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Removal of heavy metals using different polymer matrixes as support for bacterial immobilisation

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ABSTRACT

Great attention is focused on the microbial treatment of metal contaminated environments. Three bacterial strains, 1C2, 1ZP4 and EC30, belonging to genera *Cupriavidus*, *Sphingobacterium* and *Alcaligenes*, respectively, showing high tolerance to Zn and Cd, up to concentrations of 1000 ppm, were isolated from a contaminated area in Northern Portugal. Their contribution to Zn and Cd removal from aqueous streams using immobilised alginate, pectate and a synthetic cross-linked polymer was assessed. In most cases, matrices with immobilised bacteria showed better metal removal than the non-inoculated material alone. For the immobilisation with all the polymers, 1C2 was the strain that increased the removal of Zn the most, whereas EC30 was the most promising for Cd removal, especially when combined with the synthetic polymer with up to a ca. 11-fold increase in metal removal when compared to the polymer alone. Removal of individual metals from binary mixtures showed that there was differential immobilisation. There was greater removal of Cd than Zn (removals up to 40% higher than those showed for Zn). The results show that metal contaminated environments constitute a reservoir of microorganisms resistant/tolerant to heavy metals that have the capacity to be exploited in bioremediation strategies.

Capsule immobilisation of bacteria in the naturally occurring alginate and pectate and in a synthetic cross-linked polymer increased the Zn and Cd removal abilities from single and binary contaminated waters; the applications with the synthetic polymer were the most promising for Cd and Zn removal in single and binary mixtures.

1. Introduction

Heavy metal pollution is one of the most important environmental problems today, especially in relation to water contamination. Several industries, mining and smelting, as well as production of fuel, energy, fertilizers, metallurgy, electroplating, electrolysis, leatherworking and photography [1] produce waste and wastewaters that are discharged in water courses threatening the ecosystems and ultimately human health. Traditional methods of metal removal generally consist of physical and/or chemical approaches which are often expensive, with high energy and chemical requirements, producing high amounts of residues [2]. They are often not effective especially for low to moderate metal concentrations [3]. In this context, the search for more effective methods is necessary to reduce heavy metal contamination in waste water to environmentally acceptable levels. Biologically based, eco-friendly and economically more attractive technologies are required.

Biosorption is a method that involves the use of biological materials that form complexes with metal ions using their functional groups [4]. In the process, a chemical link between functional groups on the biosorbent and the metal ions present in solution or an ion-exchange reaction due to the high ion-exchange capacity of the biosorbent may occur [5]. Bacteria have a high surface area-to-volume ratio and can thus provide a large contact surface, which allows the interaction with metals in its surroundings [6], and have been successfully used as biosorbents [7–9]. However, studies demonstrate that sometimes living systems are inconsistent, especially when using freely suspended biomass. In fact, although freely suspended biomass can promote higher contact with the contaminants during the removal process, it is usually unpractical as a clean-up method [10]. Biopolymers are non-toxic and when used to immobilise biomass may help improve biosorption capacity and facilitate biomass separation from metal bearing solutions. This can then be a non-destructive process if necessary and allow the regeneration of biosorbents for multiple uses, as well as increasing biomass concentration [11,12]. The ion-exchange process that occurs in such polymers when exposed to water contaminated

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with metals [13] is complemented with the biosorption capacity of the immobilised microorganisms. Other alternative is the use of synthetic polymers as matrices that can control or promote bioadhesion. Potential applications for materials that are bio-adherent or bio-compatible are widespread [14]. Usually the synthesis of functional polymeric materials involves the use of a functional monomer to impart the desired characteristics to the final material and a cross-linker which will give the necessary rigidity to the polymer network. The main advantages of using these materials is the possibility to fine-tune the final properties by varying polymer composition, robustness and stability under a wide range of chemical and physical conditions.

Common matrices used to support organisms (either of natural or synthetic origin) include hydrogels [15], activated alumina and charcoal [16], kaolin [2], polyacrylonitrile [17], alginate and pectate.

The objectives of this study were to compare the use of alginate, pectate and a synthetic porous cross-linked polymer as immobilisation matrices for metal resistant bacteria species, comparing the contribution of different bacteria in the removal of the metals Cd and Zn, supplied alone and as mixed metal solutions.

2. Materials and methods

2.1. Isolation and selection of heavy metal resistant bacterial strains

Selected bacterial species were isolated from a metal contaminated site – Estarreja, Northern Portugal. Despite the high presence of metals – average levels of 835 mg Pb kg^{-1}, 66 mg Hg kg^{-1}, 26 mg Cr kg^{-1}, 37 mg Ni kg^{-1}, 16,800 mg Fe kg^{-1} and 3620 mg Zn kg^{-1} (total Zn) – the area is prolific in vegetation [18]. Several bacterial strains were isolated from the non-rhizosphere and rhizosphere soils. Soil samples were collected and serially diluted in saline solution (0.85% (w/v) NaCl) and inoculated on trypticase soy agar (TSA; Oxoid) at 30 °C. Visually different colonies selected on the basis of colony morphology and colour were further purified [19]. For this study, 3 strains isolated at pH 7 designated as 1ZP4, EC30 and 1C2, were selected based on their metal tolerance in *in vitro* screening assays. Cell morphology was tested as described by Alexander and Strete [20]. Gram staining tests were performed as described by Murray et al. [21] and Smibert and Krieg [22]. The pH range for growth was determined in buffered trypticase soy broth (TSB) adjusted at pH 3–10 (at 1 pH unit intervals). The turbidity of the cultures grown in an orbital shaker at 25 °C was measured at 610 nm. All buffer solutions used to adjust the pH of TSB were prepared from 1 M stock solutions [23]. Citrate buffer was used for pH 3–6, phosphate buffer for pH 7, Tris–HCl buffer for pH 8, and a carbonate–bicarbonate buffer for pH 9 and 10. Growth temperature ranges were determined at 15, 20, 25, 30, 37 °C on TSB and on TSA at 4, 10, and 50 °C. Extraction of genomic DNA, PCR amplification of the 16S rRNA gene and sequencing of the purified PCR products were carried out as described by Rainey et al. [24]. Cloning of the amplicons into pGEM T-Easy vector (Promega) and cycle-sequencing were performed at Macrogen Inc. (Seoul, Republic of Korea), using 16S universal bacterial primers (f27, f518, r800, r1492) [25]. The quality of the 16S rRNA gene sequences was checked manually by the use of the BioEdit program (version 7.0.5.3) [26], and the sequences were aligned against representative reference sequences of the most closely related members obtained from the National Center for Biotechnology Information database [27].

2.2. Effect of metals on bacterial growth in suspension cultures

Three hundred millilitre Erlenmeyer flasks containing 100 ml TSB supplemented with heavy metals at concentrations of 50, 100 mg L^{-1} (Cd^{2+}), 100, 250 mg L^{-1} (Zn^{2+}) and metal mixtures of 200 mg L^{-1} [100 mg L^{-1} (Cd^{2+}) + 100 mg L^{-1} (Zn^{2+})] were inoculated with the bacterial strains in order to achieve a starting optic density (OD) of 0.1 at 610 nm. The metals were applied as salts $ZnCl_2$ and $CdCl_2$. All the cultures, including controls (in triplicate), were incubated at 30 °C for 24 h at 150 rpm. Bacterial growth was monitored at time intervals by measuring the optical density at 610 nm and the specific growth rate of each strain was determined. The strains with the highest growth rate were EC30, 1ZP4 and 1C2 and were selected for further characterisation and for the uptake tests.

