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Immobilization of microorganisms in activated zeolite beads and alkaline pretreated straws for ammonium-nitrogen removal from urban river water

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ABSTRACT

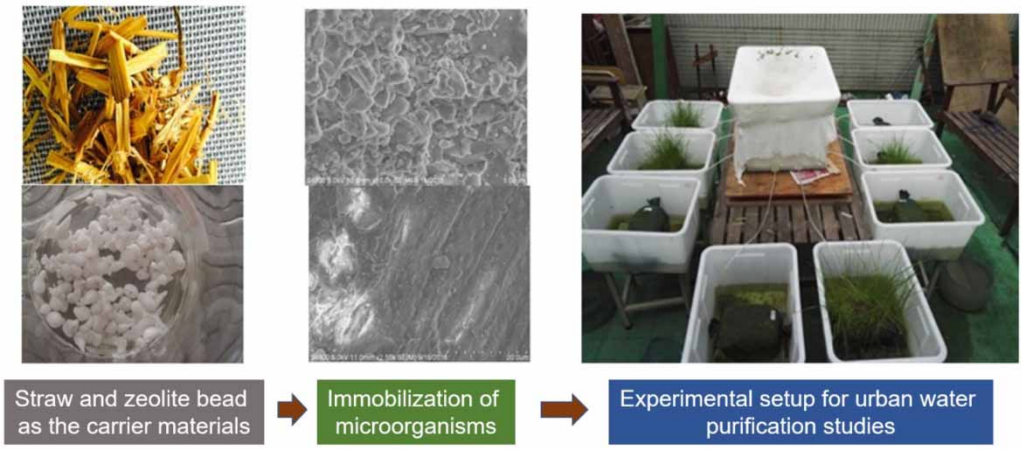
The non-treated wastewater from residential areas contains high concentrations of ammonium-nitrogen (NH_4^+-N). When discharged into the drainage water system, it deteriorates the water quality in urban rivers. This study used two types of materials to form eco-bags, using activated zeolite bead (AZB) and alkaline pretreated straw (APS), in geotextile bags for easy recovery and reuse. The AZB and APS provided the breeding habitat for the microorganisms that promoted biofilm formation on their surface. The immobilization of engineered denitrification microorganisms facilitated the removal of NH_4^+-N from the urban river water. The NH_4^+-N removal in the AZB and APS bags were in the range of 64–73%, and 56–61%, respectively, while the chemical oxygen demand (COD) removal in the AZB and APS bags ranged from 33–36%, and 30–31%, respectively. In addition, as evident from DNA and microbial community analysis, the microorganisms demonstrated a greater proclivity to grow and proliferate on the surface of AZB and APS and improved the water quality of urban rivers.

Key words: activated zeolite bead, alkaline pretreated straw, ammonium-nitrogen removal, COD removal, microbial immobilization

HIGHLIGHTS

- Activated zeolite bead (AZB) and alkaline pretreated straw (APS) were used for the immobilization of microorganism.
- Eco-bags prepared using AZB and APS in geotextile bags facilitated easy recovery and reuse.
- Engineered denitrification bacteria were used to improve the NH_4^+-N removal capacity.
- Microorganism diversity was investigated to substantiate the water quality improvement ability of AZB and APS.

GRAPHICAL ABSTRACT



INTRODUCTION

Due to an increase in population and economic activity, the amount of wastewater generated in urban residential areas is increasing at an alarming rate (Xu *et al.* 2019). In addition, in some developing countries (e.g., China and Vietnam), the existing water drainage system networks and the number of wastewater treatment plants are insufficient to meet the current demand; therefore, the untreated domestic wastewater is discharged directly into the rivers without treatment (Xu *et al.* 2019). The ammonium-nitrogen (NH_4^+-N) content of urban wastewater is high, and this has a direct impact on the survival of aquatic plants and animals in river water as well as on human health (Khalil *et al.* 2018). In order to reduce and control the amount of NH_4^+-N present in urban rivers, various techniques and technologies, including physicochemical, biological, and ecological engineering have been implemented. Typical river water purification examples include artificial aeration, mixed microbial remediation, phytoremediation, ecological floating beds, and constructed wetlands (Liu *et al.* 2016; Ling *et al.* 2017; Gao *et al.* 2018; Bai *et al.* 2020).

Gao *et al.* (2018) reported that releasing the HP-RPe-3 microbial consortium into the water and sediment layer (Chengnan river, China) resulted in NH_4^+-N and COD removal efficiencies of 20 and 38%, respectively. Liu *et al.* (2016) investigated the dynamics of dissolved oxygen (DO) profiles and the factors affecting sediment quality of polluted urban rivers under aeration conditions. It was observed from that study that the correlation between DO and NH_4^+-N removal of overlaying river water was 0.90 and 0.41, respectively. In a study conducted by Ling *et al.* (2017), the NH_4^+-N removal using several landscape plant species were determined to be 71% for *Scindapsus aureus*, 74% for water hyacinth, 67% for cockscomb, and 59% for *Salvia splendens*. In another recent study, the NH_4^+-N removal using sequential constructed wetland in Yitong river (Changchun, Jiangxi Province, China) was 80.9% (Bai *et al.* 2020). These studies clearly demonstrate that the river water quality can be enhanced using ecological technologies (Ling *et al.* 2017; Bai *et al.* 2020) and the use of artificial aeration (physical method) together with the addition of mixed microbial culture (biological method) has demonstrated significant improvement in the quality of the river water (Liu *et al.* 2016; Gao *et al.* 2018).

These methods, conversely, have only a temporary effect on water quality improvement and such improvements might only last for a short period of time. In practice, however, the survival and development of plants and microorganisms are influenced by environmental factors such as temperature, hydraulic condition of the river, and the physico-chemical and biological characteristics have a direct impact on the purification of rivers. As a result, these methods are unable to function in extreme weather conditions such as storms, droughts, and extreme cold. Constructed wetlands and floating beds are expensive to build and maintain; therefore, the required financial resources for their construction, maintenance, and replacement are not affordable in some countries due to economic reasons.

The immobilized microbial technology, i.e. the immobilization of microorganism in a suitable carrier material/matrix, is a biological method that is low in cost and has been shown to improve the efficiency of NH_4^+-N removal from wastewater by up to 50%. A recent study achieved nitrification rates of ~0.0024 mg NH_4^+-N/mg mixed liquor volatile suspended solids/h

(Shin *et al.* 2019) using microorganisms immobilized in a media composed of polyvinyl alcohol (PVA) and polyethylene. Donga *et al.* (2017) used PVA and sodium alginate for the immobilization of ammonia-oxidizing bacteria, and the NH_4^+-N removal efficiency was 90.3%. The use of immobilized microorganisms in a sequencing batch reactor resulted in an NH_4^+-N removal efficiency of 67.6% (Yang *et al.* 2020). Thus, most of the previous studies have focused on the use of expensive chemicals such as polyvinyl alcohol (PVA), zeolite, and sodium alginate to prepare the immobilized microbial beads for the removal of NH_4^+-N from wastewater (Donga *et al.* 2017; Yang *et al.* 2020).

