Thiosulphate-oxidizing Bacteria as Indicators of Sulphide Mineral Oxidation

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Abstract
Three partly-oxidized sulphur compounds, thiosulphate, trithionate and tetrathionate, were shown to be produced during the oxidation of pyrite and chalcopyrite. Since these compounds can be metabolized by thiosulphate-oxidizing bacteria, this study sought to demonstrate that these bacteria grow during the oxidation of sulphide minerals. Growth media with thiosulphate or tetrathionate were developed to detect and enumerate thiosulphate-oxidizing bacteria. It was shown that these bacteria are abundant in circumneutral, but not acidic rinse water from humidity cells containing sulphur minerals, and in mine drainage where sulphide minerals are known to be oxidized. It is concluded that the growth of thiosulphate-oxidizing bacteria is supported by sulphide mineral oxidation and that they are reliable indicators of oxidation in circumneutral drainage.

Key Words: sulphur mineral oxidation, polythionates, thiosulphate-oxidizing bacteria, indicators

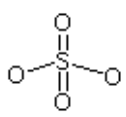
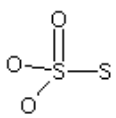
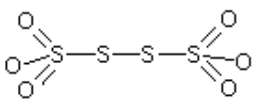
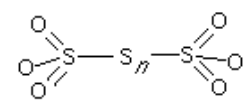
Introduction
Under most circumstances, the oxidation of sulphide minerals is ascertained by elevated conductivity and/or sulphate concentrations in mine drainage. However, these indicators are unreliable when the source rock contains low sulphide concentrations and/or mine water contacts rocks with soluble sulphate minerals in its path. Alternative indicators of sulphide mineral oxidation could be useful in these circumstances. This study sought to determine if thiosulphate-oxidizing bacteria could be used for that purpose.

Thiosalts are produced during the oxidation of sulphide minerals. Conventionally, the oxidation of sulphides is depicted as (for pyrite):

$$FeS_2(s) + 7/2O_2 + H_2O \rightarrow Fe^{+2} + 2SO_4^{-2} + 2H^+$$

However, more detailed descriptions of sulphide mineral oxidation, such as those proposed by Sand and co-workers (1996, 2001), suggest that this process is more complicated. These authors argued that pyrite, molybdenite and tungstenite (FeS_2, MoS_2 and WS_2) are oxidized through a thiosulphate mechanism, whereby thiosulphate is the first direct product of sulphide oxidation, whereas other sulphide minerals are oxidized through a polysulphide mechanism. These sulphur intermediates (Table 1) are eventually fully oxidized to sulphate, in accordance with the above equation. Others have also proposed that sulphur intermediates are produced during sulphide mineral oxidation (Nordstrom *et. al.*, 2007).

Table 1. Structure of sulphate, thiosulphate and polythionates.

Sulphate (SO_4^{2-}) 	Thiosulphate ($S_2O_3^{2-}$) 
Tetrathionate ($S_4O_6^{2-}$) 	Polythionate ($S_nO_6^{2-}$) 

These reactions became of interest to us because they could explain the presence of certain bacteria associated with sulphide mineral oxidation. It has long been known that many more bacterial species are associated with sulphide minerals than specific roles can be ascribed. For instance, Johnson (1998) listed nearly forty acidophilic prokaryotes, but assigns unequivocal roles in sulphide oxidation to only a few of them. Robbins and co-workers (2000) identified a large number of filamentous and non-filamentous bacteria, as well as fungi, ciliates, rotifers and other eukaryotes, in the highly acidic waters of Iron Mountain. Banfield and co-workers used genome-based techniques to analyze microbial communities living in ARD, and while they can define certain community structures and quantify the relative abundance of species in them, they are unable to ascribed metabolic functions to most of organisms they detect (Baker and Banfield, 2003). Druschel and co-workers speculate that ferric iron rapidly oxidizes thiosulphate in acidic environments, thereby preventing its build up as an energy source for thiosulphate-oxidizing bacteria (Druschel et. al., 2003, 2004). Mielke and co-workers demonstrated that an isolate of *Acidithiobacillus ferrooxidans* grown in the laboratory could colonize and oxidize pyrite cubes at circumneutral pH (Mielke *et. al.*, 2003).

The hypotheses put forward by Sand and the lack of knowledge about the metabolic functions for most bacteria in ARD prompted this investigation of an association between thiosulphate-oxidizing bacteria and sulphide mineral oxidation. It was hypothesized these bacteria might be indicative of sulphide oxidation if they were detected in mine drainage. Given that these bacteria will be more stable than thiosulphate or other intermediate sulphur species, they might provide a useful alternative to conductivity and/or sulphate as indicators of sulphide mineral oxidation.

