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# Utilization of Triton X-100 and polyethylene glycols during surfactant-mediated biodegradation of diesel fuel

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ABSTRACT

The hypothesis regarding preferential biodegradation of surfactants applied for enhancement of microbial hydrocarbons degradation was studied. At first the microbial degradation of sole Triton X-100 by soil isolated hydrocarbon degrading bacterial consortium was confirmed under both full and limited aeration with nitrate as an electron acceptor. Triton X-100 (600 mg/l) was utilized twice as fast for aerobic conditions ($t_{1/2}$ = 10.3 h), compared to anaerobic conditions ($t_{1/2}$ = 21.8 h). HPLC/ESI-MS analysis revealed the preferential biodegradation trends in both components classes of commercial Triton X-100 (alkylphenol ethoxylates) as well as polyethylene glycols. The obtained results suggest that the observed changes in the degree of ethoxylation for polyethylene glycol homologues occurred as a consequence of the 'central fission' mechanism during Triton X-100 biodegradation. Subsequent experiments with Triton X-100 at approx. CMC concentration (150 mg/l) and diesel oil supported our initial hypothesis that the surfactant would become the preferred carbon source even for hydrocarbon degrading bacteria. Regardless of aeration regimes Triton X-100 was utilized within 48–72 h. Efficiency of diesel oil degradation was decreased in the presence of surfactant for aerobic conditions by approx. 25% reaching 60 instead of 80% noted for experiments without surfactant. No surfactant influence was observed for anaerobic conditions.



## 1. Introduction

Over the past years biological methods have been recognized as an efficient alternative to classic remediation technologies of petroleum-contaminated sites. The biodegradation efficiency is influenced by numerous biotic and abiotic factors. Among them the bioavailability and bioaccessibility of potential carbon sources and nutrients is of major concern [1]. The hydrophobic nature of petroleum compounds, low water solubility and contaminant sorption into the soil matrix results in a notable decrease of the mass transfer rate, as most of the carbon sources are unavailable to the cells, and is regarded as a major limitation step [2].

Surfactant supplementation has been suggested as a potential approach to overcome this issue [3]. Surfactants may intensify the biodegradation process through solubilisation and mobilization of water-immiscible hydrocarbons. In this concept, the hydrophobic compound droplets are entrapped in surfactant micelles and transferred into the aqueous phase [4]. A parallel mechanism for increasing the mass transfer rate involves surfactant-induced changes in surface properties of microbial cells, which result in an enhanced affinity towards hydrocarbons. As a consequence the microbial adherence to the oil phase is increased and direct uptake of petroleum compounds may occur [5].

It was initially expected that surfactants will be responsible for an increase of surface area for potential bacterial colonization, which would lead to a higher utilization rate of dispersed hydrocarbons. While numerous researchers observed increased hydrocarbon biodegradation efficiency after surfactant supplementation [6,7], there are many contrary reports suggesting that surface active agents may inhibit the degradation rate [8–10]. The overall influence of surfactants on the progress of biodegradation processes may induce facilitation, retardation or no effect [4,11]. It was also concluded, that various issues may be responsible for the observed negative impact. One possible explanation included the toxicity of surfactants or surfactant-solubilised contaminants, which resulted in decreased microbial growth [12]. Furthermore, surfactant-mediated detachment of cells from the interfacial boundary and inhibition of direct uptake was another limiting factor [9,13]. Moreover, surfactant molecules were occasionally utilized as a preferential carbon source [10]. Recently several studies also demonstrated, that the organic compounds

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entrapped in surfactant micelles may be unavailable to microbial cells [14–17]. In our previous studies we have reported, on the example of 218 bacterial consortia, that the addition of Triton X-100 decreased the biodegradation capabilities for a large group of diesel oil-degrading consortia [18].

In order to investigate the previously observed decrease of biodegradation efficiency we carried out studies concerning the influence of Triton X-100 on diesel oil degradation carried out by a new microbial consortium under aerobic and anaerobic conditions. We focused on analyzing the fate of surfactant during the biodegradation experiments as well as possible interactions with consortium members in terms of cell surface hydrophobicity changes and toxicity.

## 2. Materials and methods

### 2.1. Chemicals

Petroleum diesel fuel (EN 590:2004) was purchased from a petrol station (PKN Orlen, Poland) and sterilized by filtration before use in experiments (Millex 0.2 μm; Millipore). Triton X-100 (Sigma–Aldrich) is a mixture of alkylphenol ethoxylates (APE) with an average degree of ethoxylation equal to 9.5. The analysis revealed approx. 0.5–1.5% content of polyethylene glycols (PEG), residues from the production process.

### 2.2. Characterization of microorganisms

#### 2.2.1. Isolation of microbial consortium

The bacterial consortium, assigned PMF1, has been isolated from a site polluted with crude oil (Czarna Górna, Poland 49.315051N; 22.662735E) under aerobic conditions employing diesel fuel as sole carbon and energy source. The isolation process was carried out on PTFE membranes, as described by Oku et al. [19].

#### 2.2.2. Extraction and amplification of bacterial DNA

After the microbial cells were disrupted by lysozyme treatment, the total DNA was extracted with the use of the Genomic Mini Plant Kit (A&A Biotechnology, Poland). The obtained DNA was then used for PCR amplification of the bacterial 16S rDNA. To amplify 16S rRNA coding sequence the oligonucleotide primers SDBac08 5′AGAGTTTGATCCTGGCTCAG3′ and Unv1492 5′ACGGCTACCTTGTTACGACTT3′ were used. PCR was performed in total volume of 25 μl containing 1× PCR buffer (10 mM Tris–HCl, pH 8.8, 2.5 mM, $MgCl_2$, 50 mM KCl and 0.1% Triton X-100), 250 ng DNA template, 0.44 μM concentration of each primer, 200 μM dNTP, 1 U DyNAzyme II DNA polymerase (Finnzymes). The amplification of 16S rDNA consisted of 15 cycles: 1 min denaturation step at 94 °C, 1 min annealing step at 56 °C, and 1 min extension at 72 °C (Biometra T Gradient thermocycler). After reaction, 4 μl samples of PCR reaction mixture were analyzed by electrophoresis in 1.0% (w/v) agarose in 1× TBE buffer (89 mM Tris–borate, 2 mM EDTA pH 8.3) and subsequently visualized by UV illumination after ethidium bromide staining.

