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Decomposition of Anionic Surface Active Substances by Bacteria from the Surface Microlayer of Lake Jeziorak Mały

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Abstract

The concentration of anionic surface active substances (ASAS) in the water of Lake Jeziorak Mały and the degradation of these substances by bacteria isolated from the surface microlayer and subsurface water were the object of the research of this paper. The concentrations of ASAS in water of this lake were at the level of about 2 mg/1. The biodegradation of ASAS was performed twice as well by microorganisms from the surface microlayer than from subsurface water. Moreover, within the surface microlayer the process of decomposing ASAS was most effectively performed by bacteria isolated from the layers of water closest to the surface (100-150 μm).

Keywords: bacterioneuston, degradation of anionic surface, active substances, detergents

Introduction

Neustonic bacteria occur in the layer of water closest to the surface in water bodies. As a result of the accumulation of organic substances, mainly lipids, proteins, polysaccharides and their derivatives, a surface film or biofilm is formed in this layer, dividing the acquatic environment from the air. There are many mechanisms and factors (adsorption, diffusion, flotation, atmospheric precipitation, anthropogenic pollution) which lead to the accumulation of large amounts of proteins, carbohydrates and lipids in the surface membrane [1,2,3]. The presence of these high-energy organic compounds creates favourable conditions for the development and accumulation of aerobic auto- and heterotrophic microflora constituting neustonic bacteria [4].

Atmospheric precipitation plays a significant role in the enrichment of the surface microlayer. Different kinds of aerosols, dust and gas in the atmosphere fall to the surface of the water as a result of gravitational sedimentation or rainfall. Before the falling matter reaches the water depths, it must pass through the surface microlayer, which has different properties from those in the water mass. Another important factor in the enrichment of the surface microlayer is the proximity of arable fields, industrial plants, lines of communication or built-up areas, from which wind and rain transport various chemical substances to water bodies. For example, aromatic hydrocarbons and heavy metals find their way into the biofilm when carried along with exhaust fumes by the wind. Pesticides and phenol-derived compounds are washed from cultivated fields in rainwater.

Chemical research until now has shown the variety of organic matter present in the surface microlayer, reaching far higher concentrations than a few centimetres below. Among organic matter, some of the surface active substances dissolve poorly in water, and as a result have a tendency to accumulate in the surface microlayer [5]. Most of the organic substances that occur in the surface microlayer lower the surface tension of the water, thanks to which they can be adsorbed within the water-air interface. On the other hand, non-organic ions raise the surface

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tension of the water and are not adsorbed but are washed into the subsurface water [6]. Daumas et al. [7] believe that organic microparticles accumulate in the microlayer in the strict sense (to 100 μm), whereas soluble biogens in the form of non-organic ions are accumulated in a slightly thicker layer of water (to 1 mm). However, Danos et al. [8] noted the stratification of the concentration of both mineral forms of nitrogen (NO_3, NO_2, NH3) and total organic nitrogen (TON) and total phosphorous (TP) in the surface microlayer. They observed the highest concentration of matter in the 50 μm layer, slightly lower in the 300 μm layer and the lowest in the subsurface water.

In connection with the polar structure of their particles, anionic surface active substances (ASAS) accumulate at the border of two phases - polar and non-polar. In natural water bodies, an example of the border between the two phases is the surface microlayer, which constitutes the border between the atmosphere and the hydrosphere and its lower-lying levels. ASAS accumulating in the surface microlayer can, depending on their concentration, constitute a source of organic carbon for heterotrophic bacteria or a factor that limits the development of micro-organisms.

For some acquatic organisms these compounds are toxic. The negative effect of detergents on bacteria mainly involves the cellular membrane being dissolved through fats being washed out. Anionic detergents are more often fatal for Gram positive bacteria, having no, or almost no, effects on Gram negative bacteria. Moreover, the presence of detergents in water is linked with the increased concentration of phosphates, which, in a short period of time, leads to the excessive eutrification of the water body [9].

Detergents, like substances used in agriculture (fungicides, herbicides, insecticides) vary as far as their susceptibility to microbiological degradation is concerned. Many of them, e.g. sodium dodecyl sulphate (SDS), undergo degradation fairly easily under the influence of bacterial activity, mainly of the *Pseudomonas* genus [10]. There are, however, other, particularly resistant substances, like cation detergents, which do not undergo biodegradation.

