Selection of diazotrophic bacterial communities in biological sand filter mesocosms used for the treatment of phenolic-laden wastewater

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Abstract

Agri effluents such as winery or olive mill wastewaters are characterized by high phenolic concentrations. These compounds are highly toxic and generally refractory to biodegradation. Biological sand filters (BSFs) represent inexpensive, environmentally friendly and sustainable wastewater treatment systems which rely vastly on microbial catabolic processes. This study aimed to assess the impact of increasing concentrations of synthetic phenolic-rich wastewater, ranging from 96 $mg.L^{-1}$ gallic acid and 138 $mg.L^{-1}$ vanillin (i.e. a total chemical oxygen demand (COD) of 234 $mg.L^{-1}$) to 2400 $mg.L^{-1}$ gallic acid and 3442 $mg.L^{-1}$ vanillin (5842 mg $COD.L^{-1}$), on bacterial communities from BSF mesocosms. This amendment procedure instigated adaptation of the bacterial communities, notably leading to the selection of a resistant diazotrophic community. This suggests that functional alterations in the bacterial communities in BSFs ensure provision of sufficient bioavailable nitrogen for the degradation of wastewater with a high C:N ratio.

Key words: Biological sand filter / Constructed wetland / Phenolic / Microbial community fingerprinting (DGGE/T-RFLP) / *nifH* gene

1. Introduction

In developing countries, agri-industrial wastewaters are frequently discharged into aquatic environments without (pre)treatments due to the lack of treatment facilities [10]. The first corollary of such practices is that water resources become contaminated and therefore unsuitable for human consumption, land irrigation, fish production and/or recreational activities. The second is that this practice constitutes a major constraint on the country's sustainable development [10]. For example, agri wastewaters are generally organic- and/or nutrient-rich effluents which can dramatically affect water quality, and notably lead to eutrophication of water systems [24].

South Africa is a water-scarce, developing country with an important and established wine industry [21] and with a young, emerging olive farming sector [26], both mainly situated in the Western Cape Province. Winery wastewater (WW) and olive mill wastewater (OMWW) are variable in nature but generally characterized by elevated Chemical Oxygen Demand (COD) levels (up to 25 $g.L^{-1}$ and 170 $g.L^{-1}$ respectively) and contaminated with (poly)phenolic compounds such as tannins [16, 29]. The (poly)phenolics are highly toxic and persist in the environment due to slow biodegradation rates [14, 23]. Therefore, strategies must be implemented to reduce the phenolic content of such wastewaters prior to discharge and/or re-use; the latter being of crucial importance in countries subject to water shortages, such as South Africa [10].

Among the various remediation (pre)treatment processes studied to treat organic-rich effluents, the use of constructed/treatment wetlands (C/TW) has been extensively investigated (for reviews see [1, 6, 9, 27]). C/TW systems are ideal for developing countries as they are cost-effective, self-sustainable, low in maintenance [10] and effective in treating simple and/or complex organic-rich contaminants such as WW and OMWW [8, 14]. The treatment

capacity of these systems relies on the synergism between plants, soil and microbial communities, thus involving abiotic (soil substratum) and biotic (plants and microorganisms) remediation mechanisms.

Phenolic compounds are phytotoxic [14] and the roles of plants in CW COD-removal capacity is controversial [27]. Therefore, in this study the impact of artificial phenolic-laden wastewater on an unplanted CW mesocosm, or biological sand filter (BSF), was investigated with an emphasis on the bacterial communities present in the BSF. Microorganisms can actively degrade phenolic compounds with several relevant enzymes being widely distributed in the microbial tree-of-life (e.g. oxygenases, dehydrogenases, laccases, peroxidases [7, 11]). However, phenolic compounds can also hamper or inhibit microbial activity and growth [18].

We have previously shown that BSF microbial communities exposed to incremental concentrations of ethanol become more tolerant of the contaminant than communities exposed to a higher concentration from outset, a phenomenon classically termed ‘microbial acclimation’ [28]. In this study, we used the more toxic and recalcitrant compounds, gallic acid and vanillin, and incrementally increased the phenolic concentration of artificial wastewater over a period of 9 weeks. Microbial community structure evolution was monitored using denaturing gradient gel electrophoresis (DGGE) analysis of the 16S rRNA gene and phenolic-selected bacterial taxa were identified with post-electrophoretic phylogenetic analysis. The study of the diazotrophic community was performed using terminal-restriction fragment length polymorphism (T-RFLP) with the *nifH* gene as phylogenetic marker [17].

