Causes and consequences of mine waste microbial community structure

Submitted by

Tomasa Sbaffi

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ……..

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Author contribution

The work in each chapter represents a collaborative effort, but the majority of work is my own. Initially primary supervision came from Dr Chris G. Bryan, who contributed to the experimental design and interpretation of results. After his departure Professor Angus Buckling became my primary supervisor and contributed to the experimental design and provided feedback on the interpretation of results. Further supervision came from Dr Pawel Sierocinski who also contributed to the experimental design in Chapter 4 and 5.

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List of abbreviations

At.	*Acidithiobacillus*
BIS	Bissoe
CHY	Chyverton Mine
CA	Caradon Hill site A
CB	Caradon Hill site B
DEV	Devon Consuls Mine
HAL	Halamanning Mine
MW	Mount Wellington site
PO	Porthtowan (Tywarnhayle Mine)
PWM	Pore Water Metals
REM	Readily Extractible Metals
StA	Saint Agnes (Trevaunance Mine)
TM	Total Bulk Metals
TR	Tretherrup site
WA	Wheal Alfred
WM	Wheal Maid

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Abstract

Acid mine drainage (AMD) is a widely studied environment in microbiology and geochemistry. However, there have been far fewer detailed studies of the microbiology and biogeochemistry of historic sulfidic mine wastes giving rise to AMD. Key questions have yet to be answered about the ecological mechanisms underlying the relationship between microbial communities and mineral substrates, the environmental features imposing selective pressure on such communities compared to nearby soils and the main ecological principles that can be used to explain such complex relationships. The South West of England has been subject to intensive mining activity, resulting in a variety of mine wastes and disused underground tunnels left undisturbed for decades. The microbial consortia inhabiting these environments make an interesting case study, as they derive from the same region and yet their similarity is unknown.

Samples of mine waste and nearby soils were collected from twelve sites in Cornwall and West Devon. Geochemistry and microbial ecology were analysed to study the environmental drivers of microbial community composition. Metals from different fractions of the samples were analysed (total, readily extractable and pore water) and their compositions related to the microbial community. The microbial ecology of most sites appeared to be largely associated with pH, and to a lesser extent to the bulk metals composition. and communities were more diverse in waste sites than nearby soils. This suggested the possibility of strong local adaptation or dispersal limitation. Information on local adaptation of consortia is potentially useful for further manipulations as it provides insights into their performance in defined conditions. Therefore, inocula prepared from the twelve mine wastes were assessed for local adaptation to sympatric and allopatric substrates via a reciprocal transplant experiment. Results revealed that, with the exemption of a few sites, microbial communities were not generally locally adapted. Bioleaching performance (pyrite dissolution) was further analysed to understand how this is improved (or not) through community mixing and coalescence. Four inocula were mixed in all possible sixteen combinations to form new coalesced inocula whose performance was tested in pyrite, showing that coalescence potentially increases performance.

The results give insights for the use of communities in biotechnologies such as biohydrometallurgy, as well as the microbial ecology of AMD-generating wastes. This study contributes to the knowledge of the microbial ecology of acidophiles in the scenario of whole communities coalescence and transplant.

1 Introduction

1.1 Microbial ecology and geology of mining ecosystems

The Earth's extreme environments host a wide array of extraordinary microorganisms that are believed to have similarities to early life on the planet (Amaral-Zettler et al., 2011). Acid mine drainage (AMD) is a common result of coal and/or metal mining worldwide caused by weathering of metal sulfides exposed during mining. AMD typically results in low-pH, high-metal, high-conductivity water that does not support megafauna life but does allow microbial life. Acid mine drainage is a widespread environmental problem primarily resulting from the oxidative dissolution of pyrite (FeS_2) and other sulfide minerals exposed to oxygen and water during mining (Nordstrom & Alpers, 1999).

Although believed to have an overall low microbial diversity, these unique environments house metabolically active, tolerant/specialist microorganisms that are well adapted to the multiple environmental stresses (i.e. acidity, high metals concentrations) encountered and are mainly responsible for the generation of these sometimes hot, acid and toxic metals-rich solutions (Baker & Banfield, 2003). *Acidithiobacillus* spp. and *Leptospirillum* spp. are widely present in AMD and many other microorganisms were isolated and observed to dominate AMD environments (Hedrich et al., 2011), other molecular-based investigations have revealed that other lesser known organisms (e.g. *Ferroplasma* spp. and *Thermoplasmata* within the Archaea, and *Leptospirillum* group III plus uncultivated *Leptospirillum* strains within the *Nitrospira* class) are dominant in certain specific mine environments and they probably have important roles in the pyrite dissolution *in situ* (Bond et al., 2000; Bond et al., 2000; Huang et al., 2011; Korzhenkov et al., 2019; Mallien et al., 2018; Tan et al., 2007). Because of their biological and geochemical relative simplicity, AMD and mine waste affected environments have the potential as model systems for quantitative analysis of microbial ecology and community function (Baker & Banfield, 2003; Denef et al., 2010). Furthermore AMD and mine waste impacted environments could indeed

host a valuable novel diversity suitable for bioprospecting novel consortia useful for biotechnology and biohydrometallurgy[1] (Johnson, 2014).

1.1.1 Mine drainage

Various authors (Blowes et al., 2015; Nordstrom, 2011) have described mine drainage as waters affected by mining and mineral processing and identify six main categories: acidic, circumneutral, basic, dilute, mineralized and saline. Acidic, basic and saline AMD are briefly presented in this section to introduce the variety of samples that are the object of this study.

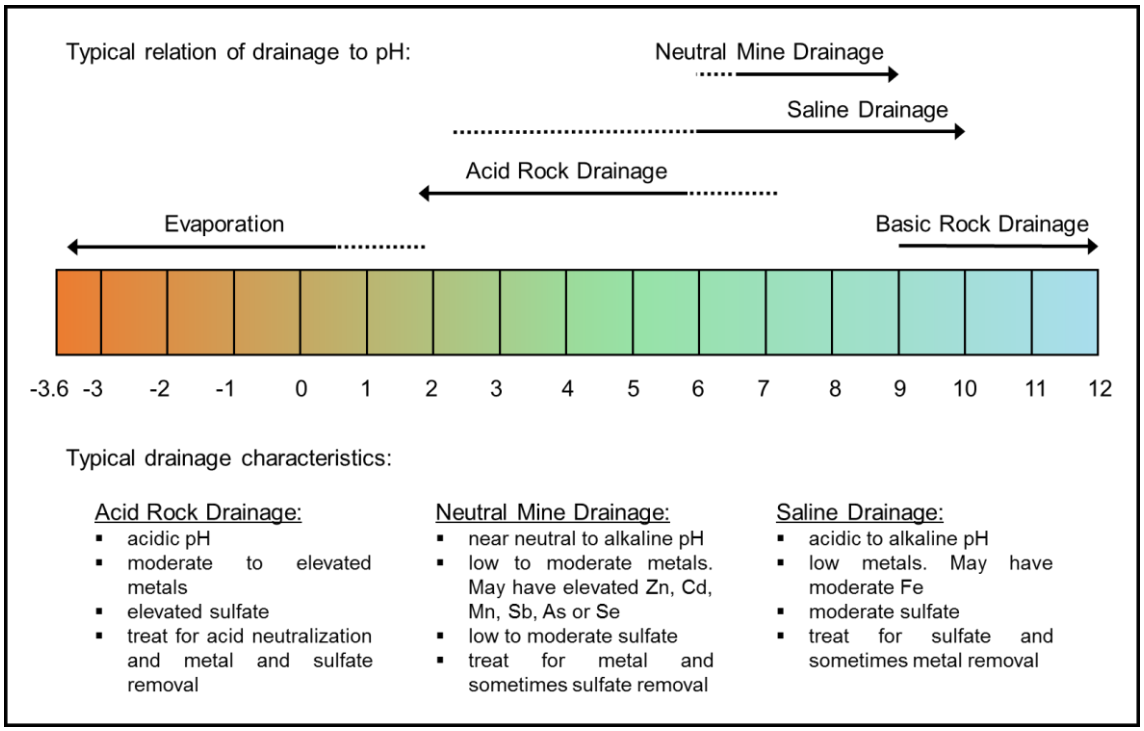


Figure 1.1. Range of pH and mine drainage characteristics. The table was modified from GARD Guide[2] and Blowes et al. (2015).

1.1.1.1 Acid mine drainage and acid rock drainage

The term 'acid rock drainage' (ARD) is widely used for any acid drainage produced from rock, whether mined or not. Acid rock drainage principally can be:

[1] "*Branch of biotechnology dealing with the study and application of the economic potential of the interactions between the microbial world and the mineral kingdom. It concerns, thus, all those engaged, directly or indirectly, in the exploitation of mineral resources and in environmental protection*" (Rossi, 1990).

[2] http://www.gardguide.com/ (GARD Guide, 2009)

- natural acid rock drainage (NARD) - the microbial weathering of naturally-exposed sulfidic minerals (Seal & Nordstrom, 2015); or
- acid mine drainage (AMD) - the microbial weathering of sulfidic materials exposed by the extractive or excavating industry .

Most ARD is AMD, and the two terms are often used interchangeably in the literature. This thesis will refer more generally to AMD and AMD sources. AMD samples usually fall in the range of pH 2–6 and sulfate is the dominant anion. For waters with pH values of 6–9, where buffering is achieved with bicarbonate equilibrium[3], most trace metals are insoluble and strongly sorbed to compounds such as iron oxides. Anionic metals and metalloids (e.g. arsenate, arsenite) are more soluble at circumneutral to basic pH because of their negative charge. At high pH values, certain metals (such as copper, zinc, tin, lead, aluminium and beryllium) become more soluble because of the amphoteric[4] nature of their compounds. The GARD Guide chart in Figure 1.1 shows the range of conditions for mine drainage.

The lowest inferred pH of acid mine drainage was - 3.6 (Nordstrom et al., 1999), which is the result of concentration through evaporation in the hot interior of the site (Iron Mountain, California). Such water is very high in dissolved constituents with a density of about 1.43 g/cm^3. There are also saline waters that are acidic but that partly depends on the definition used for saline water or salinity. Some mine drainage begins circumneutral but, because of the high ferrous iron content and insufficient alkalinity, becomes acidic as a result of the precipitation of iron hydroxides after oxidation of the ferrous iron in solution (Kirby & Cravotta, 2005).

[3] When carbon dioxide dissolves in water a small fraction is hydrated to form carbonic acid. Because it is acidic, carbonic acid dissolves calcium carbonate rocks, neutralizing the water, and forming calcium bicarbonate. Buffering is due to the presence of carbon dioxide, carbonic acid, bicarbonate ions, and carbonate ions and effectively promotes resistance to changes in pH. If acid (hydrogen ions) is added to this buffer solution (water with dissolved calcium bicarbonate), the equilibrium is shifted, and carbonate ions combine with the hydrogen ions to form bicarbonate. Subsequently, the bicarbonate then combines with hydrogen ions to form carbonic acid, which can dissociate into carbon dioxide and water. Thus, the system pH is buffered even though acid was introduced.

[4] An amphoteric compound is a molecule or ion that can react both as an acid and as a base. Many metals form amphoteric oxides or hydroxides.

1.1.1.2 Saline drainage

Both dissolved chloride concentrations and conductivity can be used to assess salinity. It can be defined more generally as the quantity of dissolved solids in water. A classification system was suggested by (Bulletin, 1958) based on sodium chloride concentration, modified in a more general way to total dissolved solids by Davis & DeWiest (1966), and modified again by Kharaka & Hanor (2003). The cutoff between saline water and brine was updated by Kharaka & Hanor (2003) using the salinity of seawater, 35,000 mg/L, as a useful reference value. A saline water is also considered to be any water with more than 10,000 mg/L dissolved solids [5] up to seawater.

Table 1.1. Classification of waters by salinity (Kharaka & Hanor, 2003).

Category	Total dissolved solids (TDS) concentration, (mg/L)
Freshwater	< 1,000
Brackish water	1,000–10,000
Saline water	10,000–35,000
Brine	>35,000

According to the classification scheme shown in Table 1.1, most AMD would be brackish water, the next most abundant would be classed as saline, and a smaller number would qualify for either freshwater or brine. Conversely, sodium chloride is rarely a major component of more superficial mine waters. An exception is made for mines near the coast that have seawater intrusion and mines that are in mid-continent areas in the vicinity of evaporite deposits commonly have high sodium chloride content. Furthermore, when mining is very deep, brackish, saline water, or brine may be encountered underlying fresh groundwater. In some mines located far from the ocean, vugs (cavity inside rock) have been discovered that contained brines dominated by sodium-calcium-chloride (Edmunds et al., 1984, 1987). Likewise, discharges from underground coal mines have been documented with elevated concentrations of alkaline and halogen elements and non-seawater Br/Cl ratio which could be attributed to same waters and residual salts in sedimentary rocks and mixing of freshwater and brines from deep-lying

[5] Total dissolved solids (TDS) is a measure of the dissolved combined content of all inorganic and organic substances present in a liquid in molecular, ionized, or micro-granular (colloidal sol) suspended form (Rhoades, 2018).

oil and gas-bearing formations (Cravotta, 2008). Likewise a coal deposit in Poland (Janina coal mine) sits in a saline aquifer and the coal mines are salt-rich (Herzig et al., 1986).

Examples of saline mine drainage are found in the Cornish environment. Cornish tin mine waters have been found to contain from 90 to 19,300 mg/L total dissolved solids (Edmunds et al., 1984, 1987; Edmunds & Shand, 2008). The increased salinity is caused by sodium–calcium-chloride type water that is thought to originate from fluid reactions with the rock. Seawater intrusion is still a viable hypothesis in addition to rock-derived salinity. The pH values range from 3.5 to 8.35 and temperatures range from 15 to 44 °C. Sulfate concentrations are less than typical seawater values (<2,700 mg/L; Millero (2001)) and generally increase with lower pH values consistent with pyrite oxidation. Two examples of mine waters reflecting the range of chemical composition are Wheal Jane and South Crofty (Table 1.2).

Table 1.2. Example of a circumneutral saline mine water from Cornwall tin mine South Crofty (Edmunds et al., 1984).

Constituent / feature	Concentration in South Crofty mine water (mM)
Temperature (°C)	41.50
pH	7.21
HCO_3^-	1.05
Cl^-	338.47
SO_4^{2-}	1.80
Ca^{2+}	58.01
Mg^{2+}	2.99
Sr^{2+}	0.47
Na^+	193.13
K^+	3.45
Li^+	17.14
Total Fe	0.08
Total Mn	0.09
Total Ni	0.003
Total Cu	0.0003

1.1.1.3 Neutral mine drainage

Neutral pH mine drainage refers to drainage waters with pH values ranging between 6 and 9 that contain other dissolved constituents, principally SO_4^{2-} and dissolved metals derived from sulfide oxidation. Neutral mine drainage occurs in settings where the acid consumption typically associated with carbonate-derived neutralization capacity is sufficient to maintain neutral pH conditions. Neutral drainage is observed at sites where the acid neutralization capacity associated with carbonate mineral content is greater than the acid generation potential associated with the sulfide content. In these settings, neutral pH conditions will be maintained throughout the duration of acid generation (Blowes & Jambor, 1990; Kirby & Cravotta, 2005; Lindsay et al., 2009). Neutral drainage is also observed in the early stages of the weathering of mine wastes with an acid generation capacity in excess of the carbonate-based neutralization capacity (Jurjovec et al., 2002). In these conditions, neutral pH conditions will continue until the carbonate mineral content is depleted to the extent that the rate of acid consumption no longer exceeds the rate of acid generation. The neutral pH period prior to the onset of acid generation is referred to as the lag period and may extend for a few months to many years, in the environment. Some waters contain potential acidity due to the presence of high concentrations of dissolved, ferrous iron. This water may become acidic downstream: spontaneous oxidation of the ferrous iron as the water becomes oxygenated leads to the formation of insoluble ferric iron, which may then precipitate as iron oxy-hydroxides, generating proton acidity in the process.

1.1.1.4 Basic mine drainage

Occasionally mine water has been found to have pH values above 9. The circumstances that can lead to such water compositions mainly include:

- low permeability rock with abundant feldspars that can react in a closed system, exchange of protons for alkalis with removal of bicarbonate through calcite precipitation (Nordstrom et al., 1989),
- evolution of groundwater to a sodium bicarbonate type through ion exchange, sulfate reduction, and organic carbon oxidation (Chapelle, 2001; Thorstenson et al., 1979),

- Dissolution of altered marls [6] that contained lime (Khoury et al., 1985).

1.1.2 Mine Waste

Mining generates waste materials that are potentially hazardous to the environment (Banks et al., 1997; Blowes et al., 2003; Johnson, 2006) as well as for human and animal health (Eisler, 2004; Li et al., 2013; Ogola et al., 2002). Given the increasing demand for metals in general and for some, such as the rare earth elements, for which new markets have arisen in recent years, humans will continue to exploit previously unexploited ore bodies, though recovery of metals from other sources, such as reprocessing mine wastes, could also provide significant quantities of metals for manufacturing industries. Future developments in the metal mining industry are likely to focus on more environment-friendly technologies that are less demanding of energy and have far smaller carbon footprints than opencast and/or deep-mining operations and using pyro-metallurgy. For example, *in situ* biomining could allow target metals to be extracted from deeply-buried ore bodies without the need to raise rocks to the surface, or to crush and mill the ore (Batterham, 2014).

Solid waste generated by metal mining can be divided into two main categories:

- waste rock,
- mine tailings.

Tailings are fine grain wastes generated during the concentration of target metal minerals from others in milled ores by methods such as froth flotation or density separation. Dumps of waste rock are composed of sand-sized particles to large stones and have less potential to generate polluting drainage waters than tailings. Waste rock covers a wide range of materials, from over-burden, to marginal material that can't be concentrated, to historic material dumped before the advent of modern concentration techniques (which is what is most common in the environment of Cornwall).

[6] Unconsolidated sedimentary rock or soil consisting of clay and lime.

Many commercially-valuable base metals, such as copper and zinc, occur as sulfide minerals, and these are often associated in ore bodies with other, relatively non-valuable minerals, such as pyrite (FeS_2), as well as other gangue minerals. The eventual presence of pyrite and other sulfide minerals in tailings wastes, as well as their fine grain size, makes them potentially highly reactive. The mechanisms involved in the oxidative dissolution of sulfide minerals have been described in many review articles and publications (Vera et al., 2013). Pyrite can be oxidized by both molecular oxygen and ferric iron, the relative importance of which depends on the solubility of ferric iron, which is pH-dependent (Evangelou & Zhang, 1995). Lime is frequently added to suppress the flotation of pyrite and, as a result, fresh mine tailing may be alkaline.

As pH declines, ferric iron becomes increasingly important as the main oxidant of sulfide minerals, for example in which pyrite is oxidized via the “thiosulfate” pathway (Vera et al., 2013):

$$6Fe^{3+} + FeS_2 + 4H_2O \rightarrow 7Fe^{2+} + S_2O_3^{2-} + 6H^+ \quad (1)$$

For the process to continue, ferrous iron has either to be re-oxidized *in situ*, or ferric iron delivered from another location. Unlike ferric iron-catalysed pyrite oxidation, the re-oxidation of the ferrous iron generated does require molecular oxygen. At low pH, this reaction is very slow. However, the presence of iron-oxidizing prokaryotes can accelerate this process by several orders of magnitude (Singer & Stumm, 1970). The thiosulfate formed in equation (1) is oxidized via various sulfur intermediates, to sulfuric acid, which again is catalysed by (sulfur-oxidizing) acidophilic prokaryotes.

$$S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+ \quad (2)$$

The sulfuric acid produced in this reaction not only increases the rate of sulfide mineral dissolution (by increasing the solubility of ferric iron) but also allows other cationic metals (including aluminum, and many transition metals) to be retained in solution.

1.1.2.1 Waste-rock piles

Waste rock is composed of the poorly mineralized rock that surrounds ore bodies, which is excavated to gain access to the ore deposit. Although the largest accumulations of waste rock are associated with open pit mining operations

(Amos et al., 2015; Lefebvre et al., 2001), many underground mines produce substantial waste rock piles. The generation of acid drainage in waste rock piles is controlled by strongly coupled interactions between:

- gas transport, water flow and solute transport;
- microbially mediated geochemical reactions;
- mineralogy of the waste materials and
- secondary reaction products.

External forcing variables, i.e. those due to variations in wind velocity or ambient climatic conditions, can drive changes in temperature, pore-gas concentration, and pore-gas pressures within the waste rock pile, influencing sulfide oxidation rates (Amos et al., 2009). Heat is generated with bacterially mediated sulfide oxidation and this can result in the development of thermally driven convective cells transporting gas, which can drive oxygen transport deep into the waste rock pile, expanding the volume of rock undergoing active oxidation (Amos et al., 2015; Lefebvre et al., 2001). A complete description of waste rock hydrology, geochemistry and mineralogy, and the potential for AMD generation in waste rock is provided by Amos et al. (2015).

Waste rock piles are non-homogeneous, containing a mixture of coarse rock and fine-grained materials, therefore a mixture of large void spaces intermingled with zones containing small pore spaces. As a result of this heterogeneity, hydrology within the waste rock pile is quite complex. Due to capillary constraints, the majority of the pore water typically flows through interconnected zones of partly saturated fine grained materials (Neuner et al., 2013). During periods of increased infiltration due to intense precipitation events, pore-water pressures can displace water from the fine-grained matrix into larger cavities, resulting in rapid macropore flow. Gas transport in waste rock piles is also affected by the grain size distribution of the rock.

Modern mining techniques favour the construction of large scale waste rock piles with a disposal technique which enhances segregation of the rock, resulting in the development of a coarse-grained rubble zone at the base of the pile and a thinner one near the pile surface. This structure enhances gas transport into the pile. Because of the presence of the large empty spaces, oxygen penetrates

deeply into the waste rock at the base of the pile, promoting sulfide oxidation reactions in this zone (Amos et al., 2015). The exothermic sulfide oxidation reactions generate heat, resulting in the development of thermally driven gas convection cells, increasing acidification and solute release in the central portion of the pile. As sulfide mineral oxidation progresses, reaction products are transported through the pile, interacting with other minerals contained in the wastes (Smith et al., 2013). These reactions can result in neutralization of acidic pore water or can result in the formation of secondary minerals and gases that accumulate along the flow paths. Subsequent dissolution of these precipitates can result in release of solutes which can then be transported through the waste rock piles to underlying aquifers or to surface water bodies.

1.1.2.2 Tailings piles

Tailings consist of wastes from concentration techniques. Tailings are finely ground and most commonly transported through pipelines and disposed of as wet slurries in impoundments or increasingly using “paste dry stack” disposal approaches. After the initial deposition of tailings, drainage of the slurry waters often occurs allowing the upper tailings to become exposed to atmospheric oxygen. Because of the fine-grained nature of the tailings, the water content in the pore spaces of tailings can remain fairly high, limiting advective transport of oxygen. In most impoundments oxygen ingress occurs through diffusion and may be restricted to the upper few meters of the tailings (Blowes & Jambor, 1990; Johnson et al., 2000; Lindsay et al., 2015; Moncur et al., 2005).

Gunsinger et al. (2006) conducted a study of gas transport in pyrrhotite-rich mill tailings at the Farley mine site, Lynn Lake, Ontario. At this site, milled tailings were segregated on the basis of grain size. As a consequence of this deposition strategy, the moisture content of the fine-grained tailings was greater than for the coarse fraction. Field measurements of pore gas oxygen concentrations and complementary mineralogical studies indicated more extensive sulfide oxidation in the coarse-grained tailings area with only limited oxidation of the fine-grained tailings. A comparison of the simulation results indicated the potential for more extensive and prolonged sulfide oxidation in the portions of the impoundment containing coarse-grained tailings, resulting in greater release of sulfate, iron and nickel.

Although gas transport in mill tailings is less rapid than is observed in waste rock piles, the sulfide content of tailings is generally greater, resulting in more extensive sulfide oxidation. In addition, the fine grained nature of the tailings limits the rate of infiltration of precipitation and can result in the transport of high concentrations of sulfide mineral oxidation products downward and laterally through the tailings. Reactions between these solutes and non-oxidized tailings, or surrounding geological materials, can result in neutralization of the pore- water pH and formation of secondary minerals.

As a consequence of the fine particle size, water flow through mill tailings is typically described using continuum flow models, acid-neutralization and secondary mineral formation reactions tend to approach equilibrium or near equilibrium conditions, resulting in the development of distinct acid-neutralization patches that are constrained by precipitation and dissolution reactions (Blowes & Jambor, 1990; Gunsinger et al., 2006; Johnson et al., 2000; Morin et al., 1988). The common acid-neutralization sequence proceeds from dissolution of calcite and dolomite, followed by dissolution of siderite, Al hydroxide phases, and Fe (III) hydroxide phases. Johnson (2000) defined acid neutralization zones within the Nickel Rim tailings impoundment (Sudbury, Ontario) and described how metal mobility was associated with pH and transitions between predominant acid-neutralization mechanisms. For example, higher concentrations of dissolved Ni were observed in regions where acid-neutralization predominantly occurred through dissolution of $Fe(OH)_3$ and $Al(OH)_3$, compared with, for example, dissolution of siderite and calcite. In addition to acid neutralization by primary and secondary carbonate and hydroxide phases, dissolution of aluminosilicate minerals also can contribute to acid neutralization (Moncur et al., 2005). Furthermore Jurjovec (2002) conducted an integrated study of acid neutralization mechanisms in tailings which combined laboratory column experiments and modelling. Results indicated the development of a series of acid neutralization *plateaux*. The release of dissolved metals from the column was closely associated with the predominant acid neutralization mechanism, with Zn, Ni, and Co released during the initial stages of the acid-neutralization sequence, whereas sharp increases in the concentrations of Cd, Pb, Al, Cr, and V were observed as carbonate minerals were depleted and the pH declined to less than 4.5.

1.1.3 Mineralogy and geochemistry

The primary source of most AMD and mine waste problems arises from the oxidation of iron sulfide minerals and subsequent release of acidity, sulfate, and dissolved metals.

Other mineral groups such as sulfates, carbonates, oxides, and aluminosilicates also release metals. Blowes & Jambor (1990) offer a classification of minerals:

1. Primary minerals: those initially present in the ore. They include the sulfide minerals initially present in the orebody, sulfide, (oxy) hydroxide and hydroxysulfate minerals associated with supergene enrichment[7], and the silicate and carbonate gangue minerals associated with the ore body.
2. Secondary minerals: form in mine workings or mine wastes. They are usually derived from oxidation reactions within the mine wastes, and include sulfate minerals (i.e. gypsum, Fe (III) (oxy) hydroxide minerals, jarosite).

1.1.3.1 Primary sulfides

The iron sulfide minerals, pyrite and pyrrhotite (respectively FeS_2 and $Fe_{(1-x)}S$ where x = 0 to 0.2), are commonly implicated in the generation of AMD. The complete oxidation of these minerals results in the generation of SO_4^{2-}, H^+ and the release of ferrous iron into solution. Ferrous iron is oxidized to ferric iron, as shown in Figure 1.2, consuming some of the acid generated from the oxidation of the sulfide.

[7] Mineral deposition process/strategy: surface oxidation produces acid that leaches metals, carrying metals downwards and re-precipitating them; in this way such process enriches the mineral.

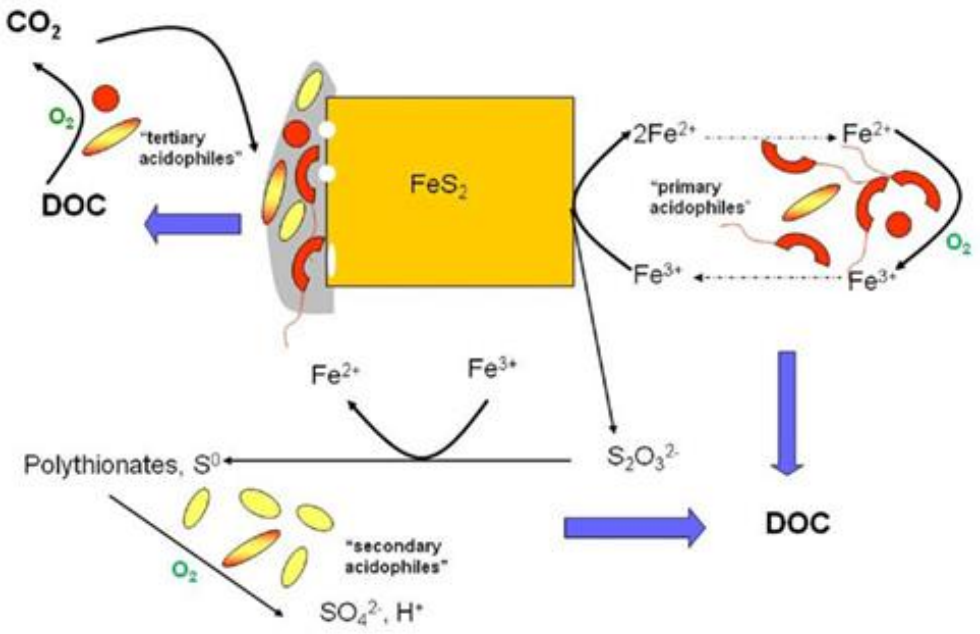


Figure 1.2. Simplified representation of the oxidative dissolution of pyrite in acidic liquor helped by the presence of acidophilic community (adapted from Johnson et al., (2012)).

Primary acidophiles are able to oxidise ferrous to ferric iron accelerating the dissolution of ferrous iron from pyrite (FeS_2). Secondary acidophiles are sulfur-oxidisers, mainly responsible for acidification (production of H^+ ions). Tertiary acidophiles are heterotrophs, thus they can metabolise dissolved organic carbon derived from decomposing dead cells and other cellular exudates and oxidise it to carbon dioxide.

The oxidation of ferrous to (soluble) ferric iron is a proton-consuming reaction. At pH above 3, the precipitation of ferric iron hydroxides and hydroxysulfates (as well as ferric iron hydrolysis) results in the generation of additional acidity. Substitution of trace elements in the pyrite and pyrrhotite structure can result in the release of high concentrations of these elements to solution. For example, increasing concentrations of Ni and Co in drainage water from waste rock at the Diavik Diamond Mine (Canada) has been experimentally attributed to the weathering and oxidation of Ni- and Co- bearing pyrrhotite (Langman et al., 2015; Neuner et al., 2013; Smith et al., 2013). Trace element-bearing sulfide minerals, commonly present in ore deposits and mine wastes can contribute to acid generation, and significantly release toxic dissolved constituents, such as As, Cd, Cu, Mo, Ni, Pb, and Zn, to mine drainage waters.

Another example is the Waite Amulet mine site (Rouyn-Noranda, Quebec, Canada), where the oxidation of the pyrite-rich sulfide mineral assembly in the tailings resulted in the generation of acidic pore water (pH < 4.0) containing high concentrations of Cu, Zn, Pb, and Ni (Blowes & Jambor, 1990). Oxidation of pyrite and arsenopyrite contained in the carbonate-rich tailings at the Delnite mine (Ontario, Canada) site resulted in neutral pH pore water containing low concentrations of dissolved Fe (less than 10 mg/L), high concentrations of sulfate (2,500–4,500 mg/L), and high concentrations of dissolved arsenate (up to 45 mg/L, 4 m below ground surface).

1.1.3.2 Secondary sulfate minerals

Secondary sulfate minerals associated with mine workings and mine wastes may provide a source of acidity, sulfate, and dissolved metals to mine drainage. One well known example of the potential for release of dissolved elements from secondary sulfate minerals (e.g. pyrite, with chalcopyrite, sphalerite) is the Iron Mountain mine in Canada (Nordstrom et al., 2000a, 2000b). Extensive sulfide oxidation released high concentrations of SO_4^{2-}, Fe, and other metals to the mine waters. Further enrichment by evaporation-concentration resulted in the development of highly acidic waters, with pH values as low as -3.6 and the formation of a range of secondary sulfate minerals (Nordstrom et al., 2000b). Controlled dissolution experiments indicate the potential for continued release of acidic water in the absence of on-going sulfide oxidation. Further, Al et al. (1994) characterized tailings derived from the Kidd Creek metallurgical site (Ontario, Canada). There, sulfide-bearing tailings are co-disposed with the residues derived from the jarosite precipitation process used at the Kidd Creek zinc refinery (containing also gypsum and iron oxyhydroxides). Co-presence of the jarosite residue and the sulfide-bearing concentrator tailings results in the reductive dissolution of the jarositic wastes, with a decline in the pore water pH and increased concentrations of dissolved sulfate, iron, zinc, and other metals (Al et al., 1994). According to the authors, ferric iron hydrolysis causes the release of acidity, following jarosite dissolution (Smith et al., 2006). More recently, increased concentrations of dissolved Ba within the sulfate-reducing zone of a tailings impoundment were observed (Lindsay et al., 2011), possibly due to the dissolution of barite concentrated by removal of sulfate via anaerobic bacterially mediated sulfate reduction.

1.1.4 Geochemical and microbiological processes

1.1.4.1 Aqueous iron oxidation

Ferric iron is a more effective oxidant than oxygen in acidic aqueous environments. The oxidation of dissolved ferrous iron to ferric iron is a key to understanding the oxidation of sulfide minerals and it is a more complex process than it might seem. The oxidation rates change by orders of magnitude depending on:

- pH,
- presence or absence of iron-oxidizing microorganisms,
- nutrient availability and mass transfer (e.g. provision of oxygen and carbon dioxide),
- hydrolysis of dissolved ferric iron,
- polymerization of ferric iron and
- precipitation.

For example, in AMD, iron can precipitate as various secondary minerals such as schwertmannite, goethite, ferrihydrite, lepidocrocite, and jarosite. Laboratory and field studies have revealed that ferrihydrite tends to dominate at pH values > 5.5, jarosite tends to dominate at pH 0.8 – 2.5, and schwertmannite tends to dominate at intermediate pH values (Bigham et al., 1996). Schwertmannite converts to goethite at the intermediate to higher pH values (2.5–5.5) (Bigham et al., 1996; Regenspurg et al., 2004; Schwertmann & Carlson, 2005) and to jarosite at lower pH values (Wang et al., 2006).

Ferrous iron concentration decreases with time by microbial iron-oxidation (Figure 1.3A). The sigmoidal shape of the curve indicates the microbial growth from the lag phase through exponential growth to exponential decay as the energy source becomes smaller and smaller (Nordstrom, 2003; Nordstrom & Campbell, 2014).

Experimentally, when ferrous iron was allowed to oxidize in 9K medium at 26 °C, over a range of pH values from 1.4 to 2.4, a rise in pH was observed for each run

(Figure 1.3C; Liao at al., 2009) due to the oxidation of ferrous iron, which consumes protons:

$$Fe^{2+} + ¼\ O_2 + H^+ \rightarrow Fe^{3+} + ½\ H_2O \quad (3)$$

Over time, the Fe^{3+} hydrolyses and decreases the pH through the release of protons:

$$Fe^{3+} + H_2O \rightarrow Fe(OH)^{2+} + H^+ \quad (4)$$

Finally, the hydrolysing iron precipitates as a mineral phase and further decreases the pH and approaches an equilibrium state:

$$Fe(OH)^{2+} + H_2O \rightarrow \alpha\text{-}FeO.OH_{(goethite)} + 2H^+ \quad (5)$$

After peaking, pH values eventually decrease because the hydrolyses of ferric iron happens more rapidly at higher pH values and the pH increase from oxidation (Eq. (3)) and cannot be distinguished from pH decrease due to hydrolysis (Eq. (4)). This increase in hydrolysis rate is related to the difference in initial pH from the pH equivalent of the first hydrolysis constant for ferric iron, pK = 2.2. This effect can be seen in the data from Kupka et al. (2007) (Figure 1.2B): an iron oxidation study with 9K medium at low temperature (5 °C). The lower temperature would also slow the rate of microbial growth so that the peaks in the pH curves would take longer to be obtained.

In both studies the rate of iron oxidation also seems to slow with lower initial pH. In Figure 1.2C, the initial rate (or the lag phase) is enhanced by the presence of iron precipitates (primarily a mixture of schwertmannite and goethite). If more ferrous iron is added to a solution similar to this when ferrous iron has gone to zero, the oxidation of ferrous iron is immediate without any lag phase in the presence of microorganisms (Blowes et al., 2015). Research continues studying the rates of ferrous iron oxidation because it is the main control on the rate of pyrite oxidation. This rate is microbially mediated at low pH values and as much as six orders of magnitude faster than the abiotic rate (Singer & Stumm, 1970). Research also keeps on investigating ferrous iron oxidation rates in circumneutral mine waters, where microbial catalysis has a minor impact as long as the environment is aerobic (Cravotta, 2015; Kirby et al., 2009).

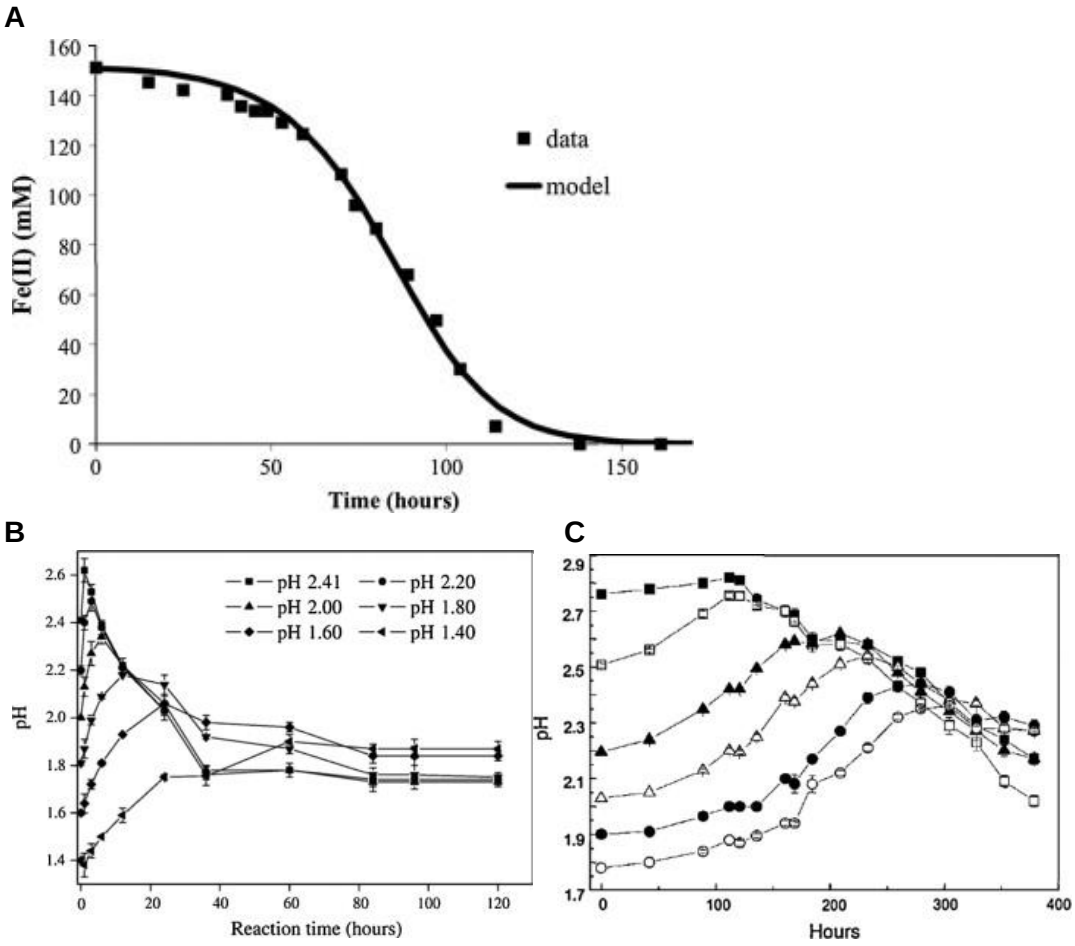


Figure 1.3. Fe oxidation and pH. A- Typical ferrous iron oxidation curve as a function of time (Darrell Kirk Nordstrom & Campbell, 2014); B- Change in pH during the oxidation of ferrous iron in 9K medium at 26 °C (Liao et al., 2009); C- Change in pH during oxidation of 9 K medium at 5 °C with initial pH designated by different symbols (∘, pH 1.8; •, pH 1.9; △, pH 2.0; ▲, pH 2.2; □, pH 2.5; ▪, pH 2.8; from Kupka *et al.*, (2007).

1.1.4.2 Pyrite oxidation

Pyrite is the most abundant sulfide mineral in the Earth's crust; it is commonly associated with coal, base metal and gold deposits. Oxidation of sulfide minerals has been the focus of extensive study because of its importance in environmental management and metallurgical processing industry. Reviews of sulfide mineral oxidation and the formation of acid mine drainage are given by many authors (e.g., Nordstrom et al., 2000a; Rimstidt & Vaughan, 2003; Rosso & Vaughan, 2006; Smith et al., 2013).

Pyrite oxidation can occur through chemical, biological, and electrochemical pathways, in oxic and anoxic systems, when mineral surfaces are exposed to

water and an oxidant (including oxygen, or ferric iron, or mineral catalysts, i.e., MnO_2). Oxidation of pyrite by atmospheric oxygen produces one mole of ferrous iron, two moles of SO_4^{2-} and two moles of H^+ for every mole of pyrite oxidized:

$$FeS_2 + {}^7/_2\, O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \quad (6)$$

The Fe(II) released may be oxidized to Fe(III) consuming one proton (Eq. (3)).

Pyrite oxidation by Fe^{3+} produces seven moles of ferrous iron, six moles of H^+ and one mole of $S_2O_3^{2-}$ for every mole of pyrite oxidised (Eq. (7)):

$$FeS_2 + 6Fe^{3+} + 3H_2O \rightarrow 7Fe^{2+} + 6H^+ + S_2O_3^{2-} \quad (7)$$

The microbial oxidation of the $7Fe^{2+}$ consumes 7 protons (Eq. (8))::

$$Fe^{2+} + {}^1/_4O_2 + H^+ \rightarrow Fe^{3+} + {}^1/_2H_2O \quad (8)$$

Furthermore, the complete oxidation of the thiosulfate produces two protons (Eq. (9)), so one mole of H^+ is produced:

$$S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2H^+ + 2SO_4^{2-} \quad (9)$$

Depending on the pH conditions, the ferric iron (not reduced during the dissolution of other minerals) may begin to precipitate: for pH 0.8 to 2.5, jarosite is formed, at increasing pH, schwertmannite is mostly precipitated but at pH > 5.5 ferrihydrite overcomes. schwertmannite tends to convert back to jarosite for pH values < 2.5 while at pH 2.5 to 5.5 converts to goethite. Depending on the secondary mineral formed, the pH may increase (jarosite) or decrease (other hydroxides).

1.1.5 Microbial ecology of mining systems

During the past decades the microbial ecology of mine related areas has mainly focused on drainage (AMD; Chen et al., 2015; Lear et al., 2009), rather than the solid phase itself (e.g. tailings, low grade material) which remain still somehow unknown (Kwong et al., 2009; Palumbo-Roe & Colman, 2010). Here is presented a brief review on the microbial ecology of those systems and the consequent interest on the microbial ecology of the solid substrates which generate acid and thus impact the biodiversity.

