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# Performance assessment and microbial diversity of two pilot scale multi-stage sub-surface flow constructed wetland systems

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## Abstract

This study assessed the performance and diversity of microbial communities in multi-stage sub-surface flow constructed wetland systems (CWs). Our aim was to assess the impact of configuration on treatment performance and microbial diversity in the systems. Results indicate that at loading rates up to 100g-$BOD_5/m^2$.day, similar treatment performances can be achieved using either a 3 or 4 stage configuration. In the case of phosphorus (P), the impact of configuration was less obvious and a minimum of 80% P removal can be expected for loadings up to 10g–$P/m^2$.day based on the performance results obtained within the first 16 months of operation. Microbial analysis showed an increased bacterial diversity in stage four compared to the first stage. These results indicate that the design and configuration of multi-stage constructed wetland systems may have an impact on the treatment performance and the composition of the microbial community in the systems, and such knowledge can be used to improve their design and performance.

**Keywords** Constructed wetlands, microbial diversity, wastewater treatment

## INTRODUCTION

The operation of constructed wetland systems (CWs) relies on a combination of physical, chemical and biological processes in which microorganisms play an important role. However, their designs are generally based on information about chemical and physical parameters with only a limited number of studies such as Calheiros et al. (2009) and Truu et al. (2009) focusing on the composition of the microbial communities in these systems. Consequently, while the design of these systems has evolved, the increased knowledge acquired by engineers has not been accompanied by an equivalent growth in understanding of the microbial ecology of these systems. Hence there is still much left to be known concerning the identities and interactions of the microbial communities associated with the treatment processes and the impact of design and operational variables on these communities. Such knowledge helps to improve the engineering design and performance of constructed wetlands and other similar systems.

Over the last decade, the emergence of molecular biological tools for studying microbial communities has made it possible to broaden our understanding of the vast diversity and interactions of microorganisms present in the complex environments of wastewater treatment systems. The availability of such molecular biological tools has resulted in remarkable insights in linking diversity and dynamics to treatment performance and process stability (Briones and Raskin, 2003). The ability to engineer CWs and other similar wastewater treatment systems to produce predictable and consistent community configurations has the potential to deliver more effective and efficient treatment systems. Recent studies have shown that it is possible to control the community diversity of a system by engineering its physical complexity (Harris et al. 2012). In this study, we assessed and compared the performance (based on loading and removal rates and impact of configuration) of two multi-stage sub-surface flow CWs and also examined the microbial composition and diversity in one of the systems. Ultimately, we are interested in being able to design and operate CWs to foster the development of specific microbial communities that can accommodate desired functional processes and make their design and operation more efficient. In this context, our objectives were: (i) to evaluate the impact of configuration on the performance of multi-stage CWs and (ii) to gain an insight into the linkage between microbial community, treatment performance and design of the CWs by analysing the microbial composition and distribution in multi-stage CWs.

## MATERIALS AND METHODS

### Systems design

The experimental set-up consists of two multi-stage CWs operated in vertical sub-surface flow mode. The two systems, referred to henceforth as systems A and B, were set up on the UCD Lyons Research Farm located in Newcastle, Co. Dublin, Ireland to treat wastewater (after settlement) emanating from the farm. The farm holds about 2000 livestock units of sheep, pigs, cattle and horses. The farm wastewater is derived from all the activities on the farm and it undergoes primary sedimentation before being pumped to a holding tank.

Table 1 gives the design summary for the two systems while figure 1 shows the field set-up of the systems. System A was a four-stage system (all stages are equal) which was reconfigured to three stages after 11 months of operation while system B was initially configured as a four-stage system (all stages are equal) and then changed to a two-stage system after 4 months. System A was reconfigured (by linking the 1$^{st}$ and 2$^{nd}$ stages together to form one single stage) in order to increase the surface contact area and decrease the pollutant loading on the stage 1. Similarly, system B showed early signs of clogging and inconsistent performances even though it was achieving good pollutant removal rate. It was therefore decided to decrease the solids loading rate and stabilise its performance by increasing the contact surface area. This was achieved by linking the 1$^{st}$ and 2$^{nd}$ stages into one stage, and also linking the 3$^{rd}$ and 4$^{th}$ stages into one stage. Consequently, system B was reconfigured as a 2-stage system.

The stages in each system were linked together using pipes connected to submersible pumps placed in each stage. The pumps were connected to a digital electronic timer which regulated the flow according to a programme schedule. Each stage in both systems was configured using 10mm gravel at the bottom up to a depth of 10cm as supporting layer. Dewatered alum sludge cakes were employed as the main wetland substrate for a depth of 65cm and then 10cm of 20mm gravel to serve as distribution layer on the top. The use of the dewatered alum sludge in CW was based on the previous laboratory studies reported by Zhao et al. (2009a,b).The dewatered alum sludge cakes used were collected fresh from the industrial filter press of a drinking water treatment plant in Southwest Dublin, Ireland where aluminium sulphate is used as coagulant. The size/length (mean±SD) of the alum sludge cakes used was 7.25±1.48 cm. Common reed, Phragmites australis, was planted on top of each stage.

