Effect of different non-ionic surfactants on the biodegradation of PAHs by diverse aerobic bacteria

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Relevance of the manuscript

This research work is relevant because it presents the remarkable positive effect of several non-ionic surfactants on the biodegradation of polycyclic aromatic hydrocarbons by different bacteria isolated from a polluted industrial soil. The work demonstrates that the rate of biodegradation can be significantly increased in presence of non-ionic surfactant although its choice must be account for the type of degrading microorganism used.

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Abstract

The aim of this work was to evaluate the effect of several non-ionic surfactants (Tween-80, Triton X-100 and Tergitol NP-10) on the ability of different bacteria (*Enterobacter* sp., *Pseudomonas* sp. and *Stenotrophomonas* sp.) to degrade polycyclic aromatic hydrocarbons (PAHs). Bacterial cultures were performed at 25ºC in an orbital shaker under dark conditions in BHB medium containing 1% of surfactant and 500 $mg \cdot l^{-1}$ of each PAH. Experiments performed with Tween-80 showed the highest cell density values and maximum specific growth rate because this surfactant was used as a carbon source by all bacteria. High degree of PAHs degradation (>90%) was reached in 15 days in all experiments. Toxicity increased at early times using Tween-80 but decreased to low levels in a short time after the firsts 24 h. On the other hand, Triton X-100 and Tergitol NP-10 were not biodegraded and toxicity kept constant along time. However, PAHs degradation rate was higher, especially by the action of *Enterobacter* sp. with Tween-80 or Triton X-100, depending on the microorganism used. Control experiments performed without surfactant showed a significant decrease in biomass growth rate with a subsequent loss of biodegradation activity likely due to a reduced solubility and bioavailability of PAHs in absence of surfactant.

Keywords: Polycyclic aromatic hydrocarbons, surfactants, biodegradation, aerobic bacteria.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are chemical organic compounds that are composed of two or more fused aromatic rings. PAHs are generally formed by incomplete combustion of fossil fuels and other organic material. They are known to be carcinogenic to humans and other organisms (Kim et al., 2001). PAHs should be undetectable in soil and groundwater under the drinking water standards and health advisory regulations of the US Environmental Protection Agency (USEPA), where 16 PAHs have been defined as priority pollutants and form a subset of “toxic pollutants” under the Clean Water Act including naphthalene, phenanthrene and anthracene (USEPA, 1972). Naphthalene is also considered a hazardous air pollutant (USEPA, 1994; USEPA 2004). These administrative regulations have encouraged scientists and engineers to identify the best remediation technologies from a variety of physical, chemical and biological methods that can be used to remove these compounds from polluted sites. Most low molecular weight PAHs are biodegradable in presence of a suitable microbial population. However, the efficiency of PAHs biodegradation is limited because these compounds have a very low aqueous solubility and vapour pressure (Luning Prak and Pritchard, 2002).

Surfactants may be useful for the bioremediation of sites polluted with PAHs since they enhance the solubility of hydrophobic compounds (Boonchan et al., 1998). Many studies have been conducted to enhance the biodegradation of PAHs using surfactants to increase their solubility by decreasing the interfacial surface tension between PAHs and the soil/water interphase. When surfactant concentration is above the critical micelle concentration (CMC), micelle aggregates provide an additional hydrophobic area in the central region of micelles enhancing the aqueous solubility of PAHs (Li and Chen, 2008). The addition of non-ionic surfactants as additives has a positive effect on PAHs biodegradation (Grimberg et al., 1996; Volkering et al., 1995). However, some negligible (Ghosh et al., 1995) or even negative effects (Laha and Luthy 1991) have been reported. Possible reasons for this, including bacteria-surfactant interactions, could be the competitive substrate utilization and the toxicity of the surfactants against the PAHs-degrading bacteria (Liu et al., 2001). So, the application

of surfactants to improve PAHs degradation may need to be optimized for any particular system for the variety of factors influencing biodegradation, including surfactants type and concentration, PAH specificity and the microorganisms present in the cultivation (Jin et al., 2007).
The main goal of the present study was to study the effect of the presence of non-ionic surfactants (Tween-80, Triton X-100 and Tergitol NP-10) on the biodegradation of low molecular weight PAHs (naphthalene, phenanthrene and anthracene) in aqueous media with diverse aerobic bacteria (*Pseudomonas* sp., *Enterobacter* sp. and *Stenotrophomona*s sp.) isolated from a microbial consortium.

2. Material and metods

2.1. Chemicals and Media

Naphthalene, phenanthrene and anthracene (all >99% purity) were employed in the degradation experiments. They were purchased from Sigma-Aldrich (Steinheim, Germany) and Fluka (Steinheim, Germany). Reagent grade dichloromethane, used such as a solvent, was supplied by Scharlau Chemie (Barcelona, Spain). Bushnell Haas Broth medium (BHB) was purchased from Panreac (Barcelona, Spain). All surfactants (Tween-80, Triton X-100 and Tergitol NP-10 were purchased from Sigma-Aldrich (Steinheim, Germany). Relevant properties of these surfactants are shown in Table 1. All chemicals used for toxicity assays were at least reagent grade.

2.2. Microorganisms

Pseudomonas sp. *Enterobacter* sp. and *Stenotrophomonas* sp. were isolated and identified from a microbial consortium obtained from a soil chronically exposed to petroleum products from a petrochemical complex in Puertollano, Spain.

2.3. Biodegradation of PAHs

Biodegradation experiments were performed in 3 replicates. 1.5 ml of the culture was inoculated in 150 ml BHB medium containing 1% w/w of the corresponding surfactant. The resulting initial molar concentration for each surfactant was 15.9 mM for Tergitol NP-10, 17.1 mM for Triton X-100 and 8.1 mM for Tween-80. Each PAH was added to a concentration of 500 $mg \cdot l^{-1}$. Erlenmeyer flasks were incubated in a refrigerated orbital shaker (Innova 40R. New Brunswick Scientific, Edison, NJ USA) at 200 rpm and 25ºC under dark conditions during 30 days. Samples were withdrawn at different times for PAHs concentration depletion and cell density monitoring. Control experiments without the addition of any surfactant were conducted for all cultures. In addition, an abiotic control experiment, in sterile conditions, without bacterial inoculation was performed in order to evaluate the contribution to the overall degradation of each PAH due to purely physicochemical processes.
Cell density was monitored by changes in the absorbance at 600 nm using a Cary-500 spectrophotometer (Varian, Palo Alto, CA, USA).
The depletion rate ($-r_i$) of each PAH (i) during the stationary phase of microbial growth was fitted to a first order kinetic model (equation 1) with satisfactory results.

$$-r_i = -\frac{dC_i}{dt} = k_{A,i} \cdot C_i + k_{B,i} \cdot C_i \qquad \text{(eq. 1)},$$

where C is the concentration of PAHs measured by HPLC analysis, 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. In order to evaluate the effect of the surfactant on the biodegradation of each PAH by different microorganisms, a two-way analysis of variance (ANOVA) with two factors, type of microorganism (four levels: *Pseudomonas*, *Enterobacter*, *Stenotrophomonas* and without inoculation) and type of surfactant (four levels: Tween-80, Triton X-100, Tergitol NP-10 and absence of surfactant), was performed for each PAH using the k_B values. In addition, a second analysis of variance with two factors, type of PAH (three levels: naphthalene, phenanthrene and anthracene) and type of microorganism (four levels: *Pseudomonas*, *Enterobacter*, *Stenotrophomonas* and all three together) was carried with the kB values from those where Tween-80 was used as surfactant. The analyses were performed using the software Statistica 6.0 (StatSoft, Inc. Tulsa, OK, USA). The variances were checked for homogeneity by the Cochran's test and Student Newman Keuls (SNK) test was used to discriminate among different treatments after significant F-test.

