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Review

Basic principles for biosurfactant-assisted (bio)remediation of soils contaminated by heavy metals and petroleum hydrocarbons – A critical evaluation of the performance of rhamnolipids

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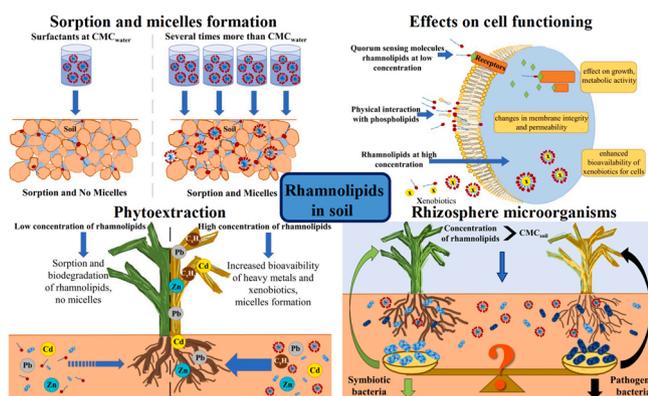
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HIGHLIGHTS

- Effective concentration of rhamnolipids in soil differs from CMC in aqueous systems.
- At high concentrations rhamnolipids may directly or indirectly cause toxic effects.
- Preferential biodegradation of rhamnolipids may reduce the removal rates.
- Rhamnolipids may cause selective shifts in soil microbial communities.

GRAPHICAL ABSTRACT



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ABSTRACT

Despite the fact that rhamnolipids are among the most studied biosurfactants, there are still several gaps which must be filled. The aim of this review is to emphasize and to indicate which issues should be taken into account in order to achieve efficient rhamnolipids-assisted biodegradation or phytoextraction of soils contaminated by heavy metals and petroleum hydrocarbons without harmful side effects. Four main topics have been elucidated in the review: effective concentration of rhamnolipids in soil, their potential phytotoxicity, susceptibility to biodegradation and interaction with soil microorganisms. The discussed elements are often closely associated

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Phytoextraction
Critical micellar concentration

and often overlap, thus making the interpretation of research results all the more challenging. Each dedicated section of this review includes a description of potential issues and questions, an explanation of the background and rationale for each problem, analysis of relevant literature reports and a short summary with possible application guidelines. The main conclusion is that there is a necessity to establish regulations regarding effective concentrations for rhamnolipids-assisted remediation of soil. The use of an improper concentration is the direct cause of all the other discussed phenomena.

1. Introduction

Biosurfactants have attracted the interest of numerous scientists for many years. One of the best known and theoretically best described groups of biosurfactants are rhamnolipids, which are mixtures of glycolipids based on rhamnose and β -hydroxyalkanoic acids (Chrzanowski et al., 2012a). The rhamnose parts in rhamnolipids represents the hydrophilic “head” part of the biosurfactant which is connected via an ether bond with the acid residue that serves as the hydrophobic “tail” (Varjani et al., 2021). There are two major forms of rhamnolipids – monorhamnolipids, which contain a single rhamnose moiety, and dirhamnolipids, which include two rhamnose units per single molecule (Soberón-Chávez et al., 2021). Both groups include a wide array of congeners and their structure depends on the number of β -hydroxyalkanoic acid moieties (a rhamnolipid may contain up to three acid units) as well as the length of the acid carbon chain, which usually ranges from 8 to 14 carbons (Jiang et al., 2020). Rhamnolipids are mainly produced by *Pseudomonas aeruginosa*, although production by other *Pseudomonas* species or even other species has also been reported (Kashif et al., 2022). They act as efficient anionic surfactants of biological origin and can reduce the surface tension of water from 72 to approx. 27 mN/m (Kashif et al., 2022). Their properties allow for pseudo-solubilization of organic compounds in the aqueous phase, formation of emulsions and modification of cell surface hydrophobicity due to interactions with microbial cells (Varjani and Upasani, 2017). This, combined with the fact that rhamnolipids also exhibit anti-microbial properties, results in a notable spectrum of possible applications, such as enhanced oil recovery and anti-corrosive action in the petroleum and gas sectors, protection of plants and stabilization of edible products in the agricultural and food sectors, pathogen control and enhanced wound healing in the medical sector, stabilization of emulsions and skin repair in the pharmaceutical and cosmetics sectors, however their involvement in bioremediation as agents which enhance environmental clean-up attempts is perhaps their most recognizable function (Kashif et al., 2022; Ambaye et al., 2021; Chafale and Kapley, 2022; Zhu et al., 2022; Li et al., 2022; Zhao et al., 2021).

Rhamnolipids-mediated remediation of soils can be successfully applied in case of both major groups of environmental contaminants: organic compounds as well as heavy metals, although the underlying removal enhancement mechanisms are different. In case of organic pollutants (mostly petroleum hydrocarbons), the major problem is associated with mass transfer limitations. Such compounds are typically characterized by low water solubility and are prone to sorption in the soil matrix. This essentially decreases their availability to microorganisms and inhibits their biodegradation rate (Wang et al., 2021a). Rhamnolipids can facilitate the biodegradation of organic contaminants via two possible mechanisms (Gaur et al., 2022a). The first is based on the solubilizing effect of biosurfactant molecules, which results in the mobilization of poorly accessible contaminants and increase of their bioavailability as well as bioaccessibility to potential degraders (Markande et al., 2021). The second mechanism is associated with promotion of direct adherence of microorganisms to organic contaminants by modulation of cellular hydrophobicity (Bao et al., 2022). Ultimately, the presence of rhamnolipids results in improved contact between the microbial cells and organic pollutants, which translates into higher microbial metabolic activity (Khoshkholgh Sima et al., 2019). Environmental clean-up in case of heavy metals requires a different

approach, as these pollutants cannot be eliminated using degradation processes. In order to avoid the uncontrolled mobilization and transport of heavy metal ions, there are also two possible scenarios for biosurfactant-assisted remediation: stabilization and soil flushing (Chen et al., 2017). Stabilization is achieved by chelation of heavy metals using biosurfactant molecules and results in limitation of their mobility due to the size and hydrophobic properties of the formed complexes, which can be subsequently collected by e.g. hyperaccumulator plants (Lopes et al., 2021). In contrast, flushing of soil is focused on the controlled removal of specific metal ions due to their coordination by rhamnolipids (Manga et al., 2021). Mobilization of relevant heavy metals, such as Cd, Ni, Zn, Fe or Pb allows to notably reduce their concentration in soil by applying soil washing processes (Lee and Kim, 2019; Johnson et al., 2021). It should be noted that both organic and inorganic pollutants often occur simultaneously, and rhamnolipids have also been investigated in co-contaminated soils (Zhang et al., 2022). For an in-depth description of interactions between biosurfactants and heavy metals or organic compounds as well as a detailed explanation of associated bioremediation strategies, please check these comprehensive reviews (Liu et al., 2018; Mishra et al., 2021; Zeng et al., 2018).

Among the many studies devoted to the production, properties and uses of rhamnolipids, there are numerous works which focus on their advantages and highlight their market potential (Liu et al., 2018; Akbari et al., 2018; Cameotra et al., 2010), e.g. the virucidal effects of rhamnolipids were recalled when COVID-19 emerged (Bakkar et al., 2021; Jin et al., 2021). However, there is a single fundamental question which has not been answered to date - namely why rhamnolipids are not used globally despite so many benefits?

The answer is a complex amalgam of several factors. The first major issue is associated with economic factors. To date, rhamnolipids have mainly been produced at high, viable concentrations by strains of the opportunistic pathogenic bacteria *Pseudomonas aeruginosa*, classified as biosafety level two (S2) from strategic substrates (e.g. sugars, plant oils or alcohols). As such, it very challenging to employ a feasible, sustainable fermentation technology due to the S2 safety regulations, limited feedstock and high purification cost of rhamnolipids, which further increase the gap to achieve actual viability. These issues, along with the possible solutions associated with improved yield, use of non-pathogenic producers, alternative feedstock as well as application of omics and gene-based analytical tools, have already been extensively discussed in the following reviews (Mishra et al., 2021; Chebbi et al., 2022; Mohanty et al., 2021; Mouafo et al., 2022; Sun et al., 2021; Irorere et al., 2017).

