Bacterial community shift and effects of the inclusion of a mixed psychrotrophic bacteria strain for sewage treatment in constructed wetland in winter seasons

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Abstract

The mechanism of wastewater treatment based on psychrophilic strains to improve the denitrification efficiency of constructed wetlands at low temperatures has already become a new hotspot. In this study, three mixed psychrophilic strains (*Psychrobacter* TM-1, *Sphingobacterium* TM-2 and *Pseudomonas* TM-3) with high capacity of denitrification were added into a vertical-flow constructed wetlands (CWs), and the effect of the mixed strains on CWs sewage treatment was evaluated. The removal efficiency of chemical oxygen demand (COD), ammonia nitrogen ($\text{NH}_4^+\text{-N}$), total nitrogen (TN) and total phosphorus (TP) was quantified to establish the degradation kinetic model and determine the best dosage of the mixed strains. The effect mechanism of the mixed strains on indigenous microbial community and the change of sewage treatment performance in low temperature constructed wetlands was clarified by high-throughput sequencing technology. The results showed that the mixed strains can effectively remove the organic pollutants (nitrogen and phosphorus) and the optimum dosage of the mixed strain was 2.5%, with average removal rates of 1.52, 2.12, 2.07 and 1.29 times than those of the control. Meanwhile, the dominant strains in the CWs were *Proteobacteria* (31.23–44.34%), *Chloroflexi* (12.04–19.05%), *Actinobacteria* (10.6–20.62%), *Acidobacteria* (8.23–11.65%), *Firmicutes* (2.23–15.95%) and *Bacteroidetes* (4.01–18.9%). These findings provide a basis for the removal of pollutants in constructed wetlands at low temperature.

1. Introduction

Constructed wetlands (CWs) are artificial constructed and controlled sewage treatment ecosystem that mimics the natural wetland, which mainly uses physical, chemical and biological technologies such as soil, artificial media, plants and microorganisms to purify sewage (Vymazal 2007; Farooqi, Basheer, and Chaudhari 2008; Breen 1990; Yu et al. 2020). For the advantages of simple process, good treatment effect and low operating cost, CWs has been widely used in many fields of wastewater treatment (Kivaisi 2001).

The composition and structure of microbial community are the main factors affected the effluent quality of constructed wetlands (Ibekwe, Grieve, and Lyon 2003; Kaushal, Wani, and Patil 2019; Hamdan, Salam, and Saikaly 2019). However, physical conditions such as low temperature affect the growth and activity of plants and population structure and metabolism of microorganisms in the CWs wastewater treatment process (Adams, Crump, and Kling 2010; Aromokeye et al. 2018; Zeng et al. 2019). The number of microorganisms and the metabolic efficiency of exogenous substances would be reduced under low temperature, so it is hard to form dominant microflora through natural selection, resulting in poor sewage treatment, or even substandard (Tang et al. 2018). Therefore, how to ensure the treatment effect of CWs under low temperature has become an urgent problem to be solved.

To date, research on the effect of bacteria organic pollutants removal under low temperature conditions in CWs is still in its infancy. The majority of the isolated strains were *Psychrotrophic* bacteria (Westlake et al. 1974; Atlas 1981; Järvinen, Melin, and Puhakka 1994; Yao et al. 2013; Vacková, Srb, and Stloukal

2011), the molecular mechanism and related theory of its low temperature adaptability has also been studied. (Järvinen, Melin, and Puhakka 1994; Guo et al. 2010; Ma et al. 2009; Ying et al. 2010). Pei *et al.* (Pei, Yang, and Tian 2010) studied the effect of *Bacillus.Subtilis* FY99-01 on denitrification efficiency in wetlands and showed that the addition of microbial strain could effectively increase the nitrate removal efficiency in wetlands. Shao *et al.* (Shao, Pei, and Hu 2013) found that adding *Paenibacillus* sp. XP1 to CWS could obviously improved the nitrogen removal efficiency at the temperature of 15 ~ 21°C, and the concentration of ammonia nitrogen and total nitrogen in the effluent has also been reduced. Therefore, screening the dominant psychrophilic high-efficiency denitrification strains will provide more feasibility for the water treatment and remediation of the constructed wetland.

