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LABORATORY STUDIES ON THE EFFECT OF HEAVY METALS ON THE SURVIVAL AND RESPIRATORY ACTIVITY OF ESTUARINE NEUSTONIC AND PLANKTONIC BACTERIA

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Abstract

Concentration of heavy metal ions (Zn, Cu, Cd, Pb) and their effect on the survival and respiratory activity of neustonic and planktonic bacteria were studied in film layer, surface microlayer and in subsurface water of estuarine Lake Gardno. Zinc, copper and lead reached their highest concentrations in surface microlayers. Cadmium, in general, was distributed homogeneously in all three studied water layers. Laboratory experiments demonstrated the highest toxicity of cadmium and lead for bacteria inhabiting Lake Gardno. Even low concentrations of those metals in culture media resulted in a rapid decrease in the survival of bacteria. Populations of bacterioneuston and bacterioplankton showed different responses to heavy metals. Neustonic bacteria showed a higher level of tolerance to various concentrations of heavy metals than did planktonic bacteria. The strongest inhibitory effect on the respiration of neustonic and planktonic bacteria was brought about by cadmium and copper.

Key words: heavy metals, bacterioneuston, bacterioplankton, survival, respiratory activity

INTRODUCTION

With the growth of industrialization, ecological and ecotoxicological problems resulting from the release of heavy metals into water ecosystems are increasing. Because microorganisms are involved in many basic ecological processes such as organic matter degradation, nutrient cycling and detoxification, the effect of heavy metals on aquatic bacteria is of great interest (Martinez et al. 1991, White et al. 1997, Ostrovskii et al. 2000). In water ecosystems, heterotrophic bacteria which are resistant to the toxic activity of heavy metals may accumulate them and transfer via a trophic chain to higher organisms by biomagnification (Fabiano et al. 1994, Niewolak et al. 1996).

High concentrations of heavy metals in water basins can affect bacterial communities. Some of the specific molecular consequences of heavy metal toxicity that have been demonstrated in bacterial systems include the production of single-strand breaks in DNA and misincorporation by DNA polymerase (Burke, Pfister 1986). However, many aquatic bacteria have developed various very efficient mechanisms for tolerating the ions of heavy metals (Barkay et al. 1992, Ron et al. 1992). Those mechanisms include transformations of metals to less toxic forms through indirect intercellular complexation, decreased accumulation, or precipitation (Harwood-Sears, Gordon 1990, Schreiber et al. 1990). Microorganisms can also reduce the toxicity of heavy metals by transforming them in the processes of chelation, oxidation, methylation and dealkylation (White et al. 1997). Some water bacteria produce several classes of specific extracellular proteins that can bind metals and allow bacterial growth to resume after initial metal-induced cessation (Rudd et al. 1983). Should this be common among the heterotrophic bacteria, it may indicate a major role of those organisms in controlling metal toxicity in water basins (Gordon et al. 1994, Chen et al. 1995).

In aquatic ecosystems, resistance to heavy metals in bacteria is often transmitted by plasmid-encoded mechanisms (Hermansson et al. 1987, Jeanthon et al.1991). There are four classic mechanisms of resistance to heavy metals encoded by plasmids: (i) inactivation (ii) impermeability, (iii) bypass, and (iv) altered target site (Foster, 1983). The plasmid-bearing strains are routinely isolated from bacteria present in water basins at frequencies from 10% to 60%. The size of naturally occurring plasmids ranges from 50 kb to 500 kb and allows them to encode self-conjugative mechanisms and metabolic functions which may have an ecological role (Lopez-Amoros et al. 1997).

The effect of heavy metals on microbial survival and activity in aquatic systems has been extensively studies in recent years. Despite numerous studies on the development of tolerance to heavy metals in planktonic (Nair et al. 1992, Lopez-Amoros et al. 1997) and benthic (Fabiano et al. 1994, Niewolak et al. 1996, Cursino et al. 1999, Reyes et al. 1999) bacterial populations, surprisingly little information (Donderski et al. 1997, 1999, Mudryk et al. 2000) is available on the resistance to heavy metals in neustonic bacteria. Hence, the object of the present study is to assess the effect of some heavy metal ions on the survival and respiratory activity of neustonic and planktonic heterotrophic bacteria inhabiting estauarine Lake Gardno.

