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The plant–microbe partnership ryegrass – *Burkholderia thailandensis* prevails over biosurfactants addition to improve hydrocarbon rhizodegradation

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ABSTRACT

Petroleum hydrocarbon soil contamination poses major environmental and health risks due to the persistence and toxicity of these pollutants. Rhizoremediation relies on synergistic interactions between plants and microorganisms and represents a sustainable and cost-effective strategy. However, its efficiency is often limited by the low bioavailability of hydrocarbons in soil. Previous research demonstrated that ryegrass (*Lolium multiflorum* L.) tolerates low concentrations of biosurfactants such as rhamnolipids, whereas higher concentrations increase hydrocarbon phytotoxicity. In parallel, *Burkholderia thailandensis* has been identified as a promising hydrocarbon-degrading and biosurfactant-producing bacterium. Building on these findings, this study investigated whether petroleum hydrocarbon rhizodegradation could be improved by combining ryegrass with *B. thailandensis* and rhamnolipids under controlled hydroponic conditions. The hydroponic system provided a simplified and well-controlled environment to isolate key biological mechanisms prior to soil application. After 120 days (2 g L^{-1} hydrocarbons; 1 × CMC_{water} rhamnolipids), the ryegrass + *B. thailandensis* treatment achieved the highest dissipation rate (70 %), particularly for C_{10}–C_{30} hydrocarbons. Biosurfactant production occurred even without rhamnolipid addition but did not enhance rhizodegradation. This indicates that endogenous biosurfactants production may already be sufficient in contaminated environments. Overall, these results question the relevance of biosurfactant supplementation, as it may increase phytotoxicity without improving rhizodegradation. They also highlight the ryegrass–*B. thailandensis* combination as a promising approach for the remediation hydrocarbon-contaminated environments.

1. Introduction

The remediation of soils contaminated with petroleum hydrocarbons remains a major environmental challenge due to the persistence, toxicity, and complexity of these organic pollutants. Hydrocarbon contamination mainly originates from industrial

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activities, oil spills, and petroleum storage. It poses significant risks to ecosystems and human health through soil and groundwater contamination (Ehis-Eriakha et al., 2025). Among available remediation strategies, bioremediation is often favored because it is cost-effective, environmentally friendly, and capable of fully mineralizing organic pollutants (Mishra et al., 2023).

Phytoremediation is a subcategory of bioremediation that exploits interactions between plant roots, associated microorganisms, and soil to degrade, extract, or stabilize contaminants (Kafle et al., 2022). In particular, the rhizosphere — the soil zone influenced by root exudates — plays a key role in stimulating microbial activity and hydrocarbon degradation (Wankhade et al., 2025). Several studies have shown that grasses, such as ryegrass (*Lolium multiflorum* L.), can enhance microbial communities in the rhizosphere and promote the rhizodegradation of petroleum hydrocarbons (Hussain et al., 2022).

The efficiency of rhizodegradation largely depends on synergistic interaction between plants and hydrocarbon-degrading microorganisms (Afzal et al., 2014). These microorganisms contribute directly to contaminant transformation and mineralization. Among the microbial traits involved, the production of biosurfactants has received considerable attention. Biosurfactants are amphiphilic compounds synthesized by certain bacteria, including *Burkholderia thailandensis*. They can increase hydrocarbon bioavailability in soils, where it is often strongly limited. By enhancing the solubility of hydrophobic compounds, particularly at or above the critical micellar concentration (CMC), biosurfactants may facilitate hydrocarbon biodegradation (Nagtode et al., 2023).

However, the effectiveness of biosurfactants such as rhamnolipids in hydrocarbon bioremediation remains debated. Several limiting factors have been identified including microbial and plant toxicity, surfactants adsorption onto soil particles, preferential substrate utilization, and micelle formation that may sequester hydrocarbons away from degrading microorganisms (Parus et al., 2023). In soil systems, relatively high biosurfactant concentrations are often required to overcome adsorption and reach the CMC. This requirement raises concerns regarding environmental compatibility, particularly in rhizodegradation systems where plants and microorganisms coexist. As a result, the net benefit of biosurfactant addition in rhizosphere-based hydrocarbon remediation remains insufficiently understood.

Although plant-assisted bioremediation, hydrocarbon-degrading bacteria, and biosurfactant-mediated solubilization have each been extensively studied, their combined and interactive effects within the rhizosphere are still poorly documented. In particular, it remains unclear whether biosurfactant addition enhances rhizodegradation under biologically compatible conditions or whether it introduces adverse effects that offset potential benefits. This lack of mechanistic understanding limits the rational optimization of integrated rhizomediation strategies.

In this context, hydroponic systems provide a simplified and highly controlled experimental framework. They allow biological interactions between plants, microorganisms, biosurfactants, and hydrocarbons to be investigated independently of soil-related constraints such as sorption heterogeneity and diffusion-limited bioavailability. While such systems do not aim to reproduce soil conditions, they are valuable tools for disentangling biological processes and identifying limiting or enhancing factors prior to extrapolation to complex soil environments.

Previous ecotoxicological studies demonstrated that ryegrass can tolerate rhamnolipids at concentrations up to $1 \times CMC_{water}$, whereas synthetic surfactants were less compatible (D'Incau et al., 2024b). Subsequent work showed that increasing rhamnolipid concentrations can exacerbate hydrocarbon-induced phytotoxicity in ryegrass. It also revealed that hydrocarbon exposure itself stimulates biosurfactant production, suggesting that external biosurfactant supplementation should be carefully evaluated (D'Incau et al., 2025). In parallel, *Burkholderia thailandensis* was identified as a promising microorganism capable of degrading complex hydrocarbon mixtures while producing biosurfactants (D'Incau et al., 2024a).

Building on these findings, the present study investigates the biological interactions between ryegrass, *B. thailandensis,* and biosurfactants under controlled hydroponic conditions. The objective was to assess their combined influence on petroleum hydrocarbon rhizodegradation. This approach represents an intermediate step toward optimizing plant–microbe interactions before large-scale application in hydrocarbon-contaminated soils.

To this end, a 120-day hydroponic rhizodegradation experiment was conducted using ryegrass, *B. thailandensis*, and commercial rhamnolipids applied at $1 \times CMC_{water}$. Petroleum hydrocarbons were introduced at an initial total concentration of 2 g L^{-1}. The specific objectives were to: (i) determine whether bacterial inoculation and/or biosurfactant addition enhances rhizodegradation under biologically compatible conditions; (ii) compare hydrocarbon dissipation patterns across treatments combining ryegrass, *B. thailandensis*, and biosurfactants; and (iii) identify biological factors that enhance or limit rhizodegradation in simplified systems, thereby providing mechanistic insights prior to future soil-based investigations.

2. Materials and methods

2.1. Reagents and chemicals

All reagents used were of analytical grade. The Hoagland nutrient solution was prepared using standard salts including KNO_3, $Ca(NO_3)_2{\cdot}4\ H_2O$, $MgSO_4{\cdot}7\ H_2O$, KH_2PO_4 and NH_4NO3. Micronutrients were also added, such as Fe(III) tartrate, H_3BO_3, $MnCl_2{\cdot}4\ H_2O$ and $ZnSO_4{\cdot}7\ H_2O$. These reagents were sourced from Sigma-Aldrich (USA) and VWR Chemicals (Belgium).

