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Original Research

Inhibiting Effect of Surfactants and Heavy Metal Ions on the Denitrification Process

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Abstract

The effect of selected surfactants and heavy metal ions on the kinetics of the process of denitrification taking place with the involvement of Bacillus *licheniformis* bacteria has been studied. The limiting concentrations of the compounds studied and the coefficients of inhibition have been determined. A kinetic model of denitrification was carried out in a lactate medium with the addition of a quaternary ammonium salt APDA has been proposed. The process has been shown to occur according to the autocatalytic model of the second order with respect to nitrates (V) and proteins and first order with respect to nitrates (III) for the subseguent irreversible reaction.

Keywords: denitrification, cationic surfactants, heavy metal ions, coefficient of inhibition

Introduction

Chemical pollution of the environment by surfactants and heavy metal ions is a result of increasing industrial activity of man. Large amounts of these pollutants when penetrating into surface water reservoirs cause foaming, reduce diffusion of the atmospheric oxygen dissolved in water and consequently lead to the death of many organisms for the deficiency of oxygen. Surfactants act as emulgators of different compounds, including oils and lubricants, and dissolve solid state pollutants insoluble in polar solvents [1]. Biodegradation of surfactants significantly depends on the type of the bacteria culture, temperature, and the structure of alkylbenzene groups [2-4]. It should be emphasised that cationic surfactants are biodegradable only in aerobic conditions. Surfactants are mainly based on quaternary ammonium salts having toxic effects on anaerobic microorganisms [5]. Their toxic effect on the environment is related to a strong interaction with biological membranes of living cells and the influence on the membrane potential having far-reaching consequences for metabolism control. The harmful effect of surfactants includes destruction of microorganisms, damage to respiratory systems, inhibition of development of reproduction of water fauna, decrease in the surface tension of water, reduction of the amount of available oxygen needed for self-purification of the natural environment.

Occurring in parallel increase in the concentration of heavy metals in the natural environment stimulates the activation of protective and adaptive mechanisms in microorganisms.

Thanks to a large variety of the adaptation mechanisms, micro-organisms can radically change the role of metals in the environment, sometimes even leading to their beneficial effect [6-8].

In concentrations higher than the threshold of toxicity, the surfactants and heavy metal ions studied lead to inhibition of the process of self-purification of ground waters and soil and delay life-sustaining processes in the environment. In view of this it is essential to determine the toxicity limits of these compounds in order to protect the natural environment against their effects.

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In this work the toxic effect of selected surfactants and heavy metal ions was analyzed on the basis of the process of denitrification being one of the most important in the natural nitrogen cycle.

Materials and Methods

The substances studied were selected surfactants (detergents, preservatives and disinfectants) and some heavy metal ions. The compounds are commonly used for washing as disinfectants, fungicides, bactericides, bleaching and chlorinating agents. The compounds studied were:

- **APDA** (Lonzabac 12.100) N-N-Bis(3-aminopropyl) dodecylo amine – disinfectant and cleaning agent, a biocide used in the food and cosmetic industry [9],
- **DDDM** didecylodimethylammonium chloride – a biocide used for washing and disinfection in the food and medical industries [10],
- **DCDMH** dichloromethylhydantoine – used for disinfection of swimming pools and closed water systems, for removal of slimes from wastes, for reduction of emission of sulphur compounds and air protection against hydrogen sulfide, for production of cleaning and disinfecting agents [11-12],
- **BCDMH** bromochlorodimethylhydantoine – the same use as that of DCDMH,
- **TClCA** trichloroizocyanuric acid –a disinfecting, oxidizing and chlorinating agent,
- **NaDClC** sodium salt of dichlorocyanuric acid [13],
- **Chloramine T** [14],
- **MMPP** magnesium monoperoxyphthalate– is a substance added to all kinds of surfactants, solvents and complexing agents, used in chemical syntheses, for bleaching and disinfection [15],
- **Glutaric aldehyde** - a biocide used in medicine for cold sterilization of surgical and dentistry instruments, in analytical chemistry, organic synthesis and polymer chemistry [16],
- **Formaldehyde** - a preserving and disinfecting agent [17], and
- **Heavy metal ions: Mn^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+}, Ni^{2+}** - used in the form of nitrate or sulphate salts.

