Research in Microbiology 169 (2018) 576–581

Contents lists available at ScienceDirect

Research in Microbiology

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

Low-level thiocyanate concentrations impact on iron oxidation activity and growth of *Leptospirillum ferriphilum* through inhibition and adaptation

Catherine J. Edward, Athanasios Kotsiopoulos, Susan T.L. Harrison*

Centre for Bioprocess Engineering Research, Department of Chemical Engineering, University of Cape Town, Rondebosch 7701, South Africa

ARTICLE INFO

Article history:
Received 19 March 2018
Accepted 26 October 2018
Available online 2 November 2018

Keywords:
Leptospirillum ferriphilum
Thiocyanate
Recycling
Mineral biooxidation
Inhibition
Adaptation

ABSTRACT

Leptospirillum ferriphilum is the dominant iron-oxidising bacterium in traditional microbial communities utilised in bioprocesses for gold recovery from sulfidic minerals. Ferrous iron oxidation activity and growth of unadapted and thiocyanate-adapted *L. ferriphilum* HT was studied in batch culture across increasing thiocyanate (SCN^-) concentrations in the range 0–2 mg/L to assess the feasibility of recycling remediated cyanidation wastewaters. Thiocyanate concentrations of 1 mg/L and 1.4 mg/L induced an inhibitory effect in the unadapted culture wherein ferrous iron oxidation rate and cell growth were compromised. A substantial lag in the onset of ferrous iron oxidation occurred at concentrations above 0.5 mg/L SCN^-, with no oxidation activity above 1.75 mg/L SCN^-. The adapted culture, however, was uninhibited across the SCN^- concentration range investigated and demonstrated a higher specific ferrous iron oxidation rate owing to reduced growth. It is postulated that SCN^- exposure in the absence of adaptation induces osmotic stress. Moreover, upregulation of genes associated with the synthesis of osmo-protectants may be responsible for the preservation of activity observed in the adapted culture. As *L. ferriphilum* is dominant within the biooxidation tank community, evidence of sustained iron oxidation activity at low-level SCN^- concentrations affirms the potential of recycling bioremediated cyanidation wastewater.

1. Introduction

The minerals industry is water intensive by nature, therefore adequate water system design and management are key in moving toward a more sustainable sector, including more sustainable minerals processing [1]. However, challenges surrounding water management are immense, more so with climate change exacerbating the frequency and intensity of water shortages and water excess [2]. As mining processes span all hydrological contexts and climate regions, the associated water demand is inherently mine site specific. Globally, the minerals processing sector is being driven to reduce freshwater extraction and minimise mine water release. This has prompted inclusion of an industrial ecology approach with movement toward zero waste and closing of water circuits, resulting in the development of water recycling technologies within mineral processing operations [3,4]. Recycling of process and wastewaters within mineral processing circuits, however, can prove difficult due to the resulting variation in water quality [3,5]. Re-use of such waters (untreated) may compromise efficiency of mineral processing operations [3,5,6].

Recycling of wastewaters within gold minerals processing and extraction processes is particularly challenging due to the potential presence of cyanide (CN^-) and cyanide reaction products within the waste streams [6]. Cyanide, and cyanide species such as ferricyanide and thiocyanate (SCN^-), are known to have a depressant effect on mineral flotation within conventional gold mining operations, thereby reducing precious metal recovery. These compounds are also known to compromise the efficacy of mineral dissolution in biomining plants, similarly reducing gold recovery [6].

Biologically-based mineral extraction processes utilise a consortium of acidophilic microorganisms to mediate the solubilisation of sulphidic minerals to enhance metal recovery [7,8]. The microbial consortium contains iron- and sulphur-oxidising organisms that actively generate leach agents, ferric iron and protons, as by-products of cellular metabolism. In gold mineral processing and

* Corresponding author.
E-mail addresses: edwcat005@myuct.ac.za (C.J. Edward), thanos.kotsiopoulos@uct.ac.za (A. Kotsiopoulos), sue.harrison@uct.ac.za (S.T.L. Harrison).

https://doi.org/10.1016/j.resmic.2018.10.003
0923-2508/

recovery, biomining technologies are utilised commercially and successfully as a pre-treatment for sulphidic refractory concentrates prior to cyanidation, in a process known as biooxidation [8,9]. This technology is well established and has proven to be robust. Complete sulphide removal through biooxidation is not essential for efficient gold recovery; however, residual sulphur species react with cyanide during gold recovery, resulting in additional cyanide consumption and SCN^- formation [10].

On recycle of water from the cyanidation and tailings circuit, the presence of residual concentrations of anions such as SCN^- and CN^- within tailings wastewaters have been reported to impact biooxidation performance detrimentally [11,12]. Historical process data suggest that the biooxidation community is highly sensitive to SCN^- with a suggested empirical limit of 1 mg/L [10,13]. Biooxidation plants, therefore, typically segregate cyanidation wastewaters and process water to avoid contamination, therein creating in a rigid water balance [6].

