sequences were analyzed using QIIME2 to produce high-resolution Amplicon Sequence Variants (ASVs). Taxonomic classification of the inferred ASVs was conducted using the SILVA reference database version 132. The Basic Local Alignment Search Tool and National Center for Biotechnology Information (NCBI) database were used to identify closely related species.

## 3. Results and discussion

### 3.1. Nitrogen removal performance

The pH, DO, and water temperature in the seawater tank were maintained at 7.4 ± 0.5, 8.4 ± 0.4 mg·L<sup>-1</sup>, and 21.1 ± 1.7°C, respectively, during the experiment. TAN exhibited a decreasing trend approximately 1 week after starting the operation, and the TAN of the tank was 0.25 mg·N·L<sup>-1</sup> on day 11 (Fig. 2). Similarly, NO<sub>2</sub><sup>-</sup>-N increased from the 10th day of operation, and NO<sub>2</sub><sup>-</sup>-N in the tank was 23.44 mg·N·L<sup>-1</sup> on the 20th day. Therefore, NO<sub>2</sub><sup>-</sup>-N was partially nitrified to NO<sub>3</sub><sup>-</sup>-N by AOBs and AOAs in the DHS reactor. The increase in NO<sub>2</sub><sup>-</sup>-N up to the 20th day of operation may be attributed to the lack of nitrification of NO<sub>2</sub><sup>-</sup>-N to NO<sub>3</sub><sup>-</sup>-N, due to the non-proliferation of NOBs. Previous studies have reported that the growth of NOBs is inhibited by salinity (de Kreuk et al., 2005; Liu et al., 2019; Zhang et al., 2023). Additionally, the growth rate of NOBs is slower than that of AOBs (Fang et al., 2009; Moussa et al., 2005). No such partial nitrification has been observed in previous start-up experiments on sewage treatment (Watari et al., 2022). Therefore, in the present study, the growth of AOBs was higher leading to the accumulation of NO<sub>2</sub><sup>-</sup>-N, highlighting the difference in the growth rates of AOBs and NOBs (details shown 3.3.3). Another factor may be that the use of artificial seawater simulating the actual RAS slows the start-up process, as no external marine NOB is introduced.

On the 11th day of operation, NH<sub>4</sub>Cl was added, as in the initial conditions, to continue the nitrification reaction (Fig. 2). After the addition of NH<sub>4</sub>Cl, the TAN in the tank decreased to less than 1.0 mg·N·L<sup>-1</sup> for 2 days. Thus, ammonia-oxidizing microorganisms grew rapidly in the sponge carrier, and NH<sub>4</sub>Cl was added when its concentration was less than 1.0 mg·N·L<sup>-1</sup>. However, NO<sub>2</sub><sup>-</sup>-N gradually accumulated in the seawater tank. Therefore, on the 21st day of operation, the HRT was extended to 3.75 min to enhance NO<sub>2</sub><sup>-</sup>-N oxidation. Subsequently, the NO<sub>2</sub><sup>-</sup>-N concentration decreased and was 9.19 mg·N·L<sup>-1</sup> in the tank on the last day of operation. After the 21st day of operation,

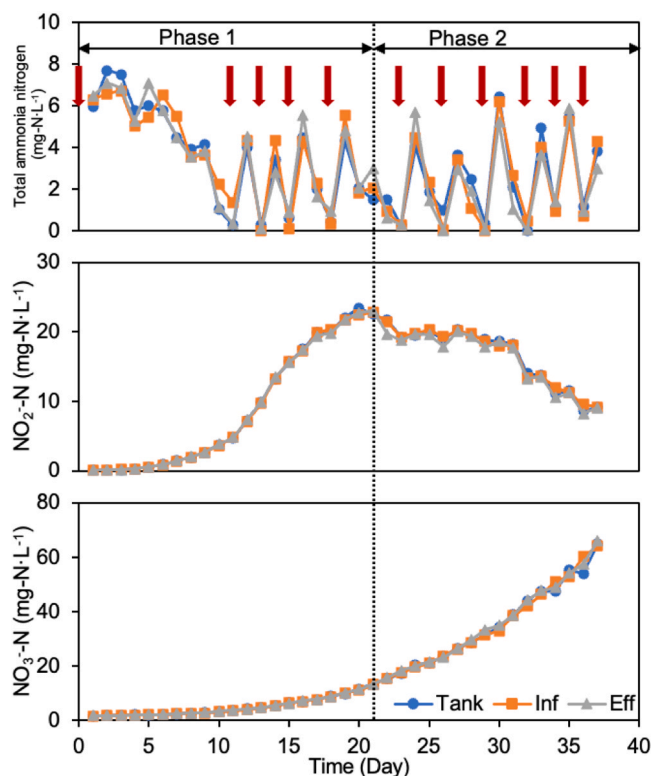


Fig. 2. Time course of TAN, NO<sub>2</sub><sup>-</sup>-N, and NO<sub>3</sub><sup>-</sup>-N through phases 1 and 2. Arrows indicate NH<sub>4</sub>Cl supplementation.

