The Dynamic Interaction between Microbial Biodiversity, Biogeochemical Activity and Sedimentary Geomorphology in the Severn Estuary

A thesis presented by

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February 2015

Declaration

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Acknowledgments

I'd like to thank Prof. Andy Weightman for all of the guidance, support and knowledge that was poured into this work over the last 4 years. Dr. Gordon Webster who provided some of the analysis within this work and who has, with his vast experience, come to the rescue countless times when I just couldn't understand what was going wrong! Thank you to the Geomicrobiology group in Earth Sciences for their help and contributions, including all those who went out on that cold boat in the Severn Estuary to go sampling. Shaun Thomas, who performed the geochemical analysis of the Severn Estuary sediments and Erwan Roussel, Barry Cragg, Gordon Webster, Xiahong Tang, Roberta Gorra, Henrik Sass and R.John Parkes who kindly allowed me to use their thermal gradient experiment sediments, they were much appreciated.

I'd like to thank all those (there's too many to count) who have passed through the Weightman, Kille, Marchesi and Mahenthiralingam labs who have made such a supportive and friendly environment in our little corner of Main Building. In particular, the Friday Picnic lot, Mel Buckley, Dan Pass and Craig Anderson, who knew how to make the most of a lunch hour and were there when I needed a long, distracting chat. And not forgetting Joan who is absolute magic!

My friends have put up with so much while I've been doing this PhD including tears, excuses and general moaning, so thank you! Mel, Fred and Lee, you know how to have a good time. And most importantly Laura, Charlie and Justin, thank you for the box!

My much loved family, Rhiannon, Cerys, Rob, my Nan, Dilys, and Kate have been amazing, providing motivational talks and trips away for my sanity! Jen and Mike, thanks for the house and for all the welsh cakes.

Gareth, you got me through this with your fantastic cooking skills, computer related expertise and a good reality check from time to time. Much love! So amaze!

Last, but by no means the least, my mum, Carol, who wanted to see this thesis completed more than anyone, and who was always there with so much love and support, this is for you.

Summary

The Severn Estuary, UK is an important model estuarine environment due to its hyper-tidal range, leading to dynamic sediment environments. This work investigated the diversity and relationship with geochemistry of Severn Estuary sediment prokaryotic communities, which have not been previously described in detail. Focus was placed on the diversity and distribution of the largely uncultivated *Chloroflexi,* which were detected in high abundance in the deep subsurface but previous work has failed to address the diversity of subdivisions in dynamic surface sediments.

Three geophysically different sampling sites were analysed from intertidal, shallow water and deep water areas of the Severn Estuary. Molecular profiling methods using the 16S ribosomal RNA (rRNA) gene, such as denaturing gradient gel electrophoresis (DGGE), ribosomal intergenic spacer analysis (RISA), quantitative PCR (qPCR) and 454 pyrosequencing were used. Novel qPCR and pyrosequencing methods were designed to target *Chloroflexi* subdivisions. Each of the sampling sites was characterised by differing prokaryotic communities depending on sediment turbidity and geochemistry, though the most abundant phyla, *Proteobacteria*, *Firmicutes* and *Chloroflexi,* were constant. The novel methods revealed surprising abundance and diversity of *Chloroflexi* subdivisions in Severn Estuary sediments, dominated by *Anaerolineae,* on a more detailed scale than previously reported in the literature.

Further experiments described how sediment prokaryotic community structure and function changed over a wide temperature range and over 100 days. Slurries of Severn Estuary intertidal sediments were incubated between 1 - 80^{o}C. A critical temperature window of 43^{o}C indicated a shift in the bacterial community to thermophilic spore-forming *Firmicutes* and from heterotrophic to autotrophic sulphate reduction. In the *Archaea* community, methanogenesis shifted from chemoorganotrophic to chemolithotrophic dependent metabolism. These results extend our knowledge of the geochemical changes in temperature dependent sediment communities, which is important for the modelling of climate susceptible habitats, such as coastal sediments.

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Abbreviations

ANAMMOX – Anaerobic oxidation of methane
ANOVA – Analysis of variance
AODC – Acridine orange direct count
BLAST – Basic local alignment search tool
BSA – Bovine serum albumin
bp – Base pairs
cbsf – Centimetres below the surface
cDNA – Complementary DNA
CO_2 – Carbon dioxide
CTAB - Cetyltrimethylammonium bromide
DEPC – Diethylpyrocarbonate
DGGE – Denaturing gradient gel electrophoresis
DNA - Deoxyribonucleic acid
dNTPs - Deoxyribonucleotides
EDTA - Ethylenediaminetetraacetic acid
EU – European Union
H_2 - Hydrogen
HCl – Hydrochloric acid
MCG – Miscellaneous Crenarchaeota Group
MID – Multiplex identifier
mRNA – messenger RNA
NaCl – Sodium chloride
NaOH – Sodium hydroxide
NCBI – National Centre for Biotechnology Information
NGS – Next generation sequencing
NMDS – Non-metric multi-dimensional scaling
OTU – Operational taxonomic unit
PCR – Polymerase chain reaction
ppt – Parts per thousand
QIIME - Quantitative insights into microbial ecology
qPCR – Quantitative polymerase chain reaction
rdh – Reductive dehalogenase
RISA – Ribosomal intergenic spacer analysis
RNA - Ribonucleic acid
rRNA – Ribosomal RNA
RT – Room temperature
SAR202 – Sargasso Sea Group 202
SDS - Sodium dodecyl sulphate
SRB – Sulphate reducing bacteria

TAE Buffer - Tris-acetate EDTA buffer
TE Buffer – Tris-EDTA buffer
Tris - Tris (hydroxymethyl)aminomethane
UPGMA - Unweighted pair group method with arithmetic mean
UV – Ultra violet

Chapter 1 - General Introduction and Background

1.2 Marine Environments

Approximately 75% of our planet is covered by marine waters and below these are the marine subsurface environments, thought to be one of the largest ecosystems on our planet (Morono et al., 2009). A number of different aquatic habitats are placed under the umbrella term of a marine environment. These include coastal habitats such as estuaries, intertidal zones, salt marshes and coral reefs as well as ocean habitats such as Deep Ocean, continental shelves, benthic zones and hydrothermal vents. Both coastal and oceanic habitats further include overlying waters at various depths and surface and deep subsurface sediments. The unifying factor in these marine environments is the salinity which ranges from brackish (0.5 to 29 parts per thousand (ppt)) to saline or seawater (30-50 ppt). At the other end of the salinity spectrum are freshwater habitats such as rivers, lakes, streams and glaciers which have low concentrations of dissolved salts and ions (<0.5 ppt). An estuary is typically a gradient of salinity from fresh water at the immediate river mouth to marine water at the sea, with brackish waters in between (Purdy et al., 2002b). This mixing leads to a variety of very interesting and unique habitats.

1.2.1 Estuaries and their Sediment Geomorphology and Geochemistry

Approximately two thirds of the world's human population live near coastal areas such as estuaries which indicates the importance of these places to society (Duarte et al., 2013). Coastal environments are shaped by waves, tides, currents and weathering, leading to erosion and sediment transport. Estuaries are a feature of coastal environments defined as a brackish, partially closed body of water with inputs from freshwater rivers and streams and open to a sea (Pritchard, 1967). Within an estuary ecosystem there are many different habitats. Intertidal zones and mudflats lead into deeper marine environments, often highly perturbed by currents and tides. These are often preceded by saltmarshes that are frequently flooded by the tides.

There are also the vast sediment layers beneath the water which are unique in their influences from land, sea and river, freshwater and marine, which leads to extremely high influxes of nutrients from a variety of sources making estuaries one of the most biologically productive regions in coastal areas (O'Sullivan et al., 2013; Poremba et al., 1999).

As well as salinity gradients along an estuary's length there can also be gradients of chemicals. Ogilvie et al. (1997) found nitrate and organic compounds were high at the estuary head with influence from rivers, whereas chloride and sulphate were found in higher concentrations towards the sea. There are also further chemical gradients with depth. Generally, marine subsurface sediments are vertically stratified into distinct geochemical layers whereby electron acceptors are consumed in order of decreasing energy production (Figure 1.1; Canfield et al., 2005; Froelich et al., 1979). This leads to a sequence of different respiratory processes from aerobic metabolism at the very surface to manganese, nitrate, iron and sulphate reduction and finally methanogenesis. In estuarine sediments, oxygen is quickly depleted, within millimetres of the surface, and so these alternate electron acceptors are required for the degradation of organic matter by anaerobic geochemical process such as sulphate reducing prokaryotic communities (Böttcher et al., 2000; Wilms et al., 2006a). Sulphate reduction, methanogenesis and denitrification are the dominant processes in coastal and estuarine sediments including the Severn Estuary (Dong et al., 2002; O'Sullivan et al., 2013; Wellsbury et al., 1996). Though, for example, the sulphate reduction zone in deep marine sediments may stretch over 40 m of depth, in tidal and estuarine sediments this is often compacted to less than 1.5 m (Engelen and Cypionka, 2009). The geology and geochemistry of a sediment environment influence the prokaryotic communities inhabiting it (Huber et al., 2007; Inagaki et al., 2003). Fermenting (the conversion of sugars to acids, gases or alcohols) and syntrophic (where one species uses the products of another species for nutrients) bacteria are the main constituents of anaerobic sediments (Selje and Simon, 2003).

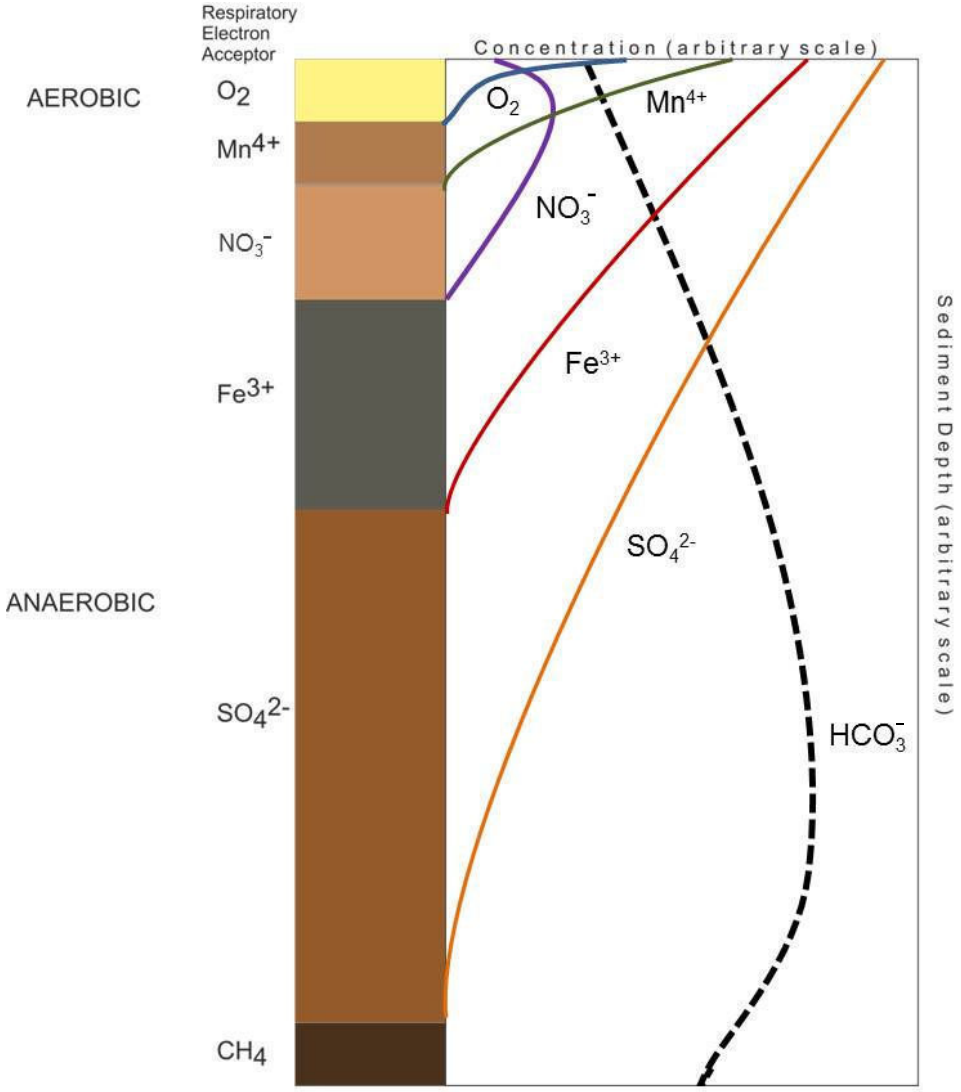

Figure 1.1 - The sequential use of highest to lowest energy respiratory electron acceptor species with increasing depth below the sediment surface in marine sediments. Adapted from Canfield et al. (2005).

