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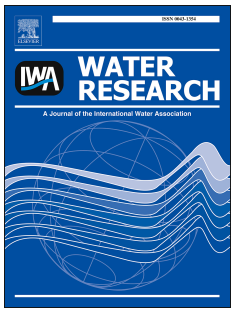

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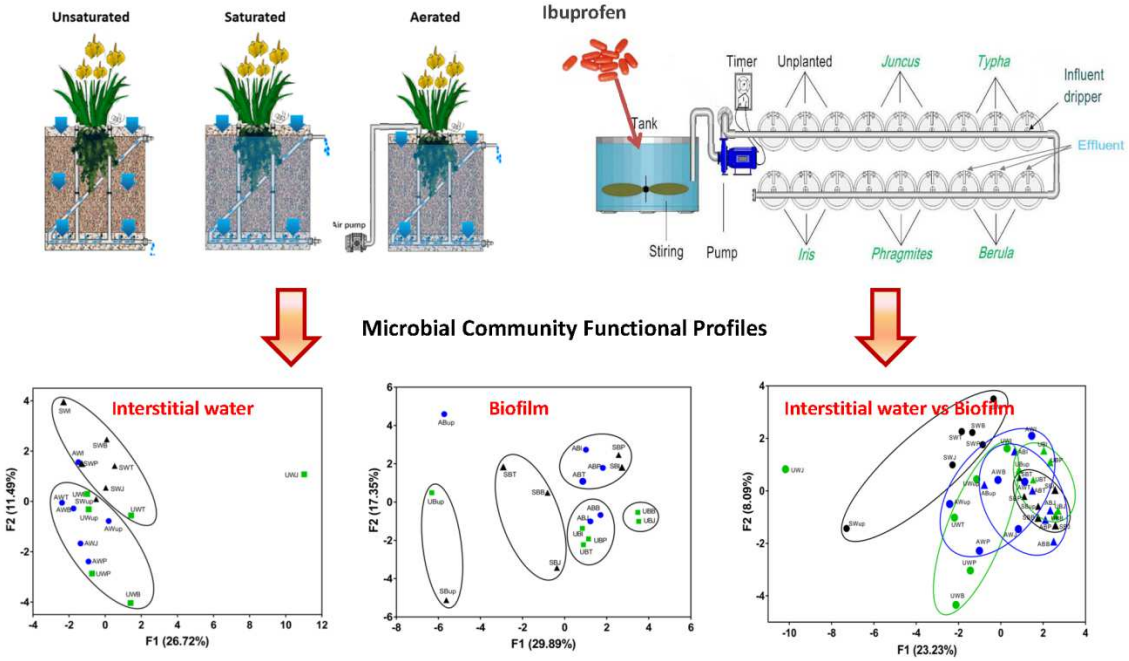

Impacts of design configuration and plants on the functionality of the microbial community of mesocosm-scale constructed wetlands treating ibuprofen

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Abstract

Microbial degradation is an important pathway during the removal of pharmaceuticals in constructed wetlands (CWs). However, the effects of CW design, plant presence, and different plant species on the microbial community in CWs have not been fully explored. This study aims to investigate the microbial community metabolic function of different types of CWs used to treat ibuprofen via community-level physiological profiling (CLPP) analysis. We studied the interactions between three CW designs (unsaturated, saturated and aerated) and six types of mesocosms (one unplanted and five planted, with *Juncus*, *Typha*, *Berula, Phragmites* and *Iris*) treating synthetic wastewater. Results show that the microbial activity and metabolic richness found in the interstitial water and biofilm of the unsaturated designs were lower than those of the saturated and aerated designs. Compared to other CW designs, the aerated mesocosms had the highest microbial activity and metabolic richness in the interstitial water, but similar levels of biofilm microbial activity and metabolic richness to the saturated mesocosms. In all three designs, biofilm microbial metabolic richness was significantly higher (p<0.05) than that of interstitial water. Both the interstitial water and biofilm microbial community metabolic function were influenced by CW design, plant presence and species, but design had a greater influence than plants. Moreover, canonical correlation analysis indicated that biofilm microbial communities in the three designs played a key role in ibuprofen degradation. The important factors identified as influencing ibuprofen removal were microbial AWCD (average well color development), microbial

metabolic richness, and the utilization of amino acids and amine/amides. The enzymes associated with co-metabolism of L-arginine, L-phenyloalanine and putrescine may be linked to ibuprofen transformations. These results provide useful information for optimizing the operational parameters of CWs to improve ibuprofen removal.

Keywords: Ibuprofen; Community-level physiological profiling (CLPP); Wetland plants, Horizontal subsurface flow; Vertical flow; Forced aeration

1. Introduction

Constructed wetlands (CWs) have been developed as an eco-friendly and cost-effective technology to remove not only conventional contaminants (such as organics, total nitrogen and phosphate) (Wu *et al.*, 2014) but also pharmaceuticals from wastewaters (Verlicchi and Zambello, 2014). It is generally recognized that the removal efficiency of pharmaceuticals in CWs is at least as good as that observed in conventional wastewater treatment processes (Li *et al.*, 2014, Huang *et al.*, 2015). Pharmaceutical removal in CWs is typically attributed to a combination of substrate sorption, phytoremediation, and microbial degradation processes (Dordio and Carvalho, 2013). Among these processes, however, microbial degradation has been demonstrated to be the main process for pharmaceutical removal in CWs (Hijosa-Valsero *et al.*, 2016, Zhang *et al.*, 2017b), and this has been shown for several different types of CW design configurations (Zhang *et al.*, 2017a). Therefore, a better understanding of the microbial community inside CWs would be beneficial for optimizing or improving the performance of CW systems in removing pharmaceuticals (Deng *et al.*, 2011).

CW design has been demonstrated to be an important factor affecting the composition of microbial communities (Button *et al.*, 2015, Lv *et al.*, 2017a). For example, Button *et al.* (2015) observed significantly different microbial community metabolic function between unsaturated vertical and saturated horizontal flow CWs used to treat domestic wastewater. Lv *et al.* (2017a) also found that the microbial

community metabolic function in unsaturated CWs significantly differed from that in saturated CW when treating the pesticide tebuconazole. From the years 1998 to 2016, 32 studies focused on the microbial communities of CWs and their role in the removal of emerging contaminants (Weber, 2016). Of these studies, none investigated the effects of CW design on microbial community metabolic function when treating pharmaceuticals. In this study, three CW experimental setups (unsaturated, saturated and saturated with aeration) were used to mimic the typical full-scale designs of horizontal subsurface flow, vertical flow, and horizontal subsurface flow with forced aeration systems, respectively. Horizontal subsurface flow and vertical flow are the more common designs for wastewater treatment in Europe, but the use of forced aeration in wetland systems to improve treatment performance is receiving more attention (Murphy *et al.*, 2016). For the first time, a CW mesocosm designed with forced aeration is directly compared with the unsaturated and saturated design.

Plants are also a crucial component of CWs. The presence of plants has been shown to influence the development of the microbial community by providing surface area for microbial attachment, releasing oxygen into the rhizosphere or secreting root exudates containing various enzymes and carbon-containing metabolites (Bais *et al.*, 2006, Dordio and Carvalho, 2013, Button *et al.*, 2015). Recent studies have reported that the presence of different plant species altered the metabolic function of the microbial community in CWs that were used to treat

106 diluted fish farm sludge (Bissegger *et al.*, 2014, Button *et al.*, 2016a) or
107 pesticide-contaminated water (Lv *et al.*, 2017b). However, studies of plant effects on
108 microbial community metabolic function have mostly been in the context of
109 saturated CWs (Weber and Legge, 2011, Weber *et al.*, 2011, Button *et al.*, 2016a, Lv
110 *et al.*, 2017b). Thus, the plant effects on microbial community metabolic function still
111 remain unclear for other CW designs, such as unsaturated and aerated CWs.

112 Additionally, previous studies have generally analyzed the microbial
113 communities in water (Ibekwe *et al.*, 2003, Lloyd *et al.*, 2004, Lv *et al.*, 2017b) or
114 biofilm separately (Ishida *et al.*, 2006, Copcia *et al.*, 2010, Li *et al.*, 2016a, Li *et al.*,
115 2016b), even though microorganisms are present in both interstitial water and
116 biofilm attached on the substrate / plant roots. Only a few previous studies have
117 addressed the microbial community in water and biofilm samples together (Gagnon
118 *et al.*, 2007, Weber and Legge, 2013, Lv *et al.*, 2017a), but these mainly investigated
119 saturated CWs, whereas only Lv *et al.* (2017a) studied microbial communities in
120 unsaturated CWs. Thus, the similarity / dissimilarity of microbial communities in
121 water and biofilm samples in different CW designs is still poorly understood.

