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Field Research on Nitrogen Removal Performance of Aerobic Denitrifiers in Source Water Reservoir by Mixing Aeration

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ABSTRACT

Changes of total nitrogen (TN), organic matter, microbial metabolic activity, aerobic denitrifying bacteria and aerobic denitrifying genes (*nir*S and *nir*K) in water and surface sediments under mixing aeration were studied in Heihe Jinpen Reservoir. Results showed that compared with the control area, the total nitrogen concentration of water and surface sediments in the enhanced area decreased by 29.7%~49.2% and 17.9%. Metabolic activity of microorganisms increased by more than 14%, the utilization rate of carbon source increased by 30% and the McIntosh diversity index of microorganisms increased by 20%. The number of aerobic denitrifiers and genes of aerobic denitrifiers were also increased by 20%. Illumina high-throughput DNA sequencing results showed that the proportion of aerobic denitrifiers such as *Acidovorax* increased from 0.01% to 0.15%, showing an increase by 15 times. The production practice showed that mixed aeration can improve the metabolic activity and denitrification characteristics of indigenous aerobic denitrifiers, providing theoretical support and technical guarantee for denitrification and carbon removal of slightly polluted source water.

INTRODUCTION

More and more lakes and reservoirs have become the sources of drinking water in cities, and most lakes and reservoirs have problems of eutrophication of different degrees (Huang et al. 2014). Higher concentrations of nitrogen and phosphorus led to excessive algae reproduction, which not only endangers the aquatic ecosystem of lakes and reservoirs, but also threatens the safety of water supply. Excess nitrogen in the water also produced certain disinfection by-products, which directly affects human health (Kang et al. 2014). Physical, chemical and biological methods have been reported for nitrogen removal. However, both physical and chemical methods have problems of high cost and incomplete removal of nitrogen. More and more attention has been paid to biological nitrogen removal (Song et al. 2014, Sun et al. 2010, Patureau et al. 1998, Chen et al. 2015).

It is generally believed that nitrogen removal mainly depends on bacterial autotrophic nitrification and heterotrophic denitrification, and nitrification was carried out under aerobic conditions; denitrification was carried out under anaerobic conditions, and finally nitrogen was formed to N_2, achieving the purpose of removal (Patureau et al. 2001). Strict anaerobic condition was required for denitrification, which made denitrification process difficult to be used in drinking water treatment. Robertson (1982) first reported aerobic denitrification at the beginning of 1980s. Aerobic denitrifying bacteria using oxygen and nitrate as electron acceptors provided a new perspective for biological denitrification of drinking water. Aerobic denitrification and nitrification existed simultaneously in a system and can provide a certain basicity to balance the acidity produced by nitrification. Robertson separated aerobic denitrifying bacteria from desulfurization and denitrification wastewater treatment systems (Robertson et al. 1982). Other studies showed that aerobic denitrifying bacteria were also isolated from marine sediment (Gao et al. 2010) and constructed wetland system (Coban et al. 2015). In recent years, more and more aerobic denitrifying bacteria

have been isolated from lake sediments: three aerobic denitrifying bacteria H-30, X-10 and C-30 with total nitrogen removal rate of more than 80% under laboratory conditions were separated by Kang et al. (2014); 14 strains with high aerobic denitrification characteristics from reservoir sediments, mainly *Acinetobacter* spp. and *Nova* spp. were isolated and enriched by Zhou et al. (2016). The removal rates of TN (total nitrogen) and TOC (total organic matter) were 80.42% and 98.30% respectively. The isolation and screening of many aerobic denitrifying bacteria not only enriched and improved the theory of aerobic denitrification, but also laid a foundation for its engineering application (Huang et al. 2015, Xu et al. 2018, Epsztein et al. 2016, Alzate et al. 2016).

In order to improve the water quality, water-lifting aerators were installed in the reservoirs to supply oxygen to the water column, which provided a good experimental platform for the study of aerobic denitrification. Nitrate nitrogen concentration in the mixed aeration area decreased from 1.71 mg/L to 0.80 mg/L, and the TN removal rate reaching 38.33% were observed in a previous study (Zhou et al. 2016). Under the conditions of production scale of mixed aeration, the mechanism of water quality improvement, especially the removal of TN, was explored at the genetic level from the perspective of microorganisms, which provided theoretical support and technical support for the application of aerobic denitrifying bacteria in engineering and the nitrogen removal of water source reservoirs.