MATERIALS AND METHODS

The study was carried out in estuarine Lake Gardno (Fig.1) situated in the World Biosphere Reserve - Słowiński National Park (Northern Poland). The lake is very shallow (1.3 m average depth) but it covers a large area (2.500 ha). Its small depth and large area, together with lack of practically any wind shields, makes possible full mixing of lake waters, both vertical and horizontal. Therefore, the lake can be regarded as a polymictic basin where no thermal or oxygen stratification is observed. Emerging macroflora covers 4% of the surface of the lake, forming an offshore belt 20-200 m wide, which is inhabited by many bird species. The main species of macrophytes are: *Typha angustifolia, Phragmites australis, Scirpus lacustris* and *Schoenoplectus lacustris*. Lake Gardno is characterised by conditions that are intermediate between marine and inland environment.

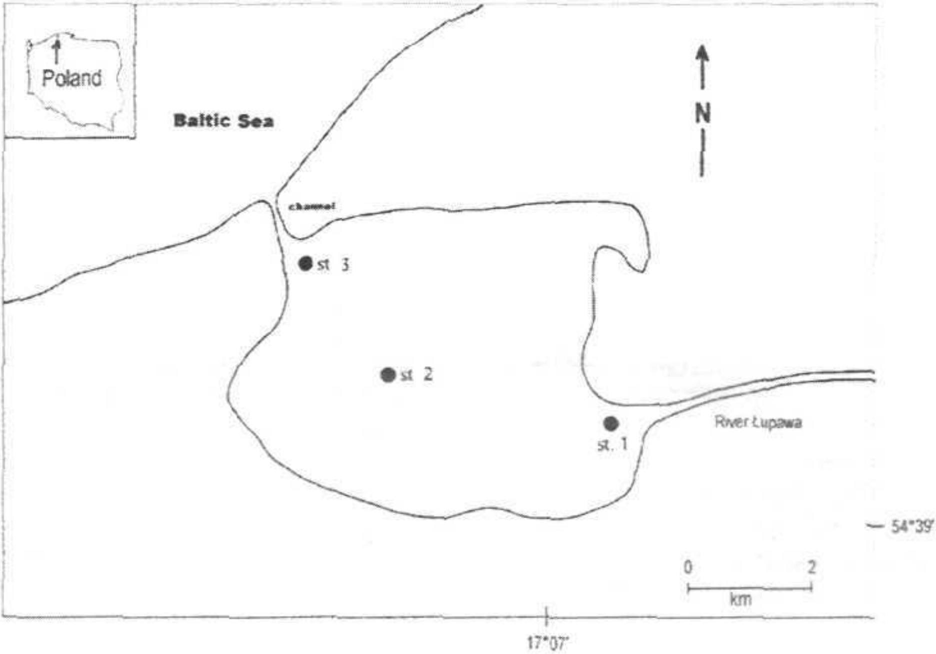

Fig. 1. Sampling stations location in Lake Gardno

On the one hand, it is supplied by waters of the River Łupawa, on the other hand, via a 1.3-km channel, it is connected with the Baltic Sea, which results in seawater abundantly penetrating into the lake. Therefore, the water of the lake, or at least its parts, acquires the quality of seawater, resulting in 2-5‰ salinity. Consistently with the Venetian system, Lake Gardno can be classified as a mixo-oligohaline type (0.5-5.0 ‰) (Dethier 1992).

