

Heavy metal tolerance and removal potential in mixed-species biofilm

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ABSTRACT

The aim of the study was to examine heavy metal tolerance (Cd^{2+} , Zn^{2+} , Ni^{2+} and Cu^{2+}) of single- and mixed-species biofilms (*Rhodotorula mucilaginosa* and *Escherichia coli*) and to determine metal removal efficiency (Cd^{2+} , Zn^{2+} , Ni^{2+} , Cu^{2+} , Pb^{2+} and Hg^{2+}). Metal tolerance was quantified by crystal violet assay and results were confirmed by fluorescence microscopy. Metal removal efficiency was determined by batch biosorption assay. The tolerance of the mixed-species biofilm was higher than the single-species biofilms. Single- and mixed-species biofilms showed the highest sensitivity in the presence of Cu^{2+} (*E. coli*-MIC 4 mg/ml, *R. mucilaginosa*-MIC 8 mg/ml, *R. mucilaginosa*/*E. coli*-MIC 64 mg/ml), while the highest tolerance was observed in the presence of Zn^{2+} (*E. coli*-MIC 80 mg/ml, *R. mucilaginosa*-MIC 161 mg/ml, *R. mucilaginosa*-*E. coli*-MIC 322 mg/ml). The mixed-species biofilm exhibited better efficiency in removal of all tested metals than single-species biofilms. The highest efficiency in Cd^{2+} removal was shown by the *E. coli* biofilm (94.85%) and *R. mucilaginosa* biofilm (97.85%), individually. The highest efficiency in Cu^{2+} (99.88%), Zn^{2+} (99.26%) and Pb^{2+} (99.52%) removal was shown by the mixed-species biofilm. Metal removal efficiency was in the range of 81.56%–97.85% for the single- and 94.99%–99.88% for the mixed-species biofilm.

Key words | biofilms, *Escherichia coli*, heavy metal, mixed-, *Rhodotorula mucilaginosa*, single-

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INTRODUCTION

In the natural environment, biofilms have a great impact on our daily life (Elias & Banin 2012). Our current understanding of the physiology and complexity of the mixed-species biofilms are still in progress. Although the mixed-species biofilms represent the dominant form in the environment (Elias & Banin 2012), previous studies were still based mainly on the studies of the individual laboratory biofilms (Hall-Stoodley *et al.* 2004; Høiby *et al.* 2010).

The single-species biofilms were up to 65 times more tolerant to the influence of heavy metals than planktonic cells (Harrison *et al.* 2005b, 2006). The mixed-species biofilm were more tolerant to stressors such as antibiotics, disinfectants, heavy metals etc., than the single-species biofilm (Golby *et al.* 2014; Jahid & Ha 2014). The ability of microbial communities to cooperate and survive the impact of antimicrobial agents explains the tolerance in the mixed-species biofilms (Elias & Banin 2012). This ability turns the mixed-species biofilm into a practical tool that has potential

in bioremediation of contaminated environments (Golby *et al.* 2014).

One of the most serious environmental problems has been heavy metal pollution. Conventional methods of metal removal have proved to be ineffective when the concentration of the metal ions was low (1–100 mg/l) (Wang & Chen 2006). Biosorption presents a modern low-cost method that uses bacteria, algae, yeasts, filamentous fungi, etc. (planktonic form) (Fu & Wang 2011), but the use of biofilms is still in the process of evaluation. A successful bioremediation process relies on understanding the interactions between microbes and contaminants (Zhang *et al.* 1995). Biofilms that live in contaminated environments have usually developed effective multiple defence mechanisms for survival (Harrison *et al.* 2007). Regardless of the fact that heavy metals cause toxic effects, tolerance to heavy metals is a natural phenomenon that depends on the various conditions in which biofilms develop (Harrison

et al. 2007). In recent years, attention has been devoted to examining the biofilms' biosorption potential (Harrison *et al.* 2006; Quintelas *et al.* 2008, 2009). To the authors' knowledge, one of the first studies on the effect of heavy metals on a mixed bacterial biofilm was published by Golby *et al.* (2014). In addition to bacteria, the efficiency of metal removal by yeast biofilm has been studied (Basak *et al.* 2014).

