Microbial Community Metabolic Profiles in Saturated Constructed Wetlands Treating Iohexol and Ibuprofen

Yang ZHANG [1,2,3,a], Tao LYU [1,4,a], Liang ZHANG [1], Mark BUTTON [5,6], Carlos A. ARIAS [1,7], Kela P. WEBER [5], Jianghong SHI [2], Zhanghe CHEN [3], Hans BRIX [1,7], Pedro N. CARVALHO [1,7,8*]

1 Department of Bioscience, Aarhus University, Aarhus 8000C, Denmark
2 School of Environmental Science and Engineering, Southern University of Science and Technology, Shenzhen 518055, PR China
3 College of Life Science, South China Normal University, Guangzhou 510631, PR China
4 School of Animal, Rural and Environmental Science, Nottingham Trent University, Nottinghamshire NG25 0QF, UK
5 Department of Chemistry and Chemical Engineering, Royal Military College of Canada, Kingston, ON K7K 7B4, Canada
6 Fipke Laboratory for Trace Element Research, Earth, Environmental and Geographic Sciences, University of British Columbia Okanagan, Kelowna V1V 1V7, BC, Canada
7 WATEC – Centre for Water Technology, Aarhus University, Aarhus 8000C, Denmark
8 Department of Environmental Science, Aarhus University, Frederiksborgsvej 399, 4000 Roskilde, Denmark

Abstract

The aim of the present study was to elucidate the microbial community metabolic profiles in saturated constructed wetland (CW) mesocosms planted with five different wetland plant species fed with water individually spiked with 100 μg L^{-1} ibuprofen or iohexol. Community-level physiological profiling (CLPP) using Biolog Ecoplates was performed and coupled with the assessment of water quality parameters (water temperature, pH, DO and TOC, TN, NH_4-N, PO_4-P removal efficiency). The microbial community metabolic profiles (microbial acitivity, richness, and carbon source utilization), as well as the water quality parameters revelead similar trends among the control mesocosms and the mescocosms fed with water spiked with iohexol and ibuprofen. Significant differences were observed between the planted and unplanted mesocosms and between seasons

* The corresponding author. [a] These authors contributed equally to this work.

(summer and winter) within each of the feeding lines (control, iohexol or ibuprofen). The microbial community metabolic profiles in the saturated CW were shaped by plant presence and plant species, while no negative impact of iohexol and ibuprofen presence was noticed at the 100 μg L^{-1}. In addition, the microbial activity and richness were generally higher in planted mesocosms than in the unplanted systems in the summer. For the first time, a positive correlation between iohexol removal and the microbial community metabolic profiles (activity, richness and amines and amides utilization in summer, and carbohydrates utilization in winter) in the saturated mesocosms was observed. Putrescine utilization in the summer and D-cellobiose, D,L-alpha-glycerol phosphate in winter were linked with the metabolic processing of iohexol, while glycogen in summer and L-phenylalanine, Glycyl-L-glutamic acid in winter were linked with ibuprofen removal efficiency in the saturated CW.

Keywords: carbon source, community-level physiological profiling, pharmaceuticals, plant species, season, water treatment

1. Introduction

Emerging organic pollutants, such as pharmaceuticals have been detected in the aquatic environment (Xu et al., 2007). Toxic effects of these compounds have been observed in aquatic organisms (Mottaleb, 2015), which could eventually pose a risk to human health and the environment (Hernando et al., 2006; Safe, 2000). Ibuprofen is an anti-inflammatory agent frequently used and demonstrated to be toxic to aquatic organisms at the μg L^{-1} concentration level (Ericson et al., 2010; Santos et al., 2010). Ibuprofen has been quantified in WWTP effluent on the order of ng to μg L^{-1} (Pal et al., 2010). Iohexol is an iodinated contrast media agent commonly used in hospitals. Even though no direct evidence has shown adverse

effects on aquatic organisms or humans, high concentrations are found in European WWTPs, which has led to general concern and further research requirements (Loos et al., 2013). Overall, pharmaceutical removal has been a principal issue of wastewater treatment over the last decade.

There is a general consensus that constructed wetlands (CWs), as a sustainable and cost-efficient alternative for wastewater treatment, can also efficiently degrade a number of pharmaceuticals (Verlicchi and Zambello, 2013). The relevant removal mechanisms of pharmaceuticals in CWs have been attributed to phytodegradation, plant uptake, biodegradation, sorption to the substrate, and photodegradation, depending on the CW design used (García et al., 2010; Li et al., 2014). Previous studies have shown that ibuprofen is a biodegradable compound within CWs (Dordio et al., 2010; Zhang et al., 2016), and more specifically in saturated CW mesocosms (Zhang et al., 2016). Seitz et al. (2008) showed that iohexol can be recalcitrant and only partially removed by ozonation. However, we demonstrated that iohexol can be efficiently removed from hydroponic solution by *Phragmites australis (*Zhang et al. 2015), as well as in saturated constructed wetlands (Zhang et al., 2016). Additionally, the mass balance and regression analysis pointed to biodegradation as the main mechanism for its removal, while for both compounds ibuprofen and iohexol, the removal rates differed according to plant species (Zhang et al., 2016). However, previously, it was not possible to conclude whether the biodegradation resulted from microbial degradation and/or metabolization within plant tissue is still unclear. Up to 2016, from the 32 publications studying pharmaceutical and microbial communities in CWs, only 7 sudies have looked at microbial community function (Weber, 2016). The majority of these studies were looking to understand the impacts of compounds such as antibiotics on the inherent microbial community and any potential long-term impacts to CW health and water treatment capabilities. However, the role of microbial community fucntion and metabolic pathways for pharmaceutical biodegradation in CWs are still unknown.

Previous research has shown that plant species selection has an influence on the microbial community functional profiles in CWs treating pharmaceuticals (Zhang et al., 2017). Lyu et al. (2016) demonstrated that plant species determined the microbial community profiles in CWs with no impact from the presence of pesticides. As for concentration, Weber et al. (2011) showed the presence of ciprofloxacin had an adverse effect on the microbial communities in CWs at a concentration of 2 mg L^{-1}. However, relatively little is known as to whether pharmaceutical presence at realistic environmental concentrations (ng L^{-1}-μg L^{-1}) can result in a discernable microbial community response. For example, by impacting the behavior of plants that shape the biofilm microbial community and subsequent microbial biodegradation. Additionally, season is widely accepted as a main factor for influencing microbial community in CWs (Stein and Hook, 2005), including when pesticides are present in the treated water (Lyu et al., 2016). However, research on seasonal effects on microbial communities in CWs treating pharmaceuticals has not yet been conducted. There is a need for better understanding the microbial community metabolic profiles in CWs, both in relation to the role of plants and seasonality. In addition, little is known regarding the relationship between microbial community function and the biodegradation of pharmaceutical compounds, including iohexol and ibuprofen.

The main aim of the present study was to elucidate the microbial community metabolic profile in saturated CW mesocosms planted with five different wetland plant species fed with water individually spiked with ibuprofen and iohexol in both summer and winter. In addition, the relationship between the microbial community metabolic profiles and pharmaceutical removal was assessed.

2. Materials and methods

2.1 Experimental setup

The experiment was conducted under a glass-roof for protection against rain and snow but ensuring outdoor environmental conditions. Five emergent plant species were used: *Typha latifolia, Phragmites australis, Iris pseudacorus, Berula erecta* and *Juncus effusus.*. Full details of the experimental setup are described elsewhere (Zhang et al., 2016). Briefly, eighteen mesocosms (5 planted, 1 unplanted, triplicated) filled with quartz sand (particle size 0.5-1 mm with average porosity of 37%), were connected to a 350 L influent tank via a PE pipe (Fig 1). The influent water was prepared with tap water, 100 mg L^{-1} "Pioner Grøn" (Brøste Group, Denmark) N: P: K full strength nutrients and acetic acid (20 mg L^{-1} total organic carbon (TOC)). Dedicated influent tanks were used for each pharmaceutical spiking and for the uncontaminated control. The compounds were spiked in the influent tanks periodically, as needed to replenish the tank capacity. The systems have been previously tested with 10 and 100 µg L^{-1} pharmaceutical concentration level, and subject to different hydraulic loading rates (HLRs) (0.7 - 13.8 cm d^{-1}) in both summer and winter as detailed elsewhere (Zhang et al., 2016). For the present study, mesocosms were sampled in September 2014 (Summer) and in March 2015 (Winter) after two weeks' stabilization at a HLR of 3.4 cm d^{-1} and a pharmaceutical exposure concertation of 100 µg L^{-1}. Sampling was performed at the end of each season to avoid disturbances to system performance. The daytime in summer and winter was 14 h and 6 h, respectively. The average measured air temperature and relative humidity were 26.7 ± 4.3 ℃ and 51.8 ± 12.7% respectively in summer and 6.1 ± 2.2 ℃ and 82.3 ± 5.4% respectively in winter.

