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RESEARCH ARTICLE

# Application potential of aerobic denitrifiers coupled with a biostimulant for nitrogen removal from urban river sediment

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## Abstract

Aerobic denitrifiers coupled with a denitrification agent were applied in the sediment of an urban river for the bioremediation of nitrogen pollution. The results revealed that 14.7% of the total nitrogen in the sediment was removed after 115 days of treatment and the nitrate nitrogen concentration removal rate was enhanced in the overlying water. Compared with the control, the total transferable nitrogen in the sediment increased from 0.097 to 0.166 mg/g, indicating that more nitrogen is likely to be involved in the biogeochemical cycling of nitrogen. Increased urease activity indicated the possible further potential of nitrogen biodegradation, while the decreased protease pointed to the low concentration of protein remaining in the sediment. Sequencing revealed that the bacterial community diversity in the sediment increased significantly after 43 days of treatment and that the effect persisted. Compared with other microcosms, the dominant phyla in the sediment after 43 days were *Firmicutes*, *Elusimicrobia*, *Spirochaetae* and *Fibrobacteres*; whereas, after 115 of treatment, the dominant bacteria were *Nitrospirae*, *Deferribacteres* and *Chloroflexi*. The dominant bacteria in the sediment are mainly associated with nitrogen cycling and thus contributed considerably to nitrogen removal in the sediment. Overall, the direction of species succession was similar to natural succession; namely, there were no undesirable ecological risks involved. This study highlights the possible benefits and feasibility of using bioaugmentation technology coupled with biostimulation to remediate nitrogen-polluted sediments.

**Keywords** Aerobic denitrification · Biostimulation · Nitrogen removal · Sediment · Enzymatic activity · MiSeq high-throughput sequencing

## Introduction

Nitrogen (N) is the most abundant chemical element in the atmosphere and is also one of the essential components of the many key biomolecules for life. Economic development, urbanisation and other human activities have doubled the amount of nitrogen entering terrestrial ecosystems, resulting in the continuous deterioration of water quality and threatening the safety of drinking water supplies (Camargo and Alonso 2006). The significant accumulation of nitrogen has become a key factor causing eutrophication in receiving waters, one of the most serious environmental problems globally. Large amounts of nitrogen from wastewater are accumulated in river sediments, constituting the threat of secondary pollution to the aquatic ecosystem. Sediment can continuously release contaminants to the overlying water, thus delaying the recovery of eutrophic environments (SØndergaard et al. 2007).

One of the most promising treatment options is bioremediation, due to its low maintenance cost, effective performance and reduced environmental impacts (Farhadian et al. 2008). Bioremediation involving bioaugmentation and/or biostimulation has been considered the most advantageous technique to treat contaminated sediment (Tyagi et al. 2011). Bioaugmentation involves the addition of an active contaminant-degrading culture to a contaminated site to

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enhance the degradation of unwanted compounds during bioremediation (Chang et al. 2012). It has proven to be a successful technology for the removal of nitrogen in wastewater in both laboratory and field applications. Compound bacterium was applied to control the eutrophication in the Cihu Lake, Hubei Province, China. The field study showed that there was an increase in transparency and reduction in total nitrogen (TN) of water after the remediation (Chen et al. 2007). Sun et al. (2009) found that the ecological floating bed enhanced by immobilised denitrifying bacteria was 17.2% of TN, 2.6% of $NH_4^+$–N and 62.8% of $NO_3^-$–N higher than that in the individual floating bed in the lab-scale study. Results of the bioaugmentation with specialised bacteria in a pilot-scale biological contact oxidation ditch system showed that removal efficiencies of TN and $NH_4^+$–N increased from 25.9 to 50.3% and from 34.5 to 60.1%, respectively, and that the bacterial community was considerably enriched (Jiao et al. 2011). While treating the effluent of the primary clarifier at the 4th wastewater treatment plant in Xi'an, China, bioaugmentation with nitrifiers in a full-scale anaerobic-anoxic-oxic system shortened the recovery time of nitrification activity at low temperatures (Pei et al. 2014). At bench-scale level, bioaugmentation with *Bacillus* sp. was efficient in the $NH_4^+$–N removal of the fish processing wastewater from 558 to 60 mg/L within 5 days of treatment (Sarnaik et al. 2015). Microbial degradation plays an important role in nitrogen removal, and traditional treatment processes rely on nitrification by autotrophs under aerobic conditions and denitrification by heterotrophs under anaerobic conditions. However, the reaction steps are usually constrained by the presence of dissolved oxygen (DO) which makes the process impractical in an urban river.

Aerobic denitrification was discovered in the 1980s, leading to a novel method for nitrogen removal, namely, one without a limitation on oxygen (Robertson and Kuenen 1983). Compared with conventional nitrogen removal systems, bacteria capable of combined heterotrophic nitrification and aerobic denitrification could oxidise ammonium to nitrite and reduce nitrite and nitrate to $N_2$ simultaneously. Aerobic denitrification is defined as the co-respiration or co-metabolism of $O_2$ and $NO_3^-$. Their utilisation of organic substrates and their tolerance to oxygen allow them to perform simultaneous nitrification and denitrification (Zhang et al. 2012b; Li et al. 2015). Currently, many studies focus on the characteristics and applications of aerobic denitrifiers in the treatment of wastewater and urban river. Pai et al. (1999) found that the aerobic denitrifier T6 could be applied as a supplement to a reactor, with the nitrogen removal rate reaching 360 mg N/(g MLVSS d), demonstrating its promising application for real wastewater treatment. Chen et al. (2015) successfully treated municipal wastewater in a pilot-scale sequencing batch reactor (SBR) with heterotrophic-aerobic nitrogen-removing bacteria, showing the stable and effective removal of carbon and nutrients. Newly isolated indigenous aerobic denitrifiers were added to water samples from Lake Taihu, and $NH_4^+$–N and $NO_3^-$–N removal efficiencies reached 60 and 75%, respectively, indicating the potential of utilising remediation on eutrophic lakes (Guo et al. 2013).

Many environmental factors, including temperature, nutrients and co-substrates, may influence the biodegradation of pollutants in the environment (Park et al. 2011). Biostimulation, a bioremediation technique, supplies the nutrients or the suitable conditions to stimulate indigenous microorganisms and accelerate the biodegradation of contaminants in the environment (Garcia-Blanco et al. 2007). In recent times, biostimulation coupled with bioaugmentation has been widely developed to improve pollution remediation. Results from the application of *Rhodococcus erythropolis* in the biodegradation of polycyclic aromatic hydrocarbons (PAHs) in mangrove sediment suggested that the combination of biostimulation and bioaugmentation is the most effective treatment, as compared to natural attenuation, biostimulation and bioaugmentation alone (Lang et al. 2016). Xiong et al. (2017) found that the degradation of 2,4,6-tribromophenol (TBP) in water and sediment microcosms was enhanced by bioaugmentation with *Bacillus* sp. GZT and that the addition of glucose or yeast extract could effectively stimulate TBP degradation. In a reservoir system, indigenous aerobic denitrifiers were enhanced in situ by water lifting and aeration, causing the TN removal rates of the water system and surface sediment to reach 21.74–52.54 and 39.82–42.92%, respectively (Zhou et al. 2016).