Kinetic study was carried out at 37°C at pH 7.5 in closed glass reactors containing a lactate medium composed of: [g/dm^3] KNO_3 = 10; $Fe(NO_3)_3 \cdot 9H_2O$ = 0.44; NH_4Cl = 0.25; $MgSO_4 \cdot 7H_2O$ = 0.5; $CaCl_2$ = 0.05; $Na_2HPO_4 \cdot 12H_2O$ = 2.5; sodium lactate 10.16 and microelements ($MnSO_4 \cdot H_2O$ = $3.47 \cdot 10^{-4}$; H_3BO_3 = $0.86 \cdot 10^{-4}$; $CuSO_4 \cdot 5H_2O$ = $1.2 \cdot 10^{-4}$; $Co(NO_3)_2 \cdot 6H_2O$ = $0.28 \cdot 10^{-4}$; $Zn(NO_3)_2 \cdot 6H_2O$ = $0.1 \cdot 10^{-4}$; $(NH_4)_6MoO_{24} \cdot 4H_2O$ = $0.18 \cdot 10^{-5}$; $NaHSeO_3$ = $0.88 \cdot 10^{-7}$; $Ni(NO_3)_2 \cdot 6H_2O$ = $0.18 \cdot 10^{-5}$). The medium was inoculated with a 4% volume of the inoculum collected after 24 h of bacteria growth. To the reactors a certain amount of the compound tested was added and the rate of denitrification was established by measuring the time changes of the concentrations of N-NO_3^- and N-NO_2^- [18].

The concentration of nitrates (V) was measured potentiometrically using an ion-selective electrode "DETECTOR" [19].

The concentration of nitrates (III) was measured spectrophotometrically on a spectrophotometer Beckman DU-640, at a wavelength of 520 nm [20].

The concentration of proteins was measured spectrophotometrically by the Lowry method using the Foulin reagent. The absorption was measured at 750 nm on a Becman DU-640 spectrophotometer [21].

The denitrifying bacteria from the genus Bacillus were isolated from the garden soil samples collected near Poznań and were identified according the Boergey key [22].

Results and Discussion

In the first stage of the kinetic study the limiting concentrations of the compound studied were established, that is the tolerated, toxic limits and the range of inhibiting concentrations (Table 1).

The least toxic compound is magnesium monoperoxyphthalate (MMPP), which is tolerated by bacteria up to a concentration of 1000 ppm. Its toxic activity begins from concentrations above 3000ppm. The most toxic are mercury ions as at the concentration of 0.8 ppm they totally inhibit the process of denitrification. The presence of the other heavy metal ions Cd, Zn, Pb, Ni also has a strong inhibiting effect on denitrification as already at low concentrations they significantly prolong the reduction time. The presence of the aldehydes studied (from 2 ppm) and cationic surfactants in the form of quaternary ammonium salts (APDA, DDDM, TClCA, NaDClC) also has a significant inhibiting effect.

The observed differences in the bacteriostatic effects of the biocides tested can be related to a different rate of reaching an equilibrium of hydrolysis, depending on environmental conditions. In the range of tolerated concentrations of the compounds studiem bacteria using a certain adaptative-protective mechanism adapt to the media with the substance added and can still be involved in the process of denitrification. A different degree of biotransformation of these compounds can be related to their chemical structure as it is known that metabolism of branched organic compounds is inhibited by the products of biotransformation.

At the next stage of the study the inhibition coefficients characterizing the substances studied were determined. Applying the Dixon method the relations between the reaction rate 1/v and the inhibitor concentration "i" were obtained for different concentrations of the organic substrate in order to conclude the inhibition type (Fig. 1).

