Supplementary material

Cadmium and lead impact on biological phosphorus removal: metal partition and adsorption evaluation in wastewater treatment processes

Number of tables: 3
Number of figures: 5
Number of texts: 4
Number of pages: 12

Table S1 List of chemicals used in this study

Purpose	Chemical	Brand
Synthetic wastewater	Sodium propionate (CH_3CH_2COONa)	Sigma-aldrich
	Dipotassium hydrogen phosphate (K_2HPO_4)	VWR
	Potassium dihydrogen phosphate (KH_2PO_4)	Merck
	Calcium chloride ($CaCl_2 \cdot XH_2O$)	Merck
	Magnesium sulfate heptahydrate ($Mg_2SO_4 \cdot 7H_2O$)	BDH
	Ammonium chloride (NH_4Cl)	Merck
	Allylthiourea	Sigma-aldrich
	Copper (II)sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$)	Merck
	Iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$)	Sigma-Aldrich
	Manganese (II) chloride tetrahydrated (98%, $MnCl_2 \cdot 4H_2O$)	Sigma
	Sodium molybdate dihydrate (99.5 %, $Na_2MoO_4 \cdot 2H_2O$)	-
	Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$)	Sigma-Aldrich
	Cobalt (II) chloride ($CoCl_2 \cdot 6H_2O$)	Sigma-Aldrich
	Potassium iodide (99 %, KI)	Sigma-Aldrich
	Boric acid (H_3BO_3)	Sigma-Aldrich
	Etilendiaminotetraacetic acid (EDTA)	Sigma-Aldrich
		Merck
Heavy metals experiments and analysis	Cadmium chloride ($CdCl_2$, >99 %)	Sigma-aldrich
	Lead nitrate ($Pb(NO_3)_2$, >99%),	Acros Organics
	Standard solution of Cd (1000 ppm), Standard solution of Pb (1000 ppm), Ammonium phosphate ($(NH_4)_2HPO_4$, 40 %)	Analytical standards
Analytical determinations	Potassium antimonyl tartrate trihydrate	Sigma-aldrich
	Ammonium molybdate	Applichem,
	Ascorbic acid	Sigma-Aldrich
	Benzoic acid	MP Biomedicals
	Poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid)	Sigma-aldrich
	Tris(hydroxymethyl)aminomethane ($C_4H_{11}NO_3$)	Saveen verner AB
	β-Nicotinamide adenine dinucleotide phosphate disodium salt (NADP, >97%)	Sigma-aldrich
	P1,P5-Di(adenosine-5′) pentaphosphate pentasodium salt (Ap5A)	Sigma-Aldrich
	Sodium phosphate glass (poly P, type 45)	Sigma-aldrich
	Adenosine 5′-diphosphate sodium salt (ADP)	Sigma-aldrich
	4-Nitrophenyl phosphate disodium salt hexahydrate,	Sigma-aldrich
	Adenosine 5′-triphosphate disodium salt hydrate (ATP)	Sigma-aldrich
	Hexokinase from *Saccharomyces cerevisiae*	Sigma-aldrich
	Glucose-6-phosphate dehydrogenase (G6PDH)	Sigma-aldrich
	Magnesium chloride ($MgCl_2$)	Acros Organics
	NADPH	Sigma-Aldrich
	4-Nitrophenol	Sigma, Sweden
	Sodium chloride	Honeywell GmbH

	Bovine soluble albumin (BSA)	Sigma-aldrich
	Bicinchoninic acid solution	Sigma-aldrich
	Chloroform	Merck
	Methanol	Sigma-Aldrich
	Sulfuric acid	Sigma-Aldrich
	Nitric acid	Sigma Aldrich
	Hydrochloric acid	Merck
	Sodium hydroxide pellets (NaOH)	Sigma-aldrich
	Potassium hydroxide pellets (KOH)	Merck
	Ethanol (98 %, C_2H_6O)	Solveco
	Total organic carbon kits (LCK385)	Hach Lange
	Membrane-filtered deionized water was obtained from a Millipore system (18.2 MΩ·cm^{-1})	Millipore, France

