



## Stability investigations of zinc and cobalt precipitates immobilized by *in situ* bioprecipitation (ISBP) process

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### ABSTRACT

*In situ* bioprecipitation (ISBP), which involves immobilizing the metals as precipitates (mainly sulphides) in the solid phase, is an effective method of metal removal from contaminated groundwater. This study investigated the stability of metal precipitates formed after ISBP in two different solid–liquid matrices (artificial and natural). The artificial matrix consisted of sand, Zn (200 mg L<sup>-1</sup>), artificial groundwater and a carbon source (electron donor). Here the stability of the Zn precipitates was evaluated by manipulation of redox and pH. The natural system matrices included aquifer material and groundwater samples collected from three different metal (Zn and Co) contaminated sites and different carbon sources were provided as electron donors. In the natural matrices, metal precipitates stability was assessed by changing aquifer redox conditions, sequential extraction, and BIOMET<sup>®</sup> assay. The results indicated that, in the artificial matrix, redox manipulation did not impact the Zn precipitates. However the sequential pH change proved detrimental, releasing 58% of the precipitated Zn back into liquid phase. In natural matrices, the applied carbon source largely affected the stability of metal precipitates. Elemental analysis performed on the precipitates formed in natural matrix showed that the main elements of the precipitates were sulphur with Zn and Co.

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### 1. Introduction

Heavy metal contamination of soil and groundwater due to anthropogenic sources such as non-ferrous industrial sectors, is a worldwide problem. In general, more than 60% of contaminated sites in the world have problems associated with the presence of toxic metals such as Cd, Pb, Cu, Zn, Hg, Co and Ni. The leaching of metals by infiltrating rainwater from the polluted soil eventually contaminates the groundwater [1]. In the last decades, a lot of research has been accomplished to develop efficient technologies such as chemical precipitation, ion exchange, adsorption, physical separation, electrochemical separation etc. for the treatment of metal-polluted groundwater and effluents [2]. However, most of these methods are pump and treat (in case of contaminated groundwater), which become particularly impracticable for huge contaminated zones where large volumes of groundwater need to be pumped and treated.

Due to the widespread extent of this problem, the non-invasive and relatively low cost *in situ* remediation technologies have gained momentum in the past few years. Some of them are phytoremediation, application of amendments and isolation by physical barriers [3]. Biotechnology also offers an interesting possibility of *in situ* bio-

precipitation (ISBP) of metals. ISBP involves amendment of a carbon source (electron donor) for metal bioprecipitation via the formation of metal sulphides, a process mediated by sulphate reducing bacteria (SRB) [4]. The SRBs, when provided with a suitable electron donor, reduce the available sulphate to sulphides, which then concomitantly precipitate the heavy metals and immobilize them in the solid phase (soil).

It is well known that the ISBP processes for metal remediation from groundwater, does not change the total metal concentration, but removes the metals from liquid phase and immobilizes them to solid phase. Consequently the study of mobility and bioavailability of immobilized metals over a period of time and *vis-a-vis* changing environmental conditions becomes essential. Though metal sulphide bindings are considered strong and immobile, these may show large variations under the influence of varying environmental conditions [5]. There have been a few studies on metal precipitates stability after application of *in situ* stabilization techniques such as *in situ* chemical precipitation (ISCP) conjugated with phytostabilization [6], immobilization with zerovalent iron [7] and cyclonic ash and compost [8]. In these studies toxicity characteristic leaching procedure, sequential extraction, leaching tests, bioavailability tests and mineralogical methods have been applied for stability investigation. To the best of our knowledge no studies have been reported to investigate the stability of Zn and Co precipitates formed after completion of ISBP process.

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In our earlier works, we investigated the feasibility of ISBP process for metal immobilization in microcosm tests with artificial sand–groundwater (ASG) matrix [9]. Here various parameters such as amount of sulphate and nutrients were varied to induce ISBP. Furthermore, in another study [10], ISBP investigations were also done in natural matrices with aquifer material and groundwater (NAG) from three different metal contaminated sites. The groundwater of these sites was contaminated mainly with Zn and Co. Here, a series of carbon sources were screened for inducing ISBP in microcosm tests. After observing ISBP in both matrices (artificial and natural, i.e. ASG and NAG, respectively) two main research questions that have been addressed in the present study are—(i) How stable are the metal precipitates formed after ISBP, both in artificial and natural matrices? (ii) How can the metal precipitate stability be studied? The hypothesis was that the metal precipitates formed after ISBP are stable with respect to changing environmental conditions. To execute this hypothesis, the objectives formulated for this study were: to investigate the stability of metal precipitates, formed after ISBP in (a) ASG matrix and (b) NAG matrices. Investigating the stability in both artificial and natural matrices was essential because though the artificial systems are not always representative of natural conditions but still give a fair idea of the phenomenon due to defined composition. Furthermore, the need for conducting the study in a natural matrix arises because the artificially spiked metals in artificial systems may form discrete sulphide phases which may not be the case in natural systems. In both these matrices the stability investigations were done on the microcosm setup that were obtained from previously described ISBP experiments [9,10]. In ASG matrix, the stability was examined by manipulating the redox and pH in the microcosms. In NAG matrices, the impact of applied carbon source on stability of formed metal precipitates was investigated by using aquifer redox treatment, sequential extraction, and bioavailability of the metals. Further, elemental analyses in the form of SEM-EDX were also conducted in NAG matrices.

## 2. Materials and methods

### 2.1. Tests performed in artificial sand–groundwater matrix (ASG)

A series of microcosm experiments, with varying experimental conditions, were initially started to induce the ISBP [9]. This was done by adding 200 mL of deoxygenated simulated groundwater to 80 g of quartzite sand in 250 mL acid-washed glass bottles under strictly anaerobic conditions. The groundwater contained ( $\text{mg L}^{-1}$ )  $\text{KHCO}_3$  (50.1),  $\text{NaHCO}_3$  (42),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (55.5),  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (47.6),  $\text{ZnCl}_2$  (416.9), sodium lactate (3500) and resazurin (1). The concentrations ( $\text{mg L}^{-1}$ ) of various components in microcosm tests were varied as follows: sulphate (1000–5000) and nutrients including  $\text{NH}_4\text{Cl}$  (0–100),  $\text{KH}_2\text{PO}_4$  (0–50),  $\text{FeCl}_2$  (0.2) and yeast extract (1–100).  $\text{ZnCl}_2$  and sodium lactate were not added to some conditions that served as controls. The details of ISBP activity have been described previously [9]. In this article, we present the results of stability investigations carried out, in the above described microcosm setups, after completion of ISBP process.

