

Full Length Research Paper

Evaluation of the importance of bacteria in vertical flow microcosms using streptomycin sulfate as the bactericide

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Accepted 26 April, 2013

The importance of bacteria in microbial communities of vertical flow microcosms was investigated using streptomycin sulfate as the bactericide. The bacterial loss reduced significantly total carbon, nitrogen and phosphorus contents enriched in substrate microorganisms ($P < 0.05$), but did not change the Shannon index of the microbial community ($P > 0.05$). In addition, the bacterial loss reduced the microbial utilization of both amines/amides and amino acid groups in Ecoplates ($P < 0.05$), but this was not the case for utilization of carbohydrates, carboxylic acids, polymers and miscellaneous compounds ($P > 0.05$). Meanwhile, a decreased removal of $\text{NH}_4^+\text{-N}$ or $\text{NO}_3^-\text{-N}$ in wastewater due to the bacterial loss was observed as well ($P < 0.05$). The current study highlighted the importance of bacteria in both transformations and removals of nitrogen compounds in constructed wetlands.

Key words: Constructed wetland, Streptomycin application, microbial biomass, metabolic patterns, pollutant removals.

INTRODUCTION

The relative importance of different microbial populations in biological processes is difficult to ascertain because of the myriad interactions among the biological communities (Krauter et al., 2005). In the laboratory, soil can be sterilized and specific microbial populations such as bacteria, fungi can be added to investigate the functions or effects of a specific microbial group on a specific nutrient cycling (Ingham and Coleman, 1984). However, some lab experiments rarely mimic the natural situation because of the difficulty in understanding completely the diversity of microorganisms that exists in actual environments.

Removal or reduction of microbial groups such as bacteria provides a useful mean for examining the function of a specific microbial group by applying narrow-spectrum biocides. Beare et al. (1992) observed a large decrease in the litter decomposition rate when streptomycin sulfate

(a bactericide) was applied to soils. Austin et al. (2006) reported that the nitrifier loss derived from the application of nitrapyrin (a chemical inhibitor of autotrophic nitrifiers) greatly altered the nitrogen cycling in a steppe soil. Therefore, the selective inhibition or removal of microbial groups has become a mean to investigate the relative contribution of microorganisms such as bacteria to biochemical processes in the environments.

Constructed wetlands (CWs) are engineered systems designed to remove pollutants from various contaminated waters (Faulwetter et al., 2009). Bacteria are believed to be a key biotic factor in driving pollutant removals in CWs (Calheiros et al., 2009; Sleytr et al., 2009), since they are involved in the mineralization processes of organic pollutants or the transformation processes of inorganic pollutants in wastewaters (Faulwetter et al., 2009). How-

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ever, some debates have been stirred. It is claimed that fungi are very important in mediating pollutant removals and biochemical processes of CWs. Liang et al. (2003) showed that the fungal number in CWs was more positively related to total nitrogen removal than the bacterial number. Recently, Song et al. (2010) also provided the relevant evidence in which the denitrification rate was not related to both structure and quantity of the denitrifying communities in CW systems. Meanwhile, Seo and DeLaune (2010) showed that the denitrification mediated by fungi in wetlands contributed more to the greenhouse gas emission under aerobic or moderately reducing conditions than those driven by bacteria.

To directly examine the importance of bacteria in CW systems, thirty vertical flow microcosms were established in the field, and streptomycin sulfate was applied as a bactericide to gradually exclude bacteria from the substratum in the microcosms. We investigated experimentally: (1) to what extent the bacterial loss can alter microbial parameters such as biomass and carbon metabolic patterns in CW systems; (2) what pollutant removals are predominantly decreased when the bacterial loss occurs in microcosm systems?