From a practical view point, when these beads are added/released into the flowing river water, they contribute to water pollution and ecological degradation for the duration of the period in which they are decomposing. Some of the methods previously employed include the use of synthetic and inert materials with high absorption capacities; nevertheless, such treatments would increase the cost of water restoration projects. Hence, a thorough feasibility and comparative analysis between natural and artificial types of immobilized for NH_4^+-N removal should be carried out in laboratory/pilot scale experiments before implementing them in real river environments.

This study evaluates the application of natural (alkaline pretreated straw, APS) and artificial (activated zeolite bead, AZB) materials for the immobilization of microorganisms and ascertain the NH_4^+-N and COD removal efficiency from polluted river water. In the conventional approach, the microorganism-immobilized beads are released directly into the river bed at the bottom of the river bed (Wang *et al.* 2008), which has the potential to have a negative impact on the river water quality. To overcome this situation, eco-bags made of geotextiles containing AZB or APS were used in this study because the bags can be more easily recovered and repurposed than traditional trash bags. By analyzing the microbial community distribution and its characteristics, the mechanism of NH_4^+-N and COD removal by AZB and APS bags was determined.

MATERIALS AND METHODS

Preparation of the immobilized microorganisms

Rice straws were cut into pieces of approximately 1 and 2 cm in length and soaked in a 2% NaOH solution for 30 min. A total of 24 hours had passed before the samples were removed and washed repeatedly with distilled water until the pH level was close to neutral. Later, the straw pieces were dried at 35 °C in a dryer, resulting in the formation of APS (Figure 1(a)). Conversely, the AZBs (Figure 1(b)) were made from a mixture of polyvinyl alcohol (PVA), sodium alginate, calcium carbonate, silica, calcium chloride, boric acid, and zeolite powder, according to a protocol previously described in the literature (Xiao *et al.* 2011).

A 1:1 mixture of PVA and water was prepared and dissolved in a water bath at 85 °C for 24 hours to form the PVA gels, which were then dried. After that, the gelling agents, such as sodium alginate, calcium carbonate, silicon dioxide, and zeolite powder were added (Table 1) and the contents were allowed to cool for 4 h.

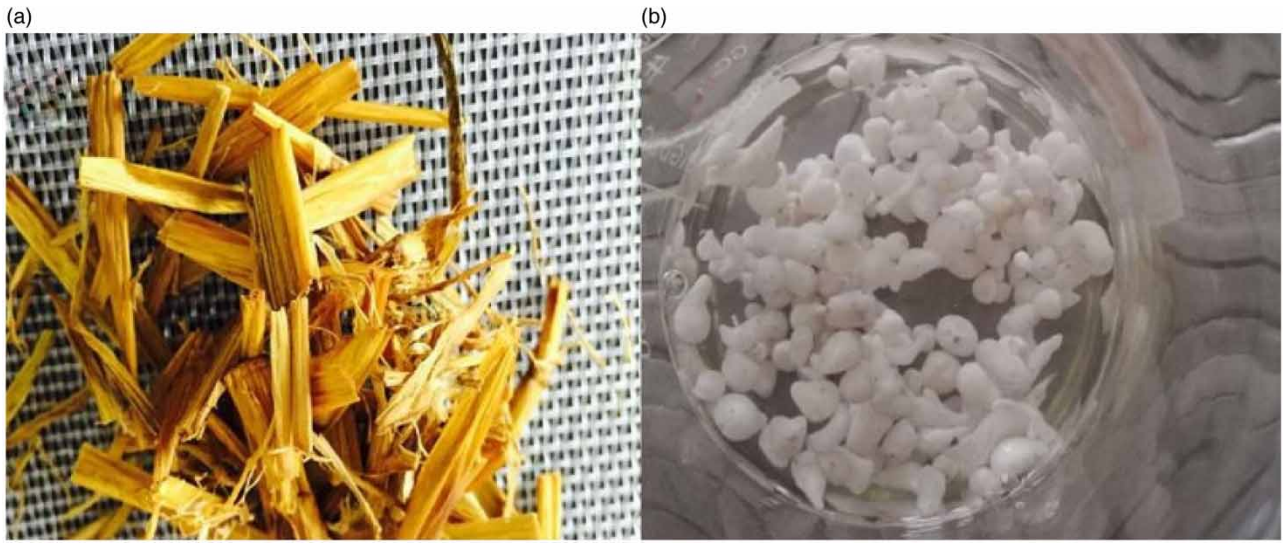


Figure 1 | Production of (a) alkaline pretreated straws; and (b) activated zeolite beads.

Table 1 | The composition of chemicals used in the gel, additive and cross-linking agents for preparing the immobilized beads

Gel agent		Additive agents		Cross-linking agent		
PVA	Sodium alginate	$CaCO_3$	SiO_2	$CaCl_2$	H_3BO_4	Zeolite powder
6 g	0.5 g	0.5 g	3.5 g	4 g	7.5 g	4 g

Thereafter, the PVA gel was added dropwise using a peristaltic pump to a solution containing 4% calcium chloride, boric acid and water (pH 7.5) which served as the cross-linking agent. The resultant mixture was stirred well using a magnetic stirrer and beads with diameters ranging from 2 to 5 mm were obtained. The beads were washed three times with saline solution before being used. Four APS bags, four AZB bags, and two sand bags were placed in ten plastic containers ($75 \times 55 \times 45$ cm^3), labeled as 1# to 10# (Table 2).

For 10 days, the bags were placed in the container and the engineered microorganisms and the denitrifiers were used to improve microorganism immobilization on the surface of APS and AZB. The engineered consortia contain 95–99% of *Leuconostoc*, *Lactobacillus*, *Pseudomonas*, *Alcaligenes*, and *Methylobacillus*, based on the technology developed by Shangde Biological Technology Co., Ltd (Danyang, Jiangsu, China). *Pseudoxanthomonas*, *Bacillus*, *Limnohabitans*, *Thermomonas*, *Pseudomonas*, and *Hydrogenophaga* genes made up 87–93% of the denitrification consortia (Table 3).

For 3–4 days, four bags 1–4# were immersed in the denitrification microbial agent, while four bags 5#–8# were immersed in the engineered microbial agent, respectively. The engineered microorganism's media composition includes soluble starch (5 g), peptone (5 g), glucose (10 g), yeast extract (5 g), magnesium sulfate (0.5 g), potassium dihydrogen phosphate (1 g), potassium nitrate (1 g), ferrous sulfate (0.01%), and calcium chloride (0.01%) in 1 L distilled water. In 1 L of deionized water, a similar denitrification culture medium contained 1 g $(NH_4)_2SO_4$, 2 g KNO_3, 63 mg potassium chloride, 6 g sodium citrate ($C_6H_5Na_3O_7{\cdot}2H_2O$), 1 g K_2HPO_4, and 0.2 g magnesium sulfate heptahydrate (distilled). Following preparation, the denitrification culture medium was sealed with clean gauze and sterilized in a high-pressure steam boiler at 121 °C for 20 min before cooling to room temperature for storage (25–28 °C). After the microorganism immobilization process was completed, the ten bags were placed in the device to evaluate their water purification ability.