Bioremediation of CN- and SCN-containing waters has been considered in several locations [14,15]. The ASTER™ process (Activated Sludge Tailings Effluent Remediation) has been found to be able to treat cyanidation wastewater successfully, resulting in 0.02–0.1 mg/L residual SCN^- in the effluent stream, well below the suggested limit [16]. This has been confirmed in the laboratory over a wide range of operating conditions and SCN^- loading rates [17]. The ASTER™ process has been coupled to several biooxidation plants with success, renewing to the desire to recycle the treated water within the biooxidation plant. Due to the perceived sensitivity of the biooxidation culture to SCN^-, it is important to understand the effect of low-level SCN^- concentrations on key microbial species if wastewater is to be recycled successfully.

Leptospirillum ferriphilum (*L. ferriphilum*) is the key iron oxidising bacterial species within the biooxidation community, readily producing the ferric iron lixiviant as part of mineral dissolution mechanism [18,19]. While other iron oxidising species have been identified in the mixed microbial culture [20,21], *L. ferriphilum* is recognised to present the highest ferrous iron oxidation rate and to maintain the lowest ferric iron concentrations, thus historically it has been considered to be the ferrous iron oxidiser of choice in tank bioleaching. To date, no definitive studies have been conducted on the impact of SCN^- on the microbial activity of *L. ferriphilum*.

This paper aims to determine the impact of increasing SCN^- concentrations on the iron oxidation capacity and growth of *L. ferriphilum* to assess tolerance and microbial response. Moreover, this study aims to investigate the potential for thiocyanate-adaptation via persistent exposure to low-level concentrations of SCN^- and the resulting effect on microbial activity. The results of this study will inform the potential for recycle of tailings water and the process operating conditions.

2. Materials and methods

2.1. Bacterial culture and growth medium

The *L. ferriphilum* HT culture used in this study was isolated from the BIOX® stock cultures maintained in the CeBER labs. It was maintained in 150 mL Erlenmeyer shake flasks (30 mL working volume) on a modified DSMZ 882 nutrient medium comprised of 0.132 g/L $(NH_4)_2SO_4$, 0.053 g/L $MgCl_2.6H_2O$, 0.027 g/L KH_2PO_4, 0.147 g/L $CaCl_2.2H_2O$ adjusted to a pH of 1.2 with concentrated H_2SO_4 (98%). The medium was then supplemented with 4 g/L ferrous iron and 1 mL trace element solution [22]. The culture was maintained in a shaking incubator at 45 °C and agitated at 140 rpm. The culture was transferred daily (using 20% inoculum volume) to sustain culture activity and purity. Prior to commencement of experimental work, it was confirmed by qPCR analysis that the culture was 99.9% *L. ferriphilum* using the HT primer set described by Tupikina et al. [23].

2.2. Analytical methods

The bacterial cell concentration was enumerated twice daily via direct counting using an Olympus CX-10 using a THOMA counting chamber under phase contrast at 1000× magnification.

Ferrous and ferric iron concentrations were quantified using the 1–10 phenanthroline colorimetric assay [24] on a Thermo Scientific GENESYS 20 spectrophotometer.

2.3. Adaptation to thiocyanate

Adaptation to thiocyanate was established via low-level introduction of SCN^- wherein *L. ferriphilum* was initially exposed to 0.25 mg/L SCN^-. Following sustained activity at this concentration, the exposure concentration was increased incrementally. Successful adaptation to 0.5 mg/L SCN^- was achieved, efforts to establish adaptation at higher SCN^- concentrations resulted in performance instability.

2.4. Experimental design

2.4.1. Preliminary scoping study

The scoping study was conducted in multiwell plates (MWPs) using Greiner Bio-one CELLSTAR® 12 Well Suspension Culture Plates (4 mL volume per well). The study was used to investigate the effect of thiocyanate (SCN^-) on ferrous oxidation activity over a broad SCN^- concentration range. Concentrations of 0 mg/L, 0.25 mg/L, 0.5 mg/L, 0.75 mg/L, 1 mg/L, 1.25 mg/L, 1.5 mg/L, 1.75 mg/L, 3 mg/L, 5 mg/L, 7 mg/L and 10 mg/L were evaluated. Each well was charged with basal salt nutrient medium, 5 g/L of ferrous and ferric iron, the designated volume of SCN^- stock solution (20 mg/L) and inoculated with 1×10^7 cells/mL of the unadapted *L. ferriphilum* culture such that a total working volume of 3 mL was achieved. To mitigate evaporative losses, each MWP was fitted with an Aera-Seal™ film and placed in a humidified container [25]. The MWPs were incubated in a shaking incubator at 45 °C and agitated at 140 rpm. Over the time course of the experiment, 10 μL samples were withdrawn at appropriate time intervals for measurement of ferrous iron concentration via colorimetric assay.

2.4.2. Batch experiments

The effect of SCN^- on ferrous (Fe^{2+}) utilization and microbial growth was investigated in MWPs at discrete SCN^- concentrations of 0.1 mg/L, 0.5 mg/L, 1 mg/L, 1.4 mg/L, 1.75 mg/L and 2 mg/L. Each well was charged with nutrient medium, ferrous and ferric iron, the designated concentration of SCN^- and inoculated with 1×10^7 cells/mL of the adapted or unadapted *L. ferriphilum* culture such that a total working volume of 3 mL was achieved and incubated as outlined above. Over the course of the experiment, 10 μL samples were withdrawn at appropriate time intervals for measurement of Fe^{2+} utilization as per the scoping study. Cell numbers were enumerated twice daily to evaluate cell growth. Moreover, microbial morphology was monitored to verify culture purity.