122 To the best of our knowledge, the direct comparison of the functionality of
123 microbial communities in these three CW designs with different plant species in a
124 single experiment has never been conducted before. The aim of this study is to
125 investigate the effects of CW design (unsaturated, saturated and aerated),
126 mesocosm type, and sample type (interstitial water or biofilm) on microbial

community metabolic function using community-level physiological profiling (CLPP) analysis in mesocosms designed to treat wastewater containing ibuprofen. Additionally, the correlation between microbial community metabolic function and water quality parameters (including ibuprofen removal) was further analyzed. CLPP is a relatively easy and information rich methodology which has been widely employed for studying the functionality of microbial community in CWs (Zhao *et al.*, 2010, Weber and Legge, 2011, Bissegger *et al.*, 2014, Button *et al.*, 2015, Lv *et al.*, 2017b). Ibuprofen (physico-chemical properties shown in Table S1) was chosen as a model compound due to its wide-spread use and because it can be systematically detected in operational field-scale systems (Kahl *et al.*, 2017, Vymazal *et al.*, 2017). Ibuprofen is known to be an easily degraded compound under aerobic conditions (Zwiener and Frimmel, 2003, Hijosa-Valsero *et al.*, 2010), and it has been proposed as a marker of wastewater contamination (de Sousa *et al.*, 2014).

2. Materials and methods

2.1. Experimental regime

A total of 54 mesocosms were used and operated under conditions simulating three different CW designs: unsaturated, saturated, and saturated with aeration (Zhang *et al.*, 2017a). For each CW design, 18 mesocosms were used (Fig. S1a), evenly divided into six mesocosm types in triplicates based on plant presence and species: unplanted, and planted with *Juncus effusus* (*Juncus*), *Typha latifolia*

148 (*Typha*), *Berula erecta (Berula), Phragmites australis* (*Phragmites*) and *Iris*
149 *pseudacorus* (*Iris*). The experimental setup had been continuously operated for
150 around 1.5 years under water-saturated conditions (Zhang *et al.*, 2017b). When the
151 present experiment started (Summer 2016), the plants were fully established and in
152 their second growth-season. Before the beginning of the present work, the
153 mesocosms operational mode was modified to allow for unsaturated and aerated
154 conditions (Fig. S1b) by changing outlet positions and including a forced aeration
155 system.

156 Each mesocosm (Fig. S1b) consisted of a black plastic container (diameter of
157 20 cm, height of 20 cm) with a surface area of 0.03 m^2. The mesocosm substrate
158 consisted of the following layers: a 4 cm-layer of coarse gravel (ø 8–12 mm, 1,900 g)
159 on the bottom, a geotextile, a 10 cm-layer of washed quartz filter sand (ø 0.5-1 mm,
160 porosity 37%, 5,700 g), and a 4 cm-layer of coarse gravel (ø 8–12 mm, 1,700 g) on
161 top to avoid exposing the filter layer to light. The effective volume of each
162 mesocosm was 1.25 L.

163 The water influent was loaded onto the top surface of the substrate through
164 a PE pipe (Ø 16 mm) fitted with pressure-compensated drippers (0.5 L/h) and
165 connected to a timer-controlled pump. The influent trickled through the substrate to
166 a collection system at the bottom. In the unsaturated design, this water was
167 evacuated from the bottom, whereas in the saturated and aerated-saturated designs
168 it was evacuated from an upper outlet placed just below the surface of the substrate

169 (Zhang *et al.*, 2017a). In the aerated-saturated design (thereafter referred to as
170 aerated design), air (2.2 L/min) was continuously injected to the bottom of the
171 mesocosms by a perforated hose connected to an air pump (SLL-40, SECOH Shanghai
172 Mec Ltd).

173 The CW mesocosms were setup under a glass roof to prevent any influences
174 from precipitation while still being exposed to natural conditions in temperature,
175 humidity and daily light exposure. The systems were fed with synthetic wastewater
176 prepared following our previous studies (Zhang *et al.*, 2017b, Lyu *et al.*, 2018): tap
177 water enriched with an N: P: K fertilizer (total-N (TN), 19.3 mg/L; P, 2.3 mg/L; Mg, 3.0
178 mg/L; K, 15.4 mg/L and S, 3.9 mg/L) (Brøste Group, Denmark) and acetic acid (20
179 mg/L total organic carbon (TOC)) to supply nutrients and carbon. The experiment
180 lasted 76 days, during which the first 30 days served as an acclimation period. During
181 this acclimation period, nutrients and 10 µg/L of ibuprofen were continuously fed
182 into the systems at a hydraulic loading rate (HLR) of 3.4 cm/d, before any sampling
183 took place. Commercial ibuprofen was purchased from a local pharmacy and its
184 exact concentration in the commercial product was determined prior to performing
185 the experiment, in order to adequately dose the wastewater. During the following
186 experimental period of 46 days, two ibuprofen concentration levels (10 and 100 µg/L)
187 and four HLRs (1.8, 3.4, 6.9 and 13.8 cm/d) were used as described by Zhang *et al.*
188 (2017a) (Fig. S2). Afterwards, the systems ran continuously at a HLR of 3.4 cm/d and

189 an ibuprofen concentration of 100 µg/L for one week. Samples for the current study
190 were then collected at a single time point on day 83.

191 **2.2. Sampling strategy**

192 At day 83, influent and effluent water samples were collected from each
193 mesocosm to measure physical-chemical parameters (water temperature (water T),
194 pH, dissolved oxygen (DO) and electrical conductivity (EC)), nutrients (total organic
195 carbon (TOC), total nitrogen (TN), ammonium (NH_4^+-N), nitrate (NO_3^--N), and
196 phosphate (PO_4^{3+}-P) and ibuprofen levels before microbiological sampling. Water
197 temperature, pH, DO, and EC were analyzed immediately after sampling using
198 portable meters (Multi-Parameter Meter HQ40d, and sensION+ EC5, HACH, USA).
199 NH_4^+-N, NO_3^--N, and PO_4^{3+}-P were measured by QuikChem Methods® (10-107-06-3-D,
200 10-107-04-1-C, and 10-115-01-1-A, respectively) on an automated flow injection
201 analyzer (QuikChem FIA+ 8000 Series, Lachat instruments, Milwaukee, USA). TN and
202 TOC were analyzed by the TNM-1 unit of a TOC-V analyzer (Shimadzu, Japan).
203 Ibuprofen was determined by high-performance liquid chromatography (HPLC) with
204 a diode array detection (DAD) (Thermo Scientific Ultimate 3000) after solid phase
205 extraction, following pre-established methods (Zhang *et al.*, 2017b).

206 After collecting water samples for the above described parameters, sampling
207 was conducted for the microbial communities. Interstitial water samples were
208 collected from the saturated and aerated designs after each mesocosm had been
209 shaken for one minute. For the unsaturated mesocosms, the outlet of each

mesocosm was first plugged and then the mesocosms were filled up with tap water before being shaken. Afterwards, the mesocosm outlet was unplugged, the first 20-30 mL of water was discarded and the remaining interstitial water was collected in a 1-L sterilized amber bottle. Tap water (and the associated filling process) used in this study was regarded as a compromise to ensure a representative sample at a single time point, as opposed to what would be a composite collection over a period of sampling. For the biofilm microbial community, cores (10 mm Ø) were taken from the first 0–10 cm layer of the substrate in each mesocosm after removing the top gravel, and thereafter stored in two 50 mL sterilized falcon tubes. The collected substrate samples contained a mix of media-associated and rhizosphere biofilm, and will hereafter be referred to as 'biofilm'. All the interstitial water and biofilm samples were processed within a 5-h period after collection.

2.3. Community level physiological profiling

The community-level physiological profile (CLPP) of the microbial community from each mesocosm sample was analyzed using BIOLOG™ Ecoplates (Biolog Inc. Hayward CA, USA). A BIOLOG™ Ecoplate includes 31 different carbon sources and one control well, in triplicate (96 wells in total) (Table S2) (Weber and Legge, 2009). Water samples were inoculated directly, while substrate-attached biofilm was detached prior to CLPP analysis. We detached the biofilm following the method described by Weber and Legge (2010b). First, sand from each mesocosm (25 g) was mixed with 100 mL of sterile 10 mM phosphate buffer solution (pH 7) in a 500 mL

231 brown bottle. Afterwards, bottles were shaken at 100 rpm for 3h at 30 ^{o}C. The
232 suspension was then diluted with phosphate buffer solution 10 times (Button *et al.*,
233 2016b). Ecoplate inoculation and incubation were conducted based on the protocol
234 described by Weber and Legge (2010a). First, 100 µL of interstitial water or diluted
235 suspension from each sample was injected into the microplate wells. Subsequently,
236 the microplates were placed on an orbital shaker at 100 rpm at 20 ^{o}C. The incubation
237 of the plates lasted 60h, during which the plates were analyzed every 6h in a Biolog
238 MicroStation reader (Biolog Inc. Hayward CA, USA) for the absorbance at 590nm. It
239 should be noted that the same type of mesocosm (three replicates, in each design)
240 were incubated on a single plate (using the carbon source replicates).

