1 *Review*

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

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

22 **Keywords:** microbial sulphide oxidation, corrosion, mine waste and water remediation, biofilm
23 development, inhibition of acid mine and rock draiange

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1. Introduction

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

32 *1.1 General Mine Waste Management Practices*

33   The present approaches to mine waste management focus on keeping water and oxygen away
34 from waste depositories, where they are considered the main drivers of weathering. To slow the
35 infiltration of water and oxygen, waste rock is stacked and compacted and/or sealed with engineered
36 covers. For tailings, water covers have provided some reduction of oxygen penetration, slowing
37 oxidation of sulphides. Vegetation covers assisted by adding organics and bactericide may have a
38 longer-lasting effect, as recently assessed with aerial photography [4]. Control over mineralized rock
39 surface or weathering has also been pursued by adding bactericides, phosphate materials, and other
40 chemicals to develop a coating over the mineral surface. These additions have worked for a while,
41 but later become unstable [5]. One of the most intensely pursued measures was the use of phosphate-

containing materials, such 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 km^2 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.
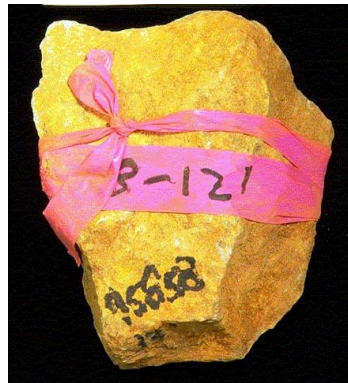
2. Approaching the geo-microbiological challenge

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

82   be defined as the process by which organisms modify their own (and other organisms')
83   environment.
84   If the niche construction is operating in these extreme habitats, then it might be operating in
85   metal leach operations. As an example, Gibraltar Mines, in British Columbia Canada reported
86   cessation of their leach dump [24]. What caused the oxidation processes to stop? This event was an
87   opportunity to observe niche construction in a primarily oxidizing habitat. Rocks from the dump
88   were obtained and investigated with Scanning Electron Microscopy (SEM) with Energy Dispersive
89   Spectroscopy (EDS) (Figure 1).
90   **Figure 1:** One of the rocks from the sections of the Gibraltar leaching dump.
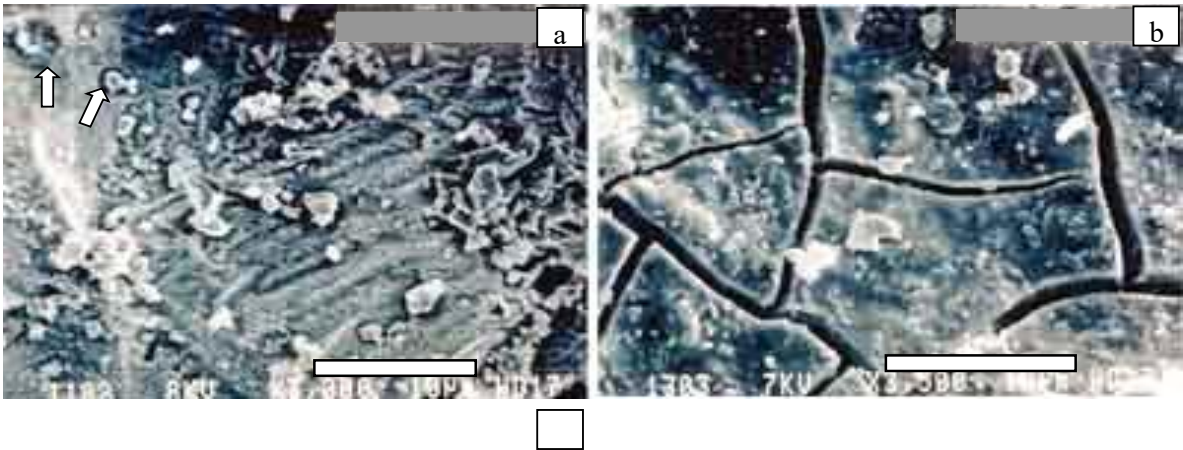

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92   The surface revealed a coating, comprised mainly of iron-hydroxide, with trace amounts of
93   phosphate, and numerous microbial colonies. These observations confirmed our belief that
94   microbiological niche construction might be operating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

