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RESEARCH ARTICLE

Treatment performance and microorganism community structure of integrated vertical-flow constructed wetland plots for domestic wastewater

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Abstract In order to investigate the treatment performance and microorganism mechanism of IVCW for domestic wastewater in central of China, two parallel pilot-scale IVCW systems were built to evaluate purification efficiencies, microbial community structure and enzyme activities. The results showed that mean removal efficiencies were 81.03 % for COD, 51.66 % for total nitrogen (TN), 42.50 % for NH_4^+-N, and 68.01 % for TP. Significant positive correlations between nitrate reductase activities and TN and NH_4^+-N removal efficiencies, along with a significant correlation between substrate enzyme activity and operation time, were observed. Redundancy analysis demonstrated gram-negative bacteria were mainly responsible for urease and phosphatase activities, and also played a major role in dehydrogenase and nitrate reductase activities. Meanwhile, anaerobic bacteria, gram-negative bacteria, and saturated FA groups, gram-positive bacteria exhibited good correlations with the removal of COD (p=0.388), N (p=0.236), and TP (p=0.074), respectively. The IVCW system can be used to treat domestic wastewater effectively.

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Introduction

Nitrogen and phosphorus pollution from domestic wastewater has become an urgent problem in the world, especially in developing countries (Li et al. 2009; Tsihrintzis and Gikas 2010; Mina et al. 2011). Constructed wetland, as an ecological engineering technology, has been proved to be able to remove nitrogen and phosphorus effectively (Vymazal 2002; Zhang et al. 2009; Tsihrintzis and Gikas 2010; Saeed and Sun 2011). In recent years, treatment performance of various types of constructed wetland for domestic wastewater has been studied intensively (van de Moortel et al. 2009; Kotti et al. 2010; Saeed and Sun 2011; Wang et al. 2011; Gikas et al. 2011; Stefanakis and Tsihrintzis 2012).

Zhang et al. (2009) have reported that the most limiting factors of Constructed wetlands application in China is the limited land resources. Integrated vertical-flow constructed wetland (IVCW), as a new, land saving, efficient wetland technology, has been successfully applied in China and Europe (Perfler et al. 1999; Liang et al. 2003; Zhou et al. 2009; Wu et al. 2011). Until now, most of this application has been focused on treatment of polluted water bodies, and researches on long-term treatment performance of IVCWs for domestic wastewater were rarely reported.

Numerous studies showed that wastewater purification in the constructed wetlands are mainly attributed to the metabolism of microbes and enzyme activities in substrate (Martens et al. 1992; Liang et al. 2003; Duarte et al. 2008; Weaver et al. 2012). In this study, two parallel pilot-scale IVCW systems were employed to investigate treatment performance, microbial community structure, and enzyme activities for domestic wastewater and the results will give references for future design, application, and operation management of the constructed wetland. Specific tasks are: (1) evaluate treatment performance at a hydraulic loading rate of 125 mm/day; (2) investigate yearly microbial community structure change; (3) identify the correlation among microbial community structure, enzyme activities, and pollutant removal efficiencies.

Materials and methods

Experimental setup

Two parallel pilot-scale IVCW systems were set up near Donghu Lake in Wuhan, China (30° 33′N, 114° 23′E; elevation, 27 m). Each with a down-flow chamber (1 m×1 m×1 m) in series with an up-flow chamber (1 m×1 m×1 m). The two chambers were separated by a wall but connected at the bottom, hence the water flow could be induced automatically from down-flow chamber to up-flow chamber and then out of the wetlands once a new inflow was added. Consequently, the chambers were saturated during the operation; the wastewater can make the most of limited land resources (Fig.1a). Gravel of 10–20 mm diameter was filled to a depth of 50 and 40 cm for the down-flow chamber and up-flow chamber, respectively, followed by a 35-cm-thick layer of 2–10 mm diameter gravel. The porosity of the substrate was estimated to be 0.40 and the effective volume of the wetland bed was 0.6 m^3.

Based on our previous study, two species of macrophytes, *Arundo donax* and *Canna indica*, were planted at a density of 6 plants/m^2 in the down-flow and up-flow chambers, respectively. The plants were fully grown during the experiments until the winter (Fig. 1 b, c, d).

Operation conditions

The influent was induced intermittently in every IVCW unit per day to yield a hydraulic loading rate of 125 mm/day, and the theoretical hydraulic retention time (HRT) was 2.4 days.

The study was carried out from 23rd April 2011 to 23rd February 2012, with an average ambient temperature of 22.03 °C. In order to minimize variability in the experiment, simulated domestic wastewater was used in the experiment. The influent characteristics are summarized in Table 1.

Samples collected

The influent and effluent water samples were collected from 23rd April 2011 to 23rd February 2012. The sampling frequency was scheduled for once every 2 days during the first 20 days, once every 4 days during the middle 30 days, and once a week during the last 250 days.