#### 2.2.3. Construction of clones libraries of bacterial 16S rDNA

PCR amplicons were cloned into pGEMTEasy vector (Promega). The *Escherichia coli* JM109 (Promega) used for clone library construction was grown in Luria–Bertani broth (LB) for 16 h at 37 °C. Transformed *E. coli* cells were grown overnight on LB agar plates containing 100 μl/ml ampicilin, 100 μl of 100 mM isopropyl-β-D-tiogalactopyranoside and 20 μl 50 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside at 37 °C. To establish the diversity within the each group of clones white colonies were picked randomly.

#### 2.2.4. PCR–RFLP analysis

Recombinant pGEMTEasy-16SrDNA plasmids were purified with Plasmid Mini kit (A&A Biotechnology). The clones were screened for different inserts of 16S rDNA by PCR and restriction mapping (RFLP – restriction fragment length polymorphisms) using the restriction enzymes FastDigest *Hinf*I and FastDigest *Taq*I (Fermentas). Digestion reactions were performed in final volume of 20 μl at 37 and 65 °C, respectively. The restriction fragments were separated by electrophoresis in polyacrylamide gel (8% PAA, 1× TBE buffer). The resolved DNA was visualized by silver staining. The gels were fixed for 10 min in 10% ethanol, rinsed three times with distilled water, incubated for 10 min in 1% $HNO_3$ (v/v), rinsed three times with distilled water, impregnated for 30 min with $AgNO_3$ (0.2% w/v) containing 0.04% formaldehyde, thoroughly washed with water and visualized with $Na_2CO_3$ (3% w/v) containing 0.02% formaldehyde. Staining was stopped by 10 min incubation in 10% (v/v) $CH_3COOH$.

The selected plasmid DNA samples with different RFLP pattern of insert were sequenced. For complete sequence determination three primers were used SDBac08, Unv1492 and F515 (5′GTGCCAGCAGCCGCGGT-3′). Whole 16S rRNA coding sequence was assembled using VectorNTI Software (Invitrogen) and contigs corresponding to samples with different restriction patterns were submitted to the National Center for Biotechnology Information for similarity searches in GeneBank (BLASTN) for taxonomic identification.

#### 2.2.5. Preparation of the inoculum

The consortium has been stored at −80 °C in a 30% (v/v) glycerol stocks. To prepare an inoculum, stock suspension was transferred (1 ml) to a 300-ml Erlenmeyer flask containing 50 ml mineral medium (composition given in [20]) and diesel fuel (0.5%, v/v), and was cultivated for 24 h at 25 °C. Later, 1 ml aliquot of the cell suspension was transferred to a new enrichment flask and the culture was grown for 3 days in the same conditions. This step was repeated three times and cells from the last enrichment were centrifuged at 10,000 × *g*, washed twice with 40 ml of mineral medium and resuspended. In all steps aerobic conditions were provided. After the enrichment procedure of the consortium stock the cultures were set up to an $OD_{600}$ of $0.1 \pm 0.01$ for each experimental condition. Polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) revealed that the community in the glycerol stock was the same as the communities in the enrichment cultures (data not shown). The initial relative abundance of the bacterial groups present in the consortium has been determined by the use of TaqMan probes and the method previously described by Cyplik et al. [21].

#### 2.2.6. Growth on Triton X-100

The potential toxicity of Triton X-100 towards the consortium grown on Na–succinate (4 g/l) as a sole source of carbon was studied, in order to exclude all possible interference from diesel fuel hydrocarbons. The toxicity was quantified by the effect of the surfactant on the microbial growth rates, according to the procedure described by Heipieper et al. [22]. Triton X-100 was added to exponentially growing cells in concentrations ranging from 1 to 1000 mg/l. The growth inhibition was defined as the percentage of the growth rates of cultures with surfactants and that of control cultures without surfactants. Effective concentrations of the compounds causing 50% growth inhibition were expressed as $EC_{50}$.

### 2.3. CSH

Cell surface hydrophobicity was measured as described by Owsianiak et al. [18].

### 2.4. Biodegradation experiments

#### 2.4.1. Full aeration

Loosely closed 250-ml Duran-Schott bottles covered with aluminum foil contained 50 ml of mineral medium and diesel oil at 1.5% (v/v) and/or Triton X-100 (150 or 600 mg/l). Initial inoculum was adjusted to $OD_{600}$ 0.1 ± 0.01 by adding approximately 1 ml of dense cell suspension from the preculture grown aerobically on diesel fuel, and cultivation was carried out at 25 °C at 120 rpm. Each day, a set of three replicates was sacrificially sampled for hydrocarbon analyzes. Cultures lacking biomass served as blanks to account for abiotic loses.

#### 2.4.2. Limited aeration with nitrate

Tightly closed 250-ml Duran-Schott bottles contained 50 ml of mineral medium ($NH_4Cl$ 1.0 was replaced with $NaNO_3$ 1.0 and $Na_2MoO_4$ 0.0005 g/l). Limited aeration was achieved by flushing with nitrogen to remove dissolved oxygen. While this method allows removing most of the oxygen, traces might be left that partially catalyze oxidative attack on surfactant compounds. Cultivation conditions were the same as in the fully aerobic setup, with cultures lacking biomass serving as blanks to account for abiotic loses. An independent set of duplicates run in parallel was set up to for nitrate and nitrite analyzes.

### 2.5. Analytical procedures

#### 2.5.1. GC-FID diesel oil determination

Residual hydrocarbons were extracted with hexane (ISO, 2000) and analyzed with gas chromatography (GC-FID), as described by Owsianiak et al. [20].

#### 2.5.2. Analyzes of nitrate and nitrite

Nitrate and nitrite depletion was determined according to a method previously described by Cyplik et al. [21].

#### 2.5.3. Preparation of the modified Dragendorff reagent

The modified Dragendorff reagent tetraiodobismuthate(III) complex in the presence of barium ions was used to precipitate the ethoxylates. In order to prepare the reagent, 1.2 g of a base bismuth(III) nitrate, 150 ml of water-free acetic acid, 100 g of barium chloride, 50 g of potassium iodide and 5 g of an anhydrous sodium hypophosphite dissolved in a 1000 ml flask filled with redistilled water.

