Campus sewage treatment in multilayer horizontal subsurface flow constructed wetlands: Nitrogen removal and microbial community distribution

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Abstract

Horizontal subsurface flow constructed wetlands (HSCWs) are widely used for wastewater treatment. The objective of this study was to assess the effects of substrate size selection and layout optimisation on pollutant removal and microbial community distribution responses in HSCWs. Three pilot-scale constructed wetlands (CWs) were established at Guilin University of Technology, China, to treat campus sewage. The three CWs included monolayer (CW1), three-layer (CW2), and six-layer (CW3) substrate structures with the hydraulic conductivity of the substrate increasing from the surface to the bottom in the multilayer CWs. Under an aerial influent loading rate of 0.38 m per day (volumetric loading rate of 0.63 per day), CW3 exhibited the highest removal performance for chemical oxygen demand (COD), NH_4^+-N, and total nitrogen (TN), with mean values of 81, 81, and 74%, respectively, followed by CW2 (68, 71, and 60%, respectively) and CW1 (56, 46, and 41%, respectively). Nitrification was demonstrated to be the limiting factor of TN removal, and higher TN removal performance in the multilayer CWs was attributed to the higher proportions of nitrifiers, including ammonia-oxidising bacteria (AOB) and nitrite-oxidising bacteria (NOB). Moreover, 454-pyrosequencing showed a significantly different spatial distribution of the N-transforming microbial community in multilayer HSCWs with substrate layout optimisation.

Abbreviations

ADB, autotrophic denitrification bacterium; **ANOVA**, analysis of variance; **AOA**, ammonia-oxidizing archaea; **AOB**, ammonia-oxidizing bacterium; **COD**, chemical oxygen demand; **CW**, constructed wetland; **HDB**, heterotrophic denitrification bacterium; **HRT**, hydraulic retention time; **HSFCW**, horizontal subsurface flow constructed wetland; **NOB**, nitrite-oxidizing bacterium; **ORP**, oxidative reduction potential; **OUT**, operational taxonomic unit; **PC**, principal component; **PCA**, principal components analysis; **TN**, total nitrogen

Keywords: Campus wastewater, Multilayer constructed wetland, Nitrogen-transforming bacteria, Substrate structure

1. Introduction

Constructed wetlands (CWs), and especially horizontal subsurface flow constructed wetlands (HSCWs), are widely used for wastewater treatment owing to their low construction cost and simple management [1]. Secondary effluent from municipal wastewater treatment that is discharged into surface water natural end-receivers contains residual chemical oxygen demand (COD) and nitrogen (N) compounds. CWs can operate as 'pre-receivers' for the refinement of discharged wastewater [2]; however, dead zones and clogging are common, resulting in preferential pathways [3] that can limit contaminant transfer between aerobic, anoxic, and anaerobic zones in the wetland bed, which may decrease treatment performance. This poor hydrodynamic behaviour is commonly caused by improper CW design. Given the increasing application of CWs and increasingly strict water quality standards, there is a growing need to improve CW design.

Pollutant transformation and biodegradation in CWs mainly occurs in the substrate-associated biofilm, making the substrate one of the most important components responsible for pollutant removal in CWs [4, 5]. Previous studies have attempted to optimise substrate arrangement in HSCWs by incorporating granular heterogeneity to improve hydraulic performance, resulting in higher pollutant removal abilities [6]. Theoretically, the new configuration of substrate, which setting substrate with larger size and higher hydraulic conductivity at the bottom layers and substrate with smaller size and lower hydraulic conductivity at the above layers, can help to avoid the dead zones at the bottom and provide an even flow path in the wetland bed [7]. Finally, uniform water flow patterns would improve pollutant biodegradation owing to higher biofilm/pollutant contact. Following modelled simulations, a system incorporating this concept was built and was found to achieve higher pollutant removal over the short term [7]; however, the stability of treatment performance over long term operation, as well as the spatial characteristics of pollutant removal in multilayer CWs remains to be evaluated.

