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Research Article

Adaptive Biofiltration Strategy for an Evolutionary Relic: Assessing Bacterial Consortia for Nitrogen Management in *Huso huso* Aquaculture

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Recirculating aquaculture systems (RASs) represent a sustainable solution for intensive fish production, offering 90%–99% water savings compared to conventional systems while preventing nutrient contamination of natural waterways. The accumulation of nitrogenous compounds, driven by factors, like overfeeding, fish waste, and insufficient water exchange, can cause major problems in aquaculture production. This study investigated the optimization of biofiltration systems for nitrogen removal in RAS through a factorial design examining two bacterial species (*Nitrosomonas* and *Nitrobacter*), two substrates (sand and straw), and two cell densities in a beluga (*Huso huso*) sturgeon farm. Results demonstrated distinct functional specialization, with the most effective total ammonia nitrogen (TAN) reduction (upto 85%) achieved by *Nitrosomonas* in straw and sand systems, while *Nitrobacter* in straw systems showed 70% nitrite (NO_2^-) removal. Substrate–microbe interactions were critical, revealing that sand provided an optimal environment for *Nitrosomonas*, whereas straw optimized *Nitrobacter*-driven nitrite reduction. These findings provide actionable guidelines for designing targeted biofiltration systems in RAS, with sand–*Nitrosomonas* recommended for ammonia-dominated systems and straw–*Nitrobacter* for nitrite mitigation. The study advances sustainable aquaculture practices by demonstrating how substrate-specific microbial optimization can simultaneously improve water quality management and environmental protection.

Keywords: biofiltration optimization; nitrogen removal; Nitrosomonas–Nitrobacter synergy; recirculating aquaculture systems (RAS); sturgeon aquaculture; water conservation technology

1. Introduction

The quality of water resources is perpetually threatened by a variety of anthropogenic activities, including industrial discharges, agricultural runoff, urbanization, climate change, and population growth [1]. Environmental pollution has now become the foremost concern in the world; conversely, scientific community is in pursuit of an explanation for pollution and its primary cause [2]. Recirculating aquaculture systems (RASs) have emerged as a critical solution for sustainable fish production, particularly in water-scarce regions, by reducing freshwater consumption by 90%–99% compared to conventional aquaculture while preventing nutrient pollution of natural ecosystems [3, 4]. This technology, with conceptual foundations dating back decades [5], is especially vital for arid countries facing severe water constraints, where traditional aquaculture's environmental footprint is untenable. By

integrating advanced biofiltration, RAS effectively converts toxic nitrogenous wastes (ammonia and nitrite) into less harmful nitrate, achieving effluent standards that comply with international regulations such as the EU Water Framework Directive [6].

The core of this water treatment process relies on harnessing specific nitrifying bacteria to drive the nitrification cycle. The efficacy of RAS hinges on biofilter performance, where nitrifying consortia (*Nitrosomonas* and *Nitrobacter*) sequentially oxidize ammonia to nitrate. While this microbial process is well-characterized in wastewater treatment [7], its optimization in sturgeon RAS requires substrate-specific adaptations. The performance of these essential bacteria is, in turn, heavily dependent on the biofilter substrate, which provides the physical surface for biofilm formation. Straw and sand substrates, for instance, differentially influence bacterial biofilm formation and nitrification rates due to variations in surface area, porosity, and organic carbon content [8]. Therefore, selecting an optimal substrate is critical for enhancing the specific nitrifying bacteria responsible for maintaining water quality. Emerging approaches combine these substrates with advanced oxidative treatments (e.g., photocatalysis) to enhance nitrogen removal [9], though practical implementation demands empirical validation under operational RAS conditions. Studies demonstrate that *Nitrosomonas europaea* achieves optimal ammonia oxidation at cell densities of 10^4–10^5 cells/mL, while *Nitrobacter winogradskyi* shows peak nitrite conversion at slightly higher concentrations (10^5–10^6 cells/mL) due to its lower growth yield [10]. The timing of bacterial inoculation is equally critical, with biofilm maturation typically requiring 2–3 weeks for complete nitrification capacity [11]. Recent advances in microbial consortia management suggest staged introduction of ammonia-oxidizing bacteria (AOB) before nitrite-oxidizing bacteria (NOB) can prevent nitrite accumulation during system startup [12], a protocol particularly relevant for sensitive species like sturgeon.

