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Cultivation of Extremely Acidophilic Archaea from an Acid Mine Drainage Site

Jason Tyler

A thesis submitted for the degree of

Master of Science by Research (MScRes)

in the School of Natural Sciences

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UNIVERSITY

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Thanks as well to my friends and family for their support during this project.

“*The role of the infinitely small in nature is infinitely great.*”

– Louis Pasteur

Abstract

The archaeal Domain is understudied and under-sampled, comprising much of the uncultivated majority of microorganisms known as microbial "dark matter". Metagenomic studies have revealed a large diversity of archaea remaining uncharacterised through lack of cultivation, and a dominance of *Thermoplasmatales* related archaea in Acid Mine Drainage (AMD) sites. The AMD site at Parys Mountain (Wales, UK) is dominated by the uncultured archaeon "E-Plasma", and the present work aims to cultivate these organisms. Archaeal Richmond Mine Acidophilic Nano-organisms (ARMAN) related organisms were also detected in this ecosystem in association with *Cuniculiplasma divulgatum*. Since no representatives of ARMAN related organisms have been validly described through cultivation, it is the aim of the present work to cultivate these organisms to uncover details regarding their physiology.

Enrichment cultures were established with samples from the Parys Mountain AMD site using five different polypeptide substrates. The microbial content was monitored during cultivation using PCR based techniques and 16S rRNA gene amplicon sequencing. Bacterial and archaeal sequence affiliations were checked with the GreenGenes and National Centre for Biotechnology Information (NCBI) databases respectively. No significant enrichment of "E-Plasma" was detected in the first cultivation attempts, but ARMAN related organisms were detected in later cultures. Subsequent cultivation experiments focussed on their enrichment.

The microbial composition of cultures was affected by pH and the choice of polypeptide substrate used during cultivation. Archaea of the order *Thermoplasmatales* grew in different quantities in all cultures, with a higher abundance in more acidic conditions. The bacterial content of cultures resembled similar AMD ecosystems, with mostly *Actinobacteria*, *Proteobacteria* and *Firmicutes* represented. After nine months the ARMAN related organism Mia14 was enriched to 35.39 % in a culture containing elemental sulfur at 37.5 °C under microaerophilic conditions with a pH of 1.7. This represents the highest abundance of these organisms to have been cultivated. The removal of sulfur yielded a lower Mia14 content and no additional supplements stimulated its growth further. Here, it is proposed that Mia14 has an association with sulfur and a possible association with *Leptospirillum* sp. Previous studies are supported by the present work regarding its dependence on *C. divulgatum* as a host organism. The role of sulfur in the metabolism of Mia14 is unclear: it may be reduced or oxidised as an energy source, or act as an attachment target for cells.

The importance of exploring the diversity of microbial "dark matter" through cultivation is emphasised by the present work. Despite the domination of "E-Plasma" in the environment, cultivation attempts proved unsuccessful. This represents the difficulties associated with *Thermoplasmatales* cultivation, as highly specific growth conditions are required. The lack of essential nutrients may have prevented the stimulation of growth, possibly due to a symbiosis or other conditions which were not replicated in cultures. With the advent of new cultivation methodology, further cultivation attempts may be made in relation to "E-Plasma". Further research using the present cultures will continue to investigate the physiology and metabolism of both Mia14 and *C. divulgatum*.

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1. Literature Review

1.1 Introduction

The three-domain system of classification used today consists of the *Archaea, Bacteria* and *Eukarya* as proposed by Woese & Fox (1977). Much of the research involving prokaryotes since then has concentrated on bacteria, with archaea being comparatively neglected. This may be partly due to their difficulty of cultivation, with only six out of the 27 proposed phyla having cultivated representatives presently (Baker *et al.*, 2020). Archaea are ubiquitous in the environment (Chaban *et al.*, 2006; DeLong & Pace, 2001) and many are extremophilic: these include the thermophilic archaeon *Pyrococcus furiosus*, whose DNA polymerase has been used in some PCR applications due to its stability at high temperatures (Lundberg *et al.*, 1991); the halophilic *Haloquadratum walsbyi* which has captured interest with its flat, square cell morphology (Oren *et al.*, 1999) and the extremely acidophilic archaea of the order *Thermoplasmatales*, which are the focus of the present work.

1.2 Overview of *Thermoplasmatales* Diversity

The archaeal Order *Thermoplasmatales* includes the most acidophilic prokaryotic organisms described so far. This is characteristic of the Order, as all species are extreme acidophiles. *Thermoplasmatales* members are further characterised by typically being thermophilic, heterotrophic and cell wall lacking (Huber & Stetter, 2006), with some exceptions. Due to the lack of a cell wall, most species have irregular pleomorphic cell shapes (Huber & Stetter, 2006; Golyshina *et al.*, 2000; Golyshina *et al.*, 2016b; Itoh *et al.*, 2007). Currently, nine species comprising six genera have been described within the order. The phylogenetic relationship of described species within the *Thermoplasmatales* order is shown in Figure 1.1 (Golyshina *et al.*, 2016b).

1.2.1 Genus *Thermoplasma*

The first member of the order *Thermoplasmatales* to be described was *Thermoplasma acidophilum*, which was isolated from a coal refuse pile in Indiana, USA, by Darland *et al.* (1970). It is a thermophilic heterotrophic facultative anaerobe, growing at optimum conditions of 59 °C and pH 1-2 (*ibid.*). *Thermoplasma volcanium* was later described by Segerer *et al.* (1988) using samples taken from solfatara fields in the Azores, Iceland, Indonesia, Italy and the USA. The organism is facultatively anaerobic, heterotrophic and thermophilic, and grows optimally at 60 °C and pH 2 (*ibid.*). The genus

contains two species that vary in GC content of DNA, with *T. acidophilum* having 46 mol % and *T. volcanium* having 38 mol % (*ibid.*).

1.2.2 Genus *Picrophilus*

Two species have been described from the genus *Picrophilus*, which were isolated from solfataric fields in northern Japan (Schleper *et al.*, 1995). The genus includes the most acidophilic organisms to have been described, *Picrophilus oshimae* and *Picrophilus torridus*. Members of this genus are unique within the *Thermoplasmatales* order as they possess an S-layer, which contributes toward their extremely acidophilic physiologies by providing an additional barrier to prevent proton influx to the cells, thereby aiding their survival at such low pH levels (Fütterer *et al.*, 2004; Quatrini & Johnson, 2018; Schleper *et al.*, 1996). The presence of an S-layer also provides cells with irregular coccoid morphologies, unlike other *Thermoplasmatales* members whose cells are pleomorphic (Huber & Stetter, 2006; Itoh *et al.*, 2007; Schleper *et al.*, 1995). They are thermophilic, heterotrophic, obligate aerobes, with both species having optimal growth conditions of 60 °C and pH 0.7 (Schleper *et al.*, 1995). Growth of *P. torridus* has been observed at pH -0.06 (*ibid.*), making it the most acidophilic prokaryotic organism to have been described to date.

1.2.3 Genus *Ferroplasma*

Ferroplasma acidiphilum is the only species to have been described in its genus (although "*Ferroplasma acidarmanus*" has also been proposed but not validated (Dopson *et al.*, 2004)). *F. acidiphilum* is one of two mesophilic members of the order *Thermoplasmatales* (Golyshina *et al.*, 2000). It was isolated from a pilot plant in Russia used for the bioleaching of pyrite ore from Bakyrtchik, Kazakhstan. It grows optimally at 35 °C and pH 1.7, and is an obligate aerobe (*ibid.*). It oxidises Fe^{2+} as an energy source and fixes carbon heterotrophically (Golyshina *et al.*, 2017b). It is likely that this organism has a significant role in biogeochemical cycling of sulphur in acidic environments (Golyshina & Timmis, 2005) and the organism may have a possible involvement in bioleaching applications.

1.2.4 Genus *Thermogymnomonas*

Thermogymnomonas acidicola was isolated in 2007 from a solfataric field in Japan. It is an obligate aerobe, and is heterotrophic and thermophilic, growing optimally at pH 3.0 and 60 °C (Itoh *et al.*, 2007) making it the least acidophilic member of the order *Thermoplasmatales* to have been currently described. It is the only species to have been described in its genus, and a family name has not currently been assigned to this genus (Itoh, 2015).

1.2.5 Genus *Acidiplasma*

Two species have been described within the genus *Acidiplasma*. *Acidiplasma cupricumulans* (previously *Ferroplasma cupricumulans*) (Hawkes *et al.*, 2006) was transferred to this genus based on similarity of 16S rRNA sequence identity levels with *Acidiplasma aeolicum* (Golyshina *et al.*, 2009). Both *A. cupricumulans* and *A. aeolicum* are facultatively anaerobic, moderately thermophilic chemo-organotrophs, capable of oxidising Fe^{2+} in aerobic conditions, reducing Fe^{3+} in anaerobic conditions, and growing on organic substrates (Golyshina *et al.*, 2009; Hawkes *et al.*, 2006). *A. cupricumulans* was isolated from a chalcocite bioleaching site in Myanmar, and grows optimally at 53.6 °C and pH 1 - 1.2 (Hawkes *et al.*, 2006). *A. aeolicum* was isolated from a hydrothermal pool in Italy, and grows optimally at 45 °C and pH 1.4 – 1.6 (Golyshina *et al.*, 2009).

1.2.6 Genus *Cuniculiplasma*

Cuniculiplasma divulgatum is the most recent member of the order *Thermoplasmatales* to have been described, and it is the only member of its genus. Two strains were isolated from acidic water bodies at copper mine sites in the UK and Spain (Golyshina *et al.*, 2016b). It is the second mesophilic member of the order *Thermoplasmatales* to have been described (Golyshina *et al.*, 2000; Golyshina *et al.*, 2016b) and is a heterotrophic facultative anaerobe, growing optimally at pH 1 – 1.2 and 37 – 40 °C, with one strain growing at temperatures as low as 0 °C, making it the most psychrotolerant member of its order to have been described to date (Golyshina *et al.*, 2016b).

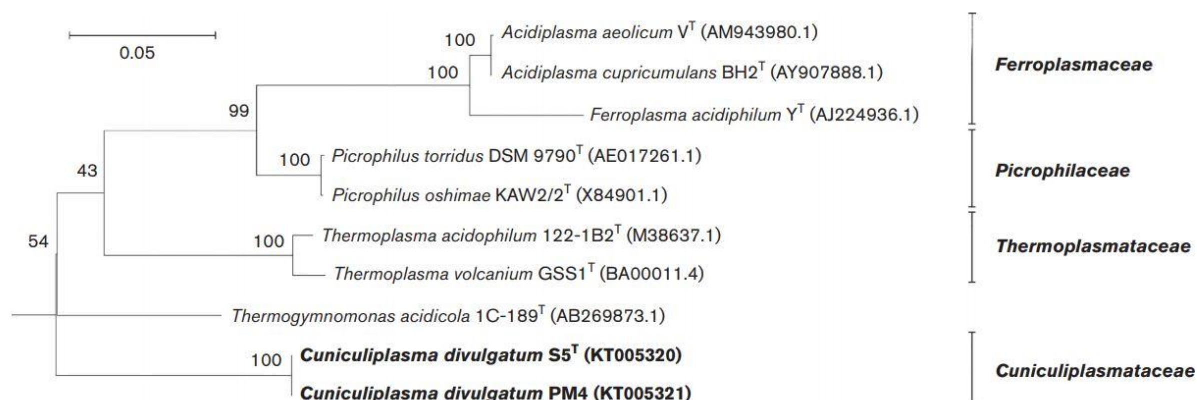

Figure 1.1: Phylogeny of the order *Thermoplasmatales*. Image from Golyshina *et al*. (2016b). All described species are shown, with both strains of *C. divulgatum*. Phylogenetic placement of each species is determined from 16S rRNA sequence identity levels. Accession numbers of strains are shown in brackets, and family names are shown to the right.

1.3 Adaptations for Acidophilic Life

In order to thrive in low pH environments, acidophilic archaea possess specialised cell membranes. These are composed of tetraether lipid monolayers, whereby isoprenoid molecules are linked by ether bonds (Baker-Austin & Dopson, 2007; Van De Vossenberg *et al.*, 1998). This provides an increased resistance to acid hydrolysis compared to the ester bonds in phospholipid bilayer membranes of bacterial and eukaryal cells (Golyshina & Timmis, 2005; Salwan & Sharma, 2020), and limits archaeal cell membrane lysis at low pH ranges. Proton influx into cells is restricted by the establishment of a Donnan potential (Baker-Austin & Dopson, 2007), with a large number of K^+ ion pumps causing the rate of K^+ ion influx to exceed that of proton efflux (Fütterer *et al.*, 2004). This results in a chemiosmotic gradient being generated across the membrane (Baker-Austin & Dopson, 2007; Crossman *et al.*, 2004). Active transport of excess protons out of cells also contributes to maintaining this chemiosmotic gradient (Baker-Austin & Dopson, 2007; Michels & Bakker, 1985).

If excessive proton influx does occur, the cytoplasm of acidophilic archaeal cells is able to act as a buffer for protons (Baker-Austin & Dopson, 2007), as various amino acids present in the cytoplasm can take up and release protons according to the internal pH of the organism (Zichlinsky & Matin, 1983). Acidophilic archaea additionally possess a high number of genes responsible for chaperone protein production compared to non-acidophilic microorganisms (Angelov & Liebl, 2006): in the event that DNA becomes damaged from exposure to low pH levels, which repair denatured proteins and damaged DNA (Baker-Austin & Dopson, 2007; Crossman *et al.*, 2004; Fütterer *et al.*, 2004; Singhal *et al.*, 2020).

1.4 Acid Mine Drainage Sites

Recent research regarding acidophilic archaea has included Acid Mine Drainage (AMD) sites. These systems are formed when abandoned mines fill with water, allowing exposed sulfidic minerals (e.g. pyrite) to undergo oxidative dissolution, forming acidic water bodies rich in metal ions (Johnson & Hallberg, 2003). These waters may flow from mines in streams, thereby forming an AMD site (Méndez-García *et al.*, 2015). In most cases, the acidification process of AMD waters is catalysed by microbial activity (Baker & Banfield, 2003; Johnson & Hallberg, 2003; Skousen *et al.*, 2019).

Some of the best studied AMD sites include Richmond Mine in Iron Mountain (California, USA); the Tinto River (Spain); the Drei Kronen und Ehrt pyrite mine (Germany); Cae Coch and Mynydd Parys (Parys Mountain), both in North Wales (UK). Iron Mountain was mined for copper, gold, silver, zinc and pyrite until 1963 (Druschel *et al.*, 2004; Johnson, 2012), after which microbial activity caused the

pH of subterranean waters to drop to -3.6 (Nordstrom *et al.*, 2000). The microbial diversity was found to be dominated by *Ferroplasma*, *Leptospirillum* and *Thiobacillus* species (Edwards *et al.*, 2000; Méndez-García *et al.*, 2015), generally with *Leptospirillum* spp. responsible for nitrogen fixation and *Ferroplasma* spp. responsible for iron oxidation (Johnson, 2012). The Tinto River in southwest Spain has a high iron and sulfate content due to the oxidation of pyrite, and an average pH of 2.3 (Sanchez-Andrea *et al.*, 2011). As such it contains an interesting microbial ecosystem with members of the order *Thermoplasmatales* represented alongside the main bacterial phyla *Acidobacteria, Actinobacteria*, *Bacterioidetes, Firmicutes* and *Proteobacteria* (*ibid*.). The Drei Kronen und Ehrt pyrite mine is partially flooded, with oxygen content and pH varying between three distinct areas within the mine (Ziegler *et al.*, 2013). It contains acidic biofilms (pH range of 2.2 – 2.6) dominated by iron oxidising bacteria with some presence of uncultured *Thermoplasmatales* related organisms (Ziegler *et al.*, 2009; 2013). Cae Coch is a sulfur mine through which rainwater drains, collecting a high content of iron and sulfur in solution (Johnson, 2012). There is variation between microbial communities inhabiting different regions of the mine (Johnson, 2012; Kimura *et al.*, 2011) with droplets on the ceiling containing autotrophic microorganisms, dominated by iron and sulfur oxidising bacteria, and heterotrophic archaea dominating acidic pools within the mine (Kimura *et al.*, 2011).

<u>1.5 ARMAN Related Organisms</u>

Archaeal Richmond Mine Acidophilic Nano-organisms (ARMAN) were first detected in Richmond Mine (Baker *et al.*, 2006), and have since been detected in many other acidic environments (Golyshina *et al.*, 2019b). They are representative of microbial "dark matter" – this constitutes the uncultivated majority of microorganisms (Golyshina *et al*., 2019b; Rinke *et al.*, 2013) that are known to exist through the use of metagenomic studies, but whose diversity has not yet been explored with cultivation attempts. The taxonomic status of ARMAN related organisms remains to be fully determined but two candidate phyla were proposed within the group: "*Candidatus* Micrarchaeota" and "*Ca.* Parvarchaeota" (Baker *et al.*, 2006). Both candidate phyla are included in the proposed DPANN superphylum (an acronym corresponding to the first five phyla included therein: Diapherotrites; Parvarchaeota; Aenigmarchaeota; Nanoarchaeota and Nanohaloarchaeota) (Dombrowski *et al*., 2019; Rinke *et al.*, 2013), whose validation through cultivation is currently represented only by *Nanoarchaeota* (Huber *et al.*, 2002). Their presence has been detected in several AMD sites (Baker *et al.*, 2006; Chen *et al.*, 2018; Golyshina *et al.*, 2017a; Krause *et al.*, 2017; Ziegler *et al.*, 2013) and some cultivation experiments have revealed an association with *Thermoplasmatales* members (Golyshina *et al.*, 2017a; Krause *et al.*, 2017).

1.6 Parys Mountain

Parys Mountain is an abandoned deep copper mine located on the island of Anglesey, North Wales (UK). It was operational from the Bronze Age until 1950, and was the largest copper mine in Europe in the late 18th century (Vernon, 1996). Upon its closure, drainage valves were blocked and the mine flooded forming a subterranean lake, which became acidic due to the oxidative dissolution of pyrite and other sulfidic minerals (Johnson, 2012). This water body was retained by a concrete dam, and concerns grew regarding its structural integrity, as the nearby town of Amlwch may have been flooded with acidic water, should the dam have failed. This led to the partial draining of the mine in order to relieve stress on the dam (Coupland & Johnson, 2004), which allowed the microbial diversity of Parys Mountain to be studied in detail.

C. divulgatum was isolated and described from the Parys Mountain AMD system, with its genome subsequently analysed (Golyshina *et al.*, 2016a; 2016b). It represents the successful cultivation of the *Thermoplasmatales* dubbed "G-Plasma", members of a group of uncultured *Thermoplasmatales* called Alphabet Plasmas. This group was first detected in Richmond Mine (California, USA) by Baker & Banfield (2003) and were given the designation of "A-Plasma" through to "I-Plasma". Golyshina *et al.* (2017a) found that *C. divulgatum* interacts with ARMAN related organisms and they depend on each other for survival, as it was suggested that there could be a significant exchange of genetic material and metabolites between these organisms in the Parys Mountain AMD system (Golyshina *et al.*, 2019a).

The archaeal diversity of the Parys Mountain AMD site was previously investigated with the construction of a 16S rRNA library, and clades of uncultured *Thermoplasmatales* were detected in sediment samples (Tyler, 2018, Undergraduate Dissertation). It is likely that the uncultured Alphabet Plasma "E-Plasma" was represented by one of these clades, as it is the dominating organism in the Parys Mountain AMD ecosystem, comprising 43.5 % of the total microbial abundance in sediment samples (Korzhenkov *et al.*, 2019) (Figure 1.2).

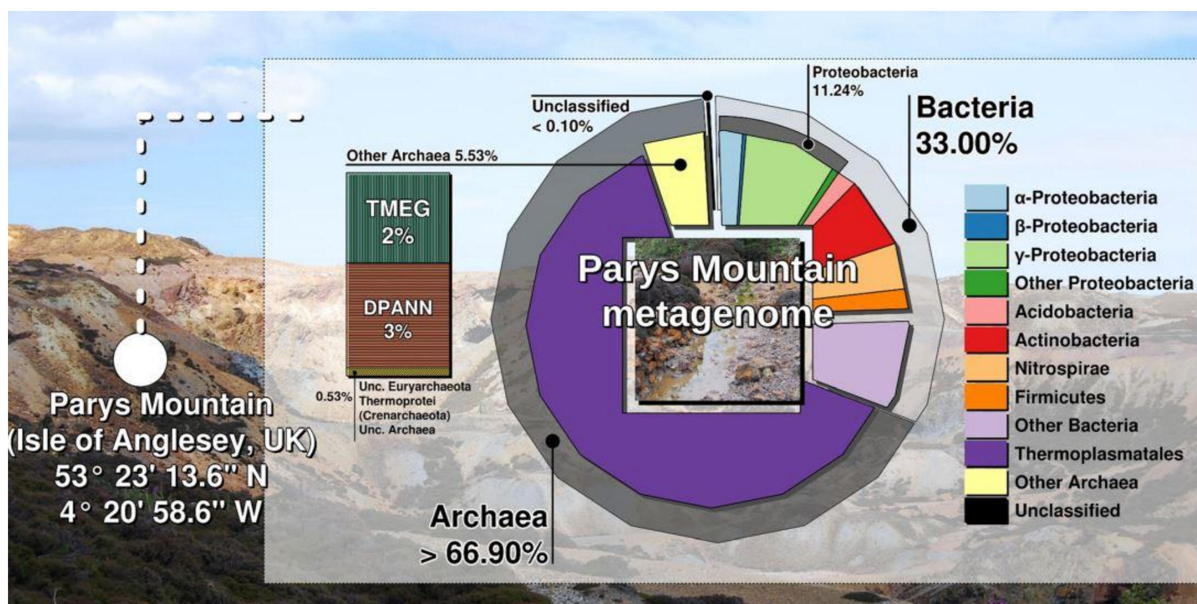

Figure 1.2: Microbial Composition of the Parys Mountain AMD Site. Image from Korzhenkov *et al*. (2019). The polyhedron represents microbial community composition according to metagenomic sequencing data, with colours representing taxonomic classification. The AMD system includes mainly archaea of the order *Thermoplasmatales*, of which 43.5% comprises the uncultured Alphabet Plasma "E-Plasma".