2.3. Synthetic cross-linked polymer synthesis

Polymers were prepared by mixing in a 100 ml glass bottle 40 g ethylene glycol dimethacrylate, 0.37 g N,N-diethylamino ethyl methacrylate, 2 g polyethylene glycol 35,000, 40.37 g N,N-dimethylformamide and 0.85 g 1,1′-azobis cyclohexanecarbonitrile. The mixture was bubbled with nitrogen for 5 min and sealed with Teflon coated caps. Polymerisation took 20 min and was initiated using an UVAPRINT 100 CVI UV source with a 0.163 W/cm^2 intensity [28]. The resulting polymer monolith was crushed manually in a mortar with a pestle and the particles in the range 200–500 μm collected using sieves from Endecotts, UK. Polymers were then washed with methanol overnight in a sohxlet apparatus in order to remove any unreacted monomers and the polyethylene glycol and after dried at 60 °C during 6 h. Polymers were produced with weak alkaline monomers in order to promote bacterial adhesion. The composition of the polymer was adapted from Barral et al. [29].

2.4. Bacterial immobilisation

The bacterial strains (EC30, 1ZP4 and 1C2) were grown in 300 ml Erlenmeyer flasks containing 100 ml TSB until the cell biomass reached an OD of 1.0 (610 nm). Cells were harvested by centrifugation at 6000 rpm for 15 min and the bacterial pellet weighed and washed using sterile ultra-pure water. The harvested biomass was re-suspended in 25 ml sterile Universal bottles containing 5 ml of saline solution (0.85%, w/v).

For Ca-alginate and Ca-pectate, the bacterial inoculum was immobilised under aseptic conditions, using the method described by Escamilla et al. [30] and Montes and Magana [31] with some modifications. The inoculum [OD = 1 (610 nm), which represented a fresh weight of 74 mg for 1C2, 108 mg for 1ZP4 and 128 mg for EC30, in a volume of 100 ml] was adjusted in a volumetric cylinder to 1:1 inoculum/polymer ratio by using alginic acid (Sigma) or polygalacturonic (Sigma) 4% (w/v) concentrated. The solution was homogenized and forced though a needle template (gauge for ±3 mm beads) with a peristaltic pump (Watson–Marlow Bredel, Wilmington, Mass.) flowing at 10 ml m^{-1}, and the droplets were collected in a sterile gel inducer solution of 3.5% (w/v) $CaCl_2$. After soaking for 1 h, the liquid was decanted and the spherical beads were washed with sterile ultra pure water. In aseptic conditions the beads were then packed into sterile 6 ml fritted SPE tubes (Supelco) with a filter. An adaptor cap (Phenomenex) was fitted to each of the tubes. For the synthetic polymer, 1 g was packed in sterile 6 ml fritted SPE tubes (Supelco) containing a filter under aseptic conditions. Bacterial biomass was then added to the tube (fresh weight of 150 mg). An adaptor cap (Phenomenex) was fitted to each of the tubes. Tubes were then left to settle for 1 h at room temperature. An additional alternative method was used with the synthetic polymer. The bacterial strains were grown in 300 ml Erlenmeyer flasks containing 100 ml TSB and 3 g of the synthetic polymer until cells grew to 1.0 OD (610 nm). Cells and polymer were then harvested by centrifugation at 6000 rpm for 15 min and the bacterial

Table 1
Characteristics of strains 1ZP4, EC30 and 1C2.

Characteristic	1ZP4	EC30	1C2
Colony pigmentation	White	White	Pearly white
Cell morphology	Rod	Rod	Rod
Gram	–	–	–
Growth temperature (°C)			
Range	10–40	10–40	10–40
Optimum	25–30	30	25
pH for growth			
Range	5–9	5–9	5–9
Optimum	7	7–8	6–7

and polymer pellet was weighted. Under aseptic conditions 1.5 g of the pellet containing the bacterial biomass and the synthetic polymer was packed in sterile 6 ml fritted SPE tubes (Supelco) with filter. An adaptor cap (Phenomenex) was fitted to each of the tubes. Tubes were then left to settle for 1 h at room temperature.

In every case, polymers were washed prior use and recirculation was made until OD of washing solution was bellow 0.1 (610 nm).

2.5. Heavy metal uptake tests

For metal uptake batch experiments, 5 ml of a solution (pH ranging from 6.50 to 7.01) containing 100 mg L^{-1} of Cd^{2+}, Zn^{2+} or a mixed metal solution containing 100 mg L^{-1} of each of the metals was added to the polymer packed tubes – metals for the solutions preparation were applied as their salts $ZnCl_2$ and $CdCl_2$. Three sequential cycles of 5 ml were tested for each treatment, with an average contact time of 2 min. Outlet solutions were collected filtered using a Puradisc 25 Syringe Filter (Whatman) and the amount of residual metal present in solution was measured by atomic absorption spectrophotometry in a Hitachi Z-8100 Atomic absorption spectrophotometer, with Zeeman correction.

2.6. Statistical analysis

Each treatment was comprised of 3 replicates. Statistical analysis was performed using the SPSS program (SPSS Inc., Chicago, IL Version 15.0). The data were analysed through variance analysis (ANOVA). To detect the statistical significance of differences ($P < 0.05$) between means, the Tukey test was performed.

3. Results

3.1. Bacterial strains

The tested phenotypic characteristics of strains 1ZP4, EC30 and 1C2 are given in Table 1. The pH and temperature ranges for growth of the strains were similar. Full length (about 1250–1450 bp) 16S rRNA of strains 1ZP4, EC30 and 1C2 were sequenced and the closest affiliation according to sequencing were for strain 1ZP4 *Sphingobacterium* sp. MG2 (AY556417), for EC30 *Alcaligenes* sp. S-SL-5 (FJ529025) and for 1C2 *Cupriavidus* sp. 2CSa-12 (GU167923).

3.2. Growth of 1ZP4, EC30 and 1C2 in the presence of heavy metals

Growth curves for strains 1ZP4, EC30 and 1C2 in the presence of Zn^{2+} are shown in Fig. 1. At the concentrations tested, Zn^{2+} had only a small effect on their growth. Growth of strains 1ZP4, EC30 and 1C2 was significantly reduced when TSB medium contained Cd^{2+} (Fig. 1). 1C2 was the strain most affected by the presence of Cd. Remarkably, none of the tested strains showed a significant lag phase. Final biomass concentration was lower when 100 mg L^{-1} of Cd^{2+} was applied (Fig. 1).