Bacteria inhabiting the surface microlayer are exposed to the action of many unfavourable physical and chemical factors. Inhabiting the outermost layer of the water body, they are particularly exposed to UV radiation and to sudden changes in temperature. In connection with the mutagenic action of UV, changes in temperature and salinity, the presence of phenol-derived compounds and heavy metals and also the high level of nutrients, the taxonomic and physiological differences between bacteria in the biofilm and the water depths are determined.

The presence of different types of organic and non-organic compounds, whose higher concentration is noted in the surface microlayer, undoubtedly determines the specific biochemical processes and their intensity. Hence, the aim of this paper was to study the occurrence and concentration of anionic surface active substances in the water of the surface microlayer and to define the ability of neustonic bacteria to decompose these compounds.

Materials and Methods

Study Area

The studies were conducted on the strongly eutrophicated lake Jeziorak Mały, which lies within the town of Hawa and is part of the Ilawa Lake District. The surface area of the water body is 26 ha, and the maximum depth is 6.4 m. This lake is joined by a shallow and narrow (1.5m) chanel at its northern part to Lake Jeziorak.

Sampling

Samples of water were taken for tests in spring (May), in summer (July) and in autumn (October) from three differentiated sites (Fig. 1). Water from the surface microlayer was taken

1) with the aid of a glass plate collecting a layer of water 100 μm in thickness,
2) with the aid of a plexiglass plate collecting a layer of water 150 μm in thickness,
3) with the aid of a Garrett net 1 with a mesh diameter of 65 μm collecting a layer of water 250 μm in thickness,
4) with the aid of a Garrett net 2 with a mesh diameter of 200 μm collecting a layer of water 350 μm in thickness.

Samples of subsurface water from a depth of 20 cm were taken with a sterile glass pipette with the aid of an automatic Pippet-boy pump (De Ville). At the same time as taking samples for microbiological research, water was taken to determine the concentration of superficially active anion substances. The samples of water were put into sterile glass bottles, and placed in a container with ice (inside which the temperature did not exceed +7°C) and transported to the laboratory. The time from the moment of taking the samples to their analysis did not exceed 6 hours.

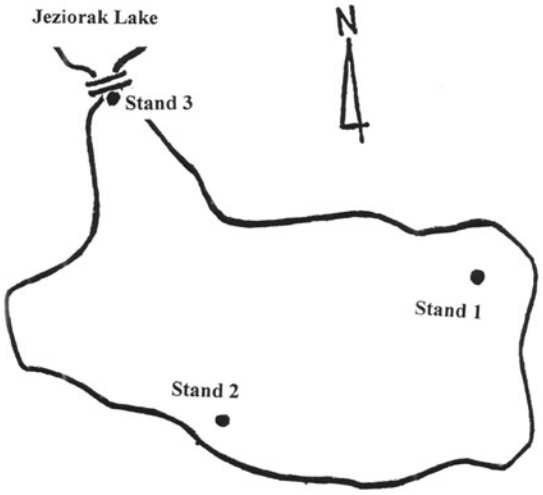

Fig. 1. Outline of the Jeziorak Mały Lake.

Determining Microbiological Parameters

The total number of bacteria (TNB) in the tested samples was determined using the method of direct counting on membrane filters [11]. The number of heterotrophic bacteria (Total Viable Count - TVC) was determined using the method of spread plates using Plate Count Agar (Difco). Incubation was conducted for 6 days at 20°C.

Determining the concentration of anionic surface active substances (ASAS) after the samples were brought to the laboratory, 100 ml of each sample was measured into a series of separatory funnels with a capacity of 250 ml and neutralized to pH 7.0, with 1M NaOH. The concentration of ASAS was determined in samples prepared according to Hermanowicz [12]. The method used involves the creation of a blue-coloured complex compound as a result of the reaction of the cation component of methylene blue with the anion component of the detergent according to the formula:

$$\underset{\text{anion}}{R\text{-}SO_3^{1-}} + \underset{\text{cation}}{BM^{1+}} \text{ -» } \underset{\text{coloured complex}}{R\text{-}SO_3BM}$$

After the extraction of detergent from the tested sample and the measurement of the absorbance of the coloured complex at a light wavelength of $\lambda = 652$ nm, a reading of the concentration of ASAS from the standard curve was carried out using a Marcel Pro S 330 spectrophotometer.

