ARCHIVES OF ENVIRONMENTAL PROTECTION

vol. 37 no. 1 pp. 75 - 88 2011

PL ISSN 0324-8461

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# HEAVY METALS IN SURFACE MICROLAYER IN WATER OF LAKE GARDNO

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**Keywords:** Surface microlayer, heavy metals, estuarine lake, seasonal changes.

**Abstract:** In the present paper we show results of our research on the contents of selected heavy metals in the surface and subsurface water layers of the estuarine lake Gardno. Obtained results show that there are substantial differences between concentrations of lead, copper and zinc in surface microlayer and subsurface waters. We observed that the surface microlayer is capable of accumulating much higher amounts of these metals than the subsurface waters. Analysis of seasonal changes in heavy metal concentrations in surface microlayers and subsurface waters shows some periodicity of these changes.

## INTRODUCTION

The surface microlayer is a thin layer, of several houndred μm found in the contact interface between water and the atmosphere [8, 10, 41]. This layer covers 3/4 of the Earth surface. It represents a significant part of water environment creating independent subecosystems on the boundary of the atmosphere-hydrosphere exchange. It is sometimes named "the last millimeter of ocean" [31]. It is created on the surface in all water ecosystems. This surface microlayer in a water reservoir is a specific chemical and physical environment, different from subsurface water. It constitutes a unique ecotone in relation to its physical, chemical and biological manageability [1, 27, 28], markedly different from subsurface water [4]. This monomolecular surface water layer is a common boundary layer with an enormous importance to the two phases: water and the atmosphere [10, 30]. For this reason it becomes variable in time and in space in terms of exchange processes [2, 11, 41]. Exchange of substance and energy is a very important process in the biogeochemical element cycles. The exchange process between the atmosphere and hydrosphere plays a key role in the water environment and determines global radiation balance [12, 31, 42]. This exchange takes place thanks to the same biological, chemical and radiatical transformations in two environments [11, 15].

Apart from physical factors, the microorganism metabolic activity in the water surface microlayer also influences its chemical composition [9, 46]. Research on water ecosystems shows a clear difference in concentrations of different substances between the surface microlayer and subsurface water. Usually concentrations of individual water

ingredients in the microlayer are higher than in water depths [1, 2, 9, 10, 15, 30, 36], and the same is true also for metal concentrations [6, 8, 23, 33, 34]. Hardy *et al.* [16] analyzed the influence of such factors as chemical microlayer composition, transport and forms of metals as well as the time spent in the microlayer on the actual levels of metals in the microlayer. Possible enrichment mechanisms in surface microlayer water in the case of metals were discussed by Liss [26]. Special attention needs to be paid to metal transport by air bubbles, containing on their surface active material which can bind metal ions from water, to metal association by insoluble organic forms and binding of metals from water and from air in metalorganic complexes. It has an essential influence on the vital condition of organisms. High levels of heavy metals found in the surface microlayer are toxic not only to neuston organisms, but also to larval fish and fry as well as other water organisms, which take food from the surface layer water. In many cases, as for example in the case of pelagic fish, their spawn develops directly in the surface microlayer [46]. The goal of the conducted research was to investigate seasonal changes in climax metal selection in the surface microlayer of Lake Gardno in terms of water salinity.

## MATERIAL AND METHODS

Lake Gardno is situated in the Słowiński National Park, in the middle part of the Baltic coast (Fig. 1).

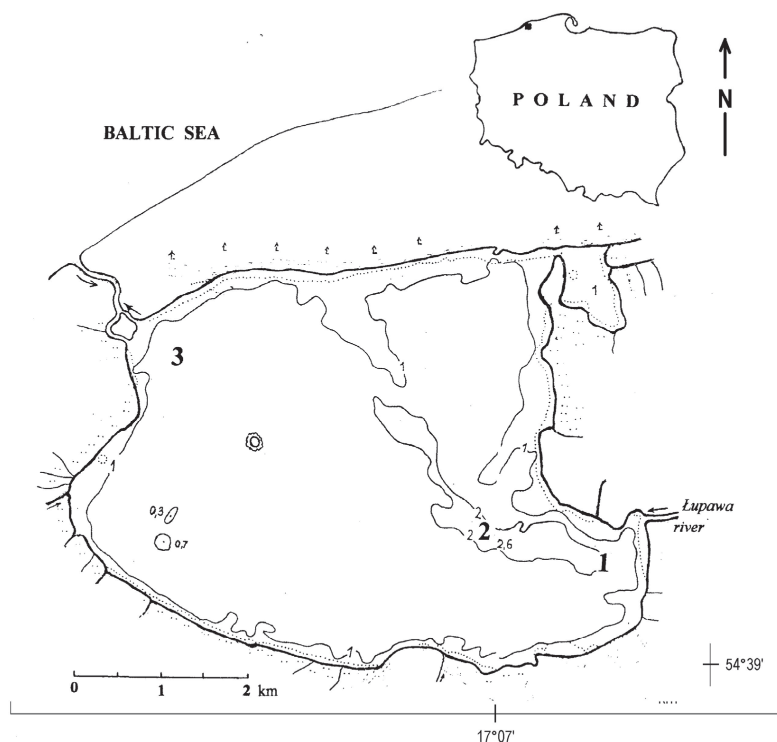


Fig. 1. Lake Gardno, northern Poland, with locations of sampling stations

This shallow lake (mean depth 1.3 m) is a full mixing lake [43, 44, 45]. Lake Gardno is the second estuary lake in Poland as regards surface [40]. From the Baltic coastline the lake is separated by a narrow land strip up to 2 km wide. Water is supplied by the Łupawa River, which discharges to Gardno in its south-eastern part (Fig. 1), and as well as by many little tributaries. The difference of water levels between the lake and the sea is not large (about 26 cm), it facilitates exchange of water by the estuary section of the Łupawa to the sea. In the north-eastern part of the lake this section is in the channel form, about 10 m wide, to join it with the sea [44].

Samples of surface microlayer and subsurface water in Lake Gardno were taken from three places (Fig. 1) once a month, from March 1999 to October 2000. Two sampling techniques were applied: glass plate [18] and polyethylene Garrett net [14]. Both methods differed in the thickness of microlayer sample collection. Samples of the thinnest microlayer (90–120 μm) were taken using a glass plate and were named Film (F), while those of the layer collected with a Garrett net at a depth of 200–320 μm were denoted as Garrett Film (FG). Samples of subsurface water (SUB) were taken from a depth of about 15 cm with use of polyethylene containers. Volume of water sample was divided into 3 sub-samples, thickened at 70°C and mineralized with $HNO_3$ *trace pure* and $HClO_4$ *trace pure* (ratio of acids 5 : 1). Mineralization was carried out in a Maxidiges MX 350 Prolabo microwave mineralizer coupled with a microcomputer designed to control the program of power and time of mineralization [35, 47]. After a full sample oxidation standardization to the constant volume of demineralized water was performed samples were kept in a laboratory refrigerator at a temperature of 4°C until measurement time [19, 32].

Determination of zinc was conducted in an Atomic Absorption Spectrophotometer Carl-Zeiss Jena AAS3 in oxy-acetylene flame. Analyses were conducted at a wavelength of 213.9 nm [5, 50]. Assays of cooper and lead were also performed with an Atomic Absorption Spectrophotometer Carl-Zeiss Jena AAS3, but this time with an attachment of an EA3 electromagnetic atomizer and automatic sample feeding. Analyses were conducted at a wavelength of 217.0 nm for lead and 324.8 nm for copper, respectively [5, 50]. The Work programme for the electromagnetic atomizer was established according to: [7, 13, 49]. For the selected series of 30 sub-samples Pb and Cu assays were conducted using two apparatuses: GBC Avanta Σ with an attachment of a GBC GF 3000 graphite furnace and of a PAR 3000 auto sampler (Medical University in Gdańsk) as well as a Carl-Zeiss Jena AAS3 apparatus. However, for Zn assays an AAS Philips Pu 9100 apparatus working in the flame version (Medical University in Gdańsk) and a Carl-Zeiss Jena AAS3 apparatus were used. For the purpose of verification of results two reference materials were used, i.e. SCP Science EnviroMAT™ & AgroMAT™ ES-H-1 Ground Water and EU-H-1 Waste Water [39]. Analytical methods recovering previously prepared central pattern of metal subjected to the same treatment as samples were applied. Lead concentration in the reference sample was higher by 8.6% than the average reported by other certificating laboratories. For copper and zinc these differences were 4.3% and 4.6%, respectively. It was used also to recover the previously prepared middle standard of the metal subjected to the same preparation as the sample. Magnitude of the recovery for lead was 91.3%–109.3%, for copper it was 92.3%–104.3 % and for zinc it was 95.4%–105.3 %, respectively.

