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RESEARCH PAPER

Optimizing Nitrifying Bacteria and Biofilter Media for Enhanced Nitrification in Aquaponics Systems

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Abstract

Efficient nitrification by microbial communities is crucial for maintaining water quality in aquaponics systems. This study optimized the performance of nitrifying bacteria—*Nitrosomonas* (Ns) and *Nitrobacter* (Nb) by investigating inoculum volume, bacterial ratio, and biofilter substrate configuration using fish wastewater. Initial tests with a 1:1 Ns:Nb ratio at inoculum volumes of 1.0, 1.5, and 2.0 mL L^{-1} showed that 1.5 mL L^{-1} achieved the highest nitrification efficiency. Further optimization revealed that a 2:1 Ns:Nb ratio (1.0 mL Ns: 0.5 mL Nb) maximized ammonia and nitrite oxidation (89.15% and 91.34%, respectively). Using this ratio, substrate type and volume fraction (12.5%, 25%, 37.5%) were evaluated, with plastic bottle caps (PBC) at 37.5% delivering superior nitrogen removal: NH_4^+ (98.5%), NH_3–N (97.6%), NO_2^- (79.2%), and NO_3^- (96.1%). Water quality parameters remained within optimal ranges (DO: 1.2–3.9 mg L^{-1}; pH: 7.18–8.53; temperature: 20.1–21.9°C). The high surface area and buoyancy of PBC promoted enhanced microbial activity. These results offer practical strategies for improving sustainable nitrification and microbial efficiency in aquaponics systems.

Introduction

Nitrification is a fundamental biological process in which ammonia (NH_3/NH_4^+) is oxidized to nitrite (NO_2^-) by Nitrosomonas (AOB) and subsequently to nitrate (NO_3^-) by Nitrobacter (NOB) (USEPA, 2007). This conversion is essential in wastewater treatment because it transforms toxic nitrogen compounds into less harmful forms, thereby protecting aquatic organisms and stabilizing water quality (Li et al., 2022). Application of AOB and NOB effectively reduces ammonia and nitrite accumulation while increasing nitrate availability (Dwiardani et al., 2021). However, uncertainties remain regarding optimal inoculum volume, treatment duration, and AOB:NOB ratios in aquaponics systems. Ammonium oxidation is generally slower than nitrite oxidation (Yao & Peng, 2017), and although a theoretical 2:1 AOB:NOB ratio has been proposed due to AOB dominance, experimental validation under aquaponics conditions is limited (Ramdhani et al., 2013).

Nitrification efficiency is strongly influenced by environmental factors including temperature, pH, dissolved oxygen (DO), alkalinity, salinity, and ammonia availability (Chen et al., 2018; Zhang et al., 2021). Optimal activity typically occurs near 20°C, declining below 10°C (Chen et al., 2018), with differential responses of AOB and NOB observed between 20–25°C (Soliman & Eldyasti, 2016). A pH range of 6–8, supported by adequate alkalinity, favors stable nitrification (Evans

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& Sober, 2015), while DO concentrations above 2 mg/L are critical due to oxygen competition between AOB and NOB (How et al., 2018; Yang, 2019). Moreover, nitrifiers can tolerate salinity up to 4%, supporting their application across diverse wastewater systems (Gao et al., 2019; Navada et al., 2019). Sustained NH_4^+ availability is also necessary to maintain stable nitrifier populations (Quoc et al., 2021).

In aquaculture systems such as aquaponics, wastewater rich in suspended solids, organic matter, and nutrients can deteriorate water quality and compromise fish health (Ahmad et al., 2021). Elevated ammonia and nitrite impair growth, swimming performance, and induce oxidative stress, potentially leading to mortality (Sikora et al., 2022; Liu et al., 2024). Efficient nitrification is therefore essential for mitigating nitrogen toxicity and enhancing system resilience. In integrated aquaponics, nitrate generated through nitrification serves as a plant nutrient, while in biofloc systems, maintaining favorable conditions for nitrifiers ensures effective nitrogen removal (Robles-Porchas et al., 2020).

Biofilter substrate properties critically influence microbial attachment, biofilm formation, and overall nitrification performance (Rodríguez-Gómez et al., 2021). Adequate surface area-to-volume ratios enhance filtration, organic matter degradation, and nitrogen transformation (Yep & Zheng, 2019; Bracino et al., 2021; Vanderzwalmen et al., 2022). Although materials such as volcanic stone, ceramic media, and nano-structured substrates show theoretical potential for ammonia removal, their practical efficiency and economic feasibility in aquaponics remain underexplored (Wu et al., 2022; Yang et al., 2022). Furthermore, integrated optimization of nitrifier ratios and substrate configuration specific to aquaponics systems has limited attention.

Accordingly, this study isolates and screens aquaponics-adapted nitrifying strains, determines the optimal inoculum volume and Nitrosomonas to Nitrobacter ratio, and evaluates different biofilter media including coarse gravel, plastic bottle caps, and composite mixtures for their capacity to support microbial colonization and nitrification. By integrating biological and structural optimization, the study aims to enhance nitrogen removal efficiency and improve water quality stability in aquaponics wastewater treatment systems.

Materials and Methods

Description of the study area

The study was conducted at Jimma University (7.6753° N, 36.8373° E), situated at an average altitude of 1780 m above sea level. The area is characterized by a warm climate, with mean annual minimum and maximum temperatures of 14°C and 30°C, respectively, and annual rainfall ranging from 1138 to 1690 mm.

