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Research Article

BACTERIAL POPULATION DYNAMICS IN WASTE OILY EMULSIONS FROM THE METAL-PROCESSING INDUSTRY

Paweł Kaszycki[1], Paulina Supel[1], Przemysław Petryszak[1]

[1] Department of Biochemistry, Institute of Plant Biology and Biotechnology, Faculty of Horticulture, University of Agriculture in Kraków, 29. Listopada Ave., 31-425 Kraków, Poland, e-mail: p.kaszycki@ogr.ur.krakow.pl; p.supel@ogr.ur.krakow.pl; p.petryszak@ogr.ur.krakow.pl

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ABSTRACT

Oil-containing wastewaters are regarded as main industrial pollutants of soil and water environments. They can occur as free-floating oil, unstable or stable oil-in-water (O/W) emulsions, and in the case of extreme organic load, as water-in-oil (W/O) emulsions. In this study two types of oily effluents, a typical O/W emulsion marked as E1 and a W/O emulsion E2, both discharged by local metal processing plants were examined to test their toxicity to microbial communities and the ability to serve as nutrient sources for bacterial growth. The organic contaminant load of the samples was evaluated on the basis of chemical oxygen demand (COD) parameter values and was equal to 48 200 mg $O_2 \cdot dm^{-3}$ and >300 000 mg $O_2 \cdot dm^{-3}$ for E1 and E2, respectively. Both emulsions proved to be non toxic to bacterial communities and were shown to contain biodiverse autochthonous microflora consisting of several bacterial strains adapted to the presence of xenobiotics (the total of $1.36 \cdot 10^6$ CFU$\cdot cm^{-3}$ and $1.72 \cdot 10^5$ CFU$\cdot cm^{-3}$ was determined for E1 and E2, respectively). These indigenous bacteria as well as exogenously inoculated specialized allochthonous microorganisms were biostimulated so as to proliferate within the wastewater environment whose organic content served as the only source of carbon. The most favorable cultivation conditions were determined as fully aerobic growth at the temperature of 25 ºC. In 9 to 18 day-tests, autochthonous as well as bioaugmented allochthonous bacterial population dynamics were monitored. For both emulsions tested there was a dramatic increase (up to three orders of magnitude) in bacterial frequency, as compared to the respective initial values. The resultant high biomass densities suggest that the effluents are susceptible to bioremediation. A preliminary xenobiotic biodegradation test confirmed that mixed auto- and allochthonous bacterial consortia obtained upon inoculation of the samples with microbiocenoses preselected for efficient hydrocarbon biodegradation led to a decrease in the organic pollution level.

Keywords: bioremediation, biostimulation, oily emulsions, petroleum-derived contaminants, autochthonous microorganisms, microbial consortia.

INTRODUCTION

Oily emulsions are common waste products of petrochemical, metal and food processing industries as well as transportation and domestic activities [Coca et al. 2011, Abdel-Raouf 2012]. These pollutants bear severe environmental risk due to their high organic load and chemical-physical characteristics that endanger human health and have strong negative impact on soil and aquatic life. Among these, oily effluents discharged by metal manufacturing facilities are particularly hazardous since they are chemically very complex [Effluent Limitations... 2003, Coca et al. 2011] and may be colonized with a variety of microorganisms (bacteria and fungi), including human pathogens [Saha, Donofrio 2012]. Four main types of fluids are used in metal processing technologies i.e. straight (neat) oils, soluble oils, semisynthetic fluids, and synthetic fluids

[Geier, Lessmann 2006, MacAdam et al. 2012]. Straight oils are usually very dense, water non-miscible preparations, composed primarily of mineral oil with numerous organic additives including surfactants [Geier, Lessmann 2006, Coca et al. 2011]. When discharged, they can become admixed with the aqueous phase, and finally form water-in-oil (W/O) emulsions in which oil acts as a dispersion medium. The latter three fluid types make up a dominating category of the so-called water-based fluids, applied as oil-in-water (O/W) emulsion suspensions in which water serves as a continuous phase, constituting up to 95% of the emulsion volume. Mineral oil-based O/W emulsions may contain a number (up to 60 of the total of 300 identified) of chemical additives [Rabenstein et al. 2009] that include emulsifiers, corrosion inhibitors, antifoam and anti-fog substances, heavy metal ions, pressure agents, stabilizers, buffers, lubricants, and, in particular, a variety of biocides [Geier, Lessmann 2006, Bakalova et al. 2008]. Biocides are added to reduce the risk of microbial contamination that often affects workers at their workplaces [Geier, Lessmann 2006] and causes metalworking fluid biodeterioration and emulsion breakdown [da Silva et al. 2001, Theaker, Thompson 2010].

