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Suplementary materials

Influence of biosurfactants on the efficiency of petroleum hydrocarbons biodegradation in soil

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Description of the samples presented in the Figure 1:

Samples inoculated with the bacterial consortium MC and enriched with a selected biosurfactant (1 g/dm³):

1. soil G6 + MC + γ-PGA
2. soil G6 + MC + surfactin
3. soil G6 + MC + rhamnolipids
4. soil G6 + MC + mixture PSR
5. soil G6 + MC + JBR425
6. soil G6 + MC + JBR320

Samples inoculated with the bacterial consortium MC and enriched with a selected biosurfactant (5 g/dm³):

7. soil G6 + MC + γ-PGA
8. soil G6 + MC + surfactin
9. soil G6 + MC + rhamnolipids
10. soil G6 + MC + mixture PSR
11. soil G6 + MC + JBR425
12. soil G6 + MC + JBR320

Control samples enriched only with the selected biosurfactant (1 g/dm³), without inoculation:

13. soil G6 + γ-PGA
14. soil G6 + surfactin
15. soil G6 + rhamnolipids
16. soil G6 + mixture PSR
17. soil G6 + JBR425
18. soil G6 + JBR320

Control samples enriched only with the selected biosurfactant (5 g/dm³), without inoculation:

19. soil G6 + γ-PGA
20. soil G6 + surfactin
21 soil G6 + rhamnolipids
22. soil G6 + mixture PSR
23. soil G6 + JBR425
24. soil G6 + JBR320

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Reference samples:

25. blanc sample – non-inoculated, no additives
26. soil G6 + bacterial consortium MC – control sample inoculated only with the MC consortium

Methods description

Respirometric test

Respirometric measurements were used to assess the aerobic biodegradation potential by monitoring oxygen consumption in soil samples under different enrichment conditions [1]. The experiments were conducted using the OxiTop® Control system. For the measurements, 30 g of soil (sample G6) was placed in sealed respirometric bottles. Depending on the experimental setup, the bottles were supplemented with: a bacterial consortium enriched with selected biosurfactants (at concentrations of 1 or 5 g/dm³), biosurfactants alone (1 or 5 g/dm³) (control samples), the bacterial consortium alone (control sample), or sterile water (blank sample). The bottles were then tightly sealed with measurement heads and incubated in a thermostatic chamber at 20°C for 90 days. The OxiTop® Control system recorded the pressure in the closed system every two hours. The data were transferred via an IR interface to the OxiTop OC 110 controller for graphical and statistical analysis using Achat OC software. Oxygen consumption (expressed in mg O_2/dm³) was calculated based on the pressure decrease using the following equation [2-4]:

$$m_{O_2} = \frac{M(O_2)}{RT_m} \cdot (V_g + \alpha \frac{T_m}{T_0}) \cdot \Delta p$$

Where: $m(O_2)$ – mass of consumed oxygen [mg/dm³], Δp – pressure drop in the vessel [Pa], Vg – volume of free gas [m³], $M(O_2)$ – molar mass of oxygen [kg/mol], R – gas constant [J·mol^{-1}·K^{-1}], T_m – measurement temperature [K], T_0 – reference temperature (273.15 K),

α – absorption coefficient (0.03103).

At the end of the 90-day incubation period, the obtained oxygen consumption data were used to evaluate the potential synergistic effect between the bacterial consortium and the biosurfactants. For this purpose, the Synergitic Index (SI) was calculated using the following formula:

$$\text{Synergitic Index (SI)} = \text{Synergitic index (SI)} \ \frac{\text{Effect(MC+B)}}{\text{Effect(MC)}+\text{Effect(B)}}$$

Where: *Effect(MC+B)* – measured biological activity (oxygen consumption) in the system inoculated with both the bacterial consortium (MC) and biosurfactants, *Effect(MC)* – activity in the system inoculated with the bacterial consortium MC alone, *Effect(B)* – activity in the system with biosurfactants alone.

SI values greater than 1 indicate a synergistic interaction between the components. This parameter was used to identify combinations showing enhanced biodegradation potential due to cooperative action.

Chromatographic analyses

TPH Extraction and Quantification

For the analysis of TPH in soil, 10 g of air-dried soil were placed in Erlenmeyer flasks and subjected to solvent extraction using dichloromethane (POCH S.A., Poland). The extraction was performed in three successive steps (3 × 20 mL), each with 15-minute ultrasonic agitation in a Sonoswiss SW 6H ultrasonic bath (30 kHz). The use of ultrasound-assisted extraction improved recovery rates, achieving approximately 95.9%, as verified using o-tert-phenyl as an internal recovery standard [4-5].

Following extraction, polar co-extracts were removed via solid-phase extraction (SPE) using Bakerbond Florisil® columns (No. JTB- 7213-03). The eluates were concentrated under reduced pressure using a rotary evaporator, redissolved in 1 mL of dichloromethane, and subjected to chromatographic analysis.