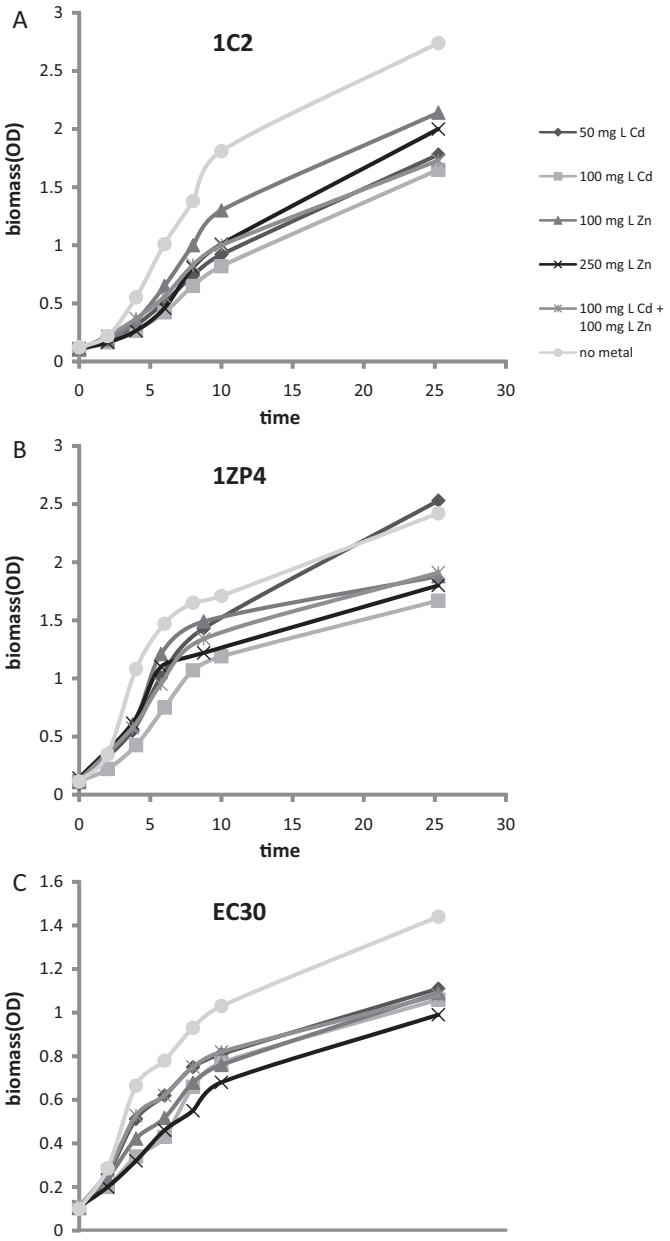


Fig. 1. Growth curves of strains 1C2 (A), 1ZP4 (B) and EC30 (C) under 50 and 100 mg Cd /L, 100 and 250 mg Zn/L, 100 mg Cd + 100 mg Zn/L and no metal.

When a metal mixture was used growth of strain 1C2 was visibly reduced (Fig. 1), which can possibly be attributed to the presence of Cd. On the other hand, the metal mixture had less effect on the growth of strains EC30 and 1ZP4. In fact, for strain EC30, part of the exponential growth phase was similar to the control growth (Fig. 1).

3.3. Removal of single metals in solution by different matrices and immobilised bacterial strains

3.3.1. Removal of Zn

The matrix type and bacterial immobilisation had a significant ($P < 0.05$) effect on Zn removal. In general, the treatments that included bacteria showed significantly ($P < 0.05$) better Zn removal than the matrices on their own, as shown by the significantly lower concentrations of Zn in the outlet of the cartridges. ANOVA two way test results were, in summary, after the

first removal cycle, $F_{Zn(matrix)} = 434$ ($P < 0.001$), $F_{Zn(bacteria)} = 1124$ ($P < 0.001$) and $F_{Zn(matrix \times bacteria)} = 154$ ($P < 0.001$); for the second cycle $F_{Zn(matrix)} = 446$ ($P < 0.001$), $F_{Zn(bacteria)} = 725$ ($P < 0.001$) and $F_{Zn(matrix \times bacteria)} = 253$ ($P < 0.001$); and for the third cycle $F_{Zn(matrix)} = 69.4$ ($P < 0.001$), $F_{Zn(bacteria)} = 175$ ($P < 0.001$) and $F_{Zn(matrix \times bacteria)} = 58.5$ ($P < 0.001$).

For each specific matrix (alginate, pectate, synthetic polymer and incubated synthetic polymer), the effect of the bacterial application on Zn removal was determined using one way ANOVA. In the alginate matrix, generally inoculation with strain EC30 immobilised in alginate gave the best immobilisation of this metal (Table 2). The removal varied significantly ($P < 0.05$) within cycles of metal application, showing that a clear relationship between the repeated use and the removal efficiency cannot generally be drawn for alginate. For pectate-based treatments, generally strain 1ZP4 was the best strain. However, in by the third cycle there was no difference between treatments ($P < 0.05$). Removals of Zn by the synthetic polymer matrix based treatments are also shown in Table 2. In general, strain 1C2 was more active when combined with the synthetic polymer. Over time (1–3 cycles) this combination became less efficient at removing this metal. When the bacterial cells were incubated with the synthetic polymer prior to packing, again strain 1C2 was the best treatment and *t* significantly ($P < 0.05$) enhanced Zn removal in this matrix (Table 2). Overall, strain 1C2 immobilised on the synthetic polymer (PY + 1C2) was the best treatment and was significantly ($P < 0.05$) better (up to 76% more metal removed), than the other treatments especially in cycles 1 and 2. Effective removal was also observed for the polymer with EC30 (PY + EC30) and for both these combinations when bacteria were incubated with the polymer (PYInc + 1C2 and PYInc + EC30).

Adsorption efficiencies to bacterial biomass per unit weight of cells were estimated and are shown in Table 4 for each bacterial treatment. For Zn removal in single solutions, higher adsorption levels were obtained for the PYInc + EC30 mixture, with an efficiency of 2.2 mg Zn g^{-1} bacterial cells.

3.3.2. Removal of Cd

The matrix type and bacterial strain immobilisation had a significant ($P < 0.05$) effect on Zn removal (two-way ANOVA). In all cycles, the treatments that included bacteria showed significantly ($P < 0.05$) better Cd removal than when the matrices were used alone. Test results were for the 1st cycle $F_{Cd(matrix)} = 756$ ($P < 0.001$), $F_{Cd(bacteria)} = 1524$ ($P < 0.001$) and $F_{Cd(matrix \times bacteria)} = 135$ ($P < 0.001$); for the second cycle $F_{Cd(matrix)} = 185$ ($P < 0.001$), $F_{Cd(bacteria)} = 630$ ($P < 0.001$) and $F_{Cd(matrix \times bacteria)} = 272$ ($P < 0.001$); and for the third cycle $F_{Cd(matrix)} = 45.2$ ($P < 0.001$), $F_{Cd(bacteria)} = 645$ ($P < 0.001$) and $F_{Cd(matrix \times bacteria)} = 209$ ($P < 0.001$).

As for Zn, Cd removal was compared for each specific matrix treatment alone and with immobilised bacterial strains. Strain EC30 immobilised in alginate was shown to significantly immobilise this metal (Table 3). The behaviour of these combinations of alginate-bacteria was also analysed throughout the cycles and it generally varied with time, with significant ($P < 0.05$) differences in the removal efficiencies between the three cycles. Strains 1ZP4 and 1C2 immobilised in pectate significantly ($P < 0.05$) increased Cd removal. The behaviour of these pectate-bacteria combinations varied throughout the cycles. Immobilisation with strain EC30 in the synthetic polymer gave a 11-fold increase in the removal of Cd when compared with the polymer alone; additionally, all the treatments showed a significant ($P < 0.05$) decrease of removal efficiency of Cd throughout the cycles, similarly to what happened for Zn (Table 3) When the bacteria were incubated with the synthetic polymer prior to packing, no specific treatment was found to be more effective than any other. However, strains EC30 and 1C2 immobilised directly with the polymer matrix improved removal (Table 3). For all cycles, strain EC30 immobilisation onto the synthetic polymer (PY + EC30) was the best treatment.

Cadmium adsorption efficiencies per unit weight of cells (Table 4) in single solutions were determined and higher adsorption levels were also obtained for the PYInc + EC30 mixture, with an efficiency of 2.8 mg Cd g^{-1} bacterial cells.

