Horizontal subsurface flow constructed wetland for removal of high-loaded ibuprofen from wastewater under tropical climate

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**HORIZONTAL SUBSURFACE FLOW CONSTRUCTED
WETLAND FOR REMOVAL OF HIGH-LOADED
IBUPROFEN FROM WASTEWATER UNDER TROPICAL
CLIMATE**

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SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING

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SUMMARY

Performance of a mesocosm-scale horizontal subsurface flow constructed wetland (HSSF-CW) system for the treatment of synthetic wastewater with high-loaded ibuprofen (IBP) was studied over 342 days under tropical conditions. Compared to an unplanted bed (control), the planted bed showed high removal efficiencies of chemical oxygen demand (COD), ammonium nitrogen ($\text{NH}_4^+\text{-N}$), total phosphate (TP) and IBP without adverse impacts caused by the high loading of IBP. *Typha angustifolia* positively influenced the reduction of $\text{NH}_4^+\text{-N}$, TP and IBP in the experiment.

The uptake of IBP to the roots of *Typha angustifolia* and its following translocation, transformation and accumulation in plant tissues were demonstrated to suggest the good ability of *Typha angustifolia* for IBP removal in HSSF-CW. The nonuniform distribution of the three IBP metabolites, namely, carboxylic acid ibuprofen (CBX IBP), 2-hydroxy ibuprofen (2-OH IBP) and 1-hydroxy ibuprofen (1-OH IBP), were observed in the lamina and sheath tissues of *Typha angustifolia*. It indicates the places where the IBP metabolites were likely to be conjugated and compartmentized thus reducing the toxicity to the essential cellular processes of leaves. The degradation process of IBP in the tissues of *Typha angustifolia* is consistent with the reported detoxification process of plants including three steps as chemical modification, conjugation and compartmentation when countering xenobiotics. Taking IBP transformation into account, the normalized IBP level accumulated in the leaves of *Typha angustifolia* exhibited uniform in HSSF-CW.

The bacterial evenness and diversity in the wetlands suggest the stronger resistance of the bacterial communities in the planted bed to the high loading of IBP in comparison to those in the unplanted bed. Certain sulfate-reducing bacteria were thought to be involved in the anaerobic biodegradation of IBP in the rhizosphere, such as *Cytophaga sp.*, Sphingobacteriales, and Cytophagales in the planted bed, while *Cytophaga sp.* and *Desulfobacterium sp.* in the unplanted bed.

To date, the available studies on HSSF-CWs for the removal of IBP at trace levels (1 to 25 µg/L) are limited and less so for high-loaded IBP. Removal behaviour of IBP along the flow path in HSSF-CWs under a high loading remains unknown. This study is believed to be one of the pioneer investigations on this issue. In addition, this thesis work is the first research study on the IBP removal mechanisms with regard to plant uptake and microbial degradation in the rhizosphere in HSSF-CWs planted by *Typha angustifolia*. The measurement of accumulation and distribution of IBP and its three metabolites in the leaves of *Typha angustifolia* is the first in wetland research. Moreover, the investigation on the probable bacteria species responsible for the microbial degradation of IBP in HSSF-CWs is another pioneer research focus.

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LIST OF SYMBOLS

1/3L	1/3 length of the treatment section along the flow path
2/3L	2/3 length of the treatment section along the flow path
1-OH IBP	1-hydroxy ibuprofen
2-OH IBP	2-hydroxy ibuprofen
APX	Ascorbate peroxidase
AS	Activated sludge
AVG	Average
CA-IBP	Carboxy-ibuprofen
CAT	Catalase
CBX IBP	Carboxylic acid ibuprofen
CE	Collision energy.
COD	Chemical oxygen demand
Cond	Conductivity
C_u	D_{60}/D_{10} , uniformity coefficient
D_{10}	The diameter at which 10% of material can pass through sieve
D_{60}	The diameter at which 60% of material can pass through sieve
DGGE	Denaturing gradient gel electrophoresis
DL	Detection limit
DO	Dissolved oxygen
EF	Enantiomeric fraction
E_H	Pielou's evenness index
ESI	Electrospray ionization
GPX	Guaiacol peroxidase
H	Shannon diversity index
HRT	Hydraulic retention time
HSSF-CW _s	Horizontal subsurface flow constructed wetlands
HUSB	Hydrolytic upflow sludge bed
IBP	Ibuprofen
K_d	Coefficient for permeability
K_{ow}	Octanol-water partition coefficient

L	Lamina of leaves; Lipid content of root tissue
LECA	Light expanded clay aggregate
$\log D_{ow}$	Logarithm octanol-water distribution coefficient
$\log K_{ow}$	Logarithm octanol/water partition coefficient
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MBR	Membrane bioreactor
Milli-Q	Trademark created by Millipore Corporation to describe ultrapure water
MW	Molecular weight
NH_4^+-N	Ammonia nitrogen
n.a.	Not available
n.r	Not removed.
OH-IBP	Hydroxy-ibuprofen
ORP	Oxidation reduction potential
OTU	Operational taxonomic unit
P	Planted bed
PCR	Polymerase chain reaction
pK_a	Dissociation constant
QL	Quantification limit
R	Recovery of target compound
r^2	Regression coefficient
RCF	Root concentration factor
RGR	Relative growth rate of plant
ROIs	Reactive oxygen intermediates
RSD	Relative standard deviation
RT	Retention time
S	Richness refers to the total number of the identifiable bands in a sample lane; Sheath of leaves
SD	Standard deviation
SF-CWs	Surface flow constructed wetlands
SPE	Solid phase extraction
SSE	Sea sand extraction
S/N	Signal to noise ratio
SOD	Superoxide dismutase

S_w	Solubility
TAE	Tris base, acetic acid, and EDTA
Temp	Temperature
TP	Total phosphorus
UP	Unplanted bed
VSSF-CWs	Vertical subsurface flow constructed wetlands
W	Water content of root tissue
WWTPs	Wastewater treatment plants

CHAPTER 1 INTRODUCTION

1.1 Background

Today, pharmaceuticals of different therapeutic classes are being consumed in large quantities for both human beings and domesticated animals (Jones et al. 2001, 2007, Uslu et al. 2013). Trace pharmaceutical residues have been found to be widespread in the aquatic environment (Kaplan 2013, Cizmas et al. 2015). Growing concerns are being raised on this issue to study the source, behaviour, fate, risk, and control of these kinds of emerging pollutants (Richardson 2007, Jiang et al. 2013).

The main route of pharmaceutical compounds into the aquatic environment is by the way of municipal wastewater. Many types of pharmaceutical compounds cannot be completely metabolized and ingested in the bodies of humans and animals. As a result, pharmaceutical metabolites, conjugates and their native forms are excreted together with urine and faeces into the sewage system (Jones et al. 2002, Carballa et al. 2004, Zhang et al. 2008b). In households, the expired and unused pharmaceutical compounds are commonly disposed with household waste or discarded into sinks or toilets (Zhang et al. 2008b). The pharmaceutical compounds disposed into sinks or flushed down the toilets in households are then introduced to the sewage system. Moreover, to a small but significant extent, wastewater from hospitals or pharmaceutical manufacturers also adds to the total loading of pharmaceutical compounds in the municipal wastewater (Jones et al. 2001, Fent et al. 2006, Santos et al. 2013). In municipal wastewater treatment plants (WWTPs) where the conventional treatment technologies are not specially designed for the removal of pharmaceutical compounds, it has been observed that most of the pharmaceuticals cannot be readily and fully removed (Carballa et al. 2004, Paxéus 2004, Lindqvist et al. 2005, Joss et al. 2006, Sim et al. 2010, Petrie et al. 2013, Kim et al. 2014). Consequently, various types of pharmaceutical compounds and their metabolites are released into surface waters, ground waters, coastal waters, and other recipient water bodies through the effluent of WWTPs (Lindqvist et al. 2005, Vieno et al. 2005, Kim et al. 2007, Jelic et al. 2011, Uslu et al. 2013). In addition, there are also some other direct pathways such as the runoff or wastewater effluent from farms,

household wastewater from small communities, and wastewater from rural regions (Focazio et al. 2008, Matamoros et al. 2009a, Anderson et al. 2013, Carvalho et al. 2013).

Upon entering the aquatic environment, the pharmaceutical compounds and their metabolites may pose potential risks to the health of aquatic life as well as human beings even at trace levels (Carlsson et al. 2006, Fent et al. 2006, Gagné et al. 2006, Pomati et al. 2006, Zuccato et al. 2006, Farré et al. 2008, Corcoran et al. 2010, Uslu et al. 2013, Ortiz de García et al. 2014). The adverse impacts on the aquatic communities include the impairment of renal, gill and liver in fish (Fent et al. 2006, Gagné et al. 2006), development of pathogen resistance (Zuccato et al. 2006), feminization of male fish (Fent et al. 2006, Corcoran et al. 2010), and decrease in plankton diversity (Farré et al. 2008). In the case of the toxic effects on the health of human beings, the degenerative and inflammatory reactions have been observed in the liver of humans under the exposure to diclofenac (Fent et al. 2006). In addition, the proliferation inhibition effects were found for the growth of human embryonic cells upon an injection of a pharmaceutical mixture (atenolol, carbamazepine, ciprofloxacin, furosemide, ibuprofen, sulfamethoxazole, etc.) (Pomati et al. 2006). At present, the information available on the ecotoxicology of pharmaceutical compounds is scarce and the potential risks related to the presence of pharmaceuticals in water environment are still under debate (Jones et al. 2004).

In recent years, certain advanced technologies including advanced oxidation processes (ozonation, photolysis and heterogeneous photolysis, Fenton and photo-Fenton, sonolysis, electrochemical oxidation, etc.), activated carbon adsorption, membrane separation and membrane bioreactor have been studied to evaluate their feasibility for the removal of pharmaceuticals from wastewater (Singh et al. 2008, Mestre et al. 2009, Naddeo et al. 2009, Rossner et al. 2009, Klammerth et al. 2010, Sipma et al. 2010, Molinos-Senante et al. 2013, Da Silva et al. 2014, Ziyilan and Ince 2014). However, the relatively high costs of these advanced technologies make large-scale application cost-prohibitive. Therefore, resorting to low-cost alternative technologies for the treatment of pharmaceuticals in wastewater is of great

significance, especially in the poor regions. For this purpose, constructed wetlands which are low-cost in construction, operation and maintenance are attracting growing interest in their application for the removal of pharmaceutical pollutants from wastewater.

Over the past several decades, constructed wetlands have been demonstrated to be an efficient technology for the treatment of conventional pollutants (e.g. COD, phosphorus and ammonium) and non-conventional pollutants (e.g. heavy metals) in a variety of wastewaters such as domestic wastewater, agricultural wastewater, industrial effluent water, leachate, mine drainage, urban runoff and contaminated ground water (Cooper et al. 1996, Vymazal et al. 1998, Sundaravadivel and Vigneswaran 2001, Stottmeister et al. 2003, Davies et al. 2008, Kadlec and Wallace 2009, Vymazal 2009, García et al. 2010, Choudhary et al. 2011, Wojciechowska 2013). However, removal of pharmaceutical contaminants from wastewater using constructed wetlands is really a fresh application field. The feasibility of constructed wetlands to remove pharmaceutical contaminants from wastewater requires in-depth understanding on the removal efficiencies, the associated removal mechanisms, the influences of design and environmental factors and the resultant health risks. Reported literatures on these issues are limited at present and much more concern is in requirement.

1.2 Objectives

The main objective of this study was to investigate the performance of a horizontal subsurface flow constructed wetland (HSSF-CW) system for the treatment of synthetic wastewater with high-loaded ibuprofen (IBP) and to study the associated removal mechanisms involving plant uptake and microbial degradation. Three metabolites of IBP such as carboxylic acid ibuprofen (CBX IBP), 2-hydroxy ibuprofen (2-OH IBP) and 1-hydroxy ibuprofen (1-OH IBP) were analyzed to lend support for in-depth understanding the removal mechanisms of IBP.

The specific objectives were:

- (1) To investigate the performance of the HSSF-CW system for the treatment of IBP and certain conventional pollutants such as chemical oxygen demand (COD), ammonium nitrogen ($\text{NH}_4^+\text{-N}$) and total phosphate (TP) under the presence of a high concentration of IBP (about 100 $\mu\text{g/L}$). The impacts of *Typha angustifolia* on the removal of the target pollutants were assessed through the comparison studies between a planted bed and an unplanted bed.
- (2) To analyze and demonstrate the contribution of plant uptake and microbial degradation in the rhizosphere to IBP removal through monitoring the concentrations of IBP and its three metabolites (CBX IBP, 2-OH IBP and 1-OH IBP) in the interstitial water and the leaves of *Typha angustifolia*.
- (3) To investigate the evenness and diversity of bacterial communities over time and to establish the phylogenetic tree of bacteria so as to understand the bacterial development in the wetland system and to find the probable bacteria responsible for the microbial degradation of IBP.

1.3 Scope

This thesis consists of seven chapters and an appendix. Chapter 1 includes the introduction to the background and objectives of the study. Chapter 2 presents a review of the reported literature on the application of constructed wetlands as an alternative secondary wastewater treatment system or a wastewater polishing process for the removal of pharmaceuticals from wastewater. The associated removal mechanisms from the perspectives of substrate, plants and microbes are also discussed. Chapter 3 describes the wetland system, the chemicals used in the experiment, the regime for operation and sampling, and the analytical methods.

The experimental results are presented in chapters 4-6. Chapter 4 focuses on the performance of the investigated HSSF-CW system. Chapter 5 deals with the contribution of plant uptake and microbial degradation in the rhizosphere to IBP removal. Chapter 6 provides an in-depth understanding on the development of

bacterial communities in the wetland system and the bacterial species responsible for the microbial degradation of IBP.

Chapter 7 is about the conclusions and recommendations for this research work. The appendix contains the detailed information on the physicochemical properties of the reported pharmaceutical compounds referred to in Chapter 2.

CHAPTER 2 LITERATURE REVIEW

2.1 Widespread Occurrence of Pharmaceuticals in the Aquatic Environment

Pharmaceuticals have been widely found in the aquatic environment such as wastewater, surface water, ground water and drinking water around the world. Related research studies have been conducted in a number of countries including China, France, Germany, Greek, South Korea, Spain, Switzerland, UK and USA (Luo et al. 2014). Some of the examples are given below to obtain an understanding on the widespread occurrence of pharmaceuticals in various aquatic environments.

Wang et al. (2015b) detected 28 pharmaceutical compounds covering 5 therapeutic categories in the urban rivers in Beijing, Changzhou and Shenzhen, China, at a mean concentration level ranging from 0.4 to 166 ng/L. This study demonstrated that the effluent water from WWTPs was the major pathway to introduce pharmaceuticals into the rivers. In addition, it was found that antibiotics occupied about half of the total contamination level. Kim et al. (2007) conducted a study on the occurrence of 14 pharmaceuticals in the surface waters (including several rivers and lakes) and the effluent water from WWTPs in South Korea. They found that the mean concentrations were from below detection to several hundred ng/L (~134 ng/L in surface waters and ~2630 ng/L in effluents). Morasch et al. (2010) investigated the occurrence of 36 pharmaceuticals in the influent and effluent wastewaters from WWTPs and the raw drinking water in Vidy Bay, Switzerland. The corresponding mean concentrations were in the range of 0.05 to 35 µg/L in the influent, 0.02 to 46 µg/L in the effluent and 0.001 to 0.4 µg/L in the raw drinking water, respectively. Kasprzyk-Hordern et al. (2008) found the presence of 41 pharmaceuticals in River Taff and River Ely in South Wales, UK, at concentrations ranging from <0.5 to 968 ng/L and <0.5 to 1089 ng/L, respectively. This study suggested that the pharmaceutical concentrations in the river water mainly relied on the water dilution extent caused by the rainfall. Besides, the study results showed that the discharge of effluent water from WWTPs was the key reason to induce the pharmaceutical pollution in river. Loraine and Pettigrove (2006) detected four

pharmaceuticals in the raw drinking water in Southern California, USA, at a mean concentration of 0.58 to 9.22 µg/L, two of which were found in the treated drinking water at a mean concentration level of 0.93 to 4.95 µg/L. The occurrence and concentrations of the pharmaceutical contaminants highly depended on seasons.

Luo et al. (2014) have reviewed the occurrence of micropollutants in the aquatic environment such as sewage, surface water, ground water and drinking water. They summarized that the effluent water from WWTPs was the major pathway to introduce pharmaceutical contaminants into surface waters. Among the 18 common pharmaceuticals in surface waters, caffeine, gemfibrozil, ibuprofen and ketoprofen were detected at high concentration levels with maximum values of 1121.4, 17.0, 36.8 and 9.8 µg/L, respectively. The corresponding maximum concentrations of these four pharmaceuticals in the influent to WWTPs were 209.0, 17.1, 603.0 and 8.6 µg/L, respectively, while they were 43.5, 5.2, 55.0 and 3.9 µg/L in the effluent from WWTPs. It is thought that in some cases rainfall was able to cause pharmaceutical emission or intensify combined sewer overflow resulting in higher levels of pharmaceuticals in surface waters. In comparison to surface waters, ground water was less polluted by pharmaceuticals at a concentration level below 100 ng/L. Besides, in this review paper, it has been reported that most of the pharmaceuticals were below the limit of quantitation or limit of detection in drinking water and the maximum concentrations of the most detected pharmaceuticals were below 100 ng/L.

2.2 Potential Ecotoxicity of Pharmaceuticals

The widespread presence of pharmaceuticals in the aquatic environment has attracted growing concern on the potential ecotoxicity to aquatic organisms as well as human beings (Corcoran et al. 2010, Cizmas et al. 2015). However, the potential effects caused by the pharmaceuticals in the aquatic environment remain lacking of knowledge, especially on the chronic toxicity.

According to the available research work to date, it indicates that the continuous exposure of aquatic organisms and human beings to pharmaceuticals in the aquatic

environment pose the carcinogenic, mutagenic and reproductive risks (Cizmas et al. 2015). The relative ecotoxicity may include inhibition of enzymes or hormones, histological and cytological changes, behavioral effects and reproductive disorders (Corcoran et al. 2010, Cizmas et al. 2015). For instance, the research conducted by Parolini et al. (2013) showed that triclosan brought notable acute toxicity to the freshwater bivalve (*Dreissena polymorpha*), inducing lesions to hemocyte DNA. Brodin et al. (2013) found that oxazepam in surface waters could increase the feeding rate, reduce the sociability and heighten the activity of wild European perch (*Perca fluviatilis*). Galus et al. (2013) observed that the embryo production of adult zebrafish (*Danio rerio*) was decreased when exposing to a pharmaceutical mixture of acetaminophen, carbamazepine, gemfibrozil and venlafaxine. Besides, the inhibition effects on the growth of human embryonic cells (HEK293) were confirmed by Pomati et al. (2006), which were induced by a pharmaceutical mixture of atenolol, bezafibrate, carbamazepine, cyclophosphamide, ciprofloxacin, furosemide, hydrochlorothiazide, ibuprofen, lincomycin, ofloxacin, ranitidine, salbutamol and sulfamethoxazole.

2.3 Physicochemical Properties of the Reported Pharmaceuticals

After reviewing 42 papers, it was found that 115 pharmaceutical compounds (18 categories) have been investigated for their removal by applying constructed wetlands as an alternative secondary wastewater treatment system or a wastewater polishing process. The physicochemical properties of the reported pharmaceutical compounds are presented in Table A in the Appendix including their chemical formula, chemical structure, molecular weight (MW), dissociation constant (pK_a), solubility (S_w , 25°C), octanol-water partition coefficient as $\log K_{ow}$, and octanol-water distribution coefficient as $\log D_{ow}$ (pH=7.4). The data in Table A were collected from various internet databases such as ChemSpider, Wikipedia, DrugBank, ChemIDplus Advanced, and Hazardous Substances Data Bank.

2.4 Classification of Constructed Wetlands

The constructed wetlands for the treatment of pharmaceutical contaminants in wastewater can be categorised into three types including the surface free water constructed wetlands (SF-CWs), the horizontal subsurface flow constructed wetlands (HSSF-CWs), the vertical subsurface flow constructed wetlands (VSSF-CWs), and the hybrid constructed wetlands (hybrid CWs). A SF-CW normally consists of a shallow basin or channel with rooted or floating plants in which water flows freely over the packed substrate layer or the impermeable bottom liner (Figure 2-1a). For a HSSF-CW, influent water is fed to the inlet zone of the wetland and flows horizontally through the treatment zone under the surface of substrate layer. The effluent water is finally collected at the outlet zone (Figure 2-1b). For a VSSF-CW, water is fed onto the substrate surface of the wetland and flows vertically from the top layer down through the substrate until it reaches the bottom layer (Figure 2-1c). The hybrid CWs are normally laid in two or three stages via a combination of two or more constructed wetlands or in the form of a combination of constructed wetlands with lagoons or facultative ponds in series or in parallel.

2.5 Constructed Wetlands as an Alternative Secondary Wastewater Treatment System for Pharmaceutical Removal

Application of constructed wetlands as an alternative secondary wastewater treatment system for the removal of pharmaceutical pollutants from wastewater has been attracting growing interest in many countries, for example, Canada, China, Denmark, Italy, Portugal, Singapore, Spain, and USA (Table 2-1). The main design parameters of the reported constructed wetlands on this issue are summarized in Table 2-1 and the related removal efficiencies of the target pharmaceuticals are listed in Table 2-2. Both the Tables 2-1 and 2-2 include the source references.

Figure 2-1. Typical structures of constructed wetlands (a) SF-CW; (b) HSSF-CW; (c) VSSF-CW (Li et al. 2014)

2.5.1 Design Parameters of Constructed Wetlands as an Alternative Secondary Wastewater Treatment System for Pharmaceutical Removal

As can be seen in Table 2-1, the wastewaters treated using constructed wetlands as a secondary wastewater treatment system mainly have included municipal wastewater, rural wastewater, household wastewater, swine wastewater, and synthetic agricultural or municipal wastewater. Most wastewaters except for the synthetic wastewater were preliminarily or primarily treated by using septic tank, screen, homogenization tank, settling tank, hydrolytic upflow sludge bed (HUSB) or aerobic reactor. The target pharmaceutical pollutants in the wastewaters were mainly the analgesic/anti-inflammatory drugs, antibiotics, beta-blockers, diuretics, lipid regulators, psychiatric drugs, stimulants/psychoactive drugs and veterinary drugs, of which the analgesic/anti-inflammatory drugs such as diclofenac, ibuprofen, ketoprofen, naproxen and salicylic acid appeared to be of particular interest. The size of the investigated constructed wetlands listed in Table 2-1 was defined according to planar size as microcosm-scale (surface area: $< 0.5 \text{ m}^2$), mesocosm-scale (surface area: $0.5 \text{ to } 5 \text{ m}^2$), pilot-scale (surface area: $5 \text{ to } 100 \text{ m}^2$), and full-scale (surface area: $> 100 \text{ m}^2$).

Certain important design parameters have been found to influence the performance of constructed wetlands for the treatment of pharmaceuticals contaminants (Table 2-1). These design parameters are presented as followed.

(1) Water depth

In the SF-CWs, the water depth was typically kept at no more than 30 cm except for the gravel SF-CWs in which 10 to 25 cm of free water covered 25 to 60 cm of gravel layer (Table 2-1). In the HSSF-CWs, the water depth was usually maintained 5 cm below the surface of substrate layer which was about 30 to 60 cm deep. However, Reyes-Contreras et al. (2011) and Ranieri et al. (2011) designed the water depth to be the same as the level of substrate as 50 cm and 60 to 65 cm, respectively. Hussain et al. (2011) controlled the water depth at 20 cm below the surface of substrate layer which was 60 cm deep. In the VSSF-CWs, the water depth was kept

at just under the surface of substrate layer (Carvalho et al. 2013). No further information was available because of the very limited relevant reported studies.

(2) Hydraulic conditions and plants

Different hydraulic retention times (HRT) were designed for different constructed wetlands. For instance, HRT of 2 to 6 days was adopted in the SF-CWs, 2 to 6 days in the HSSF-CWs, 1 to 2 days in the VSSF-CWs and 2 to 15 days in the hybrid CWs (Table 2-1). In the studies reviewed, both continuous and batch operation modes were investigated. Also, in some studies, planted constructed wetlands were compared with the unplanted ones aiming to study the influence of vegetation. According to Table 2-1, various types of vegetation were selected, of which the most popular plants were *Phragmites spp.* and *Typha spp.* The initial plant density in several studies was designed as 10 to 50 plants/m².

(3) Substrate characteristics

Gravel was the most popular substrate used in constructed wetlands, normally designed as D₆₀ of 3 to 8 mm, D₁₀ of 0.5 to 4 mm, C_u of 0.8 to 2 and initial porosity of 40 to 45% (Table 2-1). Some other kinds of substrates were also used and they included lava rock, sandy soil, sandy clay loam, soil/red soil, stone, volcanic rock and zeolite. For example, Ranieri et al. (2011) designed three substrate layers of 10 cm soil, 20 cm stone and 30 to 35 cm gravel (from top to bottom) in their experimental HSSF-CWs.