Chloroform and dichloromethane used for biosurfactant and hydrocarbon extraction were purchased from Carlo Erba Reagents (France). Molecular biology-grade water was obtained from Corning (USA). Additional reagents included Coomassie Brilliant Blue, hydrochloric acid (37 %), sodium azide, sodium chloride, sodium tetraborate, methylene blue, phenol–chloroform–isoamyl alcohol (25:24:1), Tris-HCl, disodium EDTA, Triton X-100, TSB, glycerol and lysozyme. These chemicals were obtained from Sigma-Aldrich (USA). Ethanol (absolute) and hydrogen peroxide (30 %) were supplied by VWR Chemicals (Belgium).

Quantitative PCR (qPCR) was performed using SsoAdvanced™ SYBR® Green Supermix (Bio-Rad Laboratories, USA). Primers

targeting 16S and 18S rRNA genes were purchased from Sigma-Aldrich (USA).

Rhamnolipids (R90), used as model biosurfactants, were supplied by AGAE Technologies (USA). They were of analytical grade. Their critical micellar concentration (CMC) was determined as 0.03 ± 0.005 g L^{-1} at ~20 °C using the oil-spreading test method (D'Incau et al., 2024a).

The petroleum hydrocarbon source was collected from a fuel storage contaminated site in Roanne, France. Although originating from refined fuel, the mixture was hereafter referred to as "crude oil" due to its complexity and weathered condition. Sampling and characterization were previously described in detail by D'Incau et al. (2024a).

2.2. Seed germination and plant priming

Lolium multiflorum L. seeds (Magellan-Bio, France) were surface-sterilized using 10 % hydrogen peroxide. They were then germinated for 72 h on moistened paper at 20 °C in the dark. Seeds with emerged radicles were inserted into moss and transferred to 3 L of Hoagland medium.

Plants were grown in opaque trays inside a phytotronic chamber under controlled conditions (20/15 °C day/night, 70 % relative humidity, 16/8 h light cycle, 350 μmol m^{-2} s^{-1}). The priming phase lasted 20 days. An air pump provided continuous oxygenation. Opaque trays were used to prevent algal growth (Fig. S. 1).

2.3. Bacterial inoculum preparation

Burkholderia thailandensis E264 (DSM 13276, DSMZ-German Collection) was selected based on its ability to degrade complex hydrocarbons and produce biosurfactants (D'Incau et al., 2024a). The strain was revived from −80 °C glycerol stocks. Cells were cultured in Hoagland medium with 4 % (v/v) glycerol at 28 °C and 125 rpm for 13 days. Cultures were then centrifuged at 5500 rpm for 10 min. Pellets were washed three times with physiological saline solution (*i.e.,* 9 g L^{-1} NaCl). Cells were finally resuspended in 20 mL of Hoagland medium.

2.4. Rhizodegradation experiment set up

Experiments were conducted in 500 mL Erlenmeyer flasks. Each flask was filled up to the base of the neck with 550 mL of Hoagland medium (sterilized by autoclaving). The medium was supplemented with 0.2 % (v/v) crude oil filtered through a 0.2 μm membrane. The protocol was adapted from Chaîneau et al. (2000). Each flask contained a glass tube to physically separate the plant roots from the floating crude oil layer (Fig. S. 2). Flasks were covered with aluminum foil to limit the development of photosynthetic organisms. Four treatments were established to assess the effects of the ryegrass-microbe-biosurfactant system on hydrocarbon rhizodegradation. All treatments contained ryegrass (*Lolium multiflorum*), but differed in bacterial inoculation and rhamnolipid addition. The treatments were as follows: (i) RG – ryegrass only, serving as a plant control; (ii) RG+RL – ryegrass combined with rhamnolipids; (iii) RG+Bth – ryegrass inoculated with *B. thailandensis*; and (iv) RG+Bth+RL – ryegrass inoculated with *B. thailandensis* and supplemented with rhamnolipids.

Rhamnolipids were added to relevant treatments at 0.03 g L^{-1} ($1 \times CMC_{water}$). Treatments containing *B. thailandensis* received 0.3 mL of bacterial suspension to achieve a final density of 1×10^7 CFU mL^{-1}. Incubations were carried out for 60 or 120 days. All experiments were maintained under the same environmental conditions as the priming phase. Evaporation was monitored and compensated using filtered water or nutrient solutions when required. Flasks were gently shaken three times per week (125 rpm for 20 min). Six replicates were used per treatment and sampling time. Due to the destructive nature of hydrocarbon extraction, three flasks were dedicated to hydrocarbon analyses. The remaining three flasks were reserved for biological measurements. Plant growth and anionic biosurfactants were measured on the six replicates whenever subsampling was possible. Microbial biomass and qPCR analyses were conducted on biological flasks only. This split design was imposed by the destructive nature of hydrocarbon and microbial pellet extractions and aimed at preserving independence between analytical datasets. In addition, three extra systems were prepared at the beginning of the experiment to determine the initial total hydrocarbon concentration.

2.5. Flow cytometry evaluation of microbial growth

Samples (1 mL) were collected after 5, 60, and 120 days. They were diluted in sterile 9 g L^{-1} NaCl solution and stained with 2.67 μM SYTO 9 (Thermo Fisher Scientific) for 10 min.

Analyses were performed using an Attune NxT cytometer (Thermo Fisher Scientific). A 488 nm laser was used, with emission detected using 530/30 nm (green) and 695/40 nm (red) filters. Autofluorescence from chlorophyll was distinguished using specific gating strategies (R1 for total SYTO 9-positive cells, R2 for red fluorescence; Figs. S. 3 and S. 4). Absolute quantification was achieved using reference counting beads. Three technical replicates were acquired for each biological replicate.

2.6. Microbial DNA extraction

At each time point, approximately 500 mL of culture medium was centrifuged at 5500 rpm for 10 min. Pellets were stored at −20 °C in 2 mL of 9 g L^{-1} NaCl solution.

DNA extraction was performed using a modified phenol–chloroform–isoamyl alcohol (PCI) protocol. Cells were lysed using Tris-

HCl, EDTA, Triton X-100, and lysozyme. Samples were incubated at 37 °C and treated with proteinase K. Two successive PCI extractions were performed. DNA was precipitated with isopropanol, washed with 70 % ethanol, air-dried, and resuspended in 50 μL of molecular-grade water. DNA concentrations were measured spectrophotometrically (BioSpec-nano, Shimadzu). The DNA extracts were stored at −20 °C until qPCR analysis.

2.7. Microbial density by qPCR

Bacterial density was quantified using primers 341 F and 534 R targeting the 16S rRNA gene. Fungal density was assessed using primers Fung5F and FF390R targeting the 18S rRNA gene.

Each 20 μL qPCR reaction contained 10 ng of template DNA, SsoAdvanced™ SYBR® Green Supermix (Bio-Rad), and primers. Primer concentrations were 2 μM for 16S rRNA and 0.5 μM for 18S rRNA. Amplification was performed on a MyiQ2 thermocycler (Bio-Rad) under the following conditions: 98 °C for 2 min; 35 cycles of 98 °C for 5 s, 60 °C (16S rRNA) or 50 °C (18S rRNA) for 30 s, and 72 °C for 30 s; final elongation at 72 °C for 5 min; followed by a melt curve from 60 to 98 °C. Cycle threshold (Ct) values were converted into gene copies per ng of DNA using standard curves.

2.8. Nutrient quantification by ion chromatography

Weekly samples (1 mL) were collected from inside the glass tube. Major anions and cations (Cl^-, NO_3^-, PO_4^{3-}, SO_4^{2-}, NH_4^+, Ca^{2+}, Mg^{2+}, K^+, and Na^+) were quantified by ion chromatography using a Metrohm 940 IC Vario system. Nitrate and phosphate losses were compensated by adding filtered NH_4NO_3 or KH_2PO_4 solutions when necessary.

