Microbiology of a wetland ecosystem constructed to remediate mine drainage from a heavy metal mine

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Abstract

A pilot passive treatment plant (PPTP) was constructed to evaluate the potential of a composite wetland system to remediate acidic, metal-rich water draining the former Wheal Jane tin, in Cornwall, England. The treatment plant consists of three separate and controllable composite systems, each of which comprises a series of aerobic wetlands for iron oxidation and precipitation, a compost bioreactor for removing chalcophilic metals and to generate alkalinity, and rock filter ponds for removing soluble manganese and organic carbon. To understand the roles of microorganisms in remediating acid mine drainage (AMD) in constructed wetland ecosystems, populations of different groups of cultivatable acidophilic microbes in the various components of the Wheal Jane PPTP were enumerated over a 30-month period. Initially, moderately acidophilic iron-oxidising bacteria (related to Halothiobacillus neapolitanus) were found to be the major cultivatable microorganisms present in the untreated AMD, though later heterotrophic acidophiles emerged as the dominant group, on a numerical basis. Culturable microbes in the surface waters and sediments of the aerobic wetlands were similarly dominated by heterotrophic acidophiles, though both moderately and extremely acidophilic iron-oxidising bacteria were also present in significant numbers. The dominant microbial isolate in waters draining the anaerobic compost bioreactors was an iron- and sulfur-oxidising moderate acidophile that was closely related to Thiomonas intermedia. The acidophiles enumerated at the Wheal Jane PPTP accounted for 1% to 25% of the total microbial population. Phylogenetic analysis of 14 isolates from various components of the Wheal Jane PPTP showed that, whilst many of these bacteria were commonly encountered acidophiles, some of these had not been previously encountered in AMD and AMD-impacted environments.

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1. Introduction

Many metals of commercial value occur as metal sulfides, which are often found in association with the most abundant sulfide mineral, pyrite (FeS₂). Additionally, variable amounts of pyrite are found in coal deposits. Mining of these ores and coals exposes the
pyrite to oxygen and water, which, coupled with microbial activity, leads to the formation of waters that are highly enriched with sulfate, aluminium and a range of heavy metals, the most significant of which is iron (Johnson, 2003). Other toxic elements, such as arsenic, may also be present in mine waters at elevated concentrations.

The terms “acid mine drainage” (AMD) or “acid rock drainage” (ARD) are often used to describe such waters, though these terms are somewhat misleading in some cases as AMD may have circum-neutral pH at its point of discharge from a mine or mine adit, or else contain sufficient dissolved alkaline species (principally bicarbonate) to neutralize acidity resulting from biotic or abiotic processes (Banks et al., 1997). In many cases, however, mine water discharges are acidic, due to proton production associated with the oxidation of pyrite and other metal sulfides, and to the hydrolysis of dissolved metals (iron, aluminium and manganese). Fuller accounts on the genesis and chemistries of AMD are given elsewhere (Banks et al., 1997; Nordstrom, 2000).

The impact of AMD on neighbouring streams and rivers can be very dramatic. The (often) low pH and high osmotic potential of AMD, the presence of toxic metals and metalloids, and the formation and deposition of particulate materials (such as iron and aluminium hydroxides) can result in stress and death of indigenous populations, particularly higher life-forms such as fish, resulting in reduced biodiversity. In addition, mine drainage impacted water courses cannot be used as a source of domestic or industrial water. There is, therefore, a pressing need either to halt or limit the production of AMD on site or, if this is not achievable, to treat AMD prior to its release into the wider environment.

Given that abandoned mine sites may cover very large areas, it is difficult to inhibit the formation of AMD. Therefore, treatment of the AMD is usually the only option. Active treatment of mine drainage usually consists of addition of lime (CaO) to raise the pH and cause the precipitation of metals as hydroxides. Such an approach is capital intensive, requires a ready supply of neutralising agent (which is often quarried and shipped over long distances), uses a large amount of energy, and requires people to manage the treatment process. There is, therefore, a large interest in developing “passive” remediation systems that are based on wetland ecosystems.

