Characterization of microbial communities in wetland mesocosms receiving caffeine-enriched wastewater

Dongqing Zhang¹ · Jinxue Luo^{2,3} · Zarraz May Ping Lee¹ · Richard M. Gersberg⁴ · Yu Liu^{1,5} · Soon Keat Tan^{1,5} · Wun Jern Ng^{1,5}

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Abstract A 454 high-throughput pyrosequencing approach was used to characterize the structures of microbial communities in wetland mesocosms receiving caffeine-enriched wastewater at a concentration of 250 $\mu\text{g L}^{-1}$. The removal efficiencies of caffeine in the planted beds (93.0 %) were significantly ($p < 0.05$) higher than those in the unplanted beds (81.4 %). Bacterial diversity was decreased by 25 and 22.4 %, respectively, in both planted and unplanted mesocosms after 210-day operation. The results of taxonomic analyses suggested that chronic exposure of wetland ecosystems to caffeine could lead to moderate shifts in microbial community composition. In total, 2156 operational taxonomic units (OTUs) were generated and 20 phyla comprising 260 genera were identified. The major phylogenetic groups at phylum level included *Firmicutes* (39 %), *Actinobacteria* (25.1 %), *Proteobacteria* (17.1 %), *Synergistetes* (5.6 %), and *Chloroflexi* (5.5 %).

Bacilli and *Synergistia* increased in abundance in the planted mesocosms, while for the unplanted mesocosms, *Actinobacterial*, *Clostridia* and *Betaproteobacteria* exhibited increased proportion under the exposure of caffeine. At genus level, *Propionibacterium*, *Staphylococcus*, *Bacillus*, and *Streptococcus* were found to be increased in abundance after caffeine treatment. As for the response of fungal community to caffeine enrichment, genus like *Cladosporium*, *Emericellopsis*, *Aspergillus*, and *Phoma* were found to be resistant to caffeine disturbance. When compared to the microbial community between planted and unplanted mesocosms, a distinct community profile for both bacteria and fungi community was observed. The presence of plants had a remarkable effect on the structure of microbial community, helping buffer against the stress associated with caffeine exposure.

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Dongqing Zhang and Jinxue Luo contributed equally to this work.

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✉ Wun Jern Ng
WJNg@ntu.edu.sg

Dongqing Zhang
dqzhang@ntu.edu.sg

¹ Advanced Environmental Biotechnology Center, Nanyang Environment and Water Research Institute, Nanyang Technological University, 1 Cleantech Loop, No. 06-10, Singapore 637141, Singapore

² Key Laboratory of Environmental Biotechnology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, People's Republic of China

³ CAS.WEE (Beijing) Eco-Engineering Technology Institute Co., Ltd, Beijing 100035, China

⁴ Graduate School of Public Health, San Diego State University, Hardy Tower 119, 5500 Campanile, San Diego, CA 92182-4162, USA

⁵ School of Civil and Environmental Engineering, Nanyang Technological University, N1-01a-29, 50 Nanyang Avenue, Singapore 639798, Singapore

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Introduction

Caffeine (3,7-dihydro-1,3,7-trimethyl-1 h-purine-2,6-dione), a purine alkaloid, is the most commonly used legal drug in the world and has been added onto the US Environmental Protection Agency's list of high production volume of chemicals (Buerge et al. 2003; Mohapatra et al. 2006). In a recent review on the occurrence and fate of 137 micropollutants, Verlicchi and Zambello (2014) reported that caffeine has typically been detected in wastewater treatment plants (WWTPs) at concentrations ranging from 0.22 to 209 $\mu\text{g L}^{-1}$ in influent and up to 43.5 $\mu\text{g L}^{-1}$ in effluent. Since conventional WWTPs are not uniquely designed for the elimination of micropollutants (Joss et al. 2006), caffeine has been inevitably discharged into aquatic receiving systems (Buerge et al. 2003; Kolpin et al. 2002). Once discharged, due to its high solubility in water (37.5 g L^{-1}) and high hydrophilicity with a low $\log K_{\text{ow}}$ value of -0.07 (Thomas and Foster 2005), caffeine, a non-ionizable and polar compound, can act as a persistent chemical in the environment (Rosal et al. 2009). Despite its low concentrations in aquatic systems, ecotoxicological studies have revealed that caffeine could still exert chronic adverse effects on aquatic ecosystems (Martinez Gomez et al. 2015; Moore et al. 2008). Results of caffeine toxicity tests revealed LC_{50} levels of 182 mg L^{-1} for *Daphnia magna* (Solomon et al. 1996), 130–190 mg L^{-1} for *Xenopus laevis* (Moore et al. 2008), and 200 mg L^{-1} for *Platyonus patulus* (Martinez Gomez et al. 2015). Additional studies have engendered increasing heightened concern about caffeine's prevalence in combination with other chemical compounds in wastewaters (Moore et al. 2008). Therefore, the potential harm of this alkaloid has drawn great interest in the research on caffeine elimination and its degradation in the aquatic environment.

Over last decade, a number of studies have shown that treatment using constructed wetlands (CWs) can remove the stimulant caffeine with high removal efficiencies, e.g., 97 and 99 % for horizontal and vertical subsurface flow CWs (Matamoros et al. 2009) and 83–99 % for free-floating and submerged wetlands (Matamoros et al. 2012). With a low K_{ow} value of -0.07 , caffeine should not adsorb well to sediment (Thomas and Foster 2005). Therefore, the primary removal mechanism for caffeine is biological in origin. Although plant roots can take up caffeine which may be then translocated to shoot tissues (Zhang et al. 2013), biodegradation by microbes associated with the plant rhizosphere is considered as the most important pathway for caffeine removal (Hijosa-Valsero et al. 2010; Matamoros et al. 2012; Zhang et al. 2014). Microorganisms inhabiting CWs play an important role in

biogeochemical cycling, making them key components of bioremediation processes (Truu et al. 2009). In CWs, the co-existence of variety of microbial communities among water, substrate media, and plant compartments might offer suitable ecological niches to support a diversity of metabolic pathways leading to micropollutant biodegradation (Hijosa-Valsero et al. 2010). Because wastewater treatment is for the most part a biodegradation-driven process, characterizing the structure of microbial communities is the key to understanding the biological processes that occur in wetland systems and can provide a basis for the rational design and operation of these engineered ecosystems (Dong and Reddy 2010). Additionally, understanding the structure of microbial communities may allow the precise prediction of the specific microbial players responsible for the degradation of organic pollutants (Johnsen et al. 2002). Although the bacterial diversity in CWs has been described in a few studies (Ligi et al. 2014; Arroyo et al. 2015), up to the present, the microbial diversity of treatment wetlands has not been well addressed, and there is still little understanding of the role that the higher aquatic plants play in influencing the microbial community structure in treatment wetlands (Stottmeister et al. 2003).