The systems were operated as subsurface flow using a tidal flow operation strategy. The tidal flow strategy allows the matrices of the systems to be filled with wastewater and completely drained afterward to enhance aeration (Babatunde, 2007). Wastewater from the farm activities was collected from the holding tank and pumped into a $10m^3$ tank. Appropriate dilution was then carried out to achieve desired concentration before the wastewater was gravity-fed into an underground tank with a ball-float valve control. The underground tank served as the influent tank from where the wastewater is pumped to the two systems. The concentration of influent wastewater to the systems was gradually increased in order to allow time for the system to stabilize and for the reeds to grow. Accordingly, the influent wastewater had a range of pollutant concentrations which were $BOD_5$ (31-968 mg/L), COD (124-1634 mg/L), $PO_4$-P (2.8-60 mg-P/L), TN (16-273 mg-N/L) and SS (25-633 mg/L). There were three cycles per day and each cycle consists of four hours of wastewater contact in each stage and four hours of rest during which wastewater is drained out (to the next stage) and the stage is left to rest.

**Wastewater analysis**

Wastewater samples were collected from the feed tank and from each stage of each of the two systems. The samples were analysed for COD (both total and soluble COD, (sCOD)), $BOD_5$ (Lovibond OxiDirect apparatus, Lennox, UK), Total Phosphate (Ascorbic acid method, Clesceri et al. (1998)) $PO_4$-P, Total Nitrogen (Persulfate method, Clesceri et al. (1998)), $NH_4$-N and SS. Except where indicated, all analyses were carried out using a Hach DR/2400 spectrophotometer according to its standard operating procedures. From the water quality data, the pollutant loading rate ($g/m^2$.day) was determined by multiplying the hydraulic loading rate (HLR, $m^3/m^2$.d) by the influent pollutant concentration (mg/L) while the pollutant removal rate ($g/m^2$.day) was determined by multiplying the HLR by the difference in concentration between the influent and the effluent.

**Microbial analysis - sample collection and DNA extraction**

Alum sludge samples from stages 1 and 4 of system A, prior to reconfiguration, were collected from the top 5 cm of the sludge layer in each stage and transported in sterile tubes within an ice box to the laboratory. Duplicate DNA extractions were performed from 200 mg of each sample was extracted using the DNA soil kit as recommended by the manufacturer (Qiagen) and DNA samples were stored at -80 $^oC$.

**Construction of 16S rDNA clone libraries**

PCR amplification of the 16S rDNA gene was carried out in 50 µl reactions with GoTaq Flexy DNA polymerase (Roche) using primers 63F (5'-CAGGCCTAACACATGCAAGTC) and 1389R (5′-ACGGGCGGTGTGTACAAG) as recommended by the manufacturer. The reaction mixture was preincubated at 95 $^{o}$C for 5 min and subsequently subjected to 30 cycles of 94 $^{o}$C for 30 s, 60 $^{o}$C for 45 s, 72 $^{o}$C for 120 s followed by an incubation at 72 $^{o}$C for 7 min. PCR products were purified using the QIAquick PCR purification kit (Qiagen) as recommended by the manufacturer. DNA quantification was done by UVspectrophotometry in a NanoDrop Spectrophotometer (Thermo Scientific). PCR products obtained from replicated DNA samples were pooled. Clone libraries were constructed into the pDrive vector using the QIAGEN PCR cloning kit as per the manufacturer's instructions and transformed into QIAGEN EZ competent cells (Qiagen). Insert- containing clones were selected in LB agar plates containing 100µg/ml ampicillin and 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (80µg/ml). 96 clones per library were screened by restriction fragment length polymorphism (RFLP) using MspI restriction enzyme (New England Biolabs, MA). Clones with different restriction profiles were sequenced using primer 63F at GATC-Biotech (Germany). Trimming of sequences to remove low quality ends was performed with DNABaser v4 (Heracle BioSoft S.R.L.). Sequences were deposited in the GeneBank under accession numbers KF990413 and KF990471

**Phylogenetic analysis of 16S rDNA sequences**

The nucleotide sequences were compared to entries in the GenBank data base at the National Center for Biotechnology Information using the programme BLASTN (Altschul et al. 1990). Chimeric sequences were detected and removed by ChimeraSlayer using the identify_chimeric_seqs.py pipeline implemented in QIIME 1.6 (Haas et al. 2011; Caporaso et al. 2010). Nucleotide sequences were aligned with the ClustalW2 software package (Thompson et al., 2002; Larkin et al. 2007). The phylogeny of the sequences was analyzed by calculating a distance matrix according to the Jukes-Cantor model (Jukes and Cantor, 1969) followed by the construction of a phylogenetic tree using the neighbour-joining method (Saitou and Nei, 1987) as implemented in the program Treecon (Van de Peer and De Wachter, 1995). Matrices of genetic distances between sequences were generated with the DNADIST program of the Phylip package (Felsenstein, 1989). Rarefaction curves of numbers of observed OTUs and Shannon diversity, including their confidence intervals, were generated

by assigning operational taxonomic units (OTUs) at genetic distances of 2 and 16% with the DOTUR software using the furthest neighbour method (Schluss and Handelsman, 2005).

## RESULTS AND DISCUSSION

### Overall systems performance

The loading and removal rates for systems A and B, respectively, both before and after reconfiguration are presented in Table 2 (a & b). Overall, system B was subjected to much higher loading over the duration of the field trials. The results also indicate that the removal rates obtained in both systems closely mirrored the loading rates irrespective of their configuration. Consequently, the highest removal rates were obtained in system B. This clearly demonstrates the impact of configuration and loading rates on treatment efficiency and removal rates achievable in CWs. Although it is often argued that CWs typically require a low HLR and a long hydraulic retention time (HRT) to achieve efficient pollutant removal, debate on the relationship between HLR and removal rates still continues for conditions with high HLR. This is partly due to a lack of criteria which defines what is meant by high or low HLR.