Determination of microbial populations

In order to determine the microbial diversity in the biofilms, the biofilm samples were collected from AZB plant bags, APS bags, APS plant group, and blank control sand bags (9#). DNA sequencing was performed on four biofilm samples using next-the generation sequencing (NGS) technology (Ge & Yu 2017). It consisted primarily of three steps: (1) target fragment

Table 2 | Microbial communities present in the different microbial agents and their process-based functions

Genera	Functions
Engineered microbial agents	
Leuconostoc	Organic compound degradation
Lactobacillus	Denitrification
Pseudomonas	Aerobic denitrification, heterotrophic nitrification
Alcaligenes	Aerobic denitrification, heterotrophic nitrification
Methylobacillus	Denitrification
Denitrification microbial agents	
Pseudoxanthomonas	Denitrification
Limnohabitans	Nitrification
Bacillus	Aerobic denitrification, heterotrophic nitrification
Thermomonas	Denitrification
Pseudomonas	Aerobic denitrification, heterotrophic nitrification
Hydrogenophaga	Denitrification

Table 3 | Different sets of experiments with the immobilized microorganisms and their conditions

Number	Microorganisms coupled strains	Fill	Planting	Group
1#	Denitrification bacteria	Activated zeolite beads	No	A
2#	Denitrification bacteria	Activated zeolite beads	Yes	A
3#	Denitrification bacteria	Alkali-treated straw	No	B
4#	Denitrification bacteria	Alkali-treated straw	Yes	B
5#	Engineered bacteria	Activated zeolite beads	No	C
6#	Engineered bacteria	Activated zeolite beads	Yes	C
7#	Engineered bacteria	Alkali-treated straw	No	D
8#	Engineered bacteria	Alkali-treated straw	Yes	D
9#	No	Sand	No	E
10#	No	Sand	Yes.	E

amplification via polymerase chain reaction (PCR), (2) Illumina high-throughput sequencing, and (3) biological information analysis. The biofilm samples were examined using an electron microscope (model XQC-II-1; Ningbo Gamry Optical Instrument Co., Ltd, Shanghai, China).

Determination of water quality

The fresh water for the experiment was collected from Southeast University, Nanjing, China. The pollutant concentrations in the water samples were measured in terms of the COD, NH_4^+-N, total nitrogen (TN), and total phosphorus (TP) that were in the range of 5.2–6.8, 1.55–2.13, 2.21–3.17, and 0.053–0.065 mg/L, respectively. The volume of fresh water in each experimental pot was set at 20 L (Figure 2).

The following chemicals were added to domestic wastewater in each pot to simulate the features of polluted river water: $C_6H_{12}O_6$–100 mg, NH_4Cl–200 mg, KNO_3–50 mg, and K_2HPO_4–10 mg. Three water samples were collected once every 2 days for a total of 20 days and analyzed in the laboratory. To keep the total volume of water in each pot constant, a similar amount of fresh water was added to each pot. The COD concentration was determined using the potassium permanganate method (GB11892-1989). A UV spectrophotometer (L1611002, Shanghai Jinghua Technology Instrument Co., Ltd, Shanghai, China) was used to measure the water quality parameters such as ammonium-nitrogen (HJ535-2009), TN and TP (HJ636-2012). All these analyses were done in accordance with the Standard Water Quality Determination procedure from the People's Republic of China.

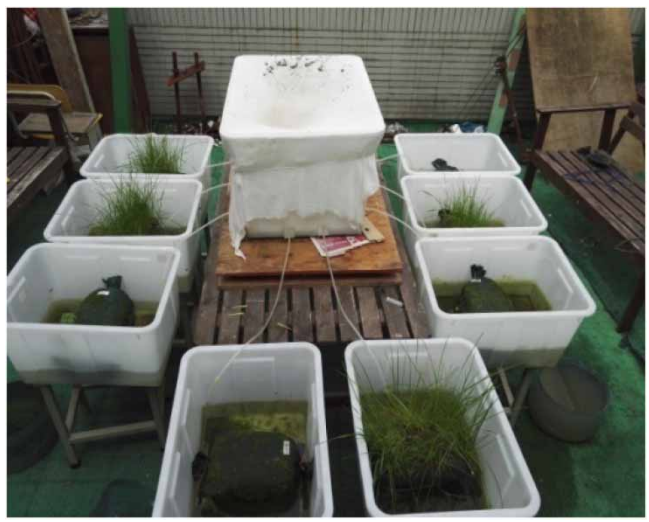


Figure 2 | Experimental containers for water quality improvement studies.

RESULTS AND DISCUSSION

Biofilm formation on APS and AZB

Microorganisms were able to colonize and form the biofilm on the surfaces of APS and AZB during the culture period as a result of this reproduction and breeding activity (Figure 3(b) and 3(d)). The roughness of the surface of APS and AZB (Figures 3(a) and 3(c)) is advantageous for bacterial adhesion (Engel *et al.* 2020). Engel *et al.* (2020) investigated biofilm formation on five different dental restorative materials using scanning electron microscopy and concluded that increasing roughness increases the surface area for bacterial attachment. The roughness of the APS and AZB surfaces may increase the exposed area, which may improve the NH_4^+-N adsorption ability (Faraj *et al.* 2020). Faraj *et al.* (2020) investigated the properties of sewage sludge, waterworks sludge, and composite sorbents, as well as their adsorption ability, and discovered that adsorption material's adsorbent efficiency can be increased by having a large exposed surface area. In addition, adsorption occurred on the APS and AZB surfaces, causing the suspended particles, nutrients and microorganisms to adhere to the surfaces.

It has been reported that the van der Waals forces, hydrogen bonding, electrostatic interactions, and pore filling, among other mechanisms, may play a role in zeolite and straw adsorption on a variety of surfaces (Arbuznikov *et al.* 1998; Suo *et al.* 2019). The APS and AZB both have numerous pores, which could allow a significant amount of nutrients to accumulate in the carriers' internal pores (Xue *et al.* 2017; Cahyono 2018; Khalil *et al.* 2018). According to Wu *et al.* (2019) and Cahyono (2018), the pore size of zeolite (as an adsorbent) ranges from 2 to 50 nm. Furthermore, the average APS pore size was ascertained to be between 7.89 and 8.16 nm. It is possible that the abundance of nutrients on the APS and AZB surfaces will promote the growth of microorganisms. According to Khalil *et al.* (2018), the maximum NH_4^+ adsorption by APS (at pH = 7.5) was 2.9, 3.5, and 4.5 mg/g, respectively, at temperatures of 25 °C, 35 °C, and 45 °C, respectively, while the maximum $NH4^+$ adsorption by APS (at pH = 7.5) was 2.9, 3.5, and 4.5 mg/g at temperatures of 25 °C, 35 °C, and 45 °C, respectively.

