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Design of remediation pilot plants for the treatment of industrial metal-bearing effluents (BIOMETAL DEMO project): Lab tests

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ABSTRACT

Current research is the first part of the BIOMETAL DEMO project, funded by the European Union, focusing on the development of complementary and modular biotreatment processes for the removal of metal ions from industrial effluents (issued from mining industry, surface treatment and ceramics). Metal bio-precipitation (in form of metal sulfides and metal phosphates) using sulfate-reducing bacteria (SRB), phytase and phosphatase enzymes, biosorption using sugar-beet pulp, algal biomass and alginate- or chitosan-based composites (immobilizing polyethyleneimine derivatives by encapsulation) have been extensively studied using synthetic and industrial effluents. These preliminary results allow designing a treatment flow-sheet including pre-treatment of the effluent using biologically-assisted precipitation followed by biosorption (for mining effluents that contain sulfate an-ions). For surface treatment and ceramics effluents, bio-precipitation combined with biosorption processes and physicochemical pretreatment followed by biosorption process represent, respectively, a promising solution. The carbon-source for the SRB process, the optimization of process parameters and the biosorbents have been selected taking into account both the technical performance and the economic viability.

Keywords:

Metal bio-removal

Bio-precipitation

Sulfate-reducing bacteria

Phytase, acid phosphatase

Biosorption

Integrated industrial wastewater treatment

1. Introduction

Heavy metal pollution is one of the most important environmental problems today even threatening human life. A large number of industries produce and discharge wastes containing different heavy metals into the environment, such as mining and smelting of metalliferous ores, surface finishing industry, energy and fuel production, fertilizer and pesticide industry and application, metallurgy, iron and steel, electroplating, electrolysis, electro-osmosis, leatherworking, photography, electric appliance manufacturing, metal surface treating, aerospace and atomic energy installation, etc. (Wang and Chen, 2009). Among these, the following four appear as the main priority targets, particularly in the industrialized world (Volesky, 2003): (a) acid mine drainage (AMD)—associated with mining operations; (b) electroplating industry waste solutions (growth industry); (c) coal-based power generation

(throughput of enormous quantities of coal); and (d) nuclear power generation (uranium mining/processing and special waste generation). Three kinds of heavy metals retaining more attention: (i) Metals with environmental impact, and toxic metals (such as Hg, Cr, Pb, Zn, Cu, Ni, Cd, As, Co, Sn, etc.); (ii) precious and strategic metals (such as Pd, Pt, Ag, Au, Ru, etc); and (iii) radionuclides (such as U, Th, Ra, Am, etc) (Wang and Chen, 2009).

The European Directive 76/464/EEC (Dangerous Substance Discharges) established two lists of substances classified as hazardous (European Commission, 1976). The List I identified 129 substances (among them mercury and cadmium and their compounds) to be eliminated from the environment because of their toxicity and their high bio-accumulating effect. The List II contained those substances (among them other metals and their compounds) that have a detrimental impact on the environment but may be present within a given area, depending on the characteristics and location of this area. The Directive also sets up a legal framework for the elimination or reduction of discharges of dangerous substances to inland and coastal waters.

A large number of industries across Europe, which are mostly equipped with physicochemical treatment plants (chemical precipitation, flocculation, etc.) for their heavy metal polluted wastewaters,

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produce a minimum level of metals in solution of 0.5–5.0 mg L⁻¹, well above the maximum admissible concentrations (order of magnitude of microgram per liter) permitted by current or proposed European legislative restrictions for discharges of soluble heavy metals into public watercourses. For this reason, innovative tertiary metal treatment technologies are needed to comply with these directives. Traditional biotechnology for wastewater treatment processes relies on the largely empirical use of ill-defined mixed populations of microorganisms in biological reactors effecting biodegradation of organic and nitrogenous waste materials (Hamer, 1997) and removal of soluble forms of heavy metals (Sterrit and Lester, 1986; Ziolkó et al., 2011). The activated sludge process relies upon the self-immobilizatory property of the microorganisms (flocculation) within microbially produced polymeric material, to co-precipitate microbial cells and metals within the flocs. Although some active metal uptake occurs, metal “insolubilization” is primarily a fortuitous adsorptive process onto the extracellular polymers and cell surfaces (Ziolkó et al., 2011), while the primary metabolic functions of the bioreactor and continued microbial growth are sensitive to metal toxicity. Although many and significant improvements have been made to understand the microbiology and kinetics of mixed-culture waste-treatment systems (Girault et al., 2011), the use of a monoculture in a separate system greatly increases the specific metal uptake. For example, in the case of *Zoogloea ramigera* the increase in cadmium (Cd) accumulation was 3000-fold in a pure culture as compared to activated sludge (Sterrit and Lester, 1986). The most practical approach may consist in employing a filter of immobilized biomass operating as a continuous metal adsorbent to high final load. Once treated, the metal waste is concentrated in either a small volume of solid material or desorbed into a small volume of liquid concentrate for easy metal recovery or disposal (Bhide et al., 1996).

All of the microbe-metal accumulative interactions described to date are encompassed within three types of mechanisms, each of which has particular merits and disadvantages. These mechanisms are metal uptake by non-living biomass (biosorption), metal removal by immobilized living cells (bioaccumulation/bio-precipitation) and growth-decoupled enzymatically mediated metal uptake (bio-precipitation) (Tsezos, 2014). In the last case, an important advance is the identification and exploitation of the enzyme(s) catalyzing the reaction(s) responsible for metal deposition from aqueous solutions. Although the provision of substrate(s) under physiologically permissive conditions is necessary, these requirements may be less stringent than for metabolizing or growing cells. Furthermore, an enzymatic process is easier to optimize and quantify; the principles of applied enzyme kinetics can then be used in a defined system that can be applied predictively to large-scale processes. At laboratory scale, this degree of sophistication has been achieved in the cases of acid phosphatase-mediated metal bio-precipitation on immobilized whole cells of a *Serratia* sp. NCIMB 40259 (Macaskie et al., 1992; Roig et al., 1995a; Roig et al., 1995b; Macaskie et al., 1997; Roig et al., 1997) and phytase-mediated metal bio-precipitation on immobilized cells of *E. coli* ATCC 33965 (Paterson-Beedle et al., 2010). This process is a hybrid approach which exploits the advantages of ion exchange processes, biosorption and growing microorganisms without the disadvantages of neither (Macaskie and Dean, 1989).

To fulfil the EU Directives, the BIOMETAL DEMO project uses a hybrid innovative tertiary treatment, based on bio-precipitation and biosorptive processes for removal and recovery of metals from different industrial wastewaters, such as acid mine drainages, electroplating wastewaters and metal contaminated wastewater from frits and glazes in ceramic tile manufacture. These waters, sharing several common physicochemical characteristics, namely acid pH, metal cations and sulfate and other anions in solution, low presence or absence of organic matter, will be treated both at laboratory scale and at in situ pilot demonstration plants, by means of metal bio-precipitation and biosorption processes acting synergistically, with the aim of reducing their metal pollution below the European maximum permissible concentrations

to be discharged into the aquatic environment. Section I in the Additional Material Section describes the organization and management of the research project.

The first active agents evaluated are the Sulfate-Reducing Bacteria (SRB) which have the ability to obtain energy by dissimilatory sulfate reduction to sulfide and thus sulfide can react with metals forming precipitates (Postgate, 1984). In this sense, bioreactors, enriched with SRB and supplemented with appropriate organic compounds, can be used for the treatment of waters contaminated with metals and sulfate, such as generally occurs in Acid Mine Drainages (AMD) (Lewis, 2010; Martins et al., 2010; Sanchez-Andrea et al., 2014). However, generally these waters are scarce in organic compounds necessary to feed SRB and therefore they must be supplemented with appropriate sources of these compounds to be treated in such processes. Over the years different materials have been successfully tested to feed SRB, as for example: tannery wastes (Boshoff et al., 2004), cheese serum and wine wastes (Martins et al., 2009), wastes from sweetmeat production (Das et al., 2013), and animal manure (Zhang and Wang, 2014). The objective of CCMAR's team in the project BIOMETAL DEMO is to optimize processes based on SRB to integrate the treatment of metal wastewaters, namely by: (1) selecting low-cost organic substrates with carbon compounds suitable as electron donors for the activity of SRB; and (2) determining the best operational conditions to treat metal contaminated waters in continuous flow systems using SRB bioreactors fed with the selected organic substrates.

The second active agents evaluated by the team from the University of Salamanca for metal uptake from the aqueous environment by bio-precipitation are phytases and acid phosphatases (phosphohydrolase enzymes), properly immobilized, which catalyze the hydrolysis of organic phosphate donor molecules, such as phytic acid, phytates (Mullaney and Ullah, 2003) and β -glycerophosphate (Roig et al., 1997), incorporated into the metal solution, releasing inorganic phosphate (HPO_4^{2-}), thus allowing the metal cations (M) to precipitate, stoichiometrically, as MHPO_4 or $\text{M}_3(\text{PO}_4)_2$ on the immobilized or supported enzyme. The process of metal uptake from solution relies upon the in situ cumulative deposition of insoluble metal phosphates (Macaskie et al., 1992; Roig et al., 1995a; Roig et al., 1995b; Roig et al., 1997; Paterson-Beedle et al., 2010). The phyto-catalyzed hydrolysis of phytic acid produces local high concentrations of inorganic phosphate; the solubility product of the corresponding metal phosphates can be exceeded in the presence of soluble metals.