MATERIALS AND METHODS

Sampling Sites and Sampling Methods

As shown in Fig. 1, Heihe Jinpen Reservoir (latitude: 34°13′N- 34°42′N, longitude: 107°43′E-108°24′E) was a canyon shaped reservoir located at the foot of Qinling Mountains. Heihe Jinpen Reservoir was the important drinking water source of Xi'an City (its water supply accounted for 80% of the total drinking water of Xi'an City). The total reservoir capacity of Heihe Jinpen Reservoir is 2.0×10^8 m^3 and the effective reservoir capacity is 1.8×10^8 m^3. The main function of the reservoir was water supply and auxiliary functions were flood control and irrigation. Eight water-lifting aerators were installed in the main reservoir area. Detailed introduction is given in the previous report (Zhou et al. 2017). Enhanced area was in the middle of the main reservoir area, which was affected by the operation of the water-lifting aerator, represented by E. The control area was about 2 km away from the main reservoir area, and was less affected by water-lifting aerator, represented by C, as shown in Fig. 1.

Water samples were drawn every 3 days. Water temperature, dissolved oxygen (DO) and pH were measured *in situ* by HACH DS5 multi-parameter water quality analyser. Water quality indicators such as total nitrogen (TN), nitrate nitrogen, total phosphorus (TP) and total organic carbon (TOC) were measured in the reservoir field laboratory according to the "Water and Wastewater Monitoring and Analysis Methods (4^{th} edition)". Surface sediments were sampled once a week with a columnar sampler. Surface sediments of 0.5 cm were sampled and stored at 4°C.

Methods for Determination of Chemical Indicators of Water and Sediments

TN and nitrate nitrogen were determined by AA3 continuous flowing analyser (SEAL, Germany); TOC was determined by TOC-L analyser of Shimadzu, Japan. Sediment samples were dried, ground and sifted. TN in sediments was digested

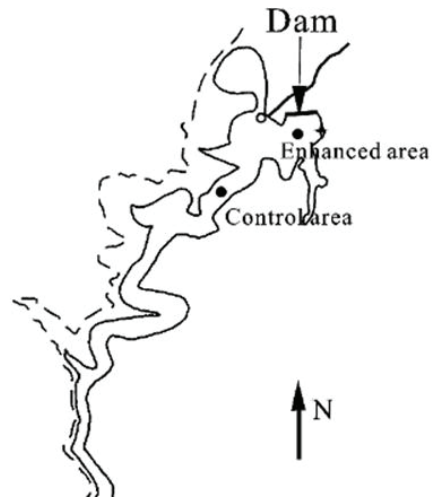

Fig. 1: Distribution map of mixing aeration area (Enhanced area) and reference area (Control area) of Heihe Jinpen Reservoir.

at 121°C for 1 hour and then determined by zinc-cadmium reduction spectrophotometry (Jiyeon et al. 2016). Organic matter in sediments was determined by potassium dichromate method (Jiyeon et al. 2016).

Methods for Determination of Microbial Metabolic Activity and Carbon Source Utilization

Microbial samples were collected at the same time as water samples for determination of chemical indicators. Microbial metabolic activity, carbon source utilization and McIntosh diversity index were measured by Biolog ECO microporous plate. Microbial metabolic activity was expressed by AWCD (average well colour development). AWCD = $\Sigma(C_{590} - C_{750})/31$. Carbon source utilization (dimensionless) was calculated by the average absorbance of microorganisms to each carbon source in each pore (Fang et al. 2016). For example, amino acid = $(C_{\text{L-arginine}} + C_{\text{L-asparagine}} + C_{\text{L-phenylalanine}} + C_{\text{L-serine}} + C_{\text{L-threonine}} + C_{\text{L-glutamic-acid}})/6$. McIntosh diversity index was also calculated by absorbance.