Materials and Methods

The production of polythionates from sulphide minerals was tested under anaerobic conditions in sealed 35 mL serum bottles. The bottles were filled with a sterile mineral salts solution (Table 2) adjusted to pH 8.4, and supplemented with 1 g purified pyrite and 1 g chalcopyrite, and 1% of an inoculum of sulphur-based denitrifying bacteria. Ground pyrite and chalcopyrite were first acid-washed, then washed with acetone and finally rinsed with distilled water before use. The medium was deoxygenated by sparging with oxygen-free nitrogen before filling the bottles. The inoculated bottles were sealed and incubated at room temperature and were sampled periodically to measure polythionates by ion chromatography.

Table 2. Composition of mineral salts solution.

Constituent	Amount, per Litre
NH_4Cl	0.24 g
$NaHCO_3$	0.24 g
KH_2PO_4	0.06 g
$MgSO_4$	0.3 g
$Ca(NO_3)_2$	0.1 g
Bromocresol green (BMG) pH indicator	Pinch

Thiosulphate-oxidizing bacteria were detected using a solid-phase most probable number (MPN) method A variety of media were tested, designed to differentiate between bacteria that can use thiosulphate or tetrathionate as an energy source. No carbon was provided for growth. Given that oxidation of sulphur intermediates would acidify the growth medium, both weakly and strongly buffered medium were used during the study (Table 3). Both media also received 1 mL/L of a trace elements stock solution (Table 4). The presence of thiosulphate-oxidizing bacteria was determined by growth of colonies and the associated change in medium pH shown by the pH indicator dye.

Table 3. Composition of strongly buffered growth medium for enumerating sulphur-oxidizing bacteria.

Constituent	Weakly-buffered	Strongly-buffered
NH_4Cl	0.24 g	0.4 g
$NaHCO_3$	0.24 g	4.0 g
KH_2PO_4	0.06 g	0.2 g
$MgSO_4$	0.3 g	0.5 g
$Ca(NO_3)_2$	0.1 g	0.1 g
Yeast Extract	0.01 g	0.01 g
Purified agar	8.0 g	8.0 g
$Na_2S_2O_3$ or $K_2S_4O_6$	1.0 g[a]	1.0 g[a]
Bromocresol green (BMG) pH indicator	0.02 g	0.02 g

[a] Thiosulphate or tetrathionate were added from filter-sterilized stock solutions after the medium was autoclaved and partly-cooled.

Table 4. Composition of trace element stock solution.

Constituent	Amount, per Litre
HCl (25% v/v)	6.5mL
$FeCl_2.4H_2O$	1.5 g (dissolved in acid)
$CuCl_2.2H_2O$	15 mg
$Na_2MoO_4.2H_2O$	25 mg
$NiCl_2.6H_2O$	25 mg
H_3BO_3	60 mg
$ZnCl_2$	70 mg
$MnCl_2.4H_2O$	100 mg
$CoCl_2.6H_2O$	120 mg

Water samples from several sources, including columns, humidity cells and mine drainage, were analyzed for the presence of both bacteria and thiosalts. The column and humidity cells contained rock different mine sites in North America (the identity of these mines is confidential, by request) and were operated at two commercial laboratories. The rock ranged in sulphide-sulphur content from 0.33 to 5.8% and in sulphate leaching rates from 0.07 to 274 mg SO_4/kg/week. The mine locations were chosen according to the characteristics of their drainage. Mine A is known to produce acid rock drainage and was used as a positive control for both parameters of study. Mine B lacks any acid rock drainage and was used as a negative control. Mine C was suspected of hosting sulphide oxidation but there was uncertainty concerning future production of acid rock drainage. Information on water chemistry was available for Mine A and B, but not for Mine C.

Samples were initially taken from a large number of humidity cells from three different mines to present a snapshot of bacterial populations over a wide range of conditions. Subsequently, different sets of humidity cells were sampled biweekly to observe the bacteria population dynamics over time. Both newly started humidity cells (less than 20 weeks old) and long running cells (100 weeks old and upward) were sampled.