Sturgeon aquaculture exemplifies the urgent need for such innovations. As ancient lineages facing extinction due to overfishing and habitat destruction [13], sturgeons (e.g., *Huso huso*), a species critically and highly valued for producing beluga caviar on a world scale, now rely heavily on RAS for conservation-oriented production. Traditional pond systems fail to address the species' sensitivity to water quality fluctuations, whereas RAS provides precise environmental control to mitigate stress and disease [11]. However, ammonia toxicity remains a critical challenge in these closed systems, with suboptimal biofiltration leading to gill damage, immunosuppression, and mortality [14].

Substrate selection profoundly influences biofilter performance through both physical and biochemical mechanisms. Straw substrates, with their high cellulose content (38%–42% dry weight) and porous structure (surface area 2.5–3.1 m^2/g), promote thicker biofilm formation but may release organic carbon that stimulates heterotrophic competition [15]. In contrast, sand substrates (particle size 0.5–2.0 mm, porosity 30%–35%) favor nitrifier dominance due to superior oxygen diffusion [16], though their lower specific surface area (0.8–1.2 m^2/g) requires careful hydraulic loading optimization.

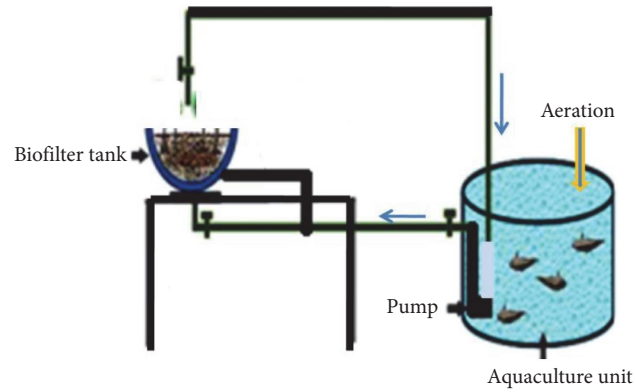

Figure 1: Schematic design of biofiltration system.

Hybrid systems combining straw's biofilm support with sand's oxidative potential show particular promise, achieving 20%–30% higher nitrification rates than single-substrate designs [17]. These material characteristics directly impact the colonization patterns of *Nitrosomonas* and *Nitrobacter*, which exhibit distinct attachment preferences based on surface hydrophobicity and nutrient gradients [18].

This study evaluates the synergistic effects of bacterial strains (*Nitrosomonas* vs. *Nitrobacter*), substrate types (straw vs. sand), and cell densities on nitrogen removal efficiency in sturgeon RAS. By identifying optimal biofilter configurations, we aim to advance both aquaculture productivity and environmental sustainability, addressing water scarcity and pollution simultaneously.

2. Materials and Methods

The study was conducted at the Glin Moghadam Brothers' sturgeon farm located in Ramsar, Iran. The experimental setup consisted of 16 polyethylene tanks with capacities 50 liters and lasted for 60 days. A semi-intensive water beluga sturgeon farm outflow (10 kg/m^3) was feeding our experimental tank (as a closed-loop system) to have the real water discharging. The fish were fed a maintenance ration of 1% of body weight per day to minimize waste accumulation, and less than 10% fresh water was added weekly to compensate for evaporative losses, maintaining the system's closed-loop integrity. Water was prefiltered through a 100 μm mesh net before being delivered to the tanks via a 1-inch diameter gravity-fed pipeline system (Figure 1).