The second crucial problem is more subtle and stems from the fact that expectations regarding the effect of rhamnolipids may not meet the challenges of reality. The advantages of rhamnolipids as compounds of bio-based origin are often extolled to a point that eludes all criticism. A clear example of this phenomenon is the division between surfactants of natural origin and synthetically produced surfactants, which is often reduced to the assumption that the former only possess advantages, while the latter exhibit disadvantages (Shao et al., 2017; Sáenz-Marta et al., 2015). This is in line with the common misconception that BIO products (plastic, chemical, etc.) are always better, more sustainable and cleaner. There is a logic error in such reasoning, since the properties of a surface-active molecule are mainly determined by its amphiphilic, hydrophilic-hydrophobic nature and to a much lesser extent by its origin. Moreover, the “bio is always better” concept consistently ignores

the developments in the sectors of synthetic biodegradable surfactants or the use of bio-based compounds for such synthesis, which have been made over the years (Bhadani et al., 2017; Villandier and Corma, 2010).

In fact, a critical analysis of the underlying limitations of rhamnolipids allows to identify many issues that have been overlooked or only partially analyzed during previous research. This review is focused on highlighting the following aspects which have been identified as possible predicaments associated with the use of rhamnolipids for enhanced recovery of heavy metals and biodegradation of hydrocarbons in soil systems:

- I. Effective concentration of rhamnolipids in soil;
- II. Phytotoxicity of rhamnolipids;
- III. Biodegradability of rhamnolipids;
- IV. Impact of rhamnolipids on the soil microbiome.

The aim of this review is to emphasize and to indicate which issues should be taken into account in order to achieve efficient rhamnolipids-assisted biodegradation or phytoextraction without harmful side effects.

2. Effective concentration of rhamnolipids in soil

2.1. Potential issues and questions

1. There is a lack of guidelines regarding the applicable ranges of concentrations during rhamnolipids-assisted bioremediation attempts.
2. The concentration of rhamnolipids in soil may be too low to achieve the expected effect.
3. What is the critical micelle concentration of rhamnolipids in soil systems?

A typical characteristic of surfactants is the ability to form micelles when a certain specific concentration, called the critical micelle concentration (CMC), is exceeded. At this point, further increases in surfactant concentration do not translate into a decrease of surface tension, only further micelle formation (Cui et al., 2008). However, the critical micelle concentration (CMC) values are valid only for pure aqueous systems (Wen et al., 2010). Any introduction of sorbent into such a solution will result in sorption of the surfactant. Soil is composed of mineral and organic components, each of which exhibits greater or lesser sorption properties. Therefore, for micelle formation to occur, the surfactant concentration must be increased by at least a concentration

equal to the sorption loss. In soil, micelles will form at much higher concentrations that are even several times higher compared to CMC values for pure water. This translates into the need to determine the concentration at which micelles will form in the actual soil system.

In order to visualize the extent of this problem, a simple experiment was conducted in which the concentrations of rhamnolipids needed to form micelles in aqueous and soil systems were compared (please see Fig. 1, the experiment is further described in the Supplementary Materials). For the pure aqueous system, the CMC_{water} value is in the range of 50–100 mg/L and corresponds well to the values reported by other authors (Wen et al., 2010; Gunawardana et al., 2011). It is important to note that the ratio of individual rhamnolipids present in the commercial JBR-425 formulation may vary, which will translate into a wider range of CMC values. The purity of rhamnolipids was also found to have a significant effect - lower purity generally results in higher CMC (Kłosowska-Chomiczewska et al., 2017). The presence of mineral and organic sorbents in the soil causes a huge loss of rhamnolipids, and the concentration at which micelles form is several times higher than for the aqueous system. For the copper smelter soil used in the experiment, the CMC_{soil} value was achieved at a concentration equal to 500 mg/L, which means that the CMC_{soil} is higher by approx. one order of magnitude than CMC_{water} ($5-10 \times CMC_{water}$). In this respect, rhamnolipids are no different from any other synthetic surfactants. For example, in their study Li et al. determined that the CMC_{soil} values of Triton X-100 and Brij 35 were 2.75 and 6.31 times higher compared to their CMC_{water} values respectively (Li et al., 2014).

An analysis of research works focused on the rhamnolipids-assisted extraction, phytoextraction, remediation, phytoremediation and bioaugmentation was conducted in terms of the concentrations used and the ability to form micelles (see Table 1) (Wen et al., 2010; Gunawardana et al., 2011; Gusiati et al., 2021; Maier et al., 2001; Posada-Baquero et al., 2020; Wang et al., 2021b; Mekwichai et al., 2020; Barajas-Aceves et al., 2015; Zhen et al., 2019; Ram et al., 2019; Jensen et al., 2011; Jia et al., 2020; Liao et al., 2016; Liduino et al., 2018; Ptaszek et al., 2020; Lu et al., 2017). The studies mainly involved removal of heavy metals (65%) or petroleum hydrocarbons (30%), with a single report addressing both pollutants and included short-term (18 h) as well as long-term (210 days) experiments. The applied concentrations of rhamnolipids ranged from 1 mg to 22.5 g/L in case of extraction treatments and from 4 mg to 7 g/kg of soil in case of remediation treatments. A single application of the biosurfactant was the predominant form of application (90% of studies) with only two studies opting for regular dosing (after 7 or 14 days). The scientific reports can

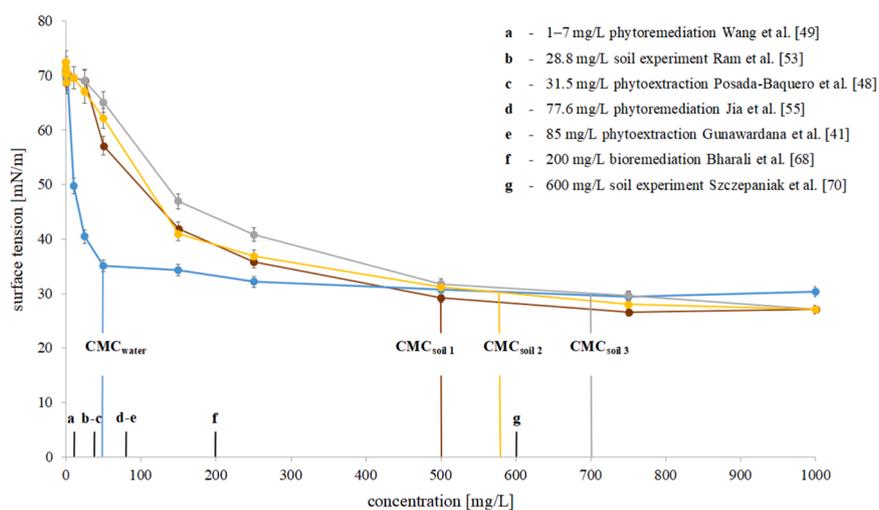


Fig. 1. Comparison of micelle-forming ability of rhamnolipids in aqueous (CMC_{water}) and soil (CMC_{soil}) environments along with an indication of the range in which other authors have conducted research. Three different types of soil were used for determining the CMC_{soil} values: soil 1 (this study), soil 2 (Woźniak-Karczewska et al., 2019), soil 3 (Woźniak-Karczewska et al., 2022). For more information please see the Supplementary Materials.

Table 1
Summary of the results of extraction, phytoextraction, remediation, phytoremediation and bioaugmentation experiments carried out using rhamnolipids with respect to the concentrations used and micelle forming ability.

