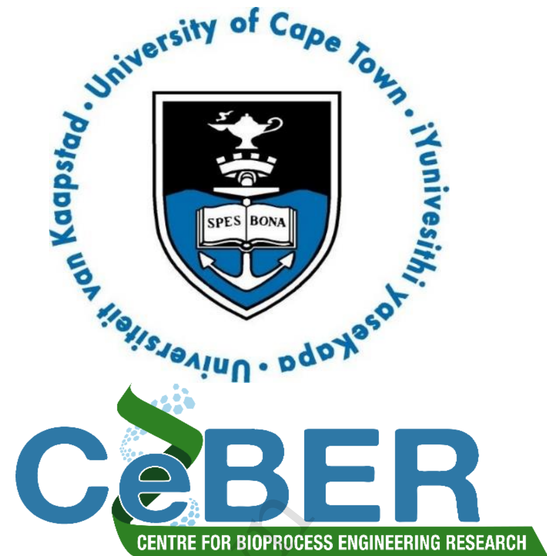

The effect of pH and dissolved organic carbon on the growth kinetics of *Ac. cupricumulans* JTC3 and *L. ferriphilum* HT pertinent to the BIOX® process

Leo Maritz

In fulfilment of the requirements for the degree of Master of Science in Engineering

Supervisor: Prof. S.T.L Harrison

Department of Chemical Engineering
Faculty of Engineering and the Built Environment
University of Cape Town

University of Cape Town

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Abstract

The BIOX® process was designed to pre-treat refractory gold-bearing concentrates to enable gold recovery. It, and related, processes are well accepted commercially. An iron- and sulfur-oxidizing microbial consortium provide lixiviants for dissolution of mineral sulfides. The autotrophic iron-oxidizing bacterium, *Leptospirillum ferriphilum* HT*,* has been regarded as the dominant iron-oxidizer within the BIOX® culture since establishing this technology in the mid-1980s; however recent studies of the BIOX® consortia present in commercial BIOX® plants worldwide revealed a dominant presence of heterotrophically-inclined archaea including the iron-oxidizer, *Acidiplasma cupricumulans* JTC3. In this study, the effect of physicochemical factors, such as pH and temperature, and the presence of dissolved organic carbon on the growth and ferrous iron oxidation kinetics of these two microorganisms were investigated as potential driving forces behind the compositional shift in the BIOX® consortia.

Biokinetic performance of *L. ferriphilum* HT-dominant and *Ac. cupricumulans* JTC3-dominant batch cultures were investigated using a basal salt media supplemented with 10 $g.L^{-1}$ ferrous iron. Yeast extract was supplied as organic substrate for the archaeon. Growth curves were constructed from cultivation in Thomson™ 24-well microtitre plates, measuring microbial growth by direct cell counting and ferrous iron concentration by the spectrophotometric 1-10 phenanthroline assay. Biokinetic performance of *L. ferriphilum* HT was investigated at the lower (40°C) and upper (45°C) temperature ranges typical of the BIOX® plant operating window and compared against the performance of *Ac. cupricumulans* JTC3 at 45°C, as a function of pH in the range pH 0.7-1.7. The effect of dissolved organic carbon on *L. ferriphilum* HT was assessed by spiking cultures with either yeast extract (0.1-0.5 $g.L^{-1}$), 10-50% spent *Ac. cupricumulans* JTC3 culture filtrate or 1-30 $mg.L^{-1}$ glycolic acid, acetic acid or pyruvic acid. The influence of organic carbon on *Ac. cupricumulans* JTC3 was investigated in a similar fashion by cultivating it in yeast extract, spent *L. ferriphilum* culture filtrate and in cultures spiked with 1-30 $mg.L^{-1}$ glycolic acid, acetic acid and pyruvic acid.

The biokinetic performance of the *L. ferriphilum* HT decreased substantially at 45°C and the detrimental effect of elevated temperature was exacerbated at pH < 1.3. Conversely, the highest *Ac. cupricumulans* JTC3 growth and volumetric oxidation rates were determined at pH < 1.3 and decreased with increasing pH. Considering the effect of organic compounds, complete inhibition of *L. ferriphilum* HT was observed in 0.5 $g.L^{-1}$ yeast extract, 25% spent culture filtrate (0.079 $g.L^{-1}$ DOC) and cultures spiked with 30 $mg.L^{-1}$ glycolic acid, and 10 $mg.L^{-1}$ acetic and pyruvic acid. No growth of *Ac. cupricumulans* JTC3 was observed in cultures lacking organic substrate or in cultures supplemented with only spent *L. ferriphilum* culture filtrate. Complete inhibition was observed in cultures grown in 50% spent culture filtrate supplemented with yeast extract. The contrasting effect of acid stress at pH < 1.5 on these two microorganisms at 45°C may contribute to the compositional shift observed in the archaeal-dominant BIOX® reactors whereas the effect of dissolved organic carbon (DOC) on these two microorganisms emphasizes the importance of microbial diversity in establishing robust biomining processes and the symbiotic relationship between organic-sensitive chemolithotrophs and their heterotrophic counterparts within the microbial consortia.

Acknowledgements

It is with great pleasure that I can submit this MSc dissertation. Conducting an MSc degree in the unfamiliar, interdisciplinary field was no simple feat, but attempting this while simultaneously juggling a part-time job (as a guesthouse chef at Beulah Lodge) and later full-time employment (as a Research Associate at Synexa Life Sciences) really pushed my limits and helped build a lot of individual character. While I take much pride in (finally) completing this MSc milestone, I cannot do so without acknowledging the amazing people around me for all their support and encouragement on this journey.

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Glossary of Terms

Term	Definition
Acidophile	Microorganisms that thrive in acidic environments
Biofilm	An adherent microbial consortium embedded within a slimy extracellular matrix composed of extracellular polymeric substances, typically attached to a surface.
Bioleaching	Microbe-mediated solubilization and recovery of metals from ore bodies and mineral concentrates
Biomining/ biohydrometallurgy	The process/field of application of using microorganisms to extract metals of economic interest from ore bodies, waste rock and mineral concentrate.
Bio-oxidation	Microbe-mediated mineral decomposition, where the target metal remains in an insoluble form.
Cyanidation	A hydrometallurgical technique used to solubilize target metals, most notably gold.
Electronic waste	Discarded electrical or electronic devices, appliances and scrap components.
Extreme Acidophile	A microorganism which has a pH optimum for growth at pH ≤ 3.0.
Flotation	A method used in mineral processing to separate and concentrate ores by altering their surfaces to a hydrophobic or hydrophilic condition.
Gangue material	Commercially worthless material that surrounds or is associated with a desired mineral in an ore deposit.
Lixiviant	A liquid medium used to selectively extract a target metal from the ore or mineral.
Microbial consortia	Two or more microbial species living symbiotically.
Moeity	A specific group of atoms within a molecule that is responsible for characteristic chemical reactions of that molecule
Planktonic	Single-cells, free-floating microorganisms
rDNA fingerprinting	DNA-based genetic tool used for determining the identity of a certain microorganism or group of microorganisms within a community of microorganisms.
Refractory mineral	Minerals which have high melting points and are resistant to deformation and softening at high temperatures.
Solids loading	The amount of suspended solids in a liquid substance.
Tailings	Finely ground ore residuals that remain after the mill process has removed the valuable metals from the ore.
Waste rock	Bedrock that has been mined and no longer of economic interest.

Acronyms and Abbreviations

ATP	Adenosine triphosphate
COD	Chemical oxygen demand
DOC	Dissolved organic carbon
EDTA	Ethylenediaminetetraacetic acid
E-waste	Electronic waste
gDNA	Genomic Deoxyribonucleic Acid
MWP	Microwell plate
NADH	Nicotinamide adenine dinucleotide + Hydrogen
pDNA	Plasmid DNA
PGM	Platinum Group Metals
pmf	Proton-motive force
rDNA	Ribosomal Deoxyribonucleic acid
RISC	Reduced Inorganic Sulfur Compounds
TE	Tris-EDTA
VFA	Volatile Fatty Acid
$-r_{Fe}{}^{2+}$	Volumetric ferrous iron oxidation rate
$q_{Fe}{}^{2+}$	Specific ferrous iron oxidation rate
μ_{max}	Maximum specific growth rate

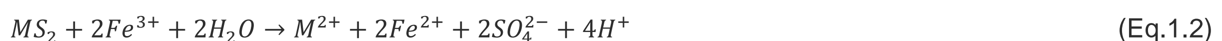
1 Introduction

1.1 Background: Biohydrometallurgy

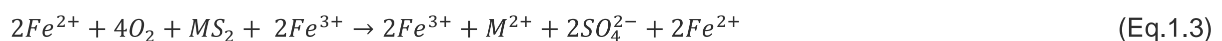
In recent years, the mining industry has been challenged to balance the increasing demand for metal-based commodities, the rapid depletion of high-grade ores and increasingly stringent environmental impact legislation. Factors such as these have driven the mining industry to look for more cost-effective and “greener” means of mineral processing as well as to invest in technologies, such as biohydrometallurgy, that can handle complex ores (Brierley, 2010). Biohydrometallurgy (also known as biomining) refers to mineral processing methods which rely on acidophilic microorganisms to orchestrate the recovery of metals from minerals, including low-grade refractory minerals, as well as electronic waste (E-waste). Biomining also provides a means of processing niche ores containing complex polymetallic minerals or minerals high in penalty elements (arsenic, antimony, and bismuth) which are not well-suited for conventional pyrometallurgical processes (Neale *et al*., 2011; Johnson, 2014).

The microbially-mediated recovery of metals occurs through either bioleaching or bio-oxidation processes. In bioleaching processes, the metal of interest is solubilized during mineral dissolution whereas bio-oxidation processes function in exposing insoluble metal particles such as gold or PGMs trapped within the mineral matrix, making them more accessible for lixiviant extraction (Brierley, 2001; Rawlings 2005). The reaction occurs indirectly through cyclical biological and chemical oxidation sub-processes. Biological oxidation refers to microbe-mediated oxidation of ferrous iron to produce ferric iron (Eq.1.1) whereas chemical oxidation refers to the oxidation of the sulfide mineral by ferric iron attack, thereby regenerating the ferrous iron (Eq. 1.2). (Sand *et al*., 2001; Rawlings, 2002). Although the biological and chemical reactions are connected through the transferral of ferrous and ferric iron, these reactions occur independently and can therefore be uncoupled and investigated independently (Boon and Heijnen, 1997).

$$2Fe^{2+} + O_2 + 4H^+ \rightarrow 2Fe^{3+} + 2H_2O \quad \text{(Eq.1.1)}$$



$$MS_2 + 2Fe^{3+} + 2H_2O \rightarrow M^{2+} + 2Fe^{2+} + 2SO_4^{2-} + 4H^+ \quad \text{(Eq.1.2)}$$



$$2Fe^{2+} + 4O_2 + MS_2 + 2Fe^{3+} \rightarrow 2Fe^{3+} + M^{2+} + 2SO_4^{2-} + 2Fe^{2+} \quad \text{(Eq.1.3)}$$

The combination of Eq. 1.1 & 1.2 summarizes the overall reaction which occurs during sulfide mineral oxidation, though the mineral oxidation pathway varies with the chemical composition of the mineral.

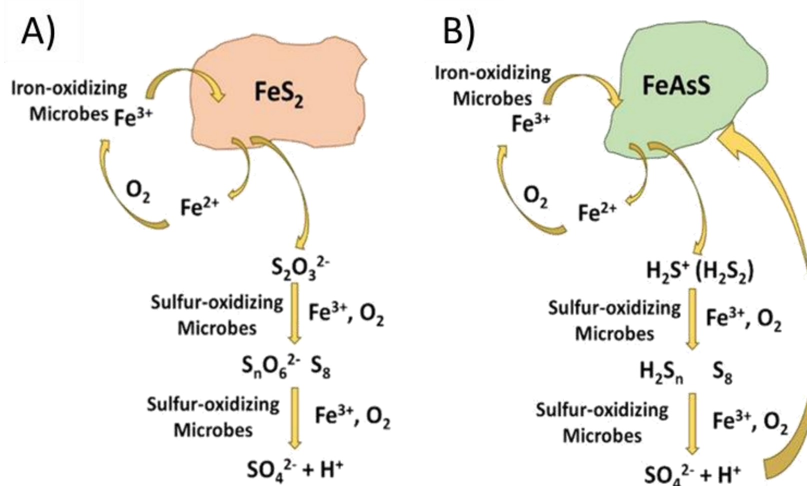
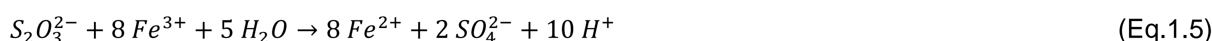


Figure 1.1: Mechanism of sulfide mineral oxidation occuring through A) the thiosulphate intermediate pathway and B) the polysulfide intermediate pathway (adapted from Schippers *et al*. 1996)

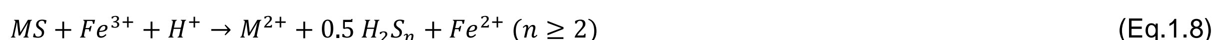
Figure 1.1 illustrates a general schematic of the two oxidation pathways that have been reported for sulfide mineral oxidation. The pathways are classified on the basis of the sulfidic intermediates produced during mineral oxidation, namely thiosulfate and polysulfides (Sand et al., 2001). When ferric ions react with acid-insoluble disulfide minerals, such as pyrite (FeS_2), the disulfide group ($-S_2$) within the mineral is oxidized to thiosulfate (Figure 1.1.A) and the associated metal cation is liberated. The sulfur intermediate is then further oxidized to tetrathionate, trithionate and/or polythionates (Eq. 1.4), however these intermediates are very unstable in the ferric-rich liquor and readily decompose to form elemental sulfur or undergo further oxidation to form sulfuric acid (Eq.1.5). During the oxidation of arsenopyrite (FeAsS), pyrrhotite (Fe_7S_8) and other acid-soluble sulfide minerals (Figure 1.1.B), the sulfur moiety present in the mineral matrix then either undergoes proton attack to form hydrogen sulfide, or a combined proton and ferric attack which results in the formation of a free disulfide species (Eq. 1.8). The liberated disulfide is then oxidized further to form unstable polysulfide intermediates which is subsequently oxidized to elemental sulfur (Eq. 1.9). The elemental sulfur produced from these pathways is either released into the leach liquor as a free aggregate or settles as a passivating layer on the mineral surface (Mustin *et al.*, 1993). In addition, the elemental sulfur and associated sulfur intermediates produced may undergo complete oxidation through biological means via sulfur oxidizing microorganisms (Eq.1.6 & Eq.1.7) (Crundwell, 1988; Sand *et al.*, 2001; Schippers *et al.*, 1996).

$$FeS_2 + 6\,Fe^{3+} + 3\,H_2O \rightarrow S_2O_3^{-2} + 7\,Fe^{2+} + 6\,H^+ \quad \text{(Eq.1.4)}$$


$$S_2O_3^{2-} + 8\,Fe^{3+} + 5\,H_2O \rightarrow 8\,Fe^{2+} + 2\,SO_4^{2-} + 10\,H^+ \quad \text{(Eq.1.5)}$$

$$S_2O_3^{2-} + 2\,O_2 + H_2O \rightarrow 2\,SO_4^{2-} + 2\,H^+ \quad \text{(Eq.1.6)}$$

$$0.125\,S_8 + 1.5\,O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+ \quad \text{(Eq.1.7)}$$


$$MS + Fe^{3+} + H^+ \rightarrow M^{2+} + 0.5\,H_2S_n + Fe^{2+}\ (n \geq 2) \quad \text{(Eq.1.8)}$$

$$0.5\,H_2S_n + Fe^{3+} \rightarrow 0.125\,S_8 + Fe^{2+} + H^+ \quad \text{(Eq.1.9)}$$

Mineral dissolution may be further characterized according to the microbe-mineral interaction. Microorganisms may interact via contact with the mineral by attaching to the mineral surface via biofilm formation, allowing for localized production of the lixiviant (Boon *et al*, 1995; Crundwell, 2003). Alternatively, planktonic, unattached microorganisms may also facilitate mineral dissolution by contributing to the ferric iron content of the leach liquor (Rawlings, 2002; Brune and Bayer, 2012).

Microbe-mineral interactions play a vital role in biomining process design and therefore variety of different strategies have been utilized to conduct bioleaching and bio-oxidation processes, although most of which can be broadly categorized as either irrigated or tank-based operations. In irrigated operations, the large low-grade ore or waste rock is either stacked in large “dumps” (dump bioleaching) or crushed before being stacked into “heaps” which are designed to optimise leaching (heap bioleaching). The dumps or heaps are then irrigated with a dilute sulfuric acid solution enriched with the bioleaching microbial consortia which is used to inoculate the stacked mineral bodies. The enriched sulfuric acid solution subsequently interacts with the metal-bearing sulfide minerals and gangue material, allowing the microorganisms present in the liquor to interact with (and adhere to) the mineral surface as the leachate liquor percolates through the stacked ore or waste rock (Rawlings *et al*., 2003; Tupikina *et al*., 2013; Sand *et al*., 2001).

On the contrary, tank-based operations are typically used to recover metals from milled mineral concentrate, which is fed as a slurry into large agitated and aerated stirred-tank bioreactors. The agitation provides a relatively homogenous environment within the vessel and allows for excellent contacting to be maintained between the leach liquor and the mineral surface while aeration ensures that the microbial consortia within the bioreactors are supplied with sufficient O_2 and CO_2. The solids loading of the slurry also impacts the microbial consortia within the bioreactors as high solids loading (< 20% w/v) leads to an increase in shear force which can be detrimental to cell viability (particularly cell-wall-lacking microorganisms and Gram-negative bacteria). Fresh concentrate is continuously fed into the primary bioreactors to ensure that an active and stable mineral-oxidizing microbial community is maintained in the vessels. The slurry, impregnated with an active microbial consortium, is transferred from the primary bioreactors into a series of secondary bioreactors where further ferric leaching and sulfide oxidation occurs. Process temperature and pH within the train of bioreactors is controlled to jointly optimize growth of the mineral-oxidizing microbial consortium and mineral oxidation and minimize risk of mineral surface passivation (van Aswegen *et al*., 2007). Although the controlled conditions and intimate contacting within the bioreactors provides higher dissolution rates compared to irrigated bioleaching technologies, the tank-based bio-oxidation technologies are inherently more expensive than their irrigation-based counterparts, as they require a greater capital investment for construction as well as higher energy input for preparation of the mineral concentrate and operation of the aerated stirred-tank bioreactors. Therefore, to date, tank-based processes are primarily used for the recovery of precious metals barring a few exceptions (Brierley and Brierley, 2001; Rawlings *et al*., 2003).

Tank-based biomining technologies were initially developed for the pre-treatment of refractory gold ores in the mid-1980’s to the late 1990’s and has led to the emergence of several dominant tank bio-oxidation technologies, most notably the BIOX® process and the BACOX™ process (Rawlings, 2011). BACOX™

was originally developed at the Youanmi Mine in Western Australia by the BacTech Mining Corporation, which has since been incorporated into the REBgold Corporation (Canada). The technology was developed using a moderately thermophilic microbial consortium (45-55°C) to facilitate the reclamation of gold and base metals from mine tailings and waste rock left by previous mining operations (Rawlings *et al*., 2003; Rawlings 2011, Watling, 2008). The BIOX® process was developed by Gencor Ltd. and has since changed ownership to BHP, BHP-Billiton, Goldfields and Biomin Ltd. before being acquired by the current technology titleholder, Outotec and is used primarily used for the pre-cyanidation treatment of gold-bearing refractory mineral concentrates (Brierley and Brierley, 2001; Clark *et* al., 2006; Smart *et al*., 2017).

Tank-based biomining technologies have been subsequently adapted for the leaching of base metals such as copper, nickel, cobalt and zinc (Clark *et al*., 2006; Brierley and Brierley, 2001). An example of this is the BioNIC™ technology, which was developed by BHP Billiton for the recovery of nickel using a mesophilic microbial consortium (Clark *et al*., 2006). BHP Billiton (in a joint venture with Alliance copper and in partnership with Codelco) were also key drivers in the development of BioCOP™ which was used in a pilot plant established at the Chuquicamata Mine (Chile) to recover copper from chalcopyrite concentrate. Due to refractory nature of chalcopyrite, the BioCOP™ technology must operate at high temperatures (> 70°C) using extremely thermophilic archaea to leach copper from the mineral concentrate (Brierley and Brierley, 2013). Other tank-based technologies have also been developed for the reclamation of base metals on a commercial scale, such as the BROGIM® process by the Kasese Cobalt Company at the Kilembe Mine in Uganda to recover cobalt (Morin and d'Hugues, 2007).

The abovementioned examples illustrate how tank-based biomining technologies have become more widespread, globally, and increasingly versatile with regards to metal reclamation (i.e. used in the recovery of both precious metals and base metals). However, in terms of commercial tank-based technologies currently in operation, the BIOX® process has proven to be the most successful. The BIOX® technology has been implemented in commercial operations for over three decades with 13 BIOX® plants commissioned worldwide.

1.2 The BIOX® process

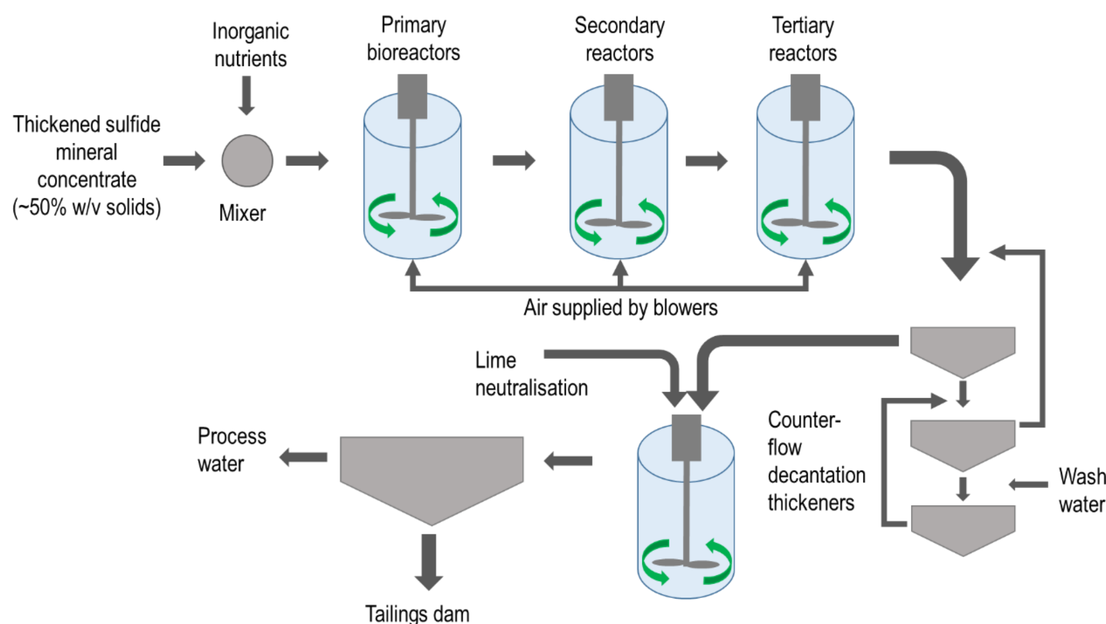


Figure 1.2: Typical flow sheet for the BIOX® process (adapted from van Aswegen *et al*., 2007)

The general process flow of the BIOX® process (Figure 1.2) consists of several stirred-tank bioreactors (primary reactors) operating in parallel, which in turn feed into secondary and tertiary reactors configured in series. The sulfidic mineral concentrate used in the BIOX® process is prepared by crushing and milling the refractory ore (typically 80% passing 75 µm diameter with a minimum diameter of 150 µm) followed by concentration by flotation. The concentrate is circulated through a regrind circuit. Following regrinding, the concentrate is thickened to a pulp density of roughly 50% (w/v) solids loading before being pumped into the BIOX® stock tank (van Aswegen *et al*., 2007). The concentrate slurry is supplemented with essential inorganic nutrients and fed continuously into the primary bioreactors (inoculated with the BIOX® microbial culture) at a pulp density of ~20% (w/v) solids loading. The overall bioreactor residence times range from 4 to 6 days with more than half of the duration spent in the primary reactors to allow for a stable microbial population to be established and to prevent subsequent microbial washout (Rawlings, 2007; van Aswegen *et al*., 2007). The slurry is then fed through the train of secondary and tertiary bioreactors where the bio-oxidation process is completed. The leached gold concentrate is then collected and washed in order to reduce the high ionic load (primarily due to high concentrations of ferric iron). Removal of these ions assists with downstream processing as it decreases lixiviant consumption during cyanidation. The concentrate slurry is washed in a counter current decantation (CCD) circuit and dissolved ionic species, including ferric iron and arsenic, are removed from the overflow liquid stream by precipitation in order to stabilise the arsenic species before disposal (van Aswegen *et al.* 2007; Rawlings 2007). The thickened and washed concentrate proceeds to cyanidation to extract and recover the gold before the barren solids also report to the tailings.

Table 1.1: Operating parameters of the BIOX® process (adapted from van Aswegen *et al.,* 2007)

Operating Parameters	
Temperature	*40°C - 45°C*
pH	*1.2-1.8*
% solids in the feed	*20%-30%*
Dissolved oxygen	*≥ 2.0 mg.L^{-1}*
Retention time	*4-6 days*
Nutrients	*Fertilizer type (N:P:K)*

The typical BIOX® operating parameters (Table 1.1) were optimized to favour pyrite and arsenopyrite oxidation (van Aswegen *et al.*, 2007). Although the temperature of the operating window was designed for a pH range of 1.2-1.8 and temperature range of 40 to 45°C (van Aswegen *et al.*, 2007; Mahmoud *et* al., 2017), fluctuations in temperature and acidity may exceed the set operating window when using a high-sulfur mineral feed as sulfide mineral oxidation is an acid-producing exothermic reaction (van Hille et al., 2013).

1.3 The BIOX® microbial consortium

The microbial consortia used in the BIOX® process are composed primarily of iron-oxidizers and sulfur-oxidizers which are responsible for the generation of ferric iron and sulfuric acid, respectively. Determination of the dominant microorganisms present in the BIOX® microbial consortium was initially conducted using traditional microbiological techniques which indicated that the consortia consisted primarily of chemolithotrophic bacteria, namely *Acidithiobacillus thiooxidans, At. ferrooxidans* and *L. ferrooxidans* (van Hille *et* al., 2013; Rawlings 2011). This was consistent with microscopic observation. The emergence of more advanced analytical techniques, such as polymerase chain reaction and 16S rDNA fingerprinting, emerged in the 1990's which allowed for a more detailed and comprehensive identification of microorganisms present in mixed-microbial communities (Pizarro *et al.*, 1996).

Further characterization of the mesophilic BIOX® microbial consortia was conducted by Rawlings et al. (1999) who reported an iron-oxidizing *Leptospirillum* sp. and sulfur-oxidising *Acidithiobacillus spp.* to be be the dominant microorganisms present in the BIOX® culture. Moreover, the findings indicated that under typical mesophilic tank-leaching conditions (high ferric iron content, temperature between 37°C-42°C, pH < 2.0) the *Leptospirillum sp.* (classified as *Leptospirillum* group II) would outcompete *At. ferrooxidans,* originally perceived to be the dominant iron-oxidizer in the BIOX® consortia (Rawlings *et al.*,1999). The microbial composition and the role of the *Leptospirillum* sp. within the BIOX® consortia was then further assessed in a laboratory-scale study conducted by Coram and Rawlings (2002). The findings from this study led to the identification of *L. ferriphilum* as the dominant iron-oxidizer and *At. caldus* as the dominant sulfur-oxidizer within the BIOX® culture.

A speciation review of the BIOX® microbial consortia inhabiting industrial BIOX® bioreactors in South Africa, Ghana and Khazakstan eluded to a significant compositional shift which was characterized by lower than expected populations of autotrophic iron-oxidizers and a prevalence of heterotrophic archaea (van Hille *et al.*, 2011). Further investigation of the BIOX® consortia was conducted in

laboratory-scale bioreactors mimicking the operating conditions of the industrial bioreactors (van Hille *et al.* 2013). The laboratory-scale investigation revealed a similar trend in the compositional shift of the BIOX® consortia, characterized by reduced levels of *L. ferriphilum* and a prevalence of heterotrophic archaea (such as *Ac. cupricumulans* JTC3 and *Ferroplasma spp.*) as well as Gram positive heterotrophic bacteria, such as *Sulfobacillus thermosulfoxidans*, (van Hille *et al.*, 2013; Harrison, 2016). Although the dynamic composition of the microbial consortium appeared to be influenced by the bioreactor operating conditions, longitudinal studies have demonstrated that the reduced abundance of *L. ferriphilum* and increasing prevalence of archaeal species (including *Ac. cupricumulans*, *Ferroplasma spp.* and *Thermoplasmotales*) was a consistent, overall trend within the BIOX® consortia (Smart *et al.* 2017). The compositional shift in the BIOX® consortia may be attributed to the physicochemical conditions within the BIOX® operating window, of which temperature has already been identified as a contributing factor behind the observed shift in the consortia through a laboratory study conducted by van Hille *et al.* (2013). Although extensive research has been conducted on the effects of pH on the iron-oxidizing acidophiles (Baker-Austin and Dopson, 2009; Ojumu and Petersen, 2011; Penev and Karamanev, 2010, Watling *et al.*, 2013; Nemati *et al.*, 1998), the effect of pH as a selective pressure on keystone iron-oxidizing species in the BIOX® consortia has not been well defined within the context of the BIOX® operating window.

The prevalence of heterotrophic archaea within the bioreactors also requires further investigation as their dominance suggests sufficient DOC is present within a bioreactor to sustain the heterotrophic microbial community. Although an inorganic concentrate slurry is fed into the bioreactors, the necessary DOC may have derived from cell-lysate and exudates produced by the autotrophs within the bioreactors (Okibe *et al.*, 2003). Unlike their heterotrophic counterparts, chemolithotrophic acidophiles may be acutely sensitive to the presence of DOC constituents in the media, particularly the presence of organic acids (Borichewski, 1966; Tuttle *et al.*, 1977; Vardanyan *et al.*, 2017). Therefore, the presence of DOC's in the media had been postulated to have an antagonistic effect by potentially inhibiting growth of chemolithotrophic bacteria while simultaneously promoting growth of archaea and other heterotrophic acidophiles. The postulated effect of DOC's would also contribute to the observed compositional shift within the BIOX® consortia as well as the consortia within contemporary biomining technologies, especially intensive tank-based technologies in which a large fraction of the microbial consortium is planktonic and hydraulic residence times are short.

1.4 Summary

Iron-oxidizing microorganisms play a fundamental role in biomining processes through the generation bioreactors, suggested a similar trend in the compositional shift within the BIOX® consortia, where *Ac. cupricumulans* JTC3 was identified as one of the prevalent archaeal species inhabiting the bioreactor (van Hille *et al.*, 2011, 2013; Harrison, 2016; Smart et al., 2017). Although both types of microorganisms are capable of oxidizing ferrous iron, *L. ferriphilum* is a very efficient iron-oxidizer and demonstrates very high affinity for ferrous iron which is essential for maintaining the highly positive redox potential (primarily determined by the ratio of ferric iron to ferrous iron in the solution) necessary to promote mineral decomposition (Rawlings *et* al., 1999; Coram and Rawlings, 2002). Therefore, an

understanding of selective pressures, such as those imposed by physical and chemical stresses, that may trigger the compositional shift in the BIOX® iron-oxidizing community is required to improve stability of the microbial consortia, robustness of the BIOX® process as well as to inform process optimisation.

of ferric iron which catalyses the decomposition of metal-bearing sulfide minerals. Chemolithotrophic bacteria that can tolerate high levels of ferric iron and capable of scavenging ferrous iron at low concentrations, namely *L. ferriphilum*, were considered to be key the iron-oxidizers in the BIOX® microbial consortia (Coram and Rawlings, 2002). However, further investigation of the microbial ecology within commercial BIOX® bioreactors eluded to relatively low levels of *L. ferriphilum* present in the consortia and a prevalence of heterotrophic iron-oxidizing archaea. Findings from a laboratory-scale study performed by van Hille *et al.* (2013), emulating the operating conditions of the commercial Scope and constraints

The aim of this study was to investigate the biokinetic performance of two prominent iron-oxidizing microorganisms, *L. ferriphilum* HT and *Ac. cupricumulans* JTC3, which have been isolated from the BIOX® bioreactors and studied in separate *L.ferriphilum* HT– and *Ac. cupricumulans* JTC3-dominant batch cultures. This investigation focusses on the influence of physicochemical factors, namely: temperature, pH and the presence of dissolved organic carbon (particularly organic acids) on microbial growth and activity. These cultures are characterised by variations in the observed lag times, microbial growth rates, ferrous iron oxidation rates and biomass yields achieved by these microorganisms. The insight gained from this study is required to provide a better understanding of how these factors influence microbial dynamics amongst the iron-oxidizers present in the BIOX® consortia and inform adjustments required to correct process outages. Furthermore, a better understanding of the microbial dynamics within the consortia allows for further optimization of the operating window for a more robust microbial community and improved bio-oxidation performance.

The study was performed using separate *L.ferriphilum* HT-dominant and *Ac. cupricumulans* JTC-dominant batch cultures cultivated in 24-well microwell plates (MWPs). Studying monocultures cultivated in 24-well MWP's provides a high-throughput means of investigating the effects of temperature, pH and DOC on the biokinetic performance of these microorganisms. Despite the speed and versatility that MWP batch cultures provide, this approach is met with several noteworthy limitations. The microorganisms are cultivated as continuous cultures in the BIOX® bioreactors and so the growth kinetics obtained under process conditions may differ from those obtained by these microorganisms under batch culture conditions. Furthermore, this approach does not provide insight into mutualistic, commensal or ammensal interactions between these two iron-oxidizers or factor in the impact that symbiotic and other inter-species relationships may have on their biokinetic performance (Okibe and Johnson, 2004).

In summary, this study aims to provides insight on how changes in temperature, pH and DOC within the BIOX® operating window influence the growth and performance of two significant iron-oxidizing microorganisms associated with the BIOX® consortia by providing biokinetic data on each individual species. These data inform the nature of the competition between the species and hence their likely

dominance. However, this study does not explore the microbial interactions between species in a mixed culture. Hence, it provides the starting point for unravelling the dynamics in the microbial communities present; however, it is not expected to reflect fully the performance of the mixed microbial consortia present within a BIOX® bioreactor as further unpacking of commensal and ammensal microbial interactions, beyond the scope of this dissertation, is required to complete the study.

1.5 Objectives

The findings from this study will have relevance to other bioleaching and bio-oxidation processes as well, particularly for tank processes. This study aims to contribute to the understanding of microbial dynamics of iron-oxidizing acidophiles involved in biohydrometallurgical processes by addressing the following objectives:

- To investigate the effect of pH on the biokinetic performance of *L. ferriphilum* at the upper and lower temperatures of the BIOX® operating window (45°C and 40°C).
- To compare the effect of pH on the biokinetic performance of *L. ferriphilum* and *Ac. cupricumulans* and predict the impact of pH on the dominance of these two microorganisms within the range of conditions investigated.
- To investigate the inhibitory effect of dissolved organic carbon on the growth and activity of *L. ferriphilum*, focussing primarily on the effect of yeast extract, organic acids and archaeal (*Ac. cupricumulans* JTC3) spent culture filtrate.
- To investigate the role of organic carbon on the growth and activity of *Ac. cupricumulans* JTC3 using yeast extract, organic acids and chemolithotrophic (*L. ferriphilum* HT) spent culture filtrate.

1.6 Thesis structure

Chapter 1 introduces the context and objectives for the study and positions this body of research within the field of biohydrometallurgy (i.e. biomining). This chapter also addresses the scope of the research as well as the limitations associated with conducting a high-throughput comparative biokinetic study of *L. ferriphilum* and *Ac. cupricumulans*.