A $2 \times 2 \times 2$ factorial design was implemented to evaluate three key independent variables: (1) nitrifying bacterial strains (*Nitrosomonas* and *Nitrobacter*), (2) substrate materials (straw and sand), and (3) bacterial densities (10,000 and 100,000 cells/L), with two replicates per experimental condition. The bacterial strains were obtained from three commercial sources purchased from the market (BIOZYM Company, USA; Stability, USA; Glosso Factory, Iran) along with a locally isolated strain from the Institute of Scientific Research and Industrial of Iran [19], which was included to evaluate its efficacy against international products under identical operational conditions. Bacterial cultures were prepared in nutrient liquid medium and incubated for 72 h at laboratory temperature in a shaker incubator to achieve the

Table 1: Performance of different biofilter configurations in nitrogen removal for beluga sturgeon recirculating aquaculture systems in one factorial design with eight groups.

	Substrate	Bacteria	Cell density	Mean ± SD (TAN) ppm	Mean ± SD (NO_3^-) ppm	Mean ± SD (NO_2^-) ppm	Mean ± SD (pH)
1	Straw	*Nitrosomonas*	10,000 (cell/L)	$0.295^{d*} \pm 0.03$	$12.60^{a} \pm 1.56$	$0.260^{ab} \pm 0.014$	$7.40^{a} \pm 0.00$
2	Straw	*Nitrosomonas*	100,000 (cell/L)	$0.350^{cd} \pm 0.32$	$14.00^{a} \pm 1.70$	$0.265^{ab} \pm 0.035$	$7.45^{a} \pm 0.35$
3	Straw	*Nitrobacter*	10,000 (cell/L)	$0.625^{abc} \pm 0.06$	$12.80^{a} \pm 1.84$	$0.095^{d} \pm 0.007$	$7.40^{a} \pm 0.14$
4	Straw	*Nitrobacter*	100,000 (cell/L)	$0.705^{ab} \pm 0.02$	$14.15^{a} \pm 4.17$	$0.075^{d} \pm 0.007$	$7.25^{a} \pm 0.07$
5	Sand	*Nitrosomonas*	10,000 (cell/L)	$0.445^{bcd} \pm 0.04$	$13.70^{a} \pm 2.12$	$0.305^{a} \pm 0.050$	$7.35^{a} \pm 0.07$
6	Sand	*Nitrosomonas*	100,000 (cell/L)	$0.300^{d} \pm 0.04$	$17.05^{a} \pm 0.21$	$0.235^{b} \pm 0.021$	$7.60^{a} \pm 0.28$
7	Sand	*Nitrobacter*	10,000 (cell/L)	$0.770^{a} \pm 0.11$	$16.20^{a} \pm 1.13$	$0.210^{b} \pm 0.014$	$7.55^{a} \pm 0.07$
8	Sand	*Nitrobacter*	100,000 (cell/L)	$0.735^{ab} \pm 0.02$	$16.25^{a} \pm 1.06$	$0.145^{c} \pm 0.035$	$7.10^{a} \pm 0.14$

Note: *Mean values sharing the same superscript lowercase letters within a column indicate no statistically significant differences ($p > 0.05$) according to Duncan's multiple range test.

required inoculum volumes of 2–3 L [19]. Substrate materials (straw and sand) were selected based on their surface properties and biofilm formation potential [20], while bacterial densities were maintained through biweekly replenishment of nutrient medium [10]. Almost half of containers (50 L volume) were filled with straw and sand; however, the straw tanks have been a lot lighter in weight than sand tanks. This configuration provided a controlled platform for evaluating microbial community dynamics and their nitrogen removal capabilities, serving as a model system for potential industrial-scale applications.

Daily water quality monitoring was conducted using Hach instrumentation: total ammonia nitrogen (TAN) was measured via DR3900 Spectrophotometer (Method 10031 for salicylate-based TAN determination) after 0.45-μm filtration, alongside NO_2^-–N and NO_3^-–N (Methods 10206 and 10020, respectively). Dissolved oxygen (DO; ±0.1 mg/L) was recorded with an HQ40d meter (LDO101 probe), while pH (±0.01) and temperature (±0.1°C) were measured using an HQ11d meter (PHC10101 electrode). Conductivity/total dissolved solids (TDS) were monitored with an HQ430d multimeter (CDC40101 cell). Due to the stable rearing conditions maintained throughout the beluga culture experiment (60 days), water quality parameters in outlet area remained almost consistent across the study period as follows: TAN = 0.68–0.82 mg/L, nitrite (NO_2^-–N) = 0.32–0.40 mg/L, nitrate (NO_3^-–N) = 11–20 mg/L, DO = 5.2–6.5 mg/L, pH = 7.0–7.7, and TDS = 255–330 mg/L. Water quality parameters (TAN, NO_2^-, NO_3^-, DO, pH, and TDS) were monitored weekly to assess system stability, with the final sampling (Day 60) serving as the primary dataset for experimental conclusions.