The horizontal substrate samples from five representative sites of the IVCW systems (as shown in Fig. 2) were collected and composited once every 2 months from 1st May 2011 to 1st January 2012 (Zhou et al. 2009; Zhang et al. 2011). All litter was removed and the samples were taken to the laboratory in sealed polypropylene bags and stored in

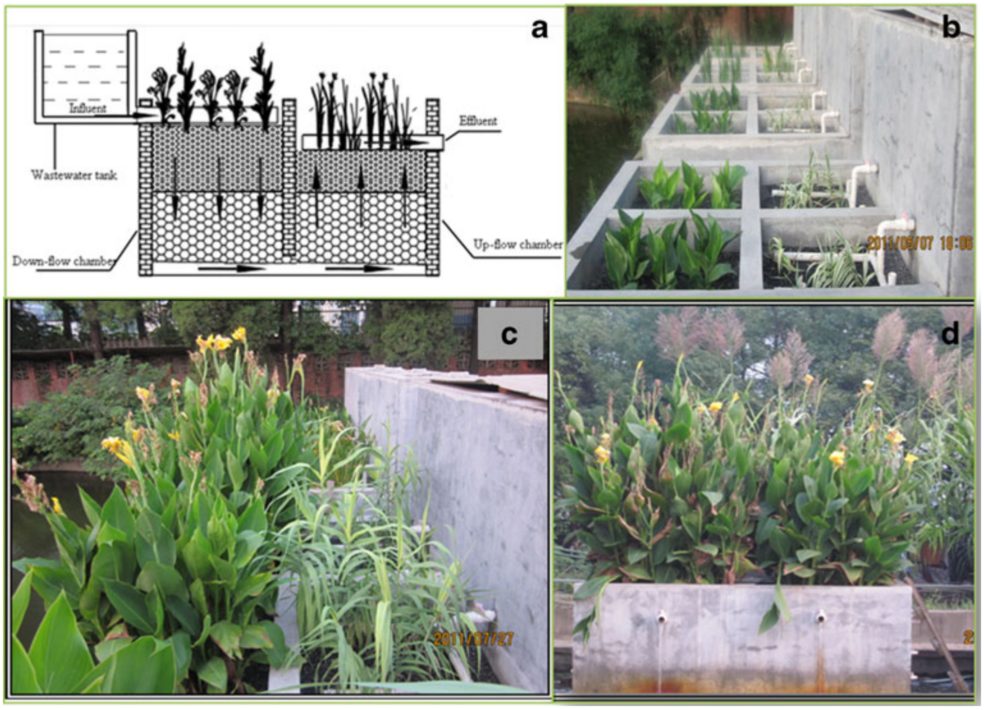

Fig. 1 **a** Schematic diagram of IVCW; **b** IVCWs after 2 weeks' operation; **c** IVCWs after 3 months' operation; **d** IVCWs at the end of November

Table 1 Characteristics of the influent and effluent

Parameters	Influent		Effluent
pH	7.56±0.43		7.59±0.45
DO (mg/L)	2.53±1.74		1.58±0.60
T (°C)	22.03±8.51 (ambient)		20.47±8.70
Conductivity (μs/cm)	399.92±41.32		508.25±67.72
Parameters	Influent concentration (mg/L)	Effluent concentration (mg/L)	Removal efficiency (%)
COD	106.94±17.98	19.74±8.60	81.03±10.16
TN	11.54±1.55	5.53±1.47	51.66±13.96
NH_4^+-N	8.34±1.70	4.80±1.03	42.50±16.83
NO_3^--N	1.37±0.86	0.24±0.27	–
NO_2^--N	0.11±0.12	0.02±0.04	–
TP	1.00±0.17	0.32±0.17	68.01±17.45

n=100. The data was presented as mean value±standard deviation

a freezer at −20 °C until analysis of microbial community structure, or at 4 °C until analysis of enzyme activities.

Water quality analysis

pH, electrical conductivity (EC), dissolved oxygen (DO), and temperature were determined using Orion 5-star portable pH/conductivity/DO multimeter (Thermo Fisher Scientific Company, USA). Chemical oxygen demand (COD) (DRB 200, Hach, USA), total nitrogen (TN), ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), nitrite nitrogen (NO_2^--N) and total phosphorus (TP) were measured according to the standard methods (SEPA, 2002).

Pollutant removal efficiency was calculated by the percentage of deduction in concentration for each pollutant as follows: removal efficiency=$(1-C_{\mathrm{eff}}/C_{\mathrm{inf}})\times 100$ %, where C_{inf} and C_{eff} are influent and effluent concentrations in milligrams per liter.