1.1.5.1 Microbiology of bioleaching

The microbial leaching of metal cations from low-grade sulfidic ores is known as bioleaching (Schippers et al., 2010) and applied in biohydrometallurgy (or biomining). Such process is driven by acidophilic consortia, iron- and sulfur-oxidizing Bacteria and Archaea that are ubiquitous at sites of mineral oxidation (Johnson, 2006). The conversion of the insoluble metal sulfides into soluble metal sulfates by these microorganisms generally proceeds over a minimum pH range of 0.5–2.0, and can occur at various ambient temperatures ranging from low to mesophilic (35°C to 40°C) and moderately thermophilic (50°C) to extremely thermophilic (over 65°C) (Rawlings, 2002). The microorganisms catalysing the bioleaching reaction are specific for the different temperature ranges. Many studies have investigated the microbial diversity present in mesophilic and moderately thermophilic bioleaching systems (Bathe & Norris, 2007; Groudeva et al., 2013; Norris et al., 2013). Some widely present and known organisms are the iron- and sulfur-oxidizing *Acidithiobacillus ferrooxidans*, the sulfur-oxidizing *Acidithiobacillus thiooxidans* and the iron-oxidizing *Leptospirillum ferrooxidans* and *Leptospirillum ferriphilum* which dominate mesophilic bioleaching processes (Coram & Rawlings, 2002; Hallberg & Johnson, 2001). Most studied microorganisms in moderately thermophilic bioleaching operations are the sulfur-oxidizing *Acidithiobacillus caldus*, the iron- and sulfur-oxidizing *Sulfobacillus thermosulfidooxidans* and the iron-oxidizing *Acidimicrobium ferrooxidans* (Norris et al., 2013; Rawlings et al., 1999). Archaea such as the iron-oxidizer *Ferroplasma acidiphilum* have also been shown to be present at these moderately thermophilic temperatures (Golyshina et al., 2000; Golyshina & Timmis, 2005; Okibe et al., 2003).

Mikkelsen et al. (2006) assess that bioleaching at temperatures above 65°C is only performed by archaea. Culture-dependent studies have demonstrated that genera such as the hyperthermophiles *Acidianus* spp, *Sulfolobus* spp and *Metallosphaera* spp (order Sulfolobales) appear to be the most efficient mineral sulfide oxidizers (Burton & Norris, 2000; Johnson, 1998). More recently, molecular ecology has allowed the exploration of the archaeal diversity present in thermophilic bioleaching systems (Chen et al., 2015; Mikkelsen et al., 2006; Norris et al., 2013), their presence suggests that these microorganisms substantially enhance the extent and the rate of metal extraction from low-grade

ores (Lindström et al., 2003; Norris et al., 2013; Romano et al., 2001; Stott et al., 2003). High temperature bioleaching has indeed potential to lower operating costs by limiting the costs for cooling the operations (Brierley & Brierley, 1999; Holmes, 1998; Rawlings, 1998).

Early in biomining related research, *At. ferrooxidans* was inaccurately thought to be the primary microbiological catalyst in mesophilic biomining processes (Bosecker, 1997), until authors found out that it was less important in numerous commercial bioleaching processes (Rawlings et al., 1999), and that the (at the time) newly isolated *L. ferrooxidans*, in combination with *At. thiooxidans* or *At. caldus*, dominated commercial mesophilic bioleaching processes (Rawlings et al., 1999). Recent studies have shown that *At. ferrivorans* can be psychrophilic to mesophilic and not able to be prominent at higher temperatures, even in the presence of oxidizing pyrite or geothermal waters with temperatures 25–70 °C (Hallberg et al., 2010; Mykytczuk et al., 2011). At such temperatures and lower pH values less than 1.5, *Leptospirillum* is more common as an iron-oxidizing bacterium. The introduction of routine molecular biology use to evaluate the microbial diversity and the different strains abundance, allowed some authors (e.g. Mikkelsen et al. (2006)) to describe novel systems. They showed two thermophilic bioleaching consortia composed exclusively of Archaea, and novel species of Sulfolobales (*Stygiolobus azoricus*-like species) dominated both cultures studied while close relatives of well-studied *Sulfolobus* species occur only in minor proportions. The work from Chen et al. (2014) and Wang et al. (2014) suggested that different community structures occur at different temperatures, and that chalcopyrite bioleaching should be inoculated and operated at high temperature in order to allow thermophiles to become the dominant microorganism in the system.

1.1.5.2 Advances in AMD and mine waste microbiology

Research in AMD microbiology has advanced the understanding of dominant microbial processes in mine environments that promote toxic metal mobility (Baker & Banfield, 2003; Johnson, 2012; Schippers et al., 2010) and for applications in hydrometallurgy and metals recovery (Hallberg, 2010; Rawlings, 2002). Schippers et al. (2010) compiled more than seventy microbiological studies of sulfidic mine wastes and heap leach piles. Robbins (2000) reported eighty six genera or species that live in waters with pH <4.5. Baker & Banfield

(2003) constructed a phylogenetic tree of prokaryotic 16S rRNA genes from studies on acid mine drainage and bioleaching sites and highlighted that all proteobacterial lineages were represented (α, β, γ and δ). Thermoplasmatales (Archaea) were also shown and both obligate autotrophic and facultative iron-oxidizing archaea have been found associated with AMD. A few eukaryotes were reported (Baker & Banfield, 2003) which complement those described for Iron Mountain, California (Robbins, 2000). In their research a synergic activity of autotrophs and heterotrophs was proposed.

One important finding is that archaea and leptospirilli are more common at the source of oxidizing pyrite where more extreme conditions of pH and temperature are likely to exist than along the gradient (Baker & Banfield, 2003; Huang et al., 2011; Tan et al., 2009). For moderate conditions of pH and temperature in mine tailings, the microbial communities can vary substantially from site to site from bacterial domination to comparable bacterial and archaeal populations (Kock & Schippers, 2008). Larger proportions of archaea compared to bacteria are found in extremely acidic environments (Nordstrom et al., 200) but generally AMD results dominated by bacteria in environments adjacent to mine waste: for example, Chen et al. (2015) found abundant *Acidithiobacillus* spp., *Leptospirillum* spp. and *Acidiphilium* spp. exhibiting high transcriptional activities and that such AMD microorganisms adapted to the different environmental conditions by regulating the expression of genes involved in multiple *in situ* functional activities.

One detailed chemical and microbiological investigation of downstream variations during Fe(II) oxidation was reported by González-Toril et al. (2011). A 1.2 km stream starting at the La Zarza-Perrunal mine in the Iberian Pyrite Belt was sampled at three points. The initial pH was 3.1 with Fe(III)/Fe(Total) = 0.11 and containing some sulfate-reducers but increased in iron-oxidizers along the downstream. *Leptospirillum* spp., *At. ferrooxidans*, and Thermoplasmata were found at all downstream locations. Iron-reducers were found throughout the river. The most downstream sample had a pH of 1.9 and contained Fe(III)/Fe(Total) = 0.99.

How microorganisms can tolerate low pH waters with high metal concentrations has interested microbiologists for a long time. The reviews by Golyshina & Timmis (2005) and Baker-Austin & Dopson (2007) described how acidophiles have in common highly impermeable cell membranes, reversed membrane potentials

that deflect inflow of protons, and rapid proton pumps that pump excess protons out of the cell. Numerous investigations have been reported on microbial life in the Rio Tinto mining area of south-western Spain (Gonzalez-Toril et al., 2003; López-Archilla & Amils, 1999). The dominant prokaryote in the river are *Leptospirillum* spp. and several strains were identified (García-Moyano et al., 2008). A comparison of microbial communities between the sediment and the water column showed some similarities in strains but higher cell density and higher richness occurred in the sediment (García-Moyano et al., 2012). Another study of an extremely acidic pyritic leachate (pH = 0.61–0.82, 134 g/L of sulfate) from San Telmo mine in the Iberian Pyrite Belt was found to be dominated by *Ferroplasma* spp. (Thermoplasmata) followed by minor amounts of leptospirilli and acidithiobacilli (Sánchez España et al., 2008).

Bacteria and archaea that catalyse the oxidative dissolution of pyritic minerals can do this without having physical contact with the mineral (non-contact leaching) but in most cases they attach to the sulfides, forming biofilms with corrosion of the minerals (contact leaching; Gehrke et al., 1998). Other bacteria that live in acidic mine waters also attach to minerals and form biofilms, including species of heterotrophic acidophiles that reduce ferric iron, rather than oxidize ferrous iron (Ghauri et al., 2007). These, in theory, have a protective influence on sulfide mineral oxidation as they can control the availability of the main chemical oxidant involved at low pH (ferric iron). Various studies (Johnson, 2014; Johnson et al., 2008) showed that, by colonizing pyrite grains by heterotrophic iron-reducing bacteria (*Acidiphilium* spp. and *Acidocella* spp.) before exposing them to autotrophic iron- and sulfur-oxidizing acidithiobacilli, it was possible to reduce pyrite dissolution by *ca.* 80%, even under conditions believed highly aggressive (pH < 2 and oxygen-saturated shake flask cultures). Interestingly, planktonic-phase pyrite-oxidizing bacteria were more abundant in cultures where the pyrite had been initially colonized with the heterotrophs, compared to fresh pyrite, possibly because biofilm formation by the heterotrophs limited the ability of the acidithiobacilli to attach to the minerals (“bioshrouding”).

Recently research is focusing on prediction attempts rather than observation and description which is allowed by the combined use of molecular tools and mathematical modelling toward a predictive model-based understanding of the distribution mechanisms of acidophilic microorganisms in the extreme AMD

system (Kuang et al., 2016). At the same time new interest is arising towards the environmental analysis finalized to help environmental protection, especially relatedly to the effect of soil recovery strategy and amendments of historic mine waste (Hesse et al., 2018; Kasemodel et al., 2019; Zornoza et al., 2016). Mapping the distribution of heavy-metal contamination and microbial communities in these soils is a first step in understanding effects of long-term, heavy-metal contamination at a basic trophic level (Beattie et al., 2018).

1.1.6 Cornish mining sites

Abandoned mines are amongst the most significant pollution threats in Britain. Mining legacy for coal, metal ores and other minerals dates to the Bronze Age. Thousands of mines have been abandoned, especially in South West England and Wales and currently discharge mine-water containing heavy metals and other pollutants into watercourses. More recently closed mines are still filling up with groundwater and will start discharging in the future (Mayes et al., 2010). Rivers in England, Wales and Scotland have been at risk of failing to meet their Water Framework Directive targets of good chemical and ecological status because of abandoned mines (Johnston et al., 2008). These rivers carry some of the biggest discharges of metals such as arsenic, cadmium, iron, copper and zinc to the seas around Britain. Seventy-two per cent of failures to achieve the cadmium quality standard in freshwater are in mined areas. In some areas, important drinking water supply aquifers were polluted or threatened by plumes of sulfate and chloride while currently alternative drinking water is used (Johnson & Thornton, 1987; Moon, 2010; Pirrie et al., 2000; Thomas, 1980).

Mining in Cornwall and Devon in the south west of England began in the early Bronze Age (approximately 2150 BC) and ended with the closure of South Crofty tin mine in Cornwall in 1998. Tin and later copper were the most productive of the metals extracted: some tin mining continued long after mining of other metals had become unprofitable. Historically extensive tin and copper mining has occurred in Cornwall and Devon, as well as arsenic, silver, zinc and a few other metals. Since 1998 there are no active metalliferous mines remaining. However, tin deposits still exist in Cornwall as well as remains of the mining legacy in the environment and tunnels (Table 1.3).

Table 1.3. List of main mines and mining impacted sites of the South West of England (UK). In bold are highlighted the biggest mines, underscored the sites taken in consideration in the microbiological survey at Chapter 1 and the experiments of this thesis.

Site name	***Type***	***Area***	***Year of closure***
Bissoe area	Environmental mine waste site	A	/
Consolidated Mines	Copper/Tin mine	A	1857
Dolcoath mine	Copper/Tin mine	A	1998
East Pool mine	Tin mine	A	1945
Great Wheal busy	Copper/Tin mine	A	1920s
Mount Wellington Tin Mine	Tin mine	A	1941
South Crofty	Copper/Tin mine	A	1998
Tretherrup	Copper mine	A	/
Wheal Frances	Tin mine	A	1918
Wheal Gorland	Copper/Tin mine	A	1864 (1906 legal closure)
Wheal Jane	Tin mine	A	1985 (1992 legal closure)
Wheal Peevor	Copper/Tin mine	A	1918
Halamanning	Environmental mine waste site	B	1858
Poldice mine	Copper/Tin mine	B	1930
Wheal Alfred	Copper mine	B	/
King Edward Mine	Copper/Tin mine	C	1881
Saint Agnes coast (Trevaunance mine)	Environmental mine waste site	C	/
Tywarnhayle Mine	Tin mine	C	1906
Wheal Coates	Tin mine	C	1914
Wheal Kitty	Tin mine	C	1930
Chyverton mine	Environmental mine waste site, Lead, Gold, Copper mine	D	1886
Caradon Hill	Environmental mine waste site	E	1886
Devon Great Consuls	Copper mine	E	1903
Ding Dong mines	Tin mine	E	1915
Fowey Consols mine	Copper mine	E	/
Botallack	Copper/Tin mine	F	1895
Cape Cornwall mine	Copper mine	F	1883
Geevor Tin Mine	Tin mine	F	1990
Levant Mine and Beam Engine	Copper/Tin mine	F	1919
Wheal Owles	Tin mine	F	1893
Great Wheal Fortune	Tin mine	G	1950s
Rinsey	Tin mine	G	1882
Wheal Vor	Copper/Tin mine	G	1910
Poldark Mine	Tin mine	H	1893
Wheal Metal	Tin mine	H	1874

In this study eight main areas are highlighted and illustrated in Figure 1.4:

A. Central area
B. Western Cornish area
C. Saint Agnes area
D. Chyverton area
E. Devon border area
F. Rinsey area
G. Western Cornish area, in proximity of the sea
H. Poldark area

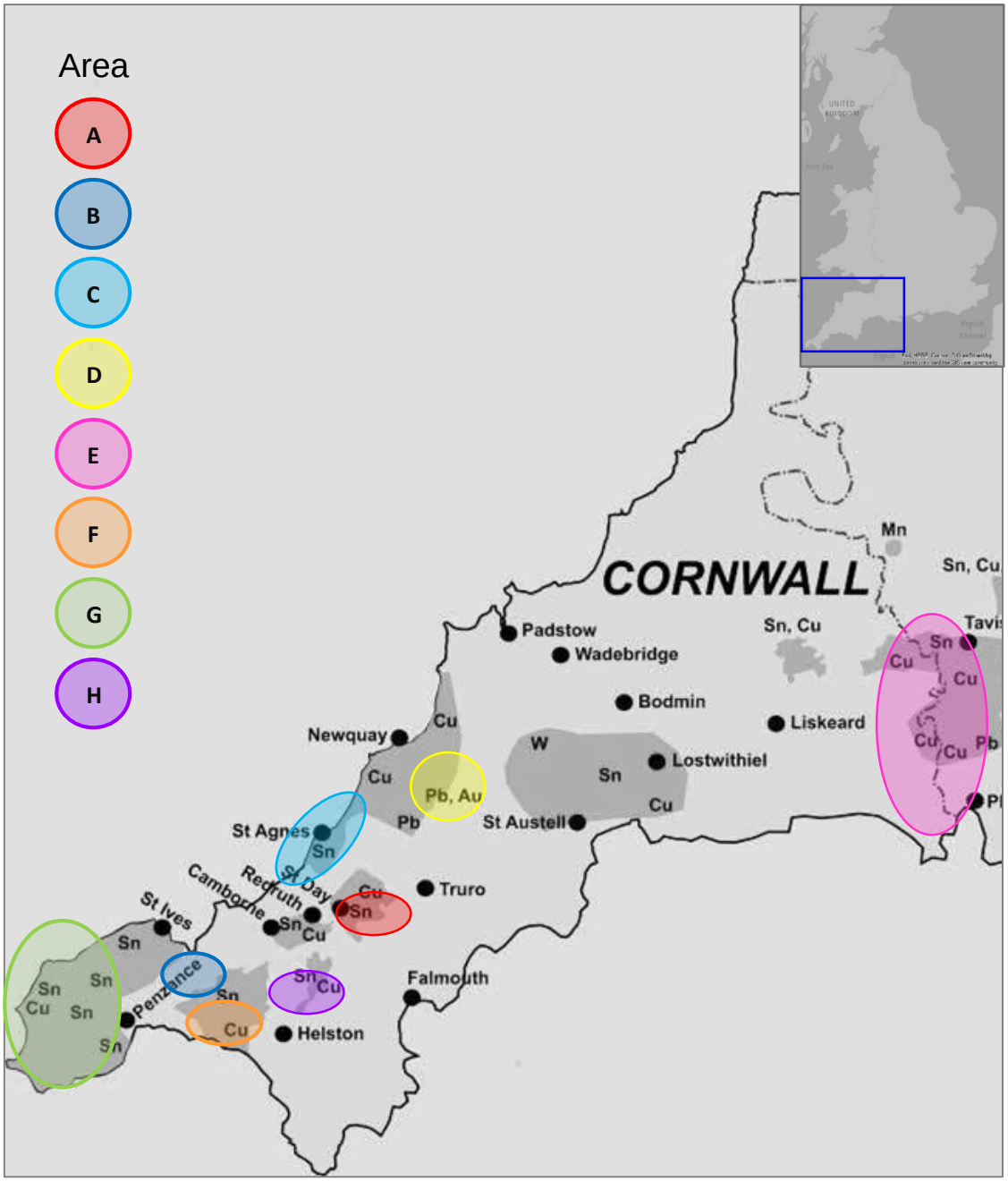


Figure 1.4. Map of South West of England (UK) highlighting the main localities and metals mined in the area (Pb = lead, W = tungsten, Sn = tin, Cu = copper, Mn = manganese). In transparent circles are indicated the areas listed in section 1.1.6 and in the legend. The map is modified from www.nmrs.org.uk ("Cornwall & Devon Mines - Northern Mine Research Society").

1.2 Thesis rationale

The microbial ecology of many environments has been widely investigated for diversity, function and relationship between biota and substrate. Unlike such ecosystems, little is known about the microbial ecology of mining environments. Indeed, of the research done to date, most has focussed on the liquid component (acid mine drainage) and little on the microbial ecology and activity in the solid substrates that give rise to AMD.

It is known that there is a strong link between the environment and its microbiology; the distribution of microorganisms is determined by the environment, and as a consequence they can be locally adapted. In AMD-generating systems, there is presumably an interplay between the two: the microbiology (specifically the community activity) affects the environment through the dissolution of metal-bearing sulfides (leading to increased acidity and soluble metal concentration) and *vice-versa*.

This thesis addresses questions which are key to a future calibrated use of acidophilic communities in a variety of applications, and an example for the use of communities deriving from other environments. It is asked whether such communities are locally adapted, if there are major geochemical features of the environment they are adapted to and finally the possible application of the understanding of the microbial adaptation to manipulate the structure and thus the function of microbial communities.

Initially, microbial communities from the Cornwall and West Devon Mining Landscape (UNESCO) were investigated together with geochemical parameters from their sites of origin. The model area has been subject to past mining activity, resulting in a variety of mine wastes left undisturbed for decades along with underground disused tunnels. For these reasons, the consortia housed by such environments were optimal to the aim of the study, their connectivity and similarity is unknown despite deriving from the same region. The association of the microbial communities with hosting contaminated environments as opposed to control soil from each site was tested to identify the variables showing a relationship with the community composition. A relationship between the geochemistry and the microbial ecology of mine waste was found, leading to the hypothesis of communities adaptation to their home environment.

It was evaluated whether communities were locally adapted with a dedicated reciprocal transplant experiment to test if they performed relatively better on their home environment than away.

Once assessed that there was not generalised local adaptation and that the mineral substrate was a driving factor for the performance of communities, a common garden experiment was designed using common experimental conditions to test if the community metabolic function could be increased by increasing the diversity of a consortia, mixing communities together, effectively removing dispersal barriers (coalescence experiment).

The search for consortia to be used in biotechnology is called bioprospecting. Bioprospecting through classical microbiological methods is useful, especially for the isolation and of new strains and strain-by-strain testing. However the approach offered in this study aimed at implementing the classical methods with a range of tests that both measure performance and implement a potential inoculum. This study aims at contributing to the knowledge of the microbial ecology of acidophiles in the scenario of whole communities' coalescence and transplant.

2 Materials and Methods

This chapter describes the materials and methods that were used routinely throughout the research project. Modifications or additions to these are noted in the relevant chapters. All chemicals used in this study were supplied by Sigma-Aldrich (U.K.) or Thermo Fisher Scientific (U.S.A.) and were of analytical reagent grade or molecular biology grade, unless otherwise stated. Good Laboratory Practice was followed throughout and aseptic techniques were used where appropriate. All the laboratory analysis and experiments were done in the Microbial Ecology Laboratory at the Environment and Sustainability building (Penryn Campus, University of Exeter, UK), unless otherwise stated.

2.1 Microbial cultivation-based techniques

Liquid medium based flasks and vials were used to cultivate extremely acidophilic (pH <3), and moderately acidophilic (pH 3-6) microorganisms. Culture incubation was carried out aerobically, unless elsewhere stated, at 26°C, and involved orbital shaking at 100 rpm. All media were heat-sterilized by autoclaving for 20 min at 121°C or filter sterilized through sterile 0.2 µm cellulose-nitrate membranes (Whatman, U.K.). All water used for cultivation purpose was reverse osmosis (RO) grade or Ultra-Pure (PURELAB flex 2, UV System, Elga, UK).

2.1.1 Liquid media

Table 2.1. Composition of the basal salt solution.

Reagent (gL^{-1})	Basal medium salts (BM), in g/L
$(NH_4)_2SO_4$	1
$MgSO_4 \cdot 7H_20$	0.4
KH_2PO_4	0.2

All flasks and vials used for the purpose of this thesis were set up with the Basal Medium (BM) described in Table 2.1. This medium was added to either pyrite or environmental mine waste (1 - 4 % w/v).

All liquid media were heat-sterilized and allowed to cool before use. Fine grain pyrite (Py, FeS_2) and other environmental mineral substrates were heat-sterilised in flask or vial together with basal medium (BM). Additions or amendments to these standard media are detailed where relevant.

2.2 Analytical techniques

2.2.1 Modified ferric chloride assay

The ferric chloride assay was modified by Govender et al., (2012), from Schnaitman et al. (1969). In the present study the modified assay was used in all experiments. The volumes were proportionally decreased from 2.0 mL to 0.2 mL, as the method was performed in 96-well Microtiter Microplates (Thermo Fisher Scientific, U.S.A.), and a BioTek® plate reader (either the PowerWave XS or the Synergy 2 model, U.S.A.) was used to read the absorbance at 400 nm wavelength. In each plate for analysis, a set of Fe (III) standards were also read. The data were then obtained from the standard curve defined by the standards.

2.2.2 Atomic absorption spectrophotometry (AAS) and inductively coupled plasma mass spectrometry (ICP-MS)

Concentrations of dissolved iron were determined against metal-free blanks using a Fast Sequential Atomic Absorption System (240FS AA, Varian now Agilent, U.S.A.) using a fuel-lean air/acetylene flame. Standards and blanks were prepared in the same matrix as the sample (1% HCl, vol/vol).

Samples were analysed using Inductively Couple Plasma-Mass Spectrometry (ICP-MS) for 22 elements using 0.5 g of split sample leached in hot *Aqua regia* before analysis at the Camborne School of Mines (CSM) Laboratories (Penryn, Cornwall, UK). The samples were digested in a 2:2:2 mixture of ACS grade concentrated HCl, concentrated HNO_3 and deionised water. Samples were digested for 1 h in a hot water bath. Sample solutions were analysed on an Agilent 7700 Series ICP mass spectrometer.

2.3 Microscopy

2.3.1 Phase-contrast microscopy

Microorganisms were visualised and enumerated by phase-contrast microscopy using a Unilux – 1 optical microscope (Kyowa, Japan) equipped with a Phase DM 40 x lens unit (total magnification 400x).

Phase-contrast microscopy was used for microbial abundance estimation using a counting cell (Thoma or Neubauer improved, Blaubrand®, Germany), a minimum of 64 fields were counted. The following calculations were used to determine cells abundance:

- Thoma counting chamber

$$Cells\ counted\ /\ (squares\ counted * 4.00E-03) = cells/L$$

- Neubauer counting chamber

$$Cells\ counted\ /\ (squares\ counted * 5.00E-05) = cells/L$$

2.4 Flasks and vials experiments

2.4.1 Experiments set up

Flask and vial experiments were incubated in orbital shakers (Panasonic MIR-S100C) at 26°C in a constant temperature room. Flasks were secured to the shaker platform via magnetic cubes and vials were either shaken in racks or during big volume experiments in plastic boxes secured with magnets to the shaker's metal plate.

2.4.1.1 Bioleaching experiment sampling

Bioleaching experiments were set up to examine the ability of a community to bioleach mineral substrates.

At t = 0, the flasks or vials were swirled to evenly disperse the solid phase and 1.5 mL samples were taken from flasks, 0.5 mL samples were taken from vials. Flask samples were processed as follows:

- A small aliquot (*ca.* 10 µL) was taken for microscopy (enumeration).

- Samples were centrifuged at 13000 rpm for 3 min, 0.9 mL supernatant was carefully removed and stored in 1:1 (v/v) HCl
- The remaining ~ 0.5 mL in the tube was used for pH measurement.

Vial samples were processed as follows:

- ~ 200 µL were pipetted in a 96-wells plate for total Fe, Fe(II) and Fe(III) measurement and:
    - The plate was centrifuged for 1 min at 3200 rpm.
    - Supernatant was used for the modified chloride assay.
- ~ 300 µL was put in Eppendorf tubes for microscopy (enumeration) and pH measurement;

During the experiment described in Chapter 4, due to the large number of vials, a method for flow-cytometry abundance estimation was implemented (see Chapter 6) and 50 µL samples were taken from the wells, after pipette mixing and prior centrifugation.

Flasks were weighed and incubated. Before each subsequent sampling, flasks were re-weighted and sterile RO water added to compensate for water lost through evaporation during incubation. Vials were not weighed nor was evaporation taken in consideration during these experiments.

2.5 Environmental sample collection and analysis

Solid-phase samples were collected in sterile containers, transported to the laboratory and processed as soon as possible.

To measure pH, 1 g samples of moist soil or mine waste were added to 2.5 mL RO water and left standing at room temperature for 1 h. the pH of the liquor was then measured.

Concentrations of readily extractable metals (REM) were used as a proxy for readily mobile (bioavailable) metals. REM concentrations were determined by shaking 5 g (wet weight) solid material in 100 mL 0.1 M H_2SO_4 using am orbital shaker at 120 rpm at room temperature, for 1 h. Aliquots from the extracts were

centrifuged at 15,600 *x g* for 3 min. Supernatants were preserved in 1:1 HCl (v/v) at 4°C until further use. Metal concentrations were determined by AAS (Fe) or ICP-MS analysis.

Dry weights and, from these, θm (moisture %) values, were determined by drying 2 to 5 g wet samples in an oven at 105°C overnight, until constant weight. Differences in weight before and after drying represented the amount of water the samples contained. Θm is expressed as a percentage value and is given by dividing the mass of water by the mass of dry spoil, multiplied by 100.

Where material was to be used in bioleaching experiments, a quantity was homogenized, mixed, dried at 60°C until constant weight and stored at RT.

When environmental populations were to be used as inocula for experiments or plating, to detach microorganisms from the sample matrix, 5 g sample were added to 100 mL BM solution (pH adjusted to environmental) in a 250 mL Falcon tube, the tube was shaken by hand and vortexed at max power for 1 min. The containers were left to settle. Once most of the solid phase had settled, the resultant liquor was serially diluted and used to inoculate experiments.

2.6 Biomolecular techniques

All water used in reagents for biomolecular applications was Nuclease-Free water, pH 8.0 (Ambion).

2.6.1 DNA extraction

DNA was extracted either from environmental or experimental whole microbial communities.

DNA from solid-phase environmental samples was extracted using the PowerMax® DNA Soil Isolation Kit (2.5 – 10 g sample) and/or the PowerLyzer PowerSoil® DNA Isolation Kit (0.25 – 0.5 g sample). Biomass from enrichment cultures (pyrite and other mineral substrates) was harvested by centrifugation (1-2 mL, 5-6 x 10^3 *g*, 45 min) and DNA was extracted using the PowerLyzer PowerSoil DNA isolation kit (QIAGEN, Germany). Both kits were used as per the manufacturer instructions, applying the following modifications:

- Beads solution and C1 solution of the kit were added as soon as the frozen sample was out of the freezer and before the first thawing step at 62°C in water bath.
- pH of samples was checked during the first thawing step using pH strips (Thermo Fisher Scientific, U.S.A.) as a precaution due to acidity of samples, and in case too low, it was promptly corrected with 1 M NaOH (twice filtered; 0.22 µm filter).
- The bead beating was done with:
    - FastPrep®-24 Classic Instrument (MP Biomedicals, U.S.A.) in case of 2.5 to 10 g samples, 2 cycles of 2.5 minutes at 4.0 m/s in 15 mL tubes.
    - QIAGEN Tissue Homogenizer in case of 0.25 to 0.5 g samples or in case of enrichment cultures' samples, 1 cycle of 3 min plus 1 cycle of 2.5 min
- Two more freeze-thawing steps (-80°C or dry ice box for a minimum of 30 minutes for bigger samples, 10 min for smaller samples, 62°C in a thermic bath for 10 minutes).
- DNA was eluted in 60 µL warm elution buffer solution (62°C) and left standing for at least 15 minutes before elution; when the PowerMax kit was used elution was done with 2 mL C6 solution.
- Extracted DNA was stored at – 20°C for a week, then at -80°C until further use.

2.6.2 Polymerase chain reaction (PCR)

PCR was used to amplify the 16S rRNA genes of Archaea and Bacteria. Bacterial 16S rRNA genes were amplified using the 27fG:1492rG primer pair (primers appended with the "G" suffix have an additional guanine residue on the 5' end, see Table 2.4. Archaeal 16S rRNA genes were amplified using the 20fG:1492rG primer pair. All primers used are listed in Table 2.3. In order to amplify the DNA of Bacteria and Archaea, at the same time the modified primer pair 515f:806r (Apprill et al., 2015; Caporaso et al., 2011; Walters et al., 2016) was used. Primers were manufactured and supplied by Eurofins Scientific (U.K.).

PCR reactions composition is described in Table 2.3. All reagents used for PCR are produced by Thermo Fisher Scientific. PCR reactions were carried out in a Veriti™ 96-Well Thermal Cycler (Applied Biosystems™, U.S.A.).

Bacterial 16S rRNA genes were amplified using “straight”, as opposed to “touchdown”, PCR as follows: initial denaturation at 95°C for 5 min; 20 -30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and polymerisation at 72°C for 90 s; followed by a final extension period at 72°C for 10 min.

Table 2.2. PCR reaction components.

Component	Volume per each reaction (µL)
MGW	17.1
Polymerase buffer	2.5
dNTPs (10 mM)	2.5
DNA Polymerase (5 U/µL)	0.15
F primer (10 µM)	0.625
R primer (10 µM)	0.625
DMSO (100%)	0.5
DNA template	1
Total (DNA included)	25

Table 2.3. Primers used. f = forward; r= reverse; * Primers with an additional guanine on the 5’ end (referred to as ‘G’ primers) and used to increase ligation efficiency during cloning.

Primer	Sequence (5’ → 3’)	Target gene	Reference
27f	AGAGTTTGATC(A/C)TGGCTCAG	Bacterial 16S rRNA	(Lane, 1991)
20f	TCCGGTTGATCC(T/C)GCC(A/G)G	Archaeal 16S rRNA	(Orphan et al., 2000)
1492r	TACGG(C/T)TACCTTGTTACGACTT	Bacterial/archaeal 16S rRNA	(Lane, 1991)
515f	GTGYCAGCMGCCGCGGTAA	Bacterial/archaeal 16S rRNA	(Caporaso et al., 2011; Parada et al., 2016)
806r	GGACTACNVGGGTWTCTAAT	Bacterial/archaeal 16S rRNA	(Apprill et al., 2015; Caporaso et al., 2011)

Archaeal 16S rRNA genes were amplified by straight PCR as follows: initial denaturation at 95°C for 5 min; 20-30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and polymerisation at 72°C for 90 s; followed by a final extension period at 72°C for 10 min.

2.6.3 Quality check of PCR products and extracted DNA

2.6.3.1 Gel electrophoresis

Gel electrophoresis was used to check the success of PCR reactions and the quality of extracted DNA. The gel contained 1% (w/v) electrophoresis-grade agarose in TAE buffer (from 50 x concentrate, Sigma-Aldrich). The agarose was dissolved using a microwave oven and cooled before the addition of 0.005% v/v ethidium bromide. The gel was cast in a suitable tray with a comb to produce sample loading wells. PCR products were mixed in a 5:1 ratio with a 6 x concentrate DNA loading buffer (Thermo Fisher Scientific). Samples were loaded into the wells and GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific) was loaded into the first and last wells as a reference. A constant voltage, variable amperage, current was applied across the gel causing the DNA to migrate (usually 100-120 mV). When sufficient migration had occurred to allow good separation of DNA fragments the DNA-ethidium bromide complex was visualised using UV light (G:BOX, Syngene, U.K). Archaeal and bacterial 16S rRNA genes amplified using the above primer pairs yield a single band corresponding to roughly 13.5 Kb in length.

2.7 Illumina sequencing

Extracted DNA quantity was checked via Qbit (Invitrogen) and electrophoresis in gel. Community analysis was performed via Illumina MiSeq sequencing at the Centre for Genomic Research at the University of Liverpool. In this section of the chapter methods used by the CGR are described.

2.7.1 Clone libraries from environmental DNA

2.7.1.1 PCR and ligation reactions

Environmental DNA deriving 16S rRNA genes (V4 hypervariable zone) from extracts were amplified by PCR as previously described. For each sample 2 µL of DNA sample entered a first round PCR using custom 16S primers (see Table 2.4), with the following condition: 15 cycles for denaturation at 95°C for 20 s, annealing at 65°C for 15 s, extension at 70°C for 30 s and a final extension at 72°C for 5 min.

PCR primers used:

Forward:
5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNGTGCCAGCMGCCGCGGTAA-3'
Reverse:
5'-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT-3'

The primer design incorporates a recognition sequence to allow a secondary nested PCR process. Samples were purified with AMPure SPRI Beads (Agencourt) before entering the second PCR run, done to incorporate Illumina sequencing adapter sequences, containing the indexes for samples' identification (i5 and i7). Fifteen cycles of PCR were performed using the same conditions as above. PCR products were purified using AMPure SPRI Beads before being quantified using Qubit (Invitrogen) and evaluated using the Fragment Analyzer. Successfully generated amplicon libraries were taken forward.

These final libraries were pooled in equimolar amounts using the Qubit and Fragment Analyzer data and size selected on the Pippin prep using a size range of 300-600 bps. Libraries were pooled using the Mosquito robot (TTP Labtech, U.K.). The quantity and quality of each pool was assessed by Bioanalyzer (Agilent, U.S.A.) and subsequently by qPCR using the Illumina Library Quantification Kit (Kapa) on a Roche Light Cycler (LC480II) according to manufacturer's instructions.

The template DNA was denatured according to the protocol described in the Illumina cBot User guide and loaded at 11 pM concentration. To help balance the complexity of the amplicon library, 15% PhiX was spiked in.

The sequencing was carried out on one lane of an Illumina MiSeq, at 2 x 250 bp paired-end sequencing with v2 chemistry.

2.7.2 Bioinformatic analysis

2.7.2.1 Initial processing and quality check of the sequence data

Initial processing and quality assessment of the sequence data was performed using an in-house pipeline (developed by Richard Gregory at the Centre for Genomic Research, Liverpool).

Briefly, base-calling and de-multiplexing of indexed reads was performed by CASAVA version 1.8.2 (Illumina) to produce 40 samples from the 1 lane of sequence data, in fastq format. The raw fastq files were trimmed to remove Illumina adapter sequences using Cutadapt version 1.2.1 (Martin, 2011). The option “-O 3” was set, so the 3' end of any reads which matched the adapter sequence over at least 3 bp was trimmed off.

The reads were further trimmed to remove low quality bases, using Sickle version 1.200 with a minimum window quality score of 20. After trimming, reads shorter than 10 bp were removed. If both reads from a pair passed this filter, each was included in the R1 (forward reads) or R2 (reverse reads) file. If only one of a read pair passed this filter, it was included in the R0 (unpaired reads) file. All trimmed read data for the complete datasets as well as detailed statistics on the read trimming were made available from the following URL:

http://www.cgr.liv.ac.uk/illum/LIMS10379Results_de70122adc4659c2/

The analysis described was performed to assess the quality of the reads, later analysis uses R1 and R2 raw reads.

To remove PCR primer sequences that could potentially introduce an artificial level of complexity in the samples, the raw reads were subjected to a Cutadapt trimming step. Due to the design of the Illumina sequencing adapters, the PCR primer sequence is located downstream of the sequencing adapter sequence,

therefore with this step both Illumina adapters and sequencing primers are removed.

To improve base quality in both read pairs, sequencing errors were corrected in both forward and reverse reads using the error-correct module within SPAdes sequence assembler, version 3.1.0 (Bankevich et al., 2012). The expected size of the amplicon covering variable region V4 is ~250bp. Therefore, 2 × 250bp reads should overlap almost entirely. Read pairs were aligned to produce a single sequence for each pair that would entirely span the amplicon using PEAR (version 0.9.10, (Zhang et al., 2014)). This applies an optimal quality trimming to each sequence mate to obtain the best possible alignment. Additionally, sequences with uncalled bases (Ns) were removed. To remove sequences originated from potential PCR primer dimers or from any spurious amplification events, a size selection between 200 bp and 600 bp was applied to each merged sequence set.

Fragmented PhiX phage genome was added to the sequence library in order to increase the sequence complexity (which can be low for amplicon sequencing and prevents the Illumina technology from functioning correctly). PhiX sequences are not indexed, so should not be associated with index reads after de-multiplexing. However, a very small proportion of PhiX reads can become associated with an index (likely as a result of 'cross-talk' between adjacent clusters on the sequencing flow-cell). To remove these sequences, each sample was compared with the complete PhiX sequence (GenBank gi9626372) using BLASTN (Altschul et al., 1990) .Sequences matching PhiX (E-value < 10-5) were filtered out of the dataset.

2.7.3 Metagenomics analysis

Sequences passing the filters for each sample were merged into a single file. This final sequence file, plus its own metadata file describing each sample, was used for the metagenetics analysis by using a custom pipeline based on QIIME 1.9.0 (Caporaso et al., 2010a). The Greengenes 13.8 database of ribosomal RNA genes ((McDonald et al., 2012); obtained from the QIIME web site, http://qiime.org/home_static/dataFiles.html) was used for the whole analysis.

2.7.3.1 Defining operational taxonomic units (OTUs)

In order to identify and quantify particular species, (e.g. genera, families, etc.), described by the generic term “operational taxonomic units” (OTUs), several steps were undertaken. Sequences containing Ns were discarded to exclude low quality sequences. The most basic operation in this process is the clustering of similar sequences into groups, to define OTUs at 99% similarity. However, two sources of error in the sequences can lead to incorrect clustering, generally resulting in an overestimate of the number of OTUs. These errors are:

- Errors in individual bases, resulting from PCR errors and/or base-calling errors during sequencing, although many may be already corrected in the previous step.
- “Chimeras”, which are 'mosaic' sequences arising from portions of two or more sequences, often due to template switching during PCR amplification.

To account for base error, an “error correction” step is included. This is carried out by clustering sequences at a given (99%) identity and generating a consensus sequence for the cluster. OTU-picking was carried out based on the results of two different clustering algorithms; VSEARCH1.1.3 (a free tool implementing the UCLUST algorithm in USEARCH (Edgar, 2010)) and SWARM (Mahé et al., 2014). Each of these were run independently and the results were merged to obtain a non-redundant set of sequences (using 100% sequence identity). These two tools implement very different algorithms; hence using both should minimize the possibility of loss of OTUs during this step. VSEARCH was run using the '--clustersmalmem' option with a threshold of 99% identity; SWARM was used with default settings. Errors are expected to be rare compared to correct sequences. Therefore, clusters containing few sequences are more likely to be the result of errors compared to clusters containing many sequences. Therefore, for both algorithms, a minimum cluster size filter was applied to remove clusters containing fewer than 2 sequences.

To account for chimeras, two steps were included: reference-based chimera detection and *de novo* chimera detection. The former uses a database of sequences as potential ‘parent’ sequences from which chimeras may derive. The latter uses the most common sequences in the data itself as potential 'parents' (hence *de novo*). Both filters are run using the VSEARCH and the respective

database. The output consists of a filtered set of sequences defined as ‘the sequences not identified as chimeras by either filter’. Once these correction steps have been carried out, the resulting clusters are defined as the OTUs.

To calculate the abundance of each OTU, sequences were then clustered with the OTUs using a minimum similarity threshold of 97% for the entire length of the sequence, using 'usearch_global' implemented in VSEARCH.

After OTU-picking, taxonomic assignment of each OTU was carried out using the QIIME script assign_taxonomy.py, using *rdp classifier* (Q. Wang et al., 2007) to match a representative sequence from each OTU to a sequence from the database. The pick_rep_set.py was used to select the most abundance sequence within each OTU's cluster to use as the representative sequence.

The analysis resulted in a phylogeny of the OTUs and an OTU table file (in ‘biom’ format, for details see http://biom-format.org/), which contains the OTU information for each sample as well as taxonomic information on the OTUs and sample metadata. The number of sequences clustered to any of the newly identified OTUs for each sample is summarised in Table 2.5. Overall, the minimum number of sequences aligned to any OTU in a sample was about 97% of the read passing the initial filters steps.

Due to the low number of reads present, all of the blank-kit negative control samples were excluded from further analysis. For the same reason, 'Sample 32 CA3' was also removed.

3 Microbial ecology of mine waste

3.1 Introduction

3.1.1 Variables driving microbial diversity in AMD and analogue extreme environments.

Acid mine drainage and associated environments have often been described as harbouring limited microbial diversity and functional complexity compared to soil environments (Hogsden & Harding, 2012; Lear et al., 2009; Simmons et al., 2005; Wassel & Mills, 1983). Molecular studies have documented spatial and seasonal variations in microbial populations and communities in AMD environments, and suggested as major environmental determinants: conductivity and rainfall (Edwards et al., 1999), pore water pH (Lear et al., 2009) and oxygen gradient (González-Toril et al., 2011) at local scale, possibly due to site-specific geochemical characteristics. Temperature and, to a lesser extent, pH have been reported to be the main drivers of microbial community composition and dynamics in wide-breadth molecular studies investigating other extreme environments such as geothermal areas (Miller et al., 2009; Ward et al., 1998), subsurface (Johnson, 2012; Macalady et al., 2007; Yanagawa et al., 2014), and deep ocean sediments (Danovaro et al., 2017; Hassenrück et al., 2016). Moreover, differences in microbial community composition exist between the alkaline (Miller et al., 2009) and acidic (Mathur et al., 2007) hot springs in Yellowstone National Park (USA) suggesting pH as a major diversity driver.

Contrasting examples have been found in sulfate-rich (Stout et al., 2009) and iron-rich (Mathur et al., 2007) acidic hot springs, as they are dominated by iron-oxidizing acidophiles such as *Acidithiobacillus* spp., despite diverse temperatures (13.9–100.6 °C). Nevertheless, temperature still represents a driving factor for adaptive evolution in microbial populations, i.e. in aquatic environments (García et al., 2018). Salinity can have an effect and determine the microbial composition of extreme environments. Numerous meta-analyses have identified salinity as the main environmental factor shaping the distribution of diversity across different habitat types (Lozupone & Knight, 2007; Tamames et al., 2010) and AMD (Edwards & Goebel, 1999). Although, some authors (Kuang

et al., 2013) found that salinity (EC values) had a weaker effect than pore water pH.

Environment (as well as chance) plays a key role in determining microbial community composition (i.e. Stegen et al., 2012; Zhou et al., 2013). As previously described, key variables have been identified that are important predictors of the distribution of microorganisms, notably pH. It is less clear if metal contamination generates “universal” selection pressures, such that communities in metal-contaminated environments tend to converge in terms of microbial composition. This hypothesis was investigated by comparing community composition in mine waste sites and paired nearby soil control sites from the same localities. A comparison of community composition of contaminated and uncontaminated sites is integrated with in depth analysis of the relationship between community composition and geochemical features of the samples.

