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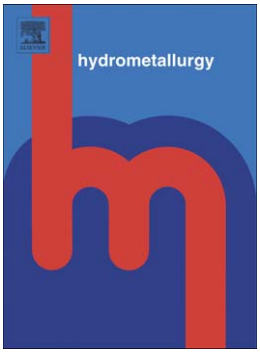

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The effects of nitrate on substrate utilisation by some iron(II)- and sulfur-oxidising *Bacteria* and *Archaea*

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ABSTRACT

The influence of nitrate on batch cultures of selected *Acidithiobacillus*, *Sulfobacillus*, *Acidianus*, *Sulfolobus* and *Metallosphaera* species capable of utilising iron(II) and reduced sulfur substrates was examined. Nitrate was added to cultures in media containing both ferrous ions and tetrathionate. The presence of nitrate resulted in decreased planktonic cell numbers, increased microbial lag times and lower ferrous ion and tetrathionate utilisation rates. These results varied with nitrate concentration, species and adaptive history. Based on the results of batch culture tests, nitrate was added to chalcopyrite concentrate bioleaching experiments to control the redox potential. Bacteria in bioleaching tests at 30 and 45 °C exhibited adaptation to nitrate resulting in high redox potentials. However, when archaea were used in bioleaching tests at 60 °C, ferrous ion oxidation was suppressed in the presence of nitrate. At a nitrate concentration of 20–30 mM, a redox potential of 430–460 mV (*vs* Ag/AgCl) was maintained during the 10 week experiment. At this redox potential copper extraction was increased by 20 % compared to a culture without nitrate, potentially offering a method of redox control for high-temperature stirred-tank bioleaching with archaea.

Keywords: bioleaching; ferrous ion oxidation; nitrate

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1 Introduction

In South America, some copper ore deposits contain small amounts of sodium or potassium nitrate that dissolve in the dilute sulfuric acid used to irrigate managed heaps of low-grade ores. Heap leach solutions are cycled through purification, concentration and recovery plants in order to produce a copper product. Very often, that process is a combination of solvent extraction (SX) and electrowinning (EW). Those cations and anions that are not removed during downstream processing are returned to the heap circuit and, with time, they build up in the pregnant leach solutions (PLS). For heap leach-SX-EW circuits, the main problem associated with the presence of nitrate anions (NO_3^-) in PLS is the degradation of SX reagents during downstream processing. Referring to negative experiences on SX-EW performance at Lomas Bayas, Radomiro Tomic and El Tesoro operations in northern Chile, Scheffel (2002) commented that while SX reagent degradation by nitrate was a recognized problem by reagent suppliers, test programmes instigated at the time did not identify the magnitude of the problem. Nitrate concentrations up to 32 g L^{-1} (0.5 M) were reported by Virnig et al. (2003a) and, more specifically, up to 37 g L^{-1} (0.6 M) was reported for the PLS at Lomas Bayas operation (Kordosky, 2011)

The oxidative power of nitrate is well known and the benefits of using sodium nitrate to dissolve minerals were recognised more than 130 years ago. Stetefeldt (1883) proposed the use of a mixture of dilute sulfuric acid and sodium nitrate to dissolve silver and copper sulfides. Much more recently, and relevant to the present application, Hard (1975) described a process for *in situ* leaching of copper sulfides using sodium nitrate in sulfuric acid (solution pH 0.5–1.5) and Lingane et al. (1977) proposed sodium nitrate-sulfuric acid injection for the in *situ* leaching of copper sulfide ores that lacked sufficient pyrite to generate acid during leaching. Most recently, Arias (2003) added sodium nitrate (the oxidant) to sulfuric acid solution (pH 1.7) and used the mixture to irrigate heaps of copper ores. The detrimental effects of nitrate on the SX process were avoided by recycling the leach liquor to a fresh heap and thus reducing the concentration of nitrate prior to SX. The test data showed that the mixture was more effective (an increase of 50%) than a bacterial leach conducted over the same time frame.

The impact of nitrate ions on microorganisms inhabiting heaps has received less attention, perhaps because the presence of nitrate salts is not widespread in sulfide ores. However, heaps are increasingly being constructed of lower grade and more complex ores which contain greater impurity contents. Early studies, using *Acidithiobacillus (At.) ferrooxidans* as the test organism, showed that 6 g L^{-1} (0.07 M) sodium nitrate inhibited ferrous ion oxidation by 40 % and that ferrous ion oxidation was completely inhibited at 8 g L^{-1} (0.1 M) (Razzell and Trussel, 1963). In a similar study, up to 2.5 g L^{-1} (0.03 M) sodium nitrate slowed ferrous ion oxidation but the bacteria “were able to adapt gradually to higher nitrate concentrations” (Tuovinen et al., 1971). The concentration of sodium

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nitrate required to inhibit ferrous ion oxidation by 50 % relative to a control was 0.085 g L^{-1} (0.001 M) at pH 0.94 and 2.125 g L^{-1} (0.025 M) at pH 3 (Alexander et al., 1987) and nitrate was more inhibitory to ferrous ion oxidation by *At. ferrooxidans* than to sulfur oxidation (Harahuc et al., 2000a; Razzell and Trussel, 1963). Niemelä et al. (1994) reported that sodium nitrate (0.006 M) inhibited ferrous ion oxidation by a mixed culture of native microorganisms enriched from mine water. Recently, du Plessis *et al*. (2007) suggested that as little as 1.5 g L^{-1} (0.024 M) NO_3^- in process water could be deleterious to bioleaching.

One of the drivers for the development of high-temperature, stirred-tank bioleaching (Batty and Rorke, 2006) using archaea was the rapid extraction of copper from chalcopyrite ($CuFeS_2$) concentrates, especially those containing deleterious contaminant elements. In this technology, depending on the mineral concentrate and the solids loading, the microorganisms must tolerate high concentrations of cations and sulfate anion and concomitant high ionic strength, which differ through the reactor series during processing. Three archaeal genera are commonly represented among ferrous ion- and sulfur-oxidising acidophiles in high temperature bioleaching systems: *Acidianus (A.)*, *Metallosphaera (M.)* and *Sulfolobus (S.)* (Norris, 2007; Mikkelson et al., 2006; Sandström and Petersson, 1997). No data on the effect of NO_3^- on archaeal ferrous ion or reduced inorganic sulfur compound oxidation were found.

Knowledge of the behaviour of bioleaching organisms inhabiting complex environments is increasing, but little is known of the impact of contaminants upon bacteria or archaea that can utilise both ferrous ions and reduced sulfur substrates. Historically, most studies were focused upon the preference of the bacterium for a substrate when grown in media with both ferrous ions and elemental sulfur (Sand, 1989; Landesman et al., 1966) or ferrous ions and a reduced inorganic sulfur compound such as tetrathionate (Shiers et al., 2013a; 2013b; 2010). However, among those dual substrate studies, the role that complex solution composition may play in substrate utilisation preferences and patterns has not received attention. In the present study, the growth and activity of the bacteria *At. ferrooxidans*, *Sulfobacillus(*Sb.*) acidophilus*, *Sb. sibiricus* and *Sb. thermosulfidooxidans* and of the archaea *A. brierleyi*, *M. hakonensis* and *S. metallicus* in media containing tetrathionate, ferrous ions and potassium nitrate were examined. In addition, the effect of potassium nitrate on copper extraction from chalcopyrite at temperatures appropriate to each species was investigated. The results obtained augment data from previous studies using dual substrate media and increase understanding of microbial behaviour in bioleaching systems.

2 Materials and Methods

All chemicals were analytical grade reagents (AR) unless otherwise stated and all solutions were prepared with de-ionised water. Preparation and transfer of sterile solutions, sub-culturing, inoculation of test flasks and sampling took place in a laminar flow hood using aseptic technique.

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2.1 Culture preparation and maintenance

All cultures were grown in a basal salts medium supplemented with yeast extract (BSM-YE). The medium was prepared by dissolving per litre of deionised water: 1.5 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4.7H_2O$, 0.25 g KH_2PO_4 and the solution pH adjusted to pH 1.8±0.05 with 18 M H_2SO_4. The prepared medium was sterilised in an autoclave at 121 °C, 120 kPa for 30 minutes. A 10% w/v yeast extract solution was prepared by dissolving 3 g in 30 mL of basal salts medium, after which that solution was sterilised by passage through a 0.2 µm pore size membrane (Pall P/N60301). An aliquot (1 mL) of the yeast extract solution was added to the basal salts medium (1 L).

Cultures of *At. ferrooxidans* (DSM 14882^T) were grown at 30 °C in BSM-YE medium supplemented with either 9.71 g L^{-1} $FeSO_4.7H_2O$ (35 mM) or 0.75 g L^{-1} $K_2S_4O_6$ (2.5 mM) as the primary energy source. Similarly, *Sb. thermosulfidooxidans* (DSM 9293^T), *Sb. acidophilus* (DSM 10332^T) and *Sb. sibiricus* (DSM 17363^T) were grown in each medium at 45 °C. Iron(II)- and tetrathionate-adapted cell lines of *A. brierleyi* DSM 1651^T, *M. hakonensis* and a tetrathionate-adapted cell line of *S. metallicus* DSM 6482^T were grown at 60 °C but a iron(II)-adapted cell line could not be established for *S. metallicus*, which required a minimum of approximately 1 mM tetrathionate for the oxidation of iron(II). This amount was established in ancillary tests conducted under the conditions described above and is consistent with the reported reduced inorganic sulfur requirement for ferrous ion oxidation by *S. metallicus* (Bathe and Norris, 2007).