Table 2-1. Design parameters of the constructed wetlands as an alternative secondary wastewater treatment system for pharmaceutical removal

No.	References	Influent Water and Target Pharmaceuticals	Preliminary/ Primary Treatment	Size and Type of CW System	Configuration and Hydraulic Conditions	Plants
1	Anderson et al. (2013)	Rural wastewater (Canada) Carbamazepine, Gemfibrozil, Sulfamethoxazole, Sulfapyridine	Septic tank	Full-scale Hybrid lagoon and SF-CW in series	Two lagoons in series: Each was 134 m long, 134 m wide and 2.3 m deep, with a water depth of 1.5m and a storage volume of 29400 m ³ . Connecting channel: 0.7 km long between lagoon and SF-CW. SF-CW: Five channel rows were in a snaking configuration with a water depth of 15 to 30 cm.	<i>Typha spp.</i>
2	Ávila et al. (2014a)	Municipal wastewater (Spain) Acetaminophen, Diclofenac, Ibuprofen	Screening and Imhoff tank	Full-scale Hybrid VSSF-CW, HSSF-CW and SF-CW in series	VSSF-CW was 317 m ² with substrate layers (top to bottom) of 0.05 m sand (1-2 mm), 0.6 m siliceous gravel (4-12 mm), and 0.15 m siliceous gravel (25-40 mm). HSSF-CW was 229 m ² with a 0.4 m siliceous gravel layer (4-12 mm) and a 0.35 m water level at a HRT of 2.3 d. SF-CW was 240 m ² with a 0.3 m water level and a 0.2 m siliceous gravel layer at a HRT of 5.1 d.	VSSF-CW and HSSF-CW: <i>Phragmites australis</i> SF-CW: <i>Typha spp.</i> , <i>Scirpus spp.</i> , <i>Iris pseudacorus</i> , <i>Carex flacca</i> , <i>Cyperus rutundus</i> , <i>Juncus spp.</i>
3	Ávila et al. (2014b)	Rural wastewater (Germany) Diclofenac, Ibuprofen	Septic tank	Pilot-scale VSSF-CW	Four VSSF-CWs were used for comparison: two sand-based unsaturated, one gravel-based unsaturated, and one gravel-based saturated. Each wetland was 2.75 m long, 2.4 m wide and 0.85 m deep with an initial plant density of 5 plants/m ² .	<i>Phragmites australis</i>

Table 2-1 (Continued)

No.	References	Influent Water and Target Pharmaceuticals	Preliminary/ Primary Treatment	Size and Type of CW System	Configuration and Hydraulic Conditions	Plants
4	Ávila et al. (2013)	Municipal wastewater (Spain) Acetaminophen, Diclofenac, Ibuprofen	Coarse screen followed by a hydrolytic upflow sludge bed (HUSB) or a conventional settling tank	Mesocosm-scale Hybrid HSSF-CWs	Three treatment lines were control line (settler-wetland permanently saturated), batch line (settler-wetland operated with saturated/unsaturated phases), and anaerobic line (HUSB-wetland permanently saturated). Each treatment line consisted of two small wetlands in parallel (0.95 m long, 0.7 m wide and 0.45 m deep each) which were connected to a big wetland in series (1.5 m long, 1.1 m wide and 0.5 m deep). Wetland kept a water depth of 25 cm and a 30 cm gravel layer ($D_{60}^a=7.3$ mm; $C_u^c=0.83$) with an initial porosity of 40% at a HRT of 3.5 d.	<i>Phragmites australis</i>
5	Ávila et al. (2010)	Municipal wastewater (Spain) Diclofenac, Ibuprofen, Naproxen	Coarse screen followed by a HUSB	Mesocosm-scale Hybrid HSSF-CWs	The system consisted of two small wetlands in parallel (0.65 m ² each) which were connected to a big wetland in series (1.65 m ²). Wetland kept a water depth of 25 cm and a 30 cm gravel layer ($D_{60}=5$ mm) with an initial porosity of 40% at a HRT of 3.5 d.	<i>Phragmites australis</i>
6	Carranza-Díaz et al. (2014)	Municipal wastewater (Germany) Caffeine, Carbamazepine, Diclofenac, Ibuprofen, Ketoprofen, Triclosan	n.a. ^d	Pilot-scale HSSF-CW	Wetland was 5.64 m ² (4.7 m long and 1.2 m wide) with a water depth of 50 cm and granular medium of medium gravel (8-16 mm) and coarse gravel (16-32 mm) at a HRT of 5.5 d.	<i>Phragmites australis</i>

Table 2-1 (Continued)

No.	References	Influent Water and Target Pharmaceuticals	Preliminary/ Primary Treatment	Size and Type of CW System	Configuration and Hydraulic Conditions	Plants
7	Carvalho et al. (2013)	Swine wastewater (Portugal) Enrofloxacin, Tetracycline	-- ^e	Microcosm-scale VSSF-CW	Seven microcosms were planted with an initial plant density of 40 plants each and nine were unplanted (0.4 m long, 0.3 m wide and 0.3 m deep). Substrate layers consisted of 10 cm roots' bed substrate, 2 cm lava rock and 4 cm gravel (top to bottom) and water depth was just below the substrate surface (100% flooding) in a batch loading mode.	<i>Phragmites australis</i>
8	Conkle et al. (2008)	Municipal wastewater (USA) Acetaminophen, Atenolol, Caffeine, Carbamazepine, Cotinine, Fluoxetine, Gemfibrozil, Ibuprofen, Metoprolol, Nadolol, Naproxen, Propranolol, Sotalol, Sulfamethoxazole, Sulfapyridine	--	Full-scale Hybrid aerated lagoon and SF-CW in series	Three aerated lagoons in series were 183 m long, 61 m wide and 3 m at a HRT of 9 d each. Water in SF-CW was evenly distributed across the width by percolation through a crushed gravel bed at a HRT of 1 d. 60% of effluent water was recycled and 40% was disinfected in a ultra-violet irradiation channel and afterwards pumped into the adjacent natural wetland for polishing before discharged into lake.	<i>Phragmites australis</i> , <i>Hydrocotyle spp.</i>
9	Hijosa-Valsero et al. (2011b);	Municipal wastewater (Spain)	Settling tank (ST) or HUSB	Mesocosm-scale	Five treatment lines each consisted of two CW tanks in series in which the first CW included two parallel compartments (93 cm long, 55 cm wide and 45 cm deep each) and the second CW was 134 cm long, 100 cm wide and 80 cm deep. Gravel depth was 30 cm and water depth was 25 cm at a HRT of 3.5 d with an initial plant density of 50 plants/m ² .	<i>Phragmites australis</i>
10	Hijosa-Valsero et al. (2011c)	Caffeine, Carbamazepine, Diclofenac, Ibuprofen, Ketoprofen, Naproxen, Salicylic acid		Hybrid HSSF-CWs		

Table 2-1 (Continued)

No.	References	Influent Water and Target Pharmaceuticals	Preliminary/ Primary Treatment	Size and Type of CW System	Configuration and Hydraulic Conditions	Plants
11	Hijosa-Valsero et al. (2011a);	Municipal wastewater (Spain)	Homogenization tank	Mescosm-scale	Wetland container was 130 cm long, 80 cm wide and 50 cm deep.	
12	Hijosa-Valsero et al. (2011c);	Hijosa-Valsero et al. (2010b and 2011c) and Reyes-		SF-CW (soilless)	Container kept 30 cm water at a HRT of 2.1 d without gravel bed with an effluent pipe at the top. Floating macrophytes were selected with an initial density of 50 plants/m ² .	<i>Typha angustifolia</i>
13	Hijosa-Valsero et al. (2010b);	Contreras et al. (2012): Caffeine, Carbamazepine,		SF-CW (with gravel)	25 cm water covered 25 cm gravel layer ($D_{10}=4$ mm) at a HRT of 3.3 d with an effluent pipe at the top and an initial plant density of 50 plants/m ² .	<i>Typha angustifolia</i>
14	Reyes-Contreras et al. (2012)	Diclofenac, Ibuprofen, Ketoprofen, Naproxen, Salicylic acid		SF-CW (with gravel)	25 cm water covered 25 cm gravel layer ($D_{10}=4$ mm) at a HRT of 5.1 d with an effluent pipe at the bottom and an initial plant density of 50 plants/m ² .	<i>Typha angustifolia</i>
		Hijosa-Valsero et al. (2011a): Amoxicillin, Ampicillin, Clarithromycin, Doxycycline, Erythromycin, Lincomycin, Sulfamethoxazole, Sulfadimethoxine, Trimethoprim		SF-CW (with gravel, unplanted)	25 cm water covered 25 cm gravel layer ($D_{10}=4$ mm) at a HRT of 6.1 d with an effluent pipe at the bottom.	Unplanted
				SF-CW (soilless)	Container kept 30 cm water at a HRT of 2.9 d without gravel bed with an effluent pipe at the top. Floating macrophytes were selected with an initial density of 50 plants/m ² .	<i>Phragmites australis</i>
				HSSF-CW	Substrate was a 45 cm gravel layer ($D_{10}=4$ mm) and water was kept 40 cm at a HRT of 2.5 d with an effluent pipe at the bottom and an initial plant density of 50 plants/m ² .	<i>Phragmites australis</i>
				HSSF-CW (unplanted)	Substrate was a 45 cm gravel layer ($D_{10}=4$ mm) and water was kept 40 cm at a HRT of 2.6 d with an effluent pipe at the bottom.	Unplanted

Table 2-1 (Continued)

No.	References	Influent Water and Target Pharmaceuticals	Preliminary/ Primary Treatment	Size and Type of CW System	Configuration and Hydraulic Conditions	Plants
15	Hijosa-Valsero et al. (2011c);	Municipal wastewater (Cubillas de los Oteros in Spain)	Bar screen and septic tank	Full-scale	FP was 1073 m ² with a water depth of 1.6 m at a HRT of 7.59 d.	<i>Lemna minor</i>
16	Hijosa-Valsero et al. (2010a)	Caffeine, Carbamazepine, Diclofenac, Ibuprofen, Ketoprofen, Naproxen, Salicylic acid		Hybrid facultative pond (FP), SF-CW and HSSF-CW in series	SF-CW was 44 m ² with a 30 cm gravel layer (6-8 mm) and a water depth of 40 cm at a HRT of 1.2 d. HSSF-CW was 585 m ² with a 55 cm gravel layer (6-8 mm) at a HRT of 5.7 d.	<i>Typha latifolia</i> <i>Salix atrocinerea</i>
		Municipal wastewater (Bustillo de Cea in Spain)	Bar screen and coarse solid tank	Full-scale	FP was 230 m ² with a water depth of 1.5-2 m at a HRT of 4.21 d.	n.a.
		Pharmaceuticals were the same as the above.		Hybrid facultative pond (FP), SF-CW and HSSF-CW in series	SF-CW was 210 m ² at a HRT of 3.53 d. HSSF-CW was 362.5 m ² at a HRT of 3.16 d.	<i>Typha latifolia</i> <i>Salix atrocinerea</i>
17	Hussain et al. (2012)	Synthetic agricultural wastewater (Canada)	--	Mesocosm-scale	Container was a high-density polyethylene half-pipe (6.1 m long, 1.5 m wide and 0.75 m deep) with a surface area of 9.29 m ² and a volume of 4.17 m ³ .	<i>Phalaris arundinacea</i> ,
18	Hussain et al. (2011)	Monensin, Salinomycin, Narasin		Hussain et al. (2012): SF-CW Hussain et al. (2011): HSSF-CW	Six SF-CWs kept 0.15 m water (HRT: 4.1 d) over 0.6 m substrate in which three beds were filled with sandy clay loam soil and the others were filled with sandy soil. Three HSSF-CWs kept 0.2 m of water (HRT: 2.2 d) below the substrate (sandy soil) surface (0.6 m) at the outlet.	<i>Typha latifolia</i>

Table 2-1 (Continued)

No.	References	Influent Water and Target Pharmaceuticals	Preliminary/ Primary Treatment	Size and Type of CW System	Configuration and Hydraulic Conditions	Plants
19	Liu et al. (2013a)	Swine wastewater (China) Ciprofloxacin HCl, Oxytetracycline HCl, Sulfamethazine	Aerobic reactor	Mesocosm-scale VSSF-CW	Two VSSF-CWs were 1 m ² each at a HRT of 30 h with substrate layers of 20 cm red soil, 30 cm volcanic rock or zeolite and 10 cm gravel (top to bottom) and with an initial plant density of 15 plants/m ² .	Hybrid <i>pennisetum</i>
20	Matamoros et al. (2009a)	Household wastewater (Denmark) Caffeine, Carbamazepine, Diclofenac, Furosemide, Ibuprofen, Ketoprofen, Naproxen, Salicylic acid	Settling tank	Full-scale and pilot scale HSSF-CW, VSSF-CW	Five full-scale HSSF-CWs' surface areas were 500 m ² (180 inhabitants), 470 m ² (80 inhabitants), 940 m ² (220 inhabitants), 1800 m ² (213 inhabitants) and 4000 m ² (280 inhabitants), respectively. Four pilot-scale VSSF-CWs' surface areas were 16 m ² (4 inhabitants), 15 m ² (2 inhabitants), 15 m ² (variable inhabitants) and 15 m ² (4 inhabitants), respectively.	n.a.
21	Matamoros et al. (2008a)	Synthetic municipal wastewater -- (Spain) Carbamazepine, Clofibric acid, Ibuprofen	--	Mesocosm-scale HSSF-CW	Container was 0.93 m long, 0.59 m wide and 0.52 m deep with a 35 cm gravel layer (initial porosity: 40%; D ₆₀ =3.5 mm; C _u =1.7) and a water depth of 30 cm in a batch operating mode.	<i>Phragmites australis</i>
22	Matamoros et al. (2007)	Municipal wastewater (Denmark) Caffeine, Carbamazepine, Diclofenac, Ibuprofen, Naproxen, Salicylic acid	Three chamber settling tank	Pilot-scale VSSF-CW	Wetland was 5 m ² with a 0.2 m bottom gravel layer (8-16 mm) and a 0.8 m top gravel layer (0-4 mm) (D ₁₀ =0.55 mm; D ₆₀ =3.1 mm). Water was distributed through four 40 mm perforated pipes.	<i>Phragmites australis</i>

Table 2-1 (Continued)

No.	References	Influent Water and Target Pharmaceuticals	Preliminary/ Primary Treatment	Size and Type of CW System	Configuration and Hydraulic Conditions	Plants
23	Matamoros and Bayona (2006)	Municipal wastewater (Spain)	Screen and Imhoff settling tank	Pilot-scale	Two parallel CWs (54-56 m ² each) were used for comparison in which one bed was with a water depth of 0.5 m, an aspect ratio of 2 : 1 and a slope of 1%, while the other bed was with a water depth of 0.3 m, an aspect ratio of 2.5 : 1 and a slope of 2.5%. Substrate was a gravel layer (D ₆₀ =3.5 mm; C _u =1.7; porosity: 40%) 5 cm above the water surface. Plant initial density was 3 plants/m ² .	<i>Phragmites australis</i>
24	Matamoros et al. (2005)	Matamoros and Bayona (2006): Caffeine, Diclofenac, Ibuprofen, Ketoprofen, Naproxen, Salicylic acid Matamoros et al. (2005): Carbamazepine, Clofibric acid, Ibuprofen		HSSF-CW		
25	Ranieri et al. (2011)	Municipal wastewater (Italy) Paracetamol	--	Pilot-scale HSSF-CW	Three HSSF-CWs were <i>Phragmites</i> bed, <i>Typha</i> bed and unplanted bed (3 m wide, 5 m long, 9.4 m ³ each) with a water depth of 0.6 m-0.65 m and substrate layers of 0.1 m soil, 0.2 m stones and 0.30-0.35 m gravel (top to bottom). Side banks were 45° inclination and bottom slope was 1%. Bottom liner was a bentonite liner which included three layers of an upper geo-textile, a lower geo-textile and sodic powdered bentonite.	<i>Phragmites australis</i> , <i>Typha latifolia</i>
26	Reyes-Contreras et al. (2011)	Municipal wastewater (Spain) Caffeine, Carbamazepine, Ibuprofen, Ketoprofen, Naproxen, Salicylic acid, Triclosan	Upflow anaerobic sludge blanket (UASB)	Pilot-scale Hybrid SF-CW and HSSF-CW in series	The two wetlands were 75 m ² each with a water depth of 0.5 m, a gravel layer (size: 6-8 mm; initial porosity: 0.45; depth: 0.3m in the SF-CW and 0.5m in the HSSF-CW), and an initial plant density of 2 plants/m ² at a HRT of 55 h.	<i>Juncus effusus</i>

Table 2-1 (Continued)

No.	References	Influent Water and Target Pharmaceuticals	Preliminary/ Primary Treatment	Size and Type of CW System	Configuration and Hydraulic Conditions	Plants
27	Xian et al. (2010)	Swine wastewater (China) Sulfadiazine, Sulfamethazine, Sulfamethoxazole	--	Microcosm -scale SF-CW	Container was 0.5 m long, 0.4 m wide and 0.4 m deep with a working depth of 0.3 m and a capacity volume of 60 L. Foam plate for macrophytes floating was 0.5m long, 0.4 m wide and 0.015 m thick, with 16 rounded holes (4 × 4, 2 cm in diameter). Eight treatment beds were two Dryan beds, two Waseyutaka beds, two Tachmasari beds and two unplanted beds.	Ryegrass (<i>Lolium</i>): Dryan, Waseyutaka, Tachimasari
28	Zhang et al. (2012a)	Synthetic municipal wastewater (Singapore) Zhang et al. (2012a and 2012b): Caffeine, Carbamazepine, Clofibric acid, Diclofenac, Ibuprofen, Ketoprofen, Naproxen, Salicylic acid Zhang et al. (2011): Carbamazepien, Diclofenac, Ibuprofen, Naproxen	--	Mesocosm -scale	Container was 1.2 m long, 0.6 m wide and 0.6 m deep with a gravel depth of 30 cm and a water depth of 25 cm at a HRT of 2 d or 4 d. Plants initial density was 14 plants/m ² .	<i>Typha angustifolia</i>
29	Zhang et al. (2012b)					
30	Zhang et al. (2011)			HSSF-CW		

Notes: ^a D₆₀: the diameter at which 60% of material can pass through sieve. ^b D₁₀: the diameter at which 10% of material can pass through sieve. ^c C_u: D₆₀/D₁₀, uniformity coefficient. ^d n.a.: not available. ^e -- : no existence.

2.5.2 Removal Efficiencies of Pharmaceuticals in Constructed Wetlands as an Alternative Secondary Wastewater Treatment System

The efficiencies of pharmaceutical contaminants removed by constructed wetlands are important performance indicators of constructed wetlands for the secondary treatment of pharmaceuticals in wastewater. As can be seen in Table 2-2, the removal efficiencies of 36 reported pharmaceutical compounds are summarized for the SF-CW, HSSF-CW, VSSF-CW and hybrid CW system, as well as the conventional WWTPs. Certain pharmaceutical compounds with concentration levels in wastewater below the detection limit are not listed in Table 2-2.

Based on the data in Table 2-2, one can deduce that the mean removal efficiencies of pharmaceuticals may be used to categorize the ease of pharmaceutical removal as the readily removable (mean removal efficiency > 70%), the moderately removable (50 to 70%), the low removable (20 to 50%) and the hardly removable (< 20%). The readily removable pharmaceuticals include acetaminophen, salicylic acid, sulfadiazine, sulfadimethoxine, sulfamethazine, sulfamethoxazole, sulfapyridine, trimethoprim, atenolol, metoprolol, furosemide, caffeine and tetracycline. The moderately removable pharmaceuticals are ibuprofen, naproxen, doxycycline and gemfibrozil. The low removable pharmaceuticals contain diclofenac, ketoprofen, amoxicillin, clarithromycin, triclosan, sotalol, clofibrate acid and carbamazepine. Ampicillin, erythromycin and lincomycin are hardly to remove.

Except for the removal efficiencies of ciprofloxacin HCl, oxytetracycline HCl, nadolol, cotinine, enrofloxacin, monensin, narasin and salinomycin which have not been reported for conventional WWTPs, by comparing the removal efficiencies of other pharmaceuticals in the constructed wetlands and conventional WWTPs, it indicates that the performance of the constructed wetlands appears to be as good as or even better than that of the conventional WWTPs. It can be inferred that constructed wetlands demonstrate good potential as an alternative secondary wastewater treatment system for the removal of pharmaceutical contaminants from wastewater.

A Comparison of the removal efficiencies of pharmaceuticals in different types of constructed wetlands (Table 2-2) indicates that the VSSF-CW exhibits more efficient for the treatment of diclofenac, ibuprofen, naproxen, salicylic acid and caffeine than the wetlands with other configurations. It may be attributed to the better oxygenation in unsaturated flow and the less sensitivity to overloading in VSSF-CW (Matamoros et al. 2007, 2009a). However, the superior performance of VSSF-CW for the removal of pharmaceuticals over other types of constructed wetlands is still inconclusive.

HSSF-CW is perhaps the most investigated constructed wetland applied separately or combined in hybrid CW system for pharmaceutical removal. As reported in Table 2-2, the HSSF-CW is comparable efficient to remove diclofenac, clarithromycin, sulfamethoxazole and carbamazepine. The hybrid CW system showed better performance for acetaminophen, diclofenac, ibuprofen, naproxen, and sulfamethoxazole compared to that in HSSF-CW. In comparison with the SF-CW, the HSSF-CW exhibited more efficient in the removal of ketoprofen, naproxen, salicylic acid, doxycycline, sulfadimethoxine, trimethoprim, caffeine, monensin, narasin and salinomycin.

Ávila et al. (2013) and Zhang et al. (2012b) found that batch operation mode was favourable for pharmaceutical removal attributable to the higher redox status generated by the alternating cycles of unsaturation and saturation. Hijosa-Valsero et al. (2011b), however, observed little difference between a batch flow and a continuous flow strategy. Zhang et al. (2012a) found that the efficiencies of their target pharmaceuticals removed by a mesocosm-scale HSSF-CW system were linearly proportional to the HRT, but the difference became less obvious when the HRT was long enough (Zhang et al. 2011). Moreover, first-order kinetics was demonstrated fit well for the decay of ibuprofen, ketoprofen, caffeine and clofibric acid in HSSF-CW systems (Matamoros and Bayona 2006, Zhang et al. 2012a).

Table 2-2. Efficiencies of pharmaceuticals removed by constructed wetlands as an alternative secondary wastewater treatment system

Pharmaceutical compounds	Removal Efficiencies (%)				
	SF-CW	HSSF-CW	VSSF-CW	Hybrid CW system	Conventional WWTPs
Analgesic/ anti-inflammatory					
Acetaminophen (Paracetamol)		47- >99 (14)		>99(19); >95 (20); 99 (50)	> 90 (39); 98- >99 (40); 70-90 (41); 96 (43); 72-97 (44); >99 (46)
Diclofenac	0-50 (1,2, 3)	0-35 (1, 2, 3); 21 (7); 0-45 (8); 41-44 (9, 10); 48-55 (11); 49-55 (10); 14 (51)	53-73 (16); 58-63 (49)	32-70 (20); 0-71 (21); 78-87 (22); 89 (50)	17 (24, 25); 69 (27); 22 (29); 7-63 (30); 0-70 (31); 33 (35); 49 (38); 17-33 (41); 39-75 (42); 35 (43); 0-80 (44); 31 (46); 42 (47)
Ibuprofen	45-95 (1, 2, 3)	5-55 (1, 2, 3); 65 (7); 62-80 (8); 81 (13); 68-72 (9, 10); 71-80 (11); 72-83 (10); 52 (12); 13 (51)	89 (7); 55-99 (16); 98- >99 (49)	>99(19, 50); 52-85 (20); 0-99 (21); 42-89 (22); 8-99 (23); 3 -42(48)	60-70 (26); 90 (22, 27, 28); 96 (29, 35); 99 (30); 87 (38); 87-93 (41); 100 (42); 88(43); 56-85 (44); 80-87 (45); 94 (46); 98 (47)
Ketoprofen	35-50 (1, 2, 3)	10-20 (1, 2, 3); 90 (7); 0-69 (8); 91-96 (9); 91-92 (10); 94-95 (10); 20 (51)		0-37 (21); 77-81 (22); 5 -42 (48)	23 (25); 30 (28); 65(29); 68 (38); 70-84 (41); 83-89 (42); 72-97 (44); 52-72 (45); 85 (46); 55 (47)
Naproxen	25-75 (1, 2, 3)	25-85 (1, 2, 3); 45 (7); 80-90 (8); 81-93 (9, 10); 83-91 (11); 82-91(10); 14 (51)	62-89 (16); 92 (7)	98(19); 0-95 (21); 73-82 (22); 99 (23); 30 -53 (48)	82 (24, 25); 40-55 (6); 66 (27); 83 (28); 89 (22); 93 (29, 38); 91-95 (42); 67 (43); 72-97 (44); 43-71 (45); 86 (46); 64 (47)
Salicylic acid	35-90 (1, 2, 3)	50-85 (1, 2, 3); 95 (7); 92-98 (8); 86-90 (9); 88-94 (10);	85-98 (16); 87 (7)	0-97 (21); 94-97 (22); 53-70 (48)	80 (22); 88 (25); 95 (28); 99 (32, 38); 94 (43); >99 (46)
Antibiotics					
Amoxicillin	27-45 (4)	6-21 (4)			35 (4);
Ampicillin	0-32 (4)	n.r. (4)			7 (4); n.r. (44)
Ciprofloxacin HCl			82-85 (17)		
Clarithromycin	11-50 (4)	31-32 (4)			18 (4)
Doxycycline	47-75 (4)	71-79 (4)			61 (4)
Erythromycin	n.r. (4)	0-64 (4)			n.r. (4); 25 (35); -17 to 50 (40)

Table 2-2 (Continued)

Pharmaceutical compounds	Removal Efficiencies (%)				
	SF-CW	HSSF-CW	VSSF-CW	Hybrid CW system	Conventional WWTPs
Lincomycin	n.r. (4)	n.r. (4)			24 (4); 64-80 (40)
Oxytetracycline HCl			91-95 (17)		
Sulfadiazine	98-99 (5)				20-82 (44)
Sulfadimethoxine	67-99 (4)	99 (4)			53 (4); 20-82 (44)
Sulfamethazine	>99 (5)		68-73 (17)		7-17 (41); 20-82 (44)
Sulfamethoxazole	59-80 (4); 89-92 (5)	73-87 (4)		91 (19)	32-66 (30); 24 (35); 60 (4); 66 (38); 0-88 (44); 83 (46)
Sulfapyridine				76 (19)	95 (28)
Triclosan		8 (51)		7-48 (48)	58 (29); 91-93 (42)
Trimethoprim	65-96 (4)	95-99 (4)			14 (4); 49 (29); 69 (35); 74 (38); 67-80 (40); 67-89 (41); 22-56 (44); 70 (46)
Beta-blockers					
Atenolol				93 (19)	50-80 (29); 84 (35); 73-79 (36); 58 (37); 83-87(41); 71-77 (44)
Metoprolol				92 (19)	83 (27); 30-65 (29); 65 (35); 29-31 (36); 17 (37); 3-67 (44)
Nadolol				77 (19)	
Sotalol				30 (19)	48 (35); 26-27 (36); 66 (37); 58-76 (44)
Diuretics					
Furosemide		71 (7)			77 (46)
Lipid regulators					
Clofibric acid		34-39 (9, 10); n.r. (13)			51 (27); 52 (35); 49 (38); 0-81 (44); 50 (47)
Gemfibrozil				61 (19)	69 (27); 75 (29); 65 (38); 56 (43); 0-81 (44)

Table 2-2 (Continued)

Pharmaceutical compounds	Removal Efficiencies (%)				
	SF-CW	HSSF-CW	VSSF-CW	Hybrid CW system	Conventional WWTPs
Psychiatric drugs					
Carbamazepine	0-50 (1, 2, 3)	20-40 (1, 2, 3); 38 (7); 27-28 (9, 10, 11); 5 (12); 16 (13); 25 (51)	20-26 (16);	0-58 (21); 5 -18 (48)	7 (27); 8 (24, 25, 33); 15 (22); 30 (29, 38); -43 to 14 (30); 0-30 (31); -13 (43); 0-40 (44); 7-11 (45); 13 (46)
Stimulants/ Psychoactive drugs					
Caffeine	25-99 (1, 2, 3);	60-99 (1, 2, 3); 97 (7); 94-99 (8); 84-93 (9, 10); 66 (51)	82-99 (16); 99 (7)	>99 (19); 14-99 (21); 95-96 (22); 55 -60 (48)	86 (22); 93 (28); 94 (29); 99 (24, 25, 33); >99 (34); 87-93 (40); 83-90 (41); 89 (43); 85-100 (44); 44-75 (45)
Cotinine				99 (19)	
Veterinary drugs					
Enrofloxacin			94(18)		
Monensin	27-34 (6)	37-43 (15)			
Narasin	29-36 (6)	42-57 (15)			
Salinomycin	30-32 (6)	41-56 (15)			
Tetracycline			98(18)		66-90 (44)

Notes: ^a n.r.: not removed.

1: Hijosa-Valsero et al. (2010b); 2: Hijosa-Valsero et al. (2011c); 3: Reyes-Contreras et al. (2012); 4: Hijosa-Valsero et al. (2011a); 5: Xian et al. (2010); 6: Hussain et al. (2012); 7: Matamoros et al. (2009a); 8: Matamoros and Bayona (2006); 9: Zhang et al. (2012a); 10: Zhang et al. (2012b); 11: Zhang et al. (2011); 12: Matamoros et al. (2008a); 13: Matamoros et al. (2005); 14: Ranieri et al. (2011); 15: Hussain et al. (2011); 16: Matamoros et al. (2007); 17: Liu et al. (2013a); 18: Carvalho et al. (2013); 19: Conkle et al. (2008); 20: Ávila et al. (2013); 21: Hijosa-Valsero et al. (2011b); 22: Hijosa-Valsero et al. (2010a); 23: Ávila et al. (2010); 24: Heberer (2002a); 25: Heberer (2002b); 26: Carballa et al. (2004); 27: Ternes (1998); 28: Camacho-Muñoz et al. (2012); 29: Bendz et al. (2005); 30: Clara et al. (2005); 31: Zhang et al. (2008b); 32: Ternes et al. (2004); 33: Heberer et al. (2002); 34: Ternes et al. (2001); 35: Ternes et al. (2007); 36: Maurer et al. (2007); 37: Vieno et al. (2007); 38: Miège et al. (2009); 39: Matsuo et al. (2011); 40: Li et al. (2011); 41: Tewari et al. (2013); 42: Samaras et al. (2013); 43: Kosma et al. (2010); 44: Lin et al. (2009); 45: Santos et al. (2009); 46: Kasprzyk-Hordern et al. (2009); 47: Kimura et al. (2007); 48: Reyes-Contreras et al. (2011); 49: Ávila et al. (2014b); 50: Ávila et al. (2014a); 51: Carranza-Díaz et al. (2014)

2.6 Constructed Wetlands as a Wastewater Polishing Process for Pharmaceutical Removal

Applications of constructed wetlands as a wastewater polishing process for the removal of pharmaceutical pollutants from wastewater have been reported in Denmark, Korea, Portugal, Sweden, Spain, UK and USA (Table 2-3). The number of the research studies on this application is relatively low compared to those publications on the application of constructed wetlands as an alternative secondary wastewater treatment system for pharmaceutical removal. The main design parameters of the reported constructed wetlands on this research focus are summarized in Table 2-3 and the related removal efficiencies of the target pharmaceuticals in different constructed wetland systems are listed in Table 2-4.