1.7 Uncultured *Thermoplasmatales*

Current knowledge of archaeal diversity is lacking, owing to the fact that archaea are generally under-sampled and understudied, and the majority of archaea are uncultured (Rinke *et al.*, 2013) due to their difficulty of isolation. Ziegler *et al*. (2013) detected several uncultured *Thermoplasmatales* through the enrichment and subsequent sequencing of 16S rRNA genes from environmental samples of biofilms within the Drei Kronen und Ehrt mine (Germany). Their presence was limited to anoxic areas of the biofilm, and it was hypothesized that they had a role in the carbon cycle of this ecosystem. Justice *et al.* (2012) additionally observed that some uncultured *Thermoplasmatales* clones together with ARMAN related organisms were likely to be largely involved with carbon cycling in similar anaerobic, acidic biofilms. Uncultured *Thermoplasmatales* may therefore have some unidentified involvement in biogeochemical cycling within their ecosystems.

The Alphabet Plasma group includes a number of uncultured genera of *Thermoplasmatales*. Predictions about the physiology and metabolism of these organisms were made by Yelton *et al.* (2013) based on metagenomic data. For "G-Plasma", they predicted the presence of an S-layer cell wall, iron oxidation, and methylotrophy. However as "G-Plasma" was later isolated and described by Golyshina *et al.* (2016b) as *C. divulgatum*, these predictions were found to be incorrect. Through

cultivation it is possible to examine the physiological characteristics and ecological roles of an organism; therefore it is necessary to test metagenomic predictions through cultivation techniques to gather a comprehensive understanding of the organisms.

1.8 Research Objectives

There is a large diversity of uncultured archaea in the Parys Mountain AMD ecosystem, with the most abundant organism being "E-Plasma" (Korzhenkov *et al.*, 2019). This represents a gap in current scientific knowledge, and the isolation and cultivation of this organism will allow physiological predictions previously made through metagenomic analysis (Yelton *et al.*, 2013) to be checked. The study of this organism will additionally contribute to understanding the biology, taxonomy, physiology and molecular adaptations of the order *Thermoplasmatales*. This is important as the order is represented in acidic environments globally (Golyshina *et al.*, 2019a; Méndez-García *et al.*, 2015) and their role in acidic ecosystems may be further studied through cultivation. This may provide an insight into the archaeal involvement in cycling of carbon, iron or sulfur in these environments. This order also represents the most acidophilic prokaryotes to have been currently described (Angelov & Liebl, 2006; Bertoldo *et al.*, 2004; Quatrini & Johnson, 2018; Schleper *et al.*, 1995; 1996) and studying their biology is important for understanding the limits of life on our planet. This also represents an investigation into the diversity of microbial "dark matter", which is important as the extent of current scientific knowledge here is lacking (Baker *et al.*, 2020; Rappé & Giovannoni, 2003; Rinke et al 2013). Additionally, since the Parys Mountain acidic stream drains into the Irish Sea, it is important to study the dominant organism of this ecosystem, as understanding the processes involved with the pollution of this environment (Jarvis & Mayes, 2012; Mayes *et al.*, 2009) is key to solving this problem.

It is hypothesised in the present work that it is possible to isolate archaea known to be present in the Parys Mountain AMD ecosystem by varying cultivation conditions. It is the first aim of the present work to cultivate and isolate the uncultured *Thermoplasmatales* "E-Plasma" and to evaluate any problems associated with this process. A second aim of this project is to analyse both the archaeal diversity of the Parys Mountain AMD ecosystem, and more generally that of microbial "dark matter". Details of interactions between organisms present in this ecosystem will provide additional information about their ecology, physiology and metabolism, and provide information regarding some elemental cycling occurring in the ecosystem. Microbial community interactions in AMD systems may also be further investigated in this way, possibly providing information about their roles in the pollution originating from these sites.

2. Materials and Methods

All experimentation was conducted using laboratory facilities at the Environment Centre Wales (ECW) and the Centre for Environmental Biotechnology (CEB) in Bangor, Gwynedd from 24/10/18 until 19/9/19.

2.1 Sample Preparation

2.1.1 Collection of Samples

Samples were collected on 30/10/18 from an acidic stream located on Parys Mountain (Mynydd Parys), Anglesey, UK (GPS location 53.38708, -4.34968) (Figure 2.1). Water saturated sediment samples with algae were taken at a depth of 3 cm, with a temperature of 15 °C, a pH of 1.8 and an Eh of 440 mV.

Figure 2.1: AMD Site at Parys Mountain. The sample site located on Parys Mountain (Mynydd Parys), Anglesey, UK (Geographical co-ordinates 53°23'13.6"N 4°20'58.6"W). The photograph was taken during the sample collection (30/10/18) and the vegetation surrounding the stream is heather.

2.1.2 Preparation of Media and Substrates

A literature search was conducted to determine the appropriate growth media and polypeptide substrates for enrichment of samples. These were chosen according to their usage in other reports of *Thermoplasmatales* cultivation experiments and their availability from suppliers (see Table 3.1).

Two 1 L solutions of *Sulfolobus* Medium (DSMZ M88) were prepared (Table 2.1). One medium was adjusted to pH 1.7, and the other to pH 1.15, with the addition of concentrated H_2SO_4.

Table 2.1: Composition of DSMZ M88 *Sulfolobus* Medium (*https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium88.pdf*).

Component	Amount
$(NH_4)_2SO_4$	1.30 g
KH_2PO_4	0.28 g
$MgSO_4 x 7H_2O$	0.25 g
$CaCl_2 x 2H_2O$	0.07 g
$FeCl_3 x 6H_2O$	0.02 g
Distilled water	1000.00 ml*

* made up to 1 L total volume

Five 100 ml solutions of various polypeptide substrates at 10 %w/v concentration were prepared with distilled water. These were BD Bacto™ Yeast Extract (Catalogue no. 212750); BD Bacto™ Yeast Extract with BD Bacto™ Tryptone (Catalogue no. 211705) in a 1:1 ratio; BD BBL™ Yeast Extract (Catalogue no. 211931); BD Difco™ Yeast Extract (Catalogue no. 210929); and BD BBL™ Beef Extract (Catalogue no. 212303). Substrate solutions were then autoclaved for one hour.

2.2 Assessment of Cultures

Five enrichment cultures were initially established, each containing a different substrate. Cultures each contained 10 ml M88 medium (pH 1.7), 10 µl SL10 trace elements (Table 2.2), 100 µl Sigma-Aldrich Kao and Michayluk vitamin solution (Catalogue no. K3129) (Table 2.3), and 100 µl of each respective substrate. Each culture was inoculated with 0.5 g sample material and incubated at 37.5 °C under microaerophilic conditions for three weeks, after which microscopic observations were taken.

Table 2.2: Composition of SL10 Trace Elements.
(*https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium320.pdf*).

Component	Amount
HCl (25 %v/v; 7.7 M)	10.00 ml
$FeCl_2 \times 4 H_2O$	1.50 g
$ZnCl_2$	70.00 mg
$MnCl_2 \times 4 H_2O$	100.00 mg
H_3BO_3	6.00 mg
$CoCl_2 \times 6 H_2O$	190.00 mg
$CuCl_2 \times 2 H_2O$	2.00 mg
$NiCl_2 \times 6 H_2O$	24.00 mg
$Na_2MoO_4 \times 2 H_2O$	36.00 mg
Distilled water	990.00 ml

Table 2.3: Composition of Sigma-Aldrich Kao and Michayluk Vitamin Solution.
(*https://www.sigmaaldrich.com/technical-documents/protocols/biology/vitamin-mixtures.html*).

Component	Amount (mg/L)
p-Aminobenzoic acid	0.02
L-Ascorbic acid	2.00
Biotin	0.01
D-Calcium pantothenate	1.00
Choline chloride	1.00
Folic acid	0.40
myo-Inositol	100.00
Nicotinamide (Niacinamide)	1.00
Pyridoxine • HCl	1.00
Riboflavin	0.20
Thiamine • HCl	1.00
Vitamin A (Retinol)	0.01
Vitamin B12	0.02
Vitamin D (Cholecalciferol)	0.01

Enrichment cultures were transferred using the treatments shown in Table 2.4. Compositions of additional supplements are shown in Tables 2.5 and 2.6. Three treatments were used for each of the five initial enrichment cultures. Cultures were transferred to new media three times, incubated at 37.5 °C under microaerophilic conditions and monitored with microscopic observation. After two months DNA was isolated from cells for use in DNA barcoding. Cultures were subsequently transferred to fresh media once each month for the next three months.

Table 2.4: Conditions of First Enrichment Culture Treatments shown per 10 ml of media, with an aliquot from each culture used to inoculate new media upon transferral.

Culture treatment 1		**Culture treatment 2**		**Culture treatment 3**	
Component	*Volume*	*Component*	*Volume*	*Component*	*Volume*
M88 pH 1.7	10 ml	M88 pH 1.15	10 ml	M88 pH 1.15	10 ml
SL10	10 µl	SL10	10 µl	SL10	10 µl
Vitamins*	100 µl	Vitamins*	100 µl	Vitamins*	100 µl
Substrate	100 µl	Substrate	100 µl	Trace Elements for *T. acidophilum*	10 µl
Inoculate	2.5 ml	Inoculate	2.5 ml	WME SS	10 µl
				Cysteine	20 µl
				SyntheChol	5 µl
				Substrate	100 µl
				Inoculate	2.5 ml

* “Vitamins” refers to Kao and Michayluk vitamin solution (Table 2.3).

Table 2.5: Composition of Trace Elements for *T. acidophilum* (*https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium158.pdf*).

Component	**Amount**
$FeCl_3$ x 6 H_2O	1.93 g
$MnCl_2$ x 4 H_2O	0.18 g
$Na_2B_4O_7$ x 10 H_2O	0.45 g
$ZnSO_4$ x 7 H_2O	22.00 mg
$CuCl_2$ x 2 H_2O	5.00 mg
Na_2MoO_4 x 2 H_2O	3.00 mg
$VOSO_4$ x 5 H_2O	3.80 mg
$CoSO_4$ x 7 H_2O	2.00 mg
Distilled water	1000.00 ml*

* made up to 1 L total volume

Table 2.6: Composition of WME SS trace elements
(*https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium141.pdf*).

Component	Amount
Nitrilotriacetic acid ($C_6H_9NO_6$)	1.50 g
$MgSO_4$ x 7 H_2O	3.00 g
$MnSO_4$ x H_2O	0.50 g
NaCl	1.00 g
$FeSO_4$ x 7 H_2O	0.10 g
$CoSO_4$ x 7 H_2O	0.18 g
$CaCl_2$ x 2 H_2O	0.10 g
$ZnSO_4$ x 7 H_2O	0.18 g
$CuSO_4$ x 5 H_2O	0.01 g
$KAl(SO_4)_2$ x 12 H_2O	0.02 g
H_3BO_3	0.01 g
Na_2MoO_4 x 2 H_2O	0.01 g
$NiCl_2$ x 6 H_2O	0.03 g
Na_2SeO_3 x 5 H_2O	0.30 mg
Na_2WO_4 x 2 H_2O	0.40 mg
Distilled water	1000.00 ml*

* made up to 1 L total volume

2.2.1 DNA Isolation

Following the incubation of the third enrichment culture transfers, biomass was collected by centrifuging 20 ml from each culture at 9600 rpm for 15 min. DNA was then isolated from cells using the Zymo Research ZymoBiomics™ DNA Miniprep Kit (Catalogue no. R2002) according to the manufacturer protocol. Vortex mixing time was increased to 10 min for more effective cell lysis, the amount of water used for DNA elution was decreased to 20 µl to obtain more concentrated samples, and the final purification step was omitted as it was not necessary. Isolated DNA was stored at 4 °C.

The success of DNA isolation was checked using gel electrophoresis. 0.8 %w/v agarose gel was prepared using 0.4 g agarose, 50 ml 1X TBE buffer (per 10 L distilled water: 109 g tris-acetate, 55 g boric acid, 7.44 g Na_2EDTA) and 7.5 µl Sigma-Aldrich™ Safe View (CAS number 99643-38-6, formula

$C_{21}H_{28}N_4$). 2.5 µl DNA samples were mixed with 0.5 µl loading buffer and loaded onto the gel. 3 µl Invitrogen™ 1kb+ DNA ladder (Catalogue no. 10787018) was also included (Figure 2.2). The gel was electrophoresed at 100 V for 20 min and visualised with UV light using the Bio-Rad GelDoc™ system. The concentration of isolated DNA samples was subsequently measured using the Qubit™ dsDNA BR Assay Kit (Catalogue No. Q32853).

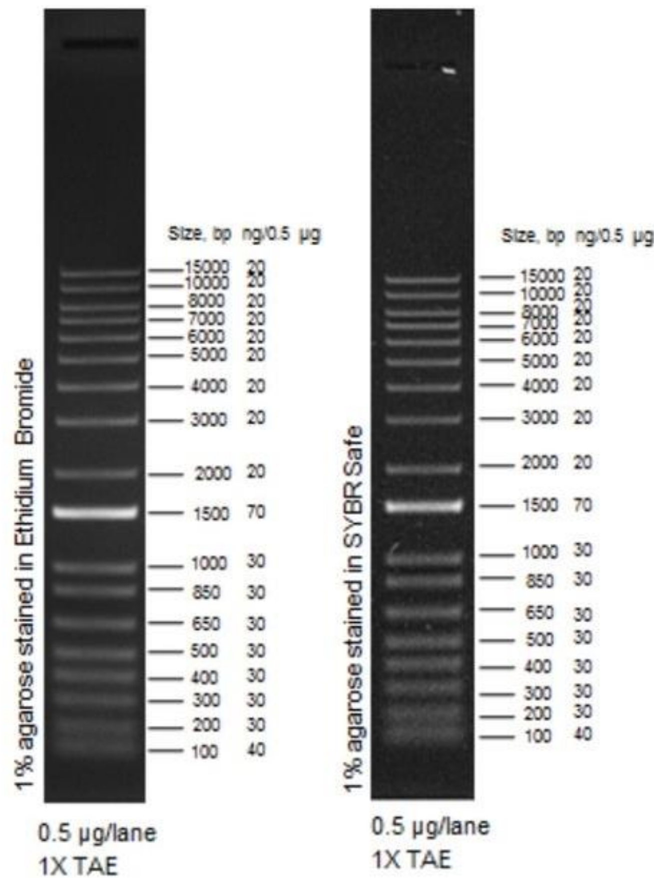

Figure 2.2: DNA Ladder DNA fragment length markers of the Invitrogen™ 1kb+ DNA ladder (Catalogue no. 10787018). Image from *https://www.thermofisher.com/order/catalog/product/10787018*.

2.2.2 PCR Amplification of DNA

PCR was conducted on DNA using specific primers in different combinations corresponding to the V4 variable region of the 16S rRNA genes. This process was repeated to obtain duplicate results for each DNA sample. The forward primer (F10) was the same for each primer pair, with the sequence ACAGCCACCCAT CGA ACTCCTACGGGAGGCAGCAG (Fadrosh *et al.*, 2014). Reverse primer sequences are shown in Table 2.7. Primers additionally included an Illumina Linker sequence, which was the same

for each forward primer (CAAGCAGAAGACGGCATACGAGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT) and reverse primer (AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT) (*ibid.*).

PCR reactions were prepared for each sample, using 1 µl F10 primer, 1 µl R1 – R15 primer (each at 10 µM concentration) (Table 2.7), 1 µl DNA isolated from cultures, 5 µl Bioline MyTaq™ Red Reaction Buffer (Catalogue no. BIO-37112), 0.25 µl Bioline MyTaq™ Red DNA Polymerase and 16.75 µl H_2O.

Table 2.7: Primer Combinations for First Barcoding showing primers used in PCR amplification of 16S rRNA fragments from DNA isolated from cultures.

Tube No.	DNA Sample*	F Primer	R Primer	R primer sequence†
1	Bacto YE 1	F10	R1	CCTAAACTACGG GGACTACHVGGGTWTCTAAT
2	Bacto YE 2	F10	R2	TGCAGATCCAAC GGACTACHVGGGTWTCTAAT
3	Bacto YE 3	F10	R3	CCATCACATAGG GGACTACHVGGGTWTCTAAT
4	Bacto YE + TR 1	F10	R4	GTGGTATGGGAG A GGACTACHVGGGTWTCTAAT
5	Bacto YE + TR 2	F10	R5	ACTTTAAGGGTG A GGACTACHVGGGTWTCTAAT
6	Bacto YE + TR 3	F10	R6	GAGCAACATCCT A GGACTACHVGGGTWTCTAAT
7	BBL YE 1	F10	R7	TGTTGCGTTTCT TC GGACTACHVGGGTWTCTAAT
8	BBL YE 2	F10	R8	ATGTCCGACCAA TC GGACTACHVGGGTWTCTAAT
9	BBL YE 3	F10	R9	AGGTACGCAATT TC GGACTACHVGGGTWTCTAAT
10	Difco YE 1	F10	R10	ACAGCCACCCAT CTA GGACTACHVGGGTWTCTAAT
11	Difco YE 2	F10	R11	TGTCTCGCAAGC CTA GGACTACHVGGGTWTCTAAT
12	Difco YE 3	F10	R12	GAGGAGTAAAGC CTA GGACTACHVGGGTWTCTAAT
13	BBL BE 1	F10	R13	GTTACGTGGTTG GATA GGACTACHVGGGTWTCTAAT
14	BBL BE 2	F10	R14	TACCGCCTCGGA GATA GGACTACHVGGGTWTCTAAT
15	BBL BE 3	F10	R15	CGTAAGATGCCT GATA GGACTACHVGGGTWTCTAAT

* “YE” = Yeast Extract, “TR” = Tryptone and “BE” = Beef Extract.

† Sequences are shown excluding the Illumina Linker sequence region of primers and H, V and W are ambiguous primer bases (Fadrosh et al., 2014).

PCR was then run on the samples. The thermal cycling conditions were 95 °C for 2 min (initial denaturation) followed by 30 cycles at 94 °C for 40 s (denaturation); 54 °C for 30 s (annealing); 72 °C for 30 s (extension) and a final extension step at 72 °C for 5 min.

The success of PCR was checked with the electrophoresis of PCR products on agarose gel using the same method as previously described, with the exception of loading 2 µl samples into wells, and omitting the loading buffer as this was already present in PCR product samples.

PCR products were purified using gel electrophoresis. 1.2 %w/v agarose gel was prepared using 50 ml TBE buffer, 0.6 g agarose and 7.5 µl Sigma™ Safe View. 10 µl samples of each PCR product were combined into pools according to DNA band size and electrophoresed at 90 V for 40 min. 3 µl Kb1^{+} DNA ladder was also included (Figure 2.2).

DNA bands were extracted from the gel using the Qiagen Qiaex® II Gel Extraction Kit (Catalogue No. 20051), following the manufacturer protocol. The DNA concentration of purified PCR products was then measured with the Qubit™ dsDNA BR Assay Kit (Catalogue No. Q32853).

2.2.3 DNA Barcoding Analysis

The Illumina MiSeq system was used for sequencing barcoded PCR amplicons, following the manufacturer protocol. This procedure was done by Marco Distaso. Sequences were subsequently assembled by Evgenii Lunev and Rafael Bargiela, who additionally determined the taxonomy of bacterial sequences from the DNA barcoding procedure using the Green Genes database (*https://greengenes.secondgenome.com/*) based on 16S rRNA sequence identity levels. The affiliation of archaeal sequences was manually determined with the National Centre for Biotechnology Information (NCBI) BLASTn search tool (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*). Archaeal sequences were aligned with the 16S rRNA sequences of members of the order *Thermoplasmatales*, retrieved from the NCBI database, using ClustalW (Thompson *et al.*, 1994). A rooted phylogenetic tree was constructed using MEGA 7 (Kumar *et al.*, 2016), using the methanogen *Methanosarcina barkeri* for outgroup comparison. Independently barcoded duplicate repeats of each dataset were merged to provide the mean abundance of taxa present. R Studio 3.6.0 was used for further biodiversity analysis of sequence data (http://www.rstudio.com/) using the R programming language (https://www.r-project.org/). The statistical analysis was conducted using the Phyloseq package (McMurdie & Holmes, 2013) and figures were produced using the ggplot 2 package (Wickham, 2016).

2.3 Cultivation of Archaea

Further cultivation steps were taken to promote the presence of metagenomic variant "E-Plasma" in culture. Two cultures (from Table 2.7, tubes 1 and 7) were each transferred to 50 ml of new media. These were previously grown using Bacto and BBL yeast extracts respectively, and both at pH 1.7 (treatment 1, see Table 2.4). The same conditions were maintained for this transferral step as in prior cultivation.

The growth of these cultures was tested with various media and substrates. 1 L of *Thermoplasma volcanium* medium (DSMZ M398) (Table 2.8) and 1 L of *Ferroplasma acidiphilum* (9K) medium (DSMZ 874) (Table 2.9) were prepared and adjusted to pH 1.7 with the addition of concentrated H_2SO_4.

Table 2.8: Composition of DSMZ M398 *Thermoplasma volcanium* medium (*https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium398.pdf*).

Component	Amount
KH_2PO_4	3.00 g
$MgSO_4x7H_2O$	1.00 g
$CaCl_2x2H_2O$	0.25 g
$(NH_4)_2SO_4$	0.20 g
Distilled water	1000.00 ml

* made up to 1 L total volume

Table 2.9: Composition of DSMZ 874 *Ferroplasma acidiphilum* (9K) medium (*https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium874.pdf*).

Component*	Amount
Solution A:	
$MgSO_4x7H_2O$	0.40 g
$(NH_4)_2SO_4$	0.20 g
KCl	0.10 g
KH_2PO_4	0.10 g
Distilled water	950.00 ml
Solution B:	
$FeSO_4x7H_2O$	25.00 g
10 N H_2SO_4	5 ml
Distilled water	45 ml

* Solutions A and B were prepared and autoclaved separately before being combined.

