Characterizing methanogen and methanotroph diversity, abundance and activity within the Hampshire-Avon catchment

A thesis submitted for the degree of PhD

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Summary:

Methane (CH_4) is an important greenhouse gas and research into its production and oxidation by microbial communities is crucial in predicting their impact in future climate change. Here, potential rate measurements, quantitative real-time polymerase chain reactions (Q-PCR) of *pmoA*, *mcrA* genes and next generation sequencing, were applied to characterize methanogen and methanotroph community structure, abundance and activity in the Hampshire-Avon catchment, UK. Soil and river sediments were taken from sites across different underlying geologies based on their baseflow index (BFI); from low (chalk) to medium (greensand) to high BFI (clay). In general, methane oxidation potentials (MOP) and methane production potentials (MPP) were greater in river sediments compared to soils (particularly higher in clays). Sequence analysis identified *Methanococcoides, Methanosarcina* and *Methanocorpusculum* as candidates driving methanogenesis across all river geologies. *Methylocystis* was also found to predominate in all the river sediments and may be a key methane oxidiser.

In soils microcosms, MOP doubled when temperature was increased from 4^{o}C to 30^{o}C (in greensand soils sampled in summer but not winter). In long-term *in-situ* field warming experiments, MOP was unaffected by temperature in the clay and chalk soils, whereas MOP increased by two-fold in the greensand soils. In both microcosms and field warming experiments *pmoA* abundance was unchanged.

In soil microcosms amended with nitrogen (N) and phosphate (P), high N and low P concentrations had the greatest inhibition on methane oxidation in clay soils, whilst chalk and greensand soils were unaffected. The *pmoA*

gene abundance was also the highest in chalk soils (<2.98 x10^5 gene copies g^{-1} dry weight soil) and was unchanged across treatments. However, in the greensand and clay soils, *pmoA* gene abundance fluctuated with treatment. In long-term field N and P manipulations, regardless of treatment, clay soils had the highest MOP followed by chalk and Greensand. There was also a 10-fold increase in *pmoA* gene abundance across all treatments, and geology.

The findings of this research demonstrated that CH_4 production and oxidation in soils and sediments can be tied to different underlying geologies, with clay geologies having the highest CH_4 production and oxidation. In addition, soil temperature changes are found to likely be secondary factors affecting methanotrophs, with MOP only increasing with temperature if CH_4 is abundant. N and P additions to soils had an overall negative effects on methanotrophy in clay soils but an overall positive effect in chalk soils, and no effect on greensand soils. These results may enable more targeted catchment management strategies to be performed to mitigate future increases in CH_4 concentrations.

Acknowledgements

I would like to express my immense gratitude to my supervisors, Corinne Whitby and Dave Nedwell, whose knowledge, guidance, and (infinite) patience have made this thesis possible. Thanks also to everyone in Lab 3.07, 5.28, and 5.32, in particular our technicians, Tania, Farid, and John for offering their technical expertise. Thanks also to my fellow PhD students, Scott, Gordon, Dave, Tom, Aisha, Ben, Jess, Ahmed, Tiv'kaa, Daniel (black), Daniel (white), Nikoletta, Duncan, Matt, James, Yaz, and Guilia for your friendship and your morale support ~~and for carrying me home after a night on the slash.~~ Many thanks also to the postdocs and academics, Boyd, Maddy, Anne, Myrsini and Alex for lending their expertise and advice to this project and life in science. Thanks also to the members of the NERC Macro Nutrient Cycle grant, Drs Kate Hepell, Katrina Lansdown, Prof Mark Trimmer, James Stockdale and Prof Phillip Ineson for their help in collecting the relevant soil and sediment background data.

To Aisha, I'm grateful that I had the opportunity to know you and to be your friend. Whatever awaits beyond this brief period of existence, I hope someday we can meet again. So until then, Rest in Peace, wherever and whenever you might be.

To my parents, siblings and nieces, thank you for making the time to help and support me during these four long years. I love you all.

Abbreviations

CH_3-S-CoM- Methyl-coenzyme M

DGGE- Denaturing Gradient Gel Electrophoresis

DNA- Deoxyribonucleic acid

dNTP- Deoxyribonucleotide

EDTA- Ethylenediaminetetraacetic acid

GHG- Greenhouse gas

mcrA- Methy-coenzyme M reductase component alpha gene

MCR- Methyl-coenzyme M reductase

MMO- Methane Monooxygenase

MOP- Methane Oxidation Potential

MPP- Methane Production Potential

NMS- Nitrate minimal salt

PCR- Polymerase chain reaction

pMMO- Particulate methane monooxygenase

pmoA- Particulate methane monooxygenase gene

Q-PCR- Quantitative polymerase chain reaction (a.k.a. real-time PCR)

RNA- Ribonucleic acid

rRNA- Ribosomal ribonucleic acid

RUMP ribulose monophosphate pathway

SIP- Stable Isotope Probing

sMMO- Soluble methane monooxygenase

SRB- Sulfate Reducing Bacteria

TAE- Tris acetate-EDTA

F_{430}- Cofactor F_{430}

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Chapter 1: Introduction: Methane sources and sinks

Global warming is mainly due to an increase in the concentration of atmospheric greenhouse gases (GHG). After carbon dioxide (CO_2), methane (CH_4) is the second most important GHG, contributing to ~30% of positive global Radiative Forcing (RF) (IPCC, 2002). Moreover, methane at a current atmospheric concentration of 1.77 µmol mol^{-1} is responsible for 16% of the global warming, due to its relatively high global warming potential (Serrano-Silva et al 2014). Of particular concern, is that atmospheric CH_4 concentrations are reported to be increasing at a rate of 0.003 µmol CH_4 mol^{-1} air $year^{-1}$ (Khalil *et al.*, 2007) and with further predicted increases in global temperature, suggests there will be further increases in atmospheric CH_4 (Kvenvolden, 2002). This is due to large quantities of CH_4 trapped in arctic areas, (either under ice or in ice as methane hydrate) (**Figure 1.1**), being released if these regions begin defrosting (Kvenvolden, 2002). The predicted amount of trapped CH_4 in hydrates is between 7,500-400,000 Tg. Thus, even a small release of this source of CH_4 could potentially increase RF considerably (McGeehin *et al.*, 2008).

It has been estimated that total annual emissions of CH_4 are around 500 Tg $year^{-1}$ emitted from both natural and anthropogenic sources (Whalen 2005) (**Figure 1.1**). Whilst natural sources account for around 40% of global CH_4 emissions, the majority of CH_4 emissions come from anthropogenic activity such as industry, landfill sites, natural gas production, livestock, agriculture etc. (Conrad, 2009; Karakurt and Aydiner, 2012).

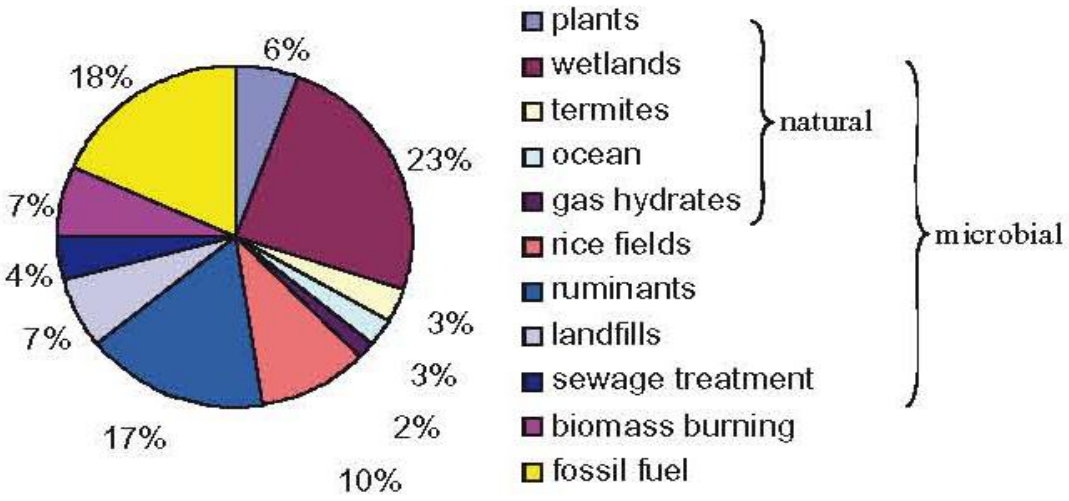


Figure 1.1: Sources of CH_4 emissions (Conrad, 2009)

In terms of natural sources and sinks of CH_4, forests are an important sink (Grunwald *et al.*, 2012), whilst wetlands are a major biological source (Conrad, 2009), contributing 145 Tg CH_4 $year^{-1}$ (Whalen, 2005). Of particular importance are flooded rice paddies, which are among the largest sources of atmospheric CH_4 contributing approximately 18% of total global emissions (IPCC, 2013). Temperate soils which contain one-third of the global organic C pool are also important in the global budget of CH_4 (Post *et al.*, 1982; Jungkunst, 2010).

1.1: Factors affecting CH_4 fluxes in terrestrial environments

Terrestrial environments play an important role in the CH_4 cycle as methanotrophy (oxidation of CH_4) and methanogenesis (production of CH_4) take place within them (Serrano-Silva *et al*., 2014). Soils (depending on the conditions), can be a source or a sink of CO_2 and CH_4. For example, pristine soils are primarily a sink for CH_4 and sequester as much carbon as they emit CO_2, but due to the impact of human activities, such as agriculture, soils are

often a source for GHG emissions (Christiansen *et al.*, 2012).

There are many factors controlling CH_4 fluxes in terrestrial environments including: whether conditions are aerobic or anaerobic, redox potential, electron acceptors, substrate availability, temperature, water availability and depth of the water table, soil pH, salinity, fertilizer applications, trace metals, competitive inhibition, vegetation, and CO_2 concentrations (Dalal *et al.,* 2008). The concentration and type of organic matter as well as O_2 concentration are major determining factors for CH_4 production (Dalal *et al.*, 2008). In contrast, fertilizer additions such as urea have been shown to decrease CH_4 emissions in rice paddy fields (Sethunathan *et al.*, 2000). Increased CO_2 concentrations and high temperatures have been shown to increase methanogenesis and decrease CH_4 oxidation in tropical rice soils (Das and Adhya, 2012). Thus, understanding the relationship between methanotrophy and methanogenesis in terrestrial environments will contribute to better ability to manage CH_4 emissions. The two key groups of organisms governing methanogenesis and methane oxidation are methanogens and methanotrophs, producers and oxidizers of CH_4 respectively (**Figure 1.2**) (Nazaries *et al.*, 2013).

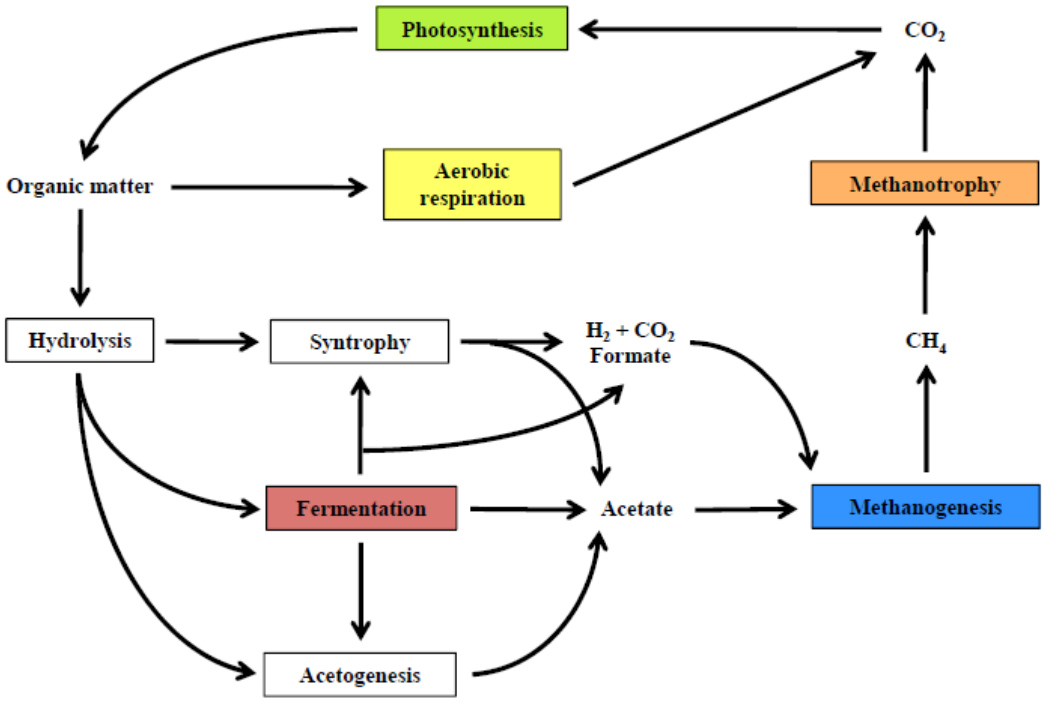


Figure 1.2: Reduction and oxidation cycle of carbon (Nazaries *et al.*, 2013).

1.2: Microbiology and Biochemistry of Methanogenesis

Methanogens are a diverse group of CH_4 producing Archaea, belonging to the Kingdom Euryarchaeota (**Figure 1.3**) (Bang and Schimtz, 2015). They are the only microorganisms known to produce CH_4 as a catabolic end product. Interest in methanogens stemmed originally from emission of CH_4 in landfills and sediments (reviewed in Garcia *et al.*, 2000). More recently, research into methanogen diversity and activity in the environment has become more intensive (Nazaries *et al.* 2013).

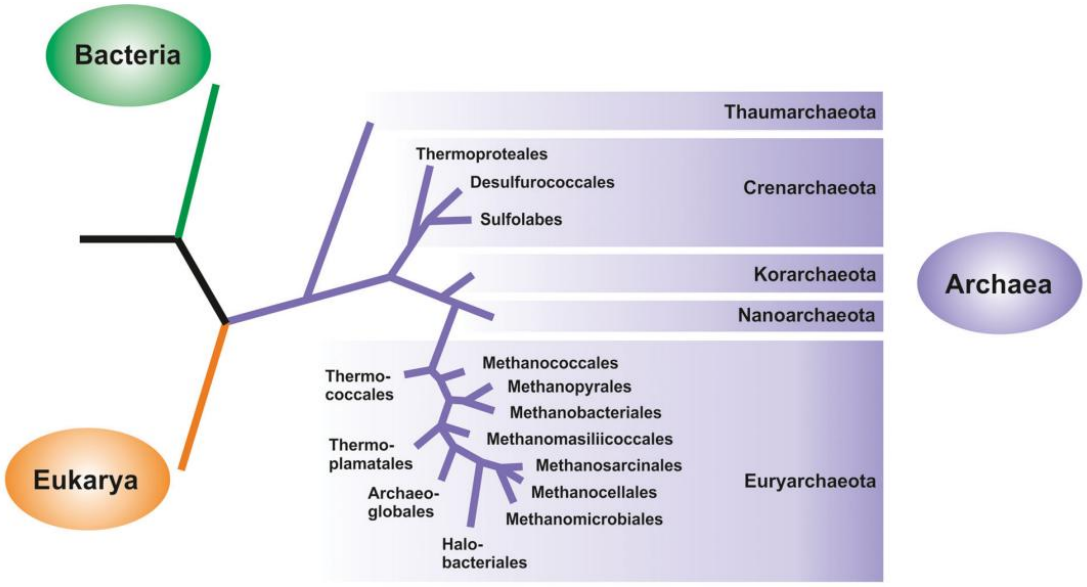


Figure 1.3: Phylogenetic tree showing seven major methanogen orders within the domain Archaea and kingdom Euryarchaeota (Bang and Schimtz, 2015).

Methanogens are ubiquitous in the environment and can be found in peat bogs, the digestive tract of almost all animals, ruminants, freshwater habitats, hydrothermal vents, petroleum reservoirs, anaerobic sewage digesters, rice paddy fields and sediments (Thauer *et al.*, 2008). Methanogenic microorganisms are strict anaerobes and therefore their growth and activity takes place only at low redox potentials such as in waterlogged soils or anoxic sediments (Smith *et al.*, 2003). Methanogens however, may also persist in oxic zones and are readily activated when wet anoxic conditions become available (Angel *et al.* 2011; 2012*)*.

1.2.1: Taxonomy and Phylogeny of Methanogens

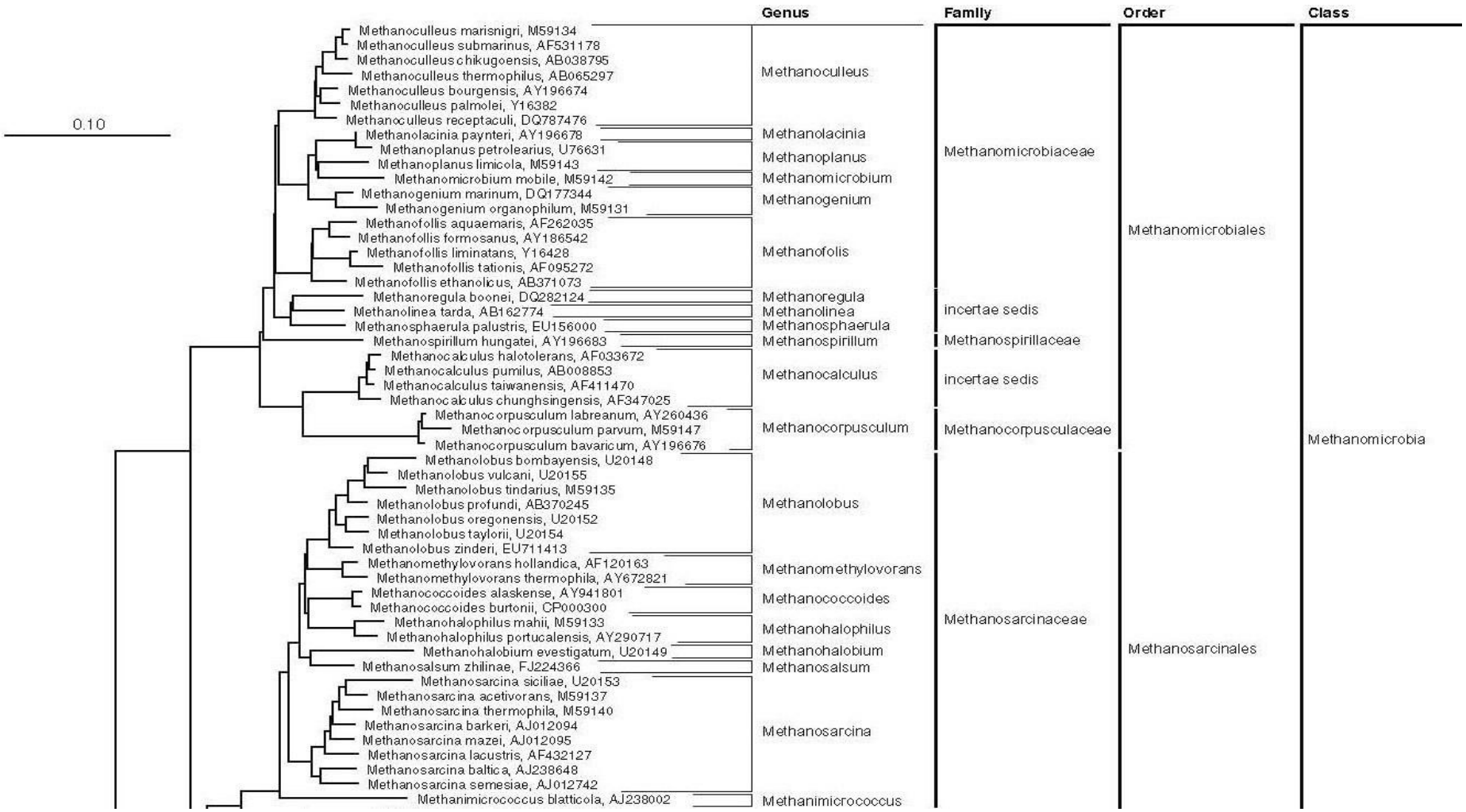
Methanogens are a relatively diverse group of microorganisms with more than 100 species identified thus far (Narihiro and Sekiguchi, 2011). Overall the species are distributed amongst 33 genera (Narihiro and

Sekiguchi, 2011). The various genera of methanogens are diverse in morphology, including rods, cocci, spirilla, and chains (Smith and Baresi, 1989).

There are four classes of methanogens; Methanomicrobia, Methanobacteria, Methanococci, and Methanopyri (**Figure 1.4**) (Narihiro and Sekiguchi, 2011). These four classes are further split into 6 orders; Methanobacteriales, Methanocellales, Methanococcales, Methanopyrales, Methanomicrobiales, and Methanosarcinales (**Figure 1.4**) (Narihiro and Sekiguchi, 2011), and more recently a seventh putative order named Mx was described (Borrel *et al.*, 2013).

Methanogens under the order Methanobacteriales have pseudomurein cell walls, containing C_{20} and C_{40} isopranyl glycerol ethers, and are non-motile (Bonin and Boone, 2006). Two families fall within this order (i.e. Methanobacteriaceae and Methanothermaceae), with species occupying diverse environments, from deep ground waters to bovine rumen and volcanic springs (Bonin and Boone, 2006). The methanogens in the order Methanococcales have proteinaceous cell walls, containing C_{20} isopranyl glycerol ethers (with C_{40} isopranyls glycerol ethers only present in *Methanocaldococcus jannaschii*) (Whitman and Jeanthon, 2006). All Methanococcales species are hydrogenotrophic and have been isolated from marine and coastal environments and are separated into Methanococcaceae and Methanocaldococceae (Whitman and Jeanthon, 2006). Methanogens under the order Methanomicrobiales are also hydrogenotrophic and contain the families Methanomicrobiaceae, and Methanospirillaceae (Garcia *et al.*, 2000).

Methanogens have also been categorized into three different classes (Bapteste *et al.,* 2005); Class I, which includes the orders Methanobacteriales, Methanococcales and Methanopyrales. Class II, which contain the order Methanomicrobiales and Class III, which is made up of the order Methanosarcinales (Anderson *et al.*, 2009). The evolutionary relationship of the three Classes was shown to be separated by a branch of non-methanogenic archaea (Bapteste *et al.*, 2005). However, it has been suggested that methanogenesis evolved in a common ancestor in all three branches, before the non-methanogenic archaea lost their original CH_4 generating function (Gribaldo and Brochier-Armanet, 2006). Functionally, all the classes are largely similar, with very narrow substrate utilizations. However, Class I methanogens have several absent proteins (that are present in the other two Classes), e.g. cofC (cofactor F420 synthesis), comA, comB, and comC, (Coenzyme M synthesis), and hmdI (used during methanogenesis) (Graham *et al.*, 2002; Bapteste *et al.*, 2005).


Genus
Family
Order
Class
0.10
Methanoculleus marisnigri, M59134
Methanoculleus submarinus, AF531178
Methanoculleus chikugoensis, AB038795
Methanoculleus thermophilus, AB065297
Methanoculleus bourgensis, AY196674
Methanoculleus palmolei, Y16382
Methanoculleus receptaculi, DQ787476
Methanolacinia paynteri, AY196678
Methanoplanus petrolearius, U76631
Methanoplanus limicola, M59143
Methanomicrobium mobile, M59142
Methanogenium marinum, DQ177344
Methanogenium organophilum, M59131
Methanofollis aquaemaris, AF262035
Methanofollis formosanus, AY186542
Methanofollis liminatans, Y16428
Methanofollis tationis, AF095272
Methanofollis ethanolicus, AB371073
Methanoregula boonei, DQ282124
Methanolinea tarda, AB162774
Methanosphaerula palustris, EU156000
Methanospirillum hungatei, AY196683
Methanocalculus halotolerans, AF033672
Methanocalculus pumilus, AB008853
Methanocalculus taiwanensis, AF411470
Methanocalculus chunghsingensis, AF347025
Methanocorpusculum labreanum, AY260436
Methanocorpusculum parvum, M59147
Methanocorpusculum bavaricum, AY196676
Methanolobus bombayensis, U20148
Methanolobus vulcani, U20155
Methanolobus tindarius, M59135
Methanolobus profundi, AB370245
Methanolobus oregonensis, U20152
Methanolobus taylorii, U20154
Methanolobus zinderi, EU711413
Methanomethylovorans hollandica, AF120163
Methanomethylovorans thermophila, AY672821
Methanococcoides alaskense, AY941801
Methanococcoides burtonii, CP000300
Methanohalophilus mahii, M59133
Methanohalophilus portucalensis, AY290717
Methanohalobium evestigatum, U20149
Methanosalsum zhilinae, FJ224366
Methanosarcina siciliae, U20153
Methanosarcina acetivorans, M59137
Methanosarcina thermophila, M59140
Methanosarcina barkeri, AJ012094
Methanosarcina mazei, AJ012095
Methanosarcina lacustris, AF432127
Methanosarcina baltica, AJ238648
Methanosarcina semesiae, AJ012742
Methanimicrococcus blatticola, AJ238002
Methanoculleus
Methanolacinia
Methanoplanus
Methanomicrobium
Methanogenium
Methanofolis
Methanoregula
Methanolinea
Methanosphaerula
Methanospirillum
Methanocalculus
Methanocorpusculum
Methanolobus
Methanomethylovorans
Methanococcoides
Methanohalophilus
Methanohalobium
Methanosalsum
Methanosarcina
Methanimicrococcus
Methanomicrobiaceae
incertae sedis
Methanospirillaceae
incertae sedis
Methanocorpusculaceae
Methanosarcinaceae
Methanomicrobiales
Methanosarcinales
Methanomicrobia

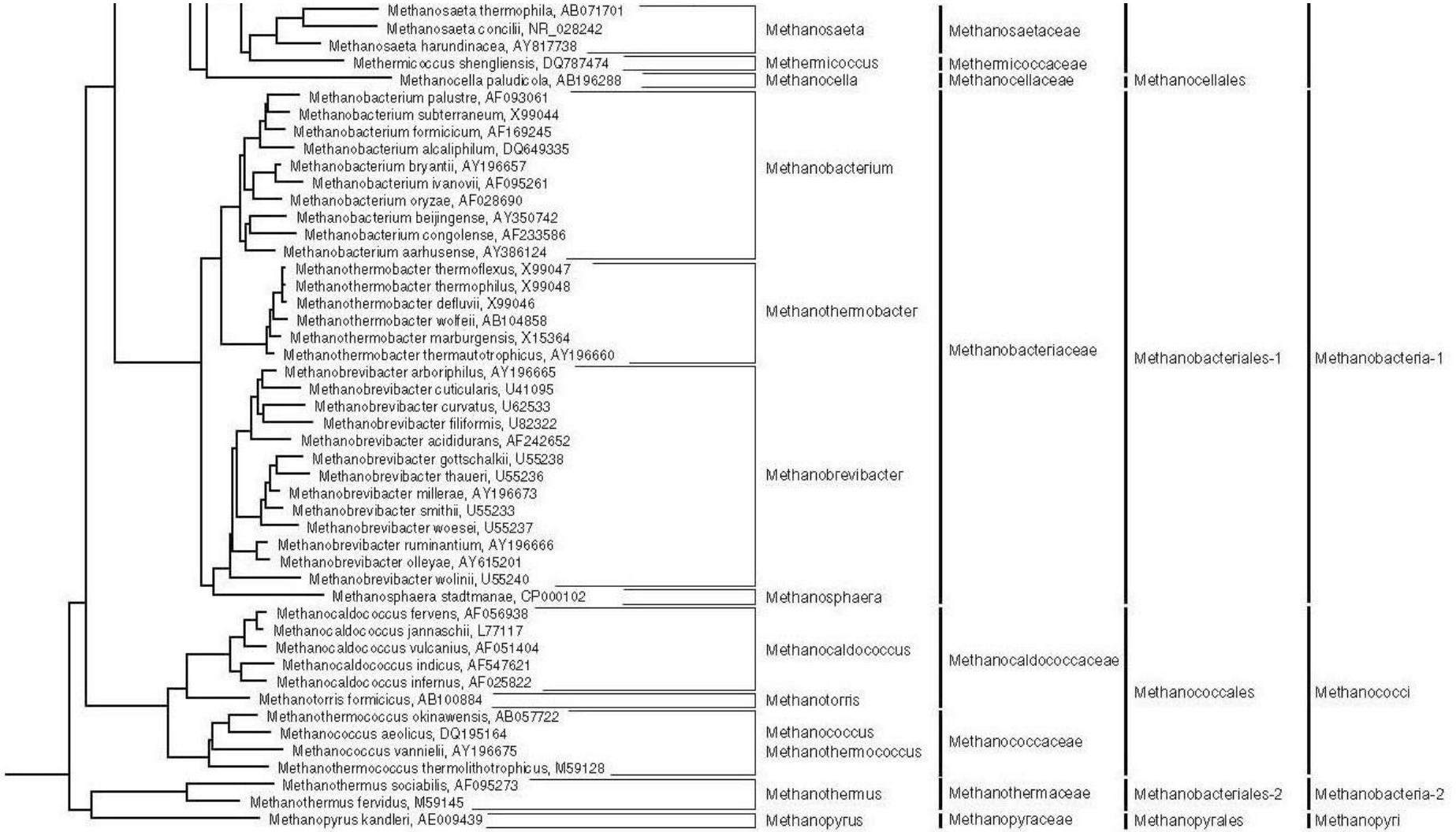


Figure 1.4: 16S rRNA phylogenetic tree of methanogens (Narihiro and Sekiguchi 2011).

1.2.2: Biochemistry of Methanogenesis and Methanogens

Methanogenesis comprises a four-step process during the degradation of complex organic compounds to CO_2 and CH_4 i.e. step 1) Hydrolysis of carbon polymers; step 2) Acidogenesis via Bacterial fermentation; step 3) Acetogenesis of metabolites from fomenters; step 4) Methanogenesis from acetate, H_2 and CO_2 (**Figure 1.5**) (Appels *et al.*, 2008).

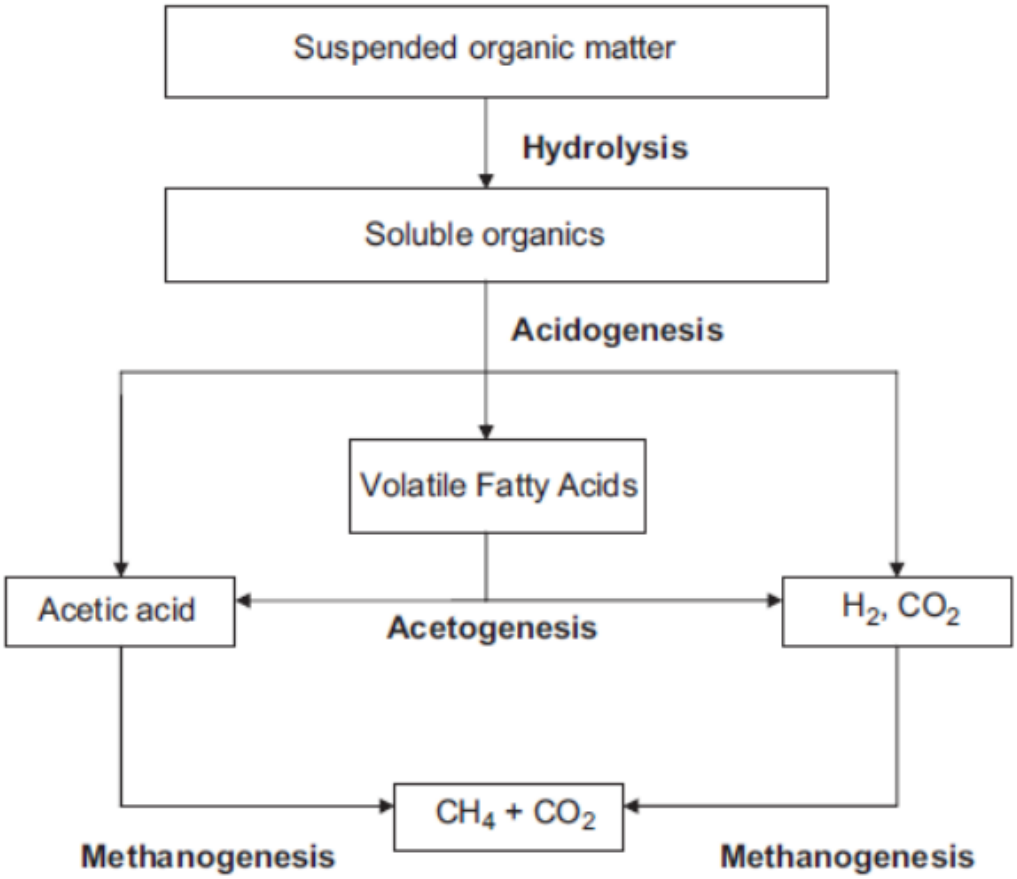


Figure 1.5: Stages leading to methanogenesis (Appels *et al.*, 2008)

Methanogens are therefore dependent on other microorganisms to provide the suitable substrates and reducing conditions necessary to survive, and so function as part of a larger microbial community (Topp and Pattey, 1997). Methanogens have been further divided into five groups according to the substrates they use (Garcia *et al.*, 2000; Le Mer and Roger, 2001):

i) hydrogenotrophs ($4H_2 + CO_2 \Rightarrow CH_4 + 2H_2O$); use H_2 as electron donors during various stages of CH_4 production, with CO_2 as a carbon source (e.g. *Methanobacterium aggregans*)

ii) formatotrophs ($4HCOOH \Rightarrow CH_4 + 3CO_2 + 2H_2O$); utilizes formate as an electron source (e.g. *Methanobrevibacter arboriphilus*)

iii) acetotrophs ($CH_3COOH \Rightarrow CO_2 + CH_4$); utilizes acetate as an electron donor as well as a carbon source (e.g. *Methanosarcina barkeri*)

iv) methylotrophs ($4CH_3OH \Rightarrow 3CH_4 + CO_2 + 2H_2O$, $4CH_3NH_2 + 2H_2O \Rightarrow 3CH_4 + CO_2 + 4NH_4^+$); utilizes various methyl compounds e.g. methanol and methylamine as electron donors and/ or carbon sources (e.g. *Methanolobus profundi*)

v) alcoholotrophs ($4CH_3CHOHCH_3 + CO_2 \Rightarrow CH_4 + 4CH_3COCH_3 + 2H_2O$); utilizes various alcohols as both electron donors and C source, although CO_2 also usable as C source (e.g. *Methanosphaera stadtmanae*)

It has been suggested that methanogens that use acetate, mainly as C source can contribute between 70-90% of total CH_4 production, although, this amount will vary depending on the *in-situ* environmental conditions (Garcia *et al.*, 2000; Norina, 2007). For example, freshwater sediments and flooded paddy soils produce around 70% of biogenic CH_4 from acetoclastic methanogens (Conrad, 2005). Members of Class III methanogens only use acetoclastic and methyloclastic routes of methanogenesis (Bapteste *et al.,* 2005). Acetoclastic conversion to CH_4 is the dominant pathway for methanogenesis in detritus rich environments (e.g. anaerobic digesters, peat bogs) and approximately two thirds of CH_4 is produced this way in these environments (Jetten *et al.*, 1992). The remaining third of methanogenesis is

due to hydrogenotrophy, with methylotrophy making up the smallest fraction of methane production.

The order Methanosarcinales contains all acetotrophic and methylotrophic methanogens, including Methanosarcinaceae and Methanosaetaceae(Garcia *et al.*, 2000). Acetoclastic methanogenesis ferments acetate (as well as pyruvate) in order to produce CH_4 (Madigan and Martinko, 2006). In this pathway acetate is first converted into acetyl CoA, either by creating acetyl phosphate from acetate, by acetate kinase, followed by the addition of CoA to the acetyl phosphate by phosphotransacetylase to produce acetyl CoA, or Acetyl-CoA synthase converts acetate to acetyl-AMP then converts acetyl-AMP to acetyl-CoA, when CoA is present (White, 2006). The former is seen in *Methanosarcina spp.* and the latter is observed in *Methanothrix s*pp (Jetten *et al.*, 1992). The methyl group in acetyl-CoA is eventually used to methylate THMP (steps 13-9, **Figure 1.6**) and from there it follows the remaining pathway to CH_4 production (steps 6-8 **Figure 1.6**) (White, 2006).

Hydrogenotrophic methanogenesis utilizes H_2 as an electron donor with CO_2 as a carbon source when producing CH_4. CO_2 is incorporated into the cell and through a series of reduction reactions in the acetyl-CoA pathway leads to CH_4 as an end product (**Figure 1.6**) (White, 2006). The methanogen species within the order Methanococcales are all hydrogenotrophic and have been isolated from marine and coastal environments (Garcia *et al.*, 2000). Methanogens under the order Methanomicrobiales are also hydrogenotrophic and contain the families Methanomicrobiaceae, and Methanospirillaceae (Garcia *et al.*, 2000).

Methanogens utilising the methylotrophic pathway can use several methyl compounds, including methanol. The general reduction pathway in presence of H_2 can be summed as:

$$CH_3OH + H_2 \Rightarrow CH_4 + H_2O$$

Or when no H_2 is present CH_3OH can reduced by CH_3-SCoM reductase:

$$4CH_3OH \Rightarrow 3CH_4 + CO_2 + HCO_3^-$$

Other methyl compounds include methylamine, dimethylamine, trimethylamine, methylmercaptan, and dimethylsulfide.

Additionally, formate and carbon monoxide can be used as an alternate to CO_2 (Zinder and Anguish, 1992). Whether utilizing acetate or H_2 and CO_2 to produce CH_4, the process of CH_4 production is the only method of acquiring ATP for methanogens. Thus functional genes regulating methanogenesis are well conserved amongst this group of microorganisms (Luton *et al.*, 2002). In addition to carbon, all methanogens use NH_4^+ as N source, however four orders of methanogens, i.e. Methanobacteriales, Methanococcales, Methanomicrobiales and Methanosarcinales have demonstrated the ability to fix N_2 gas and contain the N fixation gene (*nif*) (Leigh, 2005).

1.2.3: Methyl coenzyme M (CH_3-SCoM)

Methyl coenzyme M (CH_3-SCoM) acts as an intermediate for the primary substrates from the various pathways (Lovley *et al.*, 1984), and CH_3-SCoM acts as the final substrate in the methanogenesis pathway (**Figure 1.6**). The enzyme CH_3-SCoM reductase (MCR) is a nickel-containing enzyme found in all known methanogenic Archaea and is responsible for the conversion of CH_3-SCoM to CH_4 (Prakash *et al.*, 2014). MCR constitutes 5-12% of methanogen cellular protein

(Bokranz *et al.*, 1988) and which is further made of three components A, C and a small co-factor B, with component C thought to be the site for methyl reduction (Cram *et al.*, 1987). Component C is composed of three subunits; α, β, and γ which encoded by the *mcrA, mcrB* and *mcrG* genes respectively (Grabarse *et al.*, 2001). Sequencing of the *mcrA* gene revealed an evolutionary relationship between Archaeal anaerobic methanotrophs (ANME) and methanogens (Shima and Thauer, 2005) (See Section 1.3.4).

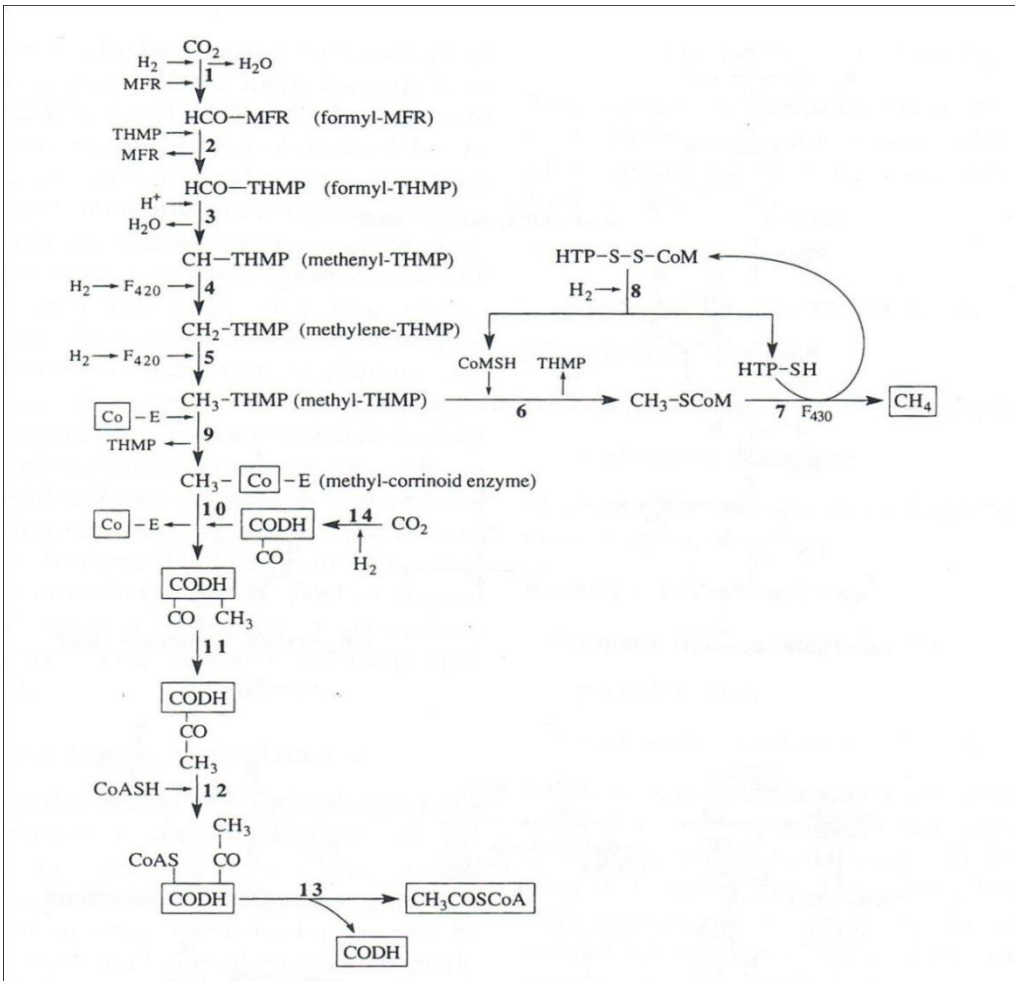


Figure 1.6: Acetyl CoA pathway in methanogens. Key stages in CH_4 production in this pathway are the incorporation of the methyl group from CH_3-THMP into CoMSH forming CH_3-SCoM (reaction 6). The methyl group in CH_3-SCoM is then reduced by methyl coenzyme M reductase into CH_4 (reaction 7). Reaction 1: Reduction of methanofuran (MFR) into formic acid (formyl-MFR). Reaction 2: Transfer of formyl

group to carrier protein tetrahydromethanopterin (THMP). Reaction 3-5: Reduction of formyl-THMP into methyl-THMP (CH_3-THMP). Reaction 6-8: Methyl reductase system. Reaction 9-13: CH_3-THMP alternatively donates CH_3 to form acetyl CoA (White, 2006).

1.2.4: Physiology and Ecology of Methanogens

Extensive research has been conducted on methanogens in both natural and modified ecosystems, in order to better understand their role in the environment. For soils, researchers have concentrated largely on common sources of CH_4 such as rice fields, landfill sites, and swamps, as well as other environments such as termite mounds (**Figure 1.1**) (**Table 1.1**) (Conrad 2009). Depending on the habitat, the composition of methanogens can vary widely (Garcia *et al.*, 2000). Peat soils have been extensively studied, as the accumulation of decayed plant matter and high water content provides ideal conditions during decomposition for methanogens, making them a prime source for CH_4 (White *et al.*, 2008). Yavitt *et al.* (2006) found that peat land was dominated by Methanomicrobiales, with Methanosarcinaceae relegated to the deepest sections of the peat, suggesting a prevalence of hydrogenotrophy in these soils.

Table 1.1: Summary of the key methanogens found in different environments

Environment	Methanogens	Key notes	Reference
Landfills	Methanobacteriales Methanosarcinales Methanomicrobiales	Major source of CH_4	Luton *et al.*, 2002 Lalouis-Carpentier *et al.*, 2006
Rice paddy	*Methanosarcina* *Methanocellales* *Methanobacteriales* *Methanomicrobiales*	Major source of CH_4, fertilizer use changes methanogen diversity, dry wet cycle affects CH_4 efflux	Großkopf *et al.*, 1998 Conrad *et al.*, 2012 Liu *et al.*, 2016
Peat soil	Methanomicrobiales Methanosarcinaceae	Majour source of CH_4, major store of organic C, arctic peat bogs potentially cause positive feedback on thawing	Yavitt *et al.*, 2006 Kotsurbenko *et al.*, 2007
Rumens	*Methanobrevibacter* *Methanosphaera*	Major source of CH_4, methanogens affected by feed quality	Zhou *et al.*, 2010 King *et al.*, 2011 Lee *et al.*, 2013
Lakes	*Methanomicrobium* *Methanobacterium* *Methanosarcina* *Methanosaeta*	Significant source of CH_4	Mach *et al.*, 2015
Rivers	*Methanomicrobium* *Methanobacterium* *Methanosarcina* *Methanosaeta*	Significant source of CH_4	Mach *et al.*, 2015
Hydrothermal vents	*Methanothermobacter* *Methanothermus* *Methanothermococcus* *Methanocaldococcus* *Methanotorris* *Methanopyrus*	Thermophilic methanogens (up to 110oC)	Jones *et al.*, 1989 Eecke *et al.* 2012 Slesarev *et al.*, 2002
Various crude oil environments	*Methanosaeta* *Methanoculleus* *Methanobacterium*	Terminal stages of crude oil degradation, use as energy source	Berdugo-Clavijo and Gieg, 2014

Rice paddy fields are a similarly important habitat for methanogens, as the stagnant water saturated soils provide conditions favoured by methanogens (Conrad *et al.*, 2012). Methanogen species composition within rice paddies differed between different geographic locations, including species from Methanobacteriales, Methanocellales and Methanosarcinaceae, although the latter was noted to be a dominant group across the different locations (*Großkopf et al.,* 1998*;* Conrad *et al.*, 2012). Landfill sites have been extensively investigated as another major CH_4 source and methanogen habitat; several methanogen species have been found including species from Methanobacteriales, Methanosarcinales, and Methanomicrobiales (Luton *et al.*, 2002), with Methanosarcinales found to be a major methanogen present in landfill effluent, comprising up to 65% of the sequences identified in a study by Laloui-Carpentier *et al. (*2006). Furthermore, additions of hydrogen and formate to effluent samples showed increases in Methanomicrobiales but Methanosarcinales remained dominant (Laloui-Carpentier *et al.*, 2006). Several less prominent soil sources of methane have also been investigated; the order Methanomicrobiales has been found to be abundant in nutrient rich fens, whilst Methanosaetaceae dominated in less nutrient rich fens (Godin *et al.*, 2012), and within aerated upland soils the genera *Methanocella* and *Methanosarcina* were found to be dominant, with two thirds of methanogenesis being attributed to the latter (Angel *et al.*, 2012)***.***

Another major source of CH_4 is emissions from ruminants, especially those from cattle farming, with genera such as *Methanobrevibacter* and *Methanosphaera* being dominant in the gut of various breeds of cattle (Zhou *et al.*, 2010; King *et al.*, 2011; Lee *et al.*, 2013). Research has looked into lowering the amount of CH_4 emitted by ruminants through various strategies; Zhou *et al. (*2009) found that

variation of the feed in cattle diet has a significant effect in selecting for different methanogenic communities and subsequent CH_4 emissions, with low feed efficiency cattle (high CH_4 emission) having double the population of *Methanobrevibacter* and *Methanosphaera* when compared to high feed efficiency cattle (low CH_4 emission). Another novel approach to reducing CH_4 production in ruminants is the development of vaccines that allow the immune system of ruminants to naturally target methanogens (Wright *et al.,* 2004). One set of vaccines led to an effective 7.7% reduction in CH_4 whilst only targeting 20% of the overall methanogen population. Such strategies have run into problems however, as a different vaccine set by Wright *et al. (*2009), eliminated 52% of the methanogen population, but overall CH_4 emissions increased by 18%, suggesting that the vaccine targeted low CH_4 producing methanogens rather than strains that produce high amounts of CH_4.

Methanogens are found to be ubiquitous within aquatic environments, and they are present in lakes (Zhu *et al.*, 2012), rivers (Zeleke *et al.*, 2013), and seas (Chronopulou *et al.*, 2017). In a study by Grossart *et al.* (2011) water samples taken from the depth profile of a lake showed a noticeable increase in CH_4 and methanogens as depth increased. This also coincided with a transition from oxic to anoxic conditions as depth increased. Interestingly, Grossart *et al.* (2011) also noted that production of CH_4 was able to continue in the oxygenated parts of the water column. This was attributed to a syntrophic relationship between the methanogens and photoautotrophic bacteria, where the methanogen is bound to the bacteria, and nutrients were directly obtained from their partner (Grossart *et al*., 2011). Other syntrophic relationships have also been suggested by Tang et al. (2014) where CH_4 production may be tied to the production of hydrogen by nitrogen fixation. The

concentrations of substrates in the waters of aquatic systems were also found to be important in determining methanogen populations; a study by Banning *et al.* (2005) of a brackish lake showed that low concentrations of acetate, shifted the community towards hydrogenotrophic methanogens of the Order Methanomicrobiales becoming more dominant, although acetotrophic Methanosarcinales were still present (Banning *et al.*, 2005). The high water content within the sediments of aquatic environments also provide ideal anoxic conditions for methanogens and it is where the vast majority of methanogens would be found (Shelley *et al.*, 2015). As with the water column, the differences in depths of sediments also create distinct CH_4/O_2 profiles within aquatic environments, although differences in anoxic and oxic zones can be mere centimetres apart, depending on the composition and size fragments of the sediment (Shelley *et al.*, 2015).

Marine environments have also been investigated for methanogenesis although not to the same extent as inland water bodies (Karl *et al.*, 2008). The observations made in marine environments have been similar to those found in inland water systems, in particular, a study by Karl *et al.* (2008) also found methanogenesis occuring in the oxic parts of the marine water column. However, production of CH_4 was not tied to methanogenesis as previously described here, instead CH_4 is generated from the decomposition of an organophosphorus compound, methylphosphonate, by various heterotrophic bacteria (Karl *et al.*, 2008). Within Marine sediments, the more traditional pathways of methanogenesis has been found, with *Methanosarcinaceae sp.* making up 85% of total *mcrA* sequences (Chronopoulou *et al.*, 2017).

Methanogenesis have also been found to occur in crude oil reservoirs as well as environments contaminated by crude oil waste, with genera such as

Methanosaeta (acetotrophy), *Methanoculleus*, and *Methanobacterium* (hydrogenotrophy) responsible for generating CH_4 (Berdugo-Clavijo and Gieg, 2014). Methanogenesis within crude oil systems are potentially important in treating oil spills, as part of attempts at bioremediation (Callaghan *et al.*, 2013), or as a means to gain extra energy from low yield/residual oil reservoirs by converting the remaining oil into CH_4 (Gieg *et al.*, 2008).

Although most methanogens are mesophilic and are able to function in temperatures ranging between 20-40ºC (Garcia *et al.*, 2000)**,** some methanogens are tolerant to more extreme temperatures. For example, the genera *Methanothermobacter, Methanothermus, Methanothermococcus, Methanocaldococcus, Methanotorris* and *Methanopyrus* are extreme thermophiles that grow at >80ºC in environments such as hydrothermal vents (Jones *et al.*, 1989*;* Eecke *et al.,* 2012). The order Methanopyrales also includes one genus of novel hyperthermophilic methanogens (growing at 110^{o}C) (Slesarev *et al.*, 2002). Due to their relatively simple nutrient requirements, as well as their ability to survive such extreme conditions, researchers have suggested methanogens within these environments were potentially among the first single cell organisms to exist on Earth (Martin *et al.*, 2008). Psychrophilic methanogens, on the opposite temperature extreme, have also been identified in arctic conditions; a study by Zhang *et al. (*2008) within a Tibetan Zoige Wetland, found methanogenic species capable of producing methane at 0^{o}C. Other extreme environments have also been studied; members of *Methanohalobium, Methanohalophilus* and *Methanolobus* which are extreme halophiles are able to grow in hypersaline environments, that have NaCl concentrations of up to 4.3 M (Nazaries *et al.*, 2013). There are also reports of moderately acidophilic and alkaliphilic methanogens that function in pH ranges

between 5.6-6.2 and 8.0-9.2 respectively (Bräuer *et al.* 2006; Zhilina *et al.* 2013). Although the diversity and activity of methanogens in the environment have been investigated extensively, there are still considerable gaps in knowledge on their interactions in the environment and with other microbial communities (Nazaries *et al.*, 2013).

1.2.5: Application of molecular techniques on methanogens

The developments in molecular techniques over the past decades has lead to a rise in the use of these techniques in the ecology of various microbial organisms and methanogens are no exception to this (Narihiro and Sekiguchi, 2011). PCR amplification and sequencing of methanogens using 16S rRNA specific to methanogens and the functional gene *mcrA* have been extensively used, with a vast array of primers developed over the years to target these genes (Narihiro and Sekiguchi, 2011). The use of primers and probes targeting the 16S rRNA gene has been the most heavily utilized, as databases for such sequences are extensive and well maintained, and also allows an insight into other non-methanogens organisms that might play a role in competing with methanogens for resources or providing substrates (e.g. H_2/ acetate). However targeting 16S rRNA genes can be problematic as sequences are sometimes inaccurate and the presence of target organisms may not reflect their *in vivo* activity. Nevertheless, 16S rRNA gene sequencing and amplification approaches targeting methanogens have been useful in various studies: in a study by Wright and Pimm (2003), various primers were successfully developed for use in general PCR and sequencing protocols that cover a majority of known methanogens.

In a study by Hook *et al.* (2009), 16S rRNA gene primers Met630F and Met803R were used in Q-PCR, which allowed for accurate quantification of methanogens in dairy cow rumen. However this primer set has been noted to cover non-methanogens (Wallace *et al.*, 2014). To overcome this several studies used 16S rRNA gene primer sets more specific to certain Orders of methanogens; Yu *et al.* (2005) developed primers specific for Methanomicrobiales, Methanobacteriales, Methanococcales, and Methanosarcinales, and have been successfully utilized in several studies ranging from dairy cow rumen (Danielsson *et al.,* 2012) to Anerobic digesters (Bartell *et al.,* 2015).

The use of the *mcrA* functional gene in molecular ecology has also been extensive as, in addition to the previously mentioned importance of its coding for MCR, it is almost exclusively found in methanogens and is thus a greater indicator of methanogen activity, abundance and diversity compared to using just the 16S rRNA gene. Early primer sets developed for the *mcrA* gene were successfully used on various environmental samples, including landfills (Luton *et al.* 2002), rice paddy fields (Ma *et al.*, 2012), peat bogs (Juottonen *et al.*, 2006), and anaerobic digesters (Steinberg and Regan 2009).

Similar to the 16S rRNA gene, Q-PCR has also seen widespread application on the *mcrA* gene (Shigematsu *et al.,* 2004; Colwell *et al.,* 2008; Nunoura *et al.,* 2008; Steinberg and Regan, 2008). Shigematsu *et al.* (2004) were among the first to successfully utilize Q-PCR on the *mcrA* gene in anaerobic digesters, however the primer sets were limited to only 3 genera of methanogens. Steinberg and Regan (2008) developed a new primer set derived from those created by Luton *et al.* (2002), resulting in a primer set, mlas and mcrArev, with a much broader coverage of known methanogens. In addition, Steinberg and Regan (2008) also developed

TaqMan probes for TaqMan Q-PCR, which allowed for more accurate quantification than Q-PCR that utilizes Sybr Green.

Both 16S rRNA and *mcrA* gene primers and probes have also been successful in obtaining identity of important methanogens within the environment via various fingerprinting, sequencing and other molecular methods, including denaturing gradient gel electrophoresis (Tabatabaei *et al.*, 2009), terminal restriction fragment length polymorphism (T-RFLP) (Lueders *et al.*, 2003), and fluorescent *in-situ* hybridization (FISH) (Tabatabaei *et al.*, 2009). The introduction of NGS (next generation sequencing) methods is promising to provide additional insights as they allow for more sequencing depth compared to older Sanger based methods, providing millions of sequence reads in a given environmental sample (Goodwin *et al.,* 2016). Wilkins *et al.* (2015) successfully used 454 pyrosequencing on anaerobic digesters to characterise the methanogen community, and correlated methane production and the *mcrA* transcripts. Snelling *et al.* (2014) utilized Illumina's sequencing by synthesis (SBS) Miseq system to study the methanogen population within sheep rumen, targeting both 16S rRNA and *mcrA* genes, and found SBS to be able to detect the majority of methanogens down to the species level.

1.3: Microbiology and Biochemistry of Methane Oxidation

Biological CH_4 oxidation is performed by methanotrophic microorganisms (methanotrophs) either aerobically by aerobic methanotrophic bacteria or anaerobically by a consortium of anaerobic Archaea and associated anaerobic bacteria (anaerobic CH_4 oxidation) (Ettwig *et al*., 2010). Methanotrophs are a physiologically distinct group of Gram-negative bacteria that can utilize CH_4 as the

sole carbon source (Hanson and Hanson 1996). Methanotrophs are currently the only known biological sink for CH_4, preventing approximately two thirds of the 1500 Tg CH_4 produced each year from reaching the atmosphere (Thauer 2011). It has been found that 90% of the atmospheric CH_4 is oxidized by OH radicals and that 5% is re-mineralized by methanotrophs (reviewed in Thauer 2011). This makes methanotrophs an important and unique set of organisms in the carbon cycle.

Methanotrophs can be found in a wide variety of environments where there is an interface between oxic and anoxic conditions and includes soils, peat bogs, rice paddies, marine and freshwater sediments, soda lakes (Semrau *et al.*, 2010; Wendland *et al.*, 2010). However, optimal activity for most methanotrophs is in environments with almost neutral pH, mesophilic temperatures and low salinities (Le Mer and Roger, 2001).

The majority of biological oxidation occurs in the oxic environments by aerobic methanotrophs i.e. 600 Tg CH_4 oxidized per year versus 300 Tg^{-1} year CH_4 oxidized per year in anaerobic environments (Thauer, 2011). It is currently considered that 80% of CH_4 produced in soils by methanogens is oxidised by methanotrophic bacteria at the soil surface (Hanson and Hanson, 1996; Conrad *et al.*, 2007). However, there are still uncertainties on the capacity of terrestrial sinks to absorb methane, due to limited knowledge of the microbial community dynamics in these environments (Berrittella and van Huissteden, 2010; Nazaries *et al.*, 2013; Veraart *et al.,* 2015).

1.3.1: Taxonomy and Phylogeny of Methanotrophs

The known aerobic methanotrophs belong to the Bacterial phyla Proteobacteria and Verrucomicrobia (Dunfield *et al.*, 2007). The Proteobacteria

methanotrophs can be further subdivided into Type I and Type II (**Figure 1.8**), whereby Type I methanotrophs are members of the family Methylococceae and belong to the Gammproteobacteria (Bowman *et al.*, 1993), whilst Type II methanotrophs belong to the Alphaproteobacteria and include several genera in the family *Methylocystaceae* and *Beijerinckiaceae* (Bowman *et al.*, 1993). In addition to Types I and II there is a further cluster historically known as Type X, which also falls within the Gammaproteobacteria but are in a distinct group from Type I. However, methanotroph taxonomy has since been revised and Type I and Type X methanotroph species have been included together in the family *Methylococcaceae* (Bowman *et al.*, 1993; Bowman *et al.*, 1995).

There are currently over 23 genera of methanotrophs divided between the types; *Methylobacter*, *Methylomicrobium*, *Methylomonas*, *Methylocaldum*, *Methylosphaera*, *Methylothermus*, *Methylosarcina*, *Methylohalobius*, *Methylosoma*, *Clenothrix*, *Clonothrix*, *Methylocaldum*, *Methylogaea* and *Methylococcu*s which belong to Type I and *Methylocystis*, *Methylosinus*, *Methylocella*, *Methyloferula* and *Methylocapsa* which belong to Type II (McDonald *et al.,* 2008). Whilst Type X includes *Methylococcus* spp*.* (Hanson and Hanson 1996). The Type I methanotrophs within the genus *Methylocella* were also the first to be confirmed as a facultative methanotroph, with some species of both *Methylocapsa* and *Methylocystis* subsequently classified as facultative methanotrophs as well (Dedysh and Dunfield, 2011). Only Type I Alphaproteobacteria methanotrophs contain species that are facultative, thus far all Type II Gammaprotreobacteria are obligate methanotrophs (Semrau *et al.*, 2011).

More recently *Methylacidiphilium* has also been identified as a methanotroph inhabiting acidic environments and falls within the Phylum Verrucomicrobia (Dunfield

et al., 2007). Several uncultured methanotrophs have also been found through molecular techniques within upland soils and have been classified as Upland Soil Cluster (USC) (Kolb *et al.,* 2005). Both Verrucomicrobia and USC methanotrophs are important considerations in methanotroph research as both occupy niche environments (Geothermal area and forest soils respectively), both belong to seperate classes to the original Alpha and Gammaproteobacteria methanotrophs, and neither are detected by common molecular probes for the typical Type I and Type II methanotrophs due to this phylogenetic divergence (McDonald *et al.*, 2008).

Among the first methanotrophs within the phylum Verrucomicrobia to be identified was *Methyloacidiphilum infernorum*, a species originally discovered in acidic volcanic mud from the New Zealand Geothermal area of Tikitere, with several independent studies finding the genus in similar environments (Dunfield *et al.*, 2007; Pol *et al.*, 2007; Islam *et al.*, 2008). Other species that have been documented include *Methylacidiphilum kamchatkense* discovered in a Russian hot spring in Kamchatka (Islam *et al.*, 2008) and *Methylacidiphilum fumariolicum* (Pol *et al.*, 2007). Examining the phylogeny of *Methylacidiphilum* through the *pmoA* gene shows that *Methylacidiphilum* diverged from a common ancestor to the Proteobacteria methanotrophs and retained an ortholog of the *pmoA* gene, along with the subsequent methanotrophic abilities, rather than obtaining the gene via other mechanisms such as horizontal gene transfer (Op den Camp *et al.*, 2009).

USC methanotrophs, noted to be an important group of high affinity methanotrophs within several systems such as forest soils and grassland, include several species inferred by sequencing as all currently known taxa are unculturable at this time (Knief, 2015). Several related taxons fall within this group, which are further divided into two major clades USCα and USCγ (Knief, 2015). USCα species

are related to the Type II Alphaproteobacteria, and can be split into the groups USCα sensu stricto (in the strict sense), which is a clade as defined by the original RA14 cluster as described by Holmes *et al.* (1999), and USCα sensu lato (in the loose sense) which includes all related taxa to USCα including USCα, Jasper Ridge Cluster 1 (JR1), and Moore House Peat cluster (MHP) (Shrestha *et al.,* 2012). USCγ is closely related to the Type I Gammaproteobacteria, and like USCα can also be split into USCγ sensu stricto, a clade including the original USCγ discovered by Knief *et al.* (2003) and USCγ sensu lato, which includes USCγ and USCγ like taxa including JR2 and JR3 (Knief, 2015). Comparatively USCα is slightly more diverse than USCγ, with the number of species level OTUs in the USCα at 133, whereas USCγ includes 98 species level OTUs (Knief, 2015).

Uncultured methanotrophs have also been found in aquatic environments and have been noted to be divergent from USC clades (Dumont *et al.*, 2014). There are currently 2 clusters associated with lakes (Lake Cluster 1 and Lake Cluster 2) as well as one aquifer cluster (Dumont *et al.*, 2014). All three groups are related to Type I methanotrophs and are relatively less diverse than USC with 17 species level OTUs within the LC1, 2 OTUs within LC2, and 9 OTUs within the aquifer cluster (Knief, 2015).

Methanotrophs have also been categorized according to their affinity and capacity to oxidize methane whereby there are methanotrophs with low affinity but high capacity, and methanotrophs with high affinity but low capacity (Bender and Conrad 1992). Low affinity CH_4 oxidation shows a K_m in the µmol L^{-1} range whist high affinity CH_4 oxidation is performed by methanotrophs with a K_m in the nmol L^{-1} range and enables oxidation at atmospheric concentration (Lau *et al.*, 2007). Therefore, low affinity organisms take up large concentrations of CH_4 much more slowly,

whereas high affinity organisms have the ability to take up CH_4 directly from the atmosphere but in low concentrations (Reay *et al.*, 2005). The latter group have yet to be identified via traditional culture based methods and inferences of their existence has relied heavily on molecular techniques such as Phospholipid Fatty Acids (PLFA)-SIP to determine high affinity communities (Maxfield *et al.*, 2006; 2009).

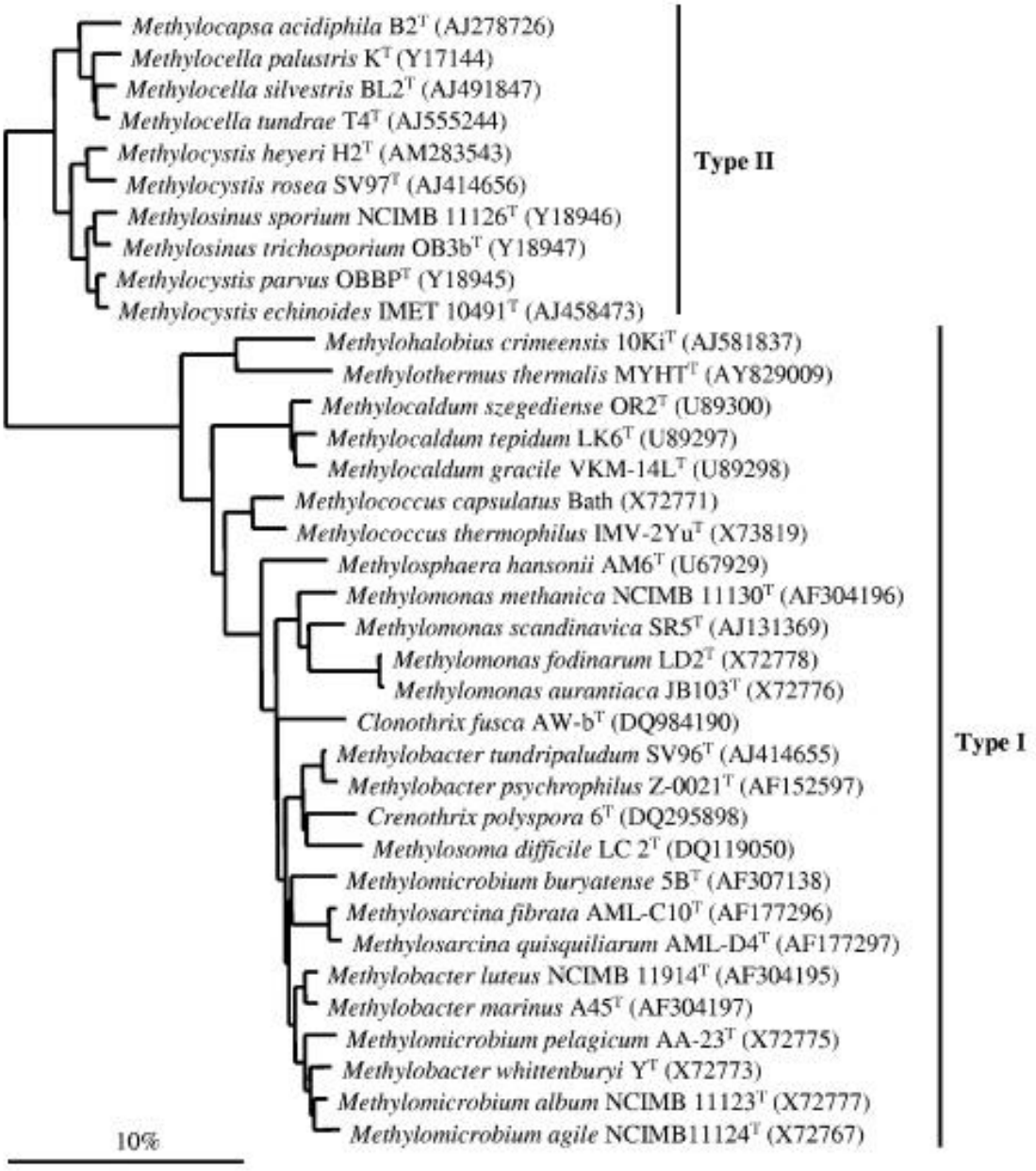


Figure 1.8: 16S rRNA phylogenetic tree of methane oxidizing bacteria (McDonald *et al.,* 2008).

1.3.2: Biochemistry of Methane Oxidation and Methane Oxidisers

Methanotrophic bacteria belong to a group of methylotrophs which are aerobic bacteria that can metabolise one-C compounds such as CH_4, methanol, formaldehyde, methylated amines, halomethanes and methylated compounds containing sulphur (Hanson and Hanson, 1996). It has also been reported that they may be able use organic substrates more complex than CH_4 such as acetate (Semrau *et al.*, 2011).

The oxidation of CH_4 to CO_2 involves the conversion of CH_4 into methanol (CH_3OH) (**Figure 1.9**). The methanol is then converted into formaldehyde (HCHO), by methanol dehydrogenase, where it is either integrated into the cell or further oxidized into formate (HCOOH) (Serrano-Silva *et al.*, 2014). Formate is finally oxidized to CO_2 by formate dehydrogenase, a NAD^+ dependent enzyme present in all known methanotrophs (Chistoserdova *et al.*, 2004; White 2006).

There are two known pathways by which formaldehyde is formed; the serine pathway or the ribulose monophosphate pathway (RuMP). The key differences between Type I and Type II methanotrophs is how they incorporate formaldehyde into cells during methane oxidation; Type I uses the Ribulose Monophosphate Pathway RuMP pathway whereas Type II methanotrophs use the serine pathway (**Figure 1.9**). Type X methanotrophs are similar to Type I and Type II in that they have characteristics of both types (i.e. utilizing both the RuMP pathway and ribulose 1,5-biphosphate for carbon assimilation, but also contain enzymes from the serine pathway (e.g. ribulose biphosphate carboxylase) (Hanson and Hanson 1996).

Methanotrophs utilizing the serine pathway produce acetyl-CoA which is metabolised into succinate by a series of additional steps and then assimilated into cell material (White 2006). The serine pathway can be simplified into the following

reaction;

$HCHO + CO_2 + 2NADH + 2H^+ + 2ATP + CoASH \Rightarrow$ acetyl-CoA + NAD^+ + 2ADP + 2Pi

In the RuMP pathway, there are 3 stages; Stage 1 creates fructose-6-phosphate (F6P) from the condensation of ribulose-5-phosphate and formaldehyde. F6P is then used in stage 2 where it is used to produce pyruvate for cell material, and phosphoglyceraldehyde (PGALD) for stage 3. Stage 3 incorporates F6P and PGALD to recycle ribulose-5-phosphate for further reactions in the RuMP pathway (White 2006).

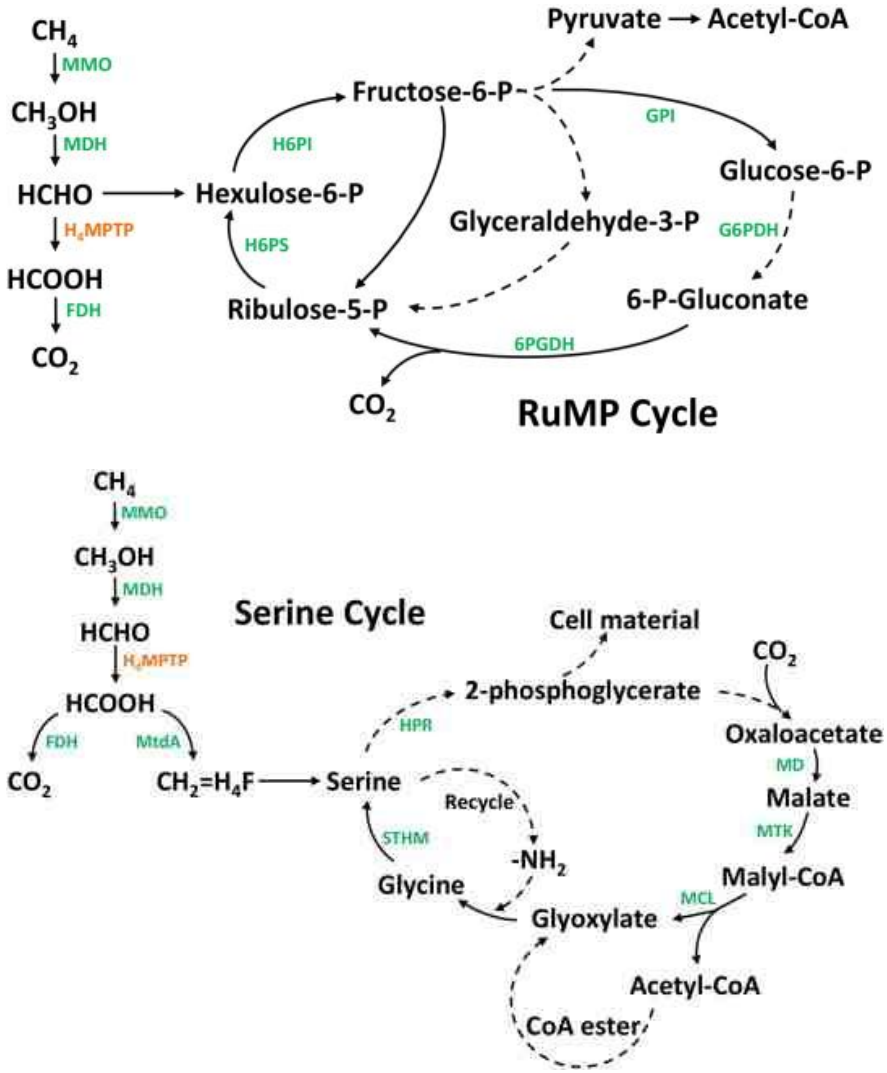


Figure 1.9: Methane oxidation leading to either the RuMP or Serine pathways (Fei *et al.*, 2014)

1.3.3: Methane Monooxygenase (MMO)

The oxidation of methane to methanol is catalyzed by the enzyme methane Monooxygenase. There are two versions of methane Monooxygenase, a soluble methane Monooxygenase (sMMO) that is secreted, and a particulate methane Monooxygenase that is bound to the membrane (pMMO) and methanotrophs possess either one or both enzymes (Murrell *et al.,* 1998). The sMMO has wider substrate specificity than pMMO, including hydrocarbons such as trichloroethylene and hence there has been much research interest in the use of methanotrophs for industrial applications (Jenkins *et al.*, 1994).

The pMMO is a copper-containing enzyme, with three polypeptides; an alpha subunit (PmoB), a beta subunit (*PmoA*) and a gamma subunit (PmoC). The pMMO is encoded by the *pmoA* gene, which has been used in molecular ecology studies to determine MOB community structure and function (McDonald *et al.*, 2005). The pMMO shares some homologies with the enzyme ammonia monooxygenase (AMO) found in ammonia oxidizing bacteria (AOB) indicating a shared ancestry (Holmes *et al.*, 1995). Due to this homology to AMO, MOBs have been shown to oxidize ammonia, albeit less efficiently than AOBs (Bédard and Knowles, 1989). However, ammonia monooxygenase found in ammonia oxidizing Archaea (AOA) were found to be only distantly related to pMMO (Stahl and de la Torre, 2012). Several methanotrophs have also been shown to contain functional genes that code for nitrogenases (*nifH*) in both Type I and Type II MOBs, so they are also able to fix nitrogen (Auman *et al.*, 2001).

1.3.4: Anaerobic oxidation of methane and Archaeal anaerobic methanotrophs (ANME)

Although methanotrophy is usually found in aerobic environments, (especially at the interfaces between aerobic and anaerobic zones), it has been discovered that anaerobic CH_4 oxidation may also occur in marine environments (Strous and Jetten, 2004). Anaerobic methane oxidation is responsible for approximately 75% of marine methane oxidation (Strous and Jetten, 2004). The mechanisms proposed for the process are reverse methanogenesis, acetogenesis and methylogenesis (Caldwell *et al.*, 2008). The most well known is the reverse mechanism of methanogenesis which takes place when a sulfate-reducing bacterium (SRB) depletes the hydrogen concentration so that CH_4 concentration is higher than hydrogen resulting in the oxidation of CH_4 to CO_2 (Caldwell *et al.*, 2008; Wendland *et al.*, 2010). The process is also called sulfate-dependent CH_4 oxidation. The process requires a syntrophic reaction with the Archaea and SRB. One mechanism proposed is as follows:

$$CH_4 + 2H_2O \Rightarrow CO_2 + 4H_2 \quad (CH_4 \text{ oxidation})$$

$$SO_4^{2-} + 4H_2 + H^+ \Rightarrow HS^- + 4H_2O \quad \text{(sulfate reduction)}$$

$$SO_4^{2-} + CH_4 \Rightarrow HCO_3^- + HS^- + H_2O \quad \text{(Net reaction)}$$

Consequently methanotrophic Archaea can be found where CH_4 and sulfate are present such as the sulfate-methane transition zone (SMTZ) as well as other environments like marine benthic zones and more extreme environment such as hydrothermal vents (Serrano-Silva *et al.*, 2014).

To date no ANMEs have been cultured and their identification has been reliant on molecular techniques. Based on 16S rRNA gene sequences three

sequence clusters have been defined for Archaeal anaerobic methanotrophs (ANME): ANME-1, ANME-2, and ANME-3 (Thauer, 2011). These ANME sequences have also been identified as being phylogenetically related to both Methanobacteriales (in the case of ANME-1) and Methanosarcinales (in the case of ANME-2 and ANME-3) (Thauer, 2011). These Archaeal anaerobic methanotrophs were identified in syntrophic association with a sulfate-reducing Deltaproteobacteria (Serrano-Silva *et al.*, 2014). However, in a study by Milucka *et al.* (2012), ANME-2 was shown to perform dissimilatory sulfate reduction without the participation of syntrophic SRBs.

It is hypothesized that ANME initiates the reversal of the last reaction in methanogenesis, i.e. the reduction of methyl-coenzyme M (CH_3-SCoM) by methyl-coenzyme M reductase (MCR). This hypothesis is supported by the recovery of *mcrA* genes from sediments obtained from anaerobic oxidation of CH_4 zones or enrichment cultures and were assigned to either ANME-1 or ANME-2 (Knittel and Boetius, 2009). Several genes involved in methanogenesis have also been detected in ANME-2a enrichments such as the *mtr* genes (subunits A-H) which encodes for tetrahydromethanopterin S-methyltransferase, that catalyzes CH_3-THMP and Co-M into CH_3-SCoM and THMP (Wang *et al.*, 2014).

In addition to sulfate reduction based anaerobic CH_4 oxidation, iron and manganese oxides can also be used as electron acceptors (Beal *et al.*, 2009). This has been noted in some marine environments where CH_4 oxidation did not correlate with SO_4^{2-} reduction (Joye *et al.*, 2004). This has been attributed to the presence of riverine sourced metal oxide deposits, such as ferrihydrite and bimessite, an iron containing and manganese containing mineral respectively (Beal *et al.*, 2009). Similar to SO_4^{2-} reduction, both iron oxide and manganese oxide are reduced by

syntrophic iron and manganese reducing bacteria (Beal *et al.*, 2009), although it was noted that some ANME are also able to reduce iron and manganese on their own. Thermodynamically, both iron and manganese based CH_4 oxidation provide more potential Gibbs free energy than sulfate; sulfate reduction provides ΔG = -14 kJ mol^{-1}, whereas ferrihydrite reduction provides ΔG = -270.3 kJ mol^{-1} and bimessite provides ΔG = -556 kJ mol^{-1} (Beal *et al.*, 2009). Although more energetically favourable, Beal *et al.* (2009) notes that oxidation of CH_4 via these two pathways occur at a slower rate.

