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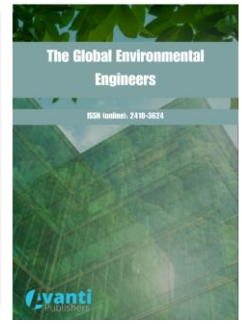

Denitrification Performance and Nitrogen Removal Pathways in Constructed Wetlands Treating Simulated Mariculture Wastewater

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ABSTRACT

To address the problem of eutrophication in nearshore waters caused by nitrogen pollution from marine aquaculture effluent and to investigate the patterns of denitrification in constructed wetlands under simulated marine aquaculture conditions, this study constructed a vertical subsurface flow constructed wetland system. Using simulated marine aquaculture effluent as the treatment substrate, the study systematically examined the effects of hydraulic retention time (HRT), salinity, and wetland substrate type on denitrification efficiency. The experimental results indicate that the constructed wetland demonstrated excellent removal efficiency for NH_4^+-N, NO_3^--N, and NO_2^--N. The optimal operating parameter combination was an HRT of 12 h, C/N ratio of 8, and a salinity of 25‰, achieving an NO_3^--N removal rate of 82.15% NH_4^+-N removal reached 77.5%, and NO_2^--N accumulation was controlled below 0.35 mg/L. This study elucidated the denitrification patterns in constructed wetlands treating simulated seawater aquaculture effluent, optimized operational parameters, and provided a theoretical basis and technical support for the ecological treatment of seawater aquaculture effluent, aligning with the research needs in the field of environmental engineering for efficient, low-cost pollution control technologies.

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1. Introduction

In recent years, the global marine aquaculture industry has experienced rapid growth, becoming a key pillar industry for ensuring food security and promoting the growth of the fisheries economy. In 2022, the output value of China's marine aquaculture reached 463.884 billion RMB, with total production continuing to rise; however, the large volumes of effluent generated during the aquaculture process have also become one of the primary sources of environmental pollution in nearshore waters [1]. Effluent from marine aquaculture is rich in pollutants such as nitrogen, phosphorus, organic matter, and suspended solids. In particular, nitrogen pollutants (NH_4^+-N, NO_3^--N, and NO_2^--N) often exceed regulatory limits; their direct discharge can lead to eutrophication in nearshore waters, triggering ecological disasters such as red tides and harmful algal blooms [2]. Disrupt the marine ecological balance, and meanwhile threaten the survival of aquaculture organisms as well as human health and safety. Currently, denitrification technologies for seawater aquaculture effluent are primarily categorized into physical, chemical, and biological methods. Although physicochemical methods (such as adsorption, membrane separation, and chemical precipitation) offer advantages such as high treatment efficiency and rapid reaction rates, they also have drawbacks including high operating costs, a tendency to cause secondary pollution, and operational complexity, making them unsuitable for the low-cost treatment requirements of large-scale seawater aquaculture effluent [3]. Biological methods have become the mainstream technology for denitrification in seawater aquaculture effluent treatment due to their environmental friendliness, low cost, and consistent denitrification performance [4]. Constructed wetlands, as a typical ecological biological treatment technology, are widely used in the field of wastewater treatment due to their simple construction, ease of maintenance, low energy consumption, and ecological restoration capabilities [5]. Constructed wetlands are ecosystems that utilize the synergistic interaction of wetland plants, substrate, and microorganisms to remove pollutants through processes such as physical retention, chemical adsorption, and biological transformation [6]. The denitrification process in these systems primarily involves plant uptake, substrate adsorption, and microbial transformation, with microorganism-mediated denitrification serving as the core process for nitrogen removal and accounting for the majority of total nitrogen removal in constructed wetlands. Compared to freshwater constructed wetlands, seawater aquaculture effluent possesses unique characteristics, including high salinity and a low carbon-to-nitrogen (C/N) ratio. The high-salinity environment inhibits the activity of denitrifying bacteria, while the low C/N ratio limits the smooth progression of denitrification reactions. Consequently, constructed wetlands face challenges such as low denitrification efficiency and poor operational stability when treating seawater aquaculture effluent, and the patterns of denitrification also differ significantly from those observed in freshwater systems [7]. Denitrification in constructed wetlands is a complex biochemical process primarily mediated by denitrifying bacteria, which reduce nitrate and nitrite to nitrogen gas (N_2) under anoxic conditions, thereby removing nitrogen from the water [8]. Research on denitrification in constructed wetlands has primarily focused on optimizing operational parameters, improving substrates, and selecting suitable plants [9, 10]. In terms of operational parameters, hydraulic retention time (HRT), influent carbon-to-nitrogen ratio (C/N), and salinity are key factors influencing denitrification efficiency. Studies have shown that extending HRT increases the contact time between pollutants and the wetland system, promoting thorough denitrification; however, excessively long HRT can lead to reduced operational efficiency and increased land requirements [11]; The C/N ratio is a major limiting factor in denitrification. An appropriate C/N ratio provides denitrifying bacteria with an adequate carbon source, thereby increasing the denitrification rate, whereas a low C/N ratio results in incomplete denitrification and the accumulation of nitrite nitrogen [12]; High-salinity environments can disrupt the cell membrane structure of denitrifying bacteria, inhibit their metabolic activity, and reduce denitrification efficiency; however, some halotolerant denitrifying bacteria can adapt to a certain range of salinity through self-regulation [13].