Observations in the early 1980s that the quality of mine water was significantly improved as it flowed through natural, sphagnum moss-dominated wetlands led to the idea that constructed wetlands could be used to remediate AMD (Wieder and Lang, 1982). Subsequently, much work in the development and engineering of wetlands to treat mine water discharges was carried out at the (then) U.S. Bureau of Mines, and the majority of early facilities were installed to treat coal mine drainage in the Appalachia region of the eastern USA (Hedin et al., 1994). Wetlands have been successfully applied in many locations to treat mine drainage (Y ounger et al., 2002), but they are usually applied to coal mine drainage, which is relatively low in metals and only mildly acidic to alkaline compared to AMD from metal mines.

Following an accidental release of approximately 50,000 m³ of metal-laden acidic water from the Wheal Jane tin mine (Younger, et al., 2004), an experimental passive treatment plant was built to evaluate the efficacy of wetlands to treat more aggressive AMD from a metal mine such as Wheal Jane. This pilot passive treatment plant (PPTP) was subjected to a 2-year period of evaluation of the plant for Wheal Jane AMD remediation. Subsequently, the PPTP became the focus of a multi-disciplinary research project aimed at understanding the fundamental principles of AMD remediation using this composite wetland approach. Given that the geochemical reactions leading to AMD remediation are microbiologically driven, the microbiology of each of the components of the PPTP was studied. In this study, the cultivatable populations of acidophiles in each of the various components of the treatment systems have been characterised in terms of numbers and types found in each of the treatment system components. Phylogenetic and some physiological information is also provided on some of the more important acidophiles that were encountered.

2. Materials and methods

2.1. Site description and sampling

A full description of the pilot passive treatment plant at Wheal Jane is given elsewhere in this issue
Inflowing AMD

Pretreatment (if any)

Aerobic cells (5) 
Fe oxidation and precipitation/As removal

Compost bioreactor 
chalcopyllic metal removal/alkalinity generation

Rock filter 
Mn removal/dissolved organic carbon removal

Tailings impoundment

Fig. 1. Flow chart of the Wheal Jane pilot passive treatment plant (“PPTP”) components and their principle function. The inflowing AMD received no pre-treatment (in the “LF system”), was lime dosed to pH 5 (“LD system”) or passed through an anoxic limestone drain (“ALD system”).
Wheal Jane; mine water taken mid-way down aerobic ponds 1, 3 and 5; compost bioreactor effluents (corresponding to A9, B16 and C7 in fig. 3 of Whitehead and Prior, 2004); and rock filter effluents (corresponding to A13, B20 and C11 in fig. 3 of Whitehead, 2004). In addition to AMD samples, the loose sediments (mainly precipitated iron (oxy)hydroxides) of the aerobic ponds underlying the mine water samples were collected.

2.2. Enumeration and isolation of acidophilic microbes

Enumeration of extremely acidophilic iron- and sulfur-oxidising bacteria was performed by serial dilution and spread plating on iron overlay ("Feo") and iron tetraionate overlay ("FeS\textsubscript{o}Q") plates both at pH 2.5 (Johnson, 1995). Two other solid media variants used include a 5 mM ferrous sulfate/5 mM sodium thiosulfate/0.025% (w/v) tryptone soya broth (TSB) overlay ("FeT\textsubscript{o}Q") solid medium with a final pH of about 4.5 to enumerate and isolate "moderately acidophilic iron-oxidising bacteria (Hallberg and Johnson, 2003a). Acidophilic heterotrophs were enumerated using yeast extract (0.02% w/v) overlay plates (pH 3.0, "YE\textsubscript{O}3", and pH 4.0, "YE\textsubscript{O}4") in which the usual heterotroph used in the underlayer of the bi-layered gel (Acidiphilium sp. SJH) was replaced with Acidocella sp. WJB-3, which does not utilise yeast extract (Hallberg et al., 1999).

Water samples were serially diluted in a sterile basal salt solution (Johnson, 1995) at pH 3.5 prior to plating. Sediment samples (approximately 5 g wet weight) were placed into 5 mL of the basal salts solution and were shaken at 1000 rpm in a test tube shaker (IKA-VIBRAX, Janke and Kunkel) for 1 h at 20 °C. After allowing the solids particles to settle by standing for 5 min, the resulting solution was serially diluted in the basal salts and plated.