Rhodotorula mucilaginosa biofilm showed noticeably more resilience in the presence of heavy metals than corresponding planktonic cells (Grujić *et al.* 2017). For this reason, the aim of the study was to test the influence of heavy metals on single- and mixed-species biofilm, formed of *R. mucilaginosa* and *Escherichia coli*. The hypothesis was that with the establishment of the synergistic interaction between *R. mucilaginosa* and *E. coli*, the mixed-species biofilm will show better results. Pb^{2+} and Hg^{2+} tolerance of single- and mixed-species biofilm (*R. mucilaginosa* and *E. coli*) was tested (Buzejić *et al.* 2016). Except for Buzejić *et al.*'s (2016) study, the heavy metal tolerance of single- and mixed-species biofilms has not been tested in the presence of other metal ions. Therefore, the aim of our study was to examine the heavy metal tolerance of the single- and mixed-species biofilm formed by *R. mucilaginosa* and *E. coli* in the presence of heavy metals Cd^{2+} , Zn^{2+} , Ni^{2+} and Cu^{2+} and to determine Cd^{2+} , Zn^{2+} , Ni^{2+} , Cu^{2+} , Pb^{2+} and Hg^{2+} removal efficiency.

METHODS

Microorganisms

Two species of microorganisms isolated from the environment were used in this study (*R. mucilaginosa* and *E. coli*). The *E. coli* strain was a gift from the Institute for Public Health, Kragujevac, Serbia. The *R. mucilaginosa* strain was identified by the test for rapid identification of yeast API 20 C AUX (Biomerieux, France). Tryptic Soy Broth (TSB, Difco) was chosen as the growth medium for both strains (Adam *et al.* 2002).

Preparation of metal solutions

Metal tolerance of the *R. mucilaginosa*, *E. coli* and *R. mucilaginosa/E.coli* biofilms was tested in the presence of metal ions, Cd^{2+} , Zn^{2+} , Ni^{2+} and Cu^{2+} , originating from $CdSO_4$, $ZnSO_4$, $NiSO_4$ and $CuSO_4$, salts (Sigma-Aldrich, St Louis, MO, USA). Biosorption potential was tested in the presence

of two extra metal ions: Pb^{2+} and Hg^{2+} , originating from $Pb(NO_3)_2$ and $HgCl_2$ salts (Sigma-Aldrich). Working solutions were prepared in TSB medium from stock solutions, no more than 60 min before use. Based on previous research and the preliminary test, a range of tested concentrations was selected in which the lowest concentration does not lead to a significant response (compared to control) and the highest concentration causes a 100% test response of the organism. Standard antibiotics (amphotericin and tetracycline) were used as the positive control. Based on previous studies on microbial biosorption potential, the selected concentration was 100 µg/ml for each metal (Basak *et al.* 2014).

Biofilm formation

To test heavy metal tolerance, the *R. mucilaginosa* and *E. coli* biofilms were formed in polystyrene microtitre 96-well plates (Sarstedt, Germany). One hundred µl of suspension (OD_{520} 0.8) was added in every well of the plate. To form the mixed *R. mucilaginosa/E. coli* biofilm, an equal amount of suspension was mixed immediately before use (Adam *et al.* 2002).

To test biosorption potential, the single- and mixed-species biofilms were formed on coverslips 22 × 22 mm, that were immersed in the wells of microtitre plates with the nutritive medium and suspension (McFarland 0.5 for bacteria; McFarland 1.0 for yeast; to form the mixed *R. mucilaginosa/E. coli* biofilm, an equal amount of suspension was mixed immediately before incubation) (Sternberg *et al.* 2014).

Heavy metal tolerance of tested biofilms and quantification

After the incubation period, the tested biofilms were treated with heavy metals and antibiotics. Quantification was performed using crystal violet (CV) assay according to the method described by Almeida *et al.* (2011) with certain modifications. The effect of the tested metals was followed during 72 hours. The contents of the plates (after 24 h, 48 h and 72 h), where the biofilms were formed, were removed and 50 µl of methanol 98% (vol/vol) was added. After 15 min the methanol content was removed and the plates were allowed to dry at room temperature. Then, to each well, 50 µl of CV was added (5 min). Plates were washed with sterile distilled water and 100 µl of glacial acetic acid, 33% (vol/vol) was added to each well of the plate. The minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) were determined by reading the optical

density (OD_{570}) using a microplate reader (Rayito, China). All tests were performed in triplicate and the mean value was calculated.

