Synergistic Combination of Plants and Microbial-Rich Substrates Improves Water Quality in an Integrated Plant-Substrate System

ARISSARA SOPAWONG[1], FATIMAH MD YUSOFF[1,2,*], MUTA HARAH ZAKARIA[1], S.M. NURUL AMIN[1], HUI TENG TAN[2], AMALIA MOHD HASHIM[3]

[1]Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Selangor, Malaysia
[2]Laboratory of Aquatic Animal Health and Therapeutics, Institute of Bioscience, Universiti Putra Malaysia, Serdang, Selangor, Malaysia
[3]Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Serdang, Selangor, Malaysia

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Abstract

Nutrient enrichment is one of the main reasons causing water quality deterioration and eutrophication in aquaculture systems, such as tanks, ponds, and natural water bodies where cage aquaculture is located. The integration of aquatic plants and substrates synergistically utilises nutrients for the development of biofilm and plant growth, thus improving the water quality in tanks. The experiment was performed in three trials to assess the use of commercial plants integrated with substrates in enhancing nutrient removal in nutrient-enriched mesocosms. Among the plants, the combination of water spinach and lemon basil exhibited significantly higher ($P < 0.05$) nutrient removal without causing adverse effects on plant growth when compared to the other treatments. For the substrates, the lava rock and bamboo showed the highest ($P < 0.05$) periphyton development and productivity. The integration of water spinach and lava rock resulted in the highest ($P < 0.05$) nutrient removal and plant relative growth rates indicating the positive effects of synergistic interaction between plants and microbial-periphyton colonised substrates. A total of 27 bacterial phyla (mainly non-pathogenic) were identified in the integrated water spinach and lava rock substrate treatment. Microbial community structure analysis showed that Proteobacteria, Planctomycetota, Verrucomicrobiota and Bacteroidota were the main groups found in water, roots of water spinach, and substrates. The highest ($P < 0.05$) bacterial diversity was observed in the substrates, followed by plant roots and water. This study illustrated that the water quality could be significantly improved by integrating suitable plants and microbial-periphyton colonised substrates in tanks.

Keywords: bioremediation, nutrient removal, aquatic plants, substrates, water quality

Introduction

Aquaculture plays a vital role in generating income and promoting economic growth, particularly in Asia where it accounts for 89% of the global aquaculture production (FAO, 2018). The ever-growing demand for fish and fishery products has led to an increase in aquaculture production and an unprecedented level of intensification (Joffre et al., 2018; Malone and Newton, 2020). Despite being the most rapidly expanding global food sector, discharges of untreated aquaculture waste are nutrient-rich contributing to pollution and eutrophication of the surrounding water bodies (Zhang et al., 2018; Liu et al., 2021; Hu et al., 2022). Recycling and enhancement of nutrient utilisation by integrating aquatic plants and substrates can be an alternative way for water purification and water quality improvement (Liu et al., 2021; Li et al., 2022a; Chen et al., 2023b).

To address this issue, various conventional and novel methods using physical, chemical, and biological processes such as bioremediation, bioflocs, biofiltration, biocoagulation and bioflocculation have been applied to improve water quality in aquaculture systems resulting in optimal yields and minimal wastes (Abbas et al., 2021; Nhi-Cong et al., 2021; Geng et al., 2022; Chiquito-Contreras et al., 2022; Chen et al., 2023a). In addition, different aquaculture production systems like aquaponics, integrated multitrophic aquaculture (IMTA), and recycling aquaculture systems (RAS) have been established to recycle aquaculture wastes and reduce the discharge into the natural ecosystems (Ekawati et al., 2021; Sopawong et al., 2023). Microbial communities formed in biofilms on substrates play important roles in decomposing organic wastes and generating inorganic nutrients for plant growth. Among the different methods, nutrient removal by using biological processes has been applied worldwide due to their advantages such as low cost, simple maintenance, and being environmentally friendly (Anawar and Chowdhury, 2020; Nedjimi, 2020).

Bioremediation uses natural biological agents such as bacteria, fungi and plants mainly to control or remediate various contaminants into less toxic forms (Ramírez-García et al., 2019; Behbudi et al., 2021). Wang et al. (2022) and Li et al. (2022b) used bacterial-microalgae consortia to improve water quality in aquaculture systems. The bioremediation technology is eco-friendly and cost-effective for cleaning up contaminated sites (Ramírez-García et al., 2019). Microorganisms use contaminants or waste nutrients as an energy source in the bioremediation process and convert contaminants or waste nutrients to harmless or useful inorganic forms through their metabolic processes (Ramírez-García et al., 2019; Behbudi et al., 2021).

The selection of plants and substrates is essential for achieving effective nutrient removal in aquaculture systems. Commercial plants such as water spinach and basil are widely used in hydroponics systems due to a range of desirable traits (Andriani et al., 2019; Sakdasri et al., 2019; Knaus et al., 2020). These traits include robust root systems, high nutrient uptake, fast growth, vegetative propagation, economic importance, and non-invasive species (Endut et al., 2016a; Su et al., 2019; Shahid et al., 2020). Further, the combination of different plant species can be applied for nutrient removal due to their synergistic effects on root systems leading to increased nutrient removal efficiency (Su et al., 2019). However, some factors such as light, nutrients and space can have an impact on plant development as well as nutrient removal efficiency (Estim et al., 2019; Saufie et al., 2020).

In addition to plants, the microbial-periphyton communities on substrates can synergistically remove nutrients from the water. Khatoon et al. (2007), Fontanarrosa et al. (2019) and Saufie et al. (2020) demonstrated that biofilm communities formed on substrates could further enhance nutrient sequestration. Thus, substrates have been introduced into the water column for biofilters containing biofilms with microbial-periphyton communities (Fontanarrosa et al., 2019; Liu et al., 2021). To be effective in nutrient sequestration and biofilm development, the substrates should have certain criteria such as high porosity, large surface area, long-term durability, ease of installation, resistance of unclogging, affordability, local availability, and being free of chemicals and contaminants (Yang et al., 2018; Omar et al., 2023). Substrate options meeting these criteria include bio-ball, bamboo, polyvinyl chloride (PVC), bio-ring, coconut coir, zeolite, limestone and volcanic rock (Fontanarrosa et al., 2019; Anix et al., 2020; Llario et al., 2020; Liu et al., 2021). Moreover, microorganisms on substrates are involved in multiple processes, including nitrification, organic matter decomposition, denitrification, and phosphorus mineralisation (Li et al., 2019; Liu et al., 2021, Chen et al. 2023b).

The integrated plant-substrate system is the combination of aquaponic and bioremediation concepts. In aquaponics, aquaculture wastewater is used as fertilisers to grow plants, whereas bioremediation uses microorganisms to decompose organic wastes into useful inorganic compounds which are recycled and used by the primary producers. Consequently, integrated aquatic plants with the substrates can be an alternative practice to promote microhabitat and improve nutrient utilisation with the symbiosis of plants and bacteria on the substrates (Yep and Zheng, 2019; Shahid et al., 2020). Li et al. (2019) and Liu et al. (2021) reported that the development of various organisms on the immobilised substrate/biofilm integrated with aquatic plants could be an efficient and environmentally friendly approach to improve nutrient utilisation in a water body.

Thus, the efficiency of commercial plant-substrate integration in nutrient removal, water quality improvements, plant biomass production, and biofilm communities should be investigated to improve aquaculture systems and contribute to a sustainable aquaculture industry. Previous studies examined the effects of plants and substrates on nutrient removal efficiency and periphyton development separately (Anix et al., 2020; Knaus et al., 2020; Lyu et al., 2020). Considering the lack of information on the synergistic effects of an integrated commercial plant-substrates system, the present study was conducted to investigate the effects of water spinach (*Ipomoea aquatica*) and lemon basil (*Ocimum basilicum* × *O. americanum*) with lava rock and bamboo substrates and their different combinations on nutrient removal, periphyton productivity and microbial communities in a plant-substrate based system.