2.4. HPLC Analysis

The simultaneous analysis of naphthalene, phenanthrene and anthracene in the culture media were performed in a reversed-phase C18 column (Luna C18(2), 7.5 cm x 4.6 mm I.D., 3 μm particle size. Phenomenex, Torrance, CA, USA) with its corresponding guard column. The HPLC system was a ProStar 230 (Varian. Palo Alto, CA, USA) equipped with a quaternary pump and photodiode array UV/Vis and fluorescence detectors. 10 μl of filtered cultivation media were injected and then eluted from the column at a flowrate of 0.8 ml/min using an acetonitrile:water gradient program as follows: isocratic 60:40 (0-2 min), gradient to 75:25 (2-14 min), isocratic 75:25 (14-15 min), gradient to 100:0 (15-16 min). The content of each PAH were calculated by an external standard technique from a standard curve of peak area vs. concentration using a wavelength of 254 nm.

2.5. Toxicity assays

The toxicity monitoring during PAHs degradation experiments was carried out following the method proposed by Greene et al. (1988) using the Microtox^{TM} test with the bioluminescent bacterium *Vibrio fischeri*, following the protocol developed by Microbics Corporation (1992). Toxicity was expressed as the percentage of the *V. fischeri* inhibition after 15 minutes of incubation with the assayed sample at 15ºC.

2.6. Total organic carbon and pH

Total organic carbon (TOC) analyses during biodegradation experiments were performed using a TOC-VCSN analyzer (Shimadzu, Japan). Samples were injected into the oven at 680ºC. The combustion was catalyzed by Pt/Al_2O_3 catalyst. The CO_2 produced was measured by NDIR method.
Monitoring of pH during PAHs-biodegradation experiments was performed by a GPL21 pH-meter (Crison. Barcelona, Spain).

3. Results

3.1. Effects of different surfactants on the bacterial growth

The growth curves of the different bacteria during the PAHs biodegradation experiments (Figure 1) show the expected latent, exponential and stationary phases. Depending on the

surfactant used, the length of these phases was different. The end of the exponential phase was reached approximately after 48 hours in experiments performed in presence of Tween-80 while 150 hours were necessary for Triton X-100 and Tergitol NP-10. The stationary phase was usually completed after 15 days of incubation in all experiments. According to previous experiments, the three strains used in the present work were able to use Tween-80 as their sole carbon source. On the other hand, growth was not detected in presence of Triton X-100 and Tergitol NP-10 under the same conditions (data not shown).
Biodegradation experiments carried out with PAHs showed that the growth rates were greater in media using Tween-80 as the surfactant, while the lower growth was achieved in those cultures performed without surfactant. In addition, the highest growth (Figure 1) and maximum specific growth rate (Figure 2) was observed when a consortium of the three previously isolated bacteria (*Pseudomonas* sp., *Enterobacter* sp. and *Stenotrophomonas* sp.) was employed.

3.2. Biodegradation of PAHs

Figures 3, 4 and 5 show, respectively, the biodegradation of naphthalene, phenanthrene and anthracene in the medium during the biodegradation experiments for different surfactants and bacteria. The addition of any surfactant increased the solubility, approximately, in a factor of 100 for naphthalene and 1000 for phenanthrene and anthracene (solubility in water of naphthalene, phenanthrene and anthracene at 25ºC are 30 $mg \cdot l^{-1}$, 1.3 $mg \cdot l^{-1}$and 0.073 $mg \cdot l^{-1}$, respectively).
Most of PAHs added especially phenanthrene and anthracene, stayed undissolved at the beginning of each experiment. However, during the exponential growth phase, as soluble PAHs were consumed, precipitated PAHs were progressively solubilised. Once the exponential growth phase finished, all precipitated PAHs had already been transformed into solubilised form (data not shown). So, from that time, PAHs measurements in each culture corresponded to the whole amount of PAHs in the medium. In all cases, the values of the biodegradation rate constant (k_B) were calculated in the stationary growth phase, where the PAHs measurements were the true total concentration of PAHs.
Naphthalene was rapidly biodegraded and removed between 200-400 hours depending on the surfactants and bacteria used (Figure 3). Using surfactants, PAHs were almost completely depleted after 30 days. The rate of naphthalene biodegradation was higher than phenanthrene and anthracene for each of the surfactants tested (Table 2).
From the results of the abiotic control (Figures 3, 4 and 5), it was observed that both phenanthrene and anthracene did not show a significant degradation in absence of microorganisms ($k_A \approx 0$ for phenanthrene and anthracene) while naphthalene showed a progressive degradation at the same condition, although this degradation rate was significantly slower than that observed in presence of PAHs-degrading bacteria. The values of k_B are shown in Table 2. The ANOVA performed with k_B values for each PAH (Table 3) showed that, in all three cases, interactions between both factors (i.e., type of microorganism and type of surfactant) were clearly significant, as it can be seen from the statistical parameters for naphthalene ($F_{6,24} = 135.49$, $p < 0.001$, $n = 36$), phenanthrene ($F_{6,24} = 326.92$, $p < 0.001$, $n = 36$) and anthracene ($F_{6,24} = 70.62$, $p < 0.001$, $n = 36$). In all experiments, the presence of surfactant significantly improved all the biodegradation process. For the biodegradation of naphthalene (Table 2), the best treatment was achieved with Tween-80 and the bacterial consortium, followed by *Enterobacter* sp. (SNK $p < 0.05$). Using this surfactant, *Pseudomonas* sp. showed the poorest naphthalene biodegradation capability among all three strains (SNK $p < 0.05$). For phenanthrene, Tween-80 and *Enterobacter* sp. showed the best results. However, *Pseudomonas* sp. yielded better results than *Stenotrophomonas* sp. for both

Tween-80 and Triton X-10 (SNK $p < 0.05$). For the biodegradation of anthracene, *Enterobacter* sp. was again the best strain, although no significant differences were observed when using Tween-80 and Triton X-10 (SNK $p < 0.05$). With respect to the use of Tergitol NP-10, the best biodegradation rate for phenanthrene and antrhacene was achieved by *Pseudomonas* sp., while the efficiency of *Enterobacter* sp. was significantly reduced compared to experiments where Tween-80 was used (SNK $p < 0.05$). These data were consistent with the results from the cell growth measurements.

3.3. Toxicity

The toxicity screening during the PAHs degradation process (Figure 6) showed a small increase during the exponential growth phase. This effect was likely due to metabolites formed and secreted into the medium. However, an overall decreasing trend was observed when Tween-80 was used as surfactant. In the case of other surfactants, the toxicity was constant during the time course of the experiment. This was due to the fact that Tween-80 is biodegradable and it is used as carbon source by the microorganisms while Tergitol NP-10 and Triton X-100 are not metabolised by the PAHs-degrading bacteria, showing that these surfactants were toxic for *V. fischeri*.
For the experiments performed with Tween-80 and the consortium of three bacteria, the toxicity decreased until values below 50%. These data show that the addition of surfactants decrease the toxicity of the medium.

3.4. TOC and pH

For a complete description of the biodegradation process, monitoring of pH and TOC from the supernatant were performed (Figures 7 and 8, respectively) during the 30 days of the incubation of each experiment. TOC evolution was similar in all experiments showing that at early times of the incubation, TOC increased reaching a maximum and then, sharply decreased along the stationary phase. At the end of the experiment TOC values slightly increased probably due to the release of compounds in the cultivation media during the death phase. In the experiment in absence of surfactant low TOC values in solution were reported and their evolution was slower than in experiments performed in presence of surfactants.
In all cases, pH increased during the exponential phase. For the experiments with Tween-80 as surfactants pH increased from 7.0 to 9.0 approximately. While, with the others surfactants pH reached values of 7.8. At the end of experiments pH decreased lightly.

