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Indicator Microorganisms and Pathogens Removal Function Performed by Copepods in Constructed Wetlands

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Abstract Removal efficiency of indicator and pathogenic microorganisms in constructed wetlands were analyzed, and microorganisms removal function performed by copepods was determined. The results showed that the constructed wetlands effectively reduced *Escherichia coli*, fecal streptococci, total coliforms, and fecal coliforms, the *Salmonella* spp. removal efficiency was relatively low and the *Clostridium perfringens* removal was the least. At copepods concentrations of 3.0×10^2/L, and 6.0×10^2/L, high die-off rates were observed for indicator and pathogenic microorganisms compared to the control group, and indicator and pathogenic microorganisms in samples with higher concentration of copepods decreased much more rapidly than those in samples with lower concentration. These results suggest that predation by copepods is an important mechanism for the removal of bacteria in constructed wetlands.

Keywords Constructed wetlands · Copepods · Indicator microorganism · Pathogen

Constructed wetlands (CWs) have proven to be a promising treatment alternative for developing countries (Kivaisi 2001; Vymazal 2002; Kaseva 2004; Korkusuz et al 2005; Song et al. 2006). They have low investment and operation costs, produce high quality effluent with less dissipation of energy, and are relatively simple to operate (Mantovi et al. 2003; Ayaz and Akca 2001; Song et al. 2003). Studies of constructed wetland systems show that removal percentages of suspended solids (SS), Biological oxygen demand (BOD_5), and Chemical oxygen demand (COD) are generally high whereas removal percentages of nutrients (N and P) are often lower and more variable.

Recently, attention has been focused on the ability of constructed wetlands to reduce human pathogens in wastewater. Constructed wetlands have been found to reduce microorganisms with varying but significant degrees of effectiveness. There have been several studies reporting on microbial water quality improvement using constructed wetlands (Green et al. 1997; Gersberg et al. 1987; John 1984; Karpiscak et al. 1996; Song et al. 2006). Song et al. (2006) reported 99.7% reduction of total coliforms and 99.6% reduction percent of fecal coliforms from a full-scale constructed wetlands system. In a multi-species (bulrush, cattail, black willow, and cottonwood) wetland system, the reduction of total and fecal coliforms were 98 and 93 percent, respectively, and enteric viruses were reduced by 98 percent (Karpiscak et al. 1996).

Predation is an important mechanism for the removal of bacteria from wastewater in constructed wetlands, especially in free water surface constructed wetlands. Processes of ciliates predation in the horizontal subsurface flow CW (Decamp and Warren 1998) and in percolated sand columns (Eisenmann et al. 1998; Wand et al. 2007) were studied. Although a few researchers have identified predatory protozoa, mini-metazoa such as nematodes and rotifers can prey upon some bacteria and pathogens (Green et al. 1997), little is known about copepods predation on indicator bacteria and pathogens.

In this study, indicator microorganisms such as total coliforms, faecal coliforms, *Escherichia coli*, and fecal streptococci, and pathogens such as *Clostridium perfringens* and *Salmonellae* were analyzed from various components of a free water surface constructed wetland. In

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addition, indicator organisms and pathogens removal function performed by copepods in constructed wetlands was also investigated. The expected result of the project is to determine predation by copepods as a potential mechanism for the removal of indicator and pathogenic microorganisms from wastewater treated in the constructed wetlands. To the best of our knowledge, this issue has never been the subject of specific research attention for constructed wetlands.