2.2 Sampling strategy

The three working lines (ibuprofen, iohexol and unspiked control) were sampled in both seasons for water quality parameters (as described elsewhere (Zhang et al., 2016)) and for community-level physiological profiling (CLPP). For the CLPP, before collection, each mesocosm was shaken laterally. Afterwards, the initial interstitial water was discharged from the bottom outlet of the mesocosm. The

first 20 - 30 mL of each sample were discarded and the remaining water sample collected in a 1 L amber bottle. Due to material and time constrains for the summer period, samples were only assessed for the influent tank, unplanted, *Typha* and *Phragmites* planted mesocosms. For winter all mesocosms (unplanted, *Typha, Phragmites, Iris, Juncus* and *Berula*) and the influent tank samples were processed. Influent samples were collected directly into an amber bottle from the storage tank in both summer and winter. All samples were carefully labeled and refrigerated before further processing (max. 2 hour interim period).

2.3 Water quality analysis

Measurements of water quality parameters (pH, water temperature, dissolved oxygen (DO)) were performed *in-situ* using dedicated probes (HQ40d HACH, Denmark). Nutrient analysis was conducted in the lab following standard methods as follows. Total organic carbon (TOC) and total nitrogen (TN) were analysed with a TOCV analyser (TOC-V, Shimadzu, Japan) and ammonium (NH_4-N), nitrate (NO_3-N) and phosphate (PO_4-P) by an automated flow injection analyser (QuikChem FIA+ 8000 Series, Lachat instruments, Milwaukee, USA). Iohexol and ibuprofen were concentrated by solid phase extraction (SPE) and analyzed by a high-performance liquid chromatography (HPLC) system equipped with a diode array detector (DAD) (Ultimate 3000, Thermo Scientific, Denmark). Methods are extensively described in Zhang et al. (2016).

2.4 Community-level physiological profiling

Community-level physiological profiling (CLPP) was performed using BIOLOG™ Ecoplates (Biolog Inc., CA, USA). Inoculation and incubation protocols have been previously described in detail by Weber and Legge (2010). Briefly, 100 µL of interstitial water sample was used to inoculate each well of the BIOLOG™ Ecoplate. Plates were incubated at room temperature in an orbital digital shaker (VWR International, PA, USA) at a speed of 90 rpm in the dark. Optical density readings

were taken with a FLUOstar Omega microplate reader (BMG LABTECH, Offenburg, Germany) at an absorbance of 590 nm every 6 h for 88 h in summer and for 57 h in winter. It should be noted that each BIOLOG™ Ecoplate represented one mesososm type, the three analytical replicates within one BIOLOG™ Ecoplate were substituted by the three true mesocosms replicates.

2.5 Data analysis

The analysis of the CLPP data was performed as previously by Weber et al. (2007) and Weber and Legge (2009). Incubation times of 88 h, 57 h and 62 h were identified as the metric for CLPP data analysis in summer, winter and seasonal comparison, respectively. Prior to analysis, all data were tested for normality and homoscedasticity. Briefly, the absorbance readings of each specific time point were Taylor transformed and used to calculate the average well color development (AWCD) and richness. The thirty-one carbon sources were divided into five broader 'guild' groups (Table S1).

One-way ANOVA was used to compare the significant difference in water quality parameters, AWCD, richness and carbon source utilization within seasons, working lines and mesocosms. Principal component analysis (PCA) was conducted with the Xlstat (version 18.07, Addinsoft, Paris, France) for the analysis of the differences between sample groups. The statistical significance of groups observed in the PCA was tested with permutational multivariate analysis of variance (PERMANOVA) using the free paleontological statistic software package PAST (Hammer et al., 2009). Canonical correlation analysis was applied to assess the correlation between water quality parameters and microbial community metabolic profiles (AWCD, richness, and utilization of five carbon source 'guild') of each sample. This approach was further complemented with Pearson's correlation analysis (Digrado et al., 2017). Within the significant results, the correlation coefficient r was interpreted as: strong correlation ($r \geq |0.7|$) and a

moderate correlation ($|0.5| \leq r < |0.7|$) (Cohen, 1988; Milton et al., 2011). All the significance testing was performed to $p<0.05$.

3. Results

3.1 Water quality parameters

The water quality parameters (water temperature, pH, DO, and TOC, TN, NH_4-N, PO_4-P, ibuprofen and iohexol removal efficiency) of the mesocosms effluent are shown in Table 1. Statistical analysis demonstrated that there was no significant difference in the parameters measured between the working lines (ibuprofen, iohexol and unspiked control). For example, in the summer, the DO for the unplanted mesocosms for the control line and iohexol lines were 7±1 and 6±2 respectively. Significant differences existed between the mesocosms and seasons within each working line for the water temperature, pH, DO and the removal efficiency of TOC, TN, NH_4-N, PO_4-P and pharmaceutical. The pH was significantly higher in the influent and unplanted mesocosms than the planted mesocosms. For example, for the ibuprofen line in the summer the unplanted mesocosms had a pH of 7.8±0.2, which was statistically larger ($p<0.05$) than a pH of 7.3±0.3 for the *Phragmites* mesocosms. Statistical differences between season was also observed for some parameters. An exhaustive list will not be made here (See Table 1 for details) however a select number of comparisons will be highlighted. The DO showed was significantly higher in the planted mesocosms when compared to the unplanted mesocosms, and this was more pronounced in winter than in summer. The removal efficiency of TOC was significantly higher in the planted mesocosms than the unplanted and this was more pronounced in winter than in summer. The removal efficiency of TN, NH_4-N and PO_4-P were significantly higher in the planted mesocosms than the unplanted, and this was more pronounced in summer than in winter. Lastly, the removal efficiency of the pharmaceuticals was significantly higher in some planted mesocosms, when compared to the unplanted mesocosms.

For example, in winter the ibuprofen removal was 27±12% for the unplanted mesocosms, which was statistically lower than 94±3% for *Juncus*, but not statistically different than 43±13% for *Phragmites*.

3.2 Microbial community metabolic profiles

In summer, the microbial activity (AWCD) and richness (Fig 2A and 2D) were significantly different between working lines, but only for the influent and unplanted mesocosms. For example, the richness of the ibuprofen line in the summer was 15±1.5, which was statistically greater than 10±3 for the control line. Thus, microbial activity and richness were similar among the planted mesocosms in the three working lines. In addition, in the iohexol line, the microbial activity was higher in the planted (*P. australis* and *T. latifolia*) mesocosms, than the unplanted systems.

In winter, the microbial activity and richness (Fig 2B and 2E) showed only significant differences between working lines for the *T. latifolia* mesocosms, where activity and richness were higher in the iohexol line (richness of 21±1.5) than in the control (richness of 10±6). For each working line, no significant differences were observed between the different mesocosms (unplanted and planted) or plant species.

Significant differences in microbial activity and richness between summer and winter were observed in most of the mesocosms and influent samples, except for the microbial activity in *T. latifolia* mesocosm in the control and ibuprofen line, the richness in the influent and *T. latifolia* mesocosm samples of the ibuprofen line, and in the *T. latifolia* and *P. australis* mesocosm samples of the control line (Fig 2C and 2F).

3.3 Carbon source utilization

The carbon source utilization in summer, winter and the seasonal comparison for each guild are shown in Fig 3A-C. Some differences can be observed in the carbon

source utilization (guilds) between the lines spiked with pharmaceuticals in comparison to the control working line (Fig 3A and 3B). These differences are more pronounced for the influent (in both seasons). Differences in carbon utilization between working lines are also observed throughout the different mesocosms in both seasons, but no global patterns (common to both pharmaceuticals) could be distinguished. In summer, the polymers and amines and amides utilization from the influent was significantly lower in the iohexol line compared with the control line, while the polymers utilization in the influent was significantly higher in the ibuprofen line (Fig S1). In winter, the amines and amides utilization was significantly lower in the ibuprofen line than in the control line in the influent (Fig S2).

