Effect of surfactants on PAH biodegradation by a bacterial consortium and on the dynamics of the bacterial community during the process

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Published on:

Bioresource Technology 102 (2011), 9438–9446

DOI: 10.1016/j.biortech.2011.07.066

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Abstract

The aim of this work was to evaluate the effect of a non-biodegradable (Tergitol NP-10) and a biodegradable (Tween-80) surfactant on growth, degradation rate and microbial dynamics of a polycyclic aromatic hydrocarbon (PAHs) degrading consortium (C2PL05) from a petroleum polluted soil, applying cultivable and non cultivable techniques. Growth and degradation rate were significantly lower with Tergitol NP-10 than that with Tween-80. Toxicity did not show any significant reduction with Tergitol NP-10 whereas with Tween-80 toxicity was almost depleted (30%) after 40 days. Regarding to the cultured bacteria, *Pseudomonas* and *Stenotrophomonas* groups were dominant during PAH degradation with Tergitol NP-10, whereas *Enterobacter* and *Stenotrophomonas* were dominant with Tween-80. DGGE analyses (PRIMER and MDS) showed that bacteria composition was more similar between treatments when PAHs were consumed than when PAHs concentration was still high. Community changes between treatments were a consequence of *Pseudomonas* sp., *Sphingomonas* sp., *Sphingobium* sp. and *Agromonas* sp.

Keywords: Microbial consortium dynamics, surfactants, polycyclic aromatic hydrocarbons, PAH-degrading consortium, DGGE, cloning.

1. Introduction

Polycyclic aromatic hydrocarbons (PAH) are a group of organic pollutants composed of two or more fused aromatic rings, produced by natural and anthropogenic sources. Besides being toxic, carcinogenic and mutagenic compounds, the semi-volatile properties of some PAH make them highly mobile throughout the environment (air, soil and water). In addition, PAH have a high trophic transfer and biomagnification within the ecosystems due to the lipophilic nature and the low water solubility that decreases with molecular weight (Clements et al., 1994). The importance of preventing PAH contamination and the need to remove PAH from the environment has been recognized institutionally by the Unites States Environmental Protection Agency (US-EPA) which has proposed 16 PAH as priority pollutants including naphthalene, phenanthrene and anthracene. Currently, governmental agencies, scientist and engineers have focused their efforts to identify the best methods to remove, transform or isolate these pollutants through a variety of physical, chemical and biological processes. Most of these techniques involve expensive manipulation of the pollutant transferring the problem from one site or phase to another (e.g. to the atmosphere in the case of cremation) (Haritash and Kausshik, 2009). However, microbial degradation is one of the most important processes that PAH may undergo compared to others such as photolysis and volatilization. Therefore, bioremediation can be an important alternative to transform PAH to less or not hazardous forms with less input of chemicals, energy and time (Haritash and Kaushik, 2009).

Most of the contaminated sites are characterized by the presence of complex mixtures of pollutants. Microorganisms are very sensitive to low concentrations of contaminants and respond rapidly to environment perturbations (Andreoni et al., 2004). Therefore, microbial communities chronically exposed to PAH tend to be dominated by those organisms capable of use PAH as carbon and energy source (Gallego et al., 2007). Even in areas previously unpolluted there is a proportion of microbial community composed by PAH degrading

bacteria able to degrade PAH (Surridge et al., 2009). These microbial communities subjected to a polluted stress tend to be less diverse, depending on the complexity of the composition and the time of exposure (MacNaughton et al., 1999). The biodegradation of hazardous compounds by bacteria, fungi and algae has been widely studied and the success of the process will be due, in part, to the ability of the microbes to degrade all the complex pollutant mixture. However, most of the PAH degradation studies reported in the literature have used versatile single strains or have constructed an artificial microbial consortium showing ability to grow with PAH as only carbon source by mixing together several known strains (Ghazali et al., 2004). Nevertheless, pure cultures and synthetic microbial consortia do not represent the natural behaviour of microbes in the environment, since the cooperation among the new species is altered. In addition, changes in microbial communities during pollutant biotransformation processes are still not deeply studied. Microbial diversity in soil ecosystems can reach values up to 10 billion microorganisms per gram and possibly thousands of different species, although less than 10% can be culturables (Torsvik and Ovreas, 2002). Therefore, additional information on biodiversity, ecology, dynamics and richness of the degrading microbial community can be obtained by non-culturable techniques, such as DGGE. In addition, small bacteria cells are not culturable whereas, large cells are supposed to account 80% of the total bacterial volume (Nannipieri et al., 2003). Therefore, despite their low proportion, culturable bacteria can provide essential information about the structure and functioning of the microbial communities. With the view focused on the final bioremediation, culture-dependent techniques are necessary to obtain microorganisms with the desired catabolic traits for bioaugmentation processes in polluted soils. The PAH degradation is limited by their low aqueous solubility but surfactants which are amphypatic molecules enhance the solubility of hydrophobic compounds (Kolomytseva, et al., 2009). Previous

works (Bautista et al., 2009) have shown that efficiency of a consortium named C2PL05 composed by PAH degrading bacteria was significantly higher using surfactants.

One of the main goals of the current work was to understand if culturable and non culturable techniques are complementary to cover the full richness of a soil microbial consortium. A second purpose of the study was to describe the effect of different surfactants (biodegradable and non-biodegradable) on growth rate, PAH degradation rate and toxicity reduction of a bacterial consortium (C2PL05). The microbial consortium C2PL05 was isolated from a soil chronically exposed to petroleum products, collected from a petrochemical complex. Finally, the work is also aimed to describe the microbial dynamics along the biodegradation process as a function of the surfactant used to increase the bioavailability of the PAH.

2. Material and methods

2.1 Chemicals and media

Naphthalene, phenanthrene, and anthracene (all 99% purity) were purchased from Sigma–Aldrich (Steinheim, Germany) and Fluka (Steinheim, Germany). Reagent grade dichloromethane and n-hexane were supplied by Scharlau Chemie (Barcelona, Spain). Surfactants (Tween-80 and Tergitol NP-10) used were supplied by Sigma-Aldrich (Steinheim, Alemania). Bushnell-Haas Broth medium (BHB) was purchased from Panreac (Barcelona, Spain) and its composition is: 0.2 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.02 $g \cdot l^{-1}$ $CaCl_2 \cdot 2H_2O$, 1.0 $g \cdot l^{-1}$ $KHPO_4$, 1.0 $g \cdot l^{-1}$ K_2HPO_4, 1.0 $g \cdot l^{-1}$ NH_4NO_3, 0.05 $g \cdot l^{-1}$ $FeCl_3$. Luria-Bertani media (LB), glucose and phosphate buffer saline (PBS) were purchased from Panreac (Barcelona, Spain).

2.2 PAH degrader consortium C2PL05

The consortium C2PL05 was obtained from a soil sample in a petrochemical complex in Puertollano, Spain. To obtain the consortium, 1g of sieved soil (<2 mm) was resuspended in 10 ml of PBS and incubated during 12 h in an orbital shaker (Innova 40, New Brunswick Scientific. Edison, NJ, USA) at 150 rpm and 25ºC under dark conditions. After that, 1.5 ml of the supernatant was inoculated in 50 ml of BHB broth (pH 7.0) containing 1%wt Tween-80 as surfactant and naphthalene, phenanthrene and anthracene (each at 250 mg l^{-1}) as carbon source. Then, the culture was incubated at 150 rpm and 25ºC under dark conditions until the exponential phase was completed. This was confirmed by monitoring the cell density by absorbance at 600 nm in a spectrophotometer (Spectronic Genesys, Thermo Fisher Scientific. Loughborough, Leicestershire, UK). Then, the consortium was stored at 4ºC to stop growth. At the beginning of each experiment, 500 µl of the stored consortium (≈ 1.6 AU) was inoculated in Erlenmeyer flasks.

