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Toxicity of anionic and cationic surfactant to *Acinetobacter junii* in pure culture

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The harmful effects of surfactants to the environment are well known. Abstract: We were interested in investigating their potential toxicity in a pure culture of Acinetobacter junii, a phosphate (P)-accumulating bacterium. Results showed a high acute toxicity of sodium dodecyl sulfate (SDS) and hexadecyltrimethylammonium bromide (HDTMA) against A. junii. The estimated EC_{50} values of the HDTMA for the inhibition of CFUs in the pure culture of A. junii was $3.27 \pm 1.12 \times 10^{-7}$ mol L⁻¹ and for the inhibition of the P-uptake rates $2.47 \pm 0.51 \times 10^{-6}$ mol L⁻¹. For SDS, estimated EC₅₀ values for the inhibition of CFUs in the pure culture of A. junii was 5.00 \pm 2.95 \times 10⁻⁶ mol L⁻¹ and for the inhibition of the P-uptake rates $3.33 \pm 0.96 \times 10^{-4}$ mol L⁻¹. The obtained EC₅₀ values in the standardised yeast toxicity test using Saccharomyces cerevisiae were $3.03 \pm 0.38 \times 10^{-4}$ and $4.33 \pm 0.32 \times 10^{-5}$ mol L⁻¹ for SDS and HDTMA, respectively. These results emphasized the need to control concentrations of surfactants entering the activated sludge system. The negative effects of these toxicants could greatly decrease populations of P-accumulating bacteria, as well as eukaryotic organisms, inhabiting activated sludge systems, which in turn could result in the decrease of the system efficiency. © Versita Warsaw and Springer-Verlag Berlin Heidelberg. All rights reserved.

Keywords: bacteria, enhanced biological phosphate removal, surfactant, toxicity, yeast

1 Introduction

Surfactants are amphipatic compounds consisting of both a hydrophobic region (alkyl chains of various length, e.g. alkylphenyl ethers, alkylbenzenes, etc.) and a hydrophilic region (e.g. carboxyl, sulfate, sulfonates, phosphates etc.) [1]. Their harmful effects on the environment have been well characterized, and include remobilization of organic

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pollutants and inhibition of enzyme activity such as microbial dehydrogenase and algae nitrogenase [2]. Sodium dodecyl sulfate (SDS) is an anionic surfactant that is used in household products such as toothpastes, shampoos, shaving foams and bubble baths [3]. Hexadecyltrimethylammonium bromide (HDTMA) is a cationic surfactant widely used in hair conditioning products. In addition, HDTMA provides a buffer solution for the extraction of DNA and is also one of the components of the topical antiseptic cetrimide [4]. Because of their widespread use, surfactants are released in abundance into the environment, particularly in wastewaters. As a result, surfactants represent potential toxicants to organisms inhabiting activated sludge systems.

The dominant bacteria in activated sludge systems are aerobic heterotrophs. Phosphate (P) accumulating bacteria are normally present in activated sludge, but in minority due to their low growth rates [5]. These bacteria play an important role in an enhanced biological phosphorus removal (EBPR) from wastewaters [6], which have become the preferred process over the chemical P removal. Eutrophication is a worldwide water pollution problem, and limiting excess of P in aquatic environments is widely used as a control strategy [7]. According to the recent metagenomic analyses [8], bacteria from the genus Acinetobacter are present as a minor component of activated sludge and P-removal is carried out primarily by unculturable species such as Accumulibacter phosphatis. However, bacteria from the genus Acinetobacter have become the model organism for EBPR since their isolation from a P-removing activated sludge plant [9]. Their capacity to remove P was the highest among all the P-accumulating isolates, although Acinetobacter spp. were present in extremely low numbers in the activated sludge plant [5].

It is critical to determine the effects of potential toxicants that can influence the growth and metabolism of P-accumulating bacteria found in activated sludge systems. It is also crucial to investigate the potential toxicity against eukaryotic organisms that have important roles in the activated sludge process.

2 Materials and Methods

2.1 Microorganisms

A culture of the P-accumulating bacterium *Acinetobacter junii* 1532 was obtained from the Deutsche Sammlung von Microorganismen und Zellkulturen GmbH. The yeast strain *Saccharomyces cerevisiae* ATCC 64252 was used for the yeast toxicity test.

2.2 Synthetic wastewater

The composition of the synthetic wastewater (prepared in 1 L of distilled water) was as follows: Na-propionate 300 mg; peptone 100 mg; MgSO₄ 10 mg; CaCl₂ 6 mg; KCl 30 mg; yeast extract 20 mg; KH₂PO₄ 88 mg. The pH of the synthetic wastewater was adjusted to 7.00 \pm 0.02 with 1 M HCl or 1 M NaOH before autoclaving (121°C/15 min).