#### 2.1.1. Wet laboratory stability tests (redox and pH manipulation) for the artificial sand–groundwater matrix (ASG)

The ISBP activity took 5–9 months for completion in various conditions and afterwards stability investigations were carried out by manipulating redox and pH in a few representative conditions. Redox was manipulated to simulate the changing redox conditions in the environment. For this, air flushing tests were conducted in order to achieve an oxic environment ( $E_h$  above +200 mV) from the initial anaerobic environment ( $E_h$  around –150 mV). Air was

flushed in the liquid phase (in all studied conditions) for 8 h until no sulphide smell was detected anymore and the samples were then allowed to stand for 24 h. Afterwards the samples were taken for metal (Zn), sulphate and pH analysis, which was done regularly until 8–9 weeks. To investigate the sequential effect of low pH, it was manually adjusted to 5 with HCl (30%) in the 8th or 9th week. During the following days, the pH was regularly measured and adjusted back to 5 (with 30% HCl), if required. The pH and metal concentrations were measured regularly for 9–16 weeks (after starting pH manipulation) depending upon the leaching of metals.

### 2.2. Tests performed in natural aquifer material–groundwater matrices (NAG)

These investigations were conducted with natural aquifer material and groundwater from three different heavy metal contaminated sites in Belgium, named hereafter as sites 1, 2 and 3. Microcosms were started with 40 g aquifer material suspended in 200 mL groundwater (250 mL serum bottles,  $L/S=5$ ) under nitrogen atmosphere. Due to the different characteristics observed for the three sites, individual feasibility ISBP tests were performed with various carbon sources that were chosen on the basis of their cost, anticipated performance effectiveness, defined composition and easy availability [10]. For groundwater of site 1, which was contaminated with Zn (up to  $500 \text{ mg L}^{-1}$ ) three different electron donors i.e., glycerol, sodium lactate containing additional nitrogen and phosphorous (referred as lactate (N/P) hereafter) and molasses were tested for inducing ISBP. For site 2, where the groundwater was contaminated with Zn (up to  $2000 \text{ mg L}^{-1}$ ) six different electron donors were tested. These involved two slow release compounds i.e., polylactate ester (PLE) and soy oil, the waste product molasses and the chemical agent calcium polysulphide ( $\text{CaS}_x$ ). Further, glycerol and lactate (N/P) were also tested. For groundwater of site 3, which was characterized with Co contamination ( $30\text{--}300 \text{ mg L}^{-1}$ ), ISBP was induced with all the carbon sources similar to site 2. Furthermore, three additional conditions with cheese whey and zero-valent iron (ZVI) with and without extra e-donor (lactate) were also investigated for site 3. Two controls (without carbon source supplementation) viz. biotic control (natural attenuation or NA) and abiotic control (with formaldehyde addition) were also examined. In case of sites 1 and 2, the soil and aquifer material were collected from a depth of 30–32 m-bg (below ground) and in site 3, samples from a depth of 4–7 m-bg were used. The additional details of ISBP microcosms setup and results have been reported previously [10]. In this article, we present the results of stability investigations carried out, in the above described microcosm setups (NAG), after completion of ISBP process.

#### 2.2.1. Wet laboratory stability tests for the natural aquifer material–groundwater matrices (NAG)

The ISBP activity took 6–9 months for completion in NAG matrix and afterwards stability investigations in all the conditions were assessed by the following methods.

**2.2.1.1. Aquifer redox treatment.** For each aquifer redox treatment 4 g of aquifer material (after completion of ISBP batch tests) was mixed with 16 mL non-contaminated groundwater ( $L/S=4$ ). In this treatment metal precipitates from different samples were exposed to three different atmospheres (in parallel) viz. aerobic ( $80\% \text{ N}_2 + 20\% \text{ O}_2$ ), anoxic ( $80\% \text{ N}_2 + 20\% \text{ CO}_2$ ) and anaerobic ( $100\% \text{ N}_2$ ), for about 10 min by using a sterile needle distribution system. The samples were then kept on a shaker for 24 h. This was followed by a second exposure to either an aerobic, anaerobic or anoxic atmosphere for 10 min and then kept 24 h on shaker. The samples were then centrifuged at  $4000 \times g$  for 10 min, subsequently filtered

**Table 1**  
pH and Zn concentration ( $\text{mg L}^{-1}$ ) in artificial matrix after completion of ISBP and stability investigations.

No.	Name	Conditions	ISBP investigation				Stability investigations					
			Before ISBP		After ISBP completion		After air flushing		After 8–9 weeks		After pH manipulation	
			[Zn]	pH	[Zn]	pH	After 24 h				After 9–16 weeks	
							[Zn]	pH	[Zn]	pH	[Zn]	pH
1	Standard condition	$\text{SO}_4^{2-}$ (1000) + Zn (200) + lactate (2800) + $\text{NH}_4\text{Cl}$ (10) + $\text{KH}_2\text{PO}_4$ (5) + Yeast extract (10)	187	5.1	0.95	8.5	0.95	8.7	0.95	8.7	31.2	5.1
2	High sulphate	$\text{SO}_4^{2-}$ (5000) + Zn (200) + lactate (2800) + $\text{NH}_4\text{Cl}$ (10) + $\text{KH}_2\text{PO}_4$ (5) + Yeast extract (10)	175	5.2	0.01	8.6	0.02	8.7	0.09	8.6	103	5.6
3	No zinc	$\text{SO}_4^{2-}$ (1000) + Zn (0) + lactate (2800) + $\text{NH}_4\text{Cl}$ (10) + $\text{KH}_2\text{PO}_4$ (5) + Yeast extract (10)	1.02	5.1	0.01	8.7	0.02	8.8	0.19	8.8	0.02	5.2
4	Low nutrients	$\text{SO}_4^{2-}$ (1000) + Zn (200) + lactate (2800) + $\text{NH}_4\text{Cl}$ (1) + $\text{KH}_2\text{PO}_4$ (0.5) + Yeast extract (10)	175	5.1	0.01	8.5	<0.02	8.5	10.8	8.4	40.5	4.9
5	No ammonium chloride and potassium dihydrogen phosphate	$\text{SO}_4^{2-}$ (1000) + Zn (200) + lactate (2800) + $\text{NH}_4\text{Cl}$ (0) + $\text{KH}_2\text{PO}_4$ (0) + Yeast extract (10)	187	5.0	0.15	8.7	<0.02	8.6	3.62	8.5	45.6	5.3
6	High yeast extract and no nutrients	$\text{SO}_4^{2-}$ (1000) + Zn (200) + lactate (2800) + $\text{NH}_4\text{Cl}$ (0) + $\text{KH}_2\text{PO}_4$ (0) + Yeast extract (100)	181	5.1	0.04	8.5	<0.02	8.3	4.93	7.4	24.1	5.09

The values in brackets are concentrations of the indicated component in  $\text{mg L}^{-1}$ .

over a 0.45  $\mu\text{m}$  filter and taken for metal analysis. The aquifer redox treatments in three different atmospheres led to a pH change of 0–1.5 units in various conditions.

