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Heavy metals effect on the growth of bacterial population isolated from the polluted waters of the river Arieş

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Abstract. Ten isolates of *Escherichia coli* were isolated from the polluted water of Arieş river (Romania) and were checked for their heavy metal tolerance. Pollution sources of the Arieş river are various, starting with mining activity, householding activity, as well as the anthropic uncontrolled factors etc. The predominant heavy metal sources in the Arieş river are the acide mine waters drained from tailings and from deposed sediments in the upper course of the river. Because heavy metals are increasingly found in microbial habitats due to natural and industrial processes, microbes have evolved, developing several mechanisms to tolerate the presence of heavy metals. Thus, in this study tolerance mechanisms of *Escherichia coli* strains isolated from the Arieş river water for metals such as copper, zinc, and manganese have been identified and described in detail.
Key Words: *Escherichia coli*, heavy metal resitance, mining pollution, Arieş river.

Rezumat. Zece tulpini de *Escherichia coli* au fost izolate din apele poluate ale Râului Arieş (România) şi testate în ceea ce priveşte rezistenţa lor la metale grele. Sursele de poluare de pe cursul râului Arieş sunt multiple, cum ar fi activitatea minieră, activităţi menajere, factori antropici, etc. Principala sursă a acumulării unor concentraţii crescute a metalelor grele în apa râului o constitue apele reziduale miniere provenite de la iazurile de decantare şi haldele de steril de pe cursul superior al râului. Deoarece metalele grele se gasesc în concentraţii din ce în ce mai crescute în habitatele microbiene rezultate din procese naturale şi industriale, microorganismele au elaborat diverse mecanisme pentru a tolera prezenţa metalelor grele. Astfel, în studiul de faţă au fost identificate şi descrise mecanisme de toleranţă faţă de metale ca şi cuprul, zincul şi manganul în cazul unor tulpini de *Escherichia coli* izolate din apa râului Arieş.
Cuvinte cheie: *Escherichia coli*, rezistenţă la metale grele, poluare minieră, râul Arieş.

Introduction. Widespread in the environment, in soil, water, human and animal gut, *Escherichia coli* is one of the sanitary water quality indicators. Its presence in water samples indicates a source of faecal pollution and increase the likelihood of digestive and urinary diseases if these waters are used as drinking water, for irrigation, or for recreational purposes. Current issues are related to the increased resistance of bacterial strains to a wide range of antibiotics and heavy metals present in the environment.

Heavy metal tolerance in the environment may contribute to the maintenance of antibiotic resistance genes by increasing the selective pressure of the environment. There is evidence (Spain 2003) that a correlation exists between metal tolerance and antibiotic resistance in bacteria because of the likelihood that resistance genes to both (antibiotics and heavy metals) may be located closely together on the same plasmid in bacteria and are thus more likely to be transferred together in the environment.

Because of the prevalence of antibiotic resistant pathogenic bacteria, infectious diseases are becoming more difficult and more expensive to treat; thus we need to not only be more careful to the drastic overuse of antibiotics in our society, but also more aware of other antimicrobials, such as heavy metals, that we put into the environment.

Heavy metal salts represent a very serious form of pollution for surface waters due to their toxicity and stability. They can induce disorders of the biological balance, with negative consequences over the various uses of water. The degree of pollution is dependent upon the nature of mine waste, the hydrological link between mines and local rivers, and the local physicochemical environment. Thus, according to this, in the water of the Arieş river increased quantities of trace metals (Cu, Zn and Pb) were detected (Ash et al 2002; Barberio et al 2001; Costanzo et al 2005; Ohlsen et al 2003; Sayah et al 2005). The values of heavy metal salts concentration are higher in the upstream sampling points as compared to those registered in the downstream of the river. It can be affirmed that these elevated values are a consequence of the pollutants accumulation as a result of mining activities.

The effect of copper ions on E. coli cells growth. There are data that evidence the resistance of *E. coli* bacterial strains to copper ions. There is evidence of clinical isolates of *E. coli* (Eleks & Higney 1970) and of coliform germs, isolated from domestic wastewaters, which manifest resistance to copper ions (Koditschek & Guyre 1974; Varma et al 1976).

There is even evidence that *E. coli* cells need small amounts of copper for growth, however, at high concentrations copper is toxic. Thus, the cellular transport mechanisms, the use of copper ions by the microorganisms for development and the mechanisms of resistance have become of major interest, especially because increasingly more strains of *E. coli* from different media have been found to exhibit multi-resistance to heavy metals and to antibiotics (Bodoczi 2011, 2012). It became increasingly more difficult to control and treat diseases with multidrug-resistant strains within the human and also animal populations.