More recently, 454 high-throughput pyrosequencing technology has become an effective method to fully explore the microbial diversity in aquatic systems (Roesch et al. 2007; Roh et al. 2010). Compared to conventional molecular technologies such as fluorescence in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), and terminal restriction fragment length polymorphisms (T-RFLP), high-throughput pyrosequencing approach can improve read lengths allowing for a highly accurate phylogenetic-based classification of individual reads (Ye et al. 2011). Particularly, it can reveal the presence of unculturable organisms and low-abundance organisms that are usually overshadowed by dominant populations in the aquatic environment (Roh et al. 2010). This technology has been widely used to investigate the microbial community structure in a variety of environments, such as those from submerged membrane bioreactors (Ma et al. 2013), activated sludge processes (Ye et al. 2011), and forest soils (Buée et al. 2009). However, very few studies using such a pyrosequencing approach have addressed the microbial structure in wetland ecosystems receiving pharmaceutical compounds (Zhao et al. 2015). Therefore, in the present study, we used high-throughput pyrosequencing approach to gain comprehensive taxonomic information and characterize the microbial profile under the disturbance of caffeine at the concentration of 250 $\mu\text{g L}^{-1}$. Table 1 presents the physicochemical properties of caffeine. The primary objective of this research was to determine the effect of caffeine on the structure of microbial community and the role that the higher aquatic plants play in determining the composition of these bacterial communities in CWs.

Table 1 Physicochemical properties of caffeine

	Caffeine
Structure
IUPAC name	1,3,7-trimethyl-1H-purine-2,6(3H,7H)-dione3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione
CAS no.	58-08-2
Formula	C ₈ H ₁₀ N ₄ O ₂
Molecular weight	194.19 g mol ⁻¹
Chemical function	Stimulant drug
Metabolism	Demethylation by CYP1A ₂ ^a
Water solubility	2.16E + 004 mg L ⁻¹ (25 °C) ^a
Half life	5 h ^a
pK _a	10.4 ^a
Log K _{ow}	-0.07 ^a
Log D _{ow}	-0.55 ^b

^a Physical Properties (PHYSPROP) database^b ChemAxon Marvin Calculator software was used to calculate log D_{ow}

Materials and methods

Description of the mesocosm-scaled CWs

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

The four mesocosms were separated among two groups, one spiked with caffeine while the other serving as control. The dimensions of each (made of fiberglass) was 1.2 m (L) × 0.6 m (W) × 0.6 m (D). The mesocosms were filled with a substrate which was composed of two layers, a bottom layer of gravel (300 mm, D₆₀=4–10 mm) and an upper layer of loamy sand (100 mm; 75 % of sand particles with D₆₀=1–2 mm, 23 % silt particles, and 2 % clay particles; Supplementary Fig. 1). At the bottom of each mesocosm, a horizontal drainage pipe (0.4 m long and 50 mm in diameter) with outlet valves was located for effluent sampling.

System operation and sampling regime

The experiment lasted 210 days. All the systems were acclimatised for 90 days with daily flooding using synthetic wastewater so as to allow the microbial biofilms to develop prior to the start of the study. The preparation of synthetic wastewater and caffeine stock solution was described by Zhang et al. (2012a, b). Synthetic wastewater was rapidly filled in each bed from the tank surface of one side. Water depth was maintained at 5 cm below gravel surface to ensure subsurface flow. Holding tanks of 150-L capacity served as the reservoir for dispensing synthetic wastewater. Batch modes were conducted at a hydraulic loading rate of 2.8 cm day⁻¹, which resulted in a hydraulic retention time (HRT) of 4 days. Water depth was kept at 5 cm below the gravel surface.

Wetland effluent samples were collected on a weekly basis in order to measure dissolved oxygen (DO), conductivity, chemical oxygen demand (COD), NH₄⁺-N, phosphate (PO₄³⁻), and caffeine concentrations. Every 2 months, the substrate media were collected from the four different wetland mesocosms for microbial community analysis. In each planted and unplanted system, three samples of substrate media were taken at a depth of 5 cm from the surface at random positions in the mesocosm and places in a polyethylene bag. All samples were placed on ice until arrival in the laboratory for

further processing. In the laboratory, three samples in each bag were mixed completely to produce a homogeneous sample for each of the mesocosms. All visible plant materials were removed before homogenization. After mixing, subsamples were taken from each plastic bag and transferred to a microtube (2 mL) for DNA extraction. Tubes were stored in a freezer (at -80°C) until molecular analysis was conducted.

Analytical procedures for physicochemical parameters and caffeine

Caffeine (99.9 % purity) was obtained from Sigma-Aldrich (Singapore). HPLC-grade solvents (acetonitrile, methanol, hexane, and ethyl acetate) were obtained from Fisher (USA). Solid phase extraction (SPE) cartridges (GracePure™ C18; 500 mg, 6 mL) were from Grace Davison Discovery Sciences (Belgium). The HPLC column (Inertsil ODS; 35 μm ; 4.6×150 mm) was purchased from Alpha Analytical (Singapore).

Physicochemical parameters analyzed within 24 h using spectrophotometer (HACH-DR 3800, USA) based on Standard Methods (APHA 1998) included COD, nitrate nitrogen ($\text{NO}_3\text{-N}$), ammonium nitrogen ($\text{NH}_4\text{-N}$), and PO_4^{3-} . DO, pH, and conductivity were all measured with a portable Multi-Parameter Digital Meter (HACH-HQ40d, USA).