In this way, iron(II)- and tetrathionate-adapted cell lines were established for six of the seven test species. Cultures were grown on their respective media for up to five sub-cultures (3 weeks) before inoculation into dual-substrate test media with different concentrations of potassium nitrate.

2.2 Dual substrate media with potassium nitrate

A bulk volume of sterile BSM-YE medium supplemented with 9.71 gL^{-1} (35 mM) $FeSO_4.7H_2O$ and 0.75 g L^{-1} (2.5 mM) $K_2S_4O_6$ was prepared. Aliquots (100 mL) of dual-substrate medium were transferred to 250 mL Erlenmeyer flasks. An aliquot of medium was removed from each flask, analytical grade potassium nitrate dissolved in it, and the solution filter sterilised (0.2 µm pore size membranes) back into the appropriate flask. The nitrate concentrations investigated varied between species but was in the range of 0–5.05 g L^{-1} potassium nitrate (0–50 mM nitrate ion). Flasks were inoculated with either iron(II)- or tetrathionate-adapted cells, initial cell density approximately 1×10^6 cells mL^{-1}, and incubated at the temperature appropriate to the test species. Aliquots (3 mL) were removed periodically under sterile conditions for solution analysis and bacterial enumeration.

2.3 Copper extraction from chalcopyrite

The $CuFeS_2$ concentrate (91–106 µm size fraction) used in bioleaching tests contained Cu, 24.5%; Fe, 25.9%; S, 28.6% and Si, 5.19%. The concentrate was washed twice with hot ethanol, then twice with boiling water to remove residual flotation reagents, and then dried at 30 °C for 24 h. Dried concentrate was representatively sampled A 25 g subsample was then removed and homogenized to provide samples for test flasks and analysis. Test flasks containing 100 mL of BSM–YE with added

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nitrate (0–50 mM) and 1.0 $g_x CuFeS_2$ were inoculated. The inocula for bioleaching tests at 30 and 45 °C were prepared using tetrathionate-adapted cell lines of *At. ferrooxidans*, the three *Sulfobacillus* species and the two archaeal species, all in equal numbers, to give initial total concentrations in the test flasks of 1×10^6 cells mL^{-1}. Tests run at 60 °C had an inoculum comprised of tetrathionate-adapted *S. metallicus* and *A. brierleyi* in approximately equal numbers, with a starting concentration in the test flasks of 1×10^6 cells mL^{-1}. Flasks were incubated using an Innova orbital-shaking incubator (150 rpm). After countering evaporation using deionised water, samples were removed periodically from flasks, cells enumerated on a subsample, and samples refrigerated pending further measurements (pH, ORP, Cu, polythionates, and nitrate concentrations).

2.4 Solution Analyses

Planktonic cells were counted using a Helber Bacterial Counting Chamber (Thoma ruling, 0.02 mm cell depth) mounted in a phase contrast microscope (detection limit, 0.5×10^5 cells mL^{-1}). It was assumed that planktonic biomass represented the bulk of the bacterial population with no significant sessile cell population. A calibrated pH meter fitted with a glass membrane electrode (Ionode model GL20) was used to measure solution pH. Solution potential was measured using a redox electrode (Ionode model IP1306, $Ag/AgCl_{(ref)}$) and ferrous ion concentrations were calculated as described by Watling et al. (2008). The redox probe was calibrated on an aliquot of the medium before tetrathionate was added. Potassium nitrate in the selected concentration range did not alter the calibration constants determined for the ferrous ion analysis. Copper was determined in solution using atomic absorption spectroscopy. Polythionate concentrations were quantified using a Waters 2695 HPLC separation module utilising an Ionpac AS16 ion exchange column. Polythionates were detected using a Waters 2996 Photodiode Array Detector at 214 nm with the exception of trithionate, detected at 192 nm (Jeffrey and Brunt, 2007). A pump flow rate of 1.5 mL min^{-1} was used and the column temperature was maintained at 25 °C. A 0.15 M sodium perchlorate solution was used as the eluent. Spectra were integrated using "Empower" software. Nitrate concentrations were quantified using a Dionex ICS-3000 HPLC system with conductivity detector. Samples were initially passed through a CIRA (11AS) separation module and then through an Ionpac AS19 column. A pump flow rate of 1 mL min^{-1} and temperature of 30 °C were maintained. All solution measurements reported in figures represent a single measurement.

3 Results and discussion

3.1 Cell growth in the presence of nitrate.

Final cell numbers decreased with increased nitrate concentration in the test media (Figure 1). Nitrate concentrations did not change significantly over the course of the batch cultures. A comparison of initial and final nitrate concentrations during the batch culture growth demonstrated a variation of 2–5 % (Data not shown).

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Iron(II)-adapted cultures of the three *Sulfobacillus* species grew at nitrate concentrations of up to 40 mM. At the highest nitrate concentration (40 mM), cell numbers of *Sb. acidophilus* and *Sb. sibiricus* decreased to approximately half those present in nitrate-free control. The impact of nitrate on *Sb. thermosulfidooxidans* growth was greater, with cell numbers in the presence of 40 mM nitrate being reduced by over 90% of those present after substrate utilisation in nitrate-free control. *At. ferrooxidans* was the most sensitive of the test bacteria to the presence of nitrate in growth medium, final cell numbers being 73% at 10 mM nitrate and no cell growth measured at 20 mM and greater.

Iron(II)-adapted archaea were more sensitive than the bacteria to the presence of nitrate in growth media. Final cell numbers for *A. brierleyi* and *M. hakonensis* in growth media containing 10 mM nitrate were 45 and 24% of the numbers attained in the nitrate-free control and no growth occurred at 20 mM nitrate or greater.

Tetrathionate-adapted cultures of *Sb. acidophilus* and *Sb. sibiricus* grew in media containing up to 40 mM nitrate, cell numbers decreasing as the nitrate concentration was increased. Final cell numbers were 67 and 69% for *Sb. acidophilus* and *Sb. sibiricus*, respectively, compared with their numbers in nitrate-free medium. Overall, these two species had the highest final cell numbers, compared with the other bacterial and archaeal species tested. The 'pairing' of *Sb. acidophilus* and *Sb. sibiricus* in terms of their responses to nitrate for both iron(II) and tetrathionate utilisation is consistent with the results of Shiers et al. (2010) for growth in nitrate-free dual-substrate media. In that earlier work it was found that *Sb. thermosulfidooxidans* and *Sb. thermotolerans* responded similarly to the selected growth conditions but differently to the *Sb. acidophilus* and *Sb. sibiricus* pair. Both *At. ferrooxidans* and *Sb. thermosulfidooxidans* grew in media containing up to 30 mM nitrate but not at 40 mM nitrate.

Tetrathionate-adapted cultures of *A. brierleyi* and *M. hakonensis* grew in the presence of up to 40 mM nitrate but final cell numbers at the highest nitrate concentration were lower by >80% in both cultures, compared with those in nitrate-free control. While tetrathionate-adapted *S. metallicus* grew in media with up to 40 mM nitrate, cell numbers dropped to 28% of those attained in nitrate-free medium.

Previous studies examining changes to culture behaviour in the presence of nitrate were focused on rate kinetics and neglected to report changes in cell numbers (e.g., Sarcheshmehpour et al., 2009; Blight and Ralph, 2008; Harahuc et al., 2000b; Suzuki et al., 1999; Niemelä et al., 1994). The effect of nitrate on cell numbers for chemolithotrophic cultures in growth media with ferrous ions and tetrathionate have not been reported previously. Lower cell yields were reported in investigations of other anionic impurities, such as chloride (Gahan et al., 2010), consistent with the results of the present study.

The lower cell yields obtained in media with added nitrate may be explained using the mechanism proposed by Alexander et al. (1987) and Suzuki et al. (1999), specifically that anionic species can migrate to the cell cytoplasm where they cause acidification. The uncoupling of substrate

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oxidation and cell generation, a cell response that occurs under stressful conditions and allows greater amounts of energy to be directed to the maintenance of cell homeostasis, may also contribute to the lower cell numbers measured. Such a response has been observed in the presence of heavy metals (Roy and Mishra, 1981), low concentrations of carbon dioxide (Kelly and Jones, 1978), after exposure to organic compounds (Collinson et al., 2011) and at low temperatures (Leduc et al., 1993) and may account for reduced cell yields in the present study. Nitrate concentrations did not change markedly over the course of the batch culture experiments. If resistance mechanisms are utilised by the test microorganisms, the persistence of nitrate in solution indicates that such mechanisms do not involve nitrate removal or degradation.

