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ORIGINAL RESEARCH

Assessment of enhanced phytoremediation of shrimp aquaculture wastewater by endophytic bacteria-inoculated floating treatment wetlands

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Abstract In Vietnam, the major problem for the sustainability of the shrimp aquaculture industry is the management of shrimp aquaculture wastewater. A promising solution to remediate pollutants in shrimp aquaculture wastewater is to use floating treatment wetlands (FTWs) in combination with pollutant-degrading bacteria. Hence, this study aimed to evaluate the effect of inoculation of endophytic bacteria isolated from Man Trau grass on the remediation of shrimp aquaculture wastewater in FTWs vegetated with grass (*Brachiaria mutica*). The results showed that FTWs only vegetated with *B. mutica* removed organic and inorganic pollutants from shrimp aquaculture wastewater and a significant reduction was observed for total dissolved solids (TDS), chemical oxygen demand (COD), ammonium (NH_4^+-N), nitrate (NO_3^--N), and phosphate (PO_4^{3-}-P) with efficiency is 60, 71, 80, 65, and 73%, respectively. Interestingly, the efficiency removal was further improved by the inoculation of endophytic bacteria. The combination of plants and bacteria produced the highest removal of COD (73%), NH_4^+-N (96%), NO_3^-–N (80%), PO_4^{3-}–P (81%), Cu (95.3), and Fe (93.4%). Moreover, the inoculated bacteria were also validated their presence in water as well as in roots and shoots of the vegetated plant. In addition, the treated water output met the wastewater discharge standards of the World Health Organization. Therefore, the results suggest endophyte-assisted FTWs are a suitable approach for the remediation of shrimp aquaculture wastewater.

Keywords *Brachiaria mutica* . Shrimp aquaculture wastewater . FTW . Endophytic bacteria . Ammonium removal

Introduction

In Vietnam, a major agriculture sector is the aquaculture industry, which will increase in the future to support the growing world population (Quyen et al. 2020; Anh et al. 2021). However, using highly nutritious feeds and other chemical products in this vital sector has raised many new environmental problems for aquatic ecology and systems, typically the toxicity of nitrogenous wastes such as ammonia, nitrite, and nitrate to aquatic lives (Mora-Ravelo et al. 2017; Anh et al. 2021). Hence, managing properly these environmental problems is the key to the sustainable development of the aquaculture industry, particularly is shrimp

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cultivation.

There are three mechanisms including physical, chemical, and biological methods (Wang et al. 2008) have been applied to minimize the negative impact of harmful wastes like ammonia, nitrite, and nitrate in aquaculture, but two first methods present some limitations such as high cost, the accumulation of chemical substances in an aquaculture pond, and the un-completed removal of nitrite in the aquatic environment as well (Wang et al. 2008; Lynch et al. 2015). Hence, a biological method using microorganisms (bacteria, fungi, algae) and plants to remove harmful wastes in contaminated sites is an alternative to manage effectively the aquaculture wastewater to reuse it that save the limited freshwater resources (Afzal et al. 2014; Lynch et al. 2015; Nhan et al. 2020). Among biological methods, a promising one to remediate the aquaculture wastewater is to use floating treatment wetlands (FTWs) that constructed by soil-less buoyant mats with holes to vegetate plants so that the root directly contacts the wastewater underneath the mat. This method had been applied and showed a high efficiency of N and P removal improving the quality of the aquaculture wastewater (Lynch et al. 2015).

Notably, in FTWs, the pollutants can be removed from the contaminated water by the association between plants and bacteria (Chang et al. 2013; Zhang et al. 2014) and vice versa (Afzal et al. 2014; Khan et al. 2013). In that theme, some microorganisms in the water can colonize on the root surface or root rhizosphere to produce a biofilm (Zhang et al. 2014). On the other side, several bacteria by penetrating the root can colonize within it and/or move to the aerial parts; they were called endophytic bacteria (Afzal et al. 2014). These indicate the microorganisms play an important role in the remediation. However, it is still unclear if the inoculated bacteria present the same characteristics at different components (water, root, and shoot) of FTWs.

