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ASSESSING THE IMPACT OF HEAVY METALS AND ANTIBIOTICS ON BACTERIAL ISOLATES FROM WASTEWATER TREATMENT PLANT

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ABSTRACT. The microorganisms isolated from water samples at the wastewater treatment facility Kruševac (Serbia) were screened for resistance to heavy metals and antibiotics. Isolates were identified using morphological and biochemical tests, as well as MALDI-TOF. The resistance to heavy metals was evaluated by determining the minimal inhibitory concentration (MIC) and the minimal microbicidal concentration (MMC) using the microdilution method. The minimal inhibitory concentration and disc diffusion methods were used to assess antibiotic resistance. Isolates were identified as *Morganella morganii* PMFKG-K1, *Klebsiella oxytoca* PMFKG-K2, *K. oxytoca* PMFKG-K3, *K. oxytoca* PMFKG-K4, *Serratia liquefaciens* PMFKG-K5, *S. liquefaciens* PMFKG-K6, *Escherichia coli* PMFKG-K7, and *Bacillus cereus* PMFKG-K8. The obtained results indicated that most isolates in planktonic form showed resistance in the presence of heavy metals such as Pb^{2+}, Zn^{2+}, Cu^{2+}, and Mn^{2+}, but showed sensitivity in the presence of Cd^{2+}, Hg^{2+}, and Cr^{6+} and antibiotics. All isolates exhibited desirable characteristics for potential use in bioremediation.

Keywords: bacterial isolates, wastewaters, identification, heavy metals, antibiotics

INTRODUCTION

Industrialization and urbanization are the main sources of several types of hazardous waste. According to the literature, the most important environmental pollutants are heavy metals, which have become a serious threat to all life forms, including microorganisms (TABAK *et al.*, 2005). Not all metals are toxic to microorganisms; some of them are important for their normal functioning, playing key roles in numerous cellular processes and being part of the structure of the cell membrane, proteins, and DNA (WALDRON and ROBINSON, 2009). However, in high concentrations, they can also be toxic to cells and disrupt the metabolism of microorganisms. The toxicity of a metal depends on its biological availability and absorbed dose, and the main mechanisms by which metals harm microorganisms are the disruption of enzymatic activity, the presence of reactive oxygen species, DNA damage, and inhibition of

transcription and translation (HASSEN *et al.*, 1998). In addition to the affinity of heavy metals to bind to thiol groups and macro biomolecules (NIES, 1999), the toxicity of heavy metals also depends on environmental factors such as pH and oxygen, which affect the solubility and availability of heavy metals (SCHULZ-ZUNKEL and KRUEGER, 2009). Because heavy metals cannot be completely removed from nature, microorganisms attempting to survive and avoid cellular damage in an environment contaminated with heavy metals develop a variety of mechanisms to combat their toxic effects. This property of microorganisms is used in bioremediation, and hazardous metals are converted into less toxic metals with their assistance (GADD, 2000).

Because metals do not degrade, are stable, and remain in nature for a long time, they can exert pressure and influence the spread of antibiotic resistance genes through mechanisms such as co-resistance, cross-resistance, and co-regulation (ASHBOLT *et al.*, 2013). NGUYEN and colleagues (2019) compared data from different studies and discussed the correlation between resistance to heavy metals and antibiotics registered in microorganisms living in different environments. They concluded that this phenomenon is more common in sediments than in water bodies. This is thought to be due to dilution in aquatic environments, while sediments are more like biofilms (NGUYEN *et al*., 2019). Whether these bacteria are found in soil or water, they pose a major threat to human health because horizontal gene transfer results in the propagation of antibiotic resistance genes, creating a community of microorganisms that are resistant to antimicrobials (HARRIS *et al*., 2012). Some studies have investigated the exchange of antibiotic resistance genes between environmental bacteria and pathogenic bacteria (SEIER-PETERSEN *et al*. 2014; DIN *et al.*, 2021), while others have found no correlation between elevated concentrations of metal ions and antibiotic resistance (DEREDJIAN *et al*., 2011; GHAZISAEEDI *et al.*, 2020).

There are numerous studies examining the effect of heavy metals and antibiotics on planktonic cells of microorganisms, that have been isolated from different areas (HASSEN *et al*., 1998; SELVI *et al*., 2012; GRUJIĆ *et al*., 2018; TAHMOURESPOUR, 2021). Based on the effect that heavy metals have on microorganisms, the current study evaluates their further application in bioremediation processes. Specifically, this study aims to investigate the resistance of selected microorganisms isolated from wastewater treatment plant to certain concentrations of heavy metals or antibiotics.