Materials and Methods

Experimental design

Three different trials were carried out to evaluate the efficiency of nutrient removal, periphyton development and microbial communities on selected plants and substrates in a tank system (Fig. 1). The first trial (Trial 1) compared nutrient removal efficiency by using three selected commercial plants; water spinach (*Ipomoea aquatica*; WS), lemon basil (*O. basilicum* × *O. americanum*; LB), and aromatic basil (*Ocimum basilicum*; AB) in 6 L tanks (16 cm length × 25 cm width × 15 cm depth) to choose the best plant. There were eight treatments with three replicates including the control (Fig. 1) based on a single plant (TP_1, TP_2, TP_3), the combination of two plant species (TP_4, TP_5, TP_6), the combination of three plant species (TP_7) and the control (C). To select the best substrate, the second trial (Trial 2) allowed five different types of substrates; bamboo stripes (TS_1-BO), lava rock (TS_2-LR), bio-ball (TS_3-BA), polyvinyl chloride pipe (TS_4-PVC) and bio-ring (TS_5-BR) to be colonised by periphyton development in triplicates (Fig. 1). The third trial (Trial 3) investigated the integration of the selected plant (from Trial 1) and substrate (Trial 2) on their nutrient removal ability using pond water in a nutrient-enriched mesocosm consisting of a 30 L tank (28 cm length × 40 cm width × 27 cm depth). There were five different treatments including the control, each with triplicates integrated lemon basil with bamboo (TPS_1-LB + BO), water spinach with bamboo (TPS_2-WS + BO), lemon basil with lava rock (TPS_3-LB + LR), water spinach with lava rock (TPS_4-WS + LR) and control (C) (Fig. 1).

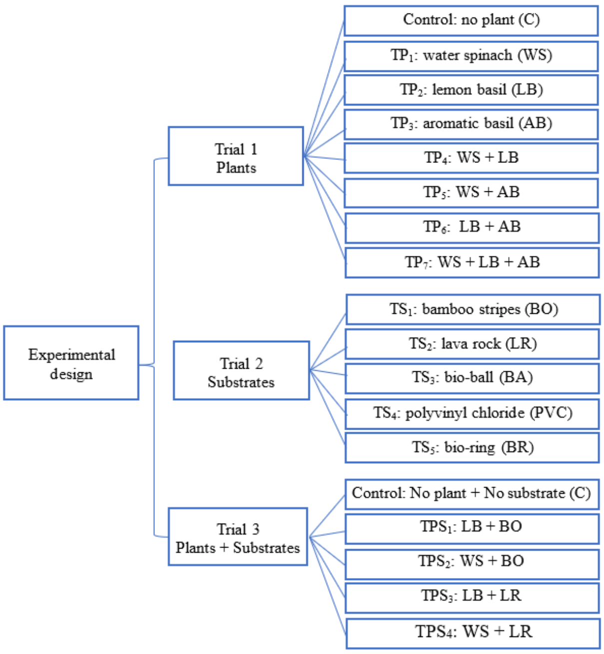

Fig. 1. Flow chart of the experimental design for nutrient removal by selected commercial plants and substrates in an integrated plant-substrate system. TP = trial plants; TS = trial substrates; TPS = trial plants + substrates.

Trial 1 – Nutrient removal by different plants

The plant species used in this study were water spinach (WS), lemon basil (LB), and aromatic basil (AB) placed in eight treatments including the control in replicates. There were no plants in the control tank. The experimental unit consisted of twenty-four 6-L aquaria filled with 5.5 L treated water (tap water fertilised with 3 $mg.L^{-1}$ of potassium nitrate (KNO_3), 1 $mg.L^{-1}$ of monopotassium phosphate (KH_2PO_4) and 2 $g.L^{-1}$ of commercial microelement (Agrostar, Malaysia). The addition of nitrogen, phosphorus and microelements was used to imitate the nutrient-rich aquaculture pond water with known nutrient concentrations; 40 $mg.L^{-1}$ total nitrogen (TN) and 1 $mg.L^{-1}$ total phosphorus (TP). The experiment was conducted for 30 days and there was no water exchange during the experimental period.

Trial 2 – Periphyton development by different substrates

Five substrate types in triplicates using a randomised complete block design were installed in the tank. Each replicate contained 52 pieces of each substrate type. Since samples from the substrates were collected four times during the experimental period (60 days), four nylon strings (13 pieces of substrates on each string) were used to tie the substrate per each replicate. The strings were hung vertically by fixing them to a circular metal string, and a wooden stick supported this metal string on the upper part of the tank (Fig. 2). The substrates were installed into the 1200 L tank which had been filled with 1000 L of fertilised pond water from the Universiti Puta Malaysia Farm (Latitude: N2°59'30.2"; Longitude: E101°42'57.8"), and periphyton communities were allowed to colonise the substrates for 14 days. The pond water was filtered with a 500-micron mesh net and fertilised with urea (10 $g.m^{-3}$) and triple superphosphate (TSP, at 5 $g.m^{-3}$) at the beginning and monthly intervals during the entire experimental period (60 days) for periphyton development (Azim et al., 2001; Chang et al., 2012). Aeration was provided to maintain dissolved oxygen above 5 $mg.L^{-1}$ during the experimental period without disturbing periphyton development on the substrates.

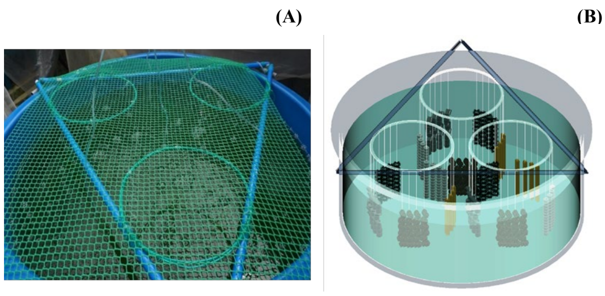

Fig. 2. The overall profile of substrate installation in randomised complete block design; (A): top view of substrate installation; (B): side view of substrate installation.

Periphyton sampling commenced 15 days after the substrate installation and continued at fortnightly intervals throughout the experimental period for the determination of periphyton identification and biomass. Ten pieces of each substrate from each replicate were selected randomly, and periphyton on each piece of the substrate was collected by scraping the substrates. After sampling, the substrates were returned to their original positions, marked, and excluded from subsequent sampling. The scraped sample was resuspended in 80 mL sterile double distilled filtered water for periphyton identification. The periphyton identification and enumeration were carried out using a Sedgewick-Rafter (SR) counting cell (Genex, UK) under an optical microscope (Zeiss, Germany).

Trial 3 – The integration of a plant and a substrate for reducing nutrients in culture water

The selected plants and substrates based on high nutrient removal and periphyton development from Trials 1 and 2 were used for Trial 3. Consequently, plants; water spinach (*I. aquatica*; WS) and lemon basil (*O. basilicum × O. americanum;* LB) while substrates; lava rock (LR) and bamboo (BO) were used in the plant-substrate system to investigate their suitable integration for nutrient removal. The fertilised pond water was prepared by using pond water from the Universiti Puta Malaysia farm (Latitude: N2°59'30.2"; Longitude: E101°42'57.8"). The pond water was filtered with a 500-micron mesh net and thereafter fertilised with urea (10 g.m^{-3}) and triple superphosphate (TSP, at 5 g.m^{-3}) at the beginning to initiate the nutrient-rich culture water in the plant-substrate system (Azim et al., 2001; Chang et al., 2012). Subsequently, the fertilised pond water (25 L) was transferred into each 30 L aquarium. The experiment was conducted for 25 days and there was no water exchange during the experimental period. Four air stones were used to supply aeration in each tank throughout the experimental period.

The substrates (larva rock and bamboo), each with a diameter of approximately 2–4 cm were washed with deionised water and sterilised (autoclave at 121 °C with 15 psi) before installing them into the aquaria, whereas the plant seeds were germinated for three weeks until leaves and roots developed. After adding 25 L of pond water into each aquarium, every four ropes with 13 pieces of each substrate type were hung in a vertical position at a distance of 15 cm apart. The plants (10 ± 0.6 cm shoot length) were then transplanted into each aquarium except the control with the density of four plants per aquarium by hanging them in between the substrates (Fig. 3).

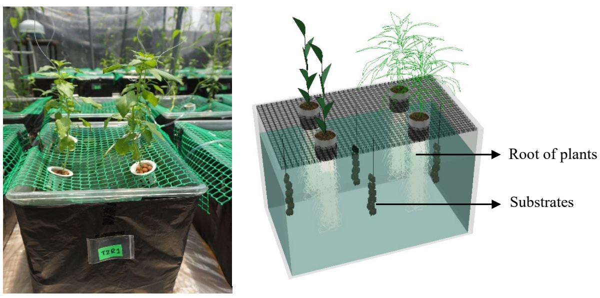

Fig. 3. The overall profile of integrated plants with substrates in a plant-substrate system; (A): top view; (B): side view.