2.3 Experimental design and treatments conditions

To evaluate the influence of Tween-80 and Tergitol NP-10 (biodegradable and non-biodegradable surfactant, respectively) on the degrading capacity of the consortium C2PL05, as well as the evolution of its microbial community, two different treatments, each in triplicate, were carried out. The replicates, were performed in 100 ml Erlenmeyer flasks with 50 ml of BHB medium (pH 7.0), Tween-80 or Tergitol NP-10 as surfactants (1% v/v), a mixture of naphthalene, phenanthrene and anthracene in n-hexane (final concentration 500 $mg \cdot l^{-1}$) and 500 µl of the C2PL05 consortium ($8.8 \cdot 10^8$ heterotrophic $cells \cdot ml^{-1}$ and $4.4 \cdot 10^7$ PAH degrading $cells \cdot ml^{-1}$ for the treatments with Tween-80; $7.3 \cdot 10^5$ heterotrophic $cells \cdot ml^{-1}$ and $2.4 \cdot 10^3$ PAH degrading $cells \cdot ml^{-1}$ for treatment with Tergitol NP-10). The replicates were incubated in an orbital shaker (Innova 40) at 150 rpm and 25ºC under dark conditions during 45 days. Previously to inoculate the consortium, the Erlenmeyer flasks were shaked overnight

to reach the solubility equilibrium of PAH and to allow the complete evaporation of n-hexane. **S**amples, vigorously shaking to ensure homogeneity, were withdrawn twice a day for 45 days except for the initial 24 hours where the sampling frequency was higher. Cell growth, PAH (soluble and precipitated), toxicity and number of heterotrophic and PAH degrading cells were measures in all samples. To study the dynamic of the microbial consortium through cultivable and non-cultivable methods, samples were withdrawn at 0, 15 and 30 days.

2.4 Bacterial growth, MPN and toxicity assays

Bacterial growth was monitored by changes in the absorbance of the culture media at 600 nm using a Spectronic Genesys spectrophotometer. According to the Monod equation (Eq. 1), the specific growth rate μ is essentially equal to μ_{max} when substrate limitation is avoided.

$$\mu = \frac{\mu_{max} \cdot S}{K_S + S} \qquad \text{(eq. 1)}$$

Therefore, from the above optical density data, the maximum specific growth rate (μ_{max}) was estimated as the logarithmized slope of the exponential phase applying the following equation (Eq. 2):

$$\frac{dX}{dt} = \mu \cdot X \qquad \text{(eq. 2),}$$

where; μ_{max}, is the maximum specific growth rate, K_s is the half-saturation constant, S is the substrate concentration, X is the cell density, t is time, and μ is the specific growth rate. In order to evaluate the ability of the consortium to growth with surfactants as only carbon source, two parallel treatments were carried out at the same conditions than the two treatments above described, but in absence of PAH.

Heterotrophic and PAH-degrading population from the consortium C2PL05 were enumerated during the PAH degrading process, comparing the effect of Tergitol NP-10 and

Tween-80 as surfactants. The estimation was performed by using a miniaturized MPN technique in 96-well microtiter plates with eight replicate wells per dilution. Total heterotrophic microbial population was enumerated in 180 µl of Luria Bertani (LB) medium with glucose (15 $g \cdot l^{-1}$) and 20 µl of the microbial consortium. PHAs-degrading population were counted in BHB medium (180 µl including the surfactant), 20 µl of a mixture of phenanthrene, anthracene and naphthalene in hexane (each at a final concentration of 500 $mg \cdot l^{-1}$) and 20 µl of the microbial consortium in each well. The MPN scores were transformed into density estimates, accounting for their corresponding dilution factors.

The toxicity was monitored during PAH degradation and estimations were carried out using the Microtox assay with the bioluminescent bacterium *Vibrio fischeri*. Three controls considered as 0% inhibition were prepared with the photobacterium and 2% NaCl (v/v) and three blanks as 100% inhibition containing only 2% NaCl (v/v). Samples were salted with NaCl (2% v/v final concentration) and the toxicity was expressed as the percentage of the *V. fischeri* inhibition after 15 min of incubation at 15ºC. To study the toxicity of the medium caused by PAH when the surfactants were not added, toxicity evolution was measured from a treatment with PAH as carbon source and degrading consortia but without surfactant under same conditions previously described.

2.5 PAH monitoring

In order to compare the effect of the surfactant on the PAH depletion rate, naphthalene, phenanthrene and anthracene concentrations in the culture media were analysed using a reversed-phase C18 column (Luna C18(2), 7.5 cm length x 4.6 mm I.D., 3 µm particle size. Phenomenex, Torrance, CA, USA), following the method described elsewhere (Bautista et al., 2009). The concentration of each PAH was calculated from a standard curve based on

peak area at 254 nm. The apparent first-order kinetic constant (k_B) due to biotic processes was calculated by applying Eq. 3:

$$-r_i = -\frac{dC_i}{dt} = k_{Ai} \cdot C_i + k_{Bi} \cdot C_i \qquad \text{(eq. 3)}$$

where C is the PAH concentration, k_A is the apparent first-order kinetic constant due to abiotic processes, k_B is the apparent first-order kinetic constant due to biological processes, t is the time elapsed and the subscript i corresponds to each PAH. Degradation caused by abiotic processes was determined by control experiments carried out in triplicate, in 100 ml Erlenmeyers flask with 50 ml of BHB medium (pH 7.0), Tween-80 (1%wt), naphthalene, phenanthrene and anthracene (each at 500 $mg \cdot l^{-1}$) without any microbial inoculum, in an orbital shaker (Innova 40) at 150 rpm and 25ºC under dark conditions. PAH concentration in the control experiments were analyzed using the HPLC system described previously. The values of k_A for each PAH were calculated by applying Eq. 2, considering $k_B \approx 0$, since no bacterial consortium was inoculated.

The amount of precipitated and bioadsorbed PAHs was measured after centrifugation of the samples. Then, dichloromethane was added to the pellet and this extraction was repeated three times and the fractions pooled. The solvent was evaporated using a nitrogen flow and the extract was dissolved into a known volume of acetonitrile for HPLC analysis.

2.6 DNA extraction from cultured bacteria and phylogenetic analysis for characterization of the PAH degrader consortium

Samples from cultures of the bacterial consortium C2PL05 during the PAH degrading process were collected to identify the effect of the surfactants (Tergitol NP-10 and Tween-80). To get about 20-30 colonies isolated at each collecting time, samples of each treatment were streaked onto Petri plates with BHB medium and purified agar and were sprayed with a mixture of naphthalene, phenanthrene and anthracene in n-hexane (final concentration 500

$mg \cdot l^{-1}$) as carbon source. The Petri plates were incubated at 25ºC under dark conditions. The isolated colonies were transferred onto LB agar-glucose plates in order to increase microbial biomass for DNA extraction, and stored in 50% glycerol (v/v) at -80ºC. In total, 91 degrading colonies from the treatment with Tween-80 and 83 degrading colonies from the treatment with Tergitol NP-10 were isolated.

Total DNA was extracted using Microbial DNA isolation kit (MoBio Laboratories. Solano Beach, CA, USA) to perform the molecular identification of the PAH-degrader isolated cultured (DIC). Amplification of the 16S rRNA coding region of the DNA was performed as described by Viñas et al., (2005) using the primers 16F27 (5'-AGAGTTTGATCMTGGCTCAG-3') and 16R1488 (5'-TTACCTTGTTACGACTTCAGG-3') and sequenced using the same primers. Sequences were edited and assembled using ChromasPro software version 1.42 (Technelysium Pty Ltd. Tewantin, Australia).