Brachiaria mutica is the salt-tolerant grass that is commonly used to remove pollutants from contaminated soil and water because they can withstand stress conditions in the wastewater due to their extensive root system and biomass (Ijaz et al. 2015; Nandakumar et al. 2019). However, FTWs using grass (*B. mutica*) as a cheap and low-cost method to remove pollutants from aquaculture wastewater have not been investigated. Moreover, the combination of plants and endophytic bacteria in FTWs for the pollutant remediation of aquaculture wastewater has not been studied. Therefore, this study aimed to evaluate the effect of endophytic bacteria in FTWs vegetated *B. mutica* for the remediation of pollutants (NH_4^+, NO_3^-, and PO_4^{3-}) in aquaculture wastewater, to identify the efficiency for pollutant removal, and to determine the persistence of the inoculated endophytic bacteria in different components (root, shoot and water) of the FTWs.

Materials and methods

Shrimp aquaculture wastewater effluent collection and characterization

Shrimp wastewater sample was collected from shrimp farming in June 2021 from Nam Dinh, Vietnam. The raw shrimp wastewater sample was determined some important parameters such as pH, electrical conductivity (EC), COD, total dissolved solids (TDS), DO, NO_3^--N, NH_4^+-N, PO_4^{3-}-P and different metals (Fe, Cu) as described earlier (Eaton et al. 2005).

Inoculated strain of Endophytic bacteria

Two endophytic *Bacillus* strains (*Bacillus velezensis* MT50, *B. amyloliquefaciens* MT51), previously isolated from Man Trau grass (*Eleusine indica*) and characterized for salt-tolerance and ammonium removal in synthesized shrimp aquaculture wastewater, were used in this study. These strains were grown overnight in Luria-Bertani (LB) broth. The pellets of bacterial cells were collected by centrifugation and then prepared the bacterial mixture by mixing equal numbers of cells of each strain (10^7 CFU/ml each). 100 ml of this mixture was inoculated in each FTWs microcosm experiment.

Experimental setup of FTWs

Batch microcosm experiments were carried from June to July 2021 at VNU Central Institute for Natural

Resources and Environmental Studies (VNU-CRES), Vietnam. Experiments were designed in twelve 30 L polyethylene tanks (L×W×H, 56.4×37.9×20.5 cm). Hydroponic mats (50.8×38.1×7.6 cm) were prepared from a polyethylene sheet. Each floating mat has five holes (5 cm diameter) that were used to vegetate cuttings of *B. mutica* (10 cuttings/ each hole) (Fig. 1). The cuttings were rooted and developed in a polyethylene tank containing 25 L tap water for one month, after that the tap water was replaced by the same amount of shrimp aquaculture wastewater and a bacterial mixture (100 mL) was added to the treatments where applicable. Different treatments (in triplicates) were performed in the covered tanks including shrimp aquaculture wastewater without *B. mutica* and bacterial inoculation (T1), shrimp aquaculture wastewater with *B. mutica* only (T2), shrimp aquaculture wastewater with *B. mutica* and bacterial inoculation (T3), and shrimp aquaculture wastewater with bacterial inoculation only (T4).

Shrimp aquaculture wastewater remediation

Treated shrimp aquaculture wastewater samples were collected to determine the effect of plants, bacteria, and plant-bacteria partnerships on the remediation of shrimp aquaculture wastewater. Different parameters were determined including pH, EC, total dissolved solids (TDS), and temperature of water samples were measured using Milwaukee Mi805 Portable pH/EC/TDS/Temperature Meter (Mi805 Milwaukee, CO, USA). Dissolve oxygen (DO) was identified by Milwaukee Dissolved Oxygen Meter (MW 600, CO, USA). The other parameters such as NO_3^--N, NH_4^+-N, PO_4^{3-}-P, COD, Cu, and Fe were determined by using the detergent solution accompanied with a multi-parameter photometer (HI83399-02, Hana, Romania) as per the introduction of the manufacture. Physical and chemical parameters (pH, temperature, DO, TDS, EC) were observed daily monitoring in 14 days of the experimental setup. For other parameters (chemical oxygen demand (COD), ammonium-nitrogen (NH_4^+-N), nitrate-nitrogen (NO_3^--N), phosphate (PO_4^{3-}-P), iron (Fe), and copper (Cu)), treated water samples were collected at the 1 day, 3 days, 7 days, 10 days and 14 days after experimental setup.