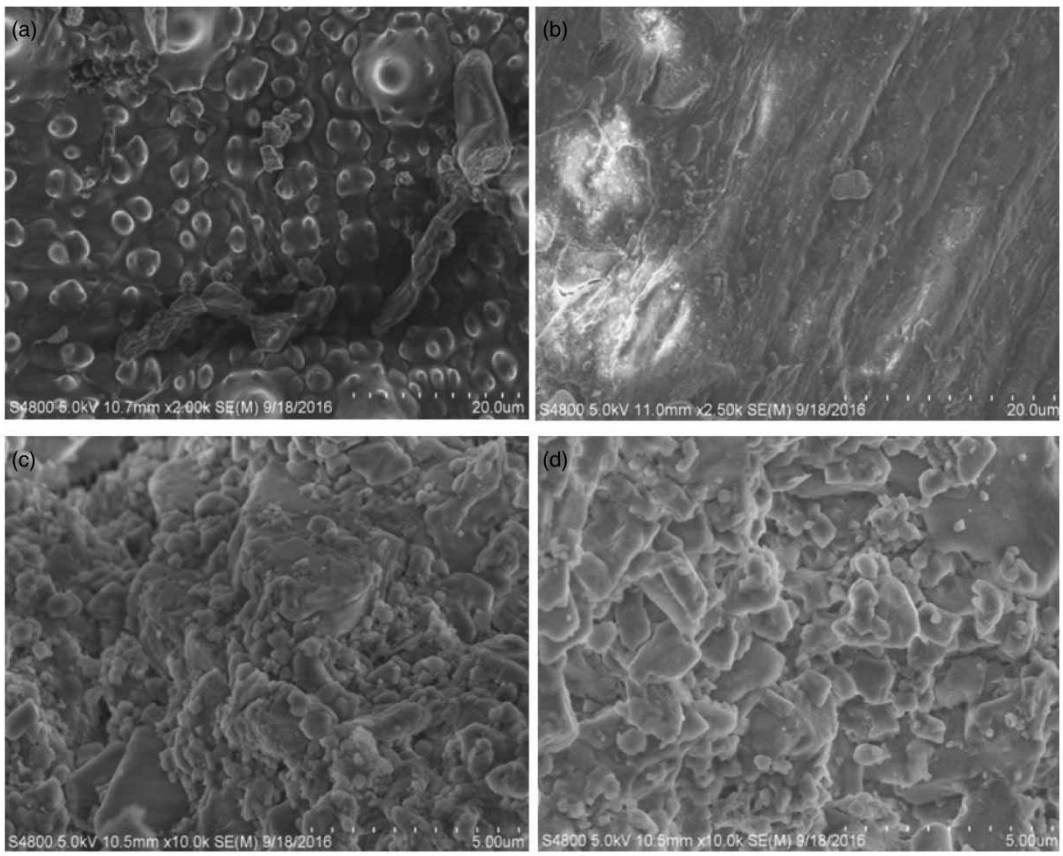


Figure 3 | The surface of the alkaline pretreated straws before (a) and after (b) biofilm formation; and the surface of activated zeolite beads before (c) and after (d) biofilm formation (Photograph was made using an electron microscope.)

In addition, as high-density nutrients were available on the APS and AZB surfaces, the bacteria actively used their flagella and pili to move forward to the surfaces (Kanematsu & Barry 2015). The bacteria used cell adhesion structures such as pili to attach to and remain on the APS and AZB surfaces.

Bacteria-secreted extracellular polymeric substances (EPS) play a major role during their long-term adhesion to the APS and AZB surfaces (Kanematsu & Barry 2015; Voběrková *et al.* 2016). The EPS, according to Voběrková *et al.* (2016), are responsible for both adhesion and cohesion interactions, while proteins serve as a carbon and energy source, and extracellular DNA is responsible for the formation of biofilm. As seen from Figure 3, stable biofilm formation was observed as a result of the attachment of free-floating microorganisms and nutrients to the APS and AZB surfaces.

COD removal

The average COD removal (%) using AZB and APS bags in A, B, C, D and E groups is shown in Table 4.

The average COD removal efficiencies in the A, B, C, D, and E groups were 33 and 36%, respectively, whereas they were 29 and 35% in the B group. In the C group, they were 30 and 31%, respectively. In the D group, they were 31 and 32%, respectively (Figure 4).

Leuconostoc aided in the degradation and absorption of organic wastes (Cogan & Jordan 1994). According to the authors, *Leuconostoc* spp. are capable of transporting and metabolizing glucose to carboxylic acid, ethanol, and carbon dioxide. Nitrifying (*Limnohabitans*) and denitrifying bacteria (*Lactobacillus, Methylobacillus, Pseudoxanthomonas, Thermomonas, Hydrogenophaga*), as well as the aerobic denitrifying/heterotrophic microorganisms (*Pseudomonas, Alcaligenes* and *Bacillus*) have contributed significantly to remove a wide variety of pollutants and enhance the water quality (Cogan & Jordan 1994; Rajta *et al.* 2019; Baskaran *et al.* 2020). Baskaran *et al.* (2020) showed that heterotrophic nitrifying bacteria could grow on organic carbon and create hydroxylamine, nitrite, and nitrate as byproducts from the nitrification reaction. According to Rajta *et al.* (2019), heterotrophic denitrifying bacteria use organic carbon as a source of growth and an electron donor throughout the denitrification process. Through organic compound breakdown, nitrification, and denitrification processes, the microorganisms in the biofilm contribute to the reduction of nutrient and organic concentrations, thereby removing COD from the river water.

The COD removal efficiencies were greater in groups A, B, C, and D (29–36%) than in group E (28%) (25–32%). Organic substances were used by heterotrophic bacteria present in APS (A, C) and AZB (B, D) biofilms for cell growth and energy production. The COD removal by microorganisms such as *Aeromonas* sp., *Pseudomonas* sp., and *Bacillus* sp. were in the range of 77% to 93% after a 12-day incubation period (Oljira *et al.* 2018). It has been established that microorganisms growing on sand particles used in a biofilter can remove up to 50% of the COD from a water source (Hua *et al.* 2003). However, in this study, it was noticed that the sand bags were unsuccessful as a biofilter and had a reduced COD removal efficiency. The COD removal was greater in the integrated *L. perenne* grass sand bag 10# (32%) than in sand bag 9# (25%). *L. perenne* grass planted in a built wetland has been demonstrated to absorb soluble nutrients straight from the water, with COD removal efficiencies

Table 4 | Effect of AZB and APS bags on the water purification capacity for COD, NH_4^+-N and TN