3.2 Effect of nitrate on substrate utilisation

Changes in substrate utilisation patterns varied between test species, adaptive history and nitrate concentration. These differences are discussed with respect to changes in lag times and the time required for complete utilisation of either ferrous ions or tetrathionate. Figure 2 provides an explanation of how the raw data from each batch culture are represented in Figures 3 and 4 illustrating substrate utilisation by bacteria and archaea, respectively, in which a set of test cultures at different nitrate concentrations are compared. The start and end times of ferrous and tetrathionate utilisation are presented, providing an indication of oxidation rate. The time period between inoculation and measurable utilisation of either ferrous ions or tetrathionate provide an indirect measure of lag time. In some cases substrates were not completely utilised in the experimental time frame; for these, the amounts utilised are shown as percentages of the original concentrations in the growth media.

3.2.1 Ferrous ion utilisation

Nitrate had two main effects on ferrous ion utilisation by microorganisms, increased lag times and reduction in the mean oxidation rate, illustrated by extended times for complete utilisation and, in some cases, incomplete utilisation (Figures 3 and 4). The occurrence and extent of the effects were species-dependent but less dependent on growth history. For example, ferrous ion oxidation lag times increased with increased nitrate concentration but *At. ferrooxidans* and *Sb. thermosulfidooxidans* were more sensitive to the presence of nitrate than *Sb. sibiricus* and *Sb. acidophilus* (Figure 3). In general, there were larger increases (with increasing nitrate concentration) in ferrous ion oxidation lag times for tetrathionate-adapted cell lines than for Iron(II)-adapted cell lines.

Archaeal lag times were of similar duration to that of each nitrate-free test for iron(II)-adapted *A. brierleyi* and tetrathionate-adapted *A. brierleyi*, *M. hakonensis* and *S. metallicus*. *M. hakonensis* had a longer lag time at 10 mM nitrate and failed to oxidise ferrous ion at higher concentrations (Figure 4). Lag times for tetrathionate-adapted cell lines were less affected by nitrate concentration but ferrous ion utilisation was slow and incomplete at 20 mM nitrate for both *A. brierleyi* and *S. metallicus*. For these species, inhibition of ferrous ion oxidation occurred after partial substrate utilisation, the extent of which was proportional to the nitrate concentration (Figure 5).

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Increased lag times and reductions in ferrous ion oxidation rates, together with reduced cell replication rates, caused by the presence of nitrate are known for bacteria but no comparative data for the archaea have been found. In an early study (Razzell and Trussel, 1963), using batch cultures of *At. ferrooxidans* in pH 1.6 media, 70 and 94 mM nitrate inhibited ferrous ion oxidation by 40% and 100%, respectively. Alexander et al. (1987), using *At. ferrooxidans* as the test organism, reported that 1 mM nitrate inhibited ferrous ion oxidation rate by 50% at pH 0.94. Nitrate (65 mM) reduced the cell replication rate of an undefined mixed mesophilic culture of ferrous ion oxidising microorganisms to 65% of that estimated for cells cultured in minimal medium (pH 1.5) but, more importantly, the culture was not viable beyond three sequential subcultures (Blight and Ralph, 2008). Ferrous ion oxidation by a mixed mesophilic microbial culture was inhibited for more than 11 days (the duration of the experiment initiated at pH 1.5) in the presence of nitrate (6 mM and 12 mM) during the bioleaching of a pyrrhotite-rich, polymetallic black schist (Niemelä et al., 1994). Sarcheshmehpour et al. (2009) reported that 7.2 mM nitrate increased the lag time to approximately four days before the start of ferrous ion oxidation by a defined mixed culture, initially containing *At. thiooxidans*, *At. ferrooxidans* and *Leptospirillum ferrooxidans* in the ratio 2:4:4, in bioleaching tests (initially pH 1.8) on four different pyrite-rich, low-grade copper ores.

3.2.2 Tetrathionate utilisation

As was the case for ferrous ion oxidation, the two main effects of nitrate on tetrathionate utilisation by the test bacteria were increased lag times and reduced mean tetrathionate utilisation rates with increased nitrate concentrations (Figure 3). The most sensitive to the presence of nitrate were *At. ferrooxidans* and *Sb. thermosulfidooxidans*. Tetrathionate-adapted *At. ferrooxidans* tolerated up to 20 mM nitrate but oxidised <50% of the tetrathionate at 30 mM. The main effect was considerably decreased utilisation rates in the presence of nitrate. Tetrathionate-adapted *Sb. thermosulfidooxidans* tolerated up to 30 mM nitrate but exhibited both increased lag times and decreased oxidation rates with increased nitrate concentration. Neither species utilised tetrathionate in the presence of 40 mM nitrate. For tetrathionate-adapted *Sb. sibiricus* and *Sb. acidophilus*, neither lag times nor tetrathionate oxidation rates were greatly changed in the range 10-40 mM nitrate.

Tetrathionate-adapted *M. hakonensis* was the most tolerant to nitrate; increased nitrate concentrations resulted in little change in lag times or in tetrathionate oxidation rates (Figure 4). Lag times were largely unaffected but tetrathionate utilisation rates for both *A. brierleyi* and *S. metallicus* slowed with increased nitrate concentrations. Of the archaea, only *S. metallicus* utilised tetrathionate in the presence of 40 mM nitrate.

The effects of nitrate on cultures in dual-substrate media containing ferrous ions and tetrathionate have not previously been described. Suzuki et al. (1999) reported that the presence of 10 mM nitrate caused a reduction of greater than 50% in the rate of oxidation of elemental sulfur by *At. thiooxidans* and Harahuc et al (2000b) used respirometry to study the inhibition of elemental sulfur oxidation by sulfur-adapted *At. ferrooxidans* in the presence of up to 500 mM nitrate. In the latter

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study, *At. ferrooxidans* tolerated up to 100 mM nitrate but higher nitrate concentrations caused significant reductions in sulfur oxidation. Lag times prior to sulfur oxidation were not estimated in either of these studies.

3.2.3 Effect of nitrate on polythionate speciation during tetrathionate utilisation

The formation of pentathionate, hexathionate and trace amounts of thiosulfate was detected in all test cultures where tetrathionate utilisation occurred and polythionate speciation patterns were similar regardless of the nitrate concentration. Polythionates could no longer be detected shortly after complete utilisation of tetrathionate for all test species, except *Sb. thermosulfidooxidans* and iron(II)-adapted *M. hakonensis*. The data for *Sb. thermosulfidooxidans* in nitrate-free, dual-substrate medium (Figure 6a) provide a typical example of polythionate utilisation. However, in cultures of *Sb. thermosulfidooxidans* (and *M. hakonensis*, data not shown), high nitrate concentrations inhibited the utilisation of polythionates (Figure 6b). While the tetrathionate was exhausted slowly (Figures 6a and 6b), the pentathionate and hexathionate formed during tetrathionate utilisation persisted through the latter half of the experiment. The data may indicate that enzymatic hydrolysis of tetrathionate takes place, but that the rates of pentathionate and hexathionate hydrolysis are markedly reduced or inhibited.

Tetrathionate hydrolase is an acid-stable enzyme with a reported optimum of pH 3 (Bugaytsova and Lindstrom, 2004) located in the cell periplasm, except for *At. ferrooxidans* where it is thought to be an outer-membrane protein (Kanao et al., 2007). Several products are generated when tetrathionate or pentathionate is hydrolysed and a number of reaction stoichiometries have been suggested from studies utilising different *At. ferrooxidans* strains (de Jong et al., 1997b; Sugio et al., 1996), *At. thiooxidans* (Tano et al., 1996), *At. caldus* (Bugaytsova and Lindstrom, 2004) or *Acidiphilium acidophilum* (de Jong et al., 1997a; Meulenberg et al., 1993). The tetrathionate hydrolysis reaction (reaction (1)) initially proposed by Meulenberg et al. (1993) and the pentathionate hydrolysis reaction (reaction (2)) (de Jong et al., 1997a, b) are analogous. The only soluble reduced sulfur product formed via this mechanism is thiosulfate but twice as much colloidal sulfur (S^0) is generated from pentathionate hydrolysis.

$$S_4O_6^{2-} + H_2O \rightarrow S_2O_3^{2-} + S^0 + SO_4^{2-} + 2H^+ \quad (1)$$

$$S_5O_6^{2-} + H_2O \rightarrow S_2O_3^{2-} + 2S^0 + SO_4^{2-} + 2H^+ \quad (2)$$

It is not known why nitrate would specifically inhibit pentathionate and hexathionate hydrolysis by the tetrathionate hydrolase enzyme in *Sb. thermosulfidooxidans* and iron(II)-adapted *M. hakonensis*.

3.3 Bioleaching: Copper extraction from chalcopyrite

Bioleaching tests using chalcopyrite concentrate were undertaken to examine the effect of nitrate addition on the solution chemistry and copper extraction. The targeted ORP window (Figures

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7–9) was based on a brief review of public-domain data pertinent to controlled-redox leaching of chalcopyrite.