2.9. Anionic biosurfactant quantification

Anionic biosurfactants were quantified at 60 and 120 days using the methylene blue complexation method. The protocol followed that described by D'Incau et al. (2025). Briefly, 2 mL of culture medium was diluted 1:1 with Hoagland solution and acidified to pH 2.3 with 0.1 M HCl solution. Samples were extracted with five volumes of chloroform. The extract (4 mL) was mixed with methylene blue (200 μL) and demineralized water (4.9 mL), shaken, and allowed to settle. Absorbance of the organic phase was measured at 638 nm using a calibration curve prepared with commercial rhamnolipids (0–0.15 g L^{-1}).

2.10. Ryegrass morphological measurements

After 60 and 120 days, ryegrass shoots and roots were harvested and rinsed. Measured parameters included total fresh weight (TFW), root and shoot length (RL, SL), dry weights (RDW, SDW), and total dry weight (TDW = RDW + SDW). Plant water content (PWC) was calculated as (TFW – TDW)/TDW.

2.11. Total hydrocarbon extraction and quantification

Hydrocarbons were extracted from the entire 550 mL culture medium. Sequential dichloromethane extractions (2 × 200 mL followed by 1 × 150 mL) were performed. Extracts were combined and evaporated under nitrogen. The residue was adjusted to 500 mL with dichloromethane.

GC-FID analyses were carried out using an Agilent 7890 A system equipped with a DB-5MS column (60 m × 0.25 mm i.d.,0.10 μm film thickness). One microlitre of sample was injected in splitless mode at 300 °C. The GC oven temperature program was as follows: 40 °C (held for 1 min) to 325 °C at 10 °C min^{-1}, with a final hold of 28 min. Helium was used as the carrier gas at a constant flow of 1.5 mL min^{-1}. Detector FID was heated at 300 °C. Air, hydrogen and helium (reference gas) were delivered at 350-, 40- and 27-mL min^{-1}, respectively. Hydrocarbon fraction concentrations (C_{10}–C_{40}) were quantified against a mineral oil calibration curve. Hydrocarbon fractions were categorized as C_{10}–C_{12}, C_{12}–C_{16}, C_{16}–C_{22}, C_{22}–C_{30}, C_{30}–C_{36}, and C_{36}–C_{40}. Retention time ranges for each fraction were determined using an *n*-alkanes standard mixture (C_{10}–C_{40}, Restek) injected at the beginning and end of each analytical sequence. When anionic biosurfactants were detected, their contribution to the GC-FID signal (C_{10}–C_{40}) was subtracted using a calibration curve prepared with commercial rhamnolipids.

2.12. Statistical analyses

Statistical analyses were conducted using R (v4.4.1) within RStudio. Treatment effects were assessed using two-way ANOVAs followed by Tukey HSD *post-hoc* tests (p-value < 0.05). Normality (Shapiro-Wilk), homoscedasticity (Levene), and residual independence (Durbin-Watson) were verified. Non-conforming data were log-transformed or analyzed using permutation tests. Relationships among microbial growth, *n*-alkane concentration, plant growth, and anionic biosurfactant-related variables were explored using multiple factor analysis (MFA). Two-way MANOVA was used to evaluate the effects of *B. thailandensis* and rhamnolipids.

3. Results

3.1. Evolution of medium culture composition

3.1.1. Hydrocarbon concentration and composition

Regular additions of nutrient solutions for all treatments ensured that mineral conditions in the culture medium were maintained for biodegradation until the end of incubation. No nutrient limitation was detected after 60 and 120 days. Major anions (Cl^-, NO_3^-, PO_4^{3-}, SO_4^{2-}) and cations (NH_4^+, Ca^{2+}, Mg^{2+}, K^+, Na^+) remained within the expected range for all treatments (data not shown).

The total hydrocarbon concentration (THC) is presented in Fig. 1. The initial THC was 1.87 g L^{-1}, close to the target value of 2 g L^{-1}. A significant decrease in THC was observed over time for all treatments (p-value < 0.001). ANOVA revealed significant effects of incubation time and a significant interaction between incubation time and treatment (p-value < 0.001 and p-value < 0.05, respectively). After 60 and 120 days of cultivation, all rhizodegradation treatments exhibited significantly reduced THC levels compared with the initial concentration. Among them, the RG+Bth treatment reached the lowest concentration (0.57 $\pm$ 0.12 g L^{-1}) after 120 days. This value was significantly lower than in all other treatments (p-value < 0.05). These results indicate that the combination of ryegrass and *B. thailandensis* most strongly enhanced hydrocarbon removal at the end of the experiment.

Dissipation rates averaged 52 % and were similar across all treatments after 60 days of culture. The values were 52 %, 57 %, 50 % and 48 % for RG+Bth, RG+Bth+RL, RG and RG+RL, respectively. After 120 days, these rates remained comparable ($\sim$ 55 % on average), except for the RG+Bth treatment. This treatment reached a dissipation rate of 70 %.

To investigate degradation in more detail, the concentrations of hydrocarbon fractions were analyzed at 60 and 120 days (Fig. 2). The initial mixture was dominated by C_{12}–C_{16} (40 %) and C_{16}–C_{22} (34 %), with smaller contributions from other fractions. The lightest hydrocarbons (C_{10}–C_{12}) showed a 94.5 % reduction after 60 days and 99.6 % after 120 days. No treatment effect was detected for this fraction (p-values > 0.1; Fig. 2a). This fraction is relatively soluble and readily accessible to microorganisms. Its shorter-chain hydrocarbons are more easily metabolized and are likely subject to preferential biodegradation. Volatilization may also contribute significantly to this rapid decrease.

For the C_{12}–C_{16} fraction (Fig. 2b), ANOVA showed significant effects of both time and treatment (p-values < 0.05). At 60 days, concentrations were similar across treatments, averaging 0.33 g L^{-1}. However, at 120 days, a significant reduction was observed in RG+Bth compared to other treatments (p-value < 0.05). This indicates that ryegrass combined with *B. thailandensis* enhanced dissipation of this fraction.

Intermediate fractions C_{16}–C_{22} and C_{22}–C_{30} (Fig. 2c,d) also decreased over time in all treatments. In RG+Bth, concentrations tended to be lower at 120 days (C_{16}–C_{22}: 0.31 g L^{-1}; C_{22}–C_{30}: 0.10 g L^{-1}). However, these differences were not statistically significant (p-values > 0.05). These results suggest a potential but non-significant benefit of the RG+Bth treatment for the dissipation of these mid-weight hydrocarbons.