241 **2.4. Data analysis**

242 The CLPP data was analyzed according to Weber *et al.* (2007) and Weber and
243 Legge (2009). The time point for analysis was selected to maximize the variance
244 between well responses and minimize the number of absorbance values above 2.0,
245 as values above 2.0 are above the linear absorbance range. Accordingly, a time point
246 of 48h was selected for the CLPP data analysis of interstitial water and
247 substrate-attached biofilm samples, and comparisons between interstitial water and
248 biofilm samples. The average well color development (AWCD) and the number of
249 carbon sources utilized (richness) were calculated according to Button *et al.* (2015).
250 To analyze substrate utilization patterns, the 31 carbon sources were classified into
251 five groups (guilds) as suggested by Weber and Legge (2009): polymers,

carbohydrates, carboxylic & acetic acids, amino acids and amines/amides. To compare the differences in the microbial communities within each design and among the three designs, principal component analysis (PCA) was performed on the basis of the differences in carbon source utilization patterns (CSUPs). All the CSUP data were processed by Taylor transformation after assessing for normality, homoscedasticity and linear correlations (Weber *et al.*, 2007). One-way permutational analysis of variance (PERMANOVA) with both Bray-Curtis and Euclidean distance was employed to assess the differences among the microbial samples in the PCA plots. PERMANOVA analysis was carried out using the free paleontological statistic software package PAST (Hammer *et al.*, 2001). The relationships between microbial community metrics (AWCD, richness and guild utilization) and the different water metrics (water temperature, EC, DO, pH, TOC, TN, NH_4^+-N, TP and ibuprofen removal) were analyzed by canonical correlation analysis (CCA). This approach was further complemented with Pearson's correlation analysis to test which correlations from the CCA were significant (p<0.05) (Digrado *et al.*, 2017). Within the significant results, the correlation coefficient r was interpreted as: strong correlation ($r \geqslant |0.7|$) and a moderate correlation ($|0.5| \leqslant r < |0.7|$) (Cohen, 1988, Milton *et al.*, 2011). All pollutant removal rates were calculated after taking evapotranspiration into account (Zhang *et al.*, 2017a). One-way Analysis of variance (ANOVA) and a post hoc Tukey's HSD test were used to assess differences in CLPP with respect to CW design (unsaturated, saturated and aerated) and mesocosm type (one unplanted and five planted mesocosm) at the 95% confidences level (p<0.05).

Two-way ANOVA was used to assess the effects of CW design and mesocosm type on typically measured water parameters (pH, EC, DO, SAT), ET and pollutant removal (TOC, TN, NH_4^+-N, TP and ibuprofen) at the 0.05 significance level. ANOVA, PCA, Pearson's correlation analysis and CCA were conducted using the XLSTAT Pro® statistical software (XLSTAT, Paris, France).

3. Results

3.1. Water quality parameters

All mesocosms had similar water temperatures (17.9-22.9 ^oC), pH (7.6-9.2) and EC (559-916 μs/cm) (Table 1). DO in the unsaturated and aerated mesocosms (5.6-7.6 mg/L) was higher than in the saturated mesocosms (3.7-6.3 mg/L), with the exception of aerated mesocosms planted with *Phragmites* and *Iris* (1.4-2.4 mg/L). As described in our previous study (Zhang *et al.*, 2017a), the plant vitality was not influenced by the ibuprofen presence. Differences in ibuprofen removal among the three CW designs were only observed in the unplanted mesocosms, where forced aeration improved ibuprofen removal, resulting in higher removal (65%) than in the unsaturated and saturated designs (45% and 35%, respectively). The unplanted mesocosms in the unsaturated and aerated designs showed higher TOC, TN and NH_4^+-N removal (45-61%, 59-75% and 38-67%, respectively) compared to the saturated design (51%, 21% and 20%, respectively). Additionally, ibuprofen was not detected in the mesocosm substrate. The planted mesocosms had similar levels of

295 nutrients removal amongst the three designs with some exceptions for TOC and TN.
296 CW design significantly affected all the measured parameters (water temperature,
297 pH, EC, DO, TOC, TN, NH_4^+-N, TP and ibuprofen removal) ($p<0.05$) (Table S3). The
298 mesocosm type (unplanted and different plants) also significantly influenced all of
299 the conventional parameters except for TOC and TN removal ($p<0.05$). Furthermore,
300 a significant interaction effect between CW design and mesocosm type was
301 observed for all conventional parameters (Table S3) ($p<0.05$).

302 **3.2. Metabolic profiles of interstitial microbial communities**

303 For the interstitial water samples, the unsaturated CW design, *Iris, Berula* and
304 unplanted mesocosms had, in general, higher microbial activity and metabolic
305 richness than those planted with *Juncus*, *Typha* and *Phragmites* (Fig. 1). In the
306 saturated and aerated designs, higher microbial activity and metabolic richness were
307 found in the planted mesocosms than in the unplanted mesocosms. Comparing the
308 mesocosm type among the three designs, the highest and lowest microbial activity
309 and metabolic richness were generally observed in the aerated and unsaturated
310 mesocosms, respectively (Fig. 1).

311 When considering CSUPs of the microbial communities in the interstitial
312 water samples using a PCA ordination, two distinct groups (Fig. 2) were determined
313 with the exception of *Juncus* in the unsaturated designs, which was independent. All
314 of the planted mesocosms in the saturated design were grouped with unsaturated
315 *Typha* mesocosms and aerated *Iris* mesocosms. The rest of mesocosms from the

316 unsaturated and aerated designs were grouped together. The carbon source
317 utilization (guilds) among the different designs and mesocosm types (Fig. S3) did not
318 reveal any clear trends.

319 The effects of plants on the microbial community metabolic function for each
320 design were further investigated separately (Fig. S4). In the unsaturated design,
321 three groups were distinguished: *Juncus* mesocosms were independent, unplanted
322 grouped with *Phragmites* mesocosms, *and Typha*, *Berula* and *Iris* mesocosms were
323 grouped. In the saturated design, unplanted mescosms were independent, *Juncus*
324 grouped with *Typha* mesocosms, and *Berula* grouped with *Phragmites* and *Iris*
325 mesocosms. In the aerated design, two slightly different groups were found:
326 unplanted and *Phragmites* mesocosms, and all the other planted mesocosms.

327 **3.3. Metabolic profiles of biofilm microbial communities**

328 In the unsaturated design, the microbial activity and metabolic richness in the
329 planted mesocosms tended to be higher than in the unplanted mesocosms (Fig. 3).
330 In the saturated design, higher microbial activity and metabolic richness were also
331 observed in the planted mesocosms compared with the unplanted mesocosms.
332 Furthermore, significantly higher microbial activity and metabolic richness ($p<0.05$)
333 was found in the *Phragmites* and *Iris* mesocosms than in the *Juncus*, *Typha* and
334 *Berula* mesocosms. In the aerated design as well, the microbial activity and
335 metabolic richness in the planted mesocosms were significantly higher ($p<0.05$) than
336 in the unplanted ones. In addition, the microbial activity and metabolic richness was

lower in *Berula* than in the other planted mesocosms. Comparisons within the same mesocosm type among the three CW designs showed lower microbial activity and metabolic richness in the unsaturated than in the saturated and aerated designs with the exceptions of lower microbial activity and metabolic richness in saturated unplanted mescosms and lower microbial activity in the saturated *Typha* mesocosms. The saturated and aerated designs had similar levels of microbial activity and metabolic richness.

The CSUPs of the microbial communities in the biofilm samples were further analyzed (Fig. 4). Five distinct groups were determined with the exception of an independent point for the unplanted aerated mesocosms: 1) unsaturated and saturated unplanted mesocosms; 2) saturated *Juncus*, *Typha* and *Berula* mesocosms; 3) aerated and saturated *Phragmites* and *Iris* mesocosms grouped with aerated *Typha* mesocosms; 4) unsaturated *Typha, Phragmites* and *Iris* mesocosms grouped with aerated *Juncus* and *Berula* mesocosms; and 5) unsaturated *Juncus* and *Berula* mesocosms. No clear trends can be gleaned from the patterns of carbon source utilization (guilds) between CW design and mesocosm type (Fig. S5). However, there was a clear increase in the number of significant differences (p<0.05) between CW designs in the biofilm samples when compared with those from the interstitial water.

PCA ordination was also performed separately for each design (Fig. S6). In the unsaturated design, unplanted mesocosms were independent. *Juncus* and *Berula*

mesocosms formed a separate group from *Typha*, *Phragmites* and *Iris* mesocosms. In the saturated design, unplanted mesocosms were also independent and two groups, one with *Juncus, Typha* and *Berula* mesocosms and other with *Phragmites* and *Iris* mesocosms were differentiated. In the aerated design, a similar separation pattern with that of the unsaturated design was observed.

