Review

Acid Rock Drainage or Not—Oxidative vs. Reductive Biofilms—A Microbial Question

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Abstract: Measures to counteract AMD generation need to start at the mineral surface, inhibiting mineral-oxidizing, acidophilic microbes. Laboratory and long term field tests with pyrite-containing mining wastes, where Carbonaceous Phosphate Mining Waste (CPMW) was added, resulted in low acidity, and near neutral drainage. The effect was reproducible, and confirmed by several independent research groups. This was shown to involve an organic coating, likely a biofilm. The biofilm formation was confirmed when CPMW was added to lignite coal waste with an initial pH of 1. Forty five days after the addition, the coal waste was dominated by heterotrophic microorganisms in biofilms. A review of the scientific literature supports that CPMW has physical and chemical characteristics which are capable of inducing a strong inhibitory effect on sulphide oxidation by forming an organic coating over the mineral surface. CPMW characteristics appear to provide the cornerstone of a new technology for the reduction of sulphide oxidation in mine wastes. An hypothesis for testing this technology is presented which could result in an economical and sustainable approach to mine waste and water management.

Keywords: microbial sulphide oxidation, corrosion, mine waste and water remediation, biofilm development, inhibition of acid mine and rock drainage

1. Introduction

As early as 1556 Agricola [1] declared that acid mine drainage (AMD) is a curse of mining. To date, no sustainable options exist to curtail the curse. Global estimates of tailings production from milling ore are several hundred thousand tons per day [2]. In addition, open pit mining generates rock waste covering areas that can be hectares or square kilometers in extent. This has led to potential conflicts with the available, arable land. Public concern about AMD, in connection to water shortages and land demands requires serious attention [3].

1.1 General Mine Waste Management Practices

The present approaches to mine waste management focus on keeping water and oxygen away from waste depositories, where they are considered the main drivers of weathering. To slow the infiltration of water and oxygen, waste rock is stacked and compacted and/or sealed with engineered covers. For tailings, water covers have provided some reduction of oxygen penetration, slowing oxidation of sulphides. Vegetation covers assisted by adding organics and bactericide may have a longer-lasting effect, as recently assessed with aerial photography [4]. Control over mineralized rock surface or weathering has also been pursued by adding bactericides, phosphate materials, and other chemicals to develop a coating over the mineral surface. These additions have worked for a while, but later become unstable [5]. One of the most intensely pursued measures was the use of phosphate-
containing materials, such as a natural phosphate ore, (commonly referred as NPR ore), which was tested in coal waste piles outdoors and in the laboratory [6-16]. An iron-phosphate coating formed on the mineral surface reducing oxygen access and limiting oxidation. The dosage of NPR ore added was based on the expected stoichiometric reaction between the iron released from pyrite weathering and the phosphate concentration in the ore. By measuring acidity/alkalinity in the effluent, the effect of the addition was determined. The additions reduced the acidity generated, but the lowest dosage of phosphate ore produced better results than higher dosages. The approach was abandoned by industry for various reasons. But, the results were intriguing, possibly indicating microbial involvement. If a heterotrophic microbial biofilm were present, it might explain the results. This concept fit well into the ecological engineering approach which we pursued for decommissioning mine waste and water management [17]. Reducing the oxidation rate within tailings and waste-rock depositories would boost the effectiveness of producing an economically-sustainable decommissioning technology.

1.2 The challenges in mine waste management.

Wilson [18] summarized the physical, chemical and geotechnical limitations of handling mine wastes, given the vast accumulations of waste rock and tailings. However, he failed to acknowledge the role of microbes which increase the oxidation rate by orders of magnitude, which is well documented. The ubiquitous nature of these microbes, along with the dramatically increased surface area of exposed pyrite in mine wastes, guarantees that oxidation reactions will overwhelm any potential reductive microbial metabolism. In addition, the sulphide oxidation reaction is exothermic, which ultimately leads to steaming or even burning waste rock piles [19,20]. Another challenge is that of the scale (square km, m, µm, or even nm) at which these processes take place. This was highlighted by Lüttge and Arvidson [21] in their book on kinetics of water-rock interactions at different scales. The authors suggest that looking at only one scale can easily lead to misinterpretations of the reactivity of processes on the mineral surface. For mine waste management, the scales can span 6-12 orders of magnitude - from the mineral surface to the vast expanses of ha or km² of waste rock and tailings deposits.

Microbial metabolism alters the reaction kinetics of weathering, while altering the topography of the mineral surface. Microbes and their exudates act on mineral surfaces by generating corrosion pits and/or biofilms, which can change the electrical charge of the surface. Oxidation of pyrite in waste rock and tailings occurs for thousands of years, with or without covers of any kind. From a practical perspective, given that microbes are a major accelerating factor, these powerful geo-microbiological processes must be of primary concern in mine waste management.

2. Approaching the geo-microbiological challenge

Ecologists view mine waste areas as extreme ecosystems. These ecosystems are defined as areas where most life forms find it hard to survive. Chemo-lithotrophic microbes, however, are a dominant feature of these habitats. They survive and even flourish under harsh physical and chemical conditions. Given these challenges, the fundamental question is, can these oxidative habitats be gradually altered to more normal reductive environments? The use of ecological principles such as niche construction [22,23] might hold the answer. Niche construction can
be defined as the process by which organisms modify their own (and other organisms')
environment.