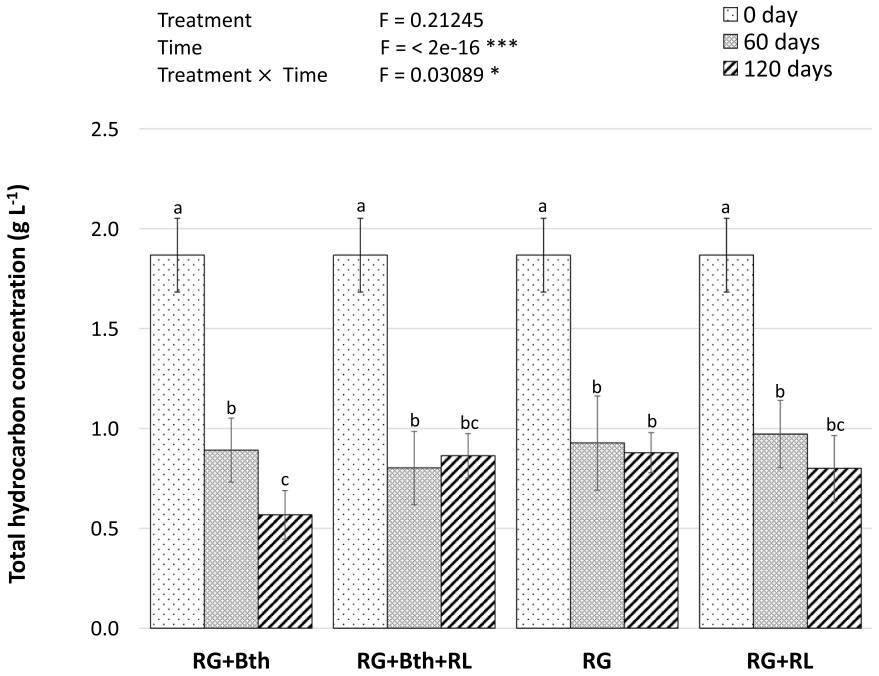


Fig. 1. Evolution of total hydrocarbon concentration. *RG* Ryegrass, *Bth B. thailandensis*, *RL* Rhamnolipids. Different letters indicate statistically significant differences at the 5 % level among treatments using a logarithmic transformation on data and Tukey HSD with p-values < 0.05 and $n = 3$. Significant levels of F statistics:. $p < 0.1$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

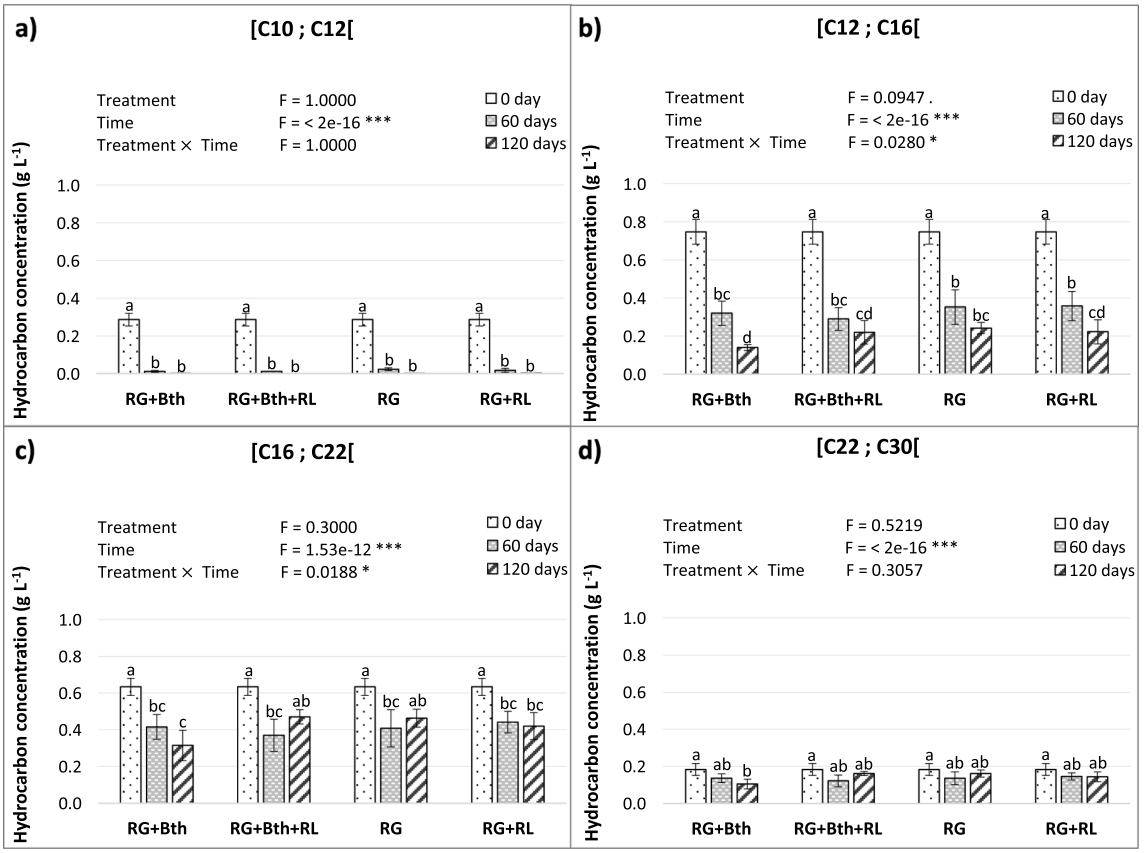


Fig. 2. Hydrocarbon concentration for individual fractions **a)** [C10; C12[, **b)** [C12; C16[, **c)** [C16; C22[and **d)** [C22; C30[. *RG* Ryegrass, *Bth B. thailandensis*, *RL* Rhamnolipids. For each fraction, different letters indicate statistically significant differences at the 5 % level among treatments using a logarithmic transformation or a permutation test on data and Tukey HSD with p-values < 0.05 and $n = 3$. Significant levels of F statistics:. $p < 0.1$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

The heavier fractions (C_{30}–C_{36} and C_{36}–C_{40}) represented less than 1.5 % of the initial mixture. They did not change significantly over time in any treatment (p-values > 0.1). This suggests strong recalcitrance under the tested conditions.

In summary, the combination of ryegrass and *B. thailandensis* (RG+Bth) was the only treatment that significantly enhanced hydrocarbon dissipation after 120 days. The effect was clearest for the C_{12}–C_{16} fraction. The lighter hydrocarbons (C_{10}–C_{12}) were mainly lost through volatilization, while heavier compounds remained largely unaffected.

3.1.2. Biosurfactant concentration

Firstly, it is important to note that anionic biosurfactants were initially present in the hydrocarbon mixture. This resulted in 44 mg L^{-1} at day 0 in treatments without rhamnolipid addition. In treatments supplemented with rhamnolipids (RL) at 1 CMC, 30 mg L^{-1} of commercial rhamnolipids were added. This corresponded to 74 mg L^{-1} at day 0.

Anionic biosurfactant concentrations (Table 1) increased between 0 and 60 days in all treatments except RG+Bth+RL, which showed a slight decrease (63.23 mg L^{-1}). At 60 days, the highest concentration was recorded in the RG+RL treatment (101.79 mg L^{-1}), followed by RG (78.11 mg L^{-1}), RG+Bth+RL (63.23 mg L^{-1}) and RG+Bth (61.69 mg L^{-1}). Between 60 and 120 days, concentrations generally declined across all treatments. The lowest value was observed in RG+Bth at 120 days (26.55 mg L^{-1}). Statistically significant variations (p-value < 0.05) after 120 days were detected in the RG+Bth+RL treatment compared to initial levels.

3.2. Evolution of microbial biomass

3.2.1. Microbial growth by flow cytometry

Microbial concentrations (Table 1) measured by flow cytometry at 60 and 120 days did not differ significantly among treatments or over time (ANOVA, p-values > 0.05). Despite this, an overall increase in microbial concentration was observed between 60 and 120 days in most treatments. RG+Bth was the exception, with lower values at 120 days. The highest microbial concentration was recorded in the RG+RL treatment at 120 days (5.10×10^4 microorganisms μL^{-1}). The lowest was observed in RG+Bth at 120 days (8.90×10^3

Table 1
Evolution of anionic biosurfactant concentrations, microbial biomass and ryegrass growth parameters in the medium culture for each treatment (*RG* Ryegrass, *Bth B. thailandensis*, *RL* Rhamnolipids). Different letters indicate statistically significant differences at the 5 % level among treatments using Tukey HSD with p-values < 0.05 and $n = 6$ (except for microbial biomass parameters with $n = 3$). Asterisks (*) indicate a significant difference at the 5 % level compared to the initial anionic biosurfactant concentration in the medium using Kruskal-Wallis test, p-value < 0.05 and $n = 6$.