The third active agent tested for metal uptake by biosorption is that provided by natural biomass (brown marine algae) and the agricultural industry (sugar-beet pulp and hemp by-products) which are abundant, cheap and easy available biosorbents (Gadd, 2009). Brown algae exhibit an excellent sorption uptake over a wide variety of metals (Hackbarth et al., 2014; Keshtkar et al., 2015). The metal sorption uptake differs depending on the type of alga used: brown algae practically duplicate the sorption capacity of red ones and triplicate the sorption performance of green algae (Romera et al., 2007). Brown algae have a complex cell wall, rich in mucilaginous polysaccharides (alginate and sulfated fucoidans). These polysaccharides contain functional groups, especially carboxyl groups, involved in metal recovery (Davis et al., 2003). Other functional groups present in the algal cell wall are: amino, sulfhydryl and sulfonates. Sugar-beet (*Beta vulgaris* L.) pulp is a residue of the sugar industry (low cost product to be re-valorized). Sugar-beet pulp has high pectin content (15–30%), but these pectins have poor binding properties due to their high degree of methylation and low molecular weight (for example sorption capacities as: 0.45 mmol Cd g⁻¹ and 0.53 mmol Pb g⁻¹). Pectins are polysaccharides cross-linked with cellulose and hemicellulose fibers. It is a polysaccharide composed of galacturonic acid units with (1,4) bonds and rhamnose units with secondary chains branches (Reddad et al., 2002). The pectin extraction process from the pulp has been optimized to produce suitable gels with porosity, chemical stability and mechanical strength as to be used as biosorbent and support in fixed bed columns (Mata, 2006). The first

Table 1

Tested organic substrates allowing good SRB activity in one month and respective prices.

Substrate	Dilution	Max. SO ₄ ²⁻ reduction (%)	Price ^a (€/t)
Molasses from orange (without preservative) (Orange juice factory)	1:200	68	80
Molasses from sugar beet (Sugar factory)	1:200	85	140
Carbocal (Sugar factory)	10% (w/v)	53	5
Sugared water (from a homogenization tank) (Candy factory)	1:5	79	not set
Sewage water (WWTP - lagoon system - first lagoon)	1:5	81	0
Olive Mill Wastewater (decanted or not) (Olive oil press)	1:5	93	0

^a Data provided by suppliers in 2014.

objective of this work was the study of Zn biosorption in fixed-bed column using brown alga *Fucus vesiculosus* and sugar beet pulp with different flow rate and bed height. The second objective was to evaluate the performance of serial columns of the selected biomasses using solutions similar to real polluted water streams.

The fourth active agent tested for metal uptake by biosorption consists in biopolymers such as chitosan and alginate (used alone or as encapsulating agents for polyethyleneimine derivatives). The ARMINES-Ecole des mines d'Alès partner has developed over the last two decades a research on metal sorption using chitosan and alginate. For example, in the case of chitosan the presence of amine groups makes possible (Guibal, 2004; My Linh et al., 2016): (a) the binding of metal cations through complexation/chelation mechanisms (free electronic doublet of amine groups) at near neutral pH (Vieira et al., 2011); and (b) the electrostatic attraction/ion exchange binding of metal anions in acidic solutions (Sicupira et al., 2010). In the case of alginate-based materials binding occurs on the carboxylic groups (guluronic and mannuronic acids) of the biopolymer by ion-exchange and chelation (Lagoa and Rodrigues, 2009; Plazinski, 2012; Chiew et al., 2016). The objectives were to: (a) design sorbents with improved selectivity for target metals in complex solutions under specific forms (gel beads) that facilitate their application in fixed-bed columns; (b) test, in a first step, their sorption properties in batch systems; and (c) evaluate the feasibility of their application in fixed-bed column systems. Polyethyleneimine (PEI) and its derivatives (obtained by grafting new functional groups such as amino-acid, sulfur compounds or tannic acid) have shown good sorption properties for metal ions (Kislenko and Oliynyk, 2002; Khunathai et al., 2013; Gao et al., 2014). Their sorption properties may be controlled, especially in terms of uptake kinetics, by limitations to intraparticle diffusion. To reduce this impact it is necessary reducing the size of sorbent particles at the expense of difficulties in: (a) recovering, at large scale, spent sorbent after metal binding; and (b) using the material in fixed-bed columns (clogging, head loss). Another solution may consist in encapsulating the micro- or nano-particles of sorbent in a suitable matrix such as biopolymer (chitosan or alginate). In this case the challenge is optimizing both the confinement of micro- or nano-particles and the mass transfer properties of composite materials. A series of PEI-derivatives was encapsulated in chitosan or alginate. PEI was chemically modified by grafting amino-acids (such as cysteine, histidine) or tannic acid, by cross-linking with glutaraldehyde or by reaction with carbon disulfide (Bertagnolli et al., 2015a; Bertagnolli et al., 2015b). Herein are described the synthesis of PEI-glutaraldehyde crosslinked, PEI-histidine and PEI-CS₂ and their encapsulation in alginate and chitosan capsules. The sorption properties of these composites are evaluated for heavy metal cations (such as Cu(II), Zn(II), Cd(II), Ni(II) and Pb(II)).

2. Materials and methods

Experimental procedures have been fully described in the Section II of Additional Material Section. Materials and methods, including analytical procedures and characterization of materials are commented with extended details.

Experimental data were regularly repeated and the observed differences were systematically below 10%.

3. Results and discussion

3.1. SRB process

3.1.1. Selection of SRB consortium and low-cost substrate for carbon supply

The most efficient SRB consortia in terms of sulfate-reduction and metals resistance was the one previously reported by CCMAR's team (Martins et al., 2009), which was collected from lagoon system sludge of the WWTP located between Faro and Olhão (Algarve, Portugal). This consortium was selected for all the following tests in this project.

In the cultures with growth media in which the organic substrates to be tested were the only added carbon compounds, six tested substrates (Table 1) allowed good results in terms of sulfate reduction (>50%), indicating activity of SRB, in one month. Three of these substrates are

Table 2

Summarised results for the performance of hitherto studied processes treating Acid Mine Drainage (AMD) in continuous flow systems with different organic substrates as carbon and electron donors (see a detailed version of this table in Additional Material Section - Table AM1).

System	Organic substrates	Parameter	Units	Initial AMD	Reactor effluent	
					Average	St. Dev
I Treating São Domingos AMD collected on January	Ethanol	pH	Sorensen scale	2.50	6.09	0.11
		[Fe]	mg.L ⁻¹	44	<0.2	-
		[Al]	mg.L ⁻¹	138	2.70	0.66
		[Zn]	mg.L ⁻¹	28	0.10	0.17
		[Cu]	mg.L ⁻¹	30	<0.4	-
II Treating São Domingos AMD collected on July	Sewage water	[SO ₄ ²⁻]	mg.L ⁻¹	1848	270	164
		pH	Sorensen scale	2.50	6.12	0.54
		[Fe]	mg.L ⁻¹	178	1.05	0.30
		[Al]	mg.L ⁻¹	211	<0.1	-
		[Zn]	mg.L ⁻¹	38	0.42	0.17
III Treating Quinta do Bispo AMD collected on April	Ethanol	[Cu]	mg.L ⁻¹	21	<0.4	-
		[SO ₄ ²⁻]	mg.L ⁻¹	2323	1301	281
		pH	Sorensen scale	2.90	6.67	0.10
		[Fe]	mg.L ⁻¹	19	<0.2	-
		[Al]	mg.L ⁻¹	42	1.21	0.34
III Treating Quinta do Bispo AMD collected on February	Ethanol	[Mn]	mg.L ⁻¹	24	19.27	1.30
		[U]	mg.L ⁻¹	9.4	1.32	0.12
		[SO ₄ ²⁻]	mg.L ⁻¹	1944	352	153
		pH	Sorensen scale	2.90	6.58	0.11
		[Fe]	mg.L ⁻¹	2.8	<0.2	-
IV Treating São Domingos AMD collected on July	Olive mill wastewater	[Al]	mg.L ⁻¹	37	<0.1	-
		[Mn]	mg.L ⁻¹	15	11.83	1.83
		[U]	mg.L ⁻¹	3.5	1.18	0.36
		[SO ₄ ²⁻]	mg.L ⁻¹	1128	275	292
		pH	Sorensen scale	2.50	5.41	0.27
IV Treating São Domingos AMD collected on July	Olive mill wastewater	[Fe]	mg.L ⁻¹	178	5.23	2.99
		[Al]	mg.L ⁻¹	211	0.25	0.06
		[Zn]	mg.L ⁻¹	38	<0.1	-
		[Cu]	mg.L ⁻¹	21	<0.4	-
		[SO ₄ ²⁻]	mg.L ⁻¹	2323	1423	91
		Total phenols	mg.L ⁻¹	-	28.40	0.85

industrial co-products with associated costs (molasses from orange and from sugar beet and Carbocal), which price is presented in the 4th column of Table 1. Thus, treatment processes based on bioreactors with SRB using any of them as carbon and electron donors have to consider their respective costs. Assuming the use of the dilutions that revealed promising results for both tested molasses (1:200) and taking into account the prices per ton of each of them and their densities: 1.2 Kg L⁻¹ for orange molasses and 1.4 Kg L⁻¹ for beetroot molasses, the purchase price associated to each one when treating 1 m³ of water contaminated with metals can be estimated respectively as 0.48 and 0.98 €. The other tested substrate with associated costs is Carbocal. Although a higher amount of this product is necessary to support a good activity of SRB, comparing with both molasses, its costs per amount of treated water is in the same range due to its lower price per ton (see Table 1). Therefore, the costs associated to the purchase of Carbocal for SRB based treatment processes can be estimated in the same way as 0.5 m³ of treated water.