There is little comparable data investigating the oxidation of chalcopyrite with nitrate in a mild sulfuric acid background. Sokić et al. (2009) demonstrated rapid chalcopyrite concentrate dissolution, however nitrate concentrations ranged from 150–900 mM and the experimental temperature was 80 °C, well in excess of the current study. Other studies utilising nitrate or nitric acid performed leaching under aggressive conditions, with comparatively high temperatures and oxidant concentrations (Gok and Anderson 2013, Bjorling et al. 1976). As the current study utilised low nitrate and sulphuric acid conditions it was assumed that nitrate present (50 mM maximum) did not influence copper extraction as a chemical oxidant.

3.3.1 Bioleaching at 30 ºC

Tests were inoculated using a mixed culture of the eight test species (bacteria and archaea) initially containing equal numbers of each species and a total cell count of 1×10^6 cells mL^{-1}. Cell numbers, solution ORP and copper extraction were monitored for 12 weeks (Figure 7).

As expected, cell numbers were lower in bioleaching media with increased concentrations of nitrate and the increased lag time was also evident from the results (Figure 7a). Nevertheless, solution ORP exceeded the targeted window in all inoculated tests. An increased period of iron(II) oxidation was evident at higher nitrate concentrations but the more tolerant species in the mixed culture adapted to the conditions after several weeks (Figure 7b). Adaptation by *At. ferrooxidans* to nitrate with increased ferrous ion oxidation rate has been noted previously; a mixed culture of native and culture collection strains was initially inhibited by 29 mM nitrate adapted slowly to higher nitrate concentrations (Tuovinen et al., 1971). Copper extractions never exceeded approximately 27% and delayed ‘starts’ in leach curves reflected increasing microbial lag times (Figure 7c). No cells were observed in the abiotic controls, the solution ORP remained below 400 mV and extraction was approximately 12%, in the 30 ºC experiment, consistent with the well documented slow chalcopyrite dissolution rates at low temperatures (Watling, 2013).

3.3.2 Bioleaching at 45 ºC

The bioleaching experiment at 45 ºC replicated the conditions of the 30 ºC bioleaching test in all respects except temperature. The maximum cell density measured decreased with increased nitrate concentration, as did the lag time (Figure 8a). A two-week lag period in cell growth indicated that the dominant species at 45 ºC was less sensitive to the presence of nitrate than was the case for the 30 ºC experiment where lag periods up to 4 weeks were observed. Solution ORP again exceeded the targeted window in all inoculated tests (Figure 8b) and adaptation to the presence of nitrate was evidenced by the sharp increase in ORP after 2–3 weeks; at 30 ºC. Copper extractions in all 45 ºC

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tests were higher than those for the 30 ºC experiment, consistent with the well-known temperature dependence of chalcopyrite dissolution, but there was no well-defined region of 'enhanced dissolution rate'.

3.3.3 Bioleaching at 60 ºC

The results of 10-week bioleaching tests inoculated with equal numbers of *A. brierleyi* and *S. metallicus* (initial cell density 1×10^6 cells mL^{-1}) confirmed that nitrate in the range of 10–50 mM strongly inhibited growth, with greatly reduced cell numbers for all nitrate-amended media (Figure 9a). The results confirm the sensitivity of both species to the presence of nitrate in growth media (Figure 1). In the 60 ºC bioleaching experiment, there was little evidence of adaptation to the presence of nitrate. As a consequence, solution ORP decreased with increased nitrate concentration and at 20–40 mM, remained within the targeted window (Figure 9b). For those tests where some archaeal growth occurred (0–40 mM nitrate), copper extractions of 70% or more were calculated, and enhanced chalcopyrite dissolution occurred in the presence of 20–30 mM nitrate yielding up to 95% extraction in 10 weeks (Figure 9c).

3.4 Nitrate, bioleaching and a possible REDOX-control strategy

During the oxidative ferric-sulfate leaching of chalcopyrite, reaction products such as elemental sulfur, ferric hydroxysulfates like jarosite or polysulfides that form and/or accumulate on mineral surfaces may influence chalcopyrite dissolution rate. This phenomenon is often referred to as 'passivation' and has been extensively studied using electrochemical and other surface-sensitive techniques (see recent reviews by Li et al., 2013, Debernardi and Carlesi, 2012 and Klauber 2008). One common finding from many studies is that copper extraction from chalcopyrite in acidic ferrous/ferric sulfate media at moderate temperatures (at atmospheric pressure) can be accelerated by employing conditions of low ORP. As a consequence, a variety of strategies to control ORP during chalcopyrite dissolution have been proposed for use with tank, vat and heap leaching (Watling, 2013 and references therein). In laboratory studies, control of the oxygen supply is the strategy most often employed to control solution ORP (Ahmadi et al., 2010; Gericke et al., 2010; Sandstrom et al., 2005; Third et al., 2002; Ahonen and Tuovinen, 1993). In one study, potassium permanganate was added (Kametani and Aoki, 1985) and in another, a current was applied to a 20% w/v chalcopyrite slurry (Ahmadi et al., 2010). Values for the optimal ORP range between 380–505 mV, most cited researchers recommending an ORP of 420–450 mV vs Ag/AgCl.

The addition of nitrate to microbial growth media in the present study had the common effect of inhibiting iron(II) oxidation, which in a bioleaching system should impact on the Fe(III):Fe(II) ratio with a trend towards lower ORP. For both the mesophilic *At. ferrooxidans* and the moderately thermophilic *Sulfobacillus* species, the inhibition of cell replication and iron(II) oxidation increased with increased nitrate concentrations and resulted in reduced solution ORP (Figures 7 and 8) but

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nevertheless still higher than the targeted ORP window. The reduced numbers of cells were still sufficient to generate ferric ion concentrations in excess of what was required and there was evidence of adaptation during the prolonged bioleaching tests. Copper extractions from chalcopyrite concentrate did not exhibit a clearly defined enhancement for any nitrate concentration.

The concept of exploiting iron(II) oxidation inhibition using anions in base metal processing is not new. Harahuc et al. (2000a) attempted to extract zinc selectively from pyritic tailings using *At. ferrooxidans* and *At. thiooxidans* and mixtures of them in the presence of potassium nitrate (10–100 mM) but the results were equivocal. In 14 day tests with 10 mM nitrate, extractions were (as % Zn/Fe): 104/24 *At. ferrooxidans*, 61/19 *At. thiooxidans* and 84/37 mixed culture, compared with 93/55, 57/18 and 87/58, respectively, in nitrate-free media. Overall, higher nitrate concentrations were less effective. Using similar methodology, the selective extraction from a complex sulfide ore of copper and zinc over iron was also disappointing, more so for copper than for zinc. In 21 day tests with 10 mM nitrate, extractions were (as % Zn/Cu/Fe): 37/14/<1 *At. ferrooxidans* and 100/15/14 *At. thiooxidans*, compared with 75/14/23 and 84/19/23, respectively, in nitrate free media.

In the proposed application of nitrate as a REDOX-control strategy, although the approach might be successful in the short term, the variety of responses, including adaptability, exhibited by the *Sulfobacillu*s species, different strains of *At. ferrooxidans* and some mesophilic mixed cultures suggests that it would not be effective to use nitrate to partially inhibit iron(II) oxidation and establish the optimal solution ORP conditions and, more importantly, maintain the conditions for prolonged periods in continuous reactors such as tanks or heaps operated at ambient to moderate temperatures and colonised predominantly with bacteria.

The results obtained using the archaea at higher leaching temperature are more promising. While cell numbers were depressed from about 12×10^7 to 4×10^7 cells mL^{-1} in the presence of 10–40 mM nitrate compared with the nitrate-free control, there were sufficient numbers to regenerate the required ferric ion oxidant and establish a solution ORP within the targeted ORP window (Figure 9). Copper extractions were enhanced in media with 20–30 mM nitrate and exceeded 90% in 10 week tests. There was no evidence of archaeal adaptation leading to increased iron(II) oxidation rates and increased solution ORP. The results are consistent with the literature on the ORP- and temperature-dependence of chalcopyrite dissolution in the ferric sulfate system (Watling, 2013 and references therein). Thus it is proposed that a REDOX-control strategy could be developed for high-temperature (atmospheric pressure) chalcopyrite bioleaching using archaea with controlled nitrate additions at 20–30 mM.

The results obtained for laboratory-scale, batch tests using generic leach conditions are not comparable to those possible in the pilot and demonstration-scale BioCOP technology (Batty and Rorke, 2006). Just as was the case during the decade-long development of high temperature copper

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concentrate processing, a technology incorporating ORP control by inhibition of iron(II) oxidation using nitrate will need to be optimised in a continuous reactor with longer-term adaptation to the targeted operating conditions than was possible in the present study. High copper extractions must be achieved with considerably shortened residence times. The concomitant development of degradation-resistant extractants or indirect means of minimising the effect of nitrate on extractants (e.g., Campbell et al., 2013; Virnig et al., 2003b; 2013) should enable the downstream purification and concentration of copper from nitrate-containing leach solutions.