Isolation and preliminary screening of nitrifying bacteria were carried out in the Microbiology Laboratory, Department of Biology. Optimization experiments for Nitrosomonas (Ns) and Nitrobacter (Nb) were conducted under controlled indoor conditions using polypropylene containers in the Aquaculture and Fisheries Laboratory, with fish wastewater obtained from an established aquaponics system. Finally, the performance of the optimized nitrifying consortia across different biofilter media was evaluated within a greenhouse-based aquaponics system to assess practical application under semi-controlled production conditions.

Sample Collection, Media Preparation and Processing

Collection and Preservation of Samples

Water and soil samples were collected from the aquaculture pond and surrounding area of Jimma University to isolate Nitrosomonas and Nitrobacter. Pond water was sampled using sterile glass bottles, while surface soil was aseptically collected from 15 cm depth using a sterile drill and spatula. This depth was selected because the upper 10–20 cm soil layer contains the highest microbial biomass and nitrifying activity due to optimal oxygen, organic matter, and root influence, whereas deeper layers show reduced microbial abundance (Bui & Henderson, 2021; Johnston et al., 2024). Samples were transported immediately to the Microbiology Laboratory and stored at 4°C until analysis.

Media Preparation, Serial Dilution, and Incubation

Selective media for *Nitrosomonas* and *Nitrobacter* were prepared using modified Winogradsky medium for the two nitrification phases (Odokuma & Akponah, 2008; Zhang et al., 2018). Media pH was adjusted to 7.75 (0.1 M NaOH/HCl), boiled, autoclaved at 121°C for 15 minutes, and poured into sterile Petri dishes (five per isolate). Stock solutions (90 mL peptone water) were prepared and autoclaved, after which 10 g soil or 10 mL water samples were added separately and homogenized. Serial dilutions (10^{-1}–10^{-5}) were performed using sterile dilution blanks. From each dilution, 0.1 mL aliquots were spread onto respective selective media and incubated aerobically at 32°C for four days.

Isolation, Screening, and Processing

Pure cultures were obtained through repeated sub-culturing following Pepper and Gerba (2015). Colonies were inoculated into nutrient broth, re-streaked on nutrient agar, incubated at 32°C for 48 hours, and maintained on slants at 4°C. Primary screening included colony morphology, Gram staining, and biochemical tests (SIM, catalase), tolerance assessment at pH 6–8 and 0.5–4% NaCl. Secondary screening evaluated nitrification efficiency under wastewater-relevant conditions (pH, temperature, turbidity, and salinity). Isolates showing high tolerance (pH 6–8; 3.5–4% salinity) and strong performance were activated in nutrient broth at 32°C for 48 hours. Equal volumes of both isolates were mixed aseptically and used for subsequent indoor optimization experiments on inoculum concentration and substrate type.

Preparation of Fish Wastewater, Substrate Collection and Processing

Preparation of Fish Wastewater in Greenhouse Tanks

Fish wastewater was generated in greenhouse tanks to assess nitrogen removal efficiency of Nitrosomonas and Nitrobacter. A 50 L tank was filled with tap water and stocked with six adult Clarias gariepinus following Clols-Fuentes et al. (2023). Fish were fed a custom-formulated diet (38% protein, 5% lipid, 30% carbohydrate, 1% minerals, 1% vitamins) optimized using linear programming (Nath & Talukdar, 2014). The feed incorporated locally sourced and sustainable ingredients—poultry manure, rumen liquor, cattle blood, and Azolla pinnata—to ensure balanced nutrition and cost-effectiveness. Feeding was conducted twice daily (9:00 AM and 4:00 PM) at 5% body weight for three weeks. A total of 32 L wastewater was collected: 6 L for inoculum volume optimization, 8 L for Ns:Nb ratio determination, and 18 L for substrate performance evaluation. Prior to each experiment, baseline nitrogen compounds (NH_4^+, NH_3-N, NO_2^-, NO_3^-) and key water quality parameters (DO, pH, temperature, EC, TDS, alkalinity, and salinity) were measured.

Substrate Collection and Experimental Design

Substrate Collection and Preparation

Locally available biofilter media coarse gravel (CG) and plastic bottle caps (PBC) were selected based on high surface area, adequate interstitial space, and material stability. Substrates (25–35 mm diameter) were washed, counted, and autoclaved prior to use. This size range aligns with standard coarse media (20–63 mm) commonly applied in recirculating aquaculture systems to support nitrification through enhanced surface area and oxygen diffusion (Rodríguez-Gómez et al., 2021). Substrates were grouped as CG, PBC, and a 1:1 mixture, and evaluated under bottom, floating, and mixed configurations to determine their influence on nitrification efficiency.

Experimental Design for Bacterial and Substrate Optimization

Five indoor experiments (in duplicate) were conducted using screened Nitrosomonas (Ns) and Nitrobacter (Nb). Optimization was performed in three sequential phases: (I) inoculum volume, (II) Ns:Nb concentration ratio, and (III) substrate type and packing density. Nitrogen compounds and water quality parameters were measured at 12-hour intervals (0–72 h). Substrate size, number, surface area (SA), and surface-area-to-volume (SA:V) ratios were calculated for performance comparison.