After one week, cultures were filtered using Millex-GS™ 0.22 µm syringe filters with mixed cellulose ester membranes (Catalogue No. SLGS033) to remove bacterial cells. Five variants were prepared for both cultures (see Table 2.10), each inoculated with 2 ml of the corresponding filtrate. Three variants were prepared with 10 ml M88 medium, one with 10 ml 9K medium, and one with M398 medium. 1 g Sigma-Aldrich pyrite (FeS_2) (Catalogue no. 12068-85-8) was added to one of the M88 variants, and 1 g Sigma-Aldrich sulfur (S_8) (Catalogue no. 7704-34-9) to another. Each variant additionally contained 100 µl Kao and Michayluk vitamin solution, 100 µl substrate (Bacto or BBL yeast extract) and 10 µl SL10 trace elements. Cultures were then incubated at 37.5 °C under microaerophilic conditions.

Table 2.10: Conditions of Second Enrichment Culture Variants shown per 10 ml of media, with an aliquot from each culture used to inoculate new media upon transferral.

9K variant		**M88 variant**		**M398 variant**		**Pyrite variant**		**Sulfur variant**	
Component	*Volume*	*Component*	*Volume*	*Component*	*Volume*	*Component*	*Volume*	*Component*	*Volume*
9K	10 ml	M88	10 ml	M398	10 ml	M88	10 ml	M88	10 ml
SL10	10 µl	SL10	10 µl	SL10	10 µl	SL10	10 µl	SL10	10 µl
Vitamins*	100 µl	Vitamins*	100 µl	Vitamins*	100 µl	Vitamins*	100 µl	Vitamins*	100 µl
Substrate	100 µl	Substrate	100 µl	Substrate	100 µl	Substrate	100 µl	Substrate	100 µl
Inoculate	2 ml	Inoculate	2 ml	Inoculate	2 ml	Inoculate	2 ml	Inoculate	2 ml
						Pyrite	1 g	Sulfur	1 g

* "Vitamins" refers to Kao and Michayluk vitamin solution (Table 2.3).

Microscopic observations were taken after one week of incubation, and any cultures with a high proportion of visible bacterial cells were filtered again using a 0.22 µm syringe filter. Cultures were then transferred to 25 ml of new media using the same conditions for each variant, inoculated using an aliquot from the corresponding previous culture, and incubated at 37.5 °C under microaerophilic conditions for one week. Light micrographs were then taken of cultures and DNA was isolated for use in DNA barcoding. Cultures were transferred to 15 ml of new media after five weeks using the same conditions for each variant, and incubated at 37.5 °C under microaerophilic conditions for four weeks.

2.3.1 DNA Isolation

DNA was then isolated from cultures and checked with gel electrophoresis using the same methods as previously described. Several alternative methods were used in an attempt to isolate DNA from some unsuccessful variants, checking each time with gel electrophoresis using the same methods as previously described. Samples of cultures were centrifuged slowly at 500 rpm for 30 s to precipitate

substrate particles prior to DNA isolation, but this was unsuccessful. Samples of cultures were centrifuged at 9000 rpm for 15 min to collect biomass in pellets, which were then each washed in 1.5 ml M88 and boiled at 97 °C for 10 min to lyse cells. PCR was then conducted directly from biomass, but this was also unsuccessful. Samples of cultures were filtered with a 0.45 µm syringe filter to attempt to remove substrate particles, but no pellet was then visible upon centrifugation. Samples of cultures were boiled to lyse cells and centrifuged to precipitate substrate particles and cell fragments prior to PCR, but this was also unsuccessful. Isolation of DNA from the unsuccessful variants was not deemed possible, so these variants were excluded from further investigations.

2.3.2 PCR Amplification and Barcoding of Successfully Isolated DNA

PCR was conducted on successfully isolated DNA samples using the same methods as previously described. The same forward primer (F10) and reverse primers (R2-R10) were used as previously described, in the combinations shown in Table 2.11.

Table 2.11: Primer Combinations for Second DNA Barcoding (First Duplicate) used in PCR amplification of 16S rRNA fragments from DNA isolated from cultures.

Tube No.*	DNA Sample†	F Primer	R Primer	R primer sequence‡
2	JT1 M88	F10	R2	TGCAGATCCAAC GGACTACHVGGGTWTCTAAT
3	JT1 M398	F10	R3	CCATCACATAGG GGACTACHVGGGTWTCTAAT
5	JT1 Sulfur	F10	R5	ACTTTAAGGGTG A GGACTACHVGGGTWTCTAAT
7	JT7 M88	F10	R7	TGTTGCGTTTCT TC GGACTACHVGGGTWTCTAAT
8	JT7 M398	F10	R8	ATGTCCGACCAA TC GGACTACHVGGGTWTCTAAT
10	JT7 Sulfur	F10	R10	ACAGCCACCCAT CTA GGACTACHVGGGTWTCTAAT

* Tubes 1, 4, 6 and 9 contained unsuccessful variants, so are omitted from this Table.

† "JT1" variants were inoculated using the culture Bacto YE 1 and "JT7" variants were inoculated using the culture BBL YE 1 from previous experiments (see Table 2.7).

‡ Sequences are shown excluding the Illumina Linker sequence region of primers. H, V and W are ambiguous primer bases (Fadrosh *et al.*, 2014).

This process was repeated to obtain duplicate results using the forward primer F11 (sequence TGTCTCGCAAGC CGA GTGBCAGCMGCCGCGGTAA) (Fadrosh *et al.*, 2014) in combination with the reverse primers shown in Table 2.12 (R16-21). Primers additionally included the same Illumina Linker sequences as previously described, and was the same for each forward and reverse primer (*ibid.*).

Table 2.12: Primer Combinations for Second DNA Barcoding (Second Duplicate) used in PCR amplification of 16S rRNA fragments from DNA isolated from cultures.

Tube No.*	DNA Sample†	F Primer	R Primer	R primer sequence‡
1	JT1 M88	F11	R16	TACCGGCTTGCA ACTCA GGACTACHVGGGTWTCTAAT
2	JT1 M398	F11	R17	ATCTAGTGGCAA ACTCA GGACTACHVGGGTWTCTAAT
3	JT1 Sulfur	F11	R18	CCAGGGACTTCT ACTCA GGACTACHVGGGTWTCTAAT
4	JT7 M88	F11	R19	CACCTTACCTTA TTCTCT GGACTACHVGGGTWTCTAAT
5	JT7 M398	F11	R20	ATAGTTAGGGCT TTCTCT GGACTACHVGGGTWTCTAAT
6	JT7 Sulfur	F11	R21	GCACTTCATTTC TTCTCT GGACTACHVGGGTWTCTAAT

* Tubes 1, 4, 6 and 9 contained unsuccessful variants, so are omitted from this Table.

† “JT1” variants were inoculated using the culture Bacto YE 1 and “JT7” variants were inoculated using the culture BBL YE 1 from previous experiments (see Table 2.7).

‡ Sequences are shown excluding the Illumina Linker sequence region of primers. H, V and W are ambiguous primer bases (Fadrosh *et al.*, 2014).

PCR was then run on the samples. The thermal cycling conditions were 95 °C for 2 min (initial denaturation) followed by 30 cycles at 94 °C for 45 s (denaturation); 50 °C for 1 min (annealing); 72 °C for 30 s (extension) and a final extension step at 72 °C for 5 min.

The same methods as previously described were then used to check the success of PCR with gel electrophoresis, purify PCR products, and run DNA barcoding on samples.

2.4 Cultivation of ARMAN Related Organisms

Further cultivation steps were taken to promote the presence of ARMAN related organisms in culture. Five carbohydrate and protein supplements were prepared to test if growth of cultures could be enhanced. 10 %w/v solutions of Sigma-Aldrich Pullulan (Catalogue no. 9057-02-7) (formula $(C_6H_{12}O_5)_n$); Trehalose (Catalogue no. 585-91-1) (formula $C_{12}H_{22}O_{11}$); Galactan (Catalogue no. 9000-40-2) (formula $(C_6H_{10}O_5)_n$); Collagen (Catalogue no. C4243) and 1,6-Anhydro-β-D-Glucose (Catalogue no. 498-07-7) (formula $C_6H_{10}O_5$) were each prepared. These were used to establish six variants in parallel (shown in Table 2.13), inoculated with the culture “JT1 M398” from Table 2.11. Each variant contained 5 ml M398 medium, 50 µl Bacto yeast extract, 50 µl Kao and Michayluk vitamin solution, 5 µl SL10 and 1 ml inoculate. Five variants each additionally contained 50 µl of a carbohydrate or protein

supplement, and one was kept as a control variant. The six cultures from previous archaeal cultivation experiments (see Table 2.11) were also transferred to 20 ml new media using the same conditions as previously described (Table 2.10), with the addition of two parallel variants with sulphur removed.

Table 2.13: Conditions of Third Enrichment Culture Variants including additional carbohydrate and protein supplements, and inoculated with the culture containing the largest proportion of ARMAN organisms (“JT1 M398” from Table 2.11).

Control variant		**Pullulan variant**		**Trehalose variant**	
Component	*Volume*	*Component*	*Volume*	*Component*	*Volume*
M398 Medium	5 ml	M398 Medium	5 ml	M398 Medium	5 ml
SL10	5 µl	SL10	5 µl	SL10	5 µl
Vitamins*	50 µl	Vitamins*	50 µl	Vitamins*	50 µl
Bacto YE†	50 µl	Bacto YE†	50 µl	Bacto YE†	50 µl
Inoculate	1 ml	Pullulan	50 µl	Trehalose	50 µl
		Inoculate	1 ml	Inoculate	1 ml
Galactan variant		**Collagen variant**		**1,6-Anhydro-β-D-Glucose variant**	
Component	*Volume*	*Component*	*Volume*	*Component*	*Volume*
M398 Medium	5 ml	M398 Medium	5 ml	M398 Medium	5 ml
SL10	50 µl	SL10	5 µl	SL10	5 µl
Vitamins*	50 µl	Vitamins*	50 µl	Vitamins*	50 µl
Bacto YE†	50 µl	Bacto YE†	50 µl	Bacto YE†	50 µl
Galactan	50 µl	Collagen	50 µl	1,6-Anhydro-β-D-Glucose	50 µl
Inoculate	1 ml	Inoculate	1 ml	Inoculate	1 ml

* “Vitamins” refers to Kao and Michayluk vitamin solution (Table 2.3)

† “YE” refers to yeast extract.

The new cultures were all incubated at 37.5 °C under microaerophilic conditions for three weeks, after which microscopic observations were taken. Cultures from previous archaeal cultivation experiments (see Table 2.11) were transferred to new media using the same conditions as previously described. A parallel variant was also prepared for each of the cultures containing sulfur, in which the sulfur was removed. After an additional five weeks of incubation, biomass was collected from cultures showing good growth for use in DNA isolation and barcoding. These cultures were then transferred to 15 ml new media using the same conditions as previously described.

2.4.1 DNA Isolation and PCR Amplification

DNA was isolated from cultures showing good growth after eight weeks of cultivation. These were five of the cultures from previous archaeal cultivation experiments, including the sulfur and no sulfur variants as shown in Table 2.14. DNA was isolated using the same methods as previously described, with the addition of washing each pellet of biomass in 1.5 ml M88 media after centrifugation of samples. The success of DNA isolation was checked using gel electrophoresis using the same methods as previously described.

PCR was then conducted on isolated DNA samples using the same methods, thermal cycle programme and component volumes as previously described, but using different primer combinations. This process was repeated to obtain duplicate results for each DNA sample. The forward primer (F2) was the same for each primer pair, with the sequence TGCAGATCCAAC GTGBCAGCMGCCGCGGTAA (Fadrosh *et al.*, 2014). Reverse primer sequences are shown in Table 2.14. Primers additionally each included Illumina Linker sequences, as previously described.

Table 2.14: Primer Combinations for Third DNA Barcoding (First Attempt) used in PCR amplification of 16S rRNA fragments from DNA isolated from cultures.

Tube No.	DNA Sample	F Primer	R Primer	R primer sequence†
1	JT7 M398	F2	R1	CCTAAACTACGG GGACTACHVGGGTWTCTAAT
2	JT1 Sulfur	F2	R2	TGCAGATCCAAC GGACTACHVGGGTWTCTAAT
3	JT1 No Sulfur*	F2	R3	CCATCACATAGG GGACTACHVGGGTWTCTAAT
4	JT7 Sulfur	F2	R4	GTGGTATGGGAG A GGACTACHVGGGTWTCTAAT
5	JT7 No Sulfur*	F2	R5	ACTTTAAGGGTG A GGACTACHVGGGTWTCTAAT

* “No Sulfur” denotes the removal of sulfur from variants four weeks prior to DNA isolation.

† Sequences are shown excluding the Illumina Linker sequence region of primers. H, V and W correspond to ambiguous primer bases (Fadrosh *et al.*, 2014).

2.4.2 PCR Re-Amplification

The success of PCR was checked with gel electrophoresis as previously described, but no DNA bands were visible. PCR products were then re-amplified by repeating PCR on the PCR products, using the same primer combinations as prior (see Table 2.14). PCR re-amplification products were then checked using gel electrophoresis using the same methods as previously described, and this time DNA bands were visible. The same methods as previously described were then used to purify PCR re-amplification

products and run DNA barcoding on samples. However, upon analysis these DNA barcoding results were dubious, so it was deemed necessary to repeat DNA barcoding of these samples.

Cultures were subsequently transferred to new media and incubated for an additional 4 weeks, after which biomass was collected from cultures. DNA was isolated by conducting PCR directly from biomass pellets as an alternative method. 1.5 ml samples from each culture were centrifuged at 12000 rpm for 15 min. The supernatants were then discarded and the process was repeated until pellets were visible. Pellets were then boiled at 95 °C for five min to lyse cells, and PCR was conducted using the prepared biomass samples using the same component volumes and thermal cycle as previously described. The forward primers F15 (sequence CGTAAGATGCCT ATGA GTGBCAGCMGCCGCGGTAA) and F16 (TACCGGCTTGCA TGCGA GTGBCAGCMGCCGCGGTAA) (Fadrosh *et al.*, 2014) were used in combination with the reverse primers shown in Table 2.15. Primers additionally included the same Illumina Linker sequences as previously described, which were the same for each forward and reverse primer (*ibid.*). DNA samples from the previous DNA isolation procedure (Table 2.14) were additionally included to repeat the PCR reaction using different primer combinations. Duplicate repeats were also prepared for each DNA sample, using the primer combinations shown in Table 2.15. Positive and negative controls were additionally included in the PCR procedure.

Table 2.15: Primer Combinations for Third DNA Barcoding (Second Attempt) used in PCR amplification of DNA, with (A) and (B) representing duplicate repeats.

Tube No.	Name*†	DNA Source‡	F Primer	R Primer	R Primer Sequence
1	JT7 M398 (A)	Boiled Pellet	F15	R1	CCTAAACTACGG GGACTACHVGGGTWTCTAAT
2	JT7 M398 (B)	Boiled Pellet	F15	R2	TGCAGATCCAAC GGACTACHVGGGTWTCTAAT
3	JT1 Sulfur (A)	Boiled Pellet	F15	R3	CCATCACATAGG GGACTACHVGGGTWTCTAAT
4	JT1 Sulfur (B)	Boiled Pellet	F15	R4	GTGGTATGGGAGA GGACTACHVGGGTWTCTAAT
5	JT1 No Sulfur (A)	Boiled Pellet	F15	R5	ACTTTAAGGGTGA GGACTACHVGGGTWTCTAAT
6	JT1 No Sulfur (B)	Boiled Pellet	F15	R6	GAGCAACATCCTA GGACTACHVGGGTWTCTAAT
7	JT7 Sulfur (A)	Boiled Pellet	F15	R7	TGTTGCGTTTCTTC GGACTACHVGGGTWTCTAAT
8	JT7 Sulfur (B)	Boiled Pellet	F15	R8	ATGTCCGACCAATC GGACTACHVGGGTWTCTAAT

9	JT7 No Sulfur (A)	Boiled Pellet	F15	R9	AGGTACGCAATTTC GGACTACHVGGGTWTCTAAT
10	JT7 No Sulfur (B)	Boiled Pellet	F15	R10	ACAGCCACCCATCTA GGACTACHVGGGTWTCTAAT
11	JT1 M398 (A)	Boiled Pellet	F15	R11	TGTCTCGCAAGCCTA GGACTACHVGGGTWTCTAAT
12	JT1 M398 (B)	Boiled Pellet	F15	R12	GAGGAGTAAAGCCTA GGACTACHVGGGTWTCTAAT
13	Pullulan (A)	Boiled Pellet	F15	R13	GTTACGTGGTTGGATA GGACTACHVGGGTWTCTAAT
14	Pullulan (B)	Boiled Pellet	F15	R14	TACCGCCTCGGAGATA GGACTACHVGGGTWTCTAAT
15	Trehalose (A)	Boiled Pellet	F15	R15	CGTAAGATGCCTGATA GGACTACHVGGGTWTCTAAT
16	Trehalose (B)	Boiled Pellet	F15	R16	TACCGGCTTGCAACTCA GGACTACHVGGGTWTCTAAT
17	Collagen (A)	Boiled Pellet	F15	R17	ATCTAGTGGCAAACTCA GGACTACHVGGGTWTCTAAT
18	Collagen (B)	Boiled Pellet	F15	R18	CCAGGGACTTCTACTCA GGACTACHVGGGTWTCTAAT
19	Galactan (A)	Boiled Pellet	F15	R19	CACCTTACCTTATTCTCT GGACTACHVGGGTWTCTAAT
20	Galactan (B)	Boiled Pellet	F15	R20	ATAGTTAGGGCTTTCTCT GGACTACHVGGGTWTCTAAT
21	1,6-Anhydro-β-D-Glucose (A)	Boiled Pellet	F15	R21	GCACTTCATTTCTTCTCT GGACTACHVGGGTWTCTAAT
22	1,6-Anhydro-β-D-Glucose (B)	Boiled Pellet	F15	R22	TTAACTGGAAGCCACTTCT GGACTACHVGGGTWTCTAAT
23	JT7 M398 (A)	Isolated DNA	F15	R23	CGCGGTTACTAACACTTCT GGACTACHVGGGTWTCTAAT
24	JT7 M398 (B)	Isolated DNA	F15	R24	GAGACTATATGCCACTTCT GGACTACHVGGGTWTCTAAT
25	JT1 Sulfur (A)	Isolated DNA	F16	R1	CCTAAACTACGG GGACTACHVGGGTWTCTAAT
26	JT1 Sulfur (B)	Isolated DNA	F16	R2	TGCAGATCCAAC GGACTACHVGGGTWTCTAAT

27	JT1 No Sulfur (A)	Isolated DNA	F16	R3	CCATCACATAGG GGACTACHVGGGTWTCTAAT
28	JT1 No Sulfur (B)	Isolated DNA	F16	R4	GTGGTATGGGAGA GGACTACHVGGGTWTCTAAT
29	JT7 Sulfur (A)	Isolated DNA	F16	R5	ACTTTAAGGGTGA GGACTACHVGGGTWTCTAAT
30	JT7 Sulfur (B)	Isolated DNA	F16	R6	GAGCAACATCCTA GGACTACHVGGGTWTCTAAT
31	JT7 No Sulfur (A)	Isolated DNA	F16	R7	TGTTGCGTTTCTTC GGACTACHVGGGTWTCTAAT
32	JT7 No Sulfur (B)	Isolated DNA	F16	R8	ATGTCCGACCAATC GGACTACHVGGGTWTCTAAT

* Culture names list the original culture used for inoculation, followed by the type of variant, and a letter denoting duplicate repeats.

† "Sulfur" and "No Sulfur" variants were cultivated using M88 medium, and additional supplement variants (tube numbers 13 – 22) were inoculated with JT1 and grown using M398 medium.

‡ DNA sources are labelled for each tube: these are boiled biomass pellets collected from cultures; and DNA previously isolated using a DNA isolation kit (see previous description of methods).

The success of PCR was checked using gel electrophoresis with a larger volume of agarose gel (150 ml) separated into three groups, each using the same methods as previously described with the exception of electrophoresing gels at 100 V for 30 min. Upon visualisation with UV light, some DNA bands were visible. The unsuccessful PCR products were re-amplified using the same methods and primer combinations as previously described (Table 2.15). PCR re-amplification products were checked using gel electrophoresis but no DNA bands were observed. Data was therefore not deemed to be recoverable from the unsuccessful samples, so they were not included in further analysis.

Successful PCR products were purified with gel extraction using the same methods as previously described, with the modification of electrophoresing gel at 75 V for 45 min. This was done to improve the precision with which DNA bands could be excised from the gel. DNA barcoding was then conducted using the purified PCR products using the same methods as previously described. The cultivation methodology of the present work is graphically summarised in Figure 2.3.