3.4. Removal of binary mixtures of metals by matrices and immobilised bacterial strains

The ability of the bacterial tested strains to take up metals from binary mixtures was then determined. Strain EC30 was best at removing Cd from the binary mixtures, regardless of immobilising system used (see Table 3). All the treatments showed significant ($P < 0.05$) variations in the removal efficiencies of Cd throughout the cycles, according to one-way ANOVA performed on data. For Zn, strain EC30 immobilised in the alginate matrix improved the differential uptake ($P < 0.05$) (Table 3), while strain 1ZP4 enhanced metal uptake when immobilised in pectate. Strain 1C2 was best at removing Zn from the binary mixtures when using the synthetic polymer. Overall, strain 1C2 + PY was best at differentially taking up Zn. As previously observed, by the third cycle metal removal was much less than in the earlier cycles.

Zinc and Cd adsorption efficiencies per unit weight of cells in the binary solution were also determined (Table 4) and the best performance was found for the treatments PYInc + 1C2 and PY + 1C2 for Zn, with an adsorption level of 1.8 mg Zn g^{-1} cells, and for P + 1C2 and A + 1C2 for Cd, registering efficiencies of 2.2 mg Cd g^{-1} cell.

Zn removal in single (Zn) and binary (Zn + Cd) mixtures in each treatment were also compared pair wise using the *t*-test (Table 2). For all matrices and cycles, differences in the ability to remove Zn were observed between simple and binary contamination scenarios, which seem to indicate that the performance of the treatments is influenced not only by the concentration but also by the metal feed composition. The same procedure was used for Cd removal in single (Cd) and binary (Zn + Cd) solutions (Table 3). As in the case of Zn, for all matrixes and cycles, differences in Cd removal were observed between simple and binary contamination scenarios.

Cd and Zn removal in the binary mixture were compared using the *t*-test. Results showing levels of the metals in the outlet (in mM) are presented in Fig. 2 for alginate, and indicate that levels of Cd in the outlet were always significantly ($P < 0.05$) lower than those of Zn. For pectate based combinations, the same trend was observed (Fig. 3). With the exception of 1C2 immobilised to the synthetic polymer treatment, that presented no significant ($P < 0.05$) differences in Cd and Zn removal in cycle 1 (Fig. 4), levels of Cd at the outlet were significantly ($P < 0.05$) lower than those of Zn in the polymer based treatments (Figs. 4 and 5), decrease that showed to be of up to 65%. It seems thus that generally the tested bacteria–matrix combinations had higher affinity for Cd when a binary mixture was present.

4. Discussion

The aim of the work was to assess the effect of bacterial immobilisation in metal removal, and to compare the efficiency of bacteria + polymer combinations in order to understand which combinations were most appropriate for use in the clean-up of Cd and Zn contaminated waters.

4.1. Removal of individual metals by immobilised bacterial matrices

Metal sequestration by a sorbent may be due to one or a combination of the following processes: ion exchange, physical adsorption, chemisorptions, complexation or microprecipitation

Table 2
Levels of Zn in the outlet for each treatment (mg Zn L^{-1}).

Treatment	Round 1		Round 2		Round 3	
	100 mg Zn/L	100 mg Zn + 100 mgCd/L	100 mg Zn/L	100 mgZn + 100 mg Cd/L	100 mg Zn/L	100 mg Zn + 100 mg Cd/L
A	$97.4 \pm 0.1^{gh,D}$	$92 \pm 2^{ef,C} \neq$	$84 \pm 2^{ef,B}$	$84 \pm 6^{fg,BC}$	$82 \pm 1^{ef,B}$	$76 \pm 0^{abc,AB}$
A + 1C2	$83.4 \pm 0.5^{ef,C}$	$81 \pm 2^{de,B}$	$87.8 \pm 0.3^{f,B}$	$89.8 \pm 0.8^{g,C} \neq$	$87,1 \pm 0.6^{f,C}$	$83 \pm 2^{abc,C}$
A + 1ZP4	$52 \pm 3^{c,A}$	$66 \pm 5^{c,A} \neq$	$84 \pm 2^{efv,B}$	$75.7 \pm 0.09^{df,AB} \neq$	$79 \pm 4^{def,B}$	$79 \pm 2^{abc,BC}$
A + EC30	$64.9 \pm 0.2^{d,B}$	$70 \pm 2^{cd,A} \neq$	$69 \pm 2^{d,A}$	$74 \pm 3^{d,A}$	$71.0 \pm 0.8^{cd,A}$	$73.9 \pm 0.8^{abc,A} \neq$
	$^{***}F = 513$	$^{***}F = 44.2$	$^{***}F = 67.1$	$^{**}F = 14,5$	$^{***}F = 35.6$	$^{**}F = 16.3$
P	$91 \pm 1^{gh,C}$	$99 \pm 2^{f,C} \neq$	$79.4 \pm 0.7^{e,B}$	$77 \pm 4^{df,A}$	$74 \pm 2^{cde,A}$	$65 \pm 0^{a,A} \neq$
P + 1C2	$79 \pm 2^{e,B}$	$77.8 \pm 0.4^{d,B}$	$80 \pm 3^{e,B}$	$83.5 \pm 0.6^{fg,B}$	$79 \pm 6^{def,A}$	$82.425 \pm 0.005^{abc,D}$
P + 1ZP4	$41 \pm 2^{b,A}$	$44 \pm 2^{b,A}$	$68 \pm 3^{d,A}$	$74 \pm 2^{cd,A} \neq$	$79.9 \pm 0.8^{defg,A}$	$75.5 \pm 0.6^{abc,B} \neq$
P + EC30	$80.28 \pm 0.03^{e,B}$	$74 \pm 3^{cd,B} \neq$	$80 \pm 1^{e,B}$	$76.8 \pm 0.3^{df,A} \neq$	$77 \pm 2^{de,A}$	$79.7 \pm 0.5^{abc,C} \neq$
	$^{***}F = 588$	$^{***}F = 386$	$^{***}F = 21.0$	$^{**}F = 10.4$	$^{NS}F = 2.14$	$^{***}F = 734$
PY	$102.05 \pm 0.05^{h,C}$	$101.4 \pm 0.6^{f,C}$	$105.5 \pm 0.8^{h,D}$	$106.2 \pm 0.6^{h,D} \neq$	$109.0 \pm 0.4^{g,B}$	$108.6 \pm 0.3^{c,A}$
PY + 1C2	$26 \pm 4^{a,A}$	$22 \pm 6^{a,A}$	$31.9 \pm 0.5^{a,A}$	$42,0 \pm 0.1^{a,A} \neq$	$74 \pm 8^{cde,A}$	$72.3 \pm 0.2^{abc,A}$
PY + 1ZP4	$44 \pm 4^{b,B}$	$46 \pm 2^{b,B}$	$68 \pm 2^{d,C}$	$73 \pm 2^{cd,C}$	$99 \pm 1^{g,B}$	$101 \pm 1^{abc,A}$
PY + EC30	$22 \pm 2^{a,A}$	$35 \pm 12^{b,AB}$	$50 \pm 2^{c,B}$	$64 \pm 5^{bc,B*}$	$65 \pm 4^{c,A}$	$76 \pm 6^{abc,A}$
	$^{***}F = 477$	$^{***}F = 82.6$	$^{***}F = 1118$	$^{***}F = 305$	$^{***}F = 64.6$	$^{NS}F = 1.41$
PYInc	$96 \pm 4^{gh,C}$	$101 \pm 1^{f,C}$	$96 \pm 1^{g,C}$	$103.99 \pm 0.06^{h,C}$	$101 \pm {}^{g,D}$	$106 \pm 4^{bc,C}$
PYInc + 1C2	$28 \pm 4^{a,AB}$	$18.9 \pm 0.5^{a,A} \neq$	$41 \pm 4^{b,A}$	$48 \pm 7^{a,A}$	$51 \pm 3^{b,B}$	$67 \pm 2^{ab,A} \neq$
PYInc + 1ZP4	$37 \pm 4^{b,B}$	$44 \pm 3^{b,B}$	$47.5 \pm 0.4^{c,B}$	$44 \pm 4^{a,A}$	$79 \pm 3^{def,C}$	$79 \pm 3^{abc,B}$
PYInc + EC30	$25 \pm 3^{a,A}$	$21 \pm 1^{a,A}$	$38.7 \pm 0.4^{b,A}$	$60 \pm 2^{b,B} \neq$	$39 \pm 4^{a,A}$	$70 \pm 2^{abc,A}$
	$^{***}F = 277$	$^{***}F = 1503$	$^{***}F = 520$	$^{***}F = 140$	$^{***}F = 254$	$^{***}F = 118$
	$^{***}F = 404$)	$^{***}(F = 172)$	$^{***}(F = 387)$	$^{***}(F = 108)$	$^{***}(F = 84)$	$^{*}(F = 2.52)$