A detailed focus on the seasonal effect (Fig 3C) reveals that the influent and unplanted mesocosms had higher variation between summer and winter. The utilization of polymers, carboxylic acid and acetic acids, as well as amino acids, tended to be higher in the influent and unplanted mesocosms in the winter (Fig S3).

3.4 Principal component analysis

The PCA ordination of the microbial community carbon source utilization patterns of the microbial community samples from all the mesocosms and the influent are shown in Fig 4. PERMANOVA analysis was used to test the statistical significance of observed groupings. In summer (Fig 4A), the samples from the influent, as well as from *T. latifolia* formed independent groups, while samples from *P. australis* and the unplanted mesocosms were grouped together. In winter (Fig 4B), the influent, *B. erecta* and *T. latifolia* mesocosms formed independent groups, while all other samples grouped together (unplanted, *P. australis, I. pseudacorus* and *J. effusus*). The samples showed groupings associated with mesocosm type (plant presence and plant species) instead of pharmaceutical presence (working line).

For the seasonal comparison (Fig 4C), the results were similar to those seen in summer and winter independently (Fig 4A and 4B). The samples from the influent and the unplanted mesocosms in winter, as well as *T. latifolia* in summer formed independent groups. Samples from *T. latifolia* and *P. australis* in winter grouped together, while all the other remaining samples grouped together.

3.5 Correlation analysis

Canonical correlation analyses between water parameters (water temperature, pH, DO, the concentrations of TOC, TN, NH_4-N, PO_4-P, and ibuprofen and iohexol removal efficiency) and microbial community metabolic profiles (AWCD, richness, and utilization of five carbon source 'guild') are shown in Table 2. In the control line, moderate ($|0.5| \leq r < |0.7|$) to strong ($r \geq |0.7|$) negative correlation was observed in summer between the activity and richness and the concentrations of TN, NH_4-N and PO_4-P in the samples, while no significant correlations were observed for the winter. In the iohexol line, strong ($r \geq |0.7|$) negative correlation was observed in summer between the concentrations of TN, NH_4-N and PO_4-P and AWCD and richness. Besides, the iohexol removal was also positively correlated with AWCD (r=0.78), richness (r=0.78), the amines and amides utilization in summer (r=0.71) and the carbohydrates utilization in winter (r=0.69). In the ibuprofen line, ibuprofen removal was observed moderately negatively correlated with the polymers utilization in summer (r=-0.62) and the amino acids utilization in winter (r=-0.52).

Table 3 presents the Pearson correlation with the specific individual carbon sources of interest. The iohexol removal efficiency had a strong positive correlation (r=0.893) with putrescine (amines and amides guild) in summer, and moderate positive correlation with D-cellobiose (r=0.537), and D,L-alpha-glycerol phosphate (r=0.583) (carbohydrates) in winter. While the ibuprofen removal efficiency had a strong negative correlation (r=-0.846) with glycogen (polymers) in summer, and moderate negative correlation with L-phenylalanine (r=-0.531), and Glycyl-L-glutamic acid (r=-0.531) (amino acids) in winter.

4. Discussion

In the present study, the microbial community metabolic profile in the influent was different between the working lines (Fig 3C). The influent had some sitting period in the tank and there was visual biofilm development, therefore there are clear signals of an adaption of the communities to the pharmaceutical presence. It appears that ibuprofen had a positive effect while iohexol a negative effect on both activity and richness in summer (Fig 2A, D), typically when it is generally assumed that higher temperatures favour microbial activity. Differences in the influent profiles are also observed with season (Fig 2C, F).

Seasonal differences were observed both in water parameters and in the microbial community function among the samples from influent, but also *T. latifolia*, *P. australis* and unplanted mesocosms. In winter, plant growth dramatically decreased. Consequently, the DO, removal efficiency of TN, NH_4-N and PO_4-P as well as microbial community metabolic function in CW reflect the decreased plant activity. The microbial community function in winter was higher than summer. The higher utilization of carbon sources in winter could be explained by: i) the temperature coefficient (Q10) - the assay temperature (room temperature) is higher than the in situ winter temperature; ii) the biofilm in the mesocosms, although being 3.5 months old, was not completely mature; iii) development/maturation of biofilm in the influent tank over time; iv) the inherent variably with the tap water used; and v) a probable combination of several of the previous explanations. The microbial community metabolic profiles in the effluent of the mesocosms shows no significant difference (for most of the samples) between the working lines (Fig 3A, B). However, when looking for seasonal effects (Fig 3C), high variations are only observed in the influent and unplanted mesocosms. Therefore, it can be concluded that both *T. latifolia* and *P. australis* have shaped and stabilized the microbial community function in both seasons. Additionally, unplanted mesocosms and influent grouped separately from the

planted mesocosms in both summer and winter (Fig 4C), which suggests a shift in microbial community function when the influent passed through the planted mesocosm. It has been shown before that unplanted wetlands have a greater shift in their interstitial microbial community (in comparison to planted wetlands) over different seasons in response to a C:N:P ratio change in wastewater (Zhao et al. 2010).

The significant difference between planted mesocosms in activity and richness in summer (Fig 2B, E) as well as carbon source utilization profiles (Fig 3B) and groupings (Fig 4) demonstrate that not only plant presence but also plant species shapes the microbial function in the mesocosms. In the present study, *P. australis* exhibited a higher microbial activity and had higher carbon source utilization (higher value of AWCD and richness) in summer. In addition, for winter where data for more plant species is available, *T. latifolia* and *B. erecta* show distinct groupings (Fig 4B). The overall difference in microbial communities between planted and unplanted mesocosms, as well as different plant species has different probable justifications. Plants can release oxygen in the rhizosphere, as well as exude different compounds (organic acids, sugars, enzymes) that consequently condition the ecological functions of the attached microbial community (Brix, 1997; Lai et al., 2012). Different plants have different exudation profiles (Zhai et al., 2013) and consequently shape distinct microbial consortium. In addition, the mechanical attachment to plant root is a conditioning factor itself. As different plants have different structures and consequently will have different root morphology for microorganisms to attach. This difference in roots between plant species leads to subsequent differences in microbial community composition. (Faulwetter et al., 2013; Zhai et al., 2013). Calheiros et al. (2009) has shown diverse and distinct microbial communities in two series of two-stage CWs planted with *T. latifolia and P. australis.*

Another clear result, is that no toxic effect was observed for the iohexol and ibuprofen on the saturated CW mesocosms at the tested 100 μg L^{-1} level. No effects

in the microbial community of saturated CW mesocosms has been also demonstrated for similar levels (100 μg L^{-1}) of pesticides (imazalil and tebuconazole) (Lyu et al., 2016). Exceptions were only observed for the influent and unplanted mesocosm samples in summer and the *T. latifolia* mesocosm samples in winter. In summer, plants are more active providing a greater buffering capacity for the rhizosphere and minimizing any potential impact of ibuprofen and iohexol. While in winter, all the plants were withered due to the cold environment (<10 ℃) and reduced light time (around 6h), likely resulting in a different rhizosphere ecosystem dynamic.

