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Enhanced Nitrogen Removal from a Recirculating Aquaculture System Using a Calcined FeS_x-Packed Denitrification Bioreactor

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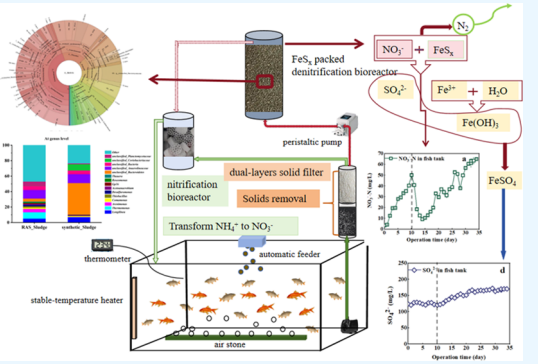
ABSTRACT: In this study, a recirculating aquaculture system (RAS) was constructed, and a denitrification bioreactor was installed to enhance nitrogen removal. In addition, the nitrogen removal performance of the system was investigated. FeS_x was prepared by calcining iron (Fe) and S^0 powder, which was used as an electron donor for denitrification. In the phase using simulating aquaculture wastewater, the concentrations of NO_2^--N and NH_4^+-N in the RAS were lower than 0.20 and 0.50 mg/L, respectively, and NO_3^--N gradually accumulated without the operation of the FeS_x-packed denitrification bioreactor. After introducing cultured fish and operating the denitrification bioreactor, NO_2^--N and NH_4^+-N in the fish tank were lower than 0.01 mg/L and lower detection limit, respectively, and the NO_3^--N removal efficiency was 79.04%. After 24 days of operation, the SO_4^{2-} concentration was lower than 200 mg/L, and the pH was stable at around 7. The survival rate of fish was 95%, and they grew 6 to 7 cm at the end of the experiment. The average weight gain of fish was 5.31 g, and the culture density increased from the initial 10 to 26.54 kg/m^3. Microbial community structure analysis showed that the diversity in the denitrification bioreactor operated in the RAS (RAS_Sludge) was higher than that in the reactor operated using synthetic wastewater (Synthetic_Sludge) due to the introduction of organic matter. *Thermomonas*, *Longilina*, *Arenimonas*, and *Thiobacillus* were dominant in RAS_Sludge, while *unclassified* genera were dominant in Synthetic_Sludge. Functional genes in RAS_Sludge and Synthetic_Sludge were predicted based on Functional Annotation of Prokaryotic Taxa, revealing differences in genes related to denitrification as well as sulfur and iron oxidation. This study provides a theoretical basis for the application of FeS_x-based autotrophic denitrification technology in RASs, promoting it from theoretical research to engineering practice.



1. INTRODUCTION

Recirculating aquaculture systems (RASs) are a high-density factory-type aquaculture method.[1] Compared with traditional aquaculture systems, the water consumption in the RAS is reduced by 90−99%,[2] leading to high aquaculture yield, energy savings, environmental protection, and safe and reliable aquatic product quality. In the RAS, ammonium (NH_4^+-N) excreted by the fish is typically transformed to less toxic nitrate (NO_3^--N) by microbial activity in bioreactors.[3] However, with the long-term operation of high-density RASs, the concentration of NO_3^--N in aquaculture water has increased, reaching 100−1000 mg of N/L.[4] When the concentration of NO_3^--N reaches 125 mg/L, it can have toxic effects on cultured fish.[5] Torno et al. studied the toxic effects of different concentrations of NO_3^--N (0−500 mg N/L) on perch, finding that higher concentrations of NO_3^--N led to higher fish mortality.[6] In addition, when the oxygen distribution is uneven, incomplete denitrification can occur in the corners or pipelines of the culture pond, generating nitrite (NO_2^--N) that is more toxic to cultured fish.[7] Water quality indicators in Australia and New Zealand suggest that the NO_3^--N concentration in freshwater and saline water aquaculture should be lower than 50 and 100 mg N/L, respectively.[8] Because of this, removing NO_3^--N from RASs is of great significance for improving the quality of aquatic products.

There are various methods for removing NO_3^--N from water, including ion exchange,[9] reverse osmosis,[10] absorption,[11] and biological denitrification.[10] Biological denitrification has a low cost, high removal efficiency, and low production of secondary pollutants.[12] In denitrification, NO_3^--N serves as an electron acceptor and is sequentially reduced to NO_2^--N, nitric oxide (NO), nitrous oxide (N_2O), and nitrogen gas (N_2).[13] Electron donors are required to perform denitrification; however, electron donor concentrations in the RAS are usually inadequate for complete denitrification, meaning that external electron donors must

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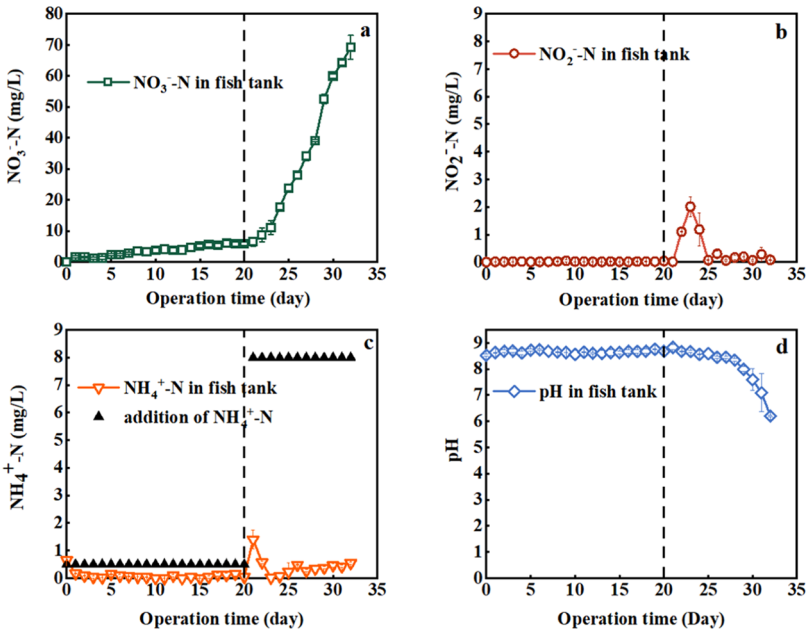