3.1.2 The Cornish Mining Heritage as a natural laboratory

The mineral diversity of Cornwall has its origins in metalliferous deposits associated with the intrusion of the Cornubian batholith, and its alteration and weathering products (Alderton, 1993; Dines, 1956; Embrey & Symes, 1987). The county has 507 mineral species, of which 40 are type localities of valid minerals (mindat.org[8]; “Cornwall, England, UK”): it is one of the prominent mining areas of the UK. Britain has a legacy of metalliferous mining going back to pre-Roman times and the mining and processing of metal ores has inevitably caused some effects on the environment. Cornwall and Devon have been extensively mined for tin, copper and other metals for centuries, with the closure of the last Cornish tin mine in 1998 (South Crofty) (Palumbo-Roe & Colman, 2010). Remains of the long mining history are widespread in the area. Anthropogenic activity has long-lasting effect on soil microbial communities (Beattie et al., 2018). In mine waste heaps and abandoned underground mines the extreme conditions allow acidophilic microbial communities to live. They tend to shift from lithotrophic

[8] Mindat.org is a website and database focusing on minerals and their localities, deposits, and mines worldwide. This information is entered by volunteers worldwide and verified by a team of experts.

metabolisms to heterotrophy with the aging of the system (Chodak et al, 2009). Mine waste sites are potentially sources of extreme pollution (Banks et al., 1997; Blowes et al., 2003; Johnson, 2014; Sun et al., 2018). This results from the production of highly acidic, metal-rich effluents when microbial communities catalyse the dissolution of pyrite and related minerals. Generally, acidophilic communities are studied because of their metabolic potential and their diversity offers the chance to gather consortia which could be tailored for future studies or laboratory experimentation. Given the remarkable amount and diversity of metals in mine wastes, acidophilic species presence and community composition hinge on a balance between their ability to oxidise some metals and the genetic resistance to toxic amount of the same metals or others. Therein the differential responses of communities may be a consequence of environmental variation, but alternatively or simultaneously, community composition and metabolisms can impact the ecosystem functioning. If so, there is a chance to manipulate the community composition and, through that, to influence its function and/or *vice versa*.

3.1.3 The focus on mine waste which generates AMD

In this study the extent to which key environmental variables correlate with mine waste and soil microbial community structure was investigated, with a focus on the diversity in metal composition of the samples. Numerous studies have characterised the composition and function of mine-related communities, mostly referring to AMD (Chen et al., 2016; Gao et al., 2019; Huang et al., 2016; Johnson et al., 2002; Kimura et al., 2011; Kuang et al., 2013; Kuang et al., 2016; Liu et al., 2014; Nancucheo & Johnson, 2012; Quatrini & Johnson, 2018; Sbaffi et al., 2017; Sun et al., 2019; Tardy et al., 2018). Some studies investigated deeper into the metabolic potential of such communities (Denef et al., 2010; Mosier et al., 2013; Mueller et al., 2010, 2011). However, previous studies have not determined the extent of how metal contamination imposes similar selection pressures on different communities, because they have not been compared to appropriate controls with sufficient replication. To this end, this study also investigates the composition of paired control soil samples from the soil outside the signalled area of the mine waste sites.

3.1.4 Experimental design and purpose.

Twelve sites, subjected to long term natural weathering, were used in this study as natural model ecosystems to investigate the relationship between microbial community composition of mine derived substrates and their particular geochemical characteristics. Further, the concept of a core microbial diversity common to all the waste sites was explored.

Literature about former Cornish mining locations (Moon, 2010) and wider South West area were consulted together with scientific and local experts and five main interest zones were identified, also listed in Chapter 1, Section 1.1.6:

A. Central area,

B. Western Cornish area,

C. Saint Agnes area,

D. Chyverton area,

E. Devon border area.

The area was explored and 12 site selected (Table 3.1). The selection of sampling sites was done taking in consideration the weathering conditions of the site, to have a good range of weathered sites.

This project aims at defining the variability of acidophilic communities in the selected area (not at fully characterizing the diversity in a specific site), describing the relationship between metals and community structure and finally the potential for each consortium for metals tolerance. The sampling strategy aims at representing the maximum variability from each site, and the “soil-like control samples” aim at highlighting the differences between mine waste and soil microbial communities.

Table 3.1. Description of sampling sites and sample codes.

Map point	Code name	Site name	Site level[9]	Location	Latitude Longitude (decimal)	Zone	Geochem Analysis Samples	Microbial ecology Samples
1	WM	Wheal Maid	O	Cornwall	50.237871, -5.158189	A	WM 1 WM 2 WM ctrl	WM 1 WM 2 WM ctrl
2	BIS	Bissoe	O	Cornwall	50.234961, -5.136995	A	BIS 1 BIS 2 BIS ctrl	BIS 1 BIS 2 BIS ctrl
3	HAL	Halamanning	O	Cornwall	50.128520, -5.411741	B	HAL 1 HAL 2 HAL ctrl	HAL 1 HAL 2 HAL ctrl
4	WA	Wheal Alfred	O	Cornwall	50.183708, -5.391806	B	WA 1 WA 2 WA ctrl	WA 1 WA 2 WA ctrl
5	StA	Trevaunance (Saint Agnes)	O	Cornwall	50.320191, -5.199024	C	StA 1 StA 2 StA ctrl	StA 1 StA 2 StA ctrl
6	PO	Tywarnhayle (Porthtowan)	U	Cornwall	50.279929, -5.230375	C	PO 1 PO 2 PO ctrl	PO 1 PO 2 PO ctrl
7	MW	Mount Wellington	U	Cornwall	50.233111, -5.141672	A	MW 1 MW 2 MW ctrl	MW 1 MW 2 MW ctrl
8	CHY	Chyverton Mine	O	Cornwall	50.317912, -5.101100	D	CHY 1 CHY 2 CHY ctrl	CHY 1 CHY 2 CHY ctrl
9	TR	Tretherrup	U	Cornwall	50.207009, -5.200947	A	TR 1 TR 2 TR ctrl	TR 1 TR 2 TR ctrl
10	DEV	Devon Consuls	O	Devon	50.537387, -4.220202	E	DEV 1 DEV 2 DEV ctrl	DEV 1 DEV 2 DEV ctrl
11	CA	Caradon South Mine	O	Cornwall	50.504215, -4.450131	E	CA 1 CA 2 CA ctrl	CA 1 CA 2 CA ctrl
12	CB	Caradon Hill	O	Cornwall	50.506036, -4.428505	E	CB 1 CB 2 CB ctrl	CB 1 CB 2 CB ctrl

[9] O = overground, U = underground (soil control samples are all overground).

3.2 Materials and methods

3.2.1 Sampling

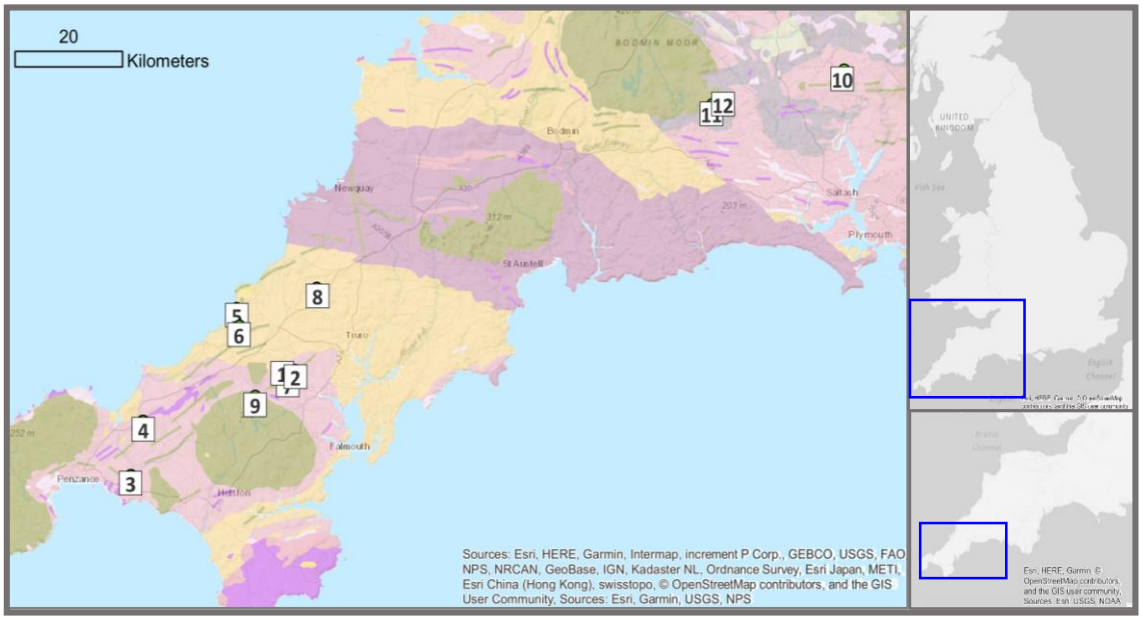


Figure 3.1. Sampling sites in South West England, UK. Main areas colour code: Upper Devonian; Lower Devonian; Middle and upper Devonian; Serpentinite; Granite.

Samples from twelve sites were collected during the winter 2015/16 (Table 3.1). The sampling strategy was designed to capture microbial diversity variability of mine waste in the large area (South West of England), not to represent the diversity of each site/location alone. For each site, two mine waste and one soil (control) sampling points were chosen. The control samples were selected outside the mining area: where the mining site was delimited by a fence the control sample was harvested outside of the fence. Where there was no signalling, the borders of the mining waste site were defined judging by eye and always at a distance of at least 100 m from the mine waste sampling point; soil was harvested where soil-like features were visible, for instance where vegetation was starting again. In the last case of underground sites, the control soil was harvested at least 100 m far from the entrance of the tunnels. Triplicate samples (*ca*. 50 g) were collected from each sample point and stored in 50 mL sterile Falcon tubes.

All samples were divided into aliquots as soon as they arrived in the laboratory, on the same day of sampling, as follows: 2 x 5 g, 2 x 2.5 g and 4 x 0.5 g for DNA extraction; 2 g for flasks enrichments; 3 – 5 g for wet/dry weight; 5 g for readily extractible metals; 1 g for pH; 5 g for pore water metals/total metals. Prior aliquoting, replicates homogenisation and the first two steps (eDNA extraction and enrichment aliquots) of the procedure were done under sterile conditions. Samples were not sieved, but large (> 10 mm) stones were removed.

3.2.2 Mine waste geochemical analysis

Samples were used to extrapolate three datasets regarding metals: readily extractible metals (REM; Bryan et al., 2006) which represent a proxy for metals availability/leachability; pore water metals (PWM), the metals which are dissolved in the pore water and those immediately soluble in water, and total metals (TM). For readily extractible metals, 5 g of wet samples aliquots were shaken at 120 rpm in an orbital shaker (SSL1 Orbital shaker, Stuart, Cole Parmer, U.S.A) in 100 mL 0.5 M H_2SO_4 (Bryan et al., 2006). At the end of the light digestion, liquid samples were collected, centrifuged at 15,700 × *g* to pellet any waste residual particulates and the supernatants were stored at 4°C in 1% HCl (1:1 vol/vol) until further analysis. Total and pore water metals (TM and PWM respectively) were estimated through a process that allowed to discern the two measures, as it follows. Deionized water (5 mL) was added to ~ 5 g (wet-weight, exact mass noted) mine waste/soil aliquots (in pre-weighed Falcon tubes). Tubes were twice inverted (or briefly vortexed at 600 rpm, 4 × *g,* when the material was fine and compact) to break up the agglomerated material and dilute the pore water into the bulk solution (Govender et al., 2014; Govender et al., 2015). The tubes were centrifuged at 150 × *g*, for 15 min and the supernatant (containing diluted PWM) was harvested and filtered (0.22 µm) in order to remove residual particles and stored at 4°C in 1% HCl (1:1 vol/vol) until ICP-MS analysis. The remaining undisturbed bulk pellet was dried at 60°C to constant weight. When the pellets were dried the tubes were weighed to calculate the dry weight of the samples and the moisture percentage. Interstitial water volume was calculated as the difference between wet and dry weight of the tubes. The dry pellet was preserved at RT until further analysis for total metals content (total digestion in *aqua regia*

performed by the Camborne School of Mines chemistry laboratory). Soluble metal concentrations in the resulting leachates/supernatants was determined by ICP-MS (Inductively Coupled Plasma - Mass Spectrometer, 7700 Series, Agilent, U.S.A.) at the Camborne School of Mines chemistry laboratory. Measurements of waste and soil pH were done in triplicate as previously described in Chapter 2.

3.2.3 DNA isolation and 16S rRNA gene amplicon library generation.

DNA extraction aliquots were stored at -80°C until processed. Total genomic DNA (gDNA) was extracted using the PowerMax DNA isolation kit (Mobio, now QIAGEN, U.S.A) (Caporaso et al., 2011), following the manufacturer's instructions, applying three freeze/thawing steps after mechanical lysis with QIAGEN Tissue Homogenizer (detailed protocol in Chapter 2). For the 16S rRNA gene amplicon sequencing, a 291 bp conserved fragment from the V4 hypervariable region was targeted using the primers (515F: 5 '-GTGYCAGCMGCCGCGGTAA - 3', 806R: 5'- GGACTACNVGGGTWTCTAAT - 3') (Apprill et al., 2015; Caporaso et al., 2011; Parada et al., 2016) with a pool of indexed primers suitable for multiplex sequencing with Illumina technology (Caporaso et al., 2011). DNA was quantified by fluorometric (Qbit, Invitrogen), checked for quality by gel electrophoresis, and by PCR, as previously described in Chapter 2. Extracted gDNA was sent to the Centre for Genomic Research at University of Liverpool where the clone library was prepared. Genomic DNA (5 ng) was mixed with 0.25 µl of each 16S primer (10 µM) and 0.5 µl of each of the nested primers (10 µM). KAPA amplification mix (2 ×) was used and the final volume was 20 µl. A negative control of water eluted from the FastDNA spin kit was also included. The samples were amplified at the following conditions: 98 °C for 2 s (one cycle), 95 °C for 20 s, 65 °C for 15 s, 72 °C for 30 s (25 cycles), 72 °C for 5 min (one cycle), and 4 °C hold. The samples were then cleaned up using Agencourt Ampure XP beads (Beckman Coulter) at a ratio 1:1. The products were eluted in 12 µl 10 mM Tris pH 7.5. The samples were analysed by Qubit fluorometry and Bioanalyser.

3.2.4 Sequencing and bioinformatic analysis

Amplicon sequencing was performed by Illumina MiSeq technology at the Centre for Genomic Research (University of Liverpool). Each pool of amplicons was sequenced at 2 × 250 bp paired-end sequencing with chemisty v2. Sequence data analysis was done using an adjusted pipeline; Casava v1.8.2 and Cutadapt v1.2.2 were used to perform the basecalling, de-multiplexing and trimming of the indexed reads (Caporaso et al., 2010a; Martin, 2011; Reeder & Knight, 2010). Filtered read pairs were analysed, and assembled into a single sequence by Flash (Magoc & Salzberg, 2011), then Qiime v1.8 was used for metagenomic analysis (Caporaso et al., 2010a). Clustering sequences at 97% of similarity generated 1,298 OTUs, the *de novo* OTU-picking and their quantification was done by using USEARCH (Edgar, 2010) v7.0. Sequences falling below the 97% similarity threshold for any of the OTUs clusters were removed from further analyses, to act as a filter against potential artefacts caused by sequencing error. The Greengenes database of ribosomal RNA gene sequences (McDonald et al., 2012) v13.8 was used as reference for chimera detection and taxonomy assignments. The taxonomic assignments for each OTU was performed by using Qiime v1.9.0 and RDP classifier (Wang et al., 2007).

3.2.5 Statistical analyses

Mild outliers were identified visually during a first analysis of the whole dataset of abiotic variables, according to Zuur & Ieno (2009). These were nevertheless included in the analysis so as not to lose information, as the present investigation is of descriptive nature and the variability of the samples was the focal point of the analysis. The impact of the environmental variables on diversity (alpha) was determined using Generalized Linear Models (applying a link = *log* to a Gamma family distributed errors) and environmental variables fitted as independent variables, such as group factor (mine waste samples or soil-like controls). Mantel test (Mantel, 1967) with univariate data was also used to screen and analyse the relationship between environmental variables and both α and β – diversity. A one-sample t-test was used to analyse differences among simple groups of replicates. These analyses were carried out using the *stat*, *phyloseq* (McMurdie & Holmes, 2013), and *vegan* (Oksanen et al., 2019) packages in R. The same were used to

calculate alpha [10] (Whittaker, 1960, 1972) and beta-diversity [11] (Jost, 2007; Whittaker, 1960) and to produce two-dimensional ordinations: principal coordinates' analysis (PCoA), principal component analysis (PCA), and non-metric multidimensional scaling (NMDS) plots using the relative abundances of taxa for each sample. For beta-diversity analysis, the *betadisper* function in the *vegan* package was used to test for multivariate homogeneity of group dispersions using a permutational approach (Anderson, 2006) and the *adonis*[12] function in the same package was used to relate microbial composition of samples and main environmental parameters (Anderson, 2001).

A similar approach was used to analyse the metals diversity and compare the extent of variability of readily extractible, pore water and total metals. The relative composition in metals of the three datasets was used to calculate Bray-Curtis dissimilarity matrices, consequently used to produce a two-dimensional ordination: principal coordinates analysis (PCoA). In this case as well, the *betadisper* function in the *vegan* package was used to test for multivariate homogeneity of group dispersions using a permutational approach (Anderson, 2006).

[10] Alpha–diversity is the mean species diversity in sites or habitats at a local scale, i.e. in each sampling site in this study (Whittaker, 1960).

[11] Beta–diversity is the extent of change in community composition, or degree of community differentiation, in relation to a complex-gradient of environment, or a pattern of environments. Whittaker (1972) proposed several ways to quantify beta diversity. In its simplest form beta diversity is defined as the ratio between gamma (regional) and alpha (local) diversities.

[12] *Adonis (Vegan, R platform*) is a function for the analysis and partitioning sums of squares, using dissimilarities, of a multivariate data set, also called PERMANOVA. It analyses the differences in the composition and/or relative abundances of organisms of different species (variables) in samples characterised by different groups, treatments or independent variables (adapted from Anderson, 2001).

3.3 Results

3.3.1 An overview of microbial communities

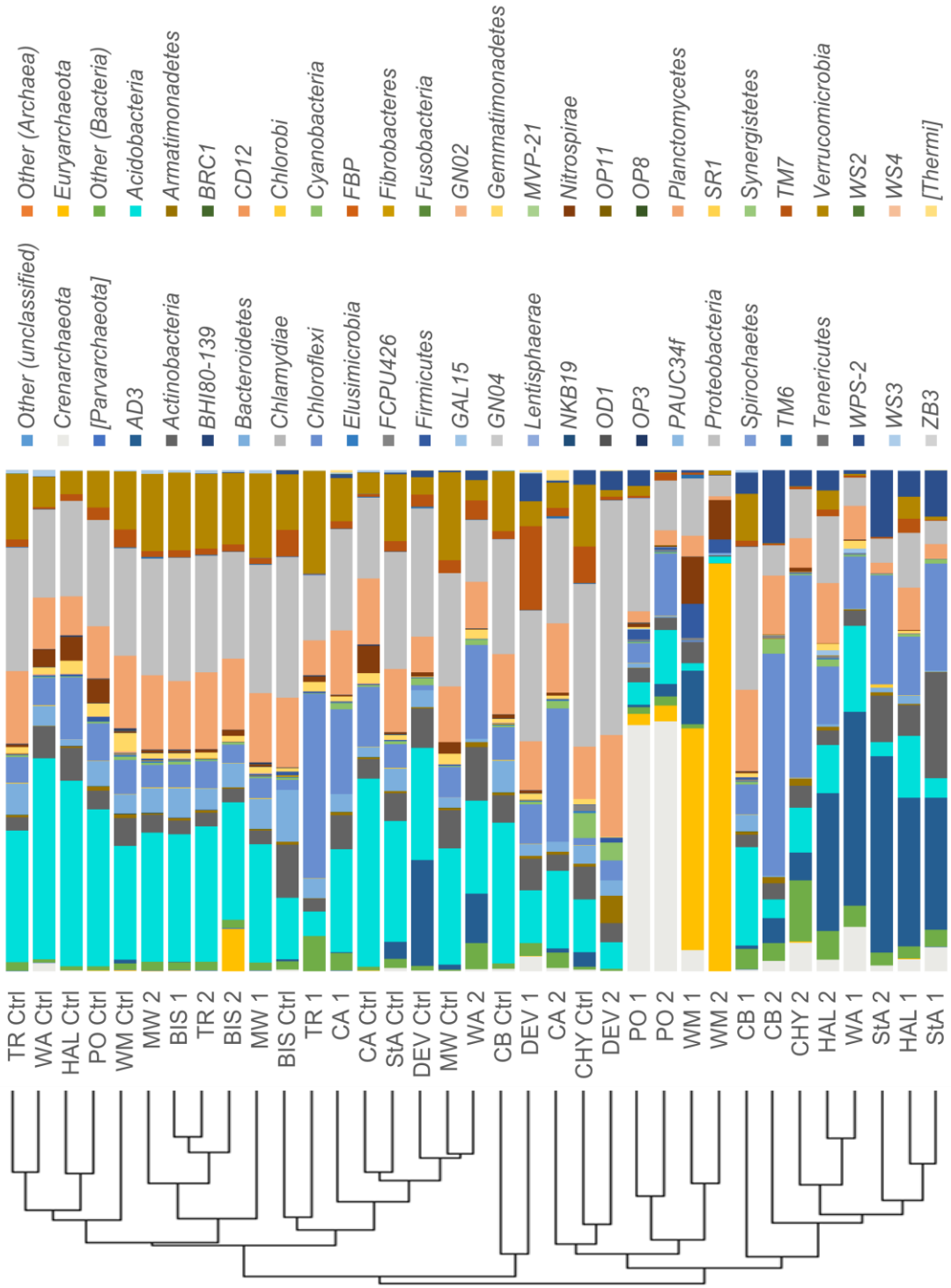


Figure 3.2. Community microbial composition of all sites at phylum level, coupled with hierarchical cluster analysis dendrogram.

Within 12 abandoned mining sites, including both mine waste and soil samples, 22 phyla were detected (Figure 3.2; all phyla were represented both in mine waste and in soil samples, i.e. there were no phyla unique to mine waste or controls). Two phyla, Proteobacteria and Acidobacteria, dominated these ecosystems (19% and 17.5% respectively of total average relative abundance), contributing 8 of the 22 most abundant genera (based on > 1% average relative abundance). Of the γ-diversity[13] (Whittaker, 1960), 71% of the of the whole study area was represented by Proteobacteria, Acidobacteria, Chloroflexi (11.1%), Planctomycetes (10.5%), Verrucomicrobia (7.4%), and candidate division AD3 (6.5%). Porthtowan (PO) and Wheal Maid (WM) were unlike the other sites, being mainly inhabited by archaea. Some phyla were present only in a sub-set of samples, e.g. Nitrospirae were co-occurring with Archaea and only abundant in Wheal Maid (WM) samples. Other phyla, like Acidobacteria, were instead more ubiquitous, and evenly spread among all samples, including both mine waste and soils.

3.3.2 Microbial diversity and clustering within and between samples

The two groups (mine waste and soil controls) diverge significantly in composition (*adonis*, Pr >t: 0.028 for the factor *group* indicating if the sample is mine waste or soil-like control at OTU level). Multivariate homogeneity of dispersion[14] for waste samples and soil-like controls was analysed to determine if control and mine waste samples differ in beta-diversity (M. J. Anderson et al., 2006). At phylum (and slightly less at species) level, the samples show different dispersion compared to the controls (Anova p-value 0.002). Beta-diversity among waste samples is significantly higher compared to the control samples' beta-diversity (Figure 3.3). The analysis is partially biased by the unbalanced design (waste samples n = 23, soil samples n = 12). Wheal Maid and Porthtowan waste samples

[13] Gamma-diversity is the total species diversity in a landscape, i.e. all sites in this study considered together.

[14] Betadisper {vegan} function in R allows to analyse the multivariate homogeneity of groups' dispersion. Non-Euclidean distances between objects and group centroids are handled by reducing the original distances to principal coordinates (M. J. Anderson et al., 2006).

are more strongly different from the main cluster driving the variability of the mine waste cluster. Overall, mine waste communities cluster together and show greater between-community diversity than surrounding soils.

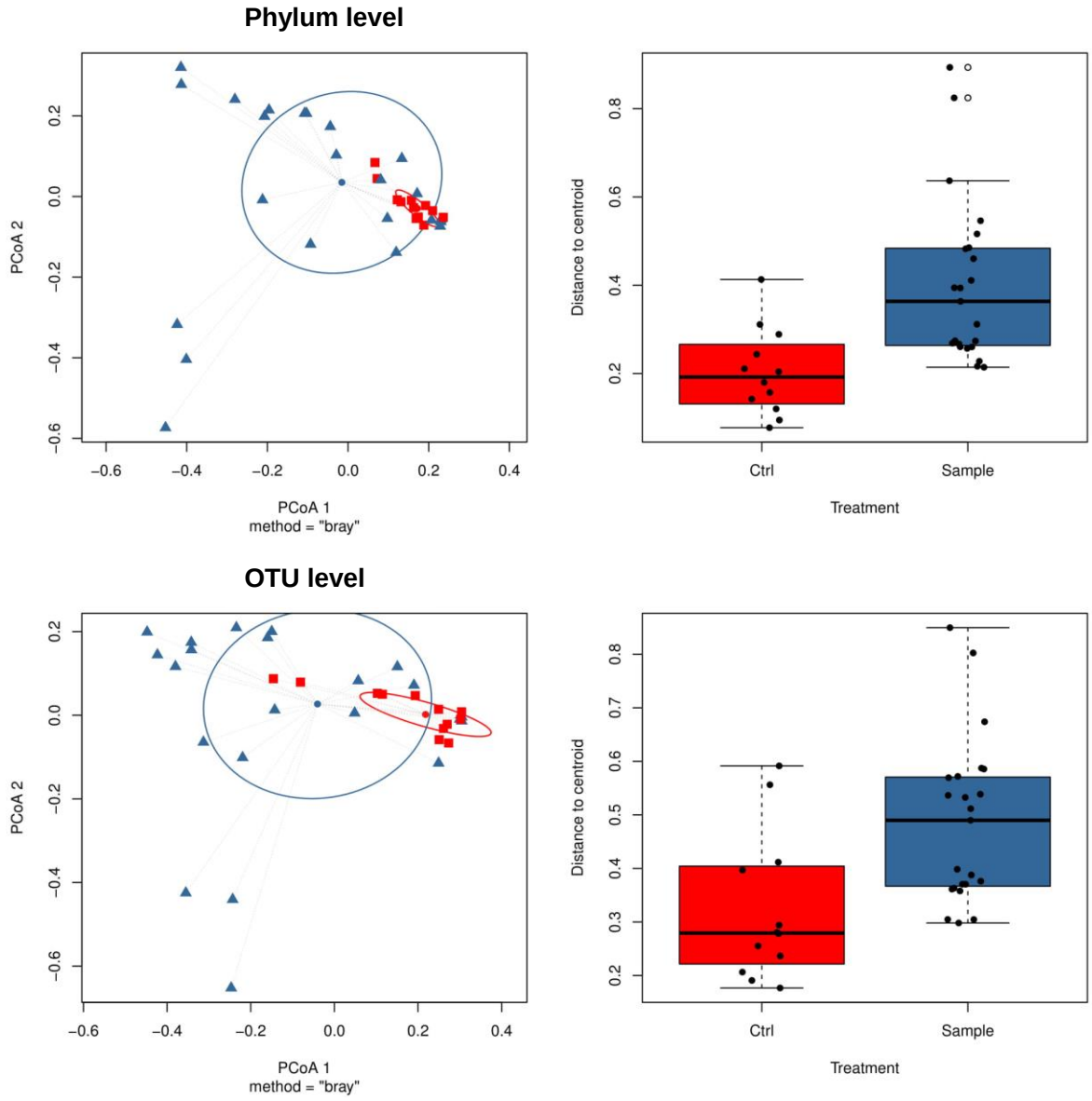


Figure 3.3. PCoA analysis showing the homogeneity of multivariate dispersion of soil-like control (■) and mine waste (▲) microbial communities samples and beta diversity as the distance of points from the groups centroid (● and ●), mine wastes beta diversity appears higher than soil-like control beta diversity both at phyla and species level (Anova p-value: 0.002 and 0.003 respectively). Ellipses representing the 1-standard deviation around the group centroids. Distances are represented from samples to centroids (grey dashed line).

3.3.3 Samples similarity between and within sites

Distance of samples to the centroid of either of the two groups (mine waste samples and nearby soils) was used as a proxy for intra - site diversity and the distance between centroids of sites-related grouping unit was used as a proxy for diversity amongst sites. Microbial diversity between sites was smaller than diversity within sites (Figure 3.4). On the base of this, the presence of a core microbiome is proposed (see Section 3.3.6). In fact when plotted the dispersion of microbial diversity for each site (including both mine waste and soil samples), most sites' clusters overlap largely and indicate variability among waste samples and soil control (Appendix Figure 2). Some sites, for instance Mount Wellington samples (soil and wastes) cluster closely, indicating that soil samples from such sites were not distant to mine waste samples in terms of microbial composition. The similarity between samples from the same site is not higher than their similarity to samples from other sites.

3.3.4 Variables mainly shaping the diversity in mine waste and surrounding soils

The sums (by mass) of all metals for each of the three types of metal measurements (readily extractible, pore water and total metals) were calculated. The variables considered were: moisture percentage and pH (abiotic variables) plus community percentage of diversity shared by each sample with the other waste samples or soils[15] (biotic variable). The described data were used to run a PCA (principle component analysis) and investigate the possible collinearity of selected abiotic variables and the shared composition percentage at species level (Figure 3.5A). The first two PCs explain 62.3% of variability; the variables applied to the PCA allow the discrimination of control samples and mine waste samples groups, although partially overlapping. Soil control samples as a group are defined by decrease in pore water metals and total metals (as a sum) and

[15] Shared composition percentage indicates the percentage of the community composed by OTUs that are shared by each waste sample with the other waste samples and by soil control samples with other soil control samples. In other words, it represents the percentage of the sample that forms the core biodiversity of all the waste samples or soil controls.

increasing pH and percentage of shared community. Using PC1 as a proxy for metal composition were obtained similar results and a stronger collinearity between pH and percentage of shared OTUs.

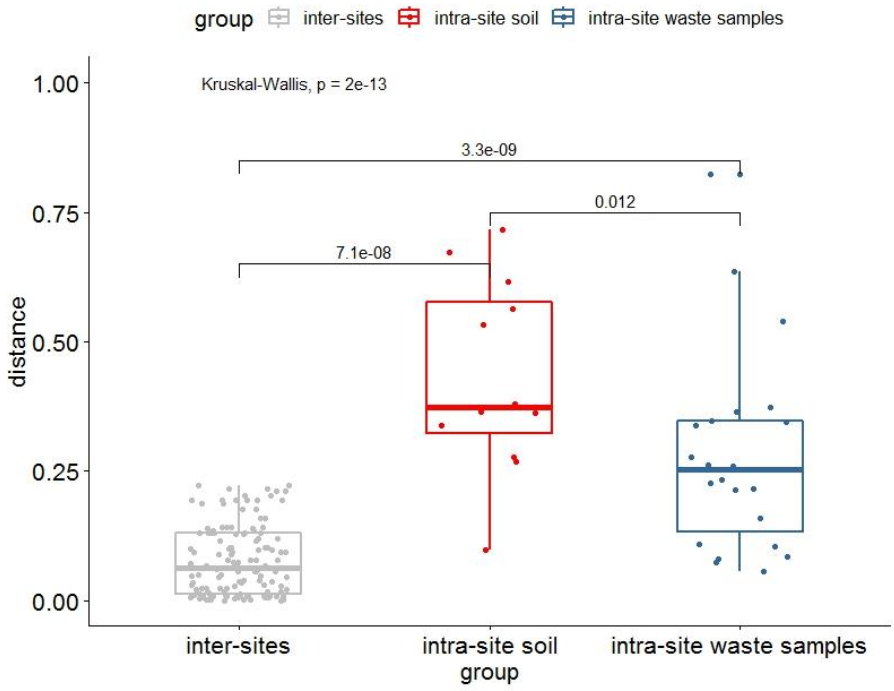


Figure 3.4. Diversity distance (to centroid of group) between sites (inter-sites) is smaller than distance between samples of the same site (intra-sites) to the respective centroid.

3.3.4.1 Principal Component 1 as a proxy for metals contamination.

Because of strong collinearity among heavy metals, a PCA on centred and scaled data with each one of the three metals matrices (REM, PWM and TM) was carried out (Figure 3.5A and B). Most metals loaded positively on the first principal component (PC1; Figure 3.6A), which was subsequently used as proxy for total metal loading, as previously shown by Hesse et al. (2018).

Here it is suggested the term “metallocity” for such a measure. The sum of metals is a useful proxy for the discrimination of samples but it can be biased by the iron concentration which is much higher than all the other metals. The first principal component of PCA ordination including the three matrices of metals concentration previously described were used as a proxy for *total metals loading* of the samples and to test the relationship between metals and pH. Readily

extractible metals (PC1) showed a positive linear relationship with pH while a negative relationship was detected between pore water metals (PC1) and pH (Figure 3.6B). PCA analysis applied to the geochemical features of all samples (REM-PC1, TM-PC1, PWM-PC1, pH, moisture percentage) and shared community percentage (Figure 3.5B) shows a separation of the two groups, waste samples and soil controls with pH collinear to shared community percentage, pore water metals and readily extractible metals showing a negative and a positive correlation with pH, respectively.

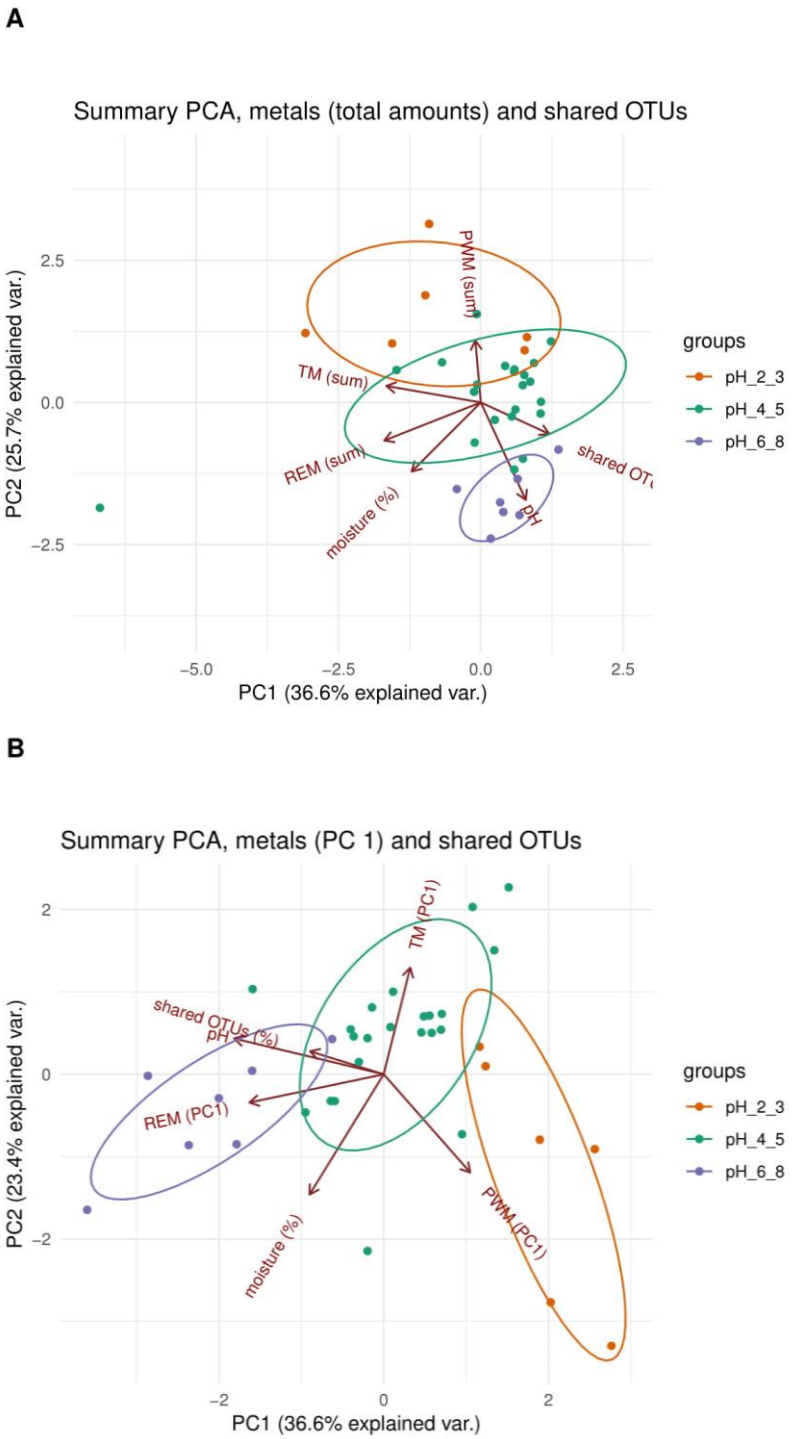


Figure 3.5. Principal component analysis summarizing abiotic and biotic factors (affecting the microbial community compositions) including either total amount of metals or the proxy PC1, explaining 62.3% and 60% of variability (A and B respectively). Higher pH samples are defined by a higher REM (sum), percentage of shared microbial community composition and moisture (non-transformed, scaled and centred data).

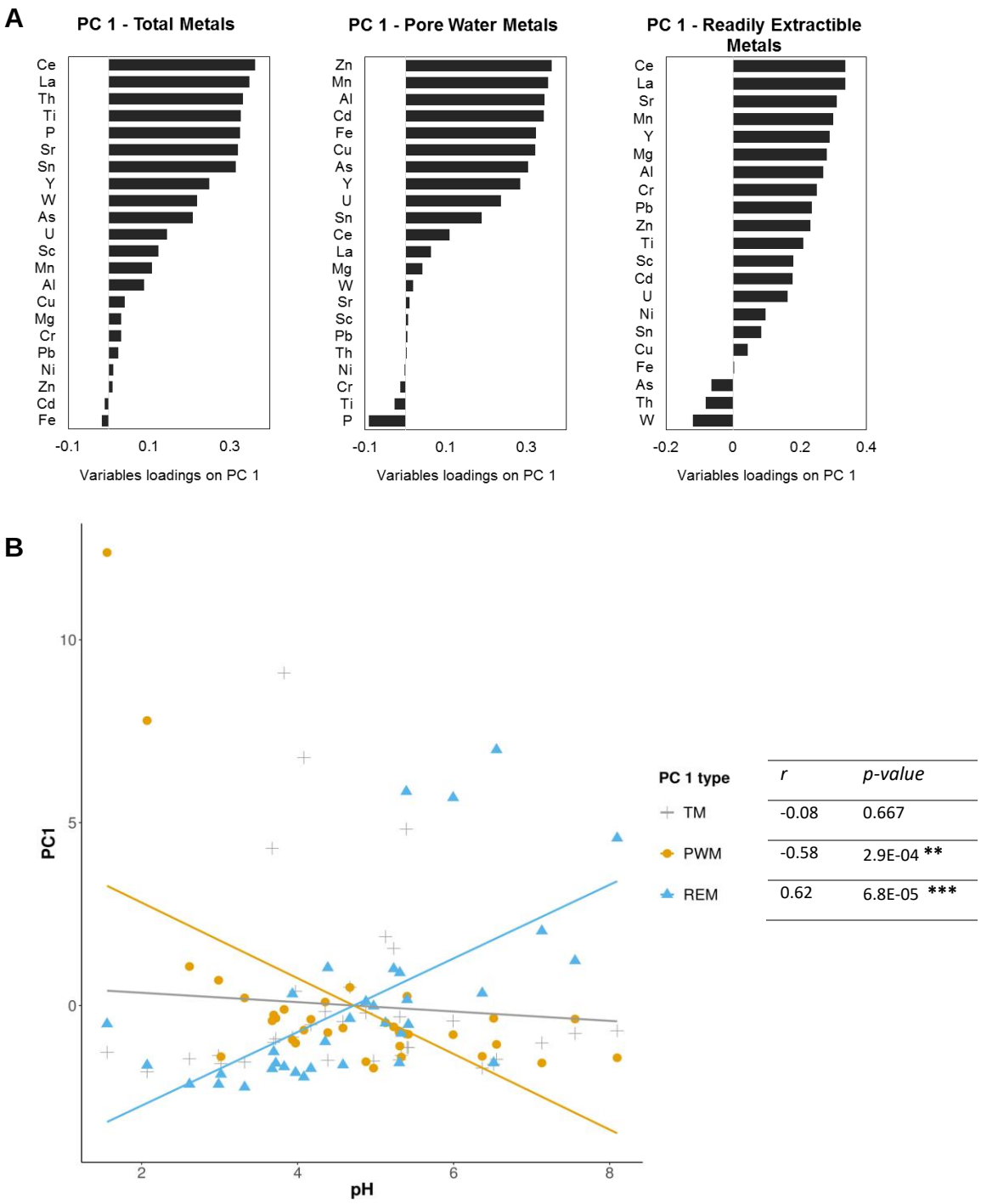


Figure 3.6. A - Variables loading on the first principal components from PCA analysis were mainly positive. B - Positive relationship linking PC1-readily extractible metals and pH, negative between PC1-pore water metals and pH.

3.3.5 Variables relationship with microbial diversity and composition

Table 3.2. Significant correlations between community structure / diversity and single variables (Mantel test).

Y (OTUs level)	Group	Substrate	Ind. Variable	Mantel statistic r	Significance	Method
Alpha diversity	Env		pH	0.4767	**0.012**	Pearson
Alpha diversity	REM	soil	Cd	0.4089	**0.041**	Pearson
Alpha diversity	PWM	mine waste	Ti	0.2193	**0.021**	Pearson
Alpha diversity	REM	mine waste	Th	0.1791	**0.034**	Pearson
Alpha diversity	TM	mine waste	P	0.3005	**0.001**	Pearson
Alpha diversity	TM	mine waste	Th	0.2171	**0.029**	Pearson
Alpha diversity	TM	mine waste	Ti	0.2208	**0.017**	Pearson
Composition	Env		pH	0.7183	**0.001**	Pearson
Composition	PWM	soil	Al	0.4947	**0.046**	Pearson
Composition	PWM	soil	Cu	0.5078	**0.038**	Pearson
Composition	REM	soil	As	0.5108	**0.033**	Pearson
Composition	REM	soil	Fe	0.4158	**0.04**	Pearson
Composition	TM	soil	As	0.5212	**0.038**	Pearson
Composition	TM	soil	La	0.5015	**0.044**	Pearson
Composition	TM	soil	Ni	0.3636	**0.043**	Pearson
Composition	TM	soil	P	0.5218	**0.026**	Pearson
Composition	TM	soil	Sr	0.5103	**0.031**	Pearson
Composition	TM	soil	Ti	0.5224	**0.024**	Pearson
Composition	TM	soil	W	0.5052	**0.029**	Pearson
Composition	TM	soil	Y	0.4901	**0.057**	Pearson
Composition	TM	soil	Zn	0.4215	**0.046**	Pearson
Composition	PWM	mine waste	Th	0.4323	**0.009**	Pearson

Besides summarising all the metals-related variables in a PC- based proxy, it was also tested if the microbial diversity and composition of communities would relate with the single metals via Mantel test (Eilers et al., 2012; Mantel, 1967). Only few variables appeared to correlate with the microbial composition and alpha diversity of the acidophilic communities observed (Table 3.2 and Appendix Table 1). Alpha diversity seemed to be mostly associated to pH, readily extractible metals (Cd and Th) and total metals (P, Th and Ti). A *glm* model was used to assess effects on alpha diversity and showed that it is defined by the group (mine waste or soil sample), REM and PWM (gamma regression, Pr > t: 0.047, 0.034 and 0.08, respectively).