Quantitative and qualitative analysis TPH, individual n-alkanes (n-C6–n-C44) and isoprenoid hydrocarbons (pristane and phytane) was carried out using a Perkin Elmer Clarus 500 gas chromatograph equipped with a flame ionization detector (FID). The separation was performed on a fused silica RTX-1 capillary column (30 m × 0.53 mm; Restek, USA), with helium as the carrier gas at a constant flow rate of 20 mL·min^{-1}. The oven temperature program was as follows: initial isothermal hold at 30 °C for 2 min; ramp to 105 °C at 10 °C·min^{-1}; increase to 285 °C at 5 °C·min^{-1}; isothermal hold at 285 °C for 5 min. [4-7]. The injector and detector temperatures were set at 290 °C and 320 °C, respectively. Calibration for TPH quantification was based on a certified standard (BAM K010; Tusnovic Instruments). Quantification of individual hydrocarbons was performed using certified standard mixtures: Supelco D2807 (n-C6–n-C44) and A029668 (Fuel Oil Degradation Mix: n-C17, pristane, n-C18, phytane). The method showed good repeatability, with standard deviations ranging between 1.53 and 4.32%. Based on the concentrations of TPH and individual n-alkanes determined in the soil samples, the biodegradation rate of these compounds was calculated using the following equation:

$$\textbf{Biodegradation [\%]} = \frac{Cn{-}x_0 - Cn{-}x}{Cn{-}x_0} * \mathbf{100}$$

Where: $Cn\text{-}x_0$ - initial concentration of TPH or n-alkane in the soil before bioremediation [mg/kg DM]. C_n - concentration of TPH or n-alkane in the soil after the bioremediation [mg/kg DM].

In addition, biodegradation coefficients C17/Pr and C18/Ph were calculated as indicators of biodegradation, using the following formulas:

$$\text{n-C17/Pr}=\frac{c(n-C17)}{c(Pr)}$$

Where: c(n-C17) – concentration of n-c17 in soil [mg/kg DM], c(Pr) – concentration of pristane in soil [mg/kg DM],

$$\text{n-C18/Ph}=\frac{c(n-C18)}{c(Ph)}$$

Where: c(n-C18) – concentration of n-c18 in soil [mg/kg DM], c(Ph) – concentration of phytane in soil [mg/kg DM],

PAHs Extraction and Quantification

The quantification of polycyclic aromatic hydrocarbons (PAHs) in soil samples was carried out using a modified QuEChERS extraction procedure, as described in the study by Wojtowicz (2022) [8], which also provides detailed information on method validation, analyte recovery, and statistical treatment of the data.

For each analysis, 10 g of air-dried soil were transferred into 50 mL polypropylene centrifuge vials. Subsequently, 5 mL of distilled water and 10 mL of acetonitrile

(Chempur, Poland) were added, followed by a salt mixture containing magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl) (SST640, Interchim, France). The vials were sealed and manually shaken for 5 minutes to ensure efficient extraction. The samples were then centrifuged at 3500 rpm for 10 minutes using an MPW-223e centrifuge (MPW, Poland).
The organic supernatant was purified using a Clean-up Kit (No. JO3937, Interchim, France), followed by filtration through 0.2 μm nylon syringe filters [6-7].
Chromatographic analysis of PAHs was conducted on a Vanquish Core HPLC system (Thermo Scientific, USA) equipped with a UV-VIS detector and a fluorescence detector (FLD), operated in series and supported by an autosampler. Separation was achieved on a NUCLEODUR C18 PAH column (125 × 4 mm, 3 μm; Macherey-Nagel, Germany). The injection volume was 10 μL. The mobile phases used were methanol (eluent A, 70% purity, POCH S.A., Poland) and acetonitrile (eluent B, 99% purity, POCH S.A., Poland). The analysis was conducted with a flow rate of 1.5 mL/min, using the following gradient program: 20–50% B (1.5 min), 50–100% B (1 min), hold at 100% B (1 min), decrease to 0% B (3 min), and final hold at 100% A (3 min) [6-8].
For the quantitative determination of PAHs, a certified standard solution (PAH Standard, Ref. No. 722393, Macherey-Nagel, Germany) was used. Analyses were performed on contaminated soil samples spiked with a certified reference material (PAHs by HPLC 40g, SQC017-40G, Sigma Aldrich, USA), ensuring high accuracy and comparability of results across matrices. Based on the concentrations of total and individual PAHs determined in the soil samples, the biodegradation rate of these compounds was calculated using the following equation:

$$\textbf{Biodegradation [\%]} = \frac{Cn{-}x_0 - Cn{-}x}{Cn{-}x_0} * \mathbf{100}$$

Where: $Cn\text{-}x_0$ - initial concentration of given PAH or total PAHs in the soil before bioremediation [mg/kg DM]. C_n - concentration of given PAH or total PAHs in the soil after the bioremediation [mg/kg DM].