Identification of inoculated bacteria in the component of FTWs

At the end of the experiment, the presence of the inoculated bacteria was determined in different components of the FTWs. The samples of treated shrimp wastewater of T3 and T4 treatments were collected to screen for the inoculated bacteria by plating on LB agar plates. Moreover, the root and shoot of *B. mutica* in the T3 experiment were also collected and used to screen for the inoculated bacteria as well. These plant samples were surface sterilized as the method described by Trung et al. (2021) and were homogenized in 0.9% NaCl solution. The homogenized solution was also plated on the LB agar plates. The inoculated plates were kept at 37°C overnight to allow the bacteria grow. Then 100 isolated colonies were used for molecular identification by Restriction Fragment Length Polymorphism (RFLP) analysis, in which the identity of the

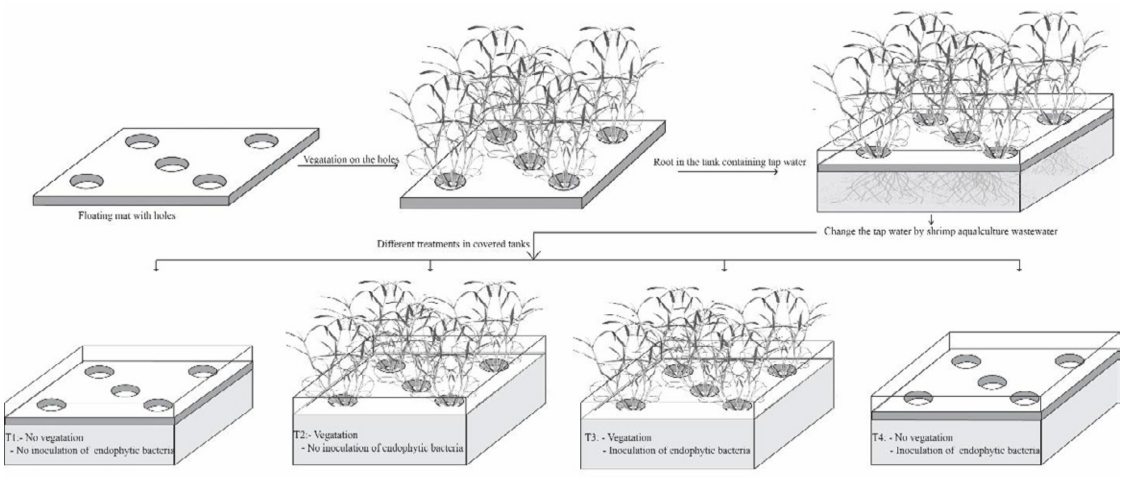

Fig. 1 Different components of floating treatments wetlands and experimental setup

isolated bacteria was compared with the inoculated strains.

Statistical analysis

Data analysis was performed by using SPSS software package version 17.0. The one-way analysis of variance (ANOVA) was used to compare data (three replicates of each treatment) among treatments. Duncan's multiple range test was used to determine the significance of the results.

Results and discussion

Shrimp aquaculture wastewater characteristics

The data analysis of shrimp wastewater effluent from Nam Dinh showed high values of NO_3^--N, NH_4^+-N, PO_4^{3-}-P, COD compared to National Standards (QCVN 08–MT 2015; QCVN 01-BYT 2009) and also World Health Organization (2006) (Table 1). These suggest that before discharge this shrimp wastewater effluent in the river, the removal of these indicators should be done.