2.1 Reproducable and replicable outdoor and laoratory experiments

96   To see if these results could be reproduced with other mine rocks, controlled experiment was
97   set up outdoors with sulphidic waste rock from a northern Quebec Cu/Zn mine. The waste rock
98   experiments used 2.5 tonnes of sulphidic waste rock with sulphides up to 15 % in 70 L plastic drums
99   (Table 1a). To these drums 8 L of phosphate mine waste rock, not ore but CPMW Carbonaceous
100   Phosphate Mining Waste, previously referred to as NPR. (Natural Phosphate Rock) was added on
101   top of the sulphidic rocks originating from the same mine used by [6-16]). The mineralogy was
102   identical but the utilization of a waste to treat another waste is attractive. The application on top of
103   the drums and not mixed throughout, simulated application onto waste rock lift, a practical aspect
104   easily implemented by the mine operator. It was assumed that rain would transport this niche-
105   altering substance to the mineral surface. The substance would then act on the surface by 1) providing
106   some neutralization by virtue of its carbonate content and 2) providing nutrients to modify the
107   microbial consortia, driving the mineral surface environment from oxidative to reductive. The
108   phosphate waste dosage added was driven by economics of the mine operation ($0.05/t of ore mined),
109   not by the stoichiometry of the sulphide/iron content. Effluent from the drums was collected
110   intermittently for 2.7 years, after which the experiment was dismantled. Forty percent of the 8 L of
111   CPMW added was recovered unreacted. The carbonate neutralization was insufficient to account for
112   the effluent quality. The rocks were stored in an industrial basement, until needed for further tests.
113   The mineral surfaces of rocks from the outdoor drum experiment were investigated with the same
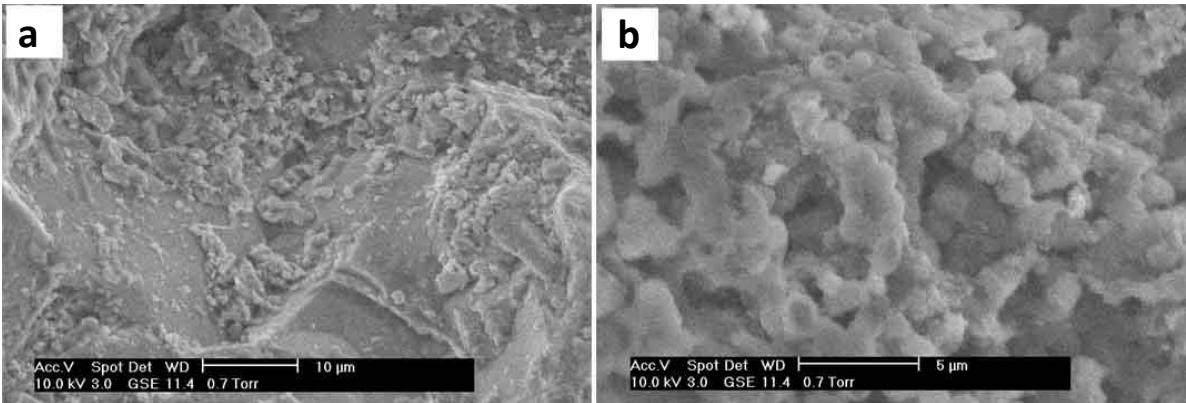114   SEM as were the rocks from the Gibraltar leach pile at the University of Toronto.

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118 Figure2a: SEM images on the surface of the waste rocks in the absence (a) or presence of NPR (b)
119 after 989 days outdoor exposure and storage (scale bars: 10μm). Surface corrosion by microbes was
120 observed in the experiment (see arrows on Figure 1a). In the presence of NPR addition, coating with
121 classical desiccation crack were recognized on the surface.

122

123 Figure 2b: ESEM images of the revived biofilms (at pH 7) on the surface of pyrite-rich waste rocks
124 isolated from drum experiments [27]. After 7 days, microbes (cocci) originally present in the drums
125 were revived and they reformed biofilms on the waste rock surface (a). Mineral precipitates could
126 also be observed on the rock surface in close association with the microbial cells, suggesting that
127 biomineralization could occur on the bacterial cell walls and EPSs (b).

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131   The differences between no CPMW and those with were striking. Electron microscopic
132   investigations revealed the expected microbial corrosion pits with microbes (Figure 2a)in contrast to
133   a coating with desiccation cracks covering the sulphides (Figure 2b). Funding for a postdoc was
134   arranged at the University of Ottawa which facilitated a detailed investigation of the desiccated
135   coating and it's revival as shown in Figure 2b. It was expected that during storage the coating would
136   vanish we re-exposed the rocks again, now stored for 4.5 years but without CPMW. Initially some
137   acid was generated, but shortly the effluent recovered to provide an circumneutral pH with low
138   acidity. The details of both outdoor exposures are summarized in [25]. Rocks from the same
139   experiment were now also shipped to the University of Western Australia as part of the P933
140   program[28]. There the mineral surface was intensely investigated with the electron microscopy,
141   quantifying the thickness of the coating, it's oxygen content and its composition. In addition CPMW
142   was added to Red Dog (Alaska, USA) mining wastes which resulted in the same findings – an organic
143   coating formed [26-29].
144
145   With these findings on the surfaces of the rocks from drum experiment, sufficient evidence had
146   emerged that the organic coating was indeed a biofilm and now was clearly the potential cause of
147   the improved effluents. However laboratory results do not necessarily translate to large-scale field
148   tests and even less to the whole waste depository.   Hence experimentation on a larger scale in the
149   field and in different field conditions is absolutely necessary, as mine waste environments are not
150   composed of static, indigenous microbial populations. This is particularly the case, as in all wastes
151   the mineral surface relevant in generating the weathering products are somewhat similar. Therefore
152   if nice constructions a fundamental evolutionary microbial process should occur in all types of
153   wastes. The microbial populations change their composition and growth dynamics with the physical
154   and chemical conditions in the wastes. Microbial populations, not the individual microbe, respond
155   to ecological processes as previously mentioned [22, 23]. Nevertheless field experiments are not well
156   received in science, as they cannot be replicated. Recently, however, large-scale field experiments
157   have gained recognition in ecology. Barley and Meeuwig [30] state that "Large-scale Un-Replicated
158   Natural Experiments" or LUNEs have aided in the formulation of hypotheses at ecologically-realistic
159   scales. These LUNEs have made positive contributions to conservation policy, providing powerful
160   insights into cosmology, evolution and geology.