3.1.2. Tests on industrial effluents

3.1.2.1. Acid mine drainage (AMD). Since the CCMAR team had previous experience in using SRB to treat AMD water from São Domingos mine, Mértola, Portugal, a sample of this water collected in January 2015 (pH = 2.4, [SO₄²⁻] = 1520 mg L⁻¹, [Cu] = 15 mg L⁻¹, [Zn] = 21 mg L⁻¹, [Fe] = 87 mg L⁻¹, [Al] = 153 mg L⁻¹) was used as a reference in the tests aiming to select suitable low-cost materials to feed SRB bioreactors in metal's removal processes for AMD waters.

Independent tests with five of the six selected materials listed in Table 1 (all except the sugared water) dissolved in AMD water were carried out in anaerobic batch cultures inoculated with SRB after a previous pH neutralization with limestone. Despite this previous pH neutralization, three of the six tested materials (molasses from orange, molasses from sugar beet and olive mill wastewater) led to an acidification (pH = 4 to 5) that prevented SRB activity. Nevertheless, consistently with previous studies (Barros et al., 2009), some tests in batch systems with these materials dissolved in AMD and with a supplement of calcite

tailings as a neutralizing agent confirmed that they are efficient organic substrates to feed SRB in the treatment of this type of waters contaminated with metals (provided that pH is maintained neutral). In all these batch tests the analyzed metals remained partially dissolved after the initial pH neutralization ([Fe] = 0.3 to 5 mg L⁻¹; [Cu] = 0.2 to 13 mg L⁻¹ and [Zn] = 1.2 to 9 mg L⁻¹), but, at the end of the experiments, their concentrations were below the maximum recommended values for irrigation waters in Portugal: [Fe] < 5 mg L⁻¹; [Cu] < 0.2 mg L⁻¹ and [Zn] < 2 mg L⁻¹ (Portuguese Government, 1998). Considering these good results regarding the removal of metals from AMD water in batch experiments with SRB fed with the five substrates tested, further experiments in continuous flow systems using this contaminated water and these substrates as carbon sources have been initiated and are currently ongoing.

3.1.2.2. Zinc electroplating industries. The main metals to treat in the wastewaters from the zinc electroplating industry participant in this project, pit discharge ("vertido del foso") and zinc line discharge ("vertido de zincado"), are zinc and iron. The wastewater "vertido del foso", with pH lower than 1 and concentrations of zinc and iron above 5 g L⁻¹, was considered too extreme to be treated by processes based on the use of SRB, while "vertido de zincado", with physical and chemical conditions less extreme, was used for tests to evaluate the feasibility of metal's removal with SRB based systems.

Two samples of "vertido de zincado", VZ1 (pH = 2.3, [Zn] = 546 mg L⁻¹, [Fe] = 10.3 mg L⁻¹ and [SO₄] = 116 mg L⁻¹) and VZ2 (pH = 2.0, [Zn] = 345 mg L⁻¹, [Fe] = 111 mg L⁻¹ and [SO₄] = 54 mg L⁻¹), were used for tests despite their relatively low concentrations of sulfate. The results obtained with sample VZ1, suggest that the metals can be removed by the action of SRB, provided that this wastewater is supplemented with sulfate and organic compounds suitable for these microorganisms as electron donors for anaerobic respiration. However, the results obtained with sample VZ2 suggest a toxic composition for SRB. In this case, even when supplemented with organic substrates having suitable carbon compounds and enough sulfate, the sample VZ2 could not be treated with these microorganisms. Consequently, the

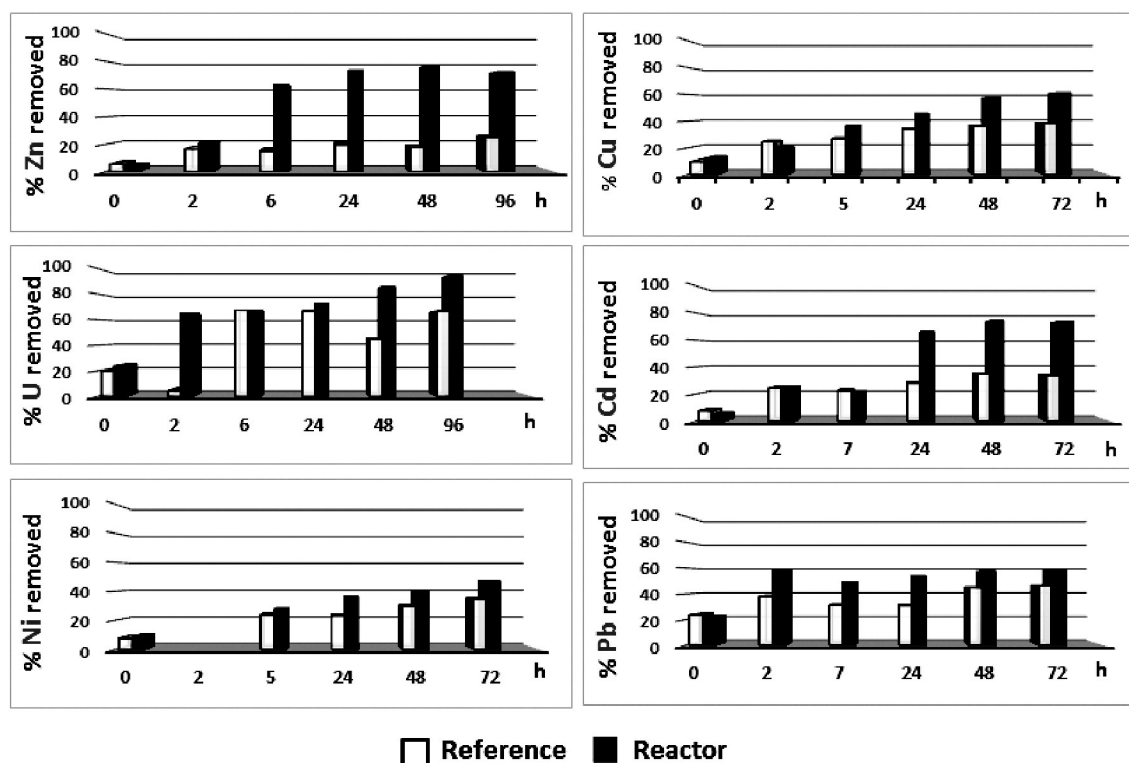


Fig. 1. Metal removal from mono-component synthetic wastewaters by means of immobilized phytase bioreactor (■) and reference reactor (□). Phytic acid 10⁻³ M.

experiments aiming to treat the wastewater “vertido de zinco” using SRB based systems were discontinued.

3.1.2.3. Ceramic industry. The main metals to treat in the wastewaters from the ceramics industry participant in this project are zinc and boron. Although it is possible to remove the former by its precipitation as zinc sulfide using SRB based processes, the same is not expected for the second. This was confirmed in batch experiments carried out during this project: SRB had good activity in the growth medium supplemented with boron (50 mg L^{-1}), but were unable to cause any decrease in the concentration of this element. Therefore, seeing that SRB based processes could only treat partially the effluents from this industry, further tests to treat them with such processes were not performed.

3.1.3. Tests in column systems on industrial effluents

Optimization of SRB based processes in continuous flow for the removal of metals is under way only with AMD waters for the reasons presented above with the results obtained during the first phase of the project. Four continuous flow systems with SRB in columns are currently being used in metals removal experiments. Two of these systems are being used to optimize the treatment of AMD from São Domingos mine using as carbon sources the zero-cost organic materials selected at initial stage (sewage water and olive mill wastewater). A third system is being used to treat also AMD from São Domingos mine, but using a pure organic compound (ethanol) known as suitable carbon and electron source for this SRB community. The other system was assembled to initiate experiments using SRB based processes for the treatment of AMD water from an Uranium mine - Quinta do Bispo ($\text{pH} = 2.5$ to 3 , $[\text{SO}_4^{2-}] = 1500$ to 2000 mg L^{-1} , $[\text{U}] = 3$ to 10 mg L^{-1} , $[\text{Al}] = 30$ to 60 mg L^{-1} , $[\text{Fe}] = 50$ to 200 mg L^{-1}), also using ethanol as the carbon source for SRB. The characterization of these four SRB processes under study is presented in Table 2.

The reactor fed with AMD from São Domingos mine and ethanol (System I - Table AM1 in Additional Material Section) began to work even before this project started, since August 2013, and it is still working efficiently. This reactor was used to develop improvements in the system that led to the use of the three modules referred in the materials and methods and has been optimized to remove metals and sulfate to concentrations below the limits for irrigation water in Portugal (Vitor et al., 2015).

The reactor fed with AMD from São Domingos mine and sewage water (System II - Table AM1 in Additional Material Section) started processing on October 2015 and its operational conditions are still being optimized. Up to now the system is able to remove metals from this water to concentrations below the limits for irrigation water in Portugal, but the sulfate reduction efficiency is not yet satisfactory since values above the limit for irrigation water (575 mg L^{-1}) are still being obtained. This is probably due to the type of carbon compounds present in this organic substrate, which may be complex molecules that need to be decomposed into smaller molecules suitable for the SRB anaerobic respiration. Trying to take advantage of using mixed consortia to improve the system performance; recently the flow rate was reduced aiming to allow other bacteria in the reactor to have time to eventually decompose those molecules, thus creating optimal conditions for SRB.