The literature review presented in Chapter 2 provides an overview of biomining and the role that it plays as a more environmentally friendly alternative to the conventional means of metal recovery. The review also addresses the mechanisms behind biologically mediated mineral oxidation and the role of iron-oxidizing acidophiles in biomining applications with emphasis on tank-based technologies, specifically the BIOX® process. The development and history of the BIOX® technology is then addressed as well as the general process flow sheet and operating parameters of the BIOX® process. In addition, the composition of the BIOX® microbial consortia is then explored in the review; identifying and comparing the dominant iron-oxidizing microorganisms present in BIOX® cultures isolated from both commercial bioreactors and laboratory-scale BIOX® bioreactors. Lastly, the review provides a general overview on the physiology and metabolism (specifically carbon and ferrous iron metabolism) of two noteworthy iron-oxidizers present in the BIOX® consortium, namely *L. ferriphilum* HT and *Ac. cupricumulans* JTC3 and

how physicochemical factors, such as temperature, pH and the presence of dissolved organic carbon, influence these iron-oxidizing acidophiles.

Chapter 3 provides a detailed description of the materials and methods associated with the batch culture kinetic study of *L. ferriphilum* HT and *Ac. cupricumulans* JTC3 performed in 24-well microwell-plates.

Chapter 4 illustrates and compares the effect of pH (across the pH range of pH 0.7-1.7) on the biokinetic performance of *L. ferriphilum* HT and *Ac. cupricumulans* JTC3. The results show the effect of pH on the biokinetic performance of *L. ferriphilum* HT cultivated at 40°C (lower limit of the BIOX® operating temperature) as well as the cumulative effect of heat and acid stress on the biokinetic performance of *L. ferriphilum* HT and *Ac. cupricumulans* JTC3, when cultivated at 45°C (upper limit of the BIOX® operating temperature). The findings were used to infer how the dominance relationship between these two iron-oxidizing microorganisms would shift as a function of pH under the experimental conditions examined.

Chapter 5 illustrates the effect of dissolved organic carbon on the biokinetic performance of *L. ferriphilum* HT and *Ac. cupricumulans* JTC3*. L. ferriphilum* HT's sensitivity towards dissolved organic carbon was explored by observing growth and activity of the microorganism in the presence of yeast extract and select organic acids, namely glycolic acid, acetic acid and pyruvic acid. *L. ferriphilum* HT growth was also investigated in *Ac. cupricumulans* JTC3 spent culture filtrate to determine if sufficient DOC was present in the spent culture to elicit an inhibitory effect on *L. ferriphilum* HT. Similarly, the growth of *Ac. cupricumulans* JTC3 on L. *ferriphilum* HT spent culture filtrate was then also investigated to determine if sufficient organic carbon was present in the filtrate to support growth of the heterotrophic archaeon. Furthermore, the effect of yeast extract and select organic acids (glycolic acid, acetic acid and pyruvic acid) on the growth and performance of *Ac. cupricumulans* JTC3 was also investigated.

Chapter 6 addresses the conclusions that can be drawn from the effect of pH and temperature on the biokinetics of *L. ferriphilum* and *Ac. cupricumulans* and the contrasting effect that the presence of DOC has on the two microorganisms. Recommendations were then derived for the optimal conditions for *L. ferriphilum* growth and activity within the BIOX® operating window while limitations of the study were made explicit. Considerations were also put forward regarding whether or not *L. ferriphilum* dominance is essential for the success BIOX® process.

2 Literature Review

2.1 Characterization of acidophiles involved in biomining processes

2.1.1 Identifying microbial consortia for biomining processes

Biomining processes are orchestrated by a diverse community of microorganisms which exhibit different metabolic and physiological characteristics and fulfil different roles surrounding the mineral dissolution process (Okibe and Johnson, 2004). However, these microorganisms share common traits which allow them to thrive under extremely adverse conditions characterized by low pH, highly positive redox potentials, elevated concentrations of heavy metals, metalloids and other solutes (Rawlings and Johnson, 2007; Johnson, 2014). Despite their integral role in the development of biomining technology, the role of microorganisms within these processes was not recognized until the mid-20th century (Johnson, 2008).

Most of the extensive microbial characterization work has been done on relatively few microorganisms isolated from biomining cultures. The bacterium *Ferrobacillus ferrooxidans*, later reclassified as *Thiobacillus ferrooxidans* and again as *Acidithiobacillus ferrooxidans* (Temple and Colmer, 1951, Rawlings, 2011), has been the most extensively characterized iron- and sulfur-oxidizer to date. Traditional plate-based enrichment and isolation techniques, followed by batch culture cultivation of the isolate, provided a selection bias for *At. ferrooxidans* owing to the abundance of ferrous iron typically present in these culture methods which appeared to have over-emphasized the abundance of the bacterium in biomining processes and its role in biological mineral oxidation. *At. ferrooxidans* subsequently dominated the microbiological research prior to the 1990's and has since become one of the most extensively-researched chemolithotrophic acidophiles associated with sulfide mineral dissolution (Watling, 2016; Johnson, 2014). Numerous acidophilic, iron-oxidizing bacteria and archaea have since been identified; these have shown vast metabolic diversity, encompassing autotrophs as well as facultative and obligate heterotrophs (Johnson *et al.*, 2012). Rawlings and Johnson (2007) proposed a convenient means of categorizing acidophiles within the microbial consortia in accordance to their ecological role within the community (Figure 2.1). Iron-oxidizing and sulfur-oxidizing chemolithotrophs were classified as primary and secondary prokaryotes, respectively, whereas the heterotrophically-inclined microorganisms were classified as tertiary prokaryotes. However, it is worth noting that these categories may overlap as some metabolically versatile species may be classified within more than one category (Johnson, 2014).

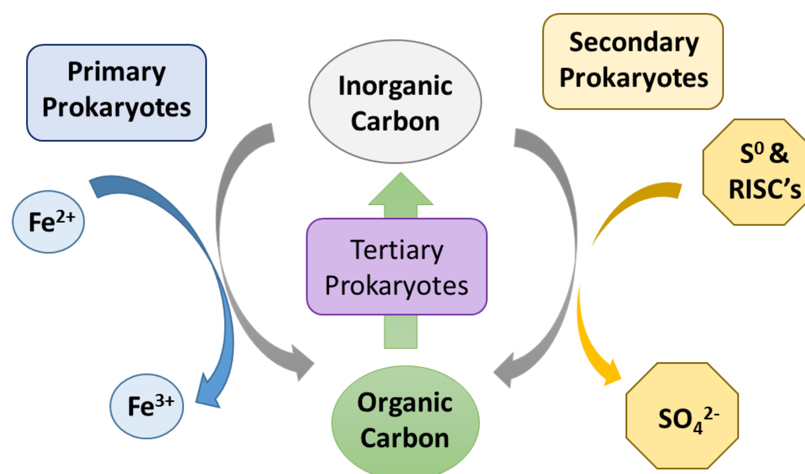


Figure 2.1: Schematic Diagram representing the relationship between primary, secondary and tertiary prokaryotes (adapted from Johnson, 1998.

Primary prokaryotes fulfil the role of oxidizing ferrous iron, thereby producing the mineral lixiviant, ferric iron. Conversely, secondary prokaryotes mediate the oxidation of elemental sulfur and reduced inorganic sulfur compounds (RISC's) produced during sulfide mineral oxidation. The sulfuric acid generated during sulfur/RISC oxidation is required for maintaining the acidic environment as well as removing precipitated sulfur which may passivate the mineral surface. From an ecological perspective, the tertiary prokaryotes in biomining microbial consortia function as heterotrophic scavengers, oxidizing dissolved organic carbon released into the leachate liquor by other members of the microbial consortia (Watling, 2016; Johnson, 2014). In doing so, these microorganisms may potentially assist autotrophic acidophiles within the consortia by scavenging metabolites and other forms of exuded dissolved organic carbon which may have a toxic effect on the autotrophs or by possibly producing extracellular proteins that may enhance microbial interaction with ferrous iron (Chi *et al., 2007)* . The apparent symbiotic relationship which exists between these microorganisms has been reported in several studies where the growth and activity of chemolithotrophs has been shown to increase when co-cultured together with heterotrophic acidophiles (Arkesteyn and De Bont, 1980; Okibe *et al*., 2004; Vardanyan *et al*., 2017; Merino *et al*., 2016; Liu *et al*., 2011).

2.1.2 Constructing microbial consortia for biomining processes

The physico-chemical environment in which the microbially-mediated mineral oxidation occurs greatly influences the natural composition of the consortia. In the case of irrigated biomining processes - temperature, aeration, mineral type, nutrient availability and biofilm formation are inherently heterogenic in nature and vary both temporally and spatially (Rawlings and Johnson, 2007; Brierley and Brierley, 2013). The microbial consortia colonizing heaps are therefore highly diverse and prone to compositional shifts during the lifetime of the heap (Minnaar et al., 2010; Tupikina et al., 2011, 2013; Govender *et al*., 2015; Johnson, 2008). Conversely, biomining technologies which utilize stirred-tank bioreactors create an environment with a higher degree of homogeneity which is controlled within a set operating window (Johnson, 2008; Rawlings, 2002). The controlled, homogenous conditions within such bioreactors creates a largely uniform (spatially), diverse, microbial consortium which is typically dominated by several acidophilic microorganisms. However, the composition of the consortia within the highly

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homogenous environment still remains dynamic and may shift in response to changes in the physico-chemical nature of the leachate solution and the mineral surface, most notably observed in continuous stirred-tank bioreactor configurations in which washout of slow-growing microorganisms may occur readily (Okibe *et al.*, 2003; Norris, 2007; Smart et al., 2017).

Shifts in the microbial composition may also impact mineral oxidation performance through supply of lixiviants and removal of potential passivation layers, therefore, manipulation of the microbial consortia may be required to optimize mineral solubilisation. This may be achieved via a 'top-down' approach whereby an adapted microbial consortium is created by placing selective pressures on the existing indigenous microflora to favour growth of specific microorganisms within the operating window. The composition of the consortia adapts through inter-species competition, in accordance to the selective pressures placed on the consortium under the conditions of the operating window (Norris, 2007; Rawlings and Johnson, 2007), as well as through mutualism, commensalism and ammensalism. Manipulation of the microbial consortium can also be achieved using a 'bottom-up' approach whereby the bioreactor is inoculated with select, adapted microbial strains which are known to thrive in a physicochemical environment tailored to specific biomining operations. The 'bottom-up' approach is especially useful for refractory mineral bio-oxidation processes operating under extreme thermophilic conditions which may be too hostile for most indigenous species of microflora occurring in these environments. Most commercialised tank-based processes use a combination of both approaches as a pre-adapted inoculum or starter-culture is used to assist with bioreactor colonisation and reduce the start-up time, but naturally-occurring microflora are continuously introduced into the bioreactors via the non-sterile mineral feed (Rawlings and Johnson, 2007; Clark and Norris, 1996; Donati *et al.*, 2016).

2.1.3 Microbial consortia within the BIOX® process

The BIOX® culture was initially characterized in the early 1980's using traditional isolation techniques. The findings indicated a dominant presence of mesophilic autotrophs, most notably *Acidithiobacillus spp.* and *Leptospirillum spp.* (Rawlings and Silver, 1995; Dew *et al.*, 1997); however, this traditional approach did not provide a true representation of the microbial composition as only a fraction of the existing microorganisms could be isolated and identified. Morphological studies of the BIOX® tanks at this time did, however, confirm the abundance of *Acidithiobacillus spp.* and *Leptospirillum spp.* A review of the microbial speciation within the Fairview BIOX® bioreactors was conducted by Rawlings in the late 1990s using PCR-based analytical techniques and species-specific 16S rDNA sequences. The findings indicated that a dominant presence of autotrophic chemolithotrophs within the BIOX® reactors; however, the composition of the dominant species differed from what had been originally reported. A *Leptospirillum spp.* was reported as the major iron oxidiser in the consortium. Its dominance was accredited to its ability to utilise low concentrations of ferrous iron and maintain a very high redox potential which favours pyrite leaching (van Scherpenzeel *et* al., 1998; Rawlings et al., 1999).

A further study of the *Leptospirillum* spp. present in the BIOX® consortia was performed by Coram and Rawlings (2002), leading to the characterization of the dominant *Leptospirillum* spp. as *L. ferriphilum,* which has since been recognized as one of the key iron-oxidizers present in mesophilic and moderately

thermophilic tank-bio-oxidation processes (Coram and Rawlings, 2002; van Niekerk 2012; Smart *et al.*, 2017). The bacterium is an aerobic, ferrous-oxidizing autotroph which exhibits a high affinity for ferrous iron and is capable of scavenging ferrous ion at highly positive redox potentials (up to +770 mV at pH 2.0) and at iron concentrations exceeding 50 $g.L^{-1}$ (Rawlings *et al.*,1999; Coram and Rawlings, 2002; Bryan et al., 2012; Smart *et al.*, 2017). *L. ferriphilum* is a thermotolerant mesophile with a reported optimal growth temperature of 38.5°C, however certain strains, such as *L. ferriphilum* HT, have been shown to grow at temperatures exceeding 45°C (Coram and Rawlings, 2002; Franzmann *et al.*, 2005) and as high as 49°C (van Hille et al.,2013).

In addition to *L .ferriphilum*, several other dominant microbial species have been identified in the microbial consortia present in BIOX® and contemporary mesophilic tank-based bioreactors, however the relative abundance of these species may vary as the consortia experience compositional shifts during the course of the bio-oxidation process (Okibe *et* al., 2003; van Hille *et* al., 2011, 2013; Smart et al., 2017). This phenomenon was demonstrated by Okibe *et al.* (2003) in a pilot-scale process consisting of three in-line CSTRs operating at 45°C under similar conditions used in the BIOX® process. Their findings suggested that the predominantly-chemolithotrophic microbial consortia dominated by bacteria such as *At. caldus* and *L. ferriphilum* shifted towards a consortium dominated by heterotrophic *Ferroplasma*-like archaea as the process progressed (Okibe *et al.*, 2003; Rawlings, 2005). These findings were supported by more recent speciation review of the BIOX® microbial consortium obtained from the BIOX® plants located in Ghana (Obuasi), South Africa (Fairview), Australia (Fosterville) and Kazakhstan (Suzdal) as well as the BIOX® stock culture (van Hille *et al.*, 2011, 2013; Harrison, 2016). The speciation results revealed that the microbial community present in these BIOX® cultures consisted of a low abundance of *L. ferriphilum* and a dominant representation of heterotrophic archaea belonging to the Thermoplasmatales order. Moreover, many of the archaea species identified within these bioreactors belong to the *Ferroplasmaceae* archaeal family which are characterized by their ability to use ferrous iron as an energy source iron-oxidizing. (Golyshina e*t al.*, 2005; van Hille *et al.*, 2013; Okibe *et al.*, 2003). The genera *Ferroplasma*, *Thermoplasma* and *Acidiplasma* are the most common archaeal species identified to date in the continuous industrial BIOX® reactors (unpublished data, CeBER labs, UCT). This differs from the consistent trend observed in the feed-and-draw lab-scale BIOX® reactors under conditions of temperature, pH and residence time similar to the large-scale reactors where *L. ferriphilum* and *At. caldus* form the dominant species of the bacterial-dominated consortia which typically also contains an observable fraction of *Ac. cupricumulans* and small amounts of *Ferroplasma* and *Thermoplasma sp.* (Smart *et al.*, 2017).

Ac. cupricumulans is a moderate thermophile with an optimum temperature of around 53-55°C reported for the BH2 and JTC3 strains (Hawkes *et al.* 2006; Franzmann *et al.* 2005; Rautenbach, 2007). This archaeal species was originally discovered during an extensive investigation of the microbial bioleaching community associated with the Myanmar Ivanhoe Copper Company Limited (MICCL) chalcopyrite heap bioleaching operations in Australia. The archaeon was initially classified as *Ferroplasma cyprexacervatum* and later re-classified as *Ferroplasma cupricumulans* (BH2) by Hawkes *et al.* (2005). Further developments within the taxonomic classification of the *Ferroplasmaceae* family

led to the reclassification of the archaeon as *Acidiplasma cupricumulans* (Golyshina *et al.* 2009). Different strains of *Ac. cupricumulans* have been isolated in numerous natural and artificial acidic environments worldwide, including areas associated with hydrothermal, geothermal and anthropogenic activities (Henneberger *et* al., 2006; Golyshina, 2011). A strain of *Ac. cupricumulans* (*Ac. cupricumulans* JTC3) has been identified and isolated from a laboratory-scale BIOX® bioreactor, but due to the highly conserved 16S rRNA sequence amongst closely-related *Acidiplasma* species (Figure 2.2), classification has been somewhat ambiguous with some investigators recognizing the *Ac. cupricumulans* JTC3 strain as an entirely separate species *Acidiplasma* JTC3 (Golyshina *et* al., 2009; Bulaev *et* al., 2015; Rautenbach, 2007). For the purpose of this study, this organism was referred to as *Ac. cupricumulans* JTC3 as reported by Smart *et al.* (2016), Ngoma *et al.* (2015) and van Hille *et al* (2013).

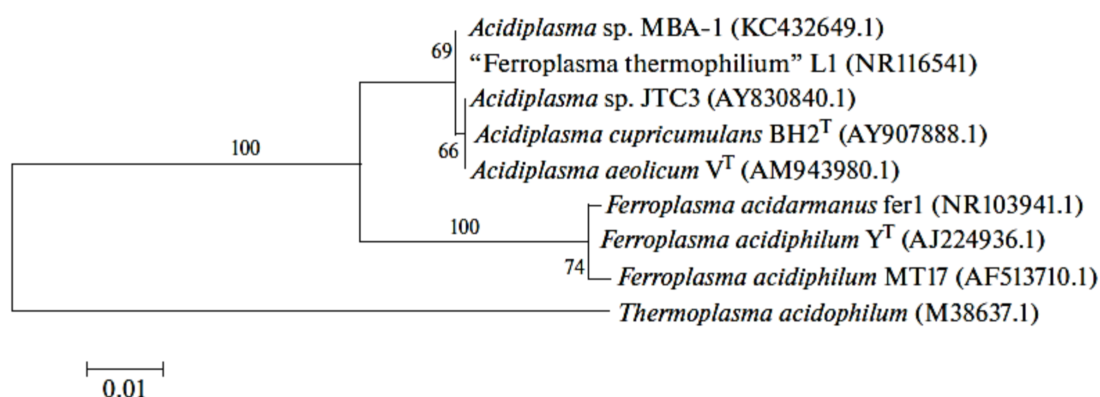

Figure 2.2: Phylogenic tree linking closely-related isolates within the Ferroplasmaceae family of the Thermoplasmatale order. Scale illustrates phylogenic distances between related isolates (Taken from Bulaev et al., 2015)

The optimal growth conditions of *Ac. cupricumulans* JTC3 of 53°C at pH 1.5 have been reported for the archaeon (Rautenbach, 2007; Hawkes *et al.*, 2004). Rautenbach (2007) also reported microbial growth (albeit very slow) of *Ac. cupricumulans* JTC3 in the absence of an organic supplement and decreased growth when cultivated with CO_2-scrubbed air which suggests that *Ac. cupricumulans* JTC3 may have exhibited the capacity for chemo-mixotrophic growth. Furthermore, gene sequences encoding known carbon fixation enzymes have been identified within the archaeon's genome (Chen *et al.* 2016). Chemo-mixotrophic growth was also observed by Cardenas *et al.* (2009) in closely related archaeal species who postulated that these microorganisms were using a "chimeric" carbon fixation pathway which incorporated CO_2 and organic carbon.

2.2 Biological oxidation of ferrous iron by acidophilic microorganisms

2.2.1 Redox couples and chemolithoautotrophic growth of the iron oxidisers

Chemolithotrophs, such as *L. ferriphilum*, rely on ferrous iron as their sole source of energy. Under favourable conditions, microbial growth is tightly coupled to oxidation of ferrous iron which has been demonstrated using both ferrous sulphate (DiSpirito and Tuovinen, 1982) and pyrite (Roy and Mishra,

1981). However, the relationship between microbial growth and substrate oxidation becomes uncoupled in the presence of inhibitors, such as organic acids, or by physico-chemical stresses such as unfavourable pH and temperature (Nemati *et al*., 1998; Alexander *et al*., 1987; Penev and Karamanev, 2010; Dempers *et al*., 2003; Baker-Austin and Dopson, 2009).

During biological oxidation, ferrous iron is readily oxidized to ferric iron and the liberated electron used to drive microbial growth. Ferrous iron oxidation must occur under aerobic conditions as the Fe^{2+}/Fe^{3+} redox couple has a very positive standard electrode potential which requires an energetically favourable terminal electron acceptor, such as molecular oxygen (Rawlings and Johnson, 2007; Johnson *et al*., 2012). At pH 7.0 the redox potential of the O_2/H_2O couple is 820Mv; however, this redox couple is pH-dependent and increases to the region of 1100-1120 mV at pH 2.0 (Ferguson and Ingledew, 2008; Johnson *et al*., 2012). In addition, the high levels of sulphate anions in the media appear to stabilize the ferrous cations in solution, thereby further lowering the redox potential of the Fe^{2+}/Fe^{3+} couple from 780 mV to a region of 650-691 mV (Lazaroff, 1983; Nemati *et al*., 1998). The difference in redox potential between the Fe^{2+}/Fe^{3+} and the O_2/H_2O redox couple is still relatively small and therefore large quantities of ferrous iron must be metabolised by the microorganisms in order to produce relatively little cellular biomass and energy. In addition to ATP, chemolithotrophs also exhibit a high demand for reductive cofactors, such as NAD(P)H, which are essential for carbon fixation and other anabolic reactions. However, NAD(P)+ is a weak oxidant to (-320 mV) and therefore the transfer of electrons from ferrous ion to NAD(P)+ is thermodynamically unfavourable and does not occur unassisted. The assistance required to facilitate this “uphill” flow of electrons occurs through a form of chemiosmosis which requires the generation and maintenance of a proton motive force (pmf) (Ingledew 1982; Nemati *et al,* 1998). While the extracellular pH of the surrounding environment is typically below pH 2.0, the intracellular cytoplasmic pH in acidophiles is maintained at a near-neutral pH (~pH 6.5). This results in the generation of a transmembrane pH gradient and electrical potential, which together constitute the pmf (Nemati *et al*., 1998). The acidic conditions therefore function as a nutritional necessity as the pmf-driven transmembrane flow of protons is essential for the production of NAD(P)H and the autotrophic assimilation of inorganic carbon.

2.2.2 Classical pathway for biological ferrous iron oxidation

The mechanistic pathway for biological ferrous iron oxidation has been most extensively studied in the model chemolithotrophic acidophile, *At. ferrooxidans*. The bacterium contains a unique operon which contains a cluster of genes involved in the electron transport chain. The genes code for several proteins belonging to the cytochrome superfamily and a low molecular weight copper-containing protein, rusticyanin, which may be expressed in abundance (up to 5% of the total cell protein) in ferrous iron-supplemented *At. ferrooxidans* (Rawlings, 2005). Rusticyanin appears to be a pivotal component of the electron transport pathway, forming the branch point between the “uphill” and “downhill” paths of the electron transport pathway (Figure 2.3). The protein functions as an electron reservoir, readily accepting electrons from the cytochrome electron acceptor exposed on the outer membrane (Cyc2) and

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channelling them into the electron transport pathway. This also ensures that Cyc2 remains in a fully oxidized state and ready to extract electrons from ferrous ions (Rawlings, 2005).

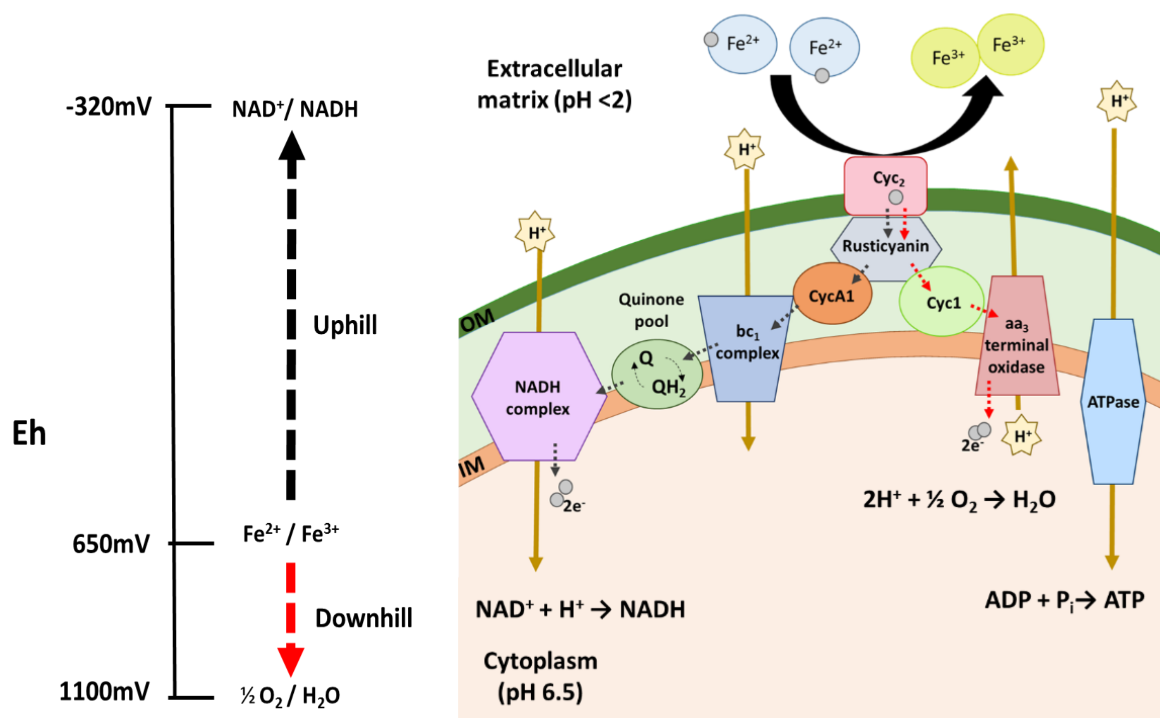

Figure 2.3 Schematic diagram of the classic model for biological oxidation of ferrous iron by *At. ferrooxidans* (adapted from Bonneyfoy and Holmes, 2012). The black arrows represent the "uphill" electron transport pathway whereas the red arrows indicate the "downhill" pathway

Findings from genomic and metagenomic analyses of acidophilic microbial communities indicated that the molecular complexes involved in biological ferrous oxidation were not conserved amongst iron-oxidizing chemolithotrophs (Tyson *et al*., 2004; Rawlings, 2005). Dissimilar ferrous iron oxidation pathways have been constructed for other acidophiles, including *Ferroplasma spp.* and *Leptospirillum spp.* which differ from the classical pathway constructed for *At. ferrooxidans* (Johnson *et al*., 2012; Bonnefoy and Holmes, 2012). The differences observed in these electron transport pathways suggest that ferrous oxidation may have emerged independently amongst different microorganisms as a result of convergent evolution. The presence of different ferrous iron metabolic pathways, alluded to in Table 2.1, may explain the differences in ferric iron sensitivity and substrate affinity exhibited between different iron-oxidizing acidophiles, most notably *L. ferriphilum* and *At. ferrooxidans* (Rawlings *et al*., 1999).

Table 2.1 Key respiratory complexes involved in biological ferrous oxidation (Bonnefoy and Holmes, 2012)

Bacteria	***Pathway***	***Outer membrane***	***Periplasm***	***Inner membrane peripheral***	***Inner membrane***
At. ferrooxidans	*Downhill*	*Cyc2*	*Rus A*	*Cyc1*	aa_3 *oxidase*
	Uphill	*Cyc2*	*Rus A*	*CycA1*	bc_1 *complex*
Leptospirillum spp.	*Downhill*	Cyc_{572}	Cyc_{579}	*Unidentified cytochrome/s*	aab_3 *oxidase*
	Uphill	Cyc_{572}	Cyc_{579}	*Unidentified cytochrome/s*	bc_1 *complex*
Archaea	*Pathway*	*Membrane peripheral*		*Inner membrane*	
Ferroplasma spp.	*Downhill*	*Sulfocyanin*		cbb_3 *oxidase*	
	Uphill	*N.D.*		*N.D.*	

In the model constructed for *Leptospirillum spp.*, the electron extracted from ferrous iron is transferred to a periplasmic cytochrome c protein (Cyc_{579}). This protein appears to be analogous to rusticyanin with regards to its function as the branch point of the bifurcated electron transport pathway (Figure 2.3). In the "downhill" pathway, electrons are transferred to an integral inner-membrane terminal oxidase, cbb_3, which transfers the electron to molecular oxygen. However, in the "uphill" pathway, electrons are transferred from Cyt_{579} to a bc_1 protein which supplies electrons to the NADH dehydrogenase complex via an intermediary quinone pool (Bonnefoy and Holmes, 2012). In the case of *Ferroplasma spp.*, electrons were thought to be extracted from ferrous iron by sulfocyanin, a copper-containing protein which is closely related to rusticyanin, on the plasma membrane. The electrons are transferred from sulfocyanin to the cytoplasmic peripheral protein, cbb_3 terminal oxidase. Unlike *Leptospirillum* spp., the pathway used by *Ferroplasma* spp. lacks any identifiable electron carriers responsible for "uphill" electron transport required for NAD(P)H generation. However, the existence of this pathway is not essential as heterotrophic microorganisms are capable of regenerating reductive cofactors through the oxidation of organic carbon (Bonnefoy and Holmes, 2012).

2.3 Physico-chemical factors and their effect on iron-oxidizing acidophiles

2.3.1 Effect of dissolved organic carbon (DOC)

The presence of DOC plays an important role in structuring the composition of the BIOX® consortia which may be derived by either endogenous sources, such as microbial exudates, or exogenous sources, such as organic contaminants in the mineral feed (Okibe *et al.,* 2003). In laboratory cultures, DOC is typically derived from cell debris and organic exudates released into the leachate liquor by members of the microbial consortia (Borichewski, 1966; Nancucheo and Johnson, 2009). Okibe and Johnson (2004) reported batch culture DOC concentrations of 88 ±17 $mg.L^{-1}$ in *L. ferriphilum* spent culture and over 100 $mg.L^{-1}$ when *L. ferriphilum* was co-cultured with *At. caldus*. Although the presence of DOC in the leachate liquor may provide an essential carbon source for the tertiary prokaryotes within the consortia, its organic constituents may also hinder the growth of autotrophic acidophiles within the consortia (Okibe and Johnson, 2004; Borichewski, 1966; Schanitman and Lundgren, 1965). The inhibitory effect of DOC has been attributed largely to organic acids and the presence of these compounds within the spent culture of chemolithotrophic cultures has been well-documented (Schanitman and Lundgren, 1965; Borichewski, 1966; Arkesteyn and De Bont, 1980; Nancucheo and Johnson, 2009). In addition to organic acids, amino acids (most notably aspartic acid) may also elicit an inhibitory response on chemolithotrophic acidophiles; however, the total amino acid content reported in spent culture is typically very small portion of the overall DOC content (Arkesteyn and De Bont, 1980). Conversely, organic acids such as pyruvic acid, acetic acid and glycolic acid have been identified as more abundant organic constituents present in acidophile spent culture. Although present in the spent culture in very low concentrations, organic acids act as very potent inhibitors of chemolithotrophic acidophile growth. Pyruvic acid and acetic acid have both been reported to inhibit growth of several

chemolithotrophic species at concentrations below 10 mg.L^{-1} (Aston *et al.*, 2009; Borichewski, 1966) whereas Nancucheo and Johnson (2009) observed complete inhibition of *L .ferriphilum* in the presence of 38 mg.L^{-1} glycolic acid.

Table 2.2 Chemical properties of glycolic acid, acetic acid and pyruvic acid

Organic acid	Chemical class	Molecular weight (g.mol^{-1})	pKa
Pyruvic Acid	2-oxo monocarboxylic acid	88.06	2.45
Acetic Acid	Simple monocarboxylic acid	60.05	4.76
Glycolic Acid	2-hydroxy monocarboxylic acid	76.05	3.83

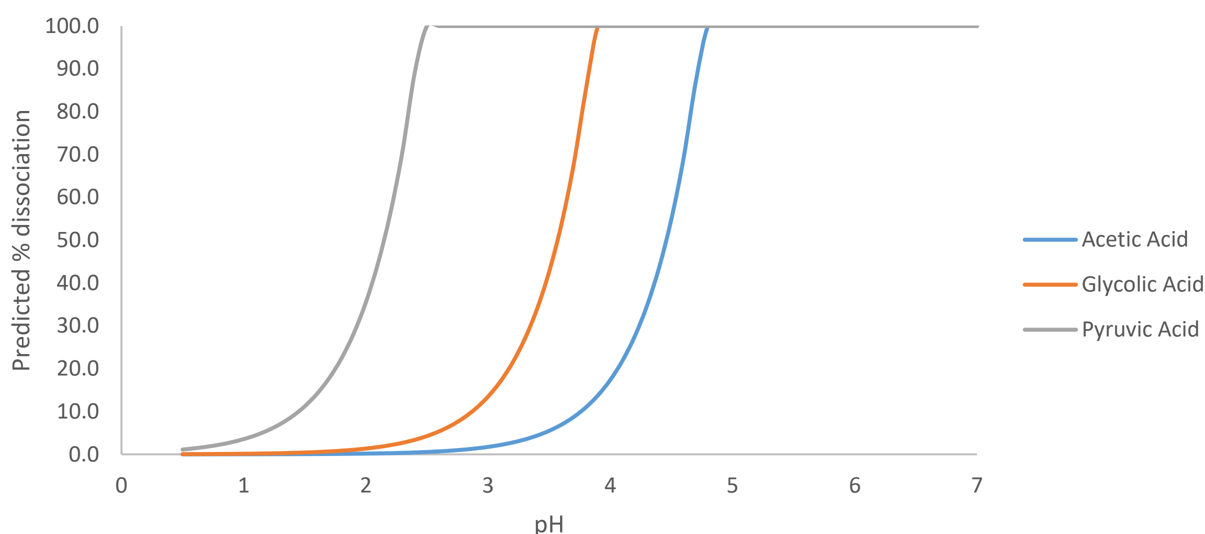

Figure 2.4: Percentage dissociation of the select organic acids as a function of pH

The inhibitory effects are also dependent on the chemical properties of the organic acids (Table 2.2). Ingledew and Poole (1982) noted that under extremely acidic conditions (pH < 3), weak organic acids primarily exist in their fully-protonated form which causes them to exhibit a near-neutral charge, allowing them to diffuse across the cell membrane more easily and infiltrate the cell. The influx of organic acids results in the disruption of the respiratory electron transport chain as the near-neutral cytoplasmic pH would encourage the highly-protonated organic acids to dissociate upon entering the cell (as illustrated by the difference in percentage dissociation shown in Figure 2.4), resulting in cytoplasmic acidification and weakening of the pmf (Ingledew and Poole, 1982). Therefore, factors such as the dissociation constant, K_a, of the organic acid and the pH of the surrounding environment greatly influence the toxicity of organic acids (Alexander *et al.*, 1987).

2.3.2 Effect of temperature

Sulfide mineral oxidation is an exothermic reaction and therefore heat generation is an inevitable by-product of the biomining process. The increase in temperature has a significant effect on the chemical oxidation reaction rate, causing the reaction rate of mineral oxidation to increase roughly two-fold with

every 10°C increase in temperature (Rawlings, 2003). In addition, temperature also plays arguably the greatest role in defining the microbial consortia and thus, temperature provides a means of further categorizing microbial members of the biomining consortia (Breed *et al*., 1999; Johnson, 1998). Microorganisms which exhibit a temperature optimum within the range of 20-40°C are collectively referred to as mesophiles and consist primarily of Gram-negative chemolithotrophic autotrophs, such as *Acidithiobacillus spp.* and *Leptospirillum spp.* However, iron and sulfur oxidizing Gram-positive bacteria such as *Sulfobacillus thermosulfidooxidans* and several Ferroplasma-like archaeal species have also been observed under these conditions (Johnson, 1998; Rawlings, 2005). Microorganisms which favour a higher temperature range of 40-60 °C are referred to as moderate thermophiles and consist primarily of Gram-negative bacterial species such as *At. caldus*, *L. ferriphilum* (Coram and Rawlings, 2002), Gram-positive bacteria such as *Sulfobacillus spp.* and *Acidimicrobium spp.* (Okibe *et al*., 2003; Cleaver *et al*., 2007) as well as heterotrophic archaea, such as *Ferroplasma spp., Thermoplasma spp.* and *Acidiplasma spp.* (van Hille *et al*., 2013). Extremely thermophilic microorganisms thrive at temperatures greater than 60°C and consist almost entirely of archaea. Actively growing archaeal species such as *Sulfolobus spp.* and *Metallosphaera spp.* have been reported in tank-based biomining processes operating at between 65°C and 80°C (Rawlings *et al*., 2003; Rawlings 2005; Mikkelsen *et al*., 2006). The temperature range designed for the BIOX® operating window is 40-45°C, but due to the exothermic nature of sulfide mineral oxidation and the need to curtail operating costs, the commercial BIOX® bioreactors typically operate within a temperature range of 42-45°C (van Hille *et al*., 2011; van Niekerk, 2012). Factors such as high-sulfur grade in the mineral feed can cause the temperature to fluctuate above the defined range, however since sulfuric acid production is associated with the exothermic breakdown of sulfidic minerals, increased mineral decomposition may trigger spikes in temperature and acidity within the reactor. A lab-scale study of BIOX® reactors performed by van Hille *et* al. (2013) highlighted the influence of temperature in defining the composition of the microbial consortia, where a shift in microbial dominance from *L. ferriphilum* to *Ac. cupricumulans* was observed at temperatures > 45°C. Moreover, the cumulative effect of heat and acid stress appeared to have exacerbated the shift in microbial dominance, spurred by an increased sulfide content in the mineral feed (van Hille *et al*., 2013).