All of the 16S rRNA gene sequences were edited and assembled by using. BioEdit software version 4.8.7. BLAST search (Madden et al., 1996) was used to find nearly identical sequences for the 16S rRNA sequences determined. Sequences were aligned using the Q-INS-i algorithm (Katoh and Toh, 2008) of the multiple sequence alignment software MAFFT version 6.611 aligning sequences in a single step. Sequence data obtained and 34 sequences downloaded from GenBank were used to perform the phylogenetic trees. Sequence divergence was computed in terms of the number of nucleotide differences per site between of sequences according to the Jukes and Cantor algorithm (1969). The distance matrix for all pairwise sequence combinations was analyzed with the neighbour-joining method (NJ) of phylogenetic tree construction with 1000 bootstrap replicates by using PAUP version 4.0B10. Maximum parsimony (MP) was also analyzed using PAUP 4.0B10 as is described in Molina et al., (2009). Sequences of *Aquifex piruphilus* were used as out-group according to previous phylogenetic affiliations (Viñas et al., 2005).

2.7 Denaturing gradient gel electrophoresis from microbial consortium during PAH degrading process

Non culture dependent molecular techniques, such as denaturing gradient gel electrophoresis (DGGE), were performed to know the effect of the surfactant on the total biodiversity of the microbial consortium C2PL05 during the PAH degradation process and compared with the initial composition of the consortium. The V3 to V5 variable regions of the 16S rRNA gene were amplified using the primers set 16S 518F and 16S 338R-GC, according to ExTaq HS DNA polymerase protocol (Promega Corp. Madison, WI, USA). Primers 338R-GC included a GC clamp at the 5´end (5´-CGC CCG CCGCGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3´). 20 µl of PCR product was loaded onto a 10% (wt/vol) polyacrilamide gels that was 0.75 mm tick with a denaturing gradient of 35-65% (100% denaturant contained 7 M urea and 40% formamide). DGGE was performed in 1xTAE buffer (2.0 M Tris-acetate, 100 mM Na_2EDTA; pH 7.4) using a DGGE 2401 system (CBS Scientific Co. Del Mar, CA, USA) at 80 V and 60ºC for 16 h. Gels were stained for 45 min in 1xTAE buffer containing Syber-Gold (500 $\mu l \cdot l^{-1}$) and viewed under UV light. Predominant bands in DGGE gel were excised with a sterile razor blade and diluted in 50 µl of deionized water overnight at 4ºC. Due to impossibility of reamplified bands, DNA of the bands was cloned in the pGEM-T Easy Vector (Promega, Madison, WI). Sequence of this PAH-degrader uncultured bacterium (DUB) were edited and assembled as described above and included in the matrix to perform the phylogenetic tree as described previously using the identification code DUB.

2.8 Statistical analyses

The maximum specific growth rate (μ_{max}) and the kinetic constant of PAH biodegradation (k_B) were evaluated by both one and two-way analysis of variance (ANOVA) using Statistica 6.0 software (Statsoft Inc. Tulsa, OK, USA) to determine differences between PAH (naphthalene, phenanthrene and anthracene) and surfactants (Tween-80 y Tergitol NP-10). Prior to analyses, Cochran's *C*-test was used to check the assumption of homogeneity of variances. Student-Newman-Keuls test (SNK) was used to discriminate among different treatments after significant F-test. Differences in microbial assemblages were graphically evaluated for each factor combination (surfactant and time) with non-metric multidimensional scaling (MDS) using PRIMER software. SIMPER method was used to identify the percent contribution of each band to the dissimilarity or similarity in microbial assemblages between and within combination of factors. Based on Viejo (2009) bands were considered "highly influential" if they contributed to the first 70% of cumulative percentage of average dissimilarity/similarity between/within combination of factors.

3. Results and discussion

3.1 Bacterial growth and toxicity media during biodegradation of PAHs

Since some surfactants can be used as carbon sources, cell growth of the consortium was measured with surfactant and PAH and only with surfactant without PAH to test the ability of consortium to degrade and grow with both surfactants (Fig. 1A). The microbial consortium C2PL05 growth was significantly lower with Tergitol NP-10 than that reached with Tween-80 which showed the best cell growth with a maximum density (Fig. 1A). In addition, the growth curve with PAH and Tergitol NP-10 showed a longer latent phase (36 hours) than with PAH and Tween-80 (< 12 hours). The specific growth rate (μ_{max}) of the consortium C2PL05 was significantly higher (Table 1A) with Tween-80 than that with Tergitol NP-10. **The results showed that Tween-80 was biodegradable for consortium**

C2PL05, since that surfactant was used as the only carbon source (Fig. 1A). Finally, when using Tergitol NP-10 as the only carbon source, growth was not observed so that this surfactant was not considered biodegradable for the consortium.

Toxicity test (bioluminescence inhibition in *Vibrio fischeri*) indicates that high values observed during the PAH degrading process with Tergitol NP-10 is caused at the initial time by both PAH and surfactant (Fig. 1B). However, when PAH are totally consumed (40-45 days) toxicity still remained high and constant which means that toxicity is only due to the Tergitol NP-10 (Fig. 1B). The toxicity of PAH + biodegradable surfactant (Tween-80) treatment decreased as the PAH and the surfactant were consumed and was almost depleted (30%) after 40 days of cultivation. The toxicity showed a slight increment at the beginning of the degradation process (Fig. 1B) as a consequence of the potential accumulation of intermediate PAH degradation products (Molina et al., 2009).

The residual total concentration of three PAH of the treatments with surfactants and the treatments without any surfactants added, is shown in Fig. 2. The consortium was not able to consume the PAH when surfactants were not added. PAH biodegradation by the consortium C2PL05 was higher and faster (15 days) with Tween-80 than with Tergitol NP-10 (40 days). In all cases, when surfactant was used, no significant amount of PAHs were detected in precipitated or bioadsorbed form at the end of each experiment, which means that all final residual PAHs were soluble.

According to previous works (Bautista et al., 2009; Molina et al., 2009) these results confirm that this consortium is adapted to grow with PAH as only carbon source and can degrade PAH efficiently when surfactant is added. According to control experiments (PAHs without consortium C2PL05) phenathrene and anthracene concentration was not affected by any abiotic process ($k_A \approx 0$ h^{-1}), in the case of naphthalene, some degree of abiotic depletion was measured during the controls, yielding an apparent first-order abiotic rate constant of

$2.7 \cdot 10^{-3} \pm 7 \cdot 10^{-5}$ h^{-1}. This value was accounted for the calculation of the biodegradation rate constant (k_B) for naphthalene in the treatments, so this not influence in the high biodegradation rate of naphthalene for Tween-80 treatments. The biotic depletion rate (k_B) of the three PAH was significantly higher for Tween-80 than that calculated for Tergitol NP-10 (Table 1B). There were no significant differences between PAH for Tergitol NP-10 ($2 \times 10^{-3} \pm 4 \times 10^{-4}$) whereas in the case of Tween-80, the value of k_B for naphthalene ($3 \times 10^{-2} \pm 6 \times 10^{-4}$) was higher than that for phenanthrene and anthracene ($1 \times 10^{-2} \pm 4 \times 10^{-4}$).

