Research in Microbiology 169 (2018) 552–557

Contents lists available at ScienceDirect

Research in Microbiology

journal homepage: www.elsevier.com/locate/resmic

Original Article

The significance of pH in dictating the relative toxicities of chloride and copper to acidophilic bacteria

Carmen Falagán [1], D. Barrie Johnson*

School of Natural Sciences, Deiniol Road, Bangor University, LL57 2UW, Bangor, UK

ARTICLE INFO

Article history:
Received 12 March 2018
Accepted 6 July 2018
Available online 19 July 2018

Keywords:
Acidophilic bacteria
Chloride
Copper
Membrane potentials
pH

ABSTRACT

The ability of acidophilic bacteria to grow in the presence of elevated concentrations of cationic transition metals, though varying between species, has long been recognized to be far greater than that of most neutrophiles. Conversely, their sensitivity to both inorganic and organic anions, with the notable exception of sulfate, has generally been considered to be far more pronounced. We have compared the tolerance of different species of mineral-oxidizing *Acidithiobacillus* and *Sulfobacillus*, and the heterotrophic iron-reducer *Acidiphilium cryptum*, to copper and chloride when grown on ferrous iron, hydrogen or glucose as electron donors at pH values between 2.0 and 3.0. While tolerance of copper varied greatly between species, these were invariably far greater at pH 2.0 than at pH 3.0, while their tolerance of chloride showed the opposite pattern. The combination of copper and chloride in liquid media appeared to be far more toxic than when these elements were present alone, which was thought to be due to the formation of copper–chloride complexes. The results of this study bring new insights into the understanding of the physiological behaviour of metal-mobilising acidophilic bacteria, and have generic significance for the prospects of bioleaching copper ores and concentrates in saline and brackish waters.

1. Introduction

Acidophilic microorganisms grow optimally in low pH environments (<3, in the case of extreme acidophiles) which can vary greatly in temperature and concentrations of dissolved solutes [1]. Generally, both extremely acidic natural (e.g. solfatara fields) and anthropogenic (e.g. metal mine-impacted sites) environments tend to contain relatively little organic carbon, and chemolithotrophy (the ability to grow using inorganic electron donors) is a very common trait amongst indigenous prokaryotes. Acidity in many of these environments derives from the oxidation of zero-valent (elemental) sulfur (ZVS) or sulfide minerals, and consequently the dominant anion present is almost invariably sulfate, though elevated concentrations of chloride have also been found in some locations (e.g. [2]). Direct dissolution of metal sulfides and indirect dissolution of acid-labile alumino-silicates and other minerals results in many low pH environments containing highly elevated concentrations of transition and non-transition metals, and metalloids such as arsenic [2,3]. While pH values can vary considerably (e.g. as reported within the Richmond mine at Iron Mountain, California [4]) they are subject to control by two buffering systems: sulfate/bisulfate (SO_4^{2-}/HSO_4^-; pK_a 1.9), and that associated with the precipitation and dissolution of ferric hydroxysulfate minerals, such as schwertmannite (equation (1)). The latter is responsible for maintaining the pH of acidic ferruginous streams and rivers (such as the Tinto river in south-west Spain) at ~ 2.5 [5].

$$8\ Fe^{3+} + SO_4^{2-} + 14\ H_2O \leftrightarrow Fe_8O_8(OH)_6(SO_4) + 22\ H^+ \quad (1)$$

Acidophilic prokaryotes have been described as being more tolerant of cationic transition metals but far less tolerant of organic and inorganic anions (other than sulfate) than their neutrophilic counterparts (e.g. [3, 6, 7]). However, this generalisation hides the fact that some cationic metals (e.g. monovalent silver) are highly toxic, and that different species can display great differences in tolerance to metals, such as strains of *Leptospirillum ferrooxidans* and *Leptospirillum ferriphilum* which differ in copper sensitivities by two orders of magnitude [8]. This generic trait has been ascribed to the fact that, in contrast to neutrophilic prokaryotes that are "negatively charged", acidophiles tend to have positive membrane

* Corresponding author.
E-mail addresses: c.falagan@exeter.ac.uk (C. Falagán), d.b.johnson@bangor.ac.uk (D.B. Johnson).
[1] Present address: CSM, College of Engineering, Mathematics and Physical Sciences, University of Exeter, Penryn, Cornwall TR10 9FE, UK.

https://doi.org/10.1016/j.resmic.2018.07.004

potentials (ΔΨ, the charge difference between either side of the cell membrane [6]). This is achieved by intracellular accumulation of benign inorganic cations such as potassium and is necessitated by the fact that, by growing in low pH liquors and maintaining cytoplasmic pH values close to neutral, acidophiles have extremely large pH gradients (ΔpH) between either side of their membranes which need to be maintained even when cells are not metabolically active to avoid acidification of the cytoplasm, resulting in cell death. The trans-membrane proton electrochemical potential (Δρ, or proton motive force) derives from the combination of membrane potential and pH gradient (equation (2) [6]).