161   *2.2 LUNEs, Large Un-replicable Natural Experiments*

162   Our field tests or LUNEs ranged in size from 0.5 to 1.5 ha, an area large enough to be
163   ecologically-relevant. Plots were set up on uranium and pyrrhotite tailings in Ontario Canada, and
164   on a spill area of zinc concentrate in Newfoundland (Table 1a). Sulphide content ranged from 1 to
165   95%. CPMW was ploughed about 10 to 20 cm into the tailings surface and the plots were covered
166   with straw chips or horse manure and seeded to prevent erosion of the surface during rain events.
167   The dosage of CPMW was calculated based on the costs of transporting lime otherwise used to
168   generate an erosion control cover. Details of the all the field tests and the methodology by which the
169   porewater of the tailings samples were assessed are given in [31-38]. After the one-time application
170   of CPMW, 3.1-3.8 years elapsed before sampling which secured 5 to 10 cm vertical sections below the
171   root zone. It was expected that the CPMW would lead to a hardpan below the seeded surface. Often
172   during intense rain events or snow melt the grass cover is destroyed as acid drainage rises from

173   below the root zone as the water can not drain, due to low hydraulic conductivity below the root
174   zone and particularly when the tailings are still frozen. A hard pan below the roots would prevent
175   that acid drainage from rising and generally reduce infiltration of atmospheric precipitation and
176   promote run-off.

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

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

189

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

	Uranium	Pyhrrotite	Polymetalic	Waste rock
Length of time (years)				
First Exposure (field/outdoors)	3.75	3.25	3.18	2.7
Storage indoors	6.5	5.5	0.5	4.5
Monitoring of Effluent/sol(1:5 w/v)	1.83	1.83	2.87	2.7
Selected Samples				
Waste rock ((ϕ) 0.01-0.25m)/ NPR (ϕ) 4 to <0.04 mm Appl. Rate	--------- 30 kg m² ------------		1:4 *(w/w)*	115kg.ton^{-1}
Number of measurements[a]	7/7	7/7	8/8	58/115
General Site Characteristics				
Hydraulic conductivity (cm.s^{-1})	10^{-5}	10^{-8}	10^{-5}	10^{-0}
Site specific sulphide %	2	85	6-8	4-15

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Elements	Units	Uranium		Pyhrrotite		Polymetalic concentrator spill tails		Waste rock	
	time	Control N=1	CPMW N=1	Control N=1	CPMW N=1	Control N=2	CPMW N=2	Control N=3	CPMW N=6
Al	mg/L	50	<0.005	870	120	89	5	3.98	0.42
Ca	mg/L	560	630	500	490	485	510	41	122
Cu	mg/L	0.59	0.001	0.68	17	86	0.07	14	4.13
Fe	mg/L	18	0.01	43	0.1	1053	0.02	3.77	0.32
P	mg/L	0.03	0.05	0.22	6.9	0.16	0.04	0.20	0.13
S	mg/L	630	510	4460	1060	3020	500	126	123
Zn	mg/L	0.98	<0.005	9.3	5.3	2085	22	78	22
pH		2.67	6.751	3.061	3.84	3	5	4.06	6.09
Cond.	uS/cm	3410	1682	7180	4030	6725	1690	n.a.	n.a.
Eh	mV	734	584	758	661	784	467	n.a.	n.a.
Acidity	mgCaCO$_3$/L	656	39	6715.4	1090	5544	87	257	76

n.a. - Not available

193

194   Acidity values reflect metal content and pH / Eh are indicative of the degree of oxidation. For
195 analysis of the elemental content only good quality supernatants selected along with statistically
196 derived control samples (no CPMW additions). As the monitored number of supernatants was large
197 due to economic constraints this reduction of samples had to be made (Table 1b). The reductions in
198 acidity values in assumed to be treated samples compared to the control samples were remarkable.
199 Further, the pH increases are reflected in the reductions in metals in the effluents. The importance
200 of these partly empirical results is that both the un-replicable and the controlled replicable effluents
201 show order of magnitude differences in element concentrations. It is proposed that this is not a
202 coincidence but that it reflects the effect of the additions of CPMW.