In CWs, N removal primarily occurs through nitrification and denitrification [8]. Nitrification is mainly carried out by ammonia-oxidising bacteria (AOB) and nitrite-oxidising bacteria (NOB), which are capable of transforming ammonia directly to nitrite and nitrate, respectively. Denitrification consists of the transformation of nitrate or nitrite into gaseous forms by heterotrophic denitrification bacteria (HDB) and autotrophic denitrification bacteria (ADB) [9, 10]. In addition, anaerobic ammonium oxidation (Anammox) bacteria, which are capable of autotrophic ammonium oxidation with nitrite as the terminal electron acceptor in anoxic/anaerobic conditions, are also known to exist within CWs [11]; however, a

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better understanding of the spatial distribution of these N-transforming microbial communities as a response to substrate layout optimisation is needed to improve our understanding of N removal mechanisms and to further refine the design and application of CWs.

The objectives of this study were to determine the effects of substrate size selection and layout optimisation on 1) the spatial characteristics of pollutant removal and on 2) microbial community distribution along the water flow path in three HSCWs. The study was carried out on one monolayer and two multilayer (three- and six-layer) pilot-scale HSCWs treating sewage from a university campus. 454-pyrosequencing of the 16S rRNA gene was conducted to determine the abundance and structure of the N-transforming microbial community.

2. Materials and methods

2.1 Experimental setup

The present study was conducted in three parallel pilot-scale HSCWs, located at Guilin University of Technology, southwest China (N25°28′, E110°31′). The systems were constructed in December 2012. A detailed description of the experimental setup can be found in Bai et al. [7]. Briefly, all systems were 2.0 m long, 1.2 m wide, 0.7 m high, and had a water depth of 0.6 m (Fig. 1). Each CW was divided into an influent distribution zone (0.2 m long), a main reaction bed (1.6 m long), and an effluent collection zone (0.2 m long). The three CWs differed in terms of the quartz sand size and layout (Supporting Information Table S1): 1) CW1 had a monolayer substrate of quartz sand (size: 0–6 mm; hydraulic conductivity (K): 65 m per day); 2) CW2 consisted of three equal layers, each 0.2 m thick, with K values of 64, 36, and 26 m per day from the bottom layer to the surface layer; and the corresponding sand has diameters of 1--3, 0.4–0.6, and 0–0.4 mm, respectively; 3) CW3 comprised of six equal layers, each 0.1 m thick, with K values of 176, 75, 55, 43 and 26 m per day from bottom layer to the surface layer, and corresponding sand sizes of 4--6, 2--4, 1--2, 0.6--0.9, 0.4--0.6 and 0–0.4 mm, respectively.

The experimental site was protected by a glass roof, in order to avoid the precipitation effect, but also received the natural variations in light exposure and temperature. The influent was real sewage generated by the university campus and pre-treated in a septic tank (Fig. 1). The three CWs shared a 200 L influent tank, and influent was controlled using a speed adjustable pump that maintained a flow rate of 0.72 m^3 per day (areal loading rate of 0.38 m per day and volumetric loading rate of 0.63 per day). The physicochemical parameters of the influent pollutant concentrations and loading rate are shown in Table 1. For each CW, two PVC pipes were placed 0.4 m (W1) and 1.6 m (W2) away from the influent at a depth of 0.4 m from the top of the system (Fig. 1). All PVC pipes were equipped with valves for water sampling. *Canna indica*, the macrophyte species chosen for analysis, was introduced at a density of 20 strains per m^2. The study was conducted in the summer (1 May to 31 July 2015), when the air temperature ranged from 17 to 30°C. These systems had previously run for approximately two years under the same operational conditions.

2.2 Water sampling and analysis

During the 12 weeks' experimental period, water samples (500 mL each) were collected from all CWs once a week. Each CW had four water sampling locations (triplicate for each site), including the influent,

effluent, and two sampling pipe valves (W1 and W2 in Fig. 1). The temperature, pH, and oxidation reduction potential (ORP) were measured in situ by a multi-parameter portable meter (HANNA HI9828). Water samples were stored in sterile plastic bottles and transported under cooling conditions (5°C) to the laboratory for COD, NH_4^+-N, NO_3^--N, NO_2^--N, and TN analysis within 24 h using standard methods of APHA [12].

2.3 Substrate sampling and analysis

2.3.1 Sampling

Samples of substrate for 454-pyrosequencing analyses were collected at the end of the study (31 July 2015) using a push core sampler (ø 5 cm, 100 cm in length). At each CW, substrate samples were taken from locations at 0.4 and 1.6 m from the influent (S1 and S2 in Fig. 1), at a depth of ~0.4 m. After removing visible roots and debris, samples were stored in sterile plastic bags, placed on ice, and transferred immediately to the laboratory. Each substrate sample was then homogenised (i.e., mixed) manually and divided into three replicates. A total of 18 samples were used for the analyses of microbial communities.