$$\Delta\rho = \Delta\Psi - 59 \cdot \Delta pH \quad (2)$$

While being positively charged confers an inherent resistance to cationic metals, it facilitates the influx of permeable anions into both active and resting cells, causing these to be problematic to acidophiles (Fig. 1). Influx of chloride ions into acidophile cells can cause the collapse of ΔΨ values, and therefore of proton motive force, as well as a general poisoning of the cytoplasm [6]. In many situations, this potential problem is not an issue that indigenous acidophile populations encounter since, as mentioned, chloride-rich extremely acidic environments are relatively rare. Increasingly, however, it is of major consequence in the major biotechnology that utilizes these extremophiles – the bio-processing of sulfide mineral ores and concentrates (biomining). Many primary ores that could be potentially biomined are located in arid and semi-arid areas where water availability is limited and quality is often poor [9]. Irrigating heaps and dumps with acidic brackish or saline waters could limit or totally inhibit indigenous acidophiles carrying out their primary role in catalysing the oxidative dissolution of sulfide minerals, the ongoing regeneration of ferric iron from ferrous, as iron-oxidizing acidophiles are generally more prone to chloride inhibition than other groups, such as sulfur-oxidizing chemolithotrophs and heterotrophic acidophiles [9]. The iron/sulfur-oxidizing gammaproteobacterium *Acidihalobacter prosperus* (named originally as *Thiobacillus prosperus*) has been reported to grow optimally on ferrous iron as electron donor in liquid media containing 20 g L^{-1} of NaCl (~340 mM Cl^-) [10,11]

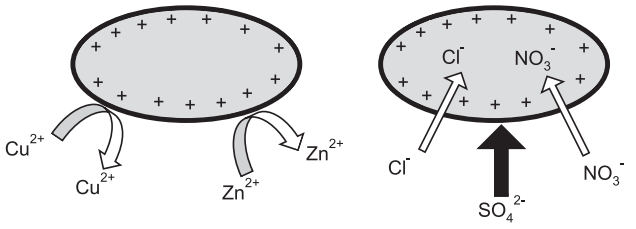

Fig. 1. Schematic representation of the generic influence of membrane potentials developed by acidophilic prokaryotes on their tolerance and sensitivity to cationic transition metals and non-metallic anions. Sulfate differs from other inorganic anions, such as chloride, in being relatively non-permeable.

though its bioleaching potential is somewhat compromised by its relative sensitivity to transition metals such as copper [10].

A previous report [12] found that 0.5–1% of viable acidophilic iron-oxidizing acidophiles in the Tinto river, which contains relatively little (typically <10 mM) chloride, were able to grow on selective solid media containing 500 mM NaCl, with ferrous iron as the sole electron donor. One of the salt-tolerant isolates (strain ST2) was identified as a strain of *Acidithiobacillus* (*At.*) *ferriphilus*, a recently described species which, like other iron-oxidizing acidithiobacilli, has been reported to tolerate elevated concentrations of transition metals [13] and would therefore appear to be a suitable candidate for bioleaching with saline/brackish lixiviants. However, while strain ST2 could grow in liquid media containing up to 800 mM NaCl with ZVS as electron donor, growth in liquid media containing ferrous iron did not occur in salt concentrations above 350 mM. This apparent anomaly prompted further experiments with *At. ferriphilus* ST2, and subsequently more generic research into pH-related chloride and copper tolerance in acidophilic prokaryotes.

2. Materials and methods

2.1. Bacteria and culture media

Eight bacterial strains, including six species and two genera of mineral-oxidizing acidophiles, were used in the current work (Table 1). All of the strains were able to use ferrous iron, ZVS and hydrogen as electron donors except *At. ferriphilus* ST2 which, like other strains of this species, does not use hydrogen [13], and the obligate heterotroph *Acidiphilium* (*A.*) *cryptum* strain SJH. The chemolithotrophic acidophiles were maintained in a liquid medium containing 10 mM ferrous iron, basal salts and trace elements [14] (supplemented with 0.02% w/v yeast extract for the *Sulfobacillus* strains) at pH 2.0 in shake flasks maintained under atmospheric air. Strains that could grow on hydrogen were also maintained in 50 μM ferrous iron, basal salts and trace elements (pH 2.0; with and without yeast extract) medium in 25 mL universal bottles in a sealed 2.5 L jar under H_2/CO_2-enriched air. The latter was prepared by adding 10 mL of water to a mixture of 1.3 g sodium bicarbonate, 0.3 g sodium borohydride and 0.1 g citric acid, in an open 25 mL universal bottle, which was also placed in the sealed jar [15]. The obligate heterotroph *A. cryptum* SJH was routinely sub-cultured in a liquid medium containing 5 mM glucose, basal salts and trace elements, pH 2.5. All of the *Sulfobacillus* strains were moderately thermophilic and were incubated at 45 °C, while the mesophilic acidithiobacilli and *A. cryptum* SJH were grown at 30 °C.

2.2. Bioreactor cultures of A. ferriphilus ST2

At. ferriphilus ST2 was grown in 5 mM ferrous iron liquid medium in a 2.3 L (1 L working volume) aerated (at ~1 L min^{-1}) and stirred (50 rpm) bioreactor (Electrolab Ltd., UK) at pH values

Table 1
Acidophilic bacteria used in the current work.