NOBs grew in the reactor and carried out a nitrification reaction where  $\text{NO}_2^-$ -N was converted  $\text{NO}_3^-$ -N.  $\text{NO}_2^-$ -N accumulation was observed in this study; however, it has been reported that the toxicity of  $\text{NO}_2^-$ -N to fish is greatly reduced by chloride (Gutiérrez et al., 2019; Navada et al., 2021).  $\text{NO}_3^-$ -N showed an increasing trend from the beginning of operation, and at the end of operation, the  $\text{NO}_3^-$ -N concentration in the tank was  $64.9 \text{ mg-N}\cdot\text{L}^{-1}$ . In this experiment,  $10 \text{ mg-N}\cdot\text{L}^{-1}$   $\text{NH}_4\text{Cl}$  was added to the seawater tank 10 times (total  $100 \text{ mg-N}\cdot\text{L}^{-1}$ ) to sustain the nitrification reaction. Nevertheless, the total amount of  $\text{NO}_2^-$ -N and  $\text{NO}_3^-$ -N in the seawater tank on the last day of the experiment was  $74.1 \text{ mg-N}\cdot\text{L}^{-1}$ . Previous studies have reported that  $\text{NO}_3^-$ -N decreases due to denitrification reaction, facilitated by the anaerobic conditions inside the sponge (Shitu et al., 2023; Watari et al., 2021a). In our experiment, the decrease in  $\text{NO}_3^-$ -N may be attributed to denitrification, driven by similar anaerobic conditions. Therefore, our results confirmed both nitrification and denitrification reactions occurred in the DHS reactor after 37 days of operation. Liu et al. (2019) used influx of high loads of  $\text{NH}_4^+$ -N ( $50 \text{ mg}\cdot\text{L}^{-1}$ ) to shorten start-up in seawater and found that  $\text{NH}_4^+$ -N was less than  $5 \text{ mg}\cdot\text{L}^{-1}$  and  $\text{NO}_2^-$ -N accumulated within 16 days in the trickling filter and 43 days in the MBBR. Moreover, a batch study on the transition of freshwater biocarriers to seawater (32%) reported that nitrification activity did not recover for 90 days after the transition to seawater (Gonzalez-Silva et al., 2021). In the present experiment, TAN decreased to  $0.25 \text{ mg-N}\cdot\text{L}^{-1}$  on the 11th day of operation and thereafter decreased to less than  $1.0 \text{ mg-N}\cdot\text{L}^{-1}$  every 2 days in phase 1 after adding  $\text{NH}_4\text{Cl}$ . After the HRT change (phase 2),  $\text{NH}_4\text{Cl}$  was added approximately every 3 days to sustain the nitrification reaction. Based on this observation, we inferred that the partial nitrification reaction performed by halophilic microorganisms after 11 days of operation signifies an early start-up. In addition,  $\text{NO}_2^-$ -N showed a decreasing trend from day 21 onward, which is considered a rapid result for a water treatment system with seawater (Liu et al., 2019; Zhang et al., 2023). Based on these results, the DHS reactor could be operated with a long HRT to enhance the activity of NOB and shorten the start-up time for the transition of the microbial community from freshwater to seawater using synthetic seawater. Furthermore, the long-term process performance of freshwater-inoculated DHS reactors under seawater conditions should be evaluated.