#### 2.5.4. Determination of Triton X-100

The culture broth (3 ml) was centrifuged at 8228 g for 15 min using Costar® Spin-X® centrifuge tube filters (0.22 μm nylon membrane). The cell and diesel oil-free samples were injected into centrifuge tubes (10 ml) and 1 ml of the modified Dragendorff reagent was added. After 10 min the precipitate was separated by centrifugation (25,200 × *g*). The isolated precipitate was rinsed with glacial acetic acid (3 × 1 ml) to remove the residual Dragendorff reagent. 1 ml of the dissolving solution (0.1 M EDTA and 0.5 M sodium acetate) was added to the precipitate. Afterwards 1.2 ml of the complex-forming solution (potassium iodide and sodium hypophosphite in the presence of hydrochloric acid) was added and the solution was transferred into a quartz cuvette. The spectrum of the obtained solutions was measured in a range of 200–900 nm against redistilled water.

#### 2.5.5. HPLC/ESI-MS analysis of Triton X-100 residues

Ethoxylates were separated from water samples by sequential extraction with ethyl acetate (APE) and chloroform (PEG) as previously described by Szymanski and Lukaszewski [23]. The extracts aliquots were used for analyses. The HPLC/ESI–MS analyses were performed using a UltiMate 3000 RSLC (Dionex Sunnyvale, CA, USA), coupled with 4000 QTRAP LC/MS/MS Hybrid Triple Quadrupole/Linear Ion trap mass spectrometry (AB SCIEX Foster City, CA, USA), Hypersil GOLD column (1.9 μm × 100 mm × 2.1 mm, Thermo Fisher Scientific Inc.). The column temperature was 35 °C. The injection volume was 10 μl. Compounds were eluted with 5 mM of ammonium acetate (A) and methanol (B) as the mobile phase, with linear gradient of 30–0% A over 15 min, and flow rate of 200 μl/min. Respective ethoxylated homologues were not separated, which allowed to obtain a signal of each homologue on one mass spectrum. The mass spectra were recorded in the *m*/*z* range of 250–1000 amu, in the first quadrupole and positive-ion mode. The electrospray source potentials were: capillary 3.5 kV, lens 0.65 kV, extractor 10 V and cone voltage 36 V. The source temperature was 120 °C and the desolvation temperature 400 °C. Nitrogen was used as the nebulising and desolvation gas at the flow rates of 100 and 200 l/h, respectively.

### 2.6. Statistical analysis

Data was analyzed via analysis of variance (ANOVA) to detect significant differences between the treated groups and control. The probability of $\alpha$ (type I error) was 5.00% ($P < 0.05$).

Dependence of the amount of Triton X-100 and PEG on the time of the process t in the examined system was described with an exponential equation:

$$y = (y_0 - y_\infty)\exp(-kt) + y_\infty$$

where $y_0$ is the amount of investigated chemicals when time is zero; $y_\infty$ is the amount of investigated chemicals remaining after an infinitely long time period, which represents the non-biodegradable fraction; $k$ is the rate constant, which characterizes the biodegradation process of the biodegradable fraction, expressed in $h^{-1}$. The half-life of the biodegradable fraction of Triton X-100 and PEG was computed as:

$$t_{1/2} = \frac{\ln(2)}{k}$$

Non-linear estimation method according to Levenberg–Marquardt was used (STATISTICA 6.0 PL software).

## 3. Results

### 3.1. Characterization of the microbial consortium

The results of 16S rRNA identification and initial relative abundance tests showed, that the isolated bacterial consortium consists of: *Achromobacter* sp. (6%), *Citrobacter* sp. (10%), *Pseudomonas fluorescens* (2%), *Comamonadaceae* (53%), *Alcaligenes* sp. (10%), *Rhodococcus* sp. (10%), *Sphingomonas* sp. (9%). The isolates belong to species commonly associated with crude oil-contaminated sites [24].

### 3.2. Biodegradation of Triton X-100

#### 3.2.1. Microbial growth on Triton X-100

In order to investigate potential toxicity, the PMF1 consortium was cultivated in the presence of Triton X-100 at concentrations ranging from 1 to 1000 mg/l. Most of the biodegradation studies dealing with surfactant degradation are carried out within the range of APE concentrations 5–1000 mg/l [25–27]. Chen et al. [28] performed isolation of APE degrading strains with the use of 500 mg/l of Triton X-100. Triton X-100 toxicity tests towards the PMF1 consortium showed, that using up to 600 mg/l of this surfactant resulted in a relatively low biomass decrease (approx. 20%

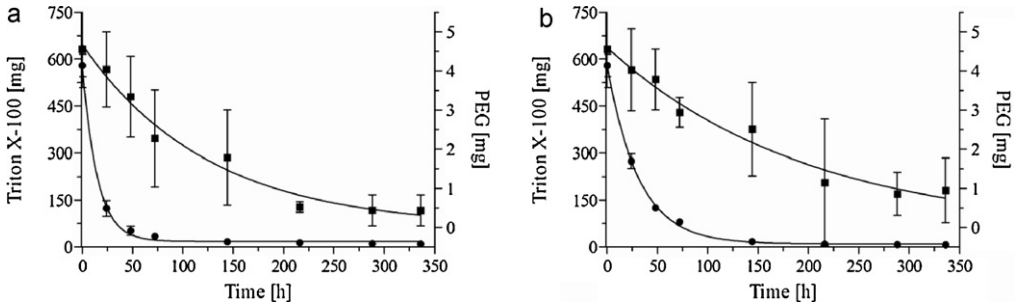


**Fig. 1.** Biodegradation of Triton X-100 and PEG under different aeration conditions. (A) Triton X-100 and PEG, aerobic; (B) Triton X-100 and PEG, anaerobic.