The reactor fed with AMD from São Domingos mine and olive mill wastewater (System IV - II - Table AM1 in Additional Material Section) was assembled on July 2015. This system is also able to produce water with metal concentrations legally acceptable for irrigation in Portugal. However, although set with a relatively long retention time (200 h), the efficiency in sulfate reduction is low and therefore optimization tests are still ongoing. The most probable reason for this is the reactor acidification to levels ($\text{pH} = 5.4$) near the limits suitable for SRB, caused by the addition of olive mill wastewater. This tendency was also observed in previous studies with other carbon sources and can be solved by filling the reactor with a mixture of coarse sand and limestone (Costa et al., 2009). Another issue to be taken into account in this case is the

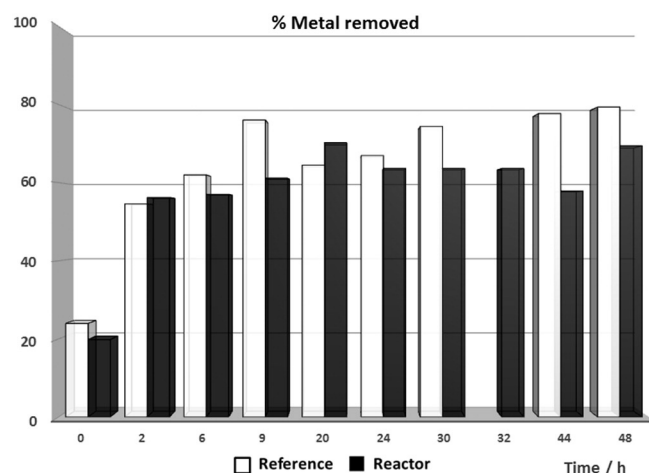


Fig. 2. Overall metal removal from a synthetic multicomponent aqueous solution by biosorption by keratin and with added phytic acid 10^{-5} M .

concentration of phenols, the primary pollutant of olive mill wastewaters, which diminished to a ten part of the initial value, but still remained about 10 times higher the emission limit (0.5 mg L^{-1}) in the Portuguese law for wastewater discharges (Portuguese Government, 1998).

The reactor fed with AMD from Quinta do Bispo mine and ethanol as carbon source (System III - Table AM1 in Additional Material Section) was assembled in June 2014 and has been in operation for more than one year, confirming for the first time the possibility of using SRB based processes to treat this water. Currently this reactor is being optimized using wine wastes, another low-cost organic substrate easy to obtain in this mine region and tested with success at CCMAR (before this project) in the treatment of artificial AMD without uranium (Costa et al., 2009).

3.2. Phytase and phosphatase precipitation process

3.2.1. Hydrolysis of phytic acid catalyzed by immobilized phytase and its application to metal bio-precipitation from synthetic metal-bearing wastewaters

First, after verifying the effects of pH and temperature on the immobilized phytase activity, it was decided to work at room temperature and pH 5.5 (5 mM acetic/acetate buffer), close to the optimum values for phytase activity and those of the industrial wastewaters. If necessary, the buffered pH was further adjusted with dilute HCl or NaOH during the kinetic runs. The storage/operational stabilities of the enzymatic reactor was also established, maintaining 50% phytase

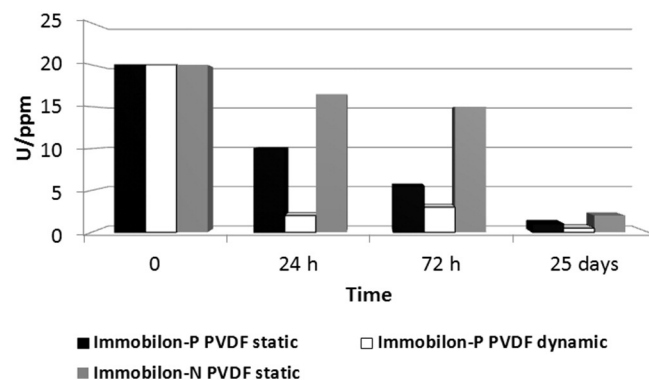


Fig. 3. Evolution of the concentration of uranium in aqueous solution after incubation (in static and orbital agitation) with the acid phosphatase immobilized in PVDF membranes in the presence of 5 mM β -GP.

Metal multicomponent aqueous solution + 50 mM β -glycerophosphate + *Amycolatopsis*

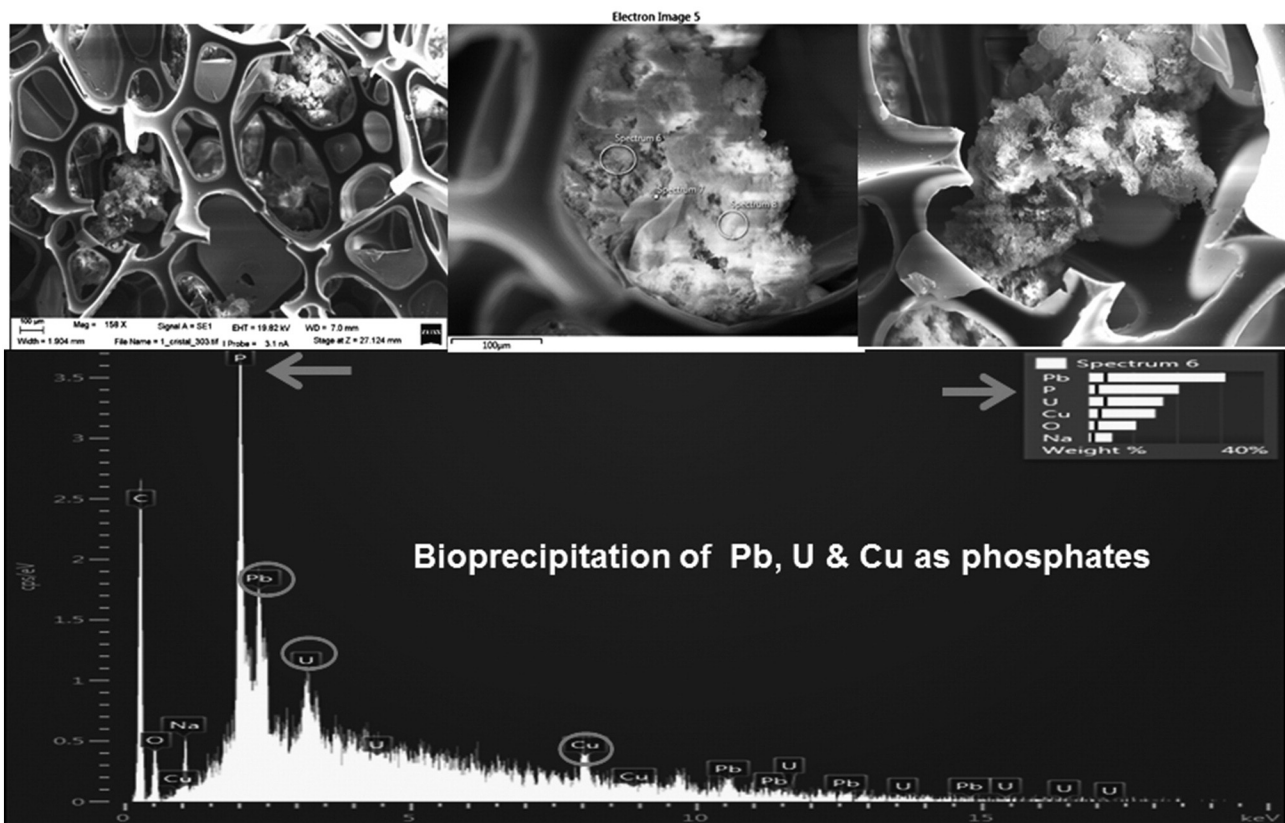
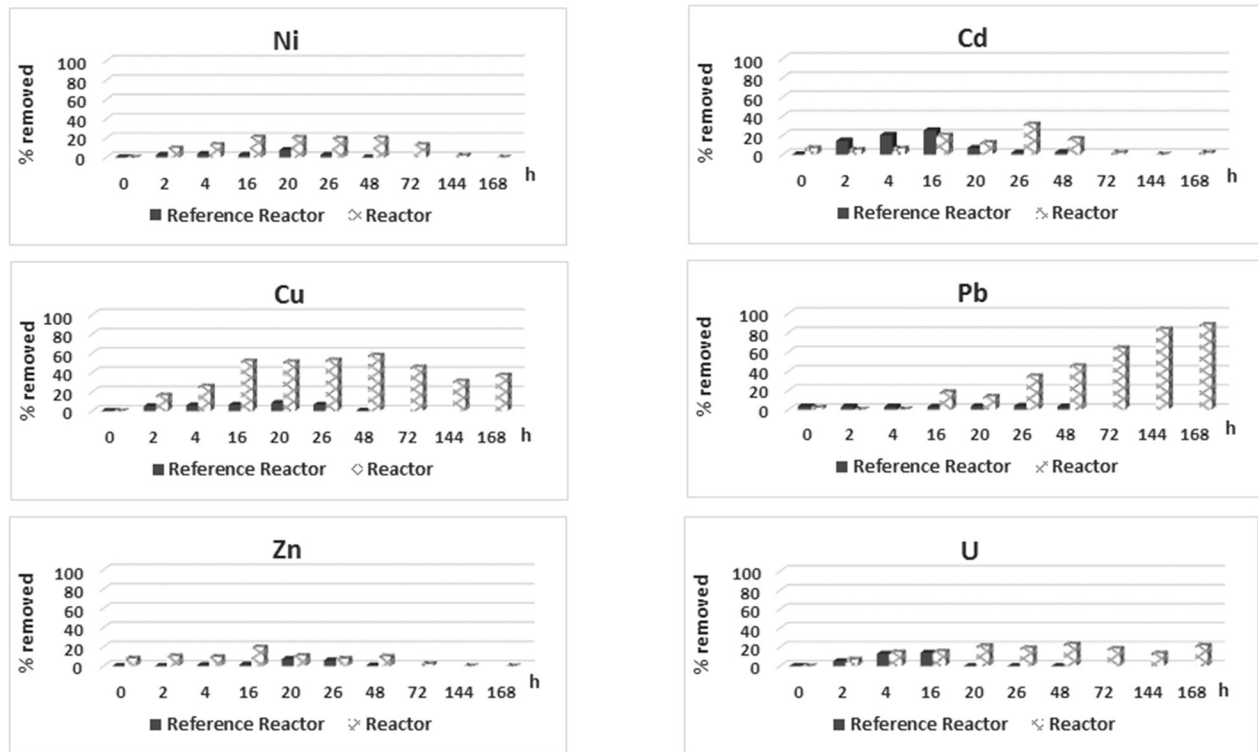


Fig. 4. Preliminary results of kinetics of bio-precipitation of Pb, U and Cu by *Amycolatopsis tucumanensis* cells adsorbed on polyurethane foam cubes when the 120 mg L⁻¹ metal multicomponent water with added 50 mM β -Glycerophosphate is recirculated for 7 days through the packed bed bioreactor. Scanned electronic photomicrographs and EDAX of the precipitates are included.