Physical-chemical parameters of water

Physico-chemical parameters such as temperature, dissolved oxygen (DO), and pH levels were measured *in situ* using YSI 556 Handheld Multiparameter (USA) between 0900 and 1000 h. Water samples were collected at five points; four from the corners and one from the middle of each experimental tank for nutrient analyses, then pooled and kept in clean bottles and immediately stored in an icebox upon arrival in the laboratory. Water quality parameters including total nitrogen (TN), total ammonia-nitrogen (TAN), total phosphorus (TP), soluble reactive phosphorus (PO_4-P), nitrite-N and nitrate-N (NO_2 + NO_3-N), were analysed according to APHA (2005). The nutrient removal was calculated using the following equation (Endut et al., 2016b):

$$\text{Nutrient removal (\%)} = \left[\frac{\text{Initial concentration of nutrients at the beginning of experiment} - \text{Final concentration of nutrient at the end of experiment}}{\text{Initial concentration of nutrients at the beginning of experiment}}\right] \times 100$$

Physical characteristics of the substrates

To assess their physical characteristics, all the substrates were cleaned and crushed into small pieces of 2–3 mm. Thereafter, small pieces of the substrates were kept in an oven at 60 °C to ensure constant weight before determining their physical characteristics, including specific surface area (m^2.g^{-1}), micropore volume (cm^3.g^{-1}), and external surface area (m^2.g^{-1}). Specific surface area, micropore volume, and micropore size were measured using nitrogen BET isotherms on a Micromeritics TriStar II Plus surface area and porosity analyser at the Institute of Advanced Technology (ITMA), Universiti Putra Malaysia (UPM).

Plant sampling and analysis

The plant seeds were obtained from a commercial source (Green World Genetics Company, Malaysia) and germinated in the greenhouse for three weeks to develop healthy roots and true leaves before transplanting them into experimental tanks (Kim et al., 2018). After one week of germination, plants were supplied with a commercial hydroponics fertiliser (City farm, Malaysia) to maintain adequate nutrient contents for their growth. Thereafter, the plants with 4–5 true leaves (10 ± 0.2 cm shoot length) were transplanted into the treatment tanks. At the beginning and end of the experiment, the plants were sampled by measuring their heights and weights (including roots). Growth parameters such as dry and wet weight, survival rate, and relative growth rate were recorded for growth performances. The relative growth rate (RGR) of exponentially growing plants is represented by the equation (Chairuangsri et al., 2014):

$$\text{Relative growth rate (RGR)} = \frac{[\text{Ln mean final dry weights} - \text{Ln mean initial dry weights}]}{\text{Culture period (days)}} \times 100$$

Microbial community analysis

Sample collection and DNA extraction

At the end of the Trial 3, the water, substrates, and plant roots were collected for bacterial community analysis based on the highest nutrient removal efficiency (TPS_4). Each sample was placed into a sterile tube with 30 mL of sterile water and transported to the laboratory on ice. Samples were immediately vortexed until adhering particles had been visibly removed. After the vortex, the samples were centrifuged at 3,000 ×g for 15 min (Yurgel et al., 2019). The supernatant was decanted, and the pellets were transferred into 1.5 mL Eppendorf tubes with 15 % glycerol and stored at –80 °C until processing for DNA extraction. Genomic DNA was extracted for all samples by using the FastDNA™ Spin Kit for soil (Thermo Fisher Scientific), following the manufacturer's instructions. All extracted DNA was stored at -80 °C until further processing.

High-throughput Illumina Miseq sequencing platform

In Trial 3, six DNA samples, including water, substrates, and plant roots (two from each compartment) from treatment 4 (TPS_4 - WS + LR) were processed and sent for sequencing to Apical Scientific Sdn. Bhd., Malaysia. The DNA samples were processed by amplicon library preparation using two-steps PCR according to the Illumina's 16S metagenomic library preparation guideline. Bacterial 16S rRNA gene of the selected regions (16S V3-V4) was amplified using locus-specific sequence primers with overhang adapters as follows; forward overhang: 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[locus-specific sequence], reverse overhang: 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[locus-specific sequence]. All the PCR reactions were carried out with REDiant 2X PCR Master Mix (1st BASE).

Statistical analyses

The data of nutrient removal, periphyton density and relative growth rate were determined and expressed as the mean ± standard error (SE). Treatment means were compared by one-way analysis of variance (one-way ANOVA). Bioinformatics analysis was performed using R package V3.6.1. The differences in the abundance of bacterial 16S rRNA genes and genera were evaluated by one-way ANOVA. The Tukey comparison test was used to compare means to identify significant (0.05) differences among treatments.

Results

Trial 1

Physical parameters and nutrient removal

During the growth of the various plant species for the entire experimental period of 30 days, the water quality was measured at seven time points (Fig. 4). The dissolved oxygen (DO) ranged from 5.45 mg.L^{-1} to 5.78 mg.L^{-1} and remained stable throughout the experiment (Fig. 4A). The pH values during the experiments showed fluctuations between 6.09 and 7.46, whereas the pH values in the treatment groups were lower than the control (without plant) and decreased over time (Fig. 4B). Water temperature ranged from 25.73 °C to 31.60 °C in all treatment groups including the control (Fig.4C).

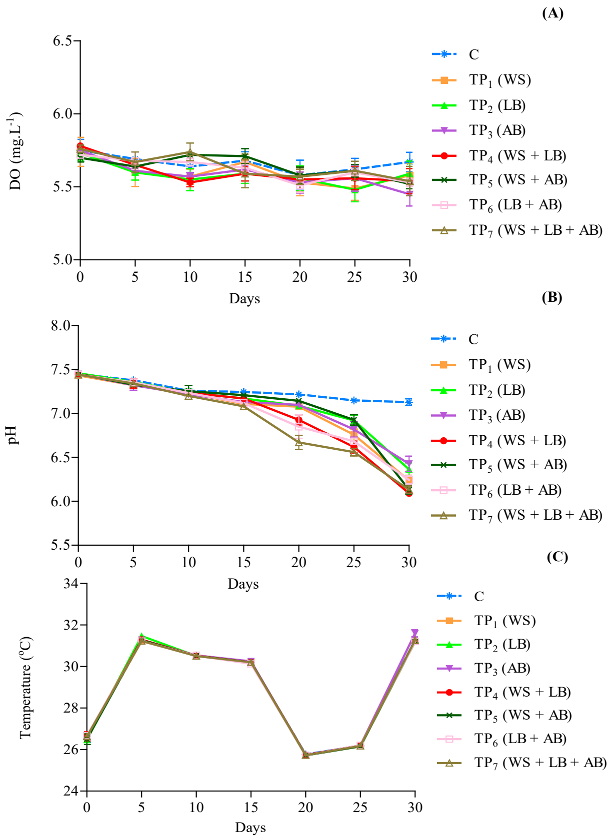

Fig. 4. Fluctuations of physical parameters; (A) dissolved oxygen; DO; (B) pH; and (C) temperature in different treatments; control (C); TP = trial plants; water spinach (WS); lemon basil (LB); aromatic basil (AB) during the 30-day trials. The values are mean ± standard error (vertical bar), n = 3.

The percentage of nutrient removal was significantly affected by the combination of plants compared to the single plant treatments (Table 1). The highest ($P < 0.05$) nutrient removal (39.08 % of total nitrogen, 88.06 % of total ammonia nitrogen, 72.61 % of nitrite + nitrate nitrogen, 82.06 % of total phosphorus, and 77.75 % of soluble reactive phosphate) was found in TP_4 (WS + LB), followed by TP_7 (WS + LB + AB), TP_5 (WS + AB), and TP_6 (LB + AB). The lowest nutrient removal was found in the control (C - no plant). In terms of single plant treatments, water spinach (TP_1-WS) showed significantly higher ($P <$ 0.05) nutrient removal compared to aromatic basil (TP_3-AB) but did not show a significant difference ($P >$ 0.05) when compared to lemon basil (TP_2-LB) (Table 1).

Table 1. Percentage of nutrient removal by different plant species, single or in combination during the study period.