**2.2.1.2. Sequential extraction.** Aquifer material (after ISBP tests) was used for sequential extraction procedure. The protocol developed by Tessier et al. [11] was used with some modifications. Only three operationally defined fractions i.e., carbonate fraction, Fe–Mn oxide fraction (reducible) and organic/sulphide fraction were extracted with following reagents: 1 M  $\text{CH}_3\text{COONa}$  for extracting metals bound to the carbonate phase; 0.04 M  $\text{NH}_2\text{OH}\cdot\text{HCl}$  in 25% acetic acid for metals associated with reducible Fe–Mn oxides and intermittent heating, with  $\text{H}_2\text{O}_2$  and  $\text{HNO}_3$  followed by extraction with 3.2 M  $\text{CH}_3\text{COONH}_4$  for metals bound to sulphide or organic matter.

**2.2.1.3. BIOMET<sup>®</sup> assay.** BIOMET<sup>®</sup> bioassay was applied to the aquifer material of all different conditions in batch. This test allows to determine the bioavailability of heavy metals. Bacterial availability of Zn was assessed using a bacterial biosensor strain *Cupriavidus metallidurans* AE1433. This strain contains a fusion between *czcS* gene encoding the Cd, Zn and Co resistance [12] and the promoterless *luxCDABE* reporter system of *Vibrio fischeri*. This resulted in inducible light production controlled by the *czcS* genes in the presence of bioavailable Zn, Cd or Co (BIOMET<sup>®</sup>) [13]. The light emission from samples, standard, and blank assays were measured every 30 min for at least 8 h at 23 °C (1 s well<sup>-1</sup>) using an Anthos lucy1 luminometer (Mikrotek Laborsysteme GmbH). Data measured by the luminometer were processed using the MIKROWIN software (version 3.0), as described by Corbisier et al. [14]. The details of soil sample preparation and bioluminescence measurements have been explained in detail previously [15].

### 2.2.2. Elemental analysis

To identify the chemical composition of the fine precipitate fraction elemental analysis was conducted by scanning electron microscopy (SEM) in combination with energy dispersive X-ray (EDX) analysis. The analysis was performed on a JEOL JSM 6340-F Scanning Electron Microscope equipped with an XFLASH silicon drift detector allowing a detection of all elements from B (5) to Am (95). The element identification was performed with the ESPRIT analysis software. Besides point analyses, also mappings of the major elements of the precipitate was carried out.

### 2.3. Physico-chemical analysis

pH and redox measurements were performed using a hand held WTW Multi 340i Set, after calibrating the pH meter at pH 7 and 4 under nitrogen atmosphere. Groundwater samples for metal analyses were filtered over a 0.45- $\mu\text{m}$  filter, acidified by pure  $\text{HNO}_3$  (2%, v/v), and analyzed by ICP-AES (Jarrell-Ash Autocomp 750). Additional measurements for site characterization were conducted according to methods described in Vanbroekhoven et al. [16].

## 3. Results and discussion

### 3.1. Stability study in artificial sand–groundwater matrix (ASG)

The results of stability investigations for ASG matrix are presented in Table 1. It is evident that after completion of ISBP activity, which took 5–9 months, Zn concentration in the liquid phase decreased by 99–100% and the metals got retained in solid phase (aquifer material) as precipitates. As mentioned in Section 2.1.1, we conducted air flushing for 8 h in all the studied conditions, in order to vary the redox conditions in the test setup. After 24 h of completion of air flushing, Zn concentration did not show much

variation and remained quite low (<0.02–0.95 mg L<sup>-1</sup>). Further, after 8–9 weeks of air flushing, no increase in Zn concentration was observed in the conditions 1–3 (standard, high sulphate and no Zn conditions, respectively), while some increase (up to 10 mg L<sup>-1</sup>) in Zn concentration was observed in conditions 4–6 (low nutrients, no ammonium chloride and potassium dihydrogen phosphate and high yeast extract concentration, respectively). It has also been previously reported that zinc sulphide show limited dissolution in oxic conditions [17] and approximately 70% ZnS dissolution requires more than 100 days of oxygen exposure [18]. Unlike redox, the sequential pH manipulation had a strong impact on Zn mobility. After 9–16 weeks of pH manipulation, presence of Zn was observed in liquid phases of all the conditions except obviously in condition 3, where no Zn was added. A possible explanation for these findings is the subsequent formation of metal hydroxides after air flushing (redox manipulation) at pH 8.5–8.7, which dissolved when the pH was lowered. A decrease of pH also tends to reduce the sorption of metals to certain minerals. Moreover, the fact that cationic metals become more mobile as pH decreases is the well-known result of the dissolution of metal-hydroxides, -oxides, -carbonates or -phosphates and the diminished adsorption at cation exchange surfaces [19].

### 3.2. Stability investigations in natural aquifer material–groundwater matrices (NAG)

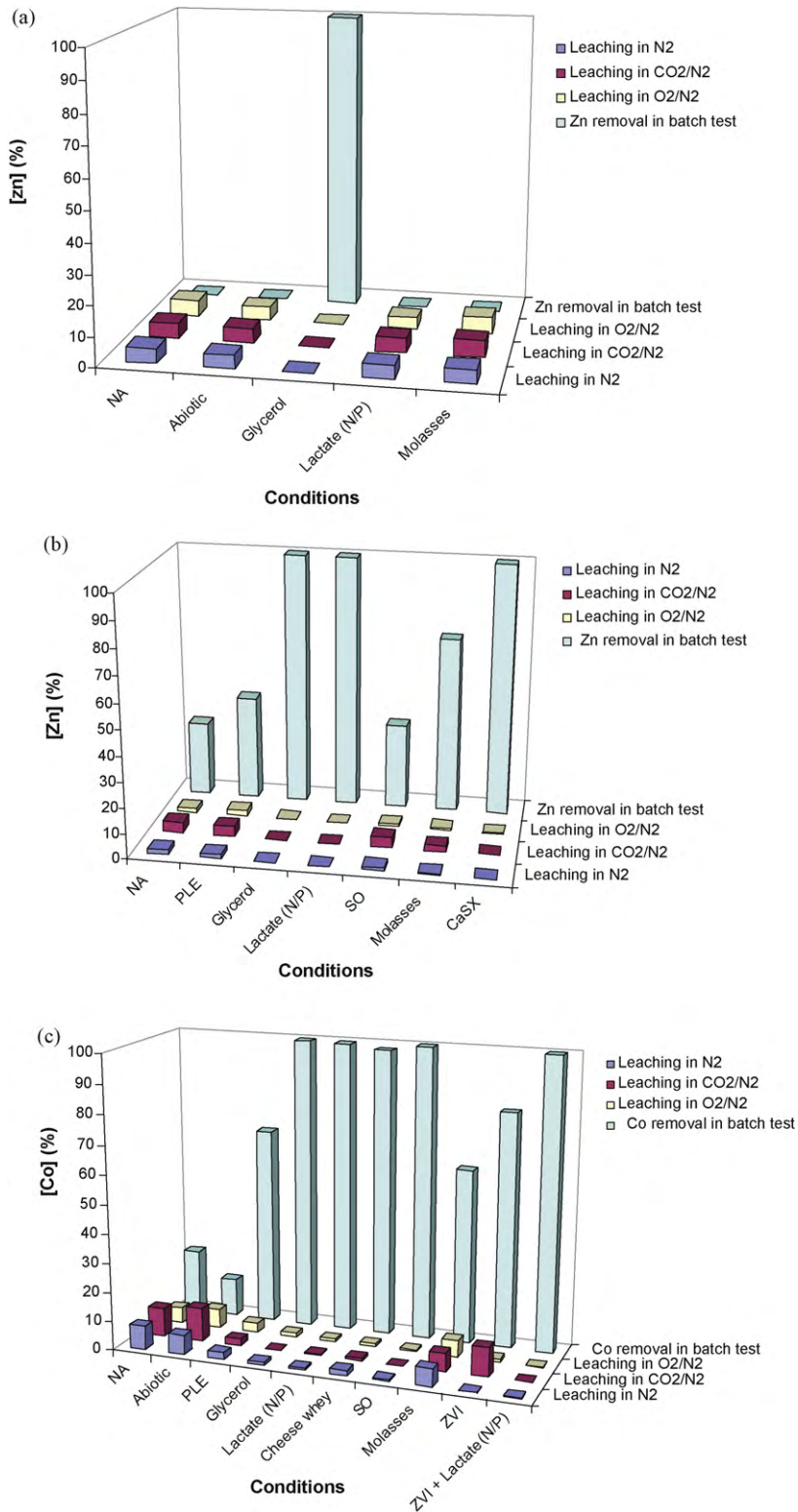
It is generally agreed that laboratory studies with artificially spiked elements are not always representative for 'natural' conditions and investigations in natural systems are indeed required. In addition, artificial systems are spiked with metals which are added in an ionic form and may react to form discrete sulphide phases. In natural contaminated sediments, absolutely pure metal sulphides may not be formed, even when elevated sulphide concentrations are present. They can also be incorporated as trace elements in Fe-monosulphides and pyrite [20]. Therefore, this study was further extended to natural aquifer material from three metal contaminated sites.