In order to draw up a standard graph of ASAS, a preliminary solution of sodium dodecyl sulphate (SDS) was prepared. 0.1 g SDS was dissolved in distilled water in a measuring flask with a capacity of 1 1. Then 10 ml of the preliminary solution was transferred to a 100 ml volumetric flask and filled to the mark with distilled water. The resulting standard solution contained 0.01 mg SDS per 1 ml. Into the next 100 ml volumetric flasks, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, and 15.0 ml of standard SDS solution were measured out and filled up to 100 ml with distilled water, which gave the equivalent of the following SDS values in the sample: 0.005, 0.01, 0.025, 0.05, 0.075, 0.1, and 0.15 mg. Solutions prepared in this way were next transferred to a series of separatory funnels with a capacity of 250 ml. To each separatory funnel was added 10 ml chloroform and 25 ml methylene blue reagent. The contents of the separatory funnels were shaken intensively for 30 seconds and left until layers separated out. The lower layer of chloroform dyed blue was transferred to the second series of separatory funnels.

The extraction of SDS with chloroform was repeated three times, using 10 ml of chloroform each time. Then, to the chloroform extracts collected in the second series of separatory funnels, 50 ml of rinsing solution was added with the following composition: 6.8 ml H_2SO_4, 50 g $NaH_2PO_4 \cdot H_2O$, $H_2O_{(dist)}$ to 1000 ml.

All of this was again shaken energetically for 30 seconds and left for layers to separate out, after which the lower chloroform layer was transferred to volumetric flasks with a capacity of 50 ml. If necessary, the volume of the extracts in the flasks was supplemented with chloroform up to 50 ml. Then, using a Marcel Pro S 330 spectrophotometer, the absorbance of the extracts with regard to a blank sample containing pure chloroform was measured at a light wavelength of $\lambda = 652$ nm.

Determining the biodegradation of AS AS by neustonic bacteria in order to determine the degree of biodegradation of ASAS, isolated bacterial strains were incubated on liquid medium with the following composition: peptone - 1.0 g, yeast extract - 2.0 g, $MgSO_4 \cdot 7H_2O$ - 2.5 mg, $FeCl_3 \cdot 6H_2O$ - 0.25 mg, $CaCl_2$ - 27.5 mg, phosphate buffer - 10 ml (according to pos. a), H_2O - to 1000 ml, pH -6.8-7.2

a) phosphate buffer was prepared using: KH_2PO_4 - 8.5 g, K_2HPO_4 .21.75 g, NH_4Cl - 2.5 g, $Na_2HPO_4 \cdot 2H_2O$ - - 33.4 g, $H_2O_{(dist)}$ to 1000 ml.

Then sterile medium, 50 ml in volume, was inoculated with the tested strains of bacteria. Incubation was conducted for 72 hours at 20°C. After incubation, the resulting culture was used for further tests on the biodegradation of ASAS, i.e. SDS, which were conducted in Erlenmeyer flasks with a capacity of 250 ml containing 100 ml sterile medium as above, to which was added 500µ SDS solution at a concentration of 1 g/1 (the final concentration in the cultures was 5 mg/1).

Medium thus prepared was inoculated with 1 ml of the culture of the tested strain. The inoculation was a culture of the strain brought up to optical density A = 0.5, whose absorbance measurement was conducted using a Specol spectrophotometer at wavelength $\lambda = 560$ nm. Sterile medium not containing SDS was used to dilute the culture.

The inoculated medium with the addition of SDS was incubated for 24 hours at 20°C. After incubation 5 ml of post-culture liquid was taken from individual flasks in order to determine the concentration of bacterial protein in the culture. The remaining post-culture liquid was transferred to separatory funnels with a capacity of 250 ml. The sample was neutralised to pH = 7.0 and the concentration of the remaining SDS was determined in it after [12]. The concentration of SDS in the sample was read from the standard curve.