3. Results

3.1. Preliminary scoping study

A preliminary study was conducted to determine the threshold SCN^- concentration at which microbial activity was completely inhibited. Low-level SCN^- concentrations (0 mg/L, 0.25 mg/L, 0.5 mg/L, 0.75 mg/L, 1 mg/L, 1.25 mg/L, 1.5 mg/L and

1.75 mg/L) were selected to mimic residual SCN^- concentrations reportedly found in suspensions of bioremediated cyanidation tailings and wastewaters [16,17]. Higher-level concentrations (3 mg/L, 5 mg/L, 7 mg/L and 10 mg/L) were selected to observe threshold SCN^- concentration tolerance as well as to replicate concentrations potentially occurring in the event of perturbations in the post-cyanidation bioremediation circuit. The scoping study results, shown in Fig. 1, indicated that low-level concentrations of SCN^- (0.25–1.75 mg/L) did not impede the ferrous iron oxidation ability of *L. ferriphilum*. However, it was noted that, despite complete ferrous iron utilization, the presence of SCN^- in concentration range from 0.75 mg/L to 1.75 mg/L induced a lag in the onset of oxidation activity. Furthermore, the oxidation lag time was noted to increase with increasing SCN^- concentration. Concentrations above 1.75 mg/L resulted in negligible iron oxidation activity, signalling the onset of complete inhibition above these concentrations. The inhibitory effect of SCN^- may further be observed in the tabulated ferrous iron oxidation rates in Table 1. The data indicated that exposure of *L. ferriphilum* to SCN^- concentrations of 0.25 mg/L and 0.5 mg/L affected the ferrous iron oxidation rate negligibly; however, exposure to concentrations between 0.75 mg/L and 1.75 mg/L resulted in an average reduction in oxidation rate of *ca.* 18.1%.

3.2. Effect of increasing SCN^- concentration on ferrous oxidation activity and growth of L. ferriphilum

This study is focused on achieving and characterizing adaptation to thiocyanate through exposure, therefore conditions showing complete inhibition cannot be used. Complete inhibition was observed at 3 mg/L SCN^-, a 30-fold increase over the SCN^- concentrations typical in bioremediated effluents (>0.1 mg/L), hence the SCN^- concentration range used for further investigation was refined. A lower SCN^- concentration range was therefore selected for in-depth study: 0 mg/L, 0.1 mg/L, 0.5 mg/L, 1 mg/L, 1.4 mg/L, 1.75 mg/L and 2 mg/L. Batch experiments were conducted at these discrete concentration points such that the influence of SCN^- on the growth and activity of *L. ferriphilum* culture could be determined.

L. ferriphilum exposed to SCN^- concentrations between 0 mg/L and 1.4 mg/L exhibited complete ferrous iron utilisation within 53 h (Fig. 2). Exposure to SCN^- concentrations above 1.4 mg/L resulted in negligible oxidation activity during the course of the experiment, indicating complete microbial inhibition (Fig. 2). It

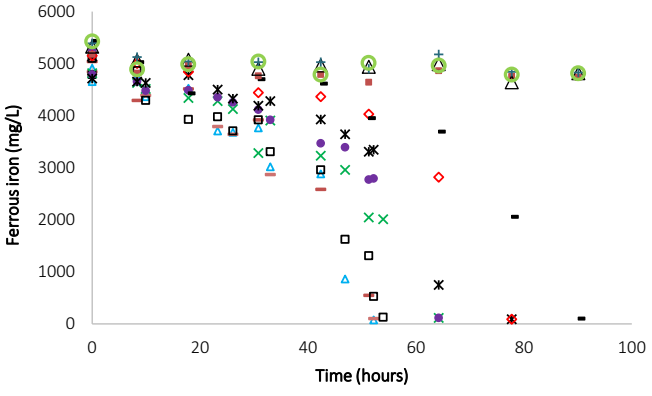

Fig. 1. Ferrous iron utilisation plot for *Leptospirillum ferriphilum* (*L. ferriphilum*) grown at 45 °C exposed to increasing concentrations of SCN^-: 0 mg/L (△), 0.25 mg/L (--), 0.5 mg/L (□), 0.75 mg/L (X), 1 mg/L (●), 1.25 mg/L (✳), 1.5 mg/L (◊), 1.75 mg/L (-), 3 mg/L (•), 5 mg/L (△), 7 mg/L (+), 10 mg/L (○) ($N = 2$).

Table 1
Linear ferrous oxidation rates of unadapted *L. ferriphilum* exposed to increasing concentrations of SCN^- ($N = 2$).