Experiment I: Optimization of Ns:Nb Volume

At a fixed 1:1 ratio, three inoculum volumes were tested in 1 L fish wastewater: T1 = 1 mL L^{-1} (0.5 mL each), T2 = 1.5 mL L^{-1} (0.75 mL each), and T3 = 2 mL L^{-1} (1 mL each) (Figure 1). Reductions in NH_4^+, NH_3-N, NO_2^- and increases in NO_3^- were used to determine the optimal volume, which was applied in Experiment II.

Experiment II: Optimization of Ns:Nb Concentration

Using the optimal inoculum volume identified in Experiment I (1.5 mL L^{-1}), four Nitrosomonas to Nitrobacter ratios were tested in 1 L of fish wastewater (duplicate containers): T1= 5:1 (1.25 mL Ns : 0.25 mL Nb), T2= 2:1 (1 mL Ns : 0.5 mL Nb), T3= 1:2 (0.5 mL Ns : 1 mL Nb), and T4= 1:5 (0.25 mL Ns : 1.25 mL Nb) (Figure 2). Nitrogen compounds (NH_4^+, NH_3-N, NO_2^-, NO_3^-) and water quality parameters were monitored at 24-hour intervals to determine the most effective Ns:Nb combination. The optimal ratio was subsequently used in substrate optimization experiments.

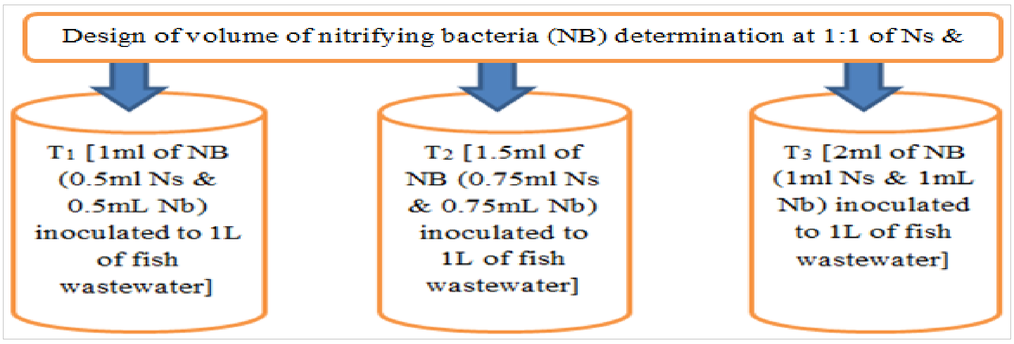

Figure 1. Illustration for the volume of nitrifying bacteria determination at 1:1 in an indoor experiment.

Experiment III: Substrate Optimization

Three biofilter substrates—coarse gravel (CG), plastic bottle caps (PBC), and 1:1 mixtures were evaluated at three volume occupancy levels: T1= 12.5%, T2= 25% and T3= 37.5%, representing a gradient of packing densities for biofilter performance assessment (Table 1). Substrate measurements were conducted in standardized cylindrical beakers (r= 5 cm, h= 12 cm; total volume≈ 942 cm³) to ensure uniform geometry. Submerged heights, surface areas (SA), volumes (V), and surface area-to-volume ratios (SA:V) were calculated using cylindrical formulas (Planini & Vollmer, 2008):

$$\text{Surface Area (SA)} = 2\pi r^2 + 2\pi rh$$

$$\text{Volume (V)} = \pi r^2 h$$

$$\text{Surface Area-to-Volume Ratio (SA:V)} = (2\pi r^2 + 2\pi rh) / (\pi r^2 h) = 2/h + 2/r$$

Where, *r* is the radius of the beaker, *h* is the height of the beaker, SA is expressed in cm², V in cm³, and SA:V in cm^{-1}.

This allowed precise quantification of substrate surface availability, a critical factor for microbial colonization and biofiltration efficiency, and maintaining methodological consistency.

Experimental Setup and Application

A controlled indoor setup (Figure 3) was used to evaluate biofiltration performance of three substrates—coarse gravel (CG), plastic bottle caps (PBC), and a 1:1 mixture—in aquaponics wastewater. Each substrate was tested at three volume occupancies: T1= 12.5%, T2= 25%, and T3= 37.5%, with two replicates per treatment (18 experimental units total).

Substrates were added as 10, 20, and 30 units for T1, T2, and T3, producing approximate dry heights of 1.5, 3.0, and 4.5 cm, respectively. Each beaker contained 1 L of aquaponics wastewater and an equal concentration of Nitrosomonas and Nitrobacter. This standardized design ensured consistency across treatments, allowing direct comparison of substrate type, quantity, and packing density on nitrification efficiency.

Measurement of Organic Matter and Wastewater Quality

Prior to experiments, nitrogenous compounds—NH_4^+, NH_3-N, NO_2^-, and NO_3^-—and turbidity in fish wastewater containing screened Nitrosomonas and Nitrobacter were measured using the high-precision Palintest Photometer 7500 Bluetooth (Palintest Ltd, 2020).

Water samples were collected in duplicate at 12-hour intervals over 72 hours to determine optimal bacterial volumes, concentration ratios, and substrate conditions. Nitrogen compounds were analyzed with Palintest reagent tablets according to manufacturer protocols, using distilled water as a blank:

- NH_4^+ and NH_3-N: 10 mL sample with Ammonia Tablets No. 1 & 2; 10 min color development; measured at photo modes 62 and 04.