Elevated concentrations of copper ions are toxic for aerobic microorganisms (Nies 1999). Toxic levels are reached when copper bound to the cell membrane and catalyzes superoxide free radical formation (Rodriguez et al 1993). The levels of cell toxicity can only be determined by monitoring the growth of bacteria cultures in the presence of heavy metals at different concentrations (Diaz et al 1996).

The concentrations of the copper ions considered to be toxic do not cause the death of all the cells in culture, some have only altered they're functions (Domek et al 1987). Researches indicate that the elevated concentrations of copper ions, even toxic concentrations, can induce entry into a state in which the cells are viable; however, they can not be grown in cell culture (Liu & Kwasniewska 1981; Gupta et al 1995). This state of latency occurs as a response to stressors in the environment and it is considered as a long time survival mechanism which was first observed in Gram-negative bacteria (Gauthier 2000).

The effect of Zn ions on E. coli cells growth. Zinc is one of the metallic ions essential for living organisms, but elevated levels of this metal can be toxic and have inhibitory effects on bacterial growth. Intracellular concentration of zinc in *Escherichia coli* cells remains constant in the case of environments that are both poor and rich in this metal (Outten et al 2001).

Mechanisms of bacterial resistance and tolerance to zinc have been demonstrated by Choudhury & Srivastava (2001) and Nweke et al (2007).

A study conducted by Yao et al (2005) shows that zinc in low concentrations stimulate growth of *Escherichia coli* strains in a culture medium, while the elevated concentrations have an inhibitory effect.

The effect of Mn ions on E. coli cells growth. Manganese is a growth inhibitor for *Escherichia coli*, but dampening is depending on the concentration of the metal in the environment. Mutant strains of *E. coli* have been isolated and they were showing resistance to the inhibitory effect of manganese in culture medium (Silver et al 1972).

The aim of this study was to determine the growth rate of the isolated *E. coli* strains sampled from the polluted water of the Arieş river in the presence of heavy metals (Co, Zn and Mn). In this respect, the optical density has been determined.

Materials and Methods. The water samples necessary for this study were taken seasonally (in January, April, July and October) from 10 sampling points upstream and downstream of the main towns the river passes through (Abrud, Baia de Arieş, Sălciua, Turda and Luncani). The main polluting sources in the area were also taken into consideration when establishing the sampling points.

The water samples were drawn from the river bed at 1 m distance from the shore, being stored in glass containers previously sterilized at 180 °C, for 60 minutes, according to SR ISO 5667-6/97.

For the performance of optical density, there were isolated *E. coli* strains from the Arieş river water on Chapman TTC culture media (Carpa et al 2014). Strains were confirmed as *E. coli* by biochemical tests. For optical density assessing subcultures were made subsequently on agar medium with different concentrations of heavy metals.

Optical density (absorbance) measured spectrophotometrically can be used to determine the concentration of bacteria in suspension. The light that passes through the cell suspension is scattered. A high degree of scattering indicates the presence of large numbers of bacteria. It can thus determine the optical density at a specific wavelength related to the various bacterial growth log phase.

For the measuring the optical density, the *E. coli* strain of control ATCC 25922 and strains isolated from the Arieş river waters on a Chapman TTC medium, and identified as *E. coli* through biochemical tests, were grown at 35 °C temperature and continuously shaken at 150 rpm, in condition of light, on a minimal M9 medium with glucose and in the presence of heavy metals (Cu, Mn, Zn) at different concentrations.

Thus, for $CuSO_4$ the three chosen concentrations were: Cu I (0.15mg/L); Cu II (0.07mg/L) and Cu III (0.02mg/L). For the $ZnCl_2$ the concentrations used were Zn I (0.034 mg/L); Zn II (0.02 mg/L); Zn III (0.002 mg/L) and for $MnCl_2$ the concentrations used were Mn I (1.2 mg/L); Mn II (0.06 mg/L); Mn III (0.008 mg/L).

Results and Discussion. For reading the obtained results, the indirect method has been used by measuring the turbidity of the bacterial culture spectrophotometrically at a wavelength of 600 nm to 1 mL of bacterial culture. Measures were made at 0, 2, 4, 6 and 24 hours of growth.

In this study the optical density was measured at λ=600 nm also for *E. coli* isolates from Arieş river and the standard *E. coli* ATCC 25922 strain too (Figure 1).

Growth curves were performed. The results obtained have been reported to the controls and standard strain of *E. coli* ATCC 25922.