3.4. Metabolic profile differences between interstitial and biofilm microbial communities

Since we cannot compare and normalize the AWCD of the different sample types (interstitial water and biofilm) (Weber and Legge, 2013), only the microbial community metabolic richness was used for making comparisons between interstitial water and biofilm based on differing CW design (unsaturated, saturated, aerated) and mesocosms type (unplanted and different plant species). Microbial metabolic richness was higher in the biofilm than in the interstitial water for all three designs (Fig. 5). However, this difference had a greater range in the unsaturated design (1.4–41.5 fold higher) than in the saturated (1.3–2.3 fold higher) and aerated designs (1.1–1.7 fold higher).

In the PCA ordination, the microbial community metabolic profiles of the interstitial water and biofilm were grouped by design and sample type (Fig. 6). The differences (Euclidean distance) between the interstitial water and biofilm were greatest in the saturated design, followed by unsaturated and then aerated designs (Fig. 6). When analyzing the three designs separately (Fig. S7), distinct groupings

379 between interstitial water and biofilm samples were again observed. A detailed
380 analysis of the guild utilization (Fig. S8) differentiated by sample type reveals that,
381 although not always statistically significant, microbial communities in the interstitial
382 water had higher carbohydrate utilization but lower amino acid and amine/amide
383 utilization than biofilm in all mesocosm types.

384 **3.5. Canonical correlation analysis**

385 The correlation between water metrics (temperature, EC, pH, DO, TOC, TN,
386 NH_4^+-N, TP and ibuprofen removal) and the interstitial water or biofilm microbial
387 community metrics (AWCD, richness, and guild utilization) was analyzed using CCA
388 (Fig. 7). The correlation matrix of the CCA analysis is shown in Table S4.

389 In the unsaturated design, there were no significant correlations in the
390 interstitial water. In the biofilm samples, however, the microbial activity and
391 metabolic richness were moderately positively correlated with the NH_4^+-N and
392 ibuprofen removal (r=0.6 ~ 0.8). Furthermore, polymer utilization was strongly
393 negatively correlated with ibuprofen removal (r=-0.8), whereas amine/amide
394 utilization was strongly positively correlated with ibuprofen removal, respectively
395 (r=0.7).

396 For the saturated design, microbial activity and metabolic richness were
397 strongly negatively correlated with pH in the interstitial water samples (r=-0.7). In
398 addition, carbohydrate utilization was moderately negatively correlated with
399 ibuprofen removal (r=-0.5). In the biofilm samples, microbial activity and metabolic

richness were moderately positively correlated with EC, TP and ibuprofen removal (r=0.5 ~ 0.8), strongly positively correlated with TN, NH_4^+-N removal (r=0.7 ~ 0.8), and moderately negatively correlated with pH (r=-0.5 ~ -0.7). Carboxylic & acetic acid utilization were moderately negatively correlated with ibuprofen removal (r=-0.6). Amino acid (r=0.7) and amine/amide (r=0.5) utilization were strongly and moderately positively correlated with ibuprofen removal, respectively.

In the aerated design, microbial activity and metabolic richness were moderately negatively correlated with pH in the interstitial water (r=-0.5). In addition, polymer utilization was moderately negatively correlated with ibuprofen removal (r=-0.5). In the biofilm samples, microbial activity and metabolic richness were moderately negatively correlated with pH (r=-0.5 ~ -0.7). Additionally, polymer utilization was moderately negatively correlated with ibuprofen removal (r=-0.6) and carboxylic & acetic acid, amine/amide and amino acid utilization were again moderately and strongly positively correlated with ibuprofen removal, respectively (r=0.6 ~ 0.7).

4. Discussion

The unsaturated, aerated and saturated CW designs efficiently removed pollutants (TOC, TN, NH_4^+-N, TP and ibuprofen) from the synthetic wastewater. For ibuprofen, 35-97% of the influent ibuprofen was eliminated in all mesocosms. The planted mesocosms in general had similar ibuprofen removal among the three

421 designs, but the use of aeration in saturated CW designs improved ibuprofen
422 removal in the unplanted mesocosms. Moreover, the presence of plants promoted
423 ibuprofen removal, resulting in higher efficiency than in unplanted mesocosms
424 (68-97% vs. 35-65%, respectively). According to our previous study (Zhang *et al.*,
425 2017a), the contributions of substrate sorption (<0.6%) and ibuprofen accumulation
426 in the plant tissues (1-5%) to ibuprofen removal were minor, and microbial
427 degradation was identified as the main pathway for ibuprofen removal in the three
428 designs. Thus, the microbial metabolic function profiles in the different mesocosms
429 may be key to explaining differences in system performance.

430 In the interstitial water samples, the lowest microbial activity and
431 metabolic richness were found in the unsaturated design. The hydraulic regime and
432 the sampling procedure most probably contributed to reduced microbial counts in
433 suspension (Lv *et al.*, 2017a). In a previous study, Button *et al.* (2015) observed lower
434 interstitial water microbial activity and metabolic richness in pilot-scale unsaturated
435 vertical flow CWs when compared to saturated horizontal subsurface flow CWs
436 treating domestic wastewater. Lv *et al.* (2017a) also found lower interstitial water
437 microbial activity and metabolic richness in unsaturated than in saturated CW
438 mesocosms processing the pesticide tebuconazole. In our study, the highest activity
439 and richness' were observed in the aerated mesocosms. The use of aeration in
440 saturated mesocosms promoted higher microbial activity and metabolic richness
441 than in the saturated mesocosms, potentially due to the physical shear stress from

442 the aeration that increased detachment of biofilm from the substrate into interstitial
443 water (Button *et al.*, 2015).

444 Regarding the biofilm samples, the lower microbial activity and metabolic
445 richness in the unsaturated mesocosms can also be explained by the hydraulic
446 regime. The conditions in the unsaturated design tend to continuously flush out
447 detached biofilm. Also the availability of pollutants/nutrients in pulses may also
448 affect the biofilm composition (Lv *et al.*, 2017a). Biofilm in saturated and aerated
449 mesocosms had similar levels of microbial activity and metabolic richness. The
450 similar water-saturated conditions and availability of nutrients may be the reason for
451 this similarity in microbial activity (Fig. 3). The present study is consistent with
452 Button *et al.* (2015), who observed similar microbial activity in the biofilm of
453 horizontal flow CWs and aerated horizontal flow CWs when treating domestic
454 wastewater.

455 The comparisons between interstitial water and biofilm samples for each
456 design consistently reveal significantly higher (p<0.05) microbial metabolic richness
457 in the biofilm than in the interstitial water (Fig. 5). This result is in agreement with
458 the findings by Weber and Legge (2013), who found much lower microbial metabolic
459 richness for the interstitial water microbial communities than those in the biofilm in
460 saturated CW mesocosms. In the present study, the microbial metabolic function
461 profiles between the interstitial water and biofilm were also significantly different
462 (p<0.05) in the saturated design (Fig. S7). This trend was also observed by Weber and

463 Legge (2013) and Lv *et al.* (2017a). Our study is the first to report this similar
464 separation of microbial activity and metabolic richness between interstitial water
465 and biofilm communities in unsaturated and aerated designs.

466 Comparing differences in the microbial metabolic function profiles between
467 the interstitial water and biofilm in the three designs, the highest dissimilarity was
468 observed in the saturated design, and the lowest dissimilarity was found in the
469 aerated design. This might be due to the fact that in the saturated design, the
470 reduced hydraulic disturbance and the long residence time provide stable conditions
471 for biofilm development (Lv *et al.*, 2017a), leading to the clearly significant difference
472 between the interstitial water and biofilm microbial metabolic function profiles. In
473 the aerated design, the air diffusion creates a continuous turbulent flow within the
474 mesocosms, likely resulting in the detachment of biofilm (Boog *et al.*, 2014, Button
475 *et al.*, 2015). This may lead to more mixing and thus more similar microbial
476 metabolic function profiles in the interstitial water and biofilm, as we found in the
477 aerated mesocosms.

478 In addition to CW design effects, the presence of different plant species also
479 shaped different microbial metabolic function profiles in both the interstitial water
480 and biofilm. However, such effects were only clear when running separate PCAs for
481 each design (Figs. S4 and S6). When considered within each design, microbial activity
482 and metabolic richness in both interstitial water and biofilm communities were
483 generally higher in the planted than in the respective unplanted mesocosm. This

might be due to plant presence providing surface area for the development of microbial communities, and oxygen release and low-molecular weight root exudates to stimulate their growth (Bais *et al.*, 2006, Dordio and Carvalho, 2013, Button *et al.*, 2015). Previous studies have demonstrated that microbial community activity and metabolic richness in the interstitial water is altered by the presence and species of plants in pilot saturated CWs treating synthetic wastewater (Zhao *et al.*, 2010) or in saturated CWs treating tebuconazole (Lv *et al.*, 2017a, Lv *et al.*, 2017b). Furthermore, a recent study has demonstrated that the presence and species of plants shaped biofilm microbial activity and metabolic richness in both saturated and unsaturated CWs (Lv *et al.*, 2017a). We have also verified that plants shape the microbial community for aerated systems. However, it was not evident which of the five species we tested has the strongest effects on microbial communities in interstitial water and biofilm, and strongest effects in increasing pollutant removal.