Table 3

Zinc biosorption in columns charged with 1 g of biomass (sugar-beet (P) and *F. vesiculosus* (F)) with different feed flow rate, 1 mL min⁻¹ (1) and 2 mL min⁻¹ (2).

Column	Time (h)	Treated metal (mg)	Adsorbed metal (mg)	Adsorption (%)	q _c (mg g ⁻¹)	Saturation rate (mg h ⁻¹)
PI	3	77.3	14.5	18	14.531	4.8
P-2	3	155.7	18.6	12	18.6	6.2
FI	5	128.9	47.7	37	47.7	9.5
F-2	5	259.5	66.9	26	66.9	13.4

activity after 175 days and 4 reutilization cycles. The molar ratio of phosphate released/initial phytic acid was 4.0, i.e. 4 mol of inorganic phosphate are released by partial hydrolysis from a phytic acid molecule (total hydrolysis may release up to 6 molecules of inorganic phosphate). Regarding the reference reactor (packed with keratin fibers), no phosphate molecules were released from phytic acid, indicating no spontaneous hydrolysis of phytic acid. With regards to metal removal from wastewaters, in an initial stage, the bioprocess studied was operated by recirculating at 1 mL min⁻¹ and pH 5.5 synthetic water (250 mL) with 20 mg L⁻¹ of one metal and 10⁻³ M phytic acid through 3 g of the phytase-keratin biocatalyst packaged within each bioreactor, checking the hydrolysis of phytic acid and the bio-precipitation process of the selected metal. In parallel, an identical aqueous solution of the metal and phytic acid was recirculated through a reference bioreactor packaged with the same mass of keratin support in order to check the possible processes of metal biosorption on keratin and the non-enzymatic hydrolysis of phytic acid.

The results are shown in Fig. 1, where the removal of each metal from the solution is found to be a process of biosorption, and in the enzymatic bioreactor an increased metal removal by the sum of a biosorption and bio-precipitation processes. Furthermore, after 48 h, no further substantial metal removal was found. The maximum metal removal performance from the aqueous solution by biosorption + bio-precipitation follows the sequence U (100%) > Zn (80%) > Cd (75%) > Cu (60%) = Pb (60%) > Ni (50%).

The following study was carried out to check the metal removal by the immobilized phytase bioprocess when passing through the bioreactor 500 mL of a multicomponent (6 metals) aqueous solution at pH 5.5 and when adding 10⁻³ M phytic acid. The first step was the dissolution, one by one, of salts of 6 different metals into water, obtaining a synthetic aqueous solution of 20 mg L⁻¹ Zn, 20 mg L⁻¹ Cu, 20 mg L⁻¹ Ni, 20 mg L⁻¹ U, 40 mg L⁻¹ Cd and 40 mg L⁻¹ Pb. The second step was to dissolve 0.3302 g of phytic acid into the previous aqueous solution with an overall metal concentration of 160 mg L⁻¹, then a whitish colloidal precipitate appears immediately, which is filtered with 0.45 mm pore size and metals concentrations in the filtrate were analyzed. As a consequence of the insolubility and stability of various phytate-metal complexes (Maddaiah et al., 1964; Vohra et al., 1965), such analysis showed that chemical precipitation of metals by 10⁻³ M phytic acid removed 75% of the overall metal content from the solution, and specifically 96% U, 93% Pb, 85% Zn, 70% Cu, 69% Cd and 46% Ni (data not shown).

Considering that this research line is looking for bio-precipitation of metals, this chemical precipitation of metals represents a competition and a potential risk for the bioprocess being postulated as a good

Table 4

Zinc biosorption in columns charged with sugar-beet pulp (P) and *F. vesiculosus* (F) using different amounts of biomass (1 or 3 g) with an inlet flow rate of 1 mL min⁻¹.

Column	Time (h)	Treated metal (mg)	Adsorbed metal (mg)	Adsorption (%)	q _c (mg g ⁻¹)	Saturation rate (mg h ⁻¹)
PI g	3	77.3	14.5	18	14.5	4.8
P3 g	3	77.3	36.5	47	12.2	12.2
FI g	5	128.9	47.7	37	47.7	9.5
F-3 g	7	186.5	113.5	61	37.8	16.2

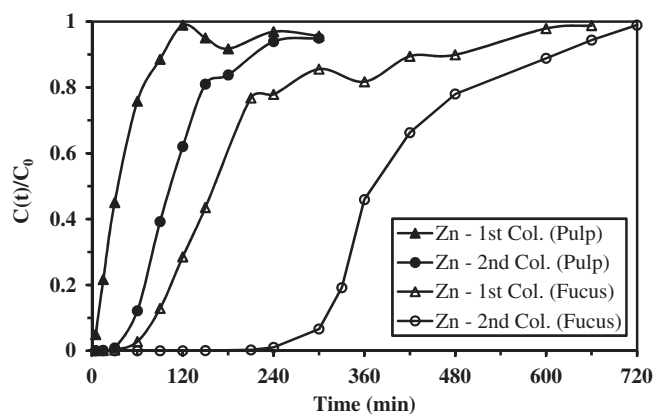


Fig. 5. Comparison of Zn biosorption from Goñabe wastewaters between single column and serial columns using *F. vesiculosus* and sugar beet pulp.

candidate for metal removal from aqueous solutions. The immediate objective was to work at phytic acid concentration low enough to prevent such direct chemical precipitation of metals.

In this sense, a series of aqueous solutions, each one with the six metals under study, at 20 mg L⁻¹ of each metal and an overall metal content of 120 mg L⁻¹, were subjected to the presence of different phytic acid concentrations from 10⁻⁶ M up to 5.10⁻⁴ M. Phytic acid concentrations lower than 10⁻⁵ M prevent the chemical precipitation of metals and at higher than 5.10⁻⁵ M the metal precipitates are visible.

Afterwards, a multicomponent aqueous solution of 6 metals (at 20 mg L⁻¹ each one) in 5 mM acetic/acetate buffer pH 5.5 with added 10⁻⁵ M phytic acid was passed at 1 mL.min⁻¹ and room temperature for 48 h through the immobilized phytase (3 g packed phytase-keratin) and reference (3 g packed keratin) bioreactors. The results are shown in Fig. 2 where it looks that metal removal

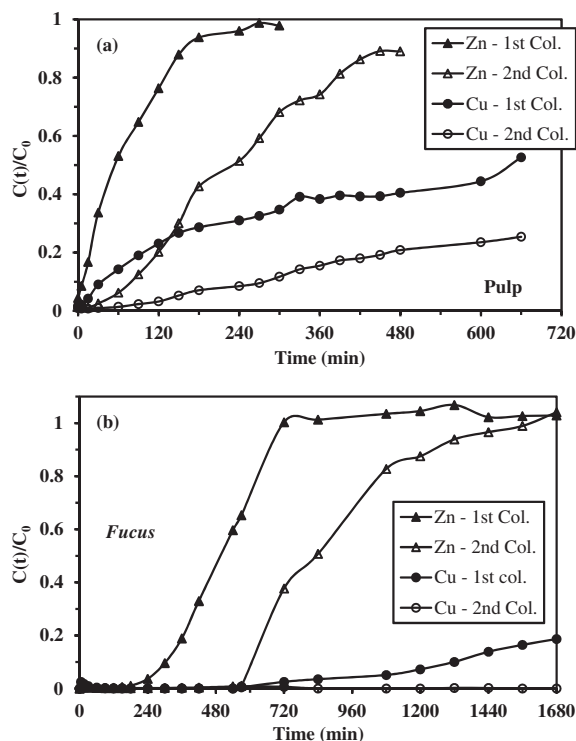
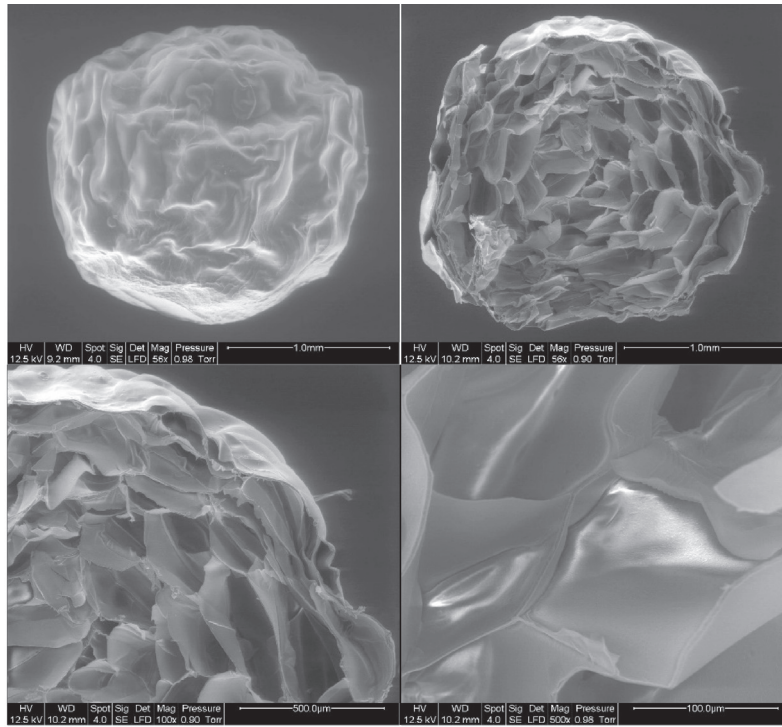


Fig. 6. Comparison of Zn and Cu biosorption from Sao Domingos wastewaters between single column and serial columns using (a) sugar beet pulp and (b) *F. vesiculosus*.