2. Materials and Methods

2.1. Experimental Setup and System Construction

This study employed a vertical subsurface flow constructed wetland as the experimental setup. This type of wetland offers advantages such as high treatment efficiency, a small footprint, and strong resistance to shock loads [14]. Furthermore, as water flows vertically through the substrate layer, it creates a favorable anoxic environment that promotes denitrification, making it suitable for denitrification treatment of effluent from marine aquaculture. The specifications for a single wetland column in the experimental setup are as follows: 15 cm in diameter and 45 cm in height. The wetland column is divided into three sections from top to bottom: the inflow zone, the substrate layer, and the outflow zone. Inflow Zone: Located at the top of the wetland column, 10 cm in height, it features an inlet. A constant-flow pump is used to control the inflow rate, and the zone is filled with gravel to ensure uniform water distribution; Substrate Layer: Located in the middle of the wetland column, 20 cm in height, this is the core component of the constructed wetland, serving to support microorganisms and adsorb pollutants. The bottom of the substrate layer is lined with a 10 cm thick layer of river pebbles (particle size 5–8 mm) to act as a support layer, preventing substrate loss while also facilitating water distribution; Outflow Zone: Located at the bottom of the wetland column, with a height of 15 cm, this zone features an outflow outlet. The height of the outlet is adjustable to control the water level within the wetland column, ensuring the substrate layer remains submerged to create an anoxic environment. A drain port is installed at the bottom of the outflow zone to empty the water and substrate from the wetland column after the experiment.

2.2. Experimental Materials

This study selected a common wetland substrate: a zeolite-biochar composite substrate (volume ratio 1:1), in which both the zeolite and biochar were commercially available products, and the gravel was natural gravel. The specific parameters are as follows: Gravel: particle size 3–5 mm, primarily composed of silica, and possessing good mechanical strength; Zeolite-biocarbon composite substrate: Zeolite and biocarbon were uniformly mixed in a 1:1 volume ratio. This substrate combines the adsorption properties of zeolite with the carbon supply capacity of biocarbon, enabling synergistic enhancement of denitrification efficiency. All substrates were washed, dried, and sieved prior to use to remove impurities and dust, ensuring their purity and uniformity.

2.3. Preparation of Simulated Mariculture Tail Water

Simulated aquaculture effluent was prepared with the following key pollutant parameters: NO_3^--N 20 mg/L, NO_2^--N 0.5–1.0 mg/L, NH_4^+-N 8 mg/L, COD 80-120 mg/L, salinity 15-35‰, and pH 7.5-8.5. The simulated effluent was prepared using deionized water, synthetic sea salt, and analytical-grade reagents. The specific preparation method is as follows: Add synthetic sea salt to deionized water, stir thoroughly, and adjust the salinity to the concentration required for the experiment; add potassium nitrate (KNO_3), sodium nitrite ($NaNO_2$), and ammonium chloride (NH_4Cl) to adjust the concentrations of NO_3^--N, NO_2^--N, and NH_4^+-N in the water, ensuring the TN concentration meets experimental requirements; Add glucose ($C_6H_{12}O_6$) as a carbon source, adjust the COD concentration, and control the C/N ratio of the influent; use hydrochloric acid (HCl) or sodium hydroxide (NaOH) to adjust the pH of the water to 7.5–8.5. Stir thoroughly, then let stand for 24 hours. Once the water has stabilized, it is ready for use in the experiment. The water quality parameters of the simulated effluent are essentially consistent with those of actual marine aquaculture effluent, accurately reflecting the denitrification performance of constructed wetlands in practical applications.