SCN^- (mg/L)	Linear Fe^{2+} oxidation rate (mg/L/h)
0.00	168.6
0.25	160.4
0.50	166.6
0.75	140.5
1.00	135.2
1.25	138.8
1.50	137.1
1.75	138.5
3.00	n.d
5.00	n.d
7.00	n.d
10.0	n.d

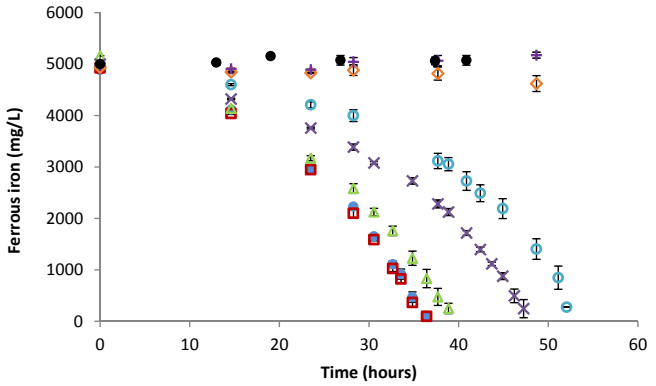

Fig. 2. Ferrous utilisation plot of unadapted *L. ferriphilum* exposed to increasing concentrations of SCN^- at 45 °C: 0 mg/L (●), 0.1 mg/L (□), 0.5 mg/L (△), 1 mg/L (X), 1.4 mg/L (○), 1.75 mg/L (◊), 2 mg/L (+), Abiotic control (●). Error bars represent the standard error of triplicate samples ($N = 3$).

was noted that *L. ferriphilum* exposed to 0.1 mg/L SCN^- remained relatively unaffected across all activity metrics. Moreover, it retained a comparable yield to that of the control test, indicating that this concentration was not inhibitory (Table 2). A marginal increase in oxidation lag time was observed at an exposure concentration of 0.5 mg/L whereas concentrations of 1 mg/L and 1.4 mg/L SCN^- were found to induce an extensive lag time, increasing by 47% and 81% respectively. Furthermore, exposure to a SCN^- concentration of 0.5 mg/L resulted in a 17% reduction in the maximum ferrous iron oxidation rate as well as a marginal decrease (*ca.* 4%) in the overall oxidation rate. The maximum oxidation rate was most significantly affected at a concentration of 1.4 mg/L SCN^-, with a 24.7% reduction in rate. Exposure to SCN^- > 0.5 mg/L resulted in a pronounced decrease in overall oxidation rate which was found to correspond to the significant increase in ferrous iron oxidation lag time. The net biomass yield was found to remain somewhat constant across the SCN^- concentrations 0–1 mg/L. However, exposure to a SCN^- concentration of 1.4 mg/L resulted in a 31.2% decrease in biomass yield (Table 2). The overall specific oxidation rate was found to decrease between SCN^- concentrations of 0.5 mg/L and 1 mg/L however, the specific oxidation rate at an exposure concentration of 1.4 mg/L increased by approximately 54% relative to that of the control, in line with the small decrease in oxidation rate but large decrease in biomass concentration. The overall volumetric cell growth rate was noted to decay linearly at SCN^- concentrations above 0.5 mg/L, decreasing by *ca.* 15% and *ca.* 60% at 1 mg/L and 1.4 mg/L, respectively (Fig. 3).

Table 2
Overall and maximum ferrous oxidation rates, ferrous oxidation lag time and biomass yield of unadapted *L. ferriphilum* exposed to increasing concentrations of SCN^- with standard deviation represented on triplicate measurements ($N = 3$).

SCN^- (mg/L)	Overall Fe^{2+} oxidation rate (mg/L/h)	Max. Fe^{2+} oxidation rate (mg/L/h)	Fe^{2+} oxidation lag time (h)	Overall specific oxidation rate (10^{10} mg Fe^{2+}/cell/h)	Yield (mg biomass/mg Fe^{2+})
0.00	131.3 ± 0.52	349.8 ± 16.8	18.17 ± 0.21	9.70	0.028
0.10	132.6 ± 0.98	356.3 ± 24.4	17.70 ± 0.11	8.60	0.032
0.50	126.4 ± 4.49	290.0 ± 19.9	18.83 ± 2.23	7.52	0.034
1.00	100.6 ± 6.48	305.5 ± 84.2	26.77 ± 5.13	6.18	0.034
1.40	84.4 ± 4.63	263.3 ± 15.3	32.97 ± 1.93	10.72	0.017
1.75	n.d	n.d	n.d	n.d	n.d
2.00	n.d	n.d	n.d	n.d	n.d

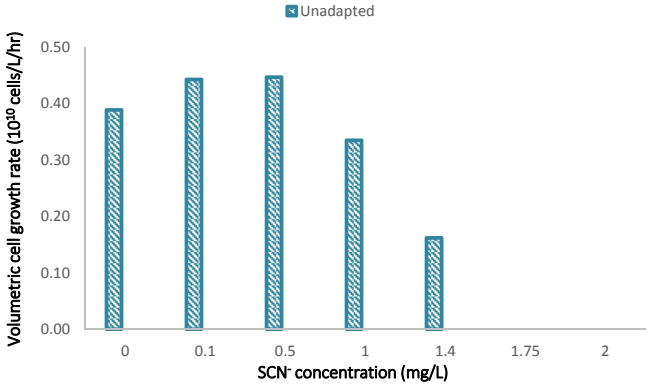

Fig. 3. Overall volumetric growth rate of unadapted *L. ferriphlum* in cells/mL/h exposed to increasing concentrations of SCN^- over time course of experiment.

3.3. *Effect of increasing SCN^- on ferrous oxidation activity and growth of adapted* L. ferriphilum

Batch experiments were conducted on the thiocyanate-adapted *L. ferriphilum* culture (0.5 mg/L SCN^-) to elucidate whether adaptation would improve tolerance to low-level SCN^- exposure. Additionally, the effect of sustained exposure to SCN^- is expected to better simulate the impact of recycling bioremediated tailings water within the biooxidation circuit. A SCN^- concentration range of 0–2.0 mg/L was used.