Parameters (%)	Treatments							
	Control (No plant)	TP_1 (WS)	TP_2 (LB)	TP_3 (AB)	TP_4 (WS+LB)	TP_5 (WS+AB)	TP_6 (LB+AB)	TP_7 (WS+LB+AB)
Total nitrogen (TN)	1.27 ± 1.71^{d}	23.68 ± 3.54^{c}	22.77 ± 2.18^{c}	22.06 ± 1.41^{c}	39.08 ± 0.55^{a}	33.03 ± 1.00^{ab}	28.38 ± 1.21^{bc}	38.65 ± 0.72^{a}
Total ammonia nitrogen (TAN)	5.15 ± 0.47^{e}	84.75 ± 0.45^{c}	84.01 ± 0.26^{c}	82.40 ± 0.18^{d}	88.06 ± 0.33^{a}	86.67 ± 0.27^{ab}	85.34 ± 0.22^{bc}	88.02 ± 0.32^{a}
Nitrite + Nitrate nitrogen ($NO_2 + NO_3$-N)	-10.53 ± 1.1^{e}	41.49 ± 0.1^{c}	39.69 ± 1.01^{cd}	37.32 ± 1.07^{d}	72.61 ± 0.60^{a}	62.12 ± 0.2^{b}	59.52 ± 0.13^{b}	70.09 ± 0.10^{a}
Total phosphorus (TP)	-3.88 ± 3.99^{c}	74.88 ± 0.57^{ab}	74.13 ± 1.35^{ab}	73.61 ± 0.37^{b}	82.06 ± 1.26^{a}	76.74 ± 0.88^{ab}	78.52 ± 1.23^{ab}	79.07 ± 0.69^{ab}
Soluble reactive phosphorus (PO_4-P)	-2.75 ± 1.57^{e}	68.45 ± 0.67^{cd}	66.73 ± 0.68^{d}	63.34 ± 0.29^{d}	77.75 ± 1.18^{ab}	73.80 ± 1.66^{abc}	72.76 ± 1.47^{bc}	78.89 ± 0.38^{a}

Data are presented as mean ± standard error of seven sampling dates with triplicates (n = 3). Means in a row followed by different superscripts are significantly different ($P < 0.05$).
TP = trial plants; WS = water spinach; LB = lemon basil; AB = aromatic basil.

Plant growth performance

The seedlings in all tanks grew rapidly and uniformly with no mortality throughout the experiment. Over the 30-day growth period, the treatment with low plant density (single and two combination treatments) exhibited good plant growth development. Among the three species of plants, water spinach (WS) exhibited the best growth development compared to other species under all conditions (Fig.5). The plant relative growth rate (RGR) was significantly higher ($P < 0.05$) in single plant treatments (TP_1-water spinach, WS; TP_2-lemon basil, LB; TP_3-aromatic basil, AB) and two-combined plant treatments (TP_4-WS + LB, TP_5-WS + AB, TP_6-LB + AB) compared to the three-combined plant treatment (TP_7-WS + LB + AB) (Fig. 5). However, there was no significant difference ($P > 0.05$) in RGR of water spinach, lemon basil, and aromatic in single and two-combined plant treatments (Figs. 5A and 5B). In three-combined plant treatment (TP_7- WS + LB + AB), the water spinach showed significantly higher ($P < 0.05$) RGR, while both lemon basil and aromatic basil showed significantly lower ($P < 0.05$) RGR with reduced growth on their emergent parts compared to water spinach (Fig. 5C).

Trial 2

Physical characteristics of substrates

Among the different types of substrates, TS_2 (lava rock) had significantly ($P < 0.05$) higher specific surface area (8.18 $m^2.g^{-1}$) than the other substrates (Table 2). In addition, the higher micropore volume (20.01 × 10^{-3} $cm^3.g^{-1}$) and external surface area (7.65 $m^2.g^{-1}$) of lava rock further suggested that this substrate was significantly ($P < 0.05$) more porous than the other substrates.

Natural productivity

Substrates with higher surface area showed better development of periphyton communities. In this study, lava rock substrate (TS_2-LR) showed the highest ($P < 0.05$) periphyton density compared to the other substrates but was not significantly different ($P > 0.05$) compared to bamboo (TS_1-BO). The lowest ($P < 0.05$) periphyton density was found on PVC (TS_4-PVC) and bio-ring (TS_5-BR). The periphyton development increased with time. The mean phytoperiphyton density on day 45 was significantly higher ($P < 0.05$) than that of the other sampling dates (Fig. 6). Mean phytoperiphyton density started to increase sharply in all substrates from day 15 and reached the highest ($P < 0.05$) peak on day 45. In terms of zooperiphyton density, the highest ($P < 0.05$) peak was found in all the substrates on the first sampling date (day 15). Afterward, zooperiphyton density of all the substrates gradually declined until the last sampling date on day 60 (Fig. 7).

Trial 3

Physical parameters and nutrient removal

In the Trial 3, the physical parameters (DO, pH, and temperature) were measured at five-day intervals (six times) during the 25-day experimental period. Dissolved oxygen and temperature in all treatments fluctuated in the same trend as the control (Fig 8). Mean values of DO varied between 6.82–7.50 $mg.L^{-1}$, and mean values of temperature levels ranged between 25.60–32.36 °C, which were considered optimum levels for aquaculture and plant growth. Mean pH values were lower in the treatments (ranged from 6.06–7.07) compared to the control (6.45–7.07) but all pH values were suitable for aquaculture activities (Fig. 8b).

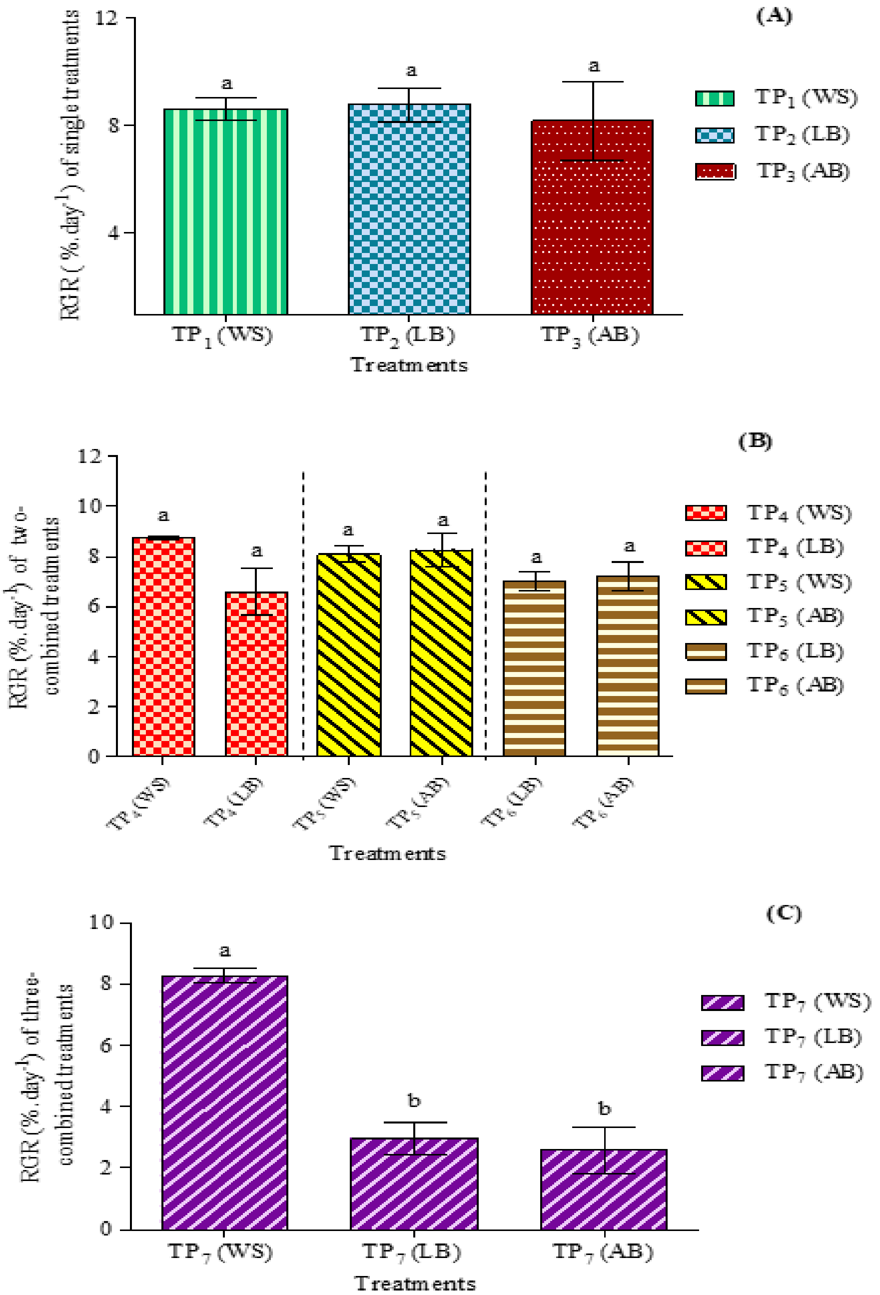

Fig. 5. Comparison of relative growth rate (mean ± standard error, n = 3) values of different plants in different treatments: (A) Single plant treatments; (B) Two-combined plant treatments; (C) Three-combined plant treatment. Different letters on the bar indicate significant differences ($P < 0.05$) among treatments (A). Different letters on the bar indicate significant differences ($P < 0.05$) within the treatments (B and C). TP = trial plants; WS = water spinach; LB = lemon basil; AB = aromatic basil.