Results are expressed as mean ± S.D. ($n = 3$). Means for each treatment in the same column with different lowercase letters are significantly different from each other ($P < 0.05$) according to the Tukey test. For each round, the test results are shown with the test statistics and as: NS, non-significant at the level $P < 0.05$; (*) significant at the level $P < 0.05$; (**) significant at the level $P < 0.01$; (***) significant at the level $P < 0.001$. For each matrix (alginate, pectate, polymer and incubated polymer) results of one way ANOVA are also shown with the test statistics and as: NS, non-significant at the level $P < 0.05$; (*) significant at the level $P < 0.05$; (**) significant at the level $P < 0.01$; (***) significant at the level $P < 0.001$. Means for the same matrix type in the same round with different uppercase letters are significantly different from each other ($P < 0.05$) according to the Tukey test. Results of the comparison between results for different effluents (Zn and Zn + Cd) for each treatment are shown and when means of Cd + Zn in each round have a $\neq$ signal they are significantly different from means of outlet Zn ($P < 0.05$) according to the *t*-test.

Table 3
Levels of Cd in the outlet for each treatment (mg Cd/L).

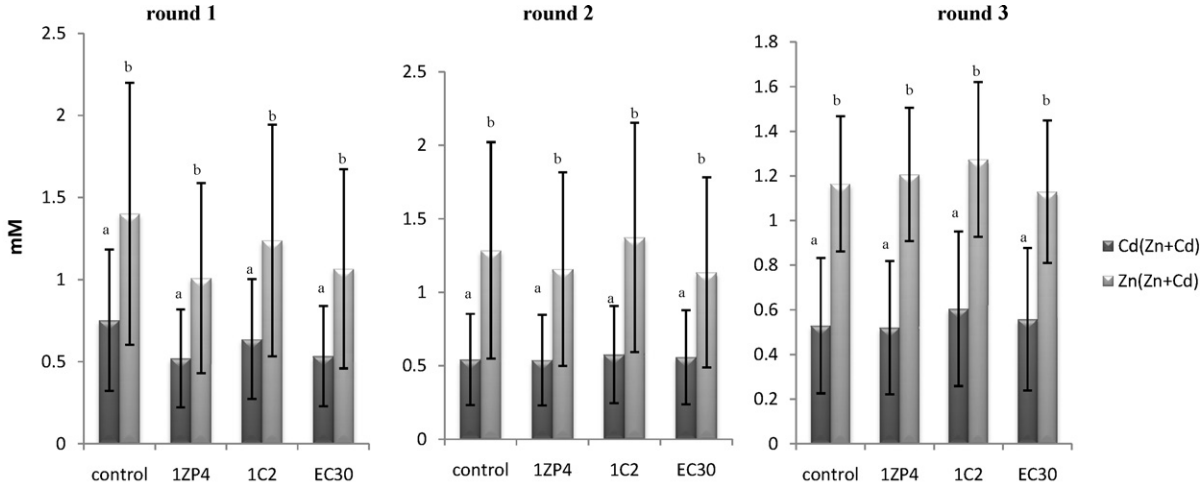
Treatment	Round 1		Round 2		Round 3	
	100 mg Cd/L	100 mg Zn + 100 mg Cd/L	100 mg Cd /L	100 mg Zn + 100 mg Cd/L	100 mg Cd/L	100 mg Zn + 100 mgCd/L
A	$88 \pm 2^{f,C}$	$85 \pm 2^{h,A}$	$61.5 \pm 0.3^{f,B}$	$61 \pm 2^{def,A}$	$63.1 \pm 0.1^{e,B}$	$60 \pm 1^{a,AB} \neq$
A + 1C2	$67.9 \pm 0.2^{e,B}$	72 ± 1^{gB}	$68 \pm 2^{fg,C}$	$65 \pm 2^{ef,A}$	$69 \pm 1^{e,B}$	$68.0 \pm 0.9^{bcde,C}$
A + 1ZP4	$63.3 \pm 0.4^{de,B}$	$58.47 \pm 0.05^{d,A} \neq$	$64 \pm 1^{fg,BC}$	$61 \pm 2^{def,A}$	$66 \pm 5^{e,B}$	$58 \pm 3^{a,A}$
A + EC30	$47 \pm 4^{c,A}$	$60 \pm 1^{de,A} \neq$	$45 \pm 2^{d,A}$	$63 \pm 1^{ef,A} \neq$	$48 \pm 1^{cd,A}$	$62.7 \pm 0.4^{abc,B} \neq$
	$^{***}F = 147$	$^{***}F = 239$	$^{***}F = 144$	$^{NS}F = 3.18$	$^{***}F = 35.6$	$^{***}F = 22.4$
P	$92 \pm 3^{f,C}$	$86 \pm 1^{h,B} \neq$	$54 \pm 2^{e,A}$	$58 \pm 3^{de,A}$	$65.7 \pm 0.3^{e,A}$	$64 \pm 7^{abcd,AB}$
P + 1C2	$63 \pm 1^{de,AB}$	$64.1 \pm 0.4^{ef,A}$	$69.2 \pm 0.7^{g,B}$	$69 \pm 1^{f,C}$	$65 \pm 1^{e,A}$	$69.6 \pm 0.2^{de,B} \neq$
P + 1ZP4	$58 \pm 3^{d,A}$	$61.9 \pm 0.8^{def,A}$	$64.8 \pm 0.8^{fg,B}$	$64.9 \pm 0.5^{ef,B}$	$65 \pm 2^{e,A}$	$57 \pm 3^{a,A} \neq$
P + EC30	$68 \pm 4^{e,B}$	$64 \pm 3^{f,A}$	$64 \pm 5^{fg,B}$	$59.92 \pm 0.07^{def,A}$	$64 \pm 2^{e,A}$	$61 \pm 1^{a,AB}$
	$^{***}F = 87.7$	$^{***}F = 76.0$	$^{***}F = 18.7$	$^{***}F = 34.8$	$^{NS}F = 1.03$	$^{*}F = 6.32$
PY	$91.9 \pm 0.3^{f,C}$	$98.24 \pm 0.03^{i,C} \neq$	$92.46 \pm 0.07^{h,D}$	$96.9 \pm 0.2^{g,C} \neq$	$95.7 \pm 0.5^{f,C}$	$100 \pm 2^{f,C} \neq$
PY + 1C2	$21 \pm 1^{b,B}$	$40 \pm 1^{c,B} \neq$	$36 \pm 3^{c,C}$	$63.8 \pm 0.9^{ef,B} \neq$	$49 \pm 3^{cd,B}$	$69 \pm 2^{cdeB,} \neq$
PY + 1ZP4	$6 \pm 2^{a,A}$	$38.41 \pm 0.05^{c,B} \neq$	$25 \pm 4^{bc,B}$	$33 \pm 4^{a,A}$	$46 \pm 4^{b,B}$	$61.7 \pm 0.7^{ab,A} \neq$
PY + EC30	$5 \pm 1^{a,A}$	$23 \pm 1^{b,A} \neq$	$15.8 \pm 0.7^{a,A}$	$38 \pm 6^{a,A} \neq$	$31 \pm 1^{a,A}$	$58 \pm 3^{a,A} \neq$
	$^{***}F = 3860$	$^{***}F = 5269$	$^{***}F = 680$	$^{***}F = 431$	$^{***}F = 353$	$^{***}F = 295$
PYInc	$101.65 \pm 0.05^{g,C}$	$101.25 \pm 0.05^{i,C} \neq$	$107.6 \pm 0.2^{i,C}$	$106 \pm 3^{g,C}$	$105.3 \pm 0.7^{g,D}$	$105 \pm 1^{f,D}$
PYInc + 1C2	$18 \pm 3^{b,AB}$	$18.5 \pm 0.5^{ab,A}$	$25 \pm 3^{b,A}$	$46 \pm 7^{bc,AB} \neq$	$30 \pm 2^{a,A}$	$69 \pm 1^{cde,B} \neq$
PYInc + 1ZP4	$19 \pm 5^{b,B}$	$37 \pm 3^{c,B} \neq$	$37 \pm 5^{c,B}$	$37 \pm 3^{ab,A}$	$54.6 \pm 0.8^{d,C}$	$72.2 \pm 0.7^{e,C} \neq$
PYInc + EC30	$11 \pm 3^{a,A}$	$16.4 \pm 0.5^{a,A} \neq$	$22 \pm 2^{ab,A}$	$52 \pm 7^{cd,B} \neq$	$41 \pm 2^{b,B}$	$61.6 \pm 0.6^{ab,A} \neq$
	$^{***}F = 528$	$^{***}F = 2052$	$^{***}F = 476$	$^{***}F = 96.3$	$^{***}F = 1607$	$^{***}F = 1112$
	$^{***}(F = 404)$	$^{***}(F = 172)$	$^{***}(F = 387)$	$^{***}(F = 108)$	$^{***}(F = 84)$	$^{*}(F = 2.52)$