		COD		NH_4^+-N		TN	
		11.1 ± 0.8 (mg/L)		10.6 ± 0.7 (mg/L)		13.7 ± 0.6 (mg/L)	
Group		Average (%)	Stdev (%)	Average (%)	Stdev (%)	Average (%)	Stdev (%)
A	1#	33	4	64	9	59	8
	2#	36	5	73	9	62	6
B	3#	30	5	62	8	61	8
	4#	35	3	76	8	65	6
C	5#	31	4	56	7	49	6
	6#	30	3	61	8	53	7
D	7#	32	2	60	8	54	7
	8#	31	5	61	8	56	7
E	9#	25	3	48	5	43	6
	10#	32	2	57	5	52	5

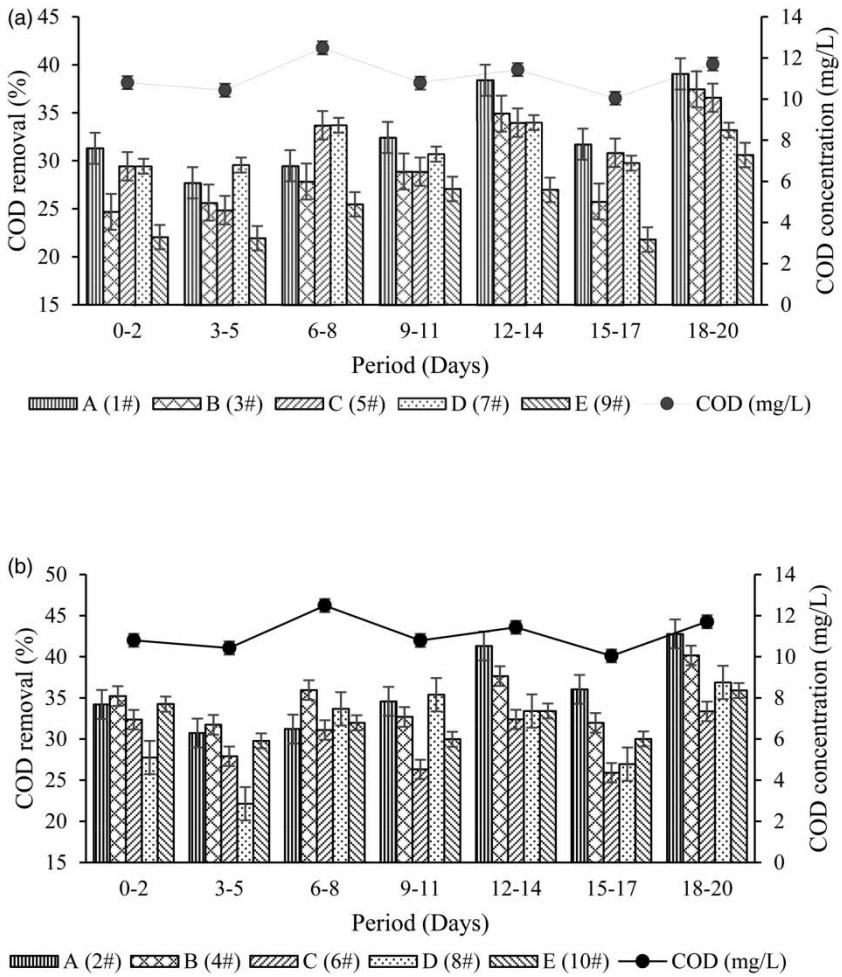


Figure 4 | COD removal profiles: (a) without plant, and (b) with plant.

ranging from 31 to 56% at a temperature of 10 °C (Ren *et al.* 2016). The results of this study established that microorganism immobilization on APS and AZB was important and practically applicable for COD removal from water.

Elimination of ammonium and nitrogen

The average removal of NH_4^+-N in the A, B, C, D, and E groups was between 64 and 73%, 62 and 76%, 56 and 61%, 60 and 61%, and 48 and 57%, and the average removal of NH_4^+-N in the A, B, C, D, and E groups was between 64 and 73%, 62 and 76%, respectively (Figure 5). The average TN removed in the A, B, C, D, and E groups was 59–62%, 61–65%, 49–53%, 54–56%, 43–52%, and 43–52%, respectively (Figure 6). The elimination of NH_4^+-N was significantly greater in groups A, B, C, and D (56 and 73%, respectively) than in group E (48 and 57%).

Nitrification and denitrification are two processes by which bacteria removes nitrogen from aqueous environments (Zhang *et al.* 2016, 2020). The ammonium ion (NH_4^+-N) is transformed to nitrite during the nitrification process by *Nitrosomonas* bacteria, which is then turned to nitrate by the nitrification bacteria (*Limnohabitans*) (Baskaran *et al.* 2020). Baskaran *et al.* (2020) created an effective microbial culture for the treatment of nitrogenous wastes from shrimp cultivation and showed that *Nitrosomonas*, *Nitrosopumilus*, and *Limnohabitans* all play a significant part in the oxidation of ammonium-nitrogen to nitrite ($2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 4H^+ + 2H_2O$).

The most important mechanism for nitrogen removal during denitrification is the conversion of nitrate or nitrite to gases (NO_3, NO_2, NO, N_2O, N_2), with nitrate serving as an electron acceptor for energy production during the process (Zhang *et al.* 2016). *Pseudomonas*, *Alcaligenes*, and *Bacillus* are all capable of aerobic denitrification. They can decrease nitrate, nitrite, nitric oxide and nitrous oxide using both oxygen and nitrate as the oxidizing agents (electron acceptors) (Zhang *et al.* 2011; Khanichaidecha *et al.* 2019; Yang *et al.* 2020).