4. Discussion

The results obtained in these experiments show that the addition of surfactants in the PAHs biodegradation have a positive effect, increasing the solubility of PAHs and allowing their progressive biodegradation up to concentration levels lower than the initial values. This effect was clearly observed considering the low solubility and biodegradation degree of PAHs when surfactant was not added. The abiotic depletion of naphthalene in presence of 1% w/w Tween-80 ($k_A = 2.2 \cdot 10^{-3} \pm 6.0 \cdot 10^{-5}$ h^{-1}), not observed for both three-ring PAHs ($k_A \approx 0$), was likely due to volatilisation caused by a higher vapour pressure of naphthalene (11.6 Pa) compared to that of phenanthrene (0.13 Pa) and anthracene (0.08 Pa). However, in presence of PAHs-degrading strains, naphthalene depletion was significantly faster than that in the abiotic control experiment demonstrating that degradation of naphthalene was mainly due to biological processes.
Of the three surfactants studied, Tween-80 was the one that produced higher degree of PAHs

degradation. Moreover, the progressive decrease in toxicity and the larger bacterial growth seem to favour a significant reduction in naphthalene, phenanthrene and anthracene. For Triton X-100 and Tergitol NP-10 the results obtained were favourable and similar in both cases. However, the biodegradation degree and rates were lower compared to the results using Tween-80. For both Triton X-100 and Tergitol NP-10 the complete degradation of phenantrene and anthracene was not achieved after 30 days of incubation.

Some studies have suggested positive effects similar to that reported in the present work both for Tween-80 and TritonX-100 (Kim et al., 2001; Yang et al., 2003; Cheng and Wong, 2006) and for Tergitol NP-10 (Volkering et al., 1995; Grimberg et al., 1996). However, other authors (Laha and Luthy, 1991, 1992; Yuan et al., 2000) reported inhibitory effects in the bacterial biodegradation activity when the surfactants were added in concentrations over the CMC, likely due to an excess of micelles of the pollutant and a reduction of the bioavailability (Mulligan et al., 2001). However, this effect was not observed in the present study, since the addition of surfactants high above CMC (Table 1) produced positive results. The larger bacterial growth and PAHs-biodegradation rate observed in presence of Tween-80 during PAHs biodegradation experiments could be due to the fact that this surfactant was biodegradable and can be used as an additional carbon source for the microorganisms (Franzetti et al., 2006; Boonchan et al., 1998; Yoshimura, 1986; Swisher, 1987), although the surfactant biodegradation could compete for the substrates (Tiehm, 1994). The fact that the rest of surfactants do not present growth in absence of PAHs does not imply that they are not biodegradable, but it is possible that the bacteria in charge of its degradation are absent in our media.

The growth of *Pseudomonas* sp., *Enterobacter* sp. and *Stenotrophomonas* sp. with PAHs as carbon source mean that these microorganisms are potential PAHs biodegraders. The results showed that in most cases that growth rate of *Enterobacter* sp. and its PAHs biodegradation rate were higher than that produced by the other bacteria. Probably it was due to a better metabolism of the initial organic compounds, since *Enterobacter* sp. was identified during the first phase of the growth of a consortium obtained from a highly oil-polluted soil. Furthermore, the rates of degradation and growth observed for *Pseudomona*s sp. and *Stenotrophomonas* sp. were also significant. Many authors have studied the genes involved in the PAHs-degradation metabolism for naphthalene (Dunn and Gunsalus, 1973; Kuhm et al., 1991; Whyte et al. 1997; Filonov et al., 2006), phenanthrene (Balashova et al., 1999; Puntus et al., 2008) and anthracene (Jacques et al., 2005) or other aromatic hydrocarbon with higher molecular weight (Boonchan et al., 1998; Juhasz et al., 2000). Anyway, the highest values of growth and degradation of PAHs were obtained when the three bacteria work together. Probably this fact is due to the existence of a microbial succession during the incubation. Since the initial compounds could be metabolized by *Enterobacter* sp. and the intermediate products formed could be metabolized by *Pseudomonas* sp. or *Stenotrophomonas* sp. doing the degradation process more effective.

The PAHs degrading efficiency depends on the molecular weight and the number of aromatic rings (Warmer and Peters, 2005), since, the solubility in water, volatility and persistence of PAHs decrease when molecular weight and the number of aromatic rings increase (Juhasz and Naidu, 2000; Kanaly and Harayama, 2000; Dimitriou-Christidis et al., 2007). The results showed that the evolution of the PAHs during the biodegradation process followed the order of solubility of these pollutants: naphthalene > phenanthrene > anthracene (Figures 3, 4 and 5). The degradation of both three-ring hydrocarbons was not associated to any abiotic processes since degradation of phenanthrene and anthracene was not observed from the abiotic control experiments. In the case of naphthalene, the depletion measured in this abiotic control was likely due to volatilization because of its higher vapour pressure (11.6 Pa at 298 K) than phenanthrene and anthracene (0.13 Pa and 0.08 Pa at 298 K, respectively). However,

the overall rate of naphthalene depletion was higher than that observed in control experiments, so that the biological contribution was significant. The large increase in the degradation rate (eq. 1) of each PAH in presence of surfactant was not only due to the large increase in PAHs solubility, but to a concomitant increase in the value of the kinetic constant (Table 2). The significant interactions between type of microorganism and type of surfactant showed by the ANOVA performed for each PAH prove that the choice of the best surfactant to increase the biodegradation efficiency depends on the microorganism or consortium used as well as the nature of the hydrocarbon to be removed.

Most studies report a depletion of target components without considering the toxicity during the process. Figure 6 shows that at the begining of each experiment toxicity was constant or even slightly increased as a consequence of the potential accumulation of intermediate products formed (Grifoll et al., 1995). Biodegradation experiments using *Pseudomonas* sp. with Triton X-100 or Tergitol NP-10 did not show a significant decrease in toxicity along the whole curse of both experiments (Figure 6). Although toxic effects produced by chemical surfactants on bacterial communities have been reported (Kim et al., 2001), in this work, this effect was nor observed, at least to a large extent, since *Pseudomonas* sp. showed capability to degrade PAHs almost completely. This means that these two surfactants were not toxic for *Pseudomonas* sp. but induced toxicity for *V. fisheri*, i.e., the bacteria used for the toxicity assay. However, when Tween-80 was used as surfactant, a significant decrease in toxicity was achieved (Figure 6) since, not only PAHs were depleted but, all three strains are capable to metabolize the surfactant. In the absence of surfactant a lower decrease in toxicity was shown. The use of the bacterial consortium was more efficient than individual bacteria reaching lower values of toxicity.

The small decrease measured in TOC (Figure 7) confirmed the PAHs biodegradation. The partial mineralization of PAHs and intermediate products to CO_2 could explain this trend. Nevertheless the results showed two increases in TOC levels during the biodegradation process. The first one, from the begining of the experiment, could be caused by the formation of intermediate degradation products and the solubilisation of PAHs precipitated in the media along the exponential growth phase. In the case of the biodegradation experiments using Tween-80 as surfactant, that effect was even larger (Figure 7A) since Tween-80 was biodegraded by all three strains used. At the end of the experiment, when the death phase was taking place, a second increase in TOC could be due to the release of intracellular matter in the broth during this phase phase. In absence of surfactant, the low solubility of PAHs produced very low values of TOC in aqueous solution.

Finally, pH evolution was shown in Figure 8. An increase in pH was observed in all experiments, probably due to intermediate products formed. At the beginning of each experiment metabolites bearing hydroxyl groups increase the pH of the culture media (Bossert and Bartha, 1984; Juhasz and Naidu, 2000; Habe and Omori, 2003; Luan et al., 2006; Puntus, 2008). The higher pH increase using Tween-80 can be due to the secreted metabolites to the medium by the biodegradation pathway of this surfactant (Dibble and Bartha, 1979).