Bioaugmentation combined with biostimulation has been widely used in wastewater treatment and urban river remediation. However, few studies have investigated the effects of aerobic denitrifiers coupled with a biostimulant for the remediation of urban river sediment. A biostimulant applied in microbial bioremediation provides minerals, nutrients and enzymes to indigenous microorganisms for a prolonged period, thereby increasing the contaminant removal efficiencies and limiting nutrient release in the sediment (Subha et al. 2017). The aim of this study was to investigate the feasibility and mechanism of nitrogen removal by aerobic denitrifiers coupled with a biostimulant. The enzymatic activities were studied to reflect the microscopic changes of sediment. Particular attention was paid to the interactions between the biostimulant, inoculated microorganisms and indigenous microbial communities, revealing the possible mechanisms for nutrient removal during remediation.

## Materials and methods

### Materials

#### Microbial consortium preparation

The aerobic denitrifiers were isolated from activated sludge in a SBR reactor. The activated sludge sample (1 mL) was

inoculated in Luria-Bertani medium (LB medium). After enriching for three times, 1 mL of liquid culture was tenfold serially diluted to $10^{-9}$, and 100 μL aliquots of each dilution were spread onto solid bromothymol blue medium (BTB medium). After 48-h incubation at 30 °C, blue cloudy colonies were isolated and then purified by repeated streaking on BTB medium. Purified strains were second screened, and identification of heterotrophic nitrification and aerobic denitrification abilities was performed. After these procedures, one strain was selected for remediation studies based on its good heterotrophic nitrification and aerobic denitrification abilities (Fig. S1). It was then preserved at temperatures below 4 °C and sub-cultured every 6 months. The results from a 16S rDNA sequence analysis revealed that the strain showed a 99% similarity with *Citrobacter* sp. (KP068655.1), affiliated to the phylum *Proteobacteria*. Based on the previous study (Wang 2011) and the pollution characteristics of sediment, the concentration of the bacterial solution is $10^8$ CFU/mL, and the optimal dosages for bioaugmentation are 0.03% (bacterial solution volume/sediment volume) in the overlying water and 0.09% in the sediment, respectively.

### Biostimulant

Treatments with biostimulant were carried out using a commercially available, legally approved product. Based on the previous study on effects of different biostimulant (Lv 2016), we chose a denitrification agent (Bio-Form, LLC, China) consisting of some enzymes and nutrients as natural bioactivators for denitrifying bacteria. Based on indications by the manufacturer and results from previous study (Lv 2016), the optimal dosage of the denitrification agent is 0.08 g/L (denitrification agent mass/sediment volume) when the denitrification agent is used to remediate polluted sediment for biostimulation.

### Design of the microcosm

The size of the device used in this experiment was 25 cm × 25 cm × 80 cm. The effective depth of the device was 70 cm and the total volume was 43.75 L. Two water tanks used for storing influent and collecting effluent were set above and below the device, respectively. Rubber hoses were used to connect the device and the water tanks. All the influent entered the device following the gravity flow design, and the flow was maintained at 9.96 ± 0.1 L/(h $m^2$) by adjusting the water-stopping clip on the rubber hose. The effluent was collected in the water tank below the device and pumped to the water tank above the device with a peristaltic pump. The water was thus recirculated in the laboratory-scale microcosm to simulate the flow of an actual river (Fig. 1).

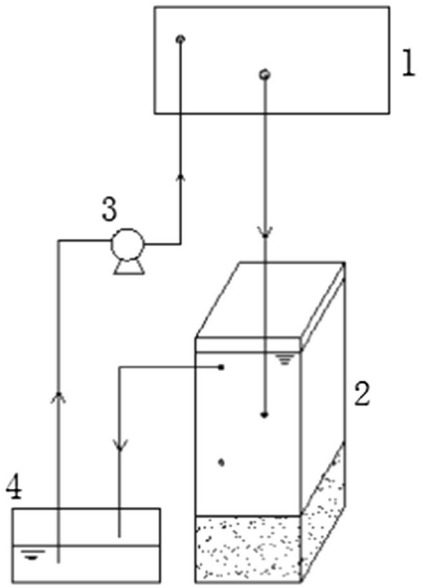


**Fig. 1** Schematic diagram of the microcosm. 1 Water tank for storing influent. 2 Device. 3 Peristaltic pump. 4 Water tank for collecting effluent

### Sediment used in the experiment

The sediment used in the experiment was collected from the original river bed of the Hai River in Tianjin, China. Before the river diversion, this ecosystem mainly suffered from fishpond pollution, farmland runoff and domestic wastewater including effluent of septic systems from the nearby villages. The sediment was evenly stirred to ensure homogeneity before detection and being put into the devices. The main quality of sediment was 1.09–1.14 mg/g of TN, 6.7–7.2 mg/g of total organic carbon (TOC), 6.93–7.61 of pH and (− 68.2) to (− 19.8) of redox potential (ORP).

## Experimental procedures

Four experimental treatments were designed in duplicate for the purposes of this study: T1 = natural attenuation as the control, T2 = bioaugmentation with aerobic denitrifiers, T3 = biostimulation with denitrification agents (0.08 g/L) and T4 = a combination of aerobic denitrifiers and a biostimulant (0.10 g/L). Considering that some biostimulants were consumed by the aerobic denitrifiers, the dosage of the biostimulant increased to 0.10 g/L during bioaugmentation and biostimulation coupling. The microcosms were created using the homogenised sediment and clean water. When the sediment and clean water were placed into the devices, the poured sediment was very fluffy and high turbidity was observed in the overlying water in the initial period. The water samples were analysed for TN, $NH_3$–N and $NO_3^-$–N every 3 days. After the sediment stood for 2 weeks, the sediment precipitated to the bottom of the devices and the overlying water was with high transparency. Moreover, there were no significant differences

on the water quality of the four devices. Based on some literatures (Li et al. 2007; Jiang et al. 2012; Pourabadehei and Mulligan 2016) and the conditions of the old Hai River, the heights of the sediment and the overlying water in the microcosm were 13 and 57 cm, respectively. As all the pollutants came from the sediment, the closed system helped to show the nitrogen transformation between the sediment and overlying water clearly, without the impact of pollutants in river water. The experiments were conducted for about 4 months, and the water temperature ranged from 15 to 28 °C during the treatment. Denitrification agents were injected directly into the water, with syringes, on the first day of the experiment. At the same time, aerobic denitrifiers were injected into the overlying water and sediment directly with syringes. Water and sediment samples were taken from the same height in every device and five individuals with equal amounts were homogenised to obtain a representative sample for every microcosm. Sampling of water and sediment was not conducted on the same day to avoid the impact of turbulence.

## Physiochemical analysis

The water samples were analysed for TN, $NH_3$–N and $NO_3^-$–N, using the standard methods described by the National Environment Protection Agency of China (NEPAC 2002). The water temperature and pH were measured in situ using a pH meter (Ohaus Instrument (Shanghai) Co. Ltd). The TN in the sediment was measured following the Modified Kjeldahl method (SAC 2014). TOC of the sediment was determined using the combustion oxidation nondispersive infrared absorption method (SAC 2009). The ORP of the sediment was determined in situ with a Hach FJA-6 ORP Meter.

