Purification effect evaluation of the designed new volcanic soil adsorption material containing bioreactor for eutrophic water treatment

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Abstract
The purpose of this study was to investigate the purification effect of a new adsorption material containing bioreactor and the critical role of viable but non-culturable (VBNC) bacteria in a eutrophication ecosystem. Major water quality parameters of the prepared eutrophic water were determined, and the microbial community was analyzed during 2 years. The results showed that removal rates of total phosphorus (TP), total nitrogen (TN), chlorophyll-a (Chl-a), and chemical oxygen demand (COD) were 90.7–95.9%, 84.5–92.4%, 87.9–95.8%, and 68.3–82.7%, respectively, indicating the high efficiency of the bioreactor in the eutrophic water treatment. Although the bioreactor had been operated for 2 years, water from the treatment group was much clearer and odorless than from the control group, exhibiting the long service life of the bioreactor. Stopping operation in August caused significant decrease of the removal rates of major water quality parameters \((p < 0.05)\). This operational stop event and high temperature in summer exerted a dual effect on the bioreactor, whereas the impact could be minimized when the bioreactor was running. Moreover, the total bacteria under +Rpf (active resuscitation-promoting factor) treatment were higher than under −Rpf (inactive resuscitation-promoting factor) treatment, implying that Rpf could resuscitate VBNC bacteria in the eutrophication ecosystem. Nine strains of VBNC bacteria were isolated based on the BLAST results of the 16S rRNA gene. Also, these bacteria might contribute to the eutrophic water treatment based on their functions of phosphorus collecting and denitrification. These results provided new insights for engineering technology innovations, and consequently these findings had benefits in eutrophic water treatment.

Keywords Eutrophication · Microbial community · Water quality parameters · Removal rates · Viable but non-culturable (VBNC) bacteria · Resuscitation-promoting factor (Rpf)

Introduction
In recent decades, the aquatic ecosystem has suffered serious problems of water eutrophication due to anthropogenic pollution and climate change events (Le et al. 2010; Smith 2003; Kosten et al. 2012; Andersen et al. 2020; Freeman et al. 2020). Eutrophication is often accompanied by rapid occurrences of harmful algal blooms (HABs), especially of *Chlorella*, cyanobacteria (Wang et al. 2019a), and diatoms (Paerl et al. 2016, 2019; Huisman et al. 2018; Woolway and Merchant 2019; Kim et al. 2020), which can threat other aquatic life and change the color and/or odor of the water body (Mousavi and Khodadoost 2019). Therefore, eutrophic water can finally lose its original functionality, such as aquatic food production and safe-drinking water supply (Huisman et al. 2018). Moreover, eutrophication is a worldwide environmental
problem and has been occurring in many countries, such as Israel (Geisler et al. 2020), Mexico (Caballer and Vazquez 2020), and the USA (Tomasko et al. 2020). In fact, over 75% of the closed water bodies (e.g., lakes, ponds, and reservoirs) in Africa, Asia, and Latin America have deteriorated severely and experimented eutrophication events since the 1990s (UN-Water 2018).

The evaluation of the trophic state of eutrophic water has become a research hotspot in the water ecology community based on major water quality parameters, including total phosphorus (TP), total nitrogen (TN), chlorophyll-a (Chl-a), and chemical oxygen demand (COD) (Chao Rodriguez et al. 2014; Smith and Schindler 2009; Carlson 1977). Therefore, it has been reported that the excessive accumulation of N and P in an aquatic ecosystem is closely related to eutrophication, which can cause greater primary production (Ahlgren et al. 2005; Feuchtmayr et al. 2009) and hinder the restoration process of the eutrophic ecosystem (Banerjee 2016). Consequently, these events can result in hyper-eutrophication and subsequent water quality deterioration (Ahlgren et al. 2005). At the same time, Chl-a and COD are the key indicators of eutrophic water, which reflect the eutrophication level and the biomass of HABs uncovering the eutrophication phenomena. Therefore, the systematic monitoring of the trophic state in the aquatic ecosystem can effectively evaluate the degree of eutrophication and assess the feasibility of new technologies for eutrophic aquatic ecosystem restoration.

Previous studies in eutrophic aquatic ecosystem restorations reported that reductions of external nutrient inputs could fail to alleviate the eutrophication (Paerl et al. 2016; Wang et al. 2019a; Lurling and Mucci 2020). Also, since numerous studies have shown the importance of that effective removal of excessive N and P in the aquatic ecosystem balance (Gruber and Galloway 2008; Domangue and Mortazavi 2018; Stoliker et al. 2016), the potential impacts of this event should be taken into considerations for eutrophic water treatment (Paerl et al. 2019; Wang et al. 2019a; Qin et al. 2020). Therefore, physical, chemical (Schauer et al. 2003), and biological (Liu et al. 2012; Liu et al. 2014; Petersen et al. 2014; Wu et al. 2015; Dave and Modi 2019; Yi et al. 2020) methods had been adopted in extensive research about eutrophic water treatment during the past decades. However, it was difficult for these methods to meet the standards of high frequency for eutrophic water treatment, especially for projects with high wingspan due to financial and labor-intensive challenge reasons, biotic and abiotic restrictions, and high risk of secondary pollution (Liang et al. 2014). Therefore, new ecological techniques have been considered to be the most promising technologies in the eutrophic water treatment.

