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Toxic effects of dissolved heavy metals on *Desulfovibrio vulgaris* and *Desulfovibrio* sp. strains

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Abstract

Biological treatment of metal-containing wastewaters with sulphate-reducing bacteria (SRB) is an attractive technique for the bioremediation of this kind of medium. In order to design a suitable engineering process to address this environmental problem, it is crucial to understand the inhibitory effect of dissolved heavy metals on these bacteria. Batch studies were carried out to evaluate the toxic effects of several heavy metal ions [Cr(III), Cu(II), Mn(II), Ni(II) and Zn(II)] on two cultures of SRB (*Desulfovibrio vulgaris* and *Desulfovibrio* sp.). The experimental data indicate that SRB show different responses to each metal. At the highest metal concentration tolerated for each metal, the precipitation levels for *D. vulgaris* were as follows: 24.7%-15 ppm Cr(III), 45%-4 ppm Cu(II), 60%-10 ppm Mn(II), 96%-8.5 ppm Ni(II) and 9%-20 ppm Zn(II). The corresponding values for *Desulfovibrio* sp. were: 25.5%-15 ppm Cr(III), 71%-4 ppm Cu(II), 66.2%-10 ppm Mn(II), 96.1%-8.5 ppm Ni(II) and 93%-20 ppm Zn(II). Results obtained in batch studies will be taken into account for the subsequent design of a sulphate-reducing bioreactor to reduce levels of heavy metals present in different types of contaminated media.

Keywords: Sulphate-reducing bacteria; *Desulfovibrio vulgaris*; *Desulfovibrio* sp.; Heavy metals; Tolerance; Bioprecipitation

1. Introduction

The presence of heavy metals in the environment represents a serious threat to the environment and human life. Current and past mining activity, as well as various industrial discharges, have contributed large quantities of acid wastewaters to the environment [1]. These waste streams usually contain high levels of sulphate and dissolved metals. The most widely used method to treat such effluent is chemical neutralization followed by the precipitation of metals. This method is expensive and generates large amounts of residual sludge. Biological treatment of these acidic and metal-containing wastewaters is an attractive alternative. The main advantage of these systems over chemical neutralization is that large volumes of sludge are not generated and the metal precipitates in the form of insoluble compounds such as oxides or sulphides. Among the biological treatment methods, the selective precipitation of metals with biologically produced H_2S has been proposed as a possible process [2,3].

Sulphate-reducing bacteria (SRB) are heterotrophic microorganisms that require strictly anaerobic conditions and a redox potential of less than −200 mV. The main organic carbon/energy substrates utilized by the fastest growing organisms (*Desulfovibrio* species) are low molecular mass organic acids, such as lactic or acetic acid, and alcohols, such as ethanol [4,5]. The pattern of carbon dissimilation is essentially the same in all cases in that the organic substrate is oxidized either completely to CO_2 or to some intermediate compound [6]. Under anaerobic conditions, SRB carry out the oxidation of simple organic compounds by using sulphate as a terminal electron acceptor—the sulphate is reduced to sulphide. The generation of sulphide produces reducing conditions, removal of acidity and the precipitation of metals from solution as sulphides. This property makes these bacteria suitable for the removal of acidity and metals from contaminated effluents [7]. The method consists of two stages: (1) the production of H_2S by SRB and (2) the precipitation of metals by the biologically produced H_2S, a reaction that produces insoluble metal sulphides that can be easily separated from a

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solution [2,7,8].

$$\underset{(e^- \text{ donor})}{\text{Organic matter}} + \underset{(e^- \text{ acceptor})}{SO_4^{2-}} \rightarrow 2\,CH_3COO^- + HS^- + HCO_3^- \quad (1)$$

$$Me^{2+} + HS^- \rightarrow MeS \downarrow + H^+ \quad (2)$$

Numerous heavy metals are toxic to microorganisms – including SRB – due to their capacity to deactivate enzymes by reacting with their functional groups, to denature proteins, and to compete with essential cations. The ability of this group of bacteria to immobilize heavy metals depends on the concentration of the metal in solution, which can cause a reduction in the metabolic activity or can even be toxic to the bacteria (causing death). This fact led to several studies that focused on determining the inhibitory effect of heavy metals on different cultures of sulphate-reducing bacteria with the aim of applying these microorganisms in metal reduction treatment processes [9,10]. The reported toxic concentrations of heavy metals to sulphate-reducing bacteria range from a few ppm (mg/L) to as much as 100 ppm [11]. Hao et al. [12] studied the toxic concentrations of several heavy metals for a mixed culture of sulphate-reducing bacteria: Zn (25–40 ppm), Pb (75–80 ppm), Cu (4–20 ppm), Cd (>4–20 ppm), Ni (10–20 ppm) and Cr (60 ppm). Utgikar et al. [11] reported that the effect of heavy metals on SRB can be stimulatory at lower concentrations and toxic/inhibitory at higher concentrations.