### 3.2. Nitrification rate of retained sludge

Fig. 3 shows the nitrification rates of the DHS-retained sludge on

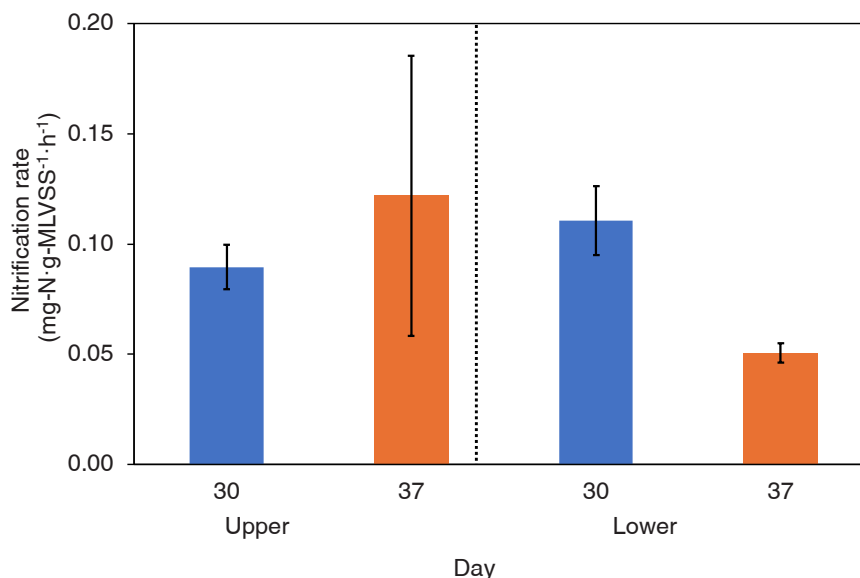


Fig. 3. Nitrification rate of the retained biofilm on days 30 and 37.

various days. The nitrification rates in the upper and bottom layers of the DHS on the 30th day of operation were  $0.090 \pm 0.010$  and  $0.11 \pm 0.016 \text{ mg-N}\cdot\text{g-MLVSS}^{-1}\cdot\text{h}^{-1}$ , respectively. The high nitrification activity in the bottom part of the reactor was also evident from previous microbial analyses of sewage-treated DHS reactors (Kubota et al., 2014; Nomoto et al., 2018). The nitrification rates were  $0.12 \pm 0.064$  and  $0.051 \pm 0.0043 \text{ mg-N}\cdot\text{g-MLVSS}^{-1}\cdot\text{h}^{-1}$  on the 37th day in the upper and bottom layers, respectively, indicating that the nitrification rate increased in the upper layer of the DHS reactor. This increase was attributed to the growth of nitrifying bacteria over time. A previous study investigating the nitrification rate tests of DHS with various pore sizes using seawater found that the nitrification rate increased with each elapsed time, with  $0.031 \text{ mg-N}\cdot\text{g-MLVSS}^{-1}\cdot\text{h}^{-1}$  at 23 days of operation and  $0.061 \text{ mg-N}\cdot\text{g-MLVSS}^{-1}\cdot\text{h}^{-1}$  at 324 days (Furukawa et al., 2019). In general, reactors used for seawater RAS are known to have lower nitrification activity than those used for freshwater RAS (Wang et al., 2016). Watari et al. (2021b) found that nitrifying bacteria accounted for approximately 10% of the total bacteria, whereas the sponge carriers used in this study had an average of 25% nitrifying bacteria during the experimental period. Therefore, the nitrification rates obtained in this experiment were higher in all cases than those reported by Furukawa et al. (2019) for the 23rd operation day, suggesting that the nitrification rates were almost equivalent to those observed on the 324th day by Furukawa et al. (2019).

### 3.3. Microbial community structure analysis

#### 3.3.1. Microbial community structure at the phylum level

Fig. 4 shows the top ten phyla observed in each sample. On day 1, Crenarchaeota (upper layer: 23.9%, bottom layer: 26.1%), Firmicutes (upper: 23.8%, bottom: 22.2%), Proteobacteria (upper: 16.0%, bottom: 15.8%), Bacteroidetes (upper: 9.4%, bottom: 7.6%), Actinobacteria (upper: 9.3%, bottom: 9.3%), and Planctomycetota (upper: 6.3%, bottom: 6.8%) were the predominant phyla in sponge carriers. The phylum Proteobacteria, which is important for the nitrification process, was dominant in the DHS reactor (Kubota et al., 2014). It is commonly found in the water treatment equipment used in RAS systems (Schreier et al., 2010). Crenarchaeota has been found in freshwater RAS biofilters, and some of its species are involved in ammonia oxidation (Pedersen et al., 2009). Several species belonging to Planctomycetota is anammox bacterium that oxidizes ammonia under anaerobic conditions (Strous et al., 1999). The presence of Planctomycetota may be attributed