If the niche construction is operating in these extreme habitats, then it might be operating in
metal leach operations. As an example, Gibraltar Mines, in British Columbia Canada reported
cessation of their leach dump [24]. What caused the oxidation processes to stop? This event was an
opportunity to observe niche construction in a primarily oxidizing habitat. Rocks from the dump
were obtained and investigated with Scanning Electron Microscopy (SEM) with Energy Dispersive
Spectroscopy (EDS) (Figure 1).

**Figure 1:** One of the rocks from the sections of the Gibraltar leaching dump.

The surface revealed a coating, comprised mainly of iron-hydroxide, with trace amounts of
phosphate, and numerous microbial colonies. These observations confirmed our belief that
microbiological niche construction might be operating here.

### 2.1 Reproducable and replicable outdoor and laboratory experiments

To see if these results could be reproduced with other mine rocks, controlled experiment was
set up outdoors with sulphidic waste rock from a northern Quebec Cu/Zn mine. The waste rock
experiments used 2.5 tonnes of sulphidic waste rock with sulphides up to 15 % in 70 L plastic drums
(Table 1a). To these drums 8 L of phosphate mine waste rock, not ore but CPMW Carbonaceous
Phosphate Mining Waste, previously referred to as NPR. (Natural Phosphate Rock) was added on
top of the sulphidic rocks originating from the same mine used by [6-16]). The mineralogy was
identical but the utilization of a waste to treat another waste is attractive. The application on top of
the drums and not mixed throughout, simulated application onto waste rock lift, a practical aspect
easily implemented by the mine operator. It was assumed that rain would transport this niche-
altering substance to the mineral surface. The substance would then act on the surface by 1) providing
some neutralization by virtue of its carbonate content and 2) providing nutrients to modify the
microbial consortia, driving the mineral surface environment from oxidative to reductive. The
phosphate waste dosage added was driven by economics of the mine operation ($0.05/t of ore mined),
not by the stoichiometry of the sulphide/iron content. Effluent from the drums was collected
intermittently for 2.7 years, after which the experiment was dismantled. Forty percent of the 8 L of
CPMW added was recovered unreacted. The carbonate neutralization was insufficient to account for
the effluent quality. The rocks were stored in an industrial basement, until needed for further tests.
The mineral surfaces of rocks from the outdoor drum experiment were investigated with the same
SEM as were the rocks from the Gibraltar leach pile at the University of Toronto.
Figure 2a: SEM images on the surface of the waste rocks in the absence (a) or presence of NPR (b) after 989 days outdoor exposure and storage (scale bars: 10μm). Surface corrosion by microbes was observed in the experiment (see arrows on Figure 1a). In the presence of NPR addition, coating with classical desiccation crack were recognized on the surface.

Figure 2b: ESEM images of the revived biofilms (at pH 7) on the surface of pyrite-rich waste rocks isolated from drum experiments [27]. After 7 days, microbes (cocci) originally present in the drums were revived and they reformed biofilms on the waste rock surface (a). Mineral precipitates could also be observed on the rock surface in close association with the microbial cells, suggesting that biomineralization could occur on the bacterial cell walls and EPSs (b).
The differences between no CPMW and those with were striking. Electron microscopic investigations revealed the expected microbial corrosion pits with microbes (Figure 2a) in contrast to a coating with desiccation cracks covering the sulphides (Figure 2b). Funding for a postdoc was arranged at the University of Ottawa which facilitated a detailed investigation of the desiccated coating and its revival as shown in Figure 2b. It was expected that during storage the coating would vanish we re-exposed the rocks again, now stored for 4.5 years but without CPMW. Initially some acid was generated, but shortly the effluent recovered to provide an circumneutral pH with low acidity. The details of both outdoor exposures are summarized in [25]. Rocks from the same experiment were now also shipped to the University of Western Australia as part of the P933 program[28]. There the mineral surface was intensely investigated with the electron microscopy, quantifying the thickness of the coating, its oxygen content and its composition. In addition CPMW was added to Red Dog (Alaska, USA) mining wastes which resulted in the same findings – an organic coating formed [26-29].

With these findings on the surfaces of the rocks from drum experiment, sufficient evidence had emerged that the organic coating was indeed a biofilm and now was clearly the potential cause of the improved effluents. However laboratory results do not necessarily translate to large-scale field tests and even less to the whole waste depository. Hence experimentation on a larger scale in the field and in different field conditions is absolutely necessary, as mine waste environments are not composed of static, indigenous microbial populations. This is particularly the case, as in all wastes the mineral surface relevant in generating the weathering products are somewhat similar. Therefore if nice constructions a fundamental evolutionary microbial process should occur in all types of wastes. The microbial populations change their composition and growth dynamics with the physical and chemical conditions in the wastes. Microbial populations, not the individual microbe, respond to ecological processes as previously mentioned [22, 23]. Nevertheless field experiments are not well received in science, as they cannot be replicated. Recently, however, large-scale field experiments have gained recognition in ecology. Barley and Meeuwig [30] state that “Large-scale Un-Replicated Natural Experiments” or LUNEs have aided in the formulation of hypotheses at ecologically-realistic scales. These LUNEs have made positive contributions to conservation policy, providing powerful insights into cosmology, evolution and geology.

