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# Responses of microbial abundance and enzyme activity in integrated vertical-flow constructed wetlands for domestic and secondary wastewater

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ABSTRACT

Although micro-organisms play a significant role in pollutant removal in constructed wetlands, little is known on the effect of wastewater-quality properties on microbial characteristics. In this study, two groups of integrated vertical-flow constructed wetland microcosms were applied to treat synthetic domestic wastewater and synthetic secondary effluent. The effects of wastewater-quality properties on microbial features were assessed. Results showed that higher values of microbial indicators were observed in the systems with domestic wastewater and in down-flow cells. Redundancy analysis revealed that organic matter concentration and temperature were two critical determinants influencing the microbial features.

*Keywords:* Integrated vertical-flow constructed wetlands (IVCWs); Domestic wastewater (DW); Secondary wastewater (SW); Microbes; Enzyme activities; Water-quality properties

## 1. Introduction

Constructed wetland (CW) is an alternative method for treating and improving quality of various types of wastewater, and has been used for ecological restoration [1–3].

The pollutant removal mechanisms in CW, involving the combinations of physical, chemical, and biological processes, take place through the interactions of plants, substrates, and adhered micro-organisms [4,5]. However, micro-organisms play a crucial role in pollutant transformation and degradation processes [1,6,7]. It was noted that the presence of extracellular enzymes in CWs, primarily deriving from micro-organisms, is important in the biogeochemical transformation and cycling of substance and energy [7,8,10]. In addition, microbial abundance and potential of nitrification/denitrification are also effective indicators of microbial processes. It was well realized that a more comprehensive understanding on microbial population and activity was favorable to explore wastewater purification mechanisms within CWs [11,12]. Increasing evidences indicated that these microbial indices can be applied as potential

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indicators for water-quality maintenance function and CW management [10,12–14]. Nonetheless, to date, the information on microbial indicators in CWs is relatively limited [15].

Integrated vertical-flow constructed wetlands (IVCWs) have been successfully applied to treat domestic and secondary wastewater [16–19]. It is expected that the microbial parameters mentioned above can be markedly affected by wastewater type due to different constituents. Accordingly, in this study, two groups of integrated vertical-flow constructed wetlands (IVCWs) were established to treat domestic and secondary wastewater, respectively, and microbial abundances, enzyme activities (dehydrogenase, fluorescein diacetate hydrolase, catalase, urease, nitrate reductase, and acid phosphatases), and potentials of nitrification and denitrification in the IVCWs were investigated, and the purposes were (1) to analyze the microbial features responding to two types of wastewater; and (2) to assess the role of wastewater-quality properties in regulating these microbial indicators.

## 2. Materials and methods

### 2.1. Experimental setups

Two groups of outdoor integrated vertical-flow constructed wetlands (IVCWs) were set up and fed with domestic and secondary wastewater, respectively, with two replicates for each type of wastewater. The IVCW diagram is shown in Fig. 1, and details were discussed in Chang et al. [17,18].

*Arundo donax var. versicolor* and *Canna indica* were transplanted in down-flow and up-flow cell, respectively, at a density of 9 plant/$m^2$.

### 2.2. Wastewater and operation conditions

Secondary wastewater was simulated based on the references [20]. For the purpose of more reasonable comparison, similar levels of TN and TP were applied in the domestic wastewater. Thus, the wastewater could be characterized as low-strength [21]. The water-quality parameters of the two type of wastewater are listed in Table 1. The wastewaters were intermittently fed into the IVCWs at a hydraulic loading rate of 12.5 cm/d.

### 2.3. Water sampling and analysis

The experiment was conducted from April 2011 to February 2012. Water samples of the inflow and outflow were collected weekly, and pH, temperature (T), electric conductivity (EC), and dissolved oxygen (DO) concentration were measured *in situ* using an Orion 5-star portable multimeter (Thermo Fisher Scientific Company, USA). Then the samples were taken to laboratory, and TN, ammonium ($NH_4^+$), nitrate ($NO_3^-$), nitrite ($NO_2^-$), and TP were determined according to the standard methods [22]. Chemical oxygen demand (COD) was analyzed by a spectrophotometer (DR/2010, Hach Co., Loveland, CO, USA).