For chlorine ions argentometry was applied according to Standard Methods [37]. Enrichment factors of water surface microlayers for heavy metals in relation to subsurface water was calculated using equations:

For film: $EF_F = C_F/C_{SUB}$

For Garret film: $EF\ f_{FG} = Cf_{FG}/C_{SUB}$

where $C_F$, $Cf_G$, $C_{SUB}$ – concentration of heavy metals in relation to water layer [15].

Pearson's correlation coefficient was calculated using the "Statistica" program [38].

## RESULTS

In the period from March 1999 to October 2000 the concentrations of three heavy metals (Cu, Zn, Pb) were analyzed in surface water microlayers and subsurface water in Lake Gardno. Table 1 presents medium concentrations of copper in both microlayers and in subsurface water in individual sampling stations on the lake.

Table 1. Mean concentrations recorded for Cu, Pb, and Zn (μg dm$^{-3}$) in individual analyzed layers and respective calculated enrichment factors

| Cu | F | FG | SUB | $EF_{F/SUB}$ | $EF_{FG/SUB}$ |
|---|---|---|---|---|---|
| 1 | 34.8 | 19.6 | 14.5 | 5.43 | 2.86 |
| 2 | 33.6 | 11.4 | 6.1 | 8.43 | 3.19 |
| 3 | 56.9 | 21.0 | 13.9 | 8.49 | 3.57 |
| Pb | | | | | |
| 1 | 41.8 | 24.9 | 8.5 | 4.92 | 2.93 |
| 2 | 31.5 | 22.9 | 8.6 | 3.66 | 2.66 |
| 3 | 46.6 | 34.9 | 8.8 | 5.3 | 3.97 |
| Zn | | | | | |
| 1 | 26.3 | 24.4 | 13.7 | 1.92 | 1.78 |
| 2 | 30.3 | 26.4 | 12.7 | 2.39 | 2.08 |
| 3 | 38.1 | 30.5 | 13.3 | 2.86 | 2.29 |

The highest concentration of copper (mean 56.9 μg dm$^{-3}$) was observed in film at position 3 (F, Fig. 2D), which was exposed the most to the effect of sea water.

Medium concentration of chlorine ions in this area amounted to 730 mg dm$^{-3}$ [43] and was five times higher than at location 1, on which water from Lake Gardno is influenced by the ground factor, because water from the Łupawa River flows into the lake there. However, the copper content in Garrett's film water (FG) and from the subsurface at location 1 was the same as at location 3 (FG and SUB) and amounted to 20 and 21 μg dm$^{-3}$ (Garrett's film) and 14 μg dm$^{-3}$ (subsurface). The lowest level of copper was observed in water at location 2, which was characterized by the biggest depth. The copper content in this microlayer (FG) and subsurface water (SUB) at location 2 was nearly two times lower than at the other locations (Table 2, Fig. 2 E–F).

In the case of lead (Fig. 3) a similar distribution of its concentration in the analyzed water layers was found as it was found for copper.

The highest concentration of lead was found at location 3 (F – 46.6, FG – 34.9, SUB – 8.8 μg dm$^{-3}$), the lowest at location 2 (F – 31.5, FG – 22.9, SUB – 8.6 μg dm$^{-3}$, Tab 2). We need to stress here a much higher lead concentration in Garrett's film at location 3 (34.9 μg dm$^{-3}$) than that in Garret's film at location 1 (24.9 μg dm$^{-3}$), i.e. by about 10 μg dm$^{-3}$. In the case of copper, its concentration at location 3 was higher by merely about 2.6 μg dm$^{-3}$.

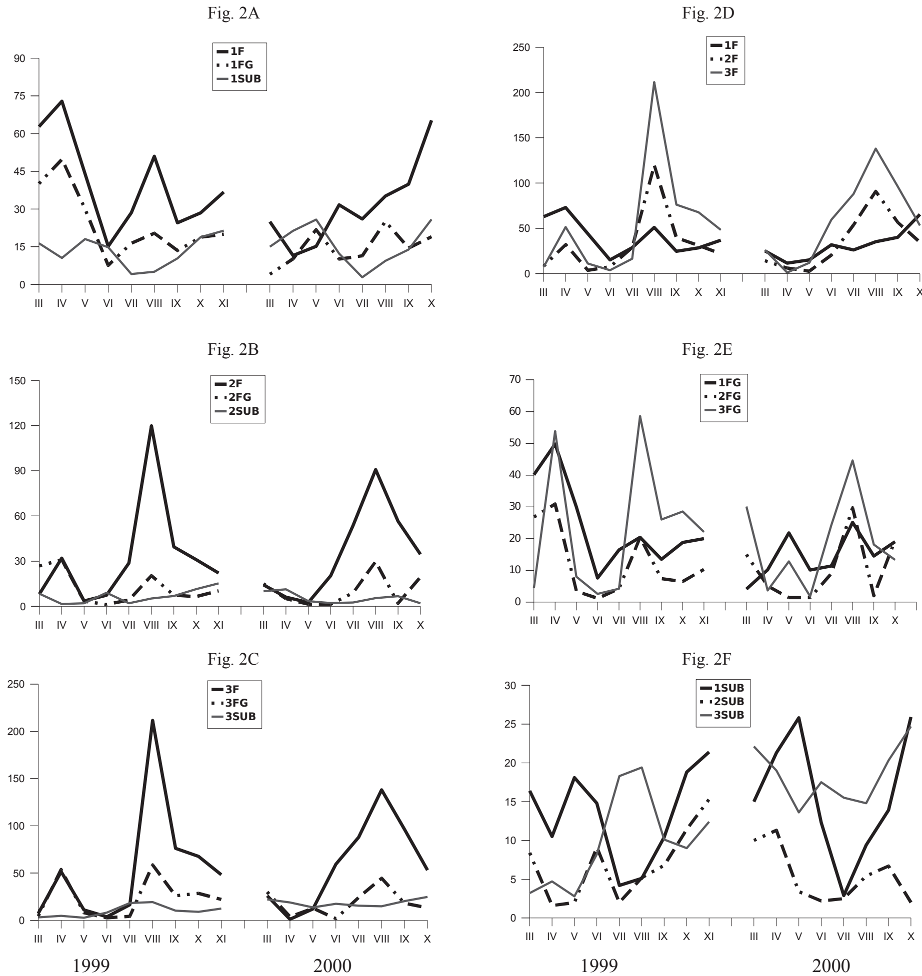


Fig. 2. Seasonal dynamic of copper concentration change (μg Cu dm$^{-3}$) in surface microlayer ans subsurface water of Gardno lake in 1991 (III-XI) and 2000 (III-X) year

The concentration of this metal in subsurface water at all analyzed locations was similar and amounted to about 8.6 μg dm$^{-3}$ (Tab. 1).

In both water surface microlayers (F and FG) a progressive increase in zinc concentration was observed going from location 1 to location 3 (Tab. 1, Fig. 4), that is as in the case of an increasing concentration of chlorine ions.

In the case of zinc concentration at location 3, it amounted to F – 38.1, FG – 30.5, SUB – 13.3 μg dm$^{-3}$, at position 1 to F – 26.3, FG – 24.4, SUB – 13.7 μg dm$^{-3}$, respectively. This metal concentration in subsurface water, similarly to lead, was similar at all the locations and amounted to about 13 μg.dm$^{-3}$.

In all cases the concentrations of analyzed metals was often highest in film, i.e. the thinnest microlayers (105 ± 15 μm). A multiple of concentration in film and in Garrett film with relation to subsurface water is reflected in values of enrichment factor in Table 1.