Table 2. Physical characteristics of substrates.

Physical parameters	Substrate types				
	TS_1 (Bamboo)	TS_2 (Lava rock)	TS_3 (Bio-ball)	TS_4 (PVC)	TS_5 (Bio-ring)
Specific surface area ($m^2.g^{-1}$)	0.45 ± 0.02^c	8.18 ± 0.01^a	0.18 ± 0.02^e	0.36 ± 0.01^d	0.55 ± 0.01^b
Micropore volume [a] ($cm^3.g^{-1}$)	$3.65{\times}10^{-3} \pm 0.21^b$	$20.01{\times}10^{-3} \pm 0.03^a$	$1.61{\times}10^{-3} \pm 0.02^d$	$0.25{\times}10^{-3} \pm 0.01^e$	$2.25{\times}10^{-3} \pm 0.03^c$
External surface area ($m^2.g^{-1}$)	1.61 ± 0.02^b	7.65 ± 0.01^a	0.73 ± 0.02^d	1.63 ± 0.01^b	0.96 ± 0.01^c

Data are presented as mean ± standard error (n = 3). Means in a row followed by different superscripts are significantly different ($P < 0.05$); a Single point desorption total pore volume of pores less than 2.06×10^{-5} nm width at $p/p° = 0.90$. TS = trial substrates.

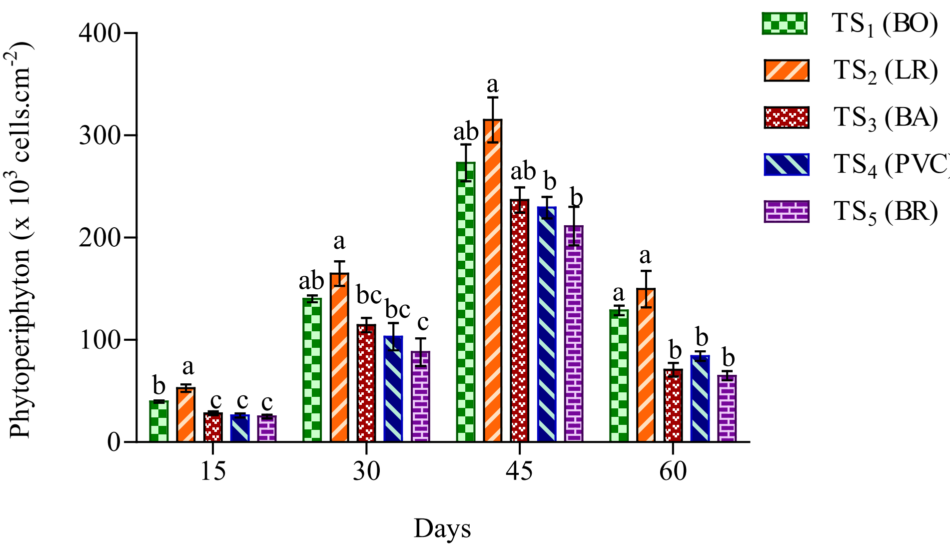

Fig. 6. Mean ± standard error (SE) values of fortnightly phytoperiphyton in different sampling days with bamboo (TS_1-BO), lava rock (TS_2-LR), bio-ball (TS_3-BA), polyvinyl chloride pipes (TS_4-PVC), bio-ring (TS_5-BR) substrates in the culture tank. Vertical bars indicate means ± SE (n = 3). Statistically significant differences ($P < 0.05$) within the same days are denoted by different letters. TS = trial substrates.

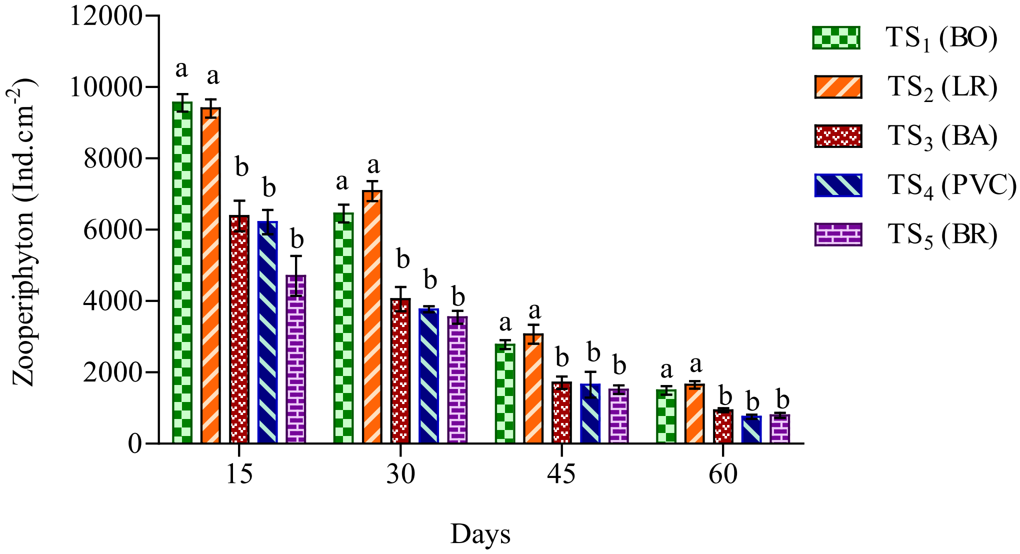

Fig. 7. Mean ± standard error (SE) values of fortnightly zooperiphyton in different sampling days with bamboo (TS_1-BO), lava rock (TS_2-LR), bio-ball (TS_3-BA), polyvinyl chloride pipes (TS_4-PVC), bio-ring (TS_5-BR) substrates in the culture tank. Vertical bars indicate means ± SE (n = 3). Statistically significant ($P < 0.05$) differences within the same days are denoted by different letters. TS = trial substrates.

The integration of water spinach and lava rock (TPS_4-WS + LR) showed the best integration for nutrient removal (Table 3). Percentage of nutrient removal in the presence of plants and substrates was significantly ($P < 0.05$) higher than in the control (Table 3). The highest ($P < 0.05$) nutrient removal (88.21 % of total ammonia nitrogen, 67.13 % of nitrite nitrogen and 24.23 % of soluble reactive phosphorus) was found in TPS_4 (WS + LR) followed by TPS_2 (WS + BO), TPS_3 (LB + LR) and TPS_1 (LB + BO).

Plant growth performances

Water spinach exhibited significantly higher ($P < 0.05$) growth under all conditions compared to lemon basil. There was no plant mortality recorded during the 25-day study period. Among the treatments, water spinach integrated with lava rock (TPS_4-WS + LR) and bamboo (TPS_2-WS + BO) showed significantly higher ($P < 0.05$) relative growth rate (RGR) than the lemon basil integrated with lava rock (TPS_3-LB + LR) or with bamboo (TPS_2-LB + BO) (Fig. 9).

Bacterial community composition

The highest nutrient removal was found in the integrated water spinach and lava rock (TPS_4-WS + LR) in Trial 3. Therefore, TPS_4 (WS + LR) was selected for

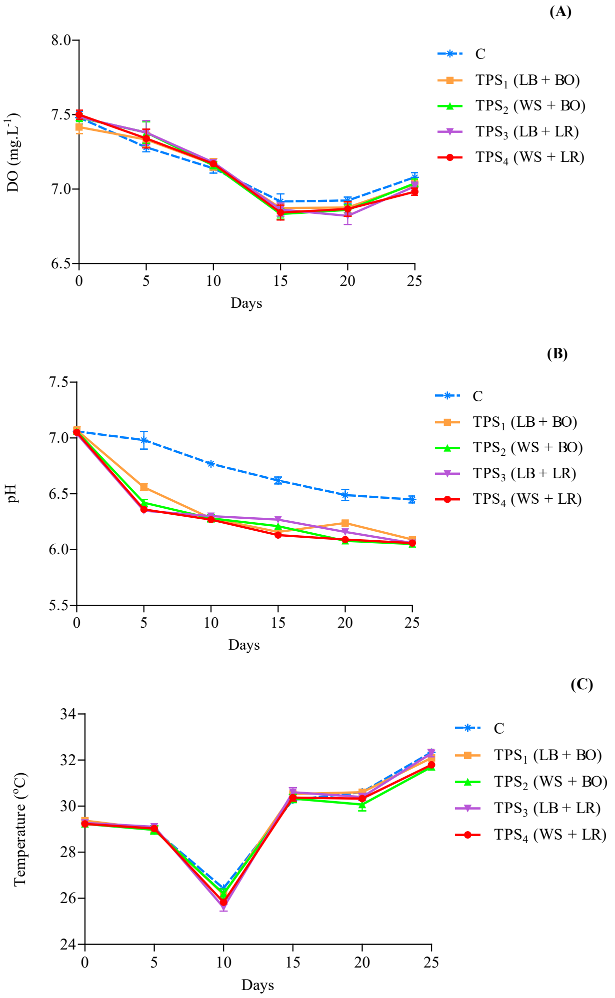

Fig. 8. Fluctuations of physical parameters; (A) dissolved oxygen; DO; (B) pH; and (C) temperature in different treatments; control (C); water spinach (WS); lemon basil (LB); aromatic basil (AB), bamboo (BO), and lava rock (LR). Values are mean ± standard error (vertical bars), n = 3.