Table S2 Composition of the trace elements solution[a] according to Chen et al. (2014)

Chemical	Concentration (g·L^{-1})
$CuSO_4 \cdot 5H_2O$	0.03
$FeCl_3 \cdot 6H_2O$	1.50
$MnCl_2 \cdot 4H_2O$	0.12
$Na_2MoO_4 \cdot 2H_2O$	0.06
$ZnSO_4 \cdot 7H_2O$	0.12
$CoCl_2 \cdot 6H_2O$	0.15
KI	0.18
H_3BO_3	0.15
EDTA	10

[a]The solution was adjusted to pH 7.5

Text S1. Crude extract preparation

For obtaining the cell extracts, sludge samples were centrifuged at 10 000 rpm for 5 minutes (Eppendorf, Germany). The supernatant was discarded and then the cells were re-suspended in 20 mM Tris-HCl pH 7.5. All cell suspensions were kept on ice while working. The re-suspended cells were sonicated using a 3 mm titanium probe sonicator VP400s (Dr. Hielscher GmbH, Germany) for 2 minutes. Thereafter, the tubes were centrifuged at 13 000 rpm for 15 minutes and the supernatants were kept on ice until analysis or frozen at -20 ºC.

Text S2. Determination of total proteins, exopolyphosphatase (PPX) and polyphosphate kinase (PPK) enzyme activities

The total content of proteins was measured by the bicinchoninic acid (BCA) method (Walker 2009). Briefly, 0.1 mL of cell extract was added to the BCA working reagent and incubated in a water bath at 37 ºC for 30 minutes. After incubation, samples were analyzed in a spectrophotometer at 562 nm (Biowave II-WPA, Nordic Biolabs, Sweden). The working reagent was a mixture of the BCA and 4 % w/v $CuSO_4$ following a mixing volume ratio of 1:0.02. The blank was the BCA working agent. The range of the calibration curve was between 0.2-1 $mg \cdot mL^{-1}$ (calibration curve equation: $y = 0.009x + 0.0354$). The detection limit was 0.082 $mg \cdot mL^{-1}$.

The PPX enzyme activity was determined following the procedure described in previous reports (Cox et al. 1981, Zheng et al. 2011b). The reaction had a final volume of 1 mL and contained 0.5 M Tris-HCl buffer (pH 6.5), 5 mM $MgCl_2$ and 2.5 mM *p*-nitrophenyl phosphate and 100 µL of cell extract. After 45 min incubation at 30 ºC, 2 mL of 0.5 M KOH was added to terminate the reaction, followed by measuring the absorbance at 405 nm (Biowave II-WPA, Nordic Biolabs, Sweden). The standard for the calibration curve was 4-nitrophenol and

the range of calibration was from 0.02 to 0.1 mM; calibration curve equation: y = 7.9867x +0.0289. The PPX specific activity was defined in terms of units·mg^{-1} protein.

The PPK enzyme activity was determined following the procedure described by previous reported studies (Robinson and Wood 1986, Zheng et al. 2011b). The reaction contained 100 mM Tris-HCl (pH 6.5), 8 mM $MgCl_2$, 200 mM Glucose, 0.5 mM NADP, 150 µg of Type 45 poly-P, 1 unit of hexokinase, 1 unit of glucose-6-phosphate dehydrogenase (G6P-DH), 0.2 mM P1,P5-di(adenosine-5')-pentaphosphate (Ap5A) and 150 µL of the cell extract in a total volume of 1 mL. The Ap5A was included to inhibit the adenylate kinase (ADK). The kinetic reaction was started by adding ADP, which had a final concentration of 1 mM. The produced NADPH was measured at 340 nm according to Zhang et al. (2000) in a UV-visible spectrophotometer (Shimadzu, Japan). The data was collected from zero to 600 seconds using the software UV Probe version 2.1 (Shimadzu, Japan), from which the range of processed data was from 150 to 300 seconds. The calibration curve was made with NADPH in a range of 0.02-0.1 mM; calibration equation curve: 3.5767x – 0.0002. The PPK activity was expressed in terms of units·mg^{-1} protein.