The kinetic study was performed on the sample with APDA in the presence of sodium lactate, whose concentration varied as 5.08, 10.16 and 20.32 g/dm^3. On the basis of the measured concentrations of nitrate and nitrite nitrogen the rate of the reaction was found. The point of

Table 1. The limiting concentrations of the compounds studied determined on the basis of a kinetic study of denitrification taking place with the use of Bacillus *licheniformis* bacteria (37°C).

compound	concentration [ppm]		
	tolerated	inhibiting	toxic
APDA	<2	2 -8	>8
DDDM	<0.1	0.1 – 0.4	>4
DCDMH	<25	25 - 100	>200
BCDMH	<40	40 - 200	>250
TClCA	<2	2 - 8	>8
NaDClC	<2	2 - 8	>8
Chloramine T	<15	15 - 60	60
MMPP	<1000	1000 – 3000	>3000
Glutaric aldehyde	<2	2 – 20	>20
Formaldehyde	<2	2 – 50	>50
Mn^{2+}	<6	6 – 1150	>1150
Ni^{2+}	<5	5 – 40	>40
Zn^{2+}	<0.65	0.65 – 1.5	>1.5
Cd^{2+}	<0.5	0.5 – 2.5	>2.5
Hg^{2+}	<0.45	0.45	0.80
Pb^{2+}	<8	8 – 40	170

intersection of the lines showing the relations between the reaction rate and the inhibitor concentration brought the information on the type of inhibition.

The points in Fig. 1. correspond to the calculated initial reaction rate 1/v as a function of APDA concentration. The lines were obtained by the regression method using the computer program Sigma Plot 2000. The lines obtained for different initial concentrations of the inhibitor are extrapolated to the same point on the axis of abscissa, which means that the inhibition is of a non-competitive type.

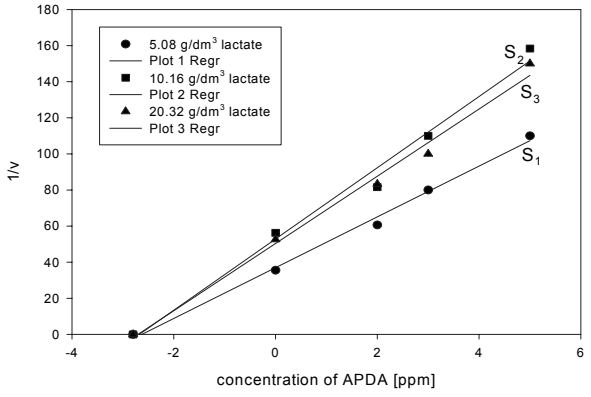

Fig. 1. The initial rate of denitrification versus the APDA concentration used for determination of the type of inhibition in the system.

Having established the type of inhibition, the coefficients of inhibition K_i have been calculated.

The relation between the rate of the reaction and the inhibitor concentration for the process of non-competitive inhibition is described by the equation.

$$v = \frac{V_{\max} \cdot s \cdot K_i}{(K_m + s)(K_i + i)} \tag{1}$$

where:

v – reaction rate [mg·dm^3·h^{-1}]

V_{max} – maximum rate of reaction in the presence of enzymatic catalysts [mg·dm^3·h^{-1}]

s – substrate concentration [mg/dm^3]

i – inhibitor concentration [mg/dm^3]

K_m – Michaelis constant

K_i – inhibition constant.

The inhibition constant K_i is defined as the concentration of the inhibitor at which the reaction rate is decreased to half of its maximum value.