The total transferable nitrogen (TTN) in the sediment was also investigated. It mainly consists of four transferable nitrogen forms: ion-exchangeable form (IEF-N), weak-acid extractable nitrogen (WAEF-N), strong-alkali extractable nitrogen (SAEF-N) and strong-oxidant extractable nitrogen (SOEF-N). The different transferable nitrogen forms in the sediment were determined according to the method described by Wang (2013). The sequential extraction methods are shown in Table 1. The amount of TN in each fraction was monitored using the method described above.

## Extracellular enzyme activity assays

According to the method suggested by Guan (1986), urease activity was determined spectrophotometrically (578 nm) using urea as the substrate. Three grams of soil in 2-mL methylbenzene were pre-treated for 15 min. A 10-mL urea solution (10%) and a 20-mL citrate buffer (pH 6.7) were added to the pre-treated soil. They were then incubated in a constant temperature incubator at 30 °C for 24 h. Following incubation, the mixture was filtrated and 4 mL of sodium phenate and 3 mL of sodium hypochlorite were added to the filtrate. The urease activity was expressed as milligrammes of ammonia nitrogen released per gram of soil per hour [mg/(g h)].

The protease activity was measured spectrophotometrically using refined casein as the substrate (Lin 2010). One gram of soil was mixed with a 20-mL refined casein solution and 1 mL of methylbenzene in a hermetically sealed flask, which was then incubated for 48 h at 30 °C. Following the incubation, 20 mL of trichloroacetic acid was added to the mixture to precipitate protein and then a 5-mL sodium carbonate solution (0.55 mol/L) and 1 mL of Folin-Phenol reagent were added to the supernate. Following a water-bath heating at 30 °C for 30 min, the protease activity was quantified by measuring absorbance at 680 nm using the spectrophotometer. The measurement was expressed as milligrammes of amino acid released per gram of soil per hour [mg/(g h)].

## Microbial community analysis

To explore the composition of the sediment microbial communities in the microcosms, sediment samples were collected at 43 and 115 days. The bacterial genomic DNA was extracted using FastDNA® SPIN Kit for Soil (MP Biomedicals, USA) following manufacturer instructions. After their extraction, the DNA samples were amplified by PCR using the primers 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT), targeting the V3–V4 region of the bacterial 16S rDNA genes. All PCR products were sequenced using the Illumina Miseq PE300 platform (Illumina, Inc., CA, USA) by Beijing Auwigene Tech Ltd. (Beijing, China).

**Table 1** Sequential extraction methods of different forms of transferable nitrogen

| Transferable nitrogen forms | Extraction (time) | Binding forms of N |
|---|---|---|
| IEF-N | 1.0 mol/L KCl (2 h) | Loosely adsorbed to sediment |
| WAER-N | pH = 5 HAc-NaAc (6 h) | N bound to $CaCO_3$ |
| SAEF-N | 0.1 mol/L NaOH (17 h) | N bound to Fe-Mn oxides |
| SOEF-N | 0.24 mol/L NaOH +20 g/L $K_2S_2O_8$ (2–3 h), 110–115 °C (1 h) | Organic-binding N |

The sequences were less than 100 bp in length after quality trimming and the sequences containing ambiguous base calls (N) or that had quality scores < 90% and > Q20 were removed. The taxonomic classification of the effective sequences was determined using the RDP Classifier against a database derived from Silva119 (http://www.arb-silva.de). All of the Illumina sequencing raw data were deposited under the National Center for Biotechnology Information (NCBI) accession nos. PRJNA388981. Rarefaction curves were generated using the software MOTHUR and R, based on the observed operational taxonomical units (OTUs). To compare the diversity of the communities between the different samples, principal component analysis (PCA), a heatmap and a redundancy analysis (RDA) were performed using the software R.

### Analytical methods

To check repeatability, some experiments were randomly run in duplicate and the withdrawn samples were analysed in triplicate to estimate random error of the measurements. The deviations between the runs performed were always lower than 10% for all results.

The experimental results were also statistically analysed using one-way ANOVA (SPSS 21.0). Significant differences were accepted at $P < 0.05$. Statistical analyses were also performed using Microsoft Excel 2013.

## Results and discussion

### Effects on nitrogen removal

Nitrogen removal by biological means is considered to be a result of nitrification and denitrification processes. It is a widely adopted approach due to its ease of implementation, high efficiency and low costs. The DO values in the overlying water varied from 4.27 to 5.49 in the four microcosms during the experimental time. During 4 months of monitoring, the four experimental treatments had different effects on the nitrogen removal in the overlying water and the sediment. As shown in Fig. 2a, $NH_4^+$–N in the four microcosms increased sharply in the initial 15 days, then decreased with time and finally remained stable. The increase of the $NH_4^+$–N concentrations in the initial 15 days resulted from the release of pollutants from the sediment. In the T2 and T4 microcosms, bioaugmentation with aerobic denitrifiers would stimulate the metabolism and growth of the indigenous microbes in the sediment, and thus more ammonia may be produced from the ammonification of organic nitrogen and released from the sediment to the overlying water. However, biostimulation with denitrification agents targeting denitrifying bacteria may have little effect on ammonifiers in the sediment. The $NH_4^+$–N concentration in the T3 microcosm was lower than that in the T2 and T4 microcosms in the initial 15 days. In the T4 microcosm, the $NH_4^+$–N concentration decreased to 1.0 mg/L after 20 days of treatment and the $NH_4^+$–N removal efficiency was 27.9% at 110 days. Compared with other