Microbial communities are a key factor for pollutants removal/transformation/depletion in CWs. Zhang et al. (2016) demonstrated that both ibuprofen and iohexol could be removed efficiently in saturated mesocosm CWs and biodegradation was the main mechanism. However, no evidence could justify whether the compounds biodegradation was attributed to the plant and/or microbial community activity. In fact, literature addressing the correlation between pharmaceutical removal and microbial function in CWs is sparse. In the present study, to the best of our knowledge, it is the first time that iohexol removal efficiency was correlated with microbial function. Iohexol showed a clear correlation with microbial activity (AWCD) and richness, the more active and diverse the community, the more removal was observed. In the present study, the canonical correlation analysis, and Pearson correlation analysis showed iohexol removal efficiency not only positively correlated with carbon source guilds, but the specific carbon source utilized. Putrescine, an amine, is a low-molecular-weight nitrogenous base known to play a wide range of functions in different cell types (Wunderlichová et al., 2014). Zhang et al. (2017) has found that putrescine was correlated with ibuprofen removal. However, in the present study it did not correlate with ibuprofen, only iohexol. Nevertheless, L-arginine was correlated with ibuprofen removal in both works. The present study covered different seasons and a single CW design (saturated conditions), while the previous study

by Zhang et al. (2017) only focused on ibuprofen and was performed in the summer in mesocosms mimicking different CW designs (unsaturated, saturated and aerated). The potential link of putrescine consumption with pharmaceutical biodegradation seems relevant, but at this stage the specific metabolic vector driving the process is unclear. Glycyl-L-glutamic acid, an amino acid was presently correlated with ibuprofen removal in the winter. In addition, the carbohydrates, D-cellobiose, and D,L-alpha-glycerol phosphate were positively correlated with iohexol removal in winter. These carbohydrates are known to be easily degraded in CWs (Meng et al., 2014), but are now for the first time linked with iohexol removal. Altogether, results point to the need for a better understanding of how and why the metabolism of different carbon sources may be linked to pharmaceutical biodegradation. High-throughput sequencing should be further explored to reveal the bacterial related to ibuprofen and iohexol degradation.

Bringing the results to the perspective of practitioners, CWs have been implemented for years and have been subject to effects of organic micropollutants even before we could measure these types of compounds. Ibuprofen and iohexol were presently demonstrated not to have an impact up to 100 μg L^{-1}. There is now increasing evidence demonstrating no measurable toxic effects for both plants (Carvalho et al., 2014) and microbial communities (Caracciolo et al., 2015) in the ranges where pharmaceuticals are detected in the aquatic environment (pg to μg L^{-1}). Therefore, we should not fear for the lifespan of existing CW systems treating domestic wastewater. Nevertheless, care should be used if considering the use of CWs to treat wastewater from the pharmaceutical industry where levels may get closer to toxicity thresholds. From a treatment perspective, plants provide a buffering capacity to CWs, helping to establish a rhizosphere and develop a mutually beneficial relationship with the microbial community over different seasons. Plants, due to, either or both, root exudation and mechanical attachment do shape the microbial function and have a key role in enhancing pharmaceutical removal. Whether it be plant selection or optimization of operating conditions

required to extend certain microbial metabolic pathways is not yet entirely clear and as such more research is needed. In addition, analysis of degradation products, followed by an attempt to close the mass balance of the parent compound, should be included in the future to help elucidate the extent of the different biodegradation mechanisms.

5. Conclusions

The difference in microbial community metabolic function among five wetland plant species and between seasons in saturated CW treating the pharmaceuticals ibuprofen and iohexol was compared. It was shown that iohexol and ibuprofen at the μg L^{-1} concentration level does not influence the microbial community of the different planted mesocosms. Plant presence was the main factor influencing the microbial function in the present study, providing a buffering capacity and reducing the seasonal effect in the microbial community. It was found for the first time that the removal of iohexol was correlated with microbial function, in both the summer and winter. In addition, utilization of specific carbon sources was linked with iohexol and ibuprofen removal. Unveiling microbial degradation pathways will likely play a central role in the further optimization of CW systems.

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The Microbial Community Metabolic Profiles in Saturated Constructed Wetland Treating Ibuprofen and Iohexol

Yang ZHANG[1,a], Tao LV[2,a], Pedro N. CARVALHO[2*], Liang ZHANG[2], Mark BUTTON[3], Carlos A. ARIAS[2], Kela P. WEBER[3], Jianghong SHI[1], Zhanghe Chen[4], Hans BRIX[2]

1 School of Environmental Science and Engineering, Southern University of Science and Technology, Shenzhen 518055, PR China

2 Department of Bioscience, Aarhus University, Aarhus 8000C, Denmark

3 Department of Chemistry and Chemical Engineering, Royal Military College of Canada, Kingston, ON K7K 7B4, Canada

4 College of Life Science, South China Normal University, Guangzhou 510631, PR China

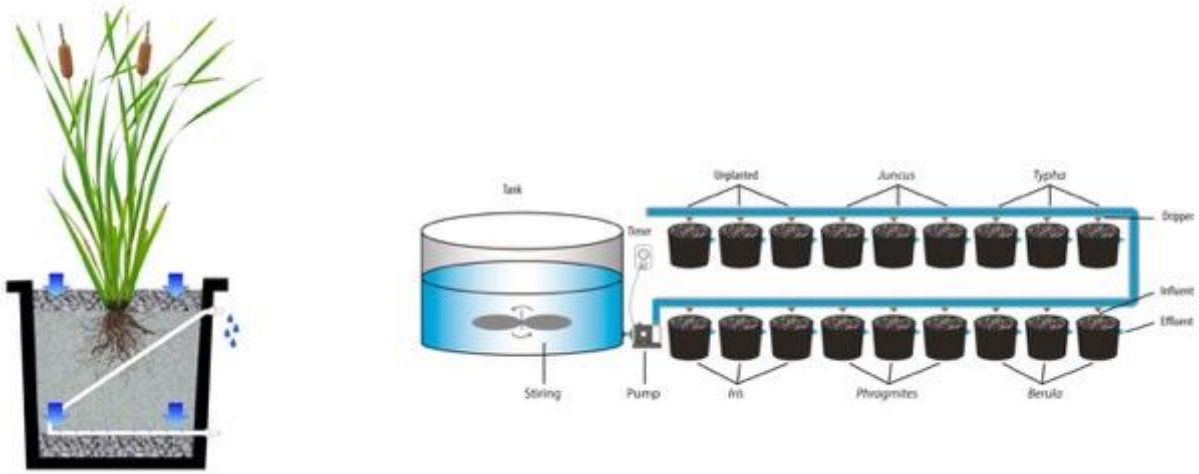

Fig. 1 Schematic of the mesocosm and working line in the experiment.