Recently, several studies have reported that new adsorption materials and microbial communities also played important roles in water purification (Ding et al. 2011; Zhou and Wang 2010). Simultaneously, viable but non-culturable (VBNC) bacteria were frequently found in dyeing workshops (Jin et al. 2017), pharmaceutical wastewater (Li et al. 2014), and polychlorinated biphenyls (PCBs) (Su et al. 2014) polluted soils in recent years. Moreover, the VBNC bacteria can survive under extreme environments by converting into the viable but non-culturable, and these bacteria can become culturable by the addition of the resuscitation promoting factor (Rpf) (Ding et al. 2011; Mukamolova et al. 2002; Serpaggi et al. 2012). Previous studies had certified that several VBNC bacteria could be recovered by Rpf, including high G + C gram-positive bacteria, low G + C gram-positive bacteria, and gram-negative bacteria (Ding et al. 2011; Yu et al. 2015). However, whether VBNC bacteria survive in eutrophic water and VBNC bacteria possess certain environmental purification functions have not been reported until now.

The purpose of this study is to design a multi-stage tandem type bioreactor containing a new volcanic soil adsorption material. The purification effect of the bioreactor on eutrophic water (TP, TN, Chl-a, and COD) and the functional microbial communities, especially for VBNC bacteria, has been assessed and analyzed based on seasonal changes. This work contributed to further understand the microbial clustering in the sewage treatment systems and provided new insights for enhancing the efficiency of eutrophic water treatment by improving the engineering technology.

Materials and methods

Preparation of the eutrophic water samples

The eutrophic water sample for the experiment was composed of a synthetic nutrient matrix mixed with raw water from a eutrophic lake (Xinyue Lake, Jinhua, China). The ratio of synthetic nutrient matrix and raw water was 97:3 (V/V). The initial compositions of the synthetic nutrient matrix were as follows: beef extract (1.0 g), yeast extract (1.0 g), K2HPO4 (0.272 g), KH2PO4 (0.456 g), and water (1.0 L). The initial water quality parameters of the eutrophic water sample were TP = 22.4 mg/L, TN = 16.5 mg/L, Chl-a = 218.5 μg/L, and COD = 202.4 mg/L. The initial pH value of the eutrophic water sample ranged from 10 to 11. The eutrophic water sample (100 L) in the recycling treatment of the bioreactor was denoted as the treatment group, and another eutrophic water sample (100 L) was considered the control group and left under natural conditions.

The novel adsorption material

The new adsorption material in the bioreactor was a mixture of several efficient adsorption materials, which was mainly composed of volcanic soil, bentonite, and zeolite. The volcanic soil came from around 1 m below the mountain topsoil of...
Niigata, Japan. The main mineral elements of the new material (the volcanic soil sample) were CaO, MgO, K_2O, SiO_2, H_3PO_4, Fe_2O_3, Al_2O_3, and N (Table S1). The new adsorption material had a granular appearance, and its diameter ranged from 2 to 4 mm visualized in a scanning electron microscope (25,000×) (Olympus, Japan) (Fig. 1). The surface area of 1 L novel adsorption material can be up to 21,476 m^2, and it is suitable to set a microbial habitat in the micron-scale holes. Therefore, when sewage flows through the bioreactor, the new adsorption materials can effectively absorb chemicals and biostimulate the microbial community.

**Construction and operation of the bioreactor**

The self-designed bioreactor was composed of nine 550-mL tower tanks, and every group of three were grouped in series (Fig. 2). The eutrophic water sample flowed into the bottom of the first tower tank and transferred into the bottom of the subsequent tower tank from the top of the former tank after flowing through the filter plate and the new adsorption material (200 g). Then, the bioreactor-treated water returned into the reservoir through a reflux pipe, and it was mixed for the next purification.

Usually, eutrophication occurs in spring, summer, autumn, and in less degree in winter, so three running and two stopping stages were established during the experiment. A 2-year stable operation scheme of the bioreactor was enacted as follows: 1 year running before summer—operational stop in summer—running in autumn—stopping in winter—running after winter. The details for the actual operation and the environmental temperature are shown in Table 1. In this study, FY, SY, and TY represented the first year, the second year, and the third year of the experiment, respectively.

