



## Characterization of bacterial communities in wetland mesocosms receiving pharmaceutical-enriched wastewater



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### ABSTRACT

A 16S rRNA gene multiplex 454 pyrosequencing approach was used to characterize the structure of the bacterial community in subsurface flow constructed wetlands planted with *Typha angustifolia* and unplanted wetland mesocosms receiving ibuprofen-enriched wastewater at a concentration of 250  $\mu\text{g L}^{-1}$ . The removal efficiency of ibuprofen (IBP) in the planted beds (78.5%) was significantly higher than that in the unplanted beds (57.9%) ( $P \leq 0.05$ ). The presence of ibuprofen significantly ( $P \leq 0.05$ ) reduced the diversity of the bacterial community. In total, 20 phyla comprising 265 genera were identified. The most predominant phyla were Firmicutes (27.6%), Actinobacteria (26.0%) and Proteobacteria (24.9%), making up 57–95% of the 16S rDNA sequences. Other dominating phyla in wetland soils were Chloroflexi (7.3%), Bacteroidetes (2.7%), Synergistetes (2.1%), and Acidobacteria (2.0%). A distinct profile of bacterial community in the wetland mesocosms was observed after IBP enrichment. IBP-enrichment enhanced the relative abundance of Actinobacteria, Bacilli and  $\gamma$ -Proteobacteria at class level. The efficient ibuprofen removal observed in this study suggested that the IBP-enriched wetland systems may have selected a restricted group of bacteria that was able to survive best in the disturbed condition and participate in the IBP biodegradation. Our findings also indicated that the presence of macrophytes may have a vital impact on the microbial community and planted wetlands may be more robust and buffered against significant shifts in microbial composition in response to IBP-disturbance.

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### 1. Introduction

Pharmaceutically active compounds (PhACs) have been regarded as emerging contaminants and provoked increasing global concern because of their continuous introduction into the aquatic environment (Daughton and Ternes, 1999; Ternes et al., 2007). Centralized wastewater treatment plants (WWTPs) have been frequently pointed out as major point sources for PhAC discharge (Carballa et al., 2004; Ellis, 2006). Since conventional WWTPs have not been specifically designed to remove PhACs, the elimination of PhACs in WWTPs is often incomplete (Joss et al.,

2006). As a consequence, significant amounts of pharmaceutical residues are suspected to enter rivers, streams and surface waters through the effluents of WWTPs (Carballa et al., 2004). Despite their low concentrations (ranging from parts per billion to parts per trillion), these compounds may still exert chronic adverse effects on aquatic organisms in receiving waters and possibly pose a human health risk if the reclaimed water is returned to the water supply (Fent et al., 2006; Ternes et al., 2007).

In this regard, alternative approaches, especially eco-friendly strategies for the elimination of micropollutants are matters of great interest. Recently, a number of studies have shown that constructed wetlands (CWs) have great potential for the removal of PhACs (Hijosa-Valsero et al., 2010; Zhang et al., 2012b). In wetland systems, the intrinsic physical, chemical and biological processes may occur simultaneously for water quality improvement, including volatilization, sorption and sedimentation, phytodegradation,

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and plant uptake (Zhang et al., 2014). In particular, the complex microbial communities, which are created by the interactions between water, soil, plant and microorganisms, play a key role in wetland biogeochemical cycles and are mainly responsible for contaminant degradation in CW system (Ibekwe et al., 2007; Ligi et al., 2014). Therefore, an understanding of the diversity and structure of microbial community is important for proper functioning of a wetland system, and characterizing the shifts in the structure of microbial community may shed light on prediction of the microbial populations responsible for the degradation of the target organic pollutants (Johnsen et al., 2002). However, to date, only a limited number of studies have focused on microbial community investigations in CWs (Tietz et al., 2007; Truu et al., 2009). And the microbial diversity of treatment wetlands has remained relatively uncharacterized, and the interactions between soil and microorganisms in wetlands are also poorly understood (Stottmeister et al., 2003). In addition, significantly missing from the literature is the influence of PhACs on microbial structure and the response of the microbial community to the presence of PhACs in wetland systems.

The application of culture-independent approaches based on the analysis of 16S ribosomal RNA (rRNA) genes including fluorescence in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), and clone library have led to new insights into microbial processes in CWs and can provide direct evidence for the presence of specific microorganisms influencing pollutant removal (Ibekwe et al., 2007; Truu et al., 2009). More recently, next generation 454 pyrosequencing approach, which is operated as a high-throughput sequencing tool with longer read length, has become an effective method to fully explore the microbial diversity in the environment (Roesch et al., 2007; Roh et al., 2010). In particular, this technology can provide a better understanding of the low-abundance microorganisms that are usually overshadowed by dominant populations in the environment (Roh et al., 2010). To date this approach has been used to study microbial communities in activated sludge in WWTPs (Zhang et al., 2012c), fresh water sediment (Wang et al., 2012), and submerged membrane bioreactors (Ma et al., 2013). To the best of our knowledge, there are no studies using high-throughput pyrosequencing technology to investigate the microbial communities in treatment wetlands in response to pharmaceutical-enriched wastewater.