Oxidation of CH_4 via iron oxide reduction is carried out through the following equation:

$$CH_4 + 8\ Fe(OH)^3 + 15H^+ \Rightarrow HCO_3^- + 8Fe^{2+} + 21H_20$$

Oxidation of CH_4 via manganese oxide reduction is carried through the following equation:

$$CH_4 + 4MnO_2 + 7H^+ \Rightarrow HCO_3^- + 4Mn^{2+} + 5H2O$$

Alternatively, anaerobic oxidation of methane can be coupled to nitrate reduction, and has also been recently described (Serrano-Silva *et al.*, 2014). This has been demonstrated by the bacterium Candidatus Methylomirabilis oxyfera, which can oxidise CH_4 using the aerobic methanotrophic pathway under anaerobic conditions (Ettwig *et al.*, 2010). *Methylomirabilis oxyfera* was discovered as a species belonging to the newly discovered NC10 clade, and was originally found in nitrate based anaerobic methane oxidation incubations, and was thought to function with an Archaeal species that was phylogenetically related to methanogens and

ANME-2 (Ettwig *et al.,* 2008). *M. oxyfera* is confirmed to oxidise CH_4 via the aerobic pathway by the presence of the complete gene for pMMO, as well as oxidation being inhibited by the presence of acetylene, an inhibitor for pMMO (Ettwig *et al.*, 2012). The proposed pathway in *M. oxyfera* that allows this involves the reduction of nitrite, to nitric oxide (NO), and then to N_2 gas, which provides the molecular O_2 from NO to the aerobic methanotrophic pathway (Ettwig *et al.*, 2010) (**Figure 1.10**).

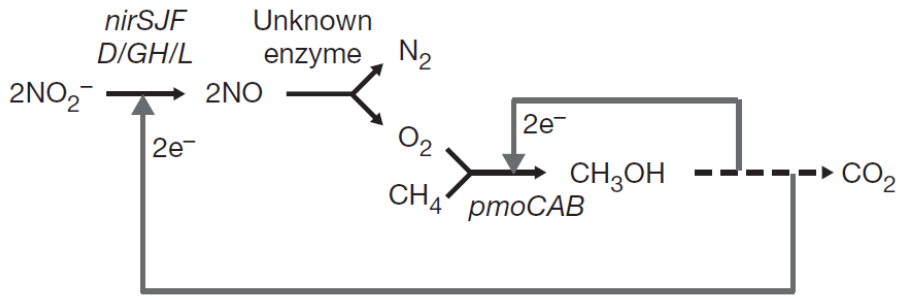


Figure 1.10: Proposed oxygen production pathway for methanotrophy in *Methylomirabilis oxyfera* (Ettwig *et al.*, 2010). Unknown enzyme is thought to be a putative NO dismutase.

The lack of nitrous oxide (N_2O) reduction and its enzyme, N_2O reductase, within the *M.oxyfera* pathway has led to proposal that a novel NO dismutase performs disproportionation of NO into N_2 and O_2 (Ettwig *et al.*, 2012). The overall reaction can be summed as:

$3CH_4 + 8NO_2^- + 8H^+ \Rightarrow 3CO_2 + 4N_2 + 10H_2O$ ($\Delta G^{0'} = -928$ kJ mol^{-1} CH_4).

Although *M. oxyfera* is able to reduce nitrite on its own, It has also been suggested that the ANME/methanogen related archaeon contributes to nitrite reduction, as the combination of the NC10 group and the archaeon, had a 30% higher nitrite reduction rate (Hu *et al.*, 2009). Additionally Hu *et al. (*2011) found that when nitrite became high enough, it eventually led to toxic conditions for *M oxyfera*.

Nevertheless, the ability of methanotrophs to obtain O_2 in this manner in anaerobic conditions may lead to important changes in how methane cycling is viewed.

Nitrite reduction coupled to CH_4 oxidation was found to not be exclusive to *M. oxyfera*; a recent study by Haroon *et al. (*2013) showed that Candidatus Methanoperedens nitroreducens, a candidate methanotroph belonging to ANME-2d, was also coupled to nitrite reduction, however as it is an ANME and therefore utilizes reverse methanogenesis, the nitrite was used as a terminal electron acceptor, rather than a source for O_2.

1.3.5: Physiology and Ecology of Methanotrophs

The diversity and distribution of the methanotrophs have been investigated in many environments most commonly where CH_4 is readily produced e.g. rice fields and landfills. Hutchens *et al.* (2004) found that Type I *Methylomonas*, and *Methylococcus,* and Type II *Methylocystis* and *Methylosinus* were responsible for the oxidation for CH_4 in the Movile cave, demonstrating that methanotrophs can survive in a chemoautotrophic environment (Hutchens *et al.*, 2004). Chen *et al.* (2007) found both Type I (i.e. *Methylomonas, Methylosarcina* and *Methylobacter*) and Type II (i.e. *Methylocella* and some *Methylocystis*) methanotrophs in landfills. Similar findings have been reported in other landfill sites (Cébron *et al.*, 2007).

Although both Type I and Type II methanotrophs were found in rice paddies (Bodelier *et al.*, 2000), Type II methanotrophs have been found to persist throughout the flooding and drainage of fields during and after growth seasons, which may be due to Type II methanotrophs being able to survive on substrates other than CH_4 (Henckel *et al.*, 2000). In an acidic forest soil, where CH_4 production is not as high as

some other environments e.g. peat bogs, the predominant active methanotrophs were *Methylocella*, *Methylocapsa* and *Methylocystis*, which again were Type II methanotrophs being able to survive on low CH_4 (Radajewski *et al.*, 2002). NC10 methanotrophs belonging to USC Forest soil cluster was also found in forest soils, as USC are high affinity methanotrophs, and able to oxidize CH_4 at atmospheric concentrations normally found in these environments (Kolb *et al.*, 2005).

Wetlands have also been investigated as the CH_4 concentrations in these soils are quite high due to the anoxic conditions created by water saturation (Berritella and van Huissteden, 2010). Wetland soils treated with nitrate and ammonia showed that the abundance of Type II methanotrophs decreased but not Type I, showing these systems are susceptible to changes in nutrients (Siljanen *et al.*, 2012). Some wetlands have also been shown to have no detectable representatives of Type II methanotrophs but were instead dominated by Type I including *Methylobacter* and *Methylococcus* (Yun *et al.*, 2010). It was noted that the lack of Type II could potentially be due to PCR bias against low amounts of Type II sequences rather than absence of Type II (Yun *et al.*, 2010).

An extensive study on upland soils, encompassing different soil types, land use and plant cover found that 70% of soils were dominated by *Methylocaldum, Methylosinus,* and *Methylocystis* (Knief *et al.*, 2003). Methanotrophs have also been highlighted for use in bioremediation of hydrocarbon pollutants, such as trichloroethylene, as some methanotrophs contain sMMO, which have a broader substrate specificity than pMMO (Jenkins *et al.*, 1994). A recent review by Semrau (2011) highlighted this difference, and showed sMMO was able to degrade various halogenated alkanes, alkenes and aromatic compounds, whereas pMMO showed much narrower substrate usage.

Methanotrophy has also been extensively studied in various river and lake sediments, as their high water content creates an ideal anaerobic environment for methanogens, which in turn sustains methanotrophs (He *et al.*, 2012; Stanley *et al.*, 2016). In one study by Costello *et al.* (2002), CH_4 oxidation was largely attributed to Type I methanotrophs, which were dominant over Type II in the lake sediments (Costello *et al.*, 2002). Shelley *et al.* (2015) showed that CH_4 oxidation occurred readily in river sediments, and that rates were dependant on the sediment particulate sizes, i.e. that fine sediments increased CH_4 production and subsequent CH_4 oxidation. He *et al.* (2012) was also able to demonstrate differences between the water column and water to sediment interface, with *Methylocystis* present throughout the former, and *Methylobacter*, *Methylosoma*, and *Methylomonas* present in the latter.

Some methanotrophic bacteria have also been recovered from extreme environments such as very acidic (with pH value of 1) or very alkali environments (with a pH value of 11) (Dunfield, 2007). For example, acidic peatlands and forests, with pH ranging from 3-5, are important sources of atmospheric CH_4, and *Methylocella, Methylocapsa* and *Methylocystis* have all been isolated from such environments (Dedysh *et al.*, 2002; 2007). *Methylocapsa acidiphila* and *Methylocella palustris* have also been found in moderately acidic soils, with both species operating at pH of 4.2 to 7.2, and pH of 4.5 to 7.0 respectively (Dedysh *et al.*, 2000; 2002). Semrau *et al.* (2011) suggested that the acidic nature of these soils, provided favorable conditions to facultative methanotrophs (e.g. *Methylocella*), as alternative energy sources such as acetate, are in more readily available protonated forms at low pH. In alkaline environments, the first known alkaliphilic methanotrophs were from soda lakes, including the species *Methylomicrobium alcaliphilum* and

Methylomicrobium buryatense which were able to function at pH levels of up to 10 (Khmelenina et al, 1997, Kalyuzhnaya et al., 2001). Further research also showed that methanotrophs can survive in alkaline soils the genera *Methylosinus*, *Methylocystis, Methylobacter,* and *Methylosoma* able to oxidize CH_4 at pH levels of 9.4 (Han *et al.,* 2009).

Methanotrophs have been found in a wide range of temperatures in the environment, ranging from 0-72^{o}C (Dunfield, 2001). For example, thermophilic/thermotolerant methanotrophs such as *Methylococcus capsulatus* Bath, *Methylocaldum* spp. and *Methylothermus thermalis* have all been isolated from various hotsprings with temperatures above 50^{o}C (Whittenbury *et al.*, 1970; Bodrossy *et al.*, 1999; Tsubota *et al.*, 2005). Although thermophilic methanotrophy was usually associated with Archaeal ANMEs rather than aerobic methanotrophs (Merkel *et al.,* 2013), the Methylacidiphilales methanotrophic Bacterial clade encompasses both thermophilic and acidophilic abilities. Several species have been found including *Methylacidiphilum infernorum*, *Methylacidiphilum kamchatkense,* and *Methylacidiphilum fumariolicum* (Dunfield *et al.*, 2007; Pol *et al.*, 2007; Islam *et al.*, 2008). All species were isolated from very similar environments, being in or near geothermally active sites, which were also -highly acidic (Dunfield *et al.*, 2007; Pol *et al.*, 2007; Islam *et al.*, 2008).

Methylacidiphilum infernorum has been found in the New Zealand geothermal area of Tikitere, and were able to actively oxidize CH_4 at *pH* between 2.0 to 5.0 and was active at temperatures up to 60^{o}C (Pol *et al.*, 2007). *Methylacidiphilum fumariolicum,* which was discovered in soils of an Italian mud volcano, was able to survive even harsher acidity levels, being able to oxidize CH_4 at pH levels of 0.8, although growth at high temperature was similar to *M. infernorum,* between 40-65^{o}C

(Dunfield et al. 2007). *Methylacidiphilum kamchatkense* was discovered in a Russian hotspring and had similar acidity and temperature requirements a *M. infernorum*, with growth and CH_4 oxidation at pH levels of 2 and temperatures of 60^oC (Islam *et al.*, 2008).

Further studies into the *Methylacidiphilum* genus showed additional insights into their physiology; *M. fumariolicum* was noted to require rare earth metals to grow, including lanthanum (Ln), cerium (Ce), praseodymium (Pr) and neodymium (Nd) (Pol *et al.*, 2013), *M. kamchatkense* can survive on H_2/CO_2 without CH_4 as a "Knallgas" bacterium (Mohammadi *et al.*, 2017), and all three are hypothesised to be autotrophic in nature (Sharp *et al.*, 2012). Even so, their physiology is still largely unknown and research is still ongoing (Pol *et al.*, 2013).

At the polar opposite of thermophiles, psychrophilic aerobic methanotrophs have been found to in various environments; a review by Trotsenko et al. (2005) noted several methanotroph species have been found in the various cold environments including *Methylobacter psychrophilus, Methylosphaera hansonii, Methylocella palustris, Methylocella silvestris, Methylocella tundrae, Methylocapsa acidiphila* and *Methylomonas scandinavica*, with several species able to operate at temperatures between 0-15^oC These methanotrophs may become important at mitigating potential increases in previously trapped CH_4 being released from thawing permafrost soils (Trotsenko et al., 2005).

1.3.6: Application of molecular techniques on methanotrophs

Similar to the previous section regarding methanogens, the use of molecular methods to research methanotroph ecology has been extensive, and several of the

issues regarding the use of 16S rRNA and functional genes in Section 1.2.5 also apply here. Both 16S rRNA and functional genes of methanotrophs are used, and there are extensive databases categorizing their sequences (McDonald *et al.*, 2008). The 16S rRNA gene has been successfully used in various studies, which encompass many of the same environments that methanogens are found in, including rice fields (Cai *et al.,* 2016), peat bogs (Kip *et al.,* 2011), landfills (Chen *et al.*, 2007), grassland (Shrestha *et al.,* 2012), and forest soils (Henckel *et al.,* 2000).

The use of functional genes in examining methanotroph communities is complicated by the two variants of MMO not being universal across all methanotrophs, requiring the development of primers targeting *mmoX* and *pmoA* for sMMO and pMMO respectively, in order to obtain a more complete understanding of methanotroph communities (McDonald *et al.,* 2008). The use of primers targeting the *pmoA gene* have seen wide spread use, as pMMO is found across the majority of methanotrophs and in particular targets obligate methanotrophs, which is useful in environments where CH_4 is abundant e.g. landfills. Primers targeting the *pmoA* gene have been utilized in PCR (McDonald *et al.,* 2008) and Q-PCR (Rahalkar *et al.*, 2009; Tavormina *et al.*, 2010). Problems have been noted with the use of *pmoA* gene due to its homology with the *amoA* gene, with some primers (e.g. A189f and A682r) that have a broad coverage also picking up *amoA* genes (Holmes *et al.* 1995). To resolve this, some studies utilize multiple primers that are more specific to certain groups of methanotrophs e.g. A189f with a different reverse primer such as A650r or mb661r (Bourne *et al.,* 2001). Additionally Verrucomicrobia and USCα require their own unique primer sets due to their pMMO's evolutionary divergence from other methanotrophs (Sharp *et al.*, 2012; Lima *et al.,* 2014).

Targeting the *mmoX* gene for amplification has also been done (although not to the same extent as *pmoA*), as facultative methanotrophs, e.g. *Methylocella* species, only contain sMMO and would otherwise be missed if only *pmoA* primers were used (McDonald *et al.* 2008). The *mmoX* gene has been successfully amplified by using primers for PCR (Cheng *et al.*, 1999), Sybr Green Q-PCR (Kolb *et al.*, 2003) and Taqman Probe Q-PCR (Kolb *et al.*, 2003). Several studies have also used the *pmoA* and *mmoX* genes together to study the shifts between communities of facultative methanotrophs and obligate methanotrophs (Kolb *et al.*, 2003; Cébron *et al.,* 2007). The *mcrA* gene can also be used to investigate methanotrophy, as ANME utilize a reverse methanogenesis mechanism to anaerobically oxidize CH_4, which is also encoded by the *mcrA* gene (Zhou *et al.*, 2014)

As with methanogens, several fingerprinting, sequencing and other molecular techniques have also been utilized, including DGGE, T-RFLP (Horz *et al.*, 2001), and FISH (Kalyuzhnaya *et al.*, 2006). The use of NGS has also become more prevalent, with 454 Pyrosequencing (Lüke and Frenzel, 2011), Illumina SBS (Saidi-Mehrabad *et al.*, 2012), and Ion Torrent sequencing (Mohammadi *et al.*, 2017) all utilized to examine various methanotroph communities.

One particular molecular technique pioneered through researching methanotrophs is Stable Isotope Probing (SIP), whereby CH_4 gas made of a heavier isotope of carbon (^{13}C) is fed into the headspace of a container with the samples being investigated. As the heavier CH_4 is taken up by the methanotrophic community, the ^{13}C is incorporated into the genetic material of the target organisms, and after DNA is extracted, the differences in size between the natural ^{12}C and ^{13}C can be separated out through differential centrifugation, and the identity of the organisms that take up the heavier CH_4 can be quantified and sequenced

(Radajewski *et al.*, 2000). This technique has been successfully tested on several environments; Cébron *et al.* (2007) utilized SIP with *pmoA*, *mmoX,* and 16S rRNA amplified and sequenced from the heavy DNA fractions. He *et al.* (2012) performed SIP on arctic and subarctic lake water and sediments, and quantified the *pmoA* gene with Sybr green Q-PCR, and Sharp *et al.* (2014) obtained methanotroph sequences within sediments of geothermal springs.

1.4: Environmental factors affecting methanogens and methanotrophs.

There are many environmental factors controlling CH_4 fluxes such as temperature, water availability, fertiliser amendments, pH, salinity, competitive inhibition (e.g. from SRBs), redox potential, electron acceptors, concentration of CH_4 and O_2, vegetation, macronutrient concentrations and availability such as $CH_4$and CO_2 (Hanson and Hanson, 1996; Serrano-Silva *et al.*, 2014). Future predicted increases in climate change will have a severe influence on these factors (both directly and indirectly) which in turn, will impact on the microbial biodiversity, abundance and function of methanogens and methanotrophs (Nazeries *et al.*, 2013). Sections 1.4.1-3 focuses specifically on how macronutrients, water availability and temperature may affect methanogen and methane oxidising Bacterial communities.

1.4.1: Effect of carbon, nitrogen, phosphorous on methanogens and methanotrophs in soils

Carbon, nitrogen and phosphorous are important macronutrients that affect microbial communities as a whole (Nazeries *et al.*, 2013). As methanogenesis is reliant on the microbial community decomposing carbon to CO_2 and other small methyl compounds, the concentration and type of organic matter available will affect

overall methanogenesis and subsequent methanotrophy (Nazaries *et al.*, 2013). For example, large quantities of less complex small-chained carbon compounds result in increased CH_4 emissions (Wagner *et al.*, *2005;* Guérin *et al.*, 2008*;* Ye *et al.*, 2011). Carbon compounds other than CH_4 can also affect methanotrophs; acetate and ethanol are preferentially taken up over CH_4 within the metabolic pathway of methanotrophs, leading to lowered CH_4 oxidation (Wieczorek *et al.*, 2011).

In general, nitrogen (N) limitation is not often observed in relation to methanogenesis (Bachoon and Jones, 1992). However, the effect of different nitrogen compounds on methanotroph growth varies, and is often tied closely to the nitrogen species and amount present in the environment (Nazaries *et al.*, 2013). For example, ammonium inhibits methanotrophy more strongly at higher concentrations (Bodelier and Laanbroek, 2004; Mohanty *et al.*, 2004), but at lower concentrations can stimulate methanotrophs (Yang *et al.*, 2011). This is probably due to the enzyme MMO (which initiates the oxidation of CH_4) binding to the NH_4^+ and react with it as it is similar in size and structure to CH_4 (Schimel, 2000). However, contradictory reports have been found with different soils, which may be due to different types of methanotrophs responding differently to NH_4^+ (Serrano-Silva *et al.*, 2014). Specifically, NH_4^+ inhibits Type II methanotrophs (e.g., *Methylocystis* sp.) but Type I methanotroph (e.g. *Methylomicrobium, Methylobacter*) are stimulated (Mohanty *et al.*, 2006). Both nitrite and nitrate inhibit methanogen and methanotroph growth (Wang and Ineson, 2003; Reay and Nedwell, 2004; Tugtas and Pavlostathis, 2007; Bodelier and Laanbroek, 2004).

The impact of phosphate on methanotrophy and methanogenesis has been uncertain as, depending on quantity and type of P, it can either stimulate or inhibit (Park *et al.*, 1991; Adhya *et al.*, 1998; Conrad *et al.*, 2000; Venkata Mohan *et al.*, 2008; Song *et al.*, 2012; Zheng *et al.*, 2013). For methanogenesis, Medvedeff *et al.* (2014) noted an increase in methane emissions when P was added in the form of Na_2PHO_4 to samples from a P limited wetland, which was likely due to P stimulating the syntrophic microbial community that provide substrates for the methanogens. However, Adhya *et al.* (1998) found that addition of P to rice paddy in the form of single superphosphate inhibited methanogenesis. This was attributed to the presence of sulfate as, SRB outcompete methanogens for H_2 and acetate, due to sulfate reduction having a lower ΔG than methanogenesis (Adhya *et al.*, 1998). Several studies had conflicting reports on the effect of phosphate on methanotrophs. Alam *et al. (*2015) found inhibition in paddy soil at 0.1M of phosphate, but stimulation of methanotrophy at 0.2 and 0.4 µM. Jugnia *et al.* (2008) found that 18-60 mg P kg^{-1} soil also stimulated methanotrophy in landfills, whereas Burke *et al.* (2012) found no significant effect with P addition.

1.4.2: Effect of water content on methanogens and methanotrophs in soils

A major environmental factor affecting both methanogenesis and methanotrophy is water content in soils (Nazaries *et al.*, *2013*). In wetlands for example, the depth of the water table has an important effect on CH_4 emissions and positive correlations between depth of water table and CH_4 emissions have been reported (Smith *et al.*, 2003; von Arnold *et al.*, 2005). The creation of anoxic zones during flooding periods allows methanogens to operate and produce methane. This has been observed in soils that are largely dry throughout the year (and thus better

aerated) becoming methanogenic once incubated under wet conditions without addition of extra organic content (Angel *et al.,* 2011; 2012).

Soil moisture content is a critical factor for methane oxidation (Jugnie *et al.*, 2008). Several studies report a soil moisture content <20% water holding capacity (WHC) rapidly decreases CH_4 oxidation rates (Bender and Conrad, 1995; Jäckel *et al.*, 2001). Otherwise, at high moisture content, methanogenesis is favored due to lower diffusion of O_2. However, CH_4 oxidation has been reported in environments with a WHC >60%, which could be attributed to the presence of aerobic microsites and/or anaerobic CH_4 oxidation (Khalil and Baggs, 2005). Studies using ^{13}C-labeled CH_4 have shown CH_4 oxidation and production occurring simultaneously in wet soils (Khalil and Baggs, 2005).

Aerobic methanotrophs will, despite the presence of CH_4, be inhibited due to the lack of oxygen but will resume oxidation of the built up CH_4 once water content is reduced (Le Mer and Roger, 2001). This cycle of methanogenesis followed by rapid methanotrophy after flooding and drainage is characterized best in rice paddy fields, where the cultivation of rice requires long periods of flooding fields for rice plants to grow, followed by drainage once crops are harvested (Henckel *et al.*, 2000). However, aerobic methane oxidation can still occur during flooding, as methanotrophs can access oxygen at the water to soil interface, as well as oxygen that is transported below the flooded soils via roots, which creates a small oxic zone around the plant rhizosphere (**Figure 1.11**) (Le Mer and Roger, 2001). After rainfall events, methanotrophic activity has been shown to be stimulated in deserts and semiarid regions, and CH_4 oxidation increases (McLain and Martens, 2004). Where water is abundant, such as in tropical forests, CH4 oxidation occurs largely in the dry

season, most likely due to an increase in oxygen concentration and a faster diffusion of CH_4 into the soil (Nazaries *et al*., 2013).

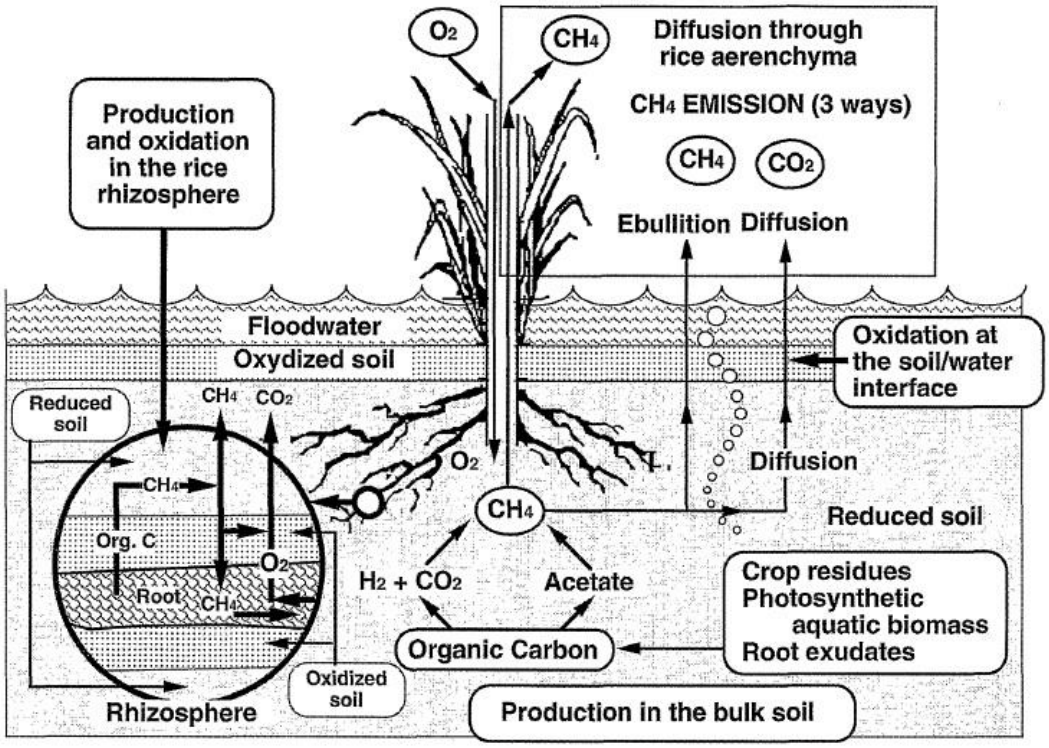


Figure 1.11: Diagram of CH_4 flux and emissions in a rice paddy field, showing methane oxidation occurring around rhizosphere of rice plants and at the oxidized soil-water interface (Le Mer and Roger, 2001).

1.4.3: Effect of temperature on methanogens and methanotrophs

Temperature is a major driver of microbial community and their function (Classen *et al.*, 2015). Elevated temperatures have been shown to increase methanogenesis and decrease CH_4 oxidation in some tropical soils (Das and Adhya, 2012). Therefore, in such tropical soils one would predict increasing CH_4 emissions with increasing atmospheric CO_2 and temperature due to global warming (Das and Adhya, 2012). This is related to a decrease in soil redox potential and an increase in substrates such as acetate favouring the growth of methanogens, whilst methanotrophic bacteria populations decrease (Das and Adhya, 2012).Temperature

variations not only affects methanogenesis, but can also select for the type of methanogenesis used (Nozhevnikova *et al.*, 2007). For example, low temperatures selector acetoclastic methanogenesis, with hydrogenotrophy being prevalent at higher temperatures (Glissman *et al.*, 2004). Although no mechanistic explanation has been offered for this difference, it is possible that it may be linked to differences in acetogenic communities under different temperatures, as excess acetate build up causes an inhibition of acetotrophic methanogens (Nozhevnikova *et al.*, 2007). Higher temperatures in some soils also decrease water content and can increase aerobic oxidation and decrease methanogenesis (section 1.4.2.)

1.4.4: Factors affecting methanogens and methanotrophs within rivers

In addition to soil systems, riverine systems are also an important consideration in global C and CH_4 cycling. A recent review by Stanley *et al.* (2016) estimates that river systems contribute upwards to 28.5 Tg CH_4 $year^{-1}$ (Stanley *et al.*, 2016). River systems have broadly similar considerations to soils when looking into the factors that affect CH_4 flux. Organic matter is an important factor as the eventual breakdown of C compounds lead to methanogenesis and methanotrophy (Stanley *et al.*, 2016) with several studies of sediments linking the changes of *in-situ* CH_4 concentrations to the availability of C (Wu *et al.,* 2007; Baulch *et al.,* 2011; Crawford *et al.*, 2014). Temperature differences within river sediments also affect CH_4 cycling (Stanley *et al.*, 2016), although research suggests that other *in-situ* factors such as limited substrate/nutrient availability often over-ride temperature effects (Shelley *et al.,* 2015). Nutrient availability in the form of N and P in sediments has not been extensively examined, however nutrient availability will have similar One major difference between soil and river CH_4 is the role of hydrology in transporting nutrients

for methanogens and methanotrophs (Stanley *et al.*, 2016). Methanotrophs are able to survive away from active methanogenic sites, as CH_4 is dissolved in water and carried to other sections of the river for oxidation (Stanley *et al.*, 2016). Anthropogenic activity also have significant effects on CH_4 in river systems. This includes damming, as these structures slow hydrological flow, depositing organic matter and nutrients into sediments and forming new large bodies of water (Chen *et al.*, 2011). Despite these differences river systems are inescapably tied to soil systems, as land-use such as from agriculture can add an over abundance of organic matter, N and P by leaching into soil and washed into river sediments. Currently there is a lack of focus on how such additions will effect CH_4 cycling (Stanley *et al.*, 2016).

1.5: Macronutrient Cycle Project and Rationale

The Macronutrient Cycle project aimed to study the macronutrients, Carbon (C), Nitrogen (N) and Phosphorous (P) and their interactions within the Hampshire-Avon catchment in England (NERC project code: NE/J011959/1). The Hampshire-Avon catchment covers an area approximately 1750 km^2 spanning across the counties of Dorset, Hampshire and Wiltshire. The geology is predominantly chalked but other formations including clay and Greensand are also found within the area (**Figure 1.12**).

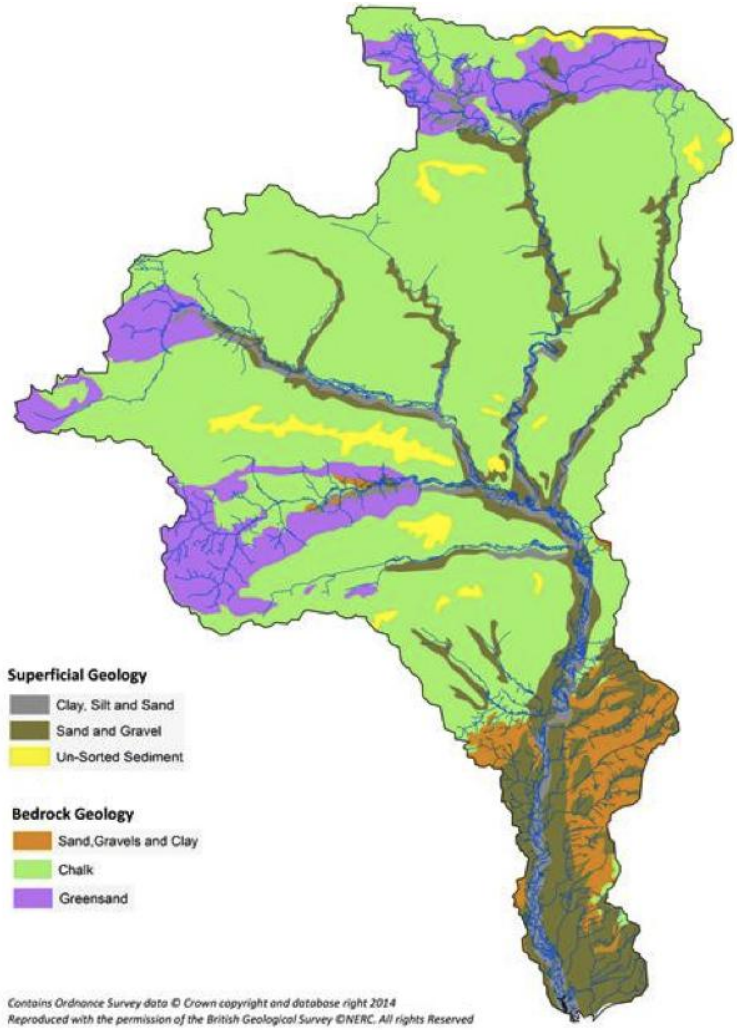


Figure 1.12: Underlying geology of the Hampshire-Avon catchment (image courtesy of http://www.hampshireavoncatchmentpartnership.org.uk/maps)

Base Flow Index (BFI) is a measure of how much groundwater contributes to the overall baseflow of a river and is influenced by the underlying geology. For example, chalk, which is highly permeable, has a BFI of up to 0.9, whereas clay, which is less permeable, has a BFI of ~0.35 (Richards, 1994). The Hampshire-Avon catchment spans different sub-catchments with contrasting geologies across an increasing BFI from clay to chalk (**Figure 1.13**).

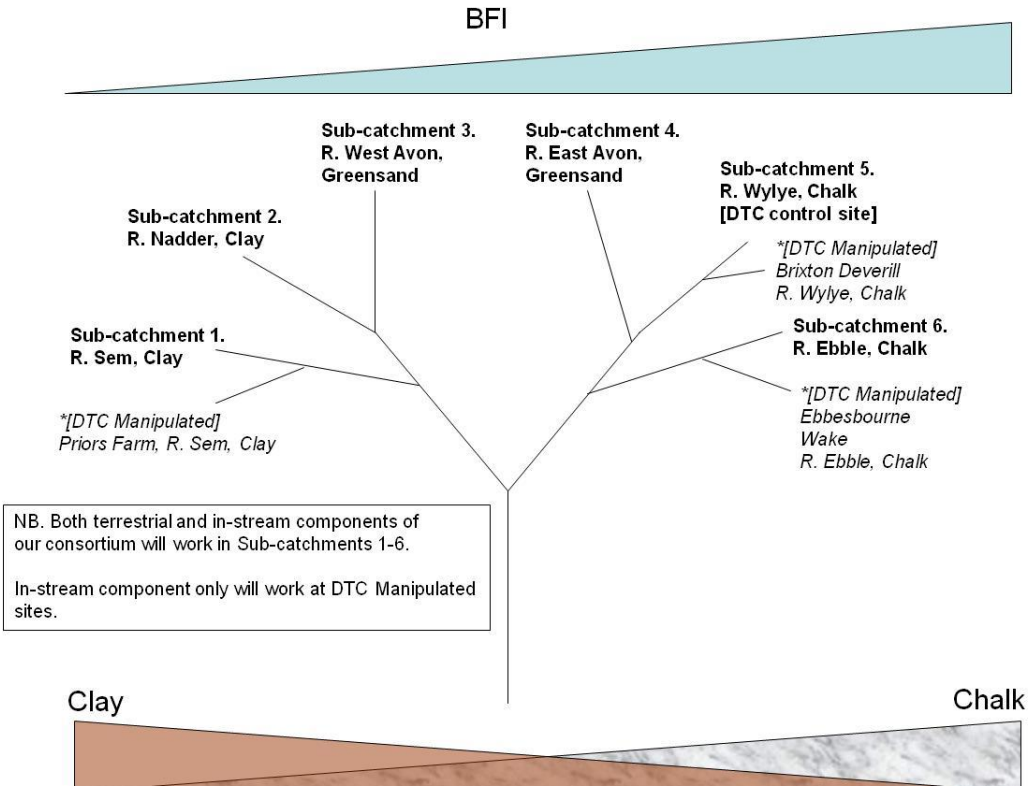


Figure 1.13: Hampshire-Avon catchment showing different sub-catchments spanning contrasting geologies. DTC- Defra Test Catchment monitoring station (NERC project code: NE/J011959/1).

Rainfall will also influence the BFI and rainfall in the catchment averages 810 mm annually (Jarvie *et al.*, 2005). Land use is predominantly agricultural with 75% of land used as farmland (Collins and Grant, 2011). The majority of industrial activity is light and concentrated in major population areas. Agricultural usage includes cereal crops, cattle, and sheep farming, with arable land concentrated in the upper catchment and grazing grasslands in the lower Avon area (Jarvie *et al.*, 2005). Therefore, anthropogenic inputs of N and P, such as from fertilizer application, and wastewater treatment discharge may enter the catchment, polluting soil and river systems and creating anoxic conditions. Such activities will have an impact on the diversity of the microbial communities and their processes and potentially lead to

increases in CH_4 and other GHG emissions. Furthermore, future climate trends predict an increase in temperatures, and thus result in changes in dry/wet cycles through the seasons creating additional uncertainty in GHG emissions (McGeehin *et al.*, 2008).

Currently there is a lack of information on how such climatic changes will impact upon carbon and nitrogen cycling and the microbes that drive these transformations at the catchment level. More specifically, there is a degree of uncertainty on how methanogens and methanotrophs might respond to such changes in global climate (Nazaries *et al.*, *2013*). Therefore, there is a need to have a clearer understanding of the factors driving methanogenesis and methane oxidation. Such information is important in determining future policies and land management to help mitigate any further changes in global climate. The overall central hypothesis to the NERC Macronutrient Project is that not only does lateral hydrological exchange (a function of underlying geology and human influence) play a key role in modulating and transforming the flux of C from catchment to coast, but that the resultant flux and dynamics of C is key to regulating the cycling of N and P.

1.6: Project Specific Hypotheses

1. Increased soil water content leads to increased anoxic conditions, which will stimulate methane production, and *mcrA* gene expression.
2. Clay geologies with low Base Flow Index (BFI) will be less permeable to water and methane production and *mcrA* gene abundance will increase, whilst chalk geologies with a higher BFI will be more permeable to water and methane production and *mcrA* gene abundance will decrease.

3. Increased temperature will stimulate the activities of methanotrophs and methanogens leading to an increase in methane oxidation, methanogenesis and increased *pmoA* and *mcrA* gene abundances.
4. Addition of N, P, and C (either by human input, or natural seasonal fluctuations) will stimulate methanogenesis and methanotrophy; leading to changes in methanogen/methanotroph community diversity and increased *mcrA*/*pmoA* gene abundances.
5. Increased CH_4 concentrations will cause methane oxidation to increase leading to an increase in methanotroph diversity and *pmoA* gene abundance, particularly the *pmoA* genes from low affinity methanotrophs.
6. In low CH_4 concentrations, methane oxidation will decrease, leading to an increase in methanotroph diversity and *pmoA* gene abundance particularly from high affinity methanotrophs.

1.7: Project aims

1. To investigate seasonal changes in the diversity, abundance and activities of methanogens and methanotrophs in relation to CH_4 production and oxidation.

2. To investigate the effect of underlying geology on the diversity, abundance and activities of methanogens and methanotrophs in relation to methane oxidation and methanogenesis.

3. To determine the effect of changes in N/P on methanotroph and methanogen community diversity, abundance and activity in soils and sediments.

4. To determine the effect of changes in temperature on methanotroph and methanogen community diversity, abundance and activity in soils and sediments.

These hypotheses will be tested via a combination of field sampling and microcosm studies. The field sampling campaigns will target the Hampshire-Avon river catchment and will sample soils and sediments at three sites overlying different geologies (i.e. greensand, chalk, and clay).

Chapter 2: Seasonal variation of Bacterial and Archaeal communities across the Hampshire-Avon catchment.

2.1: Introduction

The microbial biodiversity within soils and sediments are important drivers of ecosystem function (Wall *et al.,*2013; Philippot *et al.*, 2013; Singh *et al.*, 2014; Foshtomi *et al.*, 2015). However, microbial diversity may be influenced by various abiotic (e.g. pH, water content, temperature) and biotic factors (e.g. fauna, flora, detritus, nutrient availability) (Wall *et al.*, 2013). A key difference between soils and sediments is water content. Sediments are often continually saturated whilst the water content in many soils changes seasonally and is a key factor in determining microbial composition and activity (Ma *et al.*, 2014). In soils, water allows motile microbes to migrate through the soil matrix, determines the flow of nutrients and acts as a barrier to gas exchange (Young and Ritz 2005). Microbial community diversity may change according to water content and in general such changes are larger at the extremes of high or low water content (Stres *et al.,* 2008; Carson *et al.,* 2010; Ma *et al.,* 2014).

The effect of soil pH on microbial populations is also important with studies suggesting soils at neutral pH having higher diversity and that diversity decreases as pH becomes either increasingly acidic or alkaline (Tiago *et al.,* 2004; Rousk *et al.,* 2010; Fierer *et al.,* 2012). Nutrient availability has also been attributed to spatial and temporal differences in soil microbial diversity (Hopkins and Gregorich, 2005; Verastegui *et al.*, 2014).

In soils, microbial composition varies between soil types and a wide diversity of bacteria from various phyla have been detected including Alphaproteobacteria,

Betaproteobacteria, Gammaproteobacteria, Verrucomicrobia (Roesch *et al.,* 2007; Hansel *et al.,* 2008). For example, Bacterial diversity within some chalk soils has been shown to be lower due to the lower water-holding capacity and higher inorganic carbon compared to clay soils (Girvan *et al.*, 2003). In mixed silt and clay soils, the microbial community diversity was greater compared to sandy soils due to lower pore connectivity in the clay (Carson *et al.*, 2010). Archaea in soils are generally composed of Euryarchaeota and Thaumarchaeota, with many Thaumarchaeota species formerly classified as Crenarchaeota (Brochier-Armanet *et al.,* 2008) (Nicol *et al.*, 2003; Ochsenreiter *et al.*, 2003; Roesch *et al.*, 2007; Hansel *et al.*, 2008; Bates *et al.,* 2011). Since several Thaumarchaeota are acidophilic, Thaumarchaeota tend to dominate soils with lower pH (Lehtovirta *et al.*, 2009). Lower C:N ratio also may select for Thaumarchaeota (Bates *et al.*, 2011). Euryarchaeota in soils include all methanogens, the Class Thermoplasmata, as well as Halobacteria, and have been found in various terrestrial environments, including rice paddies, grassland, and forests, with controlling factors including pH, water content, and nitrogen content (Hu *et al.*, 2013).

Bacterial diversity within freshwater sediments has been extensively studied and similar to soil river sediments are also heavily affected by nutrient availability, pH, and other factors (Liu *et al.*, 2015; Zeglin, 2016). A meta study by Zeglin (2016), found sediments were predominantly made of Alpha, Beta, Delta, and Gamma-Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Planctomycetes, and Verrucomicrobia. The relative abundance of these Bacterial clades although persistent in most rivers does vary between different river systems as well as whether sediment, biofilm or water column was sampled (Zeglin, 2016)

Previous research on temperate river systems suggests that Thaumarchaeota are generally the most abundant Archaea with Euryarchaeota making up the smaller fraction of the community (Abreu *et al.*, 2001; Herfort *et al.*, 2009). However other aquatic systems such as sediments from shrimp farm and coastal waters have shown Euryarchaeota being dominant over Thaumarchaeota (Shao *et al.*, 2004; Stoica and Herndl, 2007.)

2.1.1: Aim

The overall aim of this chapter was to determine seasonal changes in Bacterial and Archaeal community composition and 16S rRNA gene abundance in soils and sediments in relation to physico-chemical parameters (e.g. total organic carbon content (TOC), nutrient content, pH, soil moisture content etc.).

2.1.2: Hypotheses

In this Chapter it was hypothesized that:

1) In soils and sediments, Bacterial and Archaeal 16S rRNA gene diversity will change seasonally, increasing in the summer and decreasing in the winter.

2) In soils and sediments, differences in underlying geologies will lead to significant differences in Bacterial and Archaeal 16S rRNA gene diversity whereby impermeable clays will have lower microbial diversity compared to permeable sands and chalks.

3) In soils, increased water content during the winter months will lead to a decrease in Bacterial but not Archaeal 16S rRNA gene diversity.

The specific objectives in this chapter were to:

1) Measure changes in physico-chemical parameters of soils and sediments (e.g. cation and anion concentrations, pH, soil water content, total organic carbon, and temperature) over a seasonal cycle in soils and sediments from different underlying geologies.

2) Determine changes Bacterial and Archaeal community composition over a seasonal cycle in soils and sediments from different underlying geologies.

2: Materials and Methods

2.2.1: Sampling sites

Soil and riverine sites in the Hampshire-Avon catchment that encompassed three geology types (chalk, greensand and clay) and a range of baseflow index (BFI) (from 0.2-0.9, **Table 2.1,** see Section 2.2.3) were selected as part of a large NERC Macronutrient Cycle Project (NERC Reference: NE/J011959/1). The location of soil and sediment sampling sites are shown (**Table 2.1**).

Table 2.1: Terrestrial and riverine sampling sites from across the Hampshire Avon catchment. Data provided by NERC Macronutrient Cycle group (NERC Reference: NE/J011959/1).

Site Code	Tributary (Latitude /Longitude)	Geology	Environment Type	Baseflow index
AS1	Sem (N 51° 3' 18.99", W 2° 9' 24.66")	Clay	River	0.207
AS2	Sem (N 51° 2' 44.84", W 2° 6' 37.40")	Clay	River/ Terrestrial	0.551
AS3	Sem (N 51° 3' 58.43", W 2° 8' 37.48")	Clay	River	0.635
GN1	Nadder (N 51° 2' 44.16", W 2° 6' 36.97")	Greensand	River	0.732
GA2	Avon (West) (N 51° 19' 10.42", W 1° 51' 44.06")	Greensand	River/ Terrestrial	0.814
GA3	Avon (West) (N 51° 18' 23.16", W 1° 49' 26.94")	Greensand	River	0.868
CE1	Ebble (N 51° 1' 39.16", W 1° 55' 18.66")	Chalk	River	0.885
CW2	Wylye (N 51° 3' 58.43", W 2° 8' 37.48")	Chalk	River/ Terrestrial	0.905
CA3	Avon (East) (N 51° 18' 13.11", W 1° 48' 36.34")	Chalk	River	0.838

2.2.2: Terrestrial sites and soil sampling.

Three terrestrial sites that were on or near farmland with a river running alongside were selected. These were Whitecliff Farm (CW2 chalk soil), Share Farm (AS2 clay soil), and Puckshipton House (GA2 greensand soil) (**Table 2.1**, **Figure 2.1**). The clay site at Share Farm (**Figure 2.1, panel A**) had two rivers, the Sem (AS2) and the Nadder (GN1), flanking the sampling site and was a grazing site for cows during the summer months. The greensand site at Puckshipton House (**Figure 2.1**, **panel B**) bordered the Avon (West) and was directly next to a grazing site for horses. The chalk site at White Cliff Farm **Figure 2.1**, **panel C**) was located on a grazing area for sheep, with the river Wylye running through the field (**Figure 2.1**). All sites were covered with grass and subjected to grazing from livestock.

A 40 x 40 m sampling plot was established at each soil site (**Figure 2.1**, Panels A, B, and C). Each plot comprised of three transects with sampling points along each transect, 20 m apart (**Figure 2.1A-C**). Triplicate surface soil samples (i.e. 0-5 cm depth) were collected seasonally from each of the three terrestrial sites between 2013-2014 (i.e. February, April, August and November 2013, March, May, July, and November 2014) using a circular steel coring rod (30 cm length 2 cm diameter). Each individual soil section was wrapped in sterile foil, placed in a sterile 20 mL tube and transported back to the laboratory in a vapour shipper vessel (SC 4/2V, Chart Industries, US). Additional surface soils (approximately 15-20 g) were placed into sterile 20 mL universals and stored at 4^{o}C and, within 1 week, CH_4 oxidation potential and physico-chemical properties were measured. Subsections from the surface samples (approximately 3 g) were placed in pre-weighed 120 mL serum bottles, capped and flushed with N_2 gas (0.5 bar pressure) in the field, to

maintain an anoxic headspace for CH_4 production potential measurements (See Chapter 3 Section 1.3).

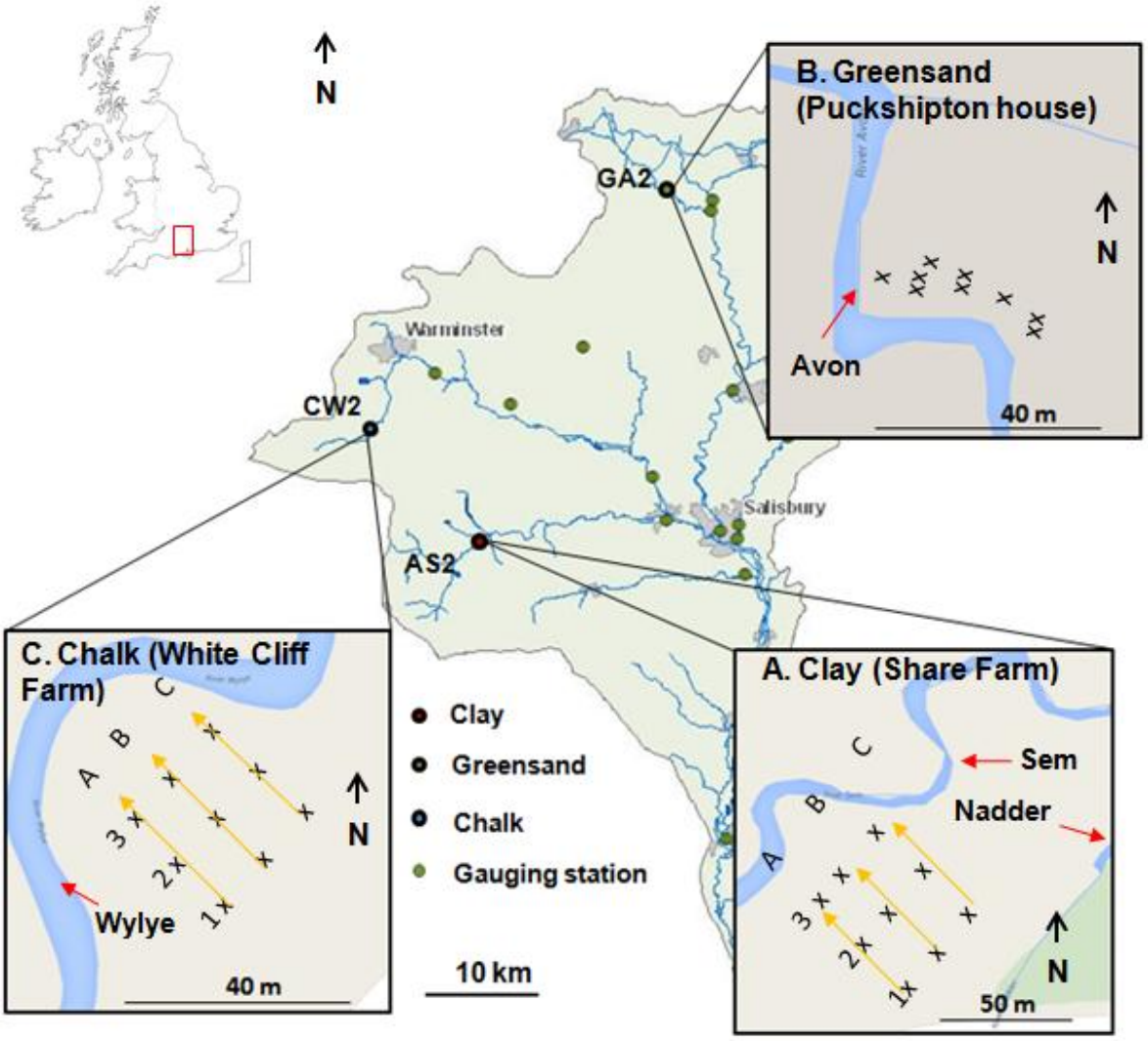


Figure 2.1: Map of Hampshire-Avon terrestrial sampling sites. Panels A, B and C show Transects A, B, and C, each transect contains triplicate sampling points (denoted 1, 2 and 3). Red arrows point to nearby rivers. Image modified from the Environmental Agency http://apps.environment-agency.gov.uk/river-and-sea-levels/120717.aspx.

2.2.3: River sites and sediment sampling.

Nine river sites were selected within the Hampshire-Avon catchment (**Table 2.1**, **Figure 2.2**). River sites were selected based on their underlying geology chalk (CE1, CW2, CA3), clay (AS1, AS2, AS3), greensand (GN1, GA2, GA3) and base flow index (BFI) (**Table 2.1**, **Figure 2.2**).

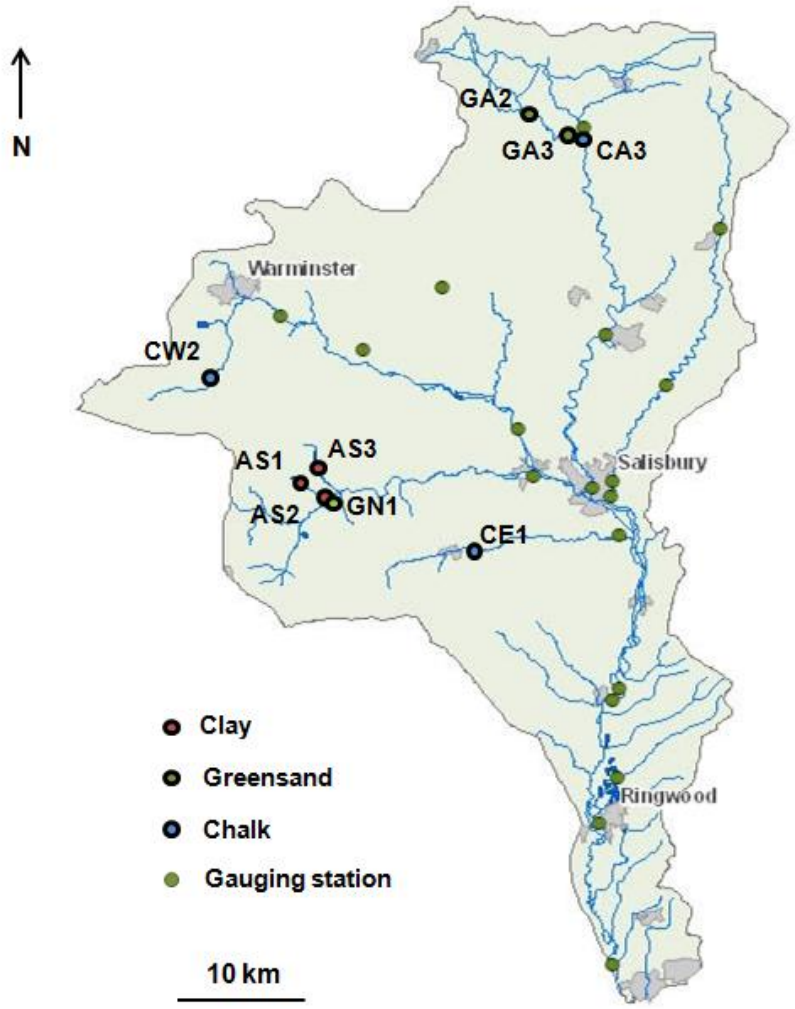


Figure 2.2: Map of Hampshire-Avon catchment and the nine river sites. Site codes listed in **Table 2.1** Image modified from http://apps.environment-agency.gov.uk/river-and-sea-levels/120717.aspx.

The BFI for the nine river sites ranged between 0.8-0.9 (chalk), 0.7-0.8 (Greensand) and 0.2-0.6 (clay) (**Table 2.1**). Therefore, in terms of decreasing BFI, the riverine sites were chalk > greensand > clay.

Sites AS1, AS2, and GN1 were located next to grazing sites for cows. Sites AS2 and GN1 was where the river Sem and Nadder merged into one stream. GA2 was located next to a small grazing site for horses. GA3 and CA3 were near a forested area and away from agriculturally developed areas. CE1 was located next to farmland, and CW2 was a grazing site for sheep (**Figure 2.2**).

River sediments were collected from all nine river sites in February, April, August, November 2013 and three river sites (AS2, GA2, CW2) in March, May, July, and November 2014. Five large sediment cores up to a depth of approximately 0-5 cm were taken at each site using a sterile plastic 8 cm diameter corer. Three subcores (15 g) were removed from the large sediment core for CH_4 oxidation and production potentials and physico-chemical measurements. Three smaller subcores (3 g) were placed in a sterile 20 mL universal tube and stored in a vapour shipper (SC 4/2V, Chart Industries, US) for molecular analyses.

2.2.4: Physico-chemical analyses.

Note: Physico-chemical data i.e. TOC, pH, temperature, anion/cation concentrations, were taken by Dr Hepell (Queen Mary, UK) and Scott Warren (University of Essex). As of writing, pH and temperature of sediments were not made available for this thesis.

Temperature measurements of soils were taken *in-situ* with a thermometer (76 mm insertion thermometer, Fisher brand, UK). The pH of soil was measured by weighing out ~10 g of dry soil, slurried with 25 ml of de-ionized water, and measured

with a pH probe (Jenway 3510 pH meter, Bibby Scientific Ltd. UK). To determine the percent water content of soil, between 1-2 g of soil samples was dried in an 80^{o}C oven (Model IH-150, Thermo Scientific, UK) for 48 hours until a constant weight was obtained.

Total organic carbon (TOC) content of soil and sediments was measured using a Shimadzu TOC-SSM (Shimadzu UK Ltd., UK) following the manufacturer's instructions. Samples were acidified using 1M HCl to remove inorganic carbon and dried overnight at 80^{o}C in an oven (Model IH-150, Thermo Scientific, UK) before measurement.

For cation and anion analysis 2 g of dried soils/sediments were placed into sterile 20 mL universals and 20 mL of sterile deionized water was added. The samples were then sonicated in a sonicating water bath (F5 Minor Ultrasonicator, Decon, UK) for 30 minutes and filtered using acetate free 0.2 µm filters (Chromafil PES 20/25, Macherey Nagel, Germany). Samples were analysed using a Dionex IC system (Thermo Scientific, UK) for both cations (4 mm CSRS column, column flow 1ml min^{-1}, 20mM MSA, column temperature 30^{o}C, detector temperature 35^{o}C) and anions (2mm ASRS column, column flow rate 0.25 ml min^{-1}, 0-10 mM gradient, column temperature 30^{o}C, detector temperature 35^{o}C, MilliQ water, hydroxide cartridge).

Porosity of soils was calculated using the formula shown in **Appendix I.**

2.2.5: Growth of pure cultures of methanotrophs and methanogens.

Pure cultures of *Methylococcus capsulatus* strain Bath (Type I), *Methylocystis parvus* strain OBBP (Type II), and *Methylosinus trichosporium* strain OB3B (Type II) were obtained from Professor Colin Murrell (University of East Anglia, Norwich, UK).

Cultures were grown on Nitrate Minimal Salt (NMS) agar plates (ATCC medium: 1306 Nitrate mineral salts medium). Serum bottles (120 mL) containing NMS liquid media (30 mL) were capped with a rubber butyl stopper and inoculated with single colonies of methanotrophic bacteria from NMS plates. CH_4 was then injected to make up ~10,000 ppm of CH_4 headspace (1% headspace). To maintain oxic conditions, 10-20 mL of headspace was removed and replaced with 20 mL of sterile air followed by 0.9 mL of 100% (v/v) CH_4. NMS cultures were incubated, statically at 30^{o}C in the dark with visible increase in turbidity following 1-2 weeks of regular CH_4 injections.

Pure cultures of *Methanosarcina baltica* strain GS1-A (DSM: 14042) and *Methanococcoides alaskenses* strain AK-5 (DSM: **17273**), were obtained from DSMZ (DSMZ, Braunschweig, Germany). Cultures were grown in 120 ml sterile serum bottles containing liquid DSMZ 141 medium and 80%/20% of N_2/CO_2 headspace. Bottles were incubated statically at 20^{o}C during which the headspace was degassed daily.

2.2.6: DNA extraction from pure cultures, soil and sediment samples.

DNA was extracted from methanotroph and methanogen pure cultures using the Griffith method (Griffiths *et al.,* 2000*)*. DNA was extracted from soils and sediments using PowerSoil DNA extraction kit according to the manufacturer's instructions (Cambio, Cambridge, UK).

2.2.7: PCR-DGGE analysis of Bacterial and Archaeal 16S rRNA genes in soils and sediments.

Bacterial and Archaeal 16S rRNA gene PCR amplifications were performed using the primers and PCR conditions as described by Muyzer *et al.* (1993) and Yu *et al.* (2008) respectively (**Table 2.2**). All PCR amplifications were carried out in triplicate for each sample using a GeneAmp 2720 thermocycler (Applied Biosciences, UK) in 50 µl reactions as follows: 1x RedTaq ready mix (Sigma-Aldrich, UK), 0.2 µM Forward/Reverse primer, and 1 µg template DNA.PCR products were examined using a 1% (w/v) 1x TAE agarose gel (Tris acetate 0.04M, 0.1mM EDTA, pH 8.0) loaded with a 1 Kb ladder (GeneRuler 1Kb DNA ladder, Thermo Scientific), and stained with ethidium bromide (10 mg L^{-1} final concentration). Gels were analysed using a UV Gel Doc (AlphaImager® EP, Alpha Innotech, Canada). Denaturing Gradient Gel Electrophoresis (DGGE) was performed according to the method of Muyzer *et al.* (1993) for both bacteria and Archaea. Resulting gels were then analyzed, with a reference sample loaded for sample comparison and presence/absence was recorded and analyzed (see section **2.2.9**).

Table 2.2: Primers used for Bacterial and Archaeal 16S rRNA gene amplification.

Primer	Gene target	Sequence (5' - 3')	Cycling conditions	References
341f-GC (forward)	Eubacteria 16S rRNA	CCTACGGGAGGCAGCAG[a]	x1 cycle: 5 min 94^{o}C x 35 cycles: 30 s 95^{o}C, 30 s 55^{o}C, 30 s 72^{o}C	Muyzer *et al.* (1993)
518r (reverse)	Eubacteria 16S rRNA	ATTACCGCGGCTGCTGG	x1 cycle: 5 min 72^{o}C	
Arc344f-GC (forward)	Archaeal 16S rRNA	ACGGGGYGCAGCAGGCGCGA[a]	x1 cycle: 5 min 94^{o}C x 35 cycles: 30 s 95^{o}C, 1 min 60^{o}C, 30 s 72^{o}C	Yu *et al.* (2008)
A650r (reverse)	Archaeal 16S rRNA	GTGCTCCCCCGCCAATTCCT	x1 cycle: 7 min 72^{o}C	

[a] GC clamp 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGC-3'

2.2.8: Illumina MiSeq preparation and analysis

Amplicon sequencing libraries of 16S rRNA and functional genes for soils and sediments were prepared by Dr McKew and Dr Chronopoulou (University of Essex). An initial 28 cycle amplification was performed using primers described in **Table 2.2**, but without GC clamps, and the PCR conditions described in section 2.2.7. Amplified DNA was cleaned using a magnetic Solid Phase Reversible Immobilization (SPRI) beads according to the manufacturer's instructions (AMPure XP, Agencourt). Nextera indexes i5 and i7 (Illumina, UK) were attached through an 8 cycle amplification. DNA concentration was quantified using a full-spectrum microvolume fluorospectrometer (Nanodrop 3300, Thermo Scientific, UK) and PCR products pooled to equimolar concentrations. Samples were quality checked using a DNA 1000 kit with a 2100 Bioanalyzer (Agilent) and sequenced by TGAC (The Genomic Analysis Centre, Norwich UK). Sequencing was performed on an Illumina MiSeq platform (Illumina, US) with a MiSeq V3 reagent kit (2 x 300 bp). Raw sequences were demultiplexed and quality filtered by Dr Alex Dumbrell using the QIIME pipeline (QIIME Linux x64 ver. 1.9.0). Operational taxonomic assignments of filtered reads were done using USEARCH (Edgar, 2010). Bacterial and Archaeal taxonomy was assigned using the RDP classifier by Dr Alex Dumbrell (Wang *et al.,* 2007). OTUs were defined as having a cut-off of 97% similarity. Resulting OTUs with less than 100 reads across all samples were excluded from some analyses (e.g. MDS) to account for computational limitations of software and hardware (e.g. too many samples causes software to crash). Relative abundance of OTUs at different taxonomic levels i.e. phylum, kingdom, order, class, family, genus were generated by David Clarke using the QIIME pipeline (University of Essex).

2.2.9: Phylogenetic analysis

Sequences of the 50 most abundant OTUs were aligned with MUSCLE and trimmed in MEGA 6 (Tamura *et al.*, 2013). Nucleotide positions with gaps in aligned sequences were deleted if more than 10% of samples contained them. The sequences from the subsequent data was then used for phylogenetic trees. Representatives within the classes of each of the 50 abundant OTUs (as identified by RDP) were taken from the NCBI database and added into the phylogenetic tree for comparison (see **Appendix VI**). Phylogenetic trees were constructed using Maximum-Likelihood of aligned sequences, using the Tamura-Nei model for nucleotide substitution and bootstrapped 1000 times, using MEGA 6 (Tamura *et al.*, 2013). The relative abundance of sequences between each month and geology were grouped into classes and drawn as pie charts in the final phylogenetic tree.

2.2.10: Statistical analysis

A multivariate ANOVA was performed in XLSTAT (version 2014.5, Addinsoft) and STAMP (version 2.1.3, Parks and Beiko) and used to test differences in the 50 most abundant bacterial/Archaeal 16S rRNA gene sequences by comparing them between different geologies (i.e. Clay vs Chalk vs Greensand), the different months (e.g. February vs August), and the different geologies within each month (e.g. February Clay vs February Chalk vs April Clay etc), with results that were significantly different ($P< 0.05$) further analysed downstream with a Tukey HSD test. Pearson's correlation tests were also used to test for correlations between Archaeal/bacterial communities and physico-chemical factors using XLSTAT (version 2014.5, Addinsoft) (Pearson's correlation results in **Appendix VII**). DGGE data of soils and sediments were used to compare community diversity between

geologies and month by converting presence/absence (1/0) of DGGE bands into a binary similarity matrix using Dice's correlation coefficient. The similarity matrix was then plotted onto a Multi-Dimensional Scaling plot using an absolute model with Kruskal's stress (1) test within the XLSTAT package (version 2014.5, Addinsoft). MDS plots with stress above 0.25 are considered to be randomly distributed, and MDS plots approaching 0 are considered to be less random. Stacked boxplots were constructed for relative abundance of Archaeal and Bacterial 16S rRNA sequences using R Studio (version 0.99.875) with codes provided by David Clark (University of Essex).

2.3: Results

2.3.1: Soil porosity and sediment BFI

Soil porosity measurements showed that chalk soils were the most porous (0.61), followed by clay (0.55) and greensand (0.47). BFI measurement shows that clay sediments had the lowest BFI and greensand sediments were only slightly lower than chalk (**Table 2.1**)

2.3.2 Physico-chemical analysis of 2013 soils

Background Physico-chemical data was collected on soils over the 2013 seasonal cycle and presented in **Table 2.3**. Overall mean *in-situ* temperature remained relatively similar across all soil sites (**Table 2.3**). Temperature increased from 0.4^{o}C in February, to 20^{o}C in June which fell again to 7^{o}C in November. Although pH was taken only in November 2-13, subsequent pH measurements in 2014 (**Table 2.9**) showed that pH remained stable throughout the year i.e. pH of 5 in clay, pH of 7 in greensand and pH 7.5 in chalk. Overall TOC within soils was lowest within the month of August (36.9 to 52.8 mg C g^{-1} dry weight soil), and lowest within the clay soils (36.9 to 49.2 mg C g^{-1} dry weight soil) and highest in greensand (52.8 to 81.8 mg C g^{-1} dry weight soil). Water content also changed between seasons, with the highest water content in February (53.5 to 76.9%) decreasing to the lowest water content in July (19.6 to 34.5%) and increasing again in November (38.1% to 44.9%). Differences between geologies also showed water content being lowest usually in clay (19.6 to 53.5 %), with both greensand (23.9 to 73.9%) and chalk (34.4 to 76.9%) being higher.

Table 2.3: Physico-chemical data for soils sampled in February, April, July and November 2013 soils (± Standard error of the mean, *n*=3). pH data provided by Dr Liang Dong (University of Essex). N.A. = no data available.

	Month	Total organic Carbon (mg C g^{-1} dry weight soil)	Mean water content (%)	Mean pH	Mean Temperature(^{o}C)
Clay	**February**	N.A.	53.5 (±1.0)	N.A.	0.4*
	April	N.A.	41.6 (±0.7)	N.A.	12*
	August	36.9 (±1.6)	19.6 (±3.6)	N.A.	20 (±0.5)
	November	49.2 (±4.0)	38.1 (±6.7)	5.1	7 (±0.5)
Greensand	**February**	N.A.	73.9 (±4.9)	N.A.	0.4*
	April	N.A.	39.6 (±4.9)	N.A.	12*
	August	52.8 (±3.7)	23.6 (±3.5)	N.A.	20 (±0.5)
	November	81.8 (±4.6)	41.1 (±3.7)	7.2	7 (±0.5)
Chalk	**February**	N.A.	76.9 (±2.8)	N.A.	0.4*
	April	N.A.	44.1 (±2.9)	N.A.	12*
	August	51.1 (±3.3)	34.4 (±3.3)	N.A.	20 (±0.5)
	November	62.1 (±4.8)	44.9 (±0.6)	7.6	7 (±0.5)

***** Temperatures from Met Office for 2013 England South http://www.metoffice.gov.uk/climate/uk/summaries/2013

Anion and cation concentrations for the 2013 soils were measured throughout the year and presented in **Tables 2.4 and 2.5**. The anions acetate (1.2 to 1.6 µmol g^{-1} dry weight soil), chloride (0.1 to 0.7 µmol g^{-1} dry weight soil), formate (2.7 to 5.5 µmol g^{-1} dry weight soil) and phosphate (0.1 to 1.1 µmol g^{-1} dry weight soil) generally decreased from February to November across all geologies, with greensand soils generally having higher concentrations of anions compared to other soils in February (0.1 to 5.5 µmol g^{-1} dry weight soil). Nitrate varied greatly between geologies and months with no clear pattern, but was higher than nitrite. Nitrite itself was generally the least abundant anion with only April Chalk soils being notably higher (0.72 µmol g^{-1} dry weight soil day^{-1}). Sulfate followed a similar pattern to acetate and other anions, however sulfate increased to its highest levels in November after July (1.39 to 2.08 µmol g^{-1} dry weight soil)

Cations, with the exception of ammonium in July chalk soils, was present throughout the different months and geologies. There was no strong overall pattern for cation distribution, with the exceptions of a decreased concentration of all cations in July across most geologies, and calcium being generally higher in the chalk soils (excluding February, 15.5 to 22.9 µmol g^{-1} dry weight soil).

Table 2.4: Anion concentration in soil sampled February to November 2013 (± Standard error of the mean, *n*=3)

	Anion concentration (µmol g^{-1} dry weight soil)						
Soil (February)	**Acetate ($C_2H_3O_2^-$)**	**Chloride (Cl^-)**	**Formate ($HCOO^-$)**	**Nitrate (NO_3^-)**	**Nitrite (NO_2^-)**	**Phosphate (PO_4^{3-})**	**Sulfate (SO_4^{2-})**
Clay	1.2 (±0)	0.5 (±0)	3.3 (±0)	0.2 (±0)	0 (±0)	0.1 (±0)	1.2 (±0)
Greensand	1.5 (±0)	0.1 (±0)	5.5 (±0)	2.1 (±0)	0 (±0)	0.7 (±0)	1.1 (±0)
Chalk	1.6 (±0)	0.7 (±0)	2.7 (±0)	0.2 (±0)	0.01 (±0)	1.1 (±0)	0.6 (±0)
Soil (April)	**Acetate ($C_2H_3O_2^-$)**	**Chloride (Cl^-)**	**Formate ($HCOO^-$)**	**Nitrate (NO_3^-)**	**Nitrite (NO_2^-)**	**Phosphate (PO_4^{3-})**	**Sulfate (SO_4^{2-})**
Clay	0.01 (±0)	0.3 (±0)	0.02 (±0)	0.4 (±0)	0.2 (±0)	0.1 (±0)	0.7 (±0)
Greensand	0.01 (±0)	0.1 (±0)	0.04 (±0)	0.7 (±0)	0 (±0)	0.8 (±0)	0.7 (±0)
Chalk	0.03 (±0)	0.1 (±0)	0.01 (±0)	0.7 (±0)	0 (±0)	0.3 (±0)	0.7 (±0)
Soil (July)	**Acetate ($C_2H_3O_2^-$)**	**Chloride (Cl^-)**	**Formate ($HCOO^-$)**	**Nitrate (NO_3^-)**	**Nitrite (NO_2^-)**	**Phosphate (PO_4^{3-})**	**Sulfate (SO_4^{2-})**
Clay	0.03 (±0)	0 (±0)	0 (±0)	0.3 (±0.1)	0 (±0)	0.0 (±0)	0.2 (±0)
Greensand	0.04 (±0)	0 (±0)	0 (±0)	0.6 (±0.4)	0.01 (±0)	0.0 (±0)	0.2 (±0)
Chalk	0.04 (±0)	0 (±0)	0 (±0)	1.0 (±0.2)	0.03 (±0)	0.0 (±0)	0.2 (±0)
Soil (November)	**Acetate ($C_2H_3O_2^-$)**	**Chloride (Cl^-)**	**Formate ($HCOO^-$)**	**Nitrate (NO_3^-)**	**Nitrite (NO_2^-)**	**Phosphate (PO_4^{3-})**	**Sulfate (SO_4^{2-})**
Clay	0 (±0)	0.1 (±0)	0 (±0)	1.0 (±0.1)	0.01 (±0)	0.00 (±0)	2.0 (±0.1)
Greensand	0.01 (±0)	0.03 (±0)	0 (±0)	2.4 (±0)	0.02 (±0)	0.00 (±0)	1.4 (±0.1)
Chalk	0.03 (±0)	0.2 (±0)	0 (±0)	1.6 (±0)	0.01 (±0)	0.00 (±0)	2.1 (±0)

Table 2.5: Cation concentration in soil sampled February to November 2013 (± Standard error of the mean, *n*=3)

Cation concentration (µmol g^{-1} dry weight soil)

Soil (February)	Ammonium (NH_4^+)	Calcium (Ca^{2+})	Magnesium (Mg^{2+})	Potassium (K^+)	Sodium (Na^+)
Clay	0.7 (±0)	4.0 (±0)	0.6 (±0)	0.8 (±0)	0.5 (±0)
Greensand	2.8 (±0)	9.3 (±0)	0.4 (±0)	1.3 (±0)	0.6 (±0)
Chalk	0.1 (±0.1)	1.8 (±0.3)	0.1 (±0.01)	0.3 (±0.1)	0.5 (±0.2)

Soil (April)	Ammonium (NH_4^+)	Calcium (Ca^{2+})	Magnesium (Mg^{2+})	Potassium (K^+)	Sodium (Na^+)
Clay	1.2 (±0)	14.1 (±0)	0.7 (±0)	2.2 (±0)	0.3 (±0)
Greensand	3.4 (±0)	9.6 (±0)	0.5 (±0)	2.0 (±0)	0.9 (±0)
Chalk	0.0 (±0)	15.5 (±0.1)	3.5 (±0.3)	0.8 (±0.1)	4.9 (±0.3)

Soil (July)	Ammonium (NH_4^+)	Calcium (Ca^{2+})	Magnesium (Mg^{2+})	Potassium (K^+)	Sodium (Na^+)
Clay	0.7 (±0)	11.2 (±0)	0.5 (±0)	2.5 (±0)	0.2 (±0)
Greensand	0.0 (±0)	0.4 (±0.1)	0.1 (±0.)	0.2 (±0.1)	0.4 (±0.1)
Chalk	0.0 (±0)	21.5 (±0.1)	0.9 (±0.)	0.5 (±0.1)	3.4 (±0.2)

Soil (November)	Ammonium (NH_4^+)	Calcium (Ca^{2+})	Magnesium (Mg^{2+})	Potassium (K^+)	Sodium (Na^+)
Clay	1.8 (±0)	3.4 (±0)	0.6 (±0)	1.6 (±0)	0.8 (±0)
Greensand	0.1 (±0.1)	2.0 (±0.3)	0.1 (±0)	0.3 (±0.1)	0.2 (±0.1)
Chalk	0.0 (±0)	22.9 (±0)	1.2 (±0)	1.1 (±0)	5.2 (±0)

2.3.3: Physico-chemical analysis of 2013 sediments

TOC of sediments was taken from February and August 2013 sediments. Overall, by geology TOC tended to be higher in greensand sediments (36 to 57.2 mg C g^{-1} dry weight soil), and by month TOC tended to be higher in August, with the exception of clay sediments which were higher in February (8.71 mg C g^{-1} dry weight soil) (**Table 2.6**).

Anion and cation concentrations for the 2013 sediments were measured in February and August by Dr Hepell (Queen Mary) and Scott Warren (University of Essex) and presented in **Table 2.7** and **Table 2.8**. Overall anion concentrations (**Table 2.7**) were low for acetate, chloride, formate, nitrite, and phosphate across all months and geologies. Nitrate was the most abundant anion and was higher in chalk sediments and decreased from February to August. Sulfate remained largely the same between the months and geologies.

Cation concentrations (**Table 2.8**) were abundant across the different months and geologies with the exception of ammonium which was largely absent throughout. Both magnesium and potassium remained largely unchanged between months and geologies. Calcium decreased slightly from February to August, and was most abundant in chalk sediments in February (28.2 µmol g^{-1} dry weight sediment). Sodium on the other hand increased from February to August and was abundant in August clay soils (8.28 µmol g^{-1} dry weight soil).

Table 2.6: TOC of sediment sampled in February and August 2013 sediments (± Standard error of the mean, *n*=3), N.A: Data not available. TOC data provided by Scott Warren (University of Essex).

	Month	Total organic Carbon (mg C g^{-1} dry weight soil)
Clay	**February**	8.7 (±3.3)
	April	10.9 (±5.5)
	August	5.6 (±2.6)
	November	N.A.
Greensand	**February**	36.0 (±11.4)
	April	29.4 (±10.8)
	August	57.2 (±25.7)
	November	32.7 (±10.2)
Chalk	**February**	13.0 (±8.0)
	April	7.7 (±2.7)
	August	24.4 (±27.2)
	November	7.4 (±3.3)

Table 2.7: Anion concentration sampled in February and August 2013 sediments (± Standard error of the mean, *n*=3). Anion data provided by Dr Hepell (Queen Mary, UK)

Anion concentration (µmol g^{-1} dry weight soil)							
Sediment (February)	**Acetate ($C_2H_3O_2^-$)**	**Chloride (Cl^-)**	**Formate ($HCOO^-$)**	**Nitrate (NO_3^-)**	**Nitrite (NO_2^-)**	**Phosphate (PO_4^{3-})**	**Sulfate (SO_4^{2-})**
Clay	0.04 (±0)	0.1 (±0)	0.01 (±0)	1.2 (±0.3)	0.0 (±0)	0.0 (±0)	1.3 (±0.5)
Greensand	0 (±0)	0.1 (±0)	0.00 (±0)	3.9 (±0.2)	0.0 (±0)	0.1 (±0)	2.2 (±0.1)
Chalk	0.01 (±0)	0.1 (±0)	0.00 (±0)	5.0 (±0.4)	0.0 (±0)	0.0 (±0)	1.9 (±0.3)
Sediment (August)	**Acetate ($C_2H_3O_2^-$)**	**Chloride (Cl^-)**	**Formate ($HCOO^-$)**	**Nitrate (NO_3^-)**	**Nitrite (NO_2^-)**	**Phosphate (PO_4^{3-})**	**Sulfate (SO_4^{2-})**
Clay	0.0 (±0)	0.1 (±0)	0 (±0)	0.6 (±0.2)	0.0 (±0)	0.0 (±0)	2.5 (±0.7)
Greensand	0.0 (±0)	0.1 (±0)	0 (±0)	1.3 (±0.1)	0.0 (±0)	0.0 (±0)	1.7 (±0.1)
Chalk	0.0 (±0)	0.02 (±0)	0.0 (±0)	2.3 (±0.1)	0.0 (±0)	0.0 (±0)	1.12 (±0.3)

Table 2.8: Cation concentration sampled in February and August 2013 sediments (± Standard error of the mean, *n*=3). Cation data provided by Dr Hepell (Queen Mary, UK).

Cation concentration (µmol g^{-1} dry weight soil)					
Sediment (February)	**Ammonium (NH_4^+)**	**Calcium (Ca^{2+})**	**Magnesium (Mg^{2+})**	**Potassium (K^+)**	**Sodium (Na^+)**
Clay	0.0 (±0)	12.0 (±2.7)	1.5 (±0.4)	1.5 (±0.2)	2.2 (±0.5)
Greensand	0.0 (±0)	26.3 (±1.6)	1.2 (±0.1)	1.1 (±0.2)	2.6 (±0.1)
Chalk	0.0 (±0)	28.2 (±0.7)	0.8 (±0.1)	0.7 (±0.3)	2.2 (±0.3)

Sediment (August)	**Ammonium (NH_4^+)**	**Calcium (Ca^{2+})**	**Magnesium (Mg^{2+})**	**Potassium (K^+)**	**Sodium (Na^+)**
Clay	0.0 (±0)	15.5 (±0.8)	3.0 (±0.5)	1.2 (±0.4)	8.3 (±2.2)
Greensand	0.0 (±0)	19.3 (±0.7)	1.10 (±0)	0.9 (±0.2)	5.1 (±0.4)
Chalk	0.0 (±0)	15.6 (±0.5)	0.8 (±0.1)	0.4 (±0.14)	3.1 (±0.4)

2.3.4: Physico-chemical analysis of 2014 soils

Background Physico-chemical data was performed on all soils over the 2014 seasonal cycle and the data is presented in Table 3. In general, the mean *in-situ* soil temperatures were consistent across all sites sampled in the same month (**Table 2.9**). The mean *in-situ* soil temperatures increased from 7^{o}C (± 0.5^{o}C) in March to between 20-22^{o}C (± 0.5^{o}C) in July (**Table 2.9**). Generally, the mean soil pH for all sites remained largely unchanged throughout the year with chalk and greensand between 7.5-7.8 and 6.8-7.3 respectively, whilst the clay soil was more acidic between 4.8-4.9 (**Table 2.9**). In general, for each month sampled, the percentage water content for the chalk sites was greater than the greensand and clay sites ($P<0.001$). The mean percentage water content of soil decreased seasonally across all sites (between 60-80% in March decreasing to between 31-46% in July) (**Table 2.9**). In general, TOC fluctuated throughout the year at all sites. Total Organic Carbon (TOC) was the lowest in the clay soils in May (46.6 mg C g^{-1} dry weight soil) but the highest in November (130 mg C g^{-1} dry weight soil). TOC in the greensand sites was 42.8 mg C g^{-1} dry weight soil in March increasing to 95.8 mg C g^{-1} dry weight soil in November. The TOC in the chalk soils was the highest in May (104.5 mg C g^{-1} dry weight soil) and decreased to 57.5 mg C g^{-1} dry weight soil in March (**Table 2.9**).

