The microbiology of passive remediation technologies for mine drainage treatment

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Abstract

Water draining abandoned mines or mine sites is often acidic and contains relatively high concentrations of dissolved metals and sulfate. Such water (AMD) arises from the oxidative dissolution of sulfidic minerals, such as iron pyrite (FeS_2), exposed by mining activities. As AMD production can occur over a very long time scale, passive remediation systems are becoming increasingly important in the treatment of this wastewater. The passive remediation technology of choice to treat AMD from coal mines and coal spoils is compost wetlands. Using molecular-based techniques (based mainly on the 16S rRNA gene), we have followed, from the start-up phase, the changes in microbial populations of wetlands filled with different compost materials. While differences in the populations of the wetlands were evident initially, the populations converged as the wetlands aged. Microbes found to dominate in the early stages of operation were mainly cellulolytic, while sulfate-reducing microbes were detected in the later stages. The microbial composition data are being compared with those from mature wetlands to gain a fuller understanding of the evolution of microbial populations in compost wetlands.

1 Introduction

One legacy of mining activity is the pollution of local water courses. Water draining abandoned mines (both coal and metal) or solid mine wastes

(spoils or flotation tailings) often contains relatively large amounts of heavy metals. Such water can have variable pH depending on the availability of buffering salts (usually in the form of carbonate), even though it is often referred to as acid mine drainage (AMD). When AMD enters surrounding ground or surface waters, the oxidation and precipitation of metals such as iron, aluminium and manganese in AMD generates acidity, negatively impacting the receiving waters. More complete details on the formation and nature of AMD can be found elsewhere (Bank et al. 1997; Nordstrom 2000; Johnson 2003).

The low pH, combined with the higher solubility of metals at lower pH, causes severe toxicity to life within waters that are impacted by AMD. Additionally, as the iron (and aluminium) precipitate, they form a yellow/orange coating on water course surfaces, which prevents reproduction of macroscopic life and disrupts the food chain for higher life forms such as fish. Also, water that has been impacted by mine drainage is unsuitable for use as domestic or industrial water supplies. For these reasons, there is an urgent need to improve technologies that, in the long term, inhibit AMD formation or if this is not possible to treat AMD prior to release into the environment.

Treatment of AMD is currently achieved by either active or passive means (Johnson and Hallberg 2004). Active treatment entails the addition of chemical reagents such as lime (calcium oxide) to neutralize the AMD and to cause the precipitation of metals in solution. Since active treatment is costly, in terms of both capital and operating costs, and requires continual monitoring, passive treatment of AMD is becoming increasingly an attractive option. Passive treatment of AMD often takes advantage of the ability of microorganisms to generate alkalinity and immobilize metals, essentially reversing the formation of AMD. The major mechanisms by which microbes generate alkalinity is though solid-phase iron reduction (Eq. 1) and sulfate reduction (Eq. 2, where CH_2O represents organic matter), which also leads to metal immobilization as solid sulfides.

$$Fe(OH)_3 + 3H^+ + e^- \rightarrow Fe^{2+} + 3H_2O \quad (1)$$

$$SO_4^{2-} + 2CH_2O + 2H^+ \rightarrow H_2S + 2H_2CO_3 \quad (2)$$

Although passive treatment schemes vary in engineering design and construction (Younger et al. 2002), several of the more successful are compost based wetlands. These wetlands have been constructed such that the AMD passes through compost that has been added to drive the microbial sulfate reduction. While these systems are generally successful, various questions still need to be addressed about their operation, including the rates at which key biochemical reactions occur and the mechanism by

which, and rates at which, carbon supply to the sulfate reducing bacteria occurs. Such questions will help to make more sound predictions of longevity of compost-based wetlands.

To begin addressing these questions, we have taken advantage of three pilot-scale wetlands that were newly constructed at an abandoned coal mine near Aspatria, in Cumbria, U.K. The wetlands were constructed to test the suitability of different substrates, including dried sewage sludge, paper pulp mill waste or a mixture of the two, to treat coal spoil runoff. Using molecular based techniques, we have followed the changes of the microbial population during the initial period of operation of the three wetlands, as well as following changes of the sulfate-reducing bacteria that were present. We also identified the dominant microbes in the wetlands and the SRB that were detected.