Aerobic Denitrifying Bacteria Count and Denitrifying Functional Gene Quantification

Through gradient dilution, the number of aerobic denitrifying bacteria in enhanced area and control area was determined by plate counting method. The preparation method of gradient dilution solution is as follows: 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 0.2 mL diluent was added to solid denitrification screening medium (The composition of the medium was: CH_3COONa, 0.10 $g \cdot L^{-1}$; $NaNO_3$, 0.02 $g \cdot L^{-1}$; $K_2HPO_4.3H_2O$, 0.02 $g \cdot L^{-1}$; $CaCl_2$, 0.01 $g \cdot L^{-1}$; $MgCl_2.6H_2O$, 0.01 $g \cdot L^{-1}$; agar, 20 $g \cdot L^{-1}$, and the pH was adjusted to 7.2 (Kang et al. 2018). Culturing of bacteria was carried out for 5 days at 30°C, then counted and calculated.

The copies of denitrifying functional genes *nir*S and *nir*K were determined by quantitative PCR. 2-L water samples were filtered by 0.22 μm filter membrane and extracted by a rapid DNA kit. The amplification sequence of *nir*S and *nir*K genes is given in Table 1. QPCR was tested by ABI 7500 of Life Technologies, USA, and the amplification reaction method was found in the previous study (Zhou et al. 2016).

Table 1: Primer sequences for gene amplification.

Gene	Primer sequence
*nir*S	cd3aF, 5′-GTSAACGTSAAGGARACSGG-3′
	R3cd, 5′-GASTTCGGRTGSGTCTTGA-3′
*nir*K	F1aCu, 5′-ATYGGCGGVCAYGGCGA-3′
	R3Cu, 5′-GCCTCGATCAGRTTRTGGTT-3′

Illumina High Throughput Sequencing

2-L water sample was filtered by 0.22 μm filter membrane and stored at ultra-low temperature. The determination was completed by Meiji Biology in Shanghai.

Operating Conditions of Water-Lifting Aerator

Destroying water stratification and improving water quality was the purpose of the operation of water-lifting aerator. In this stage, the algal density of surface layer of the reservoir was only about 200×10^4 cells/L, which was quite low, and there was no control of algae. The compressed air flow rate of each water-lifting aerator was 30-40 m^3/h, and the air supply of 8 water-lifting aerators was controlled in this range. During this researching period, 8 water-lifting aerators operated 24 hours a day, uninterruptedly. The power of the air compressor (Anruiko, Bengbu) was 132 kW, 2975 r/min, dual-purpose equipment.

RESULTS AND DISCUSSION

Changes of TN and Organic Compounds in Water and Surface Sediments

The basic situation of water quality in the control area and enhanced area before the operation of the water-lifting aeration system is given in Table 2. Before the operation of the system, water quality of the two areas tended to be consistent. For example, the results of October 10 water sample showed that the TN concentrations of the two areas were 1.81 $mg \cdot L^{-1}$ and 1.70 $mg \cdot L^{-1}$, respectively, and the difference between the two areas was only 0.11 $mg \cdot L^{-1}$, with a difference of about 6%. The TOC concentrations of the two regions were almost the same. The density of aerobic denitrifying bacteria (ADB) was 4.34×10^4 $cfu \cdot mL^{-1}$ and 4.03×10^4 $cfu \cdot mL^{-1}$, respectively, with a difference of 7%. According to the above analysis, there was a slight difference in the concentrations of TN, TOC and aerobic denitrifying bacteria between the two areas before the operation of the aeration system, and the difference was less than 10%.

In this study, the surface water temperature in the enhanced area decreased from 17.1°C (Oct-15) to 14.8°C (Nov-02), and the bottom water temperature increased from 10.1°C (Oct-15) to 14.3°C (Nov-02). DO increased from 0 $mg \cdot L^{-1}$ (Oct-15) to 9.8 $mg \cdot L^{-1}$ (Nov-02) in the enhanced area. While there was still a temperature difference of 6°C between the upper and lower water layers in the control area. On Nov-02, the concentration of DO in the control area was still 0 $mg \cdot L^{-1}$ (Nov-02) in the bottom water, which indicated that the operation of the water-lifting aeration system accelerated the damage of water stratification and had a significant effect on mixing aeration.