203

204   *2.2 Characteristics and composition of CPMW*

205   As some of the CPMW grains had different colors, a size fractionation was also carried out to
206 determine if all particles had a similar composition. All fractions were analyzed for their elemental
207 composition but no relevant differences between the grain sizes is evident as all elements measured
208 are above the detection limits with the exception of Co in the two finest fractions (Table 2a).
209   The, dissolution and solubility of the elements contained in the CPMW were assessed by
210 leaching the grains in 0.1 N sulfuric acid, distilled and rain water (Table 2b). In this table, the
211 elements are classified as major nutrients or as co-factors in enzyme catalytic reactions. Although
212 the smallest particles (Tyler mesh passing 270-400 or 0.053 to 0.037 mm) contained lower nutrient
213 concentrations, the concentrations which are released are above detection limits likely sufficient to
214 support growth. The leachability of the CPMW was addressed by decanting and refilling with acid,
215 rain and distilled water through 8 cycles. Ten grams of CPMW gavel was stirred for 1 min with a
216 magnetic stirrer in a beaker in 100 mL of the leachate solutions. The slurry was allowed to settle for
217 a minimum of 0.5 h   and the supernatant was decanted (first 3 decant cycles) which time was
218 increased to17 h. A stable, but increased pH value in the leachate after addition of the acid (pH 1.4)
219 was used to signal the end of decanting. The water from all decant cycles was collected as a mixture
220 and analyzed for elemental content (Table 2b) There was no difference in the concentrations of
221 between stirred and non-stirred solutions. Details of the methodology are given in [37] from which
222 the data of Table 2a and 2b have been extracted

223

224   **Table 2a:** Particle size distribution and elemental content of CPMW (mg/g).

	Ca	P	Fe	Mg	K	Zn	Mn	Cu	Mo	Pb	Co
Fine Gravel 45%	68400	24400	1550	600	400	15.7	4.49	1.84	2.47	0.45	0.11
Coarse Sand 52%	37400	13300	1230	650	200	8.74	3.05	1	1.39	0.27	0.07
Medium 2%	1600	433	300	0.2	0.08	1.1	1.02	1.55	0.04	0.04	0.01
Fine Sand 0.70%	533	200	100	0.23	0.12	0.41	0.36	0.49	0.02	0.02	0
Silt 0.30%	150	3	4.3	1	0.16	0.24	0.16	0.84	0.01	0.02	0
Un-Fractionated	304000	47100	5900	3630	158	388	28.6	29.7	n.a.	n.a.	n.a.

n.a. = not analyzed.

Table2b. Release of elements from NPR 0.1N in sulphuric acid distilled water and rain. Column one (sum of decants) samples were stirred with H_2SO_4, then rested for 17 h.

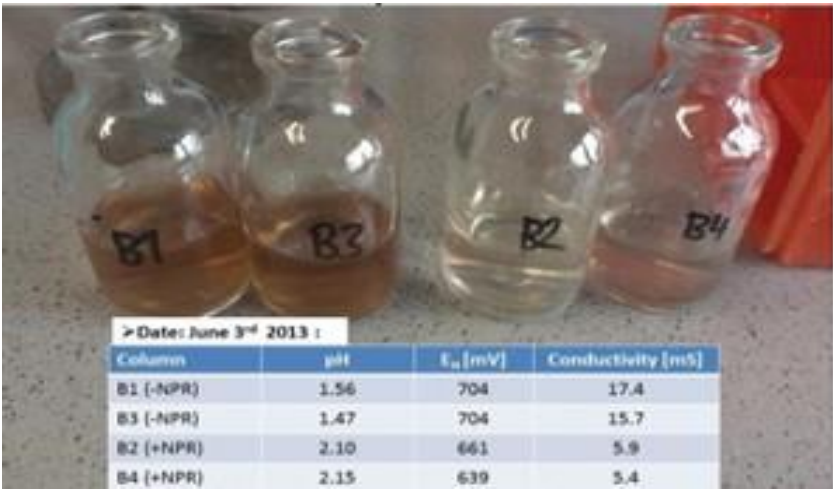|	mg/L	Sum of decants 0-8	Standing H_2SO_4	Standing DH_2O	Alkalinity mg $CaCO_3$ meq/L	Rain
Major Nutrients	Ca	620	680	510	33.6	6.65
	P	400	490	9.8	0.09	-0.06
	Fe	5.37	11.9	0.21	0.043	0.052
	Mg	18	31	0.6	1.6	1.23
	K	3.7	8.9	0.7	-4	8
Co-Factors	Zn	0.25	0.5	0.026	0.075	0.04
	Mn	0.094	0.16	0.003	-0.005	0.015
	Cu	0.017	0.09	>0.001	0.022	-0.003
	Mo	0.015	0.029	0.018	n.a.	n.a.
	Pb	>0.002	>0.002	>0.002	n.a.	n.a.
	Co	1.58	0.035	-0.001	n.a.	n.a.

n.a. = not analyzed

2.3 Which microbial groups forme the organic coating 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

242   were iron-oxidizing acidophiles in mono-layered biofilms. The elemental content of the column
243   effluents was not chemically analyzed due to economic constraints, but in the presence of CPMW,
244   the effluent was clear, which is not the case in its absence (Figure 3). The pH improved by about 0.5
245   units and the redox value and conductivity were lower (Figure 3).