Figure 1. Changes in concentrations of (a) NO_3^--N, (b) NO_2^--N, (c) NH_4^+-N, and (d) pH in Phase I.

be added to guarantee effective NO_3^--N removal. Based on the types of electron donors, biological methods are divided into heterotrophic and autotrophic denitrification, where organic carbon is an electron donor in the former while inorganic matter is an electron donor in the latter.

Compared to heterotrophic denitrification, autotrophic denitrification avoids organic carbon addition and has low biomass yields, being more applicable in the RAS. Inorganic electron donors, such as hydrogen gas,[13] reduced sulfur species,[13,14] and reduced iron species,[15] have been widely investigated in various wastewater. Pyrite is considered a potential electron donor for denitrification because of its low cost, lack of need for additional alkalinity, and low SO_4^{2-} production.[16] However, there are still shortcomings in its application, including a low denitrification rate[14] and metal leaching (copper, lead, cadmium, arsenic, and zinc).[17] Various strategies have been put forward to improve denitrification performance such as prolonging contact time,[18] pickling pretreatment,[19] and using nanosized natural pyrite.[17] Although metal pollutants are rarely leached from pyrite,[17] aquatic organisms will accumulate metals, eventually causing harm. Because of this, natural pyrite is not suitable as a denitrification electron donor in RASs. In our preliminary experiment, iron−sulfur compounds (FeS_x) were prepared by calcining iron (Fe) and S^0 powders at a certain proportion, which were used to enhance the denitrification performance and applicability in RASs. FeS_x requires low alkalinity, has a low SO_4^{2-} yield, and takes advantage of the pH buffering capacity of natural iron and sulfur while avoiding the disadvantages of natural iron and sulfur compounds. To date, calcined FeS_x has not been applied in RASs.

In this study, an RAS was constructed, and a denitrification bioreactor packed with calcined FeS_x was added to remove NO_3^--N. The objectives were to investigate the denitrification performance of the FeS_x-packed denitrification bioreactor in the RAS, monitor the health of cultured fish, and perform microbial analysis to reveal function genes in the denitrification bioreactors operated both in the RAS and in synthetic RAS wastewater.

2. RESULTS AND DISCUSSION

2.1. Phase I: Simulated Aquaculture Wastewater. The water temperature of the fish tank was 20 ± 2 °C, and the dissolved oxygen (DO) was 7.15 mg/L, which were all within the suitable growth range of the cultured fish. The changes in NO_3^--N, NO_2^--N, NH_4^+-N, and pH in the fish tank during the operation phase of simulated aquaculture wastewater are shown in Figure 1. At this stage, nitrification took place in the system, transforming NH_4^+-N to NO_3^--N; 1 g NH_4^+-N was consumed along with 4.34 g of oxygen and 7.14 g of alkalinity, resulting in 4.43g of NO_3^--N[20] (eq 1).

$$NH_4^+ + 1.83O_2 + 1.97HCO_3^- \rightarrow 0.0244C_5H_7O_2N + 0.976NO_3^- + 1.86CO_2 + 2.90H_2O \quad (1)$$

In the first 20 days, low NH_4^+-N was added to the system to start up the nitrification bioreactor with 0.50 mg/L added to the fish tank every day. In the first 5 days, there was little change in the NO_3^--N concentration in the fish tank, which increased slowly to an accumulated amount <2 mg N/L (Figure 1a). According to eq 1, when 0.5 mg of N/L NH_4^+-N was added, the theoretical yield of NO_3^--N was 2.22 mg of N/L. The accumulated amount of NO_3^--N in the first 5 days was less than the theoretical value, indicating that nitrification was still weak and nitrifying bacteria were still in the initial stage of growth. From the fifth to the 20th day, the concentration of NO_3^--N increased from ∼2 to ∼6 mg N/L, which was higher than the theoretical yield, indicating that nitrification was gradually enhanced. In the later stage, the concentration of NH_4^+-N was increased to make the nitrification system mature rapidly, using a daily dosage of 8 mg N/L (about 3.3 times the calculated theoretical NH_4^+-N conversion). At this time, the accumulation of NO_3^--N increased rapidly, reaching 72.13 mg of N/L.