Microbial community beta diversity (β-diversity) was significantly and positively correlated with low concentration pore water metals (Ce, Cr, La, Sc, Sr, Th), Fe

in readily extractible and total metals and Ni in total metals (Table 3.2 and Appendix Table 1, Mantel test). To relate microbial composition of samples and main environmental parameters, *adonis* (*vegan*, R platform) was used. Two *adonis* models better correlated with the community composition dataset: 1: diversity distribution is defined by pH, moisture percentage, *group* factor (mine waste or soil like sample) and PWM (permanova, Pr > t: 0.02, 0.03, 0.02 and 0.09 respectively); 2: diversity distribution is defined by REM and *group* factor (permanova, Pr > t: 0.03 and 0.02 respectively).

3.3.6 Geochemical pressure shaping the presence of the core microbiome

The extent of microbial diversity overlap (shared OTUs) between mine waste and respective nearby soils was visualised for each site (Appendix Figure 2); generally, the soil-like control samples per each site shared part of their diversity with one or both waste samples; in Appendix Figure 2 this is shown as the overlap among circles. Some sites showed a smaller overlap between samples and control samples (WM, WA, PO and MW). The presence of a core microbiome in the whole sampling area was so hypothesised and the percentage of shared community among samples and among control samples was calculated, considering samples and controls as two separate groups (Figure 3.7). Soil controls shared significantly more community percentage with each other (75%) than mine waste samples (60%; t-test, p–value = 0.0018). As previously shown, Wheal Maid and Porthtowan sites' samples are the ones that mostly diverge from the main diversity cluster. The shared proportion of community among waste samples could not be explained by any of the factors included in the analysis, while soil samples diversity appeared to be driven by pH and PWM (1st Principal Component), (p value < 0.05, *envfit* {vegan}, R) (Figure 3.8).

Table 3.3. Weighted versus unweighted 75% -representing community.

Core microbiome components, mine waste samples	*% in samples (average)*
Bacteria, Chloroflexi (p), Thermogemmatisporaceae (f)	4.9
Bacteria, AD3 (p), JG37-AG-4 (cl)	4.2
Bacteria, Verrucomicrobia (p), Chthoniobacteraceae (f), DA101 (g)	3.3
Bacteria (unmatched)	3.1
Bacteria, Acidobacteria (p), iii1-15 (o)	3.1
Bacteria, WPS-2 (p)	3.1
Bacteria, Chloroflexi (p), Ellin6529 (cl)	2.2
Bacteria, Planctomycetes (p), Gemmataceae (f)	2.0
Bacteria, Planctomycetes (p), Gemmatales (o), Isosphaeraceae (f)	1.8
Bacteria, Actinobacteria (p), Acidimicrobiales (o)	1.7

Core microbiome components, soil-like samples	*% in samples (average)*
Bacteria, Acidobacteria (p), iii1-15 (o)	9.3
Bacteria, Verrucomicrobia (p), Chthoniobacteraceae (f), DA101 (g)	3.8
Bacteria, Chloroflexi (p), Ellin6529 (cl)	2.7
Bacteria, TM7 (p), TM7-1 (cl)	2.3
Bacteria, Verrucomicrobia (p), Pedosphaerales (o)	2.2
Bacteria, Proteobacteria (p), Sinobacteraceae (f)	1.9
Bacteria, Planctomycetes (p), Phycisphaerae (cl), WD2101 (o)	1.7
Bacteria, Acidobacteria (p), iii1-8 (cl), 32-20 (o)	1.6
Bacteria, Planctomycetes (p), Gemmatales (o), Isosphaeraceae (f)	1.5
Bacteria, Planctomycetes (p), Gemmatales (o), Gemmataceae (f)	1.4

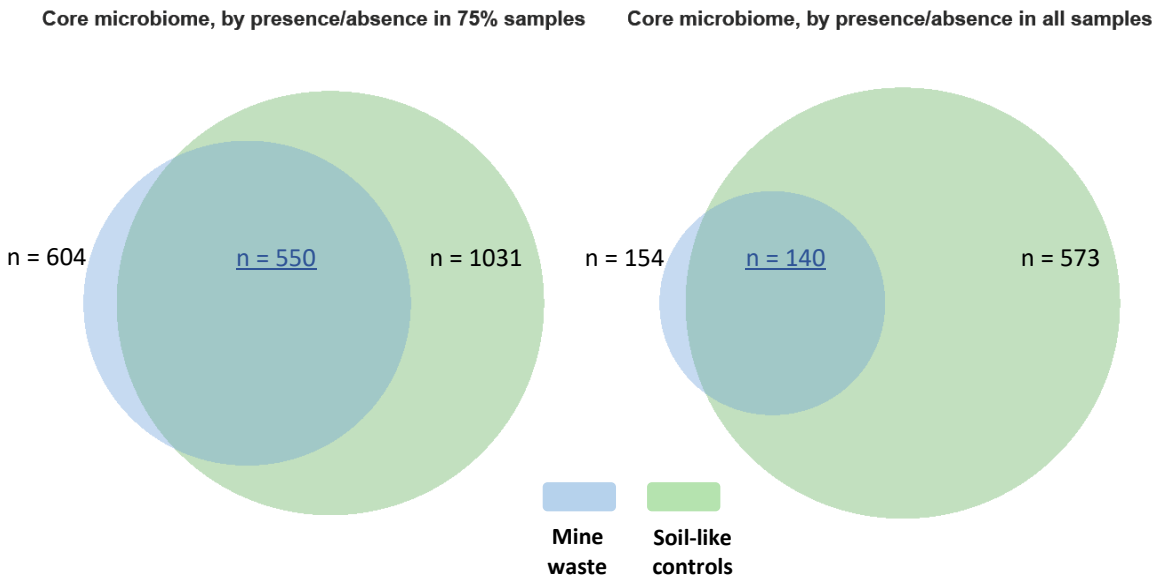


Figure 3.7. Venn diagrams representing the intersection of mine waste and soil-like core microbiomes. Presence/absence of OTUs shared by 75 percent of all mine waste samples and soil-like controls separately was used to describe the microbiome shared by both groups either in 75 percent of samples or all samples. In the above table main OTUs are listed per each core microbiome, in blue are highlighted the most relevant OTUs shared by all samples in this study.

The presence of a core microbiome common to both samples and controls was also visualised with Venn diagrams (Figure 3.7) showing the overlap of diversity between all samples and all soil controls either considering all OTUs or the ones representing 75% of the weighted diversity. Furthermore the diagram shows that control soils present much higher diversity, despite clustering closer in NMDS and PCoA (Figure 3.3) ordinations. In detail, in Figure 3.7 the green zone represents the soil adapted OTUs, the blue-green zone (n = 550 and n = 140) includes the generalist OTUs and the only blue part (n = 54 and n = 14) represents the mine waste specialists.

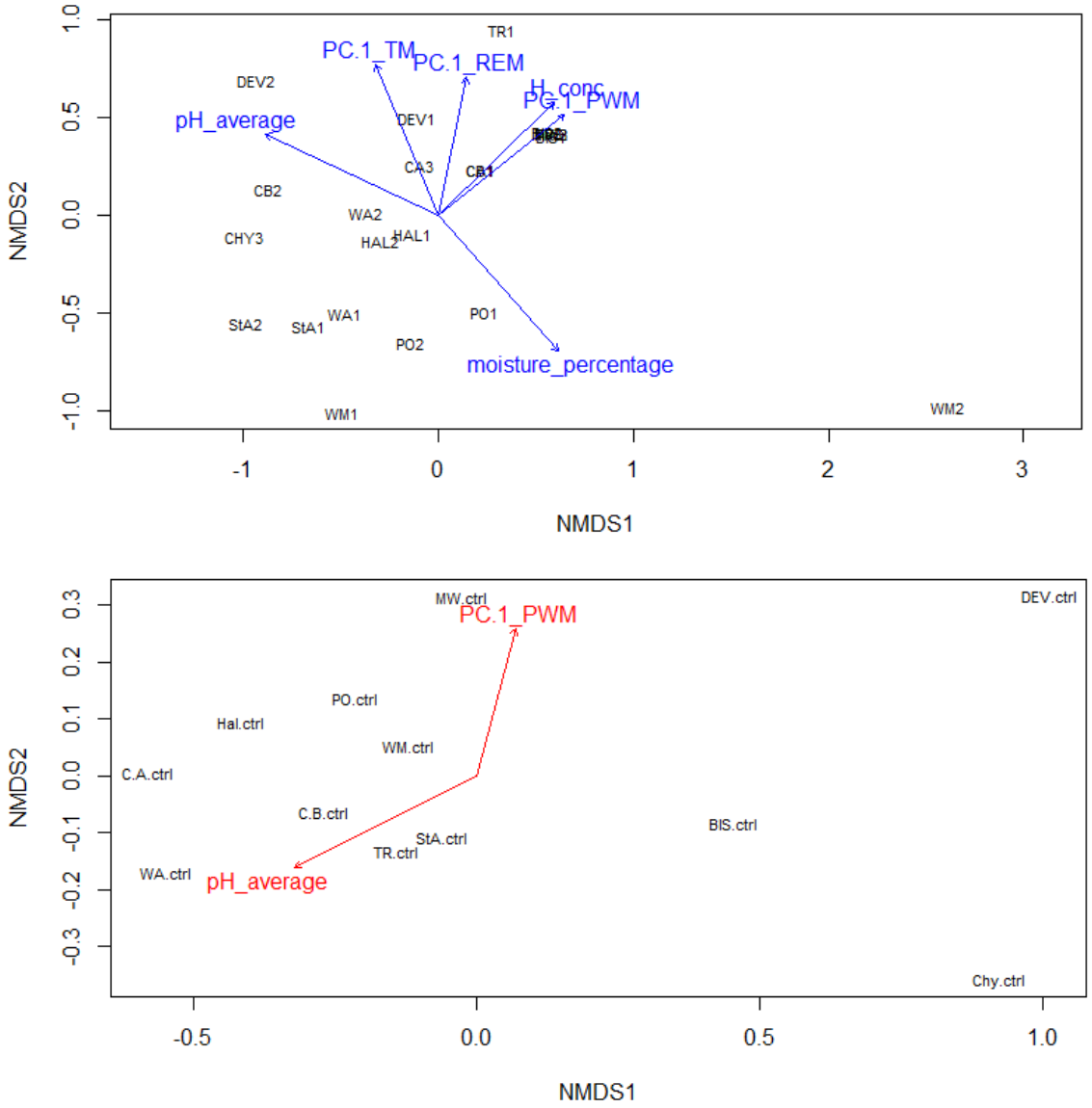


Figure 3.8. Core microbiome in mining related environments is defined by no significant variables, regarding waste samples (upper) and pH and pore water metals (PWM) regarding soil-like controls (lower, red arrows = p-value < 0.05, envfit {vegan}, R).

3.3.7 Mine waste and nearby soils geochemical differ in readily extractible metals.

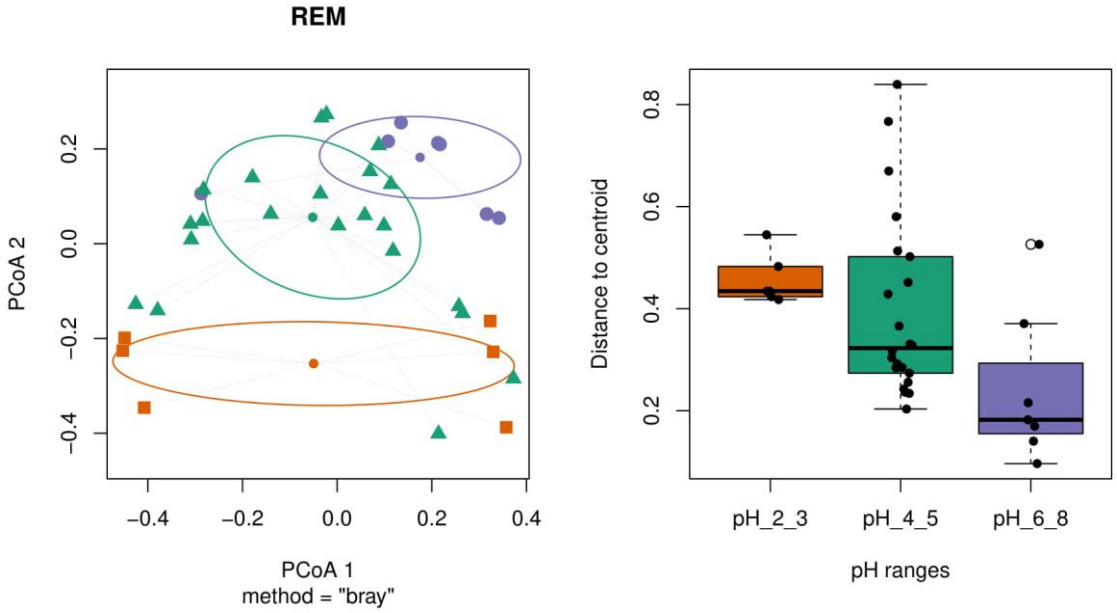


Figure 3.9. Distribution of samples according to metals diversity reflected pH ranges (A) and homogeneity of variance of three pH ranges (orange-2.0-3.9, green- 4.0-5.9 and purple- 6.0-8.0) for readily extractible metals (B). Readily extractible metals homogeneity of variance [16] comparison between pH ranges as distance to the distributions' centroids indicated that dispersions are different in general, that highest pH has the smaller dispersion and that the metals composition vary across the pH groups (*betadisper* Pr> F: 0.04; pH 6 to 8 / 2 to 3 *p adjusted*: 0.05; pH 6 to 8 / 4 to 5 *p adjusted*: 0.08; *adonis* Pr > F: 0.005; *vegan*, R platform).

The diversity of readily extractible metals (metallocity) correlated positively with pH (Figure 3.6 B) and it was found that their distribution differs between mine waste and soil-like samples. Metals composition diversity was checked for homogeneity among samples and control samples (Figure 3.10 A). Total metals diversity was higher in waste samples than in soils but overlaps the soil controls distribution, the two groups were not significantly different in composition. Pore water metals distribution shows the same diversity among samples and controls,

[16] Homogeneity of variance is an assumption underlying both t tests and F tests (analyses of variance, ANOVAs) in which the population variances of two or more samples are considered equal. The test compares the variances of the considered groups (here pH ranges).

not differing in either diversity or homogeneity (Figure 3.10 B). Readily extractible metals diversity discriminates the samples and soil-like control samples in two clusters that show different metals diversity and a non-homogeneous variance among the two groups, waste samples show a higher variability (Figure 3.10 C).

More in depth, the ordination of all samples based on readily extractible metals was represented by grouping by increasing pH ranges (from 2 to 3.9, from 4 to 5.9, and from 6 to 8, Figure 3.9). This showed that the samples in the highest pH range differ in composition and homogeneity from the lower pH range. The distance of all samples to the centroid decreases with increasing of pH, so does total metals diversity (Figure 3.9). It is important to note that pH is a log scale of proton activity; therefore, the range of protons concentration between pH 6 and 8 is exponentially lower than the range of protons concentration between pH 2 and 3. The variability of pore water metals according to pH groups is homogeneous, there is no relation of pH to the distance to the centroid of the groups. Readily extractible metals showed a different composition in the three groups and their diversity was decreasing with increasing pH.

Across sites total metals showed a variable diversity (Appendix Figure 3C), in general non-homogeneous among sites, the metals diversity was mostly variable in low pH sites for total metals, apart from Tretherrup and Wheal Alfred sites. The lower variability indicates lower difference among the three samples, soil-like controls included. In terms of pore water metals (Appendix Figure 3B), the composition in metals does not show a signature by site, exception made for the site MW, PO, TR, indicating a low difference between samples and control sample in the site. Mount Wellington site appears to have a pore water metals composition diverging from the others and clustering together (Appendix Figure 3B) when the samples were ordinated by site (PCoA ordination). The most noticeable metals composition signature among sites is represented by readily extractible metals, for which diversity of metals is lower in TR and WA sites (Appendix Figure 3A). Such sites are characterised by a minor difference in metals composition between samples and control samples (Figure 3.10 C-D, *betadisper* Pr> F: 0.018; *adonis* Pr > F: 0.004, both *vegan*, R platform).

In general readily extractible metals appeared to be predicting more efficiently the distinction between waste samples and soil controls, pH ranges and individual sites.

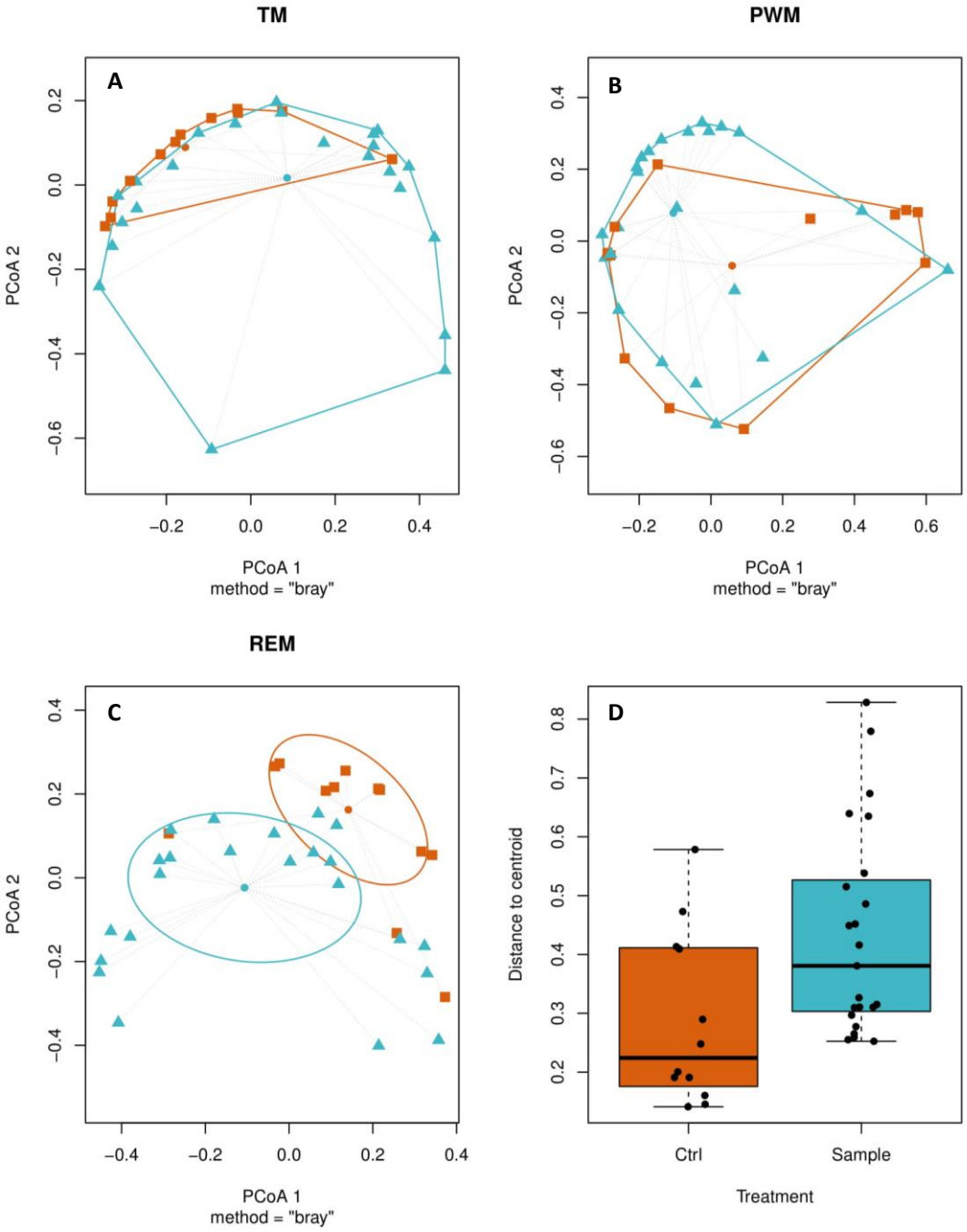


Figure 3.10. Metals diversity distribution and homogeneity of variance in soil-like controls and waste samples (orange and blue ellipses/hulls respectively), for: A - total metals, B - pore water metals, C - readily extractible metals. D – Readily extractible metals homogeneity of variance comparison between soil-like controls and waste samples as distance to the distributions' centroids indicates that in waste samples there is a major beta diversity of metals and the two groups differ in composition (*betadisper* Pr> F: 0.018; *adonis* Pr > F: 0.004, both *vegan*, R platform).

3.4 Discussion

Here a survey was conducted to determine if mine waste sites impose similar selection pressures on hosted microbial communities, relatively to paired control environments. The opposite was found: although they clustered together with respect to paired soil controls, mine waste sites showed much greater between-community diversity. This suggests that either different mine waste imposes very different selection pressure on microbial inhabitants allowing their differentiation; or different organisms are ecologically equivalent between waste samples, hence distribution is driven more by dispersal and chance events (e.g. Kerr et al., 2002).

Recent research have used extensive genomic sequencing (Denef & Banfield, 2012) or proteomics (Mueller et al., 2010) to investigate how rapid adaptive evolution may have assisted in the maintenance of the dominant populations in local AMD communities and how the physiologies of the dominant or less abundant organisms change along environmental gradients (case study, Richmond Mine, Iron Mountain, California) (Goltsman et al., 2015; Méndez-García et al., 2015). While this analysis has focused on difference in the composition of communities, there is likely to be variation within species between sites that can't be detected. Rapid evolution is suggested to be important in AMD communities. This rapid evolution may also have contributed to compositional differences, if different taxa dominate at different sites as consequence of differential rate of adaptation.

Mining-impacted environments considered in the present study are all artificial; caused by human action. They differ from other "extreme" environments because abandoned mine heaps are mainly superficial and even when they are sub-superficial disused sites, and are not completely isolated as they communicate with the outside, therein the exposition (or formation) of such microbial communities is fairly recent. Mining-related environments are considered extreme as they feature both high quantity of metals and acidity coupled with eventual lack or absence of oxygen under the superficial strata. Metals oxidation state and availability are affected by pH and moisture content (Brümmer, 1986; Ledin & Pedersen, 1996). Previous studies have assessed the relationship between pH as one main driver for microbial diversity and composition and it is known that pH ultimately affects metals composition, therein the present

approach attempts at describing the effect that pH and metals, as some main features of the environment play on the microbiota. The influence of the environmental variables has been scarcely investigated in mine waste over a wide range of weathering levels. It is unknown whether mine geochemical characteristics are predictive, of their associated microbial ecosystems. The diversity and composition of microbial communities from diverse and geographically separated acidic mining environments in Southwest England (UK) was characterized. The number of samples surveyed significantly expanded our knowledge of the broad trends of microbial distribution in acidic mine waste environments and nearby soils, being also one first example of solid substrate focused survey of this kind.

3.4.1 Microbial diversity

3.4.1.1 Beta diversity

Few paradigms of diversity are known and valid in acidic extreme conditions such as those defining acidic, metal-rich mines wastes; one paradigm is that such environments are characterised by low diversity (Keller & Zengler, 2004). However, the present results challenge this paradigm by finding the opposite is true in the studied environment. Beta diversity of waste samples was higher than control samples as revealed by the analysis of diversity dispersion at phylum level and at species level showing that the geochemical conditions of the Cornish area favoured mine waste related biodiversity. There was greater β-diversity in the mine waste sites than the soil-like controls. This result contrasts with acid mine drainage and associated environments harbouring reduced microbial diversity and functional complexity (Hogsden & Harding, 2012; Lear et al., 2009; Simmons et al., 2005; Wassel & Mills, 1983). Similar results in terms of diversity extension have been stated in other extreme environments such as hydrothermal vents (Brazelton et al., 2010) and acidic hot spring (Bohorquez et al., 2012; Power et al., 2018; Zaremba-Niedzwiedzka et al., 2017), where rare taxa accounted for most of the observed diversity, suggesting that microbial diversity could be higher than expected in some specific sites with complex interactions among environmental variables and microorganisms. Barriers to dispersal between populations may allow them to diverge through local adaptation or random

genetic drift as already demonstrated with extremophiles, specifically hyperthermophilic archaea (Whitaker et al., 2003).

From a practical point of view, soil control samples were harvested outside the mine waste areas and from patches that were outside of mine waste impacted sites as described in the methods section. Nevertheless, previous studies reported the whole south west area of UK as strongly containing high metals concentrations (Camm et al., 2004; Healy, 1995; Johnson & Hallberg, 2005; Johnston et al., 2008; Neal et al., 2005; Pirrie et al., 1997, 2002; Pirrie et al., 2003; Sbaffi et al., 2017; Whitehead et al., 2005). With regard to beta diversity being lower in soils than in waste samples, microbial heterotrophs inhabiting soil-like control samples, although present, might be struggling to grow in the contaminated soils and this might explain the scarce distinction between control and samples and mostly the major separation between groups in the Un-Weighted UniFrac compared to the weighted analysis. An alternative hypothesis is that acidophiles might have adapted to the soil environments, by occupying niches left free by struggling obligate heterotrophs, helped by a favourable more flexible metabolism (i.e. mixotrophy), especially in the unstable environment.

Mine waste samples and controls were distinguished in terms of presence/absence of OTUs, while results show the presence of a core microbiome, a certain similarity among most of waste samples and a lower β-diversity of control samples, indicating the presence of rare taxa in the taxonomical separation between waste samples and soil-like controls, high in diversity but representing each very low percentages of the microbiomes. Furthermore, sites were found to be more similar between each other than samples of the same site between each other, and this effect is more evident for soil-like controls than for waste samples. Permutational Anova confirmed that variable “site” is not significantly correlated with beta - diversity at OTUs or at phylum level - and this is due to the bias imposed by control samples which are indeed supposed to be diverging the most in composition but also the waste samples in general are not defined by sites. These results indicate that generally there is not a signature community composition by site and when there is, it includes the soil control sample (in the Mount Wellington case) suggesting a strong metals contamination of the nearby soil.

3.4.1.2 Core microbiome diversity

On the basis of what explained in the Section 3.3.6 and of the prevalence of reasonably low diversity in all samples (soil samples included) the hypothesis of a strong core community was put forward. Considering the long term weathering of the region and the variability of the substrates, this result was surprising. One relevant aspect is that the microbiota might derive from the rocks beneath the surface, and taken to the surface by mining activities, which is one potential reason for the common diversity and origin of the biota. This might mean that the speciation could be occurring and still only detectable from an intra specific point of view, as for instance in Porthowan underground site.

The same variables involved in shaping the microbial composition of whole communities were fitted in classical NMDS ordination for the sole "core" diversity. The variables' fit increased for core-only diversity, suggesting that environmental features (e.g. moisture percentage) have a more profound effect on generalists than they do on the whole community structure. These consistent outcome was presumably due to the strong selective pressures of extremely acidic conditions that determine survival of lineages. Dispersal limitation and past environmental conditions can drive genetic divergence of microbial assemblages from geographically separated sites (Martiny et al., 2006). In a wide survey at national scale, Kuang et al. (2013) find that the overall microbial diversity patterns were better predicted by contemporary environmental variation than by geography. The author points out however that the relative influence of historical versus environmental factors could be due to the sampling scale (Martiny et al., 2006), as wider scale studies show a strong effect of dispersal (Papke et al., 2003; Whitaker et al., 2003). The core community retrieved on the Cornish meta-community[17] (Leibold et al., 2004) possibly could be the result of common environmental conditions rather than the residue of a past common ancestral community separated by geographical barriers.

3.4.1.3 Taxa associated with mining-impacted environments

Proteobacteria show a wide variety in the types of metabolism, most members are facultative or obligate anaerobic, chemolithoautotrophic, or heterotrophic. In

[17] An ecological metacommunity is a set of interacting communities which are linked by the dispersal of multiple, potentially interacting species (Leibold et al., 2004).

south China AMD (Kuang et al., 2013), Betaproteobacteria were predominant in assemblages under moderate pH conditions, whereas Alphaproteobacteria, Euryarchaeota, Gammaproteobacteria and Nitrospira showed strong adaptation to more acidic environments, similarly to the present results. In a deep investigation of New Zealand geothermal springs, proteobacterial genera present were mostly characterised as aerobic chemolithoautotrophs, utilising either sulfur species and/or hydrogen for metabolism and the prevalent genus (62.9%) was *Acidithiobacillus* (Power et al., 2018). On the contrary, in the present study the most abundant proteobacterial genus was an unidentified Alphaproteobacterium. *Acidithiobacillus* was not the main genus retrieved, suggesting the domination by an alternative uncultivated functional equivalent taxa or that *Acidithiobacillus* is not a dominating taxon in well-weathered mine waste, as also suggested by Bryan (2006). *Acidithiobacillus* spp. often come to dominate enrichment cultures and are easy to detect through classical microbiology. As stated in the Introduction section on this thesis, in the past this has led to a bias: it was assumed that it dominated mine-impacted sites. However, this study further confirms that it is not the case.

Acidobacteria are ubiquitous and abundant phylum of soil microbial communities, whose abundance relative to other bacterial taxa was previously reported to strongly and negatively correlate with soil pH (Jones et al., 2009). Most Acidobacteria retrieved were unknown and did not match any known class, the ones known were Koribacteraceae, they are not a wide spread taxa but it was found in pristine areas close to revegetated iron mining sites in Brazil (Vieira et al., 2018) and were dominants in semidecidual forest, and other woodlands ecosystems (Pershina et al., 2015). Kleinsteuber et al. (2008) found Acidobacteria subdivision 1 to dominate the bacterioplankton of Lauchhammer acidic lake in Germany and to be strive in the hypolimnion (interface between the sediment and the water column) where more organic carbon was available. The growth of soil Acidobacteria of subdivision 1 is believed to be favoured by slight to moderate acidity (Eichorst et al., 2007; Sait et al., 2002), supported by culture-independent surveys revealing high abundances of Acidobacteria in bogs, forest soils, soils and wetlands impacted by AMD waters or coal waste (Brofft et al., 2002; Dedysh et al., 2006; Hallberg et al., 2006a; Edwards, Goebel, 1999). Soil Acidobacteria of subdivision 1 are found in major abundance in mildly acidic to

acidic soils (Eichorst et al., 2007). Authors (Stevenson et al., 2004) found that increased carbon dioxide partial pressures or low pH could improve the cultivability of soil Acidobacteria. Acidobacteria blooming in AMD systems are thought to be adapted to more acidic conditions (pH 2–3) than Acidobacteria from soil (Hallberg et al., 2006b; Edwards, Goebel, 1999; Kishimoto et al., 1991).

The phylum Chloroflexi contains isolates with a diversity of phenotypes, including aerobic thermophiles, which use oxygen and grow in high temperatures; anoxygenic phototrophs, (green non-sulfur bacteria); and anaerobic halorespirers (Hiraishi, 2008), which use halogenated organics (such as the toxic chlorinated ethenes and polychlorinated biphenyls) as electron acceptors. Phylum Chloroflexi members are often found dominating mining sites, i.e. Miyun County, Beijing City, China (Hong et al., 2015), where it composed over 12% of the entire meta-community. The phylum was also detected in 16S clone libraries from the former (well-weathered) Parc and Mynydd Parys mines in North Wales, UK (Bryan, 2006). This is in line with our findings: Chloroflexi represent 11.1% of the γ-diversity of the Cornish meta-community here analysed. Their presence is also reported in uranium mine waste contaminated and non-contaminated sites close to uranium mines (Dhal et al., 2011) or in the soils around the world's largest antimony mine have, contaminated by high concentrations of Sb and As (N. Wang et al., 2018). Chloroflexi are also thought to pioneer taxa on unvegetated basalts (Dunfield & King, 2004; King et al., 2008), and as vegetation cover and organic carbon increase during biological succession, they are replaced by Proteobacteria (Dunfield & King, 2004; Weber & King, 2010). Iron precipitation not only comes from microbial oxidation of iron. Biomass formation may also contribute to iron deposition (Battin et al., 2007). Furthermore Chloroflexi are known to produce long filaments and to be often associated with biogenic iron oxides (Fru et al., 2012), so they can favour iron oxide formation (Wang et al., 2011, 2014). In this survey Chloroflexi were present in most samples. However, it is interesting to note that they were scarce (in terms of relative abundance) in the two underground samples (MW, PO). This might be supported by their apparent photoautotrophic metabolism requiring sunlight.

Planctomycetes and Verrucomicrobia are part of the PVC bacterial superphylum (Planctomycetes - Verrucomicrobia – Chlamydiae; Van Niftrik & Devos, 2017), many of which feature a lack of peptidoglycan. They are an important component

of most environments, including the human gut. They were found in all sites' samples apart from mine waste samples in CHY, PO, StA and WM. Some members of Planctomycetes and Verrucomicrobia are interestingly among the taxa shared by mine waste samples within each other, by nearby soil samples within each other and by mine waste and respective control soil samples.

Candidate divisions WPS-2 and AD3 were earliest identified from polychlorinated biphenyl polluted soil (Nogales et al., 2001) and sandy surface soil (Jizhong Zhou et al., 2003). AD3 has been identified in more studies than WPS-2 (Costello et al., 2009; Kelly et al., 2010; Kavamura et al., 2013; Serkebaeva et al., 2013; Singh et al., 2012), mainly geothermal areas. Both phyla are usually present in very low abundances in soil. Candidate division AD3 and WPS-2 were uniquely reported being dominant in an ice-free polar desert in Antarctica (M. Ji et al., 2016) and the present survey is one second example where the taxa is very abundant in part of the samples.

Functional diversity is not retrievable from this diversity survey. Trait-based or functional biogeography assessed by metagenomics (Raes et al., 2014) or wide-ranging functional gene arrays (i.e. GeoChip; He et al., 2010) represent a promising strategy to address. In this analysis active community members could not be distinguished from the dormant taxa, which may be metabolically inactive because of the unfavourable environmental conditions and thus less sensitive to environmental change but potentially important for the maintenance of a useful diversity and functional redundancy, relevant for resilience and resistance to stresses(Fierer, 2017; Gihring et al., 2011; Headd & Engel, 2014; Kepner & Pratt, 1994; Whitman et al., 2006).

3.4.2 Geochemical analysis of mine waste

Principal component analysis including geochemical variables besides shared diversity percentage revealed that as a general trend, waste samples are defined by pore water metals (PWM) and total metals (TM), while soil control samples by shared core percentage, pH, moisture percentage, and less strongly by readily extractible metals (REM). This agrees with the different appearance of soil-like samples, they do contain more pore water and the higher pH present defines a minor ability of the rain to leach out metals, also the organic matter present might

act as an adsorbent for metals and contribute chelating them (Kobielska et al., 2018). Higher PWM in waste samples is justified by the lower pH and suggests that active continuous leaching of metals occurs.

3.4.2.1 Metals and pH

The total metal concentrations were broadly the same, independent of pH. However, PWM decreased with increasing pH, whereas REM increased. This might be explained by the fact that at higher pH, metals have precipitated in secondary mineral phases that are readily dissolved in the REM protocol. Conversely, at lower pH, the increased acidity prevents the formation of such secondary minerals (precipitates). This result is consistent with other studies reporting pH as the primary environmental predictor of microbial diversity in several ecosystems (Fierer, 2017; Lauber et al., 2009; Valentín-Vargas et al., 2014), including extreme ecosystems (Lear et al., 2009; Power et al., 2018)

The pH has significant influence on microbial community composition as it affects proton gradients across the cellular membrane and drastically alters nutrient availability, metal solubility and organic carbon characteristics in the environment (Christensen & Christensen, 2000). Additionally, the physiology of cells is affected by pH and less tolerant species will decrease their relative abundance in the community composition when pH increases. Optimum pH for growth can vary considerably among cultivated acidophilic species or even between phylogenetically very similar strains isolated from acidic mining environments (Edwards, 2000; Golyshina et al., 2000). Recent quantitative proteomic analyses proposed pH-specific niche partitioning of prokaryotes ability, confirming the importance of pH and related geochemical factors in regulating acidophilic microbial community structure and function (Belnap et al., 2011; Mueller et al., 2011). The variable pH was also identified as one of the general selective pressures in soil (Fierer & Jackson, 2006; Griffiths et al., 2011; Lauber et al., 2009; Rousk et al., 2010; Whitman et al., 2006). Authors such as Kuang et al. (2013) recently revealed environmental variation as the major factor explaining community differences in AMD in south China showing that microbial diversity estimates were largely correlated with pH, and also identified solution pH as a strong predictor of relative lineage abundance. Contemporary environmental variation rather than geographical distance in extreme AMD systems was a predictor for microbial diversity patterns. The named study (Kuang et al., 2013;

Liu et al., 2014) was quite complete but only took in consideration the microbial community from liquid AMD and not the origin of it (solid substrates) so a strict comparison is not possible.

Here, it is demonstrated that pH had the most significant effect on diversity across all samples measured. When samples were split into pH increments, the variable REM only significantly constrained diversity.

Total metals concentrations (TM dataset) did not allow to discern mine waste and soil controls. It is not surprising that waste samples presented higher diversity of total metals nor the partial overlap regarding total metals composition as it was hypothesized that soils are actually contaminated by nearby mine waste. Higher pH samples had a significantly lower diversity in total metals compared to lower pH samples (data not shown), this relates to the fact that generally higher pH samples were likely to be soil-like samples or at an advanced level of weathering. Unexpected results regarded pore water metals, they do not differ in composition and in diversity among waste samples and soil-like controls, and moreover pH appears not to have an effect on the ordination nor on the diversity of pore water metals. As a site, Mount Wellington showed a signature ordination concerning pore water metals only, indicating a different composition (rich in Fe) shared both by waste samples and soil-like control of this site. This might additionally indicate a strong contamination of the nearby soils from Mine Wellington mine waste.

Readily extractible metals diversity was slightly higher in waste samples and the composition in waste and control samples was different. Additionally pH showed an effect on both the composition and the diversity of readily extractible metals. Lower pH range was correlated with a higher diversity for REMs. Probably un-weathered ores/soils (showing low pH values) preserved a high amount of metals which are not strongly chelated, so their availability for bioleaching is favoured by low pH. High-pH samples share a lower diversity of total metals (bulk metals), indicating a possible previous environmental release of metals and a higher level of weathering.

The pH effect was also slightly stronger at phylum level than at species level, shaping more distinguishable OTUs clusters. The variable pH correlated strongly with both alpha diversity and composition of the communities and showed a significant negative correlation with readily extractible metals. Interestingly,

some authors (Kuang et al., 2013; Liu et al., 2014) find that microbial diversity generally increases along the pH gradient, but also observe a moderately higher diversity in the lowest pH level (pH <2.0). According to the same authors, such finding may be related to the higher organic carbon contents in a few samples in low pH group, as high carbon can promote higher species diversity in soil (Zhou et al., 2002) and marine sediments (Stach et al., 2003). It is proposed instead that most extremophile organism find a more available niche along the decreasing pH gradient but peaking at low pH and possibly a higher level not covered by our samples.

Whereas aqueous samples typically exhibit a more homogenous chemistry and community structure, the heterogeneity of non-liquid samples (e.g. particle size, depth, nutrient composition) is known to affect microbial population plus a high component of the soil and soil-like microbiome is known to be dormant and or in a steady state (Fierer, 2017; Gihring et al., 2011; Headd & Engel, 2014; Kepner & Pratt, 1994; Whitman et al., 2006). This might explain the difficulty to retrieve relationships linking the whole microbial community and most geochemical single features in solid matrices in mine-related environments. From this comes our approach using both single variables and datasets (REM, PWM and TM). The analysis of solid samples extends the results of previous work that mainly focused on biofilms or on the aqueous component of AMD, moreover it allows the identification of sincere community–substrate relationships in these environments.

Previous research demonstrated that correlation does not necessarily always occur between spatial proximity and physicochemistry (Power et al., 2018), and this is additionally demonstrated for solid substrates where heterogeneity is even more marked. Geochemical features are more driving than geographical distance (sites distribution of samples) and pH is the most influencing amongst all geochemical variables.

3.5 Conclusions

This investigation attempted at providing an insight into the regional scale patterns of microbial distribution in mine-impacted habitats, especially underexplored solid substrates. Sites with large variability in geochemical

features were selected and, to enhance variety, included in the survey were the neighbouring soils of mines.

One main emerging feature is the higher beta diversity in mine waste samples compared to nearby soil-like matrices. This matches a wider diversity in metals composition (pore water metals) in the waste samples, thus suggesting a close interaction between microbial diversity and metals potential availability. This can be explained by the radiation of specialists in the extreme environment, as opposed to the constrained diversification/survival of generalists in soil conditions. Therefore, for diversity the mine waste environment is more favourable than the soil. High diversity can imply redundancy of functions, as the systems considered are pushed by acidity and heavy metals concentrations not to allow a huge amount of functions, or could imply the diversification of functions/resistances/metabolisms relatedly to the metals diversity. The community beta-diversity is also driven by pH.

These results suggest sub-community requirements starting from a meta-community point of view, therefore providing insight into previously understudied microorganism–niche interactions in such specific habitats at a regional scale but, as an environment type also spread all around the world (Schuchová & Lenart, 2020).

Future investigations specifically focusing on the active members of the community will allow revealing more definite patterns of microbial diversity. That is pivotal, as active taxa share crucial roles in the functioning of mine waste ecosystems. Evaluation of microbial community dynamics at fine temporal scales and over relatively long periods of time within a diverse range of mine waste communities would gather novel insight and greater predictive power to the microbial diversity patterns in such extreme environments.

The relative importance of selection and chance in driving between-community diversity in mine waste communities is addresses in the next chapter, by determining if communities are locally adapted to their substrates.

4 Testing for local adaptation of acidophilic communities: a reciprocal transplant experiment

4.1 Introduction

The abundance and diversity of individuals and species in natural microbial communities facilitates their rapid adaptation to changing environments. Biogeography is the study of the distribution of organisms across time and space.

The distribution of microbial populations is determined by a number of processes. A common framework to understand community assembly is the distinction between deterministic (niche-driven) processes such as environmental selection and those that are stochastic (neutral, i.e. dispersal; Evans et al., 2017; Vellend et al., 2014). Because microorganisms have high dispersal rates, large population sizes, fast growth rates and a propensity for dormancy, communities were traditionally thought to assemble deterministically. However, recent characterizations of microbial communities provide evidence that along with environmental selection, stochastic processes can be important drivers of microbial community assembly (i.e. Ofiţeru et al., 2010; Zhou et al., 2013). Evans et al. (2017) examined in what way interactions of dispersal with drift and selection would alter the reliability of microbial communities assembling. In their simulated, realistic, individual-based model of decomposers, dispersal rate significantly altered patterns of community composition and stronger selection led to major stochasticity in microbial composition.

The role of stochasticity in community assembly can be enhanced by both high and low dispersal rates. Low or limited dispersal can introduce/enhance stochasticity in microbial communities (Bell, 2010; Lindström & Östman, 2011; Logue & Lindström, 2010; Martiny et al., 2006), potentially through increased drift (Hanson et al., 2012; Wang et al., 2013).

On the other side, high rates of dispersal can induce mass effects[18]. Through mass effects, high dispersal rates can obscure selection making microbial communities more homogeneous than expected by chance and less predictable with respect to environmental variables (Leibold et al., 2004).

That said, deterministic patterns still play a key role in patterns in microbial spatial distribution. One important factor is the extent to which microorganisms are specialists or generalists with respect to different ecological niches (Kassen, 2002). Specialisation will likely mean that microorganisms are locally adapted, (i.e. perform better in their own environment than a foreign environment; Kawecki & Ebert, 2004). In addition to stochastic processes, local adaptation is likely a key factor determining biogeography in all organisms.

In spatially heterogeneous environments evolution may lead to the adaptation of populations and communities to specific local conditions. In fact, local adaptation can result from ecological selection (i.e. species sorting), but also from evolution (i.e. different populations locally adapting) and this has been observed too in acidic environments (Kuang et al., 2013; Quatrini & Johnson, 2018).

Local adaptation theory is typically applied to metapopulations: groups of populations that interact with one another through migration (Hanski, 2005). A large part of the experimental research on microbial local adaptation has focused on individual genotypes (or populations) as units of adaptation, because experimentally discerning microbial communities in nature remains difficult (Kraemer & Boynton, 2017). Fewer studies have focused on patterns of local adaptation of entire communities, but examples include communities of mycorrhizal fungi to local plants and of microbial communities to heavy metal stress (respectively, Hoostal et al., 2008; Ji et al., 2013; Johnson et al., 2010).