The adapted culture was found to display improved tolerance to SCN^-, reflecting negligible lag time across all SCN^- exposure concentrations (Fig. 4 and Table 3). Thiocyanate tolerance was further exemplified via the adapted culture's ability to oxidise ferrous iron efficiently at SCN^- concentrations that were observed to inhibit the unadapted culture completely, specifically 1.7 mg/L and 2.0 mg/L. Furthermore, the adapted culture was found to retain both a constant maximum and average iron oxidation rate across all SCN^- concentrations in the range tested, demonstrating rates comparable to those of the unadapted culture. This compared closely to the unadapted culture's average oxidation rate at non-inhibitory exposure concentrations ($\leq$0.1 mg/L). Conversely, the overall specific rates of the adapted culture were found to be *ca.* 10-fold higher than that of the unadapted culture. Biomass yield from substrate, although somewhat constant, was found to be almost 10-fold smaller than the yield data of the unadapted culture. The overall volumetric cell growth rate (Fig. 5) was significantly lower relative to that of the unadapted culture; however, the rate was found to be constant irrespective of SCN^- concentration.

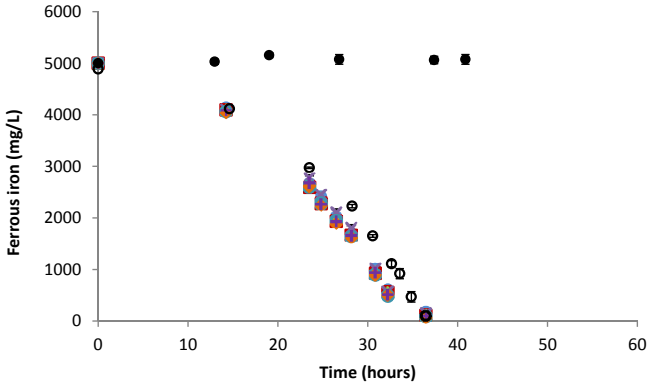

Fig. 4. Ferrous utilisation plot of thiocyanate-adapted *L. ferriphilum* exposed to increasing concentrations of SCN^- at 45 °C: 0 mg/L (●), 0.1 mg/L (□), 0.5 mg/L (△), 1 mg/L (X), 1.4 mg/L (○), 1.75 mg/L (◊), 2 mg/L (+), Abiotic control (●), compared to unadapted *L. ferriphilum* culture exposed to 0 mg/L SCN^- (○). Error bars represent the standard error of triplicate samples ($N = 3$).

4. Discussion

4.1. *Unadapted* L. ferriphilum

Thiocyanate elicited a significant inhibitory response at low concentrations of SCN^- (1–1.4 mg/L), illustrating its extreme toxicity to *L. ferriphilum* cultures previously unexposed. Without adaptation, ferrous iron oxidation rate, overall growth rate and biomass yield were found to decrease with increasing SCN^-, most significantly at a concentration of 1.4 mg/L. Additionally, oxidation lag time was found to increase with increasing SCN^-, most notably at a concentration of 1 mg/L and above. Dopson et al. [26] observed similar results with the archaeal extreme thermophile, *Sulfolobus metallicus* (*S. metallicus*). Data indicated that iron oxidation activity of the archaea was reduced by 23% when exposed to 0.5 mg/L SCN^-, whilst it remained unaffected at an exposure concentration of 0.05 mg/L. Chalcopyrite leaching tests using *S. metallicus* were also found to exhibit an increased lag time and reduced oxidation rate when exposed to a SCN^- concentration of 1 mg/L relative to the control. Though *S. metallicus* is an archaeal species, this study illustrates that the toxic effect of SCN^- becomes increasingly pronounced with concentration, even at low-level concentrations (0.5–1 mg/L). Such increases in lag time have been demonstrated as a common response to various process stresses (e.g. acid stress, shear stress, osmotic stress) in both the mesophilic and thermophilic bioleaching cultures [27,28].

L. ferriphilum is an acidophilic microorganism and is therefore physiologically and functionally equipped to survive within low pH environments and maintain the pH gradient across the cytoplasmic membrane. Regulation of this pH gradient is essential as it is linked to cellular bioenergetics and is a significant contributor to the proton motive force [29,30]. Although acidophiles have impermeable cell membranes, compounds such as organic acids and anions are known to permeate the cell membrane and dissipate the

Table 3
Overall and maximum ferrous oxidation rates, ferrous oxidation lag time and biomass yield of thiocyanate-adapted *L. ferriphilum* exposed to increasing concentrations of SCN^- with standard deviation represented on triplicate measurements ($N = 3$).

