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Heavy metals effect on the *E. coli* biofilms isolated from the polluted waters of Arieş river (Romania)

Andreea Bodoczi-Florea

Technical College, 48, Piaţa Basarabiei Street, 401122, Turda, Cluj, Romania, andy13_florea@yahoo.com

Abstract. Objective: Biofilms are surface-attached microbial communities consisted by multiple layers of cells embedded in a hydrated matrix. Biofilms are dynamic microbial communities in which transitions between planktonic and sessile modes of growth occur interchangeably in response to different environmental cues. The effects of three trace metals (copper, manganese and zinc) at different concentrations on *Escherichia coli* strains isolated from the water of Arieş river (Romania) were compared using epifluorescence microscopy. Data showed that the heavy metals affected not only the cells viability but also the biofilm formation according to the growing concentration of the metals in the media.
Key Words: biofilms, heavy metals, *E. coli*, epifluorescence microscopy.

Rezumat. Obiectiv: Biofilmele sunt comunităţi microbiene ataşate la suprafeţe solide, formate din mai multe straturi de celule încorporate într-o matrice hidratată. Biofilmele sunt comunităţi microbiene dinamice în care tranziţia dintre formele planktonice şi cele sesile de creştere se produc alternativ ca şi răspuns la diverşi factori de mediu. În lucrarea de faţă a fost studiat efectul a trei metale grele (cupru, mangan şi zinc), fiecare metal la trei concentraţii diferite, asupra unor tulpini *Escherichia coli* izolate din apa râului Arieş (România). Biofilmele astfel formate au fost studiate cu microscopul cu epifluorescenţă. Rezulatele obţinute au arătat că metalele grele au afectat nu doar viabilitatea celulară ci şi formarea biofilmelor în concordanţă cu creşterea concentraţiei metalelor în mediul de cultură.
Cuvinte cheie: biofilme, metale grele, *E. coli*, microscopie cu epifluorescenţă.

Introduction. Microbial cells attach to inert surfaces and produce exopolymers that facilitate their adhesion. Cells will grow, divide, colonize the surface and form microcolonies. The size and the number of microcolonies increases and result a biofilm (Surdeanu et al 2006).

A general characteristic of biofilm communities is that they tend to be significantly more resistant to antibiotics and antimicrobial stressors, including those represented by host-defense responses, than planktonic bacteria of the same species (Stewart & Costerton 2001; Gilbert et al 2002; Stewart 2002).

Bacterial biofilms have been used in the industrial wastewater treatment with high content in copper ions (Qureshi et al 2001). Lethola et al (2004) showed that the bacterial communities' structure in a biofilm and the cells number are influenced by the type of the substrate. Biofilm formation was higher on the surface of galvanized steel coupons than on the surface of copper coupons, the copper ions influenced the bacterial communities by the reduction of the microorganism's number.

Temporal bacterial community changes in biofilms were studied in river Garone in S-W part of France (Lyautey et al 2005). The data obtained showed a significant relationship between the community structure and environmental conditions suggesting that bacterial communities were mainly influenced by the seasonal changes (temperature, light) and hydrodynamic stability.

Tien et al (2009) have conducted several approaches to determine the possibility of biofilms applicability for biomonitoring water quality. Two river ecosystems were

analyzed to determine different biofilm development. Results obtained showed that the chemical oxygen demand was found to directly increase bacterial growth or indirectly affect growth a week later. One-month colonization biofilms were the most sensitive to change of water quality, and had the greatest number of significant relationships to physico-chemical and biological parameters. This suggested that 1-month colonization biofilms were applicable for biomonitoring water quality

Zogaj et al (2001) have showed the existence of a phenotypic diversification in the bacterial communities from a biofilm due to the microorganisms adaptabilities to the micromedia conditions and as a response to the selective pressions. There are data that suggest the detachment of some bacterial strains from the biofilm and that they return to the planktonic form. Sauer et al (2002) demonstrated the dispersion process to *P. aeruginosa* induced as a result of the modification of the culturing condition (rapid decrease of the culture media pH). The dispersion mechanism in a biofilm is of great importance in the antibiofilm therapy.

Corrosion is one of the major concerns caused by the biofilms presence in the industry. There exists evidence that sulphur reducing bacteria contribute to metal pipes corrosion. Studies were conducted on mixt bacterial communities in biofilms (Butterfield et al 2002) and suggest that the adsorption of humic substances by iron oxide containing corrosion products (CPs) can stimulate and/or support biofilm development and did not affect the similar planktonic communities.