Bacterium	Metabolism	Reference
At. ferrooxidans^T	Fe^{2+}/ZVS/H_2- oxidizing obligate autotroph	[19]
At. ferridurans^T	Fe^{2+}/ZVS/H_2- oxidizing obligate autotroph	[20]
At. ferriphilus strain ST2	Fe^{2+}/ZVS- oxidizing obligate autotroph	[13]
At. thiooxidans DSM 103717	ZVS/H_2- oxidizing obligate autotroph	[21]
S. thermosulfidooxidans^T	Fe^{2+}/ZVS/H_2- oxidizing facultative autotroph	[22]
S. thermosulfidooxidans strain BOR3	Fe^{2+}/ZVS/H_2- oxidizing facultative autotroph	This study
S. acidophilus strain BOR2	Fe^{2+}/ZVS/H_2- oxidizing facultative autotroph	This study
A. cryptum strain SJH	Fe^{3+}-reducing obligate heterotroph	[23]

set at between 2.0 and 3.0 (controlled by automated addition of sulfuric acid or sodium hydroxide) and at a constant temperature of 30 °C. The relatively low concentration of ferrous iron used was to minimize formation and accumulation of ferric iron precipitates.

To assess the tolerance of *At. ferriphilus* ST2 to chloride at different pH values, cultures were first grown in ferrous iron medium containing 500 mM NaCl at pH 3.0. Samples were withdrawn from the bioreactor at regular intervals and residual concentrations of ferrous iron determined using the Ferrozine assay [16]. Following this, a series of experiments were set up using the same medium but with the bioreactor maintained at increasingly lower pH values (down to pH 2.2). To assess tolerance to copper at different pH values, this acidophile was first grown in a shake flask culture containing 10 mM ferrous iron, 100 mM copper sulfate, basal salts and trace elements. This was used to inoculate a bioreactor culture containing the same medium (with ferrous iron lowered to 5 mM) and maintained at pH 2.0. Following completion of iron oxidation, the experiment continued by replacing the growth medium and setting the bioreactor to increasingly higher pH values.

2.3. pH-related tolerance of copper and chloride of other Acidithiobacillus *spp. and* Sulfobacillus *spp. grown on hydrogen*

Experiments were carried out using hydrogen as sole electron donor for those strains of acidophilic bacteria that were known to grow on this inorganic energy source (Table 1). A major advantage of doing this was that, in contrast to using either ferrous iron or reduced sulfur (or ZVS), pH changes during growth on hydrogen tended to be <0.1 pH unit, thereby allowing multiple small volume batch cultures to be set up simultaneously rather than to be restricted to bioreactor cultures. Liquid media containing basal salts and trace elements and varying concentrations of NaCl (5–800 mM) or $CuSO_4$ (1–500 mM) were prepared at pH 2.0 and 3.0, and sterilized by filtration through 0.2 μm pore-size polyethersulfone (PES) membrane filters (Fisher, UK) to avoid pH changes caused by heat sterilization. Duplicate small-volume (5 mL) cultures in 25 mL universal bottles were then inoculated with the type strains of *Acidithiobacillus ferrooxidans, Acidithiobacillus ferridurans* or *S.ulfobacillus* (*S.*) *thermosulfidooxidans*, or with *Acidithiobacillus thiooxidans* DSM 103717, *S. thermosulfidooxidans* strain Bor 3 or *S.ulfobacillus acidophilus* strain Bor 2. The unwashed inocula came from cultures pre-adapted to copper or chloride, and were added at 2% v/v to give initial cell densities of ~10^6 mL^{-1}. The bottles were placed in 2.5 L air-tight jars and incubated under H_2/CO_2-enriched air, as described above. The sealed jars were incubated on shaking platforms at either 30 °C (mesophilic strains) or 45 °C (moderately thermophilic strains). Cultures were incubated for either one week (the acidithiobacilli) or two weeks (the sulfobacilli) when they were withdrawn and growth estimated by measuring OD values at 600 nm (against copper-containing blanks, where appropriate). Optical density measurements provided a rapid and accurate measure of biomass in hydrogen-grown cultures as, in contrast to cultures grown on ferrous iron or reduced sulfur, there was no interference with a strongly-coloured metabolic product (ferric iron) or colloidal sulfur. Correlations between cell numbers and OD_{600} values were evaluated for each acidophile used, during exponential growth. In the case of *Acidithiobacillus* spp., and OD_{600} value of 0.5 was equivalent to between 1.0 and 2.1 × 10^9 cells mL^{-1}. For *A. cryptum* SJH this was slightly lower (8.9 × 10^8 cells mL^{-1}), and for the sulfobacilli (which formed larger cells than the Gram-negative acidophiles used), an OD_{600} of 0.5 corresponded to ~2.5–7.0 × 10^8 cells mL^{-1}.

2.4. pH-related tolerance of copper and chloride of A. cryptum *strain SJH grown on glucose*

Cultures of *A. cryptum* SJH were grown in liquid media containing 5 mM glucose, basal salts and trace elements, at either pH 2.0 or 3.0. Initial tests were carried out to establish the maximum concentrations of copper (at pH 2.0) and chloride (at pH 3.0) at which this heterotrophic acidophile could grow. Following this, an experiment was set up using a similar protocol to that described in Section 2.2 in which growth of *A. cryptum* SJH was assessed at pH 2.0, 2.5 and 3.0 in media containing different concentrations of copper or chloride.