METHODS AND MATERIALS

Microcosm design

Thirty vertical flow microcosms were established in the campus of Taizhou University, Zhejiang Province, in Eastern China from January to March of 2011. Each microcosm consisted of a cylinder with a 13.5 cm inside diameter and a 120 cm height made of high-density polyvinyl chloride (PVC). The cylinder was impermeable, lightweight, rigid, and highly resistant to acids, bases, and biological degradation (Chen et al., 2001). Similar to the design of the wetland system used in our previous study (Zhang et al., 2011), each microcosm was filled with fine sand (particle diameter 1–2 mm) in the top layer (40 cm depth), coarse sand (particle diameter 4–6 mm) in the middle layer (30 cm depth), and gravel (particle diameter 30–45 mm) in the bottom layer (30 cm depth). Three openings with screw caps were established on the outside of each filled material layer along the height of the cylinder to irrigate the wastewater with streptomycin by layer. The wastewater used in this study was the effluent from a pig breeding farm near Taizhou University. The wastewater had an average chemical oxygen demand (COD) = 60.24 mg L⁻¹, biochemical oxygen demand (BOD₅) = 48.19 mg L⁻¹, ammonia nitrogen (NH₄⁺-N) = 1.29 mg L⁻¹, nitrate nitrogen (NO₃⁻-N) = 0.62 mg L⁻¹, total nitrogen = 12.37 mg L⁻¹, soluble phosphate = 0.36 mg L⁻¹, respectively. The nutgrass (*Cyperus rotundus*) was selected as the candidate species in the microcosms due to its rapid growth and tolerance to high concentration of pollutants (by our observation), and its planting density was three individuals per microcosm. After the microcosm construction was completed, each microcosm was pulse-irrigated with 7.8 L of wastewater without streptomycin. The wastewater remained in each microcosm for 0.5 days and then was drained empty. To develop sufficiently plant and microorganisms in microcosms, this irrigation program above started from the middle of March of 2011 and operated repeatedly till the middle of July.

Streptomycin treatment

Streptomycin sulfate can inhibit a broad spectrum of bacteria with

relatively few direct effects on other organisms (Schatz et al., 1944), thus was chosen as the bactericide in this study. According to the suggestion from Seo and DeLaune (2010), the application concentration of streptomycin sulfate was individually established as 0.0, 1.0, 1.5, 2.0, 2.5 and 3.0 mg g⁻¹ sand substrate. The different doses of streptomycin sulfate were individually dissolved in wastewater, and the wastewaters with different doses of streptomycin sulfate were then distributed into the specific microcosms in five replicates in the middle of July. To ensure significant effects of streptomycin application doses on the bacteria loss, all the microcosms were repeatedly irrigated with the wastewaters with different doses of streptomycin for at least three times by using the same irrigation program of microcosms above.

Sampling

After the streptomycin application had been completed, 1000 mL of influent and effluent in each microcosm were taken in triplicates for water quality analysis. Except for the water samples for BOD₅ analysis, other samples received 1.5 mL of concentrated sulfuric acid to stop the biotic reactions occurring in wastewater, and temporarily stored in a refrigerator at -20°C to determine other water quality parameters.

Once the water samples were collected, triplicate of substrate samples in each microcosm were collected to a depth of 30 cm using a 5 cm diameter of PVC pipe, since Salomo et al. (2009) showed that the substrate layer of 30 cm was the optimum area for microbial distribution and metabolic activities in the vertical flow CW systems. These substrate samples from each microcosm were mixed to create a composite sample. The composite samples were sieved (2 mm) and were immediately placed into Ziploc™ bags. Substrate samples were temporarily stored in a refrigerator at 4°C for two days to analyze microbial parameters.