Chang et al. (2007) reviewed HLRs used in CWs and noted that HLRs ranging from 0.14 to 1.54 $m^3/m^2.d$ have been considered to be very high. In comparison to the HLRs of 0.29 – 0.56 $m^3/m^2.d$ used in this study, the two systems can be considered to be operated at high HLR. Notwithstanding, the pollutant removal rates achieved were comparable to those obtained in other studies. Furthermore, the results presented in Table 2 indicate that the removal rates seemed to be proportional to the loading rates over time. For most of the pollutants, both systems required between two to three months from start up to achieve appreciable removal rates. Thereafter, the removal rates tend to follow the trend of the loading rates. The only exception was for P, and both systems showed efficient P removal as soon as they began to receive flow irrespective of their configurations, and even when vegetation was sparse and the microbial communities were just being established.

In particular, P removal in both systems was much higher in comparison to the typical removal of 20-30% reported in many similar CWs (Brix and Arias, 2005). This confirms the excellent capacity of the alum sludge substrate used for P removal in the CWs. However, it should be noted that the systems have only been operated for a relatively short time (< 2 years), and therefore, the adsorption capacity of the alum sludge was not exceeded. In a separate publication (see Zhao et al. 2009b), we have considered the adsorption proportion of

P removal in the system and estimated the life time of the systems with respect to P removal to be 9-40 years for domestic wastewater and 2.5 – 3.7 years for high P wastewater such as the animal farm wastewater used in this study. In practice, multi-stage treatment system is usually applied, therefore, the lifetime can be expected to be longer. Furthermore, removal rates for TN were comparatively low for both systems especially during the start-up phase even though considerable $NH_4$-N removal rates were achieved. On one hand, the comparatively lower removal rates obtained may be attributed to the relatively longer time required (compared to the time required to achieve high P removal) to establish high and stable nitrogen removal rates. This is corroborated by findings from a similar study investigating long-term nitrogen removal behaviour of a two-stage CWs, in which it was reported that increased and stable nitrogen elimination was obtained from the third year of operation (Langergraber et al. 2011, 2014). On the other hand, in CWs, the central pathway for nitrogen removal is nitrification followed by denitrification (Babatunde, 2007). This suggests that under conditions of high HLR used in this study, incomplete nitrogen removal may have occurred as evidenced by the high nitrification rates and poor total nitrogen removal rates (Babatunde et al., 2011a, b).

**Impact of configuration on removal rates**

In order to examine the effect of the different configurations on the performance of the systems, a plot of the loading versus removal rates was constructed for the different configurations (Figure 2). With respect to the removal of organics ($BOD_5$ and COD), the plots show that a more stable treatment performance was achieved with system A especially with the 4 stages configuration. On the other hand, while higher removal rates were achieved in system B with the 2 stage configuration, the performance was less stable as evident by the considerable scatter of the points. Furthermore, the plots indicate that at loading rates up to 100g-$BOD_5$/$m^2$.day, similar treatment performances were obtained for system A (both 3- and 4-stage configuration) and system B (4-stage configuration only). This implies that for design purposes, similar treatment performances with respect to the removal of organic compounds can be achieved using a 3-stage configuration and at loading rates up to 100g-$BOD_5$/$m^2$.day. However, there are considerable differences in the treatment performances of the different configurations beyond the 100g-$BOD_5$/$m^2$.day. In all cases, system B with the 2-stage configuration had the highest loading rate and consequently the highest removal rate. However, it had the least stable performance.

Regarding nutrients removal, there was considerable variation in the performance of the different configurations for N and P removal. In the case of P, the impact of configuration was less obvious with respect to both TP and $PO_4$-P. For all configurations, a minimum of 80% P removal can be expected for loadings up to 10g–P/$m^2$.day. However, system B in the 2-stage configuration proved less effective at loadings above 10g–P/$m^2$.day, which suggests that 10g–P/$m^2$.day might be the effective loading rate for the systems. On the other hand, there were noticeable differences in the performance of the different configurations for N removal. For both TN and $NH_4$-N, system B with the 2-stage configuration demonstrated to be the least effective. Furthermore, while system A in the 4-stage configuration proved to be efficient for $NH_4$-N removal, it had a less stable performance for TN removal. This indicates that there was incomplete N removal in both systems irrespective of the configuration.

**Bacterial community analysis and linkage to treatment performance**

Given the differences in nutrient concentrations, resulting from removal along the different stages of System A, we hypothesized the presence of distinct bacterial communities in each stage. It should be noted that our investigation was focused on the first and last stages of system A before reconfiguration (i.e. stages 1 and 4) because they were expected to harbour the largest differences in the system. . 16S rDNA clone libraries were constructed from the first and last stages (i.e. stages 1 and 4) of system A. A total of 44 and 47 clones with different RFLP profiles were retrieved from the respective libraries and sequenced. The phylogenetic relationship among retrieved sequences was used to group them into Operational Taxonomic Units (OTUs) at different genetic distances. Bacterial diversity was estimated for each library at phylogenetic distances (PD) of 2 and 16% using the Shannon Index of diversity (*H'*). While the diversity at PD of 2% was slightly higher in the last stage, *H'* = 3.39 (95% CI 3.16 – 3.61), than in the first stage, *H'* = 3.00 (95% CI 2.74 - 3.26),, the diversity at PD of 16% was considerably higher in stage 4 , *H'* = 2.65 (95% CI 2.42 - 2.88), as compared to stage 1, *H'* = 1.98 (95% CI 1.73 – 2.24). This is congruent with analysis of rarefaction in which the number of different related OTUs at a given genetic distance was recorded as a function of the number of analysed sequences (Figure 3). The sequencing of 44 different clones from stage 1 yielded 25 and 10 OTUs at genetic distances of 2 and 16%, respectively. In contrast, the sequencing of 47 clones from stage 4 produced 33 and 18 different OTUs at the above genetic distances. While the rarefaction curve at 16% difference in stage 1 is about to reach an asymptotic behaviour, this was not the case for stage 4. These results show that the bacterial diversity increased between the first and the last stages. This indicates that the

design and configuration of the CW determines the composition of the bacterial communities, and hence the performance of the treatment in each stage.