2.3.1 Bacteria toxicity test

The bacteria were pre-grown on nutrient agar (Biolife, Italy, composition in g per 1 L of distilled water: beef extract 3; peptone 5; agar 15) for 24 h at $30 \pm 0.1^{\circ}$ C. The biomass was then resuspended in 10 ml of 0.3% NaCl and 1 ml of mixed suspension was distributed in a series of bottles, each containing different concentration of SDS or HDTMA per 100 ml of synthetic wastewater. The concentrations were 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} mol L⁻¹ for each surfactant. The control bottle contained no toxicant. The bottles were sealed with a sterile gum cap and thereafter purged with filtered air while incubated in a water bath and shaken at $30 \pm 0.1^{\circ}$ C for 24 h.

2.3.2 Yeast toxicity test (YTT)

The YTT is a rapid, simple, reliable, sensitive and cost effective method for presumptive estimations of the toxicity of wastewaters against eukaryotic organisms [10, 11]. In this study an advanced broth bottle syringe method was used [12]. In brief, this test is based on the fact that the yeast *S. cerevisiae* is able to ferment sucrose to carbon dioxide. This fermentation takes place in a closed bottle containing a liquid medium. Gas produced during the fermentation process of sucrose presses out the equivalent volume of liquid to the open syringe. If sucrose is combined with some toxicant that influences the yeast and thwarts the fermentation, the amount of created carbon dioxide is reduced in comparison to the control, or it is not formed at all. By measuring the volume of liquid pressed out, the amount of gas produced and the intensity of fermentation could be estimated indirectly. The higher the toxicity, the reduction of produced gas will be higher [10–12].

The liquid medium for YTT was inoculated with *S. cerevisiae* culture as described previously [12]. The control bottle contained distilled water, and other bottles contained different concentrations of SDS and HDTMA ranging from 10^{-2} to 10^{-7} mol L⁻¹ each. Inoculated bottles were incubated in the dark at $28.0 \pm 0.1^{\circ}$ C for 24 h. After incubation the volume of liquid pressed out was measured. The results were expressed as EC₅₀ (effective concentration of toxicant which inhibits the fermentation for 50%).

2.4 Analytical methods

The pH-value in the synthetic wastewater was measured with a WTW 330 SET. The samples were filtered before P measurements using Sartorius nitrocellulose filters, with a pore diameter of 0.2 μ m. The P (P-PO₄³⁻) concentration in the synthetic wastewater was measured spectrophotometrically in a DR/2500 Hach spectrophotometer using the molybdovanadate method (Hach method 8114).

The viable cell counts were performed by aseptically placing one ml of the sample in a tube containing 9 ml of sterile 0.3% NaCl, prior to performing serial dilutions of each sample $(10^{-1} \text{ to } 10^{-9})$ used for the future inoculation. Volumes of 0.1 ml were plated (spread plate method) onto nutrient agar (Biolife, Italy) and plates were incubated at $30 \pm 0.1^{\circ}$ C for 72 h. After incubation, the bacterial colonies were counted and reported as colony-forming units (CFUs) per one ml of synthetic wastewater. Neisser stain was performed to confirm poly-P granules in cells of *A. junii*.

All measurements were done in triplicate. Results were statistically analysed using the Statistica program [13].

3 Results and discussion

The results demonstrated a high acute toxicity of both surfactants against A. junii and S. cerevisiae even at very low concentrations. The key parameters used in this study, which determine the activity of the P-accumulating bacteria in the P removal process, are the P-uptake rate per bacterial cell and the number of cells employed in the EBPR process. The HDTMA cation is toxic and impacts the heterotrophic activities of Gramnegative aerobic bacteria [4]. Therefore, we presumed its high toxicity against A. junii.

In control reactors containing no surfactant, the final CFU was $58.2 \pm 2.8 \times 10^6 \text{ ml}^{-1}$ and the bacterial cultures achieved a P-uptake rate of $1.25 \pm 0.02 \times 10^{-10} \text{ mg P CFU}^{-1}$. The HDTMA proved to be especially toxic and exhibited around 100% inhibition of both CFU and P-uptake rate at concentrations of 10^{-5} mol L⁻¹ and higher. The estimated EC₅₀ values of the HDTMA for the inhibition of CFUs in the pure culture of *A. junii* was $3.27 \pm 1.12 \times 10^{-7} \text{ mol L}^{-1}$ and for the inhibition of the P-uptake rates $2.47 \pm 0.51 \times 10^{-6} \text{ mol L}^{-1}$ (Figure 1). There was a significant positive correlation between the inhibition of the CFUs and of the P-uptake rates (r = 0.870; p < 0.05). The results shoved that CFUs are more sensitive to the presence of HDTMA than the resulting P-uptake rates.