Numerous microbial species were found to inhabit metalworking fluids, especially the water-based emulsions [van der Gast et al. 2003, Bakalova et al. 2007, Saha, Donofrio 2012]. Furthermore, many authors point to a very limited strain detectability employing standard microbiological culture-based techniques due to the presence (up to 95% of the total population) of the so-called VBNC-type strains (*viable but non-cultivable*). Thus, alternative, culture-independent identification methods are being proposed [van der Gast et al. 2003, Selvaraju et al. 2008, Gilbert et al. 2010, Saha, Donofrio 2012, Trafny 2013]. Many of the hitherto identified strains were found strongly pathogenic, *e.g.* those of the genera *Mycobacterium, Klebsiella, Aspergillus* and *Staphylococcus* [Bakalova et al. 2007, Wang et al. 2007, Saha, Donofrio 2012]. It should be stressed here, however, that the preventive usage of biocidal chemicals may dramatically enhance the resultant environmental toxicity of the emulsions and can make the biological treatment of these wastewaters impossible. On the other hand, the occurrence of non-pathogenic strains of such genera as *Pseudomonas, Bacillus, Agrobacterium, Acinetobacter, Alcaligenes, Stenotrophomonas, Methylobacterium, Rhodococcus* and many others [van der Gast et al. 2003, Saha, Donofrio 2012] might be beneficial in terms of the potential use of these autochthons for bioremediation purposes [Cheng et al. 2005, Bakalova et al. 2007, Saha, Donofrio 2012].

Together with the microbiological risk, the presence of aggressive chemical agents and oil-derived xenobiotics in water-miscible emulsions makes these fluids particularly hazardous. They can cause adverse health effects for humans, and when released to the environment they become strongly ecotoxic, negatively affecting natural ecosystems [Tillie-Leblond et al. 2011, Mirer 2010, Grijalbo et al. 2013, Lazarević et al. 2013, Gerulova et al. 2011].

Taking into account the above information regarding great chemical variability, broad range of contamination load, and microbiological diversity of the waste emulsions discharged by the metal industry facilities it is of high importance to perform appropriate toxicity and biodegradability testing of effluents originating from different sources. The results of such tests should bring valuable data necessary to elaborate optimal disposal/cleanup strategies.

The aim of this study was to test whether the mineral oil-based waste effluents of the metal-processing industry, generated upon technological processes as oily emulsions with high organic load, can be biologically treated using auto- and/or allochthonous bacterial communities. This aim was achieved by examining toxicity of the emulsions, evaluating their colonization with indigenous microorganisms, and testing both the conditions and growth potential of autochthonous and allochthonous bacteria.

MATERIALS AND METHODS

Oily emulsions

Two types of oily emulsions discharged by local metal processing plants were examined for their toxicity, occurrence of indigenous microflora and susceptibility to colonization with allochthonous microorganisms:

- **E1 -** a spent metalworking fluid (MWF) consisting of mixed hydrocarbons and surfactants as well as other chemical additives, a typical oil-in-water (O/W) emulsion discharged from a metal cutting and forming process. It was a whitish, dense, homogeneous liquid, with no smell, characterized by a high organic contaminant

load determined as the chemical oxygen demand parameter (COD) of 48 200 mg $O_2 \cdot dm^{-3}$.

- **E2 -** an oily effluent generated upon metal cooling and machine tool lubrication, discharged as a water-in-oil emulsion (W/O) with a very high organic content (COD above 300 000 mg $O_2 \cdot dm^{-3}$). The sample was light grey with dark surface smudges and detectable smell of petroleum-like products, a dense liquid, heterogeneous and unstable, easily undergoing spontaneous phase separation.

The detailed characteristics of both emulsions are given in Table 1.

Microbiological monitoring

Emulsion samples were typically incubated at aerobic conditions in 300 cm^3 flasks rotary-shaken with laboratory thermostated shakers, each flask containing 50 cm^3 of a tested fluid. Specific experimental setups are given in the text.