[18] The expression “mass effects” refers to an extension of sink-source dynamics that is applied to multispecies metacommunities when migration from high- to low-fitness communities alters the recipient community’s dynamics (Mouquet et al., 2005; Shmida & Wilson, 1985).

4.1.1 Local adaptation theory

The theory of local adaptation indirectly assumes that the relative fitness of the compared populations (or communities) vary in space, with populations having relatively higher fitness in their own compared to foreign environments. This is possibly true in most, if not all, natural systems, because the local environment of each community is composed of biotic and abiotic factors that typically vary in time and space (Gandon et al., 1998).

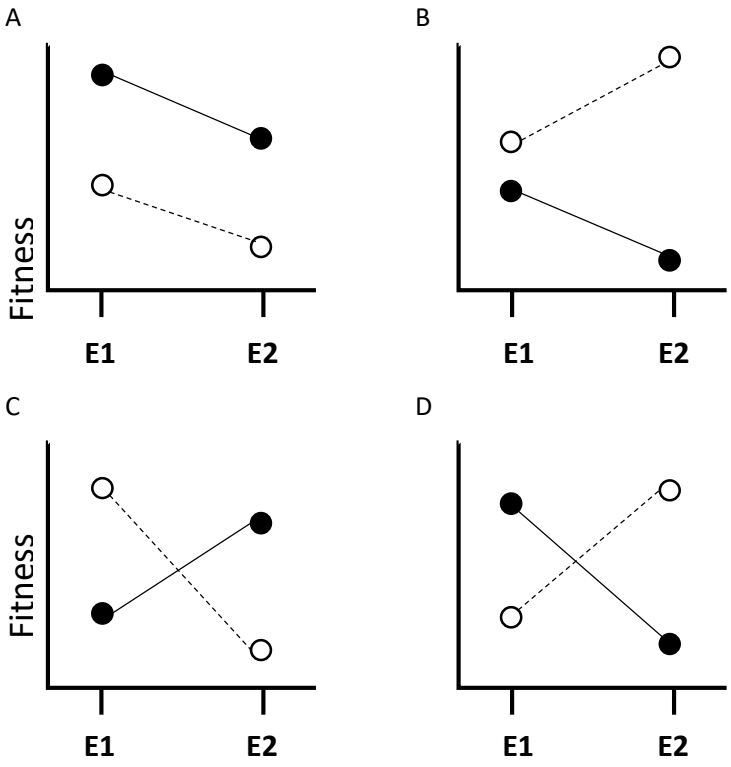


Figure 4.1. Genotype fitness in different environments. Here, Genotype 1 (●) was isolated from Environment 1, Genotype 2 (○) from Environment 2. (A) No significant G x E: Genotypes are not locally adapted according to both 'home vs. 'away' and 'local vs. foreign' frameworks; (B) significant G x E but no rank order in fitness. Genotypes are adapted according to 'home vs. away' but not 'local vs. foreign' framework; (C) significant G x E, rank order of fitness changes, but foreign genotypes are fitter: local maladaptation; (D) significant G x E, rank order of fitness changes and genotypes fittest in their local environment: local adaptation according to 'home vs. away' and 'local vs. foreign' framework. The schematic figure was adapted from Kraemer & Boynton (2017).

Local adaptation is typically quantified in the following ways:

1. In *Home vs. away* scenario, local adaptation is inferred when a genotype's mean fitness is larger in its origin environment than in remote environments according to Kawecki & Ebert (2004). (Figure 4.1B). This approach was used to infer local adaptation in (Belotte et al., 2003). A limitation of the approach is that some environments may universally support higher growth rates than other environments, and hence local adaptation may be obscured

2. The second situation is detectable with a *Local vs. foreign* analysis. It can differentiate local adaptation from environmental effects (Figure 4.1A- D). Local adaptation is proposed when genotypes have higher mean fitness in their home habitats than do competitors originated from other environments (Figure 4.1D). Kawecki & Ebert (2004) suggest that that a metapopulation is locally adapted only when all populations are locally adapted (following a local vs. foreign criterion) and (Blanquart et al., 2013) add that such criterion is likely to be valid when deme quality effects are reduced and that less likely to be fulfilled the more populations are included in an experiment. This is because some populations may be universally fitter than others, and hence local adaptation may be obscured

In this study, the term local adaptation will not be used to describe the situation when both conditions are satisfied the adaptor being the microbial community, because this is not always the case. Specifically, Sympatry–allopatry (SA) is way of summarising local vs foreign or home vs away scenarios; the average fitness of sympatric population–environment combinations is compared to the average fitness of allopatric population–environment combinations. It indicates the overall adaptive 'fit' between populations and their environments (Blanquart et al., 2013; Koskella et al., 2011). Local adaptation is verified if it does for most genotypes. Common frameworks of local adaptation experiment is summarised in Table 1.

Observational studies (see Table 4.1) indicate whether population and community patterns are consistent with local adaptation, but they do not allow a comparison between mean fitness in sympatry and allopatry, which is crucial to unambiguously draw conclusions about local adaptation A local genotype's presence alone is not a reliable proxy for its fitness (Kraemer & Boynton, 2017).

Fierer & Jackson (2006) showed significant correlations between microbial community structures and soil pH on a wide spatial scale (continental), suggesting that communities could be locally adapted to the environmental variable. Hundreds of observational studies have been conducted, in a range of backgrounds correlating microbial communities with environmental conditions, but relatively few were fitness is directly measured in own and foreign environments in a full reciprocal design. Such correlations are important to define the scale and environmental conditions for selection and help planning appropriate tests. Experimental studies, on the other hand, can test the hypotheses generate from observational studies

Table 4.1. Experimental designs employed to detect local adaptation (adapted from (Kraemer & Boynton, 2017))

Experimental design	*Type of study*	*Manipulation*	*Covariate measured*	*Obtainable results*	*Power to detect local adaptation*
Population structure	Observation	-	-	Genetic or phenotypic structuring, Evidence of positive selection	Low
Biogeography	Observation	-	Spatial distance	Population structure correlated with spatial distance or environmental variable	Low
Common garden	Experimental	Move genotypes to the same environments	Genetic distance	Differences between Genotypes and G x E	Medium
Home vs. Away	Experimental	Move genotypes to different environments	Environmental variable	Differences between Environments and G x E	Medium
Local vs. Foreign	Experimental	Move genotypes to different environments	Genetic distance and environmental variable	Differences between Genotypes, Environments, G x E (if applied in strict sense)	High
Sympatric - Allopatric	Experimental, summary of local vs foreign and home vs away methods	Move genotypes to different environment, reciprocally	-	Average difference between sympatric and allopatric combinations	High

Experimental studies of Local adaptation in nature has been extensively studied in macro systems (Anderson et al., 2013; Fraser et al., 2011; Hereford, 2009; Leimu & Fischer, 2008; Sanford & Kelly, 2011) through reciprocal transplant experiments: measuring performance in both sympatric and allopatric environments. However, it is challenging to study microbial local adaptation in nature: microorganisms are difficult to follow in the field (Kraemer & Boynton, 2017) and genetic manipulation is often used to investigate microbial evolution in the laboratory, but it is impractical or illegal to use in the field (Boynton et al., 2017).

Microbial studies conducted in the natural environment report either no significant genotype-by-environment interaction (Boynton et al., 2017; Kraemer & Kassen, 2015), significant changes in fitness between local and foreign environments without changes in genotypes' own fitness (Leducq et al., 2014) or maladaptation to local sympatric conditions (Fox & Harder, 2015; Kraemer & Kassen, 2016; Rengefors et al., 2015). Local adaptation of bacteria to ocean environmental niches, to different levels of heavy metal contamination in lake water and to soil conditions (Belotte et al., 2003; Hoostal et al., 2008; Z. I. Johnson et al., 2006) has been reported.

Most information on microbial local adaptation derives from useful experimental effort of cultivable microorganisms in laboratory environment, which makes it difficult to extend experimental results to really uncover what happens in a natural system and complex communities (Kraemer & Boynton, 2017). Kraemer & Boynton (2017) explain that observational studies of microbial diversity can provide evidence for or against an evolutionary process but rely on correlational findings instead of direct hypothesis testing (e. g. Fierer & Jackson, 2006; Fuhrman et al., 2008). An exception may be where aspect of the environment can be unambiguously controlled in the laboratory, such as measuring the local adaptation of phage to sympatric and allopatric bacteria isolated from soil, but measured *in vitro* (Vos et al., 2009).

In summary, Evidence for local adaptation in microbial systems comes from many observational and much less numerous experimental studies, mainly focusing on metapopulations and rarely considering a whole community context or the metacommunity.

4.1.2 Key ecological affecting on local adaptation

Scale

Scale can affect adaptation in terms of biotic component (e.g. community, metacommunity, microbial populations, metapopulations and species) and environmental scales (e.g. different spatial and temporal scales, diverse environments). Kraemer & Boynton (2017) affirms that observational research prior experimentation can help targeting the appropriate scale for experimental detection. Local adaptation can occur at scale of population, species and communities (driven by both ecology and evolution) and each requires being studied to understand and predict microbiomes composition. Bacteria can respond to selective pressures which are heterogeneous across very small to very large geographic distances; thus, the spatial structuring of bacterial populations and communities is likely to differ greatly across traits, species, and systems and it is to be considered as a continuum (Koskella & Vos, 2015). Likewise, given the rapid rate at which bacterial populations can respond to local selection, their rate of adaptation may be more limited by the speed of environmental changes, rather than actually by the adaptive potential of populations. (Koskella & Vos, 2015) argue that the rate of evolution, variable among environments, should fall across a continuum of rapid to relatively slow population- and community-level change.

Co-evolution

Biotic factors might drive local adaptation as well as abiotic environmental factors, Organism can be locally adapted to each other, and this may be magnified by coevolution (the reciprocal adaptation of populations) by establishing interspecific relationships (Brockhurst et al., 2003; Brockhurst & Koskella, 2013; Leybourne & Cameron, 2006). While co-evolution can occur among any interacting species or genotypes, the majority of work on microbial coevolution has been conducted in host–pathogen systems (Buckling & Rainey, 2002; Ebert, 1994; Gomez & Buckling, 2011; Greischar & Koskella, 2007), There is a slight tendency for parasites to be locally adapted to their hosts (and hence hosts locally maladapted), presumably because of their greater evolutionary potential, but this finding is by no means ubiquitous.

4.1.3 Local adaptation at community level

Traditional studies of local adaptation have tracked the fitness of individual genotypes within a species. However, in nature genotypes coexist, coevolve and the result of local adaptation is also affected by such relationships, therein, especially when the culturing of strains is difficult (i. e. extremophiles), it might be more appropriate to test for community responses to environmental shifts. Few studies considered a whole community prospective: Arbuscular mycorrhiza fungi were found showing high local colonization ability across different environments and some authors (Hoostal et al., 2008; Ji et al., 2013; Johnson et al., 2010) found community-level bacterial local adaptation to the presence or absence of heavy metals in lake water by measuring extracellular enzyme activities as a proxy for adaptation. In another case, local adaptation of the community was compared with the adaptation of individual isolates (Lawrence et al., 2016): whole communities were showing local adaptation but their respective isolates showed no such patterns, indicating that local adaptation may differ widely across levels of analysis and might be, mostly in nature, a property of the whole community unit rather than of populations or might just be driven by main player taxa.

Fitness is a quality of genotypes (Orr, 2009), and it may be conceptually difficult to extend fitness concepts to a whole community, as different species ecologically diverse might show frequency-dependent dynamics. Community local adaptation may represent the average local adaptation across all genotypes present, or may be driven by a few abundant genotypes (Koskella & Vos, 2015).

4.1.4 Measuring LA of acidophilic communities

Here, the local adaptation of acidophilic whole communities in microcosms where the main environmental factors were maintained (mineral substrate and pH) is investigated. Mesocosms and microcosms are a step further to uncovering what happens in nature although complex mesocosm might allow low replication. The acidophilic microbial metacommunity represent a good model of study, because of the many interactions within it (Johnson, 2016; Quatrini & Johnson, 2018), which motivates the interest into looking at the whole community. Many examples of microbial interactions have been described, *in vivo* and *in vitro*, in low pH

environments and laboratory simulations (i.e. Chen et al., 2016). One mutualism example well displaying the interplay of carbon, iron, sulfur cycling and a co-culture performing with greater efficiency than isolates alone, is the case of *Acidithiobacillus thiooxidans* and *Ferrimicrobium acidiphilum* growing in inorganic media with the mineral pyrite as sole energy source (Bacelar-Nicolau & Johnson, 1999). *At. thiooxidans* is an autotroph (oxidizes reduced sulfur but not iron), while *Fm. acidiphilum* is an iron-oxidising heterotroph (oxidizes iron but not reduced sulfur and requires a source of organic carbon). Together, but not alone, they can unlock the energy available from oxidizing pyrite. *Fm. acidiphilum* starts the process by generating ferric iron, which attacks the mineral and releases small amounts of reduced sulfur making it available as electron donor by *At. thiooxidans*, fuelling its fixation of carbon dioxide into organic carbon. Some of the carbon is leaked and assimilated by *Fm. acidiphilum*, facilitating its continued oxidation of iron and dissolution of pyrite. Acidity produced from sulfur oxidation (by *At. thiooxidans*) is of mutual benefit to both acidophiles (Quatrini & Johnson, 2018). Furthermore investigating the local adaptation of such communities might lead to results with practical applications, for instance it could be useful better pairing inocula and substrates to optimise their activity (for example in biohydrometallurgy or bioremediation).

4.1.5 Rationale

The main aim of the experiment was to test the local adaptation of the communities by determining if communities performed more efficiently on their own substrate in comparison with foreign communities, and equally if performance was greater on local *versus* foreign substrate. The efficiency of each experiment was defined by two main response variables: Fe-oxidation extent and total Fe solubilisation. A second aim of the experiment was to assess the presence of one or more better performing communities that can over-perform the majority of locally adapted communities.

The data deriving from the experiments were also used to define the extent of the optimal range of working conditions for the single communities (performance plasticity). In bioprospecting terms, one objective of the present study is to offer

an instrument to explore the functions of a range of microbial communities and explore their potential for bio-hydrometallurgy or bioremediation purpose.

4.2 Materials and methods

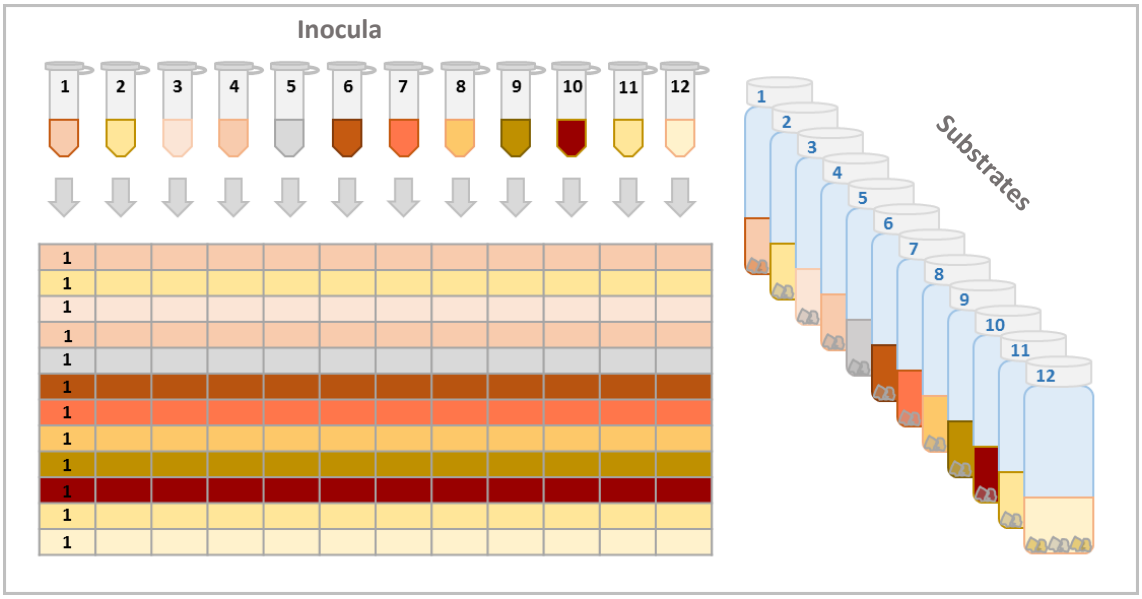


Figure 4.2. Experimental design, showing the distribution of inocula on substrates in the reciprocal transplant experiment.

4.2.1 Experimental design and purpose

Twelve inocula, deriving from the maintenance flasks described in Chapter 3, were reciprocally tested in twelve treatment conditions: the original "environments" of the communities (meaning the original waste rock debris and same pH, Figure 4.2 and Table 4.2). Each community was tested on each substrate independently and each experiment was performed in three replicates. Performance was evaluated by measuring changes in pH and soluble iron concentration and speciation.

When all communities from all experiments had passed the growth exponential phase, all the experiments were sub-cultured in fresh vials. Maintenance flasks community analysis was used as a t = 0.

4.2.2 Vial preparation

Mine waste samples harvested from South West England were dried, screened for bigger particles (> 0.5 cm, removed) and preserved at RT in safely closed Falcon tubes. Glass vials were filled with 10 mL BM and 1% substrate, loosely closed with plastic caps and sterilized by autoclaving (121°C, 15 min).

The cells abundance of twelve non-pyrite flasks, described in Chapter 3 was estimated by direct count at the phase microscope using a Thoma counting chamber. Inocula were collected, from the flasks, in sterile Eppendorf tubes, the tubes were centrifuged at 800 x g for 1 min, the supernatants harvested and centrifuged at 6000 × *g* for 40 min. The pellets of cells were then re-suspended in appropriate pH adjusted (H_2SO_4) BM. Concentrations of inocula were equalised by dilution to 4.13×10^6 cells/mL and 1 mL of each cells suspension was added to the respective vial as inoculum.

4.2.3 Experiment

Vials were agitated in orbital shakers at 100 rpm for the length of the experiment. Vials were weekly opened and periodically sampled according to Figure 4.2. During each sampling, vials were shaken vigorously, let settle for 1 min and 500 µL from each vial were collected in sterile conditions (vertical flow laminar hood).

Sampling effort was consisting in the following assay:

- Concentration of soluble Fe(II) and Fe(III) using the ferric chloride assay.

No modifications were applied to methods described in Chapter 2.

In this study, local adaptation was measured through two proxies: the total iron solubilised (tot Fe) and the percentage of ferric iron (% Fe ox). Total solubilised iron was calculated as the difference from the iron solubilised at the end of the experiment and the iron present at the beginning of the experiment, it was expressed as a standardised value, against the total iron contained in the substrate (value obtained from Total Metals, see Chapter 3). The percentage of oxidised iron (Fe^{3+}) was calculated as a percentage from the absolute value of total iron solubilised in the end of the experiment. Both total and oxidised iron were measured via the ferric Fe-chloride assay, modified from Govender et al.

(2012), as explained in Chapter 2. Two other variables were considered: pH value and cells abundance. The variable pH was collinear with Fe solubilisation, cells abundance that was measured was representing planktonic cells mainly and the author decided not to take it in consideration.

The variable chosen (total Fe solubilised and the % of Fe oxidised) represent the ability of the community to thrive in such mine waste environments. A variety of metabolisms are present in the system but due to the lack in organic carbon, heterotrophic microorganisms would feed on the dissolved organic carbon (DOC) deriving from cells exudates and dead cells either deriving from their own metabolic pool or from the Fe cycling pool described in Figure 1.2 of Chapter 1; in both cases increasing biomass would depend on the Fe cycle–derived cells and their performance. It follows that Fe solubilisation and oxidation abilities well summarise the ability of the communities performance.

4.2.4 Community analysis

Flasks were also sampled at the time (0) of the experiment described in Chapter 5, for DNA extraction and MiSeq-based community analysis. Basal media flasks were used as maintenance flasks for the original conditions of the communities. In the present study they are considered suitable for representing the t (0) of this experiment as well. Differences within the communities' compositions of flasks experiments and environmental communities are highlighted in the results section of Chapter 5.

4.2.5 Statistical analysis

All statistical analysis and most calculations were done on the platform R as described in Chapter 2.

Starting inocula were derived from maintenance flasks as described in Chapter 3, for each site, as explained, two different samples were selected and equal amounts of sample and substrate were used to set up maintenance flasks. At the start of the experiment, the flasks were sampled for inoculating the experiments and the inocula were sequenced. Unfortunately sequencing was not successful

for some samples and it was decided to use the environmental community composition as the time (0) inocula composition, by averaging the structures of the two samples from each site. In Chapter 3, some maintenance flasks structure clustering close to the environmental community structure are shown.

Hierarchical cluster analysis was performed using Ward's method (Ward, 1963), applied on the matrix describing all calculated inocula structure at phylum level, scaled.

To test for the presence of a best performing (based on iron metabolism, described above) inoculum or substrate an anova analysis was run, as a comparison against a base mean, such comparison shows if samples perform above or under the average both for total solubilised Fe and for oxidised iron (%), and both for inocula and substrates.

To visualise local adaptation results paired t-tests were done within each couple of sympatric/allopatric groups per each site, plots were shown only for *local vs foreign* scenario. The same boxplots were paired with further boxplots (Figure 4, C and D) showing only the mean values and where the local groups were normalised to zero, to show whether the allopatric mean was superior or inferior compared to the locals, despite the significance of statistical tests.

Independent variables and covariates were transformed if believed appropriate. In terms of statistical modelling, local adaptation can be described by an equation decomposing the variation of fitness into the main effects of genotype (here, the community) and environmental quality (here, mine waste substrate), plus the interaction between each genotype and environment (G x E):

$$\text{Fitness} = \text{Genotype} + \text{Environment} + \text{Genotype x Environment} \quad (2)$$

Local adaptation is one possible scenario within the interaction term, which represents the variation in genotypes' fitness relative to one another across environments. Such interaction term is independent of overall environmental quality or genotype performance (Blanquart et al., 2013) and it is tested by substituting the interaction term with the factor Sympatric/allopatric, as follows:

$$\text{Fitness} = \text{Genotype} + \text{Environment} + \text{Sympatric/Allopatric} \quad (3)$$

To be noted that this method combines both measures of local adaptation (*Home vs away* and *Local vs foreign*), and remove the potentially confounding effects of

each (i.e. some environments being universally better than others, and some communities being universally better than others).

4.3 Results

4.3.1 Inocula

The reciprocal transplant experiment was done using twelve environmental mine waste substrates and the twelve respective inhabitant communities. The mine wastes were described in Chapter 3 and presented a high variability in metal composition, readily extractible metals and total metals.

The initial microbial community composition is shown in Figure 4.3. The composition was calculated as an average of the composition of the two samples per site described in Chapter 3. Ward hierarchical clustering is applied and k set at 5, in this way the result grouping is as follows: (1) MW and BIS; (2) DEV, CB, CA and TR; (3) CHY, StA, HAL and WA; while (4) WM and (5) PO both represent respectively single groups.

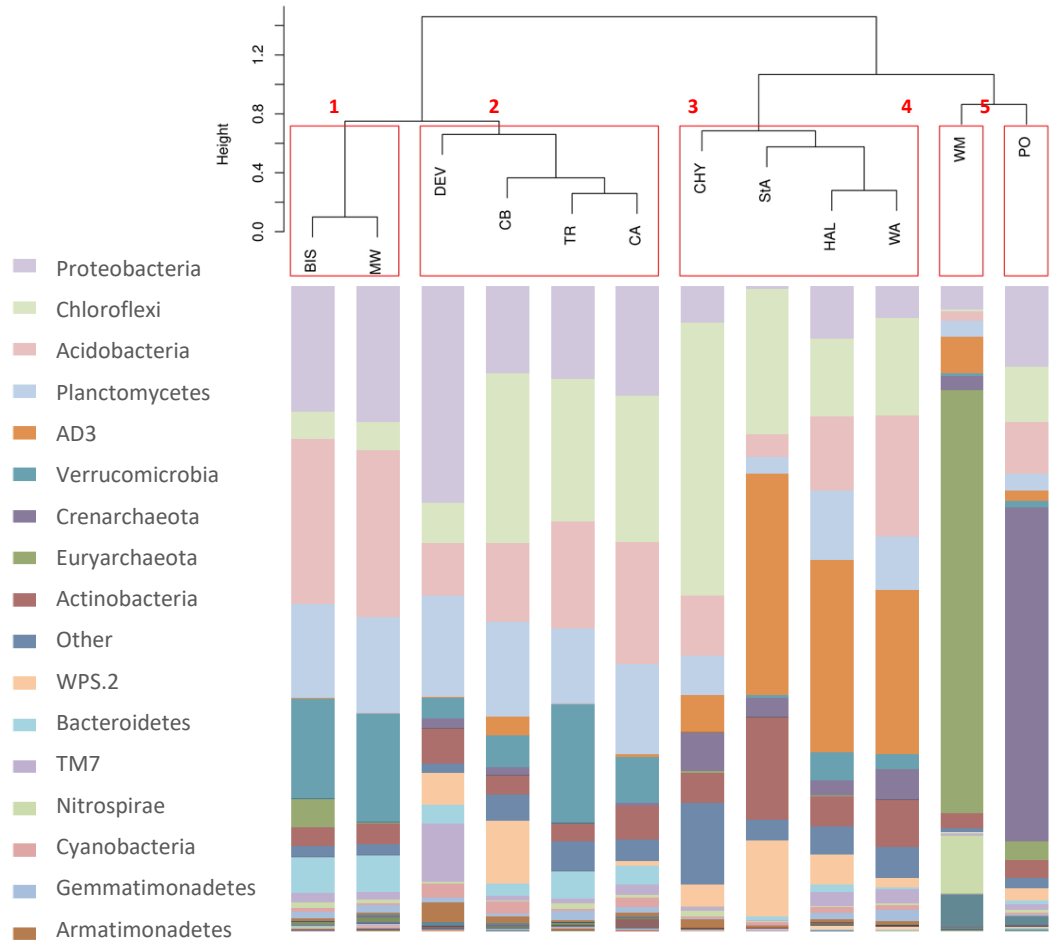


Figure 4.3. Communities' composition at the start of the experiment at phylum level, coupled with Ward Hierarchical Clustering dendrogram (k =5), in the red squares and labels the clusters identified.

The Wheal Maid and Porthtowan microbial communities were, for the most part (65% and 53%, respectively), dominated by archaea: Euryarchaeota and Crenarchaeota, respectively. Group (1) was defined by the absence of AD3, the higher abundance of Verrucomicrobia, low Chloroflexi. Group (2) showed higher Chloroflexi, the presence of WPS2, lower Acidobacteria and Verrucomicrobia. Group (3) was notably defined by a higher abundance of AD3, Chloroflexi, and lower proteobacteria, Acidobacteria and Planctomycetes, higher Actinobacteria.

4.3.2 Local adaptation

4.3.2.1 Inocula and substrates favouring good performance (Fe metabolism)

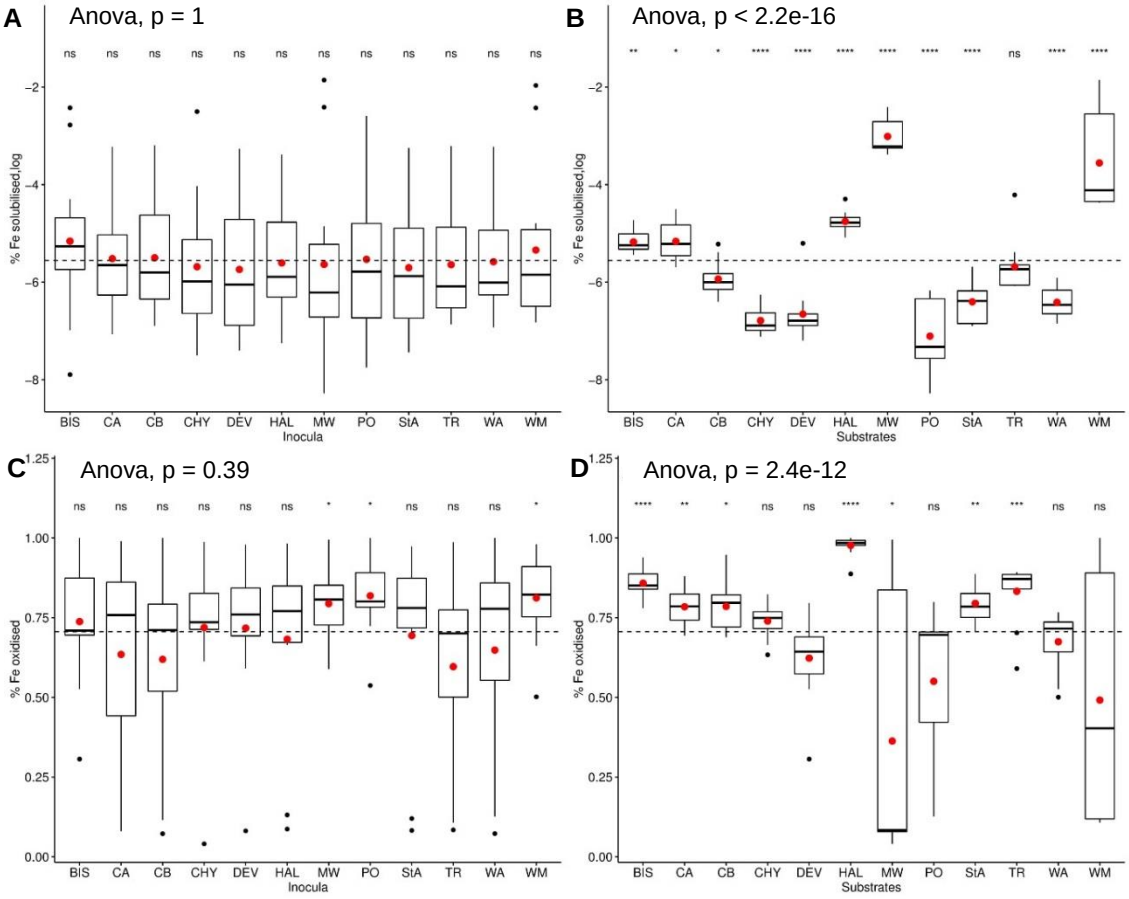


Figure 4.4. Substrate rather than inocula affect performance as iron solubilisation (A and B) and oxidation (C and D). The average performance (A and C) and by substrates (B and D) is compared to the average performance (dotted lines) via ANOVA. Significant comparisons are indicated on top, ns=non-significant, when p >0.05.

Local adaptation was studied in terms of performance by crossing inocula and substrates one to one in all 144 combinations, in three replicates. To analyse the performance of the communities in different substrates the absolute values were standardized for total solubilised iron by dividing for the total iron concentration obtained in the chemical analysis of the mineral (Total metals, see chapter 3).

The mineral oxidation performance is represented by the percentage of oxidised Fe on the total Fe solubilised.

I then compared the performance proxies for all inocula and all substrates to investigate the presence of best inocula performing well in all substrates or best

substrates, allowing a good outcome for most inocula. The inocula and substrates performance were compared to an average baseline (Figure 4.4). The test resulted significant for almost all substrates which are all less performing than average apart from two (Wheal Maid and Mount Wellington) relatively to iron solubilised (Anova, $p < 2.2e-16$) and almost all significantly better than average (apart from Devon, Mount Wellington, Porthtowan and Wheal Maid) regarding Fe oxidisation (Anova, $p = 2.4e-12$). Inocula were mostly non-significantly different from the base mean, regarding total solubilised iron (Anova, $p = 0.99$) and Fe oxidisation (Anova, $p = 0.39$); however regarding percentage iron oxidised only Mount Wellington, Porthtowan and Wheal Maid were significantly better than average (t-test against base mean, respectively: $p = 0.018$, $p = 0.011$, $p = 0.03$). Substrates were instead clearly distinctively correlating with good or low performance from the inocula. Bissoe, Caradon A, Halamanning, Mount Wellington and Wheal Maid were performing above average substrates (i.e. were "better" substrates, t-test against base mean, respectively: $p = 9.8e-05$, $p = 0.005$, $p = 3.5e-08$, $p = 8.3e-11$, $p = 3.1e-05$) regarding solubilised iron, all the others were performing below average, exception made for Tretherrup which was not significantly different from the mean. Regarding oxidation of iron, Bissoe, Caradon A, Caradon B, Halamanning, St Agnes and Tretherrup are resulting above average while Mount Wellington was below average and the remaining sites' substrates were not significantly different from the mean base (t-test against base mean, respectively: $p = 4.3e-07$, $p = 0.001$, $p = 0.007$, $p = 7.0e-12$, $p = 3.0e-04$, $p = 7.0e-04$, $p = 0.17$).

4.3.2.2 Sympatric versus allopatric local adaptation test

To test for local adaptation, the performance of the local community was compared (for each substrate) to the performance of the allopatric (i.e. "foreign") communities. , normalizing by the total amount of iron in the wastes (results are presented as log-transformed percentage or percentages) to avoid accounting, in this analysis, for the wide difference among wastes.

Results show a significant substrate-by-inoculum interaction (see Table 4.2, Interaction field). It is then investigated if such result reflected local adaptation overall by replacing the interaction term in the model with the factor sympatric/allopatric. The model (Table 2, last line) significantly showed the presence of local adaptation for the ability to oxidise Fe (%) while was not

significant for the ability to solubilise Fe. Such result implies a partial local adaptation, as only reflected by one performance proxy variable. On the basis of such result, this study explored the individual interactions in more detail.

Table 4.2. Modelling outputs

MODELS	*Y*	*Factor*	*Df*	*Sum Sq.*	*Mean Sq.*	*F value*	*Pr(>F)*	
PERFORMANCE	y=Tot Fe solubilised	Inoculum	11	11.4	1.04	3.384	0.000168	***
		Substrate	11	688.3	62.57	203.843	< 2.00E-16	***
	y= Fe (III) oxidised	Inoculum	11	2.005	0.1823	3.619	6.69E-05	***
		Substrate	11	14.638	1.3308	26.422	< 2.00E-16	***
INTERACTION	y=Tot Fe solubilised	Inoc :Substrate	121	76.3	0.63	3.685	< 2.00E-16	***
	y= Fe (III) oxidised	Inoc :Substrate	121	14.993	0.1239	6.365	< 2.00E-16	***
SYMPATRIC vs ALLOPATRIC	y=Tot Fe solubilised	Inoculum	11	11.4	1.04	3.377	0.000173	***
		Substrate	11	688.3	62.57	203.412	< 2.00E-16	***
		A_S	1	0	0.04	0.135	0.713857	
	y= Fe (III) oxidised	Inoculum	11	2.005	0.1823	3.7	4.86E-05	***
		Substrate	11	14.638	1.3308	27.01	< 2.00E-16	***
		A_S	1	0.5	0.4996	10.14	**0.00156**	**

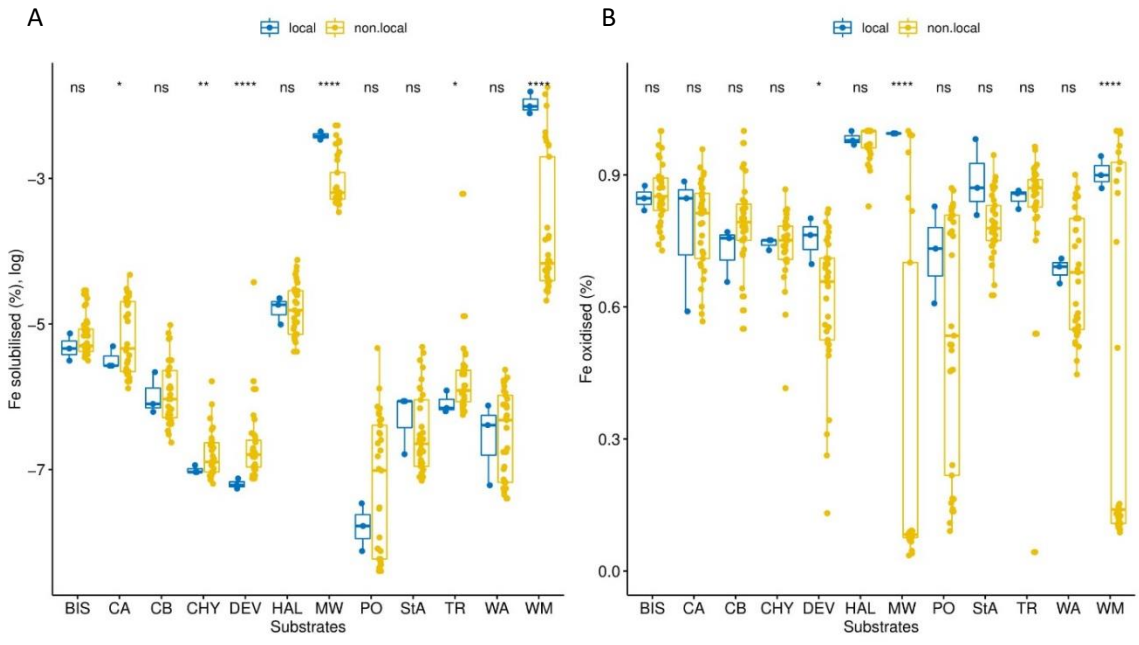


Figure 4.5. Comparison between sympatric (●) and allopatric (●) inocula per substrate, regarding Fe solubilisation (A) and Fe oxidation (B). T-test statistics is shown on top of comparisons. Local adaptation of inocula is verified in three cases, for the MW, StA and WM substrates for Fe solubilisation and DEV, PO, HAL, MW, StA, WA and WM for Fe oxidation as average; significant comparisons are indicated on top, ns=non-significant, when p >0.05).

Local adaptation by “local *vs* foreign” definition happens when the sympatric community performs better than the allopatric community, in the present experimental sets this was the case for Mount Wellington, Wheal Maid communities ($p < 0.05$) and St Agnes (n. s.), regarding Fe solubilisation (Figure 4.6 A and 4.6 C).

Four communities (CA, CHY, DEV and TR) performed significantly worse on their own substrate (local *maladaptation*) than the other inocula for Fe solubilisation.

4.3.3 Substrates association with the best performing inocula

To investigate a possible correlation between the performance of inocula and of substrates a Pearson correlation test was run, considering both the dependent variables: total solubilised iron and the total oxidised iron (%). Results showed a negative correlation between the performance of inocula and substrates regarding total oxidised Fe (Figure 4.5, Pearson; $R = -0.69$, $p = 0.013$), implying that the best substrates do not host the best communities. Moreover a non-significant correlation concerning total solubilised Fe was found (Figure 4.5 A, Pearson; $R = 0.35$, $p = 0.27$), if significant this would have demonstrated that concerning this variable, the best substrates host the best performing communities. The substrates which are most easily oxidised did not host the inocula that performed oxidisation the best.

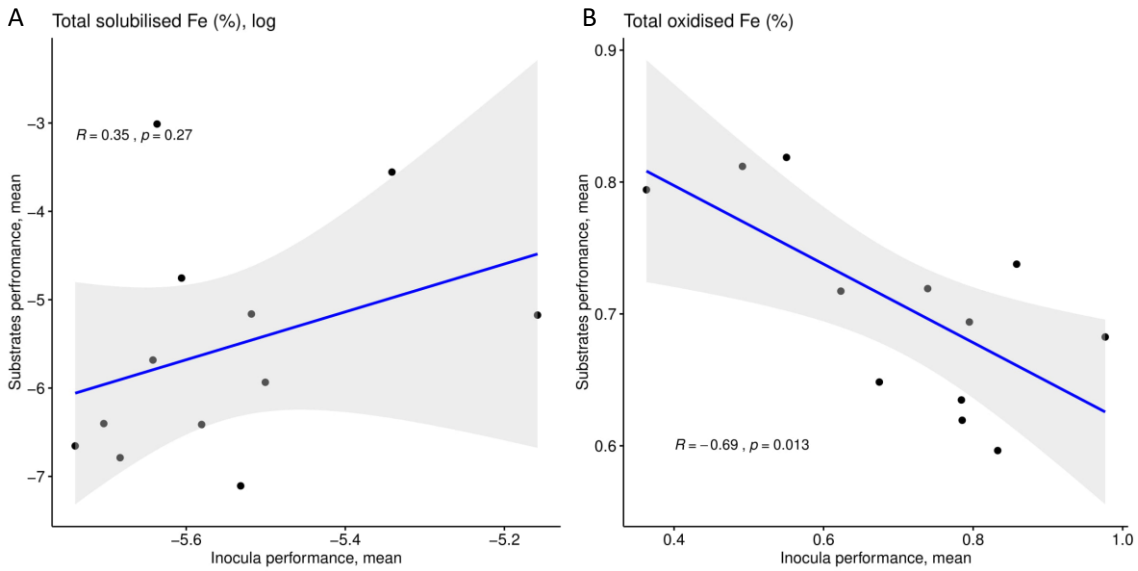


Figure 4.6. Correlation between inocula performance and substrates quality. Averaged values of the performance proxies (A, total solubilised iron and B, total oxidised iron) were tested for correlation. For a better visualisation the axes were not equalised. The performance of inocula negatively correlates with the performance of substrates for total oxidised Fe (B, Pearson; R = -0.69, p = 0.013); while there is no significant correlation for total solubilised Fe (A, Pearson; R = 0.35, p = 0.27).

4.3.4 Community composition and performance

The relationship between the community composition of inocula and performance was tested and it showed a low but significant correlation (Mantel statistics R: 0.345, p: 0.024). Some OTUs showed a relationship with the performance variables. A NMDS ordination coupled with the performance proxies (*envfit*, R platform, Appendix Figure 3) showed that Euryarchaeota (Thermoplasmata and unidentified Euryarchaeota) clustered towards the increasing experimental variables shown as the biplot, indicating a relationship between increasing performance and the presence of such taxa. On the other side, taxa clustering at the lower end of the vectors were: Thermogemmatisporales and Chloroflexi, indicating a relationship with decreasing performance. As previously shown (Figure 4.3), best performing inocula were Wheal Maid, which similarly to Porthtowan clusters on its own and both along the oxidised iron vector; Bissoe, Mount Wellington and Tretherrup inocula group along the solubilised iron vector. Devon, Caradon A and Caradon B inoculua cluster close to each other, such as Wheal Alfred and Halamanning group and St Agnes and Chyverton cluster. The

three last clusters described all were shown relating to lower performance in the NMDS ordination coupled with experimental variables analysis.

4.4 Discussion

Inocula and substrates were tested in a reciprocal transplant experiment, by cross inoculating all substrates with all inocula, singularly (including the sympatric community) in order to assess local adaptation at the level of single communities and at meta-community level, that is of the other communities of the metacommunity on reciprocal environments. The measures of adaptation consider different levels of performance (Fe metabolism) of the communities and response from the environments against sympatric or allopatric units (environments and communities). In the present model, the optimal environment is the one which maximises the performance of both its sympatric community and the allopatric ones. Similarly, the optimal community-phenotype is the one which maximizes performance against its sympatric (own) environment and the allopatric ones. From an application point of view, local adaptation is not necessarily an optimal trait for the use of a community in biotechnology; it could be more desirable to have a community which adapts rapidly to many environments.

In Figure 4.3 inocula composition is presented and despite showing diversity, as also remarked in Chapter 3, all the inocula were theoretically able to oxidise and solubilise Fe in order to thrive in mine waste, because all of them originated from sulfidic mine wastes and all of them included genera known for such properties. The prologue to this study comprised the environmental screening completed in the Cornish area (South West UK), which drove to the planning of this experiment, hinting at a possible adaptation of some communities related to environmental pH and readily extractible metals. The proxies for community performance were iron oxidation and solubilisation, respectively informing the ability to oxidise Fe (II) gaining energy and the ability to keep the system producing Fe (III) by enhancing the acidity and oxidise RISCs.