The major characteristics of aquifer material and groundwater from three different sites are presented in the Supplementary Table 1. The ISBP activity results of NAG have been summarised in Supplementary Table 2 and reported previously in detail [10]. It was concluded that for site 1 glycerol was the appropriate carbon source as it resulted in 96–100% Zn removal. For site 2 both glycerol and lactate (N/P) were suitable electron donors and resulted in 100% Zn removal. Molasses and calcium polysulphide ( $\text{CaS}_x$ ) also resulted in significant Zn removal. In case of Co (site 3), though most of applied carbon sources responded well, glycerol, lactate (N/P), cheese whey and soy oil appeared to be the most appropriate sources, resulting in 97–100% Co removal. In the following sections results of stability tests conducted by various methods with aquifer and groundwater obtained from previously performed ISBP tests for these three sites will be reported.

#### 3.2.1. Wet laboratory stability tests

**3.2.1.1. Aquifer redox treatment.** The changes in physico-chemical conditions (especially redox potential) are often associated with short-term changes in the speciation of heavy metals, which can make them more available for uptake by organisms and for leaching [20]. The effect of aquifer redox treatment (aerobic, anoxic and anaerobic) in NAG matrices is presented in Fig. 1a–c. Fig. 1a shows the effect of the aquifer redox treatment on Zn concentration for aquifer material of site 1. It is evident that up to 5% Zn leached at various redox conditions (aerobic, anoxic and anaerobic) in case of control (NA and abiotic), lactate (N/P) and molasses as carbon source whereas no Zn leached in case of glycerol. Fig. 1a also shows





**Fig. 1.** Percentage metal (Zn for sites 1 and 2 and Co for site 3) leached into liquid phase during aquifer redox treatment (a) site 1; (b) site 2; (c) site 3. On the y axis, the metal (%) leached under aerobic (N<sub>2</sub>/O<sub>2</sub>), anoxic (N<sub>2</sub>/CO<sub>2</sub>) and anaerobic (N<sub>2</sub>) atmosphere is represented (NA, natural attenuation; PLE, polylactate ester; SO, soy oil; ZVI, zero-valent iron; CaS<sub>x</sub>, calcium polysulphide).

that 100% Zn was removed (precipitated) in batch ISBP tests with glycerol as opposed to other conditions where no Zn was removed after ISBP. Therefore, the leached Zn in conditions other than glycerol comes from aquifer material which contained 41 mg kg<sup>-1</sup>-dm (dry matter) Zn [10]. The results of site 2 (Fig. 1b) show that Zn did not leach into the liquid phase with the two carbon sources, gly-

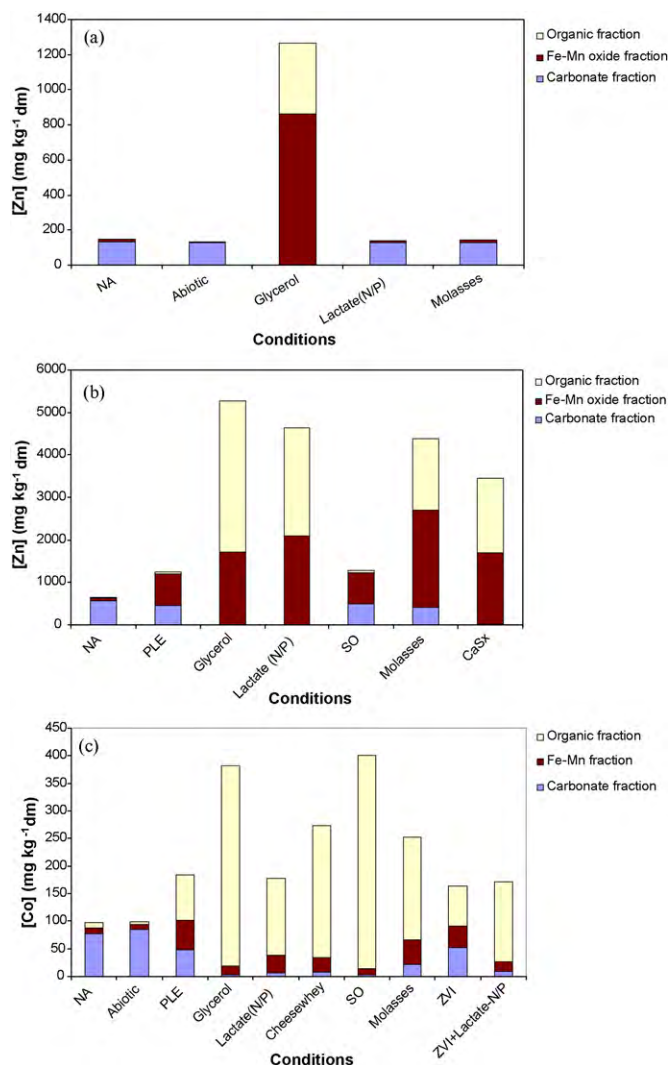
cerol and lactate (N/P) and the chemical polysulphide (CaS<sub>x</sub>). Also, as shown in Fig. 1b, in these conditions, 100% Zn was removed in batch ISBP tests [10]. However in NA, PLE, soy oil and molasses some amount of Zn (up to 4%) was leached under anoxic conditions. The results of redox treatment method for site 3 (Fig. 1c) revealed that Co did not leach from the solid phase (precipitate,

aquifer material) into the liquid phase (groundwater) with glycerol, lactate (N/P), cheese whey and soy oil under any redox condition. In NA and abiotic condition and the conditions with PLE and molasses, Co was leached into the liquid phase under all the redox conditions. In the condition with zero-valent iron without substrate (electron donor), Co was leached under anoxic condition ( $N_2/CO_2$ ) but not under anaerobic conditions ( $N_2$ ) and aerobic conditions ( $N_2/O_2$ ).