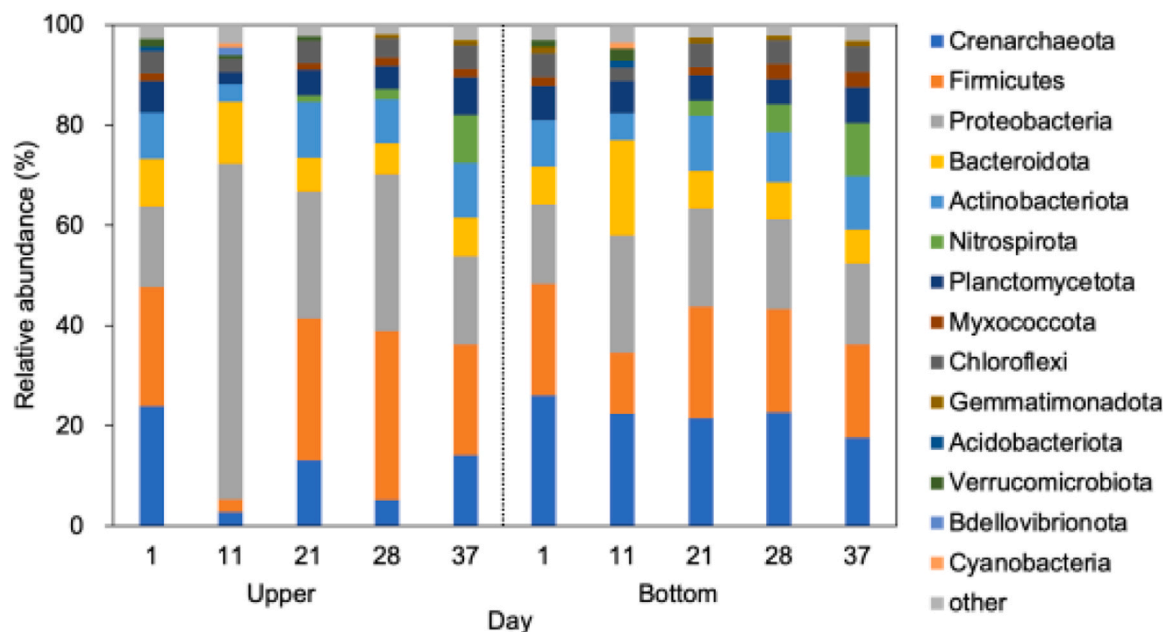


Fig. 4. The top ten phyla were detected in each sample.

to the anaerobic conditions inside the sponge carrier, which were involved in some proportion of the nitrogen removal. The Nitrospirota converts  $\text{NO}_2\text{-N}$  to  $\text{NO}_3\text{-N}$ . It comprised 1.2 % and 3.1 % of the microorganisms in the upper and lower layers, respectively, on the 21st day of operation. On the 37th day of the operation, the proportions were 9.5 % and 10.5 %, respectively. In this experiment,  $\text{NO}_2\text{-N}$  accumulated until the 21st day of operation, suggesting that Nitrospirota grew during the same period.

3.3.2. Nitrifying bacteria community in the sponge-retained sludge

Fig. 5 shows the abundance of nitrifying bacteria (> 1.0 %) at the genus level. *Candidatus Nitrosocosmicus*, an AOA, was predominantly detected, with an abundance of 23.9 % and 25.8 % in the upper and lower layers, respectively, on day 1. Previously, AOAs were considered to have a higher affinity for ammonia than AOBs, which confers an advantage in ammonia-limited environments (Martens-Habbena et al.,

2009). Therefore, AOA *Candidatus Nitrosocosmicus* could dominate the freshwater RAS DHS sponge and contribute to nitrification under low ammonia concentration conditions. However, on day 37, its abundance decreased to 14.0 % and 17.6 % in the upper and lower layers, respectively. This decrease in the abundance of *Candidatus Nitrosocosmicus* may have led to an increase in influent ammonia concentration. In addition, a previous study reported that *Candidatus Nitrosocosmicus oleophilus* grew in the temperature range of 25–30°C (Jung et al., 2016) and the DHS reactor obtained seed sponges operated at ambient temperature. Since the water temperature during our experiment was 16–24°C, we inferred that the detected *Candidatus Nitrosocosmicus* could not proliferate, and its abundance decreased over time. Nevertheless, the bottom layer exhibited a higher abundance of *Candidatus Nitrosocosmicus* than did the upper layer, suggesting that a portion of the nitrification process was performed by this AOA. On the 21st day of operation, more than 1.0 % of *Nitrosomonas* AOBs were detected (upper