The biofiltration experiment was conducted in a RAS system comprising 16 tanks (50 L each). This setup operated as a closed-loop system, where water was continuously recirculated and treated by the biofilters without a traditional water exchange. After a 20-day bacterial acclimation period, a constant flow rate of 1 L/h per tank (16 L/h total) was maintained, ensuring a hydraulic retention time (HRT) of approximately 2.5 h in the biofilter—optimal for nitrifying bacteria to prevent excessive TAN/NO_2^- accumulation. The experimental design employed a 2 × 2 × 2 factorial structure with two replicates per treatment. Data homogeneity was verified using Bartlett's test. A three-way ANOVA was conducted using SPSS 27 to assess main and interaction effects between bacterial strain, substrate type, and bacterial density. Post hoc comparisons used Duncan's multiple range test at $\alpha = 0.05$. Pearson's correlation was employed to assess interrelationships among dependent variables.

3. Results and Discussion

The experimental results (Table 1 and Figure 2) demonstrated significant variations in TAN reduction across different treatment groups. Notably, the two most effective configurations for ammonia removal were both based on *Nitrosomonas*, with Groups 1 (straw + *Nitrosomonas* at 10,000 cells/mL) and 6 (sand + *Nitrosomonas* at 100,000 cells/mL) exhibiting the highest efficacy, achieving mean TAN concentrations of 0.295 and 0.300 ppm, respectively. These values were significantly lower ($p < 0.05$) compared to other treatments, particularly those involving *Nitrobacter* (Groups 3, 4, 7, and 8), which showed higher residual TAN levels (0.625–0.770 ppm). The superior performance of *Nitrosomonas*-inoculated treatments aligns with its well-documented role as an ammonia-oxidizing bacterium (AOB), which directly converts ammonia to nitrite [21, 22].

The substrate type also influenced TAN removal, as straw-based systems (Group 1) performed comparably to sand-based systems (Group 6). This robust performance of *Nitrosomonas* on both substrates demonstrates that organic carbon availability in straw did not substantially hinder its ammonia-oxidizing activity under these experimental conditions. This contrasts with some previous studies where high organic matter was reported to suppress nitrification due to heterotrophic competition [23, 24]. The successful use of straw here highlights a key novelty of our study, demonstrating that this natural, low-cost substrate can be a viable alternative to conventional synthetic media in RAS biofiltration, challenging the prevailing assumption about its inhibitory effects on nitrifiers. Therefore, our findings indicate that under controlled conditions, straw can serve as a viable substrate without impeding ammonia oxidation. The higher cell density (100,000 cells/mL) in Group 6 did not significantly enhance TAN removal over Group 1