**Water quality parameters detection**

The major water quality parameters of eutrophic water samples were detected every 3 months. Three replicates were set for the control and treatment groups. TP and TN contents were measured by the ammonium molybdate spectrophotometric method based on the Chinese National Standards GB11893-89 (1990) and HJ636-2012 (2012), respectively. Chl-a concentration was detected by spectrophotometry (Hong et al. 2008; Xu et al. 2021). The COD of samples was analyzed based on the HACH vial-high range (20 mg/L to 1500 mg/L) Method 8000 (HACH Company 2019).

**TP detection**

The eutrophic water sample was adjusted to neutral, and water sample (25 mL) was then transferred into a 50-mL plug scale tube. About 4 mL of a K_2S_2O_8 solution was added into the plug scale tube for digestion in the autoclave (MLS-3750, SANYO, Japan). Two hours later, when the temperature dropped to 80°C, the plug scale was removed and diluted to the tick mark with ultrapure water. Also, ascorbic acid (1 mL) was added to the diluted digestion solution and mixed completely. Thirty seconds later, 2 mL of a molybdate solution was added to the plug scale and placed for 15 min at room temperature. The absorbance was determined at 700 nm by using UV spectrophotometry (UV-7504, Xinmao, China). The TP content was calculated according to the following formula:

\[
TP \text{ (mg/L)} = \frac{m}{V} \tag{1}
\]

where “m” was the P content in the water sample (μg), and “V” was the volume of the water sample (mL).

**TN detection**

The pH of the eutrophic water sample was adjusted to 5–9, and then 10 mL of a water sample was transferred into a 25-mL colorimetric tube. After, 5 mL of a solution with alkaline potassium persulfate was added into the colorimetric tube for digestion in the autoclave (MLS-3750, SANYO, Japan). The colorimetric tube was taken out and cooled down when the pressure dropped to 80°C. One milliliter of hydrochloric acid (V_HCl:V_H2O = 1:9) was added into the digestion solution and diluted to the tick mark with ultrapure water. Then the samples were mixed thoroughly. The absorbance was determined at 200 and 275 nm by using UV spectrophotometry (UV-7504, Xinmao, China) with water as the reference solution. The absorbance was calculated as follows: \( A = A_{220} - A_{275} \). Also, the content of TN was calculated according to the following formula:

\[
TN \text{ (mg/L)} = \frac{m}{V} \tag{2}
\]

where “m” was the N content in the water sample (μg), and “V” was the volume of the water sample (mL).
When the volume of the water sample was 10 mL, the detection limit and determinate range of this method were 0.05 mg/L and 0.20–7.0 mg/L, respectively. The standard curve equation is shown in Fig. S2, where the correlation coefficient (r) was 0.9992.

**Chl-a detection**

First, the eutrophic water sample (1000 mL) and 1% MgCO₃ (5 mL) were mixed completely, and 200 mL of the mixture was filtered with 0.45-μm glass fiber filter film. After the filtration, the filter film was clamped out with clean tweezers and folded in half twice. Then, the filter film was added into a centrifuge tube with 10 mL 95% ethanol and incubated at 4°C for 20 h. The centrifuge tube was centrifuged at 3000 rpm for 15 min (VC-15SP, Takara, Japan), and then the absorbance was determined at 649, 665, and 750 nm by using UV spectrophotometry (UV-7504, Xinmao, China). The content of Chl-a was calculated according to the following formula (Wintermans and Mots 1965):

\[
\text{Chl-a} = \left(13.7 \times (A_{665} - A_{750}) - 5.76 \times (A_{649} - A_{750})\right) \times (E/F) \times L
\]

where “E” was the extraction volume (mL), “F” was the filtration volume (L), and “L” was the width of the cuvette (mm).

The detection limit of this method was lower than 0.025 μg/L (Hong et al. 2008).

**COD detection**

The eutrophic water sample (2.5 mL), K₂Cr₂O₇ solution (1.5 mL), and Ag₂SO₄ solution (3.5 mL) were mixed completely in a HACH tube. Then, the mixture was digested under 150°C for 2 h, and the absorbance was determined after cooling down 30 min for COD testing (DR1010, HACH, USA).

The wavelength accuracy and photometric linearity were ±1 nm and ±0.002 A (0–1 A), respectively. The repeatability of the photometric measurement was ±0.005 A (0–1 A). The error range was changed with the value of COD and the standard was 15–150 mg/L ≤ 8% and 100–1000 mg/L ≤ 4%. The standard curve equation is shown in Fig. S3.