A single strain consequently causes unstable sewage treatment purification effect through poor viability. After entering the actual polluted environment, the survival and degradation capacity of dissociated microorganisms are easily affected by external factors, such as temperature, plants and so on. A large number of microorganisms will be lost, making it difficult to control its long-term treatment effect. As a result, it is found that the function of two or more microbial strains to combine the dominant strains has a better synergistic effect than a single strain (Tang et al. 2018; Ying et al. 2010). In previous research, Ying *et al.* (Ying et al. 2010) explored the ammonia nitrogen removal rates of the three cold-resistant strains into the constructed wetland system in cold areas to remove the winter wastewater. When the ambient temperature was 6°C, the ammonia nitrogen removal rates of the three strains was 57.7%, 59.0% and 58.7% respectively at the concentration of 5% fungicide. Under the same condition, the removal rate was increased to 67.2% after using a mixture of the three strains. Therefore, the addition of mixed bacteria enhanced the denitrification effect of low temperature constructed wetlands. However, how to add cryogenic mixed microorganisms and the behavior of exogenous microorganisms in the environment and their impact on the environment is also an important issue related to the use of degradable bacteria, which has attracted more and more attention.

In this study, three kinds of psychrophilic strain were mixed into the constructed wetland according to a certain proportion, and a stable microecosystem was formed through the synergistic action of many kinds of low-temperature microorganisms. In order to give better play to its sewage treatment capacity and adaptability to nutrients and environmental conditions, construct a low-temperature microbial wastewater treatment kinetic model in constructed wetlands, using high-throughput sequencing technology, through different taxonomic levels of research. Analyze the community structure and composition of microorganisms in constructed wetlands, combined with the kinetic model of low temperature microbial wastewater treatment in constructed wetlands, better and more accurately reflect the effects of exogenous low-temperature mixed microorganisms on indigenous microbial diversity in constructed wetlands and more accurately analyze the mechanism of microbial pollutant removal in constructed wetlands, in order to improve the sewage treatment capacity and sustainable purification effect of constructed wetlands in winter. The purpose of this study is to provide theoretical basis and scientific support for the low-temperature sewage treatment of constructed wetlands, so as to provide some guidance and reference for the application and promotion of constructed wetlands in the cold conditions in the north.

2. Materials And Method

2.1. Design and operation of vertical-flow constructed wetland

The constructed wetland (Fig. 1) was completed in March 2015. It was divided into five small-scale plot(SSP), each plot (1 m²) was designed with a vertical flow structure. The sewage of the vertical constructed wetland was evenly distributed from the surface of the block, vertically down, through the soil layer, slag layer and gravel layer to the bottom of the pool, and discharged through the collection pipe. Each plot consisted of three different particle size distribution layers: gravel in the bottom 150mm depth (40 ~ 50mm diameter), slag in the middle 250mm (5 ~ 10mm diameter), and top 300mm (in the down-flow chamber) brown soil (0 ~ 4mm diameter). And all pipes are stainless steel (diameter 75mm). In order to distribute the water evenly, the inlet pipe has a hole (5mm diameter) on the bottom and spreads on the surface. *Oriental cattail Typha orientalis Presl* was planted in the sample plots. The hydraulic retention time was set to 5 days.

This influent water was taken from Qufu water co. LTD, and its parameters were shown in Table 1.

Table. 1 Parameters of Inflow of the MSP

Index	Influent	Effluent
pH	7.2 ~ 7.8	7.0 ~ 7.4
temperature (°C)	4 ~ 12	6 ~ 12
DO (mg·L ⁻¹)	3.4 ~ 3.8	0.2 ~ 0.4
TSS (mg·L ⁻¹)	2.1 ~ 2.5	not detected
COD (mg·L ⁻¹)	170.5 ~ 200.9	not detected
NH ₄ ⁺ -N (mg·L ⁻¹)	46.8 ~ 50.8	not detected
TN (mg·L ⁻¹)	81.7 ~ 90.9	not detected
TP (mg·L ⁻¹)	12.8 ~ 14.2	not detected

DO: dissolved oxygen; TSS: total suspended solids; COD: chemical oxygen demand; NH₄⁺-N: ammonium nitrogen; TN: total nitrogen; TP: total phosphorous.