Stage 1 was dominated by species belonging to the phyla Proteobacteria and Bacteroidetes, representing 72.7 and 25% of the total number of the sequenced clones, respectively. In addition, a single clone related to the phylum Chloroflexi was also retrieved (Figure 4). Although Proteobacteria and Bacteroidetes also accounted for the majority of species present in stage 4 (74.5% Proteobacteria and 8.5% Bacteriodetes of the sequenced clones), stage 4 was more diverse in composition, with species belonging to the phylum Acidobacteria (8.7%), and the Candidate division TM7 (6.5%) present (Figure 5). Based on the design and operation of the system, stages 1 and 4 have the same hydraulic loading rate but different organic and pollutant loading rates, with the highest loading rate occurring in stage 1. Consequently, there are a number of design induced differences in parameters affecting bacterial growth between the two stages, including differences in the concentrations and nature of electron donors and acceptors as well as differences in sources of carbon and nitrogen. These undoubtedly will have resulted in the development of different microbial communities in stages 1 and 4.The Proteobacteria were the most diverse phylum in both stages. However, proteobacterial diversity in stages 1 and 4 was not the same (Figures 4 and 5). While γ-proteobacteria was the most diverse class in stage 1 (7 phylotypes representing 50.5% of sequenced clones), α-proteobacteria displayed greater diversity in stage 4 (15 phylotypes representing 48.9% of the sequences). Within the γ-proteobacteria in stage 1, the family Xanthomonadaceae was the most diverse accounting for 34.1% of the total number of bacterial species present. Species belonging to this family have been isolated from different soils such as arable, landfill, iron mines, hydrocarbon-contaminated soils, compost, river sediments and seashore sand. Therefore, the predominance of Xanthomonadaceae in stage 1 might be explained by its relatively high pollutant loading in comparison to stage 4.

Although β-proteobacteria were present in both stages, there were differences in the diversity of β-Proteobacterial species in the two stages. Results indicate that while 13.6% of the phylotypes corresponded to β-proteobacteria in stage 1, only 6.5% of the phylotypes of stage 4 belonged to this class. Most interestingly, the phylogenetic affiliations of the members of this class in stage 1 are bacterial species previously isolated from water environments and wastewater treatment plants. Moreover, Rhodocyclales and Comamonadaceae, which are known to play roles in the denitrification in activated sludges (IWA, 2009) accounted for 8.7% and 4.9% of the total diversity, respectively, in stage 1. In contrast, OTUs belonging to the β-proteobacteria retrieved from stage 4 were related to an unclassified member of β-

proteobacteria and Polynucleobacter sp the last of which is commonplace in freshwater (Hahn et al. 2005).

## CONCLUSION

The impact of different configurations on the treatment performance of two multi-stage constructed wetland systems (CWs) was assessed. Results showed that removal rates were proportional to loading rates over time in both systems. At loadings up to 100g-$BOD_5/m^2$.day, similar treatment performance was achieved for both the 3 and 4 stages configuration. In the case of P, a minimum of 80% P removal can be expected for loadings up to 10g–$P/m^2$.day irrespective of the configuration. Analysis of the clone libraries of the first and last stage of the 4-stage CW suggested an increased bacterial diversity in the last stage and pointed to a link between nutrient removal and community composition. Future work, using community fingerprinting and deep sequencing using Next Generation Sequencing technologies, will provide a more comprehensive view of the linkage between design, treatment performance and microbial community of constructed wetlands.

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Table 1 Design summary of the constructed wetland systems

| Parameter | System A | System B |
|---|---|---|
| Net treatment area ($m^2$) | 3.42 | 2.34 |
| System configuration | 4 stages connected in series (for the first 11 months)<br>3 stages connected in series (onwards from the 11$^{th}$ month, 1$^{st}$ stage gravel filled) | 4 stages connected in series (for the first 4 months)<br>2 stages connected in series and with effluent recirculation (onwards from the 4$^{th}$ month) |
| Flow regime | Downward fill and drain | Downward fill and drain |
| System hydraulic loading rate ($m^3/m^2.d$) | 0.29 (4 stages)<br>0.38 (3 stages) | 0.32 (4 stages)<br>0.56 (2 stages) |
| Hydraulic retention time per stage (hrs) | 4 | 4 |
| Media configuration (top to bottom) | 0-10cm (distribution layer, 20mm gravel); 10-75cm (main layer, dewatered alum sludge cakes);75-85cm support and drainage layer, 10mm gravel) | 0-10cm (distribution layer, 20mm gravel); 10-75cm (main layer, dewatered alum sludge cakes);75-85cm support and drainage layer, 10mm gravel) |