The rate of a biochemical reaction is described by the Michaelis equation:

$$v = -\frac{ds}{dt} = -\frac{V_{\max} \cdot s}{K_m + s}$$

At an appropriately chosen high concentration of the substrate, the maximum reaction rate is obtained, then eq. (1) becomes:

$$v = \frac{V_{max} \cdot K_i}{K_i + i} \quad (2)$$

The value of Ki was found by the graphical method proposed by Dixon, in which the dependence of the reaction rate 1/v on the inhibitor concentration 'i' is plotted. The point at which the extrapolated line intersects the x-axis is the searched values of K_i as follows from the linearization of eq. (2):

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{i}{V_{max} \cdot K_i}$$

The reaction rate "v" was calculated from the ratio $\Delta s/\Delta t$, where Δs is the loss of nitrates in the initial phase of the process that is in the first 20% of the process duration. Fig.2 illustrates the method of determination of K_i for the sample containing APDA.

The inhibition coefficients calculated for all the biocides and heavy metals studied are collected in Table 2.

Analysis of the data shown in Table 2 indicates a close correlation between the inhibition coefficient and the inhibitor concentration. If the bacteria responsible for the process of denitrification are resistant to high concentrations of the compounds studied, the value of the inhibition coefficient K_i, is high, as in the presence of MMPP K_i=137.5 and the inhibiting concentration range is 1000-3000 ppm or in the presence of Mn^{2+}, when K_i=101.77 and the inhibiting concentration ranges from 6 to 1150 ppm. If the bacteria are sensitive to low concentrations of the compounds studied the inhibition coefficient is low, as in the presence of Hg^{2+}, whose inhibiting concentration is 0.45 ppm, K_i = 0.18, or in the presence of DDDM, whose inhibiting concentration is 0.1-0.4 ppm, and the corresponding value of K_i is 0.26.

Therefore, in the systems containing the same source of carbon and the same initial concentration of nitrates, the value of K_i depends on the inhibitor concentration. The values of K_i decrease with increasing sensitivity of the bacteria studied to the inhibiting effect of the introduced inhibitors.

The kinetic model of the process of denitrification in the presence of an inhibitor was proposed on the basis of the analysis of the sample with APDA. The following scheme of the subsequent reactions was assumed:

$$NO_3^- \xrightarrow{k_1} NO_2^- \xrightarrow{k_2} N_2$$

where:
k_1, k_2 – the rate constants.

The model of an autocatalytic reaction of second order towards nitrates (V) and protein and of first order towards nitrates (III) was considered.

$$\frac{d[NO_3^-]}{dt} = -k_1[NO_3^-][B]$$

$$\frac{d[NO_2^-]}{dt} = k_1[NO_3^-][B] - k_2[NO_2^-]$$

$$\frac{dB}{dt} = Yk_1[NO_3^-][B]$$

where:
$[NO_3^-]$ –the concentration of nitrate nitrogen [g/dm^3]
$[NO_2^-]$ – the concentration of nitrite nitrogen [g/dm^3]
[B] –the concentration of protein [g/dm^3]
Y – the coefficient of protein increase [mg of increased protein /mg of reduced NO_3^-].

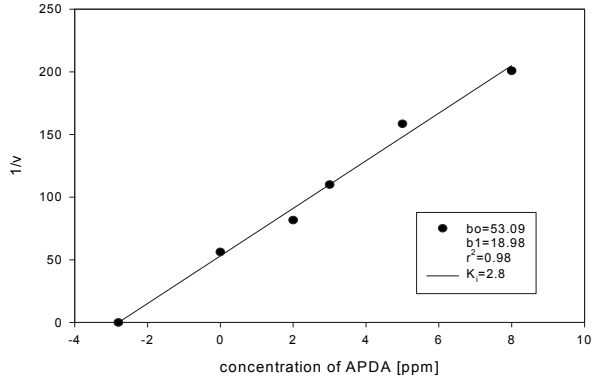

Fig. 2. The initial rate of denitrification versus the APDA concentration used for determination of the inhibition coefficient K_i.