SCN^- (mg/L)	Overall Fe^{2+} oxidation rate (mg/L/h)	Max. Fe^{2+} oxidation rate (mg/L/h)	Fe^{2+} oxidation lag time (h)	Overall specific oxidation rate (10^{10} mg Fe^{2+}/cell/h)	Yield (mg biomass/mg Fe^{2+})
0.00	132.2 ± 16.6	291.9 ± 23.4	8.92 ± 2.83	86.3	0.0032
0.10	134.0 ± 12.5	289.6 ± 23.9	8.96 ± 4.12	53.6	0.0051
0.50	134.0 ± 24.9	294.4 ± 5.5	9.78 ± 1.80	87.5	0.0031
1.00	132.9 ± 16.6	310.2 ± 26.3	10.86 ± 1.28	63.5	0.0043
1.40	134.8 ± 8.3	294.4 ± 5.20	8.77 ± 2.34	77.0	0.0036
1.75	135.3 ± 4.2	314.9 ± 30.2	8.83 ± 2.83	98.4	0.0028
2.00	134.3 ± 4.2	320.7 ± 17.4	9.19 ± 2.30	104.8	0.0026

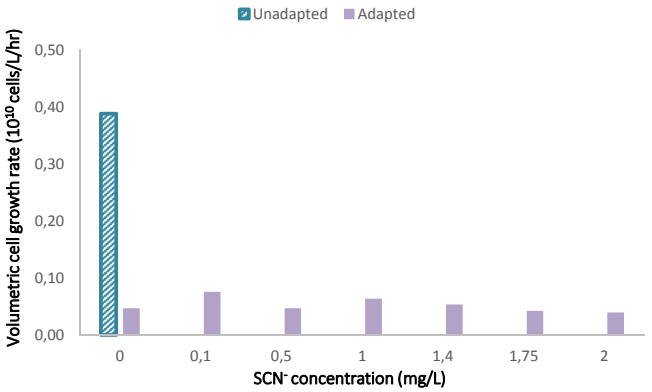

Fig. 5. Overall volumetric growth rate of thiocyanate-adapted *L. ferriphlum* in cells/mL/h exposed to increasing concentrations of SCN^- over time course of experiment compared to overall volumetric growth rate of unadapted *L. ferriphilum* exposed to 0 mg/L SCN^-.

transmembrane potential, a phenomenon which is exacerbated at low pH conditions [31]. Reduction in the transmembrane potential prompts the influx of protons into the cell, resulting in acidification of the cytoplasm thereby disrupting the pH gradient across the membrane [32]. This detrimentally affects microbial growth and activity [30]. Studies on acidophiles, specifically *A. thiooxidans*, have illustrated that various permeant anions abolish the cellular transmembrane potential under acidic conditions therein prompting protons to leak into the cell [31]. Moreover, the degree of inhibition by anions at low system pH was found to follow the same order as that of the Hofmeister series $SCN^- > NO_3^- > Cl^- > H_2PO_4^- > HSO_4^-$, thus highlighting the acute toxicity of SCN^-. However, few studies have been conducted exclusively on the impact of SCN^- therefore the observed inhibitory response of *L. ferriphilum* to SCN^- may be analogous to that induced by osmotic stress via exposure to more commonly studied anions, such as chloride (Cl^-) or nitrate (NO_3^-). The toxic effect of Cl^- on acidophilic organisms implicated in biooxidation and bioleaching has been extensively studied due to the prevalence of saline ground water in some biomining environments [4,9,33–35]. Gahan et al. [34] illustrated that the addition of Cl^- up to 10 g/L resulted in an associated increase in lag time and reduction in maximum ferrous iron oxidation rate of *L. ferriphilum* within batch systems. Chloride concentrations of 12 g/L and 13 g/L were noted to result in microbial arrest. Chemostat studies illustrated that exposure of *L. ferriphilum* to $Cl^- \leq 3$ g/L resulted in reduced growth rates and a concomitant reduction in biomass yield [34]. This response to Cl^- exposure, however, is not specific to *L. ferriphilum*. Various type strains of biomining organisms have similarly exhibited reduced cell growth and impeded ferrous iron oxidation activity in the presence of Cl^-, albeit sensitivities varied between genera [36]. These findings correlate closely to the trends observed in this study with respect to the impact of SCN^- toxicity. Yet it must be noted that Cl^- concentrations investigated are more than 1000-fold the SCN^- concentrations evaluated, thus highlighting the acute toxic effect exerted on *L. ferriphilum* by the presence of SCN^-.

Although *L. ferriphilum* demonstrated sensitivity to SCN^- similarly to Cl^-, the culture was observed to utilise the available ferrous iron completely at concentrations up to 1.4 mg/L, despite the reduction in cell growth and biomass yield. This may indicate that the energy requirement had possibly been directed toward cell maintenance or survival as opposed to growth, thus accounting for the reduced biomass yield at inhibitory concentrations. Various *L. ferriphilum* strains have been sequenced and found to contain at least four pathways by which trehalose, an osmo-protectant may be synthesized [37,38]. Additionally, group II *L. ferriphilum* species have also been identified to contain genes for synthesis of ectoine, another osmo-protectant [37,38]. Evidence of such genes may account for the reduction in cell number, specifically in response to an osmotic stress due to the presence of anions such as Cl^- or SCN^-, as energy would be diverted to synthesis of osmo-protectants [36].