The available electron acceptors for redox reactions in each anoxic layer create individual bacterial and archaeal communities that can metabolically exploit this geochemistry and therefore characterise these layers (Canfield et al., 2005; Heijs et al., 2008; Wilms et al., 2007). The scale of the marine sediment environment and the activity of prokaryotes in carbon, nitrogen and sulphur cycling means that sediments and their communities play a central role in global geochemical cycles. For example, marine coastal ecosystems account for up to 55% of global sediment organic matter oxidation (Weston and Joye, 2005). The further implication of these activities is that of the positive and negative effects of prokaryotic communities and marine sediments in climate change (Romankevich et al., 2009). The dynamic geomorphological changes in estuarine sediments, brought about by tides and turbidity, also have a major impact on the prokaryotic communities that reside there, due to the mixing of sediments and resupply of nutrients and oxygen (O'Sullivan et al., 2013; Webster et al., 2010).

1.2.2 Prokaryotes in Marine and Estuarine Subsurface Habitats

The importance of marine subsurface habitats is often greatly underestimated, and marine sediments were long thought to be biologically lifeless and frankly uninteresting (Fry et al., 2008). We now know that approximately one tenth of all living organisms on Earth, and about half of all prokaryotic life, reside in marine sediment habitats (Morono et al., 2009). Cell numbers in global marine subsurface sediments are believed to be in the order of 2.9 $x10^{29}$ to 3.5 $x10^{30}$ cells (Kallmeyer et al., 2012; Whitman et al., 1998). Numbers of prokaryotic cells decrease logarithmically with sediment depth in many other sediment types including coastal marine sediments (Figure 1.2; Inagaki et al., 2003; Parkes et al., 1994; Schippers and Neretin, 2006). The range of cell numbers discovered in marine sediments is illustrated in Table 1.1 and Figure 1.2. Microorganisms have been discovered in numbers up to 10^5 cells/cm^3 1,000 m below the seafloor (Inagaki et al., 2003; Parkes et al., 1994). When cultivation independent techniques were first applied to marine sediments, a wealth of prokaryotic diversity was uncovered with potentially unique metabolic

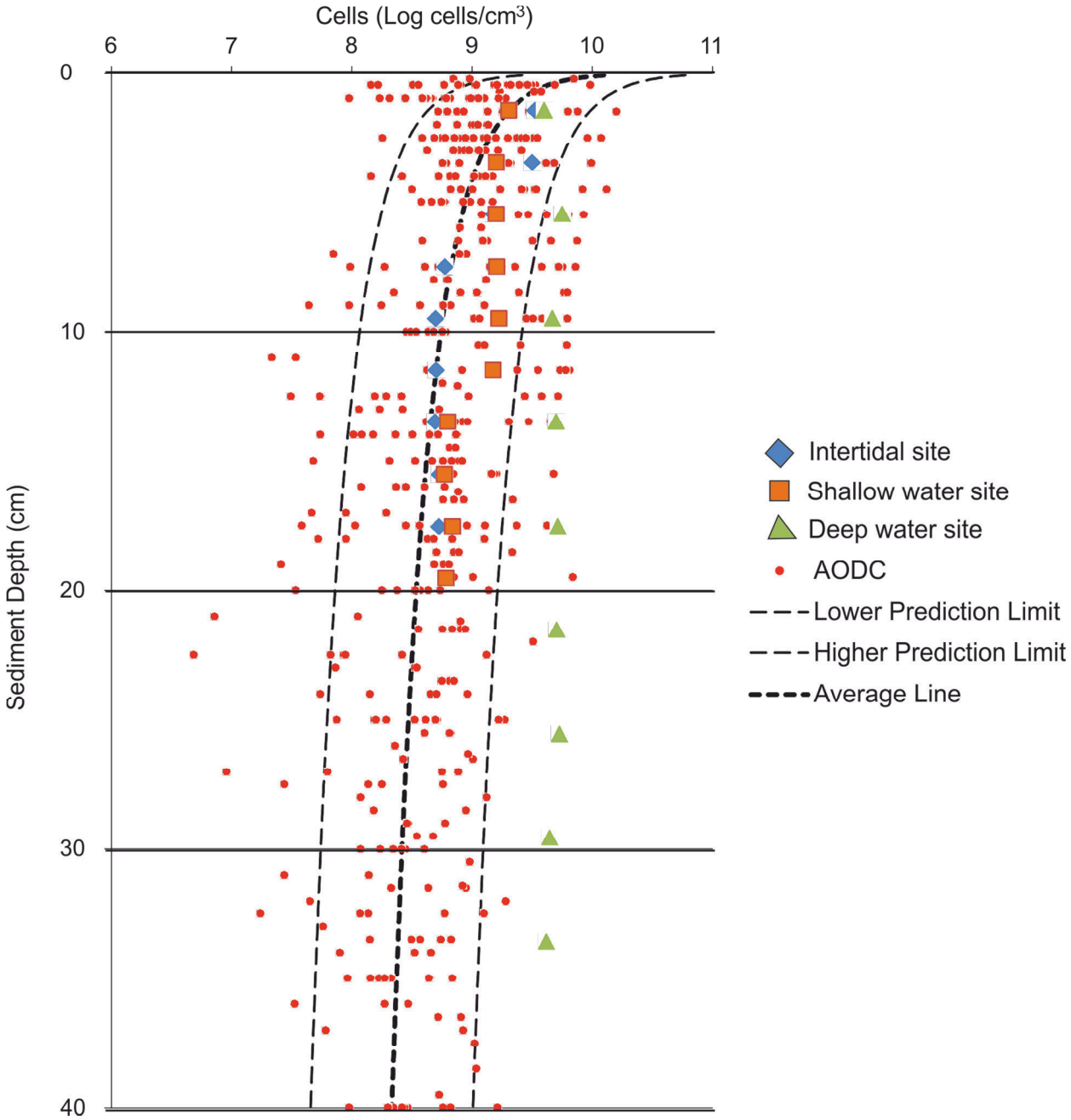

Figure 1.2 - The distribution of cell numbers with increasing sediment depth, from a range of sediment environments, including the deep subsurface, surface, and coastal sediments. Cell counts, by Shaun Thomas, Cardiff University, from the three Severn Estuary surface sediments sites included in Chapters 3 and 4 of this study (Figure 3.10), are indicated by coloured shapes (see key). Cells counted by acridine orange direct counts (AODC). Upper, lower and average prediction lines are included. AODC prediction model originally described by Parkes et al. (1994) and graph adapted from B. Cragg (personal communication).

Table 1.1 - The range of estimated cell numbers in near surface sediment environments
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Site	*Bacteria* Count[a]	*Archaea* Count[a]	Quantification Method[b]	Reference
Water column, Severn Estuary	2.2×10^5 cells/ cm^3		AODC	Joint and Pomroy, 1982
Aust Warth sediment, Severn Estuary	1×10^9 cells/ cm^3		AODC	Wellsbury et al., 1996
Tidal flat, German Wadden Sea	1×10^7 cells/ g of sediment	1×10^6 cells/ g of sediment	q-PCR	Wilms et al., 2006a
Near-surface sediments, Peru Margin	1×10^8-10^{10} copies/ cm^3	1×10^7-10^8 copies/ cm^3	q-PCR	Schippers and Neretin, 2006
Coastal subseafloor sediments, Sea of Okhotsk	1.2×10^7 cells/ cm^3		AODC	Inagaki et al., 2003
Near surface sediment, Forearc basins off Sumatra	1×10^8-10^{10} copies/ cm^3	1×10^7-10^9 copies/ cm^3	q-PCR	Schippers et al., 2010
Near surface sediment, Ocean margin sediments	1×10^8 cells/ cm^3	1×10^7 cells/ cm^3	q-PCR	Schippers et al., 2005
Sediment 1 mbsf[c], Peru Margin	Approx 1×10^4 copies/ng of DNA	Approx 9×10^3 copies/ng of DNA	q-PCR	Biddle et al., 2008
Methane seep sediments	-	1×10^7-10^8 copies/ cm^3	q-PCR	Inagaki et al., 2004

[a] Copies of 16S rRNA genes (qPCR) or total prokaryotic cell counts (AODC)
[b] AODC – Acridine orange direct counts; qPCR – Quantitative polymerase chain reaction
[c] Mbsf – meters below the surface

potential (Rochelle et al., 1994). Marine prokaryotes play an immensely important role in global biogeochemical cycles, as they are the main processors of bioactive elements and aid in the creation and degradation of biomass and have made marine sediments the Earth's largest reservoir of organic carbon (15 000x10^{18} g C; Fry et al., 2008; Teske, 2005).

Due to the natural gradients of chemicals, organic carbon and salinity, as well as large temperature ranges, physicality such as turbidity, pollution and eutrophication that are commonly seen in estuaries, prokaryotes in both sediment and the water column have been intensely investigated (Ogilvie et al., 1997). The gradients and fluctuations which give rise to many different habitats create a natural laboratory that allows researchers to access dynamic habitats in a natural environment (Bernhard and Bollmann, 2010). These gradients and micro-habitats have been linked to shifts in the microbial communities in these sediments (Bernhard and Bollmann, 2010; Crump et al., 2004). However, due to the extreme dynamic nature of estuaries, it has proved difficult to tease out the exact effect of these different gradients on individual taxa or indeed the effects of taxa on generation and maintenance of these gradients.

Specifically, much research has focused on the communities responsible for the dominant biogeochemical processes occurring in marine sediments; e.g. sulphate reduction, methanogenesis, nitrification, denitrification and anaerobic ammonium oxidation (ANAMMOX). Much of this work has been focused on the effects of salinity gradients on the activity and structure of the prokaryotic community. The analysis of both sulphate reducing bacteria (SRB) and their functional genes in estuarine sediments has been widely discussed, particularly in the Colne Estuary, UK (Nedwell et al., 2004; O'Sullivan et al., 2013; Purdy et al., 2002a). This is due to the dominance of this process; 50 -60% of total organic matter degradation is accounted for by sulphate reduction in estuarine sediments, influenced by the high concentrations of sulphate found in seawater (Jørgensen, 1982; O'Sullivan et al., 2013; Wellsbury et al., 1996). These studies agree that the abundance, diversity and activity of SRB is much greater at the marine estuary mouth than at the freshwater head, due to the higher sulphate concentrations and

salinity (Kondo et al., 2004; Leloup et al., 2006; Nedwell et al., 2004; Purdy et al., 2002a). The major species involved appear to be *Desulfobulbus* and *Desulfobacterium,* which are ubiquitous in marine and freshwater habitats, and the *Desulfotobacteria* and *Desulfovibrio* specifically in marine habitats (Oakley et al., 2010; Purdy et al., 2002a).

Similar work on the activity and diversity of archaeal methanogen communities along salinity gradients has frequently been carried out in conjunction with SRB analysis (Nedwell et al., 2004; O'Sullivan et al., 2013; Purdy et al., 2002b). Sulphate reduction and methanogenesis often occur in competition to each other with sulphate reduction common in marine, sulphate laden habitats and methanogenesis prospering in freshwater habitats (Lovley and Klug, 1983; Nedwell et al., 2004; Purdy et al., 2002b). This is because SRB have a higher affinity than methanogens to their shared substrates, hydrogen and acetate (Lovley et al., 1982; Nedwell et al., 2004). A group of generalist methanogenic *Archaea*, *Methanogenium* and the more highly specialised *Methanosaeta* have been described (Purdy et al., 2002b). O'Sullivan et al. (2013) also found anaerobic methanotrophs (ANME) were present at the sulphate methane transition zone (SMTZ) as these were able to oxidise methane using sulphate. Further to this, O'Sullivan et al. (2013) found that though SRB and methanogens usually inhabit specialised geochemical niches (Canfield and Thamdrup, 2009), the tidal nature of the estuary greatly effected these communities by homogenising the sediments, preventing specialised prokaryotes from occupying fixed niches.