Text S3. Determination of polyhydroxyalkanoates (PHA)

Polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) were determined by gas chromatography following a modification of the procedure described by Oehmen et al. (2005). First, 4 mL of sludge samples were freeze-dried (Lyph-lock 12, Labconco, NinoLab, Sweden). The weighed freeze-dried cells were placed in a tightly sealed glass-reaction tube and 1 mL of chloroform was added, followed by 1 mL of a solution of methanol, 3 % sulfuric acid and 500 mg·L^{-1} of benzoic acid. The samples and standards were digested for 3 h at 100 ^{o}C. After cooling at room temperature, 0.5 mL of distilled water was added and shaken for 30

seconds. After the separation of the phases, the organic phase (chloroform phase) was transferred to GC-vials for further analysis in a Varian gas chromatograph (model 430-6C, USA) coupled to a Varian autosampler (CP-8400). The GC was equipped with a VF-1ms column (dimensions 15 m·0.25 mm·0.25µm, P/N: CP8907, Agilent J&W GC column). For analysis, 3 µL of sample was injected to the GC with a split ratio of 15:1. The injector had a temperature of 250 ºC, the oven temperature was set to 80 ºC for 1 minute, then increased to 120 º at 10 ºC·min^{-1}, followed by an increase to 270 ºC at the rate of 45 ºC·min^{-1} and held for 3 minutes. Helium was used as the carrier at a flow of 1.5 mL·min^{-1}. The flame ionization detector (FID) was set to 300 ºC. The calibration curve was obtained using poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) as the standard, and the weight ratio of PHB:PHV was 0.88:0.12. Calibration equation curves: a) PHB: y 1.572x + 0.0505, b) PHV: y = 1.1952x + 0.0275. The data was processed by the Galaxie software version 1.9.302.530. The final content of PHA was a result of the sum of PHB and PHV.

Text S4. Extraction of extra-polymeric substances (EPS)

The EPS was extracted by heat extraction to obtain the loosely-bound EPS and the tightly-bound EPS as described by Li and Yang (2007). A sludge suspension (10 mL) was dewatered by centrifugation (refrigerated benchtop centrifuge with fixed-angle rotor SIGMA 3-16 PK, Labex, Sweden) in polyethylene tubes at 4500 rpm for 15 min. The pellet was re-suspended in 10 mL of 0.05 % w/v NaCl that was preheated in a water bath at 70 ºC to ensure that the sludge suspension reached an immediate temperature of 50 ºC. The suspension was vortexed for 1 minute and centrifuged again at 4500 rpm for 15 min. The supernatant was transferred to another tube and regarded as the LB-EPS fraction. The remaining pellet was again re-suspended in the tempered 0.05 % w/v NaCl solution and kept in the water bath for 30 min at

60 ºC. Afterwards, the suspension was centrifuged, and the supernatant was regarded as the TB-EPS fraction. Both fractions were stored at 4 ºC until analysis.