Table 2. Values of enrichment factors for film and Garrett film recorded for Cu, Pb, Zn at Lake Gardno sampling locations

| | $EF_{F/SUB}$ | $EF_{FG/SUB}$ | $EF_{F/SUB}$ | $EF_{FG/SUB}$ | $EF_{F/SUB}$ | $EF_{FG/SUB}$ |
|---|---|---|---|---|---|---|
| Cu | st.1 | | st. 2 | | st. 3 | |
| Mean | 3.54 | 1.84 | 8.64 | 3.23 | 4,45 | 2,08 |
| Minimum | 0.54 | 0.27 | 0.54 | 0.13 | 0.06 | 0.10 |
| Maximum | 10.00 | 4.74 | 23.04 | 19.31 | 10.94 | 11.45 |
| Mean from 3 locations | | | | | 5.54 | 2.38 |
| | $EF_{F/SUB}$ | $EF_{FG/SUB}$ | $EF_{F/SUB}$ | $EF_{FG/SUB}$ | $EF_{F/SUB}$ | $EF_{FG/SUB}$ |
| Pb | st.1 | | st. 2 | | st. 3 | |
| Mean | 5.01 | 2.86 | 3.72 | 3.12 | 4.80 | 3.71 |
| Minimum | 1.09 | 0.54 | 1.33 | 0.55 | 0.53 | 0.69 |
| Maximum | 17.05 | 7.92 | 10.60 | 13.80 | 19.61 | 12.68 |
| Mean from 3 locations | | | | | 4.51 | 3.23 |
| | $EF_{F/SUB}$ | $EF_{FG/SUB}$ | $EF_{F/SUB}$ | $EF_{FG/SUB}$ | $EF_{F/SUB}$ | $EF_{FG/SUB}$ |
| Zn | st.1 | | st. 2 | | st. 3 | |
| Mean | 2.43 | 2.07 | 1.52 | 1.36 | 2.10 | 1.68 |
| Minimum | 0.72 | 0.53 | 0.50 | 0.39 | 0.33 | 0.14 |
| Maximum | 10.10 | 8.45 | 3.30 | 2.53 | 5.69 | 3.69 |
| Mean from 3 locations | | | | | 2.01 | 1.70 |

This factor for copper in film ($EF_F$) ranged from 0.06 (location 3) to 23.04 (location 2), and in Garrett film ($EF_{FG}$) from 0.10 (location 3) to 19.31 (location 2). The highest value of these factors was observed at location 2 and amounted for film to a mean of 8.64 and for Garett film to 3.23 (Table 2). In Garrett film the difference between respective factors was considerably smaller than in film. It seems that the reason for this difference is the copper source which is accumulated in microlayers. Most probably this element originates mainly from the atmosphere, because enrichment factors for film at location 2 are almost three times bigger than for Garret film, at location 1 and 3 two times bigger (Tab. 2). What is more, correlation factors for copper contents in the depths of water and contents in film, at locations 1 and 2 were statistically non-significant, which showed that the main source of this metal in studies on microlayers in this area should be searched for elsewhere – most probably in the atmosphere (Tab. 3).

Table 3. Pearson's correlation coefficient (n = 17). Bold-face print showed statistically significant correlations

| | Cu | | Pb | | Zn | |
|---|---|---|---|---|---|---|
| 1PW | 1F | 1FG | 1F | 1FG | 1F | 1FG |
| | -0.03 | 0.03 | 0.15 | 0.41 | **0.71** | **0.87** |
| 2PW | 2F | 2FG | 2F | 2FG | 2F | 2FG |
| | -0.17 | -0.09 | 0.42 | 0.07 | **0.45** | **0.60** |
| 3PW | 3F | 3FG | 3F | 3FG | 3F | 3FG |
| | 0.3 | 0.02 | 0.19 | -0.09 | 0.41 | 0.36 |

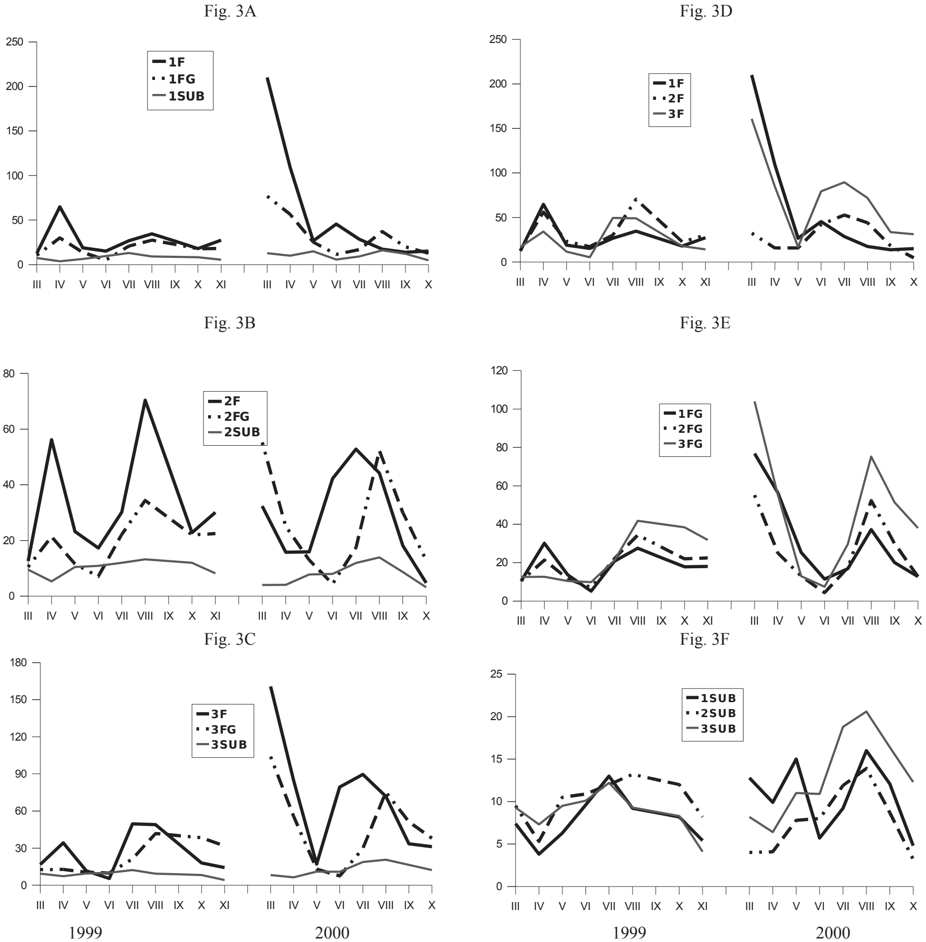


Fig. 3. Seasonal dynamic of lead concentration change (μg Pb $dm^{-3}$) in surface microlayer ans subsurface water of Gardno lake in 1991 (III-XI) and 2000 (III-X) year

It is confirmed by the lowest concentration of copper in subsurface water at location 2, but all the same the concentration in film is similar to that at location 1. Only at location 3 the value of the correlation coefficient of 0.30 indicates that in sea water an essential influence on copper accumulation in surface microlayers is also played by its concentration in deep water.

In 84% of cases the enrichment factor of film for copper was bigger than for Garret film. This result showed that the thinner microlayer has a much bigger ability to accumulate cooper than the thicker microlayers.