3.2 Molecular characterization of the cultured bacteria of the consortium C2PL05 and dynamics during the PAH degradation

The identification of cultured microorganisms and their phylogenetic relationships are key to understand the biodegradation and ecological processes in the microbial consortia. From the consortium C2PL05 grown with Tween-80, 91 strains were isolated and sequenced. From them, 7 different genotypes of PAH-degrading cultures (DIC-1 JA, DIC-2 JA, DIC-5 JA, DIC-6 JA, DIC-7 JA, DIC-8JA and DIC-9JA) were identified by 16S rRNA. For the treatment with Tergitol NP-10, 83 strains were isolated and sequenced and 6 different genotypes were identified (DIC-1 RS, DIC-2 RS, DIC-3 RS, DIC-4 RS, DIC-5 RS and DIC-6 JA). One of the isolated cultures from Tergitol NP-10 showed an identical sequence to one of the strains grown with Tween-80, therefore the previous code (DIC-6JA) was kept. Table 2 show a summary of the PAH-degrader cultures identification. The aligned matrix contained 1576 unambiguous nucleotide position characters with 424 parsimony-informative. Parsimony analysis of the data matrix yielded 10 parsimonious trees with CI = 0.609 and RI = 0.873. In the parsimonic consensus tree, 75.8% of the clades were strongly supported by boostrap values higher or equal to 70% (Fig. 3). All cultivable strains identified were γ-proteobacteria (gram-negative) and were located in three clades, *Pseudomonas* clade,

Enterobacter clade and *Stenotrophomonas* clade. These results are consistent with those of Viñas et al., (2005), who observed a strong dominance of gram negative bacteria in PAH contaminated soil during the bioremediation process. In *Pseudomonas* clade (Fig. 3), DIC are located in three clearly groups. So, DIC-2RS and DIC-3RS were grouped with *P. frederiksbergensis*, which has been previously described in polluted soils (e.g. Holtze et al., 2006) showing ability to reduce the oxidative stress generated during the PAH degrading process. DIC-1JA, DIC-2JA (Tween-80) and DIC-1RS (Tergitol NP-10) were nested in very solid group characterized by the presence of the type strain *P. koreensis,* previously studied as an agricultural soil species (Kwon et al., 2003) and DIC-5RS was located in *P. putida* group, well known by their capacity to degrade high molecular weight PAH (Samantha et al. 2002), to produce surfactants (Kruijt et al., 2009) and to resist high temperature and salinity (Egamberdieva and Kucharova, 2009). So, several species of *Pseudomonas* (e.g. *P. putida*, *P. fluorescens*) have been widely studied in bioremediation (Molina et al., 2009) and **the present results** confirmed that it was the most representative group with the non biodegraded surfactant treatment. DIC-7JA, DIC-8JA and DIC-9JA (Tween-80) which were identified as *E. cloacae* (Table 2) belonged to the *Enterobacter* clade with a strongly statistic support (Fig. 3). In this clade DIC-4RS (Tergitol NP-10) is genetically related with *E. ludwigii* which has been recently described as relevant medical species (Hoffman et al., 2005) but completely unknown his PAH degrading capacity. *Enterobacter* genus has been traditionally studied by its animal gut symbiotic function, but rarely recognized as a soil PAH degrading group (Toledo et al., 2006). In this phylogenetic tree, *E. cloacae* and *E. ludwiggi* were not resolved. This result is according to Roggenkamp, (2007) who consider necessary to use more molecular markers within *Enterobacter* taxonomical group in order to contrast the phylogenetic relationships. In addition, *Enterobacter* genera may not be a monophyletic group (Kampfer et al., 2005). Therefore, more phylogenetic studies need to be done to clarify

the species concept within this group. Finally, DIC-5JA (Tween-80) and DIC-6JA, isolated from experiments using both surfactants, (Tween-80 and Tergitol NP) are clearly belong to type strain *Stenotrophomonas* clade, genetically close to *S. maltophilia*^{T} (Table 2) which has been described as PAH-degrader (Zocca et al., 2004).

With respect to the dynamics of the microorganisms isolated from the microbial consortium C2PL05 (Fig. 4), *Pseudomonas* (DIC-1RS, DIC-2RS and DIC-5RS; Figure 4A, 4B) with a percentage around 60 % and *Stenotrophomonas* (only DIC-6JA; Fig. 4A and 4D) with presence of 90%, were dominant groups during the PAH degrading process with Tergitol NP-10, at 0 and 30 days in the case of *Pseudomonas* and at 15 days in the case of *Stenothrophomonas*. *Enterobacter* (DIC-4RS; Fig. 4A, 4D) only was present at the end of the process with a percentage around 40%. With Tergitol NP-10, *Pseudomonas* sp. group was dominant, coincident with the highest relative contribution of PAHs degrading bacteria to total heterotrophic bacteria at the beginning (33% of contribution) and at the end of the degradation process (41%). However, *Enterobacter* (DIC-7JA, DIC-8JA and DIC-9JA; Fig. 4E and 4H) with a maximum presence of 98% at 0 days and *Stenotrophomonas* (DIC-6JA, Fig. 4E and 4G) with a maximum presence of 85% at the end of the process were dominant with the biodegradable Tween-80. Thus, *Enterobacter* sp. seems to start the PAH degradation process and *Stenotrophomonas* to finish it, but at 15 days, three groups coexist within a contribution ranging 20% to 50% (Fig. 4E). Therefore, in agreement with other authors (Colores et al., 2000), the results of the present work confirm changes in the bacterial (cultured and non-cultured) consortium succession during the PAH degrading process driven by surfactant effects. According to Allen et al., (1999), the diversity of the bacteria cellular walls may explain the different tolerance to grow depending on the surfactant used. Previous works (Piskonen and Itävaara, 2004) have shown the capacity of some bacteria to use both surfactants (Tergitol NP-10 and Tween-80) as carbon sources. However, in agreement with

recent studies (Bautista et al., 2009), the present work confirms that Tergitol NP-10 is not degradable by the consortium C2PL05. These results showed a drastic change of the consortium composition after the addition of surfactant.

3.3 Biodiversity and evolution of the non-cultivable bacteria of the consortium during PAH degradation.

The most influential DGGE bands to similarity 70% of contribution according to the results of PRIMER analyses were cloned and identified, allowing to know the bands and species responsible of similarities and dissimilarities.

SIMPER procedure (Clarke 1993) was used to identify the percentage contribution (%) that each band made to the measures of the Bray-Curtis similarity between treatments at each surfactant (Tween-80 and Tergitol-NP) and time (initial time, after 15 and 30 days). Bands were selected as 'important' to be identified if they contributed to the first 70% of cumulative percentage of average similarity between treatments. Summary of the identification process are shown in Table 2. Phylogenetic relationship of these degrading uncultured bacteria was included in the previous parsymonious tree (Fig. 3). In total, 11 uncultured bacteria were identified. DUB-4RS, DUB-6RS, DUB-8RS and DUB-11RS were located in the *Pseudomonas* clade but these uncultured bacteria were no grouped with a particular species of the genus. DUB-5RS, DUB-7RS were identified as *Sphingobium* sp. and DUB-10RS as *Sphingomonas* sp. and located in the *Sphingobium* and *Sphingomonas* clade, respectivelly. DUB-2RS was nested in *Bradyrhizobium* clade because was identified as *Bradyrhizobium* sp. and this clade was supported by the type strain *B. japonicum.* In the same way, DUB-1RS identified as Uncultured *Nitrobacteria* was located in the *Nitrobacteria* clade, belonged to *N. hamadeniensis* type strain. Finally, DUB-3RS and DUB-9RS were not identified with a particular genus, so they were located in a clade composed by uncultured bacteria. The

phylogenetic relationship of these degrading uncultured bacteria allows expanding knowledge about the consortium composition and process development. Some of them belong to α-proteobacteria: DUB-5RS and DUB-7RS were related to *Sphingobium* group and DUB-10R with *Sphingomonas* clade thought this relationship should be confirmed considering the low boostrap values. *Sphingomonas*, is a genus frequently isolated as PAH degrader (Jing et al., 2007) and important in the degradation of phenanthrene metabolites (Tao et al., 2007). Similarly, *Sphingobium* sp. has been described as PAH degrader, specifically in phenanthrene degradation process (Jing et al., 2007). DUB-2R belonged to *Bradyrhizobiaceae* phylogenetic group and although *Bradyrhizobium* are genera barely described as PAH degrading bacteria, some studies based on PAH degradation by chemical oxidation and biodegradation process have described that this plant-associated bacteria are involved in the degradation of extracting agent used in PAH biodegradation techniques in soils (Rafin et al., 2009). DUB-1RS is a genotype related to *Nitrobacter* clade. **However, *Nitrobacteria* has not been described** as PAH degrader but this bacteria transform nitrites in nitrates from the oxidation of nitrites (Modrogan et al., 2010) and it is likely involved in the nitrites oxidation process when the bioavailability of PAH in the media are low and so, it is not toxic for this bacteria. Finally, DUB-8RS, DUB-6RS and DUB-11RS showed a high similitude with *Pseudomonas* strain, though the phylogenetic relationship with *Pseudomonas* clade of DUB-11RS should be confirmed.