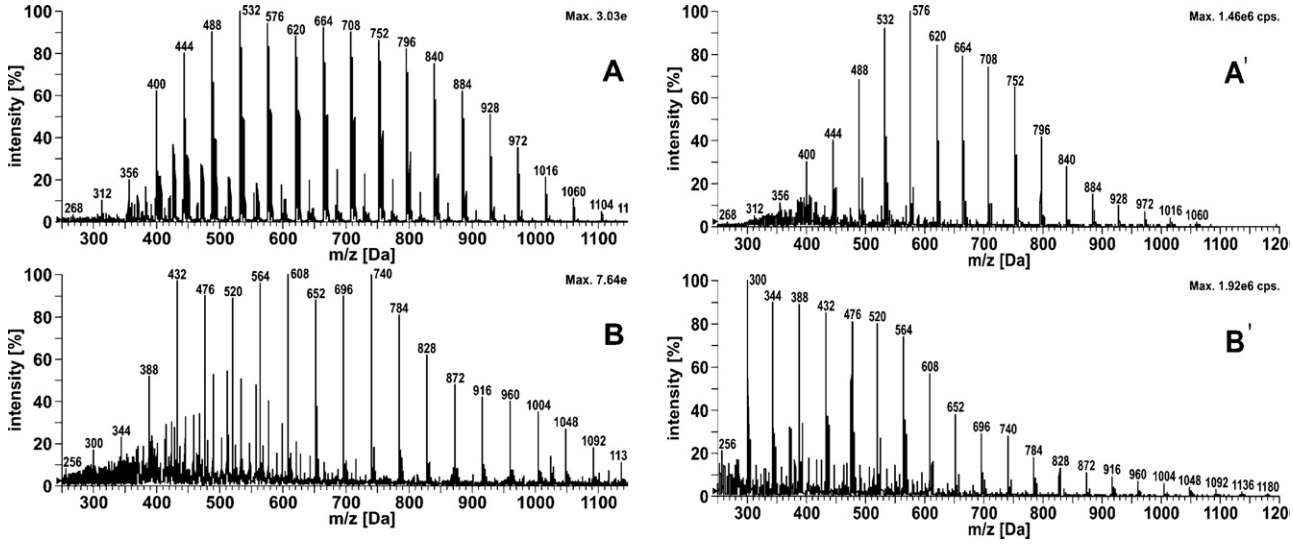


**Fig. 2.** Changes in the degree of ethoxylation profiles for Triton X-100 (A – initial; A′ – final) and PEG (B – initial; B′ – final) homologues before and after the biodegradation process.

decrease compared to growth on 150 mg/l), whereas using higher concentrations caused greater inhibition of microbial growth ($EC_{50}$ at 900 mg/l). For further experiments Triton X-100 was used at a concentration of 600 mg/l, which corresponds to approx. 4× CMC, in order to eliminate potential errors concerning the relative concentration of the biodegradation products.

### 3.2.2. Biodegradation of Triton X-100 and PEG

Possible utilization of commercial Triton X-100 by the bacterial consortium as a primary carbon source was studied under different aeration regimes. The consumption of both APE and PEG could be observed, as shown in Fig. 1.

Biodegradation of Triton X-100 was rapid and was twice as fast for aerobic conditions ($t_{1/2}$ = 10.3 h) compared to anaerobic conditions ($t_{1/2}$ = 21.8 h). During aerobic growth Triton X-100 was degraded within 2 days, while the remaining 30 mg were dissipated slowly. After 3 additional days similar results were observed for anaerobic cultures. The initially slow anaerobic biodegradation of diesel fuel was reflected by slow dissipation of nitrate and production of nitrite. After 5 days the depletion of nitrate was notable and a significant increase in nitrite production could be observed. Although traces of oxygen might have partially catalyzed an oxidative attack on the surfactant molecules, we expect that most of the degradation was carried out using nitrate and nitrite as electron acceptors. Overall, after 14 days of biodegradation the initial concentration of Triton X-100 was reduced from 590 to 6 mg/l under both aeration conditions.

On the other hand PEG compounds were metabolized at a notably slower rate. The initial concentration of PEG was reduced from 4.3 to 0.53 mg/l during aerobic growth. Similar to results obtained for Triton X-100 the degradation rate was decreased under anaerobic conditions, reaching a final concentration of approx. 1.06 mg/l. These results correspond to significantly higher half-life time values (78.4 h during aerobic growth and 116.7 h during anaerobic conditions). As a result, the PEG content increased after the biodegradation process to 8.55 and 17% of initial mass for aerobic and anaerobic conditions accordingly. The employed mathematical model includes the presence of the non-biodegradable fraction (indicated by $y_\infty$), whereas the obtained decay constant ($k$) as well as half-life values were calculated for the biodegradable fractions. The non-biodegradable fractions of Triton X-100 were at 3.1 and 1.7% for aerobic and anaerobic studies, accordingly (see Table 1).

### 3.2.3. Changes in homologue profiles of Triton X-100 and PEG

In order to investigate the details behind the biodegradation mechanisms of Triton X-100 and PEG, the samples were subjected to HPLC/ESI-MS analysis. Based on the obtained results, the changes in the profiles corresponding to shifts in ethoxylation degree (EO) were observed for both APE and PEG homologues.

The profiles representing the degree of ethoxylation of Triton X-100 before and after the biodegradation process changed significantly in terms of composition (Fig. 2). A general decrease of homologues within the ethoxylates mixture was observed, with the sole exception of $APE_8$. This was especially notable for compounds with EO ranging from 4 to 6 and from 13 to 17. The compounds with a higher degree of ethoxylation (20–22) were not detected after the biodegradation process, presumably due to complete degradation.

**Table 1**
First order degradation constants for samples containing Triton X-100 and PEG during aerobic and anaerobic growth.

| Biodegradation experiment | | Model coefficient | | | | |
|---|---|---|---|---|---|---|
| Chemical | Condition | $y_0$ | $y_\infty$ | $k$ [$h^{-1}$] | $t_{1/2}$ [h] | $R^2$ |
| Triton X-100 | Full aeration | 579.3 | 17.81 | 0.067 | 10.34 | 0.993 |
| | Limited aeration with nitrate | 579.1 | 10.07 | 0.032 | 21.81 | 0.994 |
| PEG | Full aeration | 4.711 | 0.139 | 0.008 | 78.36 | 0.820 |
| | Limited aeration with nitrate | 4.629 | 0.216 | 0.006 | 116.7 | 0.825 |

Overall, the intensity of oligomers with low EO numbers increased while the intensity of oligomers with high EO number decreased (Fig. 3).