4 Summary

Differences in planktonic cell numbers, microbial lag times and ferrous ion and tetrathionate utilisation rates vary with species and adaptive history. In most cases, the presence of nitrate causes a change in microbial substrate utilisation patterns compared with growth in nitrate-free medium. In general, cell replication is inhibited in the presence of nitrate.. Iron(II) oxidation is inhibited to a greater extent than tetrathionate oxidation. The mechanism of anionic inhibition proposed by Alexander et al. (1987) and Suzuki et al. (1999) does not account for the different sensitivities to nitrate measured for sulfur and iron(II) oxidation. Thus there may be a direct nitrate interference on one or more discrete oxidative pathways.

Data from the bioleaching tests support and augment those from the substrate utilisation tests. Despite cell replication being inhibited, cell densities were sufficient to regenerate a sufficient ferric ion concentration for chalcopyrite oxidation at all temperatures. Bacteria (30 and 45 ºC tests) adapt to the presence of nitrate. Increased iron(II) bio-oxidation results in solution ORP values greater than the targeted ORP window for enhanced copper extraction. There is little effect of nitrate on final copper extractions and no well-defined condition of enhanced copper extraction.

At 60 ºC, archaeal growth and iron(II) oxidation rates are suppressed by nitrate to the extent that, at 20-30 mM nitrate, the targeted ORP window was established in the range 430-460 mV and maintained during the 10 week experiment. No archaeal adaptation to nitrate was evident. A well-defined region of enhanced copper extraction occurred in the range of 20-30 mM nitrate. It is proposed that a REDOX-control strategy for enhanced copper extraction could be developed for high-temperature (atmospheric pressure) chalcopyrite bioleaching in continuous stirred reactors using archaea, based on controlled nitrate additions to the bioleaching slurry.

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Highlights

- Bacteria showed signs of adapting to nitrate in growth media
- Archaea did not adapt to nitrate in growth media
- Nitrate offers a potential redox control strategy for high temperature bioleaching

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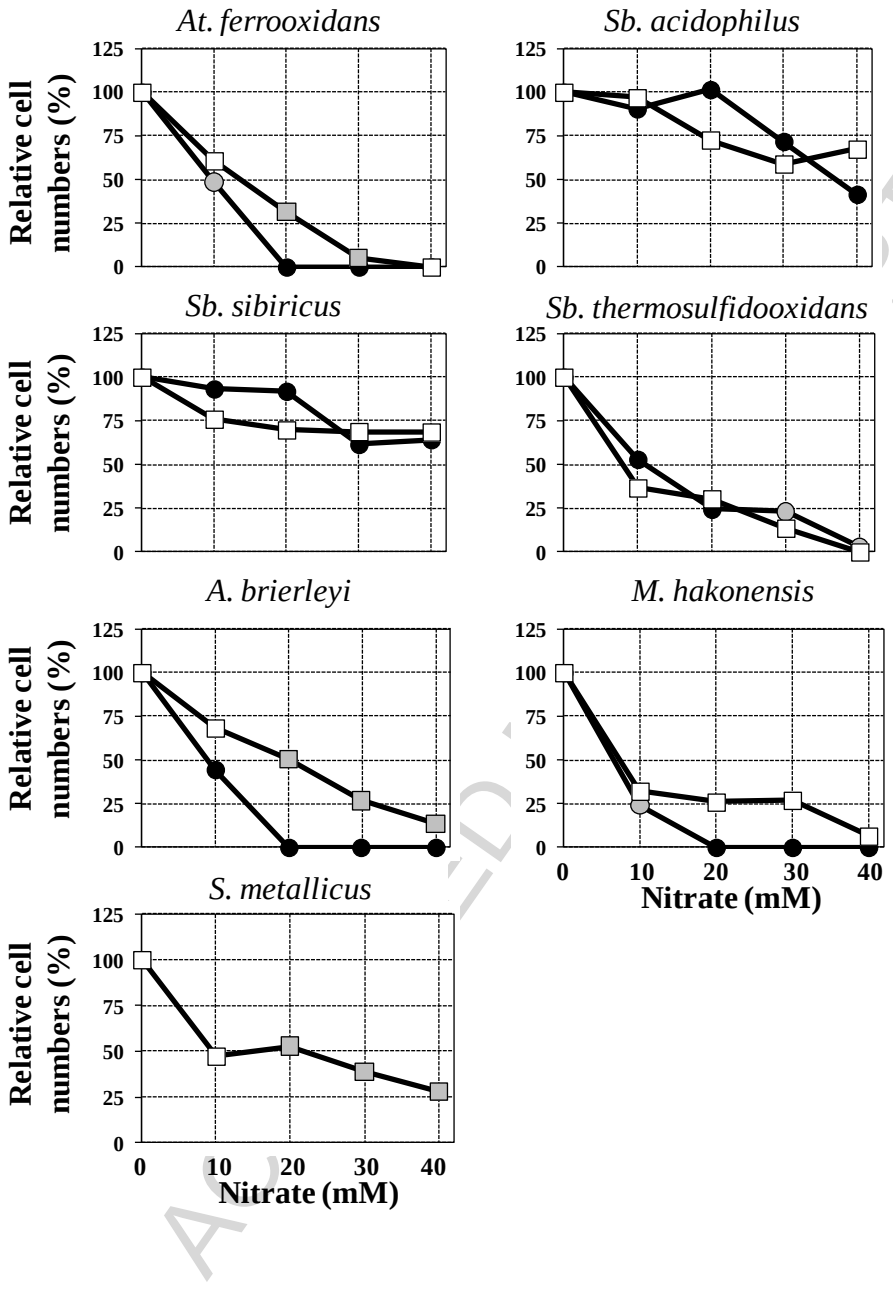

Figure 1. The effect of nitrate concentration on planktonic cell numbers upon complete substrate exhaustion. Tests were inoculated with iron(II)- (●) and tetrathionate-adapted (□) cell lines. Symbols are shaded grey for those batch cultures where oxidation of either substrate was incomplete in the time frame of the experiment (180 hours).

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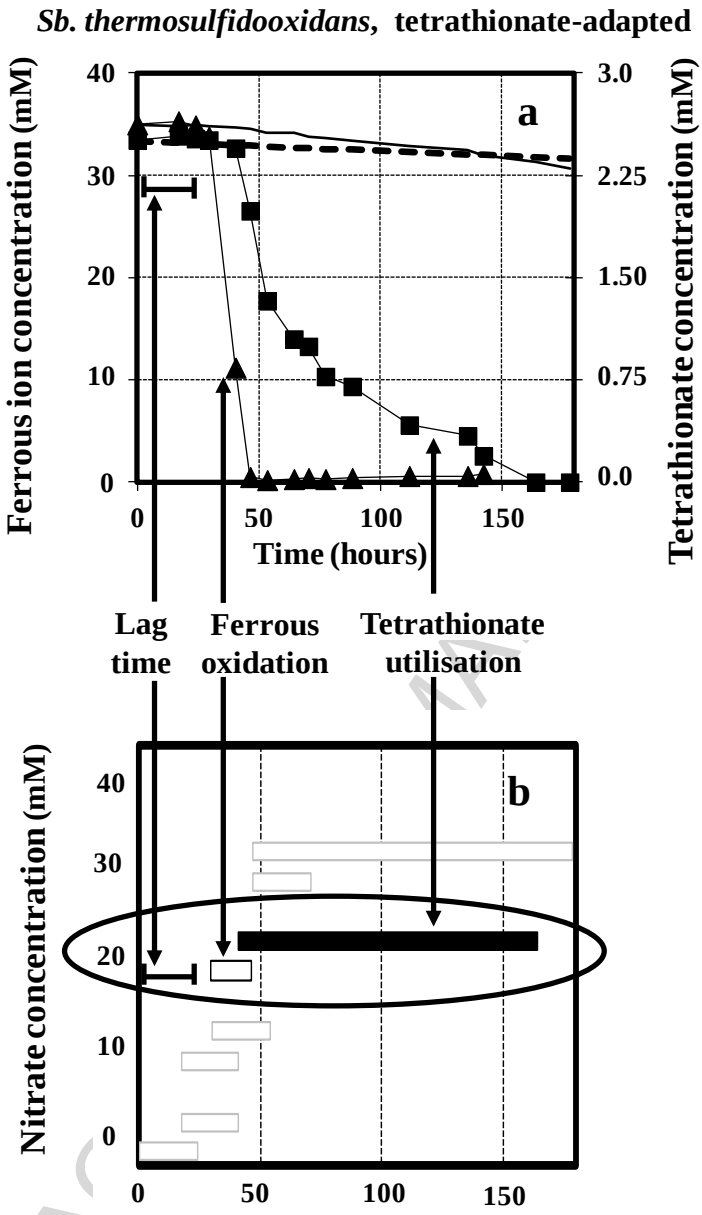

Figure 2. Example data showing the relationships between experimental data and subsequent representations of substrate utilisation for each species. (a) Ferrous ion ▲ and tetrathionate ■ concentrations for inoculated tests; background oxidation of ferrous ion (solid line) and tetrathionate (broken line) versus time in uninoculated tests. (b) Lag time |——|; duration of ferrous ion oxidation (open bar); duration of tetrathionate oxidation (solid bar) for a given nitrate concentration. Incomplete oxidation is indicated by the percentage utilisation at the end of the experiment shown alongside the relevant open or closed bar.