The concentration of caffeine was analyzed according to procedures previously described (Zhang et al. 2012b). In brief, 500 mL of effluent wastewater was filtered through a $0.45\text{-}\mu\text{m}$ glass fiber membrane filter (Millipore, USA), and then SPE was conducted prior to chromatographic analysis. Water was percolated through the SPE cartridges at a flow rate of approximately 10 mL min^{-1} . The SPE cartridges were then eluted with 5 mL ethyl acetate. The eluent was then concentrated to ca. 400 μL in a nitrogen stream and then reconstituted to 500 μL with methanol.

A Shimadzu Ultra Fast Liquid Chromatography (UFLC; Shimadzu, Japan) instrument equipped with a SPD-M20A diode array detector (DAD) was used for caffeine quantitative analysis. Chromatography was accomplished using an Inertsil ODS-3 (4.6×150 mm, $5\text{ }\mu\text{m}$) HPLC column protected by a C18 guard column (Alpha Analytical, Singapore).

Chromatography data acquisition and management was done using a “Shimadzu LCSolution program.”

DNA extraction and polymerase chain reaction

Approximately 0.25 g of the substrate media was the used for DNA extraction. DNA for genomic analysis was first extracted using a Fast DNA Spin Kit for Soil (MP Biomedicals, USA). DNA purity was verified by the 260/282 and 260/230-nm absorption ratios, measured using a ND-2000 spectrophotometer (NanoDrop Inc., Wilmington, DE). Then, the DNA purity was analyzed using agarose gel electrophoresis (1.1 %) stained with ethidium bromide solution. No additional purification procedure was needed for any of the samples.

Both 16S (bacteria) and 18S (fungal) ribosomal DNA fragments were amplified using a DNA thermocycler (Eppendorf, Germany) with universal primers for the V1–V3 regions of the 16S and 18S rRNA genes (Table 2). Polymerase chain reaction (PCR) amplification was performed according to the methodology and conditions given in Zhang et al. (2015).

Processing of pyrosequencing data

DNA samples were analyzed by Research and Testing Laboratory (Lubbock, TX) using bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) (Dowd et al. 2008). The MOTHR bioinformatics software package (version 1.3) was used for processing the sequencing (Schloss et al. 2009). First, the raw reads were trimmed for adapters, bar codes, and primers. Then, the trimmed sequences were screened for length (minimum = 200), homopolymers (maximum = 8), ambiguous bases (0 allowed), and quality (average = 25). Sequences passing these criteria were then aligned against the non-redundant SILVA ribosomal RNA database (Quast et al. 2013). Chimera detection was carried out with UCHIME within MOTHR followed by taxonomic classification of bacterial sequences using Ribosomal Database Project (RDP) training set as reference, with a confidence value of 80 %. If sequences showed similarities below these criteria, they were classified into unidentified groups for each taxonomic rank. A distance matrix was constructed at a 3 %

Table 2 Oligonucleotide primers used for polymerase chain reaction (PCR) amplification

	Primer consensus sequence Position (length)	Reference
Bacteria—16S rDNA		
Forward position	Gray 28F (5'-GAGTTTGATCNTGGCTCAG-3')	(Baker et al. 2003)
Reverse position	Gray 519R (5'-GTNTTACNGCGGCKGCTG-3')	
Fungi—18S rDNA		
Forward position	funSSUF (5'-TGGAGGGCAAGTCTGGTG-3')	(Foster et al. 2013)
Reverse position	funSSUR (5'-TCGGCATAGTTTATGGTTAAG-3')	

dissimilarity. After all the sequence processing, a total of 20,482 remaining sequences were clustered into operational taxonomic units (OTUs) using the furthest neighbor method. The closest relative for representative sequence of each OTU was identified using RDP SeqMatch. A phylogenetic tree was created using MEGA6 with bootstrap calculation using a nucleotide substitution model.

All DNA sequences had been deposited to the Sequence Read Archive of NCBI. The accession numbers of sequence data packets are SRR2177520, SRR2177522, SRR2177522, SRR2182154, SRR2182155, SRR2182157, SRR2182159, SRR2182160, SRR2182171-SRR2182175, SRR2182224, SRR2182226, SRR2182368-SRR2182370, SRR2182372, SRR2182428, SRR2182438, SRR2182469, SRR2182472-SRR2182474, and SRR2182493.

Statistical analysis

The total number of bacterial 16S ribosomal DNA (rDNA) sequences was 27,458, and the average value was 2112. Four-hundred fifty-four high-throughput pyrosequencing technology provided great unique sequence coverage (>95 %) for each individual sample. The libraries were then normalized by randomization to the smallest library to avoid unequal sampling bias when computing the diversity indices. For the calculation of relative abundance, α -diversity indices (Shannon-Wiener indices (H'), Simpson's diversity indices, and Shannon evenness indices (J')) of each sample, the R program (v.2.14.2, <http://www.r-project.org/>) was used with the output of MOTHUR. Non-metric multidimensional scaling (NMDS) was created using Bray-Curtis similarity matrices with PRIMER-E (version 6) in order to visualize the effect of caffeine on the bacterial community profile. Statistical

differences in bacterial community structure and composition between planted and unplanted mesocosms were conducted by assessing significance ($p < 0.05$) through analyses of variance (one-way ANOVA; SPSS v.15.0).

Results and discussion

Performance of wetland systems

Results of the analyses of the conventional parameters in the CW mesocosms are summarized in Table 3. As has been previously reported for other treatment wetlands (Zhang et al. 2012a), the overall average removal efficiencies of traditional parameters (e.g., COD, NH_4^+ -N, NO_3^- -N, and PO_4^{3-}) were higher in the planted beds compared to the unplanted beds. Similarly, the removal efficiencies of caffeine in the planted beds (93.0 %) were significantly ($p < 0.05$) higher than those in the unplanted beds (81.4 %). In a previous review conducted by Verlicchi and Zambello (2014), average caffeine removal efficiencies of 84 % in HSSF CWs and 62 % for the unplanted wetlands were reported. By comparison, the relatively higher removal efficiencies for caffeine observed in the present study (81.4 and 93.0 % in the unplanted and planted mesocosms) might be due to the tropical environment, since microorganisms living in CWs typically attain their optimal activity at 25 °C (Truu et al. 2009).