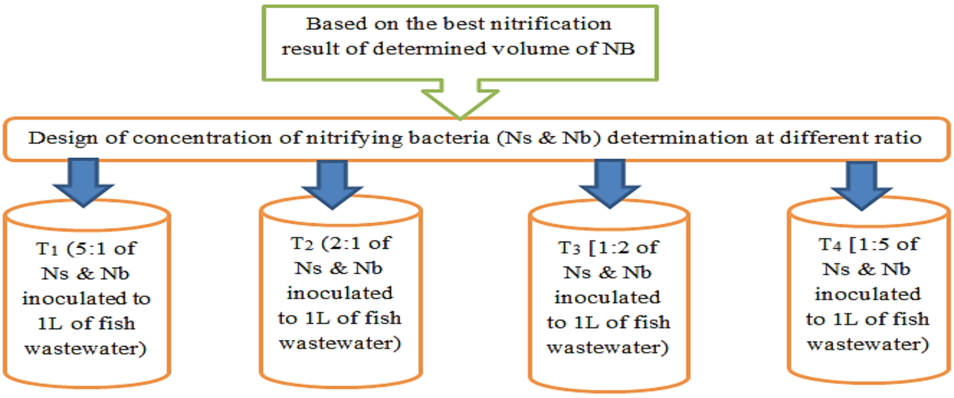

Figure 1. Illustration for the determination of concentration of nitrifying bacteria at different ratio in an indoor experiment.

Table 1. Substrate heights in wastewater, surface areas, and surface area-to-volume (sa:v) ratios for different biofilter substrates and packing densities

Types of Substrates	H in Wastewater (cm)			Surface Area (cm²)			SA:V Ratio (cm^{-1})		
	T1 (12.5%)	T2 (25%)	T3 (37.5%)	T1 (12.5%)	T2 (25%)	T3 (37.5%)	T1 (1:8)	T2 (1:4)	T3 (3:8)
Coarse gravel (CG)	1.0	1.5	2.0	188.4	204.1	219.8	0.20	0.22	0.233
Plastic bottle caps (PBC)	0.5	1.0	1.5	172.7	188.4	204.1	0.18	0.20	0.22
Mixed (50% CG+50% PBC)	0.75	1.25	1.75	180.55	196.25	211.95	0.19	0.21	0.23

Key: H= Substrate height submerged in wastewater; SA= Surface area; V= Volume of beaker (942 cm³); SA:V= Surface area-to-volume ratio.

- NO_2^-: 10 mL sample with Nitricol tablet; 10 min reaction; measured at photo mode 64.
- NO_3^-: 20 mL sample with nitrate powder and tablet; shaken, settled, 10 mL aliquot analyzed after 10 min; measured at photo mode 63.

Key wastewater parameters influencing nitrification-DO, pH, temperature, EC, TDS, and salinity were measured concurrently using a Palintest photometer and calibrated pH meter during bacterial and substrate optimization trials.

Calculation of Nitrifying Bacteria Efficiency

The efficiency of the nitrifying bacteria in transforming toxic nitrogenous compounds (NH_4^+, NH_3-N, and NO_2^-) into nitrate (NO_3^-) was quantified based on established methodologies described by Nugroho et al. (2016), considering the following pathway:

NH_4^+ and NH_3 (toxic) *Ns* NO_2^- (toxic) *Nb* NO_3^- (non-toxic up to 10 mg/L)

Efficiency was determined using the following formulas:

$$\text{Reduction Efficiency (\%)} = ((IC - FC) / IC) \times 100$$

$$\text{Production Efficiency (\%)} = ((FC - IC) / FC) \times 100$$

Where, IC= Initial concentration of the nitrogenous compound in wastewater (mg/L), FC= Final concentration of the nitrogenouscompound in wastewater (mg/L).

Data Collection and Analytical Methods

Nitrogen compounds (NH_4^+, NH_3-N, NO_2^-, NO_3^-) were quantified using the Palintest Photometer 7500 (Palintest Ltd, 2020) following manufacturer protocols. Duplicate samples were analyzed at 12-hour intervals over 72 hours using reagent tablet methods with distilled water blanks for accuracy. Key water quality parameters—dissolved oxygen (DO), pH, temperature, electrical conductivity (EC), total dissolved solids (TDS), alkalinity, salinity, and turbidity—were measured concurrently during bacterial and substrate optimization trials.

Results

A total of 22 nitrifying bacterial isolates were obtained, including 9 *Nitrosomonas* (Ns) and 13 *Nitrobacter* (Nb) strains. Among these, 5 Ns were isolated from pond water (pwNs) and 4 from soil (sNs), while 6 Nb originated from soil (sNb) and 7 from pond water (pwNb). All isolates exhibited similar morphological characteristics. Based on key nitrification-related parameters—pH tolerance, salinity tolerance, and turbidity—10 isolates (5 Ns and 5 Nb) were selected for further study. Four isolates were motile and six non-motile. All Ns were catalase-positive, whereas four Nb were catalase-negative and one catalase-positive. The selected isolates grew within a pH range of 6–8 and tolerated salinity levels up to 3.5–4% NaCl. Turbidity measurements using the Palintest Photometer 7500 (mode 48) showed values ranging from 245–340 FTU for Ns and 270–370 FTU for Nb. Although higher salt concentrations inhibited most nitrifiers, the selected strains demonstrated tolerance to elevated salinity and lower pH, supporting their suitability for fish wastewater treatment applications.