Table 2a Mean pollutant loading and removal rates (in $g/m^2.d$) for system A

| | | 4-stage | | | | | | | | | | | 3-stage | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Parameter | Rate | Feb | Mar | Apr | May | Jun | Jul | Aug | Sep | Oct | Nov | Dec | Jan | Feb | Mar | Apr | May |
| $BOD_5$ | loading | nd | 12.0 | 13.1 | 31.9 | 89.2 | 69.5 | 89.1 | 68.8 | 119.1 | 201.4 | 119.3 | nd | nd | 204.7 | 215.3 | 39.9 |
| | removal | nd | 7.5 | 7.1 | 21.8 | 70.6 | 48.2 | 64.9 | 37.9 | 87.6 | 149.8 | 89.0 | nd | nd | 111.2 | 136.4 | 14.1 |
| COD | loading | 207.6 | 140.1 | 102.0 | 141.1 | 109.8 | 189.3 | 200.4 | 285.0 | 376.3 | 202.3 | 207.6 | nd | 342.8 | 398.6 | 456.6 | 179.7 |
| | removal | 53.7 | 58.0 | 49.8 | 93.0 | 67.9 | 151.1 | 139.3 | 168.2 | 253.8 | 147.3 | 53.7 | nd | 116.7 | 270.3 | 305.1 | 97.3 |
| sCOD | loading | nd | 166.8 | 67.3 | 57.0 | 82.9 | 65.7 | 109.4 | 119.9 | 183.1 | 304.1 | 108.6 | nd | 191.5 | 295.3 | 268.2 | 131.9 |
| | removal | nd | 36.5 | 15.3 | 14.1 | 46.7 | 35.9 | 83.2 | 78.0 | 129.9 | 234.8 | 82.7 | nd | 71.4 | 175.7 | 172.9 | 64.2 |
| SS | loading | 92.1 | 72.3 | 41.7 | 33.6 | 29.3 | 29.8 | 42.1 | 49.6 | 66.7 | 100.5 | 78.3 | nd | 92.3 | 110.9 | 210.6 | 62.3 |
| | removal | 55.2 | 37.4 | 23.7 | 24.3 | 22.9 | 21.4 | 33.9 | 40.0 | 44.0 | 58.9 | 38.7 | nd | 33.1 | 64.5 | 125.0 | 24.3 |
| TP | loading | 4.7 | 4.8 | 3.1 | 2.8 | nd | 2.8 | 5.0 | 7.3 | 9.6 | 8.6 | 6.4 | 9.4 | 10.8 | 7.8 | 11.6 | 9.4 |
| | removal | 3.8 | 4.2 | 3.0 | 2.5 | nd | 2.6 | 4.7 | 6.8 | 8.9 | 7.2 | 4.9 | 7.9 | 9.4 | 6.9 | 9.9 | 7.9 |
| $PO_4$-P | loading | 7.4 | 3.9 | 2.8 | 5.2 | 4.5 | 3.8 | 6.9 | 7.8 | 9.5 | 7.9 | 5.3 | nd | 7.6 | 8.0 | 8.8 | 10.3 |
| | removal | 6.1 | 3.5 | 2.6 | 4.8 | 4.3 | 3.5 | 6.5 | 7.5 | 9.2 | 6.9 | 3.8 | nd | 7.0 | 6.7 | 7.2 | 8.7 |
| TN | loading | 24.4 | 20.5 | 25.4 | 12.5 | 27.7 | 24.7 | 58.3 | 60.9 | 63.1 | 38.2 | 31.4 | nd | 45.0 | 47.4 | 70.4 | 38.0 |
| | removal | 2.9 | 0.9 | 11.6 | 4.4 | 17.4 | 15.1 | 38.3 | 22.8 | 24.5 | 27.9 | 17.0 | nd | 27.8 | 28.8 | 45.3 | 22.4 |
| $NH_4$-N | loading | 23.7 | 21.0 | 11.0 | 13.4 | 18.6 | 17.6 | 37.2 | 47.1 | 51.1 | 31.6 | 26.5 | nd | 28.0 | 62.3 | 59.4 | 30.1 |
| | removal | 16.5 | 7.5 | 7.1 | 11.7 | 16.6 | 15.4 | 33.6 | 43.5 | 47.0 | 24.8 | 12.9 | nd | 16.0 | 27.1 | 36.9 | 11.2 |