2.3.2 DNA extraction

DNA was extracted from 0.5 g of each substrate sample using the PowerSoil DNA Isolation Kit (MoBio, USA) according to the manufacturer's protocols. Extracted genomic DNA was detected by using 1% agarose gel electrophoresis to examine its integrity and approximate concentration.

2.3.3 Pyrosequencing and Illumina high-throughput sequencing

Pyrosequencing and Illumina high-throughput sequencing were conducted at Sangon Biotech (Shanghai, China). Microbial DNA was amplified using a set of primers targeting the V3 + V4 hypervariable region (approximately 440 bp) of the 16S rRNA gene before pyrosequencing. The forward primer was 515F (GTGCCAGCMGCCGCGGTAA) and the reverse primer was 909R (CCCCGYCAATTCMTTTRAGT). The 16S rRNA gene sequencing obtained from the Illumina MiSeq 2×300 bp and the raw data were quality checked in Prinseq. To compare samples at the same sequencing depth, normalisation of the sequence number was conducted by randomly extracting at least 40 000 sequences from each sample for subsequent analysis using the Mothur software package (v.1.30.1). After removing barcodes and primers, the sequences were assigned at different taxonomic levels (from domain to genus) based on comparison to the SILVA dataset. High-quality sequences were processed using Uclust to generate operational taxonomic units (OTUs) with a 97% sequence similarity threshold. The abundance and diversity of microorganisms were classified using the RDF Classifier, which is based on Bergey's taxonomy at the genus level.

2.4 Statistical analyses

Comparison of COD, NH_4^+-N, and TN concentrations among the sample sites were performed using one-

way analysis of variance (ANOVA). The N-transforming microbial community distribution was analysed and visualised through principal components analysis (PCA). Both ANOVA and PCA were run using the SPSS.21 software package (Boston, Mass: International Business Machines, 2012).

3. Results and Discussion

3.1 Pollutant removal and water quality

The ORP increased from influent (mean −158 mV) to effluent (range = −91 to −111 mV). The improved oxidation potential of the water may reflect natural oxygen diffusion from the air and the oxygen release function of wetland plants. However, it should be noticed that the effluent is the composite sample mixed by the water from the whole CW. The HSFCWs always present different ORP in different wetland bed layers, which is higher in the top layers than in the bottom [13]. Moreover, the oxygen can be pumped into the wetlands by plant roots and form the micro aerobic-anoxic-anaerobic environment near the rhizosphere, which may still provide a suitable condition for nitrification and denitrification process [14]. Thus, further analysis of the nitrogen transforming microbial community was conducted by the molecular biology method in the present study and explained below (Sections 3.3 and 3.4). The mean effluent pH values of the three CWs were ~7.3 and showed no significant differences with the influent. The stable pH values indicate the potential buffer capacity of CWs during wastewater treatment. Notably, the pH and ORP were not significantly different between of the three CWs, suggesting that multilayer CWs do not influence water quality for campus sewage treatment.

At sampling position W1, CW2 and CW3 showed higher removal efficiencies (43 to 52%) of COD and NH_4^+-N than did CW1 (26 to 33%); however, the TN removal efficiencies of all three CWs were similar (16 to 19%) at W1 (Fig. 2). The removal of these pollutants showed similar patterns for all CWs, significantly increasing from sampling position W1 (16 to 52%) to position W2 (38 to 78%). The removal efficiencies of COD and NH_4^+-N were not further increased from W2 to the effluent; however, TN removal efficiencies improved significantly from W2 to the effluent. Overall, generally significantly higher removal efficiencies of COD, NH_4^+-N, and TN were achieved in CW3 (means of 81, 82, and 76%, respectively), followed by CW2 (means of 69, 71, and 67%, respectively) and CW1 (means of 56, 47, and 49%, respectively).