Parameter analyses

Microbial biomass analysis

Carbon, nitrogen and phosphorus contents enriched in microorganisms were determined using a chloroform fumigation-extraction technique. Ten grams of fresh sand sample was fumigated with chloroform for 24 h at 25°C to analyze microbial biomass carbon and phosphorus. After the fumigation, the organic C in substrate samples was extracted with 0.5 mol L⁻¹ K₂SO₄ and determined using a heated K₂Cr₂O₇-H₂SO₄ digestion. The K_{EC}-factor was 0.35 indicating that 35% of the biomass carbon was extractable as organic C (Sparling and West, 1988). The phosphorus in the samples was extracted with 0.5 mol L⁻¹ NaHCO₃ and measured photometrically (UV2102-PC photoelectric photometer, UNICO) at 882 nm. The biomass phosphorus was determined from the difference of the extractable inorganic phosphorus contents between the fumigated and non-fumigated samples. A K_{EP}-factor of 0.4 was used, meaning that 40% of the phosphorus in the microbial biomass was rendered extractable as the inorganic phosphorus (Brookes et al., 1982). One hundred and fifty grams (150 g) of samples was fumigated with chloroform for 10 d at 25°C, and the organic nitrogen was extracted with 2 mol L⁻¹ KCl. The extractant was then measured photometrically at 570 nm following the ninhydrin reaction. The K_{EN}-factor was 3.1 indicating that one µg of biomass nitrogen was equivalent to 3.1 µg of the ninhydrin-reactive nitrogen in 1 g of dry substrate (Amato and Ladd, 1988).

Carbon metabolic patterns

The cell supernatant of the sand sample was created using 10 g of sand sample and a 0.85% NaCl solution, and was diluted with a sterile NaCl solution to a mean inoculum density of 10⁶-10⁷ cells

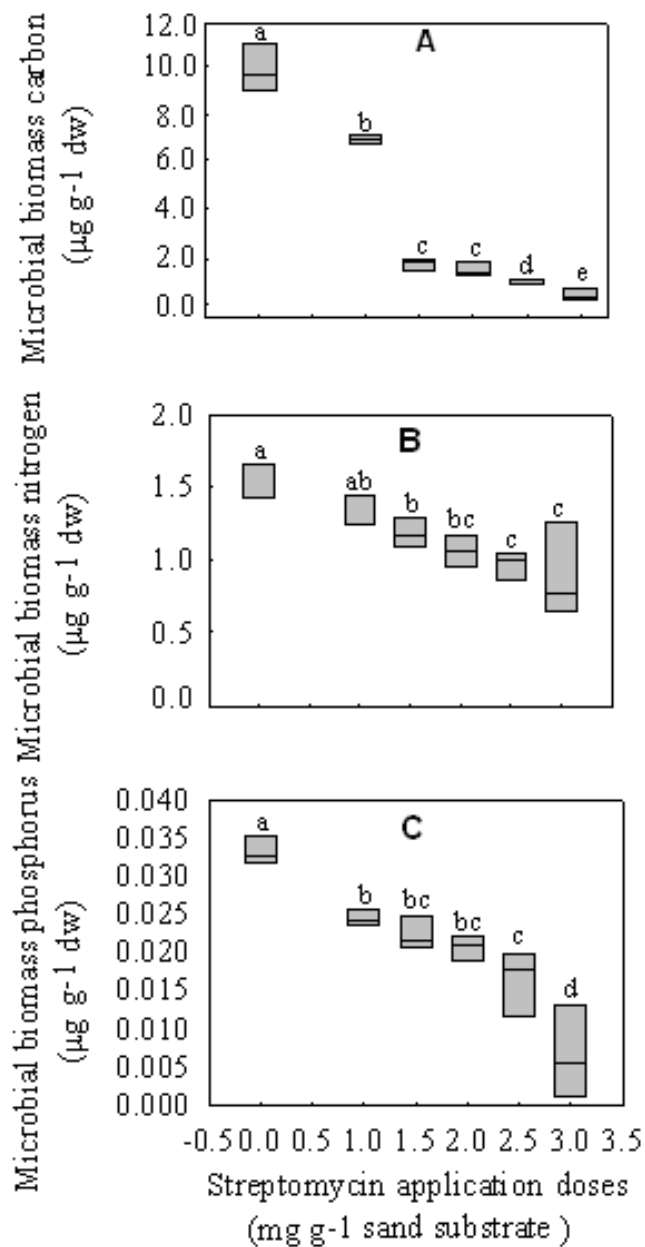


Figure 1. Effects of the streptomycin sulfate application on microbial biomass carbon (A), nitrogen (B) and phosphorus (C) contents enriched in microorganisms of the microcosms (replicated samples = 5). The lower and upper boxes are the lower quartile and upper quartile, respectively. The same lowercase letters indicate the non-significant differences between the streptomycin application doses, while different letters indicate significant differences at 0.05 level.