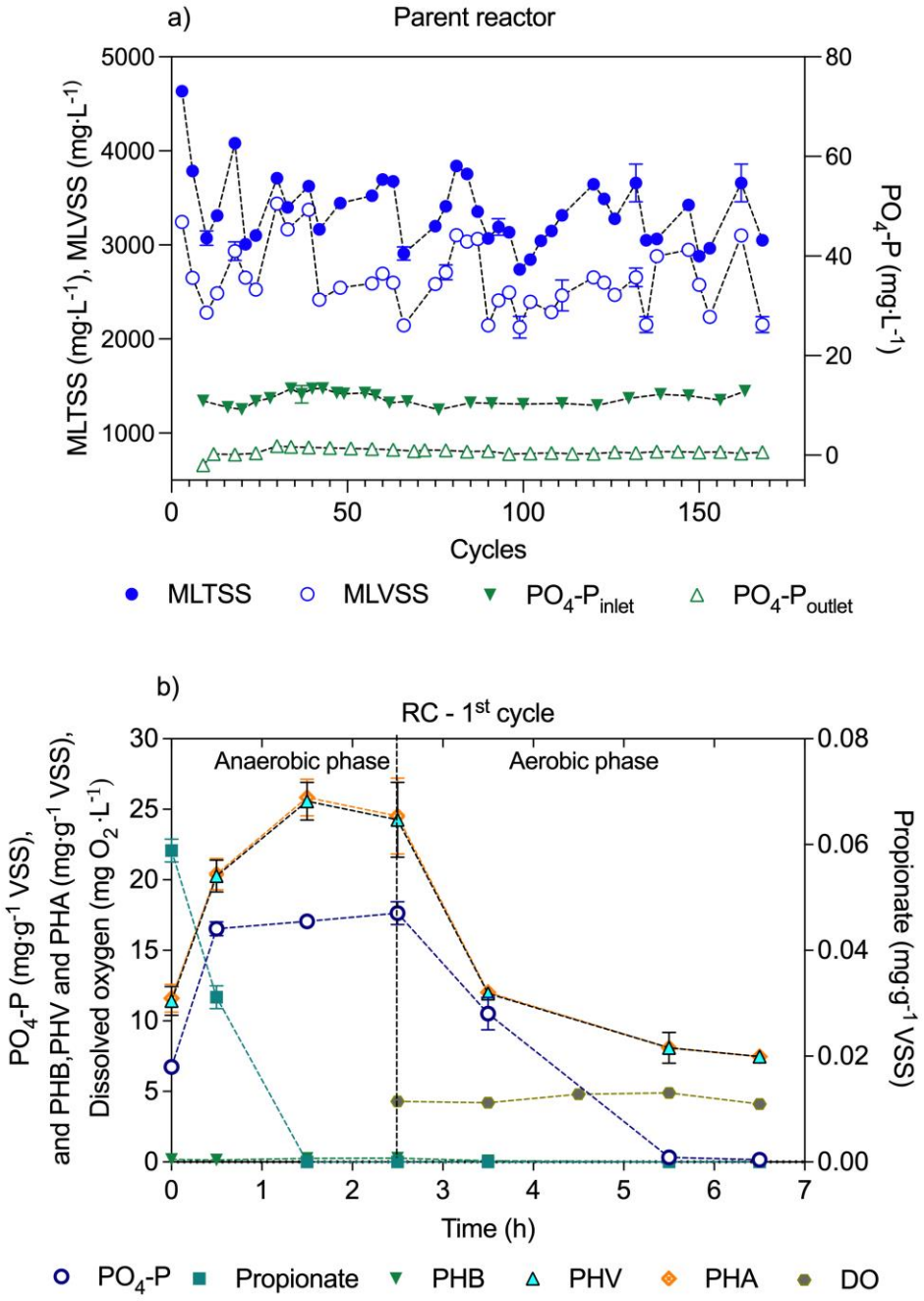

Figure S1 (a) Mixed liquor total suspended solids (TSS), mixed liquor volatile suspended solids (VSS), values of PO_4-P in the inlet and outlet ($mg \cdot L^{-1}$) of the parent reactor. (b) Typical variations of PO_4-P (mg $P \cdot g^{-1}$ VSS), propionate uptake (mg Propionate$\cdot g^{-1}$ VSS), poly-hydroxybutyrate ($mg \cdot g^{-1}$ VSS), polyhydroxyvalerate ($mg \cdot g^{-1}$ VSS) and polyhydrokyalkanoates (PHA, $mg \cdot g^{-1}$ VSS, which is the sum of PHB and PHV) of a normal EBPR process (1st cycle for RC). The vertical dotted line at time = 2.5 h indicates the change from anaerobic to aerobic conditions. Experimental conditions: COD = 150 $mg \cdot L^{-1}$, P = 10 $mg \cdot L^{-1}$, T = 22 °C, pH = 6.5 ± 0.5, and dissolved oxygen (DO) ranging from 4-5 mg $O_2 \cdot L^{-1}$

