

Short Communication

Characterization of bacterial communities of a constructed wetland in cold conditions

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One of the most useful tools for bacterial characterization in wastewater treatment plants is the fluorescence in-situ hybridization (FISH) methodology. Although it has been commonly used in activated sludge (see e.g. Juretschko et al., 1998) and trickling filters (see e.g. Okabe et al., 1999) almost no evidence on its application to low-cost systems as constructed wetlands is found in the literature (Flood et al., 1999; Silyn-Roberts and Lewis, 2001). Moreover, studies on these systems are usually carried out during the growing season and no data is usually available on the winter period, which is the time of year for which low-cost treatment systems are designed as they have to maintain their removal efficiency even in harsh conditions. In the present study we have employed FISH in different units of a low-cost wastewater treatment system, formed by a stabilization pond followed by in-series free-water surface and sub-surface constructed wetlands. The aim of this study was to describe the winter microbial community composition in each of these units and their relation with the wastewater characteristics.

The system is a mixed constructed wetland where water passes consecutively through a stabilization

pond (SP), a free water surface system (FWS) planted with *Typha latifolia* and a sub-surface wetland (SSF) planted with willows (*Salix atrocinerea*) (Fig. 1). Further details on the process are presented elsewhere (Ansola et al., 2003). The studied plant was located in Bustillo de Cea, in the northwest of León (Spain). It was designed for rural domestic wastewater treatment with an average flow of 56.3 m³/d during the study period. Total area of the system was 890 m² and the theoretical hydraulic retention time for each unit was 4.21, 3.53 and 3.16 days for the SP, FWS and SSF respectively. Despite the fact that the SP was designed to allow phytoplankton growth, a thick layer of the floating macrophyte *Lemna minor* was observed during the study.

Four samples were taken bi-weekly between November 2001 and February 2002. One liter of water was collected in each sampling point and then cooled and carried to the laboratory where samples were centrifuged and purified following Manz et al. (1994). Each sample was then divided in two parts; the first one was fixed with a 3% paraformaldehyde (PFA) solution for Gram-negative bacteria, and the second one was fixed by addition of ethanol to a final concentration of 50% (v/v) for Gram-positive bacteria, both for 16 h at 4°C. After this, the aliquots were stored in a 1 : 1 mixture of phosphate-buffered saline (PBS) and 96% ethanol at –20°C (Manz et al., 1994).

The hybridization step was done according to Manz

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Fig. 1. The wastewater treatment plant in Bustillo de Cea (León, Spain).

Sampling points. 1, Raw wastewater; 2, pretreatment outflow; 3, stabilization pond outflow; 4, free water surface wetland outflow; 5, final effluent of the plant. SP, stabilization pond; FWS, free water surface wetland; SSF, sub-superficial wetland.

et al. (1992). The following probes were used: general probe for most eubacteria (EUB 338); probes for the α , β , γ and δ subclasses of *Proteobacteria* (ALF1b, BET42a, GAM42a and SRB 385 respectively). Probes specific for the *Cytophaga-Flavobacterium* subclass (CF319a), and for the Gram-positive bacteria with high G+C content (HGC69a). With regard to the nitrifying bacteria the following probes were used: NEU 23a for most of halophilic and halotolerant ammonia-oxidizers, Nm II and Nmo 218 for *Nitrosomonas communis* and *N. oligotropha* lineages, Nsv 443 for *Nitrosospira* cluster, NmV, specific for the *Nitrosococcus mobilis* lineage and Ntspa 662 for the nitrite-oxidizing *Nitrospira* cluster (see Gieseke et al., 2001; Manz et al., 1992; Mobarry et al., 1996 for further details on probe sequences and hybridization conditions). All probes were labeled either with tetramethylrhodamine-5-isothiocyanate (TRITC) or fluorescein (Roche Molecular Biochemicals, Germany). Sample-containing slides were mounted in an antifading solution (Fluoroguard, Bio Rad) for best viewing with the epifluorescence microscope.