In recent years, several studies have evaluated the precipitation of heavy metals in real wastes (mine waste piles, acid mine drainage) by sulphate-reducing bacteria in batch and continuous systems. Kim et al. [7] described batch and column studies that were conducted to evaluate the feasibility of inoculating mine waste piles with SRB in order to neutralize the acidic supernatant and decrease the heavy metal levels. Batch incubation led to a decrease in the dissolved concentration of Cd, Cu, Ni and Zn in the supernatant to undetectable levels. Furthermore, continuous flow column experiments gave metal removal efficiencies greater than 99% for Cd, Cu and Zn and 87% for Ni. Foucher et al. [2] proposed a process that used SRB to treat acid mine drainage on the laboratory pilot scale. In this system, a fixed-bed bioreactor was used in conjunction with a gas-stripping column. Cu and Zn could be selectively recovered at pH 2.8 and 3.5, respectively. Ni and Fe could also be removed at pH 6.0 by sulphide precipitation. Sulphate reduction and metal precipitation (as a sulphide) are significant aspects of some successful large-scale processes for the biotechnological removal of metals. In some cases, this process is combined with a prior metal solubilization step [5]. Bioleaching using sulphuric acid, produced by sulphur-oxidizing bacteria, was followed by the precipitation of leachate metals by sulphate-reducing bacteria [13].

The purpose of the work described here was to study the tolerance of two cultures of sulphate-reducing bacteria (*Desulfovibrio vulgaris* and *Desulfovibrio* sp.) that occur in mining environments [14] to several heavy metal ions [Cr(III), Cu(II), Mn(II), Ni(II) and Zn(II)]. These metallic ions were selected due to they are present in a real contaminated effluent from the zone. The study was carried out by following bacterial growth and sulphate uptake. Moreover, the ability of SRB to precipitate these heavy metals in an artificially contaminated solution was evaluated by measuring the decrease in the dissolved metal concentration. This work involved an initial batch study, which forms part of a wider research programme focused on the application of this process in continuous mode to remove or reduce heavy metals present in real contaminated effluent that occurs in the industrial zone around Cadiz.

2. Materials and methods

2.1. SRB cultures

The bacterial strains used in this study were *D. vulgaris* (ATCC 29579) and *Desulfovibrio* sp. (ATCC 49975). These cultures were maintained in modified Postgate B medium (9 mL) (Table 1) in 10 mL sealed glass bottles. Medium was sterilised before pour it into the bottles at 121 °C during 20 min and allowed to cool down to room temperature. Ten percent (v/v) of inoculum was then added to the Postgate B medium. Bottles were sealed immediately in order to give the anaerobic conditions that are promoted by reducing compounds (ascorbic acid, thioglycolic acid). SRB cultures were incubated at 30 °C for 24 h. The formation of ferrous sulphide, which was detected as a black precipitate, indicate that bacterial growth had taken place and the bottles were then stored at 4 °C.

2.2. Medium and cultivation conditions

Experiments with heavy metals were carried out using the modified nutrient Postgate C medium (Table 1), which contains a high sulphate concentration. This medium does not contain Fe(II) to allow the evaluation of the precipitation of other metal under investigation.

The medium was adjusted to pH 7.5 ± 0.5 and was placed into 50 mL Pyrex glass bottles. These vessels were capped with crimped aluminium butyl rubber stoppers and sterilised in an autoclave [15]. The bottles were allowed to cool down to room temperature and they were spiked with metal solutions that had previously been sterilised by membrane filtration (pore size 0.22 μm). Chromium, copper, manganese, nickel and zinc sulphate standard solutions were used to obtain several concen-