Table 2. Inhibition coefficients characterizing the process of denitrification in a lactate medium into which the compounds tested were introduced.

compound	K_i [ppm]
APDA	2.8
DDDM	0.26
DCDMH	12
BCDMH	21.9
TClCA	2.38
NaDClC	1.85
Chloramine T	8.42
MMPP	137.5
Glutaric aldehyde	2.14
formaldehyde	1.95
Mn^{2+}	101.77
Ni^{2+}	6.08
Zn^{2+}	0.46
Cd^{2+}	0.39
Hg^{2+}	0.18
Pb^{2+}	7.05

The calculations were performed using the packet MicroMath Scientist.

Figures 3-7 show a very good comparison between the experimental and theoretical results, which supports the choice of the kinetic model. All the kinetic parameters of the process are given in Table 3.

For all APDA concentrations the correlation coefficients are very high and close to 0.99. The rate constants of particular stages of the subsequent reactions k_1 and k_2 decrease with increasing concentrations of APDA. The rate constants of the first stage k_1 for all concentrations of APDA used are higher than those of the second stage (by 28% for the standard, by 39% for APDA at 2ppm, 65% for APDA at 3ppm, 38% for APDA at 5 ppm). The exception is the reaction in the presence of APDA at 8 ppm, which is already toxic for denitrifying bacteria, for the reaction with a long induction period (t_o=30 h), at which k_2 is higher than k_1. The coefficient of protein increase Y decreases with increasing biocide concentration in all systems studied.

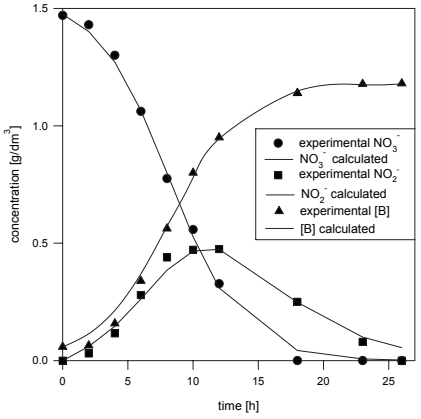

Fig. 3. Comparison of the experimental results (points) with theoretical predictions (lines) obtained on the basis of the kinetic model proposed for the process of denitrification taking place in a standard medium with the involvement of Bacillus licheniformis bacteria (standard) (37°C, pH 7.5, C/N = 2.33).

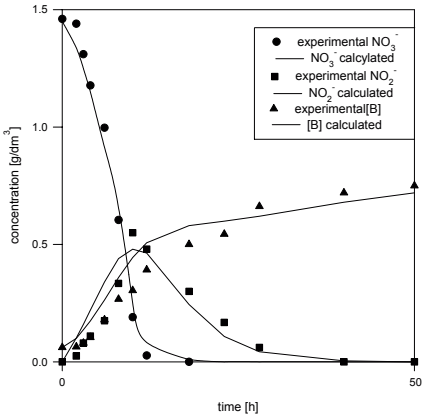

Fig. 4. Comparison of experimental data (points) with the theoretical predictions (lines) obtained, assuming the kinetic model proposed for the process of denitrification taking place in a standard medium containing 2 ppm APDA with the use of Bacillus licheniformis bacteria.

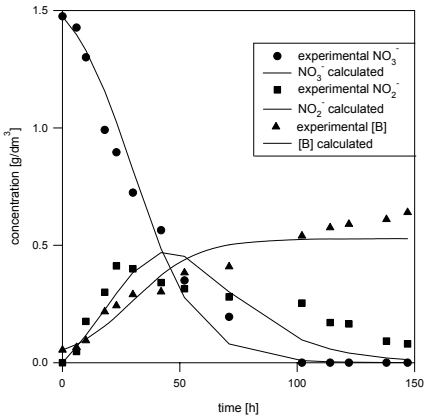

Fig. 5. Comparison of the experimental data (points) with the theoretical predictions (lines) obtained assuming the kinetic model proposed for the process of denitrification taking place in a standard medium containing 3 ppm APDA with the use of Bacillus licheniformis bacteria.