The enrichment factors of surface microlayer water in the case of lead ranged from 0.53 in film (location 3), to 19.61 (location 3), but in Garrett film they ranged from 0.54 (location 1) to 13.80 (location 3, table 2). The mean difference in their value was much

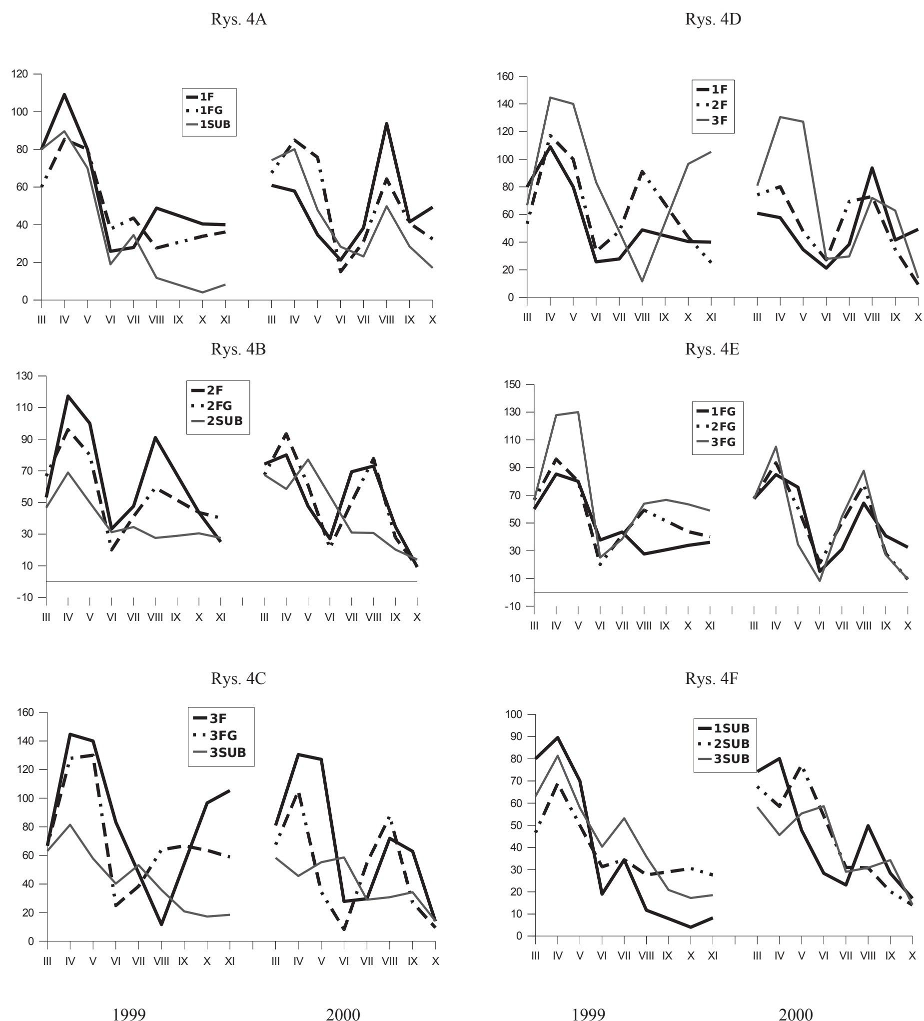


Fig. 4. Seasonal dynamic of zinc concentration change (μg Zn $dm^{-3}$) in surface microlayer ans subsurface water of Gardno lake in 1991 (III-XI) and 2000 (III-X) year

smaller than in the case of copper, for film the mean values at locations 1 and 3 were comparable. However, the lowest enrichment factor was observed at location 2, in contrast to that of copper. In the case of Garrett film the mean enrichment factors recorded at individual samplings stations were much closer than in film.

Calculated linear correlation coefficients for lead were usually positive. At the second location for line F and location 1 for microlayer FG a low correlation coefficient was recorded. It suggests a much bigger influence of subsurface water on lead accumulation in the microlayer than that of copper. Only at location 3 this influence was negligible.

Frequency of higher lead concentrations in the surface microlayer than in subsurface water was much higher than in the case of copper and exceeded 88% and for film 100%. The frequency of cases when lead concentration in film was higher than in Garrett film

was 61%. The lowest frequency (88%) was observed for zinc. Zinc was characterized by the smallest enrichment factors at location 2, which was comparable for both microlayers and for film it amounted to 1.52, while for Garrett film it was 1.36 (Tab. 2). The highest value of these factors was observed at location 1 (2.43 for film and 2.07 for Garrett film). Frequency of cases in which EF > 1 for film and Garrett film amounted to 88% at location 1, 94% at location 2 and about 80% at location 3, respectively.

At the level of significance p = 0.05 and n = 17 the correlation coefficient for the content of zinc in the surface microlayer in water of Lake Gardno and the content of this metal in the subsurface microlayer showed a significant value (Tab. 3). It indicates that zinc accumulating in microlayers comes mainly from water depths.

The changes in copper concentration in the analyses of surface layers of water in Lake Gardno in the course of a year are presented in Figure 2. Completely different changes in proportions were observed at location 1 than at locations 2 and 3 (Figs. A–C). At location 1 the highest copper concentration was observed in March and April 1999 (about 68 μg $dm^{-3}$ in film and 45 μg $dm^{-3}$ in Garrett film) while in October 2000 it was 65 and 19 μg $dm^{-3}$, respectively. In 1999 from spring to autumn declining trends were observed in contents of this metal in microlayers (in film from 62 in March to 37 μg $dm^{-3}$ in November), and in 2000 there was an increasing trend (from 25 to 65 μg $dm^{-3}$). However, at locations 2 and 3 the highest copper concentration in microlayers always occurred in the summer period, reaching its maximum in August. Particularly high concentrations were observed for film at location 3, about the highest concentration of chlorine ions (mean 730 mg $dm^{-3}$), in which the highest was observed in 1999 (about 211 μg $dm^{-3}$). In August 1999 and 2000 valuables of enrichment factors for this microlayer adopted the highest values (about 10). Figures 2A–C showed clearly the influence of land and marine factors on copper concentrations in investigated surface microlayer waters. Both film (Fig. 2D) and Garrett film (Fig. 2E) in terms of copper concentrations in the summer season showed the lowest values at location 1, in the area of the Łupawa River gate region, so in this area the lowest $Cl^-$ ion concentration was found (mean 83 mg $dm^{-3}$). However, the highest concentration of this metal was observed at location 3, in the sea water gate zone, thus the highest concentration of chlorine ions (mean 730 mg $dm^{-3}$). Results for sea water changed conditions in surface microlayers, were conducive to accumulation of considerable copper amounts. Probably the reason for such a situation is connected with neuston marine organisms, which have a bigger ability to accumulate heavy metals.

Figure 2F shows changes of copper concentration in subsurface water at individual locations. In the investigated period this concentration in this water layer changed in the smallest range from 1.6 to 25.9 μg $dm^{-3}$. Regularity of these changes in the course of a year may not be identified. Also significant differences were observed between locations. Only in the case of location 1 the highest concentration of this metal in spring and autumn may be explained by the biggest water quantity being brought to the lake in that time by the Łupawa River. It may be stated that in most cases the lowest copper concentrations in subsurface water were observed in the summer period (locations 1 and 2) or towards the end of spring (location 3). A probable reason for this was the utilization of copper by water organisms as an element essential to vegetation and it may also result from its intensified migration to surface microlayers of water.

Changes of copper concentration in Garrett film in this lake were similar to those in film. They are characterized by two concentration maximums, one of them occurring in

spring and the other in summer. Often the concentration of this metal in film is much more higher than in Garrett film. Similarly to subsurface water, minimal copper concentrations in both microlayers were observed in May and June, and sometimes in April.

Lead contents in surface microlayers of Gardno waters changed showing two maximums – in spring and summer (Figs. 3A–F). In the analyzed summer period the maximum was found in August. In June the lowest lead concentration was observed in May 2000 (Fig. 3D). We need to stress here much higher concentrations of this metal in microlayers in spring 2000, especially at locations 1 and 3. At location 1 the spring maximum in film was five times bigger than in the summer, while at position 3 it was two times bigger.

Similarly to copper, also lead concentration in microlayers depended on the location where samples were taken. In the summer period both in film and Garrett film the lowest concentration of this metal was observed at location 1, while the highest at location 3 (Fig. 3D–E). Probably in this case the reason was also the difference in properties of sea water and those of fresh water.

Seasonal changes of lead concentration in individual surface water layers are shown in Figures 3D–F. They take a similar course in lake grounds and are characterized by two maximum concentrations, occurring in spring and summer.