The protocol of Hicks (Hicks et al., 1992) for dual staining of samples with 4,6-diamidino-2-phenylindole (DAPI) and fluorescent rRNA oligonucleotide probes was slightly modified. Staining was performed in darkness for 7 min at a final concentration of 0.33 $\mu\text{g/ml}$ DAPI after the hybridization and washing steps.

Statistical analysis was carried out using Statistica package (StatSoft, 1995). ANOVA and Tukey's Test for post-hoc analysis was performed to test differences between sampling points. Data on percentages were arc sen transformed to fulfill ANOVA assumptions and the non-parametric Kruskal-Wallis test was applied in

Table 1. Summary of chemical values for each of the sampling points (mean of four samples).

Parameters	P 1	P 2	P 3	P 4	P 5
pH (units)	8.1	8.3	6.9	5.5	7.2
Dissolved oxygen (mg/L)	3.5	4.1	1.0	3.8	3.3
BOD ₅ (mg O ₂ /L)	189	28	48	80	29
COD (mg O ₂ /L)	759	118	199	162	174
TSS (mg/L)	794	101	384	141	126
NTK (mg N/L)	93	12	18	12	18
NH ₄ ⁺ (mg N/L)	40	7.8	12	6.6	11

See Fig. 1 for point location. BOD: biological oxygen demand, COD: chemical oxygen demand, TSS: total suspended solids, NTK: total nitrogen (adapted from Cortijo, 2003).

other cases.

Mean chemical values of the sampling points during the study period are shown in Table 1. Water temperature during the study ranged from 5°C to 9°C. Pretreatment using an oversized grit chamber and coarse bar screens was very effective in removing the high suspended solids concentration and their associated organic matter (sampling point P2). Most values increased again after the *Lemna*-covered stabilization pond (P3) due to the decomposition of *Lemna* from the bottom of the pond. The FWS unit (P4) seems to be effective in removing TSS and their associated COD but also in producing BOD. The SSF unit (P5) was effective in removing BOD but no significant changes were observed for the rest of the variables. Organic loading using a mean flow of 56 m³ d⁻¹ gives mean values of 7, 12 and 10 g BOD₅ m⁻² d⁻¹ for SP, FWS and SSF respectively, slightly higher than that recom-

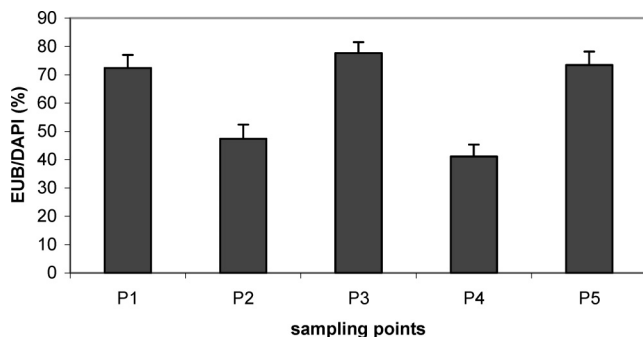


Fig. 2. Mean EUB/DAPI ratio and standard error for each sampling point.

mended for wetland systems (USEPA, 2000).