Microbiologically influenced corrosion (MC) of steel has been attributed to the activity of biofilms that include anaerobic microorganisms such as iron-respiring bacteria, yet the mechanisms by which these organisms influence corrosion have been unclear. Inhibition of corrosion is due to the reduction of ferric ions to ferrous ions and increased consumption of oxygen; both are direct consequences of microbial respiration. Dubiel et al (2002) describe how biofilms comprising iron-reducing bacteria use Fe(III) for the respiration process in the absence of the oxygen and may reduce rather than accelerate the corrosion rate of steel.

Formation of biofilms allows microbial pathogens to create a safe sanctuary in which sessile cells remain in a protected environment. However, cells within a biofilm may be also confronted with adverse environmental conditions (*i.e.* reduced nutrient availability, accumulation of toxic waste products) so that dispersion of cells would be beneficial for survival. Furthermore, this release of cells from the original biofilm community is required to generate novel communities at new locations. It follows that gaining knowledge about mechanisms regulating biofilm dispersion, at both the physiochemical and molecular levels can potentially lead to better strategies for the prevention and treatment of biofilm-associated infections.

The aim of this study consist in the establishment of the heavy metal impact on *E. coli* isolates biofilms recovered from Arieş river water samples based on the presence of dead or live cells.

Material and Methods. This study shows how heavy metals may affect the *E. coli* biofilms. In this purpose, the effect of trace metals over the microbial cells viability and the availability of bacterial strains to form biofilms, was followed.

Test organisms. The micrororganisms used in this study were the *E. coli* strains isolated from the water of the river Arieş (Romania), and a standard strain of *E. coli* ATCC 25922. Working cultures were incubated aerobically for 24 h at 37 °C in 10 mL peptone media; stock cultures were made in Tryptone Soya Agar (TSA; Oxoid) plates, stored at 4°C.

Heavy metals. The heavy metal impact on the *E. coli* isolates was investigated and in this respect, were used heavy-metal salts in three different concentrations (see Table 1).

In order to obtain biofilms, the *E. coli* strains isolated from the Arieş river waters have been used according with the standard methods, in a peptone culture media with three different concentrations of the heavy metals (Cu, Zn, Mn). The heavy metals` concentrations were established according to the registered concentration in the water of the Arieş river by the physico-chemical analysis (see our previous research: Bodoczi &

Carpa 2010, but also: Bodoczi 2009, 2010, 2011). In this study we used heavy-metals salts: $CuCl_2$, $ZnSO_4$ and $MnCl_2$.

Table 1

The types and composition of the studied heavy-metals

Heavy-metals salts	*Used amount*		
$CuCl_2$	Cu I	Cu II	Cu III
	0.15 mg/L	0.07 mg/L	0.02 mg/L
$ZnSO_4$	Zn I	Zn II	Zn II
	0.034 mg/L	0.02 mg/L	0.002mg/L
$MnCl_2$	Mn I	Mn II	Mn III
	1.2 mg/L	0.06 mg/L	0.008mg/L

The solid surface used for this study was stainless steel. The stainless steel was cut into 2.5 x 6 cm plates, cleaned with 70% ethanol, and was then rinsed in distilled water and sterilized by autoclaving. Samples were stored in Petri dishes.

Biofilm culture. To obtain the biofilms from the bacterial cultures there were made seedings in the nutrient broth, after night.

Each strain of *E. coli* was grown in fresh peptone medium (Drăgan-Bularda 2000) in the presence of the three heavy metals at the three different concentrations. Each microbial suspension was adjusted to approximately 10^8 cells/mL using the Thoma (Helber) counting chamber. The obtained bacterial suspensions were poured on the stainless steel surface so as to cover the surfaces in Petri dishes (Pap 2002). Biofilms were produced by sedimentation of the bacterial suspension of stainless steel plates for 24 h at 37 °C. Plates were rinsed with sodium chloride solution to remove non-adherent bacteria. From each strain we made a control sample. The samples surfaces were rinsed with sterile distilled water.

The *E. coli* bacterial biofilms of 24 hours exposed to different concentrations, were colored with acridin oramge (acridin orange – 0.02 g, distilled water- 100 mL) (Robbins & Marcus 1963). The samples were examined using epifluorescence microscope and photographed at 475 nm.

In the presence of the acridin-orange the living cells will be colored in green and the dead cells in orange (Francisco et al 1973). The control surfaces were colored similarly as the samples.

Results and Discussion. Numerous physical and chemical agents can action as stress factors over the microbial population, altering the biological synthesis (proteins, DNA, ARN) and the membrane functions (the process of transport through the membrane) producing in most of time not the death of the cells, but the alteration of cellular function that reduce the adaptability and the functioning capacity of the cells. There have been made studies on how the salinity, the pH of the culture media, irradiations, low temperature or freezing affects the *E. coli* cells (Anderson et al 1979). Few studies have been conducted concerning the effect of heavy metal salts on these germs of faecal pollution that at low concentration may act as stressors (Cenci et al 1985).