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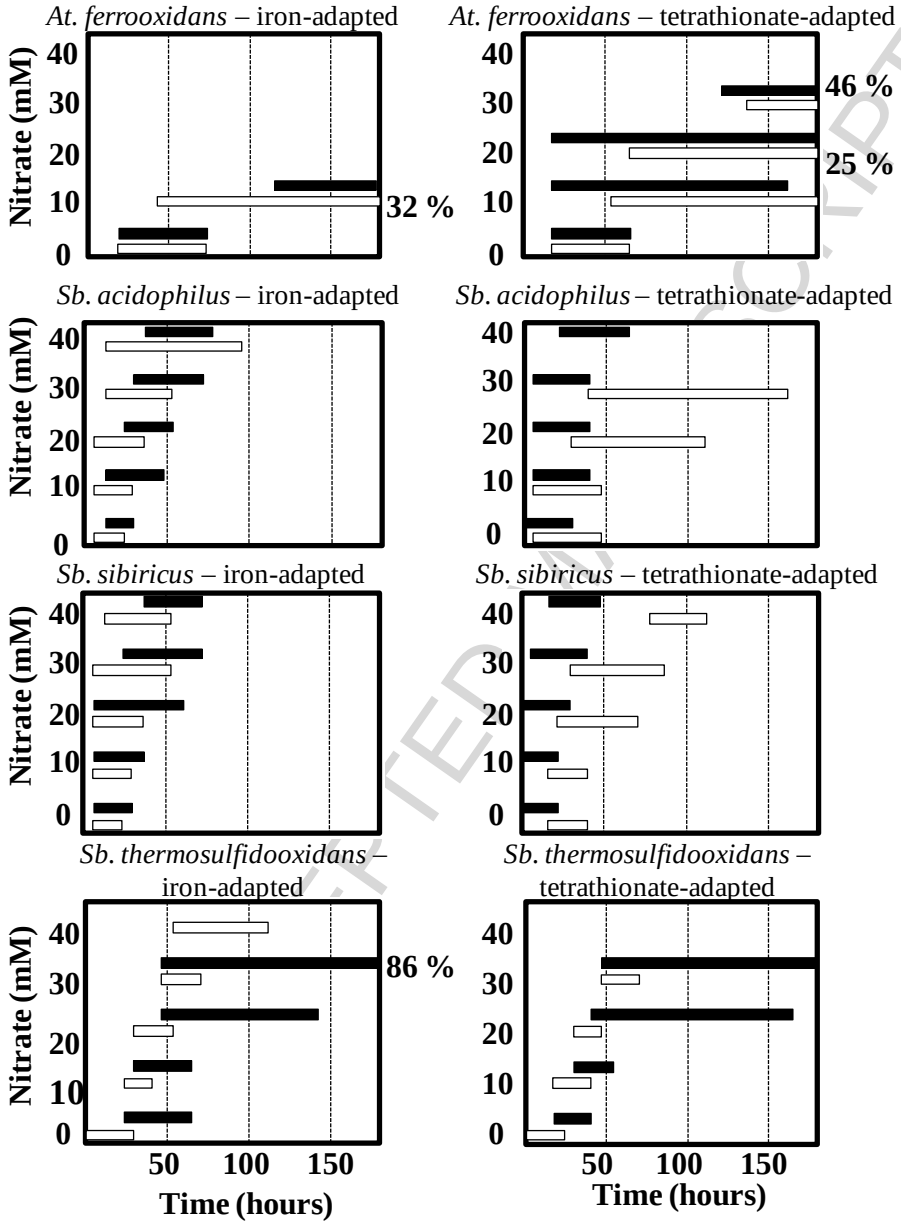

Figure 3. The effect of nitrate on chemolithotrophic bacteria with respect to the start and completion of ferrous ion (open bar) and tetrathionate (closed bar) utilisation. In those tests where oxidation of either substrate was incomplete in the experimental time frame, the percent oxidised relative to the starting substrate concentration is reported.

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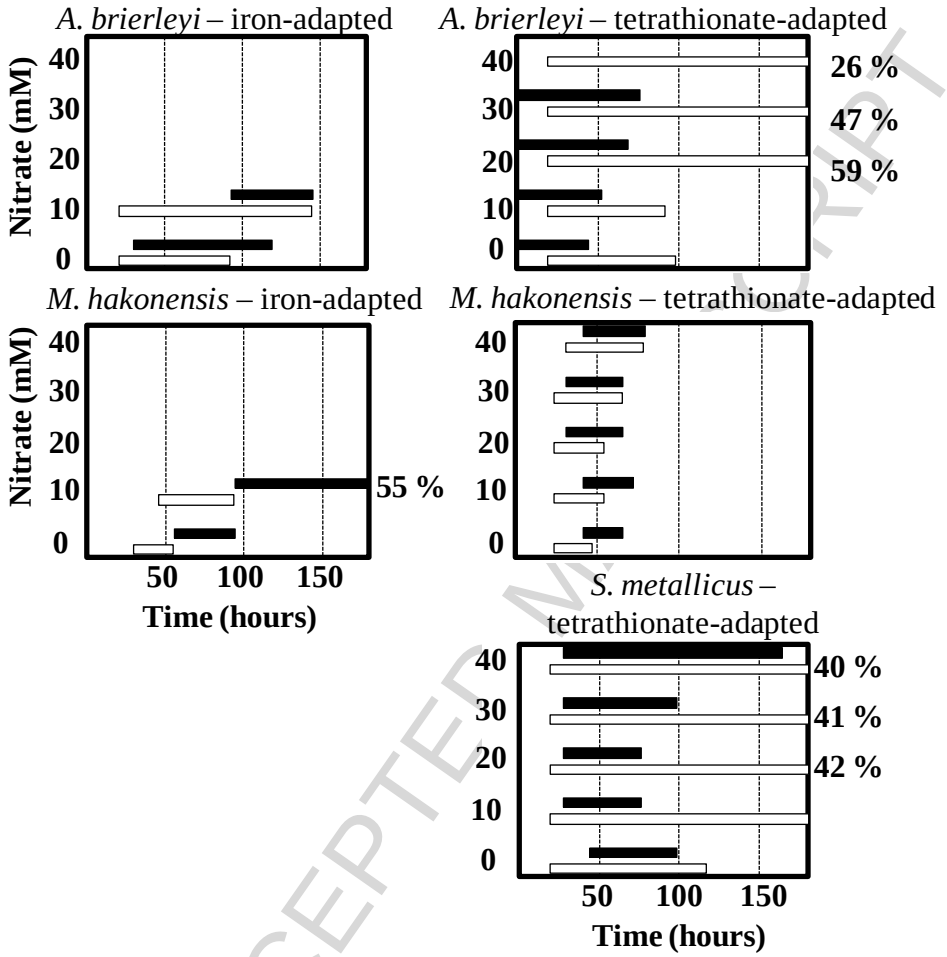

Figure 4. The effect of nitrate on archaea with respect to the start and completion of ferrous ion (open bar) and tetrathionate (closed bar) utilisation. In those tests where oxidation of either substrate was incomplete in the experimental time frame, the percent oxidised relative to the starting substrate concentration is reported.

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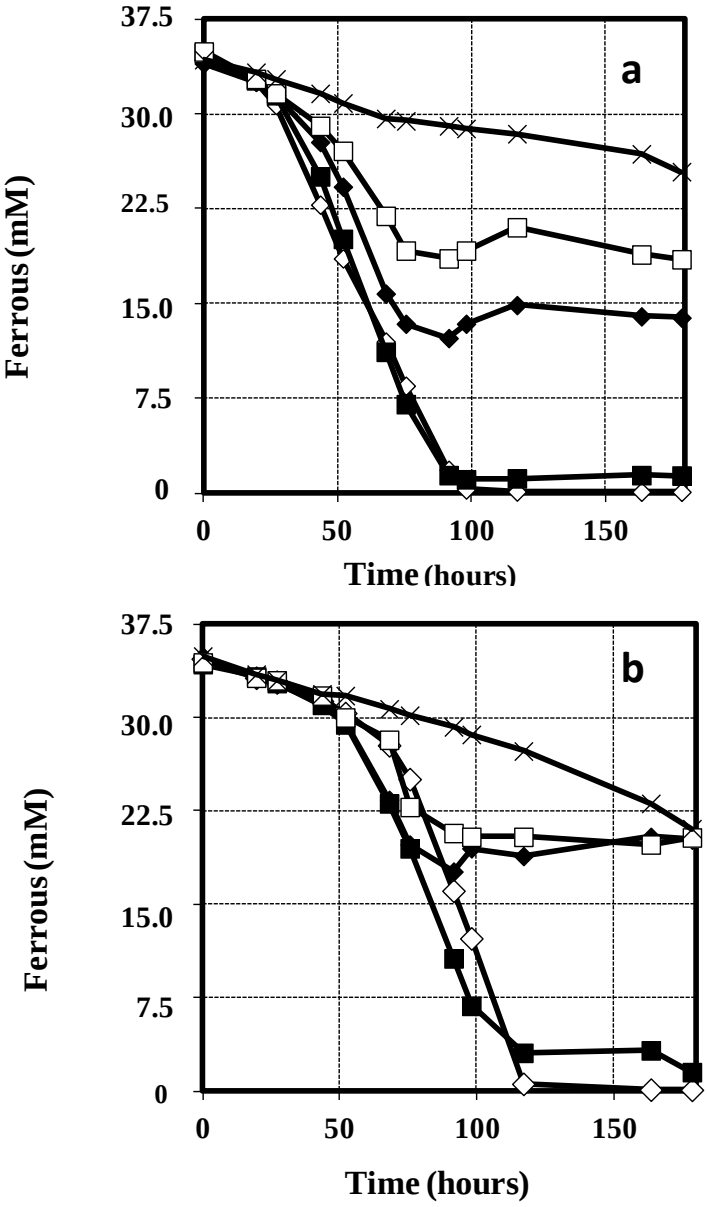