### 2.4. Substrate sampling and analysis

Substrate samples were collected on four dates during the experimental period: June 20, 2011 (Jun.); August 25, 2011 (Aug.); October 28, 2011 (Oct.); December 26, 2011 (Dec.). Five sub-samples were taken from about 10 cm depth of each cell and were thoroughly mixed as one representative sample, and a total of 8 samples were collected (4 IVCW systems

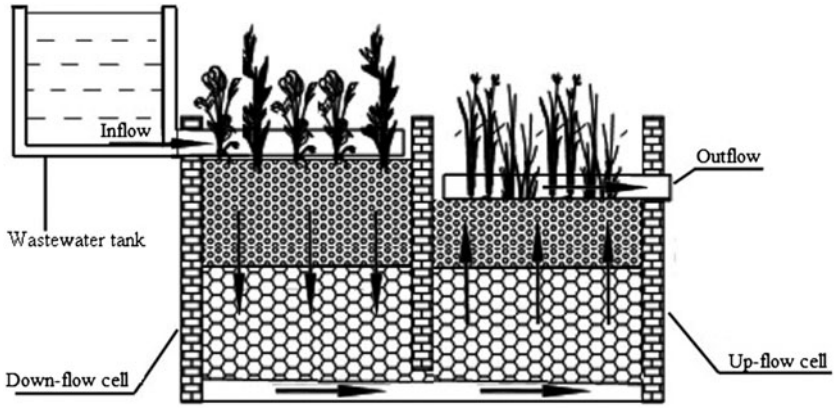


Fig. 1. Diagram of the IVCW system.

Table 1
Water qualities and pollutant removal efficiencies of the IVCWs on the four substrate sampling dates (Mean values of the two parallel systems)

| | Domestic wastewater | | | | | | | | Secondary wastewater | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Jun. | | Aug. | | Oct. | | Dec. | | Jun. | | Aug. | | Oct. | | Dec. | |
| Parameter | In | Out | In | Out | In | Out | In | Out | In | Out | In | Out | In | Out | In | Out |
| pH | 7.93 | 8.03 | 7.61 | 7.55 | 7.22 | 7.22 | 7.61 | 7.83 | 8.18 | 8.14 | 7.89 | 7.68 | 7.52 | 7.35 | 8.07 | 8.14 |
| DO (mg $L^{-1}$) | 2.54 | 1.19 | 1.44 | 1.30 | 0.61 | 1.22 | 0.98 | 2.68 | 5.23 | 1.40 | 5.32 | 1.91 | 4.94 | 2.09 | 7.55 | 4.56 |
| $T$ (℃) | 29.2 | 30.4 | 28.5 | 29.5 | 18.0 | 17.9 | 6.6 | 6.2 | 28.8 | 29.9 | 28.3 | 29.3 | 17.8 | 17.7 | 6.7 | 6.3 |
| EC (μs/cm) | 379 | 478 | 367 | 484 | 337 | 495 | 420 | 605 | 369 | 430 | 357 | 441 | 330 | 479 | 430 | 580 |
| COD (mg $L^{-1}$) | 95.3 | 9.5 | 101 | 12.9 | 115 | 15.3 | 112 | 16.9 | 18.1 | 5.3 | 18.2 | 6.9 | 19.0 | 4.5 | 17.5 | 8.3 |
| Removal efficiency (%) | 90.0 | | 87.2 | | 86.7 | | 84.9 | | 70.7 | | 62.1 | | 76.3 | | 52.6 | |
| TN (mg $L^{-1}$) | 11.6 | 5.61 | 10.9 | 5.55 | 13.2 | 6.83 | 12.4 | 9.02 | 12.6 | 4.55 | 12.4 | 2.21 | 12.9 | 2.60 | 13.1 | 13.6 |
| Removal efficiency (%) | 51.6 | | 49.1 | | 48.3 | | 27.3 | | 63.9 | | 82.2 | | 79.8 | | −0.04 | |
| Organic N (mg $L^{-1}$) | 3.24 | 0.12 | 3.76 | 0.48 | 4.20 | 0.67 | 5.83 | 1.29 | 0.81 | 0.06 | 1.12 | 0.00 | 1.01 | 0.15 | 0.73 | 1.05 |
| Removal efficiency (%) | 96.3 | | 87.2 | | 84.0 | | 77.9 | | 92.6 | | 100.0 | | 85.1 | | −43.8 | |
| $NH_4^+$ (mg $L^{-1}$) | 7.28 | 5.30 | 6.82 | 5.01 | 8.33 | 5.91 | 6.08 | 7.05 | 0.10 | 0.00 | 0.00 | 0.00 | 0.05 | 0.18 | 0.30 | 0.05 |
| Removal efficiency (%) | 27.2 | | 26.5 | | 29.1 | | −16.0 | | – | | – | | – | | – | |
| $NO_3^-$ (mg $L^{-1}$) | 0.99 | 0.19 | 0.15 | 0.06 | 0.62 | 0.25 | 0.49 | 0.60 | 11.5 | 4.30 | 11.2 | 2.11 | 11.8 | 2.17 | 12.0 | 12.5 |
| Removal efficiency (%) | 80.8 | | 60.0 | | 60.0 | | −22.4 | | 62.6 | | 81.2 | | 81.6 | | −0.04 | |
| $NO_2^-$ (mg $L^{-1}$) | 0.09 | 0.00 | 0.17 | 0.00 | 0.05 | 0.00 | 0.00 | 0.08 | 0.24 | 0.19 | 0.08 | 0.10 | 0.04 | 0.10 | 0.07 | 0.00 |
| TP (mg $L^{-1}$) | 1.02 | 1.21 | 1.23 | 0.70 | 1.22 | 0.61 | 1.14 | 0.66 | 1.03 | 0.68 | 1.25 | 0.38 | 1.20 | 0.36 | 1.13 | 0.85 |
| Removal efficiency (%) | −18.6 | | 43.1 | | 50.0 | | 42.1 | | 34.0 | | 69.6 | | 70.0 | | 24.8 | |