Phytoremediation of pollutants from shrimp aquaculture wastewater

The result showed a significant removal of total dissolved solids (TDS), chemical oxygen demand (COD), ammonium (NH_4^+-N), nitrate (NO_3^--N), and phosphate (PO_4^{3-}-P) from shrimp aquaculture wastewater in experiment contained the floating mats vegetated with *B. mutica* (T2) compared to the control (T1) were obtained with efficiency is 60, 71, 80, 65, and 73%, respectively. It is consistent with the previous reports in which the vegetated plants present a high efficiency in remediation of pollutants in aquaculture wastewater (Raharjo et al. 2015; Omitoyin et al. 2017; Mohd Nizam et al. 2020). These can be explained by the naturally metabolic and hydraulic processes of plants (Raharjo et al. 2015; Omitoyin et al. 2017; Mohd Nizam et al. 2020). Especially, the addition of endophytic bacteria into the FTWs vegetated with the same plant species

Table 1 Mean water quality of raw and treated shrimp aquaculture wastewater collected from Nam Dinh, Vietnam after two weeks

Parameters	RAW (Wastewater only)	T1 (Wastewater only)	T2 (Wastewater + Plant only)	T3 (Wastewater + Plant and Endophytic bacteria)	T4 (Wastewater + Endophytic bacteria only)	National Standards**	WHO Guidelines***
Temperature (°C)	25.38 ± 0.05 c	26.56 ± 0.13 c	28.16 ± 0.14 a	28.75 ± 0.12 a	27.14 ± 0.32 b	NG*	NG
pH	6.92 ± 0.11 c	7.12 ± 0.03 c	8.11 ± 0.21 a	8.21 ± 0.17 a	7.34 ± 0.03 b	5.5 – 9	6 - 9
EC (µs/cm)	1115 ± 4.13 c	1113 ± 0.71 c	1106 ± 2.31 b	1104 ± 1.87 a	1107 ± 0.15 b	NG	NG
TDS (mg/l)	629 ± 1.39 c	614.5 ± 5.61 c	247 ± 4.37 a	246 ± 8.14 a	441 ± 4.51 b	< 1000	1500
DO (mg/l)	4.54 ± 0.31 c	4.75 ± 0.07 c	5.8 ± 0.14 a	5.7 ± 0.21 a	5.2 ± 0.03 b	≥ 2	≥ 2
COD (mg/l)	106 ± 1.37 e	68 ± 1.43 d	30 ± 4.24 b	28.87 ± 3.12 a	35 ± 1.52 c	< 30	< 50
NO_3^--N (mg/l)	17.57 ± 0.37 c	15.72 ± 0.02 d	6.15 ± 0.03 b	3.55 ± 0.02 a	6.54 ± 0.07 c	< 15	< 30
NH_4^+-N (mg/l)	9.12 ± 0.14 c	8.39 ± 0.01 c	1.81 ± 0.03 b	0.32 ± 0.01 a	1.92 ± 0.03 b	<0.9	< 5.0
PO_4^{3-}-P (mg/l)	76.32 ± 3.5 d	70.84 ± 0.26 d	20.63 ± 1.71 b	14.57 ± 1.71 a	48.53 ± 1.71 c	<0.5	< 15
Fe (mg/l)	0.52 ± 0.11 c	0.51 ± 0.12 c	0.031 ± 0.14 a	0.029 ± 0.15 a	0.31 ± 0.13 b	<2.0	<5.0
Cu (mg/l)	0.61 ± 0.012 b	0.60 ± 0.014 b	0.04 ± 0.003 a	0.04 ± 0.002 a	0.57 ± 0.012 b	<1.0	<0.2

a, b, c Each value is mean ± SD. Values in the same row with the same letter are not significantly different as determined by Duncan's test ($P < 0.05$).

(*) Abbreviations: COD: Chemical Oxygen Demand, DO: Dissolved Oxygen, TDS: total dissolved solids, EC: electrical conductivity . NG: Not given

(**) (QCVN 08-MT, 2015): National Technical Regulation on Surface Water Quality, Vietnam. Excepted for TDS, that referenced from (QCVN 01-BYT, 2009): National technical regulation on drinking water quality, Vietnam.

(***) (World Health Organization, 2006): A compendium of standards for wastewater reuse in the Eastern Mediterranean Region from WHO, for Jordanian Standard.

(T3) produced a dramatically better remediation of aquaculture wastewater (such as COD (removed 73%), NH_4^+-N (removed 96%), NO_3^-–N (removed 80%), and PO_4^{3-}–P (removed 81%)) than other FTWs having only plant (T2) or bacterial inoculation (T4). These might due to (1) the function of inoculated bacteria in degrading the pollutants in the effluent improving the plant growth (Shehzadi et al. 2014; Ijaz et al. 2015); and (2) the plant generated an optimal microenvironment around their roots beneficial for the microbial activities in thriving and removing the pollutants (Van de Moortel 2008; Ijaz et al. 2015).