The microbial activity and metabolic richness in the biofilm in the three designs showed a larger number of moderate and strong correlations with conventional parameters and ibuprofen removal than in the interstitial water (Fig. 7 and Table S4). Also, a larger number of correlations between guild utilization and ibuprofen removal were observed in the biofilm than in the interstitial water, indicating a more crucial role for biofilm microbial communities in pollutant removal than interstitial water microbial communities. Lv *et al.* (2017a) also previously reported that biofilm microbial community contributed more to pollutant removal

505 than interstitial water microbial communities in unsaturated and saturated
506 mesocosms. Microbial degradation is clearly a main pathway for ibuprofen and
507 nutrient removal in these systems (Zhang *et al.*, 2017a, Zhang *et al.*, 2017b).

508 In the unsaturated design, none of the studied water parameters correlated
509 with the biofilm community. In the saturated design, pH and EC were the main
510 driving factors of the biofilm community (Table S4), while in the aerated design, pH
511 was the only driving water parameter.

512 Notably, ibuprofen removal was generally positively correlated with amino
513 acid and amine/amide utilizations by the biofilm microbial communities in all three
514 designs. When analyzing the specific carbons in the two guilds (see Table S2), we
515 observed that the utilizations of L-arginine and L-phenyloalanine (amino acids guild)
516 and putrescine (amines/amides guild) were positively correlated with ibuprofen
517 removal (see Fig. 8). Sakultantimetha et al. (2011) reported that L-arginine addition
518 promoted removal of the biocide tributyltin by sediment microorganisms because
519 L-arginine is an effective enzyme inducer, which stimulates enzyme production and
520 then facilitates tributyltin degradation by microorganisms. With respect to
521 L-phenyloalanine, Lawrence et al. (2005) found that ibuprofen removal and
522 L-phenyloalanine utilization were positively correlated in riverine biofilms by CLPP
523 analysis, but they were not able to provide an explanation for this result. Different
524 enzymes are responsible for different degradation/catabolic pathways. Thus, we

525 speculate that the enzymes associated with the L-arginine, L-phenyloalanine and
526 putrescine co-metabolism may be linked with ibuprofen transformations.

527 The present findings were obtained from mesocosm-scale CWs fed with
528 synthetic wastewater. Synthetic wastewater instead of real wastewater was chosen
529 for the advantage of reducing interferences during chemical analysis. Thus, pollutant
530 removal performance is expected to be different from full-scale systems where
531 typical pollutants will have higher concentrations (depending on the type of
532 wastewater being treated) and pharmaceuticals would typically exist as a mixture.
533 Regarding the microbial community dynamics, for practical reasons, mesocosms
534 systems are easier to sample for biofilm than full-scale systems. For comparison
535 purposes of design and plant species, mesocosms provide an improved insight into
536 the specific factors being studied and have a lower spatial variability than full-scale
537 systems, while keeping a tight control on the experimental variables. In addition, it is
538 known that after a state of initial difference based on inoculum community profiles
539 (0-6 days) (and different wastewater feeds) the community starts shifting towards an
540 equilibrium state with unplanted and planted mesocosm CLPP groupings (after 3
541 months) (Weber and Legge, 2011). Thus, the present microbial differentiation among
542 designs and mesocosms type observed are expected to be the same for a real
543 wastewater scenario. The presently observed correlation between ibuprofen
544 removal and specific carbon sources/different enzymatic pathways is exciting. A
545 more complex wastewater would have resulted in a more complex carbon usage

546 pattern, and this finding may not have been possible. The usage of synthetic
547 wastewater and single compounds seems an acceptable compromise when starting
548 to study metabolic degradation pathways. Further studies need be conducted to
549 assess to what extent co-metabolic degradation may be promoted in real CW
550 systems and what factors may affect it (e.g. season, pharmaceuticals concentration,
551 loading rate, bed depth).

552

553 **5. Conclusions**

554 In this study, the functionality of microbial community in the interstitial water and
555 biofilm collected from different three CW designs (unsaturated, saturated and
556 aerated) and CW mesocosm types (unplanted and five different planted categories)
557 were systematically analyzed in a full factorial design.

558 - The unsaturated mesocosms had the lowest microbial activity and metabolic
559 richness, both in the interstitial water and in the biofilm samples. The aerated
560 design had the highest microbial activity and metabolic richness in the
561 interstitial water, and had similar microbial activity and metabolic richness in the
562 biofilm to the saturated design. The microbial metabolic richness in the
563 interstitial water was significantly lower than that in the biofilm in all three
564 designs.

- CW design and the presence and species of plants influenced the microbial metabolic function in both interstitial water and biofilm. However, plant effects were sometimes masked and/or diluted by the effects of design. .

- Clear correlations between microbial metabolic functional profiles and pollutant removal, namely ibuprofen, were found with the biofilm, indicating that the biofilm microbial communities played a key role in ibuprofen removal. Moreover, microbial AWCD, metabolic richness and utilization of amino acids and amine/amides were the main factors influencing ibuprofen removal by biofilm.

- The enzymes associated with L-arginine, L-phenyloalanine and putrescine co-metabolism may be associated with ibuprofen transformations in CWs.

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724

Table 1. Water parameters measured from the unsaturated, saturated and aerated saturated mesocosms treating Ibuprofen-containing wastewater (mean ± standard deviation)*.

CW Design	Mesocosm type	Water temperature (°C)	pH	EC (µs/cm)	DO (mg/L)	TOC removal efficiency (%)	TN removal efficiency (%)	NH_4^+-N removal efficiency (%)	TP removal efficiency (%)	Ibuprofen removal efficiency (%)
Unsaturated	Influent	18.2 ± 0.1	9.0 ± 0.2	603 ± 55	2.3 ± 0.5	-	-	-	-	-
	Up	18.2 ± 0.1	8.6 ± 0.2	666 ± 18	6.1 ± 0.3	45 ± 10	59 ± 20	67 ± 14	84 ± 10	45 ± 1
	J	18.2 ± 0.1	8.5 ± 0.1	783 ± 96	6.8 ± 0.6	57 ± 4	89 ± 18	97 ± 3	91 ± 16	93 ± 9
	T	18.5 ± 0.2	8.4 ± 0.1	616 ± 31	6.4 ± 0.4	65 ± 11	85 ± 24	95 ± 2	85 ± 17	92 ± 2
	B	18.3 ± 0.1	8.6 ± 0.2	612 ± 7	6.5 ± 0.6	69 ± 1	69 ± 19	91 ± 5	78 ± 20	79 ± 12
	P	18.2 ± 0.1	8.6 ± 0.1	604 ± 25	6.7 ± 0.3	58 ± 11	87 ± 14	94 ± 2	69 ± 11	92 ± 6
	I	17.9 ± 0.1	8.2 ± 0.2	559 ± 90	6.2 ± 0.9	64 ± 14	98 ± 3	97 ± 2	95 ± 7	97 ± 1
Saturated	Influent	18.3 ± 0.1	8.7 ± 0.1	628 ± 52	1.5 ± 0.1	-	-	-	-	-
	Up	18.2 ± 0.1	8.9 ± 0.1	649 ± 12	3.7 ± 0.3	51 ± 8	21 ± 13	20 ± 1	22 ± 8	35 ± 10
	J	18.2 ± 0.2	8.2 ± 0.2	673 ± 32	6.3 ± 0.5	72 ± 5	95 ± 6	83 ± 14	71 ± 38	96 ± 3
	T	22.9 ± 0.6	8.6 ± 0.2	676 ± 60	5.5 ± 0.1	86 ± 2	99 ± 2	96 ± 2	89 ± 8	97 ± 2
	B	18.5 ± 0.5	8.4 ± 0.1	567 ± 47	5.7 ± 0.7	76 ± 12	80 ± 17	72 ± 19	80 ± 14	86 ± 8
	P	18.3 ± 0.1	8.3 ± 0.2	916 ± 56	5.8 ± 0.8	71 ± 10	99 ± 1	96 ± 10	98 ± 1	93 ± 3
	I	18.5 ± 0.1	7.6 ± 0.3	649 ± 23	4.3 ± 0.1	37 ± 3	86 ± 14	80 ± 14	46 ± 25	82 ± 1
Aerated	Influent	18.3± 0.1	9.0 ± 0.1	610 ± 49	3.6 ± 0.5	-	-	-	-	-
	Up	18.4 ± 0.1	8.9 ± 0.1	633 ± 14	5.6 ± 0.2	61 ± 2	75 ± 1	38 ± 10	-41 ± 5	65 ± 2
	J	18.3 ± 0.1	9.2 ± 0.1	735 ± 25	7.6 ± 0.1	72 ± 6	99 ± 1	96 ± 4	99 ± 1	95 ± 1
	T	18.6 ± 0.3	8.8 ± 0.6	639 ± 90	5.7 ± 0.1	64 ± 12	98 ± 3	86 ± 15	95 ± 8	97 ± 5
	B	18.3 ± 0.1	9.0 ± 0.4	629 ± 13	6.8 ± 0.2	86 ± 10	88 ± 10	81 ± 10	50 ± 74	93 ± 5
	P	18.1 ± 0.1	8.0 ± 0.1	606 ± 23	2.4 ± 1.4	77 ± 7	86 ± 4	69 ± 11	21 ± 43	68 ± 7
	I	18.5 ± 0.7	7.7 ± 0.4	624 ± 8	1.4 ± 0.5	23 ± 5	88 ± 13	74 ± 34	-28 ± 33	93 ± 1

*Up, J, T, B, P and I represent unplanted, *Juncus*, *Typha*, *Berula*, *Phragmites* and *Iris* mesocosms (triplicates), respectively.