Microbial community structure analysis

Fatty acid methyl esters (FAME) of substrate samples were analyzed using a mild alkaline methanolysis method (Schutter and Dick 2000). Fifteen milliliters of 0.2 M potassium hydroxide (KOH) in methanol was added into a 35-mL centrifuge tube containing 3 g of freeze-dried substrate. The contents of the tubes were mixed and incubated at 37 °C for an hour, during which ester-linked fatty acids were released and methylated. The tubes were vortexed every 10 min during the incubation period. Then, 3 mL of 1.0 M acetic acid was added to neutralize the pH of the tube contents. EL-FAMEs were partitioned into an organic phase by adding 10 mL hexane followed by centrifugation at 4,500 rpm for 15 min. After the hexane layer was transferred into a clean glass tube, the hexane was evaporated under N_2 steam. In the final step, FAMEs were dissolved in three aliquots of 200 μL of hexane: methyl-*tert* butyl ether (1:1) and transferred to an amber vial for gas chromatography

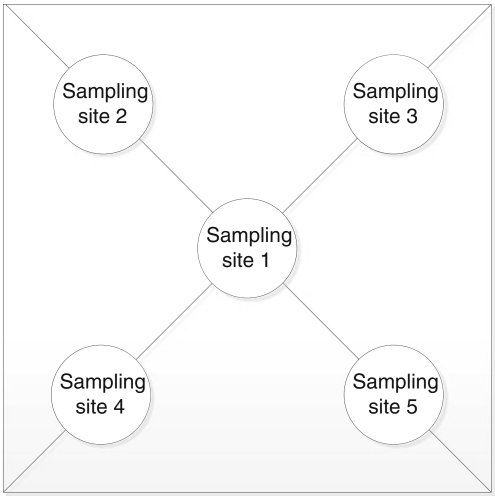

Fig. 2 The substrate sampling site of each chamber in IVCW

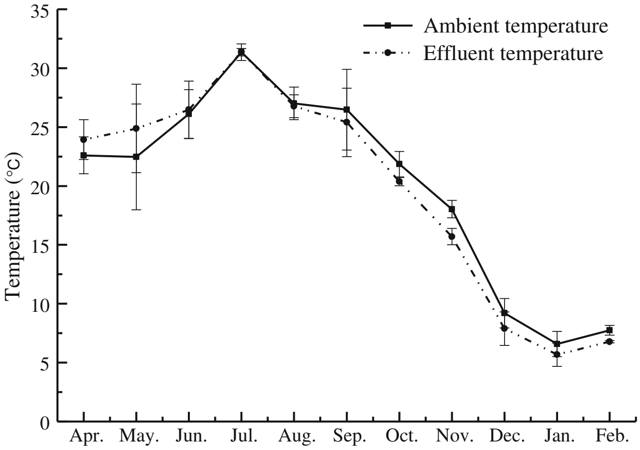

Fig. 3 Variations of ambient and effluent temperature throughout the experimental period