**Data processing**

The removal rates were calculated through the following equation:

\[
R = \left(1 - \frac{C_{t}}{C_{0}}\right) \times 100\%
\]  

### Table 1: Operation status of the bioreactor.

| Duration    | Stage     | FR (mL/s) | HRT (h) | Temperature (°C) |
|-------------|-----------|-----------|---------|------------------|
| June (FY)–May (SY) | Running  | 0.85      | 1.8     | 0–34             |
| June (SY)–August (SY) | Stopping | 0.00      | 0.0     | 24–34            |
| September (SY)–November (SY) | Running | 0.85      | 1.8     | 15–23            |
| December (SY)–February (TY) | Stopping | 0.00      | 0.0     | 0–15             |
| March (TY)–August (TY) | Running  | 0.85      | 1.8     | 15–34            |

Note: Geographical location of Jinhua: 29° 00′ 17.37″ N, 119° 29′ 54.84″ E
where “R” was the removal rate (%) of the major water quality parameter, and “C” and “C₀” were the concentrations of the major water quality parameter in the treatment and control group, respectively. A T-test was performed using the SPSS 19.0 software. The results were expressed as the mean ± SD (standard deviation).

**Assessment of the most probable number (MPN) method for bacteria identification in the bioreactor**

Bacteria were isolated from the bioreactor using the most probable number (MPN) method (McCray 1915). The VBNC bacteria were isolated by adding Rpf proteins from *Micrococcus luteus* culture supernatants (Ding et al. 2012; Su et al. 2014). The liquid medium consisted of peptone (5.0 g), yeast extract (0.5 g), glucose (5.0 g), sodium chloride (2.5 g), and distilled water (1.0 L). The medium was kept within a pH of 7.0–7.2, including the active Rpf (+Rpf) or inactive Rpf (−Rpf) factors, and the volume ratio of liquid medium and +Rpf−Rpf was 4:1. Three replicates were set for both +Rpf and −Rpf treatments. The bacterial solution was prepared by mixing 1 g of the new adsorption material and 9 mL of 0.9% saline solution. After, this bacterial solution (0.25 mL) and liquid medium (2.25 mL) were mixed. Then, the mixture was gradually diluted from 10⁻¹ to 10⁻⁷ with the prepared liquid medium. The bacterial solution with different dilutions was incubated at 30°C, and the turbidity of the bacteria solution was measured by a microplate reader at the wavelength of 660 nm (OD 660). When the microbiota was in the stationary phase, turbidity or non-turbidity of the tube was recorded as positive and negative. The total number of bacteria under +Rpf and −Rpf treatments were determined by the MPN table.

**Rpf effect and VBNC bacteria status evaluation**

VR was the ratio of the total amount of bacteria under +Rpf treatment and under −Rpf treatment, and it was used to evaluate the activity abundance of Rpf. A VR value greater than 5 indicated that there had dominant bacteria in the VBNC state, which was sensitive to Rpf.

Also, a denaturing gradient gel electrophoresis (DGGE) of the diluted bacteria solution from 10⁻¹ to 10⁻⁶ in +Rpf and −Rpf treatments were performed to identify the presence of VBNC bacteria. Genomic DNA was extracted by using the Takara® MiniBEST Bacterial Genomic DNA Extraction Kit Ver.2.0 (Takara Bio Inc., Dalian, China), and the 16S rDNA V3 region was amplified by the following primers: 8F, 5'-AGAGTTTGATCCTGGCTCAG-3', and 1492R, 5'-GGCTACCTTGTTACGA-3' (Turner et al. 1999). The sequences of 16S rRNA gene were blasted in NCBI (http://www.ncbi.nlm.nih.gov/) and EzTaxon server (http://www.eztaxon.org/) to identify the genera of bacteria. The phylogenetic tree of all isolated bacteria was constructed by MEGÀ 5.0. The neighbor-joining method was selected (Saitou and Nei 1987). The evolutionary distance matrix was calculated using Kimura’s two-parameter method (Kimura 1980), and the bootstrap values were set for 1000 replications (Felsenstein 1985).

**Results**

**Removal effect of TP in the bioreactor**

The TP concentrations in the eutrophic water sample (both in the control and treatment groups) and the removal rate of the bioreactor were shown in Fig. 3. The TP concentrations ranged from 0.91 to 2.06 mg/L and 20.09 to 23.38 mg/L in the treatment group and control group, respectively. All the TP concentrations in the treatment group were significantly lower than in the control group (p < 0.05), independently if the bioreactor was running or stopping. In addition, the TP removal rates of the bioreactor ranged from 90.7 to 95.9%, indicating that the bioreactor could efficiently reduce the TP concentration of the eutrophic water. For instance, the TP removal rate of TY in February was 92.8%, and it was significantly lower than the removal of SY in November (94.2%) and TY in May (94.4%) (p < 0.05). Therefore, this finding could indicate that both the operational stop of the bioreactor affected TP elimination. Meanwhile, the TP removal rate of SY in August was 90.7%, but it was significantly lower than the other months (p < 0.05). This event could suggest that both the operational stop of the bioreactor and high temperature were the two key factors for influencing the TP removing. Interestingly, the TP removal rate of SY in March (95.9%) was significantly higher than the other detection months (p < 0.05), implying that the bioreactor had the best purification effect in spring.
p
SY in August was 84.5% and was significantly lower than the other detection months. Different capital letters indicate significant differences at $p<0.05$ level between control and treatment groups at the same detection month. The black bar in treatment group represented the TP concentrations of the eutrophic water sample when the bioreactor was stopping.