Two types of samples were collected from each mine location or humidity cell. One sample was intended for bacterial analysis and was placed in sterile, 1.7mL Eppendorf test tubes. The second sample from each site was intended for thiosalt analysis and was placed in 1.7mL Eppendorf test tubes containing 0.1g of analytical-grade Dowex AG2-X8 strong anion exchange resin. This resin is known to be specific for thiosalts (Druschel *et al.*, 2003), which allows for separation and recovery of both thiosulphate and

polythionates. Sample volumes were accurately measured and applied to the resin before shipping, which preserved all thiosalts until their analysis. Upon receipt by the analytical lab, the resin was drained, rinsed once with water and drained again. Samples were then stored until analysis could be performed. Without the resin, it is likely that these labile sulphur intermediates would be lost from water samples.

Two different analyses were performed for polythionates. Polythionates were detected and quantified by ion-chromatography by Placer Dome Research Centre, using a proprietary method. This method was used for measuring polythionates in rinsate from columns and humidity cells, and in the test for polythionate production from pyrite and chalcopyrite. Polythionates were also quantified using a UV spectrophotometer (Thermospectronic Genesys 6) at 220nm wavelength, based on the absorbance spectra for thiosulphate and tetrathionate and the lack of absorbance at this wavelength from other compounds in solution. This method was calibrated using purified samples as well as samples analyzed separately by ion-chromatography. All the tests using resins and the polythionates elution protocol were analyzed by UV spectrophotometry.

Results

Thiosulphate production – columns and humidity cells

Rinsate from several columns and humidity cells were tested for thiosulphate. Column and cell sources were selected to provide a range of sulphide contents and oxidation rates as noted in Table 5.

Table 5. Characteristics and thiosulphate production of humidity cells.

Sample ID	Sulphide (Weight%)	Rinsate pH	Sulphate leach rate (mg/kg/wk)	Thiosulphate (mg/L)
COLUMN 4	N/A	8.16	1.4	15.6
COLUMN10	0.59	8.12	15.3	15.7
HC3	4.46	7.6	90	12.3
HC5	5.69	7.55	167	12.5
HC7	4.98	7.58	274	11.0
HC10	5.8	7.62	197	12.1
HC11	0.33	7.97	0.07	11.8
HC26	N/A	8.07	69.2	14.1
HC49	N/A	8.56	0.5	10.0

Thiosulphate was detected in rinsate from every column and humidity cell at concentrations of 10-15 mg/L. These results indicate that thiosulphate is formed during the oxidation of sulphide minerals, as suggested by Sand and co-workers (2001).

Thiosulphate production – purified pyrite and chalcopyrite

Ground purified pyrite and chalcopyrite received a culture of sulphur-based denitrifying bacteria, which is known to oxidize sulphide minerals using nitrate instead of oxygen, and the kinetics of thiosulphate production were followed. The oxidation of these minerals leads to a decrease in solution pH and to the production of thiosulphate and polythionates at concentrations between 5-50 mg/L (Figure 1).

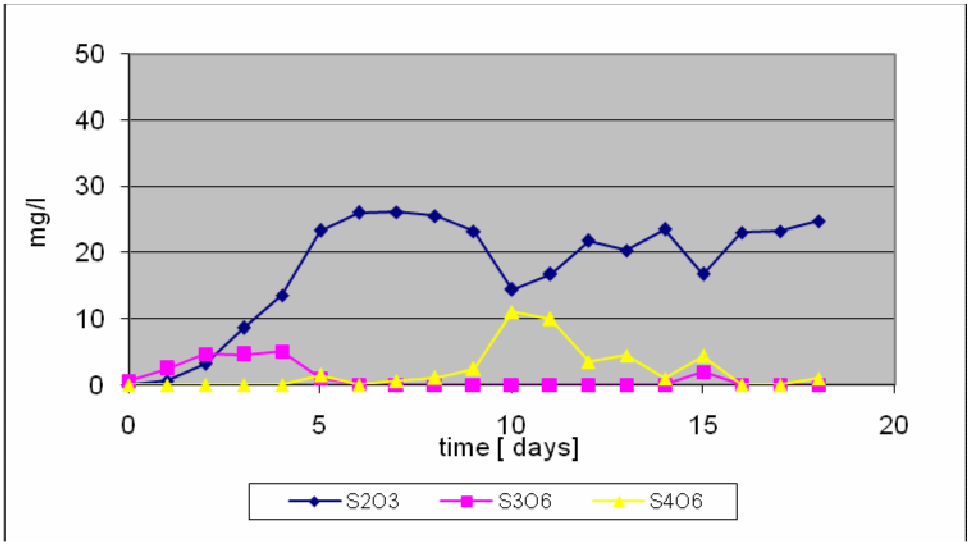


Figure 1. Concentrations of polythionates during the oxidation of a pyrite and chalcopyrite mixture.