Porosity of soils was highest in chalk soils (0.61), followed by clay soils (0.55) and was lowest in Greensand soils (0.47) (**Table 2.9**).

Table 2.9: Physico-chemical data for soils sampled in March, May, July and November 2014 soils (± Standard error of the mean, *n*=3). pH data provided by Dr Dong (University of Essex).

	Month	**Total organic Carbon** (mg C g^{-1} dry weight soil)	**Mean water content** (%)	**Mean pH**	**Mean Temperature** (^{o}C)
Clay	March	94.9 (± 8)	60.5 (± 4.)	4.9 (± 0.5)	7 (± 0.5)
	May	46.6 (± 5.7)	51.4 (± 4.1)	4.8 (± 0.5)	12 (± 0.5)
	July	50.7 (± 2.9)	31 (± 2.4)	4.85 (± 0.5)	20 (± 0.5)
	November	130 (±2)	44.2 (± 2.9)	4.91 (± 0.5)	10 (± 0.5)
Greensand	March	42.9 (± 3.2)	63.4 (± 7.2)	7.33 (± 0.5)	7 (± 0.5)
	May	78.8 (± 7.3)	44.8 (± 8.2)	6.84 (± 0.5)	12 (± 0.5)
	July	56.5 (± 7.4)	43.8 (± 7.7)	7.12 (± 0.5)	20 (± 0.5)
	November	95.8 (±12)	42.8 (± 5.1)	7.22 (± 0.5)	9 (± 0.5)
Chalk	March	57.6 (± 7.8)	80.6 (± 6.0)	7.81 (± 0.5)	7 (± 0.5)
	May	104.6 (± 2.2)	62.6 (±10.2)	7.51 (± 0.5)	12 (± 0.5)
	July	99.4 (± 2.2)	46.3 (± 6.7)	7.7 (± 0.5)	22 (± 0.5)
	November	100.3 (±14.9)	65.5 (± 4.5)	7.6 (± 0.5)	10 (± 0.5)

Anion and cation concentrations for the 2014 soil samples were measured and presented in **Table 2.10** and **Table 2.11**. Concentrations of acetate, chloride, formate, phosphate and sulfate, saw overall increases from February to May, but then decreased from May until November (**Table 2.10**). Overall pattern of anion concentrations also showed the highest anion concentrations tended to be in chalk soils. Nitrate were once again more abundant than nitrite throughout the months and geologies, with the highest concentrations found in November greensand and chalk soils (7.63 to 7.93 µmol g^{-1} dry weight soil).

Concentrations of ammonium (**Table 2.11**), calcium, potassium and magnesium all saw similar initial increases from March to May, before decreasing from May to November. Sodium concentrations on the other hand were relatively more stable from March to July but concentrations also dropped in November.

Table 2.10: Anion concentration in soil sampled March, May, July and November 2014 soils (± Standard error of the mean, *n*=3)

Anion concentration (µmol g^{-1} dry weight soil)

Soil (March)	Acetate ($C_2H_3O_2^-$)	Chloride (Cl^-)	Formate ($HCOO^-$)	Nitrite (NO_2^-)	Nitrate (NO_3^-)	Phosphate (PO_4^{3-})	Sulfate (SO_4^{2-})
Clay	1.1 (±0.1)	1.8 (±0.1)	1.7 (±0.1)	0.0 (±0)	1.4 (±0.1)	0.0 (±0)	0.6 (±0)
Greensand	2.3 (±0.3)	1.7 (±0.1)	1.2 (±0.2)	0.2 (±0.1)	1.3 (±0.1)	0.3 (±0.1)	0.4 (±0)
Chalk	1.1 (±0.4)	2.0 (±0.1)	1.5 (±0.3)	0.6 (±0.3)	0.9 (±0.3)	0.1 (±0.1)	0.5 (±0.1)

Soil (May)	Acetate ($C_2H_3O_2^-$)	Chloride (Cl^-)	Formate ($HCOO^-$)	Nitrite (NO_2^-)	Nitrate (NO_3^-)	Phosphate (PO_4^{3-})	Sulfate (SO_4^{2-})
Clay	7.8 (±0.8)	2.5 (±0.2)	6.1 (±0.4)	0.0 (±0)	0.4 (±0.1)	1.0 (±0.4)	5.5 (±1.2)
Greensand	9.3 (±1.9)	2.7 (±0.4)	8.3 (±2.5)	0.0 (±0)	0.6 (±0.1)	1.5 (±0.3)	3.2 (±0.8)
Chalk	13.3 (±1.3)	3.0 (±0.4)	11.7 (±1.1)	0.0 (±0)	1.0 (±0.4)	2.0 (±0.7)	5.9 (±0.9)

Soil (July)	Acetate ($C_2H_3O_2^-$)	Chloride (Cl^-)	Formate ($HCOO^-$)	Nitrite (NO_2^-)	Nitrate (NO_3^-)	Phosphate (PO_4^{3-})	Sulfate (SO_4^{2-})
Clay	1.3 (±0.1)	2.5 (±0.1)	0.8 (±0.2)	0.0 (±0.0)	0.5 (±0.01)	0.2 (±0.1)	0.5 (±0)
Greensand	1.9 (±0.2)	2.5 (±0.2)	0.8 (±0.3)	0.6 (±0.8)	0.7 (±0.10)	0.2 (±0.1)	0.4 (±0)
Chalk	3.7 (±0)	2.4 (±0)	2.8 (±0)	0.1 (±0)	0.6 (±0)	0.2 (±0)	0.5 (±0)

Soil (November)	Acetate ($C_2H_3O_2^-$)	Chloride (Cl^-)	Formate ($HCOO^-$)	Nitrite (NO_2^-)	Nitrate (NO_3^-)	Phosphate (PO_4^{3-})	Sulfate (SO_4^{2-})
Clay	0.2 (±0)	0.7 (±0)	0.1 (±0)	0.0 (±0)	0.8 (±0)	0.1 (±0)	0.3 (±0)
Greensand	0.6 (±0)	0.8 (±0)	0.2 (±0)	1.0 (±0.1)	7.7 (±0.1)	0.2 (±0)	0.4 (±0)
Chalk	0.0 (±0)	1.1 (±0)	0.0 (±0)	0.2 (±0.2)	7.9 (±0)	0.2 (±0)	0.6 (±0)

Table 2.11: Cation concentration in soils sampled March, May, July and November 2014 soils (± Standard error of the mean, *n*=3)

	Cation concentration (µmol g^{-1} dry weight soil)				
Soil (March)	**Ammonium (NH_4^+)**	**Calcium (Ca^{2+})**	**Magnesium (Mg^{2+})**	**Potassium (K^+)**	**Sodium (Na^+)**
Clay	0.5 (±0)	1.7 (±0.2)	0.5 (±0.8)	0.9 (±0)	3.3 (±0.1)
Greensand	0.7 (±0)	6.4 (±0.6)	0.4 (±0.1)	1.6 (±0. 7)	2.9 (±0.1)
Chalk	0.8 (±0.1)	7.9 (±0.7)	0.3 (±0)	0.9 (±0.1)	2.9 (±0.1)
Soil (May)	**Ammonium (NH_4^+)**	**Calcium (Ca^{2+})**	**Magnesium (Mg^{2+})**	**Potassium (K^+)**	**Sodium (Na^+)**
Clay	6.6 (±1.1)	8.0 (±2.3)	2.4 (±0.4)	1.6 (±0.6)	3.7 (±0.2)
Greensand	4.2 (±0.6)	26.0 (±7.5)	1.4 (±0.2)	1.9 (±0.5)	3.7 (±0.2)
Chalk	5.6 (±0.5)	46.4 (±6.7)	1.3 (±0.3)	1.6 (±0.6)	3.4 (±0.1)
Soil (July)	**Ammonium (NH_4^+)**	**Calcium (Ca^{2+})**	**Magnesium (Mg^{2+})**	**Potassium (K^+)**	**Sodium(Na^+)**
Clay	0.8 (±0.1)	2.0 (±0.2)	0.7 (±0.1)	1.2 (±0.2)	3.7 (±0.2)
Greensand	0.5 (±0.1)	6.4 (±0.2)	0.4 (±0.2)	1.4 (±0.9)	3.2 (±0.2)
Chalk	0.8 (±0.1)	9.3 (±1.3)	0.4 (±0)	1.0 (±0)	3.0 (±0.1)
Soil (November)	**Ammonium (NH_4^+)**	**Calcium(Ca^{2+})**	**Magnesium (Mg^{2+})**	**Potassium (K^+)**	**Sodium (Na^+)**
Clay	0.6 (±0.1)	1.0 (±0.1)	0.3 (±0)	0.5 (±0.1)	1.7 (±0.1)
Greensand	0.5 (±0.1)	8.4 (±0.2)	0.4 (±0)	0.6 (±0.1)	1.7 (±0)
Chalk	0.3 (±0)	7.9 (±0.8)	0.4 (±0.1)	0.8 (±0.2)	2.2 (±0.1)

2.3.5: Physico-chemical analysis of 2014 sediments

Anion and cation concentrations for the 2014 sediment samples were measured and presented in **Table 2.13** and **Table 2.14**. Overall concentrations of anions varied widely between each anion species and no clear pattern was observed, except for nitrate which was once again shown to be more abundant than nitrite throughout the samples, and anion concentrations in clay sediments tended to be higher. Cation concentrations were also as disparate as anions, other than clay sediments again, generally having higher cation concentrations.

Table 2.12: TOC of sediment sampled in February and August 2014 sediments (± Standard error of the mean, *n*=3). N.A. data not available.

	Month	**Total organic Carbon (mg C g^{-1} dry weight sediment)**
Clay	February	N.A.
	April	21.3 (±3.5)
	August	47.3 (±18.5)
	November	N.A.
Greensand	February	33.0 (±1.5)
	April	32.9 (±0.6)
	August	20.1 (±3.1)
	November	12.5 (±3.4)
Chalk	February	N.A.
	April	0.99 (±1)
	August	18.8 (±3.3)
	November	4.62 (±1.6)

Table 2.13: Anion concentration in sediment sampled from March, May, July and November 2014 (± Standard error of the mean, *n*=3). N.A= no data available. *****= No data due to flooding

Anion concentration (µmol g^{-1} dry weight sediment)

Sediment (March)	Acetate ($C_2H_3O_2^-$)	Chloride (Cl^-)	Formate ($HCOO^-$)	Nitrite (NO_2^-)	Nitrate (NO_3^-)	Phosphate (PO_4^{3-})	Sulfate (SO_4^{2-})
Clay	N.A.*	N.A.*	N.A.*	N.A.*	N.A.*	N.A.*	N.A.*
Greensand	N.A.*	N.A.*	N.A.*	N.A.*	N.A.*	N.A.*	N.A.*
Chalk	0.4 (*n*=1)	4.5 (*n*=1)	0 (±0)	0 (±0)	0.5 (*n*=1)	0.02 (*n*=1)	0.1 (*n*=1)

Sediment (May)	Acetate ($C_2H_3O_2^-$)	Chloride (Cl^-)	Formate ($HCOO^-$)	Nitrite (NO_2^-)	Nitrate (NO_3^-)	Phosphate (PO_4^{3-})	Sulfate (SO_4^{2-})
Clay	0.6 (±0.2)	2.6 (±0.2)	0.9 (±0.2)	0.0 (±0)	0.3 (±0.1)	0.0 (±0.01)	2.3 (±0.3)
Greensand	0.5 (±0)	2.5 (±0.1)	0.4 (±0)	0.0 (±0)	1.3 (±0.2)	0.0 (±0)	1.1 (±0.3)
Chalk	0.5 (±0.3)	1.7 (±0.9)	0.3 (±0.2)	0.0 (±0)	1.9 (±1.3)	0.1 (±0.1)	0.4 (±0.2)

Sediment (July)	Acetate ($C_2H_3O_2^-$)	Chloride (Cl^-)	Formate ($HCOO^-$)	Nitrite (NO_2^-)	Nitrate (NO_3^-)	Phosphate (PO_4^{3-})	Sulfate (SO_4^{2-})
Clay	1.4 (±0.3)	3.2 (±0.3)	0.3 (±0.2)	0.0 (±0)	0.3 (±0)	0.0 (±0)	1.0 (±0.2
Greensand	0.3 (±0)	2.8 (±0.3)	0 (±0)	0.0 (±0)	0.3 (±0)	0.1 (±0)	2.6 (±0.6)
Chalk	0.2 (±0)	2.3 (±0)	0 (±0)	0.0 (±0)	0.4 (±0)	0.0 (±0)	0.3 (±0)

Sediment (November)	Acetate ($C_2H_3O_2^-$)	Chloride (Cl^-)	Formate ($HCOO^-$)	Nitrite (NO_2^-)	Nitrate (NO_3^-)	Phosphate (PO_4^{3-})	Sulfate (SO_4^{2-})
Clay	N.A.*	N.A.*	N.A.*	N.A.*	N.A.*	N.A.*	N.A.*
Greensand	0 (±0)	1.5 (±01)	0 (±0)	0.2 (±0)	0 (±0)	0.1 (±0)	1.1 (±0)
Chalk	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.

Table 2.14: Cation concentration in sediment sampled from March, May, July and November 2014 (± Standard error of the mean, *n*=3) N.A= no data. N.A.*****= No data due to flooding

Cation concentration (µmol g^{-1} dry weight sediment)					
Sediment (March)	**Ammonium (NH_4^+)**	**Calcium (Ca^{2+})**	**Magnesium (Mg^{2+})**	**Potassium (K^+)**	**Sodium (Na^+)**
Clay	N.A.*	N.A.*	N.A.*	N.A.*	N.A.*
Greensand	N.A.*	N.A.*	N.A.*	N.A.*	N.A.*
Chalk	0.1 (±0.1)	1.7 (±1.7)	0.0 (±0)	0.2 (±0.2)	0.9 (±0.9)

Sediment (May)	**Ammonium (NH_4^+)**	**Calcium (Ca^{2+})**	**Magnesium (Mg^{2+})**	**Potassium (K^+)**	**Sodium (Na^+)**
Clay	0.7 (±0.2)	3.5 (±0.2)	0.4 (±0)	0.7 (±0)	3.5 (±0.1)
Greensand	0.4 (±0.1)	3.8 (±0.3)	0.1 (±0)	0.8 (±0)	3.2 (±0)
Chalk	0.5 (±0.3)	3.7 (±2.0)	0.1 (±0.1)	0.5 (±0)	2.3 (±1.1)

Sediment (July)	**Ammonium (NH_4^+)**	**Calcium (Ca^{2+})**	**Magnesium (Mg^{2+})**	**Potassium (K^+)**	**Sodium (Na^+)**
Clay	0.6 (±0.3)	5.9 (±0.8)	0.8 (±0.2)	1.5 (±0.3)	5.0 (±0.3)
Greensand	0.5 (±0.3)	8.5 (±1.7)	0.3 (±0.1)	3.8 (±2.6)	3.4 (±0.2)
Chalk	0.5 (±0)	2.9 (±0)	0.1 (±0)	0.5 (±0)	2.9 (±0)

Sediment (November)	**Ammonium (NH_4^+)**	**Calcium (Ca^{2+})**	**Magnesium (Mg^{2+})**	**Potassium (K^+)**	**Sodium (Na^+)**
Clay	N.A.*	N.A.*	N.A.*	N.A.*	N.A.*
Greensand	0.1 (±0)	3 (±0.1)	0.1 (±0)	0.5 (±0)	1.9 (±0.1)
Chalk	N.A.	N.A.	N.A.	N.A.	N.A.

2.3.6: Bacterial 16S rRNA gene diversity in soils

DGGE analysis of Bacterial 16S rRNA genes for soils sampled in February and August was performed **(Appendix Figure A3.1-A3.4)**. In general, there was good reproducibility obtained between replicates.

An MDS plot of the Bacterial 16S rRNA gene DGGE band profiles of soils sampled in February and August is presented in **Figure 2.3**. In February, the soil Bacterial communities generally formed distinct clusters based on geology, whereas in August these clusters were less distinct with several bands (**Figure 2.3**).

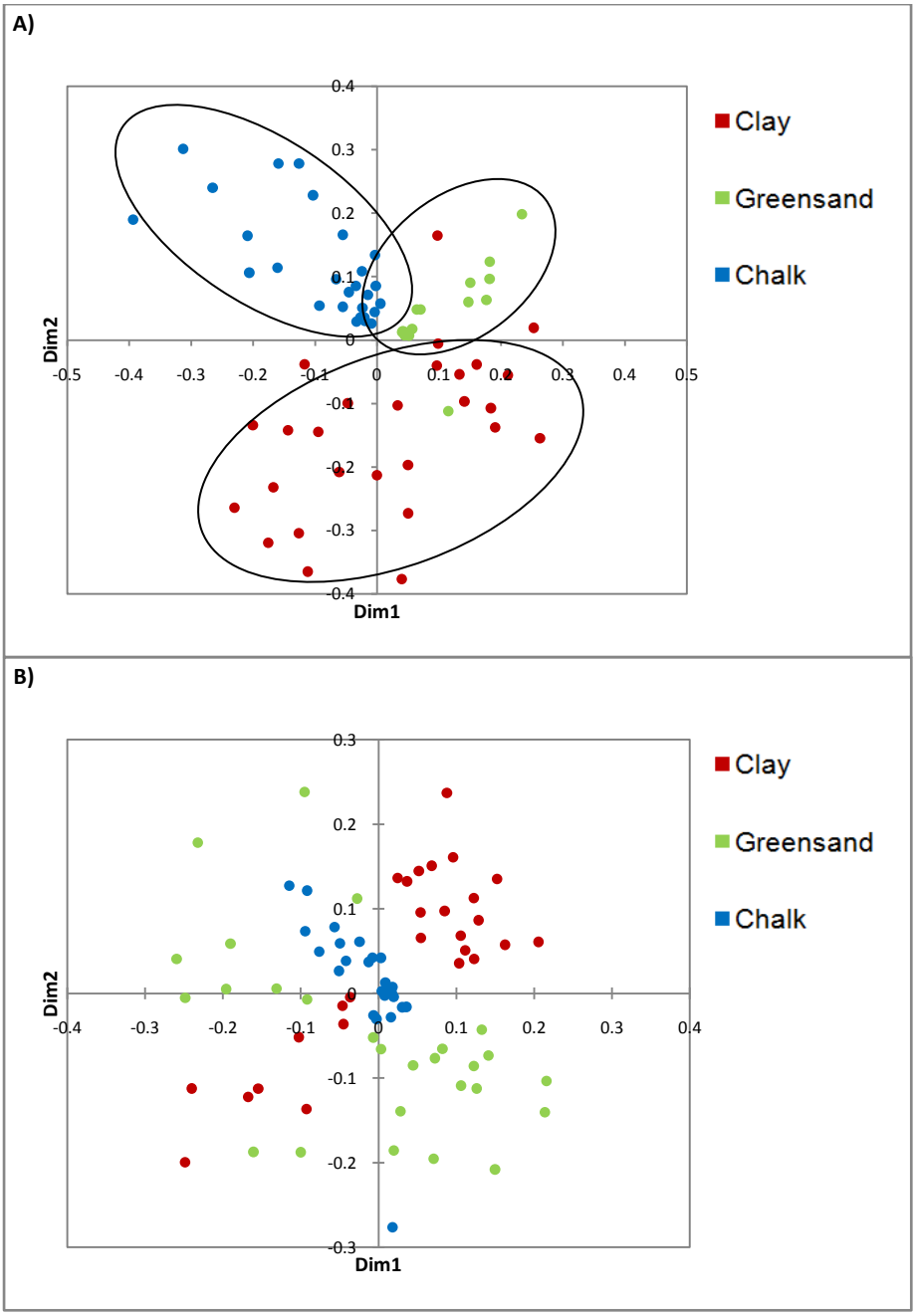


Figure 2.3: MDS plot of soil DGGE bands in February (A) (Kruskal's stress (1) = 0.208) and August (B) (Kruskal's stress (1) = 0.277) soils.

MDS plots comparing the DGGE band profiles of Bacterial 16S rRNA gene for each soil geology are presented in **Figure 2.4 A-C**. Across all geologies, the Bacterial communities present in the sediments sampled in February were generally distinct from those in August (ANOVA, *P*< 0.001) (**Figure 2.4 A-C**).

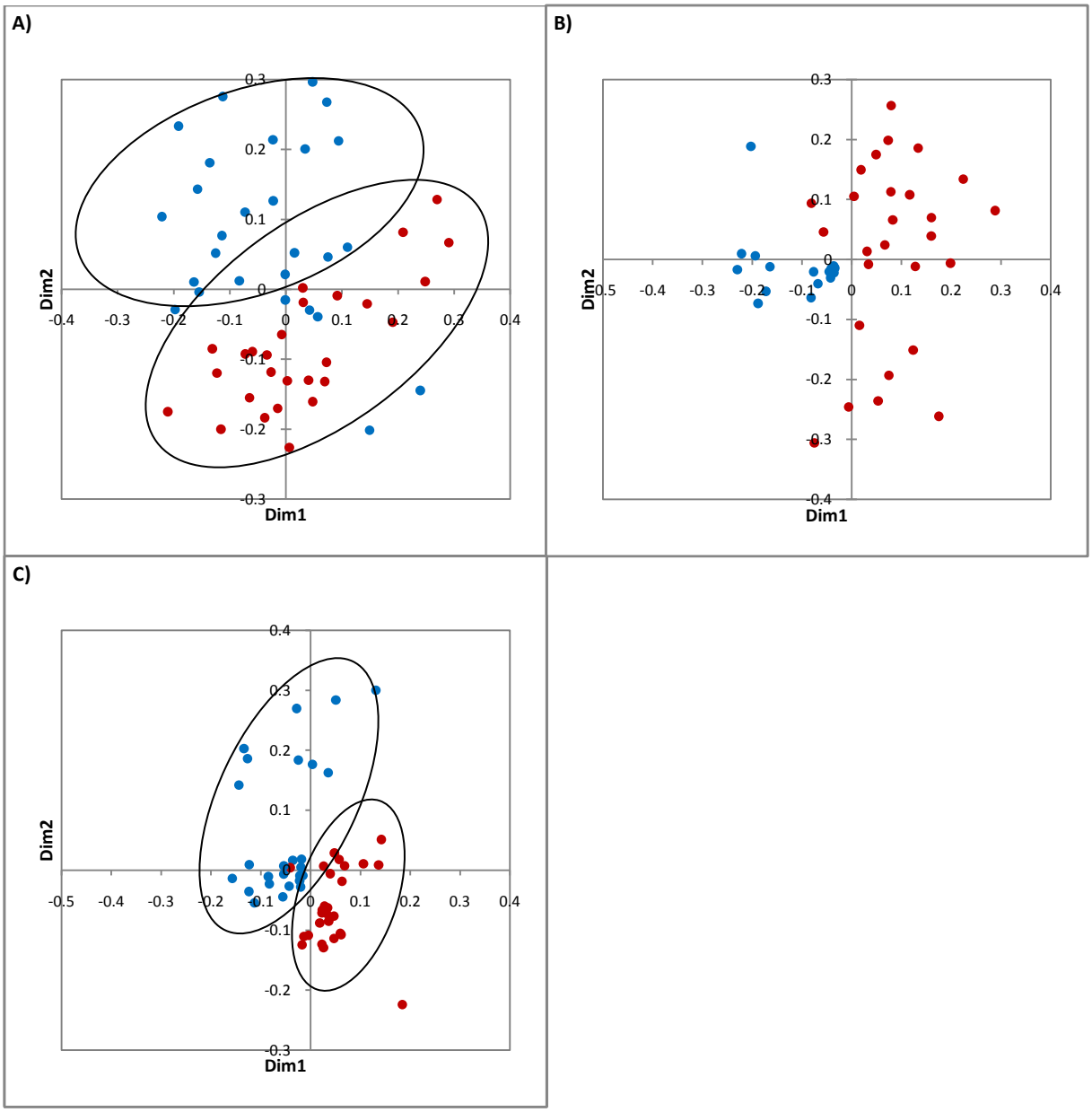


Figure 2.4: MDS plot of soil Bacterial 16S rRNA DGGE bands in February (blue) and August (red) clay (A) (Kruskal's stress (1) = 0.255), Greensand (B) (Kruskal's stress (1) = 0.216), and chalk (c) (Kruskal's stress (1) = 0.141).

Illumina MiSeq sequence analysis of Bacterial 16S rRNA genes obtained from the soils is shown in **Figures 2.5-2.7.** In soils, amplification of Bacterial 16S rRNA showed a diverse range of organisms with 18646 OTUs at the genus level across all samples. A rarefaction curve was constructed from 5609 OTUs with over 100 reads

(**Appendix Figure A2.1**). A stacked bar plot of the relative abundance (%) of Bacteria at the order level was made and presented in **Figure 2.5**.

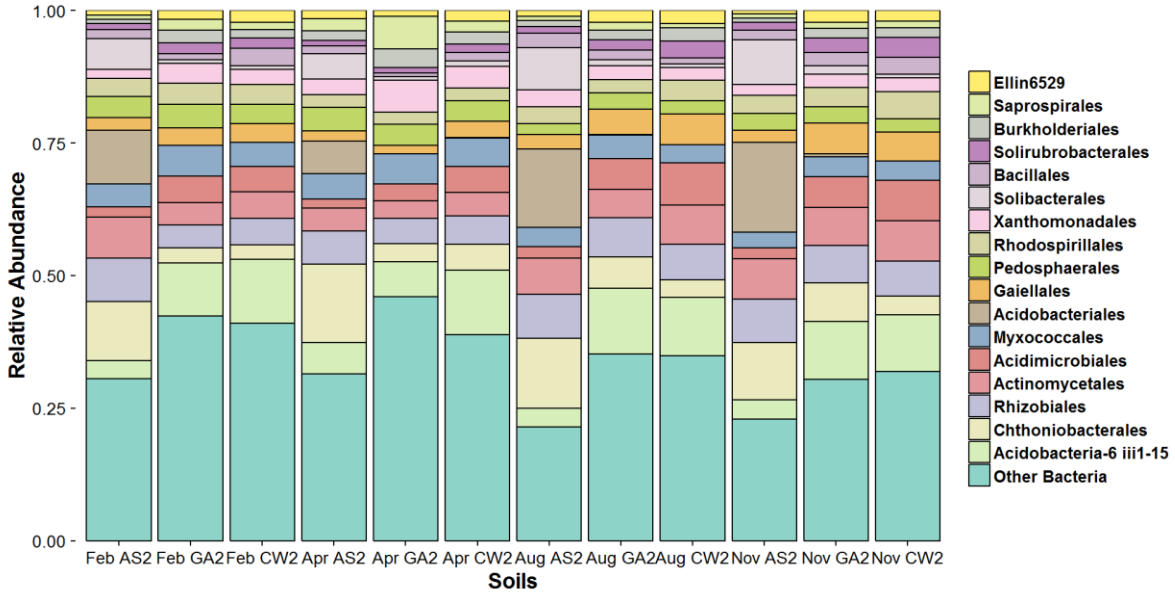


Figure 2.5: Stacked bar plot of relative abundance of different orders of Bacteria within clay (AS2), Greensand (GA2), and chalk (CW2) soils in February (Feb), April (Apr), August (Aug), and November (Nov) 2013. Other Bacteria include OTUs <1% of total abundance and clades only identifiable as Bacteria (also <1%).

Across all geologies and months, the majority of Bacterial orders made up less than 1% of the total Bacterial community and were collapsed into a single group as "Other Bacteria", which included unidentified Bacterial clades (0.3% of total relative abundance) identified only as Bacterial sequences. There were 330 different orders that had made up the <1% grouping including one identifiable methanotroph order Methylacidiphilales (<0.1% of total abundance). The bacteria orders within the <1% grouping primarily fell within the phyla of Acidobacteria, Bacteriodetes, Chloroflexi, Proteobacteria, TM7, and Verrucomicrobia. Overall, many of the <1% Bacterial orders shifted significantly between months and geologies (ANOVA, $P < 0.05$), with most orders generally being more abundant in clay soils (Tukey tests, $P <$

0.05), and a large majority of orders having higher abundance in April (Tukey tests, $P < 0.01$).

For bacteria that were >1% abundance, comparisons by geology showed significant differences in Bacterial orders (Tukey tests, $P < 0.049$), with the exception of Actinomycetales, Bacilli, Pedosphaerales, and Rhodospirales. A major difference between the geologies is the order Acidobacteriales, which formed a larger portion of the clay soils compared to the other sites and made up 6.4% to 16.9% of the Bacterial community (<0.01% in chalk/greensand soils) (Tukey tests, $P < 0.001$). On the other hand Acidomicrobiales was lower in abundance in clay geologies (1.8% to 2.2%) compared to other geologies (3.7% to 8.0%) (Tukey test Chalk $P < 0.05$, Greensand $P < 0.001$). The order Solibacterales and Chthoniobacterales were also higher in clay soils (4.7 to 8.4% and 10.9% to 14.9% respectively) compared to Greensand or chalk (Tukey tests, $P < 0.02$).

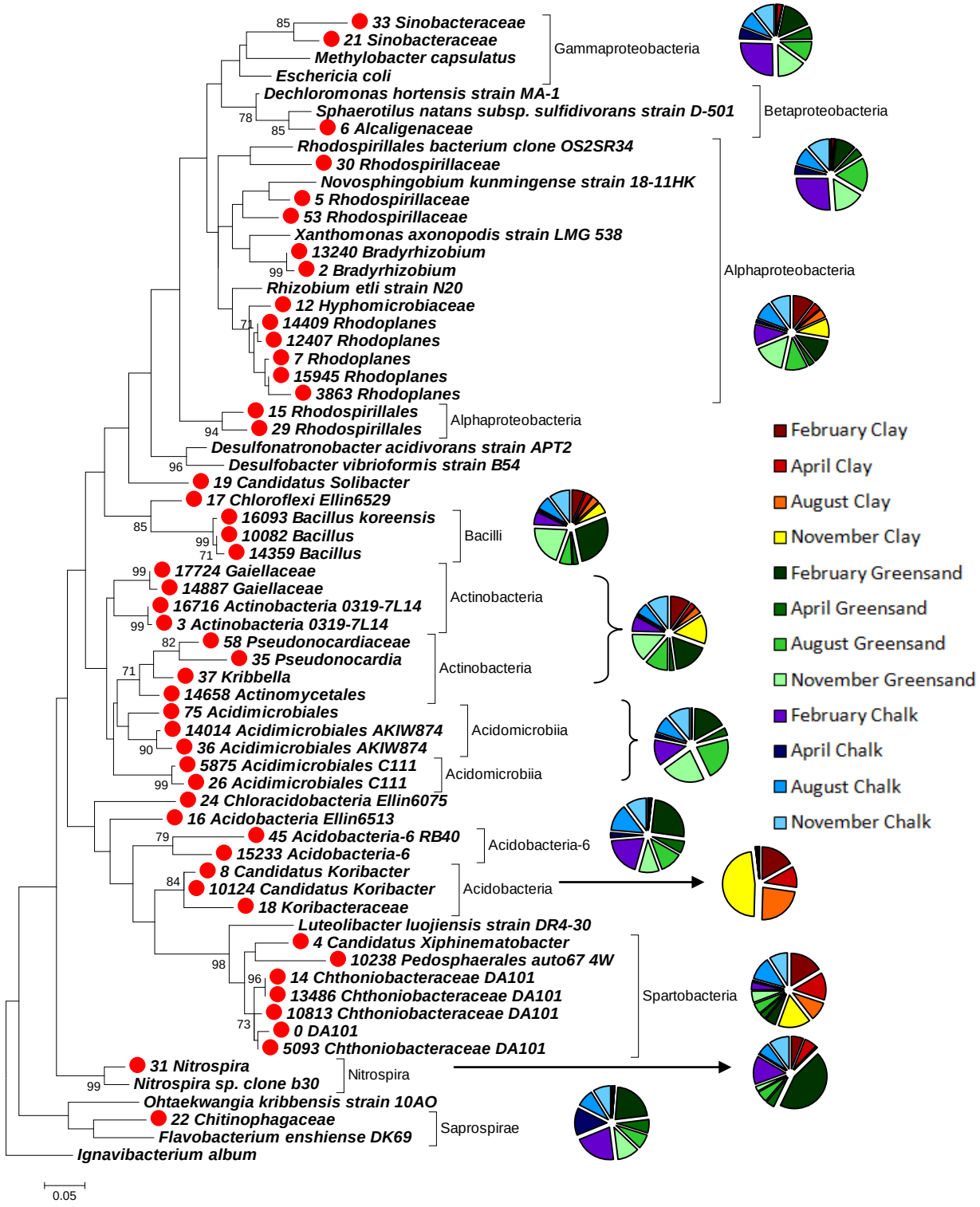


Figure 2.6: Maximum-Likelihood tree of the 50 most abundant Bacterial OTUs (red circle) within soils compared to closest sequence hits on NCBI. Only bootstrap values of >70% are presented. Pie charts represents relative abundance (%) of major classes of bacteria.

Overall, seasonal shifts in Bacterial orders, across all geologies, were significant for 11 of the 18 major orders (Tukey tests, *P*= 0.038) (see **Appendix Table A6.1**). Sequences of the 50 most abundant OTUs in soils was used to construct a phylogenetic tree with pie charts representing the relative abundance (%) of the classes within each geology across the months (**Figure 2.6**). Analysis of these sequences showed they could be separated into 11 classes; Acidobacteriia (3 OTUs), Acidobacteria-6 (2 OTUs), Acidimicrobiia (4 OTUs), Actinobacteria (8 OTUs), Alphaproteobacteria (13 OTUs), Baccilli (3 OTUs), Betaproteobacteria (1 OTU), Gammaproteobacteria (1 OTU), Nitrospira (1 OTU), Saprospirae (1 OTU), and Spartobacteria (7 OTU) (**Figure 2.6**). Distribution of Bacterial classes between chalk and Greensand were similar, whereas clay soils diverged greatly. Acidobacteriia and Saprospirae were the most dominant classes in clay soils, but were the least abundant in Chalk and Greensand (**Figure 2.6**). Between geologies the classes Betaproteobacteria (52%), Gammaproteobacteria (51%), and Saprospirae (52%) were found predominantly in the chalk soils, Alphaproteobacteria (41%), Bacilli (57%), Actinobacteria (44%), Acidomicrobia (65%), Acidobacteria-6 (52%) and Nitrospira (57%)were found predominantly in greensand soils, and Acidobacteriia (98%) and Spartobacteria (53%) were mainly found within clay soils. Between months, Acidobacteria-6 (45%), Betaproteobacteria, Saprospirae (43%), Nitrospira (65%), and Gammaproteobacteria (42%) were dominant in February soils, and Alphaproteobacteria (35%), Acidobacteriia (49%), and *Acidimicrobiia* (36%) were most abundant in November. April generally had the lowest Bacterial abundance relative to other months (4% to 21%), whereas August was usually 2nd or 3rd most abundant (12% to 32%).

An NMDS of Bacterial OTUs against geologies and months was constructed using 5609 OTUs that were 100 reads and above (this was to make it computationally viable on R, without crashing the software) (**Figure 2.7**). Clay soils (circled) were clustered furthest away from the other soils sampled (**Figure 2.7A**). The distance between the clay and the chalk/Greensand sites was due to a large cluster of OTUs unique to the clay soils (**Figure 2.7B**), most likely Acidobacteriia and Spartobacteria (**Figure 2.6**).

Except for April chalk and Greensand soils, the remaining chalk and Greensand samples were clustered closer together (**Figure 2.7A**), with a smaller grouping of OTUs in the April soils seeming to separate from the larger clusters (**Figure 2.7B**).

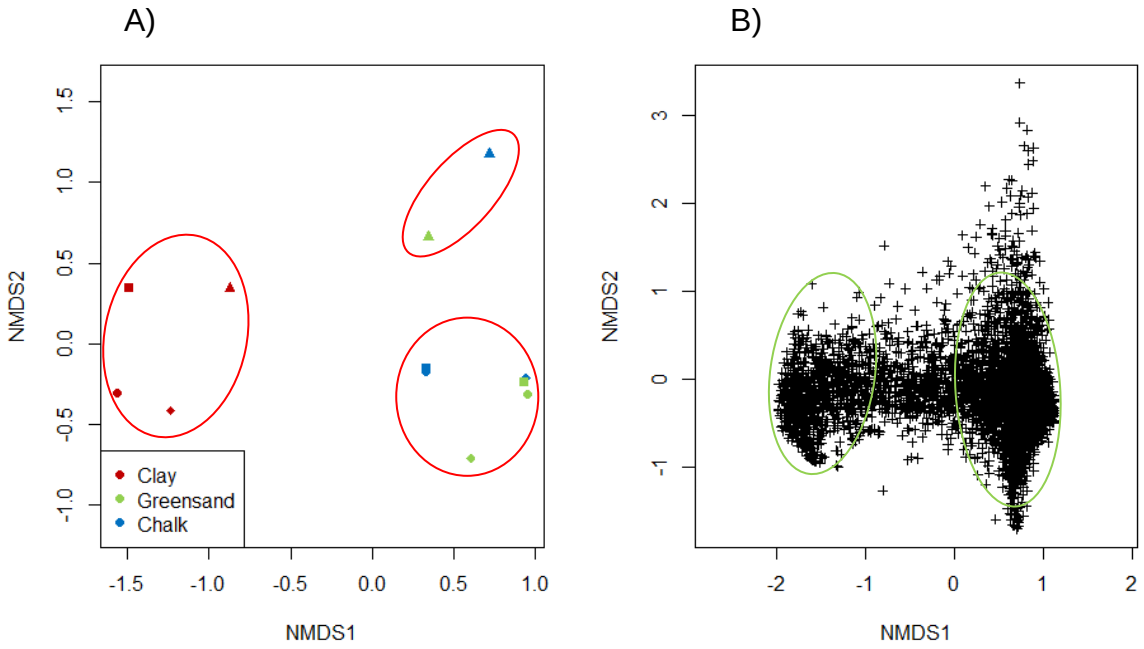


Figure 2.7: Non-metric multidimensional scaling plots of Bacterial OTUs in relation to soil sites. A) NMDS of sites according to OTU abundance B) NMDS of OTUs according to abundance at sites. Circle= November, Square= August, Triangle = April, Diamond = February

Diversity revealed by Shannon and Simpson indices showed that Bacterial diversity changed little between geologies of the same month, however differences between months was more pronounced with the lowest diversity within November soils (H= 6.799 to 7.668, D= 0.996 to 0.999) and the highest within August soils (H= 6.802 to 7.668, D= 0.996 to 0.999) (**Appendix Table A3.1**).

2.3.7: Bacterial 16S rRNA gene diversity in sediments

PCR-DGGE analysis of Bacterial 16S rRNA genes for triplicate sediments sampled in February and August was performed and the band profiles are presented in **Appendix Figures A3.5 and A3.6**. In general there was good reproducibility obtained between replicates and sediments had high diversity across all sites in both February and August.

An MDS plot derived from the Bacterial 16S rRNA gene DGGE band profiles of sediments sampled in February and August is presented (**Figure 2.8**). In February, both greensand and clay sites clustered together whereas those Bacterial communities from the chalk sites were more distinct (**Figure 2.8A**). In August however, the Bacterial communities from each geology formed discrete clusters from each other (**Figure 2.8B**).

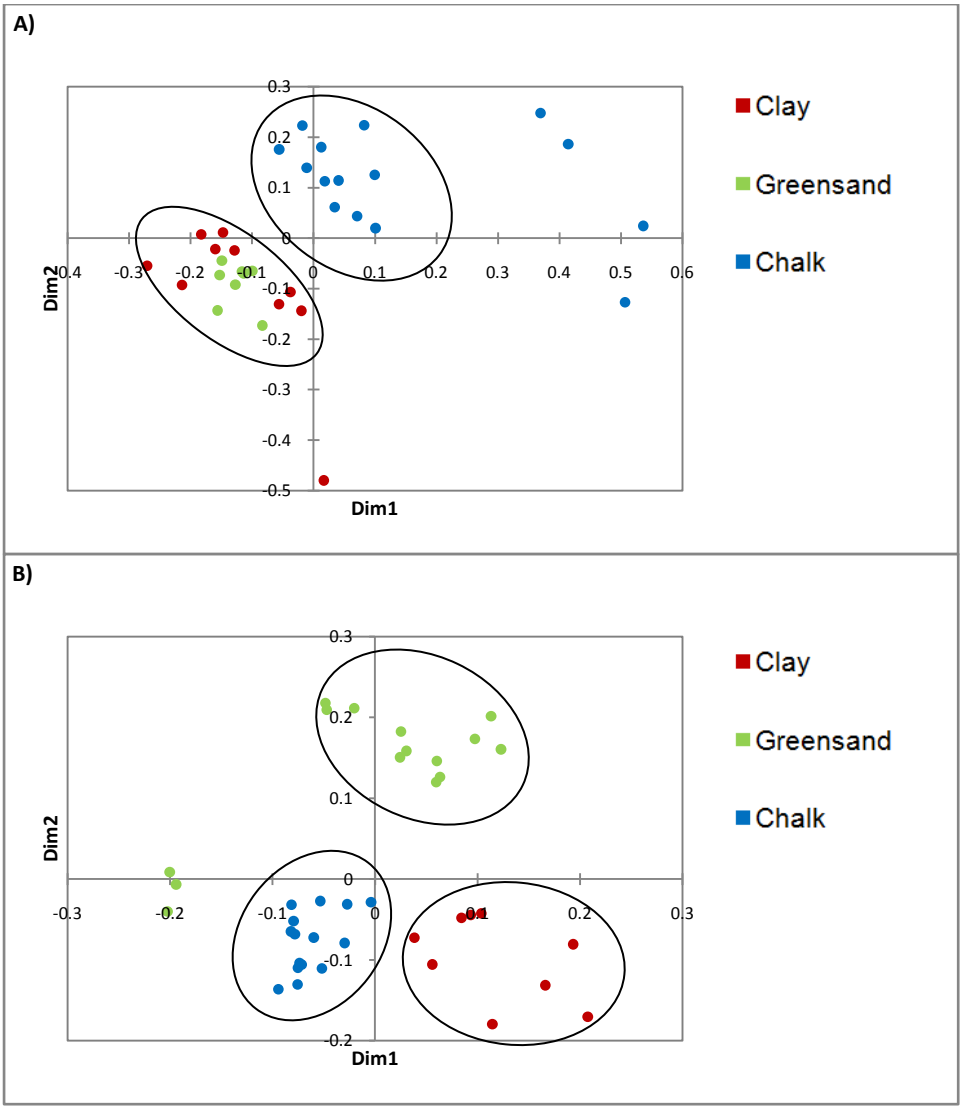


Figure 2.8: MDS Scaling plot of sediment Bacterial 16S rRNA gene DGGE band profiles in February (A) (Kruskal's stress (1) = 0.138) and August (B) sediments (Kruskal's stress (1) = 0.167).

An MDS plot comparing geology between months derived from the Bacterial 16S rRNA gene DGGE band profiles of sediments sampled in February and August is presented (**Figure 2.9**). Across all geologies, the Bacterial communities present in the sediments sampled in February were generally distinct from those in August (ANOVA, *P*<0.0001) (**Figure 2.9 A-C**).

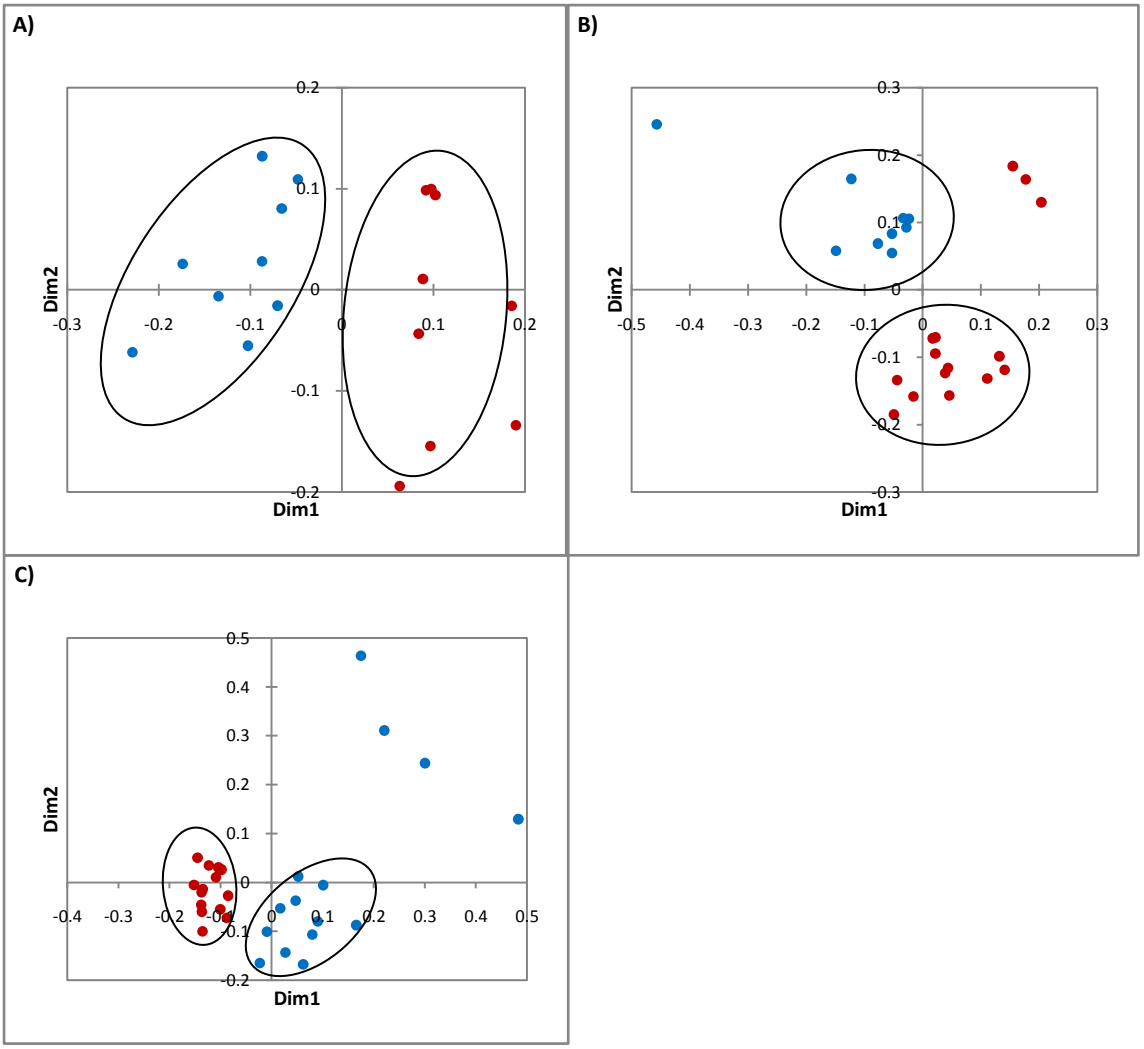


Figure 2.9: MDS plot of Bacterial 16S rRNA DGGE bands in river sediments in February (blue) and August (red) clay (A) (Kruskal's stress (1) = 0.157), Greensand (B) (Kruskal's stress (1) = 0.120), and chalk (c) (Kruskal's stress (1) = 0.139).

Illumina MiSeq sequence analysis of Bacterial 16S rRNA genes obtained from the sediments is shown in **Figures 2.10-2.12.** In sediments, amplification of Bacterial 16S rRNA genes generated a total of 46,568 unique OTUs across all samples. A rarefaction curve was constructed from 2768 OTUs with over 100 reads (**Appendix Figure A2.2**). A stacked bar graph of relative abundance of Bacterial 16S rRNA was

constructed by assigning the full sequence set at the taxonomic order level (**Figure 2.10**). Orders with less than 1% of total abundance or were unclassified/classified only as bacteria were collapsed into a single clade denoted as "Other Bacteria".

Excluding the unidentified bacteria, the Bacterial community that made up <1% of abundance included Bacterial orders within the phyla Acidobacteria, Chloroflexi, Cyanobacteria, Deinococci, Proteobacteria (including classes Beta, Delta, and Epsilon), Spirochaetes, TM7 and Verrucomicrobia. Bacterial orders that were <1% of total abundance shifted significantly between geologies and months ($P< 0.05$). Overall, when comparing geologies, most Bacterial orders were the least abundant in greensand sediments (Tukey tests, $P< 0.05$). Comparisons between months showed a significant increase in Bacterial orders in August compared to February (Tukey tests, $P< 0.05$).

The most abundant group of OTUs within February and August sediments were unidentified Bacteria comprising between 10.9 to 29.9% and 9.9 to 22.9% of the community respectively, however there was no significant difference in unclassified bacteria between the samples, whether comparing between geologies or months. There was a small significant difference in unclassified bacteria, if each river was compared individually, however, only river GA2 in February was significantly different from 3 other rivers (August CW2, February GN1, and February CE1). The second most abundant groups included Burkholderiales, Bacteriodetes, Gammaproteobacteria, Betaproteobacteria and Deltaproteobacteria. The most abundant OTUs in February were related to Burkholderiales, Rhodocyclales, and Sphingobacteriales, whilst in August the most abundant orders were Acidobacteriia, Burkholderiales, and Sphingobacteriales.

Between rivers of the same geology, GN1 and GA2 had a bigger difference in community composition in February, with "Other Bacteria" constituting 30% of total abundance in GN1 compared to 51% in GA2. Between the clay rivers in February, "Other Bacteria" abundance decreased from AS1 (52%), to AS2 (42%), then AS3 (38%), whilst in chalk rivers the relative abundance of "Other Bacteria" were largely similar (42 to 45%).

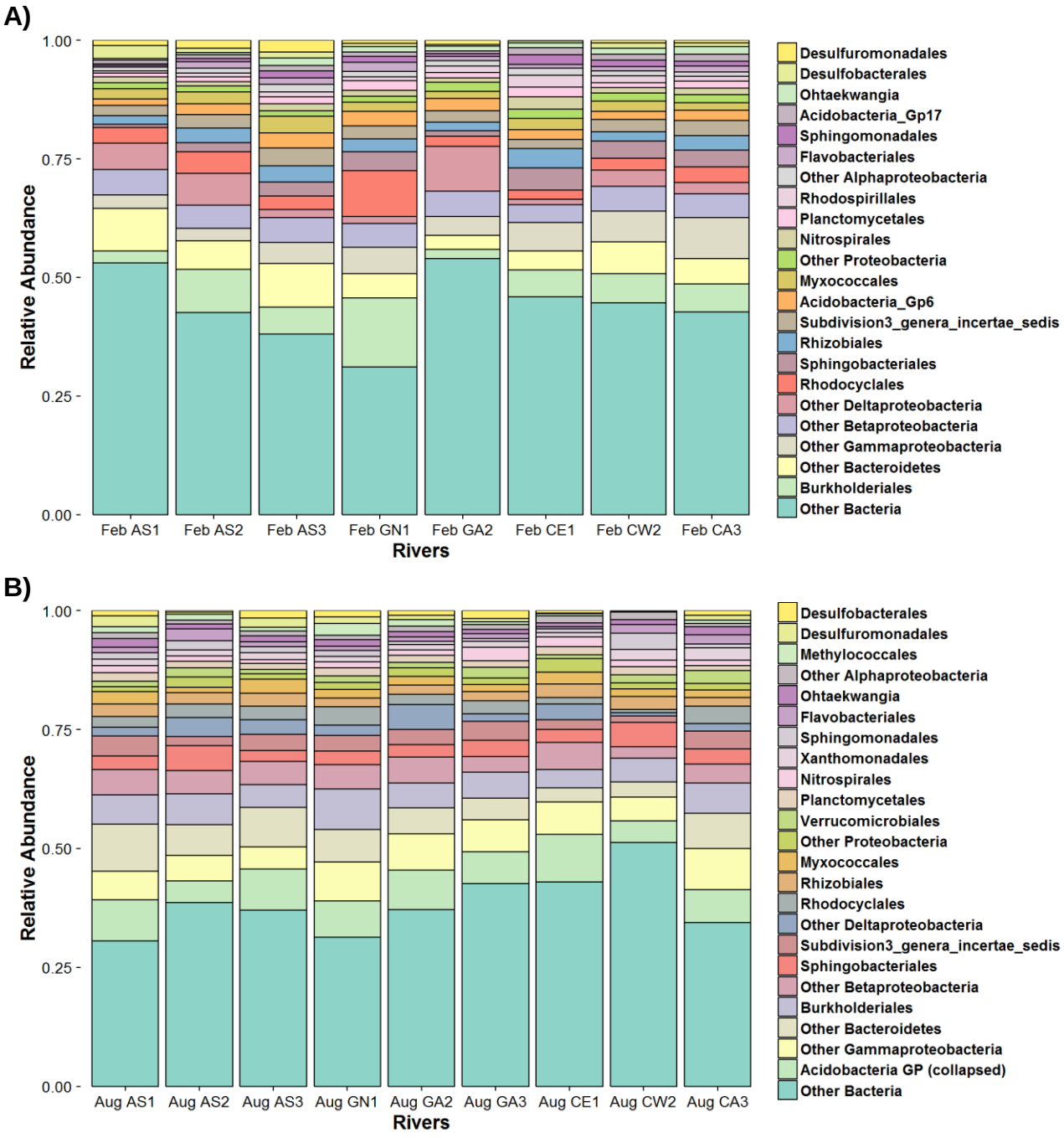


Figure 2.10: Stacked bar plot of relative abundance of Bacterial 16S rRNA genes obtained from sediments in February (A) and August (B). Sequences higher than order are prefixed with "Other". Clay (AS1-3), Greensand (GN1, GA2-3), and chalk (CE1, CW2, CA3). February (Feb), August (Aug).

A phylogenetic tree of the 50 most abundant Bacterial OTUs was constructed with NCBI sequences representative of their inferred classes (**Figure 2.11**). The percent relative abundance of each class across month and geology are represented by pie charts. Sequences grouped into 9 classes with most of these sequences grouped with the classes Betaproteobacteria and Gammaproteobacteria, with the remaining sequences divided among the classes Alphaproteobacteria, Acidobacteria, Deltaproteobacteria, Ignavibacteria, Nitrospira, Subdivision 3 and Verrucomicrobia, with Bacteriodetes phylum as a separate group (**Figure 2.11**). Betaproteobacteria clade contained only 4 OTUs identifiable at the genus level, the remaining sequences were identifiable only at or above the family level. Most of the OTUs in the Gammaproteobacteria were not identifiable below the order level, with only 4 identified at the genera level as either *Xanthomonas* or *Methylobacter*. Bacteroidetes included 3 sequences from the genus *Ohtaekwangia*, and 3 unidentified Bacteroidetes. Alphaproteobacteria contained 3 sequences at the genus level 2 from *Novosphingobium* and 1 from *Rhizobium*. Within the Deltaproteobacteria no sequences were identifiable below the family level. Ignavibacteria only contained 1 sequence identified as from the genus *Ignavibacterium.* All sequences in the class *Nitrospira* fell under the genus *Nitrospira*. The only 2 sequences from the class Acidobacteriia were both from the same genus, Acidobacteriia GP-17. Between geologies the classes Betaproteobacteria (40%), Gammaproteobacteria (43%), Ignavibacteria (41%), Nitrospira (39%), Acidobacteriia (45%) and Subdivision 3 (38%), were predominantly found in greensand sediments, Bacteriodetes (40%) and Alphaproteobacteria (37%) in clay sediments, and Deltaproteobacteria (38%) in chalk sediments. Between months, all Bacterial classes favoured sediments in August (21% to 32% clay, 22% to 39% greensand, 12% to 30% chalk) with the

exception of Deltaproteobacteria in February chalk sediments (26%). Between geologies the classes Betaproteobacteria (40%), Gammaproteobacteria (43%), Ignavibacteria (41%), Nitrospira (39%), Acidobacteriia (45%) and Subdivision 3 (38%), were predominantly found in greensand sediments, Bacteriodetes (40%) and Alphaproteobacteria (37%) in clay sediments, and Deltaproteobacteria (38%) in chalk sediments. Between months, all Bacterial classes favoured sediments in August (21% to 32% clay, 22% to 39% greensand, 12% to 30% chalk) with the exception of Deltaproteobacteria in February chalk sediments (26%).

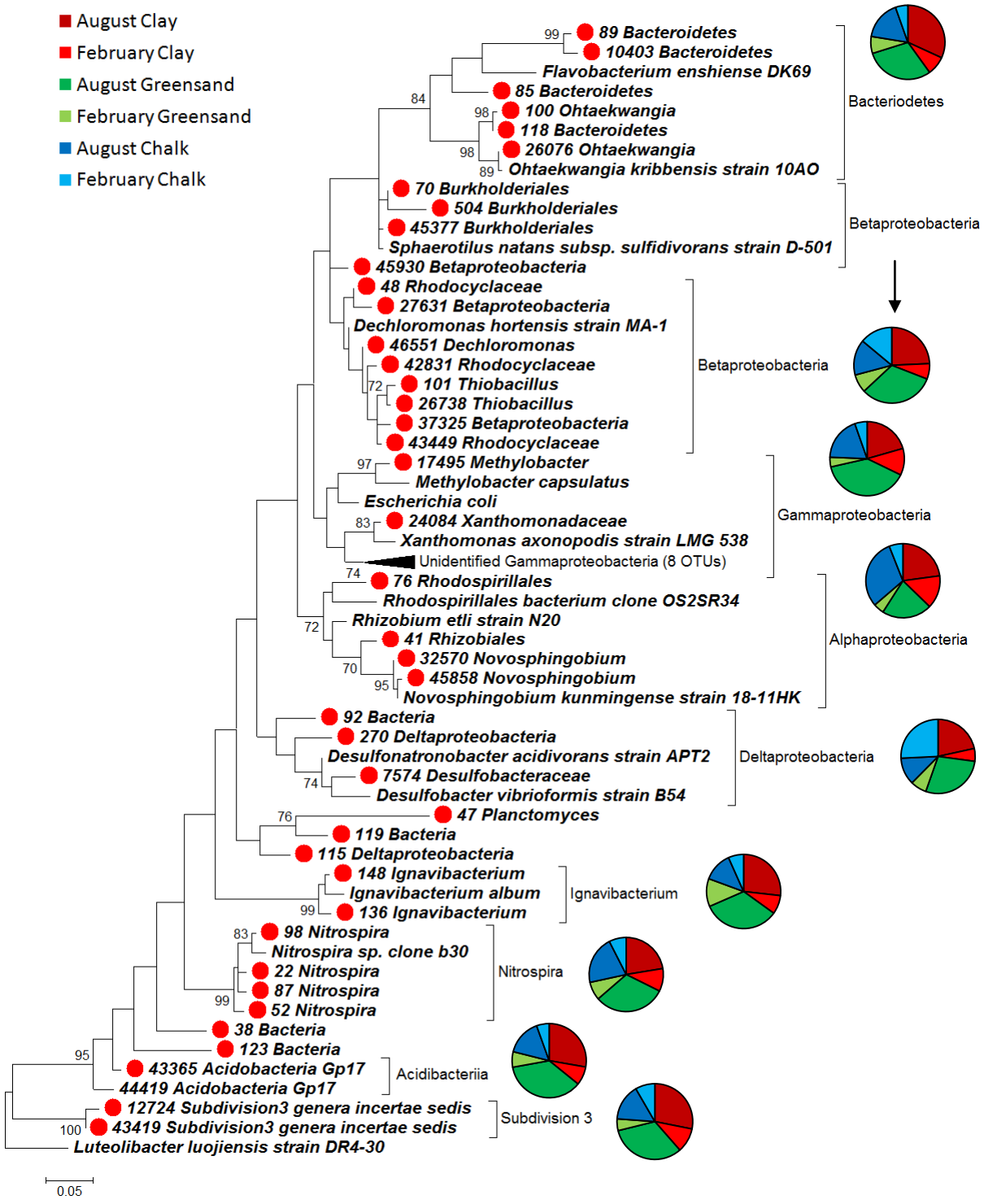


Figure 2.11: Phylogenetic tree of the 50 most abundant Bacterial OTUs (red circle) within sediments compared to closest sequence hits on NCBI. Only bootstrap values of >70% are presented. Pie charts represent relative abundance (%) of major classes of bacteria.

An NMDS was constructed from 2768 sequences used for the rarefaction curve (**Figure 2.12**).

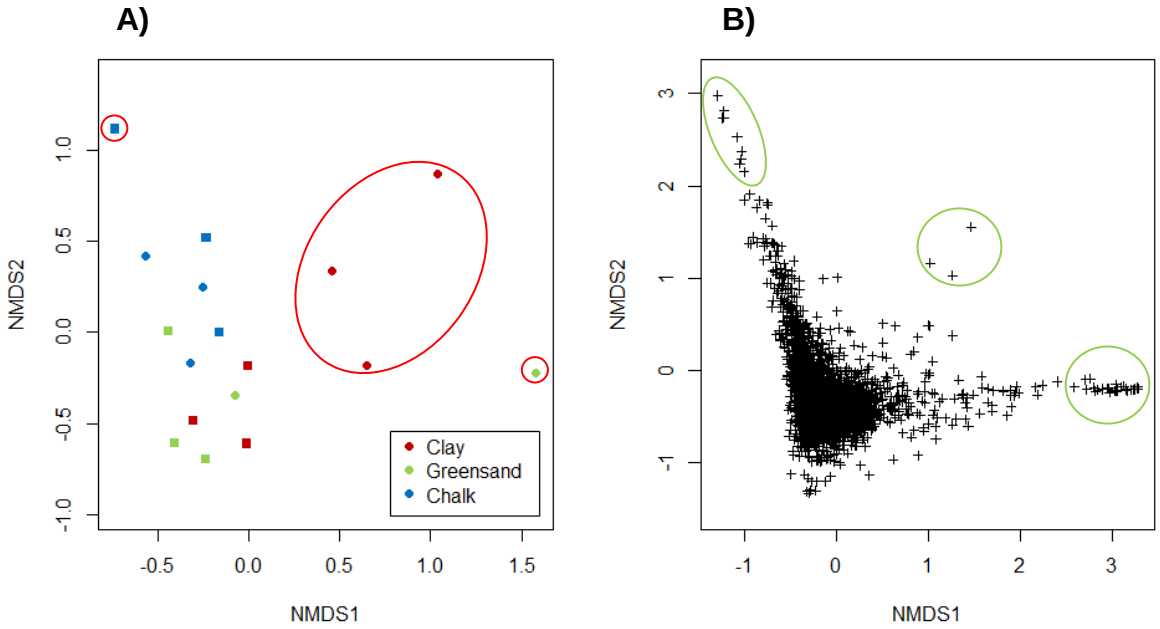


Figure 2.12: Non-metric multidimensional scaling plots of Bacterial OTUs in relation to river sites. A) NMDS of sites according to OTU abundance B) NMDS of OTUs according to abundance at sites. Squares= August, Circles = February

Bacterial communities within February clay sediments clustered separately from the other sediments (red circled). One of the Greensand sediments from February (red circled) and one chalk sediment from August (red circled) were also distantly separated from the other sediments (**Figure 2.12A**). The distribution of OTUs suggests that the differences between the February Clay sediments and the other samples was due to a small number of outlying OTUs (green circled) (**Figure 2.12B**).

Diversity of Bacterial samples according to both Shannon and Simpsons indices were similar across geologies in February (H= 6.626 to 6.772, D= 0.989 to 0.996), whereas August chalk sediments were lower than the other two geologies

(H= 5.787 to 6.063, D= 0.991 to 0.994). August sediments also tended to have a slightly lower diversity than those found in February (**Appendix Table A3.2**). Of interest to this thesis were the detection of methanotrophs in both soils and sediments, including the orders Methylacidiphilales, the genera *Methylosinus*, *Methylosoma*, and *Methylobacterium* which will be discussed further in Chapter 3.

2.3.8: Archaeal 16S rRNA gene diversity in soils

PCR-DGGE analysis of Archaeal 16S rRNA genes for triplicate soils sampled in February and August was performed (**Appendix Figure A3.7-A3.12**). In general, there was good reproducibility obtained between replicates. An MDS plot of the Archaeal 16S rRNA gene DGGE band profiles of soils sampled in February and August is presented (**Figure 2.13**). In February discrete clusters were found between geologies which were less distinct in August.

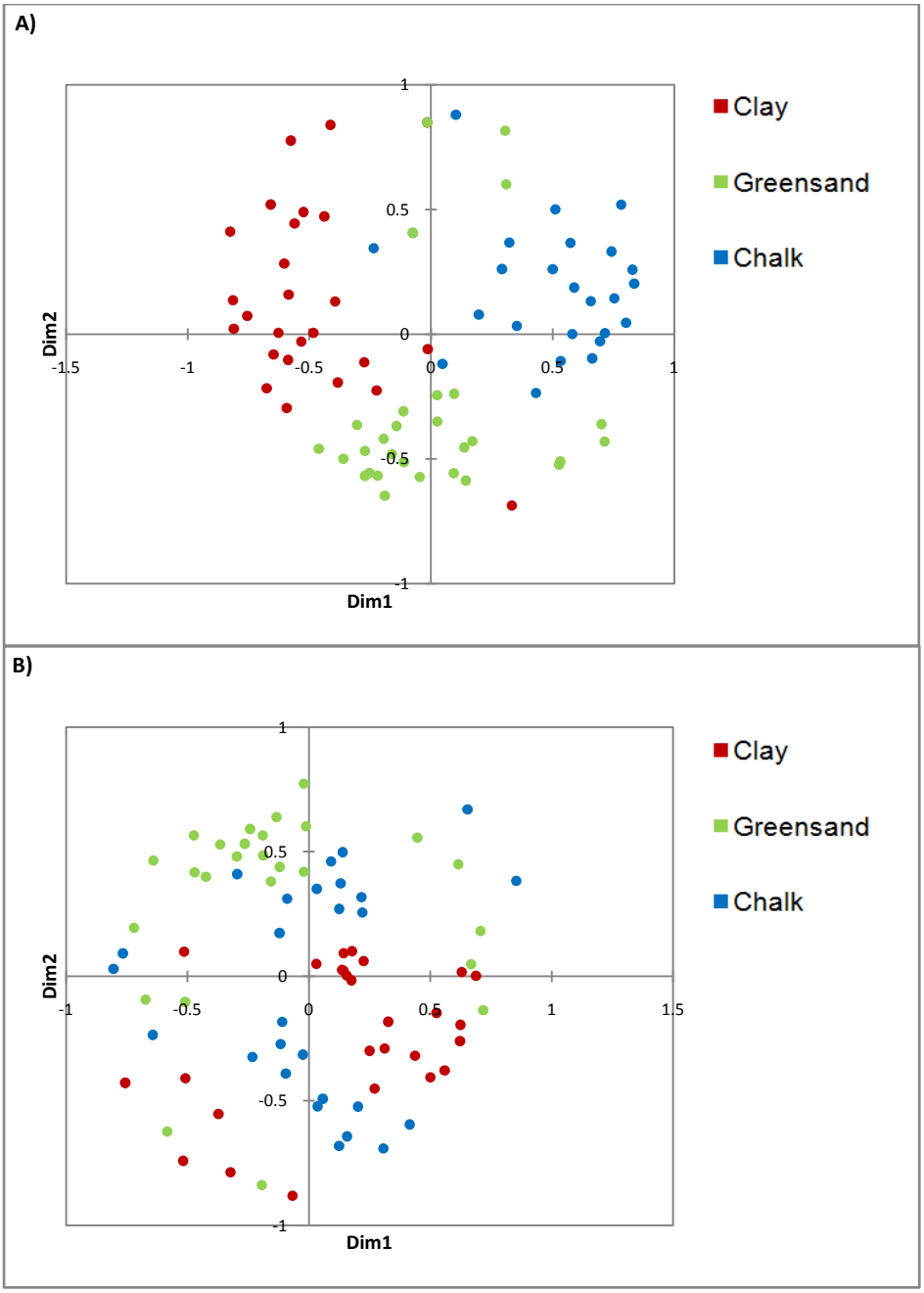


Figure 2.13: MDS Scaling plot of Archaeal 16S rRNA DGGE bands in February (A) (Kruskal's stress (1) = 0.278) and August (B) (Kruskal's stress (1) = 0.305) soils.

An MDS plot comparing geology between months derived from the Archaeal 16S rRNA gene DGGE band profiles of soils sampled in February and August is presented (**Figure 2.14**). Across all geologies, the Bacterial communities present in the sediments sampled in February were generally distinct from those in August (ANOVA, *P*< 0.001) (**Figure 2.14 A-C**).

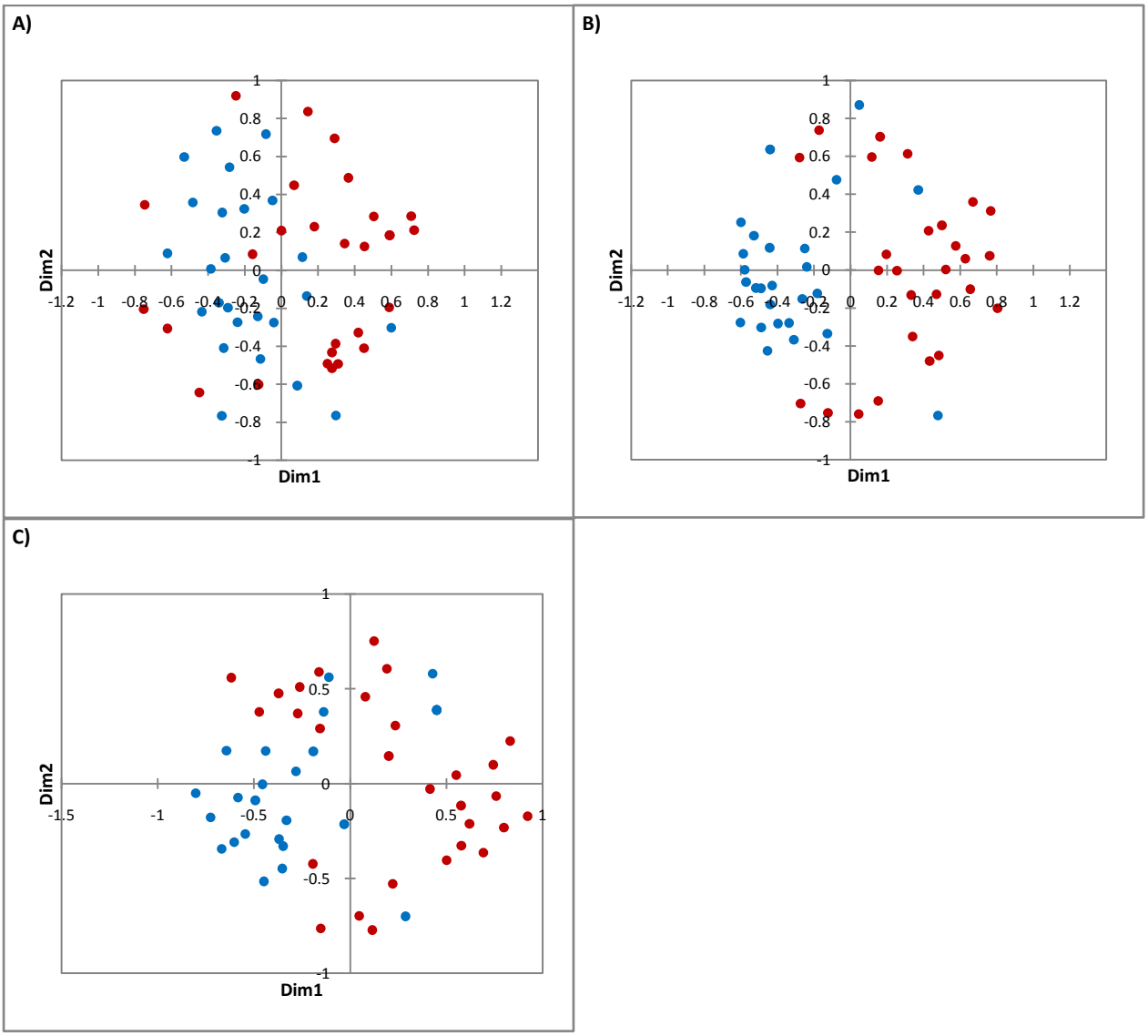
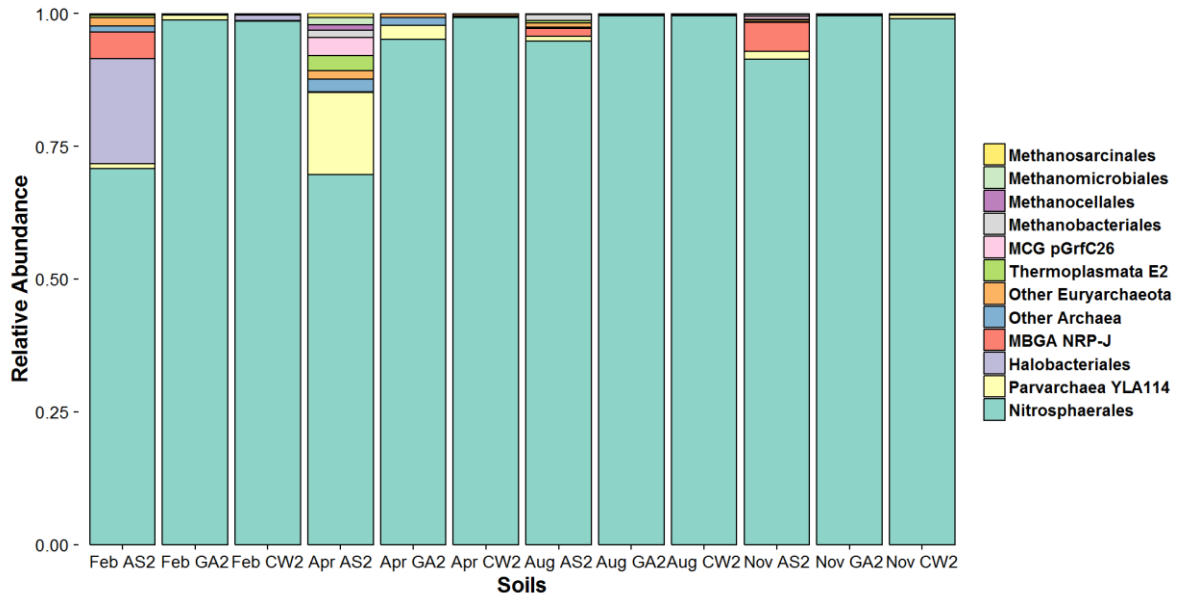


Figure 2.14: MDS plot of Archaeal 16S rRNA DGGE bands in February (blue) and August (red) clay (A) (Kruskal's stress (1) = 0.304), Greensand (B) (Kruskal's stress (1) = 0.301), and chalk (c) (Kruskal's stress (1) = 0.277) soils.

The Archaeal community composition and relative abundance data in soils obtained using Illumina MiSeq are shown in **Figure 2.15-2.16**. In soils, amplification of Archaeal 16S rRNA showed there were 2606 unique OTUs across all samples. A rarefaction curve was constructed from using the entire set (**Appendix Figure A2.3**).

Relative abundance of Archaeal sequences was made from the Archaeal sequences grouped at the Order level (**Figure 2.15**)

A)



B)

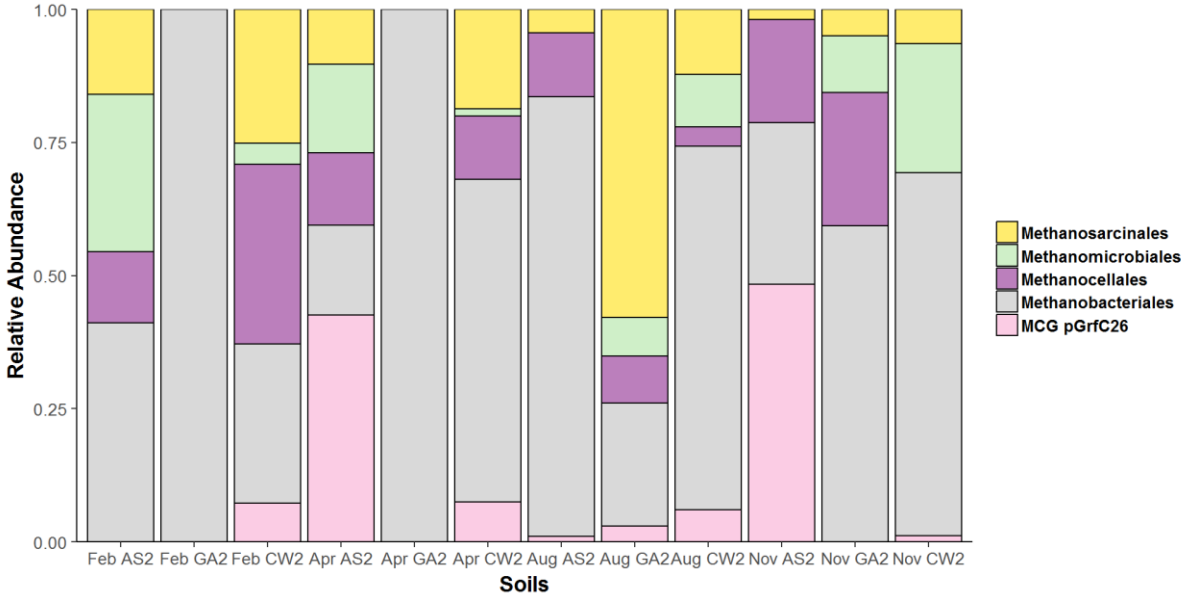


Figure 2.15: Stacked bar plot of relative abundance of different orders of A) All Archaeal orders B) Orders with <1% of overall Archaeal abundance, within clay (AS2), Greensand (GA2), and chalk (CW2) soils in February (Feb), April (Apr), August (Aug), and November (Nov) 2013.

Archaeal diversity within soils was dominated exclusively by the order Nitrososphaerales which made up 69.7% to 99.6% of the total OTU. Parvarchaea YLA114, Halobacteriales (confirmed through BLASTn search), and MBGA NRP-J were the next most abundant orders, but only in February AS2, April AS2 and November AS2 respectively. Other detectable sequences included four methanogen orders, the order Thermoplasmata, and other unidentified Euryarchaeota/Archaea. Methanogens are further analysed in Chapter 3. Orders of less than <1% showed several Methanobacteriales was present across all soils, and was normally the most abundant, especially in the greensand soils (GA2).