Determination of Optimal Inoculum Volume: Using a 1:1 Ns:Nb ratio, inoculum volumes were evaluated. Treatment 2 (T2, 1.5 mL/L) achieved the highest nitrification efficiency, with reductions of 77.17% in NH_4^+, 78.51% in NH_3-N, and 37.5% in NO_2^-, alongside a 90.79% increase in NO_3^- (Table 2). Final concentrations

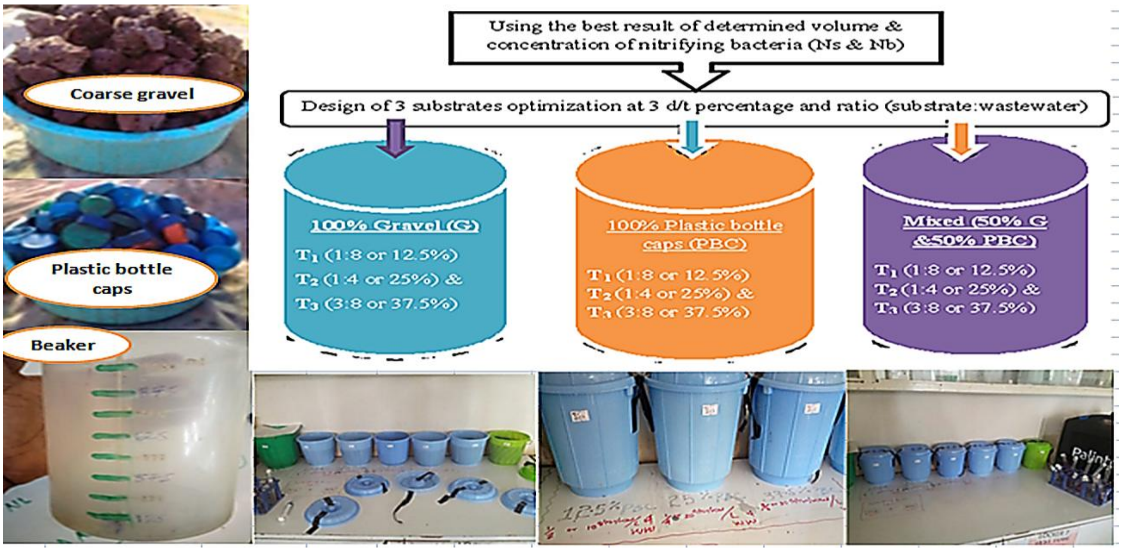

Figure 3. Experimental setup illustrating substrate optimization using coarse gravel, plastic bottle caps, and composite media for enhanced nitrification efficiency in controlled aquaponics wastewater treatment.

in T2 were 0.29 mg/L (NH_4^+), 0.26 mg/L (NH_3-N), 0.15 mg/L (NO_2^-), and 3.04 mg/L (NO_3^-). In contrast, T1 (1 mL/L) showed lower performance, with residual NH_4^+ (0.62 mg/L), NH_3-N (0.53 mg/L), and reduced NO_3^- production (2.78 mg/L), indicating insufficient ammonia oxidation at lower inoculum volumes. These findings confirm that 1.5 mL/L is the optimal inoculum volume and selected for subsequent ratio optimization.

Optimization of Nitrosomonas:Nitrobacter Ratio: At a fixed inoculum volume of 1.5 mL/L, four Ns:Nb ratios were tested: T1 (5:1), T2 (2:1), T3 (1:2), and T4 (1:5) (Table 3). T2 (2:1) exhibited the highest nitrification efficiency, achieving reductions of 89.15% (NH_4^+), 87.1% (NH_3-N), and 50% (NO_2^-), with 91.34% NO_3^- production. Time-course monitoring over 72 hours (12-hour intervals) confirmed consistent superiority of T2. Fold-change reductions reached 4.38–9.21 for NH_4^+, 4.65–8.86 for NH_3-N, and a 10.86–11.55-fold increase in NO_3^-. Nitrite accumulation occurred in T1 (3.31-fold) and T3 (2.5-fold), reflecting imbalanced nitrifier concentrations. Final residual levels in T2 were lowest for NH_4^+ (0.14 mg/L), NH_3-N (0.16 mg/L), and NO_2^- (0.13 mg/L), with the highest NO_3^- (3.35 mg/L). Conversely, T4 showed elevated residual NH_4^+ (0.71 mg/L) and NH_3-N (0.69 mg/L), and minimal NO_3^- production (1.63 mg/L). Thus, a 2:1 Ns:Nb ratio (1 mL Ns and 0.5 mL Nb per liter) was determined and applied in substrate experiments.

Water Quality During Volume and Ratio Optimization: Key parameters measured included DO (1.43–3.78 mg/L), pH (7.2–8.47), temperature (20.2–21.8°C), EC (471–531 µS/cm), TDS (306–405 mg/L), alkalinity (141–406 mg/L), and salinity (0.15–0.21 ppt) (Table 4). Increased inoculum volume elevated oxygen demand. Significant differences were observed in DO, temperature, EC, and TDS during volume determination, while salinity showed no significant variation. During optimization, significant differences occurred in DO, pH, temperature, EC, TDS, and alkalinity.