The profiles of PEG homologues also differ notably (Fig. 2). The compounds with a higher degree of ethoxylation (15–26) were depleted and a significant increase of ethoxylates with 6–8 EO units in the molecule was detected. This resulted in a shift of the PEG profile towards a lower degree of ethoxylation (Fig. 3). Nevertheless, it is worth noticing that no increase or accumulation of short-chained and low-mass molecules (EO 1–2) was detected. This may lead to a conclusion, that the observed changes in the PEG profile occurred as a consequence of the 'central fission' mechanism during Triton X-100 biodegradation, as proposed by Franska et al. [25].

### 3.3. Biodegradation of diesel oil

The microbial consortium was used during diesel oil biodegradation tests with and without Triton X-100 (150 mg/l), which corresponds to the CMC value [18]. Since oxygen may be a limiting factor in terrestrial and aquatic systems, the performance of the consortia with full aeration was compared to the performance with limited oxygen. The concentration of both diesel oil and Triton X-100 was monitored. The results are shown in Fig. 4.

During aerobic growth the diesel oil biodegradation rate was lower for surfactant-supplemented samples (Fig. 4A). After two weeks the diesel oil removal efficiency reached about 80% for pure diesel oil samples, compared to 60% achieved for samples containing diesel oil and Triton X-100. These results fit well with the biodegradation patterns observed during our previous experiments [18]. On the contrary, diesel oil biodegradation during anaerobic growth was much slower, reaching about 30% after 14 days and no apparent differences between samples with and without surfactants were observed (Fig. 4B). This corresponds to a complete depletion of diesel oil within approximately 60–70 days calculated from the slope of initial part of biodegradation curves (days 1–4) by fitting linear regression model. Similar results were obtained by Cyplik et al. [21], where complete degradation of diesel under anaerobic conditions was expected to be accomplished within 75 days.

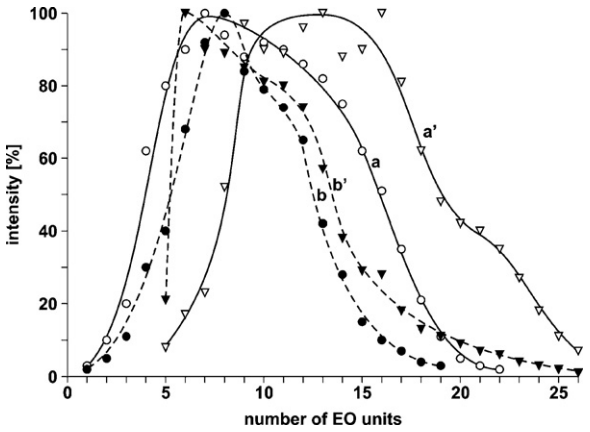


**Fig. 3.** Shifts in the degree of ethoxylation for Triton X-100 (a – initial; b – final) and PEG (a′ – initial; b′ – final) homologues before and after the biodegradation process (on the base of intensity for adequate homologues on ESI-MS spectra).

The concentration of Triton X-100 significantly decreased in all studied samples during the early stages of diesel oil biodegradation. Under anaerobic conditions Triton X-100 was dissipated at a similar rate compared to aerobically grown cultures. In both cases the surfactant content decreased to 20 mg/l after 2 days. Anaerobic biodegradation was accompanied by nitrate depletion and subsequent nitrite production and depletion (data not shown).

### 3.4. Cell surface hydrophobicity

Initially the microbial consortium exhibited hydrophilic properties, with an average CSH value between 20 and 30%. In the presence of surfactant marginal shifts towards both more hydrophobic and hydrophilic properties occurred. Overall no correlation between CSH changes and the addition of Triton X-100 was observed.

## 4. Discussion

In order to investigate the possible inhibition causes reviewed by Paria [4] the influence of Triton X-100 on the microbial consortium was studied. Efroymson and Alexander [29] reported a decrease of the bacterial adherence to hydrocarbons upon the addition of this surfactant, which in turn completely prevented the mineralization of hexadecane dissolved in heptamethylnonane. The analysis of growth on Triton X-100 as a sole carbon source revealed, that the consortium exhibited tolerance towards this surfactant within a range of concentrations 1–1000 mg/l. Even at the highest concentrations the microorganisms displayed growth, which may suggest that the toxic effect of sole APE was marginal. This does not exclude the possible surfactant-induced toxicity enhancement of diesel compounds. Bramwell and Laha [30] suggested, that the mobilization of potentially toxic hydrocarbons due to surfactant solubilisation may also contribute to the observed decrease of biodegradation efficiency. However the rapid dissipation of Triton X-100 (Fig. 1) and no effect of surfactant supplementation observed under anaerobic conditions (Fig. 4B) seem to contradict this concept. Wong et al. [9] reported that the biodegradation rate of phenanthrene by *Bacillus* sp. was decreased in the presence of synthetic surfactants (Tween 80 and Triton X-100). The Authors observed growth on Tween 80 but not on Triton X-100. Although primary utilization of these compounds was possible, the Authors concluded that the observed inhibition of biodegradation was a result of surfactant-induced reduction of direct contact between microbial cells and phenanthrene. Our studies concerning the influence of Triton X-100 on CSH revealed no apparent correlations. This may be attributed to the fact, that the consortium consisted of several different species, and while some of them may be affected by the presence of surfactants, other were resistant. Therefore the effect of the surfactant was not unequivocal. Overall it was concluded, that changes in CSH did not have a significant impact on diesel oil biodegradation efficiency. The isolated microbial consortium was highly hydrophobic, with

