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Prevalence of heavy metal and antibiotic resistance in bacterial isolates from wastewater and receiving aquatic environments

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ABSTRACT

Industrialisation and the continuous urban development are an important cause of release of heavy metals and antibiotic products into aquatic environments, affecting both water quality and human health. In this study, antibiotic and heavy metals resistance exhibited by strains isolated from different types of wastewater and freshwater receiving system were assessed. The results revealed that 40% of the isolates were resistant to AMC, 30% to KZ and TE, 25% to FOX and CAZ, 20% to CRO and SXT, 15% to ATM, CIP, PIP and IMP, 10% to NN, and 5% to CTX and AN. Majority of aquatic bacterial isolates showed multiple metal resistance (MMR), from two to six heavy metals in different combinations. The heavy metals resistance profile showed that 15% of the isolates were resistant to mercury, 40% to copper, 75% to chromium and 80% to zinc. All strains (100%) were resistant to cadmium and aluminium. Most of tested strains tolerated various concentrations of heavy metals and the minimum inhibitory concentration (MIC) ranged from 15.6 µg/ml for Hg to ≥125 µg/ml for Cu, Zn, Cr, Al and Cd. These results of the present study have shown that different types of wastewater represent an important source of heavy metal and antibiotics resistant bacteria and contribute to the spread of biological emerging contaminants into aquatic ecosystems, highlighting the need for further measures of water quality monitoring.

Keywords: *antibiotic resistance, heavy metals resistance, wastewater, urban influent.*

1. INTRODUCTION

In recent decades, the widespread and uncontrolled use of antibiotics, both in human and veterinary medicine, and in agriculture, has exerted a major impact on bacterial communities, leading to the selection of antibiotic resistant bacteria, both in the clinic and in environment [1], which indicates the need to investigate natural antibiotic resistance pools. On the other hand, the presence of toxic heavy metal contaminants in aquatic environments, arising from the discharge of untreated effluents into water bodies, is one of the most important environmental pollution issues [2,3]. Moreover, metal polluted industrial effluents discharged into wastewater treatment plants could lead to high metal concentrations in the activated sludge [4,5] and local microbial populations became adapted to the toxic concentrations of heavy metals [6,7].

Recent studies [8,9] point out that water pollution (nitrogen, heavy metals) can favour the selection and dissemination of integron-like gene structures that are supposed to play an essential role in the development of multiple antibiotic resistance, creating real platforms for grouping and integrating gene cassettes [10,11]. Integrons are modular structures with a major role in the evolution of antibiotic resistance. Integrons contain adjacent co-expressed genes that confer multi-resistance to antibiotics of different classes and resistance to other pollutant compounds (heavy metals, biocides). Due to the genetic organization, the presence of a pollutant that is the substrate of a certain resistance mechanism located on the integron, will also co-select for other co-expressed antibiotic resistance mechanisms and will lead to maintaining them in the bacterial population. For example, Class I integrons containing the quaternary ammonium salt resistance gene (*qac*) also maintain antibiotic resistance (*sulI*) as a consequence of water contamination with antimicrobial agents (disinfectants, detergents etc.) [12,13].

The main factor contributing to the horizontal transfer of antibiotic resistance genes is the selective pressure exerted by the intensive use of antibiotics for human and veterinary treatment, resulting in an increasing number of bacterial strains resistant to an increasing number of antibiotics [14]. High selective pressure facilitates the acquisition of antibiotic resistance genes, which can lead to an increase in the incidence of resistant bacteria, allowing rapid evolution and global scale dissemination [15]. Furthermore, the occurrence of sub-inhibitory antibiotic concentrations in the environment may accelerate the horizontal transfer and dissemination of antibiotic resistance genes in the environment [16]. Cross-resistance to heavy metals and antibiotics and/or the association of antibiotic resistance genes with resistance determinants to heavy metals (co-resistance) on mobile genetic elements can also favour the persistence and durability of resistance gene pools, even in the absence of selective pressure exerted by the antibiotic [17,18].

The aim of this study was to assess the incidence of heavy metal and antibiotic resistance patterns in bacterial isolates from different types of wastewater and aquatic environments which received effluents from urban sewage.