nd indicates no data

Table 2b Mean pollutant loading and removal rates (in $g/m^2.d$) for system B

| | | 4-stage | | | | 2-stage | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Parameter | Rate | Feb | Mar | Apr | May | Jun | Jul | Aug | Sep | Oct | Nov | Dec | Jan | Feb | Mar | Apr | May |
| $BOD_5$ | loading | nd | 13.2 | 19.0 | 17.8 | 212.8 | 134.2 | 178.2 | 132.9 | 229.9 | 363.3 | 230.4 | nd | nd | 301.7 | 317.3 | 58.8 |
| | removal | nd | 9.0 | 9.5 | 4.6 | 103.0 | 67.9 | 105.3 | 56.6 | 104.2 | 249.2 | 175.3 | nd | nd | 175.6 | 96.0 | 34.2 |
| COD | loading | nd | 202.3 | 161.6 | 145.2 | 298.4 | 212.1 | 365.6 | 387.0 | 550.4 | 726.6 | 390.6 | nd | 392.8 | 587.4 | 672.9 | 264.9 |
| | removal | nd | 43.1 | 63.6 | 35.6 | 126.0 | 105.2 | 200.9 | 232.1 | 226.9 | 502.0 | 327.3 | nd | 332.4 | 404.0 | 345.8 | 170.2 |
| sCOD | loading | nd | 199.8 | 72.6 | 65.6 | 173.4 | 126.9 | 211.2 | 231.5 | 392.4 | 587.3 | 282.8 | nd | 264.9 | 435.1 | 395.2 | 194.3 |
| | removal | nd | 50.2 | 27.0 | 7.5 | 65.7 | 48.3 | 98.5 | 130.1 | 206.4 | 459.9 | 221.8 | nd | 170.8 | 284.8 | 119.3 | 124.9 |
| SS | loading | 101.6 | 79.7 | 50.2 | 41.7 | 65.2 | 65.5 | 81.2 | 95.8 | 128.9 | 194.1 | 151.2 | nd | 149.2 | 163.4 | 310.4 | 91.8 |
| | removal | 48.3 | 39.3 | 25.2 | 16.4 | 30.9 | 32.8 | 34.1 | 63.6 | 64.0 | 113.3 | 106.7 | nd | 114.2 | 67.5 | 220.4 | 40.9 |
| TP | loading | 5.1 | 5.3 | 3.4 | 3.1 | nd | 5.4 | 9.7 | 14.1 | 18.6 | 16.6 | 12.4 | nd | 15.7 | 16.0 | 11.5 | 17.1 |
| | removal | 4.1 | 4.7 | 3.0 | 2.5 | nd | 3.7 | 7.0 | 11.7 | 14.3 | 13.2 | 10.2 | nd | 15.7 | 13.9 | nd | 15.0 |
| $PO_4$-P | loading | 8.2 | 4.3 | 3.1 | 6.5 | 9.2 | 7.4 | 13.3 | 15.0 | 18.4 | 15.3 | 10.1 | nd | 9.2 | 11.7 | 13.0 | 15.2 |
| | removal | 6.9 | 3.6 | 2.7 | 5.3 | 7.7 | 4.7 | 9.4 | 12.9 | 14.6 | 11.9 | 7.9 | nd | 6.3 | 10.0 | 12.2 | 12.3 |
| TN | loading | 26.9 | 22.6 | 28.0 | 13.8 | 61.0 | 47.7 | 112.6 | 121.5 | 114.4 | 73.9 | 60.6 | nd | 91.7 | 55.5 | 103.8 | 56.0 |
| | removal | 1.3 | 2.1 | 15.0 | 5.8 | 27.6 | 24.8 | 32.2 | 34.3 | 37.9 | 52.7 | 42.7 | nd | 81.2 | 15.2 | 63.2 | 38.1 |
| $NH_4$-N | loading | 23.6 | 20.5 | 12.1 | 15.8 | 39.0 | 34.0 | 71.9 | 90.9 | 98.7 | 61.1 | 51.2 | nd | 119.1 | 164.0 | 156.3 | 79.1 |
| | removal | 14.0 | 6.1 | 6.5 | 12.6 | 18.2 | 17.3 | 24.6 | 61.3 | 53.3 | 44.7 | 37.7 | nd | 53.1 | 50.2 | 30.2 | 22.0 |