2.6.1 Design Parameters of Constructed Wetlands as a Wastewater Polishing Process for Pharmaceutical Removal

As shown in Table 2-3, the wastewater treated by constructed wetlands for polishing purpose mainly have included the secondary or tertiary effluent water from municipal or rural WWTPs and the water from rivers which received effluent discharge of WWTPs. The target pharmaceutical contaminants in wastewater were mainly the analgesic/anti-inflammatory drugs, antiallergic drugs, antibiotics, antidiabetics, antidiysenterics, antifungals, antihypertensives, anti-senile dementia drugs, barbiturates, beta-agonists, beta-blockers, diuretics, hormone inhibitors, lipid regulators, psychiatric drugs, receptor antagonists, stimulants/psychoactive drugs and veterinary drugs. The commonly investigated pharmaceutical compounds were diclofenac, ibuprofen, ketoprofen, naproxen, sulfamethoxazole, triclosan, atenolol, clofibric acid, carbamazepine and caffeine. The size of the constructed wetlands listed in Table 2-3 involved microcosm-scale, pilot-scale and full-scale, of which the full-scale constructed wetlands were of most interest.

The popular constructed wetlands adopted for wastewater polishing treatment were the SF-CWs and the hybrid SF-CWs (Table 2-3). In the research studies of Llorens

et al. (2009), Matamoros et al. (2012a), Matamoros et al. (2008b) and Zarate Jr et al. (2012), the investigated SF-CWs were designed with a water depth of 0.05 to 2 m and a surface area in the range of 0.2 to 100 ha. The target wastewater was retained for 3 to 30 days in these SF-CW systems and was finally discharged to the surrounding rivers or lakes. In the hybrid SF-CWs reported by Breitholtz et al. (2012), Gross et al. (2004), Lee et al. (2011) and Park et al. (2009), two or more stages of constructed wetland basins were laid out in series or in parallel with a water depth of 0.1 to 1 m, covering a surface area in the range of 0.36 to 130 ha. The target wastewater was retained for 6 hours to 14 days in these hybrid systems and was finally discharged to the surrounding river, lake or sea. In addition, SF-CWs could also be combined with other types of constructed wetlands or ponds to form a hybrid system. For instance, the hybrid system investigated by Matamoros and Salvadó (2012) consisted of two parallel ponds (2 ha. of area and 1 m of water each) followed by three parallel deep SF-CWs (0.8 ha. of area and 0.5 m of water each) and a large shallow SF-CW (4.5 ha. of area and 0.2 m of water). The target wastewater was retained for 4 days and 8.5 days in the ponds and the SF-CWs (including the deep and the shallow SF-CWs), respectively. Finally, the effluent water from this hybrid system was discharged to an 18 ha. of receiving pond.

Dordio et al. (2009a, 2010) and Verlicchi et al. (2013) studied HSSF-CWs to remove the pharmaceuticals from secondary effluent water of WWTPs. Their HSSF-CWs were designed with a respective water depth of 0.3 m and 2.2 m, and a surface area of 0.3 m² and 28 m², respectively. Reif et al. (2011) investigated a pilot-scale VSSF-CW in batch operation mode to treat the effluent water from a WWTP in USA. The VSSF-CW was designed with substrate layers of 0.15 m filter grade sand, 0.15 m pea gravel (10 mm), 0.05 m gravel (20 mm) and 0.6 m gravel (40 to 50 mm).

In all the reported constructed wetlands, various types of plants were used, of which the popular plants were *Phragmites spp.* and *Typha spp.* (Table 2-3).

Table 2-3. Design parameters of the constructed wetlands as a wastewater polishing process for pharmaceutical removal

No.	References	Influent Water and Target Pharmaceuticals	Size and Type of CW System	Configuration and Hydraulic Conditions	Plants
1	Breitholtz et al. (2012)	Effluent water from four WWTPs (Eskilstuna, Nynäshamn, Oxelösund and Trosa, Sweden) 92 target pharmaceuticals, of which 54 were detected: Alfuzosine, Alprazolam, Amitriptyline, Atenolol, Atorvastatin, Azithromycine, Bezafibrate, Bisoprolol, Buprenorphine, Bupropion, Carbamazepine, Citalopram, Clarithromycine, Clindamycine, Clotrimazole, Codeine, Desloratadine, Diclofenac, etc.	Full-scale Hybrid SF-CWs	Eskilstuna WWTP (activated sludge biological treatment) consisted of five parallel basins - three parallel basins (1 m deep), covering an area of 28 ha. at a detention time of 6-7 d. Plant coverage was ca. 20%. Nynäshamn WWTP (sequencing batch reactor (SBR) biological treatment) consisted of two inflow basins - two parallel CW basins (used alternatively) - overland flow area, covering an area of 28 ha. at a detention time of 1.5-2 weeks. Oxelösund WWTP (SBR biological treatment) consisted of one inlet basin - two parallel CW systems (each of which consists of two basins which were used alternatively) - a joint basin, covering an area of 24 ha. at a detention time of 6 d. Trosa WWTP (activated sludge biological treatment) consisted of an overland flow area - a collecting basin - three serial CW basins, covering an area of 6 ha. at a detention time of 8 d.	<i>Ceratophyllum demersum</i> , <i>Elodea spp.</i> , <i>Glyceria maxima</i> , <i>Myriophyllum spicatum</i> , <i>Nymphaeaceae</i> , <i>Schoenoplectus lacustris</i> , <i>Typha spp.</i> <i>Carex riparia</i> , <i>Carex spp.</i> , <i>Ceratophyllum demersum</i> , <i>Elodea spp.</i> , <i>Myriophyllum spp.</i> , <i>Potamogeton spp.</i> , <i>Scirpus sylvaticus</i> , <i>Typha spp.</i> <i>Carex spp.</i> , <i>Elodea spp.</i> , <i>Phragmites australis</i> , <i>Potamogeton spp.</i> , <i>Typha spp.</i> <i>Phragmites spp.</i> , <i>Scirpus spp.</i> , <i>Typha spp.</i>

Table 2-3 (Continued)

No.	References	Influent Water and Target Pharmaceuticals	Size and Type of CW System	Configuration and Hydraulic Conditions	Plants
2	Dordio et al. (2010)	Effluent water from a rural	Microcosm-scale	Container was 0.6 m long, 0.5 m wide and 0.4 m deep with a 0.3 m substrate layer of light expanded clay aggregates (LECA).	Dordio et al. (2010): <i>Typha spp.</i>
3	Dordio et al. (2009a)	Dordio et al. (2010): Carbamazepine, Clofibric acid, Ibuprofen Droid et al. (2009a): Atenolol	HSSF-CW	Water level was kept just below the LECA surface in a batch operating mode. Initial plant density was 20 plants/m ² .	Droid et al. (2009a): <i>Phragmites spp.</i> , <i>Typha spp.</i>
4	Gross et al. (2004)	Water from the Santa Ana River (USA) to which the effluent water from the upstream WWTPs was introduced. Carbamazepine, Gemfibrozil, Ibuprofen, Ketoprofen, Naproxen	Full-scale Hybrid SF-CWs	Wetlands consisted of four subsections each of which included inter-connected cells such as East Cells (22%), South Cells (28%), North Cells (26%) and West Cells (24%), with a water depth of 0.6 m-0.76 m and a total covering area of 130 ha. Water flow of the East Cells was split into two separate streams for entering South Cells (60%) and North Cells (40%), respectively, and afterwards recombined and passed through the West Cells. HRT was 100 h.	<i>Scirpus spp.</i> , <i>Typha spp.</i> , <i>Polygonum spp.</i> , <i>Najas marina</i> , <i>Zannichellia palustris</i> , <i>Lemna spp.</i>

Table 2-3 (Continued)

No.	References	Influent Water and Target Pharmaceuticals	Size and Type of CW System	Configuration and Hydraulic Conditions	Plants
5	Lee et al. (2011)	Effluent water from the Damyang wastewater treatment plant (Korea)	Full-scale	Wetlands consisted of two basins in series including Acorus wetland and Typha wetland (30 m wide, 120 m long and 0.13 m deep each) at a HRT of 6 h.	<i>Acorus spp.</i> ,
6	Park et al. (2009)		Hybrid SF-CWs		<i>Typha spp.</i>
		Lee et al. (2011): Atenolol, Caffeine, Carbamazepine, Diclofenac, Glimepiride, Ibuprofen, Naproxen, Sulfamethoxazole Park et al. (2009): Atenolol, Carbamazepine, Diazepam, Diclofenac, Dilantine, Sulfamethoxazole, Triclosan, Naproxen			
7	Llorens et al. (2009)	Part of the effluent water from the Granollers urban WWTP (Spain)	Full-scale	Wetland was 1 ha. (maximum length and width: 189 m and 53 m respectively) with a water depth of 0.3-0.4 m for planted shallow zones and 1.5 m for unplanted deep zones at a HRT of 1 month.	<i>Phragmites australis</i> ,
8	Matamoros et al. (2008b)		SF-CW		<i>Typha latifolia</i>
		Llorens et al. (2009): Carbamazepine, Clofibric acid, Diclofenac, Ibuprofen, Ketoprofen, Naproxen Matamoros et al. (2008b): Carbamazepine, Clofibric acid, Diclofenac, Flunixin Ibuprofen, Ketoprofen, Naproxen			

Table 2-3 (Continued)

No.	References	Influent Water and Target Pharmaceuticals	Size and Type of CW System	Configuration and Hydraulic Conditions	Plants
9	Matamoros and Salvadó (2012)	Effluent water from the Empuriabrava WWTP (Spain) Caffeine, Carbamazepine, Diclofenac, Ibuprofen, Ketoprofen, Naproxen, Triclosan	Full-scale Hybrid pond and SF-CW in series	Ponds consisted of two parallel cells each of which was 2 ha. and 1 m deep at a HRT of 4 d. SF-CWs included three parallel deep cells and a large shallow cell in series in which the deep cells were 0.8 ha. and 0.5m deep each and the shallow cell was 4.5 ha. and 0.2 m deep. The HRT of SF-CWs was 8.5 d.	<i>Phragmites australis</i> , <i>Typha latifolia</i>
10	Matamoros et al. (2012a)	Water from Aarhus River and Lyngbygaards River (Denmark) which were subjected to the effluent water of the upstream WWTPs. Caffeine, Carbamazepine, Diclofenac, Furosemide, Ibuprofen, Ketoprofen, Naproxen, Triclosan	Full-scale SF-CW	Wetland was 100 ha. with a water depth of 0.05 to 2 m at a HRT of 3 to 20 d (average 7 d).	<i>Nymphaea alba</i> , <i>Schoenoplectus lacustris</i>
11	Reif et al. (2011)	Effluent water from a membrane reactor (MBR) and an activated sludge (AS) system (UK) Carbamazepine, Diclofenac, Ibuprofen, Naproxen	Pilot-scale VSSF-CW	Wetland was about 1 m deep and consisted substrate layers of 0.15 m filter grade sand, 0.15 m pea gravel (10 mm), 0.05 m gravel (20 mm) and 0.6 m gravel (40-50 mm) (from top to bottom). Wetland was fed 8 times per day for 10 minutes at a rate of 125 mL/min.	<i>Phragmites spp.</i>

Table 2-3 (Continued)

No.	References	Influent Water and Target Pharmaceuticals	Size and Type of CW System	Configuration and Hydraulic Conditions	Plants
12	Verlicchi et al. (2013)	Effluent water from a large municipal WWTP (Italy) 73 target pharmaceuticals, of which 55 were detected: Acetaminophen, Codeine, Diclofenac, Ibuprofen, Indomethacin, etc.	Pilot-scale HSSF-CW	Wetland was 28 m long, 1 m wide and 0.7 m deep at the inlet and to 1.75 m deep at the outlet at a HRT of 1 d. Substrate was a gravel layer (8-10 mm) with a porosity of 33%. Water was gravity-fed from a 10 m ³ equalization tank which was 3 m above the ground.	<i>Phragmites australis</i>
13	Zarate Jr et al. (2012)	Effluent from the Pecan Creek WWTP (USA) Triclosan	Pilot-scale SF-CW	Wetland was a continuous channel separated by three land berms covering a total area of 0.21ha. (46 m×46 m) with a storage volume of 570000 L at a HRT of 4.3 d.	<i>Ceratophyllum demersum</i> , <i>Lemna spp.</i> , <i>Paspalum spp.</i> , <i>Pontederia cordata</i> , <i>Potamogeton spp.</i> , <i>Sagittaria graminea</i> , <i>Typha latifolia</i> ,
14	Zhu and Chen (2014)	Effluent water from WWTP (China) Carbamazepine, Caffeine Diclofenac, Ibuprofen Sulfamethoxazole, Trimethoprim	Full-scale Hybrid HSSF-CWs and SF-CWs in series	HRT was 1.6 d. The hybrid CW system consisted of three subsystems in series: Subsystem I (HSSF-CWs): 190 m ² . Subsystem II (Hybrid HSSF-CWs and SF-CWs): 2534 m ² . Subsystem III(SF-CWs): 950 m ² .	Subsystem I : <i>Hydrocotyle verticillata</i> , <i>Myriophyllum spicatum</i> Subsystem II : <i>Cyperus haspan</i> , <i>Cyperus alternifolius</i> , <i>Thalia dealbata</i> , <i>Canna lily</i> , <i>Iris pseudacorus</i> , etc. Subsystem III: <i>Typha latifolia</i> , <i>Arundo donax L.</i>

2.6.2 Removal Efficiencies of Pharmaceuticals in Constructed Wetlands as a Wastewater Polishing Process

For the pharmaceutical compounds listed in Table 2-4, except for the inconsistent or low removal efficiencies of some pharmaceutical compounds obtained by Breitholtz et al. (2012), Matamoros et al. (2012a) and Verlicchi et al. (2013), the readily removable pharmaceuticals include diclofenac, ibuprofen, ketoprofen, triclosan and atenolol. The moderately removable pharmaceuticals are naproxen and caffeine, while the poorly removable pharmaceuticals include sulfamethoxazole, clofibrilic acid and carbamazepine. Nevertheless, it is not feasible to compare the performance of different types of constructed wetlands due to the limited amount of data on the removal efficiencies for each type of constructed wetland to date.

Breitholtz et al. (2012) studied four full-scale hybrid SF-CWs systems with different configurations for the removal of pharmaceutical contaminants and observed that some pharmaceuticals in the effluent water were at higher levels than those in the influent water (Table 2-4). Such a phenomenon might be attributable to the measurement variability resulted from the occurrence of pharmaceuticals close to the limit of detection and the daily fluctuations of pharmaceutical concentrations caused by intermittent loading. Moreover, it could also be explained by the mechanism that certain pharmaceuticals were metabolized to glucuronides or other conjugated metabolites and then converted back to the parent compounds by enzymatic processes after a period (Breitholtz et al. 2012).

Dordio et al. (2009a, 2010) observed that more than half of the initial level of pharmaceuticals (atenolol, carbamazepine, clofibrilic acid and ibuprofen) was eliminated during the first 6 hours and in the next period of 6-96 hours the first-order kinetics equation fitted well the pattern of pharmaceutical removal. Matamoros et al. (2008b) found that their target pharmaceuticals (carbamazepine, diclofenac, flunixin, ibuprofen, and ketoprofen) were removed with higher efficiencies during the first part of the system in comparison with that in the latter part, showing a spatial trend of the removal efficiencies.

Table 2-4. Efficiencies of pharmaceuticals removed by constructed wetlands working as a wastewater polishing process

Pharmaceutical compounds	SF-CW	HSSF-CW	VSSF-CW	Hybrid CW system
Analgesic/ anti-inflammatory				
Acetaminophen		45 (12)		
Buprenorphine				-2956 to 58 (7)
Codeine		57 (12)		29-75 (7)
Diclofenac	85 (1); 3 (2); 73-96 (3)	5 (12)	78-79 (6)	24-36 (7); 86-98 (8); 38-85 (9); 62 (10); 5 (13)
Ibuprofen	96 (1); 19 (2); 95-96 (3)	82-96 (4); 28 (12)	66-72 (6)	5-88 (7); 79-97 (8); 55 (10); 59 (11); 92 (13)
Indomethacin		45 (12)		
Ketoprofen	98 (1); 97-99 (3)	18(12)		3-56 (16); 97-99 (8)
Mefenamic acid		20 (12)		
Naproxen	72 (1); 4 (2); 52-92 (3)	36 (12)	39-45 (6)	34-75 (7); 72-96 (8); 71-82 (9); 67(10)
Phenylbutazone		55 (12)		
Propyphenazone		-19 (12)		
Orphenadrine				-19 to 54 (7)
Salicylic acid		7 (12)		
Tramadol				-12 to 26 (7)
Antiallergic drugs				
Desloratidin				47-72 (7)
Diphenhydramine				46-50 (7)
Fexofenadine				-26 to 28 (7)
Antibiotics				
Azithromycine		86 (12)		-350 to 2 (7)
Ciprofloxacin		67 (12)		
Clarithromycine		7 (12)		-58 to 58 (7)

Table 2-4 (Continued)

Pharmaceutical compounds	SF-CW	HSSF-CW	VSSF-CW	Hybrid CW system
Clindamycine				-103 to 13 (7)
Enoxacin		38 (12)		
Erythromycin		-78 (12)		
Metronidazole		100 (12)		
Nifuroxazide		100 (12)		
Norfloxacin		52 (12)		
Ofloxacin		84 (12)		
Roxithromycin		-43 (12)		
Spiramycin		58 (12)		
Sulfadiazine		-17 (12)		
Sulfamethazine		100 (12)		
Sulfamethoxazole		16 (12)		-104 to 12 (7); 28-53 (9); 23 (10); 13 (13)
Tilmicosin		51 (12)		
Triclosan	50 (2)			74-93 (8); 67-99 (9)
Trimethoprim		38 (12)		25-86 (7), -11 (13)
Antidiabetics				
Glibenclamide		23 (12)		
Glimepiride				20 (10)
Antidysenterics				
Loperamide				38 (7)
Antifungals				
Clotrimazol				-18 (7)
Fluconazole				-40 to 43 (7)
Antihypertensives				
Diltiazem				30-88 (7)

Table 2-4 (Continued)

Pharmaceutical compounds	SF-CW	HSSF-CW	VSSF-CW	Hybrid CW system
Dipyridamol				80-100 (7)
Eprosartan				24-75 (7)
Hydrochlorothiazide		63 (12)		
Ibersartan				3-58 (7)
Telmisartan				4-87 (7)
Verapamil				69-84 (7)
Anti-senile dementia drugs				
Memantin				5-32 (7)
Barbiturates				
Butalbital		42 (12)		
Pentobarbital		32 (12)		
Phenobarbital		17 (12)		
Beta-agonists				
Clenbuterol		11 (12)		
Salbutamol		100 (12)		
Terbutaline				-11 to 83 (7)
Beta-blockers				
Atenolol		93-95 (5) 48 (12)		27-53 (7); >97 (9); 77 (10)
Bisoprolol				22-36 (7)
Metoprolol		11 (12)		-3 to 30 (7)
Propranolol		38 (12)		
Sotalol		5 (12)		-21 to 17 (7)
Timolol		-8 (12)		

Table 2-4 (Continued)

Pharmaceutical compounds	SF-CW	HSSF-CW	VSSF-CW	Hybrid CW system
Diuretics				
Furosemide	52 (2)	35 (12)		
Hormone inhibitors				
Finasteride				-394 to -141 (7)
Lipid regulators				
Atorvastatin		100 (12)		4 (7)
Bezafibrate		27 (12)		-21 to 41 (7)
Clofibric acid	34 (1); 32-36 (3)	48-75 (4) 100 (12)		
Fenofibrate		100 (12)		
Gemfibrozil		22 (12)		
Mevastatin		53 (12)		
Pravastatin		52 (12)		
Rosuvastatin				-16 to 60 (7)
Psychiatric drugs				
Alprazolam				-130 to 99 (7)
Amitriptyline				6-84 (7)
Bupropion				-135 to 43 (7)
Carbamazepine	39 (1); 3 (2); 30-47 (3)	88-97 (4); -4 (12)	-0.9-26 (6)	-19 to 21 (7); 5-51 (8); -12 to 65 (9); 16 (10); -3 (13)
Citaprolam				45-97 (7)
Dilantin				7-39 (9)
Diazepam				-10 to 0 (9)
Fluoxetine		-1 (12)		
Hydroxyzine				50-75 (7)

Table 2-4 (Continued)

Pharmaceutical compounds	SF-CW	HSSF-CW	VSSF-CW	Hybrid CW system
Levomepromazine				92 (7)
Lorazepam		12 (12)		
Maprotilin				17 (7)
Mianserin				87 (7)
Mirtazapine				29-72 (7)
Oxazepam				-26 to 21 (7)
Paroxetine		100 (12)		-83 to 74 (7)
Perphenazine				13 (7)
Sertraline				0.1-94 (7)
Venlafaxine				17-65 (7)
Zolpidem				24-52 (7)
Receptor antagonists				
Alfuzosine				55-96 (7)
Cimetidine		24 (12)		
Famotidine		100 (12)		
Loratadine		29 (12)		
Ranitidine		41 (12)		-39 to 92 (7)
Stimulants/ Psychoactive drugs				
Caffeine	3 (2)			47-86 (8); 53 (10)
Naloxone				-116 to 84 (7); 83 (13)
Veterinary drugs				
Flunixin	0-64 (3)			

Notes: 1: Llorens et al. (2009); 2: Matamoros et al. (2012a); 3: Matamoros et al. (2008b); 4: Dordio et al. (2010); 5: Dordio et al. (2009a); 6: Reif et al. (2011); 7: Breitholtz et al. (2012); 8: Matamoros and Salvadó (2012); 9: Park et al. (2009); 10: Lee et al. (2011); 11: Gross et al. (2004); 12: Verlicchi et al. (2013); 13 Zhu and Chen (2014)

2.7 Removal Mechanisms Related to the Substrate, Plants and Microbes in Constructed Wetlands

2.7.1 Substrate

(1) Substrate Selection

Substrate, which is also known as the support matrix, is an important component in constructed wetlands, more so in subsurface flow constructed wetlands. The substrate in a constructed wetland not only provides support for the growth of plants and microbes, but also interacts directly with pollutants through sorption. The physical and chemical characteristics of substrate include mineralogical composition, granulometric and hydraulic properties, acid-base and surface charge properties, content of organic matter, and sorptive properties (Dordio and Carvalho 2013). These characteristics determine the partitioning of the pollutants between the water and the solid phase, and also determine the type of interactions between the pollutants and the substances on the solid surface (Dordio and Carvalho 2013). Therefore, selecting appropriate substrate is of significant importance to enhance the performance of constructed wetlands for pollutant removal.

Selection of substrate for constructed wetlands is based on certain important parameters including the mechanical and chemical resistance, size distribution and sorptive capacity. The mechanical and chemical resistance of a substrate should be able to withstand the operation conditions including the flow rate and the chemical compositions of wastewater, and shouldn't release contaminative matter into the wastewater or toxic substance to the plants and microorganisms (Dordio and Carvalho 2013). The size distribution of substrate should satisfy the requirements of porosity (e.g. 30 to 45% for coarse sand or gravel) (Cooper et al. 1996, Sundaravadivel and Vigneswaran 2001, Kadlec and Wallace 2009) and hydraulic conductivity (approximately 10^{-3} to 10^{-4} m/s) (Cooper et al. 1996, Vymazal et al. 1998, Sundaravadivel and Vigneswaran 2001) in the constructed wetlands. Appropriate porosity and hydraulic conductivity used in constructed wetlands can allow for a uniform distribution of influent water and a good collection of effluent water, and can also provide space for the wastewater treatment processes as well as

the gas exchange processes. Moreover, appropriate porosity and hydraulic conductivity can prevent the short-circuiting of flow or the development of channeling which could lead to poor contact of wastewater with the constructed wetland system (Netter 1994). Generally, the particle sizes of the substrate materials are in the range of 0 to 30 mm. For instance, the sizes of gravel commonly used are less than 12 mm (Vymazal et al. 1998, Sundaravadivel and Vigneswaran 2001). In addition, there are two important criteria to assess the size distribution of substrate including the effective particle size (D_{10} , normally greater than 0.3 mm) (Vymazal et al. 1998) and the uniformity coefficient (D_{60}/D_{10} , normally larger than 4) (Vymazal et al. 1998). The sorptive capacity of substrate should be suitable for the removal of target contaminants from wastewater. Sorption of pollutants onto the surface of substrate involves different mechanisms such as hydrophobic partitioning, electrostatic interaction, van der Waals interaction, ion exchange and surface complexation (Tolls 2001, Haberl et al. 2003, Reddy and DeLaune 2008, Pei et al. 2012, Dordio and Carvalho 2013). Non-polar organic pollutants can be preferentially adsorbed to the substrate materials which are rich in organic matter such as soil, compost and agricultural wastes through hydrophobic process. Polar or ionic pollutants are dominantly adsorbed to the substrate materials (e.g. some types of clay) via electrostatic interactions or ionic exchange.

(2) Substrate Materials

A wide variety of substrate materials has been used in constructed wetlands for wastewater treatment. The popular substrate materials are gravel, sand, crushed rock, gravel/sand mixture and soil. According to Table 2-1 and Table 2-3, the frequently used substrate for the removal of pharmaceutical is gravel. Gravel has most been found to be efficient for sorption of pharmaceuticals that are refractory to biodegradation but with relative high hydrophobicity, for example, carbamazepine (Hijosa-Valsero et al. 2011c). However, there are scarce data at present to derive in-depth understanding of the sorption performance of gravel and the associated mechanisms for pharmaceutical removal in constructed wetlands.

It has been reported in some laboratory batch experiments that light expanded clay aggregate (LECA) is a good sorbent for the acidic pharmaceutical compounds (e.g. clofibric acid and ibuprofen) and neutral pharmaceutical compounds (e.g. carbamazepine) (Dordio et al. 2009c). Dordio et al. (2007) compared three different substrate materials (LECA, expanded perlite and sand) for the removal of clofibric acid from water. Their experimental results indicated that LECA appeared to have a high sorption capacity to remove clofibric acid, while the expanded perlite only showed a limited sorption capacity and the sand did not exhibit any sorption capacity at all. Considering the alkaline nature of LECA, electrostatic interactions in the case of the acidic pharmaceuticals have been hypothesized to account for the affinities of these compounds towards the surface of LECA. Regarding the neutral compounds, van der Waals interactions might be more relevant. Dordio et al. (2009a) observed that LECA also exhibited a strong sorption capacity for atenolol which was alkaline and positively charged. Electrostatic interactions are not able to explain such a phenomenon, but ion exchange might be responsible to some extent for the removal of atenolol by LECA.

Soil is another type of common substrate material used in constructed wetlands. Hussain et al. (2012) carried out some laboratory batch experiments to study the removal efficiencies of three antibiotics including monensin, salinomycin and narasin in two SF-CWs filled with different soil substrates. Higher removal efficiency was found for sandy soil compared to that for sandy clay loam soil. These authors explained that the higher relative hydraulic conductivity of the sandy soil could favour water movement in the soil matrix causing a greater opportunity of soil-to-contaminants interactions. On the other hand, the sandy clay loam soil that contained higher organic matter content exhibited a lower infiltration rate within the soil medium. However, their research results did not imply that the sorptive capability of the sandy soil was better than that of the sandy clay loam soil. Lertpaitoonpan et al. (2009) demonstrated that higher coefficient values (K_d) could be observed for soils with higher organic carbon in the batch sorption process of sulfamethazine onto five soils (organic carbon content was in the range of 0.1 to 3.8%).