and 2 samples collected for each system) and taken back to the laboratory and stored at 4℃. The samples collected in down-flow and up-flow cell were regarded to be contacted with the influent and effluent of each IVCW, respectively. Meanwhile, relevant water samples along with substrate samplings were also collected simultaneously and analyzed (Section 2.3), and their quality properties are listed in Table 1. The moisture content of substrate sample was determined by drying to a constant weight at 70℃ in an air oven.

#### 2.4.1. Microbial population enumeration

The number of culturable heterotrophic bacteria, fungi, and actinomyces were measured by ten-fold serial dilution plate count method [23]. Beef extract peptone, martin agar, and improved Gause's I synthetic medium were employed to be the selective isolation agar medium for bacteria, fungi, and actinomyces, respectively. Triplicate plates were set for each indicator. Then the bacteria were incubated at 37℃ for 1 d, fungi at 28℃ for 3–4 d, and actinomyces at 28℃ for 5 d. Plates containing 30–100 colonies were used for counting. Microbial quantity was expressed as colony-forming units (CFUs) $g^{-1}$ substrate (dry weight).

#### 2.4.2. Enzyme activity analysis

Dehydrogenase activity (DHA) was analyzed according to Li et al. [23] and Tabatabai [24]. Briefly, 20 mL of 0.5% triphenyl tetrazolium chloride (TTC) and 5 g of substrate were incubated in a tube at 37℃ for 24 h. TTC could be reduced by micro-organisms to triphenyl formazan (TPF), which could be extracted by toluene and determined colorimetrically at 485 nm. The DHA was expressed as μg TPF $g^{-1}$ substrate (dry weight) 24 $h^{-1}$.

Fluorescein diacetate hydrolase (FDAH) was determined with a protocol adapted from Adam and Duncan [25]. Briefly, 100 μL of fluorescein diacetate (FDA, 2 mg/mL) and 5 g of substrate were incubated with phosphate buffer (0.5 mM, pH = 7.6) in a tube for 3 h at 30℃. After the reaction was stopped with 0.5 mL of chloroform/methanol (2:1 v/v), the solution was centrifuged at 6,000 g for 5 min and the supernatant was colorimetrically assayed at 490 nm. One unit indicates the mount of fluorescein (μg) produced through the hydrolysis of FDA by the micro-organisms in 1 g substrate (dry weight) in 3 h.

Catalase activity (CA) was measured titrimetrically [21,26]. About 3% $H_2O_2$ was added into the samples and placed at 4℃ for 30 min and then the residual $H_2O_2$ was titrated with 0.002 mol/L $KMnO_4$ in the

presence of $H_2SO_4$, and the CA was expressed as mL $KMnO_4$ consumed $g^{-1}$ substrate (dry weight)·$h^{-1}$.