Plate counts are given as colony forming units (CFU)/mL for water samples and for sediment samples as CFU/g dry weight sediment (obtained by drying a known amount of sediment at 70 °C for 48 h).

Bacteria were tentatively identified based on morphological characteristics of the resulting colonies. In many cases, bacteria could not be identified using established protocols (Johnson and Roberto, 1997) and were thus chosen for phylogenetic analysis based on 16S rRNA gene sequences (see below). The identity of those acidophiles that could be determined was also confirmed by 16S rRNA gene sequencing.

2.3. Total microbial counts

Microbes in water samples were fixed on site and microbes in a portion of the basal salts following shaking of the sediment samples were fixed with 3% (v/v) paraformaldehyde in phosphate buffered saline (pH 7.4). A known volume of the fixed cell suspensions was filtered through a 25 mm black polycarbonate membrane (0.2 μm pore size) and the cells retained on the membrane were stained for 10 min with the DNA-binding dye 4',6-diamidino-2-phenylindole (DAPI; 2 ml of a 1 μg/ml solution). Membranes were washed three times with 10 ml deionised H\textsubscript{2}O, briefly dried and placed onto glass slides. The filters were viewed using a Nikon ECLIPSE E600 microscope and a minimum of 100 cells per field was counted.

2.4. Phylogenetic analysis of novel acidophiles

Representative isolates identified by colony morphology were purified by repeated single colony isolation on the solid medium they were originally isolated on, prior to growth in 5 ml of the liquid version of the isolation medium. Following 5 to 7 days incubation, the cells were harvested by centrifugation (13,000 rpm; 10 min). Cell pellets were washed twice in sterile TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0); oxalic acid (10 mM, final concentration) was added to cultures of iron-oxidisers to dissolve ferric iron precipitates prior to washing in TE buffer. Cell pellets were resuspended in 20 μl of a lysis solution (0.05 M NaOH+0.25% SDS) and heated at 95 °C for 10 min before 180 μl of autoclaved deionised water was added.

The 16S rRNA gene from each isolate was amplified by PCR following a ‘Touchdown’ (Don et al., 1991) protocol as follows: denaturation for 30 s at 95 °C; annealing for 30 s at 55 °C, dropping by 1 °C each two cycles for 20 cycles and elongation at 72 °C for 90 s, followed by an additional 15 cycles with annealing at 45 °C and a final 10 min incubation period at 72 °C. Dimethyl sulfoxide (at 2%, v/v) was routinely included in the PCR mix, as this has been
found to increase the reliability of 16S rRNA gene amplification with some acidophilic microorganisms. The primers used were the eubacterial specific primers 27f and 1492r (Lane, 1991).

The resulting products were cloned into pGEM-T Easy vector (Promega) and sequenced commercially (MWG Biotech, Germany) using the T7 and SP6 primers and conserved primers in the 16S rRNA gene (Lane, 1991). The 16S rRNA gene sequences obtained were compared to other sequences in GenBank using the BLAST search algorithm (Altschul et al., 1997), and selected sequences were aligned to those from the Wheal Jane isolates for phylogenetic analysis using Thompson et al., 1997. These alignments were used to construct a distance matrix (Jukes and Cantor, 1969) for phylogenetic tree construction by neighbour joining (Saitou and Nei, 1987). Phylogenetic trees were viewed using Treeview software (Page, 1996). The DNA sequences obtained in this study were deposited in GenBank under the following accession numbers: WJ13, AY495953; WJ25, AY495954; WJ51, AY495955; WJ52, AY495956; WJ64, AY495957; WJ67, AY495958; WJ69, AY495959; WJ71, AY495960; and WJS0, AY495961.

