

Article



# Effect of Initial Cell Concentration on Bio-Oxidation of Pyrite before Gold Cyanidation

Ka Yu Cheng <sup>1,2</sup>, Caroline C. Rubina Acuña <sup>1</sup>, Naomi J. Boxall <sup>1</sup>, Jian Li <sup>3</sup>, David Collinson <sup>3</sup>, Christina Morris <sup>1</sup>, Chris A. du Plessis <sup>4</sup>, Natalia Streltsova <sup>5</sup> and Anna H. Kaksonen <sup>1,6,\*</sup>

- <sup>1</sup> Land and Water, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Floreat, WA 6014, Australia; kayu.cheng@csiro.au (K.Y.C.); caroline.rubinaacuna@csiro.au (C.C.R.A.); Naomi.Boxall@csiro.au (N.J.B.); christina.morris@csiro.au (C.M.)
- <sup>2</sup> School of Engineering and Information Technology, Murdoch University, Perth, WA 6150, Australia
- <sup>3</sup> CSIRO Mineral Resources, Waterford, WA 6152, Australia; jian.li@csiro.au (J.L.); david.collinson@csiro.au (D.C.)
- <sup>4</sup> Lhoist, Business Innovation Centre, Rue de l'industrie 31, B-1400 Nivelles, Belgium; chris.duplessis@lhoist.com
- <sup>5</sup> Vintage94 Pty Ltd., Floreat, WA 6014, Australia; strel@iinet.net.au
- <sup>6</sup> School of Biomedical Sciences, University of Western Australia, Crawley, WA 6009, Australia
- Correspondence: anna.kaksonen@csiro.au

**Abstract:** Bio-oxidation of refractory sulfidic gold minerals has been applied at the commercial scale as a pre-treatment to improve gold yields and reduce chemical consumption during gold cyanidation. In this study, the effect of initial cell concentration on the oxidation of pyritic gold ore was evaluated with four aerated bioreactors at 30 °C with 10% pulp density and pH maintained at 1.4 with NaOH. Results of NaOH consumption and changes in soluble Fe and S concentrations indicated that increasing the initial cell concentration from  $2.3 \times 10^7$  to  $2.3 \times 10^{10}$  cells mL<sup>-1</sup> enhanced pyrite oxidation during the first week. However, by day 18 the reactor with the lowest initial cell concentrations, sulfide-S and pyrite contents in the residues, and subsequent gold leaching of the bio-oxidation residues by cyanidation. Overall, the results showed that the cell concentration was clearly beneficial during the initial stages of oxidation (first 7–8 days).

Keywords: bio-oxidation; cell concentration; cyanidation; gold; pyrite

# 1. Introduction

Pyrite is one of the most widely distributed sulfides in the earth and is commonly found as a gangue mineral in association with valuable minerals [1]. Many gold deposits are sulfidic in nature and contain pyrite, arsenopyrite, and pyrrhotite. Refractory gold ores harbour finely disseminated gold particles encapsulated by a sulfide matrix that makes the gold inaccessible to lixiviants. Therefore, pre-treatment of the sulfide matrix is required to improve gold yields and reduce chemical consumption during gold leaching [2].

Conventional pre-treatment for refractory sulfidic gold ores include ultrafine grinding, roasting, and pressure oxidation. However, these methods are associated with several disadvantages such as high investment costs, low gold recovery, and environmental risks [3]. Biological pre-treatment is attractive for low-grade gold ores that are too expensive to process with conventional approaches and for ores that contain impurities, such as arsenic that foul conventional processing equipment [2]. Bio-oxidation has been used at a commercial scale since 1986 as a pre-treatment for refractory pyritic gold ores, before the leaching of gold with chemical lixiviants such as cyanide [2]. The bio-oxidation utilises the activity of acidophilic iron- and sulfur-oxidising microorganisms that generate ferric iron (Reaction 1) and sulfuric acid (Reaction 2), respectively. The biogenic ferric iron oxidises pyrite (Reaction 3) and other metal sulfides, and the released sulfur-intermediates



Citation: Cheng, K.Y.; Acuña, C.C.R.; Boxall, N.J.; Li, J.; Collinson, D.; Morris, C.; du Plessis, C.A.; Streltsova, N.; Kaksonen, A.H. Effect of Initial Cell Concentration on Bio-Oxidation of Pyrite before Gold Cyanidation. *Minerals* 2021, *11*, 834. https://doi.org/10.3390/min11080834

Academic Editor: Karel Folens

Received: 2 July 2021 Accepted: 27 July 2021 Published: 31 July 2021

**Publisher's Note:** MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



**Copyright:** © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). are oxidised by microbes to generate sulfuric acid, which further solubilises acid-soluble minerals [2] The x in reaction 3 is typically in the range of 15,000–150,000.

$$4 \operatorname{Fe}^{2+} + \operatorname{O}_2 + 4 \operatorname{H}^+ \to 4 \operatorname{Fe}^{3+} + 2 \operatorname{H}_2 \operatorname{O}$$
(1)

$$2 S^0 + 3 O_2 + 2 H_2 O \rightarrow 2 H_2 SO_4$$
 (2)

$$\operatorname{FeS}_{2}[\operatorname{Au}_{(1/x)}] + 14 \operatorname{Fe}^{3+} + 8 \operatorname{H}_{2}O \to 15 \operatorname{Fe}^{2+} + 2 \operatorname{SO}_{4}^{2-} + 16 \operatorname{H}^{+} + [\operatorname{Au}_{(1/x)}]$$
(3)

Existing industrial-scale bio-oxidation processes used for refractory sulfidic gold minerals consist of a cascade of agitated aerated tanks in series, with an overall residence time of approximately four days [4]. A typical bio-oxidation tank configuration is that the concentrate is fed to two or three primary oxidation tanks in parallel, all of which report to a series of secondary tanks in sequence (Figure 1). The prevailing active microbial cell concentrate pulp inflow, (2) cell growth rate, (3) cell death rate, and (4) washout rate [5]. The reason why bio-oxidation process flow sheets are configured with primary oxidation tanks in parallel is to allow sufficient residence time (typically 2 days) for cell growth, as there is practically no influx of cells with the pulp feed is relatively high, and a lower residence time per tank (typically less than a day per tank) is sufficient to sustain the oxidation conditions conducive for pyrite oxidation.



**Figure 1.** A typical bio-oxidation tank configuration used for sulfidic gold ore concentrates. Adapted from [4].

Industrial mineral bio-oxidation processes are similar to industrial wastewater treatment processes in terms of their utilisation of biocatalytic activity to oxidise feed stocks and desire to achieve high oxidation rates with short hydraulic retention times and a small footprint. However, mineral bio-oxidation processes fundamentally differ from industrial wastewater treatment processes in several ways, such as the type of materials to be oxidised (inorganic minerals vs. organic wastes and ammonia), operating pH (acidic vs. close to neutral), and type of microorganisms used (mainly chemolithoautotrophic iron and sulfur oxidising microbes vs. heterotrophic microbes and autotrophic ammonia oxidiser). The most relevant difference for the topic of this article is that in existing industrial-scale sulfide mineral bioreactor processes, there is no separation of biomass retention time from process hydraulic retention time. This means the cells contained in the bio-oxidation process flow with the pulp slurry and therefore have the same tank residence time. This fundamentally differs from the activated sludge industrial wastewater treatment process, where the biomass retention time in the process is greater than the hydraulic retention time [5]. In such activated sludge processes, this is achieved by recovering cells at the discharge from the oxidation processes and returning a large portion of the cells into the oxidation process. This significantly increases the efficiency and robustness of the oxidation process as the influx rate of cells is substantially increased and the process therefore does not need to rely only on cell growth rate within the reactor. This enhanced efficiency reduces the size of the reactor compared to what would be required if the cell recovery and return mechanisms were not in place. This mechanism is not used in industrial bio-oxidation processes, mainly because bio-oxidation cells do not readily flocculate and settle, as is the case in wastewater treatment processes. Cell recovery is therefore more complex, although can be achieved, and has been suggested in the patent literature [6].