Water samples were taken in June 2000 from three stations (Fig. 1) representing various environmental conditions: a freshwater zone (near the River Łupawa inflow) (site 1), a seawater zone (site 3), and a mixed zone in the mid-lake (site 2). The samples were taken from three water layers. The film layer (FL) samples (thickness of 90 ± 17μm) were taken with a 30 x 30 cm glass plate (Harvey, Burzell, 1974), the surface layer (SL) samples (thickness of 242 ± 40 μm) were collected with a 40 x 50 cm Garrett polyethylene net (24 mesh net of 2.54 cm length) (Garrett, 1965). Glass plate and polyethylene net were rinsed with ethyl alcohol and distilled sterile water prior to sampling. Water from subsurface layer (SUB) was taken at the depth of about 10 – 15 cm. All water samples were collected into sterile glass bottles and stored in an ice-box, where the temperature did not exceed 7^0C until they were used for analysis. The time between sample collection and the analyses usually did not exceed 6 - 8 h.

The concentration of heavy metals in the studied water layers was determined according to Hać et al. (1998). Immediately after collection, water samples were fixed with nitric acid until pH 2 was reached. In the laboratory, the samples were condensed by evaporation, and subsequently mineralized in the mixture of nitric acid and chloric acid in the microwave mineralizer Maxidigest MX 350 Prolabo. After mineralization, the samples were condensed in the process of chelation with ammonium pyrrolidinedithiocarbamate (APDC), and extraction with methylisobutylketone (MIBK). The final determination of the concentration of Zn, Cu, Pb, and Cd was performed on an atomic absorption spectrophotometer ASS - Zaiss.

In order to determine the effect of heavy metals on the survival of neustonic and planktonic heterotrophic bacteria, iron - peptone agar (IPA) medium was used (Ferrer et al. 1963). The following heavy metals in the form of their salts (Merck AG) were used in the experiments: $ZnSO_4$, $CuSO_4$, $CdCl_2$, and $Pb(NO_3)_2$. Dilutions of stock metals were prepared in distilled water. Final concentrations ($\mu g \cdot l^{-1}$) of ions of particular heavy metals in IPA medium were as follows: Zn^{2+} (0.0, 16.0, 32.0, 64.0, 128.0, 256.0, 512.0), Cu^{2+} (0.0, 50.0, 100.0, 150.0, 200.0, 250.0, 300.0), Cd^{2+} (0.0, 7.0, 17.0, 34.0, 123.0, 153.0, 225.0), Pb^{2+} (0.0, 20.0, 40.0, 80.0, 160.0, 240.0, 320.0) (Hornor, Hilt 1985, Jeanthon , Prieur1990, Nogueira et al. 1993).

The collected surface (FL and SL layers) and subsurface (SUB) samples of water were diluted with sterile buffered water (Daubner 1967) and inoculated by the spread method in three parallel replicates on IPA medium with various concentrations of heavy metals. IPA medium containing no heavy metals was used as control. After a 7-day cultivation at 20^0 C, the colonies of neustonic (FL, SL) and planktonic bacteria (SUB) were counted and the results were calculated as a number of bacteria per 1 ml of water. Subsequently, 10 bacterial colonies from each water layer and each collection site, which grew on media free of heavy metals, were picked from the plates and transferred to semiliquid IPA medium (5.0 g of agar per l^{-1} of medium). The cultures maintained on this medium after purity control were kept at 4^0 C and used for further investigation in order to determine the effect of heavy metals on respiratory activity of those bacteria.

In order to determine the effect of heavy metals on respiratory activity of neustonic and planktonic bacteria, oxygen uptake was measured in Clark's electrode (Rank Brothers Ltd. Model 10) (Konopka, Zakharova 1999). Respiratory activity of 9 bacterial strains from each water layer and sampling site was determined. Pure cultures of bacteria were multiplied on IPA agar slants for 48 - 72 h at 20°C. Subsequently, they were washed off from the slants with phosphate buffer (0.01 M, pH 7.0), centrifuged at 15,000 rpm for 15 min and washed twice with the buffer. The washed bacteria were resuspended in the same buffer and adjusted to the turbidity of 4 in MacFarland standard. Typically, 1 ml of such suspension contained 10^9 bacteria.