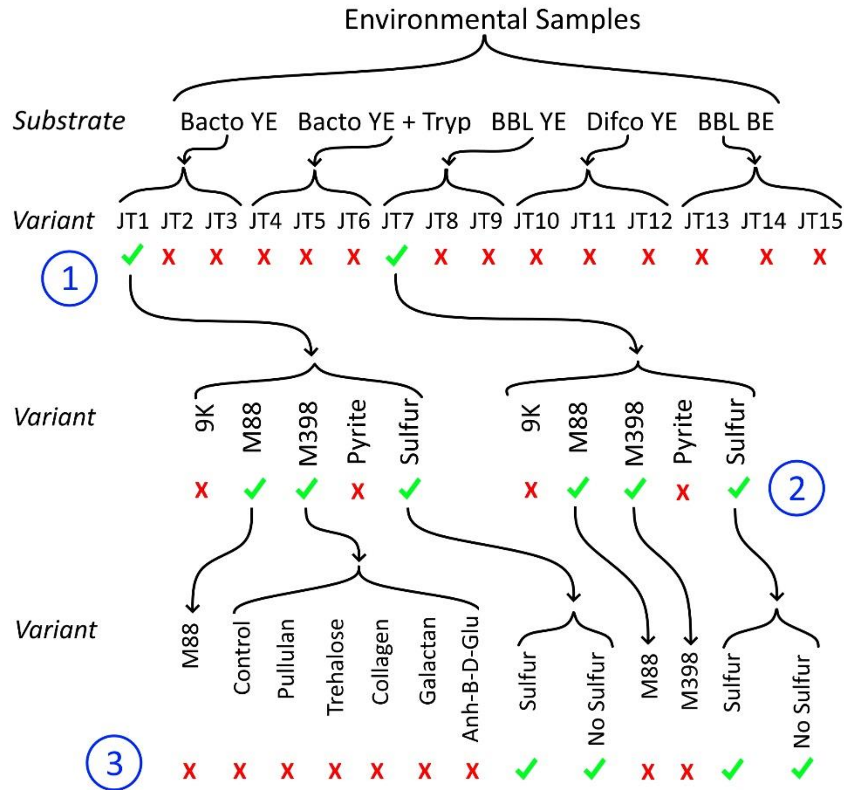

Figure 2.3: Flow Chart Summary of Cultivation Methodology. Arrows represent the continued use of cultures and brackets represent the inoculation of parallel variants. Successful results (in the first barcoding experiments: those containing the desired organisms in detectable abundances; in the second and third barcoding experiments: those from which DNA was successfully isolated) are represented by ticks and unsuccessful results are represented by crosses. Circled numbers represent: (1) "E-Plasma" was present in two cultures; (2) DNA barcoding was successful with six cultures; (3) DNA barcoding was successful with four cultures. 1,6-Anhydro-β-D-Glucose is here abbreviated to "Anh-B-D-Glu".

2.4.3 Storage of Cultures

13 cultures did not contain the organisms desired for further analysis in this project, so were stored as glycerol stock. Cultures were centrifuged at 9000 rpm for 15 min before removing all but 2.5 ml of supernatant. 500 µl of 87 %v/v glycerol solution was then mixed with each culture. Cultures were then stored at -80 °C.

3. Results

3.1 Cultivation of Microorganisms

Details regarding the cultivation conditions and physiologies of previously described members of the order *Thermoplasmatales* were acquired from the description papers for each validated species (Table 3.1) (Darland *et al.*, 1970; Golyshina *et al.*, 2000; Golyshina *et al.*, 2009; Golyshina *et al.*, 2016b; Hawkes *et al.*, 2006; Itoh *et al.*, 2007; Schleper *et al.*, 1996; Segerer *et al.*, 1988).

Table 3.1: Conditions of Cultivation for Validated Species shown for each member of the order Thermoplasmatales to have been described, with details of the physiology of each species additionally included.

Species	Physiology	Substrate	Medium	Temp/ °C	pH	TA*
Thermoplasma acidophilum	facultative aerobe, heterotrophic	Yeast extract, glucose	Darland Medium	59	1-2	-
Thermoplasma volcanium	facultative anaerobe, heterotrophic	Yeast extract, meat extract, glucose	Darland Medium	60	2	-
Thermogymn-omonas acidicola	obligate aerobe, heterotrophic	Yeast extract, glucose, mannose	M88	55	2.5	-
Ferroplasma acidiphilum	obligate aerobe, autotrophic	Yeast extract	Modified 9K	35	1.7	SL10, Fe^{2+}
Cuniculiplasma divulgatum	facultative anaerobe, organoheterotrophic	Beef extract, peptides	Modified M88	37-40	1-1.2	SL10
Acidiplasma aeolicum	facultative anaerobe, chemo-organotrophic	Yeast extract, glucose	9K	45	1.4-1.6	SL10
Acidiplasma cupricumulans	facultative anaerobe, chemomixotrophic	Yeast extract	9K	53.6	1-1.2	-

Picrophilus oshimae	obligate aerobe, heterotrophic	Yeast extract, glucose	Darland Medium	60	0.7	-
Picrophilus torridus	obligate aerobe, heterotrophic	Yeast extract, glucose	Darland Medium	60	0.7	-

* "TA" = Trace Elements.

All previously described members of the order *Thermoplasmatales* were cultivated using polypeptide substrates. Most species were cultivated with the use of yeast extract as a substrate in enrichment cultures, with beef extract used for *C. divulgatum*, and meat extract additionally used for *T. volcanium*. Polypeptide substrates were therefore used in the enrichment of environmental samples from the Parys Mountain AMD system in the present work. The conditions of the enrichment cultures could not be considered oligotrophic as the substrates were rich in nutrients such as carbon and nitrogen. Three yeast extracts (YE) from different manufacturers were included, as well as beef extract (BE) and tryptone, the latter being used only in combination with Bacto yeast extract in a 1:1 ratio. Data regarding the nutritional composition of each substrate were compiled from the BD Biosciences bio-nutrients technical manual, as shown in the modified Table 3.2 (*https://www.bdbiosciences.com/documents/bionutrients_tech_manual.pdf*).

The details of cultivation conditions for previously described archaea as shown in Table 3.1 provided information regarding the suitability of conditions for the initial establishment of enrichment cultures. M88 medium was used for the enrichment of environmental samples, as this medium was previously used for the cultivation of similar organisms from the same site (Golyshina *et al.*, 2016b). The organisms studied in the present work were sampled from a mesophilic environment; a temperature of 37.5 °C was therefore used for all cultivation experiments, as mesophilic archaea were previously cultivated at this temperature (*ibid.*). Trace elements SL-10 were additionally used during cultivation experiments for the same reason. The pH of the sample site was 1.7, so a variant of each culture was first established using the same pH. Further culture variants were later established with a more acidic pH of 1.1 in an attempt to reduce the amount of bacterial growth and to improve the enrichment of archaea in cultures.

Table 3.2: Comparison of the Nutritional Composition of Polypeptide Substrates with mean values shown for the combination of Bacto Yeast Extract and Bacto Tryptone (data from *https://www.bdbiosciences.com/documents/bionutrients_tech_manual.pdf*).

Composition	**Substrate***					
	Bacto YE	*Bacto Tryp*	*Bacto YE + Tryp*	*BBL YE*	*Difco YE*	*BBL BE*
Total Nitrogen (%w/w)	10.9	13.3	12.1	11.4	10.7	12.4
Amino Nitrogen (%w/w)	6.0	5.3	5.7	6.9	6.0	2.3
AN/TN†	0.55	0.4	0.5	0.6	0.56	0.19
Carbohydrate (mg/g)	163.3	4.3	83.8	67.6	108.2	56.1
NaCl (%w/w)	0.1	0.0	0.1	0.2	0.0	0.3
pH (1% Solution)	6.7	7.3	7.0	7.0	7.0	6.9
Calcium (µg/g)	130	256	193.0	230	191	264
Iron (µg/g)	55.3	23	39.2	62.1	57.9	27.4
Magnesium (µg/g)	750	195	472.5	799	558	285
Potassium (µg/g)	31950	3257	17603.5	58013	59240	28793
Sodium (µg/g)	4900	33910	19405	1003	1244	18510
Chloride (%w/w)	0.38	0.06	0.2	0.07	0.13	0.0
Sulfate (%w/w)	0.09	0.33	0.2	0.65	1.02	0.53
Phosphate (%w/w)	3.27	2.58	2.9	3.73	2.7	3.22

* Yeast extract is abbreviated to “YE”, Beef extract is abbreviated to “BE”, and Tryptone is abbreviated to “Tryp”.

† AN/TN ratio = degree of protein hydrolysis.

There is variation in nutritional composition between each polypeptide substrate. Bacto YE has the highest carbohydrate and chloride content; and the lowest calcium and sulfate content. The combination of Bacto YE and Bacto Tryptone in equal proportions provides the highest sodium content and the lowest potassium content. BBL YE has the highest amino nitrogen, iron, magnesium and phosphate content; and the lowest carbohydrate and sodium content. Difco YE has the highest

potassium and sulfate content, and the lowest total nitrogen, NaCl and phosphate content. BBL BE has the highest total nitrogen, NaCl and calcium content; and the lowest amino nitrogen, iron, magnesium and chloride content. Total nitrogen, phosphate and pH levels are relatively constant between substrates, while other nutrients show a greater amount of variation between substrates: BBL BE has an amino nitrogen content less than half that of other polypeptide substrates; the potassium content in BBL YE and Difco YE is approximately double that of most other substrates; sulfate content is approximately twice as high in Difco YE as in BBL YE and BBL BE; and carbohydrate content is approximately three times higher in Bacto YE than in BBL BE.

15 enrichment cultures were established using environmental samples, each with different cultivation conditions (see Table 3.3). Cultures were transferred to new media three times over a two month incubation period, after which DNA barcoding was used to determine the microbial composition of enrichment cultures (Figure 3.1). During the cultivation process, growth of cultures was monitored with microscopic observation. Before the first transferral of cultures, both cocci and rod shaped cells were observed in varying proportions among cultures, with some motility observed in a small number of cells. After further incubation, prior to DNA isolation, the density of cells was observed to be higher. Rods of varied morphology and cocci of various cell sizes were observed, with proportions varying between cultures. Some cells formed chains or groups, and some motility was observed.

Table 3.3: Cultivation Conditions of First Enrichment Cultures established using microorganisms from environmental samples, also showing the mean abundance of archaeal growth in each culture.

Culture	Substrate	pH	Additional Supplements*	Archaeal abundance/ %
JT01	Bacto YE	1.7	no	0.66
JT02	Bacto YE	1.1	no	30.08
JT03	Bacto YE	1.1	yes	37.01
JT04	Bacto YE + Tryp	1.7	no	28.40
JT05	Bacto YE + Tryp	1.1	no	77.28
JT06	Bacto YE + Tryp	1.1	yes	8.50
JT07	BBL YE	1.7	no	3.71
JT08	BBL YE	1.1	no	82.86
JT09	BBL YE	1.1	yes	66.90

JT10	Difco YE	1.7	no	0.10
JT11	Difco YE	1.1	no	78.60
JT12	Difco YE	1.1	yes	85.04
JT13	BBL BE	1.7	no	35.67
JT14	BBL BE	1.1	no	0.67
JT15	BBL BE	1.1	yes	93.36

* "Additional Supplements" refers to the inclusion of trace elements for *Thermoplasma acidiphilum*, WME SS, Cysteine and SyntheChol in cultures (for details, see section: Methods).

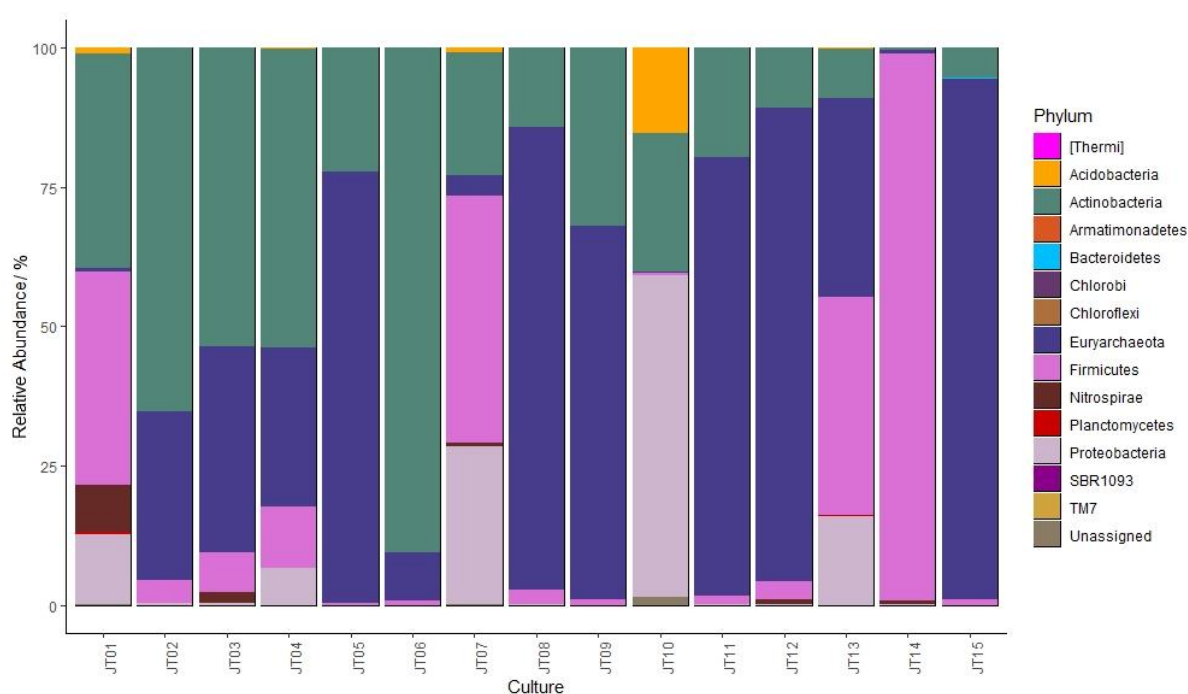

Figure 3.1: Microbial Composition of First Enrichment Cultures. Mean relative abundances of 16S rRNA reads (n=2) present in enrichment cultures after two months of cultivation are shown in a 100 % stacked bar chart according to the phylum classification level. The conditions of each enrichment culture are shown in Table 3.3.

Growth of archaea was detected in all cultures, but in some cultures their abundance was less than 1 % (JT01, JT10 and JT14). Two of these cultures were grown using a higher pH, and the cultures with over 50 % archaeal growth (JT05, JT08, JT09, JT11, JT12 and JT15) were grown using a lower pH: this indicates that the cultivation of archaea requires a highly acidic medium. Archaeal growth exceeded

80 % in cultures containing BBL YE, Difco YE and BBL BE. This suggests a high nitrogen, potassium, phosphate, or sulfate content may promote archaeal growth. *Actinobacteria* grew in most cultures, but were more abundant in cultures containing Bacto YE, or Bacto YE and Tryptone. This suggests that high proportions of carbohydrates, chloride or sodium may promote their growth. *Proteobacteria* grew almost exclusively in less acidic cultures, and their abundance was greatest with the use of Difco YE. This suggests that a high potassium or sulfate content promotes their growth. *Firmicutes* had a substantial abundance in cultures JT01, JT07, JT13, and dominated culture JT14, which contained the substrates Bacto YE, BBL YE, Difco YE and BBL BE respectively. This suggests that the organisms grow preferentially at a higher pH, but that in more acidic conditions a higher total nitrogen or calcium content may be needed to promote their growth. All relative abundance data for taxa shown (Figure 3.1) are mean values averaged from two duplicate barcoding repeats conducted using the same DNA samples.

The growth of metagenomic variant “E-Plasma” was not significant in any cultures, and the two cultures containing it (JT01 and JT07) each had low proportions of archaeal growth (0.66 % and 3.71 % respectively). Despite the lack of significant growth, both cultures were subjected to further cultivation experiments in an attempt to further enrich the growth of “E-Plasma”.

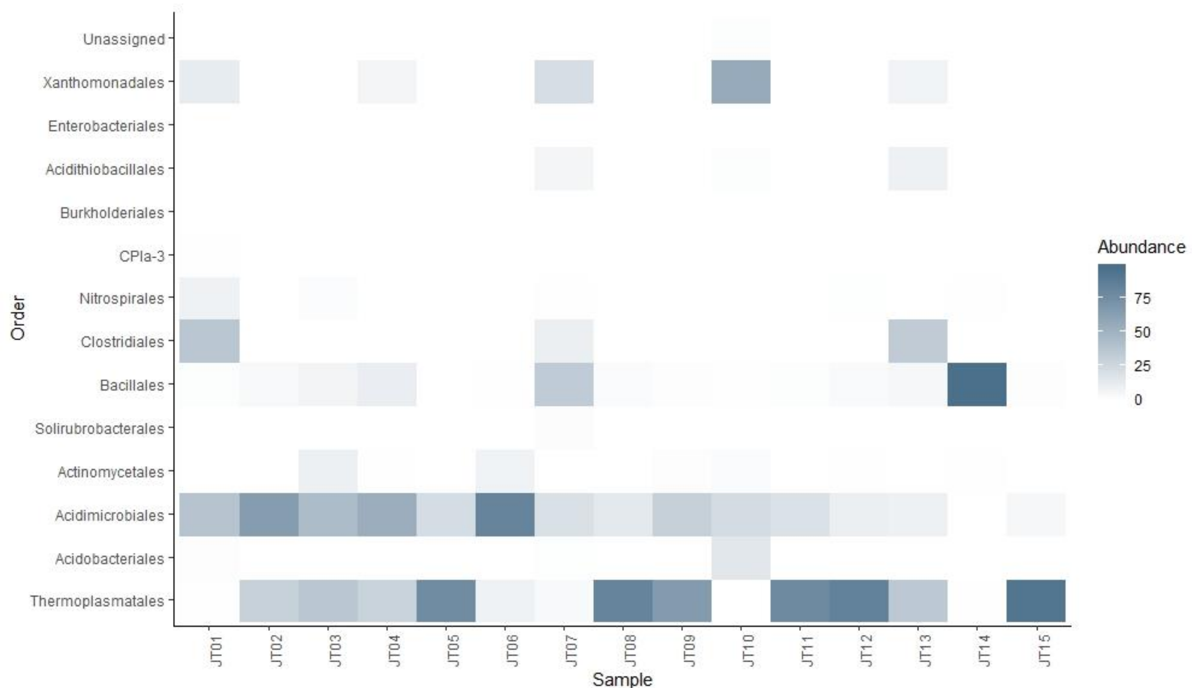

Figure 3.2: Heat Map of OTU Abundance in First Enrichment Cultures. Number of reads are represented by a colour gradient (low = white, high = blue). Taxa are shown according to the order classification level. Grouping of taxa on the Y axis represents their similarity of abundance in cultures, calculated using the Bray-Curtis method of NMDs ordination. OTUs that represent less than 0.1 % of reads in at least one sample are not shown to improve readability of the data.

The abundance of organisms in each culture is represented in Figure 3.2 according to the order classification level. All archaea present were of the order *Thermoplasmatales*, and most of the *Actinobateria* were of the order *Acidimicrobiales*. These were typically the two most abundant taxa present across all cultures. Iron oxidising bacteria (*Nitrospirales*) were mostly present only in low abundances, with their abundance being highest in culture JT01. *Proteobacteria* content was mostly comprised of the order *Xanthomonadales*, which was the most abundant taxon in culture JT10. *Firmicutes* were present in all cultures, comprised of the orders *Bacillales* and *Clostridiales*, the former of which was more abundant overall in cultures, and dominated culture JT14. Unassigned taxa were generally only present in trace amounts in all cultures, but were highest in culture JT10.

A Non-Metric Multidimensional scaling (NMDs) plot is shown in Figure 3.3, where the variation in microbial diversity between cultures is presented.

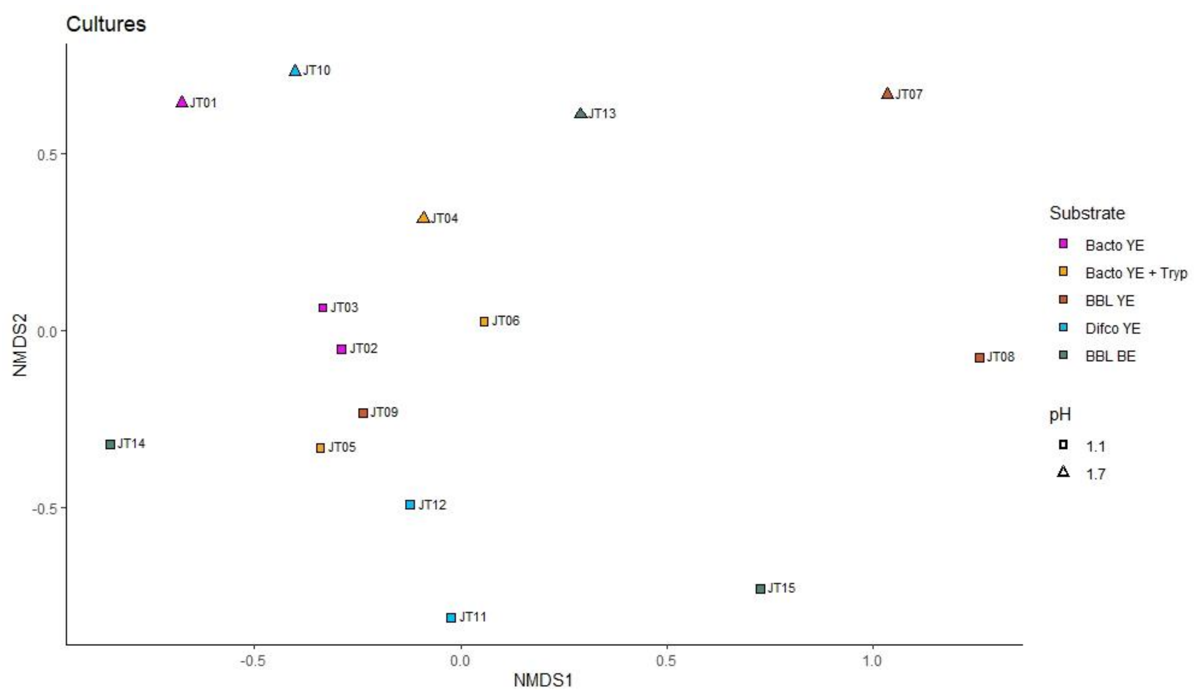

Figure 3.3: Multivariate Analysis of First Enrichment Cultures. NMDs ordination was plotted using the Bray-Curtis method. More acidic cultures (pH 1.1) are represented by triangular points and less acidic cultures (pH 1.7) are represented by square points. The polypeptide substrate used in each culture is shown by colour (magenta = Bacto YE; yellow = Bacto YE with Tryptone; orange = BBL YE; blue = Difco YE; green = BBL BE).