Both surfactants analyzed are classified by the presence of formally charged groups in its head. The head of SDS carries a negative net charge (anionic) whilst HDTMA carries a positive net charge (cationic). We presumed some HDTMA-bacteria interactions, considering the bacterial negative net charge. The cationic surfactants adsorb strongly to the surfaces of cells through a combination of hydrophobic and electrostatic interactions [14]. In the wastewater treatment plant, adsorption of the surfactants to insensitive organisms, dead cells in flocs, etc., helps to protect live and susceptible cells. These mechanisms of surfactant adsorption suggest that the observed EC_{50} values for A. junii represent the upper limits. The P group also carries a negative net charge, and interactions between HDTMA molecules and P groups were expected. Therefore, we prepared the blanks of the synthetic wastewater containing different concentrations of HDTMA without bacteria. In these blanks the P precipitation close to the theoretical molar stoichiometric ratios was observed. The amounts of P precipitated in the systems (from initial P load $22.45\pm0.86~{\rm mg~L^{-1}})$ were 22.45, 10.53, 1.88 and 0.2 mg ${\rm L^{-1}}$ at HDTMA concentrations of 10^{-2} , 10^{-3} , 10^{-4} and 10^{-5} mol L⁻¹, respectively. At lower HDTMA concentrations, the observed precipitation of P was negligible. P precipitations with HDTMA were taken into account when calculating the percent of bacterial inhibition.



Fig. 1 The profile of the HDTMA toxicity to colony forming units (CFU) (a) and P-uptake rates (b) in the pure culture of *Acinetobacter junii*. t_0 CFU (10⁶ CFU ml⁻¹) = 17.34 ± 3.69; t_0 P-PO₄ (mg L⁻¹) = 22.38 ± 0.83.



Fig. 2 The profile of the SDS toxicity to colony forming units (CFU) (a) and P-uptake rates (b) in the pure culture of *Acinetobacter junii*. t_0 CFU (10⁶ CFU ml⁻¹) = 15.11 ± 7.53; t_0 P-PO₄ (mg L⁻¹) = 22.34 ± 0.72.

The SDS exhibited around 100% inhibition of both CFU and P-uptake rate at concentrations of 10^{-3} mol L⁻¹ and higher. The estimated EC₅₀ value for the inhibition of CFUs in the SDS-treated culture of A. junii was $5.00 \pm 2.95 \times 10^{-6}$ mol L⁻¹. The inhibition of the P-uptake rates was $3.33 \pm 0.96 \times 10^{-4}$ mol L⁻¹ (Figure 2) in the SDS treatment. The inhibition of the CFUs and P-uptake rates has a significantly positive correlation (r = 0.828; p < 0.05). The results showed again that CFUs were more sensitive to the presence of SDS than the resulting P-uptake rates. The observation that viability of A. junii is more sensitive to both surfactants than the P-uptake rates indicates that the P-accumulation may be a survival mechanism in unfavourable conditions of growth, such in the case of P starvation [15]. Neisser stain at the end of the experiments confirmed the presence of poly-P granules in approximately 90% of live A. junii cells. The decreased P-accumulation as a result of surfactants represents one important mechanism of surfactant toxicity against P-accumulating bacteria. The resulting lower CFUs of A. junii at the end of aerobic phase, if again returned to the anaerobic conditions can result in a lower EBPR efficiency of the system.

The results of this study are in agreement with other results obtained from various studies on the toxicity of anionic surfactants. Both growth and nitrogen fixation of the cyanobacterium *Gloeocapsa* were inhibited at 50 ppm of SDS, corresponding to 1.73×10^{-4} mol L⁻¹ [16]. In another study consisting of various taxa, the EC₅₀ value obtained for the bacterium Vibrio fischeri was 2.6 mg L^{-1} of SDS, corresponding to 9.02×10^{-6} mol L⁻¹. The V. fischeri proved to be the most sensitive organism in the study evaluating different species of bacteria, algae, crustaceans, echinoderms and fish [3]. Higher organisms also showed sensitivity to the surfactants, with symptoms including acute toxicity (microorganisms) [3, 16], behavioural changes (fish) [17], and skin damage (humans) [18, 19]. The sewage sludge isolates A. johnsonii and Oligotropha carboxidovorans [20] showed complete loss of viability during the treatment with 0.07 and 0.7 mg ml⁻¹ $(1.92 \times 10^{-4} \text{ and } 1.92 \times 10^{-3} \text{ mol } \text{L}^{-1})$ of cetyltrimethylammonium bromide (CTAB), and near 50% and 20% of viability during the treatment with 0.2 and 2 mg ml⁻¹ of SDS $(6.94 \times 10^{-4} \text{ and } 6.94 \times 10^{-3} \text{ mol } \text{L}^{-1})$, respectively. The tested strain of A. junii showed a higher sensitivity to SDS and comparable sensitivity to HDTMA when compared with the above mentioned isolates against CTAB.