Richness and diversity of microbial communities

In this study, a 97 % similarity cutoff was used to delineate OTUs in the downstream analysis using the pyrosequencing approach. Among the large diversity of sequences in the 16S

Table 3 Average concentrations of traditional parameters and caffeine, as well as removal efficiencies (percent in the parentheses)

	Inlet	Outlet			
		Planted HSSF CWs		Unplanted HSSF CWs	
		Control	Caffeine treatment	Control	Caffeine treatment
Conventional parameters					
pH	7.7±0.2	6.9±0.1	6.6±0.2	6.5±0.2	6.3±0.3
Conductivity (S m ⁻¹)	457±13	423±36	405±47	391±51	479±63
DO (mg L ⁻¹)	7.6±0.5	4.0±1.6	3.7±2.0	3.1±0.9	2.9±1.4
COD (mg L ⁻¹)	306±17	18.8±3.1 (93.9 %)	22.4±6.1 (92.7 %)	38.4±8.9 (87.5 %)	49.8±14.1 (83.7 %)
NH ₄ ⁺ -N (mg L ⁻¹)	28.4±4.1	6.1±1.9 (78.5 %)	5.7±2.1 (79.9 %)	10.5±3.3 (63.0 %)	11.7±4.8 (58.8 %)
NO ₃ ⁻ -N (mg L ⁻¹)	9.5±0.9	1.6±0.3 (83.2 %)	1.3±0.4 (86.3 %)	4.5±0.6 (52.6 %)	3.1±0.4 (67.4 %)
PO ₄ ³⁻ (mg L ⁻¹)	22±2.8	9.1±2.6 (58.6 %)	8.5±2.1 (61.3 %)	11.8±3.7 (46.4 %)	12.4±3.7 (43.4 %)
Pharmaceutical compounds					
Caffeine (μg L ⁻¹)	250	—	17.5±8.7 (93.0 %)	—	46.5±17.1 (81.4 %)

“±” represents standard deviation

Table 4 Summary of sequencing data for initial, control (C), and different treatment (T)

	Initial	Planted HSSF CWs						Unplanted HSSF CWs					
		Control			Treatment			Control			Treatment		
		P1_C	P2_C	P3_C	P1_T	P2_T	P3_T	S1_C	S2_C	S3_C	S1_T	S2_T	S3_T
Bacteria													
OTU numbers	16	142	83	78	137	35	40	19	25	56	33	45	49
Chao richness	23	415.25	122.18	117.06	404.63	63.5	117	21	25.5	123.67	37.5	55.46	67.75
Shannon evenness (J')	0.7	0.95	0.94	0.9	0.96	0.66	0.8	0.8	0.54	0.84	0.77	0.82	0.68
Shannon diversity (H')	1.93	4.77	4.15	3.92	4.65	2.36	2.94	3.36	3.47	3.4	2.7	3.11	2.64
Fungi													
OTU numbers	11	106	15	12	124	12	10	10	16	20	–	10	13
Chao richness	12.5	164	8.5	18	178	22	25	17.5	12.5	35	–	26.5	49
Shannon evenness (J')	0.32	0.45	0.15	0.13	0.61	0.59	0.33	0.47	0.03	0.30	–	0.25	0.32
Shannon diversity (H')	0.76	2.12	0.20	0.32	2.93	1.46	0.76	1.07	0.08	0.78	–	0.68	0.84

(i) The letter “P” represents planted HSSF CWs; (ii) the letter “S” represents unplanted HSSF CWs; (iii) the letter “T” represents treatment; (iv) the letter “C” represents control; and (v) the numbers “1,” “2,” and “3” represent the sampling time

rRNA gene libraries, 2156 OTUs have been identified at a 3 % distance across the 12 samples and 652 OTUs were found to be shared among two or more samples. Most of the samples from the control beds presented higher OTU numbers than those in the caffeine-treated beds for both the bacterial and fungal communities after the libraries were normalized by randomization to smallest library (Table 4).

The analytical results of Shannon-Weiner’s indices (H') showed that bacterial diversity decreased by 25 and 22.4 % in both planted and unplanted wetland mesocosms after exposure to caffeine. On the contrary, Shannon-Weiner’s indices (H') for the fungal community in the mesocosms enriched with caffeine were higher ($p > 0.05$) than that in the control beds. A variety of previous studies have calculated H' values in aquatic ecosystems, and the values typically range from 0.5 to 5, with values of 0.5–2.0 indicating low diversity and values of 2.0–5.0 indicating high diversity (Garrido et al. 2014). In the present study, most of H' values for the wetland bacterial communities were higher than 2.0 and were in a range of 1.93–4.77, suggesting a relatively wide range of bacterial diversity. However, most of H' values for the fungal community were lower than 2.0 (with a range of 0.20–2.12), implying a relatively lower diversity compared to bacterial community.

Rarefaction curves, which describe the species richness as a function of sampling effort at a 0.03 distance cutoff, are plotted with sequence number of 16S rRNA against OTU numbers in different wetland mesocosms (Supplementary Fig. 2). Rarefaction curves generated for 16S rRNA bacteria (a) and 18S rRNA fungi (b) are based on taxonomic pyrosequencing in different wetland mesocosms, calculated by MOTHUR with a 3 % distance cutoff. For most of rarefaction curves shown in Supplementary Fig. 2, their flat slopes

indicate that a large fraction of the species diversity has been sampled.

Similarity of microbial community structure

NMDS allows a visualization of the distance relationships among the samples and provides an insight into their level of dispersion (Fig. 1). A noticeable difference between planted and unplanted as well as control and caffeine-treated mesocosms was observed in the present study. Using 0.6 as a benchmark (McLellan et al. 2010), for the planted mesocosms, all the control samples were clustered together and did not group with the caffeine-treated mesocosms, implying a notable dissimilarity in bacterial community between the caffeine-treated mesocosms and the control beds (Fig. 1b). This condition was even more pronounced in the last sampling period. For the unplanted mesocosms, the bacterial communities retrieved from the caffeine-treated mesocosms are also clustered together, but the dissimilarity between caffeine-treated and untreated systems was not as obvious with S1_C and S2_T clustering together with a similarity of 60 % and S3_C and S3_T clustering together at a 50 % similarity (Fig. 1c). Therefore, although our results suggest strong evidence for the shifts in community structure as a result of caffeine exposure, the existence of some notable outliers in these similarity plots indicates that such a conclusion cannot be definitive and points to the need for further lines of inquiry down this path.