Recently, certain biosorbents such as rice husk, granulated cork and pine bark have been considered as alternatives to the common substrate materials in constructed wetlands attributable to their low cost, easy disposal by incineration and economical value of reuse. Dordio et al. (2011c) investigated the sorption capacity of granulated cork for the removal of ibuprofen, carbamazepine and clofibric acid from water. Their laboratory experimental results showed that the granulated cork exhibited a good sorption quality and the three target pharmaceuticals were removed in the order as: ibuprofen > carbamazepine > clofibric acid. In comparison with LECA, granulated cork exhibited a much larger specific sorption capacity for all the target pharmaceuticals.

(3) Sorption Kinetics and Isotherms

Sorption kinetics and isotherms have been investigated to describe the sorption process of pharmaceuticals to certain kinds of sorbent materials. Dordio et al. (2009a, 2010, 2011c) found that more than half of the initial atenolol, carbamazepine, clofibric acid and ibuprofen were able to be removed through adsorption during the initial period of 6 hours. Their results indicated that the Freundlich equation modelled the data better than the Langmuir equation. This observation implied the significant role of the heterogeneity of sorbent material and the possible lateral interactions among the sorbed molecules. Xu et al. (2009) also observed that the Freundlich equation could describe well the adsorption process of pharmaceutical compounds in agricultural soils. Degradation of their target pharmaceutical compounds followed first-order exponential decay kinetics with an adsorption affinity order as followed: triclosan > clofibric acid > naproxen > diclofenac > ibuprofen. Yu et al. (2013) confirmed that the Freundlich equation and the first order decay kinetics fitted well the adsorption process of carbamazepine, triclosan and gemfibrozil to three different types of sorbents including Palmdale sand, Imperial Valley clay and Washington Palouse loam.

(4) Substrate pH

The pH of substrate materials could play a significant role in the sorption capacity of substrate. Vasudevan et al. (2009) found that the overall sorption efficiency of

ciprofloxacin could be affected by a difference in pH. Hussain and Prasher (2011) found an inverse correlation between the pH of substrate and the sorption efficiency of three antibiotics (monensin, salinomycin, and narasin). Sassman and Lee (2007) also observed an inverse relationship between the pH of soil and the sorption coefficient of lasalocid and monensin. Lertpaitoonpan et al. (2009) explained that soil pH inversely affected sorption process of sulfamethazine due to the different ionization states of sulfamethazine under different pH values. When the pH was less than 7.4, hydrophobic partitioning possibly accounted for the removal of the non-ionized form of sulfamethazine. When pH was above 7.4, the decrease in sorption of sulfamethazine was probably attributable to the predominance of the anionic form of sulfamethazine. A similar trend was also obtained for sulfonamides where the conversion of the neutral/cationic form of sulfonamides to the anionic form above pH 7.5 resulted in a lower sorption to soils (Kurwadkar et al. 2007).

(5) Competitive Sorption

Competitive sorption phenomenon may occur if multiple pharmaceutical compounds and some other wastewater contaminants are present in the water at the same time (Dordio et al. 2009c, Conkle et al. 2010). The sorption capacity of a pharmaceutical compound may be reduced by another pharmaceutical compound or/and other wastewater contaminants due to the competition for preferred binding sites on the solid matrices in constructed wetlands. Thus, such a competition effect may be of concern in constructed wetlands when loading with a wide range of pharmaceutical compounds.

2.7.2 Plants

(1) Plant Uptake and Metabolism

Plants in constructed wetlands play an important role in direct uptake of many organic contaminants from wastewater. The organic contaminants pass through the root epidermis, cortex and endodermis to reach the root xylem of plants (Tanoue et al. 2012, Dordio and Carvalho 2013). The Casparian strip is an impervious layer surrounding the root endodermis that prevents pollutants from passing through the

root endodermis via apoplastic flow. Therefore, the pollutants must pass through the selectively permeable membranes in the cells of root endodermis via symplastic route to the root xylem (Tanoue et al. 2012). In the cell membranes of the root endodermis, there may not exist specific transporters for the xenobiotic organic compounds (e.g. pharmaceuticals) to move into the plants tissues, but the uptake and translocation of such compounds within plants can be simply driven by diffusion (passive uptake) (Dietz and Schnoor 2001, Dordio et al. 2011a, Dordio and Carvalho 2013).

The diffusion process of pharmaceutical compounds into plant tissues is dependent on the physicochemical characteristics of the compounds such as hydrophobicity (logarithm octanol/water partition coefficient: $\log K_{ow}$), water solubility and concentration (Stottmeister et al. 2003, Dordio and Carvalho 2013). Normally, the pharmaceutical compounds with a $\log K_{ow}$ of 1 to 4 are lipophilic enough to travel through the lipid bilayer of plant cell membranes and also water soluble enough to move into the cell fluids of plants, indicating a high bioavailability to plants (Dietz and Schnoor 2001, Schröder and Collins 2002, Stottmeister et al. 2003, Dordio and Carvalho 2013). For example, carbamazepine with a $\log K_{ow}$ of 2.45 has been reported to be easily taken up by the roots of *Typha spp.* and then transported from roots to stems and leaves, finally showing the largest level of accumulation in leaves (Dordio et al. 2011c). On the contrary, diclofenac whose $\log K_{ow}$ of 4.51 is higher than 4 exhibited a limited ability to be taken up and translocated within the roots and shoots of *Scirpus validus* (Zhang et al. 2012c).

Regarding the nonionizable, polar, highly water soluble organic compounds, Dettenmaier et al. (2009) observed that caffeine could be taken up by plant roots and translocated to shoots, consistent with the findings of Matamoros et al. (2012b) and Zhang et al. (2013a). Liu et al. (2013b) found a similar phenomenon for ciprofloxacin HCl, oxytetracycline HCl and sulfamethazine which have the $\log K_{ow}$ below 1. The authors explained that translocation of such organic compounds were most likely to be driven by the transpiration process. Furthermore, they also observed the positive relationship between the concentrations of pharmaceuticals

and the accumulation levels inside the plants. Their experimental results showed that the total accumulation level of ciprofloxacin HCl, oxytetracycline HCl and sulfamethazine in *Phragmites australis* were 13834, 6901 and 2047 ng/g dry weight respectively at the source concentration of 1000 µg/L in comparison to 345, 165 and 24 ng/g dry weight respectively at the source concentration of 0.1 µg/L.

After being taken up into plants tissues, the translocated pharmaceutical compounds may be degraded through the metabolism processes (known as phytodegradation). The metabolism processes might involve some biochemical reactions including transformation of parent organic compounds, conjugation of metabolites with macromolecules, and incorporation of conjugated products into plant cell walls and vacuoles (Reinhold et al. 2010, Dordio and Carvalho 2013). Dordio et al. (2011b) detected a metabolite (10, 11-dihydro-10, 11-epoxycarbamazepine) of carbamazepine in the leaf tissues of *Typha spp.* demonstrating the occurrence of carbamazepine metabolism within the plant tissues. Liu et al. (2013b) observed the conversion of ciprofloxacin HCl and oxytetracycline HCl to their epimers such as tetracycline, enrofloxacin, chlortetracycline and ofloxacin in plants.

(2) Plant Rhizosphere

Plants in constructed wetlands play another significant role in the development of microbial population which can be supported by the rhizodeposition products such as exudates, dead cell material and mucigels, inducing various biological processes in the rhizosphere (Cronk 1996, Stottmeister et al. 2003, Calheiros et al. 2009). Matamoros et al. (2012b) found that the predominant removal process of ibuprofen was microbial degradation which was possibly related to the biofilms attached to the surface of plant roots. In addition, certain plant exudates may also work as catalytic agents for the degradation of organic compounds (Dordio and Carvalho 2013).

Most of the plant species used in constructed wetlands are able to release oxygen around root tips and on young laterals (Stottmeister et al. 2003). An oxidative protective film with a thickness of 1 to 4 mm may be formed on the root surface to

protect sensitive areas of roots from being damaged by the toxic components of wastewater (Stottmeister et al. 2003). The oxygen released in rhizosphere can also favour the oxidative transformation of pollutants in wastewater (Sundaravadivel and Vigneswaran 2001) and promote the development of aerobic microbes in the rhizosphere causing more efficient biodegradation of organic contaminants (Cronk 1996, Dordio and Carvalho 2013). The continuous release of oxygen from the roots of plants may counterbalance the chemical and biological oxygen consumption in the rhizosphere.

(3) Influence on Pharmaceutical Removal

The presence of plants in constructed wetlands played a positive role in the removal of pharmaceutical compounds such as amoxicillin, ampicillin, erythromycin, diclofenac, caffeine, carbamazepine, clofibric acid, ibuprofen, ketoprofen, naproxen, salicylic acid, sulfadiazine, sulfamethazine and sulfamethoxazole (Dordio et al. 2009a, Dordio et al. 2010, Hijosa-Valsero et al. 2010b, Xian et al. 2010, Hijosa-Valsero et al. 2011a, Hijosa-Valsero et al. 2011b, Hijosa-Valsero et al. 2011c, Zhang et al. 2011, Zarate Jr et al. 2012, Zhang et al. 2012a, Zhang et al. 2012b).

For the popular vegetation--*Typha spp.* and *Phragmites spp.* used in constructed wetlands, the experiment results of Hijosa-Valsero et al. (2010b, 2011c) indicated that *Phragmites australis* showed a better performance than *Typha angustifolia*, although the *Typha* wetland bed exhibited a more ideal plug flow behaviour than the *Phragmites* wetland bed as reported by Ranieri et al. (2011). In contrast, Dordio et al. (2009a) found that *Typha spp.* was superior to *Phragmites australis* for pharmaceutical removal, attributable to the higher transpiration rate of *Typha spp.* Zarate Jr et al. (2012) studied the accumulation of triclosan within plant tissues including roots and shoots, and their experimental results showed species-specific differences among different plants such as *Pontederia cordata* and *Sagittaria graminea*, indicating the significant impacts of plant species on the removal of pharmaceuticals.

(4) Toxicity of Pharmaceuticals to Plants

Toxicity to plants caused by exposure to pharmaceutical compounds can be evaluated by analyzing the root activity, the relative growth rate of plant ($RGR = (\ln W_1 - \ln W_0)/(t_1 - t_0)$, where W_0 and W_1 are the initial and final weights of plant respectively and $(t_1 - t_0)$ is the duration time of experiment), and the concentrations of photosynthetic pigments such as carotenoid and chlorophyll in plants tissues. Liu et al. (2013b) assessed the potential effects of ciprofloxacin HCl, oxytetracycline HCl and sulfamethazine on *Phragmites australis* at concentrations of 0.1, 1, 10, 100 and 1000 $\mu\text{g/L}$ in hydroponic solution. Their experimental results illustrated that a toxic effect on root activity and leaf chlorophyll could be observed when at high concentrations ($> 10 \mu\text{g/L}$). Dordio et al. (2009b) observed that RGRs of the *Typha spp.* plants exposed to clofibric acid at spiked concentrations of 0.5, 1.0 and 2.0 mg/L were higher than that of the control. They attributed this phenomenon to the fact that the *Typha spp.* plants exposed to clofibric acid had a much more developed root system than that of the control plants, implying the stimulation effects of clofibric acid on the root growth of *Typha spp.* These authors also found no significant differences between the concentrations of photosynthetic pigments in the plants exposed to clofibric acid than those in the control plants. However, when Dordio et al. (2011b) used *Typha spp.* to remove carbamazepine in an aqueous medium, the RGRs of *Typha spp.* plants exposed to carbamazepine at concentrations of 0.5, 1.0 and 2.0 mg/L were significantly below those of the control plants in both the initial 7 days and even after 14 days. However, a distinct trend of approaching the average RGR level of the control plants was observed after 21 days. Their observation indicated that the initial presence of carbamazepine negatively affected the plants' normal growth, but the plants appeared to be able to tolerate the chemical after a period of adaptation. Dordio et al. (2011a) also found a similar phenomenon for the RGRs of *Typha spp.* plants in hydroponic solution spiked with ibuprofen concentrations of 1.0 and 2.0 mg/L . These authors further observed that no significant differences existed in the carotenoid and chlorophyll b of the plants exposed to ibuprofen at all concentrations (0.5, 1.0 and 2.0 mg/L) from those of the control plants. However, significant differences were found between the chlorophyll a and total chlorophyll of the plants exposed to ibuprofen at a

concentration of 2.0 mg/L as compared to the control plants, implying a sign of toxicity.

In addition to the RGR and the photosynthetic pigments, the alteration of antioxidant enzyme activities in plant tissues can also serve as an indication of the phytotoxicity caused by pharmaceuticals. Xenobiotics like pharmaceutical compounds can induce an over-production of reactive oxygen intermediates (ROIs) which have the potential to damage biomolecules such as lipids, proteins and DNA and can ultimately lead to cell death (Mittler 2002). The capacity of plants to tolerate the oxidation burst caused by the over-production of ROIs is related to the activation of the antioxidant enzymatic system to scavenge and eliminate the ROI excess. The antioxidant enzymes like catalase (CAT), superoxide dismutase (SOD), guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) have been reported for the plants which were subjected to different sources of abiotic stress (drought, excessive light and xenobiotic) (Mittler 2002). Increased activities of the antioxidant enzymes measured in the study of Dordio et al. (2009b) indicated that clofibric acid induced an oxidative burst in the tissues of *Typha spp.* (roots and leaves), but the antioxidant enzymatic system appeared able to cope with it. Dordio et al. (2011b) also demonstrated that the expected production of excessive ROIs induced by exposure to carbamazepine could be effectively counteracted by the antioxidant enzymatic system. Regarding the study on the effects of ibuprofen on the antioxidant enzymatic system of *Typha spp.*, Dordio et al. (2011a) observed a continuous increase in the activity of superoxide dismutase in the roots and leaves of *Typha spp.* when the injection concentrations of ibuprofen increased. For the activities of catalase and guaiacol peroxidase in the roots and leaves of *Typha spp.*, an increase was observed at the ibuprofen concentration of 0.5 mg/L and a decrease occurred at the ibuprofen concentration of 1.0 mg/L both with a higher level than that of the control plants. However, a continuous decrease was observed at the ibuprofen concentration of 2.0 mg/L with a lower level than that of the control plants, revealing some inhibition effects induced by ibuprofen. Liu et al. (2013b) found that the activity of peroxidase in leaf tissues significantly increased with the increasing concentrations of antibiotics ($> 0.1 \mu\text{g/L}$) and then slightly declined

when the concentrations of antibiotics $> 10 \mu\text{g/L}$. However, the activities of catalase and superoxide dismutase in the leaf of *Phragmites australis* declined significantly as the dosages of target antibiotics increased, indicating the potential toxicity of antibiotics to these two kinds of antioxidants enzymes.

2.7.3 Microbes

(1) Biodegradation Mechanism

Microbes in constructed wetlands generally play a main part in the transformation and mineralization of nutrients and organic pollutants (Sundaravadivel and Vigneswaran 2001, Kadlec and Wallace 2009, Truu et al. 2009, Zhu et al. 2010, Zhu et al. 2011, Zhu et al. 2013). Biodegradation of organic compounds by microbes in constructed wetlands may take place in both the aerobic and anaerobic environments involving the activities of various kinds of microorganisms, for example, heterotrophic bacteria, autotrophic bacteria, fungi (basidiomycetes and yeasts) and specific protozoa (Kadlec and Wallace 2009).

One of the important factors that would influence strongly the microbial degradation process is the chemical structures of organic compounds (Dua et al. 2002, Reddy and DeLaune 2008). For those with simple structures which possess high water solubility and low adsorptivity, the organic compounds can be easily degraded by microorganisms. It is due to the similarity of these organic compounds to the naturally occurring compounds that are usually used as energy sources by microorganisms. For the xenobiotic organic compounds like pharmaceuticals which have the different structures from the naturally occurring compounds, they may be slowly degraded by microbes due to the lack of suitable genes for degradation in microbes. However, this does not mean that non-specific enzymes are not able to degrade xenobiotic organic compounds. The degradation of such organic compounds by non-specific enzymes may still take place at a slower rate through co-metabolism reactions that do not necessarily support the growth of microbes (Seffernick and Wackett 2001). Degradation of xenobiotic organic compounds by microbes in constructed wetlands may also be affected by the temperature, pH,

oxygen and redox potential, substrate, vegetation, nutrient availability and presence of toxic substances (Reddy and DeLaune 2008, Calheiros et al. 2009, Salomo et al. 2009, Truu et al. 2009, Li et al. 2010).

According to the literature, aerobic biodegradation is thought to be the main contributor to the microbial degradation of ibuprofen (Matamoros et al. 2005, Matamoros et al. 2008a, Ávila et al. 2010, Hijosa-Valsero et al. 2010a, Hijosa-Valsero et al. 2010b, Hijosa-Valsero et al. 2011c, Reyes-Contreras et al. 2012, Ávila et al. 2013), salicylic acid (Hijosa-Valsero et al. 2010b, Hijosa-Valsero et al. 2011c, Reyes-Contreras et al. 2012) and sulfamethoxazole (Hijosa-Valsero et al. 2011a), while anaerobic biodegradation is thought to be responsible for naproxen (Ávila et al. 2010) and caffeine (Hijosa-Valsero et al. 2010b, Hijosa-Valsero et al. 2011c, Reyes-Contreras et al. 2012). However, for the biodegradation of diclofenac, there are two different opinions as follows: 1) high redox potential could promote degradation of diclofenac (Hijosa-Valsero et al. 2010b, Hijosa-Valsero et al. 2011c, Ávila et al. 2013); and 2) the removal efficiency of diclofenac could be enhanced in anaerobic environments (Ávila et al. 2010). All the removal mechanisms presented above were inferred from the published studies and have not been explicitly demonstrated. Therefore, at present, it is still difficult to describe the actual removal mechanisms of pharmaceutical compounds in constructed wetlands.

(2) Enantioselective Degradation

Certain pharmaceutical compounds such as ibuprofen and naproxen that contain an asymmetric carbon atom (chiral carbon atom) usually have two enantiomeric forms (S_{form} and R_{form}). The enantiomeric fraction ($EF = S_{\text{form}} / (S_{\text{form}} + R_{\text{form}})$) (Matamoros et al. 2009b, Hijosa-Valsero et al. 2010a) which is compound-dependent can be used to evaluate the occurrence of enantioselective degradation in wastewater treatment system by studying the change in the trend of EF. A change in the trend of EF during wastewater treatment indicates that the enantioselective degradation of target pharmaceutical occurs mainly attributable to biodegradation processes since the enantioselective degradation is not expected for the processes of sorption and photodegradation (Wong 2006).

Enantioselective degradation of ibuprofen in VSSF-CW was reported by Matamoros et al. (2009b) that the S_{form} ibuprofen was degraded faster than the R_{form} ibuprofen under aerobic conditions resulting in the reduction of EF. Such a phenomenon was consistent with the experimental results of Hijosa-Valsero et al. (2010a). However, EF didn't change under anaerobic conditions (e.g. in HSSF-CW) due to the similar degradation rates of S_{form} ibuprofen and R_{form} ibuprofen. Owing to the different EF trends of ibuprofen under aerobic and anaerobic conditions, it can be inferred which kind of biodegradation pathway (aerobic biodegradation or anaerobic degradation) plays a predominant part in the elimination of ibuprofen in constructed wetlands. For naproxen, Matamoros et al. (2009b) observed that the naproxen EF decreased in both prevailing aerobic and anaerobic conditions (e.g. in hybrid CW systems) without significant differences. Their findings implied similar enantioselective degradation kinetics of naproxen under aerobic and anaerobic conditions.

(3) Metabolites

Evaluation of the biodegradation metabolites of pharmaceutical compounds in constructed wetlands is of great importance to elucidate the microbial degradation mechanisms, but available data are very limited to date. Matamoros et al. (2008a) investigated the behaviour of two metabolites of ibuprofen including hydroxy-ibuprofen (OH-IBP) and carboxy-ibuprofen (CA-IBP) in a HSSF-CW system to assess the relative contribution of aerobic and anaerobic pathways to the biodegradation of ibuprofen. Their experimental results indicated that the OH-IBP and CA-IBP only contributed to 5% of the degraded IBP and the accumulation of these two metabolites in the microcosms was negligible. The observation was possibly attributed to the similar kinetics of their consumption and formation. However, both OH-IBP and CA-IBP can be produced in aerobic environments and only CA-IBP can be detected under anaerobic conditions (Zwiener et al. 2002). By analyzing the removal percentage of ibuprofen attributable to the aerobic and anaerobic pathways using Equation 2.1 and Equation 2.2, the authors suggested that the aerobic biodegradation pathways was predominant for ibuprofen removal.

$$\text{Aerobic - ratio} = (C_{\text{OH-IBP}} + xC_{\text{CA-IBP}})/(C_{\text{OH-IBP}} + C_{\text{CA-IBP}}) \quad (2.1)$$

$$\text{Anaerobic - ratio} = yC_{\text{CA-IBP}}/(C_{\text{OH-IBP}} + C_{\text{CA-IBP}}) \quad (2.2)$$

Where:

$C_{\text{CA-IBP}}$ -- Concentration of CA-IBP

$C_{\text{OH-IBP}}$ -- Concentration of OH-IBP

x -- Percentage of CA-IBP obtained in the aerobic pathways, 0.4%

y -- Percentage of CA-IBP obtained in the anaerobic pathways, 1.8%

(4) Influence of Pharmaceuticals on Microbes

Enriching pharmaceutical pollutants into constructed wetlands may influence the activities and development of microbes in constructed wetlands. Weber et al. (2011) investigated the influence of ciprofloxacin on the distribution of bacterial communities in several mesocosm-scale constructed wetlands which were planted with *Phragmites australis*. Their findings showed that the presence of ciprofloxacin caused an initial adverse effect on the bacterial communities in the constructed wetlands, thereby weakening their ability to assimilate anthropogenic carbon-based compounds. However, after an acclimation period of 2-5 weeks, the bacterial communities recovered and returned to their normal functionality. Helt et al. (2012) assessed the impacts of ciprofloxacin on the antibiotic resistance of the interstitial bacterial community (total culturable heterotrophs) and selected faecal indicators (*Escherichia coli* and *Enterococcus* spp.) in constructed wetlands with the same design as those used by Weber et al. (2011). Helt et al. (2012) observed that the antibiotic resistance level of the interstitial bacteria community peaked at 7 days following the ciprofloxacin exposure and decreased thereafter, and also found that the antibiotic resistance level of the faecal indicators increased.

2.8 Conclusions

Constructed wetlands have been attracting growing attention to their application for the removal of pharmaceutical contaminants from wastewater. Based on the published literature on the feasibility of constructed wetlands as a means to treat pharmaceutical contaminants in wastewater, one can conclude that constructed

wetlands hold great potential of being used as an alternative secondary wastewater treatment system or as a wastewater polishing process. Nevertheless, it is true that little data to date exist to provide persuasive demonstration of the performance and effectiveness of constructed wetlands in these applications. There are knowledge gaps in several aspects including the removal efficiencies of pharmaceuticals in constructed wetlands, the removal mechanisms involved, the toxicity to constructed wetlands caused by pharmaceuticals and the influence of certain important parameters (configuration design, hydraulic mode, temperature and seasonality, oxygen and redox potential, pH, etc.). Moreover, most studies were conducted at a microcosm-scale or mesocosm-scale. Thus, further research work is required to provide more comprehensive and convincing evidence in larger laboratory-scale, pilot-scale or full-scale constructed wetlands. In addition, current constructed wetlands applied for the treatment of pharmaceutical pollutants in wastewater are mainly the conventional types of constructed wetland including SF-CWs, HSSF-CWs, VSSF-CWs and hybrid CW system. Several novel constructed wetlands such as tidal flow constructed wetlands, anti-sized constructed wetlands and the dewatered alum sludge based constructed wetlands can also be investigated.

CHAPTER 3 MATERIALS AND METHODS

3.1 Experimental Wetland System

A mesocosm-scale horizontal subsurface flow constructed wetland (HSSF-CW) system was set up at Nanyang Technological University (NTU) in Singapore. The wetland system was installed in a shelter environment which included a clear roof and netting on all sides. The system consisted of a wastewater holding tank and two HSSF-CW beds (Figure 3-1). The wastewater holding tank which was made of polyvinyl chloride (PVC) plastic had a volume of 200 L (1000 mm in depth and 500 mm in diameter) and was covered with a fabric mesh to maintain aerobic condition. The two HSSF-CW beds were made of fiberglass and were identical in dimensions and configuration (Figure 3-2a). One of the beds was planted with *Typha angustifolia* while the other was unplanted and used as a control. The wetland bed was 1200 mm long, 600 mm wide and 600 mm deep, and filled with a 300 mm-depth layer of gravel with an initial porosity of 45%.

Figure 3-1. Site picture of the HSSF-CW system

There were three functional sections in the wetland bed: inlet section (gravel size: 20-24 mm), treatment section (gravel size: 4-10 mm), and outlet section (gravel size: 20-24 mm). The influent water was pumped from the wastewater holding tank onto the surface of inlet section through ether-based polyurethane pressure tubing (3.2 mm in inner diameter, Cole-Parmer, USA). The effluent water was channelled to a PVC collection pipe (50 mm in inner diameter and 400 mm in length) located at the bottom of the outlet section. Water level in the mesocosm was kept 50 mm below the gravel surface by using water level control pipe (25 mm in height) which was a standing PVC pipe located near to the outlet of the wetland bed. The emergent macrophyte *Typha angustifolia* in the planted bed was planted with an initial density of 15 plants/m².

(a)

Figure 3-2. Schematic sketch of the layout of mesocosm-scale HSSF-CW system (a) and positions of sampling pipes in the treatment section of wetland bed from the plan view (b) and the longitudinal view (c). Unit: mm

In the treatment section, two perforated PVC pipes (20 mm in inner diameter) were vertically inserted in the gravel layer 300 mm apart (Figures 3-2b and 3-2c). These two pipes named as sampling pipe I and sampling pipe II were used to collect the interstitial water and sediment mixture samples at the $1/3$ length location ($1/3L$) and the $2/3$ length location ($2/3L$) of the treatment section along the flow path. Thus, the treatment section was essentially divided into three zones by the two sampling pipes: upstream $1/3$ zone (from inlet to the $1/3L$), middle $1/3$ zone (from $1/3L$ to $2/3L$) and downstream $1/3$ zone (from $2/3L$ to outlet). Moreover, these two open-

top sampling pipes were able to work as ventilation pipes to enhance aeration for the wetland bed to some degree.

3.2 Chemicals

For the synthesis of wastewater, glucose (anhydrous), ammonium sulfate (99+%), potassium dihydrogen phosphate (98+%), magnesium sulfate heptahydrate (99+%), calcium chloride dihydrate (99-105%), sodium carbonate (98%), and sodium hydrogen carbonate (99%) were purchased from Alfa Aesar (Singapore). Ibuprofen (IBP) was supplied by Sigma-Aldrich (Singapore).

In order to perform internal standard calibration, 2-hydroxy ibuprofen (2-OH IBP), 1-hydroxy ibuprofen (1-OH IBP) and ibuprofen-d₃ (IBP-d₃) were acquired from Sigma-Aldrich (Singapore), while carboxylic acid ibuprofen (CBX IBP) was provided by Toronto Research Chemicals (Canada). The physicochemical properties of IBP, CBX-IBP, 2-OH IBP and 1-OH IBP in terms of chemical structure, chemical formula, dissociation constant (pK_a) and octanol-water partition coefficient as Log K_{ow} were reported in Table 3-1.

Table 3-1. Physicochemical properties of target compounds

Compound	Chemical Structure	Chemical Formula	pK _a ^a	Log K _{ow} ^b
Ibuprofen (IBP)		C ₁₃ H ₁₈ O ₂	4.91	3.97
Carboxylic acid ibuprofen (CBX-IBP)		C ₁₃ H ₁₆ O ₄	3.97	2.78
2-hydroxy ibuprofen (2-OH IBP)		C ₁₃ H ₁₈ O ₃	4.63	2.37
1-hydroxy ibuprofen (1-OH IBP)		C ₁₃ H ₁₈ O ₃	4.55	2.69

^a Dissociation constant. ^b Octanol-water partition coefficient.