2. Material and Method

2.1. Set up and feeding/amendment procedures of the pilot-scale Biological Sand Filters (BSFs)

The BSF mesocosms consisted of two identical polyethylene tanks (length 173 cm / width 106 cm / depth 30 cm), each containing river sand (1% clay, 7% silt, 4% fine sand, 12% medium sand and 76% coarse sand), to a volume of ~0.5 m^3, void space of 0.08 m^3 and a depth of 0.3 m, and were set up as previously described [19, 28]. The BSFs were maintained in an outdoor, undercover environment in order to avoid exposure to precipitation events. The systems were operated in a hybrid mode of vertical and horizontal subsurface flow. Bi-weekly inundation (i.e. intermittent flow fed BSF), followed by gradient-directed drainage ensured that the mode of operation was biased towards classical vertical subsurface flow. This mode of operation is considered to favor aerobic microbial processes and thus increase C/BOD removal potential [5].

One replicate (A) served as a control and the other (B) served as the test BSF. Both received a bi-weekly basal influent solution consisting of 0.3 g yeast extract and 0.3 g D (+) glucose dissolved in 12.5 L tap water, for the duration of a 3 month equilibration period [19] and experimental periods. This basal nutrient amendment procedure provided a C:N:P ratio of 32:7:1 (influent COD = 24 mg L^{-1}, N = 5.5 mg L^{-1} and P = 0.76 mg L^{-1}). This low nutrient supply was designed to maintain an oligotrophic state, as oligotrophic systems are more reactive to changes in nutrient status such as contamination with wastewater [25]. During the experimental period, BSF B was amended with gallic acid and vanillin for 9 weeks, with the concentration being increased every three weeks [29]. Initially, 96 $mg.L^{-1}$ gallic acid and 138 $mg.L^{-1}$ vanillin was added (day 0 to week 3), followed by 488 $mg.L^{-1}$ gallic acid and 688 $mg.L^{-1}$ vanillin (week 4 to 6) and finally 2400 $mg.L^{-1}$ gallic acid and 3442 $mg.L^{-1}$ vanillin

(Week 7 to 9). The resultant corresponding COD values were 234 $mg.L^{-1}$, 1176 $mg.L^{-1}$ and 5842 $mg.L^{-1}$ respectively.

2.2. Sediment sampling

A 30 mm internal diameter PVC sediment corer was used to recover samples without disturbance of the sediment stratification. Triplicate sample cores were taken from the inlet and the outlet of each CW at the start of the experiment (Day 0) and after 3, 6 and 9 weeks of the experiment. The surface (0-3 cm) and deep (15-20 cm) fractions of each sample were thoroughly mixed and ~1 g wet weight sediment was retained at –80°C for subsequent molecular analysis.

2.3. Metagenomic DNA extraction and quantification

Metagenomic DNA was extracted from 0.5 g of samples (wet weight) with the Powersoil DNA isolation kit according to the manufacturer's instructions (MoBio laboratories, USA). The concentration of the DNA-samples was measured with a NanoDrop spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA).

2.4. PCR amplification, purification and restriction

2.4.1. 16S rRNA gene

All polymerase chain reactions (PCR) were carried out in a Perkin Elmer Thermocycler (Gene Amp PCR system 6700). Bacterial 16S rRNA encoding genes were amplified by PCR using the universal primers E9F (5′-GAGTTTGATCCTGGCTCAG-3′) and U1510R (5′-GGTTACCTTGTTACGACTT-3′). PCR was carried out in 50 µl reaction volumes. Each reaction contained 1X PCR buffer, 0.2 U Dream*Taq*™ polymerase (Fermentas, USA), 200

μM of each dNTP, 0.5 μM of each primer, 0.1% BSA and between 5 to 10 ng of metagenomic DNA. PCR amplification was carried out as follows : 4 min at 94°C for denaturation; 30 cycles of 30 s at 94°C, 30 s annealing at 52°C and 105 s at 72°C; and a final elongation step of 10 min at 72°C. A nested-PCR was performed using 1 μL of the amplicon obtained with the 16S rRNA primer set E9F/U1510R with the primer set 341F-GC (5'-CCTACGGGAGGCAGCAG-3') / 514R (5'-ATTACCGCGGCTGCTG-3') as follows [13]: 94°C for 4 min; 20 cycles - 94°C for 45 s; 65°C for 45 s; 72°C for 60 s additional 20 cycles - 94°C for 30 sec; 55°C for 30 sec; 72°C for 60 sec; and a final elongation step at 72°C for 10 min. PCR amplification with 341f-GC/534r was performed by using a 50 μl total volume mixture containing 0.2 U Dream*Taq*™ polymerase (Fermentas, USA), 1X PCR Buffer, 200 μM of each dNTP, 0.5 μM of each primer and 0.1% BSA. For the DGGE analysis, a 40mer GC-clamp (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG) was added to the 5' ends of the forward primers 341F.