2 Materials and Methods

2.1 Site description and sampling

The pilot-scale wetlands at the Aspatria site were constructed in October, 2002, by IMC Group Consulting Ltd. The site consisted of three 7.5 m wide by 27 m long surface-flow ponds (5-10 cm water depth), each filled with a different substrate, through which the coal spoil run-off passed (Fig. 1). The water chemistry of the influent to the three ponds is given in Table 1.

On site measurements included temperature, pH, redox (given against the standard hydrogen electrode potential, Eh), dissolved oxygen (DO), and conductivity were made with a YSI556 multimeter (Yellow Springs Instruments, Ohio, U.S.A.). Grab samples for chemical analysis were taken in sterile plastic sampling tubes and were filtered on-site for sulfate analysis (nitrocellulose, 0.2 μm pore size). Samples for ferrous iron analysis were added directly (or diluted first with 10 mM H_2SO_4 if necessary) to the FerroZine reagent prepared according to Lovley and Phillips (1987). Water samples were transported on ice to the laboratory.

Surface (1-5 cm) sediment material was sampled from the centre of each pond on the first trip, and 3 random surface sediment samples were taken from each pond on the second sampling event. The samples were collected into sterile plastic sample bottles and transported on ice to the laboratory, where they were processed within 12 hours. Pore water was extracted from the sediment by centrifugation and filtration (as above). The pore water was either immediately analysed or was frozen (-20ºC).

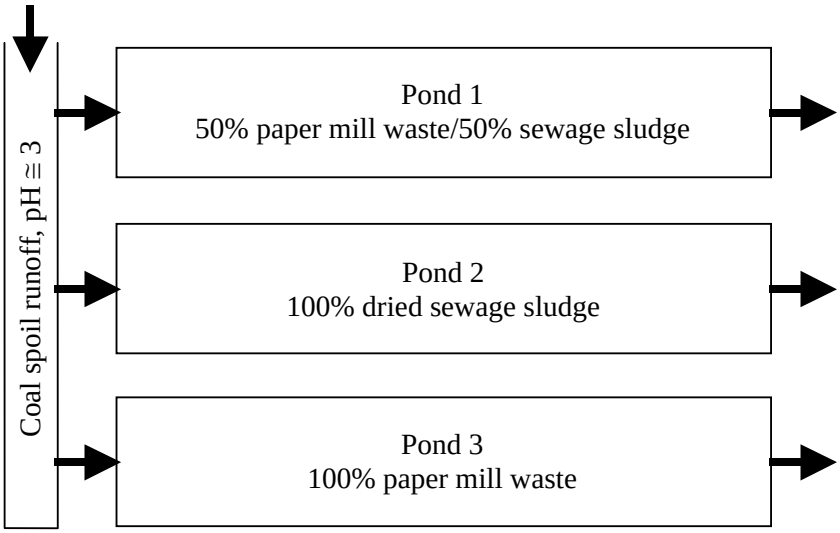

Fig. 1. Schematic diagram of the Aspatria pilot-scale wetland site. The arrows show direction of the coal spoil runoff flow. Each pond measures 27 m by 7.5 m (not drawn to scale).

2.2 Chemical analyses

Ferrous iron concentration in water samples was determined by measuring the FerroZine-ferrous iron complex at 562 nm in a Cecil 1011 spectrophotometer within 24 hours after sample collection (the coloured complex is stable for at least 96 hours). Total soluble iron was determined by adding excess ascorbic acid and remeasuring the absorbance, diluting the samples with 10 mM H_2SO_4 if necessary. Sulfate was determined turbidimetrically (as insoluble $BaSO_4$) using the Hydrocheck system (WPA, U.K.).

2.3 Molecular analysis

DNA was extracted from 0.5 g of sediment samples using the UltraClean™ Soil DNA Kit (MO BIO Laboratories, Inc., U.S.A.). Further purification was achieved with polyvinylpolypyrrolidone (Berthelet et al. 1996).

Amplification of 16S rRNA genes from the purified DNA by the polymerase chain reaction was as described previously (Okibe et al. 2003). The genes encoding the α-subunit of the adenosine-5'-phosphosulfate reductase gene (*apsA*), encoding for a subunit of an enzyme involved in sulfate reduction, were amplified from Aspatria DNA as described previously (Friedrich, 2002) except that the annealing temperature was 45ºC.