Results are expressed as mean ± S.D. ($n = 3$). Means for each treatment in the same column with different lowercase letters are significantly different from each other ($P < 0.05$) according to the Tukey test. For each round, the test results are shown with the test statistics and as: NS, non-significant at the level $P < 0.05$; (*) significant at the level $P < 0.05$; (**) significant at the level $P < 0.01$; (***) significant at the level $P < 0.001$. For each matrix (alginate, pectate, polymer and incubated polymer) results of one way ANOVA are also shown with the test statistics and as: NS, non-significant at the level $P < 0.05$; (*) significant at the level $P < 0.05$; (**) significant at the level $P < 0.01$; (***) significant at the level $P < 0.001$. Means for the same matrix type in the same round with different uppercase letters are significantly different from each other ($P < 0.05$) according to the Tukey test. Results of the comparison between results for different effluents (Cd and Zn + Cd) for each treatment are shown and when means of Cd + Zn in each round have a $\neq$ signal they are significantly different from means of outlet Cd ($P < 0.05$) according to the *t*-test.

Table 4
Adsorption of metal per unit weight of cells for each treatment (mg Zn g^{-1} cell).

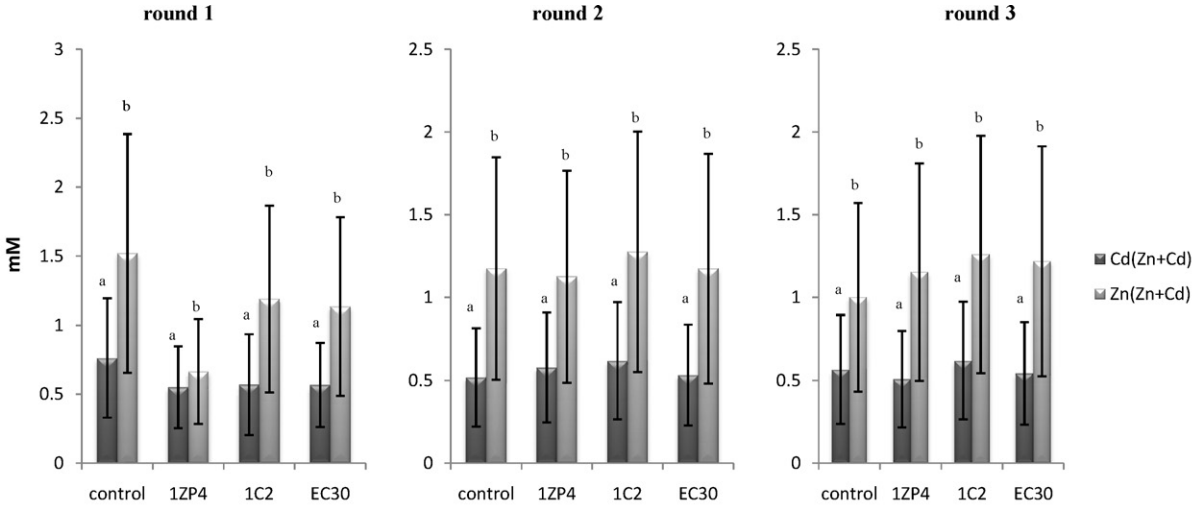
Treatment	Zn		Cd	
	100 mg Zn/L	100 mg Zn + 100 mg Cd/L	100 mg Cd/L	100 mg Zn + 100 mg Cd/L
A + 1C2	0.9 ± 0.1	1.0 ± 0.3	2.15 ± 0.09	2.2 ± 0.2
A + 1ZP4	1.3 ± 0.7	1.2 ± 0.3	1.6 ± 0.1	1.89 ± 0.09
A + EC30	1.2 ± 0.1	1.1 ± 0.1	2.1 ± 0.1	1.49 ± 0.06
P + 1C2	1.4 ± 0.2	1.3 ± 0.2	2.3 ± 0.2	2.2 ± 0.2
P + 1ZP4	1.7 ± 0.8	1.7 ± 0.7	1.7 ± 0.2	1.8 ± 0.2
P + EC30	0.82 ± 0.08	0.9 ± 0.1	1.4 ± 0.1	1.5 ± 0.1
PY + 1C2	1.9 ± 0.8	1.8 ± 0.7	2.2 ± 0.4	1.4 ± 0.4
PY + 1ZP4	1.0 ± 0.8	1.2 ± 1.0	2.5 ± 0.6	1.9 ± 0.4
PY + EC30	1.8 ± 0.6	1.4 ± 0.6	2.8 ± 0.4	2.0 ± 0.5
PYInc + 1C2	2.0 ± 0.3	1.8 ± 0.7	2.5 ± 0.2	1.9 ± 0.7
PYInc + 1ZP4	1.5 ± 0.6	1.5 ± 0.6	2.1 ± 0.5	1.7 ± 0.6
PYInc + EC30	2.2 ± 0.2	1.7 ± 0.8	2.5 ± 0.5	1.9 ± 0.7

Results are expressed as mean ± S.D. ($n = 3$). Averages presented considered removal efficiencies observed for the three rounds.