2.2 LUNEs, Large Un-replicable Natural Experiments

Our field tests or LUNEs ranged in size from 0.5 to 1.5 ha, an area large enough to be ecologically-relevant. Plots were set up on uranium and pyrrhotite tailings in Ontario Canada, and on a spill area of zinc concentrate in Newfoundland (Table 1a). Sulphide content ranged from 1 to 95%. CPMW was ploughed about 10 to 20 cm into the tailings surface and the plots were covered with straw chips or horse manure and seeded to prevent erosion of the surface during rain events. The dosage of CPMW was calculated based on the costs of transporting lime otherwise used to generate an erosion control cover. Details of the all the field tests and the methodology by which the porewater of the tailings samples were assessed are given in [31-38]. After the one-time application of CPMW, 3.1-3.8 years elapsed before sampling which secured 5 to 10 cm vertical sections below the root zone. It was expected that the CPMW would lead to a hardpan below the seeded surface. Often during intense rain events or snow melt the grass cover is destroyed as acid drainage rises from.
below the root zone as the water can not drain, due to low hydraulic conductivity below the root zone and particularly when the tailings are still frozen. A hard pan below the roots would prevent that acid drainage from rising and generally reduce infiltration of atmospheric precipitation and promote run-off.

The time between the set up and the sampling, the length of storage of the samples and the monitoring of the supernatant at laboratory temperatures and with free access of oxygen as well relevant characteristics of the tailings are given in Table 1a along with the same parameters of the replicable outdoor waste rock drum experiment.

The tailings samples were stored for 0.5 to 6.5 years in coolers with access to air after which time slurries (1:5 w:v) were prepared with an magnetic mixer. The Eh, pH and conductivity were measured in the supernatant. As the measurements of acidity/alkalinity consumed supernatant, the volume used was replaced to keep the supernatant volume the same throughout the monitoring period, which lasted up to 2.9 years (Table 1a). These long storage and monitoring times with oxygen access were to destroy any possible biofilm and would, although indirectly support the longevity of the inhibitory effect observed in the supernatant.

Table 1a: Summary of experimental timelines and characteristics of sulphidic tailings and waste rock (both data extracted from [33, 36 and 37]).

<table>
<thead>
<tr>
<th></th>
<th>Uranium</th>
<th>Pyrrotite</th>
<th>Polymetalic</th>
<th>Waste rock</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Length of time (years)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First Exposure (field/outdoors)</td>
<td>3.75</td>
<td>3.25</td>
<td>3.18</td>
<td>2.7</td>
</tr>
<tr>
<td>Storage indoors</td>
<td>6.5</td>
<td>5.5</td>
<td>0.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Monitoring of Effluent/sol(1:5 w/v)</td>
<td>1.83</td>
<td>1.83</td>
<td>2.87</td>
<td>2.7</td>
</tr>
<tr>
<td><strong>Selected Samples</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Waste rock (() 0.01-0.25m)/NPR () 4 to &lt;0.04 mm Appl. Rate</td>
<td>-------- 30 kg m² --------</td>
<td>1:4 (w/w)</td>
<td>115kg.ton⁻¹</td>
<td></td>
</tr>
<tr>
<td>Number of measurements(a)</td>
<td>7/7</td>
<td>7/7</td>
<td>8/8</td>
<td>58/115</td>
</tr>
<tr>
<td><strong>General Site Characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydraulic conductivity (cm.s⁻¹)</td>
<td>10⁻⁵</td>
<td>10⁻⁸</td>
<td>10⁻⁵</td>
<td>10⁰</td>
</tr>
<tr>
<td>Site specific sulphide %</td>
<td>2</td>
<td>85</td>
<td>6-8</td>
<td>4-15</td>
</tr>
</tbody>
</table>
Acidity values reflect metal content and pH / Eh are indicative of the degree of oxidation. For analysis of the elemental content only good quality supernatants selected along with statistically derived control samples (no CPMW additions). As the monitored number of supernatants was large due to economic constraints this reduction of samples had to be made (Table 1b). The reductions in acidity values in assumed to be treated samples compared to the control samples were remarkable. Further, the pH increases are reflected in the reductions in metals in the effluents. The importance of these partly empirical results is that both the un-replicable and the controlled replicable effluents show order of magnitude differences in element concentrations. It is proposed that this is not a coincidence but that it reflects the effect of the additions of CPMW.

### 2.2 Characteristics and composition of CPMW

As some of the CPMW grains had different colors, a size fractionation was also carried out to determine if all particles had a similar composition. All fractions were analyzed for their elemental composition but no relevant differences between the grain sizes is evident as all elements measured are above the detection limits with the exception of Co in the two finest fractions (Table 2a).

The dissolution and solubility of the elements contained in the CPMW were assessed by leaching the grains in 0.1 N sulfuric acid, distilled and rain water (Table 2b). In this table, the elements are classified as major nutrients or as co-factors in enzyme catalytic reactions. Although the smallest particles (Tyler mesh passing 270-400 or 0.053 to 0.037 mm) contained lower nutrient concentrations, the concentrations which are released are above detection limits sufficient to support growth. The leachability of the CPMW was addressed by decanting and refilling with acid, rain and distilled water through 8 cycles. Ten grams of CPMW gavel was stirred for 1 min with a magnetic stirrer in a beaker in 100 mL of the leachate solutions. The slurry was allowed to settle for a minimum of 0.5 h and the supernatant was decanted (first 3 decant cycles) which time was increased to 17 h. A stable, but increased pH value in the leachate after addition of the acid (pH 1.4) was used to signal the end of decanting. The water from all decant cycles was collected as a mixture and analyzed for elemental content (Table 2b) There was no difference in the concentrations of between stirred and non-stirred solutions. Details of the methodology are given in [37] from which the data of Table 2a and 2b have been extracted.