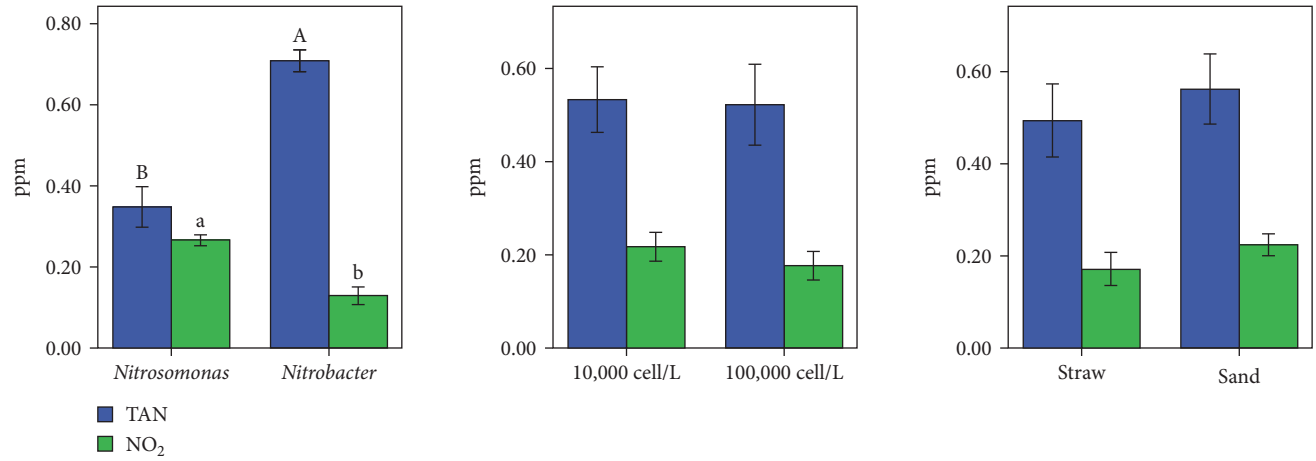

FIGURE 2: Influence of main effects of all three factors (type of bacteria, density, and substrate) employed in this study on variables TAN and NO_2^-. Significant differences ($p < 0.05$) were labeled with uppercase letters (A, B) for TAN, and lowercase letters (a, b) for NO_2^-.

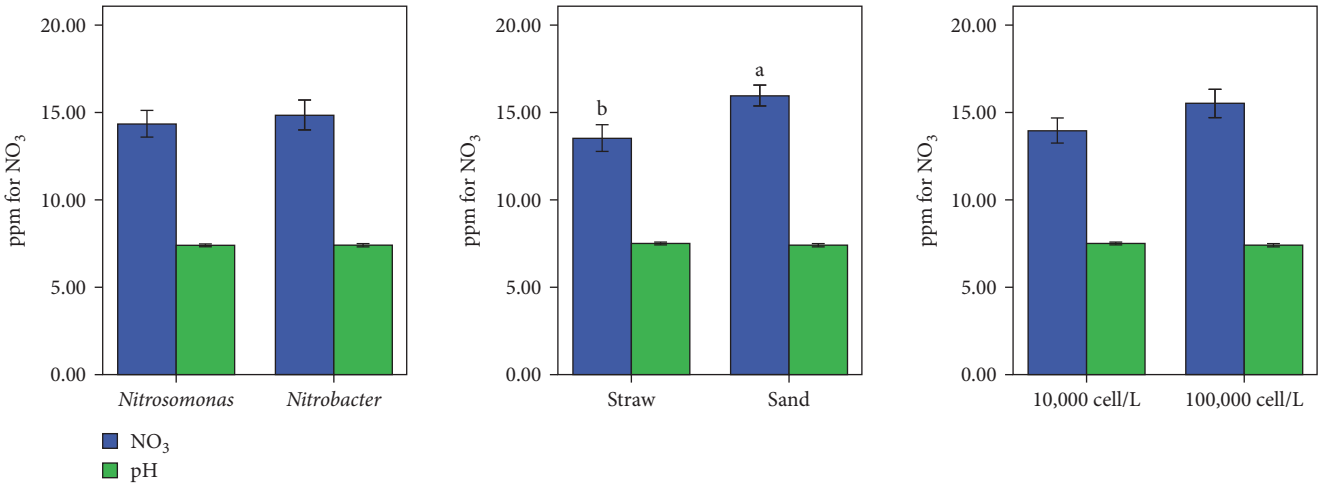

FIGURE 3: Influence of main effects of all three factors (type of bacteria, density, and substrate) employed in this study on variables pH and NO_3^-. Significant differences ($p < 0.05$) were labeled with lowercase letters (a, b) for NO_3^-.

(10,000 cells/mL), implying that beyond a threshold, increasing *Nitrosomonas* concentration may not proportionally improve ammonia oxidation, possibly due to substrate limitation or metabolic feedback inhibition [25, 26]. Therefore, the choice between sand and straw for ammonia removal can be based on other factors, such as availability or cost, without sacrificing nitrification performance.