The variations of TN and organic matter concentrations in the enhanced area during operation are given in Table 3. The DO concentration in the water body, especially in the bottom water, increased gradually with the operation of the system. After 19 days of operation, TN and TOC in different water layers in the enhanced area reduced sharply. The TN concentration in the bottom water body decreased from 2.36 $mg \cdot L^{-1}$ to 1.20 $mg \cdot L^{-1}$, with a reduction rate of 49.2%, and the TOC reduction rate in vertical water body also reached more than 22.5%. Compared with enhanced area, in the control area the highest removal rate of TN was only 7.7%, and the removal rate of TOC 6.5%.

Compared with the control area, the removal rate of TN and TOC in the enhanced area was higher. In order to further explore that where nitrogen and organic matter gone, surface sediment samples were also taken under the corresponding water column of the sampling site and their TN and OC were determined. As presented in Table 3, the concentration of TN and OC in surface sediment also decreased by 17.9% and 20.5% respectively. However, the concentration of TN and OC in surface sediment of the control area was almost the same.

Based on the above results, not only in the water, but also in the surface sediment nitrogen and organic matter in the enhanced area have been greatly reduced, and the accumulation of nitrite nitrogen was not found. Previous studies showed that the activities of indigenous microorganisms and community structure have changed during this process, and the presence of aerobic denitrifying bacteria have been found. Biolog technology was used to explore metabolic activity of microorganisms in water and surface sediments and utilization of different carbon sources in this study.

Microbial Metabolic Activity and Carbon Source Utilization in Water and Surface Sediments

Biolog ECO microporous plate was used to measure microbial metabolic activity in water and surface sediments (gradient dilution 10^4 times) in enhanced area and control area, respectively, on Oct-15 at the beginning and on Nov-02 at the end of the operation of the water-lifting aeration system. Results are shown in Fig. 2, and the microbial metabolic activity of water and surface sediments in enhanced area have been greatly increased. For example, at 144-hour time, after 19 days of mixed aeration, the microbial metabolic activity in water increased by 14.3%, while that in the control area increased by only 3%. As for the surface sediments, the microbial metabolic activity in the enhanced area increased by 14.9%, while that in the control area increased by only 6%.

Thirty-one different carbon sources available to microorganisms were classified into 6 categories. The utilization rates of microorganisms in water and surface sediments were determined by using Biolog ECO microporous plate as given

Table 2: Distribution of TN, TOC and aerobic denitrifying bacteria in water and surface sediments in control area and mixing aeration area before operation of the system.

	Indicators	Sites	Oct-01	Oct-05	Oct-10
Water	TN ($mg \bullet L^{-1}$)	E	1.74	1.68	1.81
		C	1.68	1.65	1.70
	TOC ($mg \bullet L^{-1}$)	E	3.51	3.58	3.62
		C	3.60	3.58	3.56
	ADD ($\times 10^4$ cfu/$mg \bullet L^{-1}$)	E	4.32	4.35	4.34
		C	3.84	4.24	4.03

Table 3: Total nitrogen and organic matter concentrations in water and surface sediments and their reduction rates in mixing aeration area.

	Indicators	Sites	Oct-15	Oct-21	Oct-28	Nov-02	Reduction rate（%）
Water	TN (mg/L)	0.5m	1.72	1.48	1.39	1.21	29.7
		40m	1.75	1.56	1.42	1.23	29.7
		90m	2.36	2.09	1.53	1.20	49.2
	TOC (mg/L)	0.5m	3.69	3.02	2.87	2.72	26.3
		40m	3.42	3.09	2.88	2.65	22.5
		90m	4.02	3.56	3.08	2.85	29.1
Sediment	TN (mg/L)	0.5m	1532	1482	1314	1258	17.9
	OC (%)	0.5m	3.42	2.95	2.79	2.72	20.5

in Table 4. The results showed that the utilization rates of carbonyl compounds, amino acids, esters, alcohols, amines and carboxylic acids increased by 39.7%, 66.7%, 62.5%, 33.8%, 69.2% and 7.9%, respectively by the end of mixed aeration. In the control area, the utilization rate of different carbon sources increased by only 15%, and the utilization rate of some carbon sources by microorganisms decreased slightly. This may be due to the further anoxia of water body and the further decrease of water temperature. The utilization of different carbon sources by microorganisms in sediments showed the same trend as that of water body, and increased by 28.7%, 29.3%, 36.4%, 37.5%, 37.1% and 5.2% respectively. The above results were in accordance with the TOC determination, indicating that the utilization of organic matter in water and sediment was accelerated by the increase of microbial activity.