2.4.2. Bacterial *nifH* gene

PCR amplification of the bacterial *nifH* gene was performed using the primer set PolF (5'-TGCGA[C/T]CC[G/C]AA[A/G]GC[C/G/T]GACTC-3') / PolR (5'-AT[G/C]GCCATCAT[C/T]TC[A/G]CCGGA-3') and the PCR conditions previously described [17]. For T-RFLP analyses (section 2.6) the forward primer PolF was 5'-end FAM-labelled, and the PCR products were purified using the NucleoSpin purification kit as directed by the supplier (GE Healthcare, UK). Purified PCR products (200 ng) were digested with the restriction enzyme *Hae*III at 37°C overnight.

2.5. Denaturing gradient gel electrophoresis (DGGE)

PCR amplicons obtained with the nested primer sets (341F-GC/534R) were analyzed by DGGE. Amplicons were separated on 16.5/16.5 cm, 1 mm 9% (wt/vol) polyacrylamide (37.5:1 acrylamide:bisacrylamide) gels with varying denaturing gradients (100% denaturant was 7 M urea and 40% (vol/vol) formamide). Gels were prepared using a gradient former and were cast according to manufacturer's specifications (BioRad Laboratories, USA). Electrophoresis was performed using the DCode DGGE system (BioRad Laboratories, Inc. USA) and was carried out at 100 V for 16 hrs at 60 °C (1600 Vh) in 1X TAE buffer. Gels were stained in 0.5 µg/ml EtBr in 1X TAE for 20 min and visualized on an AlphaImager 3400 imaging system.

DGGE gel pictures were processed with Gelcompar II 5.0 software (Applied Maths, Belgium). The DGGE banding patterns were compiled and band matching was performed in order to obtain large data matrixes based on the presence/absence of DGGE bands. The complete banding pattern of each profile was considered for comparison. Similarity between fingerprints was calculated with the cosine coefficient and Ward's hierarchical clustering algorithm.

DGGE-bands affected by experimental phenolic additions (i.e. only present and/or intense in the phenolic contaminated bacterial community structure) were excised using sterile surgical blades and eluted in 50 µL of filter-sterilized water at 4 °C overnight. One microlitre of the supernatant was then re-analyzed by PCR and DGGE to eliminate any residual contamination by 'parasite' bands. The remaining PCR products (~25 µL) were purified using the GFX^{TM} PCR DNA and gel band purification kit as directed by the supplier (GE Healthcare, UK). The purified PCR products from DGGE bands were sequenced with a Hitachi 3730xl DNA Analyzer (Applied Biosystems).

2.6. *nifH* T-RFLP

The evolution of the nitrogen-fixing bacterial community structure was assessed by T-RFLP fingerprinting using the *nifH* gene as a marker. The precise length of T-RFs was determined by capillary electrophoresis according to the molecular weight standard GS500 and with an acceptable error of ±1 bp. T-RFLP patterns and quality were analyzed using the freeware PeakScanner™ (version 1.0) (Applied Biosystems, https://products.appliedbiosystems.com). Peak height was used to characterize each unique T-RF, and valid T-RF peaks (between 35 and 360 bp) from triplicate T-RFLP profiles were identified, compiled and aligned to produce large data matrices using the online software T-REX (http://trex.biohpc.org/) [4]. T-RFs with intensities lower than 1% which may have originated from background interferences were excluded from the matrixes.

2.7. Statistical Analysis

All T-RFLP statistical analyses were performed with the software Primer 6 (Primer-E Ltd, UK). To visualize the effect of phenolics on the diazotrophic bacterial community profile, non-metric multidimensional scaling (MDS) plots were created using Bray-Curtis similarity matrices of square root transformed T-RFLP data reflecting relative operational taxonomic unit (OTU) abundance. Two-dimensional MDS plots were used, where the distance between points reflects the degree of similarity between the microbial community profiles in the samples. In these plots, the percentage of similarity determined by cluster analysis is displayed by ellipses. An analysis of similarity (ANOSIM), performed on the resemblance matrix, was used to test for differences in bacterial community structure between predefined groups [3]. The Shannon ($H' = -\Sigma pi/\ln pi$) and eveness ($E=H'/\ln S$) diversity indexes were calculated from the *nifH* gene T-RFLP profiles. *pi* corresponds to the relative abundance of T-RFs '*i*' in the T-RFLP profiles, and S to the richness; i.e. the total number of T-RFs detected.

