Cellular strategies against metals exposure and metal localization patterns linked to phosphorus metabolic pathways in *Ochrobactrum anthropi* DE2010

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Abstract

Cytotoxic, chemical, biochemical, compositional, and morphometric responses against heavy metal exposure were analyzed in *Ochrobactrum anthropi* DE2010, an heterotrophic bacterium isolated from Ebro Delta microbial mats (Tarragona, NE Spain). Several parameters of effect and exposure were evaluated to determine tolerance to a range of Cd(II), Pb(II), Cu(II), Cr(III) and Zn(II) concentrations. Moreover, removal efficiency, polyphosphate production and metal localization patterns were analyzed. High resistance till 20 mM for Zn and 10 mM for the other metals, and removal capacity till 90% for Pb(II) and 40% for Cr(III) were showed in *O. anthropi* DE2010. Moreover, polyphosphate production was strongly correlated with heavy metal concentration, and three clear cell localization patterns of metals were evidenced with compositional and imaging techniques: (i) extracellular in polyphosphate granules for Cu(II), (ii) in periplasmic space forming crystals with phosphorus for Pb(II), and (iii) intracytoplasmic in polyphosphate inclusions for Pb(II), Cr(III) and Zn. Both the high resistance and metal sequestration capacity, highlight the great potential of *O. anthropi* DE2010 for bioremediation strategies, especially in Pb and Cr polluted areas.

Keywords: active process; bacterium; bioaccumulation; biomineralization; heavy metal; polyphosphate production; sequestration

1. Introduction

Heavy metals are persistent pollutants widely spread in ecosystems worldwide. Among them, non-essential heavy metals such as Pb and Cd have no known role in biological systems, often inducing high toxic effects in biota

even at low concentrations and times of exposure (Olmedo et al. 2013; revision in Abtahi et al. 2017; revision in Yilmaz et al. 2018; Rani et al. 2019; Zhu et al. 2020). In contrast, low amounts of essential metals such as Cu, Cr and Zn are necessary for the right metabolic functioning of plant and animal cells, but they turn out to be hazardous when environmental levels and/or body burdens increase (revision in Nagajyoti et al. 2010; Prashanth et al. 2015; Hirve et al. 2020).

Especially sensitive areas are deltas, fragile coastal wetlands with unique species and ecosystems highly disturbed both by pollutants transported through the river and by *in situ* anthropogenic impacts (Bruins et al. 2000; Selvin et al. 2009; Masindi and Muedi 2018). The protected area of Ebro Delta (Tarragona, NE Spain) has historically polluted by industry, agriculture, hunting and domestic effluents, becoming a representative example of the environmental status of deltas worldwide (Mañosa et al. 2001; Sánchez-Chardi and López-Fuster, 2009; Dhanakumar et al. 2015). Consequently, increases of metals such as Cd, Pb, Cu, Cr, and Zn were reported in waters, soils, plants, and animals of this coastal wetland (revision in Mañosa et al. 2001). Deltas are also the suitable habitats for microbial mats formed by different microorganisms, mainly phototrophs (algae and cyanobacteria) and heterotrophs (bacteria), with crucial ecological functions such as sediment stabilization (Seder-Colomina et al. 2013; Millach et al. 2019). Several microorganisms have also been reported as highly efficient capturing heavy metals both in natural habitats and axenic laboratory cultures (Zhang et al., 2013; Coelho et al. 2015; Chaturvedi et al. 2015; Yin et al., 2016; Li et al., 2018; Maleke et al., 2019; revision in Yin et al.,2019). However,little is known about their specific strategies of immobilization and