The preparation of mixed psychrophilic strains suspension—The *Psychrobacter* TM-1 (GenBank acceptance number KR083014), *Sphingobacterium* TM-2 (GenBank acceptance number KR083015) and *Pseudomonas* TM-3(GenBank acceptance number KR083016) were incubated in the LB liquid culture

medium until their absorbance reaching 1.2 ~ 1.6. Then centrifuged in the centrifuge, discard the supernatant, and dilute the three kinds of bacteria with physiological saline. Repeat this process three times to remove nutrients from the culture medium. Finally, the absorbance of the solution was adjusted to 1.2 with normal saline water, and then the bacterial solution was mixed according to the volume ratio of 1:1:1. In early November 2018 (temperature of the water was 4 ~ 12°C), bacterium suspension of mixed psychrophilic strains ($4.4 \times 10^9 \text{ mL}^{-1}$) was injected into the vertical-flow constructed wetland system, with 0.5 ~ 5.0% volume ratio of sewage, and the concentration was B, C, D, E from low to high. And also there was a control treatment which called A that does not culture any psychrophilic bacteria. Then, the concentrations change of COD, $\text{NH}_4^+\text{-N}$, TN and TP were monitored for five months in the effluent. Meanwhile, the soil samples of five plots were collected by the plum shaped sampling method, respectively, packed in a sterile sealed bag for microbial diversity analysis.

All the parameters of water were analyzed according to the standard methods (APHA, 1998). To do the statistical analysis and draw on the determination about the results by using software Origin8.6 and SPSS19.

2.2. Microbial DNA extraction and sequencing

Microbial genomic DNA was isolated using a PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) kit. DNA products were evaluated by electrophoresis in 1% agarose gels. Nanodrop was used to test the quality and concentration of DNA. The V3–V4 hypervariable regions of the 16S rRNA gene were subjected to high-throughput sequencing by Beijing Auwigene Tech, Ltd (Beijing, China) using the Illumina Miseq Reagent Kit v3 (600 cycle)(PE300) sequencing platform (Illumina, Inc., CA, USA). And raw sequencing data were processed by Beijing Auwigene Tech, Ltd. (Beijing, China) using the pipeline tools QIIME(http://qiime.org/scripts/assign_taxonomy.html).

3. Monod Kinetic Model

The denitrification efficiency of mixed psychrophilic bacteria in vertical flow constructed wetland was simulated by simplified Monod kinetic model (Saeed and Sun 2011). It is assumed that the degradation of pollutants follows Monod kinetics, and the vertical flow constructed wetland is a continuous stirred reactor (CSTR). Therefore, the Monod equation can be expressed as:

$$K = \frac{q(C_{in} - C_{out})(C_{half} + C_{out})}{C_{out}} \quad (1)$$

In the formula above, K is the constant removal rate of the largest area ($\text{g} \cdot (\text{m}^2 \cdot \text{d})^{-1}$), q is the hydraulic loading rate ($\text{m} \cdot \text{d}^{-1}$), C_{in} is the influent nitrogen concentration ($\text{mg} \cdot \text{L}^{-1}$), C_{out} is the effluent nitrogen concentration ($\text{mg} \cdot \text{L}^{-1}$), and C_{half} is the semi-saturation constant of the limiting factor.

4. Results And Analysis

4.1. Effect of mixed strains in different dosages on sewage treatment

After running continuously for five months, the removal effects of mixed *psychrophilic* bacteria suspension with different dosage on the sewage index of vertical flow wetland is shown in Fig. 2.

The results show that the mixed *psychrophilic* bacteria suspension in the vertical flow system with different dosages had an excellent effect on the treatment of sewage in winter. The removal efficiencies of COD, $\text{NH}_4^+\text{-N}$, TN and TP increased with the dosage (0.5 ~ 5.0%). When the dosage increased from 1.5–5.0%, the pollutants removal rate significantly increased; however, the pollutants removal rate was very little increased as the dosage increased from 2.5–5.0%. Although the pollutants removal rate of 5.0% dosage is the highest, the increase is not obvious, and excess bacteria were used; therefore, considering the operating cost, the optimal dosage should be 2.5%.