		RG		RG+RL		RG+Bth		RG+Bth+RL	
Incubation time (day)		**60**	**120**	**60**	**120**	**60**	**120**	**60**	**120**
Anionic biosurfactant concentration (mg L^{-1})		78.11 ± 48.13 a	55.30 ± 19.29 a	101.79 ± 82.84 a	42.29 ± 17.07 a	61.69 ± 47.17 a	26.55 ± 9.34 a	63.23 ± 62.08 a	42.96 ± 6.32 a *
Microbial concentration (microorganisms μL^{-1})		3.39×10^4 ± 2.50×10^4 a	4.92×10^4 ± 2.41×10^4 a	2.99×10^4 ± 1.94×10^4 a	5.10×10^4 ± 3.91×10^4 a	1.17×10^4 ± 7.85×10^3 a	8.90×10^3 ± 2.96×10^3 a	1.76×10^4 ± 2.15×10^4 a	3.35×10^4 ± 2.61×10^4 a
Bacterial density (16S rDNA GC ng^{-1} DNA)		4.07×10^7 ± 2.46×10^7 a	7.06×10^7 ± 5.98×10^7 a	1.47×10^8 ± 1.76×10^8 a	1.99×10^8 ± 1.66×10^8 a	1.54×10^7 ± 3.35×10^6 a	7.28×10^7 ± 8.06×10^7 a	6.56×10^7 ± 4.38×10^7 a	6.93×10^7 ± 5.28×10^7 a
Fungal density (18S rDNA GC ng^{-1} DNA)		1.97×10^5 ± 9.02×10^4 a	2.45×10^5 ± 2.63×10^5 a	3.67×10^5 ± 3.71×10^5 a	3.95×10^5 ± 1.78×10^5 a	1.29×10^5 ± 2.32×10^4 a	5.06×10^5 ± 3.20×10^5 a	3.91×10^5 ± 2.56×10^5 a	3.54×10^5 ± 2.40×10^5 a
Growth parameters	Shoot length (cm)	15.00 ± 2.81 b	21.80 ± 6.76 ab	15.17 ± 2.71 b	22.67 ± 4.55 ab	14.83 ± 5.60 b	19.33 ± 6.41 ab	15.00 ± 1.79 b	27.33 ± 2.42 a
	Shoot dry weight (g)	0.16 ± 0.09 a	0.18 ± 0.08 a	0.21 ± 0.13 a	0.32 ± 0.32 a	0.22 ± 0.25 a	0.96 ± 1.49 a	0.18 ± 0.14 a	0.89 ± 0.83 a
	Root length (cm)	23.00 ± 5.83 ab	17.00 ± 2.68 bcd	26.00 ± 5.97 a	17.17 ± 2.64 bcd	21.00 ± 5.90 abc	12.83 ± 2.79 d	28.33 ± 3.44 a	15.00 ± 3.46 cd
	Root dry weight (g)	0.07 ± 0.05 a	0.05 ± 0.03 a	0.09 ± 0.07 a	0.17 ± 0.20 a	0.15 ± 0.23 a	0.19 ± 0.26 a	0.05 ± 0.03 a	0.27 ± 0.19 a
	Total fresh weight (g)	4.65 ± 3.86 a	1.42 ± 0.74 a	3.22 ± 2.07 a	2.78 ± 2.74 a	4.09 ± 4.75 a	4.81 ± 6.59 a	2.51 ± 0.53 a	7.13 ± 5.09 a
	Total dry weight (g)	0.23 ± 0.12 a	0.23 ± 0.11 a	0.30 ± 0.20 a	0.50 ± 0.44 a	0.37 ± 0.48 a	1.15 ± 1.65 a	0.23 ± 0.15 a	1.16 ± 0.91 a
	Plant water content (mL)	26.71 ± 34.67 a	5.77 ± 2.64 a	10.48 ± 3.01 a	4.86 ± 2.41 a	12.62 ± 4.77 a	4.95 ± 2.88 a	13.41 ± 8.71 a	5.31 ± 0.67 a

microorganisms μL^{-1}). Additionally, microbial concentrations measured at 5 days averaged 7.84×10^2 microorganisms μL^{-1} (data not shown). This represents a 30-fold increase by day 60 and a 45-fold increase by day 120 (ANOVA, p-values < 0.001).

3.2.2. *Microbial density by qPCR*

Bacterial and fungal densities were quantified by qPCR targeting 16S and 18S rDNA at 60 and 120 days (Table 1). The highest bacterial density (1.99×10^8 GC ng^{-1} DNA) was observed in RG+RL at 120 days, and the lowest (1.54×10^7 GC ng^{-1} DNA) in RG+Bth at 60 days. No significant differences were detected among treatments or sampling times (p-values > 0.05). This suggests that neither rhamnolipids nor *B. thailandensis* increased bacterial density under these conditions. As for flow cytometry, these microbial endpoints were based on $n = 3$ biological replicates.

Fungal densities (18S rDNA) similarly showed no significant variation with treatment or time (p-values > 0.05). The highest value (5.06×10^5 GC ng^{-1} DNA) occurred in RG+Bth at 120 days. However, the differences were not significant. Fungal abundance increased up to fourfold between 60 and 120 days but was unaffected by rhamnolipid or *B. thailandensis* addition.

3.3. *Evolution of ryegrass growth*

Ryegrass development was evaluated across all treatments after 60 and 120 days (Table 1). Average shoot length increased significantly over time (p-value < 0.001). It increased from 15.00 ± 3.29 cm at 60 days to 22.83 ± 5.73 cm at 120 days. The longest shoots (27.33 ± 2.42 cm) were observed in the RG+Bth+RL treatment. Shoot dry weight showed no significant differences between treatments or incubation time (p-value > 0.05). Mean biomass nevertheless increased over time (from 0.19 ± 0.16 g to 0.59 ± 0.88 g). This may reflect heterogeneous stress responses.

In contrast, root length decreased significantly over time for all treatments (p-values < 0.05). Mean values decreased from 24.58 $\pm$ 5.79 cm at 60 days to 15.5 $\pm$ 3.3 cm at 120 days. The highest root lengths were measured in RG+Bth+RL and RG+RL treatments after 60 days (28.33 $\pm$ 3.44 cm and 26.00 $\pm$ 5.97 cm, respectively). The shortest roots were found in RG+Bth at 120 days (12.83 $\pm$ 2.79 cm). Root dry weight remained statistically unchanged across treatments and time points (p-values > 0.05). It averaged 0.09 $\pm$ 0.12 g at 60 days and 0.17 $\pm$ 0.19 g at 120 days.

Total fresh weight, dry weight, and plant water content also showed no significant differences (p-values > 0.05). Trends were observed over time, with higher dry weight and lower fresh weight and water content. Overall, these results indicate suboptimal ryegrass development under hydrocarbon contamination. Root growth was the most affected endpoint.

3.4. Multivariate analysis

A multiple factor analysis (MFA) was performed on the dataset after 120 days of incubation. Variables were grouped into four categories: microbial growth (microbial concentration: MICRO, bacterial and fungal densities: 16S rDNA and 18S rDNA), *n*-alkane concentrations (fractions C_{10}–C_{12}, C_{12}–C_{16}, C_{16}–C_{22}, C_{22}–C_{30}, C_{30}–C_{36}, C_{36}–C_{40}), plant growth (shoot/root length and dry weight: SL/RL and SDW/RDW, plant water content: PWC), and anionic biosurfactant concentration (BIOSURF). The first two dimensions of the MFA explained 33.9 % and 23.5 % of the total inertia, respectively. Together, they explained 57.4 % of the data variability (Fig. 3). This indicates that the factorial plane captures most major trends.