MATERIALS AND METHODS

Isolation and identification of microorganisms

The wastewater samples were collected from the wastewater treatment plant in Kruševac (Serbia) at the beginning of May 2022. Wastewater samples were taken from the biological reactor and the secondary sedimentation tank. The colonies of bacterial species most prevalent in the examined samples were isolated and grown on differential and selective mediums for further research. Pure cultures were obtained by screening the selected isolates with the method of exhaustion. Cultures were incubated at 26ºC and 37ºC for 24 hours before the examination of morphological and biochemical characteristics. Morphological characteristics (shape, Gram staining) were examined using a light microscope (Olympus, U-RFLT-T, GmbH, Germany). The examination of biochemical characteristics was carried out using catalase, oxidase, citrate, indole, H_2S, lactose, sucrose, glucose, dextrose, urease, and Methyl Red/Voges Proskauer (MR/VP). The identification of bacterial isolates was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Resistance of planktonic cells to heavy metals and antibiotics

Preparation of the solutions of metals and antibiotics

Resistance of planktonic cells was tested in the presence of heavy metals (Pb^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Cd^{2+}, Hg^{2+} and Cr^{6+}) originating from the $Pb(NO_3)_2$, $ZnSO_4$, $CuSO_4$, $MnCl_2$, $CdSO_4$, $HgCl_2$, and $K_2Cr_2O_7$ salts (Sigma-Aldrich, St. Louis, MO, USA), as well as antibiotics [ampicillin (Am) (ATB Pharma, Belgrade, Serbia), azithromycin (He) (Hemofarm, Vršac, Serbia), cefepime (C) (Hemofarm, Vršac, Serbia), ceftriaxone (Az) (Hemofarm, Vršac, Serbia), ertapenem (E) (MERCK SHARP & DOHME D.O.O., Belgrade, Serbia), levofloxacin (Le) (Zentiva Pharma d.o.o., Belgrade, Serbia), tetracycline (T) (Pfizer Inc., USA)]. All metal compounds and antibiotics were dissolved in sterile distilled water.

Microdilution method

The microdilution method was used for the assessment of the effect of selected heavy metals and antibiotics on the planktonic growth of isolated bacteria (SARKER *et al*., 2007). 100 µL of TSB was added to each well of the 96-well plate. In the first row of the plate, 100 µL of the stock solution of the tested substances was added, and then serial dilutions were made. The concentration range for Pb^{2+}, Cu^{2+} and Mn^{2+} was from 4 to 0.031 mg/mL, for Zn^{2+} and Cd^{2+} from 20 to 0.156 mg/mL, for Hg^{2+} and Cr^{6+} from 0.5 to 0.003 mg/mL, while the obtained concentration range for all antibiotics was from 0.5 to 0.003 mg/mL. After that 2.5 µL of the glutathione (TwinLab) which served for neutralization of heavy metals was added to the part of the plates where heavy metals were dispensed, while 10 µL of resazurin (Alfa Aesar GmbH & Co., KG, Karlsruhe, Germany) was added to all wells of the plate. Sterile 0.85% saline was used to create initial suspensions of a few fresh colonies that were taken from slant agar. Bacterial suspensions were obtained from cultures, incubated for 24 h at 26ºC, and adjusted around 10^6 CFU/mL by dilution. 10 µL of suspension of each tested microorganism was added to the plates. Each plate included growth control and sterility control. The plates were incubated for 24 h at 26ºC and after that, MIC values were determined, while MMC values were determined after 48 h. Based on the color shift of resazurin, it was determined that there is a minimum inhibitory concentration of the dissolved material at which microorganisms' growth is inhibited. All measurements were made in triplicate, and values were constant. The minimum lethal concentration (the concentration that did not allow the growth of microorganisms on solid agar) was determined by transferring 10 µL of the sample without a color change to solid agar.

Disk-diffusion method

The resistance ability of isolates to antibiotics was determined by using the disc diffusion method. Bacterial suspensions were prepared by the direct colony method. Initial bacterial suspension contained about 10^8 CFU per mL. After suspensions were made, an inoculum of bacteria was swabbed onto the surface of Mueller–Hinton agar plates. Saturated filter paper discs, with a standardized concentration of antibiotics, were placed on the surface of plates, and the size of the inhibition zone around each disc was recorded after 24 h of incubation. The discs contained the following four antibiotics with various ways of action: tetracycline (30 µg); streptomycin (10 µg); cefotaxime (5 µg) (Biolab, Budapest, Hungary), and chloramphenicol (30 µg) (Torlak, Belgrade, Serbia). The interpretation of zones of inhibition (mm) was conducted according to EUCAST (2023). The ranges of inhibition zones for Enterobacteriales according to EUCAST (2023) are: to cefotaxime sensitive ≥20, resistant ≤17; and to chloramphenicol sensitive ≥17, resistant ≤17. Considering that for Enterobacteriales range of inhibition zones for streptomycin and tetracycline are not included in susceptibility test

reports of EUCAST (2023), tested isolates were classified as sensitive or resistant to these antibiotics according to the diameter of inhibition zone given in the standard antibiotic disc chart (streptomycin sensitive ≥15, resistant ≤11; tetracycline sensitive ≥19, resistant ≤17) (FERREIRA DA SILVA *et al*., 2007; FOUAD, 2011). Since tested antibiotics did not have defined clinical breakpoints for *Bacillus* spp. in EUCAST guidelines, we also classified *B. cereus* as sensitive or resistant according to the diameter of inhibition zone given in the standard antibiotic disc chart (cefotaxime sensitive ≥23, resistant ≤15; chloramphenicol sensitive ≥18, resistant ≤12; streptomycin sensitive ≥15, resistant ≤11; tetracycline sensitive ≥19, resistant ≤17) (FERREIRA DA SILVA *et al*., 2007; FOUAD, 2011).