localization patterns as well as their morphological responses against metal exposure. Some phototrophic (*Microcoleus chthonoplastes* DE2006, *Scenedesmus* sp. DE2009, *Geiltherinema* sp. DE2011) and heterotrophic (*Paracoccus* sp. DE2007, *Micrococcus luteus* DE2008, *Ochrobactrum anthropi* DE2010) microorganisms from Ebro Delta mats have been tested in axenic laboratory cultures for analyzing their ability to capture metals such as Cr(III), Pb(II), and Cu(II) (e.g. Burnat et al. 2009; Burgos et al. 2013; Maldonado et al. 2010a,b; Puyen et al. 2012; Millach et al. 2015; Villagrasa et al. 2019, 2020a). Interestingly, all these isolated microorganisms have the capacity to sequester metals externally (biosorption) in extracellular polymeric substances (EPS), becoming especially high in *Micrococcus luteus* DE2008 and Cu(II) and Pb(II) metals (Puyen et al. 2012). Additionally, some of them, mainly phototrophic, have also demonstrated the capacity to accumulate metals intracellularly (bioaccumulation) in polyphosphate (polyP) inclusions, being especially interesting for bioremediation of contaminated environments. Among those microorganisms, the gram-negative heterotrophic bacterium *O. anthropi* DE2010 has recently emerged as an interesting species due to relevant genomic findings concerning polyP production and heavy metal concentration and its high efficiency to remove and to accumulate Cr(III) in intracytoplasmic polyphosphate (polyP) inclusions and EPS (Villagrasa et al.,2020a, b). This species easily grows in liquid and solid cultures and could become a suitable model for experimental studies of heavy metals. However, its capacity and efficiency in capturing and accumulating essential and non-essential heavy metals with ecotoxicological interest remain laking. Taking this into consideration, a multi-analytical approach assessing several parameters related

to Cd, Pb, Cu, Cr, and Zn effect and exposure was performed using microbiological cell counts using optical profilometer (OP), growth curves, minimal inhibitory concentration (MIC), and half-maximal inhibitory concentration (IC_{50}). Moreover, analytical chemistry (inductively coupled plasma optical emission spectrometer (ICP-OES)), and analytical and morphometric high-resolution (HR) microscopy (transmission electron microscopy (TEM) and field emission scanning electron microscopy (FESEM)) techniques have been applied in qualitative and quantitative manners.

With all this in mind, the main goals of the present study with this bacterium *O. anthropi* DE2010 exposed to a range of Cd, Pb(II), Cu(II), Cr(III) and Zn concentrations were: (i) to analyze bacterial responses against metal exposure quantifying cell survival, uptake efficiency and removal capacity at 24 h after growing exposed to a single metal dose; (ii) to evaluate ultrastructural changes due to metal exposure; (iii) to localize metals at nanoscale showing patterns related to polyP production and structure as a mechanism to immobilize potentially toxic elements; and (iv) to discuss the potential applications of this species in metal immobilization.

2. Materials and methods

2.1 Microorganism, single heavy metals stock solutions and culture sample preparations

O. anthropi DE2010 isolated from *Scenedemus* consortium from Ebro Delta microbial mats was recently characterized and identified (Villagrasa et al. 2019). Bacterium was cultured in Luria-Bertani (LB) rich medium containing

122 tryptone (10 g L^{-1}), yeast extract (5 g L^{-1}), sodium chloride (10 g L^{-1}) and
123 bacteriological agar (15 g L^{-1}) at 27 ºC (pH 7.0).

124 Stock solutions of each heavy metal (50 mM) were prepared in sterile
125 double deionized water from the following salts: Cd from cadmium chloride
126 (Acros Organics), Pb(II) from lead nitrate (Merk), Cu(II) from copper sulphate
127 (Merk), Cr(III) from chromium nitrate (Sigma-Aldrich), and Zn from zinc sulphate
128 (Riedel-deHäen). Then, experimental solutions were freshly prepared by diluting
129 the stock solutions in LB medium to obtain the tested concentrations: 0.5, 2, 5,
130 7, and 10 mM for Cd, Pb(II), Cu(II) and Cr(III); and 2, 5, 10, 15, and 20 mM for
131 Zn. The pH of all experimental solutions was adjusted at 5.5 for preventing
132 heavy metal precipitation.

133 For all experiments, unpolluted (0 mM) and polluted cultures were
134 prepared at the same conditions for each heavy metal in the following manner:
135 2 mL of 24 h culture of *O. anthropi DE2010* grown in LB (OD_{600}) ranging
136 between 1.4-1.6 (approximately 10^{10} cfu mL^{-1}) were inoculated into 18 mL of LB
137 liquid medium with the different tested concentrations for each heavy metal
138 (final volume 20 mL). All cultures were incubated in an orbital shaker (Infors HT,
139 Ecotron) (150 rpm) at 27 ºC during 24 h. The pH of all the cultures was adjusted
140 at 5.5 for preventing heavy metal precipitation.