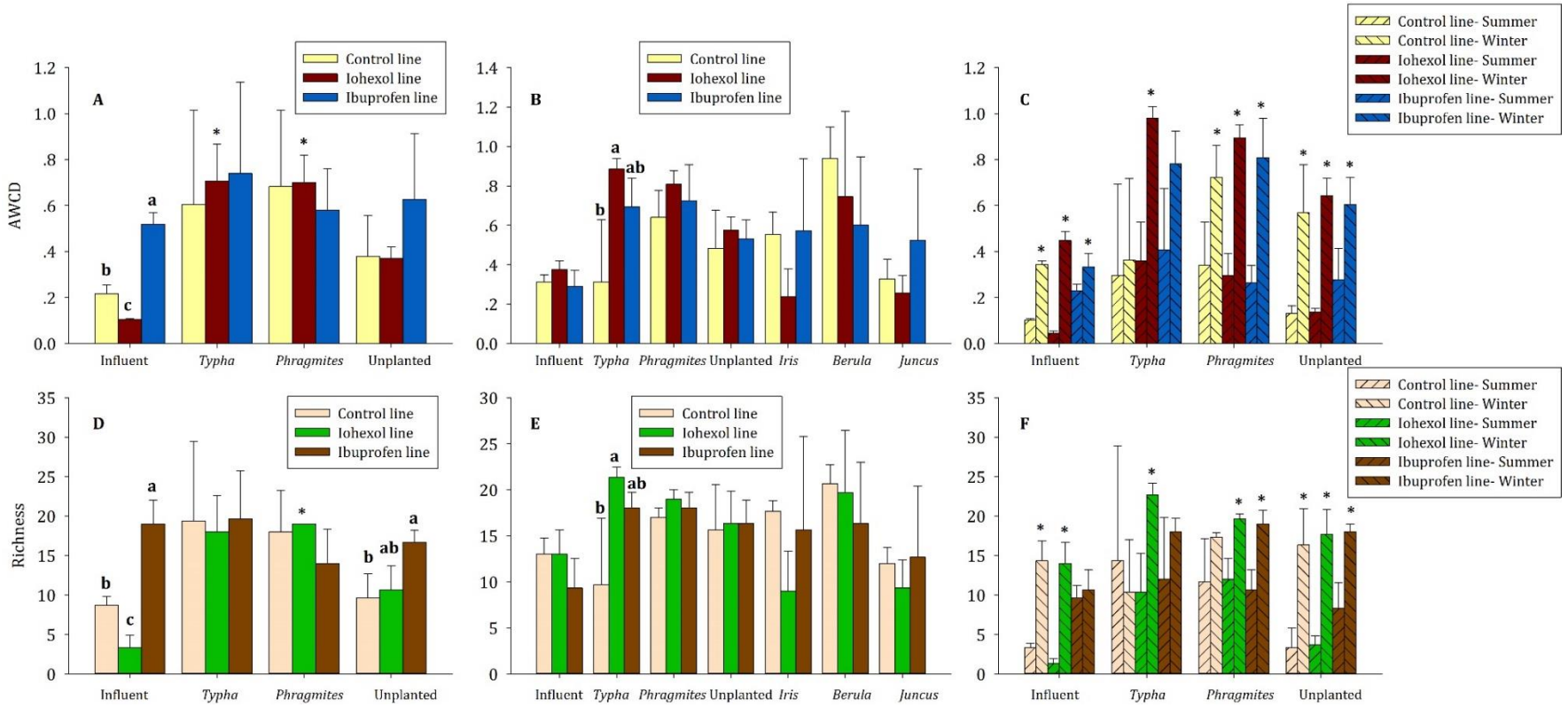

Fig. 2 Average well colour development (AWCD) and richness of different mesocosm types (influent, *Typha*, *Phragmites*, *Iris*, *Berula*, *Juncus*, unplanted) from control, iohexol and ibuprofen working lines for summer (A, D), winter (B, E) and seasonal comparison (C, F). The three columns within each group represent control, iohexol and ibuprofen working line from left to right, respectively. The different lowercase letters above the bar indicate significant difference between the workinglines for a given sample type ($p<0.05$). The asterisks in A, B, D and E indicate significant difference with the corresponding unplanted controls for a given working line, and in C and F indicate significant difference between summer and winter ($p<0.05$).

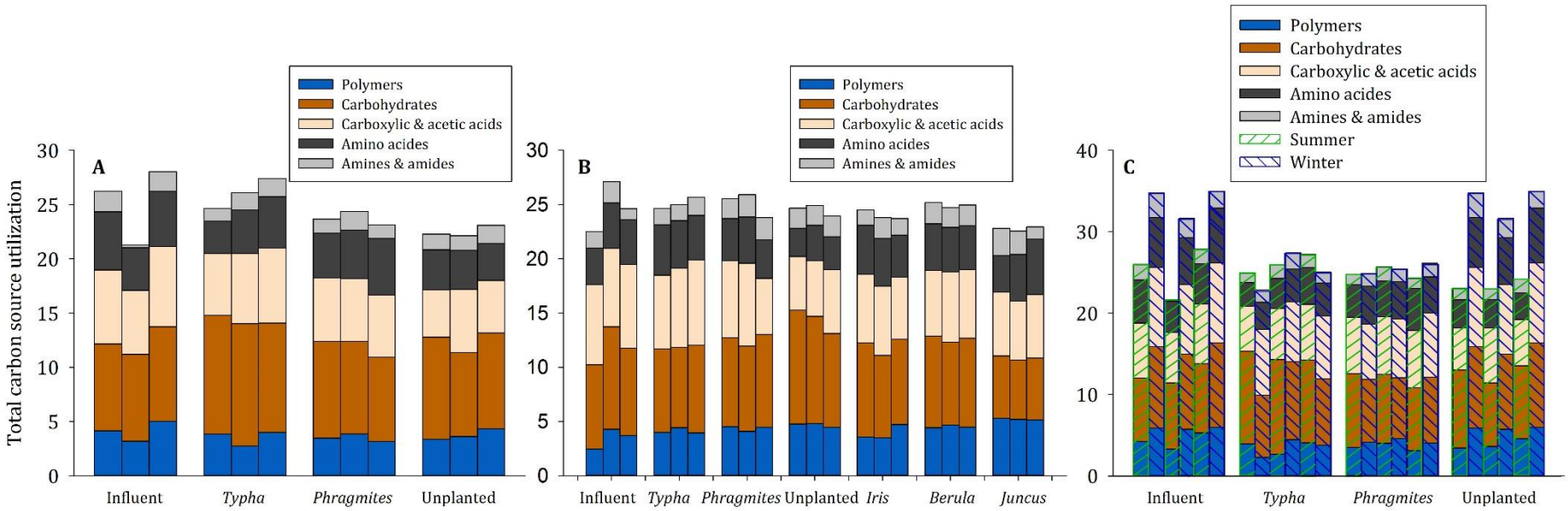

Fig.3 Carbon source utilization response for the different guilds – polymers, carbohydrates, carboxylic and acetic acids, amino acids and amines and amides in summer (A), winter (B) and seasonal comparison (C). The columns within each group represent control, iohexol and ibuprofen working line from left to right, respectively.

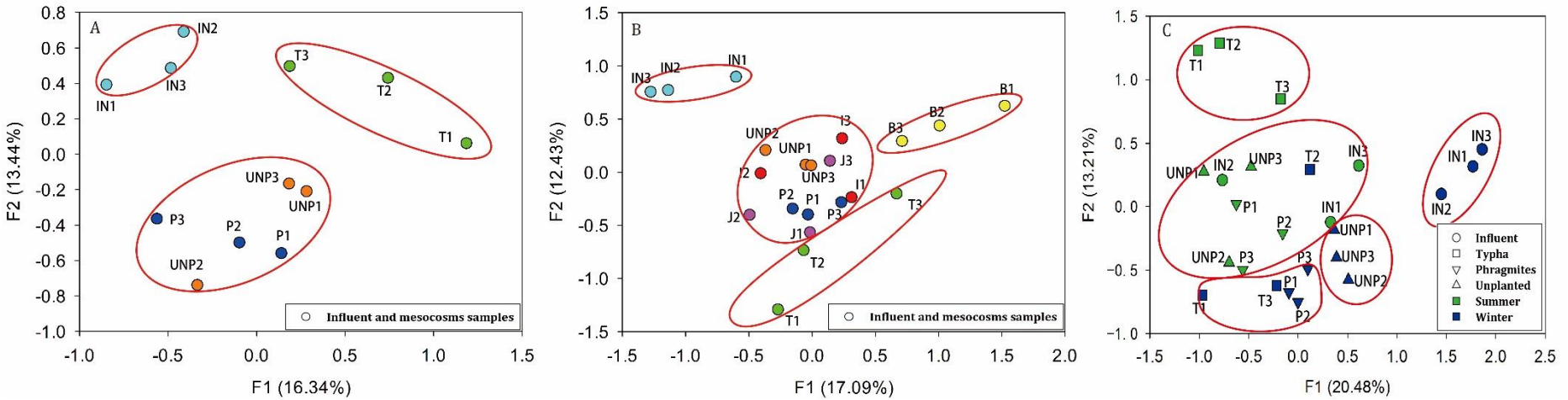

Fig. 4 PCA ordination of the Taylor transformed CLPP data of the influent (●) and the different mesocosm samples (●*Typha*, ●*Phragmites*, ●unplanted) in summer (A), (●Influent, ●*Typha*, ●*Phragmites*, ●*Iris*, ●*Berula*, ●*Juncus,* ●unplanted) in winter (B) and seasonal comparison of the influent, *Typha*, *Phragmites* and unplanted samples (C). 1, 2, 3 represent control, iohexol and ibuprofen working line, respectively. The single object in the plot represents an average of the three mesocosm replicates analysis. The different ovals indicate the groupings are statistically significant different ($p<0.05$, PERMANOVA).