2. EXPERIMENTAL SECTION

Bacterial isolation. Four samples of wastewater (urban, hospital, poultry farm) and two of surface water (Table 1) were collected according to ISO 19458/2006 [19] using sterile glass bottle, all sampling points being located in the southeast part of the country, in and around Bucharest, the capital of Romania and the first economic centre of the country. The isolation and identification of bacterial strains was based on standardized membrane filtration method using cellulose nitrate filters of 0.45 μm pore size, Millipore, USA (SR EN ISO 8199/2008) [20]. For further study, 10 yellow-orange colonies with a yellow halos (lactose-positive bacteria) on Lactose TTC Agar with Tergitol 7 medium (Merck) were randomly selected from each sample (SR EN ISO 9308-1/2004/AC/2009) [21]. Representative colonies were purified on Trypticase soy agar. Preliminary identification of strains obtained in pure culture was based on Gram staining and oxidase reaction. The bacterial isolates were further identified using biochemical tests API 20E (bioMérieux). Strains that could not be identified at the genus level have been eliminated from this study. The identified strains were preserved at -70^{0}C in LB broth supplemented with 15% (v/v) glycerol at -70^{0}C. In the current study, among a total of 60 environmental isolates, 20 species of Gram-negative bacteria were identified and selected for further study.

Table 1. Sources of water samples

Sampling points	Geographical coordinates	Type of the water sample
Dambovita River - WWTP upstream	Long.:26.219730^o Lat.: 44.395552^o	Surface water – upstream of the WWTP
Urban WWTP influent	Long.:26.231728^o Lat.: 44.395100^o	Polluted with domestic and industrial wastewater
Urban WWTP effluent	Long.:26.236542^o Lat.: 44.395946^o	Treated effluent from urban WWTP
Dambovita River - WWTP downstream	Long.:26.239748^o Lat.: 44.396379^o	Surface water - downstream of the WWTP
Poultry farms sewage	Long.:25.881163^o Lat.: 44.344264^o	Treated effluent (primary sedimentation only) from poultry farms
Bucharest hospital sewage	Long.:26.158853^o Lat.: 44.462296^o	Untreated hospital effluent

Antibiotic resistance pattern. Antibiotic susceptibility testing was performed by agar disk diffusion in accordance with the Clinical and Laboratory Standards Institute (2011) [22] recommendations, using standard discs (bioMérieux) for different groups of Gram-negative bacteria, i.e.: ampicillin (AMP, 10), piperacillin (PIP, 100), cefazolin (KZ, 30), cefoxitin (FOX, 30), cefotaxime (CTX, 30), ceftriaxone (CRO, 30), ceftazidime (CAZ, 30), amoxicillin-clavulanic acid (AMC, 20+10), ticarcillin-clavulanic acid (TIM, 75+10), aztreonam (ATM, 30), imipenem (IMP, 10), amikacin (AN, 30), tobramycin (NN, 10), trimethoprim-sulfamethoxazole (SXT, 1.25+23.75), ciprofloxacin (CIP, 5) and tetracycline (TE, 30). After 24 h of incubation at 37^{0}C, organisms were classified as sensitive (S), intermediate (I) or resistant (R) based on CLSI break points. Intermediate strains were included in the resistant class.

MIC determination. The resistance level of bacterial strains to the increasing concentrations of heavy metals [Cu^{2+} ($CuCl_2$), Zn^{2+} ($ZnCl_2$), Cr^{2+} ($CrCl_2$), Al^{2+} ($AlCl_2$), Cd^{2+} ($CdCl_2$), Hg^{2+} ($HgCl_2$)] was evaluated by the serial microdilution method in Mueller Hinton broth using 96-wells microtiter plate. The wells contained 100 μl of MH broth plus heavy metals at binary concentration ranging from 0.9 to 500 μl/ml. Subsequently, the wells were inoculated with 20 ml of microbial suspension with a McFarland density of 0.5. For each test was also performed a positive control of bacterial growth (wells containing only culture medium inoculated with microbial suspension) and a control of medium sterility (wells containing only culture medium). After incubating the plates at 37°C for 24 hours, the degree of microbial culture growth in the presence of binary concentrations of the heavy metal salts was quantified by spectrophotometric measurement at 620 nm. The lowest metal concentration, which inhibited the bacterial growth, was referred to as MIC (minimum inhibitory concentration). The levels of heavy metals in water samples were determined using an atomic absorption spectrophotometer (Solaar M5) with a mixture of air and acetylene for flame combustion.