Table 1
Composition of Postgate B and Postgate C media (g/L) for maintenance and metal experiments of sulphate-reducing bacteria

g/L	Postgate B	Postgate C
KH_2PO_4	0.5	0.5
NH_4Cl	1.0	1.0
Na_2SO_4	–	4.5
$CaSO_4{\cdot}2H_2O$	1.26	–
$MgSO_4{\cdot}7H_2O$	2.0	0.06
Sodium lactate	3.5	6.0
Yeast extract	1.0	0.25
Ascorbic acid	0.1	–
Thioglycolic acid	0.1	–
$FeSO_4{\cdot}7H_2O$	0.5	–

trations of each ion: Cr(III) 1–15 ppm, Cu(II) 0.9–9 ppm, Mn(II) 1–10 ppm, Ni(II) 1–17 ppm and Zn(II) 5–20 ppm. At this point, 10% (v/v) of inoculum was added to each sample. Previously, cells were washed with fresh Postgate C medium to avoid the incorporation of sulphide to each experiment. After inoculation, the final pH of the complete medium was 7.00 ± 0.20 except in Cr (III) experiments where pH was around 6.66 ± 0.20. Oxygen was purged from the bottles with N_2 gas before and after inoculation in order to achieve anaerobic conditions.

Bottles that did not contain inoculum or metal were prepared as control samples. Experiments were conducted in triplicate and cultures were incubated statically at 30 °C for a maximum of 14 days and were sampled for analysis of sulphate uptake, metal concentration and biomass growth.

2.3. Analytical methods

Free bacterial population was determined by counting in a Neubauer chamber in conjuction with an optical microscope (Olympus BH-2). The results are reported as cell $\times 10^6$ mL^{-1} [15]. The sulphate concentration in the medium was determined by a turbidimetric method, samples were filtered (0.22 μm) to avoid the interference of biomass in the measurement [16]. The dissolved metal concentrations of samples were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Iris Intrepid—Thermo-elemental Series 11393, Model 14425501) [17]. Previously, samples were filtered to remove biomass and metal precipitates. Then they are acidified (HNO_3 2N) and stored at 4 °C until the measurement.

3. Results and discussion

The tolerance study for sulphate-reducing bacteria (*D. vulgaris* and *Desulfovibrio* sp.) involved sampling every 3–4 days during incubation and determination of bacterial growth, sulphate concentration and dissolved metal concentration for each sample. In general, it was found that bacterial growth is affected by the presence of the metal ions studied and that the lag phase of these cultures increases as the metal concentration increases. The behaviour observed in the presence of increasing concentrations of each metal was very similar and a representative example is shown in Fig. 1a and b for the evolution of bacterial population for *Desulfovibrio* species in the presence of Cu(II).

The metal concentration also influences the sulphate-reducing capacity of SRB. An increase in the metal concentration in solution led to a decrease in the sulphate reduction rate and this effect was accompanied by a low level of metal precipitation. A representative example is presented in Fig. 2a and b and again shows data for Cu(II).

Significant differences in bacterial growth and sulphate reduction were not observed between control cultures (*D. vulgaris* and *Desulfovibrio* sp.) but the addition of metal produced marked differences in the behaviour of the two strains.

As mentioned in the introduction, the ultimate aim of this work was to evaluate metal precipitation by *Desulfovibrio* strains and, for this reason, only data corresponding to precipitation

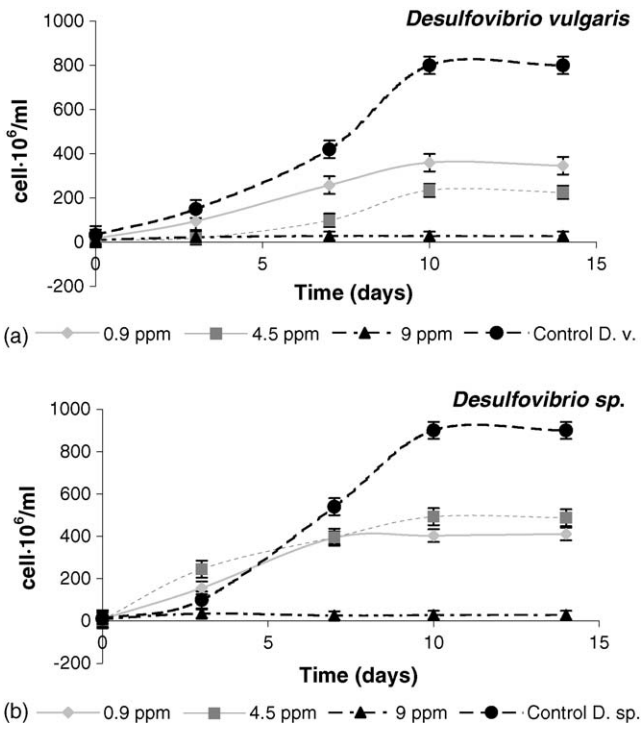

Fig. 1. Evolution of bacterial population in the presence of Cu(II) (0.9, 4, 9 ppm and control) for *Desulfovibrio vulgaris* (a) and *Desulfovibrio* sp. (b).

percentage versus time for a range of concentrations for each metal are presented.