The EUB/DAPI ratio reveals the amount of microorganisms belonging to the *Eubacteria* domain present in a sample (Manz et al., 1994). Despite the fact that this ratio is commonly used in this kind of study and it has been used here for a better comparison with other authors, it is important to notice that the ratio can be affected by several factors as the abundance of non-bacterial organisms, the potential presence of some bacterial groups not detected by the EUB 338 probe and specially by the low level of ribosomes in potentially low-activity environments as the one under study (Amann et al., 1995; Bouvier and del Giorgio, 2003; Daims et al., 1999). Differences between points 1, 3 and 5 were statistically significant from points 2 and 4 (ANOVA, $p < 0.001$) (Fig. 2). The EUB/DAPI ratio values were nearly 75% for the 1st, 3rd and 5th sampling points (raw influent, SP effluent and FWS effluent respectively), these values being similar to those found in activated sludge systems (Bouvier and del Giorgio, 2003; Manz et al., 1994); in contrast, the EUB/DAPI ratio for the 2nd and 4th sampling points (SP influent and FWS effluent respectively) was below 50%. This decrease could probably be due to the aforementioned factors affecting the EUB 338 probe. The comparison of these results with those from Reinoso (2003) and Cortijo (2003), who studied DAPI counts, total and faecal coliforms and faecal streptococci in the same samples, showed the same up and down patterns. All three faecal indicators increased one log unit from point 2 to point 3, decreased one log again in point 4 and increased once again in point 5. This therefore supports the hypothesis that bacteria abundance and their activity was lower in points 2 and 4 than in 1, 3 and 5.

This set of probes for Proteobacteria subclasses,

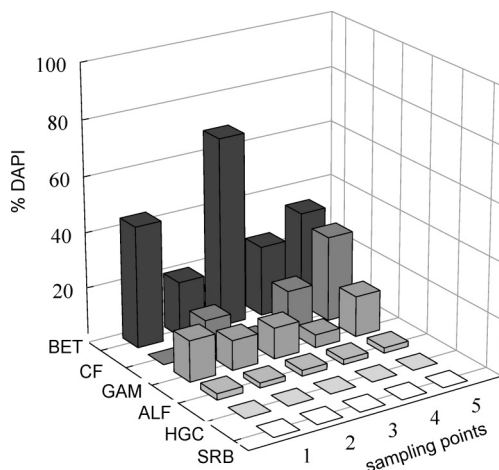


Fig. 3. Percentage of total bacteria that hybridised with ALF 1b (ALF), BET24a (BET), GAM42a (GAM), SRB 385 (SRB), CF 319a (CF) and HGC 69a (HGC) probes.

and for Cytophaga-Flavobacter (CF) and HGC bacteria have been widely used in wastewater treatment bacterial community characterization (Manz et al., 1994; Okabe et al., 1999). The specific probe/DAPI ratio was selected for a better comparison with the few studies on FISH in constructed wetlands (Flood et al., 1999; Silyn-Roberts and Lewis, 2001). In the present study the bacterial community was generally dominated by the *Betaproteobacteria* (average % of DAPI: $38.3\% \pm 19.1$) (Fig. 3), similar values to those reported in activated sludge (e.g. Manz et al., 1994). The *Betaproteobacteria* showed the same fluctuations as the EUB 338 probe, with the lowest values in points 2 and 4. Differences between sampling points were significant for β ($p < 0.001$) and CF ($p < 0.01$) probes, although not significant for α and γ subclasses. The proportion of γ and α subclasses (mean %DAPI of $10.5\% \pm 3.7$ and $1.9\% \pm 0.20$ respectively) were lower and in a more or less constant proportion in all points (Fig. 3). These percentages, like the β/γ ratio variation, were similar to those reported by Flood et al. (1999) in a FWS biofilm, showing a decrease in β (62 to 41%) and an increase in γ (11 to 34%) from the inlet to the outlet of a wetland, respectively. In our system, the β/γ ratio evolved from a 3 : 1 value in the influent to 7 : 1 in the SP effluent, decreasing to the initial proportion in the final effluent. *Betaproteobacteria* were proportionally more abundant in the raw influent and in the effluent of the stabilization pond (P3) probably in relation with the high production of suspended solids in this point as consequence of duckweed degradation.