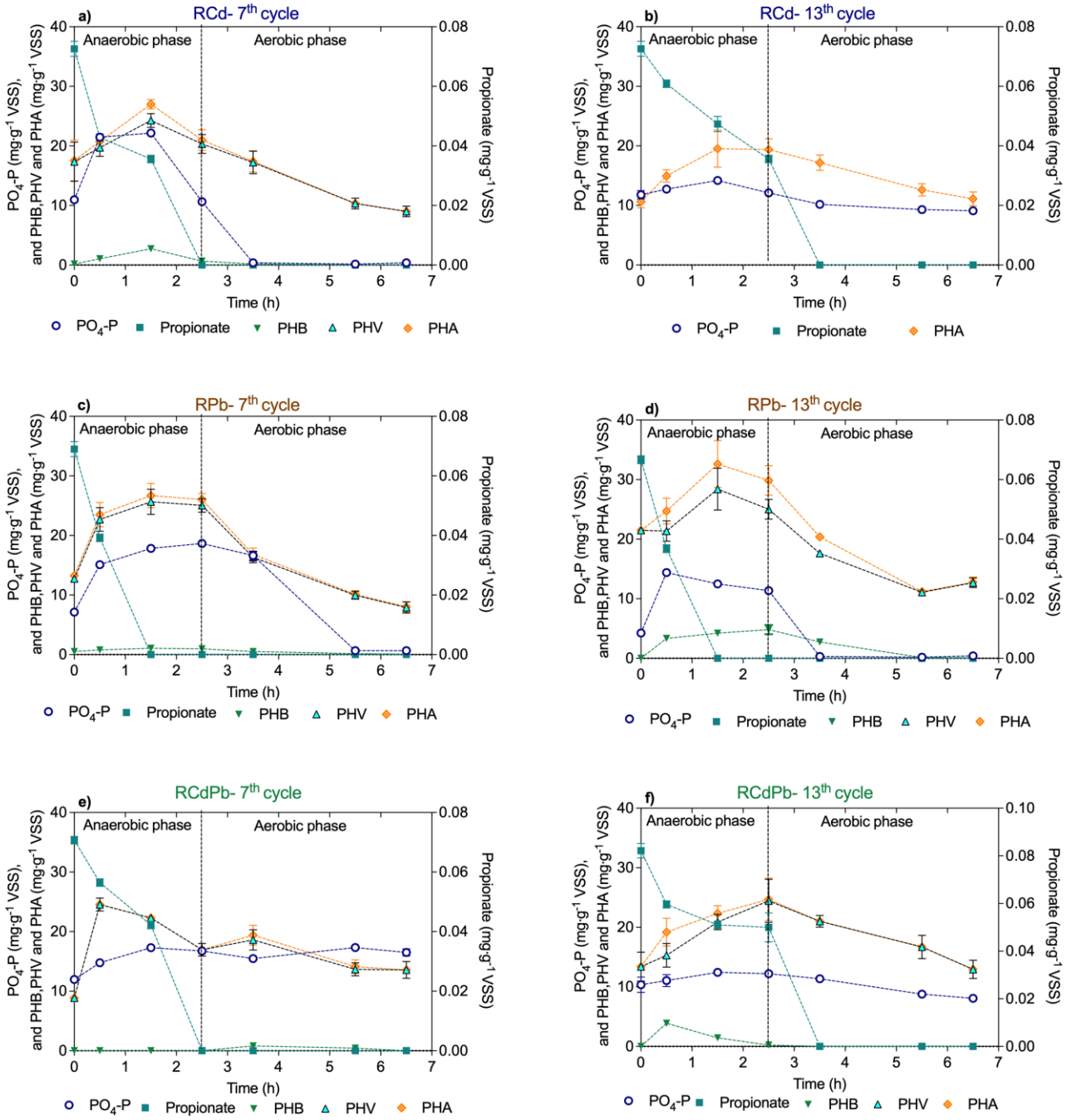

Figure S2 Fluctuations of phosphorus (mg P·g^{-1} VSS), propionate (mg Propionate·g^{-1} VSS), polyhydroxybutirate (mg·g^{-1} VSS), polyhydroxyvalerate (mg·g^{-1} VSS) and polyhydrokyalkanoates (PHA, mg·g^{-1} VSS, which is the sum of PHB and PHV) during the 7th and 13th cycle for (a,b) RCd (EBPR+Cd^{2+}), (c,d) RPb (EBPR+Pb^{2+}), (e,f) RCdPb (EBPR+Cd^{2+}+Pb^{2+}). The vertical dotted line at time = 2.5 h indicates the change from anaerobic to aerobic conditions. Experimental conditions: COD = 150 mg·L^{-1}, P = 10 mg·L^{-1}, Cd (2 mg·L^{-1}) = 18 µM = Pb (3.7 mg·L^{-1}), T = 22 °C, pH = 6.5 ± 0.5.

Table S3 Effect on PPK and PPX enzymes in EBPR in the presence of metals reported on some studies along with this study. Highlighted values in black are the relative activities expressed in percentage and values highlighted in blue are those values where either an activation or inhibition of the enzyme is observed

Metal	Concentration (µM)[a]	PPX[1]		PPK[1]		Observations	Reference
		Enzyme activity measured in the control	Relative activity (%) in presence of the metal[b]	Enzyme activity measured in the control	Relative activity (%) in presence of the metal[b]		
Cd^{2+}	8.9	0.024[c]	**112**	0.258[c]	**98**	Long-term experiment (90 days)	Chen et al. (2014)
	88.9		**108**		**49**		
	18	0.14[d]	**131**	0.64	**94**	Short-term (Cycle 7&13)[d]	This study
Pb^{2+}	18	0.14[d]	**75**	0.64	**129**	Short-term (Cycle 7&13)[d]	This study
$Cr_2O_7^{2-}$	23.2	103.75[e] µg p-nitrophenol·g $VSS^{-1}·h^{-1}$	**124**	Not measured	Not measured	Cycle 1-5[e]	Fang et al. (2012)