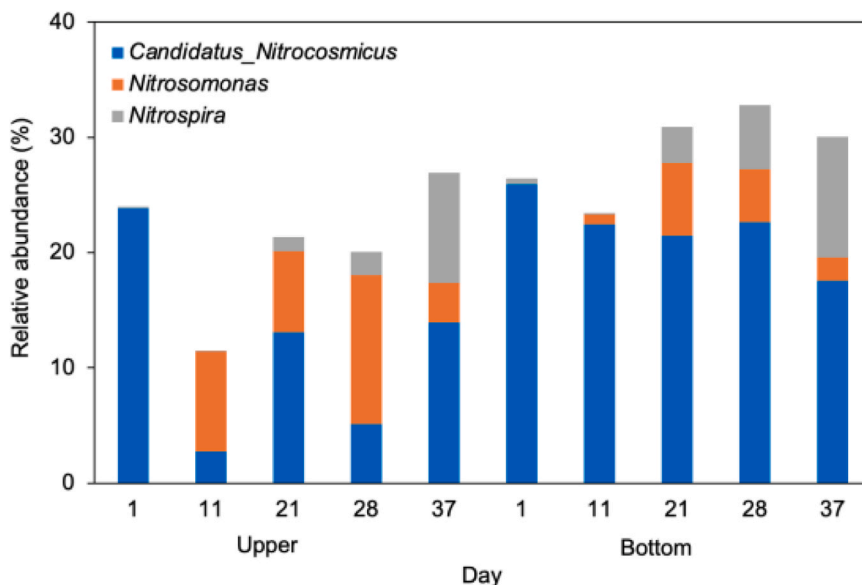


Fig. 5. The relative abundances of nitrifying microorganisms on days 1, 11, 21, 28, and 37.

layer: 7.1 %, bottom layer: 6.3 %), indicating that *Nitrosomonas* is a common AOB genus with marine species (Oshiki et al., 2020). Therefore, we inferred that *Nitrosomonas* shifted to a seawater-adapted microbial community on the 21st day of the operation. During the entire experiment, the abundance of AOB was higher in the bottom part of the DHS, which was previously reported in a DHS reactor treating domestic sewage (Watari et al., 2022). NOB *Nitrospira* was detected during the same period (upper layer: 1.2 %, bottom layer: 3.1 %), and its abundance was 9.5 % and 10.5 % in the upper and bottom layers, respectively, on the 37th day of operation. The observed *Nitrospira* spp. were similar to *Candidatus Nitrospira defluvi* and *Nitrospira marina*, which are known as NOB (Watson et al., 1986). The growth trend of *Nitrospira* was considered to follow the same rationale described in Section 3.3.1.

#### 4. Conclusions

This experiment used artificial seawater as a sponge carrier in a DHS reactor deployed in a freshwater RAS to establish a simple and start-up method. Our main findings were as follows: TAN showed a decreasing trend in approximately 1 week, and the conversion of  $\text{NO}_2^-$ -N to  $\text{NO}_3^-$ -N started on the 21st day of operation, indicating a complete nitrification reaction. The microbial community structure transitioned from a freshwater-nitrifying community to a seawater-nitrifying community on the 21st day of operation, followed by the growth of halophilic microorganisms. This promising method for preparing an RAS involves inoculating a sponge with freshwater microorganisms and adding  $\text{NH}_4\text{Cl}$  to develop a nitrification DHS reactor under seawater conditions. Although this approach shows promise, additional research is necessary to optimize the HRT process for the formation of effective biofilms, which could reduce the start-up period.

#### CRedit authorship contribution statement

**Penpicha Satanwat:** Writing – review & editing. **Hirotohi Netsu:** Methodology, Investigation. **Takahiro Watari:** Writing – original draft, Supervision, Methodology, Investigation. **Mami Nagai:** Investigation. **Takumi Akamine:** Writing – original draft, Investigation. **Takashi Yamaguchi:** Supervision, Methodology, Conceptualization. **Masashi Hatamoto:** Writing – review & editing. **Carlos Riquelme:** Writing – review & editing. **Nur Adlin:** Writing – review & editing

#### 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. Takahiro Watari reports financial support was provided by Japan Science and Technology Agency. 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.

#### Data Availability

Data will be made available on request.