141 2.2 Minimal inhibitory concentration (MIC), growth curves and half maximal
142 inhibitory concentration (IC_{50})

143 MIC of each heavy metal assayed was determined in triplicate adding 10
144 µL (one drop) of each experimental metal solution (concentrations tested in a
145 range of 0.5-25 mM) onto LB agar plates surfaces in which *O. anthropi* DE2010

was just before spread over. MIC is considered as the metal concentration at which no bacterial growth was detected in the drop zone (Luli et al. 1983) after bacterial growing at 27 ºC during 48 h.

For growth curves assays, aliquots *of O. anthropi* DE2010 were dispensed in a 96-well microplate (20 µL per well), achieving the different tested metal concentrations (0, 0.5, 2, 5, 7, and 10 mM for Cd, Pb(II), Cu(II) and Cr(III); and 0, 2, 5, 10, 15, and 20 mM for Zn) in final volume per well of 200 µL. Blank samples (bacterial free LB medium exposed or not with metals) and a control (bacterial LB medium without metal) were included in each 96-well microplate (Villagrasa et al. 2020a). The *O. anthropi* DE2010 growth was determined in a Varioskan plate reader (Thermo Fisher Scientific) by turbidity measurements (λ= 600 nm) every 30 min at 27 ºC during 24 h. The half maximal inhibitory concentration (IC_{50}) from samples was determined for each heavy metal sample as described by Volpe et al. (2014).

2.3 Cell counts by Optical profilometer (OP)

All exposed and non-exposed *O. anthropi* DE2010 samples were prepared in glass slides with surface coated with poly-L-lysine (Sigma-Aldrich) depositing 8 µL of sample inside a 1 cm^2 square and then spreading onto the surface creating a thin monolayer of bacterial cells. Samples were fixed with temperature and coated with a thin layer of Au-Pd using E5000 Sputter Coater (Bio-Rad) to improve their contrast. Quantitative surface measurements of bacterial cells were obtained using an OP Leica DCM 3D (Leica microsystems) with dual technology (confocal and interferometric). Triplicates of vertical scanning interferometry images with an area of 250.64x190.90 μm^2 were

randomly obtained for each sample and analyzed in quality topography mode using Leica map DCM 3D, version 6.2.6561 (Leica Microsystems).

2.4 Metal quantification by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES)

Cd, Pb(II), Cu(II), Cr(III) and Zn concentrations immobilized into the cells were quantified in *O. anthropi* DE2010 cultures to measure cell uptake efficiency and their heavy metal removal capacity. All the samples were centrifuged at 5,000x *g* at 4 ºC for 20 min (Eppendorf 5804R). Resulting supernatants of those samples and blank samples were analyzed as described by Villagrasa et al. (2020a). Cd, Pb(II), Cu(II), Cr(III), and Zn concentrations were quantified at 228.80, 220.40, 327.40, 267.72, and 206.20 nm respectively, in triplicate assays using an ICP-OES spectrometer Optima 4300Dv (Perkin Elmer).

2.5 Cell lysis and quantification of polyphosphate (PolyP) production

For polyP extraction, metal and control cultures were centrifugated at 5,500x *g* at 4 ºC for 15 min, supernatants discarded, and resuspended in 50 mM Tris-HCl buffer (pH 7.0). Samples were then ultrasonicated in SONOREX (Bandelin) in an ice bath for 15 min, followed by centrifugation at 5,500x *g* at 4 ºC for 20 min to remove cell debris. The resultant supernatants were treated with a protease inhibitor cocktail tablet (Roche). The polyP content was determined through the reaction of molybdenum blue method (Ansvhutz et al. 2016) with reactive phosphorus content. All assays were performed in triplicates for each sample, and polyP production (µmol of polyP per g^{-1} dry weight of biomass) results were obtained taking into account the difference between total

194 and soluble cellular phosphorus following the protocol described by Eixler et al.
195 (2005).