On the factorial map, dimension 1 distinctly separated treatments inoculated with *B. thailandensis* (Bth+) from those without inoculation (Bth–) (Fig. 3a). However, treatments with or without the addition of rhamnolipids clustered similarly. No clear separation was observed for rhamnolipid addition along dimensions 1 or 2 (Fig. 3b).

Although the factorial map representation of the data did not clearly differentiate treatments based on rhamnolipid addition, a significant interaction between *B. thailandensis* inoculation and rhamnolipids was evidenced across the concentrations of all hydrocarbon fractions studied (C_{10}–C_{40}). This interaction was supported by the two-way MANOVA results (p-values < 0.05). Moreover, MANOVA indicated that *B. thailandensis* treatment had a significant effect on the concentration of light hydrocarbons (C_{10}–C_{12}). It also

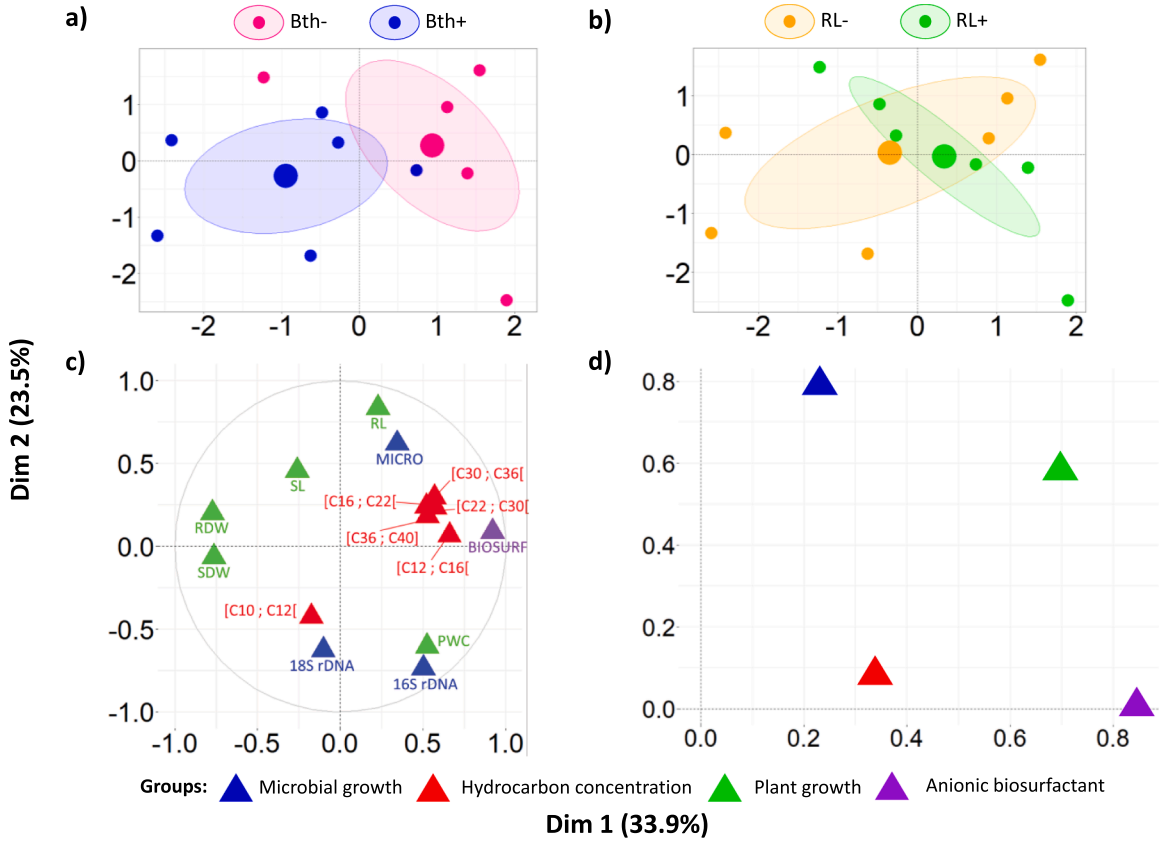


Fig. 3. Factorial maps from multiple factor analysis on four groups of variables (microbial growth, hydrocarbon concentration, plant growth and anionic biosurfactant). The numbers (in %) on the x-axis and y-axis represent the variance explained by the respective main components. **a)** Map of individuals colored according to their membership in the modalities of "*Burkholderia thailandensis*" variable (*Bth+* with *B. thailandensis*, *Bth-* without *B. thailandensis*). **b)** Map of individuals colored according to their membership in the modalities of "Rhamnolipids" variable (*RL+* with rhamnolipids, *RL-* without rhamnolipids). **c)** Representation of the variables in the factorial plane. **d)** Representation of the variable groups in the factorial plane.

affected some ryegrass growth parameters (SDW and RDW; p-values < 0.05).

The MFA variable map (Fig. 3c) revealed that variables associated with hydrocarbon concentrations from C_{12} to C_{40} and anionic biosurfactants were strongly correlated along the first dimension (Dim1). In contrast, the light hydrocarbon fraction (C_{10}–C_{12}) was not correlated with the other hydrocarbon fractions. It was positioned opposite to root length (RL) and microbial concentration (MICRO) along Dim2. This pattern indicates both an active microbial contribution to the dissipation of light hydrocarbons and a potential toxic effect of these compounds on root development. Fungal density (18S rDNA) was positively correlated with light hydrocarbons. This suggests that light-hydrocarbon dissipation was not associated with fungal abundance. No clear correlation was observed between ryegrass dry biomass and total microbial growth. This indicates that total biomass did not drive microbial abundance in this system. However, root length (RL) and plant water content (PWC) were positively correlated with microbial indicators (MICRO and 16S rDNA). These relationships suggest that some aspects of plant development supported microbial growth. Furthermore, root and shoot dry weights were negatively correlated with medium to heavy hydrocarbon fractions (C_{12}–C_{40}). This supports a toxic effect of these hydrocarbon fractions on ryegrass biomass.

Dimension 1 was mainly explained by the anionic biosurfactant (40 %) and plant growth (33 %) groups, whereas dimension 2 was mainly influenced by microbial growth (54 %) and plant growth (40 %) (Fig. 3d). These results indicate that treatments were primarilydiscriminated based on the presence or abscence of *B. thailandensis, which* mainly influenced ryegrass traits and biosurfactant levels.

Overall, the multivariate analysis confirms that *B. thailandensis* played a key role in promoting ryegrass growth and enhancing the dissipation of multiple hydrocarbon fractions. In contrast, rhamnolipid addition alone did not significantly affect microbial dynamics or hydrocarbon rhizodegradation.

4. Discussion

4.1. Bioaugmentation is more efficient than biosurfactant supplementation for recalcitrant hydrocarbon rhizodegradation

Rhizosphere processes, particularly the release of root exudates, are well documented for stimulating microbial activity and promoting hydrocarbon rhizodegradation (Hussain et al., 2018; Ruley et al., 2022). However, complete degradation of recalcitrant mixtures is rarely achieved. This limited efficiency is often attributed to low compound availability and/or chemical recalcitrance. In our hydroponic system, we evaluated whether combining ryegrass with *B. thailandensis*, a biosurfactant-producing hydrocarbon degrader (D'Incau et al., 2024a), and rhamnolipid supplementation could further enhance hydrocarbon rhizodegradation.