The pollution with trace metals, especially with heavy metals salts, became by international interest and this due to the effect that these trace metals may have on human health, as a consequence of accumulation in the food chains and water resources (Sharma & Agrawal 2005; Houston 2007; Islam et al 2007).

Numerous researches were made to evidence the way in which trace metals exercise their toxic effect over biological systems and the way in which this adapt to different stress factors (Balshaw et al 2007; Haferburg & Kothe 2007; Milner & Kochian

2008; Thompson & Bannigan 2008; Yoon et al 2008). Numerous microorganisms present specific elements that conferring their resistance to the heavy metals presence in the environment.

The aim of this study was to establish the way in which heavy metals affect the *E. coli* biofilms. In this respect, was established the way in which the presence of trace metals in the culture media affect the cells viability, or the availability of bacterial strains to form biofilms, knowing that the river Arieş water is strongly polluted with heavy metals from the waste waters resulted from mining exploitations from upstream of the river course, respectively with industrial wastes of the effluents from the downstream of the river. The results obtained are represented in Figures 1-4.

The study of the bacterial biofilms evidenced that the copper ions affect the *E. coli* bacterial communities by number reduction as a consequence of the exposure to the growing concentration of the metal in the culture media and by reducing the cell's capacity to use the substrate efficiently.

The cooper concentrations in the culture media affected also the cells viability; the number of the viable cells was decreasing with the increasing of metal concentrations (see Figure 1).

Studies made on *E. coli* evidenced a 50% growing inhibition to 1.2 mM Cu in the media (Patcharee et al 2009).

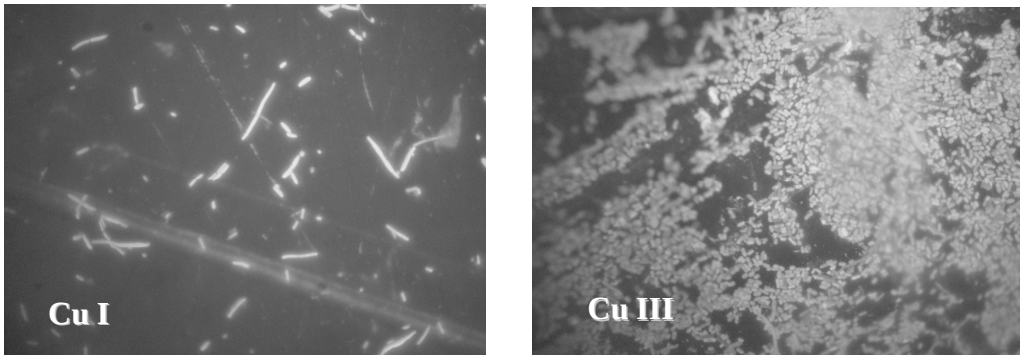

Figure 1. *E. coli* biofilms at the concentrations of Cu I (left) and Cu III (right).

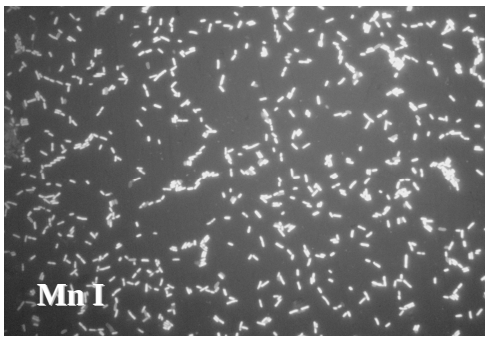

Figure 2. *E. coli* biofilms at the concentrations of Mn I.

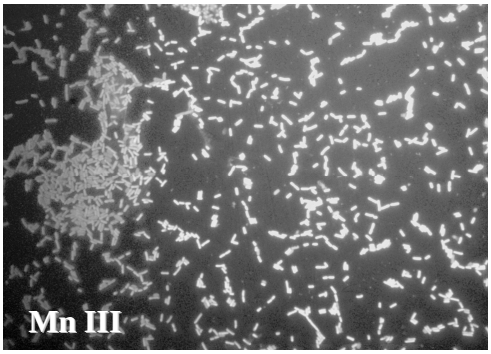

Figure 3. *E. coli* biofilms at the concentrations of Mn III.

In the case of $MnCl_2$ (Figures 2-3), it can observe that the manganese highly affected the cell's viability and the enzymatic processes, and did not reduce significantly the number of the bacterial cells. There can be observed that the highest concentrations of the manganese (Mn I) (Figure 2) in the culture media strongly affect the *E. coli* cells viability.