This article is part of an ongoing processes of evaluating components of a working hypothesis to improve industrial bio-oxidation tank processes. This working hypothesis contains the following elements:

- The pyrite oxidation rate is dependent on the microbial cell concentration up to a catalytic threshold beyond which it would not be the rate limiting factor.
- The cell concentration in primary oxidation tanks can be substantially augmented by re-introducing cells recovered from the oxidation discharge, i.e., before residue washing in counter current decantation (CCD) circuits.
- Such cell recovery and re-introduction into primary oxidation tanks would (1) reduce the residence time required in primary oxidation tanks and (2) make the process more robust at processing upsets and adverse conditions, such as the presence of detrimental elements contained in the ore or process water.

To explore the possibility of improving industrial tank bio-oxidation, a previous study investigated the effect of cell concentration on the soluble ferrous iron oxidation rate and found a direct correlation between increased cell concentration and ferrous oxidation rate [7]. In this study, the effect of cell concentration on pyrite mineral oxidation was investigated as a step towards proving or disproving the elements of the hypothesis that industrial bio-oxidation processes can be improved by utilising cell recovery and return methods, similar to that used in wastewater treatment.

## 2. Materials and Methods

#### 2.1. Microbial Culture

The mixed culture used for the experiments was cultivated by and obtained from ALS Metallurgy (Perth, Western Australia) and based on next generation sequencing of 16S rRNA genes (as conducted by an external laboratory) including *Acidithiobacillus* (*A.*) *caldus, Ferroplasma* (*F.*) *acidarmanus/acidiphilum, Leptospirillum* (*L.*) *ferriphilum,* and *Sulfobacillus* (*S.*) *thermosulfidooxidans*. The temperature ranges and optima reported in the literature for the species present in the mixed culture are: *A. caldus* (range 26 or potentially lower–54 °C, optimum 45–49 °C), *F. acidarmanus* (range 23–46 °C, optimum 42 °C)/*acidiphilum* (range 15–45 °C, optimum 35 °C), *L. ferriphilum* (range 11–49 °C, optimum 30–39 °C) and *S. thermosulfidooxidans* (range 20–60 °C, optimum 45–51 °C) [8,9]. Hence, although the 30 °C used for the pyrite oxidation experiments was not the optimum temperature for all species in the mixed culture, it was in the temperature range of all species and in the optimal range for *L. ferriphilum*, which is a key iron oxidiser that operates at high redox potentials required for pyrite oxidation.

#### 2.2. Bio-oxidation of Pyrite

#### 2.2.1. Pyrite Ores

Damp cake of master composite pyrite ore (Ore 1 + Ore 2; 50:50 (w/w)) ground to 500 µm was used for the pyrite bio-oxidation experiments. The mineralogical composition of the individual ores used for the composite ore are shown in Tables 1 and 2 and are based on quantitative X-ray diffraction (QXRD) and mineral liberation analysis (MLA). Pyrite content of the ore was 14–17 mass-%. The elemental composition of the pyrite ores and the master composite are shown in Table 3.

Mineral	Ore 1	Ore 2	Average
Non-diffracting/unidentified	25.9	41.6	33.8
Pyrite	13.4	14.7	14.1
Quartz	1.8	1.9	1.9
K-feldspar	45.7	16.6	31.2
Muscovite	8.9	6.2	7.6
Kaolinite	1.8	2.6	2.2
Alunite	0	14.0	7.0
Gypsum	2.5	0	1.3
Jarosite	0	2.4	1.2

 Table 1. Mineralogical composition (%) of the pyrite ores based on QXRD analysis.

Table 2. MLA data (as mass %) measured on +425/-850 micron screened size fraction.

Mineral	Ore 1	Ore 2 <sup>1</sup>	Average
Pyrite	11	22	17
Pyrite As1-5	0.035	0.20	0.12
Pyrite As5-10	0.053	0.021	0.037
Pyrite As10-15	0.00029	0.014	0.0071
Ti altered Pyrite	0.20	1.8	1.00
Chalcopyrite	0.033	0.012	0.023
Molybdenite	0.0022	0.018	0.010
Quartz	0.0080	1.6	0.80
K-Feldspar	67	33	50
Sanidine	2.5	2.6	2.6
Albite	0.019	0.012	0.016
Andesine plagioclase	0.069	0.020	0.045
Hornblende	0.032	0.0050	0.019
Biotite	0.086	0.26	0.17
Muscovite	1.0	3.7	2.3
Chlorite	0.0086	0.0014	0.0050
Kaolinite	4.6	9.0	6.8
Illite	11	6.8	8.9
PyrophylliteFe-			
bearing	0.37	5.6	3.0
Ca sulfate			
(anhydrite/gypsum)	0.12	0.020	0.070
Barite Sr-bearing	0.86	0.57	0.72
Alunite	0.021	11	5.5
Jarosite	0.0049	0.030	0.017
Jarosite_Al	0.090	0.48	0.29
Leucoxene type	0.20	0.42	0.31
Fe oxide	0.0094	0.010	0.0097
Rutile	0.21	0.23	0.22
Apatite	0.021	0.0016	0.011
Trolleite	0.54	0.14	0.34
Minor phases	0.026	0.026	0.026
Total	100	100	100

 $\overline{}^1$  recalculated to adjust sulfide S to assay value.

Analyte	Units	Ore 1	Ore 2	Master Composite (Average $\pm$ Standard Deviation)	
$Au_1$	g/t	1.38	3.85	$2.62 \pm 1.75$	
Au <sub>2</sub>	g/t	1.35	3.96	$2.66 \pm 1.85$	
Ag	ppm	3.000	4.5	$3.75\pm1.06$	
Al	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	9.36	9.88	$9.62\pm0.37$	
As	ppm	160	650	$405\pm 346$	
Ba	ppm	5300	2400	$3850\pm 2051$	
Be	ppm	<20	<20	<20	
Bi	ppm	<25	50	NA <sup>1</sup>	
C <sub>TOTAL</sub>	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	0.06	0.12	$0.09\pm0.04$	
CORGANIC	%	0.03	0.15	$0.09\pm0.08$	
CO3 <sup>2-</sup>	%	0.15	-0.15	$0.00\pm0.21$	
Ca	ppm	6000	<1000	NA	
Cd	ppm	<20	<20	<20	
Cl	%	0.01	0.01	$0.01\pm0.00$	
Со	ppm	20	20	$20\pm0$	
Cr	ppm	25	<25	NA	
Cu	ppm	245	185	$215\pm42$	
Fe	%	7.60	11.60	$9.60\pm2.83$	
Hg	ppm	0.1	1.0	$0.55\pm0.64$	
K	%	8.40	5.40	$6.90\pm2.12$	
Li	ppm	20	60	$40.00\pm28.28$	
Mg	%	0.28	0.12	$0.20\pm0.11$	
Mn	ppm	140	280	$210\pm99$	
Мо	ppm	240	140	$190\pm71$	
Na	ppm	2300	1000	$1650\pm919$	
Ni	ppm	20	40	$30.00 \pm 14.14$	
Р	ppm	3250	3000	$3125\pm177$	
Pb	ppm	340	380	$360\pm28.28$	
S <sub>TOTAL</sub>	%	8.72	15.40	$12.06\pm4.72$	
S <sub>SULFIDE</sub>	%	7.58	11.00	$9.29 \pm 2.42$	
SiO <sub>2</sub>	%	47.0	31.8	$39.40\pm10.75$	
Sb	ppm	23.0	17.9	$20.45\pm3.61$	
Sr	ppm	1485	1005	$1245\pm339$	
Те	ppm	3.6	9.2	$6.40\pm3.96$	
Ti	ppm	4000	5600	$4800 \pm 1131$	
V	ppm	345	365	$355\pm14$	
Y	ppm	<100	<100	<100	
Zn	ppm	120	60	$90 \pm 42$	

**Table 3.** Elemental composition of the pyrite ores and average values representing the master composite used for the pyrite oxidation experiments.