MBEC determination. The study of the influence of various heavy metal salts on the development of microbial biofilms on the inert substrate and the determination of MBEC (minimum biofilm eradication concentration) were performed using the following working protocol: isolated microbial strains were inoculated in nutrient broth (96-wells microtiter plate) in the presence of heavy metals at binary concentration ranging from 0.9 to 500 μl/ml and were incubated at 37°C for 24 h. Plates were emptied and washed with sterile physiological water. The adhered cells were fixed for 5 minutes with 100 μL of 80% methanol. After removing the methanol, the adhered cells were coloured with 1% violet crystallized alkaline solution (100 μl/well). The microbial biofilms developed were resuspended in 33% acetic acid and the suspension intensity was determined spectrophotometrically by measuring absorbance at 490 nm. The lowest concentration of the metals, which inhibited the bacterial biofilm growth, was referred to as MBEC.

3. RESULTS SECTION

The antibiotic resistance and the heavy metals resistance profile of the studied Gram-negative isolates are presented in Table 2. Both antibiotic-susceptible bacterial strains and those with different β-lactam and non-β-lactam antibiotic resistance phenotypes showed increased resistance to the studied heavy metals.

Table 2. Comparative antibiotic and heavy metals resistance profiles

Water source	Bacterial strain	Antibiotic resistance profile	Heavy metal resistance profile
WWTP upstream river	*Klebsiella pneumoniae*	S*	Zn^{2+}, Cr^{2+}, Al^{2+}, Cd^{2+}
	Enterobacter cloacae	S	Zn^{2+}, Cr^{2+}, Al^{2+}, Cd^{2+}
Urban WWTP influent	*Enterobacter cloacae*	ATM, CRO, CAZ, SXT, TE, CIP	Cu^{2+}, Zn^{2+}, Cr^{2+}, Al^{2+}, Cd^{2+}, Hg^{2+}
	K. pneumoniae	S	Zn^{2+}, Cr^{2+}, Al^{2+}, Cd^{2+}
	K. oxytoca	AMC, SXT, CIP	Cu^{2+}, Zn^{2+}, Cr^{2+}, Al^{2+}, Cd^{2+}, Hg^{2+}
Urban WWTP effluent	*K. pneumoniae*	AMC, KZ, TE, PIP	Zn^{2+}, Cr^{2+}, Al^{2+}, Cd^{2+}
	K. pneumoniae	AMC, KZ, FOX	Cu^{2+}, Zn^{2+}, Cr^{2+}, Al^{2+}, Cd^{2+}
	K. pneumoniae	S	Zn^{2+}, Cr^{2+}, Al^{2+}, Cd^{2+}
WWTP downstream river	*K. oxytoca*	AMC, SXT, TE, CIP, PIP	Zn^{2+}, Cr^{2+}, Al^{2+}, Cd^{2+}
	Citrobacter freundii	CRO, CAZ, IMP	Zn^{2+}, Cr^{2+}, Al^{2+}, Cd^{2+}
	K. oxytoca	S	Zn^{2+}, Cr^{2+}, Al^{2+}, Cd^{2+}
Hospital untreated effluent	*Escherichia coli*	S	Cu^{2+}, Zn^{2+}, Cr^{2+}, Al^{2+}, Cd^{2+}
	K. oxytoca	AMC, ATM, KZ, FOX, CRO, CAZ, TE, PIP, NN, AN	Cu^{2+}, Zn^{2+}, Cr^{2+}, Al^{2+}, Cd^{2+}, Hg^{2+}
	K. pneumoniae	AMC, KZ, FOX	Cu^{2+}, Zn^{2+}, Cr^{2+}, Al^{2+}, Cd^{2+}
	K. pneumoniae	AMC, KZ, FOX, IMP, CRO, CAZ, TE, NN, PIP, AN	Cu^{2+}, Zn^{2+}, Cr^{2+}, Al^{2+}, Cd^{2+}
Poultry effluent	*E. coli*	ATM, IMP, SXT	Cu^{2+}, Al^{2+}, Cd^{2+}
	Citrobacter freundii	CAZ, CTX, TE	Zn^{2+}, Al^{2+}, Cd^{2+}
	Citrobacter freundii	S	Al^{2+}, Cd^{2+}
	K. pneumoniae	AMC, KZ, FOX	Al^{2+}, Cd^{2+}
	K. oxytoca	S	Al^{2+}, Cd^{2+}

*S = susceptible

The results of this study revealed that 40% of the isolates were resistant to AMC, 30% to KZ and TE, 25% to FOX and CAZ, 20% to CRO and SXT, 15% to ATM, CIP, PIP and IMP, 10% to NN, and 5% to CTX and AN. It was observed among the isolates different combinations of the antibiotic patterns. For example, 35% of the isolates were resistant to three antibiotics, 15% of the isolates exhibited resistance from four to six antibiotics, whereas 10% of the isolates were resistant to ten antibiotics.