2.3.3 Effect of pH

The acidity generated through sulfide mineral dissolution also impacts the composition of the microbial community facilitating the biomining process. While these microbial communities consist exclusively of acidophiles, the level of acidity that microorganisms can tolerate may vary. The BIOX® reactors are typically colonized by extreme acidophiles (pH < 3) as the highly acidic environment assists with mineral dissolution and is required to maintain the ferric iron lixiviant in solution. Creating a highly acidic environment prevents the formation of ferric iron precipitates, such as jarosite and goethite, which decrease ferric ion availability and passivate mineral surfaces (Kaksonen *et al*., 2014; Rawlings, 2002). The pH plays an important role in the BIOX® process and is typically controlled within the range of pH 1.2-1.6 by adding limestone or sulfuric acid (van Aswegen *et al*., 2007), depending on the acid-consuming or acid-generating nature of the concentrate. However, the pH may fluctuate outside of this

range in cases of control failure or with fluctuations in the sulfide grade (van Hille *et al.*, 2013; Smart *et al.*, 2018). Oxidation of acid-soluble sulfide minerals and microbe-mediated oxidation of ferrous iron both result in an increase in pH whereas the oxidation of acid-insoluble sulfides, elemental sulfur and reduced sulfur species released during mineral decomposition lowers the pH through production of sulfuric acid. *L. ferriphilum* has been reported to grow across a broad pH range with microbial activity observed at a pH as high as 3.5 (Plumb *et al.*, 2007) and growth at a pH below 1 (Ngoma et al., 2015; Tupikina et al., 2011; Ozkaya *et al.*, 2007; Penev and Karamanev, 2010; Ojumu and Petersen, 2011; Kinnunen and Puhakka *et al.*, 2005) The findings of van Hille *et al.* (2013) suggest that the relative abundance of *L. ferriphilum* within the BIOX® culture decreased by 40% when pH dropped below pH 0.8 (at 41.5°C). The findings of Ngoma *et al.* (2015) showed that a decrease in pH < 1.0 resulted in an extended lag phase in batch culture and a decreased biomass yield. The aggravated effect of acid stress at elevated temperatures on *L. ferriphilum* was also observed by Penev and Karamanev (2010) who reported a change in cell morphology and the decoupling of ferrous oxidation and microbial growth at pH < 1.0. This decoupling phenomenon has also been reported elsewhere and has been linked to an increase in energetic requirements to maintain homeostasis within the cell (Ngoma et al., 2015; Penev and Karamanev, 2010; Dempers *et al.*, 2003). While activity was also observed across a relatively broad pH spectrum for *Ac. cupricumulans* JTC3*,* the archaeon appeared to favour more acidic conditions with growth observed across a pH range of 0.4-1.8 (Hawkes *et al.*, 2006).

Table 2.3 pH optima of *L. ferriphilum* and *Ac. cupricumulans*

Reference	Microorganism	Conditions	pH_{opt}
Breed and Hansford (1999)	*L. ferrooxidans**	*40°C, Continuous culture*	*1.3*
Ojumu and Petersen (2011)	*L. ferriphilum*	*42°C, Continuous culture*	*1.3*
Coram and Rawlings (2002)	*L. ferriphilum*	*40°C, Batch culture*	*1.4-1.8*
Rautenbach (2007)	*Ac. cupricumulans JTC3***	*52°C, batch culture*	*1.5*
Hawkes et al. (2006)	*Ac. cupricumulans BH2*	*53.6°C, batch culture*	*1.0-1.2*

**This publication reported using L. ferrooxidans before classification of Leptospirillum ferriphilum*
*** This publication reported using Ferroplasma JTC3 before classification of Acidiplasma cupricumulans*

These microorganisms appear to use different mechanisms for maintaining the near-neutral cytoplasmic pH. With regards to *L. ferriphilum*, the bacterium's genome contains a large array of genes associated with acid-tolerance, such as putative proton efflux systems and cation transporter proteins. Furthermore, Tyson *et al.* (2004) reported a relatively large percentage (11%) of *L. ferriphilum*'s proteome consisted of chaperone proteins associated with protein refolding. These proteins appear to be involved in the repair of proteins and DNA as a result of pH-related damage (Tyson *et al.*, 2004). Similar findings were reported for acidophilic archaea, namely *Picrophilus torridus*. Crossman *et al.* (2004) indicated that roughly 12% of the archaeon's genome was dedicated to transporter proteins, particularly H^+/K^+-transporting ATPase proteins which are thought to be responsible for maintaining the transmembrane pH gradient. The mechanisms for coping with the highly acidic environment are not ubiquitous and differ amongst species within the acidophilic community. Unlike in *L. ferriphilum*, the cell membrane surrounding archaea does not consist of a lipid bilayer but is instead composed of repeating isoprene units which link covalently to form a rigid tetra-ether membrane monolayer (Madigan *et*

al.,2010). The bulky isoprenoid core and ether linkages contribute to proton impermeability and make the cell membrane more resistant to acid hydrolysis (van de Vossenberg, 1998; Baker-Austin, 2009). A high proportion of acid-tolerant ferroproteins have also been identified in a closely related archaeal species which may further contribute to the acid-resistance of these microorganisms (Ferrer *et al*., 2007). The DNA structure of these microorganisms is characterised by very small genomes with high coding densities and high G+C content which appears to be another adaptation to life in extreme conditions (Futterer *et al*., 2004).

2.4 Summary

The BIOX® process was designed to pre-treat refractory gold-bearing concentrates. It and related processes are well accepted commercially. A microbial consortium of iron- and sulfur-oxidizing microorganisms provide lixiviants for sulfide mineral dissolution while heterotrophic acidophiles remove exuded organic matter which may have a detrimental effect on chemolithotrophic performance. The autotrophic iron-oxidizing bacterium, *L. ferriphilum*, has been recognised as the dominant iron-oxidizer within the BIOX® culture through morphological and molecular analysis; however, recent review of BIOX® consortia present in bioreactors at the Fairview mine (Mpumalanga, South Africa), and other commercial BIOX® plants revealed that heterotrophically-inclined iron-oxidizing archaea, such as *Ac. cupricumulans* JTC3, are currently dominant. The conditions surrounding their dominance within these bioreactors is uncertain although physico-chemical factors such as the temperature, pH and the presence of inhibitory organic compounds in the leachate liquor are postulated to provide selective pressures driving the change in the microbial consortia to favour growth of *Ac. cupricumulans* JTC3 and other archaea over *L .ferriphilum* HT.

In the case of the BIOX® operating window, temperatures typically range from 40-45°C. Multiple biokinetic studies have been reported for *L. ferriphilum* at ≤ 42°C and for *Ac. cupricumulans* at > 50°C; however, little research has been reported on the growth and performance of these two microbial strains at 45°C, the upper temperature limit for the BIOX® operating window. With regards to acid tolerance, *L. ferriphilum* has demonstrated growth across a relatively broad pH range at pH ≤ 3.5, with growth and activity observed at pH < 1.0 under optimal temperature conditions. However, it is uncertain how growth at an elevated and sub-optimal temperature (45°C) would influence the microorganism's ability to grow under such extreme acidic conditions, nor are the relative kinetics of *L. ferriphilum* as a function of pH rigorously described.

The presence of soluble organic matter in the leachate liquor may also play an important role in sculpting the microbial consortia. DOC content released into the liquor is thought to promote growth of heterotrophically-inclined archaea present in the consortia, however it is unclear what the minimal DOC requirement is to sustain these archaea, specifically *Ac. cupricumulans* JTC3. Furthermore, autotrophic chemolithotrophs are recorded to exhibit a high sensitivity towards the exuded dissolved organic carbon DOC in the acidic leachate liquor, primarily due to the presence of organic acid constituents which includes acetic acid, glycolic acid and pyruvic acid. It is unclear how strongly these compounds affect

growth of *L. ferriphilum* HT under the temperature and pH associated with the BIOX® operating window. Furthermore, little rigorous research is reported on inhibition of *Ac. cupricumulans* JTC3 by organics.

2.5 Hypothesis and Key Questions

1) *L. ferriphilum* is a thermotolerant mesophilic acidophile with a temperature optimum of 38.5°C and the ability to grow at pH < 1.0. Certain strains, such as *L. ferriphilum* HT, are capable of growing at temperatures as high as 45°C (upper limit of the BIOX® operating window) under optimal pH conditions. However, under highly acidic conditions, the growth kinetics and iron-oxidizing performance of *L. ferriphilum* HT would deteriorate due to the cumulative effect caused by heat and acid stress.
    a. What effect does pH (pH 0.7-1.7) have on the biokinetics of *L. ferriphilum* HT at the minimum temperature limit of the BIOX® operating window (40°C)?
    b. What effect does pH (pH 0.7-1.7) have on the biokinetics of *L. ferriphilum* HT at the maximum temperature limit (45°C) of the BIOX® operating window and how do the biokinetics compare to those observed at 40°C?
2) Unlike *L. ferriphilum* HT, *Ac. cupricumulans* JTC3 is an extremely acidophilic moderate thermophile with an optimal growth temperature around 53-55°C and has shown activity at a pH as low as pH 0.4. Therefore, the cumulative effect of heat and acid stress on the growth kinetics and iron-oxidizing performance of *Ac. cupricumulans* JTC3 at 45°C is expected to be lower than that on *L. ferriphilum* HT.
    a. What effect does pH have on the biokinetics of *Ac. cupricumulans* JTC3 at 45°C?
    b. As a function of pH, how do the biokinetics of *Ac. cupricumulans* JTC3 compare to that of *L. ferriphilum* HT at 45°C?
3) Autotrophic chemolithotrophs, such as *L. ferriphilum* HT, are strongly inhibited by DOC, particularly the organic acid constituents which are exuded into the media by microorganisms present in the consortia.
    a. What concentration of dissolved organic carbon is required to inhibit growth of *L. ferriphilum* HT?
    b. Do organic acids such as glycolic acid, acetic acid and pyruvic acid have an inhibitory effect on the growth of *L. ferriphilum* HT at concentrations relevant to that expected in acidophilic spent cultures?
4) Unlike, autotrophic chemolithotrophs such as *L. ferriphilum*, heterotrophically-inclined archaeal acidophiles, such as *Ac., cupricumulans* JTC3, are not as sensitive to the inhibitory effects DOC present in acidophile spent culture may benefit from the presence of DOC in the culture.
    a. Does the presence of organic waste products in the media have an inhibitory effect on *L. ferriphilum* or Ac. *cupricumulans* JTC3 or both?
    b. What is the minimum organic carbon requirement for growth of *Ac. cupricumulans* JTC3?
    c. Is the organic content exuded into the media by *L. ferriphilum* sufficient to support heterotrophic growth of *Ac. cupricumulans* JTC3?

3 Approach to Project and Methodology

3.1 Research methodology

The biokinetic study of the BIOX® reactor iron oxidizing isolates was performed by monitoring microbial growth and ferrous iron oxidation rates in separate *L. ferriphilum* HT-dominant and *Ac. cupricumulans* JTC3-dominant batch cultures. Ferrous sulphate was used as the ferrous iron substrate (i.e. no solids added) for both microorganisms and yeast extract as the organic substrate for *Ac. cupricumulans* JTC3.

Batch culture growth and substrate oxidation curves were performed in Thomson™ round-bottom 24-well microwell plates (MWPs) which had a well volume of 10.4 mL (3 mL working volume). The MWPs were sealed with adhesive air-porous sheets (ThermoFisher™) and incubated in a humidified 2 L container (using wetted paper towel). Each culture condition was loaded in triplicate (three wells) and each well was inoculated with $1x10^8$ $cells.ml^{-1}$ (See Chapter 3.3.1). Microbial growth was monitored by cell enumeration which was performed by direct cell count (see Chapter 3.3.2) whereas substrate oxidation curves were constructed by monitoring ferrous iron levels within the samples (see Chapter 3.3.3). Ferrous iron concentrations were quantified spectrophotometrically using the 1-10 phenanthroline assay adapted from the method performed by Komadel and Stucki (1988). The monitoring of pH and sampling for microbial growth and substrate oxidation were performed daily and in parallel.

3.2 Experimental Design

3.2.1 Investigating the effect of pH

In order to assess the effects of pH on the biokinetic performance of the microorganisms; parallel microbial growth and substrate oxidation curves were constructed across a pH range of 0.7-1.7 (distributed across five pH points with increments in pH of 0.2) for each of *L. ferriphilum* HT or *Ac. cupricumulans* JTC3. Substrate-supplemented working stock of the media (see Chapter 3.3.1) was prepared on the day of the experiment and sample aliquots of the media were pH-adjusted, using sulfuric acid (96% H_2SO_4), prior to inoculation. The experiment was performed at 45°C for *Ac. cupricumulans* JTC3 and at both 40°C and 45°C for *L. ferriphilum* HT.

3.2.2 Investigating the effect of DOC

The effects of DOC loading on the biokinetic performance of both microorganisms, at 45°C (pH 1.2-1.5), was considered as well as the need for organic substrate supplementation of *Ac. cupricumulans* JTC3. To assess the influence of DOC loading; microbial growth and substrate oxidation curves were created for both microorganisms using media supplemented with either 0.1-1.0 $g.L^{-1}$ yeast extract, 1-30 $mg.L^{-1}$ glycolic acid, 1-30 $mg.L^{-1}$ acetic acid or 1-10 $mg.L^{-1}$ pyruvic acid. The influence of the DOC content in spent culture was also assessed on *L. ferriphilum* HT by cultivating the bacterium in media containing *Ac. cupricumulans* JTC3 spent culture filtrate. Similarly, the influence of the DOC content in chemolithotrophic spent culture was assessed on *Ac. cupricumulans* JTC3 by cultivating the archaeon

in media containing *L. ferriphilum* HT spent culture filtrate in the presence or absence of yeast extract supplementation.

3.3 Materials & methods

3.3.1 Media and microbial stock cultures

Working stock cultures of *L. ferriphilum* HT and *Ac. cupricumulans* JTC3 were maintained in 250 mL Erlenmeyer flasks (30 mL working volume). The *L. ferriphilum* HT cultures were grown on 1x Autotrophic Basal Salt Media (Govender *et al*., 2013) whereas the *Ac. cupricumulans* JTC3 cultures were grown on 1x Archaeal Basal Salt Media (Table 3.1). Both types of basal salt media were supplemented with trace elements obtained from a 1000x trace metal stock solution (Kolmert and Johnson, 2001). The 1xAutotrophic Basal Salt and 1x Archaeal Basal Salt Media was obtained by diluting sterile, respective, 50x media stock solutions (pH 1.2) in sterilised, deionised water.

Table 3.1 Composition of Autotrophic Basal Salt and Archaeal media

1x Autotrophic Basal Salt Media		1xArchaeal Basal Salt Media	
$(NH_4)SO_4$	*0.15 g.L^{-1}*	*$(NH_4)_2SO_4$*	*0.2 g.L^{-1}*
Na_2SO_4	*0.15 g.L^{-1}*	*K_2HPO_4*	*0.1 g.L^{-1}*
$MgSO_4.7H_2O$	*0.5 g.L^{-1}*	*$MgSO_4.7H_2O$*	*0.4 g.L^{-1}*
KH_2PO_4	*0.005 g.L^{-1}*		
$Ca(NO_3)_2$	*0.014 g.L^{-1}*		
KCl	*0.05 g.L^{-1}*		

L. ferriphilum HT and *Ac. cupricumulans* JTC3 cultures were both cultivated on 10 g.L^{-1} ferrous iron obtained from 50 g.L^{-1} ferrous iron stock solution (using $FeSO_4.7H_2O$) which had been filter-sterilized by dead-end syringe filtration (0.22µm pore size). In addition to ferrous iron supplementation, the *Ac. cupricumulans* JTC3 cultures were supplemented with an organic substrate in the form of 0.5 g.L^{-1} yeast extract (Rautenbach, 2007). Working stock cultures of *L. ferriphilum* HT and *Ac. cupricumulans* JTC3 were pH-adjusted to pH 1.2-1.3 and maintained at 45°C on an orbital shaker operating at 140 rpm. The *L. ferriphilum* HT working stock cultures were sub-cultured every 2-3 days when the redox potential within the culture exceeded 600 mV whereas the *Ac. cupricumulans* JTC3 working stock cultures were sub-cultured every 3-4 days when redox potential exceeded 500 mV. Redox potential was measured using a calibrated Metrohm 827 electrode redox meter with redox potential measured with reference to a saturated Ag/AgCl electrode.

3.3.2 Cell enumeration

Cell enumeration was conducted by direct cell count at 1000x magnification using the oil-immersion objective lens (100x magnification) of an Olympus BX40 phase-contrast light microscope and a Thoma™ counting chamber. Cell counts were performed daily and in duplicate, when possible. The planktonic cell density was assumed to represent the total cell density within the samples as no particulate phase was used, *L. ferriphilum* and *Ac. cupricumulans* cultures remained in suspension and

negligible surface attachment was observed in the 24-well micro-well plates used to conduct the biokinetic experiments or the 250 mL shake flasks used for working stock cultivation.

Daily cell enumeration was performed, in duplicate when possible, on pooled sample replicates. 2 µL aliquots were taken from each well and all three replicates for each culture were pooled together within 2 mL Eppendorf tubes. 2 µl aliquots of the pooled samples was then typically diluted by a factor of 10 using H_2SO_4-acidified water (pH 1.2), depending on the number of cells counted. To reduce error, direct cell count which consisted of less than 10 cells or more than 200 cells were repeated at a lower or higher dilution factor, respectively. Direct cell counts were used to determine cell concentration using Equation 2.1:

$$C_X = \frac{C \times (\frac{N_T}{N_L})}{D \times A} \times \frac{1}{d} \times 10^3 \quad (2.1)$$

where: C_X = cell concentration (cells/mL)

C = number of cells counted in the large squares

N_T = total number of large squares = 16

N_L = number of large squares where cells were counted

D = depth of the chamber (0.02 µm)

A = total area of the chamber (1 mm^2)

d = dilution ratio

This equation can be simplified to $C_X = C \times \frac{1}{d} \times 312500$

3.3.3 Quantification of ferrous iron and ferric iron

Ferrous iron and ferric iron levels were determined for each replicate (well) for each culture. During sampling, 10 µL aliquots were taken from each well and diluted (typically 10-fold and 100-fold) in H_2SO_4-acidified water (pH 1.2) in order to dilute ferrous iron and total iron concentrations within the calibration range of 1-100 $mg.L^{-1}$. Using absorbances measured from the 1-10 phenanthroline assay, ferrous iron concentrations in each sample were extrapolated against a five-point ferrous iron calibration curve constructed using known concentrations of a ferrous iron reference standard. Once ferrous iron content in the samples had been quantified, the ferric iron present in the samples was reduced to ferrous iron by addition of hydroxyl-ammonium chloride ($HOHNH_2.HCl$) to allow for quantification of total iron present in each sample. The ferric iron concentration was calculated by subtracting the ferrous iron concentration from the respective total iron concentration. The average ferrous iron, ferric iron and total iron concentrations extrapolated for each sample was used for analysis.

3.3.4 Monitoring pH levels

Culture pH was measured daily, at room temperature from at least two replicates (wells) per culture, using a Metrohm 691 pH meter which was calibrated within the pH 1-4 range, daily, before use. The MWP sample pH was measured directly by tilting the MWP slightly and submerging the pH probe in the

relevant MWP wells. Although pH monitoring was conducted under non-sterile conditions, the pH probe was disinfected with 70% ethanol before and after each pH reading was taken to avoid cross-contamination. The culture pH was maintained to ≤0.2 pH units of the nominal pH and corrected daily by drop-wise addition (using a Pasteur pipette) of 96% H_2SO_4 to lower the pH.

3.3.5 Microbial speciation

Speciation of the *L. ferriphilum* HT and *Ac. cupricumulans* JTC3 cultures was determined by qPCR analysis. Strain-specific primers were used to identify relative abundance of *L. ferriphilum* HT and *Ac. cupricumulans* JTC3 in purified gDNA extracted from the respective working stock cultures of these two microorganisms.

3.3.5.1 Preparation of cell for gDNA extraction

The gDNA extraction was only conducted on working stock cultures which had cell concentrations exceeding 5 x 10^8 cells.mL^{-1}. A total sample volume of 10 mL was used for each extraction. Firstly 2 mL aliquots were transferred into two separate 2 mL Eppendorf tubes and the cells pelleted by centrifugation (14 000 xg for 10 min at 22°C). The supernatant was decanted, and an additional 2 mL aliquot added into each Eppendorf tube and centrifuged again in order to obtain a larger cell pellet Figure 3.1A). Finally, each pellet was resuspended in 1 mL culture and these combined into one of the tubes before repeating the centrifugation step again and discarding the supernatant to result in a pellet from the 10 mL culture (Figure 3.1B).

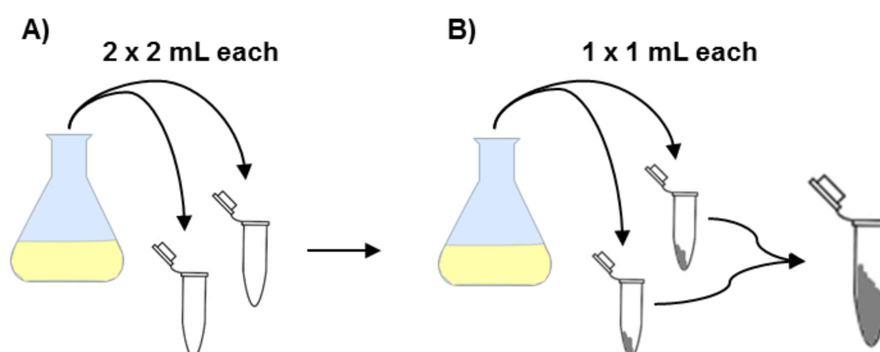

Figure 3.1: Schematic illustrating how cells were isolated for gDNA extraction from the working stock culture

Preparation of isolated cells

Residual iron was removed by washing the pellet with 10 mM citrate buffer (9.88 mM citric acid anhydrous, 0.22 mM sodium citrate dehydrate, pH 2) until a clear supernatant was obtained. This was achieved by adding 2 mL citrate buffer to the pellet, re-suspending it and centrifuging the tube for 5 minutes at 14 000 xg. The pellet then underwent two neutralization washes by re-suspending the pellet in 2 mL Tris-EDTA buffer (10 mM TrisCl, 1 mM EDTA, pH 8) before centrifuging at 14 000 xg for 5 min at room temperature. The TE buffer supernatant was also discarded and the pelleted cells were frozen at -20°C for a minimum of 12 hours prior to DNA extraction.

3.3.5.2 gDNA extraction and purification

DNA extraction was performed using the Roche High Pure PCR Template Preparation Kit. The pelleted cells were resuspended in 0.2 mL Tissue lysis buffer (Roche), 50 µg lysozyme and 2 µg DNAse-free RNase A (Fermentas) before undergoing the first of three incubation steps (37°C for 30 min).

After incubation, 40 µL Proteinase K (Roche) was added to each tube which would then undergo the second incubation step (50°C for 30 min) followed by the addition of 0.2 mL binding buffer and the third incubation step (70°C for 10 min).

A 0.1 mL aliquot of isopropanol was then added and gently mixed with the transferred supernatant. The DNA was then isolated from the isopropanol-supernatant mixture by transferring it to a combined High Pure filter/collection-tube assembly which was then centrifuged at 8000 xg for 1 min. The flow-through and the collection tube were then discarded, and the filter-tube was then fitted with a new collection tube.

The sample DNA trapped within the filter-tube was washed twice by adding 500 µL wash buffer and centrifuging the combined filter/collection-tube at 8000 xg for 1 min. The supernatant and collection-tube were then discarded, and the filter-tube was placed in a 1.5 mL reaction tube in preparation for DNA elution. The DNA was then eluted by adding 50 µL pre-warmed (70°C) elution buffer to the filter-tube and centrifuging the reaction tube at 8000 xg for 1 min. This elution step was repeated in another sterile 1.5 mL reaction tube. Sample gDNA purity and concentration was determined using a Thermo Nanodrop ND-2000 and the samples were diluted to 10 ng.μL^{-1} using RNAse-free water.

3.3.5.3 qPCR preparation and analysis of extracted gDNA

qPCR was performed using a Corbett Rotogene RG 6000 qPCR machine running on version 1.7 software (Corbett Research). A calibration curve was constructed for each run using plasmid DNA (pDNA) standards to obtain a template concentration range of 0.1-0.00001 ng/µL, in an analogous manner to the method used by Tupikina *et al.* (2013).

Each sample was analysed in triplicate using PCR reaction tubes. 14 µL MasterMix (Kapa Biosystems) was added to each replicate along with 1 µL template DNA (sample gDNA). 1 µL RNase-free water was used for the no-template control (NTC).

The conditions used for the amplification cycles were as follows: Initial denaturation step (95°C, 5 min) followed by 40 cycles of denaturation (95°C, 10s), annealing (60°C, 15s) and elongation (72°C, 20s).

3.3.5.4 Primer selection and primer-binding efficiency

Primer binding efficiency was analysed by melt curve analysis across a temperature gradient of 72°C to 95°C in 0.2°C increments. Table 3.2 illustrates the primers used for speciation of the *L. ferriphilum* and *Ac. cupricumulans* cultures.

Table 3.2: qPCR primers selection used for speciation of *L. ferriphilum* HT and *Ac. cupricumulans* JTC3

Primer title	Microorganism	5'-3' primer sequence
Universal Primers		
UniBactF335	*Universal bacteria (forward)*	*GAC TCC TAC GGG AGG CAG CA*
UniBactR937	*Universal bacteria (reverse)*	*TTG TGC GGG CCC CCG TCA AT*
UniArchF343	*Universal Archaea (forward)*	*ACG GGG IGC AIC AGG CG*
UniArchR932	*Universal Archaea (reverse)*	*TGC TCC CCC GCC AAT TCC*
Bacterial Primers		
L. ferriphilum LH	*L. ferriphilum*	*GGG GGC CTG AAT AAG GTC A*
Archaeal primers		
JTC3	*Ac. cupricumulans*	*AAG CCT AAC TTC AGA AGG CCT G*

3.4 Predicting the percentage dissociation of organic acid

The relative percentage dissociation of glycolic acid, acetic acid and pyruvic acid was determined using the same method demonstrated by Perrin *et al*. (1981) which is outlined in Equation 3.1.

$$\frac{[H^+]}{[HA]} \times 100 \tag{3.1}$$

Where,

$[H^+]$ = Dissociated Protons

$[A^-]$ = Acid conjugate.

However $[A^-] = [H^+]$, since the select organic acids all have a $[H^+]$: $[A^-]$ molar ratio of 1:1.

K_a = Dissociation constant = $\frac{[H^+][A^-]}{[HA]}$.

[HA] = Protonated organic acids. Relative concentration of protonated organic acid can be calculated as follows:

$[HA] = \frac{[H^+][A^-]}{10^{-pK_a}}$

Since, $K_a = 10^{-pK_a}$ and $K_a = \frac{[H^+][A^-]}{[HA]}$.

3.5 Quantification of organic carbon content by chemical oxygen demand (COD)

The Merck COD reagent set (CombiCheck 20, Cat. No. 1.14675.0001) was used, in conjunction with a digestion block, for determining the COD within *Ac. cupricumulans* JTC3 and *L. ferriphilum* HT spent culture. Spent culture samples were sterilized using 0.22 µm dead-end filters. The standards consisted of three potassium hydrogen phthalate solutions with respective COD values of 200, 750 and 1500 $mg.L^{-1}$. Deionised water was used as the blank. Each COD test was prepared by placing 0.15 mL

Solution A to a clean, sealable COD cell, following by slowly adding 1.15 mL Solution B. A 1.5 mL aliquot of the sample or control or standard solution was then added to the cell which was then sealed and vortexed vigorously for 10 seconds. The cells were placed in the pre-heated digestion block at 148°C for a duration of 120 min. The cells were allowed to cool, and COD was determined spectrophotometrically at a wavelength of 605 nm.

3.6 Determining the duration of lag time and maximum specific growth rate

Determining lag time provides a useful means of investigating the cell's ability to undergo metabolic adaptation (Monod, 1949). In this case, the inoculum was pre-conditioned to the cultivation conditions and therefore length of lag time can be attributed to the time required for the culture to adapt to change in the cultivation conditions (i.e. deviations in pH). The duration of the lag phase experienced during culture growth was extrapolated from the plot of natural logarithm as a function of time. A trendline was fitted to the slope indicating log-phase growth and lag time was extrapolated from the point at which this trendline intersected the logarithm of the initial cell concentration as shown in Figure 3.2A.

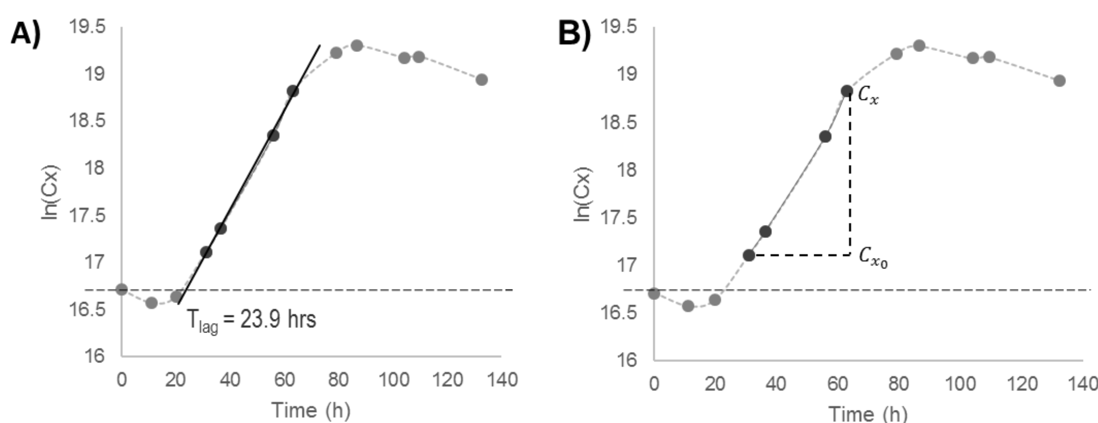

Figure 3.2: Graphical plot illustrating A) the extrapolation of lag time and B) specific growth rate determination.

Maximum specific growth rate (μ_{max}) was determined as shown in Equation 3.1 using the change in cell concentration over time during log phase growth (Figure 3.2 B).

$$\mu_{max} = \frac{\ln \left(C_x / C_{x_0} \right)}{\Delta t} \tag{3.1}$$

3.7 Determining ferrous oxidation rates and biomass yield

3.7.1 Determining volumetric oxidation rates

Volumetric ferrous oxidation rate ($-r_{Fe^{2+}}$) was calculated according to Equation 3.2 using the change in ferrous concentration over three sampling intervals during log phase of growth, as shown in Figure 3.3.

$$-r_{Fe^{2+}} = \frac{\Delta[Fe^{2+}]}{\Delta t} \quad (3.2)$$

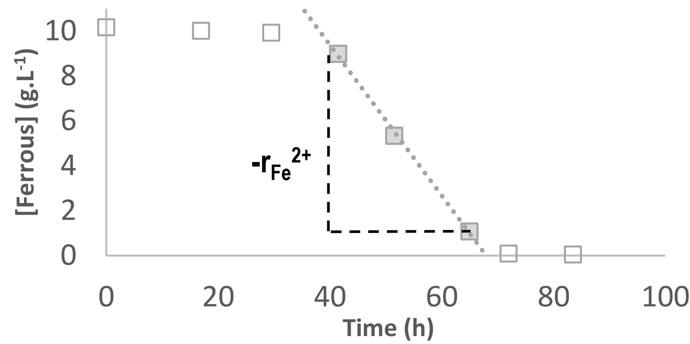

Figure 3.3: Determination of volumetric oxidation rate

3.7.2 Determining specific oxidation rates

The specific oxidation rate ($q_{Fe^{2+}}$) was determined using the volumetric oxidation rate as a function of the average cell concentration (C_x) obtained over the respective time interval (shown in Equation 3.3 and 3.4)

$$q_{Fe^{2+}} = \frac{-r_{Fe^{2+}}}{C_x} \quad (3.3)$$

or

$$q_{Fe^{2+}} = \left(\frac{\Delta[Fe^{2+}]}{\left(\frac{[C_x]_{t_1}+[C_x]_{t_2}}{2}\right)}\right)/\Delta t \quad (3.4)$$

Where,

$[C_x]_t$ refers to the cell concentration at a specific timepoint.

3.7.3 Determining biomass yield

The biomass yield ($Y_{x/s}$) on ferrous iron substrate was determined using the microbial growth and ferrous iron concentration data as shown in Equation 3.5.

$$Y_{x/s} = \frac{\Delta C_x}{\Delta Fe^{2+}} \quad (3.5)$$

3.8 Summary

The body of research was divided into two experimental blocks: Firstly, the effect of pH (Results I: The Effect of pH) and secondly, the effect of organic carbon (Results II: The Effect of Organic Carbon). The effect of pH was assessed for both microorganisms across the pH range of pH 0.7-1.7. All cultures were supplemented with 10 g.L^{-1} ferrous iron substrate (ferrous sulphate), whereas *Ac. cupricumulans* JTC3 cultures were also supplemented with 0.5 g.L^{-1} organic substrate (yeast extract) unless otherwise stated. The performance of each culture was assessed by monitoring cell concentrations and ferrous

iron levels which were used to determine biokinetic performance parameters such as: lag time, biomass yield, specific growth rate, volumetric oxidation rate and specific oxidation rate. Sampling (for cell enumeration and ferrous iron determination) as well as pH monitoring were performed at least once daily.

The effect of pH was assessed on *L. ferriphilum* HT at 40°C and reassessed at 45°C to determine the effect of acid and heat stress on the microorganism. The effect of pH was then assessed on *Ac. cupricumulans* JTC3 at 45°C and findings compared to those of *L. ferriphilum* HT at 45°C.

The effect of organic carbon was initially performed with *L. ferriphilum* HT. The microorganism's sensitivity to the presence of dissolved organic carbon was assessed by subjecting it to: 0.1-0.5 $g.L^{-1}$ yeast extract (generic dissolved organic carbon source), 10-50% *Ac. cupricumulans* JTC3 spent culture filtrate as well as media spiked with ≤ 30 $mg.L^{-1}$ organic acid (glycolic acid, acetic acid or pyruvic acid). In the assessment of the effect of organics on *Ac. cupricumulans* JTC3, the archaeon was cultivated with 0.1-0.5 $g.L^{-1}$ yeast extract to determine the optimal level of organic carbon supplementation. To assess if sufficient dissolved organic carbon was present in spent culture to support growth of the archaeon, *Ac. cupricumulans* JTC3 was then cultivated in 10-50% *L. ferriphilum* HT spent culture filtrate in the absence of additional organic carbon supplementation. The experiment was then repeated with added organic substrate (0.5 $g.L^{-1}$ yeast extract) to assess the inhibitory effect of the filtrate on the archaeon. Lastly, the inhibitory effect organic acids was assessed by spiking the archaeal media with ≤ 30 $mg.L^{-1}$ organic acid (glycolic acid, acetic acid or pyruvic acid).