<table>
<thead>
<tr>
<th>Elements</th>
<th>Units</th>
<th>Control N=1</th>
<th>CPMW N=2</th>
<th>Control N=1</th>
<th>CPMW N=2</th>
<th>Control N=3</th>
<th>CPMW N=4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al</td>
<td>mg/L</td>
<td>50</td>
<td>&lt;0.005</td>
<td>870</td>
<td>120</td>
<td>89</td>
<td>5</td>
</tr>
<tr>
<td>Ca</td>
<td>mg/L</td>
<td>560</td>
<td>630</td>
<td>500</td>
<td>490</td>
<td>485</td>
<td>510</td>
</tr>
<tr>
<td>Cu</td>
<td>mg/L</td>
<td>0.59</td>
<td>0.001</td>
<td>0.68</td>
<td>17</td>
<td>86</td>
<td>0.07</td>
</tr>
<tr>
<td>Fe</td>
<td>mg/L</td>
<td>18</td>
<td>0.01</td>
<td>43</td>
<td>0.1</td>
<td>1053</td>
<td>0.02</td>
</tr>
<tr>
<td>P</td>
<td>mg/L</td>
<td>0.03</td>
<td>0.05</td>
<td>0.22</td>
<td>6.9</td>
<td>0.16</td>
<td>0.04</td>
</tr>
<tr>
<td>S</td>
<td>mg/L</td>
<td>630</td>
<td>510</td>
<td>4460</td>
<td>1060</td>
<td>3020</td>
<td>500</td>
</tr>
<tr>
<td>Zn</td>
<td>mg/L</td>
<td>0.98</td>
<td>&lt;0.005</td>
<td>9.3</td>
<td>5.3</td>
<td>2085</td>
<td>22</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>2.67</td>
<td>6.751</td>
<td>3.061</td>
<td>3.84</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Cond.</td>
<td>uS/cm</td>
<td>3410</td>
<td>1682</td>
<td>7180</td>
<td>4030</td>
<td>6725</td>
<td>1690</td>
</tr>
<tr>
<td>Eh</td>
<td>mV</td>
<td>734</td>
<td>584</td>
<td>758</td>
<td>661</td>
<td>784</td>
<td>467</td>
</tr>
</tbody>
</table>

| Acidity | mgCaCO₃/L | 656 | 39 | 6715.4 | 1090 | 5544 | 87 | 257 | 76 |

**Table 2a:** Particle size distribution and elemental content of CPMW (mg/g).
Table 2b. Release of elements from NPR 0.1N in sulphuric acid distilled water and rain. Column one (sum of decants) samples were stirred with H₂SO₄, then rested for 17 h.

<table>
<thead>
<tr>
<th></th>
<th>Ca</th>
<th>P</th>
<th>Fe</th>
<th>Mg</th>
<th>K</th>
<th>Zn</th>
<th>Mn</th>
<th>Cu</th>
<th>Mo</th>
<th>Pb</th>
<th>Co</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fine Gravel 45%</td>
<td>68400</td>
<td>24400</td>
<td>1550</td>
<td>600</td>
<td>400</td>
<td>15.7</td>
<td>4.49</td>
<td>1.84</td>
<td>2.47</td>
<td>0.45</td>
<td>0.11</td>
</tr>
<tr>
<td>Coarse Sand 52%</td>
<td>37400</td>
<td>13300</td>
<td>1230</td>
<td>650</td>
<td>200</td>
<td>8.74</td>
<td>3.05</td>
<td>1</td>
<td>1.39</td>
<td>0.27</td>
<td>0.07</td>
</tr>
<tr>
<td>Medium 2%</td>
<td>1600</td>
<td>433</td>
<td>300</td>
<td>0.2</td>
<td>0.08</td>
<td>1.1</td>
<td>1.02</td>
<td>1.55</td>
<td>0.04</td>
<td>0.04</td>
<td>0.01</td>
</tr>
<tr>
<td>Fine Sand 0.70%</td>
<td>533</td>
<td>200</td>
<td>100</td>
<td>0.23</td>
<td>0.12</td>
<td>0.41</td>
<td>0.36</td>
<td>0.49</td>
<td>0.02</td>
<td>0.02</td>
<td>0</td>
</tr>
<tr>
<td>Silt 0.30%</td>
<td>150</td>
<td>3</td>
<td>4.3</td>
<td>1</td>
<td>0.16</td>
<td>0.24</td>
<td>0.16</td>
<td>0.84</td>
<td>0.01</td>
<td>0.02</td>
<td>0</td>
</tr>
<tr>
<td>Un-Fractionated</td>
<td>30400</td>
<td>47100</td>
<td>5900</td>
<td>3630</td>
<td>158</td>
<td>28.6</td>
<td>29.7</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td></td>
</tr>
</tbody>
</table>

n.a. = not analyzed.