In contrast to TAN reduction, Groups 3 (straw + *Nitrobacter* at 10,000 cells/mL) and 4 (straw + *Nitrobacter* at 100,000 cells/mL) exhibited the most substantial NO_2^- reduction, with residual concentrations of 0.095 and 0.075 ppm, respectively (Table 1 and Figure 2). This was expected since *Nitrobacter* is a nitrite-oxidizing bacterium (NOB) responsible for converting NO_2^- to nitrate (NO_3^-) [7, 27]. The lower NO_2^- levels in these groups confirm the functional specialization of *Nitrobacter*, whereas *Nitrosomonas*-dominated treatments (Groups 1, 2, 5, and 6) accumulated higher NO_2^- due to incomplete oxidation.

Interestingly, sand-based systems with *Nitrobacter* (Groups 7 and 8) showed intermediate NO_2^- removal (0.145–0.210 ppm), suggesting that substrate composition influences NOB activity [28, 29]. The higher cell density (100,000 cells/mL) in Group 4 marginally improved NO_2^- removal over Group 3 (10,000 cells/mL), reinforcing that NOB efficiency is density-dependent up to a certain limit [30].

All treatments resulted in substantial NO_3^- accumulation (12.60–17.05 ppm), with no statistically significant differences ($p > 0.05$) among groups (Table 1 and Figure 3), indicating complete nitrification in most systems. The highest NO_3^- levels were observed in sand-based treatments (Groups 6–8), possibly due to better oxygen diffusion in sandy substrates, facilitating full nitrification [31, 32]. The lack of significant variation in NO_3^- suggests that once NO_2^- is oxidized, further transformations (e.g., denitrification) were minimal, likely due to aerobic conditions maintained in the experiment. While nitrate is the least toxic nitrogenous waste product, its continuous buildup in a closed-loop RAS is a key consideration for long-term management. In commercial-scale systems, where concentrations can rise far beyond the levels observed here, this would

Table 2: Pearson intercorrelation coefficients between water quality parameters in beluga sturgeon recirculating aquaculture systems (RAS).

Variable	TAN	NO_3	NO_2	pH
TAN	—	0.23	–0.64**	–0.08
NO_3	—	—	–0.05	–0.20
NO_2	—	—	—	0.06
pH	—	—	—	—

Note: Correlation coefficients marked with ** are significant at $p < 0.01$ level (two-tailed Pearson correlation analysis).

necessitate management strategies such as controlled water exchange or the future integration of an anaerobic denitrification stage to convert nitrate to nitrogen gas.

pH remained stable (7.10–7.60) across all groups (Table 1 and Figure 3), with no significant fluctuations, indicating that nitrification did not drastically acidify the systems. This contrasts with some studies where ammonia oxidation led to pH drops due to proton release [33]. The stability here may be attributed to buffering from the substrates (straw may release alkalinity) or experimental conditions preventing excessive acid accumulation [34].

Our study confirms the functional specialization of nitrifying bacteria. *Nitrosomonas* proved to be the primary driver of ammonia removal, achieving its highest efficacy (upto 85% TAN reduction) in both straw and sand systems, with residual TAN concentrations of 0.295 and 0.300 ppm, respectively. In contrast, *Nitrobacter* showed a more distinct substrate preference, demonstrating superior NO_2^- removal efficiency specifically in straw systems (0.075 ppm residual, 70% removal). This indicates that while a specialized straw-*Nitrobacter* combination is crucial for nitrite mitigation, operators have flexibility in substrate choice when designing a biofilter for primary ammonia removal, as *Nitrosomonas* performed excellently on both. Therefore, the superior performance of *Nitrosomonas* across substrates offers flexibility in biofilter design, while the specialized performance of the straw-*Nitrobacter* combination is specifically recommended for nitrite mitigation [3, 35].