Xenobiotic type	Remediation process	Plant	Duration	Rham conc.	Rham conc. in relation to CMC _{water}	Monitor of rham biodegr.	Efficiency of xenobiotic removal	Research setup	Parameters	Analytical methods	Quality of analytical procedure	Conclusions	Comments	Ref.
30 g/kg total petroleum hydrocarbons in soils	phytoremediation/biodegradation	<i>Spartina anglica</i>	60 days	rham (0.5 g/kg) or 2% wt. rham modified biochar	not discussed	no	32-35%	mixture of oil contaminated and non-contaminated soil	not discussed	Soxhlet extraction, gravimetric, GC, PAHs, chlorophyll	rough analysis of degraded fractions	Interactions between microbial groups present in the soil changed after introduction of biochar and rham modified biochar.	No details on rham resilience to biodegradation and mechanism of action were presented.	Zhen et al.[52]
2% diesel oil	phytoremediation/bioaugmentation	vetiver grass (<i>Chrysopogon zizanioides</i>)	21 days	2% probably by weight	above CMC _{water} which was determined by authors as 28.8 mg/L	no	2.1 – 4.3 mg/g out of initial 7–9 mg/g	natural soil supplemented with Bushnell Hass medium	not discussed	extraction using chloroform and gravimetric method	very rough analysis of total petroleum hydrocarb.	Rham application along with bioaugmentation improved the phytoremediation efficiency of vetiver grass.	Exogenously added rham and no proof that introduced bacteria was capable of rham production. The fate of rham is unknown. Possible stimulation by N, P addition from Bushnell Hass medium. The hydrocarbon extraction efficiency may have been altered by emulsions.	Ram et al.[53]
Cu 10 mg/L, Cd 1 mg/L, Pb 5 mg/L	phytoextraction	perennial ryegrass (<i>Lolium perenne</i>)	2, 10, 30 days	85 mg/L JBR 425	1.7 × CMC _{water} = 50 mg/L	no	1–2.5-fold increase in accumulat. in roots and shoots compared to control	hydroponic Hoagland medium	not applicable	sample digestion, GF-AAS	very good	Rham was effectively increasing metal conc. in shoot in combination with citric acid, histidine, EDDS, sulfate. Rham is unlikely to facilitate increased transport to shoots and complexes formed are large and intact micelles formed would not be likely to traverse effectively through the cell membranes. Rham and sulfate were not effective for improving Pb concentrations in shoots, but significantly enhanced Cd levels in shoots.	Due to hydroponics, all interactions with the soil matrix have been omitted. Perfectly visible an average efficiency of sole rham compared to systems with other amendments.	Gunawardana et al.[41]
soil 1: Cu 1200 mg/kg, Zn 2900 mg/kg, soil 2: Cu 170 mg/kg, Zn 280 mg/kg	phytoextraction	willow (<i>Salix viminalis</i> Arresoe)	-	25 mM JBR 425 approx. 14.4 g	not discussed	no	soil 1: Cu 5.6%, Zn 6.9%, soil 2: Cu 1.5%, Zn 2.5%	calcareous soils have less than 100 ppm total hydrocarbons and <4 ppm PAHs	partially discussed	multi-step batch extraction test, ICP-MS	very good	Due to the very limited capacity to dissolve Cu and Zn, rham cannot be recommended for use in enhanced phytoextraction of heavy metal polluted, soils, at least not for calcareous soils.	The fate of rham is unknown.	Jensen et al.[54]
Cu 197 mg/kg, Zn 1550 mg/kg, Pb 342 mg/kg, Cd 2.2 mg/kg	phytoremediation	ryegrass (<i>Lolium perenne</i> L.)	50 days	0-1.0 g/kg	below and above CMC _{water}	no	migration to shoots within 9 days: Pb 98%, Cd 80%, Zn 30%, Cu 90%	heavy metal-contaminated soil	60% of field capacity, no other factors were discussed	sample digestion, GF-AAS	very good	Rham could significantly improve the activity of soil enzymes for facilitating the digestion and transformation of heavy metals from soils toward the above-ground part of ryegrass.	The fate of rham is unknown. The extraction capacity of ryegrass in this case indicates that it is a hyperaccumulator. Phytotoxicity studies and their correlation with metal accumulation are lacking.	Jia et al.[55]
crude oil 5000 mg/kg	Phytoremediation	maize (<i>Zea mays</i> L.)	90 days	2 g/L every 14 days	not discussed	no	TPH removal: 58%, saturated hydrocarb. decreased from 60% to 36%, aromatic hydrocarb. increased from 22% to 38%, asphaltene increased from 18% to 26%	artificially soil spiked with crude oil	partially discussed	gravimetric method, GC - MS	moderate due to gravimetric method	Biosurfactant amended phytoremediation may be a useful biotechnological approach for the remediation of petroleum hydrocarbon polluted soil.	The results and discussion are not consistent. As stated by the authors „the positive effects on the growth of maize grown in rham-treated soil may be due to the degradation of rham in soil, resulting in more plant growth-promoting microorganisms in the rhizosphere and better physical soil conditions for nutrition uptake by the plant“, which is not consistent with statement „the accumulation of polycyclic aromatic hydrocarbons was inhibited in the maize leaf by all selected surfactant treatments, but was facilitated in maize root by the treatments of rham“	Liao et al.[56]
TPH 18145 mg/kg, PAH 21152 µg/kg, Ni 78 mg/kg, Cr 79 mg/kg, Pb 112 mg/kg, Zn 1750 mg/kg	phytoremediation	sunflower (<i>Helianthus annuus</i> L.)	90 days	4 mg/kg	not discussed	no	TPH reduction 58%, PAH reduction 48%, Ni reduction 41%, Cr reduction 30%, Pb reduction 29%, Zn reduction 20%	soil contaminated with oily residues from petrochemical facility	80% of field capacity, other factors partially discussed	metals - sample digestion, ICP-MS, hydrocarbons - GC-FID	very good	Increase in Zn uptake by sunflowers, while Ni, Pb, and Cr uptake by sunflowers was achieved irrespective of biosurfactant supplementation.	The conc. of rham is too low to observe the complexing effect of rham with respect to heavy metals and at the same time an increase in the degradation of hydrocarbons. The fate of rham is unknown.	Lidumbo et al.[57]

(continued on next page)

Table 1 (continued)