Figure 5. Inhibition of ferrous ion oxidation in batch cultures of *A. brierleyi* (a) and *S. metallicus* (b) in the presence of 0 ◇; 10 ■; 20 ◆; 30 □; and 40 ×mM nitrate.

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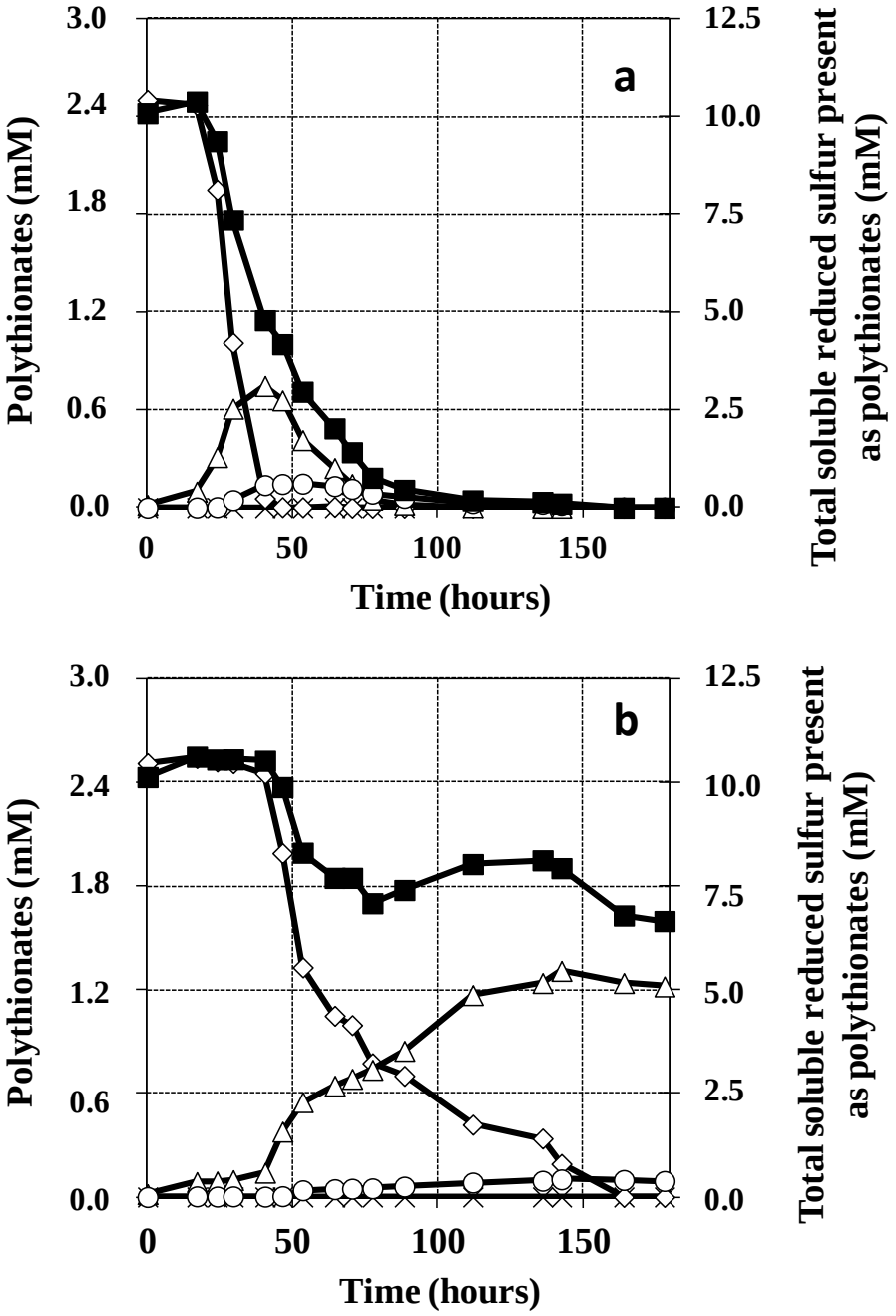

Figure 6. Total concentrations of polythionates present in batch cultures of *Sb. thermosulfidooxidans* at 0 mM (a) and 20 mM (b) nitrate. Tetrathionate ◇; pentathionate △; hexathionate ○; thiosulfate ×; and total sulfur present as polythionates ■.

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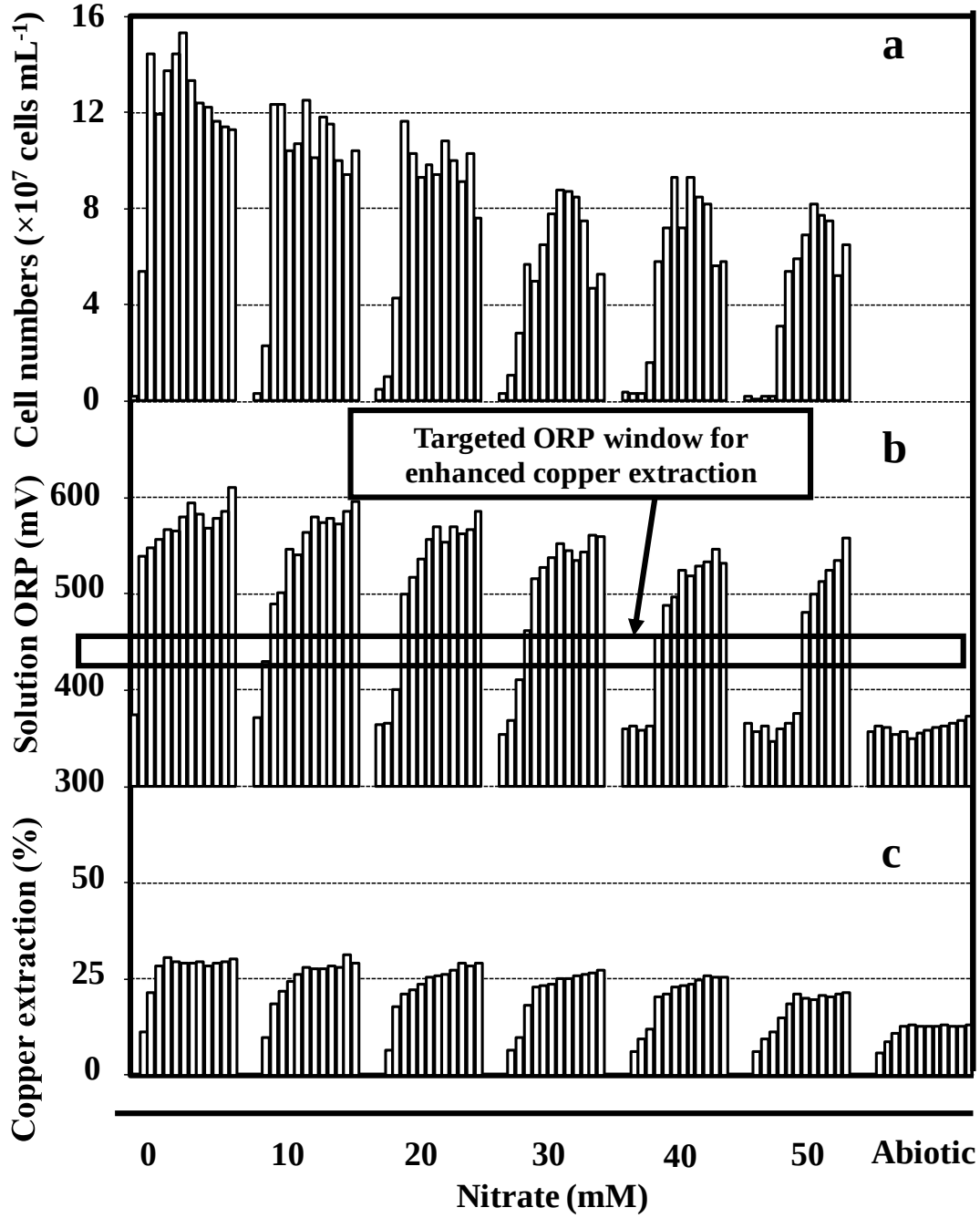

Figure 7. Copper (bio)extraction from chalcopyrite (1% pulp density) at 30 ºC using a mixed culture of bacteria and archaea in media containing nitrate. Data for cell numbers (a), solution ORP (b) and copper extractions (c). Final values for each week of the 12-week experiment are shown for each nitrate concentration. Complete copper extraction results in a concentration of 2.45 g L^{-1}.