3. Results and discussion

In BSFs, the sediment matrix (abiotic) and the microbial communities (biotic) are synergistically involved in the bioremediation processes. Indeed, in a sand-column experiment biotic and abiotic mechanisms each accounted for around 50% of phenolic removal [29]. In addition, it has been shown that acclimation/adaptation of the BSF microbial communities to phenolics and ethanol can be induced by increasing the influent strength over time [28, 29]. This paper principally focuses on the impact of the phenolic-rich synthetic wastewater on the BSF bacterial communities, as the experimental parameters associated with wastewater remediation performance have been published previously [29].

3.1. Impact of phenolic-rich wastewater on BSF bacterial community structure

When comparing the effect of pollutants on microbial communities in controlled-environment mesocosms, it is essential that similar communities are established in each system before the start of the experiment. The similarity dendrogams presented in Figure 1 show the clustering of the bacterial communities (91.3% similarity; Figure 1A) derived from the surface and deep (100% and 91.6% at the inlet and outlet respectively; Figure 1B) BSF sediments at Day 0. This confirms that the 3 month equilibration procedure generated highly similar communities in each BSF prior to experimentation (as previously demonstrated [19]), allowing confident comparative analysis of the phenolic WW impact on BSF bacterial communities.

Analysis of the DGGE patterns showed that during the course of the experiment, the bacterial communities in surface samples taken from week 3, 6 (BSFs A/B) and week 9 of the control BSF A grouped into 2 clusters sharing 58.5% similarity (Figure 1A). However, these clusters only shared 31.6% similarity with the bacterial communities present at the end of the experiment (week 9) in BSF B. These results, together with effluent analyses, demonstrate

that the functional bacterial communities in the surface of the BSF B initially adapted to increasing phenolic concentrations (up to 1176.0 mg COD.L^{-1}) while maintaining excellent removal efficiency (Table 1). However, at the highest influent COD concentration (5842.0 mg.L^{-1}; week 7-9) the effluent phenolic concentration was high (Table 1) and there was a build-up of toxic metabolites, especially catechol, in the substratum [29] which elicited large changes in the bacterial community structure. DGGE analyses of the deep sediment bacterial community structures showed three dispersed clusters with no clear impact of the phenolic-laden wastewater during the first 6 weeks of experimentation (Figure 1B), confirming adaptation of the original communities to increasing phenolic concentrations.

A spatial impact of synthetic WW on the deep sediment communities was demonstrated at week 9. At this point, the deep inlet and outlet BSF B communities shared respectively 29.4% and 73.6% similarity with those of the control (BSF A). These results strongly suggest that the selective pressure experienced by the deep outlet sediment communities was significantly lower than that experienced by the surface (inlet/outlet) and deep inlet bacterial communities. It is hypothesized that plug flow of influent may have resulted in sequential degradation of the WW from inlet to outlet, so that the bacterial communities at the deep outlet were exposed to qualitatively and/or quantitatively less toxic chemicals.

Together, these results demonstrated that despite using incremental priming, amendment with high-strength phenolic WW, such phenolic concentrations had a severe impact on the BSF bacterial communities in 3 of the 4 BSF ecological niches studied. Using a similar experimental procedure with ethanol as the sole organic contaminant, BSF bacterial communities demonstrated resistance to influent COD concentrations reaching 15800 mg.L^{-1}, consistently reducing the COD to < 100 mg.L^{-1} [28]. Comparison of these results with those obtained during amendment of the BSFs with phenolic WW clearly confirms that phenolic-

rich wastewater, such as OMWW, is highly toxic and that phenolics show a high level of recalcitrance to biodegradation [8].

3.2 Identification of phenolic-selected bacterial phylotypes

In DGGE fingerprints, each DGGE-band is considered an OTU, and its intensity is proportional to the OTU's relative abundance in the total community investigated. In this study, 9 weeks of exposure to phenolic-rich wastewater substantially modified the BSF microbial community structures, with the disappearance, appearance and/or enhancement of OTUs (Figures 1 and 2). Phenolic-selected DGGE-bands from the week 9 fingerprint of BSF B, i.e. those which intensified or were only present in the community fingerprint of BSF B, were excised for further identification. We successfully re-amplified and purified ten DGGE-bands (indicated by arrows in Figure 2), and phylogenetically identified eight by sequencing (Table 2); five from the surface samples (DGGE bands S1 to S5; Figure 2A) and three from the deep samples (DGGE bands D1 to D3; Figure 2B). Three of these were closely related to bacterial taxa with a known capability to degrade/oxidize organic and/or aromatic compounds (*Pseudomonas fluorescens* [DGGE-band S4]; *Arthrobacter* sp. [DGGE-band S3]; *Geobacter* sp. [DGGE-band D3]) [11, 32]. The selection of bacterial taxa possessing such catabolic capacities in BSF B was not unexpected, as it constitutes a direct community adaptation to the contamination [12, 15]. Four were, however, closely related to well-known nitrogen-fixing microorganisms (*Azotobacter* species / *Beijerinckia indica* [DGGE-bands S1, S5, D1, D2]).