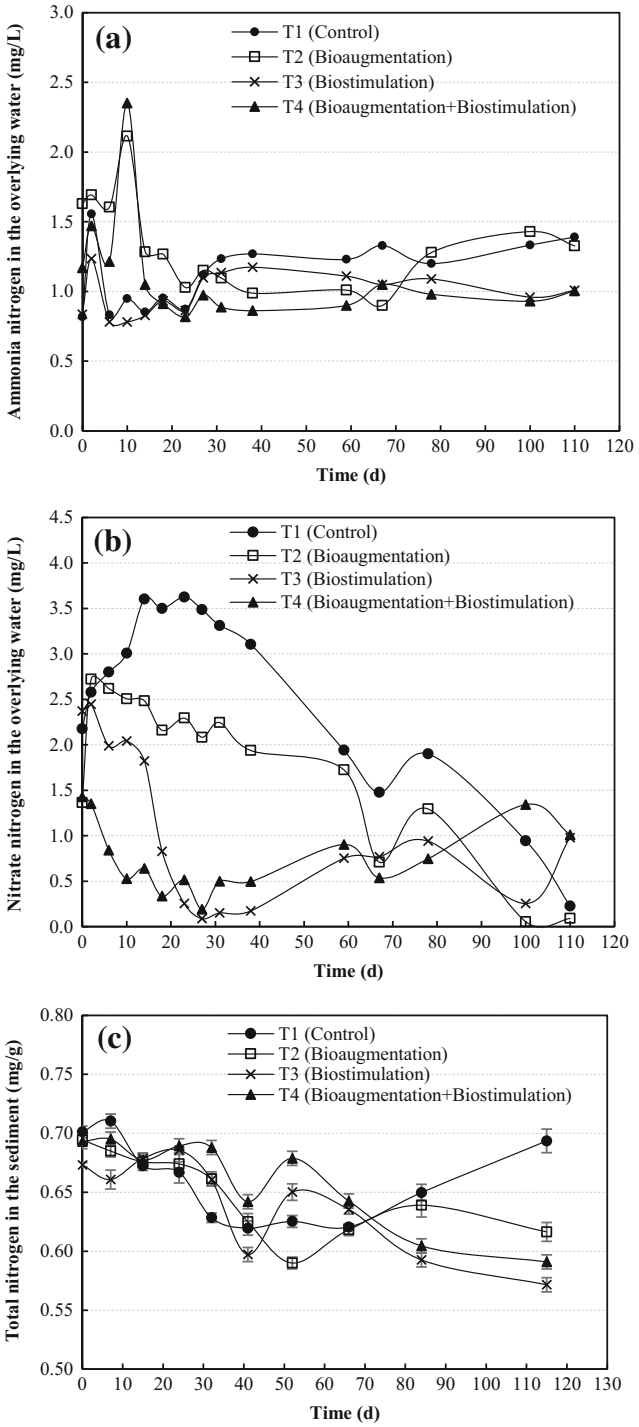


**Fig. 2** Variation in **a** $NH_4^+$–N, **b** $NO_3^-$–N in the overlying water and b TN in the sediment. (T1 natural attenuation as the control, T2 bioaugmentation with aerobic denitrifiers, T3:biostimulation with denitrification agents and T4 a combination of aerobic denitrifiers and a biostimulant)

treatments, bioaugmentation coupled with biostimulation exhibited the best effect on $NH_4^+$–N removal, indicating the increase of nitrification in the process.

Figure 2b shows the variations of $NO_3^-$-N concentrations in the overlying water after the different treatments. In the control treatment, the concentration of $NO_3^-$–N increased gradually, reaching a peak of around 3.62 mg/L at 23 days, and then decreased again with time. The variation of $NO_3^-$–N concentration in the T2 microcosm was similar to the control treatment. However, the $NO_3^-$–N concentration in the T2 microcosm was significantly lower than that in the T1 microcosm ($P < 0.05$). Compared with the T1 and T2 microcosms, the $NO_3^-$–N concentrations in T3 and T4 decreased significantly in the first 2 months ($P < 0.05$) and the treatment of bioaugmentation coupled with biostimulation showed the best performance. However, there were no significant differences among the four microcosms after 60 days of treatment ($P > 0.05$). This revealed that bioaugmentation coupled with biostimulation could enhance the removal rate of $NO_3^-$–N concentration by increasing the denitrification process, thus shortening the treatment time necessary to improve the effects of remediation.

The variations of total nitrogen in the sediment are presented in Fig. 2c. The TN concentration in the sediment showed decreasing trends in the T2, T3 and T4 microcosms. Compared to the start-up, the removal efficiencies of TN concentration in the T2, T3 and T4 microcosms were 11.3, 15.1 and 14.7%, respectively, after 115 days of treatment. During the experimental time, the temperature varied between 20 and 28 °C (under the optimal temperatures of 20–30 °C for the aerobic denitrifiers) in the initial 60 days, while it ranged between 15 and 20 °C in the later period. Variations of temperature have influence on release and precipitate of pollutants in sediment (Yang et al. 2007). In the control microcosm, the concentrations of $NH_4^+$–N and $NO_3^-$–N in the overlying water increased sharply, reaching the maximum of 1.56 and 3.62 mg/L at 2 and 23 days, respectively, then decreased with time. The TN concentration in the sediment decreased with time in the initial 30 days, then it remained stable for about 40 days and afterwards it increased gradually after 70 days of treatment, finally recovering to the original concentration. This indicates that the decrease of TN concentration in the sediment mainly resulted from the release of nitrogen from sediment to the overlying water, which caused a serious secondary water pollution. With the re-precipitation of the pollutants, the nitrogen concentration decreased in the overlying water and increased again in the sediment. In the T2, T3 and T4 microcosms, the $NH_4^+$–N and $NO_3^-$–N content in the overlying water showed increasing trends in the initial 10 days, and then decreased with time, while the TN concentrations in the sediment decreased during the experimental time. This may suggest that the nitrogen was released from the sediment to the overlying water in the initial period, while it was degraded by microbes in the microcosms with the extension of experimental time.

During 115 days of the three treatments, there were no significant differences on the removal of TN in the sediment ($P > 0.05$). However, the lowest concentration of $NO_3^-$–N was observed in the T4 microcosm, followed by T3, T2 and T1, in the initial 30 days ($P < 0.05$). Moreover, the $NH_4^+$–N content in the T4 microcosm was lower than that in other microcosms after 30 days of treatment ($P < 0.05$). Bioaugmentation with aerobic denitrifiers in this study could enhance the nitrification and denitrification processes and thus improve nitrogen removal. Biostimulation with the denitrification agent provided some essential nutrients and enzymes for the denitrifying bacteria, enhancing the denitrification process. When biostimulant was used to enhance bioaugmentation, the denitrification agent may stimulate not only indigenous denitrifying bacteria but also the introduced aerobic denitrifiers. Thus, the synergistic effect could considerably improve the nitrogen removal and show the best performance in the T4 microcosm.

## Changes in the different forms of transferable nitrogen

Sediment is an important nitrogen source to the overlying water. However, not all nitrogen fractions can be released from the sediment and cause water eutrophication. TN in sediment consists of two forms: transferable nitrogen and non-transferable nitrogen. Numerous studies show that transferable nitrogen in sediment can be involved in the biogeochemical cycling of nitrogen, whereas non-transferable nitrogen is stable and contributes little to nitrogen cycling in nature (Wang et al. 2008; Pan et al. 2011). The variations in the different forms of transferable nitrogen were also investigated in this study (Fig. 3). The result showed that the concentrations of TTN in the four microcosms decreased in the order of T4 (0.166 mg/g) > T2 (0.118 mg/g) > T3 (0.114 mg/g) > T1 (0.097 mg/g), after 115 days of treatments. This revealed that bioaugmentation coupled with biostimulation considerably increased the content of TTN in the sediment, indicating that more nitrogen had the potential to be further biodegraded. Ma et al. (2002) reported that non-transferable nitrogen could be activated and become transferable under specific conditions. Bioaugmentation with aerobic denitrifiers or biostimulation with a denitrification agent had the effect of changing the stable forms into transferable nitrogen probably by stimulating the indigenous microorganisms and changing the external conditions of sediment. The effect of single method is limited, while the combination technology exploits the advantages of biostimulation and bioaugmentation, resulting in better performance of increasing the TTN content in sediment.