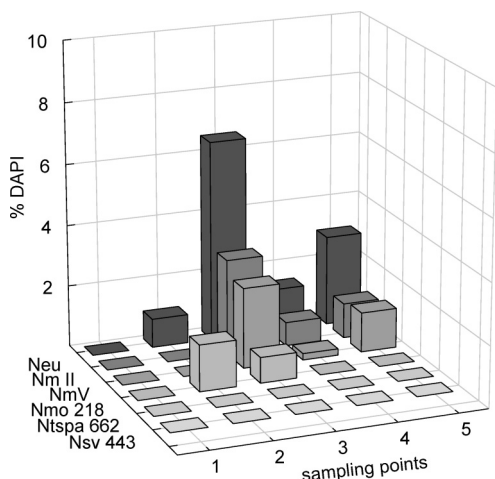


Fig. 4. Percentage of total bacteria that hybridised with NEU 23a, Nm II, Nmo 218, NmV, Nsv 443 and Ntspa 662.

CF bacteria were only detected in the 2nd, 4th and 5th sampling points, reaching values close to 20% (11.5% average) (Fig. 3), values higher than those reported in activated sludge or loaded biofilm systems which fluctuates (as %DAPI) from 1% (Kämpfer et al., 1996) to 10% (Kloep et al., 2000). Except for the final effluent, there was an inverse correlation between *Betaproteobacteria* and CF. This latter group was not present in those points where COD and TSS reached high values (1st and 3rd sampling points; Table 1). Despite the fact that HGC bacteria is commonly found in activated sludge systems (e.g. Manz et al., 1994) this group was not detected in our plant. Coinciding with similar studies carried out in activated sludge the *Deltaproteobacteria* were not detected in our system either.

Six oligonucleotide probes were used for nitrifying bacteria community characterization but positive results were only found for four of them (Fig. 4). Nitrification activity in our system seems to be carried out by *Nitrosomonas* spp. (NEU, 2.4% average), *Nitrosomonas communis* (Nm II, 1% avg.), *N. oligotropha* (Nmo 218, 0.47% avg.), and *Nitrosococcus mobilis* (NmV, 0.83% avg.). These microorganisms have often been detected in nitrogen-removal wastewater treatments plants but with higher percentages e.g. 18% in activated sludge (Juretschko et al., 1998) or 16% in an upflow biofilm system (García, 2003). The nitrite-oxidizing *Nitrospira* genus (Ntspa 662) often found in nitrifying biofilms (e.g. Okabe et al., 1999), and those groups characteristic of low ammonia concentration (Nsv 443) were not detected in our system. No nitrify-

ing bacteria was detected in the first sampling point, and the highest values were observed in the 3rd sampling point (SP effluent). Statistical differences between points were only significant for Nm II ($p < 0.01$) and Nm V ($p < 0.001$). Previous works on wetlands have focused on the biofilm communities during summer with different results. Flood et al. (1999) found 12 and 4% NEU in the inlet and outlet parts, respectively, of a low loaded wetland. Silyn-Roberts and Lewis (2001) studied a wetland as a tertiary treatment of a dairy effluent and the percentage of NEU in the biofilm fluctuated from 0 to 1.5%. It seems clear that constructed wetlands harbor a much lower percentage of nitrifying bacteria when comparing with conventional systems, being especially lower in winter. Cold water temperatures (6°C) and low oxygen levels seem to be the main reasons for the low percentages of nitrifying bacteria in our system. Moreover, these microorganisms tend to grow in bacterial biofilms (Okabe et al., 1999) being difficult to detect in the free-water flow.

Bacterial communities in the studied constructed wetland were similar to those in conventional systems as activated sludge in relation to the proportion of *Betaproteobacteria* and *Gammaproteobacteria*. The main differences were due to the low percentage or absence of nitrifying bacteria and HGC bacteria and to the higher proportions found for CF bacteria in this wetland. Comparison with other wetland systems showed similar values in the percentages and ratios of bacteria. This evidence strengthens the hypothesis that constructed wetland systems seem to harbor qualitative and quantitative differences in bacterial communities to conventional ones. The different subsystems (SP, FWS, SSF) of the treatment plant did not show a significant nitrogen-removal efficiency under winter conditions. These results support the evidence that nutrient removal in constructed wetlands is not significant, especially in winter, unless systems were properly designed for such a purpose (Tanner, 2001).

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