Materials and Methods

The free water surface constructed wetland wastewater treatment system under investigation is situated in Jiaonan, Shandong Province, China at a latitude of 35°35′–36°08′ North and a longitude of 119°30′–120°11′ East. The area is in the warm temperate zone and the climate is continental monsoon with an average annual temperature and average annual precipitation of 12.0°C and 794 mm, respectively. The system had a total area of 76.7 ha m^2 and a treatment capability of 3.0×10^4 m^3 day^{-1}. Constructed wetlands received secondary unchlorinated wastewater from Jiaonan Municipal Wastewater Treatment Facility. The treated wastewater was then pumped into a distribution system and transported into the constructed wetland. The wetland consisted of 99 treatment beds, each 140 m long and 32 m wide, with a maximum depth of 0.5 m, providing approximately 44.33 ha m^2 of surface area. All beds operated in parallel in continuous model. The hydraulic load was about 13.5 cm/day. All beds were planted with common reed (*Phragmites australis*) and a number of naturally germinated wetland plants (*Typha orientalis*, *Scirpus validus*, *Lemna minor*, etc.). During the investigation, high concentrations of copepods in jiaonan constructed wetlands bed were observed from March to June in 2007. The concentrations of copepods were as high as 3.0×10^2/L–6.0×10^2/L. The copepods in water samples were fixed by 5% formaldehyde and were confirmed as daphnia by bright field microscopy.

Wastewater was sampled from the constructed wetlands in 500 ml sterile plastic bottles. The samples were kept at 4°C during transportation. Two concentrations of copepods (3.0×10^2/L, 6.0×10^2/L) were designed and wastewater without any copepods was used as a control. The bottles were kept in the dark at room temperature. At various times, 1.0 mL samples was withdrawn from the bottles and 1:10 serial dilutions were made in Tris buffered saline before assaying for indicator bacteria and pathogen. The experiment lasted 15 days with three duplicates of each copepods concentration.

Water temperature and dissolved oxygen (DO) were taken in the field using an Orion (Model 835A) dissolved oxygen meter. The pH was measured with a model pHs-25 pH-meter. Chemical oxygen demand (COD) and Biological oxygen demand (BOD_5) were measured by the potassium dichromate-boiling method and incubation method, respectively. Total suspended solids (TSS) was measured by filter method. Total nitrogen (TN) and total phosphorus (TP) were measured by the potassium peroxysulfate decomposed method, and molybdenum blue methods, respectively. The samples for temperature, pH, COD, SS, TN, TP were processed within 24 h of collection and BOD_5 were processed within 6 h of collection.

Total coliforms (TC), Fecal coliforms (FC), Fecal streptococci (FS), and *Salmonellae* spp. were performed using standardized detection methods (Standard Method for the Examination of Water and Wastewater Editorial Board 2002). Standard methods are not available for detection of *Escherichia coli* (EC) and *Clostridium perfringens*. In those cases, detection methods based on current scientific knowledge were used. A most probable number (MPN) enrichment scheme was used to evaluate concentrations of total coliforms and fecal coliforms. *E. coli* (EC), fecal streptococci (FS), *Clostridium perfringens* and *Salmonellae* in wastewater were enumerated by the plate count method. Appropriate sample volumes, in triplicate, were checked and varied according to the group of organisms being enumerated and sample sources. Plates were incubated for 24 h at 37°C on MacConkey medium for *E. coli*, 48 h at 37°C on KF medium for Fecal streptococci, 24 h at 37°C on TSC medium for *Clostridium perfringens*, and 24 h at 35°C on SS agar for *Salmonella* spp. All bacteriological media were obtained from Hopebio (Qingdao, China). Putative *Salmonella* colonies were verified by randomly picking four to six suspect colonies from each of the respective isolation media. Presumptive Salmonella colonies were subjected to physiological and serological confirmation. Confirmation rates were used to adjust respective microbial densities. The numbers of indicator and pathogenic microorganisms were converted to $\log_{10}$ values and expressed as $\log_{10}$ CFU or $\log_{10}$ MPN per ml of wastewater.

Linear regression analyses were used to calculate die-off rates (log10 reduction per day) by the following equation: $\log_{10} N_t/N_0 = -mx + b$; where $\log_{10} N_t/N_0$ is the ratio of the $\log_{10}$ value at time t (measured in days) to the initial $\log_{10}$ value (log N_0), x is the time in days, b is the intercept value, and m is the slope.

Results and Discussion

During the sampling period, samples were collected from the Jiaonan CW system and analyzed for each of the parameters under investigation. The constructed wetlands proved efficient in reducing the levels of physical, chemical and microbial parameters associated with the incoming sewage. Table 1 presents the average inlet and outlet concentrations of each parameter.