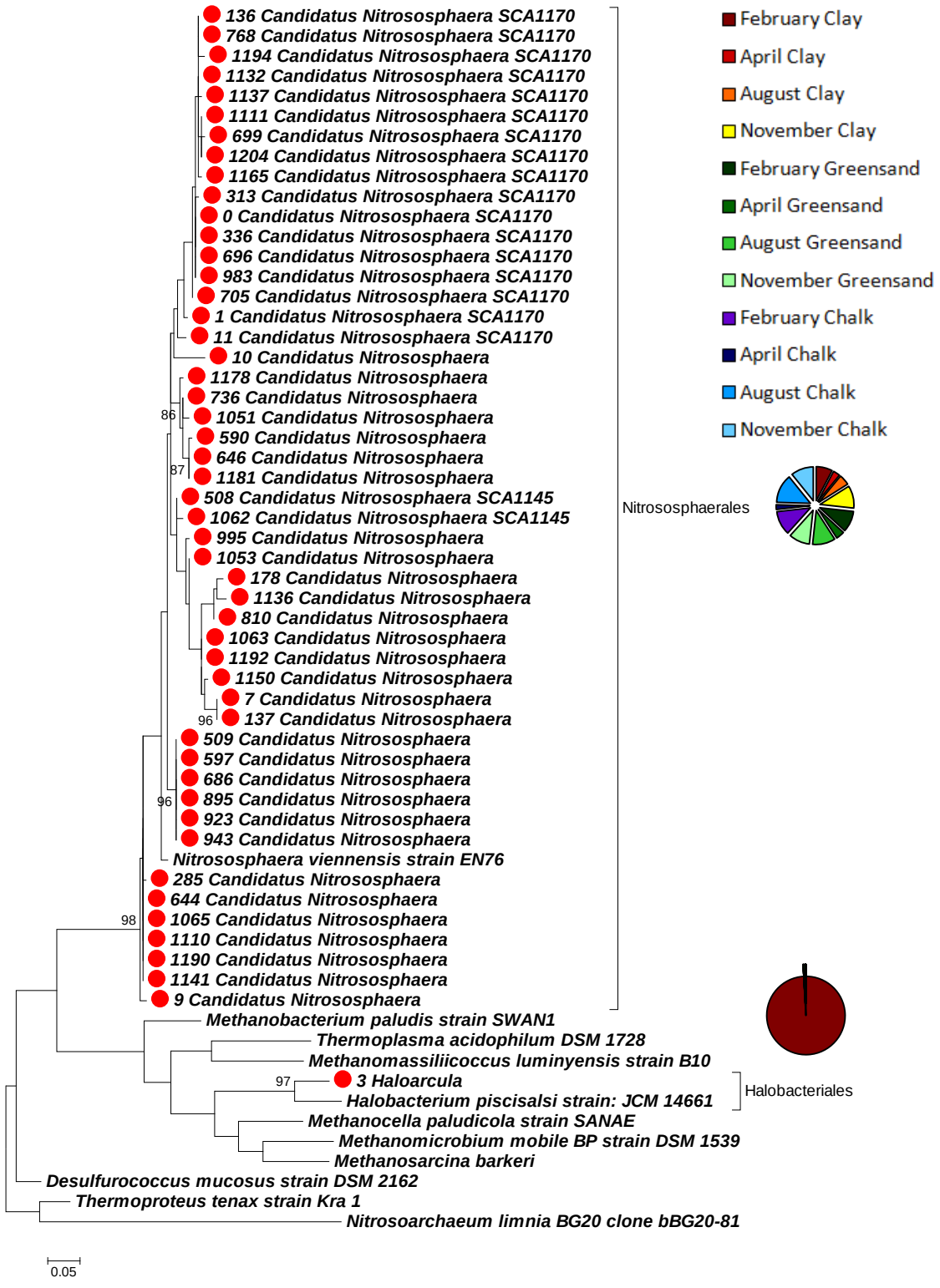


Figure 2.16: Phylogenetic tree of the 50 most abundant Archaeal OTUs (red circle) within soils compared to closest sequence hits on NCBI. Only bootstrap values of >70% are presented. Pie charts represents relative abundance (%) of major Orders of Archaea.

A phylogenetic tree of the 50 most abundant OTUs showed that the Archaeal sequences were virtually all from the *Nitrososphaera* genus, with only one sequence from *Haloarcula* (**Figure 2.16**). All the *Nitrososphaera* were also candidate organisms, with nearly half of the OTUs from Candidatus Nitrososphaera SCA1170. Nitrososphaera favoured greensand and chalk soils (35% and 37% respectively), and between months were least abundant in April soils (3% clay 4% greensand 2% chalk). Haloarcula was found almost exclusively in February clay soils (>99%).

Archaeal diversity within soils changed across months and geologies (ANOVA, P <0.001). Comparison by month showed that the lowest diversities in August (H= 3.007 to 4.617, D= 0.903 to 0.978) and highest in April (H=3.580 to 4.645, D= 0.941 to 0.978), however this change was not significant (P> 0.05). There was significant differences by geology (ANOVA, P< 0.001), clay soils tended to be lowest in diversity (H= 3.007 to 3.716, D= 0.903 to 0.941), with chalk and greensand to having similarly higher diversity (H= 4.498 to 4.862, D= 0.971 to 0.986) (ANOVA, P< 0.001) (**Appendix Table A3.3**)

2.3.9: Archaeal 16S rRNA gene diversity in sediments

PCR-DGGE analysis of Archaeal 16S rRNA genes for triplicate sediments sampled in February and August was performed and the band profiles are presented in **Appendix Figures A3.13-A3.14**. In general there was good reproducibility obtained between replicates and sediments had high diversity across all sites in both February and August. An MDS plot of the Archaeal 16S rRNA gene DGGE band profiles of sediments sampled in February and August is presented (**Figure 2.17**). An MDS plot comparing geology between months derived from the Archaeal 16S rRNA gene DGGE band profiles of sediments sampled in February and August is

presented (**Figure 2.18**). Across all geologies, the Archaeal communities present in the sediments sampled in February were generally distinct from those in August (ANOVA, *P*<0.001) (**Figure 2.18 A-C**).

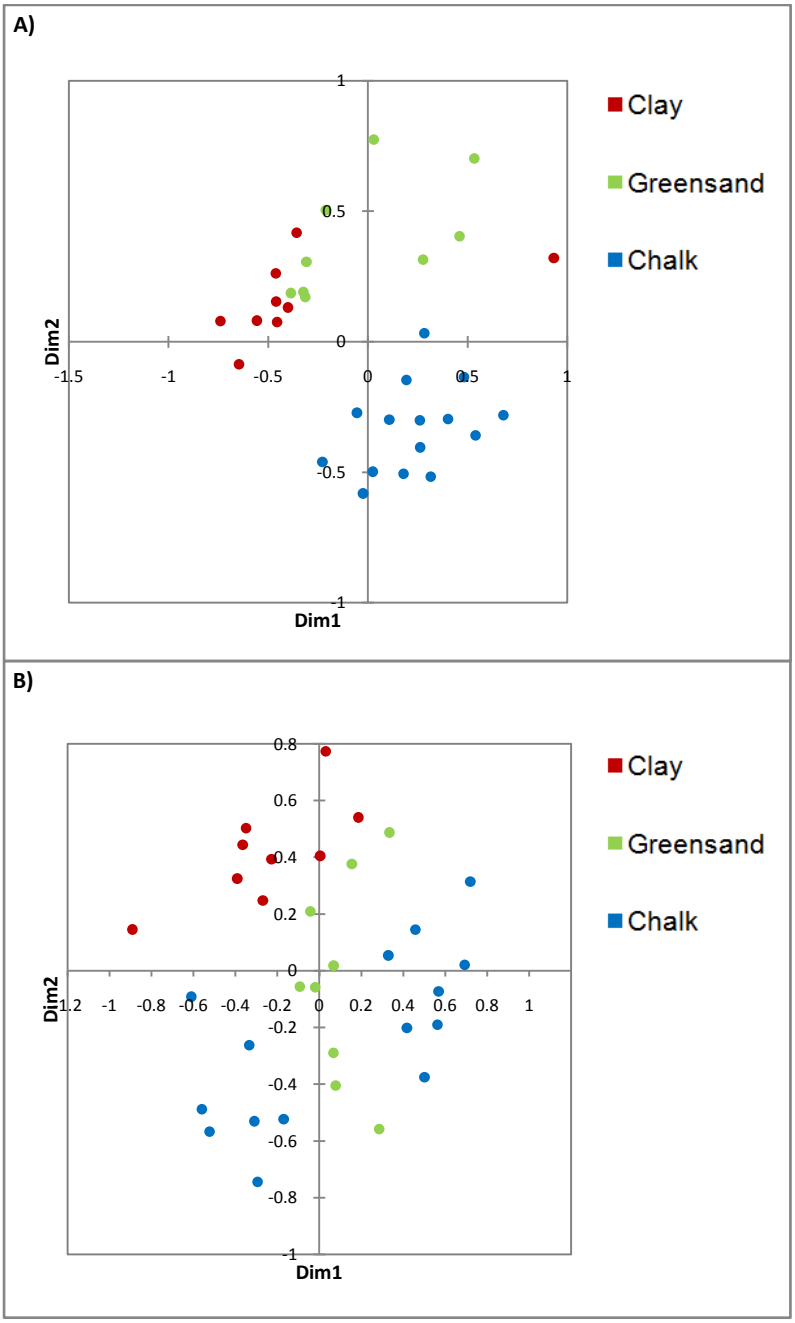


Figure 2.17: MDS Scaling plot of sediment Archaeal 16S rRNA gene DGGE band profiles in February (A) (Kruskal's stress (1) = 0.209) and August (B) sediments (Kruskal's stress (1) = 0.296).

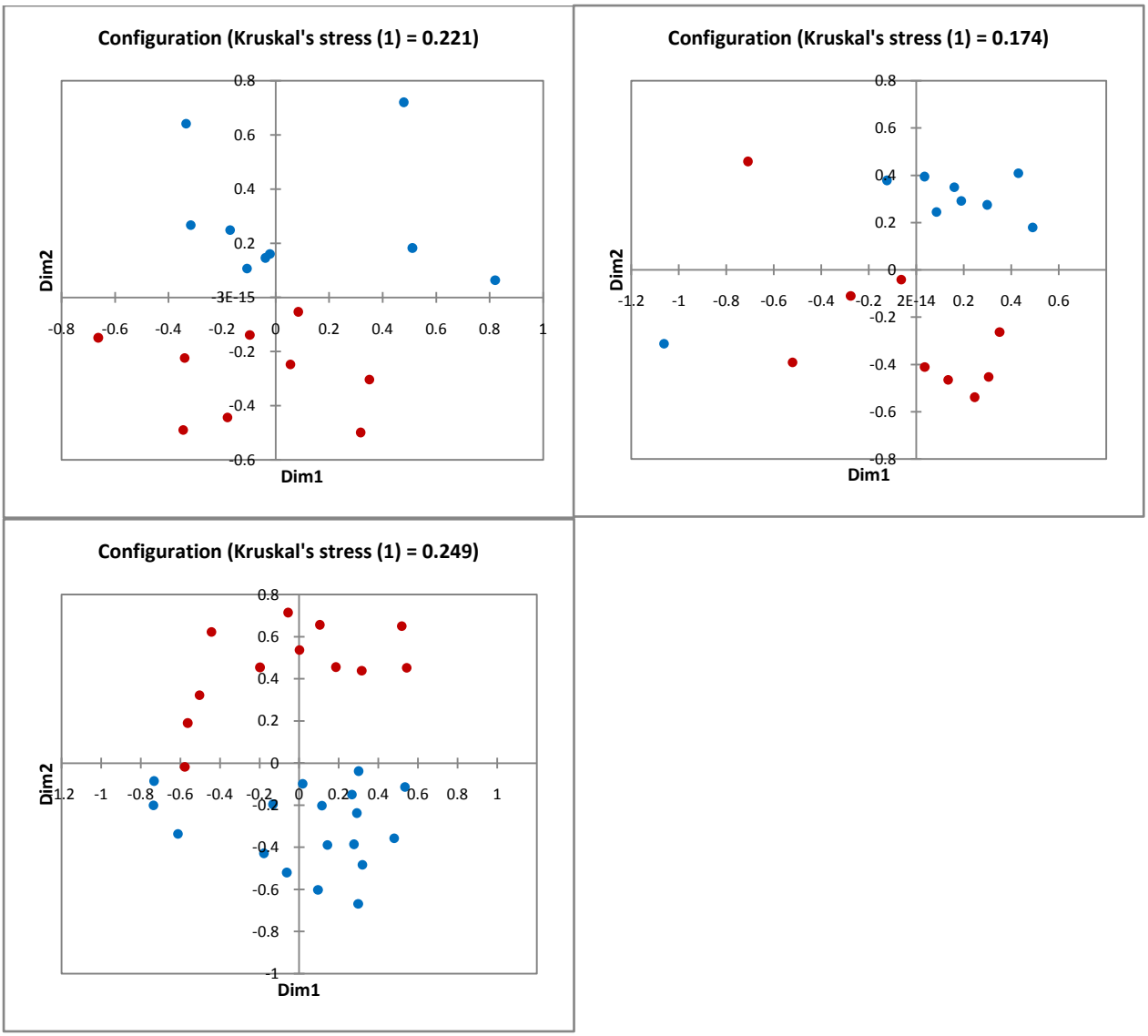


Figure 2.18: MDS plot of Archaeal 16S rRNA DGGE bands in February (blue) and August (red) clay (A) (Kruskal's stress (1) = 0.221), Greensand (B) (Kruskal's stress (1) = 0.174), and chalk (C) (Kruskal's stress (1) = 0.249) sediments.

Illumina MiSeq sequence analysis of Archaeal 16S rRNA genes obtained from the sediments is shown in **Figure 2.19-2.21.** In sediments, amplification of Archaeal 16S rRNA showed a diverse range of organisms with 4006 unique OTUs across all samples and a rarefaction curve was constructed from these OTUs (**Appendix FigureA2.4**).

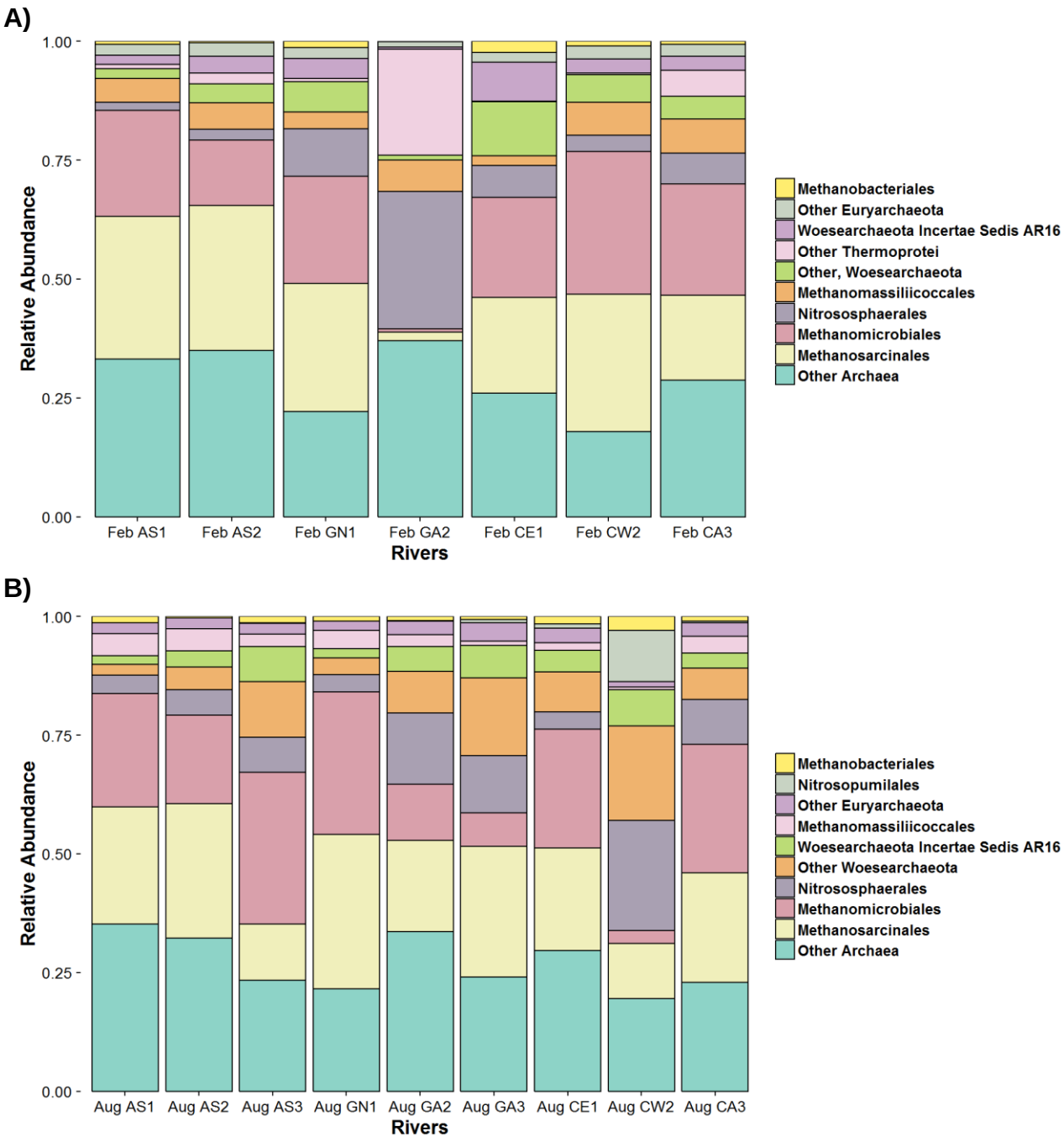


Figure 2.19: Stacked bar plot of relative abundance of different classes of Archaea within February (A) and August (B) river sediments. Clay (AS1-3), greensand (GN1, GA2-3), and chalk (CE1, CW2, CA3). February (Feb), August (Aug).

Within February sediments, the majority of OTUs in the samples were identified as being methanogens (41.3-58.9%) or belonging to no known Archaeal clade (17.9-37.1%). However, in the February GA2 sediments, Nitrososphaerales

and "Other" Thermoproteii made up a larger portion of the community (28.9% and 22.3% respectively). Within August sediments the majority of OTUs in the samples were identified as being methanogens or belonging to no known Archaeal clade (17-35%) (**Figure 2.19**) however their abundance was not significantly different when compared between months or geologies (*P*=0.228). Methanogens as a whole were the dominant taxa and belonged to two major methanogen orders; Methanomicrobiales and Methanosarcinales. Both orders were significantly different across months and geologies (*P*< 0.001), with the main difference being most samples were significantly higher in abundance when compared to Feb GA2 samples. Although unidentified Archaea were among the most abundant across all samples, they were not significantly different between months or geologies.

A phylogenetic tree was constructed using the 50 most abundant OTUs and NCBI sequences that represented their inferred order (**Figure 2.20**). The phylogenetic tree contained 8 clades and these were further separated into 6 clades at the order level, with 2 clades representing unclassified Archaea. Of the 6 identified orders 5 of them were methanogens, with most sequences belonging to either the orders Methanosarcinales or Methanomicrobiales. Within the Methanosarcinales, the majority of sequences fell under the defunct genus *Methanothrix*, which has now been reclassified to *Methanosaeta*. A separate branch within Methanosarcinales contained the genus *Methanosarcina*, and one minor branch that contained 2 sequences within the family Methanosarcinaceae. The clade identified as Methanomicrobiales is dominated by the genus *Methanoregula*, with only 2 sequences from a different genus, *Methanolinea*.

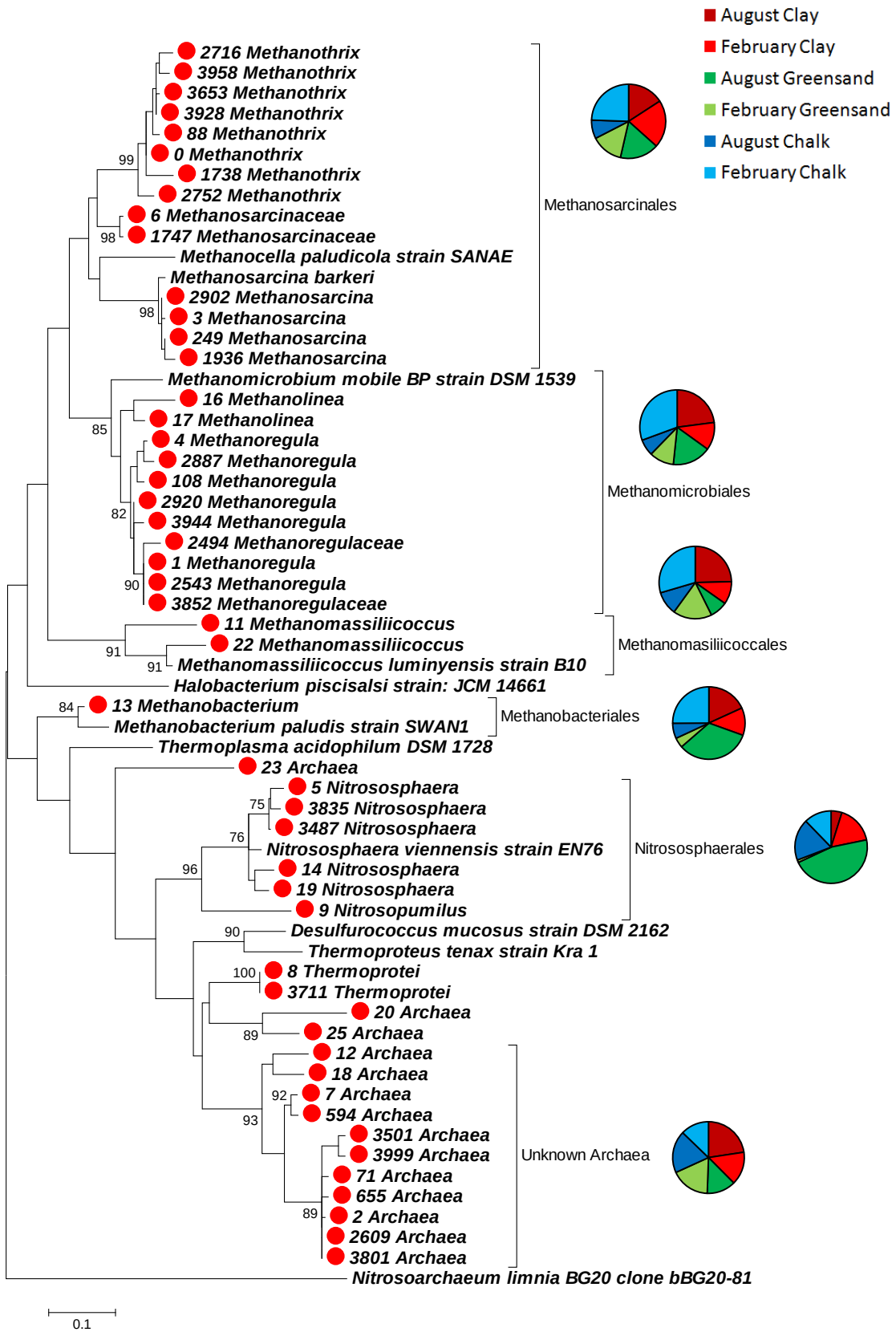


Figure 2.20: Phylogenetic tree of the 50 most abundant Archaeal OTUs (red circle) within sediments compared to closest sequence hits on NCBI. Only bootstrap values of >70% are presented. Pie charts represent relative abundance of Archaeal orders.

The orders Methanomassiliicoccales and Methanobacteriales, only contained a single genus i.e. *Methanomassiliicoccus* and *Methanobacterium* respectively. All sequences from the order Nitrososphaerales fall under the genus Nitrososphaera with the exception of one sequence branching out as the genus *Nitrosopumilus.* The remaining sequences separated into an unidentified clade of *Archaea* (**Figure 2.20**). Between geologies the orders Methanosarcinales (37% in clay), Methanomicrobiales (37% in chalk), Methanomassiliicoccales (40% in chalk), and Unknown Archaea clade (37% in clay) were found to have a small preference for clay or chalk sediments with greensand being proportionally lower in abundance (<31%). However Methanobacteriales and Nitrososphaerales tended to favour greensand sediments (38% and 47% respectively). Between months Methanosarcinales (21% clay, 24% chalk) and Methanomassiliicoccales (17% greensand, 30% chalk) were predominantly found in February sediments. In contrast August sediments were favoured by Methanomicrobiales (23% clay, 17% greensand), Methanobacteriales (18% clay, 33% greensand), Nitrososphaerales (46% greensand, 19% chalk), and Unknown Archaea (22% clay, 19% chalk). Further analysis and discussion of methanogens are presented in Chapter 3.

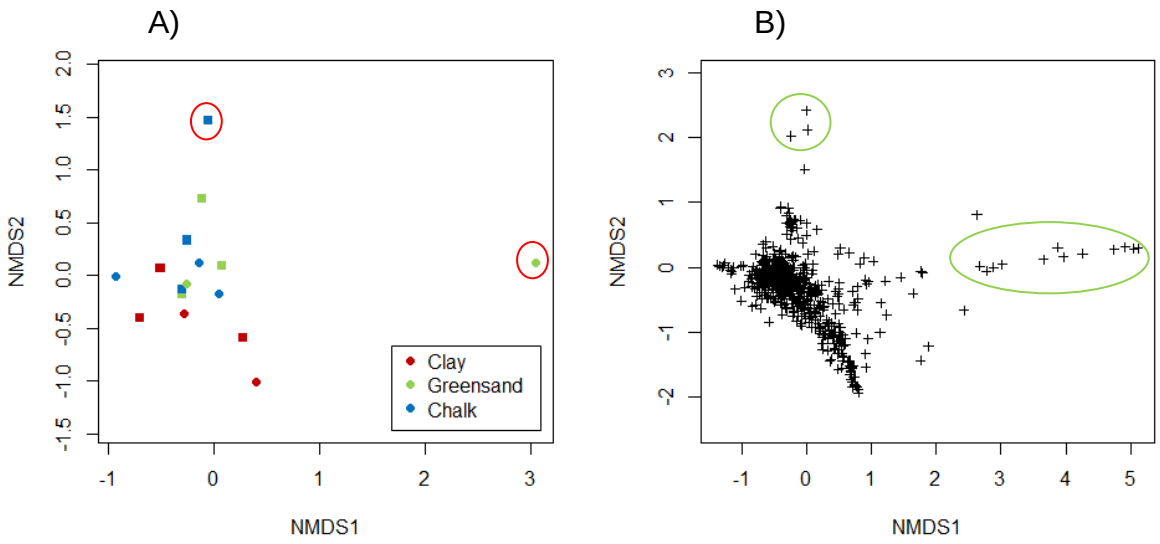


Figure 2.21: Non-metric multidimensional scaling plots of Archaeal OTUs in relation to river sites. A) NMDS of sites according to OTU abundance B-) NMDS of OTUs according to abundance at sites. August (squares), February (circles).

The NMDS shows the Archaeal communities were largely similar between the river sites and months, with the exception of one of the chalk samples from August and a greensand sample from February (red circled) (**Figure 2.21a**). The distribution of OTUs suggest the differences between the circled sites was due to a small number of outliers (green circled) (**Figure 2.21b**).

Shannon and Simpson diversity indices of Archaeal across both geologies and months were similar except for the February AS2 and Chalk CA3 samples (**Appendix Table A3.4**)

2.4: Discussion

In the seasonal sampling campaign, both Bacterial and Archaeal communities showed marked shifts from month to month and between the different geologies. Both Bacterial and Archaeal DGGE analysis showed a shift of soil Bacterial communities from February to August across all geologies and was confirmed in sequence analysis of OTUs. Sequencing of soil Bacterial community showed that diversity was similar to that of sediments in February, however Bacterial diversity was slightly higher in August soil than August sediments. In addition, community diversity lowered in April and November soils compared to February and August soils, with diversity more than halved in November. The relative abundance of the 50 most abundant Bacterial OTUs showed significant differences between the clay site and the other soils (ANOVA, $P < 0.015$). All the major identifiable Bacterial classes excluding Alphaproteobacteria, Acidobacteriia, Actinobacteria and Spartobacteria were in much lower abundance in clay compared to other sites. Acidobacteriia in particular appears to favour the clay site over the other geologies. This is likely due to the lower pH (pH <5), which many members of Acidobacteriia favour, in clay sites compared to chalk or Greensand (pH of >7). A study by Chroňáková (2015) on overwintering of cattle and their effect on grassland showed that low pH selected for Acidobacteriia and was more than halved in soils with neutral pHs. It should be noted however that several members of Acidobacteriia are also present in neutral to alkaline soils, and it is possible other factors are selecting for Acidobacteriia (Ward *et al.*, 2009). In our study Acidimicrobiia was also detected and also favour low pH however unlike Acidobacteria, Acidimicrobiia were largely absent from clay soils. This suggests factors other than pH are affecting the differences between community

composition of these two classes in soils, however no strong correlation with the two Bacterial clades was found with other abiotic factors.

DGGE in this study also revealed a shift in Bacterial communities in sediments from February to August, which was confirmed by the shifts in relative abundance of the overall OTUs, and the change in the top 50 most abundant Bacterial OTUs. This shift in community structure between seasons has been demonstrated in previous research in rivers (Staley *et al.*, 2013; Gibbons *et al.*, 2014). Sequence analysis of sediments also showed that the most abundant classes remained largely the same i.e. Alphaproteobacteria, Bacteriodetes, Betaproteobacteria, Gammaproteobacteria, Ignavibacteria and Nitrospira. Bacterial community composition in the Hampshire-Avon sediments were similar to a previous study of river sediments by Gibbons *et al.* (2014), with representatives from, Acidobacteria, Bacteriodetes, Nitrospirae, the Proteobacterias and Verrucomicrobia. Similarly a study by Staley *et al.* (2013) using Illumina MiSeq found the above classes of organisms within their river study site, suggesting that these particular classes make up the bulk of Bacterial communities in river environments. Earlier studies using clone libraries also found the same Bacterial classes as core communities within rivers (Gabriel *et al.,* 2002; Cottrell *et al.,* 2005; Newton *et al.,* 2011).

Comparisons of the most abundant Bacterial OTUs within sediments showed that changes of OTUs from month and geology were significant only for a few, which included Desulfuromonadales, Desulfobacterales, Gammaproteobacteria, Bacteriodetes and "Other" Bacteria ($P < 0.0001$ to 0.038). The influence of nitrite on community structure in sediments may well be important as some of the most abundant Bacterial OTUs were from the genus *Nitrospira*, a gram negative nitrite-

oxidizing bacterium common in freshwater and saltwater environments (Daims *et al.,* 2015), therefore *Nitrospira* potentially affects overall community structure by being the main driver of nitrifaction. Similarly the influence of sulfate on the community might be explained by several OTUS identified as Desulfobacterales and Desulfuromonadales, which can also potentially affect overall community by acting as the main sulfate reducers. Specifically, in relation to methanogens, sulfate reducers have the potential to out compete them for resources, such as hydrogen, where sulfate reduction is more energetically favourable i.e. lower ΔG than methanogenesis (Conrad *et al.*, 1986).

However, testing for direct correlations between ammonium, nitrate and nitrite with *Nitrospira* showed no significant correlations between the nitrogen species (Pearson = -0.032 to 0.182, $P>0.2$), nor could sulfate be directly linked to the two orders of sulfate reducers (Pearson = -0.147 to 0.156, $P= >0.25$).

Several genera of methanotrophs have also been detected, although only the genus *Methylobacter* (order Methylococcales) was part of the most abundant OTUs, suggesting they are the most common methane oxidizers in sediments. Previous research has found that *Methylobacter* species have been found to be dominant in littoral lake sediments (Pester *et al.,* 2004).

Archaeal community in soils did change slightly according to DGGE, however sequencing showed that the diversity of Archaea was extremely low in soils mostly due to the dominance of a single genus, Nitrososphaera, an ammonia oxidizing Archaea (Tourna *et al.,* 2011) (Figure 2.5 and 40). The over representation of a single genus could be down to errors whilst preparing for sequencing or during sequencing e.g. contamination of samples with DNA derived from monocultures or poor quality sequences. However ammonium concentrations within soils were also

higher (highest 3.4 µmol g^{-1} dry weight soil) compared to sediments (highest 0.01 µmol g^{-1} dry weight sediment), suggesting Nitrososphaerales were selected for by the environmental ammonium concentration. One interesting result from the Archaeal soil sequences was the presence of Halobacteria in February clay soils, which made up 19.8% of the Archaeal sequences. Sequences were confirmed through subsequent BLASTn search placing all genera at 99% identity similarity to NCBI sequences. Halobacteria are extreme halophiles often found in environments with saturated salt concentrations (Yadav *et al.*, 2015) and they have been found in high salt environments such as salt mines and hypersaline lakes (Fendrihan *et al.,* 2006). Species within this halophilic group require concentrations of at least 1-1.5 M of salt to grow (Oren, 2008), however these conditions are not present in our sampling sites, and Na^+ ion concentrations were lower in February clay soils than the other two geologies. It is therefore unlikely that the detected Halobacteria are native to the sites in this study. The most likely scenario would be contamination of the sample from pure Halobacteria DNA, as almost all Halobacteria sequences were in one particular February AS2 replicate (95%).

Within sediments sequencing of Archaeal communities showed methanogens were the dominant group, which contradicts previous research that suggested Thaumarchaeota as the dominant Archaeal phyla in similar temperate river systems (Abreu *et al.,* 2001). The high relative abundance of methanogens suggests that methane production will occur readily in the sediments. Methanosarcinales, Methanobacteriales, Methanomassiliicoccales, and Methanobacteriales were all identified with previous research showing. Further examination of the methanogen community will be presented in Chapter 3.

The only other major named order within the 50 most abundant Archaeal sequences were Nitrososphaerales, an ammonia oxidizing archaeon (Tourna *et al.,* 2011). Nitrososphaerales is among the most common ammonia oxidizing Archaea (AOA) and are highly abundant in other environments (Tourna *et al.*, 2011). However, as of writing, no other soil environments similar to the Avon-Hampshire catchment showed such heavy dominance of Nitrososphaerales. Nitrososphaera and other AOA are a critical component in the cycling of nitrogen in soils and sediments (Zhalnina *et al.*, 2014) and are able to oxidise ammonium by utilising the Archaeal variant of the ammonia monooxygenase (AMO), with recent studies suggesting they are of equal importance to ammonia oxidising bacteria in the first stages of nitrification (Prosser and Nicol, 2008). The relatively large abundance of AOA in this study suggests that AOA are important nitrifiers within our soils. A study by Zhang *et al.* (2011), noted a bias towards AOA in soil with pH of <4.5, however none of the soils within our study sites fall below pH 5, indeed both chalk and greensand soils are neutral to slightly alkaline. The reason for the extreme dominance of Nitrososphaerales is still uncertain.

In conclusion, this study found that in soils Acidobacteria-6, Acidimicrobiia, Actinobacteria, Alphaproteobacteria, Baccillales, Betaproteobacteria, Gammaproteobacteria, Nitrospira, Saprospirae, and Spartobacteria were the most common bacteria classes within chalk and greensand soils, whereas Acidobacteriia and Spartobacteria were more common in clay soils. Most Bacterial clades within soils were also more abundant within the month of February and the least abundant in April. Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Gammaproteobacteria, Acidobacteria, Bacteroidetes, Ignavibacteria, Nitrospira,

Subdivision 3 and Verrucomicrobia were found across all sediments which were in greater abundance in February compared to august. Whilst Bacterial communities within soils and sediments saw overlap, Archaeal communities within the same environments were considerably more skewed. In soils Archaea were almost exclusively from Nitrososphaerales, with a few OTUs identified as Halobacteriales, Parvarchaea, MBGA NRP-J and a few methanogens. In sediments methanogens were the dominating Archaea which included Methanosarcinales, Methanobacteriales, Methanomassiliicoccales, and Methanobacteriales, with the remaining Archaea belonging to Nitrososphaerales and unknown Archaea. Generally Archaea were found to be more abundant in clay and chalk (Methanosarcinales, Methanomicrobiales, Methanomassiliicoccales, and unknown Archaea) than greensand, and were also more abundant in August (Methanomicrobiales, Methanobacteriales, and Nitrososphaerales).

Chapter 3: Seasonal fluxes in methanotroph and methanogen communities across different geologies.

3.1: Introduction

Methanogens are responsible for the production of up 1000 Tg of CH_4 annually, of which 600 Tg is offset by methanotrophs (Thauer, 2011). Methanogens and methanotrophs are ubiquotous and have been found in environments such as grassland, lakes, rivers, peat bogs, rice paddy fields, and sewage treatment plants (Mer and Roger*,* 2001*;* Conrad 2009; Nazaries *et al.,* 2013). Methanogens produce CH_4 under anaerobic conditions, whereas Bacterial methanotrophs oxidize CH_4 largely under aerobic conditions. *Methylomiribilis oxyfera* is an exception however, as it is utilizes aerobic oxidation of CH_4 in anoxic conditions via denitrification (Ettwig *et al.*, 2010).

In rivers, CH_4 production and oxidation is common as the short transition from aerobic to anaerobic sediments and their near constant waterlogging make it ideal for bothprocesses (Cole *et al.,* 2007; Bastviken *et al.,* 2011). In addition water flow and runoff from surface soils may also affect CH_4 production and oxidation (Conrad *et al.,* 2009; Maeck *et al.,* 2013). Higher river flow will replenish oxygen depleted river waters and thus keep sediments more oxic, reducing CH_4 production. Conversely, slow river flow will tend to reduce turnover of deoxygenated water and thus increase anaerobic conditions and subsequently methanogenesis will increase (Maeck *et al.,* 2013).

In soils, water availability is a major factor affecting CH_4 transformations (Mer and Roger, 2001; Conrad *et al.* 2009; Nazaries *et al.,* 2013). In highly water logged soils,oxygen may deplete over time as it is not replenished by atmospheric O_2 and

such anoxic zones would favour methanogens.Conversely, a soil with low water content will allow gases to diffuse more easily, increasing O_2 availability favouring methanotrophs (Nazaries *et al.,* 2013). Water content also affects the syntrophic microbial community that contributes to methanogenesis by increasing or decreasing abundancec of e.g. acetate/hydrogen producing microbes, which in turn affects methanotrophy (Conrad 2009; Nazaries *et al.,* 2013). In flooded rice paddies, high water content results in increased CH_4 production, but once fields are drained post harvest, CH_4 oxidation may increase as conditions became more aerobic (Conrad *et al.,* 2001). Similar results have been found in grasslands where methanogens became activeupon rewetting (Angel *et al.,* 2012). Soil texture and porosity also affectssoil water transport. For example, higher clay content causes greater soil aggregation under wet conditions creating anoxic conditions favouring methanogenesis (Frey *et al.,* 2011). Solubility of gases may also become a factor in the different seasons, as increase in temperatures decreases gas solubility in water (Yamamoto *et al.*, 1976). This may lead to lower CH_4 oxidation potentials, as O_2 may be reduced when temperature is increased, whereas production of CH_4 may increase due to annoxia, as dissolved O_2 becomes depleted. However a study by Shelley et al. (2015) demonstrated that tempreature increases in river systems potentially have no effect, or increases CH_4 oxidation and production.

Currently, little is known about how the underlying geology of soils and sediments affect key processes like methanogenesis and methanotrophy.

3.1.1: Aim

The overall aim of this chapter was to determine seasonal changes in the abundance and community structure of methanogens and methanotrophs, in relation to CH_4

oxidation and CH_4 production across different underlying geologies (clay, greensand, chalk).

3.1.2: Hypothesis

In this chapter it was hypothesized that:

1) CH_4 production and *mcrA* gene abundance in soils would be higher in winter due to increased water content (which in turn will lead to increased CH_4 oxidation and *pmoA* gene abundance).

2) In sediments where water content is always saturated, CH_4 production and *mcrA gene* abundance will be greatest in the summer due to increased temperatures which in turn will lead to increased CH_4 oxidation and *pmoA* gene abundance.

3) CH_4 production and *mcrA* gene abundance will be highest in clay (least porous) compared to Greensand and chalk (more porous).

The specific objectives of this chapter was to-

1) Quantify the changes in abundance and community structure of methanogens and methanotrophs within the soils and sediments of the Hampshire-Avon catchment over a seasonal cycle across three different geologies.

2) Measure the potential CH_4 production and CH_4 oxidation rates within soils and sediments in relation to their respective community structure and functional gene abundance.

3.2: Materials and Methods

3.2.1: PCR amplification of *mcrA* and *pmoA* genes from soils and sediments.

Soils and sediments were collected and DNA extracted as previously described (See Sections 2.2.2 and 2.2.3). PCR amplification of the *mcrA* and *pmoA* genes was carried out using the primer pairs mlas/mcrArev and A189f/A650r respectively and the cycling conditions described by Steinberg and Regan (2008) and Bourne *et al.* (2001) (**Table 3.1**). Both primer sets were selected for having the broadest coverage of their respective target organisms. However in the case of A189f/A650r, several important methanotrophs including those under Verrucomicrobia, USCα and USCγ are not targeted.

Table 3.1: Primers used in this study

Primer name	Gene target	Specificity[a]	Sequence (5' - 3')	Reference
mlas (forward)	*mcrA*	Methanogens	GGTGGTGTMGGDTTCACMCARTA	Steinberg and Regan (2008)
mcrArev (reverse)	*mcrA*		TTCATTGCRTAGTTWGGRTAGTT	
A189f (forward)	*pmoA*	Methanotrophs (except Verrucomicrobia, USCα and USCγ)	GGNGACTGGGACTTCTGG	Bourne *et al.* (2001)
A650r (reverse)	*pmoA*		ACGTCCTTACCGAAGGT	

[a]Specificity refers to both forward and reverse primers.
N = A,T,G, or C; R = G or A; W= A or T.

All PCR amplifications were carried out using a GeneAmp 2720 thermocycler (Applied Biosciences, UK) in 50 µl reactions as follows: 1x RedTaq ready mix (Sigma-Aldrich, UK), 0.2 µM Forward/Reverse primer, and 1 µg template DNA.PCR products were examined using a 1% (w/v) 1x TAE agarose gel (Tris acetate 0.04M, 0.1 mM EDTA, pH 8.0) loaded with a 1 Kb ladder (GeneRuler 1 Kb DNA ladder, Thermo Scientific), and stained with ethidium bromide (10 mg L^{-1} final concentration). Gels were analysed using a UV Gel Doc (AlphaImager® EP, Alpha Innotech, Canada).

3.2.2: Q-PCR amplification of *mcrA* and *pmoA* genes

Q-PCR amplification of the *mcrA* and *pmoA* genes was carried out using the primer pairs mlas/ mcrArev, and A189f/ A650r respectively (**Table 3.1**). Q-PCR amplification for both primer sets were carried out using one cycle at 95^{o}C for 2 min followed by 40 cycles of 95^{o}C for 5 s (denaturation), 60^{o}C for 30 s (note: these conditions were used as the SENSIFast protocol has a combined annealing and elongation step), and one cycle of 60^{o}C for 5 s and 95^{o}C for 5 s (melt curve production) (Cycling conditions used according to manufacture instructions of the SensiFAST polymerase (Bioline, UK)). Amplifications were carried out in 96 well plates with 6 standards in duplicate, a No Template Control (NTC) in triplicate, and each sample triplicate was carried out with three technical replicates. Each well contained a final volume of 15 µL, with each well containing a final concentration of 1x SensiFAST Sybr No-ROX mix (Bioline, UK), 400 nM of each primer and 50 ng of DNA. Plates were run on a CFX96^{TM} Real-Time Detection System (Bio-Rad, USA). Standard curves were generated using a 10-fold serial dilution of PCR products amplified from DNA from either pure strains of methanogen (*Methanosarcina*

baltica)or methanotroph (*Methylococcus capsulatus*). PCR products were purified using a GenElute PCR Clean-up kit (Sigma, UK) and quantified using a fluorospectrometer (Nanodrop 3300, Thermo Scientific, UK). Q-PCR results were quality checked by 1) Checking for linearity of the standard curves 2) Checking cycle thresholds (Ct) of no template controls (NTC) were below 38 to 40 cycles 3) Samples with Cts within 2 cycles of NTC were deemed as being equivalent to the NTC (i.e. 0 copies of the gene) 4) Melt curves were checked between sample peaks and standard peaks to ensure target gene was amplified i.e. melt peaks from samples should be the same temperature as those in the standard, with NTCs being much lower or not present (note that some drift is expected as environmental samples are compared to standards from pure cultures) (See **Appendix IV** for examples).

3.2.3: Methane oxidation potentials of soils and sediments

To create a series of standards (i.e. 1000 ppm, 750 ppm, 500 ppm, 250 ppm, 10 ppm), laboratory grade 100% (v/v) CH_4 (British Oxygen Company, UK) was diluted in 120 mL serum bottles and injected into a gas chromatograph with a flame ionizing detector (GC-FID) Shimadzu GC-2014 GC-FID (Shimadzu, UK) fitted with a SPL liner without packing (Carrier gas was helium, injection temperature 200^{o}C, splitless injection mode, linear velocity 47.7 mL sec^{-1}, flow pressure 70 kPa, Total flow 7 mL min^{-1}, column flow 5.97 mL min^{-1}, purge flow 1mL min^{-1}). The column used was a 50 m long capillary column (column temperature 160^{o}C, internal diameter 0.53 mm, 0.1 mm film thickness). The FID was set at 200^{o}C.

Triplicate samples of surface soils (0-5 cm depth) and sediments (0-5 cm depth) were placed into sterile 120 mL serum bottles, capped with a butyl rubber stopper and CH_4 was added to make ~500 ppm headspace. Samples were

incubated statically at 20^{o}C in the dark for 9 days. Methane oxidation potential (MOP) measurements were performed every 3 days, by injecting triplicate 30 µL of the headspace of each sample replicate into the GC-FID. The volume of CH_4 injected into the GC was converted to volume of CH_4 at standard temperature and pressure using the combined gas law.

$$V2 = \frac{V1 \times P1 \times T2}{T1 \times P2}$$

Where V2 is the volume of CH_4 injected at standard pressure and temperature (STP), V1 is the volume injected at room temperature and pressure (RTP), P1 is the room pressure, T1 is the room temperature, T2 is the standard temperature, and P2 is the standard pressure. The volume of CH_4 injected at standard temperature and pressure is then used to calculate the amount of CH_4 in µmol, using the Ideal Gas Law.

$$n = \frac{P \times V}{R \times T}$$

Where *n* is the amount of CH_4 in µmol, *P* is the standard pressure, *V* is the volume injected at standard temperature and pressure, *R* is the gas constant (0.082057 µL x atm [µmol x K]) and *T* is the standard temperature. As *n* is the amount of CH_4 as an ideal gas (100% v/v), *n* is converted to the appropriate amount for the standards, e.g. 500 ppm of 1.2 µmol CH_4 is 0.0006 µmol. The amount of CH_4 is then converted to pmol of CH_4.

3.2.4: Methane production potentials of soils and sediments

Triplicate serum bottles (120 mL) containing ~5 g of surface soil (0-5 cm) or ~5 g of surface sediments were flushed on-site with 99.9% N_2 gas (British Oxygen Company, UK) for 8 minutes at 0.5 bar pressure. Nitrogen flushed serum bottles were re-flushed; half of the bottles were flushed with an 80%:20% H_2:CO_2 gas (British Oxygen Company, UK), and the other half of the bottles were flushed with an 80%:20% N_2:CO_2 gas (British Oxygen Company, UK). This was to test for methanogenesis with and without H_2 as this may select for methanogens that utilize *in-situ* substrates such as acetate. Samples were incubated statically at 20^{o}C in the dark for 9 days. Methane production potential (MPP) was measured by injecting 30 µL headspace every 3 days into a GC-FID as described in Section 3.2.3.

3.2.5: Functional gene database

DNA was prepared for MiSeq Illumina sequencing as previously described in Sections 2.2.8 using primers and cycling conditions listed in **Table 3.1**. A custom database of *pmoA*/*mcrA* functional genes was made using sequences archived in the FunGene database (http://fungene.cme.msu.edu/). Only sequences from named strains were used in the database (see **Appendix VI** for list of organisms used). Illumina MiSeq sequences of the respective functional genes were then compared with the database using the RDP Classifier in Qiime, with a cut-off of 97% similarity (carried out by David Clarke, University of Essex).

3.2.6: Phylogenetic analysis

Functional gene sequences were aligned and trimmed using the MEGA 6 program (Tamura *et al.*, 2013). Nucleotide positions with gaps in aligned sequences

were deleted if more than 10% of samples contained them. The sequences from the subsequent data was then used for phylogenetic trees. Representatives within the orders of each of the 50 abundant methanotroph/methanogen OTUs were added from the custom FunGene database (see section **3.2.5**, and **Appendix VI**). Phylogenetic trees were constructed using Maximum-Likelihood of aligned sequences, using the Tamura-Nei model for nucleotide substitution and bootstrapped 1000 times, using MEGA 6 (Tamura *et al.*, 2013). The relative abundance of sequences between each month and geology were grouped into orders and drawn as pie charts in the final phylogenetic tree.

Functional genes were checked for accuracy prior to running phylogenetic trees; sequences were converted to corresponding amino acid sequences after alignment and compared to archived amino acid sequences, and any inaccuracies e.g. sequence coding for stop codons were corrected. To ensure *pmoA* genes were not *amoA* genes, sequences were converted to amino acid sequences after alignment and amino acid sequences were compared for divergent sequences (**Appendix V**).

3.2.7: Statistical analyses

A multivariate ANOVA was used to test differences in MOP/MPP rates and *pmoA/mcrA* gene abundance between geologies and months. Results that were significantly different were then further analysed with a Tukey HSD test. Pearson's correlation tests were also used to test for correlations between MOP/MPP rates and physicochemical factors, with results presented in **Appendix VII**. XLSTAT version (2014.5, Addinsoft), and STAMP (version 2.1.3, Parks and Beiko) software package

were used for sequencing data, using multivariate ANOVA tests followed by Tukey tests for significantly different ANOVA results.

3.3: Results

3.3.1: Methane oxidation potential (MOP) in soils and sediments

MOP measurements for soil and sediments from March to November 2014 are presented in **Figure 3.1**.

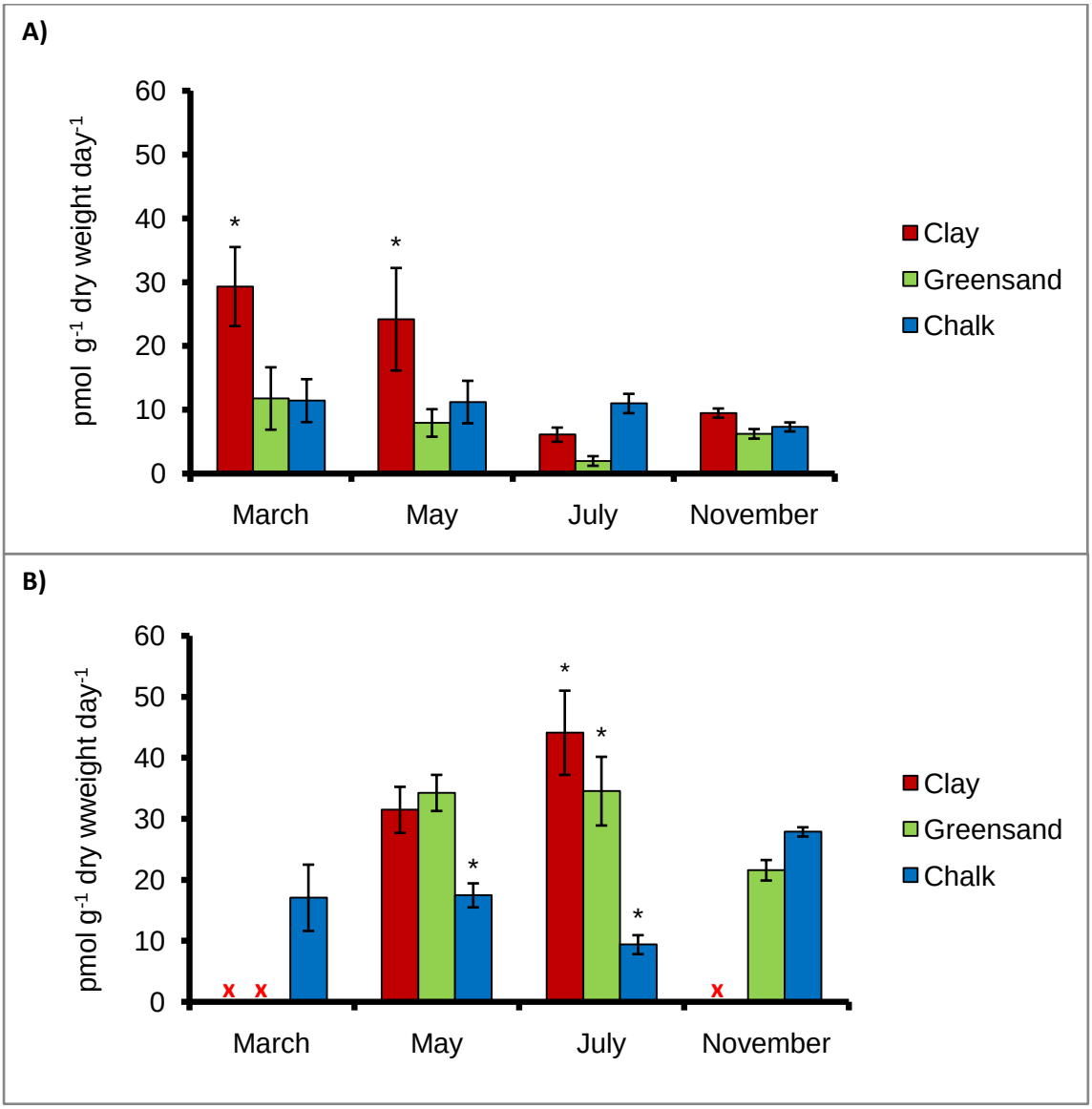


Figure 3.1: MOP of **A)** soils and **B)** sediments from March to November 2014.. **x =** Rivers inaccessible due to flooding. * = sample is significantly different ($P \leq 0.05$). Error bars represent the standard error of mean ($n = 9$)

Generally, MOP was highest in the clay soils across all months (with the exception of July) (ANOVA, $P< 0.0001$) (**Figure 3.1A**). MOP was significantly higher in the clay soils in March (29.32 pmol g^{-1} dry weight soil day^{-1}) compared to the greensand and chalk soils (Tukey test, $P< 0.032$). MOP within clay soils also subsequently decreased to <10 pmol g^{-1} dry weight soil day^{-1} in July and November (**Figure 3.1A**). The MOP in the greensand and chalk soils was generally low across all months, with the lowest MOP found in the greensand soils in July (1.97 pmol g^{-1} dry weight soil day^{-1}) (**Figure 3.1A**).

In the sediments, MOP changed significantly over time and between geologies (ANOVA, $P= 0.001$) (**Figure 3.1B**). MOP was the highest in clay sediments (44.12 pmol g^{-1} dry weight sediment day^{-1}) in July, and in particular was significantly higher compared to chalk sediments in March, May and July, as well as November greensand sediments (Tukey test, $P< 0.042$). In contrast, MOP was the lowest in chalk sediments (9.39 pmol g^{-1} dry weight sediment day^{-1}) in July, which then increased to 27.88 pmol g^{-1} dry weight sediment day^{-1} in November (**Figure 3.1B**) although this was not significant. The MOP of soils and sediments were significantly different from each other (ANOVA, $P< 0.0001$), with MOP of sediments generally higher than their corresponding soils (**Figure 3.1A and B**). Specifically clay and greensand sediments in May and July were significantly higher than most soils (Tukey test, $P <0.05$).

3.3.2: Methane production potential (MPP) in soils and sediments

The MPP of soils and sediments from March to November 2014 is presented in **Figures 3.2 and 3.3**. In general, methanogenesis under N_2 atmosphere in soils was very low throughout the year across all geologies, with a maximum of 1.19 pmol

g^{-1} dry weight soil day^{-1}in March chalk soil (**Figure 3.2A**). Methanogenesis in soils under H_2 atmosphere significantly changed with time and geology (ANOVA, $P<$ 0.0001). H_2 methanogenesis was normally highest in the clay soils (except for July), with clay soils in March having significantly higher production rates than any other soil (11.20 pmol g^{-1} dry weight soil day^{-1}) (Tukey tests, $P<0.0001$). Overall methanogenesis across all geologies decreased from March to below detectable levels in July, before increasing again to 10.47 pmol g^{-1} dry weight soil day^{-1} in November (**Figure 3.2B**). No methanogenesis under H_2 atmosphere was detected in soils sampled in July across all geologies (**Figure 3.2B**).

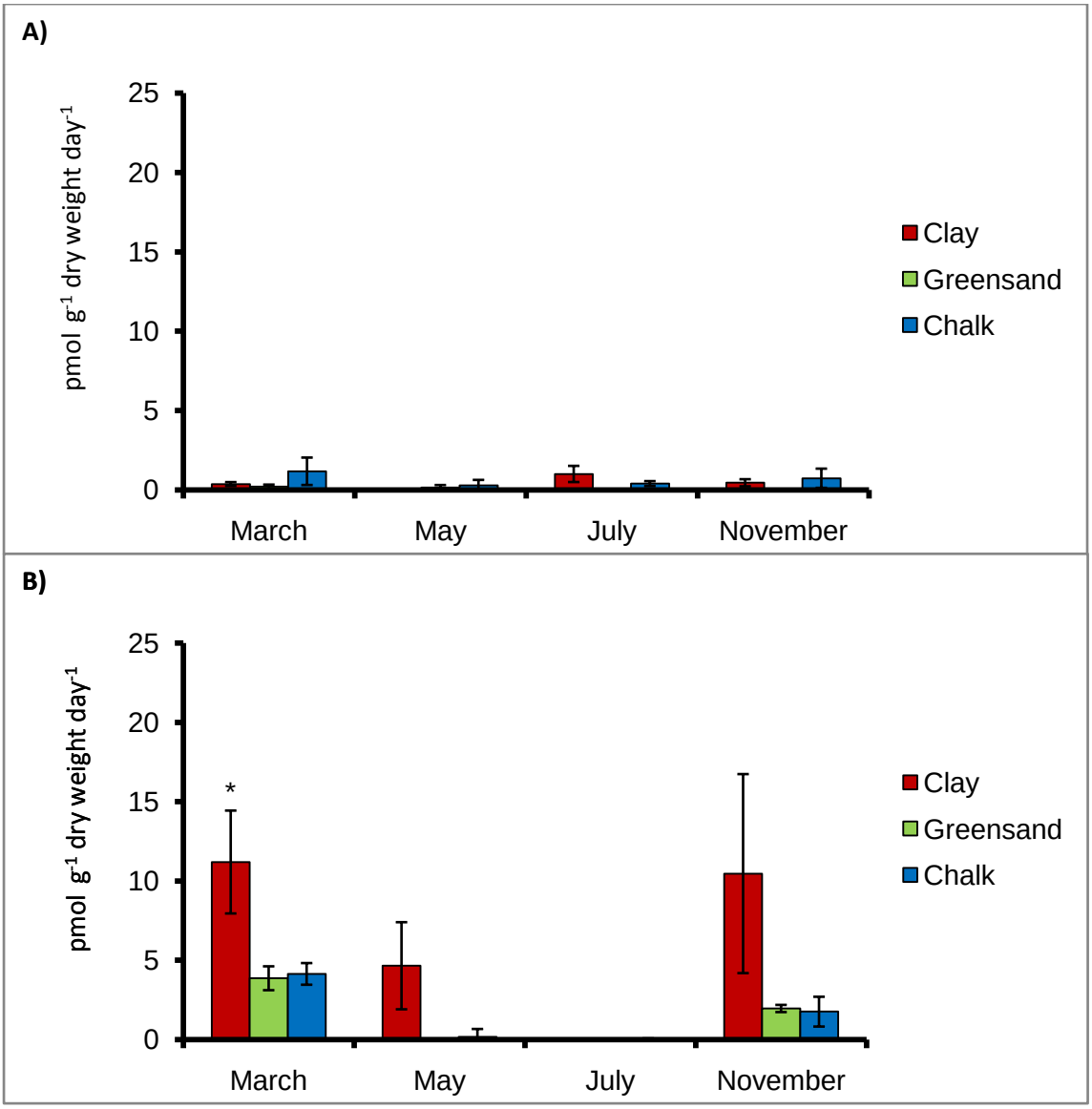


Figure 3.2: MPP under **A)** N_2 and **B)** H_2 atmosphere in soils sampled in March to November 2014.* = sample is significantly different ($P \leq 0.05$). Error bars represent standard error of the mean ($n = 3$)

In sediments, methanogenesis in both H_2 and N_2 atmosphere were detected throughout the year (**Figure 3.3 A and B**). In sediments, methanogenesis under N_2 atmosphere differed significantly between geologies and months sampled (ANOVA, $P = 0.002$). In general, methanogenesis under N_2 atmosphere was low between March and July (<53.44 pmol g^{-1} dry weight sediment day^{-1}) across all geologies but

increased in November to a maximum value of 156.54 pmol g^{-1} dry weight sedimentday^{-1} in the chalk sediments (Tukey test, P< 0.026) (**Figure 3.3A**)**.** H_2 methanogenesis also shifted significantly over time between geologies (ANOVA, P= 0.001). H_2 methanogenesis was low in March and May but was significantly higher (between 75.60 and 100.48 pmol g^{-1} dry weight sedimentday^{-1}) in July across all geologies (**Figure 3B**). In particular, sediments in July from clay, chalk, and greensand, as well May chalk sediments were significantly higher than chalk sediments in March, and clay sediments in May and greensand sediments in May (Tukey test, P< 0.021).

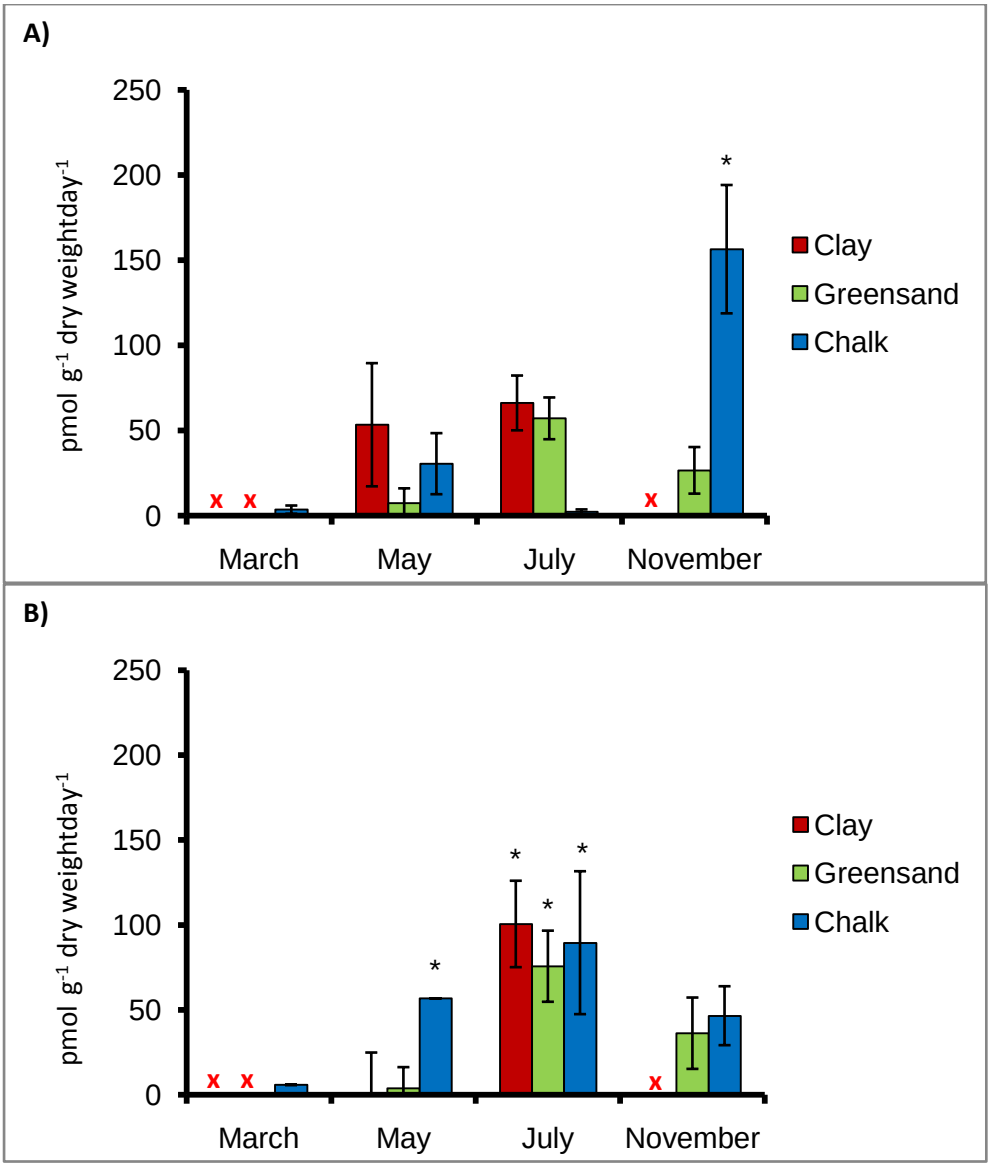


Figure 3.3: MPP under **A)** N_2 and **B)** H_2 atmosphere in sediments sampled in March to November 2014 (*n*= 3). **x** = Rivers inaccessible due to flooding. * = sample is significantly different (*P*≤ 0.05). Error bars represent standard error of the mean (*n*= 3)

3.3.3: Quantitative PCR of *pmoA* genes in soils and sediments

Q-PCR analysis of *pmoA* gene abundance in soils and sediments was performed and presented in **Figure 3.4 A and B**. In soils, the *pmoA* gene abundance varied significantly over time and geology (ANOVA, *P*< 0.0001) (**Figure 3.4A**). The

highest *pmoA* gene abundance in the soils was in July in the chalk (8.15 x10^5 gene copies g^{-1} dry weight soil) and greensand soils (3.42 x10^5 copies g^{-1} dry weight soil), compared to the clay soils which had (2.35 x10^3 gene copies per g^{-1} dry weight soil), with all three samples being significantly different than the other soil samples (Tukey tests, $P < 0.05$).

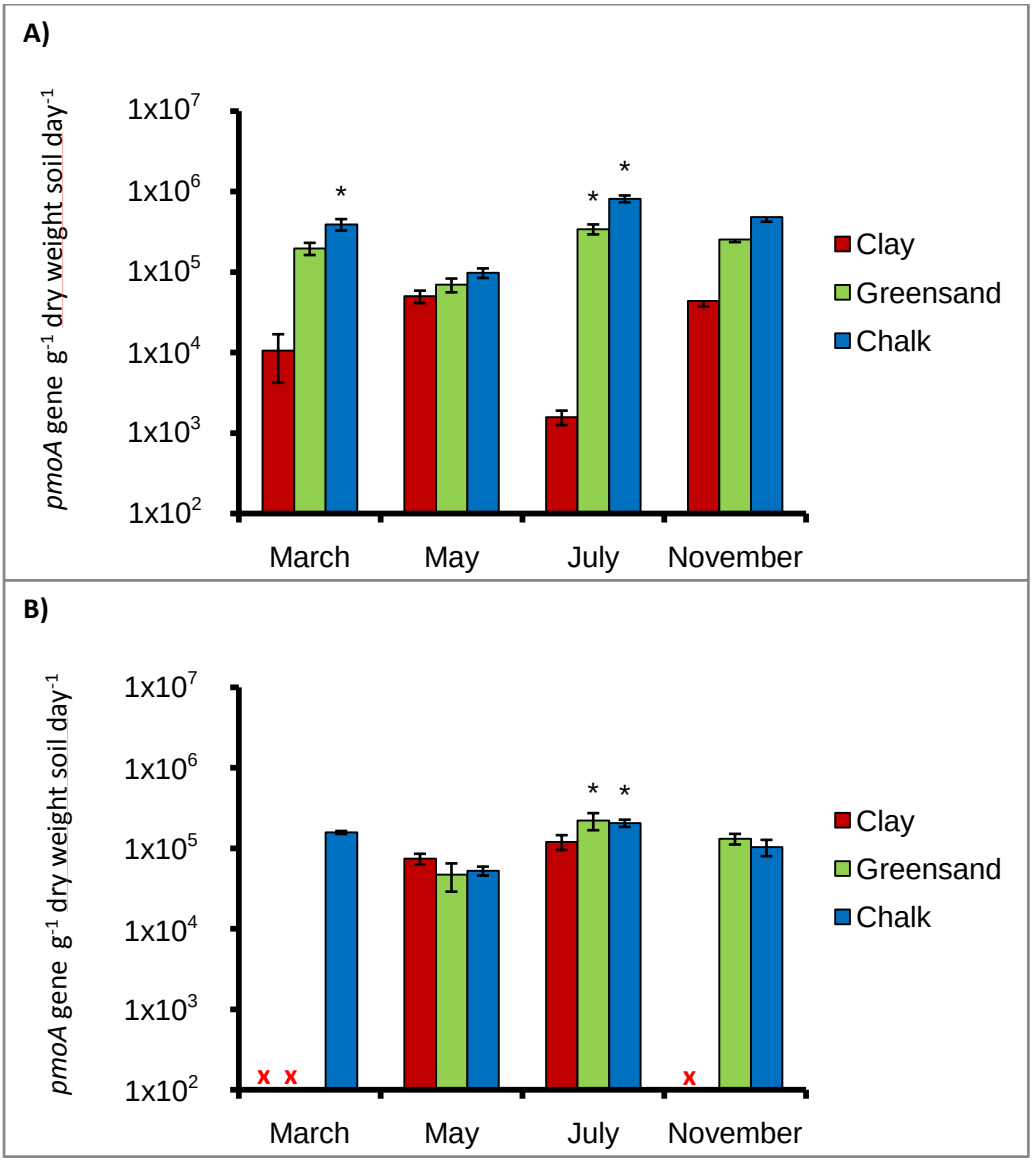


Figure 3.4: *pmoA* gene abundance in soils **A)** and sediments **B)** sampled from March to November 2014 ($n = 3$). **x** = Rivers inaccessible due to flooding. * = sample is significantly different ($P \leq 0.05$). Error bars represent standard error of the mean ($n = 3$)

In the sediments, *pmoA* gene abundance showed a significant variation across months and geologies (ANOVA, P= 0.002) (**Figure 3.4B**), however their overall change was small, with *pmoA* gene abundance in the sediments fluctuating between 1 x10^4 and 1 x10^5 gene copies g^{-1} dry weight sediment throughout the year across all geologies. Only sediments in July from the greensand sites, were significantly different from the rest of the samples (Tukey test, P< 0.049), with the exception of July chalk sediments being significantly higher than May chalk sediments (Tukey test, P= 0.048).

3.3.4: Quantitative PCR of *mcrA* gene in soils and sediments

Q-PCR analysis of *mcrA* gene abundance in soils and sediments is presented in **Figure 3.5 A and B**. In soils, *mcrA* gene abundance showed significant variations between months and geologies (ANOVA, P< 0.0001). Across the months, the abundance of the *mcrA* gene in soils tended to be higher in greensand and chalk sites compared to the clay soils (**Figure 3.5A**). In general, *mcrA* gene abundance in the chalk and greensand soils increased around ten-fold in July and November compared to March and May. The *mcrA* gene abundance was significantly higher in both the chalk (1.74 x10^4 gene copies g^{-1} dry weight soil) and greensand soils (1.97 x10^4 copies g^{-1} dry weight soil) in July and November (9.74 x10^3 gene copies g^{-1} dry weight soil, chalk and 1.38 x10^4 copies g^{-1} dry weight soil, greensand) compared to other samples (Tukey tests, P< 0.029) (**Figure 3.5A**). In the clay soils, *mcrA* gene abundance was between 1.85 x 10^2 and 1.27 x 10^3 gene copies g^{-1} dry weight soil, between March and July but increased to 5.28 x10^3 gene copies g^{-1} dry weight soil in November.

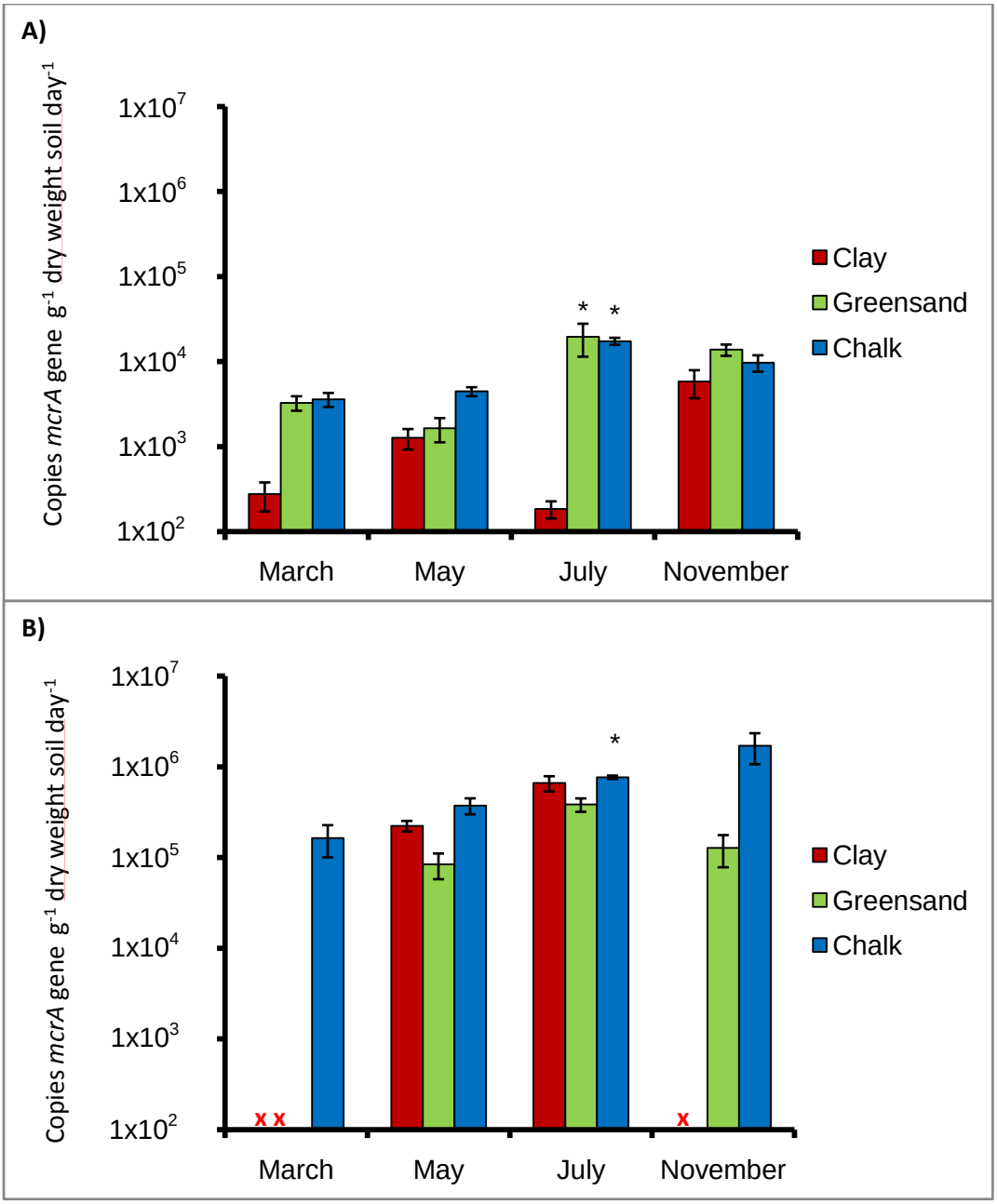


Figure 3.5: *mcrA* gene abundance in soils **A)** and sediments **B)** sampled from March to November 2014. **x=** Rivers inaccessible due to flooding. * = sample is significantly different (P≤ 0.05). Error bars represent standard error of the mean (*n*= 3)

In sediments, *mcrA* gene abundance was significantly different between months and geologies (ANOVA, *P*= 0.002) (**Figure 3.5B**). Both chalk (Tukey tests, *P*< 0.019) and clay (Tukey tests, *P*= 0.007) sediments were significantly different in

July and November compared to the other months. The *mcrA* gene abundance tended to fluctuate very little throughout the year as well as across geologies (between 8.42 x10^4 and 7.66 x10^5 gene copies g^{-1} dry weight sediment) with the only significant change from the highest *mcrA* gene abundance in the chalk sediments (7.66 x10^5 gene copies g^{-1} dry weight sediment) in November (Tukey tests, $P<$ 0.012).

3.3.5: Methanotroph 16S rRNA sequences in soils and sediments

The relative abundance (%) of methanotroph 16S rRNA gene sequences from soils is presented in **Figure 3.6.** Methanotrophic sequences in soils were grouped under 8 different genera of methanotrophs and comprised < 5.3% of total Bacterial abundance. OTUs that were not identifiable at the genus level were assigned as "Other", followed by the next taxonomic ranking. Overall "Other" Methylacidiphilales were the most abundant and found across all geologies throughout the year. "Other" Methylocystaceae were also abundant in clay soils in February, August and November although only August soils were significantly higher in abundance (Tukey test, $P<$ 0.001). "Other" Methylocystaceae was also significantly higher in clay soils, compared to other geologies (Tukey tests $P<$ 0.001). In addition, the genus *Methylosinus* was significantly higher in clay soils as well (Tukey tests, $P<$ 0.05).

A phylogenetic tree of the 16S rRNA gene from the 50 most abundant methanotroph OTUs from soils is presented in **Figure 3.7**. As several bacteria with prefix "Methylo" are not necessarily methanotrophic, genera were searched in the literature for methanotrophic properties, with methylotrophic only genera then discarded from the analysis. The percent relative abundance (%) of each order across month and geology are represented by pie charts. The 50 most abundant

methanotrophs within soils could be categorized into 2 major orders: Methylacidiphilales (43 OTUs) and Rhizobiales (7 OTUs). The order Methylacidiphilales contained mainly *Methylacidiphileae S-BQ2-57* (29 OTUs), with the remainder OTUs consisting of unknown Methylacidiphilales sequences (12 OTUs) and Methylacidiphilales LD19 sequences (2 OTUs). Half of the sequences belonging to the order Rhizobiales were unidentified Methylocystaceae, with the remaining sequences belonging to the genera *Methylosinus* (2 OTUs) and *Methylobacterium* (1 OTU). The distribution of both Rhizobiales (93%) and Methylacidiphilales (46%) were largely favoured in clay soils and least abundant in greensand soils (4% and 24% respectively). Between months Methylacidiphilales (54%) were most abundant in February, whereas Rhizobiales were more abundant in November (46%).

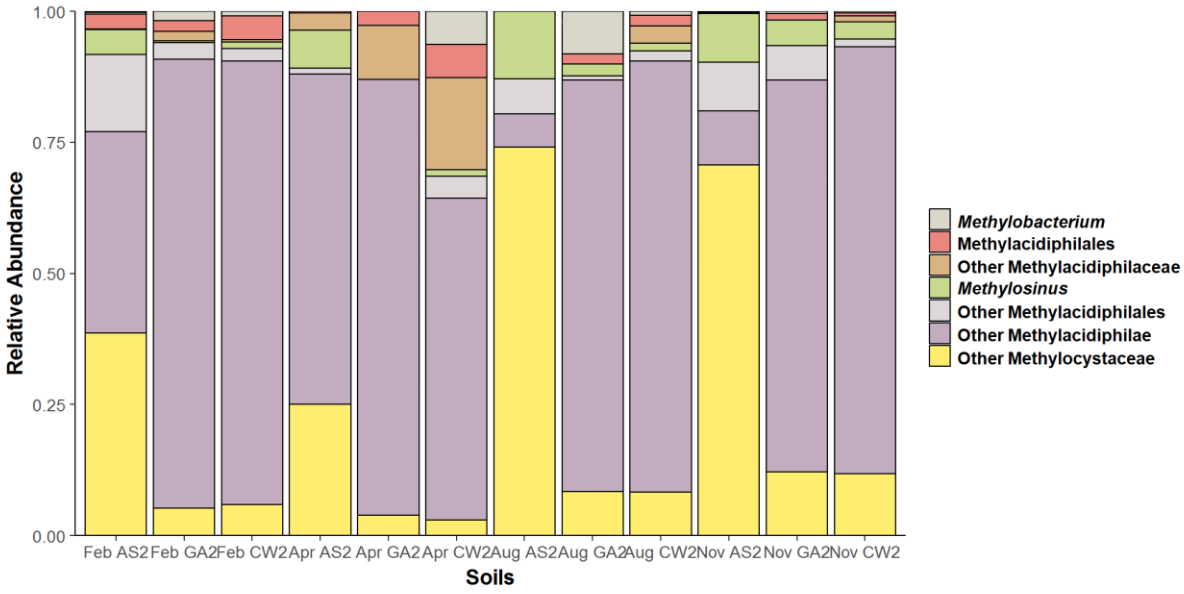


Figure 3.6: Relative abundance of methanotroph 16S rRNA gene sequences from soils sampled February (Feb), April (Apr), August (Aug), and November (Nov) 2013 soils.