mL⁻¹. A total of 125 µL of the cell suspension was added to each well in a given Ecoplate™ (Biolog, Hayward, CA). These plates were placed in a plastic box containing a water-soaked paper towel in order to minimize evaporation from the wells, and then incubated in an incubator at 28°C. The turnover of each carbon source was estimated photometrically at 590 nm using a microplate reader (GENios Pro™, Tecan, Trading AG, Switzerland). The reading (A_{590}) for each substrate sample was corrected for background absorbance by subtracting the absorbance of the control well

containing water only (Moynahan et al., 2002).

Average well color development (AWCD, indexed as total activity of microbial community) of substrate utilization at A_{590} was calculated as the average optical density across all wells per plate using $AWCD = (\sum n_i)/31$ (Zak et al., 1994), where n_i represents the relative optical density of the i well which was corrected against color development in the control well. In addition to AWCD patterns with the incubation time, 31 carbon sources were subdivided into six substrate groups, as suggested by Zak et al. (1994): carbohydrates (CH), carboxylic acids (CA), amines/amides (AM), amino acids (AA), polymers (PL) and miscellaneous (MS). The average utilization of all carbon substrates within each group at the end of a 192 h incubation was also calculated. Meanwhile, the functional diversity of the microbial community (that is, organic substrate utilization diversity, expressed as Shannon index) in each sand sample was also calculated using $H = -\sum p_i (\ln p_i)$, where p_i was the ratio of the individual carbon substrate utilization to the sum of all carbon substrate utilizations at the end of the 192 h incubation.

Water quality analysis

Water quality parameters including BOD₅, COD, NH₄⁺-N, NO₃⁻-N and phosphate were determined following the standard methods established by the American Public Health Association (Greenberg et al., 1992). Pollutant removals were calculated based on the mass balance method (Maltais-Landry et al., 2009):

$$R (\%) = [1 - (E_v \times E_c / I_v \times I_c)] \times 100$$

Where, R is the removal efficiency; E_v is the treated effluent volume; E_c is the treated effluent concentration; I_v is the influent volume and I_c is the concentration in the influent.

Data analysis

Significant difference of parameters between application doses was determined using one-way analysis of variance (ANOVA). Multiple comparisons of means were conducted using a Tukey test at $P = 0.05$. All statistical analyses were performed using SPSS statistic software (version 11.50 for Windows).

RESULTS AND DISCUSSION

The bacterial loss reduced total microbial biomass parameters in microcosms

In the current substratums, microbial biomass parameters including the carbon, nitrogen and phosphorus contents enriched in microorganisms reduced significantly by higher streptomycin application doses ($P < 0.05$, Figure 1A, B and C). This indicated that bacteria were quantitatively a dominant group within the microbial communities in CW systems (Sleytr et al., 2009; Stottmeister et al., 2003). Generally, the function of a given microbial community may be governed by the magnitude of its biomass (Griffiths et al., 2000). However, our results did not go along with a decrease in the overall metabolic activities in terms of organic substrate utilization patterns with the incubation time (AWCD, $P > 0.05$, Figure 2). A similar result was observed by Girvan et al. (2005) who found that the copper stress significantly reduced the bacterial abundance in soils, but did not greatly reduce the decom-