Data sources: ChemIDplus Advanced database (<http://chem.sis.nlm.nih.gov/chemidplus/>) and software Marvin Sketch 14.11.3.0.

For the solid phase extraction (SPE), sea sand extraction (SSE) and liquid chromatography-tandem mass spectrometry (LS-MS/MS), HPLC-grade methanol, n-hexane, hydrochloric acid (37%) and ammonium acetate 5 M solution (molecular biology grade) were bought from Merck (Singapore).

For the bacteria analysis using the method of polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE), 2X Go Taq Master Mix, 25 mM MgCl_2 , nuclease-free water and urea were provide by Promega (Singapore). 40% Acrylamide/Bis (37.5:1) and Formamide were purchased from Bio-Rad (Singapore).

3.3 Operation and Sampling

After the HSSF-CW system had been developed, acclimation of the *Typha angustifolia* plants in the mesocosms took about two weeks during which the plants were irrigated with tap water every other day. Then, the two wetland beds were continuously fed with the synthetic wastewater (without IBP) from the wastewater holding tank at a flow rate of 7.64 mL/min for 30 days. The hydraulic retention time (HRT) of the wetland system was about 4 days. The components of the synthetic wastewater were designed as 300 mg/L chemical oxygen demand (COD), 27 mg/L ammonium nitrogen (NH_4^+ -N) and 18 mg/L total phosphate (TP).

From the 30th day onward, the influent water was spiked with IBP at a concentration of 100 $\mu\text{g/L}$. Sampling campaign began on the 30th day after the system start-up. The sampling locations included the inlet (wastewater holding tank), 1/3L (sampling pipe I), 2/3L (sampling pipe II) and outlet in both the planted and unplanted beds. Once every 4 days, a 40 mL of water sample was collected from each sampling location to test the conventional parameters such as temperature (Temp), pH, electrical conductivity (Cond), dissolved oxygen (DO), oxidation reduction potential (ORP), COD, NH_4^+ -N and TP to assess the operation status of the wetland system.

After the system had adapted to the wastewater environment with IBP injection, from the 62nd day onwards, the sampling interval was changed to once every 8 days.

Besides the 40 mL of water sample for the analysis of conventional parameters, additional 50 mL of water sample was collected at each sampling location to test the concentrations of IBP and its metabolites (CBX IBP, 2-OH IBP and 1-OH IBP). Also, a 50 mL of sediment mixture was collected from the sampling pipes I and II, respectively, in both the beds for bacteria analysis. In addition, leaves of *Typha angustifolia* (one or two entire leaves) were collected from random branches of the plants around the sampling pipe I (1/3L) and sampling pipe II (2/3L), respectively. The leaf samples were separated accordingly into lamina and sheath tissues for further treatment to measure the amount of IBP and its metabolites inside.

The wetland system was continuously operated over a period of 342 days. During the main sampling period (62nd to 342nd day), the water samples and leaf samples were collected every 8 days. However, for the sediment mixture samples, the sampling campaigns were only conducted during the period of 62nd to 86th day (period 1), 126th to 150th day (period 2), 190th to 214th day (period 3), 254th to 278th day (period 4) and 318th to 342nd day (period 5). During each period, 4 sediment mixture samples were collected from the same sampling location once every 8 days. During the period 1, the 4 sediment mixture samples collected from the planted bed were labelled as P-1/3L_1-1, P-1/3L_1-2, P-1/3L_1-3 and P-1/3L_1-4 for the sampling location of 1/3L, while P-2/3L_1-1, P-2/3L_1-2, P-2/3L_1-3 and P-2/3L_1-4 for the sampling location of 2/3L. For the unplanted bed, the 4 sediment mixture samples were labelled as UP-1/3L_1-1, UP-1/3L_1-2, UP-1/3L_1-3 and UP-1/3L_1-4 for the sampling location of 1/3L, while UP-2/3L_1-1, UP-2/3L_1-2, UP-2/3L_1-3 and UP-2/3L_1-4 for the sampling location of 2/3 L. During the period 2, the 4 sediment mixture samples collected from the planted bed were labelled as P-1/3L_2-1, P-1/3L_2-2, P-1/3L_2-3 and P-1/3L_2-4 for the sampling location of 1/3L, while P-2/3L_2-1, P-2/3L_2-2, P-2/3L_2-3 and P-2/3L_2-4 for the sampling location of 2/3L. The samples collected from the unplanted bed during period 2 and the samples collected in other periods (3 to 5) can be analogized.

3.4 Analytical Methods

3.4.1 Analytical Methods for Conventional Parameters

Temperature, pH, electrical conductivity, DO and ORP were tested on site by using the HQ40d portable multi-parameter meter (Hach, Singapore). COD was determined by using the USEPA reactor digestion method with the digestion solution kit for COD (Cat. 2125815, Hach, Singapore). NH_4^+ -N was analyzed by using the salicylate method with the AmVerTM high range ammonia reagent set (Cat. 26069-45, Hach, Singapore). TP was measured by using the molybdovanadate with acid persulfate digestion method with the high range total phosphate reagent set (Cat. 27672-45, Hach, Singapore). The instruments for the analysis of COD, NH_4^+ -N and TP were the DRB200 digital reactor block and the DR 3800TM benchtop spectrophotometer (Hach, Singapore).

3.4.2 Solid Phase Extraction

The water samples (50 mL) for analysis of IBP and its metabolites were vacuum percolated through 1.2 μm glass microfiber filters (Cole-Parmer, Chicago, IL, USA) followed by 0.45 μm nylon membrane filters (Phenomenex, Torrance, CA, USA) and acidified to pH 2 with hydrochloric acid. The pretreated samples were then concentrated and cleaned with Phenomenex Strata-X (60 mg, 3 mL) cartridges (Torrance, CA, USA) for solid phase extraction (SPE). Prior to extraction, the cartridges were conditioned with 5 mL of methanol followed by 5 mL of acidic Milli-Q water (pH=2) at a flow rate of 1 mL/min. The water samples were vacuum percolated through the cartridges at a flow rate of 3 mL/min. The sample-loaded cartridges were rinsed using 5 mL of Milli-Q water and dried under vacuum for 30 min. The elution was performed with 5 mL of methanol at a flow rate of 1 mL/min. Thus, the concentration multiple of water samples was 10. The extract was filtered through 0.45 μm PTFE membrane filter (Filtrex, Singapore). Finally, 1 mL of the filtered extract was injected with 10 μL of 10 mg/L ibuprofen- d_3 for the analysis of target compounds by liquid chromatography-tandem mass spectrometry (LS-MS/MS).

3.4.3 Sea Sand Extraction

The plant leaf samples were preliminarily rinsed with dilute hydrochloric acid (1 mol/L) and additionally three times with Milli-Q water. The sea sand was washed several times with Milli-Q water followed by three rinses with methanol. Both the cleaned sea sand and plant leaves were air dried before use in laboratory (drying period: about 7 days). Thereafter, 0.5 g of dried plant leaves was placed in a quartz mortar with 2 g of sea sand and 4 mL of n-hexane. The materials were thoroughly homogenized in the mortar with pestle. The blend was then transferred into a 10 mL syringe connected with a 0.45 μ m PTFE membrane filter (Filtrex, Singapore). The packing material was covered with a circle of filter membrane (PTFE) and compressed using the syringe plunger. The filled syringe was dried under vacuum for 30 min and was eluted with 10 mL of methanol. The extract was evaporated under gentle nitrogen stream and reconstituted with 5 mL of methanol. The following filtration and analysis of the extract are the same as that mentioned in last paragraph.

3.4.4 Liquid Chromatography-Tandem Mass Spectrometry

Chromatographic separation was carried out with a Shimadzu liquid chromatography system (Kyoto, Japan) equipped with a DGU-20A₃ degasser, two LC-20AD pumps, a SIL-20AHT autosampler, a CBM-20A controller and a CTO-20AC column oven, using a Luna C18 (2) reversed phase column (75 mm $\times$ 2 mm, 3 μ m particle size) and a C18 security guard cartridge (4 mm $\times$ 2 mm) both from Phenomenex (Torrance, CA, USA). A binary mobile phase of ammonium acetate 5 mM (A) and methanol (B) was used for separation at a flow rate of 0.2 mL/min. The gradient elution was programmed as: 0-0.5 min, 5%-65% B; 0.5-6.0 min, 65%-95% B; 6.0-6.1 min, 95%-100% B; 6.1-7.0 min, 100% B; 7.0-7.1 min, 100%-5% B; 7.1-10.0 min, 5% B. Sample injection volume was 10 μ L.

The liquid chromatography instrument was coupled to a LCMS-8030 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan). Detection was performed in either positive or negative mode by multiple reaction monitoring (MRM). The

desolvation line temperature (DL temperature) was set at 250 °C, heat block temperature at 400 °C, nebulizing gas at 3 L/min and drying gas at 15 L/min. The dwell time was 60 ms. Mass parameters for MRM acquisition mode are shown in Table 3-2.

Table 3-2. Mass parameters for the analysis of target compounds in either positive or negative MRM mode

Compound	MW ^a	ESI ^b mode	RT ^c (min)	Precursor ion (m/z)	Product ion (m/z)	Q ₁ Pre Bias (V)	CE ^d	Q ₃ Pre Bias (V)
CBX-IBP	236.26	-	4.15	235.10	191.10	10	10	12
					73.00	10	15	27
2-OH IBP	222.28	+	4.67	240.30	205.10	-20	-10	-24
					163.10	-20	-20	-18
1-OH IBP	222.28	+	4.90	240.30	205.10	-20	-10	-24
					163.10	-20	-20	-18
IBP	206.10	-	6.44	205.10	161.15	16	10	30

^a Molecular weight. ^b Electrospray ionization. ^c Retention time. ^d Collision energy.

Quantification of the analytes (IBP, CBX IBP, 2-OH IBP and 1-OH IBP) in the extract from SPE and SSE was performed using an internal standard method. The internal standard method was established with a series of calibration standard solutions of target compounds at concentrations of 1000 µg/L, 500 µg/L, 100 µg/L, 50 µg/L, 10µg/L, 5 µg/L and 1µg/L. Internal standard solution of IBP-d₃ (10 mg/L) was added in each calibration standard solution at a concentration of 100 µg/L for internal standard calibration to compensate possible matrix effects. The linearity of the internal standard method exhibited good fits with a regression coefficient $r^2 > 0.99$ (Table 3-3). Detection limit (DL) and quantification limit (QL) of LC-MS/MS method for the target compounds were determined at signal to noise ratio (S/N) of 3 and 10, respectively (Table 3-3). Precision of the LC-MS/MS method in terms of repeatability (intra-day) and reproducibility (inter-day) was studied by analyzing five replicates of 100 µg/L standard solutions of target compounds (Table 3-3).

Table 3-3. Linearity, limit and precision of LC-MS/MS method

Compound	Linearity (r^2) ^a	Detection limit (DL, $\mu\text{g/L}$)	Quantification limit (QL, $\mu\text{g/L}$)	Repeatability (% RSD ^b ; n=5)	Reproducibility (% RSD; n=5)
IBP	0.9993	0.57	1.72	0.94	3.48
CBX IBP	0.9961	4.57	13.84	1.32	6.48
2-OH IBP	0.9999	6.52	19.76	1.14	1.94
1-OH IBP	0.9997	0.23	0.70	1.21	1.98

^a Regression coefficient. ^b Relative standard deviation.

Extracted ion chromatograms of a standard mixture of target compounds at 100 $\mu\text{g/L}$ are shown in Figure 3-3. CBX IBP was confirmed by the fragment ion 191.10 (Figure 3-3a). For IBP confirmation, fragment ion 161.15 was taken into account (Figure 3-3c). Although 2-OH IBP and 1-OH IBP share the same MRM transition (205.10>163.1), they can be distinguished by using their different retention time (Figure 3-3b).

(a)

(b)

(c)

Figure 3-3. MS chromatograms of target compounds at a standard concentration of 100 μ g/L, (a): CBX IBP; (b): 2-OH IBP and 1-OH IBP; (c): IBP.

3.4.5 Recoveries

Recoveries of the SPE-LC-MS/MS method for the target compounds were assessed by analyzing the water samples collected from all the sampling locations. By adding 10 µL of 50 mg/L stock standard solution of each target compound into 50 mL of water samples, the theoretical concentrations of target compounds in the extract after SPE (concentration multiple: 10) should be 100 µg/L if one doesn't consider the background concentrations. Control samples without standard injection were used to test the background concentrations of target compounds. After testing the SPE extract by LC-MS/MS, the recoveries of target compounds were calculated following Equation 3.1. The assay was repeated three times and three replicate analyses were performed for each extract. The results of the recoveries are shown in Table 3-4.

$$R = \frac{C_s - C_b}{100 \mu\text{g/L}} \times 100\% \quad (3.1)$$

Where:

R -- Recovery (%) of target compound;

C_s -- Concentration (µg/L) of target compound in the sample with standard injection

C_b -- Background concentration (µg/L) of target compound in sample without standard injection

Table 3-4. Recoveries of SPE-LC-MS/MS method for target compounds in all kinds of water samples

Compound	Water at Inlet	Recoveries % (RSD %; n=3)					
		Water at 1/3L		Water at 2/3L		Water at Outlet	
		Planted	Unplanted	Planted	Unplanted	Planted	Unplanted
IBP	100.4 (2.3)	117.6 (6.1)	108.1 (7.6)	109.4 (7.0)	103.5 (3.0)	114.1 (2.5)	108.4 (3.3)
CBX IBP	116.6 (12.4)	117.6 (11.4)	109.3 (22.5)	111.3 (19.3)	116.0 (11.4)	106.5 (16.7)	118.6 (10.9)
2-OH IBP	105.8 (4.9)	101.8 (7.3)	97.0 (10.6)	90.2 (3.4)	96.8 (5.4)	86.4 (5.7)	91.0 (2.7)
1-OH IBP	113.1 (2.8)	108.0 (9.9)	111.5 (9.1)	112.5 (7.8)	118.2 (8.6)	96.0 (16.1)	122.5 (14.7)

Recoveries of the SSE-LC-MS/MS method for the target compounds were assessed by measuring the recovery of 10 µL of the standard solution (50 mg/L) of each target compound which was added to 0.5 g of dry plant leaves (mixture of lamina and sheath tissues) that had not been exposed to pharmaceuticals. The extraction was performed with 10 mL of methanol. The extract was evaporated under nitrogen gas and reconstituted to 5 mL of methanol and filtered through a 0.45 µm PTFE membrane filter (Filtrex, Singapore). The filtered extract was analyzed by LC-MS/MS. This assay was repeated three times and three replicate analyses were performed for each extract. The results of the recoveries for the leaf samples of *Typha angustifolia* are shown in Table 3-5.

Table 3-5. Recoveries of SSE-LC-MS/MS method for target compounds in the leaf samples

	IBP	CBX IBP	2-OH IBP	1-OH IBP
Recoveries % (RSD %; n=3)	103.9 (16.5)	120.6 (9.4)	111.7 (5.4)	82.6 (29.1)

3.4.6 DNA Extraction

50 mL of sediment mixture sample collected from the wetland system was centrifuged at a speed of 10000 rpm for 10 min. The centrifugal deposits were then placed into a Lysing Matrix E tube of the FastDNATM SPIN Kit for Soil (Cat. 116560200, MP Biomedicals, Singapore). Following the instruction manual of the kit, DNA was extracted.

3.4.7 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was performed with 1-5 µL of template DNA depending on the DNA extract yield to make the DNA amount in the range of 100 pg-100 ng. The forward primer selected was BAC.GC-338F (5'CGCCCGCCGCGC GCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAG3') and the reverse primer was BAC 805R (5'GACTACCAGGGTATCTAATCC3') (Sigma-Aldrich, Singapore). Both the forward primer and reverse primer were diluted to 10 µM before use and 1 µL of each was utilized for 50 µL PCR reaction at a final concentration of 200 NM. Moreover, 25 µL of 2X Go Taq Master Mix, 3

μL of 25 mM MgCl_2 and 15-19 μL of nuclease-free water were also added into the reaction solution.

After the 50 μL of reaction solution was prepared, PCR was run by using a Bio-Rad S1000TM Thermal Cycler machine (Singapore). The touchdown PCR program was designed as initial denaturation of 95 °C for 3 min followed by 30 cycles of 95 °C, 60 °C and 72 °C for 30 seconds each, in which the annealing temperature of 60 °C was decreased by 0.5 °C per cycle to 45 °C on the 30th cycle. Then, it was followed by 30 addition cycles of 95 °C, 45 °C and 72 °C for 30 seconds each. Finally, PCR completed with a 72 °C extension step for 7 min and the PCR product was held at 12 °C until kept in -20 °C freezer.

PCR success was examined by loading 2.5 μL of PCR product with 0.5 μL of 6X DNA loading dye (Thermo Scientific, USA) into a 2% precast agarose gel containing GelRedTM nucleic acid gel stain (Biotium, USA) in 1X TAE buffer. The agarose gel was run for 30 min under 100 V using the Bio-Rad PowerPacTM Basic machine (Singapore). After the electrophoresis, the gel was taken an image by the Bio-Rad Gel DocTM XR + Imaging System machine (Singapore) to confirm the presence of approximate 500 bp band in the amplified sample and the absence of any band in the blank sample which replaced the template DNA with nuclease-free water.

3.4.8 Denaturing Gradient Gel Electrophoresis

Denaturing Gradient Gel Electrophoresis (DGGE) was performed with an 8% (weight/volume) acrylamide gel (made from acrylamide, formamide, urea, 50X TAE buffer and Milli-Q water) containing a linear denaturant gradient from 30% to 60%. The Gel was casted and run for 14 h under 85 V at a constant temperature of 60 °C by using the Bio-Rad Dcode System for DGGE (Singapore). GelRedTM nucleic acid gel stain (Biotium, USA) was used to stain the gel for 1 h. The stained gel was then put in the Bio-Rad Gel DocTM XR + Imaging System machine (Singapore) to take an image and was analyzed by using the Bio-Rad QuantityOne software.

Based on the analytical results of the QuantityOne software, the richness (S) which referred to the total number of the identifiable bands in a sample lane was determined. Besides, the Shannon diversity index (H) and the Pielou's evenness index (E_H) were calculated following Equation 3.2 and 3.3, respectively.

$$H = -\sum_{i=1}^S p_i \ln p_i \quad (3.2)$$

Where:

p_i -- Relative band intensity (proportion of peak intensity of the i^{th} band relative to the sum of peak intensities of all the bands in a sample lane).

$$E_H = H/H_{\max} \quad (3.3)$$

Where:

$H_{\max} = \ln S$, maximum diversity possible

3.4.9 Pyrosequencing

Several template DNA samples selected according to the DGGE results were sent to the Research and Testing Laboratory (Lubbock, TX, USA) for 454 pyrosequencing analysis. The volume of each template DNA sent out was 25 μL . The bacterial 16s assay chosen for the template DNA samples was 357F and 926R. Based on the OTU (operational taxonomic unit) results of pyrosequencing, some representative OTUs were checked on the Nucleotide Blast of National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov/>) and used for establishment of phylogenetic tree with the MEGA5.2 software.

3.4.10 Statistical Analysis

Experimental data were tested by using SPSS V20 software to determine the statistical differences between the planted and unplanted beds for the removal efficiencies of target pollutants and the concentrations of IBP metabolites. Comparison was viewed to have shown significant difference when $p < 0.05$.

CHAPTER 4 HSSF-CW SYSTEM PERFORMANCE

4.1 Introduction

Water-quality parameters are of great significance to evaluate the performance of constructed wetlands for wastewater treatment. In this chapter, some important water-quality parameters will be spatially analyzed along the flow path including the physicochemical parameters such as temperature (Temp), pH, electrical conductivity (Cond), DO and ORP, the common pollutants such as COD, $\text{NH}_4^+\text{-N}$, and TP, and the micropollutant IBP. The influences of plants on the removal of pollutants will be discussed according to the differences between the planted and unplanted beds.

4.2 Physicochemical Parameters

As can be seen in Table 4-1, the temperatures monitored at all the sampling locations during the sampling period (30th to 342nd day) exhibited very little fluctuation. It did not mean that there was no intra-day fluctuation of temperature. The small fluctuation of temperature measured was attributed to the fact that sampling was performed at the same time in the morning on the day of measurement. The pH values in the planted bed dropped from a neutral value at inlet to a slightly acidic value at 2/3L and were constant to the outlet. The slightly acidic conditions within the planted bed may be due to the oxidation reactions of organic matter which released hydrogen ions. Conversely, the pH values in the unplanted bed appeared a very slight decreasing trend along the flow path, which showed a relatively neutral condition. As for the electrical conductivity, a significant decrease was observed from inlet to 1/3L in both the planted and unplanted beds. Further downstream in the planted bed, a slighter decrease was found from 1/3L to outlet. Nevertheless, in the case of unplanted bed, the conductivity was almost the same from 1/3L to outlet. For the values of DO, there was a significant decrease from inlet to 1/3L in both the beds. At the downstream sampling locations, however, the DO values exhibited a slightly increasing trend

until outlet. The trend was more obvious in the case of planted bed. Similar to the DO values, the values of ORP also decreased significantly from inlet to 1/3L and then showed a slight increasing trend downstream to outlet, which was also more obvious in the planted bed.

Table 4-1. Physicochemical parameters of water quality at all the sampling locations

Sampling Locations		Temp (°C)	pH	Cond (µs/cm)	DO (mg/L)	ORP (mv)
Inlet	Avg ± SD	29.9 ± 1.7	7.47 ± 0.34	683.5 ± 77.0	7.08 ± 0.55	290.7 ± 35.1
	Min - Max	26.3 - 32.3	6.86 - 8.13	560.0 - 921.0	6.15 - 8.23	196.4 - 342.8
1/3L (P ^a)	Avg ± SD	29.7 ± 1.9	6.85 ± 0.32	569.4 ± 62.9	0.83 ± 0.54	-211.1 ± 127.5
	Min - Max	25.8 - 32.9	6.31 - 7.53	463.0 - 697.0	0.07 - 1.84	-328.3 to 119.7
2/3L (P)	Avg ± SD	29.6 ± 1.8	6.51 ± 0.35	503.5 ± 76.0	1.28 ± 0.57	-132.7 ± 101.3
	Min - Max	26.0 - 32.8	5.86 - 7.31	228.1 - 672.0	0.18 - 3.09	-248.3 to 120.1
Outlet (P)	Avg ± SD	29.2 ± 1.8	6.51 ± 0.38	470.8 ± 66.8	1.78 ± 0.72	-95.0 ± 146.1
	Min - Max	25.9 - 32.7	5.76 - 7.36	350.0 - 680.0	0.91 - 4.36	-288.0 to 230.5
1/3L (UP ^b)	Avg ± SD	29.6 ± 1.8	7.00 ± 0.41	571.4 ± 105.3	0.90 ± 0.59	-237.2 ± 97.2
	Min - Max	26.0 - 33.3	6.28 - 7.92	262.0 - 772.0	0.05 - 2.21	-333.9 to 128.8
2/3L (UP)	Avg ± SD	29.3 ± 1.8	6.99 ± 0.36	576.7 ± 63.2	1.19 ± 0.70	-210.9 ± 55.9
	Min - Max	25.9 - 32.7	6.37 - 7.62	458.0 - 734.0	0.09 - 2.57	-290.3 to -63.4
Outlet (UP)	Avg ± SD	29.2 ± 1.8	6.90 ± 0.34	577.8 ± 54.7	1.37 ± 0.60	-201.8 ± 112.1
	Min - Max	25.5 - 33.0	6.23 - 7.57	503.0 - 715.0	0.33 - 2.53	-333.8 to 126.0

^a Planted bed. ^b Unplanted bed. Number of sampling campaigns: n=44

It is known that oxygen concentration in constructed wetlands is determined by the oxygen transfer rate and the oxygen consumption rate (Vymazal et al. 2008, Nivala et al. 2013). The oxygen transfer rate is related to the dissolved oxygen concentration in the influent water, the atmospheric diffusion through unsaturated gravel layer, the plant-mediated oxygen transfer by the rhizospheric release, and the oxygen transfer at the water-biofilm interface via the ventilation pipe (Vymazal et al. 2008, Imfeld et al. 2009, Nivala et al. 2013). On the other hand, the oxygen consumption rate is mainly based on the carbonaceous biochemical oxygen demand and the nitrogenous biochemical oxygen demand (Vymazal et al. 2008, Nivala et al. 2013).

The changing trends of DO and ORP observed in the experiment and described above were largely related to the oxygen consumption demanded by the aerobic respiration and the chemical oxidation. In this study, the ORP between -215 mV to -120 mV in the planted bed and below -200 mV in the unplanted bed at all sampling locations except for inlet (Table 4-1) revealed a predominantly anaerobic environment within the wetland bed (Vymazal et al. 2008). However, there was still certain DO in water ranging from 0.8 to 1.8 mg/L (Table 4-1). The reasons might be due to the oxygen transfer through the ventilation pipes (sampling pipe I, sampling pipe II and water level control pipe) closed to where the water samples were collected. As reported in Table 4-1, both the ORP and DO were at a relatively higher level in the planted bed compared to those in the unplanted bed. The plausible interpretation is that plants might have played a positive role in oxygen transfer and produced some aerobic microenvironments in the predominantly anaerobic environment inside the planted bed.

4.3 COD

Concentrations of COD measured for the water samples collected from all the sampling locations are illustrated in Figure 4-1. As shown in Figure 4-1, the influent COD concentration varied in the range from 264 to 420 mg/L and was observed to decrease significantly at 1/3L in the planted bed. At the subsequent sampling locations in the planted bed, reduction in the concentration of COD was slight along the flow path. In the case of the unplanted bed, the changing trend of COD concentration at different sampling locations was similar to that in the planted bed. The significant reduction of COD in the unplanted bed also took place at the upstream 1/3 zone (from inlet to 1/3L). Overall, the temporal fluctuation of COD concentrations at all the sampling locations was not significant over the entire experimental period except during the initial period between the 30th day to the 50th day. It was mainly due to the reason that the wetland system took time to achieve stable conditions after start-up.

(a)

(b)

Figure 4-1. COD concentration in water monitored at different sampling locations in the planted bed (a) and the unplanted bed (b)

In Figure 4-2, based on the mean concentrations of COD at different sampling locations, the corresponding removal efficiencies of COD at different zones in the wetland bed can be compared between the planted and unplanted beds. In both the beds, under a COD loading of 355 ± 32 mg/L, the significant removal of COD was achieved at the upstream 1/3 zone (from inlet to 1/3L) with an efficiency of $83.7 \pm 10.2\%$ and $81.9 \pm 11.4\%$ in the planted and unplanted beds, respectively. The

plausible interpretation for such a phenomenon is that the high concentration of DO in the influent water (Table 4-1) and the high initial feeding concentration caused the sharp abatement of COD near the inlet location via aerobic biodegradation. At the middle 1/3 zone (from 1/3L to 2/3L), the COD was marginally removed by 7.3% and 4.0% with a cumulative removal efficiency of $91.0 \pm 9.4\%$ and $85.9 \pm 10.2\%$ in the planted and unplanted beds, respectively. The planted bed performed a little better for COD removal than the unplanted bed at the first two zones. However, it turned less effective at the downstream 1/3 zone (from 2/3L to outlet) where the removal efficiency of COD was 2.9% in the planted bed and 4.1% in the unplanted bed. Generally speaking, the difference ($p > 0.05$) between the total removal efficiency of COD in the planted bed ($93.9 \pm 6.1\%$) and the unplanted bed ($90.0 \pm 7.8\%$) was too small to assess the influences of *Typha angustifolia* on COD removal.