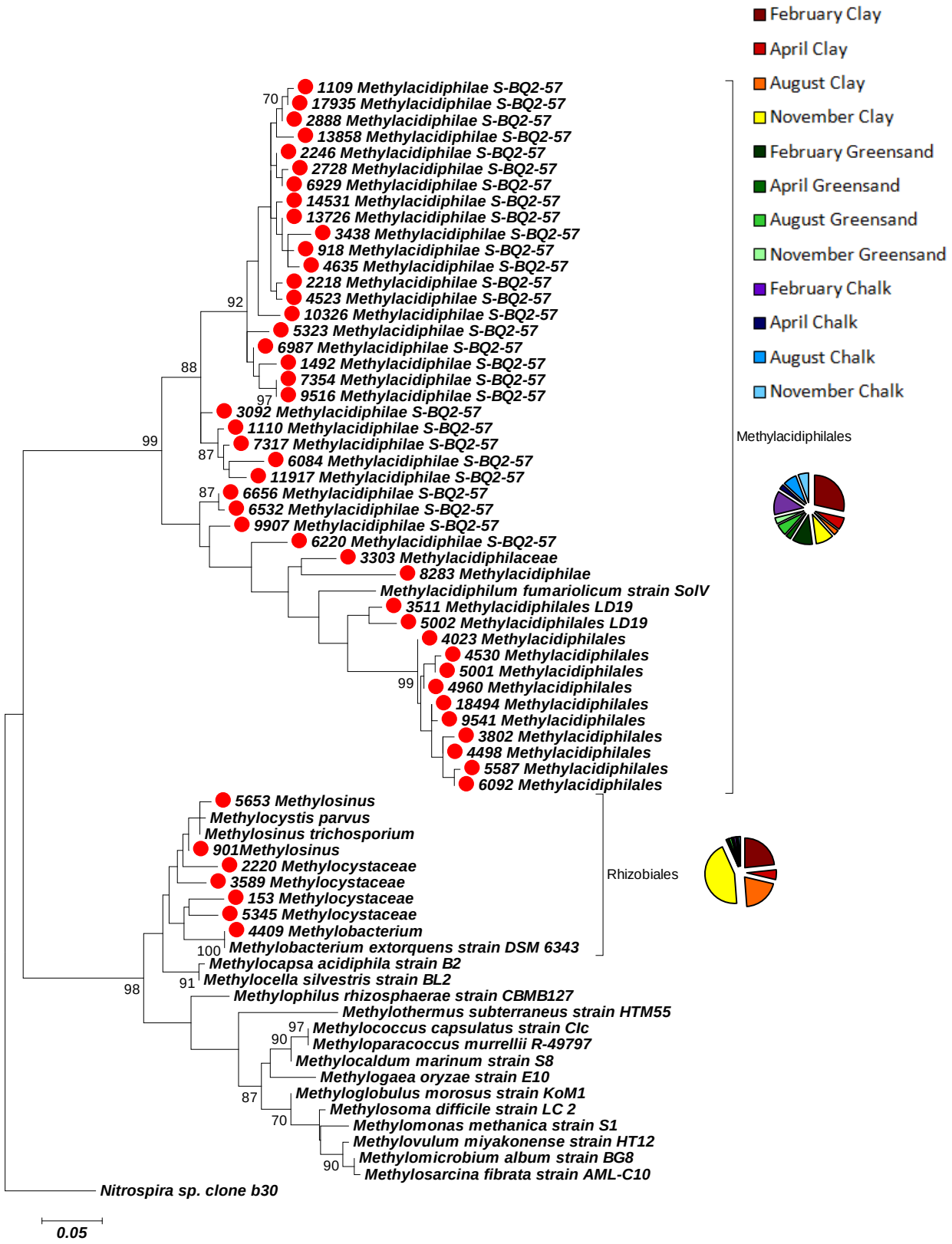
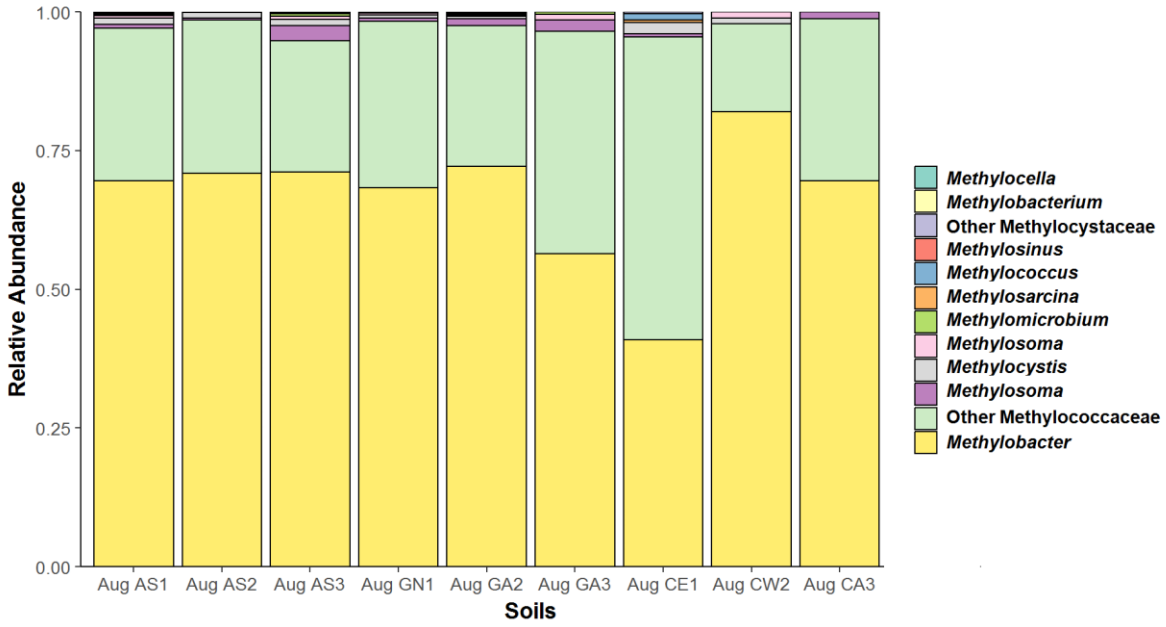


Figure 3.7: Phylogenetic tree of the 50 most abundant methanotroph 16S rRNA gene OTUs (red circle) within soils compared to closest sequence hits on NCBI. Only bootstrap values above 70% are presented. Pie charts represents relative abundance (%) of major orders of methanotrophs.

The relative abundance of methanotroph 16S rRNA gene sequences from sediments in August and February is presented in **Figure 3.8 A and B**.

A)



B)

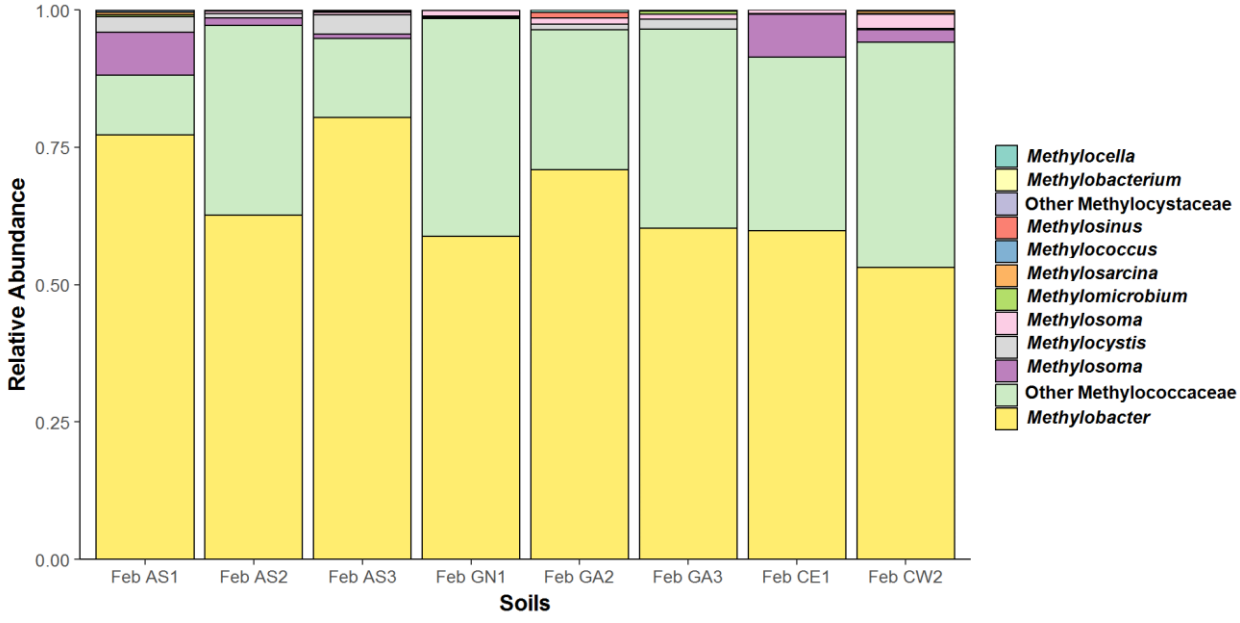


Figure 3.8: Relative abundance of methanotroph 16S rRNA gene sequences from sediments sampled in August (**A**) and February (**B**). Clay (AS1-3), Greensand (GN1, GA2-3), and chalk (CE1, CW2, CA3). February (Feb), August (Aug).

In the sediments, a total of 225 16S rRNA gene sequences were identified as methanotrophs, which were divided into 19 different genera, comprising <1.66% of the total Bacterial 16S rRNA gene sequences obtained. Sequences that could only be identified at above the genus level were labelled under the nearest taxonomic classification and prefixed with "Other". The most abundant methanotrophs were, *Methylobacter* and "Other" Methylococcaceae which were predominant across all geologies and found in both August and March, with sediments from the rivers AS1, AS3 in February, and CW2 and AS2 in August having significantly higher relative abundance than the river CE1 in August (Tukey tests, $P< 0.05$).

A phylogenetic tree of the 50 most abundant OTUs obtained is presented in **Figure 3.9**. The percent relative abundance of (%) each order across month and geology are represented by pie charts. The vast majority of OTUs were classified into three main methanotroph orders: Methylococcales (46 OTUs) which included *Methylobacter*, *Methylomonas*, *Methylosarcina* genera; Rhizobiales (4 OTUs) which contained Methylocystaceae Family,

Between geologies, both Methylococcales (59%) and Methylophilales (57%) were most abundant in clay sediments, whereas Rhizobiales were most abundant in greensand (36%) and chalk (35%). Between months, both Methylococcales (36% clay, 19% chalk) and Methylophilales (38% clay, 21% chalk) were more abundant in February clay and chalk sediments, whereas both were higher in greensand sediments in August (9% and 11% respectively). Rhizobiales were more abundant in August for clay and greensand sediments (24% and 31% respectively), but were abundant in chalk February sediments (22%).

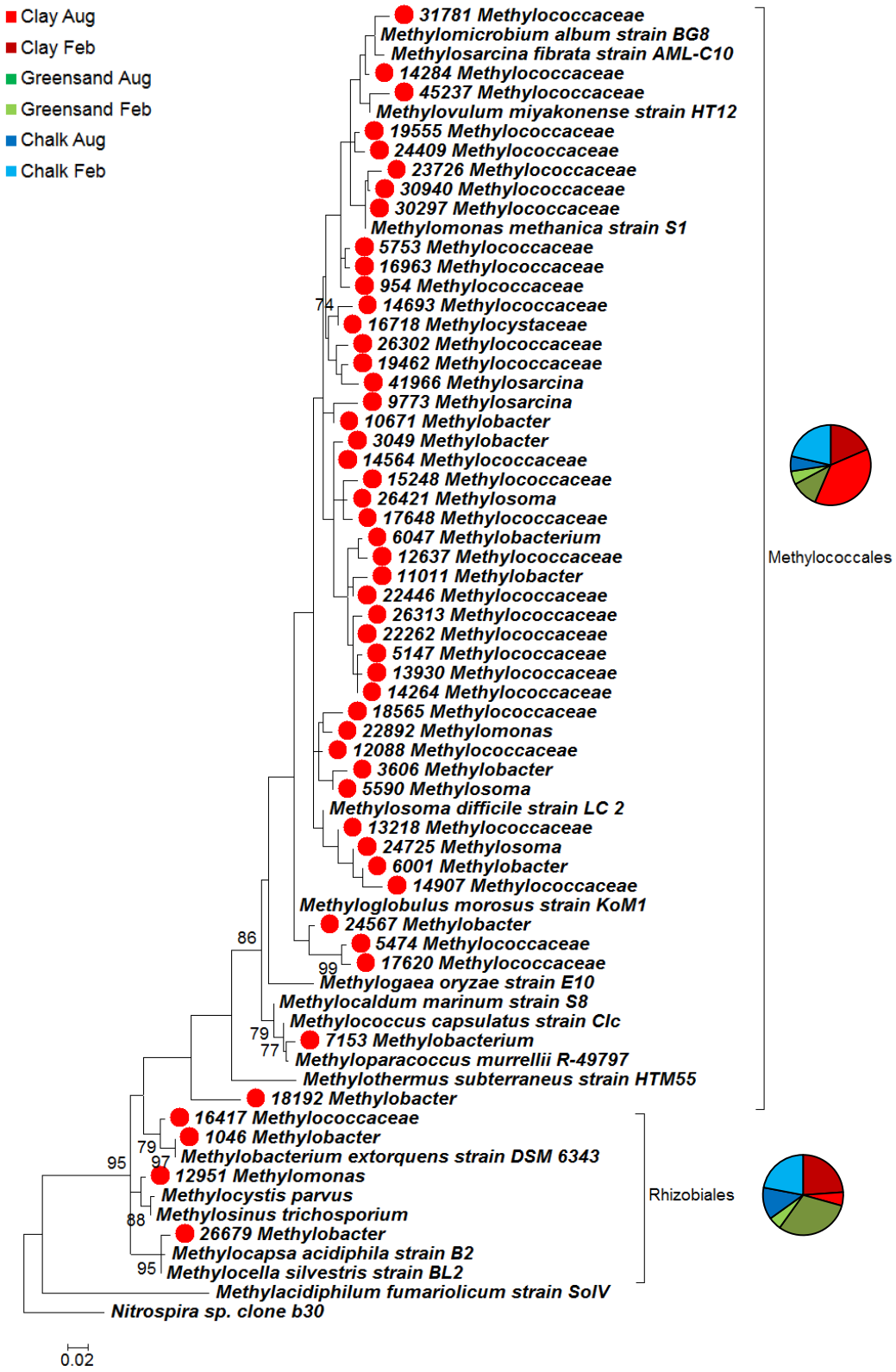


Figure 3.9: Phylogenetic tree of the 50 most abundant methanotroph 16S rRNA gene OTUs (red circle) within sediments compared to closest sequence hits on NCBI. Only bootstrap values above 70% are presented. Pie charts represent relative abundance (%) of methanotroph orders across geology and month.

3.3.6: Methanogen 16S rRNA sequences in soils and sediments

The relative abundance of methanogen 16S rRNA gene sequences in soils are presented in **Figures 3.10**. Methanogen 16S rRNA gene sequences in soils comprised <1.3% of total Archaeal sequences, with 14 genera found across all months and geologies. Relative abundance of methanogenic genera varied greatly between month and geology (ANOVA, $P< 0.0001$). Compared to other and geologies, Methanobacterium was found to be significantly more abundant in November and August clay soils (Tukey tests, $P< 0.05$), Methanobrevibacter was highest in November greensand soils (Tukey tests, $P< 0.05$), Methanomicrobiales in February greensand soils (Tukey tests, $P< 0.05$), and Methanoregulaceae (Tukey tests, $P< 0.02$).

Phylogenetic analysis of the 50 most abundant methanogen 16S rRNA gene OTUs from soils was performed and is presented in **Figure 3.11**. The percent relative abundance (%) of each order across month and geology are represented by pie charts. Methanogens were classified into five main orders; Methanobacteriales 5 OTUs, Methanomassiliicoccales 4 OTUs, Methanosarcinales 2 OTUs, Methanocellales 3 OTUs and Methanomicrobiales 3 OTUs. Between geologies all methanogen orders were most abundant in clay soils (>80%) and was lowest in chalk (<10%). Distribution across months showed April soils generally having the most abundant methanogens (43% to 59%), with the exception of Methanobacteriales which was highest in August (32%).

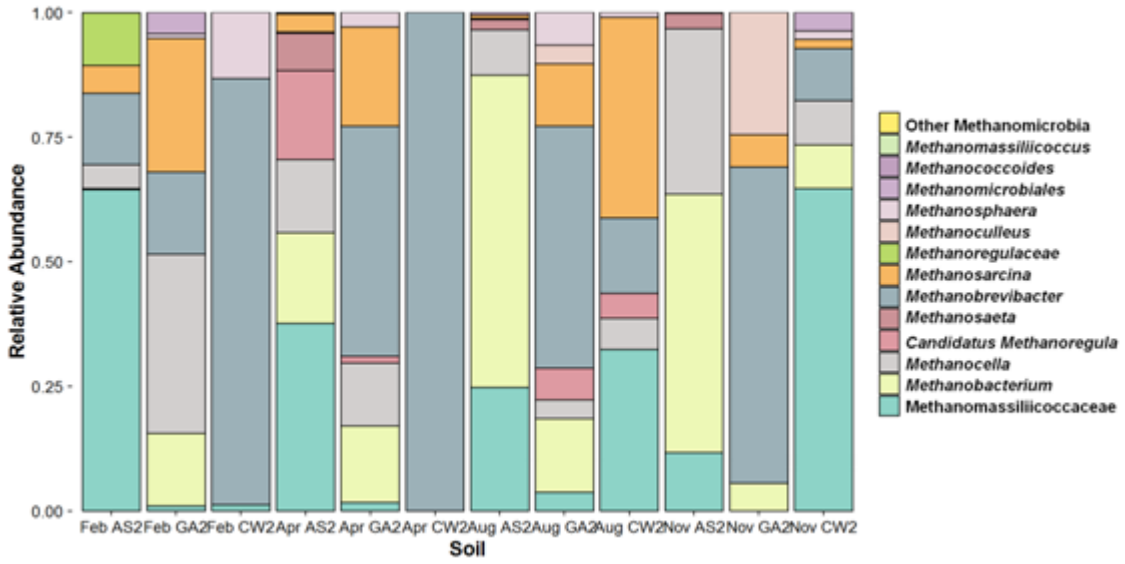


Figure 3.10: Relative abundance of methanogen 16S rRNA gene sequences from soils sampled in February, April, August, and November. Clay (AS2), Greensand (GA2), and chalk (CW2) soils in February (Feb), April (Apr), August (Aug), and November (Nov).

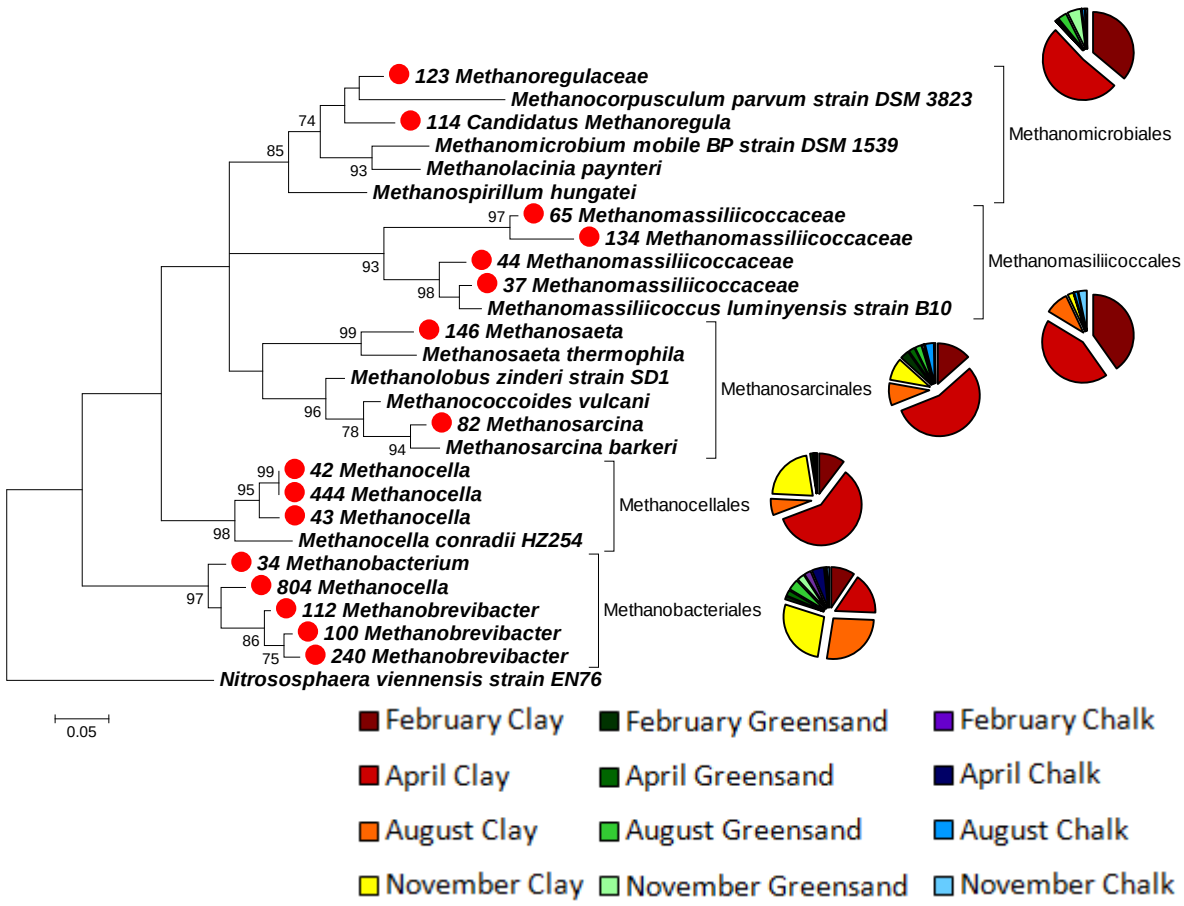


Figure 3.11: Phylogenetic tree of the 50 most abundant 16S rRNA methanogen OTUs (red circle) within soils compared to closest sequence hits on NCBI. Only bootstrap values above 70% are presented. Pie charts represent relative abundance (%) of methanogen orders across geology and month.

The relative abundance of methanogen 16S rRNA gene sequences in sediments are presented in **Figures 3.12-3.13**. Methanogen sequences made up a significant portion of sediment Archaeal OTUs comprising >50% of the total Archaeal community. Methanogen OTU sequences fell under 17 different genera. With the exception of sample GA2 greensand (sampled in March and August) *Methanoregula* (Tukey's test, $P< 0.05$) and *Methanothrix* (Tukey's test, $P< 0.05$) were dominant across geologies (**Figure 3.12A and B**). Several methanogens were only

significantly higher in a single river during a specific month however; compared to all other sediments *Methanobacterium* was significantly higher in August GA2 sediments (Tukey tests, $P< 0.05$), *Methanofollis* in February CA3 (Tukey tests, $P< 0.05$), *Methanolobus* (Tukey tests, $P< 0.05$), *Methanomassiliicoccus* in February GA2 (Tukey tests, $P <0.001$), *Methanosphaera* in August GA3 (Tukey tests, $P < 0.001$), and Other Methanosarcinaceae in February AS2 (Tukey tests, $P< 0.05$).

Phylogenetic analysis of the 50 most abundant methanogen 16S rRNA gene OTUs from sediments grouped into four orders, i.e. Methanosarcinales 26 OTUs, Methanomicrobiales 21 OTUs, Methanobacteriales 1 OTUs and Methanomassiliicoccales 2 OTUs (**Figure 3.13**). When comparing between the different river geologies, all methanogen orders were generally more abundant within chalk sediments (37% to 58%) and least abundant in greensand sediments (19% to 28%). Methanogen orders in clay sediments were the second most abundant, except for the orders Methanomassiliicoccales (42%). Within the different months, Methanosarcinales (64%), Methanomicrobiales (51%), and Methanobacteriales (54%) were more abundant in August sediments, whereas Methanomassiliicoccales (57%) were most abundant in February sediments.

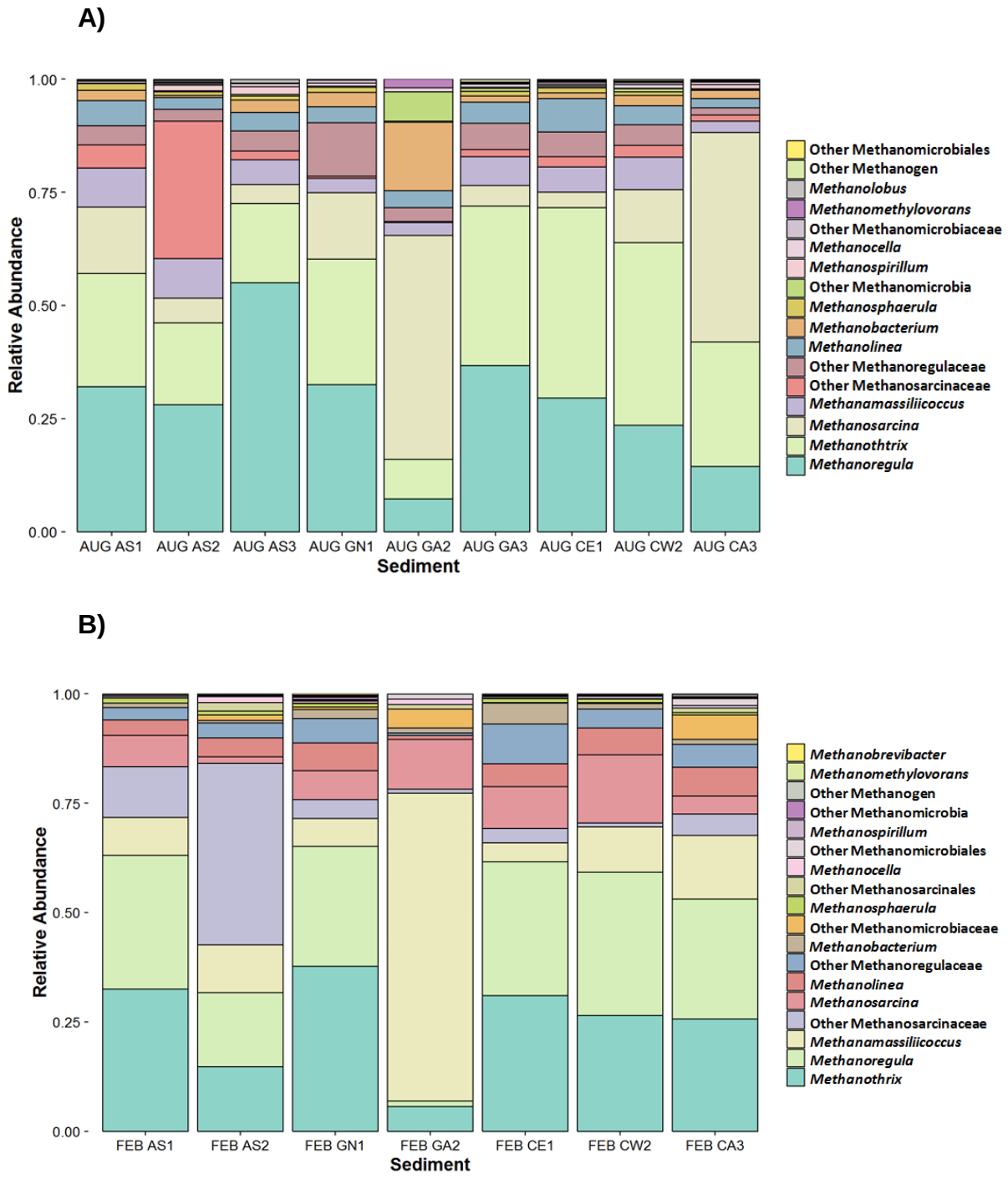


Figure 3.12: Relative abundance of methanogen 16S rRNA gene sequences from sediments sampled in August (**A**) and February (**B**).Clay (AS1-3), Greensand (GN1, GA2-3), and chalk (CE1, CW2, CA3). February (Feb), August (Aug).

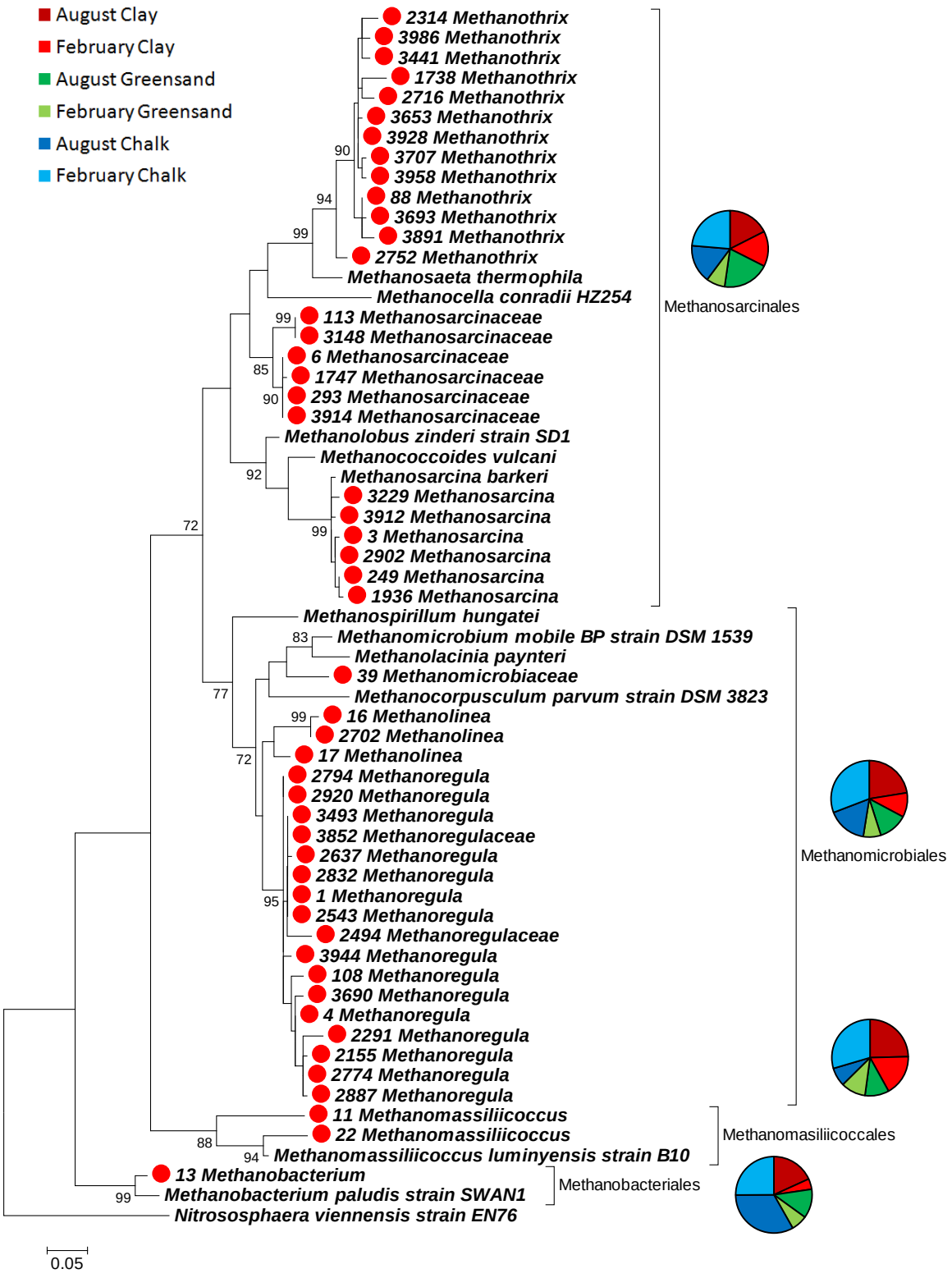


Figure 3.13: Phylogenetic tree of the 50 most abundant methanogen 16S rRNA gene OTUs (red circle) from sediments compared to closest sequence hits on NCBI. Only bootstrap values above 70% are presented. Pie charts represent relative abundance (%) of methanogen orders across geology and month.

3.3.7: Methanotroph *pmoA* gene sequences in soils and sediments

A stacked boxplot of soil *pmoA* genes was not presented as >99.9% of *pmoA* gene sequences were unassigned with remaining <0.1% classified as *Methylocystis*. This was confirmed via BLASTn of the 50 most abundant sequences, with a majority of sequences belonging to uncultured sequences, with some sequences not matched with any NCBI sequences. A phylogenetic tree was constructed from the 50 most abundant *pmoA* gene sequences were presented in **Figure 3.14**. Pie charts represent relative abundance of orders across geology and month. Between geologies, the abundance of *pmoA* genes that clustered with Rhizobiales were greatest in chalk soils (46%), whereas Methylococcales were most abundant in greensand (75%), and both groups were lowest in clay soils (22% and 0.37% respectively). Between months, both clades overall decreased from February (14% to 25%) to April (4% to 10%) increased in August (34% to 47%), and decreased again in November (32% to 35%).

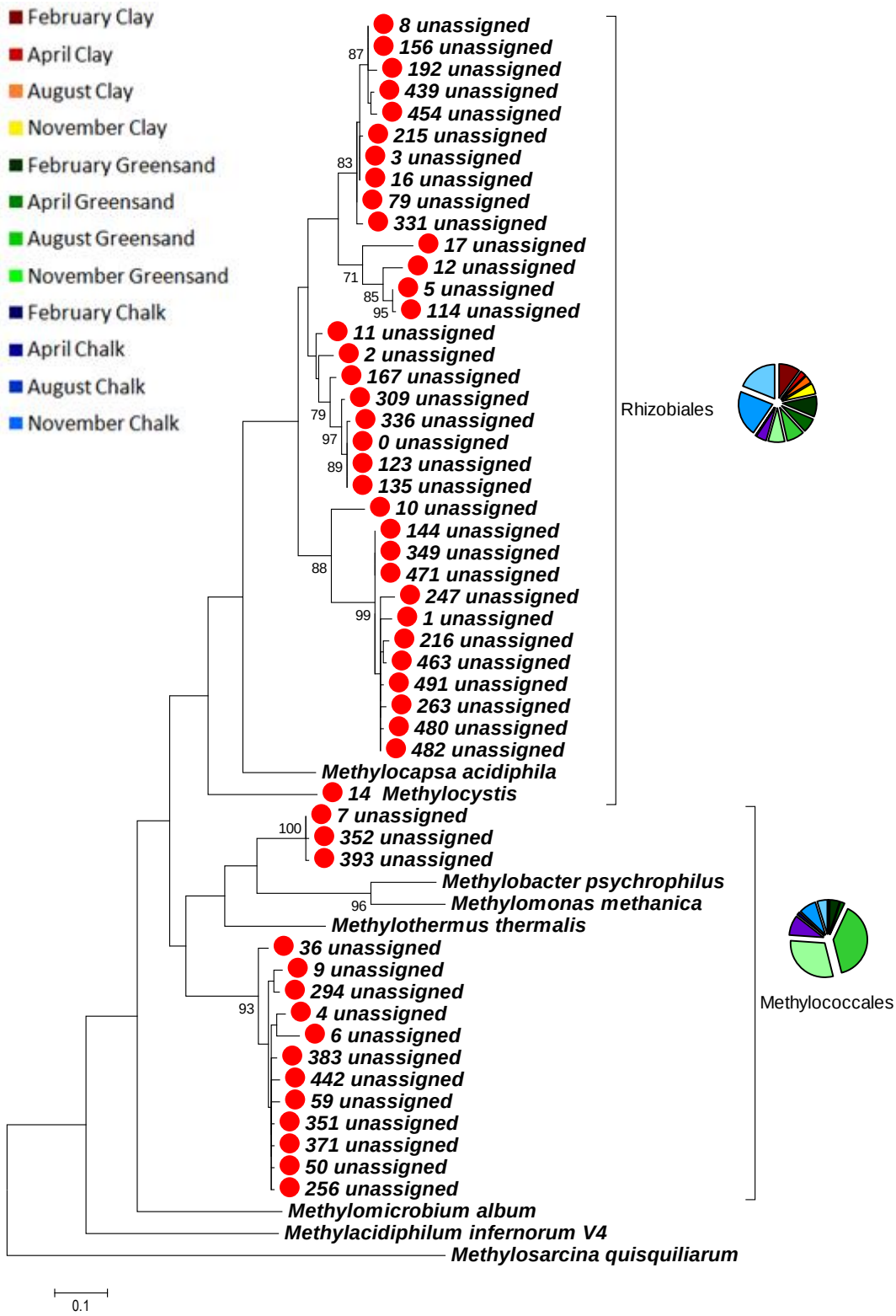
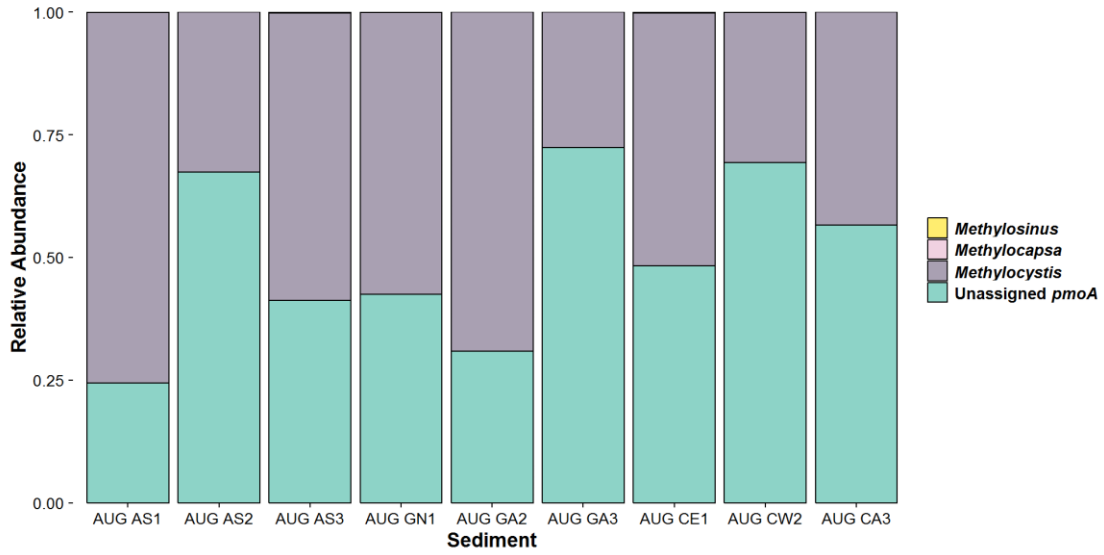


Figure 3.14: Phylogenetic tree of 50 most abundant *pmoA* gene sequences (red circles) within soil compared to closest sequence hits from custom NCBI database. Only bootstrap values above 70% are presented. Pie charts represent relative abundance (%) of methanotroph orders across geology and month.

The relative abundance of methanotroph *pmoA* genes found in sediments is presented in **Figure 3.15**. The majority of *pmoA* sequences obtained across all sediments in August and March were related to *Methylocystis* or previously unknown *pmoA* gene sequences and fluctuated in relative abundance across geologies in August (**Figure 3.15A**). In general the relative abundance of *Methylocystis* was greater in the chalk compared to clay and greensand in February. In contrast the relative abundance of unassigned *pmoA* were generally higher in clay and greensand in February compared to the chalk (**Figure 3.15B**).

Phylogenetic analysis of the 50 most abundant *pmoA* OTUs is presented (**Figure 3.16**). Several OTUs from unassigned sequences clustered with *Methylocystis* and *Methylocapsa* which overall suggested all sequences belonged to the order Rhizobiales. The *pmoA* gene was evenly distributed between February and August (50% each), however between geologies sequences were higher in abundance in clay (37%) and lowest in greensand (29%)

A)



B)

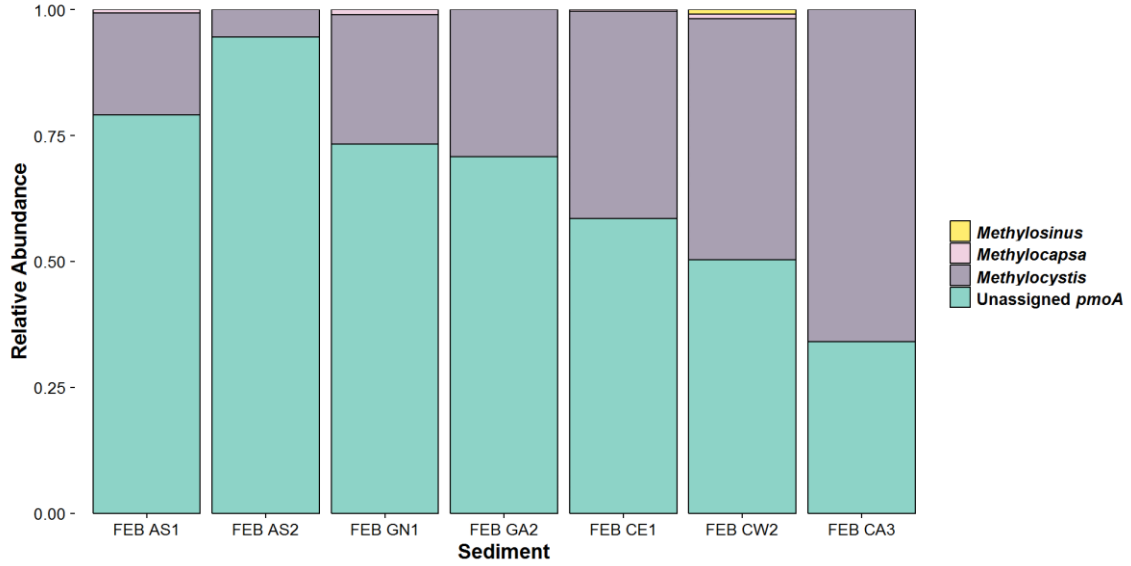


Figure 3.15: Relative abundance of *pmoA* gene sequences from sediments sampled in August (**A**) and February (**B**). Clay (AS1-3), Greensand (GN1, GA2-3), and chalk (CE1, CW2, CA3). February (Feb), August (Aug).

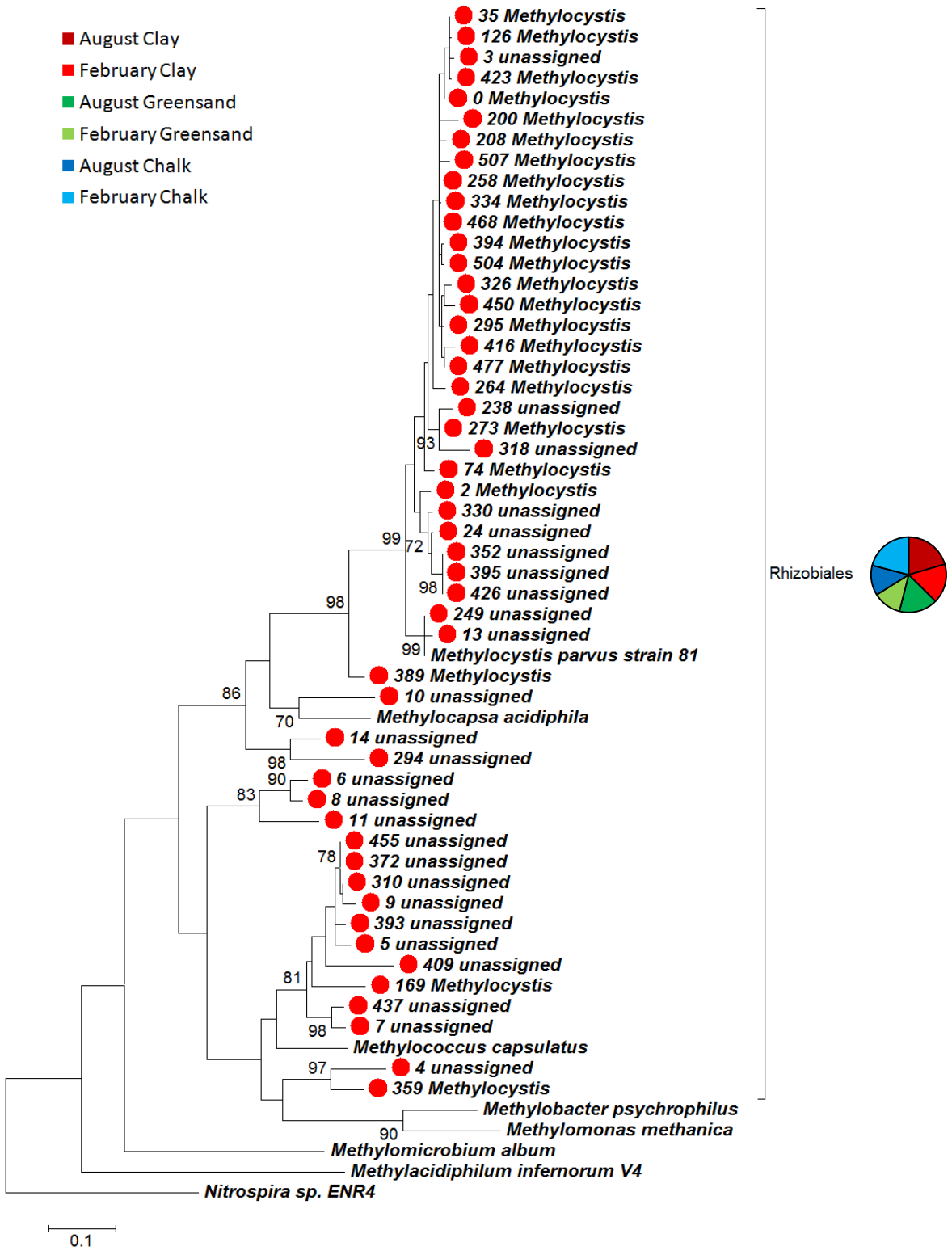


Figure 3.16: Phylogenetic tree of the 50 most abundant *pmoA* gene sequences (red circles) within sediments compared to closest sequence hits from custom NCBI database. Only bootstrap values above 70% are presented. Pie charts represent relative abundance (%) of orders across geology and month.

3.3.8: Methanogen *mcrA* sequences in sediments

The relative abundance of *mcrA* genes found in sediments is presented in **Figure 3.17A and B**. Note that 99% of sequences were "unassigned" and were excluded from the boxplot. Similar to the unassigned *pmoA* sequences, these *mcrA* sequences when put through BLASTn returned uncultured *mcrA* sequences. In general, Methanococcoides and Methanosarcina were predominant in August whilst Methanosarcina and Methanocorpusculum were predominant in February across most geologies. Methanoculleus (in March) and Methanospirillum (in August) were also present in relative high abundance.

A phylogenetic tree of the 50 most abundant *mcrA* gene sequences is presented (**Figure 3.18**). Between geologies, Methanobacteriales (55%) and Methanomicrobiales (49%) were most abundant within chalk sediments whereas Methanosarcinales were most abundant within greensand sediments (68%). Between months, all three orders were found mainly within August (59% to 73%). Sequences of *mcrA* gene in soils are not available as the PCR amplification step for the *mcrA* gene failed to amplify.

A)

B)

Figure 3.17: Relative abundance of *mcrA* gene sequences from sediments sampled in August (**A**) and February (**B**). Clay (AS1-3), Greensand (GN1, GA2-3), and chalk (CE1, CW2, CA3). February (Feb), August (Aug).

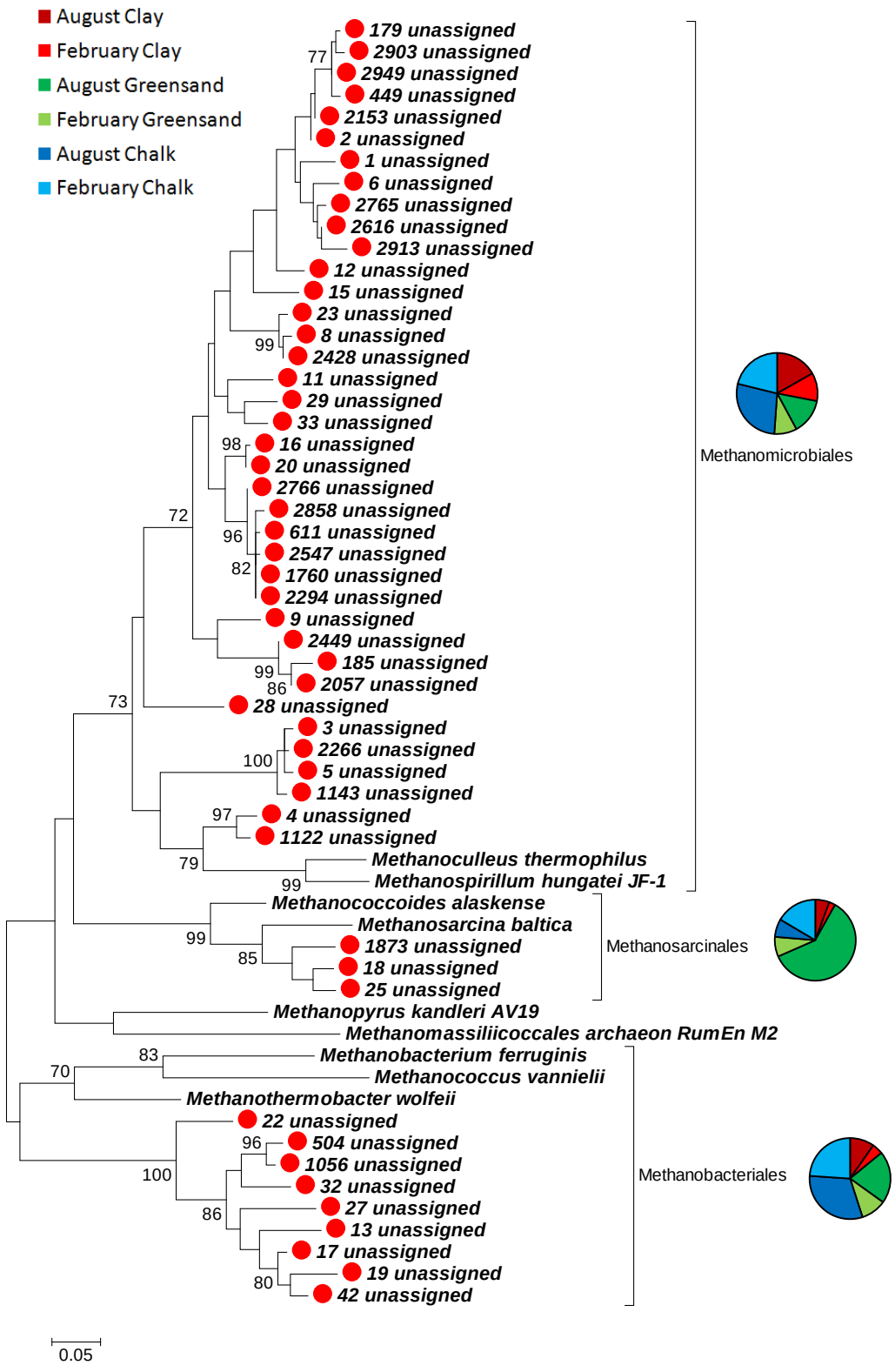


Figure 3.18: Phylogenetic tree of 50 most abundant *mcrA* gene sequences (red circles) within sediments compared to closest sequence hits from custom NCBI database. Only bootstrap values above 70% are presented. Pie charts represent relative abundance (%) of orders across geology and month.

3.4: Discussion

In this study higher MOP and MPP were found in river sediments compared to soils suggesting that these rivers are potential 'hot spots' of activity in CH_4 transformations. MOP of soils in our current study suggests that the overall oxidation potential is comparatively lower than other soils studied (See **Table 3.2**). Chan and Parkin (2001) performed similar MOP experiments with prairie soils, a closely related environment to our semi-arable grassland, at atmospheric CH_4 and saturated CH_4 conditions. In atmospheric CH_4 conditions, their MOP in prairie soils was similar to oxidation potentials within the soils in this study, with MOP at the pmol scale, however prairie soils (1790 to 2186 pmol g^{-1} dry weight soil day^{-1}) were still higher than our study sites (1.96 to 23.92 pmol g^{-1} dry $weight^{-1}$ day^{-1}). In the same study by Chan and Parkin, the soil samples closest to the MOP values in this study were from Kluver treated grassland soil and forest soil (0.24 to 600 pmol g^{-1} dry weight soil day^{-1}). Reay *et al. (*2004) tested MOP on forest soils and although CH_4 concentrations were optimized to similar levels as our current study, their measured MOP were higher at 24-96 nmol g^{-1} dry $weight^{-1}$ day^{-1}. In studies using saturated CH_4 conditions, MOP begins to dwarf the oxidation rates within this study by 2 to 3 orders; Börjesson *et al. (*2003) and He *et al. (*2012) recorded rates at the µmol scale and Preuss *et al.*'s, (2013) study in Arctic wetland soil found MOP at the mmol scale. As the closest MOP and CH_4 concentrations belong to studies that use atmospheric conditions, which are ideal for high affinity methanotrophs, this would also place the MOP of our study within the range of high affinity methanotrophy.

Table 3.2: List of methanotroph land and aquatic habitats. **+** = increase, **-** = decrease

Environment	Methanotroph genus	Methanotrophic potential (MOP)	*pmoA* abundance	Key factors	Reference
Hampshire-Avon soil	*Methylacidiphilum* *Methylobacterium* *Methylosinus*	1.96 to 23.9 pmol g^{-1} dry weight-1 day-1	1.58 x 10^3 to 8.15 x 10^5 gene copies g^{-1} dry weight soil	Ammonium (**-** *pmoA*) Sulfate (**-***pmoA*) *mcrA* (**+***pmoA*) MPP (**+** MOP)	This study
Gisburn forest land-use experiment	Not measured	24 to 96 nmol g^{-1} dry weight day^{-1}	Not measured	Nitrate (**-** MOP)	Reay *et al.*, 2004
Landfill cover soil	Type I Methanotroph Type II Methanotroph	29.3 to 39.3 µmol g^{-1} dry $weight^{-1}$ day^{-1}	Not measured	Low Temperature (**+** Type I Troph) Higher Temperature (**+** Type II methanotroph)	Börjesson *et al.*, 2003
Forest Prairie Hilltop	Not measured	Low Affinity: 0.48 nmol 16.8 mmol g^{-1} dry weight soil day^{-1} High Affinity: 0.24 pmol to 2.19 nmol g^{-1} dry weight day^{-1}	Not measured	Inorganic N (**+** low affinity MOP) (**-** high affinity MOP)	Chan and Parkin 2001
Rice Paddy soil	*Methylomirabilis oxyfera*	Not measured	8.5 × 107 to 1.0 × 10^8 gene copies g-1 dry weight soil	Total Carbon (**-***pmoA*)	Zhou *et al.*, 2014
Arctic wetland soil	Not measured	18.3 mmol day^{-1} g dry weight $soil^{-1}$	Not measured	Soil diffusivity (**+** MOP)	Preuss *et al.*, 2013

Hampshire-Avon sediment	*Methylobacter* *Methylobacterium* *Methylocella* *Methylococcus* *Methylocystis* *Methylomicrobium* *Methylosarcina* *Methylosinus* *Methylosoma* *Methylomonas*	7.79 to 17.47 pmol g^{-1} dry $weight^{-1}$ sediment day^{-1}	4.7×10^4 to 2.21×10^5 gene copies g^{-1} dry weight sediment	H_2 Methanogenesis (**+** MOP)	This study
River Lambourn	Not measured	7.47 to 134.6 nmol g-1 wet $weight\ day^{-1}$	Not measured	Light exposure (**-** MOP)	Trimmer *et al.*, 2010
Yellow River sediment	*Methylomirabilis oxyfera*	Not measured	8.63×10^3 to 1.83×10^5 gene copies g^{-1} dry weight sediment	Ammonium (**+***pmoA*) Total Organic Carbon (**+***pmoA*) P > N ratio (**-***pmoA*) pH (**-***pmoA*)	Yan *et al.*, 2015
Fine river sediment Gravel river sediment	Not measured	Fine sediment: 4.13 µmol g^{-1} dry weight sediment day^{-1} Gravel sediment: 5.76 nmol g^{-1} dry weight sediment day^{-1}	Not measured	Temperature (**+**MOP in "Fine" gravel)	Shelley *et al.*, 2015

Fewer studies have examined MOP in rivers when compared to soil systems, however overall MOP in other studies, like soils, are higher than those found in this study (**Table 3.2**). Trimmer *et al.* (2010) showed the MOP of chalk rivers had MOP between 7.47 to 134.6 nmol g^{-1} wet weight sediment day^{-1} (Trimmer *et al.*, 2010). In this study, MOP were much lower in the chalk rivers throughout the year (7.79 to 17.47 pmol g^{-1} dry $weight^{-1}$ day^{-1}). A similar river system studied by Shelley *et al.* (2015) also found MOP higher than our study, which again was at the nmol scale.

Here, methanotroph 16S rRNA and *pmoA* gene abundances were also generally lower in the soils compared to sediments across sites and throughout the year with <8.15 $x10^5$ gene copies per g^{-1} dry weight soil. Here, MOP was found to be the highest in winter months in the soils but did not correlate with *pmoA* gene abundance (P=0.975). Previous studies on soils have found varying numbers of *pmoA* gene abundances. For example, in rice fields Kolb *et al.* (2003), found 5 x 10^6 copies g^{-1} dry weight soil. Whilst another study by Ho *et al.* (2011) found *pmoA* gene abundances between 1-3 x 10^3 g^{-1} dry weight soil. It should be noted however that both these studies used different primer sets, rather than the broader range A189f and A650r used in this study. Although the primers used in this study were designed to exclude *amoA* genes, it is still possible that *amoA* genes are amplified, as the design of the primers were used against only a select few ammonia oxidizers (Bourne *et al.*, 2001).

In contrast to the soils, MOP and *pmoA* gene abundance in the sediments were significantly higher in summer months (P= 0.004). However like soil, *pmoA* gene abundance did not correlate with MOP. In this study, the majority of *pmoA* sequences obtained in soils were unknown *pmoA* gene sequences but clustered closely with sequences from *Methylocystis* and *Methylobacter* within soils, whereas

pmoA gene sequences from sediments were mostly identified as, or clustered with *Methylocystis* with a few clustering with *Methylocapsa*. As *Methylobacter, Methylocystis* and *Methylocapsa* contains facultative methanotroph species, this may explain why *pmoA* gene sequences were not correlating with MOP, as methanotrophs are likely either deriving carbon from other sources, or expressing sMMO over pMMO (Theisen and Murrell, 2005).

As sequencing between 16S rRNA and *pmoA* genes were staggered between 2013 and 2014, a direct comparison between the genes would be problematic. However what can be inferred from the similar seasons (e.g. February vs March, April vs May etc.) and geologies shows that the vast majority of sequences within soils were derived from the order Methylacidiphilales, which belong to the Phylum Verrucomicrobia. If the population of methanotrophs between 2013 and 2014 remained similar, this would suggest that the vast majority of methanotrophy would be performed by Verrucomicrobia that are not detectable with the *pmoA* primers used in this study (Oshkin *et al.,* 2014), and future additional assays will be required to determine their functional relevance within this study.

In general, MPP in both soils and sediments in this study was lower than what was found previously (**Table 3.3**). For example, MPP in a chalk sediment was between 528 to 1920 nmol g^{-1} dry weight sediment day^{-1} (Shelley *et al.,* 2015) whilst a study by Mach *et al.* (2015) measured MPP at 34 nmol g^{-1} dry weight sediment day^{-1} in a river with a mixed clayish sediment. In this study MPP throughout the year and across geologies was <100 pmol g^{-1} dry weight sediment day^{-1}. Soil MPP is also considerably lower than what is found in previous research (**Table 3.3**), with production as low as 360 pmol CH_4 g^{-1} dry weight soil day^{-1} still above the highest

measured rates in our soils (Chan *et al.*, 2001; Schäfer *et al.*, 2012 Dubey *et al.*, 2014).

Table 3.3: List of methanogen land and aquatic habitats. **+** = increase, **-** = decrease

Environment	Methanogen genus	Methanogenic potential (MPP)	*mcrA* abundance	Key factors	Reference
Hampshire-Avon soil	*Methanococcoides* *Methanoculleus* *Methanomassiliicoccus* *Methanomicrobiales* *Methanosphaera*	N2 : 0-156.5 pmol g^{-1} dry weight sediment day^{-1} H2 : 75.6 to 100.48 pmol g-1 dry weight sediment day^{-1}	1.85 $x10^2$ to 1.97 $x10^4$ copies g^{-1} dry $weight^{-1}$	Acetate (**-**) Nitrate (**+**) Phosphate (**-**) Ammonium (**-**) Formate (**-**)	This study
Peat soil	Not measured	1.1 to 595 nmol g^{-1} h^{-1} dry weight soil	1.5 $x10^4$ to 1.5 x 10^5 copies g^{-1} dry $weight^{-1}$ soil	MPP (**+** *mcrA* gene transcript) Temperature (**+** MPP)	Freitig and Prosser, 2009
Brewery waste Dairy based food waste Dairy cow waste Beef slaughter house	Not measured	H2 : 0.8 to 14.9 ml CH_4 g^{-1} VSS h^{-1}	~4.5 x 10^8 to ~1.5 $x10^9$ copies g^{-1} pellet	H2 MPP (**+** *mcrA* abundance)	Morris *et al.* 2015
Municipal waste Forest Prairie Hilltop	Not measured	H2 : 1.2 nmol to 7.2 mmol g^{-1} dry weight soil h^{-1}	Not measured	Not measured	Chan and Parkin 2001

Hampshire-Avon sediment	*Methanococcoides* *Methanobacterium* *Methanospirillum* *Methanomethylovorans* *Methanoculleus* *Methanocorpusculum* *Methanosarcina*	H2 : 6 to 89 pmol g^{-1} dry $weight^{-1}$ day^{-1} N_2 : 2 to 157 pmol g^{-1} dry $weight^{-1}$ day^{-1}	8.4 x 10^4 to 1.7 x 106 gene copies g^{-1} dry $weight^{-1}$	Water content (**+**)	This study
River Sitka	*Methanomicrobium* *Methanobacterium* *Methanosarcina* *Methanosaeta*	H_2 : 34 nmol g^{-1} dry weight sediment day^{-1}	5 to 33 nmol g^{-1} dry weight sediment	Increasing depth (**+**)	Mach *et al.*, 2015
Fine chalk river sediment Gravel chalk river sediment	Not measured	Fine sediment: 480 to 1920 nmol CH_4 g^{-1} day^{-1}	Not measured	Temperature (**+**MPP in "Fine" sediment)	Shelley *et al.*, 2015

As clay soils had the highest MPP in our study, it is possible that the lower porosity of clays would lead to more anoxic conditions, favouring methanogens (Frey *et al.*, 2011), however the measured soil porosity did not correlate with MPP (*P*= 0.172). Acetate may also play a role in methanogenesis in sediments. Despite a higher concentrations of acetate found in the soils (highest concentration 13.34 µmol g^{-1} dry weight soil) compared to sediments (highest concentrations 1.37 µmol g^{-1} dry weight sediment), methanogenesis under N_2 atmosphere was generally low or not detected in the soils throughout the year and across geologies. It is possible that other organisms were outcompeting methanogens for substrates such as acetate in the soils, which may include sulfate and iron reducing bacteria (Bethke *et al.,* 2011). In contrast to the soils, acetotrophic methanogenesis was detected in the river sediments, and hence lower acetate concentrations were found in the sediments (Chapter 2). In this study, H_2 atmosphere was the dominant treatment for CH_4 production in both soils and sediments.

Similar to MOP, the MPP was also higher in the river sediments compared to soils throughout the year. This is likely due to year round water logging in sediments causing anaerobic conditions, compared to the fluctuating water content of the soils. The more readily anaerobic conditions in sediments also mean shorter transition zones from methanogenesis to oxidation, increasing CH_4 availability for oxidation compared to soils (Nazaries *et al.,* 2013).

In this study, methanogenesis was highest in clay soils, and related to the relative abundance of methanogen 16S rRNA sequences but not *mcrA* gene abundance. This should be taken with precaution as the sequences were derived from similar seasons, but not the same year Although *mcrA* genes were not detected in any of the soils (probably below the detection limits for the assay used),

methanogen 16S rRNA gene sequences were recovered from soils and found to be related to Methanobacteriales, Methanomassiliicoccales, Methanosarcinales, Methanocellales and Methanomicrobiales suggesting that the upper soil layers had anoxic microsites that can harbour low numbers of methanogens (Verchot *et al.*, 2000). Furthermore, *mcrA* gene abundance in the majority of soils did not correlate with MPP (Pearson's correlation, *P*= 0.143). Here *mcrA* gene abundances were <1.97 x10^4 copies g^{-1} dry $weight^{-1}$ soil whereas a study by Freitig and Prosser (2009) found 1.5 x10^4 to 1.5 x 10^5 copies g^{-1} dry $weight^{-1}$ soil. The *mcrA* gene abundances in the sediments were between 8.42 x 10^4 to 1.7 x 10^6 copies g^{-1} dry $weight^{-1}$ across all sites which is 2-3 orders of magnitude lower than *mcrA* gene abundance found in mudflats in the Yangtze river, China (i.e. between 10^7-10^8 copies g^{-1} dry $weight^{-1}$ (Zeleke *et al.*, 2013). Unlike sediment *pmoA*, sediment *mcrA* did in fact correlate with H_2 MPP (Pearson's correlation= 0.511, *P*= 0.007). MPP and *mcrA* gene abundance was significantly higher in the summer months in the sediments (ANOVA, *P*= <0.0001). Here *Methanococcoides, Methanosarcina* and *Methanocorpusculum* were predominant with *Methanoculleus* and *Methanospirillum* also present in relative high abundance in the sediments. Representative species of these methanogens have been previously found in other river sediments (Xie *et al.*, 2014; Chen *et al.*, 2015, Youngblut *et al.*, 2015).

In this study, a large proportion of the *mcrA* and *pmoA* recovered were unassigned, with subsequent BLASTn searches from NCBI assigning them as being uncultured sequences, which is contrary to 16S rRNA gene sequence data which assigned up to 14 genera of methanogens in sediments. The discrepancy between the methanotroph 16S rRNA and *pmoA* gene sequences was particularly profound.

In sediments, methanotrophs under the order Methylococcales made up 46 out of 50 of the most abundant sequences and Rhizobiales made up the remaining 4 sequences, however sediment *pmoA* sequences were exclusively from the Rhizobiales genera *Methylocystis* sequences.

The likely explanation for the discrepancy is that the 16S rRNA gene has been far better studied than the functional genes and therefore has a more robust database (Santamaria *et al.*, 2012). The FunGene database derives sequences from NCBI, where the number of sequences of both *mcrA* and *pmoA* genes is comparatively lower. Snelling *et al.* (2014) had similar issues when using the FunGene database to analyse *mcrA* gene sequences, although they were identifiable as Methanobacteriales, 69% were unclassified below the order level. It is also plausible that the sequence disparity is due to the use of sMMO by the Methylococcales rather than the pMMO as several of the related sequences for Methylococcales including *Methylomicrobium* (Nakamura *et al.*, 2007), *Methylomonas* (Auman and Lidstrom, 2002) and *Methylococcus capsulatus* (Jiang *et al.*, 1993). However other sequences, such as those related to *Methylobacter* contain only pMMO, making it unlikely to be not actively transcribed during CH_4 oxidation. In addition sequences within closely related Rhizobiales 16S rRNA gene sequences also relate to sMMO containing methanotrophs e.g. *Methylocella* (Dedysh *et al.*, 2005) and *Methylocystis* (Belova *et al.*, 2011), with *Methylocella* containing only sMMO and a known facultative methanotroph. This would make it unlikely that conditions favouring sMMO would select overwhelmingly for Methylococcales and not Rhizobiales.

The discrepancy between 16S rRNA and *pmoA* gene in our soil samples is easier to explain however, as the most abundant 16S rRNA genes are derived from

Methylacidiphilales, a Verrucomicrobia methanotroph who's *pmoA* sequence is distinct from the majority of methanotrophs, and so is not covered by the A189f/A650r primer pair used in this study (Sharp *et al.*, 2014). To confirm this, further tests will be needed with *pmoA* gene primers specific for Methylacidiphilales taken into account.

In conclusion, relatively high MOP and MPP in relation to functional gene abundance in sediments indicates that river sediments are potentially more active than soils in CH_4 transformations. Methanococcoides, Methanosarcina and Methanocorpusculum are likely candidates driving methanogenesis in these sediments, with *Methylocystis* and other *Methylocystis* like methanotrophs oxidising the methane generated across the different geologies and seasons. Thus, the, river sediments in the Avon-Hampshire catchment are likely a more important source/sink for CH_4 compared to soils, however as our results for MOP and MPP are much lower than previous research, their overall importance at the larger environmental scale may be smaller.

Chapter 4: Effect of temperature on soil methanotroph communities across different geologies.

4.1: Introduction

By the end of the 21st century, anthropogenic climate change is set to increase global temperatures to around 2-4^{o}C (IPCC, 2013). Microorganisms that govern the cycling of macronutrients can be sensitive to temperature changes, yet previous research on exactly how temperature affects microbes is somewhat contradictory (Classen *et al.*, 2015). For example, Steinauer *et al.* (2015) found that temperature only increased soil microbial enzymatic function in soils that were from high plant diversity plots and that, overall, plant diversity was far more important in determining microbial function than temperature. However, in forest soils from a long term warming experiment (+5^{o}C above ambient, temperature range from 20^{o}C to -6^{o}C from summer to winter respectively), microorganisms were strongly affected by increases in temperature, whereby soil respiration increased with increasing temperature (Bradford *et al.,* 2008). Differences in seasonal temperature extremes may also be important (Bradford *et al.,* 2008).

Specifically, Methanotrophs can be sensitive to temperature changes, for example, certain taxa, such as *Methylosinus*, *Methylocystis*, *Methylomonas*, *Methylomonas* were more abundant under higher temperatures, whilst others such as *Methylomicrobium* were unaffected (Mohanty *et al.,* 2007). In another study, methanotroph communities shifted towards Type II methanotrophs at higher temperatures (Urmann *et al.,* 2009). Several studies looking into temperature effects on methanotrophs also found similar shifts between Type I and Type II methanotrophs, i.e. Type II tended to favour higher temperatures >15^{o}C (Mohanty *et*

al., 2007), whereas Type I methanotrophs favoured lower temperatures <10^{o}C (Wartiainen *et al*., 2003; Börjesson *et al*., 2004; Wagner *et al*., 2005; Lieber and Wagner, 2007; Graef *et al*., 2011; He *et al*., 2012) In rice paddies, forest soils and landfill sites, increased temperatures have resulted in increases in methane oxidation rates (Park *et al.,* 2005*;* Mohanty *et al.,* 2007). Reduction of water content within soils due to increased temperatures allows more aeration, where methane can diffuse from previously anoxic zones to aerobic zones, as well as allowing methanotrophs access to atmospheric methane and oxygen (Nazaries *et al.,* 2013).

Methanotrophs are also found near environmental temperature extremes. As CH_4 releases are expected to increase from arctic and Antarctic regions receding due to climate change, several studies have examined methanotrophs within various permafrost environments that would likely be affected by increasing temperatures to understand how these methanotrophs respond to climate change (He *et al.*, 2012). He *et al.* (2012) examined methanotrophs in arctic lake sediments which were reported to be able to function at psychrophilic temperatures (4^{o}C) although oxidation potential also increased as temperature increased, suggesting that methanotrophs were psychrotolerant rather than psychrophilic, with both Type I and Type II methanotrophs being detected. Martineau *et al.* (2012) found that the most abundant methanotrophs within a Canadian arctic soil were from Type I *Methylobacter* and *Methylosarcina* methanotrophs, however another Canadian arctic soil study by Pacheco-Oliver *et al. (*2002) found Type II methanotrophs, *Methylocystis* and *Methylosinus* were dominant.

Environments at the higher end of the temperature extreme have also been studied, including mid latitude deserts (Angel *et al.*, 2009), mud volcanoes (Sharp *et al.*, 2014) tropical soils (Das *et al.*, 2012) and hydrothermal vents (Merkel *et al.*,

2013). Angel *et al.*, found methanotrophs within soils from the Negev desert, however MOP depth profiles indicated oxidation only occurred below the surface crust, where methanotrophs avoided higher temperatures which reached 48^{o}C in summer. Sharp *et al. (*2014) recovered sequences of the Verrucomicrobia methanotroph *Methylacidiphilum* genera from geothermal environments, with samples only detected at temperature ranges between 44.1 to 81.6^{o}C. Merkel et la., (2013) recovered ANME-1 GBa sequences from hydrothermal vents covering temperatures of 50 to 70^{o}C. Das *et al. (*2012) found temperatures of 45^{o}C significantly decreased the rate of CH_4 oxidation within tropical rice paddy soil, however this was only the case when combined with a flooded treatment.

Currently, there are still some uncertainties on how temperature changes affect methanotroph communities. This lack of knowledge has implications for future changes in climate, as understanding accurately how methanotrophs respond to temperature can lead to potential CH_4 mitigation strategies.

4.1.1: Aim

The overall aim of this chapter was to determine the effect of temperature on soil methanotroph community structure, activity and *pmoA* gene abundance across different geologies.

4.1.2: Hypothesis

In this chapter it was hypothesized that:

1) Previous research showed that Type II methanotrophs became more predominant over Type I methanotrophs when temperatures increased. It is predicted that in our

soils, Type I methanotrophs will be predominant in winter samples, whereas Type II will be dominant in summer samples.

2) Increases in temperatures will cause increases in methane oxidation potential rates, with *pmoA* gene abundance in soils being higher in the summer than in the winter.

3) In the long-term field warming experiments (where temperatures are increased to 4^{o}C above ambient), there will be little change in the methanotroph community, *pmoA* gene abundance and methane oxidation potentials in the warmed soils when compared to the ambient untreated soils.

4) In permeable chalk and sands, where methane can diffuse more readily than in the impermeable clays, methane oxidation potential rates and *pmoA* gene abundance will increase with increasing temperatures.

The specific objectives of this chapter were to:

1) Establish soil temperature microcosms using soils collected in summer and winter and measure MOP, *pmoA* and 16S rRNA gene abundance and diversity.

2) Collect soils from a long-term field warming experiment where heated soils were 4^{o}C higher than ambient and measure MOP, *pmoA* and 16S rRNA gene abundance and diversity across different geologies.

4.2: Methods

4.2.1: Soil temperature microcosm experiment

Triplicate soil cores (10 g) were collected from the greensand site (Puckshipton House) in August 2014 (denoted summer) and March 2015 (denoted winter) and transported to the laboratory as described previously (see Chapter 2, Section 2.1.2). Triplicate sub-samples (3 g) were removed from each core and placed into sterile 125 ml serum bottles containing 500 ppm CH_4 in the headspace. Samples were then incubated statically at 4^{o}C, 10^{o}C, 20^{o}C and 30^{o}C. An additional set of triplicate untreated control (UC) soils were processed immediately as background controls for physicochemical analysis, MOP and molecular analyses. MOP was then measured every 3 days for 9 days as described previously (see Chapter 3, Section 3.3). Triplicate soil samples were removed at day 0 and day 9 and stored at -20ºC for molecular analyses.DNA extraction, Q-PCR amplification, and Illumina MiSeq sequencing were performed as described previously (See Chapter 2, Sections 2.1.7-2.1.8; Chapter 3, Sections 3.1.1-3.1.2). MOP measurements were performed every three days as described previously (See Chapter 3, Section 3.1.3).

4.2.2: Long-term field warming experiment

A long-term field warming experiment was set up (courtesy of Professor Ineson, Dr Stockdale, University of York, as part of the NERC Macronutrient Cycle Project). The experiment was set up at three sites: clay (AS2), greensand (GA2), and chalk (CW2). A diagram of the experimental set up is shown in **Figure 4.1**. Ceramic cores 50 cm deep and 200 cm in diameter were inserted into the plots. Triplicate control cores (i.e. at ambient temperature) were also set up. Warmed soil

cores were heated to a constant temperature of 4ºC above ambient using a heated metal grid. The experiment was run from March to August 2015. Triplicate soil samples (~5 g) were taken within 10 cm around the ceramic cores and snap frozen at -20ºC as described previously (Chapter 2, Sections 2.2.2 and 2.2.6). MOP measurements were performed as described previously (Chapter 3, Section 3.1.3). Temperature incubations for MOP were all at 20°C.

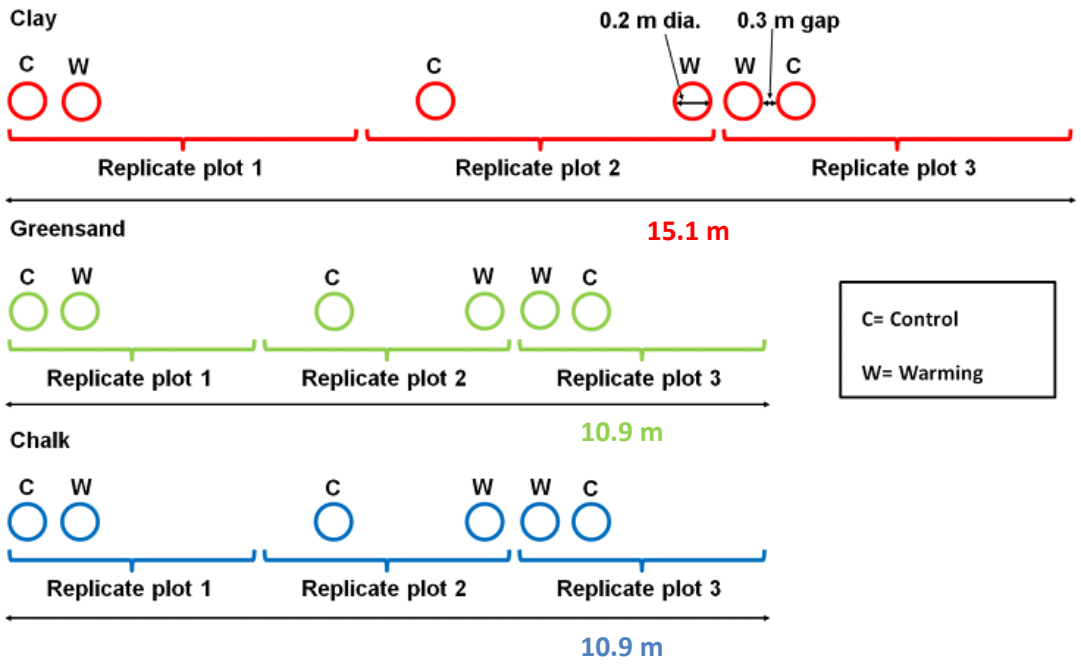


Figure 4.1: Diagram of long-term field warming experiment. Circles represent the ceramic cores inserted along each transect. C denotes control cores not warmed; W denotes soils warmed to 4ºC above ambient from March 2015 to August 2015. [Note: The clay transect is longer due to an additional flooding treatment that was not investigated in this thesis].

Each site was also equipped with an automated gantry system (SkyLine2D; Biology Electronic Services) courtesy of the University of York to take CO_2, CH_4, and N_2O gas samples every 180 s along each transect. The system comprised an

infrared gas analysis system (IRGA; model 8100, Li-Cor, Lincoln, NE, USA) and a cavity enhanced absorption spectrometer (Fast Greenhouse Gas Analyser (FGGA); model 907-0010, Los Gatos Research, Inc., Mountain View, CA, USA) (**Figure 4.2**).

Figure 4.2: Photograph showing the Skyline2D gantry system at the Greensand (GA2) site with attached analysers (image courtesy of Dr James Stockdale).

4.2.3: Physicochemical analyses

Physicochemical analyses were performed on all soils (from both temperature microcosm and long-term field warming experiments) as described previously (Chapter 2, Section 2.2.4). Note: For the anion and cation analyses, sample replicates from both soil microcosm and long-term field experiments were lost. Also due to technical issues with the DIONEX, winter soil samples were lost, and only

soils sampled from the summer months from the soil microcosm experiment were analysed.