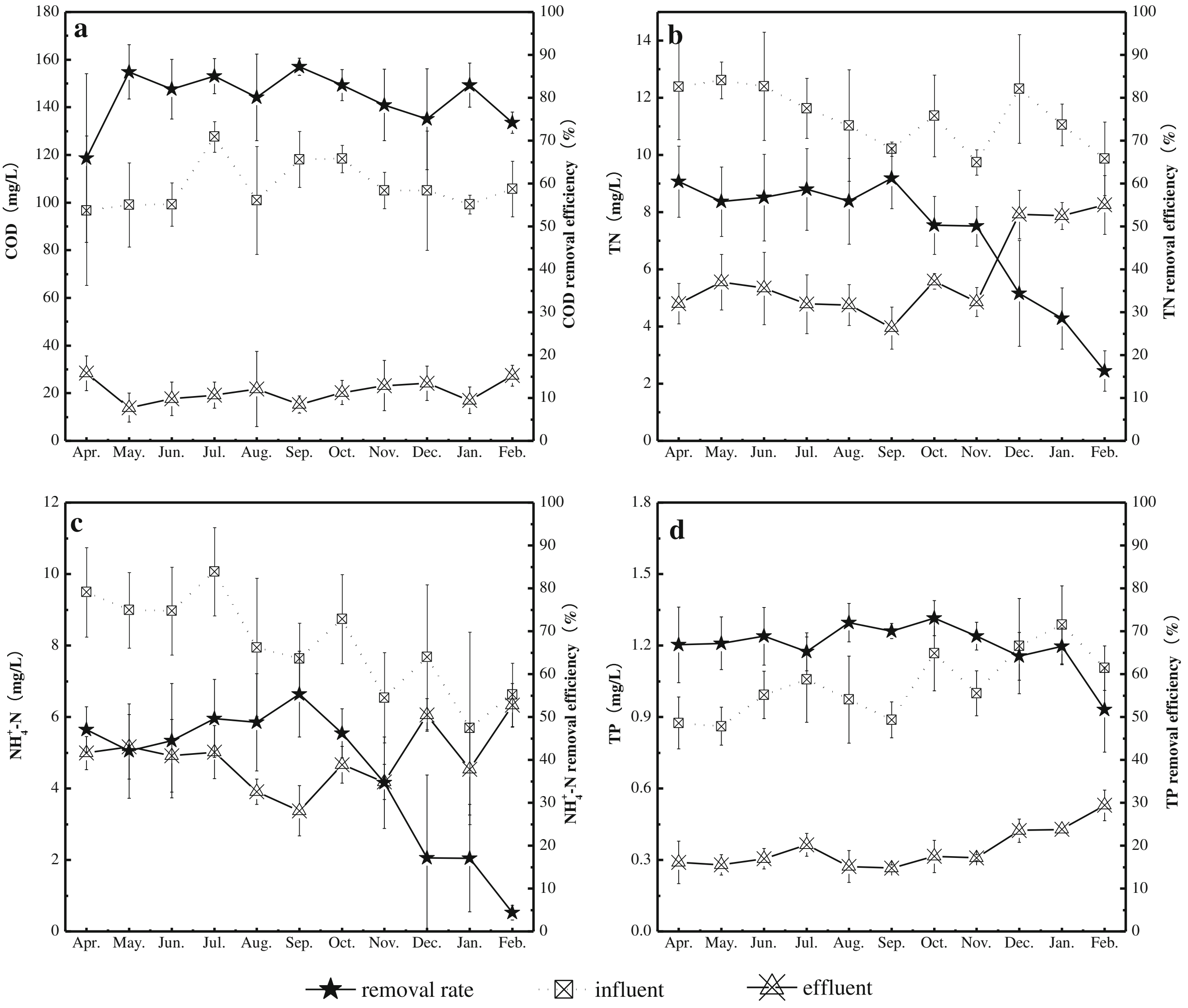

Fig. 4 Removal rates for pollutants (mean±SD, $n \geq 8$). **a** COD, **b** TN, **c** NH$_4^+$-N, **d** TP

(GC) analysis. The MIDI peak identification software (MIDI, Inc., Newark, DE) was used to identify individual fatty acids. Fatty acid peaks were identified using 26-component bacterial acid methyl ester mix (Supelco, USA).

Fatty acids (FAs) are designated by the total number of carbon atoms, followed by a colon and the number of double bonds. Then a "ω" and a number show the position of the initial double bond from the methyl end of the chain, sometimes followed by a "c" or "t" for *cis* or *trans* configuration, respectively. The prefixes "i" and "a" refer to methyl branching at the iso- and anteiso- positions, respectively. Cyclopropane FAs have the prefix "cy" (Zelles 1999).

Different microbial groups, including Gram-positive and Gram-negative bacteria, characterized by typical fatty acids with different chain structures, can be used as biomarkers for these groups (Zelles 1999). In this study, fatty acids i15:0, a15:0, 15:0, i16:0, 16:1ω9, i17:0, 17:0, cy17:0, 18:1ω9c, 18:1ω9t, and cy19:0 were chosen to represent total bacteria (Pankhurst et al. 2001). Monounsaturated FAs (MUFAs), including 16:1ω9, 18:1ω9c and 18:1ω9t, were used as indicators of Gram-negative bacteria; branched FAs, including i15:0, a15:0, i16:0 and i17:0, were used to indicate Gram-positive; Cyclopropyl FAs, including cy17:0 and cy19:0 were used as indicators of Anaerobic bacteria (Zelles et al. 1992; Wilkinson 1988; D'Angelo et al. 2005).

Enzyme activity analysis

The dehydrogenase activity in the substrate was determined by Tabatabai (1994) method. The urease activity was