4.2. *Thiocyanate-adapted* L. ferriphilum

The results presented show clearly that continued exposure to low levels of SCN^- (0.5 mg/L) led to adaptation within the *L. ferriphilum* culture to tolerate SCN^- concentrations as high as 2 mg/L, and potentially higher, representing levels some 20-fold those expected in the ASTER™-treated water. Performance of the adapted culture in the presence of 0–2 mg/L SCN^- showed consistent ferrous iron oxidation rate across the full concentration spectrum. Further, these rates reflected the volumetric ferrous iron oxidation rate of the unadapted culture in the absence of SCN^-. However, the biomass formation rate and biomass concentration was significantly compromised, leading to a substantial increase in the specific ferrous iron oxidation rate. This suggests that the adapted culture is characterised by a much-increased maintenance energy requirement to sustain the cells under adverse conditions. This may potentially reflect a major advantage for biooxidation processes as the volumetric ferric iron generation rates can be sustained with reduced growth, possibly reflecting reduced CO_2 and nutrient consumption. CO_2 mass transfer is frequently a limitation in biooxidation processes requiring supplementation or increased energy input [39].

Genomic and proteomic data indicate that *L. ferriphilum* contains various genes that allow for the survival and proliferation of the species under environmentally harsh conditions [37,38,40]. Genes relating to synthesis of compatible solutes, such as trehalose and ectoine, are hypothesised to be upregulated with persistent exposure to non-lethal SCN^- concentrations therein enabling sustained ferrous iron oxidation in the presence of SCN^-.

Successful adaptation of the dominant iron oxidising bacteria within the traditional biooxidation microbial community to non-

lethal SCN^- concentrations indicates that recycling of bio-remediated cyanidation and tailings wastewaters is a feasible process option. Active recycling of remediated waters will increase water balance flexibility, allowing inclusion of various water sources, within biomining operations without compromising process efficiency. However, the microbial kinetics in response to SCN^- exposure and adaptation must be evaluated to inform optimisation of biooxidation tank operating conditions.

Conflict of interest

None declared.

Acknowledgements

The authors gratefully acknowledge the DST and NRF of South Africa for financial support of this study through the South African Research Chair Initiative (SARChI) chair in Bioprocess Engineering held by STLH (UID 64778).