4.2.4: Illumina MiSeq sequencing

Amplicon sequencing libraries of 16S rRNA and *pmoA* genes for both temperature microcosm and long-term field warming experiments were prepared by Dr Chronopoulou (University of Essex) as described in Chapter 2, Section 2.2.7. Unfortunately samples were mislabelled and not available as of writing

4.2.5: Statistical analysis

XLSTAT statistics package (version 14.5, Addinsoft) was used for ANOVA and correlation tests. A multivariate ANOVA was used to test differences in MOP rates and *pmoA* gene abundance between geologies and months. Results that were significantly different were further analysed with a Tukey HSD test. Pearson's correlation tests were also used to test for correlations.

4.3: Results

4.3.1: Soil temperature microcosm experiment: Physicochemical Data

Physicochemical data for the temperature microcosm experiment is presented in Tables 4.1 and 4.2. In general, Total Organic Carbon (TOC) did not vary significantly between temperatures (*P*= 0.058), although TOC in summer samples (<240.4 mg C g^{-1} dry weight soil) were slightly lower than winter samples (<263.97 mg C g^{-1} dry weight soil) (**Table 4.1**). Percentage water content remained largely the same within the same months (ANOVA, *P*= 0.51), but differed between months (ANOVA, *P*= 0.033).

In general, the anion concentrations were similar across treatments (**Table 4.2**). Formate, nitrate and acetate were all zero or below detectable limits across treatments and controls. Nitrite concentrations increased to from 1.42 µmol g^{-1} dry weight soil at 4°C to 1.75 µmol g^{-1} dry weight soil at 30°C. Sulfate concentrations remained between 0.27 to 0.41 µmol g^{-1} dry weight soil across temperature treatments. Phosphate concentrations were low were between 0.08 to 0.17 µmol g^{-1} dry weight soil across temperature treatments (**Table 4.2**). For the soils incubated at temperatures from 4°C to 20°C, the ammonium concentrations were between 0.37-0.41 µmol g^{-1} dry weight soil. However, when soils were incubated at 30°C, the ammonium concentrations decreased slightly to 0.26 µmol g^{-1} dry weight soil (**Table 4.3**). Magnesium concentrations were generally low across temperature treatments, but increased with increasing temperature from 0.16 to 0.23µmol g^{-1} dry weight soil. Potassium concentrations were generally similar across temperature treatments around 0.5 µmol g^{-1} dry weight soil, Sodium was the most abundant cation across the temperature treatments between 4.03-5.60 µmol g^{-1} dry weight soil (**Table 4.3**).

Table 4.1: Physicochemical data of soils used in temperature microcosm experiments (*n*=3).

Sample (Season)	Temperature (°C)(±SE)	Mean Total Organic Carbon (mg C g^{-1} soil) (±SE)	Mean Percentage Water Content (%) (±SE)
March (Winter)	4 (±0.1)	238.5 (±10.1)	39.9 (± 3.0)
	10 (±0.1)	264.0 (± 27.9)	34.3 (± 3.2)
	20 (±0.1)	262.8 (± 55.3)	36.2 (± 2.3)
	30 (±0.1)	240.3 (± 0.7)	34.4 (± 4.0)
August (Summer)	4 (±0.1)	240.4 (± 2.5)	50.6 (± 1.4)
	10 (±0.1)	239.5 (±1.1)	50.5 (± 2.4)
	20 (±0.1)	234.8 (±1.1)	53.1 (± 3.4)
	30 (±0.1)	239.9 (± 5.5)	51.2 (± 1.5)

Table 4.2: Anion concentrations of summer soils used in temperature microcosm experiments (*n* =1).

	Anion concentration(µmol g^{-1} dry weight soil)					
Temperature (°C)	**Acetate ($C_2H_3OO^-$)**	**Formate ($HCOO^-$)**	**Nitrite (NO_2^-)**	**Nitrate (NO_3^-)**	**Phosphate (PO_4^{3-})**	**Sulfate (SO_4^{2-})**
Untreated control	0.0	0.0	0.0	1.6	0.2	0.4
4	0.0	0.0	0.0	1.4	0.1	0.3
10	0.0	0.0	0.0	1.7	0.2	0.4
20	0.0	0.0	0.0	1.5	0.1	0.3
30	0.0	0.0	0.0	0.0	0.1	0.3

Table 4.3: Cation concentrations of summer soils used in temperature microcosm experiments (*n* =1)

Temperature (°C)	Cation concentration (µmol g^{-1} dry weight soil)				
	Ammonium (NH_4^+)	**Calcium (Ca^{2+})**	**Magnesium (Mg^{2+})**	**Potassium (K^+)**	**Sodium (Na^+)**
Untreated control	0.5	2.4	0.4	1.0	4.9
4	0.4	2.3	0.2	0.6	4.0
10	0.4	2.3	0.2	0.7	5.6
20	0.4	2.3	0.2	0.6	4.2
30	0.3	2.3	0.2	0.5	4.0

4.3.2: Methane oxidation potential rates of soils from the temperature microcosms.

The Methane oxidation potential rates (MOP) of soils sampled in the summer and winter and incubated under four different temperatures are shown in **Figure 4.3**.

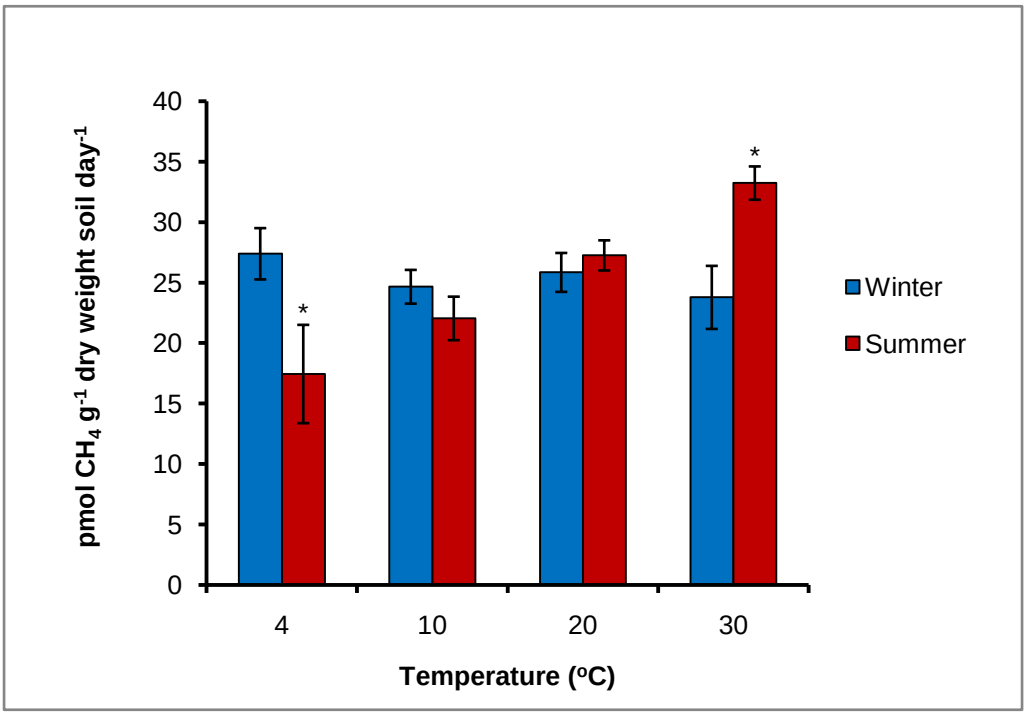


Figure 4.3: MOP of soils from temperature microcosm experiment. Error bars represent standard error of mean (*n* = 3).* = sample is significantly different (P≤ 0.05).

For soils sampled in the summer, an increase in temperature resulted in an increase in MOP from 17.45 pmol CH_4 g^{-1} dry weights oil day^{-1} at 4°C to 33.24 pmol CH_4 g^{-1} dry weight soil day^{-1} at 30°C (Pearson's correlation = 0.984, *P*= <0.0001) (**Figure 4.3**). In contrast, soils sampled in the winter soils were not significantly affected by

temperature and MOP remained between 23.78-27.39 pmol CH_4 g^{-1} dry weight soil day^{-1} across temperature treatments but with the highest MOP at 4°C (**Figure 4.3**).

4.3.3: 16S rRNA gene abundances of soils from the temperature microcosms.

The 16S rRNA gene abundance from the temperature microcosms is presented in **Figure 4.4**. In general, 16S rRNA gene abundances remained largely similar between summer and winter samples and temperature treatments (i.e. between 3.67 x 10^8 to 2.05 x 10^9 gene copy g^{-1} dry weight soil).

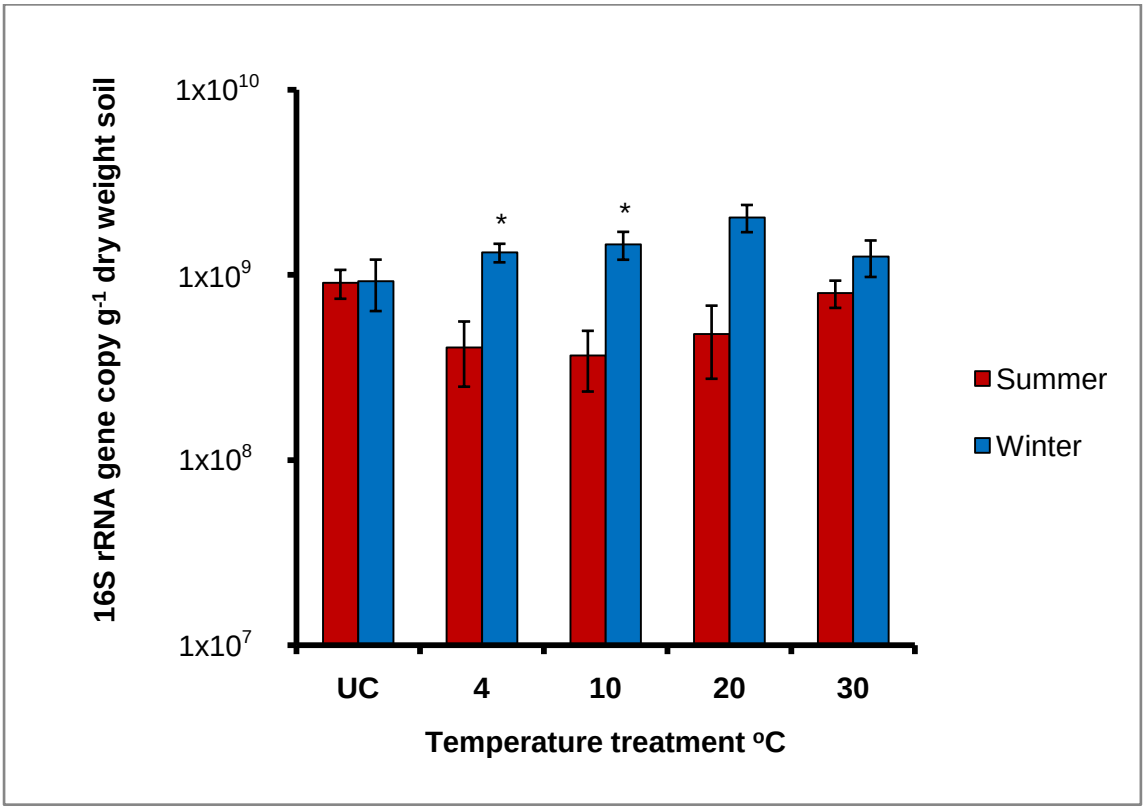


Figure 4.4: 16S rRNA gene abundances from temperature microcosm experiment. Error bars represent standard error of mean (*n* = 3) from before the start of the experiment (UC= Untreated Control) and end of experiment (4 to 30°C). * = sample is significantly different (P≤ 0.05).

There was a significant difference in 16S rRNA gene copy number between summer and winter samples (ANOVA, *P*= 0.001) whereby the soils sampled in the winter incubated at 20^{o}C had the highest gene abundance (2.05 x 10^{9} gene copy g^{-1} dry weight, Tukey HSD test, *P*= 0.001 to 0.046) compared to summer at 20^{o}C (3.67 x 10^{8} to 9.25 x 10^{8} gene copy gene copy g^{-1} dry weight).

4.3.4: *pmoA* gene abundances in soils from the temperature microcosms.

The *pmoA* gene abundance from the temperature microcosms is presented in **Figure 4.5**. In general, *pmoA* gene abundances showed no significant changes between incubation temperature (i.e. between 1.09 x 10^{3} - 5.25 x 10^{3} copies g^{-1} dry weight soil) (ANOVA, *P*= 0.232), with the exception of the Winter untreated control which had 3.27 x 10^{7} copies g^{-1} dry weight soil. As this sample was higher than any previously measured *pmoA* gene abundance, it's likely the sample was contaminated by the standard and was not included in the ANOVA.

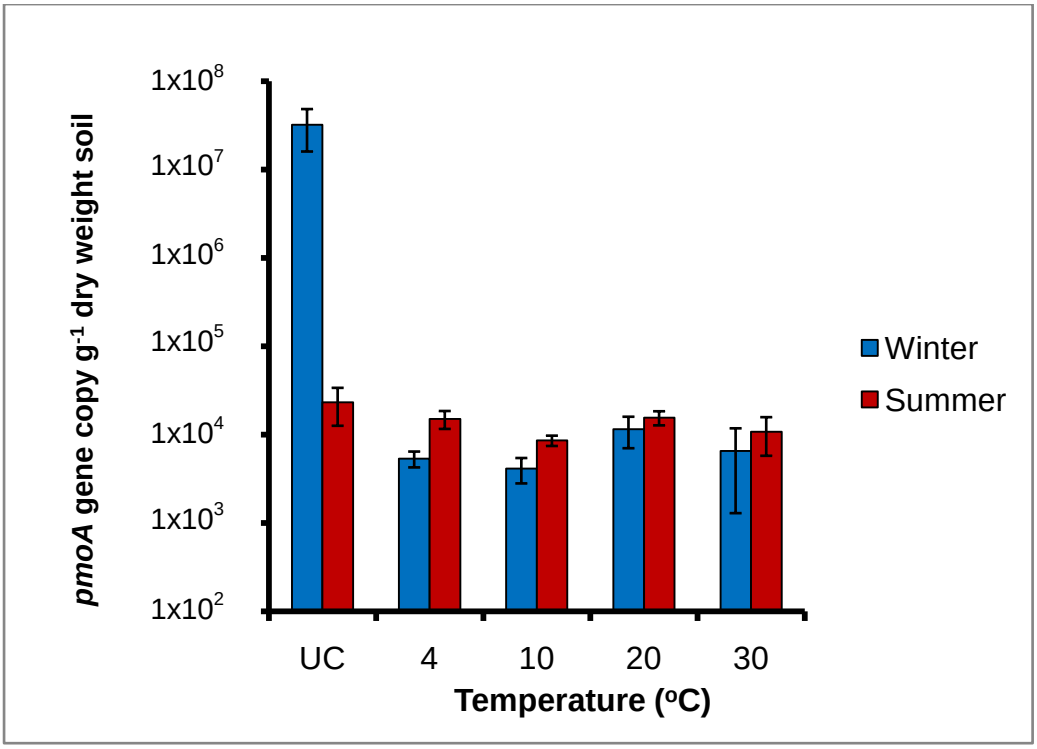


Figure 4.5: Abundance of the *pmoA* gene in summer warmed and winter warmed experiment from start of experiment (UC= Untreated Control) and end of experiment (4 to 30^{o}C). Error bars represent standard error of mean (*n* = 3) No samples were significantly different (UC excluded from ANOVA)

4.3.5: Long-term field warming experiment: physicochemical data

Physicochemical data for the long-term field warming experiments are presented in **Tables 4.4-4.6**. In general, Total Organic Carbon (TOC) did not vary significantly between the controls and the warmed plots, or between the geologies (*P*= 0.699) (**Table 4.4**). Water content also did differ significantly between geologies and treatments (P= 0.002) and was highest in chalk (66-71%) and lowest in greensand soils (37-48%). Across geologies the controls had higher percentage water content than warmed treatments (**Table 4.4**).

Nitrate and acetate concentrations were all zero or below detectable limits across treatments and controls (**Tables 4.5**). For both clay and greensand soils nitrite and phosphate concentrations were higher in the controls compared to the warmed samples whereas in the chalk samples nitrite and phosphate concentrations were approximately similar between control and warmed samples (**Tables 4.5**). Sulfate concentrations were lower in warmed samples compared to controls (greensand), but similar concentrations for the clay warmed and the respective controls, whilst in the chalk soils were higher in the warmed compared to controls (**Tables 4.5**). For both clay and greensand soils, formate concentrations were higher in the controls compared to the warmed samples, whilst in the chalk soils, formate concentrations were similar between the control and warmed samples (**Tables 4.5**).

In the clay soils, ammonium and calcium concentrations were all zero or below detectable limits across treatments and controls (**Tables 4.6**). In general, between controlled and warmed treatments, control treatments tended to have lower cation concentrations with the exception of ammonium (chalk), magnesium (greensand), potassium (clay) and sodium (greensand) (**Table 4.6**).

Table 4.4: Physicochemical data of soils from long-term field warming experiment (*n=3*)

Soil sample	Treatment	Mean Total Organic Carbon (mg C g^{-1} soil) (±SE)	Mean Percentage Water Content (%)
Clay	Control	244.3 (±0.6)	58.2 (± 4)
	Warmed	242.1 (±1.2)	49.6 (± 1.4)
Greensand	Control	242.2 (±1.3)	47.7 (± 1.4)
	Warmed	243.5 (±1.3)	36.7 (± 3.9)
Chalk	Control	241.2 (±3.0)	71.0 (± 2.8)
	Warmed	244.0 (±1.0)	66.7 (± 3.2)

Table 4.5: Anion concentrations of soils from long-term field warmed experiment. (*n=1*)

Soil Sample	Anion concentration (µmol g^{-1} dry weight soil)					
	Acetate ($C_2H_3O_2^-$)	**Formate ($HCOO^-$)**	**Nitrite (NO_2^-)**	**Nitrate (NO_3^-)**	**Phosphate (PO_4^{3-})**	**Sulfate (SO_4^{2-})**
Clay Control	0.9	5.4	1.6	0.7	3.1	0.9
Clay Warmed	0.4	4.4	1.5	0.5	2.3	0.4
Greensand Control	0.7	5.0	1.7	0.7	3.6	0.7
Greensand Warmed	0.8	9.0	0.8	3.1	2.3	0.8
Chalk Control	0.5	10.3	0.8	3.5	2.0	0.5
Chalk Warmed	0.5	10.1	0.7	2.4	3.0	0.5

Table 4.6: Cation concentrations of soils from long-term field warmed experiment. (*n=1*)

	Cation concentration (µmol g^{-1} dry weight soil)				
Soil Sample	**Ammonium (NH_4^+)**	**Calcium (Ca^{2+})**	**Magnesium (Mg^{2+})**	**Potassium (K^+)**	**Sodium (Na^+)**
Clay Control	0.0	0.0	0.7	3.1	1.5
Clay Warmed	0.0	0.0	0.7	3.1	1.6
Greensand Control	1.0	0.8	3.5	2.0	1.0
Greensand Warmed	1.8	0.9	2.7	2.2	1.0
Chalk Control	0.7	0.5	1.3	2.4	1.3
Chalk Warmed	0.7	0.5	1.5	3.8	2.3

4.3.6: Methane oxidation potentials of soils from field warming experiment.

The Methane oxidation potential rates (MOP) of soils from the long-term field warming experiment are shown in **Figure 4.6**.

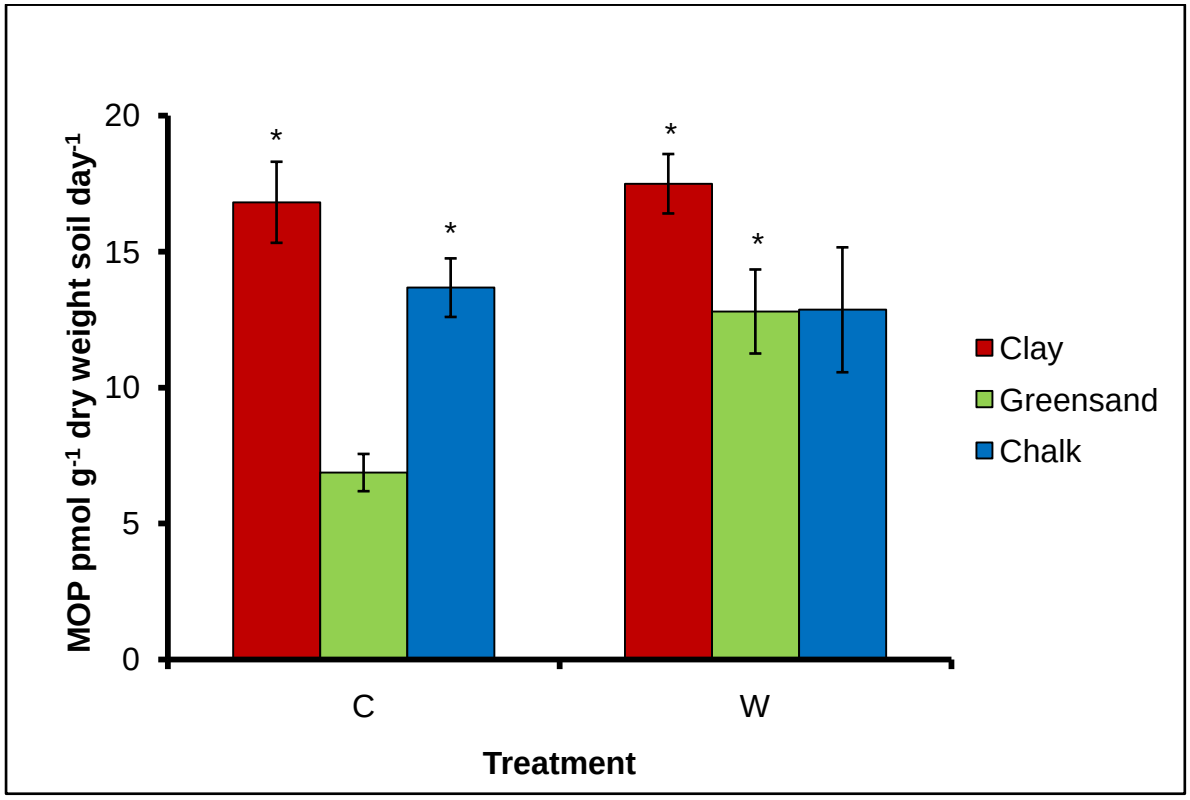


Figure 4.6: MOP of soils of soils from the long-term field warming experiment. C= Control, W= Warmed. Error bars represent standard error of mean (*n* = 3). * = sample is significantly different (*P*≤ 0.05).

Clay soils (control and warmed) had greater MOP (between 16.81 to 17.50 pmol g^{-1} dry weight day^{-1}). There was no significant difference in MOP between clay control and warmed plots (ANOVA, *P*= 0.851). MOP in Greensand soils were significantly greater in the warmed (12.80 pmol g^{-1} dry weight day^{-1}) compared to the control (6.88 pmol g^{-1} dry weight day^{-1}) (*P*= 0.007).

4.3.7. 16S rRNA gene abundances of soils from long-term field warming experiment.

The 16S rRNA gene abundances of soils from the long-term field warming experiment are shown in **Figure 4.7**.

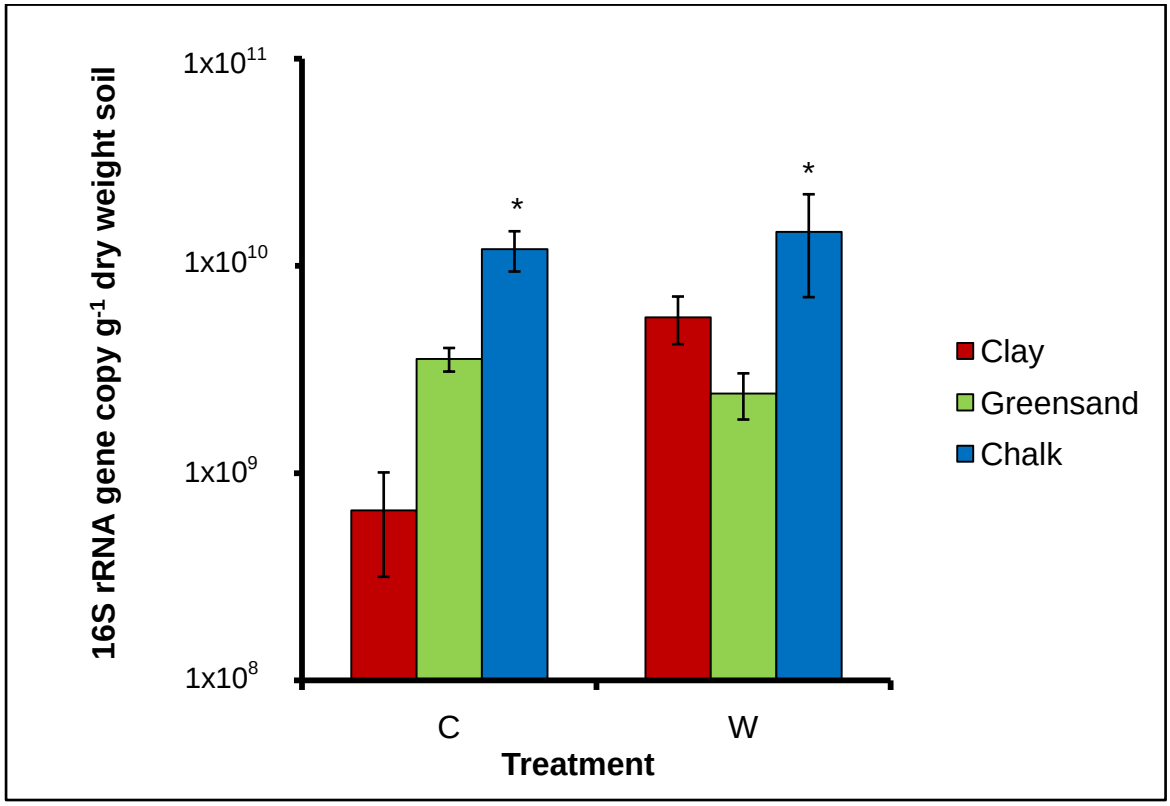


Figure 4.7: Abundance of 16S rRNA gene in field warmed experiment. C= Control, W= Warmed. Error bars represent standard error of mean (*n* = 3). * = sample is significantly different (*P*≤ 0.05).

In general, there was no significant difference in 16S rRNA gene abundance between control and warmed soils for the respective geologies (*P*= 0.066). In the control plots, 16S rRNA gene abundance were highest in chalk (6.63 x 10^8 gene copy g^{-1} dry weight soil) followed by Greensand (3.56 x 10^9 gene copy g^{-1} dry weight soil), and in clay soils (1.21 x 10^{10} 16S rRNA gene copy g^{-1} dry weight). In warmed

plots, 16S rRNA gene abundance chalk soils remained highest in the chalk, whereas 16S rRNA gene in clay rose to similar abundance with Greensand (**Figure 4.7**).

4.3.8: *pmoA* gene abundances of soils from long-term field warming experiment.

The *pmoA* gene abundances of soils from the long-term field warming experiment are shown in **Figure 4.7**.

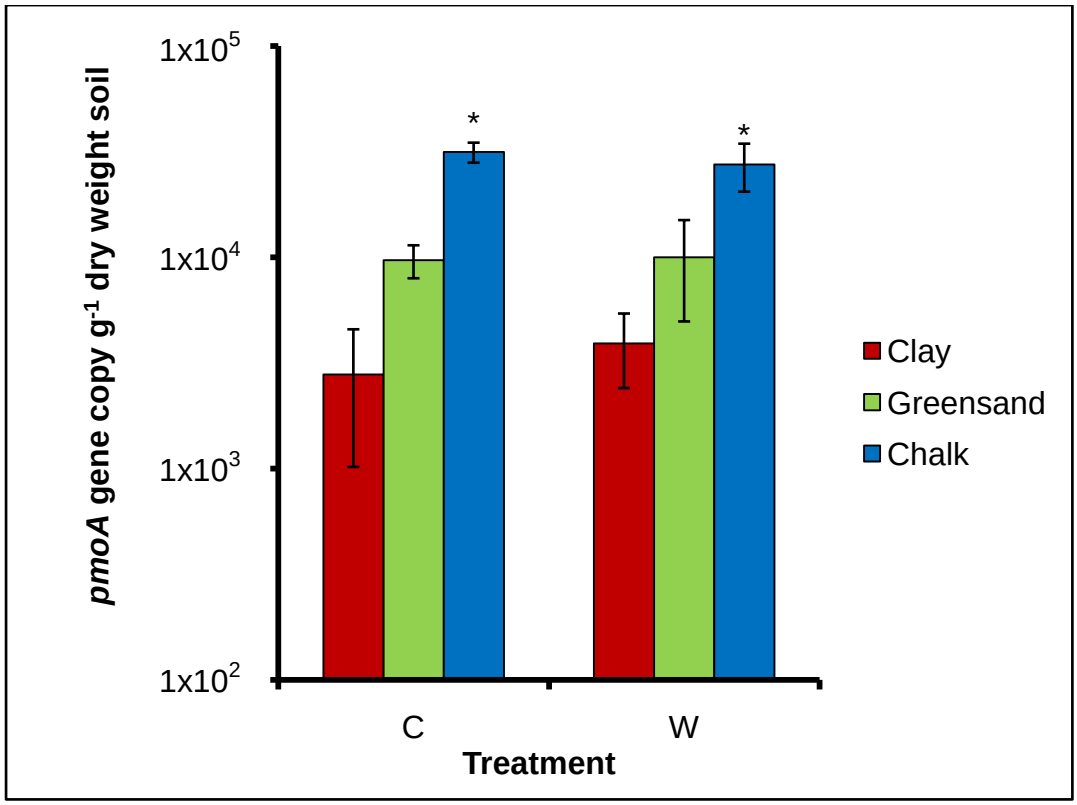


Figure 4.8: Abundance of *pmoA* gene in field warmed experiment.C= Control, W= Warmed. Error bars represent standard error of mean (*n* = 3). * = sample is significantly different (*P*≤ 0.05).

In general, there was no significant difference in *pmoA* gene abundance between control and warmed soils for the respective geologies (*P*= 0.787). In the control plots, *pmoA* gene abundance were highest in chalk (3.14 x 10^4 gene copy g^{-1}

dry weight soil) followed by Greensand (9.67 x 10^3 *pmoA* gene copy g^{-1} dry weight soil), and clay soils (2.79 x 10^3 *pmoA* gene copy number g^{-1} dry weight). The *pmoA* gene abundance from the warmed plots was 2.74 x 10^4 *pmoA* gene copy g^{-1} dry weight soil in the chalk decreasing to 9.98 x 10^3 copy g^{-1} dry weight soil in the greensand and 3.91 x 10^3 copy g^{-1} dry weight soil in the clay.

4.3.9: PCR-DGGE analysis of 16S rRNA gene in soils from temperature microcosms and long-term field warming experiment.

PCR DGGE of 16S rRNA gene in soils from temperature microcosms and long-term field warming experiments was performed and the data are shown in **Appendix 3.1**. Overall, gels showed that DGGE bands between lanes of different temperatures treatments were identical in intensity and abundance, indicating no significant shifts in the bacterial community.

4.3.10: Illumina MiSeq 16S rRNA and *pmoA* gene sequence analysis of soils from temperature microcosms and long-term field warming experiment.

Unfortunately due to time constraints and technical difficulties in running the sequencing pipeline, the 16S rRNA and *pmoA* gene MiSeq sequence data could not be analysed.

4.4: Discussion

In the temperature microcosm experiments, there was a strong positive correlation between an increase in temperature and increase in MOP from soils sampled in summer. This suggests methanotrophs in summer samples were more active at elevated temperatures and follows known enzyme kinetics, where increases in temperature increases enzyme activity (Peterson *et al.*, 2007). However, in the winter samples MOP was relatively constant, across temperatures suggesting that methanotroph activity in winter were not affected by temperature difference. This is contrary to previous research that suggests microbial communities during the summer are more resilient to temperature changes, as temperature differences tended to be greater during a summer day's cycle (Bradford *et al.* 2008), so other factors are potentially affecting the overall MOP in winter. Our findings also run contrary to a study by Steimkamp *et al.* (2001), who found temperature was only a strong influencing factor on methanotrophy for soils with *in-situ* temperatures of $<10^{o}C$; our greensand soils from summer, with *in-situ* temperature $>10^{o}C$ continued to increase in MOP in response to increasing temperature, whereas our winter soils with *in-situ* temperature $<10^{o}C$ were unaffected by temperature changes.

The most likely explanation for our results is that methanotrophs within winter samples were adapted to higher CH_4 concentrations than the amount presented in the headspace of our bottles, therefore the rate of MOP would only change when CH_4 increases. This can be seen in the previous chapter where March soils demonstrated higher MOP and MPP when compared to those from August, and overall MOP of soils from each site followed the increases of MPP (**Figure 3.2** and **3.3**). In two studies on lake sediments, Duc *et al.* (2010) and Lofton *et al.* (2014)

noted that temperature only had significant effects on MOP when CH_4 was not limited as a substrate. A study by Shelley *et al.* (2015) found similar results on river sediments where oxidation potentials were only affected by temperature in fine sediments (typically more anoxic and therefore more CH_4), compared to more aerobic gravel sediments (typically more aerobic and therefore less CH_4).

This temperature effect for when substrate is not limiting, may also explain the results of our long-term field experiment, where only greensand increased in MOP in the warmed treatments compared to the controls. Furthermore this can be expanded to the lack of change in both the clay and chalk field experiments and field sampling experiments. From the previous chapter, both clay and chalk soils demonstrated higher methanotrophy and methanogenesis (Figure 3.2), and since these two sites normally produce more CH_4, it is reasonable to infer that the lack of increase from temperature stimulation, is due to the provided CH_4 within the headspace being too low in quantity. It can be theorised that by only increasing CH_4 oxidation when CH_4 concentrations are high, methanotrophs can minimize depleting available CH_4 resources, as well as avoiding expending energy to process CH_4 when it is not advantageous.

Within bottle MOP experiments, *pmoA* gene abundance was not significantly different between winter and summer samples. This was similar to what was found study by Mohanty *et al.* (2007), where the overall *pmoA* gene abundance was similar, however different *pmoA* sequences were identified (by T-RFLP) in response to different temperatures. Therefore it is possible that in our study, the lack of response to temperature in *pmoA* genes in the bottle experiments could be due to

similar shifts in methanotroph community i.e. the abundance of psychrophilic methanotrophs at low temperatures (<10^{o}C), are outcompeted by more mesophilic methanotrophs at higher temperatures, and so net *pmoA* gene abundance remains the same. Although it must be noted that functional gene abundances do not necessarily correlate with the function they are responsible for, as gene abundance does not mean that it is being actively transcribed (Rocca *et al.,* 2014).

The abundance of *pmoA* genes within the field warmed plots also showed no correlation with MOP, however nitrite did have positive correlation with *pmoA* (Pearso*n*= 0.492, *P*= 0.038) but a negative relationship with MOP (Pearson = -0.579, *P*= 0.012). As methanotrophs can perform both nitrification (ammonium, to nitrite, to nitrate) and denitrification (nitrate to nitrogen gas) (Stein and Klotz, 2011), this correlation suggests it is possible that the process of nitrification by methanotrophs is inhibiting MOP and is removed once temperature increased in greensand soils. However, the more likely explanation remains that the lower CH_4 concentration being responsible for the lack of response to temperature during winter. As of writing, data from the *in-situ* gas emissions as well as Illumina MiSeq data (specific for samples from this experiment) were not made available for this thesis. Analysis of this data will provide further insight into the differences of the methanotroph community between the temperature treatments.

In conclusion, our short term temperature incubations showed that greensand soils were significantly affected by temperature increases albeit only in the summer samples. Abundance of *pmoA* and 16S rRNA genes were not affected by the temperature treatments suggesting methanotrophs are acclimatized to temperature

variations. Within the long-term temperature experiment showed that methanotrophy and methanotrophs are largely unaffected by the increases in temperature in chalk and clay soils. However, in the greensand soils, temperature had a strong effect on methanotrophy, both in field warmed and temperature gradient experiments, but like the chalk and clay geologies, neither *pmoA* or 16S rRNA gene were associated with temperature shifts in soils. This suggests that future global temperature increases are overall minor factor on methanotrophs in our study sites.

Chapter 5: Effects of nitrogen and phosphorous on soil methanotroph communities and methane oxidation potential rates.

5.1: Introduction

Nitrogen (N) and phosphorous (P) are key macronutrients and (via fertilizer applications) can leach into ground waters and/or surrounding rivers causing eutrophication and subsequently decrease the biodiversity in these ecosystems (Smith *et al.,* 1999). Although the effects of fertilizer additions on methanotrophs have been well studied in agricultural land, in grassland soils it has been less well characterized. Both N and P have been found to have varying effects on CH_4 oxidation; from inhibition, to no effect, to stimulation (Schnell *et al.*, 1994; Nold *et al.*, 1999; Bodelier *et al.*, 2004; Datta *et al.*, 2013; Zheng *et al.*, 2013).

In soils, ammonium, nitrate and nitrite have been shown to potentially cause a negative effect on methanotrophs (Reay *et al.*, 2004; Bodelier and Laanbroek, 2004; Siljanen *et al.,* 2012; Nazaries *et al.*, 2013). Ammonium in particular is an important factor for methanotrophs and methane oxidation, as the pMMO enzyme is homologous to the Ammonium Monooxygenase enzyme (AMO). Due to this homology of AMO and pMMO, ammonium can compete for the binding sites within pMMO and potentially inhibit methanotrophy (Yang *et al.*, 2011). At low concentrations however, ammonium can stimulate methanotrophy as it is an important source of N for methanotrophs (Bodelier *et al.*, 2000; Yang *et al.*, 2011). Ammonium can also be indirectly toxic, as by-products of nitrification, such as nitrate/nitrite, can build up and cause direct toxicity to methanotrophs (Stein and Klotz, 2011). The severity of inhibition to methanotrophy caused by ammonium also

depend on the species of methanotrophs, as some are able to effectively oxidize higher ammonium concentrations, as well as withstand toxic effects of nitrification by-products (Nyerges and Stein, 2009). Type I methanotrophs are noted to be generally unaffected or even stimulated by increasing ammonium concentrations, whereas Type II methanotrophs are usually inhibited by high ammonium concentrations (Nyerges and Stein, 2009).

Similar to ammonium, depending on the concentration, nitrate and nitrite can also be toxic to methanotrophs (Tugtas *et al.*, 2007; Dunfield and Knowles 1995). As nitrite and nitrate are by-products of nitrification, which as previously mentioned can occur in methanotrophs, some methanotrophs are able to act as denitrifiers and reduce nitrate into N_2 gas (Hoefman *et al.*, 2014). The various effects of nitrate/nitrite have been documented and found to be dependent on the species, possibly down to the strain, of methanotroph (Hoefman *et al.*, 2014); Methane oxidation within *Methylosinus trichosporium* was documented to be inhibited by nitrate addition (King *et al.*, 1994), whereas methanotrophy in *Methylomicrobium* and *Methylocaldum* was stimulated by ammonium addition (Noll *et al.*, 2008), and *Methylomonas* saw no adverse effect to CH_4 oxidation (Hoefman *et al.*, 2013). Recent research has also discovered the existence of aerobic methane oxidation coupled to denitrification (Ettwig *et al.*, 2010). This pathway is utilized by the methanotroph *Methylomirabilis oxyfera*, where aerobic CH_4 oxidation occurs in anoxic conditions, by firstly reducing nitrate to nitrous oxide, followed by dismutation of oxygen from nitrous oxide, providing O_2 to the standard methanotrophic pathway (Ettwig *et al.*, 2010). In environments occupied by *M. oxyfera*, nitrate is a necessary substrate and so stimulates methane oxidation (Ettwig *et al.*, 2010).

Phosphate effects in relation to methanotrophy are less well studied, however there is a similar level of variability on how P affects methanotrophs (Veraart *et al.*, 2015). A study on rice paddies by Alam *et al.* (2014) showed that 0.1 M phosphate additions inhibited CH_4 oxidation in soil microcosms. Whereas Gray *et al.* (2014) found that lower concentrations of phosphate (0.2 & 0.4 µM) stimulated CH_4 oxidation in permafrost soils. However, several studies using Na_2HPO_4, NaH_2PO_4, and $Ca(H_2PO_4)$ found no effect on CH_4 oxidation and methanotroph communities (Keller *et al.,* 2005, Keller *et al.*, 2006, Hütsch *et al.*, 1996). The reasons for this varying effect are not well understood, however it is suggested that such variation will likely be determined by *in-situ* adaptations, as examination of genes associated with P cycling showed no obvious phylogenetic basis (Veraart *et al.*, 2015). Several select genes were found to be exclusive to the Alphaproteobacteria/ Gammaproteobacteria methanotrophs however; Alkaline phosphatases was absent in Alphaproteobacteria methanotrophs and *Methylomiribilis oxyfera*, but present in all Gammaproteobacteria methanotrophs, whereas acid phosphatases and polyphosphate kynase were present in both Alpha and Gammaproteobacteria methanotrophs, but not *M. oxyfera* (Veraart *et al.*, 2015).

Previous work on CH_4 oxidation and methanotroph ecology has largely investigated the effects of N and P in isolation, with N compounds being extensively investigated, yet N and P co-exist in the environment, and is often added in tandem as fertilizer in agricultural practices. Keller *et al. (*2005) added combined N and P treatments in Peat soil, however no effect on CH_4 oxidation was observed, whereas a study by Zheng *et al. (*2013) found N and P, in addition to K, inhibited CH_4 oxidation. Currently little is known about how the combined N and P (at varying concentrations both individually and combined) can effect CH_4 oxidation and

methanotroph communities in grassland soils. This could be important for future agricultural practices since grassland is often used for livestock grazing and may be subjected to N and P fertilizer additions to improve the quality of grass for livestock (Defra, 2010).

5.1.1: Aim:

To determine the effect of N and P concentration (added either individually or together) on soil methanotroph community structure and activity.

The specific objectives of this chapter were to:

1) Measure the effect of different concentrations of N and P on MOP in soils
2) Measure the effect of different concentrations of N and P on *pmoA* gene abundance and methanotroph communities.

5.1.2: Hypothesis:

It is hypothesised that:

1) At higher N concentrations, both MOP and *pmoA* gene abundance will decrease.

2) At higher P concentrations, both MOP and *pmoA* gene abundance will increase.

3) At higher concentrations N and lower concentrations of P, there will be no net change in MOP and *pmoA* gene abundance compared to either N or P alone.

4) At lower concentrations N and higher concentrations of P there will be a greater increase in MOP and *pmoA* gene abundance compared to either N or P alone.

5.2: Materials and Methods

5.2.1: N and P microcosm experimental set up

Triplicate soils (25 g) were collected from the clay, greensand and chalk sites in November 2014 as previously described (Chapter 2, Section 2.2.2). Experimental soil microcosms were set up as shown in **Table 5.1**. N was added in the form of NH_4NO_3 and P was added in the form of P_2O_5, dissolved in Ultrapure MilliQ H_2O with the soil water content adjusted to 80% water holding capacity. Soil physicochemical analysis was performed as described previously (Chapter 2, Section 2.2.2). MOP were performed as previously described (Chapter 3, Section 3.2.3). PCR primers and cycling conditions for Q-PCR amplification of the *pmoA* gene were as described previously (Chapter 3, Section 3.2.2).

Table 5.1: **Experimental set up for N and P soil microcosm experiment**

*Field equivalent concentrations based on UK fertiliser application data (Seghers *et al.,* 2003, Sherestha 2010, Banger *et al,.* 2012) (Appendix V).

Sample Code	Treatment	Final concentration (of either N or P added) [a](mg N g^{-1} dry weight of soil); [b](mg P g^{-1} dry weight of soil)	Field equivalent concentration* [a](kg N ha^{-1} $year^{-1}$); [b](kg P ha^{-1} $year^{-1}$)
N1	Low conc. N only addition	0.06[a]	50[a]
N2	Medium conc. N only addition	0.12[a]	100[a]
N3	High conc. N only addition	0.25[a]	200[a]
P1	Low conc. P only addition	0.05[b]	20[b]
P2	Medium conc. P only addition	0.17[b]	75[b]
P3	High conc. P only addition	0.34[b]	150[b]
N1P1	Low conc. N plus low conc. P	0.06[a]; 0.05[b]	50[a]; 20[b]
N1P3	Low conc. N plus high conc. P	0.06[a]; 0.34[b]	50[a]; 150[b]
N3P1	High conc. N plus low conc. P	0.25[a]; 0.05[b]	200[a]; 20[b]
N3P3	High conc. N plus high conc. P	0.25[a]; 0.34[b]	200[a]; 150[b]
Control	No N or P addition	0[a]; 0[b]	0[a]; 0[b]

5.2.2: Long-term N and P Field manipulation experiment

A long-term (March to August 2015) N and P field manipulation experiment was set up (courtesy of Prof. Ineson and Dr Stockdale, University of York) at each of the clay, greensand and chalk sites, as part of the NERC Macronutrient Cycle Project (**Figure 5.1**). Lysimeters (50 cm depth) were inserted into the soil along each transect, and either nitrogen (N) or phosphate (P) or both nitrogen and phosphate (+N+P) was added (equivalent to 86 kg N ha^{-1} $year^{-1}$ as NH_4NO_3, and 20 kg P ha^{-1} $year^{-1}$ as P_2O_5) as shown in the schematic diagram (**Figure 5.1**). Each site was equipped with an automated system that took regular gas measurements of CH_4, CO_2 and N_2O as previously described (Chapter 4, Sections 4.2.3-4.2.4).

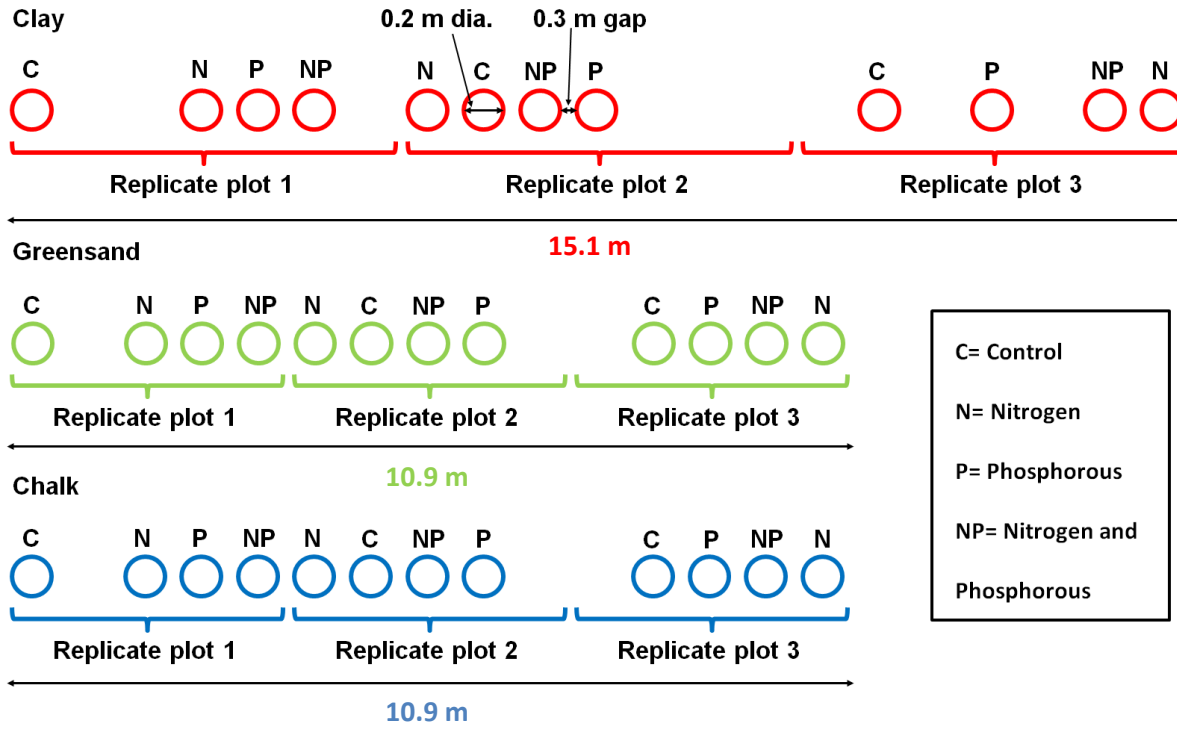


Figure 5.1: **Schematic diagram of long-term N and P Field manipulation experiment in the clay, greensand and chalk soil sites.** (Image modified courtesy of Dr Stockdale). (The clay transect is longer due to an additional flooding treatment set up by the University York that was not used in this thesis).

Soil was cored within 10 cm around each of the ceramic lysimeters using a 20 mL syringe with the end cut off. Soil cores (10 g) were collected from each site for physicochemical analysis as previously described (Chapter 2, Section 2.2.2). Unfortunately due to unforeseen circumstances with a laboratory move sample replicates for the physicochemical analyses were lost. MOP was carried out as described in Chapter 3, Section 3.1.3. Amplification of 16S rRNA and *pmoA* genes were carried out with cycling conditions as previously described (Chapter 2, Section 2.2.7-2.2.8; Chapter 3, Section 3.2.1-3.2.2)

5.2.3: Illumina MiSeq preparation and analysis

DNA extracted from both the N and P microcosm experiment and the long-term N and P Field manipulation experiment was prepared for Illumina MiSeq preparation by Dr Chronopoulou as described previously (Chapter 2, Section 2.2.8), using the same 16S rRNA and *pmoA* gene primers described previously (Chapter 3, Section 3.2.2).

5.2.4: Statistical analyses

A multivariate ANOVA was used to test differences in potential rates and functional gene abundances between geologies and nutrient amendments. Results that were significantly different were then further analysed with a Tukey HSD test. Pearson's correlation tests were also used to test for correlations between potential rates and other factors. The XLSTAT statistics package (version 2014.5, Addinsoft) was used for all statistical analyses.

5.3: Results

5.3.1: Physicochemical analysis of N and P soil microcosm experiment

Starting physicochemical parameters prior to the N and P microcosm experiments are from November 2014 seasonal soil samples and is listed in Chapter 2, **Tables 2.9**, **2.10** and **2.11**.

Total organic carbon (TOC) from the N and P microcosm experiments was similar between geologies and treatments (ranging from 223-246.6 mg C g^{-1} dry weight soil) (*P*= 0.02) (**Table 5.2**). In general, the anion concentrations were similar across treatments (**Table 5.3**). Nitrate was around 16-fold higher in the greensand soils also and also around 21-fold higher in the chalk soils amended with a high concentration of N (N3), and a high concentration of N and a low concentration of P (N3P1) compared to the controls (**Table 5.3**). Phosphate concentrations were generally similar across treatments with the exception of the greensand soils amended with medium concentrations of P, which resulted in an increase in phosphate concentrations compared to the controls (**Table 5.3**). In the greensand (High P), formate concentrations were around 5-fold greater compared to the controls.

In general, the cation concentrations were similar across treatments with the exception of ammonium and calcium, which varied with treatment and geology (**Table 5.4**). In the controls for the clay soils, ammonium concentration was zero which increased to varying concentrations up to 11.8 (±1.0) µmol g^{-1} dry weight soil across the different N and P treatments. Interestingly, calcium concentrations in the greensand soils decreased across most of the N and P treatments (by up to 2.5 fold) compared to controls (**Table 5.4**).

Table 5.2: **Total Organic Carbon (TOC) of N and P soil microcosms.** (± S.E Standard error of the mean, *n*=3).

N= Nitrogen addition, P= Phosphorous addition. Numbers 1,2,3 denotes low, medium, high concentrations respectively- See Table 5.1 for concentrations added.

		Total Organic Carbon (TOC) (mg C g^{-1} dry weight soil) (± S.E)		
Sample	**Treatment**	**Clay**	**Greensand**	**Chalk**
Control	Unamended soil	238.9 (± 2.0)	242.1 (± 1.3)	244.6 (± 2.6)
N1	Low N	244.7 (± 0.9)	242.0 (± 1.9)	245.3 (± 1.4)
N2	Medium N	242.9 (± 1.7)	242.9 (± 2.0)	245.7 (± 1.8)
N3	High N	241.0 (± 4.0)	244.1 (± 1.9)	244.8 (± 1.8)
P1	Low P	223.4 (± 6.1)	243.1 (± 1.5)	242.8 (± 3.2)
P2	Medium P	233.1 (± 5.1)	243.1 (± 0.7)	246.6 (± 0.2)
P3	High P	225.0 (± 9.9)	239.5 (± 0.8)	246.3 (± 1.0)
N1P1	Low N plus low P	233.6 (± 2.2)	244.8 (± 0.9)	232.8 (± 12.7)
N1P3	Low N plus high P	230.1 (± 3.9)	240.8 (± 0.8)	246.6 (± 0.5)
N3P1	High N plus low P	242.2 (± 2.3)	243.6 (± 1.9)	245.0 (± 1.9)
N3P3	High N plus high P	227.6 (± 4.2)	242.3 (± 1.6)	245.5 (± 1.2)

Table 5.3: **Anion concentrations of N and P soil microcosms.** (± S.E Standard error of the mean, *n*=3).

N= Nitrogen addition, P= Phosphorous addition. Numbers 1,2,3 denotes low, medium, high concentrations respectively- See Table 5.1 for concentrations added).

		Anion concentration (µmol g^{-1} dry weight soil)					
Clay Sample	**Treatment**	**Acetate ($C_2H_3O_2^-$)**	**Chloride (Cl^-)**	**Formate ($HCOO^-$)**	**Nitrate (NO_3^-)**	**Nitrite (NO_2^-)**	**Phosphate (PO_4^{3-})**
Control	Unamended soil	2.7 (±0.1)	1.7 (±0.2)	0.5 (±0.1)	1.0 (±0)	0.0 (±0)	0.2 (±0.1)
N1	Low N	3.0 (±0.4)	1.5 (±0.1)	0.7 (±0.1)	1.7 (±0.3)	0.0 (±0)	0.4 (±0.1)
N2	Medium N	3.1 (±0.4)	1.4 (±0.1)	0.6 (±0.1)	1.4 (±0)	0.0 (±0)	0.3 (±0.1)
N3	High N	1.8 (±0.1)	1.8 (±0.1)	0.6 (±0)	4.6 (±0.5)	0.0 (±0)	0.2 (±0)
P1	Low P	3.2 (±0)	1.6 (±0.1)	0.7 (±0)	1.6 (±0.1)	0.0 (±0)	0.4 (±0)
P2	Medium P	3.2 (±0.2)	1.5 (±0.1)	0.6 (±0.1)	1.5 (±0.2)	0.0 (±0)	0.3 (±0.1)
P3	High P	3.3 (±0.2)	1.6 (±0.1)	0.5 (±0.1)	1.3 (±0.1)	0.0 (±0)	0.5 (±0.1)
N1P1	Low N plus low P	1.6 (±0)	1.3 (±0.1)	0.6 (±0.1)	2.6 (±1.0)	0.0 (±0)	0.2 (±0)
N1P3	Low N plus high P	2.5 (±0.6)	1.4 (±0.1)	0.6 (±0)	1.0 (±0)	0.0 (±0)	0.1 (±0.1)
N3P1	High N plus low P	1.6	1.7	0.7	6.4	0.0	0.1

		(±0.1)	(±0.1)	(±0)	(±0.1)	(±0)	(±0)
N3P3	High N plus P	3.0	2.3	0.6	1.0	0.0	0.3
		(±0.1)	(±0.7)	(±0.1)	(±0)	(±0)	(±0.1)

Anion concentration (μmol g^{-1} dry weight soil)

Greensand Sample	Treatment	Acetate ($C_2H_3O_2^-$)	Chloride (Cl^-)	Formate ($HCOO^-$)	Nitrate (NO_3^-)	Nitrite (NO_2^-)	Phosphate (PO_4^{3-})
Control	Unamended soil	1.9	1.5	0.9	1.2	0.2	0.2
		(±0.1)	(±0.1)	(±0.1)	(±0.1)	(±0.1)	(±0.2)
N1	Low N	1.9	2.1	0.6	4.3	1.2	1.2
		(±0.3)	(±0.2)	(±0)	(±1.0)	(±1.2)	(±0.4)
N2	Medium N	1.7	1.5	0.6	8.0	0.0	0.0
		(±0)	(±0.1)	(±0.1)	(±3.2)	(±0)	(±0.7)
N3	High N	1.9	1.8	0.6	17.1	0.0	0.0
		(±0)	(±0.1)	(±0.1)	(±4.4)	(±0)	(±0.2)
P1	Low P	2.5	1.5	0.6	1.5	0.0	0.0
		(±0.1)	(±0.1)	(±0.2)	(±0.1)	(±0)	(±0.4)
P2	Medium P	4.9	1.7	1.4	5.1	6.1	6.1
		(±0.1)	(±0)	(±0.1)	(±4.0)	(±3.3)	(±0)
P3	High P	5.8	1.7	2.0	1.2	0.4	0.4
		(±0.1)	(±0.1)	(±0.2)	(±0.1)	(±0.3)	(±0.1)
N1P1	Low N plus low P	2.2	1.4	0.6	7.3	0.0	0.0
		(±0.1)	(±0.1)	(±0.1)	(±0.1)	(±0)	(±0.1)
N1P3	Low N plus high P	4.1	2.1	1.3	1.9	0.1	0.1
		(±1.0)	(±0.3)	(±0.2)	(±0.1)	(±0)	(±0.5)
N3P1	High N plus low P	1.3	1.9	0.6	18.9	0.0	0.0
		(±0.4)	(±0.1)	(±0.1)	(±1.8)	(±0)	(±0.1)
N3P3	High N plus P	4.1	2.3	1.3	1.7	0.0	0.0
		(±0.6)	(±0.3)	(±0.1)	(±0.1)	(±0)	(±0.5)

Anion concentration (µmol g^{-1} dry weight soil)							
Chalk Sample	**Treatment**	**Acetate ($C_2H_3O_2^-$)**	**Chloride (Cl^-)**	**Formate ($HCOO^-$)**	**Nitrate (NO_3^-)**	**Nitrite (NO_2^-)**	**Phosphate (PO_4^{3-})**
Control	Unamended soil	4.2	2.0	1.0	1.8	0.0	4.9
		(±0.4)	(±0.1)	(±0.1)	(±0.1)	(±0.0)	(±0.6)
N1	Low N	4.1	2.0	1.2	8.2	2.0	1.3
		(±0.3)	(±0.2)	(±0.1)	(±1.0)	(±1.5)	(±0.2)
N2	Medium N	3.0	1.8	1.1	20.0	0.0	0.8
		(±0.3)	(±0.2)	(±0.1)	(±0.9)	(±0)	(±0.1)
N3	High N	3.4	1.7	1.0	34.6	0.9	1.2
		(±0.2)	(±0.1)	(±0.1)	(±1.3)	(±0.8)	(±0.2)
P1	Low P	3.3	1.6	0.7	7.7	0.0	0.9
		(±0.8)	(±0)	(±0.1)	(±6.1)	(±0)	(±0.1)
P2	Medium P	1.4	1.0	0.2	8.8	12.1	6.2
		(±0.4)	(±0)	(±0.1)	(±0.5)	(±2.5)	(±1.0)
P3	High P	1.1	1.2	0.1	11.2	5.0	5.3
		(±0.2)	(±0.1)	(±0.1)	(±0.7)	(±2.8)	(±0.2)
N1P1	Low N plus low P	3.5	1.8	1.1	9.6	0.0	1.9
		(±0.1)	(±0.2)	(±0.1)	(±0.7)	(±0.0)	(±1.3)
N1P3	Low N plus high P	3.0	1.4	0.8	1.0	0.0	5.4
		(±0.2)	(±0)	(±0.2)	(±0)	(±0.0)	(±0.2)
N3P1	High N plus low P	4.1	1.8	1.3	36.5	0.0	1.7
		(±0.1)	(±0.2)	(±0.1)	(±0.6)	(±0.0)	(±0.4)
N3P3	High N plus P	3.2	2.2	0.9	11.7	0.0	1.2
		(±0.4)	(±0.4)	(±0)	(±5.1)	(±0.0)	(±0.1)

Table 5.4: **Cation concentrations of N and P soil microcosms.** (± S.E Standard error of the mean, *n*=3).

N= Nitrogen addition, P= Phosphorous addition. Numbers 1,2,3 denotes low, medium, high concentrations respectively- See Table 5.1 for concentrations added).

		Cation concentration (µmol g^{-1} dry weight soil)				
Clay Sample	**Treatment**	**Ammonium (NH_4^+)**	**Calcium (Ca^{2+})**	**Magnesium (Mg^{2+})**	**Potassium (K^+)**	**Sodium (Na^+)**
Control	Unamended soil	0 (±0)	6.5 (±0.2)	1.9 (±0.1)	0.9 (±0)	4.3 (±0.2)
N1	Low N	8.6 (±0.6)	9.1 (±0.3)	2.9 (±0.3)	1.1 (±0)	4.8 (±0.1)
N2	Medium N	0 (±0)	6.1 (±0.6)	1.5 (±0.2)	1.0 (±0.2)	4.1 (±0.2)
N3	High N	11.8 (±1.0)	7.9 (±0.8)	2.1 (±0.2)	1.1 (±0.1)	4.4 (±0.2)
P1	Low P	6.3 (±0.9)	5.4 (±0.8)	1.5 (±0.2)	0.7 (±0.1)	3.8 (±0.2)
P2	Medium P	6.5 (±0.6)	6.2 (±0.3)	1.8 (±0.1)	0.9 (±0)	4.2 (±0.1)
P3	High P	5.1 (±0.2)	6.1 (±0.1)	1.8 (±0.1)	0.9 (±0.1)	4.2 (±0.1)
N1P1	Low N plus low P	9.5 (±0)	6.2 (±0.3)	1.7 (±0.1)	1.0 (±0.1)	4.2 (±0.1)
N1P3	Low N plus high P	10.4 (±0.1)	7.7 (±0.3)	2.3 (±0.1)	1.3 (±0.1)	4.3 (±0.1)
N3P1	High N plus low P	0 (±0)	6.7 (±0.3)	1.8 (±0.0)	1.0 (±0.1)	4.1 (±0)
N3P3	High N plus P	11.3 (±0.6)	6.6 (±0.4)	1.7 (±0.2)	0.9 (±0)	4.0 (±0)
		Cation concentration (µmol g^{-1} dry weight soil)				
Greensand Sample	**Treatment**	**Ammonium (NH_4^+)**	**Calcium (Ca^{2+})**	**Magnesium (Mg^{2+})**	**Potassium (K^+)**	**Sodium (Na^+)**
Control	Unamended soil	10.9 (±0.2)	102.3 (±6)	3.2 (±0.1)	2.0 (±0.1)	5.6 (±0)
N1	Low N	5.47 (±0.3)	53.5 (±3.6)	1.9 (±0.4)	1.2 (±0.6)	5.1 (±0.1)

N2	Medium N	0 (±0.0)	39.5 (±1.8)	1.2 (±0)	1.9 (±0.1)	4.3 (±0.1)
N3	High N	4.2 (±0.1)	51.1 (±2)	1.5 (±0.1)	1.8 (±0.1)	5.0 (±0.1)
P1	Low P	0 (±0.0)	50.6 (±12.8)	1.4 (±0.3)	1.5 (±0.1)	4.3 (±0.2)
P2	Medium P	9.5 (±0.6)	77.0 (±4)	2.1 (±0)	1.2 (±0)	4.5 (±0.2)
P3	High P	9.2 (±0.1)	83.5 (±11.5)	2.4 (±0.5)	1.2 (±0.1)	4.5 (±0)
N1P1	Low N plus low P	5.7 (±0.4)	65.1 (±2.8)	1.8 (±0.1)	1.9 (±0.1)	5.0 (±0.1)
N1P3	Low N plus high P	10.8 (±0)	110.2 (±5.3)	3.3 (±0.1)	1.7 (±0)	5.4 (±0.1)
N3P1	High N plus low P	0 (±0)	40.4 (±10.7)	1.6 (±0.1)	2.2 (±0.4)	4.4 (±0.2)
N3P3	High N plus P	0 (±0)	60.1 (±3.3)	1.8 (±0.1)	2.3 (±0.1)	4.8 (±0.2)

Chalk Sample	**Treatment**	**Cation concentration (µmol g^{-1} dry weight soil)**				
		Ammonium (NH_4^+)	**Calcium (Ca^{2+})**	**Magnesium (Mg^{2+})**	**Potassium (K^+)**	**Sodium (Na^+)**
Control	Unamended soil	4.8 (±0.3)	25.2 (±0.7)	2.6 (±0.2)	2.3 (±0.3)	2.6 (±0.2)
N1	Low N	4.9 (±1.1)	23.7 (±2.3)	2.5 (±0.2)	2.1 (±0.1)	2.5 (±0.2)
N2	Medium N	0 (±0)	16.4 (±0.6)	1.8 (±0)	1.9 (±0.2)	1.8 (±0)
N3	High N	5.1 (±0.8)	28.6 (±4.1)	3.1 (±0.5)	2.2 (±0.1)	3.1 (±0.5)
P1	Low P	0 (±0)	21.0 (±3.6)	2.1 (±0.3)	1.8 (±0.4)	2.1 (±0.3)
P2	Medium P	1.0 (±0.3)	8.3 (±0.3)	1.3 (±0.1)	1.4 (±0.1)	1.3 (±0.1)
P3	High P	1.2 (±0.3)	9.0 (±0.4)	1.3 (±0.1)	1.5 (±0.1)	1.3 (±0.1)
N1P1	Low N plus low P	6.8 (±1.0)	18.6 (±5.0)	2.4 (±0.3)	2.3 (±0.4)	2.4 (±0.3)
N1P3	Low N plus high P	6.6 (±0.1)	22.1 (±1.7)	3.0 (±0.3)	1.5 (±0.3)	3.0 (±0.3)
N3P1	High N plus low P	0 (±0)	16.9 (±1.6)	1.7 (±0.3)	2.1 (±0.3)	1.7 (±0.3)
N3P3	High N plus P	6.2 (±0.3)	21.0 (±2)	2.8 (±0.3)	1.4 (±0.1)	2.8 (±0.3)

5.3.2: Effect of N and P additions on methane oxidation potentials in soil microcosms.

In general, MOP rates varied between treatments and geologies (**Figure 5.2**). Specifically, there was a significant increase in MOP in the chalk soils amended with medium and high concentrations of P (P2, P3), whereby MOP increased from 7.16 pmol g^{-1} dry weight soil day^{-1} (in the control soil) to <16.84 pmol g^{-1} dry weight soil day^{-1}(in the amended soils) (P< 0.0001) (**Table 5.5**). There was also a significant increase in MOP when the chalk soils were amended with a high concentration of both N and P (N3P3), whereby MOP increased from 7.16 pmol g^{-1} dry weight soil day^{-1} (in the control soil) to 20.51 pmol g^{-1} dry weight soil day^{-1}(in the amended soil N3P3) (P< 0.0001). When the chalk soils were amended with a low concentration of N and a high concentration of P (N1P3), MOP also increased (but not significantly) from 7.16 pmol g^{-1} dry weight soil day^{-1} (in the control soil) to 18.65 pmol g^{-1} dry weight soil day^{-1} (in the amended soil N1P3) (P= 0.07).

Generally in the clay soils MOP decreased across most treatments (with the exception of soils containing a low concentration of P (P1) where MOP remained largely unchanged compared to controls). Specifically, in the clay soils amended with a high concentration of N and low concentration of P (N3P1), there was a significant decrease in MOP from 13.28 pmol g^{-1} dry weight soil day^{-1} (in the control soil) to 0.82 pmol g^{-1} dry weight soil day^{-1} (in the amended soil N3P1) (P= 0.015). MOP also significantly decreased in the clay soils amended with low concentrations of N (N1), as well as soils amended with combinations of N and P (e.g. N1P1, N1P3, N3P3) (P= 0.032). Generally in the greensand soils, MOP either decreased slightly across all treatments or remained largely unchanged compared to controls. One notable observation was that in greensand soils amended with a medium concentration of P

Table 5.5: Effect of N and P treatments on MOP and *pmoA* gene abundance compared to control. **+** = increase, **-** = decrease. *N*= Nitrogen addition, P= Phosphorous addition. Numbers 1,2,3 denotes low, medium, high concentrations respectively

Geology	Treatment	Effect on MOP (pmol CH_4 g^{-1} dry weight soil)	Effect on *pmoA* (gene copy number g^{-1} dry weight soil)
Clay	N1	-9.9	-7.5×10^3
	N2	-5.3	$+9.1 \times 10^3$
	N3	-5.6	-7.5×10^3
	P1	1.4	-7.3×10^3
	P2	-4.5	$+3.9 \times 10^4$
	P3	-5.8	$+2.9 \times 10^4$
	N1P1	-8.9	-7.3×10^3
	N1P3	-8.4	$+7.6 \times 10^4$
	N3P1	-12.5	$+5.3 \times 10^4$
	N3P3	-8.5	-7.5×10^3
Greensand	N1	-4.2	-2.5×10^4
	N2	-2.9	-8.7×10^3
	N3	-2.5	-2.5×10^4
	P1	-2.1	$+1.6 \times 10^4$
	P2	-8.7	$+7.1 \times 10^4$
	P3	-0.4	$+1.2 \times 10^5$
	N1P1	-0.9	-2.2×10^4
	N1P3	-7.0	$+9.4 \times 10^4$
	N3P1	+1.2	$+2.1 \times 10^4$
	N3P3	-3.3	-2.3×10^4
Chalk	N1	-0.6	-5.5×10^3
	N2	+5.1	$+5.9 \times 10^4$
	N3	+2.4	$+8.1 \times 10^4$
	P1	+1.2	-4.4×10^4
	P2	+9.7	-3.6×10^4
	P3	+9.5	-3.6×10^4
	N1P1	-0.8	$+5.1 \times 10^3$
	N1P3	+11.5	$+5.4 \times 10^4$
	N3P1	-0.2	$+2.9 \times 10^4$
	N3P3	+13.4	$+8.6 \times 10^4$

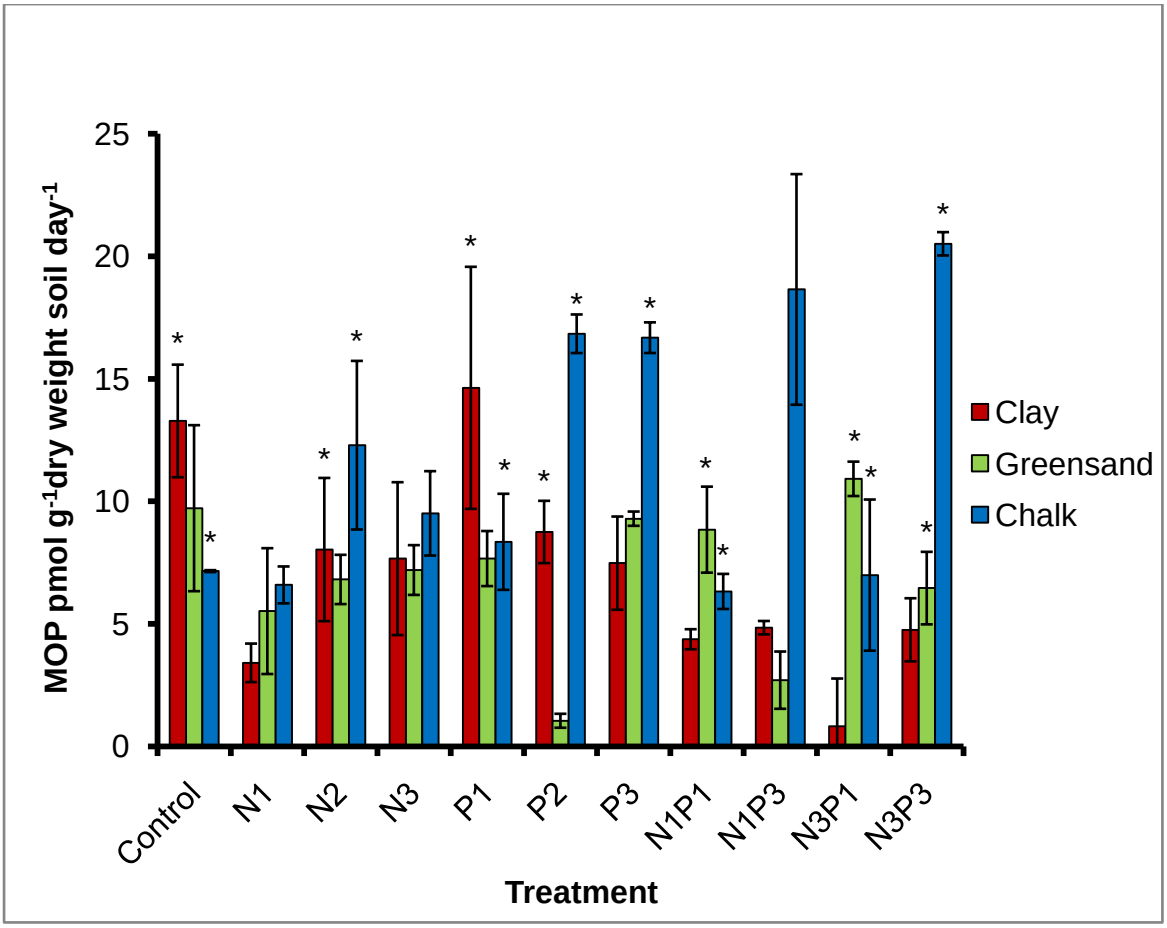


Figure 5.2: Effect of N and P additions on MOP in soil microcosms. (Error bars represent the Standard error of the mean, *n*=3). N1 low N; N2 medium N; N3 high N; P1 low P; P2 medium P; P3 high P; N1P1: Low N and low P; N1P3: Low N and high P; N3P1: High N and low P; N3P3: High N and high P. (See Table 5.1 for concentrations added). * = sample is significantly different ($P \leq 0.05$).

(P2), MOP decreased from 9.72 pmol g^{-1} dry weight soil day^{-1} (in the control soil) to 1.05 pmol g^{-1} dry weight soil day^{-1} (in the amended soil N3P1) ($P = 0.06$).

5.3.3: Effect of N and P additions on *pmoA* gene abundance in soil microcosms.

In general, the abundance of the *pmoA* gene was the highest in the chalk soils (<2.98 x10^5 gene copies g^{-1} dry weight soil) and remained largely unchanged across treatments compared to the control (**Figure 5.3**). In both the greensand and clay soils, *pmoA* gene abundance fluctuated with treatment. For example, in the clay soils treated with either low or high concentrations of N (N1, N3), the *pmoA* gene copy number decreased from 0.7 x 10^4 gene copies g^{-1} dry weight soil in the control to below detectable limits in the soils (**Figure 5.3A**). The *pmoA* gene copy number also decreased by around 10-fold in the clay soils treated with low P (P1), as well as combinations of N and P such as soils amended with low N and low P (N1P1), and high N and high P (N3P3). In contrast, *pmoA* gene copy number increased by around 10-fold in the clay soils treated with medium P (P2), high P (P3), low N and high P (N1P3), high N and low P (N3P1).

When the greensand soils were amended with a medium or high concentration of P (P2, P3) and a low concentration of N and high concentration of P (N1P3), *pmoA* gene copy numbers significantly increased by around 10-fold (*P*= 0.028) . In contrast, when the greensand soils were amended with either a low or high concentration of N (N1, N3), as well as combinations of N and P such as soils containing a low concentration of N and P (N1P1) and a high concentration of N and P (N3P3), *pmoA* gene copy numbers decreased by around 10-fold.

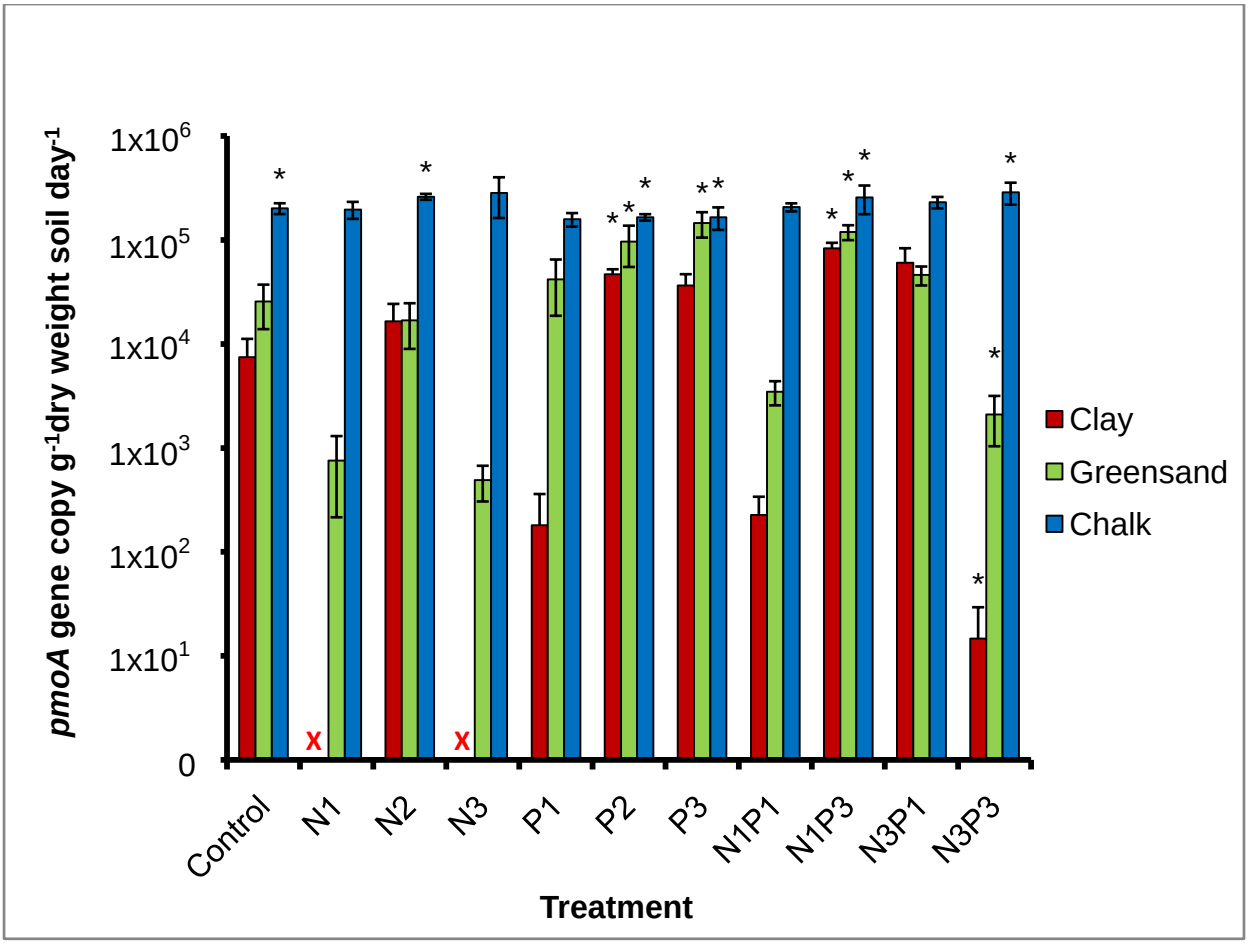


Figure 5.3: Effect of N and P additions on *pmoA* gene abundance in soil microcosms. (Error bars represent the Standard error of the mean, *n*=3). N1 low N; N2 medium N; N3 high N; P1 low P; P2 medium P; P3 high P; N1P1: Low N and low P; N1P3: Low N and high P; N3P1: High N and low P; N3P3: High N and high P. (See Table 5.1 for concentrations added). **X** = *pmoA* gene below detection threshold. * = sample is significantly different (*P*≤ 0.05).

5.3.4: Physicochemical analysis long-term N and P Field manipulation experiment

Total organic carbon (TOC) concentrations from the long-term N and P field manipulation experiment did not differ significantly between soils or treatments (*P*= 0.108) (**Table 5.6**). However, the percentage water content within each geology was

significantly different across treatments (*P*= 0.02) with the highest percentage water content in the chalk, followed by the clay and Greensand (**Table 5.6**).