On the basis of the results of the concentration of SDS read from the standard curve, the loss of SDS in the post-culture liquid was calculated and expressed as a percentage of the biodegradation of SDS by the studied bacterial strains. The decrease in the concentration of SDS (Xt) after the time of incubation t was calculated in percentages according to the following formula:

$$X_t = \frac{a - b}{a} \cdot 100$$

where: a - concentration of SDS in time t_0 = 5 mg/1, b - concentration of SDS after time t [mg/1]

The results obtained were calculated for the amount of bacterial protein in the culture, whose concentration was determined using Bradford's method [13].

Table 1. Number of bacteria in the surface microlayer and subsurface water of the Jeziorak Mały Lake.(number / 1 ml).

Date sample	Sample-taking method					Average		EC
taken	A	B	C	D	E	SM	SSW	SM/SSW
Spring (May)	32.51* 169.1**	27.50 371.0	24.33 79.8	20.48 65.3	15.67 14.3	26.21 171.3	15.67 14.3	1.67 11.9
Summer (July)	48.31 123.3	47.56 353.3	18.12 183.2	41.30 99.9	10.80 19.2	38.82 189.9	10.80 19.2	3.59 9.8
Autumn (October)	28.52 43.3	46.91 79.3	19.59 200.0	16.31 120.0	10.39 30.0	27.83 110.6	10.39 30.0	2.68 3.6

Explanation: A - sample taken using glass plate, B - sample taken using plexiglass plate, C - sample taken using Garrett net 1, D - sample taken using Garrett net 2, E - water from the subsurface layer, SM - surface microlayer (A+B+C+D/4), SSW - subsurface water, EC - enrichment coefficient, * - total number of bacteria x 10^6, ** - number of heterotrophic bacteria x 10^3.

Table 2. Concentration of anionic surface active substances (ASAS) in the water of theJeziorak Mały Lake.

Site where sample was taken	Concentration of ASAS (mg/ l)
Site 1	2.40
Site 2	1.58
Site 3	2.20
Average	2.06

Results

The data concerning the total number of bacteria (TNB) and the number of heterotrophic bacteria (TVC) in the studied samples of water are presented in Table 1. It follows from them that the maximum TNB and TVC in the surface microlayer occurred in summer. In spring and autumn the values were slightly lower, but similar. In the subsurface water the maximum TNB was found in spring, while that of TVC in autumn. The samples of water taken from the subsurface water always contained several times fewer bacteria than the samples of water from the surface microlayer.

The results of the analyses of the concentration of anionic detergents in the water of Jeziorak Mały are presented in Table 2. It follows from the data obtained that the concentrations at sites I and II were similar (2.40 and 2.20 mg/1, respectively), while at site III, situated in the middle of the long axis of the lake, the concentration of anionic detergents was considerably lower, at 1.58 mg/1. These results were used to determine the concentration of sodium dodecyl sulphate (SDS) in experiments concerning the biodegradation of these substances (anionic detergents).

Table 3 and Figure 2 present the results of tests concerning the degradation of sodium dodecyl sulphate (SDS) by strains isolated in spring, summer and autumn from the surface microlayer and the subsurface water of Jeziorak Mały. It follows from the analysis of the average values that the highest degree of degradation of a detergent used in this paper by bacteria isolated from the surface microlayer was observed in autumn and was 68.54%; a slightly lower level was noted in summer (58.46%), and the lowest in spring (56.75%). An analogous situation occurred in the case of bacteria isolated from subsurface water. The maximum degradation of SDS was also found to be by bacteria isolated in autumn (61.48%), and the minimum in spring (29.85%).

Comparing the process of degradation of SDS by bacteria isolated from the surface microlayer and subsurface water, distinct differences can be seen. It follows from the presented data that neustonic bacteria displayed a greater ability to degrade that detergent than planktonic bacteria. After a 24-hour culture of that bacteria in the presence of SDS, the degree of its degradation by strains from the surface microlayer was 61.25%, and by strains from the subsurface water 40.5%.