In a previous study conducted by Al-Abed et al. [21], the Zn present in mineral processing waste did not leach under changing redox conditions. Similar observations were reported by Carbonell-Barrachina et al. [22] where arsenic (As) solubility in sewage sludge amended soil was found static under highly aerobic and anaerobic conditions and it was attributed to arsenite precipitation by sulphide.

**3.2.1.2. Sequential extraction.** Sequential extraction was conducted in order to determine the distribution of metals in different chemical forms. Despite the limitations in terms of selectiveness of various extractants, operational factors etc., sequential extraction still provides useful information regarding the forms in which metal exists and thus indicates the mobility and the potential environmental risks [23]. In this study, the metals were separated into three operationally defined fractions namely, carbonate fraction (anaerobic), Fe–Mn oxide fraction (reducible) and organic/sulphide fraction as mentioned in Section 2.2.1.

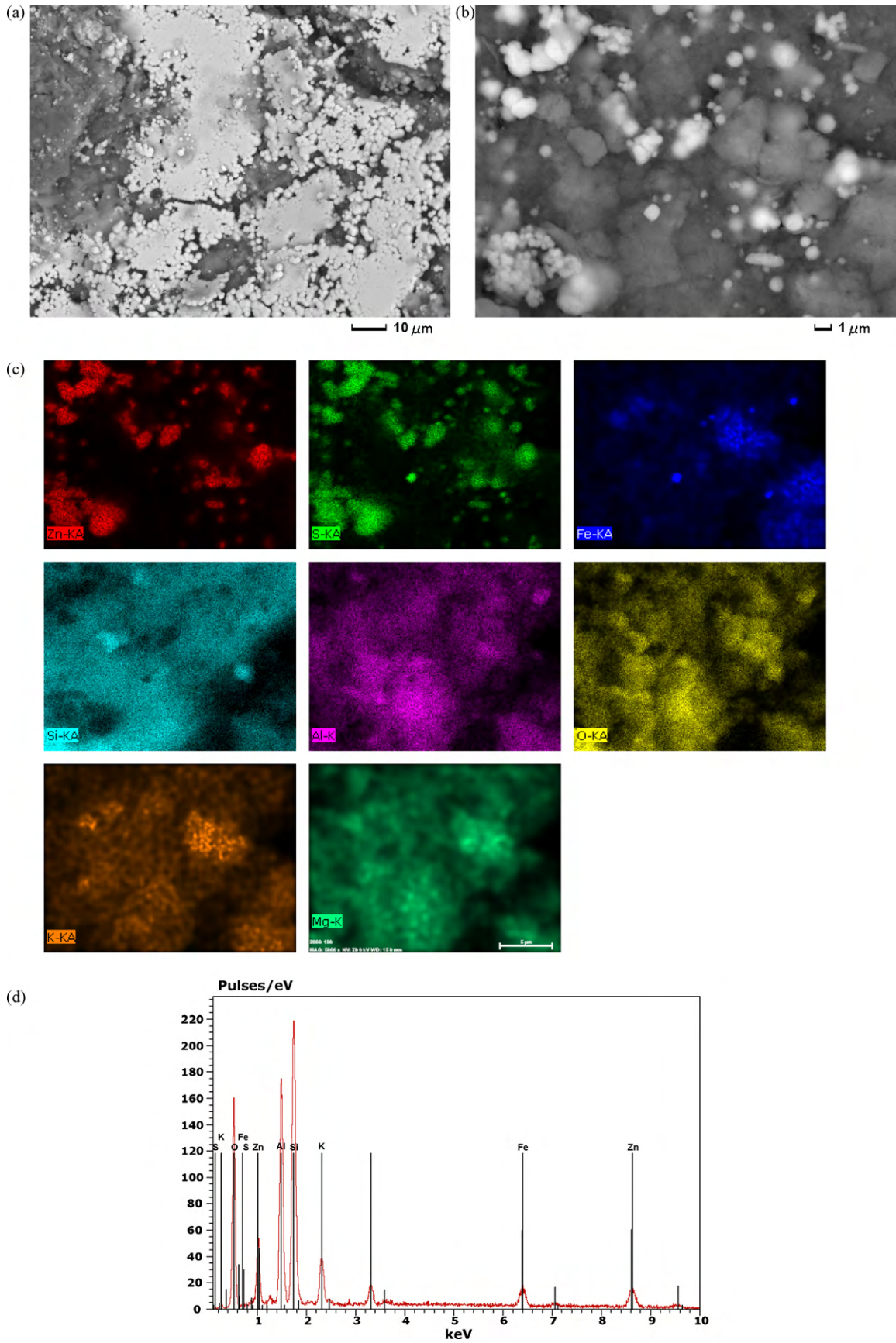
The sequential extraction results of aquifer material and groundwater for site 1 are shown in Fig. 2a. Both controls, i.e., NA and abiotic control, as well as the conditions with molasses and lactate (N/P) as carbon source showed that approximately 90% of the Zn was recovered in the pH dependent carbonate fraction. On the other hand, in case of glycerol as carbon source, most of the Zn occurred in the reducible Fe–Mn fraction (68%) and more stable organic/sulphide fraction (31%). The metal precipitates formed with application of glycerol were therefore most stable and these results corroborate well to those obtained by aquifer redox treatment where the precipitated Zn did not leach out with changing redox conditions (in case of glycerol) (Fig. 1a). Glycerol is in fact considered a good carbon source for sulphide production and metal precipitation by SRB [24]. Further, it is evident from Fig. 2b that in site 2 approximately 90% Zn in control condition (NA) and 35–40% Zn in case of PLE and soy oil appears in carbonate fraction. Fig. 2b also revealed that almost no Zn was found in carbonate fraction with glycerol and lactate (N/P) as carbon source. In case of both these carbon sources 35–45% Zn was present in Fe–Mn oxide fraction and 55–65% in organic/sulphide phase indicating that the precipitates formed in case of glycerol as well as lactate (N/P) application were most stable. The addition of  $CaS_x$  (the chemical agent) also resulted in relatively stable Zn precipitation with almost 50% Zn recovery in Fe–Mn oxide fraction and organic/sulphide fraction each. It is also important to note that the initial Zn fraction, i.e., the carbonate fraction, in the NA was converted to more stable fractions when glycerol and lactate (N/P) were used as carbon source. These results also corroborated well to the data obtained from aquifer redox treatment (Fig. 1b). The solubility of Zn remained low with changing redox conditions, because even the Zn associated with Fe–Mn fraction, would have again precipitated as sulphides after the dissolution of Fe–Mn oxides due to the presence of sulphate and suitable carbon source. Theoretically, Fe–Mn oxide fraction represents the content of metals bound to iron and manganese oxides and the characteristics of the sites 1 and 2 (Supplementary Table 1) indicate the presence of both Fe and Mn in the soil. The association of Zn with Fe–Mn oxide fraction is usually attributed to high stability constants of Zinc oxides. Zn associated with Fe–Mn fraction has widely been reported in soils [25], sludge originating from bioreactor treating acid mine drainage [23] and solids removed from permeable reactive barrier [26]. Moreover, Fe–Mn oxides are con-