As for urease activity (UA), 10 mL of 10% aqueous urea were implemented as the reaction medium and incubated with 10 mL phosphate buffer (pH 6.7), 0.5 mL toluene, and 5 g substrate at 37℃ for 48 h. Urea was hydrolyzed to ammonium, which can be assayed colorimetrically at 460 nm [27]. UA was expressed as μg $NH_4^+$ $g^{-1}$ substrate (dry weight) 48 $h^{-1}$.

Nitrate reductase activity (NRA) was detected according to Abdelmagid and Tabatabai [28]. Ten milliliter of 1% $KNO_3$ was used as the reaction medium and incubated with 10 g substrate in 50 mL phosphate buffer (pH 7.5) for 24 h at 25℃. The nitrite produced was detected colorimetrically at 540 nm. One unit indicates 1 μg nitrite formed at the presence of 1 g substrate in 24 h.

Regarding the determination of acid phosphatases activity (APA), p-nitrophenyl (pNP) phosphate was employed as the reaction medium, and the reaction was conducted at 37℃ for 1 h in acetate buffer (pH 5.8) in the presence of toluene. After terminating the reaction with 4 mL of 0.5 M NaOH, the pNP produced at the presence of phosphatases was detected spectrophotometrically at 400 nm [21,29], and APA was expressed as μg pNP $g^{-1}$ substrate (dry weight) $h^{-1}$.

#### 2.4.3. Nitrification and denitrification potentials

Nitrification potential (NP) was measured by ammonium oxidation potential followed Tang et al. [30] and Li et al. [31]. About 20 g of substrate sample and 150 mL of solution containing sufficient ammonium were placed in 250 mL plasma flasks. The solution contained per liter: 0.59 g $(NH_4)_2SO_4$, 0.14 g $K_2HPO_4$, 0.027 g $KH_2PO_4$, 1.20 g $NaHCO_3$, 0.30 g $CaCl_2{\cdot}2H_2O$, 0.20 g$MgSO_4$, 0.00625 g $FeSO_4$, 0.00625 g EDTA and 1.06 g $NaClO_3$, and the pH was 7.2. $NaClO_3$ was added to inhibit the oxidation of $NO_2^-$ to $NO_3^-$. Then the flasks were incubated at 25℃ on a shaker at 150 rpm for 24 h. During the incubation, aliquots of 5 mL were taken out to determine $NO_2^-$ concentration colorimetrically at 2, 4, 6, 10, 20, and 24 h. Then the volume loss during sampling were normalized and the angular coefficient of linear regression for the amount of nitrite produced and incubation time was calculated as NP, and expressed as μg $NO_2^-$ $g^{-1}$ substrate (dry weight) $h^{-1}$.

As for denitrification potential (DP), the test solution contained per liter: 1.44 g $KNO_3$, 2.0 g glucose, 27.2 g $KH_2PO_4$ and 34.8 g$K_2HPO_4$, and the pH was 7.2. Ten grams of substrate sample and 100 mL solution were put into a tube, and the tube was sealed and incubated at 25℃ on a shaker at 150 rpm for 24 h after creating an anoxic condition by pure $N_2$ input [31,32]. TN lost after the incubation was determined and DP was expressed as mg N lost $g^{-1}$ substrate (dry weight) 24 $h^{-1}$.

### 2.5. Data analysis

All microbial parameters were determined in triplicate and corrected with a blank and the mean values were used for calculation on the oven-dry weight basis. The differences of the mean values of all microbial parameters were detected by one-way univariate analysis of variance (ANOVA) followed by least significant difference (LSD) test employing SPSS 17.0 software package for Windows, with $p \le 0.05$ regarded as a significant difference.

In addition, to visualize the aggregated distinctions of the microbial parameter profiles among different samples and to assess the regulating role of wastewater-quality properties, a redundancy analysis (RDA) was conducted due to the previous detrended correspondence analysis (DCA) for microbial parameters exhibited a short gradient length. All microbial indicators were regarded to be the responding variables characterizing the samples and were transformed through log $(x + 1)$ to downweight large values. Wastewater-quality properties including contaminant concentrations were the explanatory variables. The analysis was performed by software package Canoco for Windows, version 4.5.

## 3. Results

### 3.1. Water-quality characteristics and pollutant removal efficiencies

The water qualities of inflow and outflow, together with pollutant removal efficiencies of the IVCWs, are listed in Table 1.