Figure caption

Fig. 1. Microbial activity based on average well color development (AWCD) (a) and metabolic richness (b) in the interstitial water samples from unplanted, *Juncus*, *Typha*, *Berula*, *Phragmites* and *Iris* planted mesocosms in unsaturated, saturated and aerated CW designs. Within each CW design, significant differences (p<0.05) among mesocosm types are marked using lower case letters. Significant differences among the three CW designs for each mesocosm are denoted by uppercase letters.

Fig. 2. Principle component analysis (PCA) plot of the microbial community based on carbon source utilization patterns in the interstitial water samples. The first letter in the three letter code (U, S and A) represents unsaturated, saturated and aerated designs; the second letter (W) represents interstitial water; and the last letter (up, J, T, B, P and I) represents unplanted (up), *Juncus* (J), *Typha* (T), *Berula* (B), *Phragmites* (P) and *Iris* (I) planted mesocosms, respectively. The indicated groups (ellipse encapsulations) are significantly different from each other (p<0.05, PERMANOVA).

Fig. 3. Microbial activity based on AWCD (a) and metabolic richness (b) for the biofilm samples from unplanted, *Juncus*, *Typha*, *Berula*, *Phragmites* and *Iris* planted mesocosms in unsaturated, saturated and aerated CW designs. Within each CW design, significant differences (p<0.05) among mesocosm types are marked using lower case letters. Significant differences among the three designs for each mesocosm are denoted by uppercase letters.

Fig. 4. Principle component analysis (PCA) plot of the microbial community based on the carbon source utilization pattern in the biofilm samples. The first letter in the three letter code (U, S and A) represents unsaturated, saturated and aerated designs; the second letter (B) represents biofilm; and the last letter (up, J, T, B, P and I) represents unplanted (up), *Juncus* (J), *Typha* (T), *Berula* (B), *Phragmites* (P) and *Iris* (I) planted mesocosms, respectively. The indicated groups (ellipse encapsulations) are significantly different (p<0.05, PERMANOVA).

Fig. 5. Microbial metabolic richness for the interstitial water and biofilm samples from unplanted, *Juncus*, *Typha*, *Berula*, *Phragmites* and *Iris* planted mesocosms in unsaturated (a), saturated (b) and aerated (c) CW designs. Asterisks represent statistically significant differences (p<0.05) between interstitial water and biofilm samples within the same mesocosm type and CW design.

Fig. 6. Principle component analysis (PCA) plot of the microbial community based on the carbon source utilization pattern in the interstitial water and biofilm samples. The first letter in the three letter code (U, S and A) represents unsaturated, saturated and aerated designs; the second letter (W and B) represents interstitial water and biofilm; and the last letter (up, J, T, B, P and I) represents unplanted (up), *Juncus* (J), *Typha* (T), *Berula* (B), *Phragmites* (P) and *Iris* (I) planted mesocosms, respectively.

Fig. 7. Canonical correlation analysis (CCA) of water quality parameters and microbial community metrics (AWCD, richness, guilds utilization) for the interstitial water and biofilm samples from unsaturated, aerated and saturated designs, respectively. Abbreviations: water T=water temperature, DO=dissolved oxygen, EC=electrical conductivity, TOC=total organic carbon removal, TN=total nitrogen removal, TP=total phosphorus removal and NH_4^+-N= ammonium removal, ibuprofen=ibuprofen removal, AWCD=average well color development, Carbs=carbohydrates, C&AA=carboxylic & acetic acids, Amino=amino acids, and A/A=amines/amides.

Fig. 8. Correlation between the utilization of L-arginine, L-Phenyloalanine, Putrescine and ibuprofen removal.

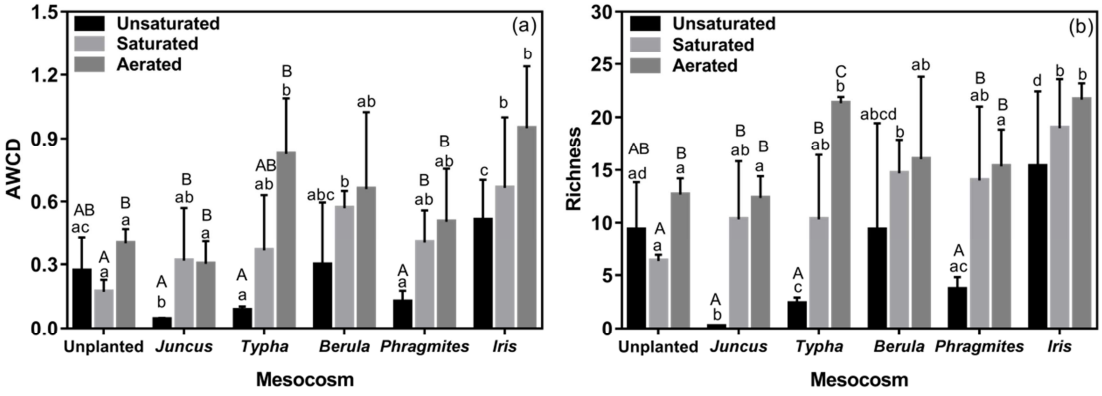


Fig. 1. Microbial activity based on average well color development (AWCD) (a) and metabolic richness (b) in the interstitial water samples from unplanted, *Juncus*, *Typha*, *Berula*, *Phragmites* and *Iris* planted mesocosms in unsaturated, saturated and aerated CW designs. Within each CW design, significant differences (p<0.05) among mesocosm types are marked using lower case letters. Significant differences among the three CW designs for each mesocosm are denoted by uppercase letters.

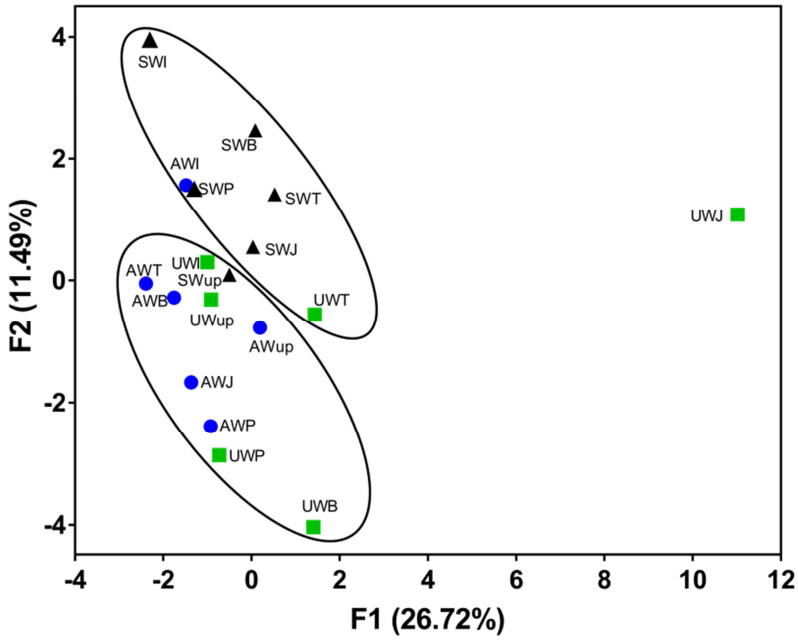


Fig. 2. Principle component analysis (PCA) plot of the microbial community based on carbon source utilization patterns in the interstitial water samples. The first letter in the three letter code (U, S and A) represents unsaturated, saturated and aerated designs; the second letter (W) represents interstitial water; and the last letter (up, J, T, B, P and I) represents unplanted (up), *Juncus* (J), *Typha* (T), *Berula* (B), *Phragmites* (P) and *Iris* (I) planted mesocosms, respectively. The indicated groups (ellipse encapsulations) are significantly different from each other (p<0.05, PERMANOVA).