Cu^{2+}	7.8	0.075[f]	**84**	0.106[f]	**294**	Long-term (100 days)	Wu et al. (2015)
	39.3		**156**		**447**		
	15.7	Not reported	-	0.26[g]	**35**	Short-term	Tsai et al. (2013)
	47.2				**27**		
	62.9				**23**		
Ag^{+}	9.3	0.09[h]	**55**	0.29[h]	**100**	1-3 days	Chen et al. (2013)
	46.3		**100**		**107**	120-130 days	
TiO_2-NPs	626	Data not available	**100**[i]	Data not available	**100**[i]	Long-term (70 days)	Zheng et al. (2011a)
ZnO-NPs	12.3	0.022[j]	**91**	0.286[j]	**101**	Short-term	Zheng et al. (2011b)
	122.8		**64**		**58**		
	614.4		**41**		**40**		
Cd^{2+}+ Pb^{2+}	36	0.14[d]	**55**	0.64[d]	**110**	Short-term (Cycle 7&13)[d]	This study

[a]For the purpose of comparison, the concentrations are expressed in µM. [b]The relative activities are expressed in percentage with respect to their controls (absence of metals). [c]Values taken from Table S2. [d]Values (control and when metals are present) were average of the values of the 7th and 13th cycle presented in Table 1 of this study. [e]Values are approximated and extracted from Figure 5a; an average activity was calculated of the data from cycle 1 to 5 (4 experimental points). [f]Values taken from Table 2. [g]Values are approximated and were extrapolated from Figure 5a. [h]Values obtained from Table S4. [i]Data was not available for the calculation, authors claimed no effect was observed on both enzymes and 100 % of remaining activity was attributed. [j]Values obtained from Table 1. [1]The units of enzyme activities are µmol NADPH·mg^{-1}·min^{-1} for PPK and µmol *p*-nitrophenol·mg^{-1}·min^{-1} for PPX, unless otherwise mentioned.