 $^{1}$  NA = not available.

# 2.2.2. Culture Medium

The pyrite oxidation experiments were carried out with 0K medium (modified 9K medium [10], which contained (g L<sup>-1</sup>): 3.0 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.50 K<sub>2</sub>HPO<sub>4</sub>, 0.50 MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.10 KCl and 0.010 Ca(NO<sub>3</sub>)<sub>2</sub>) [7]. The media was supplemented with 2.0 g L<sup>-1</sup> of ferrous iron (i.e., 10 g L<sup>-1</sup> of FeSO<sub>4</sub>·7H<sub>2</sub>O), and the pH was adjusted to 1.4.

# 2.2.3. Acid Consumption Test

For the acid consumption test, 50 g of the pyrite ore was added into two stirred tank reactors. One litre of the 0K medium pre-adjusted to the designated pH value (pH 1.4) was added to the reactors. No microbial culture was added. The reactors were stirred continuously at 500 rpm without additional aeration at 30 °C for 3 h. The pH of the reactors was monitored and controlled at the set point using Labview with the addition of  $0.5 \text{ M H}_2\text{SO}_4$ .

# 2.2.4. Microbial Culture

Cells from a mixed culture of acidophilic bioleaching microorganisms were first harvested at  $12,000 \times g$  for 15 min. The pellets were resuspended in 900 mL of 0K medium containing 2.0 g L<sup>-1</sup> ferrous iron (pH 1.4) to wash the cells and centrifuged at  $3000 \times g$  for 30 min to remove soluble organic compounds that may have been released from the cells during the first centrifugation at the higher g force. The remaining cells in the supernatant were further harvested by centrifuging at  $12,000 \times g$  for 15 min. Both cell pellets were resuspended in approximately 1.2 L of 0K medium containing 2.0 g L<sup>-1</sup> ferrous iron (pH 1.4) and the combined cell suspension was used for the bioreactor experiment.

# 2.2.5. Reactor Set Up and Operation

Pyrite oxidation experiments were conducted in four 2 L continuously stirred glass tank reactors (CSTR) (denoted as A, B, C, and D) (Figure 2) using 10 % w/v pulp density (based on dry weight) of the master composite pyrite ore. The mass of the moist ore was 128 g (equivalent to 100 g dry weight) for each reactor and the liquid volume of each reactor was 1 L. The initial concentration of ferrous iron was 2.0 g  $L^{-1}$ , and pH set point was 1.4  $\pm$  0.05. The pH of the reactor was continuously monitored and feedback-controlled to the set point value using Labview, with 1 M NaOH solution used to adjust the pH. The four reactors were inoculated with a  $10 \times$  dilution series of the concentrated mixed culture. The initial cell concentration in A was 10, 100, and 1000-fold higher than that in reactors B, C, and D, respectively (the highest initial cell number in reactor A was  $2.3 \times 10^{10} \pm 0.3 \times 10^{10}$  cells mL<sup>-1</sup>). The reactors were operated in a water bath at 30 °C. The reactors were continuously mixed with overhead stirrers (IKA RW20 digital) at a rate of 500 rpm and aerated with compressed air that had been humidified with Dreschel bottles filled with distilled water and maintained in a water bath at 30 °C. The aeration rate was kept at 1 L min<sup>-1</sup> and the liquid level in the reactors was adjusted on a daily basis by adding Milli-Q water to compensate for evaporation. Measurement of the evaporative loss was done by visually assessing the change in the liquid level (with a temporary stoppage of the stirrer) against a line drawn on the reactor wall to indicate the liquid level at the start of the experiment.



**Figure 2.** Bioreactor set up for pyrite oxidation experiments showing Dreschel bottles in water bath, stirred tank reactors in water bath, and pumps used for dosing NaOH solution.

# 2.2.6. Sampling

The solution pH, redox potential, and dissolved oxygen (DO) concentration were measured daily for each reactor. The consumption of NaOH was recorded using the Labview software every 5 min. Samples (3 mL) were taken from the reactors on weekdays and filtered through 0.2  $\mu$ m filters for ferrous iron, total soluble Fe, and total soluble S analyses. Additional samples (*ca.* 200  $\mu$ L) were taken for cell counts every 1–2 days on weekdays.

# 2.2.7. Termination of the Reactor Process and Post-Treatment of the Ore Residues

At the end of the experiments, the reactor slurries were collected into centrifuge tubes and the residues were separated from the liquor by centrifuging at  $3350 \times g$  for 5 min. The residues from the experiment were washed three times with approximately 300 mL of Milli-Q water acidified to pH 1.4 with H<sub>2</sub>SO<sub>4</sub> per wash, centrifuged at  $3350 \times g$  for 5 min after each wash to collect the solids, and dried at 60 °C for three days. Dissolved organic carbon (DOC) concentrations of the supernatants collected from the experiment were measured to evaluate the content of soluble organic compounds in the leach liquors at the end of the experiment. The dried residues were weighed and pulverised with a mortar and pestle. Fractions of residues from the experiment were analysed by quantitative X-ray diffraction (QXRD) undertaken at CSIRO (Waterford, Western Australia), and subjected to elemental analysis (N, C, Fe, S, K, Na) and gold cyanidation leach tests at ALS Metallurgy (Perth, Western Australia).

Cyanidation experiments were conducted by ALS Metallurgy for the residues from the pyrite oxidation experiment to determine the potential of gold solubilisation after bio-oxidation. The leach tests were carried out in leach bottles using roll agitation for 24 h with 60.2 g, 61.8 g, 63 g, and 56.1 g of residue for reactors A, B, C and D, respectively, and 0.25% NaCN, corresponding to 3.75 kg NaCN t<sup>-1</sup>.

#### 2.3. Analytical Methods

# 2.3.1. Solution pH, Redox Potential and Dissolved Oxygen (DO)

Reactor pH was measured using a TPS miniCHEM controller and pH probe (Ionode pH electrode, IJ44CT) that was calibrated using pH 1.68 and 4.01 buffers. Each reactor was equipped with one pH probe, which was interfaced with the Labview program via a controller box (TPS, MiniChem pH controller). The pH in the reactors was confirmed at various times during the experiment using an external TPS pH electrode interphased with a pH meter (Model smartCHEM-pH, TPS) in order to detect drift in the pH values of the reactor probes. Redox potential was measured using a TPS meter, model MC-80 and IONODE ORP combination electrode, model PRFO, serial No. 0910-063971 (Ag/AgCl reference). DO concentrations were measured using a HACH LDO HQ10 meter with an HACH LDO probe.