nd indicates no data

Figure 1 The Multi-stage constructed wetland systems

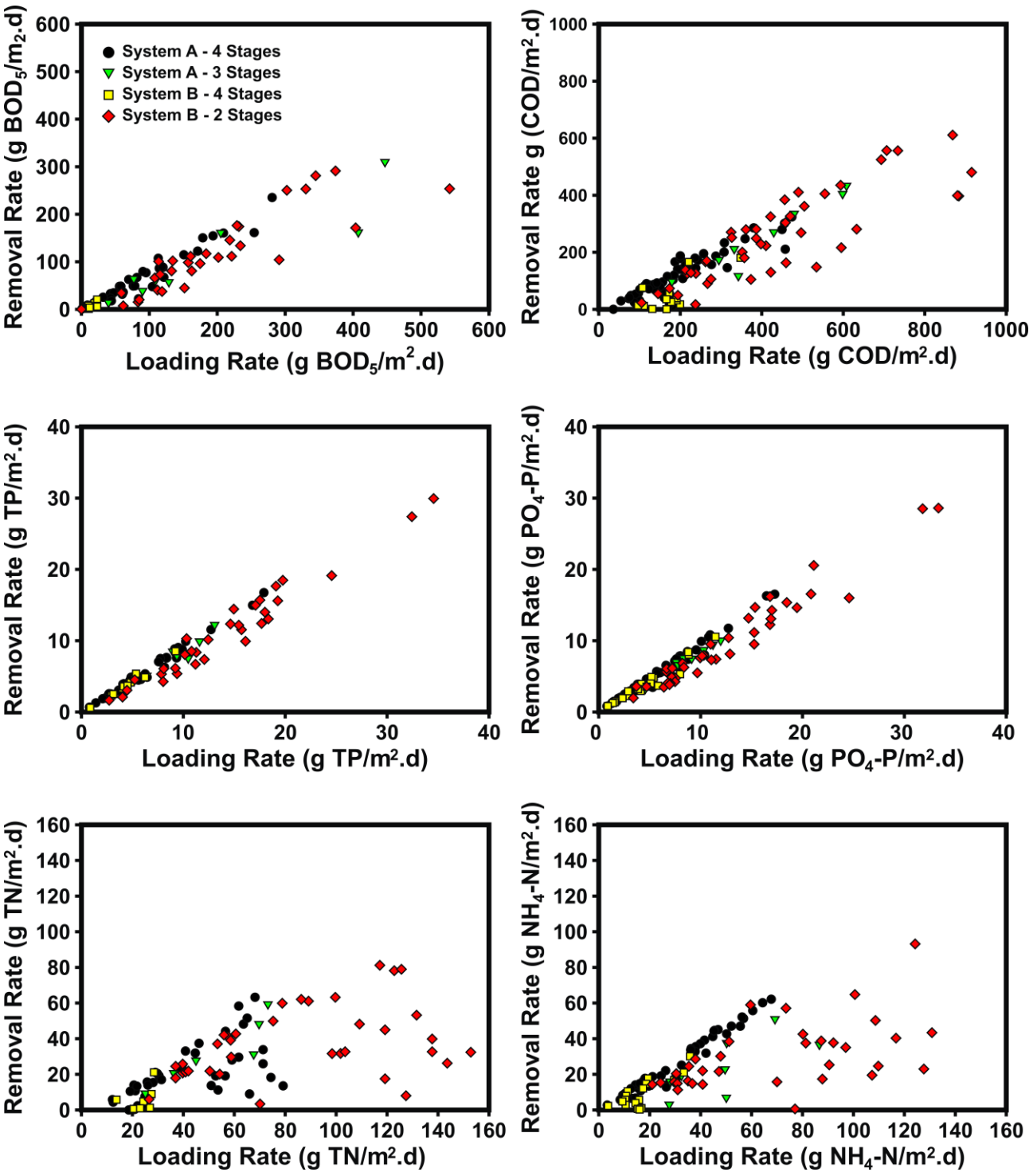


Figure 2. Plot of loading vs removal rate for selected pollutants for the different configurations of systems A and B.

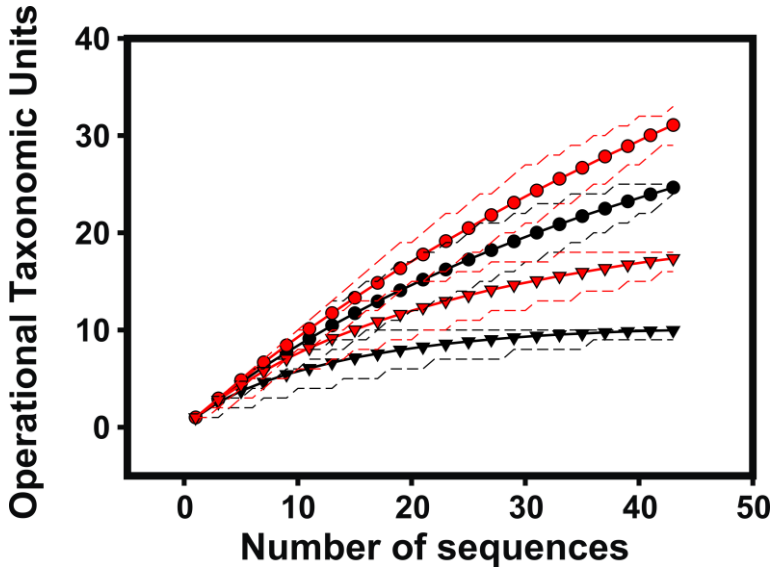


Figure 3. Analysis of rarefaction to compare the bacterial diversity associated to alum sludge media in the constructed wetland systems. 16S rDNA clone libraries were prepared from the first and last stages of the four-stage CWs. After sequencing, distance matrices were obtained with the DNADIST program of the Phylip package (Felsenstein, 1989). Rarefaction curves were generated by assigning operational taxonomic units (OTUs) obtained from stage one (black) and stage four (red) at genetic distances of 2% (circles) and 16% (triangles) with the DOTUR software package (7). Broken lines show the 95% confidence intervals.