Figure 4 shows seasonal changes in zinc contents in surface microlayers and subsurface water in Lake Gardno. In all cases a declining trend was observed for the concentration of this metal in the course of a year. Dynamics of changes were similar of for copper and lead. They were characterized by two maximums – in spring and summer. In the case of zinc, the spring maximum was much bigger than the summer one. It is particularly visible in Garrett film in 1999, when zinc concentration in summer at individual locations was almost two times bigger than in the summer. In the case of Garrett film the same relationship may be observed as for lead and copper, the highest concentration of zinc in summer being recorded at location 3, while the lowest at location 1 (Fig. 4E). Such relationships were not observed in film (Fig. 4D). However, this relationship was much more evident in spring both in film and in Garrett film. Zinc content in spring in microlayers from location 3 was much higher than at other locations. The biggest difference was noticed in spring 2000 (Fig. 4D), when zinc concentration in film from location 3 in April amounted to about 145 μg $dm^{-3}$, from location to 2–117μg $dm^{-3}$ and from location 1–109 μg $dm^{-3}$. Thus, the influence of sea water on zinc concentration in microlayers was marked more clearly in spring than in summer.

The levels of zinc concentration in subsurface water are much bigger than those of copper and lead. The concentration ranged from 4.0 to 89.6 μg $dm^{-3}$ (Fig. 4). In this case two maximums – spring and summer, were also observed. The course of these changes was similar to the seasonal changes in zinc concentration in subsurface water and in surface water at locations 1 and 2, which suggests that the main source of this metal in microlayers in a major part of the lake is its content in subsurface water.

## DISCUSSION

Heavy metals accumulate generally in surface microlayers, yielding correlation coefficients from 1 to 100 [46]. Such a big discrepancy in enrichment factors obtained results from the type of analyzed metals, the adopted sampling technique and manageability of a given water region [17, 28, 46]. To date analyses of heavy metals contained in surface

lake water microlayers have been rare, so comparable material concerns mainly sea water. When comparing empirical values discussed in this study, those presented in Table 4, reported by Cross *et al.* [3], Hardy *et al.* [16], Hong and Lin [21], Migon and Nicolas [29], we may observe that mean enrichment factors recorded in sea and ocean waters are much higher than those for fresh water.

Table 4. Enrichment factors of surface microlayer for selected heavy metals recorded in salt water according to literature date

| Region | Year | Metal | EF | References |
|---|---|---|---|---|
| Puget Sound, Washington | 1982–1983 | Cu | 10–16 | 16 |
| | | Pb | 26–65 | |
| | | Zn | 14–29 | |
| Chesapeake Bay | 1986 | Cu | 8 | 17 |
| | | Pb | 43 | |
| | | Zn | 2,6 | |
| Xiamen Bay, China | 1987 | Cu | 1–2.61 | 21 |
| Mediterannean Sea | 1994 | Pb | 19 | 29 |

The accumulation of heavy metals in surface water microlayers results from physical properties of this specific subecosystem and from collection of different chemicals and microorganisms conducive to accumulation. Physical properties include the force of surface tension, adhesive force, convection of matter from deep waters and the bottom, passive transport with air bubbles. An additional type of heavy metal accumulation in the microlayer is their binding by microsuspension (colloidal system), e.g. high quality of protein, carbon hydrate and other substances, which are also collected in this subecosystem. Also neuston organisms can produce organic components, which can yield the chelation effect for heavy metals dissolved in the surface microlayer [46]. Hunter and Liss [22] and Lion and Lacke [24] suggested that dissolving metals can be included in the complexes of ions metals with organic ligands and in this form can exist in the surface microlayer. On the other hand, a fundamental source of enrichment of the microlayer with elements is rainfall, which consists of dusts and aerosols getting into microlayers thanks to gravity. In the presented studies the enrichment of surface microlayers in waters of Lake Gardno by rainfall plays the biggest role in the case of copper and lead, where obtained correlation coefficients were low (Tab. 3). In the case of lead, except for the area with a considerable effect of sea water (location 3), its concentration in deep waters is also essential. Positive correlation coefficients for zinc, recorded in this study, showed that the analyzed surface microlayers were fed with this metal by subsurface water.

In the presented results we can notice that Zn, Pb, Cu are accumulated in major quantities in the thinner microlayer (film) at each of the discussed locations (Figs. 2–4). Also Falkowska [11] showed that heavy metals and other inorganic matter are found in higher concentrations in the microlayer partly due to a closer exchange zone, i.e. the air–water interphase. This effect is caused by better collineation of inorganic matter to remain in the thinner surface layer. In the thick layer which was sampled using the Garret net we can find a higher accumulation of phytoneuston and heterotrofic bacteria than in deep waters, which facilitates the binding of heavy metals in organic combinations with live neuston cells [22, 24, 46].

The concentrations of analyzed heavy metals in terms of their contents can be ordered as follows: Zn > Pb > Cu. This statement is true both in the situation observed in subsurface water and in the analyzed surface microlayers and is consistent with the findings for sea ecosystems reported by Hardy *et al.* [17] and Lion [25].

Observed seasonal dynamics of heavy metal concentrations change in subsurface water. The correlation observed in the case of Pb and Zn indicated the influence of subsurface water on the accumulation of these metals in surface microlayers. In the case of Cu, in surface microlayers very high concentrations in September and much lower in early spring can be observed. In that time in subsurface water decreasing concentrations of these elements may usually be observed (Fig. 2). It correlates with changes observed in microlayers in the spring period (March–May) and July–August for Pb and Zn (Figs. 3 and 4). In the case of Zn and Pb one can notice a drop of concentrations in all investigated layers during May–June and after the autumn period (Figs. 3B and 4B). Scarce scientific papers analyzed seasonal changes of assayed values in surface microlayers, especially for heavy metals. In land ecosystems Hlibricht-Illkowska [20] analyzed biogenic substances in surface microlayers. Similar study was conducted in the Bay of Gdansk by Falkowska [11], who came to a conclusion that seasonal changes showed a significant temporary fluctuation, but we may find their maximum and minimum concentrations of chemical matter in close periods. When investigating such dynamism we should take into consideration the fact that the ecological situation in lakes changes each year. In the same month but different years different meteorological conditions are found, which significantly affects live organisms, thus affecting also the circulation of matter, including heavy metals analyzed in the presented study. Changes of Zn and Cu concentrations, as elements in specific quantities essential for life, are connected with the migration of neuston microorganisms [31]. Also Pb, although harmful for neuston organisms, will be included in cell structures [24, 46].

Seasonal dynamics in discussed heavy metal contents in the surface microlayers is a physical property resultant of this specifical subecosystem, because they are essential for substance storage in this ecoton. On the other hand, the property of accumulation frequent for microneuston organisms, which include heavy metals in their organism and connected with permanent migration within daily and seasonal periods, will influence changes in their concentrations in subsurface water. The third element is precipitation, which influences this microlayer bringing in its load. In this case the dynamic of observing changes in the surface microlayer is not always consistent with changes observed in subsurface water.

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Received: February 9, 2010; accepted: December 7, 2010.

METALE CIĘŻKIE W MIKROWARSTWIE POWIERZCHNIOWEJ WODY JEZIORA GARDNO

W prezentowanej pracy są przedstawione wyniki analizy stężenia metali ciężkich w powierzchniowej i podpowierzchniowej warstwie wody estuariowego jeziora Gardno. Uzyskane wyniki wskazują, że są znaczące różnice między stężeniem ołowiu, miedzi i cynku w mikrowarstwie powierzchniowej wody a ich stężeniem w warstwie wody podpowierzchniowej. Zaobserwowano, że mikrowarstwa powierzchniowa ma zdolność do akumulowania znacznie wyższych ilości tych metali niż woda podpowierzchniowa. Analiza zmian sezonowych fluktuacji koncentracji metali ciężkich w mikrowarstwie powierzchniowej i podpowierzchniowej wody wskazuje na periodyczny charakter tych zmian.