In this experiment, the water temperature changed from 25.3 ± 0.12 ºC to 29.25 ± 0.05 ºC and from 25.4 ± 0.03 ºC to 29.35 ± 0.06 ºC for the control and treatments, respectively. It is well known that temperature plays a role in the solubility of many chemical compounds in water such as ammonia hence affecting aquatic lives. This temperature is suitable for plant growth.

Another indicator is the pH value which was fluctuated during the experiments. Notably, the pH in the FTWs with plants was slightly decreased (from 6.8 ± 0.8 to 6.4 ± 0.6 in T2 and from 6.8 ± 0.5 to 6.1 ± 0.4 in T3) than the one (around 6.8 ± 0.6) in the FTWs without plants (Table 1 and Fig. 2). This pH range is within the limits required for conserving aquatic ecology and for animals (QCVN 08-MT 2015; World Health Organization 2006). This pH reduction could be the result of (1) acidic exudates released from root (Lynchet et al. 2015; Ijaz et al. 2015) and/or (2) carbon dioxide (CO2) generated during roots respiration (Ijaz et al. 2015; Yasin et al. 2021), and/or (3) organic acids produced by the microbial metabolism (Trung et al. 2021). Moreover, a similar trend was noticed for the reduction of EC parameter in all treatments making the effluent safe for use in irrigation (Fig. 2). This EC reduction could due to the degradation and/or production of inorganic and organic compounds in the effluent (Ijaz et al. 2015). For the total dissolved solids (TDS), as can be seen in Table 1, the average concentration in T2 (247 ± 4.37) and T3 (246 ± 8.14) treatments presented a significant decrease compared to the control (T1) and with bacteria only (441 ± 4.51). These TDS values were in the range of National Standards (QCVN 08-MT 2015; QCVN 01 – BYT 2009) and WHO (2006). Also presented in Table 1, the Cu and Fe concentration showed a decreased trend after 14 days from 0.52 to 0.029 mg/l (95.3%) and 0.61 to 0.04 mg/l (93.4%), respectively.

Moreover, the results also presented the amount of the dissolved oxygen concentration (DO) of all experiments on the first day was around 4.55 ± 0.07 mg/l, which decreased in next day before steadily increased in the day after (Fig. 2). The interesting phenomenon was the measured OD values in vegetated treatments were significantly higher than OD value determined in the unvegetated treatments. This is not consistent with previous results, in which the OD in the unvegetated treatments was higher than in the vegetated treatments (Iamchaturapatr et al. 2007). This difference can be explained by the difference in the experiment designed in this study. Here, we also covered the unvegetated treatments with the floating mat that blocked the direct contact of the oxy in the air and the sunlight with the water preventing the photosynthetic activity (possibly, the algae) in the unvegetated treatment. On the other side, the vegetation of plants of the growing FTW system could have increased the diffused oxygen in the rhizosphere through the plant roots increasing the OD value (Ijaz et al. 2015; Rehman et al. 2019).

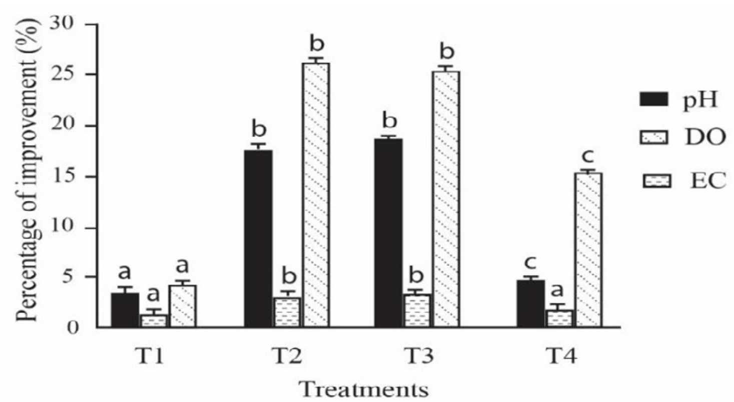

Fig. 2 Percentage improvement of pH, EC, and DO of shrimp aquaculture wastewater after two weeks with different treatments. (T1) sewage wastewater without vegetation and bacterial inoculation, (T2) sewage wastewater with vegetation only, (T3) sewage wastewater with vegetation and bacterial inoculation, (T4) sewage wastewater with bacterial inoculation only. Each value is mean of three replicates. Different letters indicate statistically significant differences between treatments at a 5% level of significance.