#### 2.3.2. Ferrous Iron

Samples for ferrous iron analysis were filtered through 0.2  $\mu$ m filters and diluted five times using 0.2 M HCl. Further dilutions were made using Milli-Q water when necessary. Ferrous iron concentrations in samples were measured spectrophotometrically using the phenanthroline method (3500-Fe, APHA, 1992). HCl (0.5 mL; 0.2 M), Milli-Q water (0.75 mL), phenanthroline solution (0.5 mL, 10 g L<sup>-1</sup>), and ammonium acetate buffer (0.25 mL; 250 g of NH<sub>4</sub>C<sub>2</sub>H<sub>3</sub>O<sub>2</sub> in 180 mL MQ-water, 700 mL glacial acetic acid) were added to 0.5 mL of diluted filtered sample. The samples were mixed, and their absorbance was measured at 510 nm. The concentration of ferrous iron in each sample was determined using a standard curve prepared from known concentrations of ferrous sulfate acidified with 0.2 M HCl.

## 2.3.3. Soluble Fe and S

Samples for soluble iron and sulfur analysis were filtered, diluted five times in 0.2 M HCl, and stored at 4 °C prior to analysis. Total iron and sulfur were measured using inductively coupled plasma-atomic emission spectrometry (ICP-AES) undertaken by the Analytical Services laboratories of CSIRO Process Science and Engineering at Waterford, Western Australia.

## 2.3.4. Dissolved Organic Carbon

Dissolved organic carbon (DOC) was measured in the leach liquors at the end of the pyrite bio-oxidation experiment. Filtered samples were diluted 10 times with Milli Q and

stored at 4 °C prior to analysis. DOC was measured using a Shimadzu TOC-VCPH total organic carbon analyser. The DOC concentrations of the samples were determined using external standards ranging from 0 to 100 mg DOC  $L^{-1}$ .

## 2.3.5. Cell Counting

For detaching cells from minerals for total cell counting, slurry samples were sonicated (30 sec on, 30 sec off) three times in a Grant Ultrasonic Bath XUBA3 at operating frequency 44 kHz, power 35 W, and power per volume 14 W L<sup>-1</sup>. After letting the solids settle for approximately 30 min, cells were counted in the supernatant using a Thoma counting chamber by phase contrast microscopy with three replicate counts using a 100× objective. If cell numbers were too high for direct cell counting, samples were diluted with phosphate buffered saline (PBS) buffer (pH 1.8) before counting. The PBS buffer was prepared as follows. The 30 × PBS solution contained 77.37 g L<sup>-1</sup> of Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, 13.1 g L<sup>-1</sup> of NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, and 226.3 g L<sup>-1</sup> of NaCl. The stock solution was diluted 1/30 with Milli-Q water and pH adjusted to 1.8 with 1 M HCl before sterilising by autoclaving or filter sterilising with a 0.8/0.2 µm gradient filter.

# 2.3.6. Moisture Content of the Ore

The moisture content of the ore was determined by drying a subsample of the moist ore at 105 °C until no more change in the weight was observed. The dried ore was cooled in a desiccator before the weight was recorded. The weights recorded before and after the drying were used to calculate the moisture content.

## 2.3.7. Quantitative X-Ray Diffraction Analysis (QXRD)

The mineralogy of the feed materials and residues from the pyrite oxidation experiment was examined by QXRD by the Analytical Services laboratories of CSIRO Mineral Resources Flagship in Waterford, WA. The samples were mixed with 10% (w/w) fluorite (CaF<sub>2</sub>) as internal standard and micronised using a McCrone micronising mill in ethanol medium for 15 min. The micronised samples were air-dried and re-ground before back-pressing into a conventional XRD sample holder. X-ray diffraction measurements were carried out in a PANalytical high resolution multipurpose powder diffractometer (Empyrean). Coα radiation was used and operated at 45 kV and 40 mA. A Bragg-Brentano High Definition (BBHD) monochromator was applied to the incident beam. A PIXcel3D proton counting X-ray detector was used to collect the data over an angular range of  $3-120^{\circ} 2\theta$  with a continuous scan mode of total 2 h scanning time. The qualitative XRD data was interpreted with the combination of PCPDFWIN (Version 2.02) and Highscore Plus (3.04) database. The mineral composition of the samples was obtained by Rietveld analysis using commercially available TOPAS (version 4.2) software from Bruker Advanced X-ray Solutions. QXRD by Rietveld analysis employed a fundamental parameters approach to line profile fitting, with the combination of known instrumental set up and published crystal structures for all crystalline phases. Spherical harmonic functions were applied to improve the fundamental K-feldspar (Orthoclase) model. The accuracy of the QXRD results was checked by comparing QXRD predicted versus observed elemental compositions for the samples.

## 2.3.8. Calculations

For pyrite oxidation experiments, the percent pyrite oxidation was calculated using Equation (4) as previously described by Kaksonen [11]:

Sulfide oxidised(%) = 
$$(m_{sulfideinore} - m_{sulfideinresidue}) \times 100/m_{sulfideinonre}$$
 (4)

where  $m_{sulfideinore} = mass$  of sulfide-S in the ore (g) and  $m_{sulfideinresidue} = mass$  of sulfide-S in the residue (g). These were calculated using equations 5 and 6, respectively:

$$m_{sulfideinore} = Sulfide_{ore}(\%) \times m_{ore}/100$$
 (5)

$$m_{sulfideinresidue} = Sulfide_{residue}(\%) \times m_{residue}/100$$
 (6)

where Sulfide<sub>ore</sub>(%) = percentage of sulfide-S in ore,  $m_{ore}$  = mass of ore used (g), Sulfide<sub>residue</sub>(%) = percentage of sulfide-S in residue and  $m_{residue}$  = mass of the recovered residue (g).

# 3. Results and Discussion

# 3.1. Acid Consumption of Pyrite Ore

Acid consumption tests were carried out with the pyrite at pH 1.6 and 1.4. The pyrite ore used for the bioreactor experiments did not consume any acid ( $0.5 \text{ M H}_2\text{SO}_4$ ) during the acid consumption test conducted using 0K medium with 2 g L<sup>-1</sup> ferrous iron at pH 1.4 and 1.6 in non-aerated stirred tank reactors at 30 °C for 3 h (Figure 3). Hence, the acid consumption by the gangue minerals in the ore was negligible within the pH range used in the bioreactor experiments.



**Figure 3.** Solution pH and cumulative acid ( $0.5 \text{ M H}_2\text{SO}_4$ ) consumption of the pyrite ore using 0K medium with 2 g L<sup>-1</sup> ferrous iron at pH values of 1.4 and 1.6 in non-aerated stirred tank reactors without inoculation of cells at 30 °C.

## 3.2. Bio-Oxidation of Pyrite

The pyrite oxidation experiment was conducted for a period of 18 days. Total cell counts and DO concentrations during the experiment are shown in Figure 4a,b, respectively. The cell density in reactor A remained relatively stable during the experiment, whereas the cell densities in reactors B and C increased during the first nine days (Figure 4a). The cell density in reactor D decreased at the start of the experiment (from day 0 to 2), possibly due to the attachment of cells onto the ore. Thereafter, the cell density in reactor D increased gradually, and approached a similar level as in reactor C at the end of the experiment. It was unclear whether the initial cell attachment would have also occurred in reactors A, B, and C, in which the cell densities could have been too high to detect a measurable change as a result of attachment. The relative order of cell densities in the reactors remained A > B > C > D throughout the experiment. The DO concentration in all reactors was maintained at 7–8 mg  $L^{-1}$  throughout the experiment (Figure 4b). It is worth noting that severe foaming was observed in reactor A, which had the highest initial cell concentration of  $> 10^{10}$  cells mL<sup>-1</sup>. This foaming phenomenon may be caused by proteins released from broken cells due to the high shear forces inside the reactor (e.g., collision by the fine ores and the propeller) [12]. As the cell concentration was very high, the likelihood of collision increased in reactor A compared to other reactors. The cells in reactor A may also have been starving due to the high cell to substrate ratio. This may have led to the death of some cells and release of organic compounds from the cells. Another possible explanation is the production of surface-active agents (biosurfactants) by the dense culture [12].



