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Mechanism-specific and whole-organism ecotoxicity of mono-rhamnolipids

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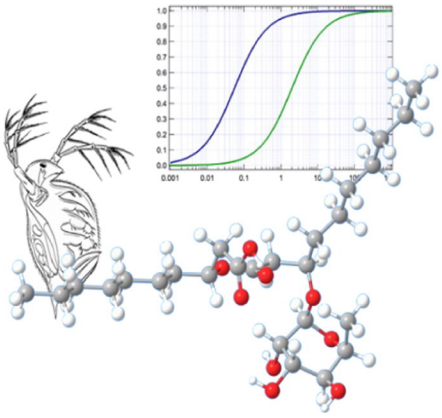
HIGHLIGHTS

- The mono-rhamnolipids caused low acute toxicity to ecotoxicological model organism.
- Mechanism-specific investigations revealed no mutagenicity and estrogenicity.
- Mono-rhamnolipids are environmental friendly alternatives to chemical surfactants.

GRAPHICAL ABSTRACT



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ABSTRACT

Biosurfactants like rhamnolipids are promising alternatives to chemical surfactants in a range of applications. A wider use requires an analysis of their environmental fate and their ecotoxicological potential. In the present study mono-rhamnolipids produced by a recombinant *Pseudomonas putida* strain were analyzed using the Green Toxicology concept for acute and mechanism-specific toxicity in an ecotoxicological test battery. Acute toxicity tests with the invertebrate *Daphnia magna* and with zebrafish embryos (*Danio rerio*) were performed. In addition, microbial and fungicidal effectiveness was investigated. Mutagenicity of the sample was tested by means of the Ames fluctuation assay. A selected mono-rhamnolipid was used for model simulations regarding mutagenicity and estrogenic activity. Our results indicate that mono-rhamnolipids cause acute toxicity to daphnids and zebrafish embryos comparable to or even lower than chemical surfactants. Rhamnolipids showed very low toxicity to the germination of *Aspergillus niger* spores and the growth of *Candida albicans*. No frameshift mutation or base substitutions were observed using the Ames fluctuation assay with the two tester strains TA98 and TA100. This result was confirmed by model simulations. Likewise it was computed that rhamnolipids have no estrogenic potential. In conclusion, mono-rhamnolipids are an environmental friendly alternative to chemical surfactants as the ecotoxicological potential is low.

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1. Introduction

Rhamnolipids are glycolipid biosurfactants which gain increasing attention for possible use in different industrial fields. They are produced by several microbial strains but the main producing species is the human-pathogen *Pseudomonas aeruginosa*. Other rhamnolipid-producing bacteria are, e.g., *Burkholderia* spp. and *Enterobacter* spp. (Abdel-Mawgoud et al., 2010; Toribio et al., 2010). The structure of rhamnolipids is highly diverse (Abdel-Mawgoud et al., 2010; Vatsa et al., 2010). In general they are composed of one or two rhamnose molecules (mono- or di-rhamnolipids) and one or two hydrophobic β-hydroxy fatty acids which can also differ in chain length or saturation level (Abdel-Mawgoud et al., 2010). Under phosphate or nitrogen limiting conditions a mixture of species characteristic rhamnolipids are produced (Abdel-Mawgoud et al., 2011). Because of their amphiphilic structure, they have properties including the reduction of surface tension and the function as emulsifier and are hence comparable to their industrially widely used chemical counterparts. These physico-chemical properties are the basis for their natural function in bacteria and their possible industrial use. For their producers, rhamnolipids promote access to poorly soluble substrates for subsequent biodegradation as well as surface motility and biofilm development (Abdel-Mawgoud et al., 2010). Additionally, they show antimicrobial activity (Sotirova et al., 2008). Potential commercial applications include the washing or cleaning industry, cosmetic industry, biomedicine and even the food industry, primarily because of their emulsifying and antimicrobial properties (Nitschke and Costa, 2007; Stipcevic et al., 2006). Also bioremediation of hydrocarbons or heavy metal contaminated sites (Mulligan, 2005) and biological plant protection against pathogenic bacteria or fungi is discussed (Vatsa et al., 2010).

Rhamnolipids together with other biosurfactants like sophorolipids have environmentally relevant advantages over chemical surfactants. They are produced from renewable resources like glucose or plant oil. A better biological degradation compared to the chemical counterparts is referred (Mohan et al., 2006; Mulligan, 2005) which is important, since several typical chemical surfactants used in washing or cleaning reagents, such as linear alkylbenzene sulfonates (LAS), leave 5–10% of their molecular structure as residues in the environment (Develter and Fleurackers, 2010).