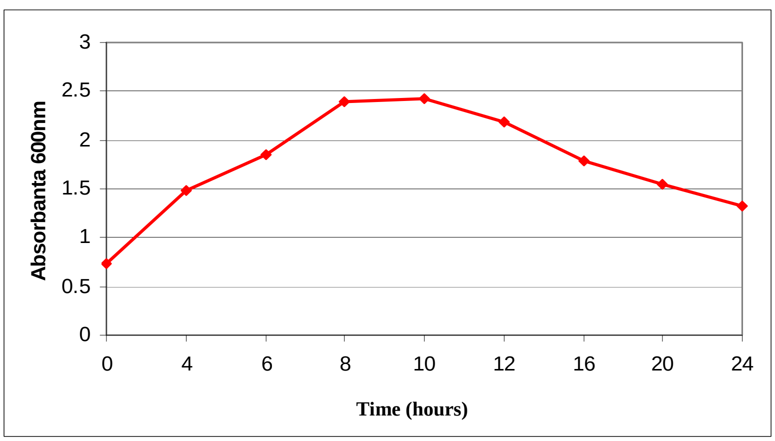

Figure 1. Optical density at the standard strain of *Escherichia coli* ATCC 25922.

Determination of the increase rate in strains of E. coli in the presence of heavy metals. *E. coli* strains growth is influenced by the metal concentration in the culture medium and exposure time. The progressive decrease of the population is evidenced from prolonged periods of exposure (over 20 hours of incubation). Similar results have been registered by Sasadhar et al (2004) and Cho et al (2002). They highlighted the harmful effect of several metals on *E. coli.*

To observe the effect of each metal on the growth of *E. coli* strains isolated from the Arieş river, they were compared considering rising highs recorded in the 8 hour growth for each of the three concentrations of the studied metals (Cu, Zn, Mn).

The results obtained were compared with those from the literature Nies 1992; Satchanska et al 2005; Rajbanshi 2008; Wang et al 2008) observing the existence of similarities. The graphics are rendered in Figure 2.

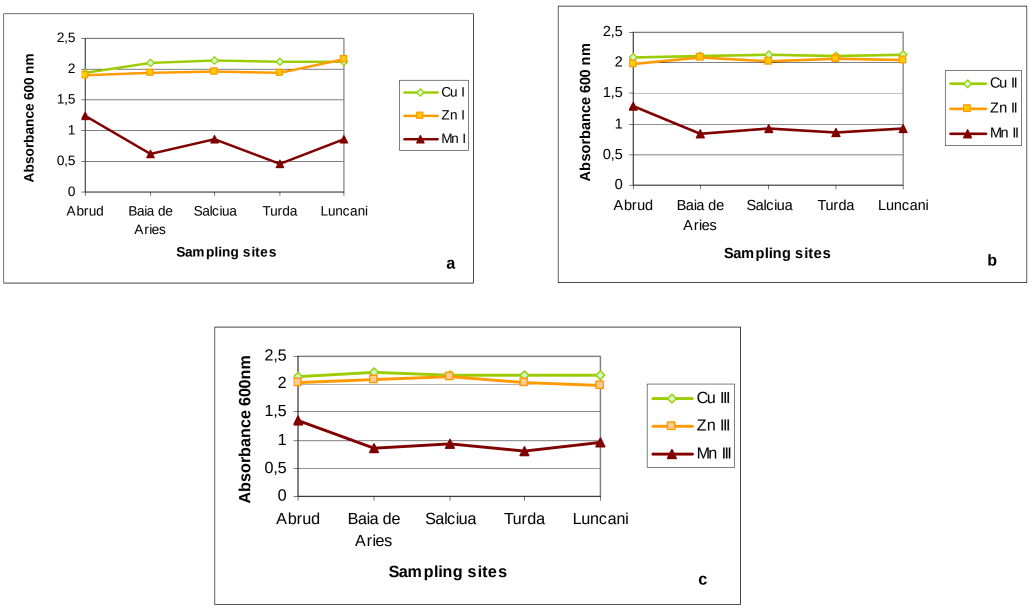

Figure 2. The registered values achieved in the 8th hour of incubation of the *E. coli* strains in the presence of the heavy-metals (Cu, Zn, Mn) at the three different concentrations (I, II, III).

Also, for each of the studied heavy-metals (Cu, Zn, Mn) the existence of growth variations according to the sampling sites, the used metal and its concentration in the culture media may be observed. The results show that inhibitory effects of three heavy metals on *E. coli* can be ranked in a decreasing order: Mn>Zn>Cu. The manganese had the highest inhibiting effect and copper had the lowest.

Taking into consideration the metal concentration in the culture media, the most inhibiting effect over the growth corresponds to the highest concentrations of the metal ions (Figure 2a). In this respect, a better growth rate in the presence of the copper and zinc ions have been registered, signifying the existence of a resistance/tolerance of the studied variants for this metals, that can be explained by the exposure of these strains to the high values of heavy-metals (over the permitted limits) in the chemically polluted river waters. This can already be associated with an induced resistance/tolerance of the *E. coli* strains to heavy metals compounds.