When the dosage of NH_4^+-N was 0.5 mg N/L, the concentrations of NO_2^--N and NH_4^+-N in the fish tank were kept low (<0.02 and <0.10 mg N/L, respectively; Figure 1b,c),

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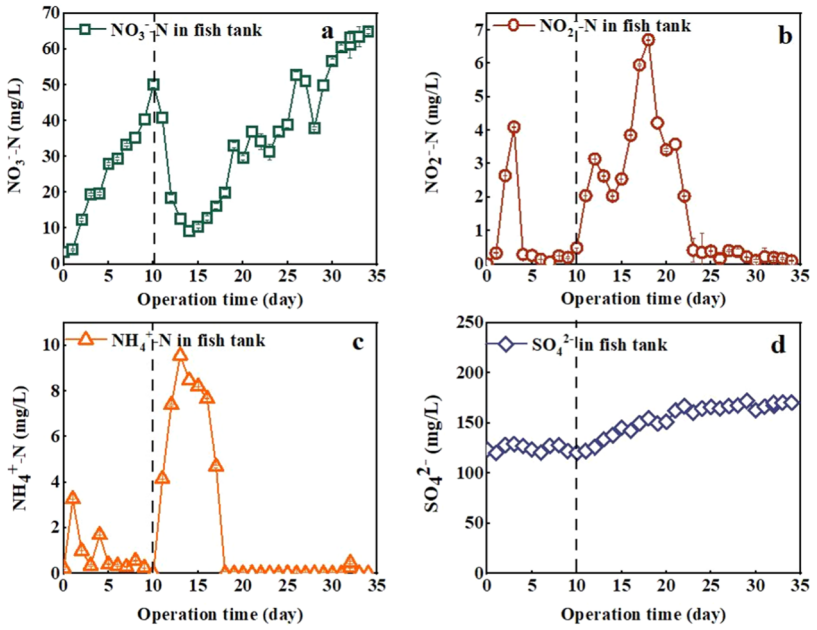

Figure 2. Changes in concentrations of (a) NO_3^--N, (b) NO_2^--N, (c) NH_4^+-N, and (d) pH in Phase II.

and the removal efficiency of NH_4^+-N reached 80%. At the later stage, when the dosage of NH_4^+-N was 8 mg/L, NO_2^--N concentration increased to 2.01 mg N/L on the fourth day before decreasing to below 0.20 mg N/L; NH_4^+-N concentration increased to 1.40 mg N/L on the second day, then decreased to below 0.50 mg N/L, with the removal efficiency reaching 93.75%, indicating that a biofilm was continuously formed. Due to the sudden increase in NH_4^+-N dosage, NO_2^--N and NH_4^+-N concentrations fluctuated for a few days but quickly recovered to a low level.

The pH in the system was maintained at around 8.50 in the first 25 days and gradually decreased to 6.03 in the next few days (Figure 1d). Nitrification consumed alkalinity, and the pH decreased in the later stage, which showed that nitrification was strong and the bioreactor was operating stably. The acceptable pH for aquatic animals varies with their type;[21] the best range for the survival and growth of carp is pH 7.5−8.0.[22] Because of this, alkalinity should be added to balance the pH in a real RAS operation.

The nitrification bioreactor ran well, and NO_2^--N and NH_4^+-N in the RAS were maintained at a low level, which would not pose a threat to the health of aquatic organisms. However, the accumulation rate of NO_3^--N was 5.51 mg/L/d. As high NO_3^--N would be harmful to the survival and growth of aquatic organisms, a denitrification bioreactor should be added to the system to reduce its concentration.

2.2. Phase II: RAS Operation after the Introduction of Fish. Because the RAS was operated in winter, heating rods were placed in the fish tank to keep the water temperature at 21 ± 3 °C. In addition, the DO was ~6.5 mg/L, which was within the suitable growth range of cultured fish. The concentration changes of NO_3^--N, NO_2^--N, NH_4^+-N, and SO_4^{2-} in the fish tank are shown in Figure 2. In the first 10 days, the FeS_x-packed denitrification bioreactor was not connected, and the concentration change trends of NO_3^--N, NO_2^--N, and NH_4^+-N in the fish tank were similar to those in Phase I. NO_3^--N accumulated rapidly, with an accumulation of 50.10 mg of N/L and an average accumulation rate of 5.00 mg/L/day within 10 days. Because of the continuous aeration in the system, there was no environment for denitrifying bacteria to grow, and so NO_3^--N accumulated. NO_2^--N first increased, reaching its highest concentration of 4.10 mg N/L on the fourth day before decreasing to <0.50 mg N/L. NH_4^+-N also showed first increased and then decreased, reaching a peak of 3.26 mg N/L on the second day before decreasing to <2.00 mg N/L. The accumulation peak of NO_2^--N was later than that of NH_4^+-N because, in the process of nitrification, NH_4^+-N is first transformed into NO_2^--N before being transformed into NO_3^--N (Figure 2b,c).

On the 11th day, an FeS_x-packed denitrification bioreactor was added to remove NO_3^--N from water. Afterward, the concentration of NO_3^--N decreased from 50.10 to 10.50 mg of N/L within 5 days, and the removal efficiency was 79%. However, after 5 days, NO_3^--N gradually accumulated, and the denitrification performance of the system decreased. Two possible reasons exist for this. The effective volume of the denitrification column was only about 250 mL, and the volume of aquaculture water in the system was 20 L; therefore, the treatment capacity of the denitrification bioreactor may not have been enough for effective NO_3^--N removal. Additionally, the RAS was operated in winter; although an insulation layer was added to the column, the temperature was still low, with an average of 20 °C, lower than the optimum temperature of denitrifying bacteria (about 30 °C). Therefore, the microbial activity may have been affected, leading to a decrease in treatment efficiency.

Although denitrification in the system was weakened, the accumulation rate of NO_3^--N in the system was slower than that in the phase without the denitrification bioreactor. After 21 days, NO_3^--N accumulated from 10.50 to 64.90 mg/L with an average accumulation rate of 2.59 mg/L/d, about half of the NO_3^--N accumulation rate without the denitrification bioreactor (Figure 2a). This indicated that the FeS_x-packed denitrification bioreactor removed NO_3^--N from water at low temperatures and slowed the increase in the level of NO_3^--N in the RAS.