Measurement of the dissolved metal concentration can serve as an indicator of the bioactivity of the SRB. In order to facilitate the discussion of the results and to evaluate the precipitation

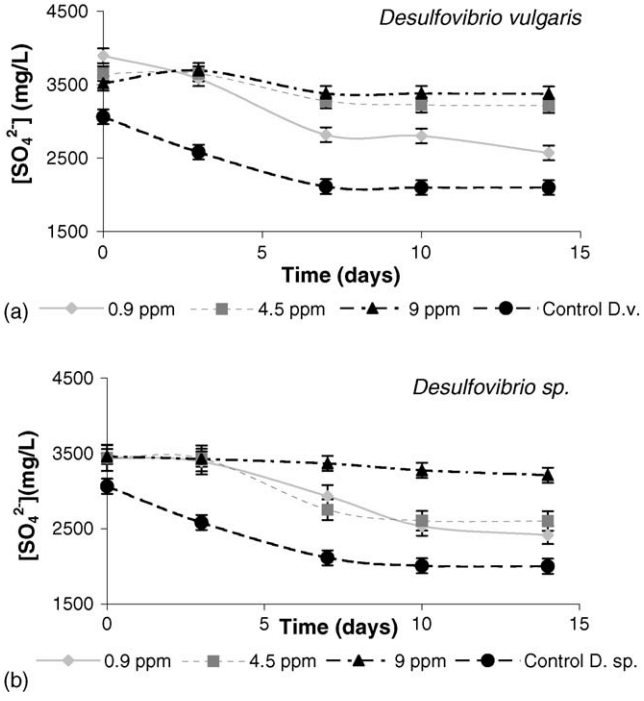

Fig. 2. Evolution of sulphate concentration in the presence of Cu(II) (0.9, 4, 9 ppm and control) for *Desulfovibrio vulgaris* (a) and *Desulfovibrio* sp. (b).

capacity of metal ions by the SRB under investigation, the bioprecipitation percentage (%BP) was defined as the percentage of precipitated metal with respect to the initial quantity of dissolved metal in the culture. This parameter was calculated using the following equation:

$$\%\mathrm{BP} = \frac{([M]_{t=0} - [M]_{t=t}) \times 100}{[M]_{t=0}} \quad (3)$$

where $[M]_{t=0}$ is the dissolved metal concentration at initial time, after inoculation and $[M]_{t=t}$ is the dissolved metal concentration at measure time.

Abiotic controls with each metallic ion were carried out to determinate the metal precipitation by non-biological mechanism, metal precipitation was minor than 1% in each case. These data has not been included in the figures due they are not noticeable.

3.1. Chromium(III)

The effects of Cr(III) ions on SRB cultures were assessed by supplementing the assays with $Cr_2(SO_4)_3$ to obtain concentrations of 1, 4.5, 9, 12 and 15 ppm Cr(III). This metal ion produces inhibition of sulphate-reducing bacteria and concentrations of Cr(III) above 1 ppm affect the bacterial growth, extend the lag phase from 2 to 4 days, and decrease the maximum growth ($400\,\mathrm{cell} \times 10^6\,\mathrm{mL}^{-1}$) in comparison to the control cultures. In addition, the metabolic activity is affected and leads to a low level of sulphate reduction. It can be seen from Fig. 3a and b that the bioprecipitation percentage increases with metal concentration. This observation can be explained in that an increase in metal concentration leads to a higher level of product formation in reaction (2), thus leading to precipitation of the metal as its sulphide. The precipitation level was very low even at the highest concentration of chromium tested (15 ppm), which led to 25% of precipitated chromium after 11 days of incubation for both strains. Concentrations of Cr(III) higher than 15 ppm were tested but they proved to be toxic for the bacteria and precipitation was not observed. A concentration of 15 ppm can therefore be considered the maximum concentration of chromium tolerated by these anaerobic cultures. This value is below the levels obtained by Hao et al. [12], who found a tolerance of 60 ppm for chromium. The difference in these values could be due to the fact that Hao et al. employed enriched SRB cultures from wastewater.