From the analysis of average values concerning the method of taking samples from the surface microlayer (Fig 3), it can be stated that the highest degree of degradation of SDS occurred in the layer with a thickness of 150 μm containing bacteria collected using a plexiglass plate (B - 77.48%); slightly lower was by bacteria from the layer with a thickness of 100 μm collected using a glass plate (A - 70.80%). The lowest degree of SDS degradation was displayed by neustonic bacterial strains collected using a Garrett 2 net, occurring in the layer of water with a thickness of 350 jam (D-36.14%).

Summarizing the collected data, it follows that the majority of bacterial strains isolated from the surface microlayer and the subsurface water, regardless of the season, degraded SDS easily. However, this compound was more actively and more strongly degraded by bacteria inhabiting the surface microlayer than those in the subsurface water.

Discussion

Some hundreds of micrometers inside the microlayer and still deeper water change the temparature, the quantity and quality of nutrients and the amount of sunlight penetrating. These factors, in all certainty, determine the development of bacteria and the kinetics of biochemical transformations. This finds a reflection in the number, spe-

Table 3. Degradation of SDS by strains from the surface microlayer and subsurface water of the Jeziorak Mały Lake (data in %).

SDS - concentration 5 mg/l						
Season	Layer of water					Average in SM
	A	B	C	D	SSW	
Spring (May)	56.90	64.26	57.75	48.10	29.85	56.75
Summer (July)	67.18	70.66	57.22	38.79	30.27	58.46
Autumn (October)	88.33	97.53	66.81	21.52	61.48	68.54
Average	70.80	77.48	60.59	36.14	40.50	61.25

Explanation: A - sample taken using glass plate, thickness of layer 100 mm, B - sample taken using plexiglass plate, thickness of layer 150 mm, C - sample taken using Garrett net 1, thickness of layer 250 mm, D - sample taken using Garrett net 2, thickness of layer 350 mm, SM - surface microlayer (A+B+C+D/4), SSW - subsurface water from a depth of 20 cm

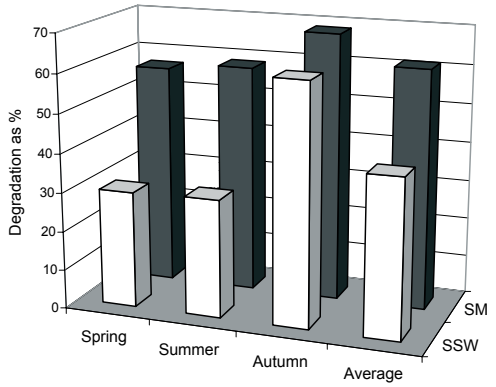

Fig. 2. Degradation of SDS by strains from the surface microlayer (SM) and subsurface water (SSW).

cies composition and physiology of bacteria inhabiting that environment [14, 15].

The results of research on the total number of bacteria (TNB) and the number of heterotrophic bacteria (TVC) showed that these parameters were always greater in the surface microlayer than in the subsurface water. The maximum number of neustonic bacteria occurred in summer (July), which is in accordance with Niewolak's research [16], and the minimum was in spring (May). The total number of bacteria in the surface microlayer was 1.25-3.59 times higher than in the subsurface water at a depth of 20 cm. Results of earlier research [17,18] confirm the data on the numerical predominance of neustonic bacteria over planktonic bacteria. But the results of research published by Niewolak [16] indicate a greater number of bacteria in the layers situated below the surface film, which the author justifies by the selective influence of UV radiation on the survival rate of bacteria. It follows from research conducted by Hermanson et al. [19], and by Maki and Herwig [20], that pigmentation and the presence of plasmids with encoded resistance to ultraviolet radiation in the cells of neustonic bacteria protect them from the photosensitising effect of sunlight, and in particular the lethal action of UV. Moreover, Sieburth [21] found that the number of coloured bacteria from the genus *Flavobacterium* reached the maximum during the period of the strongest insolation. Other bacteria were also characterized by high tolerance in relation to strong light. According to Reinheimer [22] the harmful effect of light is particularly noticeable in relation to bacteria devoid of protective pigments. Potter [23] and Jones et al. [24] contend that chromogenic bacteria occur in greater numbers in the surface film than in deeper layers of water.