2.5. Analytical methods

Ferrous iron concentration in water samples was determined colorimetrically with HEPES buffered ferrozine (Lovley and Phillips, 1987). To avoid any changes in Fe²⁺ concentration during transport of samples, they were immediately added to the ferrozine reagent on site (diluted with 10 mM H₂SO₄ if needed); control experiments showed that the iron–ferrozine complex formed was stable for at least 96 h. Total Fe was measured by atomic adsorption spectroscopy. Sulfate was measured turbidimetrically with a Hydrocheck test kit (WPA, Cambridge, UK). Dissolved organic carbon (DOC) was determined using a Protoc Analyser (Pollution and Process Monitoring, UK).

3. Results

Most of the data presented here are from one of the three component composite systems of the Wheal Jane PPTP (the LD system). While the other two systems (the “ALD” and LF systems) were also monitored throughout the study period, the general microbiological and chemical trends they displayed were similar to those of the LD system. During the latter part of the study period, the LF compost bioreactor was noted to function more effectively than the other two, which had important repercussions on water chemistries of the downstream rock filter pools as described elsewhere in this issue (Hallberg and Johnson, 2004; Johnson and Hallberg, 2004).

3.1. Physico-chemical characteristics of Wheal Jane samples

The major physico-chemical properties of the samples taken for microbiological analysis across the entire composite LD system are given in Table 1. In general, all samples taken during the 30-month period of study were acidic (pH~4 or less) with the exception of the compost bioreactor effluents. A marked decrease in total soluble iron, and changes in the relative concentrations of ferrous and ferric iron, occurred as the mine water passed through the treatment system. Redox potentials increased as mine water flowed through the aerobic cells, reflecting increasing ferric iron concentrations relative to ferrous (the ferrous/ferric couple dictating surface water Eₜ in these cells) but were significantly lower in water draining the anaerobic cells, due to poising of Eₜ by the sulfide/sulfate couple at that stage. Except for a small increase in the LD aerobic cell 1 water which is probably due to dissolution of some dolomite in the lime, concentrations of sulfate and of total dissolved ions (measured as conductivity) also showed net decreases in all three systems, again indicating that the PPTP was at least partially effective in remediating the AMD. Concentrations of DOC remained relatively small throughout the various components of the systems, except following passage through the compost bioreactors, where there was a mean increase of about 10-fold.

3.2. Enumeration of cultivatable microbes in the component sections of the PPTP

As mentioned earlier, the PPTP had been decommissioned for a period of 2 years following the initial
evaluation of the wetland system for Wheal Jane AMD remediation. In the current study, mine water was re-introduced to the PPTP on 20 March, 2000, which is referred to here as time zero. Over the subsequent 30-month period, periodic samples were taken from the various components of each of the three treatment systems of the PPTP for microbiological analysis.

The feed water, taken directly from shaft 2 of the Wheal Jane deep mine, was initially dominated by moderately acidophilic microorganisms (Fig. 2), rather than the more familiar extremely acidophilic (pH optimum ~2) iron-oxidising acidophiles such as Acidithiobacillus ferrooxidans and Leptospirillum ferrooxidans. For months 0–10, the microbial populations appeared to be quite stable (except for the extremely acidophilic sulfur oxidisers), but in samples taken during months 14 and 15, no microbes were detected on solid media inoculated with feed water AMD (detection limit was 10 CFU/ml). In contrast, total microbial counts during this period did not vary much (data not shown). The cause of this decline in

![Fig. 2. Plate counts of acidophilic microorganisms in Wheal Jane AMD samples taken from shaft 2. Microbes enumerated include moderately acidophilic iron-oxidisers (●), moderately acidophilic sulfur-oxidisers (■), extremely acidophilic iron-oxidisers (○), extremely acidophilic sulfur-oxidisers (□), and heterotrophic acidophiles (♦). The mean total microbial count of the AMD samples was 1.14×10^6 cells/ml. The time scale on the x-axis refers to months since the flow of AMD through the PPTP commenced on 20 March, 2000.](image-url)
cultivable acidophiles is uncertain, though it did coincide with the installation of new pumps for the chemical treatment plant in the shaft from where the AMD was accessed. Subsequently, the populations of cultivatable acidophiles did recover; heterotrophic isolates were found to be numerically dominant, and

![Graph](image)