Changes of Aerobic Denitrifying Bacteria and Functional Genes

The number of aerobic denitrifying bacteria and the functional genes of aerobic denitrification (*nir*K and *nir*S) in control area and enhanced area were quantitatively determined. The results are shown in Fig. 3, the average number of aerobic denitrifying bacteria in water column of sampling site in enhanced area increased from 2.14×10^4 CFU/m.L^{-1} to 5.84×10^4 CFU/m.L^{-1}, while the number of aerobic denitrifying bacteria in control area maintained at 0.98×10^4 CFU/m.L^{-1} to 1.85×10^4 CFU/m.L^{-1} during the operation of the aeration system and showed a slight downward trend.

There were two forms of nitrite reductase with different structure but with the same function: copper-containing *nir*K and iron-containing *nir*S genes. As shown in Fig. 3, the copies of *nir*K and *nir*S genes in the mixed aeration region increase gradually from 7.18×10^3 μ.L^{-1} to 9.61×10^3 μ.L^{-1}, while the copies of *nir*K and *nir*S genes in the control region remained at 6.73×10^3 μ.L^{-1} to 7.65×10^3 μ.L^{-1}, showing no significant change in number.

Previous pilot-scale experiments showed that the number of aerobic denitrifying bacteria and the denitrifying functional genes (*nir*K and *nir*S) at the control area decreased (Zhou et al. 2016), but this phenomenon was not found in this study. This may be due to the lack of carbon source in the closed test (previous study), which led to the inhibition of microbial metabolism and reproduction. This study was conducted in the reservoir field site, and the exchange between water bodies would not lead to the lack of carbon source.

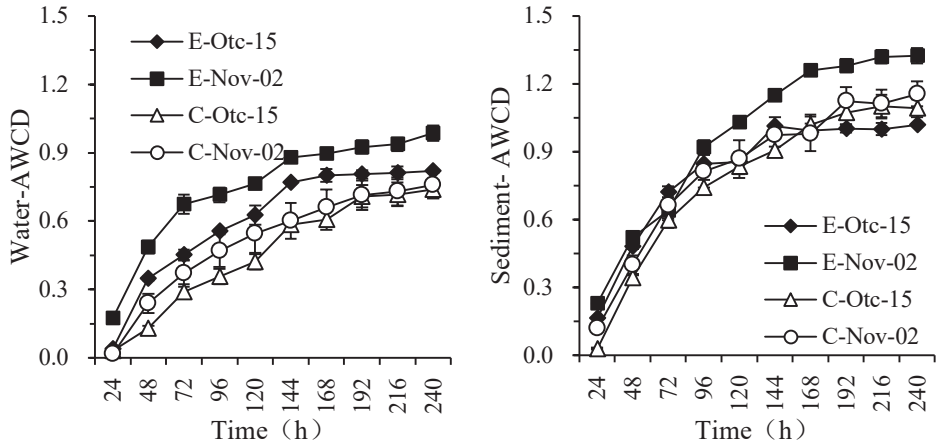

Fig. 2: Changes of AWCD in water and surface sediments in enhanced area (E) and control area (C).

Table 4: Utilization rate of carbon sources by microorganisms and U index changes in water and surface sediments in mixing aeration area.

Samples		carbonyl compounds	amino acids	esters	alcohols	amines	carboxylic acids	U index
Water	Oct-15	0.73	0.72	0.80	0.71	0.65	0.88	3.24
	Nov-02	1.02	1.20	1.30	0.95	1.10	0.95	4.28
	Increase proportion（%）	39.7	66.7	62.5	33.8	69.2	7.9	32.1
Sediment	Oct-15	0.94	0.85	0.99	0.88	0.89	1.05	5.21
	Nov-02	1.21	1.09	1.35	1.21	1.32	1.21	6.32
	Increase proportion（%）	28.7	29.3	36.4	37.5	37.1	15.2	21.3

