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IMPACT OF DIFFERENT ENVIRONMENTAL PARAMETERS UPON THE SUSCEPTIBILITY TO HEAVY METALS SALTS IN *ESCHERICHIA COLI* AQUATIC STRAINS

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Abstract

Purpose: to investigate the relationships among the expression of susceptibility to heavy metal salts, different incubation temperatures and chemical composition of the culture media in E. coli aquatic strains. 100 strains of E. coli isolated from Black Sea were investigated for the expression of resistance to different bivalent metals (Cu, Co, Mn, Zn, Ni) compounds. The experiments were performed comparatively at different incubation temperatures (22°C, 37°C and 44°C) in aerobic and anaerobic conditions, NaCl concentrations (from 0 to 10%), glucose (1.5 and 3%) and pH (5.0, 7.2 and 9.6). The metals susceptibility patterns varied with the tested parameter and the bivalent metal compounds. The temperature growth induced an increase in susceptibility of the tested strains to Zn (from 85% at 22°C, 20% at 37°C and 100% at 44°C), Mn (from 50% at 22°C, 15% at 37°C and 75% at 44°C), Cu (from 10% at 22°C, 0% at 37°C and 55% at 44°C), Co (from 10% at 22°C, 0% at 37°C and 30% at 44°C), Ni (from 0% at 22°C, 0% at 37°C and 5% at 44°C). Concerning the influence of salinity, the highest 10% NaCl induced to occurrence of susceptibility to all tested metals, followed by 2, 6 and 7% NaCl with susceptibility to 4 of 5 metals. The highest susceptibility levels to Zn and Mn was expressed in inverse order to 3, 4, 6, 7, 0, 2 and 0.5 % NaCl, while to Cu at 3, 2 and 7% NaCl. The tested strains were very resistant to Ni and Co, at the majority of tested salinities. Concerning the relationship between the chemical composition of the culture medium and the susceptibility levels to metals, the higher glucose concentration of 3% and the alkaline pH induced higher rates of susceptibility Mn, Zn and Ni. In conclusion the expression of heavy metals susceptibility features of the E. coli strains is strongly influenced by the incubation temperature and salinity, demonstrating the role of these parameters in the selection of resistance genes in the aquatic strains.

Keywords *Heavy metals salts, Esherichia coli, aquatic strains, divalent transitional metal salts*

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Introduction

Contamination of surface waters by fecal pollution constitutes a serious environmental and public health threat. In large complex systems, fecal pollution can be introduced from multiple sources, including sewage overflows, agricultural runoff, and urban storm water. Identifying and eliminating the source of contamination is not straightforward because assessment of fecal pollution generally

relies on a limited number of surface water samples to measure fecal indicator organism densities [1]. *Escherichia coli* is a type of fecal coliform bacteria commonly found in the intestines of animals and humans. The presence of *E. coli* in water is a strong indication of recent sewage or animal waste contamination. During rainfalls, snow melts, or other types of precipitation, *E. coli* may be washed into creeks, rivers, streams, lakes, or ground water. When these waters are used as sources of drinking water and the water is not treated or inadequately treated, *E. coli* may end up in drinking water [2,3]. Numerous studies provide evidence that *E. coli* can persist in the benthos environment and subsequently be detected in overlying surface waters. Residual populations were reported in one study, where fecal coliform levels in wastewater subjected to low temperatures decrease rapidly but then stabilize to 1 to 10% of the initial population size. In addition, *E. coli* that has been isolated from septic tanks has been found to be less diverse and genetically distinct than strains of *E. coli* from the inhabitants of the households served by those systems [3]. The aim of the present study was to investigate the heavy metals susceptibility profiles of *Escherichia coli* aquatic strains isolated from sea waters in different experimental conditions.