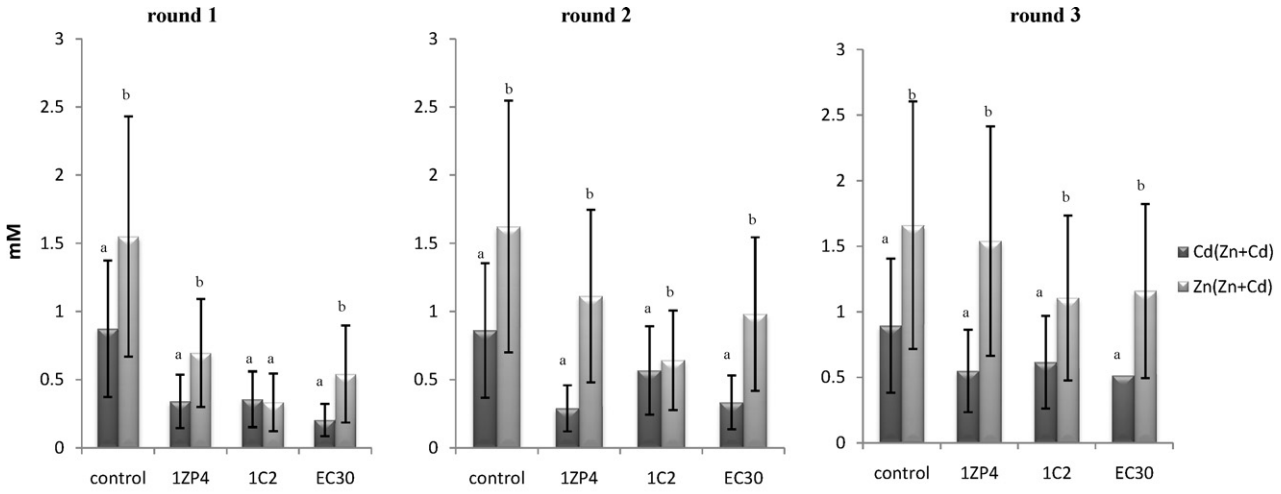
Results are expressed as mean ± S.D. ($n = 3$). Means for the same bacterial treatment in each round with different letters are significantly different from each other ($P < 0.05$) according to the t-test.

Fig. 2. Zn and Cd levels in the combined outlet (Zn + Cd) in the alginate matrix with different bacteria applications (mM).



Results are expressed as mean ± S.D. ($n = 3$). Means for the same bacterial treatment in each round with different letters are significantly different from each other ($P < 0.05$) according to the t-test.

Fig. 3. Zn and Cd levels in the combined outlet (Zn + Cd) in the pectate matrix with different bacteria applications (mM).

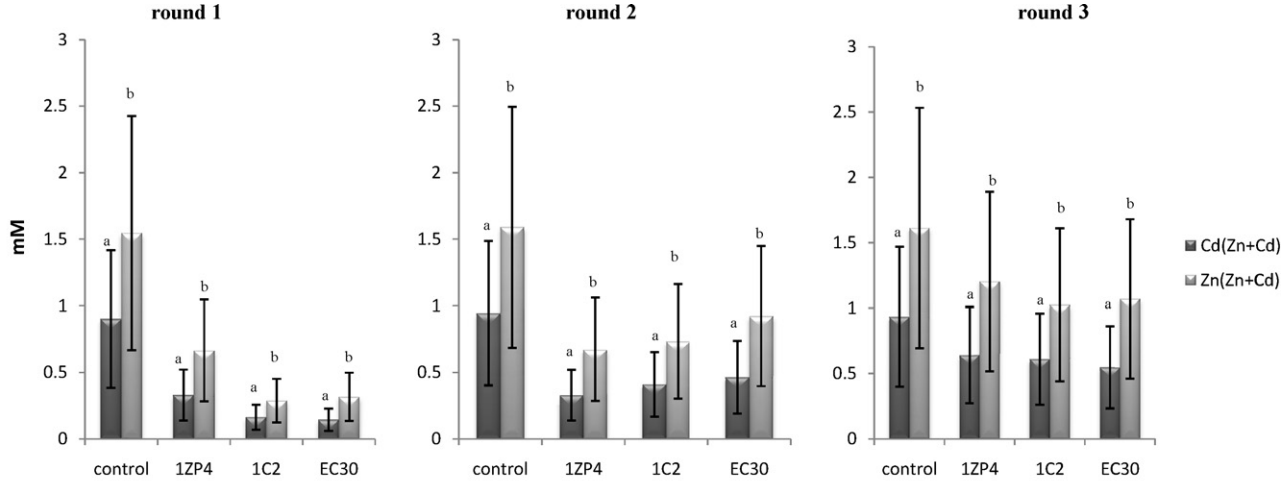


Results are expressed as mean ± S.D. ($n = 3$). Means for the same bacterial treatment in each round with different letters are significantly different from each other ($P < 0.05$) according to the t-test.

Fig. 4. Zn and Cd levels in the combined outlet (Zn + Cd) in the synthetic polymer matrix with different bacteria applications (mM).

[32]). In the case of alginate – a linear polysaccharide that can be found in many algal species [33] and which has been extensively used in metal removal studies [34] – and pectate – a pectin compound which has been used to remove Zn in aqueous solutions by Khotimchenko et al. [13] – it appears that the process of ion-exchange takes place when metal binds to this matrix [35,36].

Despite this adsorption capacity of the polymers, the present study showed that the immobilisation of bacteria increased the removal abilities of all the matrices (alginate, pectate and the synthetic polymer). In fact, bacteria have been successfully used as biosorbents [7–9] because of their small size, their ubiquity, ability to grow under controlled conditions and resilience to a wide range of contaminants [37]. Bacteria are known to produce extracellular polymeric substances which are composed by proteins, polysaccharides and uronic acid. These substances contain several functional groups like carboxyl, phosphoric, amine and hydroxyl groups [38,39]. Both the phosphoryl and carboxyl groups of the peptide chains in bacterial cell walls provide negatively charged sites in Gram-positive bacteria. For Gram-negative bacteria, such as 1ZP4, EC30 and 1C2, the phosphate groups within the lipopolysaccharides of their outer membrane are the primary sites for metal interaction, with only one of the carboxyl group in this net being free to interact with metals [37]. The process of binding of metal ions to bacteria involves electrostatic interaction between metal ions and the biomass [4] as bacteria have a net negative charge that favour the biosorption of metal [40], as observed in the present work. Further studies have shown a similar pattern when comparing the use of polymers alone and when immobilising microorganisms: For example, Sag et al. [41] have shown that when aqueous solutions of Cu were treated with Ca-alginate immobilised *Zooglea ramigera*, an increase in Cu removal occurred from 64%, for the treatment with only Ca-alginate, to 94%. Aksu et al. [11] have also shown that after long periods, the adsorption capacity of alginate immobilised *Chlorella vulgaris* exceeded that of alginate alone. Synthetic responsive polymers have also been used successfully to control the attachment of bacterial cells to surfaces



Results are expressed as mean ± S.D. ($n = 3$). Means for the same bacterial treatment in each round with different letters are significantly different from each other ($P < 0.05$) according to the t-test.

Fig. 5. Zn and Cd levels in the combined outlet (Zn + Cd) in the incubated synthetic polymer matrix with different bacteria applications (mM).