Statistical analysis

The measurement results were statistically processed using Microsoft Excel (Redmond, Washington, DC, USA).

RESULTS AND DISCUSSION

Isolation and identification of microorganisms

Eight bacterial strains were identified as *Morganella morganii* PMFKG-K1, *Klebsiella oxytoca* PMFKG-K2, *K. oxytoca* PMFKG-K3, *K. oxytoca* PMFKG-K4, *Serratia liquefaciens* PMFKG-K5, *S. liquefaciens* PMFKG-K6, *Escherichia coli* PMFKG-K7, and *Bacillus cereus* PMFKG-K8. Gram staining and biochemical assays (Table 1) revealed that isolated Gram-negative bacteria include *M. morganii*, *K. oxytoca*, *S. liquefaciens* and *E. coli*, whereas only *B. cereus* was isolated Gram-positive bacteria. The obtained results showed the analyzed strains matched with high score values with each corresponding strain type already present in the Bruker MALDI Biotyper database. Score values were between 1.78 and 2.36.

Resistance of planktonic cells to heavy metals and antibiotics

By determining the minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC), the resistance of planktonic microorganisms was tested in the presence of heavy metals (Table 2). All tested isolates showed resistance to Pb^{2+} (MIC between 2 and >4 mg/mL), Zn^{2+} (MIC between 5 and >20 mg/mL), Cu^{2+} (MIC between 0.5 and 4 mg/mL), and Mn^{2+} (MIC between 0.25 and >4 mg/mL). The lowest resistance for all tested isolates was observed in the presence of Cd^{2+} (MIC between <0.156 and 2.5 mg/mL), Hg^{2+} (MIC between 0.015 and 0.25 mg/mL), and Cr^{6+} (MIC between 0.007 and 0.25 mg/mL), except for both strains of *S. liquefaciens* PMFKG-K5 and PMFKG-K6 that showed resistance in the presence of Cd^{2+} (MIC and MMC between 1.25 and >20 mg/mL).

In the study by ORJI *et al.* (2021), the trend of heavy metal removal by the strain *Morganella* sp. WEM7 was as follows: Cu > Ni > Pb > Hg > Zn > Cd (100, 98.64, 97.48, 93.33, 80.85, 68.42%), whereas the present study showed slightly better resistance in the presence of Zn^{2+} than Hg^{2+}. RAMYA and THATHEYUS (2019) indicated that isolated *M. morganii* ACZ05 can be a very good choice for Zn^{2+} removal. *M. morganii* ACZ05 showed high MIC values for Zn^{2+}, indicating its resistance to this metal, which is consistent with our study. *M. morganii* (1Ab1) was able to reduce Cr^{6+} at a concentration of 4.6 mg/mL after being treated with raw tannery wastewater at 48 hours of incubation, pH 7.0, and a temperature of 37°C (PRINCY *et al*., 2020). In our study, *M. morganii* PMFKG-K1 was sensitive in the presence of Cr^{6+}.

Klebsiella oxytoca has been studied for its metabolic properties and is considered a promising microorganism for the bioremediation of heavy metals such as Pb, Zn, and Mn

(MUÑOZ *et al.*, 2015; BARBOZA *et al*., 2018; TAHMOURESPOUR, 2021). This is in accordance with our results for *K. oxytoca* which showed resistance in the presence of the mentioned metals. In the study by TAHMOURESPOUR (2021) it has been indicated that the isolated strain *K. oxytoca* ATHA1 also developed resistance mechanisms in the presence of Cd^{2+}, which is not the case in our study.

KUMAR *et al.* (2019) isolated and evaluated *S. liquefaciens* BSWC3 and *Klebsiella pneumoniae* RpSWC3 isolates for bioremediation effectiveness in both single and mixed cultures. These isolates showed very good bioremediation efficiency for Cd^{2+} which is in accordance with our study. *Serratia* sp. is also one of the heavy metal-resistant species isolated from the sample collected from the industrial effluents containing metallic contaminants such as Pb^{2+}, Zn^{2+}, Cu^{2+}, Ag^{2+}, and Hg^{2+} (RAMYA and BOOMINATHAN, 2017). In the study by AHMED *et al*. (2020) it is shown that *S. liquefaciens* is resistant to Cr^{6+} but not to Cd^{2+} and Pb^{2+}. The isolates *S. liquefaciens*, PMFKG-K5 and PMFKG-K6 used in the current study showed resistance in the presence of Cd^{2+} and Pb^{2+}, but not in the presence of Cr^{6+}.