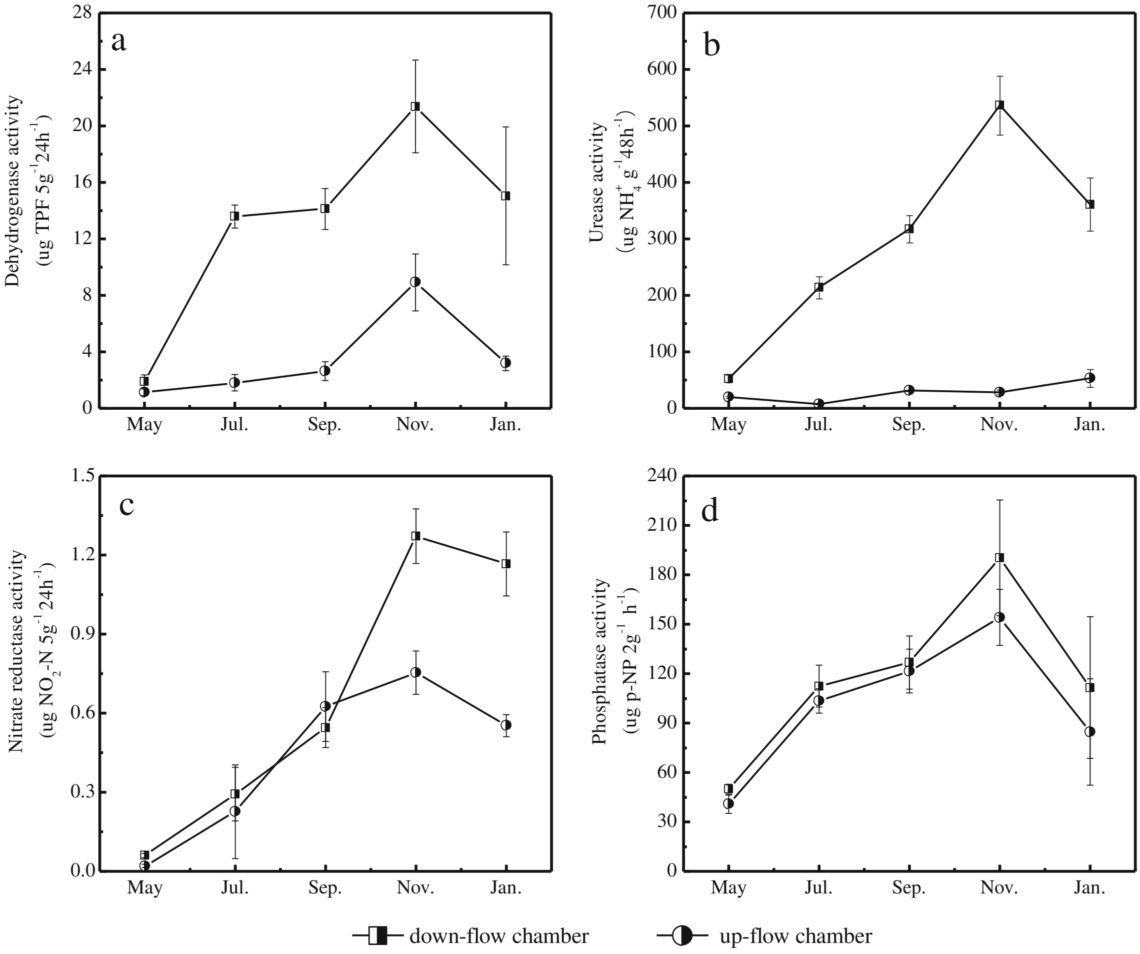

Fig. 5 Enzyme activities of the IVCW plots (mean±SD, *n*=6). **a** Dehydrogenase, **b** urease, **c** nitrate reductase, **d** phosphatase

analyzed according to Klose and Tabatabai (2000). The nitrate reductase activity was analyzed using KNO_3 as a substrate (Abdelmagid and Tabatabai 1987). The phosphatase activity was analyzed by the Schinner and von Mersi (1990) method.

Statistical analysis

The experiments data were summarized and reported as mean values±standard deviation (SD). Comparison of the averages was carried out by one-way ANOVA followed by the least significant difference (LSD) test, using the software of SPSS 16.0 (SPSS Inc., Chicago, IL, USA), significant differences was set at $p<0.05$.

The effects of microbial communities and environmental parameters on enzyme activities and pollutants removal were tested using redundancy analysis (RDA). The RDA was performed using Canoco 4.5 software (Center for Biometry, Wageningen, the Netherlands) which was fully discussed in ter Braak (1994) and Leps and Smilauer (2003).

Results

Removal efficiencies for pollutants

The mean concentrations in the effluent and removal efficiency of pollutants during the experimental period are shown in Table 1 and Figs. 3 and 4.

COD

The average COD concentrations were reduced from 106.94± 17.98 mg/L to 19.74±8.60 mg/L, with average COD removal efficiency of 81.03±10.16 %. During the experimental period, the COD removal increased early, with the highest removal of 87.25 % occurred in September, and then decreased gradually.

N

In general, TN and NH_4^+-N concentrations decreased from 11.54±1.55 mg/L and 8.34±1.70 mg/L to 5.53±1.47 mg/L

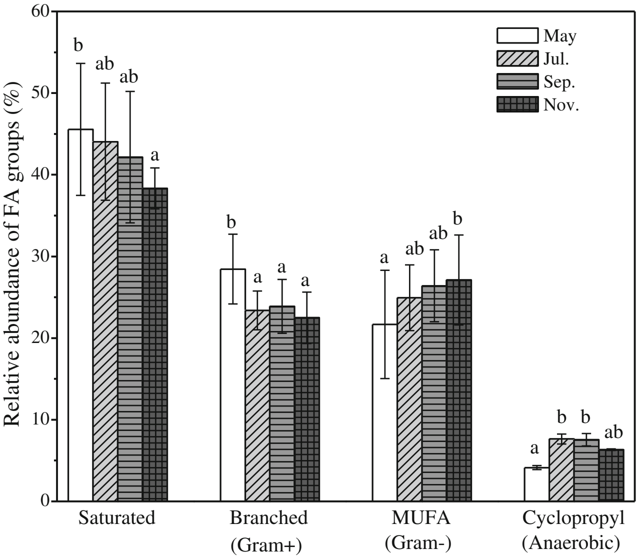

Fig. 6 Variation of FA groups of the IVCW plots

and 4.80±1.03 mg/L, respectively. Accordingly, mean removal efficiencies for TN and NH_4^+-N were 51.66± 13.96 % and 40.50±16.83 %, respectively. As for NO_3^--N and NO_2^--N, the average effluent concentrations were 0.24 and 0.02 mg/L, with removal rates of 92.56 and 97.86 %, respectively.