TPH 25758 mg/kg soil d.w.	phytoremediation/bioaugmentation	ryegrass (<i>Lolium perenne</i> L. cv. <i>Pearlgreen</i>)	112 days	5 µg/g soil d.w.	intentionally below CMC _{water}	no	TPH removal: 31%	2.5% hydrocarbon polluted industrial soil	partially discussed	GC	good	<i>R. erythropolis</i> CDEL254 increased the biomass of plant; addition of rham inhibited this effect. The use of rham and bioaugmentation does not increase the efficiency of the process. The complexity of microorganisms, plants, and pollutants interactions were reported.	The fate of rham is unknown. A possible explanation for the action of rham may be due to interactions with Quorum sensing systems.	Praszcak et al. [58]
Cd 10 mg/kg, Pb 552 mg/kg	enhanced phytoextraction in the presence of rham	maize (<i>Zea mays</i> L.)	21 days	500 mg/kg soil	not discussed	no	shoot conc.: Cd 41 mg/kg d.w., Pb 178 mg/kg d.w.	artificially polluted with Cd and Pb	discussed	sample digestion, AAS	very good	Heavy metals leaching for rham assisted phytoextraction was the lowest compared to EDTA, EDDS.	Chelant applied at a realistic dose for commercial applications. The fate of rham is unknown.	Lu et al. [59]
Cu 1652 mg/kg, Pb 291 mg/kg	remediation/extraction	maize	42 days	22.5 g/L water JBR 425	above CMC _{water}	no biodegr., 10% loss by sorption to soil	Pb removal: 64 – 73% Cu removal: 60 – 73%	copper smelter soil	discussed	sequential batch washing	good	Sequential washing increased organic carbon in the soil due to biosurfactant sorption. Micelle formation and complexation of metals were responsible for metal removal.	If we assume that CMC for rham in JBR 425 preparation is 50 mg/L, the authors used concentration of 450 CMC, despite that they still observed 10% loss of rham it means that 45×CMC was lost for sorption processes. The fate of rham is unknown.	Gusiatin et al. [46]
soil 1: Cd 1.49 mg/kg, Zn 131 mg/kg soil 2: Cd 1.56 mg/kg, Zn 139 mg/kg	phytoextraction	maize (<i>Zea mays</i>), sunflower (<i>Helianthus annuus</i>)	42 days	0-1.4 mmol/kg/week JBR 425 approx. 0-808 mg/kg/week	below, equal and above CMC _{water}	no biodegr., sorption of rham was studied	soil solution maize Cd 5 – 27 µg/L, Zn 1 – 8 mg/L, soil solution sunflower Cd 2 – 28 µg/L, Zn 0.3 – 1.1 mg/L	artificially soil spiked with Cd and Zn	insightfully discussed	batch adsorption experiments, sample digestion, ICP-OES or ICP-MS	very good	Rham can increase the solubility of Cd in soil washing, no evidence that rham will enhance Cd removal by phytoextraction. Adsorption of rham in soil at low application rates resulted in insufficient metal mobilization and subsequently inadequate Cd accumulation by plants. High doses of rham decreased plant growth. The behavior of rham in soils is highly concentration-regulated so that the window where Cd solubilisation is high but phytotoxicity is low is absent or narrow, which limits its use in phytoextraction.	Very methodical analysis addressing all possible scenarios including consideration of rham phytotoxicity, although the rham biodegradation was not taken into account.	Wen et al. [40]
soil containing: Ca 1.0%, Pb 0.1%, Zn 0.05% wt.	phytoremediation	corn (<i>Zea mays</i> Mayo Tripsano), halophyte (<i>Atriplex nummularia</i>)	21–35 days	5 mmol/kg soil approx. 2885 mg/kg	not discussed	no	corn - enhanced Cu shoot uptake from 37 to 113 mg/kg, halophyte - decreased Cu root uptake from 82 to 45 mg/kg	mine tailing waste classified as a loamy sand mixed 1:1 with a forest mulch	partially discussed	Cu – AAS analysis	good	Rham were plant specific. For corn, rham enhanced shoot uptake, for <i>Atriplex</i> , rham decreased shoot uptake.	Rham were degraded by soil populations in approx. 2 weeks, important to maintain a constant level of rham in the system.	Maiter et al. [47]
soil 1: Pb 23 864 mg/kg, Fe 21 700 mg/kg soil 2: Pb 3783 mg/kg, Fe 98 800 mg/kg	remediation/extraction	maize	18–22 h	10 mM approx. 5770 mg	not discussed	no	soil 1: Pb removal 3660 µg/g soil 2: Pb removal 540 µg/g	historically contaminated soils – wastes from local Ag, Pb, Zn, mines	partially discussed	batch soil washing experiments, Pb, Fe – AAS analysis	good	Rham are capable of metal removal far in excess of the soluble portion removed by the control extracts, efficiency is dependent on soil type and type of metal contamination.	Rham removed more than the soluble fraction of Pb.	Maiter et al. [47]
PAHs 513 ± 84 mg/kg	phytoextraction	sunflower (<i>Helianthus annuus</i> L.)	210 days	7 µg/kg soil R90, 90% pure AGAE Technologies	CMC _{water} = 31.5 mg/L	no	80-90% mineralization of fluorene, phenanthrene, anthracene, fluoranthene, pyrene	artificially heavily polluted soil combined with sand	partially discussed	Tmax, Soxhlet extraction, PAHs-HPLC analysis	good	Slowly-desorbing PAHs that remain after the initial phase of fast biodegradation were mobilized significantly by the rham. Problems with rhizosphere influence on biosurfactant action.	The fate of the rham is unknown. The CMC _{soil} is unknown, but it can be assumed that the rham conc. of 7 g/kg soil assure formation of micelles. The toxic effects on the plant have not been studied although mentioned.	Possada-Baquero et al. [48]
Cd	Phytoremediation	Siberian iris (<i>Iris sibirica</i> L.)	60 days	1–7 mg/L	not discussed	no	The accumulation of Cd in leaves, stems and roots was the highest (in root increase of 21.5 mg/kg and shoot increase of 5 mg/kg in comparison to the control) for rhamnolipids concentration <1 mg/L	Cd = 25 mg/L Cd ²⁺ as Cd(NO ₃) ₂ × 4H ₂ O nutrient solution	not applicable	sample digestion, ICP-MS	very good	Rham enhanced the accumulation of Cd in roots of <i>Iris sibirica</i> L. Rham can damage leaves, but are not harmful for roots. Rham stimulates the antioxidant enzymes, which protect plants from the harmful effect of Cd.	The fate of rham is unknown. At the conc. used, rham cannot act as surfactants, and their effect is most likely due to the biological role played by this molecule.	Wang et al. [49]
Cd 36.8 mg/kg	phytoextraction	corn (<i>Zea mays</i>)	7, 45, 80 days	2308 mg/kg, which is 4 mmol/kg a mixture of mono- and di-rham at a ratio of 55:45	CMC _{water} = 0.99 mM, approx. 577 mg/L CMC _{soil} = 35.66 mM approx. 20576 mg/L	no	Cd in the above-ground parts of corn treated with rham were 192.4 µg, control – 134.4 µg Cd removal from soil in presence of rham 3-5-fold higher compared to control	Cd(NO ₃) ₂ × 4H ₂ O	soil spiked with 36.8 mg/kg Cd	sample digestion, FAAS, ICP-MS	very good	Above-ground parts of corn accumulated Cd better than roots. The higher Cd uptake and removal efficiencies explained by the fact that biosurfactants can form complexes with Cd ions, which increases Cd mobility in soil.	The fate of rham is unknown. Very high conc. of rham, note that the soil studied is probably rich in organic compounds. There is usually a higher accumulation of metals in roots than in shoots. The authors should investigate this phenomenon further.	Mekwicheh et al. [50]
Pb 3411 µg/g, Zn 7329 µg/g, Cu 215 µg/g, Cd 55 µg/g, Cr 64 µg/g, Ni 69 µg/g	phytostabilization	<i>Acacia retinodes</i> , <i>Nicotiana glauca</i> , <i>Echinochloa polystachya</i>	5 months for shrubs 7 months for grass	Mixture of crude rham from <i>Bacillus israelensis</i>	CMC at 5% - provided by authors unable to determine real conc.	no	the highest % reductions in root, stem, and/or leaf weights occurred for <i>N. glauca</i> stem 90%, <i>A. retinodes</i> stem 83%, and <i>E. polystachya</i> root 81%	mine tailings and compost	discussed	sample digestion, AAS	good	Concentrations of heavy metals in the three plants irrigated with crude biosurfactant were not significantly different from those irrigated with water.	Probably an incorrect biosurfactant analysis - <i>Bacillus</i> does not produce rham. It is unknown what conc. was used for the study. The fate of the rham is unknown.	Barajas-Aceves et al. [51]

basically be divided into two groups.

The first group describes the positive effect of introducing rhamnolipids on the processes of biodegradation of petroleum substances or phytoextraction of heavy metals in the soil environment. Interestingly, the rhamnolipids were used at concentration below the CMC_{water} , equal to the CMC_{water} or above the CMC_{water} , but were always below the CMC_{soil} for analyzed soil samples (Gunawardana et al., 2011; Ram et al., 2019; Jia et al., 2020). The authors justified their results based on typical physicochemical properties of rhamnolipids, i.e. solubilization (Gunawardana et al., 2011; Gusiatin et al., 2021). It should however be noted, that rhamnolipids most likely did not form any micelles because they were applied at too low concentrations.

The second group of reports outlines soil experiments in which rhamnolipids were used at concentrations that ensured micelle formation (CMC_{soil}) and thus were multiple times higher than CMC_{water} (Wen et al., 2010; Gusiatin et al., 2021). The authors used high concentrations of rhamnolipids and in most cases it was possible to observe the efficient performance of rhamnolipids as emulsifiers or chelants. In this case it should be emphasized that aside from the advantageous action of rhamnolipids, negative effects were also established in some studies, which were mostly associated with phytotoxicity.

To summarize this section, two basic facts should be noted. First, the concentration at which micelles form in soil is many times higher than the concentration at which micelles form in aqueous systems. If the concentration of rhamnolipids is too low, there is no chance for micelle formation and encapsulation of xenobiotics or for the efficient complexation of heavy metals (please see Fig. 2). Most rhamnolipids will simply be more or less bound to the soil matrix. Second, rhamnolipids may also contribute to positive effective at low concentrations, however in this case they will likely function as additional carbon sources or signaling molecules, stimulating and modifying microbial activity, which should be kept in mind when interpreting results.