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#### References

- Adlin, N., Hatamoto, M., Yamazaki, S., Watari, T., Yamaguchi, T., 2022. A potential zero water exchange system for recirculating aquarium using a DHS-USB system coupled with ozone. *Environ. Technol.* 43 (2), 275–285. <https://doi.org/10.1080/09593330.2020.1784295>.
- Ahmed, N., Turchini, G.M., 2021. Recirculating aquaculture systems (RAS): environmental solution and climate change adaptation. *J. Clean. Prod.* 297, 126604. <https://doi.org/10.1016/j.jclepro.2021.126604>.
- Badiola, M., Mendiola, D., Bostock, J., 2012. Recirculating Aquaculture Systems (RAS) analysis: main issues on management and future challenges. *Aquacult. Eng.* 51, 26–35. <https://doi.org/10.1016/j.aquaceng.2012.07.004>.
- de Kreuk, M.K., Pronk, M., van Loosdrecht, M.C., 2005. Formation of aerobic granules and conversion processes in an aerobic granular sludge reactor at moderate and low temperatures. *Water Res.* 39 (18), 4476–4484. <https://doi.org/10.1016/j.watres.2005.08.031>.
- Fang, F., Ni, B.J., Li, X.Y., Sheng, G.P., Yu, H.Q., 2009. Kinetic analysis on the two-step processes of AOB and NOB in aerobic nitrifying granules. *Appl. Microbiol. Biotechnol.* 83 (6), 1159–1169. <https://doi.org/10.1007/s00253-009-2011-y>.
- FAO. 2020. The State of World Fisheries and Aquaculture 2020. Sustainability in action. Rome. <https://doi.org/10.4060/ca9229en>.
- Furukawa, A., Hatamoto, M., Kusaka, J., Kawamata, M., Mori, M., Hoaki, T., Yamaguchi, T., 2019. Evaluation of nitrification performance using nitrifying-DHS reactor with various sponge-pore sizes for breeding tank of marine aquaria. *J. Jpn Soc. Water Environ.* 42 (1), 7–12. <https://doi.org/10.2965/jswe.42.7>.
- Furukawa, A., Matsuura, N., Mori, M., Kawamata, M., Kusaka, J., Hatamoto, M., Yamaguchi, T., 2016. Development of a DHS-USB recirculating system to remove nitrogen from a marine fish aquarium. *Aquacult. Eng.* 74, 174–179. <https://doi.org/10.1016/j.aquaceng.2016.08.004>.
- Gonzalez-Silva, B.M., Jonassen, K.R., Bakke, I., Ostgaard, K., Vadstein, O., 2021. Understanding structure/function relationships in nitrifying microbial communities after cross-transfer between freshwater and seawater. *Sci. Rep.* 11 (1), 2979. <https://doi.org/10.1038/s41598-021-82272-7>.
- Gutiérrez, X.A., Kolarevic, J., Takle, H., Baeverfjord, G., Ytteborg, E., Fyhn Terjesen, B., 2019. Effects of chronic sub-lethal nitrite exposure at high water chloride concentration on Atlantic salmon (*Salmo salar*, Linnaeus 1758) parr. *Aquac. Res.* 50 (9), 2687–2697. <https://doi.org/10.1111/are.14226>.
- He, Q., Cheng, Z., Zhang, D., Main, K., Feng, C., Ergas, S.J., 2020. A sulfur-based cyclic denitrification filter for marine recirculating aquaculture systems. *Bioresour. Technol.* 310, 123465. <https://doi.org/10.1016/j.biortech.2020.123465>.