References

[1] Gunson AJ, Klein B, Veiga M, Dunbar S. Reducing mine water requirements. J Clean Prod 2012;21:71–82.
[2] World Economic Forum. The global risks report 2016. vol. 1. Geneva. 2016.
[3] Liu W, Moran CJ, Vink S. A review of the effect of water quality on flotation. Miner Eng 2013;53:91–100.
[4] Rea SM, McSweeney NJ, Degens BP, Morris C, Siebert HM, Kaksonen AH. Salt-tolerant microorganisms potentially useful for bioleaching operations where fresh water is scarce. Miner Eng 2015;75:126–32.
[5] Bahrami A, Hosseini MR, Razmi K. An investigation on reusing process water in gold cyanidation. Mine Water Environ 2007;26:191–4.
[6] Adams MD. Impact of recycling cyanide and its reaction products on upstream unit operations. Miner Eng 2013;53:241–55.
[7] Olson GJ, Brierley JA, Brierley CL. Bioleaching review, part B: progress in bioleaching: applications of microbial processes by the minerals industries. Appl Microbiol Biotechnol 2003:249–57.
[8] Harrison STL. Biotechnologies that use acidophiles. In: Quatrini R, Johnson DB, editors. Acidophiles: life in extremely acidic environments. Caister Academic Press; 2016. p. 265–84.
[9] Watling HR, Collinson DM, Corbett MK, Shiers DW, Kaksonen AH, Watkin ELJ. Saline-water bioleaching of chalcopyrite with thermophilic, iron(II)- and sulfur-oxidizing microorganisms. Res Microbiol 2016;167:546–54.
[10] van Aswegen P, van Niekerk J, Olivier W. The BIOX™ process for the treatment of refractory gold concentrates. In: Rawlings DE, Johnson DB, editors. Biomining. Berlin: Springer-Verlag Berlin Heidelberg; 2007. p. 1–32.
[11] Steyn B, Benewoe V. A practical example of recovery improvements in a bacterial oxidation plant. In: World gold conference; 2009. p. 265–70.
[12] Van Hille RP, Dawson E, Edward C, Harrison STL. Effect of thiocyanate on BIOX® organisms: inhibition and adaptation. Miner Eng 2015;75.
[13] Miller PC. The design and operating practice of bacterial oxidation plant using moderate thermophiles (the BacTech process). In: Rawlings DE, editor. Biomining – theory, microbes and industrial processes. Georgetown: Springer-Verlag Berlin Heidelberg; 1997. p. 81–102.
[14] Whitlock JL. Biological detoxification of precious metal processing wastewaters. Geomicrobiol J 1990;8:241–9.
[15] Makhotla N, van Buuren C, Olivier JW. The ASTER process: technology development through to piloting, demonstration and commercialization; 2010. p. 236–53. Available at: www.biomin.co.za/pdf/2018aster
[16] van Buuren C. BIOMIN's novel integrated technologies for optimising refractory gold ore processing solutions; Proceedings of ALTA 2014, Perth, Australia. 23-25 May 2014. 247.
[17] Van Zyl AW, Harrison STL, Van Hille RP. Determining an effective operating window for a thiocyanate-degrading mixed microbial community. J Environ Chem Eng 2017;5:660–6.
[18] Coram N, Rawlings DE. Molecular relationship between two groups of the genus Leptospirillum and the finding that *Leptospirillum ferriphilum* sp. nov. dominates South African commercial biooxidation tanks that operate at 40°C. Appl Environ Microbiol 2002;68:838–45.
[19] Rawlings DE, Tributsch H, Hansford GS. Reasons why 'Leptospirillum'-like species rather than *Thiobacillus ferrooxidans* are the dominant iron-oxidizing bacteria in many commercial processes for the biooxidation of pyrite and related ores. Microbiology 1999;145:5–13.
[20] Okibe N, Gericke M, Hallberg KB, Johnson DB. Enumeration and characterization of acidophilic microorganisms isolated from a pilot plant stirred tank bioleaching operation. Appl Environ Microbiol 2003;69:1936–43.
[21] Zhou H, Zhang R, Hu P, Zeng W, Xie Y, Wu C, et al. Isolation and characterization of *Ferroplasma thermophilum* sp. nov., a novel extremely acidophilic, moderately thermophilic archaeon and its role in bioleaching of chalcopyrite. J Appl Microbiol 2008;105:591–601.
[22] Kolmert A, Johnson DB. Remediation of acidic waste waters using immobilised, acidophilic sulfate-reducing bacteria. J Chem Technol Biotechnol 2001;76:836–43.
[23] Tupikina OV, Minnaar SH, van Hille RP, van Wyk N, Rautenbach GF, Dew D, et al. Determining the effect of acid stress on the persistence and growth of thermophilic microbial species after mesophilic colonisation of low grade ore in a heap leach environment. Miner Eng 2013;53:152–9.
[24] Komadel P, Stucki JW. Quantitative assay of minerals for Fe^{2+} and Fe^{3+} using 1,10-phenanthroline: III. A rapid photochemical method. Clays Clay Miner 1988;36:379–81.
[25] Meissner MP, Xu Z, Jones GC, Minnaar SH, Harrison STL. A novel microwell-based analytical technique for studying ferrous iron biooxidation activity. Miner Eng 2014;60:8–13.
[26] Dopson M, Sundkvist JE, Lindström EB. Toxicity of metal extraction and flotation chemicals to *Sulfolobus metallicus* and chalcopyrite bioleaching. Hydrometallurgy 2006;81:205–13.
[27] Ngoma IE, Ojumu TV, Harrison STL. Investigating the effect of acid stress on selected mesophilic micro-organisms implicated in bioleaching. Miner Eng 2015;75:6–13.
[28] Nemati M, Harrison STL. Effect of solid loading on thermophilic bioleaching of sulfide minerals. J Chem Technol Biotechnol 2000;75:526–32.
[29] Baker-Austin C, Dopson M. Life in acid: pH homeostasis in acidophiles. Trends Microbiol 2007;15:165–71.
[30] Zammit CM, Watkin ELJ. Adaptation to extreme acidity and osmotic stress. In: Quatrini R, Johnson DB, editors. Acidophiles: life in extremely acidic environments. Norfolk: Caister Academic Press; 2016. p. 49–62.
[31] Suzuki I, Lee D, Mackay B, Harahuc L. Effect of various ions, pH, and osmotic pressure on oxidation of elemental sulfur by *Thiobacillus thiooxidans*. Appl. Environ. Microbiol. 1999;65(11):5163–8.
[32] 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.
[33] Gahan CS, Sundkvist JE, Sandström Å. A study on the toxic effects of chloride on the biooxidation efficiency of pyrite. J Hazard Mater 2009;172:1273–81.
[34] Gahan CS, Sundkvist JE, Dopson M, Sandström Å. Effect of chloride on ferrous iron oxidation by a *Leptospirillum ferriphilum*-dominated chemostat culture. Biotechnol Bioeng 2010;106:422–31.
[35] Shiers DW, Blight KR, Ralph DE. Sodium sulphate and sodium chloride effects on batch culture of iron oxidising bacteria. Hydrometallurgy 2005;80:75–82.
[36] Zammit CM, Mangold S, Rao Jonna V, Mutch LA, Watling HR, Dopson M, et al. Bioleaching in brackish waters – effect of chloride ions on the acidophile population and proteomes of model species. Appl Microbiol Biotechnol 2012;93:319–29.
[37] Goltsman DSA, Denef VJ, Singer SW, VerBerkmoes NC, Lefsrud M, Mueller RS, et al. Community genomic and proteomic analyses of chemoautotrophic iron-oxidizing "*Leptospirillum rubarum*" (Group II) and "*Leptospirillum ferrodiazotrophum*" (Group III) bacteria in acid mine drainage biofilms. Appl Environ Microbiol 2009;75:4599–615.
[38] Issotta F, Galleguillos PA, Moya-Beltrán A, Davis-Belmar CS, Rautenbach G, Covarrubias PC, et al. Draft genome sequence of chloride-tolerant *Leptospirillum ferriphilum* Sp-Cl from industrial bioleaching operations in northern Chile. Stand Genomic Sci 2016;11:1–7.
[39] Bryan CG, Davis-Belmar CS, van Wyk N, Fraser MK, Dew D, Rautenbach GF, et al. The effect of CO_2 availability on the growth, iron oxidation and CO_2-fixation rates of pure cultures of *Leptospirillum ferriphilum* and *Acidithiobacillus ferrooxidans*. Biotechnol Bioeng 2012;109:1693–703.
[40] Mi S, Song J, Lin J, Che Y, Zheng H, Lin J. Complete genome of *Leptospirillum ferriphilum* ML-04 provides insight into its physiology and environmental adaptation. J Microbiol 2011;49:890–901.