The mean COD concentration decreased from 320 mgL^{-1} to 125 mgL^{-1}, a reduction of 60.9%. BOD_5 removal was slightly efficient than COD removal, decreasing from 33 mgL^{-1} to 11 mgL^{-1}, a reduction of 66.7%. The BOD_5/COD ratio in inlet and outlet decreased from 0.103 to 0.088, indicating that organic matter susceptible to biological degradation was removed by the constructed wetlands. The mean TSS concentration decreased from 70 mgL^{-1} to 30 mgL^{-1}, a reduction of 57.1%. These reductions are generally within the ranges reported in the literature for similar wetland systems (Kadlec and Knight 1996; Song et al. 2006). In contrast to other parameters, TN showed the low efficient reduction (11.1%) and TP was higher in the outflow of the constructed wetlands compared to the influent wastewater.

Table 1 Average inlet and outlet concentrations of each parameter at the Jiaonan constructed wetland system

Parameter	Influent	Effluent	Removal (%)
Physical parameter			
pH	7.50	7.92	
DO (mgL^{-1})	0.39	1.84	
TSS (mgL^{-1})	70	30	57.1
	mgL^{-1}	mgL^{-1}	%
Chemical parameter			
COD	320	125	60.9
BOD_5	33	11	66.7
TP	1.13	2.98	−163.7
TN	71.8	63.8	11.1
	Log_{10} CFU/mL	Log_{10} CFU/mL	Log_{10}
Microorganism parameter			
Total coliforms	5.20	2.45	2.75
Fecal coliforms	5.20	2.45	2.75
Escherichia coli	4.63	1.60	3.03
Fecal streptococci	4.53	1.81	2.72
Clostridium perfringens	2.40	1.76	0.64
Salmonellae	4.90	3.30	1.60

Samples were collected between 11 March and 11 June 2007

The concentrations of indicator and pathogenic microorganisms in the constructed wetlands were lower in the effluent than in the influent. All microorganisms studied were removed and/or inactivated as the water flowed through the wetland. The constructed wetlands could effectively reduce *E. coli* by 99.9%, fecal streptococci by 99.8%, total coliforms by 99.8% and fecal coliforms by 99.8%, the *Salmonella* spp. removal efficiency was relatively low at 97.5% and the *C. perfringens* removal was the least at 77.2%. Total coliform and fecal coliform reductions are similar to the mean reductions reported for municipal treatment wetlands (Gersberg et al. 1987; Reed et al. 1995; Kadlec and Knight 1996). Although reductions of fecal coliforms were encouraging when viewed on a percentage basis, caution is warranted given that average outflow concentrations of fecal coliforms were greater than 100,000 MPN/L, which did not meet the EPA of China guidelines for fecal coliforms removal.

The die-off rates for indicator microorganisms and pathogens at different copepod concentration are presented in Table 2. Compared to the natural die-off rates, the presence of copepods in the wastewater appeared to have shown positive effects on the removal of both indicator microorganisms and pathogens. The higher copepods concentration, the greater die-off rates were received. It showed the survival time can be shortened by copepods.

As can be seen from the Table 2, the die-off rates of six microorganisms varied with each other. Among the four kinds of indicator microorganism, total coliforms had the longest survival time whereas fecal streptococci had the highest die-off rate. The die-off rate at copepods

Table 2 The die-off rates of Microorganisms at copepods concentrations of 3.0×10^2/L and 6.0×10^2/L