196 2.6 Ultrastructural and analytical assessment with electron microscopy

197 A complete evaluation of ultrastructural morphometry and sub-cellular
198 metal localization was performed with four high-resolution (HR) electron
199 microscopy techniques. Metal exposed and non-exposed cultures of *O. anthropi*
200 DE2010 were centrifuged at 5,000x *g* during 20 min at 4 ºC in a refrigerated
201 centrifuge (Eppendorf 5804R), the resulting pellets were included in soft agar
202 (3% agarose) and processed following conventional transmission electron
203 microscopy (TEM) procedures optimized to this type of samples (Maldonado et
204 al. 2010a; Villagrasa et al. 2019; Solé et al. 2019). Briefly, samples were fixed
205 with 2.5% glutaraldehyde (Merck) in 0.1 M Millonig buffer (Millonig 1961) during
206 2 h, postfixed in 1% osmium tetroxide containing 0.8% potassium
207 hexoferrocyanide in Millonig buffer during 1 h, dehydrated in acetone,
208 embedded in Spurr resin, and polymerized at 60 ºC during 48 h. Ultrathin
209 sections (70 nm) of selected areas from semithin sections (1 µm) were obtained
210 with an ultramicrotome UCT7 (Leica Microsystems).

211 For ultrastructural studies with TEM, a set of ultrathin sections were
212 placed in carbon coated Cu grids (200 mesh) and contrasted following routine
213 protocol of uranyl acetate and lead citrate solutions. Randomly distributed
214 sections of at least 2 grids of each sample were analyzed in a TEM JEM-1400
215 (Jeol) equipped with an Erlangshen CCD camera (Gatan) and operating at
216 80kV.

For analytical studies, with TEM and field emission scanning electron microscope (FESEM), another set of samples were placed in carbon coated Au grids (100 mesh) and observed without contrasting in HR microscopes. For HR-TEM, samples were analyzed in a TEM JEM-2011 (Jeol) equipped with an 895 USC 4000 CCD camera (Gatan) and operating at 200 kV. Compositional and crystallographic studies of polyP aggregates (granules and inclusions) were performed with energy dispersive X-ray (EDX) analysis and selected area electron diffraction (SAED), respectively. The obtained diffraction powder ring patterns allowed us to know the kind of sample following this description: (i) amorphous (diffuse rings), (ii) crystalline (bright spots), and (iii) polynanocrystalline (small spots making up rings) (Meshi et al., 2012). For HR-SEM, the same samples were observed in a FESEM Merlin (Zeiss) operating at 2 kV and equipped with a backscattered (BSE) detector.

2.7 Statistical analysis

Quantitative data were tested both for normal distribution and homogeneity of variances with Kolmogorov-Smirnov and Levene tests, respectively. Statistical comparisons between groups were carried out by one-way analysis of variance (ANOVA), Bonferroni pairwaise test and Tukey multiple comparison *post-hoc* test. Significant differences in ANOVA, Bonferroni's and Tukey's test were accepted at $p \leq 0.05$. The analyses were performed using SPSS software (version 20.0 for Windows 7). All quantitative data are expressed as mean ± standard error of the mean.

3. Results and discussion

241 In the present study, a combination of qualitative and quantitative
242 microbiological, morphological, and analytical techniques was selected to show
243 a complete overview of the bacterium *O. anthropi* DE2010 responses to heavy
244 metals exposure.

245 3.1 Cytotoxic effect of heavy metals

246 Cytotoxic effect of heavy metals exposure in *O. anthropi* DE2010 cultures
247 was determined using IC_{50} and MIC values (Fig. S1, supplementary material).
248 Results from both parameters showed the same cellular responses against
249 each metal exposure. Then, the IC_{50} values remained in the same range (3.5
250 mM in Cd to 5 mM in Pb(II)) but being highest for Zn (10 mM). These values in
251 *O. anthropi* DE2010 were slightly higher than those obtained in environmental
252 bacteria for Cu(II) and Cd (2.65 and 4.30 mM, respectively) (Nweke et al. 2007),
253 in *Salmonella* sp. for Zn (0.8 mM) (Bestawy et al. 2013) and for Cd, Pb(II) and
254 Cu(II) of 0.005, 0.006, 0.03 mM, respectively for *Photobacterium phosphoreum*
255 T3S (Zeb et al. 2017). The MIC values obtained for *O. anthropi* DE2010 were
256 10 mM for Cd, Pb(II), Cu(II) and Cr(III) and 20 mM for Zn. These values exceed
257 the MIC obtained by *Escherichia coli* ATCC25922, which has been treated as a
258 reference in MIC assays (Bhardwaj et al. 2018). All this information pointed to
259 the high resistance of *O. anthropi* DE2010 to exposure at high concentrations of
260 heavy metals, especially to Zn, considered toxic for other microbial species.
261 these bacterial cells, such as extracellular sequestration, intracellular
262 sequestration, active export and enzymatic detoxification, which help them
263 interact with metals as well as tolerate rapid environmental changes in metal
264 levels (revision in Yin et al., 2019).
265