Figure 4-2. Mean concentration of COD and relative cumulative removal efficiency at different sampling locations in both the planted and unplanted beds

4.4 $\text{NH}_4^+\text{-N}$

Concentrations of $\text{NH}_4^+\text{-N}$ in the water samples collected from all the sampling locations are illustrated in Figure 4-3. As can be seen in Figure 4-3, the influent concentration of $\text{NH}_4^+\text{-N}$ ranged from 17.1 to 26.6 mg/L. The fluctuation of $\text{NH}_4^+\text{-N}$

concentration at all the sampling locations was not significant over the entire experimental period except for the period between the 198th day to the 270th day. During this period, the concentration of $\text{NH}_4^+\text{-N}$ at outlet in the planted bed was relatively higher than those in other periods and sometimes even higher than that at 2/3 L. This period (May to July) corresponded to the dry season in Singapore when weather was dry and sunshine was strong. The weather conditions accelerated the water losses via evaporation and transpiration in the wetland bed so that to some extent the leaves of *Typha angustifolia* turned yellow and the sprouting of new stalks was few. In addition, the problem of pests such as ants, powdery mildew and scale insects was considerably more serious during this period, which also adversely influenced the plants' growth. Under these conditions, therefore, the operation environment of the planted bed was not in its best state with relatively weak plants, resulting in lower $\text{NH}_4^+\text{-N}$ removal. After the dry season, however, the operation environment of the planted bed became better as the weather got cooler and wetter, which led to better removal of $\text{NH}_4^+\text{-N}$.

(a)

(b)

Figure 4-3. $\text{NH}_4^+\text{-N}$ concentration in water monitored at different sampling locations in the planted bed (a) and the unplanted bed (b)

As shown in Figure 4-4, $\text{NH}_4^+\text{-N}$ was loaded at a concentration of 23.0 ± 1.9 mg/L to the wetland system. Based on the mean concentrations of $\text{NH}_4^+\text{-N}$ at 1/3L, 2/3L, and outlet in the planted bed, the cumulative removal efficiencies were calculated as $20.2 \pm 10.0\%$, $49.8 \pm 10.9\%$, and $60.8 \pm 9.9\%$. In the case of the unplanted bed, the cumulative removal efficiency of $\text{NH}_4^+\text{-N}$ at the corresponding three sampling locations achieved $15.0 \pm 11.6\%$, $22.8 \pm 10.5\%$, and $30.7 \pm 7.2\%$, respectively. The removal of $\text{NH}_4^+\text{-N}$ in the unplanted bed mainly took place at the upstream 1/3 zone, whereas in the planted bed it mainly occurred at the first two zones from inlet to 2/3. This observation was plausibly attributed to the positive impacts of *Typha angustifolia* on $\text{NH}_4^+\text{-N}$ removal in the planted bed. The associated mechanisms might involve the process of uptake and assimilation by plants and the enhanced activities of microorganisms for nitrification in the rhizosphere (Vymazal et al. 2008). The microbial degradation in rhizosphere could be supported by the exudates and the oxygen released from plant roots. As a result, the total removal efficiency of $\text{NH}_4^+\text{-N}$ in the planted bed was approximately twice than that in the unplanted bed ($p < 0.05$). Moreover, at the upstream 1/3 zone in both the planted and unplanted beds, the high level of DO in the influent water (Table 4-1) and the high initial feeding concentration might also be important contributors to the aerobic process of

nitrification. Besides, the reduction in the removal efficiency of $\text{NH}_4^+\text{-N}$ at the downstream 1/3 zone in the planted bed compared to those at the first two zones might be due to the low concentration of $\text{NH}_4^+\text{-N}$ at this zone.

Figure 4-4. Mean concentration of $\text{NH}_4^+\text{-N}$ and relative cumulative removal efficiency at different sampling locations in both the planted and unplanted beds

4.5 TP

Concentrations of TP measured for the water samples collected from all the sampling locations are shown in Figure 4-5. The influent concentration of TP varied from 11.1 to 19.9 mg/L. The temporal fluctuation of TP concentrations at all the sampling locations was not significant throughout the experimental period except for the period between the 198th day to the 270th day. In this period, the effluent concentration of TP in the planted bed exhibited relative higher than those during other periods and sometimes even higher than that at 2/3L. This phenomenon was similar to the observation for $\text{NH}_4^+\text{-N}$ (Section 4.4) with the same explanation.

(a)

(b)

Figure 4-5. TP concentration in water monitored at different sampling locations in the planted bed (a) and the unplanted bed (b)

In Figure 4-6, according to the mean concentrations of TP at the different sampling locations, the cumulative removal efficiencies of TP are compared between the planted and unplanted beds. Under the TP feeding at a concentration of 15.8 ± 2.1 mg/L, in the case of the planted bed, TP was cumulatively removed by $21.6 \pm 13.6\%$, $44.6 \pm 23.0\%$, and $63.2 \pm 19.5\%$, respectively, at the three zones along the flow path. Whereas, in the unplanted bed, the cumulative removal efficiencies of TP

at these three zones were $13.7 \pm 19.4\%$, $19.9 \pm 11.9\%$, and $25.8 \pm 12.6\%$, respectively. It was noticeable that the planted bed exhibited a uniform removal efficiency for TP at these three different zones, whereas the unplanted bed showed a significant removal for TP at the upstream 1/3 zone. The process of plant uptake can plausibly account for this observation (Vymazal et al. 2008), implying the positive role of *Typha angustifolia* on the abatement of TP in the planted bed. As a result, the total removal efficiency of TP in the planted bed was more than double that in the unplanted bed ($p < 0.05$). The main reduction of TP at the upstream 1/3 zone in the unplanted bed might be attributed to the high initial feeding concentration at inlet.

Figure 4-6. Mean concentration of TP and relative cumulative removal efficiency at different sampling locations in both the planted and unplanted beds

4.6 IBP

As can be seen in Figure 4-7, the IBP concentration in the influent water ranged from 73.6 to 132.8 $\mu\text{g/L}$ during the sampling period and was mainly attributed to the experimental error during the preparation of synthetic wastewater in the wastewater holding tank every 4 days. According to the results of three experiments conducted during different periods (Table 4-2), about 3- 8% of IBP was removed in

the wastewater holding tank after 4 days. The plausible explanation for this observation was that photolysis and biodegradation of IBP took place to some small extent due to the exposure of the tank to the strong sunshine and elevated temperature during the day.

Fluctuation of IBP concentrations was also observed for the water samples collected from the sampling locations at 1/3L, 2/3L, and outlet in both the planted bed (Figure 4-7a) and unplanted bed (Figure 4-7b). Such a phenomenon was attributed to the fluctuation of IBP feeding concentration and wetland operation conditions. During the period of 198th -270th day, the concentration of IBP in the effluent water from the planted bed was relatively higher than those during other periods. This was consistent with the removal of $\text{NH}_4^+\text{-N}$ and TP described in Section 4.4 and 4.5. It was due to the less desirable conditions of the wetland system during the dry season in Singapore. After the dry season, the operation environment of the wetland system became better as the weather got cooler and wetter, which led to observed lower concentration of IBP in the effluent water.

Table 4-2. Variation of IBP concentration in the wastewater holding tank during the interval of 4 days

Days	IBP Concentration, $\mu\text{g/L}$ (Cumulative Removal Efficiency of IBP, %)		
	Experiment 1	Experiment 2	Experiment 3
Initial day	84.96	97.36	111.56
After 1 day	84.83 (0.15)	98.84 (-1.52)	112.92 (-1.22)
After 2 days	83.30 (1.95)	96.50 (0.88)	109.16 (2.15)
After 3 days	80.14 (5.67)	95.93 (1.47)	107.17 (3.94)
After 4 days	78.28 (7.86)	93.73 (3.73)	104.63 (6.21)

(a)

(b)

Figure 4-7. IBP concentration in water monitored at different sampling locations in the planted bed (a) and the unplanted bed (b)

The mean concentration of IBP in the influent water was 107.2 ± 12.8 $\mu\text{g/L}$ during the sampling period (Figure 4-8). In the case of the planted bed, the mean concentrations of IBP at the sampling locations of 1/3L, 2/3L and outlet were 78.1 ± 11.1 $\mu\text{g/L}$, 50.3 ± 11.2 $\mu\text{g/L}$ and 33.0 ± 11.9 $\mu\text{g/L}$, respectively. IBP removal at these three sampling locations was achieved with cumulative efficiency of $26.9 \pm 8.7\%$, $52.8 \pm 10.2\%$ and $69.2 \pm 10.7\%$, respectively. In the unplanted bed, the mean

concentrations of IBP at the sampling locations of 1/3L, 2/3L and outlet were $83.3 \pm 9.5 \mu\text{g/L}$, $76.1 \pm 9.5 \mu\text{g/L}$, and $68.9 \pm 9.8 \mu\text{g/L}$, respectively, with corresponding cumulative removal efficiencies of $21.8 \pm 8.6\%$, $28.6 \pm 8.0\%$, and $35.4 \pm 8.3\%$.

The synthetic wastewater contained negligible amount of suspended solids, and so the adsorption of IBP to suspended organic matter played a minor role in IBP removal even though IBP with a $\log K_{ow}$ of 3.97 (Table 3-1) exhibits a relatively high hydrophobicity. Besides, photolysis of IBP was unlikely to take place inside the wetland as a result of the absence of free water on the surface of the gravel layer. Here we discuss only the main mechanisms from the perspectives of plant uptake and microbial degradation to account for the spatial change of IBP concentrations and removal efficiencies along the flow path from inlet to outlet.

Figure 4-8. Mean concentration of IBP and relative cumulative removal efficiency at different sampling locations in both the planted and unplanted beds

As illustrated in Figure 4-8, IBP was significantly removed at the upstream 1/3 zone in the unplanted bed with a removal efficiency of 21.8% which was less than but close to that in the planted bed (26.9%). This phenomenon was most plausibly attributed to that in both the planted and unplanted beds IBP was involved in

aerobic biodegradation near the inlet where the feeding water contained a relatively high level of DO (Table 4-1). However, the oxygen transfer associated with the influent water was limited and its effects would have weakened rapidly downstream. It was also noted that there was no more efficient oxygen transfer zone in the unplanted bed except for the area in the vicinity of the ventilation pipes whose influence was relatively small. It can be seen from Figure 4-8 that the removal efficiency of IBP at the middle 1/3 zone in the unplanted bed decreased sharply (6.8%). Such a situation in the unplanted bed continued at the downstream 1/3 zone where the removal efficiency of IBP (6.8%) was similar to that at the middle 1/3 zone. Based on the analysis above, it can be deduced that in the unplanted bed the aerobic biodegradation contributed to the removal of IBP from water but was limited by the poor oxygen transfer in the bed.

In contrast to the circumstances in the unplanted bed, the positive effects of plants (*Typha angustifolia*) on the abatement of IBP were apparent in the planted bed. Although the removal efficiency of IBP at the upstream 1/3 zone in the planted bed was not much higher than that in the unplanted bed, it was significantly higher at the middle 1/3 zone (25.9%) and the downstream 1/3 zone (16.4%) compared to those in the unplanted bed. The total removal efficiency of IBP in the planted bed was almost twice than that in the unplanted bed ($p < 0.05$). It can be inferred that the microbial degradation of IBP in the planted bed was enhanced and promoted by the activities of microorganisms in the rhizosphere. Such a process was most probably related to the activities of aerobic microorganisms in the rhizosphere which were supported by the exudates and the oxygen released from the plant roots (Imfeld et al. 2009, Garcia-Rodríguez et al. 2014, Verlicchi and Zambello 2014). The preferential aerobic biodegradation of IBP within constructed wetlands has also been confirmed in some other peer studies (Matamoros et al. 2005, Matamoros et al. 2008a, Ávila et al. 2010, Hijosa-Valsero et al. 2010a, Hijosa-Valsero et al. 2010b, Hijosa-Valsero et al. 2011c, Reyes-Contreras et al. 2012, Ávila et al. 2013). Besides, plant uptake may play another important role in IBP removal (Dordio et al. 2011a). These associated mechanisms will be explained in Chapter 5. As can be seen in Figure 4-8, the removal efficiency of IBP at the downstream 1/3 zone in the planted bed decreased

when compared to those in the two upstream zones, indicating that the microbes performed less actively most probably because of the relative low concentration of IBP at this zone.

4.7 Conclusions

In this study, the HSSF-CW system was operated well in the tropical environment based on the spatial monitoring data of water-quality parameters throughout the experimental period. The ORP monitoring reveals a predominantly anaerobic environment within the wetland beds although there was still certain DO in water attributed to the oxygen transfer through the ventilation pipes. Compared to the unplanted bed, the planted bed held higher levels of both ORP and DO in water and likely produced relatively more aerobic microenvironments.

Under a COD loading of 355 ± 32 mg/L, the cumulative removal efficiencies of COD at 1/3L, 2/3L and outlet in the planted bed were $83.7 \pm 10.2\%$, $91.0 \pm 9.4\%$ and $93.9 \pm 6.1\%$, respectively, while they were $81.9 \pm 11.4\%$, $85.9 \pm 10.2\%$ and $90.0 \pm 7.8\%$ in the unplanted bed. The significant removal of COD took place at the upstream 1/3 zone in both the planted and unplanted beds. The difference between the removal efficiencies of COD in both the beds was too small to access the influence of *Typha angustifolia* on COD removal in the investigated HSSF-CW system.

The influent $\text{NH}_4^+\text{-N}$ was 23.0 ± 1.9 mg/L and was cumulatively decreased at 1/3L, 2/3L and outlet in the planted bed by $20.2 \pm 10.0\%$, $49.8 \pm 10.9\%$ and $60.8 \pm 9.9\%$, respectively, while it was cumulatively removed by $15.0 \pm 11.6\%$, $22.8 \pm 10.5\%$ and $30.7 \pm 7.2\%$ in the unplanted bed. The positive impacts of *Typha angustifolia* on $\text{NH}_4^+\text{-N}$ removal were significant.

With a TP feeding concentration of 15.8 ± 2.1 mg/L, the cumulative removal efficiencies of TP at 1/3L, 2/3L and outlet in the planted bed were achieved by $21.6 \pm 13.6\%$, $44.6 \pm 23.0\%$ and $63.2 \pm 19.5\%$, respectively, while they were $13.7 \pm$

19.4%, $19.9 \pm 11.9\%$ and $25.8 \pm 12.6\%$ in the unplanted bed. The positive role of *Typha angustifolia* plants in TP removal could account for this observation.

Under a high IBP loading of $107.15 \pm 12.79 \mu\text{g/L}$, the cumulative removal efficiencies of IBP at 1/3L, 2/3L and outlet in the planted bed were $26.9 \pm 8.7\%$, $52.8 \pm 10.2\%$ and $69.2 \pm 10.7\%$, respectively, while they were $21.8 \pm 8.6\%$, $28.6 \pm 8.0\%$ and $35.4 \pm 8.3\%$ in the unplanted bed. The significant spatial increase in IBP removal efficiencies along the flow path in the planted bed demonstrates the efficacy of employing HSSF-CW to continuously remove IBP from wastewater. During the experiment, the positive impacts of *Typha angustifolia* on the abatement of IBP were apparent. It can be inferred that the process of plant uptake and the microbial degradation in the rhizosphere enhanced the removal of IBP in the planted bed.

According to the current literature data (Table 2-2), IBP in wastewater could be removed with efficiencies: 60-75% in SF-CWs, 30-81% in HSSF-CWs, 55-99% in VSSF-CWs and 22-99% in hybrid constructed wetlands. The feeding concentrations of IBP to these constructed wetlands varied at trace levels ranging from 1 to 25 $\mu\text{g/L}$. To date, however, available literature on the removal of high-loaded IBP by constructed wetlands is very limited. This study is one of the pioneer research work to investigate the performance of constructed wetlands for treatment of high-loaded IBP. The high-loaded IBP which is oxygen consuming organic matter might mask the oxygen superiority of the planted bed in comparison to the unplanted bed. Also, the potential toxicity of IBP to the growth of plants (Dordio et al. 2011a) may limit the advantages of plants for the removal of pollutants. In this study, the operation situation of the HSSF-CW was efficient throughout the experimental period with high removal efficiencies of pollutants and was not adversely influenced by the high-loaded IBP.

The performance of the investigated HSSF-CW (planted with *Typha angustifolia*) for the removal of conventional pollutants and IBP at a HRT of 4 days was comparable to or even better than that reported in other studies which also applied

HSSF-CWs to remove high-loaded IBP from wastewater. For instance, in the experiment of Ávila et al. (2013) whose control line (Table 2-1) is close to the wetland design of this study, the removal efficiencies of COD, $\text{NH}_4^+\text{-N}$ and IBP in the first stage were about 61.2%, 39.8% and 51.9%, respectively, when at a IBP loading of $83.6 \pm 2.7 \mu\text{g/L}$. In another study of Ávila et al. (2010) (Table 2-1), the experimental results of their target mesocosm-scale HSSF-CWs which were injected with $132.0 \pm 7.0 \mu\text{g/L}$ of IBP showed that COD, $\text{NH}_4^+\text{-N}$ and IBP could be removed by 63.2%, 66.7% and 58.5% in the first stage, respectively. Besides, the positive role of plants in the removal of IBP was observed by Carranza-Diaz et al. (2014) who focused on a pilot-scale HSSF-CW planted with *Phragmites australis* at a high IBP load of 10.6-92.0 $\mu\text{g/L}$ (Table 2-1). However, IBP was only removed by 13% and 9% in the planted and unplanted beds, respectively. Additionally, the planted bed in the study didn't show a better performance for the removal of COD and $\text{NH}_4^+\text{-N}$. The removal efficiencies of COD were about 53.6% and 55.1% in the planted and unplanted beds, respectively, and there existed an increase in the effluent $\text{NH}_4^+\text{-N}$ concentration in the planted bed. The impacts induced by the high load of oxygen consuming compounds in their study seemed to mask the oxygen difference between the planted and unplanted beds.

The concentration of IBP in the effluent water from the planted bed in this study was about $33.0 \pm 11.9 \mu\text{g/L}$ close to the influent IBP concentrations reported in other studies on the performance of constructed wetlands for the removal of IBP at trace levels. Therefore, if connecting the wetland bed to another one in series, the hybrid constructed wetland system for treatment of high-loaded IBP will be more efficient. For example, the studies conducted by Ávila et al. (2010 and 2013) obtained a total IBP removal efficiency up to 99% by using hybrid HSSF-CWs in series.

CHAPTER 5 PLANT UPTAKE AND MICROBIAL DEGRADATION IN RHIZOSPHERE

5.1 Introduction

It was observed in Chapter 4 that *Typha angustifolia* played a positive role in the removal of IBP from wastewater in the studied HSSF-CW. In order to obtain an in-depth understanding on the associated mechanisms, this chapter will analyse the experimental data about the concentrations of IBP and its three metabolites (CBX IBP, 2-OH IBP and 1-OH IBP) in both the water and leaf samples. It aims to identify how the plant uptake and the microbial degradation in the rhizosphere contribute to the IBP removal in the studied HSSF-CW.

5.2 Potential of Root Uptake

The tendency of organic chemicals moving into plant roots from an external solution can be assessed by root concentration factor (RCF) (Trapp 2004, Pilon-Smits 2005). RCF refers to the ratio between the concentration of one specific organic chemical in the root tissue (ng/g) and that in the surrounding external solution ($\mu\text{g/L}$) under equilibrium conditions (Trapp 2004, Pilon-Smits 2005) and can be expressed as Equation (5.1) (Trapp and Matthies 1995). The concentration in root tissue of a certain plant depends on the concentration in external solution and the octanol-water partition coefficient (K_{ow}) of organic chemical. RCF can fairly assess the potential of IBP and its metabolites taken up by the roots of *Typha angustifolia* from the interstitial water within the planted bed. Organic chemicals with medium hydrophobicity ($\log K_{ow}$: 1 ~ 4) are readily taken up by plants through passive diffusion (Calderón-Preciado et al. 2012). These chemicals are not only hydrophobic enough to travel through the lipid bilayer of cell membranes but also hydrophilic enough to penetrate into the cell fluids (Calderón-Preciado et al. 2012). IBP and its three metabolites (CBX IBP, 2-OH IBP and 1-OH IBP) investigated in this study have the $\log K_{ow}$ values in the range from 1 to 4 (Table 3-1). A higher

concentration in the interstitial water within the planted bed would induce a highly efficiency of being taken up into the roots and vice versa.

$$RCF=W+LcK_{ow}^b \quad (5.1)$$

Where:

RCF – Root concentration factor

W – Water content of root tissue

L – Lipid content of root tissue

K_{ow} – Octanol-water partition coefficient

c and b – Coefficients to correct for difference between root tissue and octanol

As illustrated in Figure 4-8, the concentrations of IBP in the interstitial water at 1/3L and 2/3L in the planted bed were 78.1 and 50.3 µg/L, respectively, which were relatively high showing a tendency to be taken up by the roots of *Typha angustifolia*. Figure 5-1 presents the appearance of CBX IBP in the water samples during the long-term wetland operation. CBX IBP was not frequently detected and showed a low concentration level ranging from 2.1 to 8.0 µg/L and 2.3 to 4.2 µg/L in the planted and unplanted beds, respectively. The observation of CBX IBP in water demonstrates the transformation of IBP to CBX IBP. The infrequent and low-concentration presence of CBX IBP in water is most plausibly attributed to its fast biodegradation rate which has been reported in biological treatment systems (Winkler et al. 2001, Weigel et al. 2004, Ferrando-Climent et al. 2012). According to Equation (5.1), the root uptake of CBX IBP by *Typha angustifolia* from the interstitial water is considered negligible in this study.

(a)

(b)

Figure 5-1. Occurrence of CBX IBP in water samples collected at different sampling locations in the planted bed (a) and the unplanted bed (b)

The hydroxylated metabolites of IBP in the forms of 2-OH IBP (Figure 5-2) and 1-OH IBP (Figure 5-3) were observed in the water samples on each sampling date in both the planted and unplanted beds. As can be seen in Figures 5-2 and 5-3, throughout the experimental period, the concentrations of 2-OH IBP and 1-OH IBP fluctuated to some extent mainly due to the fluctuation of the influent IBP concentration and the operation conditions of wetland system. The presence of 2-

OH IBP and 1-OH IBP in the water samples demonstrates the occurrence of IBP hydroxylation in water within the studied wetland beds.

(a)

(b)

Figure 5-2. Occurrence of 2-OH IBP in water samples at different sampling locations in the planted bed (a) and the unplanted bed (b)

(a)

(b)

Figure 5-3. Occurrence of 1-OH IBP in water samples at different sampling locations in the planted bed (a) and the unplanted bed (b)

As shown in Figure 5-4, the mean concentrations of 2-OH IBP at 1/3L and 2/3L in the planted bed were 9.3 and 16.1 µg/L, respectively, which were relatively low. Uptake of 2-OH IBP to the roots of *Typha Angustifolia* from the interstitial water was considered minor according to Equation (5.1). Compared to 2-OH IBP, the mean concentrations of 1-OH IBP at 1/3L and 2/3L in the planted bed were even

lower, i.e. 1.1 and 1.2 $\mu\text{g/L}$, respectively. As such, the uptake of 1-OH IBP to the roots of *Typha angustifolia* can be nearly negligible.

Figure 5-4. Mean concentrations of 2-OH IBP and 1-OH IBP in water samples at different sampling locations in both the planted and unplanted beds

An empirical relationship between RCF and $\log K_{ow}$ of non-ionised chemicals was established by Briggs et al. as Equation (5.2). By using this equation, the root concentrations of IBP, 2-OH IBP and 1-OH IBP at 1/3L in the planted bed would be 81.4, 9.1, and 1.1 ng/g, respectively, while 52.5, 15.7, and 1.2 ng/g at 2/3L. The root concentration of CBX IBP cannot be estimated due to its low frequency of being detected in water. These estimated results are fairly consistent with the above analysis on the potential of IBP and its metabolites taken up by the roots of *Typha angustifolia*. However, the electrically-charged status of organic chemicals and the plant species may largely influence the relationship between RCF and $\log K_{ow}$ (Trapp 2004). The estimated results obtained from Equation (5.2) may be underestimated to a certain extent.

$$RCF = 0.82 + 0.03K_{ow}^{0.77} \quad (5.2)$$

Where:

RCF – Root concentration factor

K_{ow} – Octanol-water partition coefficient

5.3 Accumulation of IBP and Its Metabolites in Leaves.

As shown in Figure 5-5, IBP was detected in the leaves of *Typha angustifolia* at the two sampling locations of 1/3L and 2/3L throughout the experimental period. The presence of IBP in the leaves demonstrates that IBP was taken up by the roots of *Typha angustifolia* and was translocated from the roots to leaves. This finding is in agreement with the analytical result in the above section that IBP has a tendency to be taken up by the roots of *Typha angustifolia*. Removal of IBP by *Typha spp.* through root uptake has also been confirmed by Dordio et al. (2011a) based on the measurement of IBP concentration in external solution in a hydroponic experiment.

Figure 5-5. Concentrations of IBP in the lamina (L) and sheath (S) tissues of *Typha angustifolia* collected at 1/3L and 2/3L in the planted bed

Figures 5-6 to 5-8 present the concentrations of CBX IBP, 2-OH IBP and 1-OH IBP in the leaf tissues of *Typha angustifolia* at the sampling locations of 1/3L and 2/3L, respectively. All these three IBP metabolites were detected in both the lamina and

sheath tissues of *Typha angustifolia*. It indicates that transformation reactions of IBP happened in plant tissues (roots, leaves or both), which were enzymatically catalyzed or performed by endophytic microorganisms.

The enzymatic degradation of IBP was likely to involve the catalytic actions of monooxygenases and carboxylesterases to produce hydroxylated and carboxylated products (Figure 5-9). Such a process is regarded as chemical modification (or chemical transformation) and is a crucial phase during the detoxification process of plants when countering the phytotoxicity of organic pollutants (Sandermann Jr 1992, Sandermann 1994, Coleman et al. 1997, Pilon-Smits 2005). The modified products are usually more hydrophilic and less toxic compared to the parent compound due to the introduction of functional groups such as hydroxyl or carboxyl (Coleman et al. 1997). The lower log K_{ow} values of CBX IBP, 2-OH IBP and 1-OH IBP in comparison to IBP (Table 3-1) support this reported point of view.

The IBP biodegradation activity induced by endophytic microorganisms is considered rather low mainly attributable to the defense characteristic of plants to protect against microbial pathogens (Coleman et al. 1997, Robert and Friml 2009). For instance, the Casparian strip in the endodermis of roots can create a tight barrier to prevent the invasion of pathogens into the vasculature (Robert and Friml 2009). Another interpretation is the high concentration of CBX IBP in the leaf tissues at a magnitude of 900-1500 ng/g (Figure 5-6). CBX IBP has been reported to have a fast biodegradation rate (Winkler et al. 2001, Weigel et al. 2004, Ferrando-Climent et al. 2012). The limited endophytic microorganisms present in the leaf tissues would not significantly reduce the concentration of CBX IBP to a very low level.

Figure 5-6. Concentrations of CBX IBP in the lamina (L) and sheath (S) tissues of *Typha angustifolia* collected at 1/3L and 2/3L in the planted bed

Figure 5-7. Concentrations of 2-OH IBP in the lamina (L) and sheath (S) tissues of *Typha angustifolia* collected at 1/3L and 2/3L in the planted bed

Figure 5-8. Concentrations of 1-OH IBP in the lamina (L) and sheath (S) tissues of *Typha angustifolia* collected at 1/3L and 2/3L in the planted bed

Figure 5-9. Chemical modification reactions of IBP to produce the hydroxylated and carboxylated metabolites and the followed conjugation processes of the metabolites with glucose in the leaf tissues

Figure 5-10 shows the mean concentrations of IBP and its three metabolites (CBX IBP, 1-OH IBP, 2-OH IBP and IBP) detected in both the lamina and sheath tissues of *Typha angustifolia*. As illustrated in Figure 5-10, the mean concentrations of IBP in the leaf tissues at a magnitude of 130-180 ng/g show a roughly even distribution in the lamina and sheath tissues regardless where to collect the leaf samples. It

indicates that IBP was well transported in the plant tissues. According to the basic knowledge about the transport processes of substances in plants (Robert and Friml 2009), IBP was likely imported by passive diffusion in the apoplast through the epidermis, cortex, endodermis and pericycle of roots to the xylem and then driven by transpiration to move in the xylem sap. In the tropical setting (high temperature, strong sunshine and moderate wind), the water transportation rate of plants is considered sufficiently high to distribute IBP evenly in the xylem sap (Pilon-Smits 2005).