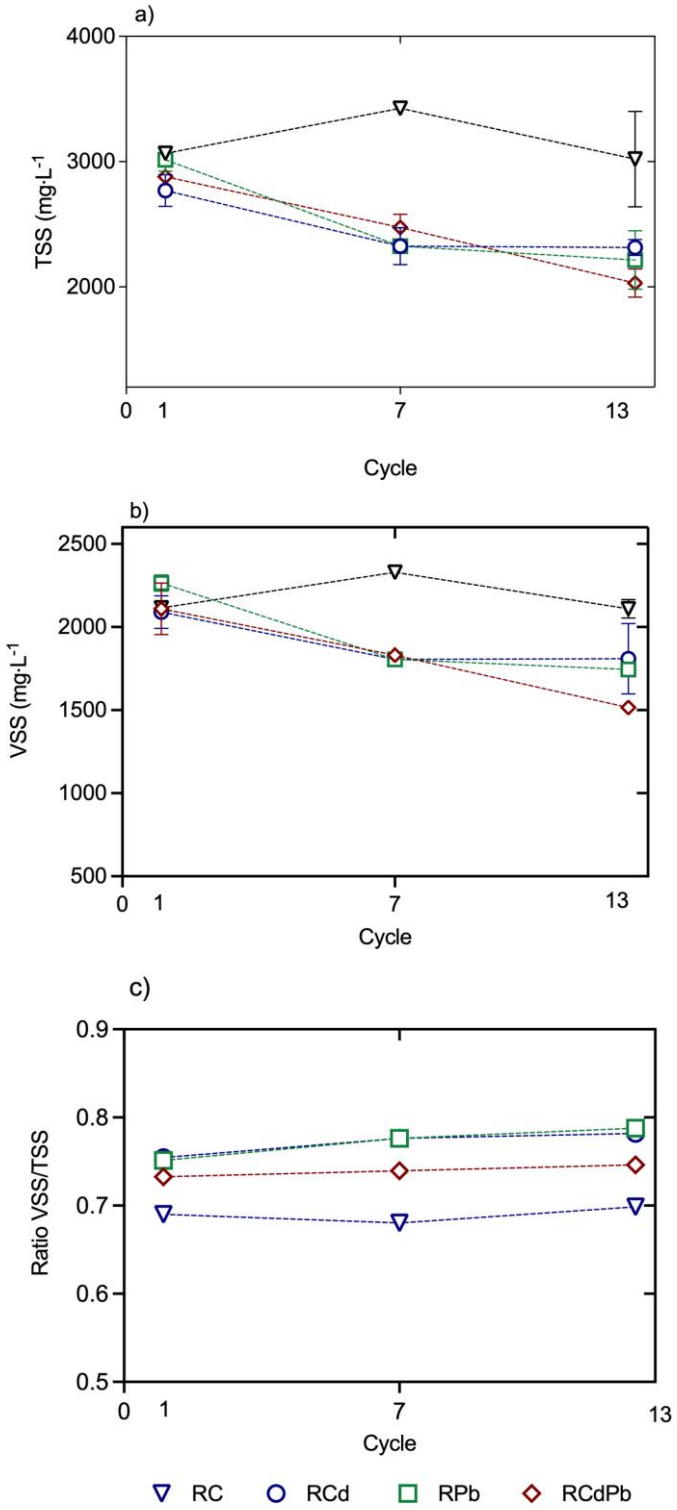

Figure S3 Variations of (a) mixed liquor total suspended solids (TSS), (b) mixed liquor total volatile solids (VSS), and (c) the ratio of VSS/TSS determined for the 1st, 7th and 13th cycle of the RC (EBPR control), RCd (EBPR+Cd^{2+}), RPb (EBPR+Pb^{2+}) and RCdPb (EBPR+Cd^{2+}+Pb^{2+}). Experimental conditions: COD = 150 mg·L^{-1}, P = 10 mg·L^{-1}, T = 22 °C, Cd (2 mg·L^{-1}) = 18 µM = Pb (3.7 mg·L^{-1}), pH = 6.5 ± 0.5