Table 5.6: Total organic carbon (TOC) concentrations and percentage water content from long-term N and P field manipulation experiment

(± S.E Standard error of the mean, *n*=3).

Soil type	Treatment	Total Organic Carbon (TOC) (mg C g^{-1} dry weight soil) (± S.E)	Percentage water content (%)
Clay	**Control**	244.3 (±0.6)	58.2 (±1)
	+N	243.8 (±1.1)	61.0 (±1.1)
	+P	243.3 (±0.9)	56.3 (±0.9)
	+N+P	243.0 (±0.2)	59.0 (±1)
Greensand	**Control**	242.2 (±1.3)	47.7 (±1)
	+N	242.2 (±0.6)	45.9 (±0.8)
	+P	244.7 (±0.5)	48.2 (±0.8)
	+N+P	240.6 (±1.5)	43.9 (±0.7)
Chalk	**Control**	241.2 (±3)	71.0 (±0.8)
	+N	242.9 (±1.9)	71.9 (±0.8)
	+P	239.7 (±2.6)	77.1 (±1.5)
	+N+P	238.5 (±0.7)	70.8- (±1)

In general, the anion concentrations were similar across treatments (**Table 5.7**). Both formate and acetate were either low or not detected across geologies and treatments. In the chalk soils, nitrate concentrations increased by around 11-fold in the +N and +N+P treatments compared to controls. In the chalk soils, nitrite also increased by up to 12-fold in all treatments compared to controls (**Table 5.7**). Cation concentrations varied widely between treatments and geologies (**Table 5.8**). Interestingly, in the chalk soils, the concentration of calcium decreased in all the treated soils compared to the control.

Table 5.7: Anion concentrations from the long-term N and P field manipulation experiment. (*n*=1).

Anion concentrations (µmol g^{-1} dry weight soil)							
Soil type	Sample	Acetate ($C_2H_3O_2^-$)	Chloride (Cl^-)	Formate ($HCOO^-$)	Nitrite (NO_2^-)	Nitrate (NO_3^-)	Phosphate (PO_4^{3-})
Clay	**Control**	0.1	0.0	1.7	0.0	0.0	1.5
	+N	0.1	0.0	2.0	0.0	0.0	1.0
	+P	0.0	0.0	2.0	0.0	0.0	2.1
	+N+P	0.0	0.0	2.2	0.4	0.9	1.4
Greensand	**Control**	0.0	0.0	2.3	1.0	0.8	1.0
	+N	0.0	0.0	2.3	1.8	0.6	1.7
	+P	0.0	0.0	1.7	0.1	0.7	0.9
	+N+P	0.1	0.0	1.5	0.0	0	1.7
Chalk	**Control**	0.8	0.0	0.0	0.7	0.5	1.3
	+N	0.0	0.0	0.2	8.4	0.6	1.4
	+P	0.0	0.0	1.0	5.1	0.4	1.4
	+N+P	0.0	0.0	1.7	5.9	0.5	1.2

Table 5.8: Cation concentrations from the long-term N and P field manipulation experiment. (*n*=1).

Cation concentration (µmol g^{-1} dry weight soil)						
Soil type	Sample	Ammonium (NH_4^+)	Calcium (Ca^{2+})	Magnesium (Mg^{2+})	Potassium (K^+)	Sodium (Na^+)
Clay	**Control**	0.9	5.4	1.6	0.7	3.1
	+N	0.4	4.4	1.5	0.5	2.3
	+P	0.7	5.0	1.7	0.7	3.6
	+N+P	0.8	9.0	0.8	3.1	2.3
Greensand	**Control**	0.5	10.3	0.8	3.5	2.0
	+N	0.5	10.1	0.7	2.4	3.0
	+P	0.4	8.4	0.7	3.2	1.8
	+N+P	0.5	4.9	1.6	0.8	3.3
Chalk	**Control**	0.5	18.4	0.8	1.3	2.4
	+N	0.3	14.7	0.6	0.9	2.4
	+P	0.3	13.1	0.5	0.8	2.5
	+N+P	0.4	14.5	0.7	1.2	2.1

5.3.5: Effect of N and P additions on methane oxidation potential in long-term N and P field manipulation experiment.

MOP within field N and P amendments is presented (**Figure 5.4**). Regardless of treatment, clay soils had the highest MOP (<21.7 pmol g^{-1} dry weight soil) followed by chalk (<17.1 pmol g^{-1} dry weight soil), and then Greensand (<12.8 pmol g^{-1} dry weight soil) (Tukey HSD P= 0.002 to P< 0.0001) (**Figure 5.4**). In the clay soils, MOP increased with all treatments compared to the control although this was not significant (P> 0.872). In the chalk soils, MOP remained largely unchanged across all treatments compared to the control. In the greensand soils although phosphate or both nitrogen and phosphate additions did not result in any significant change in MOP compared to the control, MOP did increase slightly with the addition of nitrogen only although this was not significant (P> 0.526).

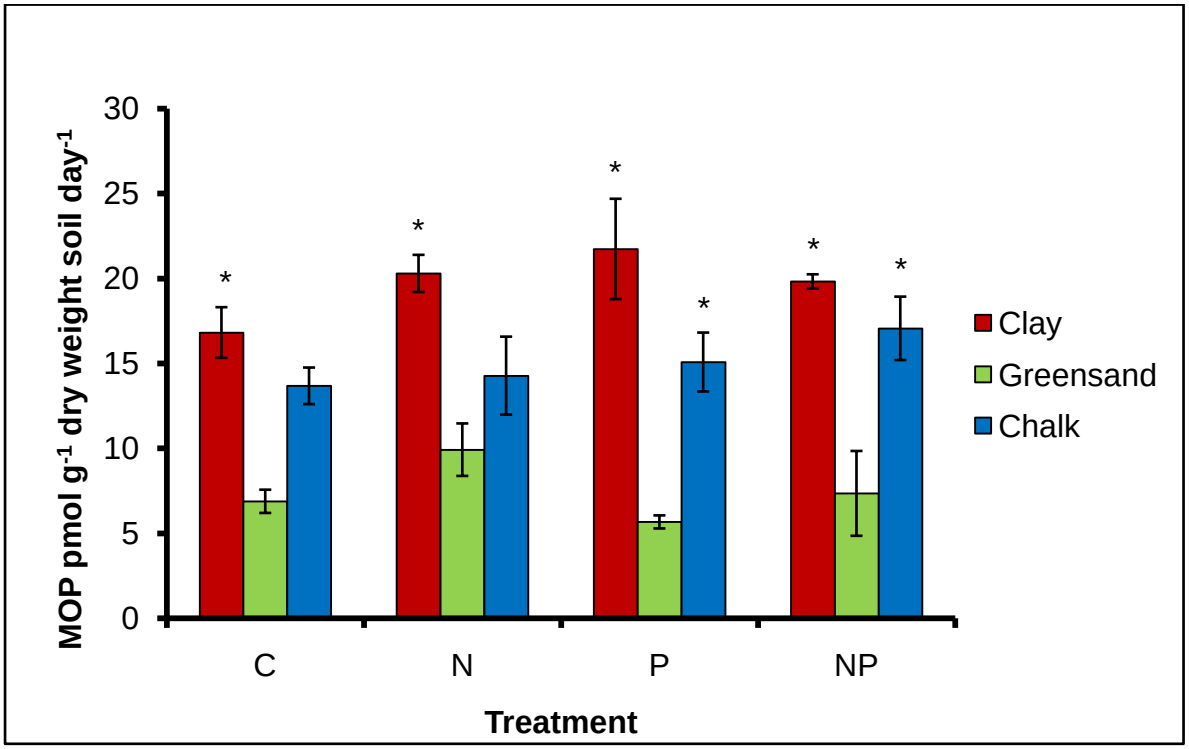


Figure 5.4: MOP in long-term N and P field manipulation experiment. C: Control, N: + Nitrogen, P: + Phosphate, NP: + Nitrogen and Phosphate. Error bars represent standard error of the mean (n=3). * = sample is significantly different (P≤ 0.05).

5.3.6: Quantitative PCR of the Bacterial 16S rRNA gene and *pmoA* gene in long-term N and P field manipulation experiment.

The abundance of the Bacterial 16S rRNA gene and *pmoA* gene are presented (**Figure 5.5 A and B**). There was no significant difference in Bacterial 16S rRNA gene abundance between geologies (ANOVA, P= 0.583). However, there was a significant increase in Bacterial 16S rRNA gene abundance across all treatments, for each geology (ANOVA, P< 0.0001). Specifically, Bacterial 16S rRNA gene abundance increased from $<1.21 \times 10^{10}$ gene copies g^{-1} dry weight soil in the unamended control to $>2.83 \times 10^{11}$ gene copies g^{-1} dry weight soil in the treated soils (Tukey's tests, P <0.044) (**Figure 5.5A**).

There was also a significant increase in *pmoA* gene abundance across all treatments, for each geology (P= 0.006). Specifically, in the greensand and chalk soils, *pmoA* gene abundance increased from 9.67×10^{3} and 3.14×10^{4} gene copies g^{-1}dry weight soil in the control to $>9.80 \times 10^{4}$ and $>1.13 \times 10^{5}$ gene copies g^{-1} dry weight soil in the treated soils (respectively) (**Figure 5.5B**). In the clay soils, the addition of both nitrogen and phosphate did not cause any significant change in the abundance of *pmoA* gene copy numbers (P= 0.978). However, the addition of nitrogen only or phosphate only caused an increase from 2.79×10^{3} gene copies g^{-1} dry weight soil in the unamended control to 3.81×10^{4} and 4.5×10^{4} gene copies g^{-1} dry weight soil in the nitrogen or phosphate treated soils respectively (P< 0.027).

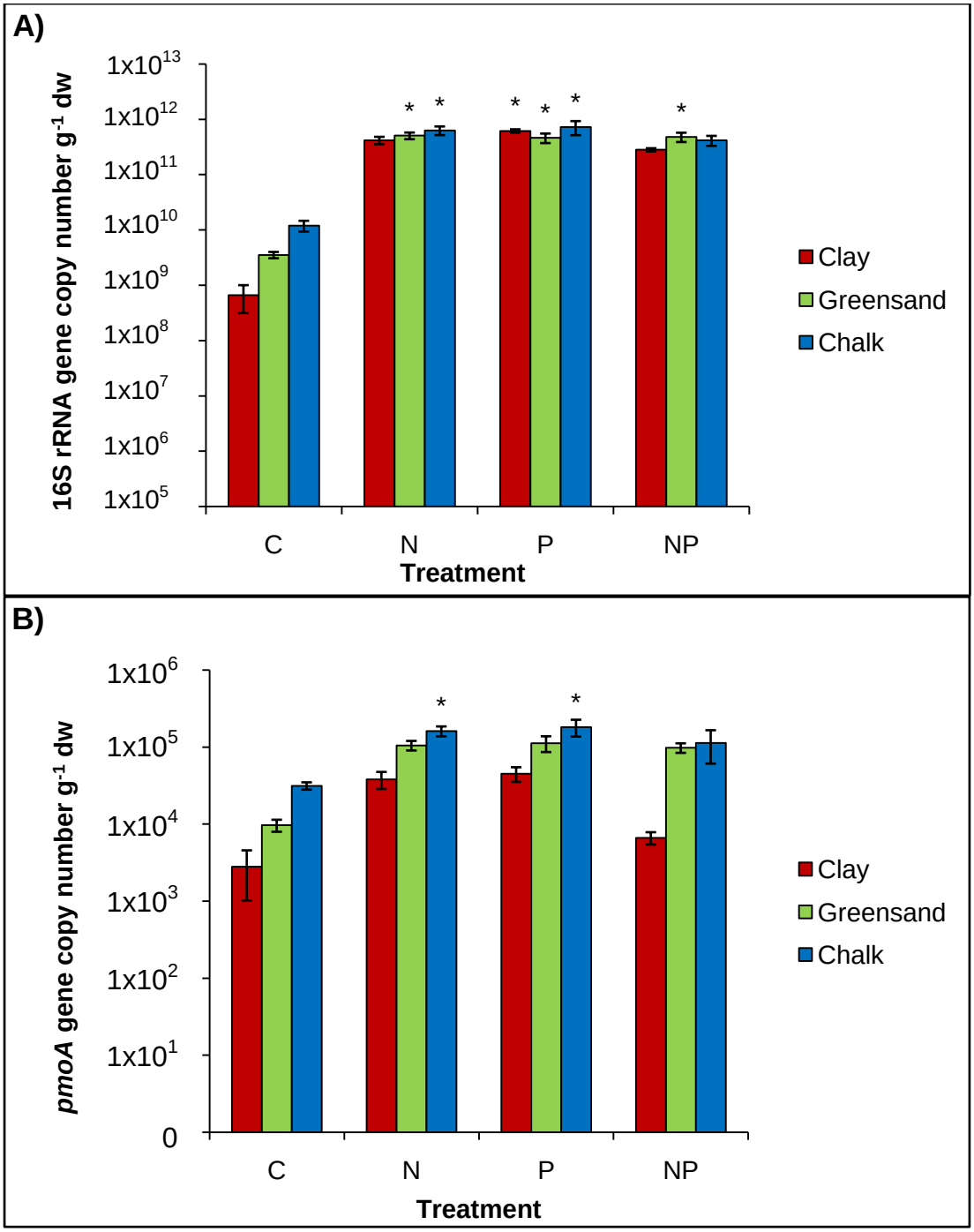


Figure5.5: Abundance of Bacterial 16S rRNA (A) and *pmoA* (B) genes in long-term N and P field manipulation experiment. C: Control, N: +Nitrogen, P: +Phosphate, NP: +Nitrogen and Phosphate. Error bars represent standard error of the mean (n=3). * = sample is significantly different ($P \leq 0.05$).

5.3.7: Illumina MiSeq 16S rRNA and *pmoA* gene data

Unfortunately DNA samples submitted for Illumina MiSeq sequencing of the 16S rRNA and *pmoA* genes were not available at the time of writing.

5.4: Discussion

In the soil microcosm experiments, it was notable that MOP generally decreased in the clay soils across most treatments, both with the addition of N as well as N and P together. This was especially the case with clay soils amended with a high concentration of N and low concentration of P (N3P1) where the greatest inhibition of MOP was observed. Generally, where MOP decreased (such as the clay soils N1P1, N3P3), there was a concomitant decrease in *pmoA* gene copy number. This suggests that the methanotroph community had probably decreased in relative abundance resulting in an overall decrease in MOP rates.

One possible explanation for the observed decrease in methanotroph abundance could be the concentration of nitrate present in the soil inhibiting the methanotroph communities. Indeed, in soils N3P1 and N1P1 there was a six-fold and >two-fold increase respectively in nitrate concentration in these samples compared to the controls. Previous research has found similar effects of nitrate inhibition on MOP rates (Reay and Nelson, 2004; Xu and Inubashi, 2004). However, an increase in nitrate concentration does not explain the observed decrease in MOP in the clay soils N1, N1P3 and N3P3 where nitrate concentration was lower than the controls. Interestingly, in these clay soils (N1, N1P3 and N3P3), ammonium concentrations were significantly greater than the controls (P= 0.0001). Thus, it may be ammonium rather than nitrate that was inhibiting MOP rates in these clay soils.

A previous study by Yang *et al.* (2011) showed that the addition of ammonium fertilizers resulted in a reduction in the ratio of Type II: Type I methane oxidising bacteria. Thus, it is possible that an increase in ammonium concentration caused a similar shift in methanotroph community structure in these clay soils resulting in the decrease in MOP rates. Alternatively, it is possible that the methanotrophs under these conditions were oxidising ammonium rather than methane. It is well known that due to the homology of the MMO enzymes have with AMO enzymes, methanotrophs can actively oxidise ammonium as well as methane, and ammonium oxidising methanotrophs have been identified from a range of different environments (Holmes *et al.,* 1995; Khmelenina *et al.,* 1999; Nyerges and Stein 2009; Zheng *et al.*, 2013).

Another possible explanation for the observed decrease in MOP in these clay soil microcosms could be due to the methanotrophs present utilising acetate rather than oxidising the methane. Indeed, acetate concentrations in soils N1P1 and N3P1 both had around 1.5-fold lower acetate compared to the controls. This suggests that the methanotrophs present in these soils were possibly utilising the acetate, reducing the acetate concentration in these samples compared to the controls. It has been previously shown that methanotrophs are able utilize acetate as a carbon source when CH_4 is low or absent (Theisen and Murrell, 2005; Rahman *et al.,* 2011; Belova *et al.,* 2011). It has also been shown previously that Alphaproteobacteria methanotrophs of the genus *Methylocella* possess only the soluble form of sMMO and are able to grow on acetate, pyruvate, succinate, malate and ethanol in addition to C1 substrates (Denys *et al.* 2005; Theisen *et al.,* 2005). Members of the Family Methylocystaceae also contain sMMO and have been shown to be able to utilize acetate as well (McDonald *et al.,* 1997; Belova *et al.,* 2011) and previously in Chapter 3, several OTUs among the most abundant methanotroph were

Methylocystaceae, supporting the idea that facultative methanotrophs are consuming acetate. In addition, the relative abundance of the *pmoA* gene in the clay soils N1P1 decreased. Thus, it is possible that members of the genus *Methylocella* containing the sMMO enzyme rather than the pMMO enzyme were present and utilising acetate in these clay soils. In another study, a member of the genus *Methylocystis* also could grow slowly on acetate in the absence of methane and adopted this approach as a survival strategy (Belova *et al.,* 2011).

Similarly to the clay soils, the greensand soil microcosms also showed a significant decrease in MOP in the soils amended with a medium concentration of P (P2) (P= 0.06), which may also be related to an increase in nitrate concentration inhibiting methanotroph activity. However, in this greensand soil sample (P2), the relative abundance of the *pmoA* gene was found to increase slightly, suggesting that the methanotroph community had increased in abundance compared to the control. Thus, another factor such as a shift in the overall community structure may be causing the observed decrease in MOP rates in these soils.

In contrast to the clay and greensand soil microcosms, there was generally, a significant increase in MOP in the chalk soils amended with a medium and high concentrations of P (P2, P3) (P= 0.0001). There was also a further increase in MOP when the chalk soils were amended with a low concentration of N and a high concentration of P (N1P3) or high concentration of both N and P (N3P3). With the exception of sample N3P3, these soils all had higher concentrations of phosphate compared to the control. It is therefore possible that in the present study, these chalk soils (P2, P3, N1P3) were phosphate limited, and the addition of phosphate in these microcosms stimulated the growth of methanotroph communities. However, in these chalk soils this observed increase in MOP did not correspond to an increase in the

relative abundance of *pmoA* gene copies, which remained largely unchanged across treatments compared to the controls.

Another possible explanation for the observed increase in MOP in the chalk soils amended with P is that either high concentrations of nitrate or ammonium were stimulating methanotroph activity. However, it is unlikely that nitrate was increasing MOP rates, as it can be argued that in some of the samples (such as N3P1) where very high nitrate concentrations were measured, MOP was similar to that of the controls. It is more likely that in these soils ammonium was stimulating methane oxidation potential rates. For example, sample N3P1 had no measurable ammonium and MOP rates were similar to that of the controls, whereas, other samples (such as N1P3 and N3P3), ammonium concentrations were around 1.5-fold greater than the controls, and the corresponding MOP rates increased. Hoefman *et al.* (2014) noted that differential tolerance to nitrogen has been observed at the genus level in *Methylomonas*, with some among the same strain being able to tolerate high concentrations of ammonium (100 mM ammonium).

Interestingly nitrite appears to be higher than nitrate in +N samples in the field experiment, notably in both the chalk and greensand experiments' +N and +NP treatments (also +P in chalk) were considerably higher. This was not the case in bottle experiments where nitrite was almost always lower than nitrate. This result is surprising as natural systems tend to have higher nitrate than nitrite, as nitrite is rapidly reduced by nitrification (Focht and Martin, 1979). Nitrite however can build up in a system if excess nitrate is introduced, as the nitrifiers are unable to reduce nitrite rapidly enough (Tiso and Schecter, 2016) or if organic carbon is low (Focht and Martin, 1979). This build-up of nitrite in our soils does not appear to affect our overall MOP in the field experiment however.

Whilst MOP in the chalk and greensand soils remained largely unchanged across all treatments with the long-term N and P field experiment, the relative abundance of the *pmoA* gene increased across all treatments. This suggests that although the relative abundance of methanotrophs increased, there was no overall increase in methane oxidation activity. In the long-term N and P field experiment, the relative abundance of the 16S rRNA gene also increased for each geology across treatments, compared to the controls. This suggests that the overall Bacterial populations had also been stimulated and increased in abundance following N and/or P additions. It is therefore possible that the methanotrophs in the clay and greensand soils were competing with other microorganisms for available substrates.

In conclusion, the long-term addition of N and P (via fertilizer application) to soils with underlying chalk geologies, are likely to stimulate methanotroph communities to oxidise more methane compared to unfertilised soils. In contrast to chalk soils, the activity of the methanotroph communities in soils with underlying greensand or clay geologies, are unlikely to be affected by long-term fertiliser applications. However, in the short-term, the application of high concentrations of N in addition with small concentrations of P in clay soils may cause decreases in MOP rates and the relative abundance of methanotrophs, that may not necessarily occur in chalk and greensand soils. Such findings have implications to future climate change, where such fertiliser management strategies of chalk grassland soils may help towards mitigating future predicted increases in CH_4 concentrations.

Chapter 6: General Discussion

Global warming and climate change have largely been attributed to increases in atmospheric greenhouse gas (GHG) concentrations. Methane is a very important trace gas because, per molecule, it absorbs infrared radiation much more strongly than carbon dioxide, and relative to carbon dioxide, methane is, per mole, 3.7 times more potent in its global warming potential (Lashof and Ahuja 1990). The majority of CH_4 emissions come from anthropogenic activities, such as industry, landfill, livestock and agriculture (Conrad, 2009; Karakurt *et al.*, 2012).

There are many environmental factors controlling CH_4 fluxes, including redox potential, electron acceptors, substrate availability, temperature, pH, fertilizer applications, trace metals, and CO_2 concentrations (Dalal *et al.,* 2008). Two key groups of organisms governing methanogenesis and methane oxidation are methanogens and methanotrophs, producers and oxidizers of CH_4 respectively (Nazaries *et al.*, 2013). Currently there insufficient knowledge on how anthropogenic activities as well as global climate change might affect methanogens and methanotrophs (Nazaries *et al.,* 2013). Therefore, a better understanding on the relationship between these two functional microbial groups in a changing environment is crucial.

This thesis focused on soils and river sediments from across three geology types (clay, chalk and greensand) in the Hampshire-Avon catchment, England. This was accomplished by the use of microcosm and field experiments in conjunction with potential rate measurements and molecular techniques to characterize methanogens and methanotrophs involved in CH_4 flux. Chapter 2 aimed to determine seasonal changes in Bacterial and Archaeal communities in soils and river sediments across

geologies in relation to physicochemical parameters. In general, shifts in Bacterial communities occurred in both sediments and soils across season and geology with several major classes detected being common to both ecosystems including *Alphaproteobacteria, Bacteriodetes, Betaproteobacteria, Gammaproteobacteria,* and *Nitrospira* as has been found elsewhere (Zwart *et al.,* 2002; Cottrell *et al.,* 2005; Newton *et al.,* 2011).

Archaeal communities on the other hand showed much larger differences between sediments and soils, with methanogens making up a large part of the overall community in sediments including Methanosarcinales, Methanomicrobiales, Methanobacteriales, Methanomassiliicoccales, and Nitrososphaerales. Methanosarcinales, Methanobacteriales and Methanomicrobiales have been found previously in various environments including soils, river sediments, lakes, and deep sea vents (Brazelton *et al.*, 2010; Beckmann *et al.,* 2011; Frey *et al.,* 2011; Conrad, 2012; Chaudhary *et al.*, 2014). The predominance of methanogens within sediments may explain the higher MPP found in sediments (Chapter 3) compared to the soils and is likely due to the higher water content of sediments compared to soils creating anoxic zones that favour methanogens.

In soils, the ammonia oxidizing Archaea was *Nitrososphaera* dominant comprising more than 80% of the total Archaeal OTUs found*;* which may be due to higher ammonium concentrations present, although this is inconclusive. *Nitrososphaera* has also been previously identified in soils (Tourna *et al.,* 2011). Interestingly, *Acidobacteriia* were predominant in the clay soils compared to the more permeable chalk and greensands. This is likely due to the lower pH (pH <5) in the clay favouring *Acidobacteriia*, compared to the neutral pH of chalks or

Greensands. Chroňáková *et al.* (2015) also showed that low pH selected for *Acidobacteriia* in soils.

In order to better elucidate the seasonal changes in methanotroph and methanogen communities across geologies, *pmoA* and *mcrA* gene abundances in relation to CH_4 production (MPP) and CH_4 oxidation potentials (MOP) were measured (Chapter 3). In general, higher MOP and MPP were found in the river sediments compared to soils suggesting that the rivers are potential 'hot spots' of activity in CH_4 transformations with Methanococcoides, Methanosarcina and Methanocorpusculum likely candidates driving methanogenesis.

In soils, methanogenesis was generally the highest in the clay soils, and was related to an increase in relative abundance of methanogen 16S rRNA sequences but not *mcrA* gene abundance. The methanogen 16S rRNA gene sequences that were recovered from soils were related to Methanobacteriales, Methanomassiliicoccales, Methanosarcinales, Methanocellales and Methanomicrobiales suggesting that the upper soil layers had anoxic microsites that can harbour low numbers of methanogens as has been found previously (Verchot *et al.*, 2000).

Here, MOP was found to be much lower than has been previously found in soils (Reay *et al.*, 2004) and *Methylocystis* and other unclassified *Methylocystis* like methanotrophs were likely oxidising the CH_4 present irrespective of geology and season. Based on 16S rRNA gene sequencing, members of the genus *Methylobacter* were the most abundant methanotroph in the river sediments. *Methylobacter,* is a Type I obligate methanotroph which has been found in various environments including freshwater rivers and lakes, wetland, and rice paddy soil

(Rahalkar and Schink, 2007; Bowman, 2015; Wei *et al.,* 2016). Here, sediments had a higher MOP and MPP compared to soils (Chapter 3). This might explain why *Methylobacter* was more prevalent in the sediments than the soils as higher CH_4 concentrations are preferred by obligate methanotrophs, whereas facultative methanotrophs would be more prevalent in environments with low CH_4 concentrations. However, this was not supported by *pmoA* gene sequencing whereby the majority of *pmoA* gene sequences recovered were unidentified but clustered with *Methylocystis* not *Methylobacter*. This was likely due to the relatively sparse identifiable *pmoA* sequence data from the functional gene databases and highlights a gap in our knowledge regarding methanotroph *pmoA* sequencing. Based on 16S rRNA gene sequencing, members of the Verrucomicrobia methanotrophs Methylacidiphilales were abundant in soils, a clade that includes (Nakatsu *et al.*, 2006).

Increased temperatures are a key issue with climate change. Chapter 4 aimed to determine the effect of temperature on soil methanotroph community structure, activity and abundance across different geologies. Soils were subjected to warming treatments both in long-term field experiments and in short-term microcosms. In general, methanotrophy and methanotroph communities were largely unaffected in chalk and clay soils, suggesting that future global temperature increases are a minor factor on methanotroph activities in these sites. However, in the greensand soils, temperature had a strong effect on methanotrophy, both in long-term field warming experiments and soil temperature microcosms with methanotrophs in summer samples potentially more active at elevated temperatures. However, in the winter samples MOP was relatively constant, across temperatures suggesting that methanotroph activity was not directly affected by an increase in temperature. This

compliments previous studies that observed no increase when samples were subjected to temperature increases (Shelley *et al.*, 2015). Both *pmoA* and 16S rRNA gene abundances were largely unaffected by temperature treatments in either the microcosm or the field experiments.

Increases in global populations will inevitably increase demands on agricultural land use. Although studies have investigated the effects N fertilizers have on methanotrophy, the effect of N and P and P alone on methanotrophs is less well understood (Veraart *et al.*, 2015). Chapter 5 aimed to determine the effect of N and P concentration (such as via fertilizer additions) on soil methanotroph community structure and activity. In Chapter 5, soils in long-term field experiments and in short-term microcosms were both subjected to typical N and P additions expected of managed grasslands in Southern England (Defra 2010). In general, the long-term addition of N and P (via fertilizer application) to clay soils potentially stimulated methane oxidation compared to the unfertilised soils. In contrast to clay soils, methanotroph activity in greensand or chalk soils, are unlikely to be affected by long-term fertilizer applications. However, in the short-term, it was shown that the application of high concentrations of N may cause MOP rates and the relative abundance of methanotrophs in clay soils to decrease, that may not necessarily occur in chalk and greensand soils. Previous research had similar conflicting results regarding N addition, where N can inhibit or stimulate methanotrophs depending on the concentration and type of N used (Noll *et al.,* 2008; Stein and Klotz, 2011; Hoefman *et al.,* 2013).

To summarise, from the current study the methanogenic and methanotrophic potential in soils and sediments of the Hampshire-Avon catchment can be divided according to geology, however the prediction that these processes will follow BFI

was not strictly true. Soils with underlying clay geology (lowest BFI) did have a higher CH_4 production potential and subsequent methanotrophy was also higher, however chalk (highest BFI) and greensand (middle BFI) had similar rates of MPP and MOP, with greensand in some instances having slightly lower rates than chalk. Comparable sediments showed similar differences from the prediction that MOP and MPP followed BFI, with clay and greensand sediments having similar higher MOP rates, whereas MPP was higher in chalk and lower in greensand. Although the methanogenic potential of soils was not tested under different temperature conditions, nor tested for different nutrient amendments, several key points can still be drawn: across all geologies, any increases in temperature that may potentially lead to increases in methanogenesis, will likely lead to concomitant increase in methanotrophy. N and P additions that lead to increases in methanogenesis will similarly be compensated by increases in MOP in chalk soils, however clay soils may result in net increases in CH_4 emissions as MOP is suppressed from N and P additions.

In conclusion, this thesis has demonstrated an advance in the understanding of seasonal changes in methanogen and methanotroph communities in environments with different geologies, as well as their responses to the effect of fertilizer addition and increases in temperature. Such findings have implications to future climate change, where such targeted fertilizer management strategies of grassland soils may help towards mitigating future predicted increases in methane concentrations.

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Appendix

Appendix I: Porosity calculations

Porosity calculations was carried out using the following formula:

$$\Phi = 1 - \frac{P_b}{P_p}$$

Where Φ is the porosity of soils, P_b is the dry bulk density of soils and P_p is the particle density of soils. Dry bulk density of soil was calculated by Dr James Stockdale using the following formula.

$$Pb = \frac{V_s}{m_{dry}}$$

Where *Pb* is the dry bulk density, V_s is the volume of soil in a cylinder (radius of 2.1 cm and a height of 5 cm), and m_{dry} is the weight of dry soil in the cylinder.

Particle density was determined by the following formula.

$$Pp = \frac{m_{dry}}{V_p}$$

Where *Pp* is the particle density, m_{dry} is the dry weight of soil and V_p is the volume of soil particles. V_p was determined by placing dried soil of a known weight (between 5 and 8 g) in a 250 mL volumetric flask of known weight. Water was then added to the volumetric flask up to the neck of the flask. The flask was then heated gently to and held at 80^{o}C until soil particles were silted to remove air filling the soil's pore space. The soil was then left to cool and settle overnight. Water was then added to the 250 mL line of the volumetric flask and then weighed. The volume of soil particles (V_p) is then the difference between the weight of 250 mL water and the weight of the water in the flask.

Appendix II: Rarefaction curves

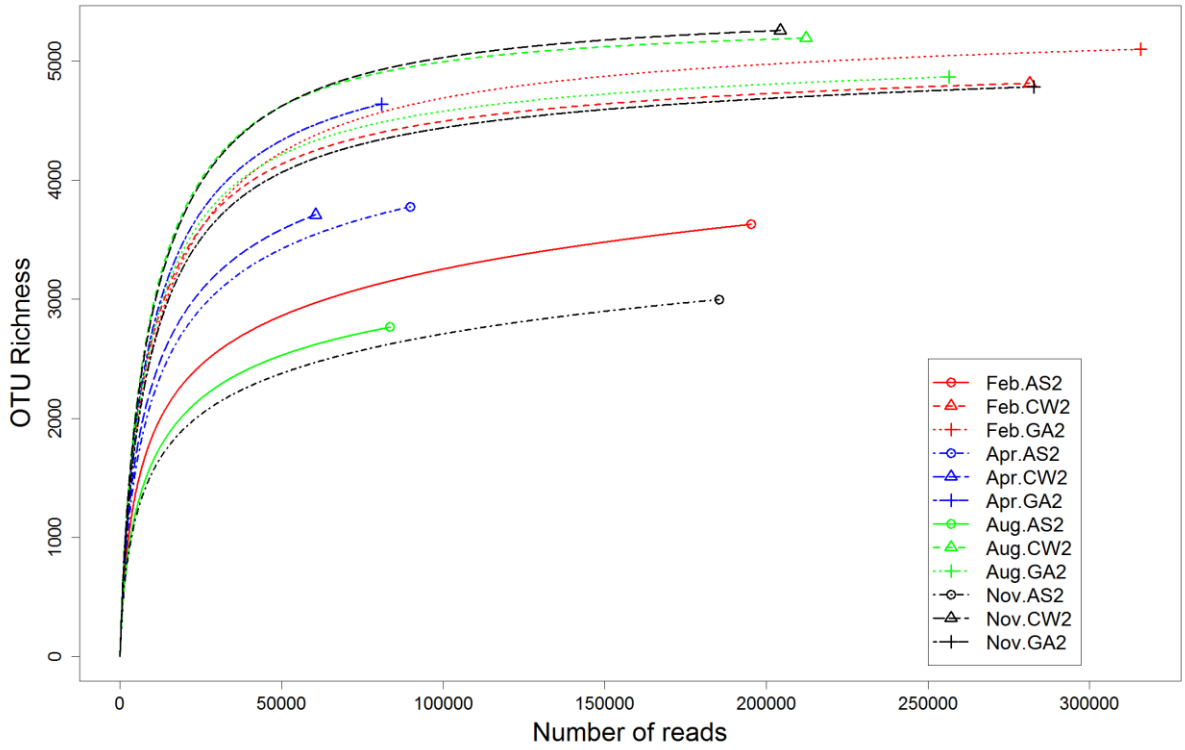

Figure A2.1 Rarefaction curve of 16S rRNA Bacterial soils. Feb = February, Apr = April, Aug= August, Nov = November. AS2 = Clay, CW2 = Chalk, GA2 = Greensand.

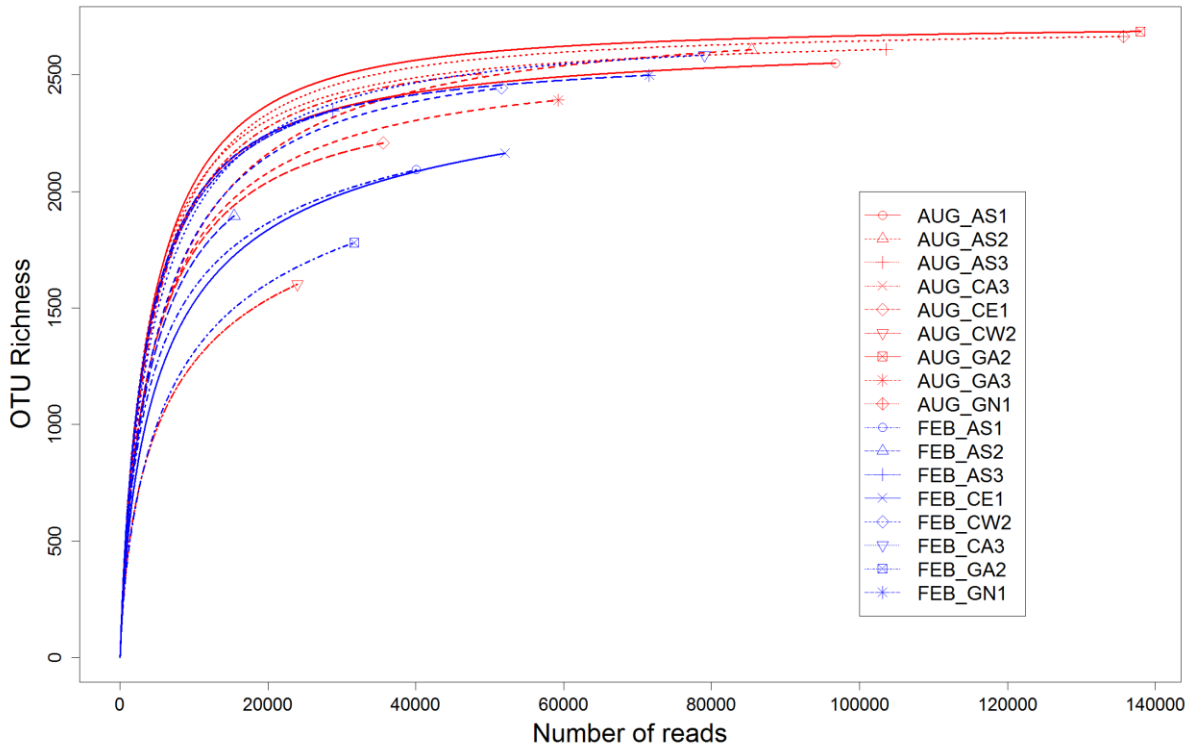

Figure A2.2 Rarefaction curve of 16S rRNA Bacterial sediments. Aug = August, Feb = February. AS1, AS2, AS3 = Clay. CA3, CE1, CW2 = Chalk. GA2, GA3, GN1 = Greensand.

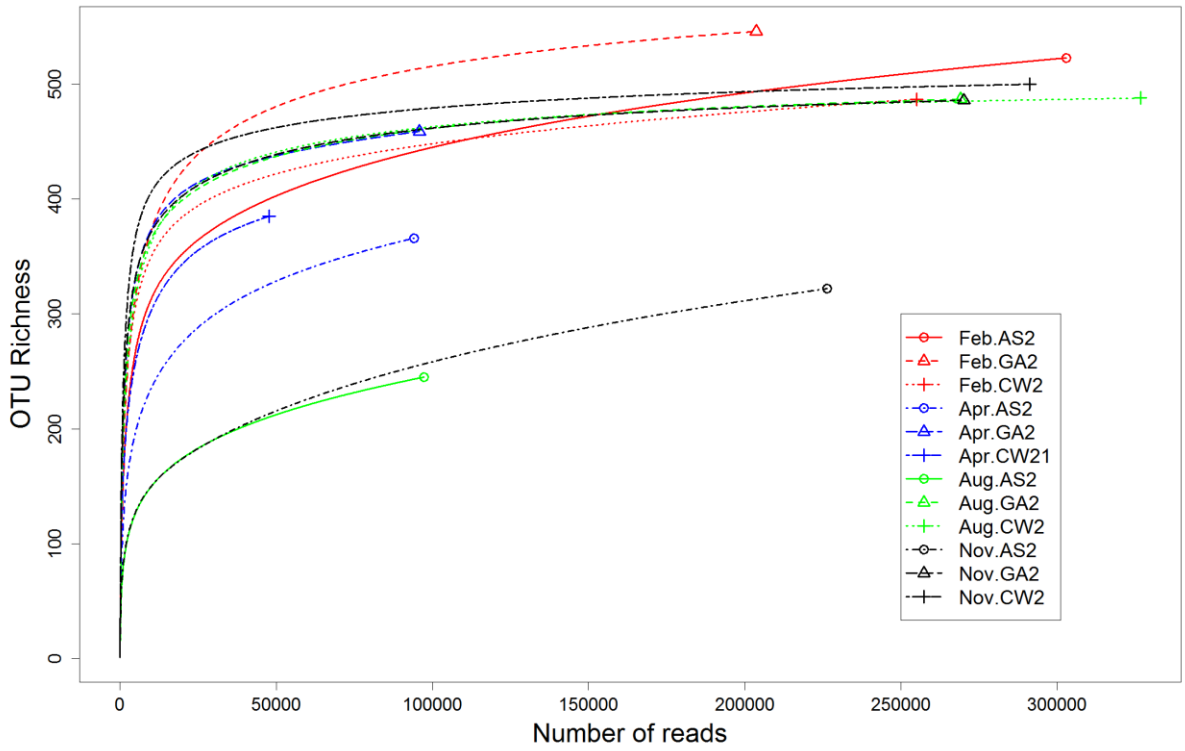

Figure A2.3 Rarefaction curve of 16S rRNA Archaeal soils. Feb = February, Apr = April, Aug= August, Nov = November. AS2 = Clay, CW2 = Chalk, GA2 = Greensand.

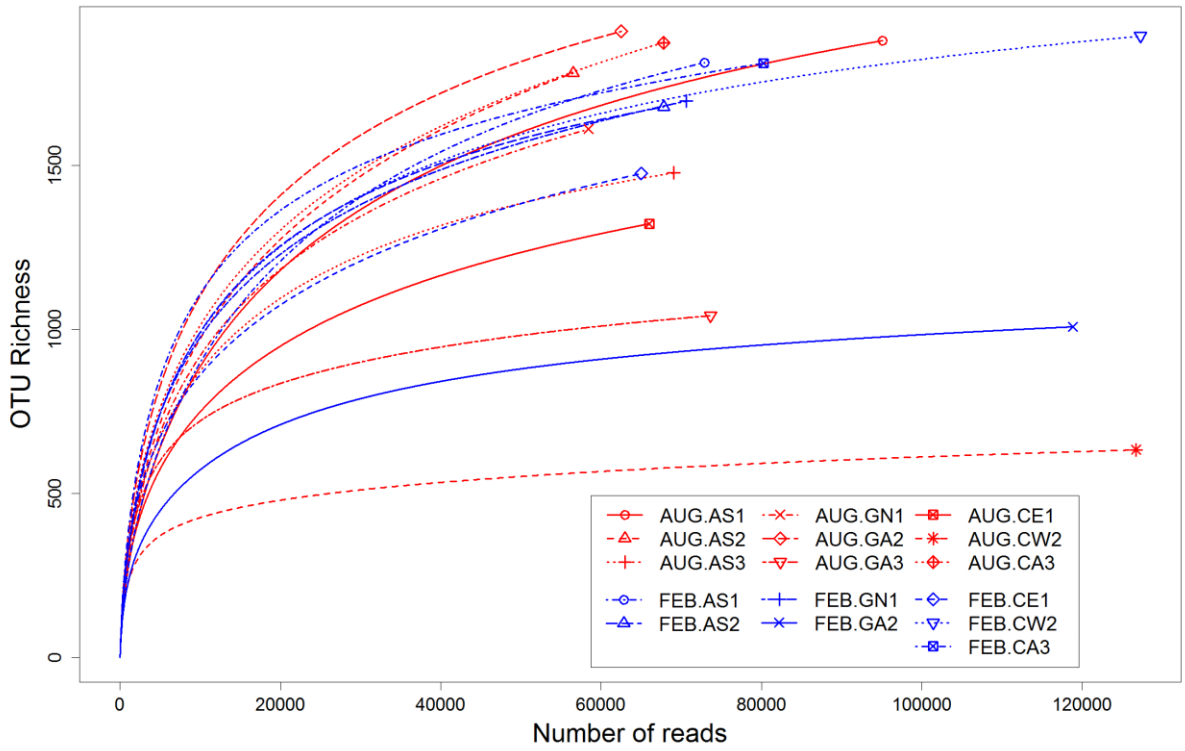

Figure A2.4 Rarefaction curve of 16S rRNA Archaeal sediments. Aug = August, Feb = February. AS1, AS2, AS3 = Clay. CA3, CE1, CW2 = Chalk. GA2, GA3, GN1 = Greensand.

Appendix III: DGGE Gels and Diversity indices

In February clay soils key bands were highlighted from 1-24. Bands 1 and 6 were present in most samples across the entire transect but tended to be lower in abundance. Bands 2, 3, 4 and E were present across most samples and were most abundant in transects A and B. Bands 7, 8, and 9 appeared in most samples but was most abundant in transect C. Band 11 was present across all samples and was highly abundant compared to 1 to 8 bands, with one band that is particularly intense (circled, **Figure A3.1**). Band 13 was present across all samples and like band 12 had one highly intense band (circled **Figure A3.1**), but was less abundant for other samples. Band 14 to 22 was present across all samples with similar intensities in their most abundant samples. Bands 23 and 24 are of similar abundance and their intensity varied slightly between geologies (**Figure A3.1**).

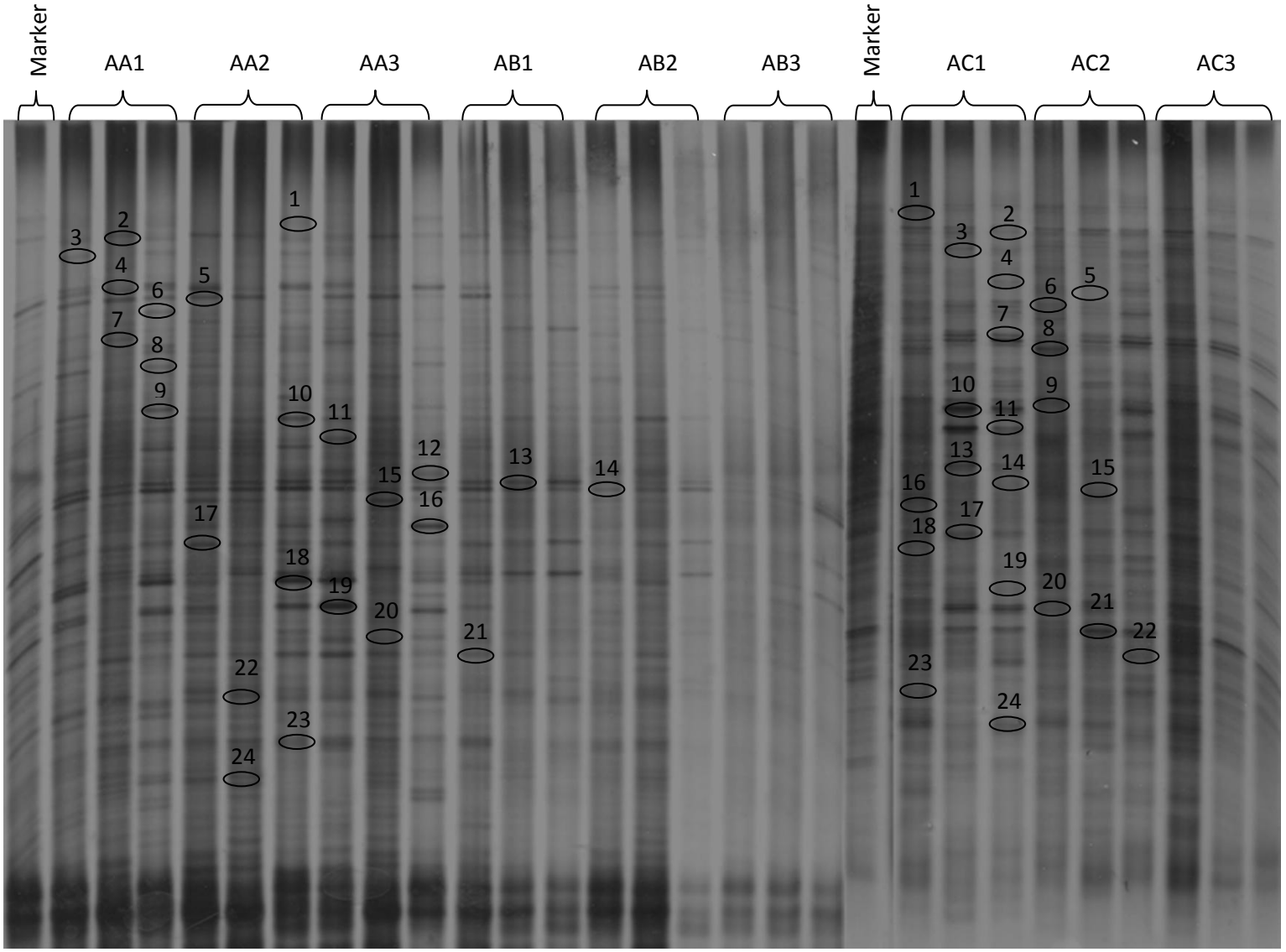

Figure A3.1: DGGE gel of Bacterial 16S rRNA gene of February clay soil sites bands of interest labelled 1-24. AA1-3= Clay transect A, points 1 to 3. AB1-3 = Clay transect B, points 1 to 3. AC1-3 = Clay transect C, points 1 to 3.

In February Greensand soils, key bands were highlighted from 1-24. Bands 1 and 2 were present but was feint compared to the majority of other bands highlighted (**Figure A3.2**). Band 3 and 4 were present across most samples and were most abundant in transects A and C. Bands 5 and 6 appeared in all samples and bands were more intense than 3 and 4 bands. Band 7 is present mainly in transect C with a single a bright band (circled **Figure A3.2**). Band 8 to 12 are present across most samples and was most abundant in the A transect. Bands 13 to 11 was present across all samples and like band 10 had one highly abundant band (circled **Figure A3.2**), but was less abundant for other samples. Band 13 to 19 is highly abundant across all samples particularly transects B and C. Band T had the highest intensity out of all the bands and were present in all samples. Bands 21, 22, 24 and 24 were similar to bands 11 to 13 and were present in all samples. Band Z was present in most samples but present mainly in Transect C (**Figure A3.2**).

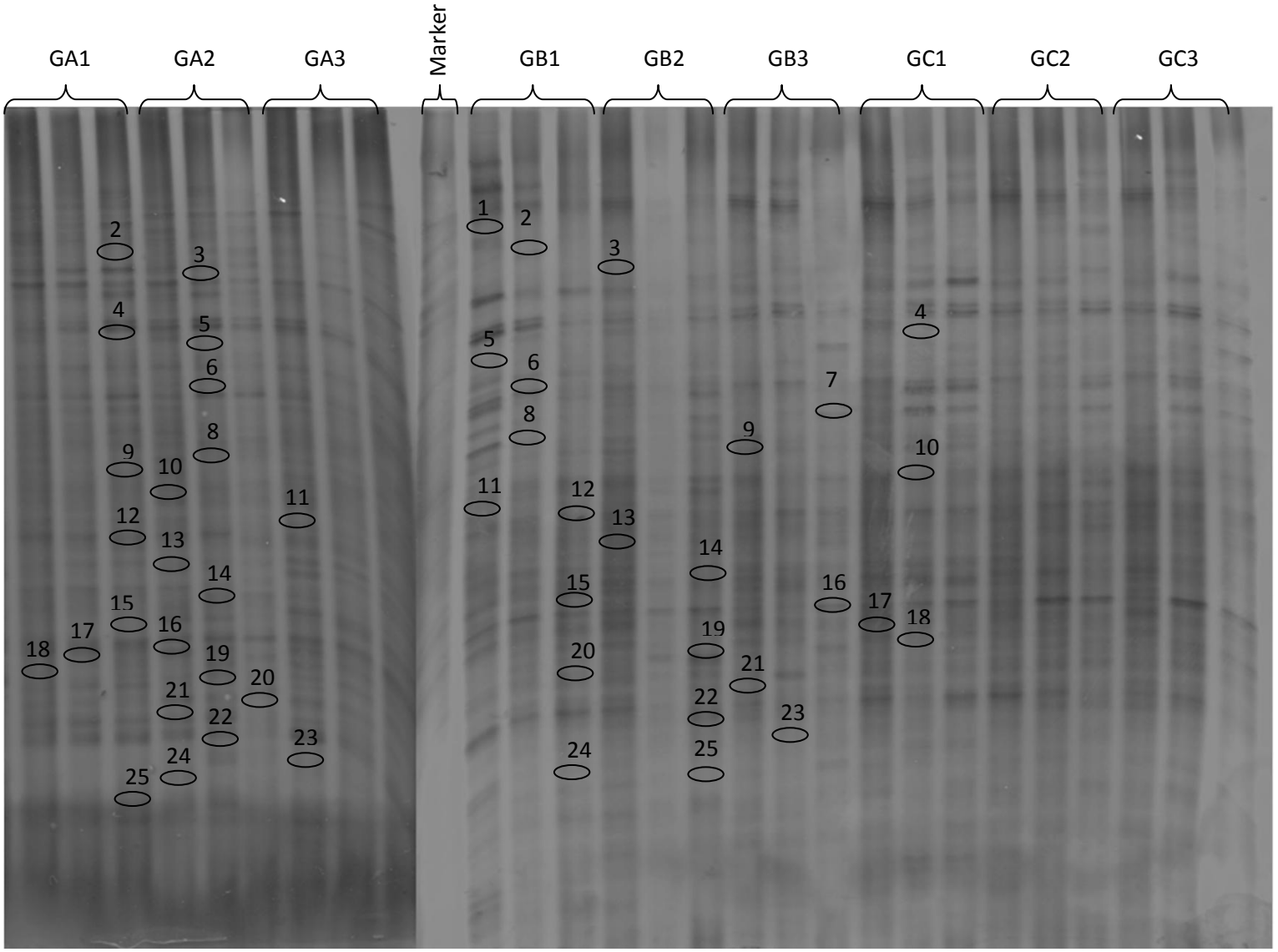

Figure A3.2: DGGE gel of Bacterial 16S rRNA gene of February Greensand soil sites bands of interest labelled 1-25. GA1-3= Greensand transect A, points 1 to 3. GB1-3 = Greensand transect B, points 1 to 3. GC1-3 = Greensand transect C, points 1 to 3

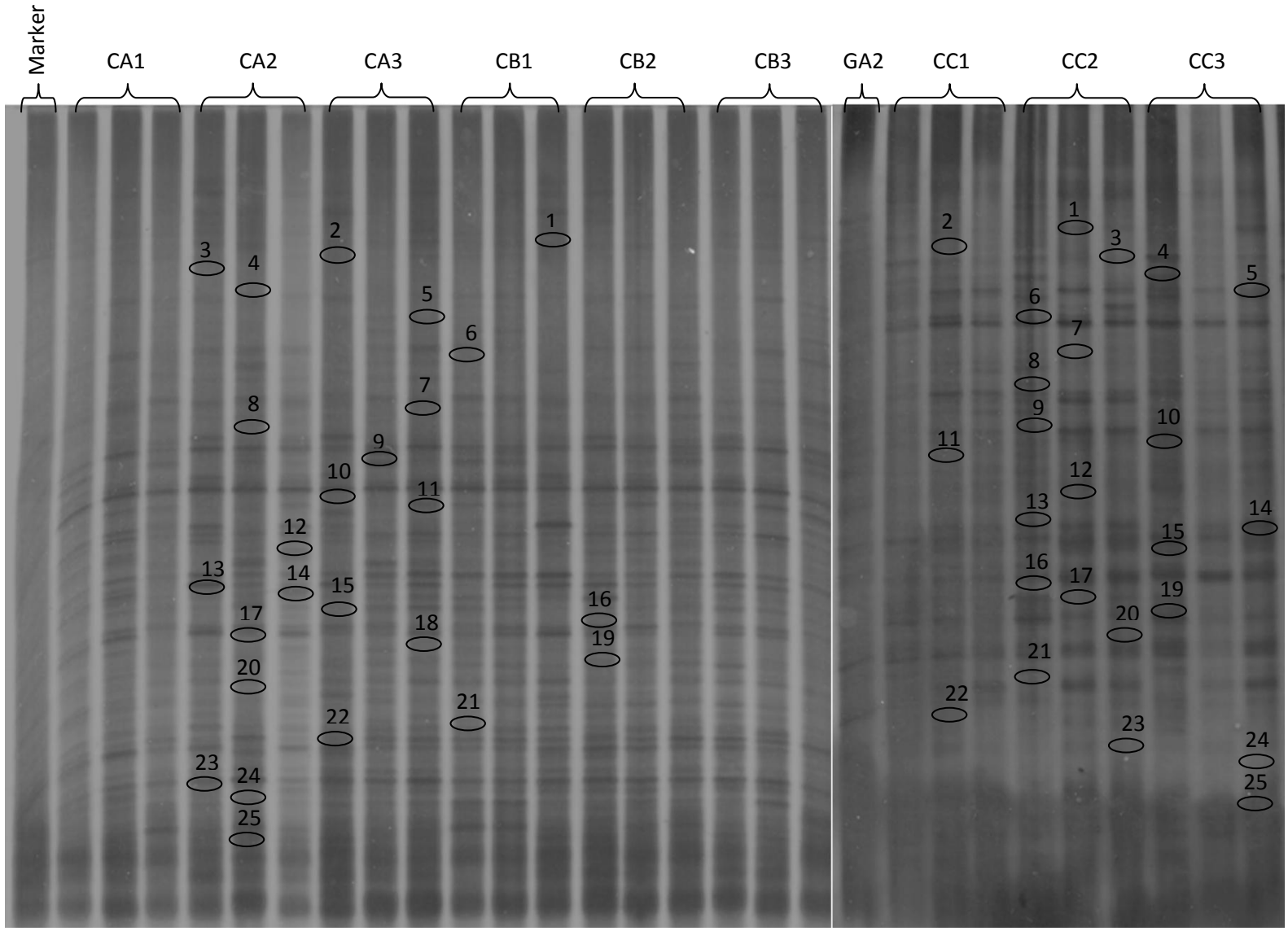

Figure A3.3: DGGE gel of Bacterial 16S rRNA gene of February chalk soil sites bands of interest labelled 1-25. CA1-3= Chalk transect A, points 1 to 3. CB1-3 = Chalk transect B, points 1 to 3. CC1-3 = Chalk transect C, points 1 to 3.

In February chalk soils, key bands were listed from 1-25. Bands 1 to 4 were feint compared to the other bands but were present in the majority of samples (**Figure A3.3**). Bands 5 and 6 were similar to bands 1 to 4 but were present across all samples. Although bands 7 and 8 were present in all samples the most intense bands were in transect C. Band 9 and 10 was present across most samples with the majority in transects A and B. Bands 11 and 12 was present across all samples, with the majority of the most intense bands in transect A and B. Bands 13, 14, and 15 were present in most samples within the A and B transect. Bands 16 to 20 were present in all the samples and at similar intensities throughout. Bands 21, 22, and 23 were present mainly in the A and B transect with bands in transect C present in lower abundance. Bands 24 and 25 were present in one or two samples in transect C but was present in all samples from transect A and B (**Figure A3.3**).

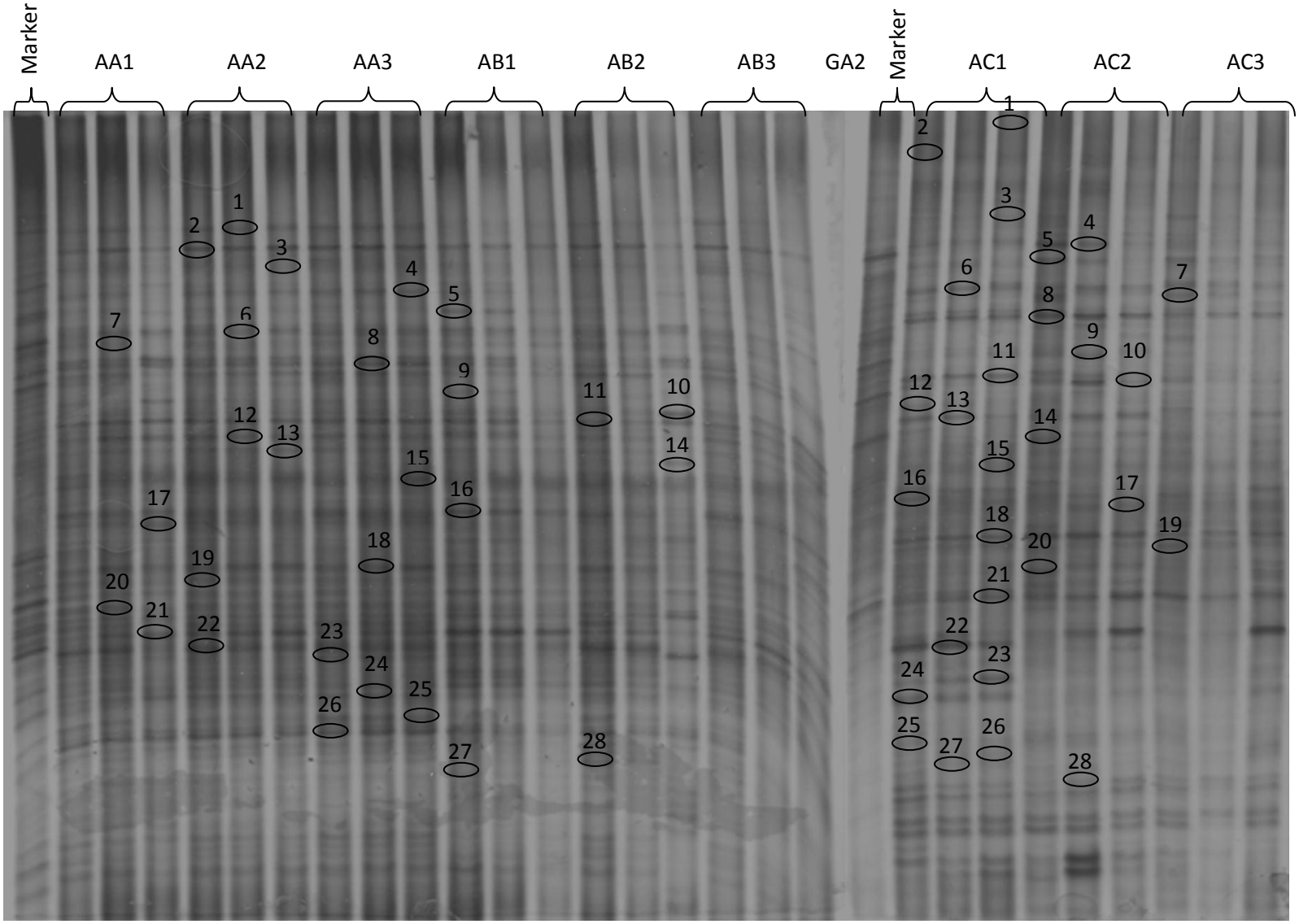

Figure A3.4: DGGE gel of Bacterial 16S rRNA gene of August clay soil sites bands of interest labelled 1-28 AA1-3= Clay transect A, points 1 to 3. AB1-3 = Clay transect B, points 1 to 3. AC1-3 = Clay transect C, points 1 to 3.

In August clay soils, bands of interest were highlighted from 1-28 (**Figure A3.4**). Bands 1 and 2 were present in all the transects but were fainter in transect C. Band 3 although present in all the samples was fainter than bands 1 or 2. Bands in E were present in all the samples but bands were fainter in transect B. Band 6 is present throughout with similar intensity in each band. Band 7 was present in all samples although those in transect C was much fainter than those in transects 1 and 2. Band 8 was present throughout at similar intensities. Band I was present faintly in the majority of samples. Bands 10 and 11 were present at roughly the same intensity in all the samples. Band 12 was present in all samples but was faint in samples from transect C. Band M was present in all samples with several high intensity bands in transect C. Band N was present in all samples at the same intensity. Bands 15, 16 and 18 were similar band intensities in transects A and B, with slightly fainter bands in transect C. Band S were high intensity in all sampels. Bands 20 and 21 were most intense in transects A and B with slightly fainter bands in transect C. Band 22 and 24 were highly abundant in transect A and slightly less abundant in transects B and C. Band 25 to AB were highly abundant in transect A and B and significantly less abundant in transect C. Bands 4 and 27 was faint across all samples (**Figure A3.4**).

In February sediments, key bands are circled and labelled from 1-15 (**Figure A3.5**). The bands highlighted were dominant across most geologies. Band 12 for example, was present across all sediments. Band 8 and 11 was also similar to band 12 although many bands in the clay site were less abundant. Band 2 was present in most river samples but was particularly abundance in the clay geology. Band 3 was also most intense in clay and in Greensand. Bands 4, 5, and 6 were less intense across the river sites overall but had between one and three high intensity bands present in all the geologies (**Figure A3.5**).

In August sediments key bands were highlighted from 1-17 (**Figure A3.6**). Band 1 and 2, were present mainly in chalk and Greensand geologies with intermittent bands found in clay (**Figure A3.6**). Band 3 and 4 were present mainly in chalk geologies and largely absent in clay and Greensand. Bands 5, 6 and 7 were present in the majority of samples across all geologies. Bands 8 to 15 were present across all geologies with bands 13 and N having the highest intensity bands. Band 17 was present across all geologies but was intermittent between samples (**Figure A3.6**).

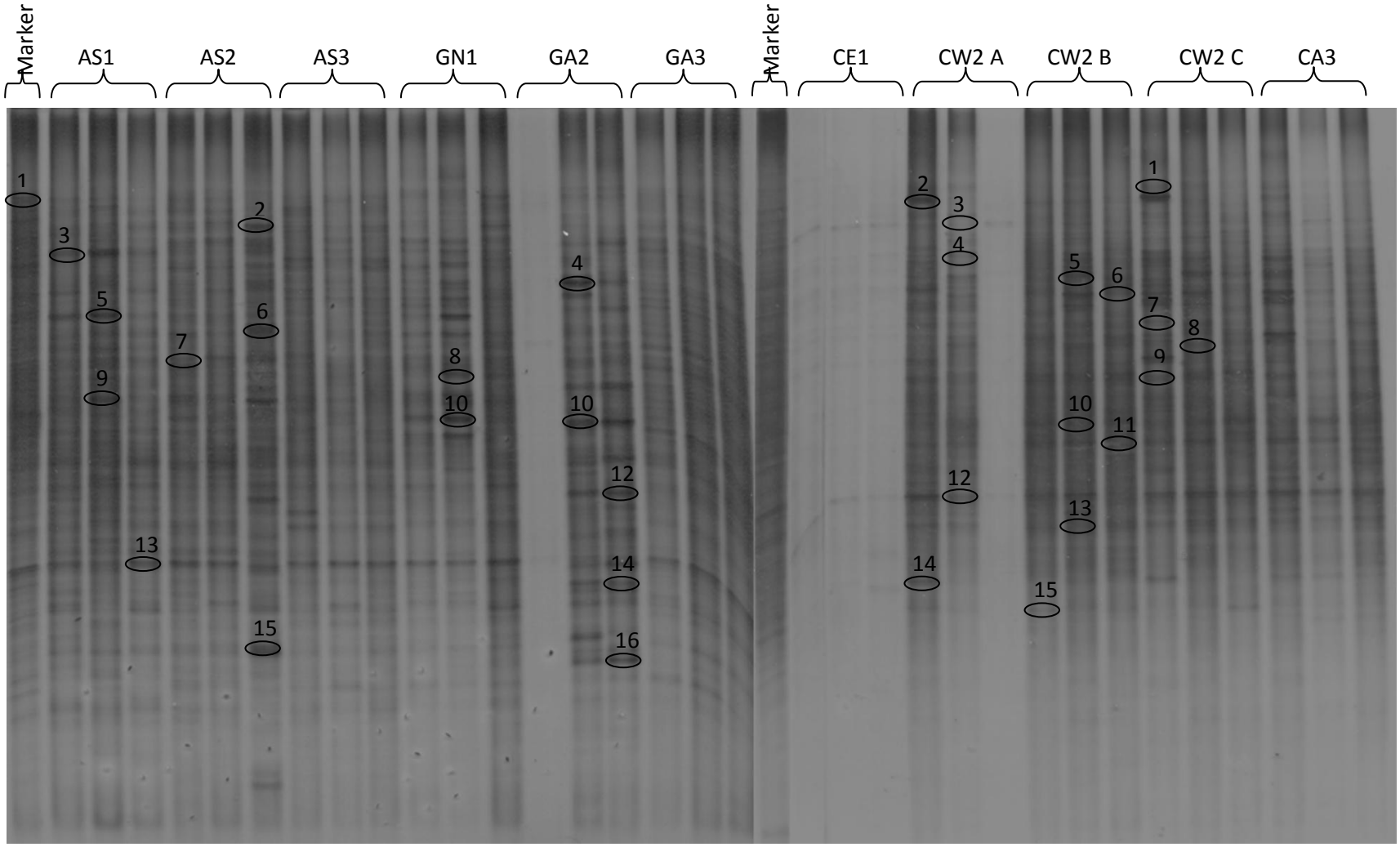

Figure A3.5: DGGE gel of Bacterial 16S rRNA genes in February sediments. AS1, AS2, AS3 are the clay sites, GN1, GA2, GA3 are Greensand sites, and CE1, CW2, and CA3 are the chalk sites (see Table 1 Section 2.2.1 for site codes). Bands of interest labelled 1-17.

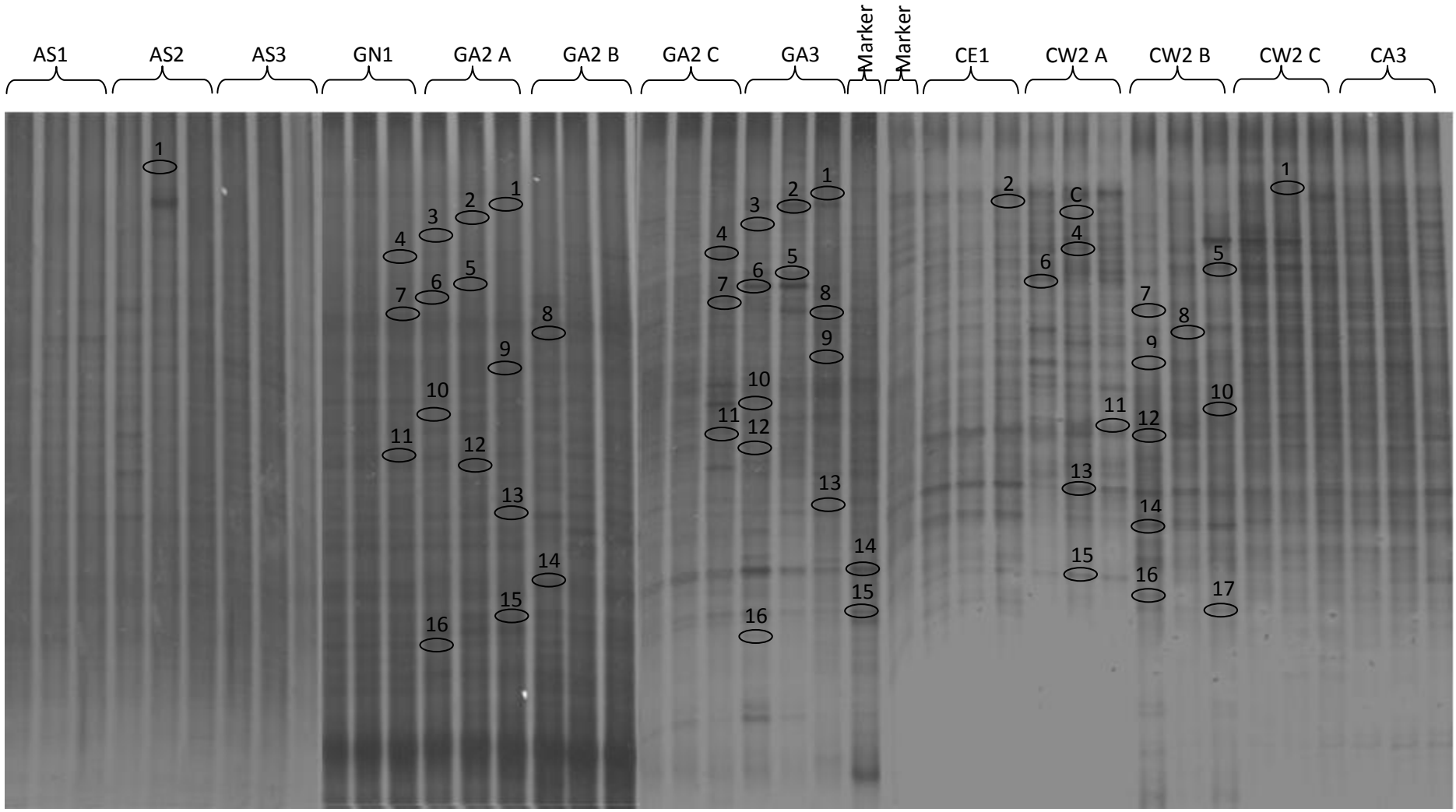

Figure A3.6: DGGE gel of Bacterial 16S rRNA gene in August sediments. AS1, AS2, AS3 are the clay sites, GN1, GA2, GA3 are Greensand sites, and CE1, CW2, and CA3 are the chalk sites (see Table 1 Section 2.2.1 for site codes). Bands of interest labelled 1-17.

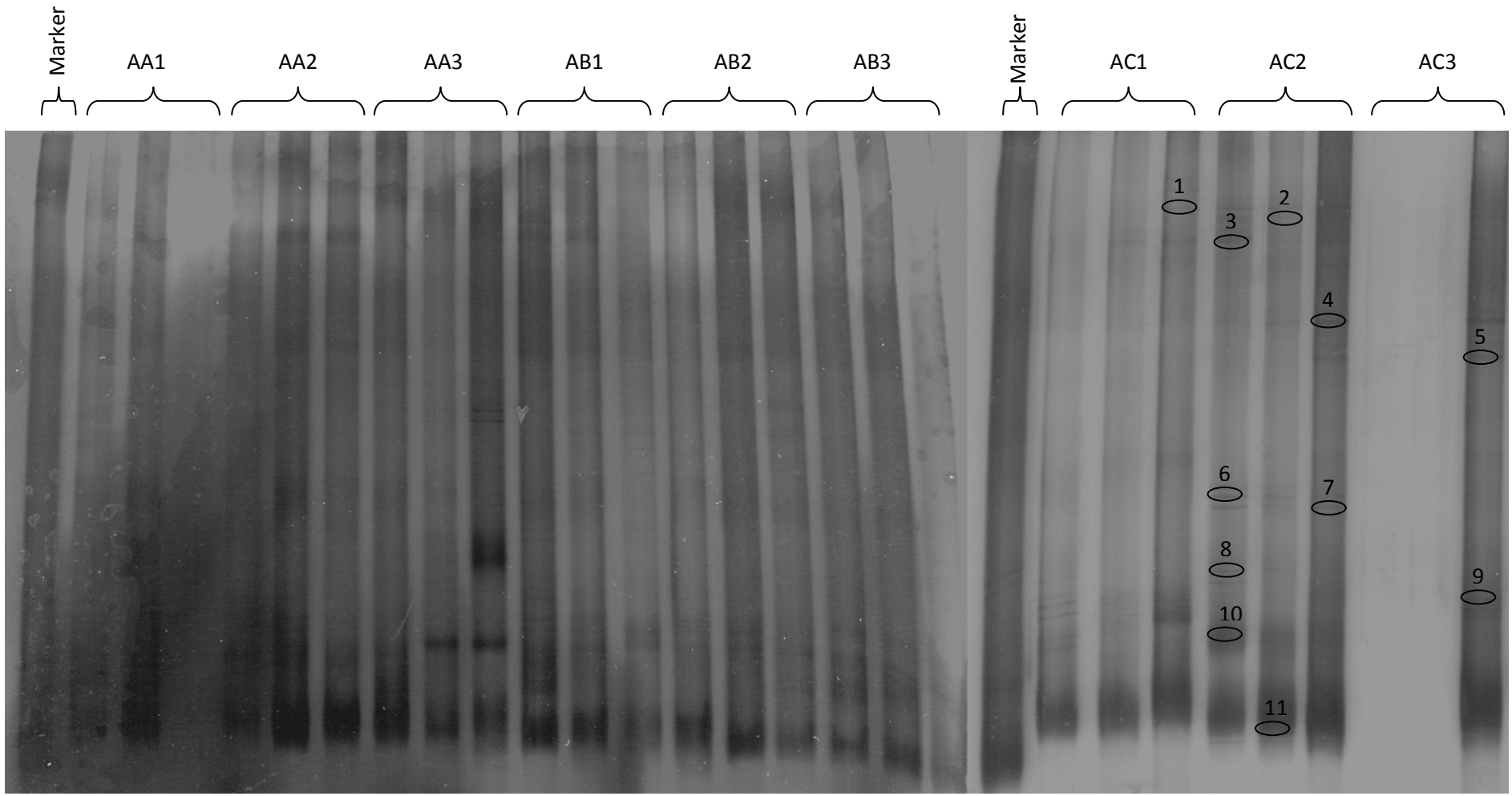

Figure A3.7: DGGE gel of Archaeal 16S rRNA gene of February clay soil sites. Bands of interest were labelled as 1 to 11.

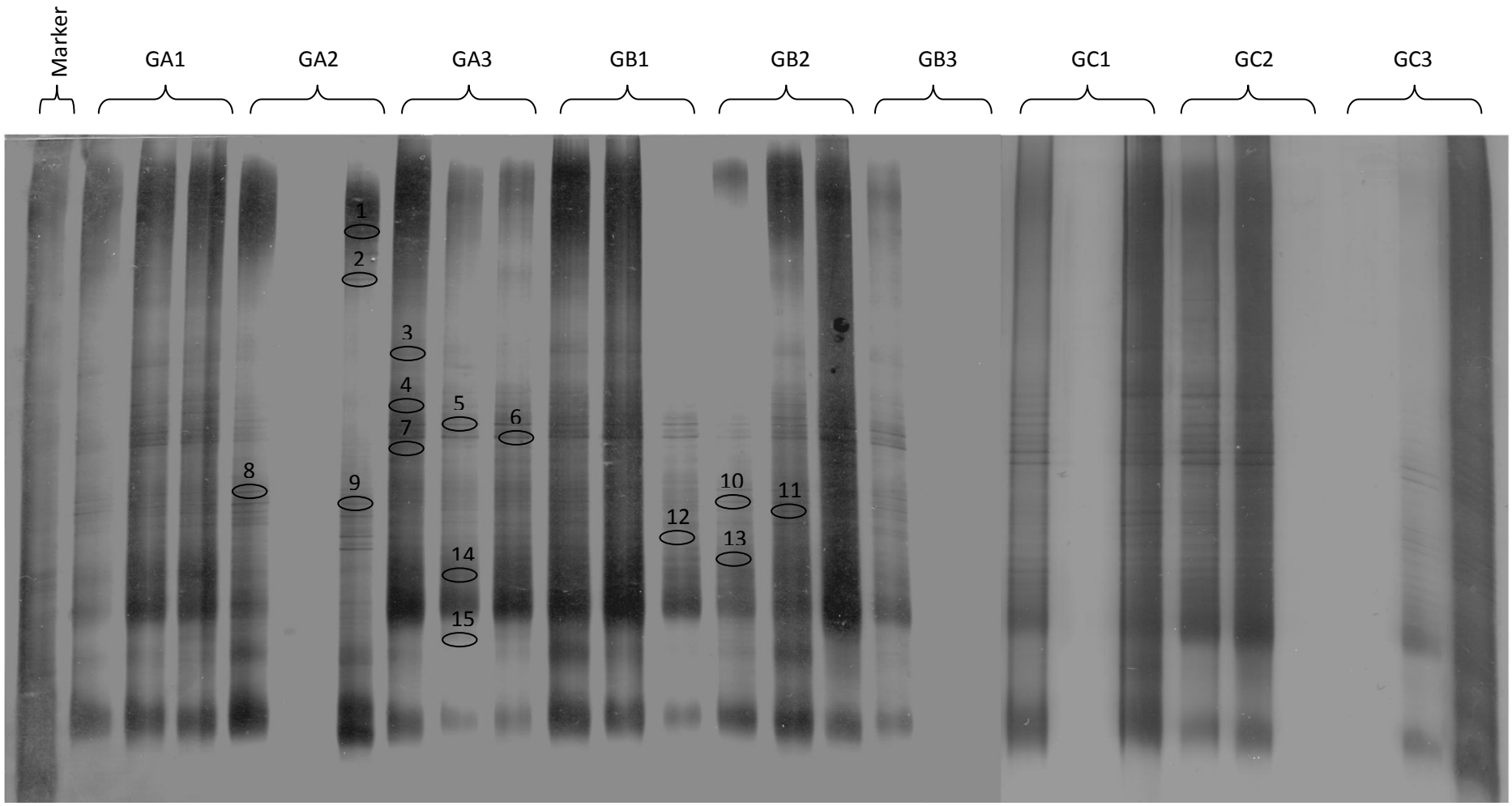

Figure A3.8: DGGE gel of Archaeal 16S rRNA gene of February Greensand soil sites. Bands of interest were labelled as 1 to 15.