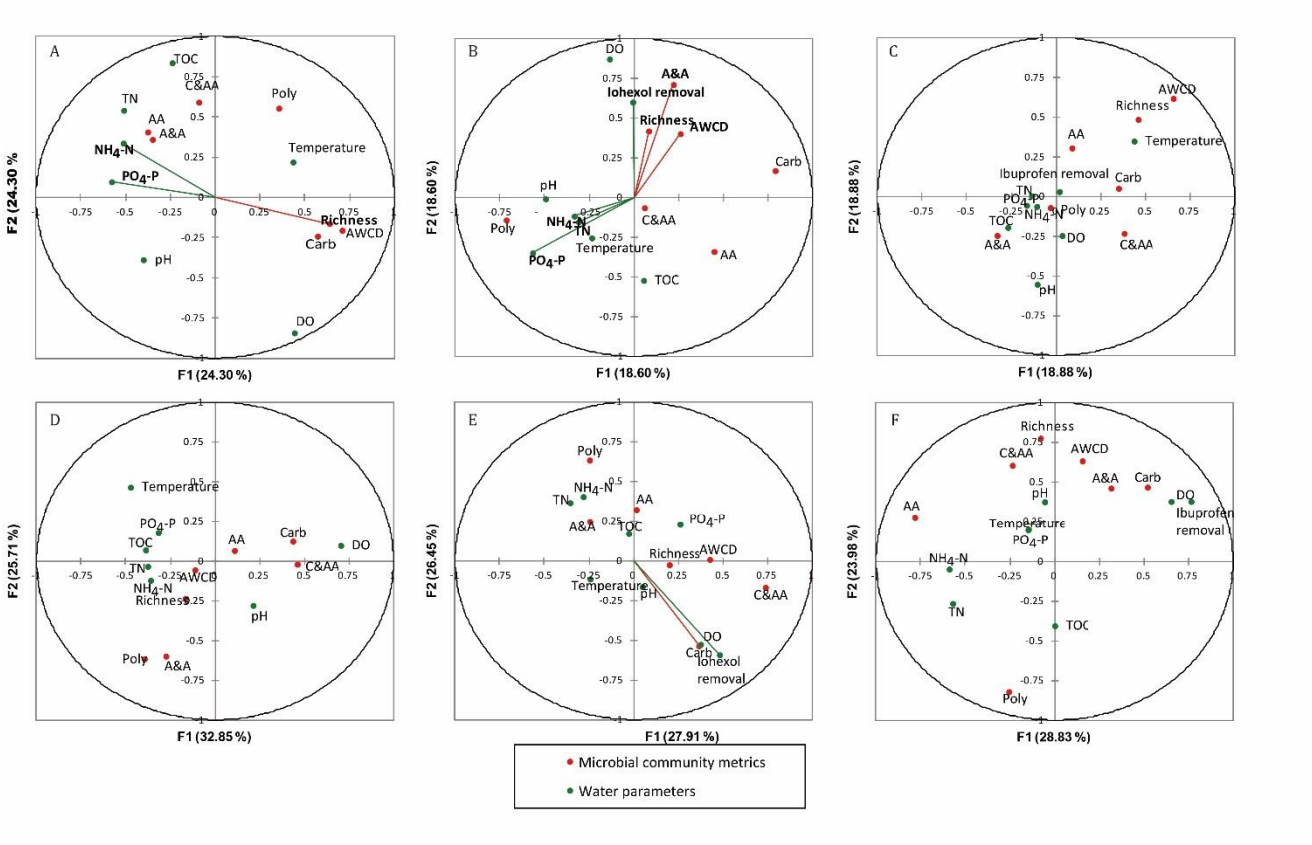

Fig. 5 Canonical correlation analysis (CCorA) between the water parameters (temperature, pH, DO and TOC, TN, NH_4-N, PO_4-P, ibuprofen (IBU) and iohexol (IOH) removal efficiency) and the microbial community metabolic metrics (AWCD, richness, and utilization of polymers (poly), carbohydrates (carb), carboxylic and acetic acids(C&AA), amino acids (AA) and amines and amides(A&A)) of all the mesocosms from the control, iohexol and ibuprofen line in summer (A,B, and C) and winter(D, E, and F), respectively.

Table 1 Overall average and standard deviation of the water quality parameters (temperature, pH, DO and TOC, TN, NH_4-N, PO_4-P, ibuprofen (IBU) and iohexol (IOH) removal efficiency) in the mesocosms (UNP:unplanted; T:*Typha*; P: *Phragmites*; I: *Iris*; B: *Berula*; J:*Juncus*) from different working lines

			Temperature (°C)	pH	DO (mg L^{-1})	TOC (%	TN (%)	NH_4-N (%)	PO_4-P (%)	IOH (%)	IBU (%)
Control line	Summer	In	21±3	7.6±0.3	4±2	--	--	--	--	--	--
		U	20±4	7.8±0.5	7±2	51	49±1	32±13	22±10	--	--
		T	22±5	7.4±0.2	8±1	55	97±4	98±4	97±6	--	--
		P	21±4	7.5±0.4	8±1	60	98±2	100.0±0.	93±10	--	--
	Winter	In	8±2	7.4±0.3	4±2	--	--	--	--	--	--
		U	6±3	7.7±0.1	8±2	54	20±1	10±7	-103±70	--	--
		T	6±3	7.5±0.1	8±2	41	60±2	49±15	20±17	--	--
		P	6±3	7.4±0.1	8±2	60	90±1	88±15	66±24	--	--
		J	6± 3	7.4±0.1	10±1	68	91±7	80±13	80±16	--	--
		B	6± 3	7.4±0.1	9±3	35	81±1	78±15	47±20	--	--
		I	6± 3	7.4±0.1	11±1	60	95±8	91±11	69±26	--	--
IOH line	Summer	In	21±3	7.6±0.3	4±2	--	--	--	--	--	--
		U	21±4	7.9±0.1	6±2	48	51±2	45±22	24±16	85±4	--
		T	18±7	7.3±0.1	8±1	66	99±1	100.0±0.	100.0±0.	87±3	--
		P	21±4	7.3±0.3	8±1	53	98±1	100.0±0.	88±11	76±8	--
	Winter	In	7±2	7.4±0.3	4±3	--	--	--	--	--	--
		U	7±3	7.7±0.1	7±1	51	6±4	8±5	-125±28	80±1	--
		T	6±3	7.4±0.1	7±1	33	74±2	45±9	18±10	82±7	--
		P	6±3	7.4±0.1	9±2	71	90±1	73±15	59±4	86±1	--
		J	7±3	7.4±0.1	11±1	80	97±1	96±4	98±1	86±9	--
		B	6±3	7.4±0.2	10±2	62	66±1	60±18	44±27	91±1	--
		I	6±3	7.4±0.1	11±1	72	98±1	95±4	72±9	89±5	--
IBU line	Summer	In	21±3	7.8±0.2	4±2	--	--	--	--	--	--
		U	21±3	7.8±0.2	6±2	53	61±2	53±23	28±19	--	50±28
		T	21±4	7.4±0.4	8±1	60	98±1	100.0±0.	99±2	--	86±14
		P	21±4	7.2±0.6	8±1	55	97±2	100.0±0.	83±17	--	67±8
	Winter	In	7±2	7.4±0.2	4±3	--	--	--	--	--	--
		U	6±3	7.7±0.1	8±2	34	18±1	11±13	-82±44	--	27±12
		T	6±3	7.5±0.2	10±1	74	98±2	66±6	18±14	--	73±7
		P	7±3	7.4±0.1	8±2	62	86±1	81±16	64±12	--	43±13
		J	6±3	7.3±0.1	11±1	63	97±1	93±4	92±5	--	94±3
		B	7±3	7.4±0.1	9±2	55	65±1	58±15	32±6	--	78±4
		I	7±3	7.4±0.1	11±2	76	97±2	94±5	46±15	--	76±7