Estuaries act as an important sink for nitrogen compounds from agricultural and industrial waste that would otherwise cause eutrophication of estuarine habitats (Dong et al., 2009; Mosier and Francis, 2008). The ability of sediment prokaryotic communities to transform these compounds and return them as nitrogen gas to the atmosphere has been well documented (Dong et al., 2002; Robinson et al., 1998). Prokaryotic communities can remove up to 50% of nitrogenous compounds by coupling of nitrification-denitrification and ANAMMOX (Mosier and Francis, 2008; Nedwell, 1999; Nicholls and Trimmer, 2009). The amount removed can vary greatly depending on the flushing time of freshwater though the estuary (Nixon et al., 1996; Ogilvie et

al., 1997). Again, the communities capable of these important processes have been monitored with respect to salinity, temperature and chemical gradients in estuaries. Nitrification, including ammonia oxidation, denitrification and ANAMMOX are performed by specialist communities from the *Betaproteobacteria, Gammaproteobacteria, Planctomycetes* and the ammonia oxidizing *Thaumarchaeota* phylum (Bernhard and Bollmann, 2010; Brochier-Armanet et al., 2012; Dale et al., 2009). Analogously to the patterns of sulphate reduction and methanogenesis, nitrification is commonly performed at the estuary mouth, denitrification at the head and ANAMMOX mostly at the estuary head in surface sediments (Cao et al., 2011; Dong et al., 2002; Dong et al., 2009; Rooks et al., 2012). As well as supply of oxygen and nitrate, temperature and seasonality have been found to have a large effect on the rates of these processes in estuarine sediments. Denitrification and ANAMMOX rates increased in winter, with lower temperatures (Dale et al., 2009; Nicholls and Trimmer, 2009; Ogilvie et al., 1997).

Whereas there has been extensive research into these specialised communities, much less work has concentrated on whole community analysis of estuarine sediments. Webster et al. (2010) and Wellsbury et al. (1996) investigated prokaryotic communities in Severn Estuary sediments (described in more detail below), and several research groups have analysed Pearl River Estuary whole bacterial and archaeal sediment communities. Sun et al. (2012) utilised denaturing gradient gel electrophoresis (DGGE) to investigate the vertical depth profile of *Bacteria* and found *Proteobacteria, Bacteroidetes, Acidobacteria, Chloroflexi, Actinobacteria, Firmicutes, Planctomycetes* and *Cyanobacteria* to be most common. *Proteobacteria* and *Bacteroidetes* were the most abundant phyla. Depth, salinity, pH, ammonium, phosphate and silicate were the main influences on these communities. Jiang et al. (2011) performed a similar study on the *Archaea* using 16S ribosomal RNA (rRNA) gene clone libraries, and found that the Miscellaneous Crenarchaeota Group (MCG) was the most abundant at all depths, but that *Methanomicrobiales* and ANME-2 were also detected at all depths at lower abundance. However, the archaeal sequences were mostly unassignable to higher phylogenetic ranks and so remained mostly unknown.

Much more work has focused on tidal flats, which are often found in, but are not specific to estuaries (Böttcher et al., 2000; Kim et al., 2008; Lee et al., 2011; Wilms et al., 2006a; Wilms et al., 2006b).

1.3 The Severn Estuary Sediment Environment

The Severn Estuary at the mouth of the River Severn is the longest river in the UK, which flows into the Bristol Channel. The estuary stretches from after the second Severn crossing at Severn Beach, Gloucestershire to between Lavernock Point, Cardiff and Sand Point, Weston-Super-Mare (Figure 1.3). The Severn catchment area is very large, reaching into mid and east Wales and much of the west of England. Many rivers drain into the estuary including the rivers Usk, Taf and Wye on the Welsh side and the rivers Avon, Yeo and Axe from England. It is estimated that approximately 77% of the sediment brought into the estuary in contributed from the Severn, Avon and Wye (Allen, 1991; Jonas and Millward, 2010). From these sources, a range of materials are introduced into the estuary, including nutrients, pollutants and microorganisms (Gao et al., 2013b).

The Severn Estuary and its environs include salt marsh, extensive intertidal flats and marine habitats, home to a number of significant plant and animal species, and as such has become an important national and international conservation area (Ballinger and Stojanovic, 2010; Burton et al., 2010). The estuary itself and surrounding habitats have been designated a Site of Special Scientific Interest, a Ramsar Site, which are internationally important wetlands, and a European Special Protection Area (Burton et al., 2010). This is mainly due to the large number of migratory bird species that winter on the wetlands such as European white-fronted goose, Bewick's swan, Shelduck, Dunlin and Redshank (Burton et al., 2010). In recent years a rapid increase in the number of fish species has been recorded (>100), which has been attributed to the general improvement in water quality since these habitat restrictions have been imposed (Henderson and Bird, 2010).

The estuary is hyper-tidal with a mean range of over 6 m, and has the second highest tidal range in the world (mean spring tide of 12.3 m), just below that of the Bay of Fundy, Nova Scotia, Canada (mean spring tide 14.5

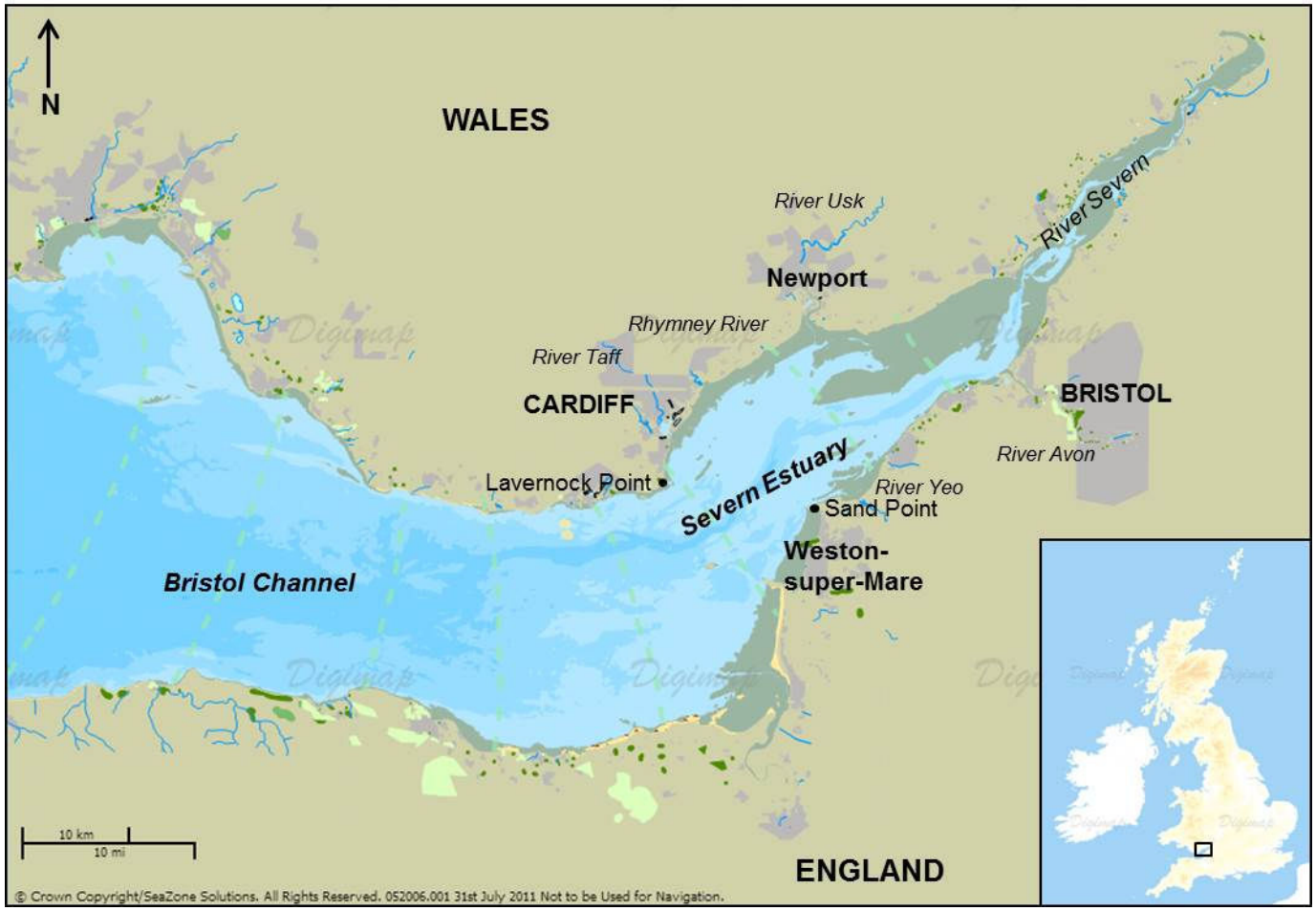


Figure 1.3 - Map of the Severn Estuary, UK. Scale 1 : 350 000. Map created March 2014 using EDINA Marine Digimap Service, http://edina.ac.uk/digimap.

metres) (Desplanque and Mossman, 2001; Kirby, 2010; Manning et al., 2010). This high tidal range is caused by its natural conical shape, unique in the UK, which funnels the North Atlantic tidal wave into the much narrower and shallower channel of the estuary and eventually the River Severn (Manning et al., 2010; Ware et al., 1972). This process creates the natural phenomenon of the Severn Bore, a large surge wave that can reach up to 3 m in height and travels 34 km up the estuary into the River Severn to Gloucester.

The hyper-tidal nature, winds, storms and sea level in the estuary play vital roles in the shaping of its sediment geomorphology. These factors lead to mixing within the waters which gives rise to homogeneity in salinity and temperature as well as in benthic and water column dwelling communities (Uncles, 1983; Uncles, 2010). The estuary bed mainly consists of rocks, gravel and sandbanks created by strong sediment movements (Green, 2009). The estuary is highly turbid along its length, due to the strong tides and currents, creating fine suspended sediments and giving the water its characteristic muddy colouring (Manning et al., 2010). Strong tidal currents and turbidity leads to the extensive turnover of the upper most sediment layers with up to 1 m of the upper sediment frequently turned over (Kirby and Kirby, 2008; Wellsbury et al., 1996). Fluid mud deposits that are able to move across the estuary bed have been recorded in the deeper parts of the main channel (Kirby, 2010; Uncles, 2010). These are known to add the mixing of the upper most sediment. The tides and strong waves also result in the erosion and deposition of sediment and mud on the mudflats and intertidal zones which varies seasonally (Allen and Duffy, 1998; Kirby and Kirby, 2008; O'Brien et al., 2000). In fact some intertidal and mudflats have been shown to be up to 1 cm higher in the spring due to increased sediment supply (Whitehouse and Mitchener, 1998).

The high tide has many important implications on the anthropogenic uses of the estuary. The Severn Estuary has the highest energy levels of any estuary in the UK and so it has been targeted for the use of tidal barrages to produce renewable energy (Kirby, 2010; Polymenakou et al., 2009). This is a highly controversial scheme and the potentially detrimental effects of the barrage

on the natural flora and fauna is much debated. The fine sediments created by tidal currents accumulate and spread pollutants throughout the estuary (Langston et al., 2010). The area in and around the estuary is exploited by industry (including nuclear power plants), agriculture, shipping, sewage systems, renewable energy schemes and also tourism and urban development. From these anthropogenic sources the range of pollutants found in the estuary is extensive. These include radionucleotides, polychlorinated biphenyls (PCBs), heavy metals (e.g. lead) and pesticides (Duquesne et al., 2006; McCubbin et al., 2001). Many of the background reference thresholds for pollutant chemicals set by OSPAR (Oslo and Paris Conventions for the protection of the marine environment of the North-East Atlantic) are exceeded in the estuary. Sewage from the 39 waste water treatment outflows and industrial effluent flow into the estuary at approximately 0.8 x 10^6 m^3/day and 0.2 x 10^6 m^3/day, respectively (Jonas and Millward, 2010). Thus both environmental and anthropogenic influences must be taken into account when considering the changing ecology of the estuary and its environs.