Thiosulphate was detected one day after the beginning of the test, and its concentrations increased steadily for seven days. Trithionate was also detected early, but its concentration decreased by the fourth day. Tetrathionate was first detected much later, after nine days.

An increase in tetrathionate on Day 10 coincided with a decrease in thiosulphate concentration. This can be attributed to the condensation of thiosulphate, as shown in the equation below:

$$2S_2O_3^{-2} + 0.5O_2 + 2H^+ \longrightarrow S_4O_6^{-2} + H_2O$$

However, trithionate cannot be formed through such a mechanism, and it was most likely formed during the oxidation of polysulphides, which Sand (1996) predicted would be produced during the oxidation of chalcopyrite.

Thiosulphate-oxidizing bacteria – humidity cells

Thiosulphate-oxidizing bacteria were enumerated in rinsate from fifty columns or humidity cells. These analyses provided a comprehensive dataset to examine the relationship between these bacteria and factors associated with sulphide mineral oxidation.

The number of thiosulphate-oxidizing bacteria in rinsate was surprisingly high, typically ranging from 10^4 to 10^6 cell/mL (Figure 2). A small number of samples contained very low cell numbers.

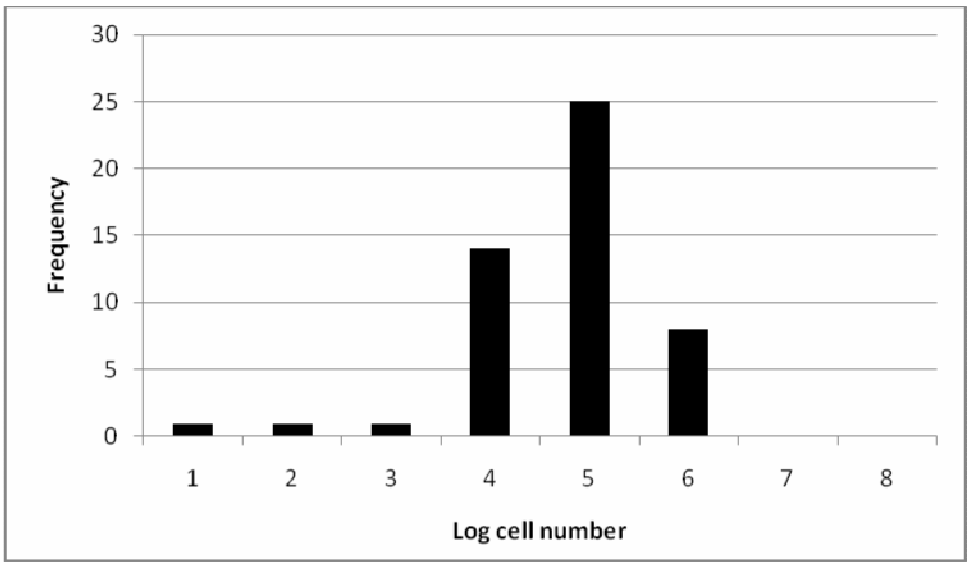


Figure 2. Frequency distribution of cell numbers in humidity cell rinsate.

There was a clear relationship between rinsate pH and cell numbers (Table 6). Cell numbers in acidic rinsate were lower by two orders of magnitude compared with numbers in neutral or alkaline rinsate. Even slightly acidic rinsate appeared to have approximately five times fewer cells than neutral rinsate.

Table 6. Thiosulphate-oxidizing bacteria numbers in relation to rinsate pH.

		Rinsate pH		Cell Numbers	
Rinsate pH range	Count	Mean	Median	Mean	Median
1-4.5	5	3.66	3.86	1,279	123
4.5-6.5	4	5.75	5.66	11,730	5,360
6.5-8.0	16	7.57	7.53	79,884	30,953
>8.0	25	8.52	8.56	75,641	44,991
1-8.8	50	7.50	8.05	64,450	31,179

The crush size of minerals in the humidity cells had no effect on cell number (correlation coefficient = 0.074). Similarly, there was no correlation between cell number and the age of the humidity cell (correlation coefficient = 0.069), sulphate concentration in the rinsate (correlation coefficient = -0.052), and the percent sulphide-sulphur in the humidity cell (correlation coefficient = -0.18).