In Figure 3.3, the distance between plot points represents variation in microbial community diversity, with cultures containing more similar microbial community compositions plotted more closely together. There is an apparent separation in variation between cultures of differing pH values: less acidic cultures are all clustered in separation from more acidic cultures. As demonstrated by Figure

3.1, there is a general tendency for archaea to grow more abundantly in more acidic conditions, and the grouping of cultures according to varied acidity shown in Figure 3.3 may reflect a reduced overall diversity from increased archaeal abundance in the more acidic cultures. While the placement of cultures in Figure 3.3 according to substrate use (represented by colour) shows less of a trend, it illustrates how the variation in microbial composition between cultures is affected by the use of different substrates. The number of more acidic cultures is twice that of less acidic cultures, as there are two variants at pH 1.1: a variant with additional supplements (Trace elements for *Thermoplasma acidiphilum*; WME SS; Cysteine and SyntheChol) and a variant without additional supplements (see section: Methods). However, this variation is not represented in Figure 3.3 as it does not improve readability, and the inclusion of this metadata adds little additional information to the figure.

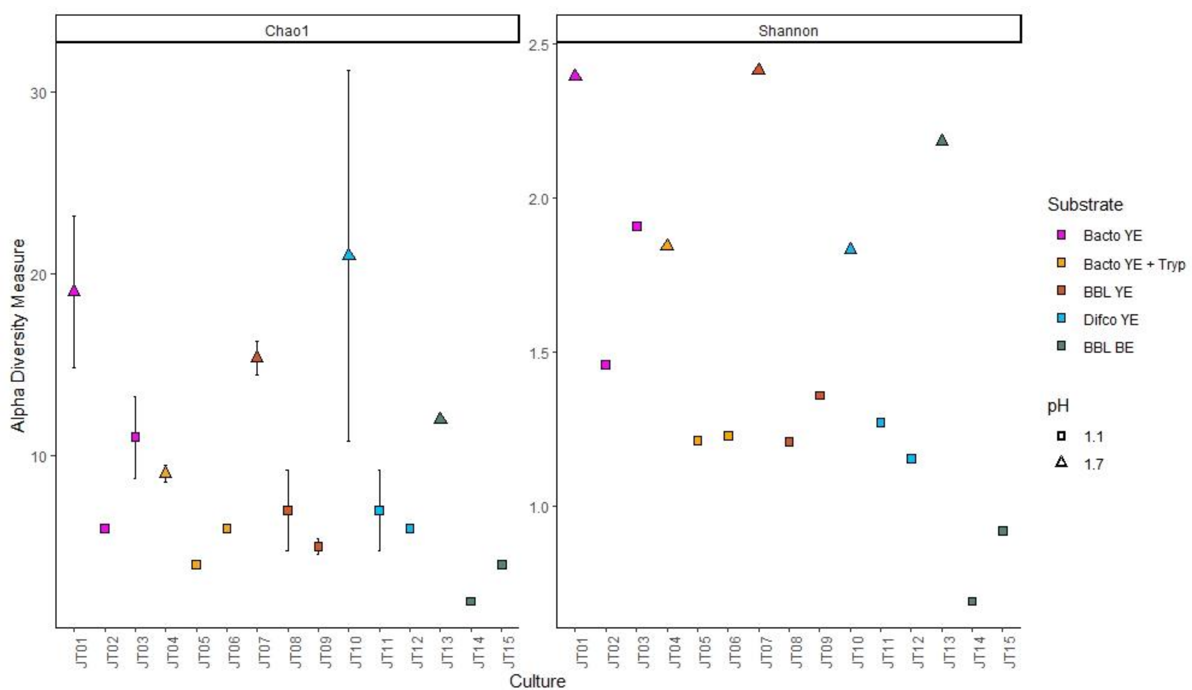

Figure 3.4: Alpha diversity of First Enrichment Cultures. Chao1 and Shannon diversity indices are shown with more acidic cultures (pH 1.1) are represented by triangular points and less acidic cultures (pH 1.7) are represented by square points. The polypeptide substrate used in each culture is shown by colour (magenta = Bacto YE; yellow = Bacto YE with Tryptone; orange = BBL YE; blue = Difco YE; green = BBL BE).

Estimates of species richness are presented using the Chao1 diversity index, and the abundance and evenness of taxa are shown using the Shannon index in Figure 3.4 across all cultures. This allows a comparison of the microbial diversity of cultures to be made. In general, less acidic cultures have a higher diversity which may be due to a higher bacterial content. Among the more acidic cultures, BBL BE has the lowest diversity, and Bacto YE the highest. Cultures JT01 and JT07 have some of the highest

measures of diversity across all cultures, and these were the two cultures found to contain "E-Plasma" in small amounts. The use of additional supplements is not portrayed in Figure 3.3 as it does not consistently affect the diversity of cultures: in cultures with Bacto YE, Bacto YE with Tryptone, or BBL YE as the substrate, the diversity is increased with the inclusion of additional supplements; but with cultures containing BBL YE or Difco YE, the diversity is reduced.

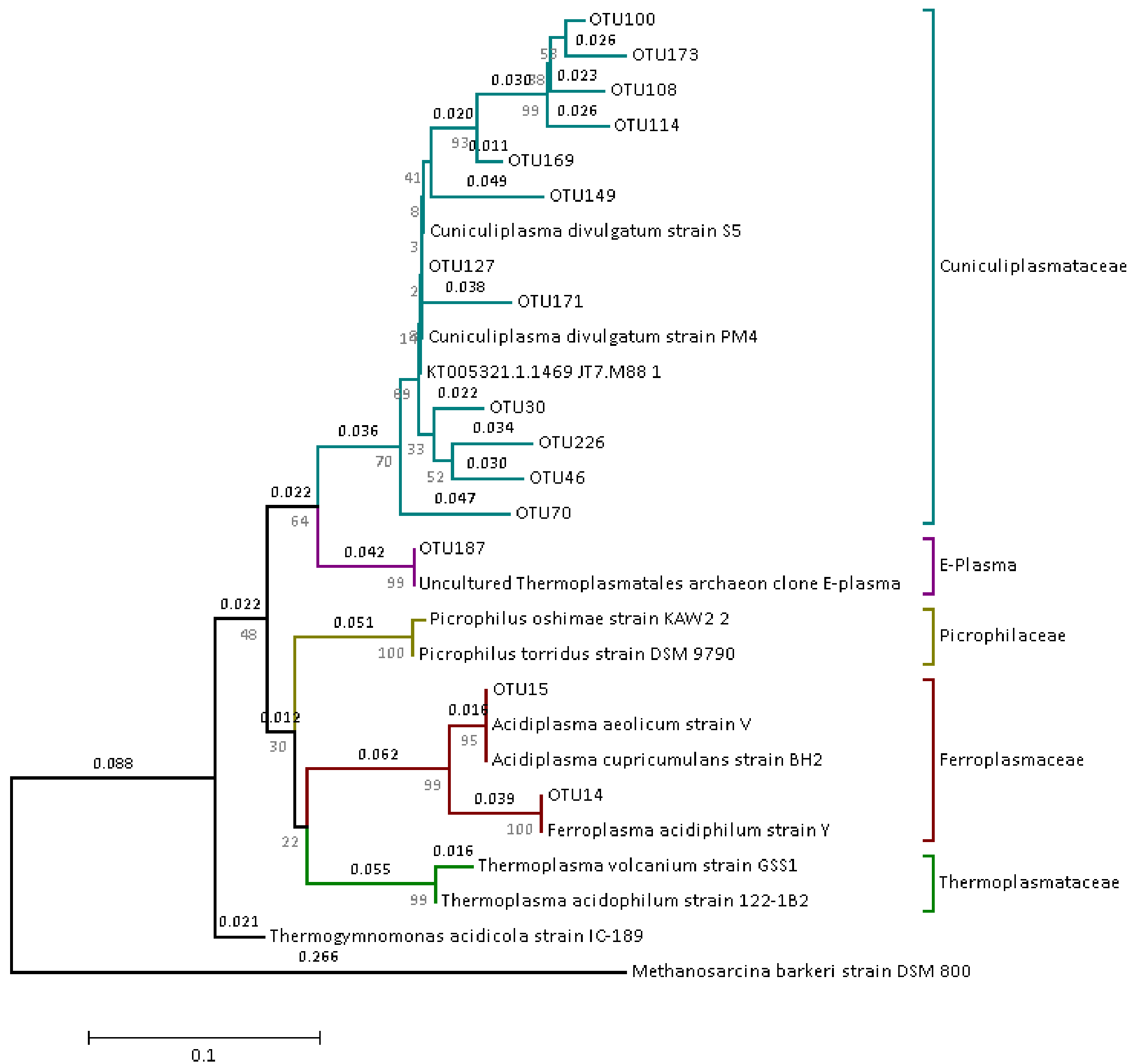

Figure 3.5: Phylogeny of Archaeal OTUs in First Enrichment Cultures. A rooted phylogenetic tree is shown, displaying archaeal 16S rRNA sequences from cultures alongside those of type strains of described members of the order *Thermoplasmatales*, with *Methanosarcina barkeri* used as an outgroup. The archaeal taxonomy at the family level is displayed using coloured brackets (blue = *Cuniculiplasmataceae*; yellow = *Picrophilaceae*; red = *Ferroplasmaceae*; green = *Thermoplasmataceae*; additionally purple = metagenomic variant "E-Plasma"). Type strain sequence data was obtained from the NCBI database. 28 nucleotide sequences were included with missing data removed for a total of 252 positions. Phylogeny was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). Branch lengths are shown in black above branches, with values shorter than 0.01 omitted, and

Bootstrap test results (500 replicates) are shown in grey below branches (Felsenstein, 1985). The sum of branch lengths is 1.15984493. The Jukes-Cantor method was used to determine evolutionary distances (Jukes & Cantor, 1969), and gamma distribution was used to model the variation rate.

The affiliation of archaeal OTU (Operational Taxonomic Unit) 16S rRNA sequences with previously described members of the order *Thermoplasmatales* is shown in Figure 3.5, with the uncultured archaeon clone “E-Plasma” additionally shown, and the methanogenic archaeon *Methanosarcina barkeri* used as an outgroup. No bacterial OTUs are included in the phylogenetic tree as the present work primarily focuses on archaea. The majority of sequences are most closely affiliated with *C. divulgatum*, with *Acidiplasma* spp., *F. acidiphilum* and metagenomic variant “E-Plasma” each represented by one sequence. No sequences are associated with *Picrophilacae*, *Thermoplasmatacae* or *Thermogymnomonas* spp. in cultures.

In summary, it was possible to cultivate acidophilic archaea from environmental samples using polypeptide substrates. All detected archaea were of the order *Thermoplasmatales*, with the majority of detected archaeal sequences being most closely associated with *C. divulgatum*. Variation in microbial compositions of acidophilic cultures is caused by both differences in the nutritional composition of each polypeptide substrate and the variation of pH, with the addition of supplements having little effect on microbial growth. Overall, the acidity of growth conditions was responsible for a greater amount of variation in microbial diversity than the type of polypeptide substrate used. The growth of archaea was more successful in more acidic cultures, while with most bacterial phyla the converse was true. While “E-Plasma” was present in minor quantities in two cultures, its growth was not significant. The most abundant bacterial phyla across all cultures included *Actinobacteria*, *Proteobacteria* and *Firmicutes*. Some presence of *Nitrospirae* and *Acidobacteria* was additionally detected.

3.2 Cultivation of Archaea

Following the first amplicon sequencing, cultures were transferred three times over three months, after which 10 cultures were stored as glycerol stock. Further cultivation experiments were conducted using the two cultures previously found to contain “E-Plasma” (cultures JT01 and JT07). Cultivation conditions were maintained using the same media and substrates as previously described for both cultures, with eight additional variants prepared using different media as shown in Table 3.4. Cultures were transferred three times over a three-week incubation period, and were monitored using microscopic observation.

Table 3.4: Cultivation Conditions of Second Enrichment Cultures. The mean abundances of members of the archaeal order *Thermoplasmatales* in cultures are presented alongside their respective cultivation conditions.

Culture*	**Medium**	**Substrate**	**Sulfur (S_8)**	**Pyrite (FeS_2)**	***Thermoplasmatales* content/ %**
JT1_9K	9K	Bacto YE	Absent	Absent	N/A*
JT1_M88	M88	Bacto YE	Absent	Absent	99.27
JT1_M398	M398	Bacto YE	Absent	Absent	96.18
JT1_PYR	M88	Bacto YE	Absent	Present	N/A
JT1_SUL	M88	Bacto YE	Present	Absent	99.64
JT7_9K	9K	BBL YE	Absent	Absent	N/A
JT7_M88	M88	BBL YE	Absent	Absent	99.51
JT7_M398	M398	BBL YE	Absent	Absent	99.92
JT7_PYR	M88	BBL YE	Absent	Present	N/A
JT7_SUL	M88	BBL YE	Present	Absent	99.83

* DNA was not successfully isolated from four cultures (indicated by N/A).

After one week of incubation, cultures were observed to contain varying proportions of both rod-shaped cells and cocci in varying sizes, so all cultures were filtered upon their first transferral to new media. After filtration most cultures were observed to be devoid of bacterial presence, with the exception of two culture variants which were each filtered a second time. Microscopic observation prior to DNA isolation revealed the exclusive growth of cocci in almost all cultures, with cell density varying between cultures. Variation in cell size was apparent within most cultures, and in culture JT1_9K (inoculated with culture JT1, using 9K medium for growth) pili were observed and some cells were additionally joined together.

DNA isolation was attempted after three weeks of cultivation. This was successful for six cultures (Table 3.4), but it was not possible to isolate DNA from any 9K or pyrite (FeS_2) variants after several attempts using a variety of methods, so these variants were excluded from further analysis. DNA barcoding was used to determine the microbial composition of successful cultures (Figure 3.6).

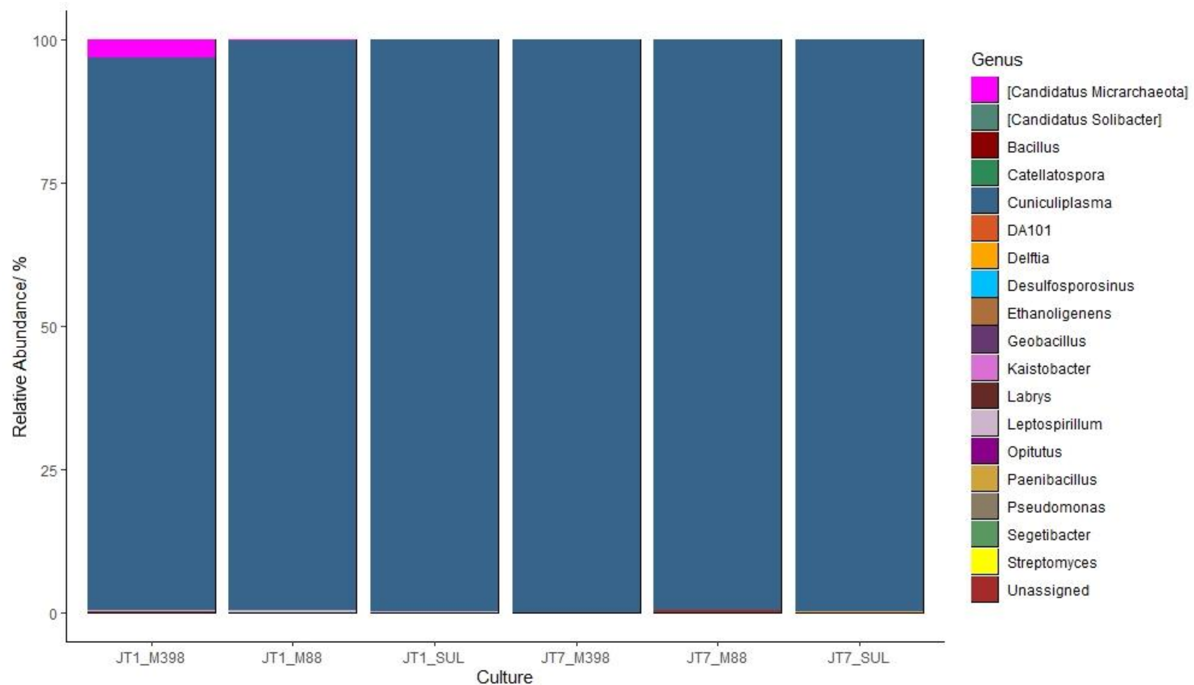

Figure 3.6: Microbial Composition of Second Enrichment Cultures. Mean relative abundances of 16S rRNA reads (n=2) present in enrichment cultures after three weeks of cultivation are shown in a 100 % stacked bar chart according to the genus classification level. The conditions of each enrichment culture are shown in Table 3.4.

Archaea were highly enriched in all cultures from which DNA isolation was successful, with an abundance of over 99 % of sequencing reads in five cultures (Figure 3.6). "E-Plasma" was not detected in any cultures, and members of the order *Thermoplasmatales* were represented only by *C. divulgatum*. The most highly enriched culture was JT7_M398, which had 99.92 % reads derived from *C. divulgatum*. This was cultivated using BBL YE and M398 medium, and was inoculated using the culture JT07 from the previous cultivation results, which had 3.71 % reads derived from *Thermoplasmatales*. Additionally there was a low abundance of ARMAN-related organisms (order *Micrarchaeales*) present in some cultures: this was highest in culture JT1_M398, which had a mean average ARMAN content of 3.15 %. ARMAN-related organisms were also present in cultures JT1_M88, JT1_SUL and JT7_M398, each with an abundance of less than 1 %. Trace amounts of bacteria were additionally detected in all cultures, mostly of the orders *Acidimicrobiales*, *Bacilliales* and *Nitrospirales*. The taxonomic status of the order *Micrarchaeales* varies between databases: The Genome Taxonomy Database (GTDB) (https://gtdb.ecogenomic.org/) lists the order within the phylum *Micrarchaeota*, whereas the NCBI (https://www.ncbi.nlm.nih.gov/) and GreenGenes (https://greengenes.secondgenome.com/) databases instead list the group as the phylum "*Ca.*

Micrarchaeota" without listing an order. All relative abundance data for taxa shown (Figure 3.6) are mean values averaged from two duplicate barcoding repeats conducted using the same DNA samples.

Table 3.5: Alpha Diversity of Second Enrichment Cultures showing Chao1 and Shannon diversity indices.

	Alpha Diversity Index	
Culture	**Chao1**	**Shannon***
JT1_M398	4	0.800
JT1_M88	2	0.693
JT1_SUL	2	0.693
JT7_M398	2	0.693
JT7_M88	2	0.693
JT7_SUL	2	0.693

* shown to three significant figures.

The microbial diversity of cultures following further cultivation experiments was reduced, as indicated by chao1 and Shannon indices in Table 3.5. This is due to the enrichment of *C. divulgatum* to over 99 % of sequencing reads in most cultures. The larger diversity observed in culture JT1_M398 is likely due to the presence of ARMAN related organisms, which was the highest of any cultures. The lack of variation between cultures containing sulfur (S_8) (JT1_SUL; JT7_SUL) and those lacking sulfur (JT1_M88; JT7_M88) with otherwise identical conditions suggests that at this stage of cultivation, it is not likely that the addition of sulfur had any significant effect on the growth of cultures.

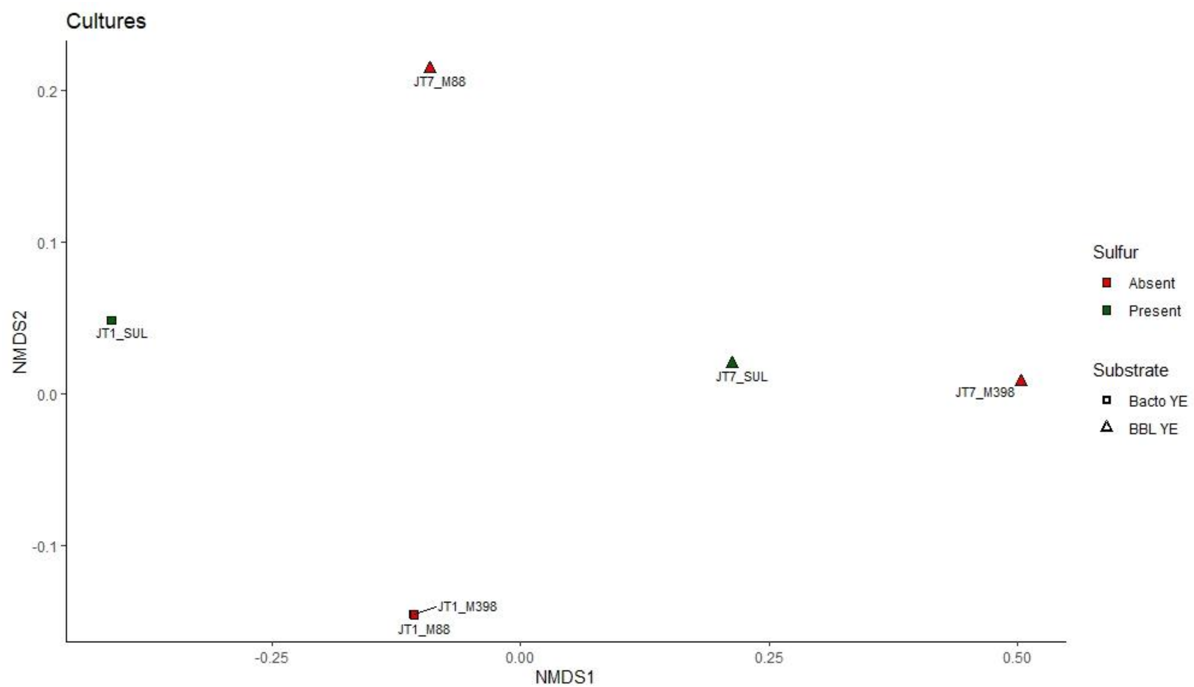

Figure 3.7: Multivariate Analysis of Second Enrichment Cultures. NMDs ordination was plotted using the Bray-Curtis method. Cultures grown using Bacto YE are represented by square points, and cultures grown using BBL YE are represented by triangular points. The addition of sulfur in each culture is shown by colour (red = absent; green = present).