2.5. Synergistic impacts of chloride and copper on the growth of At. ferrooxidans, At. ferridurans *and* At. thiooxidans

Liquid media containing 50 mM or 100 mM NaCl and $CuSO_4$, basal salts and trace elements were adjusted to pH 2.0 and 3.0 with sulfuric acid and sterilized by filtration through 0.2 μm pore-size PES membrane filters. Duplicate small-volume (5 mL) cultures in 25 mL universal bottles were then inoculated with the type strains of *At. ferrooxidans, At. ferridurans* or with *At. thiooxidans* DSM 103717. These were placed in a 2.5 L air-tight jar, and incubated under H_2/CO_2-enriched air, as described above. Bacterial growth was estimated after one week by measuring OD values at 600 nm against copper-containing blanks.

2.6. Bioleaching a copper concentrate with At. ferriphilus *ST2 in the presence of 500 mM NaCl*

A 2 L (working volume) bioreactor was set up in batch mode to assess whether *At. ferriphilus* ST2 was able to bioleach a copper concentrate at a pH value where it was able to grow and oxidize ferrous iron in the presence of 500 mM NaCl. The bioreactor vessel was filled with 1.5 L of medium containing 500 mM sodium chloride, 25 mM ferrous sulfate, basal salts and trace elements, 37.5 g of a copper concentrate containing ~30% copper (sourced from the Sossego copper mine Brazil; the concentrate contained 83% chalcopyrite, 4% bornite, 3% each of pyrite, chlorite and amphibolite, and 1% plagioclase) added, followed by 100 mL of an active culture of *At. ferriphilus* ST2, grown in 500 mM NaCl/25 mM Fe(II) liquid medium at pH 3. The bioreactor vessel was stirred at 150 rpm, aerated with ~1 L air/min, temperature set at 30 °C, and pH maintained at 3.0 ± 0.05 by automated addition of 1 M NaOH or H_2SO_4. Samples were withdrawn at regular intervals to determine concentrations of soluble iron [16] and copper [17] and to measure redox potentials. A control bioreactor culture (also maintained at pH 3 and containing 500 mM NaCl) in which pyrite (20 g L^{-1}) replaced chalcopyrite was also set up. The presence of metabolically-active/viable bacteria was tested for by plating samples onto solid ferrous iron-containing overlay medium [18].

3. Results

At. ferriphilus ST2 grew and oxidized ferrous iron in liquid medium containing 500 mM NaCl in batch cultures in a bioreactor maintained at pH 3.0. The acidophile continued to oxidize iron in the presence of 500 mM salt when the bioreactor culture pH was progressively lowered to pH 2.4 (Fig. 2). However, at pH 2.2 after a small amount of iron had been oxidized, this came to a halt and most (~70%) of the available ferrous iron remained non-oxidized. Separate tests carried out in shake flasks confirmed that ferrous iron oxidation by *A. ferriphilus* ST2 was completely inhibited in pH 2.0 medium that contained 500 mM NaCl (data not shown).

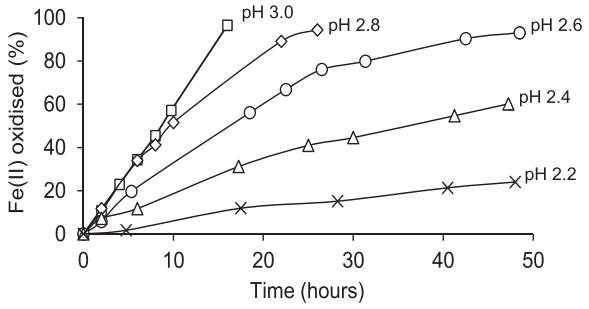

Fig. 2. Ferrous iron oxidized by *At. ferriphilus* ST2 in media containing 500 mM NaCl at set and maintained pH values.

Opposite trends were observed for copper tolerance by *At. ferriphilus* ST2 grown on ferrous iron. In pH 2.0 liquid medium this acidophile oxidized ferrous iron in the presence of 300 mM (though not 500 mM) copper, while in the same medium poised initially at pH 3.0 ferrous iron oxidation was observed in the presence of 20 mM, but not 50 mM, copper.

All six acidophiles that grew using hydrogen as sole electron donor displayed greater tolerance to chloride when grown at pH 3.0 than at pH 2.0 (Fig. 3). There were, however, major differences in the abilities of these acidophiles to tolerate chloride at both pH values, with *At. ferridurans*^{T} growing in liquid medium containing up to 700 mM NaCl at pH 3, while growth of *S. acidophilus* strain Bor 2 at the same pH was limited to 50 mM. In every case, except *At. ferrooxidans*^{T}, copper tolerance showed the opposite pH-related trend, being more toxic to the acidophilic bacteria examined at pH 3.0 than at pH 2.0 (Fig. 3). Again, there were major variations between bacteria, with the type strains of both *At. ferrooxidans* and *At. ferridurans* growing in the presence of 250 mM copper at pH 2.0, while the maximum concentration at which growth of *S. acidophilus* Bor 2 was observed at the same pH was 10 mM. Both *At. thiooxidans* DSM 103717 and *S. acidophilus* Bor 2 were extremely sensitive to copper at pH 3, with the maximum concentration at which growth was observed being 1 mM. Tests carried out subsequently (by sub-culturing into copper- and chloride-free media) confirmed that viable bacteria were recovered from all cultures where positive growth was reported, but not from those categorized as being inhibited.