Local adaptation is verified in two cases, for the Mount Wellington (MW) and Wheal Maid (WM) inocula both in terms of iron solubilisation and iron oxidation and Devon Consuls (DEV) in terms of iron oxidation as sympatric inocula in a

comparison towards allopatric communities. Maladaptation is found for Caradon A (CA), Chyverton (CHY), Devon consuls (DEV) and Tretherrup (TR). These consortia performed worst on their own substrate. Finally partial local adaptation was demonstrated regarding Fe oxidation. Consequently, it is not possible to conclude that the Cornish microbial metacommunity is locally adapted but the top performing communities (WM, MW and PO) in most substrates are also locally adapted to their sympatric environment (by *local vs foreign* definition) and among the ones presenting the lowest pH; Wheal Maid community was also standing out for community structure (high abundance of Thermoplasmata and Nitrospirae).

4.4.1 Why is there no overall local adaptation?

Some authors (Gandon & Michalakis, 2002) affirm that when the mean quality of the habitat of different populations is the same across time, an averaged measure of local adaptation in the meta-population (or meta-community) can be used (average over the different measures of local adaptation of each population or community). Thus, local adaptation becomes a property of the whole metapopulation (metacommunity) and, assuming that adaptation has a genetic basis, it can measure the distance between the variability of the environment and the distribution of adaptive genetic variation (the potential adaptability of a population or a community). It is affirmed that in this specific case, the metacommunity of Cornish mining environments is not strictly locally adapted, that is to say that the indigenous community is not, by default, the best performing.

4.4.1.1 Different age of communities and migration can undermine local adaptation.

Migration rate affects the genetic variability of the population host or parasite, in more common local adaptation studies, which in turn affects adaptation (Morgan et al., 2005). Low migration rates might enhance evolutionary potential and favour local adaptation but high migration rate can undermine local adaptation and homogenize the metacommunity. The presence of a core community (Chapter 3 of this thesis and Sbaffi et al., 2017) suggests the presence of migration in the total environment object of this work. In terms of evolution, mutation occur randomly (Loewe & Hill, 2010), in a similar way migration process is stochastic

and it affects the community composition in ways that research tries and predict (Morgan et al., 2005). In Chapters 3 and 5 it is hypothesised that community composition is affected by non-stochastic variables both biological and environmental. This study considers the initial potential variability of the communities and not the end-point diversity.

Furthermore, the communities derive from mine wastes at different levels of weathering and age, the time factor might affect not only the pH of the mine waste but also the adaptation of communities, in the long term, to their local substrates (Bryan et al., 2007).

4.4.1.2 Whole community point of view

In the case of a microbial community-environment system, the abiotic environment is subject to a high variability in time over a wide range of timescales (single generation to geological timescales), this is the case of the communities considered in this study, coming from temporally heterogenic scenarios. It derives that the stability of the environment is a pivotal factor in shaping evolutionary predictions: rapid environmental change might select for adaption (phenotypic plasticity) and more long-term change might result in evolutionary adaptation (genetic change over time; Ellis et al., 1999; Koskella & Vos, 2015).

By providing a set of extracellular useful metabolites, other members of the community might prevent the need for individual cells to produce these compounds, selecting for the loss of the responsible genes and increasing interdependence among species. In this way, a functionally diverse community can promote the rearrangement of genomes, as selection removes genes with redundant function within a community, according to the Black Queen Hypothesis (Morris et al., 2012). This process resembles the population- level process of cooperative public good production (West et al., 2006); however, in a community, different species are typically limited by different resources and so don't need to compete directly, therefore the relationship resembles that of commensalism (Koskella & Vos, 2015)**.**

A further consideration regarding a community-level study is that the variability of the microbial community is wide in terms of composition and genetic variation, therefore there could be a latent local adaptation, preserved in community functional patches (or single species) but not measurable with a whole community

functional proxy, but rather the final communities composition would be needed. A response to selection might occur as a result of a novel mutation within a population, movement of mobile genetic elements within/among populations, or movement across populations of multiple species simultaneously. Therein the challenge to better understand microbial adaptation at community-level is in measuring key parameters that govern changes in individual genomes, as well as whole communities, according to (Koskella & Vos, 2015). In this study the community is considered as a whole functioning system and focus on the whole community performance only.

Not many transplant experiment in literature were found considering a whole microbial community, most transplant experiments (focusing on biotic environments as hosts) showed that parasites perform better on sympatric than on allopatric hosts (Ebert, 1994; Kaltz & Shykoff, 1998; Koskella et al., 2000; Lively, 1989; Parker, 1985). One study based on environmental communities used as a proxy for performance extracellular enzyme activities (EEA) and observed the development of resistance mechanisms to the types and local levels of heavy metals in the environment, suggesting community–level local adaptation (Hoostal et al., 2008). However, some other experiments did not find any evidence of local adaptation (Dufva, 1996; Mutikainen et al., 2000) or even found a local maladaptation of the parasite (Kaltz et al., 1999; Oppliger et al., 1999), as it was also observed in this study.

Common environments-based studies show that larger numbers of species promote local adaptation (Kraemer & Boynton, 2017). Conversely in the environments of the present study, most adapted communities were the most acidophilic ones and thus showing lower diversity and Shannon index, H' (Sbaffi et al., 2017).

Regarding single species, a correlation between inocula composition and performance was attempted and consecutively the relationship between single OTUs (Phylum level) of the inocula and final performance, admittedly with no predictive power. Euryarchaeota (Thermoplasmata and unknown) were shown to positively correlate with good performance, in terms of presence and abundance. Thermoplasmata described in literature are all acidophiles, optimally growing at pH < 2. *Picrophilus* spp. and *Ferroplasma* spp. are in this group, where many members are defined by not containing a cell wall and by being thermophilic

(Reysenbach et al., 2001). The presence of these organisms may be selected for by the extreme environment created as a result of efficient microbial activity, and may not be the cause in itself. Nevertheless, they are probably good indicator organisms for a ‘good’ community. Currently there is no proof that such specific archaeon genus might benefit the community containing it and this result requires further investigation.

4.4.1.3 Limitations of measurement

In contrast to population-based experiments in which an ancestral clone is placed in a novel environment and is tracked over time, community-level experiments usually measure the rate of change of a focal ecosystem function due to differential species growth or death following an environmental manipulation (Koskella & Vos, 2015). From an experimental point of view, in this model, environments are fixed (substrate plus pH by treatment) but not stationary, their variability is affected by the community itself, specifically by the modules responsible for the following functions: iron oxidation, sulfur oxidation, organic carbon consumption. During the experiment the environment is closed. Natural environments are not fixed, stable nor close, there is a flux of nutrients, organic carbon and metals; acidophilic microorganisms change the environment in which they are in, and this happens at a slower rate in the natural environment due to its openness. It is indeed not easy to adapt current and past theory of local adaptation to specific systems, in this case the acidophilic community and the whole community prospective (Kraemer & Boynton, 2017).

Furthermore, the experimental environment is undeniably different from the natural one for many aspects, firstly a liquid medium (adjusting for local pH values) was used, while in nature pore water is a small percentage of the environment; environmental temperature on the days of sampling was *ca.* 10°C while the experimental temperature was 26°C in a controlled room; light was reduced to minimum to favour chemotrophic metabolisms.

Finally, laboratory conditions might pose a limitation in the measurement of local adaptation, although such bias is shared from all communities and treatments and it was attempted preventing it by pre-acclimating the inocula to flask cultivation conditions.

4.4.1.4 Environment stability

Variation and selection are highly important in the process of adaptation because variation generates the types that can be potentially favoured by selection and, ultimately, lead to adaptation.

According to theory and observations, local adaptation is more likely in large than in small populations (Blanquart et al., 2012; Leimu & Fischer, 2008) but also adaptation (viability of the population) can increase with the intensity of selection in stable environments, as more fit components can be selected (Bürger & Lynch, 1995). On the other side, Lande & Shannon (1996) showed that for the adaptation of a population living in a temporally variable environment, the genetic variance within the population is pivotal. In fact, in temporally variable environments stronger selection decreases the viability of the population. This is due to the effect of selection on the amount of genetic variance: strong selection reduces the genetic variance and thus inhibits adaptation to a temporally variable environment. Such theories do not completely match with the origin of Wheal Maid and Mount Wellington inocula: WM features the lowest diversity richness among all communities considered in this study and the lowest Shannon index as well and the community derives from an over ground mine waste site, a less stable environment in time compared to underground sites. MW diversity richness is one of the highest, as opposed to Wheal Maid, relatively to this study. MW inoculum derives from an underground site, a very stable environment (Brannen-Donnelly & Engel, 2015) and the most acidic and heavy metals rich among the studied sites. Therefore, the high diversity let us hypothesise the presence in its diversity of spores and dormant strains (Lennon & Jones, 2011).

In these cases are presented: a community with a high diversity adapted to a stable environment and a less diverse community showing local adaptation to a temporally variable environment. In the first case we would expect that more fit components of the community would be selected by the stable environment (Bürger & Lynch, 1995) while the community (MW) shows high diversity; in the second case we hypothesise that the surface heap environment whose chemical parameter are not stable in time represents a disturbance for the community that selected for a lower diversity (Buckling et al., 2000).

4.4.2 Patterns of local adaptation / maladaptation

4.4.2.1 Best and worst performing inocula and substrates susceptibility to bioleaching

Results indicated the presence of better and worse substrates that could affect the performance of most communities. Some substrates were more susceptible to bioleaching as evidenced both by oxidation and solubilisation of Fe (Bissoe, Caradon A and Halamanning), which presented lower starting pH and not to high level of readily extractible metals. St Agnes, Chyverton and Caradon B were good for oxidisation but not over the average for solubilisation while Mount Wellington and Wheal Maid were good substrates for solubilisation but not for iron oxidation. Such results could be due to the mineralogical nature of the wastes (StA, CHY and CB) and not to the lack of oxidising ability of the inocula because the Fe (II) available was oxidised from most communities; MW and WM wastes might instead chemically release a high amount of Fe (II) making it too hard to survive in such conditions and to oxidise for most populations and not many strains can thrive in such a concentration of Fe (and other heavy metals) in the substrate. Another explanation is that MW and WM substrates might be more reactive; reducing the ferric iron in solution faster than the microbial community can re-oxidise it; this would be exacerbated in a mass-transfer limited system such as vials, therefore, according to this hypothesis, the wastes wouldn't be faster in releasing Fe *per se*, but they would be consuming ferric iron faster.

Porthtowan is the only substrate significantly poorly allowing bioleaching performance below average for both performance proxies and it is hypothesised that such mine waste creates an unhospitable environment for most communities due to the very high concentration of Fe and other heavy metals (mainly Cu). No inoculum was significantly performing better on most substrates for solubilisation but Wheal Maid, Mount Wellington and Porthtowan were performing above the average in most substrates relatively to Fe (II) oxidation; this result let us hypothesise that the required level of adaptation to solubilise the substrate is higher than the one required for oxidising plus the inocula WM, MW and PO possibly include strains that either oxidise more efficiently and survive best in higher concentration of Fe (III).

The probability of adaptation to allopatric substrates depends on components of the community, their presence and their concentration.

I include a cluster analysis plot based on the geochemical features of the substrates for completeness (Figure 4.7), modified from (Sbaffi et al., 2017), to show the similarity among the substrates involved in the experiment. Wheal Maid, Bissoe and Mount Wellington substrates were characterised by lower pH and high Fe and readily extractible metals (REM) concentration; St Agnes, Caradon B, Porthtowan, Chyverton and Devon Consuls were defined by average to low pH and low REM; Tretherrup was isolated and featuring high pH and high metals; the remaining substrates (Wheal Alfred, Halamanning and Caradon A) had higher pH and high REM.

The twelve inocula microbial compositions were calculated from the environmental samples communities' data presented on Chapter 3 (two samples per each one of the twelve sites were mixed and used to inoculate twelve flasks, maintaining environmental conditions). They were here re-analysed as single communities, largely reflecting what already stated in Chapter 3. Inocula containing a high percentage of Archaea clustered closely but in two (i and ii) separate clusters (Wheal maid and Porthtowan). Bissoe and Mount Wellington (iii) were in the same group and it is not surprising as they are geographically close and share historical and geological origin. Devon Consuls, Caradon A and B samples and Tretherrup communities formed another cluster (iv), sharing a higher original pH. Chyverton, St Agnes, Halamanning and Wheal Alfred were representing the western group (v) with a higher pH and weathering. The cluster analysis was done to highlight the similarity among communities prior the experiment expecting to observe local adaptation patterns, if not global, among clusters.

Group (i) was expected to perform well in similar substrates, independently from the similarity in community composition because of the acidity of the mine waste and the presence of Proteobacteria (mainly *At. ferrooxidans*). The large presence of Chloroflexi in the group (ii), coupled with the higher pH let us predict that the communities would be preferably oxidising the substrate when pH was not low and maladapting to the most acidic groups' substrates (i, iv and v). Not much literature is available for the AD3 candidate division (Zhou et al., 2003), therein due to the average pH, it was predicted that group (iii) communities would have

performed well in terms of oxidation in similar substrates. Regarding the remaining clusters (iv and v), they derive from acidic mine waste and in prior tests they showed the ability to oxidise and solubilise pyrite, thus it was expected a good performance from such groups in a range of substrates.

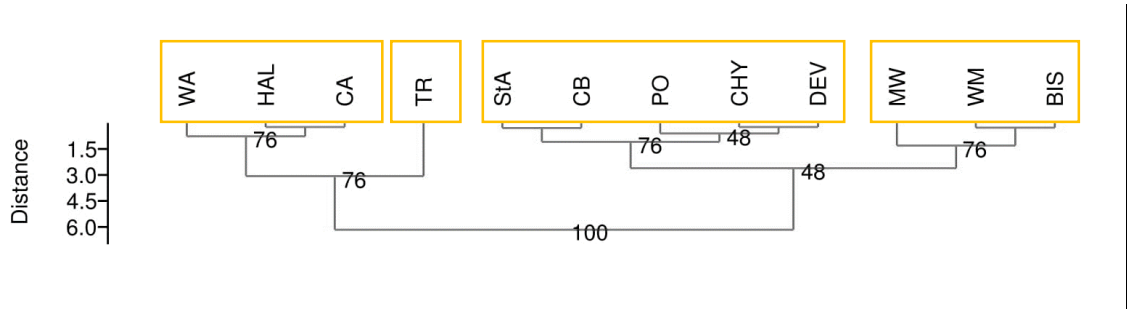


Figure 4.7. Cluster analysis of substrates' geochemical features, modified from (Sbaffi et al., 2017). Readily extractible metals composition and pH were used to perform a cluster hierarchical analysis, Ward's method, applied k = 4.

4.4.2.2 Local adaptation effect is not predictable.

Ultimately, an interaction was observed between performance and sympatric/allopatric factor, and results showed local adaptation for two inocula, not for all twelve. This last result is not predictable from the pH or the community level of diversity nor composition, as the two inocula diverge for such features. This suggests that while performance could be enhanced by moving communities and that sometimes foreign inocula might better the performance for one substrate, this might also not be the case and the performance could not change or instead decrease.

One way to overcome this issue is to try and predict the behaviour of the performance when the sympatric community is mixed with one or more allopatric communities. Such event is referred to as coalescence and it is the topic of Chapter 5, where a common substrate is used to isolate the effect of the communities' coalescence.

4.5 Conclusions

Environmental heterogeneity is required for the evolution of local adaptation. Twelve communities were selected deriving from a heterogeneous area (South west England) and tested their local adaptation to their sympatric environments. It was attempted employing a community-wide local adaptation approach with the aim of pairing it with endpoint community sequencing in the future to investigate individual populations' contribution to community fitness.

The level of variability (16S rRNA gene) within the metacommunity considered was assessed and it was observed five main groups clustering in diversity and composition. No evidence of a general local adaptation was found but two out of twelve communities showed adaptation to the sympatric substrate, specifically Wheal Maid and Mount Wellington consortia, deriving as well from the most extreme mine wastes. The conditions for local adaptation were not both satisfied, in fact the named inocula were also the best performing on most of the other substrates showing a wide adaptability in terms of oxidising potential. Some substrates were shown to correlate more than others with a better performance from inocula, especially the less acidic mine wastes and heavy metals poorest ones. Thermoplasmata were associated with successful performance and this result requires further investigation. Moreover some inocula were showing maladaptation especially from the *local vs foreign* point of view.

At every place and time there is directional selection to optimal status. Natural negative frequency-dependent selection (the more common a genotype becomes, the lower the fitness) and variation of diversity in space might ensure a continually changing optimal community in each environmental site (Kraemer & Boynton, 2017). This has not yet demonstrated in synthetic experimental systems (i.e. bioreactors and mesocosms) and further research is needed to better define selection and adaptation in acidophilic communities.

Additional studies are needed aimed at defining the potential adaptability of a community, in terms of adaptive genetic variation, more in detail. This work is suggested as a wide primer for future research on local adaptation in acidophilic communities and its involvement in applied issues.

5 Mixing multiple acidophilic communities enhances metabolic activity

5.1 Introduction

Community coalescence (the mixing of entire communities) is an ecological process widely occurring both in nature and in industrial processes reliant on microorganisms (Rillig et al., 2015; Rillig, Lehmann, et al., 2016; Rillig & Mansour, 2017). The consequences of this process are not well understood (Adams et al., 2014; Calderón et al., 2017; Hausmann & Hawkes, 2009; Livingston et al., 2013; Souffreau et al., 2014). Earlier theoretical research suggested that microbial communities could potentially behave as cohesive modules during coalescence (Gilpin, 1994; Toquenaga, 1997; Wright, 2008; Tikhonov, 2016): functional units where the single populations are more prone to i.e. cooperate within each other than with functional equals coming from other communities. This could occur when co-evolutionary links between community members are sufficiently strong (e.g. due to cross feeding between species, etc.). Theory predicts that species arising from a community with a higher community-wide productivity, rather than simply the most competitive individual species should dominate the coalescence event (Tikhonov, 2016). This means one of the coalescing communities should dominate the new assembly in terms of both the structure and function. Sierocinski et al. (2017) confirmed that this model of coalescence is true for methanogenic microbial communities, while recent research suggests that it is also valid for brackish environments where salt water communities meet fresh water ones (Rocca et al., 2019). The fact that coalescence leads to predictable structure and function in the communities has potential applied implications. If the community function optimum is associated with interests of human activity, coalescence can be used to improve industrial yields in engineered microbial communities.

If taxa are co-selected as modules, the correlation between individual community contribution and productivity is likely to break down. This is best illustrated by the extreme scenario where all taxa within a mixed community are co-selected from a single community: the mixture will be entirely dominated by a single constituent community, and hence the contribution of all other communities will be

independent of their individual productivity (i.e., they will contribute null to the mixture's composition, even though they have non-zero productivity individually). The intermediate scenario happens where co-selection occurs within two independent modules and also breaks down this correlation if one module contributes much more to community productivity than the other (Sierocinski et al., 2017). Sierocinski et al., (2017a) provided two possible scenarios representing the dominance of the most productive community described as the extremes of a continuum:

1) Consortia of multiple taxa from the same community act as semi-cohesive units and are selected together. This might arise as a result of coevolved mutualistic (or unidirectional) cross-feeding interactions (Embree et al., 2015; Großkopf & Soyer, 2016; Schink, 1997), notably between the three main components of the acidophilic community in pyrite further described (sulfur-oxidising, iron-oxidising and heterotrophic acidophiles). When a key member of a coevolved unit is selected in the coalescence, the species linked to it are selected together with it as they have an advantage over the species that never coevolved with a keystone species (MacArthur, 1970; Roughgarden, 1976; Schluter, 2000), (ecological co-selection).

2) An alternative explanation is that coevolved interactions within individual communities are unimportant, and the dominant community is simply a mix of the best individual species for each individual function within the community. In this scenario coevolved cross-feeding interactions are no more specific for taxa isolated from within a community than for taxa isolated from different communities: functionally equivalent taxa are interchangeable between communities ("survival of the fittest" in terms of species).

The same theoretical model was validated experimentally by Lu et al. (2018) using isolates, showing that collective invasions generically produce ecological co-selection of interacting species. However, data do not always support the theory (Castledine et al., 2019) and the same authors found no effect of coevolutionary history on either genotype fitness or community performance, which suggests parallel (co)evolution between communities. This experimental

approach is not easily feasible for extremophile complex communities, composed by often uncultivable/ uncultivated taxa.

Even though community coalescence in the case of microbial communities has been described relatively recently, anecdotally it has been used for a long time for practical purposes. One industrial case where coalescing communities is widely adopted is bioreactor-based bio-hydrometallurgy (for instance to bioleach copper from chalcopyrite). Typically at the start of the process, communities from various environments are mixed in order to obtain an optimal acidophilic community for the bio-leaching of metals from minerals (Rawlings & Johnson, 2007). However, the benefit approach has not been systematically investigated or approached from an evolutionary ecological perspective. Additionally, the current understanding is that acidophilic bioleaching reactor communities are very simple, dominated by two-four species while bioleaching heaps are far more complex.

The biochemistry of acidophilic communities would suggest dominance by the best-performing community following community coalescence, and hence greater average performance of mixtures versus single communities, because of strong mutualistic interactions increasing the likelihood of ecological co-selection. For example, communities that metabolise pyrite – a common sulfide mineral and a common model substrate for acidophilic communities - involve mutualisms between iron oxidising, sulfur oxidising and heterotrophic microorganisms (Figure 1.2 of Chapter 1). Pyrite dissolution can start in presence of Fe(III) then, as shown in Figure 1.2 of Chapter 1, continues by means of Fe-oxidisers which keep providing Fe(III). Pyrite dissolution releases reduced inorganic sulfur compounds (RISCs), which can be oxidised by sulfur oxidisers to produce protons, thus acidifying the system. Heterotrophic organisms are needed in the system to oxidise organic carbon, which can be toxic to acidophiles when concentrated (Fournier et al., 1998; Johnson, 1995, 2008; Johnson & McGinness, 1991; Okibe et al., 2003; Okibe & Johnson, 2004; Quatrini & Johnson, 2018). Therefore, concentrations of soluble iron (especially ferric) and protons (pH) are good proxies for the overall performance of a mineral-oxidising system.

This work attempts to test the patterns of coalescence firstly obtained from a methane producing communities (Sierocinski et al., 2018, 2017), whether they apply to acidophilic communities and to offer a new context for the coalescence

to be investigated. To test the coalescence in bioleaching communities, four different acidophilic communities from mine waste characterized by different geochemical properties were used. The communities were mixed in groups of two, three and four and grew them on pyrite, and the outcome of coalescence on community composition determined from 16S rRNA gene amplicon sequencing of component and coalesced communities. The proxies for community function used were Fe solubilisation (total iron in solution) and H^+ production (inferred from changes in pH). They represent the community ability to maintain the oxidation process going. Note that this study didn't investigate the mechanisms underpinning coalescence outcomes, for instance, the importance of co-selection versus individual selection.

5.2 Materials and methods

5.2.1 Inocula

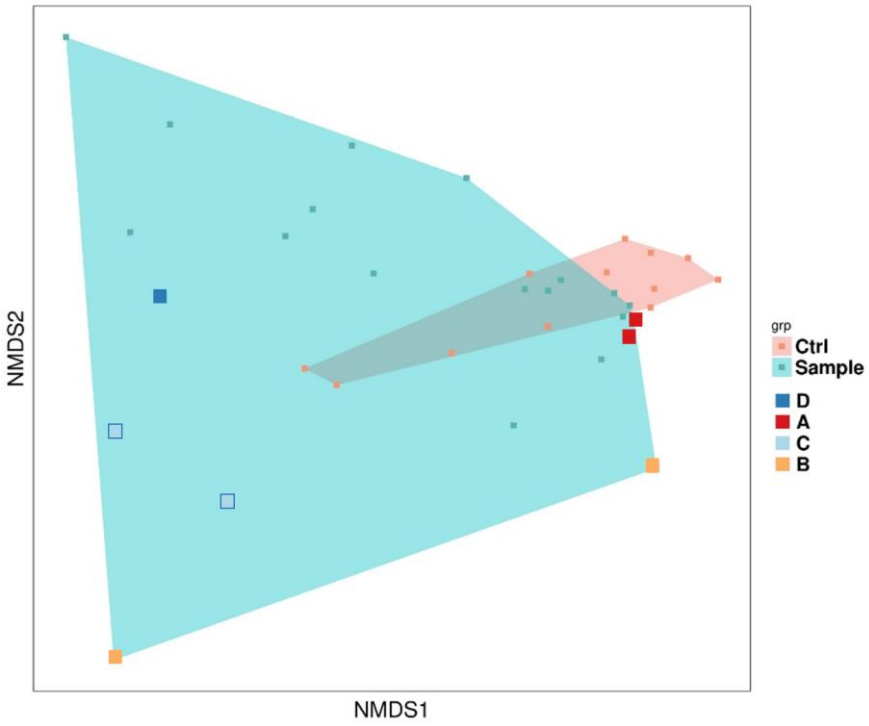


Figure 5.1. Non-metric multidimensional scaling (NMDS) of weighted OTUs abundancies from Chapter III, at genera level, all samples included: mine waste samples (cyan) and soil-like controls (pink). Highlighted (squares) the four sites' samples used as inocula in this study.

During a previous survey of the microbial diversity from mine waste from the South West of England, microbial communities of the twelve sites were described (see Chapter 3). The twelve communities were enriched in “maintenance flasks”: they were fed on their own environmental mine waste substrate with the purpose to maintain the original community structure; the twelve communities were also cultivated in “test flasks”: they were fed on pyrite (1%) flasks prepared as described in Chapter 2. This second set of flasks was used to test the performance of communities, to rank their ability to oxidise Fe and solubilise pyrite. Four consortia were selected as follows: the best performing communities in pyrite in the test described that showed the greatest compositional differences in a NMDS ordination (Figure 5.2: Mount Wellington (A), Wheal Maid (B), Chyverton (C) and Porthtowan (D)).

5.2.2 Experimental design

A reciprocal co-culturing experiment with a constant substrate (pyrite) was designed using four acidophilic microbial communities. Inocula were prepared mixing four selected original communities in all possible combination groups of 4, 3 and 2, as shown in Table 1, in equal proportions in terms of cells abundance ($1 \bullet 10^6$ cells/mL). Single community cultures were also run as a control treatment, plus non-inoculated treatment. Fe oxidation was assessed via spectrophotometric assay of Fe (III) and total Fe, together with pH once a week.

DNA samples for microbial community composition were collected from starting inocula (T_0) and end point (T_f) and prepared for sequencing (MiSeq). All treatments were run in three replicates. This study aimed at determining whether coalesced acidophilic communities were dominated by the community most efficient in isolation.

Table 5.1. Treatments of the experiment composition in coalesced and single communities.

Treatment n.	Communities mixed n.	Communities coalescing
1	4	A/B/C/D
2	3	A/B/D
3	3	A/B/C
4	3	A/C/D
5	3	B/C/D
6	2	A/B
7	2	A/D
8	2	A/C
9	2	B/D
10	2	B/C
11	2	C/D
12	1	A
13	1	B
14	1	C
15	1	D
16	0	none

5.2.3 Experimental setup

Glass vials were filled with 10 mL Basal Medium (pH 2.5) and 1% (w/v) pyrite (details in Chapter 2), loosely closed with plastic caps and sterilized by autoclaving (121°C, 15 min).

Four selected non-pyrite flasks (communities maintained in their original environmental substrate), inoculated with the communities described in Chapter 3, were checked for cells abundance by phase-contrast microscopy using a Thoma counting chamber. Inocula were collected, from the flasks, in sterile Eppendorf tubes, the tubes were centrifuged at 800 × *g* for 1 min, the supernatants harvested and centrifuged at 6000 × *g* for 40 min (which does not affect cell viability; Pembrey et al., 1999; Sheng & Liu, 2011). The pellets of cells were then re-suspended in pH 2.5 (H_2SO_4) BM and cells counted. While mixing communities, concentrations of inocula were equalised by dilution to 1×10^6

cells/mL and 1 mL of each cell suspension was added to the respective vial as inoculum.

5.2.4 Experiment workflow

Vials were agitated in orbital shakers at 100 rpm for the length of the experiment (*ca.* 2 months). Vials were sampled in sterile conditions. During each sampling, vials were shaken, left to settle for 1 min and 500 µL from each vial collected under a vertical flow laminar hood. Sampling consisted of the following assays: pH measurement and Fe(II)/Fe(III) via ferric chloride assay (Govender et al., 2012), as described in detail in Chapter 2. Briefly, such assay estimates Fe(III), then, after an oxidising process, all the Fe in the sample is revealed and measured (Total Fe Solubilised).

5.2.5 Community analysis

DNA was isolated at the starting and endpoint of the experiment for MiSeq-based community analysis. DNA was extracted from all the treatments and replicates. DNA quality was checked by fluorometry (Qubit, Invitrogen™) for quantity, gel electrophoresis for integrity and PCR for assessing the amplifiability of the desired region (V4 region of the 16S rRNA gene). When isolated DNA quantity was not sufficient, further extractions were done from frozen samples and the resulting elutes pooled together. Prior to extraction of DNA, samples were preserved at -80°C in beads solution and C1 solution from the PowerLyzer PowerSoil DNA isolation kit (QIAGEN).

5.2.5.1 DNA isolation and 16S rRNA gene amplicon library generation

Substrate aliquots for DNA extraction were stored at -80°C until processed. Total gDNA extraction was done using the DNeasy PowerLyzer PowerSoil isolation kit (Mobio, now QIAGEN, U.S.A) (Zaremba-Niedzwiedzka et al., 2017) following the manufacturer's instructions, applying three freeze/thawing steps after mechanical lysis with QIAGEN Tissue Homogenizer (detailed protocol in Chapter 2). For the 16S rRNA gene amplicon sequencing, a 291 bp conserved fragment from the V4 hypervariable region was targeted using the primers (515F: 5 ’-GTGYCAGCMGCCGCGGTAA - 3', 806R: 5'- GGACTACNVGGGTWTCTAAT - 3'), (Apprill et al., 2015; Caporaso et al., 2011; Parada et al., 2016) with a pool of

indexed primers suitable for multiplex sequencing with Illumina technology. DNA was quantified fluorometrically (Qbit, Invitrogen), checked for quality by gel electrophoresis, and by PCR, as previously described (Chapter 2). Extracted DNA was sent to the Centre for Genomic Research at University of Liverpool where the clone library was performed at Centre for Genomic Research (University of Liverpool). Genomic DNA (5 ng) was mixed with 0.25 µl of each 16S primer (10 µM) and 0.5 µl of each of the nested primers (10 µM). KAPA amplification mix (2 ×) was used and the final volume was 20 µl. A negative control of water eluted from the FastDNA spin kit was also included. The samples were amplified at the following conditions: 98 °C for 2 s (one cycle), 95 °C for 20 s, 65 °C for 15 s, 72 °C for 30 s (25 cycles), 72 °C for 5 min (one cycle), and 4 °C hold. The samples were then cleaned up using Agencourt Ampure XP beads (Beckman Coulter) at a ratio 1:1. The products were eluted in 12 µl 10 mM Tris pH 7.5. The samples were analysed by Qubit fluorometry and Bioanalyser.

5.2.5.2 Sequencing and bioinformatic analysis

Amplicon sequencing was performed by Illumina MiSeq technology at Centre for Genomic Research (University of Liverpool). Each pool of amplicons was sequenced at 2 × 250 bp paired-end sequencing with v2 chemisty. Initial processing and quality assessment of the sequenced data were performed using an adjusted pipeline; Casava v1.8.2 and Cutadapt v1.2.2 were used to perform the base calling, de-multiplexing and trimming of the indexed reads (Caporaso *et al.*, 2010; Reeder and Knight, 2010; Martin, 2011). Filtered read pairs were analysed, and assembled into a single sequence by Flash (Magoc & Salzberg, 2011), and then Qiime v1.8 was used for metagenomic analysis (Caporaso *et al.*, 2010). Clustering sequences at 97% of similarity generated 1,298 OTUs, the de novo OTU-picking and their quantification was done by using USEARCH v7.0 (Edgar, 2010). Sequences falling below the 97% similarity threshold for any of the OTUs clusters were removed from further analyses, to act as a filter against potential artefacts caused by sequencing error. The Greengenes database of ribosomal RNA sequences (McDonald et al., 2012) v13.8 was used as reference for chimera detection and taxonomy assignments. The taxonomic assignments for each OTU was performed by using Qiime v1.9.0 and RDP classifier (Q. Wang et al., 2007).

5.2.6 Statistical analyses

All statistical analysis was performed using the platform R and the package *vegan* (Oksanen et al., 2019). Non-metric multidimensional scaling (NMDS) was applied on datasets of OTUs previously scaled. The datasets included: for the inocula, OTUs abundancies from Chapter 3, (genera level) and calculated mixed inocula for the mixes of communities; for the end point OTUs abundancies from MiSeq sequencing. Categorical variables were applied to highlight treatments or continuous variables (pH, total solubilised Fe and oxidised Fe percent) were used to show the distribution of performance. To plot the experimental proxies as vectors, adding them to the ordination diagram (NMDS), the function *envfit*[19] from the package *vegan* was used. Comparisons between replicated categories was done either with Anova or t-test (when either many or two categories were compared), with appropriate correction for multiple comparison. PCA was only used to ordinate metadata or experimental data and categorical variables were applied to group treatments in the visualisation (scaled data). The schematic phylogenetic tree showed was produced using MEGA version 7 software (Kumar et al., 2016), and it is built using maximum likelihood inference and 16S rRNA gene sequences (V4 region) of the genus *Leptospirillum*.

The impact of diversity (alpha) was determined using Linear Models. These analyses were carried out using the *stat*, *phyloseq* (McMurdie & Holmes, 2013) and *vegan* packages in R. For beta-diversity estimation, the betadisper function in the vegan package was used to test for multivariate homogeneity of group dispersions using a permutational approach (Anderson, 2006; Anderson et al., 2006).

[19] The function fits environmental vectors or factors onto an ordination. The projections of points onto vectors have maximum correlation with corresponding environmental variables, and the factors show the averages of factor levels.

5.3 Results

5.3.1 Community performance

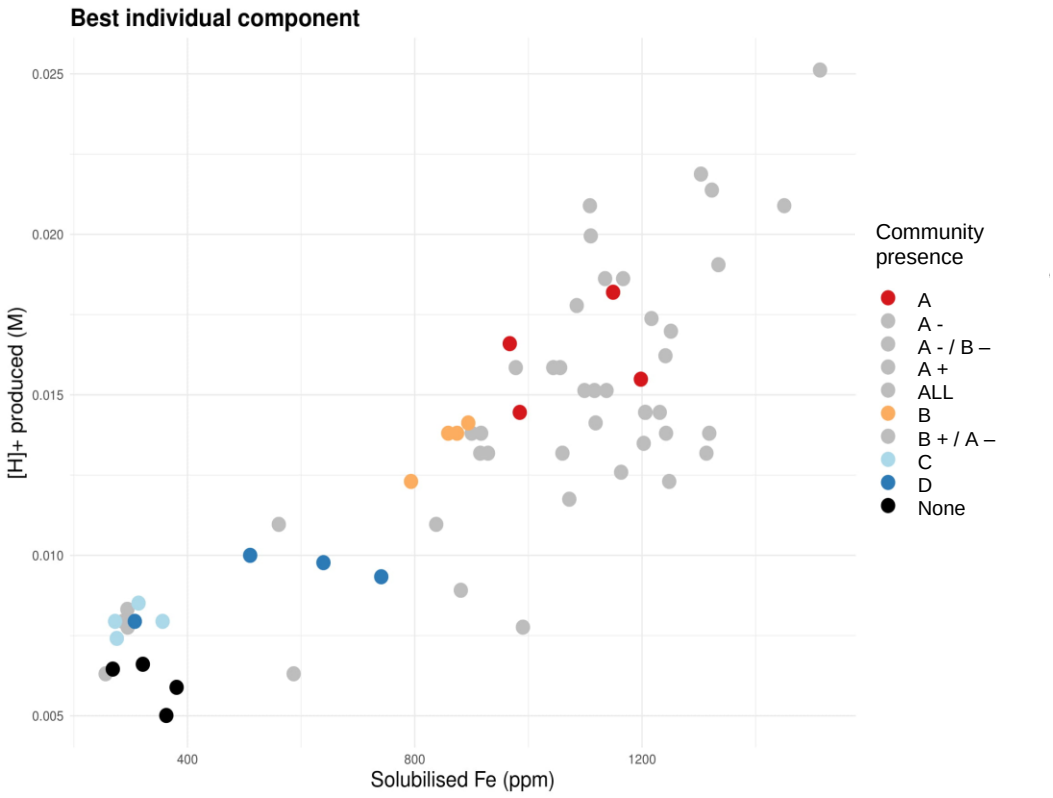


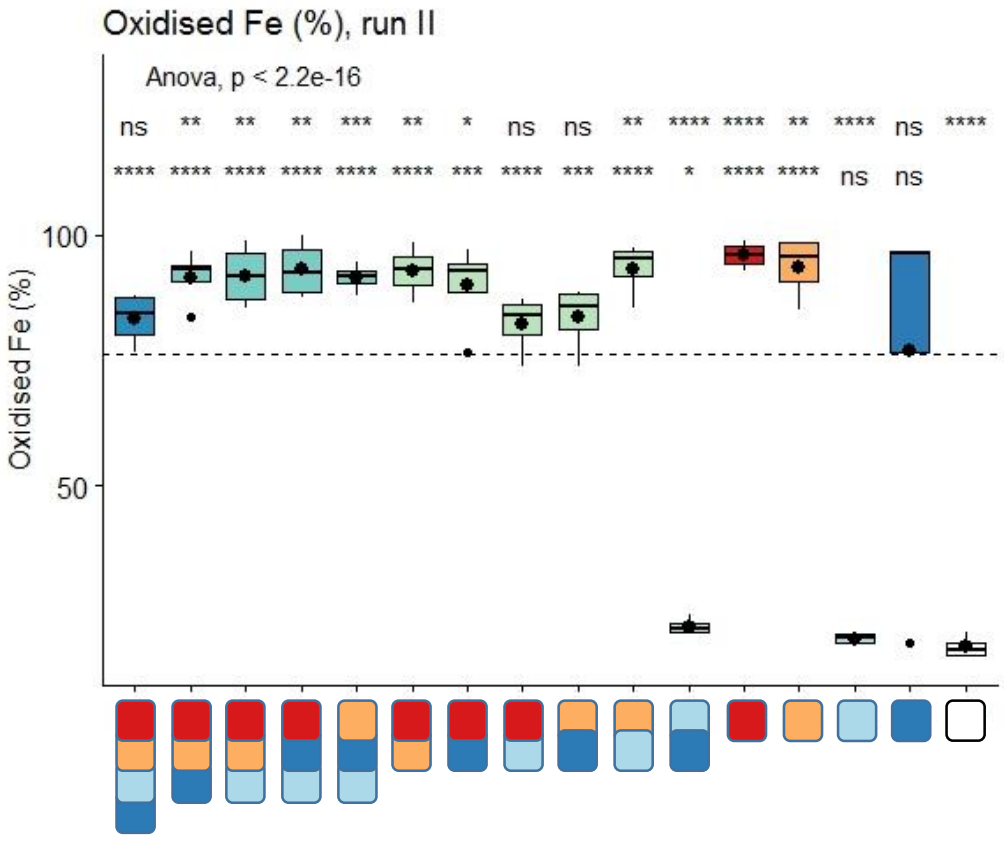
Figure 5.2. Positive linear relationship between acid production and iron solubilisation (Pearson's R = 0.85, p < 2.2e-16). The figure is highlighting the performance of non-coalesced communities, A, B, C and D in isolation.

The chosen proxies for performance were: the ability of the community to solubilise iron and the ability to produce acid through the oxidation of RISCs (reduced inorganic sulfur compounds). Unsurprisingly, there was a strong positive correlation between the two proxies' values (Figure 5.3) therefore in this work it is chosen to consider the acid production as proxy for performance for subsequent analyses. Similar results were obtained observing the iron solubilisation data (Figure 5.4B).

I first compared the performance of the communities non-inoculated controls. Acid production was significantly above the average line (which included the control treatment) only for the community "A" in isolation, while it was below the average line for the inocula "C" in isolation, "D" in isolation and the control

treatment. Most mixtures significantly outperformed the control, with the exception of “B/C/D”, which did not differ from the control.

I next conducted an ANOVA using the presence and absence of each community as explanatory variables. The presence of the community A, either in isolation or in coalesced communities resulted in increased acid production, as did the presence of community B to a lesser extent (Table 5.2). There was also an interaction between the presence of A and B, whereby the presence of A appeared to rescue a reduction in performance of mixtures containing B and other communities (Table 5.2). No other communities or their interactions significantly affected performance. A second ANOVA analysis was done using a grouping factor whose levels are illustrated in Figure 5.5, to further investigate how different communities affected the performance (*F*: 16.17; *p-value*: 2.0E-11). Tukey post-hoc test indicated that performance is equally high if the community A present, the performance in the presence of A and B is not significantly different but the only presence of the community B in the absence of A (level B+/A-) doesn’t affect the performance differently from the control or the community D and C which are producing the lowest [H^+] (Table 5.3).



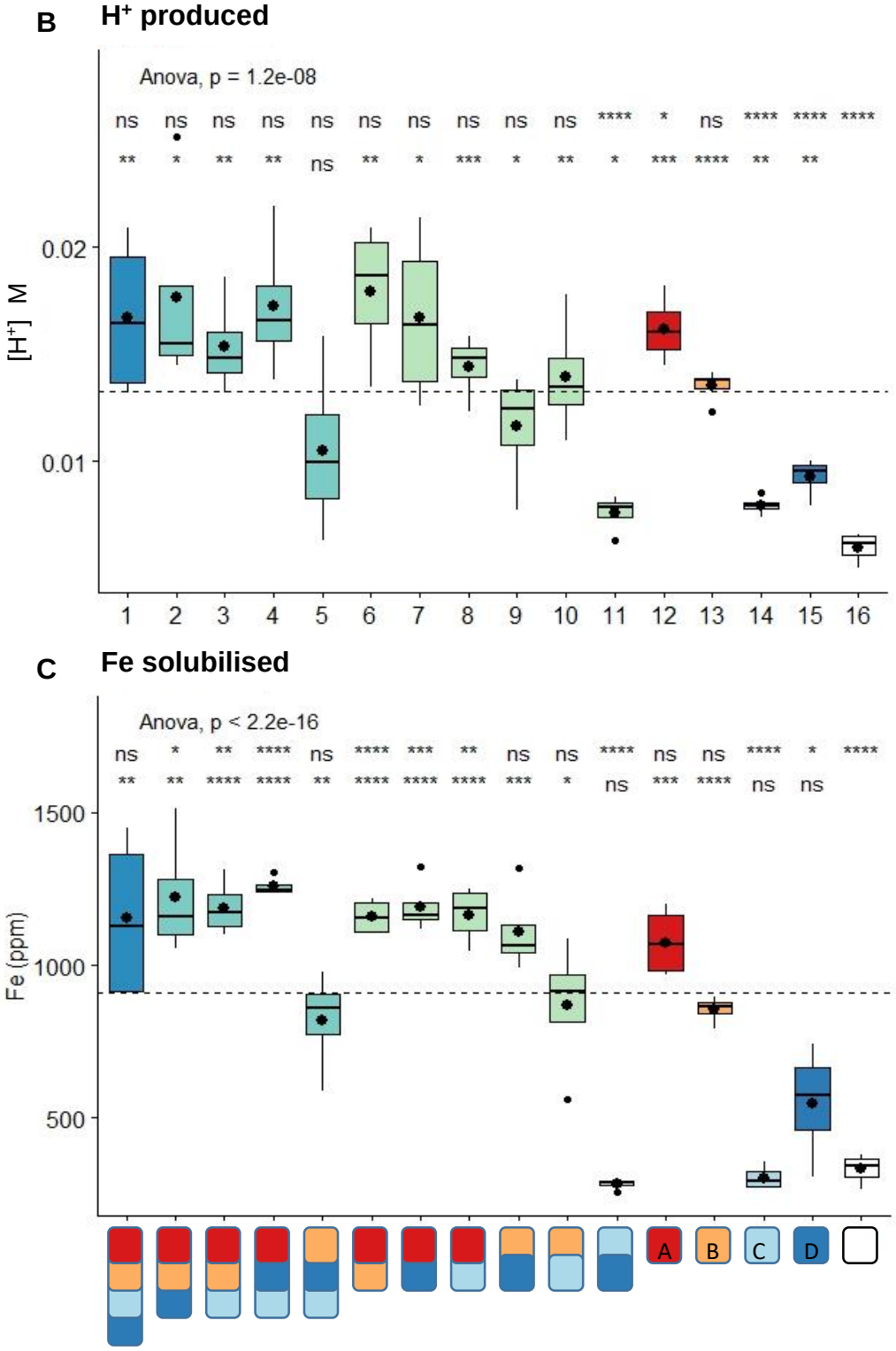


Figure 5.3. Estimated oxidised Fe (%, A), acid released (B) and solubilised Fe (C) at the experiment endpoint for the sixteen treatments. Statistics is shown between single communities-based treatments in two rows (top row is a t- test comparing all treatments to a base-mean, bottom row is a t-test comparing all treatments to the non-inoculated control; ns: non-significant; p > 0.05, *: p <= 0.05, **: p <= 0.01, ***: p <= 0.001, ****: p <= 0.0001; ●: mean value, no Bonferroni correction applied).