1.3.1 Prokaryotes in the Severn Estuary Sediment Environment

There is much research into the larger organisms in the Severn Estuary such as wild birds, fish and shellfish but there is still little knowledge of the microbial populations (Burton et al., 2010; Langston et al., 2010). The Severn Estuary is of particular interest as it receives nutrients from both land and water potentially creating intense primary production and heterotrophic activity (Joint, 1984; Morris, 1984). Also, chemicals from agriculture and industry leach into the estuary, which could have a profound effect on the prokaryotic metabolic processes as seen by Engelen and Cypionka (2009). The dramatic physical attributes are also thought to directly affect the sediment communities, including microorganisms, burrowing organisms and filter feeders in the estuarine sediments. Severn Estuary sediments have been classified as “barren” and “naturally-depauperated” due to the inability of burrowing organisms and filter feeders to colonise the highly dynamic sediments (Kirby, 2010). Up to the first 1 m of sediment can be turned over and the effect of this re-oxygenation on prokaryotic communities remains to

be seen (Wellsbury et al., 1996). The extent of the anaerobic biogeochemical zones in Severn Estuary sediments have been defined by changes in sediment pore water (Webster et al., 2010). Oxygen is depleted quickly and so alternative electron acceptors are consumed in order of decreasing energy production as previously described (Figure 1.1; Canfield et al., 2005; Froelich et al., 1979). The change from sulphate reduction to methanogenesis occurs over a relatively limited depth range of 50-60 cm below the sediment surface (Webster et al., 2010).

The first investigations into the microbial populations of Severn Estuary were by the Sabrina project, hosted by the University of Bristol (Dineley and Smith, 1975). This was partly focused on the influence of the physical and chemical aspects on primary production and phytoplankton in the water column (Joint, 1984; Joint and Pomroy, 1981). Research, through to the present, has also focused on analysis of so called 'bacterial pollution' of enterococci bacteria, and how the estuary's physical properties may affect bathing waters around the Bristol Channel (Anson and Ware, 1974; Gao et al., 2013a; Kay et al., 2005; Ware et al., 1972). Joint and Pomroy (1982) analysed activity of *Bacteria* in the water column at various points in the outer estuary near Cardiff and the Bristol Channel. They found the highest numbers of *Bacteria* and activities in the estuary itself were related to high water turbidity and currents. Further work by the same group investigated the effect of physical properties of the estuary on phytoplankton communities (Joint, 1984). In fact, all of the aforementioned research focused on the unusually high turbidity of the estuary and the consequences on microbial communities in the water column.

However, since 1984 there have been very few further studies, specifically investigating prokaryotic communities in Severn Estuary sediments. In 1994 and 1995, two groups looked at the structure and stability of microbial biofilms on intertidal mudflats in Portishead, Bristol, but neither looked at the phylogeny of the organisms there (Underwood et al., 1995; Yallop et al., 1994). Wellsbury et al. (1996) were the first to adopt molecular, rather than cultivation-based methods to examine intertidal sediments from the Severn Estuary at Aust Warth. Using acridine orange direct counts (AODC), they

reported that prokaryote numbers were consistently high (~10^9 cells/ml) in the top 8 cm, and dropped with increasing depth. Sulphate reduction was also found to be the dominant biogeochemical process (60% of the total organic matter degradation) occurring in these sediments, which agrees with other coastal sediment habitats (Jørgensen, 1983), and methanogenesis was found to contribute to approximately 1% of total organic matter degradation (Wellsbury et al., 1996). More recently, Webster et al. (2010) highlighted the vital ecological role of uncultivated prokaryotes in the tidal sediments at Portishead, Bristol. This group utilised DNA stable isotope probing (SIP) method to analyse the structure and activity of both *Bacteria* and *Archaea* in multiple geochemical zones within a tidal flat. *Proteobacteria,* such as the *Gammeproteobacteria* and *Epsilonproteobacteria*, and the *Marine Group 1 Archaea* proliferated in specific geochemical zones in the sediments, depending on the substrate given. Interestingly, no sequences related to known SRB were detected. This was the first description of archaeal communities in the Severn Estuary. The MCG and MCG-B archaeal groups were detected, and methanogen diversity was described in terms of 16S rRNA and *mcrA* gene phylotypes. Further work by Watkins et al. (2012) isolated a *Methanococcoides* strain from Portishead which was able to metabolise ethanolamine. However, little is yet known of the diversity and function of the prokaryotic communities in Severn Estuary sediments and their potential role in global biogeochemical cycles.

1.4 Cultivation-Independent Methods in Microbial Ecology

1.4.1 The Development of Cultivation Independent Techniques in Microbial Ecology

The ‘great plate count anomaly’ describes the vast underestimation of the diversity of an environmental microbial community caused by the limitations inherent in cultivation-dependent analysis (Staley and Konopka, 1985). It was noted that there was an incongruence between the number of microbes in environmental samples counted with microscopy and the number of colonies formed with culturing methods (Jannasch, 1959; Kogure et al., 1979). Torsvik et al. (1990) estimated that less than 1% of all

microorganisms in an environmental sample could be grown in the laboratory. Furthermore, it was estimated that only 0.001–0.1% of microbes can be cultivated from marine environments, such as seawater (Amann et al., 1995; Kogure et al., 1979). Thus, it is apparent that the use of cultivation-independent techniques to analyse the elusive organisms from these challenging environments is vital.

With the advent of 16S rRNA gene polymerase chain reaction (PCR) the limitations in culturing of microbes are avoided (Lane et al., 1985). 16S rRNA and functional gene PCRs have been widely used for the analysis of marine and sub-seafloor sediments with much success (Biddle et al., 2008; Colin et al., 2013; D'Hondt et al., 2004). The 16S rRNA gene has many advantages in the description of prokaryotic communities, which is why it has become so generally utilised. The gene is ubiquitous among both *Bacteria* and *Archaea* so even unknown prokaryotes can be targeted. Also, the mixture of conserved and variable regions allows the targeting of all prokaryotes or specific groups via use of appropriate PCR primers, and differentiation down to genus or species levels, respectively. Its size (approximately 1500 bp) is large enough to allow a great deal of information to be contained and, therefore, targeted by molecular approaches.

There are important limitations also to consider. Firstly, the use of PCR to amplify the sequence brings inherent biases associated with PCR such as primer bias to certain taxa or ambiguous targeting of other similar sequences (Hongoh et al., 2003). Though PCR is a powerful tool, it is limited if an environment has very low biomass. Whole genome amplification techniques have been used to successfully amplify metagenomic DNA from a low biomass communities, such as deep sediments, so that even the rarest community member is analysed (Abulencia et al., 2006). Also, the copy number of the 16S rRNA gene varies greatly between organisms with estimates between 1 – 15 copies per genome and polyploidy; some bacteria and archaea are known to have over 80 genome copies per cell (Hildenbrand et al., 2011; Punita et al., 1989; Větrovský and Baldrian, 2013). Multiple 16S rRNA gene copy numbers mean certain communities may be overestimated in their abundance, concealing slower growing communities

(Santos and Ochman, 2004). Finally, and perhaps most importantly, the detection of 16S rRNA gene sequences indicates the presence but not the activity or functionality of that organism. RNA, being a labile and transiently transcribed molecule, allows the targeting of active populations to overcome, in part, these problems (Ward, 2005). Technologies such as mRNA labelled SIP and RNA-seq allow more sensitive assays of the active prokaryotic transcriptome that have already been applied to difficult sediment environments (Dumont et al., 2011; Marioni et al., 2008).

1.4.2 Profiling and Quantification Methods to Characterise Prokaryotic Communities

Many innovative techniques have been derived from 16S rRNA gene analysis. Common methods such as denaturing gradient gel electrophoresis (DGGE), thermal gradient gel electrophoresis (TGGE) and ribosomal intergenic spacer analysis (RISA) are used in combination with 16S rRNA gene PCR to create a profile of the microbes in an ecosystem (Borneman and Triplett, 1997; Christen, 2008; Muyzer and Smalla, 1998). These methods produce distinct banding patterns for the whole or targeted prokaryotic community for further statistical or sequencing and phylogenetic analysis. Since the first use of DGGE by Muyzer et al. (1993), it has become one of the most commonly utilised tools in microbial ecology and has been used in conjunction with functional gene primers (Ascher et al., 2010; Webster et al., 2007). Fluorescence *in situ* hybridisation (FISH) uses fluorescently labelled oligonucleotides, created from 16S rRNA gene sequences, to visually identify and enumerate individual microbial cells in their natural environment (Amann et al., 2001). CARD-FISH is a method used to amplify the FISH signal for genes with low copy number or less metabolically active cells i.e. in deeper sediments. This method was used with great success by Gittel et al. (1999) to analyse the abundance of sulphate reducing bacteria in deep tidal flat sediments. DNA microarrays are another technique that allows the high-throughput analysis of gene expression in bacteria grown under specific conditions (Dedysh, 2009; Moussard et al., 2009). However, with current research focusing more on

metagenomic and barcode analysis using next generation sequencing (see below) it is likely that profiling methods will very soon be redundant.

Quantification of prokaryotic communities is often estimated using fluorescent dyes. AODC utilises the nucleic acid dye acridine orange to stain fixed living cells before counting using epifluorescence microscopy (Cragg and Parkes, 2014; Rublee and Dornseif, 1978). The AODC method has been largely replaced by the rapid method of quantitative PCR (qPCR), which uses a range of different dyes, most commonly SybrGreen, to intercalate with double stranded DNA during a PCR reaction, giving real time quantification. Coupling this with specific 16S rRNA gene PCR primers allows for the approximation of cell numbers and has allowed the estimation of cell numbers from a range of samples including deep subsurface sediments (Blazejak and Schippers, 2010; Schippers and Neretin, 2006). The coupling of functional or taxa targeting PCR primers and hybridisation probes, such as reductive dehalogenase and *Dehalococcoidia* specific primers, have given greater insight into the presence and abundance of specific functional communities (Hatt and Löffler, 2012; Ritalahti et al., 2006). The quantification of prokaryotic communities is important in the prediction of global biomass and carbon sequestration (Kallmeyer et al., 2012; Whitman et al., 1998).

1.4.3 Next Generation Sequencing (NGS) Technologies

The development of NGS methods in the early 2000s has largely led to the replacement of gene cloning and Sanger sequencing in microbial ecology research. The advantages over more traditional methods, such as cloning and profiling, are numerous and generally beneficial. For example, the most popular NGS method with microbial ecologists has been pyrosequencing by 454 Life Sciences and Roche Diagnostics, which detects the incorporation of individual bases by the release of inorganic pyrophosphate linked to production of light pulses that are detected by CCD cameras (Margulies et al., 2005; Roh et al., 2010). The benefits of pyrosequencing were longer read lengths than other technologies (approximately 700 bp), running of high throughput parallel sample using barcoded primers, greater depth of

coverage (up to 1 million reads in one run) and less biases introduced than cloning in *Escherichia coli* (Liu et al., 2012; Roh et al., 2010; Shokralla et al., 2012). In fact, numerous comparisons between clone library Sanger sequencing and pyrosequencing have found that much greater diversity is detected using the latter method (Biddle et al., 2008; Hamdan et al., 2012; Kim et al., 2008; Quince et al., 2008). Furthermore, pyrosequencing has allowed microbial ecologists access to the 'rare biosphere', those organisms in low abundance in a given environment. It is believed that this pool of unknown and uncharacterised diversity may harbour unique metabolisms with potential to buffer communities or become abundant with extreme environmental or anthropogenic change (Brown et al., 2009; Sogin et al., 2006). However, the 454 pyrosequencing platform will not be supported by Roche from 2016, and so attentions must turn to new technologies such as the newest Illumina and Ion Torrent sequencing platforms, which offer greater read lengths and depth of coverage than previous sequencing chemistries.