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Date: June 3ʳᵈ 2013 :			
Column	pH	E_H [mV]	Conductivity [mS]
B1 (-NPR)	1.56	704	17.4
B3 (-NPR)	1.47	704	15.7
B2 (+NPR)	2.10	661	5.9
B4 (+NPR)	2.15	639	5.4

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

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

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

267   *3.1 Fundamentals of biofilm formation*

268   Microorganisms populate every habitable environment on Earth. They attach to surfaces and
269   form biofilms virtually on all types of materials. Through their metabolic activity they affect the
270   chemistry and physical properties of their surroundings. In the evolutionary context these alterations
271   are known as niche construction, well documented for microbes. Life at the atmosphere-lithosphere
272   boundary is a niche that is sparsely covered by thin subaerial microbial communities, which:
273   • has adapted to all types of terrestrial/subaerial stresses such as desiccation, extreme
274   temperatures, low nutrient availability and intense solar radiation,
275   • interacts with minerals that serve both as a dwelling and a source of nutrients and trace elements,
276   and
277   • enhances weathering of rocks and soil formation.

278   Microbial biofilm communities are ubiquitous in aquatic and terrestrial ecosystems as well as
279 on man-made materials. There are initial colonizers and other species more likely to attach to existing
280 biofilms [39,40] and take part in biogenic weathering on natural rocks as well as on construction
281 materials, such as stone, steel and concrete [41-43]. Subaerial biofilm microorganisms have adapted
282 to desiccation, solar radiation and other environmental challenges by developing protective,
283 melanized cell walls, assuming micro-colonial architectures and symbiotic lifestyles [45].
284   Biofilms are the key to understanding how microbes adapted to these stresses influence and
285 interact with the physical environment. After settling and attaching to the mineral surface, cells begin
286 excreting extracellular polymeric substances (EPSs), which protect the cells from desiccation, and
287 provide a three-dimensional structure linking cells into a community. This EPS matrix controls the
288 environment around settled microbes in structural and functional manners [46].
289   The development of a biofilm involves at least 5 stages [47]. The first stage is the initial
290 attachment of cells to the surface, which in most cases involves a conditioning film of organic
291 molecules that have adsorbed to the material. In stage 2, production of EPS results in more firmly
292 adhered, irreversible attachment. In stage 3, the biofilm develops, and characteristic biofilm
293 architectures are formed. The developed biofilm architecture with stratification patterns emerges in
294 matured biofilms (stage 4). At the same time the niche development of diverse metabolic groups of
295 different microbial species and cell subpopulations within the biofilm occurs. Finally, in the last
296 developmental stage (stage 5) the dispersion of single planktonic cells from the mature biofilm is
297 observed. Those, motile, planktonic cells can be considered as pioneers that seek new suitable
298 habitats for growth and biofilm formation in their new environment. These stages apply most
299 appropriately on single-species biofilms of heterotrophic microorganisms in laboratory experiments.
300 This holds mainly for acidophilic, chemo-litho-autotrophic or bioleaching microorganisms. Those
301 groups are responsible for the oxidation of metal sulfides in mining waste and hence lead to
302 contaminated AMD and they display a simple, monolayer biofilm architecture and a low production
303 of EPSs.
304   In general, free-swimming, planktonic microorganisms in water bodies represent a minority of
305 the total number of microorganisms in aquatic systems. The remaining microorganisms form
306 biofilms and are attached to surfaces [47, 48]. They are found in soils, on inanimate mineral and rock
307 surfaces, on living tissues, decaying biomass and interfaces, such as the neuston-air-liquid phase or
308 the oil-water interphase [48]. EPSs, consisting of polysaccharides, proteins, lipids and nucleic acids,
309 are important for contact and binding of planktonic cells to material surfaces during the initiation of
310 the biofilm formation process and shape the environment of cells in established biofilms. EPSs are
311 functional components that control environmental parameters in biofilms to some extent. Microbes
312 have evolved specific organic coatings that mediate attachment to the substratum, protect the cells
313 from desiccation, firmly anchor the cells to the surface, and act as an extracellular nutrient reservoir,
314 due to presence of biodegradable material. EPSs also represent a sorption site for dissolved organic
315 molecules from the bulk solution. Due to sorption, scavenging, and diffusion-limited transport of
316 biocides through EPS, biofilm microorganisms are well protected from numerous threads, including
317 biocide application and sanitation measures [46].
318   Extracellular polymeric substances may also be considered as a digestion system, functionalized
319 by anchoring extracellular enzymes in the self-generated EPS environment. Therefore, the EPS
320 components, which are species-specific, shape the biofilm community structure and control the
321 spatial organization of microorganisms. Those can be attributed to different metabolic guilds, which
322 are involved in synergistic, cooperative but also competitive and inhibitory interactions. At the same
323 time, biofilms and EPSs also promote gene-transfer. Overall, biofilms and EPSs protect bacterial
324 populations, increase their fitness and gene pool and can enhance metabolism and growth of different
325 species in various niches [46]. Consequently, biofilm organic coatings on surfaces are a form of life
326 insurance, functionally adapted to their microbial members, specific niches and places in the
327 environment.