Fluorescence microscopy

Fluorescence microscopy was used to examine the influence of heavy metals on tested biofilms according to the method described by Kronvall & Myhre (1977) with certain modifications. In each well of a microtitre plate, 50 µl of methanol was added and incubated at room temperature until the methanol evaporated. After incubation, 50 µl of acridine orange stain (5 mg/ml) was added into the microtitre plate. After 2 min, the microtitre plate was washed with sterile distilled water. Tested biofilms were observed on the Olympus BX51 fluorescence microscope (Olympus, Shinjuku, Tokyo, Japan) and analysed using the Cytovision 3.1 software package (Applied Imaging Corporation, Santa Clara, CA, USA).

Batch biosorption assay

Batch assay was performed according to the method described by Basak *et al.* (2014). In order to study the effect of metal removal by biofilms, the batch experiments were conducted by immersing the coverslips (22 × 22 mm), with formed biofilms, in metal solution (100 µg/ml) at pH 6. Each day for 5 days, 1.5 ml of aliquots were taken and centrifuged at 10,000 rpm for 5 min. The supernatant (samples and controls) was subjected to spectrophotometer (520 nm) analysis for residual metal concentration. All experiments were performed in triplicates and their mean value was calculated. The metal removal percentage (%) was calculated from the following:

$$E(\%) = \frac{(C_i - C_r)}{C_i} \times 100 \quad (1)$$

where C_i is the initial concentration of metal ion (µg/ml) and C_r is the final concentration of the metal ion (µg/ml).

Dry weight determination of biofilms

Dry weight determination was chosen in order to examine and monitor the impact of the total biofilm mass of single- and mixed-species biofilms on metal removal. Each day for 5 days, the dry weight of biofilms was measured according to the method described by Pedersen (1982).

RESULTS AND DISCUSSION

Biofilm formation

R. mucilaginosa and *E. coli* formed a single- and mixed-species biofilm in the 96-well microtitre plate as well as on the coverslips. Biofilm production was quantified by CV and dry weight assay. The results of both tests showed that production of the *R. mucilaginosa/E. coli* biofilm was higher in relation to the single-species biofilms ($E. coli < R. mucilaginosa < R. mucilaginosa/E. coli$). The same results were obtained by Buzejić *et al.* (2016). Harrison *et al.* (2006) reported that *Candida tropicalis* could survive in unfriendly conditions due to its ability of biofilm formation. *E. coli* showed an ability of biofilm formation that was in accordance with the results of Harrison *et al.* (2005a), where different strains of *E. coli* (*E. coli* JM109, *E. coli* HM21 and *E. coli* HM22) showed potential for biofilm formation on an MBEC-HTP system. The formation of the mixed-species biofilm was influenced by numerous processes that determine the biofilm's shape and nature (Elias & Banin 2012).

Heavy metal tolerance of tested biofilms and quantification

The results of heavy metal tolerance of tested biofilms were expressed as the MIC and the MLC (Table 1). The MIC for the *E. coli* biofilm was determined after 24 h and the MLC after 48 h. The MIC for the *R. mucilaginosa* and *R. mucilaginosa/E. coli* biofilm was determined after 48 h and MLC after 72 h.

Heavy metal tolerance of the *R. mucilaginosa* biofilm was better than the *E. coli* biofilm in our work (Table 1). This was in accordance with the results of Buzejić *et al.* (2016) who had examined the biofilms' tolerance to the metal ions Pb^{2+} and Hg^{2+} . Harrison *et al.* (2006) reported that MLC_{100} for the *C. tropicalis* biofilm in the presence of Cd, Zn, Ni and Cu was observed at concentrations $>2.8 \times 10^2$ mM, 64 mM, $>4.9 \times 10^2$ mM and 1.4×10^2 mM, respectively. In our study, the obtained MLC results for the *R. mucilaginosa* biofilm, in the presence of Ni, Cu, Zn and Cd, were obtained at concentrations of 153 mg/ml, 64 mg/ml, 645 mg/ml and 834 mg/ml, which were partially in accordance with the results of the mentioned study, since the authors did not determine the MLC (larger than the maximum used concentration). In our work, the MLC was determined in the presence of all tested substances. The