NO_2^--N and NH_4^+-N first accumulated and then decreased (Figure 2b,c), with the maximum accumulation of NO_2^--N

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Table 1. α Diversity Index of Microbial Community in Denitrification Column

sample	number	OTUs	Shannon	Chao	Ace	Simpson	Shannon even	coverage (%)
RAS_Sludge	323,480	5336	5.50	5342.60	5398.72	0.02	0.64	100
Synthetic_Sludge	343,245	3744	3.68	3798.11	3912.00	0.17	0.45	100

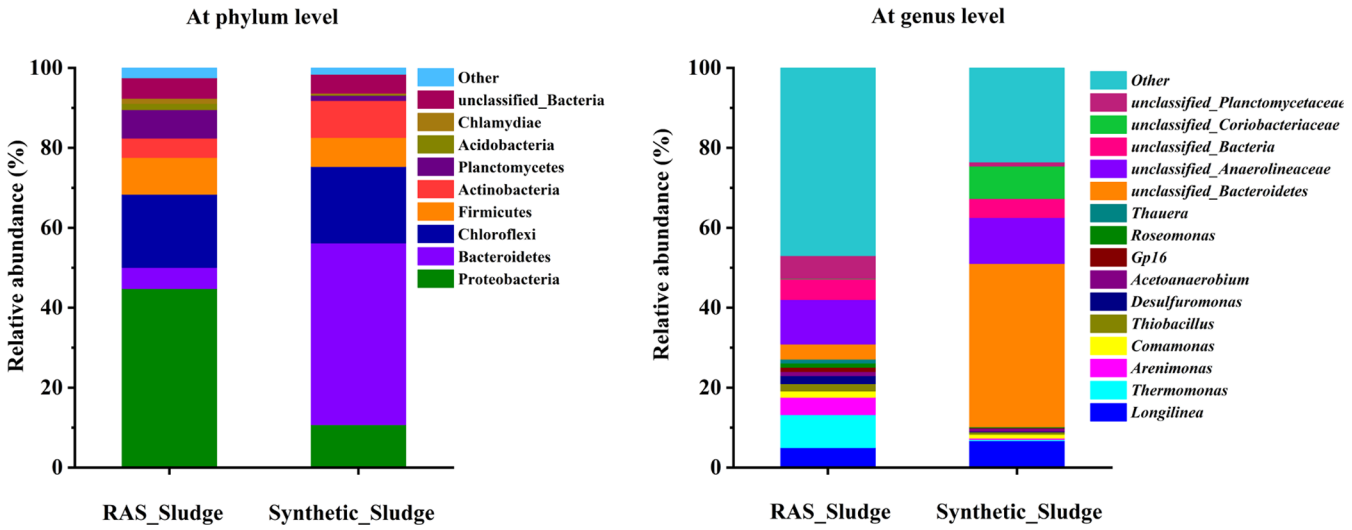

Figure 3. Bacteria community structure in RAS_Sludge and Synthetic_Sludge: (a) at the phylum level; (b) at the genus level.

reaching 6.71 mg of N/L and the peak accumulation amount of NH_4^+-N reaching 9.56 mg of N/L. NO_2^--N is one of the intermediate products of the reduction of NO_3^--N to N_2. At this time, the denitrification performance of the bioreactor was good, leading to temporary accumulation of NO_2^--N. Afterward, the NO_2^--N concentration dropped to 0.20 mg/L, which was within the healthy range for fish.[23] The NH_4^+-N might have resulted from the degradation of fish waste or uneaten fish food. Additionally, the Fe_xS packed in the denitrification bioreactor was prepared by calcining Fe and S^0 powders; there may have been some Fe powder remaining in the synthetic Fe_xS, which is a strong reducing agent that could reduce NO_3^--N to NH_4^+-N.[24] The NH_4^+-N was quickly removed by nitrification and decreased to 0.50 mg/L. The peak value of NH_4^+-N was reached in about 15 days; NO_2^--N reached its peak value later because NH_4^+-N was first converted into NO_2^--N, which was converted into NO_3^--N.

The accumulation of SO_4^{2-} in the fish tank after the addition of the denitrification bioreactor is shown in Figure 2d. In the first 10 days, when there was no denitrification bioreactor in the system, the SO_4^{2-} in the fish tank was from the added tap water, with an average concentration of 124.95 mg/L. After the denitrification bioreactor was operated, SO_4^{2-} accumulated slowly, increasing from 120.43 to 170.55 mg/L within 25 days with a total increase of 50.12 mg/L and an accumulation rate of 2.00 mg/L/d. The SO_4^{2-} was generated from FeS_x-based denitrification, in which NO_3^--N was reduced to N_2 through the oxidation of FeS_x to Fe^{3+} and SO_4^{2-}. The accumulation rate of SO_4^{2-} in the first 11 days (10th to 21st day) was 2.81 mg/L/d, higher than the 0.57 mg/L/d seen later (22nd to 34th day). This is because of the higher denitrification rate in the early stage compared to that in the later stage. However, the concentration of SO_4^{2-} in the RAS was low, with its total concentration in the system being lower than the drinking water quality standard of China (GB 5749−2022, 250 mg/L), indicating it would not cause potential health threats and chronic toxic effects to the cultured fish in the RAS.[25]

When fish were introduced into the fish tank without operating the FeS_x-packed denitrification bioreactor (0−10 days), the pH value in the fish tank dropped from about 8 to about 7, as nitrification consumed alkalinity. However, during the experiment, $NaHCO_3$ was added to buffer the alkalinity consumed by nitrification, so the pH value of the system was still maintained at a level suitable for the growth of aquatic organisms.[20] After the denitrification bioreactor was operated (10−34 days), the pH value in the fish tank was relatively stable, remaining around 7. Although the pH value dropped to 6.29 on the 20th day, it returned to 6.88 on the 24th day, remaining at around 7 thereafter. The above results showed that FeS_x, as an electron donor, successfully maintained a neutral pH, which was beneficial for denitrification of bacteria and aquatic organisms.