- Jung, M.Y., Kim, J.G., Sinnighe Damste, J.S., Rijpstra, W.I., Madsen, E.L., Kim, S.J., Hong, H., Si, O.J., Kerou, M., Schleper, C., Rhee, S.K., 2016. A hydrophobic ammonia-oxidizing archaeon of the Nitrososphaera clade isolated from coal tar-contaminated sediment. *Environ. Microbiol. Rep.* 8 (6), 983–992. <https://doi.org/10.1111/1758-2229.12477>.
- Kirishima, Y., Watari, T., Terada, S., Choeisai, P., Choeisai, K., Nguyen, T.H., Okubo, T., Nagano, A., Matsueda, T., Hatamoto, T., Yamaguchi, T., 2024. Restart performance of prolonged stopped down-flow hanging sponge reactor for treating of low-strength domestic sewage. *J. Environ. Chem. Eng.* 12 (3), 112802. <https://doi.org/10.1016/j.jece.2024.112802>.
- Kotchareon, W., Watari, T., Adlin, N., Nakamura, Y., Satanwat, P., Pungrasmi, W., Powtongsook, S., Takeuchi, Y., Hatamoto, M., Yamazaki, S., Yamaguchi, T., 2021. Effect of salinities on nitrogen removal performance of DHS-USB system and growth of *Epinephelus bruneus* in closed recirculating aquaculture system. *Int. Biodeterior. Biodegrad.* 164, 105299. <https://doi.org/10.1016/j.ibiod.2021.105299>.
- 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>.
- Liu, D., Li, C., Guo, H., Kong, X., Lan, L., Xu, H., Zhu, S., Ye, Z., 2019. Start-up evaluations and biocarriers transfer from a trickling filter to a moving bed bioreactor for synthetic mariculture wastewater treatment. *Chemosphere* 218, 696–704. <https://doi.org/10.1016/j.chemosphere.2018.11.166>.
- Losordo, T.M., Masser, M.P., Rakocy, J., 2000. Recirculating aquaculture tank production systems. Southern Regional Aquaculture Center.
- Lu, J., Zhang, Y., Wu, J., Wang, J., 2020. Nitrogen removal in recirculating aquaculture water with high dissolved oxygen conditions using the simultaneous partial nitrification, anammox and denitrification system. *Bioresour. Technol.* 305, 123037. <https://doi.org/10.1016/j.biortech.2020.123037>.
- Martens-Habbena, W., Berube, P.M., Urakawa, H., de la Torre, J.R., Stahl, D.A., 2009. Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. *Nature* 461 (7266), 976–979. <https://doi.org/10.1038/nature08465>.
- Moussa, M.S., Hooijmans, C.M., Lubberding, H.J., Gijzen, H.J., van Loosdrecht, M.C., 2005. Modelling nitrification, heterotrophic growth and predation in activated sludge. *Water Res.* 39 (20), 5080–5098. <https://doi.org/10.1016/j.watres.2005.09.038>.
- Navada, S., Gaumet, F., Tveten, A.-K., Kolarevic, J., Vadstein, O., 2021. Seeding as a start-up strategy for improving the acclimation of freshwater nitrifying bioreactors to salinity stress. *Aquaculture* 540, 736663. <https://doi.org/10.1016/j.aquaculture.2021.736663>.
- Neori, A., Chopin, T., Troell, M., Buschmann, A.H., Kraemer, G.P., Halling, C., Shpigiel, M., Yarish, C., 2004. Integrated aquaculture: rationale, evolution and state of the art emphasizing seaweed biofiltration in modern mariculture. *Aquaculture* 231 (1–4), 361–391. <https://doi.org/10.1016/j.aquaculture.2003.11.015>.