Sulphate-reducing bacteria showed the lowest precipitation levels for chromium sulphate of all the metal compounds studied.

3.2. Copper(II)

The changes in bacterial growth as a function of time for *D. vulgaris* and *Desulfovibrio* sp. with varying amounts of copper (0.9, 4 and 9 ppm) are shown in Fig. 1a and b. Bottles were supplemented with $CuSO_4$. A significant decrease in biomass population was observed on the addition of 0.9 ppm Cu(II), indicating that this concentration exerts a significant inhibitory effect on SRB cultures. It can also be seen that this inhibition becomes more significant as the dissolved metal concentration increases, and the assays with higher levels of copper reduce the ability of sulphate-reducing bacteria to precipitate the metal (Fig. 4a and b). This result is consistent with those obtained by Utgikar et al. [11], who reported that the copper concentrations

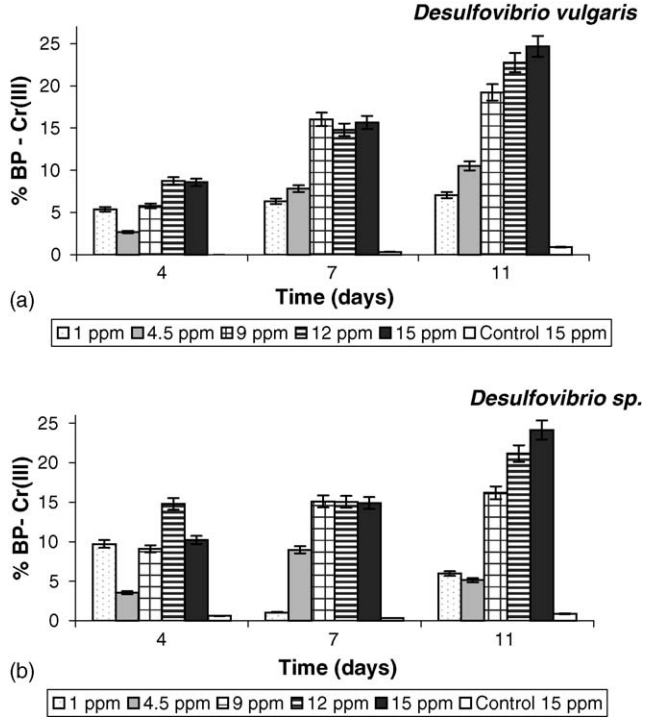

Fig. 3. Percentage of metal precipitation of Cr(III) (1, 4.5, 9, 12, 15 ppm and control) by *Desulfovibrio vulgaris* (a) *and Desulfovibrio* sp. (b).

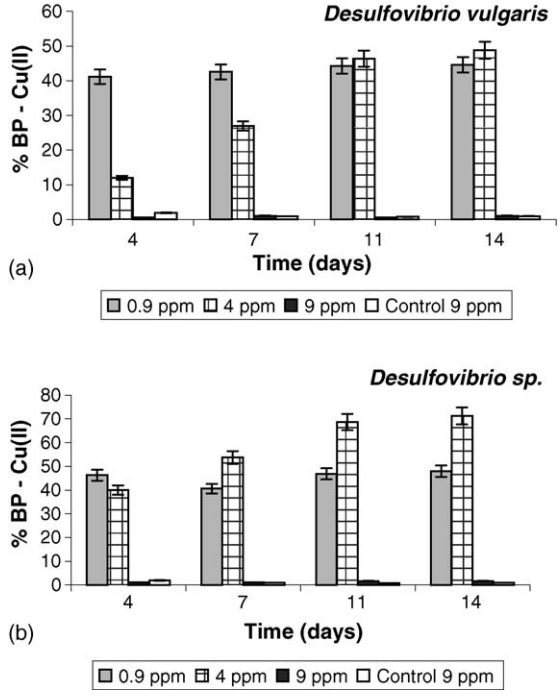

Fig. 4. Percentage of metal precipitation of Cu(II) (0.9, 4, 9 ppm and control) by *Desulfovibrio vulgaris* (a) and *Desulfovibrio* sp. (b).

decreased by 42 ± 6% for initial concentrations below 6.0 ppm and by 16 ± 5% for concentrations higher than 7.0 ppm.