The levels of surfactants used in this study and estimated EC_{50} values are equivalent to those typically found in wastewater treatment plants. The usual levels of anionic surfactants present in the wastewater are 10 mg L⁻¹ (which corresponds to 3.47×10^{-5} mol L⁻¹ of SDS) and the levels of cationic surfactants are 5 mg L⁻¹ (which corresponds to 1.37×10^{-5} mol L⁻¹ of HDTMA). SDS is known to be rapidly degraded in wastewater treatment plants and many species of bacteria are known to degrade SDS [21]. However, there are no data showing the biodegradation ability of the *A. junii* strain used in this study. Also, the results do not indicate the possible biodegradation. The toxicity of SDS is marked at very low concentrations and this would not be the case with possible biodegradation of SDS. Cationic surfactants are less readily degraded [14] and it is unlikely that bidegradation of HDTMA could influence our results. Interactions among different bacteria inhabiting activated sludge systems should also be considered. Bacterial coaggregates found in active sludge flocs also provide a protection from surfactants at lower concentrations [20]. The possibility of biodegradation and/or adsorption indeed indicates that the EC_{50} values mentioned above are upper limits, and extensive studies in the activated sludge system are needed.

The average pH values after 24 h of incubation in control reactors were 7.66, which was not significantly different than in the reactor containing 10^{-7} mol L⁻¹ of SDS or HDTMA. The pH values correlated significantly negatively with the toxicity of SDS against CFUs (r = -0.816; p < 0.05) and P-uptake rates (r = -0.997; p < 0.05) and the toxicity of HDTMA against CFUs (r = -0.988; p < 0.05) and P-uptake rates (r = -0.871; p < 0.05).

We were interested in investigating toxicity against eukaryotic organisms in addition to bacteria because of the complex composition of activated sludge, which contains not only P-accumulating bacteria but also many other bacteria, as well as a variety of eukaryotic organisms. Moreover, A. junii is not a model organism for toxicity studies, and results of a standard toxicity test such as YTT allows us to compare validity of results. In this study the yeast S. cerevisiae was used in order to estimate the toxicity of surfactants against eukaryotic organisms. The obtained EC_{50} values in YTT were $3.03 \pm 0.38 \times 10^{-4}$ and $4.33 \pm 0.32 \times 10^{-5}$ mol L⁻¹ for SDS and HDTMA respectively. The coefficients of the variation were 12.54% and 7.39% for SDS and HDTMA, respectively, which is in the range of the variation of results for the standard toxins. The results exhibited marked sensitivity of S. cerevisiae against both surfactants, with HDTMA showing increased toxicity. S. cerevisiae showed significantly lower sensitivity to SDS and HDTMA than A. junii. The results of the standard YTT correlated positively (although not significantly p > 0.05) with the observed toxicity of HDTMA against the CFUs (r = 0.987) and P-uptake rates (r = 0.913) and the toxicity of SDS against the CFUs (r = 0.935) and P-uptake rates (r = 0.971) of A. junii.

The results of this study emphasize the importance of controlling amounts of anionic and cationic surfactants in wastewaters entering the activated sludge systems. The surfactants have ambiguous effects on the environment [1] and their toxicity to A. junii is clear. Both SDS and HDTMA proved to be toxic against S. cerevisiae, indicating that the eukaryotic organisms inhabiting activated sludge could be sensitive to the surfactants as well, especially given that earlier studies have demonstrated numerous toxic effects of surfactants [1, 3, 17].

4 Conclusions

The results showed high toxicity of both anionic (SDS) and cationic (HDTMA) surfactants in a pure culture of *A. junii*, as well as in the model eukaryotic organism *S. cerevisiae*. The cationic surfactant displayed a strong interaction with the P group, indicating a possible problem in EBPR due to P precipitation. Results highlighted the need to control concentrations of these, and possibly other surfactants in activated sludge systems. Considerable amounts of surfactants are being released in wastewaters and the negative effect of these toxicants could significantly affect the role of P-accumulating bacteria, as well as eukaryotic organisms, inhabiting activated sludge with EBPR characteristics.

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