As shown in Figure 5-10, 2-OH IBP accumulated in the lamina tissues at a concentration approximately 3 times than that in the sheath tissues. Adversely, CBX IBP and 1-OH IBP accumulated in the sheath tissues at concentrations about 1.3 and 1.7 times than those in the lamina tissues, respectively. The results suggest that the three IBP metabolites accumulated and distributed unevenly inside the leaf tissues. The most plausible explanation is that these metabolites were conjugated with glucose under the catalysis of glucosyltransferases and thereafter the conjugates were compartmentized in places where they were least harmful to the essential cellular processes of leaves (Coleman et al. 1997, Pilon-Smits 2005). The conjugation process is illustrated in Figure 5-9. The functional groups (hydroxyl and carboxyl) of the metabolites make themselves able to conjugate with the endogenous organic molecules (Coleman et al. 1997, Pilon-Smits 2005). Glucose which was reported to be responsible for the conjugation with hydroxyl and carboxyl groups (Coleman et al. 1997, Pilon-Smits 2005) was the major carbohydrate in the feeding wastewater in this study. Besides, the general cellular compartments associated with compartmentation are thought to be the vacuole and the apoplast of leaf cells (Sandermann 1994, Coleman et al. 1997, Pilon-Smits 2005). Therefore, it can be deduced that 2-OH IBP was likely to enter the sheath symplast from the xylem via diffusion followed by conjugation with glucose in the cytosol and finally was transported to the vacuole or the apoplast by ATP-dependent pumps. Whereas, CBX IBP and 1-OH IBP preferred to enter the lamina symplast and then conjugated and compartmentized in the same way like that of 2-OH IBP.

Figure 5-10. Mean concentrations of IBP and its metabolites (CBX IBP, 2-OH IBP and 1-OH IBP) in the lamina (L) and sheath (S) tissues of *Typha angustifolia* collected at 1/3L and 2/3L in the planted bed

5.4 Contribution of Plant Uptake to IBP Removal

Figure 5-11 illustrates the contribution of plant uptake to IBP removal by normalizing the concentrations of CBX IBP, 2-OH IBP and 1-OH IBP in the lamina and sheath tissues of *Typha angustifolia* to their corresponding IBP concentrations. Losses due to the conjugation and compartmentation of CBX-IBP, 2-OH IBP and 1-OH IBP in leaf tissues are neglected in this case. As shown in Figure 5-11, the total normalized IBP concentrations in the lamina and sheath tissues at 1/3L were 1821.6 ng/L and 1858.1 ng/L, respectively, while they were 1969.5 ng/L and 1839.7 ng/L at 2/3L. It can be deduced that the level of IBP accumulated in the leaves of *Typha angustifolia* through plant uptake is spatially uniform in the investigated HSSF-CW.

Figure 5-11. Normalized IBP concentrations in the lamina (L) and sheath (S) tissues of *Typha angustifolia* collected at 1/3L and 2/3L in the planted bed. 1/3L-L: lamina tissues at 1/3L; 1/3L-S: sheath tissues at 1/3L; 2/3L-L: lamina tissues at 2/3L; 2/3L-S: sheath tissues at 2/3L. Different legends represent the contribution of target compounds to the normalized IBP concentrations

5.5 Contribution of Microbial Degradation in Rhizosphere to IBP Removal

As shown in Figure 5-4, the concentration of hydroxyl ibuprofen (OH IBP) including 2-OH IBP and 1-OH IBP gradually increased along the flow path in both the planted and unplanted beds, which was consistent with the continuous degradation of IBP in water. The concentration of 2-OH IBP was significantly higher than that of 1-OH IBP. These observations indicate that the two hydroxylated metabolites of IBP were not biodegraded in the HSSF-CW under the experimental conditions and that 2-OH IBP was the prominent hydroxylated metabolite. Such findings agree with the experimental results of other biological treatment systems reported (Zwiener et al. 2002, Weigel et al. 2004, Quintana et al. 2005, Collado et al. 2012, Ferrando-Climent et al. 2012).

By comparing the concentrations of OH IBP in water between the planted and unplanted beds (Figure 5-4), it exhibits that plants played a positive role in the production of OH IBP ($p < 0.05$). Furthermore, it has been noted earlier that the uptake of OH IBP to the plant roots was very low or could be neglected. Thus, the microbial degradation of IBP in rhizosphere was mainly responsible for the higher level of OH IBP in the planted bed compared to that in the unplanted bed. Such a process was most probably related to the activities of aerobic microorganisms in the rhizosphere which could be supported by the exudates and oxygen released from the plant roots (Imfeld et al. 2009, Garcia-Rodríguez et al. 2014, Verlicchi and Zambello 2014).

In order to further quantify the contribution of the microbial degradation in rhizosphere to the total IBP removal in the planted bed, the proportion of the IBP removal due to OH IBP production was analyzed using the data from Figure 4-8 and Figure 5-4. According to Figure 4-8, the IBP removal occurred in the three zones of treatment section in the planted bed along the flow path (upstream 1/3 zone, middle 1/3 zone and downstream 1/3 zone) was 29.1, 27.7 and 17.3 $\mu\text{g/L}$, respectively. As calculated from Figure 5-4, the differences between the production

of OH IBP (2-OH IBP and 1-OH IBP) in the planted and unplanted beds corresponded to 4.0, 5.2 and 0.2 µg/L of IBP in the three zones along the flow path, respectively. These differences could be the results of microbial degradation of IBP in rhizosphere. As a result, the contribution of the microbial degradation in rhizosphere responsible for the production of OH IBP to the total IBP removal in the planted bed was 13.7%, 18.9% and 1.1% in the upstream 1/3, middle 1/3 and downstream 1/3 zones, respectively. It indicates that the contribution happened predominantly in the middle 1/3 zone, secondly in the upstream 1/3 zone and minimally in the downstream 1/3 zone. The possible phytotoxicity of high-load IBP in the upstream 1/3 zone might to some extent restrict the microbial degradation of IBP in rhizosphere. The phytotoxicity induced by the high concentration of IBP decreased with the degradation of IBP along the flow path. Thus, in the following middle 1/3 zone, the relatively less phytotoxicity due to the lower concentration of IBP could enhance the microbial degradation of IBP in rhizosphere. However, in the downstream 1/3 zone, the microbial degradation of IBP in rhizosphere appeared to be limited by the low concentration of IBP due to the lack of abundant organic source. Overall, the contribution of the microbial degradation in rhizosphere responsible for the production of OH IBP to the total IBP removal in the planted bed was about 12.7%.

5.6 Conclusions

This research study represents a first attempt to investigate the issue on the contribution of plant uptake and microbial degradation in rhizosphere to the removal of IBP from wastewater by *Typha angustifolia* in HSSF-CW. In this study, the presence of IBP in the leaves demonstrates that IBP was taken up by the roots of *Typha angustifolia* and was translocated from the roots to leaves. The even distribution of IBP in the leaf tissues of *Typha angustifolia* indicates that IBP was transported well in the xylem sap of *Typha angustifolia* driven by the transpiration process.

Chemical transformation of IBP to CBX IBP, 2-OH IBP and 1-OH IBP happened in both the plant tissues and surrounding water. The three IBP metabolites in water

showed negligible tendency to be taken up by the roots of *Typha angustifolia*. Nevertheless, they were found to accumulate in the leaves of *Typha angustifolia* with an uneven distribution in the lamina and sheath tissues. The production of hydroxylated and carboxylated metabolites of IBP in the leaf tissues was mainly due to the enzymatic degradation of IBP which involved the catalytic actions of monooxygenases and carboxylesterases. Thereafter, the metabolites were conjugated with glucose under the catalysis of glucosyltransferases followed by the compartmentation in the vacuole and the apoplast of leaf cells. According to the uneven distribution of IBP metabolites in the lamina and sheath tissues, 2-OH IBP was likely to be conjugated and compartmentized in the sheath tissues, whereas CBX IBP and 1-OH IBP preferred to be conjugated and compartmentized in the lamina tissues.

The normalized IBP amount (taking its transformation into account) accumulated in the leaves of *Typha angustifolia* through the process of plant uptake was spatially uniform in the studied HSSF-CW (1821.6-1969.5 ng/g).

The contribution of the microbial degradation responsible for the production of hydroxyl ibuprofen (OH IBP) including 2-OH IBP and 1-OH IBP to the total IBP removal in the planted bed was 13.7%, 18.9% and 1.1% at the upstream 1/3, middle 1/3 and downstream 1/3 zones, respectively. Overall, the contribution of the microbial degradation in the rhizosphere responsible for the production of OH IBP to the total IBP removal was about 12.7%.

CHAPTER 6 BACTERIA DEVELOPMENT UNDER HIGH-LOADED IBP

6.1 Introduction

As presented in Chapters 4 and 5, it was inferred that microbial degradation played a significant role in the removal of IBP from wastewater in the studied HSSF-CW system. Thus, conducting an investigation on the microorganisms within the wetland beds should lead to a further understanding on the microbial degradation mechanisms of IBP. In this chapter, bacteria were the research focus and their diversity and evenness at different sampling locations along with time were studied by using the DGGE method. Based on the DGGE results, some representative DNA samples were selected for pyrosequencing analysis to identify the dominant bacterial communities and the probable bacteria that could be responsible for the microbial degradation of IBP within the studied HSSF-CW system.

6.2 Diversity and Evenness of Bacteria

The Shannon diversity index (H) and the Pielou's evenness index (E_H) of bacteria in the sediment mixture samples were calculated by using the data of richness (S) and peak intensity obtained from the image of DGGE gel. The DGGE images for the sediment mixture samples collected at the sampling locations of 1/3L and 2/3L in both the planted and unplanted beds from periods 1 to 5 are shown in Figures 6-1 to 6-5. Results of diversity and evenness of bacteria in the planted and unplanted beds during these five periods are listed in Tables 6-1 to 6-5. In these figures and tables, the samples were labelled according to the convention described in Section 3.3.

It can be seen from the Figures 6-1 to 6-5 that richness of the bacteria in the sediment mixture samples was not abundant. This observation might be attributed to the components of samples in which the interstitial water accounted for the most proportion. Sediments that normally originated from detached biofilms, plant residues and silt (brought from the soil around the plant roots) constituted a small

amount of the samples. Besides, the fact that the wetland beds were not inoculated by sludge and that the influent water was synthetic might also have contributed to the limited richness of bacteria within the wetland system which had not been operated for a substantial length of time such as years.

During any given period, four samples were collected from the same location and were reflected as four lanes on the image of DGGE gel. The lane on the DGGE gel which exhibited the highest diversity among the four lanes was marked and the corresponding DNA sample was selected and sent for pyrosequencing analysis. During period 1, the selected samples were P-1/3L_1-4, P-2/3L_1-3, UP-1/3L_1-4 and UP-2/3L_1-1 (Table 6-1). During period 2, the selected samples were P-1/3L_2-1, P-2/3L_2-4, UP-1/3L_2-1 and UP-2/3L_2-3 (Table 6-2). During period 3, the selected samples were P-1/3L_3-2, P-2/3L_3-3, UP-1/3L_3-2 and UP-2/3L_3-3 (Table 6-3). During period 4, the selected samples were P-1/3L_4-3, P-2/3L_4-2, UP-1/3L_4-1 and UP-2/3L_4-3 (Table 6-4). During period 5, the selected samples were P-1/3L_5-1, P-2/3L_5-1, UP-1/3L_5-4 (simultaneously considering the richness) and UP-2/3L_5-4 (Table 6-5).

The variations of the diversity and evenness of bacteria at the sampling locations of 1/3L and 2/3L in both the planted and unplanted beds from periods 1 to 5 are shown in Figure 6-6. In the planted bed, the values of bacterial evenness fluctuated over a small range along with time except for that in period 4 at 2/3L. The bacterial evenness ranged from 0.75 to 0.88 at 1/3L and from 0.69 to 0.97 at 2/3L. On the whole, the bacterial evenness at 2/3L was a little higher than that at 1/3L, showing a less variation in bacterial communities.

As shown in Figure 6-6, in the planted bed, a sharp decrease in the bacterial diversity was observed at 1/3L from period 1 (1.75) to period 2 (1.20), probably attributable to the pressure caused by the suddenly high-loaded IBP. As the bacterial communities acclimated to the wastewater environment in the planted bed, the bacterial diversity began to rise from period 2 and reached a peak during period 4 (1.50). As analyzed in Chapter 4, the period 4 (254th to 278th day) corresponded to

the local dry season during which the removal of $\text{NH}_4^+\text{-N}$, TP and IBP in the planted bed was relatively poor compared to that in other periods. In the subsequent period 5, the removal efficiencies of $\text{NH}_4^+\text{-N}$, TP and IBP became recovered. It implies that plant uptake was weakened during period 4, which mainly contributed to the relatively low removal of $\text{NH}_4^+\text{-N}$, TP and IBP in the planted bed. The bacterial communities responsible for the degradation of target pollutants seemed not to be significantly influenced by the weather conditions in period 4. The adverse impacts of the dry season on the bacterial diversity were likely to be lingeringly reflected during period 5, inducing a drop in the bacterial diversity (1.33).

Decrease in the bacterial diversity at 2/3L from period 1 (1.10) to period 2 (1.06) was not as obvious as that at 1/3L. Such a phenomenon indicates that the high loading of IBP had not strongly affected the bacterial communities at 2/3L possibly due to the stress relief at 1/3L. The bacterial diversity at 2/3L also rose to a peak point in period 4 (1.18) but exhibited to drop during period 5 (0.76) in a similar manner to the observation at 1/3L due to the weather reasons.

In the unplanted bed, the bacterial evenness showed a lowest point at both the sampling locations in period 3, implying that the variation in bacterial communities during this period was higher than those in other periods. During other periods, the bacterial evenness was in the range of 0.82 to 0.93 at 1/3L and 0.76 to 0.87 at 2/3L, showing a relatively stable level. At the same time, the bacterial diversity at the two sampling locations in period 3 also dropped to the lowest level. It was most probable that the bacterial in the unplanted bed were undergoing an acclimation process from periods 1 to 3, which took a longer time than that in the planted bed. It implies that the bacteria communities in the planted bed were more sensitive to their surrounding environment and were more active to make a response to the abiotic stress, showing a strong resistance to the high loading of IBP. During period 4, both the bacterial diversity and evenness at the two sampling locations increased to a peak level. The decrease in them during period 5 might be due to the adverse influences caused by the dry season in period 4.

Figure 6-1. Images of DGGE gels for the sediment mixture samples collected at the sampling locations of 1/3L and 2/3L in both the planted and unplanted beds during period 1

Table 6-1. Diversity and evenness of bacteria in the planted and unplanted beds during period 1

Planted Bed				Unplanted Bed			
	S	H	E _H		S	H	E _H
P-1/3L				UP-1/3L			
1-1	7	1.69	0.87	1-1	4	1.35	0.98
1-2	7	1.71	0.88	1-2	4	1.33	0.96
1-3	7	1.75	0.90	1-3	5	1.37	0.85
1-4	8	1.84	0.89	1-4	5	1.52	0.95
P-2/3L				UP-2/3L			
1-1	3	1.01	0.92	1-1	8	1.86	0.90
1-2	4	1.20	0.87	1-2	7	1.77	0.91
1-3	4	1.23	0.89	1-3	6	1.61	0.90
1-4	3	0.97	0.88	1-4	3	0.70	0.63

Figure 6-2. Images of DGGE gels for the sediment mixture samples collected at the sampling locations of 1/3L and 2/3L in both the planted and unplanted beds during period 2

Table 6-2. Diversity and evenness of bacteria in the planted and unplanted beds during period 2

Planted Bed				Unplanted Bed			
	S	H	E _H		S	H	E _H
P-1/3L				UP-1/3L			
2-1	6	1.60	0.90	2-1	7	1.74	0.89
2-2	3	0.93	0.84	2-2	4	1.21	0.87
2-3	4	1.08	0.78	2-3	4	1.15	0.83
2-4	5	1.18	0.74	2-4	5	1.40	0.87
P-2/3L				UP-2/3L			
2-1	3	0.98	0.90	2-1	4	1.16	0.84
2-2	3	1.07	0.97	2-2	3	0.87	0.79
2-3	3	1.02	0.93	2-3	6	1.66	0.92
2-4	4	1.19	0.86	2-4	6	1.63	0.91

Figure 6-3. Images of DGGE gels for the sediment mixture samples collected at the sampling locations of 1/3L and 2/3L in both the planted and unplanted beds during period 3

Table 6-3. Diversity and evenness of bacteria in the planted and unplanted beds during period 3

Planted Bed				Unplanted Bed			
	S	H	E _H		S	H	E _H
P-1/3L				UP-1/3L			
3-1	3	0.92	0.84	3-1	3	0.59	0.54
3-2	6	1.41	0.79	3-2	4	1.03	0.74
3-3	5	1.40	0.87	3-3	3	0.78	0.71
3-4	6	1.34	0.75	3-4	4	0.97	0.70
P-2/3L				UP-2/3L			
3-1	2	0.69	1.00	3-1	4	0.71	0.51
3-2	4	1.32	0.95	3-2	3	0.74	0.67
3-3	5	1.52	0.94	3-3	4	0.95	0.68
3-4	3	1.08	0.99	3-4	4	0.90	0.65

Figure 6-4. Images of DGGE gels for the sediment mixture samples collected at the sampling locations of 1/3L and 2/3L in both the planted and unplanted beds during period 4

Table 6-4. Diversity and evenness of bacteria in the planted and unplanted beds during period 4

Planted Bed				Unplanted Bed			
	S	H	E _H		S	H	E _H
P-1/3L				UP-1/3L			
4-1	5	1.20	0.75	4-1	7	1.83	0.94
4-2	7	1.36	0.70	4-2	7	1.72	0.88
4-3	10	1.85	0.80	4-3	5	1.50	0.93
4-4	8	1.58	0.76	4-4	5	1.50	0.93
P-2/3L				UP-2/3L			
4-1	4	0.96	0.69	4-1	5	1.43	0.89
4-2	8	1.51	0.73	4-2	6	1.49	0.83
4-3	6	1.26	0.70	4-3	6	1.54	0.86
4-4	5	1.01	0.63	4-4	4	1.28	0.92

Figure 6-5. Images of DGGE gels for the sediment mixture samples collected at the sampling locations of 1/3L and 2/3L in both the planted and unplanted beds during period 5

Table 6-5. Diversity and evenness of bacteria in the planted and unplanted beds during period 5

Planted Bed				Unplanted Bed			
	S	H	E _H		S	H	E _H
P-1/3L				UP-1/3L			
5-1	6	1.59	0.89	5-1	4	1.13	0.82
5-2	6	1.39	0.77	5-2	4	1.21	0.88
5-3	5	1.20	0.74	5-3	5	1.42	0.88
5-4	5	1.15	0.71	5-4	6	1.27	0.71
P-2/3L				UP-2/3L			
5-1	3	1.00	0.91	5-1	6	1.33	0.74
5-2	2	0.67	0.97	5-2	6	1.37	0.76
5-3	2	0.69	0.99	5-3	5	1.17	0.73
5-4	2	0.69	0.99	5-4	7	1.56	0.80

(a)

(b)

Figure 6-6. Variation of bacterial diversity and evenness at the sampling locations of 1/3L and 2/3L in both the planted bed (a) and unplanted bed (b) from periods 1 to 5

6.3 Bacterial Communities in the Planted Bed

6.3.1 Phylum Taxonomy of Bacteria in the Planted Bed

Based on the OTUs (operational taxonomic unit) analysis results of pyrosequencing for 16S rDNA, the taxonomic composition of bacterial communities at the phylum level in the planted bed from periods 1 to 5 is shown in Figure 6-7. The bacterial communities at 1/3L in the planted bed were distributed across 7 dominant phyla including Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Proteobacteria and Spirochaetes, while almost the same phyla except for Spirochaetes at 2/3L. The phyla of Acidobacteria, Actinobacteria and Spirochaetes which composed a very small part in the bacterial communities were not usually detected in all the sampling periods. The major phylum was Proteobacteria with a significant proportion of 57.0 – 83.7% at 1/3L, approximately exhibiting an increasing trend from periods 1 to 5 although there was a slight drop in period 5. At the sampling location of 2/3L, Proteobacteria also showed the highest percentage in the range from 75.2 to 90.3% with a rising tendency from periods 1 to 5. The phyla of Bacteroidetes, Chloroflexi and Firmicutes occupied about 5.3 – 12.3%, 1.6 – 4.5% and 7.3 – 16.4% at 1/3L, respectively, while they were 1.8 – 5.2%, 1.1 – 2.4% and 1.7 – 8.0% at 2/3L.

6.3.2 Phylogenetic Tree of Bacteria in the Planted Bed

The OTUs utilized to build the phylogenetic tree were selected from those that could be found in all the sampling periods. The phylogenetic tree which is used to show the relationship of bacterial communities can be seen in Figures 6-8 and 6-9 for the two sampling locations in the planted bed, respectively. The representative bacteria for the biodegradation of IBP were thought to exist at both the 1/3L and 2/3L sampling locations. Therefore, the OTUs named as BAC-HSSFCW-P2, BAC-HSSFCW-P13, BAC-HSSFCW-P22, BAC-HSSFCW-P18, BAC-HSSFCW-P8, BAC-HSSFCW-P15, BAC-HSSFCW-P4 and BAC-HSSFCW-P24 which could be observed at both the sampling locations were analyzed to find the probable bacteria responsible for the microbial degradation of IBP in the planted bed.

(a)

(b)

Figure 6-7. Bacterial communities at the phylum level at the sampling locations of 1/3L (a) and 2/3L (b) in the planted bed from periods 1 to 5

Thiomonas sp. (BAC-HSSFCW-P2) and *Thiobacillus* sp. (BAC-HSSFCW-P13) which belong to the class of Betaproteobacteria in the phylum of Proteobacteria have been reported as the bacteria able to oxidize sulfide (Hallberg and Johnson 2005, Ramírez et al. 2009, García-de-la-Fuente et al. 2011, Luo et al. 2013). *Thiobacillus* sp. was also reported to be involved in the reduction of nitrate by using sulfide as the electron donor (Pous et al. 2014). *Clostridium cavendishii* (BAC-HSSFCW-P4) and *Clostridium saccharobutylicum* (BAC-HSSFCW-P24) which belong to the genus of *Clostridium* within the Firmicutes phylum were demonstrated to have the ability to degrade glucose, starch, or xylose to hydrogen, carbon dioxide, ethanol, butanol, acetate, butyrate, lactate and acetone through fermentation process (Saint-Amans et al. 2001, Berezina et al. 2009, Noparat et al. 2011, Zhao et al. 2011). Such a process could conserve energy to support the growth of sulfide oxidizing bacteria and nitrate reducing bacteria (Wang et al. 2015a). Probably, it could explain the phenomenon that the proportion of *Thiomonas* sp. exhibited the highest abundance in comparison with other species in the planted bed (Figures 6-8 and 6-9).

Cytophaga sp. (BAC-HSSFCW-P18), Sphingobacteriales (BAC-HSSFCW-P8) and Cytophagales (BAC-HSSFCW-P15) which are within the same phylum of Bacteroidetes have been verified to possess the capability to anaerobically degrade complex organic compounds such as alkanes, benzene, toluene, ethylbenzene, tribromophenol and polycyclic aromatic hydrocarbons through the simultaneous process of sulfate reduction (Phelps et al. 1998, Rosselló-Mora et al. 1999, Pérez-Jiménez et al. 2001, Nakagawa et al. 2002, Zhang et al. 2013b). Thus, it can be inferred that *Cytophaga* sp., Sphingobacteriales, and Cytophagales might play a significant role in the microbial degradation of IBP in the planted bed. Besides, Burkholderiales (BAC-HSSFCW-P22) which belongs to the class of Betaproteobacteria in the Proteobacteria phylum was reported to be able to degrade polycyclic aromatic hydrocarbons (Guo et al. 2011), implying that it might also contribute to the microbial degradation of IBP.

Figure 6-8. Bacterial phylogenetic tree at the sampling location of 1/3L in the planted bed from periods 1 to 5 (OUT percent is shown in the brackets)

Figure 6-9. Bacterial phylogenetic tree at the sampling location of 2/3L in the planted bed from periods 1 to 5 (OUT percent is shown in the brackets)

6.4 Bacterial Communities in the Unplanted Bed

6.4.1 Phylum Taxonomy of Bacteria in the Unplanted Bed

The taxonomic composition of bacterial communities at the phylum level in the unplanted bed from periods 1 to 5 is shown in Figure 6-10. The bacterial communities at 1/3L in the unplanted bed contained 7 dominant phyla such as Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Deinococcus-Thermus, Firmicutes and Proteobacteria. At the sampling location of 2/3L, the bacterial communities were also distributed across 7 dominant phyla but they were a little different from those at 1/3L, including Acidobacteria, Bacteroidetes, Chlorobi, Chloroflexi, Firmicutes, Proteobacteria and Spirochaetes.

The phyla of Acidobacteria, Actinobacteria and Deinococcus-Thermus occupied a very small portion of the bacterial population at 1/3L and were not usually observed in all the sampling periods. The similar situation was also found for Acidobacteria, Bacteroidetes, Chlorobi, Chloroflexi and Spirochaetes at 2/3L. In both the sampling locations, only two phyla of Firmicutes and Proteobacteria were the commonly detected bacterial communities throughout the experiment in which Proteobacteria accounted for a predominant proportion. The percentage of Proteobacteria in the unplanted bed varied from 70.0 to 81.8% at 1/3L and 75.8 to 97.3% at the 2/3L. For the phyla of Firmicutes, its proportion was approximate 7.3 – 16.3% and 1.2 – 10.0% at 1/3 L and 2/3L, respectively. For the phyla of Bacteroidetes and Chloroflexi which could be found in all the sampling periods at 1/3 L, they occupied about 1.7 – 3.1% and 4.0 – 10.6%, respectively.

(a)

Figure 6-10. Bacterial communities at the phylum level at the sampling locations of 1/3L (a) and 2/3L (b) in the unplanted bed from periods 1 to 5

6.4.2 Phylogenetic Tree of Bacteria in the Unplanted Bed

In Section 6.3.2, the *Cytophaga sp.*, Sphingobacteriales, and Cytophagales which are within the phylum of Bacteroidetes were suspected to be the bacteria able to degrade IBP in the planted bed. Although Bacteroidetes phylum was not often found at 2/3L in the unplanted bed, it was selected to establish the phylogenetic tree of the bacterial communities in the unplanted bed. The Chloroflexi phylum was also selected to build the phylogenetic tree due to its high proportion during period 2 at 2/3L although it was not usually detected at 2/3L. The phylogenetic trees at the two sampling locations in the unplanted bed are shown in Figures 6-11 and 6-12, respectively. The representative OTUs named as BAC-HSSFCW-UP22, BAC-HSSFCW-UP25, BAC-HSSFCW-UP11, BAC-HSSFCW-UP3, BAC-HSSFCW-UP8, BAC-HSSFCW-UP12, BAC-HSSFCW-UP2, BAC-HSSFCW-UP20, BAC-HSSFCW-UP1 and BAC-HSSFCW-UP6 were analyzed to find the probable bacteria responsible for the microbial degradation of IBP in the unplanted bed.

Thiobacillus thioparus (*Thiobacillus sp.*) (BAC-HSSFCW-UP3) and *Thiomonas sp.* (BAC-HSSFCW-UP11) which belong to the class of Betaproteobacteria within the Proteobacteria phylum were discussed in Section 6.3.2 to state their ability of oxidizing sulfide or sulfur (Hallberg and Johnson 2005, Ramírez et al. 2009, García-de-la-Fuente et al. 2011, Luo et al. 2013). Besides, *Thiovirga sulfuroxydans* (BAC-HSSFCW-UP22) in the class of Gammaproteobacteria and *Sulfuricurvum kujiense* (BAC-HSSFCW-UP8) in the class of Epsilonproteobacteria both of which belong to the phylum of Proteobacteria have also been reported to have the capability to oxidize sulfide or sulfur (Kodama and Watanabe 2004, Vannini et al. 2008). Moreover, during the oxidation process of sulfide or sulfur, *Sulfuricurvum kujiense* could use the nitrate as the electron acceptor under anaerobic conditions to perform denitrification.