Figure 4. (a) Total cell counts and (b) dissolved oxygen (DO) concentrations in the bioreactors during the pyrite oxidation experiment.

The reactor pH values recorded via the Labview program and manually during the pyrite oxidation experiment are shown in Figure 5a,b, respectively. The volumetric and gravimetric base consumptions are shown in Figure 5c,d, respectively. The pH in reactors A, B, and C increased to between 1.46 and 1.50 within the first few hours after the start-up, likely due to the proton consumption during bio-oxidation of the ferrous iron in the growth medium (Figure 5a,b). However, the initial rise in pH was only transient and the pH values in these reactors decreased to the set point of pH 1.4 within 10 h of operation (Figure 5a). The decrease in pH was likely a combined effect of pyrite oxidation and generation of protons during ferric iron precipitation. Notable drifts (slight increases) in the readings of the pH probes were detected throughout the experiment. However, daily recalibrations of pH probes meant that the fluctuations did not impair the effectiveness of the feedback-control, which was unaffected and did not trigger any unintentional base addition. In fact, the base consumption measured by weight loss recorded for the experiment was in good agreement with the consumption recorded by the Labview program.

The base consumption in reactor D started immediately after the onset of experiment, as during this period the pH in D was lower than the set point (Figure 5a,c). This may be due to the low cell concentration, and hence slow ferrous iron oxidation that did not consume as much acid as was generated in pyrite oxidation. After the initial pH correction, the pH in D gradually increased during the first 5 days, possibly due to ferrous iron oxidation. In all other reactors, the pH initially increased as a result of more rapid ferrous iron oxidation, and hence there was no NaOH demand at the beginning of the experiments in reactors A–C. NaOH demand in reactors A and B began at 13.3 h and 21.5 h (on day 0), respectively (Figure 5c,d). A longer lag time (48 h) was observed for reactor C to start consuming NaOH (Figure 5d), most likely due to the lower cell density contained in this reactor, and therefore lower ferrous iron oxidation rate.

The base consumption patterns recorded after the first day in the pyrite oxidation experiment could be generally divided into two distinct phases, phase 1: from days 1 to 8; and phase 2: from days 8 to 18. The base consumption during the first phase followed the order of reactor A > B > C > D. This trend was expected given that the cell densities in the reactors were in the same descending order. From days 8 to 18, the cell density in reactor D increased to a level that was similar to that of reactor C and by day 18 the cumulative NaOH consumption in reactor D was similar to that of reactor B. At the end of the experiment (day 18), the order of cumulative base consumption was reactor A > D > B > C and the rate of NaOH consumption in reactor D was the highest (Figure 5c,d). The relationship between the amount of NaOH consumption measured by weight loss and the computer program in the pyrite oxidation experiment was linear as shown in Figure 6.

![](_page_10_Figure_2.jpeg)

**Figure 5.** (a) Solution pH recorded via Labview program and (b) read manually from pH monitors, (c) volumetric base consumption recoded via Labview program and (d) base consumption determined by weight change of the NaOH bottles during the pyrite oxidation experiments with reactors A–D.

![](_page_10_Figure_4.jpeg)

**Figure 6.** Relationship between the NaOH consumption measured by weight loss and the computer program in the pyrite oxidation experiment with reactors A, B, C, and D. The NaOH consumption determined by weight loss measurement was converted to volumetric consumption using the density of the 1 M NaOH solution (i.e.,  $1.04 \text{ g mL}^{-1}$ ). The computer recorded data was obtained by multiplying the flow rate of the NaOH dosing pump (mL s<sup>-1</sup>) with the cumulative switch on time (s) of the pump.

Recorded NaOH consumptions were much lower than expected, only 2.71–3.47% of theoretical NaOH consumption, assuming complete pyrite oxidation (Figure 7a). The actual NaOH consumption was also only 5.12–7.25% of that expected based on actual pyrite

oxidation values derived from the mineralogical analysis of the residues from reactors A–D (Figure 7a). According to the stoichiometry for complete pyrite oxidation (Reaction 7), 16 moles of OH<sup>-</sup> (as NaOH) would be required to neutralise the protons generated for each mole of pyrite oxidised.

![](_page_11_Figure_3.jpeg)

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+$$
 (7)

Actual NaOH consumption (mol NaOH kg<sup>-1</sup> ore (dry weight))

**Figure 7.** (a) Percent of recorded NaOH consumption versus theoretical NaOH consumption assuming complete pyrite oxidation or actual pyrite oxidation for the pyrite oxidation experiment; (b) relationship between actual pyrite oxidation or sulfide oxidation and the actual NaOH consumption in the experiment.

Considering the head ores used in the experiments contained 15% (w/w) pyrite, NaOH consumption of 80 g NaOH (per 100 g ore (dry wt.)) could be expected for complete pyrite oxidation. Hence, it was expected that the NaOH consumptions in all reactors (A to D) would be considerably higher. However, except for reactor D where the NaOH consumption appeared to be continuously increasing from day 8, all other reactors (A–C) showed signs of NaOH consumption slowing down at the end of the experiments (Figure 5c,d).

The reason for the discrepancy between the recorded and theoretical NaOH consumption is unclear. However, weak linear correlations were obtained between pyrite (and sulfide) oxidation and the actual NaOH consumption (Figure 7b). Hence, one may still consider NaOH consumption a rough surrogate indicator of pyrite oxidation for the specific head ore used in this study. Undoubtedly, more complicated reactions could have been involved in the process and as such, NaOH consumption should not be generalised for this purpose when different ores are tested. One factor could be the possible acid consumption of gangue minerals during the extended period of reactor operation as compared to the abiotic acid consumption test. Another factor contributing to the pH and NaOH consumption is the acid generated during precipitation of ferric iron as jarosite according to reaction 5, where A represents a monovalent cation such as ammonium (NH<sub>4</sub><sup>+</sup>), potassium (K<sup>+</sup>), sodium (Na<sup>+</sup>), or hydronium (H<sub>3</sub>O<sup>+</sup>) [13].

$$3Fe^{3+} + A^+ + 2SO_4^{2-} + 6H_2O \rightarrow AFe_3(SO_4)_2(OH)_6 + 6H^+$$
 (8)

Further understanding of the parameters or reactions that determine the rate and extent on the ferrous oxidation and ferric precipitation is essential, given the extent of ferric iron precipitation is dependent on the concentration of oxidised iron and the prevailing pH [14]. Ferrous iron concentration, redox potential, and total soluble Fe and S concentrations during the experiment are shown in Figure 8. The ferrous iron was nearly completely oxidised in reactors A-C within the first day, whereas in D near complete oxidation was reached on day 8 (Figure 8a). Similarly, the redox potential in reactors A–C increased rapidly within a day and remained stable at approximately +650 mV thereafter until the end of the experiment. The increase in redox potential in reactor D was substantially slower. By day 10, the redox potential in reactor D was similar to those recorded for reactors A–C (Figure 8b). The lower redox potentials in D during the first week of the experiment may also have caused a shift in the microbial community structure by allowing such microbial species that are sensitive to higher redox potentials to grow. For example, A. ferrooxidans has been reported to have a higher growth rate at redox potentials below +470 mV (vs. Ag/AgCl), whereas Leptospirillum spp. have a greater affinity for Fe<sup>2+</sup> and are less sensitive to inhibition by Fe<sup>3+</sup> at a higher redox potential [15]. A. ferrooxidans can oxidise both  $Fe^{2+}$  and reduced sulfur species whereas *Leptospirillum* can only oxidise  $Fe^{2+}$  [9]. The possible shift in the microbial community structure may have increased the pyrite oxidation rates. However, since the microbial communities were not analysed at the end of the experiment, the possible shift in the community structure remains unconfirmed. It is also possible that indigenous microorganisms present in the non-sterilised ore may have become enriched towards the end of the experiment and contributed to the bio-oxidation.