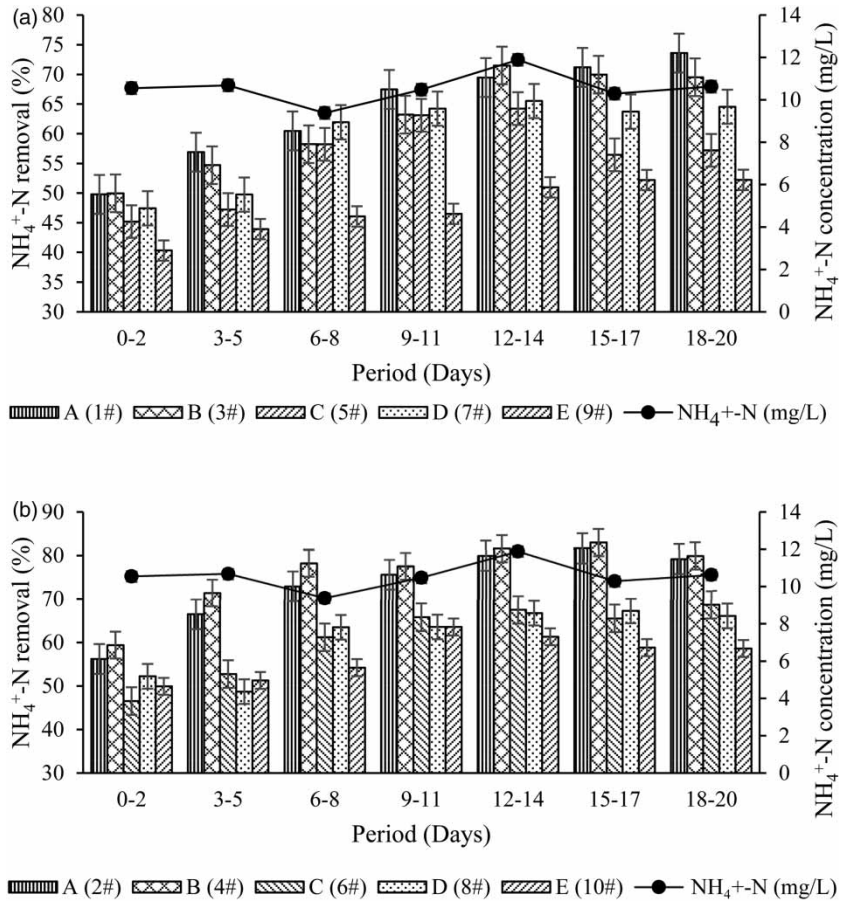


Figure 5 | NH_4^+-N removal profiles: (a) without plant, and (b) with plant.

The heterotrophic nitrification–aerobic denitrification strains *Pseudomonas* and *Bacillus* sp. are used for simultaneous nitrification and denitrification that convert ammonium-nitrogen to nitrogen gas (Zhang *et al.* 2011; Khanichaidecha *et al.* 2019). Khanichaidecha *et al.* (2019) reported nitrogen removal using a pure-culture of *Bacillus licheniformis* and discovered that NH_4^+-N could be removed from wastewater at a concentration of 30 mg/L, achieving a maximum removal of 73%. Zhang *et al.* (2011) examined the heterotrophic nitrifying ability of *Pseudomonas stutzeri* and reported a 39% conversion of NH_4^+-N to nitrogen gas over an 18-hour period. Denitrifiers were immobilized on AZB and APS, resulting in the creation of a denitrifier biofilm. The proliferation of microorganisms could have resulted in a rapid decline in the nitrogen concentration of river water (Zhang *et al.* 2020).

L. perenne absorbs nutrients during its growth and reproduction, which contributed significantly to the reduction of NH_4^+-N in the water (Ren *et al.* 2016). Conversely, the microorganisms growing on the *Rhizophora* decompose organic matter, fix nitrogen, and solubilize phosphorus, paving the path for easy nutrient absorption by the plants (Hassan *et al.* 2019). The consortia of denitrification microorganisms developed in APS and AZB bags removed more NH_4^+-N than the designed microorganisms. As a result, the application of denitrifiers for microbial immobilization on AZB and APS resulted in considerable NH_4^+-N removal. The average removal of NH_4^+-N by the eco-bag groups A and B (62 and 76%, respectively) was greater than the removal by eco-bag groups C and D (56 and 61%). The average removal of TN in the eco-bag's groups A and B (59 and 65%, respectively) was greater than in the eco-bag's groups C and D (49 and 56%). In general, the use of denitrification microbial inoculum resulted in a greater removal of ammonium-nitrogen and TN than the use of designed microbial inoculum.

Microbial growth on the eco-bags

As the application of a denitrification microbial inoculum resulted in significant removal of NH_4^+-N from water, a characterization of the microorganism community was performed in the AZB and APS. Four eco-bag samples were sequenced at

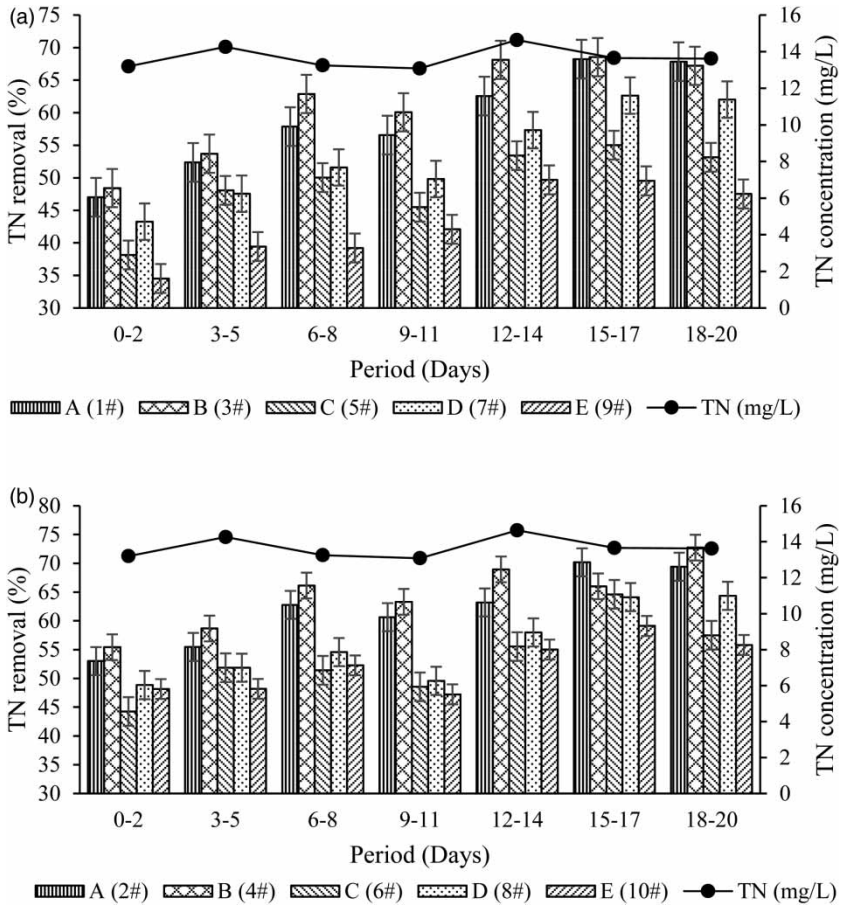


Figure 6 | TN removal profiles: (a) without plant, and (b) with plant.

Table 5 | The results of microbial biodiversity analysis (samples 2#, 5#, 6# and 10#)

Samples	Sample ID	No. of seqs	OTUs	PD of the whole tree	Chao	Shannon
2#	A1.1	35638	2932	210	3660.15	8.86
5#	A1.2	30165	2643	199	3330.18	8.92
6#	A1.3	42133	2753	201	3484.29	8.64
10#	A1.4	28512	2471	189	3224.67	8.47

high-throughput for the denitrifying bacteria–AZB plant group (2#), the denitrifying bacteria–APS group (5#), the denitrifying bacteria–APS plant group (6#), and the blank control group (10#), respectively. Table 5 and Figures 7 and 8 illustrate the results of the sequence information and diversity index.