**Removal effect of TN in the bioreactor**

Figure 4 shows the TN concentrations in the eutrophic water sample (both in the control and treatment groups) and the removal rate of the bioreactor. In general, the TN concentrations ranged from 1.32 to 2.44 mg/L in the treatment group and 13.86 to 17.50 mg/L in the control group. All the TN concentrations in the treatment group were significantly lower than in the control group ($p<0.05$), indicating that the bioreactor could effectively reduce the TN concentration of the eutrophic water. In fact, the TN removal rate of the bioreactor ranged from 84.5 to 92.4%, which could imply that the bioreactor could efficiently reduce the TN concentration in the eutrophic water sample. The TN removal rates of the treatment group were significantly lower than in the control group ($p<0.05$), at both events (i.e., operation or no operation of the bioreactor). Moreover, the TN removal rate of SY in August was 87.9%, which was significantly lower than SY in November (95.2%) and TY in May (95.8%) ($p<0.05$). This finding could indicate that the operational stop affected the TN elimination. Meanwhile, the TN removal rate of SY in August was 87.9%, which was significantly lower than the other detection months ($p<0.05$). Therefore, both the operational stop and the high temperature exerted a double effect on the TN removal. Furthermore, the TN removal rate of SY in March (95.6%) was significantly higher than the other detection months, except for FY in September (91.9%) and TY in May (91.8%), suggesting that the bioreactor had the best purification effect in spring and autumn.

**Removal effect of Chl-a in the bioreactor**

The Chl-a concentrations in the eutrophic water sample (both in the control and treatment groups) and the removal rate of the bioreactor are shown in Fig. 5a. The Chl-a concentrations ranged from 8.41 to 28.46 μg/L and 200.11 to 234.38 μg/L in the treatment group and control group, respectively. All the Chl-a concentrations in the treatment group were significantly lower than in the control group ($p<0.05$) at all time during both the operation and the non-operation periods. The Chl-a removal rates of the bioreactor ranged from 87.9 to 95.8%, which could imply that the bioreactor could efficiently reduce the Chl-a concentration of the eutrophic water. The Chl-a removal rate of TY in February was 93.7%, but it was significantly lower than SY in November (95.2%) and TY in May (95.8%) ($p<0.05$). This finding could indicate that the operational stop affected the Chl-a elimination. Meanwhile, the Chl-a removal rate of SY in August was 87.9%, which was significantly lower than the other detection months ($p<0.05$). Therefore, both the operational stop and the high temperature exerted a double effect on the Chl-a removal. Furthermore, the Chl-a removal rate of SY in March (95.6%) was significantly higher than the other detection months, except for SY in November (95.2%) and TY in May (95.8%). Thus, the bioreactor had the better purification effect in spring and autumn. Figure 5b shows the comparison of water samples between the control and treatment groups.

**Removal effect of COD in the bioreactor**

Figure 6 shows the COD concentrations in the eutrophic water sample (both in the control and treatment groups) and the removal rate of the bioreactor. The COD concentrations ranged from 32.13 to 45.11 mg/L and 185.26 to 208.42 mg/L in the treatment group and control group, respectively. All the COD concentrations in the treatment group were significantly lower than in the control group ($p<0.05$) during the whole operation and non-operation events. The COD removal rates of the bioreactor ranged from 68.3 to 82.7%, showing that the bioreactor could efficiently reduce the COD.
concentration of the eutrophic water. The COD removal rate of TY in February was 78.6% and was significantly lower than SY in November (82.7%) and TY in May (81.3%) \( (p<0.05) \), suggesting that bioreactor stopping operation affected the COD elimination. Meanwhile, the COD removal rate of SY in August was 68.3%, and it was significantly lower than SY in November (82.7%), suggesting that the bioreactor had a better purification effect in spring and autumn.