Among the four forms of transferable nitrogen, IEF-N was released from sediment to overlying water most easily,

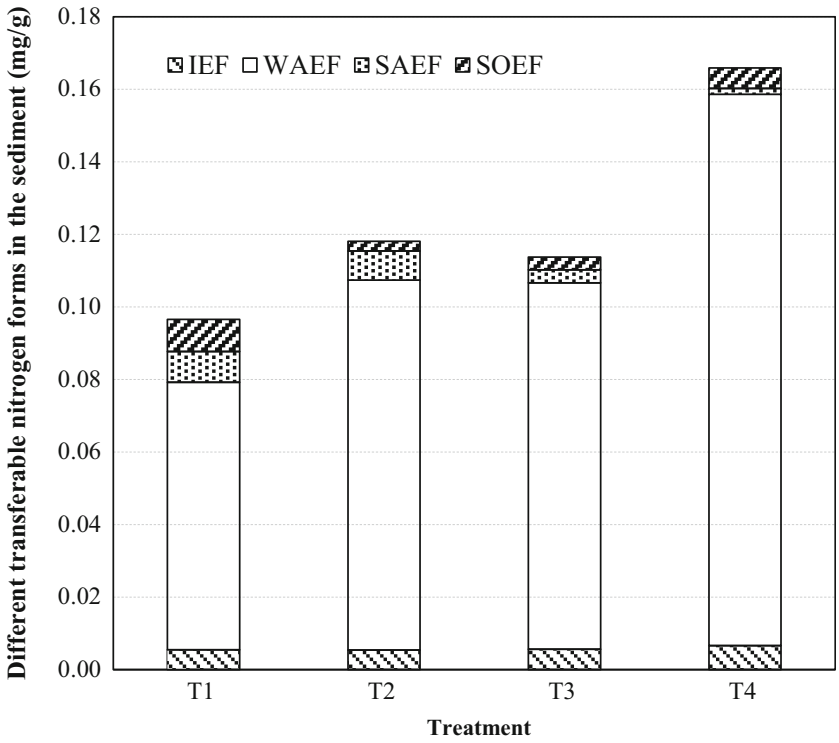


**Fig. 3** Variation in the different forms of transferable nitrogen in the sediment, after 115 days. T1 natural attenuation as the control, T2 bioaugmentation with aerobic denitrifiers, T3 biostimulation with denitrification agents and T4 a combination of aerobic denitrifiers and a biostimulant

followed by WAEF-N, SAEF-N and SOEF-N (Zhang et al. 2012a, b). As IEF-N is the most active and most unstable form of transferable nitrogen, it takes part in nitrogen cycling easily and may cause the release of nitrogen from the sediment to overlying water, raising the potential risk of secondary water pollution (Liu et al. 2015). Although the concentration of TTN in the T4 microcosm increased significantly, the IEF-N concentration in the T4 microcosm was similar across all microcosms. Low concentrations of IEF-N prevent adverse impacts on the overlying water. Lv et al. (2004) suggested that total benthos biomass had a positive relationship with SOEF-N in sediment, and that organic-binding nitrogen was easily utilised by benthos biomass. This indicated that microorganisms were active under environments with high concentrations of SOEF-N. Moreover, Wang et al. (2014) found that WAEF-N would convert to SOEF-N by mineralisation and microbial activities. After treatment with bioaugmentation coupled with biostimulation, the content of SOEF-N was higher than that after bioaugmentation /biostimulation alone. Compared with the control treatment, the concentration of WAEF-N increased from 0.074 to 0.152 mg/g in the T4 microcosm. The higher concentrations of WAEF-N and SOEF-N in the T4 microcosm could provide a sustainable environment for the microbes in the sediment.

## Extracellular enzyme activity in the sediment

The activity of the extracellular enzymes can mediate microbial decomposition in the sediment, as they determine the direction and effects of the biochemical reaction in the environment. Microorganisms obtain essential compounds from the environment for their metabolism and growth, through the enzymatic process (Moreno et al. 2003). Enzyme synthesis and secretion by microbes must consume enough nutrients and energy. Thus, the induction of enzyme synthesis and secretion is usually governed by the availability of nutrients in environment. Burns et al. (2013) found that the microbe biomass in the environment increased until all the available nutrients were consumed to satisfy the demand for metabolism and growth. At that moment, the production of extracellular enzymes was reduced and the decomposition of exogenous substrate decreased. As a biological property of sediment, enzyme activities are assumed earlier indicators of pollutant degradation, as compared to physical or chemical parameters in sediment. This is because enzymatic activities are sensitive to changes in the environment and can also be an indicator of microbial activities in the environment (Dick 1994).

The urease activities in the sediment of all samples exhibited decreasing trends overall during the experimental period (Fig. 4a). The activity of urease in the T4 microcosm declined sharply during the initial 25 days and then kept decreasing gradually. The urease activities in the T1, T2 and T3 microcosms showed variations in the first month, then decreased and finally remained stable. After 80 days of treatment, the lowest urease activity in the sediment was observed in the T3 microcosm [0.29 mg/(g h)], followed by microcosms T1 [0.48 mg/(g h)], T4 [0.66 mg/(g h)] and T2 [0.74 mg/(g h)]. Urease is an important hydrolytic enzyme and plays an

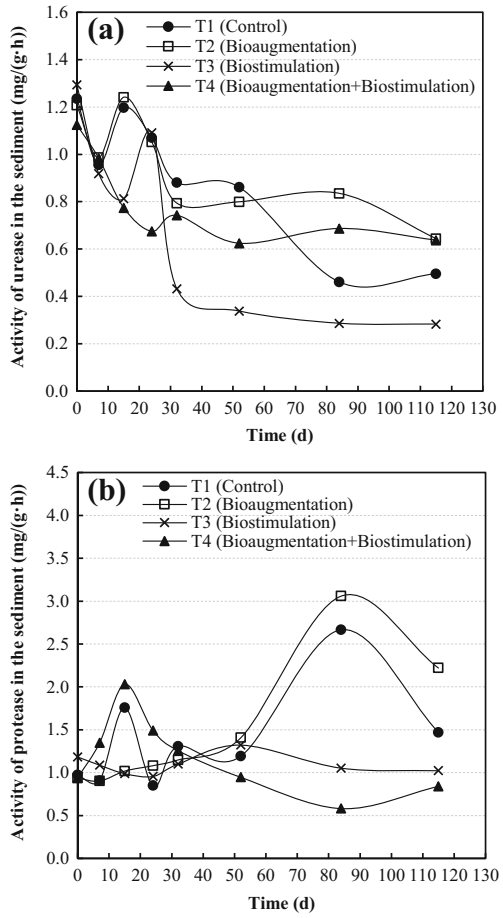


**Fig. 4** Variations in **a** urease and **b** protease in the sediment. (T1 natural attenuation as the control, T2 bioaugmentation with aerobic denitrifiers, T3 biostimulation with denitrification agents and T4 a combination of aerobic denitrifiers and a biostimulant)

important role in nitrogen cycling. The microorganisms catalyse urea, a type of organic nitrogen, by producing urease to obtain $NH_4^+$ for their growth. Reynolds et al. (1985) reported that urease activity is usually in a significant positive relationship with the concentration of total available nitrogen in sediment. The indigenous sediment microbes were stimulated by bioaugmentation and since sufficient available nitrogen was left in the sediment, the activity of urease was highest in the T2 microcosm. The lowest urease activity (in the T3 microcosm) resulted from the low concentration of available nitrogen remaining in the sediment. After the treatment with bioaugmentation and biostimulation, the available organic nitrogen decreased significantly in the first month, causing the decline of urease activity in the sediment. With the extension of the experimental time, bioaugmentation coupled with biostimulation would stimulate the metabolism and growth of the indigenous microbes in the sediment, and the increase of TTN in sediment could provide sufficient available nitrogen for microbes in the environment. This indicated that more nitrogen in the T4 microcosm had the potential to be removed with the high urease activity in the sediment.