When the dosage is 2.5%, the removal efficiency of the four indexes in the sewage by the mixed *psychrophilic* bacteria in the vertical flow system is significantly improved, and the removal rates of COD, $\text{NH}_4^+\text{-N}$, TN and TP were between 84.15 ~ 87.06%, 89.79 ~ 92.28%, 85.55 ~ 89.41% and 96.68 ~ 97.11%, respectively, and the average removal rates were 1.52, 2.12, 2.07 and 1.29 times than those of the control. Moreover, the average concentrations of COD, $\text{NH}_4^+\text{-N}$, TN, and TP in the effluent were 42.62, 4.26, 11.14 and $0.42 \text{ mg}\cdot\text{L}^{-1}$, respectively, which has reached the first grade of Chinese national discharge standard of pollutants for municipal wastewater treatment plant (GB18918-2002).

Compared to the single bacterium of *Pseudomonas flava* WD-3, the optimal dosage was greatly reduced from 6.0 % to 2.5%, and the hydraulic retention time was shortened (HRT) from 10 days to 5 days, but the average removal rates of $\text{NH}_4^+\text{-N}$ and TP were 6.4% and 6.5% higher, as the COD removal rate is consist with it(Tang et al. 2014), and the removal performance is very stable. Thereby it increased the amount of sewage treatment and reducing the cost of sewage purification. At the same time, the results fully shown that there is a mutually beneficial relationship between the bacteria, and the coordination of various microorganisms, so that pollutants can be better removed. Liu's studies have shown that the mixture of *bacillus subtilis*, *lactobacillus lactobacillus* and *lactobacillus plantarum* had higher removal rate of nitrogen and COD than the single bacteria treatment systems(K. F. Liu et al. 2010). Similarly, Moussavi & Heidarizad indicated that the addition of mixed bacteria shortens the acclimation time and aeration time of the reactor, improves the processing capacity and effect of the reactor, and enhances the impact load resistance and stability of the system(Moussavi and Heidarizad 2011). González added the selected mixed bacteria to stirred tank and fluidized-bed bioreactors directionally, the result showed that the time of domestication and aeration was shorten and the disposal ability and the effect of continuous process were improved, especially the COD removal efficiency was more than 90%(González et al. 2001).

Moreover, these studies also indicated the possibility of the utilization of the mixed bacteria to dispose the low-temperature sewage

Within the scope of optimal dosage, the statistical analysis of dosage and the purification effect of pollutants showed that the dosage of mixed *Psychrotrophic* bacteria was significantly positive correlated with the removal rate of COD, $\text{NH}_4^+\text{-N}$, TN and TP of sewage, and the correlation coefficients were 0.9870**, 0.9844**, 0.9422**, 0.9742**, respectively, **p < 0.01.

4.3. Simulation and validation of the model

Compared with the pseudo-first order kinetic model, the Monod kinetic model was more consistent with the actual situation of microbial treatment pollutants in water treated by microorganisms, so it is more suitable for the microorganism-led pollutant degradation process (Mitchell and McNevin 2001). When the above-mentioned Monod model was used to simulate the decontamination rate of mixed bacteria in vertical flow wetland, the C_{half} of this model for COD, $\text{NH}_4^+\text{-N}$, TN and TP were $20 \text{ mg}\cdot\text{L}^{-1}$, $1.0 \text{ mg}\cdot\text{L}^{-1}$ (Henze, Grady Jr., and Gujer 1987), $2.0 \text{ mg}\cdot\text{L}^{-1}$ (Giese, Khalil, and Ulery 1978) and $0.2 \text{ mg}\cdot\text{L}^{-1}$ (Henze et al. 1995). Therefore, three parameters were introduced to evaluate the merit of the model, as shown below:

$$R^2 = \frac{\left[\sum_{i=1}^N (X_i - \bar{X})(Y_i - \bar{Y}) \right]^2}{\sum_{i=1}^N (X_i - \bar{X})^2 \sum_{i=1}^N (Y_i - \bar{Y})^2}$$

efficient determination: (value range: 0–1) (2)

$$RRMES = \sqrt{\frac{1}{N} \sum_{i=1}^N (Y_i - \hat{Y})^2} / \hat{Y}$$

Relative root-mean-square error: (value range: 0–∞) (3)

$$ME = 1 - \sum_{i=1}^N (Y_i - \hat{Y})^2 / \sum_{i=1}^N (Y_i - \bar{Y})^2$$

Model efficiency: (value range: –∞–0) (4)