Substrate Optimization: Three substrates—coarse gravel (CG), plastic bottle caps (PBC), and a 50:50 mixture—were evaluated (Table 5). Increased substrate levels enhanced removal efficiency, reflecting improved surface area-to-volume ratios. Among substrates, floating PBC demonstrated the highest nitrification efficiency, followed by the mixed substrate and CG. At the optimized inoculum (1.5 mL/L; 2:1 ratio), 72-hour monitoring showed that T3 achieved the greatest reductions in reduced nitrogen species and highest NO_3^-

Table 2. Final concentrations (mg l^{-1}) and removal efficiency (%) of nitrogen compounds by nitrifying bacteria at different application volumes (1:1 ratio of Ns and Nb)

Bacterial optimization		Final concentration of N-compounds (mg L^{-1})				Efficiency of nitrifying bacteria (%)			
	Treatments	NH4+	NH3-N	NO2-	NO3-	NH4+	NH3-N	NO2-	NO3-
Volume of Ns and Nb at 1:1	T1 (1 ml L^{-1})	0.62±0.04^{a}	0.53±0.03^{a}	0.18±0.01^{a}	2.78±0.42^{b}	51.18	56.2	25	89.93
	T2 (1.5 ml L^{-1})	0.29±0.03^{b}	0.26±0.01^{a}	0.15±0.01^{a}	3.04±0.03^{a}	77.17	78.51	37.5	90.79
	T3 (2 ml L^{-1})	0.30±0.06^{b}	0.28±0.01^{b}	0.16±0.01^{a}	2.97±0.03^{a}	76.38	76.85	33.33	90.57

Table 3. Effects of different concentration ratios of Nitrosomonas (Ns) and Nitrobacter (Nb) at the selected volume of 1.5 ml/L on nitrogen compound transformation in fish wastewater.

Bacterial Optimization		Final Concentration N-compound in mg/L				Efficiency of Nitrifying Bacteria (%)			
	Treatment	NH_4^+	NH_3-N	NO_2^-	NO_3^-	NH_4^+	NH_3-N	NO_2^-	NO_3^-
Ns:Nb (5:1)	T1	0.24±0.01^{c}	0.21±0.06^{a}	0.86±0.03^{a}	1.91±0.03^{b}	81.4	83.07	3.3×	84.82
Ns:Nb (2:1)	T2	0.14±0.01^{d}	0.16±0.03^{a}	0.13±0.01^{c}	3.35±0.03^{a}	89.15	87.1	50	91.34
Ns:Nb (1:2)	T3	0.56±0.01^{b}	0.48±0.04^{b}	0.65±0.01^{b}	1.85±0.03^{b}	56.59	62.79	2.5×	84.32
Ns:Nb (1:5)	T4	0.71±0.01^{a}	0.69±0.03^{c}	0.18±0.01^{c}	1.63±0.03^{c}	44.96	44.36	30.8	82.21

Table 4. Water quality measurements recorded during the determination of volume and concentration for nitrifying bacteria (Ns and Nb).

Bacterial Optimization	Treatment	DO (mg/L)	pH	Temp (°C)	EC (µS/cm)	TDS (mg/L)	Alkalinity (mg/L)	Salinity (ppt)
Volume of Nitrifiers @ 1:1 Ns:Nb	Initial	3.78±0.00	7.20±0.00	20.2±0.00	471±0.00	306±0.00	141±0.00	0.15±0.00
	T1 (1 ml/L)	1.76±0.01	8.32±0.06	21.6±0.07	506.5±6.36	381.5±0.71	388±4.24	0.185±0.01
	T2 (1.5 ml/L)	1.68±0.01	8.39±0.04	21.6±0.07	514±2.83	392.5±2.12	394±7.07	0.185±0.01
	T3 (2 ml/L)	1.66±0.01	8.47±0.02	21.7±0.14	520±2.83	400.5±3.54	398.5±3.54	0.195±0.01
Concentration of Ns &Nb @ Ratio	Initial	3.75±0.00	7.23±0.00	20.5±0.00	476±0.00	311±0.00	143±0.00	0.17±0.00
	T1 (5:1)	1.43±0.03^{d}	8.30±0.02	21.7±0.07	518±8.49	394±4.24	394.5±4.95	0.21±0.00
	T2 (2:1)	1.50±0.02^{c}	8.35±0.04	21.8±0.14	531±2.83	405±4.24	406.5±7.78	0.21±0.01
	T3 (1:2)	1.63±0.03^{b}	8.23±0.03	21.6±0.07	499±2.83	383.5±2.12	384.5±4.95	0.21±0.00
	T4 (1:5)	1.74±0.01^{a}	8.20±0.02	21.5±0.00	491.5±4.95	379±2.83	375.5±3.54	0.20±0.01

Key: Dissolved oxygen (DO), Temperature (^{O}C), electric conductivity (EC), total dissolved solid (TDS).

production. Overall conversion improvements ranged from 4.38–9.21-fold (NH_4^+), 4.65–8.86-fold (NH_3-N), 1.60–1.86-fold (NO_2^-), and 10.86–11.55-fold (NO_3^-). Substrate optimization significantly enhanced microbial activity and nitrification performance.

Water quality parameters during substrate evaluation (Table 6) were within comparable ranges: DO (1.2–3.93 mg/L), pH (7.18–8.53), temperature (20.1–21.85°C), EC (243–388 µS/cm), TDS (157–255.5 mg/L), alkalinity (135–441 mg/L), and salinity (0.07–0.12 ppt). Treatments achieving higher nitrogen removal exhibited greater oxygen consumption. Substrate application significantly influenced water quality parameters, with ANOVA indicating significant differences ($P<0.05$) in NH_4^+, NH_3-N, NO_2^-, and NO_3^- among treatments.