Table 1 | Tolerance of single- and mixed-species biofilm in the presence of tested substances

Tested substances	Single-species biofilms				Mixed-species biofilm	
	<i>R. mucilaginosa</i>		<i>E. coli</i>		<i>R. mucilaginosa/E. coli</i>	
	MIC	MLC	MIC	MLC	MIC	MLC
Cd	52	834	26	208	208	834
Zn	161	645	80	161	322	645
Ni	76	153	38	153	307	615
Cu	8	64	4	8	64	128
Amphotericin B	0.007	0.06	0.007	0.03	0.25	0.5
Tetracycline	0.06	0.12	0.01	0.06	0.5	0.5

MIC, minimal inhibitory concentration; MLC, minimal lethal concentration. Values in the table are in mg/ml.

results in the available literature suggest that heavy metal tolerance of biofilms was time dependent (Harrison *et al.* 2005a, 2005b). Harrison *et al.* (2005a) investigated the sensitivity of the *E. coli* JM109 biofilm in the presence of heavy metal ions including Cd, Zn, Ni, Cu and Hg. The MIC was obtained at concentrations of 2.3 mM, 31 mM, 17 mM, 16 mM, and 0.04 mM. In our study, the *E. coli* biofilm showed tolerance in the presence of Cd²⁺, Zn²⁺, Ni²⁺, Cu²⁺ and the MIC was obtained at concentrations of 26 mg/ml, 81 mg/ml, 38 mg/ml and 4 mg/ml. The MIC for Cu was in accordance with Harrison *et al.* (2005a).

Mixed-species biofilms can be established by one or several different microbial species, which are the dominant form in nature. Studies confirmed that the multispecies biofilm was extremely resistant to antimicrobial treatment in comparison to the single-species biofilm (Burmølle *et al.* 2006). Our results matched with the results of the mentioned studies. Heavy metal tolerance of single- and mixed-bacterial biofilms (isolates from the sludge tailings) in the presence of metal ions, including Cu, Zn, Ni and Pb, was examined by Golby *et al.* (2014). The obtained results showed that the mixed bacterial biofilm was extremely resistant to the applied metal ions. The reported tolerance values were the following: over 20 mg/l for Pb, 16 mg/l for Zn and 3.2 mg/l for Ni. Accurate concentrations of the heavy metals' tolerance for biofilms were not determined in the mentioned study. Hence, we could say that the results of the mentioned study were in accordance with ours (Table 1). The MIC of Zn and Ni for the mixed-species biofilm (*R. mucilaginosa/E. coli*) was 323 mg/ml and 308 mg/ml, which was several times larger than in the study of Golby *et al.* (2014).

Antibiotics (amphotericin B and tetracycline) were used as the control of our experiment. Mixed-species biofilm

showed a higher tolerance to antibiotics in relation to single-species biofilm (Table 1), which was in accordance with the study carried out by Adam *et al.* (2002).

Fluorescence microscopy

The results obtained by fluorescence microscopy for the single- and mixed-species biofilm are presented in Figure 1.

The panels in the figure present the MIC of tested substances on the *E. coli*, *R. mucilaginosa* and *R. mucilaginosa/E. coli* biofilms. The results of MIC, which were obtained by reading the optical density using a microplate reader for the single- and mixed-species biofilm, were confirmed by fluorescence microscopy, which was used as visual confirmation.

Batch biosorption assay

The results of metal removal efficiency (Cd²⁺, Zn²⁺, Ni²⁺, Cu²⁺, Pb²⁺ and Hg²⁺) by single- and mixed-species biofilm, after 5 days, are presented in Figures 2 and 3.