There are limitations to the pyrosequencing method, for example general prokaryotic primers may not target all taxa in an environment (Soergel et al., 2012; Teske and Sørensen, 2008; Wang and Qian, 2009) and homopolymers of six or more bases create artefacts in sequence libraries that could be construed as novel taxa (Kunin et al., 2010). These limitations have been addressed by improving primer design (Pinto and Raskin, 2012), and bioinformatics software to target sequences with artefacts or chimeras (Haas et al., 2011; Quince et al., 2008). A further limitation that must be considered by the researcher is the amount of sequencing effort to apply to an environmental sample to detect as much of the diversity as possible. Quince et al. (2008) used a statistical method to estimate sequencing effort needed to evaluate a sample's diversity. They estimate that diverse environments such as the deep ocean and soils would need hundreds of times greater number of samples than currently processed to achieve 90% of the taxonomic diversity. Fortunately, the capability of sequencing technologies is constantly improving with the newest Illumina platforms capable of giving up to 2 billion reads per run (HiSeq 2500 platform:

http://www.illumina.com/systems/sequencing.ilmn, accessed March 2014).A range of approaches utilising pyrosequencing are currently in use in the microbial ecology field. Amplicon sequencing using 16S rRNA gene targeted primers to specifically analyse the taxonomic diversity of a prokaryotic community is widely popular. For example, this method has been applied to soils (Roesch et al., 2007), tidal flat sediments (Kim et al., 2008) and underwater freshwater springs (Ionescu et al., 2012). Amplicon sequencing has also been adapted for use with functional genes to analyse the metabolic potential in a community (Lüke and Frenzel, 2011; Sun et al., 2011).

A metagenomic approach, that is non-specific sequencing of whole community DNA, has been largely successful in identifying functional aspects of environmental communities (Biddle et al., 2008; Edwards et al., 2006; Varin et al., 2012). This is particularly useful in geochemically interesting environments, such as sediments, where the role of prokaryotes in global geochemical cycles is still being unravelled. Metatranscriptomic approaches, in which cDNA libraries are made and sequenced from total or mRNA from a prokaryotic community, are now more widely used (DeLong, 2009; Frias-Lopez et al., 2008; Poretsky et al., 2009). These methods allow the targeting of active communities only, which in ancient environments such as deep subsurface sediments is important since dead cells or ancient extracellular DNA may affect the sequencing profile (Mills et al., 2012; Newberry et al., 2004). These methods have been widely used in marine environments for both basic and applied research; for example, discovery of new drugs and biotechnologically useful enzymes (Simon and Daniel, 2011). Orsi et al. (2013) successfully used a metatranscriptomic approach to describe potential microbial metabolism in the deep biosphere (continental shelf of Peru, Ocean Drilling Program Site 1229), but *Chloroflexi* sequences were found at very low abundance. By contrast, amplicon sequencing at the same deep biosphere site indicated *Chloroflexi* was a dominant group (Biddle et al., 2008), indicating organisms with lower expression levels may be missed with mRNA based techniques.

Finally a new field of single cell genomics is becoming popular to try to elucidate the functional potential of prokaryotic populations by their functional

genes. This has already been applied to *Chloroflexi* cells from sediment environments with much success (Kaster et al., 2014; Wasmund et al., 2014b), and is further described in the next section.

1.5 The *Chloroflexi* Phylum of *Bacteria*

1.5.1 Chloroflexi *Phylogeny*

In his seminal work on comparative 16S rRNA gene sequences and bacterial evolution, Woese (1987) described the commonly termed 'green non-sulphur' bacteria as one of the eleven bacterial phyla. This phylum was later renamed the *Chloroflexi* after the type strain *Chloroflexus aurantiacus,* one of only four species to be isolated from the phylum at that time (Garrity and Holt, 2001; Pierson and Castenholz, 1974). It is a diverse and deeply branching phylum, comparable to the *Proteobacteria,* and is the deepest branching phototrophic phylum on the 16S rRNA gene phylogenetic tree (Hanada et al., 2002; Oyaizu et al., 1987). The phylum itself contains a broad range of phenotypes, from mesophiles to thermophiles, phototrophy to chemotrophy, Gram positive and negative, aerobic and anaerobic, spore-forming, gliding and non-motile species (Hugenholtz et al., 1998; Rappé and Giovannoni, 2003). The apparent ubiquity of environmental clones related to this phylum in terrestrial and marine ecosystems, as well as the human microbiome (e.g. the oral cavity (Campbell et al., 2014; Dewhirst et al., 2010), indicates the important role of the *Chloroflexi* in the natural environment (Hugenholtz et al., 1998). Few members of the phylum have been cultivated to date, and so the full metabolic potential of the *Chloroflexi* and their role in these environments is largely unclear (Costello and Schmidt, 2006; Durbin and Teske, 2011).

Currently, the *Chloroflexi* is divided into at least eight major formally described subdivisions or classes, based on 16S rRNA gene sequences, (Table 1.2 & Figure 1.4; Kawaichi et al., 2013; Morris et al., 2004). However, there has been much confusion in the literature over the division and naming of the classes within the phylum, with subphyla, subgroup, subdivision and

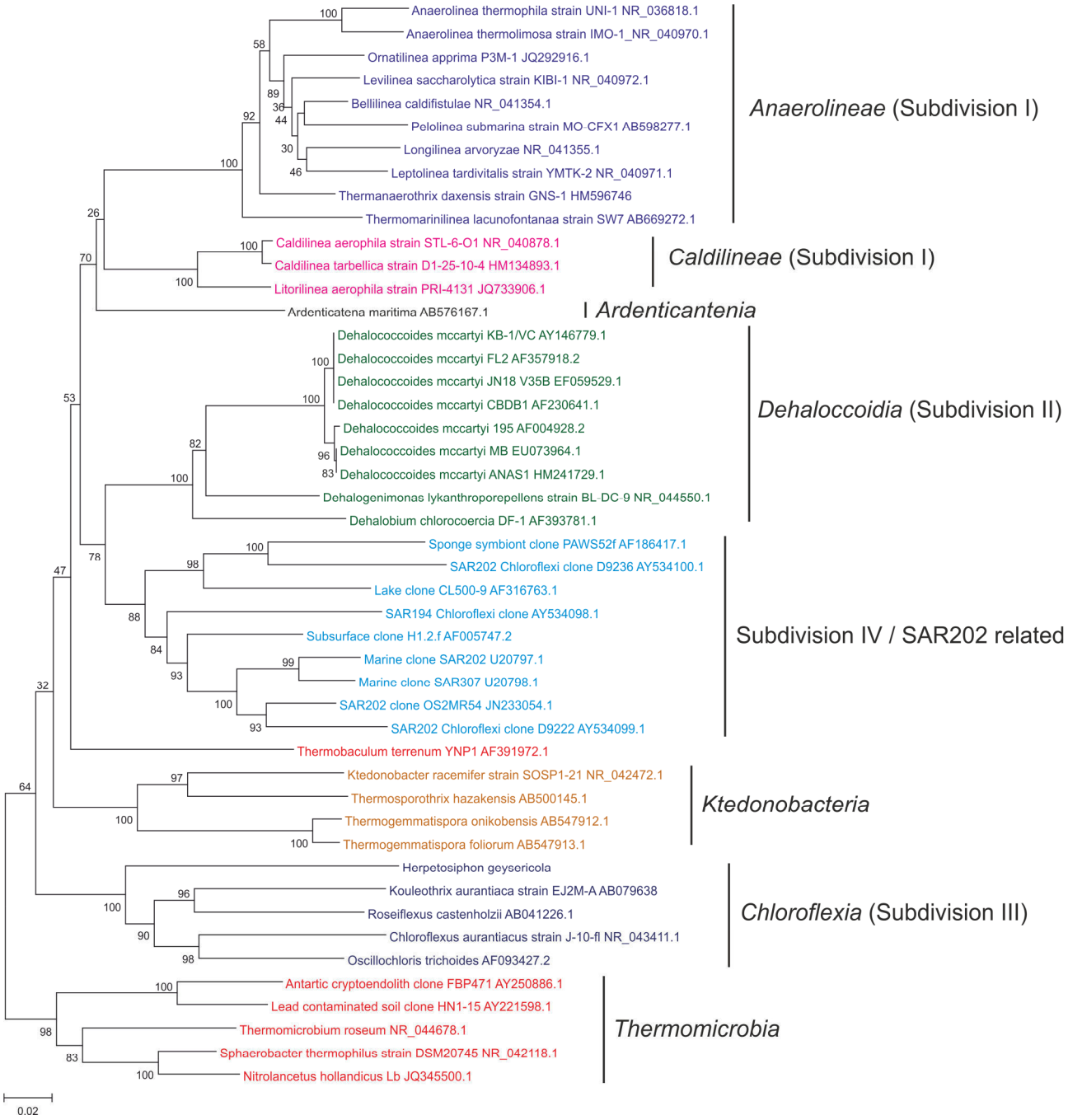


Figure 1.4 – Phylogenetic tree of 16S rRNA gene sequences from type strains and uncultivated environmental clones from the eight *Chloroflexi* subdivisions. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA5.

Table 1.2 – Description of each *Chloroflexi* class derived from cultivated representatives.

***Chloroflexi* Class**	**Cultured Type Strains**	**Isolated From**	**Aerobic/ Anaerobic Growth**	**Metabolism**	**Electron Acceptors**	**Temperature Range**	**Cell Morphology**	**Gram Stain**	**Spore-Forming**
Anaerolineae (Subdivision I)	*Anaerolinea thermophila* *Anaerolinea thermolimosa* *Bellilnea caldifistulae* *Leptolinea tardivitalis* *Levilinea saccharolytica* *Longilinea arvoryzae* *Ornatilinea apprima*	Anaerobic sludge, rice paddy soil, deep hot aquifers	Anaerobic	Fermentation	None	20-73°C	Filamentous	Negative	No
Caldilineae (Subdivision I)	*Caldilinea aerophila* *Caldilinea tarbellica* *Litorilinea aerophila*	Hot springs, deep hot aquifer	Both	Fermentation Aerobic	Oxygen	37-65°C	Filamentous	Negative	No
Dehalococcoidia (Subdivision II)	*Dehalococcoides mccartyi* *Dehalogenimonas lykanthroporepellens* *Dehalogenimonas alkenigignens*	Anaerobic reactor, groundwater, river sediment	Anaerobic	Chemoheterotrophic (Dehalorespiration)	Chlorinated alkanes	15-35°C	Discs, cocci	Negative	No

***Chloroflexi* Class**	**Cultured Type Strains**	**Isolated From**	**Aerobic/ Anaerobic Growth**	**Metabolism**	**Electron Acceptors**	**Temperature Range**	**Cell Morphology**	**Gram Stain**	**Spore-Forming**
Chloroflexia (Subdivision III)	*Chloroflexus aurantiacus* *Chloroflexus aggregans* *Chloronema giganteum* *Oscillochloris trichoides* *Heliothrix oregonensis* *Roseiflexus castenholzii* *Herpetosiphon aurantiacus*	Hot springs, association with freshwater alga	Both	Phototrophic, fermentation	Oxygen	10-67°C	Filamentous	Negative	No
Thermomicrobia	*Thermomicrobium roseum* *Sphaerobacter thermophilus* *Nitrosolancetus hollandicus*	Hot spring, thermal soil, anaerobic sludge	Aerobic	Chemoheterotrophic, chemolithoautotrophic (Nitrite oxidation)	Oxygen	43-80°C	Rods	Both	No
Ktedonobacteria	*Ktedonobacter racemifer* *Thermosporothrix hazakensis* *Thermogemmatispora onikobensis* *Thermogemmatispora foliorum*	Soil, compost, geothermal soil	Aerobic	Chemoheterotrophic	Oxygen	17-74°C	Filamentous with branched mycelia	Positive	Yes
Ardenticatenia	*Ardenticatena maritima*	Coastal hydrothermal field	Aerobic	Chemoheterotrophic (Nitrate & iron reduction)	Ferric compounds, nitrate, oxygen	30-75°C	Filamentous	Negative	No
Subdivision IV (SAR202 related)	No cultured strains	Marine sediment & water	-	-	-	-	-	-	-

class being used interchangeably. Hugenholtz et al. (1998) initially assigned the names subdivision I – IV to clades within the phylum while analysing over 8,000 bacterial 16S rRNA gene sequences to clarify the bacterial domain. Later, as more cultivated representatives were described, these subdivisions were renamed accordingly and others added as the *Anaerolineae* (Sekiguchi et al., 2003), *Dehalococcoidia* (Löffler et al., 2013)*, Chloroflexi* (Garrity and Holt, 2001), *Thermomicrobia* (Hugenholtz and Stackebrandt, 2004)*, Ktedonobacteria* (Cavaletti et al., 2006) and the unofficially named subgroup IV/SAR202 related clonal group (Morris et al., 2004). Further naming systems based on uncultivated clone members (e.g. H09, C12, G04, E05, A07, B12, C05) have also been used, leading to further confusion (Costello and Schmidt, 2006; Rappé and Giovannoni, 2003). In this work will use the official class level naming system given above and in Table 1.2 and Figure 1.4 will be used.