It has previously been reported that diazotrophs are selected in organic-contaminated sediments [2]. Such adaptive traits indicate that bioavailable N is of pivotal importance for the bioremediation of organic-rich effluents and/or for organic-contaminated whole-ecosystems to function effectively. The physiological costs for microbial communities to adapt/acclimate even to slight environmental changes can be great enough to impede ecosystem functioning

[22]. This may translate into a reduction in bioremediatory performance of biological treatment processes, such as BSFs. This is confirmed by recent results showing that, when exposed to organic-rich effluent supplemented with bioavailable nutrients (notably N), (i) BSF bacterial communities maintained their original community structures and (ii) the BSF displayed better COD removal performances than an experimental replicate which was not supplemented by N [20]. Together, this demonstrates that bioavailable N and thus diazotrophic communities are crucial in biological treatment processes.

3.3. Evolution of the diazotrophic diversity in phenolic-contaminated BSF sediments

In order to better understand the dynamics of diazotrophic bacterial communities in BSF B (contaminated with phenolic WW), a T-RFLP approach targeting the phylogenetic marker *nifH* gene was adopted (Figure 3, Table 3). The clustering of the samples in the nMDS plot presented in Figure 3 clearly demonstrates significant differences between the samples representing the week 9 BSF B communities and the remainder of the samples (ANOSIM, Global R = 0.475, P = 0.002). The mean *H'* and *E* diversity indexes were significantly lower ($p < 0.05$, $p < 0.001$ respectively) in BSF B (1.763 ± 0.259; 0.8281 ± 0.067, respectively) than in the control BSF A (2.179 ± 0.528; 0.9772 ± 0.0114, respectively) (Table 3). Together, these results clearly indicate the selection of a specific, less diverse and phenolic-resistant diazotrophic community in BSF B. In microcosm experiments, ecosystem stability (i.e. resistance and resilience) and function have been shown to be indifferent to the declining diversities of the total microbial community and of specific functional groups (denitrifiers and nitrite-oxidizers) respectively [30, 31]. Therefore, it can be hypothesized that the resistant and specific (diazotrophic) BSF bacterial communities selected by phenolics, even at the highest influent concentrations, performed sufficiently for the BSF ecosystem to maintain its essential

functions and processes (e.g. primary production and N-fixation), despite failing remediatory performances.

4. Conclusion

Phenolic-rich wastewaters are highly toxic and therefore represent a treatment challenge in biological systems. In this study, we have demonstrated that applying increasing concentrations of phenolics to a treatment BSF resulted in a bacterial community response, with a notable selection for diazotrophs. The coupling of this response to an efficient phenolic removal suggested that the adapted and phenolic-selected diazotrophic BSF bacterial communities enabled the systems to achieve a desirable nutrient balance via self-regulation and that an adequate level of bioavailable nitrogen is crucial for biological treatment processes. Such a self-regulating system obviates the need for the addition of chemicals to achieve a particular C:N ratio for optimal biodegradation, which saves on material and labour costs and decreases the likelihood of excess N in the treated effluent. Therefore, when designing organic-treatment processes involving microbial communities, the presence of a pre-adapted diazotrophic community is an important factor to consider to minimize the energetic burden involved in the *in vivo* selection of alternative metabolic capacities (such as N-fixing phylotypes), which might in turn enhance the efficiency of catabolic processes focused on the removal of the xenobiotic compounds.