In our study, it can be seen that *D. vulgaris* is more sensitive to copper concentration than *Desulfovibrio* sp. The precipitation percentage for *D. vulgaris* reaches a maximum of 45% for the experiments with 0.9 and 4 ppm. Nevertheless, in the case of *Desulfovibrio* sp. precipitation levels of 48 and 71%, respectively, are reached for the same concentrations. The difference in the behaviours is possibly due to the natural tolerance to toxic metals of the undefined strains of genus *Desulfovibrio* (*D.* sp.).

Significant precipitation was not observed in the experiment with 9 ppm of copper, meaning that this concentration is extremely toxic for SRB cultures. The values obtained indicate that copper is more toxic than chromium; i.e., exposure to copper (at 9 ppm) results in a greater inhibition among the SRB, that are shown with the stop of the bacterial growth and the sulphate uptake. This finding is consistent with the reported literature values for toxic concentrations, which are lower for copper than chromium (Hao et al. [12]). The reason for this difference in cell deactivation could be due to interactions with the bacterial culture, although further investigations into the exact mechanisms are required to obtain a deeper understanding of this phenomenon.

3.3. Manganese(II)

The effect of Mn(II), using $MnSO_4$, was studied by exposing the cultures to different metal concentrations (1, 5 and 10 ppm). *D. vulgaris* and *Desulfovibrio* sp. tolerated manganese concentrations up to 10 ppm. The precipitation levels are represented in Fig. 5a and b. It can be seen that the bioprecipitation percentage increased with the initial metal concentration and reached maximum values of 60% for *D. vulgaris* and 65.2% for *Desulfovibrio* sp. Once again, *Desulfovibrio* sp. gave a higher precipitation percentage for each concentration than the other species. Maximum precipitation was achieved after an incubation period of 10 days. Data of batch studies for this metal have not been reported in the literature and so comparisons cannot be made.

3.4. Nickel(II)

A set of experiments with $NiSO_4$ addition was carried out to study the influence of Ni(II) (1, 4, 8.5 and 17 ppm) on the bacterial activity of *Desulfovibrio* strains. The precipitation levels found in these experiments are shown in Fig. 6a and b. Nickel concentrations greater than 8.5 ppm exert a marked inhibitory effect on cultures. It was found that this effect is more significant for *D. vulgaris*, where the dissolved metal concentration did not decrease during experiments with an initial concentration of 17 ppm. *Desulfovibrio* sp. shows tolerance to concentrations below 17 ppm and requires only 4 days to reach the maximum precipitation level in each case. However, *D. vulgaris* requires 7 days to reach the maximum precipitation level. Once the maximum precipitation level had been reached it remained constant with time. These observations can be explained if one considers the metal sulphide precipitate to be acting as a barrier for the biomass already present in the culture, and this could be responsible for the cessation of the sulphate-reduction activity [18]. Once again, different behaviour is observed for the two strains in the presence of nickel at different concentrations in solution. This observation is consistent with data reported in literature

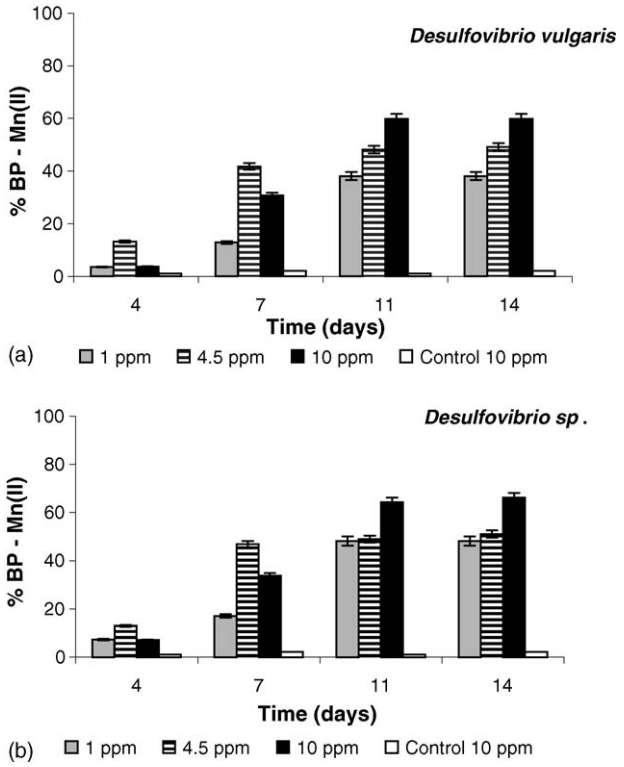

Fig. 5. Percentage of metal precipitation of Mn(II) (1, 5, 10 ppm and control) by *Desulfovibrio vulgaris* (a) and *Desulfovibrio* sp. (b).