In contrast, with respect to the fungal community, high similarity was observed between caffeine-treated and control-planted mesocosms (Fig. 1e). However, for the unplanted beds, caffeine-treated beds presented a remarkable dissimilarity to the control (Fig. 1f). Moreover, it is worth

Fig. 1 Non-metric multidimensional scaling (NMDS) analysis of microbial community phylogenetic distance at class level derived from pyrosequencing bacterial 16S rRNA genes and fungal 18S rRNA genes, **a** bacterial community for both planted and unplanted mesocosms, **b** bacterial community for planted mesocosms, **c** bacterial community for unplanted mesocosms, **d** fungal community for both

planted and unplanted mesocosms, **e** fungal community for planted mesocosms, and **f** fungal community for unplanted mesocosms. (i) Letter “P” represents planted beds; (ii) letter “S” represents unplanted beds; (iii) letter “T” represents treatment; (iv) letter “C” represents control; and (v) numbers “1,” “2,” and “3” represent the sampling time

noting that a significantly temporal variation was observed for the unplanted mesocosms over time, suggesting the dynamic microbial communities and biotic/abiotic processes throughout the whole experiment. When comparing the microbial community between planted and unplanted mesocosms, a distinct community profile for both bacteria and fungi was observed in the planted mesocosms with similarities less than 40 % relative to unplanted mesocosms (Fig. 1a, d). These findings can also be illustrated by a dendrogram which was constructed to provide a visual presentation of the dissimilarity between the different treatment regimes (Supplementary Fig. 3).

Composition of microbial community

In total, 20 bacterial phyla comprising 260 genera were identified in the present study. Across all soil samples, *Firmicutes* (averaging 39 %) represented the most abundant phyla, followed by *Actinobacteria* (25.1 %) and *Proteobacteria* (17.1 %). The other predominant phyla (>1 %) were *Synergistetes* (5.6 %), *Chloroflexi* (5.5 %), *Bacteroidetes* (2.9 %), and *Acidobacteria* (1.0 %; Fig. 2a). It was also noticed that besides the dominant microbes, as many as 3.3 % of total sequences could not be assigned to the phylum level. The

Fig. 1 (continued)

relative abundances of these groups roughly paralleled the major taxa found in wetland substrate media (Janssen 2006).

At the class level, broad taxonomic analyses revealed that a total of 46 bacterial taxa were found, and the most predominant bacteria (>1 %) were affiliated with *Bacilli* (33.4 %), followed by *Actinobacteria* (25.4 %), *Gammaproteobacteria* (8.3 %), *Clostridia* (5.6 %), *Synergistia* (4.8 %), *Anaerolineae* (4.7 %), *Alphaproteobacteria* (3.6 %), *Betaproteobacteria* (3.4 %), and *Bacteroidetes* (1.2 %; Fig. 2b).

Among the assigned 260 genera, the most abundant (>1 %) bacteria were affiliated with *Propionibacterium* (16.9 %), *Staphylococcus* (9.3 %), *Anaerolineaceae* (8.1 %), *Bacillus* (5.8 %), *Staphylococcus* (5.0 %), unclassified *Synergistaceae* (2.9 %), *Streptococcus* (2.7 %), *Escherichia/Shigella* (2.6 %), unclassified *Enterobacteriaceae* (2.1 %), *Granulicatella* (2.0 %), and *Clostridium* (1.4 %; Fig. 2c). The high abundance of *Propionibacterium* was somewhat surprising. However, Hayashi and Furusaka (1979) similarly found that the population of *Propionibacterium* in the flooded paddy soils they

studied was about 10 times higher than that of *Clostridium*, a value most similar to their respective abundance in the present study. As well, *Streptococci* were also shown to represent a major segment of the bacterial flora of indigenous to a variety of soils (Gledhill and Casida 1969). The moderate abundance shown by *Escherichia/Shigella* was also unexpected as the mesocosm's inflow in this study was synthetic wastewater presumably without fecal bacteria. However, in recent years, the growth of *Escherichia coli* in soils, sediments, and water in tropical and sub-tropical regions has been widely documented, and the organism is considered to be an established part of the soil biota within these regions (Byappanahalli and Fujioka 1998, 2004). Table 5 shows the taxonomic affiliation obtained from pyrosequencing with closest cultured matches according to the results of 16 s rDNA sequence match from RDP.

Additionally, high-throughput sequencing technology has the ability to identify a greater number of sequences than

Fig. 2 Composition of bacterial community of 16S rRNA sequences from wetland mesocosms. Relative abundance was calculated by running R program. **a** Bacteria—phylum-level distribution, **b** bacteria—class-level distribution, **c** bacteria—genus-level distribution, and **d**

fungi—genus-level distribution. (i) The letter “P” represents planted HSSF CWs; (ii) the letter “S” represents unplanted HSSF CWs; (iii) the letter “T” represents treatment; (iv) the letter “C” represents control; and (v) the numbers “1,” “2,” and “3” represent the sampling time

traditional DNA approaches, expediting the detection of more unknown sequences and those with low abundance as compared to traditional clone library approaches that, in turn, assist in the discovery of more new species (Roh et al. 2010). Therefore, it was not surprising that the percentage of unclassified bacteria has increased with the depth of classification. In the present study, significant percentage of genera such as unclassified *Anaerolineaceae* (*Chloroflexi*; 8.1 %), unclassified *Synergistaceae* (*Synergistetes*; 6.1 %), unclassified *Clostridiales* (*Firmicutes*; 2.2 %), unclassified β -*Proteobacteria* (β -*Proteobacteria*; 1.6 %), and unclassified *Bacteroidetes* (*Bacteroidetes*; 1.2 %) belong to hidden diversity of unknown or uncultured species (Table 5), suggesting that most 16S rRNA gene sequences might be from bacteria that are not associated with known genera (Janssen 2006).

In the present study, the substrate media of the wetlands were dominated by the following seven major fungal groups at genus level: *Aspergillus* (16.3 %), *Chaetomium* (14.1 %), *Phoma* (6.2 %), *Pleospora* (3.5 %), *Emericellopsis* (3.3 %), *Ganoderma* (3.0 %), and *Cladosporium* (2.2 %). It is noteworthy that uncultured fungus accounted for 51.4 % of the total fungal sequences.