A checklist for application scenarios should always include a determination of rhamnolipid CMC values for the target soil system (i). Establishing the optimal concentration of rhamnolipids is necessary to achieve the most efficient complexation of heavy metals and encapsulation of organic contaminants in micelles. To this end, the composition and type of soil should be taken into account (ii). Some studies have suggested that increased amounts of clay, soil organic matter, and aluminum, iron, and manganese hydroxides increase the adsorption of rhamnolipids in soil, which may limit the remediation efficiency (Wen et al., 2010; Ochoa-Loza et al., 2007). This is consistent with the results presented in Fig. 1, as the CMC_{soil} values are in the range of 200–500 mg/l, depending on the soil type. Therefore, it should be taken into account that the CMC in soil will be approx. one order of magnitude higher than in water due to sorption to the soil matrix. In case of combined rhamnolipid and bioaugmentation treatments, it should be

remembered that the biomass is also prone to sorption of surfactants (Maier et al., 2001; Cierniak et al., 2020), which should be factored into the analysis of effective concentrations. Other soil characteristics at the remediated site, such as pH, average temperature, porosity or cation exchange capacity, should also be investigated (iii). These parameters have a substantial impact on the behavior of rhamnolipids e.g. different assembly patterns occur at different pH ranges (Kashif et al., 2022). It is also crucial not to apply excessive amount of rhamnolipids into soil systems. This ensures lower process costs and reduces the risks of potential negative effects such as toxicity towards plants or microorganisms (both described in the next sections). Should there be a need to introduce rhamnolipids at higher concentrations, the adverse effects can be potentially mitigated by considering the use of several smaller doses over time instead of applying a high amount in a single volume (iv). Lastly, the composition of rhamnolipids should also be considered (v). As mentioned in the Introduction section, rhamnolipids are a heterogenic mixture of structurally different congeners, the content of which may notably vary. Although this statement may seem obvious, it is regrettably often omitted in case of remediation attempts. There are reports which suggest that dirhamnolipids and monorhamnolipids differ in terms of biodegradability, release of organic contaminants from micelles and stability (Phulpoto et al., 2022). This may be a subtle source of fundamental bias.

3. Phytotoxicity of rhamnolipids

3.1. Potential issues and questions

1. Rhamnolipids may exhibit phytotoxic effects when applied at higher concentrations.
2. Mobilization of organic pollutants or heavy metals by rhamnolipids may negatively influence the plants.
3. Which plant species are used for phytotoxicity assessment during rhamnolipid-assisted bioremediation?

Overall, there is a lack of data regarding the ecotoxicity of rhamnolipids used for soil treatments (Lopes et al., 2021). It is therefore difficult to distinguish the exact mechanism of rhamnolipid-induced toxicity towards plants, however two possible scenarios can be established. At the micro level, it can be expected that rhamnolipids will behave as anionic surfactants and interact with cells (Otzen, 2017). In consequence, the first scenario assumes that the amphiphilic structure of rhamnolipids will allow them to directly incorporate into bilayers of biological membranes and result in the modulation of membrane structure or binding to proteins. This is supported by studies which confirm that rhamnolipids can promote protein degradation by neutralizing positive charges (Fracchia et al., 2015).

The second scenario is based on an indirect action, namely the ability of rhamnolipids to increase the solubility or to emulsify toxic contaminants and thus to increase their bioavailability. This effect has been described by many authors (Das and Chandran, 2011; Karlapudi et al., 2018), moreover, it is often suggested that aged petroleum contaminants, mainly polycyclic aromatic hydrocarbons, can be mobilized by the introduction of rhamnolipids (Liu et al., 2021). Hence, the second mode of phytotoxic action is associated with the enhanced transport and uptake of toxic heavy metals or organic pollutants in the presence of rhamnolipids.

Available reports dealing with the phytotoxicity of rhamnolipids were summarized in Table 2 (Wen et al., 2010; Gunawardana et al., 2011; Posada-Baquero et al., 2020; Mekwichai et al., 2020; Millioli et al., 2009; Bharali et al., 2018; Marecik et al., 2012; Szczepaniak et al., 2015). It should be highlighted that among numerous research works regarding rhamnolipids, there are only few that actually address the aspects of rhamnolipids toxicity to plants. The reviewed studies mainly investigated phytotoxic effects during phytoextraction of heavy metals as well as bioremediation of hydrocarbon contaminations (75 %), the

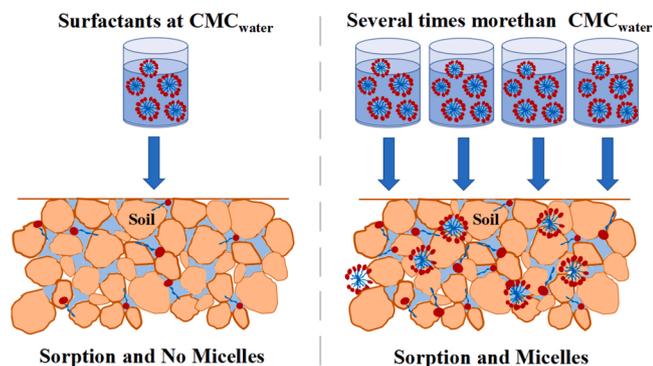


Fig. 2. Due to sorption of rhamnolipids in soil, a higher concentration of bio-surfactants is needed for micelles to form. CMC_{soil} is several times higher than CMC_{water} . The particle size of rhamnolipids and micelles does not correspond to scale.

Table 2

Summary of the phytotoxic effects of rhamnolipids against selected plants taking into account the biosurfactant concentration together with the most important conclusions from the conducted studies.

Plant	Duration	Research setup	Determined phytotox. parameters	Conc. of rham	Results	Conclusions	Comments	Ref.
lettuce (<i>Lactuca sativa</i>)	5 days	tests in Petri dishes with soil	germination index (GI), dehydrogenase activity	0 – 15 mg/g, toxic effect above 4 mg/g soil	EC ₅₀ = 4 – 6 mg/g, 10 – 15 mg/g 80% of GI inhibition	Addition of rham in a crude oil contaminated soil was very toxic to the seed germination.	Addition of rham improved bioremediation rate. CMC _{soil} was not determined.	Millioli et al. (2009)
mung bean (<i>Vigna radiate</i>), rice (<i>Oryza sativa</i>)	3 – 7 days	tests in Petri dishes without soil	GI, seed germination, root elongation	5 – 200 mg/L, below, equal and above CMC _{water} , toxic effect above CMC _{water}	GI reduction to 55 – 65% and 45-54% in mung bean and rice, respectively.	No significant hazardous impact on insect larvae, mammalian cells and skin tissues. Rham exhibit phytotoxicity	Future research on plant and animal model systems is required to establish their biocompatible nature.	Bharali et al. (2018)
maize (<i>Zea mays</i>)	42 days	pre-germination on a Petri dishes, then growth in pots with fertilized soil	plant transpiration, shoot biomass	weekly dose of 1.25, 5, 12.5 mmol rham/kg soil or single dose of 5, 25, 50 mmol rham/kg soil JBR 425	In all cases: reduction of biomass and decreased transpiration Weekly dose: stunted growth, decreased conc. of Fe, Ca, K, Mn in plant tissue Single dose: wilting, necrosis	Irrespective of the application frequency and conc. rham revealed phytotoxicity.	No evidence that rham will enhance Cd removal by phytoextraction. Adsorption of rham in soil at low application rates resulted in insufficient metal mobilization and inadequate Cd accumulation by plants. High doses of rham decreased plant growth significantly.	Wen et al. (2010)
perennial ryegrass (<i>Lolium perenne</i>)	30 days	seeds germination in glass beads with Hoagland's medium, then experiments in vials with metals –hydroponic studies	roots and shoots mass	85 mg/L JBR 425	Disorders of chlorophyll production, necrosis, leaf shedding, decreased root biomass yield up to 32%	Rham were not effective for improving Pb conc. in shoots, but significantly enhanced Cd levels in shoots in Cd-only treatments.	These studies should be contrasted with analogous studies in soil to discuss this in a broader context.	Gunawardana et al. (2011)
alfalfa (<i>Medicago sativa</i>), sorghum (<i>Sorghum saccharatum</i>), mustard (<i>Sinapis alba</i>) cuckoo flower (<i>Cardamine pratensis</i>)	7 days	germination test performed in Petri dishes, with soil, plant growth studies in pots with soil	germination index, dry matter of shoots and roots	above 75 mg/kg of wet soil JBR 425	Rham in soil: decrease of GI for alfalfa, mustard and for sorghum to 70, 71, and 41%, respectively. The cuckooflower remained unaffected. Rham in soil spiked with diesel: alfalfa <33% for 100 mg/kg rham, mustard 66-100% up to 600 mg/kg rham, sorghum – all conc. decrease of the dry matter of shoot and root for 600 mg/kg rham	Rham may potentially be toxic to the natural vegetation. Phytotoxicity seems to be species dependent. The addition of rham contributes to a significant increase of phytotoxicity in a diesel oil-contaminated soil.	Below 75 mg/kg of wet soil no toxicity of rham was observed, above CMC _{water} an increase in toxicity is seen, maximum toxicity observed for systems where rham exceed CMC _{soil} .	Marecik et al. (2012)
white mustard (<i>Sinapis alba</i>)	3 days	phytotestkit - test plates without soil	shoots and roots length	150-600 mg/L (AGAE, 90% pure)	reduction of roots length: 36% for 150 mg/L, 64% for 300 mg/L and 69% for 600 mg/L rham conc. reduction of shoots length: 18.7% for 150 mg/L, 14.3% for 300 mg/L 37.4% for 600 mg/L rham conc. wilt and strong decay	Rham could exhibit higher phytotoxicity towards white mustard seeds compared to synthetic surfactant.	Employment of surfactant for bioremediation requires careful and insightful studies in order to avoid phytotoxicity.	Szczepaniak et al. (2015)
sunflower (<i>Helianthus annuus L.</i>)	210 days	studies in pots with soil and rham added at 75 day	germination, blooming evolution, stem length of plants	7 g/kg soil R90, 90% pure AGAE Technologies		Addition of rham to the soil after the end of the sunflower ontogenetic cycle sped up the plant decline process.	No phytotoxic parameters like GI, plants length were described, there is only the general effect – wilt and decay.	Posada-Baquero et al. (2020)