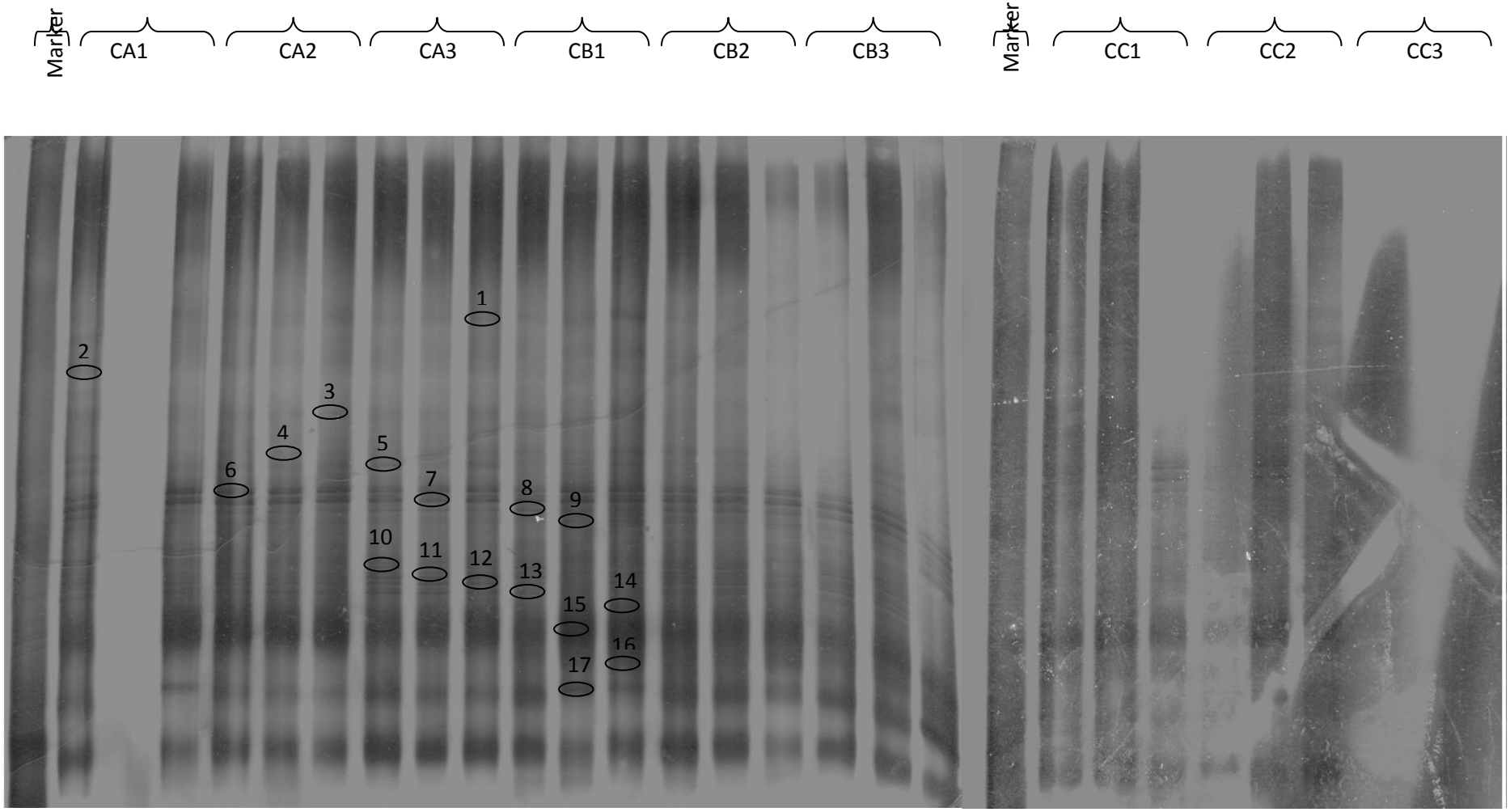

Figure A3.9: DGGE gel of Archaeal 16S rRNA gene of February chalk soil sites. Bands of interest were labelled as 1 to 17.

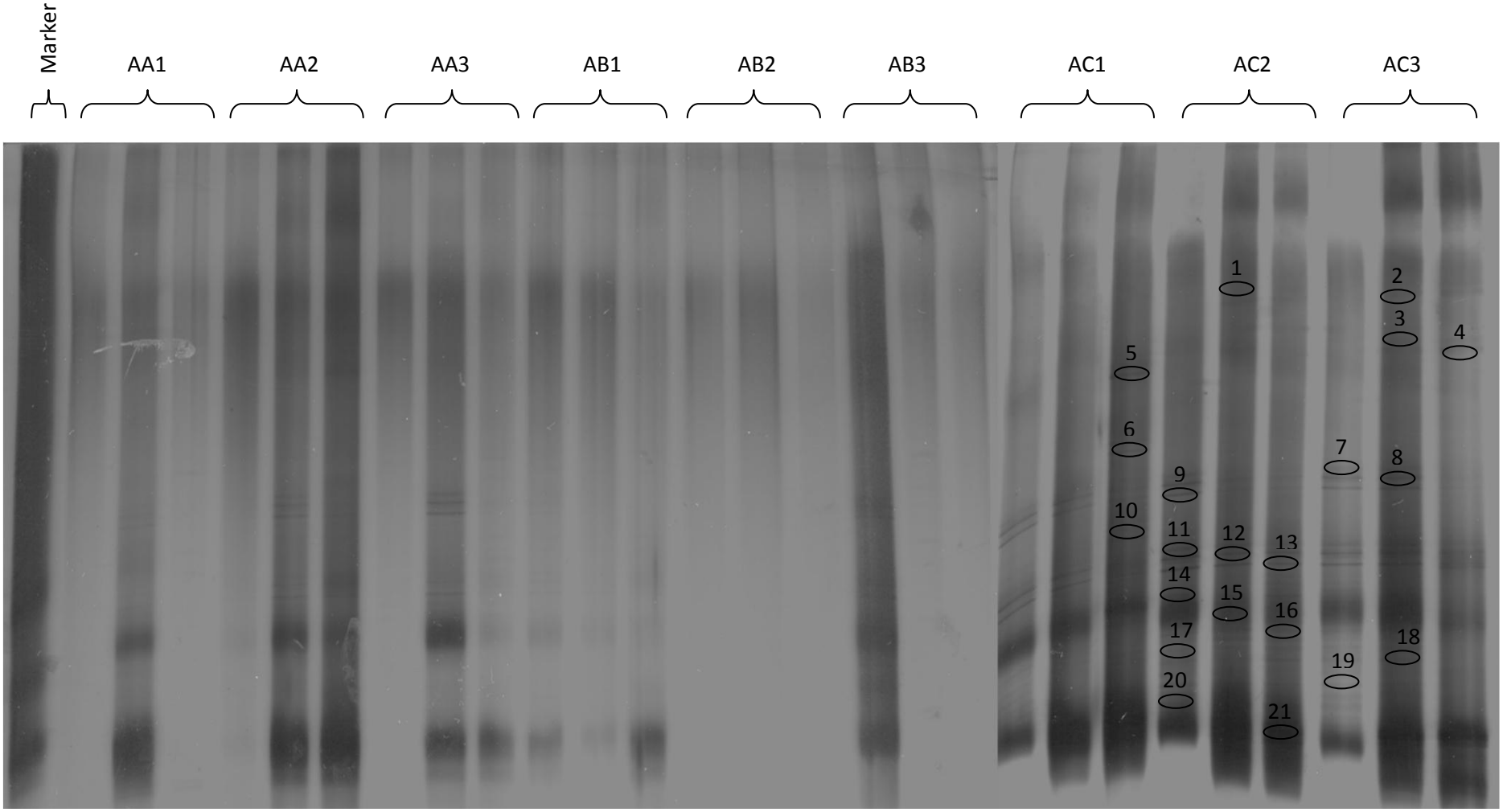

Figure A3.10: DGGE gel of Archaeal 16S rRNA gene of August clay soil sites. Bands of interest were labelled as 1 to 21.

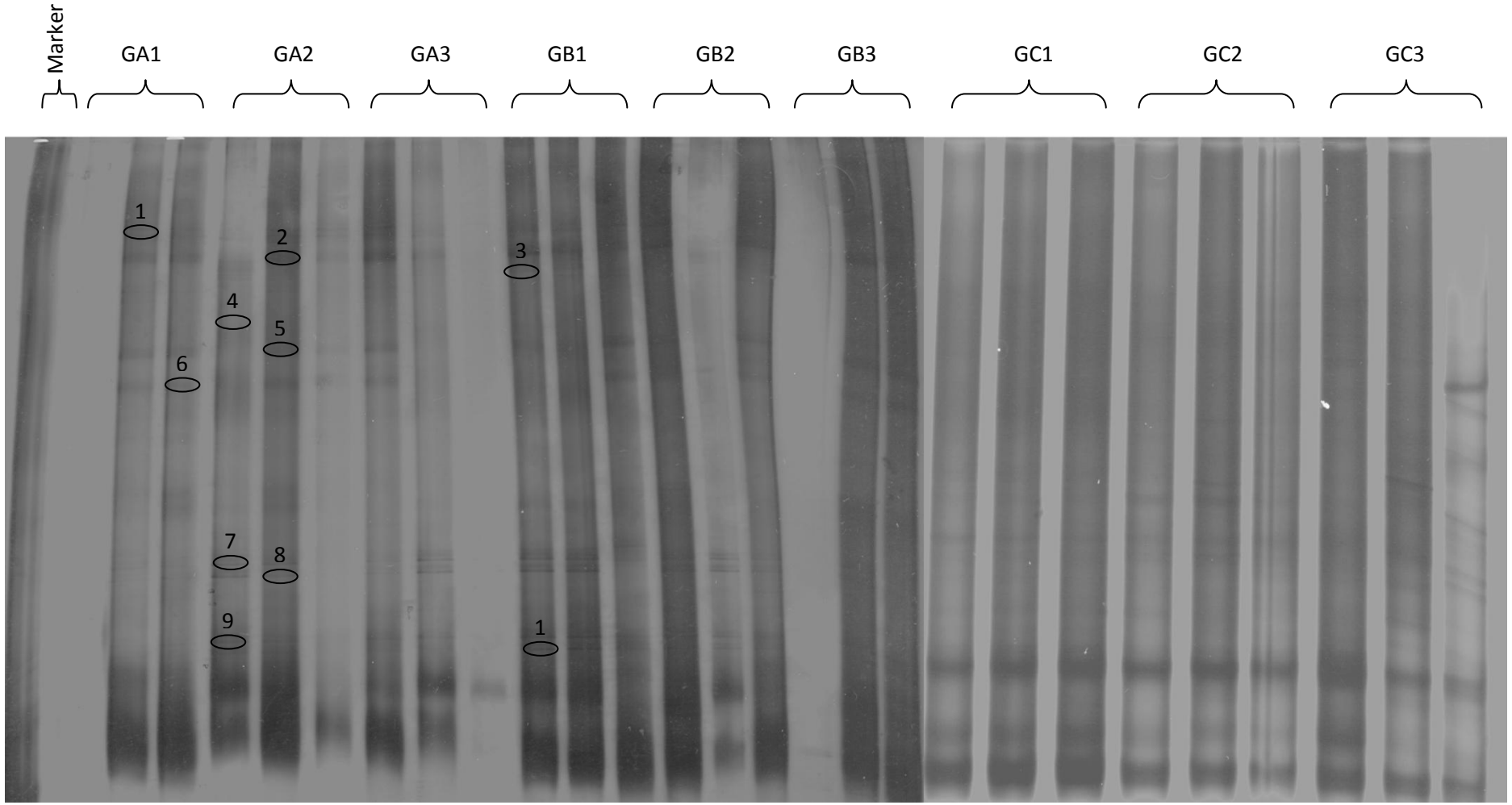

Figure A3.11: DGGE gel of Archaeal 16S rRNA gene of August Greensand soil sites. Bands of interest were labelled as 1 to 10.

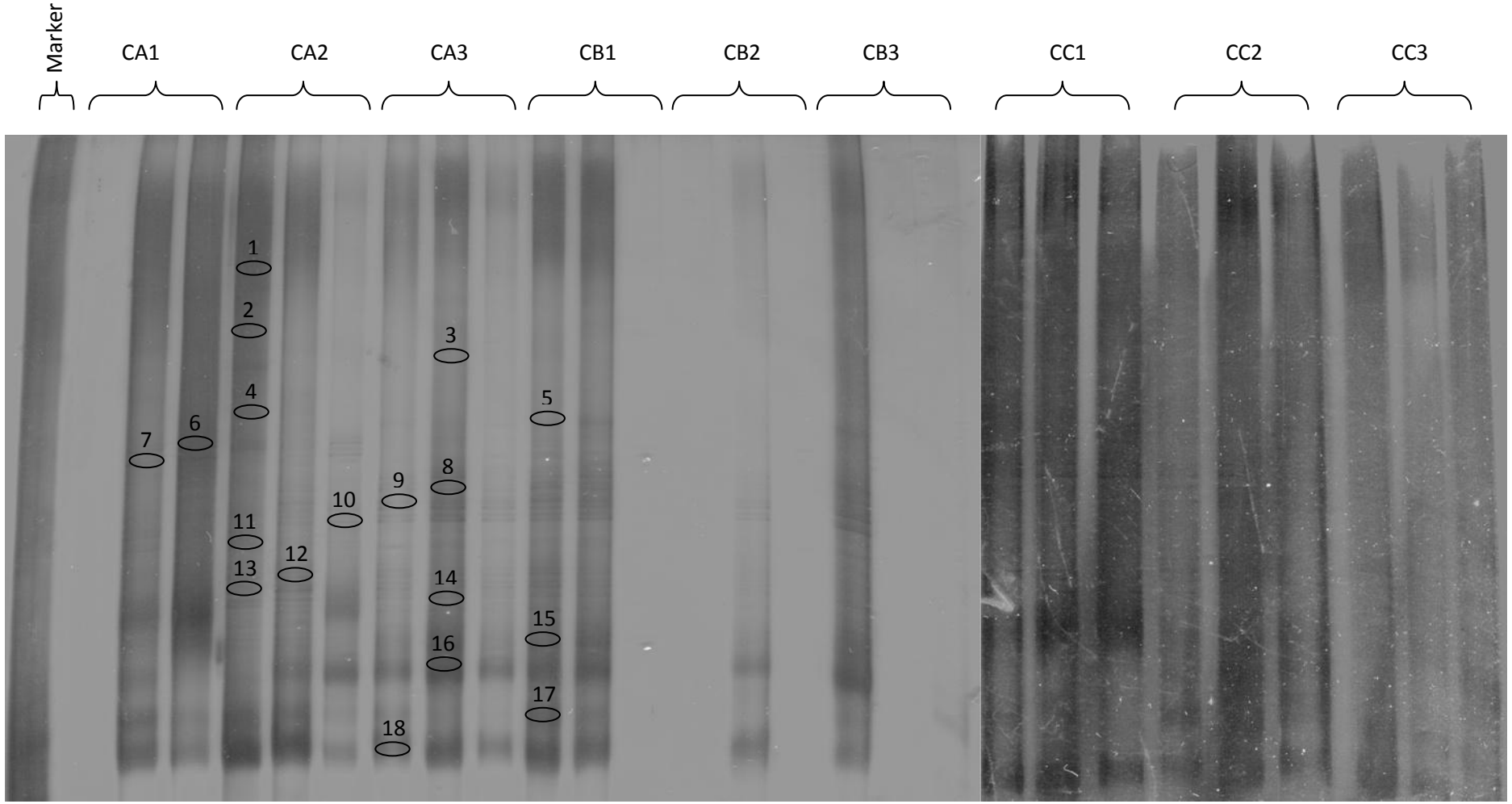

Figure A3.12: DGGE gel of Archaeal 16S rRNA gene of August chalk soil sites. Bands of interest were labelled as 1 to 8.

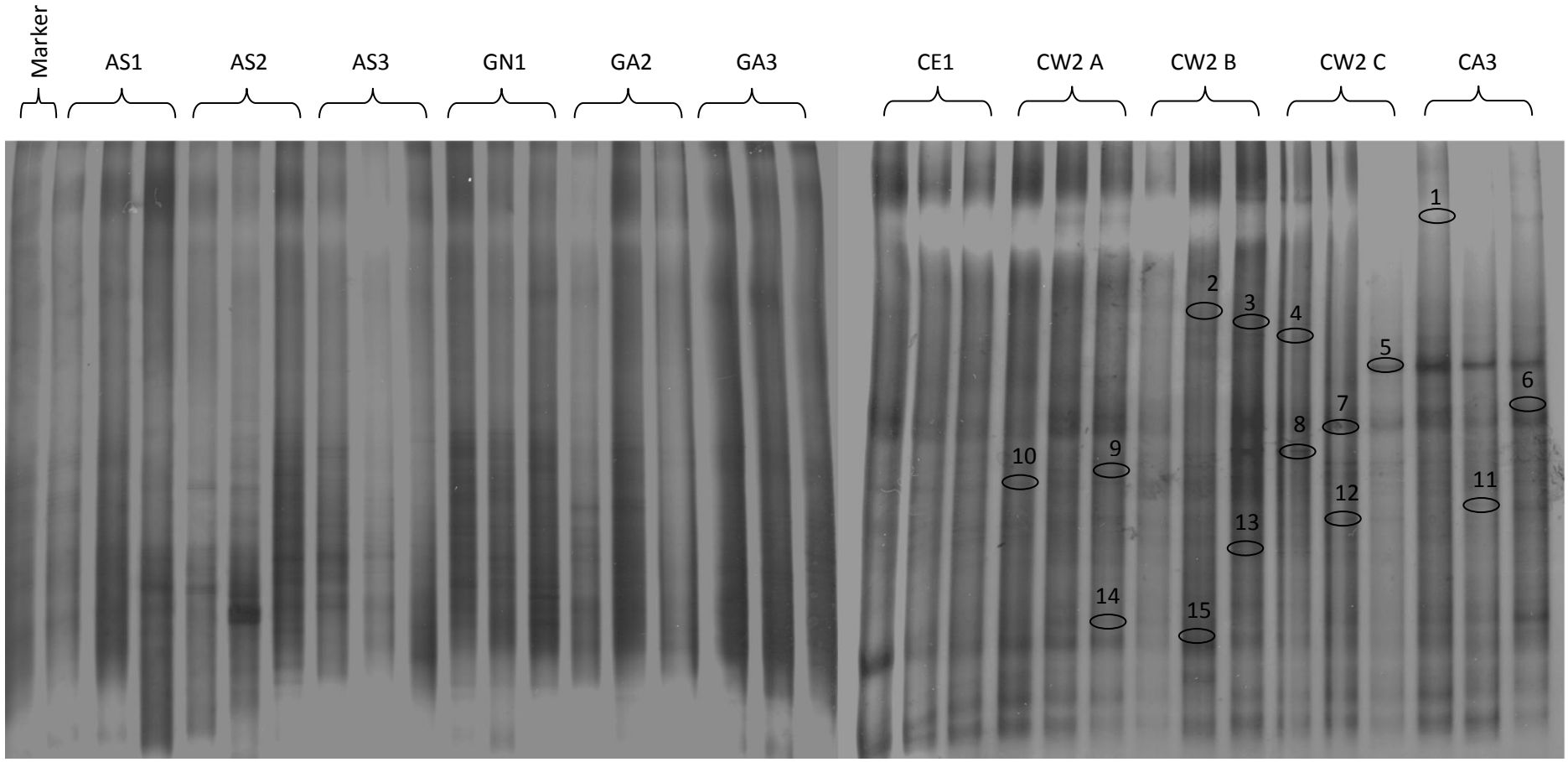

Figure A3.13: DGGE gel of Archaeal 16S rRNA gene of February sediments. Bands of interest were labelled 1 to 15.

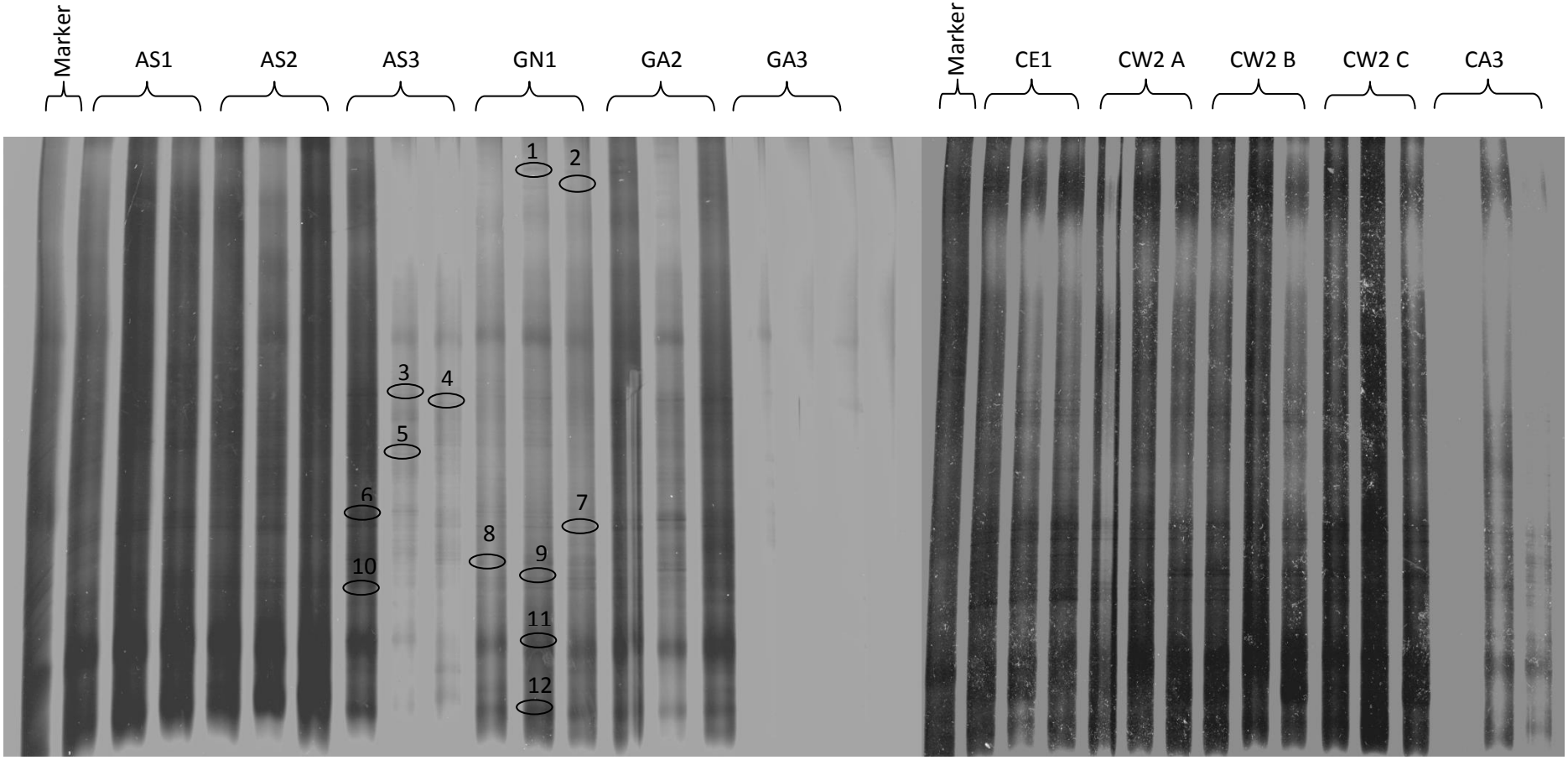

Figure A3.14: DGGE gel of Archaeal 16S rRNA gene of August sediments. Bands of interest were labelled as 1 to 12.

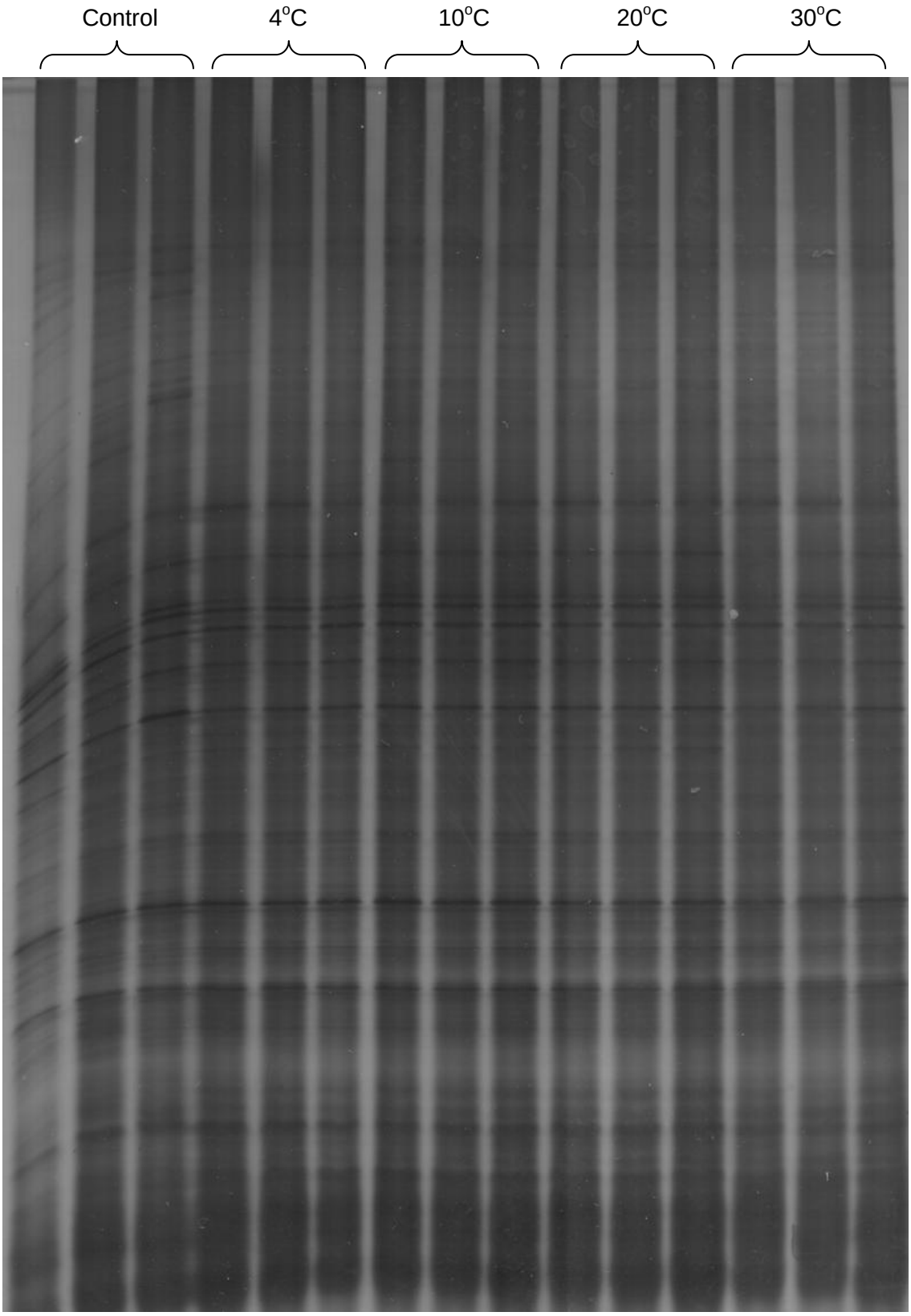

Figure A3.15: DGGE gel of temperature treated Bacterial 16S rRNA gene of summer greensand soils.

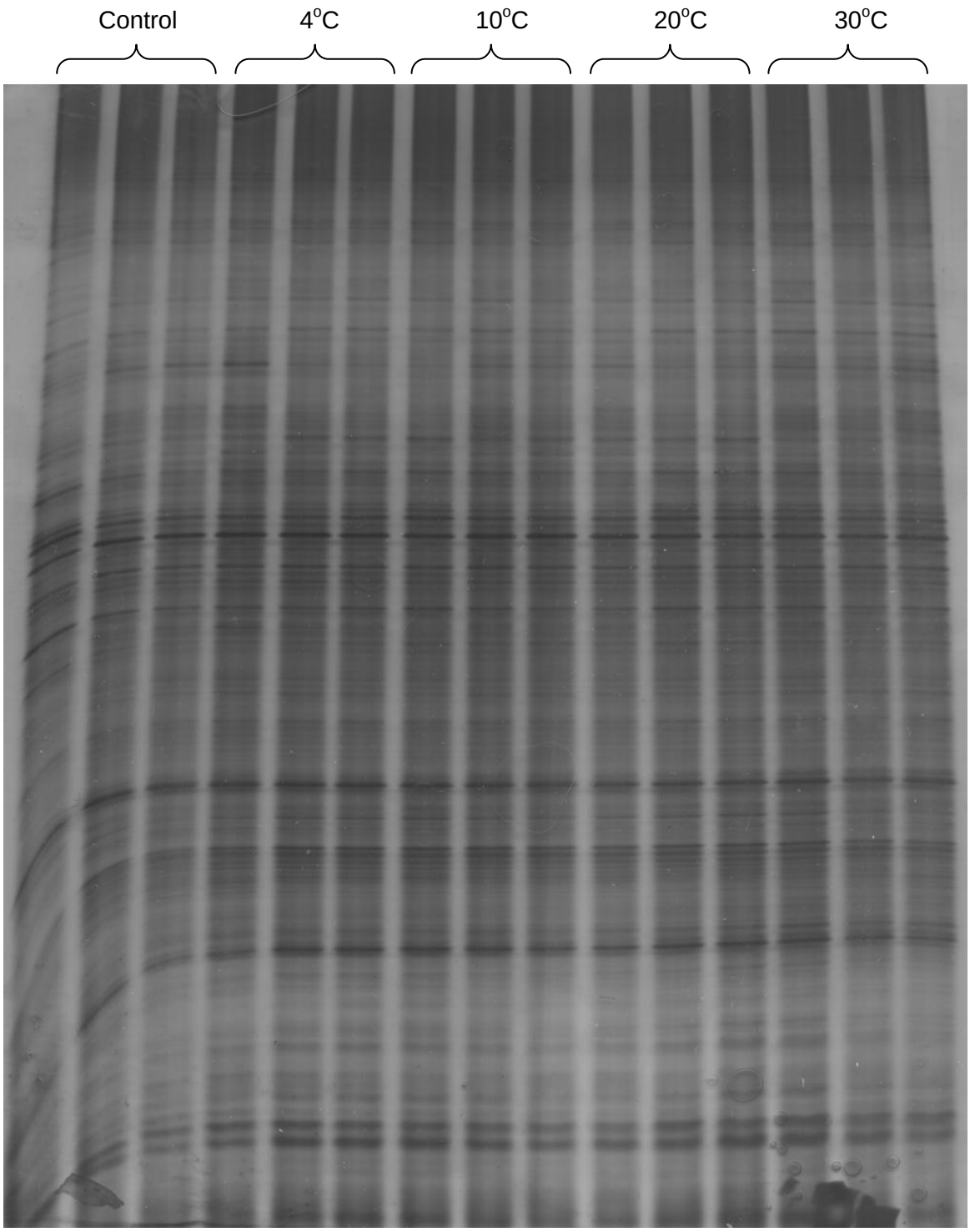

Figure A3.16: DGGE gel of temperature treated Bacterial 16S rRNA gene of winter greensand soils.

DGGE gels for both summer and winter temperature experiment samples showed no marked changes between treatments, with bands remaining the same throughout.

Table A3.1: Shannon and Simpson diversity indices of Bacterial soil community

Sample	Month	Geology	Shannon	Simpson
FEB.AS2	February	Clay	7.053	0.997
FEB.GA2	February	Greensand	7.768	0.999
FEB.CW2	February	Chalk	7.598	0.999
APR.AS2	April	Clay	7.221	0.997
APR.GA2	April	Greensand	7.296	0.999
APR.CW2	April	Chalk	7.593	0.999
AUG.AS2	August	Clay	6.802	0.996
AUG.GA2	August	Greensand	7.812	0.999
AUG.CW2	August	Chalk	7.688	0.999
NOV.AS2	November	Clay	6.799	0.996
NOV.GA2	November	Greensand	7.634	0.998
NOV.CW2	November	Chalk	7.668	0.999

Table A3.2: Shannon and Simpson diversity indices of Bacterial sediment community

Sample	Month	Geology	Shannon	Simpson
FEB.AS1	February	Clay	6.626	0.996
FEB.AS2	February	Clay	5.950	0.989
FEB.AS3	February	Clay	6.772	0.996
FEB.GN1	February	Greensand	6.537	0.995
FEB.GA2	February	Greensand	5.971	0.992
FEB.CE1	February	Chalk	6.406	0.994
FEB.CA3	February	Chalk	6.327	0.995
FEB.CW2	February	Chalk	5.254	0.984
AUG.AS1	August	Clay	6.167	0.994
AUG.AS2	August	Clay	6.385	0.996
AUG.AS3	August	Clay	5.920	0.993
AUG.GN1	August	Greensand	6.458	0.997
AUG.GA2	August	Greensand	5.460	0.975
AUG.GA3	August	Greensand	5.864	0.984
AUG.CE1	August	Chalk	5.787	0.991
AUG.CW2	August	Chalk	5.924	0.991
AUG.CA3	August	Chalk	6.063	0.994

Table A3.3: Shannon and Simpson diversity indices of Archaeal soil community

Sample	Month	Geology	Shannon	Simpson
FEB.AS2	February	Clay	3.716	0.936
FEB.GA2	February	Greensand	4.498	0.975
FEB.CW2	February	Chalk	4.626	0.981
APR.AS2	April	Clay	3.580	0.941
APR.GA2	April	Greensand	4.645	0.978
APR.CW2	April	Chalk	4.095	0.971
AUG.AS2	August	Clay	3.007	0.903
AUG.GA2	August	Greensand	4.617	0.978
AUG.CW2	August	Chalk	4.509	0.976
NOV.AS2	November	Clay	3.277	0.928
NOV.GA2	November	Greensand	4.862	0.986
NOV.CW2	November	Chalk	4.867	0.984

Table A3.4: Shannon and Simpson diversity indices of Archaeal sediment community

Sample	Month	Geology	Shannon	Simpson
FEB.AS1	February	Clay	5.127	0.985
FEB.AS2	February	Clay	5.263	0.987
FEB.AS3	February	Clay	5.445	0.988
FEB.GN1	February	Greensand	5.678	0.992
FEB.GA2	February	Greensand	5.334	0.989
FEB.CE1	February	Chalk	5.247	0.987
FEB.CA3	February	Chalk	5.129	0.986
FEB.CW2	February	Chalk	4.476	0.975
AUG.AS1	August	Clay	5.487	0.990
AUG.AS2	August	Clay	5.108	0.985
AUG.AS3	August	Clay	5.124	0.978
AUG.GN1	August	Greensand	5.442	0.990
AUG.GA2	August	Greensand	4.093	0.957
AUG.GA3	August	Greensand	5.381	0.988
AUG.CE1	August	Chalk	5.237	0.985
AUG.CW2	August	Chalk	5.679	0.992
AUG.CA3	August	Chalk	5.545	0.992

Appendix IV: Example Q-PCR Quality checks

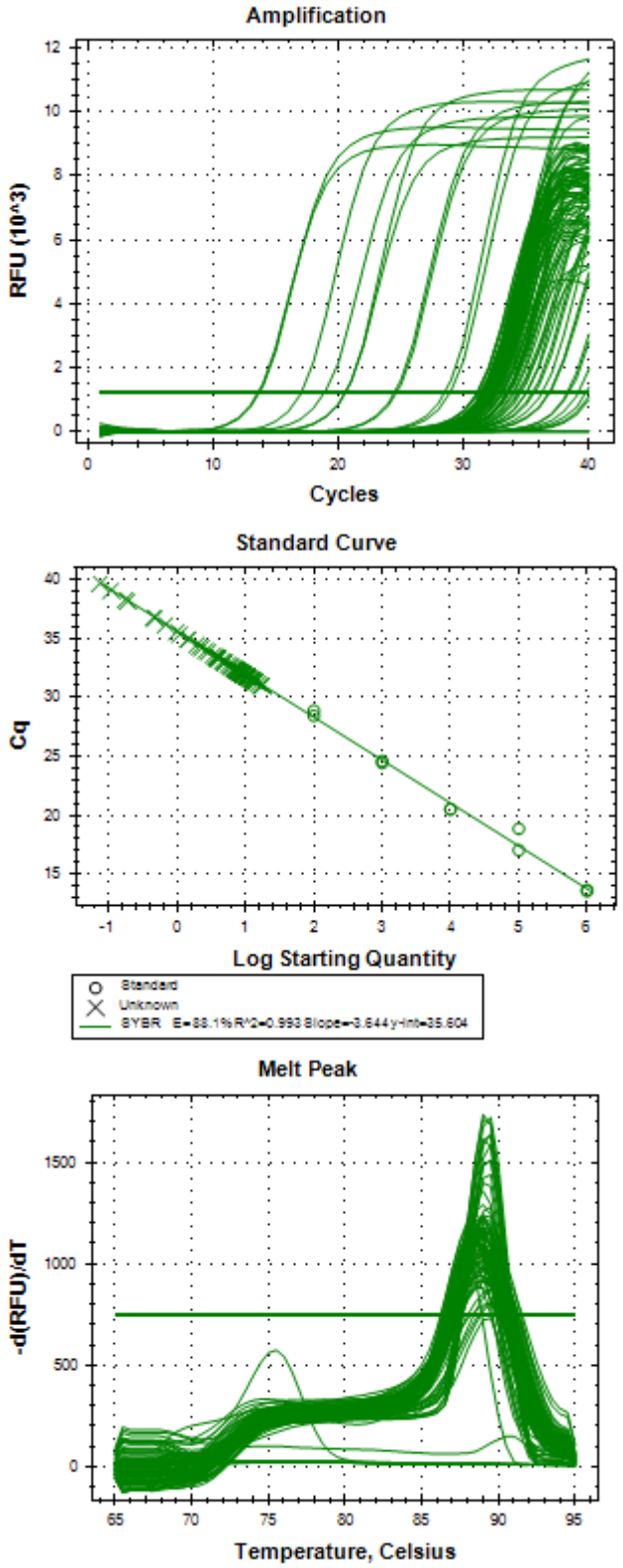

Figure A4.1: Example graphs from November soil QPCR runs A) Amplification chart B) Standard curve chart C) Melt peak charts

Appendix V: Check for homology in *pmoA* and *amoA* alignment

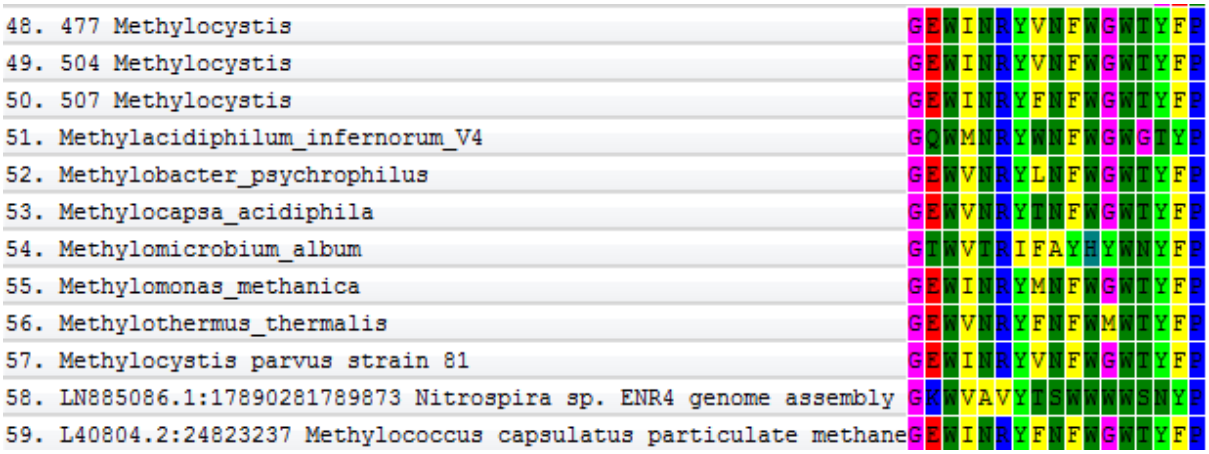

Figure A5.1: Alignment of translated sequences from known partial *pmoA* sequences (51-57) (see **Appendix Table A6.4**), samples (48 - 50), known full *amoA* sequence (58) (Accession number: LN885086), and known full *pmoA* sequence (59) (Accession number: L40804).

Appendix VI: List of sequences used in alignment and phylogenetic tree analysis

Table A6.1: List of A) Bacterial and B) methanotroph 16S rRNA genes used in sequence alignment and phylogenetic tree analysis

A) Name of organism	Accession no.
Desulfobacter vibrioformis strain B54	U12254.1
Chitinophagaceae bacterium PMP191F	PMP191F
Dechloromonas hortensis strain MA-1	AY277621.1
Desulfonatronobacter acidivorans strain APT2	GU289732.1
Eschericia coli	J01859.1
Flavobacterium enshiense DK69	JN790956.1
Ignavibacterium album	AB478415.1
Luteolibacter luojiensis strain DR4-30	NR_109500.1
Methylobacter capsulatus	L20843.1
Nitrospira sp. clone b30	AJ224041.1
Novosphingobium kunmingense strain 18-11HK	JQ246446.1
Ohtaekwangia kribbensis strain 10AO	GU117703.1
Rhizobium etli strain N20	KT883848.1
Rhodospirillales bacterium clone OS2SR34	JN233133.1
Sphaerotilus natans subsp. sulfidivorans strain D-501	FJ871054.1
Xanthomonas axonopodis strain LMG 538	X95919.1
B) Name of organism	Accession no.
Methylobacterium extorquens strain DSM 6343	AB175631.1
Methylocaldum marinum strain S8	NR_126189.1
Methylocapsa acidiphila strain B2	NR_028923.1
Methylocella silvestris strain BL2	AJ491847.1
Methylococcus capsulatus strain CIc	JN166982.1
Methylocystis parvus	AF150805.1
Methylogaea oryzae strain E10	NR_116407.1
Methyloglobulus morosus strain KoM1	JN386974.1
Methylomicrobium album strain BG8	NR_029244.1
Methylomonas methanica strain S1	NR_041815.1
Methyloparacoccus murrellii R-49797	HF558990.1

Methylosarcina fibrata strain AML-C10	AF177296.1
Methylosoma difficile strain LC 2	DQ119050.1
Methylosinus trichosporium	AF150804.1
Methylothermus subterraneus strain HTM55	AF150804.1
Methylacidiphilum fumariolicum strain SolV	AB536747.2
Methylovulum miyakonense strain HT12	AB501287.1

Table A6.2: List of A) Archaeal and B) methanogen 16S rRNA genes used in sequence alignment and phylogenetic tree analysis

A) Name of organism	Accession no.
Thermoplasma acidophilum DSM 1728	NR_028235.1
Desulfurococcus mucosus strain DSM 2162	NR_102882.1
Halobacterium piscisalsi	NR_113057.1
Methanocella paludicola strain SANAE	NR_028164.1
Methanomicrobium mobile BP strain DSM 1539	M59142.1
Thermoproteus tenax strain Kra 1	NR_044683.1
Methanobacterium paludis strain SWAN1	NR_133895.1
Nitrosoarchaeum limnia BG20 clone bBG20-81	KC357905.1
Methanomassiliicoccus luminyensis strain B10	HQ896499.1
Nitrososphaera viennensis strain EN76	NR_134097.1
Methanosarcina barkeri	M59144.1
B) Name of organism	Accession no.
Methanobacterium paludis strain SWAN1	NR_133895.1
Methanocella paludicola strain SANAE	NR_028164.1
Methanocella conradii HZ254	NR_118245.1
Methanomassiliicoccus luminyensis strain	HQ896499.1

B10	
Methanomicrobium mobile BP strain DSM 1539	M59142.1
Methanolacinia paynteri	AY196678.1
Methanospirillum hungatei	NR_074177.1
Methanocorpusculum parvum strain DSM 3823	M59147.1
Methanosarcina barkeri	M59144.1
Methanosaeta thermophila	LN868388.1
Methanococcoides vulcani	NR_133780.1
Methanolobus zinderi strain SD1	EU711413.1

Table A6.3: List of methanogen *mcrA* genes used in sequence alignment and phylogenetic tree analysis

Name of methanogen	Accession no.
Methanocaldococcus fervens AG86	CP001696
Methanocaldococcus infernus ME	CP002009
Methanocaldococcus jannaschii DSM 2661	L77117
Methanocaldococcus villosus KIN24-T80	APMM01000014
Methanocaldococcus vulcanius M7	CP001787
Methanobacterium aarhusense	AY386125
Methanobacterium aggregans	KP006500
Methanobacterium alcaliphilum	AB842184
Methanobacterium bryantii	AB542756
Methanobacterium congolense	AB542748
Methanobacterium espanolae	AB542749
Methanobacterium ferruginis	AB542745
Methanobacterium flexile	HM802935
Methanobacterium formicicum DSM 3637	AMPO01000008
Methanobacterium ivanovii	EF465107
Methanobacterium lacus	CP002551

Methanobacterium movens	HM802934
Methanobacterium oryzae	AB542752
Methanobacterium paludis	CP002772
Methanobacterium palustre	AB542753
Methanobacterium petrolearium	AB542744
Methanobacterium subterraneum	AB542754
Methanobacterium uliginosum	EF465105
Methanobrevibacter arboriphilus	AB300777
Methanobrevibacter oralis	LN876655
Methanobrevibacter ruminantium M1	CP001719
Methanobrevibacter smithii ATCC 35061	CP000678
Methanosphaera stadtmanae DSM 3091	CP000102
Methanothermobacter crinale	HQ283274
Methanothermobacter marburgensis	AB842182
Methanothermobacter tenebrarum	AB523786
Methanothermobacter thermautotrophicus	U10036
Methanothermobacter wolfeii	AB300780
Methanothermus fervidus DSM 2088	X70765
Methanotorris formicicus Mc-S-70	AB353227
Methanotorris igneus Kol 5	AB353228
Methanococcus aeolicus Nankai-3	CP000743
Methanococcus maripaludis	AB703630
Methanococcus vannielii	M16893
Methanococcus voltae A3	CP002057
Methanothermococcus okinawensis IH1	CP002792
Methanothermococcus thermolithotrophicus DSM 2095	AB353226
Methanocella arvoryzae MRE50	AM114193
Methanocella conradii HZ254	CP003243
Methanocella paludicola SANAE	AP011532
Methanocorpusculum labreanum	AY260441
Methanocorpusculum parvum	AY260444
Methanoculleus bourgensis	KF773774
Methanoculleus chikugoensis	AB703635

Methanoculleus marisnigri JR1	CP000562
Methanoculleus palmolei	AB300784
Methanoculleus thermophilus	AF313804
Methanofollis ethanolicus	AB703643
Methanogenium organophilum	AB353222
Methanoplanus limicola	AB703642
Methanoplanus petrolearius DSM 11571	CP002117
Methanolinea mesophila	AB496719
Methanolinea tarda NOBI-1	AGIY01000002
Methanoregula boonei 6A8	CP000780
Methanoregula formicica SMSP	CP003167
Methanoregula formicicum SMSP	AB479391
Methanosphaerula palustris E1-9c	CP001338
Methanospirillum hungatei JF-1	CP000254
Methanospirillum lacunae	AB517988
Methanospirillum psychrodurum	KF153053
Methanosaeta concilii GP6	CP002565
Methanosaeta harundinacea	AB679171
Methanosaeta harundinacea 8Ac	AY970348
Methanosaeta pelagica	AB679169
Methanosaeta sp. HA	LC033612
Methanosaeta thermophila PT	CP000477
Methanococcoides alaskense	AB703632
Methanococcoides burtonii DSM 6242	CP000300
Methanococcoides methylutens MM1	CP009518
Methanohalobium evestigatum Z-7303	CP002069
Methanohalophilus halophilus	AB703633
Methanohalophilus mahii DSM 5219	AB353223
Methanohalophilus portucalensis FDF-1	AB908273
Methanolobus profundi	AB703629
Methanolobus psychrophilus R15	CP003083
Methanolobus tindarius DSM 2278	AZAJ01000001
Methanolobus zinderi	EU715818

Methanomethylovorans hollandica	AY260442
Methanomethylovorans thermophila	KP109831
Methanomethylovorans uponensis	KC876049
Methanosalsum zhilinae DSM 4017	CP002101
Methanosarcina acetivorans C2A	AE010299
Methanosarcina baltica	LC015100
Methanosarcina horonobensis HB-1	CP009516
Methanosarcina lacustris Z-7289	CP009515
Methanosarcina mazei	JJPX01000018
Methanosarcina siciliae C2J	CP009508
Methanosarcina soligelidi	KJ432634
Methanosarcina spelaei	CP009503
Methanosarcina subterranea	AB288268
Methanosarcina thermophila CHTI-55	CP009502
Methanosarcina vacuolata Z-761	CP009520
Methermicoccus shengliensis	EF026570
Methanopyrus kandleri AV19	AE009439
Methanomassiliicoccales archaeon RumEn M2	LJKL01000008

Table A6.4: List of methanogen *mcrA* genes used in sequence alignment and phylogenetic tree analysis

Name of methanotroph	Accession no.
Candidatus Methylomirabilis oxyfera	FP565575
Methylocapsa acidiphila	CT005238
Methylocapsa aurea	FN433470
Methylocystis bryophila	FN422005
Methylocystis echinoides	AJ459000
Methylocystis heyeri	AM283546
Methylocystis hirsuta	DQ364434
Methylocystis parvus OBBP	U31651
Methylocystis rosea SV97	AJ414657
Methylosinus acidophilus	DQ076755
Methylosinus sporium	AJ459005
Methylosinus trichosporium OB3b	ADVE01000127
Methylococcaceae bacterium ET-HIRO	AB453962
Methylobacter marinus	EU722430
Methylobacter psychrophilus	AY945762
Methylobacter tundripaludum SV96	AJ414658
Methylocaldum gracile	U89301
Methylocaldum marinum	AB900159
Methylocaldum szegediense	U89303
Methylocaldum tepidum	U89304
Methylococcus capsulatus	AF533666
Methylogaea oryzae JCM 16910	EU359002
Methyloglobulus morosus	JN386975
Methylomagnum ishizawai	AB669168
Methylomarinum vadi	AB453964
Methylomicrobium album	EU722431
Methylomicrobium alcaliphilum 20Z	FO082060
Methylomicrobium buryatense	AF307139
Methylomicrobium japanense	AB253367

Methylomicrobium kenyense	JN687579
Methylomicrobium pelagicum	U31652
Methylomonas koyamae	AB538965
Methylomonas methanica	EU722434
Methylomonas paludis	HE801217
Methyloparacoccus murrellii	AB636304
Methyloprofundus sedimenti	KF484908
Methylosarcina fibrata AML-C10	AF177325
Methylosarcina lacus	AY007286
Methylosarcina quisquiliarum	AF177326
Methylosoma difficile	DQ119047
Methylosoma sp. TFB	GQ130273
Methylovulum miyakonense HT12	AB501288
Methylohalobius crimeensis 10Ki	AB687535
Methylomarinovum caldicuralii	AB302948
Methylothermus subterraneus	AB536748
Methylothermus thermalis	AY829010
Methylacidiphilum fumariolicum SolV	CAHT01000053
Methylacidiphilum infernorum V4	CP000975
Methylacidiphilum kamchatkense	JQ034364

Appendix VII: Statistical Analyses

Table A7.1: Summary of multiple ANOVA of major OTU groups in 2013 soils:

	Ellin6529	Saprospirales	Burkholderiales	Solirubrobacterales	Bacillales	Solibacterales
R^2	0.724	0.788	0.547	0.611	0.376	0.928
F	8.582	12.167	3.944	5.132	1.97	42.106
Pr > F	< 0.0001	< 0.0001	0.001	< 0.0001	0.062	< 0.0001
	Rhodospirillales	Pedosphaerales	Gaiellales	Acidobacteriales	Myxococcales	Acidimicrobiales
R^2	0.684	0.444	0.587	0.878	0.534	0.766
F	7.075	2.617	4.657	23.446	3.754	10.721
Pr > F	< 0.0001	0.015	0	< 0.0001	0.001	< 0.0001
	Xanthomonadales	Actinomycetales	Rhizobiales	Chthoniobacterales	Acidobacteria-6	Other Bacteria
R^2	0.682	0.536	0.534	0.651	0.834	0.751
F	7.021	3.787	3.743	6.117	16.417	9.889
Pr > F	< 0.0001	0.001	0.001	< 0.0001	< 0.0001	< 0.0001

Table A7.2A: Pearson's correlation matrix of 16S rRNA gene sequences with anions and cations in 2013 soils

Variables	H_2O%	Porosity	Acetate	Flouride	Formate	Nitrate	Nitrite	Phosphate	Sulfate
Simpson Diversity	0.05	0.09	**0.42**	0.23	**0.39**	**-0.49**	0.04	**0.48**	**-0.8**
Ellin6529	0.04	-0.25	0.01	-0.13	-0.11	0.07	0.25	0.08	-0.27
Saprospirales	0.19	**0.3**	-0.1	0.17	-0.03	-0.03	0.11	**0.53**	-0.07
Burkholderiales	0.06	0.08	-0.1	0.01	-0.08	0.11	0.17	**0.38**	-0.2
Solirubrobacterales	-0.04	-0.28	-0.06	-0.2	-0.12	**0.41**	-0.08	**-0.29**	0.23
Bacillales	0.03	-0.23	0.08	-0.05	-0.03	0.01	-0.08	-0.19	0.17
Solibacterales	**-0.34**	0.13	-0.19	-0.14	-0.13	-0.19	-0.11	**-0.44**	0.08
Xanthomonadales	0.08	0.17	-0.1	0.1	-0.04	-0.08	0.16	**0.51**	-0.19
Rhodospirillales	0.15	-0.26	0.28	0.1	0.23	**0.46**	**-0.38**	-0.2	**0.4**
Pedosphaerales	**0.47**	0.21	0.28	**0.5**	**0.34**	-0.11	0.22	**0.43**	0.13
Gaiellales	-0.1	-0.19	-0.05	-0.27	-0.13	**0.3**	-0.05	**-0.3**	0.01
Acidobacteriales	**-0.31**	0.11	-0.14	-0.1	-0.08	-0.17	-0.16	**-0.43**	0.09
Myxococcales	**0.39**	0.21	0.27	**0.42**	**0.33**	-0.14	0.2	**0.57**	-0.14
Acidimicrobiales	0.01	**-0.29**	-0.01	-0.19	-0.09	0.27	0.04	-0.03	-0.02
Actinomycetales	-0.27	-0.19	-0.1	**-0.29**	-0.14	0.14	-0.25	**-0.6**	0.24
Rhizobiales	**-0.44**	0.05	-0.28	**-0.42**	**-0.29**	-0.04	-0.19	**-0.64**	0.04
Chthoniobacterales	**-0.32**	0.18	-0.24	-0.15	-0.19	-0.15	0.02	**-0.41**	-0.02
Acidobacteria-6	0.22	-0.22	0.09	0	-0.01	0.11	**0.29**	0.24	-0.14
Other Bacteria	**0.49**	0.04	**0.33**	**0.41**	**0.33**	-0.02	0.13	**0.69**	-0.09

Values in bold are different from 0 with a significance level alpha=0.05

Table A7.2B: Pearson's correlation matrix of 16S rRNA gene sequences with anions and cations in 2013 soils

Variables	Ammonium	Calcium	Magnesium	Potassium	Sodium
Simpson	**0.48**	**-0.74**	**-0.76**	0.07	**-0.99**
Ellin6529	-0.06	0.07	**-0.4**	0.06	-0.12
Saprospirales	**0.64**	0.11	-0.17	**0.33**	-0.19
Burkholderiales	**0.42**	0.09	**-0.29**	0.21	-0.17
Solirubrobacterales	**-0.47**	**0.35**	0.06	-0.12	**0.36**
Bacillales	**-0.4**	0.16	0.11	-0.01	0.25
Solibacterales	-0.24	**-0.33**	**0.29**	**-0.34**	0.16
Xanthomonadales	**0.56**	0.05	-0.21	0.27	-0.24
Rhodospirillales	**-0.53**	**0.37**	0.25	-0.03	**0.35**
Pedosphaerales	**0.53**	0.14	0.09	**0.47**	-0.16
Gaiellales	**-0.45**	0.18	-0.13	-0.21	0.18
Acidobacteriales	-0.27	**-0.32**	**0.33**	**-0.32**	0.14
Myxococcales	**0.64**	0.03	-0.24	**0.41**	**-0.4**
Acidimicrobiales	-0.23	0.28	-0.2	0.01	0.1
Actinomycetales	**-0.65**	0.03	0.21	**-0.43**	**0.37**
Rhizobiales	**-0.51**	-0.25	0.11	**-0.54**	0.23
Chthoniobacterales	-0.12	**-0.34**	0.15	**-0.3**	0.07
Acidobacteria-6	0.09	0.28	-0.27	0.23	-0.07
Other Bacteria	**0.6**	0.18	-0.26	**0.52**	**-0.35**

Values in bold are different from 0 with a significance level alpha=0.05

Table A7.3: Summary of multiple ANOVA of major OTU groups in 2013 sediments:

	Desulfuromonadales	Desulfobacterales	Ohtaekwangia	Sphingomonadales
R2	0.340	0.259	0.034	0.105
F	6.188	4.189	0.427	1.414
Pr > F	0.007	0.028	0.658	0.263
	Flavobacteriales	Other Alphaproteobacteria	Planctomycetales	Nitrospirales
R2	0.121	0.120	0.004	0.061
F	1.654	1.638	0.048	0.784
Pr > F	0.212	0.215	0.954	0.468
	Other Proteobacteria	Myxococcales	Subdivision3 genera incertae sedis	Rhizobiales
R2	0.056	0.097	0.164	0.120
F	0.709	1.293	2.356	1.638
Pr > F	0.502	0.293	0.116	0.215
	Sphingobacteriales	Rhodocyclales	Other Deltaproteobacteria	Other Betaproteobacteria
R2	0.028	0.128	0.046	0.091
F	0.342	1.759	0.581	1.198
Pr > F	0.714	0.194	0.567	0.319
	Other Gammaproteobacteria	Other Bacteroidetes	Burkholderiales	Other Bacteria
R2	0.213	0.372	0.080	0.181
F	3.241	7.109	1.038	2.653
Pr > F	0.057	0.004	0.370	0.091

Table A7.4A: Pearson's correlation matrix of 16S rRNA gene with anions and cations in 2013 sediments

Variables	Acetate	Flouride	Formate	Nitrate	Nitrite	Phosphate	Sulfate
Desulfuromonadales	0.01	**0.39**	-0.02	**-0.40**	-0.17	-0.13	0.16
Desulfobacterales	0.20	0.06	0.13	**-0.37**	0.01	-0.02	-0.15
Ohtaekwangia	**-0.33**	0.13	**-0.39**	0.20	0.26	-0.25	**0.32**
Sphingomonadales	-0.15	-0.16	-0.18	0.14	0.05	-0.25	-0.03
Flavobacteriales	-0.20	0.20	**-0.30**	-0.06	0.07	-0.24	**0.34**
Alphaproteobacteria	-0.03	-0.17	0.01	0.25	-0.21	0.10	-0.16
Planctomycetales	-0.26	-0.04	**-0.33**	0.17	-0.09	-0.12	0.15
Nitrospirales	-0.06	-0.17	-0.14	0.18	-0.07	0.00	-0.07
Other Proteobacteria	-0.02	-0.21	-0.05	0.22	0.20	0.14	-0.09
Myxococcales	0.15	-0.12	0.19	-0.02	-0.16	0.11	-0.21
Subdivision3	-0.22	0.24	-0.28	-0.13	0.08	-0.18	**0.28**
Rhizobiales	0.01	-0.06	0.05	0.22	**-0.32**	0.17	0.02
Sphingobacteriales	**-0.28**	-0.15	**-0.39**	0.20	0.18	-0.25	0.13
Rhodocyclales	-0.03	0.18	0.09	0.02	-0.21	0.24	0.21
Deltaproteobacteria	0.14	0.26	**0.30**	-0.09	-0.13	**0.29**	0.04
Betaproteobacteria	0.14	0.21	0.10	-0.07	-0.04	0.27	0.07
Gammaproteobacteria	**-0.31**	-0.17	**-0.45**	0.25	**0.47**	-0.24	0.14
Other Bacteroidetes	0.23	0.24	-0.02	**-0.44**	0.03	-0.17	0.07
Burkholderiales	**-0.30**	0.04	**-0.28**	0.09	0.00	-0.08	**0.28**
Other Bacteria	-0.21	**-0.42**	**-0.38**	-0.23	**0.38**	**-0.62**	-0.24

Values in bold are different from 0 with a significance level alpha=0.05

Table A7.4B: Pearson's correlation matrix of 16S rRNA gene with anions and cations in 2013 sediments

Variables	Ammonium	Calcium	Magnesium	Potassium	Sodium
Desulfuromonadales	-0.06	**-0.29**	**0.51**	**0.41**	0.21
Desulfobacterales	-0.03	**-0.35**	0.14	**0.32**	0.04
Ohtaekwangia	0.21	**0.33**	0.11	-0.13	0.25
Sphingomonadales	-0.01	0.10	0.03	**-0.42**	0.01
Flavobacteriales	**0.30**	0.12	**0.30**	0.05	0.25
Alphaproteobacteria	-0.24	0.11	-0.11	**-0.31**	-0.20
Planctomycetales	-0.12	0.20	-0.01	-0.25	0.15
Nitrospirales	-0.03	0.12	-0.25	-0.17	-0.02
Other Proteobacteria	-0.11	0.19	**-0.31**	-0.23	-0.10
Myxococcales	-0.26	-0.19	0.21	-0.12	-0.19
Subdivision3	0.12	0.04	0.27	0.25	**0.29**
Rhizobiales	-0.22	0.10	0.11	-0.15	-0.14
Sphingobacteriales	0.14	0.26	-0.04	**-0.34**	0.13
Rhodocyclales	-0.02	0.06	0.18	0.24	-0.03
Deltaproteobacteria	-0.23	-0.04	0.04	**0.35**	-0.08
Betaproteobacteria	**-0.31**	-0.04	0.12	0.24	0.01
Gammaproteobacteria	**0.29**	**0.34**	-0.25	-0.27	0.17
Other Bacteroidetes	0.03	**-0.40**	**0.40**	**0.34**	0.25
Burkholderiales	0.03	0.17	0.17	-0.05	0.13
Other Bacteria	0.25	-0.17	-0.07	**-0.48**	0.25

Table A7.5: Pearson's correlation matrix of Archaeal 16S rRNA gene with anions and cations in 2013 soils

	Other Archaea	Methanosarcinales	Methanomicrobiales	Methanocellales
R2	0.425	0.532	0.259	0.480
F	2.417	3.722	1.143	3.026
Pr > F	0.023	0.001	0.359	0.006
	Methanobacteriales	MCG pGrfC26	Thermoplasmata E2	Other Euryarchaeota
R2	0.350	0.244	0.667	0.474
F	1.762	1.054	6.557	2.946
Pr > F	0.099	0.423	< 0.0001	0.007
	MBGA NRP-J	Halobacteriales	Parvarchaea YLA114	Nitrososphaerales
R2	0.827	0.319	0.585	0.495
F	15.647	1.534	4.619	3.208
Pr > F	< 0.0001	0.162	0.000	0.004

Table A7.6A: Pearson's correlation matrix of Archaeal 16S rRNA gene with anions and cations in 2013 soils

Variables	H_2O%	Porosity	Acetate	Flouride	Formate	Nitrate	Nitrite	Phosphate	Sulfate
Other Archaea	0	0.11	-0.07	0.05	-0.05	-0.24	0.11	0.02	-0.1
Methanosarcinales	-0.06	0.02	-0.13	0.02	-0.12	-0.16	0.13	-0.11	-0.1
Methanomicrobiales	-0.02	0.01	-0.08	0.03	-0.07	-0.1	0.09	-0.07	-0.06
Methanocellales	-0.09	0.03	-0.16	-0.02	-0.14	-0.15	0.09	-0.13	-0.05
Methanobacteriales	-0.22	0.04	-0.17	-0.1	-0.16	-0.18	0.02	-0.16	-0.14
MCG pGrfC26	-0.03	0.02	-0.1	0	-0.09	-0.08	0.08	-0.07	-0.02
Thermoplasmata E2	-0.07	0.03	-0.11	0.04	-0.09	-0.22	0.1	-0.14	-0.11
Euryarchaeota	-0.08	0.11	-0.04	0.02	-0.01	**-0.32**	0.07	-0.16	-0.07
MBGA NRP-J	-0.05	0.05	0.08	0	0.11	-0.18	-0.19	**-0.3**	**0.41**
Halobacteriales	0.11	0.01	0.23	0.16	0.24	-0.16	-0.07	-0.08	0.08
Parvarchaea YLA114	-0.04	0.08	-0.15	0.03	-0.12	-0.14	0.09	-0.06	-0.06
Nitrososphaerales	-0.02	-0.06	-0.06	-0.12	-0.09	0.26	0	0.16	-0.05

Values in bold are different from 0 with a significance level alpha=0.05

Table A7.6B: Pearson's correlation matrix of Archaeal 16S rRNA gene with anions and cations in 2013 soils

Variables	Ammonium	Calcium	Magnesium	Potassium	Sodium
Other Archaea	**0.33**	-0.24	-0.1	0.11	-0.19
Methanosarcinales	0.16	-0.22	-0.04	0.11	-0.1
Methanomicrobiales	0.12	-0.14	-0.03	0.08	-0.06
Methanocellales	0.1	-0.19	0.06	0.07	-0.03
Methanobacteriales	0.01	-0.26	-0.04	-0.07	-0.07
MCG pGrfC26	0.1	-0.11	0.04	0.08	-0.01
Thermoplasmata E2	0.14	**-0.28**	-0.06	0.08	-0.13
Euryarchaeota	0.18	**-0.33**	-0.07	-0.04	-0.18
MBGA NRP-J	-0.25	-0.05	**0.51**	-0.22	0.28
Halobacteriales	-0.04	-0.13	-0.03	-0.05	-0.1
Parvarchaea YLA114	0.21	-0.19	-0.02	0.13	-0.07
Nitrososphaerales	-0.09	0.26	-0.03	-0.01	0.1

Values in bold are different from 0 with a significance level alpha=0.05

Table A7.7: Summary of multiple ANOVA of major OTU groups in 2013 sediments

	Methanobacteriales	Nitrosopumilales	Euryarchaeota Other	Methanomassiliicoccales	Woesearchaeota AR16
R^2	0.217	0.428	0.231	0.411	0.220
F	2.495	6.748	2.706	6.282	2.532
Pr > F	0.045	< 0.0001	0.032	0.000	0.042
	Woesearchaeota Other	Nitrososphaerales	Methanomicrobiales	Methanosarcinales	Archaea Other
R^2	0.272	0.181	0.120	0.172	0.104
F	3.366	1.991	1.223	1.865	1.050
Pr > F	0.011	0.098	0.314	0.120	0.401

Table A7.8A: Pearson's correlation matrix of 16S rRNA gene with anions and cations in 2013 sediments

Variables	TOC	Acetate	Flouride	Formate	Nitrate	Nitrite	Phosphate	Sulfate
Methanobacteriales	0.02	-0.23	-0.1	-0.11	-0.1	0.14	**-0.31**	-0.17
Nitrosopumilales	**-0.4**	0.04	-0.02	**0.32**	**0.39**	-0.11	**0.37**	-0.06
Euryarchaeota Other	0.04	0	-0.13	-0.19	0.03	0.03	-0.11	0.05
Methanomassiliicoccales	**0.29**	**0.29**	0.2	**0.29**	0.15	**-0.49**	**0.47**	-0.01
Woesearchaeota AR16	-0.2	**-0.33**	-0.27	-0.07	-0.12	0.25	**-0.46**	-0.15
Woesearchaeota Other	**-0.3**	**-0.32**	**-0.31**	-0.17	-0.05	**0.3**	**-0.48**	-0.19
Nitrososphaerales	**-0.4**	-0.1	-0.18	-0.2	0.22	**0.3**	-0.16	-0.02
Methanomicrobiales	**0.32**	-0.1	-0.08	0.15	-0.14	-0.1	-0.1	-0.21
Methanosarcinales	0.08	-0.02	-0.02	0.07	-0.2	0.18	-0.18	-0.07
Archaea Other	0.21	0.11	0.13	-0.02	-0.16	-0.16	-0.02	0.12

Values in bold are different from 0 with a significance level alpha=0.05

Table A7.8B: Pearson's correlation matrix of 16S rRNA gene with anions and cations in 2013 sediments

Variables	Ammonium	Calcium	Magnesium	Potassium	Sodium
Methanobacteriales	-0.06	-0.06	0.02	-0.23	0.03
Nitrosopumilales	-0.11	**0.31**	**-0.44**	0.11	**-0.43**
Euryarchaeota Other	0.18	0	0.03	-0.1	0.09
Methanomassiliicoccales	**-0.37**	0.02	0.06	0.2	-0.24
Woesearchaeota AR16	**0.28**	-0.05	0.12	**-0.35**	0.01
Woesearchaeota Other	**0.36**	0.02	0.03	**-0.38**	-0.03
Nitrososphaerales	0.21	0.24	-0.17	-0.23	-0.05
Methanomicrobiales	-0.19	-0.17	0.15	-0.19	-0.04
Methanosarcinales	0.16	-0.24	-0.06	0.01	0.11
Archaea Other	-0.04	-0.2	0.14	0.14	0.15

Table A7.9: Summary of multiple ANOVA in 2014 soils

	MPP	MPP	mcrA	*pmoA*
R2	0.670	0.765	0.673	0.811
F	4.423	7.098	4.498	9.341
Pr > F	0.001	< 0.0001	0.001	< 0.0001

Table A7.10A: Pearson's correlation matrix of function/ functional gene with anions and cations in 2014 soils

Variables	MOP	MPP	*mcrA*	*pmoA*
MOP	**1**	**0.38**	**-0.25**	**-0.24**
MPP	**0.38**	**1**	-0.16	-0.09
mcrA	**-0.3**	-0.2	**1**	**0.36**
pmoA	**-0.2**	-0.1	**0.36**	**1**
Acetate	-0.1	**-0.3**	-0.11	-0.18
Flouride	**-0.3**	-0.2	**0.44**	**0.25**
Formate	-0	**-0.2**	-0.17	**-0.23**
Nitrate	**0.24**	**0.41**	-0.18	0.02
Nitrite	-0.1	0.06	0.07	0.2
Phosphate	-0.1	**-0.2**	-0.1	-0.17
Sulfate	0.15	**-0.2**	-0.17	**-0.29**
Ammonium	0.15	**-0.3**	-0.2	**-0.31**
Calcium	-0.2	**-0.2**	-0.04	-0.1
Magnesium	0.21	**-0.2**	**-0.25**	**-0.33**
Potassium	-0.1	-0.1	0	0.15
Sodium	0.11	**-0.2**	**-0.29**	**-0.3**

Values in bold are different from 0 with a significance level alpha=0.05

Table A7.10B: Pearson's correlation matrix of function/ functional gene with anions and cations in 2014 soils

Variables	MOP	MPP
MOP	**1**	**0.403**
MPP	**0.403**	**1**
WATER CONTENT	**0.211**	**0.272**

Table A7.11: Summary of multiple ANOVA of major OTU groups in 2014 sediments

	MOP	N2 MPP	H2 MPP	*mcrA*	*pmoA*
R2	0.728	0.677	0.702	0.702	0.697
F	6.032	4.722	5.305	5.294	5.183
Pr > F	0.001	0.003	0.002	0.002	0.002

Table A7.12: Pearson's correlation matrix of function/ functional gene with anions and cations in 2014soils

Variables	MOP	N2 MPP	H2 MPP	*mcrA*	*pmoA*
MOP	**1**	**0.66**	-0.16	-0.09	-0.02
N2 MPP	**0.66**	**1**	0.14	0.28	0.11
H2 MPP	-0.16	0.14	**1**	**0.62**	0.4
mcrA	-0.09	0.28	**0.62**	**1**	**0.48**
pmoA	-0.02	0.11	0.4	**0.48**	**1**
Acetate	0.37	**0.62**	-0.11	0.07	-0.26
Chloridel	-0.25	-0.17	-0.21	-0.17	-0.12
Flouride	0.2	**0.49**	**0.47**	**0.73**	0.3
Formate	0.17	0.07	-0.37	-0.34	-0.41
Nitrite	-0.01	0.19	**0.52**	**0.8**	**0.75**
Nitrate	-0.18	-0.11	-0.13	-0.36	-0.36
Phosphate	0.08	0.24	0.05	-0.02	0.06
Sulfate	0.4	0.34	-0.26	-0.12	0.4
Ammonium	0.18	**0.51**	0.07	0.23	0.3
Calcium	0.32	**0.47**	-0.04	0.12	**0.48**
Magnesium	**0.52**	**0.7**	-0.04	0.27	0.07
Potassium	**0.45**	0.19	-0.03	0.03	0.11
Sodium	**0.47**	**0.6**	0.06	0.25	0.15

Values in bold are different from 0 with a significance level alpha=0.05

Table A7.13: Summary of multiple ANOVA in bottle temperature experiments

	MOP	16S rRNA	*pmoA*	mg C/ g soil
R^2	0.283	0.663	0.392	0.372
F	1.879	9.340	3.064	2.811
Pr > F	0.156	0.000	0.232	0.055

Table A7.14: Pearson's correlation matrix of function/ functional gene with anions and cations in bottle temperature experiments

Variables	Temp	MOP	16S rRNA	*pmoA*
Temperature	**1**	**0.94**	0.48	-0.33
MOP	**0.94**	**1**	0.51	-0.39
16S rRNA	0.48	0.51	**1**	-0.21
pmoA	-0.33	-0.39	-0.21	**1**
mg C/ g soil	-0.14	-0.19	0.21	0.41
Nitrate	0.5	0.52	0.28	-0.48
Nitrite	-0.29	-0.36	0.1	0.04
Phosphate	0.48	0.53	0.05	-0.2
Sulfate	0.18	0.24	-0.1	-0.16
Ammonium	**-0.73**	**-0.67**	-0.52	0.48
Calcium	**0.83**	**0.83**	0.28	-0.3
Magnesium	**0.78**	**0.79**	0.23	-0.25
Potassium	**-0.58**	-0.47	-0.42	0.08
Sodium	-0.35	-0.24	-0.29	-0.07
H_2O	**-0.66**	**-0.69**	-0.22	0.36

Values in bold are different from 0 with a significance level alpha=0.05

Table A7.15: Summary of multiple ANOVA of field temperature experiments

	MOP	*pmoA*	16S rRNA	mg C/ g soil
R^2	0.742	0.809	0.691	0.201
F	6.911	10.145	5.357	0.605
Pr > F	0.003	0.001	0.008	0.698

Table A7.16: Pearson's correlation matrix of function/ functional gene with anions and cations in field temperature experiments

Variables	MOP	*pmoA*	16S rRNA	mg C/ g soil
MOP	**1**	-0.14	-0.19	0.19
pmoA	-0.14	**1**	**0.53**	-0.05
16S rRNA	-0.19	**0.53**	**1**	-0.14
mg C/ g soil	0.19	-0.05	-0.14	**1**
Acetate	0.1	**0.57**	0.34	-0.3
Ammonium	0.3	-0.02	0.04	0.2
Calcium	-0.3	**0.88**	**0.64**	-0.11
Magnesium	**0.63**	**-0.56**	-0.36	0.17
Nitrate	-0.15	0.02	0.22	0.25
Nitrite	**-0.51**	0.21	-0.02	-0.01
Phosphate	**-0.65**	0.31	0.06	-0.06
Potassium	**-0.76**	0	-0.15	-0.05
Sodium	0.45	0.12	0.33	0.24
Sulfate	0.33	0.32	**0.48**	0.17
H_2O	-0.19	**0.89**	**0.67**	-0.09

Values in bold are different from 0 with a significance level alpha=0.05

Table A7.17: Summary of multiple ANOVA in bottle N experiments

	MOP	*pmoA*	Ammonium	Calcium	Magnesium
R2	0.596	0.964	0.941	0.842	0.716
F	3.085	55.886	33.512	11.134	5.271
Pr > F	0.000	< 0.0001	< 0.0001	< 0.0001	< 0.0001
	Potassium	Sodium	Acetate	Chloride	Fluoride
R2	0.614	0.527	0.755	0.384	0.652
F	3.326	2.325	6.415	1.299	3.915
Pr > F	0.000	0.007	< 0.0001	0.219	< 0.0001
	Formate	Nitrate	Nitrite	Phosphate	
R2	0.775	0.878	0.452	0.774	
F	7.174	14.965	1.720	7.149	
Pr > F	< 0.0001	< 0.0001	0.057	< 0.0001	

Table A7.18: Pearson's correlation matrix of function/ functional gene with anions and cations in bottle N experiments

Variables	MOP	*pmoA*
MOP	**1**	0.05
pmoA	0.05	**1**
Ammonium	0.04	0.07
Calcium	0.11	**0.78**
Magnesium	-0.06	**-0.28**
Potassium	0.13	0.16
Sodium	0.01	**0.36**
Acetate	0.1	**0.68**
Chloride	0	0.22
Fluoride	-0.04	**0.63**
Formate	0	**0.74**
Nitrate	0.11	**0.49**
Nitrite	-0.12	0.18
Phosphate	0.16	**0.51**

Values in bold are different from 0 with a significance level alpha=0.05

Table A7.19: Summary of multiple ANOVA of major OTU groups in bottle P experiments

	MOP	*pmoA*	Ammonium	Calcium	Magnesium
R2	0.665	0.824	0.932	0.976	0.756
F	4.135	9.746	28.750	86.619	6.459
Pr > F	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
	Potassium	Sodium	Acetate	Chloride	Fluoride
R2	0.839	0.856	0.748	0.668	0.546
F	10.869	12.380	6.199	4.204	2.512
Pr > F	< 0.0001	<0.0001	< 0.0001	< 0.0001	0.004
	Formate	Nitrate	Nitrite	Phosphate	
R2	0.866	0.840	0.833	0.928	
F	13.437	10.924	10.401	26.895	
Pr > F	< 0.0001	< 0.0001	< 0.0001	< 0.0001	

Table A7.20: Pearson's correlation matrix of function/ functional gene with anions and cations in bottle P experiments

Variables	MOP	*pmoA*
MOP	**1**	**0.3**
pmoA	**0.3**	**1**
Ammonium	**0.32**	0.2
Calcium	**0.62**	**0.69**
Magnesium	**0.3**	0.23
Potassium	0.13	**0.33**
Sodium	**0.43**	**0.48**
Acetate	**0.46**	**0.43**
Chloride	**0.25**	0.12
Fluoride	**0.54**	**0.26**
Formate	**0.39**	0.2
Nitrate	**-0.32**	0.03
Nitrite	-0.05	**0.27**
Phosphate	**0.52**	**0.61**

Values in bold are different from 0 with a significance level alpha=0.05

Table A7.21: Summary of multiple ANOVA of major OTU groups in N field experiments

	MOP	*pmoA*	16S rRNA
R2	0.784	0.679	0.740
F	21.720	12.709	17.056
Pr > F	< 0.0001	< 0.0001	< 0.0001

Table A7.22: Pearson's correlation matrix of function/ functional gene with anions and cations in N field experiments

Variables	MOP	*pmoA*	16S rRNA
MOP	**1.00**	-0.22	0.08
pmoA	-0.22	**1.00**	**0.74**
16S rRNA	0.08	**0.74**	**1.00**
Acetate	0.14	**-0.36**	**-0.50**
Ammonium	0.22	**-0.62**	**-0.40**
Calcium	-0.04	**0.41**	0.01
Magnesium	0.17	**-0.55**	-0.26
Nitrate	-0.03	-0.20	0.15
Nitrite	0.05	**0.62**	**0.35**
Phosphate	-0.17	0.32	0.14
Sulfate	0.20	-0.12	0.04
Ammonium	0.26	**-0.75**	**-0.50**
Calcium	-0.08	0.31	-0.09
Magnesium	0.28	**-0.44**	-0.14
Potassium	**-0.47**	-0.20	-0.23
Sodium	0.22	-0.06	0.13

Values in bold are different from 0 with a significance level alpha=0.05