As expected, the total soluble Fe and S concentrations in all reactors increased throughout the experiment (Figure 8c,d). Initially, the total soluble Fe and S concentrations in reactors A and B increased at a similar rate. However, after the first week both total soluble Fe and S concentrations were highest in reactor B followed by reactors A, C, and D. By the end of the experiment the soluble iron and sulfur concentration in reactor D were similar to those in reactor B. However, total soluble Fe and S concentrations in reactors A and C were similar to each other, but lower than those in reactors B and D (Figure 8c,d). Overall, the total soluble Fe and S results suggested that the kinetics of bio-catalysed pyrite oxidation was not proportional to the initial cell concentrations tested in this study  $(2.3 \times 10^{10}-2.3 \times 10^7 \text{ cells mL}^{-1})$ . The higher cell density in reactor A may have enhanced jarosite precipitation, which in turn may have decreased the total soluble Fe and S concentrations (Figure 8c,d). Extracellular polymeric substances (EPS) have been suggested to contribute to changes in solution chemistry that facilitates precipitation [16].

DOC concentrations in the leach liquors at the end of the pyrite oxidation experiment were 76, 40, 27, and 30 mg L<sup>-1</sup> for reactors A, B, C, and D, respectively. The DOC concentration was highest in reactor A, followed by reactors B, D, and C. A photo of the supernatants of the reactor slurries collected at the end of the pyrite oxidation experiments is shown in Figure 9. The supernatants collected from A (with the highest initial cell density) after mineral solids separation with short centrifugation at  $3350 \times g$  for 5 min appeared to be most turbid, probably due to higher cell densities. High concentrations of dissolved organic compounds may inhibit the activity of some bioleaching microorganism and also slow down abiotic pyrite oxidation with Fe<sup>3+</sup> [17–19].

![](_page_13_Figure_1.jpeg)

**Figure 8.** (a) Ferrous iron, (b) redox potential (vs. Ag/AgCl), (c) total soluble iron, and (d) sulfur during the pyrite oxidation experiment with reactors A, B, C, and D.

![](_page_13_Picture_3.jpeg)

Figure 9. Supernatants of the reactor slurries collected from reactors (A–D) at the end of the pyrite oxidation experiments.

Figure 10 shows dried residues from the pyrite oxidation experiment, and masses of the recovered residues are shown in Table 4. The brown colour on the surface of the residues from reactor A was likely due to cell biomass recovered along with the mineral residues (Figure 10).

![](_page_13_Picture_6.jpeg)

Figure 10. Dried residues from reactors (A) to (D) (from left to right) from the pyrite oxidation experiment.

Reactor	Used Dry Ore Mass for Experiment (g)	Recovered Dry Residue (g)	Change in Solid Mass during Experiment (%)	
A	100	91.64	-8.36	
В	100	94.08	-5.92	
С	100	94.99	-5.01	
D	100	88.74	-11.26	

**Table 4.** Masses of dry feed ores and recovered residues from the pyrite oxidation experiment and percent change in solid mass during the experiment.

The elemental compositions of the residues from the pyrite oxidation experiment are shown in Table 5. The Fe, total-S, and sulfide-S contents were lower in the residues than in the feed ore, with the lowest Fe and total-S contents detected for reactor D residue, followed by reactors B, A, and C, respectively. The sulfide-S was lowest for reactor B residue, followed by reactors D, A, and C, respectively. Total-C content was highest for reactor A residue, followed by B, D, and C, respectively. This is consistent with the higher biomass amount in A residue as observed visually (Figures 9 and 10).

				Rea	ictor	
Analyte	Units	Feed Ore –	Α	В	С	D
Au	g t <sup>-1</sup>	$2.64^{1}$	3.50	2.59	2.75	2.74
Al	%	9.62	10.3	10.9	10.6	11.0
C <sub>TOTAL</sub>	%	0.09	0.93	0.39	0.24	0.30
Fe	%	9.60	5.94	5.02	6.32	4.54

Table 5. Composition of feed ore and residues from pyrite oxidation experiment.

6.90

1650

12.06

9.29

<sup>1</sup> value is an average of two replicates.

%

ppm

%

%

Κ

Na

**S**<sub>TOTAL</sub>

**S**<sub>SULFIDE</sub>

The mineralogical composition data of the feed and leached residues by Rietveld based QXRD conducted by CSIRO is listed in Table 6. A reasonably good agreement was found between the previous MLA results of the feed ore and the QXRD results obtained for feed ore when analysed in conjunction with the leach residues. The feed ore was dominantly K-feldspar type minerals, with moderate amounts of illite type and kaolinite type clays, a moderate amount of pyrite and alunite, and trace amounts of Ti mineral anatase, jarosite, barite, quartz, and gypsum.

7.73

2050

6.40

5.12

7.33

1950

5.76

4.60

7.33

1800

8.26

6.74

8.00

2050

5.48

5.08

Based on the QXRD analysis, pyrite contents in all leach residues were lower than in feed ore, with the pyrite content being lowest in the residue from reactor D, followed by reactors B, A, and C, respectively. The pyrite contents in the leach residues from reactors A–D correlated well with the sulfide-S content observed by chemical analysis (Figure 11). Dissolution of other major and moderate phases including K-feldspar, kaolinite, alunite, and illite were not obvious from the QXRD results (Table 6). However, jarosite content in reactor A-D residues was increased as compared to the feed ore, which indicated jarosite formation under the reactor conditions. The jarosite content in the residue increased with increasing initial cell concentration. As mentioned previously the presence of higher number of cells and thus more abundant EPS may have enhanced jarosite precipitation [16]. The presence of cations such as Fe<sup>3+</sup>, Al<sup>3+</sup>, K<sup>+</sup>, Na<sup>+</sup>, and NH<sub>4</sub><sup>+</sup> in sulfate-based solution in slightly acidic conditions will encourage jarosite formation [20]. Other than the soluble chemicals already present in the reactors (e.g., those present in the 0K medium and the dosed NaOH over the course of the experiment), additional cations may become available from the dissolution of pyrite, small amounts of K-feldspar minerals, and illite type minerals associated with the ore (Table 6).

Minerals (%)	<b>F</b> _10,0	Reactor			
	Feed Ore	Α	В	С	D
Quartz	1.2	1.5	1.3	1.2	1.3
Anatase	1.0	0.8	1.0	0.7	0.9
Barite	0.5	ND <sup>4</sup>	ND	ND	ND
Alunite	11.8	12.1	11.9	12.3	12.5
Jarosite	1.2	4.5	4.0	3.7	3.4
Pyrite <sup>1</sup>	15.0	7.4	6.5	9.9	5.9
K-Feldspar <sup>2</sup>	44.4	45.7	50.1	48.7	48.5
Illite <sup>3</sup>	7.6	9.7	6.8	7.2	7.4
Gypsum	0.6	0.0	0.4	0.3	0.4
Kaolinite	11.9	11.7	12.1	13.2	13.2
unaccounted	4.9	6.7	5.8	2.7	6.5
Sum	100	100	100	100	100

**Table 6.** Mineralogical composition of feed ore and residues from pyrite oxidation experiment analysed using QXRD.

<sup>1</sup> QXRD analysis predicts lower S and Fe values for the feed, indicating underestimated pyrite value for the feed; <sup>2</sup> The model for K-feldspar was modified based on the best matched model suggested by Highscore plus 3.0, spherical harmonic series (SHS) function was applied to accommodate peak broadening potentially due to the structural disorder in the feldspar mineral. <sup>3</sup> Muscovite and illite have very similar XRD, the presence of a small amount of muscovite is hard to detect by XRD; <sup>4</sup> ND = not detected.