Efficient removals for COD and organic N were achieved. Except in Dec., the reduction efficiencies of TN and TP were higher for SW than those for DW.

### 3.2. Microbial quantities

The numbers of pure-cultured bacteria, fungi, and actinomyces in the IVCWs are shown in Fig. 2.

As seen in Fig. 2, wastewater type influenced the abundances of bacteria and fungi in the IVCWs greatly, with higher quantities in units fed with DW with respect to the same cells, although the differences between up-flow cells were usually not statistically

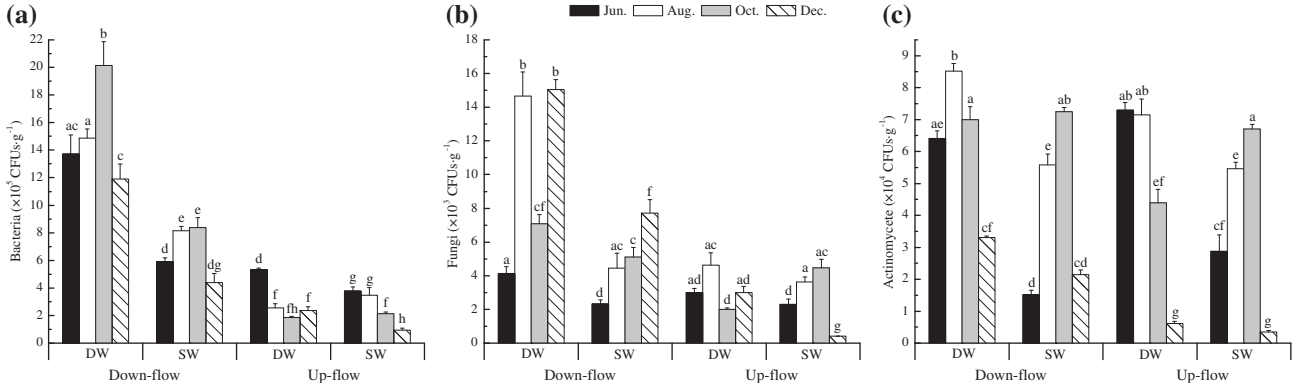


Fig. 2. Microbial quantities in the IVCWs. (a) bacteria; (b) fungi; (c) actinomyces.

significant. In each plot, numbers of bacteria and fungi, were mostly greater in down-flow cells than those in up-flow ones. However, the impact of wastewater type on the quantities of actinomyces was relatively slight.

### 3.3. Enzyme activities

Fig. 3(a)–(f) shows the activities of six kinds of enzymes related to microbial processes. It was seen that higher enzyme activities were mostly associated with IVCWs treating DW, especially for down-flow cells ($p < 0.05$). With respect to the same unit, higher values were usually found in down-flow cells. Meanwhile, the enzyme activities varied with the sampling time, with highest values detected in Aug. for DHA, FDAH, and UA, while in Dec. for CA and NRA. Almost all enzyme activities exhibited similar temporal variation trends.

### 3.4. Potentials of nitrification and denitrification

Similar to enzyme activity, higher values of nitrification and denitrification potentials were almost obtained in down-flow cells despite the wastewater type and sampling time.

As far as the influence of wastewater type, larger values of nitrification potential (NP) were associated with the IVCWs treating DW, with statistical significance detected ($p < 0.05$) except in Dec. However, as for denitrification potential (DP), in down-flow cells, higher values were found in IVCWs treating DW, but in up-flow cells fed with SW.

Similar temporal variations were also presented, with the highest values observed in Jun. and Aug. for nitrification and denitrification potential, respectively, and the lowest mostly detected in Dec.

### 3.5. Relationships among the samples, microbial parameters, and water-quality properties

The axis 1 and 2 of the RDA triplot explained 67.4% and 17.3% of the cumulative variance in the microbial parameters, respectively. The analysis was efficient due to the significant results of Monte Carlo permutation test on the first axis ($F$-ratio = 6.207 and $P$-value = 0.0020) and all axes ($F$-ratio = 12.56 and $P$-value = 0.0020).

The concentrations of COD, organic N and $NH_4^+$ were the determinant regulators for microbial indicators loading on axis 1 and temperature on axis 2. As a result, the samples of down-flow cell fed with DW were distinguished from others on the axis 1, and samples collected in Dec. were differentiated on the axis 2.