The descriptive statistics of cell counts at each metal concentration evaluated with an OP are shown in Fig. 1. Interestingly, the cell number decrease when metal concentration increase, reaching the minimum values at the highest metal concentrations (10 mM for Cd; Pb(II); Cu(II); and Cr(III); and 20 mM for Zn). According to this perfect correlation, the most evident cytotoxic effect resulting in an abrupt cell decrease, around 40 and 25% was detected between 0.5 and 2 mM for Cd and Pb(II) respectively, and more than 30% between 2 and 5 mM for the rest of metals.

Significant differences (p <0.05) in cell counts obtained with ANOVA comparison were found among all the metal concentrations for Cd (F=68.76), Pb(II) (F= 56.25), Cu(II) (F= 107.1), Cr(III) (F= 330.4), and Zn (F= 16.39). Significant reductions in cell count of 85 % for Cd, 80 % for Pb(II), 79 % for Cu(II), 84 % for Cr(III) and 47 % for Zn were observed comparing controls with samples exposed to 10 mM of each metal. These percentages agree with those obtained for IC_{50} and MICs and strongly suggest that metal toxicity for *O.anthropi* DE2010 is Cd>Cr(III)>Pb(II)>Cu(II)>Zn being the cadmium the most toxic and the zinc the least. Moreover, the presence of live cells at all metal concentrations demonstrates the high tolerance of this bacterium to deleterious effects of each of the five heavy metals strongly suggesting a similar behaviour against exposure to other potentially toxic elements.

3.2 Heavy metals removal and uptake efficiencies

Descriptive statistics of metal removal and uptake efficiency by *O. anthropi* DE2010 for each metal and concentration are shown in Tables 1 and S1 (supplementary material). The highest removal capacity found in *O. anthropi*

291 DE2010 was around 90% for Pb(II), followed by around of 40% for Cr(III). Lower
292 capacities of 20%, 10% and 3.0% were detected to remove Zn, Cd and Cu(II),
293 respectively. Moreover, similar ranges of metal removal
294 (Pb(II)>Cr(III)>Cd>Zn>Cu(II)) and uptake efficiency
295 (Pb(II)>Cr(III)>Cu(II)>Cd>Zn) were found at the highest common concentration
296 for all metals (10 mM). Significant differences ($p < 0.05$) obtained with ANOVA
297 comparison were found among all the metal concentrations for Cd (F= 20.66),
298 Pb(II) (F=13,271), Cu(II) (F= 19.53), Cr(III) (F= 1,190), and Zn (F= 22.76),
299 respectively. Moreover, Tukey multiple comparisons were labelled in Table 1.
300 Comparing between metals, *O. anthropi* DE2010 is able to capture 82-fold more
301 Pb than Cu, and their *q* values were 15-fold more for Pb (q= 1,548 mg g^{-1}) than
302 for Zn (q= 102 mg g^{-1}). Removal rates of 36 % for Cd, 18 % for Pb(II),13 % for
303 Cu(II), 39 % for Cr, 9.0 % for Zn (Chatterjee et al. 2010) and of 15 % for Cr
304 (Joutey et al. 2014) were previously described in an environmental isolate
305 bacterium and *Serratia proteamaculans*, respectively. Moreover, *q* values
306 around of 200 mg g^{-1} for Pb(II) in *Klebsiella* strain R19 (Bowman et al. 2018)
307 and of 29.80 mg g^{-1} in *Exiguobacterium* sp. ZM-2 for Cr were reported (Alam
308 and Ahmad, 2011). Comparing between these species, *O. anthropi* DE2010
309 emerges as an extremely efficient bacterium to remove heavy metals,
310 especially Pb and Cr.
311
312 3.3 Heavy metals induction of PolyP production
313 PolyP production in *O. anthropi* DE2010 cultures varied according to the
314 heavy metal and its concentration (Fig. 2). Significant differences ($p < 0.05$)
315 obtained with ANOVA comparison were found for Pb(II) (F= 77.50), Cu(II) (F=