Experiment Details

100 environmental *E. coli* isolated in Constanta, Romania from sea water. The isolation and identification of these strains was based on filter membrane method, according to SR ISO 9308-1 2000 [4]. This technique consists in filtering 100 ml water sample using a filter membrane of 47 mm diameter. The membrane is applied on Lactose TTC medium poured in 47 mm diameter Petri plates. After 48 hours incubation at 44°C, *E. coli* will develop yellow colonies on the membrane. Oxidase and indole production test were performed additionally for the identification of *E. coli* strains. From 24 h bacterial cultures, there were performed suspensions with 0.5 McFarland standard density (in PBS). From the initial suspensions, 200µl inoculum was inoculated in 1 ml broth and incubated at 5 different temperatures (4ºC, 22ºC, 37ºC, 44ºC and 56ºC), in aerobic and respectively anaerobic conditions. From the same initial suspensions, 200µl inoculum was inoculates in 1 ml broth with 3 different pH values (5.0; 7.2 and 9.6), 2 different glucose concentrations (1.5% and 3%), and 9 different concentrations of Na Cl (0%, 0,5%, 2%, 3%, 4%, 5%, 6%, 7%, and 10%). All samples were incubated at 37ºC for 24 h (5). After 24 h, the sensitivity of bacterial cultures to 5 heavy metal salts (Cu, Co, Mn, Zn, Ni) was tested by adding solutions of these heavy metals oh 0.1 g/ml concentration (in DMSO). 5µl of these solutions were tested by spotting over the bacterial cultures layer, followed by incubation at 37ºC for 24 h. The sensitivity of the tested strains was expressed by the growth inhibition zone around the bacterial culture [5]. The experiments were repeated, by varying each time another physical or chemical parameter [6-7]. Concerning the study of the influence of physical factors, we analyzed the action of temperature, of O_2 partial pressure and of pH value on the susceptibility to divalent transitional metals. For the study of the temperature influence, the previously presented experiments were repeated at different incubation temperatures: 4°C, 22°C, 37°C, 44°C and 56°C. For the study of the influence of O_2 presence/absence, the experimental variants were incubated at: 4°C, 22°C, 37°C, 44°C and 56°C, for 24 hours, in aeropauses conditions (O_2 presence), and in anaerobiosis conditions (O_2 absence). The anaerobiosis conditions were achieved by adding paraffin oil. The study the influence of pH value on the culture medium was performed by cultivating bacteria on medium with pH adjusted to the desired value, with hydrochloric acid solutions, 10% solution for pH 5.0 value and 0, 1 N sodium hydroxide solution for pH 9.6 value. The of the influence of chemical factors included the study of

the influence of glucose concentration on the culture medium, the strains being seeded in 1 ml nutrient broth with concentration of 1.5% and 3% glucose and the influence of NaCl concentration on the culture medium, the bacterial strains being seeded in 1 ml nutrient broth with the following concentrations of salt: 0%, 0.5%, 2%, 3%, 4%, 5%, 6%, 7% and 10% [6-7].

Results and Discussions

Normal biological activity of microorganisms is strongly influenced by physical and chemical environmental conditions. Biological activity is greatest when environmental conditions are optimal to the species needs, when metabolic reactions involved in the processes of growth and multiplication take place normally. Optimal conditions for microorganisms living in natural environments are very rare, but bacteria compensates by high resistance to adverse conditions and a high capacity for adaptation, compared with higher organisms [8, 9].

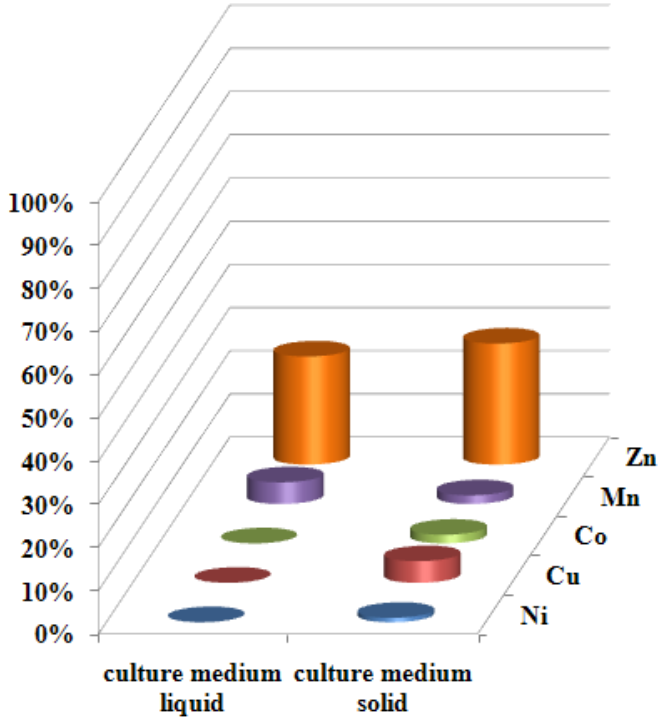

Figure 1: *The influence of the consistency of the culture medium on the antimicrobial activity (%) of divalent transitional metal salts in E. coli strains isolated from sea water.*

