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High-throughput pyrosequencing analysis of bacteria relevant to cometabolic and metabolic degradation of ibuprofen in horizontal subsurface flow constructed wetlands

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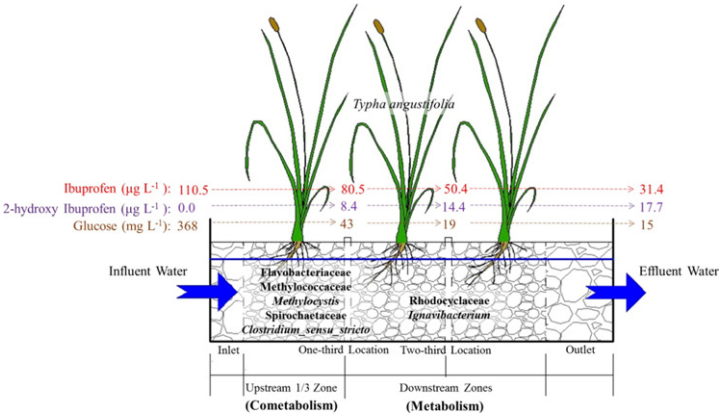
HIGHLIGHTS

- Microbial degradation mechanisms of ibuprofen in constructed wetlands were studied.
- High-throughput pyrosequencing was used to investigate the bacterial community.
- Contribution of plants to ibuprofen removal was related to microbial degradation.
- Both aerobic and anaerobic bacteria were relevant to the ibuprofen cometabolism.
- Bacteria involved in the ibuprofen metabolism were strongly associated with plants.

GRAPHICAL ABSTRACT



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ABSTRACT

The potential toxicity of pharmaceutical residues including ibuprofen on the aquatic vertebrates and invertebrates has attracted growing attention to the pharmaceutical pollution control using constructed wetlands, but there lacks of an insight into the relevant microbial degradation mechanisms. This study investigated the bacteria associated with the cometabolic and metabolic degradation of ibuprofen in a horizontal subsurface flow constructed wetland system by high-throughput pyrosequencing analysis. The ibuprofen degradation dynamics, bacterial diversity and evenness, and bacterial community structure in a planted bed with *Typha angustifolia* and an unplanted bed (control) were compared. The results showed that the plants promoted the microbial degradation of ibuprofen, especially at the downstream zones of wetland. However, at the upstream one-third zone of wetland, the presence of plants did not significantly enhance ibuprofen degradation, probably due to the much greater contribution of cometabolic behaviors of certain non-ibuprofen-degrading microorganisms than that of the plants. By analyzing bacterial characteristics, we found that: (1) The aerobic species of family Flavobacteriaceae, family Methylococcaceae and genus *Methylocystis*, and the anaerobic species of family

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Spirochaetaceae and genus *Clostridium_sensu_stricto* were the most possible bacteria relevant to the cometabolic degradation of ibuprofen; (2) The family Rhodocyclaceae and the genus *Ignavibacterium* closely related to the plants appeared to be associated with the metabolic degradation of ibuprofen.

1. Introduction

Ibuprofen, which belongs to the family of non-steroidal anti-inflammatory drugs, is among the most consumed pharmaceuticals worldwide and shows analgesic, anti-inflammatory and antipyretic effects by inhibiting the synthesis of prostaglandin (Corcoran et al., 2010; Saravanan et al., 2012). This pharmaceutical has been detected in the aquatic environment at trace concentrations up to 603 μg L^{-1} in raw wastewaters, up to 85 μg L^{-1} in treated effluents, and up to 5 μg L^{-1} in surface waters (Corcoran et al., 2010; Luo et al., 2014). It is worth noting that ibuprofen behaved as a toxic compound for the reproduction of some aquatic vertebrates and invertebrates such as *Daphnia magna* (0.5 to 50 μg L^{-1}) (Wang et al., 2016), zebrafish (>10 μg L^{-1}) (David and Pancharatna, 2009) and Japanese medaka (1 to 100 μg L^{-1}) (Flippin et al., 2007). Besides, Pomati et al. (2006) found that a mixture of 13 pharmaceuticals including ibuprofen (100 ng L^{-1}) induced an additive inhibition impact on the growth of human embryonic cells.

Due to the potential ecotoxicity of ibuprofen, its occurrence, fate, risk and control in the aquatic environment have received great attentions (Corcoran et al., 2010; Luo et al., 2014). Normally, the relatively good biodegradability of ibuprofen (Ávila et al., 2014a, 2014b) allows ibuprofen to be removed readily at a typical high efficiency of >90% in conventional biological treatment processes such as activated sludge systems (Ferrando-Climent et al., 2012; Luo et al., 2014), membrane biological reactors (Luo et al., 2014; Quintana et al., 2005), and constructed wetlands (Li et al., 2014). However, there still lacks of an in-depth understanding on the biodegradation mechanisms of ibuprofen and relevant microbial communities in different biological treatment systems.