Casein hydrolyzate (Casamino acids vitamin free - Difco) was used as a respiratory substrate in this study. This substrate ($0.5\ mg \cdot ml^{-1}$) was dissolved in phosphate buffer containing different concentrations (0.0, 1.0, 2.0, 4.0, 8.0, 20.0, 30.0, 50.0 $\mu g \cdot ml^{-1}$) of $ZnSO_4$, $CuSO_4$, $CdCl_2$ and $Pb(NO_3)_2$. Casein hydrolyzate free of heavy metals was used as control. Before measurements, the respiratory chamber of Clark's electrode was calibrated with sodium dithionate at the polarizing voltage of 6.0 V. After calibration, 1.5 ml of the bacterial suspension and 30 μl of respiratory substrate were put into the respiratory chamber. Changes in voltage on the electrode *were recorded by an analogue recorder XY Line Record TZ 5000 and stored in a* computer program BS81x - BS51x Data Recording System Ver 3.3.05. The number of measurements was set 30, taken every 6 seconds. During the measurements, the Clark's electrode was connected to a flow stabilizer of temperature, which ensured thermal stability in the respiratory chamber. Respiratory activity of each strain of bacteria was measured in triplicates. Data were corrected for endogenous respiration and the oxygen uptake was converted into $\mu l\ O_2\ h^{-1}$ per 10^9 cells.

RESULTS

The vertical distribution of heavy metals in Lake Gardno is presented in Fig 2. It can be seen clearly that the concentration of heavy metals is stratified, and there are significant differences in concentrations of those metals between surface and subsurface water layers. Zinc and copper reached their highest concentrations in the film layer (FL), whereas the highest concentration of lead was recorded in the surface layer (SL). Minimum concentrations of those metals were noted in the subsurface water layer (SUB). Cadmium, in general, was distributed homogeneously in all studied water layers.

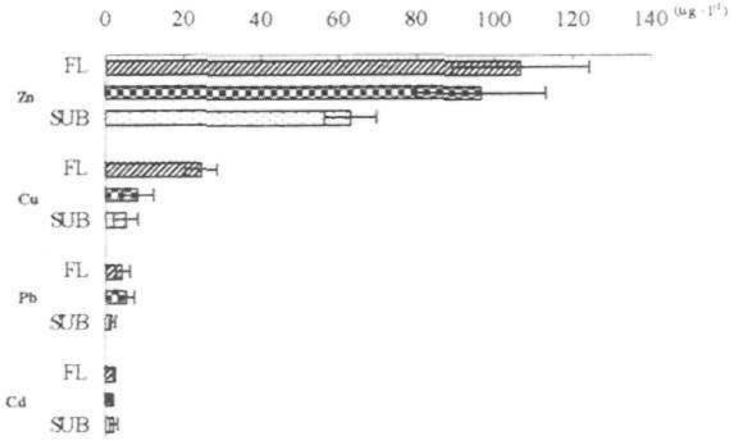

Fig. 2. Heavy metal concentrations in studied water layers.
Bars represent standard errors

Table 1 shows concentrations of heavy metals at the sampling sites in Lake Gardno. A significant gradient of heavy metal concentrations was observed between different parts of Lake Gardno. The highest concentrations of zinc and lead were noted in the seawater zone (st. 3). Maximum concentrations of copper were observed at the site close to the River Łupawa inflow (st. 1), whereas maximum cadmium concentrations were recorded in the mid-part of the lake (st. 2).