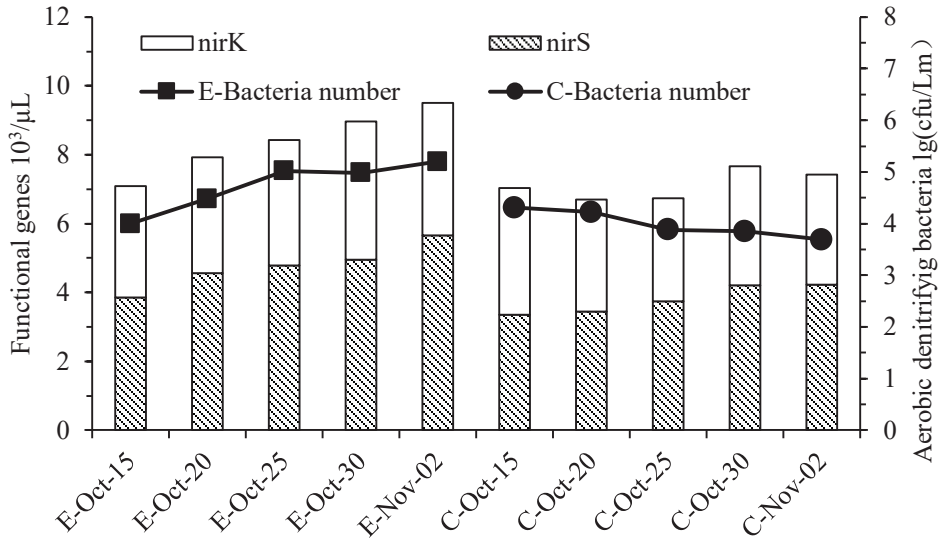

Fig. 3: Changes of aerobic denitrification genes and density of aerobic denitrifying bacteria in water in enhanced area (E) and control area (C).

High-throughput sequencing was used to analyse the proportion of aerobic denitrifying bacteria in the operation of the water-lifting aeration system. The results (Table 5) showed that after the operation of the system, the density of the main aerobic denitrifying bacteria *Acidovorax*, *Novosphingobium*, *Hydrogenophaga*, *Pseudomonas*, *Methylophaga*, *Bacteroidetes* and *Sphingomonadaceae* increased significantly. For example, the proportion of *Acidovaxor* increased from 0.01% before the operation of the system to 0.15%. The operation of water-lifting aeration system increased the proportion of aerobic denitrifying bacteria in total bacteria. This conclusion also helped to reveal the mechanism of denitrification nitrogen removal in water.

Principal Component/Redundancy Analysis of Environmental Factors and Microorganisms

Principal Component Analysis (PCA) is a powerful tool for analysing sample differences. In this paper, PCA was carried out based on environmental factors (water quality parameters) and microbial composition, respectively, to analyse the differences between enhanced and control areas in the operation of water-lifting aeration system. The results showed that the principal component analysis of the environmental factors and microbial composition can explain 95.41% and 87.19% of water quality and microbial composition respectively, which can well reflect the whole process. As shown in Fig. 4, at the beginning, there was no obvious difference in water quality and microorganisms composition between the enhanced area and the control area, and the distribution of sample sites in each depth was concentrated. However, with the operation of the aeration system, DO and T in the enhanced area changed, and the microbial population also changed. The difference between the enhanced area and the control area was more and more obvious. It was clear

Table 5: Variations of aerobic denitrifying bacteria in water in mixing aeration area (E) and control area (C).

Species/sampling sites-dates	E-Oct-15	E- Nov-02	C-Oct-15	C-Nov-02
Acidovorax	0.01%	0.15%	-	0.02%
Novosphingobium	0.01%	0.10%	0.01%	-
Hydrogenophaga	-	0.05%	0.02%	0.01%
Pseudomonas	0.01%	0.02%	0.01%	0.01%
Methylophaga	0.01%	0.06%	0.01%	-
Bacteroidetes	0.02%	0.02%	0.01%	0.03%
Sphingomonadaceae	0.01%	0.03%	0.02%	0.02%