The variations of the protease activities in the sediment are shown in Fig. 4b for all samples. The activities of protease in the T1 and T2 microcosms increased with time and reached a maximum value of 2.67 and 3.06 mg/(g h) at 84 days respectively. The protease activity in the T3 microcosm remained stable at about 1.09 mg/(g h) during the experimental time. In the T4 microcosm, the protease activity in the sediment increased sharply, reaching a maximum of 2.03 mg/(g h) at 15 days, and then began to steadily decrease. The result showed that the protease activities of the four microcosms in the sediment decreased in the order of T2 > T1 > T3 > T4, after 80 days of treatment. Protease is one of the most important enzymes in hydrolysis of organic nitrogen and can be produced by most bacteria and fungi. It can hydrolyse macromolecule proteins and polypeptides to peptide and amino acid, satisfying the demand of the metabolism and growth of microbes. Previous study found that the concentration of protein in the sediment is positively related to the activity of protease (Wilczek et al. 2004). Bioaugmentation alone may stimulate the synthesis and secretion of protease by microbes in the sediment; whereas, biostimulation alone had little influence on the activity of protease. Bioaugmentation coupled with biostimulation showed a synergistic effect on the increase of protease activity during the initial period and thus accelerated the decomposition of protein in the sediment, exhibiting the best performance. After 50 days of treatment, the lowest protease activity was observed in the T4 microcosm, indicating low concentration of protein remaining in the sediment.

## 16S rDNA sequencing

Analysis using Miseq high-through sequencing allowed the determination of bacterial species diversity and of the community shift after the treatment with bioaugmentation/biostimulation. The Shannon diversity index was used to evaluate the levels of bacterial diversity in the different samples. Compared with the control treatment, the Shannon diversity index increased significantly after the bioaugmentation/biostimulation treatment of 43 days. The Shannon diversity index of the four microcosms was in the order T2 (9.17) > T3 (9.08) > T4 (8.72) > T1 (8.37). However, the Shannon diversity index in the T4 microcosm exhibited the highest value after 115 days of treatment (9.00), followed by microcosms T1 (8.95), T2 (8.86) and T3 (8.79). This indicated that the bacterial diversity in the sediment increased after 43-d remediation of dosing the aerobic denitrifiers/denitrification agents alone; however, the effect did not persist. The addition of aerobic denitrifiers coupled with the denitrification agent showed a persistent effect on increasing the bacterial diversity in the sediment. Natural succession existed in the control; however, a longer time was necessary for the bacterial diversity to increase and to for bioremediation of the polluted sediment to be achieved.

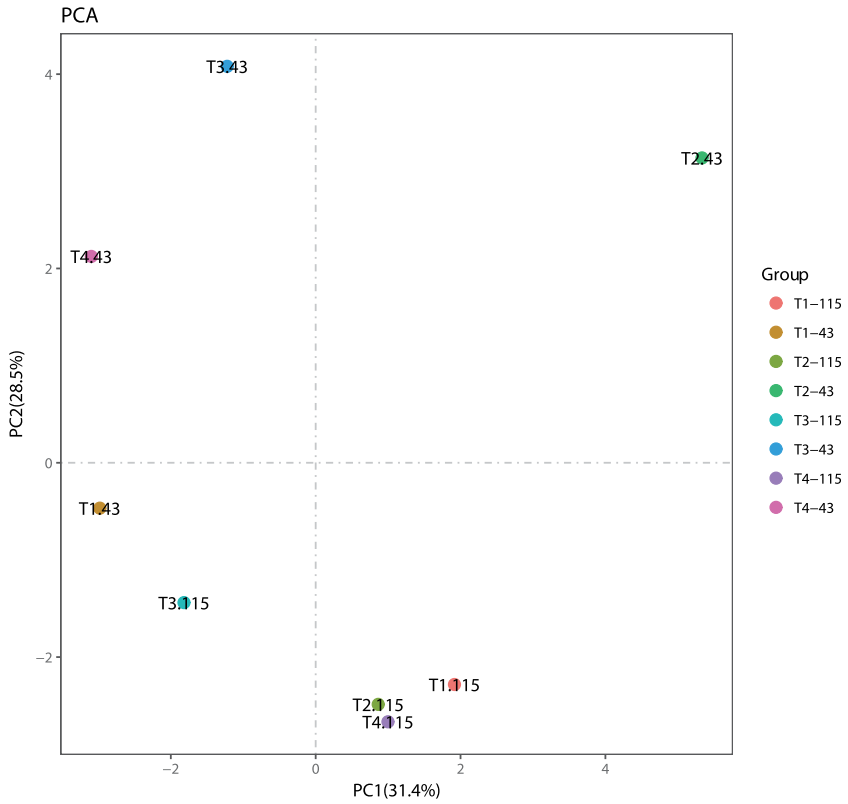


**Fig. 5** Principal component analysis (PCA) of the sediment in the four microcosms, after 43 and 115 days of treatment. The samples were named using the style 'microcosm-experimental time', e.g. T1–43 refers to the sample collected in the T1 microcosm after 43 days of treatment. (T1 natural attenuation as the control, T2 bioaugmentation with aerobic denitrifiers, T3 biostimulation with denitrification agents and T4 a combination of aerobic denitrifiers and a biostimulant)

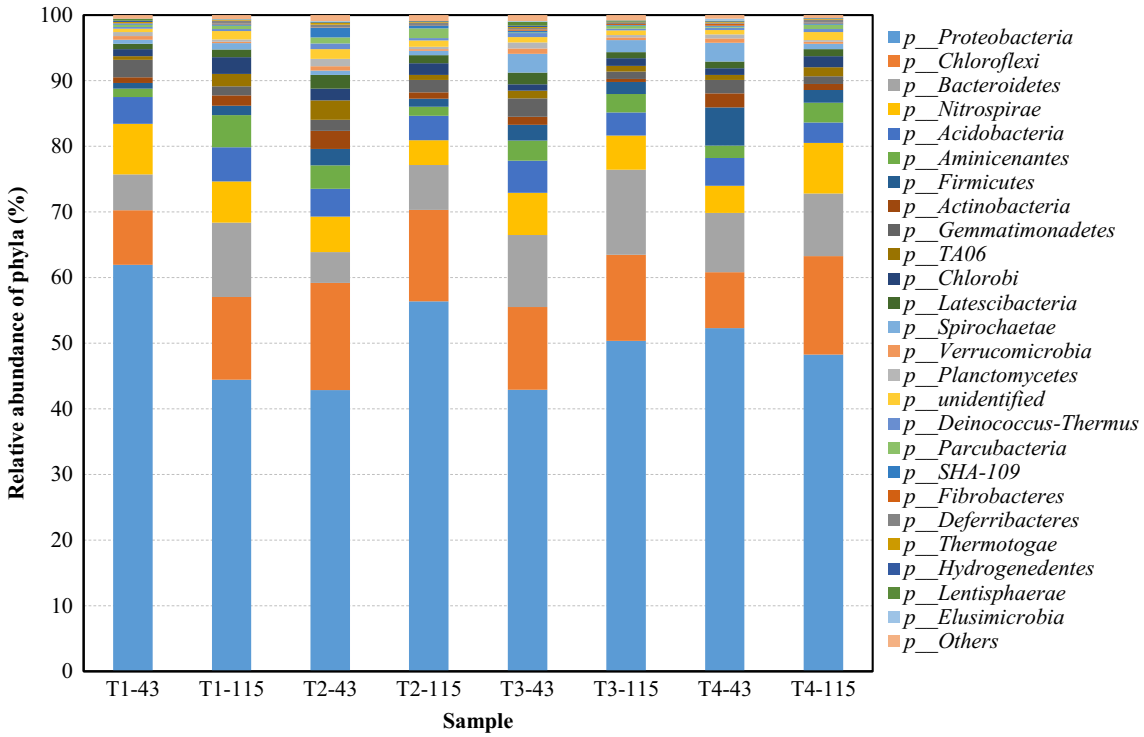


**Fig. 6** Relative abundance of the dominant bacterial phyla in the four microcosms, after 43 and 115 days. The samples were named using the style 'microcosm-experimental time', e.g. T1–43 refers to the sample collected in the T1 microcosm after 43 days of treatment. (T1 natural attenuation as the control, T2 bioaugmentation with aerobic denitrifiers, T3 biostimulation with denitrification agents and T4 a combination of aerobic denitrifiers and a biostimulant)