Table 1

Mean heavy metals concentrations ($\mu g \cdot l^{-1}$) in studied stations

Metal	Station		
	1	2	3
Zn	76.67 (70-0-80.0)	76.08 (50.0-100.0)	113.33 (70.0-140.0)
Cu	16.40 (4.2-28.6)	11.64 (2.0-28.7)	12.98 (4.2-18.3)
Pb	2.73 (1.20-7.80)	1.73 (0.80-3.20)	6.83 (3.20-9.50)
Cd	1.95 (1.41-2.49)	2.85 (1.95-4.10)	1.41 (0.33-3.03)

Figure 3 presents the effect of different concentrations of heavy metals on the survival of bacteria in the surface (FL, SL) and subsurface water layers (SUB). The data show that cadmium and lead were most toxic for neustonic and planktonic bacteria. Only in the presence of very low concentrations of lead (20 - 40 $\mu g \cdot l^{-1}$) and cadmium (7- 34 $\mu g \cdot l^{-1}$) in the medium the survival of some of the bacteria was possible. Higher concentrations of both of those metals were lethal for all neustonic and planktonic bacteria. On the other hand, bacteria inhabiting the surface and subsurface water layers were most tolerant of zinc; this was indicated by bacterial growth in the whole studied range of concentration (16 - 512 $\mu g \cdot l^{-1}$) of this metal. Data shown in Figure 3 indicate that neustonic bacteria were more tolerant of increasing concentrations of the tested heavy metals than planktonic bacteria.

At all studied sites in Lake Gardno, lead and cadmium showed the strongest inhibitory effect on the survival of bacteria (Fig. 4), whereas the tolerance of zinc and copper was much higher in bacteria isolated from various parts of the lake.

Figure 5 presents data on the effect of various concentrations of heavy metals on the respiratory activity of neustonic and planktonic bacteria. The rate of oxygen uptake measured at the concentrations of heavy metals in the respiratory substratum ranging from 1 to 20 $\mu g \cdot ml^{-1}$ was similar to that in the control, but it fell rapidly at 30-50 $\mu g \cdot ml^{-1}$. This process was most clearly seen in the case of cadmium and copper. Between neustonic and planktonic bacteria no significant differences in the effect of various concentrations of heavy metals on the intensity of respiratory processes were determined.

Small concentrations (1-20 $\mu g \cdot ml^{-1}$) of heavy metals in the respiratory substratum have not been shown to limit the respiratory activity of bacterial microflora isolated from the studied parts of Lake Gardno (Fig. 6). It was only at the concentrations of 30-50 $\mu g \cdot ml^{-1}$ that zinc, copper, lead, and cadmium showed inhibitory effect on this bacterial metabolic process. The inhibitory effect of heavy metals was the weakest in bacteria isolated from the sea zone (site 3), and the strongest in bacteria from the mid-lake (site 2).

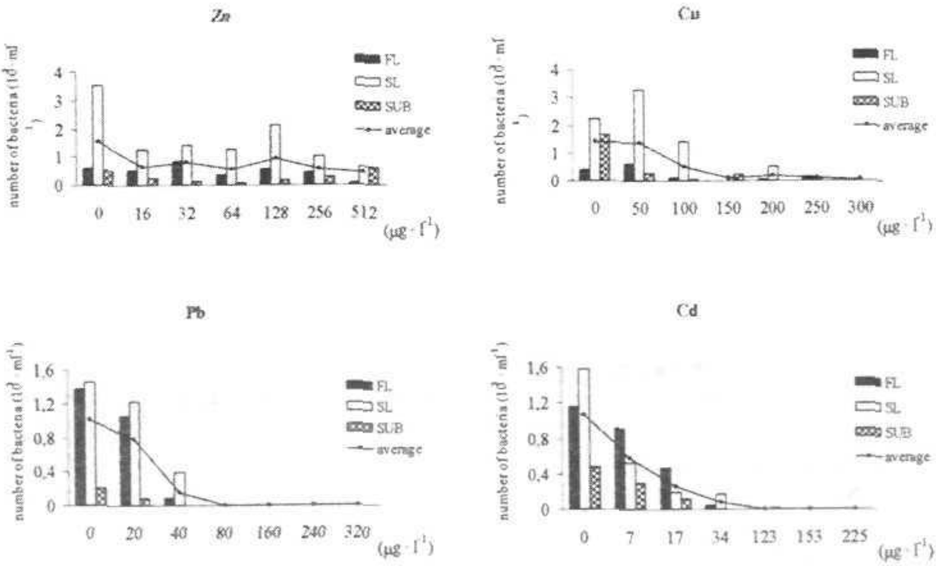

Fig. 3. The effect of different heavy metal concentrations in media on the survival of neustonic and planktonic bacteria