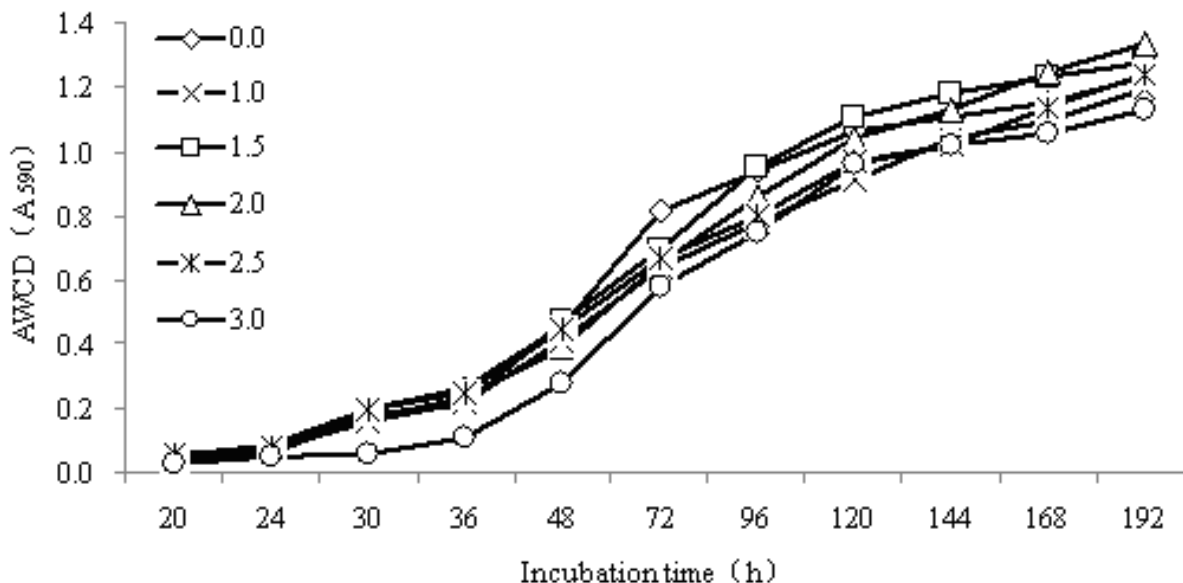


Figure 2. Average well color development (AWCD) of carbon substrate utilizations in Ecoplates with the incubation time. Numbers such as 0, 1.0, 1.5, 2.0, 2.5, 3.0 in the legend represent the different application doses of streptomycin sulfate (mg L^{-1}), respectively.

position rate of wheat shoots. They attributed this phenomenon to the functional stability of bacterial communities (that is, one function driven by a microbial community dose not easily change with the structure of the microbial community) to the copper stress. The CW systems are different from the soil systems in many aspects, such as repeated wastewater irrigation and higher nutrient loadings (Faulwetter et al., 2009). In the current study, the organic substrate utilization patterns with the incubation time did not significantly change among streptomycin application doses, which might be related to the sufficient amount of nutrients in the wastewater, since it is well known that the availability of nutrients is the determinant in mediating microbial activities. On the other hand, the combined roles of other microorganisms such as fungi in contributing to the AWCD patterns may be an alternative cause, since streptomycin has few direct target effects on these microorganisms (Schatz et al., 1944).

The bacterial loss reduced utilizations of amines/amides and amino acids in Ecoplates

The metabolic profiles derived from the BIOLOG technique have already been shown to be useful in distinguishing heterotrophic microbial communities among and within wastewater-treatment systems (Hench et al., 2004; Osem et al., 2007; Salomo et al., 2009; Zhang et al., 2010). According to the molecular characteristics of 31 carbon substrates in Ecoplates, we classified 31 carbon substrates into six carbon substrate groups including carbohydrates, carboxylic acids, amines/amides, amino acids, polymers and miscellaneous to test the response of a specific group utilization to the bacterial loss (Zak et al., 1994). Utilizations of carbohydrates, carboxylic acids,

polymers and miscellaneous compounds did not reduce significantly with the streptomycin application dose ($P > 0.05$, Figure 3A, B, C and F), thus showing that bacteria may not be the dominant drivers of these carbon substrate utilizations in the current microcosm systems (Girvan et al., 2005). We speculated that fungi might play important roles in regulating these carbon substrate utilizations (Schatz et al., 1944). Similarly, the functional diversity (Shannon index) of microbial species did not change with the bacterial loss either ($P > 0.05$, Figure 4), indicating that a great functional redundancy (that is one function can be carried out by different groups of microorganisms, Crechiosa et al., 2004) in microbial communities did presented in the current microcosms (Wohl et al., 2004). Nonetheless, the bacterial loss significantly reduced the utilizations of amines/amides and amino acids in Ecoplates at the application dose of $3.0 \text{ mg streptomycin g}^{-1}$ sand substrate ($P < 0.05$, Figure 3D and E), showing that these nitrogen substrate utilizations were controlled more by bacteria than by other microorganisms such as fungi in the substratums. Similar results from grassland studies were also reported, where a decrease in soil microbial abundance reduced some specific microbial activities such as the denitrification, nitrification and methane oxidation in soils (Griffiths et al., 2000). Austin et al. (2006) also provided other evidence that the bacterial loss greatly reduced the nitrifying activities in the steppe soils.