According to the results of the Operational Taxonomic Units (OTUs) and Chao index, the total numbers of species in the samples were as follows: 2# > 6# > 5# > 10#. The results indicated that sample 2# (denitrification bacteria–AZB plant group) included a greater number of microorganisms than sample 6# (denitrification bacteria–APS plant group). The greater number of pores on the surface of AZB than on APS may allow for more microbial attachment and proliferation (Amer *et al.* 2017; Mukherjee *et al.* 2020). Mukherjee *et al.* (2020) found that, as the ageing duration extended from 24 to 72 hours, the surface area grew from 54 to 76 m^2/g and the pore volume increased from 0.04 to 0.13 cm^3/g. The APS could only support the growth of microbes on a small layer of surface straws. In comparison to APS, the very porous nature of AZB may allow bacteria to grow and multiply both on the surface and deep within the pores (Weiß *et al.* 2013; Mukherjee *et al.* 2020). The number of microorganisms in the eco-bags 2# and 6# was greater than the number of microorganisms in eco-bag 5# (denitrifying bacteria–APS group).

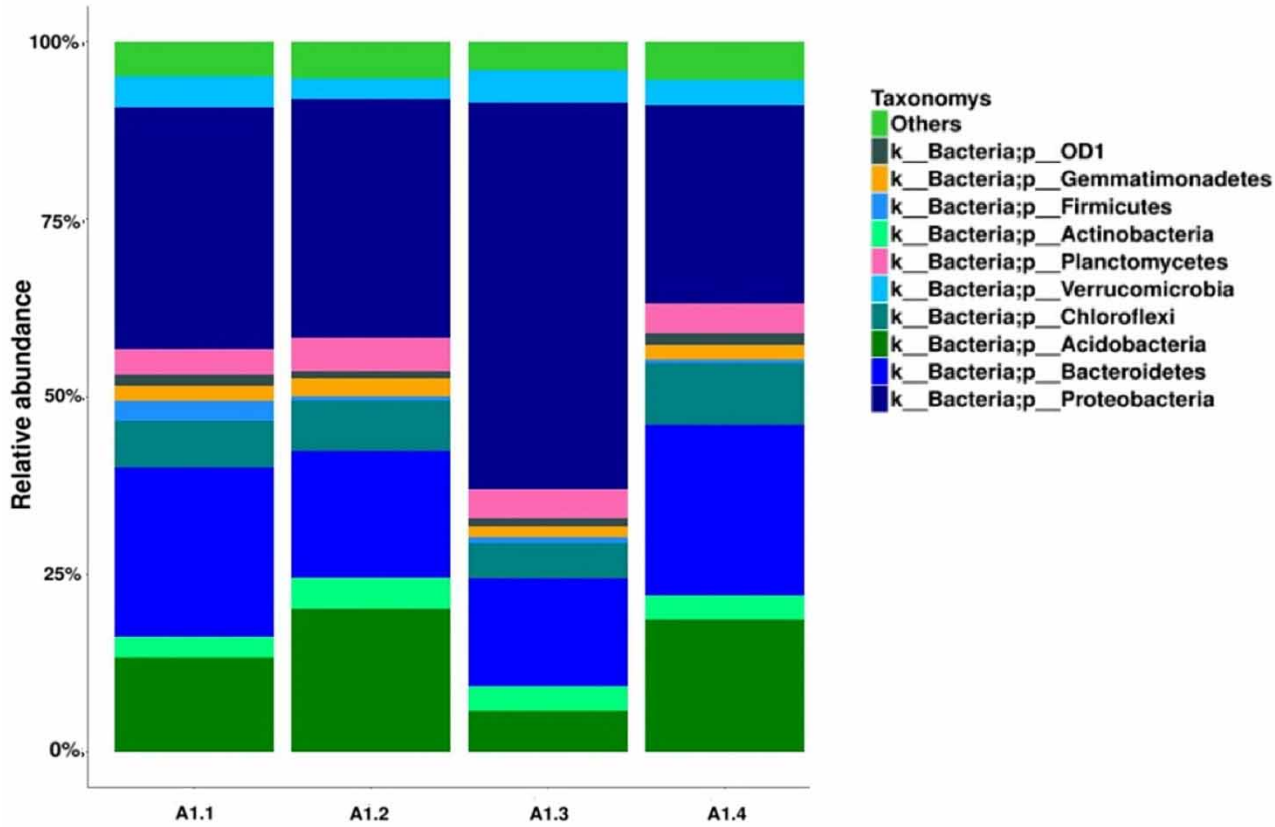


Figure 7 | The relative abundance at the phylum level in the four groups of samples.

Thus, while absorbing nutrients for growth, plant roots secrete a variety of low-molecular-weight (e.g. amino acids, organic acids, sugars, and phenolics) and high-molecular-weight (e.g. polysaccharides and proteins) organic compounds that serve as sources of essential nutrients for rhizosphere microbial growth. It has been demonstrated that intact root cells emit low-molecular-weight organic chemicals, whereas the root cap cells secrete high-molecular-weight organic compounds. These chemical substances found in the root system of plants aid in maintaining the activity of the microorganisms and the sloughing of detrimental microorganisms (Hassan *et al.* 2019). Thus, the integrated eco-bag groups 2# and 6# contained a greater quantity of microorganisms than the eco-bag group 5# without plants. Without the microbial inoculum, the sand bag 10# exhibited the lowest quantity of microorganisms. Quartz minerals dominated the silica sand particles, which have a trigonal crystal and hexagonal lattice structure (Imseeha *et al.* 2020). As a result, sand grains have a poorer ability to absorb nutrients and form biofilms than APS and AZB (Romaní & Sabater 2001; Weiß *et al.* 2013). Without the addition of the microbial inoculum, the microorganisms were derived primarily from those suspended in the water, which may have contributed to the delayed formation of biofilms in the sand bags (Romaní & Sabater 2001).

The Shannon index ranked the microbial community diversity from high to low as follows: 5# > 2# > 6# > 10# (Table 5). The microbial diversity index of the eco-bag groups 2#, 5#, and 6# was higher that of the sand bags 10# (control), indicating that the microorganisms were immobilized in APS, AZB, and the plant root system (Weiß *et al.* 2013; Hassan *et al.* 2019; Mukherjee *et al.* 2020). Ten phyla of microorganism, i.e. Proteobacteria, Bacteroidetes, Acidobacteria, Chloroflexi, Planctomycetes, Verrucomicrobia, Gemmatimonadetes, Actinobacteria and Firmicutes, OD1 were found in the biofilms on AZB and APS. Similarly, 12 genera of microorganisms, i.e. *Thermomonas*, *Flavisolibacter*, *Flavobacterium*, *Kaistobacter*, *Limnohabitans*, *Luteolibacter*, *Ramlibacter*, *Steroidobacter*, *Bacillus*, *Hydrogenophaga*, *Pseudomonas* and *Pseudoxanthomonas* have also been frequently detected in biofilms of APS and AZB. Proteobacteria had the highest relative abundance among the four groups of eco-bag samples. The fraction (%) of Proteobacteria (*Pseudoxanthomonas*, *Limnohabitans*, *Thermomonas*, *Pseudomonas*, *Hydrogenophaga*, *Kaistobacter*, *Ramlibacter and Steroidobacter*) in the groups 2#, 5#, 6# and 10# was 34, 34, 54 and 28%, respectively.