Determination of bacterial frequency, bacterial survivability and cell population dynamics were done using standard microbiological techniques based on a Koch method of culture surface plating onto agar-solidified optimal media (2.5% enriched agar, Biocorp, Poland). To check for the presence of yeast and other fungi, a Sabouraud medium supplemented with an antibiotic cocktail (Biocorp, Poland) was used. CFU (colony-forming units) were counted after 3 days of incubation at 37 °C. Cell frequencies were expressed as $CFU \cdot cm^{-3}$ of aqueous samples. In order to obtain homogeneous cell suspensions, prior to dilutions, bacterial samples were sonicated under mild conditions for 10 min with a laboratory ultrasound washer (UN-2 Unitra/Unima, Poland).

Source of allochthonous microorganisms

In the tests involving allochthonous microorganisms the studied emulsions were inoculated with the microbial consortium ZB-01 developed in Biochemistry Department of University of Agriculture in Krakow (volume proportions given in the text). This aerobic bacterial biocenosis has been widely used in environmental bioremediation projects to trigger and/or stimulate biodegradation of petroleum-oil hydrocarbons in contaminated waters and soils [Kaszycki et al. 2001, Kołoczek, Kaszycki 2006, Kaszycki et al. 2010, Kaszycki et al. 2011]. It consists of a number of environmental strains, selected and isolated over years from sites heavily polluted with organic compounds. Typically, the initial cell density of the consortium used as *inoculum* exceeded $5 \cdot 10^8$ $CFU \cdot cm^{-3}$).

Toxicity tests of waste emulsions

Emulsion toxicity tests were done based on the survivability determination of the ZB-01 bacterial consortium (see the description above) inoculated to either E1 or E2. Two variant volume proportions (effluent: ZB-01) were examined, that is 1:1 and 9:1. The samples were then incubated for 7 and 9 days, respectively, and checked for the cell population density.

Chemical oxygen demand determination

The COD analyses were carried out employing a dichromate procedure according to the protocols of standardized automated ampoule-based mineralization-analytical system Hach-Lange DR5000 (Germany).

Extraction of organic substances and determination of contamination load

The organic content of emulsion samples was extracted using petroleum ether (fractions boiling at 40–60 °C, POCh, Poland). Briefly, a 10 cm^3 sample was weighed, thoroughly mixed, acidified with 1 cm^3 18% HCl and dried with approx.

Table 1. Characteristics of the waste oily emulsions

Sample No.	Emulsion type	Source (industrial process)	pH	COD [mg $O_2 \cdot dm^{-3}$]	Ether-extractable organic fraction [$mg \cdot dm^{-3}$]	Occurrence of bacteria [$CFU \cdot cm^{-3}$]	Bacterial colony morpho-types	Occurrence of fungi & yeast [$CFU \cdot cm^{-3}$]
E1	Oil-in-Water (O/W)	mineral-oil based metal-working fluid (metal cutting and forming)	6.67	48 200	10 100	$1.36 \cdot 10^6$	4	0
E2	Water-in-Oil (W/O)	mixture of oils from machine lubricating and cooling systems	7.20	>300 000*	290 895	$1.72 \cdot 10^5$	3	0

Explanation: * – a value beyond the measurable limit.

30 g of anhydrous $MgSO_4$. The sample was then placed in an extraction thimble made of nitrocellulose (Sigma-Aldrich) and extracted (for approximately 6 h) in a 65 cm^3 Soxhlet apparatus at 40–60 extraction cycles. All the extractions were carried out at least in duplicates. After evaporating the excess solvent at 85 °C, the flasks containing extracted material were dried at 105 °C for 1.5 h, then cooled in a vacuum desiccator and finally, the residual material (high-boiling organic fraction) was weighed.

All the chemicals were of analytical grade. In microbiological analyses fully sterile conditions were applied. The mean experimental error was established based on repetitive tests as 10%, 12% and 15% for the analyses of COD, organic substances content and for the determination of bacterial cell population, respectively.