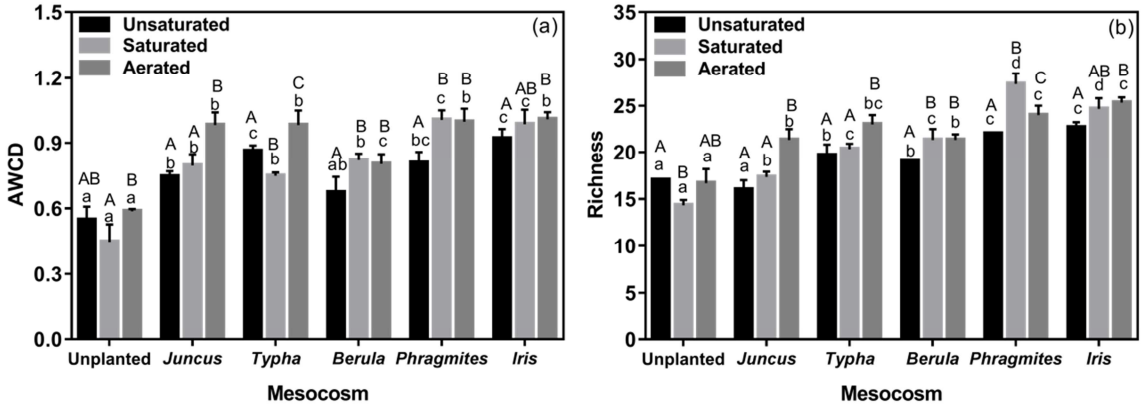


Fig. 3. Microbial activity based on AWCD (a) and metabolic richness (b) for the biofilm samples from unplanted, *Juncus*, *Typha*, *Berula*, *Phragmites* and *Iris* planted mesocosms in unsaturated, saturated and aerated CW designs. Within each CW design, significant differences (p<0.05) among mesocosm types are marked using lower case letters. Significant differences among the three designs for each mesocosm are denoted by uppercase letters.

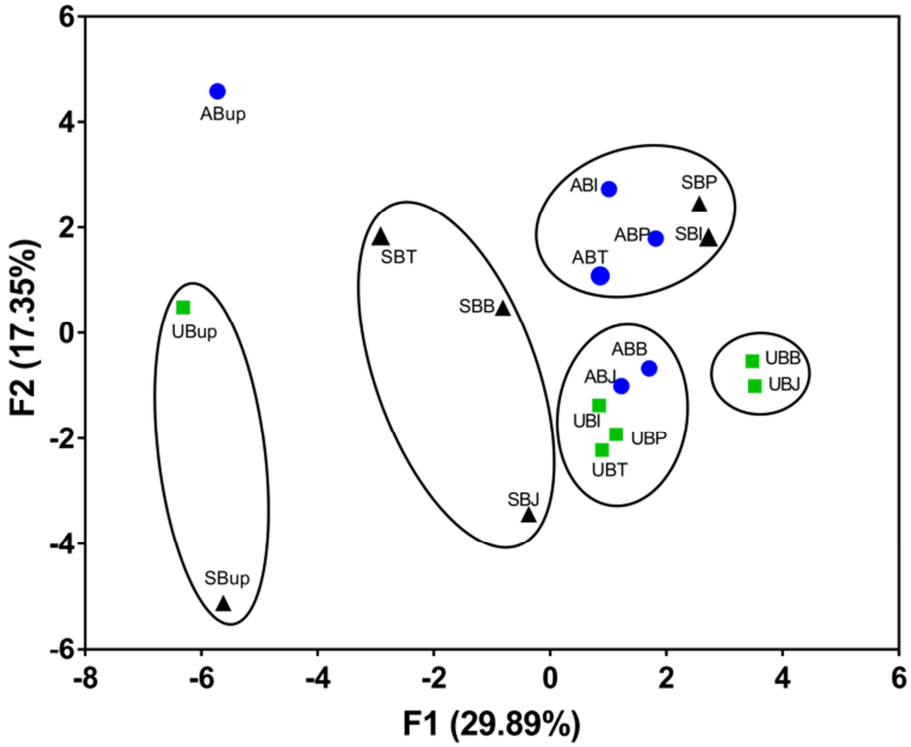
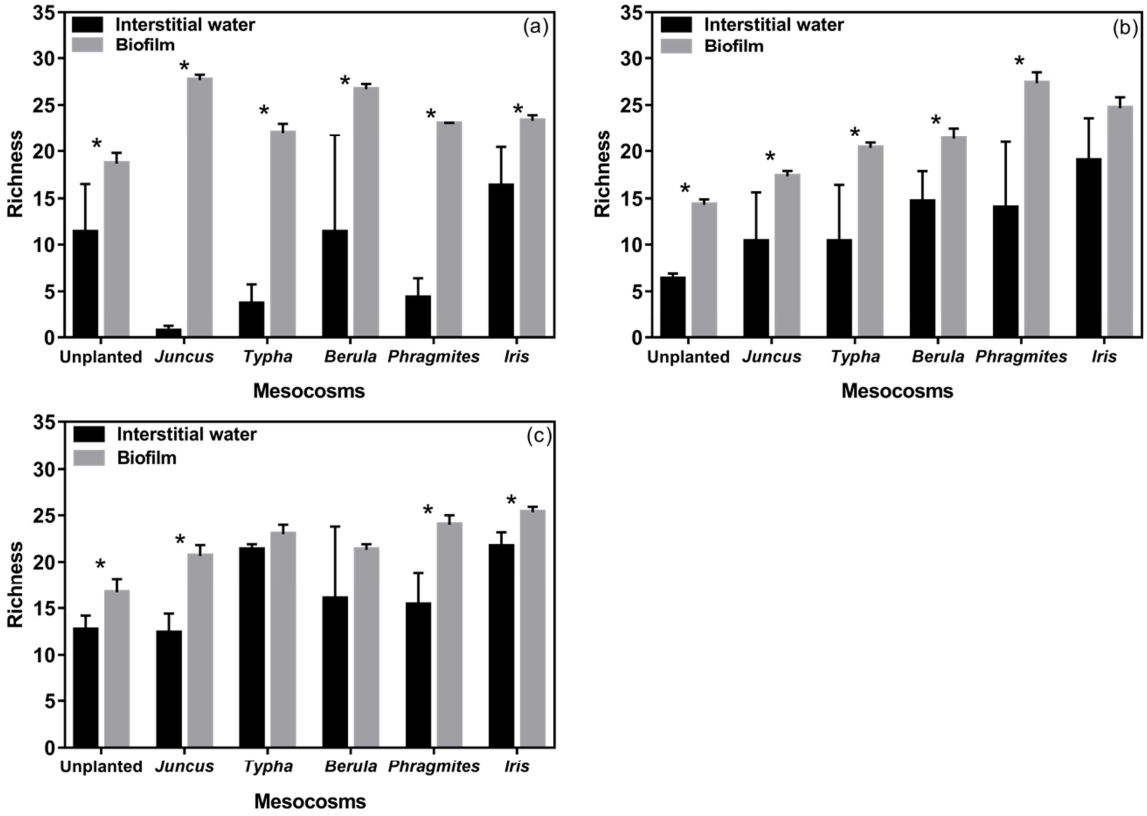


Fig. 4. Principle component analysis (PCA) plot of the microbial community based on the carbon source utilization pattern in the biofilm samples. The first letter in the three letter code (U, S and A) represents unsaturated, saturated and aerated designs; the second letter (B) represents biofilm; and the last letter (up, J, T, B, P and I) represents unplanted (up), *Juncus* (J), *Typha* (T), *Berula* (B), *Phragmites* (P) and *Iris* (I) planted mesocosms, respectively. The indicated groups (ellipse encapsulations) are significantly different (p<0.05, PERMANOVA).