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Legend to figures

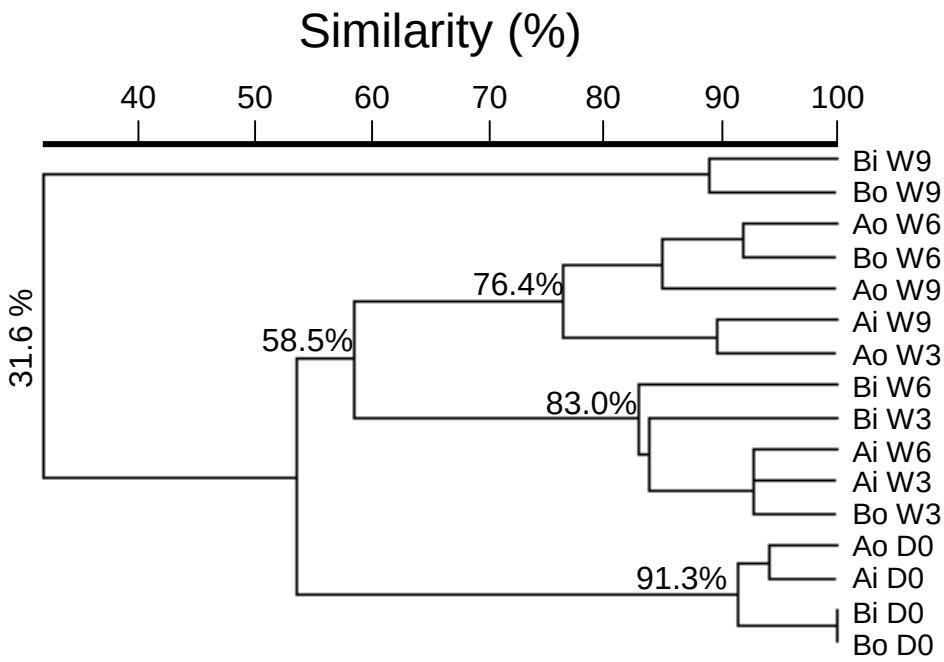
Figure 1 Dendrograms of DGGE pattern similarity of surface (A) and deep (B) sediment bacterial communities of the biological sand filters. A/B refers to the respective BSFs. i/o: inlet/outlet. D0: Day 0 / W3: Week 3 / W6: Week 6 / W9: Week 9.

Figure 2 Week 9 DGGE-gels of the surface (A) and deep (B) sediment bacterial communities of the biological sand filters. A/B refers to the respective BSFs. i/o: inlet/outlet. Arrows and their associate references indicate DGGE-bands excised, sequenced and presented in Table 2; except ND which indicates excised DGGE-bands with sequences not determined.

Figure 3 2D-Nonmetric Multi-Dimensional Scaling plot of Bray Curtis similarity of diazotrophic biological sand filter bacterial community structures determined by T-RFLP analysis of *nifH* genes (2D stress = 0.11). The ellipses around the samples describe their level of similarity (black line 20%; dashed grey 40%; dashed black 60%). △: Control surface; ▲: Phenolic contaminated surface; ○: Control deep; ●: Phenolic contaminated deep. I: inlet / O: outlet. W9: Week 9 / D0: Day 0.

Figure 1

A


Similarity (%)
40
50
60
70
80
90
100
Bi W9
Bo W9
Ao W6
Bo W6
Ao W9
Ai W9
Ao W3
Bi W6
Bi W3
Ai W6
Ai W3
Bo W3
Ao D0
Ai D0
Bi D0
Bo D0
76.4%
58.5%
83.0%
91.3%
31.6 %

B

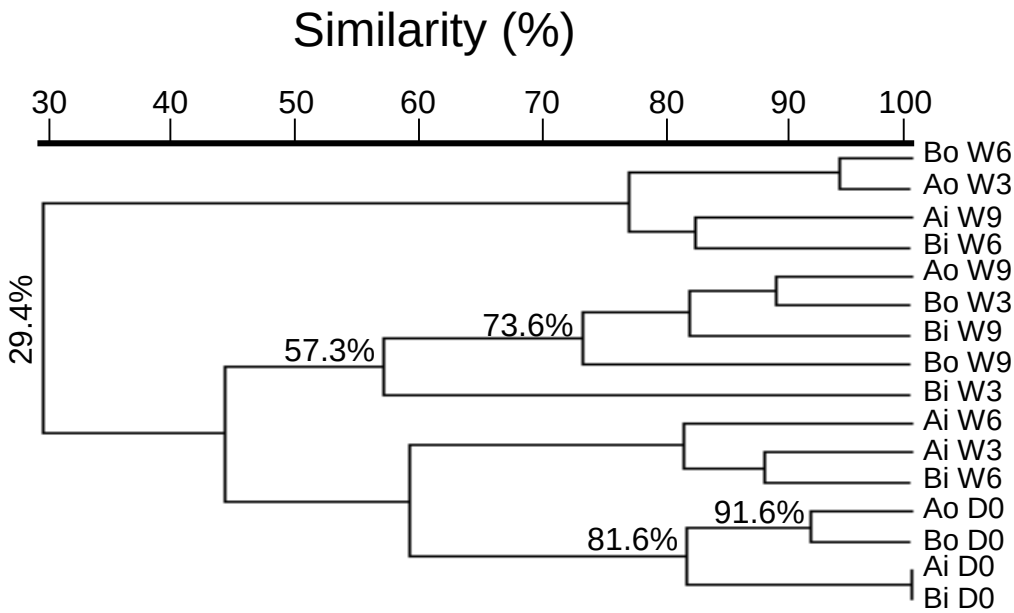
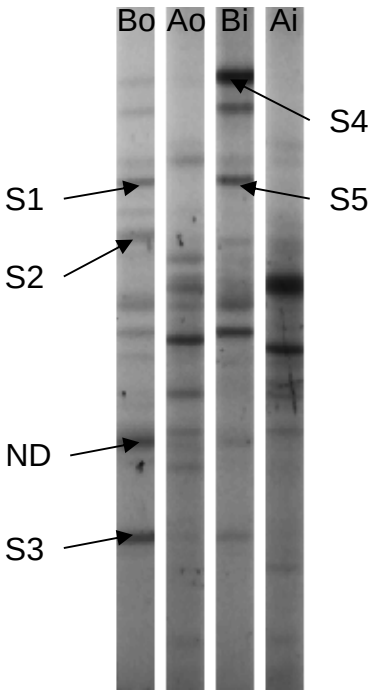