Clostridium beijerinckii (BAC-HSSFCW-UP2) which belongs to the *Clostridium sp.* in the phylum of Firmicutes was thought to be able to degrade glucose, starch, or xylose to hydrogen, ethanol, butanol, acetate, butyrate, lactate, and acetone through fermentation process (Berezina et al. 2009, Noparat et al. 2011, Zhao et al. 2011)

similar to that mentioned in Section 6.3.2. In addition, *Levilinea* sp. (BAC-HSSFCW-UP1) and *Longilinea* sp. (BAC-HSSFCW-UP6) which are in the same class of Anaerolineae and the same phylum of Chloroflexi have also been reported as the bacteria species with the ability to ferment glucose for growth to produce acetate, lactate, hydrogen, and carbon dioxide (Grégoire et al. 2011). Besides, *Enterobacter asburiae* (BAC-HSSFCW-UP3) belonging to the class of Gammaproteobacteria within the phylum of Proteobacteria was verified to be able to induce the glucose dehydrogenase for the oxidation of glucose to yield hydrogen (Gyaneshwar et al. 1999, Shin et al. 2007).

Cytophaga sp. (BAC-HSSFCW-UP12) which was discussed in Section 6.3.2 as a kind of sulfate reducing bacterium showed a possibility to perform the biodegradation of IBP and was also found in the unplanted bed. Besides, *Desulfobacterium* sp. that belongs to the family of Desulfobacteraceae in the Proteobacteria phylum was also regarded as a type of sulfate reducing bacterium with a capability to degrade complex organic pollutants (Zhang et al. 2008a). Therefore, *Desulfobacterium* sp. might be also responsible for the microbial degradation of IBP in the unplanted bed.

Figure 6-11. Bacterial phylogenetic tree at the sampling location of 1/3L in the unplanted bed from periods 1 to 5 (OUT percent is shown in the brackets)

Figure 6-12. Bacterial phylogenetic tree at the sampling location of 2/3L in the unplanted bed from periods 1 to 5 (OUT percent is shown in the brackets)

6.5 Conclusions

The bacterial evenness in the planted bed fluctuated slightly over time, suggesting that the variation in bacterial communities was relatively insignificant. For the bacterial diversity, a sharp drop was observed from periods 1 to 2 due to the pressure induced by the high-loaded IBP in the influent water. In the subsequent periods (2 to 5), it became recovered after the bacterial communities acclimated to the wastewater environment although there were some negative impacts caused by

the dry season in Singapore. In the case of the unplanted bed, both the bacterial evenness and diversity dropped from periods 1 to 3, indicating that the bacteria communities in the unplanted bed took a longer time to adapt to the wastewater environment than that in the planted bed. It implies that the bacteria communities in the planted bed were more sensitive to their surrounding environment and were more active to make a response to the abiotic stress, showing a strong resistance to the high loading of IBP.

There are only a handful reported studies which focused on the bacterial species actively involved in the microbial degradation of IBP. Murdoch and Hay (2015) cultivated a bacterium on IBP to analyze the accumulation of IBP metabolites. This bacterium which was isolated from activated sewage sludge belonged to the *Variovorax* genus within the phylum of β -proteobacteria. Their experimental findings suggested the possible metabolism of IBP via a trihydroxyibuprofen pathway. Moreover, *Sphingomonas* sp. (Kagle et al. 2009) and *Patulibacter* sp. (Almeida et al. 2013) were investigated by quantitative proteomics to identify the genes responsible for the microbial degradation of IBP. To date, *Sphingomonas* sp. which was isolated from a sewage treatment plant is the only reported bacterium with the ability to utilize IBP as the sole carbon and energy source (Murdoch and Hay 2005, 2013).

The reported bacteria responsible for the microbial degradation of IBP were not observed in the investigated HSSF-CW system. In the experiment, some sulfate-reducing bacteria were thought to be involved in the microbial degradation of IBP, such as *Cytophaga* sp., Sphingobacteriales, and Cytophagales in the planted bed, while *Cytophaga* sp. and *Desulfobacterium* sp. in the unplanted bed. Moreover, Burkholderiales observed in the planted bed might also contribute to the microbial degradation of IBP. In order to obtain an in-depth understanding on the efficiency and metabolism of these bacteria for the microbial degradation of IBP, cultivation of some bacteria strains is in requirement.

All of the above bacteria probably responsible for the microbial degradation of IBP were due to the predominantly anaerobic conditions in the investigated HSSF-CW system. The results of pyrosequencing analysis in this study were strongly related to the sediment mixture samples from which the bacteria DNA templates were isolated. However, the sediment mixture samples couldn't fully stand for all the bacterial communities in the rhizosphere of plants, especially for the aerobic bacteria which were thought to play a major role in IBP degradation (Chapters 4 and 5). It is quite difficult to collect representative samples for the aerobic bacteria from the rhizosphere in this study. Such an issue requires more effort and meticulous work in future studies.

CHAPTER 7 CONCLUSIONS AND RECOMMENDATIONS

The performance of a mesocosm-scale horizontal subsurface flow constructed wetland (HSSF-CW) system for the treatment of synthetic wastewater with high-loaded ibuprofen (IBP) was investigated over a period of 342 days under tropical conditions. The mechanisms associated with IBP removal were studied from the perspectives of plant uptake and microbial degradation. The bacteria development in the HSSF-CW system was analyzed to determine the probable bacteria responsible for the microbial degradation of IBP. The major conclusions are summarized as followed:

- (1) Compared to an unplanted HSSF-CW, the removal efficiencies of COD, NH_4^+ -N, TP and IBP in the planted HSSF-CW were high and satisfactory, indicating an efficient operation without adverse impacts caused by the high-loaded IBP. *Typha angustifolia* in the planted bed showed a positive role in the removal of NH_4^+ -N, TP and IBP.
- (2) IBP was taken up by the roots of *Typha angustifolia* followed by translocation to leaves. Chemical transformation of IBP to carboxylic acid ibuprofen (CBX IBP), 2-hydroxy ibuprofen (2-OH IBP) and 1-hydroxy ibuprofen (1-OH IBP) was observed to take place in both the plant tissues and surrounding water. The three metabolites showed negligible tendency of root uptake, but were observed to accumulate in leaves. 2-OH IBP was found to be conjugated and compartmentized in the sheath tissues, whereas CBX IBP and 1-OH IBP were found to prefer the lamina tissues. Taking its transformation into account, the normalized IBP accumulated in leaves through plant uptake was uniform in the HSSF-CW (1821.6-1969.5 ng/L). The contribution of the microbial degradation in the rhizosphere responsible for the production of hydroxyl ibuprofen (OH IBP) to the total IBP removal was about 12.7%. The observations suggest the good ability of *Typha angustifolia* for IBP removal in HSSF-CW.

- (3) The bacterial evenness in the planted HSSF-CW fluctuated slightly over time, suggesting that the variation in bacterial communities was relatively insignificant. For the bacterial diversity in the planted bed, it was sharply decreased in the initial period but recovered in the subsequent periods after the bacteria communities acclimated to the wastewater environment. As for the unplanted bed, the bacteria communities took a longer time to adapt to the wastewater environment than that in the planted bed. It indicates that the bacteria communities in the planted bed exhibited a stronger resistance to the high loading of IBP.
- (4) Some sulfate-reducing bacteria were thought to be involved in the microbial degradation of IBP in HSSF-CWs, such as *Cytophaga sp.*, Sphingobacteriales and Cytophagales in the planted bed, while *Cytophaga sp.* and *Desulfobacterium sp.* in the unplanted bed. Besides, Burkholderiales observed in the planted bed might also contribute to the microbial degradation of IBP.

For a further in-depth understanding on the performance of HSSF-CW for the removal of high-loaded IBP from wastewater and the associated removal mechanisms, the following work is recommended:

- (1) Establish a hybrid constructed wetland system by connecting the HSSF-CW to another in series to improve the performance of wetland system when countering a high loading of IBP.
- (2) Investigate the concentration of IBP accumulated in the roots of *Typha angustifolia* and in the biomass of a whole plant to better understand the process of plant uptake and to establish the mass balance on IBP.
- (3) Study on the modification, conjugation and compartmentation involved in the detoxification process of IBP in plant tissues at the cytological level to further explain the chemical accumulation differences between the lamina and sheath tissues and to evaluate the potential toxicity of IBP and its metabolites to plant.

- (4) Cultivate certain bacterial strains based on the results in this study to determine the most efficient bacterial species for the anaerobic degradation of IBP.
- (5) Look for an efficient way to collect representative samples from the rhizosphere to study the bacterial species responsible for the aerobic degradation of IBP.

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APPENDIX

Table A. Chemical structures and physicochemical properties of 115 pharmaceutical compounds treated by constructed wetlands

Pharmaceutical compound	Chemical Structure	Chemical formula	MW ^a	pK _a ^b	S _w ^c (mg L ⁻¹)	log K _{ow} ^d	log D _{ow} ^e (pH=7.4)
<i>Analgesic/Anti-inflammatory</i>							
Acetaminophen (Paracetamol)		C ₈ H ₉ NO ₂	151	9.38	1.4×10 ⁴	0.46	0.47
Buprenorphine		C ₂₉ H ₄₁ NO ₄	468	8.31	0.64	4.98	1.86
Codeine		C ₁₈ H ₂₁ NO ₃	299	8.21	1.2×10 ⁴	1.19	0.27
Diclofenac		C ₁₄ H ₁₁ Cl ₂ NO ₂	296	4.15	2.4	4.51	1.44
Ibuprofen		C ₁₃ H ₁₈ O ₂	206	4.91	21	3.97	0.58
Indomethacin		C ₁₉ H ₁₆ ClNO ₄	358	4.50	0.937	4.27	-0.16

Table A (Continued)

Pharmaceutical compound	Chemical Structure	Chemical formula	MW ^a	pK _a ^b	S _w ^c (mg L ⁻¹)	log K _{ow} ^d	log D _{ow} ^e (pH=7.4)
Ketoprofen		C ₁₆ H ₁₄ O ₃	254	4.45	51	3.12	-0.16
Mefenamic acid		C ₁₅ H ₁₅ NO ₂	241	4.20	1.12	5.12	2.37
Naproxen		C ₁₄ H ₁₄ O ₃	230	4.15	16	3.18	0.35
Orphenadrine		C ₁₈ H ₂₃ NO	269	8.91	113	3.77	1.99
Phenylbutazone		C ₁₉ H ₂₀ N ₂ O ₂	308	4.50	21.95	3.16	0.85
Propyphenazone		C ₁₄ H ₁₈ N ₂ O	230	n.a. ^f	668	1.94	1.74
Salicylic acid		C ₇ H ₆ O ₃	138	2.97	2240	2.26	-1.14

Table A (Continued)

Pharmaceutical compound	Chemical Structure	Chemical formula	MW ^a	pK _a ^b	S _w ^c (mg L ⁻¹)	log K _{ow} ^d	log D _{ow} ^e (pH=7.4)
Tramadol		C ₁₆ H ₂₅ NO ₂	263	9.41	750	2.63	0.29
<i>Antiallergic drugs</i>							
Desloratadine		C ₁₉ H ₁₉ ClN ₂	311	9.73	4	3.48	0.84
Diphenhydramine		C ₁₇ H ₂₁ NO	255	8.98	362.7	3.27	1.63
Fexofenadine		C ₃₂ H ₃₉ NO ₄	502	8.76	2.4×10 ⁻²	2.81	1.23
<i>Antibiotics</i>							
Amoxicillin		C ₁₆ H ₁₉ N ₃ O ₅ S	365	7.43	3430	0.87	-2.32
Ampicillin		C ₁₆ H ₁₉ N ₃ O ₄ S	349	7.44	439	1.35	-1.84

Table A (Continued)

Pharmaceutical compound	Chemical Structure	Chemical formula	MW ^a	pK _a ^b	S _w ^c (mg L ⁻¹)	log K _{ow} ^d	log D _{ow} ^e (pH=7.4)
Azithromycin		C ₃₈ H ₇₂ N ₂ O ₁₂	749	8.74	7.1	4.02	1.40
Chloramphenicol		C ₁₁ H ₁₂ Cl ₂ N ₂ O ₅	323	7.49	2500	1.14	1.02
Ciprofloxacin		C ₁₇ H ₁₈ FN ₃ O ₃	331	6.09	1.1×10 ⁴	0.28	-1.26
Ciprofloxacin HCl		C ₁₇ H ₁₉ ClFN ₃ O ₃	368	n.a.	n.a.	0.65	-1.35
Clarithromycin		C ₃₈ H ₆₉ NO ₁₃	748	8.99	0.34	3.16	2.06

Table A (Continued)

Pharmaceutical compound	Chemical Structure	Chemical formula	MW ^a	pK _a ^b	S _w ^c (mg L ⁻¹)	log K _{ow} ^d	log D _{ow} ^e (pH=7.4)
Nifuroxazide		C ₁₂ H ₉ N ₃ O ₅	275	n.a.	1416	1.49	0.50
Norfloxacin		C ₁₆ H ₁₈ FN ₃ O ₃	319	5.77	1.8×10 ⁵	-1.03	-1.59
Ofloxacin		C ₁₈ H ₂₀ FN ₃ O ₄	361	5.45	2.8×10 ⁴	-0.39	-1.41
Oxytetracycline HCl		C ₂₂ H ₂₅ ClN ₂ O ₉	497	n.a.	n.a.	n.a.	n.a.
Roxithromycin		C ₄₁ H ₇₆ N ₂ O ₁₅	837	9.08	0.019	2.75	2.98
Spiramycin		C ₄₃ H ₇₄ N ₂ O ₁₄	843	n.a.	n.a.	2.74	1.26

Table A (Continued)

Pharmaceutical compound	Chemical Structure	Chemical formula	MW ^a	pK _a ^b	S _w ^c (mg L ⁻¹)	log K _{ow} ^d	log D _{ow} ^e (pH=7.4)
Sulfadiazine		C ₁₀ H ₁₀ N ₄ O ₂ S	250	6.36	77	-0.09	-0.80
Sulfamethazine (Sulfadimidine)		C ₁₂ H ₁₄ N ₄ O ₂ S	278	7.59	2846	0.89	0.16
Sulfadimethoxine		C ₁₂ H ₁₄ N ₄ O ₄ S	310	6.91	343	1.63	-1.22
Sulfamethoxazole		C ₁₀ H ₁₁ N ₃ O ₃ S	253	6.16	3942	0.89	-0.54
Sulfapyridine		C ₁₁ H ₁₁ N ₃ O ₂ S	249	8.43	268	0.35	0.41
Triclosan		C ₁₂ H ₇ Cl ₃ O ₂	290	7.68	4.6	4.76	5.20
Trimicosin		C ₄₆ H ₈₀ N ₂ O ₁₃	869	n.a.	n.a.	n.a.	n.a.

Table A (Continued)

Pharmaceutical compound	Chemical Structure	Chemical formula	MW ^a	pK _a ^b	S _w ^c (mg L ⁻¹)	log K _{ow} ^d	log D _{ow} ^e (pH=7.4)
Trimethoprim		C ₁₄ H ₁₈ N ₄ O ₃	290	7.12	400	0.91	0.47
<i>Antidiabetics</i>							
Glibenclamide		C ₂₃ H ₂₈ ClN ₃ O ₅ S	494	n.a.	0.06	4.79	1.82
Glimepiride		C ₂₄ H ₃₄ N ₄ O ₅ S	491	4.32	0.08	4.70	1.00
<i>Antidysenterics</i>							
Loperamide		C ₂₉ H ₃₃ ClN ₂ O ₂	477	9.41	0.04	5.15	3.61

Table A (Continued)

Pharmaceutical compound	Chemical Structure	Chemical formula	MW ^a	pK _a ^b	S _w ^c (mg L ⁻¹)	log K _{ow} ^d	log D _{ow} ^e (pH=7.4)
<i>Antifungals</i>							
Clotrimazole		C ₂₂ H ₁₇ ClN ₂	345	6.62	0.03	6.26	4.90
Fluconazole		C ₁₃ H ₁₂ F ₂ N ₆ O	306	12.71	335.5	0.25	0.50
<i>Antihypertensives</i>							
Diltiazem		C ₂₂ H ₂₆ N ₂ O ₄ S	415	8.18	465	2.79	3.36
Dipyridamole		C ₂₄ H ₄₀ N ₈ O ₄	505	6.59	922	1.52	3.33

Table A (Continued)

Pharmaceutical compound	Chemical Structure	Chemical formula	MW ^a	pK _a ^b	S _w ^c (mg L ⁻¹)	log K _{ow} ^d	log D _{ow} ^e (pH=7.4)
Eprosartan		C ₂₃ H ₂₄ N ₂ O ₄ S	425	6.93	0.02	6.37	-0.63
Hydrochlorothiazide		C ₇ H ₈ ClN ₃ O ₄ S ₂	297	7.90	722	-0.07	-0.09
Irbersartan		C ₂₅ H ₂₈ N ₆ O	429	4.12	0.06	5.31	4.19
Telmisartan		C ₃₃ H ₃₀ N ₄ O ₂	515	3.65	3.5	6.66	3.49

Table A (Continued)

Pharmaceutical compound	Chemical Structure	Chemical formula	MW ^a	pK _a ^b	S _w ^c (mg L ⁻¹)	log K _{ow} ^d	log D _{ow} ^e (pH=7.4)
Verapamil		C ₂₇ H ₃₈ N ₂ O ₄	455	8.92	4.5	3.79	2.46
Anti-senile dementia drugs							
Memantine		C ₁₂ H ₂₁ N	179	10.7	894	3.28	0.08
Barbiturates							
Butalbital		C ₁₁ H ₁₆ N ₂ O ₃	224	8.48	1700	1.87	1.50
Pentobarbital		C ₁₁ H ₁₈ N ₂ O ₃	226	8.11	679	2.10	1.94
Phenobarbital		C ₁₂ H ₁₂ N ₂ O ₃	232	7.30	1110	1.47	1.45

Table A (Continued)

Pharmaceutical compound	Chemical Structure	Chemical formula	MW ^a	pK _a ^b	S _w ^c (mg L ⁻¹)	log K _{ow} ^d	log D _{ow} ^e (pH=7.4)
Beta-agonists							
Clenbuterol		C ₁₂ H ₁₈ Cl ₂ N ₂ O	277	9.63	3320	2.00	0.56
Salbutamol		C ₁₃ H ₂₁ NO ₃	239	10.30	1.4×10 ⁴	0.64	-2.12
Terbutaline		C ₁₂ H ₁₉ NO ₃	226	8.86	2.1×10 ⁵	0.90	-1.44
Beta-blockers							
Atenolol		C ₁₄ H ₂₂ N ₂ O ₃	266	9.60	1.3×10 ⁴	0.16	-1.76
Bisoprolol		C ₁₈ H ₃₁ NO ₄	325	9.67	2240	1.87	-0.20
Metoprolol		C ₁₅ H ₂₅ NO ₃	267	9.67	1.7×10 ⁴	1.88	-0.47
Nadolol		C ₁₇ H ₂₇ NO ₄	309	9.67	8330	0.81	-1.54

Table A (Continued)

Pharmaceutical compound	Chemical Structure	Chemical formula	MW ^a	pK _a ^b	S _w ^c (mg L ⁻¹)	log K _{ow} ^d	log D _{ow} ^e (pH=7.4)
Propranolol		C ₁₆ H ₂₁ NO ₂	259	9.42	61.7	3.48	0.79
Sotalol		C ₁₂ H ₂₀ N ₂ O ₃ S	272	9.43	5510	0.24	-1.68
Timolol		C ₁₃ H ₂₄ N ₄ O ₃ S	316	9.21	2740	1.83	-1.20
<i>Diuretics</i>							
Furosemide		C ₁₂ H ₁₁ ClN ₂ O ₅ S	331	4.25	149.3	2.03	-0.82
<i>Hormone inhibitors</i>							
Finasteride		C ₂₃ H ₃₆ N ₂ O ₂	373	2.22	11.7	3.03	3.83
<i>Lipid regulators</i>							
Atorvastatin		C ₃₃ H ₃₅ FN ₂ O ₅	558	4.33	0.63	4.24	0.74

Table A (Continued)

Pharmaceutical compound	Chemical Structure	Chemical formula	MW ^a	pK _a ^b	S _w ^c (mg L ⁻¹)	log K _{ow} ^d	log D _{ow} ^e (pH=7.4)
Bezafibrate		C ₁₉ H ₂₀ ClNO ₄	362	3.83	1.2	4.25	-1.09
Clofibric acid		C ₁₀ H ₁₁ O ₃ Cl	215	3.20	582.5	2.57	-1.20
Fenofibrate		C ₂₀ H ₂₁ ClO ₄	361	-4.90	0.25	5.19	4.80
Gemfibrozil		C ₁₅ H ₂₂ O ₃	250	4.42	5.0	4.77	1.69
Mevastatin		C ₂₃ H ₃₄ O ₅	391	4.21	4.8	3.95	3.57

Table A (Continued)

Pharmaceutical compound	Chemical Structure	Chemical formula	MW ^a	pK _a ^b	S _w ^c (mg L ⁻¹)	log K _{ow} ^d	log D _{ow} ^e (pH=7.4)
Pravastatin		C ₂₃ H ₃₆ O ₇	425	4.21	12.1	3.10	-0.88
Rosuvastatin		C ₂₂ H ₂₈ FN ₃ O ₆ S	482	4.00	18.0	2.48	-2.71
<i>Psychiatric drugs</i>							
Alprazolam (Xanax)		C ₁₇ H ₁₃ ClN ₄	309	5.08	13.1	2.12	1.92
Amitriptyline		C ₂₀ H ₂₃ N	277	9.40	0.82	4.92	2.65
Bupropion		C ₁₃ H ₁₈ ClNO	240	8.22	140.2	3.85	2.13
Carbamazepine		C ₁₅ H ₁₂ N ₂ O	236	15.96	17.7	2.45	1.90

Table A (Continued)

Pharmaceutical compound	Chemical Structure	Chemical formula	MW ^a	pK _a ^b	S _w ^c (mg L ⁻¹)	log K _{ow} ^d	log D _{ow} ^e (pH=7.4)
Citalopram		C ₂₀ H ₂₁ FN ₂ O	324	9.78	31.1	3.74	1.35
Dilantin (Phenytoin)		C ₁₅ H ₁₂ N ₂ O ₂	252	8.33	178.6	2.47	1.38
Diazepam (Valium)		C ₁₆ H ₁₃ ClN ₂ O	285	3.40	50	2.82	2.80
Fluoxetine		C ₁₇ H ₁₈ F ₃ NO	309	9.80	60.3	4.05	1.57
Hydroxyzine (Equipsoe)		C ₂₁ H ₂₇ ClN ₂ O ₂	375	7.82	428	2.36	2.25
Levomepromazine (Methotrimeprazine)		C ₁₉ H ₂₄ N ₂ OS	328	9.19	20	4.68	3.04

Table A (Continued)

Pharmaceutical compound	Chemical Structure	Chemical formula	MW ^a	pK _a ^b	S _w ^c (mg L ⁻¹)	log K _{ow} ^d	log D _{ow} ^e (pH=7.4)
Lorazepam		C ₁₅ H ₁₀ Cl ₂ N ₂ O ₂	321	13.00	80	2.39	2.47
Maprotiline		C ₂₀ H ₂₃ N	277	10.54	23.4	4.52	1.51
Mianserin		C ₁₈ H ₂₀ N ₂	264	6.92	21.5	3.35	0.77
Mirtazapine		C ₁₇ H ₁₉ N ₃	265	6.67	39.3	3.03	0.28
Oxazepam		C ₁₅ H ₁₁ ClN ₂ O ₂	287	10.61	179	2.24	2.22
Paroxetine		C ₁₉ H ₂₀ FNO ₃	329	9.77	8.5	3.10	1.48

Table A (Continued)

Pharmaceutical compound	Chemical Structure	Chemical formula	MW ^a	pK _a ^b	S _w ^c (mg L ⁻¹)	log K _{ow} ^d	log D _{ow} ^e (pH=7.4)
Perphenazine		C ₂₁ H ₂₆ ClN ₃ OS	404	7.94	23.7	4.15	3.60
Sertraline		C ₁₇ H ₁₇ Cl ₂ N	306	9.85	3.5	5.29	3.04
Venlafaxine		C ₁₇ H ₂₇ NO ₂	277	8.91	266.7	3.28	0.76
Zolpidem		C ₁₉ H ₂₁ N ₃ O	307	6.20	5.7	3.85	3.03
Receptor antagonists							
Alfuzosin		C ₁₉ H ₂₇ N ₅ O ₄	389	7.30	91.9	1.86	0.35
Cimetidine		C ₁₀ H ₁₆ N ₆ S	252	6.80	9380	0.40	-0.06

Table A (Continued)

Pharmaceutical compound	Chemical Structure	Chemical formula	MW ^a	pK _a ^b	S _w ^c (mg L ⁻¹)	log K _{ow} ^d	log D _{ow} ^e (pH=7.4)
Famotidine		C ₈ H ₁₅ N ₇ O ₂ S ₃	337	9.29	1271	-0.64	-1.03
Loratadine		C ₂₂ H ₂₃ ClN ₂ O ₂	383	4.33	0.01	5.20	5.94
Ranitidine		C ₁₃ H ₂₂ N ₄ O ₃ S	314	8.08	2.5×10 ⁴	0.27	-1.07
Stimulants/Psychoactive drugs							
Caffeine		C ₈ H ₁₀ N ₄ O ₂	194	10.40	2.2×10 ⁴	-0.07	-0.63
Cotinine		C ₁₀ H ₁₂ N ₂ O	176	8.80	1.0×10 ⁶	0.07	0.07
Fluoxetine		C ₁₇ H ₁₈ F ₃ NO	309	9.80	60.3	4.05	1.41

Table A (Continued)

Pharmaceutical compound	Chemical Structure	Chemical formula	MW ^a	pK _a ^b	S _w ^c (mg L ⁻¹)	log K _{ow} ^d	log D _{ow} ^e (pH=7.4)
Naloxone		C ₁₉ H ₂₁ NO ₄	327	7.27	1420	2.09	1.34
Veterinary drugs							
Enrofloxacin		C ₁₉ H ₂₂ FN ₃ O ₃	359	5.94	3400	0.70	1.08
Flunixin (Banamine)		C ₁₄ H ₁₁ F ₃ N ₂ O ₂	296	5.82	0.85	4.90	1.61
Monensin		C ₃₆ H ₆₂ O ₁₁	671	7.95	3.0×10 ⁻³	5.43	0.96
Narasin		C ₄₃ H ₇₂ O ₁₁	765	7.90	n.a.	6.13	3.17

Table A (Continued)

Pharmaceutical compound	Chemical Structure	Chemical formula	MW ^a	pK _a ^b	S _w ^c (mg L ⁻¹)	log K _{ow} ^d	log D _{ow} ^e (pH=7.4)
Salinomycin		C ₄₂ H ₇₀ O ₁₁	751	6.50	n.a.	8.53	2.64
Tetracycline		C ₂₂ H ₂₄ N ₂ O ₈	444	3.30	231	-1.30	-2.23

Notes: ^a MW: molecular weight; ^b pK_a: dissociation constant; ^c S_w: solubility at 25 °C (mg L⁻¹); ^d Log K_{ow}: octanol-water partition coefficient;

^e Log D_{ow}: octanol-water distribution coefficient at pH=7.4; ^f n.a.: not available

Data sources: Structure and physico-chemical data sources: ChemIDplus Advanced (<http://chem.sis.nlm.nih.gov/chemidplus/>), ChemSpider (<http://www.chemspider.com/>), DrugBank (<http://www.drugbank.ca/drugs>), Hazardous Substances Data Bank (<http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB>), Wikipedia (http://en.wikipedia.org/wiki/Main_Page).