The biokinetic assessments conducted in both experimental blocks were performed using ostensibly pure L. ferriphilum HT and Ac. cupricumulans JTC3 cultures isolated from the BIOX® stock cultures. Speciation by qPCR analysis was performed on the working stock cultures to assess purity of the microbial cultures and the findings in Appendix A.3 indicate that a high degree of purity was maintained in the Ac. cupricumulans JTC3 working stocks cultures over the course of the study (Figure 6.7). Conversely, a noticeable representation of bacterial and/or archaeal contaminants were identified in the L. ferriphilum HT working stock cultures used in this study (Figure 6.6). However, it is worth noting that although microbial contaminants may have been detected in the L. ferriphilum HT working stock cultures, observations during the cell enumeration indicated that virtually all of the microorganisms detected in the L. ferriphilum HT biokinetic experiments consisted of spiral and/or vibrio-shaped cells, which is a quintessential feature of the bacterium.

4 Results I: The Effect of pH

4.1 The effect of pH and temperature on the biokinetic performance of *L. ferriphilum* HT

The growth and activity of *L. ferriphilum* HT was investigated at 40°C, pH 0.7-1.7, to determine the effect of acid stress, within the BIOX® operating window, under near-optimal temperature conditions for the bacterium. Microbial growth was monitored until > 99% of ferrous iron oxidation was achieved, which occurred within 83.5 hours across the pH range of 0.7-1.7. These data are presented in Figure 4.1.

At 40°C, *L. ferriphilum* HT growth was observed across the entire pH 0.7-1.7 range investigated. The change in pH influenced the biokinetic performance of the bacterium. The ferrous iron concentration (Figure 4.1A) and microbial growth (Figure 4.1B) data both suggest a preferred pH range of pH 1.1-1.5 for the *L. ferriphilum* strain.

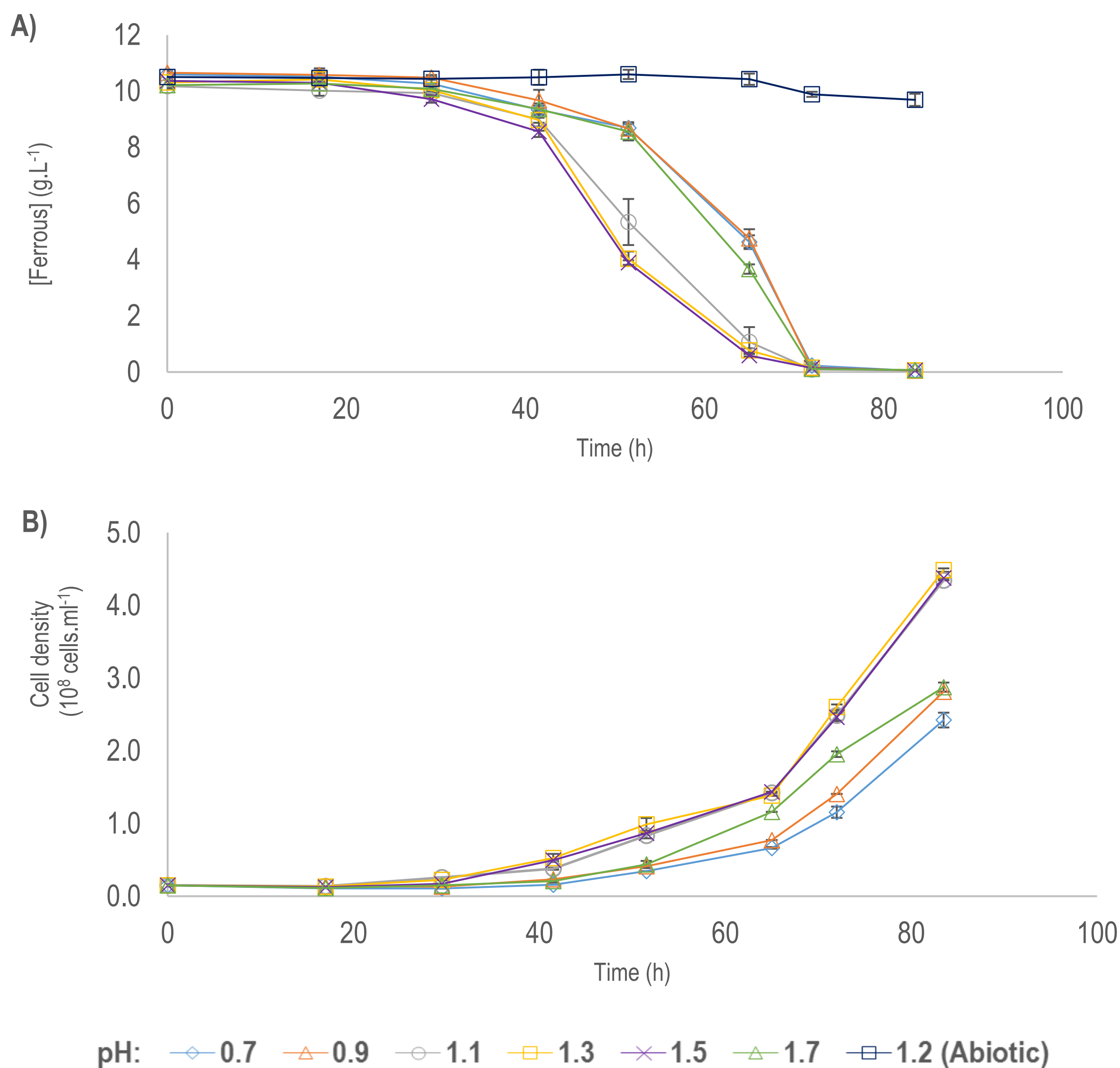

Figure 4.1: The effect of pH on the A)ferrous oxidation activity and B) microbial growth of *L. ferriphilum* HT at 40°C

Lag times were compared to assess the microorganism's ability to acclimatize to the pH conditions within each culture (Table 4.1). Shorter lag times were obtained within the pH 1.1-1.5 range, with the shortest lag time determined at pH 1.3 (13.5 h). The short lag times and high yields determined within the 'optimal' pH 1.1-1.5 range may be attributed to pre-adaptation of the *L. ferriphilum* HT working stock culture, maintained at pH 1.2 ± 0.2, which was used to inoculate the cultures. As pH deviated from the pH 1.1-1.5 range, the time required for the microbe to acclimatize increased, prolonging the lag phase. The increase in lag time was most noticeable as pH decreased below pH 1.1, with the longest lag time determined at pH 0.7 (39.6 h).

Culture pH had a noticeable impact on *L. ferriphilum* HT growth across the pH 0.7-1.7 range, as μ_{max} decreased with decreasing pH, eluding to a relationship between microbial growth and acid stress. The highest cell densities were obtained within the pH 1.1-1.5 range (4.41±0.07 x10^8 cells.mL^{-1} at the point of ferrous iron depletion), in accordance with the yield observed. Lower cell densities were achieved for cultures deviating from this pH range (pH < 1.1 and pH > 1.5), with the lowest maximum cell density determined at pH 0.7 (2.65 ± 0.23 x10^8 cells.mL^{-1} at the point of ferrous iron depletion). Similarly, the highest $-r_{Fe}^{2+}$ determined for *L. ferriphilum* HT at 40°C were achieved between 41.5–65.0 hours of incubation within the pH 1.1-1.5 range. The q_{Fe}^{2+} oxidation rates determined for the bacterium remained relatively constant across the pH 1.1-1.7 range (8.62 ± 0.27 nmol Fe^{2+}.cell^{-1}.h^{-1}) but increased with decreasing pH. The high $-r_{Fe}^{2+}$ obtained at pH 1.1-1.5 may be attributed to the shorter lag times and higher yields determined for the bacterium across this pH range. The increase in the q_{Fe}^{2+} and decrease in yields obtained under sub-optimal pH conditions may suggest decoupling of relationship between microbial growth and ferrous iron oxidation.

Table 4.1 Effect of pH on the biokinetic performance of *L. ferriphilum* HT at 40°C

pH	T_{Lag} (h)	$Y_{(x/s)}$ (cells^{-1}.μmol Fe^{2+})	$-rFe^{2+}$ (mg_{Fe}^{2+}.L^{-1}.h^{-1})	q_{Fe}^{2+} (nmol Fe^{2+}.(cell.h^{-1}))	μ_{max} (h^{-1})
0.70	*39.6*	*0.383*	*200.4*	*11.57*	*0.061*
0.90	*36.0*	*0.371*	*209.5*	*9.92*	*0.060*
1.10	*19.8*	*0.492*	*336.8*	*8.89*	*0.062*
1.30	*13.5*	*0.493*	*348.8*	*8.68*	*0.068*
1.50	*15.9*	*0.487*	*339.3*	*8.35*	*0.074*
1.70	*36.8*	*0.426*	*242.2*	*8.43*	*0.073*

Dempers *et al.* (2003) reported a similar trend for a *L. ferriphilum*-dominated mixed culture grown in a chemostat culture where biomass yield would remain relatively constant at pH 1.1-1.5 (0.0054-0.0058 mol C.(mol Fe^{2+})$^{-1}$.h^{-1}) but decreased by 33-38% at an elevated pH of pH 1.7 (0.0036 mol C.(mol Fe^{2+})$^{-1}$.h^{-1}). Furthermore, decreasing biomass yield in response to increasing acid stress has also been reported in *L. ferriphilum*-dominated batch cultures as shown in the Table 4.2 below (Ngoma *et al.*, 2015).

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Table 4.2. Effect of acid stress on the Δbiomass yield (%) on a mesophilic *L.ferriphilum*-dominated culture maintained at 35°C, receiving various concentrations of pH-neutralziing Na_2CO_3 (adapted from Ngoma *et al*., 2015).

Exposure time (h)	Control	0.34 M Na_2CO_3	0.51 M Na_2CO_3	0.68 M Na_2CO_3
1 h	*0*	*25.1*	*32.4*	*48.8*
3 h	*0*	*51.3*	*53.0*	*93.9*
24 h	*0*	*3.1*	*33.6*	*88.0*

Table 4.3 compares the μ_{max} of the current work against those reported in similar biokinetic studies. The specific growth rates determined at pH 1.7 were comparable to the μ_{max} reported by Breed *et al.* (1999) at pH 1.75. Similarly, the μ_{max} determined by Dempers *et al.* (2003) at pH 1.5 was similar to that achieved in the current work at the respective pH but differed at higher and lower pH levels. In addition, Franzmann *et al.* (2005) determined a μ_{max} of 0.1 h^{-1} for *L. ferriphilum* at pH 1.6 at 39.6°C, which is substantially higher than the μ_{max} determined in the current work around pH 1.6 (0.074 h^{-1} across the pH 1.5-1.7 range).

The difference in specific growth rates presented in Table 4.3 may be attributed to differences in experimental methodology and calculation of μ_{max}. The findings of both Breed *et al.* (1999) and Dempers *et al.* (2003) were determined using *Leptospirillum*-dominant continuous cultures and μ_{max} determined indirectly by monitoring ferrous iron oxidation rates and via off gas (O_2 and CO_2) analysis. In similar fashion to the current work, the specific growth rates reported by Franzmann *et al.* (2005) were also determined using *L. ferriphilum* batch cultures, however the reported μ_{max} was determined using the rate of ferrous iron oxidation rather than cell density, which may have also contributed to the difference in μ_{max} shown in the table below.

Table 4.3 Comparing cited specific growth rates (qFe^{2+}) achieved by *L. ferriphilum* and *Leptospirillum*-dominant cultures at 40°C

pH	0.7	0.9	1.1	1.3	1.5	1.6	1.7	1.75
Current work	*0.061*	*0.060*	*0.062*	*0.068*	*0.074*		*0.073*	
Breed et al., 1999								*0.077*
Dempers et al., 2003			*0.089*	*0.087*	*0.077*		*0.038*	
Franzmann et al., 2005						*0.100*		

In summary, growth and activity was observed under all conditions examined. The change in pH within the pH 0.7-1.7 range influenced the biokinetic performance of *L. ferriphilum* HT. Cultivation at pH 1.1-1.5 appeared to be least affected by the change in pH as illustrated by the short lag times and high biomass yields obtained by the cultures within this range. Conversely, prolonged lag times, low biomass yields and low volumetric oxidation rates were obtained at as pH deviated from this range. Furthermore, the lowest μ_{max} and $-r_{Fe}^{2+}$ were obtained at pH ≤ 0.9, highlighting the impact of acid stress on the biokinetic performance of the bacterium. The low yields and high qFe^{2+} determined at pH ≤ 0.9 may elude to the decoupling of the ferrous iron oxidation activity from microbial growth as the energetic demands for cell maintenance increase as a result of increased acid stress.

4.2 Comparing the effect of pH on the biokinetic performance of *L. ferriphilum* HT at 45°C vs 40°C

The biokinetic performance of the *L. ferriphilum* HT was also investigated as a function of pH at an elevated temperature (45°C) to emulate the upper temperature limit set for the BIOX® operating window. These data are presented in Figure 4.2. The impact of pH on growth (Figure 4.2B) and ferrous iron oxidation activity (Figure 4.2A) was exacerbated at 45°C compared to 40°C. The findings indicate that the highest microbial growth and oxidation rates were achieved at pH 1.7, decreasing with decreasing pH. The decrease in growth and activity became more pronounced at pH < 1.3. In contrast to findings at 40°C, negligible growth and oxidation activity was observed by *L. ferriphilum* HT at pH 0.7°C.

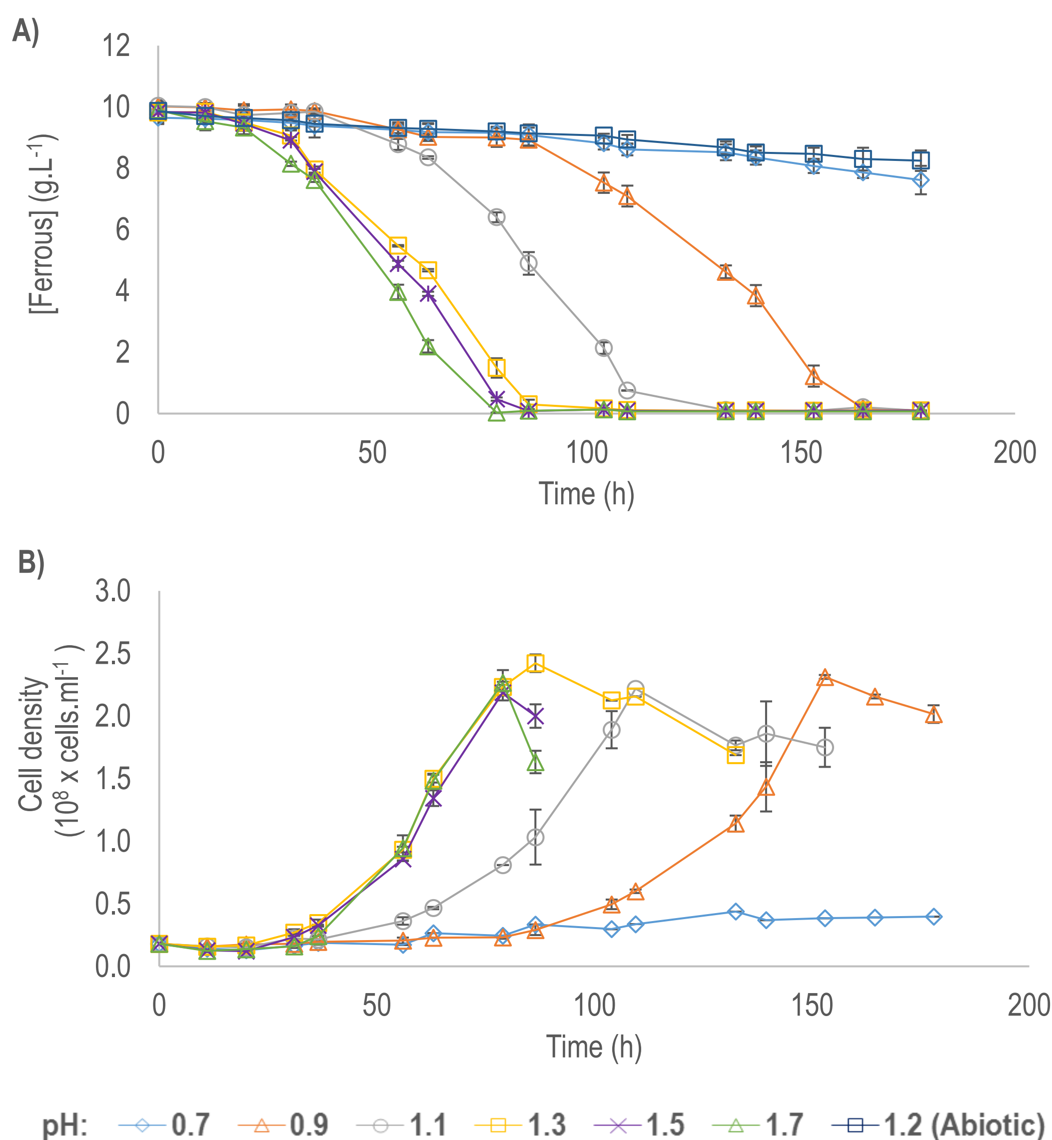

Figure 4.2: Effect of pH on the A) ferrous oxidation activity and B) microbial growth of *L. ferriphilum* HT at 45°C

Table 4.4: Ferrous iron (%) oxidized by *L. ferriphilum* HT under different pH and temperature conditions

pH	0.7	0.9	1.1	1.3	1.5	1.7
	Fe^{2+} oxidized at 40°C (%)					
After 72h incubation (%)	*97.8*	*98.6*	*99.1*	*98.5*	*98.7*	*98.9*
After 83.5h incubation (%)	*99.6*	*99.5*	*99.5*	*99.5*	*99.4*	*99.3*
	Fe^{2+} oxidized at 45°C (%)					
After 79h incubation (%)	*5.1*	*10.2*	*36.2*	*84.8*	*95.2*	*99.1*
After 86.5h incubation (%)	*5.8*	*10.8*	*51.2*	*96.9*	*99.2*	*99.1*
After 178h incubation (%)	*21.1*	*99.1*	*99.1*	*99.0*	*99.0*	*99.3*

Although microbial growth and activity was observed by *L. ferriphilum* HT at 45°C within the pH 0.9-1.7 range, the $-r_{Fe}{}^{2+}$ and $q_{Fe}{}^{2+}$ achieved by these cultures were substantially lower than their 40°C counterparts. More than 98% of the ferrous iron was oxidized within 72 hours of incubation within the pH 0.9-1.7 range at 40°C and almost complete depletion of ferrous iron levels (> 99% ferrous iron oxidized) across all pH levels investigated was observed after 83.5 hours of incubation (Table 4.4). Ferrous iron depletion was observed at 45°C within a similar time frame at pH 1.5 & 1.7 but became increasingly slower at lower pH levels. However, upon ferrous iron depletion, similar maximum cell densities were achieved at 45°C, across pH 0.9-1.7 ($2.30 \pm 0.12 \times 10^8$ cells.mL^{-1} at the point of ferrous iron depletion).

The $q_{Fe}{}^{2+}$ and μ_{max} determined for the bacterium at 45°C were all lower than the corresponding rates achieved at 40°C. Furthermore, both $q_{Fe}{}^{2+}$ and μ_{max} decreased with decreasing pH although μ_{max} remained relatively constant at pH 1.3-1.7 (Table 4.5). Together, the μ_{max},$-r_{Fe}{}^{2+}$ and $q_{Fe}{}^{2+}$ determined for the bacterium indicate that the increase in temperature had a detrimental effect on microbial growth rate and metabolic activity which became more evident at pH < 1.3. These findings suggests that, at 45°C and pH < 1.3, the combination of heat stress and acid stress had a cumulative effect on the biokinetic performance of the bacterium.

Table 4.5 Biokinetic profile determined for *L. ferriphilum* HT at 45ºC

pH	T_{Lag} (h)	$Y_{(x/s)}$ (cells/µmol Fe^{2+})	$-rFe^{2+}$ ($mg_{Fe^{2+}}.L^{-1}.h^{-1}$)	$q_{Fe}{}^{2+}$ (nmol Fe^{2+}.(cell^{-1}).h^{-1})	μ_{max} (h^{-1})
0.7	*N.D.*	*N.D.*	*N.D.*	*N.D.*	*N.D.*
0.9	*69.9*	*0.418*	*128.7*	*1.04*	*0.031*
1.1	*35.3*	*0.415*	*151.6*	*1.75*	*0.034*
1.3	*23.7*	*0.411*	*173.2*	*2.65*	*0.054*
1.5	*25.7*	*0.436*	*191.7*	*3.06*	*0.055*
1.7	*30.5*	*0.457*	*204.6*	*4.99*	*0.055*

L. ferriphilum HT demonstrated the highest μ_{max} at pH 1.3-1.7 for both temperature regimes investigated (Figure 4.3), however lower growth rates were obtained across all pHs studied at 45°C relative to the rates obtained at 40°C. Furthermore, the decrease in pH below pH 1.3 had a greater effect on μ_{max} at 45°C when compared to the corresponding μ_{max} obtained at 40°C. An 18.5% difference in μ_{max} was

observed at 40°C between pH 1.7 and pH 0.9, whereas a 43.4% difference in μ_{max} was observed at 45°C across the same pH range.

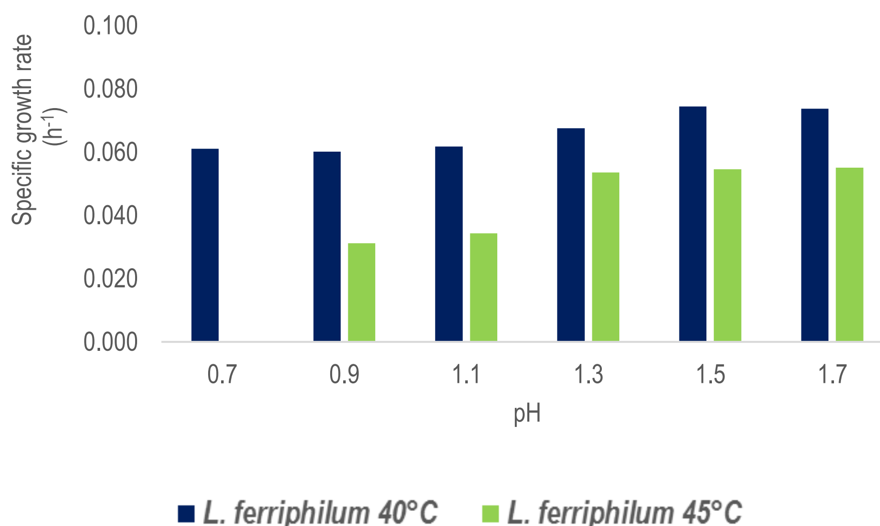

Figure 4.3: Effect of pH on the maximum specific growth rate (μ_{max}) of *L. ferriphilum* HT at 40°C and 45°C

Both the $-r_{Fe^{2+}}$ and $q_{Fe^{2+}}$ decreased with decreasing pH at 45°C. The $-r_{Fe^{2+}}$ determined for the bacterium at 45°C (Figure 4.4A) were also noticeably lower than the corresponding rates achieved at 40°C across, ranging between 15.5% lower at pH 1.7 to 55% lower at pH 1.1. Similarly, the $q_{Fe^{2+}}$ obtained at 45°C (Figure 4.4B) were all substantially lower than those determined at 40°C (over 90% lower at pH 0.9 and ~40% lower at pH 1.7).

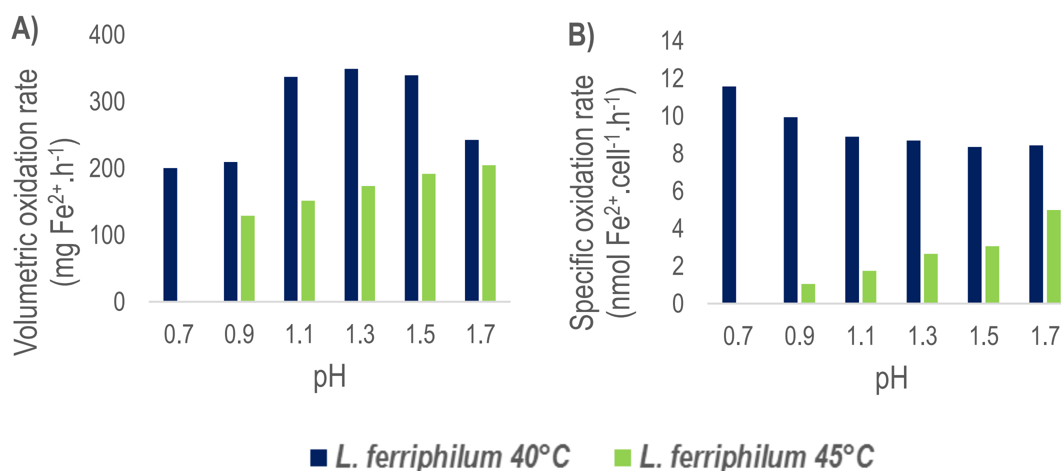

Figure 4.4: Effect of pH on the A) volumetric oxidation rate (rFe^{2+}) and B) specific oxidation rate (qFe^{2+}) achieved by *L. ferriphilum* HT at 40°C and 45°C

As shown in Figure 4.5A, the duration of lag times obtained at 45°C were longer than the corresponding lag times obtained at 40°C. Under both temperature regimes, the shortest lag time was obtained at pH 1.3 which can be attributed to pre-adaptation of the working stock used to inoculate the cultures (maintained at pH 1.2 ±0.2 at 40°C and 45°C, respectively). Lag time increased as pH deviated from this range although a more marked increase in lag time duration was determined at pH < 1.3 at 45°C.

Cultivating *L. ferriphilum* HT at an elevated temperature (45°C) may have had a detrimental impact on the μ_{max} and q_{Fe}^{2+} obtained by the bacterium, however biomass yields obtained at 40°C and 45°C remained relatively similar (Figure 4.5B). The relationship between biomass yield and pH differed across the two temperature regimes. At 40^{0}C, biomass yield appeared to be inversely proportional to the lag time duration, where cultures which displayed the shortest lag phase (pH 1.1-1.5) exhibited the highest yields. Conversely, yields remained relatively constant at 45^{0}C, but did begin to increase at pH ≥ 1.5 although the significance of this increase has yet to be tested.

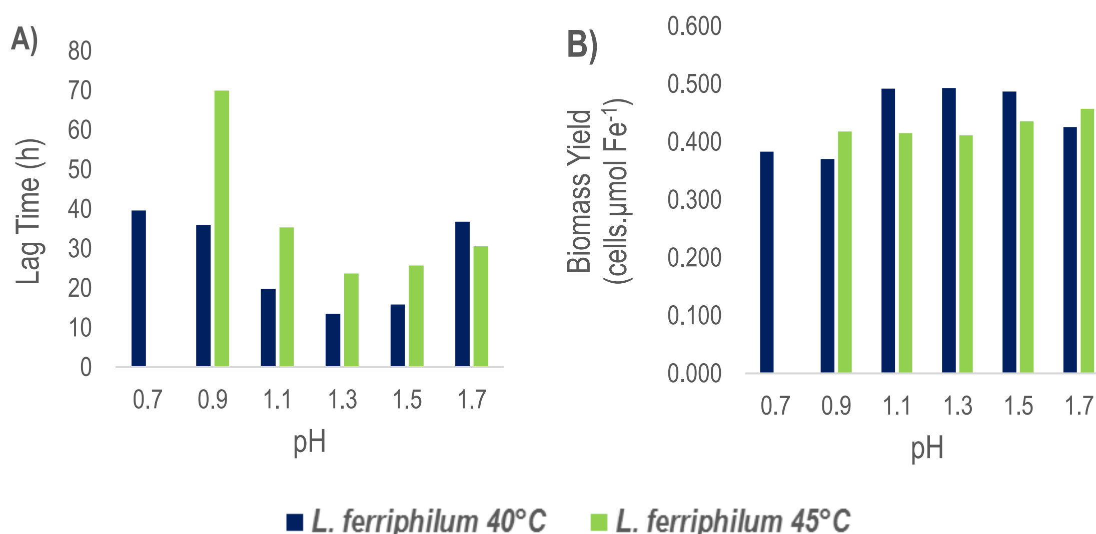

Figure 4.5: Effect of pH on A) Lag time and B) yield coefficient ($Y_{x/s}$) of *L. ferriphilum* at 40°C and 45°C

By comparing the biokinetic performance of *L .ferriphilum* HT at 40°C and 45°C, it can be seen that the increase in temperature has a detrimental effect on the bacterium, as shown by the lower $-r_{Fe}^{2+}$, q_{Fe}^{2+} and μ_{max} as well as the longer lag times determined at the elevated temperature. Moreover, the impact that the elevated temperature had on the growth and activity of the bacterium appeared to be exacerbated at pH < 1.3, suggesting that the heat and acidity may have had a cumulative effect on *L. ferriphilum* HT.

While pH did influence the bacterium's growth and activity at both 40°C and 45°C, the relationship between pH and the biokinetic performance differed across the two temperature regimes. At 45°C, the specific growth rate, oxidation rates and biomass yields all decreased with decreasing pH. At 40°C, the lowest yields, μ_{max} and $-r_{Fe}^{2+}$ were also determined at pH < 1.1, however the q_{Fe}^{2+} appeared to increase as pH decreased. The increase in oxidation activity below pH 1.1 at 40°C may be attributed to the greater energetic demands directed towards cell maintenance and survival survive under sub-optimal conditions (pH < 1.1) which comes at the expense of microbial growth. An increase in q_{Fe}^{2+} was not observed under at the elevated temperature (45°C) at pH < 1.3, possibly due to the cumulative effect of heat and acid stress under these conditions being detrimental to metabolic activity.

4.3 The effect of pH on biokinetic performance of *Ac. cupricumulans* JTC3 at 45°C

Microbial growth and oxidation activity of the *Ac. cupricumulans* JTC3 was also investigated as a function of pH across the pH 0.7-1.7 range. The experiment was performed at 45°C to emulate the upper temperature limit of the BIOX® operating window. Figure 4.6A indicates a slight decrease in the rate of ferrous iron oxidation as well as residual ferrous iron levels increasing with increasing pH (after 116 hours incubation). A residual ferrous iron concentration of 0.156 g.L^{-1} was determined at pH 0.7 whereas the residual iron concentration of 2.120 g.L^{-1} was determined at pH 1.7. The microbial growth shown in Figure 4.6B indicates that similar maximum cell densities were obtained at pH 0.9-1.5 (13.1 ± 0.9 x10^{8} cells.mL^{-1}) with the maximum cell densities decreasing as pH deviated from this range.

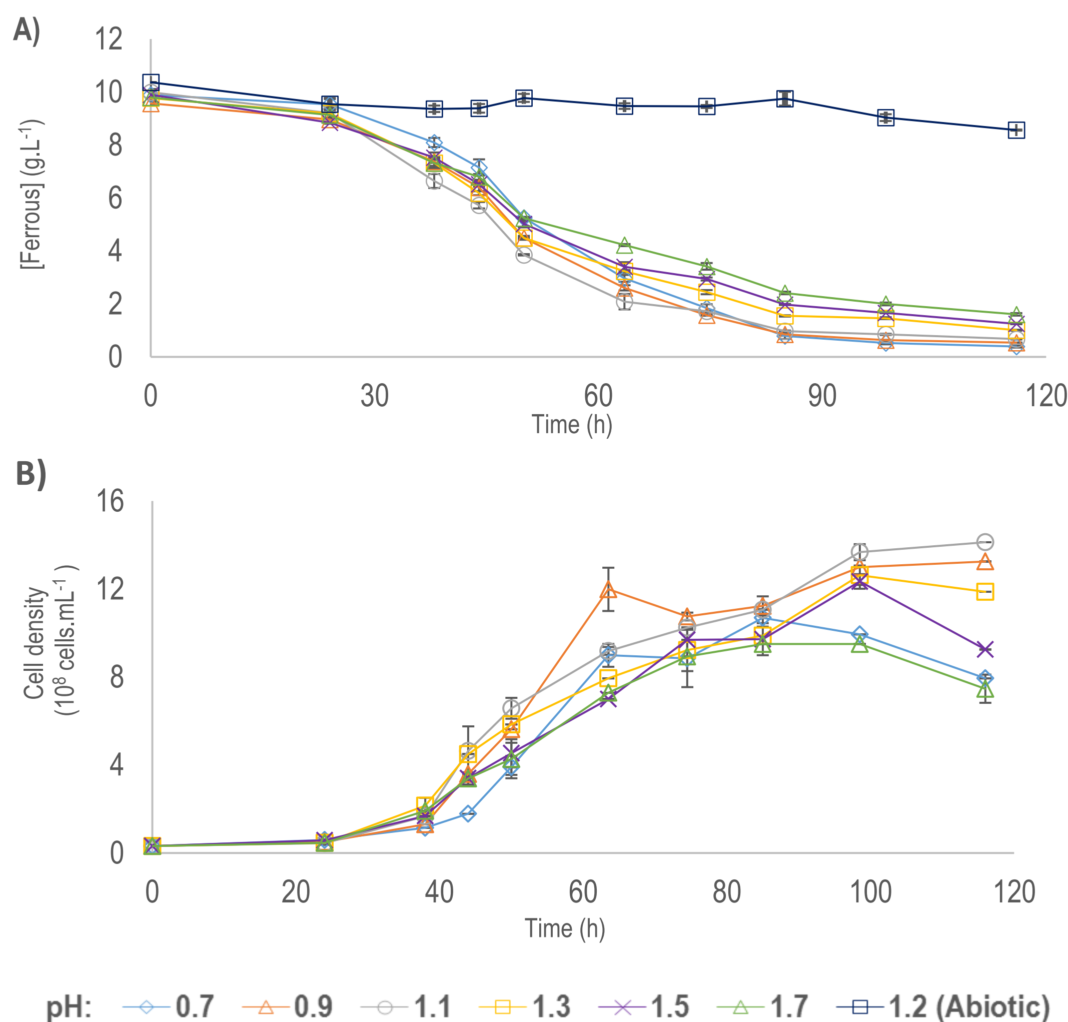

Figure 4.6: Effect of pH on the A) ferrous oxidation activity and B) microbial growth of *Ac. cupricumulans* JTC3 at 45°C

Lag times remained relatively constant across the pH 0.7-1.7 range (Table 4.6) which suggests that pH did not effect the archaeon's resilience and ability to acclimatize to the change in acidity under experimental conditions. In contrast, pH had a noticeable effect on μ_{max}, $-r_{Fe}^{2+}$ and yields achieved by the archaeon.

Although the q_{Fe}^{2+} determined for the archaeon remained relatively constant across the pH 0.7-1.7 range; pH did have a more noticeable effect on μ_{max} which subsequently influenced the $-r_{Fe}^{2+}$ achieved through its impact of cell number. The highest μ_{max} and $-r_{Fe}^{2+}$ rates were achieved at pH 0.9 and the lowest rates determined at pH 1.7 (Table 4.6).

Table 4.6: Biokinetic performance associated with microbial growth and ferrous oxidation of *Ac. cupricumulans JTC3*

pH	T_{Lag} (h)	$Y_{(x/s)}$ (cells/ µmol Fe^{2+})	-rFe2+ (mgFe2+.$L^{-1}.h^{-1}$)	qFe^{2+} (nmol Fe^{2+}.cell-1.h^{-1})	μ_{max} (h^{-1})
0.7	*21.8*	*2.144*	*236.5*	*2.68*	*0.102*
0.9	*20.0*	*2.103*	*240.7*	*3.24*	*0.122*
1.1	*21.5*	*2.439*	*232.2*	*2.62*	*0.114*
1.3	*20.9*	*2.353*	*235.0*	*2.73*	*0.083*
1.5	*17.2*	*2.413*	*208.7*	*3.12*	*0.082*
1.7	*25.9*	*2.689*	*173.8*	*2.61*	*0.067*

The μ_{max} achieved for the archaeon (Table 4.6) were comparable to those reported by Rautenbach (2007), where a μ_{max} of 0.090 h^{-1} was determined for *Ac. cupricumulans* JTC3 at pH 1.2. With regards to $-r_{Fe}^{2+}$, Hawkes *et al.* (2006) reported a similar trend where ferrous iron oxidation rate (reported as ferric iron production rate) decreased at pH < 1.0 (~19 mg.$L^{-1}.h^{-1}$ ferric iron production rate at pH 1.0 decreased to ~5 mg.L^{-1},h^{-1} at pH 1.7). Furthermore, Rautenbach (2007) reported a $-r_{Fe}^{2+}$ of 110 mg .$L^{-1}.h^{-1}$ at 52°C (pH 1.2) for *Ac. cupricumulans* JTC3 while Franzmann *et al.* (2005) reported a similar oxidation rate of 115 mg.$L^{-1}.h^{-1}$ at 55°C, pH 1.2 for *Ac. cupricumulans* BH2. Although the $-r_{Fe}^{2+}$ obtained for *Ac. cupricumulans* JTC3 (Table 4.6) were higher (~233.6 $mg_{Fe}^{2+}.L^{-1}.h^{-1}$) than those reported by Rautenbach (2007) and Franzmann *et al.* (2005), these rates are not inherently related to the specific oxidation rates of the archaeon may be attributed to differences in experimental parameters, such as cell densities and organic carbon availability (0.1 g.L^{-1} yeast extract supplementation used by Franzmann *et al.*, 2005 and Hawkes *et al.,* 2006).