(continued on next page)

Table 2 (continued)

Plant	Duration	Research setup	Determined phytotox. parameters	Conc. of rham	Results	Conclusions	Comments	Ref.
maize (<i>Zea mays</i>)	30 days	studies in pots with soil and fertilizer	total biomass, Cd shoot, root uptake	577- 2885 mg/kg approx. 1-5 mmol/kg	maize biomass treated with 4 mmol/kg rham was the highest - 32% higher than control. Slight wilting after the third week, in a presence of 4-5 mmol/kg Cd conc.: shoots uptake - 4.50 µg/ plant	4 mmol/kg rham did not exhibit toxicity to corn plants, Cd uptake and corn biomass were enhanced compared to plants treated with 5 mmol/kg rham. Rham at lower conc., did not have significant effects on Cd uptake.	Phytotoxicity was visible, but not discussed by authors.	Mekwichai et al. (2020)

rest evaluated their toxicity in general (25 %). The concentrations of rhamnolipids ranged from 5 to 600 mg /L in case of aqueous systems and from 75 mg to 15 g/kg of soil in case of terrestrial systems, with the duration of experiments between 3 and 210 days. In the majority of cases a single dose of the rhamnolipids was applied, whereas regular dosing (after 7 days). Experiments were conducted in under controlled conditions using petri dishes and pots filled with soil, more rarely with the use of phytotestkits and hydroponic systems. The assessment of toxic effects was mainly based on measurements of the germination index, root and shoot length or wet and dry biomass. Other reported effects of rhamnolipids phytotoxicity include impaired chlorophyll production, wilting, leaf drop, and reduced transpiration. The tested plant species mainly included model crops and vegetables (e.g. maize, rice, sunflower, white mustard, lettuce) or perennial plants (ryegrass, alfalfa and cuckoo flower). It should be noted that adverse effects of rhamnolipids were observed in all studies, especially when the biosurfactants were applied at higher concentrations. The susceptibility to rhamnolipids seems to be species-specific, as phytotoxicity was observed at concentrations ranging from 75 mg/kg of soil for sorghum to 2–3 g/kg of soil for maize. More importantly, notable toxic effects were observed in cases when the concentrations ranged from 200 to 600 mg/kg of soil, which corresponds with the CMC_{soil} values discussed in the previous section. Additionally, some studies reported the toxicity of sole rhamnolipids as well as increased toxicity in case of rhamnolipid-contaminant systems, which confirms that both previously described scenarios may occur in case of biosurfactant-assisted remediation.

To summarize this section, two mechanisms can be distinguished that may be responsible for the phytotoxicity of rhamnolipids. The first is a direct interaction with phospholipids in the cell membrane, and consequently changes in the effectiveness of the membrane as a selective barrier. The second mechanism is related to an increase of the bioavailability of xenobiotics and thus an increase in the concentration of toxic xenobiotics in the plant. Moreover, both mechanisms may overlap.

As mentioned previously, future application scenarios should include an in-depth analysis of rhamnolipid concentrations (i). At lower doses, the effect may be insignificant due to retention of the biosurfactant in the soil matrix, whereas the use of excessive amount may result in phytotoxicity (Fig. 3). In case of mixtures of different xenobiotics, an increase in the phytotoxic effects should be expected. For phytoextraction studies, the potential inhibitory effect towards the selected hyperaccumulator needs to be evaluated (ii). Interestingly, there is a lack of data regarding this particular research area. The few available reports confirm that rhamnolipids applied at 100 mg/L reduce the germination index of hyperaccumulator canola (*Brassica napus*) to 54 % (Gidudu and Chirwa, 2022), which confirms the necessity to explore this issue. In turn, when soil washing is considered as an in situ remediation strategy, the potential inhibitory effect of rhamnolipids towards the seeds of native plant species should be tested along with the impact of rhamnolipids-heavy metal complexes (iii). The latter is of particular

importance in case of sites co-contaminated with organic compounds and heavy metals, as the increased mobility of pollutants may result in unintended exposure. The actual moment of introducing the biosurfactant solution into the contaminated site also seems to be a factor worth consideration (iv). According to (Posada-Baquero et al., 2020), an approach based on initial development of the plant followed by application of rhamnolipids may allow to mitigate the problems associated with phytotoxicity of the biosurfactant. Recent advances in the field of biosurfactant-assisted bioremediation also suggest that the use of plant endophytes capable of producing rhamnolipids may be a promising solution to eliminate the problem of phytotoxicity and enhance the contaminant removal rate (He et al., 2020; Wu et al., 2018) (v). Nevertheless, this approach requires more in-depth research due to safety issues associated with the potential virulence of such endophytes (Kumar et al., 2013).

4. Biodegradation of rhamnolipids

4.1. Potential issues and questions

1. Biodegradation of rhamnolipids as the preferred carbon sources instead of contaminants.
2. Do rhamnolipids remain in the remediated site for a period that warrants an effect?
3. What is the actual impact of rhamnolipids on the removal efficiency during long-term treatments?

Rhamnolipids are commonly referred to as easily biodegradable, which is often emphasized as their notable advantage in terms of environmental safety (Wen et al., 2010; Gunawardana et al., 2011; Jia et al., 2020). This is understandable, taking into consideration the tightening legislation regarding environmental pollution, increasing popularity of products of natural origin as well as the public approval of sustainable development and circular economy concepts. Taking the chemical structure of rhamnolipids into account, it can indeed be expected that there will always be microorganisms capable of their degradation within complex microbiota present in a given niche. This is a well-known general rule in microbial ecology: “Everything is everywhere, but the environment selects” (Anon, 1934). Rhamnolipids will likely be decomposed into the respective sugar and acid moieties upon cleavage of the ether bond, which can be further utilized as easily-available carbon sources. Nevertheless, from the perspective of remediation efficiency the biodegradability of rhamnolipids can also become a disadvantage. Since rhamnolipids can be rapidly dissipated by microorganisms during environmental clean-up attempts, this eliminates the possibility to re-use the biosurfactant and may notably limit any expected effects associated with its application. The latter scenario can be assumed especially in case of aqueous systems, in case of which rhamnolipids will be more readily bioavailable to potential degraders, and the rate of their depletion will be all the more rapid the lower their

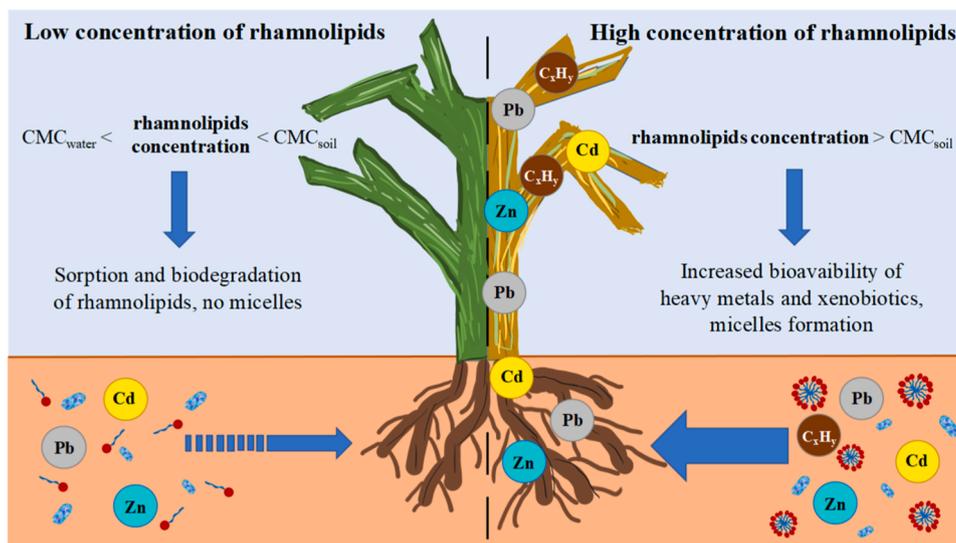


Fig. 3. Relationship between the concentration and the effective action of rhamnolipids during phytoextraction. Physical formation of micelles with xenobiotics or metal complexation ensures efficient transport and uptake of xenobiotics by plants and, as a result, their negative impact on the plant. The size of rhamnolipid micelles, complexes and metals does not correspond to scale.

concentration is. The degradation processes occurring in soil are slower than those in liquid media (Fantke et al., 2017). As such, there are reports which suggest that rhamnolipids will remain in the soil long enough to perform their targeted action (Wen et al., 2009).