Fig. 3. Numbers of cultivatable acidophiles detected in water samples taken from the aerobic cells 1 (A), 3 (B) and 5 (C) of the LD system. Microbes enumerated include moderately acidophilic iron-oxidisers (●), moderately acidophilic sulfur-oxidisers (■), extremely acidophilic iron-oxidisers (○), extremely acidophilic sulfur-oxidisers (□), and heterotrophic acidophiles (●). The mean total microbial count of the aerobic cell water samples was $1.8 \times 10^6$ cells/ml. The time scale on the x-axis refers to months since the flow of AMD through the PPTP commenced on 20 March, 2000.
moderately acidophilic iron-oxidisers recovered to similar numbers detected in samples taken during months 0–10.

As in the feed mine water, moderately acidophilic iron-oxidisers were generally more numerous than extreme acidophiles in surface waters of the aerobic

Fig. 4. Numbers of cultivatable moderately acidophilic iron-oxidisers (●), moderately acidophilic sulfur-oxidisers (■), extremely acidophilic iron-oxidisers (○), extremely acidophilic sulfur-oxidisers (□), and heterotrophic acidophiles (●) associated with the sediment samples taken from the aerobic cells 1 (A), 3 (B) and 5 (C) of the LD system. The mean total microbial count of the sediment samples was $1.8 \times 10^7$ cells/g dry weight of sediment. The time scale on the x-axis refers to months since the flow of AMD through the PPTP commenced on 20 March, 2000.
cells in the LD system (Fig. 3) among the culturable microbes. A general trend of increasing relative numbers of extremely acidophilic iron-oxidisers as the pH of the water decreased (due to hydrolysis of the oxidised iron) as it flowed through the five aerobic cells was observed. Interestingly, even though concentrations of DOC in surface waters of the aerobic cells were consistently low (mean values <3 mg/l) heterotrophic microbes were usually found to outnumber iron-oxidising autotrophs.

The acidophiles isolated from the sediment samples from the aerobic cells showed more variability, in terms of cell number fluxes and diversity of groups of acidophiles, than those in surface water samples (Fig. 4). No single group of microbes were numerically dominant, with heterotrophic microbes being found in similar numbers as iron-oxidising bacteria. Somewhat unexpectedly, although concentrations of soluble ferrous iron decreased rapidly as the water flowed through the...
aerobic cells, there was no obvious parallel decline in numbers of iron-oxidising bacteria in either sediment samples or surface waters.

Following an initial period where counts of viable bacteria in the compost bioreactor (anaerobic cell) effluent increased, the microbial populations remained relatively stable (Fig. 5). Having passed through the cells, the pH of the mine water had increased somewhat (though was generally <pH 6) and the dominant isolates were again moderate acidophiles, the majority of which were able to oxidise ferrous iron (indicated by characteristic deposition of ferric iron precipitates in the growing colonies). The next most important group, numerically, were heterotrophic acidophiles, while extremely acidophilic iron- and sulfur, were present in very low numbers (10–100/ml) in anaerobic cell effluent in the LD system, and were not detected in the LF and “ALD” systems (data not