The heavy metal removal efficiency by mixed-species biofilm was the best on all 5 days (concentrations of the heavy metal ions were reducing during this time), in comparison with the single-species biofilms. The best results showed the *R. mucilaginosa/E. coli* biofilm with a range of metal removal from 94.99% to 99.88% (the ranges of residual concentrations of metal ions were from 5.01% to 0.12%) (Figures 2 and 3). Concentrations of the heavy metal ions in the substrate were reducing during the testing time, whereby the microbial biofilms showed high efficiency, which was in accordance with the results of the study of Basak *et al.* (2014). Figures 2 and 3 show variations

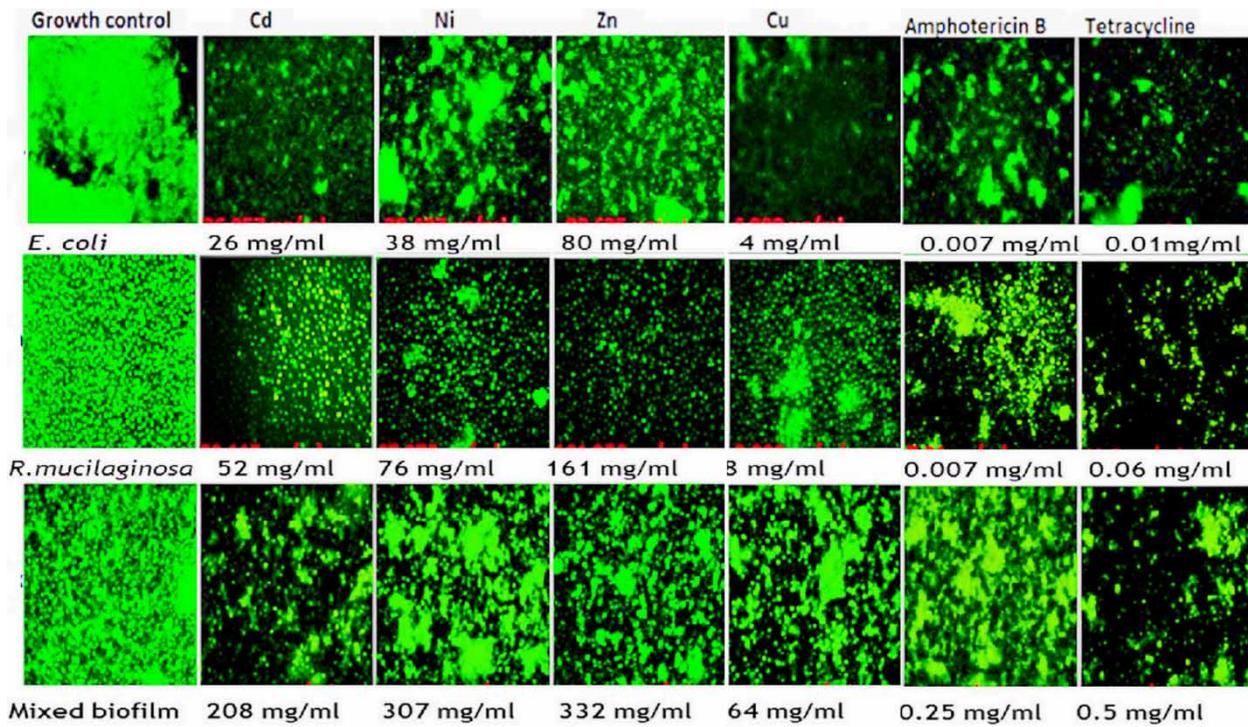


Figure 1 | The MIC results of heavy metals' tolerance for tested biofilms (*E. coli*, *R. mucilaginosa* and *R. mucilaginosa/E.coli*).