Microorganisms	Copepods concentrations					
	Control		3.0×10^2/L		6.0×10^2/L	
	Log reduction/ (day log_{10} day^{-1})	R^2	Log reduction/ (day log_{10} day^{-1})	R^2	Log reduction/ (day log_{10} day^{-1})	R^2
Total coliforms	0.251	0.830	0.264	0.812	0.337	0.773
Fecal coliforms	0.374	0.922	0.404	0.896	0.481	0.911
Fecal streptococci	2.544	0.917	2.544	0.926	4.544	0.987
Escherichia coli	0.549	0.996	1.049	0.986	1.049	0.999
Clostridium perfringens	0.284	0.975	0.303	0.988	0.374	0.981
Salmonella spp	0.371	0.956	0.454	0.955	0.580	0.943

concentrations of 0.0×10^2/L was the naturally occurring without copepod, die-off rates of all microorganisms varied from 0.251 $\log_{10}$ day^{-1} to 2.544 $\log_{10}$ day^{-1}. At copepods concentrations of 3.0×10^2/L and 6.0×10^2/L, the more rapidly die-off rates were observed. Total coliforms was 0.264 $\log_{10}$ day^{-1} and 0.337 $\log_{10}$ day^{-1}, respectively. Fecal coliforms had a slightly greater rate than total coliforms as 0.404 $\log_{10}$ day^{-1} and 0.481 $\log_{10}$ day^{-1}, respectively. For the other two indicator microorganisms, the die-off rates of *E. coli* are both 1.049 $\log_{10}$ day^{-1}, no variation were discovered as the higher copepods concentration given. Raising the concentration of copepods appeared to have little effect on inactivation of *E. coli.* The opposite phenomena were observed for fecal streptococci which had the shortest survival time among all the indicator microorganisms. When the copepods concentrations increased from 3.0×10^2/L to 6.0×10^2/L, the die-off rate magnitude raised from 2.544 $\log_{10}$ day^{-1} to 4.544 $\log_{10}$ day^{-1}.

The two kinds of pathogens, i.e., *C. perfringens* (CP), *Salmonella* spp (SM) had the similar inactivation phenomena as total coliforms and fecal coliforms but die-off rates at different concentration of copepods were magnitude lower than *E. coli* and fecal streptococci, which showed that the kinetic actions of *Salmonella* spp. and *C. perfringens* were quite differences from these indicator organisms in free-flow surface constructed wetlands. The inactivation rate of *C. perfringens* at copepods concentrations of 3.0×10^2/L and 6.0×10^2/L was 0.303 $\log_{10}$ day^{-1} and 0.374 $\log_{10}$ day^{-1}, and *Salmonella* spp was 0.454 $\log_{10}$ day^{-1} and 0.580 $\log_{10}$ day^{-1}, respectively. So the increasing of copepod concentration had much more effect on the inactivation of *Salmonella* spp. compared to *C. perfringens*.

The mechanisms of indicator and pathogenic bacteria removal in constructed wetlands include sedimentation, predation, absorption, and die-off from unfavorable environmental conditions, including UV in sunlight and temperatures unfavorable for cell reproduction. Sedimentation is thought to be one of the mechanisms of microbial reduction from wetlands used for wastewater treatment. Several studies have found that coliforms, fecal coliforms, and Salmonella tend to concentrate in sediments of polluted surface waters (Karima et al. 2004; Gerba and Mcleod 1976; Van Donsel and Geldreich 1971). Some studies have reported predation as another important mechanism in the microbial reduction process. Wand et al. (2007) reported that the protozoa may play the dominant role during the decrease of bacteria in wetlands, and *bdellovibrios*'s function was also identified whereas its significance needed to be evaluated. Decamp and Warren (1998) found that a population of 20 *Paramecium*/ml could have the potential to remove 17,760 *E. coli*/ml from the wastewater in the 8 h required for the wastewater to flow through the first third of the reed bed at the lowest retention time.

As our experiment did not evaluate the filtration and adsorption process by substrates and plants, we can not determine if the predation by copepods plays the dominant role during the decrease of bacteria. However, the existence of copepods being unfavorable for the survival of indicator microorganisms and pathogens has been proved. In this paper, with the normal natural decrease of bacteria in control group, high concentration of copepods leads to the result of bacteria decreasing rapidly. Copepods feed chiefly on small phytoplankton, organic particles, bacteria and other microorganisms. As Jiaonan CW system is a free water surface constructed wetland and copepods are phototactic, during the hydraulic retention time of wastewater purification, the most bacteria in the overlaying water can be preyed by copepods easily.

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