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Origin of isolate</th>
<th>Nearest relatives</th>
<th>Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WJ2a</td>
<td>Water from LF cell 5 on FeT</td>
<td>Iron Mountain clone TRA5-3 (AF047645) Fratia aurantia DSM 6220T (AJ010481)</td>
<td>97.9</td>
</tr>
<tr>
<td>WJ6a</td>
<td>Sediment from LF cell 5 on FeO</td>
<td>Propionibacterium acnes ATCC 6919T (AB042288) Uncultured hydrocarbon seep (AF154099)</td>
<td>99.9</td>
</tr>
<tr>
<td>WJ7a</td>
<td>Sediment from LF cell 5 on FeO</td>
<td>Acidobacterium capsulatum DSM 11244T (D26171) Iron Mountain clone TRB82 (AF047646)</td>
<td>92.6</td>
</tr>
<tr>
<td>WJ13</td>
<td>AMD from shaft 2 on FeO</td>
<td>Acidithiobacillus ferroxidans ATCC 23270T (AJ278718) At. ferroxidans ATCC 33020 (AJ278719)</td>
<td>99.6</td>
</tr>
<tr>
<td>WJ18a</td>
<td>AMD from shaft 2 on FeT</td>
<td>Halothiobacillus neapolitanus DSM 581T (AF173169) Halothiobacillus sp. W5 (X97534)</td>
<td>99.3</td>
</tr>
<tr>
<td>WJ25</td>
<td>Water from LD cell 1 on FeO</td>
<td>Uncultured bacterium clone RCP1-83 (AF523909) Iron Mountain clone BA46 (AF225450) “Ferrimicrobium acidophilum” (AF251436)</td>
<td>97.7</td>
</tr>
<tr>
<td>WJ51</td>
<td>Water from LF cell 5 on YEO3</td>
<td>Acidiphilium cryptum ATCC 33463T (D30773) A. multivorans DSM 11245T (AB006711)</td>
<td>99.6</td>
</tr>
<tr>
<td>WJ52</td>
<td>Water from LF cell 5 on YEO3</td>
<td>Acidiphilium sp. NO-17 (AF376026) Uncultured bacterium clone RCP1-25 (AF523873) A. acidiphilum ATCC 27807T (D86111)</td>
<td>99.3</td>
</tr>
<tr>
<td>WJSO</td>
<td>“ALD” anaerobic cell effluent on FeT</td>
<td>Acidithiobacillus thiooxidans ATCC 19377T (Y11596) At. thiooxidans B-S3 (X75269)</td>
<td>99.8</td>
</tr>
</tbody>
</table>

The matches for each isolate are shown along with the percentage identity. The numbers in the parentheses are GenBank accession numbers for the related sequence. A superscript “T” indicates the type strain of a given species.

Data from Hallberg and Johnson (2003a).

b Data from Johnson and Hallberg (2004).
shown). It is important to note here that numbers of moderately acidophilic iron-oxidisers increased by 2 to 3 orders of magnitude compared to numbers present in mine water that entered the compost bioreactors.

As in the compost bioreactor effluents, moderately acidophilic iron-oxidisers and heterotrophic acidophiles dominated the cultivatable population in the rock filter effluents (Fig. 6). In contrast to the other components of the PPTP, there was a marked difference in numbers of cultivatable acidophilic microorganisms present in the LF rock filter effluent and corresponding effluents in the LD and “ALD” systems. Numbers of moderately and, in particular, extremely acidophilic microbes were far smaller in the LF system than in the LD and “ALD” systems, presumably reflecting the differential performances of the compost bioreactors in the PPTP (Johnson and Hallberg, 2004).

3.3. Total microbial counts in the PPTP samples

In general, total numbers of microorganisms (from DAPI staining) were remarkably similar in water samples taken from each of the components (aerobic and anaerobic cells, and rock filter pools) in each of the three systems in the PPTP, and in sediments taken from the aerobic cells. Total numbers of microorganisms were ca. 1.8 x 10^6 cell/ml of water and ca. 1.8 x 10^7 cell/g dry weight of sediment. Corresponding counts using solid media were 2.0–9.0 x 10^5 cells/ml water and 4.6 x 10^6 cells/g dry weight sediment. Plate counts therefore accounted for approximately 1 to 5% of the total cells in the various mine water samples, up to 25% of the total cells associated with the sediment samples.