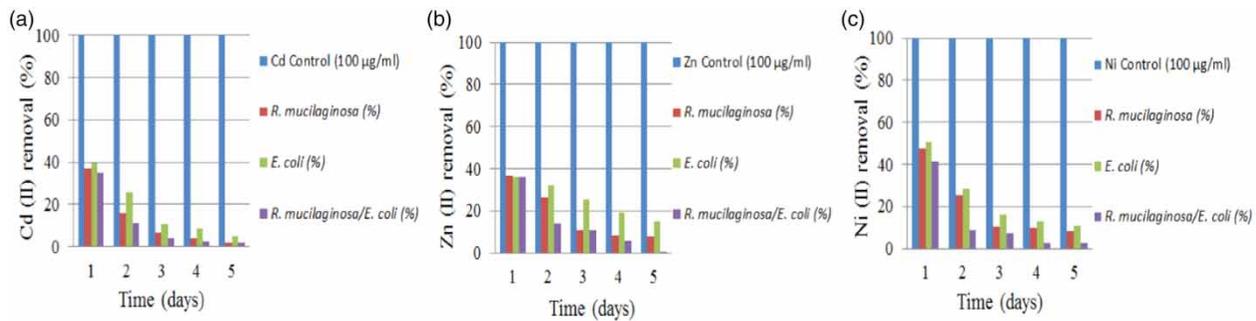


Figure 2 | The removal efficiency of (a) Cd^{2+} , (b) Zn^{2+} and (c) Ni^{2+} by single- and mixed-species biofilms.

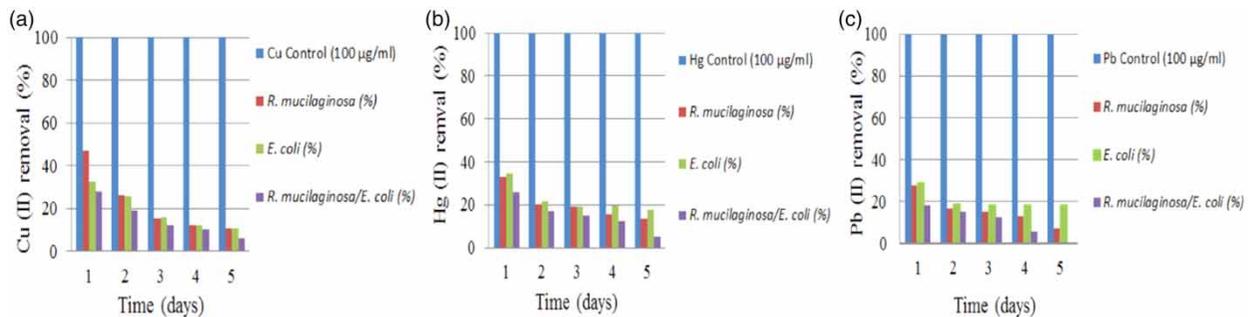


Figure 3 | The removal efficiency of (a) Cu^{2+} , (b) Pb^{2+} and (c) Hg^{2+} by single- and mixed-species biofilms.

in the speed of removing metal ions by days. It is obvious that the process of metal removal occurs in two phases. The first phase was extremely fast due to the high initial activity of biofilms as biosorbents. It was observed that the highest efficiency during the treatment was in the first 2–3 days. After that, the slow phase of metal removal occurs, whereby the change in residual metal concentrations was insignificant. Our observation was in accordance with the study of Volesky (2003). Quintelas et al. (2009) reported the percentage of Cd^{2+} and Ni^{2+} removal at different initial concentrations using the *E. coli* biofilm and kaolin. The percentage of Cd^{2+} removal on the initial concentration of 97 mg/l was 71.3%, which was in accordance with our results, where the percentage of Cd^{2+} removal efficiency after 48 h was 74.18%. The percentage of Ni^{2+} removal at the initial concentration of 101 mg/l was 45.3%. The same results were obtained in our work, where the percentage of Ni^{2+} removal efficiency after 24 h was 49.34%. Basak et al. (2014) investigated the removal of Zn^{2+} using the *Candida rugosa* and *Cryptococcus laurentii* biofilms. The removal of Zn^{2+} ions was found to be 88% and 74.2%. In our study, the percentage of Zn^{2+} removal efficiency for the *R. mucilaginosa* biofilm after 48 h was 74%, which was in accordance with the results of the mentioned study. The percentage of Zn^{2+} removal efficiency for the *R. mucilaginosa*/*E. coli* biofilm after 48 h was better (87%) than the percentage of removal efficiency of the *R. mucilaginosa* (74%) and *E. coli* (68.25%) biofilms individually. Similar results were obtained by White & Gadd

(1998), who tested the accumulation and effects of cadmium on single- and mixed-species sulfate-reducing bacterial biofilms. In our work, both biofilms formed separately and together showed extremely high biosorption potential, but the absorption amount of single-species biofilms was slightly lower than in the mixed-species biofilm (Figures 2 and 3). The amount of single- and mixed-species biofilm (Figure 4) could affect the removal of metal ions (Cd^{2+} , Zn^{2+} , Ni^{2+} , Cu^{2+} , Pb^{2+} and Hg^{2+}).

