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THE TOXICITY OF RHAMNOLIPIDS AND TWEEN 80 WITH REGARD TO WHITE MUSTARD (*SINAPIS ALBA)* SEEDS AND ACTIVATED SLUDGE MICROORGANISMS

Summary

Surfactants (surface active agents) have a wide spectrum of use in the industry, but also in agriculture. Moreover, there is a tendency to use both synthetic (e.g. Tween 80) and natural (e.g. rhamnolipids) surfactants in bioremediation of contaminated sites. In this case, a potential toxicity of surfactants towards different elements of the ecosystem should be considered. The aim of this study was to determine phytotoxicity in relation to Sinapis alba (white mustard) seeds and toxicity towards activated sludge microorganisms of rhamnolipids and Tween 80 at different concentrations. The results indicate a higher phytotoxicity of rhamnolipids compared with Tween 80. Surfactants used at the highest concentration, (600 mg⋅l^{-1}) exhibited an inhibition of roots growth by 69% and 16% (compared with a control) for rhamnolipids and Tween 80 respectively. The results of toxicity towards activated sludge microorganisms confirm the negative impact of both surfactants especially in high concentrations. In the two highest concentrations no significant difference between synthetic surfactants and biosurfactants was observed. The results suggest that rhamnolipids could not be considered as more 'eco-friendly' surfactants compared with synthetic Tween 80.
Key words*: rhamnolipids, Tween 80, toxicity, phytotoxicity, TTC*

TOKSYCZNOŚĆ RAMNOLIPIDÓW I TWEENU 80 W ODNIESIENIU DO NASION GORCZYCY *(SINAPIS ALBA)* ORAZ MIKROORGANIZMÓW OSADU CZYNNEGO

Streszczenie

Surfaktanty, czyli związki powierzchniowo czynne, charakteryzują się szerokim spektrum wykorzystania w wielu gałęziach przemysłu, ale również w rolnictwie oraz ochronie środowiska. W celu zwiększenia wydajności procesów remediacyjnych prowadzących do oczyszczania środowiska wykorzystuje się zarówno surfaktanty syntetyczne (np. Tween 80), jak i naturalne (np. ramnolipidy). Należy jednak zwrócić uwagę na potencjalną toksyczność wykorzystywanych surfaktantów wobec różnych elementów ekosystemu. Celem badań było określenie fitotoksyczności względem nasion gorczycy (Sinapis alba) oraz toksyczności wobec mikroorganizmów osadu czynnego ramnolipidów oraz Tweenu 80 używanych w różnych stężeniach. Wyniki wskazują na wyższą fitotoksyczność ramnolipidów w porównaniu z Tweenem 80 – w przypadku najwyższego zastosowanego stężenia, czyli 600 mg⋅l^{-1}, inhibicja korzeni w obecności ramnolipidów wynosi 69%, natomiast w obecności Tweenu – 16%. Uzyskane wyniki toksyczności względem mikroorganizmów osadu czynnego potwierdzają negatywny wpływ surfaktantów nasilający się wraz ze wzrostem stężenia, przy czym w dwóch najwyższych stężeniach nie uwidoczniła się istotna różnica pomiędzy ramnolipdami a Tweenem 80.
Słowa kluczowe*: ramnolipidy, Tween 80, toksyczność, fitotoksyczność, TTC*

1. Introduction

Surfactants, (surface active agents) are a group of compounds with specific characteristics due to their chemical structure. The molecules of surfactants are composed of two parts with different properties: hydrophilic (called "head") and hydrophobic (called "tail"). A polar (hydrophilic) part of surfactant has a high affinity for water and other polar liquids, and its presence confers the ability to dissolve in this kind of liquids. The non-polar (hydrophobic) part of surfactant can be dissolved in non-polar liquids, such as oils. The entire classification of surfactants takes into account a lot of factors such as chemical structure, properties, and origin [13]. The surfactants could be synthesized artificially or obtained from natural materials. Surface active agents are commonly used in cosmetic and pharmaceutical industry, paper and pulp industry, as plant protection products, textiles, paints, varnishes, adhesives.

Recently, surfactants have also been considered as agents, which could be used in environmental protection [6]. The use of surfactants during cleaning of the environment is particularly important in the case of a group of highly toxic pollutants, known as persistent organic pollutants (POPs), which include pesticides, polycyclic aromatic hydrocarbons (PAHs), chlorophenols, and polychlorinated biphenyls (PCBs). The removal of contaminants, enhanced by surfactants could be performed with the use of physiochemical methods (e.g. the flushing of soil with water or vapor) or biological methods (using microorganisms or plants). Biological methods are gaining more and more attention of scientist. Among the biological methods, particular attention is paid to the bioremediation, which consists in the decomposition of organic compounds by microorganisms. In the case of bioremediation, surfactants are primarily used to enhance the bioavailability of hydrophobic compounds and consequently to improve biodegradation process [3].