Community structure comparisons among the different samples were performed using principal component analysis (Fig. 5). The results show that microflora were well separated in the four microcosms after 43 days of treatment; whereas, the samples collected from the four microcosms after 115 days presented clusters, all located in quadrants 3 and 4. The addition of aerobic denitrifiers/denitrification agent could significantly affect the sediment community structure in the short term; however, the direction of species succession remained similar to the natural succession overall. This indicated that a transient addition of aerobic denitrifiers/denitrification agent would not have a permanent adverse influence on the indigenous microbial community or pose undesirable ecological risks.

The sediment microbial communities were also examined after they underwent the various treatments in this study. Figure 6 shows that similar microbial communities were dominant in the sediment after the different treatments. Moreover, community shifts were identified in the four microcosms during the bioremediation processes. The eight dominant microbial phyla were *Proteobacteria* (42.85–61.92%), *Chloroflexi* (8.34–16.34%), *Bacteroidetes* (5.48–12.96%), *Nitrospirae* (3.80–7.72%), *Acidobacteria* (3.13–5.18%), *Aminicenantes* (1.30–4.93%), *Firmicutes* (0.85–5.78%) and *Gemmatimonadetes* (1.14–2.80%).

The heatmap (Fig. 7) shows the community shift following the addition of aerobic denitrifiers/denitrification agent, after 43 and 115 days of treatment. Compared with the other treatments, the superior phyla in the T4 microcosm after 43 days were *Firmicutes*, *Elusimicrobia*, *Spirochaetae* and *Fibrobacteres*. Previous studies showed that *Firmicutes* bacteria are known to denitrify heterotrophically and can degrade a variety of organic pollutants such as carbon and energy sources in environment (He et al. 2016; Knowles 1982). The abundant *Elusimicrobia* can degrade glucose into lactate, acetate, hydrogen and $CO_2$, and can fix nitrogen synthesising and secreting nitrogenase. Moreover, it has a peptide degradation pathway comprising transamination reactions and leading to the formation of alanine (Zheng et al. 2016; Herlemann et al. 2009). It has been observed that *Spirochaetae* can facilitate the fermentation of carbohydrates and amino acids into acetate, hydrogen and $CO_2$ (Lee et al. 2013). *Fibrobacteres* bacteria play an important role in the conversion of lignocellulosic biomass in the biosphere (Ransom-Jones et al. 2014). After 115 d of treatment, *Nitrospirae*, *Deferribacteres* and *Chloroflexi* became the superior phyla in the T4 microcosm, as compared with the other microcosms. Huang et al. (2016) reported that *Nitrospirae* bacteria are associated with nitrite oxidation,

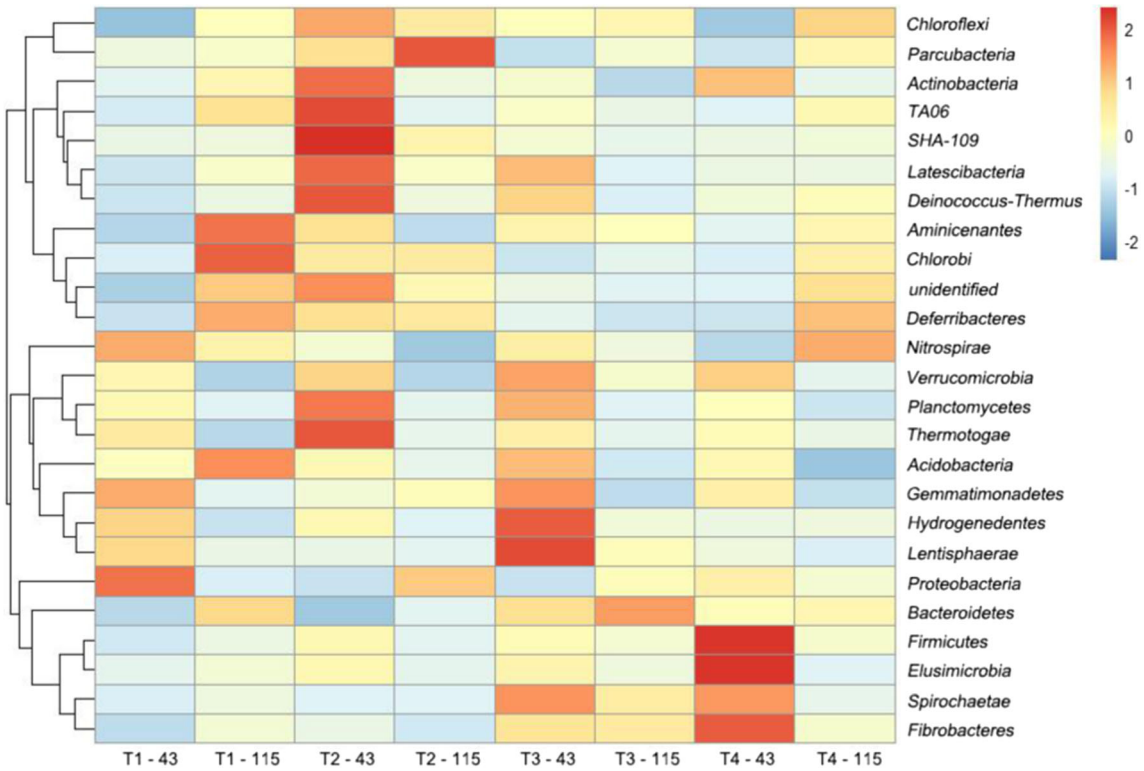


**Fig. 7** Heatmap showing the 25 most abundant microbial community phyla in the four microcosms, after 43 and 115 days of treatment. The samples were named using the style 'microcosm-experimental time', e.g. T1–43 refers to the sample collected in the T1 microcosm after 43 days of treatment. (T1 natural attenuation as the control, T2 bioaugmentation with aerobic denitrifiers, T3 biostimulation with denitrification agents and T4 a combination of aerobic denitrifiers and a biostimulant)