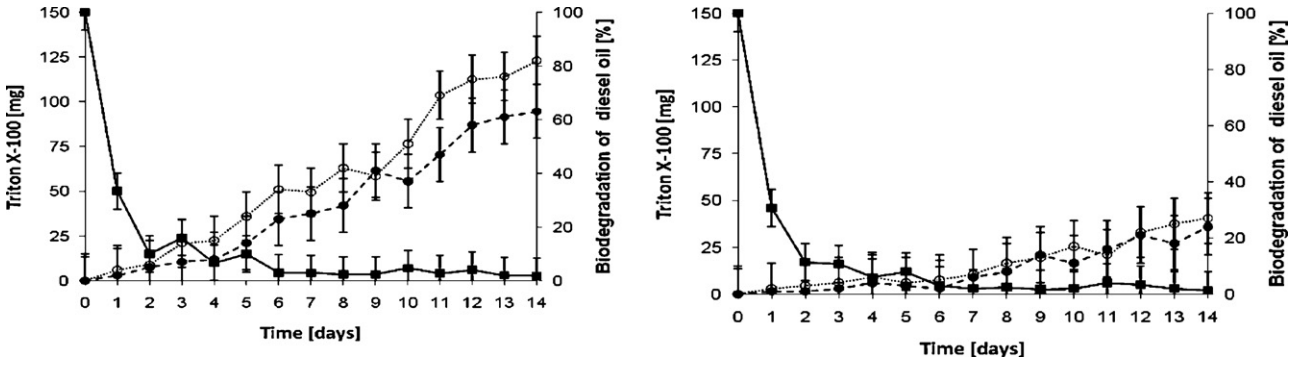


**Fig. 4.** Changes of diesel oil and Triton X-100 concentrations during biodegradation tests under different aeration conditions (A – aerobic; B – anaerobic): ■ – Triton X-100; ● – diesel oil with Triton X-100; ○ – diesel oil.

a tendency to form bacterial biofilms on interfacial boundary. As surfactants tend to accumulate on the water–oil interface, it can be expected, that biodegradation of APE will occur prior to microbial attachment and utilization of oil phase. The results obtained during surfactant-mediated biodegradation of diesel oil proved, that under aerobic conditions the rate of hydrocarbon utilization by the microbial consortium was decreased in the presence of surfactants (Fig. 4A). Since the concentration of surfactant was notably reduced after two days, the possible entrapment of diesel hydrocarbons in micelles contributing to an inhibition of bioavailability also seems unlikely. Therefore it may be concluded that the decrease of diesel oil degradation efficiency in the presence of Triton X-100 during aerobic growth was caused by preferential utilization of surfactants.

The environmental impact of APE and alkylphenols (AP) has been intensively studied over the past years [31,32]. Several surfactant biodegradation studies were carried out using microbial isolates capable of growth solely on ethoxylated compounds. Most of such isolates belong to the *Pseudomonas* and *Sphingomonas* genus [33–35]. Chen et al. [28] studied the *Pseudomonas nitroreducens* TX1 strain, which was able to grow in the presence of up to 100,000 mg/l of Triton X-100.

It has been concluded, that APE undergo three possible biodegradation pathways [36]: terminal attack on the ethoxylene chain, proceeding either by oxidation [28,37] or cleavage [38]; 'central fission', which leads to the separation of AP and PEG molecules [25]; finally, the degradation of alkyl chain is also plausible [26]. Although the utilization of APE by microorganisms has been widely recognized, there are inconsistent reports regarding the degradability of their potential metabolites [39]. Since stepwise oxidation is considered as the most popular mechanism, most researchers observed the formation of short-chained APE with a low molecular mass and carboxylated products as main metabolites. Afterwards, the most plausible step includes the degradation of the alkyl chain, resulting in the emergence of dicarboxylated products [38]. Although alkylphenols are potential intermediates during the biodegradation process, it seems that these compounds are rarely detected during aerobic growth. This may be caused by the fact, that alkylphenols appear to be readily biodegraded into further metabolites, as several recent studies confirmed both alkyl chain oxidation and ring-cleavage mechanisms [40–42]. The studies carried out by Naylor et al. [43] with the use of $^{14}C$ ring-labeled nonylphenol ethoxylates indicated, that upon ring-cleavage the intermediates were most likely incorporated into microbial cells and converted into biomass.

These findings fit well with the results obtained during this study, as no alkylphenol moieties were detected. There was also no trace of either mono- or dicarboxylated products, which may suggest the occurrence of a non-oxidative degradation mechanism, similar to the one suggested by Lu et al. [44]. However the Authors reported an apparent increase of short-chained APE concentrations (EO 1 and 2) after 14 days. On the contrary, during our studies the concentration of these compounds did not differ notably (Fig. 3). On the other hand, a significant increase of the lower EO homologues was observed for PEG compounds, which may suggest a different biodegradation pathway. The obtained results suggest that the dissipation of Triton X-100 most likely occurred as a consequence of the 'central fission' cleavage, which would lead to the emergence of PEG and alkylphenols. The presence of AP moieties was not observed during HPLC–MS studies. It might be plausible, that these compounds were utilized by hydrocarbon degrading soil isolates used in this study. It was concluded, that upon the separation of hydrophile and hydrophobe, PEG were subjected to further degradation, possibly via sequential cleavage and depolymerization. However more studies are needed to elucidate the details behind the biodegradation mechanism of APE by the PMF1 consortium.

Although the biodegradation of PEG is considered to occur at a relatively slow rate due to high energy required to cleave the ether bond [45], there are numerous studies indicating, that these compounds may serve as a carbon source for several bacterial species [46,47].

The obtained results bring about several implications. Firstly, the desired effect of surfactant-mediated solubilisation during biodegradation of petroleum hydrocarbons may be decreased due to preferential utilization of surfactant molecules by microbial consortia. Secondly, although the microbial consortium was isolated from a petroleum-contaminated site and cultivated on hydrocarbons, it exhibited the ability to utilize ethoxylated alkylphenols. After the biodegradation of APE, the alkylphenol moieties were not detected. This may suggest, that both APE and AP were utilized by the microbial consortium. PCR-DGGE tests showed that all of the consortium members were present after the surfactant-mediated biodegradation of diesel oil, which confirmed that the microbial consortium was stable. However, the presence of surfactants may result in uneven involvement of specific microbial populations in the biodegradation processes. Such quantitative changes among species may impact diesel oil biodegradation efficiency. Further studies will focus on assessing the quantitative changes in the microbial community structure, which may occur as a response to the presence of surfactants.