The diversity of the amplified genes was determined by terminal restriction fragment length polymorphism (T-RFLP) analysis (Marsh 1999). In this case, the genes were amplified as above in three independent reactions using forward primers labelled with the fluorescent molecule Cy5. The resulting amplification products were pooled prior to digestion with the restriction enzymes *Cfo* I or *Msp* I. The resulting Cy5-labelled terminal restriction fragments (T-RFs) were resolved on a CEQ8000 genetic analysis system (Beckman Coulter, U.K.) to within ± 1 nucleotide. For the purposes of this study, a T-RF was considered to represent a single microorganism, though a T-RF may in fact represent more than one microbe. Also, T-RFLP was used in a quantitative manner, but it should be noted that such analysis represents the final yield of the respective amplified genes, which is not necessarily directly proportional with the number of microbes present in the sediment sample.

Clone libraries of the 16S rRNA and *apsA* genes were constructed by ligating PCR products, generated without labelled primers, to the pGEM®-T Easy Vector (Promega) according to the manufacturer's instructions. Inserts were amplified using the vector specific primers SP6 and T7, and were screened for uniqueness by restriction enzyme digestion using the same enzymes as for T-RFLP analysis. Unique clones were sequenced on the CEQ8000 genetic analysis system with the DTCS QuickStart kit (Beckman Coulter). The sequences thus obtained, or the translated gene products in the case of the *apsA* genes, were compared to genes in public databases by BLAST searching (Altschul, 1997).

3 Results

3.1 Chemical analysis of the Aspatria wetlands

Following a period of 6 months after construction of the Aspatria wetlands, sampling of the wetlands was undertaken. The coal spoil run-off that entered the ponds was acidic, and contained elevated concentrations of iron (roughly 75% of which was ferrous) and sulfate (Table 1). The solutes (Fe, SO_4^{2-}, H^+) in the wetland influent increased from month 6 to month 8, possibly due to the lack of rainfall during this time period.

In contrast, the chemistry of the water that passed through the wetlands improved considerably in the intervening two months (Table 1). The pH of the effluents increased from a range that was similar to the influent at month 6 to near neutral by month 8 while the concentration of iron in the effluents decreased. Also, the Eh of the effluents decreased, indicating that sulfate reduction was occurring in the wetlands. This was most pronounced

in pond 1, where the greatest reduction in sulfate concentration also occurred.

Further indication of sulfate reduction was observed in the Aspatria porewater chemistry (data not shown). Here, lower sulfate concentrations, low Eh and higher pH (compared to the influent and effluents) implied that sulfidogenesis was occurring. The lack of detectable soluble sulfide in the porewaters was due to the measurable concentrations of soluble iron in the porewaters (exclusively as Fe^{2+}). Although not measured directly, an indication of the accumulation of metal sulfides was noted as an increased blackening of the sediments taken on month 8 compared to month 6.

Table 1. Water chemistry of the coal spoil influent to the Aspatria wetlands and of the effluents from the three ponds on months 6 and 8 of operation.

Water sample	pH	Eh (mV)	Sol. Fe^{2+} (mg/L)*	Tot. Sol. Fe (mg/L)*	Sulfate (mg/L)
11/02/03					
Influent	2.94	625	237	298	1512
Pond 1 Effluent	3.22	591	432	477	2011
Pond 2 Effluent	2.97	628	390	544	2204
Pond 3 effluent	3.22	592	430	491	2031
15/04/03					
Influent	2.68	679	533	694	2912
Pond 1 effluent	7.4	147	0	0	1115
Pond 2 effluent	6.8	712	53	137	2503
Pond 3 effluent	6.5	191	76	81	1912

* Sol. = soluble, that is what passes through a 0.2 μm pore-size filter.