In conclusion, this study showed the positive effect of non-ionic surfactants on the PAHs biodegradation process due to a remarkable increase in both solubility and bioavailability of these hydrocarbons, yielding higher biodegradation rates in presence of surfactants. The highest values of PAHs degradation and bacterial growth rate were obtained when Tween-80 was used as surfactant. Furthermore, this surfactant was used as a growth substrate by the bacteria. The strains used in the research showed a complete degradation of two and three-ring hydrocarbons. The best results were obtained with the use of a consortium composed of the three bacteria for the biodegradation of naphthalene and *Enterobacter* sp. for phenanthrene and anthracene.

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Table 1
Relevant properties of surfactants.

Surfactant	Molecular formula	Molecular Weight	CMC[a,b] (mM)	Density[b] (g/ml)
Tween-80	$C_{18}H_{37}(C_6H_9O_5)(OC_2H_4)_{20}OH$	1309	0.012	1.06
Triton X-100	$C_8H_{17}C_6H_4\ (OC_2H_4)_{10}OH$	625	0.232	1.07
Tergitol NP-10	$C_{15}H_{24}O(OC_2H_4)_{10}OH$	652	0.069	1.04

[a] CMC: Critical micelle concentration
[b] at 298 K

Table 2
Apparent first-order biodegradation rate constant (k_B) for each PAH with different microorganisms and surfactants (± values correspond to standard deviation of 3 replicates)

Microorganism	Surfactant	k_B (h^{-1})		
		Naphthalene	Phenanthrene	Anthracene
Pseudomonas sp.	Tween-80	$7.9\cdot10^{-3}\pm6.4\cdot10^{-4}$	$8.2\cdot10^{-3}\pm2.0\cdot10^{-4}$	$3.3\cdot10^{-3}\pm6.7\cdot10^{-4}$
	Triton X-100	$9.5\cdot10^{-3}\pm4.4\cdot10^{-4}$	$8.6\cdot10^{-3}\pm2.5\cdot10^{-4}$	$6.4\cdot10^{-3}\pm2.3\cdot10^{-4}$
	Tergitol NP-10	$1.1\cdot10^{-2}\pm3.3\cdot10^{-4}$	$5.3\cdot10^{-3}\pm3.2\cdot10^{-4}$	$5.7\cdot10^{-3}\pm3.1\cdot10^{-4}$
	No surfactant	$2.4\cdot10^{-3}\pm1.8\cdot10^{-4}$	$1.0\cdot10^{-3}\pm1.3\cdot10^{-5}$	$4.7\cdot10^{-4}\pm7.3\cdot10^{-6}$
Enterobacter sp.	Tween-80	$1.7\cdot10^{-2}\pm2.2\cdot10^{-4}$	$1.2\cdot10^{-2}\pm7.4\cdot10^{-5}$	$7.0\cdot10^{-3}\pm5.3\cdot10^{-4}$
	Triton X-100	$1.2\cdot10^{-2}\pm3.9\cdot10^{-5}$	$9.8\cdot10^{-3}\pm3.7\cdot10^{-5}$	$7.3\cdot10^{-3}\pm1.4\cdot10^{-4}$
	Tergitol NP-10	$1.1\cdot10^{-2}\pm6.6\cdot10^{-5}$	$3.3\cdot10^{-3}\pm1.3\cdot10^{-4}$	$4.4\cdot10^{-3}\pm1.2\cdot10^{-4}$
	No surfactant	$4.0\cdot10^{-3}\pm3.7\cdot10^{-4}$	$5.2\cdot10^{-4}\pm1.2\cdot10^{-5}$	$5.5\cdot10^{-4}\pm3.4\cdot10^{-5}$
Stenotrophomonas sp.	Tween-80	$1.5\cdot10^{-2}\pm7.4\cdot10^{-4}$	$7.4\cdot10^{-3}\pm1.1\cdot10^{-4}$	$5.2\cdot10^{-3}\pm4.7\cdot10^{-4}$
	Triton X-100	$1.3\cdot10^{-2}\pm1.7\cdot10^{-5}$	$5.3\cdot10^{-3}\pm1.6\cdot10^{-4}$	$3.1\cdot10^{-3}\pm1.0\cdot10^{-4}$
	Tergitol NP-10	$1.1\cdot10^{-2}\pm3.3\cdot10^{-4}$	$2.0\cdot10^{-3}\pm1.2\cdot10^{-4}$	$3.1\cdot10^{-3}\pm1.2\cdot10^{-4}$
	No surfactant	$9.8\cdot10^{-4}\pm3.2\cdot10^{-5}$	$7.8\cdot10^{-4}\pm4.7\cdot10^{-5}$	$5.2\cdot10^{-4}\pm1.5\cdot10^{-5}$
3 bacteria above	Tween-80	$2.0\cdot10^{-2}\pm3.9\cdot10^{-5}$	$9.5\cdot10^{-3}\pm1.5\cdot10^{-4}$	$6.7\cdot10^{-3}\pm5.5\cdot10^{-6}$
	No surfactant	$4.6\cdot10^{-3}\pm1.9\cdot10^{-4}$	$8.7\cdot10^{-4}\pm4.0\cdot10^{-5}$	$5.6\cdot10^{-4}\pm4.1\cdot10^{-5}$

Table 3
Analysis of variance (ANOVA) summary for the biotic degradation rate constant (k_B)

	Source	Sum of squares	D.F.[a]	F-value	p-value
Naphthalene[f]	Intercept	0.003395	1	25529.64	<0.0001
	Surfactant[b]	0.000655	3	1642.96	<0.0001
	Strain[c]	0.000073	2	274.71	<0.0001
	Interaction	0.000108	6	135.49	<0.0001
	error	0.000003	24		
Phenanthrene[g]	Intercept	0.001036	1	45705.99	<0.0001
	Surfactant[b]	0.000412	3	6053.49	<0.0001
	Strain[c]	0.000041	2	914.81	<0.0001
	Interaction	0.000044	6	326.92	<0.0001
	error	0.000001	24		
Anthracene[h]	Intercept	0.000553	1	5725.337	<0.0001
	Surfactant[b]	0.000147	3	506.049	<0.0001
	Strain[c]	0.000020	2	105.797	<0.0001
	Interaction	0.000041	6	70.616	<0.0001
	error	0.000002	24		
Tween-80[i]	Intercept	0.003605	1	21129.93	<0.0001
	PAHs[d]	0.000561	2	1645.28	<0.0001
	Strain[e]	0.000198	3	387.24	<0.0001
	Interaction	0.000114	6	111.03	<0.0001
	error	0.000004	24		

[a] Degrees of freedom
[b] Surfactant: Tween-80, Triton X-100, Tergitol NP-10 and without surfactant.
[c] Strain: *Pseudomonas* sp., *Enterobacter* sp. and *Stenotrophomonas* sp.
[d] PAHs: Naphthalene, phenanthrene and anthracene.
[e] Strain: *Pseudomonas* sp., *Enterobacter* sp., *Stenotrophomonas* sp and without inoculation.
[f] Effect of the type of strain and surfactant on the biodegradation of naphthalene.
[g] Effect of the type of strain and surfactant on the biodegradation of phenanthrene.
[h] Effect of the type of strain and surfactant on the biodegradation of anthracene.
[i] Effect of the type of PAH and strain on the biodegradation process using Tween-80 as surfactant.

CAPTION OF FIGURES

Figure 1. Effect of different surfactants on the growth of PAHs-degrading bacteria (*Pseudomonas* sp. ■, *Enterobacter* sp.●, *Stenotrophomonas* sp. ▲, and a mixture of the three above ◆) during PAHs biodegradation experiments(error bars denote standard deviation of 3 replicates). Cultures were performed using Tween-80 (A), Triton X-100 (B) and Tergitol NP-10 (C), and without surfactant (D)

Figure 2. Specific growth-rate (μ) of PAHs-degrading bacteria in different surfactants (error bars denote standard deviation of 3 replicates). Experiments with Triton X-100 and Tergitol NP-10 were not performed for the three-bacterium consortium.