The biokinetic performance of *Ac. cupricumulans* JTC3 was therefore also assessed against the reported performance of closely related archaea cultivated at a similar temperature. Okibe *et al.* (2003) reported a μ_{max} of 0.077 h^{-1} (doubling time of 9 h) at pH 1.5 for *Ferroplasma* strain MT17 (at 45°C), which was similar, albeit slightly lower, than that achieved by *Ac. cupricumulans* JTC3 in this study (0.082 h^{-1}). Golyshina *et al.* (2009) reported a μ_{max} of 0.032 h^{-1} for *Ac. aeolicum* V^T, at pH 1.6 within a temperature range of 42-45°C. While factors such as media composition may have had some influence on the μ_{max} reported for these closely-related species (e.g. 0.5 g.L^{-1} yeast extract was used in the current study whereas 0.2 g.L^{-1} yeast extract used by Okibe *et al,* 2003 and Golyshina *et al.*, 2009), the comparison also supports the postulation made by Bulaev *et al.* (2011) that the high-degree of phylogenetic similarity amongst different strains and closely-related microbial species (particularly *Acidiplasma spp.)* may not necessarily correlate with physiological similarities.

Ac. cupricumulans JTC3 displayed the highest μ_{max} and q_{Fe}^{2+} at pH 0.9 and the lowest respective rates at pH 1.7. In addition, the increase in pH may have influenced the archaeon's affinity for ferrous iron as

seen by the direct correlation between increasing residual ferrous iron concentration in the culture and increasing pH. Despite the influence of pH on the biokinetics of the archaeal cultures, the lag times obtained at 45°C across the pH 0.7-1.7 range remained relatively constant, highlighting *Ac. cupricumulans* JTC3's proficiency to acclimate to the pH experimental conditions investigated.

4.4 Comparing the effect of pH on *Ac. cupricumulans* JTC3 and *L. ferriphilum* HT at 45°C

A comparative analysis was then performed on the biokinetic performance of *L. ferriphilum* HT and *Ac. cupricumulans* JTC3 to assess the effects of pH on these two iron oxidizing microorganisms at the upper temperature limit of the BIOX® operating window (45°C).

The difference in lag times, shown in Figure 4.7A, suggests that *Ac. cupricumulans* JTC3 was able to acclimate to the different pH conditions more effectively than *L. ferriphilum* HT. The lag times determined for the archaeon remained relatively constant across the pH range 0.7-1.7 and were all shorter than the corresponding lag times determined for *L. ferriphilum* HT. Conversely, the lag times determined for the bacterium increased as pH deviated from the pH 1.3 level, with lag time increasing sharply at pH below pH 1.3. With regards to biomass yields, the yields determined for *Ac. cupricumulans* JTC3 were substantially higher than those determined for *L. ferriphilum* HT across the pH range examined (Figure 4.7B). This is partially expected owing to the smaller cell size of *Ac. cupricumulans*. The coccoid *Ac. cupricumulans* cells have a typical diameter of ~0.2-0.5 µm in whereas the vibrio or spirilla-shaped *L .ferriphilum* cells are typically 0.3 to 0.6 µm wide and 0.9 to 3.5 µm long (Hawkes *et* al., 2006; Coram and Rawlings, 2002). .

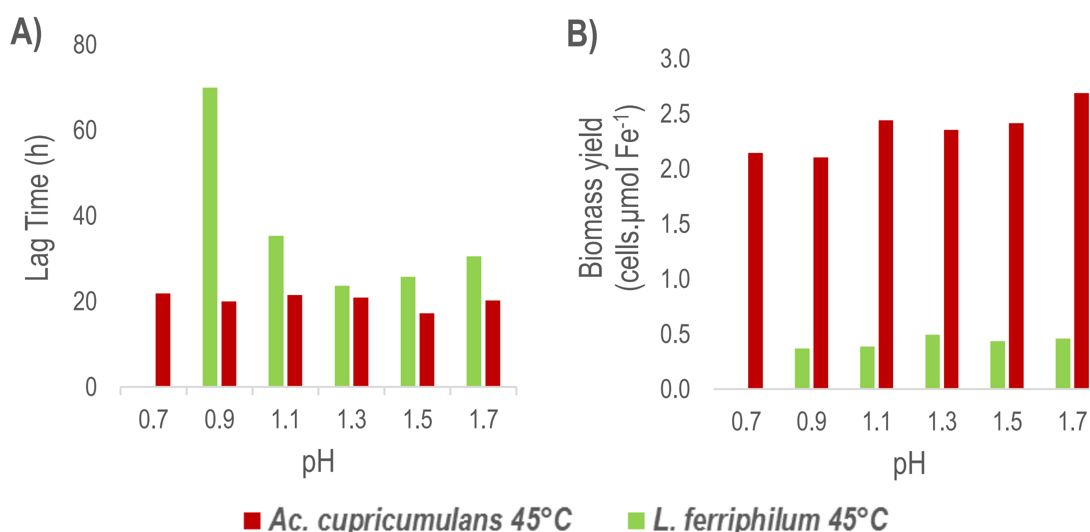

Figure 4.7: Effect of pH on the A) duration of lag time and B) yield coefficient determined for *L. ferriphilum* HT and *Ac. cupricumulans* JTC3 at 45°C

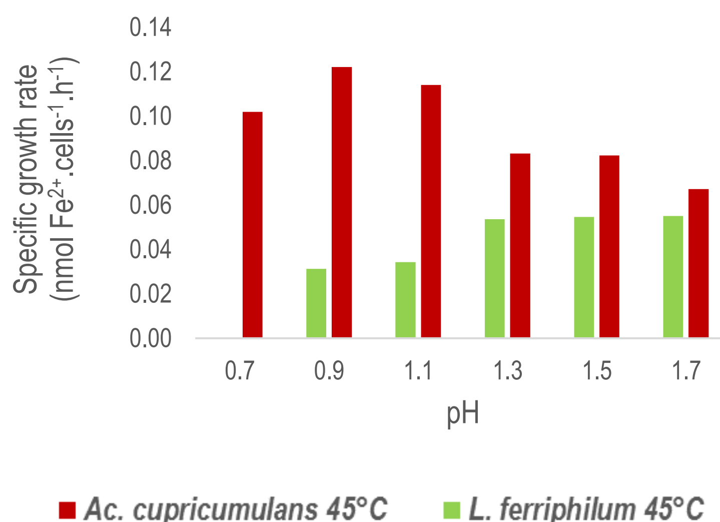

Figure 4.8: Effect of pH on the specific growth rate determined for *L .ferriphilum* HT and *Ac. cupricumulans* JTC3 at 45°C

Similarly, the archaeon also displayed higher μ_{max} than *L. ferriphilum* HT across all corresponding pH levels at 45°C (Figure 4.8). The change in pH did appear to have contrasting effects on the growth trends of these two microorganisms. The μ_{max} decreased for *Ac. cupricumulans* as pH increased from pH 0.9 to pH 1.7, whereas μ_{max} increased with increasing pH for the bacterium.

The marked difference in yields can be attributed to several factors, most notably their source of carbon and their cell size. As a heterotrophic microorganism, *Ac. cupricumulans* JTC3 utilizes an organic carbon substrate present in the medium to synthesize organic compounds whereas the autotrophic *L. ferriphilum* HT is dependent on the fixation of atmospheric carbon dioxide for the synthesis of organic compounds. The energetic burden required to synthesize organic compounds via carbon fixation is much greater than that achieved by metabolism of organic substrates (Johnson and Hallberg, 2009).

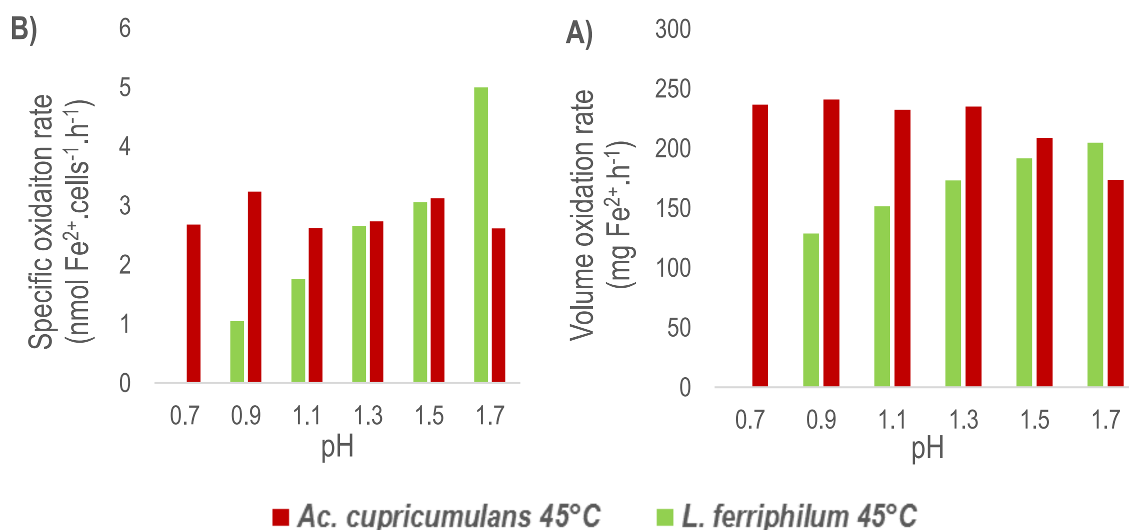

Figure 4.9: Effect of pH on the A) average specific oxidation rate and B) average volumetric oxidation rate determined for *L. ferriphilum* HT and *Ac. cupricumulans* JTC3 at 45°C

The archaeon may have been able to out-grow bacterium, but it was not able to completely out-perform it across the entire pH range examined, as shown in Figure 4.9A. At pH 1.7 the average q_{Fe}^{2+} determined for *L. ferriphilum* HT was higher than that of Ac. *cupricumulans* JTC3, however, the average q_{Fe}^{2+} determined for the bacterium decreased with decreasing pH. The q_{Fe}^{2+} rates determined for both microbes were similar at pH 1.3-1.5, however, at pH < 1.3 the average q_{Fe}^{2+} determined for the archaeon was higher than of *L. ferriphilum.*

With regards to the average $-r_{Fe}^{2+}$ determined for the two microorganisms, *Ac. cupricumulans* JTC3 achieved higher $-r_{Fe}^{2+}$ at pH 0.7-1.3 whereas both microorganisms displayed similar $-r_{Fe}^{2+}$ at pH 1.5-1.7. The $-r_{Fe}^{2+}$ performance of *Ac. cupricumulans* JTC3 benefits from the relatively high μ_{max} and its resilience towards acid stress at 45°C across the pH range.

Despite the sub-optimal growth and performance of *L. ferriphilum* HT at 45°C, the bacterium functioned as a more efficient iron scavenger than *Ac. cupricumulans* JTC3 at pH 0.9-1.7. Residual ferrous iron levels present in the cultures of the respective microorganisms were compared a were compared at the point at which negligible microbial growth was observed for all cultures and the experiment concluded (Table 4.7). After 178 hours of incubation, ≥ 99% of the ferrous iron present in the pH 0.9-1.7 *L. ferriphilum* cultures had been oxidized (< 0.1 $g.L^{-1}$ residual ferrous iron present in the cultures). Conversely, ≤ 96% of the ferrous iron had been oxidized in the *Ac. cupricumulans* JTC3 cultures after 116 hours of incubation (< 0.4 $g.L^{-1}$ residual ferrous iron present in the cultures), with the relative amount of ferrous iron oxidized decreasing with increasing pH.

Table 4.7. % Ferrous iron oxidized over the duration of the biokinetic experiment

pH	0.7	0.9	1.1	1.3	1.5	1.7
L. ferriphilum HT (178 h)	*21.1*	*99.1*	*99.1*	*99.0*	*99.0*	*99.3*
Ac. cupricumulans JTC3 (116 h)	*96.0*	*94.4*	*93.2*	*89.7*	*87.4*	*83.6*

In summary, the μ_{max} determined for *Ac. cupricumulans* JTC3 *at* 45°C were higher than the corresponding μ_{max} of *L. ferriphilum* HT across the entire pH range examined. These findings also suggest that, even at 45°C, *L. ferriphilum* HT appeared to be a more efficient iron oxidizer at pH 1.7 and a better scavenger of ferrous iron at pH 0.9-1.7 compared to *Ac. cupricumulans* JTC3. However, the bacterium's ability to oxidize ferrous iron decreased with decreasing pH due to the apparent compounding effects of acid and heat stress. Conversely, the opposite trend was determined for the archaeon who displayed an increase in ferrous iron affinity and $-r_{Fe}^{2+}$ as pH decreased. A higher $-r_{Fe}^{2+}$ was determined for *Ac. cupricumulans* JTC3 at pH < 1.5, however, as a result of the contrasting effect of pH on the oxidation activity of these two microorganisms at 45°C, both microorganisms displayed similar average $-r_{Fe}^{2+}$ at pH 1.5-1.7. Based on these findings, *Ac. cupricumulans* JTC3 may potentially out-compete and out-perform *L. ferriphilum* HT at pH < 1.5, at the upper temperature limit of the BIOX® operating window.

5 Results II: The Effect of Organic Carbon

5.1 Effect of organics on the microbial growth and ferrous oxidation activity of *L. ferriphilum* HT

5.1.1 Effect of yeast extract on *L. ferriphilum* HT

L. ferriphilum HT's sensitivity to the presence of dissolved organic carbon in the media was investigated by supplementing the cultures with 0.05 - 0.5 $g.L^{-1}$ yeast extract (pH 1.2 at 45°C). Ferrous iron concentrations (Figure 5.1A) and microbial growth rates (Figure 5.1B) were monitored to assess the effect of the added yeast extract.

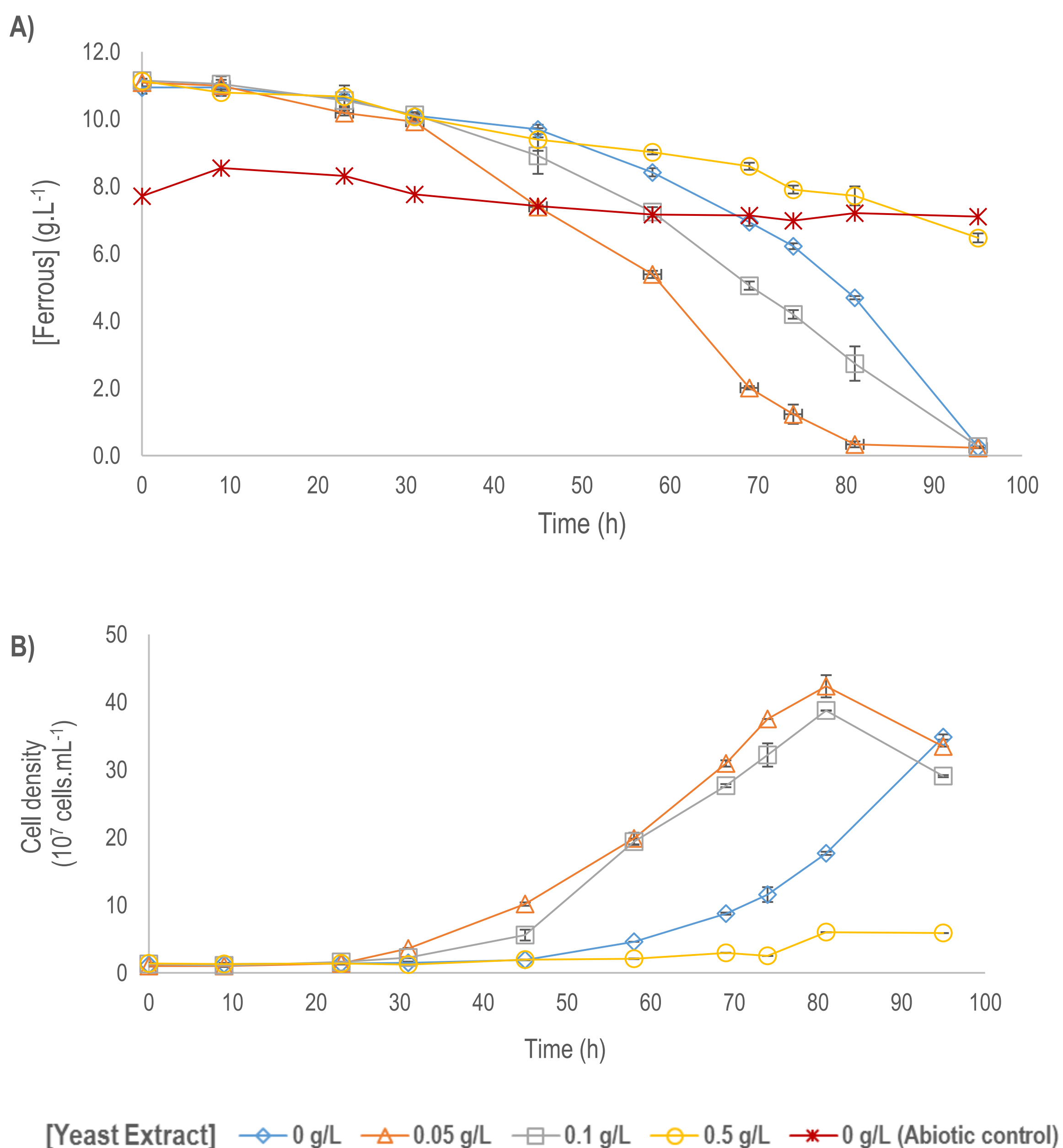

Figure 5.1: Effect of yeast extract on A) ferrous oxidation activity and B) microbial growth of *L .ferriphilum* HT

The findings from Figure 5.1 indicate that growth and activity was observed in the presence of 0.05 $g.L^{-1}$ and 0.1 $g.L^{-1}$ yeast extract while growth inhibition was observed in cultures containing 0.5 $g.L^{-1}$ yeast extract. Similar residual ferrous iron levels were present, after 95 hours of incubation, in the cultures with 0.0 $g.L^{-1}$, 0.05 $g.L^{-1}$ and 0.1 $g.L^{-1}$ yeast extract (97.6-97.9% of ferrous iron oxidized). A substantially higher level of residual ferrous iron concentration was determined in cultures containing 0.5 $g.L^{-1}$ yeast extract (41.9% ferrous iron oxidized); however, more ferrous iron was oxidized in these cultures compared to the abiotic control (7.9% ferrous iron oxidized). The gradual decrease in ferrous iron obtained in the abiotic control can be attributed to auto-oxidation of the ferrous iron under the aerobic experimental conditions (Keenan, 1969). The more marked decrease observed in cultures containing 0.5 $g.L^{-1}$ yeast extract suggests that biological oxidation occurred within these growth-inhibited cultures. Biological oxidation occurring in the growth-inhibited cultures may be attributed to metabolically active, but non-growing, *L. ferriphilum* HT present following inoculation or to slow-growing cultures.

Table 5.1: Effect of yeast extract on the biokinetic performance of *L. ferriphilum* HT

[Yeast Extract] ($g.L^{-1}$)	*Lag time (h)*	*Yield coefficient (cells / μmol Fe^{2+})*	*$-rFe^{2+}$ ($mg.L^{-1}$)*	*qFe^{2+} (nmol $Fe^{2+}.cell^{-1}.h^{-1}$)*	*μ_{max} (h^{-1})*
0	35.9	0.653	284.1	3.26	0.059
0.05	18.3	0.860	219.5	1.71	0.057
0.1	25.6	0.879	195.8	1.64	0.060
0.5	N.D.	N.D.	N.D.	N.D.	N.D.

N.D. – Not Determined

The highest average q_{Fe}^{2+} and $-r_{Fe}^{2+}$ was determined for the *L. ferriphilum* HT cultures containing no yeast extract (despite its unexpectedly-long lag time) whereas the μ_{max} remained relatively constant amongst cultures containing 0.0 - 0.1 $g.L^{-1}$ yeast extract (Table 5.1). Negligible growth was observed in cultures containing 0.5 $g.L^{-1}$ yeast extract which is consistent with the findings of Sand *et al.* (1991). Similar biomass yields and lag times were determined for the cultures containing 0.05 $g.L^{-1}$ and 0.1 $g.L^{-1}$ yeast extract whereas a lower yield and longer lag time was determined for the yeast extract absent positive control.

5.1.2 Effect of spent culture filtrate from *Ac. cupricumulans* JTC3 on *L. ferriphilum* HT

While the effect of yeast extract on *L. ferriphilum* HT illustrates the chemolithotroph's sensitivity to organic carbon, this does not provide a true reflection of the organic carbon content and composition expected in biomining leachate liquor. To address this, *L. ferriphilum* HT growth was also investigated in the presence spent culture filtrate derived from *Ac. cupricumulans* JTC3. The organic carbon content within the filtrate was determined through COD analysis (Table 5.2). It was assumed that the organic content present in the spent culture filtrate (determined by COD analysis) would consist of intracellular cytoplasmic fraction as well as exuded waste products, metabolites, and residual yeast extract.

Table 5.2: Analysis of organic content present in *Ac. cupricumulans* JTC3 spent culture filtrate

Filtrate composition	*COD (mg.L⁻¹)*
Ac. cupricumulans JTC3 spent culture filtrate	412.5
Archaeal media containing ferrous iron concentration equivalent to that in spent culture	(97.4)
Dissolved organic carbon	**315.1**

The effect of DOC derived from archaeal spent culture was then assessed by cultivating *L. ferriphilum* HT in media containing 10%, 25% and 50% *Ac. cupricumulans* JTC3 spent culture filtrate. Similar rates of microbial growth (Figure 5.2B) and ferrous iron oxidation (Figure 5.2A) were observed by the cultures containing 10% spent culture filtrate and the positive control (no added filtrate). Conversely, no growth or oxidation activity was observed in cultures with ≥ 25% spent culture filtrate.

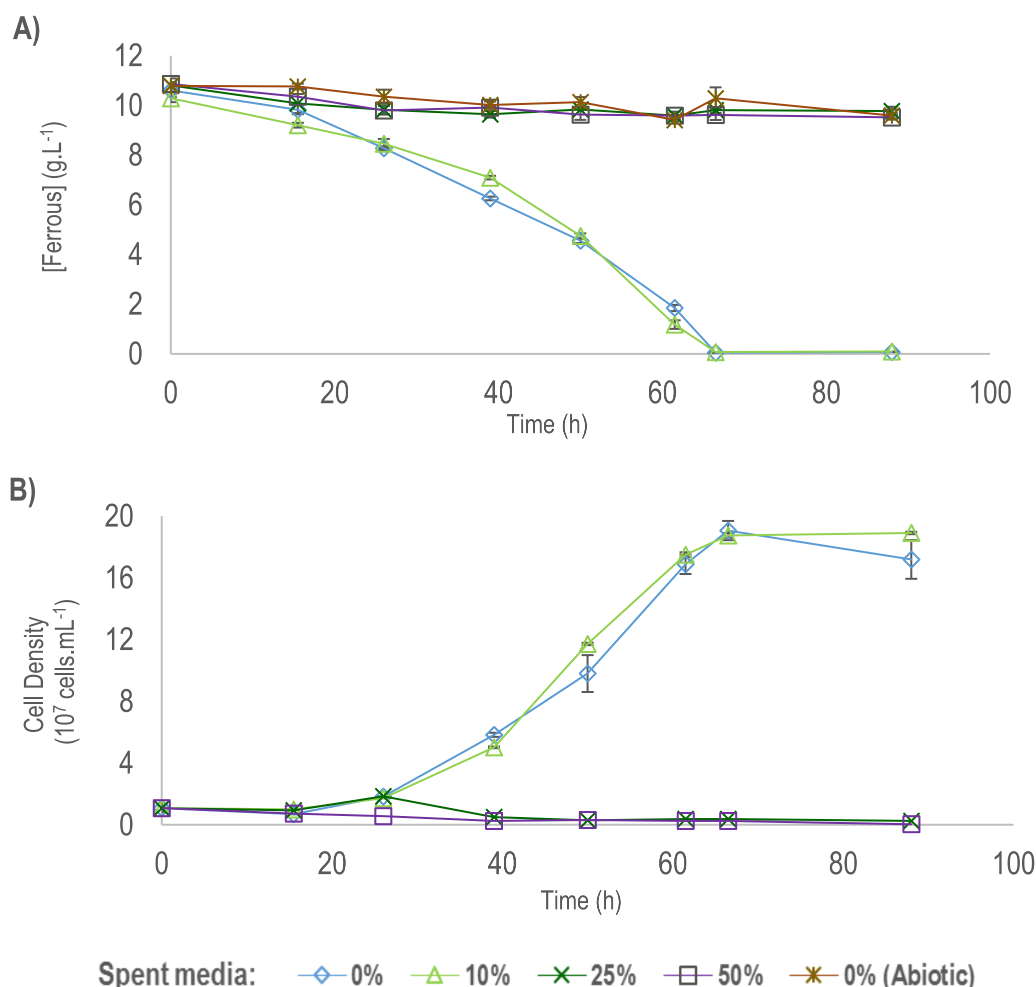

Figure 5.2: The rate of A) ferrous iron oxidation activity and B) microbial growth of *L. ferriphilum* HT grown in varying concentrations (%) of *Ac. cupricumulans* JTC3 spent culture at 45°C, pH 1.5

The findings from Figure 5.2, summarized below in Table 5.3, suggest that the addition of 10% spent culture filtrate (0.032 g.L^{-1} DOC) had negligible inhibitory effect on *L. ferriphilum* HT while complete

inhibition of *L. ferriphilum* HT was observed in cultures containing 25% (0.079 $g.L^{-1}$ DOC) and 50% filtrate (0.158 $g.L^{-1}$ DOC). Similar findings were reported by Vardanayan *et al.* (2017) for *L. ferriphilum* cultures supplemented with cell lysate prepared from an autoclaved and filtered sulfur oxidizer enriched *Acidiphilium* J1 culture. Vardanayan *et* al. (2017) observed partial inhibition of *L. ferriphilum* in the presence of 10% cell lysate (0.41 $g.L^{-1}$ DOC) and no growth with ≥ 20% cell lysate (≥ 0.83 $g.L^{-1}$ DOC). The comparison drawn between the inhibitory effect of organics in this study and that of Vardanyan *et al.* (2017) is used to illustrate the relative sensitivity of the bacterium to dissolved organic carbon derived from cell lysates and exudates. However, the difference in inhibitory concentrations determined between these two studies may be attributed to the difference in composition of the *Ac. cupricumulans* JTC3 spent culture filtrate used in the current study to the autoclaved cell lysate used by Vardanyan *et al.* (2017).

Table 5.3: Effect of *Ac. cupricumulans* JTC3 spent culture filtrate on the biokinetic performance of *L. ferriphilum* HT

Spent culture filtrate DOC conc. ($g.L^{-1}$)	*Lag Time (h)*	*Yield coefficient (cells / µmol Fe^{2+})*	*$-rFe^{2+}$ ($mg.L^{-1}.h^{-1}$)*	*qFe^{2+} (nmol $Fe^{2+}.cell^{-1}.h^{-1}$)*	*μ_{max} (h^{-1})*
0 $mg.L^{-1}$	19.34	0.442	225.8	7.23	0.063
0.032 $g.L^{-1}$ (10% filtrate)	17.84	0.420	255.2	8.10	0.061
0.079 $g.L^{-1}$ (25% filtrate)	N.D.	N.D.	N.D.	N.D.	N.D.
0.158 $g.L^{-1}$ (50% filtrate)	N.D.	N.D.	N.D.	N.D.	N.D.

N.D. – Not Determined

In summary, the *Ac. cupricumulans* JTC3 filtrate had a greater inhibitory effect on the biokinetic performance of *L. ferriphilum* HT compared to the relative effect of yeast extract (or autoclaved archaeal culture filtrate as reported by Vardanyan *et al.*, 2017). These findings emphasize that *L. ferriphilum* HT's sensitivity towards organic carbon is influenced by both the concentration and composition of the organic carbon content present in the media.

5.1.3 Effect of organic acids on microbial growth and ferrous oxidation activity of *L. ferriphilum* HT

Investigating the growth of *L. ferriphilum* HT in spent culture and in the presence of yeast extract demonstrated the influence that composition of organic carbon has on its ability to inhibit growth of *L. ferriphilum* HT.

In a study focussing on the inhibitory effects of yeast extract on acidophiles, Kishimoto *et al.* (1990) identified pyruvic acid, and other organic acid constituents of yeast extract, as major contributors to the growth inhibition observed. The inhibitory effects of organic acids on chemolithotrophs associated with bioleaching processes has been well documented with organic acids such as pyruvic acid, acetic acid and glycolic acid reported, repeatedly, as constituents within the organic content present in their spent culture (Borichewski, 1966; Schanitman and Lundgren, 1965; Aston *et al.*, 2009; Nancucheo and Johnson, 2009). In this experiment, the inhibitory effects of organic acids on the biokinetic performance of *L. ferriphilum* HT were investigated using glycolic acid, acetic acid and pyruvic acid. Although the effect of organic acids on the microorganism were assessed using the same mass concentrations of

the select organic acids (within the 1-30 mg.L^{-1} range) the molar concentrations varied as shown in the table below.

Table 5.4. Relative molar concentrations of the added organic acids

Organic acid	*Molar mass (g.mol^{-1})*	*1 mg.L^{-1} (μM)*	*10 mg.L^{-1} (μM)*	*30 mg.L^{-1} (μM)*
Glycolic acid	76.05	13.1	131.5	394.5
Acetic Acid	60.05	16.7	166.5	499.6
Pyruvic Acid	88.06	11.4	113.6	340.7

5.1.3.1 Effect of glycolic acid on *L. ferriphilum* HT

The inhibitory effects of glycolic acid, acetic acid and pyruvic acid were compared against one another as well as against the inhibitory effects of these organic acids previously reported in literature on *L. ferriphilum* and similar chemolithotrophic acidophiles. The effect of glycolic acid on the biokinetic performance of *L. ferriphilum* HT was assessed at 45°C, pH 1.5, by spiking cultures with 1-30 mg.L^{-1} of the organic acid. Ferrous iron oxidation activity (Figure 5.3A) and microbial growth (Figure 5.3B) were determined in cultures spiked with 1 mg.L^{-1} and 10 mg.L^{-1} glycolic acid, however growth inhibition was observed in cultures spiked with 30 mg.L^{-1} glycolic acid. This is consistent with the findings of Nancucheo and Johnson (2009), where complete inhibition of *L. ferriphilum* HT was reported in the presence of ~38 mg.L^{-1} glycolic acid.

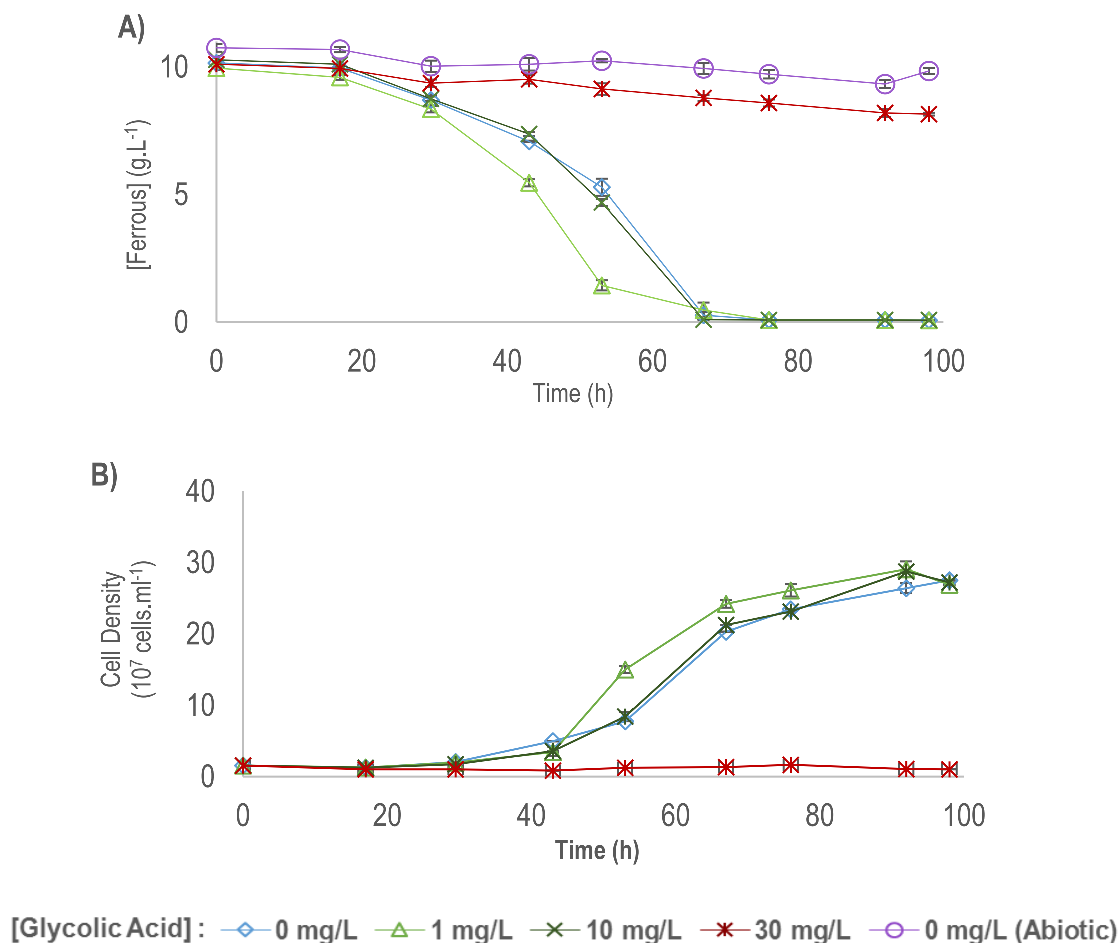

Figure 5.3: Effect of glycolic acid on A) ferrous iron oxidation activity and B) microbial growth of *L. ferriphilum* HT at 45°C, pH 1.5

Table 5.5: Effect of Glycolic acid on the biokinetic performance of *L. ferriphilum* HT

[Glycolic acid] ($mg.L^{-1}$)	Lag time (h)	Yield coefficient (cells / µmol Fe^{2+})	$-rFe^{2+}$ ($mg.L^{-1}.h^{-1}$)	qFe^{2+} (nmol $Fe^{2+}.cell^{-1}.h^{-1}$)	μ_{max} (h^{-1})
0	24.23	0.381	224.8	3.00	0.061
1	28.70	0.304	293.9	8.25	0.067
10	31.01	0.299	230.6	2.99	0.067
30	N.D.	N.D.	N.D.	N.D.	N.D.

N.D. – Not Determined

The addition of 1 $mg.L^{-1}$ and 10 $mg.L^{-1}$ glycolic acid did not appear to have a negative effect on the biokinetic performance of *L .ferriphilum* HT, with these cultures performing similarly to the positive control cultures (Table 5.5). Moreover, the results may suggest that the $q_{Fe}{}^{2+}$ and $-r_{Fe}{}^{2+}$ increased in cultures spiked with 1 $mg.L^{-1}$ glycolic acid. After 98 hours of incubation, > 99% of the ferrous iron was oxidized in cultures containing 0-10 $mg.L^{-1}$ glycolic acid. Although negligible growth was observed in the cultures containing 30 $mg.L^{-1}$ glycolic acid, the residual ferrous iron content in these cultures was lower than that observed for the abiotic control as 19.4% of the ferrous iron was oxidized in the cultures spiked with 30 $mg.L^{-1}$ glycolic acid whereas only 8.4% of the ferrous iron had undergone auto-oxidation in the abiotic control which suggests that non-growth associated biological oxidation may have occurred within these growth-inhibited cultures.