The impact of caffeine on microbial community

In this study, the results of taxonomic analyses suggested that chronic exposure of wetland ecosystems to caffeine could lead to moderate shifts in microbial community composition. Some bacteria exhibited remarkable increase in abundance after caffeine treatment, implying that these highly capable for stress-tolerance bacteria could apparently adapt best to changes in the external environment. On the other hand, other members of the microbial community may be reduced in abundance whether that be they are outcompeted (i.e., caffeine serves as food source for other species) or sensitive (i.e., caffeine acts as an antimicrobial). It has been reported in earlier studies that caffeine at a relatively low concentration was antimicrobial and resulted in inhibition of growth and death of bacterial strains like *E. coli* and other bacterial strains particularly upon addition of caffeine at log phase of growth (Dash and Gummadi 2006; Gummadi et al. 2012). Indeed, Ramanavičienė et al. (2002) found that the growth of *E. coli* was inhibited significantly at caffeine concentrations as low as 1 g/L.

Table 5 The taxonomic affiliation obtained from pyrosequencing with closest cultured matches according to the results of 16S rDNA sequence match from Ribosomal Database Project (RDP and GenBank)

OTU number	Phylogentic affiliation at phylum level	Phylogenetic affiliation at genus level	Closest relative species	NCBI accession no.	Similarity (%)	Averaging relative abundance (%)
Phylum Firmicutes						
OTU01010	<i>Firmicutes</i>	Unclassified <i>Bacillales</i>	Unclassified <i>Bacillales</i>	na	98.2	5.0
OTU00045	<i>Firmicutes</i>	<i>Bacillus</i>	<i>Bacillus vireti</i>	EU221372	100	5.8
OTU00030	<i>Firmicutes</i>	<i>Streptococcus</i>	<i>Streptococcus oralis</i>	GU045378	100	4.2
OTU00011	<i>Firmicutes</i>	<i>Granulicatella</i>	<i>Granulicatella para-adiacens</i>	AY879298	100	2.2
OTU00176	<i>Firmicutes</i>	Unclassified <i>Clostridiales</i>	Unclassified <i>Clostridiales</i>	na	75.8	2.2
OTU00002	<i>Firmicutes</i>	<i>Staphylococcus</i>	<i>Staphylococcus epidermidis</i>	AY730712	100	1.3
OTU00042	<i>Firmicutes</i>	Unclassified <i>Planococcaceae</i>	Unclassified <i>Planococcaceae</i>	na	100	0.7
OTU00269	<i>Firmicutes</i>	Unclassified <i>Planococcaceae</i> <i>incertae sedis</i>	Unclassified <i>Planococcaceae</i> <i>incertae sedis</i>	na	96.8	0.7
OTU00090	<i>Firmicutes</i>	<i>Ammoniphilus</i>	<i>Ammoniphilus</i> spp.	GQ062548	98.7	0.7
OTU00023	<i>Firmicutes</i>	<i>Sporosaricina</i>	<i>Sporosaricina globispora</i>	AB681045	97	0.6
OTU00446	<i>Firmicutes</i>	<i>Anaerococcus</i>	<i>Anaerococcus octavius</i>	Y07841	100	0.5
OTU00043	<i>Firmicutes</i>	Unclassified <i>Clostridiaceae_1</i>	Unclassified <i>Clostridiaceae_1</i>	na	97	0.5
OTU00123	<i>Firmicutes</i>	<i>Clostridium_sensu stricto</i>	<i>Clostridium chromiireducens</i>	AY228334	97	0.4
OTU00237	<i>Firmicutes</i>	<i>Geobacillus</i>	<i>Geobacillus thermodenitrificans</i>	AF114426	100	0.4
OTU00018	<i>Firmicutes</i>	Unclassified <i>Clostridiales</i> <i>Incetae_Sedis_SI</i>	Unclassified <i>Clostridiales</i> <i>Incetae_Sedis_SI</i>	na	100	0.3
OTU00117	<i>Firmicutes</i>	<i>Pelotomaculum</i>	Uncultured bacterium	AB853918	94.7	0.2
OTU00053	<i>Firmicutes</i>	<i>Peptoniphilus</i>	<i>Peptoniphilus</i> spp.	AM176529	100	0.2
Phylum Actinobacteria						
OTU00001	<i>Actinobacteria</i>	<i>Propionibacterium</i>	<i>Propionibacterium acnes</i>	AB108480	100	16.9
OTU00026	<i>Actinobacteria</i>	<i>Corynebacterium</i>	<i>Corynebacterium segmentosum</i>	X84437	100	0.9
OTU00096	<i>Actinobacteria</i>	<i>Micrococcus</i>	<i>Micrococcus luteus</i>	AF057289	100	0.9
OTU00612	<i>Actinobacteria</i>	Unclassified <i>Actinomycetales</i>	Unclassified <i>Actinomycetales</i>	na	90.2	0.7
OTU00313	<i>Actinobacteria</i>	<i>Mycobacterium</i>	<i>Mycobacterium moriokaense</i>	AJ429044	100	0.5
OTU00071	<i>Actinobacteria</i>	Unclassified <i>Actinobacteria</i>	Unclassified <i>Actinobacteria</i>	na	95.8	0.3
OTU00639	<i>Actinobacteria</i>	<i>Nocardioideis</i>	<i>Nocardioideis albus</i>	AF004989	100	0.2
Phylum Proteobacteria						
OTU00009	γ -proteobacteria	Unclassified <i>Enterobacteriaceae</i>	Unclassified <i>Enterobacteriaceae</i>	na	100	2.7
OTU00004	γ -proteobacteria	<i>Escherichia_Shigella</i>	<i>Escherichia coli</i>	AP009048	99	2.2
OTU00044	γ -proteobacteria	<i>Serratia</i>	<i>Serratia quinivorans</i>	AJ233435	100	1.7
OTU00024	β -proteobacteria	Unclassified <i>Betaproteobacteria</i>	Unclassified <i>Betaproteobacteria</i>	na	100	1.6
OTU00037	α -proteobacteria	Unclassified <i>Rhizobiales</i>	Unclassified <i>Rhizobiales</i>	na	100	1.5
OTU00694	α -proteobacteria	Unclassified <i>Proteobacteria</i>	Unclassified <i>Proteobacteria</i>	na	96.4	0.5
OTU00063	β -proteobacteria	Unclassified <i>Comamonadaceae</i>	Unclassified <i>Comamonadaceae</i>	na	100	0.5
OTU00252	γ -proteobacteria	<i>Pseudomonas</i>	<i>Pseudomonas oryzihabitans</i>	HM755542	100	0.4
OTU00379	α -proteobacteria	<i>Methylocystis</i>	<i>Methylosinus trichosporium</i>	AB648997	90.4	0.3
OTU00067	γ -proteobacteria	<i>Enhydrobacter</i>	Uncultured bacterium	GQ007555	100	0.2
OTU00256	β -proteobacteria	Unclassified <i>Burkholderiales</i> <i>incertae sedis</i>	Unclassified <i>Burkholderiales</i> <i>incertae sedis</i>	na	94.7	0.2
OTU00417	α -proteobacteria	<i>Pseudovibrio</i>	<i>Pseudovibrio denitrificans</i>	AB681263	100	0.2