Figure 3. Effect of different surfactants on the naphthalene degradation by diverse PAHs-degrading bacteria (*Pseudomonas* sp. ■, *Enterobacter* sp. ●, *Stenotrophomonas* sp. ▲, a mixture of the three above ◆, abiotic control ▽) (error bars denote standard deviation of 3 replicates). Cultures were performed using Tween-80 (A), Triton X-100 (B) and Tergitol NP-10 (C), and without surfactant (D).

Figure 4. Effect of different surfactants on the phenanthrene degradation by diverse PAHs-degrading bacteria (*Pseudomonas* sp. ■, *Enterobacter* sp. ●, *Stenotrophomonas* sp. ▲, a mixture of the three above ◆, abiotic control ▽) (error bars denote standard deviation of 3 replicates). Cultures were performed using Tween-80 (A), Triton X-100 (B) and Tergitol NP-10 (C), and without surfactant (D).

Figure 5. Effect of different surfactants on the anthracene degradation by diverse PAHs-degrading bacteria (*Pseudomonas* sp. ■, *Enterobacter* sp. ●, *Stenotrophomonas* sp. ▲, a mixture of the three above ◆, abiotic control ▽) (error bars denote standard deviation of 3 replicates). Cultures were performed using Tween-80 (A), Triton X-100 (B) and Tergitol NP-10 (C), and without surfactant (D).

Figure 6. Toxicity of culture media during PAHs biodegradation experiments using different surfactants Tween-80 (*Pseudomonas* sp. ■, *Enterobacter* sp. ●, *Stenotrophomonas* sp. ▲ a mixture of the three above ◆, abiotic control ▽), Triton X-100 (*Pseudomonas* sp. □) and Tergitol NP-10 (*Pseudomonas* sp. ○).

Figure 7. Total organic carbon (TOC) during PAHs biodegradation experiments by diverse PAHs-degrading bacteria (*Pseudomonas* sp. ■, *Enterobacter* sp. ●, *Stenotrophomonas* sp. ▲, a mixture of the three above ◆). Cultures were performed using Tween-80 (A), Triton X-100 (B) and Tergitol NP-10 (C), and without surfactant (D).

Figure 8. pH during PAHs biodegradation experiments by diverse PAHs-degrading bacteria (*Pseudomonas* sp. ■, *Enterobacter* sp. ●, *Stenotrophomonas* sp. ▲, a mixture of the three above ◆). Cultures were performed using Tween-80 (A), Triton X-100 (B) and Tergitol NP-10 (C), and without surfactant (D).

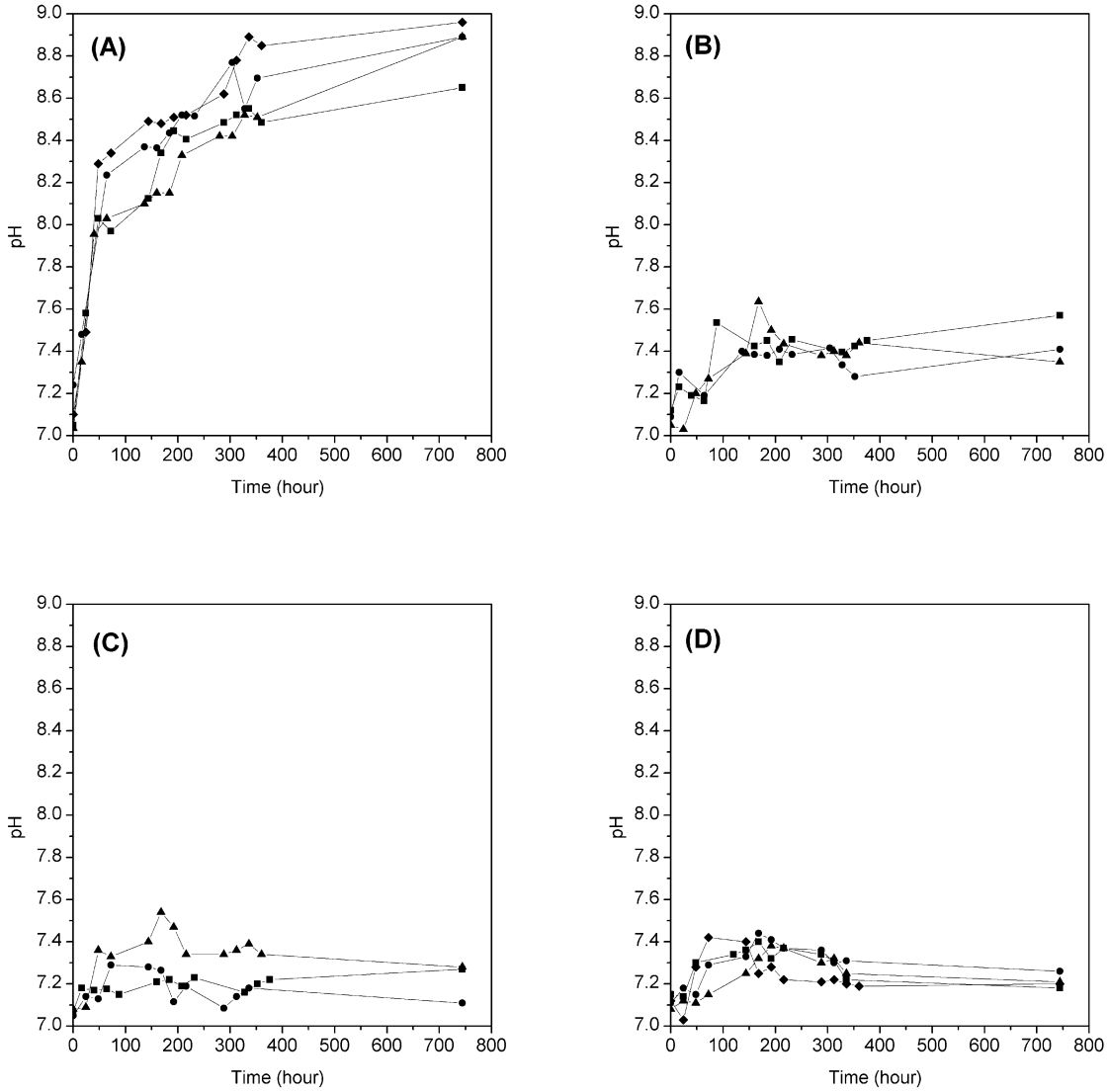


Figure 1. Effect of different surfactants on the growth of PAHs-degrading bacteria (*Pseudomonas* sp. ■, *Enterobacter* sp.●, *Stenotrophomonas* sp. ▲, and a mixture of the three above ◆) during PAHs biodegradation experiments(error bars denote standard deviation of 3 replicates). Cultures were performed using Tween-80 (A), Triton X-100 (B) and Tergitol NP-10 (C), and without surfactant (D)