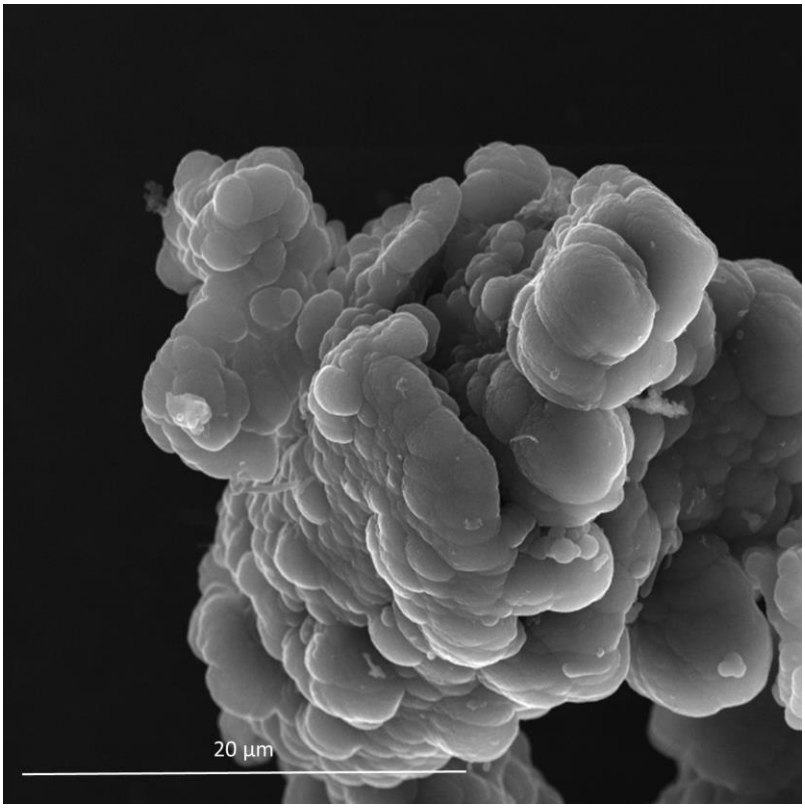

Figure S4 Secondary electron image of a floc of sludge after the 13th cycle in EBPR+Cd^{2+}+Pb^{2+} (RCdPb). Concentration of Cd and Pb was 18 µM each

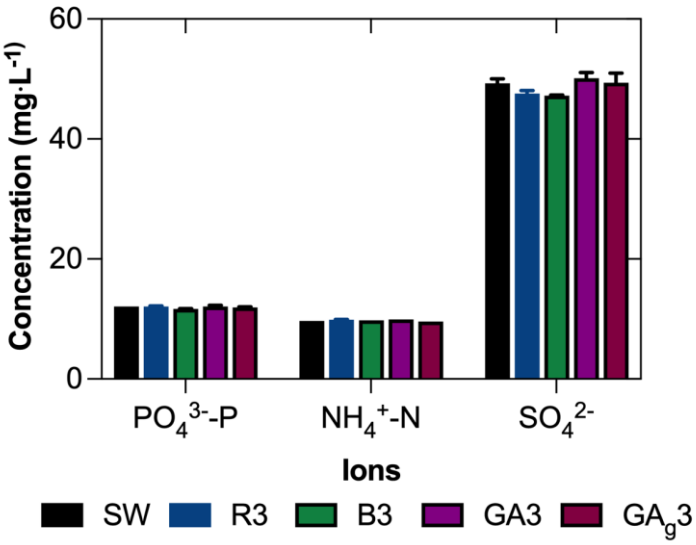

Figure S5 Concentrations of PO_4^{3-}-P, NH_4^+-N and SO_4^{2-} in mg·L^{-1} measured in synthetic wastewater before and after 3 hours of treatment with 3 adsorbents. R: resin Diaion®CR11, B: STNTs-Ch beads, GAC: granular activated carbon (Chemviron Carbon AB), GAC_g: grounded activated carbon

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