Similarity (%)
30
40
50
60
70
80
90
100
Bo W6
Ao W3
Ai W9
Bi W6
Ao W9
Bo W3
Bi W9
Bo W9
Bi W3
Ai W6
Ai W3
Bi W6
Ao D0
Bo D0
Ai D0
Bi D0
73.6%
57.3%
91.6%
81.6%
29.4%

Figure 2

A



B:

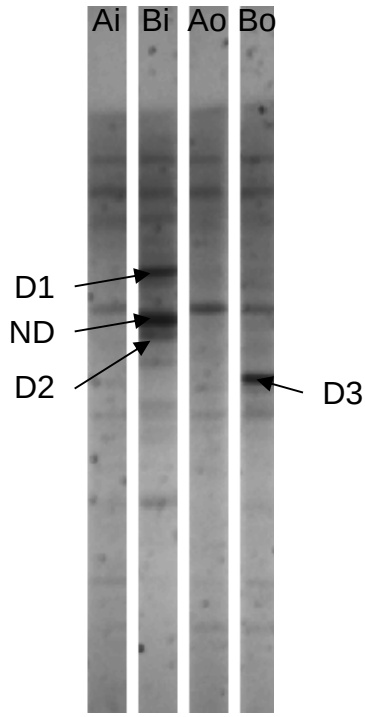


Figure 3

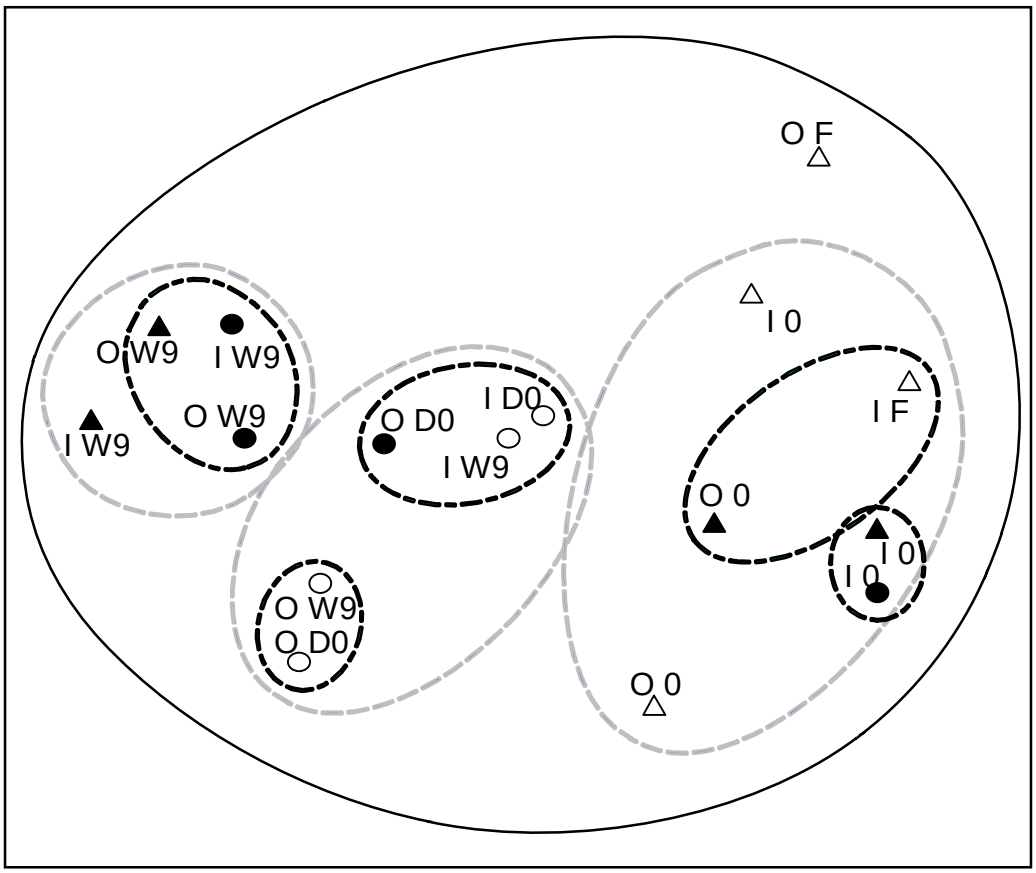


Table 1 Average removal rate and influent and effluent wastewater qualities for the WW-amended CW B during 112 days.

Parameter	Week	Influent	Effluent	Removal* (%)
COD ($mg.L^{-1}$)	0 – 3	234.0	15.7 ± 4.2	93.4
	3 – 6	1176.0	62.0 ± 18.3	95.4
	6 – 9	5842.0	573.6 ± 126.9	91.5
Total phenols (mg $GAE.L^{-1}$)	0 – 3	295.1	1.4 ± 0.5	99.5
	3 – 6	1475.6	16.4 ± 4.0	99.1
	6 – 9	7378.0	393.7 ± 75.8	95.3

*: The concentrations from the control BSF A effluent were taken into account to determine the removal rates of BSF B:

Removal efficiency % = 100 – [((BSF B effluent concentration – BSF A effluent concentration) / BSF B influent concentration) * 100]

Table 2 Sequence similarities of excised DGGE-bands shown in Fig. 2.

DGGE Band [Accession Nb]	BSF niche	Most closely related sequence [Accession number]	% of Identity (nb of base)[a]	Origin
S1 [KC609353]	Surface outlet	*Beijerinckia indica* strain SDSA-I30/2 [GU372346.1] and various *Azotobacter beijerinckii* strains [EF100152.1] [EF100151.1] [NR_042071.1]	100% (152)	Rice field soil or type strains
S2 [KC609354]	Surface outlet	*Acetobacter woodii* DSM 1030 strain [NR_074548.1]	94% (151)	Type strain
S3 [KC609355]	Surface outlet	*Arthrobacter polychromogenes* strain CT276 [HQ455829.1]	98% (151)	Rhizosphere of organic olive grove