328 *3.2 Biofilms of acidophilic, mineral-oxidizing on metal sulfide surfaces*

329   Microbes, such as acidophilic, mineral-oxidizing bacteria, are ubiquitous in the environment
330 [45]. These chemo-lithotrophs, such as *Acidithiobacillus ferrooxidans* or *Leptospirillum ferrooxidans* form
331 biofilms on metal sulfide surfaces. These oxidative biofilms formed by chemo-lithotrophic microbes
332 accelerate metal sulfide oxidation. Hence, acid rock drainage or acid mine drainage is accelerated by
333 the presence of these organisms [49, 50]. Oxidative biofilms are most likely the first ones formed on
334 exposed mineral surfaces. Bioleaching of pyrite often correlates with attached cells in the corrosion
335 pits on the mineral surface [49]. Consequently, once attachment has occurred and the EPS matrix is
336 developed, the weathering or corrosion of the metal sulfide surface is dependent on iron-oxidation
337 activity and mass transfer of dissolution products within the EPS-matrix between the mineral and
338 the cell [50, 51].
339   The attachment of cells is the first and most important process, as with this the cells begin to
340 colonize the mineral surface and persist on the metal sulfide. Motile species of acidophilic bacteria
341 colonize metal sulfides once acidic conditions prevail due to chemical and physical weathering of the
342 mineral surface and chemotactical attraction towards dissolution products of metal sulfides. Motile
343 bacterial species have also been demonstrated to sense metal sulfides, due to chemotactical sensing
344 of dissolution products that arise due to the chemical oxidation or acidolysis of metal sulfides. A
345 similar chemical sensing mechanism is hypothesized for *Acidithiobacillus ferrooxidans*[T]. This strain is
346 not motile and does not have the genes associated with flagellar and chemotactical motility.
347 However, it has a luxI/R-type Quorum Sensing (QS) cell-cell communication system, which allows
348 for concerted control of gene expression in a cell-density dependent manner [48]. The corresponding
349 Acyl-Homoserine-Lactone (AHL) signaling molecules were detected in pyrite and elemental sulfur
350 cultures of this and related strains. This finding, in combination with commonly high cell densities
351 in biofilms on mineral surfaces, suggested that cell-cell communication is involved in regulation of
352 biofilm formation of several acidophilic leaching bacteria, as it has been described for other bacterial
353 species [52-57].
354   This hypothesis has been confirmed since EPS production in *Acidithiobacillus ferrooxidans*[T] is
355 enhanced when exposed to synthetic AHLs and attachment of this species and several other strains
356 is specifically affected by the presence of AHLs with different acyl chain lengths [56,57].
357 Consequently, planktonic cells may attach passively due to electrostatic interaction. More
358 specifically, motile cells may actively sense pyrite surfaces chemotactically due to the release of
359 dissolution products at pitting sites, such as ferrous iron and reduced sulfur compounds. These
360 chemicals represent nutrients for the microbial cells. Cell-cell communication may be involved in
361 sensing established bacterial cell populations on mineral surfaces. In that context it is important to
362 note that inter-species cell-cell-communication in bioleaching bacteria was demonstrated to exert
363 effects that influence the microbial community composition in leaching habitats. Mineral colonization
364 is especially influenced in many species of acidophilic leaching bacteria by presence of AHL
365 molecules [57]. In summary, chemotactical attraction or repulsion is a possible mechanism
366 influencing attachment. However, the expression of EPS-related genes, due to sensory mechanisms
367 and cell-to-cell communication also influences cell abilities to attach. This observation highlights cell-
368 cell-communication as an important factor in niche generation and competition between iron-
369 oxidizing acidophiles. Some forms of these processes are most certainly involved in the mechanism
370 of bioleaching prevention after CPMW addition to mining waste.
371   The process of attachment has been studied extensively in pure- and mixed-cultures [58-65]. It
372 has been hypothesized that the attached microorganisms are presumably the ones which start and
373 enhance the leaching process. It has become evident, that at low concentrations of iron ions (<200
374 mg/L), biofilm cells on pyrite surfaces are exclusively relevant for the oxidation [60], since their EPS
375 accumulate the oxidative agent, ferric iron. This situation is common at the initiation of AMD
376 generation from waste rocks, when mining wastes are exposed to rainwater. With elevated levels of
377 iron ions, the activity of free-swimming, iron-oxidizing cells also becomes important. However, for
378 attached biofilm cells, the EPSs act as an enlarged reaction space between the metal sulfide surface
379 and the attached cells. In the case of mineral-oxidizing, acidophilic bacteria, such as *Acidithiobacillus*
380 *ferrooxidans* or *Leptospirillum ferrooxidans* on sulfide mineral surfaces, their EPSs are functionalized by

381 presence of glucuronic acid residues [61 - 63]. Chemical analyses of the EPSs of *A. ferrooxidans*, *A.*
382 *thiooxidans* and *Leptospirillum ferrooxidans* indicated a common composition of neutral sugars, fatty
383 acids and uronic acids, but differed with the strain and the growth substrate [61]. Iron ions were only
384 detectable in the EPSs of iron- or pyrite-grown cells, but not in EPSs of sulfur-grown cells. Pyrite
385 oxidation rates correlated with the amount of complexed iron ions in the case of *A. ferrooxidans* and
386 *L. ferrooxidans* [62, 63].
387   The uronic acid residues in the polymeric matrix of the EPS bind metal ions and exhibit a
388 preference for ferric iron ions. In turn, 2 moles of glucuronic acid residues bind 1 mole of ferric iron.
389 It is therefore accumulated in the extracellular space, directly at the cell surface. These ions provide
390 a positive charge to the cells, which results in a primary electrostatic attraction to metal sulfide
391 minerals, that exhibit a negative surface charge at pH < 2 [64]. In addition, the accumulation of the
392 oxidative agent, ferric iron, in the reaction space between biofilm cells and the sulfide mineral
393 provides an oxidative environment, coupling chemical oxidation of sulfide moieties of the mineral
394 with biological oxidation of ferrous iron. However, in addition to electrostatic, hydrophobic
395 interactions contribute to the firm attachment to metal sulfides and elemental sulfur that occurs as an
396 intermediate during bioleaching [61, 65, 66]. Consequently, attachment of leaching bacteria to metal
397 sulfides is influenced by pH and ionic strength.