**Stage 1**

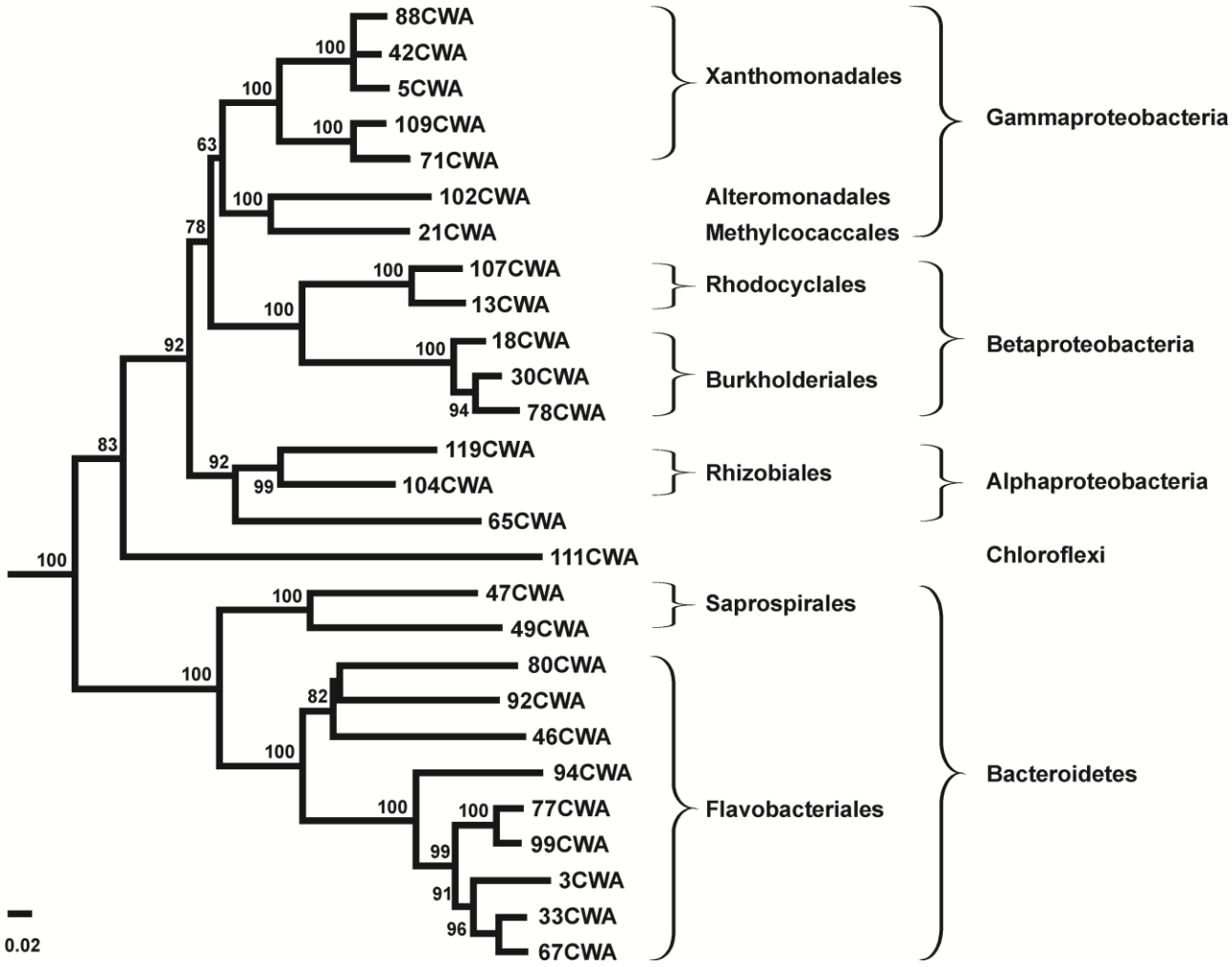


Figure 4. Phylogenetic relationships based on 16S rDNA gene sequences of uncultured bacteria associated with alum sludge media in the first stage of system A. Bootstrap values of 1000 replicates are given at branch points. The bar represents 0.02 substitutions per site.

## Stage 4

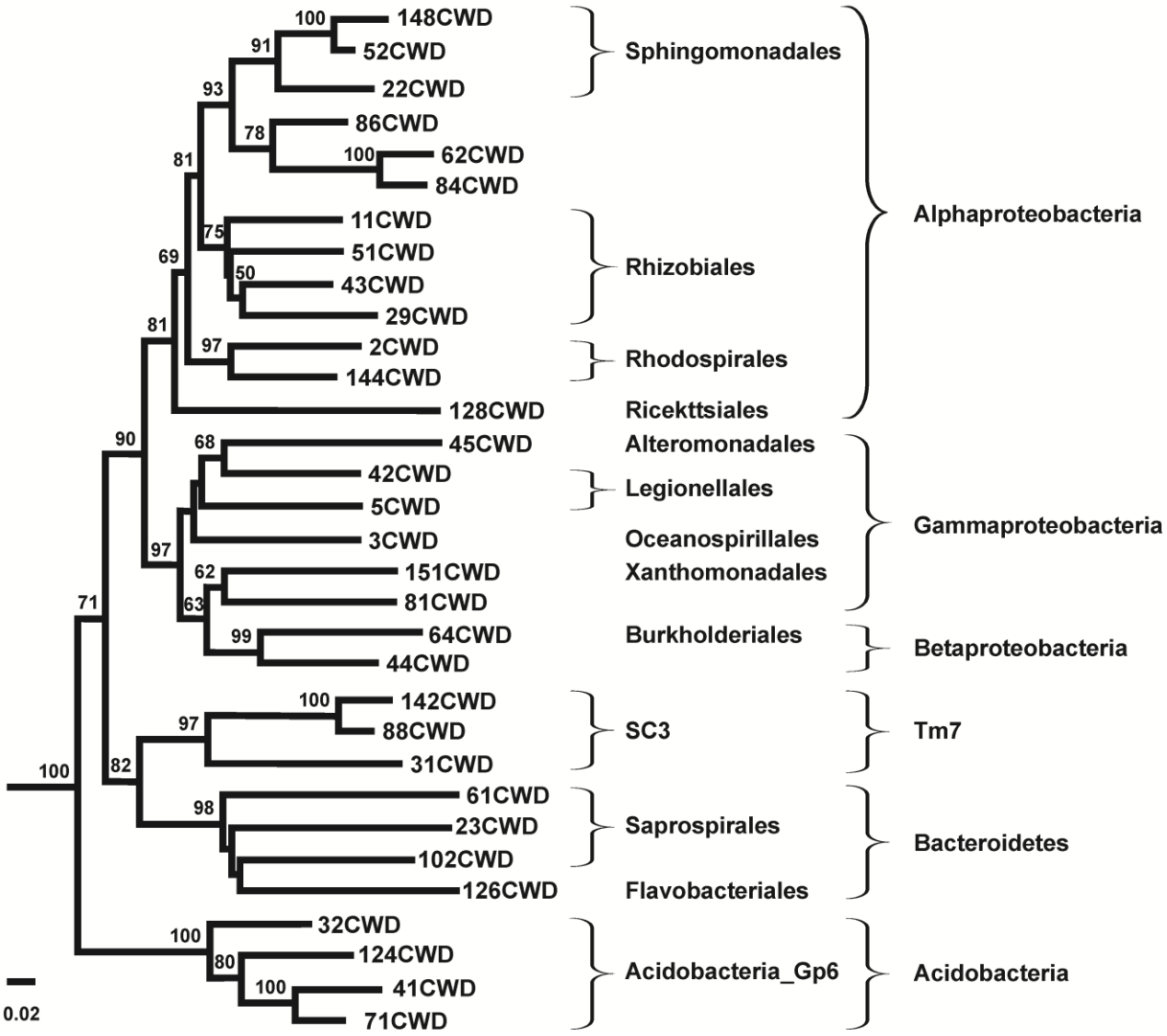


Figure 5. Phylogenetic relationships based on 16S rDNA gene sequences of uncultured bacteria associated to alum sludge in the fourth stage of system A. Bootstrap values of 1000 replicates are given at branch points. Bar represents 0.02 substitutions per site.