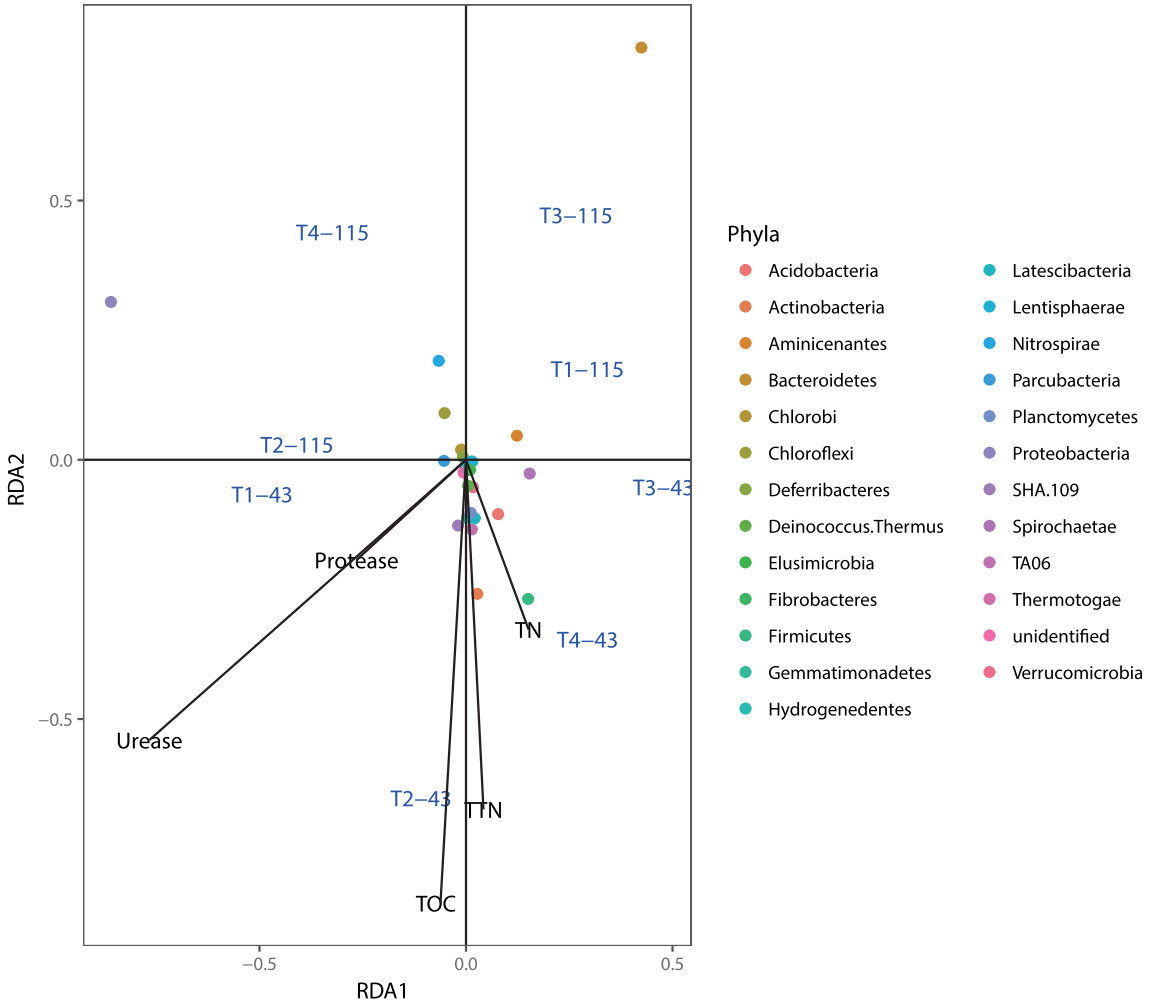


**Fig. 8** Redundancy analysis of the bacterial communities in the sediment based on phyla distribution. The samples were named using the style 'microcosm-experimental time', e.g. T1–43 refers to the sample collected in the T1 microcosm after 43 days of treatment. (T1 natural attenuation as the control, T2 bioaugmentation with aerobic denitrifiers, T3 biostimulation with denitrification agents and T4 a combination of aerobic denitrifiers and a biostimulant)

sulphur oxidation and sulphate reduction. *Chloroflexi* can induce anaerobic nitrogen removal and have the ability to utilise glucose and N-acetyl glucosamine under oxic and anoxic conditions (Miura et al. 2007). Gittel et al. (2012) found that the *Deferribacteres* possessed the functional potential to reduce nitrate. The dominant bacteria in the T4 microcosm were mainly associated with nitrogen cycling and thus contributed considerably to nitrogen removal in the sediment. Additionally, the introduced aerobic denitrifiers with low relative abundance observed did not become dominant in the sediment. Morgante et al. (2010) reported that bioremediation strategy might cause changes in indigenous community. This indicated that the introduced aerobic denitrifiers could stimulate the bacterial community shift and synergize with the indigenous microorganisms to achieve good effects on the removal of nitrogen.

A redundancy analysis (RDA) was performed to identify the relationships among the microbial functional communities, environmental variables and enzymatic activities based on the phyla level. Figure 8 showed that the majority of bacteria, such as *Actinobacteria* and *Firmicutes*, had a positive relationship with the TOC and TN concentrations in the sediment; whereas, *Proteobacteria*, *Bacteroidetes*, *Nitrospirae* and *Chloroflexi* showed a negative relationship with the concentrations of TOC and TN. Previous study showed that *Proteobacteria* play an important functional role in the sulphate and nitrate co-reduction process (Chen et al. 2017). Kampmann et al. (2012) reported that *Bacteroidetes* might play an important role in protein degradation and can adapt to rather low levels of nutrients. *Nitrospirae* and *Chloroflexi* have been found to be involved in nitrogen cycling in environment. The results also showed that the activities of urease and protease were in a positive relationship with the TTN,

instead of the total nitrogen. Moreover, there was a positive relationship between the activities of urease and protease and the abundance of the phylum *Proteobacteria*. This indicated the phylum *Proteobacteria* may play an important role in the synthesis and secretion of urease and protease. The microbial communities in the four microcosms, after 43 days of treatment (quadrants 3 and 4), was relatively different from the ones after 115 days of treatment (quadrants 1 and 2). A positive correlation appeared between the content of TOC and TN and the bacterial patterns of the sediment in the four microcosms after 43 days. However, the bacterial diversity patterns of the sediment in the four microcosms were inversely related to the pollution parameters, after 115 days of treatment. The results of the redundancy analysis suggest that the nitrogen and carbon sources were the key factors affecting the bacterial community function and composition in the sediment.

## Conclusions

In this study, aerobic denitrifiers coupled with a denitrification agent considerably enhanced nitrogen biodegradation when added to sediment and overlying water. Although most of nitrogen left in the sediment was characterised by low biodegradability, the combination technology had the effect of changing the stable forms into transferable nitrogen probably by stimulating the metabolism of indigenous microorganisms and changing the environment conditions in the sediment. As IEF-N was released from sediment to overlying water most easily, low concentrations of IEF-N have little impact on the overlying water. Furthermore, the changes of nitrogen content in the overlying water and sediment showed the good performance on the removal of nitrogen and lower secondary pollution in the overlying water. Increases of urease activity and the TTN content indicate further potential degradation of nitrogen in the sediment. Increase of bacterial diversity and variation of bacterial community structure guarantee sustainable remediation of the sediment, enhancing resistance to pollution load. Moreover, the direction of species succession was similar to that of natural succession, thus indicating that it is free of undesirable ecological risks. Therefore, aerobic denitrifiers combined with a biostimulant will be expected to be a promising strategy for improving nitrogen removal performance in sediment remediation. This study mainly focuses on the mechanism of the combination technology, showing good application potential for in situ bioremediation. In the future, pilot and field experiments will be also conducted to evaluate the effects for the in situ bioremediation engineering in an urban river.

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