## 4. Discussions

Higher TN removal achieved for SW than DW except in December might be probably due to the anoxic conditions in the wetland beds, which were resulted from the continuous water-saturated status except for the feeding time, and anoxic conditions was beneficial to nitrate removal by denitrification. No nitrate could be eliminated in December primarily due to the high DO concentration. As for the TN removal in DW, the low $NH_4^+$ removal efficiencies indicated that nitrification process was restrained. Nevertheless, satisfactory removals of COD and organic N were achieved, indicating that moderate DO-consumed process could proceed in the IVCWs.

Microbial abundances, enzyme activities, and potentials of nitrification and denitrification were widely employed as informative indicators for elucidating status of microbial ecology and ecosystem processes in CWs due to their sensitivity to environmental variations

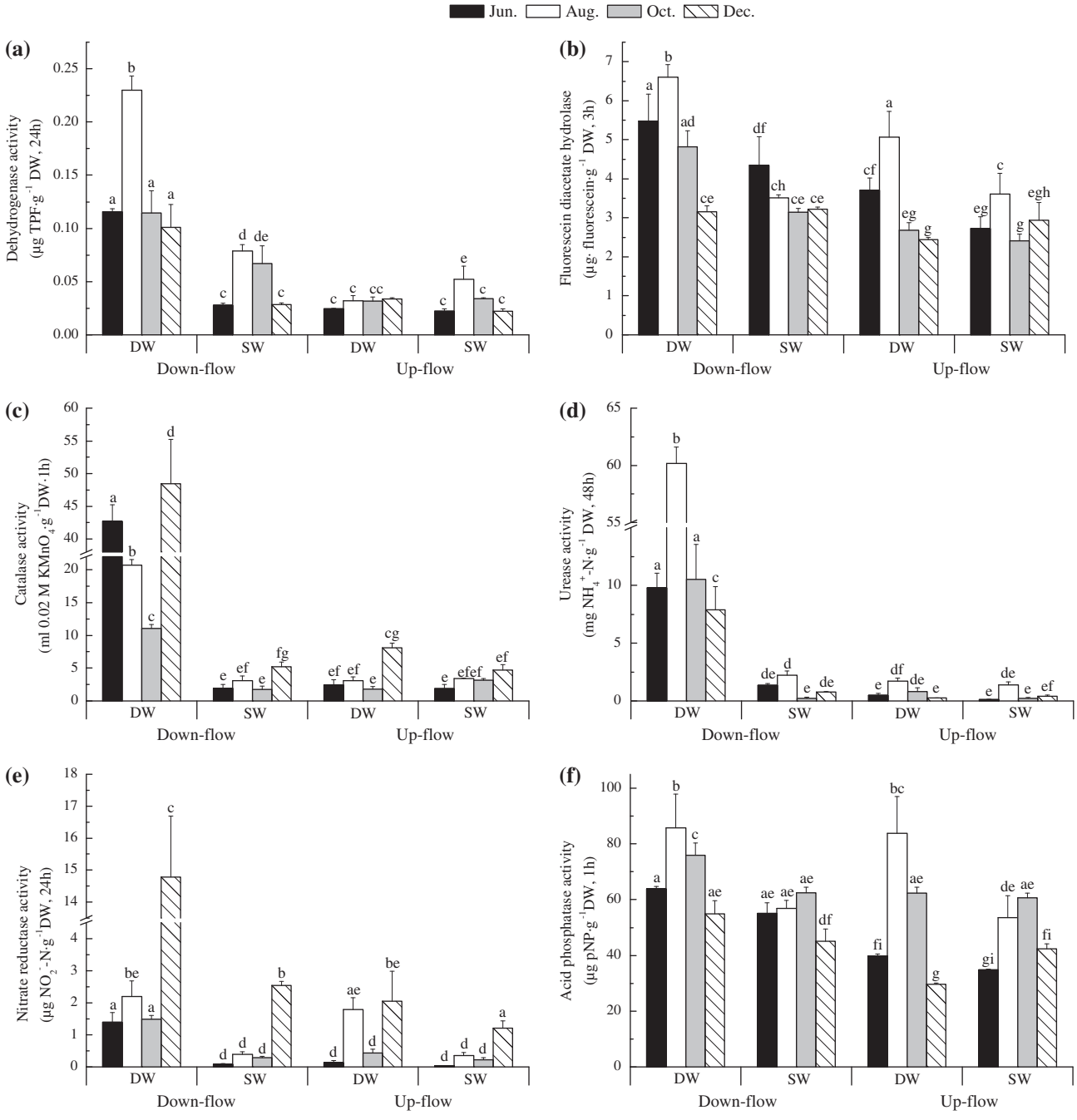


Fig. 3. Enzyme activities in the IVCWs. (a) dehydrogenase; (b) fluorescein diacetate hydrolase; (c) catalase; (d) urease; (e) nitrate reductase; (f) acid phosphatases.

and well correlation with contaminant removal function [5,10,33,34].