Regarding the recent trends, which support natural products and technologies, a special emphasis is placed on the use of biosurfactants, replacing the synthetic surfactants. Biosurfactants are surface active agents of microbial origin, produced by bacteria, fungi and yeast. They are considered to be low or non-toxic as well as biodegradable and consequently, sometimes even called as "green" surfactants [10]. Based on their physico-chemical properties biosurfactants can be divided into six groups: glycolipids, lipopeptides, neutral lipids, phospholipids, fatty acids, and polymeric. One of the best known biosurfactants are rhamnolipids [2]. Despite many studies indicating high toxicity of synthetic surfactants towards microorganisms, little is known about the influence of biosurfactants on the environment. Although rhamnolipids are generally considered as non-toxic agents, there are some literature reports indicating their antibacterial, cytotoxic and phytotoxic properties [4, 8, 12].

The aim of this study was to compare phytotoxicity and toxicity towards activated sludge microorganisms of natural (rhamnolipids) and synthetic (Tween 80) surfactants, and, to assess the safety of both compounds in bioremediation.

2. Materials and methods
2.1. Chemicals and activated sludge

During the experiment, rhamnolipids (AGAE, 90% pure) and Tween 80 (POCH) were used. Activated sludge was received from the sewage treatment plant in Koziegłowy near Poznan (Poland).

2.2. Experimental variants

The experiment was performed in the following experimental variants: 1) a control (distilled water); 2) rhamnolipids (150 mg/l of distilled water); 3) rhamnolipids (300 mg/l); 4) rhamnolipids (600 mg/l); 5) Tween 80 (150 mg/l); 6) Tween 80 (300 mg/l); 7) Tween 80 (600 mg/l).

2.3. Phytotoxicity assessment

The determination of phytotoxicity was carried out using commercially available kits (Phytotestkit, Tigret, Poland) measuring the direct effect of used surfactants on seed germination and early growth of plants. In order to assess the phytotoxicity, the following procedure was performed: test plate was filled with foam pad and filter paper and spiked with rhamnolipids or Tween 80 in concentration depending on variant. Then the second filter paper and white mustard *(Sinapis alba)* seeds were laced and the test plate was closed. The incubation was carried out at 25ºC for 3 days.

2.4. TTC analyses

In order to determine the toxicity of rhamnolipids and Tween 80 towards activated sludge microorganisms, TTC test was performed. Colourless triphenyltetrazolium chloride (TTC) after reaction with proton is reduced to red form - triphenylformazan (TF). The intense red colour (measured by the spectrophotometer), corresponds with a high activity of microorganisms [9]. The activated sludge was centrifuged and 1 ml of distilled water (for control) or surfactant (in appropriate concentration depending on variant) was added. Samples were incubated for 10 min at room temperature, and then 0,1 ml of TTC (6%) was added. After incubation (15 min, room temperature) the samples were centrifuged (8 000 rpm, 1 min) and the supernatant was removed. Next, 1 ml of ethanol (96%) was added and samples were incubated for 2 minutes at room temperature. After incubation and centrifugation (15 000 rpm, 3 min), the measurement of absorbance was performed using a spectrophotometer UV-Vis Helios lambda model (Thermo Scientific) at a wavelength of 490 nm.

2.5. Statistical analyses

All analyses were performed in three replicates. Bars represent standard errors of the mean.

3. Results and discussion
3.1. Phytotoxicity of surfactants

The obtained results indicate toxic effect of rhamnolipids towards mustard seeds (Fig. 1). Rhamnolipids toxicity was raising with increasing concentration - the lengths of roots were reduced by 36% (for 150 mg⊙l^{-1}), 64% (for 300 mg⊙l^{-1}) and 69% (for 600 mg⊙l^{-1}) compared to the control sample. In the case of shoots, the toxic effect of rhamnolipds was not significant, and the shoots lengths were decreased by 18,7% (for 150 mg⊙l^{-1}), 14,3% (for 300 mg⊙l^{-1}) and 37,4% (for 600 mg⊙l^{-1}) in comparison to the control. The obtained results are consistent with the trends presented by Marecik et al. [7], where growth inhibition was confirmed at the stage of germination of mustard, alfalfa and sorghum seeds above the concentration of 150 mg⊙l^{-1}.