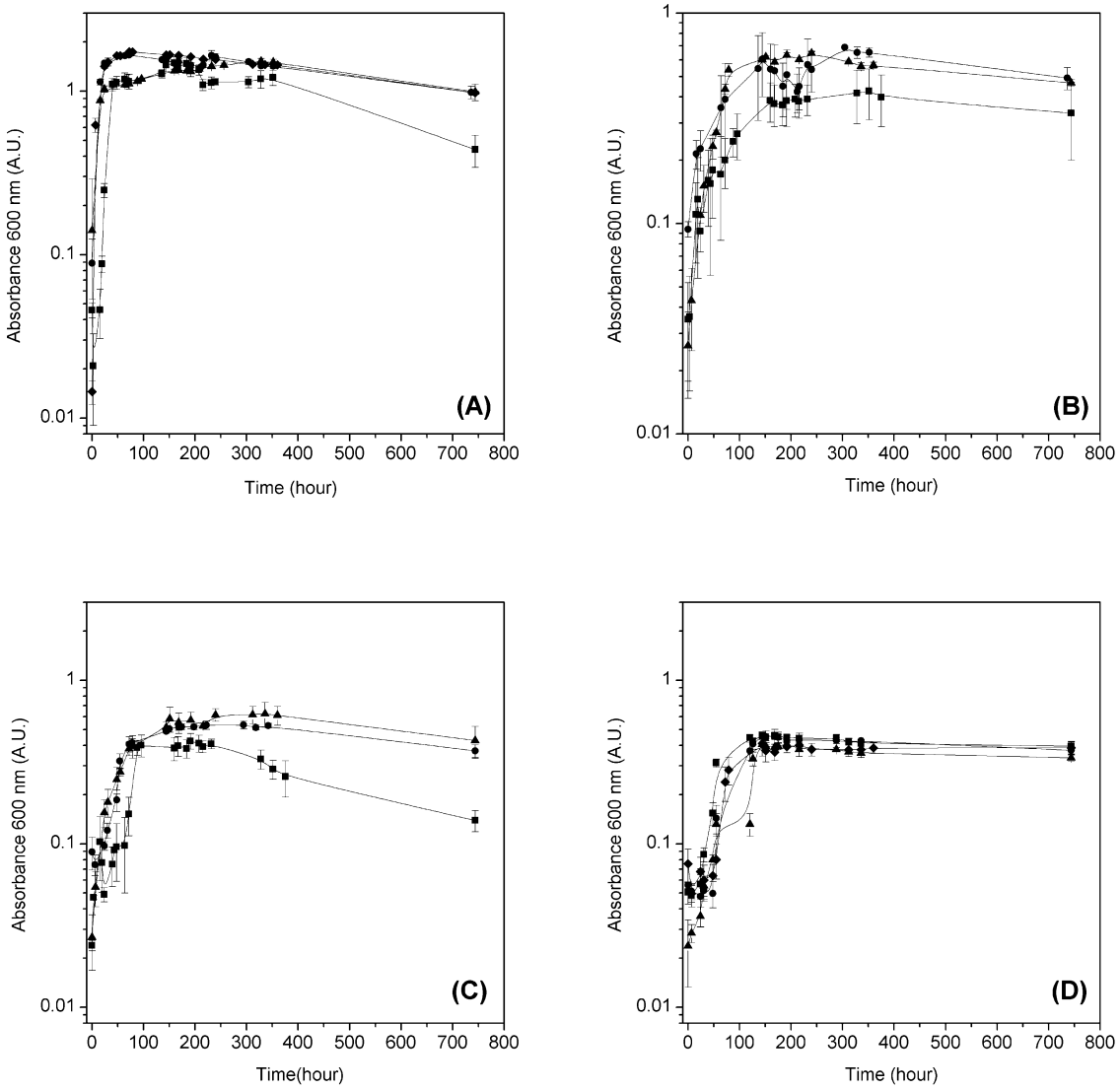
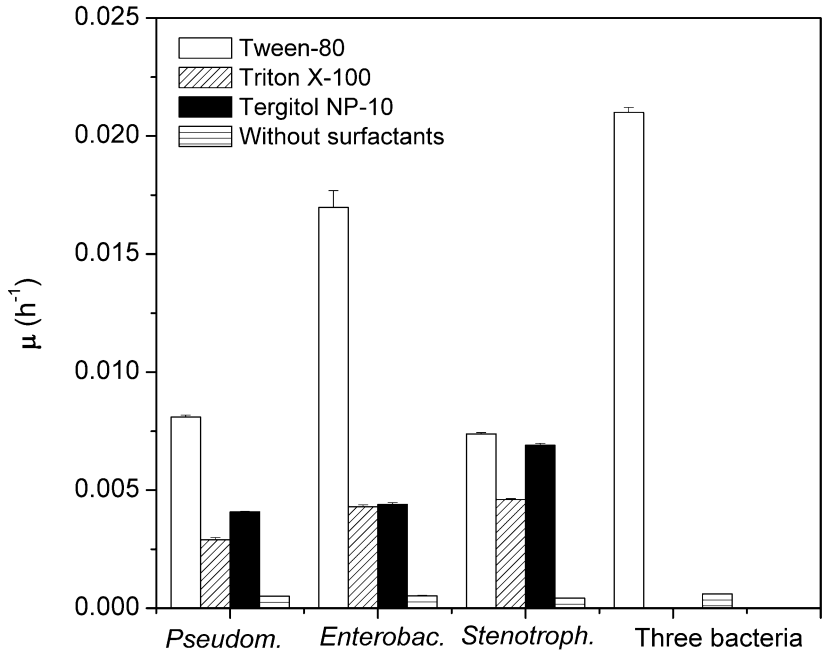


Figure 2. Specific growth-rate (μ) of PAHs-degrading bacteria in different surfactants (error bars denote standard deviation of 3 replicates). Experiments with Triton X-100 and Tergitol NP-10 were not performed for the three-bacterium consortium.



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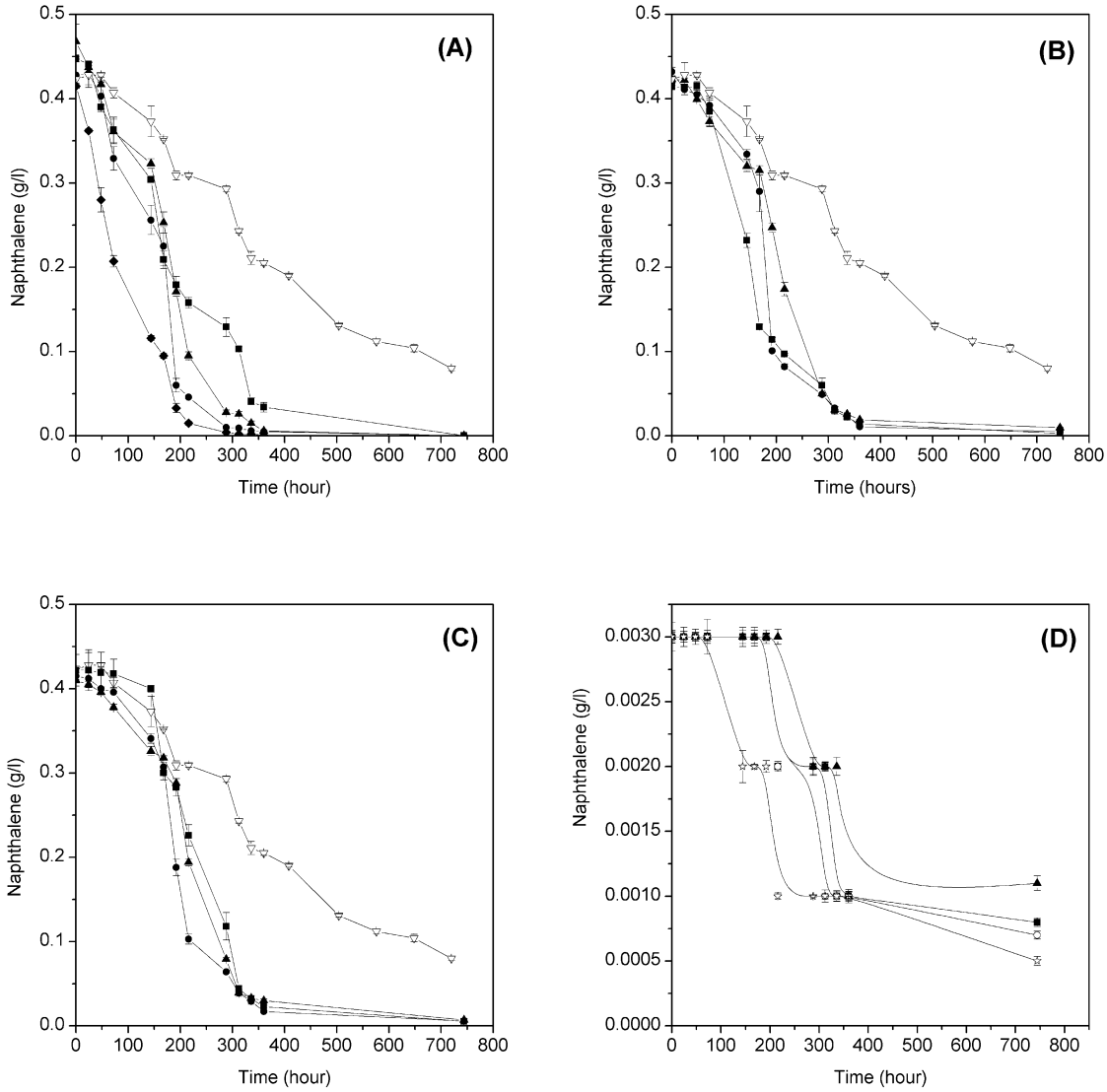