Where X_i , Y_i were the pollutant concentrations in the inflow and outflow at different time, respectively; $\bar{X}$, $\bar{Y}$ were the mean of pollutant concentrations in the inflow and outflow, respectively; and $\hat{Y}$ was the predictive value.

Simplified Monod model of the relationship between $f(C_{\text{in}}, C_{\text{out}}, q)$ and C_{out} is shown in Fig. 3. Regression fitting line slope of the maximum removal rate of pollutants ($K, \text{g}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$); and the Statistical parameters, such as R^2 , RRMSE, and ME were used to represent the deviation between the measured values of pollutants and the predicted values of the model.

The relationship between in the simplified Monod model were revealed The slope of the regression fitting line is the maximum removal rate of pollutants); statistical parameter applied to indicate the deviation

between the measured values of pollutants and the predicted values of the model. The dotted line represents 95% confidence interval, including the real regression fitting line.

Figure 3 shown, the statistical parameter R^2 of COD, $\text{NH}_4^+\text{-N}$, TN and TP were 0.6304, 0.9499, 0.9286 and 0.7739 with different dosages of mixed bacteria, both RRMES and ME were well within the expected range. The Monod model was helpful to comprehend the relationship between constructed wetland volume and pollutant concentration, and was used to collect data and run the model ("Major Subject : Environmental Engineering" 2011). From the collected data, that showed a better fit between the predicted and experimental values of pollutants removal by mixed bacteria in the vertical flow wetland. Monod described the degradation rate of pollutants as the only source of energy or carbon source, that is, the biomass produced by the consumption of each unit of carbon source (Schobinger 1976). Similarly, in the case of controlling the concentration of pollutants, Monod can also describe the degradation rate by the dosage of different microorganisms. Therefore, the simplified Monod model accurately predicted the removal efficiency of pollutants.

4.4. Bacterial community structure analysis

4.4.1 Bacterial community shift in phylum level

For 5 samples from different vertical flow systems, a total of 879,791 valid sequences were obtained after quality control. The clean sequences for A, B, C, D and E were 99,868, 266,285, 38,445, 54,430 and 46,861, respectively. The length of these valid sequences was principally about 400 ~ 440bp, accounting for 99.6% of the total number of valid sequences. The mean length of these valid sequences was nearly 420 bp.

The bacterial sequences obtained from five groups were classified into 5,863 OTUs, of which the OTUs cluster was divided into 13 phyla. As demonstrated in Fig. 4, the main phyla in the five wetland soil samples were *Proteobacteria* (31.23–44.34%), *Chloroflexi* (12.04–19.05%), *Actinobacteria* (10.6–20.62%), *Acidobacteria* (8.23–11.65%), *Firmicutes* (2.23–15.95%) and *Bacteroidetes* (4.01–18.9%), and the proportion of these bacteria generally shows a decreasing trend. *Gemmatimonadetes*, *Cyanobacteria*, *Nitrospirae*, *Verrucomicrobia*, *Planctomycetes*, *Parcubacteria* and *Saccharibacteria* were also emerged in most biosamples at a relatively low abundance.