It was reported the oxygen concentration in the effluent was a key role in reducing COD in the aquatic system (Vymazal 2010). In this study, the FTWs vegetated with *B. mutica* increased the OD value in the effluent (Fig. 2) and consequently enhanced the degradation of organic pollutants (Fig. 3). In the association with inoculated endophytic bacteria, the treatments vegetated with *B. mutica* presented the highest efficiency of COD reduction (about 73%). This result was in agreement with the report of Sun et al. (2009), in which bacterially assisted Canna-carrying FTWs significantly improved the COD removal efficiency. This might be the inoculated bacteria functioned in transforming and decomposing the organic compounds (Vymazal 2010). For example, the endophytes combined with the plant (*Brachiaria mutica*) presented a dramatical removal of organic compounds such as oil and grease (more than 53%) (Ijaz et al. 2015); or two plant species (*Brachiaria mutica* and *Leptochloa fusca*) associated with *Acinetobacter* sp. greatly enhanced the removal of sodium dodecyl sulphate (97.5%) in wastewater (Yasin et al. 2021). These results suggest the promising application of FTWs in association with endophytic bacteria to reduce COD in the aquatic system.

In terms of nutrient removal, the results presented a significant removal of NO_3^--N, NH_4^+-N, PO_4^{3-}-P in the vegetated treatments compared to the unvegetated treatments. Especially, as can be seen from Fig. 3, the highest removal efficiency of nutrients in the effluent was observed at the T3 experiment among others suggesting the plant–endophyte synergism is the key factor in the elimination of eutrophication in shrimp wastewaters. This was supported by the study of Sun et al. (2009), in which the removal efficiency of total nitrogen in 5 days increased from 50.4% (used only the *canna indica* vegetated floating bed) to 72.1% (used the *canna indica* vegetated floating bed in combination with bacterial inoculation). In this experiment, the removal efficiency of NO_3^--N, NH_4^+-N was increased from 54.63% and 80.15% to 79.79% and 96.49%, respectively, for vegetated FTWs with and without bacterial inoculation. Moreover, a similar trend was observed for PO_4^{3-}-P reduction, which was significantly higher in the vegetated FTWs inoculated with bacteria than the vegetated FTWs only. These results indicated the nutrients (NO_3^--N, NH_4^+-N, PO_4^{3-}-P) could be removed from the water through natural uptake of plant for nutrients and/or natural metabolism of the plant and associated microbes such as ammonium oxidation, nitrification (Iamchaturapatr et al. 2007; Mohd Nizam et al. 2020).

The determination of the inoculated endophytic bacteria in FTWs

It was reported that the degradation of organic pollutant in phytoremediation strongly depend on the plant-bacteria synergism and correlates with a number of contaminants-degrading bacterial communities in their