2.4. Experimental Design

This experiment investigates the denitrification patterns of simulated aquaculture effluent in constructed wetlands, employing a single-factor experimental design using the controlled variable method. While keeping basic conditions such as substrate type, pH, and temperature constant, the study sequentially examined the effects of three key parameters: hydraulic retention time (HRT), influent carbon-to-nitrogen ratio (C/N), and salinity. Gradient levels were set for each parameter, with each set of operating conditions maintained for 10 days. Water samples were collected daily from both the influent and effluent streams. Each water sample was analyzed in three analytical replicates to measure the concentrations of NO_3^--N, NO_2^--N, and NH_4^+-N. This process identified the optimal ranges for each parameter and ultimately determined the optimal combination of operating parameters for the constructed wetland to treat seawater aquaculture effluent.

2.5. Sample Collection and Detection Methods

During the experiment, influent and effluent samples were collected daily. Influent samples were collected from the outlet of the constant-flow pump, and effluent samples were collected from the outlet of the wetland column. Three replicate samples were collected for each sample, with a volume of 50 mL per sample. Immediately after collection, the samples were placed in a cooler (4°C) for storage, and testing was completed within 24 hours to prevent sample degradation from affecting the test results.NO_3^--N is determined by ion chromatography; NO_2^--N is determined by the N-(1-naphthyl)ethylenediamine spectrophotometric method; NH_4^+-N is determined by the Nessler's reagent spectrophotometric method; COD is determined by the rapid digestion spectrophotometric method; pH is determined directly using a pH meter, which is calibrated with a standard buffer solution prior to testing.

3. Results and Discussion

3.1. Effect of HRT on Denitrification Nitrogen Removal Efficiency

HRT is a key operational parameter affecting the denitrification efficiency of constructed wetlands; by altering the contact time between pollutants and the wetland system (substrate, microorganisms, and plants), it influences the extent to which denitrification occurs (Fig. **1**) [15]. This study investigated the effect of hydraulic retention time (HRT = 4, 8, and 12 h) on the system's denitrification performance. The trends in nitrogen concentrations in the influent and effluent indicate that HRT is a key parameter in regulating the system's nitrogen removal efficiency. The concentrations of nitrate nitrogen and ammonia nitrogen in the influent remained stable throughout all stages, maintaining levels of approximately 20 mg/L and 8 mg/L, respectively, providing uniform influent conditions for comparing treatment performance under different HRTs. At the HRT = 4 h stage, the system's effluent nitrate nitrogen removal rate was only about 50%; the effluent ammonia nitrogen removal rate was 50%, while the nitrite nitrogen concentration remained at around 1 mg/L. Overall nitrogen removal efficiency was relatively low, indicating that the shorter HRT could not provide sufficient reaction time for nitrification and denitrification processes. Insufficient contact between pollutants, substrate, and microorganisms prevented denitrifying bacteria from fully utilizing nitrate and nitrite in the water for denitrification, resulting in low denitrification efficiency. and the accumulation of nitrite nitrogen limits the system's denitrification performance [16]. When the HRT was extended to 8 h, the contact time between pollutants and the wetland system increased, allowing denitrifying bacteria sufficient time to carry out metabolic activities and reduce nitrate and nitrite nitrogen to nitrogen gas, thereby improving denitrification efficiency and reducing nitrite accumulation [17, 18]; the concentration of nitrate nitrogen in the effluent rapidly decreased to approximately 4 mg/L, with a removal rate increasing to 80%; the ammonia nitrogen removal rate increased to 75%; and the concentration of nitrite nitrogen also decreased simultaneously, indicating that extending the HRT provided nitrifying and denitrifying bacteria with more sufficient reaction time, thereby promoting nitrogen transformation and removal [19]. At the HRT = 12 h stage, the system's denitrification performance was further optimized, with the effluent nitrate nitrogen concentration stabilizing at approximately 3 mg/L and a removal rate as high as 85%; the ammonia