TN removal was stable from April to September, and the average removal efficiency was 58.14 %, then decreased sharply (Fig. 2b), especially after November. Meanwhile, the NH_4^+-N removal increased early, and then declined dramatically, with the maximum removal efficiency of 55.33 % occurred in September (Fig. 2c). At the end of the experiment, the poor efficiency of TN and NH_4^+-N was observed probably due to lower temperature and withered plants.

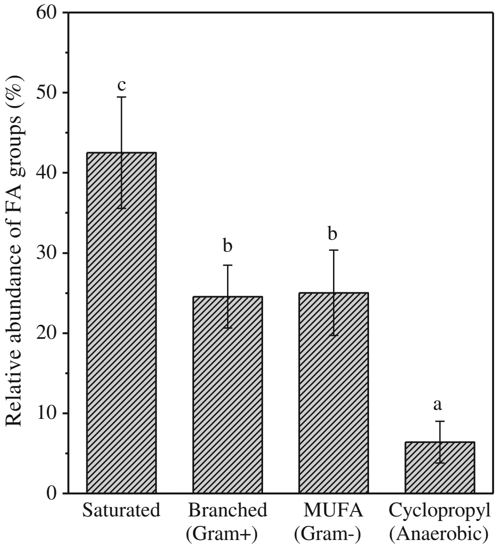

Fig. 7 The average FA groups in the substrate

TP

The average effluent TP concentration during the experimental period was 0.33±0.18 mg/L, with a mean removal efficiency of 67.52 %. The TP removal was relatively stable from April to October, and then declined gradually from November.

Enzyme activities

The activities of dehydrogenase, urease, nitrate reductase and phosphatase in the down-flow chamber and up-flow chamber of the IVCWs are summarized in Fig. 5.

During the experimental period, dehydrogenase, urease, nitrate reductase and phosphatase activities showed a single-peak pattern (November). Except phosphatase, the average enzyme activities in the down-flow chamber were significantly higher than those in the up-flow chamber ($p<0.05$).

Microbial community structure

The microbial community structure during the experimental period is shown in Figs. 6 and 7.

The relative abundance of saturated FAs were dominated in the FAME profiles (45.34~38.34 %) (Fig. 7), with the highest abundance occurred in May ($p<0.05$) (Fig. 6), followed by branched FAs (Gram-positive bacteria) and monounsaturated FAs (Gram-negative bacteria), but no significant difference ($p>0.05$) was observed between them. During the experimental period, branched FAs decreased from 24.78± 0.75 % to 19.66±0.27 %, while monounsaturated FAs increased gradually from 21.67±6.64 % to 27.13±5.51 %. Abundance of cyclopropyl FAs (anaerobic bacteria) were the lowest one (4.13~6.34 %), with the lowest abundance in May.

Discussion

Evaluation of treatment performance for pollutants

During the whole operation period, the average COD removal efficiencies were 81.03±10.16 %. It was much higher than the previous studies reported by Fountoulakis et al. (2009), in which freewater surface constructed wetland, horizontal subsurface flow CW, and rotating biological contactor, packed bed filter were applied to treat domestic wastewater with mean inlet COD of 99.6±49.4 mg/L under the same HLR of 125 mm/day; but similar to the result of Zhao et al. (2011), in which two-stage combinations of

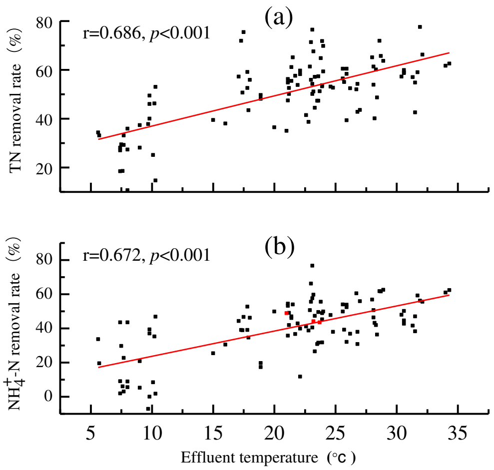

Fig. 8 Relationships between water temperature and TN and NH_4^+-N removal rates of the IVCWs. **a** TN, **b** NH_4^+-N

subsurface vertical down-flow and up-flow constructed wetland systems were applied; meanwhile, lower than the removal capacity for a mean influent COD of 510 mg/L (140 g COD/m^2 day) by using ten pilot-scale vertical-flow constructed wetlands of different design and operation characteristics operated continuously for 3 years under Mediterranean climatic conditions (Stefanakis and Tsihrintzis 2012).

The removal efficiency of TN in this study was similar to the result of Wang et al. (2011) and Wu et al. (2011), but lower than Akratos and Tsihrintzis (2007), Kotti et al. (2010) and Gikas and Tsihrintzis (2012) for the similar size. The removal rates for NH_4^+-N was similar to Chang et al. (2012), while lower than Kotti et al. (2010), Wu et al. (2011) and Xiong et al. (2011). This may be attributed to different HRT (Huang et al. 2000; Akratos and Tsihrintzis 2007; Kotti et al. 2010; Stefanakis and Tsihrintzis 2012).