The correlation matrix (Table 2) reveals significant relationships between nitrogen species and pH in the biofiltration system. The strong negative correlation between TAN and NO_2 ($r = –0.64$, $p < 0.01$) demonstrates efficient nitrification progression, where ammonia oxidation inversely relates to nitrite accumulation, consistent with established nitrification kinetics [36]. The weak positive TAN–NO_3 correlation ($r = 0.23$) suggests partial nitrate accumulation occurs independently of immediate ammonia oxidation, possibly due to nitrate recycling or differential bacterial activity [8]. Notably, the nonsignificant pH correlations with all nitrogen species ($|r| < 0.20$) indicate stable system buffering, a critical advantage of properly managed RAS that maintains pH within optimal ranges (7.0–8.0) despite nitrogen transformations [37]. These relationships collectively validate the biofilter's functional stability while highlighting the need for species-specific monitoring, particularly for the TAN–NO_2 transition phase where toxicity risks peak.

For aquaculture applications, these results provide clear operational guidance: sand-based biofilters with *Nitrosomonas* are optimal for ammonia-dominated systems, like sturgeon RAS, while straw-*Nitrobacter* combinations better address nitrite accumulation. This targeted approach outperforms non-optimized systems by leveraging substrate–microbe synergies, though direct efficiency comparisons require further controlled studies.

Future research should prioritize: (1) long-term stability under varying organic carbon loads, (2) molecular characterization of microbial dynamics [38], and (3) development of hybrid substrate systems for sequential nitrogen removal. Such advances would further enhance the sustainability of RAS, which already reduces water use by >90% compared to conventional aquaculture [3] while preventing nutrient pollution [4].

RAS represent a transformative approach to sustainable aquaculture by minimizing water consumption and preventing environmental contamination. By incorporating our optimized biofilters, RAS can achieve near-complete nitrogen removal, significantly reducing the discharge of toxic ammonia and nitrite into natural water bodies. This closed-loop design recycles >90% of system water [3], while the targeted microbial–substrate combinations we identified further enhance treatment efficiency. Such systems align with the UN Sustainable Development Goals (SDG 14) by preventing eutrophication from nutrient-rich effluents [4], while supporting intensive food production with a 5–10× lower water footprint than traditional aquaculture. The integration of these biofilters could make RAS a cornerstone for environmentally responsible protein production.

4. Conclusions

This study elucidates the critical role of substrate-microbe interactions in nitrogen removal within biofiltration systems. Under our experimental conditions, the most significant finding was the consistent high performance of *Nitrosomonas* for ammonia oxidation on both sand and straw substrates, providing operational flexibility for this critical step. In contrast, *Nitrobacter* exhibited a strong substrate preference, demonstrating superior nitrite removal efficiency specifically in straw-based systems. These findings shift the optimization paradigm from a one-size-fits-all approach to a targeted strategy based on the dominant nitrogenous waste.

The results offer a scientifically grounded framework for biofilter design. While the 50 L scale of this study necessitates validation at commercial levels, our data suggest that system operators have a viable choice between sand and straw for robust ammonia removal with *Nitrosomonas*. However, for targeted nitrite mitigation, a straw-based biofilter is indicated to maximize *Nitrobacter* activity.

Future research, building upon these findings, should prioritize: (1) scaling up these substrate-bacteria combinations in pilot-scale RAS to validate their efficacy and economic feasibility under industrial conditions, and (2) using molecular techniques to uncover the mechanisms—such as biofilm architecture and interspecies competition—that explain *Nitrobacter*'s distinct preference for straw and *Nitrosomonas*'s versatility. By focusing on these targeted questions, we can

advance the development of more efficient, tailored, and sustainable biofiltration strategies for aquaculture.

Data Availability Statement

In terms of data request, we can provide data for your perusal.

Disclosure

All the authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization, supervision: Hamid Faghani Langarudi and Mohammad Reza Ghomi. Methodology, writing – original draft preparation: Peyman Bagherian and Hamid Faghani Langarudi. Validation: Hamid Faghani Langarudi, Mohammad Reza Ghomi, Masood Ghane, and Dharmendra K. Meena. Formal analysis: Masood Ghane. Investigation: Peyman Bagherian and Masoud Hashemi. Resources, funding acquisition: Dharmendra K. Meena. Data curation: Peyman Bagherian. Writing – review and editing: Peyman Bagherian, Mohammad Reza Ghomi, Dharmendra K. Meena, and Masoud Hashemi. Project administration: Hamid Faghani Langarudi.

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