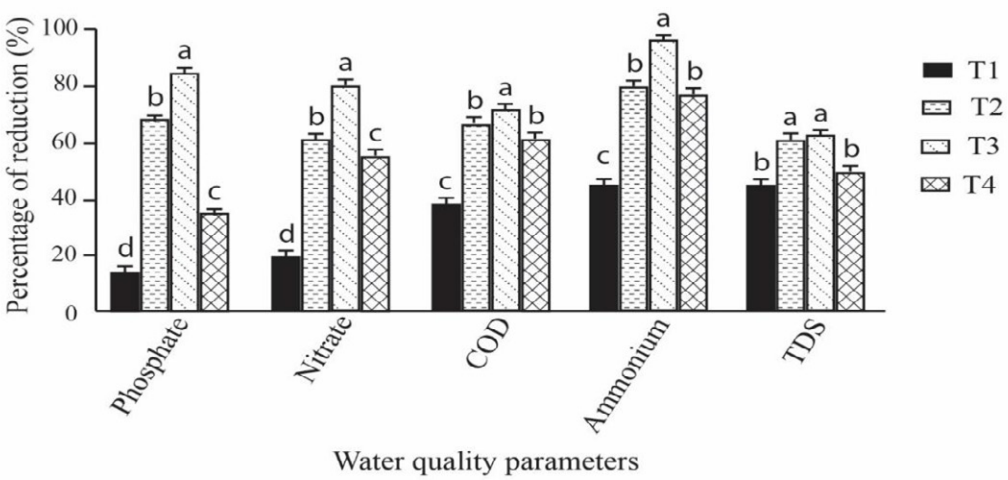

Fig. 3 Percentage removal of pollutants in shrimp farm effluent treated in two weeks in different treatments. (T1) sewage wastewater without vegetation and bacterial inoculation, (T2) sewage wastewater with vegetation only, (T3) sewage wastewater with vegetation and bacterial inoculation, (T4) sewage wastewater with bacterial inoculation only. Each value is mean of three replicates. Different letters indicate statistically significant differences between treatments at a 5% level of significance.

Table 2 Identification of the inoculated endophytic bacteria in the water, root interior, and shoot interior of *Brachiaria mutica* vegetated in FTWs

Treatments*	Water sample ($x10^3$ CFU/ml)	Root sample ($x10^3$ CFU/g)	Shoot sample ($x10^3$ CFU/g)
T3	6.72 ± 2.32	13.21 ± 2.17	7.64 ± 2.18
T4	4.24 ± 1.51	-	-

* (T3) sewage wastewater with vegetation and bacterial inoculation, (T4) sewage wastewater with bacterial inoculation only. Each value is mean of three replicates ± SD.

environment (Ijaz et al. 2015; Tara et al. 2019). In this study, the inoculated endophytes were observed not only in the water but also in the root, shoot of the vegetated *B. mutica* (Table 2). This could be because the inoculated bacteria are the endophytic bacteria that presented salt-tolerance and multiple host infection (Trung et al. 2021).

Moreover, the results also showed a relatively higher number of inoculated bacteria in the water sample collected from vegetated treatment (T3) than that in the unvegetated treatment (T4). This could be the plants produced optimal microenvironment surrounding their root for the growth of the inoculated and indigenous microbes, subsequently increased number of inoculated endophytes in T3 (Ijaz et al. 2015; Tara et al. 2019). Interestingly, the screening experiment also showed significantly higher numbers of the inoculated bacteria in the root interior ($13.21x10^3$ CFU/g) than that of shoot interior ($7.64x10^3$ CFU/g). This is consistent with the result of Fatima et al. (2015), in which the inoculated bacteria preferred the root interior to the shoot interior.

Conclusions

This study was carried out to strengthen the potential application of FTWs for a low-cost and feasible method of reducing pollutants of shrimp aquaculture effluents. The results showed that FTWs vegetated with *B. mutica* presented a significant removal of pollutants in the shrimp aquaculture effluents, especially in the combination with the bacterial inoculation the removal efficiency was even higher. In addition, the results also presented bacterial colonization of the inoculated endophytes in root and shoot of *B. mutica* that might produce the plant-bacteria interaction to enhance the removal of organic and inorganic pollutants and excess nutrients in the shrimp aquaculture effluents. These data suggest the application of endophytic bacteria in FTWs vegetated with *B. mutica* to remediate the pollutants in the shrimp aquaculture effluents.

Abbreviation COD, chemical oxygen demand; CFU, colony forming unit; DO, dissolved oxygen; FTW, floating treatment wetland; LB, Luria-Bertani; TDS: total dissolved solids, EC: electrical conductivity

Competing interests The authors declare that they have no competing interests.

Author's contribution DQT and LTA conceptualized and supervised the project, designed experiments, analysed data, and prepared original manuscript draft. TTTT activated endophytic bacteria, carried out greenhouse experiments, analyzed data. NTT was involved in final interpretation of data, and editing of the manuscript. DVV was involved in revised Tables in manuscript, and prepared the manusript. All authors have read and approved the manuscript.

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