Variation between cultures is shown using a NMDs plot in Figure 3.7, where variation between cultures is represented by distance between plot points. The data points for JT1_M398 and JT1_M88 are overlapping in placement, which may be due to these cultures containing the highest proportion of ARMAN – related organisms. The remaining variation between cultures may be due to some difference in trace amounts of bacteria present in each culture. While there is no apparent trend associated with the addition of sulfur in cultures at this stage of cultivation, there was a much higher presence of ARMAN – related organisms in cultures grown using Bacto YE as a polypeptide substrate. This is shown in Figure 3.8 by an apparent separation between triangular points (cultures grown using BBL YE) and square points (cultures grown using Bacto YE).

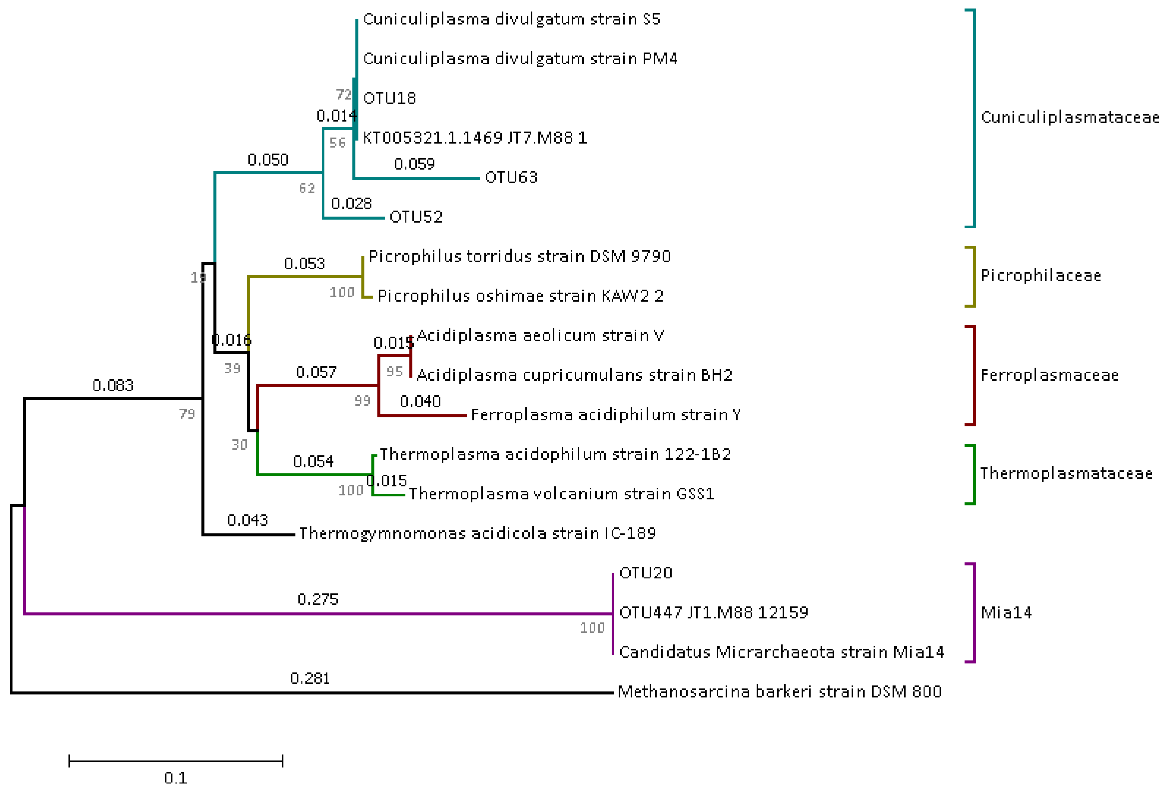

Figure 3.8: Phylogeny of Archaeal OTUs in Second Enrichment Cultures. A rooted phylogenetic tree is shown, displaying archaeal 16S rRNA sequences from cultures alongside those of type strains of described members of the order *Thermoplasmatales*, with *Methanosarcina barkeri* used as an outgroup. The archaeal taxonomy at the family level is displayed using coloured brackets (blue = *Cuniculiplasmataceae*; yellow = *Picrophilaceae*; red = *Ferroplasmaceae*; green = *Thermoplasmataceae*; additionally purple = "Ca. Micrarchaeota" strain Mia14). Type strain sequence data was obtained from the NCBI database. 18 nucleotide sequences were included with missing data removed for a total of 252 positions. Phylogeny was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). Branch lengths are shown in black above branches, with values shorter than 0.01 omitted, and Bootstrap test results (500 replicates) are shown in grey below branches (Felsenstein, 1985). The sum of branch lengths is 1.10715156. The Jukes-Cantor method was used to determine evolutionary distances (Jukes & Cantor, 1969) and gamma distribution was used to model the variation rate.

The affiliation of archaeal OTUs with previously described members of the order *Thermoplasmatales* is shown in Figure 3.8, with the uncultured "*Candidatus* Micrarchaeota" strain Mia14 additionally shown, and the methanogenic archaeon *Methanosarcina barkeri* used as an outgroup. Bacterial sequences are omitted as they are not the focus of the present work, and the abundance of reads derived from bacteria in all cultures was less than 1 %. All archaeal OTUs from cultures correspond to *C. divulgatum* and the ARMAN related organism Mia14, with no sequences found to be affiliated with other members of the order *Thermoplasmatales*.

In summary, it was not possible to successfully enrich the content of "E-Plasma" in the present cultivation experiments. Cultures containing *C. divulgatum* were enriched to an abundance of over 96 % in all cultures, and the presence of ARMAN related organisms was detected with an abundance of 3.15 % in one culture, and in trace amounts in other cultures. The ARMAN related organisms were associated mostly with Bacto YE. It was not possible to isolate any DNA from any of the culture variants containing pyrite or 9K medium. Lower diversity was observed across all cultures in comparison to previous cultivation experiments due to the reduced bacterial content. The ARMAN related organisms detected in the cultures had 16S rRNA sequences identical to Mia14.

3.3 Cultivation of ARMAN Related Organisms

Subsequent experimentation focussed on enriching ARMAN related organisms in cultures. Since ARMAN related organisms were most abundant in culture JT1_M398, the growth of this culture was tested using various additional supplements (pullulan, trehalose, collagen, galactan and 1,6-anhydro-β-D-glucose). Parallel variants were also prepared using the cultures containing sulfur (JT1_SUL and JT7_SUL) whereby sulfur was removed. Cultures were transferred four times over a three month incubation period.

Cultures were monitored with microscopic observation after three weeks of incubation. Cocci were present in all cultures, with a high cell density in all sulfur and no sulfur variants, as well as in cultures grown using M398 medium. The cultures grown using only M88 medium had a lower cell density. There was no apparent difference observed in variants of culture JT1_M398 with additional supplements, as all variants showed poor growth. After several attempts using various methods, the isolation of DNA and PCR amplicon sequencing was only successful with four cultures using PCR directly from boiled biomass pellets, as shown in Table 3.6. No results were obtained successfully through the process of re-amplification of PCR products for other cultures. The mean abundances of taxa in the successful cultures are shown in Figure 3.9. Cultures JT1_M88 and JT7_M88 were excluded from further analysis due to insufficient growth.

Table 3.6: Cultivation Conditions of Successful Third Enrichment Cultures* showing the mean abundances of members of the archaeal order *Thermoplasmatales* and of ARMAN related organisms in successful archaeal cultivation experiments.

Culture†	**Medium**	**Substrate**	**Sulfur (S_8)**	***Thermoplasmatales* content/ %**	**ARMAN content/ %**
JT1_S	M88	Bacto YE	Present	35.77	35.39
JT1_N	M88	Bacto YE	Absent	88.25	2.52
JT7_S	M88	BBL YE	Present	99.90	0.03
JT7_N	M88	BBL YE	Absent	99.99	0.00

* success defined by DNA extraction and amplification.

† DNA was not successfully isolated from 12 cultures (not shown). “JT1” and “JT7” refer to the original culture used for inoculation, while “S” and “N” refer to the presence or absence of sulfur in cultures respectively.

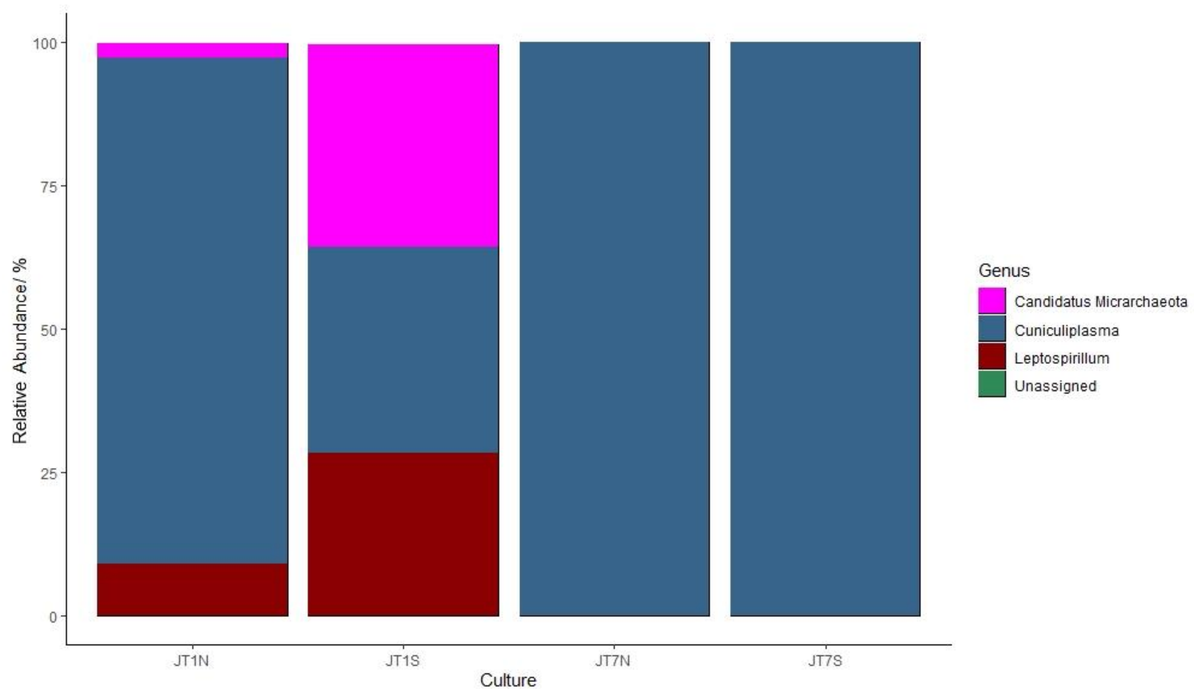

Figure 3.9: Microbial Composition of Third Enrichment Cultures. Mean relative abundances of 16S rRNA reads (n=2) present in enrichment cultures after three months of cultivation are shown in a 100 % stacked bar chart according to the genus classification level. Conditions of cultivation are shown in Table 3.6. OTUs that represent less than 0.1 % of reads in at least one sample are not shown to improve readability of the data.

The ARMAN related organisms in culture JT1_S were successfully enriched to a mean read abundance of 35.39 %, with a maximum abundance of up to 50 % in one repeat. This culture grew in Bacto YE and M88 medium, with sulfur as an additional supplement. ARMAN related organisms were present in a lower abundance in JT1_N, were almost absent from culture JT7_S and were not detected in culture JT7_N. JT1 culture variants both contained Bacto YE, which may indicate that this substrate has some importance in the cultivation of ARMAN related organisms. The growth of ARMAN related organisms was the most highly enriched in culture JT1_S which contained sulfur, while the growth of ARMAN related organisms was less successful in culture JT1_N from which sulfur was removed. This suggests that there may be an association between ARMAN related organisms and sulfur. Additionally, there is an apparent correlation between the growth of *Leptospirillum* spp and ARMAN related organisms, as proportions of both organisms appear similar in cultures JT1_S and JT1_N. This data is additionally shown by a heatmap in Figure 3.10. All relative abundance data for taxa shown (Figure 3.9) are mean values averaged from two duplicate barcoding repeats conducted using the same DNA samples.

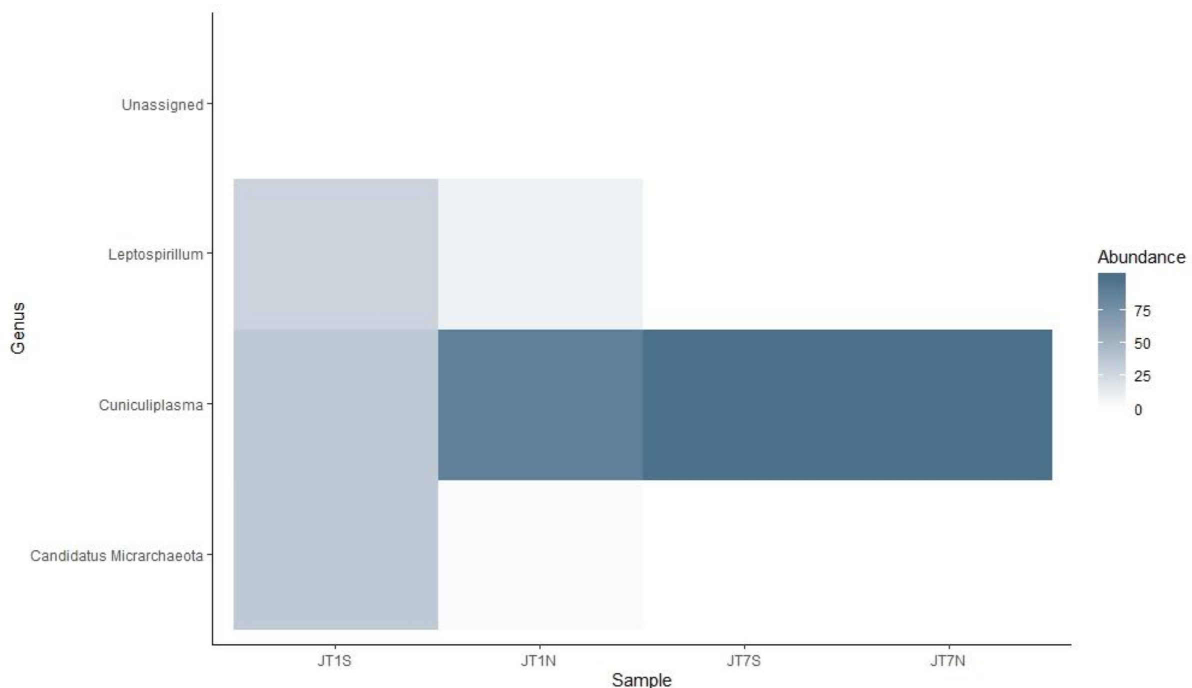

Figure 3.10: Heat Map of OTU Abundance in Third Enrichment Cultures. Number of reads are represented by a colour gradient (low = white, high = blue). Taxa are shown at the genus classification level. Grouping of taxa on the Y axis represents their similarity of abundance in cultures, calculated using the Bray-Curtis method of NMDs ordination. OTUs that represent less than 0.1 % of reads in at least one sample are not shown to improve readability of the data.

Cuniculiplasma were highly enriched in cultures JT7_S and JT7_N, and were the most abundant organism in culture JT1_N. The growth of these cultures was observed to be more rapid than those

with a higher content of ARMAN related organisms, most notably with culture JT1_M398, which previously had the highest presence of ARMAN related organisms (Figure 3.6). Culture JT1_M398 subsequently did not grow sufficiently for DNA to be isolated for the third DNA barcoding procedure, which suggests that an increased content of ARMAN related organisms may be responsible for a reduced growth rate in cultures containing *C. divulgatum*. Additionally, the apparent absence of ARMAN related organisms in culture JT1_SUL in the previous barcoding results (Figure 3.6) compared with their abundance of 35 % of sequencing reads in the third enrichment (Figure 3.9) in the same culture indicates a slow growth rate for ARMAN related organisms.

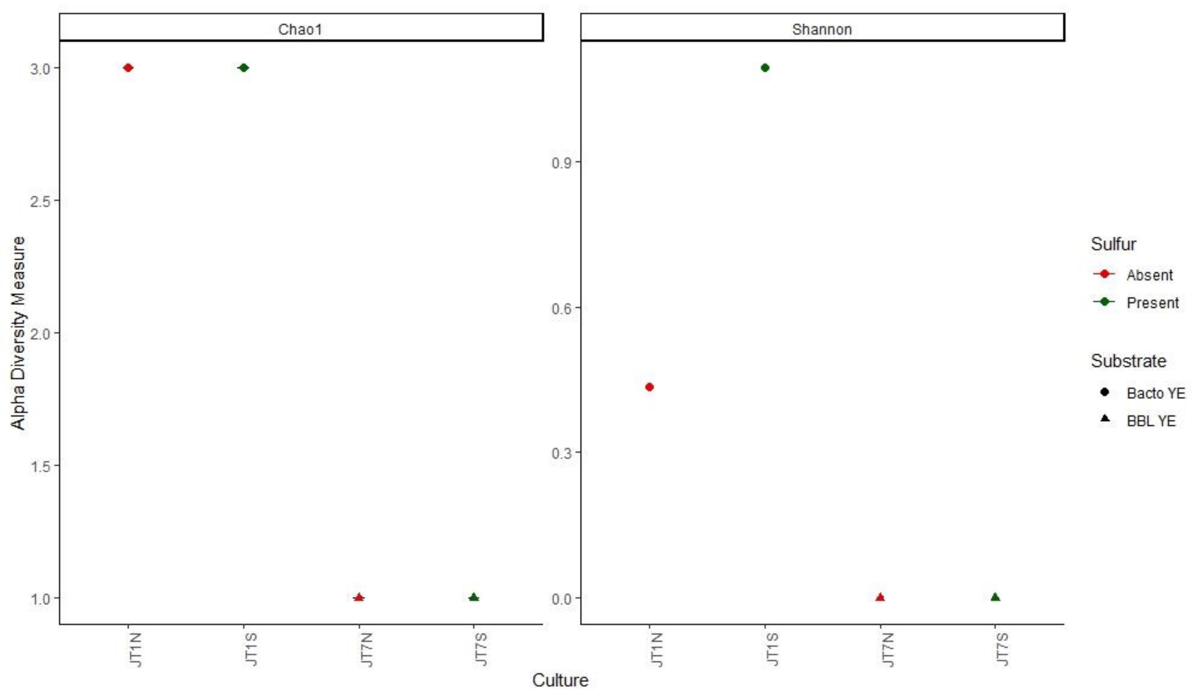

Figure 3.11: Alpha Diversity of Third Enrichment Cultures. Chao1 and Shannon diversity indices are shown. Cultures grown using the substrate Bacto YE are represented by circular points, and cultures grown using BBL YE are represented by triangular points. The addition of sulfur to cultures is indicated by colour (green = present; red = absent).

The microbial diversity of cultures JT7_S and JT7_N as indicated by Figure 3.11 is very low, as the cultures are highly enriched with *C. divulgatum* to over 99 %. The diversity of culture JT1_S is highest as roughly equal proportions of ARMAN related organisms, *Cuniculiplasma* and *Leptospirillum* are present. While the same three groups are present in culture JT1_N, the proportions of ARMAN related organisms and *Leptospirillum* are decreased resulting in a lower value for the Shannon diversity index in Figure 3.11.

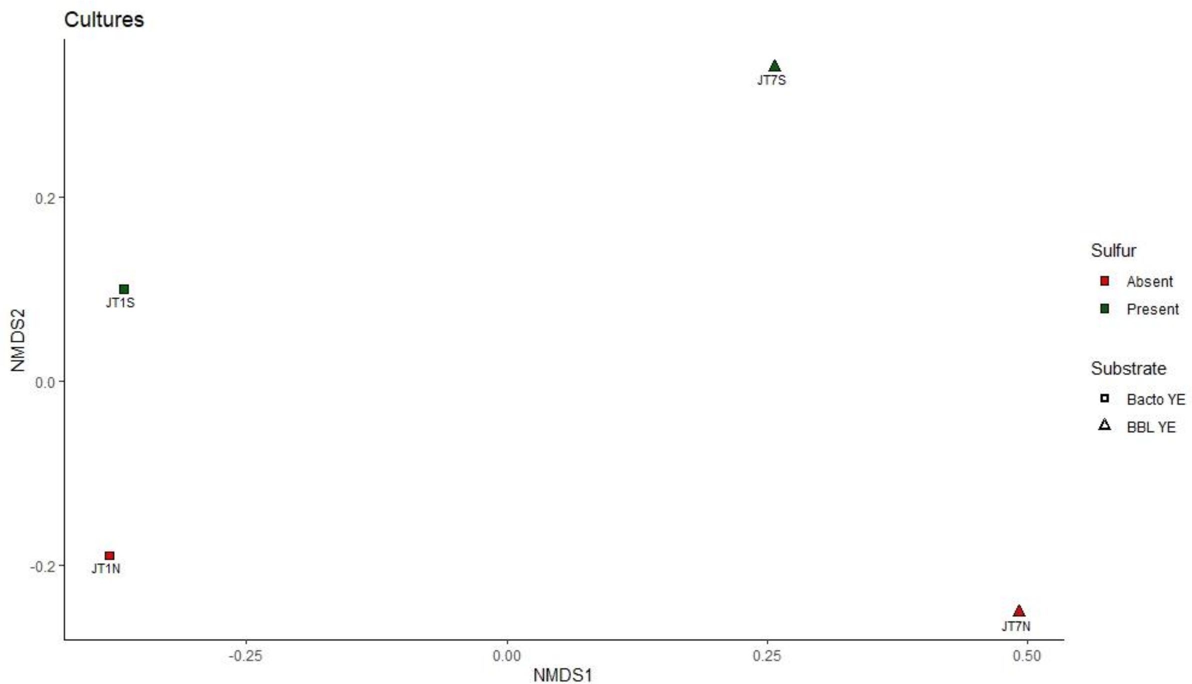

Figure 3.12: Multivariate Analysis of Third Enrichment Cultures. Variation between cultures is shown by plotting NMDs ordination using the Bray-Curtis method. Cultures grown using Bacto YE are represented by square points, and cultures grown using BBL YE are represented by triangular points. The addition of sulfur in each culture is shown by colour (red = absent; green = present).