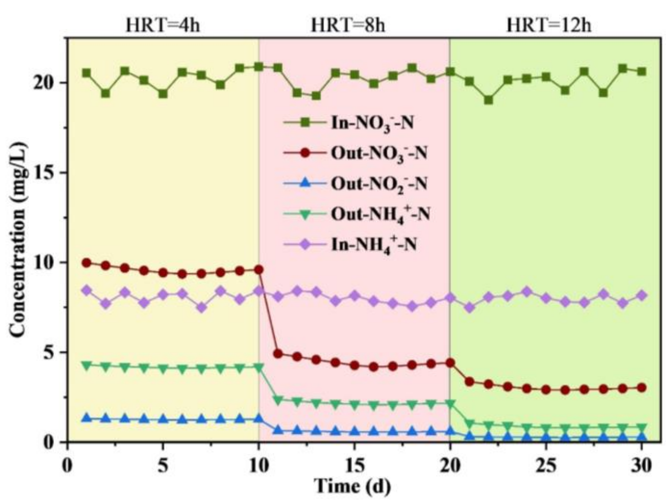

Figure 1: Changes in removal rates of NO_3^--N, NH_4^+-N and accumulation of NO_2^--N under different HRT conditions.

nitrogen concentration dropped below 1 mg/L, with a removal rate exceeding 87.5%; and nitrite accumulation virtually disappeared, achieving efficient and stable nitrogen removal. These results indicate that as HRT increases, the alternation between aerobic and anoxic environments within the system becomes more thorough, leading to more complete nitrification. The denitrification process is also enhanced due to more efficient carbon source utilization, effectively preventing the accumulation of nitrite as an intermediate product [20]. In summary, extending the HRT significantly improves the system's removal efficiency for nitrate nitrogen and ammonia nitrogen while reducing nitrite nitrogen accumulation. Under the experimental conditions, the denitrification performance was optimal at an HRT of 12 h, providing important evidence for subsequent process parameter optimization.

3.2. Effect of C/N Ratio on Denitrification Efficiency

Denitrification is a metabolic process mediated by heterotrophic microorganisms. Carbon sources serve as a vital energy source for the growth, reproduction, and metabolic activities of denitrifying bacteria, and the C/N ratio of the influent directly affects the smooth progression of the denitrification reaction (as shown in Fig. **2**) [21, 22]. In terms of COD removal efficiency, as the influent COD concentration increased in stages, the effluent COD concentration showed a corresponding upward trend, rising from approximately 30 mg/L in the initial stage to 45 mg/L in the second stage and 65 mg/L in the third stage. However, the removal rate remained consistently high (>65%), indicating that the system possesses strong buffering and degradation capabilities regarding carbon sources. As an electron donor for denitrifying bacteria, the increased concentration of organic carbon sources not only provides sufficient energy for microbial metabolism but also directly regulates the synthesis and enzymatic activity of key denitrification enzymes, laying a material foundation for improving subsequent nitrogen transformation efficiency [23]. The results of the dynamic changes in nitrogen forms show that the nitrogen concentrations in the effluent exhibit a significant decreasing trend as the COD concentration increases. In the initial stage (COD = 110 mg/L), the concentrations of NO_3^--N, NH_4^+-N, and NO_2^--N in the effluent were 9.2, 4.0, and 1.0 mg/L, respectively; When COD increased to 170 mg/L, the effluent concentrations of NO_3^--N and NH_4^+-N decreased to 6.3 and 3.0 mg/L, respectively, while the NO_2^--N concentration further decreased to 0.5 mg/L; During the high

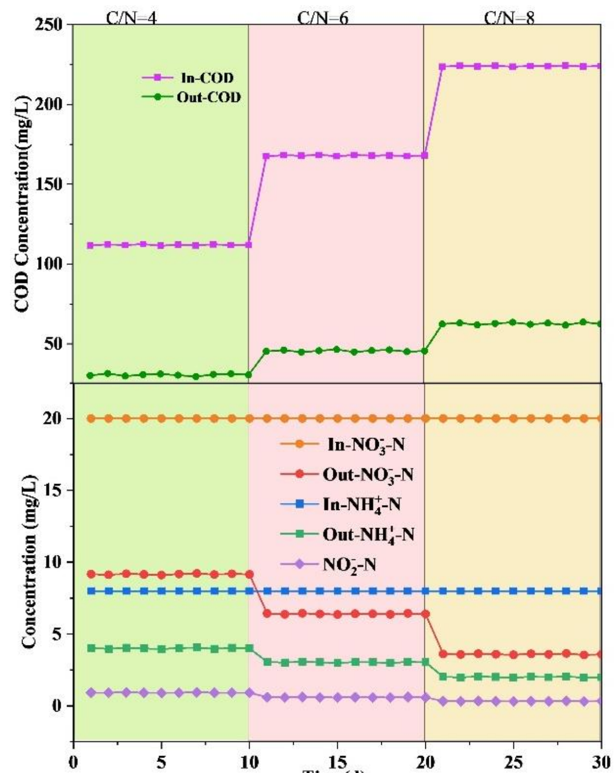

Figure 2: Changes in removal rates of COD, NO_3^--N, NH_4^+-N and accumulation amount of NO_2^--N under different C/N ratios.