316 521.7), Cr(III) (F= 671.9), and Zn (F= 679.7). Moreover, Bonferroni pairwaise
317 test were labelled in Figure 1. The levels of polyP (µmol of polyp per g^{-1} dry
318 weight of biomass) were clearly correlated with the increment of Pb(II), Cu(II),
319 Cr(III), and Zn, being 3, 3.5, 4, and 4.5-fold more in higher metal concentrations
320 compared to control. These findings agree with those obtained by Francisco et
321 al. (2011) and Andreeva et al. (2014) demonstrating that polyP concentration
322 increased in microbial cultures exposed to heavy metals. In marked contrast,
323 the concentration of polyP is practically invariable among all range of Cd
324 concentrations (Fig 2A) in spite of 10% of Cd captured by *O. anthropi* DE2010.
325 Neither induced polyP production nor Cd bioaccumulation in intracytoplasmic
326 polyP inclusions strongly suggests a different bacterial response for Cd. This
327 metal probably could be adsorbed in extracellular polymeric substances (EPS)
328 also due to the sorption ability of *O. anthropi* DE2010 recently reported for Cr
329 (Villagrasa et al. 2020).
330
331 3.4 Heavy metals localization patterns and cellular survival strategies
332 Imaging of morphological alterations and cellular localization of heavy
333 metals in *O. anthropi* DE2010 at nanoscale was performed with four high-
334 resolution microscopy techniques (Fig. 3). Ultrastructure of unpolluted cultures
335 showed typical morphology (size and shape) of bacterial cells with scarce and
336 small polyP inclusions (Figs. 3 A1 and A2), as reported in Villagrasa et al.
337 (2019). Those inclusions act as a phosphorus reservoir without detectable metal
338 content by EDX and BSE and with amorphous structure by SAED (Figs. 3 A2-
339 A4). In contrast, heavy metals exposure disturbed normal cell metabolism
340 altering the bacterial morphology. Moreover, intracellular ultrastructure indicated

different degrees of alteration, including evident cytoplasm disorganization and retraction (Figs. 3 B1-F1) as well as an increase of pleomorphic cells in Pb(II), Cr(III) and Cu(II) exposed cells (Figs. 3 C1-E1, respectively). The high toxicity of these metals in aquatic environments and their relationship with the presence of pleomorphic cells have been reported in microbial species (e.g. Hasnain and Sabri, 1992; Villegas et al. 2013; Bulaev et al. 2017).

The analytical studies with EDX and BSE demonstrated that polyP aggregates containing phosphorus are the main storage structures of metals in *O. anthropi* DE2010 cells and have metal-specific patterns of sub-cellular localization (Fig.3 B2-E2, B4-E4). Cu(II) induced granules mainly located extracellularly in the outer membrane surface (Fig. 3 D2), besides Cr(III) and Zn induced inclusions mostly in the cell cytoplasm (Figs. 3 E2 and F2, respectively), and Pb(II) in both the periplasmic space and the cytoplasm (Fig. 3 C2). In marked contrast, the results in Cd(II) exposed cultures showed no evident morphological changes and polyP inclusions evidenced no metal content (Fig. 3 B2). It must be noted that the different electron diffraction/SAED patterns obtained from the polyP aggregates showed a general amorphous type of crystallographic structure, (Figs. 3 B3, D3-F3), as often occurs in biological systems, except for Pb(II), which is crystalline (Fig. 3 C3). This particular result indicates that *O. anthropi* DE2010 not only is able to bioaccumulate Pb(II) but also can biomineralizate it highly efficiently, as a mechanism to reduce its bioavailability and, therefore, biological impact in bacterial cells.