During the experiment, the survival rate of carp was 95%; the fish showed liveliness and normal feeding, and there was no death caused by the deterioration of the water quality. At the end of the experiment, the carp had grown from 2−3 to 8−10 cm, increasing by 6−7 cm. The average weight of fish increased from 3.21 to 8.52 g, with an average weight gain of 5.31 g. The culture density increased from the initial 10 to 26.54 kg/m^3. Together, these results showed that the carp were in good health with good growth.

2.3. Microbial Analysis. Microbial samples were collected from the FeS_x-packed denitrification bioreactor operated in the RAS (RAS_Sludge) and that operated using synthetic NO_3^--N-polluted water (Synthetic_Sludge) to reveal the effects of residual organic matter from the RAS on the microbial community structure. Table 1 shows the α-diversity indexes of RAS_Sludge and Synthetic_Sludge. A total of 323,480 and 343,245 sequences were obtained from RAS_Sludge and Synthetic_Sludge, respectively. The number of operational taxonomic units (OTUs) in RAS_Sludge was higher than that in Synthetic_Sludge (5336 and 3744, respectively). The Chao and Ace indices of RAS_Sludge were both higher than those of Synthetic_Sludge, indicating that the total number of species

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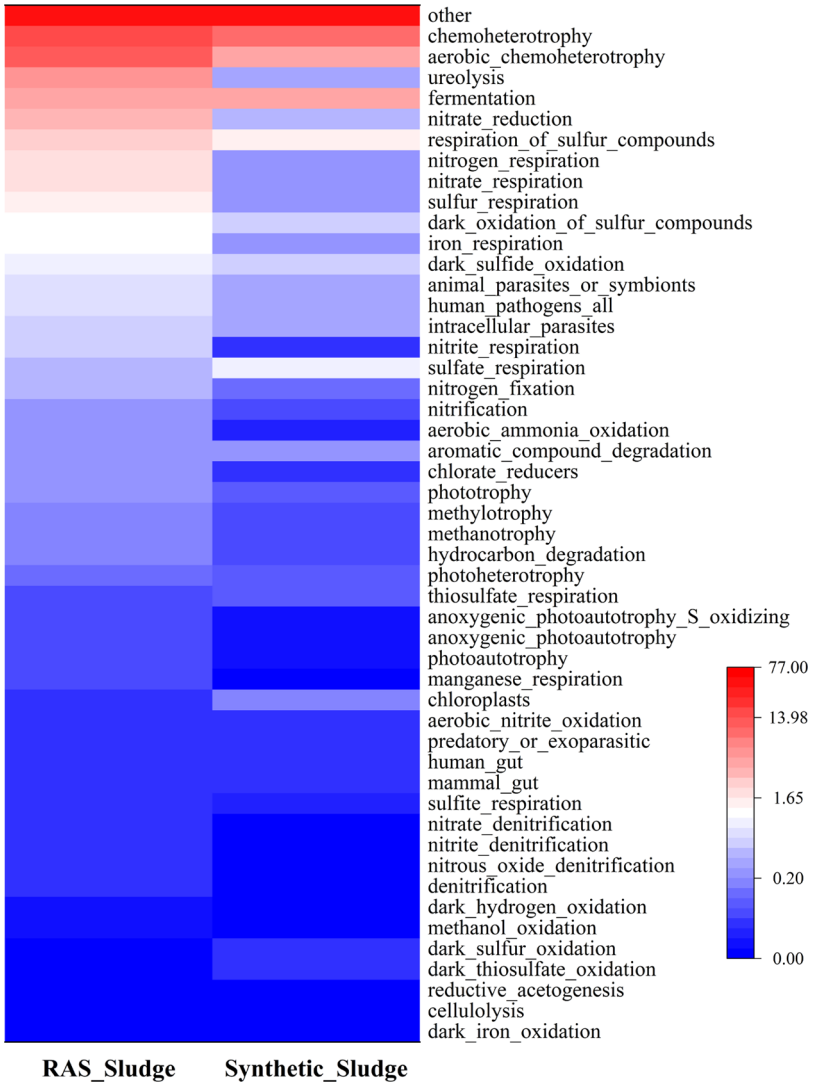

Figure 4. Heatmap of Functional Genes in RAS_Sludge and Synthetic_Sludge based on FAPROTAX.

in RAS_Sludge was greater than in Synthetic_Sludge. In addition, Shannon and Shannon even also showed that the diversity of the RAS_Sludge community was higher than that of Synthetic_Sludge. Simpson's index of RAS_Sludge was 0.07, which was much smaller than that of Synthetic_Sludge, indicating that the microbial community in RAS_Sludge was more diverse than that in Synthetic_Sludge. Although the operating environment and conditions of RAS_Sludge and Synthetic_Sludge were the same, the different water quality conditions resulted in differences in microbial community abundance and diversity between them. The FeS_x-based denitrification bioreactor operated in the RAS had higher biodiversity and increased species abundance, which was attributed to the complex components found in aquaculture water.