The increase in amounts of biofilms over the time significantly impacted the efficiency of metal removal. These observations were in accordance with a study conducted by Al-Garni et al. (2009) that investigated the biosorption characteristics of the filamentous fungi *Aspergillus fumigatus* in the removal of cadmium from aqueous solutions.

CONCLUSION

Based on the knowledge of detoxification mechanisms used by microorganisms to reduce the heavy metal toxicity, it is possible to develop efficient and environmentally friendly (bio) technologies for metal remediation. The main aim of our work was to study the effect of selected heavy metals in the context of single- and mixed-species biofilms used in development of biotechnologies suitable for metal removal. Based on the obtained results, a noticeable difference in the metal tolerance, as well as high biosorption potential between the single- and mixed-species biofilms occurs. The

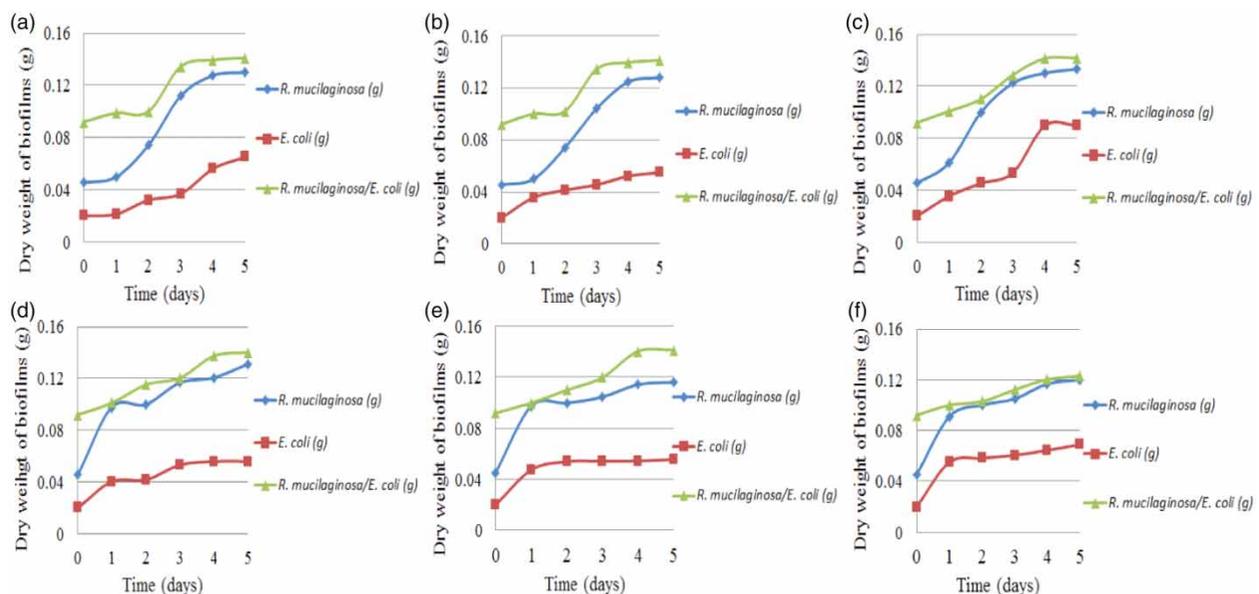


Figure 4 | The amount of single- and mixed-species biofilm in the presence of (a) Cd^{2+} , (b) Zn^{2+} , (c) Ni^{2+} , (d) Cu^{2+} , (e) Pb^{2+} and (f) Hg^{2+} .

R. mucilaginosa and *E. coli* biofilms show significant potential in the process of elimination of heavy metals from contaminated environments, whereby the mixed-species biofilm (*R. mucilaginosa/E. coli*) has greater efficiency.

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