Bacteria were also enumerated in rinsate from selected humidity cells for up to seven weeks. Both newly started humidity cells (less than 11 weeks old) and long running cells (60 weeks old and upward) were sampled, but cell numbers did not exhibit distinct patterns in any of these humidity cells.

Different growth media were tested to determine if different bacteria would be retrieved from humidity cell rinsate. A strongly-buffered medium for enumerating thiosulphate-oxidizing bacteria produced comparable results to moderately-buffered medium, though cell numbers tended to be lower. Two different media containing tetrathionate instead of thiosulphate were also tested, since tetrathionate is stable over a greater pH range than thiosulphate (pH 4-11 vs. 6-10). Although the growth patterns of tetrathionate-oxidizing bacteria were quantitatively different, they did not differ qualitatively from those

of thiosulphate-oxidizing bacteria. As such, the conclusions reached for thiosulphate-oxidizing bacteria using the moderately-buffered medium apply equally to bacteria recovered by variants of this medium.

Thiosulphate-oxidizing bacteria – mine water

The above results suggested that thiosulphate-oxidizing bacteria might be present in circumneutral mine water that has contacted oxidizing sulphide minerals. To confirm this, the drainage from three mines was analyzed for thiosulphate-oxidizing bacteria.

A number of reference sites were sampled near the above mines. The sites were known not to receive mine drainage or otherwise be influenced by mining activity. Bacteria levels at these sites were between 10,000 and 20,000 cells/mL.

Mine A produces strongly acidic mine drainage (Table 7). Cell numbers in this sample were very low (>1,000 cells/mL), consistent with what was observed in humidity cells.

Table 7. Characteristics and cell numbers of water from Mine A.

Sample ID	Water pH	Sulphate (mg/L)	Numbers of thiosulphate-oxidizing bacteria (Cells/mL)
MP	2.5-2.7	6,170-18,571	210-2,046 (average 779)

At Mine B, mine water is neutral-to-alkaline. Sampling locations were divided in two groups: one group known to have no sulphide mineral oxidation and another likely to have oxidation. The first group (WS East and West; ME, LW, and BW Lake) had alkaline water (pH 8.0) with slightly elevated conductivity and sulphate concentrations (Table 8). Numbers of thiosulphate-oxidizing bacteria were comparable to those at reference sites, averaging 19,000 cells/mL.

Table 8. Characteristics and cell numbers of water from Mine B.

Sample ID	Water pH	Conductivity (µS/cm)	Sulphate (mg/L)	Numbers of thiosulphate-oxidizing bacteria (Cells/mL)
WS EAST	8.02	664	231	26,749
ME LAKE	8.18	1305	533	4,815
WS WEST	7.93	1029	451	8,006
LW LAKE	8.07	575	206	15,105
BW LAKE	7.98	289	73	42,869
MESA A POND	8.14	690	231	23,979
MESA B POND	8.22	1620	646	45,287
MESA C POND	8.23	597	206	9,211
B1 POND	7.9	439	120	68,513
S3 POND	8.35	1070	441	239,791
S4 POND	8.31	2020	1050	16,094,631
NALT	8.3	1440	545	699,647
SHIK	8.17	1570	635	36,116

The eight other sampling locations also had alkaline waters, but varied widely in other parameters and bacterial counts. In particular, three sites (S3 and S4 Ponds, NALT) showed very high bacteria counts as well as high conductivity and sulphate concentrations. Unlike others, water at these three sites had been underground for some time and daylighted briefly before being sampled. Two other sites (MESA B Pond and SHIK) had moderately elevated conductivity, sulphate concentrations and cell numbers. Three sites

with lower conductivity and sulphate concentrations (MESA A and C Ponds, B1 Pond) had moderately elevated cell numbers.

When looking at the entire dataset, cell numbers were found to be moderately correlated with conductivity (r=0.57), but correlated with sulphate concentrations more strongly (r=0.71). Considering the many factors that could affect these parameters, these data suggest that there is a correlation between thiosulphate-oxidizing bacteria and known indicators of sulphide mineral oxidation.

At the time of sampling, sulphide mineral oxidation was suspected at Mine C, but there was little evidence for it. Our analysis for thiosulphate-oxidizing bacteria was part of a larger program aimed at resolving this question. Samples were collected from a reference site and from five seeps flowing from waste rock dumps.