2.3 Which microbial groups form the organic coating of the biofilm?

The waste rock and tailings experiments solidified our assumptions about the involvement of biofilms. The next step was to identify the groups of microbes forming the biofilm, and possibly document their growth and development. This was addressed in a bioleach experiment where CPMW was added to German lignite coal columns at the University of Duisburg-Essen, Aquatic Biotechnology, Biofilm Centre and details of the experiment are given in [39]. The coal columns were sterilized and inoculated with an acidophilic enrichment culture originating from Rio Agrio, Argentina. The microbes were maintained using pyrite as sole energy source. The microbial populations were enumerated by 'most probable number' before CPMW addition and 2, 4 and 10 weeks later. In those columns without CPMW, strong bioleaching was evident as measured by electrical conductivity (Figure 3). In those columns with CPMW, there was a shift in the microbial populations after day 45. About 90% of populations consisted of neutrophilic heterotrophs covering the pyrite surfaces with a 10 µm thick biofilm, whereas without CPMW, 99% of the populations
were iron-oxidizing acidophiles in mono-layered biofilms. The elemental content of the column
effluents was not chemically analyzed due to economic constraints, but in the presence of CPMW,
the effluent was clear, which is not the case in its absence (Figure 3). The pH improved by about 0.5
units and the redox value and conductivity were lower (Figure 3).

![Effluents from coal columns. Carbonaceous Phosphate Mining Wastes (CPMW) were added to columns B2 and B4, but not to B1 and B3. Since we know that biofilm formation is stimulated by NPR we changed the designations used in all previous publications, as we suspect the carbonaceous nature is more important than the phosphate content.](image)

**Figure 3:** Effluents from coal columns. Carbonaceous Phosphate Mining Wastes (CPMW) were added to columns B2 and B4, but not to B1 and B3. Since we know that biofilm formation is stimulated by NPR we changed the designations used in all previous publications, as we suspect the carbonaceous nature is more important than the phosphate content.

Microbial ecology describes one aspect of biofilms as spatially-organized microbial consortia consisting of many metabolic groups, which alter their activity depending on the environmental conditions. It is hypothesized that atmospheric precipitation carries small particles of the added phosphate mining waste material to the mineral surface where it promotes the proliferation and growth of protective, heterotrophic, neutrophilic microorganisms. The pH within the microenvironment on the mineral surface with nutrient-bearing CPMW (P, Ca, K) is raised and organic matter is introduced probably partially from dead chemolitho-autotrophic acidophiles. The formation of a protective biofilm by neutrophilic and heterotrophic microorganisms is induced. To date, this hypothesis is not confirmed. The experiments detailed here provide evidence for the longevity of the biofilm on the pyrite mineral surfaces. However, to understand biofilm formation and longevity, the processes of biofilm establishment and development must be evaluated in the context of the CPMW additions and its effects. Hence the literature is consulted to highlight the state of the art knowledge about biofilm formation in general and specifically has CPMW characteristics which would trigger niches construction in bioreach or weathering conditions. Below is a discussion of these topics as they relate to mine wastes and CPMW. Can bad bugs be beaten by good bug’s?

3. **General Characteristics of Biofilms and Functions**

3.1 **Fundamentals of biofilm formation**

Microorganisms populate every habitable environment on Earth. They attach to surfaces and form biofilms virtually on all types of materials. Through their metabolic activity they affect the chemistry and physical properties of their surroundings. In the evolutionary context these alterations are known as niche construction, well documented for microbes. Life at the atmosphere-lithosphere boundary is a niche that is sparsely covered by thin subaerial microbial communities, which:

- has adapted to all types of terrestrial/subaerial stresses such as desiccation, extreme temperatures, low nutrient availability and intense solar radiation,
- interacts with minerals that serve both as a dwelling and a source of nutrients and trace elements, and
- enhances weathering of rocks and soil formation.
Microbial biofilm communities are ubiquitous in aquatic and terrestrial ecosystems as well as on man-made materials. There are initial colonizers and other species more likely to attach to existing biofilms [39,40] and take part in biogenic weathering on natural rocks as well as on construction materials, such as stone, steel and concrete [41-43]. Subaerial biofilm microorganisms have adapted to desiccation, solar radiation and other environmental challenges by developing protective, melanized cell walls, assuming micro-colonial architectures and symbiotic lifestyles [45].

Biofilms are the key to understanding how microbes adapted to these stresses influence and interact with the physical environment. After settling and attaching to the mineral surface, cells begin secreting extracellular polymeric substances (EPSs), which protect the cells from desiccation, and provide a three-dimensional structure linking cells into a community. This EPS matrix controls the environment around settled microbes in structural and functional manners [46].

The development of a biofilm involves at least 5 stages [47]. The first stage is the initial attachment of cells to the surface, which in most cases involves a conditioning film of organic molecules that have adsorbed to the material. In stage 2, production of EPS results in more firmly adhered, irreversible attachment. In stage 3, the biofilm develops, and characteristic biofilm architectures are formed. The developed biofilm architecture with stratification patterns emerges in mature biofilms (stage 4). At the same time the niche development of diverse metabolic groups of different microbial species and cell subpopulations within the biofilm occurs. Finally, in the last developmental stage (stage 5) the dispersion of single planktonic cells from the mature biofilm is observed. Those, motile, planktonic cells can be considered as pioneers that seek new suitable habitats for growth and biofilm formation in their new environment. These stages apply most appropriately on single-species biofilms of heterotrophic microorganisms in laboratory experiments. This holds mainly for acidophilic, chemo-litho-autotrophic or bioleaching microorganisms. Those groups are responsible for the oxidation of metal sulfides in mining waste and hence lead to contaminated AMD and they display a simple, monolayer biofilm architecture and a low production of EPSs.