398 *3.3 Potential Effects of CPMW on oxidative biofilms on metal sulfides surfaces*

399   CPMW, due to pH-raising acidolysis and the associated release Ca and Mg ions, will affect
400 subsequent attachment of bacteria. Temperature also affects attachment. Stress through the addition
401 of chloride or copper ions to assays with un-adapted cells inhibited attachment. In contrast, the
402 presence of 1 mM glucuronic acid, glucose or galactose enhanced EPS production and colonization
403 of pyrite surfaces [60, 67, 68]. Hence, the EPS selects for a preferential attachment to metal sulfides.
404 Likewise, the mineral surface characteristics play an equally important role such as crystal structure
405 defect sites, surface imperfections and corrosion pits on the mineral surface are colonized first. In
406 general, metal sulfides are often preferentially colonized compared to gangue minerals [69].
407 Furthermore, growth conditions and growth substrate influences cell attachment. In general, pyrite-
408 grown cells, with enhanced amounts of EPS compared to cells grown with iron(II)-ions as sole energy
409 source, attach more efficiently to metal sulfides [60,61,69].
410   In another study sixteen strains of acidophilic bacteria were screened for their abilities to adhere
411 to pyrite ore, glass beads and ferric hydroxysulfates [70]. These were strains of the iron- and sulfur-
412 oxidizer, *Acidithiobacillus ferrooxidans*, the sulfur-oxidizer *Acidithiobacillus thiooxidans* and the iron-
413 oxidizer *Leptospirillum ferrooxidans*, heterotrophic acidophiles *Acidiphilium* spp. and *Acidocella* sp. and
414 moderately thermophilic iron- and sulfur-oxidizing *Sulfobacillus thermosulfidooxidans* and *Sulfobacillus*
415 *acidophilus*. Considerable variations were found between different species of acidophiles, and also
416 between different strains of the same species, in how they attached to solids. Attachment generally
417 increased with time (over 100 min) though 99% of one *A. ferrooxidans* isolate attached to pyrite after
418 just a 10 min exposure. Also it was found that most acidophiles attached more readily to pyrite than
419 to glass beads. However, attachment to ferric hydroxysulfates was highly variable, though one *A.*
420 *ferrooxidans* isolate and one heterotrophic acidophile (*Acidocella*) attached strongly to ferric iron
421 precipitates. These minerals, namely jarosites and schwertmannite occur in AMD/ARD environments
422 and are also observed in cultures of *A. ferrooxidans* and other acidophilic, iron-oxidizing species [70].
423   The demonstrated inhibiting effect of CPMW on metal sulfide oxidation certainly has multiple
424 causes. Chemical effects, due to acid consumption and formation of a mineral precipitate coating
425 alone do not explain the efficacy of CPMW as only scarce phosphate signals were noted during the
426 microscopic investigations of the organic coating. Instead, a definite cover of organics was repeatedly
427 identified in all investigations. The microbiological implications of CPMW additions have proven to
428 be important in several studies [35]. However, the mechanisms influencing microbial biofilms on
429 mining waste and CPMW particles have not been thoroughly studied. We propose a mechanism that
430 may explain the demonstrated dramatic shift of the microbial community composition on metal
431 sulfides [35], due to stimulation of a neutral pH on the mineral surface followed by heterotrophic