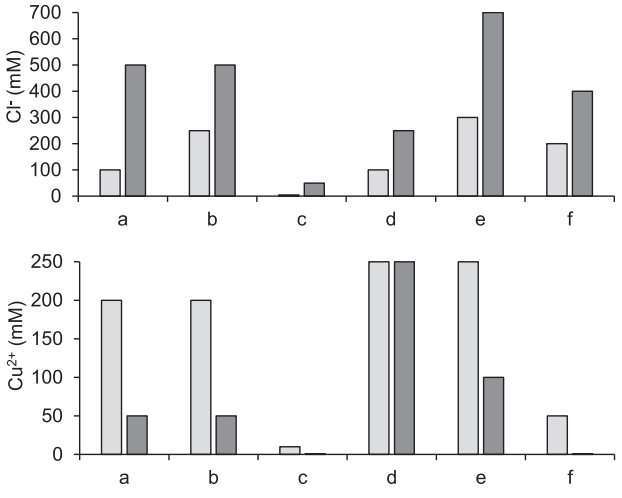

Fig. 3. Maximum concentrations of chloride and copper at which growth of the acidophilic bacteria tested was observed, with hydrogen as electron donor, at pH 2.0 (light grey bars) or 3.0 (dark grey bars); a, *S. thermosulfidooxidans*^{T}; b, *S. thermosulfidooxidans* BOR3; c, *S. acidophilus* BOR2; d, *At. ferrooxidans*^{T}; e, *At. ferridurans*^{T}; f, *At. thiooxidans* DSM 103717. Cultures were considered to show "no growth" when OD_{600} values were <0.02 (0.01 for *Sulfobacillus* spp.), and compared to that of the cation/anion free-cultures (where OD_{600} were typically >0.50). Initial OD_{600} values were ~0.01.

A. cryptum SJH grew in glucose medium at pH 2.0 that contained up to 15 mM copper, and in the presence of 500 mM NaCl in pH 3.0 liquid medium. As with the chemolithotrophic bacteria examined, the obligate heterotroph *A. cryptum* SJH was more sensitive to copper at higher pH but more tolerant of chloride (Table 2). No growth was observed at pH 2.0 in medium containing 100 mM NaCl, and the heterotroph displayed poor growth at pH 3.0 in the presence of 5 mM copper. *A. cryptum* SJH was marginally more tolerant to chloride at pH 2.5 than it was at pH 3.0 (Table 2).

Experiments carried out in which copper and chloride were added together to hydrogen-grown cultures showed that combinations of the two elements were more toxic to the three *Acidithiobacillus* spp. tested than when they were added alone (Fig. 4) at both pH 2.0 and 3.0, though *A. thiooxidans* displayed a similar tolerance to copper (at pH 2.0) in both the presence and absence of chloride.

Bioleaching of the chalcopyrite concentrate by *At. ferriphilus* at pH 3.0 in the presence of 500 mM NaCl was highly ineffective (Fig. 5), though the same concentrate was readily bioleached by this iron-oxidising acidophile under more "standard" conditions (pH 2, in the absence of added salt; data not shown). Some iron and copper was solubilised rapidly, presumably by acid dissolution of chalcopyrite and other minerals, but thereafter concentrations of both metals fluctuated, presumably due in part to formation of secondary precipitates, but showed no consistent increases. Virtually all of the soluble iron (up to 80 mM) was present as ferrous iron, which was also reflected in the relatively low redox potentials (E_H values of between +526 and +624 mV) measured during this experiment, which was anticipated since ferric iron is highly insoluble at pH 3. Relatively low redox potentials were also observed in the parallel bioreactor culture containing pyrite, though concentrations of soluble ferrous iron were much lower (6–8 mM) and, in contrast to the chalcopyrite culture, viable bacteria were present throughout the 44 day incubation period (as evidenced by plating samples on overlay medium).

4. Discussion

Data from experiments carried out in the present study help explain some previous apparently anomalous published findings, but also provide a cautionary note for how metal and chloride tolerance and toxicity data for acidophilic microorganisms are reported and interpreted. The conundrum of why a strain of *At. ferriphilus* (ST2) could not grow on ferrous iron in liquid media containing >350 mM NaCl, even though it was isolated as an iron-oxidizing colony found on a solid medium that contained 500 mM salt and grow on ZVS in liquid media containing up to 800 mM NaCl, can be put down to the different (initial) pH values of these media (2.0 and 2.8 for liquid and solid iron media, and 3.0 for the ZVS medium). Ferrous iron liquid media used for acidophiles are usually set initially at low pH values to avoid precipitation of the ferric iron generated (iron oxidation is a proton-consuming reaction) while oxidation of ZVS generates acidity so batch cultures often have initial pH values of ~2.5–3.0. Growing chemolithotrophic acidophiles in pH-controlled bioreactors can eliminate the problem of pH fluctuations associated with growth in batch cultures, as can using hydrogen as electron donor, though this is not

Table 2
Growth of *A. cryptum* SJH at pH 2.0, 2.5 and 3.0 in the presence of different concentrations of copper and chloride.