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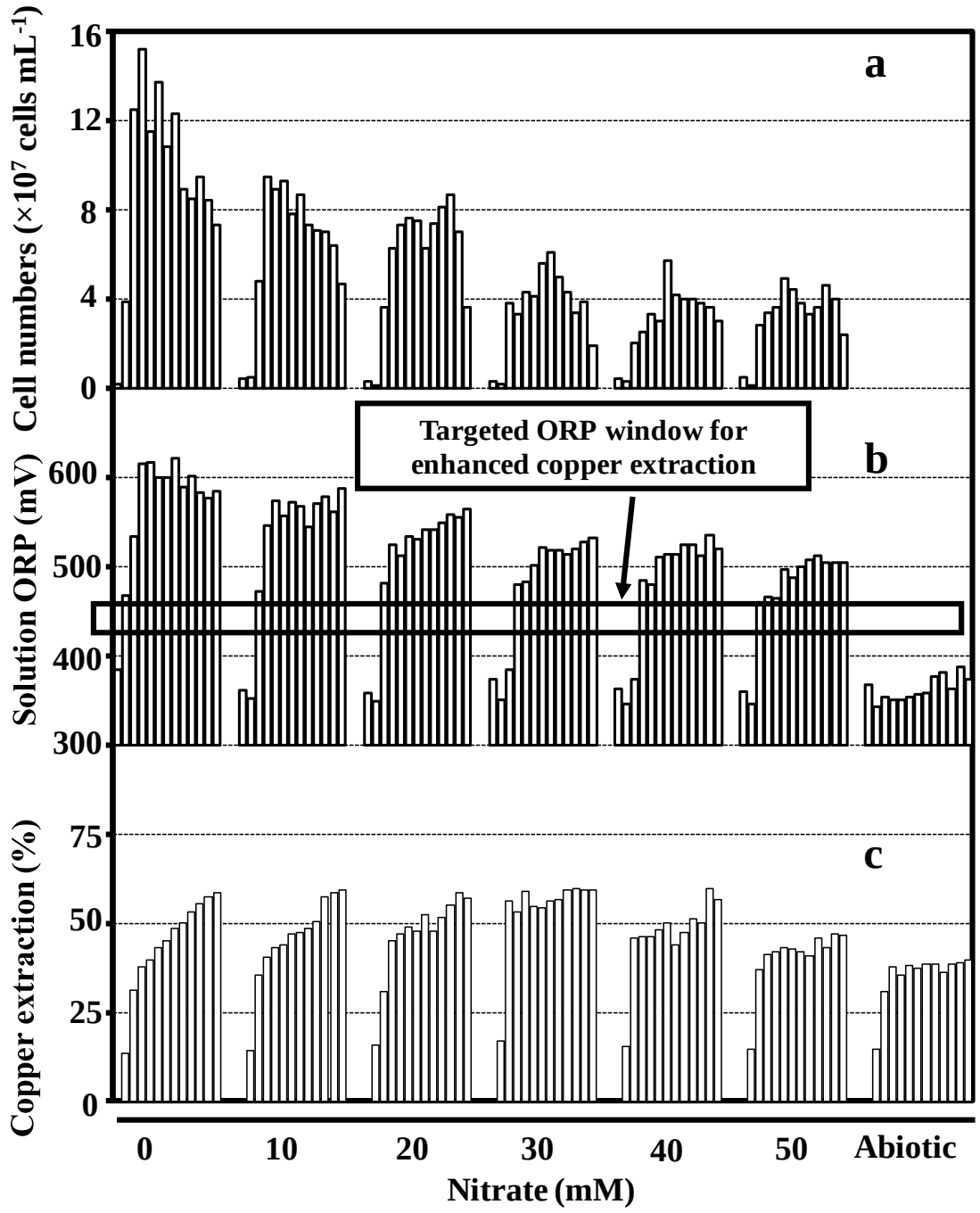

Figure 8. Copper (bio)extraction from chalcopyrite (1% pulp density) at 45 ºC using a mixed culture of bacteria and archaea in media containing nitrate. Data for cell numbers (a), solution ORP (b) and copper extractions (c). Final values for each week of the 12-week experiment are shown for each nitrate concentration. Complete copper extraction results in a concentration of 2.45 g L^{-1}.

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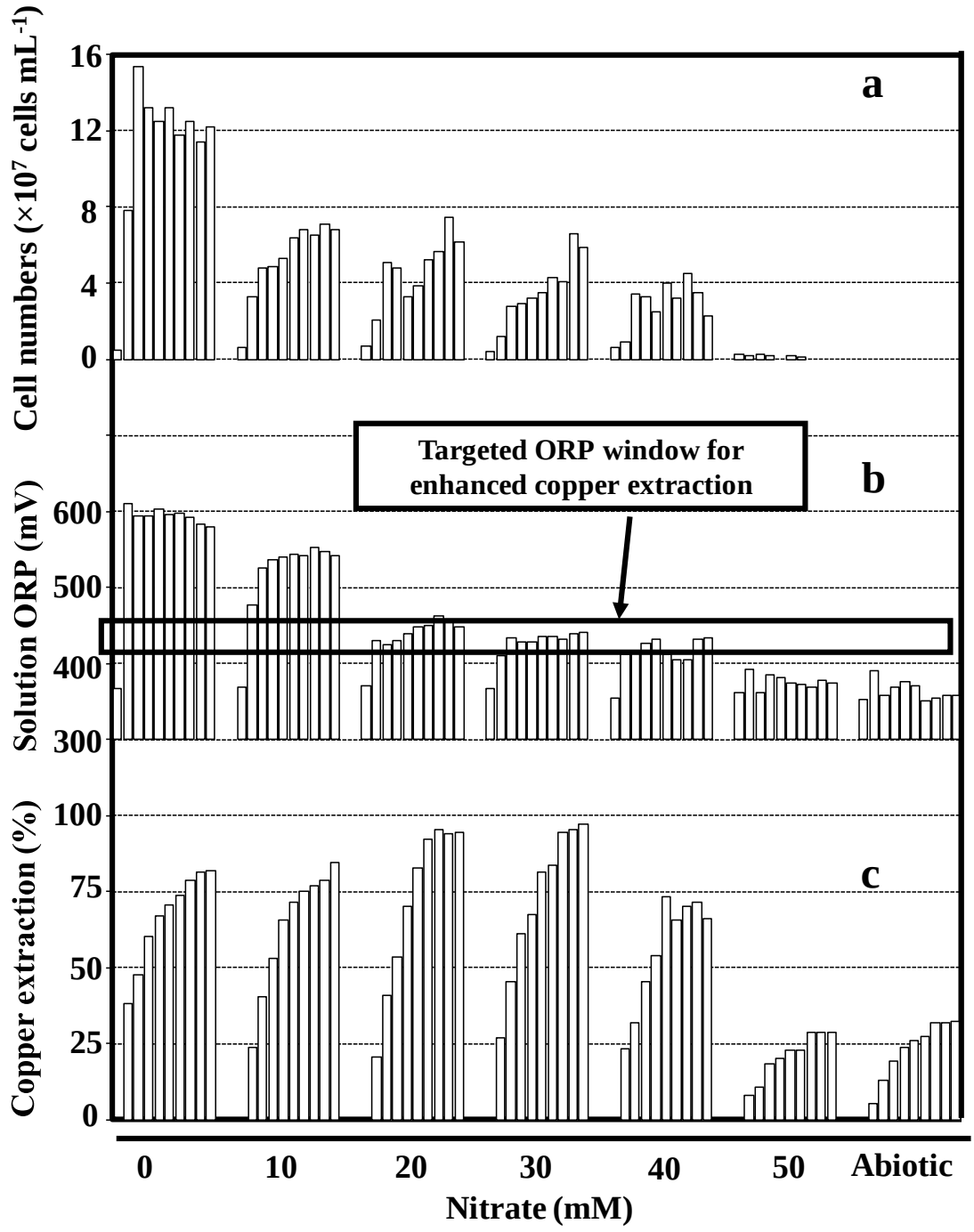

Figure 9. Copper (bio)extraction from chalcopyrite (1% pulp density) at 60 ºC using a mixed culture of bacteria and archaea in media containing nitrate. Data for cell numbers (a), solution ORP (b) and copper extractions (c). Final values for each week of the 10-week experiment are shown for each nitrate concentration. Complete copper extraction results in a concentration of 2.45 g L^{-1}.