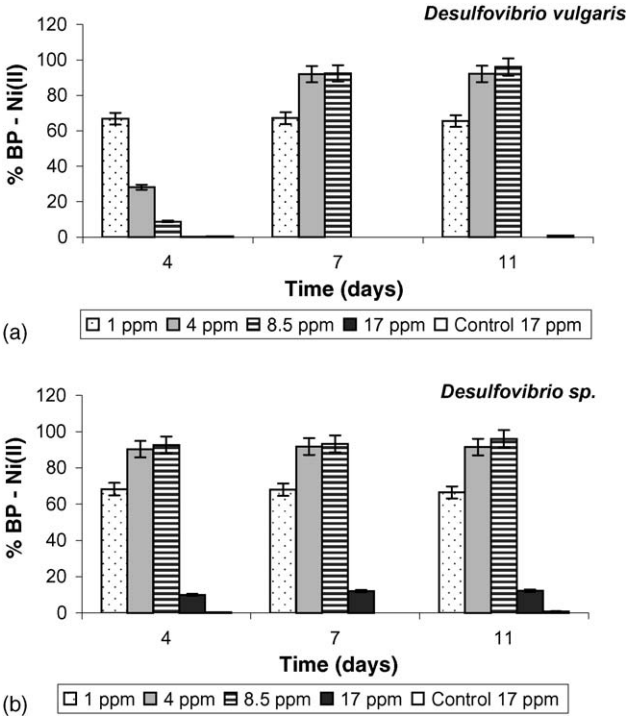

Fig. 6. Percentage of metal precipitation of Ni(II) (1, 4, 8.5, 17 ppm and control) by *Desulfovibrio vulgaris* (a) and *Desulfovibrio* sp. (b).

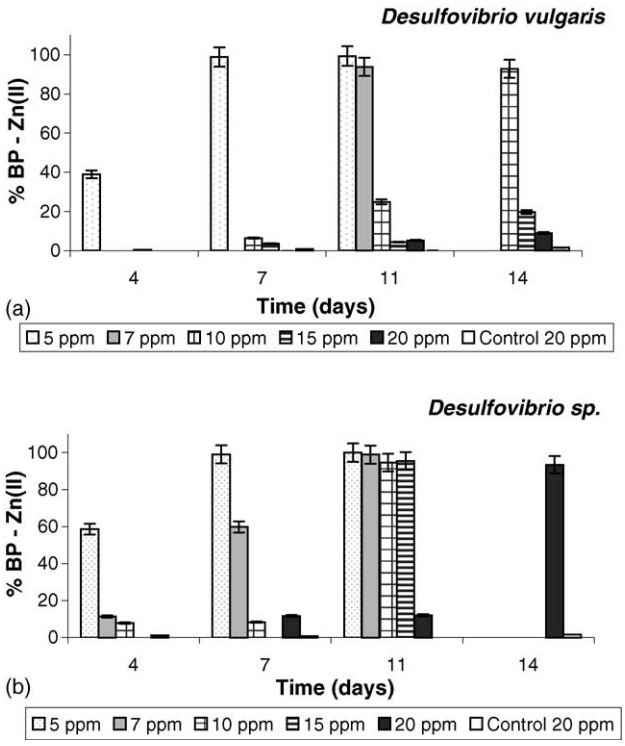

Fig. 7. Percentage of metal precipitation of Zn(II) (5, 7, 10, 15, 20 ppm and control) by *Desulfovibrio vulgaris* (a) and *Desulfovibrio* sp. (b).

(Hao et al. [11]; Poulson et al. [19]), where Ni(II) concentrations of 10 and 20 ppm are reported to be toxic for pure and mixed cultures of SRB, respectively.

3.5. Zinc(II)

Experiments carried out adding $ZnSO_4$ allow study the tolerance of *D. vulgaris* and *Desulfovibrio* sp. to Zn (II). Concentrations tested were: 5, 7, 10, 15 and 20 ppm. Bacterial growth was affected by increasing the Zn(II) concentration and this caused a delay in the lag phase. Zn(II) precipitation levels are represented in Fig. 7a and b. Once *Desulfovibrio* sp. had adapted to the dissolved metal in the medium, zinc concentrations decreased by almost 100% after 7 days for 5 ppm, 11 days for 7, 10 and 15 ppm and 14 days for 20 ppm. The behaviour found for *D. vulgaris* was completely different to that of the other strain; in the former case, incubation periods of 11 days were required to reach precipitation values above 93% in experiments with 5 and 7 ppm of Zn(II), and 14 days for 10 ppm. Experiments with concentrations of 15 and 20 ppm showed higher levels of inhibition because the overall decrease amounted to 20 and 9% Zn(II) precipitation, respectively, after 14 days of incubation. The implication of this finding is that the metabolic rate of the surviving *D. vulgaris* is affected to a greater extent by the presence of zinc than the corresponding *Desulfovibrio.* sp. culture.