The six dominant phyla of the five samples were *Proteobacteria*, *Chloroflexi*, *Actinobacteria*, *Acidobacteria*, *Firmicutes* and *Bacteroidetes*, which accounted for 83.36–89.88% of the tested phyla. The microbial changes in vertical flow systems with different concentration gradients were obvious. Many strains in *Proteobacteria*, *Firmicutes* and *Bacteroidetes* have been proved to have good purification effect on organic pollutants in sewage and have better ability of nitrogen and phosphorus removal (Peralta, Ahn, and Gillevet 2013). As shown in Fig. 4, the proportion of *Proteobacteria*, *Chloroflexi* and *Actinobacteria* in sample D and E was similar, which corresponds to the results that the treatment effect of sewage treatment in these two systems is roughly the same and better. *Proteobacteria* are the main force of sewage treatment, and the majority of *Proteobacteria* are denitrifying bacteria, among which α -

Proteobacteria is the main class of cold resistant bacteria and has a strong ability to remove nitrogen elements. β -*Proteobacteria* can use organic matter to transform into NH_3 and other substances, and then remove organic pollutants in sewage (Juretschko et al. 2002; Shapleigh 2011). The proportion of *Proteobacteria* in sample A was significantly higher than that of other vertical flow systems, which may be the *Psychrobacter TM-1* (GenBank acceptance number KR083014) and *Pseudomonas TM-3* (GenBank acceptance number KR083016) in the three strains are both *Proteobacteria*, which compete with other strains of *Proteobacteria*, and inhibit the growth of other strains, and then leads to the poor effect of sewage treatment. *Proteobacteria* always maintains a high relative abundance (> 31.23%) in each vertical flow wetland (Costa et al. 2014), which is beneficial to the removal of organic matter and nutrients, which is consistent with the results of Sun and other studies (Sun et al. 2016). The sewage treatment effect of sample D and E is better than others, and the sewage treatment effect of sample C is obviously lower than that of the former two. This is mainly because it contains more *Actinobacteria* (20.63%), which inhibits the growth of other bacteria conducive to nitrogen and phosphorus removal (Ramirez, Craine, and Fierer 2012). The number of *Firmicutes* bacteria in sample E (15.95%) was significantly more than that of the other four samples (2.23–10.04%). Compared with sample A, it was found that sample E had the greatest inhibitory effect on the diversity of indigenous microorganisms in constructed wetlands, and the wetland sewage treatment effect of sample E was the best, indicating that some bacteria in *Firmicutes* enhanced the effect of nitrogen and phosphorus removal in vertical flow systems. It has been found that *Firmicutes* can be involved in the decomposition of organic matter, which can decompose organic matter into inorganic matter and provide nutrients for microorganisms and plants, which plays an important role in sewage treatment in constructed wetlands (Wirth et al. 2012; Lim, Chiam, and Wang 2014). What's more, The sewage treatment effect of sample C is obviously lower than that of the former two, mainly because it contains more actinomycetes (20.63%), which inhibits the growth of other bacteria conducive to nitrogen and phosphorus removal (Ramirez, Craine, and Fierer 2012).

4.5 Bacterial community shift in genus level

The relative ratio of bacterial composition at genus level was shown in Fig. 5. The 20 genera with the richest species were compared with the sequencing sequences, and the heatmap of different colors were constructed. The change of relative richness can be observed intuitively through the change of color in the heatmap, and the abundance differences between different genera or the same genus in different samples can be provided in different samples (Jami et al. 2013). The vertical flow systems where the samples are located also have high diversity at the genus level. And the main bacteria are *Anaerobacillus*, *Bacillus*, *Luedemannella*, *Bryobacter*, *Iamia*, *uncultured*, *Azohydromonas*, *Geobacter*, *Caenimonas*, *Haliangium*, *H16*, *Streptomyces*, *RB41*, *Trichococcus*, *Macellibacteroides*, *Anaerolinea*, *Gaiella*, *Roseiflexus*. According to the analysis of the Fig. 5, the abundance of *Geobacter*, *Haliangium*, *Azohydromonas* and *Anaeromyxobacter* in sample A was significantly higher than that of the other four samples, *Azohydromonas* were related with nitrogen cycle, including nitrification, denitrification and nitrogen fixation (McDonald et al. 2015; Lin et al. 2018). The abundance of *Iamia*, *Anaerolinea* and *Luedemannella* in sample C was higher than that of other samples. *Luedemannella* is related to the removal of organic pollutants, belongs to *Actinobacteria* and may inhibit the growth of denitrifying