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## References


[1] B. Angelova, H.P. Schmauder, Lipophilic compounds in biotechnology – interactions with cells and technological problems, J. Biotechnol. 67 (1999) 13–32.
[2] H. Harms, T.N.P. Bosma, Mass transfer limitation of microbial growth and pollutant degradation, J. Ind. Microbiol. Biotechnol. 18 (1997) 97–105.
[3] D. Randazzo, D. Berti, F. Briganti, P. Baglioni, A. Scozzafava, P.D. Gennaro, E. Galli, G. Bestetti, Efficient polycyclic aromatic hydrocarbons dihydroxylation in direct micellar systems, Biotechnol. Bioeng. 74 (2001) 240–248.
[4] S. Paria, Surfactant-enhanced remediation of organic contaminated soil and water, Adv. Colloid Interface Sci. 138 (2008) 24–58.
[5] L. Chrzanowski, E. Kaczorek, E. Pijanowska, A. Olszanowski, The relation between rhamnolipid adsorption on yeast and bacterial strains, hydrophobicity and hydrocarbon biodegradation, Fresenius Environ. Bull. 15 (2006) 682–686.
[6] D.G. Brown, P.R. Jaffe, Effects of nonionic surfactants on cell surface hydrophobicity and apparent Hamaker constant of a *Sphingomonas* sp., Environ. Sci. Technol. 40 (2006) 195–201.
[7] M. Gerginova, J. Manasiev, N. Shivarova, Z. Alexieva, Influence of various phenolic compounds on phenol hydroxylase activity of a *Trichosporon cutaneum* strain, Z. Naturforsch. C: J. Biosci. 62 (2007) 83–86.
[8] A. Tiehm, Degradation of polycyclic aromatic hydrocarbons in the presence of synthetic surfactants, Appl. Environ. Microbiol. 60 (1994) 258–263.
[9] J.W.C. Wong, M. Fang, Z. Zhao, B. Xing, Effect of surfactants on solubilization and degradation of phenanthrene under thermophilic conditions, J. Environ. Qual. 33 (2004) 2015–2025.
[10] H. Yu, L. Zhu, W. Zhou, Enhanced desorption and biodegradation of phenanthrene in soil–water systems with the presence of anionic–nonionic mixed surfactants, J. Hazard. Mater. 142 (2007) 354–361.
[11] Z. Liu, A.M. Jacobson, R.G. Luthy, Biodegradation of naphthalene in aqueous nonionic surfactant systems, Appl. Environ. Microbiol. 61 (1995) 145–151.
[12] P.A. Willumsen, U. Karlson, P.H. Pritchard, Response of fluoranthene-degrading bacteria to surfactants, Appl. Microbiol. Biotechnol. 50 (1998) 475–483.
[13] J.D. Van Hamme, O.P. Ward, Physical and metabolic interactions of *Pseudomonas* sp. strain JA5–B45 and *Rhodococcus* sp. strain F9–D79 during growth on crude oil and effect of a chemical surfactant on them, Appl. Environ. Microbiol. 67 (2001) 4874–4879.
[14] L. Chrzanowski, L.Y. Wick, R. Meulenkamp, M. Kaestner, H.J. Heipieper, Rhamnolipid biosurfactants decrease the toxicity of chlorinated phenols to *Pseudomonas putida* DOT-T1E, Lett. Appl. Microbiol. 48 (2009) 756–762.
[15] L. Chrzanowski, M. Owsianiak, A. Szulc, R. Marecik, A. Piotrowska-Cyplik, A.K. Olejnik-Schmidt, J. Staniewski, P. Lisiecki, F. Ciesielczyk, T. Jesionowski, H.J. Heipieper, Interactions between rhamnolipid biosurfactants and toxic chlorinated phenols enhance biodegradation of a model hydrocarbon-rich effluent, Int. Biodeter. Biodegr. 65 (2011) 605–611.
[16] Z. Dai, Z. Wang, J.H. Xu, H. Qi, Assessing bioavailability of the solubilization of organic compound in nonionic surfactant micelles by dose–response analysis, Appl. Microbiol. Biotechnol. 88 (2010) 327–339.
[17] Z. Wang, Bioavailability of organic compounds solubilized in nonionic surfactant micelles, Appl. Microbiol. Biotechnol. 89 (2011) 523–534.
[18] M. Owsianiak, A. Szulc, Ł. Chrzanowski, P. Cyplik, M. Bogacki, A.K. Olejnik-Schmidt, H.J. Heipieper, Biodegradation and surfactant-mediated biodegradation of diesel fuel by 218 microbial consortia are not correlated to cell surface hydrophobicity, Appl. Microbiol. Biotechnol. 84 (2009) 545–553.
[19] S. Oku, M. Nishiyama, Y. Takao, Selective isolation of bacteria from soil with hydrophobic materials, World J. Microbiol. Biotechnol. 27 (2011) 1941–1945.
[20] M. Owsianiak, L. Chrzanowski, A. Szulc, J. Staniewski, A. Olszanowski, A.K. Olejnik-Schmidt, H.J. Heipieper, Biodegradation of diesel/biodiesel blends by a consortium of hydrocarbon degraders: Effect of the type of blend and the addition of biosurfactants, Bioresour. Technol. 100 (2009) 1497–1500.
[21] P. Cyplik, M. Schmidt, A. Szulc, R. Marecik, P. Lisiecki, H.J. Heipieper, M. Owsianiak, M. Vainshtein, L. Chrzanowski, Relative quantitative PCR to assess bacterial community dynamics during degradation of diesel and biodiesel fuels under various aeration conditions, Bioresour. Technol. 102 (2011) 4347–4352.
[22] H.J. Heipieper, B. Loffeld, H. Keweloh, J.A.M. de Bont, The cis/trans isomerization of unsaturated fatty acids in *Pseudomonas putida* S12: an indicator for environmental stress due to organic compounds, Chemosphere 30 (1995) 1041–1051.
[23] A. Szymanski, Z. Lukaszewski, Determination of poly(ethylene glycols) in environmental samples by the indirect tensammetric method, Analyst 121 (1996) 1897–1901.