**Fig. 2.** Metal concentration (Zn for sites 1 and 2 and Co for site 3) in various fractions as determined by sequential extraction procedure (a) site 1; (b) site 2; (c) site 3. Metal sequentially extracted in  $mg L^{-1}$  by different solvents is recalculated to  $mg kg^{-1} \cdot dm$ . (NA, natural attenuation; PLE, polylactate ester; SO, soy oil; ZVI, zero-valent iron;  $CaS_x$ , calcium polysulphide.)

sidered excellent metal scavengers especially for Zn, Cu and Mn [27]. Possibly, the increase in pH (from 3.6 to 6.5 after ISBP) and decrease in redox potential (from  $>300$  to  $-400$  mV) created by applying appropriate carbon source [10] facilitated the association of Zn with Fe–Mn oxide fraction. However, it has also been reported by Ngiam and Lim [28] that despite the maintenance of oxygen-free environment during the extraction of Fe–Mn oxide fraction with  $NH_2OH \cdot HCl$  during sequential extraction, oxidation of samples may take place. This may result in an over-representation of reducible fraction and an under-representation of organic/sulphide fraction in heavy metal speciation patterns. Therefore, the amount associated with Fe–Mn fraction in these results could also have originated from the more stable sulphide fraction.

In case of site 3 the sequential extraction results (Fig. 2c) indicated that approximately 80% Co was present in carbonate fraction in NA and abiotic conditions. However, in the presence of carbon source most of the Co was shifted to organic/sulphide fraction except in case of ZVI as electron donor and PLE as carbon source. Moreover, for these 'successful conditions' the initial dominant fraction, i.e., the carbonate fraction, as observed in the NA condition and abiotic control, was almost not detected anymore. The highest



**Fig. 3.** Microscopic study by SEM–EDX of the precipitate crystals formed during ISBP in batch experiments (lactate (N/P) as carbon source) for site 2; (a and b) the spheroidal structure of biogenic metal sulphide (ZnS); (c) the mapping of the image “b”; (d) the elemental composition of precipitates as analyzed by EDX.



**Table 2**  
Bioavailable Zn as measured by BIOMET<sup>®</sup> assay in precipitates of sites 1 and 2.

Conditions	Bioavailable Zn (mg kg <sup>-1</sup> -dm)	
	Site 1	Site 2
NA	164	492
Abiotic	126	n.a.
PLE	n.a.	1191
Glycerol	30	18
Lactate (N/P)	245	5
Soy oil	n.a.	408
Molasses	678	1100
CaS <sub>x</sub>	n.a.	20

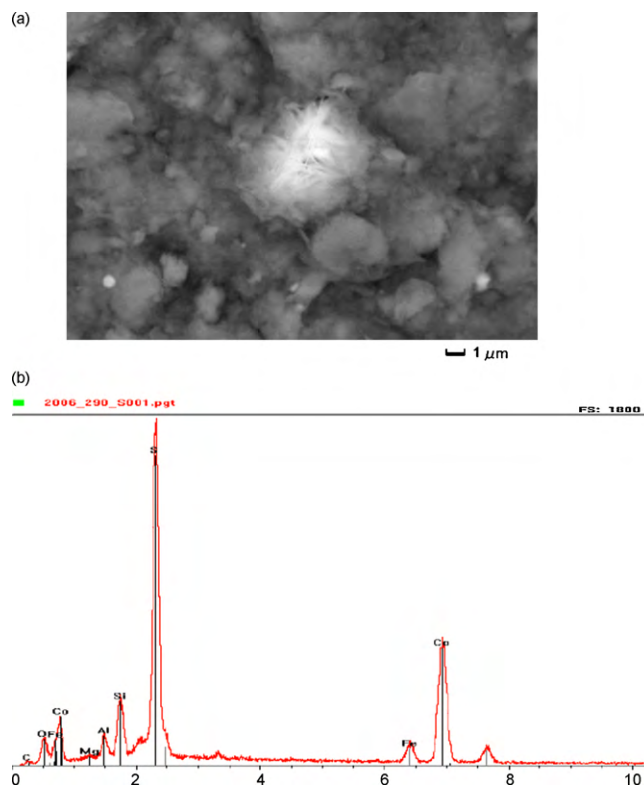
n.a., not applicable; PLE, polylactate ester; SO, soy oil; CaS<sub>x</sub>, calcium polysulphide; dm, dry matter.

affinity of organic matter/sulphides fraction for Co has been previously reported in anaerobic sludge of Upflow Anaerobic Sludge Bed reactor treating paper mill wastewater and alcohol distillery wastewater [29,30]. It is essential to mention here that in all the results of sequential extraction (Zn for sites 1 and 2 and Co for site 3), the total Zn or Co extracted is higher in the conditions of most appropriate (effective) carbon sources as compared to the other ineffective (or no carbon source) conditions. This is because most of the Zn and Co (present in liquid phase) was precipitated (into solid phase) in these conditions, therefore, the total metal extracted during sequential extraction in these conditions was high.

**3.2.1.3. BIOMET<sup>®</sup> assay.** BIOMET<sup>®</sup> assay results for sites 1 and 2 are presented in Table 2. For site 3 we could not interpret the signals very well due to interference by Ni present in the sample (8 mg kg<sup>-1</sup>-dm in aquifer material and 0.54 mg L<sup>-1</sup> in groundwater). Based upon BIOMET<sup>®</sup> assay, low bioavailable Zn was observed in case of glycerol as carbon source for site 1 and for both glycerol and lactate (N/P) in site 2. The bioavailable Zn is often correlated to the exchangeable fraction [31,32]. In this study we do not have an exact value for exchangeable fraction because the sequential extraction was conducted with an aim to investigate the stability. Therefore, only three fractions carbonate, Fe–Mn oxide and organic/sulphide fractions were extracted since the initial two fractions (water soluble and exchangeable fraction) are not stable. Further, extraction of more fractions could increase error between various fractions. Therefore, the carbonate fraction reported in Fig. 2a–c does not differentiate between exchangeably bound metals (extracted with light salts such as MgCl<sub>2</sub>, Ca (NO<sub>3</sub>)<sub>2</sub>) and acid extractable fraction. However, the sequential extraction of aquifer material of both the sites indicated an almost negligible carbonate fraction in case of glycerol for site 1 and glycerol and lactate (N/P) in site 2. This corroborated well with low bioavailability (Table 2) in both cases indicating significant stability of the metal precipitates.