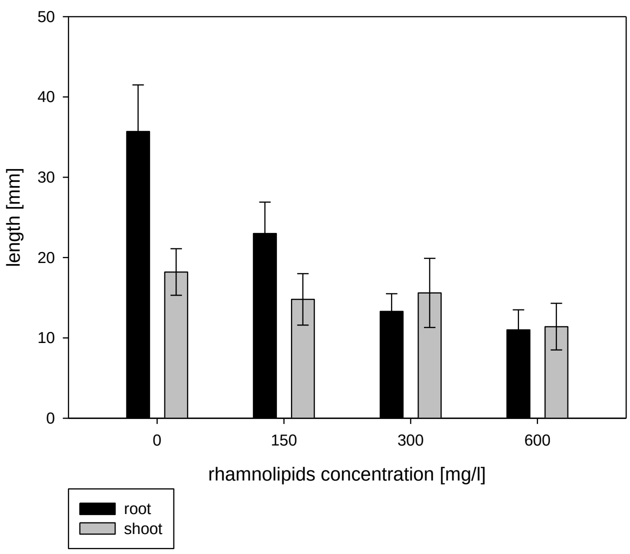

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Fig. 1. The phytotoxicity of rhamnolipids
Rys. 1. Fitotoksyczność ramnolipidów

The phytotoxicity results of Tween 80 are presented in Figure 2. A slight increase in phytotoxicity of samples containing Tween 80 compared to the controls was observed. In the presence of Tween 80 at the concentration of 150 mg·l^{-1} the roots growths were reduced by 11%, at 300 mg·l^{-1} by 8% and at 600 mg·l^{-1} by 16% compared to the control. In the case of shoots, for concentrations in the range form 150 mg·l^{-1} to 300 mg·l^{-1}, the toxic effect was less evident (approx. 5%), while for a concentration of 600 mg·l^{-1} inhibition of shoots growth reached 14%. Literature reports indicate that the use of nonionic surfactants (such as Tween 80) at high concentrations, may have a negative impact on the index of germination, seedling growth, development of the leaves as well as abnormal consumption of potassium and phosphorus transport in higher plants. On the other hand, there are also studies confirming the stimulatory effect on hormonal activity, the growth of roots and shoots, and the synthesis of chlorophyll at low, non-toxic concentrations [11]. Recent research performed by [1] studied potential application of Tween 80 during the bioremediation of contaminated soils, showed the lack of negative effects to *Medicago sativa* crops. Thus, it is very important, to choose the appropriate concentration of the surfactant for the individual process.

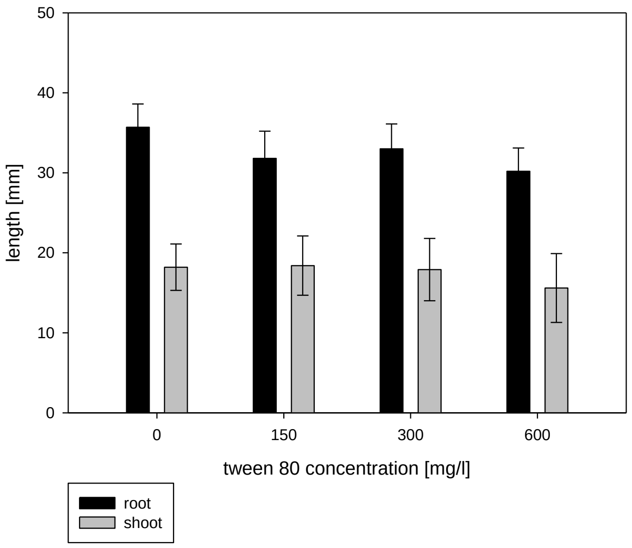

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Fig. 2. The phytotoxicity of Tween 80
Rys. 2. Fitotoksyczność Tweenu 80

The results of phytotoxicity of rhamnolipids and Tween 80 indicate that rhamnolipids could not be considered as less toxic agents compared with synthetic Tween 80. Moreover, in the case of the highest concentration (600 mg·l^{-1}) the inhibition of roots in the presence of rhamnolipids reached the value of 69%, whereas in the presence of Tween only 16%. The results suggest that rhamnolipids at higher concentration could have negative effect on plants.

3.2. Toxicity towards activated sludge microorganisms

Figure 3. shows the absorbance of the systems in the presence of rhamnolipids at various concentrations. The highest absorbance, which corresponds with the most active microbes, was observed for the control sample (without surfactants). After addition of rhamnolipids at a concentration of 150 and 300 mg·l^{-1} the absorbance decreased by approximately 16%, and the maximum decrease in absorbance was observed for the samples with addition of rhamnolipds at concentration of 600 mg·l^{-1} (a decrease by 40% compared to the control). This concentration was the most toxic towards activated sludge microorganisms.