1.5.1.1 Anaerolineae *and* Caldilinea *(Subdivision I)*

The *Anaerolineae* class was proposed by Hugenholtz and Stackebrandt (2004), named for the first cultivated representative and type strain *Anaerolineae thermophila* (Sekiguchi et al., 2003). Until this point *Anaerolineae* (or subdivision I) consisted mostly of environmental clone sequences from a large variety of habitats such as hot springs, subsurface sediments, waste water treatment works, cold tundra soils and aquifers, to name a few (Björnsson et al., 2002; Costello and Schmidt, 2006; Durbin and Teske, 2011). Yamada and Sekiguchi (2009) estimated that >70% of deposited *Chloroflexi* sequences in the RDP database (release 10.11) were related to *Anaerolineae,* and, as such, this class has the most diverse and largest number of cloned sequences of all the *Chloroflexi*. Again, this indicates an extremely important role of the *Anaerolineae* in a variety of habitats that is now, beginning to be revealed.

Recent advances in genomic research allow us to postulate the metabolic potential of difficult to cultivate organisms, such as the *Anaerolineae* and *Caldilineae,* with two unpublished whole genomes available (Table 1.3).

Table 1.3 - Description of *Chloroflexi* sequenced genomes from each subdivision.

***Chloroflexi* Class**	**Organism Name**	**Number of Genome Sequences**	**Number of Chromosomes**	**Accession Number[a]**	**Genome Size (Mb)**	**16S rRNA Gene Copy Number**	**Reference**
***Anaerolineae* (Subdivision I)**	*Anaerolinea thermophila* UNI-1	1	1	NC_014960	3.53	2	Narita-Yamada,S. et al., unpub results
***Caldilineae* (Subdivision I)**	*Caldilinea aerophila*	1	1	NC_017079.1	5.14	2	Oguchi, A. et al., unpub results
***Dehalococcoidia* (Subdivision II)**	*Dehalococcoides mccartyi* strains CBDB1,195, BAV1, VS, GT, DCMB5, BTF08, GY50, SG1	9	1	NC_007356.1, NC_002936.3, NC_009455.1, NC_013552.1, NC_013890.1, NC_020386.1, NC_020387.1, NC_022964.1	1.34 – 1.47	1	Kube et al. (2005), Seshadri et al. (2005), Copeland, A. et al., unpub results, McMurdie et al. (2009), Lucas, S. et al., unpub results, Poritz et al. (2013), Ding, C. et al., unpub results, Wang, S. et al., unpub results.
	Dehalococcoides lykanthroporepellen BL-DC-9	1	1	NC_014314.1	1.69	1	Siddaramappa et al. (2012)

[a] Reference accession number from the National Centre for Biotechnology Information

Chloroflexi Class	Organism Name	Number of Genome Sequences	Number of Chromosomes	Accession Number[a]	Genome Size (Mb)	16S rRNA Gene Copy Number	Reference
	Dehalococcoidia bacterium SCGC AB-539-J10	1	-[b]	NZ_ARPL00000000.1	1.44	1	Wasmund et al. (2014b)
	Dehalococcoidia bacterium Dsc1	1	-	NZ_JARM00000000.1	0.32	1	Kaster et al. (2014)
	Dehalococcoidia bacterium DscP2	1	-	NZ_JARN00000000.1	1.38	1	Kaster et al. (2014)
Chloroflexia **(Subdivision III)**	*Chloroflexus aurantiacus* J-10-fl	1	1	NC_010175.1	5.26	3	Copeland, A. et al., unpub results,
	Chloroflexus aggregans	1	1	NC_011831.1	4.68	3	Lucas, S. et al., unpub results
	Herptesiphon aurantiacus	1	1	NC_009972.1	6.79	2	Kiss et al. (2011)
	Roseiflexus castenholzii	1	1	NC_009767.1	5.72	2	Copeland, A. et al., unpub results,
	Oscillchloris trichoides DG-6	1	-	NZ_ADVR00000000.1	4.37	4	Kuznetsov et al. (2011)

[b] Where the number of chromosomes or 16S rRNA genes are unknown, this is a whole genome shotgun sequence and no data was available from NCBI

Chloroflexi Class	Organism Name	Number of Genome Sequences	Number of Chromosomes	Accession Number[a]	Genome Size (Mb)	16S rRNA Gene Copy Number	Reference
Thermomicrobia	*Thermomicrobium roseum*	1	1	NC_011959.1	2.00	1	Wu et al. (2009)
	Sphaerobacter thermophilus	1	2	NC_013523.1 NC_013524.1	2.74 1.25	2	Spring et al. (2010)
	Nitrolancea hollandica	1	-	NZ_CAGS00000000.1	3.89	1	Sorokin et al. (2012)
Ktedonobacteria	*Ktedonobacter racemifer*	1	-	NZ_ADVG00000000.1	13.66	8	Chang et al. (2011)
Subdivision IV (SAR202 related)	SAR202 cluster bacterium SCGC AAA240-N13	1	-	NZ_AQTZ00000000.1	1.46	-	Stepanauskas, R. et al., unpub results

Hug et al. (2013) performed ground breaking work on the community metagenomics of aquifer sediments, where they isolated three partial *Chloroflexi* genomes, including many of their metabolic genes. From analysis of one *Anaerolineae* genome, they found adaptions to both aerobic and anaerobic growth, as well as a large number of genes for the fermentation of a variety of sugar compounds and amino acids, detoxification, heavy metal redox and contaminant degradation genes (Hug et al. 2013). Together with work by Kindaichi et al. (2012), they hypothesize that this broad metabolic capability allows *Anaerolineae* to scavenge organic compounds and decaying cell debris to survive in nutrient limited, mixed aerobic and anaerobic niches such as sediments. The recently cultivated strain *Pelolinea submarina* was isolated from deep subseafloor sediments in Japan and was found to be an anaerobic fermentative organism, capable of utilising a range of carbohydrate compounds in a methanogenic community which lends weight to this hypothesis (Imachi et al., 2014).

Now twelve species from at least nine genera have been cultivated, as summarised in Table 1.2. The group mainly consists of non-motile, filamentous bacteria with both aerobic and anaerobic, chemoorganotrophic and fermentative but not photosynthetic growth (Yamada et al., 2006). Most strains were isolated in sludge reactors and waste water treatment works due to the biotechnological importance of the *Anaerolineae* in the granulation and bulking of sludge (Yamada and Sekiguchi, 2009). Recently, two strains have been isolated from a hydrothermal sea vent and marine subsurface sediments in Japan (Imachi et al., 2014; Nunoura et al., 2013). Interestingly, certain isolates have been shown to only grow or more efficiently grow in a syntrophic association with hydrogenotrophic methanogens (Sekiguchi et al., 2003; Yamada et al., 2007a; Yamada et al., 2006). This could suggest syntrophic associations with archaea in methanogenic environments, such as subsurface sediments, which warrants further investigation. The once monophyletic subdivision I has now been split into the *Anaerolineae* and *Caldilineae* classes due to differences in respiration (i.e. strict anaerobic and

facultatively aerobic growth, respectively) and poor node support in phylogenetic trees (Yamada et al., 2006).

1.5.1.2 Dehalococcoidia *(Subdivision II)*

Dehalococcoidia (Subdivision II) was originally designated *Dehalococcoidetes* by Hugenholtz et al. (1998) after the first isolated species *Dehalococcoides ethenogenes* 195 (Maymo-Gatell et al., 1997). The class was later officially renamed the *Dehalococcoidia* and the strain to *Dehalococcoides mccartyi* 195 (Löffler et al., 2013). The *Dehalococcoidia* have been one of the most intensively investigated *Chloroflexi* classes due to its dehalogenation capabilities and therefore biotechnological relevance and novel occurrence in deep subsurface sediment environments (Fry et al., 2008; Löffler and Edwards, 2006). Nine *Dehalococcoides* strains have so far been cultivated, and their genomes sequenced (Table 1.3), all of which are strictly anaerobic, Gram negative, coccoidal bacteria, able to gain energy through reductive dehalogenation of chlorinated and brominated compounds by oxidation of H_2 via the reductive dehalogenase (*rdh*) genes (Table 1.2; Cheng and He, 2009; He et al., 2005; Hug et al., 2012; Müller et al., 2004). *D. mccartyi* 195 was the first bacterium to be described that fully dechlorinate toxic tetrachloroethene to the nontoxic ethene (Maymo-Gatell et al., 1997). Another genus also exists within the *Dehalococcoidia* that contains two species: *Dehalogenimonas lykanthroporepellens* and *Dehalogenimonas alkenigignens,* which are phenotypically similar to *Dehalococcoides,* but are phylogenetically distinct (Bowman et al., 2013; Moe et al., 2009).

Though it was previously proposed all *Dehalococcoidia* relied on reductive dehalogenation, analysis of sediment *Dehalococcoidia* related metagenomes indicate further metabolic potential, such as fermentation of sugars and plant polymers, as well as potential for acetogenesis (Hug et al., 2013). Further to this, evidence of a nitrogenase operon was found in the *D. mccartyi* genome (Table 1.3; Seshadri et al., 2005). Subsequently, Lee et al. (2009) reported that *D. mccartyi* could fix nitrogen in long term growth experiments. Nitrate- and nitro-reductases and nitrogen fixing domains were also detected in *Dehalococcoidia* single cells of deep subsurface sediments in the Peru

Margin (Table 1.3; Kaster et al., 2014). This evidence suggests that *Dehalococcoidia* are more metabolically versatile that previously thought.

1.5.1.3 Chloroflexia *(Subdivision III)*

Chloroflexia (Subdivision III, renamed from *Chloroflexi* to avoid confusion with whole phylum (Gupta et al., 2012)) are phototrophic, filamentous, gliding bacterium often referred to as filamentous anoxygenic phototrophs (FAPs) (Garrity and Holt, 2001). All are phototrophs, although some species grow aerobically in the dark and anaerobically in the light (Table 1.2; Hanada et al., 2002; Keppen et al., 2000). Though several of the cultivated representatives are thermophiles, recovered from microbial mats in hot springs, a number of species are mesophilic (Hanada et al., 1995a; Hanada et al., 1995b; Holt and Lewin, 1968; Pierson et al., 1985). Recent work on the phylogeny of the *Chloroflexia* and the whole phylum has suggested that only the *Chloroflexia* and *Thermomicrobia* should be considered as part of the phylum *Chloroflexi* with the remaining subdivisions regarded as part of a superphylum (Gupta et al., 2012). Clearly, further work is needed to clarify the class and phylum level phylogeny of the *Chloroflexi.*

1.5.1.4 The Uncultivated Clone Group and SAR202 Related Chloroflexi *(Subdivision IV)*

Subdivision IV was first introduced by Hugenholtz et al. (1998) as a class level group of the *Chloroflexi* with no cultivated representatives, and this is still the case. It is referred to as subdivision, subphylum or subgroup IV or the SAR202 related *Chloroflexi*. A number of environmental 16S rRNA gene phylotypes constitute the group, in particular SAR202 bacterioplankton, originally isolated from a stratified water column of the Sargasso sea below the deep chlorophyll maximum (Giovannoni et al., 1996). Since then SAR202-related sequences have been detected in freshwater, marine water, sediments, soils and the deep subsurface (Fry et al., 2008; Morris et al., 2004). In the absence of cultivated isolates and metagenomic or single cell genomic information (unavailable for access; Table 1.2), the metabolic potential of the class is unknown.