Table 5.2. Results of the linear model: [H+] = A × B × C × D.

Explanatory variables (Dependent: $[H^+]$)	*df*	*Sum of Squares*	*Mean Square*	*F*	*p-value*	
A	1	6.7E-04	6.7E-04	86.77	**2.5E-12**	***
B	1	1.2E-04	1.2E-04	15.53	**2.6E-04**	***
C	1	1.0E-07	1.0E-07	0.01	0.907	
D	1	1.5E-06	1.5E-06	0.20	0.657	
A × B	1	6.1E-05	6.1E-05	7.93	**0.007**	**
A × C	1	2.2E-05	2.2E-05	2.86	0.097	.
B × C	1	1.5E-05	1.5E-05	1.96	0.168	
A × D	1	1.2E-05	1.2E-05	1.54	0.221	
B × D	1	8.6E-06	8.6E-06	1.12	0.296	
C × D	1	4.0E-07	4.0E-07	0.05	0.828	
A × B × C	1	2.9E-06	2.9E-06	0.38	0.543	
A × B × D	1	4.0E-07	4.0E-07	0.06	0.814	
A × C × D	1	2.1E-05	2.1E-05	2.70	0.107	
B × C × D	1	6.0E-07	6.0E-07	0.07	0.791	
A × B × C × D	1	1.8E-06	1.8E-06	0.24	0.627	
Residual	48	3.7E-04	7.7E-06			

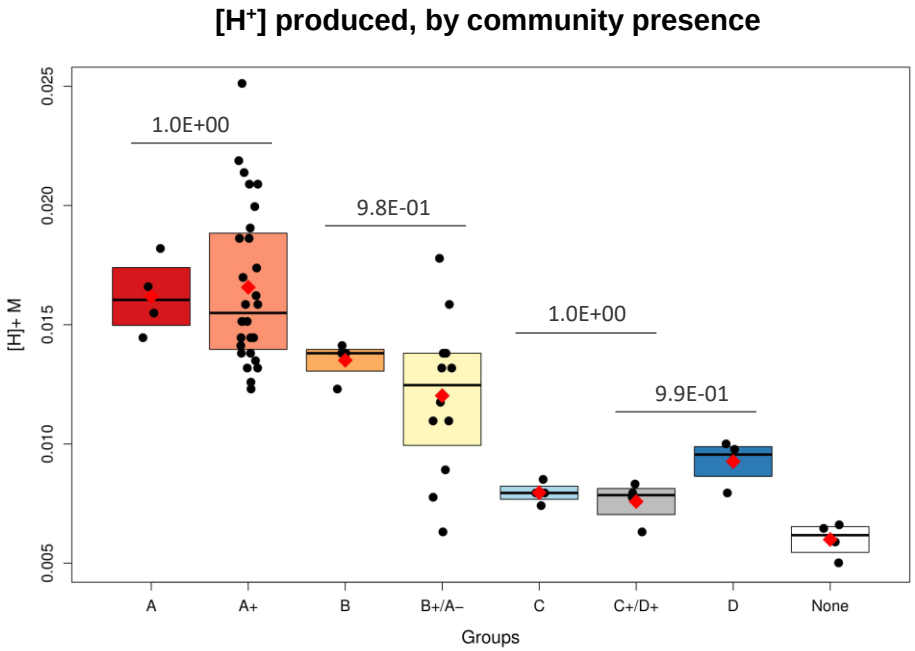


Figure 5.4. Net acid production at the endpoint of the experiment by groups representing community A and B presence. Mean performance (red rhombuses) of A containing consortia is significantly equal to the performance of the community A, the best performing community alone. Comparisons are highlighted by bars with Tukey post-hoc test results (Table 5.3).

Table 5.3. Tukey post-hoc test results of the ANOVA analysis.

	C+/D+	***A+***	***B***	***B+/A-***	***C***	***ctrl***	***D***
A	**1.3E-03**	1.0E+00	8.7E-01	1.8E-01	**2.4E-03**	**8.1E-05**	**1.8E-02**
C+/D+		**3.5E-06**	7.0E-02	1.3E-01	1.0E+00	9.9E-01	9.9E-01
A+			4.5E-01	**3.9E-04**	**8.8E-06**	**1.0E-07**	**2.1E-04**
B				9.8E-01	1.1E-01	**7.6E-03**	3.9E-01
B+/A-					2.0E-01	**9.2E-03**	6.8E-01
C						9.7E-01	1.0E+00
ctrl							7.1E-01

5.3.2 Increasing the amount of communities in a mix enhances its performance

Given that the performance of coalesced communities seemed to primarily be driven by the presence of A and B, it is perhaps unsurprising that increasing the number of communities coalesced in a mixture increased performance: Total solubilised iron (linear model, *F*: 31.24; *p-value*: 5.4e-07) and acid production (linear model, *F*: 19.34; *p-value*: 4.4e-05). Coalesced communities of 3 are statistically performing better compared to the base-mean, and all coalesced communities' levels (1, 2, 3 and 4) are performing better than the non-inoculated control as shown in Figure 5.6 A and B.

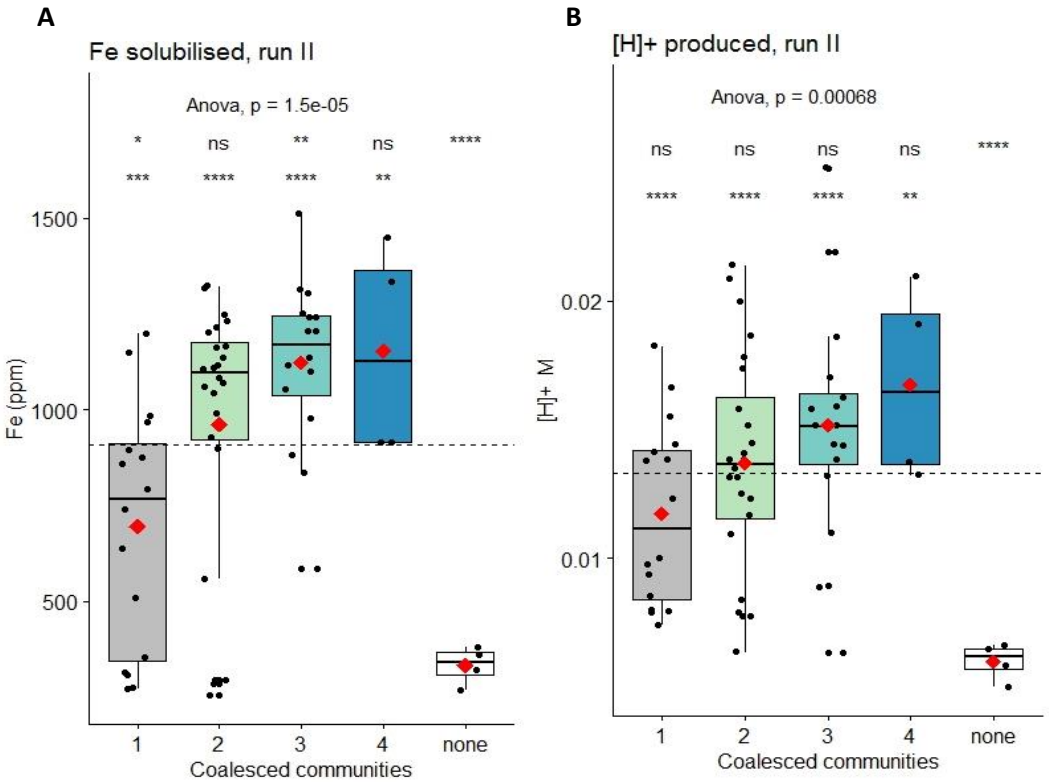


Figure 5.5. Solubilised iron (A) and net acid production (B) by increasing number of coalescing communities. Increasing the number of mixed communities enhance the performance of the systems. Statistics is shown between single communities-based treatments in two rows (top row is a t- test comparing all treatments to a base-mean, bottom row is a t-test comparing all treatments to the non-inoculated control; ns: non-significant; p > 0.05, *: p <= 0.05, **: p <= 0.01, ***: p <= 0.001, ****: p <= 0.0001; ♦: mean value, on top the result of ANOVA analysis with coalesced communities as factor).

5.3.3 Correlation between community composition and performance

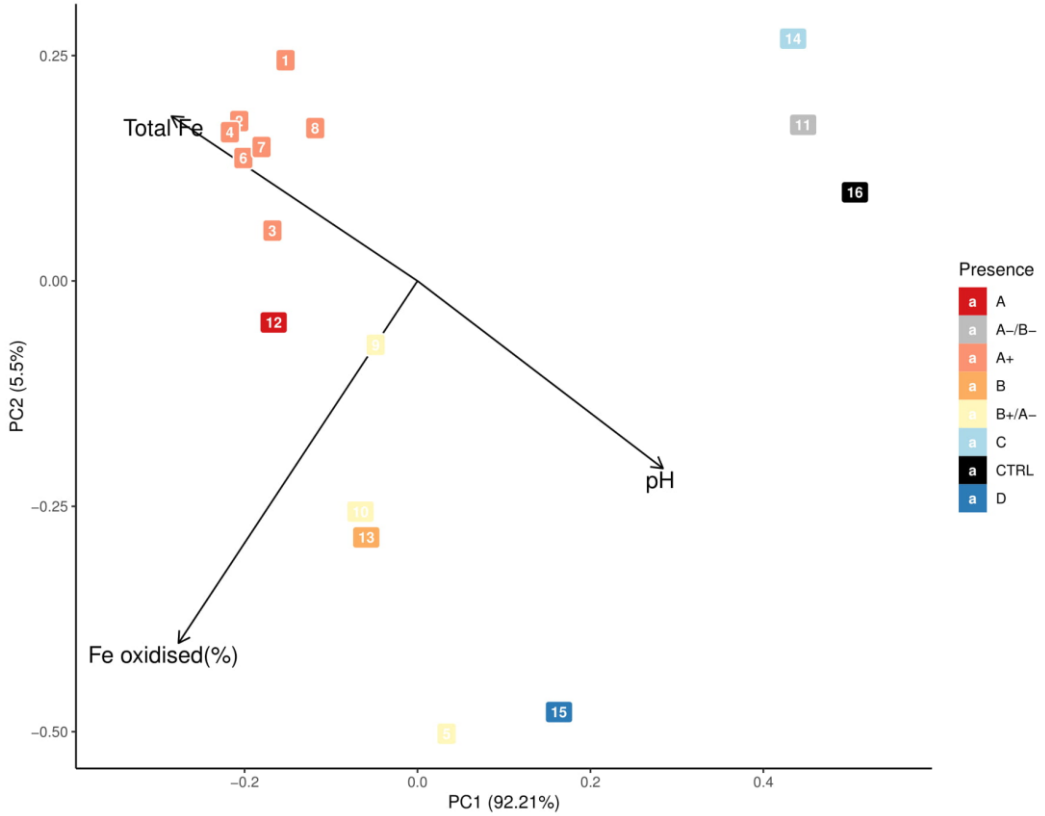


Figure 5.6. Principal component analysis of performance data. The mean of replicates performance variables was calculated by treatment, the data were scaled. The number on the symbols indicate the treatment (see Table 5.1). The presence of the community A allows a similar performance and so does the presence of the community B.

There is a clear and significant correlation between community composition at the end of the experiment and the performance of the communities (Mantel test, Pearson r = 0.516, Significance= 0.002, n=47) and a weaker, non-significant correlation between communities' calculated compositions at the start of the experiment and the averaged performance of the communities (Mantel test, Pearson r = 0.296, Significance= 0.064, n=15). The positive correlation found suggests that there is an optimal community, the community A.

To further investigate this outcome, a principal component analysis was conducted (Figure 5.7), considering three final response variables (pH, total solubilised Fe and percentage of oxidised Fe, the latter included for completeness) as proxies for performance. It showed the grouping of A-containing communities were not only better at solubilizing Fe, but also were

closer in overall performance to the community A. Communities containing B but not A (B+/A-) performed differently than the A-containing consortia. They still oxidised Fe but were not reducing pH as efficiently as and did not solubilise the pyrite at the same level. Community C and “C/D” treatment clustered close to the negative control. The plot in Figure 5.7 is a summary of performance and it clearly shows clustering patterns. In general the clustering patterns were similar to the grouping obtained by NMDS analysis on the treatment’s community compositions (Figure 5.8), confirming the results from mantel test, and the performance patterns reflect the composition of communities. Again, the positive correlation suggests that the functional outcome of coalescence is reflected by community, therefore the members of the community A might always dominate in a mixture. However, applying NNLS analysis would demonstrate this more explicitly (Sierocinski et al., 2017).

5.3.4 Community composition converges

A NMDS analysis was done (Figure 5.8) based on weighted OTUs abundancies, showing convergence in the community composition of all mixtures containing the community A and closeness to the community A in isolation. Convergence of communities imply that deterministic processes (i.e. selection) drove changes in composition. It is hypothesise that the shift in the composition of communities happened by selection for more acidophilic strains in all the treatments due to the introduction of the consortia in pyrite (and its oxidation). Previously, inocula were cultivated in “maintenance” flasks (i.e. maintained on their source substrate/mine waste, for details see Chapter 2). In Figure 5.8 it is visible that “D t(0)” and “C t(0)” cluster quite close to the environmental respective ancestor communities, meaning that the composition of the natural communities (env) and of the start of the experiment (t_0) were very close. Communities then shift towards a new equilibrium strongly defined by a less diverse structure (Figure 5.8 and Figure 5.12).

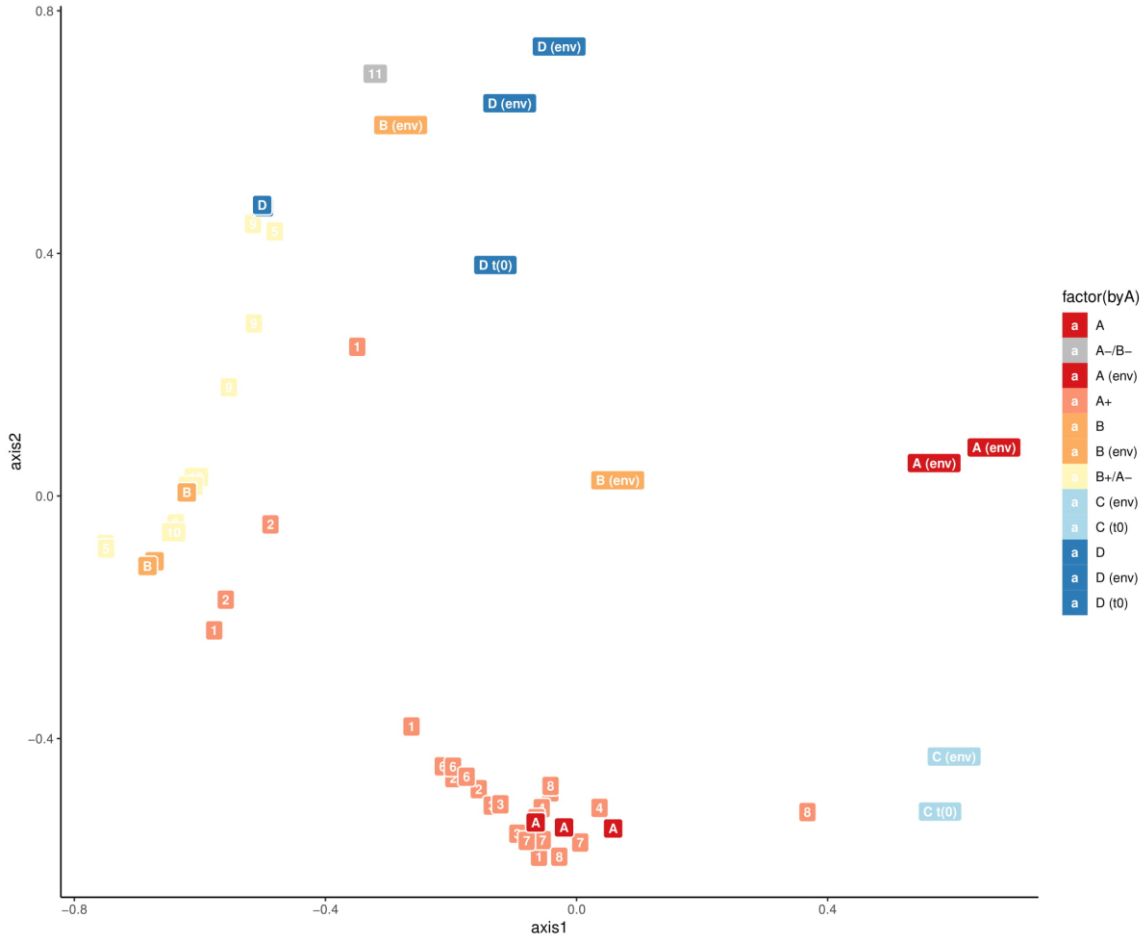


Figure 5.7. Non-metric multidimensional scaling (NMDS) of weighted OTUs abundancies showing that in community composition the consortia containing A are closely related to A itself and so the communities non-containing A but B are closely related to it. Regarding the original inocula at the start of the experiment, A t(0) and B t(0) sequencing was not successful but it is visible through the C t(0) and D t(0) that the environmental composition, A (env) and B (env) is close to the respective t (0) of the experiment. The symbol "-"indicates the absence of such community in the mix, while the symbol "+" indicates the presence of such community in the mix (i.e. A+ indicates all the inocula containing A, B+/A- indicates all the inocula containing B but not A).

5.3.5 Features of high performing communities

5.3.5.1 Relationship between performance proxy and diversity

In this study it is found that there is a negative relationship between final pH of the experimental units (as a proxy for performance) and the α-diversity of the

inocula (Figure 5.9A), hence better performing communities are here the more diverse.

5.3.5.2 Relationship between performance proxy and environmental pH

Further, there is a positive correlation of the final pH of experiments and environmental pH of the four communities (Figure 5.9B). These results show that the better performing communities are more diverse at the beginning of the experiment, and also come from an original more acidic environment, although the effect of pH (Figure 5.9 B) is less significant than the effect of diversity (Figure 5.9 B). Results suggest that a better performing community in pyrite is diverse and deriving from low pH substrate.

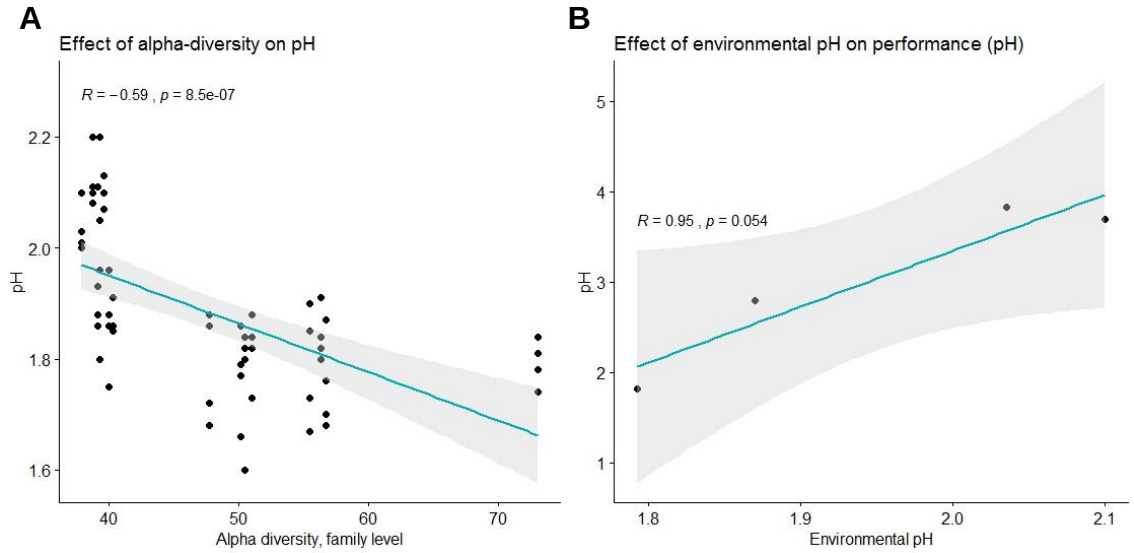


Figure 5.8. Alpha diversity of inocula is positively related to acid production as a proxy of performance for coalescing communities (Pearson's R = - 0.59, p = 8.5e-07, lm adj. R^2: 0.33). Environmental pH of the four inocula is positively related to the final pH of the experimental units and lower performance (Pearson's R = 0.95, p = 0.054).

5.3.5.3 Strains defining the communities

OTUs were filtered by abundance and fitness to the performance proxies (total solubilised iron, percentage of oxidised iron and pH) in a NMDS ordination of experimental end point with fitted variables. This highlighted the OTUs that were both most abundant and whose frequency was correlated with environmental variables ($p < 0.05$) used: pH, total Fe and Fe (III) %. Nine main OTUs were found clustering close to one of the two main poles (Figure 5.10, in red variables with $p < 0.05$), the one indicating successful performance (Tot Fe) and the other one

non-successful performance (higher pH, Figure 5.10). Six out of nine OTUs were *Leptospirillum* sp., unclassified, an abundant genus, as confirmed by the composition of experimental communities (Figure 5.12). Poor performing communities were mainly characterised by the presence of one *Leptospirillum* OTU and Acetobacteraceae, while successful ones were characterised by five *Leptospirillum* OTUs and two *Acidithiobacillus* OTUs (Figure 5.10). In Figure 5.12, it is visible that *Acidithiobacillus,* as a genus, is present in the original communities at time zero, however it only increase in the successful treatments.

Regarding the community composition, it was observed that *Leptospirillum* dominates the end point of the whole experiment while *Acidithiobacilli* are only present in well performing treatments and in the A community end point (treatment 12). D community is mainly dominated by *Leptospirillum* whereas coalesced communities formed by C and D (treatment 11) present a peculiar composition being mostly dominated by *Acidiphilium* (Figure 5.12).

Diversity notably diminish from the beginning of the experiment to the final time point (Figure 5.12).

The OTU sequences available for *Leptospirillum* characterising only good performance or low performance treatments (Table 5.4) were compared in a phylogenetic tree (Figure 5.11A). It was observed that those sequences were belonging to groups phylogenetically distinct, suggesting a possible phylogenetical divergence either among different sites or conditions. Further analysis indicated that the genus *Leptospirillum* OTUs are distributed in a non-homogeneous fashion. The OTU00002 is present in treatments 1, 2, 5, 7, 8, 9, 10, 11, 13, 15 (Figure 5.11B), representing more than 10% of the total diversity (Figure 5.11C) in treatments 1, 5, 9, 13 and 15 characterised by the presence of either community B or D. The remaining five *Leptospirillum* OTUs describe the genus presence of treatments 3, 4, 6, 7, 8, 12 (Figure 5.11B) and in the same communities they represent only less than 1% of the total diversity (Figure 5.11C).

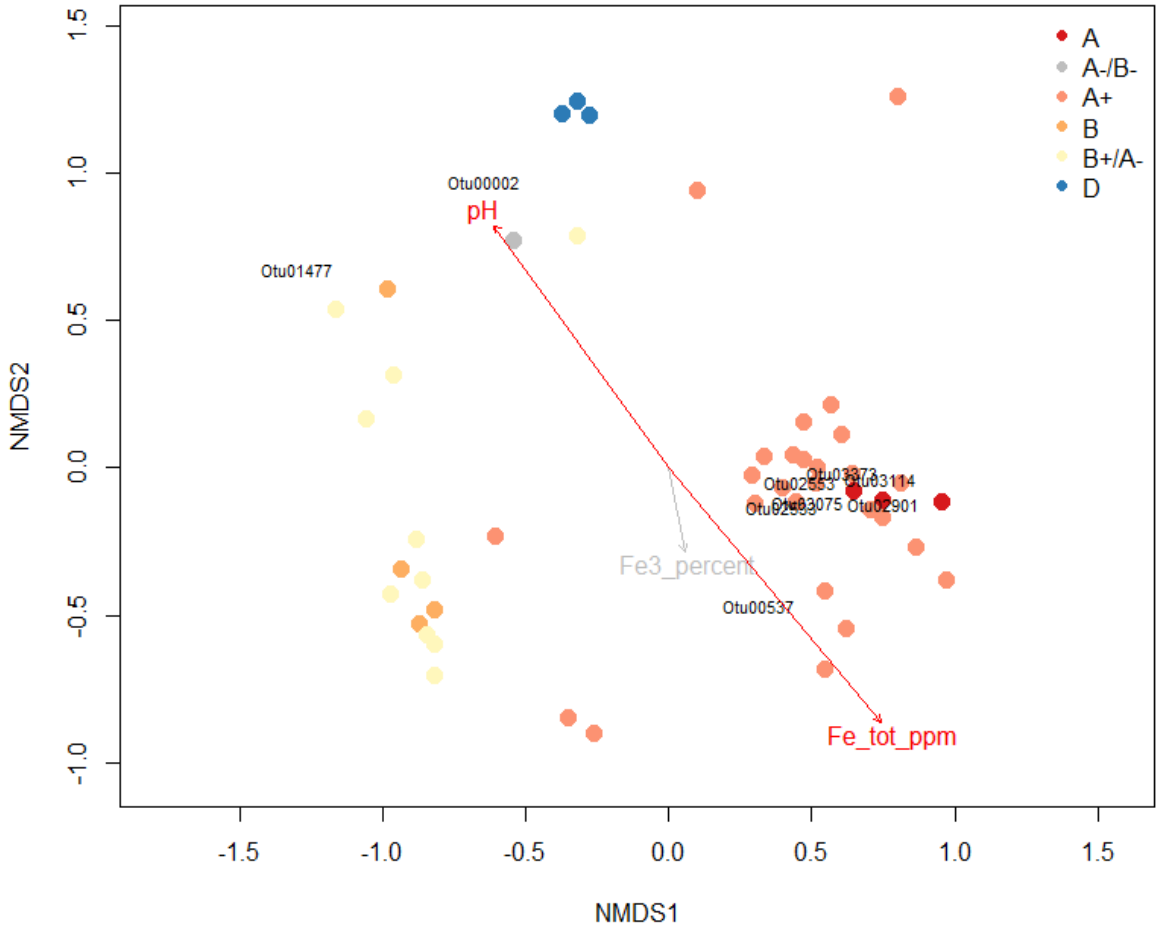


Figure 5.9. NMDS ordination plot including only end point microbial community composition (all replicates used) of the experiment. The function *envfit* (*vegan* package, R) was used to fit and add the three proxies for the performance as vectors in an ordination diagram. Fe (III) percentage: ability to oxidise all Fe dissolved in the environment. Total Fe: ability of the community to continuously oxidise Fe and therein keep on helping the mineral release iron and sulfur. Red arrows and names of the variables indicate their fit significance (red = p value < 0.05) Increasing pH is here a proxy for unsuccessful performance in this context. Main OTUs are shown and were filtered by relative abundance (0.005) and fitness to the environmental variables (0.3).

Table 5.4. OTUs showing a positive or negative correlation with performance variables

OTU code	**Correlation with performance**	**OTUs, closest match (similarity %)**
Otu00002	-	Bacteria(100); Nitrospira(100);Nitrospira (100); Nitrospirales (100); Nitrospiraceae (100); **Leptospirillum**(100) ●
Otu01477	-	Bacteria(100); Proteobacteria (100);Alphaproteobacteria(100); Rhodospirillales(100); Acetobacteraceae(100); **Acetobacteraceae**_unclassified(100)
Otu00537	+	Bacteria(100); Proteobacteria (100); Gammaproteobacteria(100);Acidithiobacillales(100); Acidithiobacillaceae(100); ***Acidithiobacillus***(100)

Otu02533	+	Bacteria(100); Proteobacteria (100);Gammaproteobacteria(100);Acidithiobacillales(100); Acidithiobacillaceae(100); ***Acidithiobacillus***(100)
Otu02553	+	Bacteria(100); Nitrospira (100); Nitrospira (100); Nitrospirales (100); Nitrospiraceae (100); ***Leptospirillum***(100) ▼
Otu02901	+	Bacteria(100); Nitrospira (100); Nitrospira (100); Nitrospirales (100); Nitrospiraceae (100); ***Leptospirillum***(100) ▼
Otu03075	+	Bacteria(100); Nitrospira (100); Nitrospira (100); Nitrospirales (100); Nitrospiraceae (100); ***Leptospirillum***(100) ▼
Otu03114	+	Bacteria(100); Nitrospira (100); Nitrospira (100); Nitrospirales (100); Nitrospiraceae (100); ***Leptospirillum***(100) ▼
Otu03373	+	Bacteria(100); Nitrospira (100); Nitrospira (100); Nitrospirales (100); Nitrospiraceae (100); ***Leptospirillum***(100); ▼

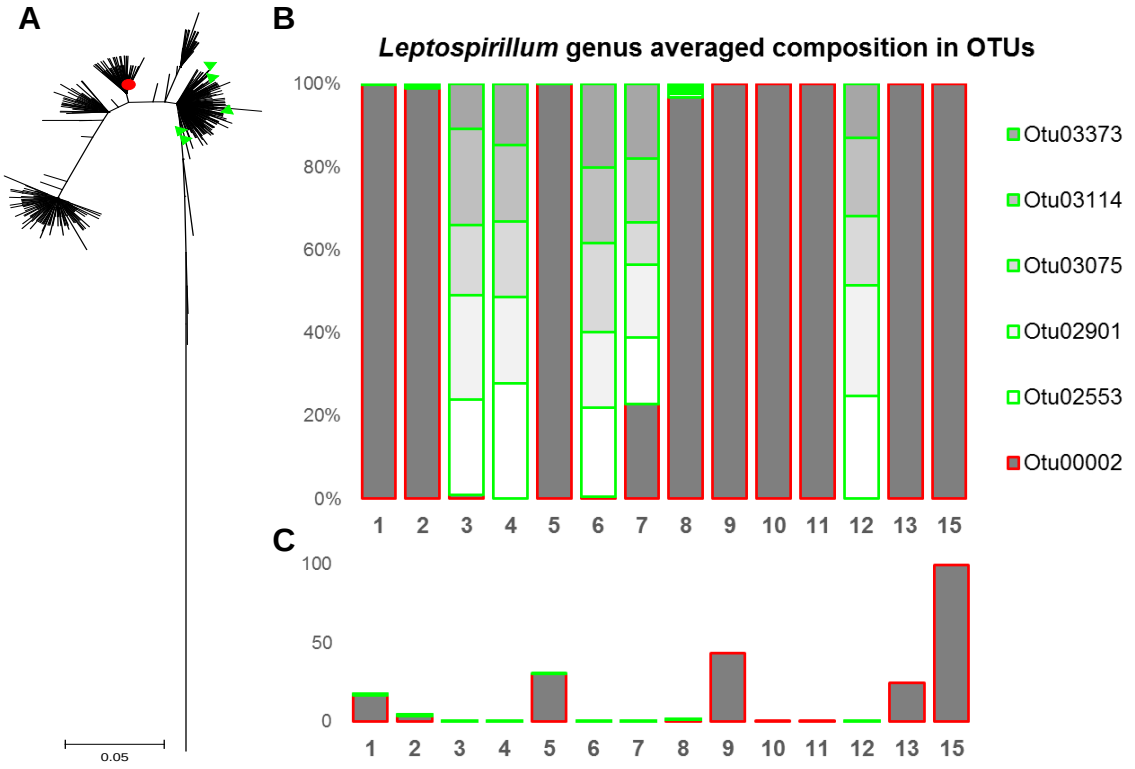


Figure 5.10. A - Schematic phylogenetic tree of *Leptospirillum* OTUs present in the study. OTUs which resulted significantly correlated with successful (▼) or unsuccessful (●) performance were highlighted (OTUs details in Table 5.4). B – *Leptospirillum* genus averaged composition in OTUs in each treatment as a percentage of the genus. C - *Leptospirillum* genus averaged composition in OTUs as a percentage of the treatment community.

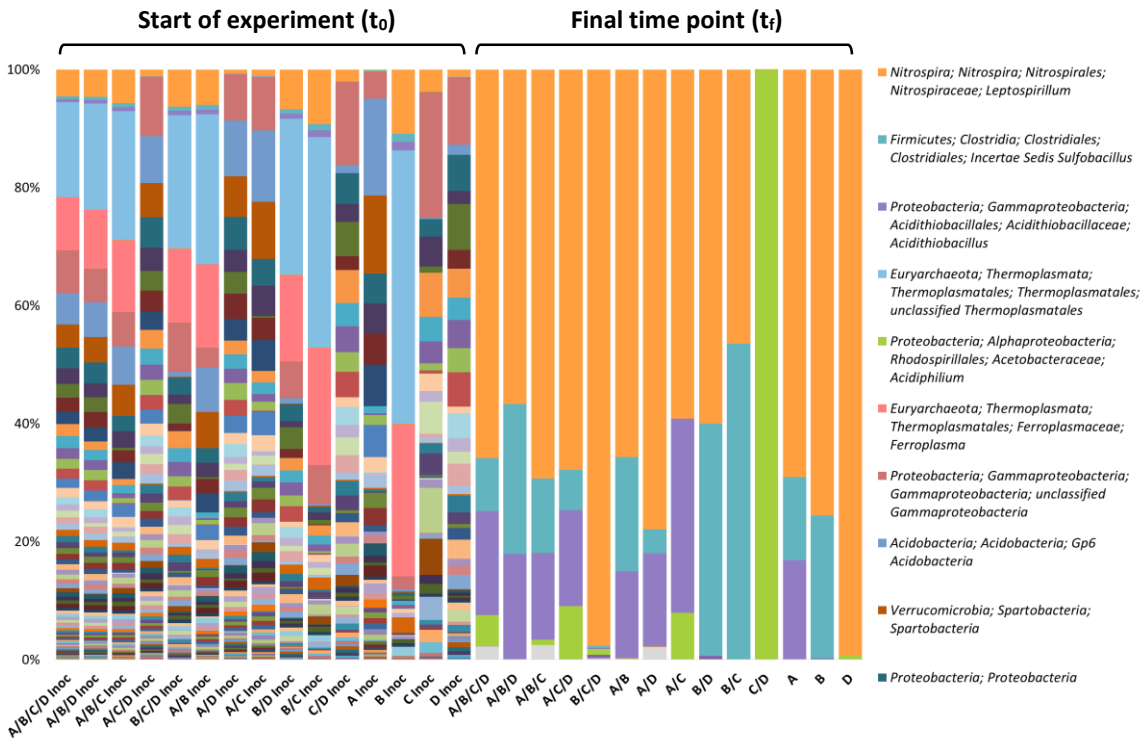


Figure 5.11. Communities' phylogenetic composition, relative abundance of complete dataset shown (averaging replicates), legend is given for the main seven groups.

5.4 Discussion

In this study it was experimentally tested the effect of whole community coalescence on performance, in the context of diverse acidophilic communities deriving from different substrates. Acidophilic communities were used as acid production is a useful proxy for the ability of the leaching community to fully exploit available resources (in this case pyrite as a source of Fe and S): pH increases because of protons released by the oxidation of sulfur and polythionates (Bryan & Johnson, 2008; Johnson, 2012; Okibe & Johnson, 2004) as shown in Figure 1.2 of Chapter 1.

This work followed the experiment in Chapter 4 showing no strict local adaptation of the communities on the sympatric substrate and it represents a further investigation on the systematic optimisation of inocula. It is investigated whether mixing communities would increase their performance by synthetically coalescing four communities in all the possible combinations. Furthermore the reasons of the found patterns are investigated.

Consistent with previous research (Sierocinski et al., 2017), these results showed that mixing communities increased the performance of the resultant coalesced, because of the dominance by the best performing community in isolation. The mixtures containing the best individual community had also the highest levels of performance. In addition there is a strong correlation between performance and the community composition at the end point, and a convergence of communities' composition towards a more restricted diversity, implying selection. The best performing communities contained relative high frequencies of sulfur-oxidisers. According to this results there is likely to be an optimal community, suggested by the positive correlation between final composition and performance which also hint at the functional outcome of the coalescence reflected by the community, as the community A always dominates in a mixture.

The focus wasn't on the detailed mechanisms implied in the coalescence of communities, for example whether co-selection has a role on the definition of the final coalesced mix or if simply all taxa within a community are the best performing in the respective function and therein are selected. In order to obtain such details it would be needed to pull apart the individual communities which is not at the moment possible, but it is a possible follow up of this thesis.

The author focused on the ecological mechanisms underpinning the observed dominance by the best performing community. As the best-performing community in isolation determined both the composition and performance of mixtures of communities, acid production should increase with increasing number of communities in a mixture. Generating this positive relationship between the number of communities and productivity could be: co-selection of the best patches from the best performing communities or it be a ‘‘sampling effect’’ (inoculating more communities increases the chance that the best-performing community will be present in the mix (Tilman et al., 2009). Mixing communities could increase performance beyond that of the maximum of single communities in some circumstances (transgressive over-yielding, Harper, 1977), in this context this happened especially when poorly performing communities in isolation benefitted from the presence of the best communities and as a general pattern (Figure 5.12).

Community A (Mount Wellington) was the best performing in isolation and also the necessary component for the success of coalesced microbial communities.

Mixes containing C and D did not show a contribution to the performance by C and/or D at the end of the experiment and this is confirmed by the treatment C/D which performed worse than community D in isolation, especially regarding the solubilisation of iron. In this case the lower performance might be related to the dilution of community D in the mix. Even the community B appeared not being able to elevate the performance of mixes (with no A) to the level of A. For performance, communities containing A were in average as productive as A, communities containing B but not A were less productive but as productive as B itself. This way, the presence of the best community could predict the performance of a coalesced mixture.

It is demonstrated a correlation between community composition at start (weaker) and end (stronger) points with performance thus indicating that the members of the communities that were selected by the conditions were indeed performing an effect on the substrates. This result reflects the importance of the composition of consortia and suggests that key species or key groups play a role on the performance. It could indeed indicate that the endpoint composition is composed by the taxa that better live in acidity and high concentration of Fe and not necessarily the taxa that actively changed the system. At t(0), greater initial diversity (in the inocula) indicates that there is potential for greater resiliance within the communities. Therefore, it is possible that bioleaching activity starts faster and in turn that environmental conditions within the flasks are modified faster. As a result, at the end point the organisms which survive the best might have been selected, even if the inital bioleaching was done by other organisms which were not as competitive in the environment they created.

Successful DNA analysis for two samples (C and D) highlighted a closeness between environmental community structure and flasks-retrieved community's structure which was important as the structure for A and B for the time point (0) was missing. Communities shifted towards a less diverse and more acidophilic structure and treatment 11 (Figure 7, in grey) highlights that even in this case, community C did not affect greatly the composition as it clusters very close to either D time (0) and D time (final). All communities containing A mainly grouped together at the end point of the experiment apart from two replicates of treatment 1 and 2 (A/B/C/D and A/B/D, respectively) which possibly influenced by B and D more, clustered closer to such single community treatments. Further, one

replicate of treatment 8 (A/C), clusters close to C (env), suggesting that in this case, the community shifted possibly towards the component C, it is believed stochastically. The same happened for the treatment 9 and one replicate of treatment 5 (B/D and B/C/D, respectively) indicating that also in this case the communities shifted majorly towards the community D's composition. These examples highlight a certain level of stochasticity in the selection of the "winning" community or of the "winning" patches of communities. One observation allowed by this result is that the selected winning patches in D are the same in other mixed communities containing D and there is not stochasticity in this.

The microbial communities selected differed considerably in their composition. Communities A and D derived from underground disused mines were mainly dominated by archaea while B and C were deriving from open air environmental mine waste tailings (further details are described in Chapter 3). The previous study in the area showed the presence of a core microbiome common to most samples but the four selected inocula were sufficiently different from each other to study the convergence of their composition and/or the prevalence of specific species (Figure 5.2). It is considered that all four communities contained the functional groups necessary to grow on pyrite.

In acidophilic leaching communities few specific functional roles important for the oxidation of pyrite can be defined and they interact within each other, heterotrophs help decontaminating the system from organic compounds which could be toxic for many acidophiles (Fournier et al., 1998; Johnson, 1995, 2008; Johnson & McGinness, 1991; Okibe et al., 2003; Okibe & Johnson, 2004; Quatrini & Johnson, 2018); hence, the role of community cohesion in shaping community performance is likely to be particularly important (Toquenaga, 1997).

Functional roles appear to have a major impact on the success of communities, *Nitrospirae* are nitrite oxidisers and can either assimilate carbon by fixation or by consumption of organic molecules, among those *Leptospirillum* sp. is strictly chemolithoautotrophic, fixing carbon using ferrous iron as their electron donor and oxygen as the electron acceptor (Chen et al., 2016; Méndez-García et al., 2015; Tyson et al., 2004) *Firmicutes* are a very wide group and some can oxidise iron and sulfur and can be heterotrophs (Chen et al., 2016; Dopson & Johnson, 2012; Méndez-García et al., 2015), *Sulfobacillus, S. thermosulfidooxidans* can exist as an autotroph or a heterotroph oxidizing sulfide minerals, in the presence

of some organic substrates or as a mixotroph; it can also be a facultative organotroph with the ability to get energy and carbon from organic compounds with or without the presence of oxygen (Dopson & Johnson, 2012). *Acidithiobacilli* are acidophilic mainly obligate autotrophs (*Acidithiobacillus caldus* can also grow mixotrophically) that use elementary sulfur, tetrathionate and ferrous iron as electron donors, they assimilate carbon from carbon dioxide (Dopson & Johnson, 2012). *Acidiphilium*, member of the *Acetobacteraceae*, is known to be able to reduce iron in presence of organic molecules or sulfur compounds to sulfuric acid and can fix carbon dioxide (Dopson & Johnson, 2012; Méndez-García et al., 2015).

Successful performance is here primarily suggested by the presence of *Acidithiobacillus*, as *Leptospirillum* is present in almost all treatments, apart from C/D treatment and *Sulfobacillus* prevailed in the treatments containing the community B conferring a good performance but not as good as in the treatments where *Acidithiobacillus* was present. As mentioned previously, it is no surprise that *Leptospirillum* can thrive in high Fe concentration conditions, and resist to high levels of Fe (III) (Rawlings et al., 1999). In the present analysis it emerges that, despite *Leptospirillum* sp. is widely represented in almost all treatments, main *Leptospirillum* genus OTUs are differentially distributed among the treatments and there is a set of five OTUs correlating with best performance, represented in treatments that contain the community A and phylogenetically are diverging from the rest of the genus clusters (Figure 5.11A). This result suggest a different origin of those OTUs as they are present when the community A is in the mixture but it is not possible to hypothesise their functional relevance as they only represent a small percentage of the communities.

According to chapter 3 of the present study, the selected sites were geochemically characterised as follows: A and D, were underground sites, usually defined by a more stable community composition and environmental features. Despite being underground sites, site A and D were quite different in geochemical features. A had low pH and high interstitial water metals' concentration (Fe, Cu, Zn, As, Cd, Pb, and U), B and D were only defined by higher moisture percentage. C had a higher pH compared to the others (pH 3.7).