toxicological analyses

Phytotoxkit™ Test (MicroBioTests Inc., Belgium)
The Phytotoxkit™ chronic toxicity assay evaluates the inhibition of seed germination and early root elongation. Three plant species, selected based on their germination and root growth rates, are used to ensure comprehensive toxicity assessment within a 72-hour incubation period. These species include the monocotyledon *Sorghum saccharatum* and the dicotyledons *Lepidium sativum* (cress) and *Sinapis alba* (white mustard). Tests were conducted in triplicate using transparent polystyrene plates under controlled conditions (25°C, darkness) [6-7, 9-12]. Inhibition of germination and root growth was calculated as:

$$I = \frac{(A - B)}{B} \cdot 100\%$$

Where: **I** – inhibition (%), **A** – seed germination or root length in control soil, **B** – seed germination or root length in test soil

Ostracodtoxkit(F)™ Test (MicroBioTests Inc., Belgium)
The Ostracodtoxkit(F)™ test is a direct contact chronic toxicity bioassay using juvenile *Heterocypris incongruens* crustaceans. Juveniles hatched from cysts over 52 hours were exposed to soil samples in six-well polystyrene microplates. Each well contained 0.5 g of soil and 2 mL of standardized algal suspension as food. Ten ostracods were introduced per well. Controls were conducted using reference soil. Incubation occurred at 25°C for

six days [6-7, 12-14]. Endpoints included survival and growth, with growth inhibition (I) calculated as:

$$I = 100 - \left(\frac{A}{B} \cdot 100\%\right)$$

Where: A- ostracod length in test soil, B=- ostracod length in control soil.

Microtox® Solid Phase Test (SDI, USA)

The Microtox® test employs the luminescent bacterium *Vibrio fischeri* to evaluate sample toxicity through bioluminescence inhibition. Bioluminescence is catalyzed by luciferase during bacterial metabolism and decreases in the presence of toxic substances. Lyophilized *V. fischeri* bacteria were stored at -20°C and rehydrated before testing. The assay involved exposing bacteria to serial dilutions of wastewater samples, with luminescence measured using the Delta Tox analyzer. The effective concentration reducing luminescence by 50% (EC_{50}) was determined; lower EC_{50} values indicate higher toxicity [6-7, 15-17]. To facilitate interpretation, EC_{50} values were converted to Toxicity Units (TU) using:

$$TU = \frac{100}{EC_{50}}$$

Higher TU values correspond to increased toxicity.

To allow for comparison across different bioassays, toxicity results expressed as inhibition percentages (Phytotoxkit™, Ostracodtoxkit(F)™) and effective concentrations (EC_{50}; Microtox®) were converted to Toxicity Units (TU). For tests reporting EC_{50} values, TU was calculated as:

$$TU = \frac{100}{EC_{50}}$$

For tests where inhibition (%) was directly measured, TU was derived from inhibition values (I, in %) as:

$$TU=I$$

where I represents the percentage inhibition of seed germination, root growth, or organism growth. This approach enables the integration of toxicity endpoints on a common scale, facilitating comparison between assays.

The key information regarding the applied bioassays is summarized in Table S1.

Table S1 Characteristics of bioindication ecotoxicological tests applied for toxicity assessment

Test	Test Organism	Type of Toxicity	Experimental Parameters	Toxicity Indicators
Phytotoxkit™	*Sorghum saccharatum*, *Lepidium sativum*, *Sinapis alba*	Acute	- Incubation time: 72 h - Temperature: 25°C - Darkness, polystyrene test plates	EC_{50} for seed germination and root elongation Converted to Toxic Units (TU)

Test	Test Organism	Type of Toxicity	Experimental Parameters	Toxicity Indicators
Ostracodtoxkit F™	*Heterocypris incongruens* (freshwater crustacean)	Chronic	- Exposure duration: 6 days - Temperature: 25°C - Carried out in 6-well polystyrene plates	Mortality (%) Growth inhibition (based on body length) Converted to Toxic Units (TU)
Microtox® Solid Phase	*Vibrio fischeri* (marine bacterium)	Acute	- Exposure time: 15 min - Temperature: ambient - Based on luciferase-catalyzed bioluminescence inhibition	EC_{50} (50% luminescence reduction) Converted to Toxic Units (TU)

Table 2 Synergitic Index (SI) for different experimental setups and biosurfactant concentrations

Sample	**Synergitic Index** (SI) Concentration of biosurfactant in the inoculating mixture (1 g/dm³)	**Synergitic Index** (SI) Concentration of biosurfactant in the inoculating mixture (5 g/dm³)
soil G6+MC+γ-PGA	1,152	1,365
soil G6+MC+surfactin	1,112	1,071
soil G6+MC+rhamnolipids	1,146	1,309
soil G6+MC+mixture PSR	1,218	1,398
soil G6+MC+JBR425	1,204	1,461
soil G6+MC+JBR320	1,195	1,455

*** All SI values are greater than 1, which indicates the presence of a synergitic effect between the bacterial consortium and the applied biosurfactants.**

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