5.1.3.2 Effect of acetic acid on *L. ferriphilum* HT

The effect of acetic acid on the biokinetic performance of *L. ferriphilum* HT was also investigated by creating growth curves from *L .ferriphilum* cultures (at 45°C, pH 1.5) spiked with 1, 10 and 30 $mg.L^{-1}$ acetic acid. The experiment was performed in parallel to the experiment investigating the effect of glycolic acid, on a shared 24-well MWP. Both experiments shared the same organic acid absent positive control and abiotic control. In similar fashion to the trend observed with glycolic acid, the addition of 1 $mg.L^{-1}$ acetic acid did not appear to have a detrimental effect on *L. ferriphilum* HT, but may have increased ferrous iron oxidation activity as seen by the increase in average $-r_{Fe}{}^{2+}$ and $q_{Fe}{}^{2+}$ in these cultures. Negligible growth was observed in the presence of ≥ 10 $mg.L^{-1}$ acetic acid, suggesting that acetic acid had a more potent inhibitory effect on *L. ferriphilum* HT compared to glycolic acid.

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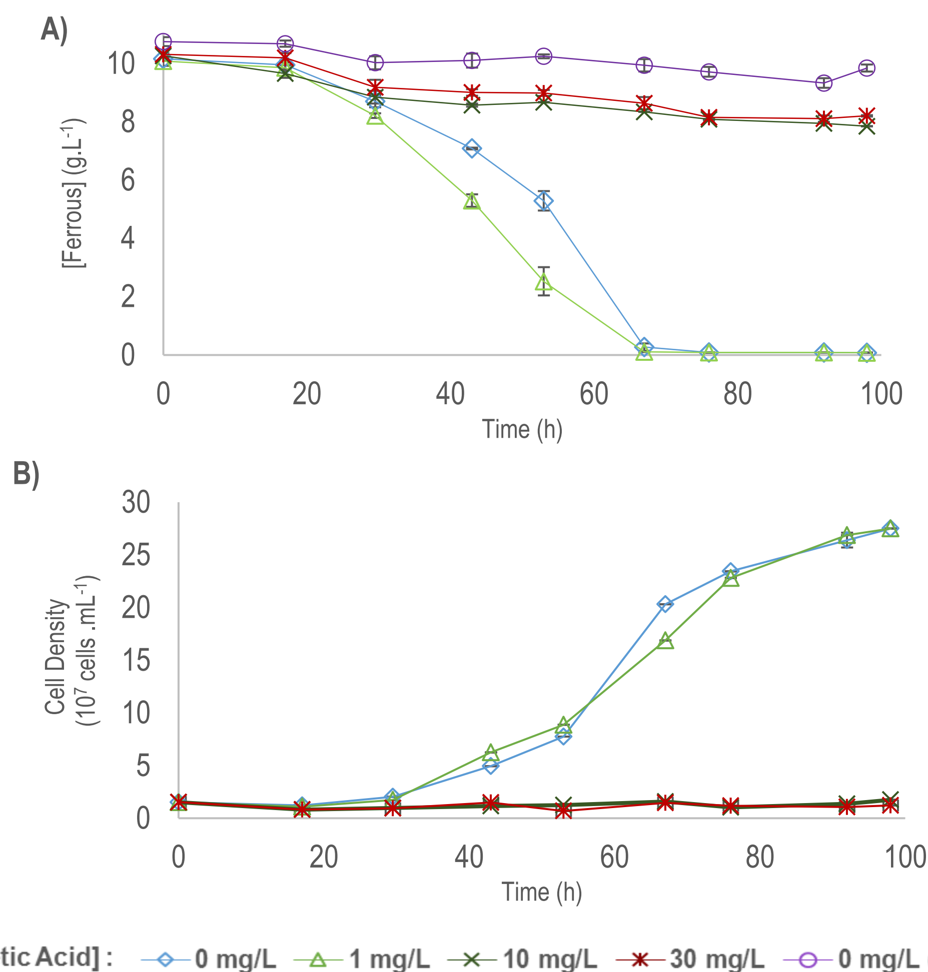

Figure 5.4: Effect of acetic acid on A) ferrous iron oxidation activity and B) microbial growth of *L. ferriphilum* HT at 45°C, pH 1.5

Table 5.6: Effect of acetic acid on the biokinetic performance of *L. ferriphilum* HT

[Acetic acid] ($mg.L^{-1}$)	*Lag time (h)*	*Yield coefficient (cells / μmol Fe^{2+})*	*$-rFe^{2+}$ ($mg.L^{-1}.h^{-1}$)*	*qFe^{2+} (nmol $Fe^{2+}.cell^{-1}.h^{-1}$)*	*μ_{max} (h^{-1})*
0	24.1	0.315	283.90	5.23	0.061
1	24.1	0.384	241.86	10.85	0.061
10	N.D.	N.D.	N.D.	N.D.	N.D.
30	N.D.	N.D.	N.D.	N.D.	N.D.

N.D. – Not Determined

The findings, summarised in Table 5.6, suggest that inhibitory concentration of acetic acid exists within the range of 1-10 $mg.L^{-1}$ for the bacterium. This is consistent with the findings of Aston et al. (2009) for a similar chemolithotrophic acidophile (*At. caldus*) shown in Table 5.8 . Although the addition of ≥ 10 $mg.L^{-1}$ acetic acid inhibited microbial growth, biological oxidation was still present in these cultures, albeit at a much lower rate. The residual ferrous iron content present in cultures spiked with 10 $mg.L^{-1}$ and 30 $mg.L^{-1}$ acetic acid were substantially lower than those obtained for the abiotic control. After 98 hours of incubation, 23.5% of the ferrous iron had been oxidized in cultures spiked with 10 $mg.L^{-1}$ acetic acid and 20.5% in the cultures spiked with 30 $mg.L^{-1}$ acetic acid compared to the 8.4% of the ferrous iron oxidized in the abiotic control.

5.1.3.3 Effect of pyruvic acid on *L. ferriphilum* HT

In addition to glycolic acid and acetic acid, the effects of pyruvic acid on the biokinetic performance of *L .ferriphilum* HT was also assessed, at 45°C, pH 1.5, by spiking *L. ferriphilum* cultures with 1 mg.L^{-1} or 10 mg.L^{-1} pyruvic acid. The effect of pyruvic acid was assessed on a separate 24-well MWP to that of glycolic acid (section 5.1.3.1) and acetic acid (section 5.1.3.2) and therefore did not share the same positive and abiotic control as the other organic acids investigated. The data is presented below in Figure 5.5 and the biokinetic rates determined from the resultant biokinetic rates presented in Table 5.7.

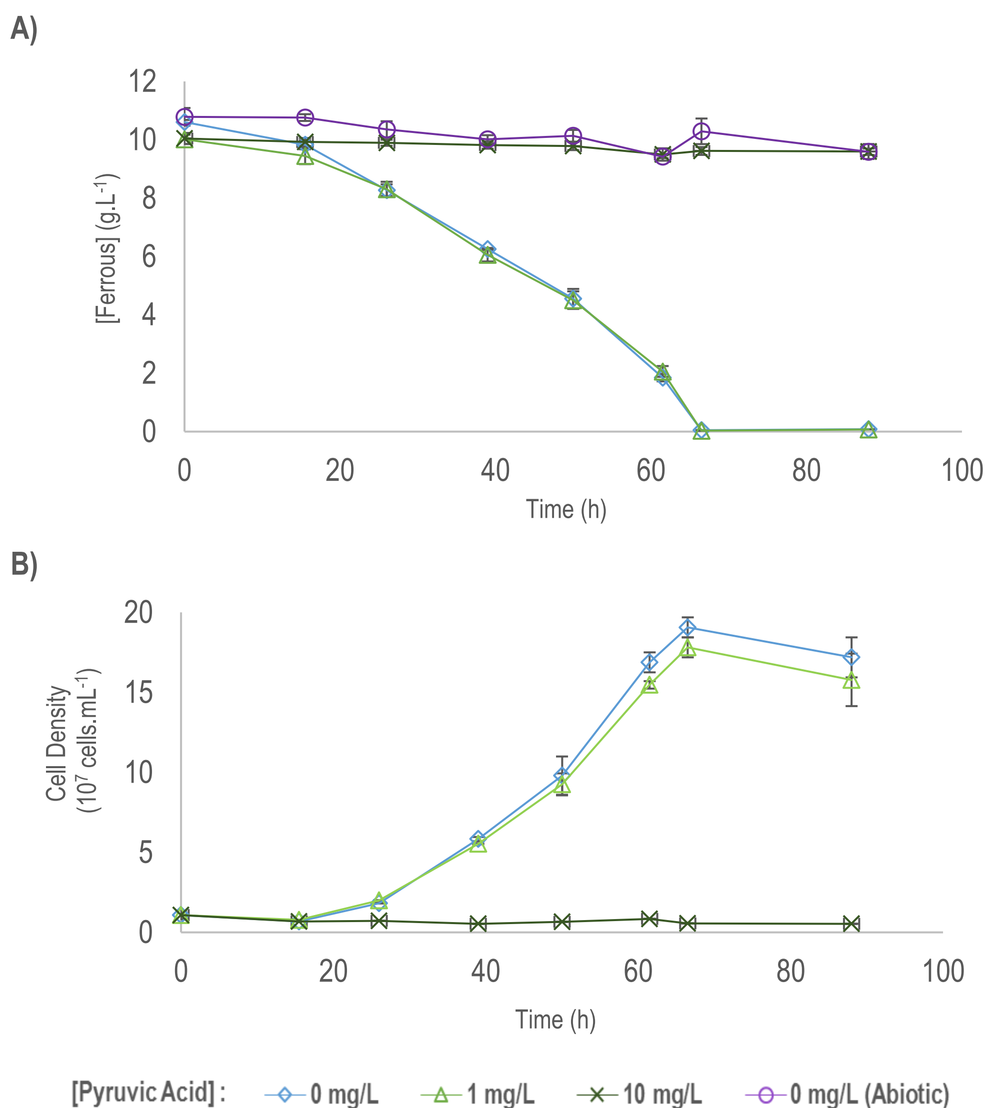

Figure 5.5: Effect of pyruvic acid on A) ferrous iron oxidation activity and B) microbial growth of *L. ferriphilum* HT at 45°C, pH 1.5

Table 5.7: Effect of pyruvic acid on the biokinetic performance of *L .ferriphilum* HT

[Pyruvic acid] (mg.L^{-1})	*Lag time (h)*	*Yield coefficient (cells / μmol Fe^{2+})*	*$-rFe^{2+}$ (mg.L^{-1}.h^{-1})*	*qFe^{2+} (nmol Fe^{2+}.cell^{-1}.h^{-1})*	*μ_{max} (h^{-1})*
0	19.3	0.388	225.8	3.70	0.063
1	17.5	0.344	219.1	4.07	0.064
10	N.D.	N.D.	N.D.	N.D.	N.D.

N.D. – Not Determined

The addition of 1 mg.L^{-1} pyruvic acid did not appear to have a substantial inhibitory effect on the biokinetic performance of the bacterium. In contrast to the findings obtained with 1 mg.L^{-1} glycolic acid or acetic acid; a substantial increase in the average $-r_{Fe}{}^{2+}$ and $q_{Fe}{}^{2+}$ was not observed in cultures spiked with 1 mg.L^{-1} pyruvic acid. Negligible microbial growth was observed in the presence of 10mg.L^{-1} pyruvic acid which is consistent with the findings of Aston et al. (2009) and Borichewski (1966) shown in Table 5.8. Although biological oxidation was observed in the all *L. ferriphilum* cultures spiked with ≤ 30 mg.L^{-1} glycolic acid or acetic acid, negligible biological oxidation was observed in the growth-inhibited cultures spiked with 10 mg.L^{-1} pyruvic acid. After 66.5 hours of incubation, > 99% ferrous iron oxidation was achieved in cultures containing 0-1 mg.L^{-1} pyruvic acid and 4.3% ferrous iron was oxidized in cultures spiked with 10 mg.L^{-1} pyruvic acid compared to the 4.6% oxidation observed in the abiotic control.

5.1.3.4 Comparing the inhibitory effects of glycolic acid, acetic acid and pyruvic acid on *L. ferriphilum* HT

The inhibitory effects of glycolic acid, acetic acid and pyruvic acid were compared against one another as well as against the inhibitory effects of these organic acids previously reported in literature on *L. ferriphilum* and similar chemolithotrophic acidophiles.

The findings shown in Figure 5.6 suggest that the addition of 1 mg.L^{-1} glycolic acid, acetic acid or pyruvic acid had no substantial detrimental effect on the biokinetic performance of *L .ferriphilum* HT while complete inhibition was observed in cultures spiked with > 10 mg.L^{-1} of any organic acid investigated. Glycolic acid exhibited the weakest inhibitory effect on *L. ferriphilum* HT. Cultures spiked with 10 mg.L^{-1} glycolic acid grew and performed at least as well as the positive control, whereas no growth was observed in the cultures spiked with 10 mg.L^{-1} acetic acid or pyruvic acid. Furthermore, the inhibitory concentrations determined for glycolic acid, acetic acid and pyruvic acid were consistent with those reported in literature for *L. ferriphilum* and similar chemolithotrophic bacteria (Table 5.8). By comparing the effects of the organic acids on a molar basis, the findings shown in Figure 5.6 suggest that pyruvic acid may have had the most potent inhibitory effect on the bacterium. Complete inhibition of *L. ferriphilum* was observed in cultures spiked with at least 113.6 μM pyruvic acid, whereas the same level of inhibition was observed with 166.5 μM acetic acid and 394.5 μM glycolic acid.

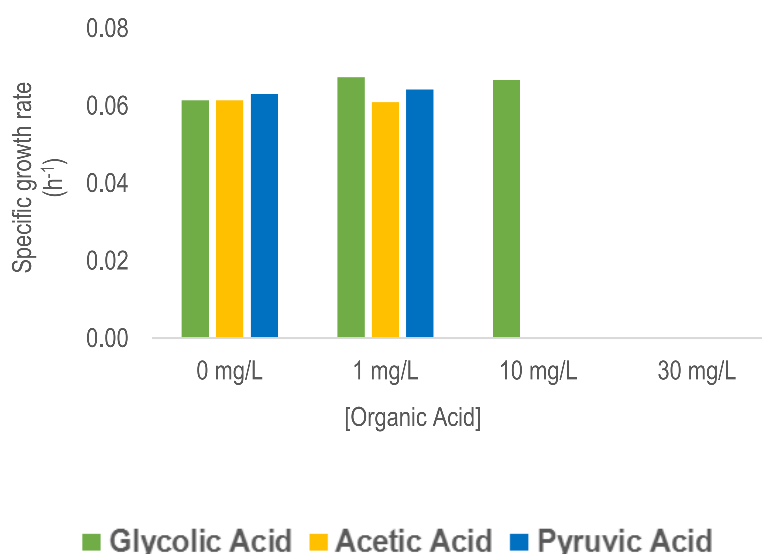

Figure 5.6: Effects of organic acids on the specific growth rate of *L. ferriphilum* HT at 45°C

Alexander *et al*. (1987) postulated that the toxicity of organic acids relates to the number of charged functional groups and dissociation constant of the acid, as well as the pH of the surrounding medium. Of the three organic acids investigated in this experiment, pyruvic acid exhibits the lowest dissociation constant (pKa 2.45) and acetic acid the highest (pKa 4.75). The experiment was conducted at pH 1.5 which is substantially lower than the pKa range of the select organic acids, resulting in these being primarily in their protonated form. Glycolic acid has a pKa of 3.83, making it a stronger acid than acetic acid (Perrin *et al*. 1981), however acetic acid appeared to have a greater inhibitory effect on *L. ferriphilum*. Differences in molecular weight may account for this as acetic acid is smaller than glycolic acid and therefore more moles of acetic acid were introduced when the organic acids were added on a mass basis as shown in Table 5.4.

Table 5.8: Inhibitory concentrations of glycolic acid, acetic acid and pyruvic acid on *L. ferriphilum* HT and similar cited autotrophic acidophiles (*Acidithiobacillus spp.*)

Reference	*Microorganism*	*pH*	*[Organic Acid] (mg/L)*	*Partial Inhibition*	*Complete Inhibition*
Current work	*L. ferriphilum* HT	pH 1.5	Glycolic Acid		30 mg.L^{-1}
			Acetic Acid		10 mg.L^{-1}
			Pyruvic Acid		10 mg.L^{-1}
Borichewski, 1966	*At. thiooxidans*	pH 1.0	Pyruvic Acid	~4.0 mg.L^{-1}	57 mg.L^{-1}
Aston et al., 2009	*At. caldus*	pH 2.5	Pyruvic Acid	3.5 mg.L^{-1}	
		pH 2.5	Acetic Acid	6.0 mg.L^{-1}	
Nancucheo and Johnson, 2009	*L. ferriphilum*	pH 1.7	Glycolic Acid	~7.6 mg.L^{-1}	38 mg.L^{-1}

In conclusion, these organic acids investigated appeared to have a far greater inhibitory effect on *L. ferriphilum* growth when compared to yeast extract or archaeal spent culture filtrate. Complete inhibition of *L. ferriphilum* HT was observed in cultures spiked with 30 mg.L^{-1} glycolic acid and 10 mg.L^{-1} acetic or pyruvic acid. By comparison, the same level of inhibition was observed in *L. ferriphilum* cultures

supplemented with 25% archaeal spent culture filtrate (0.079 $g.L^{-1}$ DOC) or spiked with 0.5 $g.L^{-1}$ yeast extract. The physicochemical environment at which biomining processes takes place may also contribute to the inhibitory effect of these compounds. Under such acidic conditions, weak organic acids exist primarily in the more toxic, fully protonated form. These findings highlight the inhibitory effect that dissolved organic carbon may have on autotrophic acidophiles, namely *L. ferriphilum* HT, and how the inhibitory effect is influenced by the composition of the DOC present in the media under the highly acidic conditions associated with biomining processes.

5.2 Effect of organics on the microbial growth and ferrous iron oxidation activity of *Ac. cupricumulans* JTC3

5.2.1 Effect of yeast extract on *Ac. cupricumulans* JTC3

Ac. cupricumulans JTC3's dependency on an organic substrate was investigated by monitoring microbial growth and ferrous iron oxidation activity in cultures supplemented with varying concentrations of a commonly-used organic substrate (i.e. yeast extract). The data resulting is presented in Figure 5.7. The findings shown in Figure 5.7 suggests that the archaeon is dependent on an organic substrate as negligible growth and activity was observed in the absence of yeast extract which is consistent with the findings of Hawkes *et al.* (2006). Moreover, the availability of the organic substrate appeared to be the growth-limiting factor as μ_{max} increased with increasing yeast extract content (within the 0.1-0.5 $g.L^{-1}$ range) as shown in Table 5.9 which is consistent with the optimal yeast extract concentration determined for this microorganism by Rautenbach (2007).

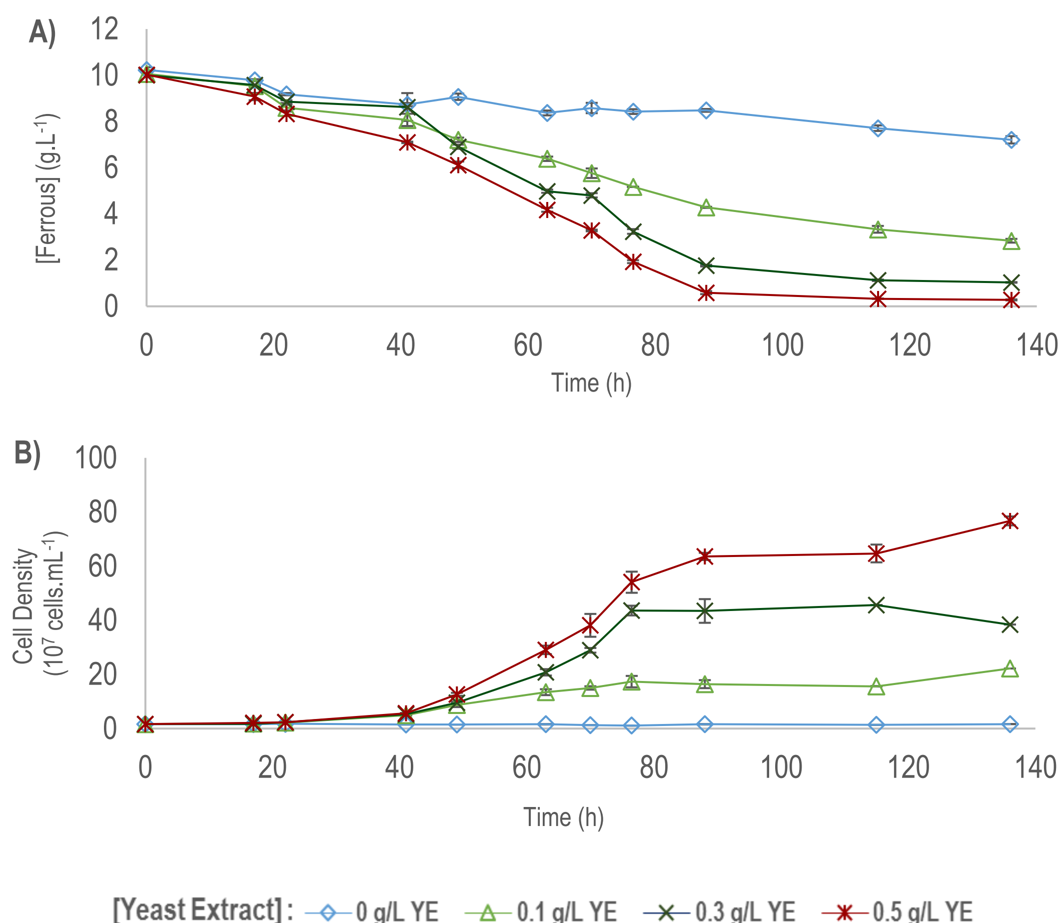

Figure 5.7: Effect of yeast extract on of A) ferrous oxidation activity and B) microbial growth of *Ac. cupricumulans* JTC3

As yeast extract content within the cultures increased, the $q_{Fe}{}^2$ appeared to decrease despite the increase in μ_{max}. The decoupling of the ferrous iron oxidation activity from microbial growth suggests that the microbe's dependency on ferrous iron as an energy source decreased as the organic substrate become more readily available. However, the increased availability of yeast extract did not appear hinder the microorganism's ability to scavenge ferrous iron as the lowest residual ferrous iron concentration was obtained by the cultures containing 0.5 $g.L^{-1}$ yeast extract. After 136 hours of incubation, 96.7% of the ferrous iron had been oxidized compared to the 88.7% and 66.9% ferrous iron oxidized by cultures containing 0.3 $g.L^{-1}$ and 0.1 $g.L^{-1}$ respectively.

Table 5.9: Effect of yeast extract on the biokinetic performance of *Ac. cupricumulans* JTC3

[Yeast Extract] ($g.L^{-1}$)	*Lag time (h)*	*Yield coefficient (cell / µmol Fe^{2+})*	*$-rFe^{2+}$ ($mg.L^{-1}.h^{-1}$)*	*qFe^{2+} (nmol $Fe^{2+}.cell^{-1}.h^{-1}$)*	*μ_{max} (h^{-1})*
0	N.D.	N.D.	N.D.	N.D.	N.D.
0.1	17.3	0.783	84.1	1.259	0.045
0.3	16.7	2.724	151.7	0.976	0.054
0.5	18.2	2.585	152.3	0.927	0.063

5.2.2 *Ac. cupricumulans* JTC3 growth on *L. ferriphilum* HT spent culture filtrate

In laboratory studies, yeast extract is commonly used as the organic substrate supplement, however the organic profile of yeast extract does not provide an accurate representation of the DOC content that these microorganisms would encounter in a biomining environment. A major source of DOC content present within BIOX® bioreactors and contemporary biomining technologies is postulated to be derived from cell lysates and exuded cell products produced by autotrophic chemolithotrophs (Okibe *et al.* 2003). Therefore, *Ac. cupricumulans* JTC3 was cultivated on *L. ferriphilum* HT spent culture filtrate to determine if sufficient organic carbon was present in the cell lysates and exudates of the chemolithotroph to support growth of the archaeon.

Table 5.10: Organic carbon content in *L. ferriphilum* HT spent culture filtrate determined by chemical oxygen demand (COD)

Filtrate composition	*COD (mg.L^{-1})*
L. ferriphilum HT spent culture filtrate	183.3
ABS media containing ferrous iron concentration equivalent to that in spent culture	(136.5)
Dissolved organic carbon	46.9

The DOC content (Table 5.10) within the *L. ferriphilum* HT spent culture filtrate (46.9 mg.L^{-1}) consisted of the clarified cell lysates and exudates. The DOC content was substantially lower than that determined in *Ac. cupricumulans* JTC3 filtrate (315.1 mg.L^{-1}), however this was expected as archaeal filtrate contained residual yeast extract whereas the *L. ferriphilum* HT culture was not supplemented with an organic carbon source.

Ac. cupricumulans JTC3 Fe^{2+} oxidation activity (Figure 5.8A) and microbial growth (Figure 5.8B) was observed in the yeast extract-supplemented positive control; however, in the absence of yeast extract supplementation, negligible growth and activity was observed in the cultures containing 10% (4.7 mg.L^{-1} DOC), 25% (11.7 mg.L^{-1} DOC) and 50% filtrate (23.4 mg.L^{-1} DOC) filtrate. The findings presented in Figure 5.7 from section 5.2.1 indicate that growth was observed in cultures supplemented with at least 0.1 g.L^{-1} yeast extract, however growth in media containing less than 0.1 g.L^{-1} organic substrate was not determined. Therefore, it is possible that the lack of growth observed in samples supplemented with ≤ 50% spent culture filtrate may be due to a lack of organic carbon available to promote growth of *Ac. cupricumulans*.

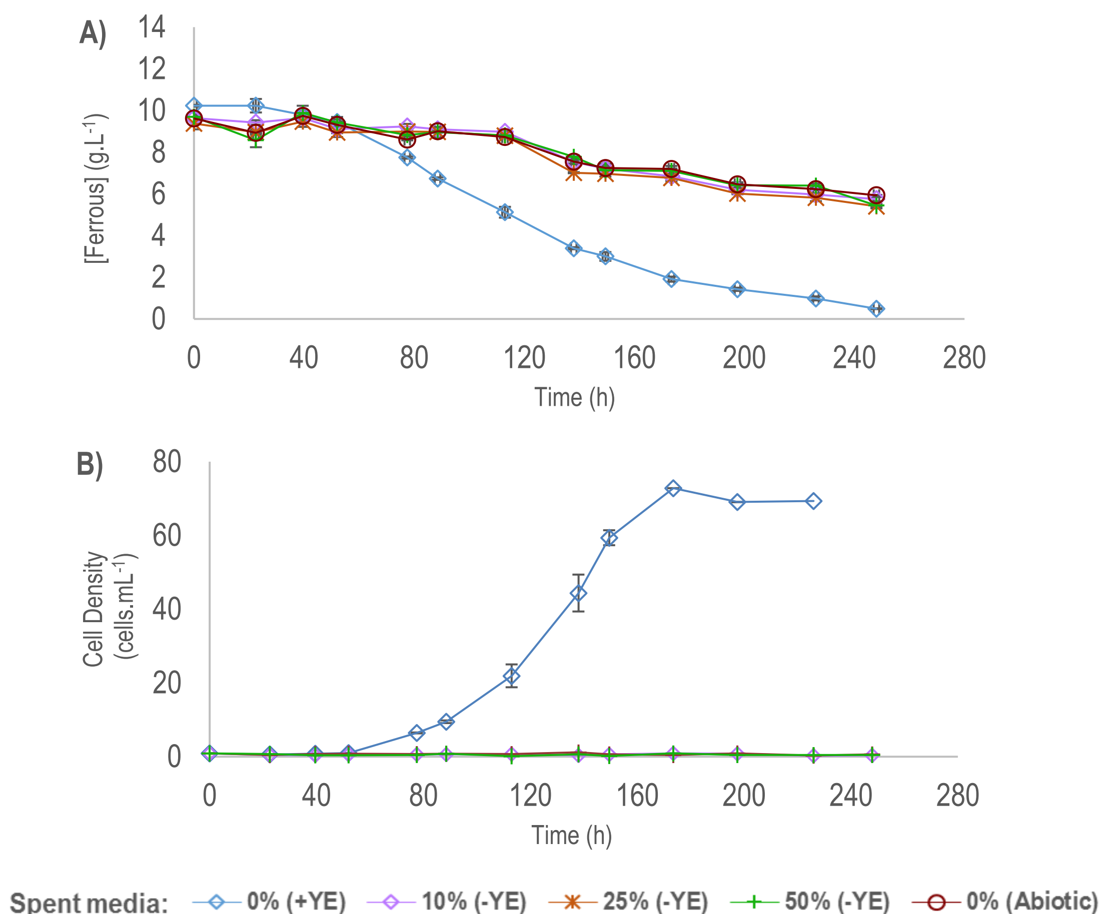

Figure 5.8: Effect of *L .ferriphilum* HT spent culture filtrate on A) the rate of ferrous oxidation and B) microbial growth of *Ac. cupricumulans* JTC3 in the absence of an additional organic supplement

Another possible cause for the lack of archaeal growth observed filtrate-grown cultures may be the presence of inhibitory molecules in the spent culture. To determine whether the lack of growth was solely due to insufficient organic carbon or potentially due to the presence of inhibitory molecules in the filtrate, *Ac. cupricumulans* JTC3 was cultivated in media containing the *L. ferriphilum* filtrate which had also been supplemented with 0.5 $g.L^{-1}$ yeast extract. The data is presented in Figure 5.9 and biokinetic rates shown in Table 5.11.

While no archaeal growth was observed in cultures containing only filtrate as an organic carbon source, microbial activity and growth was observed in cultures containing 10%, and 25% spent culture supplemented with yeast extract, which suggests that insufficient organic carbon was present in the corresponding filtrate content to support growth of the archaeon. However, no growth was observed in cultures containing 50% filtrate and yeast extract which also suggests that constituents within the spent culture had an inhibitory effect on the archaeon at higher concentrations.

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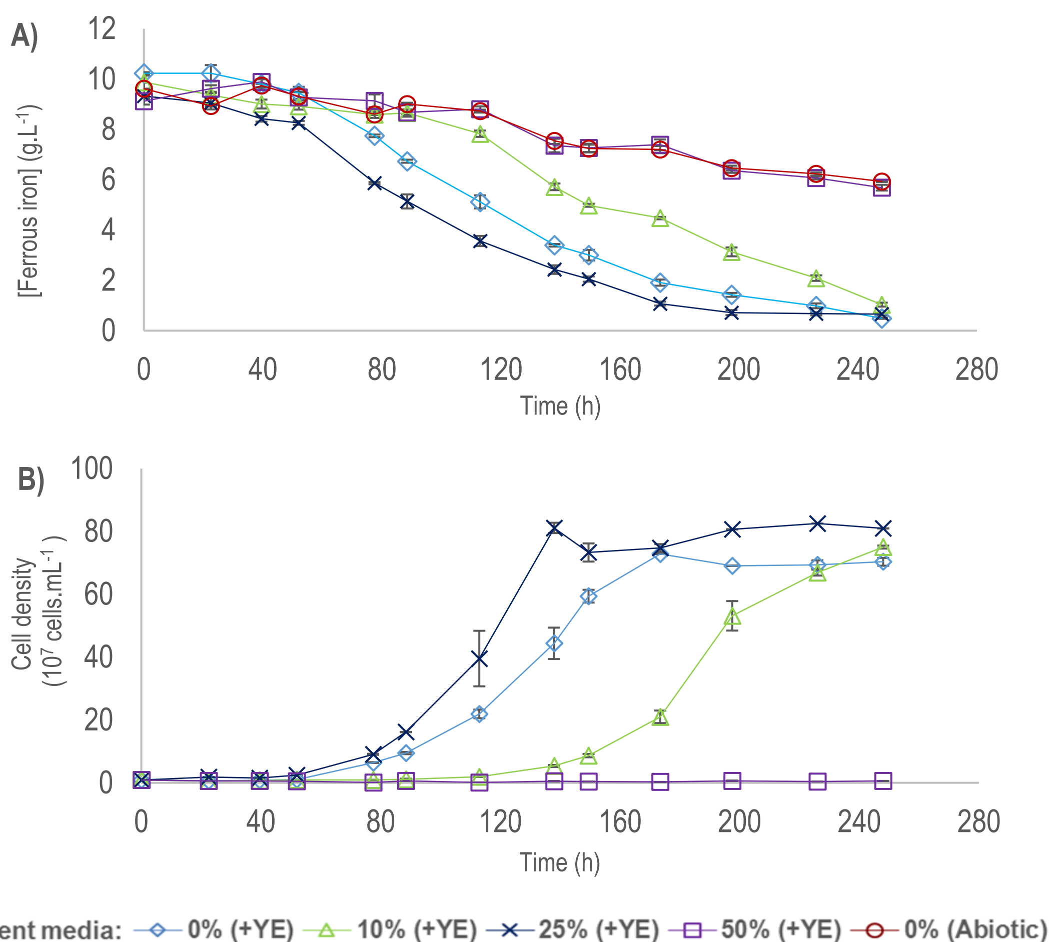

Figure 5.9: Effect of *L .ferriphilum* HT spent culture filtrate on the A) rate of ferrous oxidation and B) microbial growth of *Ac. cupricumulans* JTC3 supplemented with 0.5 g.L^{-1} yeast extract

While 50% filtrate appeared to have an inhibitory effect on the archaeon, the effect of ≤ 25% filtrate on the microorganism showed no definitive trend. The addition of 10% & 25% filtrate (supplemented with yeast extract) had contradictory effects on the biokinetic performance of *Ac. cupricumulans* JTC3. The addition of 25% filtrate showed slightly improve the biokinetic performance of the archaeon, relative to the positive control containing yeast extract only, as illustrated in

Table 5.11 by the shorter lag time and increased growth and oxidation rates. Conversely, the addition of 10% filtrate showed a detrimental effect on the archaeon as shown by the prolonged lag time, decreased μ_{max} and $-r_{Fe}^{2+}$. The increase in biokinetic performance may be attributed to factors such as the added organic substrate or a more positive redox environment as a result of the added ferric ions present in the filtrate. Since the presence of 25% filtrate did not have a negative impact on the archaeon, the prolonged lag time and decrease in microbial growth observed in the presence of 10% filtrate may have been caused by additional stress derived from external factors, such as preparation errors.

Table 5.11: Biokinetic performance of *Ac.cupricumulans* JTC3 grown in spent culture filtrate (supplemented with 0.5 g.L^{-1} yeast extract)

% filtrate + yeast extract (0.5 g.L^{-1})	Lag Time (h)	Yield coefficient *(cell / µmol Fe^{2+})*	*-rFe^{2+} (mg.L^{-1}.h^{-1})*	*qFe^{2+} (nmol Fe^{2+}.cell^{-1}.h^{-1})*	*μ_{max} (h^{-1})*
0	39.7	3.188	71.9	0.26	0.043
10 (4.7 mg.L^{-1})	86.3	3.094	55.4	0.45	0.039
25 (11.7 mg.L^{-1})	28.3	2.752	76.9	0.34	0.046
50 (23.4 mg.L^{-1})	N.D.	N.D.	N.D.	N.D.	N.D.

N.D. – Not Determined

In summary, these findings suggest that the organic carbon content present in *L. ferriphilum* HT filtrate, derived from cell lysate and other exudates, was insufficient to support growth of *Ac. cupricumulans* JTC3 which required additional organic carbon supplementation for growth. However, the lack of growth observed in cultures containing 50% filtrate supplemented with yeast extract suggests that organic constituents within the spent culture filtrate may have had an inhibitory effect on the archaeon.

In context of the BIOX® process, the findings shown in Figure 5.9 may suggest that the cell lysates and exudates produced by *L. ferriphilum* HT, alone, may not be sufficient to sustain the heterotrophic community within the BIOX® consortia and that other dominant members of the microbial consortia, such as *At. caldus* (Nancucheo and Johnson, 2009), may play a greater role in providing an organic substrate for their heterotrophic counterparts. In the bioreactor study performed by Nancucheo and Johnson (2009), the DOC content present in an *L. ferriphilum* culture maintained at pH 1.7 was a mere 7.0 mg.L^{-1} whereas the DOC content in an *Ac. caldus* culture maintained at pH 2.5 was 39 mg.L^{-1}. When the respective cultures were exposed to pH shock (by rapidly dropping pH to pH 1.0), the DOC content in the *L. ferriphilum* culture increased by ~23% whereas the DOC content in the *Ac. caldus* culture increased by ~112%.