![](_page_15_Figure_4.jpeg)

**Figure 11.** Calculated oxidation of sulfide and pyrite based on total-sulfide analysis and QXRD analysis considering mass loss during the experiment for reactors **A–D**.

Results from the extraction of gold from residues of the pyrite oxidation experiment using  $3.75 \text{ kg} \text{ NaCN t}^{-1}$  varied from 71.91% to 87.78%, as shown in Table 7. Gold extraction was highest for reactor D followed by reactors A, B, and C. By the end of the experiment, the effect of initial cell concentration was no longer evident and relatively high gold leaching was obtained for all residues as compared to ore that had not been bio-oxidised. For comparison, on average, cyanidation tests on the ore without bio-oxidation resulted in only 30–35% gold extraction (data not shown). Final cyanide concentrations after 24 h cyanidation were 0.055%, 0.055%, 0.075%, and 0.050%, and the corresponding cyanide consumptions were 2.93, 2.93, 2.63, and 3.00 kg NaCN t<sup>-1</sup> for residues from reactors A, B, C, and D, respectively.

Reactor	Gold Extraction (%)		
А	84.64		
В	83.05		
С	71.91		
D	87.78		

**Table 7.** Extraction of gold from residues of the pyrite oxidation experiment with reactors A–D using  $3.75 \text{ kg NaCN t}^{-1}$ .

A summary of the extent of pyrite oxidation in reactors A–D as estimated based on various parameters after one week and at the end of the experiment is shown in Table 8. After the first week of the pyrite oxidation experiment, the NaOH consumption implied that the increasing cell numbers could enhance pyrite oxidation. Apart from reactor A, which had lower soluble Fe and S concentrations than B, the soluble Fe and S concentrations also increased with increasing cell numbers. However, at the end of the experiment (day 18), the effect of initial cell concentrations was no longer clear. Although reactor A still showed the highest NaOH consumption at the end of the experiment, the second highest consumption was recorded for reactor D, which had the lowest initial cell numbers. Reactor D also showed the second highest soluble Fe and S concentrations just after reactor B. Sulfide-S content of the residues indicated that reactor B would have the highest pyrite oxidation, whereas based on pyrite content and gold extraction the oxidation was more extensive in reactor D. At the end of the experiment, reactor C showed the lowest pyrite oxidation based on most of the indicators listed in Table 8.

**Estimated Extent of Pyrite Estimated Extent of Pyrite** Parameter Oxidation after One Week (Day 7) Oxidation at the End (Day 18) NaOH consumption A > B > C > D $A > D \approx B > C$ Soluble Fe A > B > C > D $B > D > C \approx A$ Soluble S B > A > C > D $B > D > A \approx C$  $\rm NA^{1}$ Sulfide-S content of B > D > A >> Cresidue Pyrite content of NA D > B > A >> Cresidue Gold extraction NA D > A > B > C

**Table 8.** The order of the extent of pyrite oxidation in reactors A–D as estimated based on various parameters after one week and at the end of the experiment.

 $\overline{^{1}}$  NA = not applicable.

The fact that the highest initial cell concentration in reactor A did not result in the highest pyrite oxidation over the 18 day period could be due to the inhibitory effect of the elevated level of dissolved organic matter on microbial activity and pyrite oxidation [17,19]. Yacob et al. [19] found that the rate of pyrite oxidation by ferric iron in sterile suspensions at pH 1.8 was reduced by 87% in the presence of dissolved organic compounds produced from the microbial cells. They also affirmed that such inhibition was attributed to the complexation reaction between the organic compounds and Fe<sup>3+</sup>. Moreover, Marchand and Silverstein [17] found that the rate and extent of pyrite oxidation by *A. ferrooxidans* were limited by the presence of organic compounds, and that the growth of this iron oxidiser was notably inhibited by dissolved organic compounds.

At the start of the experiment, most cells in reactor A were likely active, which explains the rapid start-up and NaOH consumption. However, after a while the cells in reactor A may have been starving due to limited availability of substrates (ferrous iron and reduced sulfur compounds) released from pyrite. This may have caused some of the cells to die and release DOC. The foaming that was observed after a few days in reactor A may have been related to cell lysis as the DOC concentration in reactor A was higher than in other reactors at the end of the experiment.

Previous studies on the effect of initial cell concentration on the efficiency of biooxidation processes are somewhat limited in the biomining context. Okereke and Stevens [21] and Boxall et al. [7] evaluated the effect of cell concentration on ferrous iron oxidation rates at cell concentration ranges of  $3 \times 10^8$ – $9 \times 10^8$  cells mL<sup>-1</sup> and  $6.8 \times 10^7$ – $7.1 \times 10^9$  cells mL<sup>-1</sup> and temperatures of 10-30 °C and 30 °C, respectively, and reported increasing ferrous iron oxidation with increasing initial cell concentrations. The study by Okere and Stevens [21] used a culture of A. ferrooxidans and the study by Boxall et al. [7] used a similar mixed culture composed of A. caldus, Ferroplasma acidarmanus/acidiphilum, L. ferriphilum, and Sulfobacillus thermosulfidooxidans as used in the present study. Tambwe et al. [22] evaluated the desulfurisation of high sulfur coal discards using inoculated an uninoculated columns. The inoculation was conducted with a mixed culture of A. caldus and L. ferriphilum at an initial cell concentration of 10<sup>12</sup> cells per ton of coal discards. Redox potential increased more rapidly in the inoculated columns than in the uninoculated control column that relied on native microbes. However, after approximately 50 days the redox potential in the uninoculated column reached similar levels to the inoculated columns. Sulfur removal as a result of bio-oxidation after 380 days was 61.6-63.5% in the inoculated columns and 60.1% in the uninoculated column [22]. Although the applied system was different, namely an unsaturated column instead of a stirred tank reactor, the results are in agreement with those of the present study in that the prolonged operation of a sulfide ore bio-oxidation system somewhat masked the effect of the initial cell concentration on sulfide oxidation. Nevertheless, the findings of the present study revealed that increasing cell concentration is a viable strategy to enhance pyrite oxidation rate in reactors that are operated with a retention time of below 8 days.

## 4. Conclusions

During the first week of the pyrite oxidation experiment, NaOH consumption and soluble Fe and S concentrations indicated that the increasing initial cell concentration enhanced pyrite oxidation in the range of initial cell concentrations of  $2.3 \times 10^7$ – $2.3 \times 10^{10}$  cells mL<sup>-1</sup>. The oxidation rates observed within the first four days of the experimental period is most relevant to industrial bio-oxidation tank process applications and indicate support for the notions outlined in the earlier stated hypothesis that increased cell concentration increases pyrite oxidation rate. The results provide sufficient impetus for further exploration of this hypothesis, which would most sensibly focus on continuous mineral feed and reactor operation with separation of cell hydraulic retention time (via cell recovery and reintroduction into the oxidation reactor) from the overall hydraulic retention time in the system.

By contrast to the above, the data gathered after eight days of operation are relevant to bio-oxidation processes (such as heap leaching) where hydraulic residence time is extensive, i.e., in the range of months. After approximately eight days of the pyrite oxidation experiment, the effect of cell concentration was no longer clear, and by the end of the experiment, reactor D with the lowest initial cell concentration showed enhanced performance based on soluble Fe and S concentrations, sulfide-S and pyrite contents of the residues, and gold leaching.