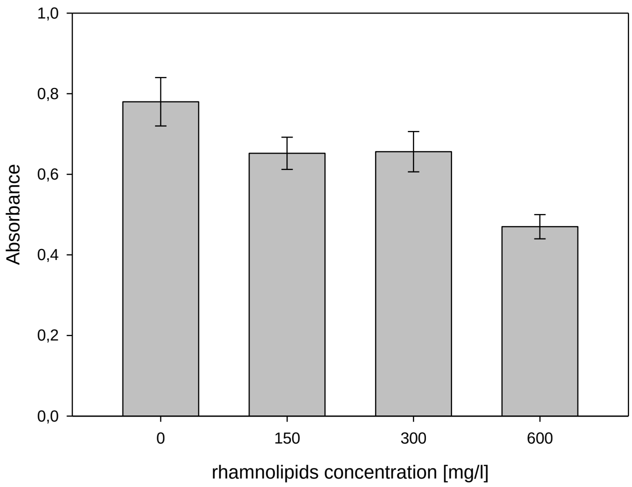

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Fig. 3. The toxicity of rhamnolipids towards activated sludge microorganisms
Rys. 3. Toksyczność ramnolipidów wobec mikroorganizmów osadu czynnego

An analogous test was carried out for samples containing Tween 80 at identical concentrations. It should be noted that the addition of surfactant at a concentration of 150 mg·l^{-1} did not affect the activity of enzymes catalyzing the oxidation reactions, since the absorbance did not decrease in comparison to the control sample. A significant decrease of absorbance (by 13%) was observed in the case of concentration of 300 mg·l^{-1}, while the highest toxicity was noticed at concentration of 600 mg·l^{-1} (41% reduction of absorbance compared with the control).

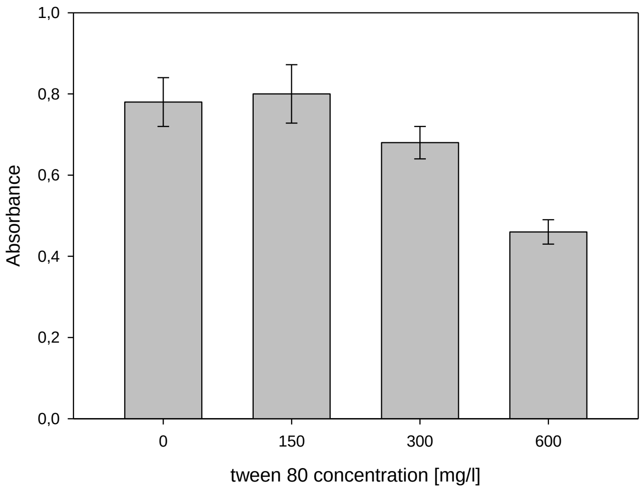

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Fig. 4. The toxicity of Tween 80 towards activated sludge microorganisms
Rys. 4. Toksyczność Tweenu 80 wobec mikroorganizmów osadu czynnego

A comparison of the toxicity of natural and synthetic surfactants towards active sludge microorganisms indicates that the difference in mentioned toxicity is not as high as in the case of phytotoxicity. Although Tween 80 did not exhibit toxicity at a concentration of 150 mg·l^{-1}, a further increase in the concentration caused a decrease in absorbance by values close to systems containing rhamnolipids. The toxicity of surfactants towards microorganisms may result from their binding with proteins and the phospholipids of biological membranes. Ivankovic and Hrenović [5] pointed out that high levels of nonionic surfactants can lead to increase in the permeability of cell membranes and cause leakage of the compounds of low molecular weight. This may disturb the functioning of cells due to damage of membrane and loss of ions and amino acids.

4. Conclusions

The results of phytotoxicity and toxicity towards activated sludge highlight the need for precise optimization of bioremediation process enhanced by surfactants in order to avoid negative effect of such compounds on the environment. In comparison to Tween 80, rhamnolipids could exhibit higher phytotoxicity towards white mustard seeds and similar toxicity towards activated sludge microorganisms. Obtained results confirm the need to select a proper type and concentration of surfactant during *in situ* bioremediation.

5. References

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The work was supported by the Polish Ministry of Science and Higher Education based on decision No 202830/E-380/M/2014 from 9.04.2014 (internal No 507.752.48).