Analysis of DGGE gel (Fig. 5) showed that treatment with Tergitol NP-10 had very few changes during biodegradation process whereas when the consortium was grown with the biodegradable surfactant Tween-80 more changes were observed. Similarity (Table 3) between treatments were compared and analyzed by type of surfactant (Tween-80 *vs* Tergitol NP-10) or by sampling time (15 days *vs* 30 days). The MDS analysis (Table 3), showed the lowest values of Bray Curtis similarity coefficient between the consortium at initial time (T_0;

not exposed to PAHs and surfactants) with the PAH and Tween-80 after 15 days (16) and 30 days (7). However, the similarity of T_0 with PAH and Tergitol NP-10 after 15 days (22) and 30 days (26) was much higher. Similarty between time treatments (15 and 30 days) within Tergitol NP-10 (56) was higher than with Tween-80 (32). The similarity within treatments with Tween-80 was mainly due to the bands 1 and 29 (Table 3; Uncultured *Nitrobacteria* and *Sphingobium* and *Pseudomonas* respectively, see Table 2), whereas the similarity within Tergitol NP-10 treatment was due to bands 1 and 30 (Table 3; Uncultured *Nitrobacteria* and Uncultured bacteria respectively, see Table 2).

The genera identified in this work have been previously described as capable to degrade the three PAH completely and efficiently with a reduction of the toxicity (Bautista et al., 2009). In the case of the treatment with Tween-80, the lower biodiversity may be caused by a few dominant species of these genera driven during the PAH degradation process by antagonist and synergic bacterial interactions and not by differences in the functional capacities. However, when consortium grows with a non-biodegradable surfactant, there is higher biodiversity of species and interaction because the activity of various functional groups can be required to deal the unfavorable environmental conditions.

4. Conclusions

The choice of surfactants to increase bioavailability of pollutants is critical for *in situ* bioremediation because toxicity can persist when surfactants are not biodegraded. Nevertheless, surfactants affect the dynamics of microbial populations in a stable PAH-degrading consortium. From the application point of view, the combination of culturable and non culturable identification techniques may let to optimize the bioremediation process. For bioaugmentation processes, culturable tools help to select the more appropriate bacteria, allowing growing enough biomass before adding to the environment. However, for

biostimulation process it is important to know the complete consortium composition to enhance their natural activities.

Acknowledgment

Authors are deeply indebted to Raquel Sanz, Laura Sánchez and Laura García and for their support during the development of the experiments. Authors also gratefully acknowledged the financial support from the Spanish Ministry of Environment (Research project 13/2006/2-1.1) and Fundación Alfonso Martín Escudero. **Authors** also thank Repsol-YPF for providing the soil samples. This work is framed within the Official Máster en Ciencia y Tecnología Ambiental from Universidad Rey Juan Carlos**.**

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Tables

Table 1. Analysis of variance (ANOVA) for the effects of surfactants on the specific growth rate, μ_{max}, (A) and for the effects of the surfactants and PAH on the biotic degradation rate, k_B, (B) of the C2PL05 consortium.

Effect (A)	Sum of squares	d.f.	F-value	p-value
Surfactant	16	1	782	0.001
Error	0.021	2		
Effect (B)	**Sum of squares**	**d.f.**	**F-value**	**p-value**
PAH	$1.5 \cdot 10^{-4}$	2	779	0.001
Surfactant	$8.2 \cdot 10^{-4}$	1	4042	0.001
PAH x Surfactant	$1.2 \cdot 10^{-4}$	2	624	0.001
Error	$2.03 \cdot 10^{-7}$	12		

Table 2. Bacteria identification and percentage of similarity from the GenBank data base. Degrading uncultured bacteria (DUB) form DGGE bands and degrading isolates cultured (DIC).

Colonies identified by cultivable techniques			
DIC	% similarity	Mayor relationship with bacteria of GenBank (acc. No)	Phylogenetic group
DIC-1RS[b]	98.0	*Pseudomonas koreensis* (NR025228)	*Pseudomonadaceae* (γ)
DIC-2RS [b]	100.0	*Pseudomonas frederiksbergensis* AY785733)	*Pseudomonadaceae* (γ)
DIC-3RS [b]	100.0	*Pseudomonas frederiksbergensis* (AY785733)	*Pseudomonadaceae* (γ)
DIC-4RS [b]	99.0 99.0	*Enterobacter ludwigii* (AJ853891) *Enterobacter cloacae* (EU733519)	*Enterobacteriaceae* (γ)
DIC-5RS [b]	99.0	*Pseudomonas putida* (EU046322)	*Pseudomonadaceae* (γ)
DIC-6JA/DIC-6RS [c]	100.0	*Stenotrophomonas maltophilia* (AY512625)	*Xanthomonadaceae* (γ)
DIC-1JA [a]	99.00	*Pseudomonas koreensis* (NR025228)	*Pseudomonadaceae* (γ)
DIC-2JA [a]	99.00	*Pseudomonas koreensis* (NR025228)	*Pseudomonadaceae* (γ)
DIC-5JA [a]	99.64	*Stenotrophomonas maltophilia* (AY512625)	*Xanthomonadaceae* (γ)
DIC-7JA [a]	99.85	*Enterobacter cloacae* (AF157695)	*Enterobacteriaceae* (γ)
DIC-8JA [a]	99.93	*Enterobacter cloacae* (AF157695)	*Enterobacteriaceae* (γ)
DIC-9JA [a]	100	*Enterobacter cloacae* (AF157695)	*Enterobacteriaceae* (γ)

Identification by non-cultivable techniques				
DUB	Band	% similarity	Mayor relationship with bacteria of GenBank (acc. No)	Phylogenetic group
DUB-1RS [a]	1	97.0	Uncultured *Nitrobacteria* sp. (AM990004)	*Bradyrhizobiaceae* (α)
DUB-2RS [b]	21	98.0	*Bradyrhizobium* sp (HQ171485)	*Bradyrhizobiaceae* (α)
DUB-3RS [b]	24	94.0	Uncultured bacterium (AY939443)	--
DUB-4RS [b]	24	100	Uncultured *Pseudomonas* sp. (HM561497)	*Pseudomonadaceae* (γ)
DUB-10RS[b]	28	98.0	Uncultured *Sphingomonas* sp. (HM438638)	*Sphingomonadaceae*(α)
DUB-11RS[a]	28	96.0	*Pseudomonas* sp (HM468085)	*Pseudomonadaceae* (γ)
DUB-6RS [b]	29	98.0	*Pseudomonas stutzeri* (HQ130335)	*Pseudomonadaceae* (γ)
DUB-8RS [b]	29	98.0	*Pseudomonas* sp (HM468085)	*Pseudomonadaceae* (γ)
DUB-5RS [b]	29	99.0	*Sphingobium* sp. (EF534725)	*Sphingomonadaceae*(α)
DUB-7RS [b]	29	98.0	*Sphingobium* sp. (EF534725)	*Sphingomonadaceae*(α)
DUB-9RS [b]	30	97.0	*Bacterium* (AJ295668)	--

[a]: DIC or DUB belonging to treatments with Tween-80
[b]: DIC or DUB belonging to treatments with Tergitol NP-10
[c]: DIC or DUB belonging to treatments with Tween-80 and Tergitol NP-10

Table 3. Bands contributing to approximately the first 70% of cumulative percentage of average similarity (%). Bands were grouped by surfactant and time.