Figure 3a shows the distribution of microbial communities at the phylum level. The dominant phyla in RAS_Sludge and Synthetic_Sludge were similar, with the top five being Proteobacteria, Bacteroidota, Chloroflexi, Firmicutes, and Actinobacteria, all of which are ubiquitous in sewage treatment systems.[26,27] These phyla were also detected in a pyrite-based autotrophic denitrification bioreactor.[28] The abundance of Proteobacteria in RAS_Sludge was 44.82%, which was 34.07% higher than that in Synthetic_Sludge; the abundance of Bacteroidota was 5.28%, which was 40.19% lower than that in Synthetic_Sludge, and the abundance of Actinobacteriota was 4.88%, 4.31% lower than that in I Synthetic_Sludge. The abundances of Chloroflexi and Firmicutes were similar in the two reactors. Proteobacteria and Bacteroidetes are very important in denitrification and are widely distributed in denitrification bioreactors.[29] Proteobacteria are often found in the process of sulfur autotrophic denitrification and are important functional bacteria in the coreduction of SO_4^{2-} and nitrate.[30] The abundance of Proteobacteria in RAS_Sludge increased, indicating that denitrification was improved when the denitrification bioreactor was added to the RAS. Bacteroidetes play a role in breaking down macromolecules during denitrification,[31] aiding NO_3^--N reduction. Chloroflexi can degrade macromolecular organic matter, and members of Firmicutes are often found in activated sludge systems and comprise a wide variety of bacteria that decompose pollutants.[27] Actinobacteria is also a common phylum in denitrification bioreactors.[28,32]

The distribution of microbial communities at the genus level is shown in Figure 3b. The dominant genera in RAS_Sludge were *Thermomonas* (8.24%), *Longilinea* (5.02%), *Arenimonas*

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(4.36%), *Thiobacillus* (1.87%), *Comamonas* (1.01%), *Desulfuromonas* (1.98%), and *Thauera* (1.01%). There were significant differences in the abundance of dominant genera between RAS_Sludge and Synthetic_Sludge. The dominant genera in Synthetic_Sludge were *Longilinea* (6.74%), *Arenimonas* (0.36%), *Thiobacillus* (0.61%), *Comamonas* (0.95%), *Desulfuromonas* (0.20%) and *Thauera* (0.049%). The most dominant genera in Synthetic Sludge mainly consisted of unclassified genera. These results showed that RAS water significantly changed the bacterial community structure. There were residual food residues and feces in the RAS, which could be used as organic substances for denitrification. *Arenimonas* is also a common bacterium in mixed nutrition systems.[29] Pang et al.[33] found that *Arenimonas* was abundant in a mixed nutrition denitrification system with pyrite and biodegradable polymer complex as electron donors. *Thermomonas* is capable of denitrification and uses inorganic carbon under organic-free conditions.[34,35] *Thermomonas* was the dominant denitrifier in the S^0 cultures; its abundance in RAS_Sludge was 8.24%, higher than that in Synthetic_Sludge, indicating that the presence of organic matter promoted its growth. *Thiobacillus*, *Desulfuromonas*, and *Comamonas* are all Proteobacteria. Compared with that of Synthetic_Sludge, the relative abundance of these genera in RAS_Sludge increased. *Thiobacillus* plays an important role in sulfur autotrophic denitrification.[36] The abundance of *Thiobacillus* in RAS_Sludge was 1.26% higher than that in Synthetic_Sludge, indicating that mixed nutrition aided the growth of sulfur autotrophic denitrifying bacteria. *Comamonas* belongs to Bacteroidetes at the phylum level and is responsible for degrading high molecular weight compounds; they are commonly found in heterotrophic denitrifying bioreactors.[37] The relative abundance of *Comamonas* in RAS_Sludge increased compared to that in Synthetic_Sludge, which might be because of organic matter in the RAS water. *Longilinea* is related to the degradation of organic matter,[38] and its abundance in RAS_Sludge and Synthetic_Sludge was above 5%. *Desulfuromonas* is also able to carry out denitrification.[34]

To gain general insights into the functional profile variation in the different sludge, functional genes in RAS_Sludge and Synthetic_Sludge were predicted based on FAPROTAX. The heatmap of functional community profiles based on FAPROTAX is shown in Figure 4. The functional genes in the two bioreactors were similar but with different relative abundances. The three most dominant genes in both RAS_Sludge and Synthetic_Sludge included those involved in "chemoheterotrophy," "aerobic_chemoheterotrophy," and "fermentation." These are common genes in seed sludge obtained from wastewater treatment tanks.[38] The relative abundance of "ureolysis" was significantly higher in RAS_Sludge than in Synthetic_Sludge, which was attributed to feces from fish. In addition, the functional genes related to the N cycle, such as "nitrate_reduction," "nitrate_respiration," and "nitrite_respiration," had a higher relative abundance in the RAS_Sludge than in the Synthetic_Sludge.

There were no significant differences in the abundances of denitrification-related genes, such as "denitrification," "nitrous_oxide_denitrification," "nitrite_denitrification," and "nitrate_denitrification." The results showed that the denitrification function genes enriched by RAS were the same as those enriched using synthetic water. The abundance of genes related to sulfur oxidation was also the same between sludge types, as well as genes related to "iron_respiration." Some aerobic functional genes, including those related to "aerobic ammonia_oxidation," "aerobic_nitrite_oxidation," and "nitrification," also had a higher abundance in the RAS_Sludge than Synthetic_Sludge. This resulted from the water pumped in the FeS_x-based denitrification bioreactor in the RAS containing DO > 5 mg/L.