The Chl-a concentrations in control and treatment group as well as the Chl-a removal rate of the bioreactor; b the real comparison between control and treatment group. Different small letters indicate significant differences at \( p < 0.05 \) level of LSD test of the removal rate under different detection months. Different capital letters indicate significant differences at \( p < 0.05 \) level between control and treatment groups at the same detection month. The black bar in treatment group represented the Chl-a concentrations of the eutrophic water sample when the bioreactor was stopping

**The OD\textsubscript{660} value of bacteria solution in both +Rpf and −Rpf treatments**

Bacteria were cultivated by using the MPN method in this study. The OD\textsubscript{660} values in the stationary phase of the bacterial solution are shown in Table 2. Moreover, no significant difference of OD\textsubscript{660} values was observed between +Rpf and −Rpf treatment at the 10\textsuperscript{-1} and 10\textsuperscript{-3} dilutions, respectively. However, the OD\textsubscript{660} values from 10\textsuperscript{-4} to 10\textsuperscript{-6} were continuously higher in the +Rpf treatment than in the −Rpf treatment, revealing that both number and bacterial species increased in function of Rpf addition. Also, the total number of bacteria attached to the new adsorption material in the +Rpf and −Rpf treatments were 2.4 × 10\textsuperscript{9} and 1.1 × 10\textsuperscript{8} cells/g, respectively. Interestingly, the V\textsubscript{R} value was 21.8, indicating that there was Rpf-sensitive dominant bacteria in the VBNC state.

**The DGGE analysis of bacterial solution in both +Rpf and −Rpf treatment**

Figure 7 shows the PCR results of the 16S rDNA V3 region in the MPN culture system (same as the previous experiment). The target bands were clear and bright, indicating that the PCR product could be used for DGGE analysis after purification.

The DGGE analysis showed that the +Rpf treatment had more bands than the −Rpf treatment, especially at the dilution from 10\textsuperscript{-2} to 10\textsuperscript{-6} (Fig. 8). This result agreed with the OD\textsubscript{660} values, indicating that Rpf had an effect on promoting bacterial resuscitation in the eutrophic water treatment system. Moreover, the bands of the +Rpf treatment at the 10\textsuperscript{-3}, 10\textsuperscript{-5}, and 10\textsuperscript{-6} dilutions appeared on the top of the lanes, whereas the bands of the same treatment at 10\textsuperscript{-2} appeared at the bottom of the lane. Therefore, these bacteria could compete with each other and could not co-exist at the same dilution.
Blast results of the 16s rDNA PCR products

In this study, 24 strains of bacteria were isolated from the bioreactor (Table 3). The isolated bacteria belonged to 11 genera, including *Bacillus*, *Brevibacillus*, *Burkholderia*, *Enterobacter*, *Lysinibacillus*, *Microbacterium*, *Micrococcus*, *Ochrobactrum*, *Paenibacillus*, and *Pseudomonas*. Most of the isolated bacteria were low G + C gram-positive (50%), followed by gram-negative bacteria (42%), and high G + C gram-positive bacteria (8%). Therefore, Rpf not only promoted the resuscitation of gram-positive bacteria, but also activated gram-negative bacteria in the bioreactor. Moreover, nine strains of VBNC bacteria were native from four genera, including *Bacillus* (3), *Burkholderia* (2), *Enterobacter* (2), and *Pseudomonas* (2). Figure 9 shows the phylogenetic tree.

Discussion

Efficient purification capacity of the new adsorption material in the bioreactor

Previous studies have shown that TP, TN, Chl-a, and COD were the major water quality parameters for evaluating the trophic state of eutrophic water (Carlson 1977; Chao Rodriguez et al. 2014; Smith and Schindler 2009; Zhang et al. 2020). Moreover, excessive accumulation of N and P in the aquatic ecosystems not only can cause high primary production levels (Ahlgren et al. 2005; Feuchtmayr et al. 2009), but also can slow the restoration process of the water ecosystem (Banerjee 2016). Consequently, this event can result in eutrophication and HABs occurrences. In addition, these previous studies showed that both Chl-a and COD were the key indicators for reflecting the degree of eutrophication. Therefore, systematic monitoring of the trophic state of eutrophic water could effectively assess the purification effect of the new adsorption material containing bioreactor. In this 2-year monitoring study, four major water quality parameters were assessed by real-time detection. The results showed that the self-designed bioreactor could efficiently remove TP, TN, Chl-a, and COD, independently if the bioreactor was running or not operating. In fact, the average removal rates of TP, TN, Chl-a, and COD reached 93.6%, 89.6%, 93.4%, and 78.5%, respectively. Also, the bioreactor revealed a long service life regardless of the high removal rate of these quality parameters. Although the bioreactor had been operating for 2 years, the...
Water from the treatment group was clear and odorless, whereas the water from the control group was cloudy and odorous. Moreover, the removal rate of other reported bioremediation methods could easily be affected due to temperature and light intensity. For example, this event was reported in aquatic plants and macrophytic algae restoration systems (Zuo et al. 2014; Xue et al. 2011) and in an Ipomoea aquatica with low-energy ion implantation system (Li et al. 2009). However, the removal rates of the designed bioreactor in this study were less influenced when the bioreactor was running, especially for TP, TN, and Chl-a, in which the removal rates were approximately or greater than 90%. Furthermore, when compared with other eutrophic water treatment systems, the bioreactor also revealed a better purification effect than these systems, such as the constructed wetlands system (Zhao et al. 2012), the integrated floating island system (Lu et al. 2014), the restoration of animals and plants system (Hua et al. 2008), and the combination treatment of bacteria and plants system (Hua et al. 2010). Therefore, the bioreactor designed in the present study could meet the demand in a large-scale eutrophication ecosystem restoration.