In general, free-swimming, planktonic microorganisms in water bodies represent a minority of the total number of microorganisms in aquatic systems. The remaining microorganisms form biofilms and are attached to surfaces [47, 48]. They are found in soils, on inanimate mineral and rock surfaces, on living tissues, decaying biomass and interfaces, such as the neuston-air-liquid phase or the oil-water interphase [48]. EPSs, consisting of polysaccharides, proteins, lipids and nucleic acids, are important for contact and binding of planktonic cells to material surfaces during the initiation of the biofilm formation process and shape the environment of cells in established biofilms. EPSs are functional components that control environmental parameters in biofilms to some extent. Microbes have evolved specific organic coatings that mediate attachment to the substratum, protect the cells from desiccation, firmly anchor the cells to the surface, and act as an extracellular nutrient reservoir, due to presence of biodegradable material. EPSs also represent a sorption site for dissolved organic molecules from the bulk solution. Due to sorption, scavenging, and diffusion-limited transport of biocides through EPS, biofilm microorganisms are well protected from numerous threads, including biocide application and sanitation measures [46].

Extracellular polymeric substances may also be considered as a digestion system, functionalized by anchoring extracellular enzymes in the self-generated EPS environment. Therefore, the EPS components, which are species-specific, shape the biofilm community structure and control the spatial organization of microorganisms. Those can be attributed to different metabolic guilds, which are involved in synergistic, cooperative but also competitive and inhibitory interactions. At the same time, biofilms and EPSs also promote gene-transfer. Overall, biofilms and EPSs protect bacterial populations, increase their fitness and gene pool and can enhance metabolism and growth of different species in various niches [46]. Consequently, biofilm organic coatings on surfaces are a form of life insurance, functionally adapted to their microbial members, specific niches and places in the environment.

3.2 Biofilms of acidophilic, mineral-oxidizing on metal sulfide surfaces
Microbes, such as acidophilic, mineral-oxidizing bacteria, are ubiquitous in the environment [45]. These chemo-lithotrophs, such as Acidithiobacillus ferrooxidans or Leptospirillum ferrooxidans form biofilms on metal sulfide surfaces. These oxidative biofilms formed by chemo-lithotrophic microbes accelerate metal sulfide oxidation. Hence, acid rock drainage or acid mine drainage is accelerated by the presence of these organisms [49, 50]. Oxidative biofilms are most likely the first ones formed on exposed mineral surfaces. Bioleaching of pyrite often correlates with attached cells in the corrosion pits on the mineral surface [49]. Consequently, once attachment has occurred and the EPS matrix is developed, the weathering or corrosion of the metal sulfide surface is dependent on iron-oxidation activity and mass transfer of dissolution products within the EPS-matrix between the mineral and the cell [50, 51].

The attachment of cells is the first and most important process, as with this the cells begin to colonize the mineral surface and persist on the metal sulfide. Motile species of acidophilic bacteria colonize metal sulfides once acidic conditions prevail due to chemical and physical weathering of the mineral surface and chemotactical attraction towards dissolution products of metal sulfides. Motile bacterial species have also been demonstrated to sense metal sulfides, due to chemotactical sensing of dissolution products that arise due to the chemical oxidation or acidolysis of metal sulfides. A similar chemical sensing mechanism is hypothesized for Acidithiobacillus ferrooxidans5. This strain is not motile and does not have the genes associated with flagellar and chemotactical motility. However, it has a luxI/R-type Quorum Sensing (QS) cell-cell communication system, which allows for concerted control of gene expression in a cell-density dependent manner [48]. The corresponding Acyl-Homoserine-Lactone (AHL) signaling molecules were detected in pyrite and elemental sulfur cultures of this and related strains. This finding, in combination with commonly high cell densities in biofilms on mineral surfaces, suggested that cell-cell communication is involved in regulation of biofilm formation of several acidophilic leaching bacteria, as it has been described for other bacterial species [52-57].

This hypothesis has been confirmed since EPS production in Acidithiobacillus ferrooxidans is enhanced when exposed to synthetic AHLs and attachment of this species and several other strains is specifically affected by the presence of AHLs with different acyl chain lengths [56,57]. Consequently, planktonic cells may attach passively due to electrostatic interaction. More specifically, motile cells may actively sense pyrite surfaces chemotactically due to the release of dissolution products at pitting sites, such as ferrous iron and reduced sulfur compounds. These chemicals represent nutrients for the microbial cells. Cell-cell communication may be involved in sensing established bacterial cell populations on mineral surfaces. In that context it is important to note that inter-species cell-cell-communication in bioleaching bacteria was demonstrated to exert effects that influence the microbial community composition in leaching habitats. Mineral colonization is especially influenced in many species of acidophilic leaching bacteria by presence of AHL molecules [57]. In summary, chemotactical attraction or repulsion is a possible mechanism influencing attachment. However, the expression of EPS-related genes, due to sensory mechanisms and cell-to-cell communication also influences cell abilities to attach. This observation highlights cell-cell-communication as an important factor in niche generation and competition between iron-oxidizing acidophilic leaching bacteria. Some forms of these processes are most certainly involved in the mechanism of bioleaching prevention after CPMW addition to mining waste.