1.5.1.5 Thermomicrobia

The *Thermomicrobia* are a strictly aerobic, Gram variable, chemoheterotrophic class that grow optimally in moderately to hyperthermophilic temperatures (Table 1.2; Botero et al., 2004). The *Thermomicrobia* was a distinct phylum containing the species *Thermomicrobium roseum*, isolated from a hot spring in Yellowstone National Park (Jackson et al., 1973). Woese (1987) recognised the phylum as related to the *Chloroflexi* but it was not officially moved as a class within the *Chloroflexi* until 2004, with the addition of *Sphaerobacter thermophilus* from the *Actinobacteria* (Hugenholtz and Stackebrandt, 2004). Recently, a nitrite oxidising species, *Nitrolancetus hollandicus,* was isolated from a nitrifying reactor, which belongs to this class (Table 1.2; Sorokin et al., 2012). This species differed from others in the *Thermomicrobia,* since it is a chemolithoautotroph that can grow at a wider temperature range (25-63^{o}C). This was the first description of nitrite oxidizing abilities in *Chloroflexi,* indicating the growing notion of the importance of *Chloroflexi* in nitrification and global biogeochemical cycles.

1.5.1.6 Ktedonobacteria

The *Ktedonobacteria* class was added to the *Chloroflexi* in 2010 after two species (*Ktedobacter racemifer* and *Thermosporothrix hazakensis*) were isolated from thermal soils and were assigned to this distinct class within the phylum (Table 1.2; Cavaletti et al., 2006; Yabe et al., 2010b). Two further species have been cultivated from soils and compost, and all four have been found to be thermophilic,Gram positive, aerobic and spore-forming (Yabe et al., 2011). This was the first description of spore-forming activity in the *Chloroflexi*. The *Ktedonobacteria* formed branched vegetative and aerial mycelia, similar to *Actinomycetes* (Yabe et al., 2010a). Though all four cultivated species were isolated from soils, sequences related to the *Ktedonobacteria* have recently been detected in deep subsurface sediments near the hydrothermal vent field Loki's Castle at the Arctic Mid-Ocean Ridge (Jørgensen et al., 2013).

1.5.1.7 Ardenticatenia

The *Chloroflexi* phylum continues to grow, with a new class added in the past year, the *Ardenticatenia* (Kawaichi et al., 2013). This class was proposed for the newly isolated *Ardenticatena maritima,* a thermophilic, chemoheterotrophic facultative anaerobe, isolated from an iron-rich coastal hydrothermal field in Japan (Table 1.2). It is the first species of *Chloroflexi* to be described which grows by dissimilatory iron- and nitrate-reduction under anaerobic conditions, and lends further weight to the role of *Chloroflexi* in the global nitrogen cycle.

1.5.2 Distribution of Chloroflexi *in the Subsurface Sediment Environment*

The *Chloroflexi* have been detected in many different environments but none have generated as much interest as their apparent dominance in some deep subsurface sediments (Biddle et al., 2008; Fry et al., 2008; Teske, 2006). In organic rich, deep sediments taken from a variety of sites, the numbers 16S rRNA gene sequences related to *Chloroflexi* reached up to 80% of the total, and account for an average of 17% of all clones from the deep subsurface (Figure 1.5; Fry et al., 2008; Kaster et al., 2014; Parkes et al., 2014). Many of the *Chloroflexi* sequences detected were related to uncultivated clones with no close phylogenetic associations to cultivated species (Webster et al., 2006). This makes prediction of their metabolic function, physiology and ecology in these habitats extremely difficult. The *Dehalococcoidia* and subdivision IV are deemed to be deep subsurface indicator organisms as these are two of the most commonly detected *Chloroflexi* subphyla (Biddle et al., 2011; Inagaki et al., 2006). The *Anaerolineae* and most recently the *Ktedonobacteria* have also been detected in deep sediments to a lesser extent (Inagaki et al., 2006; Jørgensen et al., 2013). The subphyla that were found were often stratified with depth (Durbin and Teske, 2011; Inagaki et al., 2003; Parkes et al., 2005). In the most comprehensive analysis of subdivision distribution to date, Durbin and Teske (2011) discovered a shift in subdivision dominance in South Pacific abyssal sediments. The subdivision IV related SAR202 group were abundant in the water column, whereas *Anaerolineae* and an unnamed, clonal clade VIb were more abundant in

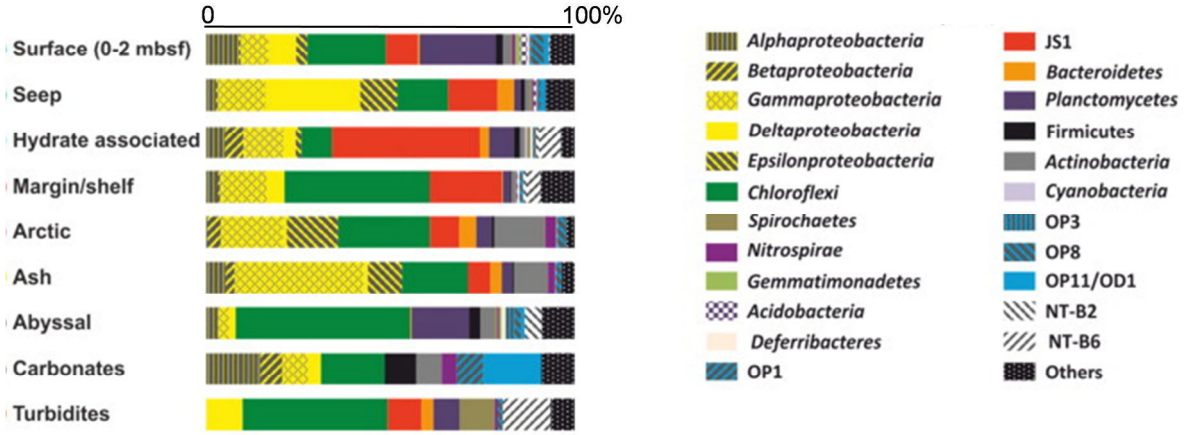


Figure 1.5 – Distribution and phylogeny of average percentage of 16S rRNA gene clone library sequences from various deep subsurface sediment environments. 205 clone libraries were used in this analysis, for full list see Supplementary Material of Parkes et al. (2014). Adapted from Parkes et al. (2014).

sediments 60-70 cbsf. By contrast, *Dehalococcoidia* and subdivision IV dominated below the oxycline (Durbin and Teske, 2011; Fry et al., 2008; Inagaki et al., 2006). These findings suggest *Chloroflexi* subdivision depth dominance may depend on nutrient richness and oxygen penetration in sediment profiles. The abundance and diversity of *Chloroflexi* in shallow surface and coastal sediments has been much less well described, as cultivation-based analysis used in the past overlooked these difficult to cultivate species (Adrian, 2009; Köpke et al., 2005; Wilms et al., 2006a). Recently, relatively high numbers of *Chloroflexi* have been detected in tidal sediments (Wang et al., 2012; Wilms et al., 2006a; Wilms et al., 2006b). Using clone libraries of German Wadden Sea sediments, a significant shift in dominance from *Proteobacteria* to *Chloroflexi* 2 mbsf was discovered that had not be seen in previous coastal sediments (Parkes et al., 2005; Webster et al., 2007; Wilms et al., 2006a). All of the *Chloroflexi* related clones from this sediment were related to *Dehalococcoidia.* Very little research has since concentrated on the specific subdivisions, or their role in these shallow sediment environments, as often the *Chloroflexi* are not specifically investigated. However, it is recognised that *Chloroflexi* communities in estuarine and tidal-flat sediments often follow the same abundance and geochemical patterns with depth as deep subsurface marine sediments and are therefore useful models for these habitats (Engelen and Cypionka, 2009).

The current hypothesis as to why the *Chloroflexi* are so abundant in some but not all deep subsurface and tidal sediments, is that these organisms utilise compounds in organic rich sediments and work syntrophically with hydrogenotrophic organisms (such as methanogens) to maintain their main substrate, hydrogen (Inagaki et al., 2006; Webster et al., 2006; Wilms et al., 2006a). Specifically, much significance has been placed on the frequent detection of *Dehalococcoidia* and dehalorespiration. Futagami et al. (2009) analysed the distribution of reductive dehalogenase (*rdh*) genes in deep subsurface sediments and concluded that dehalorespiration was an important energy yielding process in this ecosystem. Dehalogenating communities related to *Chloroflexi* have also previously been detected in

anaerobic estuarine sediments (Ahn et al., 2008). It is considered that the ability to utilise chlorinated compounds in sediments gives the *Dehalococcoidia* a competitive advantage in nutrient poor habitats. However, as previously discussed, this is a limited view, based on poor phylogenetic relationships between 16S rRNA gene sequences and *D. mccartyi* 195 (Wilms et al., 2006a). Recently, Wasmund et al. (2014b) and Kaster et al. (2014) used single cell genome analysis to predict the function of *Dehalococcoidia* in shallow surface sediments (10 cbsf) from Aarhus Bay, Denmark and deep subsurface sediments form the Peru Margin. Though the groups found evidence of great metabolic diversity, such as beta-oxidation and oxidation of aromatic compound genes, no evidence of reductive dehalogenase genes was detected. Thus, current understanding of these communities is very limited, and much more research, focusing on the diversity and role of the *Chloroflexi* subdivisions is needed.

1.6 Project Aims

The overall aim of this thesis was to investigate the prokaryotic communities present in different Severn Estuary sediment environments, with particular focus on the *Chloroflexi* phylum of *Bacteria.* Further to this, the relationship between community structure, geochemistry and depth of the sediment was analysed to improve knowledge of estuarine prokaryotic communities. Finally, since the *Chloroflexi* are a poorly studied phylum, this thesis aimed to develop methods to analyse the *Chloroflexi* subdivisions and gain insight into their distribution, diversity and function. The specific objectives were:

To develop an effective DNA extraction method for use with these estuarine sediments to produce high DNA yield and limit contamination of the sample with organic materials (Chapter 2).

To investigate the patterns of the *Bacteria* and *Chloroflexi* communities using molecular profiling techniques to compare community structure at different sediment sampling sites and with depth. This generated a base knowledge of the potentially unique prokaryotic communities present in the Severn Estuary (Chapter 3).

To develop novel profiling and qPCR assays for the *Chloroflexi* and its subdivisions to create a qualitative and quantitative representation of *Chloroflexi* communities in Severn Estuary surface sediment (Chapter 3).

To utilise 454 pyrosequencing to create an in-depth picture of the prokaryotic communities in Severn Estuary sediments for comparison with profiling results. This highlighted the so-called “rare” community members that profiling methods are not rigorous enough to detect. More specifically, deep sequencing of targeted *Chloroflexi* communities was tested and performed (Chapter 4)

To analyse the effect of temperature change on the *Bacteria* and *Chloroflexi* communities in Severn Estuary intertidal sediments using thermal gradient experiments on sediment microcosms (Chapter 5).