S4 [KC609356]	Surface inlet	*Pseudomonas fluorescens* strain X [JQ361765.1]	99% (153)	Greenhouse soil
S5 [KC609357]	Surface inlet	*Beijerinckia indica* strain SDSA-I30/2 [GU372346.1] and various *Azotobacter beijerinckii* strains [EF100152.1] [EF100151.1] [NR_042071.1]	100% (152)	Rice field soil or type strains
D1 [KC609358]	Deep inlet	*Beijerinckia indica* strain SDSA-I30/2 [GU372346.1] and various *Azotobacter beijerinckii* strains [EF100152.1] [EF100151.1] [NR_042071.1]	100% (152)	Rice field soil or type strains
D2 [KC609359]	Deep inlet	*Azotobacter salinestris* strain ATCC 49674 [JX680337.1] and *Azotobacter* sp. Msn2 [JX437936.1]	86% (151)	Tea Rhizosphere / Farm soil
D3 [KC609360]	Deep outlet	Various uncultured Geobacteraceae clones	99% (154)	Soil

[a]: The numbers in parentheses correspond to the number of based used to calculate the levels of sequence identity.

Table 3 Evolution of the *nifH* gene T-RFLP profile diversity indexes in the BSFs A (control) and B (phenolic-spiked) mesocosms.

	Control BSF A (Day 0 / Week 9)			Phenolic-contaminated BSF B (Day 0 / Week 9)		
BSF niche	S	*H'* index	*E* index	S	*H'* index	*E* index
Surface Inlet	18 / 19	2.792 / 2.841	0.9659 / 0.9649	14 / 10	2.558 / 1.948	0.9693 / 0.8461
Surface Outlet	10 / 6	2.230 / 1.738	0.9683 / 0.9701	16 / 7	2.704 / 1.419	0.9753 / 0.7291
Deep Inlet	12 / 11	2.461 / 2.367	0.9905 / 0.9870	18 / 10	2.813 / 1.977	0.9733 / 0.8588
Deep Outlet	6 / 6	1.776 / 1.768	0.9912 / 0.9868	8 / 7	2.044 / 1.709	0.9831 / 0.8785