The selection of ryegrass, *B. thailandensis*, and the experimental conditions used in this study was guided by our previous work. First, we showed that *B. thailandensis* can degrade the same complex petroleum hydrocarbon mixture and produce biosurfactants under high hydrocarbon loads (D'Incau et al., 2024a). Second, we demonstrated that ryegrass is sensitive to biosurfactant–hydrocarbon interactions under controlled conditions (D'Incau et al., 2025, 2024b). It should be emphasized that the hydroponic system used in this study was not intended to reproduce soil conditions. Instead, it provides a simplified and controlled framework. This design helps isolate biological interactions between plants, microorganisms, and hydrocarbons. It also avoids soil-related constraints such as sorption heterogeneity and diffusion-limited bioavailability.

After 60 days of cultivation, hydrocarbon dissipation rates were similar for all ryegrass treatments (~52 %). This suggests that neither biosurfactant nor *B. thailandensis* inoculation increased dissipation at this stage. This level of dissipation should be interpreted considering abiotic losses. Abiotic losses were estimated at ~25 % based on previous results obtained for the same hydrocarbon mixture (D'Incau et al., 2024a). Accordingly, biodegradation attributable to the ryegrass treatments was ~39 %. Under hydroponic conditions, dissipation of light hydrocarbon fractions (C_{10}-C_{12}) is expected to be largely dominated by abiotic processes such as surface-driven volatilization. Quantitative abiotic dissipation of the same hydrocarbon mixture under comparable conditions has been previously reported (D'Incau et al., 2024a). It was therefore not repeated here, to maintain focus on treatment-driven biological effects.

After 120 days, the RG + Bth treatment (ryegrass + *B. thailandensis*) was the only treatment to exhibit a significantly enhanced degradation yield compared to the ryegrass alone control. This modality achieved 70 % dissipation. This suggests a positive plant-bacteria interaction in the context hydrocarbon rhizodegradation. This aligns with studies reporting that rhizosphere development gradually stimulates hydrocarbon degraders (Kirk et al., 2005; Lü et al., 2024). Kirk et al. (2005) notably observed a positive impact of ryegrass on microbial abundance and diversity in soil contaminated with 31 g kg^{-1} of total hydrocarbons (TH) after 7 weeks. Similarly, our flow cytometry analyses showed a significant increase in microbial concentration of 30–45 times (p-values < 0.001) between 5 and 60–120 days. This increase occurred in all ryegrass treatments. This stimulatory effect likely results from rhizodeposits providing labile carbon sources. These substrates may have been preferentially consumed over hydrocarbons. This could delay hydrocarbon biodegradation early in experiment.

By 120 days, microbial concentrations converged across treatments ($3.57 \pm 1.95 \times 10^4$ microorganisms μL^{-1}). Most treatments showed higher microbial abundance at 120 days than at 60 days. Still, RG+Bth showed the highest dissipation despite the lowest microbial concentration (8.90×10^3 microorganisms μL^{-1}). This indicates that dissipation was not directly proportional to total microbial abundance. However, microbial abundance was assessed with $n = 3$ biological replicates due to the destructive nature of sampling. Therefore, we interpret microbial results mainly as overall trends rather than treatment-specific quantitative differences.

Despite the high dissipation rate in RG + Bth, root development was limited during the incubation period. Root growth is a known driver of rhizodegradation (Yang et al., 2023). No biomass gain was observed between 60 and 120 days (p-values > 0.05). Root length decreased in all treatments (from ~24.58 ± 5.79 cm at 60 days to ~15.50 ± 3.26 cm at 120 days). This could be due to hydrocarbon

phytotoxicity. This toxicity may be exacerbated by biosurfactants (Marecik et al., 2012; Parus et al., 2023). According to Parus et al. (2023), increased phytotoxicity can result from higher xenobiotic bioavailability and plant accumulation. The combined effects of rhamnolipids and xenobiotics remain insufficiently documented. In the present study, phytotoxicity was assessed primarily through plant growth-related parameters. These endpoints integrate plant performance and stress over time. Other physiological or biochemical markers (e.g., oxidative stress responses or photosynthetic performance) were not investigated. They were considered beyond the scope of this work. Nevertheless, our dataset provides complementary evidence for biosurfactant-associated phytotoxicity under rhizodegradation conditions.

Multifactorial analysis revealed a negative correlation between ryegrass growth (root and shoot dry weights) and biosurfactant concentration. Biosurfactant concentration was also correlated with C_{12}–C_{40} *n*-alkane concentrations. Accordingly, higher final concentrations of anionic biosurfactants in the medium were associated with lower ryegrass biomass and reduced hydrocarbon dissipation. This pattern supports that biosurfactants can enhance hydrocarbon phytotoxicity in ryegrass (D'Incau et al., 2025), ultimately leading to reduced hydrocarbon dissipation. Interestingly, RG+Bth showed the highest rhizodegradation efficiency. It also had the shortest roots and the lowest biosurfactant concentration (26.55 ± 9.34 mg L^{-1}) at 120 days. This may indicate reduced exposure to solubilized hydrocarbons. Such reduced exposure could favor more effective rhizodegradation over time.

Despite the limited growth of ryegrass, the final dissipation rate of 70 % after 120 days in the presence of ryegrass and *B. thailandensis* was relatively high. It is comparable to several hydroponic rhizodegradation studies. For instance, Chaîneau et al. (2000) reported 64–80 % total hydrocarbon (TH) degradation (initial TH concentration: 650 mg L^{-1}) after 40 days of hydroponic culture in Hoagland medium with or without maize. Similarly, Flocco et al. (2002) observed a 2.7-fold reduction in phenanthrene half-life (initial concentration: 50 mg L^{-1}) in 30-day hydroponic culture with alfalfa. Hussain et al. (2019) reported 92 % hexadecane removal (initial concentration: 150 mg L^{-1}) within 50 days using a consortium (*Acinetobacter* spp. and *Bacillus niabensis*) combined with the halophyte *Leptochloa fusca*. However, these previous studies typically involved simpler hydrocarbon mixtures and lower concentrations (0.05–0.65 g L^{-1}). In this context, while such results demonstrate the potential of plant–microbe interactions under controlled conditions, extrapolation to soil systems should be made cautiously. Hydroponic systems do not account for soil-specific processes such as sorption, spatial heterogeneity, or root–soil matrix interactions.

In contrast, our system contained a complex, weathered hydrocarbon mixture dominated by light to intermediate fractions (C_{12}–C_{30}). It also comprising up to 80 % unresolved complex mixture (UCM) (D'Incau et al., 2024a). The initial total hydrocarbon concentration was high (2 g L^{-1}). Notably, only the RG + Bth treatment showed significant depletion of these fractions. This supports confirming its enhanced rhizodegradation potential for aged mixtures. Moreover, the absence of n-C_{17} and n-C_{18} (C_{17}/pristane and C_{18}/phytane = 0) indicates prior weathering and partial degradation (Ma et al., 2007). This weathering likely contributed to the limited biodegradability of the remaining fractions. Compared with model-compound studies, our results underscore the real-world challenge of remediating aged and recalcitrant hydrocarbon mixtures.