Escherichia coli isolated from petroleum refinery effluent and utilized in the study by OAIKHENA *et al*. (2016) had a low maximum tolerance concentration for Cd^{2+} when compared to Cr^{6+}, Ni^{2+}, and Zn^{2+}. Enterobacteriaceae and Bacillaceae are facultative anaerobic bacteria able to obtain energy in the absence of oxygen and survive in extreme conditions like in refinery petroleum effluent (OAIKHENA *et al*., 2016) and during the processing of wastewater (FERREIRA DA SILVA *et al.*, 2007). Wastewater treatment plants are also important reservoirs of human and animal commensal bacteria (FERREIRA DA SILVA *et al*., 2007). In the current study, bacteria were isolated from the biological reactor and the secondary sedimentation tank where a large proportion of bacteria survive with only temporary access to oxygen (facultative anaerobes) or even survive without it (obligate anaerobes). These bacteria play a very important role in wastewater treatment by facilitating the decomposition of macromolecular organic matter into simpler compounds (CYPROWSKI *et al*., 2018). In the study by RAJBANSHI (2008) ten heavy metal resistant bacteria were isolated from the oxidation ditch of the Guheswori Sewage Treatment Plant. One of them was *E. coli*, which showed resistance in the presence of Cr^{6+}. On the other hand, *E. coli* used in the current study had the lowest resistance in the presence of Cr^{6+} compared to other tested isolates, it also showed sensitivity in the presence of Cd^{2+}, but not in the presence of Zn^{2+}. The study of NWAGWU *et al.* (2017) revealed that Pb^{2+}, Zn^{2+} and Cd^{2+} concentrations were reduced by *E. coli* to 100, 52.23, and 74.91%, respectively. The results obtained from the investigation done by DEBORAH and RAJ (2016) indicated that isolated *E. coli* had degraded tested heavy metals in different percentages: 41% (Zn), 56% (Cu), 56% (Fe), 39% (Mn), 51% (Cr), 51% (Pb), 67% (Cd), 67% (Ni), 100% (As), and 100% (Hg). The *E. coli* PMFKG-K7 strain employed in the current investigation demonstrated remarkable resistance to Pb^{2+} and Cu^{2+}, but not to Hg^{2+}.

It has been shown that bacteria from the genus *Bacillus* are able to bioaccumulate different heavy metals. Various tolerances in heavy metal resistant bacteria are frequent because heavy metals can have similar toxic mechanisms (ANUSHA and NATARAJAN, 2020). In the study by GUPTA MAHENDRA *et al*. (2014) *B. cereus*, *B. carotarum*, *B. lentus* and *B. licheniformis*, showed a wide range of resistance to different heavy metals such as Pb^{2+}, Zn^{2+}, and Cr^{6+}. ANUSHA and NATARAJAN (2020) showed that the bioremediation efficiency of *B. cereus* was found to be 91.98% (Cu^{2+}), 79.9% (Cr^{6+}), 97.17% (Pb^{2+}), 77.44% (Zn^{2+}), 81.6% (Fe^{2+}), 62.8% (Mn^{2+}) and 60.92% (Mg^{2+}) respectively. Indigenous microorganisms are very useful in the treatment of metal contaminated spaces. *B. cereus* RC-1, growing under various pH values and initial metal concentrations, was able to remove a few heavy metals, such as Cu^{2+} (16.7% maximum removal efficiency), Zn^{2+} (38.3%), Cd^{2+} (81.4%), and Pb^{2+} (40.3%) (HUANG *et al*., 2018). *Bacillus cereus* PMFKG-K8 used in the current study showed good

resistance in the presence of Pb^{2+}, Zn^{2+}, Cu^{2+}, and Mn^{2+} which is in accordance with previously mentioned studies.

Heavy metal contamination of an ecosystem led to selection pressure for antibiotic resistance in bacteria living in such conditions (TIMONEY *et al*., 1987). Genes that are involved in this resistance can be located on mobile genetic fragments that can move from one bacterium to another, either through a transformation or conjugation (HARRIS *et al*., 2012). Many investigations have shown the same resistance mechanisms that protect microorganisms and are utilized to combat the lethal effects of antibiotics and heavy metals (PAL *et al*., 2017). *Morganella morganii* used in the study by RAMYA and THATHEYUS (2019) was only resistant to Ticarcillin. The correlation between metal and antibiotic resistance in *K. oxytoca* isolated from industrial effluent was found (TAHMOURESPOUR, 2021). This occurred as a response of bacteria to the increased concentration of heavy metals discharged into industrial effluents. The results of the study by SINEGANI and YOUNESSI (2017) indicated the multiple antibiotic resistance patterns in the isolates of agricultural soils (Ampicillin, Amoxicillin, Vancomycin, Tetracycline, Doxycycline, and Streptomycin). They concluded that Gram-negative strains showed a high rate of co-resistance to Hg^{2+} and antibiotics, as well as between Zn^{2+}, Hg^{2+}, and Ni^{2+} to the β-lactam antibiotics in Gram-positive strains. *Serratia liquefaciens* and *B. cereus* used in the study by AHMED *et al.*, (2020) were multi-metal and multi-drug resistant (amoxicillin, ampicillin, cefixime, ceftazidime, and tazobactam). YAMINA *et al.* (2012) and RAJBANSHI (2008) demonstrated that *Klebsiella* sp., *E. coli* and *Bacillus* sp. isolated from wastewater exhibited co-resistance to heavy metals and antibiotics. *Bacillus* strains used in the study by YAMINA *et al.* (2012) demonstrated combined resistance to Pb^{2+}, Cd^{2+}, and Zn^{2+} and ampicillin, and *Bacillus* strains used in another study showed resistance to the highest concentrations of Pb^{2+}, Zn^{2+}, and Cu^{2+} and ceftriaxone, tetracycline, and ampicillin (SHAMMI and AHMED, 2013).