To understand the distribution of microorganisms in nature, and to develop methods to control their activities and to destroy harmful microorganisms, we have to know the influence of environment on microorganisms. Microorganisms react differently to changing environmental conditions, some being inhibited and others stimulated. The consistency of the culture medium (liquid *vs* solid) did not influence the antimicrobial activity of divalent transitional metal salts in *E. coli* strains isolated from sea water, antimicrobial activity being slightly increased when bacteria were grown in solid medium, with the exception of the Mn salt (figure 1). Temperature is one of the most important environmental factors influencing growth and survival of organisms, the rate of biochemical reactions in cells growing with it, but nucleic acids and other cellular components are sensitive to high temperatures above a certain level and can be irreversibly inactivated. Unlike the extreme temperatures that suspend any antimicrobial activity, the moderate ones are allowing the normal metabolic processes and growth and multiplication of microorganisms [11]. Each species growth corresponds to a minimum temperature (below which no growth occurs), an optimum temperature (allows the normal metabolic processes at which growth is fastest) and a maximum temperature (above which growth is impossible).

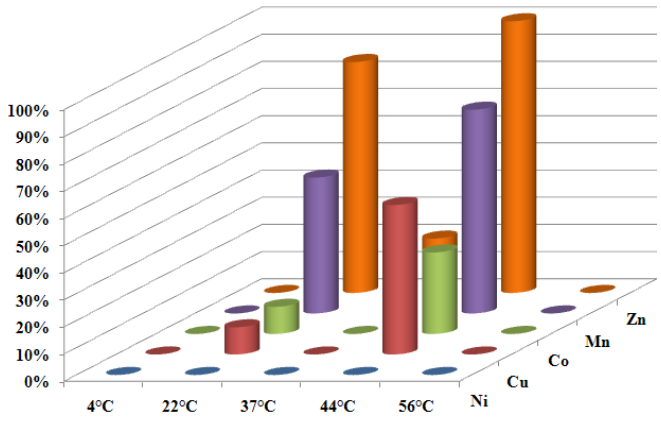

Figure 2: *Temperature's influence on the antimicrobial activity of divalent transitional metal salts (%), in aerobiosis conditions, in E. coli strains isolated from sea water.*

The levels of sensitivity were best expressed at a temperature of 44°C, followed by 22°C. The only metal that registered a total resistance phenomenon, indifferent of the bacteria cultivation temperature, was Ni (figure 2).

Incubation temperatures at 4°C and 56°C in aerobic conditions did not affect expression of bacterial sensitivity to divalent transition metal salts in *E. coli* strains isolated from sea water and in the other incubation conditions were recorded the following results: i) at physiological growth temperature (37°C) the *E. coli* strains showed a total resistance to metal salts of Ni, Cu, and Co and the other metals were registering a slight decrease in resistance to 85 Mn %, and Zn to 80%; ii) a lower incubation temperature (22°C) has brought changes in the behavior of *E. coli* strains, especially against Zn salts, to which salt the strains became 85% sensitive, followed by Mn with 50% sensitivity, and for Co and Cu salts, only 10% of the strains studied became susceptible after cultivation at 22°C; iii) incubation at a higher temperature (44°C) completely changed the behavior of studied strains, they becoming fully susceptible to Zn. In terms of sensitivity to other salts, a slight decrease in resistance to Mn (75% of the strains studied were susceptible), followed by Cu (55%) and Co (30%).

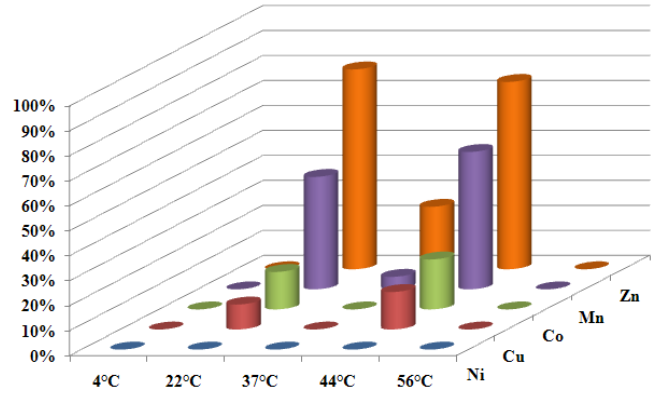

Figure 3: *Temperature's influence on the antimicrobial activity of divalent transitional metal salts (%), in anaerobiosis conditions, in E. coli strains isolated from sea water.*

At incubation temperatures of 44°C and 22°C only Zn showed a bactericidal effect. The levels of sensitivity were best expressed at a temperature of 44°C, followed by 22°C.