3.2 Microbial populations in Aspatria wetlands

After six months of operation, a survey of the microbial populations in the wetlands at Aspatria was carried out. T-RFLP analysis of the amplified 16S rRNA genes (Fig. 2) revealed that two of the three different wetlands comprised of different microbial populations; no DNA was amplified using DNA purified from pond 2 on this occasion. The amplified DNA from Pond 1 was dominated (60%) by one microbe, represented by a terminal restriction fragment of 356 nucleotides in length. In contrast, the amplified DNA from pond 3 showed more diversity than pond 1, and was dominated to a lesser extent (20%) by one microbe with a T-RF of 99

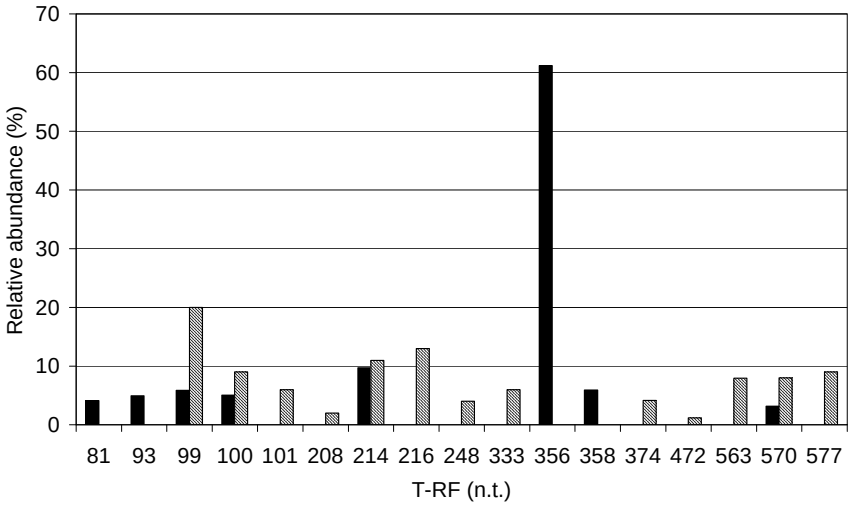

Fig. 2. Diversity and relative abundance of bacteria in Aspatria wetlands as revealed by T-RFLP analysis. 16S rRNA genes were amplified using DNA purified from pond 1 (solid bars) and pond 3 (stripped bars). The size of the terminal restriction fragments (T-RFs) is given in nucleotides (n.t.).

nucleotides. Four microbial species were apparently common to both wetlands.

On the second sampling occasion, the populations of ponds 1 and 3 had changed (data not shown). The dominant microbe in pond 1 on the first sampling occasion decreased in prevalence from 60% to 15, while the other microbes increased in predominance. The same was true for pond 3. On this occasion, DNA was amplified from pond 2 sediment samples, and T-RFLP analysis revealed it to have a different population than the other two ponds. Multiple samples taken from each of the ponds on this sampling event showed that there was little spatial variation in terms of microbial populations within a pond, indicating that this was not the cause of the observed differences in populations of the three ponds.

3.3 Identification of the dominant microbes in Aspatria pond sediment

To further identify and understand the roles of the microbes present in the Aspatria sediment samples, 16S rRNA gene libraries were constructed. Sequencing of the cloned genes revealed the majority of the microbes were cellulose-utilizing microbes (Table 2), or were unidentified microbes from

similar habitats. Sequence analysis was also able to allow assignment of one of the major T-RFs (356 n.t.) to clone ASP11.

Table 2. The nearest related microbe (and the percent identity of the two 16S rRNA genes) of the dominant cloned genes from Aspatria ponds 1 (ASP1x) and 3 (ASP3x), where x represents the clone number.

Clone	Closest relative and % homology	Comments
ASP11	*Fibrobacter* spp. ~ 92	*Fibrobacter* spp. are anaerobic cellulose degraders
ASP12	*Clostridium* spp. ~ 93	
ASP13	Soil isolate from anoxic soil 98.9	
ASP14	uncultured bacteria 97.6	found in anaerobic wastewater treatment plants
ASP15	uncultured microbe 99.2	from an anaerobic consortium
ASP16	pASP33 98.5	
ASP31	*Pseudoxanthomonas taiwanensis* 100	cellulolytic
ASP32	*Xanthomonas* spp. 94.7	96.3 % homology with pASP11
ASP33	*Brevundimonas diminuta* 90.1	*Brevundimonas* spp. are cellobiose utilizers
ASP35	uncultured bacteria 92.6	found in anaerobic wastewater treatment plants
ASP36	*Stenotrophomonas acidaminiphila* 100	aerobe from UASB reactor
ASP37	Rumen clones ~ 90	
ASP38	*Clostridium Vincentia* 98.9	*Clostridium* spp. are cellulolytic anaerobes