Table 5 (continued)

OTU number	Phylogentic affiliation at phylum level	Phylogenetic affiliation at genus level	Closest relative species	NCBI accession no.	Similarity (%)	Averaging relative abundance (%)
Phylum <i>Chloroflexi</i>						
OTU00028	<i>Chloroflexi</i>	Unclassified <i>Anaerolineaceae</i>	Unclassified <i>Anaerolineaceae</i>	na	100	8.1
OTU00101	<i>Chloroflexi</i>	Unclassified <i>Ktedonobacterales</i>	Unclassified <i>Ktedonobacterales</i>	na	66.9	0.4
Phylum <i>Synergistetes</i>						
OTU00074	<i>Synergistetes</i>	Unclassified <i>Synergistaceae</i>	Unclassified <i>Synergistaceae</i>	na	91.1	6.1
Phylum <i>Bacteroidetes</i>						
OTU00041	<i>Bacteroidetes</i>	Unclassified <i>Bacteroidetes</i>	Unclassified <i>Bacteroidetes</i>	na	97.1	1.2
OTU00204	<i>Bacteroidetes</i>	<i>Cloacibacterium</i>	Uncultured <i>bacterium</i>	DQ337018	100	1.0
OTU00344	<i>Bacteroidetes</i>	Unclassified <i>Chitinophagaceae</i>	Unclassified <i>Chitinophagaceae</i>	na	90.8	0.5
OTU00425	<i>Bacteroidetes</i>	Unclassified <i>Porphyromonadaceae</i>	Unclassified <i>Porphyromonadaceae</i>	na	100	0.2
Phylum <i>Verrucomicrobia</i>						
OTU00135	<i>Verrucomicrobia</i>	<i>Optitutus</i>	Uncultured <i>bacterium</i>	DQ404823	95.6	0.7
Phylum <i>Acidobacteria</i>						
OTU00049	<i>Actinobacteria</i>	GP4	Uncultured <i>bacterium</i>	HQ697521	90.2	0.5

na not applicable

At class level, the *Bacilli* (57.9 ± 7.1 versus 15.5 ± 1.9 %) and *Synergistia* (13.3 ± 3.4 versus 5.3 ± 0.8 %) phyla increased in abundance in the planted mesocosms, while for the unplanted mesocosms, *Actinobacterial* (39.8 ± 7.6 versus 21.1 ± 5.4 %), *Clostridia* (7.2 ± 1.1 versus 3.9 ± 0.4 %), and *Betaproteobacteria* (4.3 ± 0.6 versus 1.6 ± 0.2 %) exhibited increases after exposure to caffeine.

At the genus level, the exposure of caffeine caused an increase of total sequences of *Propionibacterium* (12.4 ± 2.8 % in the planted beds and 35.0 ± 4.3 % in the unplanted beds), compared to the control beds (11.1 ± 1.9 and 6.9 ± 1.1 %, respectively). Similarly, pyrosequencing analysis revealed a remarked increase in abundance for *Staphylococcus* after caffeine exposure (12.8 ± 2.8 versus 4.3 ± 0.8 % for planted beds and 21.5 ± 4.4 versus 1.7 ± 0.3 % for unplanted beds). The presence of caffeine also increased *Bacillus* in the planted beds (21.5 ± 4.1 versus 3.5 ± 0.5 %; Fig. 2c). A significant increase was also observed for unclassified *Synergistaceae* after caffeine exposure (18.3 ± 4.3 versus 5.3 ± 1.0 % and 0.7 ± 0.2 versus 0.1 ± 0.02 %). These specific genera have been reported in previous studies to have unique catabolic machinery for the breakdown of various contaminants (Dong and Reddy 2010; Vymazal 2007). *Bacillus*, which is ubiquitously found in wetland substrate media and capable of high resistance to environmental insults due to its ability to form spores, has been extensively reported to have the potential for solubilizing phosphate (Dong and Reddy 2010),

denitrification (Vymazal 2007), and pharmaceutical wastewater treatment in wetland systems (Chaturvedi et al. 2008). *Staphylococcus* has been commonly used in bioremediation for the treatment of various wastewaters, including chromate-contaminated industrial wastewater (Vatsouria et al. 2005), brewery wastewater (Zhang et al. 2007), and textile dye wastewater (Elisangela et al. 2009). *Synergistaceae* has been intensively found in the anaerobic processes for the treatment of various wastewaters like slaughterhouse wastewater (Palatsi et al. 2011), rice straw fermentation liquor wastewater (Ducey and Hunt 2013), industrial oil recovery wastewater (Garcia-Mancha et al. 2012), and swine wastewater (Ducey and Hunt 2013). The related changes in microbial structure that we observed most probably reflected a response of the microbial community to the disturbance of a balanced ecosystem. Moreover, the high removal efficiencies of caffeine observed throughout the study suggest that the selected microbial community not only was able to tolerate the disturbance but also participate in caffeine biodegradation. Future research must now be dedicated to finding out the underlying mechanisms that drive the community shifts and determining how these shifts may affect important wetland microbial community functions.