5.2.3 Effect of organic acids on *Ac. cupricumulans* JTC3.

Findings from Section 5.2.1 (The effect of yeast extract on *Ac. cupricumulans* JTC3) indicated that an organic substrate is essential for the growth of the archaeon. However, the findings from Section 5.2.2 (Growth of *Ac. cupricumulans* JTC3 on *L. ferriphilum* spent culture filtrate) suggest that certain organic constituents present in the spent culture filtrate may have had an inhibitory effect on the growth and activity of *Ac. cupricumulans* JTC3. The inhibitory effect of organic carbon was investigated using glycolic acid, acetic acid and pyruvic acid, which are known organic constituents in spent culture with the potential to elicit an inhibitory effect on acidophiles (Schanitman and Lundgren, 1965; Borichewski, 1966; Arkesteyn and De Bont, 1980; Nancucheo and Johnson, 2009).

5.2.3.1 Effect of glycolic acid on *Ac. cupricumulans* JTC3.

Glycolic acid's effect on the biokinetic performance of *Ac. cupricumulans* JTC3 was assessed by cultivating the archaeon in media supplemented with 0.5 g.L^{-1} yeast extract and spiked with 1, 10 and 30 mg.L^{-1} glycolic acid. The performance of these cultures was then compared against the 0 mg.L^{-1} glycolic acid positive control which was also cultivated in media supplemented with 0.5 g.L^{-1} yeast

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extract. Microbial activity (Figure 5.10A) and growth (Figure 5.10B) was observed in all glycolic acid-spiked cultures.

The findings shown in**Error! Reference source not found.** Table 5.12 suggest that the presence of 1-30 mg.L^{-1} glycolic acid in the media did not have a noticeable effect on the microorganism's ability to acclimate upon inoculation as shown by the similar lag times obtained by the glycolic acid-spiked cultures. Furthermore, microbial growth and activity remained relatively similar in the presence of 1 mg.L^{-1} and 10 mg.L^{-1} glycolic acid, whereas a slight decrease in growth and activity was observed in the presence of 30 mg.L^{-1} glycolic acid, suggesting partial inhibition. Moreover, a higher level of residual ferrous iron was also determined in the culture spiked with 30 mg.L^{-1} glycolic acid relative to those spiked with 1-10 mg.L^{-1} of the organic acid, suggesting a possible decrease in affinity for ferrous iron. Roughly 97% of the ferrous iron had was oxidized by the archaeon after 174.5 hours of incubation compared to the 94.3% ferrous iron oxidized in the culture spiked with 30 mg.L^{-1} glycolic acid.

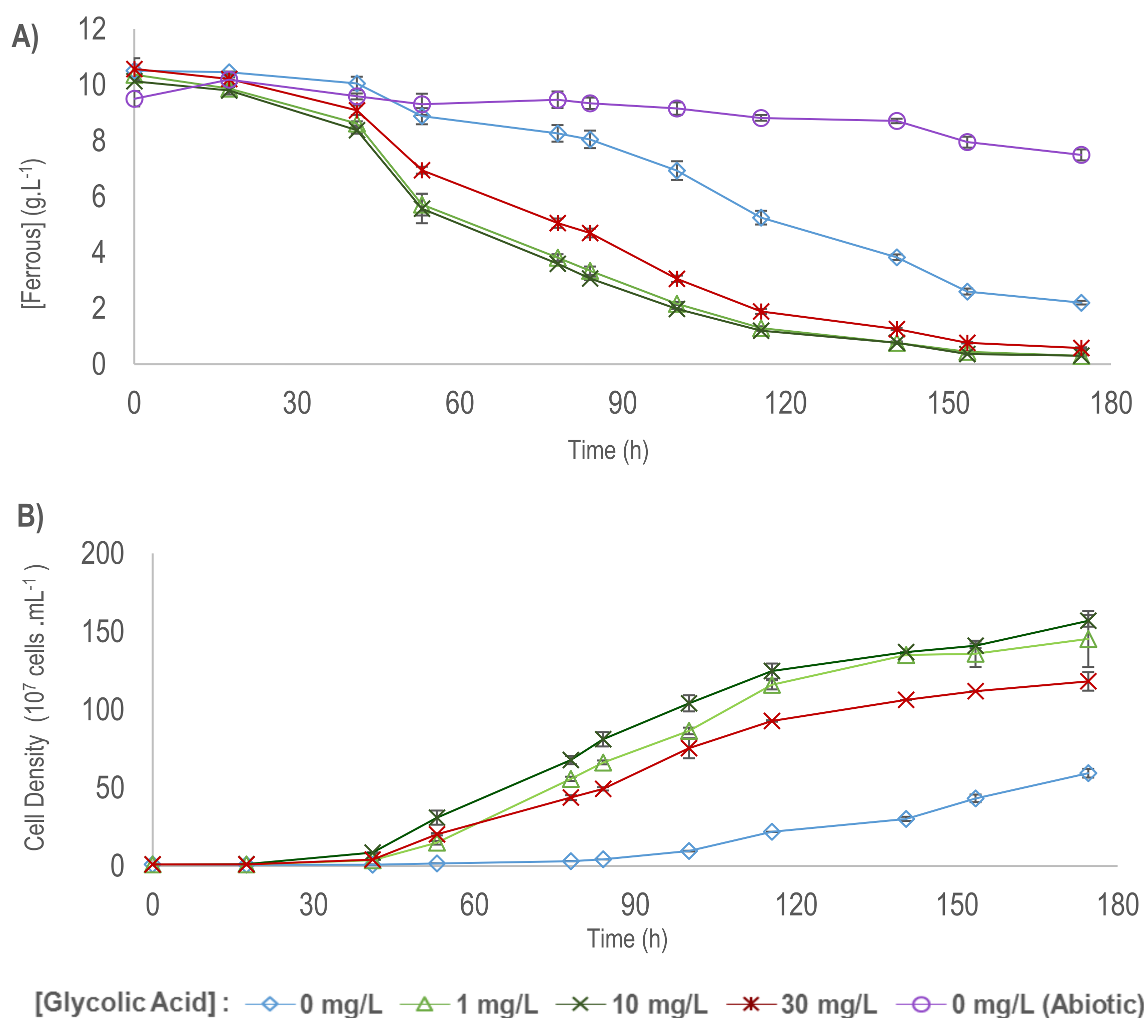

Figure 5.10: Effect of Glycolic acid on A) ferrous oxidation activity and B) microbial growth of *Ac. cupricumulans* JTC3

Table 5.12: Biokinetic performance of *Ac. curpicumulans* JTC3 cultivated with added glycolic acid

[Glycolic Acid] ($mg.L^{-1}$)	*Lag time (h)*	*Yield coefficient (cell / μmol Fe^{2+})*	*$-rFe^{2+}$ ($mg.L^{-1}.h^{-1}$)*	*qFe^{2+} (nmol $Fe^{2+}.cell^{-1}.h^{-1}$)*	*μ_{max} (h^{-1})*
0	55.7	1.204	78.4	0.27	0.052
0*	18.7	2.21	157.6	0.61	0.070
1	19.0	2.230	122.6	0.46	0.067
10	14.9	2.562	123.2	0.36	0.066
30	18.7	1.734	102.2	0.50	0.062

** Ac. cupricumulans JTC3 0 $mg.L^{-1}$ organic acid control sample data taken from Section 5.2.3.3 (Effect of pyruvic acid of Ac. cupricumulans JTC3)*

A relative comparison of the performance of the glycolic acid-spiked cultures could not be drawn against the respective positive control due to the poor performance of the 0 $mg.L^{-1}$ culture. The prolonged lag time and relatively low specific growth and oxidation rates may suggest that the integrity of the culture was compromised. The microbial growth and ferrous iron oxidation data obtained by glycolic acid absent *Ac. cupricumulans* JTC3 culture was compared to those obtained in similar *Ac. cupricumulans* cultures analysed in this study, cultivated under the same conditions (archaeal media supplemented with 0.5 $g.L^{-1}$ yeast extract and cultivated at 45°C at pH 1.3 ±0.1) as shown in Table 5.13. The combined *Ac. cupricumulans* microbial growth and oxidation data shown in

Ac. cupricumulans #1 *Ac. cupricumulans #2* *Ac. cupricumulans #5*
Ac. cupricumulans #3 *Ac. cupricumulans #4*

Figure 5.11 clearly indicate that the biokinetic performance of the 0 $mg.L^{-1}$ glycolic acid positive control differed from the other growth and oxidation curves determined for the archaeon under identical experimental conditions. The glycolic acid positive control was therefore treated as an outlier. To account for the compromised growth and activity displayed by the outlier culture, the biokinetic performance of the cultures spiked with glycolic acid were therefore compared against both the 0 $mg.L^{-1}$ glycolic acid positive control (i.e. *Ac. cupricumulans* #3) and 0 $mg.L^{-1}$ pyruvic acid positive control (i.e. *Ac.cupricumulans* #5) to assess the effect of glycolic acid on the archaeon. By comparison to the 0 $mg.L^{-1}$ shared organic acid positive control (0 $mg.L^{-1}$ pyruvic acid positive control), it is not certain if the addition of 1-10 $mg.L^{-1}$ glycolic acid had any substantial effect on the growth and activity of the archaeon as μ_{max} and $-r_{Fe}^{2+}$ and biomass yields remained relatively constant across the 0-10 $mg.L^{-1}$ range. However, a more noticeable decrease in as μ_{max} and $-r_{Fe}^{2+}$ was determined for cultures spiked with 30 $mg.L^{-1}$ glycolic acid, which may elude to partial inhibition of archaeon by glycolic acid at this concentration.

Table 5.13. Description of *Ac.cupricumulans* JTC3 cultures collectively represented in Figure 5.11

Culture ID as per Fig. 5.11	Nominal pH	[Yeast Extract] ($g.L^{-1}$)	[Organic Acid Added] ($mg.L^{-1}$)	Data Source
Ac cupricumulans #1	1.3	0.5	0	Section 4.3 (Effect of pH)
Ac cupricumulans #2	1.2	0.5	0	Section 5.2.1 (Effect of YE)
Ac cupricumulans #3	1.2	0.5	0	Section 5.2.3.1 (Effect of glycolic acid)
Ac cupricumulans #4	1.1	0.5	0	Section 4.3 (Effect of pH)
Ac cupricumulans #5	1.2	0.5	0	Section 5.2.3.3 (Effect of pyruvic acid)

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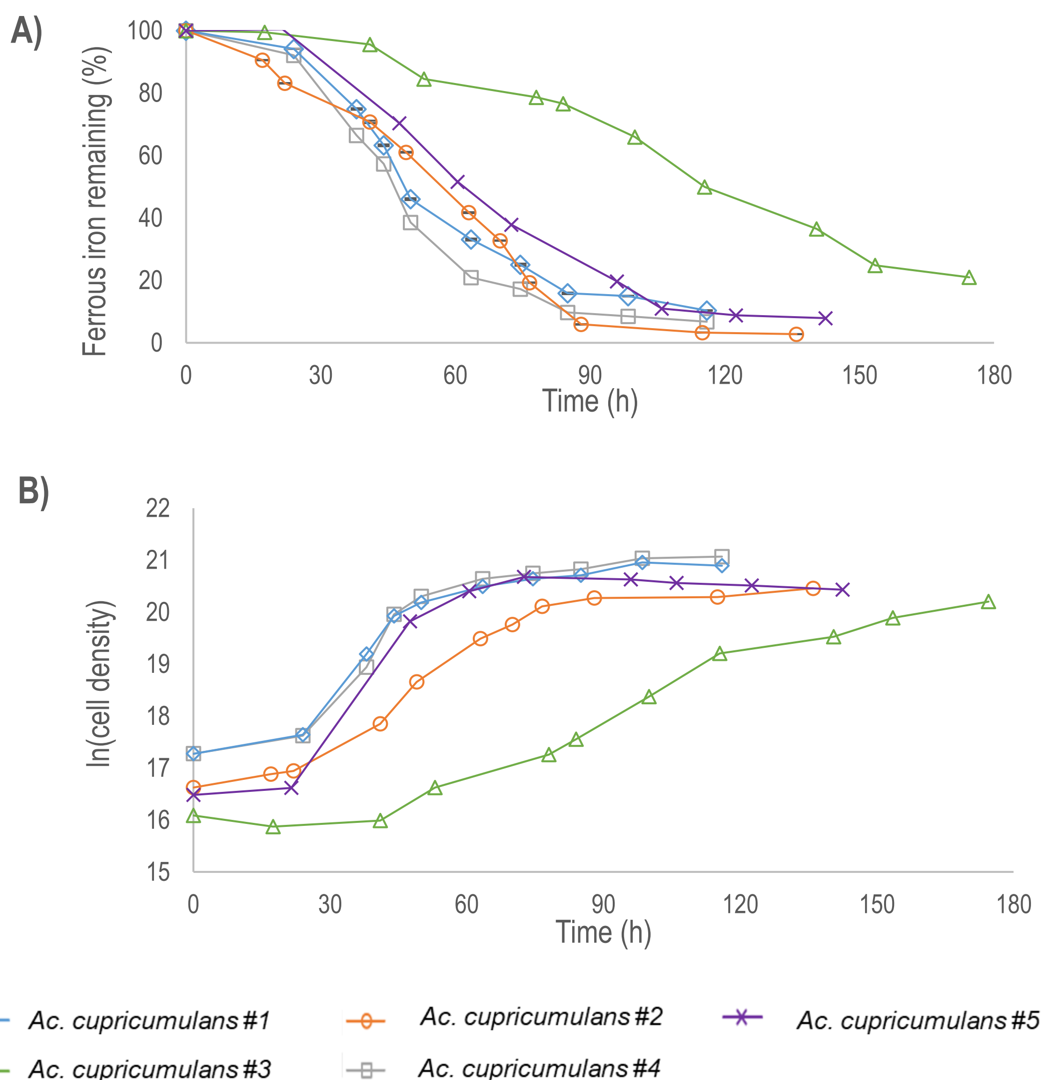

Figure 5.11. Combined A) ferrous iron oxidation curves and B) microbial growth curves for *Ac. cupricumulans* cultures supplemented with 0.5 g.L^{-1} yeast extract cultivated at 45°C.

5.2.3.2 Effect of acetic acid on *Ac. cupricumulans* JTC3

The effects of acetic acid on the biokinetic performance of *Ac. cupricumulans* JTC3 was assessed in parallel with the effects of glycolic acid (on the same 24-well MWP) and so the same abiotic control and 0 mg.L^{-1} positive control were shared across both experiments. Therefore, the positive control used to assess the effect of acetic acid also performed poorly, displaying longer lag times and lower microbial growth and oxidation rates compared to the cultures spiked with 1 mg.L^{-1} & 10 mg.L^{-1} acetic acid (Figure 5.12). The biokinetic rates determined for the cultures spiked with acetic acid (Table 5.14) were therefore compared against the shared 0 mg.L^{-1} organic acid positive control as mentioned above.

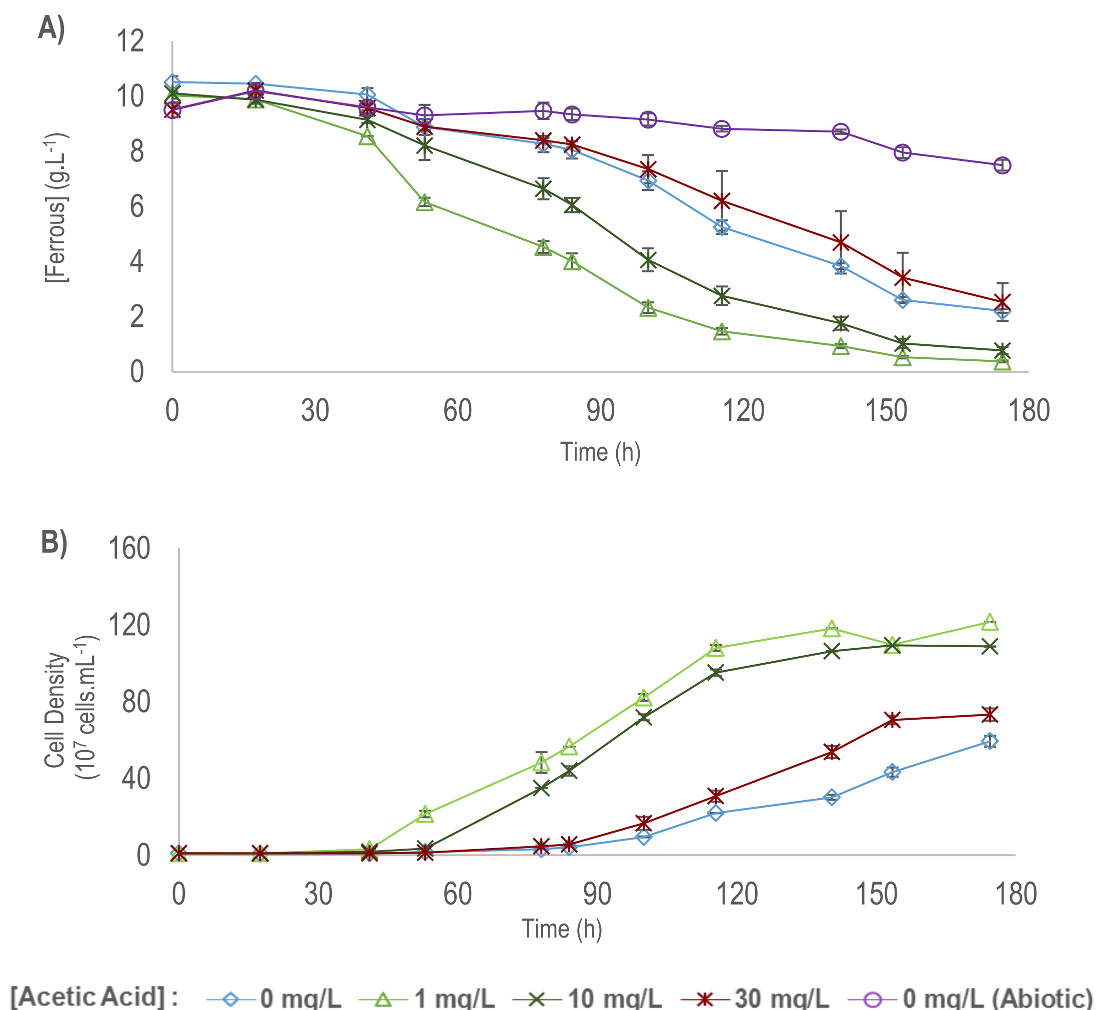

Figure 5.12: Effect of acetic acid on of A) ferrous oxidation activity and B) microbial growth of *Ac. cupricumulans* JTC3

The findings presented in Figure 5.12 indicate that growth and activity was observed in all archaeal cultures spiked acetic acid; however, unlike glycolic acid, acetic acid had a more noticeable inhibitory effect on the biokinetic performance of the archaeon at a lower concentration. The biokinetic rates shown in Table 5.14 indicate a decrease in μ_{max} and q_{Fe}^{2+} as well as an increase in lag time as acetic acid concentration increased from 1 $mg.L^{-1}$ to 30 $mg.L^{-1}$, however it is uncertain how significant the decrease in μ_{max} and q_{Fe}^{2+} may be in the culture spiked with 1 $mg.L^{-1}$ acetic acid.

In addition, the residual ferrous iron concentrations also increased with increasing acetic acid content and were higher than the corresponding concetrations determined in the cultures spiked with glycolic acid. After 174.5 hours of incubation, 96.2% ferrous iron was oxidized in the culture spiked with 1 $mg.L^{-1}$ acetic acid, 92.2% in the culture spiked with 10 $mg.L^{-1}$ acetic acid and 75.2% ferrous iron oxidized for the culture spiked with the 30 $mg.L^{-1}$ acetic acid.

Table 5.14: Biokinetic performance of *Ac. cupricumulans* JTC3 cultivated with added acetic acid

[Acetic Acid] ($mg.L^{-1}$)	Lag time (h)	Yield coefficient (cell / μmol Fe^{2+})	$-rFe^{2+}$ ($mg.L^{-1}.h^{-1}$)	qFe^{2+} (nmol Fe^{2+}. $cell^{-1}.h^{-1}$)	μ_{max} (h^{-1})
0	55.7	1.204	78.4	0.27	0.052
0*	18.7	2.21	157.6	0.61	0.070
1	20.5	1.866	105.5	0.46	0.066
10	37.9	1.702	72.0	0.41	0.059
30	45.8	2.367	73.6	0.18	0.050

** Average biokinetic rates using Ac. cupricumulans cultivated with 0.5 g.L^{-1} yeast extract (section 5.2.1) as well as the organic acid positive controls for section 5.2.3*

5.2.3.3 Effect of pyruvic acid on *Ac. cupricumulans* JTC3

The effect of pyruvic acid on the biokinetic performance of *Ac. cupricumulans* was also assessed, however this experiment was performed independently of the acetic acid and glycolic acid experiments (which were performed in parallel on the same 24-well MWP) and therefore used a different 0 $mg.L^{-1}$ positive control and abiotic control. The experiement was performed at 45°C, pH 1.2 assessing cultures spiked with 1 $mg.L^{-1}$ and 10 $mg.L^{-1}$ pyruvic acid. The data is presented in Figure 5.13.

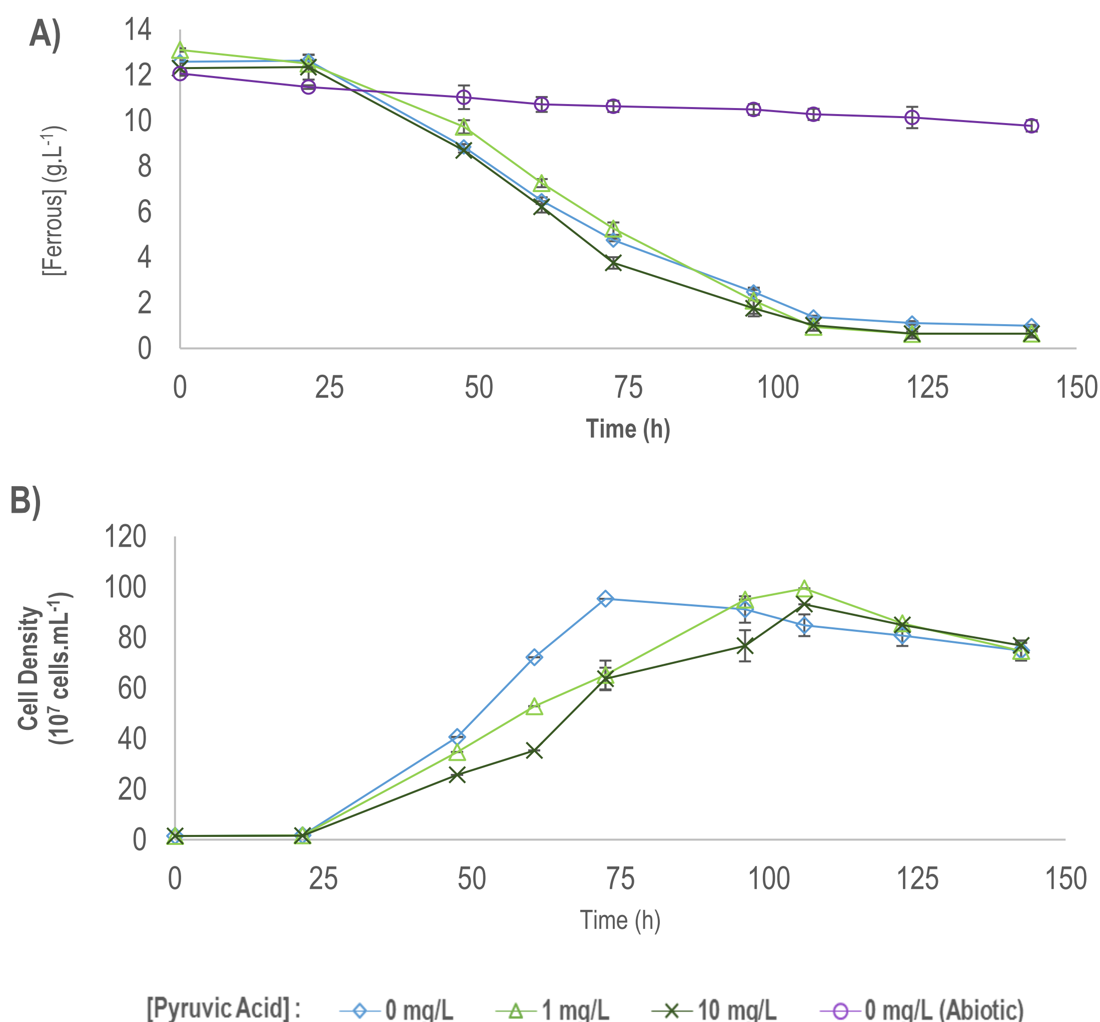

Figure 5.13: Effect of pyruvic acid on the A) ferrous oxidation activity and B) microbial growth of *Ac. cupricumulans* JTC3

Microbial growth was obtained in cultures spiked with 1-10 mg.L^{-1} pyruvic acid as indicated in Figure 5.13 above. Similar lag times time (

Table 5.15) were obtained by cultures containing 0-10 mg.L^{-1} pyruvic acid suggesting that the addition of pyruvic acid did not interfere with the inoculate's ability to acclimatise to the culture conditions. It is uncertain if the addition of 1 mg.L^{-1} pyruvic acid had any inhibitory effect on the archaeon as similar μ_{max} and $-r_{Fe}{}^{2+}$ were obtained by the 1mg.L^{-1} pyruvic acid spiked culture and the positive control, however a noticeable decrease in both μ_{max} and $-r_{Fe}{}^{2+}$, was determined the 10 mg.L^{-1} pyruvic acid spiked culture, suggesting a partial inhibitory effect caused on *Ac. cupricumulans* JTC3.

Table 5.15: Biokinetic performance of *Ac. cupricumulans* JTC3 cultivated with added pyruvic acid

[Pyruvic Acid] (mg.L^{-1})	*Lag time (h)*	*Yield coefficient (cell / µmol Fe^{2+})*	*$-rFe^{2+}$ (mg.L^{-1}.h^{-1})*	*qFe^{2+} (nmol Fe^{2+}. cell^{-1}.h^{-1})*	*μ_{max} (h^{-1})*
0	18.7	2.213	157.6	0.61	0.070
1	18.3	1.561	157.4	0.28	0.067
10	18.6	1.279	142.1	0.27	0.061

5.2.3.4 Comparing the inhibitory effects of glycolic acid, acetic acid and pyruvic acid on *Ac. cupricumulans* JTC3

Figure 5.14 provides a comparative analysis of the effects of glycolic acid, acetic acid and pyruvic acid on the specific growth rate of *Ac. cupricumulans* JTC3 (against the shared 0 mg.L^{-1} organic acid positive control). Although microbial growth was observed under all conditions examined, partial inhibition were at organic acid concentrations at least as low as 10 mg.L^{-1} for acetic acid and pyruvic acid and 30 mg.L^{-1} for glycolic acid. The partial inhibition was characterized by a decrease in μ_{max} , $-r_{Fe}{}^{2+}$ and potentially a decrease in the affinity for ferrous iron by the archaeon. In similar fashion to the inhibitory effect of organic acids determined for *L. ferriphilum* HT (Table 5.8), acetic acid and pyruvic acid appeared to have a more potent inhibitory effect compared to glycolic acid on a mass basis, however, in all cases for the archaeon, these effects were small. On a molar basis, the findings suggest that pyruvic acid may have had the most potent inhibitory effect out of the organic acids investigated as partial inhibition was determined with at least 113.6 µM pyruvic acid compared to the similar level of inhibition achieved with 131.5 µM acetic acid and 394.5 µM glycolic acid.

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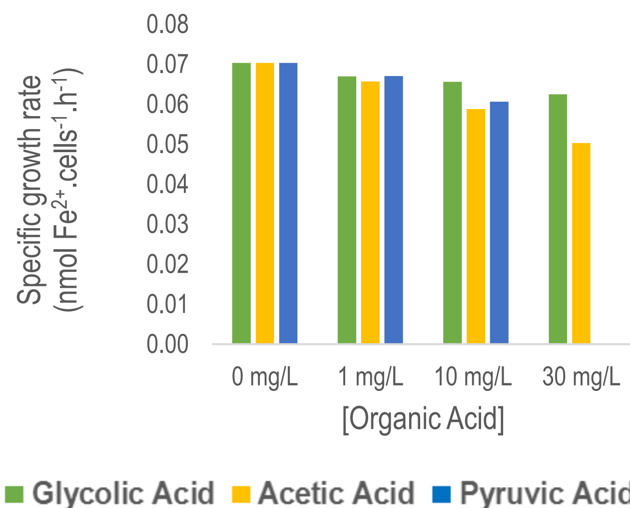

Figure 5.14. Effects of organic acids on specific growth rate of *Ac. cupricumulans* JTC3

Despite the heterotrophic nature of *Ac. cupricumulans* JTC3, these findings suggest that partial inhibition was observed in cultures containing glycolic, acetic, or pyruvic acid. The findings gathered from the effects of DOC's on these two microorganisms emphasize the importance of microbial diversity in biomining processes and the symbiotic relationship shared between organic-sensitive chemolithotrophs and their heterotrophic counterparts within the microbial consortia.

6 Conclusions:

This study focusses on two iron-oxidizing isolates, *Leptospirillum ferriphilum* HT and *Acidiplasma cupricumulans* JTC3, which form part of the mixed-microbial consortia responsible for mediating biomining processes, particularly tank-based technologies operating under moderately thermophilic conditions (around 40-45°C) such as the BIOX® process. Biological oxidation of ferrous iron plays an important role in biomining processes used for the extraction of metals from mineral sulfides as it allows for the regeneration of the sulfidic mineral oxidant, ferric iron. Although *L. ferriphilum* was recognized as the dominant iron-oxidizer in tank-based bio-oxidation processes over a long period, increasingly the prevalence of iron-oxidizing heterotrophic archaea, including *Ac. cupricumulans* JTC3 has also been observed in both lab-scale and especially in commercial tank-based bioreactors operating above 40°C. An increased relative abundance of heterotrophic archaea and decreased abundance of *L. ferriphilum* suggests that a compositional shift of the microbial consortium has occurred particularly within the commercial-scale bioreactors; however, the driving force behind the compositional shift remains uncertain. Changes in the physicochemical conditions, such as pH and temperature within the BIOX® operating window were thought to be a major contributors to the change in microbial dynamics as well as the presence of dissolved organic carbon, a potential inhibitor of *L. ferriphilum* and necessary carbon source for *Ac. cupricumulans*, which may have also influenced the relative abundance of these two microorganisms within the consortia.

6.1 Effect of pH on the growth and performance of *L. ferriphilum* HT and *Ac. cupricumulans* JTC3

L. ferriphilum HT is an extreme acidophile capable of maintaining growth across a pH range of 0.7-1.7 at a near-optimal temperature of 40°C (lower temperature limit of the BIOX® operating window) with optimal performance achieved within the pH 1.1-1.5 range. However, the bacterium's ability to withstand highly acidic conditions (pH ≤ 1.1) decreased at an elevated temperature of 45°C (upper temperature limit of the BIOX® operating window). At 45°C, growth of *L. ferriphilum* HT appeared to be sub-optimal, characterised by longer lag times and substantially lower μ_{max}, $-r_{Fe}{}^{2+}$ and $q_{Fe}{}^{2+}$ compared to those achieved at 40°C. Furthermore, the increase in lag times and decrease in became more pronounced with decreasing pH, suggesting that the heat and acid stress may have had a cumulative effect on the performance of the bacterium which was most evident at pH ≤ 1.1, with negligible growth and activity observed at pH 0.7.

Conversely, the highest μ_{max} , $-r_{Fe}{}^{2+}$ and $q_{Fe}{}^{2+}$ were determined for at pH 0.9 for *Ac. cupricumulans* JTC3, indicating that, at 45°C, the archaeon was better-suited to the highly acidic conditions (pH ≤ 1.1) compared to *L .ferriphilum* HT. The archaeon displayed substantially higher μ_{max} across the entire pH 0.7-1.7 range compared to the bacterium, however μ_{max} decreased with increasing pH. The $q_{Fe}{}^{2+}$ determined for bacterium increased with increasing pH whereas the $q_{Fe}{}^{2+}$ determined for the archaeon remained relatively constant across the pH range examined. This led to the bacterium exhibiting a higher $-r_{Fe}{}^{2+}$ at pH 1.7, whereas similar $-r_{Fe}{}^{2+}$ were determined for both microorganisms at pH 1.5 and higher $-r_{Fe}{}^{2+}$ achieved by the archaeon at pH < 1.5.

These findings would suggest that the biokinetic performance of *L. ferriphilum* HT is greatly hindered at 45°C and low pH and that it may be outcompeted by *Ac. cupricumulans* JTC3 at pH < 1.5 if co-cultured with the archaeon. However, it is important to note that the biokinetic experiments in this study were conducted using batch monocultures of *L. ferriphilum* HT and *Ac. cupricumulans* JTC3 and so competition between these two microorganisms is inferred based on the relative inherent growth rates of these species. These may differ from those obtained in a co-culture biokinetic study, should microbial interactions of mutualism, commensalism or ammensalism exist.

In summary, the findings from this study support the postulation that the cumulative effect of heat (temperatures increasing from 40°C to 45°C) and acid stress (pH decreasing from pH 1.5-1.7 to pH 1.1-1.3) at the upper temperature limit of the BIOX® operating window may well provide a major driving force, shaping the microbial consortia from an *L. ferriphilum*-dominant consortium to an archaeal-dominant consortium.

6.2 Presence of dissolved organic carbon and its effect on the growth of *L. ferriphilum* HT and *Ac. cupricumulans* JTC

The current work also focussed on *L. ferriphilum* HT's sensitivity towards dissolved organic carbon sources as well as investigating the role of organic carbon as an essential nutrient and/or inhibitor of *Ac. cupricumulans* JTC3. The findings suggest that the inhibitory effect of DOC's on *L. ferriphilum* HT

was dependent on both the concentration and composition of the DOC present in the medium. Partial inhibition of the bacterium was observed in cultures containing 0.1 $g.L^{-1}$ yeast extract while complete inhibition was observed in cultures containing 0.5 $g.L^{-1}$ yeast extract. In contrast, complete inhibition of the bacterium was observed when cultivated in media containing 25% (v/v) *Ac. cupricumulans* JTC3 spent culture filtrate (0.079 $g.L^{-1}$ DOC), suggesting that the DOC content in the filtrate may have had a greater inhibitory effect on the bacterium compared to yeast extract.

The effects of organic acids that have been identified in chemolithotrophic spent culture, namely: glycolic acid, acetic acid and pyruvic acid, were assumed to be responsible for the potent inhibitory effect observed, hence their effect on *L. ferriphilum* HT was also investigated. The organic acids appeared to have a greater inhibitory effect on the bacterium under experimental conditions, compared to the DOC effects of yeast extract and spent archaeal culture filtrate. Complete inhibition was observed in cultures spiked with individual organic acids at concentrations as low as 30 $mg.L^{-1}$ for glycolic acid and 10 $mg.L^{-1}$ for acetic acid and pyruvic acid. This supports the postulation that organic acids functions as potent organic inhibitors of *L. ferriphilum* and their presence in spent media, albeit at a low concentration, may be sufficient to elicit an inhibitory response on the bacterium.