ARCHIVES OF ENVIRONMENTAL PROTECTION

vol. 37 no. 1 pp. 89 - 98 2011

PL ISSN 0324-8461

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# MOLECULAR ANALYSIS OF MICROORGANISMS RESPONSIBLE FOR THE FIRST PHASE OF NITRIFICATION IN AN ANOXIC ENVIRONMENT

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**Keywords:** 16S rRNA gene, *amoA* gene; *β*-proteobacterial ammonia oxidizers; DGGE; nested-PCR, *Nitrosomonas*-like bacteria.

**Abstract:** Ammonia-oxidizing bacteria communities were evaluated in a completely mixed, laboratory scale membrane reactor (MBR) working under anoxic conditions for 5 months. The microorganisms in activated sludge were fed a synthetic medium containing 66–150 mg $NH_4^+$-N/l. The age of the activated sludge in MBR was 50 days and the hydraulic retention time (HRT) was 3.3 days. The estimation of the diversity and complexity of the AOB community together with the identification of the dominant bacteria in the activated sludge under anoxic conditions were performed using denaturing gradient gel electrophoresis (DGGE) and DNA sequencing. Molecular analysis of the microbial community carried out with two microbial molecular markers, 16S rRNA gene and *amoA* gene, suggested that nitrification was led by a *Nitrosomonas*-like species. In the biocenosis of the investigated bioreactor, oxygen was the crucial selective parameter. The results obtained in this work showed that a*moA* gene research is more suitable to study the stability and effectiveness of ammonia oxidation. This information emphasizes the necessity of the usage of molecular markers based on functional genes instead of ribosomal ones in order to present the actual state of the process performed in bioreactors. It was also stated that *Nitrosomonas* - like bacteria are able to perform nitritation even in anoxic environment, that is probably the reason why these bacteria are the most common AOB in different bioreactors.

## INTRODUCTION

Nitrogen concentrations over the prescribed levels in wastewater treatment effluent can cause a serious problem to receiving watercourses leading to eutrophication and being toxic to water environment [3]. It is widely known that the removal of nitrogen from wastewater is achieved mainly through a combination of nitrification and denitrification. From the aspect of the efficiency of wastewater treatment, the crucial process responsible for ammonia oxidation is nitrification. This process is led by two phylogenetically distinct groups of microorganisms: aerobic chemoautotrophic ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB).

Nitrification is an oxygen-demanding process and low oxygen concentration is the major environmental factor controlling it. It is considered that oxygen levels below 1.5 mg/l should drastically decrease the effectiveness of nitrification [22], but it was proved that wastewater treatment processes led by simultaneous nitrification/denitrification can achieve up to 80% of the total nitrification under minimal aeration conditions where no detectable oxygen concentration was observed [1].

The most abundant bacteria expected to be found in activated sludge under anoxic condition belong to the *Nitrosomonas* sp. [9]. *Nitrosomonas* sp. is known to be obligatory chemolithotrophic, ammonia-oxidizing bacteria, which is able to lead nitrification and denitrification simultaneously under limited oxygen conditions. Under anoxic conditions, nitrite is an electron acceptor for ammonia oxidation without any observed cell growth [20].

Recently molecular analyses of bacteria have provided information about bacterial physiology and ecology, which may be helpful in the improvement of wastewater technology. One of the most useful methods for bacterial diversity and community composition estimation is denaturing gradient gel electrophoresis (DGGE) [25]. Two molecular markers are used in the DGGE analysis of ammonia-oxidizing bacteria: a 16S rRNA gene and an a*moA* gene. The 16S rRNA gene is known to be a good phylogenetic marker as well as a bacterial identification molecule [10]. The a*moA* gene codes the active site subunit of the ammonia monooxygenase – enzyme responsible for ammonia oxidation, and has recently been used as an alternative AOB identification and phylogeny marker [17]. Although the use of the *amoA* gene as a molecular marker is increasing, in comparison with 16S RNA gene, this gene sequence's length and database are still not sufficient enough for AOB analysis.

The main aim of this study was a molecular analysis of *β*-proteobacterial ammonia oxidizers derived from activated sludge in order to estimate the diversity and complexity of an AOB community under anoxic conditions. Furthermore, an attempt to identify the dominant bacteria was undertaken.

## MATERIALS AND METHODS

### *Reactor details and analytical procedure*

Activated sludge from a completely mixed, laboratory-scale membrane bioreactor, with a volume of 36 l, was used in this study. The reactor was operated anoxically (oxygen level below 0.5%) with nitrification and fed with a synthetic medium containing 66–150 mg $NH_4^+$-N/l. The medium consisted of $NH_4Cl$, $NaNO_2$, $KH_2PO_4$ and $NaHCO_3$ and it was enriched with landfill leachete from Gliwice, Poland, at the range of 10–50%. The membrane (A4 Size Mat Sheet Membrane, Kubota System) with a pore size of 0.4 μm was submerged in the reactor. Activated sludge from a municipal wastewater treatment plant performing nutrient removal was used for seeding. The age of the sludge was 50 days and the hydraulic retention time (HRT) was 3.3 days in the experimental bioreactor. The level of mixed liquor suspended solids was changing at the range of 11.3–32.2 g/l during the experiment. Nitrogen compound concentrations analysis was performed as described previously [24].

***Activated sludge samples preparation and DNA extraction***

The experiment was carried out for 5 months. Activated sludge samples (volume of 10 ml) were collected at 4-week intervals from the membrane bioreactor and numbered 1 to 5 respectively. Samples were pelleted by centrifugation (5 000 × g, 10 min, 4°C) and stored at -20°C.

Total genomic DNA was extracted from 0.2 g of the activated sludge samples using a Fast DNA Spin Kit for Soil (MP Biomedicals LLC) according to the manufacturer's instructions. The amount of DNA was measured spectrophotometrically using a Biophotometer (Eppendorf) and stored at -20°C until PCR amplification.

***PCR amplification conditions***

The nested-PCR procedure was used in the study for 16S rRNA gene amplification. For this purpose two sets of primers were used (Table 1).

The first round of PCR was performed with CTO primers, enabling partial PCR amplification of 16S rRNA bacterial gene belonging to ammonia-oxidizing *β-Proteobacteria*. The PCR was carried out in a total volume of 30 µl and the reaction mixture. The PCR product was used as a template in the second cycle of nested-PCR as described previously [7, 24].

Table 1. Primers used in the study

| Target gene | Primer | Sequence | Reference |
|---|---|---|---|
| *β*-proteobacterial AOB 16S rRNA | CTO189f – ABC – GC | 5' CCG CCG CGC GGC GGG CGG GGC GGG GGC ACG GGG GGA CMA AAG YAG GGG ATC G 3'* | 7 |
| | CTO 654r | 5' CTA GCY TTG TAG TTT CAA ACG C 3'* | 7 |
| Bacterial 16S rRNA | 338F-GC | 5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG 3' | 12 |
| | 518r | 5' ATT ACC GCG GCT GCT GG 3' | 12 |
| *amoA* | Amo1-F - GC | 5' CGC CGC GCG GCG GGC GGG GCG GGG GCG GGG TTT CTA CTG GTG GT 3' | 18, 21, 24 |
| | Amo2R-TC | 5' CCC CTC TGC AAA GCC TTC TTC 3' | 18, 21, 24 |

*Degeneracies shown in bold; Y = T/C, M = A/C

Subunit A of ammonia monooxygenase gene (*amoA*) amplification was performed using primers Amo1-F- GC and Amo2R-TC, as described previously [24]. The PCR was carried out in a total volume of 30 µl and the amplification was performed using an Eppendorf thermal cycler. The PCR products were evaluated in agarose gel (0.8% w/v agarose, Promega) in 1×TBE buffer (Tris, boric acid, EDTA, pH = 8.3), stained with ethidium bromide (0.5 µg/l) in MiliQ water and photographed under UV light.

***Denaturing gradient gel electrophoresis conditions and DNA bands extraction***

DGGE was performed using the Dcode Universal Mutation Detection System (BioRad). Polyacrylamide gel (8% w/v for 16S rRNA gene, 6% w/v for a*moA* gene, 37:1 acrylamide-bisacrylamide, Fluka) with a gradient of 30–60% denaturant was prepared according to the manufacturer's guidelines.