3. CONCLUSIONS

In this study, an RAS with a calcined FeS_x-packed denitrification bioreactor was set up to investigate denitrification performance using FeS_x as an electron donor for removing NO_3^--N from the RAS. In the simulated aquaculture wastewater, the concentrations of NO_2^--N and NH_4^+-N were lower than 0.20 and 0.50 mg/L, respectively, and NO_3^--N gradually accumulated. After introducing cultured fish and operating the FeS_x-based denitrification bioreactor, the concentrations of NO_2^--N and NH_4^+-N in the fish tank were lower than 0.01 mg/L and below the detection limit, respectively, and 79.04% of NO_3^--N was removed. After 24 days of RAS operation, the concentration of SO_4^{2-} was lower than 200 mg/L. During the entire RAS operation, the pH in the fish tank was stable at around 7. Overall, the water quality in the RAS was maintained within a suitable range for cultured fish.

During the whole experiment, the survival rate of carp was 95%; the cultured fish were healthy and lively, and there was no death caused by the deterioration of the water quality. At the end of the experiment, the carp grew from 2−3 to 8−10 cm, an increase of 6−7 cm. The weight of the fish increased from 3.21 to 8.52 g, with an average increase of 5.31 g. The culture density increased from the initial 10 to 26.54 kg/m^3.

Microbial community structure analysis showed higher diversity in the denitrification bioreactor operated in the RAS (RAS_Sludge) than in that operated using synthetic wastewater (Synthetic_Sludge). The dominant genera in RAS_Sludge included *Thermomonas*, *Longilina*, *Arenimonas*, and *Thiobacillus*, while unclassified bacteria were dominant in Synthetic_Sludge. The prediction of microbial functional genes showed that the functional genes in the two bioreactors were similar but with different relative abundances due to the difference in the quality of influent water. This study provides a strategy to enhance nitrogen removal from the RAS.

4. MATERIALS AND METHODS

4.1. Experimental Fish. Common carp (*Cyprinus carpio*) is a freshwater fish with high adaptability, cold tolerance, and fast growth; it is one of the main cultured fish species in China. Carp with an average length of 2−3 cm were purchased from Zhengzhou Fishing Ground (Henan, China). Before the experiment, fish were put in 200 L of aerated tap water and were acclimated for 2 weeks to adapt to the laboratory conditions. The acclimation period used a natural light cycle, and the average water quality parameters were a temperature of 20 ± 0.5 °C, pH 8.5 ± 0.2, DO > 5 mg/L, and alkalinity of 250 ± 20 mg $CaCO_3$/L. The fish were fed twice a day, with a total daily feeding amount of 1% of the fish mass. Fish food contained crude protein ≥38%, crude fat ≥3.0%, crude fiber ≤8.0%, crude ash ≤15%, moisture ≤10%, calcium ≥1.2%, phosphorus ≥0.4%, and lysine ≥1.5%. Healthy and lively fish were randomly selected for the experiment.

4.2. Experimental Water. The water used in fish tanks was municipal tap water, which was aerated to remove

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chlorine. In the stage using simulated aquaculture wastewater, an NH_4^+-N stock solution (10 g N/L) was added to the fish tank to simulate the NH_4^+-N produced by fish, prepared by adding 38.2 g of ammonium chloride (NH_4Cl) into 1 L of deionized water. The stock solution was added to the fish tanks to achieve the desired NH_4^+-N concentrations. In the present study, final concentrations of 0.5 and 8 mg of N/L NH_4^+-N were used in the simulating stage. Fish food (2 g) was also added to simulate fish waste generated from feeding. Synthetic RAS wastewater was prepared with these components by adding 0.304 g/L $NaNO_3$, 0.0019 g/L NH_4Cl, and 0.044 g/L KH_2PO_4 to tap water, obtaining 50 mg N/L of NO_3^--N, 0.5 mg N/L of NH_4^+-N, and 10 mg P/L.

4.3. RAS Construction. Two sets of parallel RASs were established (Figure 5). Each RAS was composed of a fish tank

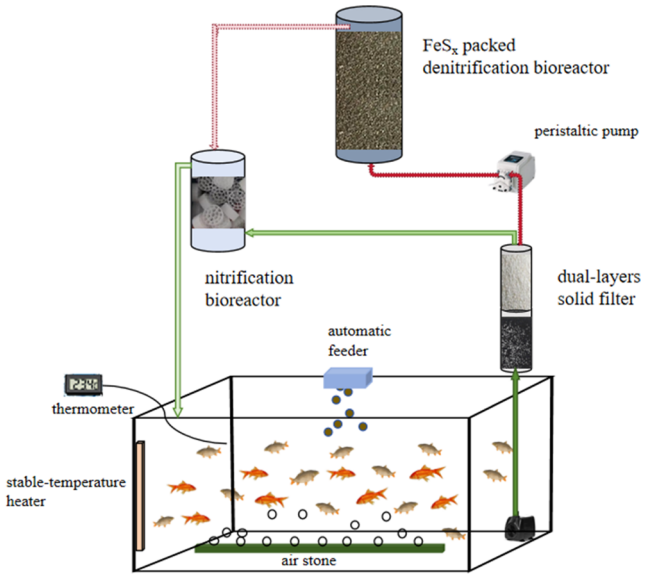

Figure 5. Experimental RAS device.

(L × W × H: 42 × 23 × 26 cm) and two water-treatment loops. The fish tank was equipped with an automatic feeder and an aeration device. During the operation, the fish tank was cleaned daily with a magnetic brush.

The first treatment loop included a dual-layer solid filter (H × D, 20 × 4 cm) and a nitrification bioreactor (H × D, 20 × 13.5 cm). The dual-layer solid filter consisted of two identical cylindrical polyethylene columns. The first layer filter was filled with perlite particles (1−2.5 mm) to remove large-particle solid waste, and the second layer filter was filled with cotton with smaller mesh to remove fine solid waste. The nitrification bioreactor was filled with a plastic filler for the attachment and growth of microorganisms, with an effective volume of 1.5 L. The nitrification bioreactor was also equipped with an aeration device. The bioreactor was inoculated with sludge from an aerobic tank (2.68 g/L). In the first treatment loop, the water in the fish tank was pumped into the dual-layer solid filter using a submersible pump to remove solid waste and then flowed into the nitrification bioreactor to transform NH_4^+-N to NO_3^--N. After treatment, the water was recycled back to the fish tank.