The isolates tested in our investigation demonstrated antibiotic sensitivity in the range of <0.003 mg/mL and 0.062 mg/mL, with no detected correlation between heavy metals and antibiotics resistance. Results obtained by the disc diffusion method showed that none of the tested isolates exhibited full resistance to the four tested antibiotics (Table 4). The examined isolates displayed sensitivity to chloramphenicol (inhibition zone in the range from 27 to 40 mm), streptomycin (inhibition zone in the range from 18 to 26 mm), and tetracycline (inhibition zone in the range from 28 to 40 mm). All isolates were sensitive to cefotaxime (inhibition zone in the range from 24 to 40 mm) except for *B. cereus* PMFKG-K8. According to CITRON and APPLEMAN (2006) *B. cereus* s.s. is typically resistant to penicillin and other β-lactam antibiotics. By our results *B. cereus* PMFKG-K8 was resistant to cefotaxime and cefepime, which are β-lactam antibiotics also.

CONCLUSION

The present study examined the effect of certain concentrations of heavy metals and antibiotics on isolates of microorganisms selected from the wastewater treatment plant Kruševac (Serbia). The identification of isolates was confirmed on MALDI-TOF, and the microdilution method was used to determine the minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) of the tested heavy metals. Minimum inhibitory concentration (MIC) and disk diffusion method were used for investigation of resistance to antibiotics.

Table 1. Biochemical reactions of tested isolates

Microorganisms	Oxidase	Catalase	Citrate	Urease	H_2S	Indole	Glucose	Sucrose	Lactose	Dextrose	VP/MR
M. morganii PMFKG-K1	-	+	-	+	-	+	+	-	-	-	-/+
K. oxytoca PMFKG-K2	-	+	+	+	-	+	+	+	+	+	+/-
K. oxytoca PMFKG-K3	-	+	+	+	-	+	+	+	+	+	+/-
K. oxytoca PMFKG-K4	-	+	+	+	-	+	+	+	+	+	+/-
S. liquefaciens PMFKG-K5	-	+	+	-	-	-	+	+	-	-	-/+
S. liquefaciens PMFKG-K6	-	+	+	-	-	-	+	+	-	-	-/+
E. coli PMFKG-K7	-	+	-	-	-	+	+	+	+	+	-/+
B. cereus PMFKG-K8	-	+	+	+	-	-	+	+	-	+	+/-

+ positive reaction, - negative reaction

Table 2. Resistance of planktonic cells to the presence of heavy metals

Microorganisms	Metals													
	Pb		Zn		Cu		Mn		Cd		Hg		Cr	
	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC
M. morganii PMFKG-K1	2	2	20	20	1	4	>4	>4	<0.156	0.312	0.125	0.125	0.125	0.125
K. oxytoca PMFKG-K2	4	4	5	5	4	4	1	1	0.625	2.5	0.25	0.25	0.166	0.25
K. oxytoca PMFKG-K3	2	4	>20	>20	2	4	0.25	0.25	<0.156	0.312	0.062	0.062	0.031	0.041
K. oxytoca PMFKG-K4	4	4	10	10	4	4	2	2	<0.156	0.312	0.062	0.104	0.031	0.041
S. liquefaciens PMFKG-K5	4	4	>20	>20	2	2	4	4	2.5	>20	0.062	0.062	0.166	0.25
S. liquefaciens PMFKG-K6	>4	>4	5	5	4	4	4	4	1.25	>20	0.104	0.104	0.25	0.25
E. coli PMFKG-K7	>4	>4	10	10	2	2	0.5	1.6	<0.156	0.416	0.052	0.052	0.007	0.01
B. cereus PMFKG-K8	2.3	>4	5	10	0.5	2	4	4	<0.156	<0.156	0.015	0.026	0.125	0.25

MIC values (mg/mL) – means inhibitory activity; MMC values (mg/mL) – means microbicidal activity

Table 3. Resistance of planktonic cells to the presence of antibiotic

Microorganisms	Antibiotics						
	Am	Az	C	E	He	Le	T
	MIC	MIC	MIC	MIC	MIC	MIC	MIC
M. morganii PMFKG-K1	0.19	<0.003	<0.003	<0.003	0.051	<0.003	<0.003
K. oxytoca PMFKG-K2	0.062	<0.003	<0.003	<0.003	0.025	<0.003	<0.003
K. oxytoca PMFKG-K3	0.051	<0.003	0.032	<0.003	0.015	<0.003	<0.003
K. oxytoca PMFKG-K4	0.051	<0.003	<0.003	<0.003	0.012	<0.003	<0.003
S. liquefaciens PMFKG-K5	0.025	<0.003	<0.003	<0.003	0.007	<0.003	0.005
S. liquefaciens PMFKG-K6	0.031	<0.003	<0.003	<0.003	0.007	<0.003	0.007
E. coli PMFKG-K7	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003
B. cereus PMFKG-K8	0.062	0.062	0.5	<0.003	0.007	<0.003	<0.003