RESULTS AND DISCUSSION

Occurrence of indigenous microflora

Both emulsions were shown to contain autochthonous microflora consisting of several bacterial strains adapted to the presence of xenobiotics. The total of $1.36 \cdot 10^6$ CFU·cm^{-3} and $1.72 \cdot 10^5$ CFU·cm^{-3} were determined for E1 and E2, respectively. Although no detailed strain identification was made, general biodiversity of the indigenous bacteria could be preliminary evaluated based on the number of distinct colony morphotypes. Four morphotypes found for E1, and three for E2 might suggest a relatively low biodiversity when compared to 8–15 colony types typically detected on plates with the multispecies microbial ZB-01 consortium (not shown). However, it should be pointed out here that many of the indigenous strains might belong to a group of non-cultivable (VBNC) microorganisms, which is a finding typical for metalworking fluids [van der Gast et al. 2003, Gilbert et al. 2010, Saha, Donofrio 2012, Trafny 2013]. In such a case the observed biodiversity would be limited and the total number of bacteria reduced due to detection of culturable strains only. Interestingly, the studied samples lacked in any yeast or fungi as could be excluded by the appropriate tests employing fungi-selective media (Table 1). Note that no fungi occurred throughout all the tests performed later.

As cited earlier, a number of studies on metalworking fluids collected from different industrial sources proved the occurrence of biodiverse indigenous microflora. According to other authors, the measured population densities varied depending on the emulsion source and ranged from 10^5 to 10^7 CFU·cm^{-3} [Veillette et al. 2004, Liu et al. 2010, Saha, Donofrio 2012] with one reported case of over 10^8 CFU·cm^{-3} [Mattsby-Baltzer et al. 1989]. Unlike our observations, fungi (mainly yeast and molds) were also detected in some samples [Liu et al. 2010, Saha, Donofrio 2012].

Toxicity tests

As might be expected from the presence of numerous autochthonous bacteria, none of the emulsions were found toxic to exogenously applied microbial consortia. Upon inoculation with the ZB-01 biocenosis, neither short-term (a 2-day toxicity test, not shown) nor long-term observations (7–9 days, see Table 2 and Figure 1) revealed any toxic effect that would lead to the loss of bacterial survivability.

Table 2. Bacterial cell population densities in emulsions E1 and E2 admixed (1:1 v/v) with the ZB-01 preparation, as determined immediately upon inoculation ("start") and after 7-day incubation at 25 °C ("end")

Sample	Start [CFU · cm^{-3}]	End (7 days) [CFU · cm^{-3}]
E1	$1.21 \cdot 10^8$	$1.48 \cdot 10^9$
E2	$1.22 \cdot 10^8$	$2.24 \cdot 10^9$

On the contrary, a strong tendency was observed for further proliferation of the allochthonous (together with autochthonous) bacteria over time for both of the examined inocula variants. The above effect is shown in Table 2 and in Figure 1 where the oil samples were inoculated at high and low initial densities (that is, ZB-01 admixed at volume proportions 1:1 and 1:9), respectively. The above results indicate that the lack of toxicity combined with microorganism biostimulation by aeration and shaking enabled bacteria to efficiently assimilate and/or biodegrade heterogeneous xenobiotics present in the treated samples. Apparently both of the studied waste effluents had not been supplemented with any biocidal agents, which might result in disabling possibility of biological treatment. Our findings are thus very important in terms of elaborating the most effective oily-waste management method since the results strongly suggest that the effluents might be susceptible to biological remediation.