3.4 Sulfate-reducing bacterial populations in Aspatria wetlands

As sulfate reduction is of major importance to AMD remediation by compost wetlands, we also focussed the sulfate-reducing bacteria in the wetlands. Since the majority of the bacteria in the wetlands were not SRB, we followed SRB populations by targeting a gene encoding an enzyme specific to sulfate reduction, the *apsA* gene. T-RFLP analysis of the *apsA* gene revealed similar populations of SRB in all three wetlands. Each was dominated by one or two T-RFs (data not shown), which were common to all three ponds.

As with the 16S rRNA gene, the identities of the SRB were revealed by sequencing the cloned genes from the wetlands (data not shown). Two

clones obtained were highly related to each other. Comparison of the eight cloned genes with those in public databases revealed that they were all members of the genus *Desulfovibrio*, but none was significantly related to any described species of this well-known genus of sulfate-reducing bacteria.

4 Discussion

Three compost wetlands were constructed at an abandoned coal mine near Aspatria, U.K., to evaluate the suitability of this technology to treat the highly acidic, metal laden water that originated in the nearby coal spoil. The three wetlands (or ponds) were filled with paper pulp mill waste, dried sewage sludge or a mixture of the two to determine their effectiveness in serving as growth and energy source for the microbial activities that are key to AMD remediation (see Eqs. 1 and 2).

Six months after flow of the coal spoil runoff through the wetlands started, a monitoring programme was initiated to study the effectiveness of the wetlands for AMD remediation and to assess microbial populations in the three ponds. In the intervening two months between sampling, the concentrations of dissolved iron, protons (measured as a decrease in pH), and sulfate in the influent AMD increased, apparently as a result of the lack of rainfall in that time period.

During the same two months there was a marked improvement in the wetland effluents. The concentration of dissolved iron dropped to between 12 to 18% in pond 2 and 3 effluents, while none was detected in pond one effluent (lower limit of iron detection is 0.01 mg/L). The decrease in iron concentration in the effluents was accompanied by a decrease in sulfate concentration and an increase in pH, with pond 1 again the best performing pond (in terms of AMD remediation).

Terminal restriction fragment analysis was used to investigate the microbial populations in these wetlands. The initial results showed that the populations differed between ponds 1 and 3 (no DNA was amplified from pond 2), both in terms of the number of different microbes detected and in terms of the relative abundance of the microbes. Two months later, the populations had changed, and the microbe that dominated the early samples was not as prevalent in the latter samples. Interestingly, the populations of the 3 ponds began to appear to be converging (with each sharing more T-RFs of the same size). The dominant microbes in ponds 1 and 3 were identified by 16S rRNA gene sequence, and in the majority were cel-

lulolytic or were closely related to microbes found in anaerobic environments, such as anaerobic wastewater treatment bioreactors.

No sulfate-reducing bacteria were identified by 16S rRNA gene analysis, presumably because they form only a minor component of the total microbial population. To detect and identify SRB in the Aspatria sediment samples, it was necessary to target the *apsA* gene, which encodes for an enzyme that is involved in the reduction of sulfate to sulfide. Analysis of the SRB communities revealed that they were very similar in each of the three wetlands on both sampling occasions. The dominant SRB detected at Aspatria were all members of the genus *Desulfovibrio*, a well-studied genus of SRB with no described species that is able to use acetate as electron donor for sulfate reduction.

To further understand the underlying principles of AMD remediation by compost wetlands, it is important to understand the microbes that are driving the key biogeochemical reactions. Aside from sulfate-reduction, it is also important to know how the compost provides the key chemicals that fuel the SRB. Our studies indicate that an important step in this pathway is cellulose degradation, and that acetate is not important for sulfate reduction at Aspatria. Further analysis of microbial communities in compost based wetlands, coupled with biochemical studies will lead to a better understanding of these reactions, and ultimately allow for rational design of wetlands and better predication of their useful lifetime.

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