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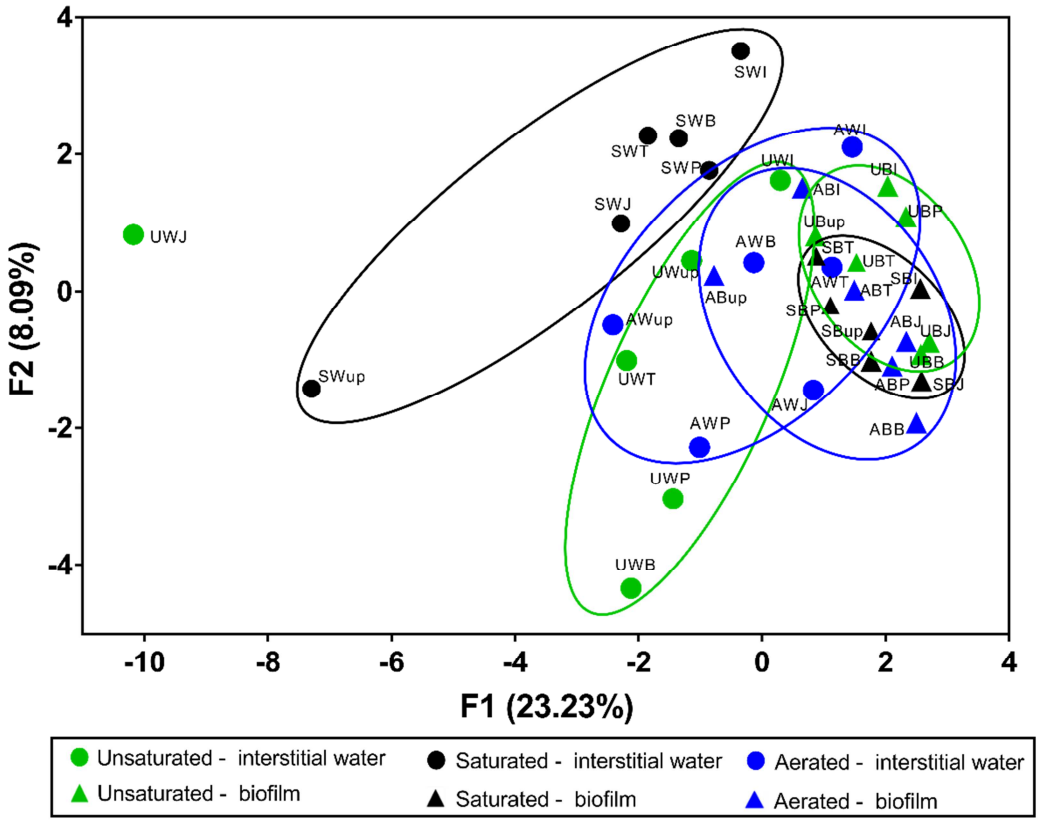
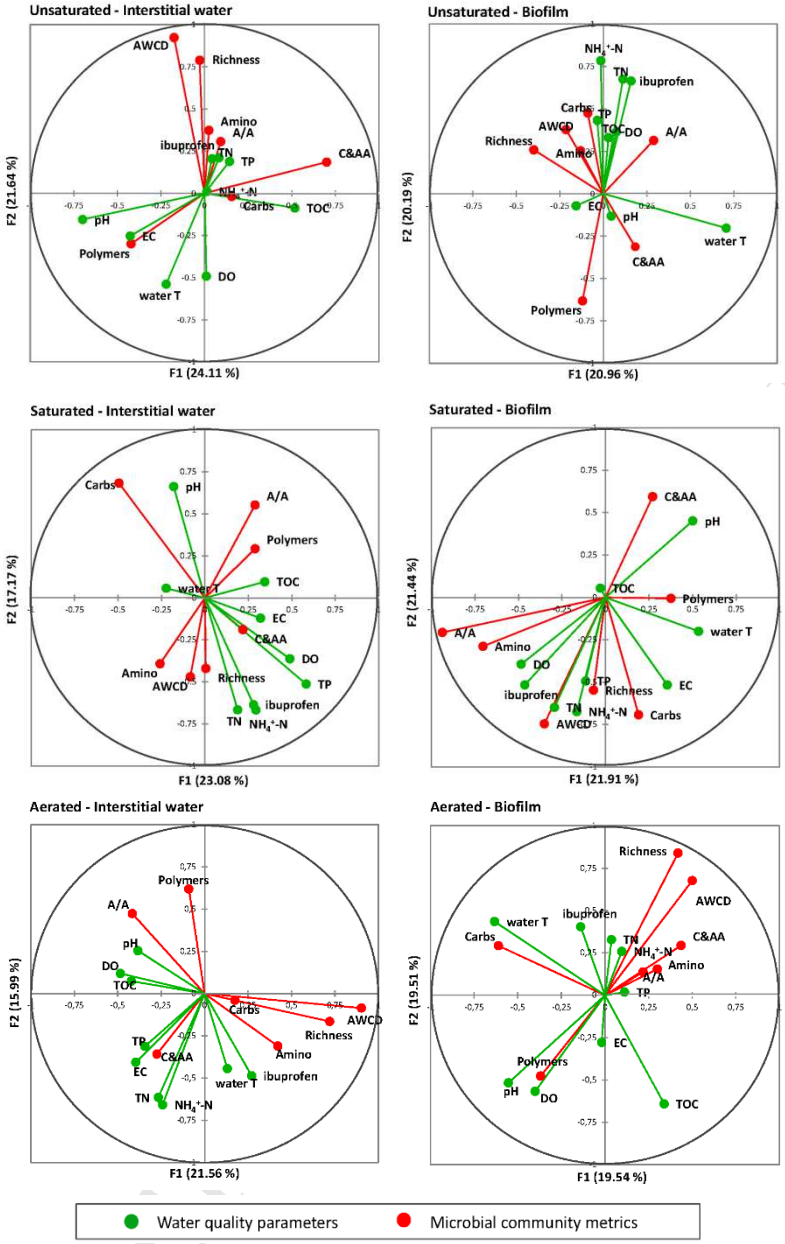


Fig. 6. Principle component analysis (PCA) plot of the microbial community based on the carbon source utilization pattern in the interstitial water and biofilm samples. The first letter in the three letter code (U, S and A) represents unsaturated, saturated and aerated designs; the second letter (W and B) represents interstitial water and biofilm; and the last letter (up, J, T, B, P and I) represents unplanted (up), *Juncus* (J), *Typha* (T), *Berula* (B), *Phragmites* (P) and *Iris* (I) planted mesocosms, respectively.



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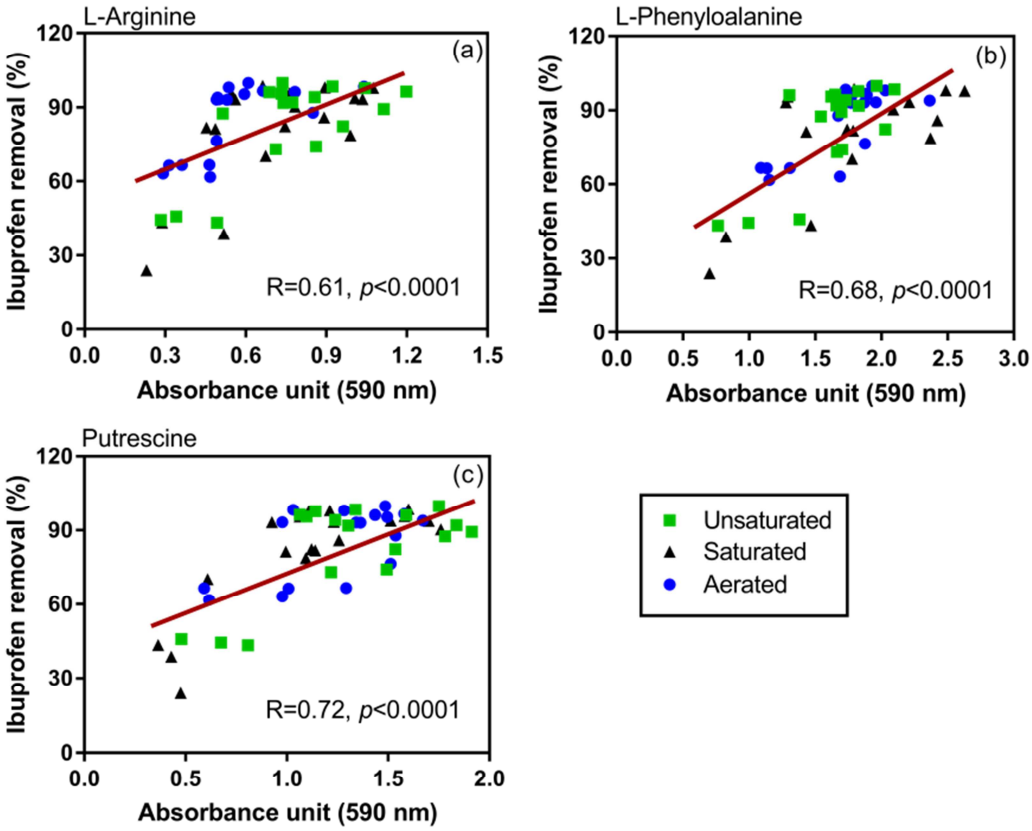

Fig. 8. Correlation between the utilization of L-arginine, L-Phenyloalanine, Putrescine and ibuprofen removal.

- Effects of CW design and plants on microbial community function were investigated
- Different microbial community function found in interstitial water and biofilm
- CW design affected microbial community function more than plants
- Biofilm microbial community plays a greater role in ibuprofen removal
- Ibuprofen degradation may be associated with different enzymatic processes