MIC values (mg/mL) – means inhibitory activity; ampicillin (Am), ceftriaxone (Az), cefepime (C), ertapenem (E), azithromycin (He), levofloxacin (Le), tetracycline (T)

Table 4. Determination of resistance to antibiotics by disk-diffusion method

Microorganisms	Antibiotics (µg)			
	C	CTX	S	TE
	30	5	10	30
M. morganii PMFKG-K1	s	s	s	s
K. oxytoca PMFKG-K2	s	s	s	s
K. oxytoca PMFKG-K3	s	s	s	s
K. oxytoca PMFKG-K4	s	s	s	s
S. liquefaciens PMFKG-K5	s	s	s	s
S. liquefaciens PMFKG-K6	s	s	s	s
E. coli PMFKG-K7	s	s	s	s
B. cereus PMFKG-K8	s	r	s	s

chloromphenicol (C), cefotaxime CTX), streptomycin (S), tetracycline (TE); r-resistant, s-sensitive

Based on the obtained results, it could be concluded that isolated bacterial species in planktonic form showed better resistance to heavy metals such as Pb^{2+}, Zn^{2+}, Cu^{2+}, and Mn^{2+} compared to Cd^{2+}, Hg^{2+}, and Cr^{6+}, as well as to tested antibiotics. Given that the tested isolates showed resistance in the presence of some heavy metals they can be further studied for their application in the process of bioremediation. In order to assess their heavy metal removal capacity, the effect of these heavy metals on individual and mixed biofilms of tested bacterial species should also be studied.

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References:

[1] AHMED, S., AKTAR, S., ZAMAN, S., JAHAN, R.A., BARI, M. (2020): Use of natural biosorbent in removing dye, heavy metal and antibiotic-resistant bacteria from industrial wastewater. *Applied Water Science* **10** (5): 1–10.2017. doi:10.1007/s13201-020-01200-8

[2] ANUSHA, P., NATARAJAN, D. (2020): Bioremediation potency of multi-metal tolerant native bacteria *Bacillus cereus* isolated from bauxite mines, kolli hills, Tamilnadu-a lab to land approach. *Biocatalysis and Agricultural Biotechnology* **25**: 101581.

[3] ASHBOLT, N.J., AMÉZQUITA, A., BACKHAUS, T., BORRIELLO, P., BRANDT, K.K, COLLIGNON, P., COORS, A., FINLEY, R., GAZE, W.H., HEBERER, T., LAWRENCE, J.R., LARSSON, D.G., MCEWEN, S.A., RYAN, J.J., SCHNFELD, J., SILLEY, P., SNAPE, J.R., VAN DEN EEDE, C., TOPP, E. (2013): Human Health Risk Assessment (HHRA) for environmental development and transfer of antibiotic resistance. *Environmental Health Perspectives* **121** (9): 993–1001. doi: 10.1289/ehp.1206316

[4] BARBOZA, N.R., CORDEIRO, M.M., SANTOS, P.A., QUEIROZ, P.S., LEÃO, V.S., GUERRA-SA, R.A. (2018): New Application for *Klebsiella oxytoca* in Bioremediation: Treatment of Manganese-Laden Wastewaters. *Water Air Soil Pollut* **229**: 19. doi: 10.1007/ s11270-017-3651-7

[5] CITRON, D.M., APPLEMAN, M.D. (2006): *In vitro* activities of daptomycin, ciprofloxacin, and other antimicrobial agents against the cells and spores of clinical isolates of *Bacillus* species. *Journal of Clinical Microbiology* **44** (10): 3814–8. doi: 10.1128/JCM.00881-06

[6] CYPROWSKI, M., STOBNICKA-KUPIEC, A., ŁAWNICZEK-WAŁCZYK, A., BAKAL-KIJEK A., GOŁOFIT-SZYMCZAK M., GÓRNY R.L. (2018). Anaerobic bacteria in wastewater treatment plant. *International Archives of Occupational and Environmental Health* **91**: 571–579. doi: 10.1007/s00420-018-1307-6

[7] DEBORAH, S., RAJ, J.S. (2016): Bioremediation of heavy metals from distilleries effluent using microbes. *Journal of Applied and Advanced Research* **1** (2): 23–28. doi: 10.21839/jaar.2016.v1i2.21

[8] DEREDJIAN, A., COLINON, C., BROTHIER, E., FAVRE-BONTÉ, S., COURNOYER, B., NAZARET, S. (2011): Antibiotic and metal resistance among hospital and outdoor strains of *Pseudomonas aeruginosa*. *Research in Microbiology* **162** (7): 689–700. doi: 10.1016/j.resmic.2011.06.007

[9] DIN, G., FAROOQI, A., SAJJAD, W., IRFAN, M., GUL, S., ALI SHAH, A. (2021): Cadmium and antibiotic-resistant *Acinetobacter calcoaceticus* strain STP14 reported from sewage treatment plant. *Journal of Basic Microbiology* **61** (3): 230–240. doi: 10.1002/jobm.202000538

[10] EUCAST (The European Committee on Antimicrobial Susceptibility Testing) (2023): Breakpoint tables for interpretation of MICs and zone diameters. Version 13.0. http://www.eucast.org. Accessed 20 May 2023.