The significantly better treatment performances of CW3 and CW2 indicated that a multilayer substrate layout in HSCWs can be a benefit for the removal of pollutants, including both COD and N. Wetland substrate layout optimisation by decreasing particle size from bottom to top may change the hydraulic conditions of the HSCWs and achieve more homogenised water flow and fewer water flow short-cuts [15]. In this case, COD and N removal through microbial biodegradation should significantly increase owing to enhanced pollutant--microbial contact [16]. The higher pollutant removal abilities of CW3, which has a multilayer substrate layout, may reflect more homogenised water flow in the wetland bed. Moreover, the multilayer configured wetland may also cause higher filtration capacity for the solid pollutants removal.

3.2 Nitrogen mass balance

Nitrogen mass balance (Fig. 3) showed that for all three CWs the proportion of NH_4^+-N decreased

significantly from influent to W2 and then continued to decrease slowly until the effluent. In contrast, the NO_3^--N percentage clearly increased from influent (3%) to W1 (20–30%), and then declined progressively until the effluent reached <3% contribution (<0.9 mg L^{-1}) in all CWs. For NO_2^--N, percentages remained below 0.3% while concentrations remained <0.1 mg L^{-1} for all samples throughout the study. The difference between TN and all other inorganic N species was considered to reflect mainly organic and particulate N forms, which showed a clear decrease from influent to W1 and then remained relatively stable along the remaining length of all CWs.

Nitrification and denitrification, which can transform NH_4^+-N to NO_3^--N (nitrification) and then into gaseous N (denitrification) to achieve final N removal, can play a crucial role in TN removal from CWs [17]. Previous studies have shown that rapid NH_4^+-N removal through nitrification mainly occurs within the front of wetland beds in traditional HSCWs, due to the initial amount of oxygen content from the influent. The high initial oxidation reduces the available oxygen for continued reactions further along the bed [18]. Here, the NH_4^+-N removal from the front section (influent to W1) of the CWs was accompanied by significant increases in NO_3^--N mass contributions (Fig. 3). This process resulted in similar TN removal for all CWs at W1 (Fig. 2). Similarly, easily degradable organic and particulate N may also degrade in the front part owing to the better aerobic conditions. The residue contents were not further removed in the CWs and do not contribute to TN removal, reflecting the recalcitrant N. In the back part of CWs, NO_3^--N concentrations significantly decreased to <0.9 mg L^{-1} at the effluent for all CWs, which may indicate thorough denitrification under relatively anaerobic conditions. Simultaneously, slowed NH_4^+-N removal in this section supports the hypothesis that nitrification is the limiting process for TN removal owing to reduced oxygenation.

3.3 Microbial community abundance and distribution

Nitrifiers (AOB & NOB), denitrifiers (HDB and ADB), and Anammox bacteria were detected in all substrate samples. In total, ~40 000 high-quality gene sequences were obtained from each sample and the classified OTUs ranged from 9830 to 11008. Rarefaction curves based on the OTUs at 3% dissimilarity (Supporting Information Fig. S1) indicated that the sequences were sufficient to reflect the diversity of the microbial communities. Thereof, around 1000 OTUs (~10% of the total) in each CW were N-transforming bacterial strains.

AOB & NOB, HDB and ADB were found to be the main N-transforming microbial communities (2 to 8% of the total OTUs; Fig. 4), which supports the theory that nitrification and denitrification were the main N-removal processes in all CWs. Moreover, denitrifiers (the sum of HDB and ADB) showed clearly higher OTU compositions than nitrifiers (the sum of AOB and NOB) in all CWs, supporting the theory that denitrifying bacteria were more abundant than nitrifiers. It can be explained by the overall anoxic condition (effluent ORP of around -100 mV) in the three wetland beds, which was suitable for the growth of denitrifiers. However, the presence, even though lower amount, of nitrifiers in the CWs may be due to the micro aerobic-anoxic-anaerobic environment near the rhizosphere created by the oxygen pumping function of the plant roots. On this basis, nitrification, not denitrification, may be the key factor in limiting TN removal. At sampling site S1, nitrifiers in multilayer CWs (165–182 OTUs) were higher than in the monolayer CW (141 OTUs); therefore, higher TN removal (Fig. 2) may reflect a greater abundance

of nitrifiers in multilayer CWs (CW2 and CW3), which can promote higher NO_3^--N through nitrification followed by removal through denitrification. Anammox bacteria only showed 4–11 OTUs (<0.5% of the total) in all samples, which indicates that Anammox bacteria could exist in CWs as a potential microbial route for equivalent close-coupled nitrification–denitrification; however, given the relatively limited abundance compared with other functional bacterial strains, their contribution toward N removal in the present study was relatively small.