The process of attachment has been studied extensively in pure- and mixed-cultures [58-65]. It has been hypothesized that the attached microorganisms are presumably the ones which start and enhance the leaching process. It has become evident, that at low concentrations of iron ions (<200 mg/L), biofilm cells on pyrite surfaces are exclusively relevant for the oxidation [60], since their EPS accumulate the oxidative agent, ferric iron. This situation is common at the initiation of AMD generation from waste rocks, when mining wastes are exposed to rainwater. With elevated levels of iron ions, the activity of free-swimming, iron-oxidizing cells also becomes important. However, for attached biofilm cells, the EPSs act as an enlarged reaction space between the metal sulfide surface and the attached cells. In the case of mineral-oxidizing, acidophilic bacteria, such as Acidithiobacillus ferrooxidans or Leptospirillum ferrooxidans on sulfide mineral surfaces, their EPSs are functionalized by
The demonstrated inhibiting effect of CPMW on metal sulfide oxidation certainly has multiple causes. Chemical effects, due to acid consumption and formation of a mineral precipitate coating alone do not explain the efficacy of CPMW as only scarce phosphate signals were noted during the microscopic investigations of the organic coating. Instead, a definite cover of organics was repeatedly identified in all investigations. The microbiological implications of CPMW additions have proven to be important in several studies [35]. However, the mechanisms influencing microbial biofilms on mining waste and CPMW particles have not been thoroughly studied. We propose a mechanism that may explain the demonstrated dramatic shift of the microbial community composition on metal sulfides [35], due to stimulation of a neutral pH on the mineral surface followed by heterotrophic
niche formation. This niche is readily provided when CPMW particles are in contact with the acid
mineral surface or leachate. Once metal sulfide oxidation begins, acidic conditions prevail on the
waste rock minerals. Consequently, particles of the CPMWs start to dissolve due to acidolysis,
releasing microbial nutrients, such as PO$_4^{3-}$, Ca$^{2+}$, Mg$^{2+}$ and CO$_2$ while consuming acid. Consequently, CPMW particles provide a micro-niche for neutrophilic bacteria in the vicinity of the acidic environments colonized by mineral-oxidizing acidophiles on the metal sulfides. The development of reducing biofilms by neutrophilic bacteria on CPMW particles or in their surroundings is facilitated by the presence of the released nutrients. Simultaneously, heterotrophic bacteria and fungi may utilize biomass from acidophilic leaching bacteria. In that context it is also interesting to note that pyrite oxidation in acidophilic mixed cultures of the chemo-lithotroph Acidithiobacillus ferrooxidans and the heterotrophic Acidiphilium cryptum is more efficient at higher initial chemolithotroph / heterotroph inoculation ratios [71]. High abundances of the heterotrophs may inhibit chemo-lithotrophs, due to utilization of EPS as carbon source and release of inhibitory metabolites [72]. Likewise, there may be release of signaling molecules [54], toxins [73], biofilm dispersal factors [74] or other inhibitory or antimicrobial metabolites from the neutrophilic, heterotrophic microbial populations that are establishing in the provided micro-environmental niche provided by the CPMW particles. These factors might also efficiently inhibit, inactivate or even utilize the biomass of the neighboring acidophilic cell population. Therefore, a self-enhancing shift in the microbial community composition, representing a niche construction, is promoted by CPMW due to the formation of a protective, reducing biofilm on waste rock material. Consequently, metal sulfide oxidation and acid production will stop and the observed improved effluent characteristics would be met.

In addition to this hypothesis, CPMW inevitably supports the formation of stable, protective biofilms [29, 39]. It is anticipated, that this observation can be explained by the release of ample Ca$^{2+}$-ions upon contact with rain water [29]. At elevated pH (> 4), Ca$^{2+}$-ions support salt-bridge binding of polysaccharides. Hence, CPMW supports coagulation of cells and the attachment of free-swimming cells to mineral-attached organic material, thereby promoting biofilms. Since heterotrophs cannot grow using metal sulfides as an energy source as chemo-lithotrophs do, they must find nutrients from elsewhere after establishing a biofilm. This biofilm and the elevated pH reduce or prevent further oxidation of the metal sulfide by mineral-oxidizing, acidophilic microorganisms. These protective biofilms have been found in laboratory experiments with CPMW [35]. Biofilms have not been identified in the samples from LUNEs, but it can be assumed that the same microbiological processes take place in the field. Low acidity and the raised pH in the supernatant of the slurries obtained from the field tailings samples are indicative of some protective biofilm. An organic coating was documented on the waste rocks of the drum experiment from Le Mine Selbaie multiple times, after different, acid-generating, storage conditions [27]. These organic coatings were found in our studies to be persistent over several years. They were drought- and frost-resistant, and revivable as documented by [29, 31]. It is evident that changes in the microenvironment at the surface of the mineral will bring about changes in biofilm metabolic activity. Consequently, influencing microbial metabolism can alter habitats over time, and these influences will then exert control over the evolution and structure of microbial communities [75]. EPS produced from one species will be shared with neighboring cells. This altruistic use of a shared resource may be an evolutionary advantage as it pushes later generations of the same species up and out into better oxygen conditions, while limiting oxygen supply to others [76].