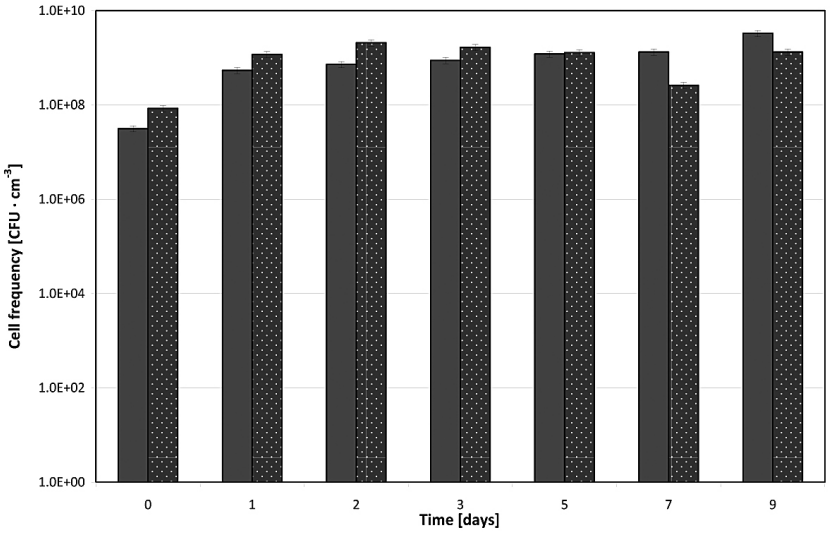

Figure 1. Emulsion toxicity test (E1 – solid bars and E2 – dotted bars): based on bacterial population observations in the samples inoculated with the ZB-01 consortium (diluted 10×)

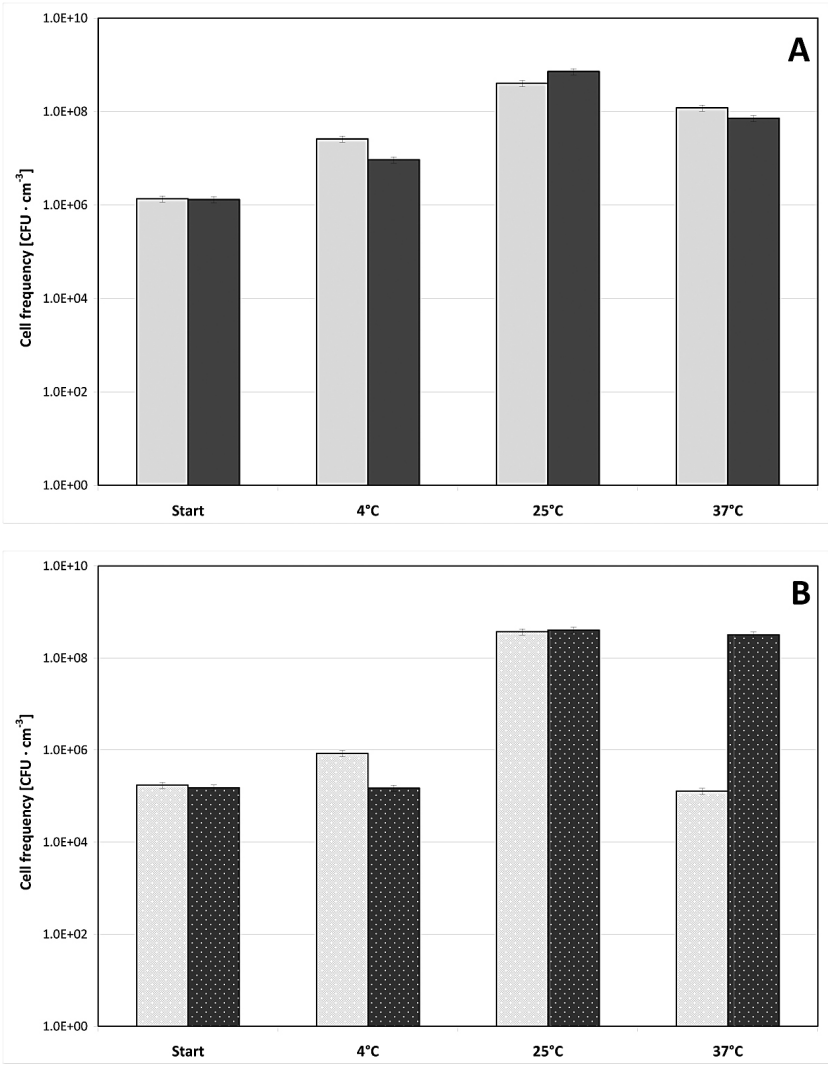

Figure 2. Temperature dependence of bacterial population determined in the E1 (A) and E2 (B) emulsions upon 6-day cultivation at 4 °C, 25 °C, and 37 °C; light and dark bars correspond to the frequency of autochthons (uninoculated samples), and autochthons + allochthons (samples inoculated with ZB-01 at 1000× dilution), respectively

Biostimulation of auto- and allochthonous microflora

In another set of experiments both the indigenous bacteria as well the introduced allochthonous microorganisms inoculated at 1000× dilution were biostimulated so as to proliferate within the emulsion wastewater environments. The cultivation conditions were described in Materials and Methods section. Since no trophic additives were supplemented it is inferred that the organic content of each emulsion served as the only source of carbon. Figure 2 presents the results of the test of temperature optima for bacteria in E1 and E2 (Figure 2 A and B, respectively). In a 6-day incubation at variant temperatures of 4 °C, 25 °C, and 37 °C a profound increase (three to four orders of magnitude) of both autochthonous and allochthonous bacterial frequency was observed, as compared to the initial population value (marked as "start"). For both effluents, the growth-stimulatory effect was pronounced the most at 25 °C and therefore any further testing was done at this temperature.