Band	Tween-80	Tergitol-NP-10	15 days	30 days
1	38.28	21.05	27.07	30.14
29	29.69			15.09
30		24.69		19
24		8.81	34.47	
27*		8.45		
21		5.16		
Cumulative similarity (%)	31.68	44.79	44.79	33.91

* Cloning was not possible

Figure Captions

Fig. 1. (A) Cell density of the consortium C2PL05 with PAH and Tween-80 (●), with Tween-80 (○), with PAH and Tergitol NP-10(■) and with Tergitol NP-10 (□). (B) Toxicity of the cultivation media during degradation of PAH by the consortium C2PL05 grown with Tween-80 (●), Tergitol NP-10 (■) and of the control experiment PAHs without surfactants (▲).

Fig. 2. Residual total PAH concentration (%wt) in the treatment with Tween-80 (■), Tergitol NP-10 (●) and without surfactant (△).

Fig. 3. Neighbour joining tree showing the phylogenetic relationship of the 16S rRNA for the PAH-degrader isolated culture (DIC) from the consortium C2PL05 with Tergitol NP (DIC-1JA – DIC-9JA) and Tween-80 (DIC-1RS – DIC-5RS) and PAH-degrader uncultured bacteria (DUB) obtained from DGGE of the consortium with both surfactant at 0, 15 and 30 days of the process. Boostrap values of neighbourjoining and parsimonus)higher than 50% are showed on the branch of the tree (NJ/MP). No incongruence between parsimony and neighbour joining topology were detected. *Pseudomonas* genus has been designated as *P.; Pantoea* genus as *Pa*. *Sphingobium* as *S.* and *Sphingomonas* as *Sp*. *Xantomona*s as *X*. and *Xyxella* as *Xy*. ^{T}= type strain.

Fig. 4. (A) Dynamics of the microbial consortium C2PL05 during PAH degradation process with Tergitol NP-10 and (E) with Tween-80 as surfactants. Isolated and identified genus were *Pseudomonas* (■), *Stenotrophomonas* (●) and *Enterobacter* (▲). Dynamics and succession of the (B) *Pseudomonas*, (C) *Stenotrophomonas* and (D) *Enterobacter* ribotypes with Tergitol NP-10 as surfactant. Dynamics and succession of the (F) *Pseudomonas*, (G) *Stenotrophomonas* and (H) *Enterobacter* ribotypes.

Fig. 5. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA gen fragments from the consortium C2PL05 with initial inoculum (lane 1), with Tween-80 at 15 (lane 2) and 30 (lane 4) days, with Tergitol NP-10 at 15 (lane 3) and 30 (lane 5) days, and molecular weight markers (lane 0). According to PRIMER analysis, similar bands between treatments (15 and 30 days) with Tergitol NP-10 (○) and between treatments (15 and 30 days) with Tween-80 (△) are shown.

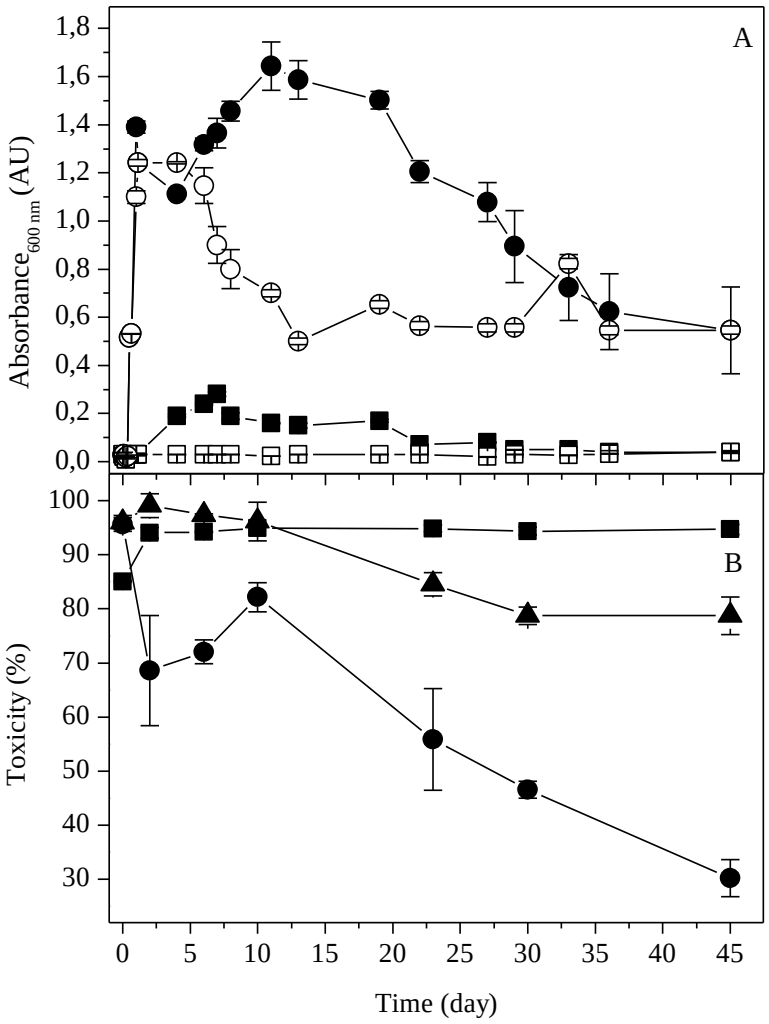
A
B
Absorbance600 nm (AU)
Toxicity (%)
Time (day)
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
100
90
80
70
60
50
40
30
0
5
10
15
20
25
30
35
40
45

Figure 1

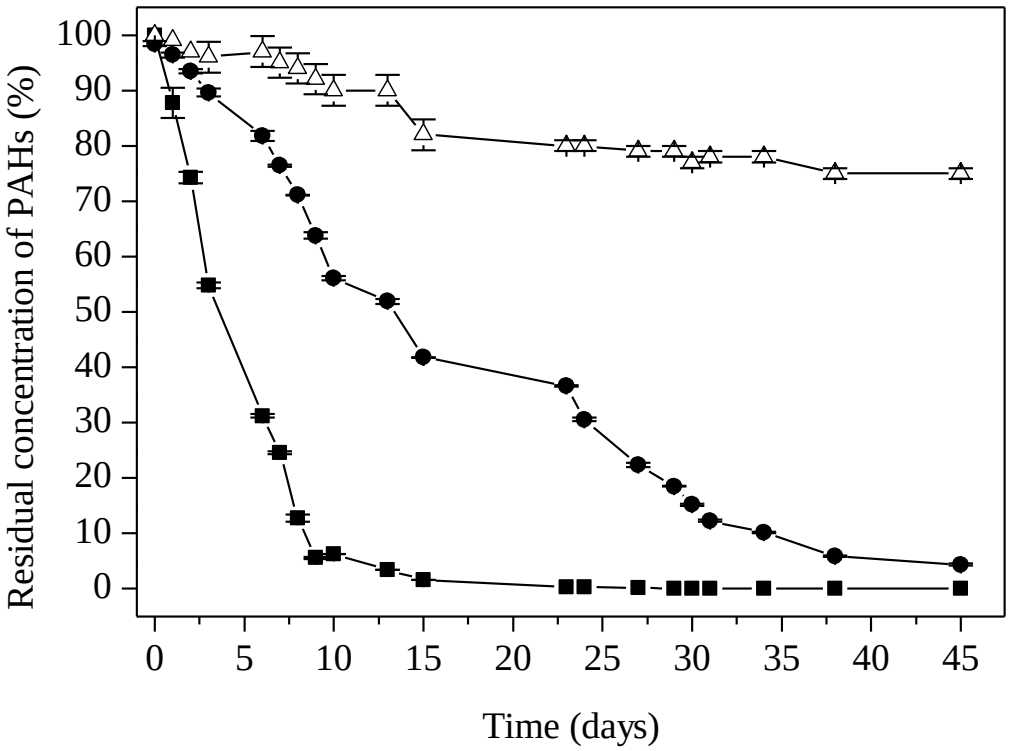
Residual concentration of PAHs (%)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
40
45
Time (days)