3.4. Isolation and phylogenetic characterisation of predominant cultivatable bacteria in the PPTP

To understand further the role of the acidophiles in remediating Wheal Jane AMD, those isolates that appeared to be important among the cultivatable microbes, either in terms of their numerical abundance or their obvious physiological traits (e.g. iron-oxidisers) were subjected to phylogenetic analysis. Numerically dominant isolates were initially differentiated on the basis of colony morphology, and were then tested for growth on the various other solid media to facilitate preliminary identification. The identity of the isolates was then determined by sequencing of the 16S rRNA gene and comparison with gene sequences of known microbes (Table 2). A range of acidophiles was detected in the Wheal Jane PPTP, and included extremely acidophilic iron-oxidising microbes such as At. ferrooxidans and L. ferrooxidans. Two important moderately acidophilic iron-oxidisers in the Wheal Jane PPTP were also identified. These include WJ18, which was numerically dominant, among the culturable microbes, in the raw AMD and was most closely related to Halothiobacillus neapolitanus (Hallberg and Johnson, 2003a), and isolate WJ68, which was the dominant cultivatable organism in compost bioreactor effluents and which was shown to be most closely related Thiomonas intermedia (Johnson and Hallberg, 2004). One Wheal Jane microbe was highly related to microbes detected in a natural wetland that was impacted by coal spoil AMD (Broffit et al., 2002), and is also related to the iron-oxidising heterotroph “Ferrimicrobium acidiphilum”. Other Wheal Jane microbes included heterotrophs belonging to the genus Acidiphilum and some that were highly related to bacteria of the genus Fratetria, a microbe not normally associated with acidic environments. The isolation of the heterotrophs WJ6, highly related to Propionibacterium acnes, and WJ7, which is remotely related to Acidobacterium capsulatum, has been described previously (Hallberg and Johnson, 2003a).

4. Discussion

The Wheal Jane pilot passive treatment plant was constructed to evaluate the potential for passive remediation of highly acidic effluent from a heavy metal mine. It is a unique facility, in that it comprises three separate composite systems, each of which can be manipulated in terms of influx flow rate of AMD and (in the cases of the LD and “ALD” systems) pH adjustment of inflowing water. This has facilitated the multi-disciplinary project, which has studied the underlying principles of AMD remediation using wetland ecosystems (Whitehead and Prior, 2004).
Using a suite of highly efficient and selective solid media (Johnson, 1995; Hallberg and Johnson, 2003a), populations of cultivatable acidophiles in the various components of the PPTP were enumerated. The feed water to the PPTP, i.e. mine water taken directly from Wheal Jane shaft 2, comprised of a mixed population of microorganisms that was initially dominated by moderately acidophilic iron-oxidising bacteria. The dominant cultivatable isolate in this feed water was related to *Halothiobacillus* spp.; their importance in the oxidation of ferrous iron in AMD has been described elsewhere (Hallberg and Johnson, 2003a). Other iron-oxidisers identified in the AMD water included the more widely documented, extremely acidophilic iron-oxidisers *At. ferrooxidans* and *L. ferrooxidans*. The prevalence of the moderately acidophilic microbes is in keeping with the higher pH of the mine water (mean of 3.7) compared to the optimal pH (~2) for growth of the more extremely acidophilic microbes.

The population of acidophiles in the rest of the components of the PPTP also reflected the physico-chemical characteristics of the sites from which they were isolated. As the water passed through the aerobic cells, where iron oxidation and precipitation occurred, the ferrous iron concentration decreased as did the mine water pH (as a consequence of ferric iron hydrolysis). This was reflected in a subtle change in the culturable microbial populations, from the moderately acidophilic microbes to the more extremely acidophilic group. These water samples were, however, dominated by heterotrophic acidophiles. The association of heterotrophic acidophiles with iron-oxidisers has been known for some time (Harrison, 1984), but they would also be expected to be found in significant numbers in a wetland ecosystem such as the Wheal Jane PPTP, where significant inputs of organic carbon arise from the wetland plants and algae and also from the compost bioreactors.

The fact that measured DOC concentrations in surface waters of the aerobic cells were invariably small (mean <3 mg/l) presumably reflects catabolism of plant- and algae-derived organic compounds by the indigenous heterotrophic microbes. While the numbers of both moderate and extreme acidophiles present in surface waters in the aerobic cells were generally small (10^2–10^4/ml), there were larger populations of both in aerobic cell sediments. Again, these were mainly acidophilic heterotrophs and iron-oxidisers, with neither the extremely acidophilic nor moderately acidophilic microbes dominating. The importance of the iron-oxidisers associated with the aerobic cell sediments to the remediation of the Wheal Jane AMD has been described elsewhere in this issue (Hall et al., 2004).