bacteria.(Ara and Kudo 2007).And *Trichococcus*, *Macellibacteroides* and *Roseiflexus* were ubiquitous in sample B. *Trichococcus*(J.-R. Liu et al. 2002) is related to sludge bulking, which is little in sample E and D. The increase of these bacteria may inhibit the growth of other cold-tolerant bacteria with good sewage treatment effect, and as a result, the effect of wetland sewage treatment is poor. The relative abundance of *Bacillus*, *Anaerobacillus*, *Streptomyces* and *RB41* in sample D and E is significantly richer than that in other samples. This directly leads to the significant improvement of the sewage treatment effect of the wetland where the sample E and D are located. *RB41* was reported to be effected on the removal of pollutants such as phosphorus(Zhang, Xu, and Zhu 2018), meanwhile, *Bacillus*, belonging to the *Firmicutes*, not only has the function of degrading COD, but also has the ability of nitrate reduction(Pei, Yang, and Tian 2010),indicating that *Bacillus* can stably exist and maintain a certain metabolic ability in wetlands, and work together with other heterotrophic and autotrophic bacteria to complete the removal of COD and nitrogen from domestic sewage. *Anaerobacillus* (C. Liu et al. 2015) is a heterotrophic denitrifying microorganism, which can reduce nitrate and release molecular nitrogen to achieve the purpose of nitrogen removal. It can be seen that when three mixed psychrophilic strains, namely, *Psychrobacter*, *Sphingobacterium* and *Pseudomonas*, were added to the Vertical-flow Constructed Wetlands, it caused significant changes in the structure and number of indigenous microorganisms. Meanwhile, when the dosage of mixed suspension of three psychrophilic strains was 2.5%, the mixed bacterial suspension of the three psychrophilic strains played a certain role in promoting the growth of *Bacillus*, *Anaerobacillus*, *Streptomyces* and *RB41* in the Vertical-flow Constructed Wetlands. The sewage treatment effect and microbial community analysis of each system showed that *Bacillus*, *Anaerobacillus*, *Streptomyces* and *RB41* play a decisive role in improving the removal efficiency of nitrogen, phosphorus and Organic pollutant. The addition of this kind of mixed bacteria suspension is beneficial to improve the treatment capacity of the Vertical-flow Constructed Wetlands to sewage, including nitrogen, phosphorus and organic matter, under the condition of low temperature.

5 Conclusion

This study investigated the effect of pollutant removal and the migration of bacterial community, and explained the pollutant removal mechanism from the point of view of microorganisms. The mixed strains can effectively remove the organic pollutants (nitrogen and phosphorus) and the optimum dosage of the mixed strain was 2.5%,with average removal rates of 1.52, 2.12, 2.07 and 1.29 times than those of the control. *Proteobacteria*, *Chloroflexi*, *Actinobacteria*, *Acidobacteria*, *Firmicutes* and *Bacteroidetes* were dominant bacteria in Vertical flow constructed wetland. three mixed psychrophilic strains was propitious to enrich *Bacillus*, *Anaerobacillus*, *Streptomyces* and *RB41*, which leads to the improvement of the effect of Vertical flow sewage treatment. The purpose of this study is to enrich bacterial information and promote the development and application of wetland sewage treatment system.

Declarations

Ethics approval and consent to participate. Not applicable.

Consent for publication. Not applicable.

Availability of data and material. All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests. None.

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Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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Figures

Figure 1

Schematic Diagram of the Vertical-flow Constructed Wetland System

Figure 2

COD, $\text{NH}_4^+\text{-N}$, TN and TP concentration in inflow and outflow with different dosages of mixed bacteria in the vertical flow wetland

Figure 3

Monod kinetics regression between inflow and outflow COD, $\text{NH}_4^+\text{-N}$, TN and TP values in the vertical flow wetland.

Figure 4

Relative ratio of the dominant bacteria at phylum level in different vertical flow wetlands (On the left is the hierarchical cluster analysis among samples, and on the right is the histogram of the community structure of the samples. The abscissa is the relative abundance of species in the sample, and the ordinate is the sample name.)

Figure 5

Thermal map analysis at genus level in different vertical flow wetland samples (On the left is the relationship clustering analysis of species. The abscissa is the genus of the sample, the ordinate is the species, and the color depth represents the abundance of the species. Only the dominant species at the top are shown here.)