Figure 4. Effect of different surfactants on the phenanthrene degradation by diverse PAHs-degrading bacteria (*Pseudomonas* sp. ■, *Enterobacter* sp. ●, *Stenotrophomonas* sp. ▲, a mixture of the three above ◆, abiotic control ▽) (error bars denote standard deviation of 3 replicates). Cultures were performed using Tween-80 (A), Triton X-100 (B) and Tergitol NP-10 (C), and without surfactant (D).

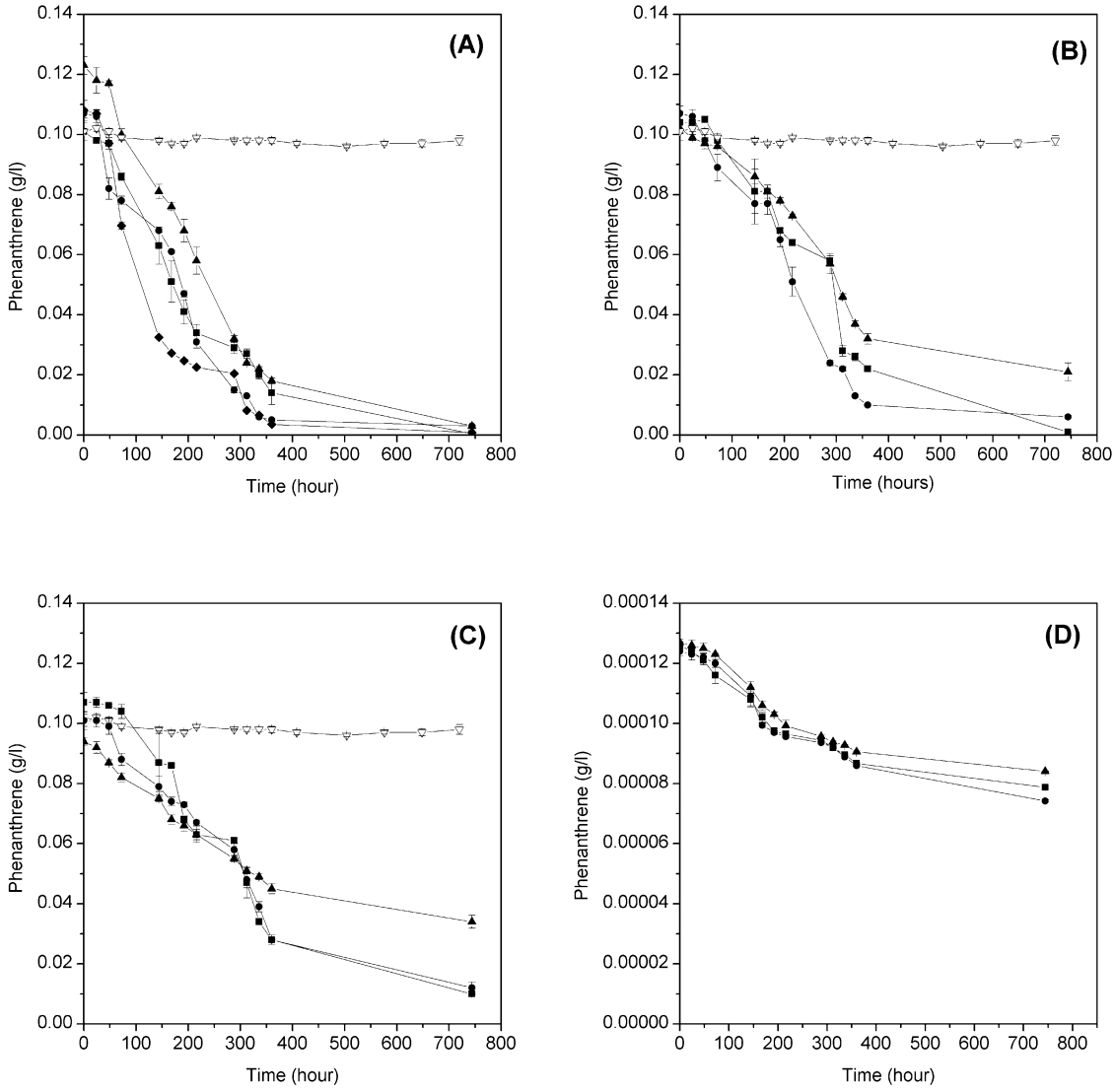
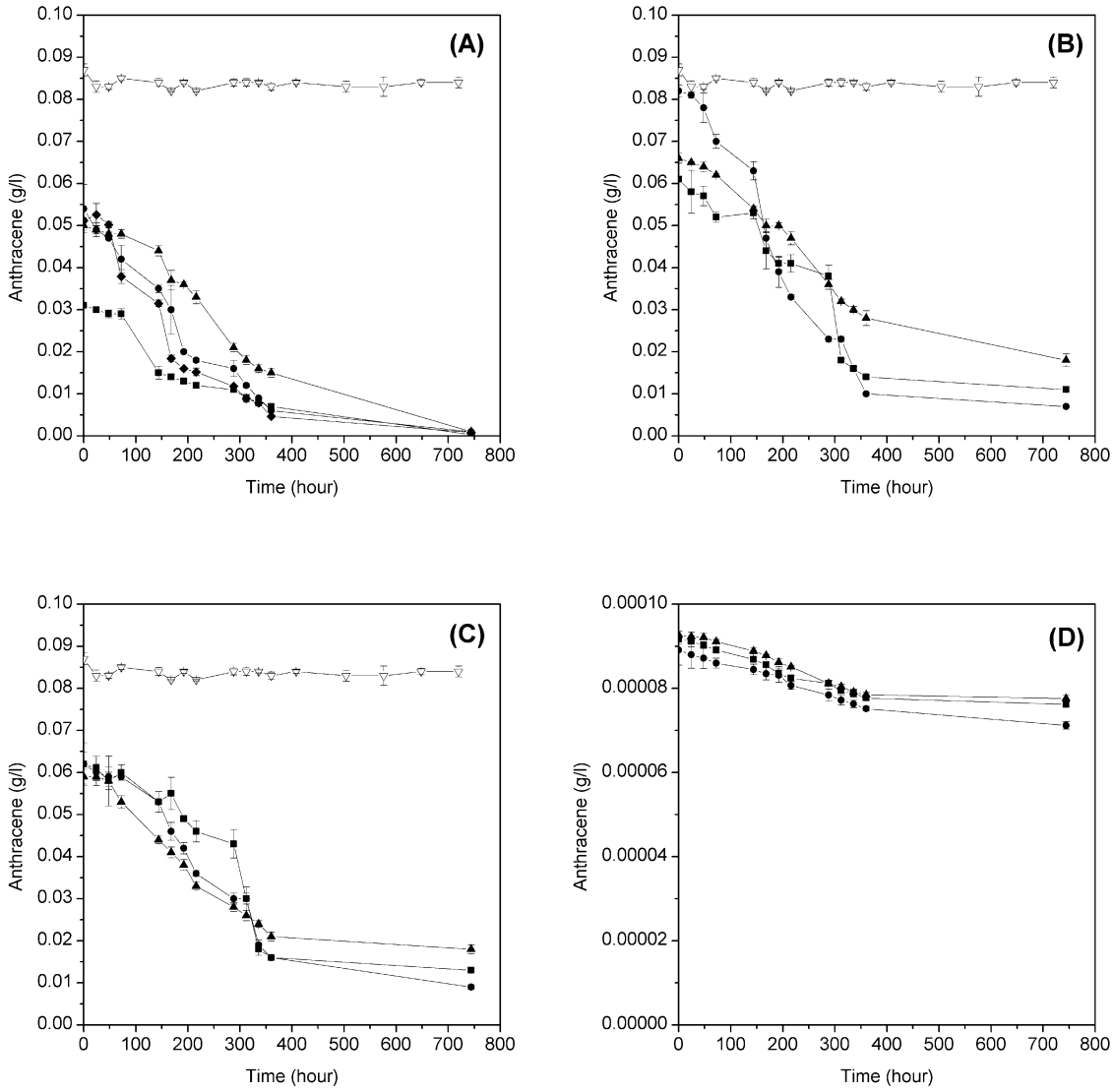