Copper				
Concentration (mM)	5	10	15	20
pH 2.0	++	++	++	+
pH 2.5	++	+	–	–
pH 3.0	+	–	–	–
Chloride				
Concentration (mM)	100	250	500	700
pH 3.0	++	++	++	–
pH 2.5	++	++	++	+
pH 2.0	–	–	–	–

++, extensive growth (OD_{600} > 0.75); +, restricted growth (OD_{600} <0.75, >0.02); –, no growth (OD_{600} < 0.02).

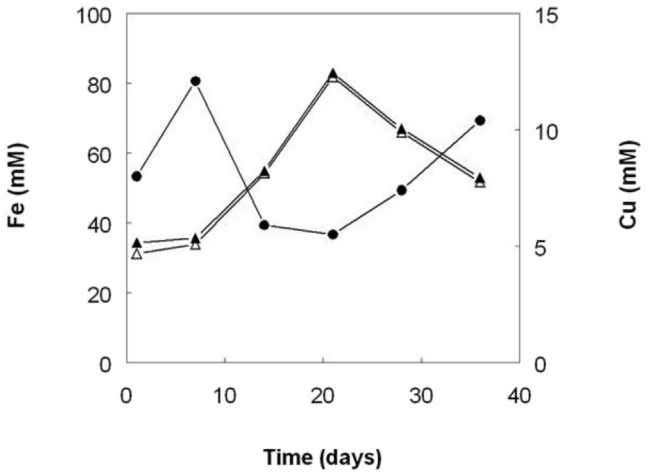

Fig. 5. Changes in concentrations of total soluble iron (▲), ferrous iron (Δ) and copper (●) in a bioreactor containing 2.5% (w/v) chalcopyrite and 500 mM NaCl, and maintained at pH 3.0 (at 30°).

appropriate for species, such as *At. ferriphilus* and *A. cryptum*, that do not use this electron donor.

Further experiments with hydrogen-grown cultures showed that other mineral-oxidizing acidophiles that had not been selected specifically on their basis of chloride tolerance could, in some cases, be cultivated to high cell densities (>10^9 cells mL^{-1}) in liquid media containing salt concentrations similar to those of marine waters, so long as the medium pH was ~3.0. At least in the case of *At. ferridurans*^{T}, the maximum concentration of NaCl tolerated was fairly close to that reported for the "benign" salt $MgSO_4$ [20] suggesting that the upper tolerance limit was more probably an osmotic potential barrier rather than one related specifically to NaCl. Chloride toxicity, however, was far more acute in extremely acidic (pH 2.0) media for all the acidophilic bacteria examined, but conversely their sensitivities to cationic copper increased as the medium pH increased.

At least a partial explanation for these observations comes from considering how different growth medium pH values impact the theoretical membrane potentials of the acidophilic bacteria investigated. Resting (metabolically-inactive) cells have Δρ values of zero, and for an acidophile with a typical cytoplasmic pH of 6.5 bathing in a pH 2.0 liquid medium (ΔpH = 4.5), according to equation (2) it has to achieve a ΔΨ of +266 mV and to maintain this in order to remain viable. If the pH of the external liquor is 3.0, ΔΨ is still positive but lower (+206 mV). Energized, or metabolically-active, cells have variable Δρ values, though +240 mV has been quoted as a typical value [6]. In this case, an acidophile cell would have a ΔΨ value of +26 mV at pH 2.0, 0 mV at pH ~2.5, and −34 mV at pH 3.0. The implications of this are: (i) at pH 2.0, acidophiles would accumulate non-specific anions such as chloride but retain an intrinsic resistance to the influx of cationic transition metals such as copper, irrespective of whether they are metabolically active or not; (ii) energized cells would attract cationic metals but repel anions at pH 3.0 but the opposite would be the case in pH 2.0 liquid media; (iii) acidophiles become increasingly susceptible to non-specific influx of anions as they become less metabolically active, and their membrane potentials become more positive. Points (i) and (ii) are supported by data from the current experiments, while point (iii) is supported by the observation that post-stationary phase cultures of acidophiles lose viability far more rapidly in media that contain chloride (D.B. Johnson, unpublished data), and a report that de-energised *At. ferrooxidans* cells are more sensitive to chloride and other inorganic anions than are energized cells [24]. It was interesting to note that the tolerance of *A. cryptum* SJH to chloride was marginally greater at pH 2.5 (when cells would be theoretically uncharged) than at pH 3.0, though this heterotroph was again far more prone to salt inhibition at pH 2.0 when cells would have had positive ΔΨ values.