The bacterial loss reduced removals of $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ in microcosms

It is claimed that the bacterial community plays a critical

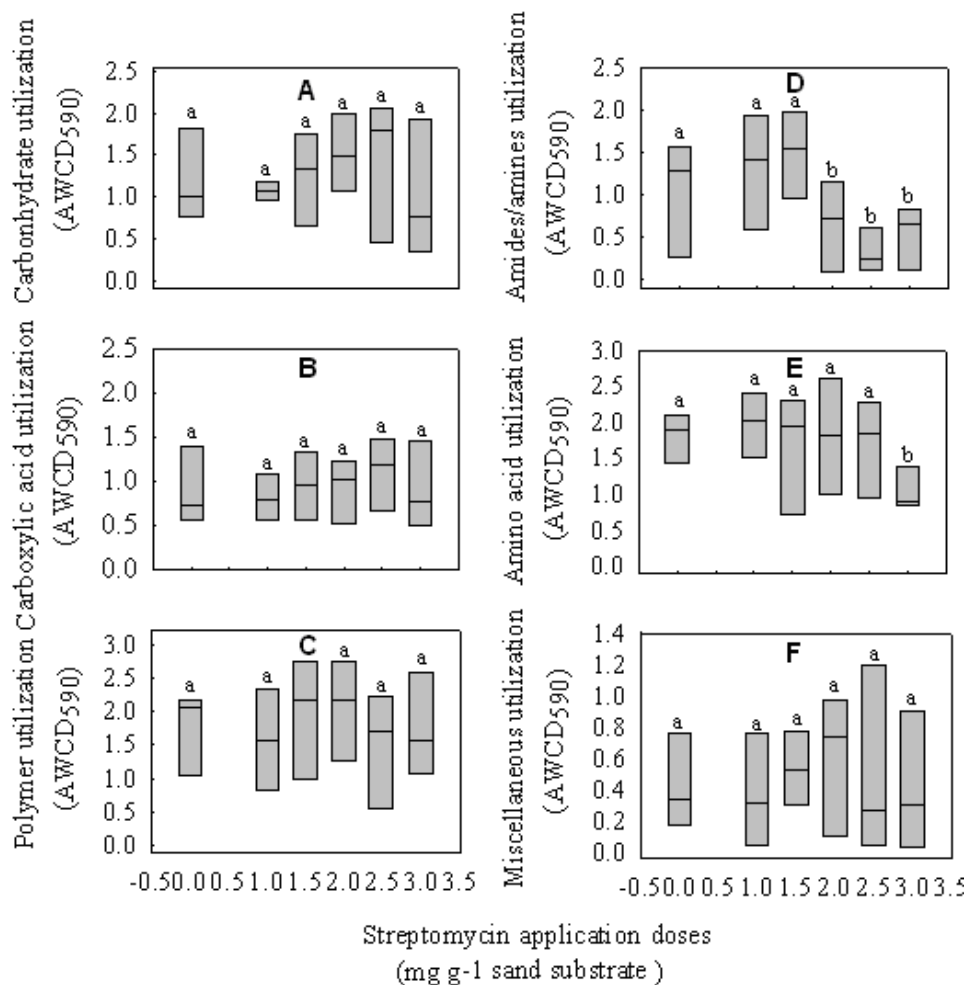


Figure 3. Effects of the streptomycin sulfate application on the utilizations of six carbon substrate groups at the end of a 192 h incubation in Ecoplates (A–F) in the microcosms (replicated samples = 5). Other explanations please see Figure 2.