Season changes and operational affect the removal rates of the bioreactor

Several studies reported that the climate alteration and season changing were the key factors for water eutrophication (Cardoso-Silva et al. 2020; Le et al. 2010; Smith 2003). Furthermore, water temperature and nutrient availability were believed to be two of the most important factors in facilitating occurrences of HABs (Beaulieu et al. 2013; Rigosi et al. 2014; Tong et al. 2019). Therefore, understanding the potential impacts of water temperatures on eutrophication and their interactions with seasonal changes were crucial to assess the stability of the designed bioreactor. In this study, a continuously running process of the bioreactor was designed in the first year, and two operational stops (summer and winter) were applied in the next year. By doing this, the effect of the season changing on the purification of the bioreactor could be comprehensively evaluated. According to these results, the removal rates of the four major water quality parameters of TY in February were significantly lower than SY in November and TY in May ($p < 0.05$), indicating that the operational stop

### Table 3 BLAST results based on the 16S rDNA gene sequences. The “*” marked strains belonged to VBNC bacteria

| Genera               | Isolated strains | VBNC strains | Isolated bacteria       | Results of blast                  | Similarity (%) |
|----------------------|------------------|--------------|-------------------------|-----------------------------------|----------------|
| Bacillus             | 9                | 3            | JLR42 Bacillus thuringiensis | 99.8                             |                |
|                      |                  |              | JLN43 Bacillus anthracis  | 100                              |                |
|                      |                  |              | JLR52 Bacillus cereus*    | 100                              |                |
|                      |                  |              | FJHR63 Bacillus pumilus   | 99.5                             |                |
|                      |                  |              | FJHN63 Bacillus pumilus   | 99.9                             |                |
|                      |                  |              | SFGR63 Bacillus pumilus*  | 99.9                             |                |
|                      |                  |              | FJHR61 Bacillus sp.       | 99.6                             |                |
|                      |                  |              | FJHN62 Bacillus sp.       | 99.6                             |                |
|                      |                  |              | JLR51 Bacillus thuringiensis* | 100                            |                |
| Brevibacillus        | 1                | 0            | JLR44 Brevibacillus parabrevis | 100                             |                |
| Burkholderia         | 3                | 2            | SFGR61 Burkholderia cepacia* | 99.7                            |                |
|                      |                  |              | FJFR61 Burkholderia cepacia* | 99.7                            |                |
|                      |                  |              | FJFN61 Burkholderia cepacia | 99.8                             |                |
| Enterobacter         | 2                | 2            | SFGR62 Enterobacter ludwigi* | 99.8                            |                |
|                      |                  |              | FJFR64 Enterobacter sp.*  | 99.9                             |                |
| Lysinibacillus       | 1                | 0            | WQSR5 Lysinibacillus macroides | 99.7                            |                |
| Microbacterium       | 1                | 0            | FJFR65 Microbacterium dextranolyticum | 99.8                         |                |
| Micrococcus          | 1                | 0            | FJFN62 Micrococcus yunnanensis | 99.8                            |                |
| Ochrobactrum         | 1                | 0            | WQSR6 Ochrobactrum medium | 99.5                             |                |
| Paenibacillus        | 1                | 0            | JLR45 Paenibacillus polymyx | 99.9                             |                |
| Pseudomonas          | 3                | 2            | FJFN63 Pseudomonas nitritireducens | 99.9                           |                |
|                      |                  |              | FJFR62 Pseudomonas sp.*   | 97.6                             |                |
|                      |                  |              | FJFR63 Pseudomonas sp.*   | 99.4                             |                |
| Sphingomonas         | 1                | 0            | FJHN61 Sphingomonas aquatilis | 99.6                            |                |
could significantly influence the purification effect of the bioreactor, despite that eutrophication was less likely to occur in winter. Furthermore, the removal rates of the four major water quality parameters of SY in August were significantly lower than the other detection months ($p < 0.05$), implying that both the operational stop and the high temperature in summer could affect the removal rates of the bioreactor. Warming was the most significant changing event of surface water bodies in summer (Rigosi et al. 2014; Piccolroaz et al. 2020), where usually phytoplankton thrive. Moreover, some functions of aquatic ecosystems such as biochemical transformations of nutrients were altered in summer (Wu et al. 2017; Ding et al. 2018; Jenny et al. 2020). In fact, this event could create a suitable environment for phytoplankton surviving (e.g., cyanobacteria) and resulted in the occurrence of HABs (Paerl et al. 2016; Freeman et al. 2020). From these results, although both the operational stop and the high temperature affected the removal rates of the bioreactor, these events had little influence on the purification effect when the bioreactor was running. Therefore, this finding showed the successful performance of the bioreactor. Also, the removal rates of the four major water quality parameters of SY in March and December were always higher than the other months, suggesting that the bioreactor achieved the best purification effect in spring and autumn.