Variations in the polarity and magnitude of membrane potentials are, however, insufficient to account for all of the results obtained both in this study (e.g. species-dependent tolerance of both copper and chloride, which showed great variation) and elsewhere. Dopson and Holmes [7] differentiated between

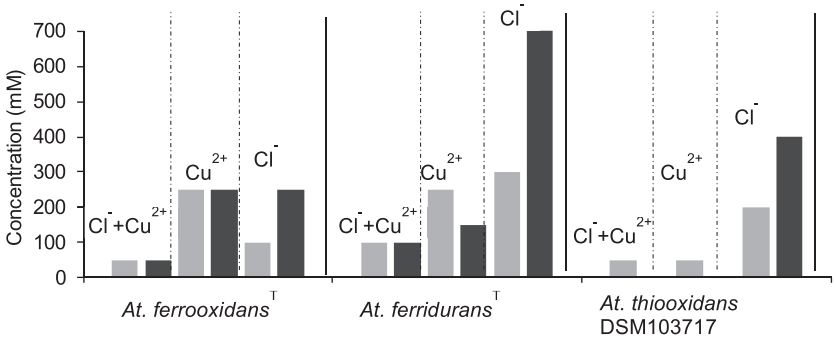

Fig. 4. Maximum concentrations of copper and chloride, added either alone or together, at which cultures of *At. ferrooxidans*^{T}, *At. ferridurans*^{T}, and *At. thiooxidans* DSM 103717 were able to grow at pH 2.0 (grey bars) or 3.0 (black bars) with hydrogen. Cultures were considered to show "no growth" when OD_{600} values were <0.02 and compared to that of the copper/chloride free-cultures (where OD_{600} were typically >0.50). Initial OD_{600} values were typically ~0.01.

passive (e.g. membrane potential barriers) and active (e.g. using efflux proteins to pump metals out of cells and conversion to less toxic forms) mechanisms exhibited by acidophiles for resisting metals and metalloids, the latter considered likely to be species (or even strain) specific. Enhanced tolerance to copper has been found, in at least one case, to correlate with the presence of greater numbers of metal-exporting proteins that may have been acquired by horizontal gene transfer [25]. Intracellular phosphatases have also been proposed as potential metal carriers, mediating the export of metals from acidophilic cells [26]. Acidophiles respond to generic osmotic stress by synthesising intracellular osmotic protectants, such as trehalose and ectoine [8,9]; less is known about their response to specific chloride stress, though maintaining and repairing damaged cell membranes and increased use of efflux pumps appears to be among these [11,27].

Transition metals can be complexed by a variety of inorganic ligands, including sulfate [28], which impacts their toxicities to acidophiles [7]. In the present study, the addition of non-inhibitory concentrations of chloride to copper-containing media at pH 2.0 greatly enhanced the sensitivities of both *At. ferrooxidans* and *At. ferridurans* to copper, and likewise including non-inhibitory concentrations of copper in pH 3.0 media appeared to lower their tolerance of chloride. Chloride acts as a ligand of both copper (I) and copper (II), forming a variety of complexes that can be positively (e.g. $CuCl^{+}$) or negatively (e.g. $CuCl_4^{2-}$) charged, or uncharged (e.g. $CuCl_2$). A variety of complexes can occur in copper chloride solutions, the relative amounts of which being determined by the relative concentrations of copper and chloride [29]. The addition of chloride to copper-containing pH 2.0 liquid medium would have produced anionic copper chloride complexes which were presumably more toxic than either Cu^{2+} or Cl^{-}, and a similar scenario (possibly involving cationic copper chloride complexes) appeared to be the case at pH 3.0.

Copper chloride complexes were also thought likely to be responsible for the ineffective bioleaching of the chalcopyrite concentrate in the presence of 500 mM salt. Even though pH 3 is generally considered too high for microbially-catalysed oxidation of sulfide minerals due to ferric iron being poorly soluble at this pH, it was thought that this might be overcome to some extent by complexing of ferric iron by chloride which would enhance its solubility. However, the main impasse appeared to be the inhibition of ferrous iron oxidation in the bioreactor culture. Even though the individual concentrations of copper and chloride were both less than those that inhibited the growth of *At. ferriphilus* ST2 at pH 3.0, their toxicity when present together, and presumably forming copper-chloride complexes, was far more acute. This finding has a generic significance for bioleaching copper ores and concentrates in saline and brackish waters.

Conflicts of interest

There are no conflicts of interest.

Acknowledgement

This work was supported by the Natural Environment Research Council, UK (Grant ref. NE/L014076/1) as part of its *Resource Recovery from Waste* programme.