Figure 3.12 illustrates how microbial growth varies according to the presence of sulfur (green points) and the use of Bacto YE as a substrate (square points). Cultures JT1_S and JT1_N have the most similarity according to the NMDs plot, as both contain ARMAN related organisms and *Leptospirillum* spp, as well as the presence of *Cuniculiplasma* observed across all four cultures. The variation between these two cultures is likely a result of the difference in proportions of the organisms present: a higher abundance of *Cuniculiplasma* is present in culture JT1_N while culture JT1_S includes a larger amount of ARMAN related organisms and *Leptospirillum* spp. Cultures JT7_S and JT7_N are placed farther apart due to their almost exclusive *Cuniculiplasma* content, however the trace amounts of ARMAN related organisms present in culture JT7_S, and the trace amounts of other unassigned organisms present in both cultures, contribute to the variation described in these cultures by Figure 3.12.

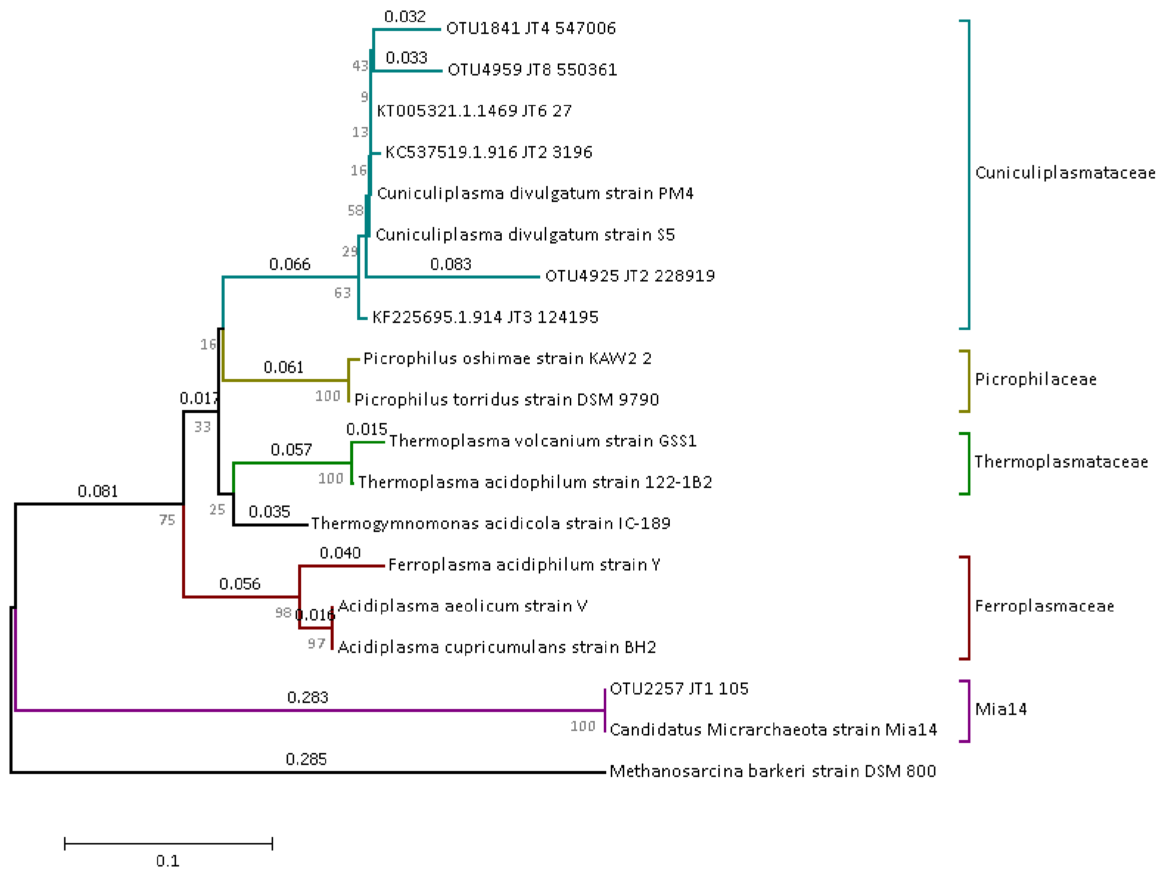

Figure 3.13: Phylogeny of Archaeal OTUs in Third Enrichment Cultures. A rooted phylogenetic tree is shown, displaying archaeal 16S rRNA sequences from cultures alongside those of type strains of described members of the order *Thermoplasmatales*, with *Methanosarcina barkeri* used as an outgroup. The archaeal taxonomy at the family level is displayed using coloured brackets (blue = *Cuniculiplasmataceae*; yellow = *Picrophilaceae*; red = *Ferroplasmaceae*; green = *Thermoplasmataceae*; additionally purple = "Ca. Micrarchaeota" strain Mia14). Type strain sequence data was obtained from the NCBI database. 19 nucleotide sequences were included with missing data removed for a total of 250 positions. Phylogeny was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). Branch lengths are shown in black above branches, with values shorter than 0.01 omitted, and Bootstrap test results (500 replicates) are shown in grey below branches (Felsenstein, 1985). The sum of branch lengths is 1.19002420. The Jukes-Cantor method was used to determine evolutionary distances (Jukes & Cantor, 1969) and gamma distribution was used to model the variation rate.

The affiliation of archaeal OTUs with previously described members of the order *Thermoplasmatales* is shown in Figure 3.13, with "*Candidatus* Micrarchaeota" strain Mia14 additionally shown, and the methanogenic archaeon *Methanosarcina barkeri* used as an outgroup. Bacterial sequences are omitted from the phylogenetic tree as the present work primarily focuses on archaea. The majority of sequences are most closely affiliated with *C. divulgatum*, with one sequence identical in 16S rRNA

affiliation to strain Mia14. No sequences are associated with the taxa *Ferroplasmaceae, Picrophilaceae*, *Thermoplasmataceae* or *Thermogymnomonas* spp. in cultures.

In summary, a culture containing the ARMAN related organism Mia14 was successfully enriched to 35.39 % of the total biomass. The culture additionally contained *C. divulgatum* and *Leptospirillum* sp-like bacteria. This culture was grown with the inclusion of elemental sulfur. A second culture containing 2.52 % Mia14 was additionally obtained, which was dominated by *C. divulgatum* and also contained *Leptospirillum* spp. This variant was grown using the same conditions, but elemental sulfur was removed from this variant. Two other variants grown using a different polypeptide substrate contained over 99.9 % *C. divulgatum* with no significant Mia14 content. The growth of the cultures containing Mia14 was slow, and no parallel variants containing any additional carbohydrate or protein supplements showed any significant growth.

4. Discussion

4.1 Composition of First Enrichment Cultures

The microbial composition of acidophilic cultures is significantly affected by the type of polypeptide substrate used in the first enrichments. A comparison of cultures grown using a variety of yeast and meat extracts indicates that a difference in manufacturer of substrates additionally affects the microbial composition of cultures. It is therefore possible to control which organisms grow in a culture by changing the substrate used during cultivation.

Archaea were associated with substrates containing nitrogen, potassium, phosphate and sulfate, with their abundance being generally higher in more acidic cultures. The most abundant archaea present in cultures were *C. divulgatum*, with some presence of *A. aeolicum* and *F. acidiphilum*– like organisms. The successful growth of these species is partly due to their optimal pH ranges all lying at or below 1.7 as shown in Table 3.1 (Golyshina *et al*., 2000; 2009; 2016b). The most abundant archaeal species in cultures was *C. divulgatum*, which was generally greater in more acidic conditions owing to its extremely acidophilic nature. The substrate BBL BE provided the highest abundance of *C. divulgatum*, and this was the substrate used previously during its isolation (Golyshina *et al*., 2016b). Some cultivation attempts for *Thermoplasmatales* members may therefore be more successful when the optimal substrate is used.

Actinobacteria were associated with substrates containing carbohydrates, chloride and sodium. This is in accordance with the recommendations from Jiang *et al.* (2016) regarding the cultivation of *Actinobacteria* using liquid media: some cultivation methods include the addition of soluble starch, peptones and NaCl, as well as polypeptide substrates. According to the BD Biosciences technical manual (*https://www.bdbiosciences.com/documents/bionutrients_tech_manual.pdf*) BBL Phytone™ Peptone contains high proportions of carbohydrates (392.9 mg/g), sodium (34 mg/g) and chloride (0.76 %w/w), as well as calcium (1001.1 µg/g) and iron (89.8 µg/g). Additionally the abundance of *Actinobacteria* was highest when Tryptone was used in conjunction with Bacto YE. Since Tryptone contains a higher proportion of sodium than other substrates (Table 3.2) this may indicate that sodium content has the most influence over the abundance of A*ctinobacteria* in the Parys Mountain ecosystem.

Proteobacteria were found to be associated with less acidic conditions, and their abundance was highest in the presence of potassium and sulfur. In acidic streamers also sampled from Parys Mountain by Hallberg *et al.* (2006) *Proteobacteria* were found to be the most abundant phylum present,

representing 80 – 90 % of the total microbial diversity. The streamers had a pH of 2.7 – 2.4, representing a less acidic environment than the sediment sampled in the present work. Their abundance is therefore more likely to be affected by acidity rather than a variation in nutritional composition, but sulfur may be involved in their metabolism owing to their presence in both sample sites at the Parys Mountain site.

Firmicutes were present in all cultures, but were mainly associated with less acidic conditions, and they dominated a culture containing a high amount of total nitrogen (12.4 %w/w) and calcium (264 µg/g) from the substrate BBL BE. Their presence in acidic metal rich ecosystems has been previously documented (Korehi *et al.*, 2014; Sanchez-Andrea *et al*., 2011) but in less acidic conditions than those in the present work (pH >2.0). It is therefore likely that variation in their presence in cultures is caused by their association with less acidic conditions. Their domination of culture JT14 (Figure 3.1) may not be a reliable result as their abundance in culture JT15 is very different, while the cultivation conditions are very similar – the only difference being the addition of extra supplements to culture JT15.

Nitrospirae are present in low abundances in all cultures, with their highest abundance in cultures containing Bacto YE. This substrate contained a high amount of iron (55.3 µg/g), so their presence is probably due to *Leptospirillum* sp.-like bacteria oxidising iron in cultures. Their presence in cultures at pH 1.1 is warranted by their extremely acidophilic nature (Corkhill *et al.*, 2008; Jafari *et al.*, 2017).

Acidobacteria were present in trace amounts in less acidic cultures, with a higher abundance in culture JT10. This contained Difco YE, with a high potassium content (59240 µg/g). Despite being represented in some acidic environments (Kielak *et al.*, 2016; Kleinsteuber *et al.*, 2016), *Acidobacteria* are often lacking in cultures (Rappe & Giovannoni, 2003). Their presence has been detected as low as pH 2 (Kleinsteuber *et al.*, 2008) but the cultures in the present work are likely too acidic for their growth.

The bacterial content in the present enrichment cultures reflects the microbial diversity studied using metagenomic analysis in this particular environment (Korzhenkov et al., 2019). The metagenomic data includes the presence of the same major bacterial phyla, although the total bacterial content of this ecosystem was determined to be less abundant at 33 % of total metagenomic reads. Other studies of different sites in Parys Mountain suggest the dominance of *Proteobacteria* and *Actinobacteria* (Kay *et al*., 2013), both of which were significantly enriched in our cultures. The microbial content of the present enrichment cultures is also typical of other AMD ecosystems (Mendez-Garcia et al., 2015) with the same phyla mostly represented in similar proportions elsewhere.

The microbial composition of three sulfidic AMD sites originating from copper mines in Botswana, Germany and Sweden were analysed by Korehi *et al.* (2014), in which the most abundant phyla present

were *Firmicutes* and *Proteobacteria* with some minor representation of *Acidobacteria*, *Actinobacteria*, *Bacteroidetes* and *Nitrospirae*. These copper mines differed from Parys Mountain in having less acidic conditions of pH 3.2 – 6.5, indicating that the bacterial content of the cultures in the present work is represented similarly elsewhere: the main difference being that *Actinobacteria* were found to be substantially more abundant in the present work. This additionally highlights the extremely acidophilic nature of the order *Thermoplasmatales*, as they are lacking in similar but less acidic environments.

4.2 Cultivation of Archaea

The cultivation of members of the order *Thermoplasmatales* from environmental samples was successful in all of the first cultivation experiments, with a large variation in the proportion of archaeal growth between cultures. Cultivation of members of this order is difficult as highly specific growth conditions are required, but archaea grew on all of the polypeptide substrates included in the cultivation experiments. The acidity of enrichment cultures determines much of the microbial diversity, as the NMDS plot in Figure 3.3 indicates a lower diversity in more acidic conditions. This is largely due to the acidophilic nature of the archaea detected here: all archaea present in these cultures are members of the order *Thermoplasmatales* and are extremely acidophilic. Their growth was associated more with cultures at lower pH values, thereby reducing the microbial diversity in these cultures as archaea became more enriched. This is additionally reflected by the Chao1 and Shannon diversity indices in Figure 3.4, with the cultures grown at pH 1.7 having higher diversity measures than those grown at pH 1.1. This is to be expected as fewer organisms have adaptations for survival in more acidic environments (Baker & Banfield, 2003; Baker-Austin & Dopson, 2007).

The high abundance of *C. divulgatum* is a notable feature of many of the present cultures (e.g. Figures 3.6 and 3.9). While there is a possible mechanism by which microbes may dominate an environment as "microbial weeds" (Cray *et al.*, 2013) this is not likely to be the case in these cultures: in the environment from which they were sampled, *C. divulgatum* is represented in a minor abundance (Korzhenkov *et al.*, 2019) and it is only through cultivation techniques that a high abundance was obtained (Golyshina *et al.*, 2016b). Since "E-Plasma" is closely related to *C. divulgatum* (Baker & Banfield, 2003; Korzhenkov *et al.,* 2019; Yelton *et al.*, 2013) and both species inhabit the same environment (Korzhenkov *et al.*, 2019), modified cultivation conditions based on those used for the isolation of *C. divulgatum* were chosen as an appropriate starting point for the attempted cultivation of "E-Plasma" in the present work. However, since *C. divulgatum* is already suited to these conditions it is not surprising that it dominated the present cultures even with modifications.

The global distribution of *Thermoplasmatales* members among AMD sites, and their dominance in acidic environments, is represented in the present work by the detection of *A. aeolicum*, *C. divulgatum* and *F. acidiphilum*–like organisms in enrichment cultures. The first detection of *C. divulgatum* (then referred to as "G-Plasma") was from Iron Mountain, CA, USA by Tyson *et al.* (2004) and despite a significant geographical distance, the genome is quite similar to that found in the environment studied in the present work (Golyshina *et al.*, 2016a; 2017a). Metagenomic studies have detected sequences associated with the organism in similarly acidic environments in Japan (Kato *et al.*, 2011), Italy (Jones *et al.*, 2012), Germany (Ziegler *et al.*, 2013), Spain (Golyshina *et al.*, 2016b) and Mexico (Chen *et al.*, 2018) among other locations (Golyshina *et al.*, 2019b). This is illustrative of the dominance of *Thermoplasmatales* members in low pH environments, as is additionally demonstrated in the present work. There is also apparent diversity among the detected sequences most closely affiliated with *C. divulgatum*, with another strain possibly represented in cultures based on 16S rRNA sequence identity levels. All phylogenetic trees (Figures 3.5, 3.9 and 3.14) show OTUs differentiated by a minimum affiliation of 93.23 % with the two validated strains of *C. divulgatum*, PM4 and S5 (Golyshina *et al.*, 2016b). This is representative of another level of diversity in the Parys Mountain AMD system.

4.3 Cultivation of "E-Plasma"

In the Parys Mountain AMD ecosystem, *C. divulgatum* is represented as a minor group in the microbial community in contrast to "E-Plasma", which comprised 43.5 % of the diversity in sediment samples (Korzhenkov *et al.*, 2019). However, cultivation attempts in the present work do not reflect these abundances. The conditions used during cultivation were modified from previous similar cultivation experiments from the same ecosystem (Golyshina *et al.*, 2016b) but these cultivation conditions seem to be selective for *C. divulgatum*. While there is currently no evidence to suggest any large difference in physiologies between *C. divulgatum* and "E-Plasma" (Korzhenkov *et al.*, 2019; Yelton *et al.*, 2013), the lack of success in the attempted cultivation of "E-Plasma" using current methodologies suggests that a component required for their growth is completely lacking in cultures.

The results of the first cultivation experiments shown in Figure 3.1 indicate that "E-Plasma" was detected in cultures JT1 and JT7, with read abundances of 0.66 % and 3.71 % respectively. Since it is possible to grow "E-Plasma" in cultures from an environmental sample with the use of polypeptide substrates, this suggests that the organism grows heterotrophically. Culture JT1 contained Bacto YE, which according to Table 3.2 contains a high proportion of carbohydrates compared to the other substrates tested in the present work. Similarly, the substrate BBL YE used in culture JT7 contains high proportions of phosphates and amino nitrogen. Both substrates additionally have a higher magnesium

content and a lower sodium content than others. Although both BBL and Bacto yeast extracts have a higher iron content than the other substrates, it was not possible to evaluate whether the oxidation of ferrous iron may be part of the metabolic activity of “E-Plasma”: further experiments conducted using the iron rich 9K medium to test iron oxidation in cultures were unsuccessful, as it was not possible to isolate DNA. This was most likely due to an insufficient number of cells present in these cultures. Phosphate content was increased with the introduction of M398 medium in subsequent cultivation experiments, but this did not increase the abundance of “E-Plasma” in cultures. This suggests that “E-Plasma” requires the presence of carbohydrates, amino nitrogen and magnesium for successful growth, with a possible lack of tolerance to sodium. Figure 3.1 also provides information about the acidophilic nature of “E-Plasma”: the only growth occurred at pH 1.7, with no growth observed in any cultures with more acidic conditions. It is therefore likely that “E-Plasma” may be less acidophilic than the closely related *C. divulgatum*, which grows optimally at pH 1.1 (Golyshina *et al.*, 2016b) while the optimal pH range for growth of “E-Plasma” may be closer to pH 1.7.

The environment dominated by “E-Plasma” contained a high proportion of sulfur in sediment and water samples from Parys Mountain (Korzhenkov *et al.*, 2019) and it was suspected that an increased phosphate content may have promoted a positive effect on the growth of “E-Plasma”, since this nutrient was abundant in both substrates on which “E-Plasma” grew according to Table 3.2. However, cultures containing “E-Plasma” were not enriched through the use of M398 media for increased phosphate levels, or with the addition of sulfur to cultures, as indicated by Figure 3.6. This was possibly due to the lack of an unknown essential nutrient, since cultivation conditions are highly specific. If a particular nutrient was missing from the cultures this may explain the lack of significant growth of “E-Plasma”: even with sufficient amounts of other important nutrients present, growth may be limited by the absence of some other nutrient from cultures. This was problematic for the cultivation of *Spirochaetes* bacteria, as the media traditionally included low amounts of tungsten (Leadbetter, 2003), which inactivates nitrogenase. However, the organisms fix nitrogen during growth (Lilburn, 2001), so cultivation success was improved by using tungsten-reduced media to increase the uptake of nitrogen by the bacteria from the media (Alain & Querellou, 2009). This illustrates how the lack of a specific nutrient in the culture (nitrogen) may prevent the growth of the organisms. In the case of E-Plasma, this missing nutrient may be provided by another organism in the environment, which may indicate a symbiosis or association between organisms which was not replicated during cultivation. This may be due to an interference in intracellular communication caused by the disruption of physical distance between microorganisms during the cultivation process in comparison to their natural state in the environment (Overmann, 2006). The possibility of a symbiosis may be supported by Figure 3.4 in which the cultures containing “E-Plasma” (JT01 and JT07) have the highest diversity indices of all cultures: if

another organism was facilitating the presence of trace amounts of "E-Plasma" in the aforementioned two cultures, its presence was not represented in any of the other cultures as is shown by their lower diversity indices.

4.4 Cultivation of ARMAN Related Organisms

In the present work, sequences were detected in cultures which had a 100 % 16S rRNA affiliation with the ARMAN related organism "*Ca.* Mancarchaeum acidiphilum" (Mia14). Their highest read abundance was first measured at 3.15 % in culture JT1_M398 after a total of six months of cultivation, with trace amounts in three other cultures as shown in Figure 3.6. It was not possible to continue monitoring culture JT1_M398 with DNA barcoding as subsequent attempts to isolate DNA were unsuccessful, most likely due to a lack of sufficient growth. However, after a further three months of cultivation, culture JT1_SUL was found to contain a 35.39 % average abundance of reads derived from Mia14, with a maximum read abundance of up to 50 % (Figure 3.9). This culture previously contained only trace amounts of Mia14.

Currently this represents the third successful attempt of cultivating ARMAN related organisms. The first was conducted by Krause *et al.* (2017) whereby a culture was established containing primarily an archaeon most closely related (91.6 % affiliation) to *T. acidicola*; as well as the acidophilic fungus *Acidothrix acidophila;* ARMAN related organisms; and *C. divulgatum*, which was highly stable over 2.5 years of cultivation. Secondly Golyshina *et al.* (2017a) established a highly enriched culture containing *C. divulgatum*, with a 20 % abundance of the ARMAN related organism Mia14. This facilitated the assembly of the only complete "*Ca.* Micrarchaeota" genome, and represented the first binary culture containing ARMAN related organisms. Thirdly in the present work, a culture was established containing on average 35.39 % Mia14, 35.77 % *C. divulgatum* and 28.84 % *Leptospirillum* spp, which currently represents the highest abundance of ARMAN related organisms to have been cultivated.