In contrast, the sensitive genera that were remarkably repressed in both planted and unplanted mesocosms after caffeine exposure were affiliated with unclassified *Anaerolineaceae* (1.3 ± 0.3 versus 0.6 ± 0.2 % in the planted beds and 25.4

± 4.5 versus 7.7 ± 1.2 % in the unplanted beds). *E. coli* (2.1 ± 0.4 %) and *Escherichia/Shigella* (2.6 ± 0.2 %) became even undetectable at the end of the experiment (Fig. 2c). Apparently, disturbance from contaminants such as caffeine will decrease the community of microbes to only those that are pollutant-tolerant so that microorganisms that cannot adapt may no longer be a significant component of the community. Marked differences in the OTU values presented for the control and caffeine-treated mesocosms were also observed in the phylogenetic tree (Supplementary Fig. 4), which was constructed based on high-throughput pyrosequencing with a maximum likelihood algorithm. This was further checked by bootstrap calculations applying the Tamura-Nei model using program MEGA 6.

As for the response of fungal community to caffeine-enrichment, genera such as *Cladosporium*, *Emericellopsis*, *Aspergillus*, and *Phoma* were found to be resistant to caffeine disturbance, while *Trichoderma*, *Chaetomium*, and *Ganoderma* were inhibited and even undetectable after caffeine enrichment (Fig. 2d). *Aspergillus* is the most common occurring genera in sewage sludge (More et al. 2010). As a result, it has been extensively reported to have potential for heavy metal biosorption (Srivastava and Majumder 2008; Srivastava and Thakur 2006; Zafar et al. 2007), biosorption of reactive dye from textile wastewater (Khalaf 2008), and biodegradation and detoxification of industrial wastewaters (Fadil et al. 2003). Antagonistic *Trichoderma* are frequently found in soils in temperate regions and have been found with the pronounced ability to secrete diverse category of enzymes such as peroxidase, laccases, and oxidases which are responsible for the biodegradation of toxic organic compounds like polycyclic aromatic hydrocarbon (PAH) (Wang et al. 2006), anthracene, and fluoranthene in treatment wetlands (Giraud et al. 2001).

The impacts of macrophytes on the microbial community

The relationship between higher aquatic plants and microbial community in the treatment process has been arguable, due to the limited evidence both in experiments and practices (Baptista et al. 2008). Certain previous studies have indicated that plants do not exert a strong effect on the structure and diversity of the microbial community (Ahn et al. 2007; Gorra et al. 2007), while other studies have reported that macrophytes might be a significant driving force in influencing the structure of microbial communities (Calheiros et al. 2009; Osem et al. 2007). In the present study, the presence of plants increased the overall diversity of the microbial community, and there were significant ($p < 0.05$) differences in richness of microbial community between the planted and unplanted treatment wetlands (Table 4). Moreover, NMDS analysis revealed distinct community profiles for both bacteria and fungi in the planted mesocosms with similarities less than 40 %

relative to unplanted mesocosms (Fig. 1a, d). This suggests that the higher aquatic plant may well play a role in the shaping of the structure of the microbial community regardless of the caffeine disturbance. This finding is also consistent with findings by Weber et al. (2008) that showed that CWs operated similarly had communities with similar diversities, or in other words, that the presence or absence of higher plant species can exert a significant effect on the overall composition of the microbial community.

In addition, the taxonomic analysis of the bacterial composition clearly indicated a distinct microbial community profile in the planted mesocosms compared to the unplanted beds. Since the process of caffeine catabolism is mostly aerobic (Gummadi et al. 2012), the presence of wetland plants may contribute to the aerobic degradation of caffeine through the release of oxygen from rhizosphere, which further promotes the interactions between plants and microorganisms. Moreover, plants can support large microbial populations in the rhizosphere through their secretion of organic exudates such as carbohydrates and amino acids (Brix 1997; Vymazal 2005). In the caffeine-treated planted mesocosms, *Bacilli* was the most predominant (25.0–63.2 %) followed by *Actinobacteria* (6.3–27.5 %) and *Synergistia* (0.2–39.8 %), while for the caffeine-treated unplanted mesocosms, *Actinobacteria* was the most abundant (27.5–60.0 %) followed by *Bacilli* (23.7–46.6 %) and *Gammaproteobacteria* (1.2–21.3 %; Fig. 2a). At genus level, regardless of the caffeine enrichment, the bacteria that were significantly ($p < 0.05$) increased in abundance in the presence of plants were affiliated to *Bacillus* (3.5 ± 0.6 % in the control and 21.1 ± 4.2 % in the caffeine-treated beds) and unclassified *Synergistaceae* (5.3 ± 1.1 and 6.3 ± 0.7 %), while in the unplanted beds, these two species were almost undetectable. Similarly, some OTUs, such as OTU 45 (*Bacillus*), OTU 46 (*Bacillales*), OTU 349 (*Rhizobiales*), and OTU 274 (*Pseudomonas*), were only present in the planted caffeine-treated mesocosms but not in the unplanted beds. On the contrary, the abundance of certain bacteria was noticeably ($p < 0.05$) decreased in the presence of the higher aquatic plant. These were *Anaerolineaceae* (25.4 ± 3.6 versus 1.3 ± 0.4 % and 7.6 ± 1.8 versus 0.6 ± 0.2 %) and *Clostridium* (3.9 ± 0.9 versus 1.1 ± 0.2 % and 1.5 ± 0.3 versus 0.2 ± 0.06 %) in both the control and caffeine-treated beds, respectively. Additionally, *Staphylococcus* also showed inhibition in the presence of plants after caffeine exposure (21.5 ± 4.8 versus 12.8 ± 3.1 %).

Conclusions

Microorganisms inhabiting CWs play a significant role in the biogeochemical cycles, making them key components of bioremediation processes. The characterization of microbial community in the planted and unplanted HSSF CWs receiving

caffeine-enriched wastewater was investigated using a high-throughput pyrosequencing approach. The diversity of the bacterial community was reduced in the wetland system exposed to caffeine at the concentration of 250 mg L⁻¹ during a 210-day period. Planted HSSF CWs exhibited significantly higher caffeine removal efficiencies compared to those in the unplanted HSSF CWs. A distinct profile of microbial community for the caffeine-enriched wetland mesocosms compared to the control was observed. The high caffeine removal efficiencies observed suggest that the selected microbial communities after caffeine enrichment were able to tolerate and adapt best to the environmental disturbance. The taxonomic analyses revealed that the presence of plant did exert significant effect on the microbial community structure.

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