The relationship between the presence of organic carbon and the biokinetics of *Ac. cupricumulans* JTC3 was also investigated. The findings suggest that *Ac. cupricumulans* JTC3 was dependent on the presence of organic carbon for growth as no growth was observed in cultures without organic substrate supplementation. Microbial growth rate and biomass yields increased with increasing yeast extract concentration within the 0.1-0.5 $g.L^{-1}$ range; however the average $q_{Fe^{2+}}$ rates decreased with increasing yeast extract content (within the 0.1-0.5 $g.L^{-1}$ range) which may elude to an increasing use of the organic substrate as an energy source in place of ferrous iron. No growth was observed in archaeal cultures containing *L. ferriphilum* HT 10-50% filtrate (0.005-0.023 $g.L^{-1}$ DOC) as the organic substrate although growth was observed when cultures containing 10-25% filtrate were supplemented with 0.5 $g.L^{-1}$ yeast extract, suggesting that insufficient organic carbon may have been present in 10-25% filtrate to sustain heterotrophic growth of the archaeon. Conversely, no growth was observed in cultures containing 50% of the bacterial culture filtrate supplemented with 0.5 $g.L^{-1}$ yeast extract which may also suggest that the filtrate contained constituents which could elicit an inhibitory response on the archaeon when cultivated in 50% filtrate (0.023 $g.L^{-1}$ DOC). Although such inhibitory constituents present in the spent culture were not identified, these inhibitory compounds were thought to be organic acids derived from cell lysates and exudates in the filtrate. The inhibitory effects of organic acids on *Ac. cupricumulans* JTC3 was then investigated, once again using glycolic acid, acetic acid and pyruvic acid as the select organic acids. The presence of organic acids had less of an inhibitory effect on the archaeon compared to that observed with *L. ferriphilum* HT. Growth was observed in cultures spiked with up to 30 $mg.L^{-1}$ glycolic acid, 30 $mg.L^{-1}$ acetic acid and 10 $mg.L^{-1}$ pyruvic acid, however partial inhibition was noted by the organic acids at these respective concentrations. *L. ferriphilum* HT's sensitivity to the presence of organic carbon and *Ac.cupricumulans* JTC3's obligatory requirement elude to the symbiotic relationship between autotrophic chemolithotrophs and their heterotrophic counterparts within the BIOX® consortium. Conversely, the growth inhibition of *L. ferriphilum* by the metabolites of *Ac. cupricumulans*,

and potentially vice versa, may demonstrate potential ammensal relationships with impacts on community dynamics reaching beyond competition.

6.3 Relevance and importance of the study

The findings from this study demonstrate how the temperature and pH range of the BIOX® operating window impact on biokinetic profiles of *L. ferriphilum* HT and *Ac. cupricumulans* JTC3. More specifically, these findings illustrate that although *L. ferriphilum* HT is capable of growing at the upper temperature limit of the BIOX® operating window, the elevated temperature appears to have a detrimental effect on the biokinetic performance of the bacterium. Furthermore, the effect of the elevated temperature on the bacterium is exacerbated as pH in the bioreactor decreases (particularly at pH < 1.3). In contrast, these conditions favoured growth of the less-efficient, but faster-growing archaeon, *Ac. cupricumulans* JTC3 which may explain the compositional shift observed in archaeal-dominant tank-based bioreactors.

With regards to the effect of DOC's on these two microorganisms, the findings highlight *L. ferriphilum* HT*'s* sensitivity towards dissolved organic carbon; most notably organic acids and the dissolved organic carbon content present in archaeal filtrate which was capable of inhibiting growth of the bacterium at very low concentrations. The findings also demonstrate *Ac. cupricumulans* JTC3's dependency on an organic carbon substrate for growth. Further, the lack of growth observed on *L. ferriphilum* HT filtrate suggests that the exudate produced by this chemolithotrophic, on its own, was insufficient to sustain growth of *Ac. cupricumulans* JTC3. In context of the BIOX® process and contemporary biomining processes, these findings suggest that *L. ferriphilum* HT cell lysates and exudates are not the primary source of organic content for the heterotrophic community within the microbial consortia and that these microorganisms are also dependent on additional dissolved organic carbon sources derived from other dominant species in the consortia, such the sulfur oxidizing *At. caldus*.

Interestingly, growth inhibition was also observed when *Ac.cupricumulans* JTC3 was cultivated on filtrate supplemented with an organic carbon substrate, indicating that organic compounds present in *L. ferriphilum* HT exudate were capable of partially inhibiting growth of the heterotrophic archaeon at a relatively low concentration and organic acids may have played a role in the inhibitory effects observed with *L. ferriphilum* filtrate. Despite demonstrating a higher tolerance than *L. ferriphilum* HT towards the select organic acids, partial inhibition was observed when *Ac. cupricumulans* JTC3 was grown in the presence of relatively low concentrations of the organic acids (30 $mg.L^{-1}$ glycolic acid and 10 $mg.L^{-1}$ acetic pyruvic acid). The findings gathered from the effects of DOC's on these two microorganisms emphasize the importance of microbial diversity in biomining processes and the symbiotic relationship shared between organic-sensitive chemolithotrophs and their heterotrophic counterparts within the microbial consortia.

6.4 Recommendations

The biokinetic profiles obtained for *L. ferriphilum* HT and *Ac. cupricumulans* JTC3 were obtained using ostensibly pure cultures grown on substrate-supplemented minimal media. This approach provides a useful, reductive approach to determine the effect of temperature, pH and organic inhibitors on the

growth and performance of these microorganisms individually. From this, an initial assessment of the likely dominant species can be determined. Research to further the insights gained should be extended using continuous culture configurations supplied with a sulfide mineral feed (e.g. pyrite) to monitor and compare sulfide mineral oxidation rate of *L. ferriphilum* HT and *Ac. cupricumulans* JTC3 at the lower and upper range of BIOX® operating window, with regards to temperature and pH. Further, the findings presented here give the intrinsic growth and ferrous iron oxidation kinetics of each species and can be used to infer that the relative abundance of these two microbes across the BIOX® operating window should no microbial interactions exist. To explore the interaction between these two iron-oxidizers, co-culturing *L. ferriphilum* HT and *Ac. cupricumulans* JTC3 in continuous culture is required. Integration of these two data sets will enable a more complete understanding of community dynamics and microbial interactions impacting the microbial dynamics between *L. ferriphilum* HT and *Ac. cupricumulans* JTC3 as a function of the BIOX® operating window.

The dominant presence of *L. ferriphilum* in BIOX®, and related processes, has been largely regarded as a key component to a healthy and well-performing mineral-solubilizing consortium. However, the results shown in this dissertation suggest that the presence and performance of iron-oxidizing archaea should not be under-estimated. Despite the archaeon's relatively low specific oxidation rates, *Ac. cupricumulans* JTC3's ability to thrive under higher temperatures and more acidic conditions may provide benefit to the process. Substrate oxidation rates achieved by *Ac. cupricumulans* JTC3 may be significantly lower than that of *L. ferriphilum* HT at the lower temperature limit (40°C), but have been shown to be competitive at 45°C and low pHs, particularly in the range pH 0.9 to 1.3. Operating at the upper temperature limit of the BIOX® operating window is expected to increase in the kinetic reactivity rates of sulfidic mineral oxidation. Further, it may assist in reducing operating expenses, such as lower cooling costs. However, a reliance on heterotrophic archaea only to facilitate this process is expected to introduce a problem surrounding organic carbon availability. In context of CSTR processes such as BIOX®, this problem may be explored by varying the operating windows for the primary and secondary reactors within the CSTR system. The operating window within the primary bioreactors could be controlled to operate primarily at 40°C to promote growth of *L. ferriphilum* and its chemolithotrophic contemporaries, while the operating temperature in the secondary reactors could be allowed to increase to 45°C to reduce costs, improve chemical reactivity rates, promote growth of heterotrophic iron-oxidizing archaea such as *Ac.cupricumulans* JTC3 and enhance process robustness.

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Appendix A:

A.1 Determining theoretical cell mass and carbon content for *Ac. cupricumulans* JTC3

Cell mass was determined using two working stock cultures of Ac. cupricumulans JTC3. The archaeal cultures were cultivated at 45°C in archaeal media supplemented with 0.5 $g.L^{-1}$ yeast extract at a pH of pH 1.2. Cell mass was determined as follows:

$$m_{cell} = \frac{m}{C_x \times V}$$

Where,

m_{cell} = mass per cell

m = total mass of sample

C_x = cell concentration

V = volume of sample

Cell concentration for each sample was determined by direct cell count (as per Chapter 3.3.2) and sample mass was determined gravimetrically. For gravimetric analysis, an aliquot (1.5-2.0 ml) of the working stock culture was transferred to a 2 ml Eppendorf tube of known mass. The cells were pelleted by centrifugation (14 000 xg for 10 min at 22°C). The supernatant was decanted, and an additional aliquot was added into the tube and centrifuged again in order to obtain a larger cell pellet (as previously shown in Figure 3.1). This process was repeated four times in order to obtain a cell pellet of quantifiable mass. The tube containing the cell pellet was then dried in a drying oven and weighed. Determination of mass was performed, in duplicate, for both archaeal working stock cultures.

Table 6.1: Determining mass per cell of *Ac. cupricumulans* JTC3

Working Stock Culture	A		B	
Replicate	1	2	1	2
Empty tube mass	1.1241	1.1242	1.1211	1.1116
Tube+cell pellet mass	1.1245	1.1247	1.1214	1.1119
net (cell pellet) mass	0.0004	0.0005	0.0003	0.0003
Cell concentration	68750000		84375000	
Total sample volume used (ml)	9	10	10	9
Cell mass	6.46465E-13	7.27273E-13	3.556E-13	3.951E-13
Average cell mass	6.86869E-13		3.75309E-13	
	5.31E-13			

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A.2 pH control during batch biokinetic experiments

Culture pH was monitored at each sampling time point and maintained at roughly ± 0.2 of the nominal pH by addition of concentrated sulfuric acid.

A.2.1 pH readings for *L. ferriphilum* HT and *Ac. cupricumulans* JTC3 biokinetic tests investigating the effect of pH.

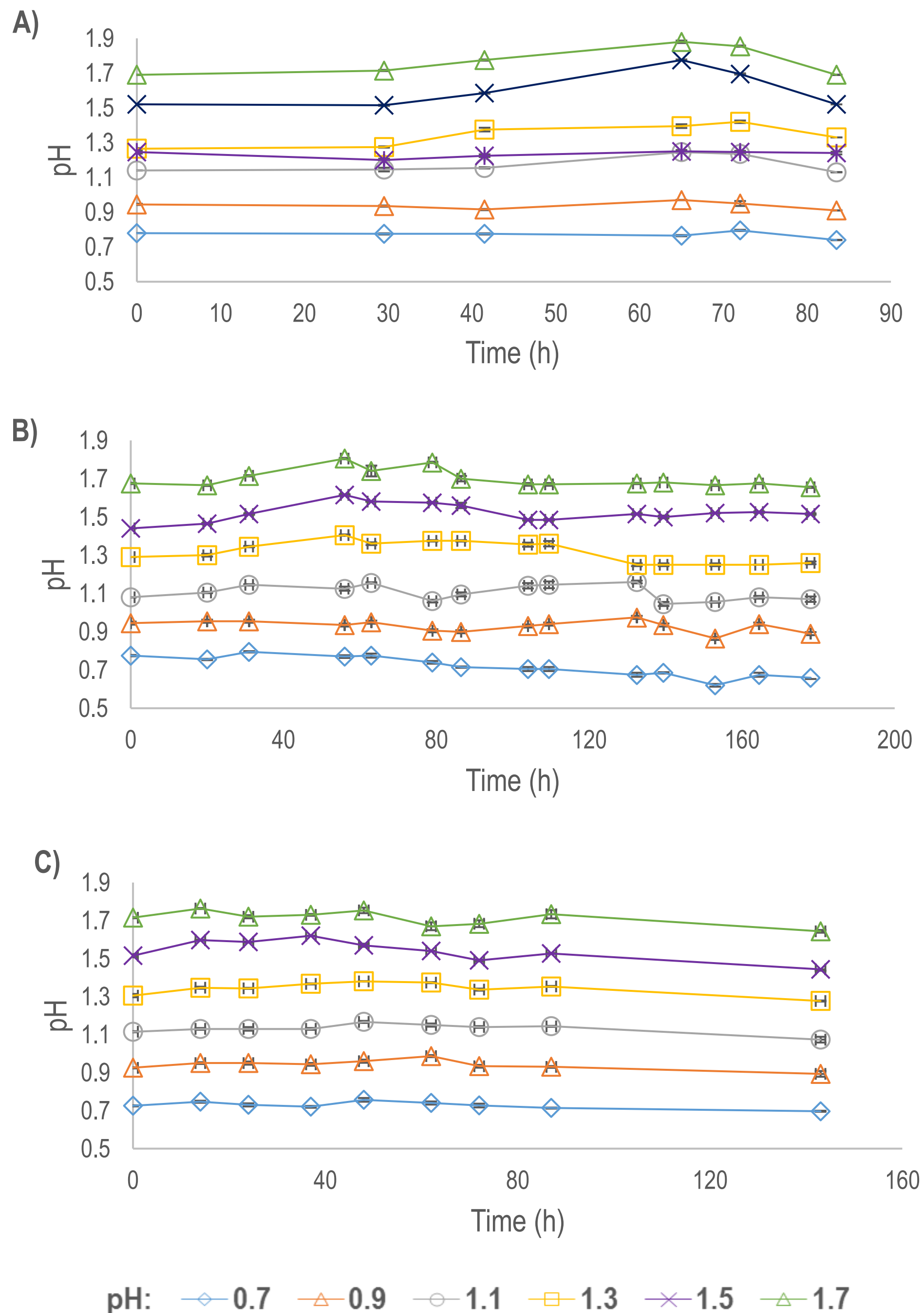

Figure 6.1: pH readings **A)** *L. ferriphilum* HT at 40°C, **B)** *L. ferriphilum* at 45°C and **C)** *Ac. cupricumulans* JTC3 at 45°C

A.2.2 pH readings for *L. ferriphilum* HT biokinetic tests investigating the effect of yeast extract and spent culture filtrate

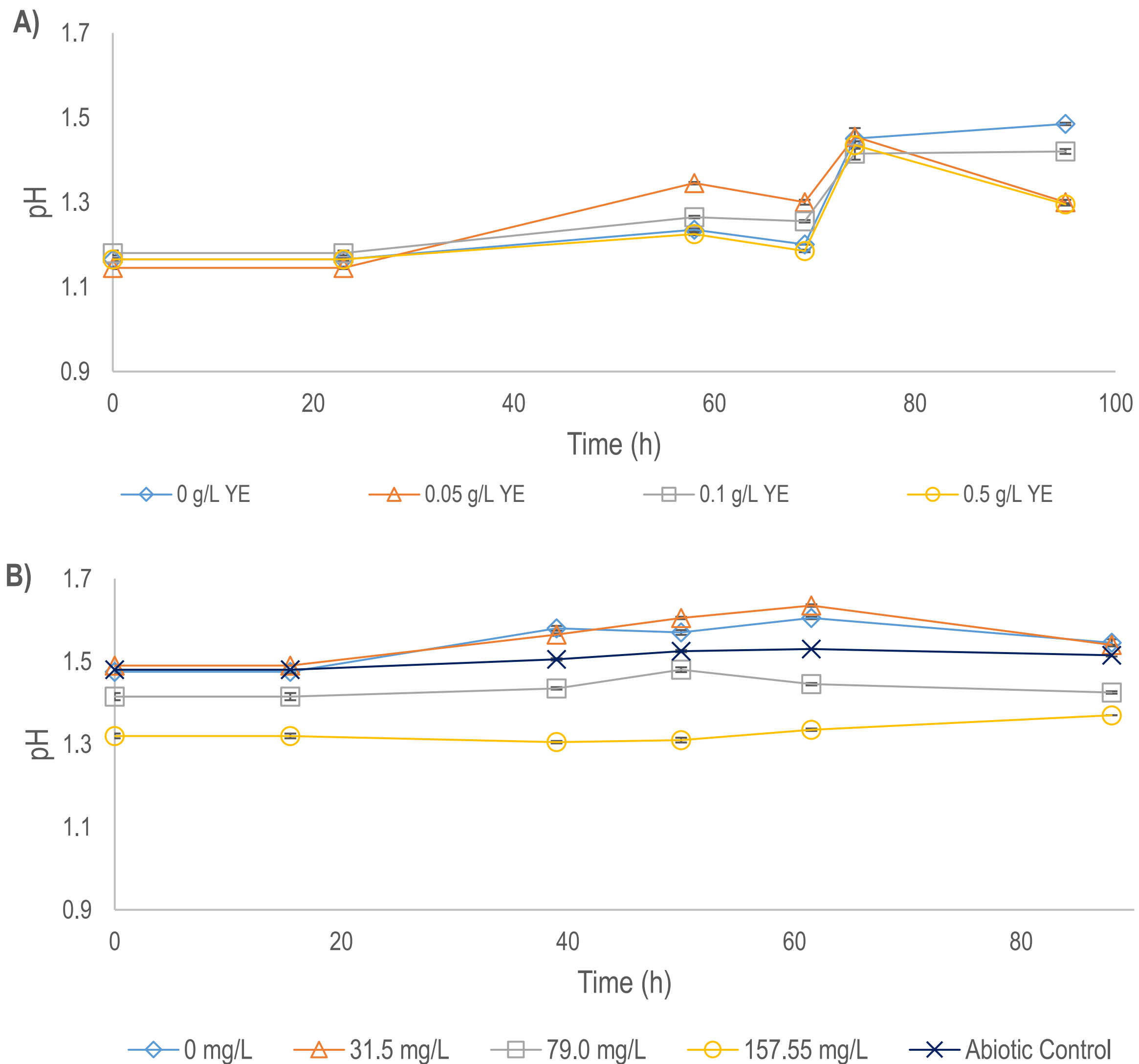

Figure 6.2: pH readings for the *L. ferriphilum* HT biokinetic tests investigating **A)** the effect of yeast extract and **B)** the effect of *Ac. cupricumulans* JTC3 filtrate

A.2.3 pH readings for *L. ferriphilum* HT biokinetic tests investigating the effect of glycolic acid, acetic acid and pyruvic acid.

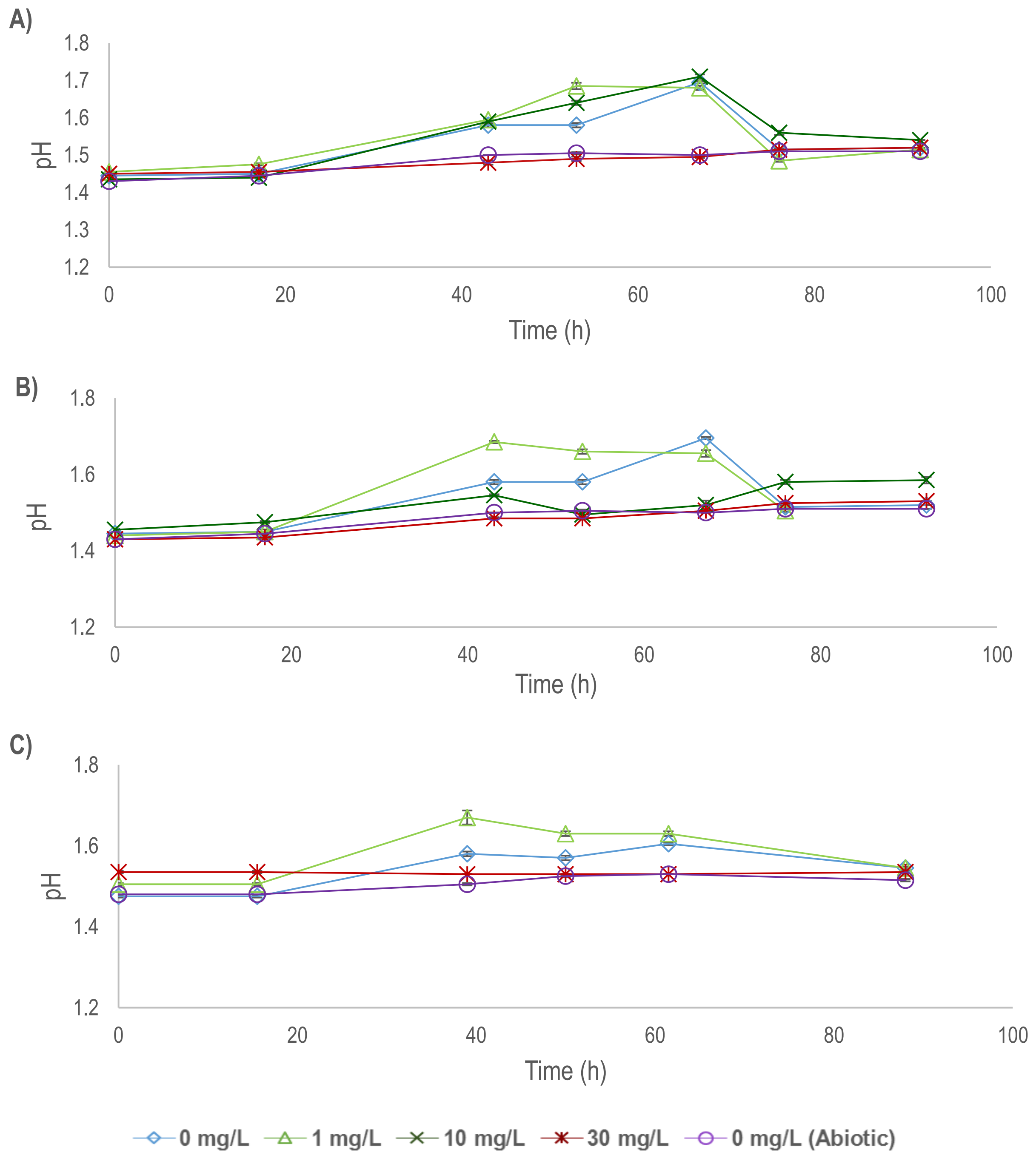

Figure 6.3: pH readings for the *L. ferriphilum* HT biokinetic tests investigating effect of **A)** Glycolic Acid, **B)** Acetic Acid and **C)** Pyruvic Acid

A.2.4 pH readings for *Ac. cupricumulans* JTC3 biokinetic tests investigating the effect of yeast extract and spent culture filtrate

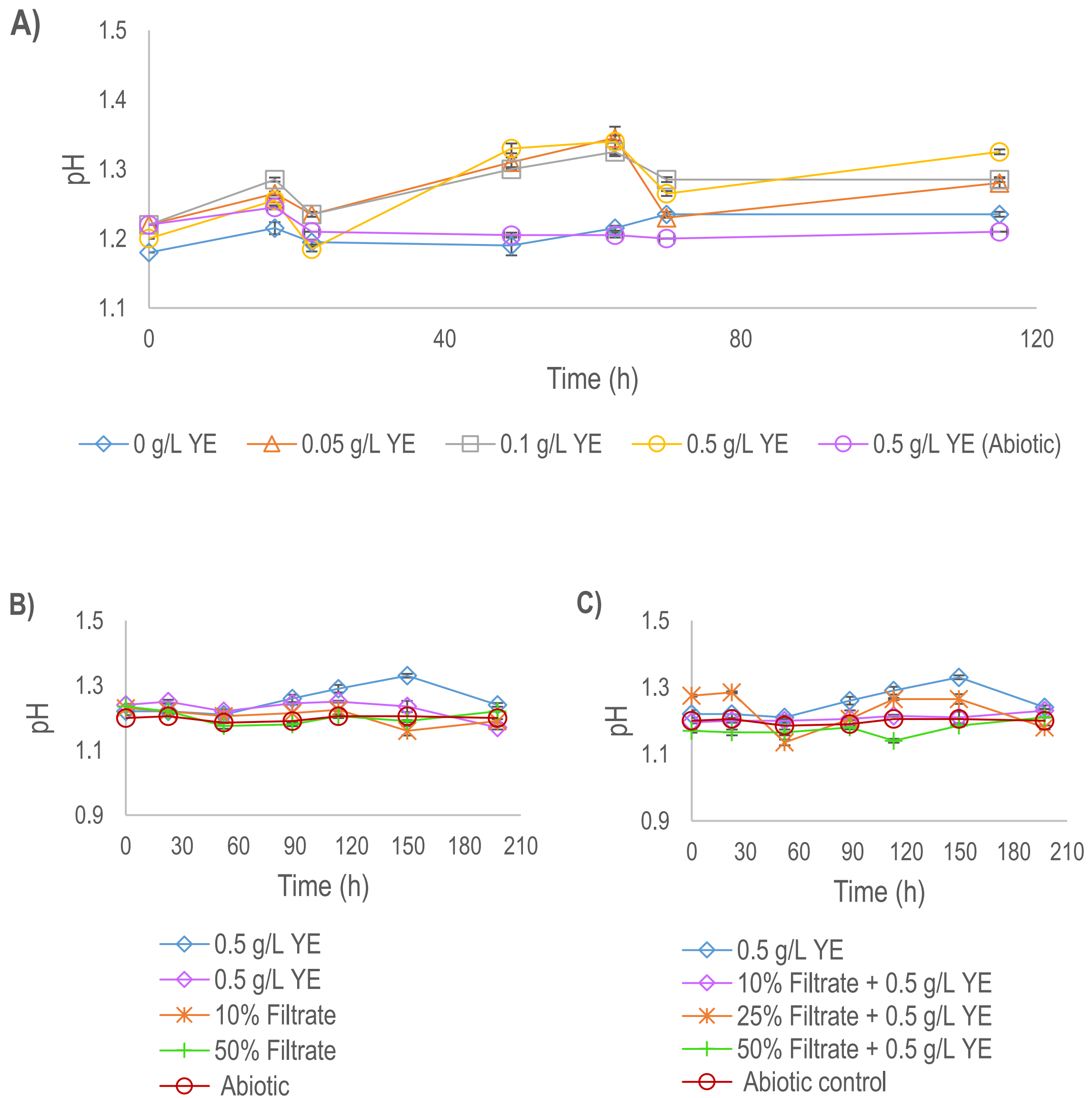

Figure 6.4: pH readings for *Ac. cupricumulans* JTC3 biokinetic tests investigating the effect of A) yeast extract, B) spent culture filtrate and C) yeast extract supplemented spent culture filtrate

A.2.5 pH readings for *Ac. cupricumulans* JTC3 biokinetic tests investigating the effect of glycolic acid, acetic acid and pyruvic acid

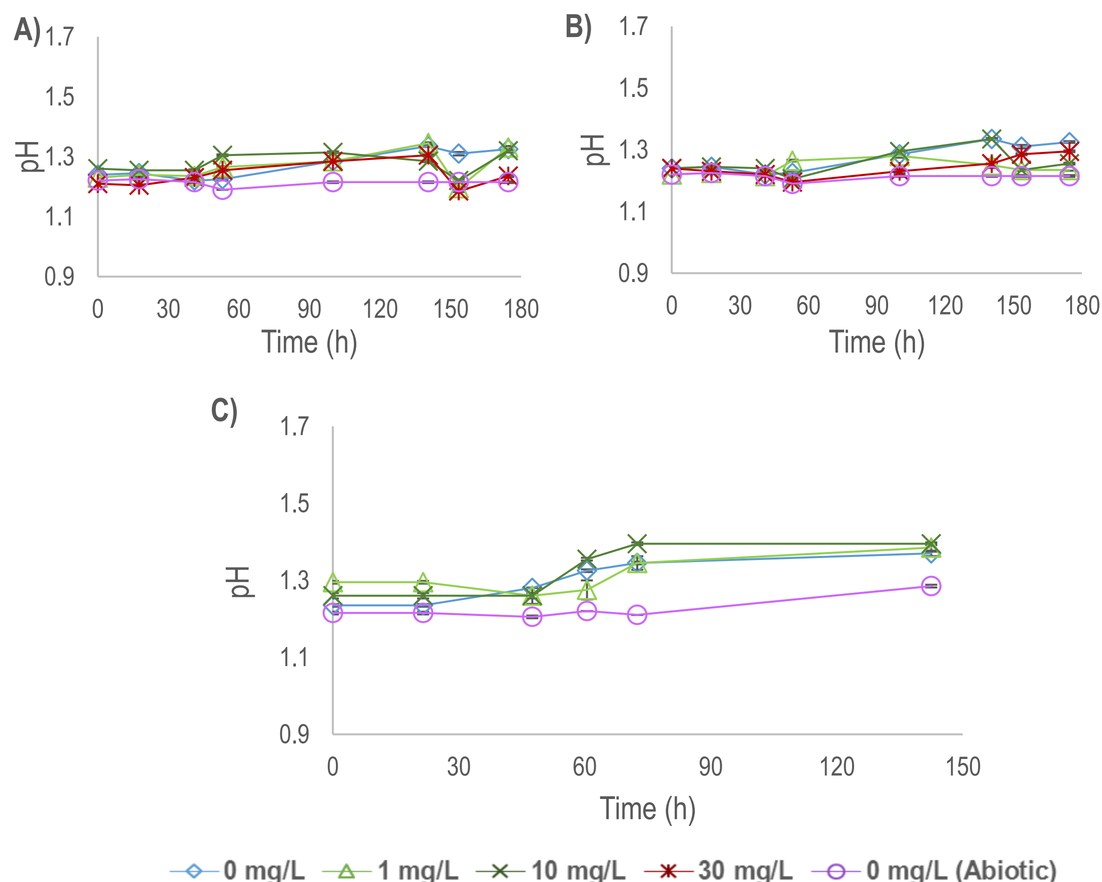

Figure 6.5: pH readings for *Ac. cupricumulans* JTC3 biokinetic tests investigating the effect of **A)** Glycolic Acid, **B)** Acetic Acid and **C)** Pyruvic Acid

A.3 Speciation of *L. ferriphilum* HT and *Ac. cupricumulans* JTC3 working cultures.

A.3.1 Speciation of *L. ferriphilum* HT working stock culture

L. ferriphilum HT speciation conducted on three consecutive sub-cultures and determined by qPCR identification. Relative abundance of *L. ferriphilum* HT within the three working stock cultures varied between 78-84%. In the working stock cultures, A & B, the remaining proportion of microorganisms present in the culture may be attributed to other bacteria (13-14% relative abundance) and, to a lesser extent, archaea (3-4% relative abundance). Conversely, the remaining proportion of microorganisms present in working stock culture C were archaeal species (~22% relative abundance).

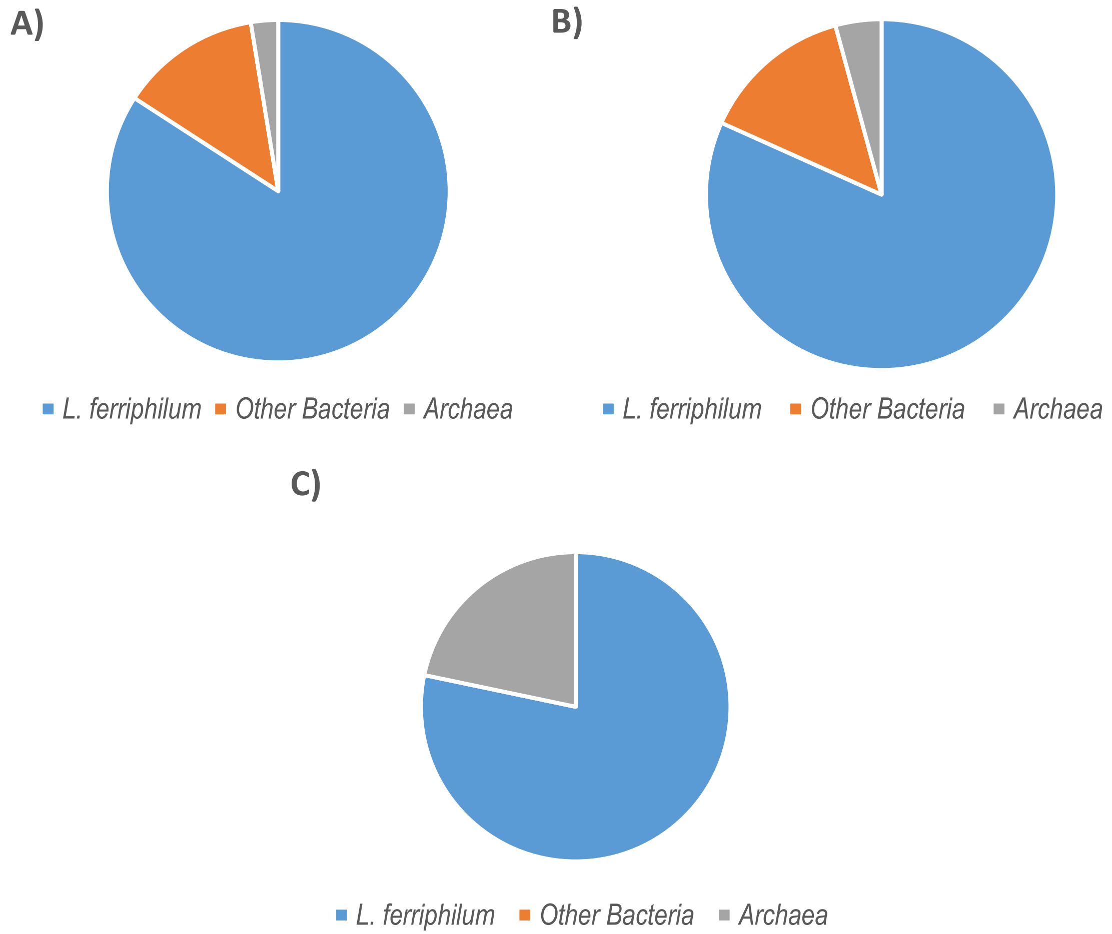

Figure 6.6 qPCR analysis of the *L. ferriphilum* HT working stock culture where **A)**, **B)** and **C)** are consecutive sub-cultures of the working stock

A.3.2 Speciation of *Ac. cupricumulans* JTC3 working stock culture

Ac. cupricumulans JTC3 speciation conducted on three consecutive sub-cultures and determined by qPCR identification. A high degree of purity was determined in all three Ac. cupricumulans JTC3 working stocks examined (> 99.9% relative abundance).

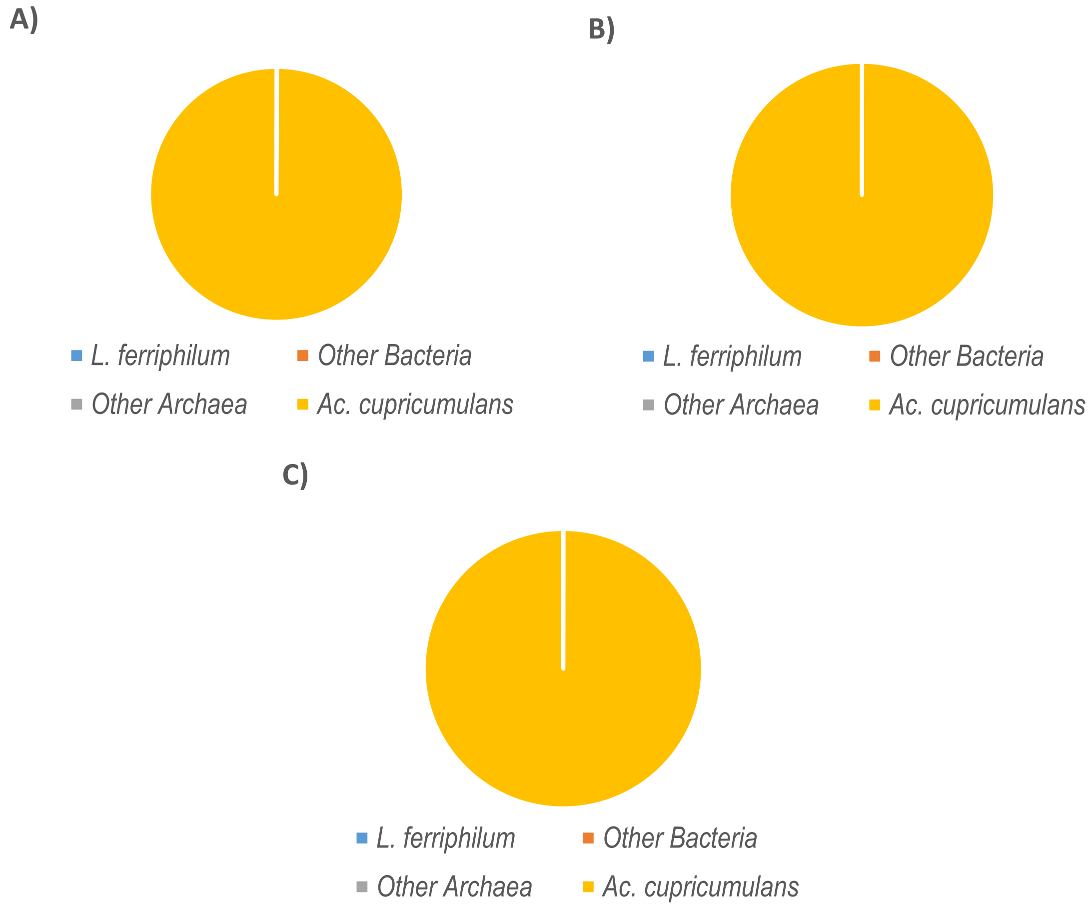

Figure 6.7qPCR analysis of the *Ac. cupricumulans* JTC3 working stock culture where A), B) and C) are consecutive sub-cultures of the working stock