The TN and NH_4^+-N removal efficiencies were decreased from November, gradually decreased temperature was the main reason (Akratos and Tsihrintzis 2007; Tsihrintzis and Gikas 2010). As shown in Fig. 8, the N removal efficiency positively related to temperatures of the effluent, demonstrating temperatures had a significant impact on N removal. Kuschk et al. (2003) and Wu et al. (2011) have reported that nitrification was inhibited when the temperature was below 10 °C and decreased sharply below 6 °C, which was in agreement with our result.

The removal mechanisms of phosphorus from wastewater in constructed wetlands were mainly through substrate adsorption, iron exchange, and plant uptake (Billore et al. 1999). During the whole experiment, the TP removal efficiency remained relatively steady, and mean TP removal efficiencies (68.01±17.45 %) was similar to the results of Chung et al. (2008), Wu et al. (2011) and Gikas and Tsihrintzis (2012), but lower than others (Xiong et al. 2011; Dong et al. 2011; Wang et al. 2011).

Relationships between substrate enzyme activities, pollutants removal, and operation time

The relationships among substrate enzyme activities, pollutants removal, and operation time are shown in Table 2.

Significantly positive correlation was found between the operation time and all the enzyme activities (p<0.001), which can be attributed to the IVCW system operation condition. For the relationship between operation time and pollutant removal, only significant negative correlation were found with TP removal (p=0.003, F=−0.545). Meanwhile, significant positive correlations between nitrate reductase activities and TN and NH_4^+-N removal efficiencies were found. The result was not similar to Li et al. (2011). The negative relationship between phosphatase activity and TP removal was also found, which can be explained by the fact that TP removal was mainly absorbed by the substrate in the constructed wetland, and phosphatase activity had inverse relationship with substrate P concentration (Wright and Reddy 2001; Allison et al. 2007).

Relationships between microbial community structures and function

Enzyme activity has been regarded as an important indicator of microbial community function (Mentzer et al. 2006; Zhang et al. 2011), and microbial community structure has been demonstrated to be closely related to specific enzyme activities (Zhang et al. 2006).

In the present study, the correlations between microbial community structure and function were analyzed by RDA, a significant association (p=0.002) was observed (Fig. 9).

The most significant diagnostic FA groups associated with the enzyme data included Gram-positive bacteria (p=0.002), anaerobic bacteria (p=0.024), Gram-negative bacteria (p=0.078) and Saturated FA groups (p=0.096). The results showed that Gram-negative bacteria were mainly

Table 2 Correlations among substrate enzyme activities, pollutant removal and operation time

Time	Phosphatase	Urease	Nitrate reductase	Dehydrogenase
	+***	+***	+***	+***
Nitrate reductase	TN	NH_4^+-N	Phosphatase	TP
	+*	+*		−**

*p<0.05; **p<0.01; ***p<0.001

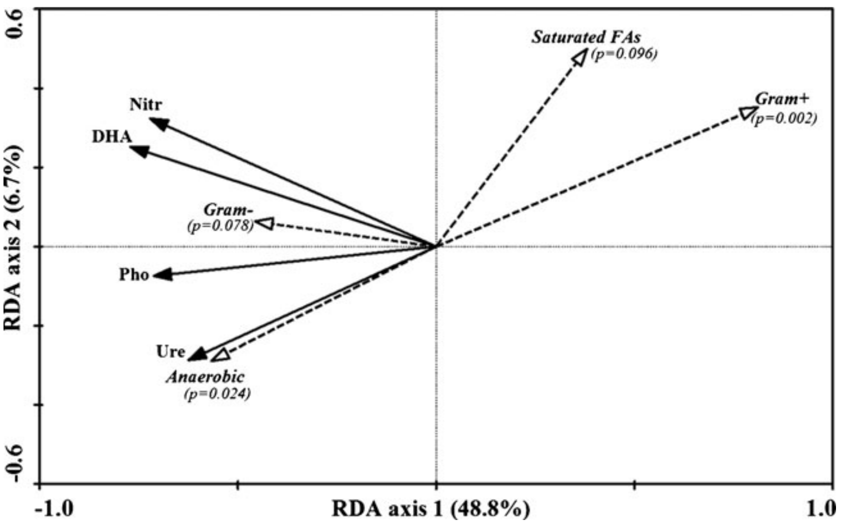

Fig. 9 The biplot of the first two RDA axes of enzyme activity and microbial communities. Enzyme activity parameters (expressed as "response variables") were presented as *black solid arrow*, whereas microbial communities (expressed as explanatory variables) were presented as *empty dashed arrow*. The microbial communities were labeled with their *p* values to show the significance of their relationship to RDA axes. In the diagram, *Gram*$^+$ for Gram-positive bacteria abundance, *Gram*$^-$ for Gram-negative bacteria abundance, *Anaerobic* for anaerobic bacteria abundance; *DHA* for dehydrogenase, *Ure* for urease, *Nitr* for nitrate reductase, *Pho* for phosphatase

responsible for urease and phosphatase activities, also important contributors for dehydrogenase and nitrate reductase activities. This finding was consistent with Zhang et al. (2011). Meanwhile, urease and phosphatase activities were also greatly impacted by the abundance of anaerobic bacteria (Fig. 9).