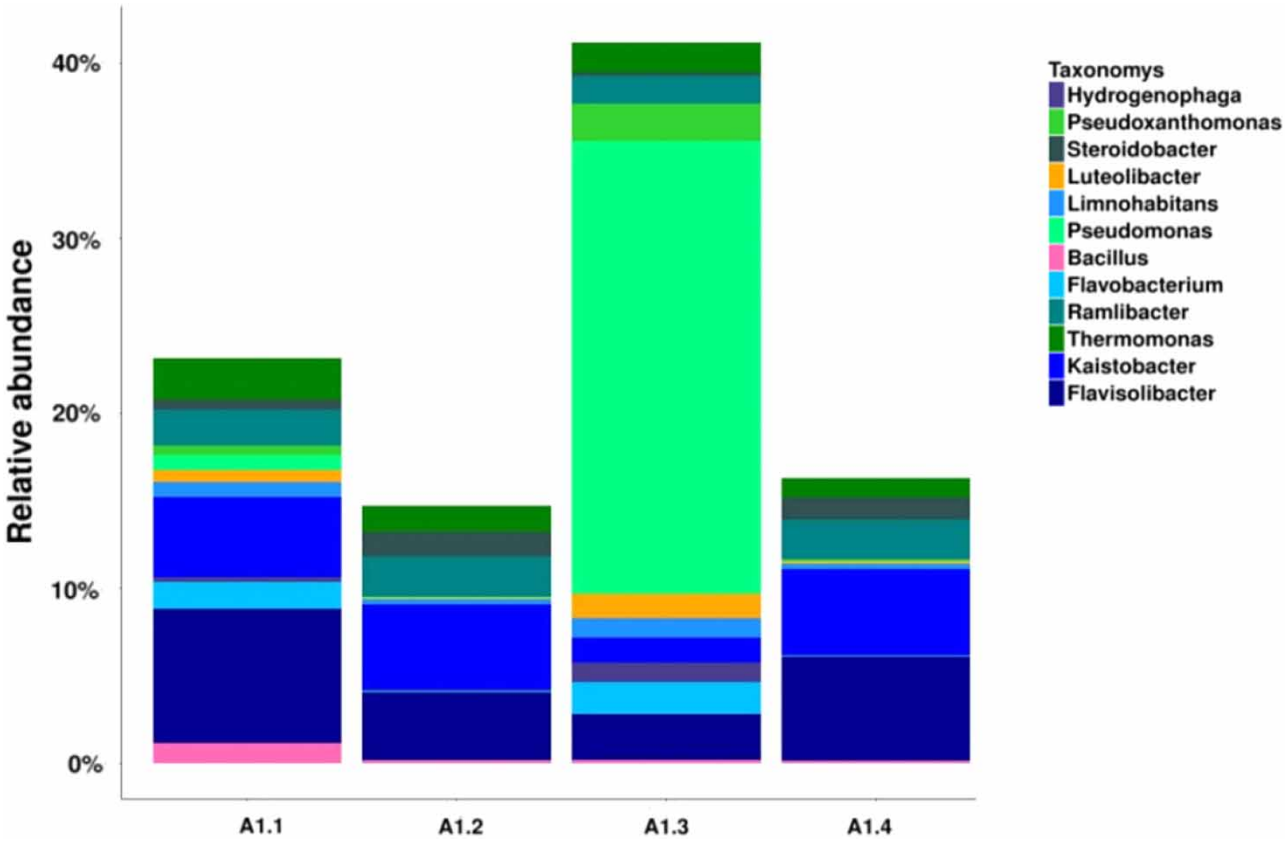


Figure 8 | The relative abundance at the genus level in the four groups of samples.

Proteobacteria are the most common prokaryotes in the water environment, and they include photosynthetic bacteria, chemoautotrophic, and chemoheterotrophic bacteria found in both aerobic and anaerobic environments (Chen *et al.* 2019; Baskaran *et al.* 2020). *Pseudomonas* belongs to the Proteobacteria, which includes both aerobic and heterotrophic denitrifying bacteria. Chen *et al.* (2019) identified genes from *Thermomonas*, *Pseudomonas*, and *Hydrogenophaga* as belonging to denitrifying bacteria. According to Baskaran *et al.* (2020), *Pseudomonas*, *Bacillus*, and *Hydrogenophaga* are aerobic denitrifying bacteria that can denitrify under aerobic conditions. To remove nitrogen from water, the nitrification and denitrification processes work in tandem. According to the sequencing results of the four bacterial species groups, the abundance of *Pseudomonas* in A1.3 (denitrification bacteria–APS plant group) was the highest at 26%, followed by A1.1 (denitrification bacteria–AZB plant group) with 1%, and the order of abundance of *Pseudomonas* was: A1.3 > A1.1 > A1.2 > A1.4. Based on these good results, straw was discovered to be the best material for the growth of *Pseudomonas* sp.

The abundance of Bacteroidetes (*Flavobacterium*, *Flavisolibacter*) in the 2#, 5#, 6#, and 10# groups was 24, 18, 15, and 24%, respectively, which have been shown to play important roles in the denitrification process (Baskaran *et al.* 2020). Flavobacterium is an important potential aerobic denitrifier (Pishgar *et al.* 2019). *Flavobacterium*-assisted heterotrophic nitrification and aerobic denitrification have been shown to have high nitrogen removal. According to Huang *et al.* (2016), *Flavisolibacter* levels have significant positive relationships with a number of denitrification genes. The findings demonstrated that the abundance of Bacteroidetes is critical for ammonium-nitrogen removal from aquatic environments.

Future research perspectives

The immobilization of microorganisms in AZB and APS has been used in this study to demonstrate their ability to remove ammonium and nitrogen from urban river water. However, the research was limited to the evaluation of the water purification abilities of AZB and APS in a laboratory setting. Further investigation is required to determine the efficacy of ecobags incorporating AZB and APS for improving water quality in the river and lake under a variety of climate and hydraulic conditions. The timing of the release of AZB and APS must be considered in order to avoid the decay of the material, which would otherwise result in the contamination of river water.

CONCLUSIONS

The immobilization of microorganisms in AZB and APS revealed a highly porous structure that provided a habitat for microbial growth and reproduction. On the AZB and APS surfaces, the engineered microorganism and denitrification microorganism inoculum were rapidly replicated. The immobilization carriers containing denitrifying microbial inoculum removed more NH_4^+-N than the engineered microbial inoculum. The results of this study demonstrated that the biofilm formed on the AZB and APS surfaces had a higher efficiency for COD, NH_4^+-N, and TN removal.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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