Co is present in small amounts in the environment, but it can become toxic when they accumulate in living cells, a mechanism still unclear. Regarding toxicity of Co in *E. coli* and *Salmonella enteritidis* strains it was demonstrated that it inhibits S and Fe compounds with an increased oxidative stress and sulfur assimilation [11]. It was observed that heat shock induced by rapid transfer of *E. coli* cells at 37°C to 5°C and kept at this temperature for 60 minutes decreased the bacterial resistance to acrylic acid and Cu ions [12-14]. In anaerobic conditions, we can observe that the levels of sensitivity were also best expressed at the temperature of 44°C, followed by 22°C. The only metal that registered a total resistance phenomenon, indifferent of the bacteria cultivation temperature, was Ni (figure 3). The growth of bacteria in aerobiosis and anaerobiosis conditions has shown that the presence/absence of O_2 did not influence the phenomenon of bacterial resistance to the studied salts. It should be noted that at 37°C, in both aerobic and anaerobic conditions, the antimicrobial activity of Ni, Co and Cu was completely eliminated, and the susceptibility rates for Mn and Zn were drastically reduced (figure 4).

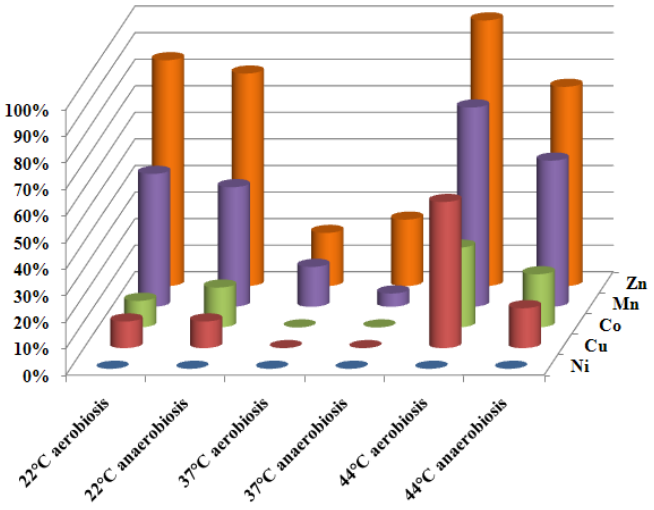

Figure 4: *The influence of the O2 presence/absence on the antimicrobial activity of divalent transitional metal salts (%), in anaerobiosis conditions, in E. coli strains isolated from sea water.*

Each microbial species corresponds to a optimum pH of development, most developing at a neutral pH (7.2

to 7.6), except *Vibrio cholerae* which develop at an alkaline pH (8.9 to 9.2), while yeasts and molds prefer an acidic pH [15]. We could notice that the levels of sensitivity were best expressed at alkaline pH; the most constant results were registered for the salts of Cu, Co, and Zn (figure 5). Mn sensitivity slightly increased at acid pH values in comparison with neutral pH, while at alkaline pH, the sensitivity for Mn and Zn significantly increased (figure 5). Ni had antimicrobial activity only at alkaline pH (figure 5).

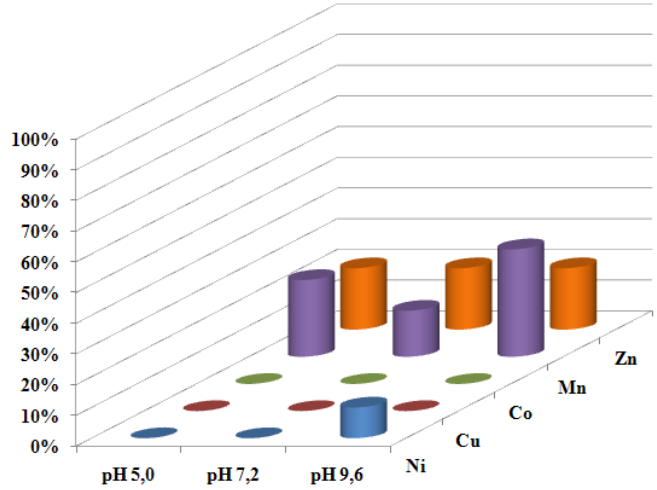

Figure 5: *The influence of the culture medium's pH variation on the antimicrobial activity of divalent transitional metal salts (%), in anaerobiosis conditions, in E. coli strains isolated from sea water.*