**The potential ability of VBNC bacteria in the restoration of the eutrophication ecosystem**

The present study reported that the eutrophication level was well associated with the activities of bacteria in the aquatic ecosystems, such as nitrogen-fixing bacteria, nitrifying bacteria, and denitrifying bacteria (Fosso-Kankeu and Mulaba-Bafubiandi 2014). In addition, the nitrogen absorbed and utilized by microbial and algae could dissolve back into the water body leading to death and decomposition events and increasing the nitrogen concentration. In fact, the $\text{NO}_3^-$-N concentration in spring was over 10 times higher than in summer in the Lake Taihu, China (Xu et al. 2010, 2015; Wang et al. 2019b). Therefore, the nitrification rates were the highest in March and the lowest in July based on stable-isotope techniques (Hampel et al. 2018), implying that the bacteria could positively take part in the eutrophic water treatment. In this study, the total bacterial count in the $+$Rpf treatment were higher than the $-$Rpf treatment, which was consistent with the OD$_{660}$ values and the DGGE results of the MPN culture system. This finding indicated that the addition of active Rpf could efficiently facilitate microbial resuscitation. Moreover, the VR value was 21.82, implying that there were Rpf-
sensitive dominant VBNC bacteria in the eutrophic water treatment system. Also, 24 bacteria were isolated from the new adsorption material and were native from 11 genera. Specifically, nine isolated strains belonged to VBNC bacteria based on the 16S rDNA BLAST results, and these bacteria were then annotated into four genera, including Bacillus, Burkholderia, Enterobacter, and Pseudomonas. Numerous studies had reported that bacteria from the genera Bacillus owned the ability of algae-lysing and heterotrophic nitrification (Kim et al. 2005), whereas Pseudomonas possessed the ability of phosphorus-accumulation and these bacteria also are present in the sewage treatment due to their nitrification and deinitrification abilities (Li et al. 2015; Srinandan et al. 2011). In addition, Bacillus and Pseudomonas are two crucial genera in the treatment of heavy metal (HMs) due to their high adsorption and transformation abilities of heavy metal ions, such as Co²⁺, Ni²⁺, and Pb²⁺ (Giridhar Babu et al. 2013; Haroun et al. 2017). Also, Burkholderia and Enterobacter were reported before to purify sewage and to promote organic matter degradation (Mceely et al. 2009; Tiar et al. 2018), respectively. Therefore, the potential of the verified VBNC bacteria of the four genera had abilities of degrading organic matter, deinitrification, phosphorus-collecting, and algae-lysing. The latter is beneficial in eutrophic water treatments. Although the definite function of the isolated bacteria remained a limitation to the best of our knowledge, the role of VBNC bacteria provided new insights for eutrophication ecosystems restoration.

Conclusions

In the present 2-year monitoring study, the purification effects of the self-designed bioreactor were investigated by detecting the major water quality parameters. First, the high removal rates of TP, TN, Chl-a, and COD were observed, indicating the efficient purification ability of the new adsorption material containing bioreactor. Secondly, although the operational stop and the high temperature in summer affected the purification effect, the impact could be minimized when the bioreactor was running. Thirdly, the bioreactor had a long service life, which met the demand for a long period of treatment. Fourthly, Rpf could resuscitate the VBNC bacteria in the eutrophication ecosystem, and these bacteria had the potential to be added in eutrophic water treatments. These results contributed to the development of engineering technology innovations for aquatic ecosystems restorations. Also, this study provided new insights for water treatment by VBNC bacteria.

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Author contribution HF performed the microbial analysis and was a major contributor in writing the manuscript. LD provided original ideas for this manuscript and participated in editing. JZ analyzed the major water quality parameters and participated in data collection. XW was responsible for supervision, writing review, and editing. All authors read and approved the final manuscript.

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Data availability The datasets used or analyzed in this study are available from the corresponding author or Linxian Ding upon reasonable request.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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