Figure 2

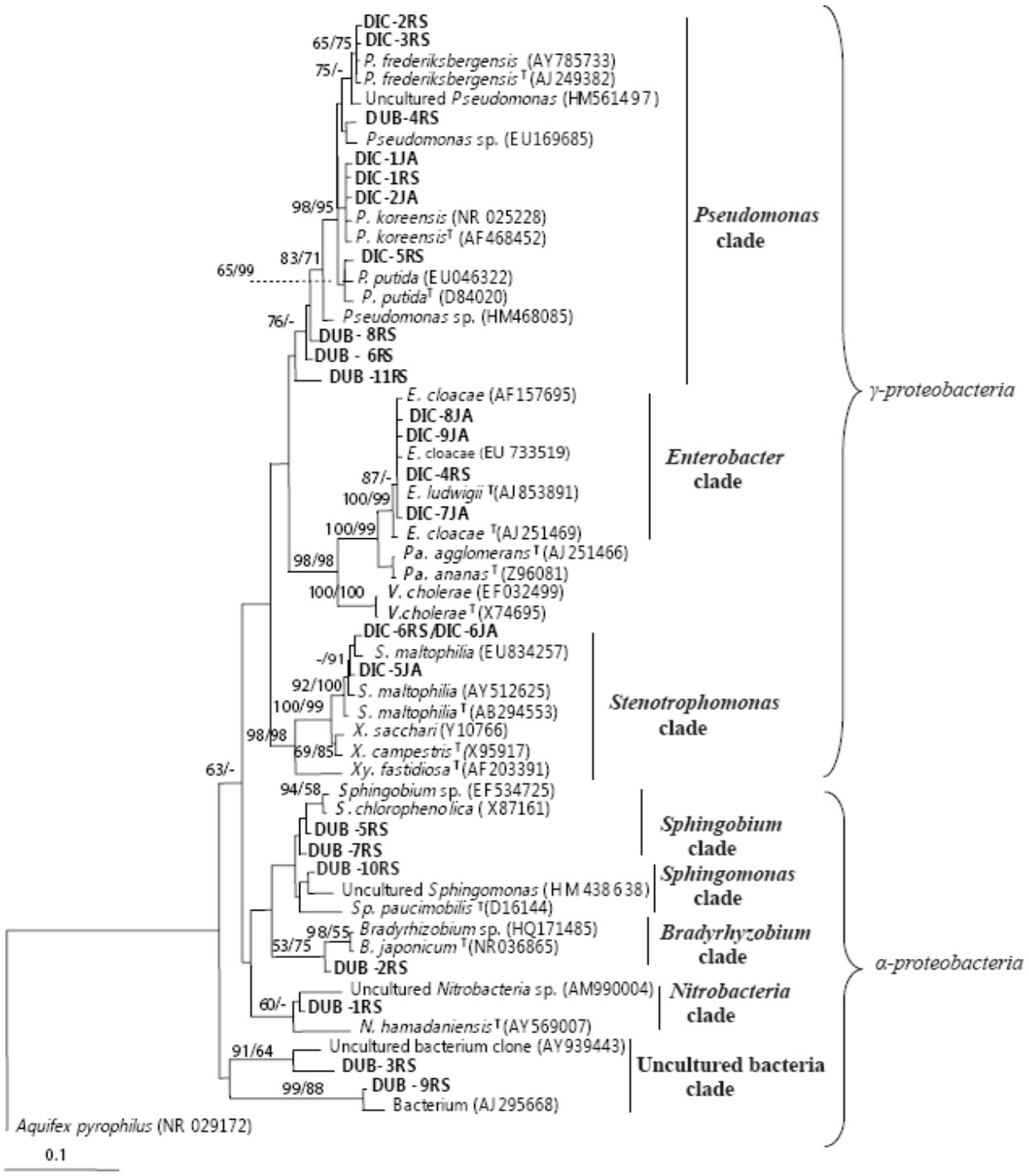
DIC-2RS
DIC-3RS
P. frederiksbergensis (AY785733)
P. frederiksbergensis T (AJ249382)
Uncultured Pseudomonas (HM561497)
DUB-4RS
Pseudomonas sp. (EU169685)
DIC-1JA
DIC-1RS
DIC-2JA
P. koreensis (NR 025228)
P. koreensis T (AF468452)
DIC-5RS
P. putida (EU046322)
P. putida T (D84020)
Pseudomonas sp. (HM468085)
DUB- 8RS
DUB - 6RS
DUB -11RS
Pseudomonas clade
E. cloacae (AF157695)
DIC-8JA
DIC-9JA
E. cloacae (EU 733519)
DIC-4RS
E. ludwigii T (AJ853891)
DIC-7JA
E. cloacae T (AJ251469)
Pa. agglomerans T (AJ251466)
Pa. ananas T (Z96081)
V. cholerae (EF032499)
V.cholerae T (X74695)
Enterobacter clade
DIC-6RS/DIC-6JA
S. maltophilia (EU834257)
DIC-5JA
S. maltophilia (AY512625)
S. maltophilia T (AB294553)
X. sacchari (Y10766)
X. campestris T (X95917)
Xy. fastidiosa T (AF203391)
Stenotrophomonas clade
γ-proteobacteria
Sphingobium sp. (EF534725)
S. chlorophenolica (X87161)
DUB -5RS
DUB -7RS
Sphingobium clade
DUB -10RS
Uncultured Sphingomonas (HM438638)
Sp. paucimobilis T (D16144)
Sphingomonas clade
Bradyrhizobium sp. (HQ171485)
B. japonicum T (NR036865)
DUB -2RS
Bradyrhyzobium clade
Uncultured Nitrobacteria sp. (AM990004)
DUB -1RS
N. hamadaniensis T (AY569007)
Nitrobacteria clade
Uncultured bacterium clone (AY939443)
DUB- 3RS
DUB - 9RS
Bacterium (AJ295668)
Uncultured bacteria clade
α-proteobacteria
Aquifex pyrophilus (NR 029172)
0.1