According to the obtained results, a similarity can be observed in the case of the strains isolated from Abrud sampling point, where, for each of the three concentrations of manganese, the maximal values were achieved in the eighth hour of incubation. It may be due to the fact that bacterial strains exhibit some tolerance/resistance mechanisms induced by the presence of the heavy metals at high concentrations in the polluted waters of the river. For Mn I, the growth was minimal in case of the strains isolated from the Turda section (λ=0.463).

According to the results obtained and compared to the controls, we can speak of a negative and inhibitor effect of the growth at high concentration of Cu on the *E. coli* isolates. However, elevated values registered in some sampling points as Luncani and Baia de Arieş at Cu II and Cu III, may be due to the presence of this metal in the natural environment due to the discharge of sewage and industrial mining, thus being able to speak of acquired resistance of *E. coli* strains at these points on the time period of exposure to this metal.

In the case of Cu I the maximum value 2.132 was registered in Sălciua sampling site, and the minimum of 2.107 in Abrud. The maximum value reached in Sălciua can be explained by the fact that at this point the water is not closely exposed to discharges from the mine drainage so in this area the water shows the effects of natural purging processes. The largest increases were recorded at the lowest concentrations of Cu in the culture media (Cu III), here maximum growth was registered in the 8 hour of incubation in Baia de Arieş sampling site, where at λ = 600 nm a value of 2.219 has been recorded, while the lowest increase was registered in Abrud (2.144).

The concentration of Zn I shows a higher increase that exceeds those of the concentration of Cu I in Luncani sampling point which correlate with elevated concentrations of Zn in river water at the sampling point, resulting from industrial activities.

The achieved results suggest the inhibitory effect of the manganese over the growth of the *E. coli* strains at each of the metal concentrations induced in the culture media. The *Escherichia coli* strains growth in the presence of the $MnSO_4$ depends on the metal concentration in the culture media and the time of exposure. At each of the three concentrations of the manganese ions in the culture media the maximal growth was achieved in the eighth hour of incubation.

In the case of the strains isolated from Abrud sampling points, the maximal growth was registered in the eighth hour of incubation at each of the used concentrations, which may be explained by the fact that bacterial strains exhibit some tolerance/resistance mechanisms induced by the presence of the heavy metals at high concentrations in the polluted waters of the river, at this point.

According to the results, a similarity can be observed in the case of the strains isolated from Abrud sampling point, where, for each of the three concentrations of manganese, the maximal values were achieved in the eighth hour of incubation. For Mn I, the growth was minimal in case of the strains isolated from the Turda section (λ=0.463).

Observing the behavior of *E. coli* strains isolated from the river Arieş characterized in terms of ability to increase in areas with heavy metals, the inhibitory effect of heavy metals on the growth of *E. coli* strains by altering their ability to effectively use the substrate can be recorded. In this respect, compared to the standard strain of *Escherichia coli* ATCC 25922 the entering in the phase of decline more quickly of the isolated strains at 10-12 hours of culture can be observed.

Also, the existence of a bacterial multiresistence was proved both for heavy metals and antibiotics (Filali et al 1999; Dalsgarrd & Guardbassi 2002; Bodoczi 2011, 2012). Comparing the results obtained in the case of *E. coli* strains studied in the Arieş river the presence of such resistance to antibiotics and heavy metals was registered on the lower course of the river due to the effects of anthropogenic influences: urbanization, industrialization, the inefficiency of waste water treatment or even the discharge of fecal household waste water directly into the river, due to lack of sewage networks in certain areas of Turda and Câmpia Turzii cities.

It vas also revealed a stronger toxic effect of heavy metals on the population of microorganisms in the water than on those in sediments.

Conclusions. Thus, the achieved results suggest the inhibitory effect of the manganese over the growth of the *E. coli* strains at each of the used concentrations of metal in the culture media. The *E. coli* strains growth in the presence of the $MnSO_4$ depends on the metal concentration in the culture media and the time of exposure.

The isolated *E. coli* variants growth and viability is influenced by the heavy-metal values in the culture media and depends on the sampling site. Thus, in that sampling area where heavy-metals were revealed in high concentration in the river water due to the mining or industrial pollution, an induced mechanism of tolerance/resistance of the bacterial strains can be observed, which emphasises an increased growth and cellular viability.

Because most of the times the high concentration of heavy-metals considered as toxic doesn't destroy the cells it was demonstrated that cells may enter in a viable but non-culturable (VBNC) condition as a response to the high contents of metals in the culture media. Based on these, we may affirm that the reduced values obtained in the case of optical density may be due to this state of the cells induced as a response to the environmental changes (the presence of the heavy-metals at different concentrations) and not as a consequence of cell death.

The excessive use of the natural resources and the growing tendencies of industrialization increase the concerns regarding the effect of heavy-metal compounds on the microbial populations and human health, as a consequence of their accumulation in the food chain and water sources.

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