4.2. Biosurfactant supplementation fails to promote hydrocarbon rhizodegradation

Although biosurfactants are widely applied in bioremediation to increase hydrocarbon bioavailability (Melikoglu, 2026), our results show that adding rhamnolipids did not improve hydrocarbon rhizodegradation. In our study, all treatments initially contained anionic biosurfactants from the hydrocarbon mixture at a concentration of 44 mg L^{-1}. In treatments without rhamnolipid supplementation, biosurfactant concentrations increased after 60 days. In contrast, concentrations remained stable or declined when commercial rhamnolipids were added. These results question the relevance of externally adding biosurfactants to hydrocarbon-contaminated systems. Such compounds may already be present *in situ*. Moreover, the addition of commercial rhamnolipids did not stimulate further biosurfactant production. It may even have reduced net biosurfactant levels over time. This highlights the difficulty of controlling biosurfactant dynamics in biologically active systems.

It should also be noted that the methylene blue complexation assay quantifies only anionic biosurfactants. It does not provide structural identification. Thus, other anionic biosurfactants besides rhamnolipids may have been produced. Non-ionic or cationic surfactants could also have been produced or consumed during incubation. This limitation further complicates interpretation. It also supports caution when proposing biosurfactant supplementation as a general strategy.

Interestingly, the lowest biosurfactant concentration (26.55 mg L^{-1}) was observed in RG+Bth at 120 days. This treatment also showed the highest dissipation rate. Several mechanisms can explain this pattern. Firstly, one could assume that enhanced rhizodegradation reduced biosurfactant concentrations through co-consumption. However, no significant differences in microbial abundance or density were observed among treatments. RG+Bth even showed the lowest microbial concentration at 120 days. Therefore, the low biosurfactant concentration is unlikely to reflect higher microbial biomass. Instead, the decrease is more consistent with co-metabolic processes. Hydrocarbons solubilized within micelles may have been degraded together with associated biosurfactants. This could lead to simultaneous consumption of both compounds. This mechanism aligns with the observed dissipation patterns. It may explain the lower biosurfactant levels in the most efficient rhizodegradation treatment.

Furthermore, rhamnolipid addition did not enhance hydrocarbon rhizodegradation after 120 days. This is consistent with Zhu and Zhang (2008), who found that ryegrass uptake of PAHs decreased with increasing biosurfactant concentration. The optimal biosurfactant concentration for PAH adsorption was around 25.8 mg L^{-1}. This value is close to the biosurfactant concentration observed in RG+Bth treatment at 120 days.

Beyond adsorption effects, rhamnolipids can impair plant development. Parus et al. (2023) described two mechanisms: disruption of membrane integrity and increased xenobiotic bioavailability. Both could reduce plant growth and root biomass (D'Incau et al., 2025, 2024b). High surfactant concentrations can also inhibit microbial activity (Liu et al., 2023). They can shift metabolism toward

surfactant consumption (De Luca and Autilio, 2021). They may also sequester hydrocarbons in micelles, reducing effective bioavailability for biodegradation (Zhu et al., 2025). The phytotoxic effects of biosurfactants, alone or in combination with petroleum hydrocarbons, have been previously investigated in detail for ryegrass under comparable conditions (D'Incau et al., 2025, 2024b). Therefore, mechanistic phytotoxicity endpoints were not re-explored here.

The shortest root and shoot lengths after 120 days were observed in RG+Bth. This treatment also had the lowest final anionic biosurfactant concentration. Factorial analysis nevertheless revealed that ryegrass biomass (dry root and shoot dry weights) was inversely correlated with biosurfactant concentration. Based on growth-related endpoints, these findings therefore reinforce the hypothesis of increased hydrocarbon phytotoxicity in the presence of biosurfactants (D'Incau et al., 2025). They also indicate that higher biosurfactant concentrations do not improve rhizodegradation efficiency in this system. Furthermore, plant growth and biosurfactant concentrations were measured with a high number of replicates ($n = 6$). This supports the robustness of the negative relationships observed between biomass, biosurfactants, and hydrocarbon dissipation.

Our data additionally suggest microbial growth plateaued by 60 days. No significant further increase was detected up to 120 days (p-values > 0.05). As microorganisms had already reached stationary phase, a higher surfactant concentration could not further stimulate biodegradation (D'Incau et al., 2024a). This may explain why presence of biosurfactants did not translate into higher dissipation by microorganisms, which were unable to process an increase in hydrocarbon solubilization. The decrease in anionic biosurfactant in rhamnolipid-supplemented treatments suggests that commercial rhamnolipids may have served as a preferred carbon source. This could counteract the intended goal of stimulating hydrocarbon degradation.

Overall, rhamnolipid supplementation did not enhance rhizodegradation. It may even have hindered it. The complexity and weathered nature of the hydrocarbon mixture likely remained the main limitation. In addition, biosurfactant-associated phytotoxicity could further constrain plant-microbe functioning. It should be noted that this experiment was deliberately conducted under hydroponic conditions. These conditions maximize hydrocarbon availability. They also isolate biological effects from soil-related limitations. Consequently, preferential biosurfactant consumption may be less pronounced in soil. Phytotoxic effects of hydrocarbons in the presence of biosurfactants may also be less severe.

On the other hand, Lin et al. (2011) reported that biosurfactant addition enhanced diesel biodegradation in contaminated soil only at the beginning of incubation. No sustained benefit was observed after 80–100 days. Similarly, Szulc et al. (2014) found that rhamnolipid addition alone did not improve diesel degradation in soil. These results suggest rhamnolipids may not be advantageous in soil either. Moreover, overcoming the strong adsorption of rhamnolipids to soil particles would require large biosurfactant quantities. This would increase costs and could compromise ryegrass growth.

5. Conclusion

This study demonstrates that the combined use of ryegrass and *Burkholderia thailandensis* (RG+Bth) effectively enhances the rhizodegradation of a complex, weathered hydrocarbon mixture, with an initial hydrocarbon concentration of 2 g L^{-1}, under controlled hydroponic conditions. Despite limited plant growth, hydrocarbon dissipation reached 70 % after 120 days. This enhancement was driven mainly by plant–microbe interactions and metabolic activity, rather than by total microbial abundance. In contrast, the addition of commercial rhamnolipids did not improve hydrocarbon degradation. It was instead negatively associated with ryegrass growth. Based on plant growth-related responses, higher biosurfactant concentrations were associated with increased hydrocarbon-induced stress. This stress likely limited rhizodegradation efficiency. Importantly, biosurfactants naturally present in the hydrocarbon mixture appeared sufficient to enable hydrocarbon solubilization. This indicates that biosurfactant production is already ubiquitous and functionally active in such systems. In this context, the key advantage of *B. thailandensis* lies in its hydrocarbon-degrading capacity rather than in its biosurfactant production. Accordingly, attempts to artificially increase biosurfactant concentrations under biologically active conditions may be counterproductive. Such additions may amplify phytotoxicity and reduce rhizodegradation performance. The conclusions of this study are based on growth-related endpoints. More detailed physiological or biological assessments of phytotoxicity were beyond the scope of this work. These aspects have already been addressed in previous studies. Overall, the optimization of rhizodegradation should prioritize robust plant–microbe partnerships, such as ryegrass and *B. thailandensis*, rather than external biosurfactant inputs. Finally, it should be emphasized that this work was designed as a mechanistic investigation under simplified hydroponic conditions. Validation under soil conditions is now required. Such studies are necessary to confirm these results and assess their applicability for field-scale remediation of hydrocarbon-contaminated environments.

CRediT authorship contribution statement

D'Incau Emmeline: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sonia Henry:** Supervision, Project administration, Funding acquisition, Conceptualization. **Sylvie Egloff:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Catherine Lorgeoux:** Writing – review & editing, Investigation, Formal analysis. **Lucas Charrois:** Validation, Methodology, Investigation, Formal analysis. **Héloïse Bazart:** Validation, Methodology, Investigation. **Stéphanie Ouvrard:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.eti.2026.104771.

Data availability

Data will be made available on request.

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