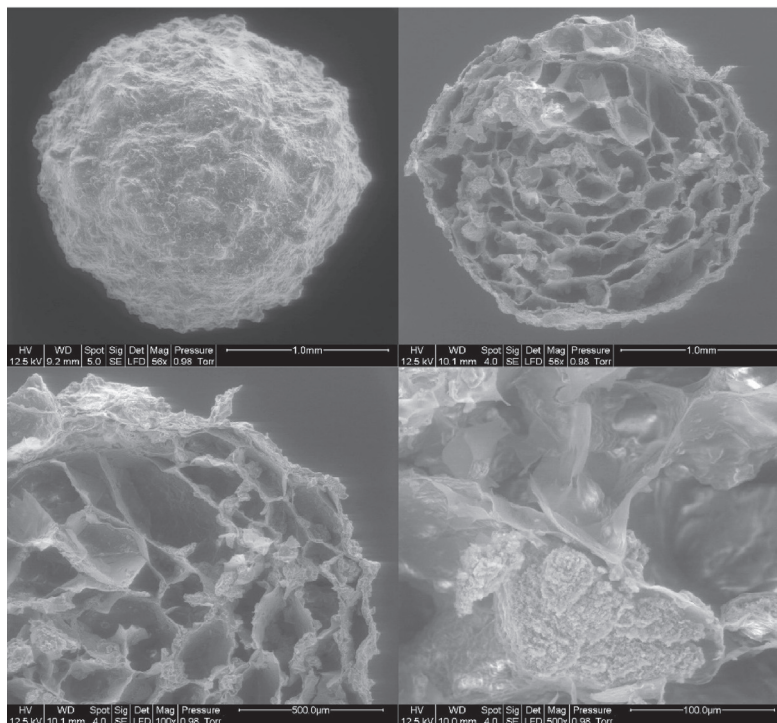
is predominantly due to biosorption process because the reference reactor is as effective for metal removal than the immobilized phytase bioreactor. After 9 h, biosorption removed about 80% of initial metal content of the aqueous solution. These results are in agreement with previous data reported (Roig et al., 2003; Gao et al., 2014).

3.2.2. Hydrolysis of β -glycerophosphate catalyzed by acid phosphatase immobilized on Immobilon-P PVDF and Immobilon-N PVDF membranes and its application to metal bio-precipitation from synthetic metal-bearing wastewaters

Given the interference of chemical precipitation of metal phytates with the process of bio-precipitation of metals investigated, it was



Alginate beads (freeze-drying: $-52\text{ }^{\circ}\text{C}$) (at different magnifications)



PEI-CS₂/alginate composite (freeze-drying: $-52\text{ }^{\circ}\text{C}$) (at different magnifications)

Fig. 7. SEM micrographs of different alginate-based sorbents.

decided to resume the research using a commercial acid phosphatase from wheat, suitably immobilized, as possible biocatalyst of metal bio-precipitation process. The substrate of the acid phosphatase is β -glycerophosphate, that does not form insoluble metal complexes but that is hydrolyzed by the enzyme to inorganic phosphate, which, in the presence of metals, would precipitate them as metal phosphates. After checking the hydrolysis of β -glycerophosphate 5 mM by acid phosphatase immobilized on PVDF membranes, a synthetic aqueous solution of 6 metals Zn, U, Ni, Cu, Cd and Pb (20 mg L^{-1} of each metal) in 5 mM acetic/acetate buffer, pH 4.5, to which was added 5 mM β -GP, was incubated in batch with the immobilized acid phosphatase. The analysis of the metals in solution during the operation time showed that only uranium is significantly removed from the aqueous solution (up to 90% at 24 h) (Fig. 3).

3.2.3. Immobilized bacteria and their ability to precipitate metals from synthetic metal-bearing wastewaters

After researching the ability to catalyze the metal bio-precipitation by the enzymes phytase and acid phosphatase, it was decided to study the bacteria *Mycobacterium psychrotolerans* and *Amycolatopsis tucumanensis* as potential agents for the bio-immobilization of metals, based on their resistance and adaptation to environments with high concentrations of metals and, particularly, on their acid phosphatase activity. A multicomponent aqueous solution of 6 metals (at 20 mg L^{-1} each one) in 5 mM acetic/acetate buffer pH 5.5 with added 50 mM β -glycerophosphate was passed at 1 mL min^{-1} and room temperature through a bioreactor packed with small cubes of reticulated polyurethane foam loaded with bacteria cells. Another identical metalized solution (+ 50 mM β -glycerophosphate) was passed through a reference bioreactor with empty cubes. The results for *Mycobacterium* show a little removal of Pb (25% at 2 h), Zn (20% at 2 h) and U (20% at 4 h) but they are much more promising for *Amycolatopsis* with 80% elimination for Pb, 60% for Cu but only 20% for U (Fig. 4).

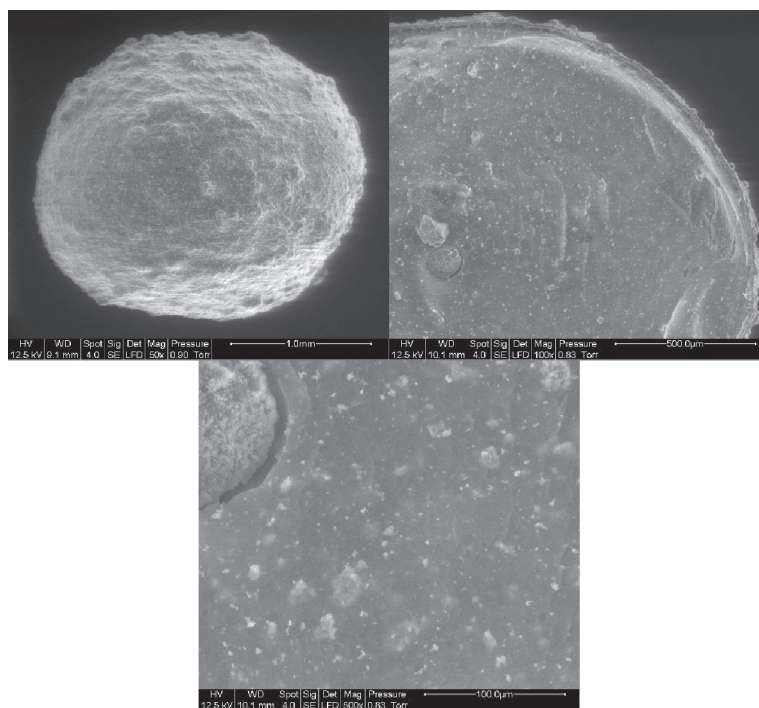
3.3. Continuous biosorption process using algal biomass and sugar-beet pulp

3.3.1. Effect of feed flow rate and bed height on breakthrough curves

The evaluation of the feed flow rate influence indicated the relationship between the column efficiency and the contact time metal-biomass. Table 3 summarizes the results obtained during the zinc treatment. A higher flow rate leads to a shorter saturation time of columns, indicating that the mass transfer zone became more narrow and the consequent lower efficiency of the columns. These results are consistent with those obtained using other biomasses during copper biosorption (Valdman et al., 2001; Christoforidis et al., 2015). The improvement of the metal adsorption in the columns performed at 1 mL min^{-1} with respect to the columns performed at 2 mL min^{-1} was likely caused by a longer contact time between the biomasses and the metal solutions.

The effect of the bed height in the zinc biosorption was studied by comparison of two columns with 1 and 3 g of *F. vesiculosus* and sugar beet pulp ($500 \text{ mg L}^{-1} \text{ Zn}^{2+}$; 1 mL min^{-1}). The biosorption parameters are shown in Table 4. An increase of the bed height by increasing the mass of biomass substantially increased the efficiency of the columns. Nevertheless, the column performance and the metal recovery decreased slightly. This effect could be due to two different factors: the pressure drop along the column, and the increase of the amount of biomass that would cause greater interferences between active binding sites.

Table 4 also shows the zinc uptake (mg.g^{-1}) of a column with 1 and 3 g of biomass. From the comparison of this parameter in both systems, with brown algae and sugar beet pulp, columns with 3 g presented lower q_c than the respective with 1 g. The uptake was lower probably due to the formation of preferential flow channels. This behavior has been previously reported in pectin xerogel columns where the decrease of copper uptake with increasing amounts of biomass may not only be due to a pressure drop along the column, but caused by greater interferences between binding sites in the adsorbent (Mata et al., 2009).



PEI-histidine/alginate composite (freeze-drying: $-52 \text{ }^\circ\text{C}$) (at different magnifications)

3.3.2. Columns in series

In order to apply these biosorption columns in the treatment of real polluted effluent, serial columns were tested with a solution similar to a mine acid drainage and another one similar to wastewaters from a galvanic industry. Two consecutive biosorption columns permitted the substitution of the saturated portions of the biosorbent in the initial columns without interrupting the effluent treatment. The solutions were drop-fed (5 mL min^{-1}) into the first of two columns in series containing 10 g of biomass (*Fucus vesiculosus* and sugar beet pulp).