carbon source phase (COD = 220 mg/L), the effluent concentrations of NO_3^--N and NH_4^+-N decreased to 3.5 and 2.0 mg/L, respectively, while the NO_2^--N concentration was nearly zero. This significant improvement in nitrogen removal efficiency is closely related to the enzymatic metabolic processes of denitrifying bacteria. The denitrification process is catalyzed synergistically by a series of key enzymes, among which nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor), and nitrous oxide reductase (Nos) are the core functional enzymes [24]. When the carbon source supply is insufficient, the lack of electron donors limits Nar activity, leading to an impeded nitrate reduction process and an elevated NO_3^--N concentration in the effluent; simultaneously, insufficient Nir activity can cause a temporary accumulation of nitrite [25]. As COD concentrations increased, an ample supply of organic carbon not only promoted the growth and reproduction of denitrifying bacteria but also significantly enhanced the activity of Nar and Nir by providing reducing equivalents (NADH) [26]. At the same time, the increased activity of Nos effectively prevented the accumulation of N_2O, ensuring the stable removal of nitrogen in the form of N_2. This finding is highly consistent with the extremely low NO_2^--N concentrations in the effluent during the high-carbon-source phase. Notably, the system simultaneously achieved the removal of NH_4^+-N, which may be related to the enhanced metabolic activity of heterotrophic nitrifying bacteria resulting from the increased carbon source. Heterotrophic nitrifying bacteria utilize organic carbon sources to convert NH_4^+-N into NO_3^--N, which is then further reduced by denitrifying bacteria, forming a synergistic denitrification pathway of heterotrophic nitrification–aerobic denitrification [27]. The increased carbon source concentration not only provides energy for the heterotrophic nitrification process but also supplies sufficient substrate for the subsequent denitrification step, thereby promoting efficient nitrogen removal.

3.3. Effect of Salinity on Denitrification Nitrogen Removal Efficiency

The high salinity of aquaculture effluent is a key factor affecting the denitrification efficiency of constructed wetlands. A high-salinity environment can disrupt the cell membrane structure of denitrifying bacteria, affect their osmotic balance, and inhibit microbial metabolic activity [28]. As shown in Fig. (**3**), as salinity increases, the COD removal efficiency of the constructed wetland system decreases. The COD concentration in the effluent rises with increasing salinity, and the system's stability in removing organic matter gradually diminishes. When salinity ranged from 15‰ to 25‰, the influent COD concentration remained stable between 215 and 225 mg/L, and the COD removal rate remained above 78%. Specifically, the average COD removal rate was approximately 81.5% at a salinity of 15‰ and approximately 79.8% at a salinity of 25‰. The relatively small variation in COD removal efficiency during this stage indicates that the inhibitory effect of low-to-moderate salinity environments on the system's organic matter removal function is weak. Salt-tolerant microorganisms within the wetland can adapt to salinity stress through mechanisms such as osmotic regulation and the synthesis of intracellular compatible solutes, thereby maintaining high organic matter degradation metabolic activity and ensuring the stability of the system's COD removal performance [29]. When salinity increased from 25‰ to 35‰, the average COD removal rate decreased from 79.8% to approximately 67.2%. This is because a high-salinity environment (35‰) significantly exacerbates osmotic stress on functional microorganisms, disrupting the integrity and permeability of microbial cell membranes, which leads to reduced activity of intracellular metabolic enzymes (such as dehydrogenases and oxidases) and a significant decline in the microorganisms' ability to oxidize and degrade organic matter; At the same time, high salinity affects the adsorption properties of the wetland substrate, weakening the physical adsorption and chemical complexation of dissolved organic matter by the substrate, which further reduces the system's COD removal efficiency [30]. Furthermore, the microbial community structure changes under high-salinity conditions: the abundance of less salt-tolerant heterotrophic bacteria decreases significantly [31], while bacterial groups that are highly salt-tolerant but have lower organic matter degradation efficiency become dominant, which also leads to a decline in COD removal efficiency.