Anionic surface active substances (anionic detergents), especially those produced now, are easily degradable compounds. Since 1965 the industry has been producing so-called "soft" detergents, which do not cause a permanent foam to remain on the surface of water bodies, and whose degradation lasts from a few to less than twenty days. From the chemical point of view these are alkylarylsulphonats with linear particles. Unfortunately, their shortcoming is their high

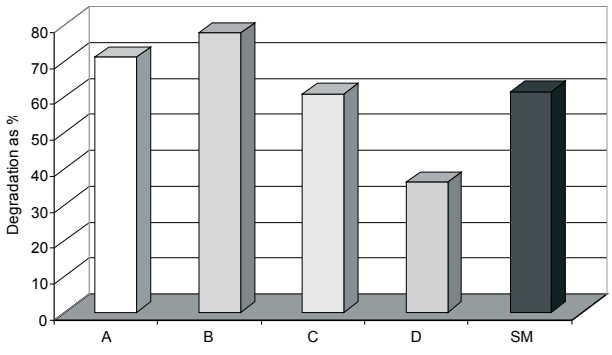

Fig. 3. Degradation of SDS by bacteria from individual layers of the surface microlayer.

Explanation: A - thickness of layer 100 μm, B - thickness of layer 150 μm, C - thickness of layer 250 μm, D - thickness of layer 350 μm, SM - surface microlayer (A+B+C+D/4).

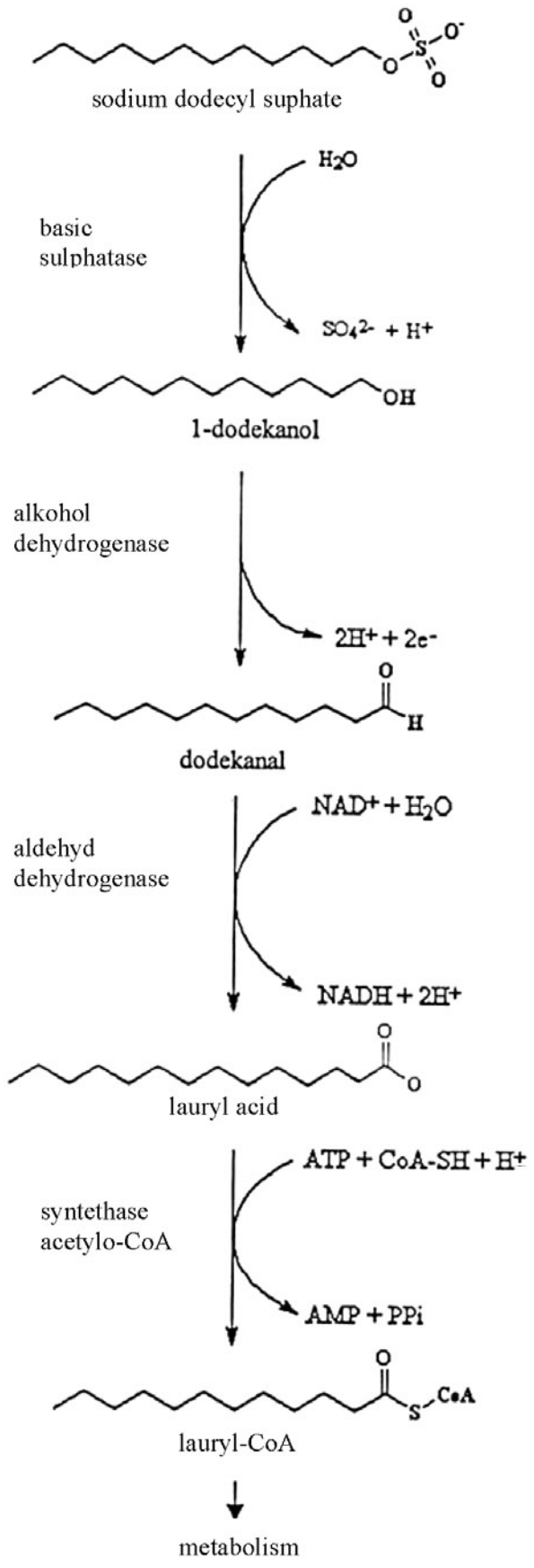

Fig. 4. Scheme of sodium dodecyl sulphate degradation.