The biosorption curves obtained using solutions with a high concentration of zinc (500 mg L^{-1}) are shown in Fig. 5. The results pointed out that the use of serial columns increases the service time of the columns resulting more useful in large scale treatments. The slope of the curves depends on the inlet concentration of each column. The first column, in contact with the more concentrated metal solution, exhibited a higher slope than the second and was the first to achieve the saturation point, indicating that it had the narrowest transfer zone.

The biosorption curves obtained in the experiments performed using solutions similar to the Sao Domingos wastewaters (containing $[\text{Zn}]_0 = 20 \text{ mg L}^{-1}$ and $[\text{Cu}]_0 = 12 \text{ mg L}^{-1}$) are shown in Fig. 6.

Since this is a more diluted solution than the Industrial Goñabe wastewater, the biosorption is more efficient and presented a longer service time. The results indicate that copper has higher affinity for the biomass than zinc ($q_{\text{max Cu pulp}} = 0,16 \text{ mmol/g}$; $q_{\text{max Cu algae}} = 0,97 \text{ mmol/g}$). This effect is especially remarkable in case of brown algae. Zinc, due to its lower affinity broke through the column faster than copper, as shown in Fig. 6.

3.4. Biosorption process ((PEI-derivative encapsulated in biopolymer matrices)

3.4.1. Characterization of PEI-derivative/biopolymer composites and sorption properties

Biopolymer capsules (free of PEI-derivative) show with SEM analysis the usual scaffold structure obtained with the encapsulation of solid particles in alginate-based materials. On the opposite hand, for both chitosan and alginate based materials the immobilization of PEI-derivative particles is characterized by the presence of more or less agglomerated aggregates between the foils of the polymer scaffold (Figs. 7 and 8).

The PEI-micro particles are efficiently entrapped in the biopolymer matrix; however, the presence of large agglomerates may induce weak availability and accessibility to the reactive groups of PEI-derivative): the synthesis procedure should take into account the optimization of solid phase dispersion in the biopolymer solution.

3.4.2. Sorption properties in batch systems

Alginate and chitosan have high affinity for metal sorption through different mechanisms involving metal complexation or ion exchange (Guibal, 2004; Papageorgiou et al., 2006; Cataldo et al., 2013). It is thus important determining the effective contribution of the PEI-derivative on metal binding properties of the composites. Experiments have been performed under comparable experimental conditions with the four alginate-based sorbents (alginate, PEI-crosslinked/alginate; PEI-histidine/alginate and PEI-CS₂/alginate capsules, Tables AM2 and AM3; See Additional Material Section) and for chitosan-based sorbents (Table AM4, See Additional Material Section) at different pH values

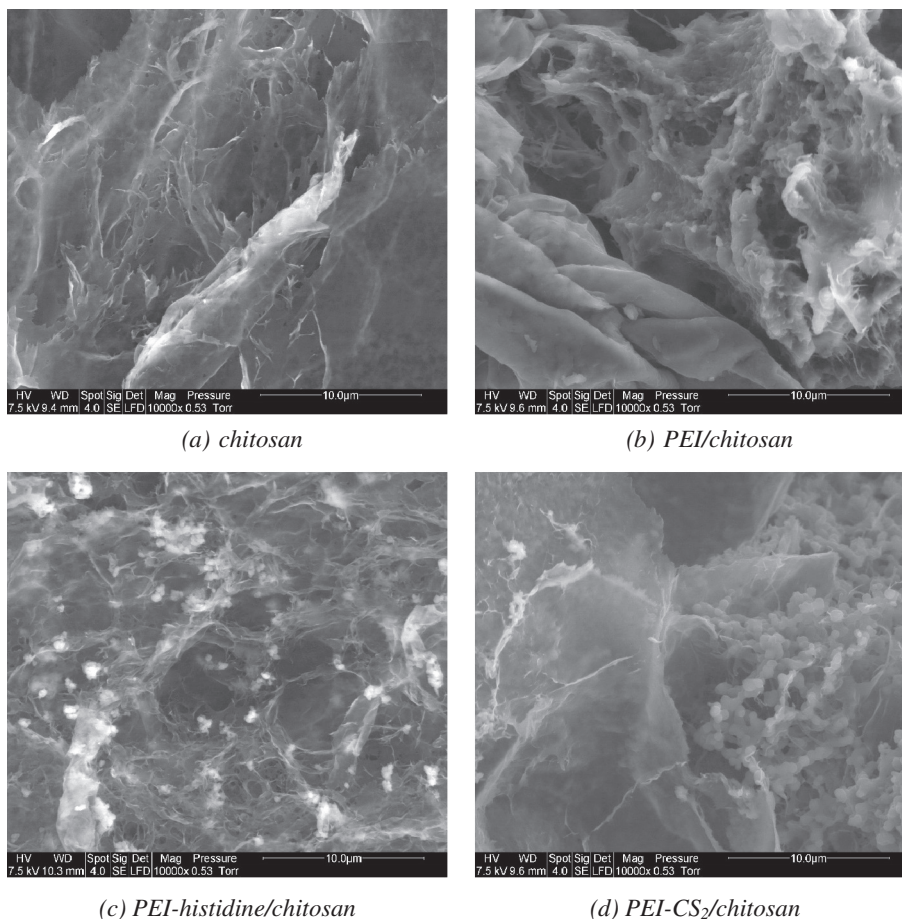


Fig. 8. SEM micrographs of chitosan-based sorbents (Magnification: $\times 10000$).

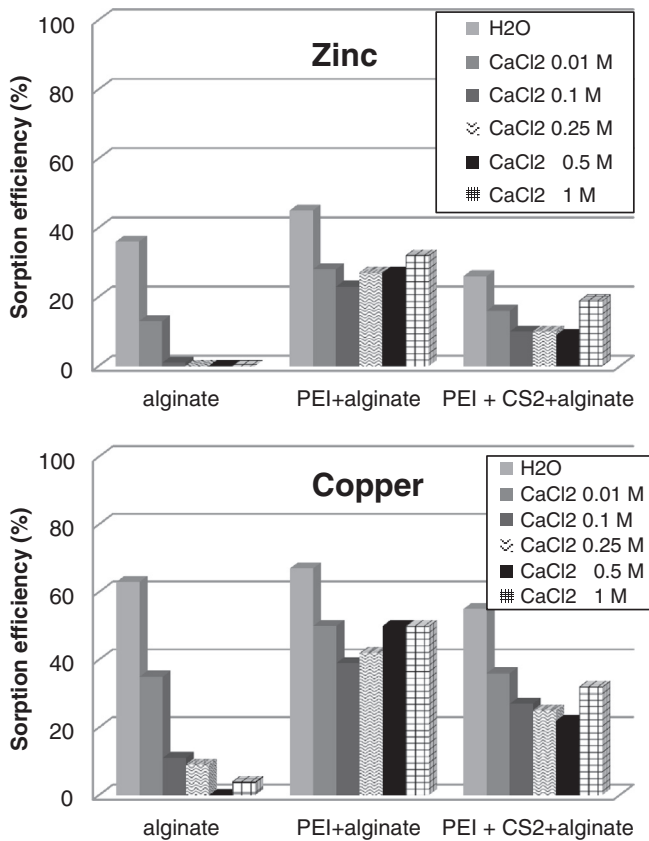


Fig. 9. Sorption of Zn(II) and Cu(II) in presence of increasing concentrations of calcium. (C_0 : 2 mmol metal L^{-1} ; SD: 1 $g L^{-1}$, pH 4).

using synthetic pure solutions. Actually, the values of sorption capacities and sorption efficiencies were roughly comparable and the incorporation of PEI-derivatives did not significantly change sorption performance for Zn(II) and Cu(II) in pure solutions. The fraction of PEI-derivative in the composite materials was close to 50% in weight. More details on the sorption properties of these different materials for a series of divalent cations are reported in Additional Material Section

(Fig. AM7 shows sorption isotherms; Figure AM8 shows uptake kinetics; Table AM5 reports the values of the parameters of the Langmuir equation). Sorbents prepared with chitosan showed lower mechanical resistance. During the sorption process some chitosan based materials were degraded and it was not possible to obtain accurate curves. In addition, the histidine derivative of PEI appears to be, in most cases, less efficient than other sorbents, regardless of the pH. Figure 9 shows the impact of increasing concentrations of calcium on metal binding. The introduction of small amounts of Ca(II) drastically reduced the sorption capacity of Zn(II) and Cu(II) by alginate material: calcium ions act as competitor ions against divalent metal cations for interacting with carboxylic groups of alginate. The CS₂-derivative of PEI, encapsulated into alginate capsules, is also strongly affected by the presence of calcium ions; however, sorption remained significantly higher than alginate beads. Dithiocarbamate-based reactive groups on PEI-CS₂ sorbent are slightly more selective to heavy metal ions than carboxylic groups for both Zn(II) and Cu(II).

The most efficient and most selective composite is the glutaraldehyde-crosslinked PEI encapsulated in alginate beads: the sorption decreased by <30% even in the presence of a large excess of calcium ions (i.e., 1 M CaCl₂). The incorporation of PEI-glutaraldehyde derivative strongly improves the selectivity of the sorbent for heavy metal ions compared to alkaline earth metals (such as Ca(II)). In order to evaluate the selectivity properties of the sorbents experiments have been performed in multi-component solutions. Fig. 10 shows the sorption of divalent cations (Zn(II), Cd(II) and Cu(II)) in single- and multi-component solutions. Whatever the sorbent, the sorption affinity depends on the composition of the encapsulation material; the sorption capacity for alginate composites decreases according Cu(II) > Cd(II) > Zn(II), whereas for chitosan composites was found Cu(II) > Zn(II) > Cd(II).