[42] demonstrating the attachment of *Hallomonas* and *Staphylococcus* strains to surface-grafted synthetic polymers. However, the amount of biosorbent, initial concentration of metal, presence of further contaminants in the aqueous solutions, structural properties of both the support matrix and the biosorbent material all affect the biosorption rate [34], rendering it difficult to compare results from different reports, and thus the main focus of this report is not to attempt such comparisons. The 3 selected strains – 1C2, 1ZP4 and EC30 – exhibited high resistance to Cd and Zn and all showed high specific growth rates when these heavy metals were present at different concentrations. Strains 1C2, 1ZP4 and EC30 are all Gram-negative and affiliated to genera *Cupriavidus*, *Sphingobacterium* and *Alcaligenes*, respectively. Many reports have shown that Gram-negative are more tolerant to heavy metals than Gram-positive bacteria. This metal tolerance can be attributed to the interactions between microbial cell wall components and heavy metal ions both contributing to metal detoxification [43–45]. In the biosorption of complex solutions, different metal ions may compete for the active sites existing on the support matrix and/or on the cell wall of the biomass. Consequently, the preference of the biomass for some metals is an important issue [46], and thus the knowledge of the growth and metal resistance patterns of the bacterial species is of great importance.

Measurement of the growth of the selected strains in the presence of Cd and Zn indicated differences in toxicity towards the bacteria among the heavy metals. Specifically, the presence of Cd^{2+} inhibited the growth of the strains tested, except for strain EC30 that showed a remarkable capacity to tolerate Cd in solution, with only a 15–20% biomass reduction. Zn^{2+} caused also a reduction in biomass production; however in a less significant degree when compared to Cd. Strain EC30 apparently was more sensitive to Zn^{2+} than to Cd^{2+}. When metal mixtures were present, the growth rate was lower than that observed when only Zn was tested. The decrease in biomass observed whenever metals were present possibly results from a decrease in the substrate utilization efficiency due to a higher energy cost of microorganisms subject to metal stress [47].

In the present study 1C2, a species affiliated to the *Cupriavidus* genera, was generally the one that most increased the removal performance of Zn (in single and binary solutions), especially when associated with the synthetic polymer. In contrast, EC30, a bacterium affiliated to the *Alcaligenes* genera, gave the most promising results for Cd removal in single and binary mixtures, especially when combined with the synthetic polymer. In fact, EC30 has also shown to be the most resistant to Cd in the tolerance study performed which may explain these results. Mondal et al. [48] reported the use a species of *Ralstonia*, phylogenetically related to *Cupriavidus*, *Ralstonia eutropha*, for the elimination of Fe, Mn, Cu, As and Zn, with removals of up to 65.2%, 72.7%, 98.6%, 8% and 99.3%, respectively, from metal contaminated water. Species from the genera *Alcaligenes* (such as EC30) have also been reported by Chang and Tseng [49] as important in immobilised biomass strategies, and Diels et al. [50] have studied the application for heavy metal removal of composite membrane reactor immobilised *Alcaligenes eutrophus* bacteria with a reduction of metals such as Cd, Zn, Cu, and Pb in solution from 100 ppm to less than 50 ppb. As for strain 1ZP4, belonging to genera *Sphingobacterium*, there is also a study from Bootham et al. [51] describing *Sphingobacterium mizutatae* as being part of a bacterial consortium used to treat metal contaminated effluents.

The removal efficiencies registered in the present report, for a contact time of 2 min, reach levels of 2.8 mg Cd g^{-1} cell and 2.2 mg Zn g^{-1} cell; a longer residence time could have allowed for higher uptakes. Arica et al. [34] used Ca alginate as a support for Zn biosorption with immobilised live and inactivated fungus *Phanerochaete chrysosporium*, and for a similar initial Zn concentration (100 mg L^{-1}) removals of ca. 20–35 mg Zn g^{-1} adsorbent were observed. In fact, these values are quite higher than those shown in this work, however the residence time was of 90 min while in the present study the average contact time was of 2 min. Also, and for solution of similar Cd initial concentration, Quintelas et al. [2] presented uptake levels of app. 10 mg Cd g^{-1} *Escherichia coli* supported on kaolin, this time for a residence time of 10 days. Nevertheless, the levels of adsorption of the tested systems will depend not only on the characteristics of the used immobilisation media, but also on the residence time of the metals in the cartridge. Sag et al. [41] analysed the effect of flow rate in the adsorption of Cu to alginate and immobilised *Z. ramigera* and have showed that an increase in the flow of five times could result in decreases in the metal removal of up to 15 times.

4.2. Removal of binary mixtures of metals by immobilised bacterial matrices

Mixtures of Cd and Zn are typically found in contaminated effluents of industrial processes [52]; additionally, from a biological point of view Cd can be transported by the same transporters as Zn [53].Nevertheless, Fan et al. [54] have shown that when using binary mixtures of Cd and Zn, the biosorption capacity of either metal was lower than that found in non-competitive conditions. However, this did not always occur in the present study. In some cases there was a differential increase in the removal abilities of either of the tested metals when present as a binary solution when compared to single solution. Such phenomenon may be explained by the hypothesis that the sorption of the other metallic contaminants in solution altered the conformation of the metal binding sites and increased the affinity of sites for that particular metal adsorption in that specific combination of matrix, bacteria and usage [10]. On the other hand, the opposite effect was observed in some cases where there was a decrease in Cd or Zn removal capacities of specific matrix-bacteria combinations. The most likely reason for this antagonistic effect may be the competition for adsorption sites on the cell and polymer surfaces. Chen et al. [10] also found that Cd uptake capacity was slightly reduced when Pb and Hg are present in solution, suggesting that in Ca-alginate immobilised *Microcystis aeruginosa* most Cd adsorption sites were specific, whereas some of these Cd binding sites were also capable of binding other metals. Despite these variations in the removal of metals in the binary mixture levels of Cd at the outlet were lower than those of Zn, and in the large majority of cases this trend was significant. The preference of a sorbent for a metal may be explained on the basis of electronegativity of the metal ions (Cd = 1.69 and Zn = 1.65, according to the Pauling scale), molecular weight (Cd = 112.4 and Zn = 65.4) and ionic radius (Cd = 95 and Zn = 74), with the first being positively related to the adsorption capacity, and the second and third being inversely related to it [2]. In the present study, electronegativity seems to play an important role in the affinity of the tested combinations to Cd, but other conditions such as ionization energy can have contributed to influence the adsorption behaviour of the metals [55].

5. Conclusions

Immobilisation of bacteria in naturally occurring and synthetic polymers increased the removal abilities of all the matrixes (alginate, pectate and synthetic cross-linked polymer), with up to 12-fold when compared to the use of the polymers alone. Strain 1C2, a species from the *Cupriavidus* genera, generally has the best capacity for increasing the removal of Zn when immobilised on any of the polymers, in single and binary solutions, especially when associated with the synthetic polymer. EC30, a bacteria affiliated to the *Alcaligenes* genera, was the most promising concerning

Cd removal in single and binary mixtures, again when combined with the synthetic polymer. Thus, the combinations that would be recommended to clean-up aqueous solutions containing Zn or Cd would be respectively 1C2 or EC30 immobilised on the synthetic polymer (PY + 1C2 and PY + EC30). Synthetic cross-linked polymers are promising matrixes and should be explored further in immobilised microbial cartridges. In this format, in addition to the promising results presented here, synthetic polymers have the added advantage of being easily reusable, unlike their natural counterparts.

Acknowledgments

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