Most parameters characterizing microbial activities were positively related to the abundances of cultivable microbes significantly, especially bacteria (Table 2), suggesting the consistency of microbial quantity and activity. Due to limited data and large distinction of quality properties of the two types of wastewater, no significant relationship between microbial parameter and pollutant removal efficiency was found in this study.

On the other hand, C, N, and P in wastewater are essential elements for the microbial growth and proliferation, thus microbial populations and activities of CW can be impacted by the level and composition of pollutants, and can be indicative of the status and

Table 2
Correlations between microbial abundances, enzyme activities and potentials of nitrification and denitrification. DHA: dehydrogenase activity; FDAH: fluorescein diacetate hydrolase activity; CA: catalase activity; UA: urease activity; NRA: nitrate reductase activity; APA: acid phosphatases activity; NP: nitrification potential; DP: denitrification potential

| Microbes | DHA | FDAH | CA | UA | NRA | APA | NP | DP |
|---|---|---|---|---|---|---|---|---|
| Bacteria | 0.806** | 0.651** | 0.584* | 0.568* | 0.291 | 0.526* | 0.601* | 0.793** |
| Fungi | 0.755** | 0.427 | 0.631** | 0.688** | 0.712** | 0.452 | 0.270 | 0.462 |
| Actinomycetes | 0.526* | 0.550* | 0.084 | 0.426 | −0.163 | 0.715** | 0.512* | 0.602* |

*Significant correlation at $p < 0.05$ level.
**Highly significant correlation at $p < 0.01$ level.

function of the CW system [10,11,35]. It was found that almost all the microbial indicators in down-flow cells were higher than those in up-flow ones, especially for the plots fed with DW, which was consistent to other reports [19,34]. It might be attributed to the higher nutrient availability in top layer of down-flow cell [36–38]. Actually, it was found that pollutant concentrations, especially organic matter, were crucial determinant regulators for microbial characteristics (Fig. 5), which partly supported the results of other references [39,40].

Enzymes, as biological catalysts, can facilitate various metabolic processes including biogeochemical cycles of nutrients and pollutants detoxification [41]. Among these microbial parameters, DHA is closely correlated with the oxidative capability of viable microbes and plays an important role in organic substance degradation [35,42]. The markedly higher DHA detected in down-flow cell treating DW might be resulted from the significantly higher organic matter content in the influent, and it was crucial to the removal of the organic substance, which was achieved principally in the top layer of the down-flow cell [43,44].

Catalase is an intracellular enzyme secreted primarily by bacteria, fungi, and plant in CW to decompose hydrogen peroxide, which is widely presented in the substrate and plant's body [45]. Furthermore, catalase activity is related closely to the strength of the process of substrate microbiology [23], thus significantly higher catalase activities in down-flow cell fed with DW might suggest much more intense microbiological process in the cell.

Urease can catalyze the hydrolysis of urea-like substances to ammonium, and plays an important role in nitrogen transformation and removal in CW [12,13]. The significantly higher urease activities in the down-flow cell treating DW (Fig. 3(d)) might probably be responsible for the efficient elimination of organic N (Table 1).