the assessment of bacterial communities. According to the analysis, a total of 27 bacterial phyla across all samples were identified using the OTU classifier (Fig. 10). Proteobacteria was one of the most abundant phyla in water and substrates of TPS_4 accounting for 36.13 % and 35.67 % of the total bacteria, respectively (Fig. 10). However, Planctomycetota (32.62 %) was the most dominant phylum in the plant roots. On the substrate, the other major phyla were Planctomycetota (14.51 %), Verrucomicrobiota (14.12 %) and Bacteroidota (10.16 %), while on the plant roots, Verrucomicrobiota (18.96 %) and Proteobacteria (18.52 %) were dominant (Fig. 10A). *Nitrospira* bacteria were found on the substrate (5.18 %), root (2.86 %) and water (0.24 %) (Figs. 10A and 10B). In addition, nitrifying and denitrifying bacteria such as *Nitrosomonas*, *Thermomonas*, *Azospira* and *Rhizobium* were also present in the TPS_4 (WS + LR).

In this study, some Nitrobacteria and denitrifying bacteria such as *Nitrospira* and *Thiobacillus* were significantly higher ($P < 0.05$) on the substrates than the root and water. *Bosea* and *Acidovorax* were significantly higher ($P < 0.05$) in the substrates but were not significantly different ($P > 0.05$) compared to the roots. *Prosthecobacter* and *Thermomonas* were also found higher on the substrates, but not significantly different ($P > 0.05$) from those in the water. However, *Prosthecobacter* was not reported in the roots in this study (Fig. 11).

Nitrosomonas and some denitrifiers such as *Dechlomonas* and *Flavobacterium* were significantly higher ($P < 0.05$) in water compared to the substrates and roots (Fig. 12). Moreover, some beneficial bacteria such as *Novosphingobium* sp., *Bacillus* sp. and *Thaiobacillus* sp. were found on the plant roots, substrate and water whereas, *Novosphigobium*, showed significantly higher ($P < 0.05$) abundance in substrates and water than in roots. *Bacillus* was significantly higher ($P < 0.05$) in the root compared to the substrates, but it was not found in the water.

Discussion

The current study showed that different plant species of water spinach, lemon basil and aromatic with their single or in combinations have a significant effect on nutrient removal. Additionally, the physical characteristics of the different five substrate types, particularly the high specific surface structure of the substrates played a prominent role in periphyton and biofilm development. Water spinach integrated with lava rock was the most effective combination for nutrient removal in aquaculture-enriched water. In

Table 3. Percentages of nutrient reduction in different treatments during the experimental period.

Parameters	Treatments				
	Control (no plant + substrate)	TPS_1 (LB + BO)	TPS_2 (WS + BO)	TPS_3 (LB + LR)	TPS_4 (WS + LR)
Total ammonia nitrogen (TAN)	33.1 ± 1.64^{d}	73.83 ± 1.23^{c}	79.78 ± 0.88^{b}	78.91 ± 0.50^{bc}	88.21 ± 0.97^{a}
Nitrite nitrogen (NO_2-N)	20.80 ± 3.26^{d}	46.05 ± 1.21^{c}	56.36 ± 1.06^{b}	53.9 ± 1.09^{bc}	67.13 ± 2.92^{a}
Soluble reactive phosphorus (PO_4-P)	5.48 ± 1.01^{d}	16.48 ± 0.77^{c}	24.92 ± 0.42^{b}	24.23 ± 0.69^{b}	30.41 ± 1.28^{a}

Data are presented as mean ± standard error of six sampling dates with triplicates (n = 3). TPS = trial plants + substrates; WS: water spinach; LB: lemon basil; LR: lava rock; BO: bamboo. Means in a row followed by different superscripts are significantly different ($P < 0.05$).

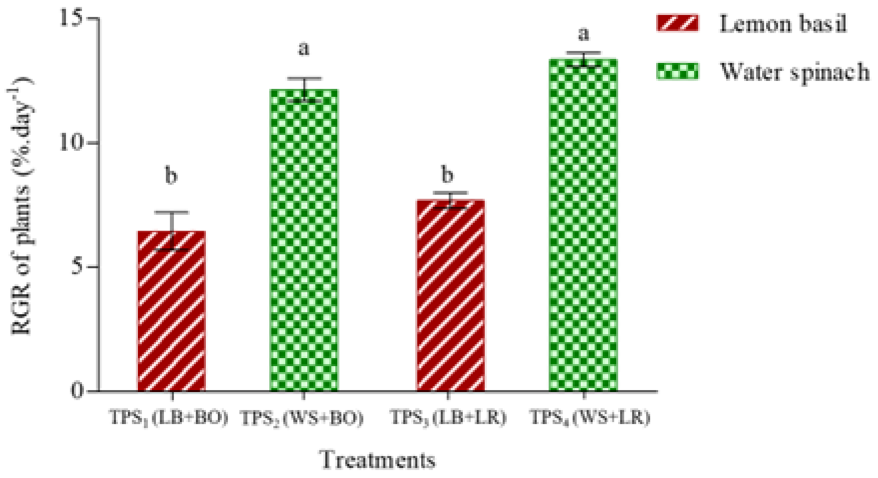

Fig. 9. Mean ± standard error (SE, n = 3) values of relative growth rate (%.day^{-1}) of lemon basil (LB) and water spinach (WS) on different substrate types of bamboo (BO) and lava rock (LR). Different letters showed a significant difference ($P < 0.05$) among the treatments.