The gel was run for 15 h at 55 V in a 1×TAE buffer (Tris, acetic acid, EDTA, pH = 8.0) at a constant temperature of 60°C. The gel was stained with SYBR Gold (1:10 000, Invitrogen) in MiliQ water for 30 min and destained in MiliQ water for 40 min, then visualized under UV light and photographed using a Kodak 1D.

***PCR products purification and sequencing***
To identify the most abundant AOB in the activated sludge samples dominant bands from DGGE fingerprint were cut off, extracted from the gel and purified using a GenElute PCR Clean-up Kit (Sigma) according to the manufacturer's instructions and sequenced. DNA sequences of the bacterial 16S rRNA gene obtained in the procedure were identified by comparison with the NCBI GenBank (National Center for Biotechnology Information) using BLAST (Basic Local Alignment Search Tool).

***Nucleotide sequence accession numbers***
The GenBank accession numbers for the partial sequences determined in this study are: FJ907322-FJ907326 (16S rRNA gene sequences) and FJ907200-FJ907202 (*amoA* gene sequences). Table 2 presents sequence characteristics of DNA derived from electrophoresis gel in this study and their similarities to the closest relatives.

Table 2. Sequence characteristics of DNA derived from electrophoresis gel and their similarities to the closest relatives

| Band | Gene | Length (bp) | Accession no. | Closest relatives (accession no.) | coverage/ identity (%) |
|---|---|---|---|---|---|
| OZ1 | *amoA* | 432 | FJ907200 | Uncultured bacterium clone *amoA* SBR JJY 63 (FJ577881) | 96/99 |
| OZ2 | *amoA* | 432 | FJ907201 | Uncultured bacterium clone *amoA* SBR JJY 63 (FJ577881) | 96/99 |
| OZ6 | *amoA* | 432 | FJ907202 | Uncultured bacterium clone *amoA* SBR JJY 63 (FJ577881) | 96/99 |
| OZR1 | 16S rRNA | 195 | FJ907322 | Uncultured *Nitrosomonas* sp. isolate DGGE gel band B1 (FJ654650) | 100/100 |
| OZR2 | 16S rRNA | 195 | FJ907323 | Uncultured *Nitrosomonas* sp. isolate DGGE gel band B1 (FJ654650) | 100/100 |
| OZR3 | 16S rRNA | 195 | FJ907324 | Uncultured *Nitrosomonas* sp. clone LEQUIA R0CTO47 (FM997806) | 100/98 |
| OZR4 | 16S rRNA | 195 | FJ907325 | Uncultured *Nitrosomonas* sp. isolate DGGE gel band B1 (FJ654650) | 100/98 |
| OZR5 | 16S rRNA | 195 | FJ907326 | Uncultured *Nitrosomonas* sp. clone LEQUIA R0CTO47 (FM997806) | 100/100 |

***Numerical analysis of the DGGE fingerprints***
The numerical analysis of DGGE fingerprints was performed using a Kodak 1D. Bacterial biodiversity was estimated on the basis of densitometric measurements and the Shannon diversity index for the samples was calculated [13].

## RESULTS

In order to promote the AOB responsible for nitrification in anoxic condition, the oxygen level was kept below 0.5%. The activated sludge was fed with synthetic medium containing 66–150 mg $NH_4^+$-N/l. The nitrogen species concentrations are presented in Fig 1. At the beginning of the experiment the effectiveness of ammonia nitrogen oxidation was at the level of 80%. From the 12$^{th}$ week of the process the effectiveness started to decrease and reached 10% at the end of the experiment.

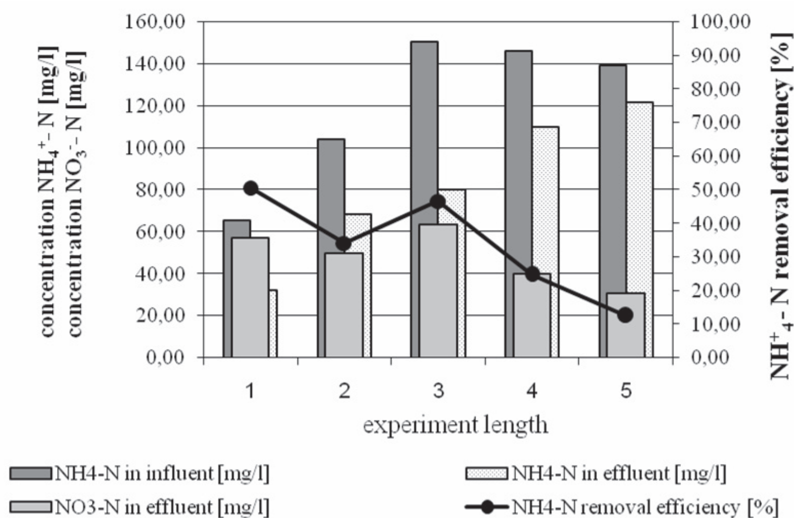


Fig. 1. Nitrogen compounds concentration and ammonia oxidation effectiveness measured for MBR bioreactor during the experiment; $NO_2^-$ concentrations in the effluent not shown due to a very low values obtained

To evaluate the effect of the treatment on the diversity of ammonia oxidizing bacteria, the total DNA obtained at each sampling time (at 4 week intervals) during the 5 months of the experiment was analyzed by PCR-DGGE. The applied approach allowed us to obtain the clear picture of bacterial structure using both primer pairs (Fig. 2). The fingerprint obtained by amplification of ammonia monooxygenase gene fragment (Fig. 2b) possessed more bands but samples were less varied than fingerprint created by resolution of 16S rRNA gene fragment (Fig. 2a).

The dominant bacteria DNA bands were extracted from the gel and underwent purification and sequencing. The analysis revealed that among all obtained sequences eight were unique and each of them corresponds to expected gene. Four sequences of 16S rRNA gene (195 bp) showed the highest similarity to DNA sequences of various uncultured *Nitrosomonas* species. The sequenced DNA of *amoA* gene fragment bands (495 bp) was similar to DNA sequences of undefined and uncultured microorganisms possessing ability of ammonia oxidation (Tab. 2).

In order to compare the diversity of the samples, the fingerprints obtained from DGGE separation of partial 16S rRNA gene fragments of ammonia oxidizers were ana-

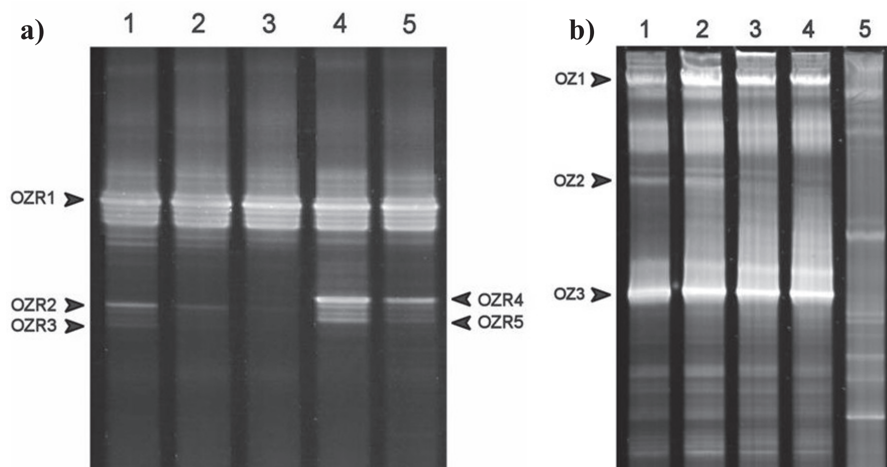


Fig. 2. DGGE gel with MBR activated sludge samples under anoxic conditions photographed in UV light for (a) 16S rRNA gene PCR products (b) *amoA* gene PCR product. Characteristics of species corresponding to labeled bands are given in Table 2

lyzed. Bacterial diversity was estimated on the basis of fingerprint optical density measurements and the Shannon diversity index for the samples was calculated. Figure 3 shows the changes of biodiversity throughout the process, the estimated values of the Shannon index obtained for samples 1 to 5 were: 2.04, 1.74, 1.84, 2.24, and 2.03 respectively.