### 3.2.2. Scanning electron microscopic analysis

Elemental analysis by SEM-EDX was conducted to confirm the mechanism of metal removal. Here we present the results for the condition lactate (N/P) as carbon source of sites 2 and 3. Fig. 3 presents the SEM-images and EDX-spectra for site 2 (lactate (N/P) as carbon source). It is visible from Fig. 3a and b that metal sulphide precipitates have mainly a spheroidal structure. It has been reported that in case of biogenic sulphides the bacterial cell walls and other biogenic materials are encrusted and fossilized by inter grown spheroids. The integration of iron supports the formation of framboidal or proto-framboidal structures [33,34]. This was confirmed by the EDX analysis (Fig. 3d), which shows the presence of zinc, iron and sulphur. Further, the EDX-mapping (Fig. 3c) also confirmed that the white precipitates (shown in Fig. 3b) indeed contain zinc, sulphur and iron. As a consequence of sulphide deposition, the formed solids on the cell wall may hinder the bacterial metabolism.



**Fig. 4.** Microscopic study by EDX-SEM of the precipitate crystals formed during ISBP in batch experiments (lactate (N/P) as carbon source) for site 3; (a) the SEM-image of the precipitate; (b) the elemental composition of precipitate as analyzed by SEM-EDX.

However, such inhibition was not observed in our system which performed well up to a 10-month period. This indicates that the inactivation of bacterial cells by metal sulphide precipitates was overcome by the regeneration capacity of the bacterial population. This phenomenon was also reported by Remoundaki et al. [35] in the biofilm of sulphate reducing fixed bed reactor.

Fig. 4a and b represents the SEM-image and the EDX-analyses of cobalt sulphide precipitates from site 3, where lactate (N/P) was used as a carbon source. The precipitate can clearly be seen in the SEM-image. EDX analysis of the precipitates surface shows the presence of Co and S as the main components, which suggests that Co has been precipitated as sulphide in crystalline form. The precipitate measured 3–4 μm, lying within the range of 2–10 μm, reported by Neculita et al. [36] for Zn, Cd, Cu and Ni sulphides formed in column bioreactors treating acid mine drainage. Normally the size of precipitates depends upon the optimal stoichiometric ratio of metal and sulphide, which is difficult to control in *in situ* treatment methods [37].

## 4. Conclusions

Based upon the research approach and methodologies applied in this work, it can be concluded that in artificial matrix (ASG), the manipulations in redox conditions did not affect the metal precipitates significantly, however, the sequential pH manipulation was detrimental. Further investigations on effect of parallel pH manipulations are required. Nevertheless while applying ISBP, this limitation should be taken into account that adsorbed or non-sulphide precipitates might come back to solution in case the surrounding environments are exposed to strong pH variations. It is noteworthy that the experimental duration (of changing redox condition or pH manipulation) is one of the most important oper-



ational parameters depending upon the solution and solid phase matrix. This implies the need of standardizing various methodologies applied to investigate the stability. Application of appropriate carbon source in the natural (NSG) matrices resulted in metal precipitates that were stable with respect to redox variations. Moreover, the bioavailability of metals also decreased after ISBP in the presence of suitable carbon source indicating the stability of metal precipitates. The elemental analysis showed the presence of metal and sulphur in the precipitates confirming that the metals were indeed precipitated as sulphides.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2010.04.119.