Figure 3

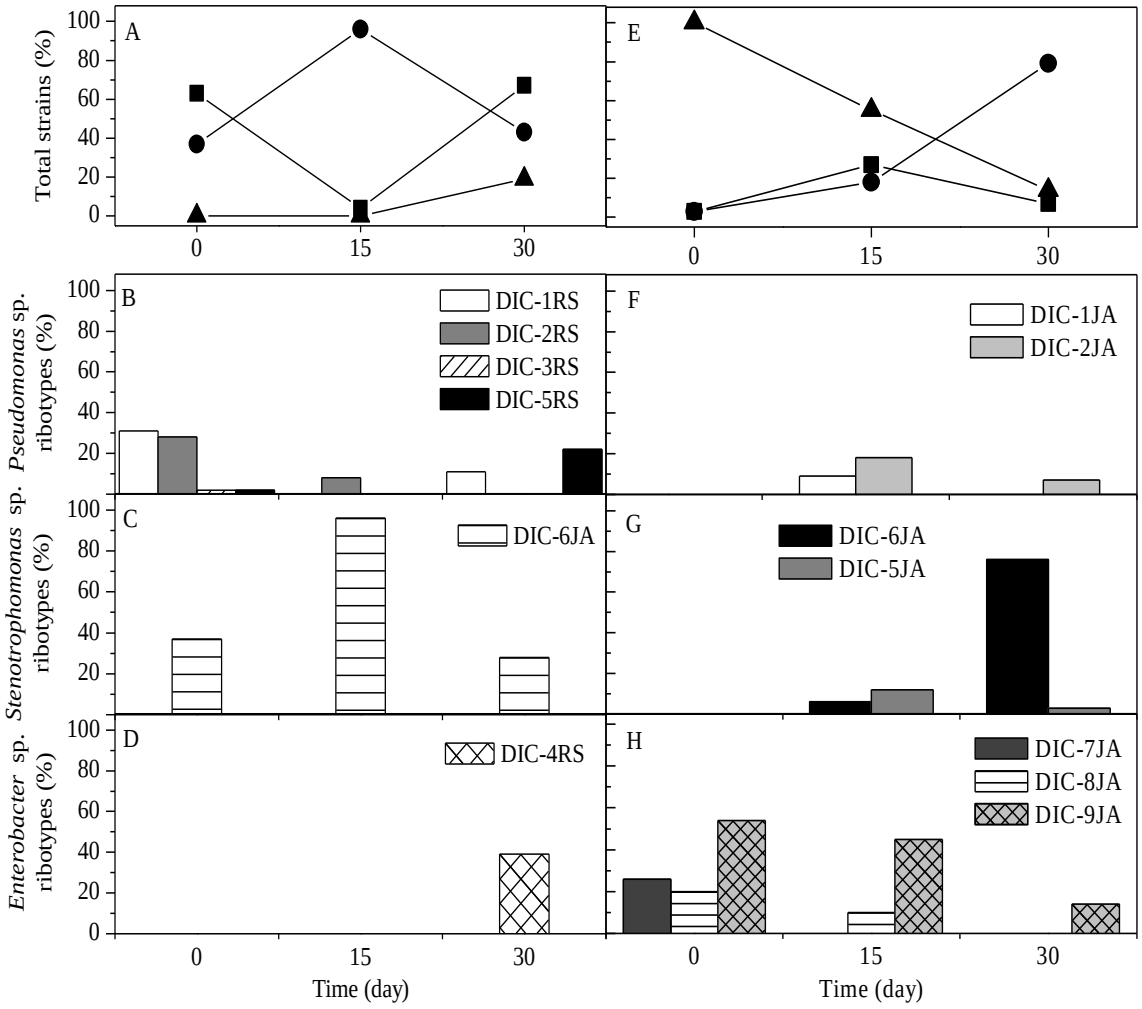
A
E
B
F
C
G
D
H
Total strains (%)
Pseudomonas sp. ribotypes (%)
Stenotrophomonas sp. ribotypes (%)
Enterobacter sp. ribotypes (%)
DIC-1RS
DIC-2RS
DIC-3RS
DIC-5RS
DIC-1JA
DIC-2JA
DIC-6JA
DIC-5JA
DIC-4RS
DIC-7JA
DIC-8JA
DIC-9JA
Time (day)
Time (day)

Figure 4

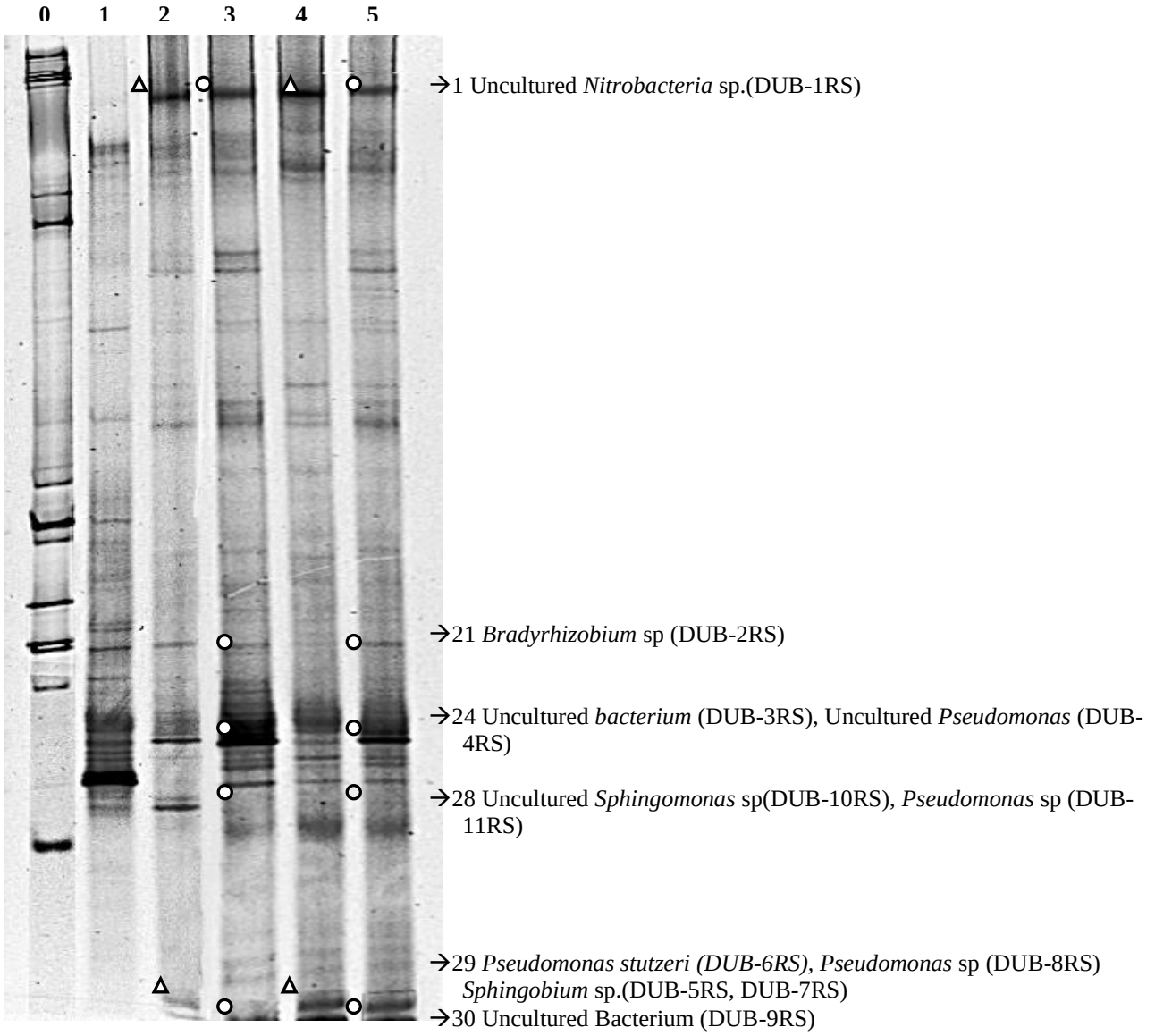
0
1
2
3
4
5
→1 Uncultured *Nitrobacteria* sp.(DUB-1RS)
→21 *Bradyrhizobium* sp (DUB-2RS)
→24 Uncultured *bacterium* (DUB-3RS), Uncultured *Pseudomonas* (DUB-4RS)
→28 Uncultured *Sphingomonas* sp(DUB-10RS), *Pseudomonas* sp (DUB-11RS)
→29 *Pseudomonas stutzeri (DUB-6RS)*, *Pseudomonas* sp (DUB-8RS) *Sphingobium* sp.(DUB-5RS, DUB-7RS)
→30 Uncultured Bacterium (DUB-9RS)

Figure 5