432   niche formation. This niche is readily provided when CPMW particles are in contact with the acid
433   mineral surface or leachate. Once metal sulfide oxidation begins, acidic conditions prevail on the
434   waste rock minerals. Consequently, particles of the CPMW start to dissolve due to acidolysis,
435   releasing microbial nutrients, such as PO_4^{2-}, Ca^{2+}, Mg^{2+} and CO_2 while consuming acid. Consequently,
436   CPMW particles provide a micro-niche for neutrophilic bacteria in the vicinity of the acidic
437   environments colonized by mineral-oxidizing acidophiles on the metal sulfides. The development of
438   reducing biofilms by neutrophilic bacteria on CPMW particles or in their surroundings is facilitated
439   by the presence of the released nutrients. Simultaneously, heterotrophic bacteria and fungi may
440   utilize biomass from acidophilic leaching bacteria. In that context it is also interesting to note that
441   pyrite oxidation in acidophilic mixed cultures of the chemo-lithotroph *Acidithiobacillus ferrooxidans* and
442   the heterotrophic *Acidiphilium cryptum* is more efficient at higher initial chemolithotroph / heterotroph
443   inoculation ratios [71]. High abundances of the heterotrophs may inhibit chemo-lithotrophs, due to
444   utilization of EPS as carbon source and release of inhibitory metabolites [72]. Likewise, there may be
445   release of signaling molecules [54], toxins [73], biofilm dispersal factors [74] or other inhibitory or
446   antimicrobial metabolites from the neutrophilic, heterotrophic microbial populations that are
447   establishing in the provided micro-environmental niche provided by the CPMW particles. These
448   factors might also efficiently inhibit, inactivate or even utilize the biomass of the neighboring
449   acidophilic cell population. Therefore, a self-enhancing shift in the microbial community
450   composition, representing a niche construction, is promoted by CPMW due to the formation of a
451   protective, reducing biofilm on waste rock material. Consequently, metal sulfide oxidation and acid
452   production will stop and the observed improved effluent characteristics would be met.
453   In addition to this hypothesis, CPMW inevitably supports the formation of stabile, protective
454   biofilms [29, 39]. It is anticipated, that this observation can be explained by the release of ample Ca^{2+}-
455   ions upon contact with rain water [29]. At elevated pH (> 4), Ca^{2+}-ions support salt-bridge binding of
456   polysaccharides. Hence, CPMW supports coagulation of cells and the attachment of free-swimming
457   cells to mineral-attached organic material, thereby promoting biofilms. Since heterotrophs cannot
458   grow using metal sulfides as an energy source as chemo-lithotrophs do, they must find nutrients
459   from elsewhere after establishing a biofilm. This biofilm and the elevated pH reduce or prevent
460   further oxidation of the metal sulfide by mineral-oxidizing, acidophilic microorganisms. These
461   protective biofilms have been found in laboratory experiments with CPMW [35]. Biofilms have not
462   been identified in the samples from LUNEs, but it can be assumed that the same microbiological
463   processes take place in the field. Low acidity and the raised pH in the supernatant of the slurries
464   obtained from the field tailings samples are indicative of some protective biofilm. An organic coating
465   was documented on the waste rocks of the drum experiment from Le Mine Selbaie multiple times,
466   after different, acid-generating, storage conditions [27]. These organic coatings were found in our
467   studies to be persistent over several years. They were drought- and frost- resistant, and revivable as
468   documented by [29, 31]. It is evident that changes in the microenvironment at the surface of the
469   mineral will bring about changes in biofilm metabolic activity. Consequently, influencing microbial
470   metabolism can alter habitats over time, and these influences will then exert control over the
471   evolution and structure of microbial communities [75]. EPS produced from one species will be shared
472   with neighboring cells. This altruistic use of a shared resource may be an evolutionary advantage as
473   it pushes later generations of the same species up and out into better oxygen conditions, while
474   limiting oxygen supply to others [76].
475   Those sharing cells are deemed to utilize less efficient alternative electron acceptors or use
476   fermentative pathways for energy conservation. The maturation of the biofilm will therefore limit
477   access of oxygen to the covered metal sulfide surface favoring reducing conditions at this critical
478   interface. De Beer et al. [77] measured the concentration of oxygen at various points in a biofilm
479   grown from an undefined consortium of microorganisms. The profiles of the concentration of oxygen
480   within the biofilm indicated that there was a large depletion of oxygen within the bacterial clusters,
481   while the voids between the clusters acted as conduits for the supply of oxygen to the lower regions
482   of the clusters. Whether minute amounts of oxygen could still be available at the CPMW-treated
483   metal sulfide surface or not is not critical, since the niche of acidophilic, metal-sulfide oxidizing

484   microorganisms has been eradicated by CPMW. The slight increase in pH locally and the manifested
485   shift in the microbial community composition is evidence in this direction. A succession of this
486   process with drainage water flowing through a waste rock pile that has been treated with CPMW
487   particles on each lift is therefore likely to occur. This succession is likely supported by transport of
488   fine CPMW particles with the water entering the wastes, along with the elements leached from
489   CPMW and the suggested presence of biogenic signaling or antimicrobial substances.

490   **4. Conclusions**
491   Our knowledge of the microbial communities involved in bioleach operations has grown
492   tremendously in the last few decades. The CPMW results presented here suggest that heterotrophic
493   biofilms inhibit acidophilic bioleaching microorganisms, and by extension stops generation of acid
494   mine drainage at its source. This approach of using CPMW as a stimulant, fostering stabile,
495   heterotrophic, reducing biofilms by acting as a neutralizing agent and releasing nutrients for
496   microorganisms in a gentle manner without risk of eutrophication, may signal a possible resolution
497   to Agricola's dilemma. We hope that this contribution assists others with bioleach operations, but it
498   might also bring about an urgently needed paradigm shift in how the mining industry approaches
499   the environmental burden associated with acid mine drainage. Acidophilic microbes have been
500   around for millennia interfering in man's quest for metals. By accepting the role of microbes in
501   mining, and approaching the wastes as microbial habitats, we can support natural, ecological repair
502   processes, and shift mine effluents from acidic to neutral pH. Carbonaceous Phosphate Mining
503   Wastes may be an important tool in that competition but other materials such as phosphate mine
504   tailings have similar effects. Laboratory test work has shown success. It is now time to proceed with
505   evaluating existing LUNEs and or start large-scale LUNEs in an operating mine.
506

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