Figure 5. Effect of different surfactants on the anthracene degradation by diverse PAHs-degrading bacteria (*Pseudomonas* sp. ■, *Enterobacter* sp. ●, *Stenotrophomonas* sp. ▲, a mixture of the three above ◆, abiotic control ▽) (error bars denote standard deviation of 3 replicates). Cultures were performed using Tween-80 (A), Triton X-100 (B) and Tergitol NP-10 (C), and without surfactant (D).



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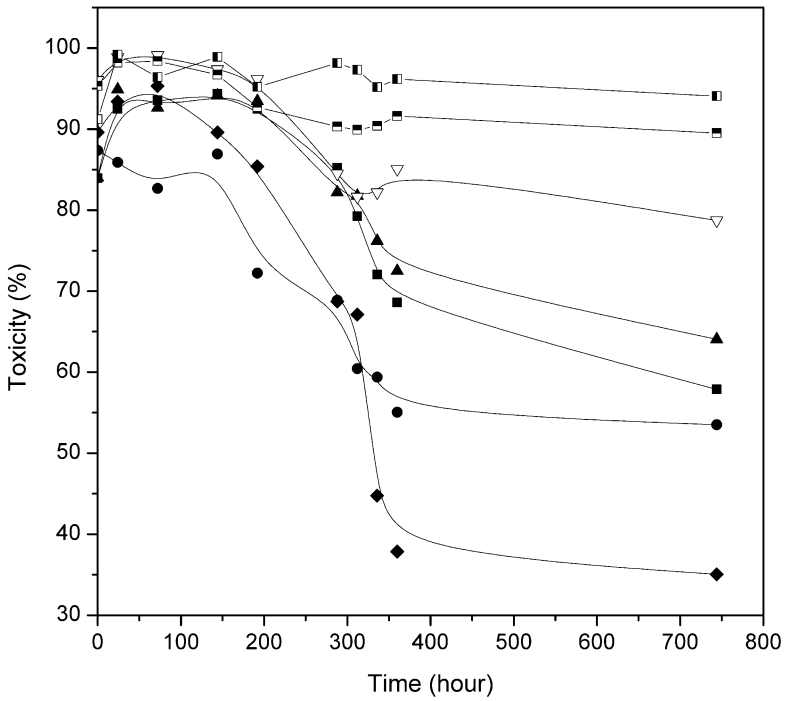

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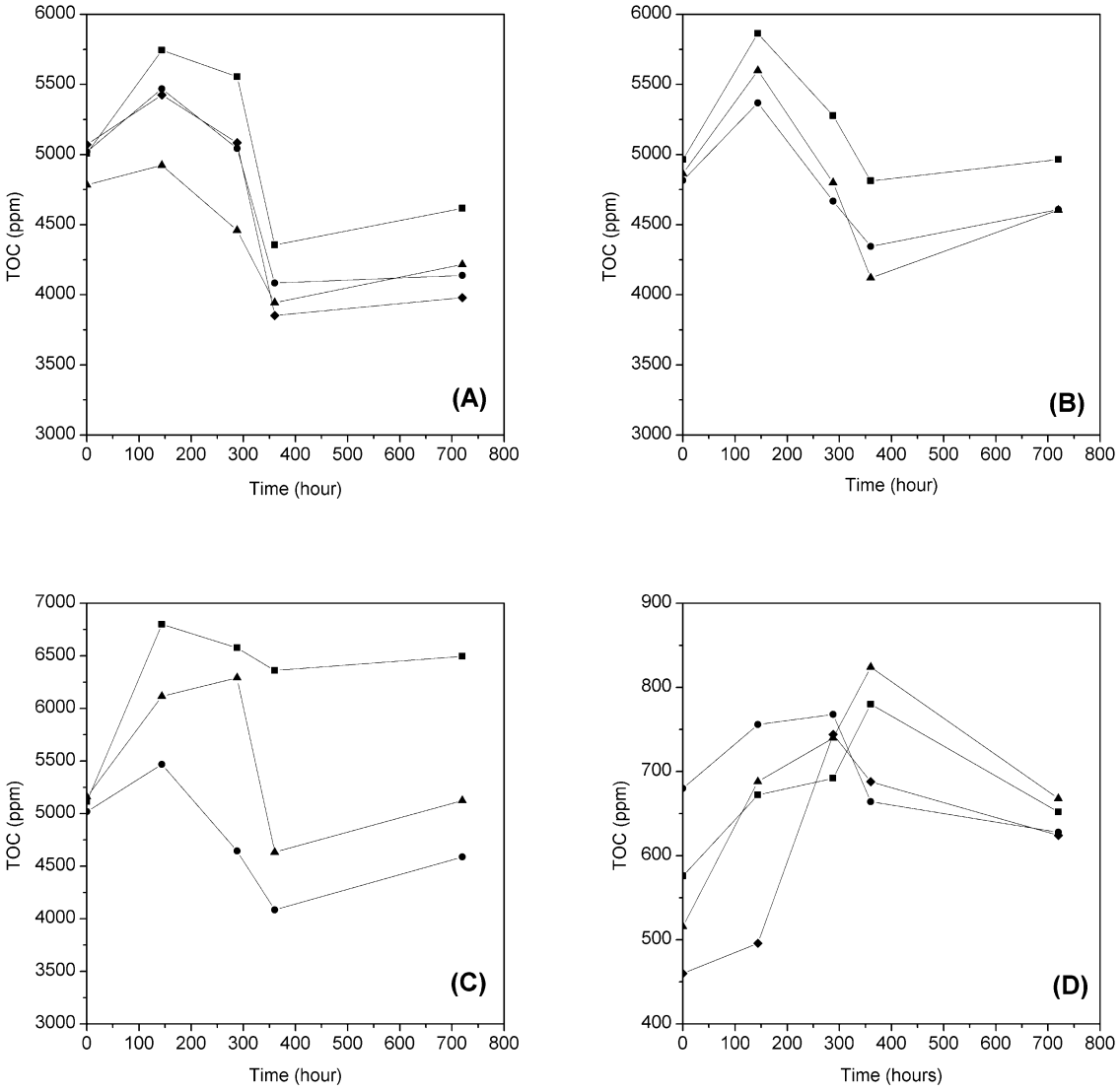

Figure 8. pH during PAHs biodegradation experiments by diverse PAHs-degrading bacteria (*Pseudomonas* sp. ■, *Enterobacter* sp. ●, *Stenotrophomonas* sp. ▲, a mixture of the three above ◆). Cultures were performed using Tween-80 (A), Triton X-100 (B) and Tergitol NP-10 (C), and without surfactant (D).