Phosphatases can catalyze the mineralization of phosphonolipid and organic P compounds into inorganic form [46]. The relatively less differences of

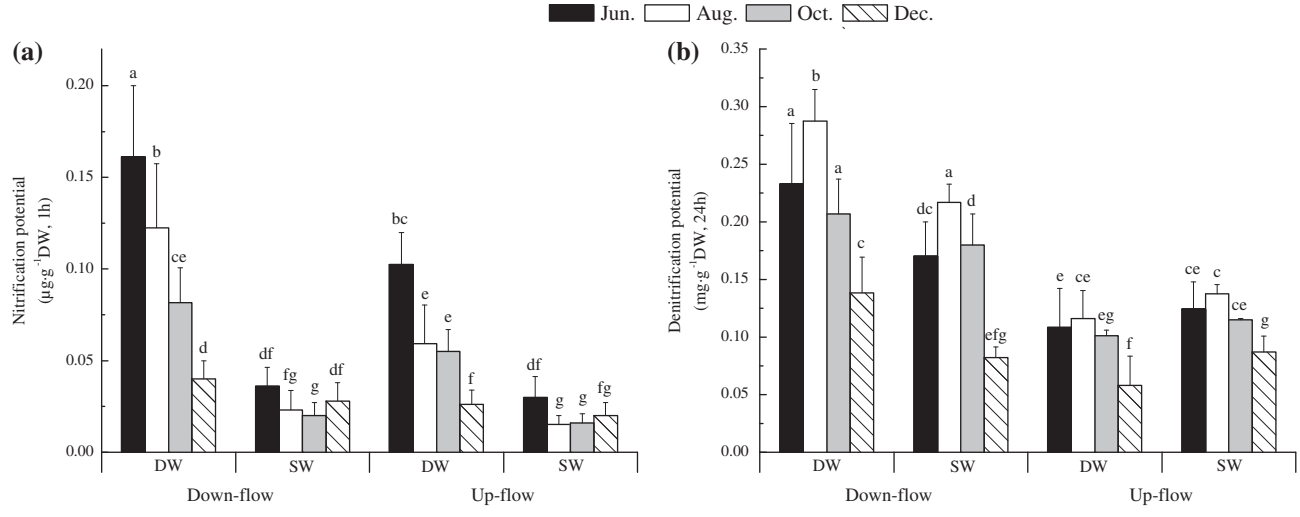


Fig. 4. Potentials of nitrification (a) and denitrification (b).

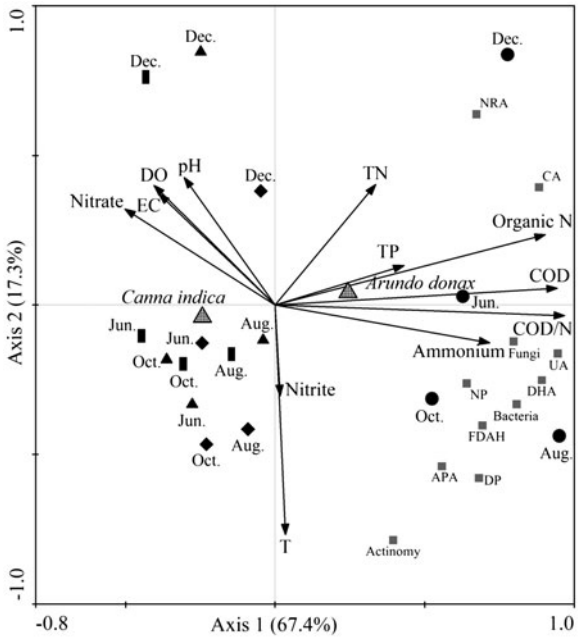


Fig. 5. Redundancy analysis (RDA) ordination triplot showing microbial indicators determined in different samples (● DW, down-flow; ◆ DW, up-flow; ▲SW, down-flow; ▮ SW, up-flow) and water-quality properties (arrows). DHA: dehydrogenase activity; FDAH: fluorescein diacetate hydrolase activity; CA: catalase activity; UA: urease activity; NRA: nitrate reductase activity; APA: acid phosphatases activity; NP: nitrification potential; DP: denitrification potential.

phosphatase activities among all cells might be attributed to that no organic P was applied as P source in the wastewaters.

Notably higher nitrification potential detected in the systems treating DW might probably be attributed to that the high availability of ammonium supported the survival and development of nitrifying bacteria [47,48]. Nevertheless, with respect to denitrification potential, organic carbon source was a more crucial regulator in comparison to nitrate availability [49,50], resulting in higher values detected in down-flow cell fed with DW (Fig. 4(b)). Consequently, nitrate was not notably accumulated in the systems treating DW owing to that denitrification process was not restrained.

## 5. Conclusions

(1) Higher values of microbial parameters were generally obtained in the IVCWs fed with domestic wastewater and in down-flow cells.
(2) Organic matter concentration and temperature were crucial determinant regulators affecting the microbial features.

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