Scanning electron micrographs of the samples taken from two bottles exposed to 20 ppm – one at initial time and other after 14 days of incubation – are shown in Fig. 8. The presence of precipitates is clearly visible in the vicinity of the bacterial cell in the cultures exposed to metal after several days of incubation and the cells themselves appear to be surrounded by a hazy cloud, most probably due to the presence of metal sulphide. Similar micrographs (not shown) were also obtained for the other samples exposed to the metals under investigation. These features were not observed in the control sample (without bacteria). It is reasonable to suppose that a fine metal precipitate is concentrated in the vicinity of the SRB and that these insoluble sulphides act as a physical barrier to the sulphate reduction activity of cells [18]. This effect could be external to the cells and non-toxic, as the SRB culture remains viable and could retain its ability to reduce sulphate in other environmental conditions. Further studies of X-ray dot maps for sulphur and metals were carried out in order to confirm this assumption.

In conclusion, the precipitation levels of the maximum tolerable concentrations (MTC), defined as the highest concentration of a metal in the medium that does not cause death of organisms [20], were generally higher for *Desulfovibrio* sp. and this indicates that this strain is more suitable for applications in the treatment of metal-contaminated media. The MTC values for each metal for *D. vulgaris* were as follows: 24.7%-15 ppm Cr(III), 45%-4 ppm Cu(II), 60%-10 ppm Mn(II), 96%-8.5 ppm Ni(II) and 9%-20 ppm Zn(II). The MTC values for *Desulfovibrio* sp. were: 25.5%-15 ppm Cr(III), 71%-4 ppm Cu(II),

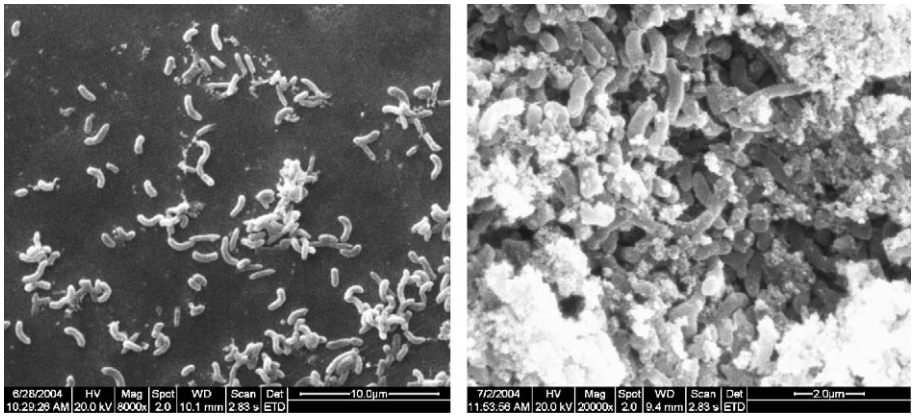

Fig. 8. Scanning electron micrographs of *Desulfovibrio* sp. exposed to 20 ppm of Zn(II): at initial time (×8000) (left side) and after 14 days of incubation (×20,000) (right side).

70%-10 ppm Mn(II), 96.1%-8.5 ppm Ni(II) and 93%-20 ppm Zn(II). The relative order for inhibitory metal concentration was Cu > Ni > Mn > Cr > Zn for both cultures.

The adverse effects caused by individual heavy metals have been studied using synthetic solutions containing Cr(III), Cu(II), Mn(II), Ni(II) and Zn(II). Acid mine drainage and other metal-contaminated effluents contain a mixture of heavy metals. Additional studies are currently in progress to determine the inhibitory effect of mixtures of heavy metals and to apply this approach to real contaminated sludge using *Desulfovibrio* strains.

The results obtained in this study, along with data reported in the literature, can be used to predict the response of SRB in an operating sulphate-reduction bioreactor for the biotreatment of AMD or heavy metal-contaminated sludge. A number of studies are currently in progress aimed at addressing these issues.

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