### References

- [1] L. Diels, K. Vanbroekhoven, Remediation of metal and metalloid contaminated groundwater, in: M.D. Annable, M. Teodorescu, P. Hlavinek, L. Diels (Eds.), *Methods and Techniques for Cleaning-up Contaminated Sites*, Springer, The Netherlands, 2008, pp. 1–23.
- [2] Z. Djedidi, J.B. Khaled, R.B. Cheikh, J.F. Blais, G. Mercier, R.D. Tyagi, Comparative study of dewatering characteristics of metal precipitates generated during treatment of monometallic solutions, *Hydrometallurgy* 95 (2009) 61–69.
- [3] C.N. Mulligan, R.N. Yong, B.F. Gibbs, Remediation technologies for metal-contaminated soils and groundwater: an evaluation, *Eng. Geol.* 60 (2001) 193–207.
- [4] L. Diels, N. van der Lelie, L. Bastiaens, New developments in treatment of heavy metal contaminated soils, *Rev. Environ. Sci. Biotechnol.* 1 (2002) 75–82.
- [5] P. Kelderman, A.A. Osman, Effect of redox potential on heavy metal binding forms in polluted canal sediments in Delft (The Netherlands), *Water Res.* 41 (2007) 4251–4261.
- [6] Y. Hashimoto, H. Matsufuru, T. Sato, Attenuation of lead leachability in shooting range soils using poultry waste amendments in combination with indigenous plant species, *Chemosphere* 73 (2008) 643–649.
- [7] J. Kumpiene, I.C. Montesinos, A. Lagerkvist, C. Maurice, Evaluation of the critical factors controlling stability of chromium, copper, arsenic and zinc in iron-treated soil, *Chemosphere* 67 (2007) 410–417.
- [8] M. Nachttegaal, M.A. Marcus, J.E. Sonke, J. Vangronsveld, K.J.T. Livi, D. Van der Lelie, D.L. Sparks, Effects of in situ remediation on the speciation and bioavailability of zinc in a smelter contaminated soil, *Geochim. Cosmochim. Acta* 69 (2005) 4649–4664.
- [9] E. Schols, R. Swennen, E. Smolders, L. Diels, B. Laenen, K. Vanbroekhoven, Stability of metal precipitation formed during immobilization of metals from polluted groundwater by means of in situ precipitation, in: *Proceedings UFZ/TNO International Conference on Soil–Water Systems*, Milan, Italy, June 3–6, 2008.
- [10] K. Vanbroekhoven, S. Van Roy, L. Diels, J. Gemoets, P. Verkaeren, L. Zeuwts, K. Feyaerts, F. van den Broeck, Sustainable approach for the immobilization of metals in the saturated zone: *in situ* bioprecipitation, *Hydrometallurgy* 94 (2008) 110–115.
- [11] A. Tessier, P. Campbell, M. Bisson, Sequential extraction procedure for the speciation of particulate trace metals, *Anal. Chem.* 51 (1979) 844–851.
- [12] D. van der Lelie, L. Verschaeve, L. Regniers, P. Corbisier, Use of bacterial tests (the VITOTOX genotoxicity test and the BIOMET heavy metal test) to analyze chemicals and environmental samples, in: G. Persoone (Ed.), *Microbiotests for Routine Toxicity Testing and Biomonitoring*, Kluwer Academic/Plenum Publishers, Amsterdam, The Netherlands, 1999, pp. 197–208.
- [13] P. Corbisier, E. Thiry, L. Diels, Bacterial biosensors for the toxicity assessment of solid waste, *Environ. Toxicol. Water Qual.* 11 (1996) 171–177.
- [14] P. Corbisier, D. van der Lelie, B. Borremans, A. Provoost, V. de Lorenzo, N. Brown, J. Lloyd, J. Hobman, E. Csöregi, G. Johansson, B. Mattiasson, Whole cell- and protein-based biosensors for the detection of bioavailable heavy metals in environmental samples, *Anal. Chim. Acta* 387 (1999) 235–244.
- [15] K. Vanbroekhoven, A. Bossus, B. Borremans, L. Diels, Determination of Bioavailable Concentrations of Mercury using the BIOMET® Bioassay at an Industrially Contaminated Site, *Senspol*, Cranfield University, UK, 2004.
- [16] K. Vanbroekhoven, S. Van Roy, C. Gielen, M. Maesen, A. Ryngaert, L. Diels, P. Seuntjens, Microbial processes as key drivers for metal (im)mobilization along a redox gradient in the saturated zone, *Environ. Pollut.* 148 (2007) 759–769.
- [17] S. Carroll, P.A. O'Day, B. Esser, S. Randall, Speciation and fate of trace metals in estuarine sediments under reduced and oxidized conditions, *Seaplane Lagoon, Alameda Naval Air Station (USA)*, *Geochem. Trans.* 3 (2002) 81–101.
- [18] K. Sukola, F. Wang, A. Tessier, Metal-sulfide species on oxic waters, *Anal. Chim. Acta* 528 (2005) 183–195.
- [19] S. Van Herreweghe, R. Swennen, V. Cappuyns, C. Vandecasteele, Chemical associations of heavy metals and metalloids in contaminated soils near former ore treatment plants: a differentiated approach with emphasis on pH<sub>stat</sub>-leaching, *J. Geochem. Explor.* 76 (2002) 113–138.
- [20] V. Cappuyns, R. Swennen, Kinetics of element release during combined oxidation and pH<sub>stat</sub> leaching of anoxic river sediments, *Appl. Geochem.* 20 (2005) 1169–1179.
- [21] S.R. Al-Abed, P.L. Hageman, G. Jegadeesan, N. Madhavan, D. Allen, Comparative evaluation of short-term leach tests for heavy metal release from mineral processing waste, *Sci. Total Environ.* 364 (2006) 14–23.
- [22] A. Carbonell-Barrachina, A. Jugsujinda, R.D. DeLaune, W.H. Patrick Jr., The influence of redox chemistry and pH on chemically active forms of arsenic in sewage sludge-amended soil, *Environ. Int.* 25 (1999) 613–618.
- [23] T. Jong, D.L. Parry, Heavy metal speciation in solid-phase materials from a bacterial sulfate reducing bioreactor using sequential extraction procedure combined with acid volatile sulfide analysis, *J. Environ. Monitor.* 6 (2004) 278–285.
- [24] J.S. Webb, S. McGinness, H.M. Lappin-Scott, Metal removal by sulphate-reducing bacteria from natural and constructed wetlands, *J. Appl. Microbiol.* 84 (1998) 240–248.
- [25] L.Q. Ma, G.N. Rao, Chemical fractionation of cadmium, copper, nickel and zinc in contaminated soils, *J. Environ. Qual.* 26 (1997) 259–264.
- [26] O. Gibert, J. de Pablo, J.L. Cortina, C. Ayora, Evaluation of municipal compost/limestone/iron mixtures as filling material for permeable reactive barriers for *in situ* acid mine drainage treatment, *J. Chem. Technol. Biotechnol.* 78 (2003) 489–496.
- [27] K.A. Yusuf, Sequential extraction of lead, copper, cadmium and zinc in soils near Ojota waste site, *J. Agron.* 6 (2007) 331–337.
- [28] L.-S. Ngiam, P.-E. Lim, Speciation patterns of heavy metals in tropical estuarine anoxic and oxidized sediments by different sequential extraction schemes, *Sci. Total Environ.* 275 (2001) 53–61.
- [29] E.D. van Hullebusch, A. Peerbolte, M.H. Zandvoort, P.N.L. Lens, Sorption of cobalt and nickel on anaerobic granular sludges: isotherms and sequential extraction, *Chemosphere* 58 (2005) 493–505.
- [30] S.I.C. Lopes, M.I. Capela, E.D. van Hullebusch, A. van der Veen, P.N.L. Lens, Influence of low pH (6, 5 and 4) on nutrient dynamics and characteristics of acidifying sulfate reducing granular sludge, *Process Biochem.* 43 (2008) 1227–1238.
- [31] C. Tibazarwa, P. Corbisier, M. Mench, A. Bossus, P. Solda, M. Mergeay, L. Wyns, D. van der Lelie, A microbial biosensor to predict bioavailable nickel in soil and its transfer to plants, *Environ. Pollut.* 113 (2001) 19–26.
- [32] W. Geebelen, D.C. Adriano, D. van der Lelie, M. Mench, R. Carleer, H. Clijsters, J. Vangronsveld, Selected bioavailability assays to test the efficacy of amendment-induced immobilization of lead in soils, *Plant Soil* 249 (2003) 217–228.
- [33] D.J. Large, N.J. Fortey, A.E. Milodowski, A.G. Christy, J. Dodd, Petrographic observations of iron, copper, and zinc sulfides in freshwater canal sediment, *J. Sediment. Res.* 71 (2001) 61–69.
- [34] J.W. Moreau, R.I. Webb, J.F. Banfield, Ultrastructure, aggregation-state, and crystal growth of biogenic nanocrystalline sphalerite and wurtzite, *Am. Miner.* 89 (2004) 950–960.
- [35] E. Remoundaki, P. Kousi, C. Joulain, F. Battaglia-Brunet, A. Hatzikioseyan, M. Tsezos, Characterization, morphology and composition of biofilm and precipitates from a sulphate-reducing fixed-bed reactor, *J. Hazard. Mater.* 153 (2008) 514–524.
- [36] C.M. Neculita, G.J. Zagury, B. Bussièrè, Effectiveness of sulfate-reducing passive bioreactors for treating highly contaminated acid mine drainage: II. Metal removal mechanisms and potential mobility, *Appl. Geochem.* 23 (2008) 3545–3560.
- [37] A.H.M. Veeken, L. Akoto, L.W.H. Pol, J. Weijma, Control of the sulfide (S<sub>2</sub>) concentration for optimal zinc removal by sulfide precipitation in a continuously stirred tank reactor, *Water Res.* 37 (2003) 3709–3717.