Overall, these results show rapid, varied, and specific responses to different metal stressors and the great importance of polyP production in metal chelation by active processes of bacterial bioaccumulation and/or

biomineralization. This metal bioimmobilization is an effective mechanism in reducing metal bioavailability, preventing and/or avoiding toxic effects. Moreover, this ultrastructural information about metal toxicity can be confirmed by metal localization in bacterial cells.

3.5 Potential applications in metal immobilization

O. anthropi DE2010 cells can rapidly respond to metals exposure using different strategies as bioaccumulation and biomineralization in combination with biosorption. These pathways were extremely efficient to quelate Pb and Cr. Also, Zn cellular bioaccumulation and the ability to store Cu(II) in external polyphosphate granules were evidenced. All of these processes can be taken into account for potential applications due to the reduction of bioavailability of these metals often highly toxic for biota in aquatic environments (Sánchez-Chardi et al. 2007; Sánchez-Chardi and López-Fuster 2009; Seder-Colomina et al. 2013). Finally, Cd biosorption in EPS physicochemical binding could be easily broken by other competitors (e.g. cations, quelator, etc.), resulting in secondary pollution when used in bioremediation strategies. All our findings with *O. anthropi* DE2010 pointed out in the high efficiency of this bacterial species to quelate metals from the environment using different metabolic pathways. These data suggest high metabolic plasticity in *O. anthropi* DE2010 (e.g Comte et al. 2013, Guerrero and Berlanga, 2016).

In addition to our promising results, more specific studies are needed to evaluate the advantages of each bacterial strategy to localizate and bind specific metals and different chemical species. Moreover, further analysis of the capacity of *O. anthropi* DE2010 to remove them in mixed metal solutions and

391 microcosm experiments are also crucial to consider the feasibility of this
392 bacterium in bioremediation processes in natural ecosystems. Up to now, our
393 results with individually high concentrations of five widely distributed heavy
394 metals strongly suggest that this bacterial species can be considered as a
395 valuable player in future bioremediation strategies with biological systems,
396 especially in Pb and Cr polluted environments, more so when concentrations of
397 these metals are lethal for other prokaryotic and eukaryotic organisms.
398
399 **4. Conclusions**
400 *O. anthropi* DE2010, isolated from polluted Ebro Delta microbial mats,
401 exhibited resistance to high concentrations of heavy metals and an unusual
402 ability to sequestrate Pb(II) and Cr(III), which is especially high for Pb(II). In an
403 active process, bacterial cells immobilized heavy metals in polyP inclusions
404 and/or granules, besides phosphorus crystalline structures to reduce their
405 biological toxic effects. Those structures followed a metal-specific pattern in cell
406 distribution.
407 In summary, *O. anthropi* DE2010 revealed specific responses as survival
408 strategies for each heavy metal exposure, including bioaccumulation (for Pb(II),
409 Cu(II), Cr(III), and Zn), biosorption (for Cd), and biomineralization (for Pb(II)).
410
411 **CRediT author statement.**

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Declaration of Competing Interest

The authors declare no conflict of interests**.**

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Figure captions

Figure 1. Polyphosphate content (µmol of polyP per g^{-1} dry weight of biomass) in the *O. anthropi* DE2010 cultures grown at increasing concentrations of Cd (A), Pb(II) (B), Cu(II) (C), Cr(III) (D) and Zn (E) (mean ± SE).

Figure 2. High resolution imaging by electron microscopy techniques: TEM (1), TEM-EDX (2), TEM-SAED (3) and FESEM BSE (4) in the *O. anthropi* DE2010 cultures grown at unpolluted culture (A); besides they grown at 10 mM of Cd (B), Pb(II) (C), Cu(II) (D), Cr(III) (E) and 20 mM of Zn (F) polluted cultures. The arrows of EDX analyses showed the representative peak of phosphorus and the assayed heavy metal, respectively. The scale bars represent 1 µm, 0.5 µm, 5 nm^{-1} and 1 µm for TEM, TEM-EDX, TEM-SAED and FESEM BSE, respectively. In the TEM figures: Cytoplasm retraction (CR); periplasmic space precipitate (PSP); pleomorphic forms (PF); polyP granules (PG) and polyP inclusions (PPI).