[11] FERREIRA DA SILVA, M., VAZ-MOREIRA, I., GONZALEZ-PAJUELO, M., NUNES, O.C., MANAIA, C.M. (2007). Antimicrobial resistance patterns in Enterobacteriaceae isolated from an urban wastewater treatment plant. *FEMS Microbiology Ecology* **60** (1): 166–176. doi: 10.1111/j.1574-6941.2006.00268.x

[12] FOUAD, Z. (2011). Antimicrobial Disk Diffusion Zone Interpretation Guide. doi: 10.13140/RG.2.2.13801.70240

[13] GADD, G.M. (2000): Bioremedial potential of microbial mechanisms of metal mobilization and immobilization. *Current Opinion in Biotechnology* **11** (3): 271–279. doi: 10.1016/s0958-1669(00)00095-1

[14] GHAZISAEEDI, F., CIESINSKI, L., BEDNORZ, C., JOHANNS, V., PIEPER, L., TEDIN, K., WIELER, L.H., GÜNTHER S. (2020): Phenotypic zinc resistance does not correlate with antimicrobial multi-resistance in fecal *E. coli* isolates of piglets. *Gut pathogens* **12** (1): 1–10. doi: 10.1186/s13099-019-0342-5

[15] GRUJIĆ, S.M., RADOJEVIĆ, I.D., VASIĆ, S.M., ČOMIĆ, LJ.R., OSTOJIĆ, A.M. (2018): Heavy metal tolerance and removal efficiency of the *Rhodotorula mucilaginosa* and *Saccharomyces boulardii* planktonic cells and biofilm. *Kragujevac Journal of Science* **40**: 217–226. doi: 10.5937/KgJSci1840217G

[16] GUPTA MAHENDRA, K., KIRAN, K., AMITA, S., SHIKHA, G. (2014): Bioremediation of heavy metal polluted environment using resistant bacteria. *Journal of Environmental Research and Development*. **8** (4): 883–889.

[17] HARRIS, S.J., CORMICAN, M., CUMMINS, E. (2012). Antimicrobial residues and antimicrobial-resistant bacteria: impact on the microbial environment and risk to human health – A review. *Human and Ecological Risk Assessment* **18**: 767–809.

[18] HASSEN, A., SAIDI, N., CHERIF, M., BOUDABOUS, A.J.B.T. (1998): Resistance of environmental bacteria to heavy metals. *Bioresource technology* **64** (1): 7–15. doi: 10.1016/S0960-8524(97)00161-2

[19] HUANG, F., WANG, Z.H., CAI, Y.X., CHEN, S.H., TIAN, J.H., CAI, K.Z. (2018): Heavy metal bioaccumulation and cation release by growing *Bacillus cereus* RC-1 under culture conditions. *Ecotoxicology and Environmental Safety* **157**: 216–226. doi: 10.1016/j.ecoenv.2018.03.077

[20] KUMAR, P., GUPTA, S.B., SONI, R. (2019): Bioremediation of cadmium by mixed indigenous isolates *Serratia liquefaciens* BSWC3 and *Klebsiella pneumoniae* RpSWC3 isolated from industrial and mining affected water samples. *Pollution* **5** (2): 351–360. doi: 10.22059/poll.2018.268603.533

[21] MUÑOZ, A.J., ESPÍNOLA, F., MOYA, M., RUIZ, E. (2015). Biosorption of Pb(II) Ions by *Klebsiella* sp. 3S1 Isolated from a Wastewater Treatment Plant: Kinetics and Mechanisms Studies. *BioMed Research International* **2015**: 719060. doi: 10.1155/2015/719060.

[22] NGUYEN, C.C., HUGIE, C.N., KILE, M.L., NAVAB-DANESHMAND T. (2019): Association between heavy metals and antibiotic-resistant human pathogens in environmental reservoirs: A review. *Frontiers of Environmental Science and Engineering* **13**: 46. doi: 10.1007/s11783-019-1129-0

[23] NIES, D.H. (1999): Microbial heavy-metal resistance. *Applied Microbiology and Biotechnology* **51** (6): 730–750. doi: 10.1007/s002530051457

[24] NWAGWU, E.C., YILWA, V.M., EGBE, N.E., ONWUMERE, G.B. (2017): Isolation and characterization of heavy metal tolerant bacteria from Panteka stream, Kaduna, Nigeria and their potential for bioremediation. *African Journal of Biotechnology* **16** (1): 32–40. doi: 10.5897/AJB2016.15676

[25] OAIKHENA, E.E., MAKAIJE, D.B., DENWE, S.D., NAMADI, M.M., HAROUN, A.A. (2016): Bioremediation Potentials of Heavy Metal Tolerant Bacteria Isolated from Petroleum Refinery Effluent. *American Journal of Environmental Protection* **5** (2): 29–34. doi: 10.11648/j.ajep.20160502.12