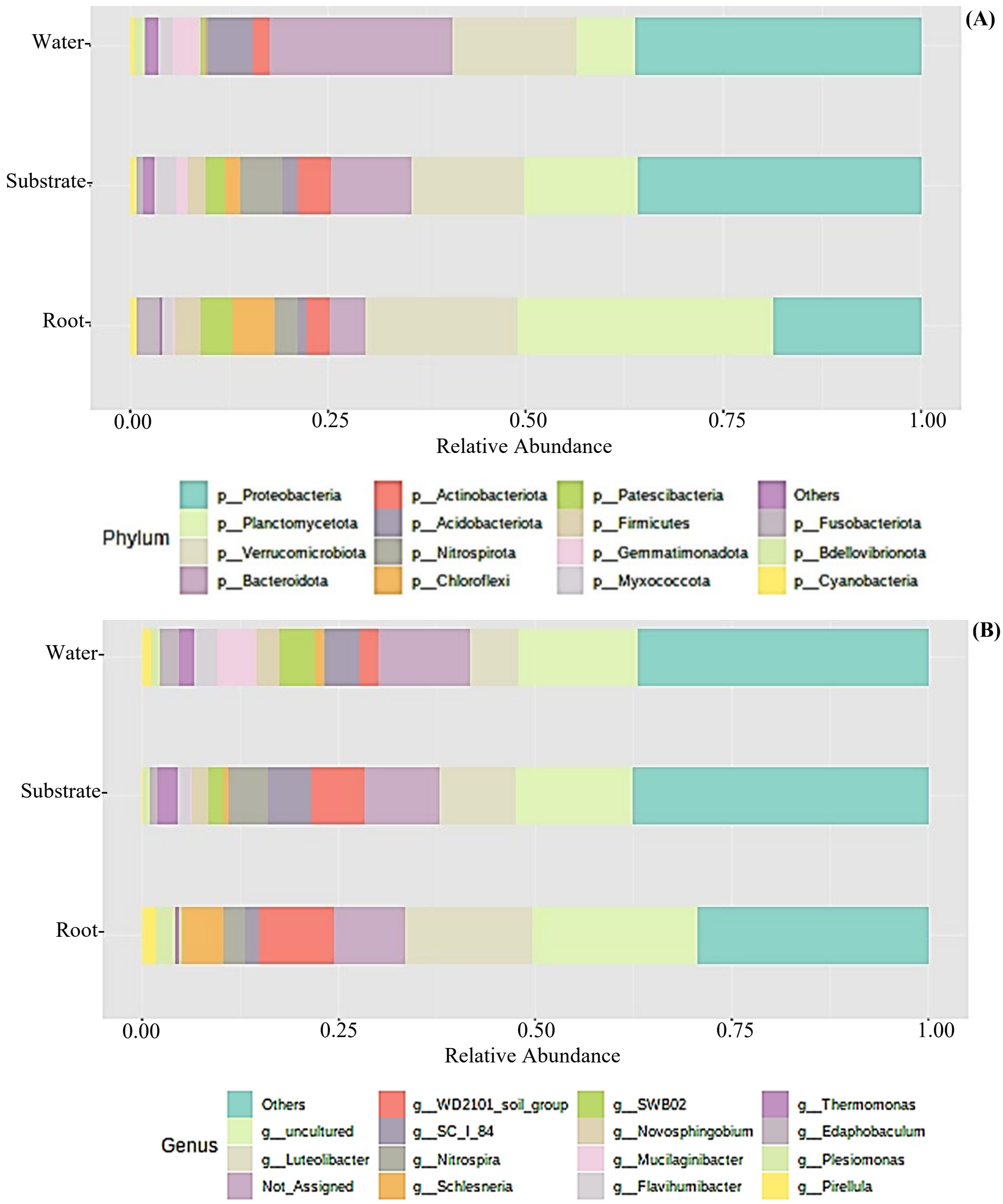

Fig. 10. Relative abundance of the top 15 bacterial phyla (A) and genera (B) in the water, on the substrate, and on the plant root of the water spinach and lava rock treatment (TPS$_4$).

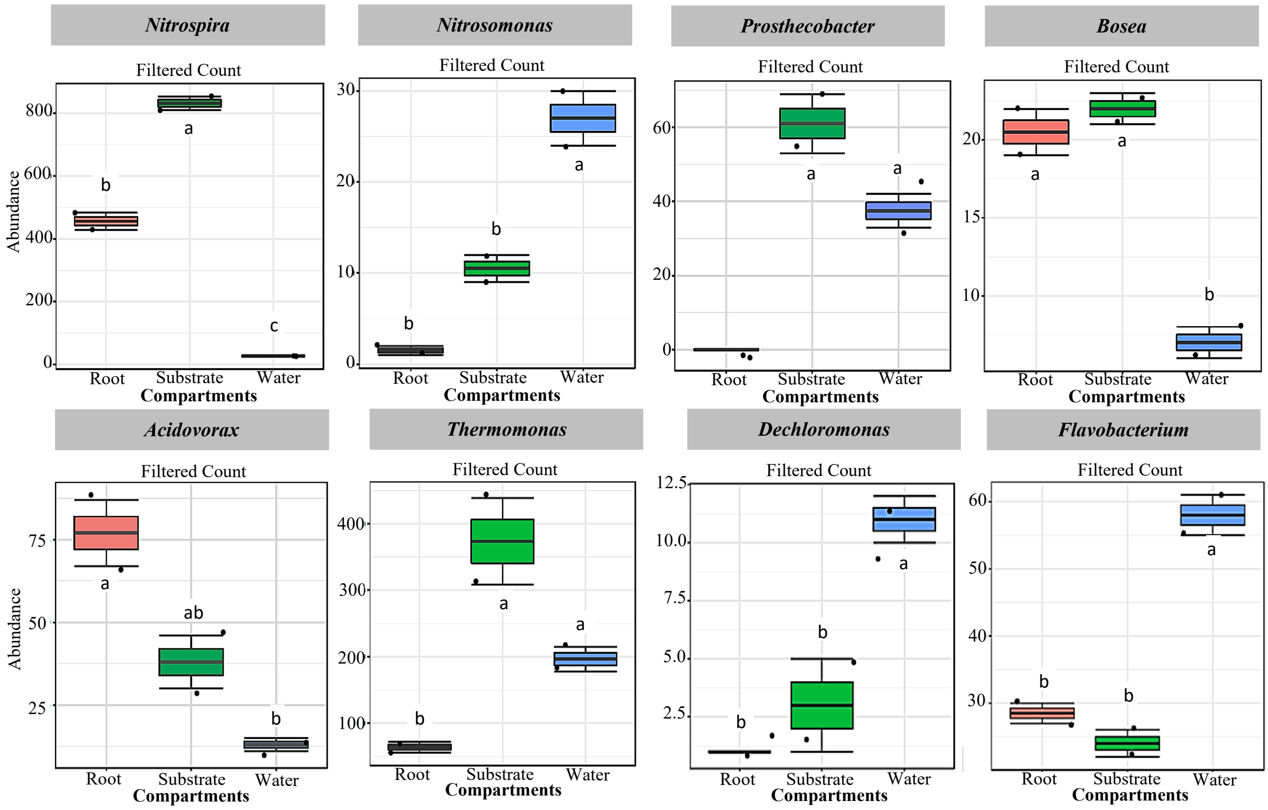

Fig. 11. Bacterial genera related to ammoxidation, nitrification, and denitrification in different compartments found in the integrated water spinach and lava rock treatment (TPS_4); root, substrate and water. Different letters indicate statistically significant differences ($P < 0.05$) among the compartments.

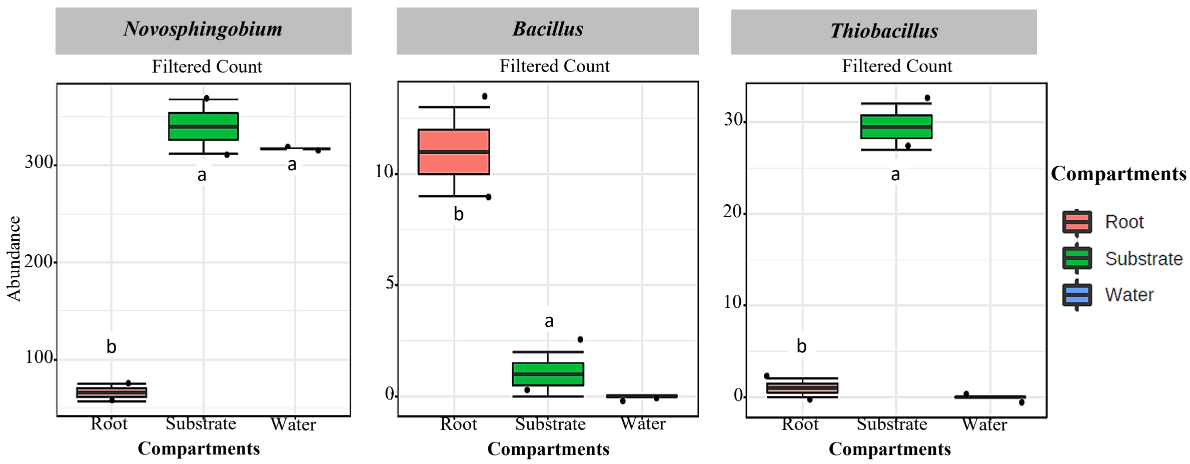

Fig. 12. Beneficial bacteria genera in different compartments found in the integrated water spinach and lava rock treatment (TPS_4); root, substrate and water. Different letters indicate statistically significant differences ($P < 0.05$) among the compartments.

fact, the nutrient removal could have been enhanced by the development of bacterial communities in different compartments, the substrates, water and plant roots (Saufie et al., 2020; Liu et al., 2021).

In Trial 1, it was found that the three plant species, especially when used in a combination of two species played a notable role in nutrient removal and growth performance without negative effects on their growth rates within the 30-day growth period. The present findings are also in line with the results by Su et al. (2019), who showed that the combination of *Salvinia natans* and *Hydrocotyle vulgaris* effectively removed nutrients (TP and TN) in the water column. Nevertheless, the present study showed that the most suitable species for removing total nitrogen (TN), total ammonia nitrogen (TAN) and total phosphorus (TP) were water spinach and lemon basil, or their combination. Plants can remove nutrients in the water column by root absorption, adsorption, and plant extraction (Wang et al., 2020; Liu et al., 2021). Further, the combination of different plant species can enhance nutrient removal efficiency due to their synergistic interaction on different physiology of root structures (Tripathi and Upadhyay, 2003; Su et al., 2019). In this study, the robust fibrous root system of water spinach, coupled with the elongated root system of lemon basil, contributes to this synergistic interaction. Therefore, plant growth and physiology are essential factors for nutrient removal efficiency (Andriani et al., 2019; Yang and Kim, 2020).