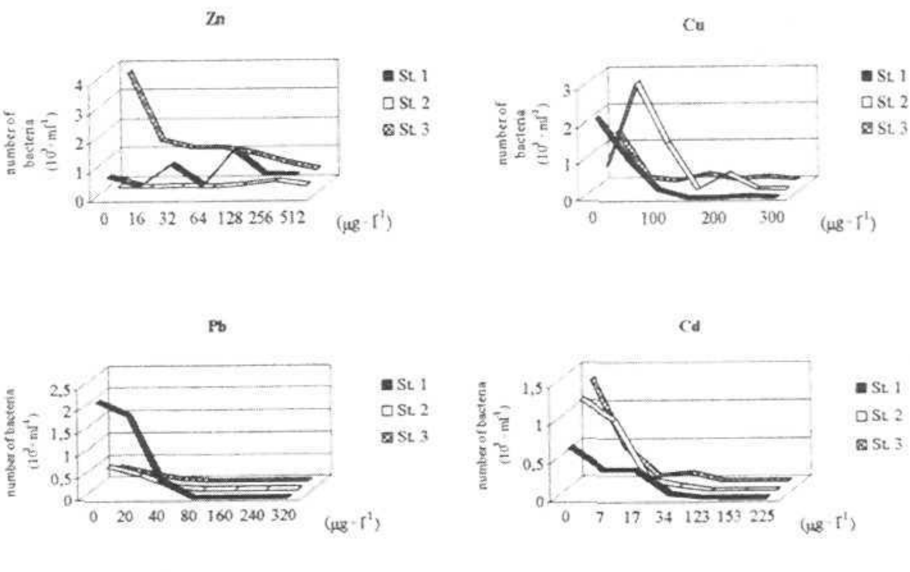

Fig. 4. Influence of elevated zinc (Zn), cooper (Cu), lead (Pb) and cadmium (Cd) concentrations on the number of bacteria isolated from different part of Lake Gardno

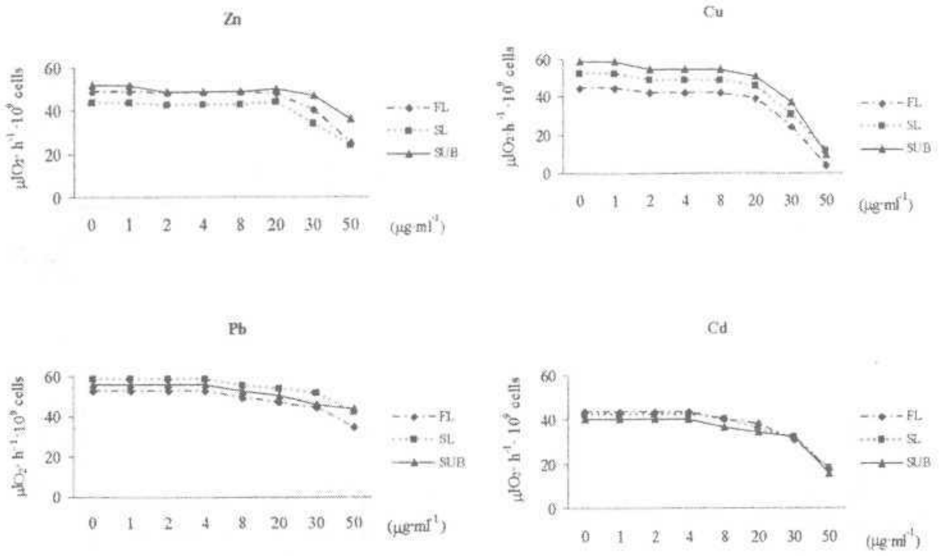

Fig. 5. Oxygen consumption by bacteria inhabiting surface microlayers (Fl, SL) or subsurface layer (SUB) at different concentrations zinc, copper, lead and cadmium