Ibuprofen (IBP), a non-steroidal anti-inflammatory drug, was selected for this study because of its widespread usage and ubiquitous occurrence in WWTPs. Verlicchi and Zambello (2014) reviewed 118 pharmaceuticals which have been frequently detected in raw urban wastewater and indicated that ibuprofen was considered as the most commonly investigated anti-inflammatory drug with the highest influent concentration of 373  $\mu\text{g L}^{-1}$  in raw urban wastewater. Although various removal mechanisms (e.g. sorption, sedimentation and plant uptake) may contribute to the total elimination of PhACs in CWs (Zhang et al., 2014), for polar acidic PhACs like IBP, microbial degradation is believed to be the most significant removal mechanism (Quintana et al., 2005). Table 1 presents the physicochemical properties of IBP. In the present study, we characterized the shift of bacterial community structure within horizontal subsurface flow (HSSF) CWs planted *Typha angustifolia* and unplanted control mesocosms receiving IBP-enriched wastewater at a concentration of 250  $\mu\text{g L}^{-1}$  using high-throughput pyrosequencing technology. More specifically, the objectives of this study are to (i) assess the treatment performance within planted and unplanted wetland mesocosms receiving IBP-enriched wastewater; (ii) characterize the shifts in diversity and structure of bacterial community in response to IBP enrichment; and (iii) assess the role that *Typha angustifolia* plays in the structure shift in bacterial communities receiving IPB-enriched wastewater. To the best of our knowledge, this is the first study to gain a fundamental

understanding of the diversity and structure of the microbial community in wetland systems receiving IBP-enriched wastewater using 454 high-throughput pyrosequencing technology.

## 2. Materials and methods

### 2.1. Description of the mesocosm-scaled CWs

Four identical mesocosms including two HSSF CWs planted with cattail (*Typha angustifolia*) at a density of 15 plant  $\text{m}^{-2}$  and two unplanted mesocosms, were set up in parallel at the campus of Nanyang Technological University, Singapore (1°22' N, 130°48' E). The location is a typical tropical environment and is generally hot and humid year round. The average temperature ranges from 23 °C to 32 °C. There is no distinct wet/dry season and most rainfall occurs during the northeast monsoon (November to January) when the rainfalls are usually sudden and heavy.

The layout of the HSSF CWs is presented in Supplementary Fig. 1. The four mesocosms were divided into two groups: one was spiked with IBP, while another one was serving as control. Each system was made of fiberglass with the dimensions of 1.2 m in length, 0.6 m in width and 0.6 m in depth. Each mesocosm was filled with 400 mm-thick substrate, which was composed of two layers: a bottom layer of gravel (300 mm,  $D_{60} = 4\text{--}10$  mm) and an upper layer of loamy sand (100 mm, 75% of sand particles with  $D_{60} = 1\text{--}2$  mm; 23% silt particles; 2% clay particles). A horizontal drainage pipe (0.4 m long and 50 mm in diameter) with an outlet valve was located at the lower edge of the mesocosms.

### 2.2. System operation and sample collection

All the systems were acclimatized for three months with daily irrigation of synthetic wastewater to allow the plant roots and biofilm development prior to the study. The synthetic wastewater was obtained by diluting the stock solution to reach initial concentrations of 300  $\text{mg L}^{-1}$  COD, 27  $\text{mg L}^{-1}$   $\text{NH}_4\text{-N}$ , 22  $\text{mg L}^{-1}$   $\text{PO}_4\text{-P}$ , 4  $\text{mg L}^{-1}$   $\text{Fe}^{3+}$ , 7  $\text{mg L}^{-1}$   $\text{Mg}^{2+}$ , and 6  $\text{mg L}^{-1}$   $\text{Ca}^{2+}$ . 50-liter of synthetic wastewater was spiked with 12.5 mg of IBP to obtain an influent concentration of 250  $\mu\text{g L}^{-1}$ . Batch modes were operated at hydraulic retention time (HRT) of 4-d resulting from a hydraulic loading rate of 2.8  $\text{cm d}^{-1}$ . Water depth was maintained at 5 cm below gravel surface.

The experiment lasted 7 months from June to December, 2013. The wastewater effluent samples were collected weekly to determine physicochemical parameters (e.g., DO, conductivity, COD,  $\text{NH}_4\text{-N}$ ,  $\text{PO}_4^{3-}$ , etc.) and IBP concentrations. Soil samples were collected every two months from four different wetland regimes for bacterial community analysis. In both planted and unplanted wetland systems, three bulk soil samples were taken at the depth of 5–10 cm from surface using an auger at random spots and combined in a polyethylene bag. All samples were kept in a cooler with ice packs to inhibit bacterial activity until further reprocessing in the laboratory. At the laboratory, each bag was homogenized manually to mix all three samples for each sampling spot. Any visible plant material was manually removed prior to homogenization. Once mixed, a subsample was taken from each bag and transferred to a microtube (2 mL) for DNA extraction. Tubes were stored at a freezer (at  $-80^\circ\text{C}$ ) until microbial molecular analysis was conducted.

### 2.3. Analytical procedures for water quality parameters and IBP concentration

Physicochemical parameters were analyzed within 24 h using spectrophotometer (HACH-DR 3800, USA) based on Standard Methods (APHA, 1998): chemical oxygen demand (COD), ammonium ( $\text{NH}_4\text{-N}$ ), nitrate ( $\text{NO}_3\text{-N}$ ) and total phosphorus (TP).

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