The assessment of heavy metal resistance level showed that 15% of the isolates were resistant to mercury, 40% to copper, 75% to chromium and 80% to zinc. All strains (100%) were resistant to cadmium and aluminium. The following decreasing trends of bacterial resistance to the tested heavy metals was found: Al^{2+} =Cd^{2+} >Zn^{2+} >Cr^{2+} >Cu^{2+} >Hg^{2+}.

Majority of aquatic bacterial isolates showed multiple metal resistance (MMR). For instance, 15% of the isolates exhibited resistance to two heavy metals, 10% to three heavy metals, while 40% of the isolates showed resistance to four heavy metals. The resistance to five heavy metals was detected in 20% of the isolates, whereas 15% of the isolates exhibited resistance to six heavy metals.

The bacterial strains isolated from different aquatic environments, with different phenotypes of antibiotic resistance, were selected and tested in the presence of various heavy metal salts for evaluating the minimum inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC) developed on inert substrate. In order to explore the relationship that occur between different heavy metal salts (binary concentrations) and microbial culture growth level and/or bacterial biofilm development, contour plots were drawn (Fig. 1).

In the case of Cu, Zn, Cr, Al and Cd, the minimum inhibitory concentration (MIC) values were ≥125 μg/ml for most of bacterial strains tested. This heavy metals concentration was much higher than the concentration of the respective heavy metals in the analysed water samples. Thus, the values of the heavy metal concentrations ranged in the water samples between 0.118 and 0.579 μg/ml for Zn, between 0.005 and 0.036 μg/ml for Cu, between 0.002 and 0.014 μg/ml for Cr and between 0.0003-0.0054 μg/ml for Cd. In the case of Hg, MIC values were lower than those for previous heavy metals, being for most strains around 15.6 μg/ml, but there were also strains sensitive to very low concentrations, of 0.9 μg/ml.

The MBEC values for tested heavy metals were also high, being for most analysed strains, of ≥250 μg/ml, which shows that in investigated aquatic environments, the presence of heavy metals, even in high concentrations, does not interfere with the ability of bacteria to survive by forming sessile microbial communities, which are favourable to adaptation of environmental condition. The resistant strains isolated from aquatic environments exhibited multiple drug resistance to beta-lactams, third generation cephalosporins, tetracyclines, folate inhibitors, aminoglycosides and fluoroquinolones, correlated with a high resistance to heavy metals. The resistance of environmental isolates to CAZ and CTX suggests that these organisms may produce extended-spectrum β-lactamases (ESBLs), typically as a consequence of plasmids acquisition which often carry other resistance genes as well [23]. It has been shown that water pollution with organic or inorganic matters (nitrogen, heavy metals, biocides) can favour the selection and dissemination of integron-like gene structures that can disseminate genes resistant to different antibiotic classes and other

compounds with antimicrobial activity (heavy metals, biocides) [24,25].