There was significant negative correlation between Gram-positive bacteria and urease and phosphatase activities, while no significant correlation between enzyme activity and Gram-negative bacteria was found (Table 3). The results were similar to the findings reported by Mentzer et al. (2006).

Relationship among microbial community structure, environmental parameters, and pollutant removal

Previous studies have demonstrated that microorganisms play different roles in the removal or transformation of

Table 3 Correlations between microbial community and environmental variables

	Anaerobic	Gram$^-$	Gram$^+$
Time			–**
DO	–**		+*
TP			+**
Urease			–**
Phosphatase			–**
Dehydrogenase			

*$p<0.05$; **$p<0.01$

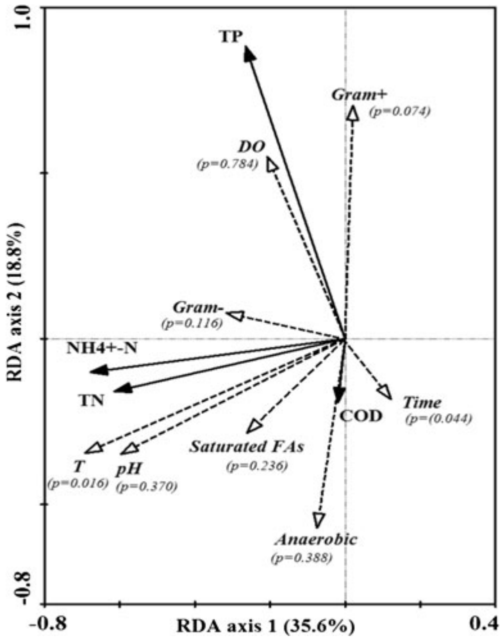

Fig. 10 The biplot of the first two RDA axes of microbial communities, environmental parameters and pollutant removal efficiencies. The pollutant removal efficiencies (expressed as "response variables" in the RDA analysis) were presented as *black solid arrow*, whereas the specific diagnostic FA groups and environmental parameters (expressed as explanatory variables) were presented as empty dashed arrow. The explanatory variables were labeled with their *p* values to show the significance of their relationship to RDA axes. The following abbreviations were used in the diagram: *Gram*$^+$ for Gram-positive bacteria abundance, *Gram*$^-$ for Gram-negative bacteria abundance, *Anaerobic* for anaerobic bacteria abundance; *Time* for operation time, *T* for average temperature of influent and effluent

pollutants in the constructed wetlands (Ahn et al. 2007; Krasnits et al. 2009). Microbial community structure has been proposed to be an important determinant of water quality improvement in the wetland systems (Calheiros et al. 2009; Faulwetter et al. 2009), and temperature (Smith et al. 2010), hydrologic regime and pollutant treatments (Mentzer et al. 2006; Steenwerth et al. 2006), plant diversity and function group richness (Zhang et al. 2010, 2011) and biotic succession (Kent et al. 2007), could strongly influence the microbial community structure.

In the present study, the effects of microbial community structure (four diagnostic fatty acid (FA) groups) and environmental parameters on pollutant removal efficiencies were also determined by RDA (Fig. 10).

The most significant explanatory variables associated with removal efficiencies of pollutants included temperature (p= 0.016), operation time (p=0.044), and Gram-positive bacteria (p=0.074). The removal of TN and NH_4^+-N were positively correlated with temperature, pH, abundance of saturated FA groups, and Gram-negative bacteria. TP removal were significantly positively correlated with the abundance of Gram-positive bacteria indicators (branched fatty acids) (Table 3), but negatively correlated with sampling time. Abundance of anaerobic bacteria was contributed to the removal of COD.

The results indicated that operation time and temperature were the key environment factor to influence removal of pollutants. Gram-positive bacteria were mainly responsible for TP removal, and anaerobic bacteria might be important contributors for COD removal, while the removal of N was primarily dependent on the abundance of saturated FA groups and Gram-negative bacteria.

Conclusions

(1) Mean removal efficiencies of 81.03 % for COD, 51.66 % for TN, 42.50 % for NH_4^+-N and 68.01 % for TP were achieved by the IVCWs to treat domestic wastewater at a loading rate of 125 mm/day under the subtropical monsoon climate, respectively. HRT and temperature was the main limited factors for nitrogen removal.

(2) Nitrate reductase activities significant positive correlated with TN and NH_4^+-N removal efficiencies, the nitrate reductase activities could be used to indicate the efficiency of nitrogen removal in IVCWs.

(3) Anaerobic bacteria, saturated FA groups, and Gram-positive bacteria were mainly responsible for COD (p= 0.388), N (p=0.236), and TP (p=0.074) removal, respectively.

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