Cells of *Escherichia coli* encounter fluctuating extracellular zinc levels and maintain zinc homeostasis by transporting excess metal out of the cell and regulating zinc uptake across the cytoplasmic membrane. There are at least fore Zn^{2+} specific transport systems characteristic to *E. coli* cells. Zinc uptake is facilitated by the combined activities of the ZnuABC and ZupT transport systems (Lewis et al 1999; Grass et al 2002). The ZnuABC system is the primary Zn^{2+} influx pathway and is induced in response to zinc deprivation (Patzer & Hankte 1998). Bacteria respond to high levels of exogenous Zn^{2+} by increased expression of the ZntA and ZitB efflux systems (Rensing et al 1997; Grass et al 2002).

In case of the Zn, heavy-metals salts in different concentration affect only the quantity of viable cells which is reduced once with the increasing metal contents Zn III → Zn I, the cell`s number, in case of the 24 hour bacterial biofilm are not affected (Fig. 4).

According to other studies (Yao et al 2005), was found that a low concentration of zinc had a promoting action on the growth of *E. coli*, but a high concentration of zinc had an inhibitory action.

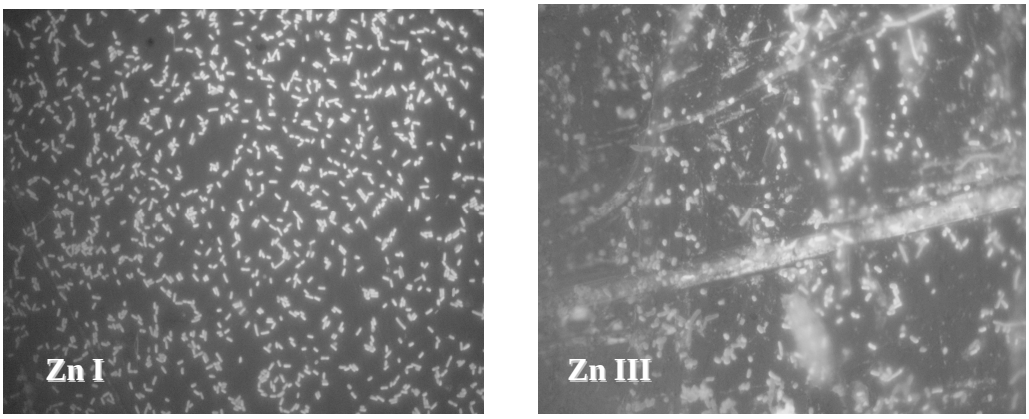

Figure 4. *E. coli* biofilms at the concentrations of Zn I (left) and Zn III (right).

Similar results have been obtained by Crane et al (2007) on entheropatogenic *E. coli* strains case in that the low concentration of Zn ions produce little or no inhibition of cells, and the higher concentrations of the metal the *E. coli* strains growth was inhibited within more than 60%. The Zn ions effects have been tested over the respiratory processes of *E. coli* cells (Kasahara & Anraku 1972). Authors observed the Zn ions effect over the oxygen rate consumption. The Zn ions inhibit completely the chemical reaction even at the low concentration of the Zn in the media (0.1M $ZnSO_4$), the oxygen rate was inhibited from 99% to 68%; the Hg and Cd ions blocked the respiratory reactions, meanwhile the Mn and Cd ions seems to have no effect. As well, the extreme concentrations of Zn in the media did not affect the *E. coli* cells viability (Hong et al 1995).

Following the Zn ions effect on the 24 h *E. coli* biofilms (Figure 4), was observed a reduction of the viable cells at the highest concentration of the Zn in the culture media. Cell viability grows with the reduction of the metal ions concentration in the media. In what view the cell numbers there was not registered significantly modifications at the three metal ions concentrations.

In the case of the 24 h old biofilms the metal ions at different concentrations affect only the number of the viable cells that are reduced significantly with the increasing metal concentrations in the culture media, and not affect de number of the existent cells.

Conclusions. The study of the obtained biofilms showed that the copper ions affect the *E. coli* bacterial communities by numerical reduction as result of the exposition to the growing concentration of Cu in the media, as well as a consequence of bacterial cells availability to use efficiently the substrate.

In the presence of $MnCl_2$, it can be observed that Mn ions affect more the cell viability and the enzymatic processes and not reduced significantly the number of the cells at the level of the biofilm. As well in the case of Zn, the metallic ions at different concentrations affected only the number of the viable cells that was reduced significantly with increasing concentration of the heavy metals in the culture media. In the case of the 24 h biofilms the number of the existent cells in the media was not affected only the cell viability.

In generally, heavy-metals at high concentrations reduce the growth and viability of the *E. coli* bacterial strains isolated from the water of the Arieş river by diminishing their ability to use the substrate efficiently.

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Author:
Andreea Bodoczi-Florea, Technical College, 48, Piaţa Basarabiei Street, 401122, Turda, Cluj, Romania, European Union; e-mail: andy13_florea@yahoo.com

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