Discussion

This study systematically optimized the biological components of nitrification in aquaponics by integrating microbial selection, inoculum management, and biofilter substrate design. Specifically, it addressed three interrelated objectives: (i) isolation and screening of aquaponics-adapted nitrifying bacteria, (ii) determination of optimal inoculum concentration and *Nitrosomonas:Nitrobacter* (Ns:Nb) ratios, and (iii) evaluation of substrate type and loading to maximize microbial colonization and nitrogen removal efficiency.

Nitrifying bacteria were successfully isolated from pond water and soil, confirming these environments as reliable reservoirs of autotrophic nitrifiers, consistent with previous findings (Gao et al., 2019). The isolates exhibited characteristic colony morphology and enzymatic profiles associated with *Nitrosomonas* (ammonia-oxidizing bacteria, AOB) and *Nitrobacter* (nitrite-oxidizing bacteria, NOB). Functionally, the screened strains demonstrated high ammonium (NH_4^+) and nitrite (NO_2^-) oxidation efficiencies, validating their suitability for aquaponics wastewater treatment. Importantly, salinity tolerance up to 4% highlights their adaptability to fluctuating ionic conditions typical of semi-recirculating systems where evaporation may concentrate dissolved salts. Comparable salt-tolerant nitrifiers have been reported in marine aquaculture biofilters (Gao et al., 2019; Dwiardani et al., 2021), reinforcing the ecological robustness of the selected strains.

Table 5. Optimization of three substrates using determined nitrifying bacteria (Ns and Nb)

		Treatments and final N-compounds in mgL^{-1}			Efficiency (%)		
Substrate Type	Parameter	T1 [1:8]	T2 [1:4]	T3 [3:8]	T1 (12.5%)	T2 (25%)	T3 (37.5%)
Coarse Gravel (cg)	NH_4^+	0.07±0.014	0.05±0.014	0.03±0.014	94.57	96.12	97.67
	NH_3-N	0.09±0.014	0.07±0.014	0.04±0.014	92.74	94.36	96.77
	NO_2^-	0.13±0.014	0.10±0.014	0.08±0.028	45.83	58.33	66.67
	NO_3^-	4.25±0.028^c	5.02±0.014^b	6.26±0.028^a	94.12	95.02	96.01
Plastic Bottle Caps (pbc)	NH_4^+	0.06±0.014	0.04±0.014	0.02±0.000	95.35	96.9	98.45
	NH_3-N	0.08±0.014	0.05±0.014	0.03±0.014	93.55	95.97	97.58
	NO_2^-	0.11±0.014	0.07±0.014	0.05±0.014	54.17	70.83	79.17
	NO_3^-	4.47±0.014^c	5.35±0.014^b	6.46±0.028^a	94.41	95.33	96.13
Mixed (50% cg+50% pbc)	NH_4^+	0.065±0.010	0.05±0.000	0.03±0.000	94.96	96.12	97.67
	NH_3-N	0.085±0.010	0.07±0.014	0.035±0.010	93.15	94.36	97.18
	NO_2^-	0.115±0.010	0.085±0.010	0.075±0.010	47.92	64.58	68.85
	NO_3^-	4.28±0.014^c	5.03±0.014^b	6.27±0.028^a	94.16	95.03	96.01

Table 6. Water quality parameters during substrate optimization using Ns and Nb

Substrate Type	Treatment	DO (mg/L)	pH	Temperature (°C)	EC (µS/cm)	TDS (mg/L)	Alkalinity (mg/L as $CaCO_3$)	Salinity (ppt)
Coarse gravel (cg)	T0 (Initial)	3.93±0.00	7.18±0.00	20.1±0.00	329±0.00	214±0.00	135±0.00	0.07±0.00
	T1 (1:8 or 12.5%)	1.72±0.01	8.28±0.04	21.4±0.01	359±2.83	232.5±3.54	413.5±17.68	0.10±0.00
	T2 (1:4 or 25%)	1.55±0.04	8.38±0.10	21.55±0.07	370±2.83	245±5.66	429±2.83	0.10±0.00
	T3 (3:8 or 37.5%)	1.24±0.01	8.53±0.26	21.75±0.07	387.5±6.36	255.5±2.12	441±16.97	0.11±0.01
Plastic bottle caps (pbc)	T0 (Initial)	3.82±0.00	7.21±0.00	20.2±0.00	248±0.00	166±0.00	136±0.00	0.09±0.00
	T1 (1:8 or 12.5%)	1.70±0.01	8.10±0.67	21.5±0.01	360.5±3.54	233.5±2.12	404.5±61.52	0.115±0.01
	T2 (1:4 or 25%)	1.53±0.03	8.22±0.08	21.7±0.14	371.5±7.78	239±2.83	411.5±65.76	0.12±0.00
	T3 (3:8 or 37.5%)	1.20±0.11	8.24±0.78	21.85±0.07	388.5±3.54	250.5±2.12	419±72.13	0.12±0.00
Mixed (50% cg + 50% pbc)	T0 (Initial)	3.82±0.00	7.27±0.00	20.1±0.00	243±0.00	157±0.00	148±0.00	0.08±0.00
	T1 (1:8 or 12.5%)	1.85±0.09	7.85±0.32	21.4±0.14	344±9.90	217±15.56	378.5±37.48	0.11±0.01
	T2 (1:4 or 25%)	1.75±0.13	8.01±0.51	21.5±0.14	354.5±14.85	227.5±34.65	400±49.50	0.11±0.01
	T3 (3:8 or 37.5%)	1.70±0.18	8.17±0.76	21.7±0.14	362±28.28	239.5±51.62	409.5±68.59	0.11±0.00

Key: Dissolved oxygen (DO), electric conductivity (EC) and total dissolved solid (TDS).