Note: "-" means undetected

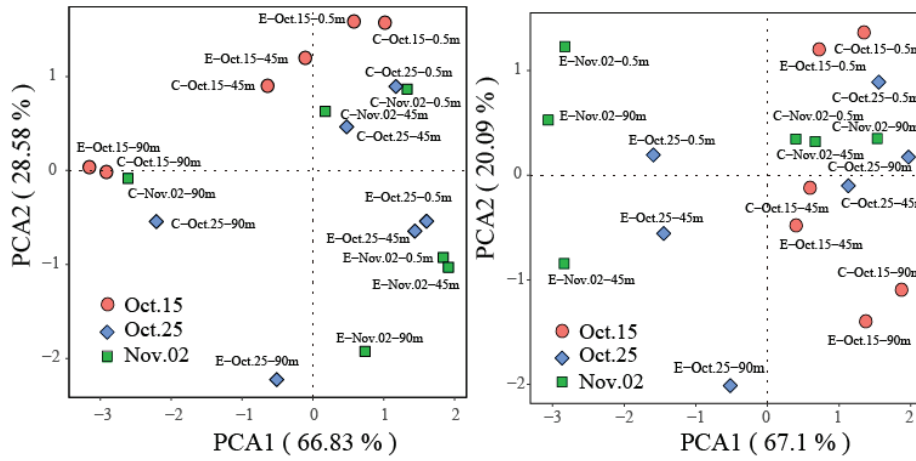

Fig. 4: Principal component analysis of environmental factors and microorganisms.

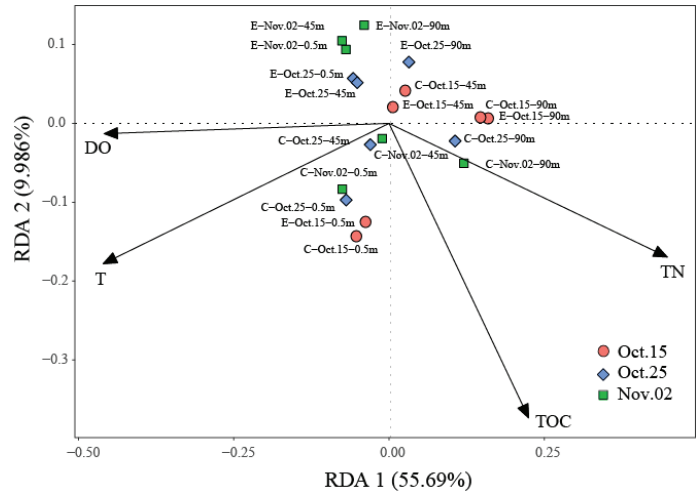

Fig. 5: Redundancy analysis of species and environmental factors during operation.

that water-lifting aeration can change water environment to regulate microbial community composition and finally improving water quality.

The relationship between microbial species and environmental factors is shown in Fig. 5. It was obvious that with the operation of the system, the differences between the enhanced area and the control area gradually increase. The correlation between environmental factors and microbial communities showed that DO, T and TN had a very significant correlation with RDA1 ($P < 0.001$), TOC and RDA1 had a significant correlation ($P < 0.05$). DO and T had a significant correlation with RDA1 ($P < 0.05$). The results of TN showed that the microorganisms in nitrogen cycle increase with the operation of water-lifting aeration. The water-lifting aeration system can control the microbial composition by changing the water environment, thus achieving nitrogen removal and improving water quality.

CONCLUSIONS

(1) *In-situ* water quality improving study was carried out in the water source reservoir at field scale. It was found that TN and TOC in the enhanced area reduced by 29.7%-49.2%, 22.5%-29.1%, and TN and OC in the surface sediment reduced by 17.9% and 20.5%, respectively, while the reduction rate in the control area was not obvious.

(2) Biolog ECO microporous plate was used to measure microbial activity and carbon utilization in water and surface sediments. It was found that mixed aeration

increased microbial metabolic activity by more than 14%, carbon utilization by more than 30% and microbial McIntosh diversity index by more than 20% in the enhanced area.

(3) The results of high-throughput sequencing and amplification of aerobic denitrifying functional genes (*nir*K and *nir*S) showed that the number of functional genes and their proportion of aerobic denitrifying bacteria in the enhanced area increased by the mixed aeration process.

(4) Mixed aeration can improve microbial metabolic activity, the number of aerobic denitrifying bacteria and the number of functional genes (*nir*K and *nir*S) and enhance their denitrification performance. Satisfying results of denitrification and carbon removal have been achieved in field experiments of reservoirs, which further enriched the mechanism and practice of denitrification and carbon removal in source water.

(5) In order to destroy the stratification of water body and the oxygen-filled the bottom in field research, the air supply can be controlled at 30-40 m^3/h from the point of view of energy saving.

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