The spatial distribution of the N-transforming microbial community among the three CWs at different sampling sites was interpreted using PCA (Fig. 5). The first two principal components (PCs) accounted for 82.79 and 15.72% of the total variation. Nitrifiers (AOB and NOB) mainly contributed to both positive PC1 and PC2. HDB clearly contributed more to the positive direction of PC1; however, ADB mainly contributed to the negative direction of PC2. Moreover, the short loading factor line of Anammox confirmed the low contribution ability of Anammox. Samples from S1 and S2 of each CW group showed significant differences. For S1, nitrifiers and HDB were most abundant in CW3, followed by CW2 and CW1. This clearly confirms a shift in the spatial distribution of N-transforming microbial communities in CW2 and CW3. In CW3, the ADB contribution increased along the direction of water flow until it replaced HDB as the dominant denitrifier. This change may have been caused by higher COD removal that resulted in insufficient carbon for heterotrophic denitrification (Fig. 2). As suggested by previous studies, microbial communities from different sites within CWs are specialised for their environment. They are fundamentally distinct from each other and exhibit significant spatial variations [8]. The results indicate that substrate layout optimisation in CWs could shift the position of N-transforming microbial communities, which could further improve TN removal abilities.

3.4 Microbial community structure and distribution

In each of the CWs, the microbial communities showed high diversity from phylum to genus level. Microbial DNA could be assigned to three major (relative abundance >10%) phylum, ten classes, 14 orders, 15 families, and 20 genera. Among them, the relative abundances of AOB & NOB, HDB, ADB and Anammox bacterial species at the genus level are further analysed and presented in Fig. 6.

3.4.1 Nitrifiers (AOB and NOB)

For all CWs, the most abundant genus was *Nitrospira* (38 to 57%), followed by *Nitrosomonas* (19 to 49%) and *Nitrosococcus* (10 to 36%). In contrast, only very few (<0.3%) *Nitrososphaera* were detected (Fig. 6). *Nitrosococcus* and *Nitrosomonas* are well known as AOB and have been considered the most important contributors to aerobic ammonia oxidation [19]. *Nitrospira* is a known NOB because it can transform NO_2^--N to NO_3^--N, and then eliminate the toxic effects of accumulated NO_2^{--}-N [19].

Nitrosococcus (AOB) dominated at S2 for CW2; although, its abundance was much lower at the other sampling sites for all CWs. *Nitrosomonas* (AOB) dominated at S1 for both CW2 and CW3, but not CW1. Previous studies have shown that ammonium oxidation is the rate-limiting step of nitrification compared with nitrite oxidation [20]. As AOB (the sum of *Nitrosomonas* and *Nitrospira*) exhibited higher abundances in CW2 and CW3 (as compare with CW1), it contributed to the higher ammonium removal efficiency.

3.4.2 Denitrifiers (HDB and ADB)

The HDB bacteria were dominated by *Denitratisoma* (28 to 62%), *Rhizobium* (8 to 42%), *Thauera* (4 to 14%), *Acidovorax* (4 to 16%), *Comamonas* (1 to 13%), and *Rhodobacter* (4 to 11%). Other groups showed lower and comparable proportions in HDB (<5%; Fig. 6). The two dominant HDBs (*Denitratisoma* and *Thauera*) belong to the Rhodocyclaceae family, members which are frequently found in wastewater heterotrophic denitrifying communities [21]. *Thauera* has been found with many hydrogen-oxidising autotrophic denitrifying microbial communities, indicating that it can grow autotrophically [9], which is consistent with its relatively high abundance at S1, where organic matter was abundant for all CWs.

The genera associated with ADB were *Thiobacillus* and *Sulfurimonas*. *Thiobacillus denitrificans* and *Sulfurimonas denitrificans* (formerly known as *Thiomicrospira denitrificans*) are the most commonly reported ADB strains [22, 23], and *Thiobacillus* was dominant (>90%) in all samples. This finding shows that combined heterotrophic and autotrophic denitrification processes can be attributed to N removal in HSCWs. Moreover, *Thiobacillus* and *Thauera* are dominant bacterial strains that cooperatively fulfil a simultaneous denitrification process [24]. The ADB abundances showed no significant differences between sampling sites S1 and S2, or between the different CWs.