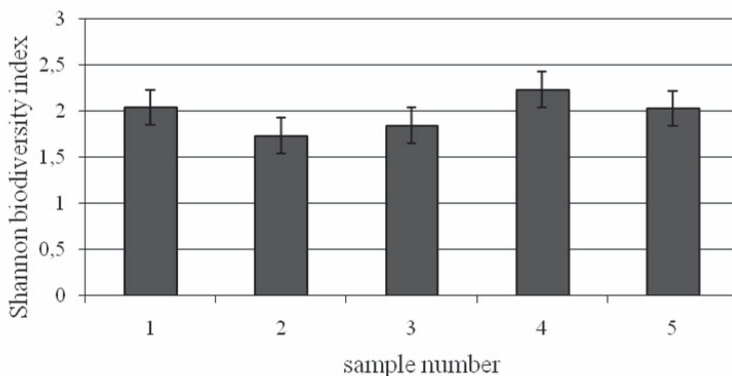


Fig. 3. Biodiversity of *β*-proteobacterial ammonia-oxidizing bacteria obtained in the study

## DISCUSSION

From the earliest stage of the research into ammonia oxidizers, chemoautotrophic nitrifying bacteria were considered to be strictly aerobic and the oxidation of ammonia led by them was thought to require dissolved oxygen [15]. Nowadays it has been demonstrated that the complete anoxic conversion of ammonia by *Nitrosomonas europaea* and *Nitroso-*

*monas eutropha* with nitrite as electron acceptor [19] is possible. Autotrophic AOB have also been found in temporarily and permanently anoxic rhizosphere habitats [5]. In the bioreactor's biocenosis investigated in this research, oxygen was the crucial selective parameter. It seems probable that oxygen stress limited the biocenosis diversity only in relation to the representatives of the genus *Nitrosomonas*. These bacteria are considered to be not only the most abundant, but also the most effective ammonia oxidizers. In this experiment the effectiveness of ammonia oxidation was low, ca. 30%. This could suggest that an uncultured group of *Nitrosomonas* sp. in the anoxic environment identified in this study was not able to lead the nitrification process at the level equal to that in aerobic conditions. Diab *et al.* [4] suggested that nitryfiers can survive oxygen limitation by changing their metabolism to a very low rate, comparable to a state of resting cells. It is highly likely that AOB cells under anoxic conditions change their physiology in such a way, however, this statement needs further research.

It cannot be excluded that other than *Nitrosomonas* sp. ammonia-oxidizing bacteria can be responsible for ammonia oxidation in anoxic conditions, but such situation is difficult to state due to the fact of the very specific primers usage. The relatively short length of the gene sequences identified in the study could also be the reason for the misidentification of bacteria. The method of bacteria identification based on gene sequences comparison may be also deficient. Yu *et al.* [23] noted previously that species affinity in the case of gene sequence based identification in the gene databases is stated when the similarity of the sequences is over 95%. In the case of bacteria, this level of differences can be significant enough for its misidentification.

The clone 16S rRNA gene – OZR1 was the only dominant present in the biocenosis during the entire length of the experiment (Fig. 2a). Clones OZR2 together with OZR4 and OZR3 together with OZR5 were identified as the most similar to the same clone sequence. Interestingly, clones OZR2 and OZR3 disappeared gradually between the first and the twelfth week of the experiment (Fig. 1 and Fig. 2a, 1$^{st}$ to 3$^{rd}$ sampling time). These clones reappeared (as OZR4 and OZR5) in the sixteenth week of the experiment (Fig. 1 and Fig. 2a, sampling time 4$^{th}$). This situation could be linked to the ammonia concentration, rising gradually to 150 mg/l (to) in the twelfth week (3$^{rd}$ sampling time), and stabilized at the level of 140-150 mg/l till the end of the experiment. It is worth noting that the effectiveness of the process decreased drastically from the twelfth week (3$^{rd}$ sampling time). Such a situation could be caused by the rebuilding of the biomass.

The changeability of the *amoA* gene sequences (Fig. 2b) obtained in the study appears to be more constant when compared with the 16S rRNA gene sequences (Fig. 2a). Despite the fact that each of the 16S rRNA gene sequences identified in the study belonged to uncultured *Nitrosomonas* sp., the a*moA* gene sequences belonged to uncultured and unidentified bacteria. This situation emphasizes the fact that anoxic nitrification can also be led by bacteria not belonging to *β-Proteobacteria* ammonia oxidizers, such as *Planctomycetes,* responsible for the Anammox process or other subclasses of *Proteobacteria*. The comparison of the DNA bands changeability in DGGE gels for the identification of genes (16S rRNA) and functional genes (a*moA* gene) showed that new sequences of *amoA* gene appeared 4 weeks after the beginning of the decline of the process's effectiveness. This could suggest that the bacteria belonging to *Nitrosomonas* sp. identified in the system were not responsible for ammonia oxidation. Such a situation stresses the fact that ammonia oxidation research should be concentrated on studies of functional genes

rather than constitutive ones, because the former are more important to the effectiveness of the process.

It has previously been stated that *Nitrosospira* sp. representatives co-exist in anoxic marine sediments with *Nitrosomonas* sp. [11] and other natural environments, especially in soil [2], sediments [8] and freshwater [6]. *Nitrosospira* sp. is also known to be present in nitrifying activated sludge, but is not commonly reported as relevant AOB in bioreactors [16]. The results obtained by Park *et al.* [14] suggest that a low dissolved oxygen level would be more favorable for *Nitrosospira* sp. In the studies presented no representatives of this genus were present in the system. These results underline the fact that the oxygen level is not the only limiting factor for AOB growth in a lab-scale anoxic bioreactor.

The biodiversity of *β*-proteobacterial ammonia oxidizers was relatively steady during the entire length of the experiment. The changes in the diversity range of 0.5 are not relevant to the stability of the process. It is necessary to mention that the biodiversity of *β*-proteobacterial AOB is *de facto* the diversity of *Nitrosomonas*-like clones.

*Acknowledgments*

*This research was supported by the Polish Ministry of Science and Higher Education, grant no: 2 P04G 086 30.*

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Received: November 15, 2010, accepted: January 15, 2011.

## ANALIZA MOLEKULARNA MIKROORGANIZMÓW ODPOWIEDZIALNYCH ZA PROWADZENIE PIERWSZEJ FAZY NITRYFIKACJI W ŚRODOWISKU ANOKSYCZNYM

W eksperymencie badano grupę bakterii utleniających amoniak w bioreaktorze membranowym całkowitego wymieszania (MBR), pracującym w warunkach anoksycznych przez 5 miesięcy. Osad czynny zasilano pożywką syntetyczną, zawierającą 66–150 mg N-$NH_4^+$/l. Wiek osadu wynosił 50 dni, a hydrauliczny czas zatrzymania – 3,3 dnia. Oszacowanie różnorodności i złożoności zbiorowiska bakterii utleniających amoniak oraz identyfikacja mikroorganizmów dominujących w badanym osadzie czynnym w warunkach anoksycznych została przeprowadzona metodą elektroforezy w gradiencie denaturacji (DGGE) i sekwencjonowania DNA. Analiza molekularna biocenozy, przeprowadzona z użyciem dwóch markerów molekularnych: genu kodującego 16S rRNA i genu kodującego monooksygenazę amonową (*amoA),* wykazała, że proces nitritacji był prowadzony przez gatunki bakterii z rodzaju *Nitrosomonas*. W biocenozie badanego bioreaktora stężenie tlenu było głównym parametrem selekcyjnym dla nitritatorów. W badaniach wykazano, że markerem molekularnym sprawdzającym się lepiej w monitoringu efektywności procesu nitryfikacji pierwszej fazy jest gen a*moA*. Te dane podkreślają, że w badaniach efektywności prowadzonego procesu zachodzącego w bioreaktorach, informacje uzyskiwane

z markerów funkcjonalnych mają większą wartość niż uzyskane z markerów rybosomalnych. Stwierdzono również, że bakterie *Nitrosomonas* sp. są zdolne do prowadzenia nitritacji w warunkach anoksycznych i prawdopodobnie z tego powodu są najczęściej spotykaną bakterią utleniającą amoniak.