In a long-term (18 day) systematic test, bacterial cell population dynamics was monitored at 25 °C upon cultivation as described above. The results presented in Figure 3 confirm earlier data indicating bacterial growth within emulsions at favorable conditions. Furthermore, at least for the case of E1 it can be clearly seen that the introduction of allochthonous strains (dark bars) led to significantly larger microbial biomass density relative to uninoculated samples (light bars). The latter observation indicates that in attempts to elaborate a biological treatment method the bioaugmented process should be preferably considered.

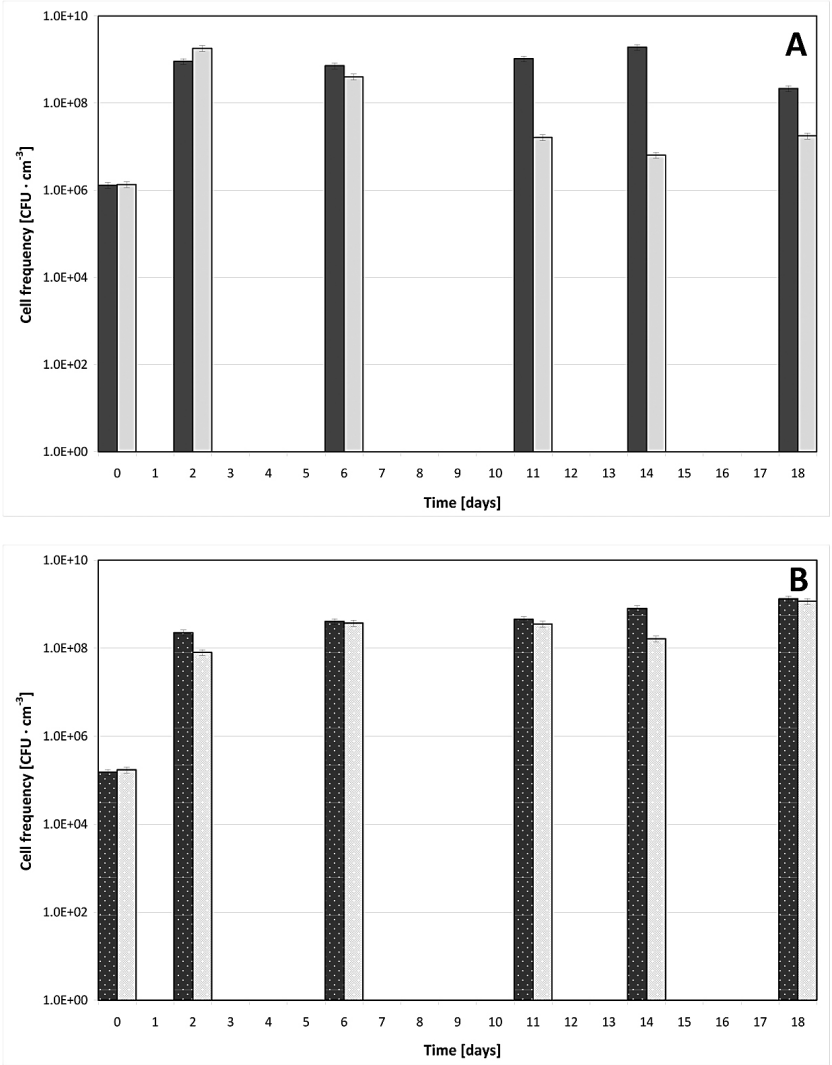

Figure 3. Bacterial cell population dynamics of the E1 (A) and E2 (B) emulsions as determined upon long-term cultivation at 25 °C; light and dark bars represent the uninoculated and inoculated samples, respectively, as in Figure 2