[24] R.C. Prince, The microbiology of marine oil spill bioremediation, in: B. Oliver, M. Magot (Eds.), Petroleum Microbiology, ASM Press, Washington, DC, 2005, pp. 317–335.
[25] M. Franska, R. Franski, A. Szymanski, Z. Lukaszewski, A central fission pathway in alkylphenol ethoxylate biodegradation, Water Res. 37 (2003) 1005–1014.
[26] T. Tanghe, W. Dhooge, W. Verstraete, Formation of the metabolic intermediate 2,4,4-trimethyl-2-pentanol during incubation of a *Sphingomonas* sp. strain with the xeno-estrogenic octylphenol, Biodegradation 11 (2000) 11–19.
[27] C. Planas, J.M. Guadayol, M. Droguet, A. Escalas, J. Rivera, J. Caixach, Degradation of polyethoxylated nonylphenols in a sewage treatment plant. Quantitative analysis by isotopic dilution-HRGC/MS, Water Res. 36 (2002) 982–988.
[28] H.J. Chen, G.L. Guo, D.H. Tseng, C.L. Cheng, S.L. Huang, Growth factors, kinetics and biodegradation mechanism associated with *Pseudomonas nitroreducens* TX1 grown on octylphenol polyethoxylates, J. Environ. Manage. 80 (2006) 279–286.
[29] R.A. Efroymson, M. Alexander, Biodegradation by an Arthrobacter species of hydrocarbons partitioned into an organic solvent, Appl. Environ. Microbiol. 57 (1991) 1441–1447.
[30] D.P. Bramwell, S. Laha, Effects of surfactant addition on the biomineralization and microbial toxicity of phenanthrene, Biodegradation 11 (2000) 263–277.
[31] J. Zhao, G. Zhang, Y. Qi, Y. Zhao, Aerobic biodegradation of alkylphenol ethoxylates, Bioresour. Technol. 97 (2006) 2478–2480.
[32] G.M. Klecka, C.A. Staples, C.G. Naylor, K.B. Woodburn, B.S. Losey, C8- and C9-alkylphenols and ethoxylates. II. Assessment of environmental persistence and bioaccumulation potential, Hum. Ecol. Risk Assess. 14 (2008) 1025–1055.
[33] E. Nishio, Y. Ichiki, H. Tamura, S. Morita, K. Watanabe, H. Yoshikawa, Isolation of bacterial strains that produce the endocrine disruptor, octylphenol diethoxylates, in paddy fields, Biosci. Biotechnol. Biochem. 66 (2002) 1792–1798.
[34] E. Nishio, H. Yoshikawa, M. Wakayama, H. Tamura, S. Morita, Y. Tomita, Isolation and identification of *Sphingomonas* sp. that yields tert-octylphenol monoethoxylate under aerobic conditions, Biosci. Biotechnol. Biochem. 69 (2005) 1226–1231.
[35] Y.W. Lin, G.L. Guo, H.C. Hsieh, S.L. Huang, Growth of *Pseudomonas* sp. TX1 on a wide range of octylphenol polyethoxylate concentrations and the formation of dicarboxylated metabolites, Bioresour. Technol. 101 (2010) 2853–2859.
[36] C.A. Staples, G.M. Klecka, C.G. Naylor, B.S. Losey, C8- and C9-alkylphenols and ethoxylates. I. Identity, physical characterization, and biodegradation pathways analysis, Hum. Ecol. Risk Assess. 14 (2008) 1007–1024.
[37] H. Sato, A. Shibata, Y. Wang, H. Yoshikawa, H. Tamura, Characterization of biodegradation intermediates of nonionic surfactants by MALDI-MS. 2. Oxidative biodegradation profiles of uniform octylphenol polyethoxylate in 18O-labeled water, Biomacromolecules 4 (2003) 46–51.
[38] J. Lu, Q. Jin, Y. He, J. Wu, J. Zhao, Biodegradation of nonylphenol ethoxylates by *Bacillus* sp. LY capable of heterotrophic nitrification, FEMS Microbiol. Lett. 280 (2008) 28–33.
[39] J. Montgomery-Brown, M. Reinhard, Occurrence and behavior of alkylphenol polyethoxylates in the environment, Environ. Eng. Sci. 20 (2003) 471–486.
[40] P.F.X. Corvini, J. Hollender, R. Ji, S. Schumacher, J. Prell, G. Hommes, U. Priefer, R. Vinken, A. Schaffer, The degradation of alpha-quaternary nonylphenol isomers by *Sphingomonas* sp. strain TTNP3 involves a type II ipso-substitution mechanism, Appl. Microbiol. Biotechnol. 70 (2006) 114–122.
[41] P.F.X. Corvini, A. Schaffer, D. Schlosser, Microbial degradation of nonylphenol and other alkylphenols – our evolving view, Appl. Microbiol. Biotechnol. 72 (2006) 223–243.
[42] J. Montgomery-Brown, Y. Li, W.H. Ding, G.M. Mong, J.A. Campbell, M. Reinhard, NP1EC degradation pathways under oxic and microxic conditions, Environ. Sci. Technol. 42 (2008) 6409–6414.
[43] C.G. Naylor, C.A. Staples, G.M. Klecka, J.B. Williams, P.T. Varineau, C. Cady, Biodegradation of [14C] ring-labeled nonylphenol ethoxylate, Arch. Environ. Contam. Toxicol. 51 (2006) 11–20.
[44] J. Lu, Q. Jin, Y. He, J. Wu, W. Zhang, J. Zhao, Biodegradation of nonylphenol polyethoxylates by denitrifying activated sludge, Water Res. 42 (2008) 1075–1082.
[45] R. Marchal, E. Nicolau, J.P. Ballaguet, F. Bertoncini, Biodegradability of polyethylene glycol 400 by complex microfloras, Int. Biodeter. Biodegr. 62 (2008) 384–390.
[46] M. Bernhard, J.P. Eubeler, S. Zok, T.P. Knepper, Aerobic biodegradation of polyethylene glycols of different molecular weights in wastewater and seawater, Water Res. 4 (2008) 4791–4801.
[47] S.E. Sharvelle, J. Garland, M.K. Banks, Biodegradation of polyalcohol ethoxylate by a wastewater microbial consortium, Biodegradation 19 (2008) 215–221.