- Nomoto, N., Hatamoto, M., Ali, M., Jayaswal, K., Iguchi, A., Okubo, T., Takahashi, M., Kubota, K., Tagawa, T., Uemura, S., Yamaguchi, T., Harada, H., 2018. Characterization of sludge properties for sewage treatment in a practical-scale down-flow hanging sponge reactor: oxygen consumption and removal of organic matter, ammonium, and sulfur. *Water Sci. Technol.* 77 (3-4), 608–616. <https://doi.org/10.2166/wst.2017.557>.
- Onodera, T., Okubo, T., Uemura, S., Yamaguchi, T., Ohashi, A., Harada, H., 2016. Long-term performance evaluation of down-flow hanging sponge reactor regarding nitrification in a full-scale experiment in India. *Bioresour Technol* 204, 177–184. <https://doi.org/10.1016/j.biortech.2016.01.005>.
- Oshiki, M., Aizuka, T., Netsu, H., Oomori, S., Nagano, A., Yamaguchi, T., Araki, N., 2020. Total ammonia nitrogen (TAN) removal performance of a recirculating down-flow hanging sponge (DHS) reactor operated at 10 to 20 °C with activated carbon. *Aquaculture* 520, 734963. <https://doi.org/10.1016/j.aquaculture.2020.734963>.
- Pedersen, L.-F., Pedersen, P.B., Nielsen, J.L., Nielsen, P.H., 2009. Peracetic acid degradation and effects on nitrification in recirculating aquaculture systems. *Aquaculture* 296 (3-4), 246–254. <https://doi.org/10.1016/j.aquaculture.2009.08.021>.
- Ruiz, P., Vidal, J.M., Sepúlveda, D., Torres, C., Villouta, G., Carrasco, C., Aguilera, F., Ruiz-Tagle, R., Urrutia, H., 2019. Overview and future perspectives of nitrifying bacteria on biofilters for recirculating aquaculture systems. *Rev. Aquacult* 12 (3), 1478–1494. <https://doi.org/10.1111/raq.12392>.
- Schreier, H.J., Mirzoyan, N., Saito, K., 2010. Microbial diversity of biological filters in recirculating aquaculture systems. *Curr. Opin. Biotechnol.* 21 (3), 318–325. <https://doi.org/10.1016/j.copbio.2010.03.011>.
- Shitu, A., Chen, W., Tadda, M.A., Zhang, Y., Ye, Z., Liu, D., Zhu, S., Zhao, J., 2023. Enhanced aquaculture wastewater treatment in a biofilm reactor filled with sponge/ferrous oxalate/biochar composite (Sponge-C(2)FeO(4)@NBC) biocarriers: performance and mechanism. *Chemosphere* 330, 138772. <https://doi.org/10.1016/j.chemosphere.2023.138772>.
- Strous, M., Fuerst, J.A., Kramer, E.H., Logemann, S., Muyzer, G., van de Pas-Schoonen, K. T., Webb, R., Kuenen, J.G., Jetten, M.S., 1999. Missing lithotroph identified as new planctomycete. *Nature* 400 (6743), 446–449. <https://doi.org/10.1038/22749>.
- T.M. Losordo, M.P. Masser, J. Rakocy, Recirculating aquaculture tank production systems Overview of Critical Considerations SRAC Publ., 451 (2) (1998).
- Tadda, M.A., Li, C., Gouda, M., Abomohra, A.E.-F., Shitu, A., Ahsan, A., Zhu, S., Liu, D., 2021. Enhancement of nitrite/ammonia removal from saline recirculating aquaculture wastewater systems using a moving bed bioreactor. *J. Environ. Chem. Eng.* 9 (5), 105947. <https://doi.org/10.1016/j.jece.2021.105947>.
- 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* 20 (2), 389–418. <https://doi.org/10.1007/s11157-021-09573-1>.
- Uemura, S., Okubo, T., Maeno, K., Takahashi, M., Kubota, K., Harada, H., 2016. Evaluation of water distribution and oxygen mass transfer in sponge support media for a down-flow hanging sponge reactor. *Int. J. Environ. Res* 10 (2), 265–272. <https://doi.org/10.22059/IJER.2016.57721>.
- Wang, C.-Y., Chang, C.-Y., Chien, Y.-H., Lai, H.-T., 2016. The performance of coupling membrane filtration in recirculating aquaponic system for tilapia culture. *Int. Biodeterior. Biodegrad.* 107, 21–30. <https://doi.org/10.1016/j.ibiod.2015.10.016>.
- Watari, T., Hata, Y., Hirakata, Y., Nguyet, P.N., Nguyen, T.H., Maki, S., Hatamoto, M., Sutani, D., Setia, T., Yamaguchi, T., 2021a. Performance evaluation of down-flow hanging sponge reactor for direct treatment of actual textile wastewater; Effect of effluent recirculation to performance and microbial community. *J. Water Proc. Eng.* 39, 101724. <https://doi.org/10.1016/j.jwpe.2020.101724>.
- Watari, T., Kirishima, Y., Choiesai, 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 Proc. Eng.* 47, 102798. <https://doi.org/10.1016/j.jwpe.2022.102798>.
- 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>.
- Watari, T., Vazquez, C.L., Hatamoto, M., Yamaguchi, T., van Lier, J.B., 2021c. Development of a single-stage mainstream anammox process using a sponge-bed trickling filter. *Environ. Technol.* 42 (19), 3036–3047. <https://doi.org/10.1080/09593330.2020.1720309>.
- Watson, S.W., Bock, E., Valois, F.W., Waterbury, J.B., Schlosser, U., 1986. *Nitrospira marina* gen. nov. sp. nov.: a chemolithotrophic nitrite-oxidizing bacterium. *Arch. Microbiol.* 144, 1–7. <https://doi.org/10.1007/BF00454947>.
- Xiao, R., Wei, Y., An, D., Li, D., Ta, X., Wu, Y., Ren, Q., 2019. A review on the research status and development trend of equipment in water treatment processes of recirculating aquaculture systems. *Rev. Aquacult.* 11 (3), 863–895. <https://doi.org/10.1111/raq.12270>.
- Zhang, W., Liu, B., Sun, Z., Wang, T., Tan, S., Fan, X., Zou, D., Zhuang, Y., Liu, X., Wang, Y., Li, Y., Mai, K., Ye, C., 2023. Comparison of nitrogen removal characteristic and microbial community in freshwater and marine recirculating aquaculture systems. *Sci. Total Environ.* 878, 162870. <https://doi.org/10.1016/j.scitotenv.2023.162870>.