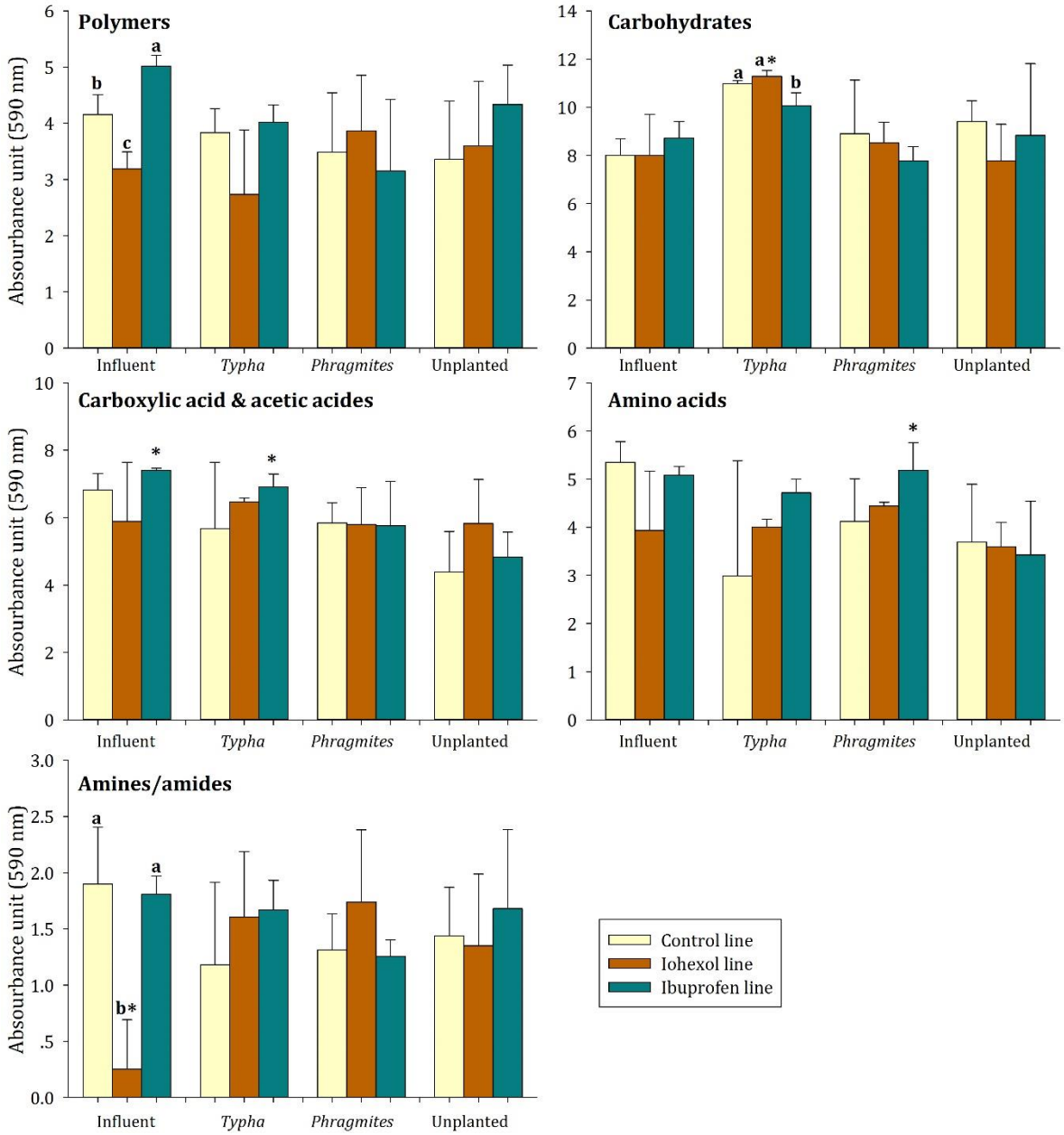

Fig. S1 Utilization of carbon sources (Guilds) of different samples (influent, *Typha*, *Phragmites*, unplanted) from control, iohexol and ibuprofen working lines in summer. The different lowercase letters above the bar indicate significant difference between the three working lines for a given type of sample. The asterisks indicate significant difference between mesocosm types for a given working line.

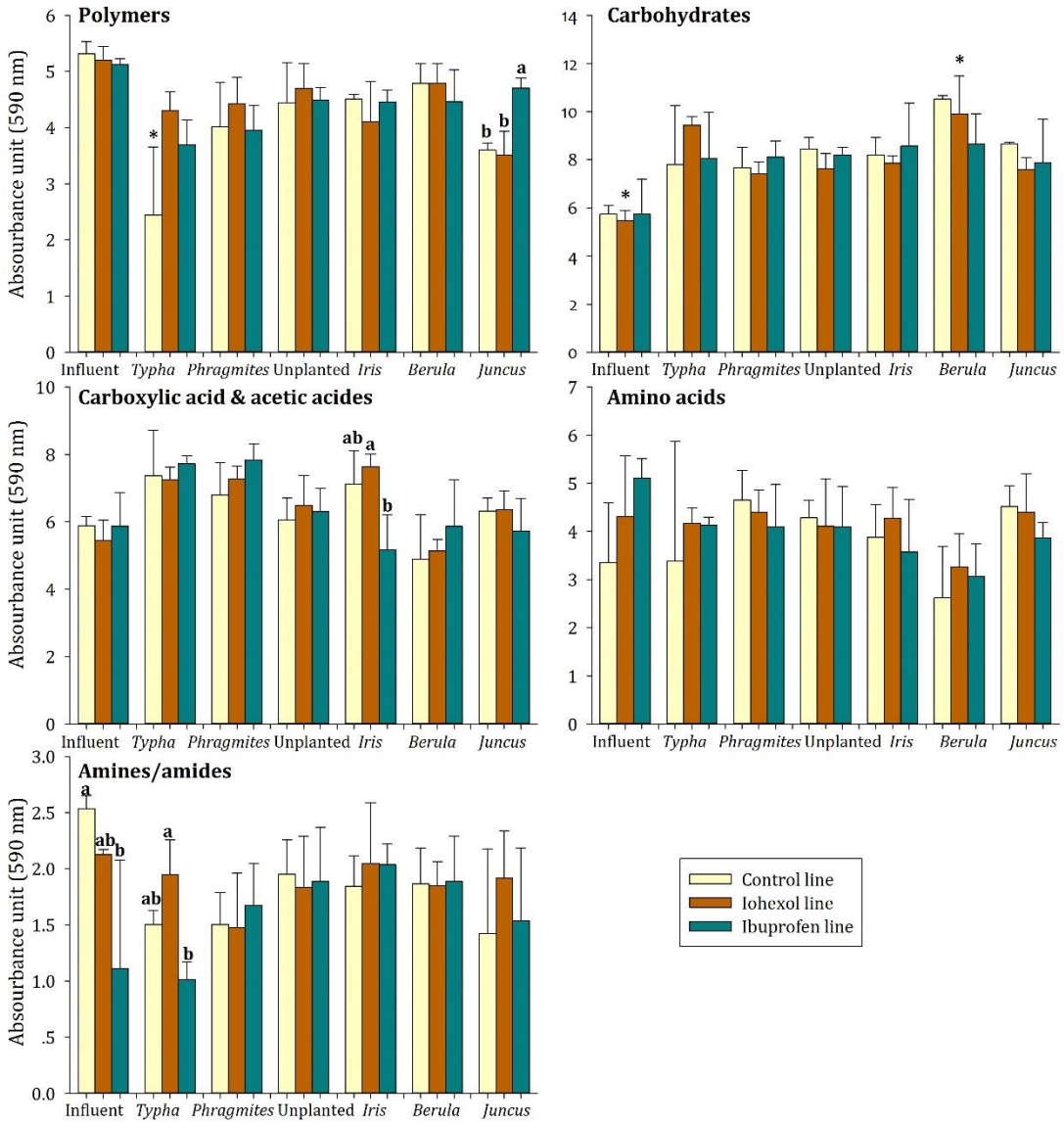

Fig. S2 Utilization of carbon sources (Guilds) of different samples (influent, *Typha*, *Phragmites*, *Iris*, *Berula*, *Juncus*, unplanted) from control, iohexol and ibuprofen working lines in winter. The different lowercase letters above the bar indicate significant difference between the three working lines for a given type of sample. The asterisks indicate significant difference between mesocosm types for a given working line.