Table 1. Pollutant removals with the streptomycin application dose in vertical flow microcosms (means \pm standard deviation, replicated samples = 5).

Streptomycin application doses (mg g ⁻¹ sand substrate)	BOD ₅ (%)	COD (%)	NH ₄ ⁺ -N (%)	NO ₃ ⁻ -N (%)	Phosphate (%)
0.0	47.91 \pm 2.8	59.89 \pm 3.5	54.82 \pm 4.5	73.15 \pm 16.5	63.38 \pm 2.5
1.0	42.04 \pm 12.5	52.56 \pm 15.6	51.66 \pm 5.5	50.42 \pm 13.3	65.88 \pm 2.2
1.5	40.95 \pm 8.5	51.19 \pm 10.6	43.46 \pm 8.6	45.82 \pm 3.7	65.95 \pm 2.8
2.0	41.65 \pm 9.2	52.06 \pm 11.5	42.89 \pm 7.5	43.72 \pm 2.9	71.63 \pm 3.4
2.5	41.03 \pm 13.5	51.29 \pm 16.9	40.77 \pm 5.2	42.14 \pm 14.2	71.29 \pm 4.9
3.0	39.61 \pm 12.0	49.51 \pm 14.5	32.87 \pm 6.1*	38.34 \pm 5.2*	66.68 \pm 5.9

Data labeled with * are significant at $P < 0.05$, as tested by one-way ANOVA. BOD-biochemical oxygen demand; COD-chemical oxygen demand.

role in regulating major pollutant removals in CW systems, leading to large effects on water quality (Hartman et al., 2008). In the current study, the bacterial loss significantly reduced the total microbial biomass, but did not result in a large reduction in the removal of BOD₅ or COD

($P > 0.05$, Table 1). These findings are in agreement with the effects of the bacterial loss on utilizations of carbohydrates, carboxylic acids, polymers and miscellaneous compounds, thus further demonstrating a limited role that the bacterial community played in the oxidation of total