Thiosulphate-oxidizing bacteria reported at 57,000 cells/mL in a sample from the reference site, which was higher than expected (Table 9). However, cell numbers in seepage were much higher and exceeded 100,000 cells/mL in four of five seeps. Based on our earlier studies, these data indicated that sulphide minerals were indeed being oxidized, though evidence for this was not directly available.

Table 9. Numbers of thiosulphate-oxidizing bacteria in seepage at Mine C.

Sample ID	Numbers of thiosulphate-oxidizing bacteria (Cells/mL)
REF-005	56,888
SEEP-018B	>2,400,000
SEEP-013	806,341
SEEP-007C	351,750
SEEP-052	169,631
SEEP-065	4,519

Geochemical and mineralogical studies on the waste rock undertaken while this study was conducted subsequently confirmed that sulphide oxidation was taking place. We were later able to obtain samples from several humidity cells that had been set up soon after our field work and analyze them for the presence of thiosulphate-oxidizing bacteria. Cell numbers were determined in rinsate from humidity cells three weeks after they were established (Table 10). Cell numbers in three humidity cells (S26-3, S28-3, and S30-3) were very high, indicating that sulphide minerals were being oxidized. The low cell numbers in S27-3 suggested that no oxidation was limited in that humidity cell, whereas the intermediate numbers in the other humidity cells indicated that oxidation was uncertain or absent in the three remaining samples.

Table 10. Thiosulphate-oxidizing bacteria in rinsate of humidity cells with waste rock from Mine C.

Sample ID	Numbers of thiosulphate-oxidizing bacteria (Cells/mL)
S4-3	53,533
S26-3	630,838
S27-3	2,638
S28-3	456,204
S29-3	45,287
S30-3	2,211,573
S31-3	68,513

Conclusions

High conductivity and sulphate concentrations in mine drainage are typically taken as necessary and sufficient evidence for the oxidation of sulphide minerals. However, such evidence would be inconclusive at mines where the sulphide-sulphur content in waste rock is low and where sulphate minerals are present in significant amounts. In that case, other, more direct evidence of sulphide mineral oxidation must be obtained. In this study, elevated numbers of thiosulphate-oxidizing bacteria are shown to be such an indicator.

The production of partially-oxidized sulphur intermediates during the oxidation of sulphide minerals was confirmed. Thiosulphate was detected in rinsate from several columns and humidity cells. In addition, thiosulphate and polythionates were shown to be produced during the biological oxidation of ground pyrite and chalcopyrite. The detection of these partially-oxidized sulphur intermediates supports the mechanisms of sulphide mineral oxidation proposed by Sand and others.

Four different growth media were tested during this study. Differences in the numbers of bacteria detected by these different media were noted, but they were not substantial and did not affect the conclusions of this study.

Thiosulphate-oxidizing bacteria were abundant in rinsate from a large number of humidity cells and columns containing sulphide minerals. Their numbers were not correlated with crush size, sulphate concentrations in rinsate or age and percent sulphide-sulphur in the humidity cell. Rinsate pH was the only factor that was strongly correlated with cell numbers. Given that thiosulphate and polythionates are labile at acidic pH, this correlation makes sense; these compounds will simply decompose before they can be metabolized and support the development of large bacterial populations. However, there is a strong association between the oxidation of sulphide minerals at circumneutral pH and the presence of these bacteria in mine drainage collected downstream. This can be beneficial at mine site where there are high background concentrations of sulphate or when there are problems with conventional indicators of mineral oxidation.

Thiosulphate-oxidizing bacteria were also detected in mine drainage. These bacteria are naturally present in the environment, as evident by their detection at between 10,000 and 20,000 cells/mL in reference samples. Their numbers increased dramatically in circumneutral or alkaline drainage of sites where sulphide mineral oxidation was suspected or demonstrated, reaching well over 100,000 cells/mL at some sites. In the case of Mine C, where sulphide mineral oxidation was suspected but not yet demonstrated, elevated numbers of thiosulphate-oxidizing bacteria provided some of the first clear evidence that oxidation was taking place. This was corroborated by geochemical studies conducted after this study, as well as analysis of rinsate from humidity cells containing samples of waste rock from Mine C.

Thiosulphate-oxidizing bacteria were present at low concentrations in acidic mine drainage, as was observed also in humidity cells. The rapid decomposition of thiosulphate and other thiosalts at acidic pH is the likely explanation for this observation.

The overall conclusion of this study is that thiosulphate-oxidizing bacteria are present in elevated numbers in mine drainage that contacts oxidizing sulphide minerals (relative to reference waters) and are therefore good indicators of oxidation at mine sites.

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