The second treatment loop was composed of a denitrification bioreactor (H × D, 35 × 5 cm) and accompanying tubing. The denitrification bioreactor was filled with 0.20 kg volcanic rock and 0.54 kg FeS_x, with an effective volume of 254 mL. The FeS_x was prepared by calcining Fe and S^0 powders at a molar ratio of 1:1. Fe and S^0 powders were thoroughly and evenly mixed before being heated in a tube furnace (KJ-T1200-S801C, Kejia). The burning process was carried out under N_2 with heating for 60 m to 450 °C and holding at 450 °C for 40 min before lowering the temperature to room temperature. Before the operation, the FeS_x-based denitrification bioreactor was inoculated with 2.90 g/L activated sludge from an anaerobic tank of a municipal sewage treatment plant. Water was pumped from the dual-layer solid filter to the denitrification bioreactor using a peristaltic pump. The effluent from the denitrification bioreactor was discharged into the nitrification bioreactor and then recirculated back to the fish tank.

4.4. Experimental Design and Operation. The experimental protocol was approved by the Animal Care and Use Committee of Henan University of Technology. The calculation of key parameters of the RAS is shown in Table 2.

Table 2. RAS Parameter Design and Calculation[a]

index	calculation method	parameter
aquaculture water (m^3)		0.02
breeding density (kg/m^3)		10
biomass (kg)	aquaculture water × breeding density	0.2
daily feeding rate (%)		1
daily feeding amount (kg)	biomass × daily feeding rate	0.002
feed protein content (%)		38
NH_4^+-N transformation amount (mg)	daily feeding amount × feed protein content × 16% × 40%	48.64

[a]16% is nitrogen content, and 40% is the amount of nitrogen converted into NH_4^+-N.

The RAS was operated in two phases (Table 3). Phase I was carried out to simulate NO_3^--N accumulation without the addition of a denitrification bioreactor. In Phase II, aquaculture fish were introduced to the fish tank. Initially, the denitrification bioreactor was not operated to determine the NO_3^--N accumulated during raising the fish. Following this, the FeS_x-based denitrification bioreactor was operated to remove NO_3^--N from the RAS.

To compare the influence of the RAS on the microbial community structure in the FeS_x-based denitrification bioreactor, another FeS_x-based denitrification bioreactor was independently operated by using synthetic NO_3^--N-polluted wastewater as a control.

The first phase was the operation with simulated aquaculture wastewater (Table 3). In the first 20 days, 10 mg of NH_4^+-N (0.5 mg of N/L) was added daily to start the nitrification bioreactor. In the last 12 days, 160 mg of NH_4^+-N (8 mg of N/L) was supplemented daily, which was about 3.3 times the calculated NH_4^+-N conversion. To keep the pH stable, sodium bicarbonate ($NaHCO_3$) was used to adjust the pH in the fish tank.

In the second phase, farmed fish were introduced to test the practicality of the RAS. After domestication, the average weight of the fish was 3.21 g/carp; the breeding density was 10 kg/m^3, and the daily feeding amount was 2 g. The RAS was operated for 10 days without denitrification during days 11−34, and the denitrification bioreactor was connected to investigate its effect on NO_3^--N removal.

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Table 3. RAS Experimental Scheme and Operating Conditions

	experiment	breeding density (kg/m^3)	NH_4^+-N supplement (mg N/L)	denitrification reactor operation (yes or no)	duration (days)
phase I	initialization phase		0.5	no	1−20
	steady stage		8	no	21−32
phase II	initialization phase	10		no	1−10
	steady stage	10		yes	11−34

The changes in temperature, pH, DO, NO_3^--N, NO_2^--N, and NH_4^+-N in the fish tank were monitored daily, as were the changes in the SO_4^{2-} concentration after the connection of the denitrification bioreactor. Due to evaporation, the amount of water in the fish tank was reduced, and dechlorinated tap water was added to the fish tank daily to ensure water level stability.

4.5. Analytical Method. *4.5.1. Chemical Analysis.* Water samples were taken from the fish tank and filtered using a 0.45 μm filter membrane for water quality analysis, including pH, DO, NO_3^--N, NO_2^--N, NH_4^+-N, and SO_4^{2-}. Portable water quality analyzers, including Thermo Scientific Eutech DO, pH value, and COND 6+, were used to measure pH and DO. Ion chromatography (ICS-900, Thermo, US) was used to determine the SO_4^{2-} concentration. The concentrations of NO_2^--N, NH_4^+-N, and NO_3^--N were measured by ultraviolet spectrophotometry (UV-5900PC, METASH, Shanghai) according to the standard methods. In addition, 20 carp were randomly taken from the fish tank at the beginning and end of the experiment, and the length and weight were measured to obtain average fish growth and weight gain.

4.5.2. Microbial Analysis. At the end of the experiment, the bioreactors were disassembled, and the FeS_x materials attached to sludge in the middle of the bioreactors were collected for microbial analysis (RAS_Sludge and Synthetic_Sludge). The microbial samples were analyzed by Shanghai Majorbio Technology Co., Ltd. The online platform of Majorbio cloud platform was used to analyze the microbial community composition.

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Notes

The authors declare no competing financial interest.

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