Concerning the glucose concentration, the high glucose concentration (3% versus 1,5%) significantly increased bacteria sensitivity to Mn and Zn salts and with only 5 % for Ni and Cu salts. The only metal not exhibiting any antimicrobial action both in low (1,5%) and in high glucose concentrations (3%) was Co (fig. 6). Microbial sensitivity to divalent transitional metal salts was strongly influenced by the salinity variations in the culture medium. Low NaCl concentrations (0% and 0,5%), as well as the 5% NaCl concentration induced the same sensitivity profile (for example, sensitivity to Mn and Zn). At3%, 4%, 6%, 7% NaCl concentrations, we noticed the presence of sensitivity patterns with 3 or even 4 markers, such as sensitibity to the salts of Zn, Mn and Cu or Zn, Mn and Ni or Zn, Mn, Ni and Cu. At the highest concentration of 10% NaCl, we noticed a lower sensitivity level for all tested divalent transitional metal salts (figure 7). Zn had bactericid activity at 3% and 4% NaCl concentrations in the culture medium (figure 7). The only NaCl concentration where Mn had bactericidal action was 3% (figure 7). Zn and Mn exhibited antimicrobial activity in all tested NaCl concentrations (figure 7).

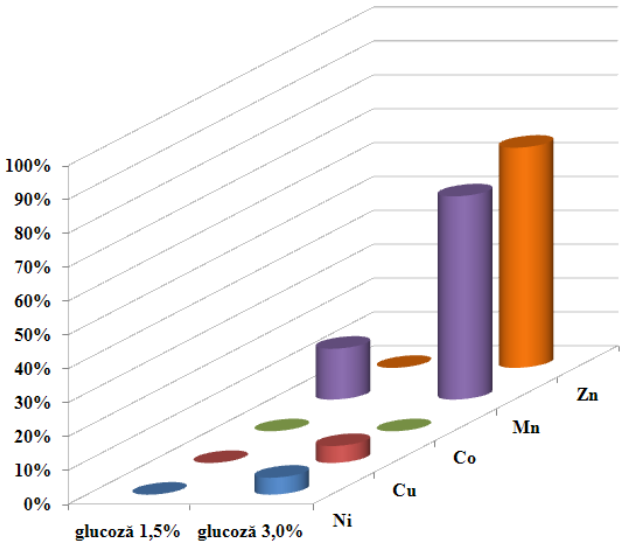

Figure 6: *The influence of the variation of the glucose concentration in the culture medium on the antimicrobial activity of divalent transitional metal salts (%), in anaerobiosis conditions, in E. coli strains isolated from sea water.*

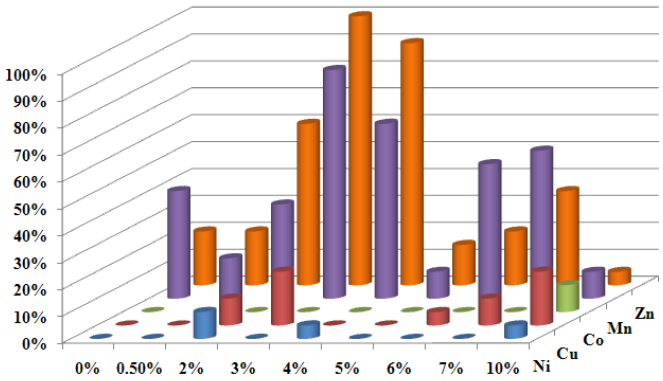

Figure 7: *The influence of the NaCl concentration variation in the culture medium on the antimicrobial activity of divalent transitional metal salts (%), in anaerobiosis conditions, in E. coli strains isolated from sea water.*

Conclusions

The sensitivity profiles of the de *Escherichia coli* strains isolated from sea water to divalent transitional metals (Ni, Co, Cu, Mn and Zn) varied depending on the experimental conditions (cultivation temperature, O_2 presence/absence, pH and glucose and NaCl concentrations in the culture medium), the highest antimicrobial activity being manifested by the Zn and Mn salts. The growth temperature, pH and salinity induced different expression levels of susceptibility to heavy metals. The highest levels of sensitivity were registered at the temperature of 44°C, alkaline pH, high glucose concentrations and average salinity, and the lowest ones at the temperature of 37°C, neutral pH, low glucose concentrations and low salinity. The lowest susceptibility rates were noticed at 37ºC, neutral pH, low glucose concentration and low salinity. The Zn salt exhibited a bactericidal effect at the incubation temperatures of 44°C and 22°C, in aerobiosis conditions, in media supplemented with 3% and 4% NaCl, and the Mn salt at 3% NaCl concentrations. The Ni salt exhibited antimicrobial activity at alkaline pH conditions (9,6) in culture media with2%, 4% and 10% NaCl, and the Co salt at 22°C and 44°C in both aerobiosis and anaerobiosis incubation conditions, in media with 10% NaCl concentration. The cultivation in aerobic/anaerobic conditions did not influence the sensitivity to various divalent transitional metal salts.

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