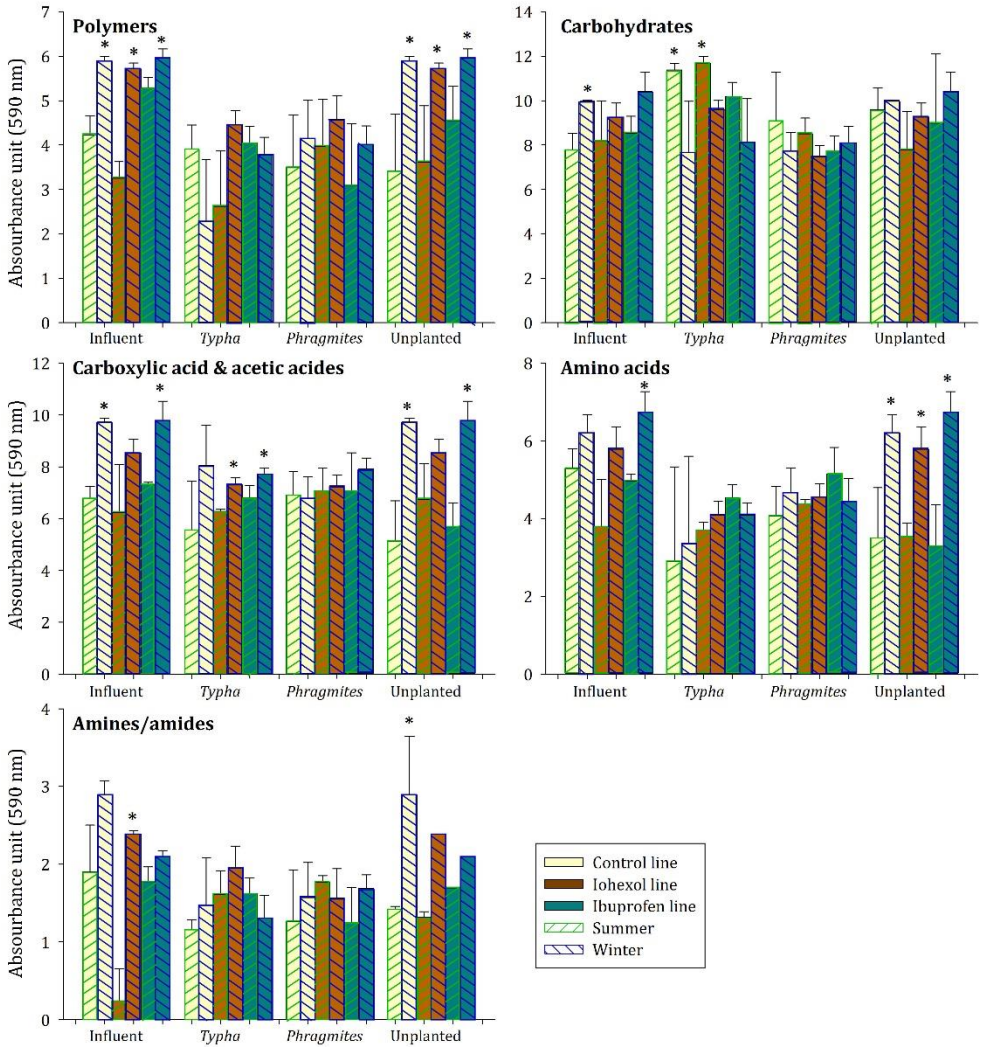

Fig. S3 Seasonal comparison of utilization of carbon sources (Guilds) of different samples (influent, *Typha*, *Phragmites*, unplanted) from control, iohexol and ibuprofen working lines. The different lowercase letters above the bar indicate significant difference between the three working lines for a given type of sample. The asterisks indicate significant difference between mesocosm types for a given working line.

Table S1 Correlation matrix of the canonical correlation analysis between water parameters (temperature, pH, DO and TOC, TN, NH_4-N, PO_4-P, ibuprofen (IBU) and iohexol (IOH) removal efficiency) and the microbial community metabolic metrics (AWCD, richness, and utilization of polymers (poly), carbohydrates (carb), carboxylic and acetic acids(C&AA), amino acids (AA) and amines and amides(A&A)) of all the mesocosms from the control (PC), iohexol (IOH) and ibuprofen (IBU) working lines in summer and winter.

Groups	Variables	Summer							Winter						
		AWCD	Richness	Poly	Carbs	C&AA	AA	A&A	AWCD	Richness	Poly	Carbs	C&AA	AA	A&A
PC	Temp	0.064	0.103	0.556	0.305	-0.219	-0.402	-0.146	0.153	0.146	0.132	0.052	-0.408	0.160	-0.083
	pH	-0.581	-0.670	-0.128	0.037	-0.659	-0.318	-0.091	-0.200	-0.073	0.023	-0.140	0.293	0.111	0.157
	EC	0.434	0.315	0.424	0.104	0.411	0.495	0.499	0.084	0.095	-0.094	-0.134	0.017	-0.269	0.370
	DO	0.520	0.542	-0.362	0.493	-0.437	-0.524	-0.490	0.172	0.125	-0.133	0.578	0.203	0.283	-0.518
	TOC	-0.318	-0.172	0.150	-0.343	**0.740**	0.451	0.263	0.000	-0.022	0.005	-0.274	-0.198	-0.204	0.445
	TN	**-0.710**	**-0.726**	0.114	-0.341	0.174	0.273	0.264	-0.232	-0.189	0.087	-0.334	-0.196	-0.121	0.486
	NH_4-N	**-0.720**	**-0.834**	0.156	-0.276	-0.135	0.173	0.274	-0.169	-0.111	0.220	-0.219	-0.276	-0.026	0.481
	PO_4-P	-0.690	**-0.847**	0.028	-0.284	-0.292	0.143	0.257	0.175	0.132	0.025	-0.075	-0.097	0.058	0.057
IOH	Temp	-0.121	-0.108	0.589	-0.271	0.123	0.064	-0.037	-0.289	-0.216	-0.141	-0.192	0.007	0.139	-0.094
	pH	-0.322	-0.238	0.630	-0.521	-0.136	-0.133	-0.056	0.243	0.235	0.105	0.129	-0.160	-0.377	-0.018
	EC	-0.378	-0.475	0.144	-0.105	-0.201	0.032	-0.292	0.216	0.318	0.272	0.163	-0.525	-0.596	0.100
	DO	0.630	0.680	0.017	0.199	0.031	-0.252	**0.711**	-0.121	-0.284	-0.584	0.534	0.306	-0.087	-0.020
	TOC	-0.358	-0.513	0.212	-0.269	0.110	-0.006	-0.394	0.307	0.418	0.349	-0.279	-0.062	-0.129	-0.028
	TN	**-0.918**	**-0.858**	0.044	-0.532	-0.212	-0.389	-0.588	0.253	0.397	0.598	-0.282	-0.405	-0.120	0.055
	NH_4-N	**-0.897**	**-0.825**	0.056	-0.528	-0.212	-0.400	-0.563	0.278	0.393	0.600	-0.226	-0.373	-0.110	0.066
	PO_4-P	**-0.825**	-0.688	0.286	-0.660	-0.208	-0.278	-0.649	0.562	0.594	0.390	0.005	0.087	-0.113	-0.183
	Removal	**0.841**	**0.895**	0.040	0.292	0.095	0.112	**0.805**	0.224	0.106	-0.483	**0.746**	0.451	-0.062	-0.271
IBU	Temp	0.368	0.422	0.389	0.253	0.154	0.479	-0.181	0.424	0.302	0.023	-0.099	-0.030	0.356	-0.009
	pH	-0.407	-0.079	0.321	-0.076	0.012	-0.607	0.363	0.138	0.269	-0.318	0.324	0.402	0.111	0.074
	EC	-0.306	-0.006	0.086	0.183	0.048	0.178	0.416	-0.509	-0.538	0.257	-0.029	-0.185	-0.227	-0.267

DO	-0.011	-0.346	-0.454	-0.117	-0.203	-0.318	-0.158	0.252	0.189	-0.449	0.511	0.009	-0.422	0.337
TOC	-0.483	-0.060	0.314	-0.111	0.519	0.448	0.350	-0.339	-0.424	0.218	-0.141	-0.108	0.038	-0.336
TN	-0.231	0.230	0.573	-0.123	0.096	-0.176	0.372	-0.290	-0.124	0.421	-0.310	-0.059	0.297	-0.075
NH_4-N	-0.206	0.191	0.541	-0.084	-0.024	-0.312	0.328	-0.198	0.034	0.271	-0.207	0.063	0.319	0.051
PO_4-P	-0.197	0.052	0.415	-0.160	-0.238	-0.447	0.266	0.143	0.170	-0.149	0.106	0.341	0.209	-0.018
Removal	0.261	-0.181	-0.475	0.114	-0.389	-0.250	-0.268	0.302	0.151	-0.399	0.468	0.066	-0.498	0.175

Bold numbers in the table indicate statistically strong correlations ($P<0.05$).