It should be highlighted that biodegradation of rhamnolipids is an aspect that seems to be often overlooked. It was not included in the experimental set-up of any study presented in Tables 1 or 2. Nevertheless, there are studies which have addressed this issue. An example is a study regarding the biodegradation of diesel and biodiesel in liquid cultures in the presence of 150 mg/L rhamnolipids, in which the biosurfactant molecules were readily dissipated by a consortium of hydrocarbon degraders in all samples within 7 days under both aerobic and nitrate-reducing conditions (Chrzanowski et al., 2012b). The biodegradation susceptibility order was established as follows: biodiesel > rhamnolipids > diesel oil with the final conclusion that rhamnolipid supplementation did not enhance the overall biodegradation efficiency of petroleum hydrocarbons. Preferential biodegradation of rhamnolipids compared to pyrene, which resulted in lower removal efficiency, was also reported by Ghosh and Mukherji (2016). Of course there are instances, when the biodegradation of rhamnolipids may be crucial in order to allow the remediation process to proceed properly. Micelles will form more resistant structures, stabilized by the contaminants present in their core. This behavior was reported in experiments in which rhamnolipids selectively encapsulated chlorophenols in micelles (Chrzanowski et al., 2011, 2009). Nevertheless, high stability of micelles contributes to poor transfer of the entrapped compounds into the aqueous phase, which may sometimes prevent microorganism from degrading the entrapped compound (Zeng et al., 2018). In such cases the biodegradability of rhamnolipids becomes a merit. Higher persistence in terrestrial systems can be confirmed by the results of biodegradation of rhamnolipids at 150 mg/kg in fine grained silty clay soil, during which the highest dissipation of biosurfactant concentration was observed between day 6th and 16th, with complete degradation within one month (Szulc et al., 2014). This slow degradation of rhamnolipids was due to sorption and desorption processes, limiting the degradation rate. In another report rhamnolipids were used at very high concentrations, well above the CMC_{soil} and the results revealed rhamnolipids losses of 10 %, which in this particular case means losses of $45 \times CMC_{water}$ solely due to abiotic processes (Gusiatin et al., 2021). The short-term positive effect of the one-time addition of the biosurfactant on diesel oil degradation was observed, however it was insignificant in a one-year perspective. Similar

conclusion was established by Lin et al. (2011) as rhamnolipids initially increased the biodegradation efficiency of diesel oil, however no significant effect was observed in the latter stages.

To summarize this section, it is important to highlight the fact that the use of low concentrations of rhamnolipids very often proved to be ineffective in phytoextraction and phytoremediation, which may result from either sorption or degradation of the biosurfactant by microorganisms present in a given niche. In case of biodegradation of organic pollutants, the utilization of rhamnolipids may result in positive short-term effects, i.e. increase of microbial biomass or enhanced metabolic capacity, however this also results in a lack of substantial long-term effects. This issue is especially important in case of recalcitrant pollutants, such as polycyclic aromatic hydrocarbons, as their metabolic breakdown requires considerably more time.

In consequence, it is highly advised to track the changes of rhamnolipid concentration during any application scenarios (i). This simply allows to verify whether the introduction of a single dose of rhamnolipids is adequate and to identify potential time-points at which an additional doses should be used. The lack of in-depth studies dedicated to monitoring the biodegradation of rhamnolipids creates an information gap in terms of sustainability and cost effectiveness. Bioaugmentation with rhamnolipid-producing bacteria is a solution worth considering in order to eliminate the problems associated with depletion of rhamnolipids (ii). Nevertheless, this strategy also faces some limitations, and introduction of non-native species into a given niche is a challenging task.

5. Impact of rhamnolipids on the soil microbiome

5.1. Potential issues and questions

1. Since rhamnolipids exhibit antimicrobial properties, they may be toxic to soil microorganisms.
2. Does the introduction of rhamnolipids result in shifts of community dynamics?
3. How does the signaling function of rhamnolipids affect the soil microbiome?

As a result of their amphiphilic structure, rhamnolipids possess antimicrobial properties. Additionally, this biosurfactant is also known for its ability to disrupt existing bacterial biofilms and inhibit their

formation. There are reports which confirm high efficiency of rhamnolipids in terms of inhibiting the growth of pathogenic bacteria, such as *P. aeruginosa*, *S. aureus*, *E. coli*, *V. cholera*, *K. pneumoniae*, *C. perfringens* or *S. typhimurium* (Varjani et al., 2021) as well as fungi and oomycetes, such as *Botrytis* sp., *Rhizoctonia* sp., *Fusarium* sp., *Alternaria* sp., *Pythium* sp., *Phytophthora* sp. or *Plasmopara* sp. (Crouzet et al., 2020). On this basis, it can be inferred that rhamnolipids may also affect the proliferation of microorganisms at the remediated site. Another characteristic feature of rhamnolipids is their participation in quorum sensing (Chrzanowski et al., 2012a; Dusane et al., 2010; Woźniak-Karczewska et al., 2017). At very low concentrations, they can affect the communication between microorganisms and thus influence the abundance and function of different microbial species (Fig. 4). Thus, two scenarios can be considered in terms of the effect of rhamnolipids on soil microbial communities. In the first, toxic effects of rhamnolipids will be observed at high concentrations, and in the second, the effects of rhamnolipids on microorganisms will result from their biodegradation (as described in the previous section) or their function as signaling molecules.

The reported minimal inhibitory concentration values of rhamnolipids for single strains of bacteria are higher and range from 0.5 to 2.5 g/L (Gaur et al., 2022b; Grzywaczyk et al., 2021), whereas those reported for single fungal strains are usually lower, at 50–250 mg/L (Borah et al., 2016; Thakur et al., 2021). Based on the data presented in Tables 1 and 2 it can be concluded that bactericidal concentrations of rhamnolipids are rarely employed and, as discussed in Section 2, the effective concentration of rhamnolipids in soil would be lower due to sorption. This may justify why rhamnolipids may serve as protective agents for plants against pathogenic fungi without simultaneously incapacitating soil bacteria. Measurements of dehydrogenase activity in soil microcosms indicated that microbial communities can tolerate up to 4 g/kg of rhamnolipids without any adverse effects, however they were affected at higher concentrations (Millioli et al., 2009). In most studies which include the analysis of bacterial community dynamics, the analysis is typically performed for contaminated samples treated with rhamnolipids, as such, it is difficult to distinguish whether the observed changes can be attributed to the biodegradation of the pollutant or the biosurfactant. However, there are a few studies which specifically investigated the effect of sole addition of rhamnolipids on the community structure. For example Posada-Baquero et al. (Posada-Baquero et al., 2020), observed a notable shift in the microbiome composition along with an overall decrease of its diversity after introducing 7 g/kg of rhamnolipids. The post-treatment community was mainly dominated by *Pseudomonas*, *Comamonadaceae*, unclassified *Enterobacteriaceae* and *Achromobacter*. The authors emphasized that the application of

rhamnolipids led to a selective stimulation of specific members of the community, some of which were not detectable in the absence of the biosurfactant. Other studies also support these findings, however different dominant bacteria were observed, e.g. *Sphingomonas*, *Altererythrobacter* and *Lysobacter* (Wang et al., 2021a) or *Gaiella*, *Solirubrobacter*, *Nocardioidea*, and *Bacillus* (Lu et al., 2019). It should be noted, that it may be difficult to observe long-term changes, as microbial systems usually tend to return to baseline after some time e.g. after 3 months, as in the study by Szczepaniak et al. (Szczepaniak et al., 2016).