In the light of rhamnolipids being promising to be established as biological alternatives to chemical surfactants, their potential entry into the environment for instance with sewage water and the resulting potential effects on the environment has to be considered. In this context, it is important to analyze effects on any biological level, from single cells over organisms and populations to ecosystems. To date a lot of knowledge exists about the most efficient rhamnolipid production processes and their potential use in industry, but data on biotests for an ecotoxicological evaluation of rhamnolipids is scarce. An emerging strategy for the investigation of potentially adverse effects is the so-called Green Toxicology, providing a framework for integrating the principles of toxicology into the enterprise of designing safer chemicals, thereby minimizing potential toxicity as early in production as possible (Maertens et al., 2014). Green Toxicology's novel utility lies in driving innovation by moving safety considerations to the earliest stage in a chemical's lifecycle, e.g., to molecular design.

Hence, the aim of this study was to achieve a ecotoxicological characterization by analyzing a mono-rhamnolipid mixture produced by the recombinant strain *Pseudomonas putida* KT2440 by means of a comprehensive (eco)toxicological test battery. Bioassays and effect based methods are important and well established tools in risk assessment to determine the toxic potential of water-born substances for the environment (Wernersson et al., 2015). To cover the whole complexity of possible modes of action, the examination regarding different endpoints and therefore the use of a whole test battery is necessary. In addition to bioanalytical tools, in silicio models can provide a first assessment of the ecotoxicological effects by correlating the physico-chemical properties based on their chemical structure with modes of action. Within such model simulations, a molecule for example is decomposed in substructural units, which are defined non-hydrogen centers with neighbor atoms in each bonding direction (atom-centered fragments, ACFs). To quantify the similarity, ACFs of a test compound is compared to a given ACF pool (Kühne et al., 2009).

In our study we focused on a selection of endpoints within such a biotest battery like acute toxicity to daphnids and zebrafish (*Danio rerio*) embryos. Also mutagenic effects, using the Ames fluctuation assay, were investigated as well as microbial and fungicidal effectiveness. Models were applied to calculate mutagenic and estrogenic activity within the software system ChemProp (UFZ Department of Ecological Chemistry, 2013). In this software, the atom-centered fragment (ACF) approach for example is used to quantify the extent of structural similarity between two compounds (Schüürmann et al., 2011). It is discussed, how biosurfactants can be compared to chemical surfactants from washing or cleaning industry on an effect level.

The achieved insight into the ecotoxicological effects of mono-rhamnolipid is promising for a necessary ecotoxicological evaluation of biosurfactant as potential emerging contaminants. Efficacy and safety findings can support the industrial establishment of these biosurfactants.

2. Material and methods

2.1. Rhamnolipid production

For rhamnolipid production the recombinant strain *P. putida* KT2440 pSynPro16 was used. The host *P. putida* KT2440 is naturally not able to produce mono-rhamnolipids. The recombinant strain contains the *rhlAB* operon isolated from *P. aeruginosa* PAO1 on the plasmid pSynPro16, which encodes the biosurfactants synthesis pathway. A mixture of different mono-rhamnolipids varying in the chain length of the β-hydroxy fatty acids was used throughout the study. For rhamnolipid production the bacteria were cultivated according to Wittgens et al. (2011) using LB-medium complemented with 10 g l^{-1} glucose and tetracycline (20 mg l^{-1}). The culture broth containing the rhamnolipids was first centrifuged with 9000 rpm for 20 min to remove cell material. Then the rhamnolipids were purified using chloroform-ethanol extraction according to Wang et al. (2007). Culture supernatant was washed three times with the chloroform-ethanol mixture (2:1 *v*/v) in a volume ratio 1:1. Afterwards the resolvent of the selected organic phase was vaporized in vacuo and finally the mono-rhamnolipids were redissolved in deionized water.

2.2. Rhamnolipid quantification

In order to determine the concentration of mono-rhamnolipids, high performance liquid chromatography (HPLC) was used (Ultimate 3000 HPLC, Dionex Corporation, Sunnyvale, USA). A reversed phase chromatography with a C-18 column Kinetex (Phenomenex, Aschaffenburg, Germany) and a gradient elution was used. The mobile phase was composed of 70% acetonitrile and 30% water with 0.2% formic acid and adjusted to a flow of 1 ml min^{-1}. For quantification of the sample components charged aerosol detection was applied (CAD-Detektor, ESA, Dionex Company, Chelmsford, USA). The amounts were calculated with a mono-rhamnolipid standard (Rha-C10-C10) within the chromatogram.

2.3. Fish embryo acute toxicity test (FET) with D. rerio

Worldwide, the zebrafish has become a popular model for biomedical research and (eco)toxicology (Strähle et al., 2012). To investigate possible embryotoxic and teratogenic effects of mono-rhamnolipids, the fish embryo toxicity test with the zebrafish (*D. rerio*) was performed.