Site A's community contains *Acidobacteria*, *Planctomycetes*, *Proteobacteria* and *Verrucomicrobia*, in Chapter 3 it is observed that this composition is close to soil

controls' structure. Site B is mainly characterised by *Euryarchaeota* and smaller percentages of other groups. Site C was mainly characterised by Chloroflexi and site D distinguished from the others greatly by Crenarchaeota (representing more than 60% of the whole community). The sample A was although among the most extreme samples, being from an underground mine and therefore rich in iron and having the lowest pH. The high diversity in species that would normally strive in such conditions was surprising. Therefore it was hypothesised that cells were in a high percentage dormant. This observation suggests that, even if dormant, a higher diversity offers the chance of a superior response to changing conditions (Hawkes & Keitt, 2015; Lennon & Jones, 2011).

When in pyrite the community drifted towards a more acidophilic composition and a diversity convergence is observed, implying selection, in the limits of the variability of the strains contained in the communities and their individual ability to cope with higher acidity and pyrite-released sulfates and Fe. Such conditions might have defined the extinction of *Euryarchaeota*, *Verrucomicrobia* and unidentified *Gammaproteobacteria*.

Strongly correlated with lower performance were different strains of *Leptospirillum* and one *Acetobacteraceae* strain, while more *Leptospirillum* strains and some *Acidithiobacillus* strains were strongly correlated with successful performance, as previously proved by (Bryan et al., 2011). This result is congruent with other communities' composition structures previously observed (e.g. Norris, 2007) plus *Acetobacteraceae* do not efficiently oxidise iron nor oxidise sulfur intermediates (Kersters et al., 2006). *Leptospirillum* was also dominating all treatments, even unsuccessful ones and one strain very common and statistically related to unsuccessful communities resulted phylogenetically branching separately from the other *Leptospirillum* strains (the ones defining better performing communities), further analysis are needed to ascertain the phylogenetical and functional drift of *Leptospirillum*.

As proxies for successful performance here only total solubilised iron and pH resulted significant statistically: oxidised iron did not result significantly connected with success and it is suggested this is because even less successful communities managed to oxidise the iron present (supposedly as they contained *Leptospirillum*) but less efficiently than *Acidithiobacillus*-containing consortia, which also can oxidise sulfur. The presence of sulfur-oxidisers appears to be key

to an optimal performance (Ma et al., 2017). This results also confirm the strong convergence to *Leptospirillum* dominated communities' structure of almost all treatments. If the experiments were further continued perhaps a higher diversification would appear and it would be visible that *Leptospirillum* domination was only part of an initial phase following the huge stress of substrate change for the communities, or on the contrary confirm that it represents the most stable community composition.

Previous research suggests that in aerobic communities, cross-feeding interactions are less important (Morris et al., 2013); studies to date (Livingston et al., 2013) suggest that asymmetric outcomes, although less extreme, may be common. This study is relative to aerobic communities but extremophile, less diverse and with possibly stronger interactions and interdependencies, additionally the limited amount of functional roles (iron-oxidisers, sulfur-oxidisers and heterotrophs) might increase the chance of competition for niches and therefore a less strong cohesion in functional modules.

Further work under a range of conditions is clearly required to determine the generality of the present findings. As it is suggested by other authors, this approach could be applied to a range of biotechnological processes using microbial communities, as well as to manipulate microbiomes in clinical and agricultural contexts (Rillig et al., 2016).

Sierocinski et al. (2017) performed an analysis of each community's contribution toward the final mixture suggesting that certain combinations of taxa within a community might be co-selected as a result of coevolved interactions. The same study also shows that performance increased with the number of inoculated communities. The present results leads to the fact that more communities coalescing deliver a higher solubilised iron and a higher acid production. In this regard, adding more communities might increase the chance of including "winning" strains and the "winning" communities; the levels of standard deviations in those data are quite wide and that the mixes of four consortia performed better in average but not significantly. Similarly in this case, had the experiment stopped later perhaps a more marked result would have been observed.

This study identified a simple method to significantly improve yield during bioleaching: inoculate the systems with a broad range of microbial communities,

and the best-performing community will eventually dominate. Such practice has actually been used in the past, although perhaps not in a reasoned way, also not always the local community is the best performing on its own substrate. This work encourages to screen in vials or flasks a wide range of communities and the relative mixes to select a smaller collection of consortia with a predictable outcome. The selected range of consortia could be consequently tested in the bigger scale that often does not allow the necessary replication. In the simplest case this would involve conducting factorial engineering experiments with and without coalescence events in the first two phases (Rillig et al., 2016). The present study also focuses on the completeness of the required functional diversity in order to have a good performance and perhaps a high redundancy of relevant functions, as it is observed the coexistence of multiple OTUs related to the same genus (i.e. *Leptospirillum*), in contrast with what is found by (Sierocinski et al., 2018), where any loss of diversity was likely to reduce performance. On the other side in most treatments the communities survived but not all functions were maintained, not necessarily the ones of human interest, such as acid production.

Microbial communities are efficient in delivering specific functions, therein this approach could be applied to a range of biotechnological processes driven by microbial communities, in the biohydrometallurgy industry primarily, in agriculture (Rillig et al., 2016) and many other future biotechnology processes reliant on microorganisms and applied synthetic microbial ecology (Mee & Wang, 2012). An important next step would involve shifting from simple monitoring of coalescence effects during microbiome engineering to the targeted manipulation of the coalescence events themselves, as according to Rillig et al. (2016a) coalescence would also help obtaining some of the variability that is essential for the subsequent selection of needed traits.

5.5 Conclusions

For the first time the behaviour and performance of coalesced microbial consortia of extremophiles was investigated. This study attempts a quantitative analysis of performance and link the community structure to it. Acidophilic environmental communities are known to have a relatively low diversity, which is further reduced by the conditions they meet when used in bioleaching or for an industrial aim. It

is shown that a selection of most fit species happens but also that such species retain a high intraspecific diversity which will be the focus of future studies.

The community most efficient at using resources in isolation dominates when more communities are mixed together, thus enhancing mixed-community productivity beyond the average of the component communities. In this experiment the most efficient communities were the more diverse and there was a relationship between α-diversity at the beginning of the experiment and performance.

It is noticed that an initial higher diversity of inocula determine a final better performance and that a lower final pH (better performance) is obtained from communities deriving from lower pH environmental conditions. Thus, the common place habit of harvesting multiple communities from a variety of substrates which are similar to the mineral to be treated could actually lead close to optimal performance.

In this study it is offered an analysis of the effect of coalescence on community performance on a simple substrate and a minimal set up, as a primer for future experimentation, regarding extremophile microbial consortia, on more complex cultivation systems and a variety of substrates or for the systematic inoculum optimisation for bigger scale experiments.

The findings obtained are relevant to the understanding of the ecological dynamics of microbial acidophilic communities as well as showing a simple method for improving communities' properties for bio-leaching.

6 Discussion and conclusion

Each chapter of this thesis contained its own detailed discussion and conclusion; the purpose of this chapter is to highlight the main findings and discuss unifying themes and wider implications.

6.1 Synopsis of each chapter:

Chapter 3:

- A higher than expected diversity was retrieved in mine waste impacted environments, including uncultivated archaea (Euryarchaeota and Crenarchaeota).
- Beta diversity was higher in mine waste samples than in nearby soil-like matrices suggesting that the mine waste microbial metacommunity is well adapted to the environmental conditions, allowing a higher diversity to proliferate; while the proximity to mine-impacted areas imposes selective pressure on nearby control soil communities limiting diversification.
- This matches a wider diversity in metals composition (readily extractible metals) of the waste samples.
- The community diversity is mainly driven by pH.
- Readily extractible metals proxy (metallocity) and pH are related in a positive linear relationship.
- There is a common core microbiome shared from waste samples and soils of the microbial metacommunity, showing that both the nearby mine waste and the microbial communities have an effect on the soil close to mine impacted environments.

Chapter 4:

- There was no general local adaptation in the metacommunity considered.
- Local adaptation was observed regarding the performance (Fe oxidation) of especially the microbial communities of the mine waste samples: Devon Consuls, Mount Wellington and Wheal Maid,

- Mount Wellington, Wheal Maid and Porthtowan were also the only inocula showing global over average performance (Fe oxidation).
- Performance was clearly defined by substrates that facilitated microbial activity or disadvantaged it.
- Best substrates didn't necessarily house the best performing inocula.

Chapter 5:

- The community presenting the best performance (Fe oxidation and Fe solubilisation) in isolation would also dominate the performance of coalesced communities.
- When more communities were mixed the performance increases along a growing trend.
- The best performing coalesced communities composition at the end of the experiment included sulfur-oxidisers acidophiles.
- The genus *Leptospirillum* dominated the end point of the experiment.

6.2 General remarks

Understanding the causes and consequences of microbial community structure in a selected environment is pivotal firstly to frame the microbial function in the whole community frame; especially as either in applied biotechnologies and in nature microorganisms thrive in communities and not in isolation: each population relying on or surviving the functional consequences of other populations.

A fundamental goal of microbial ecology is to understand what determines the diversity, stability, and structure of microbial ecosystems. The microbial context poses exceptional conceptual challenges because of the strong mutual influences between the microorganisms and their chemical environment through the consumption and production of metabolites, and the use and transformation of environmental resources. The whole community context experimental frame is gaining visibility in the last few years, following a decade of descriptive studies, correlation- based, via Next Gene Sequencing. Recently molecular technologies

allowed to uncover the effect of treatments on the microbial community structure in experiments while in the same time measure the effects of its function.

6.2.1 The descriptive environmental microbial ecology, its utility and its limits

Given the limitations of inferring processes from biogeographic patterns, (Hanson et al., 2012) suggested that studies should focus on directly testing the underlying processes. Definitely descriptive environmental microbial ecology and biogeography have limitations, such as the limited amount of variables measurable, their measure is not always comparable among different authors' methods, furthermore correlation of variables present issues too, and anyhow each single sampling/survey can only describe a really small portion of space and time of a living system. Even in extensive sampling efforts for number of samples or time points, it is difficult to be representable of a whole microbiological system, but this can be achieved, for instance and cautiously, in aquatic systems which are fluid and more homogeneous. It is extremely difficult being representable of a system when considering solid substrates, because any sampling point or even replicate is just one point in a heterogeneous space. Therefore, the sampling strategy result important and it need to be finalised to the response of a research question or better to the information gathering for experimental purpose.

In this study the aim of the sampling strategy was defining the environmental pressure on a subsample of the metacommunity in Cornwall (UK) hosted by mine waste. As a corollary the environmental effect on mine waste was compared to the effect on nearby soils and it was found that in such case the pressure is bigger and constrains the diversity. Different metals datasets were tested for correlation with communities' composition, showing that readily extractible metals were the best proxy to distinguish between soils and mine waste, while pore water and total metals were not correlating.

Chapter 3 gave important information for the understanding of following experiments, such as communities' structure, original living conditions, especially pH. In Chapter 5, a correlation between performance and environmental pH of the inocula is observed, while in Chapter 3 it is shown that readily extractible metals (PC1) co-vary with pH (Figure 1.6). This suggests that environments rich

in high readily extractible metals might harbour potential performing inocula. By observing the loadings on the PC1 it would be possible to infer what metals prevail in the loadings hence defining the PC1.

Therefore, microbial ecology can be useful as a starting point for further experimental ecology allowing to rely on a starting knowledge of the area, of the communities and eventually their relationships with metals *a priori*. It is also useful in the interpretation of experimental results.

A decade ago microbial ecology was a discipline on the verge of maturing beyond the descriptive phase. However, correlation analyses do not distinguish cause and effect and therefore, it cannot suggest that an environmental characteristic is a cause of diversity (Prosser, 2020). Recently microbial ecology analysis is finally not only explaining observed descriptive patterns based on the hypothetical processes that might generate them but it is an essential tool for experimental hypothesis testing.

6.2.2 Whole community ecology and its applications of ecological concepts in industry

Acid production and the ability of a consortium to oxidise a mineral substrate are key traits, used from the research and development for decades (Barrie Johnson, 2014; Vera et al., 2013). The present work contributes in connecting the dots between microbial experimental ecology and biohydrometallurgy proposing to use trait-designed experiments to inform and optimise the biomining process, specifically the inoculum design. In the past, for example, the objective of designing and operating stable and resilient high-performing systems could be met in unusual ways, not following the procedure: describe, explain, predict, manipulate (McMahon & Martin, 2007).

As energy and environmental constraints become more challenging, there will be greater need and incentives to reprocess low grade ore and relic mine wastes; more over the circular economy frame implies the recycling of metals from electronic and other metallic wastes and metal-rich waste waters; while *in situ* biomining could allow buried ore bodies to be economically exploited, perhaps with the help of acidophilic consortia (Johnson, 2014). This scenario hints at the

future importance of the resources optimisation and the search for “low cost” biotechnologies, such as those implicated in the field of biohydrometallurgy. It follows that the efficiency of the microbial community function would have more and more relevance as shown by studies in mesocosms imitating mineral heaps and focused on their microbial colonisation (Chiume et al., 2012).

This study gives applicable suggestions regarding the properties of the Cornish mine waste communities extendable to a variety of similar frameworks, relying on microbial function. The evolution of a consortium prior its use in technology is pivotal and affecting its adaptation to a specific substrate, surely has an effect on the performance of the consortium on the desired application. In this investigation it is observed that the communities deriving from most acidic environments were the best performing when the common garden treatment was applied (pyrite), while there was no evident local adaptation of most communities to sympatric substrates, exception made for the ones deriving from the most acidic and As-rich environments. Such results suggest that the communities previously adapted to an extreme environment conserve even in less acidic environments the main trait of acid production ability. Then it was noticed the major plasticity of most extreme communities for better performing even in ecologically distant scenarios, therefore the hypothesis that such property might be preserved by dormant species that resist in difficult environments (Hawkes & Keitt, 2015; Lennon & Jones, 2011). Furthermore community coalescence enhances the performance thanks to a best performing community which drives the composition and function of the coalesced community. This concept could be useful in applications allowing to avoid the detailed search for the best inoculum as long as a variety of consortia are tested and a subset is coalesced: the only presence of the best performing community would ensure a good performance.

6.2.3 Limitations of the work and future perspectives

Experimental work has limitations of its own, starting with the simplicity of the mesocosm/microcosm, it is possible to manipulate and decide the environmental factors to control, waiving on the openness of the natural system. In this work the author focused on a sub-section of acidophiles by using as a proxy for performance the ability of the consortia to oxidise and solubilise iron. Trait-based

experimental ecology focuses on limited traits of the community used as a model and it was decided to only focus on one portion of the acidophilic community because iron and sulfur-oxidisers are important players for the generation of acid mine drainage in the environment and the ability to maintain acidity in a system is a property widely used in the field of biohydrometallurgy (Brierley & Brierley, 2013). Another reason for such choice is the difficulty to measure cells density in a wide number of treatments and replicates because of the presence of the mineral debris it is not possible to use, for instance, a spectrophotometer for 96-wells plates, as widely used in microbial ecology. Further studies are necessary to complement the work here proposed and either include mixotrophic and heterotrophic microorganisms in the experiment or focus on such component of acidophilic communities.

As a main limitation of the experiments described in this thesis, in order to replicate the experiments in an effective number and homogeneity, it was decided to use liquid media on top of mineral substrate (2%) and such treatment might affect the structure of environmental communities. Nevertheless, Chapter 5 includes a description the community structure of environmental samples and the respective inocula maintained in liquid media but with 2% (w:v) natural substrate and in Figure 5.7 an NMDS ordination shows that the environmental samples were clustering close to the maintenance communities, thus suggesting a very similar structure.

A second limitation is the lack of knowledge in the intra-community mechanisms implied in the selection of taxa during coalescence, for the dataset analysed it was not possible to conduct the non-negative least-squares (NNLS) analysis, implemented by Sierocinski et al. (2017) which would allow to pull apart the communities and track the contribution of original coalesced and non- coalesced communities into the end point communities.

A further limitation of the experimental study is represented by the method chosen for the sterilisation of mine waste to be used in the experiments. By autoclaving the mine waste, its structure might change and differ compared to the environmental structure. One indication that the autoclaved mineral used to feed the microbial communities did not change substantially is given by the community composition of consortia sequenced after they had been cultivated in autoclaved media and mineral: their community composition did not vary substantially and in

an NMDS ordination they cluster close to their ancestor communities, indicating similar composition. Therefore the method was considered for the experiments. For future work alternative ways of sterilisation will be considered (e.g. tyndallisation).

In Chapter 4, the initial intention consisted in exploring the microbial diversity of the end point communities, especially the locally adapted towards the non-adapted, unfortunately the huge amount of samples did not allow such analysis, alongside other reasons, cost and time. Currently new systems allowing to obtain the community structure of samples in real-time (i.e. Minion), are implemented by (Jain et al., 2016), among others, and they represent a valuable resource for experimental microbial ecology, because of the short time needed to deliver results.

Such technology is most useful in the context of synthetic communities use to test ecological hypothesis. They limit the factors that impact the microbial community to a minimum, allowing their management and identifying specific community responses and traits (De Roy et al., 2014; Singh, 2010).

This study is one of the first attempts in the use of a set of whole natural microbial communities to experimentally investigate ecology and evolution of acidophiles testing communities local adaptation and coalescence. A next step in the methodologies could be the design of synthetic communities, and the comparison of their performance to the natural communities as done by (Castledine et al., 2019), in terms of populations.

6.3 Concluding remarks

Disentangling the roles of the abiotic environment, evolution history, interactions on how microbial communities are formed, maintained and function remains a crucial goal of the microbial ecology of extremophiles and has relevance to its biotechnological application.

It is found a larger diversity in mine waste environments than previously thought (e.g. Baker & Banfield, 2003; Quadros et al., 2016). When the same communities were constrained in a simple pyritic system a remarkable simplicity emerged (Goldford et al., 2018), which may permit a more fundamental understanding of

how microbial communities work than is possible through study of more complex communities.

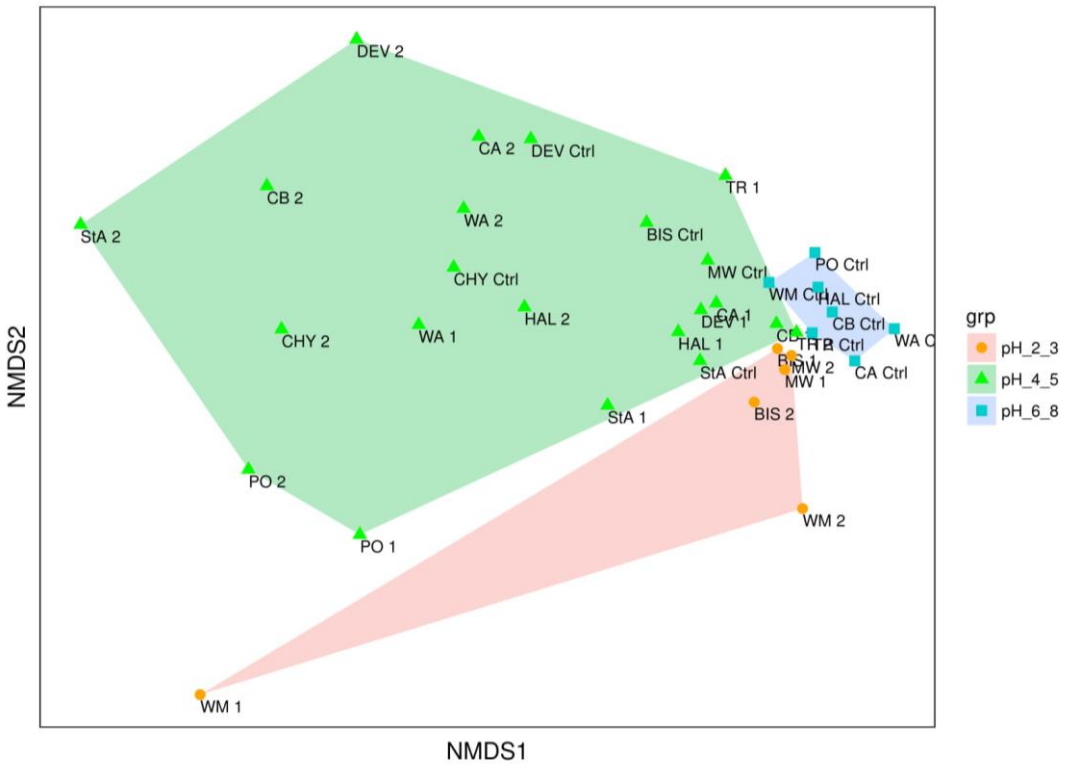
In this work contributes to the unravelling the consequences of interactions between communities and between community and substrate in a range of frameworks. Local adaptation is studied as a cause of environmental community structure, described the structure of representatives of the Cornish metacommunity and found patterns driving the diversity and consecutively the function of such communities. With the coalescence experiment at community level it is offered a first example of a replicated experiment simulating the interaction of multiple communities, regarding extremophiles. Moreover this study contributes to a new trait-based collective experimental scientific effort that follows an impressive amount of descriptive work in the past decade regarding descriptive microbial ecology, especially in extreme environments.

Appendix

Appendix Table 1. Correlation between communities' structure and single variables (Mantel tests)

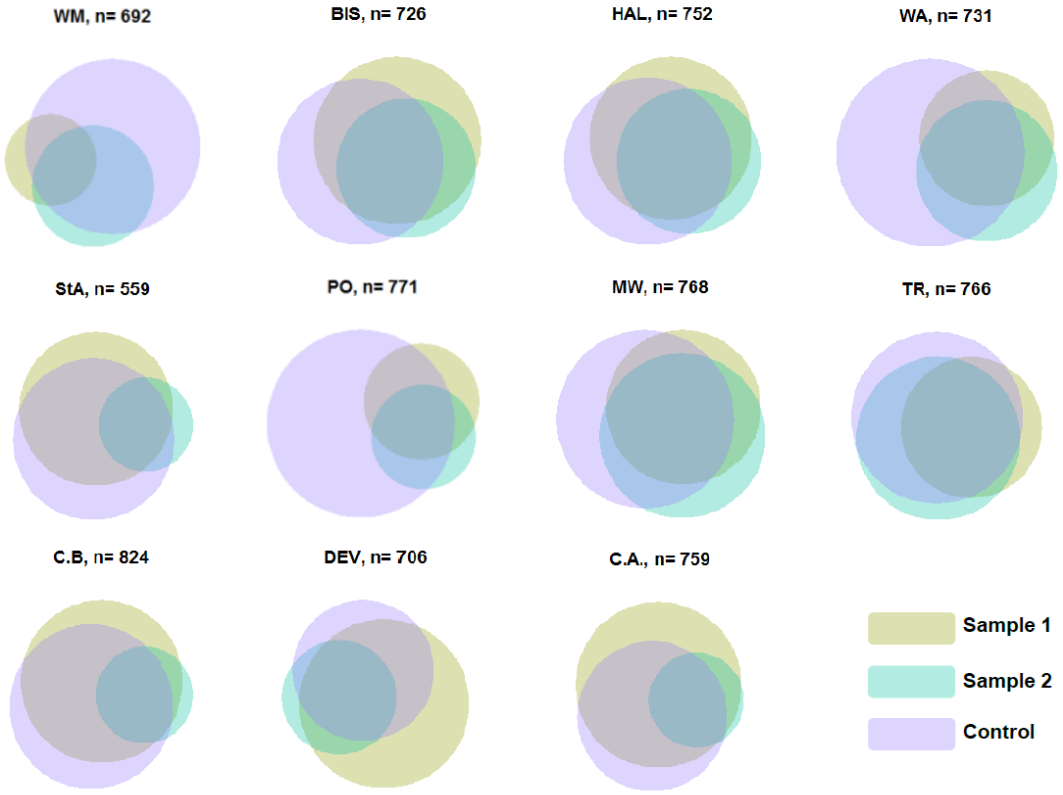
Y (OTUs level)	Group	Substrate	Ind. Variable	Mantel statistic r	Significance	Method
Alpha diversity	Env		pH	0.4767	**0.012**	Pearson
Alpha diversity	PWM	soil	Al	0.2864	0.131	Pearson
Alpha diversity	PWM	soil	Cu	0.2819	0.137	Pearson
Alpha diversity	REM	soil	As	0.2878	0.137	Pearson
Alpha diversity	REM	soil	Cd	0.4089	**0.041**	Pearson
Alpha diversity	REM	soil	Fe	0.1883	0.132	Pearson
Alpha diversity	REM	soil	Mn	0.1916	0.112	Pearson
Alpha diversity	REM	soil	Ti	0.262	**0.068**	Pearson
Alpha diversity	TM	soil	As	0.3003	0.12	Pearson
Alpha diversity	TM	soil	Fe	0.2169	0.185	Pearson
Alpha diversity	TM	soil	Sn	0.279	0.167	Pearson
Alpha diversity	TM	soil	Sr	0.3144	0.106	Pearson
Alpha diversity	TM	soil	Th	0.2982	0.14	Pearson
Alpha diversity	TM	soil	Ti	0.3007	0.111	Pearson
Alpha diversity	TM	soil	W	0.264	0.133	Pearson
Alpha diversity	TM	soil	Y	0.2636	0.159	Pearson
Alpha diversity	TM	soil	Zn	0.1894	0.165	Pearson
Alpha diversity	PWM	mine waste	Ti	0.2193	**0.021**	Pearson
Alpha diversity	REM	mine waste	Cd	0.1059	0.099	Pearson
Alpha diversity	REM	mine waste	Th	0.1791	**0.034**	Pearson
Alpha diversity	REM	mine waste	W	0.1354	0.088	Pearson
Alpha diversity	TM	mine waste	La	0.129	0.095	Pearson
Alpha diversity	TM	mine waste	P	0.3005	**0.001**	Pearson
Alpha diversity	TM	mine waste	Sr	0.1778	**0.077**	Pearson
Alpha diversity	TM	mine waste	Th	0.2171	**0.029**	Pearson
Alpha diversity	TM	mine waste	Ti	0.2208	**0.017**	Pearson
Composition	Env		pH	0.7183	**0.001**	Pearson
Composition	PWM	soil	Al	0.4947	**0.046**	Pearson
Composition	PWM	soil	Cu	0.5078	**0.038**	Pearson
Composition	REM	soil	As	0.5108	**0.033**	Pearson
Composition	REM	soil	Cd	0.409	0.159	Pearson
Composition	REM	soil	Fe	0.4158	**0.04**	Pearson
Composition	TM	soil	As	0.5212	**0.038**	Pearson
Composition	TM	soil	Ce	0.4626	0.099	Pearson
Composition	TM	soil	Cu	0.1654	0.225	Pearson
Composition	TM	soil	Fe	0.3976	0.117	Pearson
Composition	TM	soil	La	0.5015	**0.044**	Pearson
Composition	TM	soil	Ni	0.3636	**0.043**	Pearson
Composition	TM	soil	P	0.5218	**0.026**	Pearson
Composition	TM	soil	Sc	0.3614	**0.061**	Pearson
Composition	TM	soil	Sn	0.499	**0.081**	Pearson
Composition	TM	soil	Sr	0.5103	**0.031**	Pearson
Composition	TM	soil	Th	0.5165	**0.072**	Pearson
Composition	TM	soil	Ti	0.5224	**0.024**	Pearson

Composition	TM	soil	W	0.5052	**0.029**	Pearson
Composition	TM	soil	Y	0.4901	**0.057**	Pearson
Composition	TM	soil	Zn	0.4215	**0.046**	Pearson
Composition	PWM	mine waste	Ce	0.2729	**0.08**	Pearson
Composition	PWM	mine waste	La	0.3058	**0.071**	Pearson
Composition	PWM	mine waste	Ni	0.1016	0.151	Pearson
Composition	PWM	mine waste	Sr	0.3394	**0.087**	Pearson
Composition	PWM	mine waste	Th	0.4323	**0.009**	Pearson



Appendix Figure 1. NMDS ordination plot of all samples grouped by pH ranges at OTUs level.

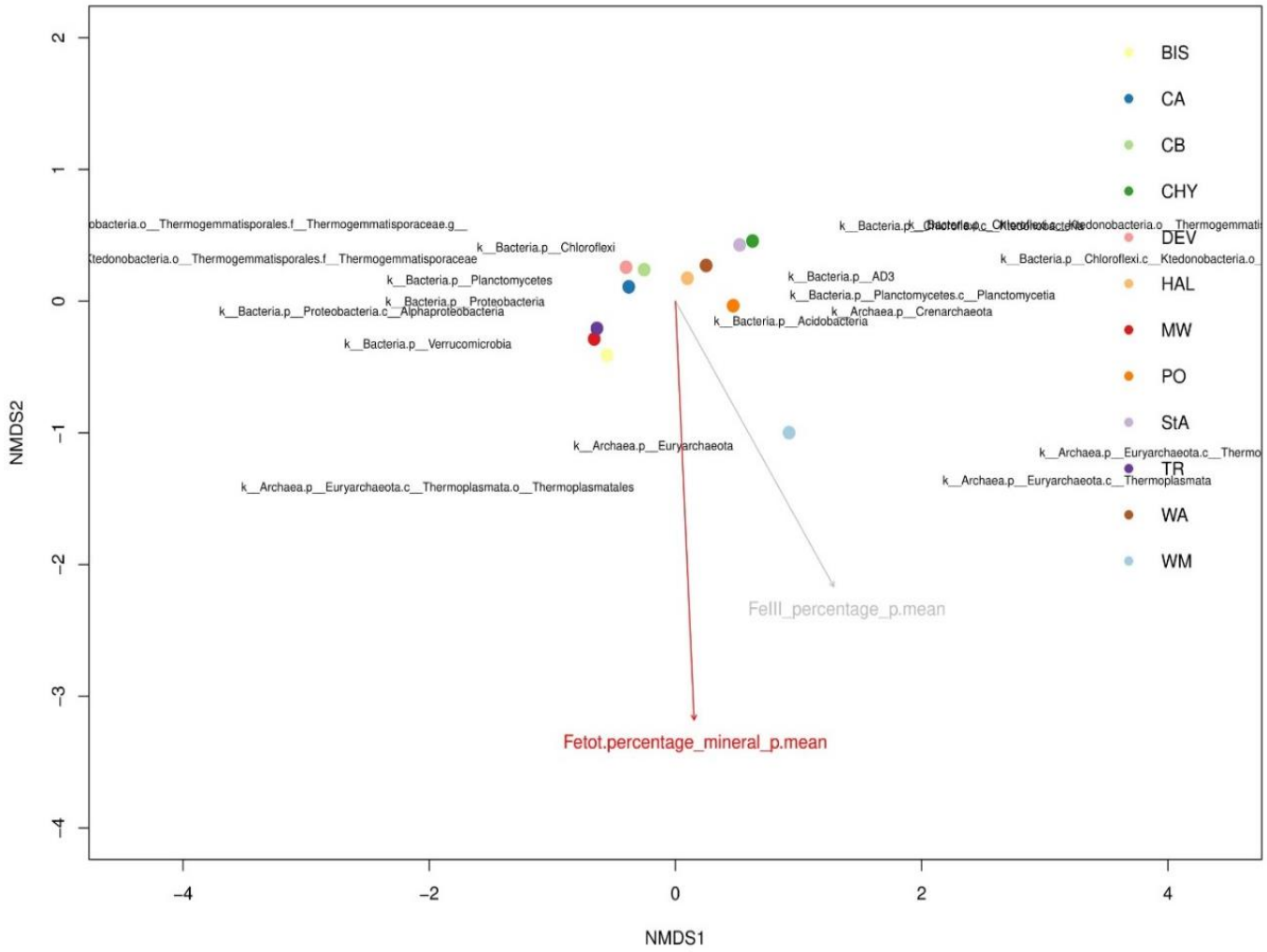
Intermediate pH samples show a higher β-diversity (non-transformed, scaled and centred data), Chapter 3.

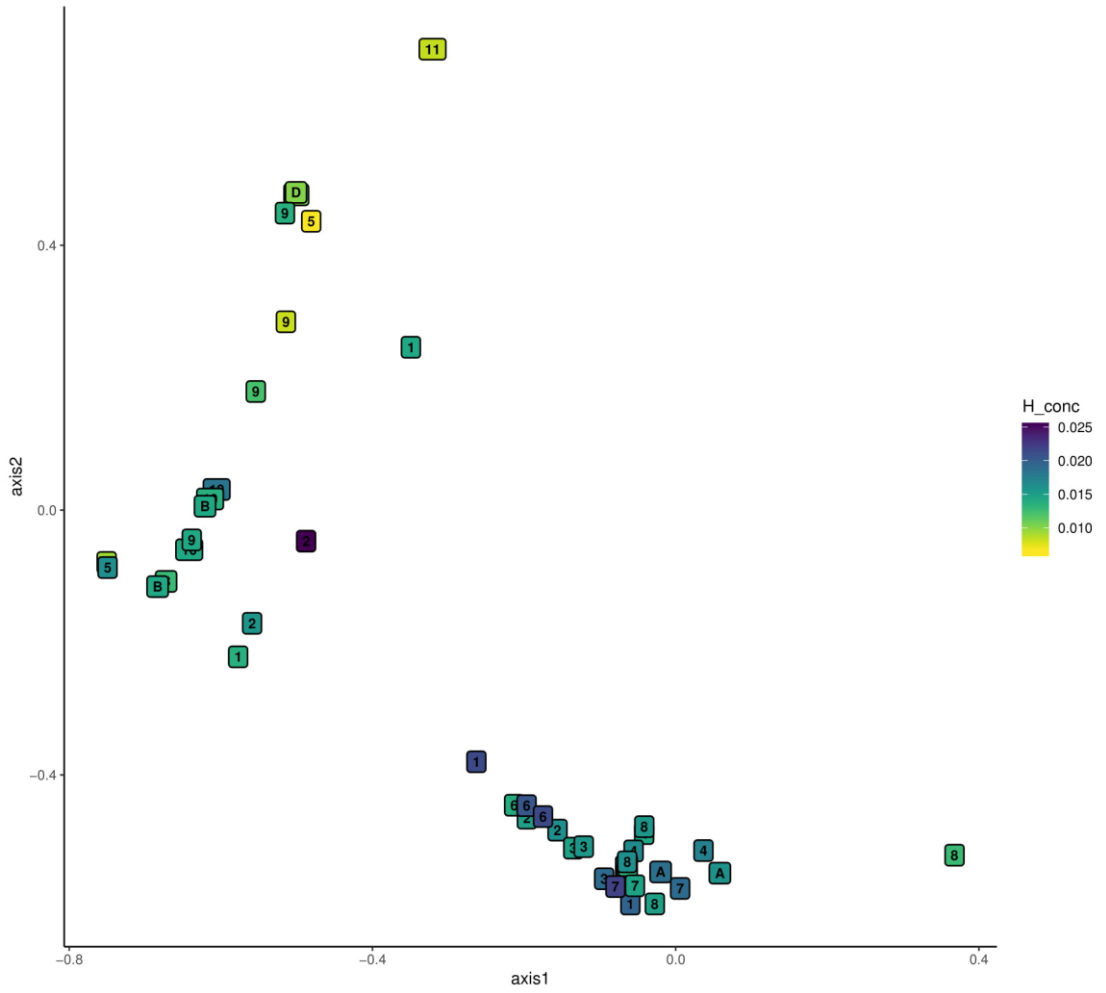


Appendix Figure 2. Microbial communities OTUs overlap between mine waste samples and soil samples (n= total number of OTUs counted in each site, non-weighted data)

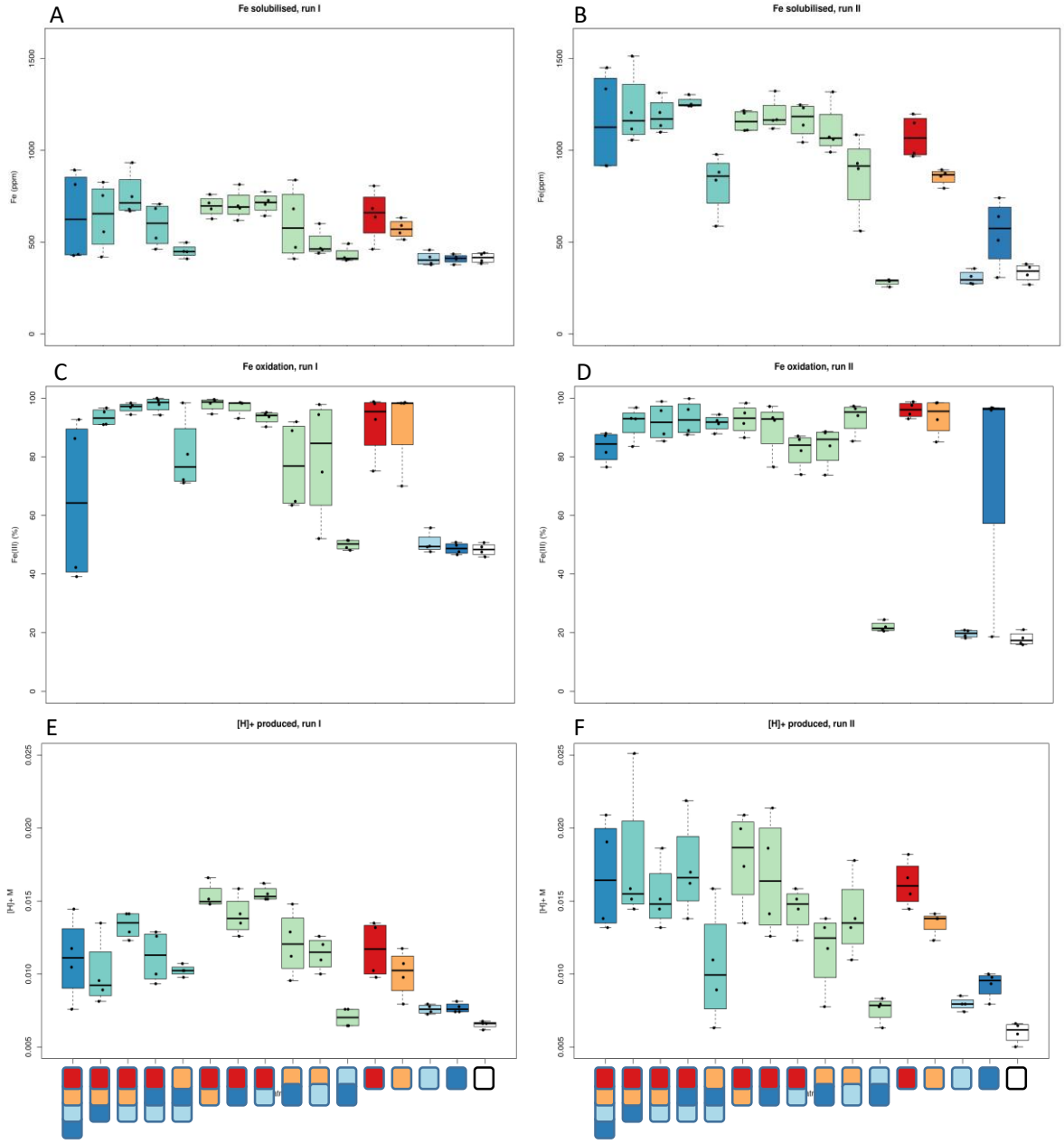
Appendix Figure 3. Non-metric multidimensional scaling (NMDS) ordination at the start of the experiment, chapter 5.

The proxies for the performance of inocula are fitted into the ordination. Total solubilised iron is the only variable which significantly fits to the ordination ($p < 0.05$). OTUs are shown, after filtering by abundance and fit to the variables. The OTUs shown gradually relate to increasing total solubilised iron: Chloroflexi and Thermogemmatisporaceae and Planctomycetes are the ones relating to lower Fe, while Euryarchaeota are the ones clustering towards increasing solubilised iron (and percentage of oxidised iron).

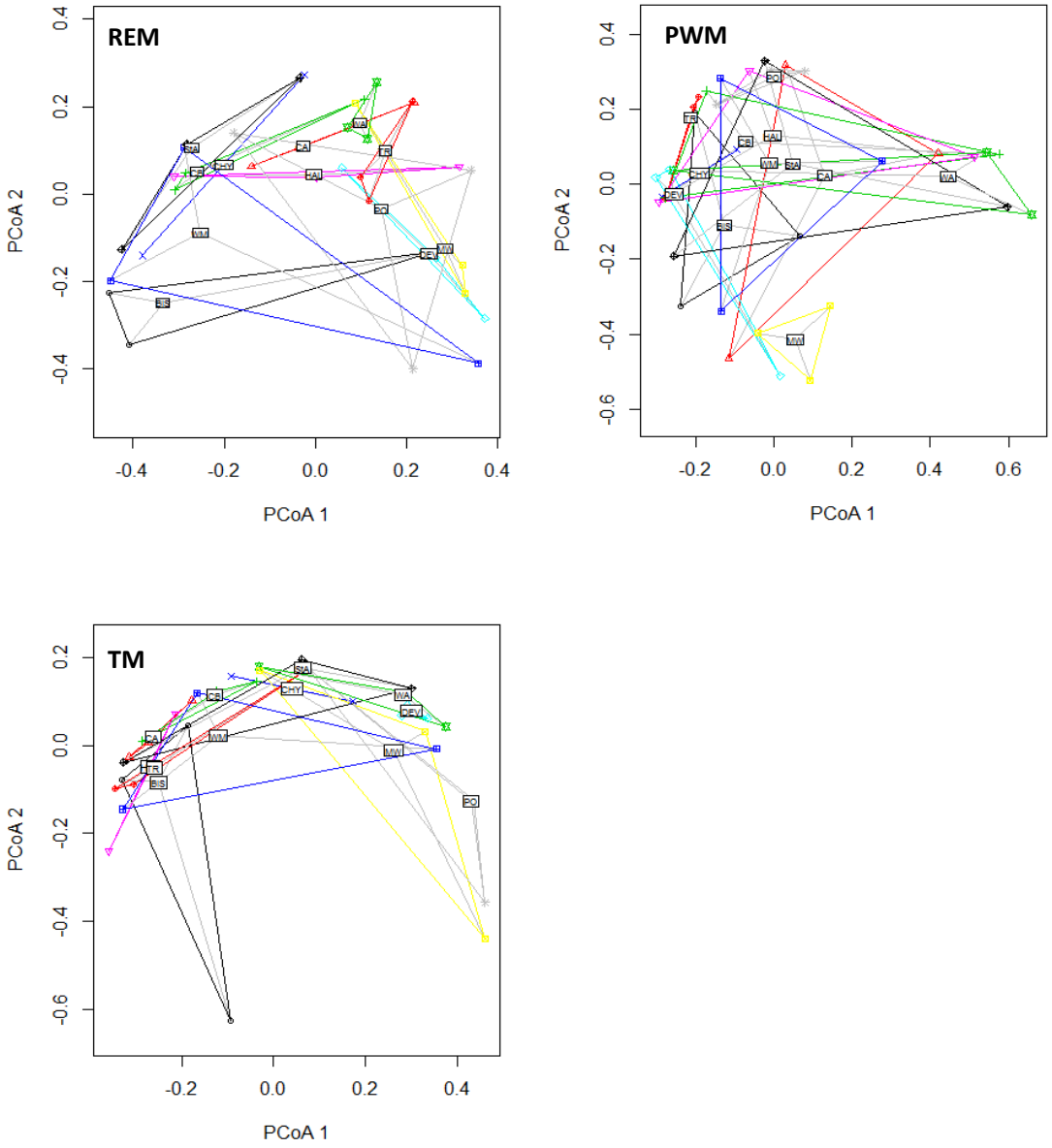




Appendix Figure 4. Acid production is highlighted in an NMDS ordination of all replicated treatments.



Appendix Figure 5. Performance of all treatments at the end of the first and final run of the coalescence experiment, Chapter 5. Total solubilised Fe at the end of run I (A) and II (B); oxidised Fe (%) at the end of run I (C) and II (D); estimated acid released at the end of run I (E) and II (D) for the sixteen treatments. The communities represented are Mount Wellington, Wheal Maid, Porthtowan and Chyverton.



Appendix Figure 6. Principal Coordinate Analysis (PCoA) ordination of mine waste and soil samples composition of metals. The analysis was repeated for the three dataset of metals composition (REM – Readily Extractible Metals, PWM – Pore Water Metals, TM – Total Metals). In bright yellow are highlighted the samples from Mount Wellington site and the hull connecting the points.

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