Optimization of microbial dosing revealed that 1.5 mL/L inoculum concentration with a 2:1 Ns:Nb ratio consistently produced superior nitrification performance. This outcome underscores two mechanistic principles: (1) higher inoculum densities accelerate biofilm establishment by reducing lag phases, and (2) a moderately AOB-dominant consortium ensures sufficient nitrite generation to sustain NOB activity without permitting toxic nitrite accumulation. These results align with recommendations to inoculate nitrifying consortia simultaneously rather than sequentially to preserve process stability (Winkler et al., 2012; Ramdhani et al., 2013). Imbalances in AOB or NOB abundance can disrupt nitrification kinetics—excess AOB may lead to nitrite buildup, whereas insufficient AOB limits substrate availability for NOB, reducing nitrate production efficiency.

Substrate optimization further clarified the structural determinants of biofilter performance. Three media—coarse gravel (CG), plastic bottle caps (PBC), and a 50:50 composite mix—were tested at 12.5%, 25%, and 37.5% volumetric loading. Across treatments, 37.5% substrate concentration yielded the highest nitrification efficiency, with PBC outperforming the alternatives. At 37.5% PBC (SA:V= 0.22), removal efficiencies reached 98.45% for NH_4^+, 97.58% for NH_3-N, and 79.17% for NO_2^-, with nitrate (NO_3^-) production of 96.13%. These findings support existing literature indicating that lightweight, floating plastic media provide enhanced surface area, oxygen diffusion, and biofilm development compared to denser, static materials such as gravel (Yep & Zheng, 2019; Rodríguez-Gómez et al., 2021). The superior performance of repurposed PBC demonstrates that low-cost materials can deliver high biofilter efficiency, offering practical advantages for resource-limited aquaponics operations.

Water quality parameters further validated system optimization. The combined treatment (1.5 mL/L inoculum, 2:1 Ns:Nb ratio, 37.5% PBC substrate) significantly enhanced ammonia and nitrite removal, thereby reducing toxicity risks to fish and plants. Stable pH values (7.2–8.5) supported nitrifier metabolism (Zhang et al., 2018), while increased electrical conductivity (EC) and total dissolved solids (TDS) reflected effective mineralization and nitrate accumulation. Dissolved oxygen (DO) ranged from 1.43–3.78 mg/L—occasionally below the ideal >2.0 mg/L threshold for aerobic nitrification (How et al., 2018)—yet substrate-mediated aeration and selection of tolerant strains sustained functional stability. Adequate alkalinity (135–441 mg/L) buffered acidification resulting from nitrification, and low salinity (0.07–0.21 ppt) with moderate temperatures (20.1–21.85°C) remained within acceptable ranges for nitrifier activity.

Conclusion

Nitrification—the biological oxidation of ammonia and ammonium to nitrate by Nitrosomonas and Nitrobacter—is essential for detoxifying fish wastewater and sustaining aquaponics systems. This study demonstrated that optimal nitrification occurred at a 2:1 Ns:Nb ratio using a combined inoculum of 1.5 mL L^{-1} (1.0 mL Nitrosomonas, 0.5 mL Nitrobacter), confirming that a higher proportion of ammonia-oxidizing bacteria enhances ammonia removal. Substrate optimization indicated that 37.5% substrate volume, providing the highest surface area-to-volume ratio, maximized bacterial colonization and nitrogen conversion efficiency. Environmental factors—dissolved oxygen, pH, temperature, and biofilter retention time—significantly influenced bacterial performance. Stationary water conditions improved ammonia and nitrite removal, emphasizing the importance of hydraulic retention and substrate contact in biofilter design. Limitations include untested nitrate uptake by plants and the effects of water flow and aeration, which could impact system-wide nutrient dynamics. Future studies should investigate microbial community structure via metagenomics, assess variable hydraulic loading rates, and integrate plant nitrate assimilation to optimize the nutrient loop. Enhancing the synergy among microbial, plant, and fish components will improve both sustainability and productivity in aquaponics systems.

Ethical Statement

This study did not involve human or animal subjects. All microbial strains were isolated from environmental (non-human) sources, and all procedures were conducted in accordance with institutional and national biosafety regulations, including the Ethiopian Biosafety Regulatory Framework (2022) and the Biosafety and Biosecurity Guidelines for Health Laboratories in Ethiopia (2023). Standard biosafety protocols were followed to ensure environmental and laboratory safety.

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Author Contribution

All authors contributed to the study design and approved the final manuscript. Firew Admasu, a PhD student, led the material preparation, data collection, and analysis, wrote the first draft, and implemented all comments and suggestions provided by the other authors. Mulugeta Wakjira, Tokuma Negisho, and Ketema Bacha played key roles as advisors throughout the research process, from designing the study to closely following the experimental activities. They actively evaluated progress, provided constructive feedback, comments, and suggestions, and contributed significantly to refining and finalizing the manuscript. Soressa Gershe assisted with data analysis, evaluation, and offered valuable comments on the manuscript.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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