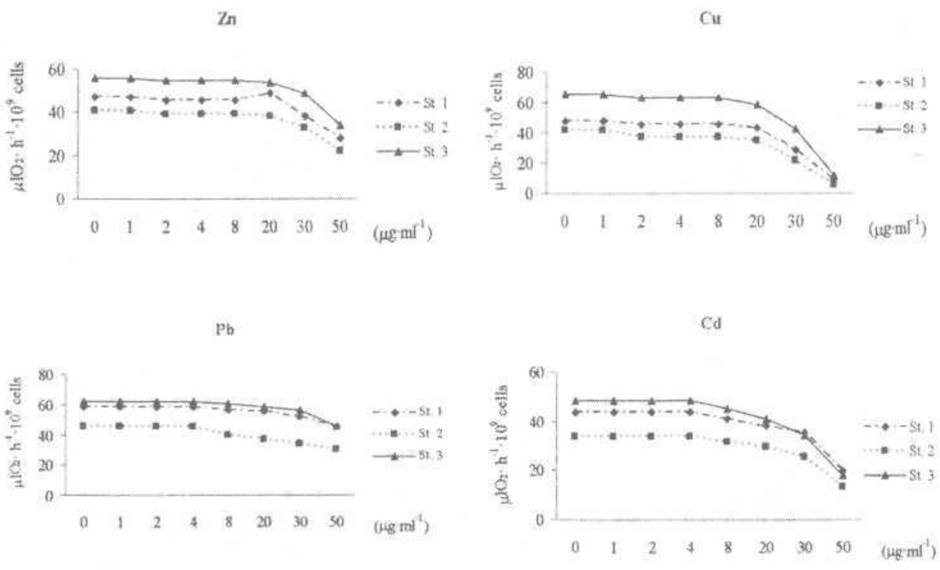

Fig. 6. Influence of heavy metals upon the respiration rate of bacteria isolated from different stations of Lake Gardno

DISCUSSION

Increase in the concentrations of heavy metals in surface microlayers relative to subsurface waters was described from various aquatic ecosystems including seas, estuaries and freshwater systems (Lion et al. 1982, Hornor, Hilt 1985, Kim 1985, Donderski et al.,1999). Concentrations of heavy metals in surface microlayers may be 10 to 100 times higher than in the underlying waters (Waldichuk 1982). Also in Lake Gardno, concentrations of lead, zinc and copper in surface water layers at times greatly exceeded the corresponding concentration in subsurface water. According to Maki (1993), accumulation of heavy metals at the air- water edge may result from molecular diffusion, floatation, surface adsorption, atmospheric aerosols and anthropogenic contamination. Piotrowicz et al. (1972) suggested that surface water microlayers can form an important site for the incorporation of heavy metals into food chains in water ecosystems.

A number of investigations into toxic effects of heavy metals on the growth and survival of aquatic bacteria have been performed, both in pure cultures and in mixed populations (Hermansson et al. 1987, Niewolak et al. 1996, Donderski et al. 1997, Lopez-Amoros et al. 1997, Mudryk et al. 2000). Results of these studies, along with data published in the present paper, reveal a diminishing bacterial survival with increasing concentrations of heavy metals in culture media. This results from the fact that many bacterial enzymes, such as proteases, phosphatases, reductases, liases, ureases, dehydrogenases, and arylosulphatases are inhibited by heavy metals (Foster 1983, Bogdanova et al. 1992, Doelman et al. 1994, Tubbing et al. 1995).

The strongest toxic effect on the survival of bacteria inhabiting Lake Gardno was brought about by cadmium and lead, while the effect of zinc and copper was relatively weak. Similarly, a high toxicity of cadmium, and a low toxicity of zinc and copper was determined by Jeanthon, Prieur (1990) and Reyes et al. (1999) for bacteria isolated from the Pacific and Skidaway Island. Also Gauthier et al. (1985) determined a considerably higher survival of bacteria exposed to zinc and copper than to cadmium in agar medium.