In particular, constructed wetlands with the properties of being ecological friendly and economical have attracted increasing interest in their application to treat the pharmaceutical pollutants in wastewaters (Li et al., 2014; Verlicchi and Zambello, 2014). In conventional constructed wetland systems, removal efficiencies of ibuprofen (1 to 25 μg L^{-1}) have been reported as follows: 60–75% in surface free water constructed wetlands (SF-CWs), 30–81% in horizontal subsurface flow constructed wetlands (HSSF-CWs), 55–99% in vertical subsurface flow constructed wetlands (VSSF-CWs), and 22–99% in hybrid constructed wetlands (Ávila et al., 2014a, 2014b; Li et al., 2014; Verlicchi and Zambello, 2014). Although excellent removals of ibuprofen were achieved in these constructed wetlands, it is still unclear to get an insight into the relevant removal mechanisms of ibuprofen, particularly, the microbial degradation mechanisms. Tran et al. (2013) have summarized that ibuprofen is mainly involved in heterotrophic cometabolism and metabolism. However, the bacteria associated with the cometablic and metabolic degradation of ibuprofen in constructed wetlands remain unknown to date.

In this study, a HSSF-CW system consisting of a planted bed with *Typha angustifolia* and an unplanted bed (control) was investigated to identify the bacteria responsible for the cometabolism and metabolism of ibuprofen. For this purpose, the temporal and spatial bacterial diversity and evenness changes in the wetland system were studied by denaturing gradient gel electrophoresis analysis and the bacterial community structure was determined by using the 454 high-throughput pyrosequencing technique. Furthermore, the degradation dynamics of ibuprofen was evaluated by monitoring the spatial and temporal variations of glucose, ibuprofen and 2-hydroxy ibuprofen (one of the predominant hydroxylated metabolites of ibuprofen) (Ferrando-Climent et al., 2012; Quintana et al., 2005; Zwiener et al., 2002). Finally, the bacteria involved in the cometabolic and metabolic degradation of ibuprofen were determined by taking into consideration the bacterial characteristics and ibuprofen degradation dynamics.

2. Materials and methods

2.1. Chemicals

Synthetic municipal wastewater was employed as the feed for the investigated wetland system, containing about 300 mg L^{-1} of chemical oxygen demand (COD), 27 mg L^{-1} of ammonium nitrogen (NH_4^+–N) and 18 mg L^{-1} of total phosphate (TP) (Zhang et al., 2012). In detail, glucose, potassium dihydrogen phosphate, ammonium sulfate, calcium chloride dihydrate, magnesium sulfate heptahydrate, sodium carbonate, and sodium hydrogen carbonate were supplied by Alfa Aesar (USA). The internal standard calibration chemicals of liquid chromatography-tandem mass spectrometry included ibuprofen (IBP), 2-hydroxy ibuprofen (2-OH IBP) and ibuprofen-d_3 (IBP-d_3), which were acquired from Sigma-Aldrich (USA). HPLC-grade methanol, hydrochloric acid (37%) and ammonium acetate 5 M solution were supplied by Merck (USA). The chemicals used for polymerase chain reaction and denaturing gradient gel electrophoresis were purchased from Promega (USA). They included the following: 2× Go Taq Master Mix, 25 mM $MgCl_2$, nuclease-free water and urea. Other chemicals such as 40% Acrylamide/Bis (37.5:1) and Formamide were acquired from Bio-Rad (USA).

2.2. Wetland setup, operation and sampling

The experimental HSSF-CW system consisting of a planted bed with *Typha angustifolia* and an unplanted bed (control) was set up at Nanyang Technological University campus in Singapore, as shown in Fig. 1. Each wetland bed with a surface area of 1.2 × 0.6 m^2 was filled with gravel (0.3 m deep). Water level was controlled at 0.05 m below the gravel surface. Two perforated pipes (20 mm i.d.) were vertically inserted into the gravel to collect samples at the locations of about one-third length (1/3L) and two-third length (2/3L) of the treatment section along the flow path. Accordingly, the wetland bed was divided into three zones including the upstream 1/3 zone (inlet to 1/3L), middle 1/3 zone (1/3L to 2/3L) and downstream 1/3 zone (2/3L to outlet).

The wetland system was operated continuously over 342 days. IBP-free synthetic wastewater (7.6 mL min^{-1}) was initially fed into the wetland beds at a 4-day hydraulic retention time. After a 30-day adaption, IBP (~100 μg L^{-1}) was continuously injected into the influent water. From the 62nd day onwards, a sampling campaign was conducted over five periods (periods 1 o 5) with 40-day interval at the inlet, 1/3L, 2/3L and outlet. The five periods were from the 62nd to 86th day (period 1), the 126th to 150th day (period 2), the 190th to 214th day (period 3), the 254th to 278th day (period 4), and the 318th to 342nd day (period 5). During each sampling period, every 8 days (including the initial day), the water samples (100 mL each) used for water-quality monitoring were collected at all sampling locations and the sediment mixture samples (50 mL each) used for bacterial DNA isolation were collected at 1/3L and 2/3L in both beds.

2.3. Analytical methods

2.3.1. Analysis of conventional water-quality parameters

In-situ measurements of water temperature, pH, dissolved oxygen (DO), and oxidation and reduction potential (ORP) were performed with an HQ40d portable multi-parameter meter (Hach, USA). By using