3.4.3 Anammox

Candidatus Brocadia and *Candidatus Anammoximicrobium*, both Planctomycetes, are commonly known to be capable of denitrifying nitrogenous compounds using ammonium as an electron donor and NO_2^- as an electron acceptor under anoxic/anaerobic conditions [22, 25]. This process is often acknowledged to be a short circuiting pathway that normally requires relatively low oxygen and organic carbon. Here, the above two Anammox bacteria were detected in all samples, with *Candidatus Brocadia* more abundant, especially in CW2 and CW3. This result supports the potential for a NH_4^+-N depuration pathway from the reaction of Anammox; however, the contribution of Anammox to TN removal requires further study.

4. Conclusions

This study investigated the influence of substrate size selection and layout optimisation on pollutant removal and microbial community characteristics in CWs. The multilayer CWs, in which the hydraulic conductivity of the substrate decreased from the bottom to surface, exhibited significantly higher removal ability of COD, NH_4^+-N, and TN. Nitrification was demonstrated to be the limiting factor of TN removal in HSCWs. The higher TN removal performance in multilayer CWs was attributed to the higher proportions of nitrifiers (AOB and NOB). Moreover, clear differences in the abundance, structure, and spatial distribution of N-transforming microbial communities were observed between multilayer and monolayer CWs. HDB tended to be more abundant in the first part of CWs, while ADB was more abundant in the end part of multilayer HSCWs.

Acknowledgements

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Table 1. Physicochemical parameters of the influent

Parameter	Value (mg L^{-1})	Areal loading rate (g m^{-2} per day)	Volumetric loading rate (g m^{-3} per day)
Water temperature (°C)	21 ± 7	--	--
pH	7.3 ± 0.3	--	--
DO	1.3 ± 0.3	--	--

ORP	--158 $\pm$ 30	--	--
COD	145 $\pm$ 36	54 $\pm$ 14	91 $\pm$ 23
NH_4^+-N	23 $\pm$ 10	9 $\pm$ 4	14 $\pm$ 6
NO_3^--N	0.9 $\pm$ 0.8	0.3 $\pm$ 0.3	0.6 $\pm$ 0.5
NO_2^--N	$\leq$0.1	$\leq$0.04	$\leq$0.06
TN	32 $\pm$ 8	12 $\pm$ 3	20 $\pm$ 5

Figure legends

Figure 1. Schematic diagram of monolayer (CW1) and multilayer (CW2, CW3) pilot-scale HSCWs for treating campus sewage. Red dots represent water (W) and substrate (S) sampling sites.

Figure 2. Removal efficiencies of chemical oxygen demand (COD; %), NH_4^+-N (%), and TN (%) along wetland beds (W1–W2–out) in constructed wetland 1 (CW1; blue bars), CW2 (red bars), and CW3 (green bars). Error bars indicate standard variation. Letters above bars denote the significant difference.

Figure 3. Mass balance of different N species (NH_4^+-N = blue bars; NO_3^--N = red bars; NO_2^--N = black bars; sum of organic and particulate N = green bars; removed N = grey bars) in each sampling site along wetland beds (In–W1–W2–out) of (a) CW1, (b) CW2, and (c) CW3. Mass balances were calculated from mean values. The sum of organic and particulate N was calculated as the difference between TN and all other inorganic N species.

Figure 4. OTUs of each N-transforming microbial community in substrate samples S1 and S2 for constructed wetland 1 (CW1), (b) CW2, and (c) CW3. Error bars indicate standard variation. Blue bars denote AOB, red bars denote NOB, green bars denote ADB, and grey bars denote Anammox.

Figure 5. PC (black circles) analysis of the relationships between sampling locations and relative abundance of each N-transforming microbial community OTUs in substrate samples (coloured circles = S1, coloured squares = S2) of the three constructed wetlands (CW1 = blue symbols, CW2 = yellow symbols; CW3 = red symbols). Arrows represent the loading factors of each variable.

Figure 6. Relative abundances (%) of N-transforming microbial genus (see colour scale on figure) in different substrate samples (S1 and S2) for (a) constructed wetland 1 (CW1), (b) CW2, and (c) CW3.