Preliminary analysis of E1 biodegradability

In order to check whether the observed dramatic increase of bacterial density correlated with the drop of the organic load due to xenobiotic carbon utilization, the E1 sample collected at the end of the test (day 18) was analyzed for the ether-extractable organic fraction content and COD (Figure 4 A and B, respectively) and compared with the initial value of untreated emulsion. Note that E2 was not examined due to its extreme load with organic compounds. In Figure 4 a decrease of both parameters can be seen, although the data obtained for COD (Figure 4 B) bear poor statistic reliability due to high experimental error and need to be further confirmed. At the same time the results presented in Figure 4 suggest that higher activity was revealed for the mixed bacterial consortium formed by both autho- and inoculated allochthons. Taken together these preliminary data, some optimization actions should be taken to improve the kinetics and the final yield of bioremediation process. Such actions are now planned to include the dilution of the waste effluent prior to biodegradation as well as inoculation with higher density of allochthonous bacteria. The results will be reported in a separate paper.

CONCLUSION

- The tested oily emulsions generated as waste products of metal industries are non toxic to bacteria and may thus be microbiologically treated.
- They are populated with biodiverse autochthonous bacterial species. These bacteria are well adapted to the emulsion environment and for that reason they appear as good candidate strains to form a biocenosis that would be capable of efficient bioremediation of the emulsions.

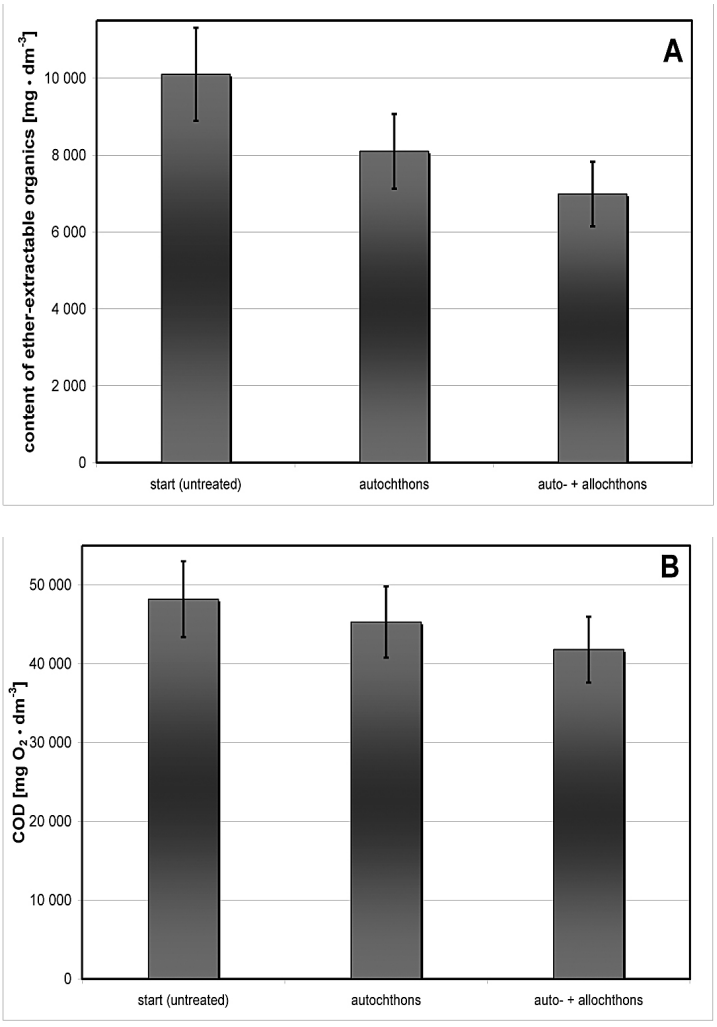

Figure 4. Results of 18-day bioremediation of undiluted E1 as observed with the method of organic compounds extraction (A) and COD determination (B)

- Microbial biomass grows upon oily xenobiotic utilization under aerobic conditions at the temperature optimum of 25 °C. The highest cell frequency is obtained when the emulsions become bioaugmented by inoculation with allochthonous bacteria preselected for efficient hydrocarbon biodegradation.
- Oily emulsion E1 can undergo biological treatment under conditions enabling bacterial proliferation, however the kinetics as well as the final yield of the bioprocess needs further optimization to make the microbiological method more economically competitive.

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