[26] ORJI, O.U., AWOKE, J.N., ALOKE, C., OBASI, O.D., OKE, B., NJOKU, M., EZEANI, N.N. (2021): Toxic metals bioremediation potentials of *Paenibacillus* sp. strain SEM1 and *Morganella* sp. strain WEM7 isolated from Enyigba Pb–Zn mining site, Ebonyi State Nigeria. *Bioremediation Journal* **25** (4): 285–296. doi: 10.1080/10889868.2020.1871315

[27] PAL, C., ASIANI, K., ARYA, S., RENSING, C., STEKEL, D.J., LARSSON, D.J., HOBMAN, J.L. (2017): Metal resistance and its association with antibiotic resistance. *Advances in Microbial Physiology* **70**: 261–313. doi: 10.1016/bs.ampbs.2017.02.001

[28] PRINCY, S., SATHISH, S.S., CIBICHAKRAVARTHY, B., PRABAGARAN, S.R. (2020): Hexavalent chromium reduction by *Morganella morganii* (1Ab1) isolated from tannery effluent contaminated sites of Tamil Nadu, India. *Biocatalysis and Agricultural Biotechnology* **23**: 101469. doi: 10.1016/j.bcab.2019.101469

[29] RAJBANSHI, A. (2008): Study on heavy metal resistant bacteria in Guheswori sewage treatment plant. *Our nature* **6** (1): 52–57. doi: 10.3126/on.v6i1.1655

[30] RAMYA, R., BOOMINATHAN, M. (2017): Isolation of *Serratia liquefaciens* as Metal Resistant Bacteria from Industrial Effluent. *International Journal of Advance Research, Ideas and Innovations in Technology* **3** (6): 1272–1275.

[31] RAMYA, D., THATHEYUS, A.J. (2019): Research article biosorption of zinc from aqueous solution by the bacterial strain, *Morganella morganii* ACZ05. *Asian Journal of Biological Sciences* **12** (4): 869–876. doi: 10.3923/ajbs.2019.869.876

[32] SARKER, S.D., NAHAR, L., KUMARASAMY, Y. (2007): Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. *Methods* **42** (4): 321–324. doi: 10.1016/j.ymeth.2007.01.006

[33] SCHULZ-ZUNKEL, C., KRUEGER, F. (2009): Trace metal dynamics in floodplain soils of the river Elbe: a review. *Journal of Environmental Quality* **38** (4): 1349–1362. doi: 10.2134/jeq2008.0299

[34] SEIER-PETERSEN, M.A., JASNI, A., AARESTRUP, F.M., VIGRE, H., MULLANY, P., ROBERTS, A.P., AGERSØ Y. (2014): Effect of subinhibitory concentrations of four commonly used biocides on the conjugative transfer of Tn916 in *Bacillus subtilis*. *Journal of Antimicrobial Chemotherapy* **69** (2): 343–348. doi: 10.1093/jac/dkt370

[35] SELVI, A.T., ANJUGAM, E., DEVI, R.A., MADHAN, B., KANNAPPAN, S., CHANDRASEKARAN, B. (2012): Isolation and characterization of bacteria from tannery effluent treatment plant and their tolerance to heavy metals and antibiotics. *Asian Journal of Experimental Biological Sciences* **3** (1): 34–41.

[36] SHAMMI, T, AHMED, S. (2013). Heavy metal tolerance and antibiotic resistance of *Bacillus* spp. isolated from two major rivers in Bangladesh. *Bangladesh Journal of Microbiology* **30** (1-2): 17–22. doi: 10.3329/bjm.v30i1-2.28448

[37] SINEGANI, A.A.S., YOUNESSI, N. (2017): Antibiotic resistance of bacteria isolated from heavy metal-polluted soils with different land uses. *Journal of global antimicrobial resistance* **10**: 247–255. doi: 10.1016/j.jgar.2017.05.012

[38] TABAK, H.H., LENS P., VAN HULLEBUSCH, E.D., DEJONGHE, W. (2005): Developments in bioremediation of soils and sediments polluted with metals and radionuclides. Microbial processes and mechanisms affecting bioremediation of metal contamination and influencing metal toxicity and transport. *Reviews in Environmental Science and Bio/-Technology* **4** (3): 115–156. doi: 10.1007/s11157-005-2169-4

[39] TAHMOURESPOUR, A. (2021): Heavy metals and antibiotic co-resistance in bacterial isolates of industrial effluents. *Journal of Water and Wastewater* **32** (5): 12–20. doi: 10. 22093/wwj. 2021.257072.3079

[40] TIMONEY, J.F., PORT, J., GILES, J. SPANIER, J. (1987): Heavy-metal and antibiotic resistance in the bacterial flora of sediments of New York Bight. *Applied and Environmental Microbiology* **36** (3): 465–72. doi: 10.1128/aem.36.3.465-472.1978

[41] WALDRON, K.J., ROBINSON, N.J. (2009): How do bacterial cells ensure that metalloproteins get the correct metal? *Nature Reviews Microbiology* **7** (1): 25-35. doi: 10.1038/ nrmicro2057

[42] YAMINA, B., TAHAR, B., MARIE LAURE, F. (2012): Isolation and screening of heavy metal resistant bacteria from wastewater: a study of heavy metal co-resistance and antibiotics resistance. *Water Science and Technology* **66** (10): 2041–2048. doi: 10.2166/wst. 2012.355.