References

[1] Quatrini R, Johnson DB. Acidophiles: life in extremely acidic environments. Haverhill: Caistor Academic Press; 2016.
[2] Macur RE, Langner HW, Kocar BD, Inskeep WP. Linking geochemical processes with microbial community analysis: successional dynamics in an arsenic-rich, acid-sulphate-chloride geothermal spring. Geobiology 2004;2:163–77.
[3] Johnson DB, Aguilera A. The microbiology of extremely acidic environments. In: Yates MV, Cindy H, Nakatsu CH, Miller RV, Pillai SD, editors. Manual of environmental microbiology. 4th ed. New York: ASM Press; 2015. 4.3.1-1–4.3.1-24.
[4] Nordstrom DK, Alpers CN, Ptacek CJ, Blowes DW. Negative pH and extremely acidic mine waters from Iron Mountain. Calif Environ Sci Technol 2000;34:254–8.
[5] González-Toril E, Llobet-Brossa E, Casamayor EO, Amman R, Amils R. Microbial ecology of an extreme acidic environment, the Tinto river. Appl Environ Microbiol 2003;69:4853–65.
[6] Norris PR, Ingledew WJ. Acidophilic bacteria: adaptations and applications. In: Herbert RA, Sharp RJ, editors. Molecular biology and biotechnology of extremophiles. Glasgow: Blackie; 1992. p. 115–42.
[7] Dopson M, Holmes DS. Metal resistance in acidophilic microorganisms and its significance for biotechnologies. Appl Microbiol Biotechnol 2014;98:8133–44.
[8] Galleguillos Perez PA. Biodiversity and stress response of extremophilic prokaryotes isolated from the Escondida Copper Mine, Chile [PhD thesis]. UK: University of Wales; 2011.
[9] Zammit CM, Watking ELJ. Adaptation to extreme acidity and osmotic stress. In: Quatrini R, Johnson DB, editors. Acidophiles: life in extremely acidic environments. Haverhill: Caister Academic Press; 2016. p. 49–62.
[10] Huber H, Stetter KO. *Thiobacillus prosperus* sp. nov., represents a new group of halotolerant metal-mobilizing bacteria isolated from a marine geothermal field. Arch Microbiol 1989;151:479–85.
[11] Dopson M, Holmes DS, Lazcano M, McCredden TJ, Bryan CG, Mulroney KT, et al. Multiple osmotic stress responses in *Acidihalobacter prosperus* result in tolerance to chloride ions. Front Microbiol 2017;7:2132.
[12] Johnson DB, Grail BM, Bonnefoy V. New insights into salt-tolerance in acidophilic iron-oxidising bacteria. Adv Mater Res 2015;1130:3–6.
[13] Falagán C, Johnson DB. *Acidithiobacillus ferriphilus* sp. nov., a facultatively anaerobic iron- and sulfur-metabolizing extreme acidophile. Int J Syst Evol Microbiol 2016;66:206–11.
[14] Ñancucheo I, Rowe OF, Hedrich S, Johnson DB. Solid and liquid media for isolating and cultivating acidophilic and acid-tolerant sulfate-reducing bacteria. FEMS Microbiol Lett 2016;363. fnw083.
[15] Hedrich S, Johnson DB. Aerobic and anaerobic oxidation of hydrogen by acidophilic bacteria. FEMS Microbiol Lett 2013;349:40–5.
[16] Stookey L. Ferrozine – a new spectrophotometric reagent for iron. Anal Chem 1970;42:779–81.
[17] Anwar MA, Iqbal M, Qamar MA, Rehman M, Khalid AM. Determination of cuprous ions in bacterial leachates and for environmental monitoring. World J Microbiol Biotechnol 2000;16:135–8.
[18] Johnson DB, Hallberg KB. Techniques for detecting and identifying acidophilic mineral-oxidising microorganisms. In: Rawlings DE, Johnson DB, editors. Biomining. Heidelberg: Springer-Verlag; 2007. p. 237–62.
[19] Kelly DP, Wood AP. Reclassification of some species of *Thiobacillus* to the newly designated genera *Acidithiobacillus* gen. nov., *Halothiobacillus* gen. nov., and *Thermithiobacillus* gen. nov. Int J Syst Evol Microbiol 2000;50:511–6.
[20] Hedrich S, Johnson DB. *Acidithiobacillus ferridurans* sp. nov., an acidophilic iron-, sulfur- and hydrogen-metabolizing chemolithotrophic gammaproteobacterium. Int J Syst Evol Microbiol 2013;63:4018–25.
[21] Quatrini R, Escudero LV, Moya-Beltrán A, Galleguillos PA, Issotta F, Acosta M, et al. Draft genome sequence of *Acidithiobacillus thiooxidans* CLST isolated from the acidic hypersaline Gorbea salt flat in northern Chile. Stand Genomic Sci 2017;12:84.
[22] Karavaiko GI, Golovacheva RS, Pivovarova TA, Tzaplina IA, Vartanjan NS. Thermophilic bacteria of the genus Sulfobacillus. In: Norris PR, Kelly DP, editors. Biohydrometallurgy, proceedings of the international symposium, Warwick 1987 science and technology letters, Kew; 1988. p. 29–41.
[23] Johnson DB, McGinness S. Ferric iron reduction by acidophilic heterotrophic bacteria. Appl Environ Microbiol 1991;57:207–11.
[24] Alexander B, Leach S, Ingledew WJ. The relationship between chemiosmotic parameters and sensitivity to anions and organic acids in the acidophile *Thiobacillus ferrooxidans*. J Gen Microbiol 1987;133:1171–9.
[25] Orellana LH, Jerez CA. A genomic island provides *Acidithiobacillus ferrooxidans* ATCC 53993 additional copper resistance: a possible competitive advantage. Appl Microbiol Biotechnol 2011;92:761–7.
[26] Remonsellez F, Orell A, Jerez CA. Copper tolerance of the thermoacidophilic archaeon *Sulfolobus metallicus*: possible role of polyphosphate metabolism. Microbiology 2006;152:59–66.
[27] Guo X, Jiang C, Luo Y, Zhang M, Poetsch A, Liu S. Proteomic and molecular investigations revealed that *Acidithiobacillus caldus* adopts multiple strategies for adaptation to NaCl stress. Chin Sci Bull 2014;55:301–9.
[28] Welham NJ, Malatt KA, Vukcevic S. The effect of solution speciation on iron-sulphur-arsenic-chloride systems at 298°K. Hydrometallurgy 2000;57:209–23.
[29] Zhao H, Chang J, Boika A, Bard AJ. Electrochemistry of high concentration copper chloride complexes. Anal Chem 2013;85:7696–703.