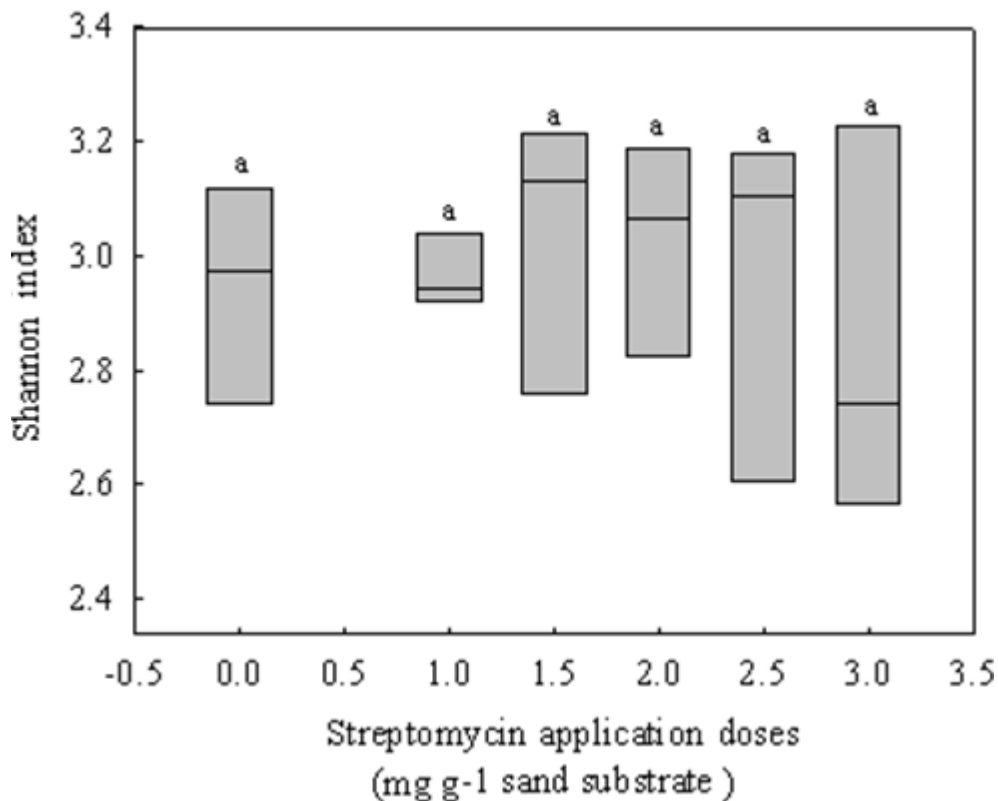


Figure 4. Effects of the streptomycin sulfate application on the functional diversity (Shannon index) computed from the utilizations of 31 carbon substrates at the end of a 192 h incubation in Ecoplates (replicated samples = 5). Other explanations please see figure 2.

organic pollutants (BOD₅ and COD) in the current microcosms. One possible explanation for no significant effects of the bacterial loss on both BOD₅ and COD removals was that fungi in microcosms played a greater role in contributing to the removals of BOD₅ and COD than bacteria, since Liang et al. (2003) and Jin and Kelley (2007) reported that the eukaryotic organisms such as fungi were also important biotic populations in controlling pollutant removals of CW systems. Meanwhile, the current study also showed that the bacterial loss did not lead to a significant decline in the phosphate removal. This might be a real case, since previous studies indicated that the phosphorus removal in CWs mainly depended on the sorption to substrates (Kelderman et al., 2007). On the other hand, an interesting finding was that the removals of BOD₅, COD and phosphate paralleled the change in the functional diversity of the microbial community, indicating that the streptomycin application might only kill some rare species which in consequence would not change main functionality of the system (Girvan et al., 2005).

Unlike the removal of BOD₅, COD and phosphate, a reduced removal of NH₄⁺-N or NO₃⁻-N at the dose of 3.0 mg streptomycin L⁻¹ wastewater was observed in the current microcosms ($P < 0.05$, Table 1). The decreased

removal of NH₄⁺-N or NO₃⁻-N with the bacterial loss was consistent with the decreased utilization of amines/amides and amino acids in the Ecoplates. These findings show that bacteria were actively involved in nitrogen transformations in the CW systems, since it is well known that the nitrification, denitrification and anaerobic ammonium oxidation driven by ammonium-oxidizing bacteria, nitrite-oxidizing bacteria, denitrifiers and anammox bacteria are important pathways for the nitrogen removals in CW systems (Lavrova and Koumanova, 2010; Lee et al., 2009). Some studies claimed that the structural or functional diversity of microbial communities in soils are important in maintaining the ecosystem stability (Bell et al., 2005; Kaffe-Abramovich and Steinberger, 2006; Wohl et al., 2004), but in the current study the functional diversity of the microbial species did not follow the decreased removals of NH₄⁺-N and NO₃⁻-N, indicating that the functional diversity of the microbial species was not necessarily the determinant for controlling the removal functions of inorganic nitrogens in CW systems (Hsu and Buckley, 2009). A similar finding was also reported by Jiang (2007) who claimed that a reduction in the bacterial species diversity had not significant effect on the decomposition of organic compounds, suggesting that a change in the bacterial diversity did not necessarily lead

to a significant functional change in the ecosystems.

Conclusions

We presented for the first time an example of the bacterial loss experiment using streptomycin sulfate as bactericide in vertical flow microcosms fed with the piggery wastewater. Our study revealed that the bacterial loss reduced microbial biomass parameters within CW systems, thus showing that bacteria are indeed the dominant microbial components within CW systems. Nonetheless, only utilizations of amines/amides and amino acids in Ecoplates and removals of $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ in wastewater were reduced by the bacterial loss, whereas utilizations of carbon compounds in Ecoplates and removals of BOD_5 , COD and phosphate in wastewater did not follow the bacterial loss. Therefore, our study provides the direct evidence in which bacteria appear merely important in transformations and removals of nitrogen compounds in CWs.

ACKNOWLEDGEMENTS

We are grateful for the funding provided by the National Science Foundation of China (No. 51279121). We wish to thank Professor Shi S. Ke for his helps in the experimental analysis.

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