3.4.3. Metal desorption

Metal desorption is generally operated by pH change using acidic solutions. Preliminary tests have shown that HCl solution can efficiently remove divalent metal cations (Fig. 11); however, simultaneously to metal desorption a significant loss of material was observed. The solution consisted in adding calcium chloride in the solution to prevent alginate dissolving and sorbent degradation. Varying HCl and CaCl₂ concentrations allowed determining the optimum conditions (i.e., 0.2 M CaCl₂ solution with pH controlled to 1.2–1.5 with HCl) for

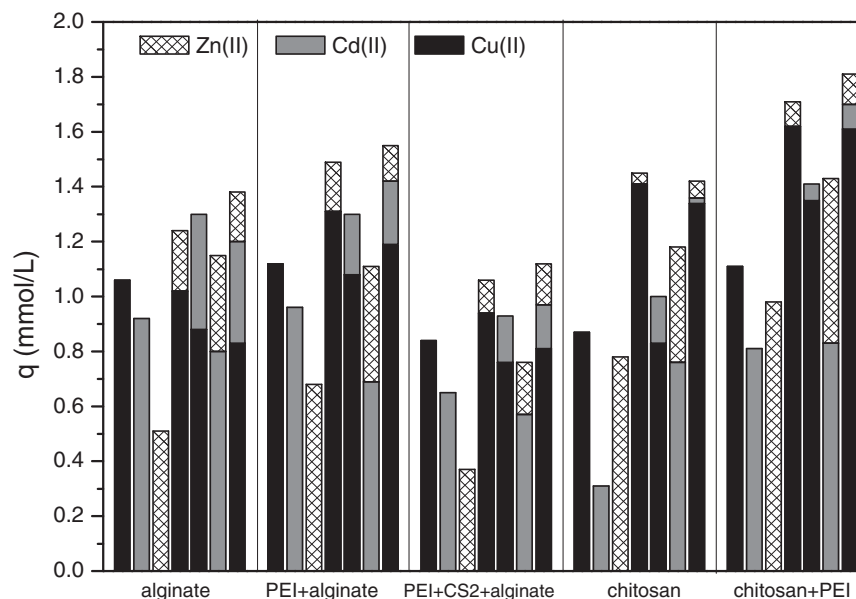


Fig. 10. Single and multi-component sorption of Zn(II), Cd(II) and Cu(II) using different sorbents (C_0 : 2 mmol each metal L^{-1} ; SD: 1 $g L^{-1}$, pH 4).

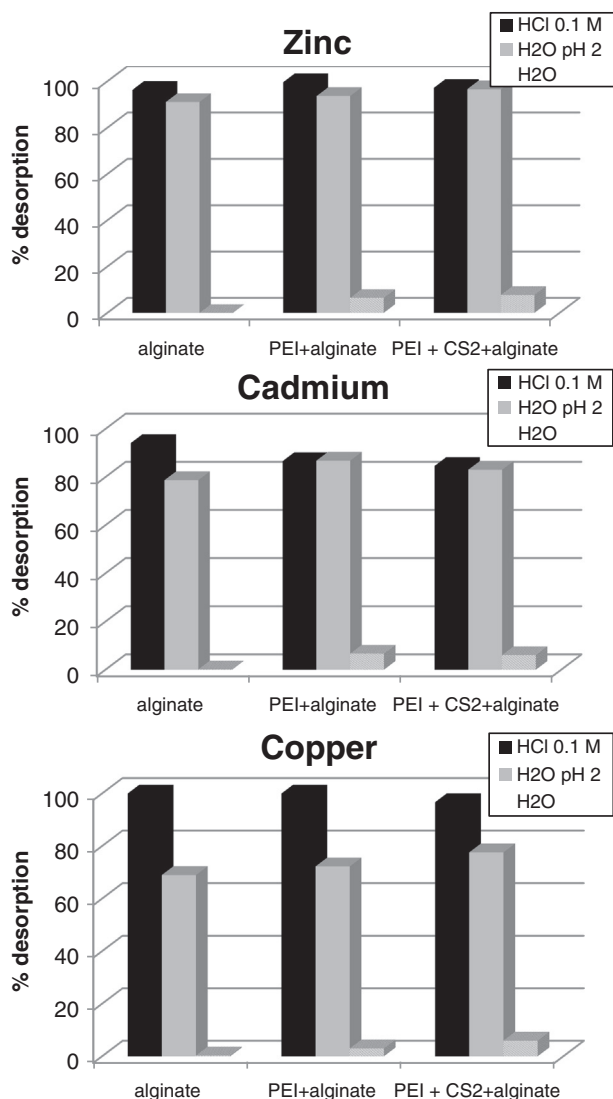


Fig. 11. Desorption tests for Zn(II), Cd(II) and Cu(II). (sorption: C_0 : 2 mmol metal L^{-1} ; SD: 1 g L^{-1} , pH 4; desorption: 10 mL of eluent) (Reprinted with permission from ACS; see Bertagnolli et al., 2016).

achieving high desorption efficiency (around 80–85%) and low loss of sorbent (below 8%).

4. Conclusion and perspectives

Five alternative zero or low-cost substrates were confirmed to be suitable sources of carbon compounds to feed SRB bioreactors in the treatment of AMD waters, allowing an efficient removal of metals: molasses from orange, molasses from sugar-beet, carbocal, sewage water (WWTP lagoon system – first lagoon) and olive mill wastewater. Yet, for using these substrates further optimization of operational conditions is needed to reach high efficiencies in sulfate removal. Experiments integrated in the project BIOMETAL DEMO are currently ongoing aiming to achieve that purpose.

The immobilized phytase hydrolyzing phytic acid, while producing inorganic phosphate which precipitates U (100%) > Zn (80%) > Cd (75%) > Cu = Pb (60%) > Ni (50%) as metal phosphates from metallized mono-component aqueous solutions, does not cause bio-precipitation of metals present in multicomponent synthetic wastewater with soluble Zn, U, Ni, Cu, Cd and Pb (at a total metal concentration of 170 mg L^{-1}). Instead what is produced is the chemical precipitation of the metals as insoluble and more stable metal phytates than the

corresponding metal phosphates. This represents, therefore, a serious competition and interference for the bio-precipitation of metal phosphates; the bioprocess is postulated as a good candidate for the removal of metals from aqueous solutions.

Consequently, after the first months of the second year of the Project BIOMETAL DEMO, a decision making was taken by the team from the University of Salamanca in the sense of considering other immobilized biocatalysts such as acid phosphatase from wheat and bacteria *Mycobacterium psychrotolerans* and *Amycolatopsis tucumanensis* with acid phosphatase activity, as new biological agents for bioprecipitating metals from wastewaters. The substrate of these three acid phosphatases is the phosphate monoester β -glycerophosphate, not forming insoluble metal complexes but which is hydrolyzed by the catalytic action of the enzyme to inorganic phosphate, which, in the presence of metals, would precipitate them as insoluble metal phosphates. Accordingly, metal-bearing wastewaters would be decontaminated of their dissolved metals by such metal bio-precipitation process. The latter hypothesis was experimentally studied in multicomponent metallized synthetic wastewater with some promising preliminary results for the bio-precipitation of metals such as Pb, U, Zn and Cu by the biocatalytic action of such bacteria.

Biosorption of heavy metal ions by inexpensive dead biomasses as brown algae and sugar beet pulp is a feasible technology for removing toxic metals from wastewaters. Adsorption curves obtained at different flow rates indicated that an increase in the flow rate decreases the efficiency of the column. In addition, an increase of the bed height increased the pressure drop in the column and the saturation time. Finally, serial columns seemed to be a suitable process for the treatment of polluted wastewaters.

Chitosan and alginate have been tested for the encapsulation of micro-particles of PEI chemically modified by a series of chemical compounds (tannic acid, amino-acids, carbon disulfide). Taking into account both stability (not shown but experiments under strong agitation showed a partial mechanical degradation of the sorbents when using chitosan), economics and technical performances alginate appears to be more appropriate for the encapsulation of PEI-derivatives. The incorporation of PEI derivatives does not significantly improve the sorption properties in synthetic pure solutions compared to alginate or chitosan beads; however, the presence of these micro-particles enhances sorption capacities in complex solutions. The composite sorbents have a preference for Cu(II) over Ni(II), Cd(II), Pb(II) and Zn(II). Divalent metal cations can be easily desorbed from saturated sorbents using $CaCl_2$ solutions (with pH adjusted in the range 1.2–1.5 with HCl). The presence of calcium chloride is necessary for reinforcing the stability of the composite material. The most promising material consists in the alginate encapsulation of PEI crosslinked with glutaraldehyde.

An alternative process has been recently developed for substituting algal biomass (associating *Fucus* sp. and *Laminaria digitata*) to alginate in the encapsulation of PEI-glutaraldehyde crosslinked sorbent. This new process would reduce the costs of sorbent manufacturing and would contribute to a cleaner synthesis since the alginate extraction from algal biomass is not required. In addition, the gelling of alginate material procedure was changed to improve the stability of the composite materials: instead of the conventional process that consists in the external ionotropic gelation an in situ ionotropic gelation procedure was developed. The new materials appear to be more stable while maintaining good sorption properties. This is the composite sorbent that will be tested on industrial effluents as a polishing treatment to algal/– beet pulp treatments (developed at Universidad Complutense de Madrid) after SRB and/or phytase precipitation processes.

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