In summary, increasing the initial cell concentration up to approximately  $2.3 \times 10^{10}$  cells mL<sup>-1</sup> was beneficial for pyrite oxidation during the first 7–8 days. Future work should explore methods to recover cells effectively and economically and compare continuously operated pyrite oxidation processes with and without cell recovery and return.

Author Contributions: Conceptualisation, K.Y.C., C.A.d.P., N.S., and A.H.K.; formal analysis, K.Y.C., N.J.B., J.L., D.C., C.M., and A.H.K.; funding acquisition, C.A.d.P., N.S., and A.H.K.; investigation, K.Y.C., N.J.B., J.L., D.C., C.M., C.A.d.P., N.S., and A.H.K.; project administration, A.H.K.; resources, C.A.d.P., N.S., and A.H.K.; software, K.Y.C.; supervision, A.H.K.; visualisation, K.Y.C., C.C.R.A., N.J.B., and A.H.K.; writing —original draft preparation, K.Y.C., C.C.R.A., and A.H.K.; writing—review and editing, K.Y.C., C.C.R.A., N.J.B., J.L., C.M., C.A.d.P., N.S., and A.H.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research received funding from CSIRO.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

- Lv, X.; Zhao, H.; Zhang, Y. Review on the Bio-oxidation of Pyrite: Implications for the Mining Industry. In *TMS 2020 149th Annual Meeting & Exhibition Supplemental Proceedings*; The Minerals, Metals & Materials Society, Ed.; The Minerals, Metals & Materials Society: Pittsburgh, PA, USA, 2020; pp. 1309–1314.
- Kaksonen, A.H.; Mudunuru, B.M.; Hackl, R. The role of microorganisms in gold processing and recovery—A review. Hydrometallurgy 2014, 142, 70–83. [CrossRef]
- Purnomo, I.; Chaerun, S.K.; Mubarok, M.Z. Biooxidation pretreatment of low grade refractory gold tailings using a sulfuroxidizing mixotrophic bacterium. *IOP Conf. Ser. Mater. Sci. Eng.* 2019, 478, 012020. [CrossRef]
- 4. Dew, D.W.; Lawson, E.N.; Broadhurst, J.L. Chapter 3: The BIOX<sup>®</sup> process for biooxidation of gold-bearing ores and concentrates.
- In *Biomining: Theory, Microbes and Industrial Processes*; Rawlings, D.E., Ed.; Springer: Berlin/Heidelberg, Germany, 1997; pp. 45–78.
  5. Tchobanoglous, G.; Burton, F.L. *Wastewater Engineering Treatment, Disposal, and Reuse*, 3rd ed.; McGraw-Hill, Inc.: Singapore, 1991; pp. 371–372.
- 6. Du Plessis, C.A. Recovery of Bioleaching Microbes. International Patent WO 03/008565 A1, 30 January 2003.
- Boxall, N.J.; Cheng, K.Y.; du Plessis, C.A.; Collinson, D.; Morris, C.; Streltsova, N.; Seaman, B.; Seaman, D.; Vollert, L.; Kaksonen, A.H. Increasing cell concentration does not affect specific ferrous iron oxidation rate in a continuously stirred tank bioreactor. *Hydrometallurgy* 2018, 181, 189–194. [CrossRef]
- Franzmann, P.D.; Haddad, C.M.; Hawkes, R.B.; Robertson, W.J.; Plumb, J.J. Effects of temperature on the rates of iron and sulfur oxidation by selected bioleaching *Bacteria* and *Archaea*: Application of the Ratkowsky equation. *Miner. Eng.* 2005, *18*, 1304–1314. [CrossRef]
- Schippers, A. Microorganisms involved in bioleaching and nucleic acid-based molecular methods for their identification and quantification. In *Microbial Processing of Metal Sulfides*; Donati, E.R., Sand, W., Eds.; Springer: Berlin/Heidelberg, Germany, 2007; pp. 3–33.
- 10. Silverman, M.P.; Lundgren, D.G. Studies on the chemolithoautotrophic iron bacterium *Ferrobacillus ferrooxidans*. 1. An improved medium and harvesting procedure for securing high cell yields. *J. Bacteriol.* **1959**, *77*, 642–647. [CrossRef] [PubMed]
- Kaksonen, A.H.; Perrot, F.; Morris, C.; Rea, S.; Benvie, B.; Austin, P.; Hackl, R. Evaluation of submerged bio-oxidation concept for refractory gold ores. *Hydrometallurgy* 2014, 141, 117–125. [CrossRef]
- 12. Delvigne, F.; Lecomte, J.-P. Foam formation and control in bioreactors. In *Encyclopedia of Industrial Biotechnology: Bioprocess, Bioseparation, and Cell Technology;* Flickinger, M.C., Ed.; John Wiley & Sons, Inc.: New York, NY, USA, 2010.
- 13. Gramp, J.P.; Jones, F.S.; Bigham, J.M.; Tuovinen, O.H. Monovalent cation concentrations determine the types of Fe(III) hydroxysulfate precipitates formed in bioleach solutions. *Hydrometallurgy* **2008**, *94*, 29–33. [CrossRef]
- Kaksonen, A.H.; Morris, C.; Rea, S.; Li, J.; Wylie, J.; Usher, K.M.; Ginige, M.P.; Cheng, K.Y.; Hilario, F.; du Plessis, C.A. Biohydrometallurgical iron oxidation and precipitation: Part I—Effect of pH on process performance. *Hydrometallurgy* 2014, 147, 255–263. [CrossRef]
- Rawlings, D.E.; Tributsch, H.; Hansford, G.S. Reasons why 'Leptospirillum'-like species rather than Thiobacillus ferrooxidans are the dominant iron-oxidizing bacteria in many commercial processes for the biooxidation of pyrite and related ores. Microbiology 1999, 145, 5–13. [CrossRef] [PubMed]
- 16. Kaksonen, A.H.; Morris, C.; Hilario, F.; Rea, S.M.; Li, J.; Usher, K.; Wylie, J.; Ginige, M.P.; Cheng, K.Y.; du Plessis, C. Iron oxidation and jarosite precipitation in a two-stage airlift bioreactor. *Hydrometallurgy* **2014**, *150*, 227–235. [CrossRef]
- 17. Marchand, E.A.; Silverstein, J. Influence of Heterotrophic Microbial Growth on Biological Oxidation of Pyrite. *Environ. Sci. Technol.* **2002**, *36*, 5483–5490. [CrossRef] [PubMed]
- 18. Okibe, N.; Gericke, M.; Hallberg, K.; Johnson, D.B. Enumeration and characterization of acidophilic microorganisms isolated from a pilot plant stirred-tank bioleaching operation. *Appl. Environ. Microbiol.* **2003**, *69*, 1936–1943. [CrossRef] [PubMed]
- Yacob, T.; Pandey, S.; Silverstein, J.; Rajaram, H. Soluble Microbial Products Decrease Pyrite Oxidation by Ferric Iron at pH < 2. Environ. Sci. Technol. 2013, 47, 8658–8665. [PubMed]
- 20. Dutrizac, J.E.; Jambor, J.L. Jarosites and their application in hydrometallurgy. Rev. Mineral. Geochem. 2000, 40, 405–458. [CrossRef]

- 21. Okere, A.; Stevens, S.E., Jr. Kinetics of iron oxidation by *Thiobacillus ferrooxidans*. *Appl. Environ. Microbiol.* **1991**, *57*, 1052–1056. [CrossRef] [PubMed]
- 22. Tambwe, O.; Kotsiopoulos, A.; Harrison, S.T.L. Desulphurising high sulphur coal discards using an accelerated heap leach approach. *Hydrometallurgy* **2020**, *197*, 105472. [CrossRef]