To summarize this section, it should be elucidated that rhamnolipids are not completely neutral in terms of their impact on the soil microbiome. At high concentrations possible inhibition of microbial activity due to toxicity should be considered, whereas at low concentrations their biodegradation or signaling function may also cause changes in the community structure. These types of interactions can be expected to overlap, hence it may be challenging to establish an unequivocal interpretation of the results.

In this regard, future application scenarios should consider the determination of community shifts in response to the introduction of rhamnolipids (i). This will ensure that appropriate taxa, e.g. species with a high biodegradation potential towards target pollutants, are not eliminated in the remediated site. Consequently, there is a need to develop cost-effective analysis methods and expand the relevant genetic database regarding potential “key players” associated with rhamnolipids-assisted bioremediation processes (ii). Another interesting issue is the interaction between rhamnolipids, plants and microorganisms in the rhizosphere (iii). It is known that plants provide their rhizosphere with root exudates such as carbohydrates, organic acids, amino acids as well as oxygen, which promote the growth of selected species of plant growth-promoting bacteria (PGPB), and these in turn produce specific substances and protect the plant against other, undesirable microorganisms (Bakker et al., 2014; Selosse et al., 2004). For example, fluorescent pseudomonads are favored for ecological crop management and soil health because of their ability to rapidly colonize roots and the rhizosphere, utilize a wide range of molecules as growth substrates, and release secondary metabolites that are crucial for native competition and growth-promoting compounds that help improve root proliferation and consequently plant growth (Sharma et al., 2007). Rhamnolipids interact with plants and influence their life processes, e.g. accumulation of reactive oxygen species and calcium influx in grapevine (Varnier et al., 2009; Yan et al., 2015) as well as antioxidative reactions in tomato (Varnier et al., 2009). The potential role of rhamnolipids as a communicator between plants and rhizosphere bacteria/endophytes or as a stress factor is a topic worthy of further exploration (Fig. 5).

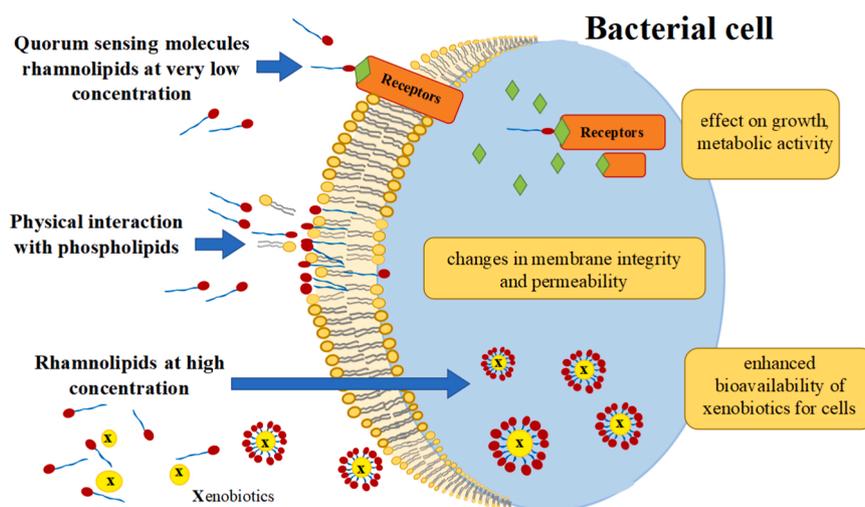


Fig. 4. Possible effects of rhamnolipids on cell function include: acting as quorum sensing molecules, as a surfactant that interacts with phospholipids, and as a surfactant that increases the availability of xenobiotics. The size of rhamnolipid micelles, complexes and xenobiotics does not correspond to scale.

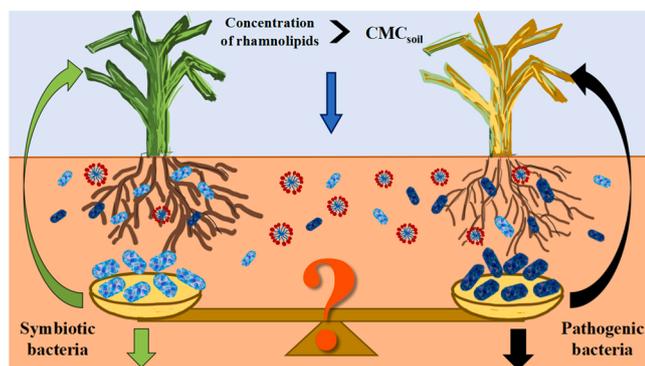


Fig. 5. The effect of rhamnolipids addition at high, effective concentrations on the soil microbiome is still unknown. Could changes in microbial balance translate into plant health and functioning during e.g. bioremediation/phytoextraction? This is an important question that has yet to be explored. The size of rhamnolipid micelles and bacteria does not correspond to scale.

6. Conclusions

This review describes the potential limiting factors for using rhamnolipids in remediation, which are often overlooked in case studies. From the perspective of effectiveness, four areas have been elucidated: effective concentration of rhamnolipids in soil, their potential phytotoxicity, susceptibility to biodegradation and interaction with soil microorganisms. The discussed elements are closely associated and often overlap, thus making the interpretation of results all the more challenging.

In summary, selection of an appropriate concentration of rhamnolipids is crucial for a successful bioremediation. While this fact is evident, it should be remembered that soil each system is different and will require different content of rhamnolipids to ensure their efficient action. If the concentration will be too high, this may result in phytotoxicity and decreased activity of soil microorganisms. On the other hand, if the concentration is too low, the biosurfactant will be rendered useless due to sorption or rapid biodegradation. The latter can potentially be beneficial, unless rhamnolipids will become a preferential carbon source compared to the target contaminant. Rhamnolipids-induced community shifts should also be determined in order to avoid selective stimulation on unwanted bacterial taxa, e.g. pathogens.

This review addresses aspects related to the evaluation of environmental benefits of rhamnolipid-assisted remediation as opposed to potential environmental costs of production and use of rhamnolipids or potential tradeoffs between economic benefits and costs of remediation were also not discussed (Søndergaard and Owsianiak, 2018; Tiso et al., 2020; Owsianiak et al., 2013). Taking into consideration the high costs for the biotechnological production of rhamnolipids (US\$1.5 and \$1500/g (Sarubbo et al., 2022)), it can be challenging to consider the use of rhamnolipids as a standard approach to enhance the overall bioremediation efficiency. Nevertheless, there is much room for improvement. In order to ensure the most profitable outcome of rhamnolipids-assisted bioremediation attempts, the following aspects should be considered in future studies:

1. There is a need to establish clear guidelines for rhamnolipids-assisted remediation of soil in terms of effective concentrations. This will require more data regarding the impact of rhamnolipids composition on their performance in soil, the correlation between soil type and parameters and rhamnolipids CMC values, optimal dosage strategies.
2. The database regarding the safe and potentially phytotoxic concentrations of rhamnolipids for bioremediation processes should be expanded, most notably in terms of influence of rhamnolipids of hyperaccumulator plants, time-window for optimal introduction of rhamnolipid solutions for phytoremediation and possible use of

rhamnolipid producing bacteria for sustainable and safe on-site production of rhamnolipids.

3. Due to the limited amount of data regarding the actual persistence of rhamnolipids in a remediated site, there is a need to develop analytical methods for a rapid and relatively easy monitoring of rhamnolipid concentration in environmental samples and evaluation of the corresponding microbial community changes.

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.

Data Availability

Data will be made available on request.

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Environmental implication

Basic principles for biosurfactant-assisted (bio)remediation of soils contaminated by heavy metals and petroleum hydrocarbons – a critical evaluation of the performance of rhamnolipids.

Critical analysis of the underlying limitations of rhamnolipids allowed us to identify the following areas that have been overlooked or only partially analyzed during previous research:

- a) effective concentration of rhamnolipids in soil;
- b) effect of rhamnolipids on metal leaching from different soil-forming phases;
- c) biodegradability of rhamnolipids;
- d) impact of rhamnolipids on the soil microbiome.

Providing solutions for these issues is crucial in order to efficiently use rhamnolipids for enhanced bioremediation of pollutants.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2022.130171](https://doi.org/10.1016/j.jhazmat.2022.130171).

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