Single and two-combined plant treatment (TP_4; WS + LB) showed significantly (P < 0.05) higher RGR compared to the three-combined plant treatment (TP_7; WS + LB + AB). The lower growth performances showed in TP_7, particularly in lemon basil and aromatic basil could be due to the competition for nutrients, light and space in three-combined plant treatment. Estim et al. (2019), reported that the lower biomass weight of Chinese white cabbage compared to *Brassica rapa* in an aquaponic system was due to competition.

In Trial 2, the periphyton biomass and nutrient composition were influenced by various factors such as algal and bacterial taxonomic composition, nutrient levels, water quality, light intensity, and substrate types (Gangadhar et al., 2017; Cao et al., 2019). This study found a significantly higher (P < 0.05) specific surface area (8.18 $m^2.g^{-1}$) and periphyton density in lava rock than the other substrates indicating that the surface of lava rock might be more suitable for the adhesion of periphyton and biofilm development. This result was similar to that reported by Huang et al. (2012), who documented that volcanic rock had a high specific area (4.72 $m^2.g^{-1}$) and number of micropores that facilitates the development of biofilm communities.

Moreover, Andriani et al. (2018) and Tanjung et al. (2019) documented that the higher surface area on the substrate facilitated more bacterial growth, which could enhance the decomposition of organic matter. The results from the current study also agree with Cao et al. (2019) and Tanjung et al. (2019), who mentioned that high surface area and large cavities on substrates could harbour a great number of bacteria as well as periphyton communities resulting in an improvement of water quality in the culture system. The highest mean density of phytoperiphyton was observed on day 45 across all substrate types, while zooperiphyton reached the peak on day 15. This is because fertiliser was introduced at the beginning of the experiments and in the subsequent months. Therefore, it increased nutrient levels in the water column, significantly influencing the enhanced growth and development of periphyton in the system (Katsiapi et al., 2016; Ramlee et al., 2021). However, the gradual decrease in periphyton density after the high peak could be related to the life cycle, self-shading, and dislodgment in the absence of grazers (Silva et al., 2014; Błędzki and Rybak, 2016).

In Trial 3, the water spinach integrated with lava rock substrate (TPS_4) removed almost 88.21 % of TAN from the water column. Sa'at and Zaman (2017) documented that phytoremediation using water spinach was able to reduce nutrients up to 80 % of all parameters by the end of the 25-day treatment, which supports the current study. A similar removal rate of 67.13 % for NO_2-N was also observed in TPS_4 (WS + LR). More than 30.41 % of the PO_4-P in the water column was removed in the TPS_4 (WS + LR) in this study, which was comparable to the values (45–70 %) reported by White and Cousins (2013). However, variations in removal rates could be attributed to different plants and substrates (Su et al., 2019; Liu et al., 2021).

Furthermore, Chang et al. (2012) suggested that additional substrates surrounding the plant root in the water column could adequately remove nutrients. The presence of substrates would benefit the growth of macrophytes and enhance the activities of the microorganisms (Cao et al., 2016; Liu et al., 2021). In this present experiment, although both TPS_2 (WS + BO) and TPS_4 (WS + LR) treatments effectively removed inorganic nitrogen and phosphorus in the water, the combination of the water spinach and the lava rock significantly enhanced (P < 0.05) the nutrient removal compared to the combination with bamboo. The results showed that adding lava rock substrate to TPS_4 (WS + LR) could effectively improve nutrient removal efficiency. The contribution of the substrate to the total nutrient removal was higher in TPS_4 (WS + LR) compared to TPS_2 (WS + BO), suggesting that substrate absorption was also another factor for high nutrient removal in the system (Levy et al., 2017). However, the effect of the plant and substrate on the higher nitrogen removal could be due to the favourable environment in lava rock for the growth of microorganisms.

Water quality parameters are congenial for plant survival, growth, and other living organisms in the system (Thorarinsdottir, 2015; Saha et al., 2016). In this study, there was no death or disease noticed in the plants during the experiment. The dissolved oxygen (DO), pH and temperature in both single and combined plant treatments were in the optimal ranges as reported in previous studies on plant growth in hydroponic systems (Saha et al., 2016; Pinho et al., 2018; Oladimeji et al., 2020). In this study, the DO levels in both treatment groups and the control showed fewer fluctuations due to oxygen supplementation provided throughout the entire experimental period. The treatment groups (with plants or substrates) tended to have a decrease in the pH compared with the control (without plants or substrates). The reduction of pH might be due to the production of carbon dioxide during rhizome breathing and the formation of organic acids (Abed et al., 2019) as well as the nitrification process of bacteria (Thorarinsdottir 2015; Li et al. 2019).

Water spinach in both TPS_2 (WS + BO) and TPS_4 (WS + LR) showed excellent growth characteristics, with a relative growth rate (RGR) at the end of the experiment significantly higher ($P < 0.05$) than that of lemon basil in TPS_1 (LB + BO) and TPS_3 (LB + BO). However, the increase in plant biomass also led to nutrient accumulation in the plants, which was indicated by a higher RGR rate of water spinach and higher quantity of N and P removed through plant uptake in the TPS_4 (WS + LR) compared to TPS_1 (LB + BO) and TPS_3 (LB + BO). In this study, water spinach showed significantly higher ($P < 0.05$) nutrient removal without adverse interactions on their growth rates under all conditions compared to lemon basil. Water spinach is considered as an aquatic plant with strong roots which can tolerate a high load of nutrients (Shahid et al., 2020). Therefore, it can perform better in terms of growth rate than lemon basil.

The results of microbial structure analysis showed that Proteobacteria, Planctomycetota, Verrucomicrobiota and Bacteroidota were the main groups found in water, roots, and substrates in the TPS_4 (WS + LR). Other studies have demonstrated that these bacteria could be responsible for nutrient removal in water (Eck et al., 2019; Sun et al., 2019). Previous studies by Eck et al. (2019) and Li et al. (2016) also reported that Proteobacteria was a major phylum, representing 34.6 % in an aquaponic and aquaculture system, which was consistent with this study. Additionally, Proteobacteria in this study was the dominant phylum found on the substrate and in the water, while Planctomycetota was the main phylum on the roots. However, Schmautz et al. (2017) reported that Proteobacteria (approximately 50 %) and Bacteroidetes (15–20 %) were the main phyla in the root zone in the aquaponics system.

Proteobacteria, Bacteroidota, Verrucomicrobiota and Nitrospirota contain some genera commonly involved in the N and P cycle. These phyla could remove NH_4^+ and NO_3^- via heterotrophic nitrification and aerobic denitrification (Azevedo et al., 2018; Kasozi et al., 2021). *Nitrospira* is a genus in Nitrospirota, which is capable of directly converting ammonium to nitrate as it is known as a total nitrifier and considered a complete ammonia oxidiser (comammox) (Daims et al., 2015; Bartelme et al., 2017). In this study, *Nitrospira* depicted a high relative abundance on the substrates which might be due to a greater specific area on the lava rock. This observation was similar to Schmautz et al. (2017), who reported that *Nitrospira* (3.9 %) was relatively high on a biofilter but low on the plant roots in an aquaponic system. The presence of plants and substrates in a fish culture system had a large influence on the composition of the bacterial community (Eck et al., 2019; Liu et al., 2021). The quantity of ammonifying bacteria, nitrifying bacteria and denitrifying bacteria including *Nitrospira*, *Bosea*, *Acidovorax*, and *Thermomonas* were high on the substrates and roots of water spinach in this study. The relative abundance of functional bacteria for nitrogen removal increased in TPS_4 (WS + LR) indicating that the microbial activities greatly contributed to the nutrient removal in the culture system with water spinach and lava rock.

Conclusion

The present study showed that the robust fibrous root system of water spinach, along with the high specific surface area of lava rock substrates containing functional bacterial assemblages, significantly improved environmental conditions in an integrated plant-substrate system. Furthermore, lava rock substrates with a high specific surface area facilitated the colonisation of periphyton and other living organisms. Hence, the integration of water spinach with lava rock in this study demonstrated synergistic effects on nutrient mitigation alongside higher plant growth. Additionally, periphyton communities, coupled with microbial nitrification and denitrification, also played a significant role in enhancing nitrogen removal in the integrated plant-substrate system.

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Author contributions: Arissara Sopawong: Conceptualisation, validation, methodology, investigation, visualisation, formal analysis, original draft, review and editing. Fatimah Md Yusoff: Conceptualisation, methodology, visualisation, review and editing, supervision, resources, project administration, funding acquisition. Muta Harah Zakaria: Writing, review and editing, supervision. S.M. Nurul Amin: Writing, review and editing, supervision. Amalia Mohd Hashim: Writing, review and editing, supervision. Hui Teng Tan: Methodology and formal analysis.

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