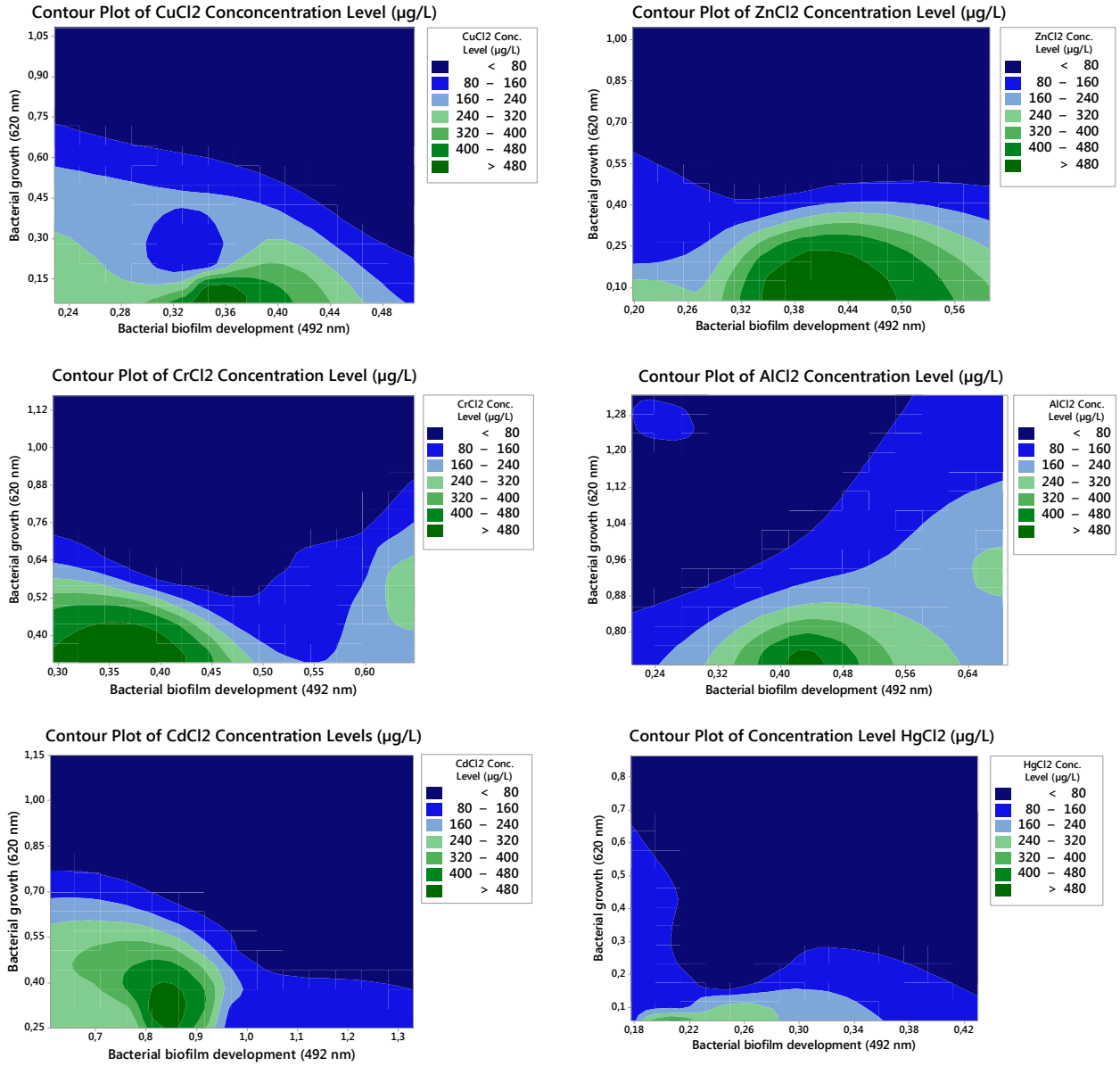

Figure 1. Microbial culture growth level (620 nm) and bacterial biofilm development (492 nm) in the presence of various heavy metal salts (binary concentrations).

Bacteria from heavy metal polluted environments have an increased prevalence of antibiotic resistance phenotypes compared to those in control areas [26]. The *tetA(41)* gene encoding tetracycline resistance, that has been discovered recently, was identified in *Serratia marcescens* strains isolated from a heavy metal polluted water stream [27], providing indirect samples of co-selection. Thus, resistance plasmids may be responsible for conferring not only antibiotic resistance but also simultaneous resistance to heavy metals, favouring the maintenance and spread of antibiotic resistance due to the selective pressure exerted by heavy metals presence [28,29].

4. CONCLUSIONS

The bacterial isolates from wastewater and receiving river, both antibiotic susceptible strains and those with different antibiotic resistance phenotypes, exhibited increased resistance to heavy metals (Cu, Zn, Cr, Al and Cd). The MIC and MBEC values, for most tested strains, were much higher than the concentration of the respective heavy metals in the analysed water samples, showing that heavy metals, even in high concentrations, do not interfere with the ability of bacteria to survive by forming sessile microbial communities, which are favourable for adaptation to environmental condition. In addition, the level of heavy metal resistance in environmental bacterial isolates could be used as a potential bioindicator of metal ecotoxicity to other aquatic organisms.

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