As the mine water passed through the aerobic cells, the ferrous iron concentration decreases, but the numbers of iron-oxidisers did not. This may be due, at least in part, to the regeneration of ferrous iron from the ferric iron by biological reduction within the sediments. Pore water samples extracted from aerobic cell sediments have been shown to contain much greater concentrations of ferrous iron than surface waters, and also to contain iron-reducing acidophilic bacteria (Hallberg and Johnson, 2003b). This has been supported by the isolation of microbes from the aerobic cells that belong to the genus *Acidiphilium*, all species of which are known to reduce ferric iron (Johnson and McGinness, 1991); similar bacteria have been found in AMD-impacted sediments elsewhere (Küsel et al., 2002).

During the passage of the mine water through the compost bioreactors, a significant change in culturable microbial population occurred. The increase of numbers of moderately acidophilic microbes in the compost bioreactor effluents, compared to those entering these cells, suggests that they are being enriched in the bioreactors. This may be due, in part, to the absence of dilution of the mine water by rainfall within these enclosed cells (as occurs in the aerobic cells), but is also due to the generation of sulfide in the compost bioreactors. The main microbe found in the effluents (WJ68) was identified as a *Thiomonas* species (Table 2, and Johnson and Hallberg, 2004). All known *Thiomonas* spp. are able to oxidise reduced sulfur compounds, such as sulfide (HS-; Moreira and Amils, 1997), and only recently has their abilities also to oxidise ferrous iron been recognised (Coupland et al., 2003). Isolate WJ68 was also shown to oxidise reduced sulfur in aerobic media. It is intriguing to speculate whether this bacterium was growing in aerobic niches within the compost bioreactors, or possibly growing anaerobically, coupling the oxidation of sulfide to the reduction of ferric, which was also noted to enter these cells (Johnson and Hallberg, 2004).
The compost bioreactor effluents also contained many heterotrophic acidophiles, including some that have been found to be members of the genus *Frateuria*. These microbes are acetogenic bacteria that have been previously only found associated with plants (Swings et al., 1984). More recently, however, they have been found in mine drainage in Norway (Johnson et al., 2001) and also in acidic (pH 4–5) soils (Curtis et al., 2002). While these microbes are considered obligately aerobic, they may well be capable of anaerobic growth by fermentation.

Even though virtually all of the soluble iron present in the raw AMD had been removed from the water following passage through the final components of the remediation systems (the rock filters), a significant numbers of iron-oxidising bacteria were detected in waters draining the PPTP. These were again mainly *Thiomonas* spp. and might have been surviving on the sulfide (and sulfur), as well as the ferrous iron, that was present in compost bioreactor effluents. This hypothesis was supported by decreasing water pH and ferrous iron concentrations, and slight increases in sulfate concentration as the mine water flowed through the rock filter ponds in the LD and “ALD” systems.

In terms of the operation of the treatment system, it is important to note that the presence of these versatile microbes in the water will compromise such a treatment system should any ferrous iron be present in these effluents as well. The iron-oxidising *Thiomonas* will catalyse the oxidation of the ferrous iron to ferric iron. The subsequent hydrolysis of the ferric iron will generate more acidity, essentially leading to a reversal of the whole AMD remediation process.

The data presented here represent the first such study of a composite wetland ecosystem constructed specifically for remediating metal-rich, acidic mine drainage. The inventory of indigenous microorganisms in the various components of the Wheal Jane PPTP has revealed some interesting facts, not least of which is the importance of novel moderately acidophilic iron- and sulfur-oxidising bacteria in remediating mine water of pH>3. Taken together with other studies published in this issue concerning the activities of the microbes in relation to the remediation process, this has broadened our understanding of the biogeochemical processes that underlie the passive treatment of metal mine drainage. Further studies, however, are needed to address such topics as to what controls the populations and activities of acidophiles in these constructed ecosystems. Also, since most of the data here are of those indigenous microorganisms that could be cultivated in the laboratory, the identities and potential roles of those microbes that were not isolated need to be elucidated, using a molecular-based approach. Such continued investigations will lead to the sound design and operation of future passive treatment plants for long term, sustainable remediation of mine drainage waters.

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**References**