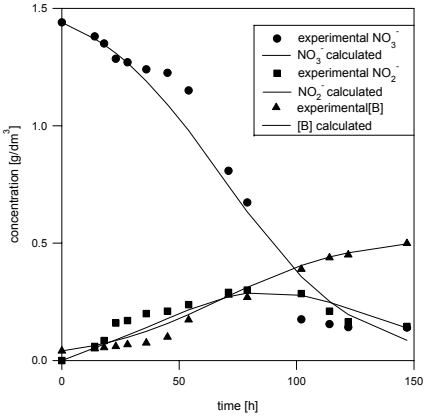

Fig. 6. Comparison of the experimental data (points) with the theoretical predictions (lines) obtained assuming the kinetic model proposed for the process of denitrification taking place in a standard medium containing 5 ppm APDA with the use of Bacillus licheniformis bacteria.

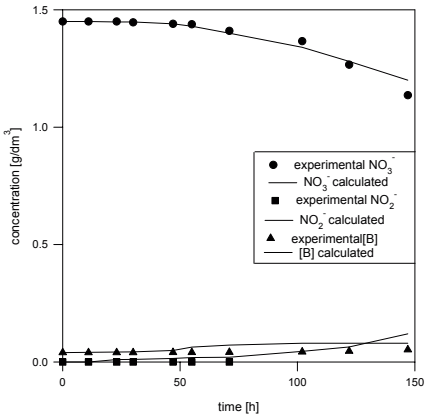

Fig. 7. Comparison of the experimental data (points) with the theoretical predictions (lines) obtained assuming the kinetic model proposed for the process of denitrification taking place in a standard medium containing 8 ppm APDA with the use of Bacillus licheniformis bacteria.

Table 3. The kinetic parameters of the process of denitrification taking place with the involvement of Bacillus *licheniformis* bacteria in a medium containing APDA at different concentrations calculated assuming the second order kinetic model (37°C, pH = 7.5, C/N = 2.33).

	Standard	2 ppm	3 ppm	5 ppm	8 ppm
t_o [h]	0	0	0	5	30
k_1 [$dm^3/g \cdot h$]	0.307 ± 0.005	0.31 ± 0.01	0.137 ± 0.008	0.069 ± 0.003	0.013 ± 0.003
k_2 [h^{-1}]	0.221 ± 0.009	0.19 ± 0.02	0.048 ± 0.006	0.043 ± 0.004	0.03 ± 0.02
Y	0.76 ± 0.01	0.51 ± 0.02	0.32 ± 0.02	0.34 ± 0.02	0.24 ± 0.14
correlation coefficient	0.993	0.989	0.982	0.993	0.999
Standard deviation	0.035	0.066	0.080	0.059	0.032
Criterion of the model selection	5.6	3.7	3.0	4.0	5.9

With increasing concentration of the biocide the values of all parameters decrease. However, at the APDA concentration of 2 ppm (limit of inhibiting activity) k_1 does not change with respect to the standard while k_2 decreases by about 13% and Y by 33%. At the APDA concentration of 3 ppm k_1 decreases by 60%, while k_2 by as much as 78% and Y by 58% relative to the standard. This means that the second stage of the reaction is more sensitive to the effect of biocide. At the toxic concentration of APDA of 8 ppm, the situation is different, that is k_1 is lower than k_2 , which means that the first stage of the reaction so the reduction of nitrates (V) to nitrates (III) is more sensitive to the compound tested.

Conclusions

The coefficients of inhibition and limiting concentrations of the compounds tested have been determined. On the basis of the reaction in the presence of APDA a kinetic model of the process of denitrification has been proposed. According to the model the process is autocatalytic of second order towards nitrates (V) and proteins, and first order towards nitrates (III) for the subsequent irreversible reaction.

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