The system's removal efficiency for nitrogen pollutants exhibited a phased pattern similar to that observed for COD: under salinity conditions of 15‰-25‰, the removal rate of NH_4^+-N remained above 70%, and that of NO_3^--N remained above 80%; the accumulated concentration of NO_2^--N consistently remained below 0.1 mg/L, with no accumulation of nitrite observed. During this stage, salinity did not significantly inhibit the functional microbial communities involved in nitrification and denitrification: regarding the nitrification process, halotolerant ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) can maintain enzyme activity through intracellular ion regulation, and the activity of ammonia monooxygenase (AMO) and nitrite oxidoreductase (NOR) was not significantly affected. both ammonia oxidation and nitrite oxidation proceeded efficiently, preventing the accumulation of nitrite nitrogen [32]; the activities of nitrate reductase (Nar) and nitrite reductase (Nir) in halotolerant denitrifying bacteria remained stable, allowing the denitrification process to proceed completely, with nitrate nitrogen being thoroughly reduced to N_2 [33]. When salinity increased to 35‰, nitrogen removal efficiency deteriorated significantly: the removal rate of NH_4^+-N dropped to 52.6%, and that of NO_3^--N fell to 68.3%; the accumulation of NO_2^--N reached a maximum of 0.78 mg/L. This is primarily because the high-salinity environment significantly reduced the metabolic activity of AOB and NOB, with NOB being far more sensitive to salinity than AOB. The activity of nitrite oxidoreductase was significantly inhibited, preventing the further conversion of nitrite nitrogen into nitrate nitrogen and resulting in a marked accumulation of nitrite; simultaneously, high salinity reduced the ammonia-oxidizing efficiency of AOB, resulting in a corresponding decrease in NH_4^+-N removal efficiency [34].

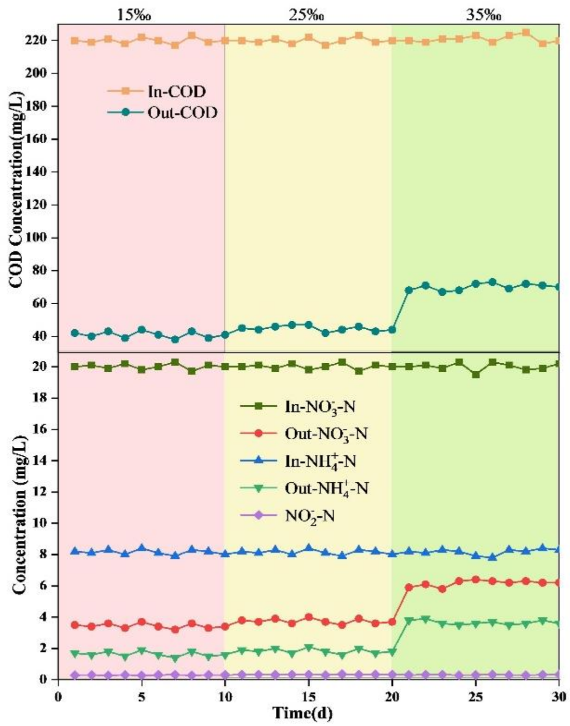

Figure 3: Changes in removal rates of COD, NO_3^--N, NH_4^+-N and accumulation amount of NO_2^--N under different salinity levels.

4. Conclusion

This study addresses the issue of eutrophication in nearshore waters caused by nitrogen pollution from marine aquaculture wastewater by constructing a vertical subsurface flow constructed wetland system to investigate the denitrification patterns of simulated aquaculture wastewater. The study examines the effects of hydraulic retention time, carbon-to-nitrogen ratio, salinity, and substrate type on denitrification efficiency, and combines microbial community analysis to elucidate the denitrification mechanism. The results show that the system exhibits excellent removal efficiency for ammonia nitrogen, nitrate nitrogen, and nitrite nitrogen. The optimal operating parameters are a hydraulic retention time of 12 hours, a carbon-to-nitrogen ratio of 8, salinity of 25‰. Under these conditions, the nitrate removal rate reached 82.15%, the ammonia nitrogen removal rate was 77.5%, and nitrite accumulation was controlled below 0.35 mg/L. This study optimized process parameters and provides theoretical and technical support for the ecological treatment of seawater aquaculture wastewater.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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