content of orthophosphates, which are released into the environment during biodegradation, thus hastening the process of eutrophication. The only organisms capable of degrading detergents are bacteria. Because each detergent is composed of a hydrophobic and a hydrophilic element, it is obvious that these compounds naturally accumulate at the border of two phases, e.g. water-air, or, in other words, in the surface microlayer. In connection with this, it is precisely neustonic bacteria that should play an important role in the degradation of these compounds. In accordance with expectations, the results obtained indicate that bacteria from both the surface microlayer and the subsurface water are capable of biodegrading anionic detergents, and thus detoxifying the environment. However, the efficiency of the degradation process conducted by bacteria from the surface microlayer is from several percent (autumn) to several dozen percent (spring) higher than among strains from the subsurface water.

The efficiency of the biodegradation of the detergent used altered distinctly according to season. Among strains from both the surface microlayer and the subsurface water the lowest efficiency was noted in spring (May) (56.75% and 29.85%, respectively) and the highest in autumn (October) (68.54% and 61.48%, respectively). This phenomenon might be caused by the gradual adaptation of microfiora to the presence of detergents or by the too high concentrations of detergents in the spring period, due to which they had a toxic effect on micro-organisms, and then, together with the decrease in the concentration of these compounds in the environment in subsequent months, the percentage of strains actively degrading anionic detergents grew. This would be in accordance with Schlegel's findings [25], according to which the rate of degradation of many organic compounds by the bacterial population is inversely proportional to their concentration. Thus, substances like detergents or aromatic hydrocarbons undergo degradation only at low concentrations, where their toxic effect is reduced. This might result from the fact that high concentrations of these compounds impede the activity of the enzymatic systems of micro-organisms, thus rendering the process of biodegradation impossible. Also, the concentration of the detergent (5 mg/1) used in the experiments in our research was twice as high as the concentration of these compounds found in the studied lake (1.58 -2.4 mg/1).

The analysis of the results obtained as regards the methods used for taking samples allows us to state that the detergent used was best degraded by bacteria obtained using the plexiglass plate B (where the average value of degradation was 77.48%). This is probably a result of the fact that it is precisely in this layer with a thickness of about 150 μm that the greatest cumulation of detergents takes place, with the hydrophile part of the particles under the water, while the hydrophobic part remains anchored in the extreme surface membrane of the surface microlayer, which is mainly composed of lipids and glycolipids. Ac-

cording to Scott and Jones [26], from the biochemical point of view, degradation of sodium dodecyl sulphate (SDS) used in this paper is initiated by the release of inorganic sulphur by basic sulphatase (Fig. 4). Then the released alcohol is oxidized to lauryl acid by alcohol dehydrogenase, coded by the operon *alk* BFGHJKL and *alk* ST, which encode supplementary proteins essential to the degradation of 5 to 12 carbon, linear alkanes. In the end lauryl acid is degraded in the process β - oxidation. The pattern described was elaborated for bacteria from the *Pseudomonas* genus, but other bacterial strains can also initiate the process of degradation of SDS or participate in later stages of its degradation [26]. Among bacteria capable of biodegrading SDS the author also mentions bacteria from the genera *Bacillus, Flavobacterium* and *Achromobacter.* The lack of analogous findings concerning the concentrations and degradation of detergents in the surface microlayer makes it impossible to conduct further discussion.

In summary, detergents, like the majority of organic compounds, undergo biodegradation in natural conditions, or in other words they undergo degradation under the influence of enzymes produced by micro-organisms. The relative ease with which SDS was degraded results from its simple, linear structure, small molecular mass and high solubility in water. Apart from that it is a widespread compound in many branches of industry used in, among other things, toothpaste, washing powders, shampoos or as an additive in food. Thus, it often finds its way into the environment, which means that it is accessible to bacteria living there and probably contributes to their adaptation.

Conclusions

1. The TNB and TVC are higher in surface microlayer than in subsurface water.
2. Bacterial cells isolated from surface microlayer and subsurface water of Lake Jeziorak Mały are able to degradate ASAS.
3. Bacteria inhabiting surface microlayer decompose ASAS more efficiently than the ones from subsurface water.

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