The growth rate of ARMAN related organisms is very slow, taking several months to be sufficiently abundant in cultures to be detected using PCR with specific primers. Despite this, the abundance of Mia14 in culture JT1_SUL represents a substantial enrichment compared to their abundance in the environment: in the Parys Mountain AMD ecosystem, Mia14 comprises 0.3 – 0.4 % of the total reads from sediment samples (Korzhenkov *et al.*, 2019). The addition of sulfur to cultures increases the success of the growth of ARMAN related organisms, as culture JT1_SUL had an average abundance of Mia14 32.87 % higher than the variant with sulfur removed (culture JT1_N in Figure 3.9) after the same duration of cultivation. This is evidence to suggest that Mia14 utilises sulfur in its metabolism.

Previously, Krause *et al.* (2017) suggested that Mia14 may utilise ferric sulfate as an electron acceptor. While their focus was primarily on ferric iron, it provides evidence of the potential utilisation of sulfates by Mia14, which would be supported by the results of the present work.

Research involving the metabolism of sulfur by archaea primarily focusses on thermoacidophiles, and the topic is less understood in comparison to bacteria. Within the order *Thermoplasmatales*, members of the genus *Thermoplasma* respire using sulfur under anaerobic conditions (Segerer *et al.*, 1987); this is accomplished with two genes responsible for sulfur reduction (anaerobic sulphite reductases AsrA/ Ta0046 and AsrB/ Ta0047) and one gene encoding sulphide-quinone reductase (Ta1129) (Ruepp *et al.*, 2000). Additionally *A. aeolicum* metabolises sulfur, as it possesses sulfur oxidoreductase (TZ01_04750) and sulfate-adenilyl transferase (TZ01_04545) genes (Bulaev *et al.*, 2017) which facilitate the oxidation of sulfur, with one strain (*Acidiplasma* sp. MBA-1) additionally having been observed to grow using elemental sulfur (Bulaev *et al.*, 2017; Muravyov & Bulaev, 2013).

The metabolism of sulfur has been further studied in the acidophilic thermophilic archaeon *Acidianus ambivalens* of the order *Sulfolobales*. It both reduces and oxidises sulfur depending on the state of cellular contact with sulfur particles: directly accessible sulfur is oxidised while indirectly accessible sulfur is reduced (Amenabar & Boyd, 2018). This is possible due to the possession of genes encoding sulfur oxygenase reductase, sulphite and thiosulfate oxidoreductases, tetrathionate hydrolase, and oxidative 5'-phosphosulfate reductase enzymes (Kletzin, 2008; Kletzin *et al.*, 2004; Laska *et al.*, 2003). The organism additionally metabolises RISC (Reduced Inorganic Sulfur Compounds) by means of tetrathionate hydrolase enzymes bound to the S-layer, with an optimal activity at pH 1 (Protze *et al*., 2011). These enzymes convert elemental sulfur equally into sulfite and sulfide compounds (Rohwerder & Sand, 2007) which are further converted into sulfates by additional enzymatic processes (Keltzin *et al.*, 2004).

Homologues of the genes responsible for sulfur metabolism reported in other archaeal species are mostly lacking in Mia14, with only the gene represented in the capacity of sulfur metabolism being sulfide-quinone oxidoreductase (Mia14_0109) (Golyshina *et al*., 2017a) which is responsible for oxidising sulfide compounds with the utilisation of quinones (Brito *et al.*, 2009). It is therefore possible that elemental sulfur acts as an electron acceptor in the metabolic processes of Mia14, and in this way sulfur may be made available as an energy source; however there is no current evidence of this process in the genome of Mia14 so proteomic studies may be required to investigate this process. It is also possible that the sulfur particles facilitate the survival of Mia14 by providing a surface for the cells to attach to. However, if the mechanism of sulfur metabolism in Mia14 is similar to that of *A. ambivalens*, the possibility of attachment to sulfur particles may suggest that elemental sulfur is instead oxidised.

The metabolic function of sulfur in Mia14 remains unclear and requires further experimentation to investigate these possibilities.

Previous cultivation experiments have suggested an anaerobic mechanism of respiration for Mia14 (Krause *et al.*, 2017), however in the present work it is apparent that these organisms are capable of growth under microaerophilic conditions, suggesting some possible aerobic respiration for ARMAN related organisms. This is supported by findings from Golyshina *et al.* (2017a). Additionally the most successful growth of Mia14 occurred in the presence of M88 medium at pH 1.7, with Bacto YE used as a polypeptide substrate. According to Table 3.2, this may suggest that Mia14 requires carbohydrates, potassium or magnesium for successful growth. There was no evidence of any enrichment of Mia14 with the addition of pullulan, trehalose, collagen, galactan or anhydrous-β-D-glucose to cultures, as no parallel variants prepared using these additional supplements showed any stimulation of growth after two months of incubation. These supplements were chosen as they mimic some nutrients present in the environment at Parys Mountain (Korzhenkov *et al.*, 2019) but due to the lack of any successful growth, it is unlikely that these carbohydrates or proteins are utilised in the metabolism of Mia14.

Mia14 grew in the presence of *C. divulgatum* in all instances, supporting the finding from Golyshina *et al.* (2017a) that *C. divulgatum* is the preferred host for Mia14. Additionally, in cultures JT1_SUL and JT1_N (Figure 3.9) *Leptospirillum*-sp like iron oxidising bacteria comprised similar proportions of cultures in comparison to Mia14. This suggests that in addition to being dependent on *C. divulgatum* as a host, Mia14 may have a possible association with *Leptospirillum* spp. These bacteria may provide metabolites and/ or elements essential for the growth of Mia14, possibly including ferric iron, which may be responsible in part for an increase in the abundance of Mia14 in these cultures. This may add bacteria to the known organisms that ARMAN related organisms interact with, alongside the fungus *A. acidophilia* (Krause *et al.*, 2017) and two *Thermoplasmatales* members (Golyshina *et al.*, 2017b; Krause *et al.*, 2017). However, it is additionally possible that the presence of *Leptospirillum* spp in cultures was coincidental: the bacterial cells may simply have persisted in cultures having passed through the filtration process, and their enrichment may have no effect on that of Mia14.

4.5 Comments on Methodology

The lack of sufficient growth in several cultures was likely the main factor contributing to the unsuccessful isolation of DNA. This was probably the reason for DNA isolation from all 9K and Pyrite variants being unsuccessful, although it is possible that some components present in these cultures

were interfering with PCR (Schrader *et al.*, 2012). This is additionally reflected by other cultures: parallel variants of the 9K cultures were prepared excluding a polypeptide substrate to test if organisms were growing heterotrophically or chemotrophically (data not shown), and despite good growth observed in these cultures, the isolation of DNA was not successful after multiple attempts with various methods. There were additionally several cultures which showed no significant growth, such as the parallel variants of cultures containing ARMAN related organisms prepared with additional supplements. This was likely due to their very slow rate of growth contributing to a lack of sufficient cells for DNA isolation to be successfully conducted.

The re-amplification of PCR products was conducted in some instances in an attempt to amplify DNA despite negative PCR results. However the subsequent DNA barcoding of these datasets provided spurious results, so this method was deemed inaccurate and unreliable in the context of the analysis of biodiversity in cultures.

Filtering cultures following the first cultivation experiments proved to be a successful method of removing the majority of bacterial cells, with the archaeal cells squeezing through the filter due to a lack of a cell wall (Golyshina *et al.*, 2016b). However, the low abundance of “E-Plasma” in cultures coupled with its complete absence following this method may suggest that it was removed during this process. Although this is a possibility, it remains more likely that its unsuccessful enrichment was due to the lack of appropriate cultivation conditions.

Next generation sequencing was used to quantify growth in the cultivation experiments, using Illumina barcoding as the main method of assessing microbial diversity. However this approach has some disadvantages: the use of short DNA fragments restricts BLAST searching, and a different result might be obtained if the entirety of the 16S rRNA gene was sequenced (Ari & Arikan, 2016). Shotgun sequencing may be preferential as information regarding every member of the microbial community is provided, but for the purposes of the present work the Illumina barcoding approach was appropriate, as it generally indicated the correct direction in which cultivation experiments should progress.

4.6 Future Research

The successful cultures grown in present work will be subjected to further research. Further experimentation involving the highly enriched cultures of *C. divulgatum* already suggests that the species may be able to grow both aerobically and anaerobically on sulfur (Personal Communication, Olga Golyshina, 2020). Additionally some subsequent cultivation experiments may focus on

categorising the potentially new strain of *C. divulgatum* that may be present in these cultures, to contribute further to the current knowledge of this species.

The physiology of “E-Plasma” remains unclear: from the present cultivation efforts it seems likely that it is mesophilic, heterotrophic, and does not oxidise iron, but further cultivation experiments must be conducted to evaluate these properties. Subsequent research may additionally focus on the possibility of any symbiotic association of “E-Plasma”: if a highly enriched culture is successfully cultivated in the future, this may involve a metaproteomic study of community interactions.

Cultivation experiments will continue with the cultures containing ARMAN related organisms, and the association of Mia14 with sulfur and *Leptospirillum* spp will be investigated further. The metabolic role of sulfur in Mia14 must also be determined and any ambiguity surrounding the bacterial association must also be tested. Additionally by determining the optimal conditions for the growth of Mia14, more highly enriched cultures may be provided. The additional supplements tested with ARMAN related organisms replicated some nutrients that may be found in the Parys Mountain AMD site; since no clear difference in the stimulation of their growth was observed, some other nutritional supplements may be tested in future research. One such possibility may be a microbial extract, since Mia14 may feed on dead cells in the ecosystem. These cultivation tests may also be conducted using “E-Plasma”, if there are any successful future cultivation attempts. Future research may increase the extent to which ARMAN related organisms are known to interact with other species, as their association with various *Sulfolobales* species is currently being investigated by Hiroyuki Sakai (Personal Communication, Olga Golyshina, 2020) and in this way our understanding of the biology of these organisms may be increased.

4.7 Conclusion

Cultivation experiments in the present work have confirmed that archaea can be grown using polypeptides, and that the choice of substrate affects the microbial composition of cultures, even down to a difference in manufacturer. The difficulty of cultivating archaea is also shown, as the environmental conditions required by these organisms are very specific.

Fundamentally this study investigated the microbial diversity of the Parys Mountain AMD site, offering an insight into the diversity and physiology of microbial “dark matter”. Archaea comprise a large proportion of the uncultured majority of microorganisms, and there is very limited knowledge of these organisms. The lack of significant growth of “E-Plasma” is an indication that current cultivation methods are not suitable for its enrichment from environmental samples. Incubation durations were

in excess of two months, which provided a sufficient opportunity for the organisms to grow. Since the media used here were similar in composition to those previously used for the cultivation of similar species, it is unlikely that the cultures were too nutrient-rich. It is possible that its dominance in the Parys Mountain AMD ecosystem is facilitated by the presence of a nutrient which was completely absent during cultivation attempts, or this may be due to an interconnection with other organisms not captured in cultures. The nutritional requirements for growth of "E-Plasma" are not shared by related species isolated from the same environment (Golyshina *et al.*, 2016a) and metagenomic studies have not revealed the components required for its growth (Yelton *et al.*, 2013). Additionally metabolic predictions (*ibid.*) were not confirmed in a pure culture (Golyshina *et al.*, 2016a) so further cultivation experiments are required for discovering this aspect of the physiology of "E-Plasma". Its successful cultivation may depend on new methods and equipment. Since metagenomic predictions do not accurately reveal details of the physiology, community interactions or ecological functions of microorganisms, cultivation of microbial "dark matter" is important in understanding their biology and exploring their diversity.

The present work represents the highest abundance of ARMAN related organisms to have been cultivated so far. Their growth was most successful when enriched with sulfur, and they grew in the presence of *Leptospirillum*-sp like bacteria. This is indicative of an association of Mia14 with sulfur, whose growth may be additionally stimulated by iron oxidising bacteria. The metabolic pathways of the respiration of sulfur in Mia14 are unknown, but the present work is confirmation of the presence of such processes in mesophilic archaea. Sulfur may be additionally used by the organisms as an attachment point.

The applied aspects of this study relate to the role of acidophilic archaea in environmental pollution from AMD sites. The *Thermoplasmatales* members are distributed globally in acidic, heavy metal-rich environments and their role therein remains to be fully understood, especially in contrast to that of bacteria. The dominance of "E-Plasma" in the Parys Mountain AMD site may indicate an involvement with biogeochemical cycling but the present work was unable to replicate this process in cultivation attempts. However the role of Mia14 in the elemental cycling of sulfur may be of some importance in this ecosystem and future research may focus on investigating this process. The present work is additionally confirmation of its heterotrophic role in carbon cycling.

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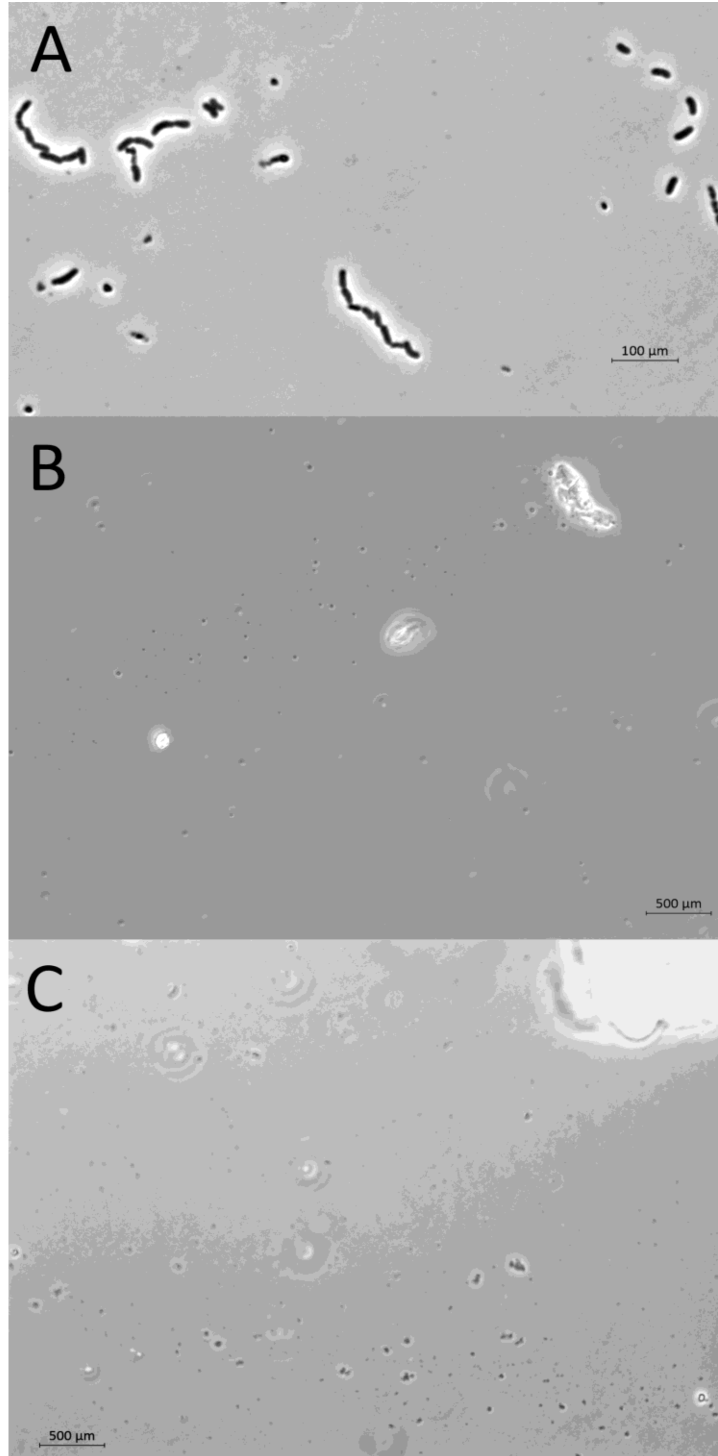
Appendices

Appendix I: Micrographs



Light micrographs at 100 X magnification were taken of enrichment cultures prior to the isolation of DNA for use in barcoding procedures. Here a selection of micrographs corresponding to the culture eventually containing the highest proportion of Mia14 are presented.

A: Light micrograph taken prior to the first DNA barcoding procedure after nine weeks of cultivation following inoculation with environmental samples. This culture corresponds to "JT01" in Figure 3.1. The culture is dominated by bacteria with some archaeal cells present.

B: Light micrograph taken prior to the second DNA barcoding procedure after three weeks of cultivation following inoculation with the cultures previously found to contain traces of E-Plasma, in this case inoculated with culture JT01. This culture corresponds to "JT1_SUL" in Figure 3.6. Mia14 is only present in trace amounts in this culture, with its abundance below 1 %.

C: Light micrograph taken prior to the third DNA barcoding procedure after four months of cultivation following the previous barcoding procedure. This culture corresponds to "JT1_S" in Figure 3.6. The average abundance of Mia14 in this culture is 35.39 %.

Appendix II: R Script

The biodiversity analysis of DNA barcoding results was conducted using R Studio. The code that was written to analyse each dataset is included here. The code was modified for each dataset to include different raw data files, but otherwise the analysis of the data remained consistent in each case. Some figures created for some datasets were omitted from the results as they did not convey any additional or useful information.

```
#############################################
#                                           #
##         JT PM Barcoding Script          ##
#                                           #
#############################################


### 1. READ DATA & CREATE PHYLOSEQ OBJECTS ###

# call libraries
library(ggplot2)
library(dplyr)
library(phyloseq)
library(devtools)
library(ggrepel)

# load data
otu_mat <- read.csv("C:/Users/h/Documents/BANGOR UNI/5. MASTER'S
DEGREE/R/first barcoding/combined average data/OTU matrix.csv")
tax_mat <- read.csv("C:/Users/h/Documents/BANGOR UNI/5. MASTER'S
DEGREE/R/first barcoding/combined average data/Taxonomy table.csv")
samples_df <- read.csv("C:/Users/h/Documents/BANGOR UNI/5. MASTER'S
DEGREE/R/first barcoding/combined average data/Sample metadata.csv")

# define row names from OTU or Culture columns, then delete the
original
row.names(otu_mat) <- otu_mat$OTU+
otu_mat <- otu_mat %>% select (-OTU)+
row.names(tax_mat) <- tax_mat$OTU+
tax_mat <- tax_mat %>% select (-OTU)+
row.names(samples_df) <- samples_df$Culture+
samples_df <- samples_df %>% select (-Culture)

# transform OTU and Taxa tables into matrixes, leave metadata as
data frame
otu_mat <- as.matrix(otu_mat)+
tax_mat <- as.matrix(tax_mat)

# transform to phloseq objects, then combine to one object
OTU = otu_table(otu_mat, taxa_are_rows = TRUE)+
TAX = tax_table(tax_mat)+
samples = sample_data(samples_df)

PM <- phyloseq(OTU, TAX, samples)+
PM
```

```
# visualise data
sample_names(PM)
rank_names(PM)
sample_variables(PM)

# at this point irrelevant data may be removed

# remove unobserved OTUs (sum 0 across all samples)
PM_prune = prune_taxa(taxa_sums(PM) > 0, PM)+
PM_prune

# normalize number of reads in each sample using median sequencing
depth
total = median(sample_sums(PM_prune))+
standf = function(x, t=total) round(t * (x / sum(x)))+
PM = transform_sample_counts(PM_prune, standf)
# number of reads used for normalisation = 6269



### 2. BAR CHART ###


# create bar chart of phya present in each culture, then remove
boundaries

phylum_colors <- c(
  "magenta", "orange","#508578", "#DA5724", "deepskyblue",
"#673770",
  "#AD6F3B", "slateblue4","orchid", "#652926", "red3",
  "thistle3", "darkmagenta","#D1A33D", "#8A7C64", "#599861")
plot_bar(PM_prune, fill="Phylum") +
  geom_bar(aes(fill=Phylum), stat="identity", position="stack")+
  scale_fill_manual(values = phylum_colors) +
  xlab("Culture")+
  theme_classic()+
  theme(axis.text.x = element_text(angle = 90))


### 3. HEATMAP ###


# merge taxa by genus
rank_names(PM)+
PM_merge <- tax_glom(PM_prune, taxrank = "Order")+
PM_merge

# to avoid being very cluttered, filter dataset

# only take OTUs that represent at least 1% of reads in at least one
sample

PM_abund <- filter_taxa(PM_merge, function(x) sum(x > total*0.001) >
0, TRUE)+
PM_abund
```

```
plot_heatmap(PM_abund, method = "NMDS", distance = "bray",
             taxa.label = "Order", taxa.order = "Order",
sample.order = sample_names(PM_abund),
             trans = NULL, low = "white", high = "skyblue4",
na.value = "white")+
  theme_classic()+
  theme(axis.text.x = element_text(angle = 90))


### 4. ALPHA DIVERSITY ###


# create Chao1 richness and Shannon diversity plots

plot_richness(PM, measures=c("Chao1", "Shannon"))+
  xlab("Culture")+
  geom_point(aes(fill = Substrate,shape = pH>1.5),size=2,stroke=1,)+
  scale_fill_manual(values=phylum_colors, breaks=c("Bacto YE",
"Bacto YE + Tryp", "BBL YE", "Difco YE", "BBL BE"))+
  scale_shape_manual(values=c(22,24), name="pH", labels=c(1.1,
1.7))+
  guides(fill = guide_legend(override.aes=list(shape=22)))+
  theme_classic()+
  theme(axis.text.x = element_text(angle = 90))


### 5. ORDINATION (NMDS) ###


# do multivariate analysis using Bray-Curtis distance and NMDS
ordination

PM.ord <- ordinate(PM_merge, "NMDS", "bray")

# plot cultures

plot_ordination(PM_merge, PM.ord, type="Samples", title="Cultures")+
  geom_point(aes(fill = Substrate,shape = pH>1.5),size=2,stroke=1,)+
  scale_fill_manual(values=phylum_colors, breaks=c("Bacto YE",
"Bacto YE + Tryp", "BBL YE", "Difco YE", "BBL BE"))+
  scale_shape_manual(values=c(22,24), name="pH", labels=c(1.1,
1.7))+
  guides(fill = guide_legend(override.aes=list(shape=22)))+
  geom_text(aes(label=sample_names(PM_merge),hjust=-0.3, vjust=0.3),
size=3)+
  theme_classic()
```