Kim (1985) who investigated the effect of heavy metals on natural bacterioneuston and bacterioplankton populations in the Baltic Sea, around Kiel Fjord, observed that a higher level of tolerance to various concentrations of heavy metals was shown by neustonic bacteria than by bacteria collected from the subsurface. The results presented in the present paper correspond to those data. Neustonic bacteria inhabiting Lake Gardno showed greater tolerance to heavy metals than planktonic ones. There are at least two possible explanations for this observation. Large amounts of organic matter accumulated in surface water (Maki 1993) could limit the toxicity of heavy metals for heterotrophic bacteria living in this special biotope. In the surface microlayer, toxic effects of heavy metals might be reduced or masked through the formation of complexes of metals with organic compounds, as they bound easily with small organic molecules (amino acids, simple sugar) and large molecules (proteins, nucleic acids) creating complex molecules (Piotrowicz et al. 1972, Albright, Wilson 1974, Lion, Leckie 1982). Also, as heavy metals are probably continuously present in the surface microlayer, bacterial adaptations to those substances may evolve (Kim 1985).

Some previous papers based on laboratory experiments (Farrell et al. 1993, Hardoyo et al. 1993, Perez-Garcia et al. 1993, Fabiano et al. 1994, Mudryk et al. 2000) determined the inhibitory effect of heavy metals on metabolic-respiratory activity of aquatic bacteria. Results presented in this paper confirm the inhibitory effect of high concentrations of heavy metals on the respiration processes in neustonic and planktonic bacteria inhabiting Lake Gardno, with the strongest effect brought about by cadmium and copper. Also Bååth et al. (1998) determnined a significant decrease in the respiration rate of bacteria in the presence of copper. Goulder et al. (1979) and Kim (1985) found that cadmium inhibited glucose oxidation by bacteria. According to Foster (1983) cadmium ions are taken up into bacterial cells where they cause rapid cessation of respiration by binding to sulfhydryl groups in proteins.

The authors of the present study are fully aware that laboratory experiments alone are not sufficient for the general assessment of the impact of heavy metals on aquatic bacteria. Such studies fail to take into account the complex role of various environmental parameters (such as organic matter ligands or the presence of other pollutants) which may reduce or increase metal toxicity. However, the data obtained may provide information on the potential effect of heavy metals on the survival and respiratory activity of bacterial communities, since so far very little has been known about the effect of heavy metals on estuarine populations of neustonic and planktonic bacteria.

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BADANIA LABOLATORYJNE WPŁYWU METALI CIĘŻKICH NA PRZEŻYWALNOŚĆ I AKTYWNOŚĆ ODDECHOWĄ ESTUARIOWYCH NEUSTONOWYCH I PLANKTONOWYCH BAKTERII

Streszczenie

W pracy przedstawiono wyniki badań dotyczące wpływu różnych stężeń metali ciężkich (Zn, Cu, Cd, Pb) na przeżywalność i aktywność oddechową bakterii zasiedlających powierzchniowe i podpowierzchniowe warstwy wody przymorskiego jeziora Gardno. Wykazano, że kadm i ołów były najbardziej toksyczne wobec bakterii neustonowych i planktonowych. Organizmy te najwyższy poziom tolerancji wykazywały wobec cynku. Stwierdzono, że bakterie zasiedlające powierzchniowe warstwy wody wykazywały wyższy poziom przeżywalności w stosunku do testowanych metali ciężkich niż mikroflora bakteryjna żyjąca w głębszych warstwach wody jeziora Gardno. Stężenie metali ciężkich w zakresie 30 – 50 μg/ml substratu oddechowego wywierało najbardziej inhibitorowy wpływ na intensywność procesów respiracyjnych badanej mikroflory bakteryjnej.