Those sharing cells are deemed to utilize less efficient alternative electron acceptors or use fermentative pathways for energy conservation. The maturation of the biofilm will therefore limit access of oxygen to the covered metal sulfide surface favoring reducing conditions at this critical interface. De Beer et al. [77] measured the concentration of oxygen at various points in a biofilm grown from an undefined consortium of microorganisms. The profiles of the concentration of oxygen within the biofilm indicated that there was a large depletion of oxygen within the bacterial clusters, while the voids between the clusters acted as conduits for the supply of oxygen to the lower regions of the clusters. Whether minute amounts of oxygen could still be available at the CPMW-treated metal sulfide surface or not is not critical, since the niche of acidophilic, metal-sulfide oxidizing
microorganisms has been eradicated by CPMW. The slight increase in pH locally and the manifested shift in the microbial community composition is evidence in this direction. A succession of this process with drainage water flowing through a waste rock pile that has been treated with CPMW particles on each lift is therefore likely to occur. This succession is likely supported by transport of fine CPMW particles with the water entering the wastes, along with the elements leached from CPMW and the suggested presence of biogenic signaling or antimicrobial substances.

4. Conclusions

Our knowledge of the microbial communities involved in bioleach operations has grown tremendously in the last few decades. The CPMW results presented here suggest that heterotrophic biofilms inhibit acidophilic bioleaching microorganisms, and by extension stops generation of acid mine drainage at its source. This approach of using CPMW as a stimulant, fostering stable, heterotrophic, reducing biofilms by acting as a neutralizing agent and releasing nutrients for microorganisms in a gentle manner without risk of eutrophication, may signal a possible resolution to Agricola’s dilemma. We hope that this contribution assists others with bioleach operations, but it might also bring about an urgently needed paradigm shift in how the mining industry approaches the environmental burden associated with acid mine drainage. Acidophilic microbes have been around for millennia interfering in man’s quest for metals. By accepting the role of microbes in mining, and approaching the wastes as microbial habitats, we can support natural, ecological repair processes, and shift mine effluents from acidic to neutral pH. Carbonaceous Phosphate Mining Wastes may be an important tool in that competition but other materials such as phosphate mine tailings have similar effects. Laboratory test work has shown success. It is now time to proceed with evaluating existing LUNEs and or start large-scale LUNEs in an operating mine.
5. References


33. Kalin, M., Improving pore water quality in reactive tailings with phosphate mining


41. Dang, H.; Lovell, C. R. Bacterial Primary Colonization and Early Succession on Surfaces in Marine Waters as Determined by Amplified rRNA Gene Restriction Analysis and Sequence Analysis of 16S rRNA Genes. Applied and Environmental Microbiology 2000, 66, 467-475.


44. Kip, N.; van Veen, J. A. The dual role of microbes in corrosion. ISME J 2015, 9, 542-551.


55. Waters, C.M.; Lu, W.; Rabinowitz, J.D.; Bassler, B.L. Quorum sensing controls biofilm formation in Vibrio cholerae through modulation of cyclic di-GMP levels and repressio of vpsT. Journal of Bacteriology 2008, 190, 2527-2536.


680 biofilm formation by Acidithiobacillus Ferrooxidans revealed by genome analysis and experimental
683 68. Bellenberg, S.; Leon-Morales, C-F.; Sand, W.; Vera, M. Visualization of capsular polysaccharide
685 69. Africa, C-J.; Harrison, S.T.L.; Becker, M.; Hille, R.Pv. In situ investigation and visualisation of
686 microbial attachment and colonisation in a heap bioleach environment: The novel biofilm reactor.
688 70. Afzal Ghauri, M.; Okibe, N.; Johnson, D. B. Attachment of acidophilic bacteria to solid surfaces:
689 The significance of species and strain variations. Hydrometallurgy 2007, 85, 72-80.
689 71. Bellenberg, S.; Florian, B.; Vera, M.; Rohwerder, T.; Sand, W. Comparative study of planktonic
690 and sessile cells from pure and mixed cultures of Acidithiobacillus ferrooxidans and Acidiphilium
692 72. Hallmann, R. Einfluss extrazellulärer, polymerer Substanzen von Leptospirillum ferrooxidans auf
dessen Bioconenose mit Acidiphilium spec. RB8, Dissertation, University of Hamburg 1996
693 73. Caiazza, N. C.; O'Toole, G. A. Alpha-toxin is required for biofilm formation by Staphylococcus
695 74. McDougald, D.; Rice, S. A.; Barraud, N.; Steinberg, P. D.; Kjelleberg, S. Should we stay or should
696 we go: mechanisms and ecological consequences for biofilm dispersal. Nature Review Microbiology
698 75. Gleeson, D.; McDermott, F.; Clipson, N. Understanding microbially active biogeochemical
699 76. Xavier, J. B.; Foster, K.R. Cooperation and conflict in microbial biofilms. Proceedings of the
701 77. De Beer, D.; Stoodley, P.; Roe, F.; Lewandowski, Z. Effects of biofilm structures on oxygen and