Chapter 2 - A Comparison of DNA Extraction Methods for use with Severn Estuary Sediments

2.1 Introduction

The ‘great plate count anomaly’ describes the vast underestimation of the diversity of an environmental microbial community caused by the limitations inherent in cultivation dependent analysis (Staley and Konopka, 1985). It was found that there was an incongruence between the number of microbes in environmental samples counted with microscopy and the number of colonies formed with culturing methods (Jannasch, 1959; Kogure et al., 1979). It was estimated that <1% of all microorganisms in an environmental sample could be grown in the laboratory (Torsvik et al., 1990). In some environments the discrepancy is extreme, it has been estimated that only 0.001–0.1% of microbes could be cultured from marine environments, such as seawater (Amann et al., 1995; Kogure et al., 1979). Thus, it was apparent that the use of cultivation independent techniques to analyse the elusive organisms from these challenging environments was vital. With the advent of 16S ribosomal RNA (rRNA) gene PCR the difficulties of cultivation of microbes was avoided (Lane et al., 1985). 16S rRNA and functional gene PCRs have been widely used for the analysis of marine and sub-seafloor sediments with much success (Biddle et al., 2008; Colin et al., 2013; D'Hondt et al., 2004). However, with the avoidance of cultivation limits come with limitations in 16S rRNA gene analysis itself.

The nucleic acid extraction method is the first and arguably the most important step in cultivation independent analysis. The method chosen inherently affects the quality of the extracted DNA. The extraction method must be able to extract DNA with high yields, good purity and with few natural contaminants to limit interference with downstream molecular applications (Schneegurt et al., 2003). Also, the extraction method must be suitable for the environmental sample to be processed (Lakay et al., 2007; Zhou et al., 1996). Sediments have been notoriously difficult environments from which to extract nucleic acids. The depth and biogeochemistry of a

sediment environment will affect the amounts of nucleic acids extracted. Deep subsurface sediments often have extremely low microbial biomass, whereas near surface and tidal environments, such as estuaries, have a comparatively greater amount of biomass (Kallmeyer and Smith, 2009). Furthermore, sediments are dynamic, containing particles that free DNA may adsorb to, and many natural inhibitors that may co-extract causing complications in molecular analysis. Sediments, like soils, are often saturated with humic acid substances, which pose great challenges in downstream molecular applications. Humic acids are a mixture of compounds created from the decay of organic material such as plants and animals (Zipper et al., 2003). These compounds often contain chemical groups similar to those found in nucleic acids which will co-purify with DNA during the extraction (Dong et al., 2006; Ogram et al., 1987). Humic acids interfere with a number of downstream processes, such as DNA quantification, by UV spectrophotometry and fluorescence, and PCR. Though the exact method of inhibition has been unclear, it is most likely by the competitive binding of phenolic groups in the humic acids to amide groups on the nucleic acid bases thus preventing the binding of the polymerase, primers or fluorescence molecules (Bachoon et al., 2001; Tsai and Olson, 1992; Zipper et al., 2004). Humic acids are also known to sequester magnesium ions that are essential for the performance of *Taq* polymerase (Tsai and Olson, 1992). Finally, humic acids have high absorption coefficients in the UV spectrum, due to ring structures similar to that of DNA bases, leading to overestimation of DNA concentration when using UV spectrophotometry (Bachoon et al., 2001).

There is no universal standard DNA extraction protocol for environmental samples (Schneegurt et al., 2003). There are many commercial DNA extraction protocols and protocols published for specific sample types. Figure 2.1 shows the four general steps in a nucleic acid extraction protocol and gives examples of the variety of different methods possible at each step. With the multitude of different combinations of steps there also come a number of positive and negative influences that the extraction technique may have on the DNA. Feinstein et al. (2009) found that the yield and purity of

1. Cell lysis

- Direct or indirect extraction
- Mechanical lysis e.g. Bead beating, sonication, freeze-thaw
- Chemical lysis
  - Enzymatic e.g. Proteinase K, lysozyme
  - Alkaline e.g. NaOH and sodium dodecyl sulphate (SDS)
  - Chaotropic salts e.g. guanidine HCl, guanidine thiocyanate, urea, and lithium perchlorate
  - Heat

2. Nucleic acid extraction

- Organic extraction e.g. phenol:chloroform
- Magnetic using silica magenite nanoparticles (Sebastianelli et al., 2008)

3. Nucleic acid precipitation

- Alcohol precipitation with salts e.g. ice cold ethanol, isopropanol
- Polyamines e.g spermidine and spermine

4. Purification

- Columns e.g. Q-sepharose (GE Healthcare), silica spin columns
- Organic often before precipitation to remove proteins e.g. phenol:chloroform:isoamylalcohol
- Gel electrophoresis
- Polyvinylpyrrolidone (PVPP)
- Electroelution
- RNase or DNase

Figure 2.1 - The main steps involved in nucleic acid extraction protocols with examples of the various methods used for each step.

extracted DNA may vary by both the sample type and by the extraction method used. Furthermore, Carrigg et al. (2007) found that the type of method used directly affected the bacterial community composition of the extracted DNA. Even the type of bacteria lysed by different techniques can be biased by the lysis method. Prokaryotic cells can be lysed while still in the environmental sample and the released DNA extracted directly, called a 'direct' method. 'Indirect' methods first remove the cells from the environmental sample and then lyse the cells. Direct methods have proven to give the greater yield by other investigations and is the most popular technique in soil and sediment research (Schneegurt et al., 2003; Wintzingerode et al., 1997). Mild lysis conditions (such as chemical lysis) will favour Gram negative cells and will not lyse the more robust Gram positive cells. On the other hand, harsher lysis steps (such as bead beating) will lyse both Gram positive and Gram negative cells, but may shear the latter's DNA making it unusable in downstream processing (Schneegurt et al., 2003). Research by Howeler et al. (2003) indicated that bead beating was a particularly effective as 95% of the cells in their samples were lysed, and so this method has prevailed in sediment based experiments (Corinaldesi et al., 2005; Schneegurt et al., 2003) .

The principle aim of this work was to find and optimise a DNA extraction method for Severn Estuary sediment. This method would give a high yield of DNA, good purity and community coverage and would also be time efficient. Extracted DNA must be pure enough to use in a variety of cultivation-independent molecular techniques to analyse the structure and function of prokaryotic communities in Severn Estuary sediments. The method chosen must introduce as little bias as possible to the microbial community composition of the sample. Also, with the potential of a large number of sediment samples to extract from, the process would preferably enable high-throughput of samples. Five extraction techniques were chosen following a search of current literature, and have been summarised in Table 2.1. As bead beating has been favoured for difficult sediments three protocols based on bead beating were chosen. Two chemical lysis based protocols were included for comparison. All methods included were 'direct' as these

Table 2.1 - A summary of the chosen DNA extraction methods to be compared in this work

Extraction Method	**Nucleic Acids Extracted**	**Cell Lysis Method**	**Purification Method**	**Samples Used in Publication**	**Reference**
FastDNA	DNA	Bead Beating	Silica Spin Filter	Marine Sediments	Webster et al., 2003
PowerSoil	DNA	Bead Beating	Inhibitor Removal Solution Silica Spin Filter	Soil and Intertidal Sediments	Inceoglu et al., 2010 Sawicka et al., 2010
Combination of FastDNA and PowerSoil	DNA	Bead Beating	Inhibitor Removal Solution Silica Spin Filter	-	This work
Peršoh	DNA & RNA	Bead Beating	$Al_2(SO_4)_3$ Chloroform:Isoamyl alcohol (24:1)	Soil	Peršoh et al., 2008
Luna	DNA	Chemical (with Proteinase K)	Phenol:Chloroform Silica Spin Filter	Marine Sediments	Luna et al., 2006
Sharma	DNA	Chemical (with Proteinase K)	Phenol:Chloroform Q-Sepharose	Soil	Sharma et al., 2007

methods give higher DNA yields than ‘indirect’, as previously discussed. One of the protocols, an amended version of the FastDNA spin protocol for soil (MP Biomedicals), is currently widely used our laboratory and elsewhere, and was chosen for comparison with the other methods. This protocol was previously optimised by Webster et al. (2003), with great success, for use with a range of deep sub-seafloor sediments with extremely low biomass. Sediment samples from different depths from two sediment cores, taken from separate sites within the Severn Estuary, were used in the extractions. As future work entails investigation of a range of tidal and marine sediments as well as a range of depths, it was considered important that the chosen extraction protocol should consistently provide good quality DNA extracts from a range of sediment types.

2.2 Materials and Methods

2.2.1 Sediment Sampling

The first sediment samples used for this investigation were taken on 23rd October 2009 from the Severn Estuary intertidal flats at Woodhill Bay, Portishead, Bristol, UK (51°29'30.94''N, 2°46'28.91''W). The core was cut into 8 samples of 5 cm depth sections in a laminar flow cabinet with a sterilised stainless steel spatula, packaged separately and stored at -20°C. The second core was taken from the shallow water in Cardiff and Penarth Roads in the Severn Estuary (51°43'58.33''N, 3°15'98.33''W) on 18th February 2011. The core was cut into 2 cm slices using a core extractor and stored in sterile 50 ml Falcon tubes at -20°C. The 0-2 cm Penarth and 0-5 cm Portishead samples were extracted from 3 separate times to produce 3 biological replicates for further analysis.

2.2.2 DNA Extraction Methods

2.2.2.1 Fast DNA Spin Protocol for Soil (MP Biomedicals)

Sediment samples weighing 0.5 g were taken under aseptic conditions in a laminar flow cabinet. An amended version of the FastDNA Spin for Soil Protocol (MP Biomedicals) protocol was used (Webster et al., 2003). Briefly, cells in sediment were mechanically broken by grinding with silica beads in a lysing tube. Sodium phosphate buffer and MT buffer were added and shaken twice for 30 s at speed 5.5 in the FastPrep Instrument (MP Biomedicals) followed by centrifugation at 16,000 x *g* for 8 min at room temperature (RT). Protein Precipitation Solution was added to supernatant, mixed by hand and centrifuged for 5 min at 16,000 x *g* at RT. Binding Matrix Suspension was added to supernatant, inverted and allowed to settle. The suspended mixture was centrifuged in a Spin Filter at 14,000 x *g* for 1 min at RT. SEWS-M (Salt Ethanol Wash) was added and again centrifuged at 14,000 x *g* for 1 minute at RT and the matrix was allowed to dry. 100 µl of DES (DNA/Pyrogen Free Water) was added to matrix and centrifuged at 14,000 x *g* for 1 min at RT; DNA was eluted into the catch tube. All extracted DNA samples from this and the other extraction protocols were stored at -20°C.

2.2.2.2 PowerSoil DNA Isolation Protocol (MoBio Laboratories Inc)

DNA was extracted from the sediment as described by the manufacturer's protocol, except the mechanical cell breaking (step 5) was replaced with two 30 s runs at speed 5.5 in the FastPrep Instrument (MP Biomedicals). Briefly, sediment samples weighing 0.25 g were weighed in the provided lysing tubes, which contained silica beads, under aseptic conditions in a laminar flow cabinet. Solution C1 was added to each tube and vortexed to mix. The samples were shaken in the FastPrep Instrument (MP Biomedicals). Following this, sediment was separated by centrifugation at 10,000 x *g* for 30 s at RT and the subsequent supernatant transferred to a clean 2 ml collection tube. After the addition of C1, mixing and centrifugation was repeated twice more and the supernatants pooled. C2 was added, mixed and incubated at 4^{o}C for 5 min. The sample was centrifuged at 10,000 x *g* for 1 min at RT and the supernatant transferred to a clean 2 ml collection tube. Solution C3 was added, mixed, incubated at 4^{o}C for 5 min and centrifuged at 10,000 x *g* for 1 min at RT again. The supernatant was removed to a clean 2 ml collection tube to which C4 was added and mixed by vortexing. 675 µl of the supernatant was loaded onto the spin filter and centrifuged at 10,000 x *g* for 1 min at RT. This loading and centrifugation step was repeated twice more. Solution C5 was added to the spin filter, centrifuged at 10,000 x *g* for 30 s at RT and the flow through discarded. The filter was dried by further centrifugation at 10,000 x *g* for 30 s at RT. The spin filter was put in a clean 2 ml collection tube, carefully avoiding C5, and 100 µl of sterile Diethylpyrocarbonate (DEPC) treated water (Fisher Scientific) was added. DNA was eluted by centrifugation at 10,000 x *g* for 30 s at RT.

2.2.2.3 Luna Protocol

The Luna et al. (2006) protocol was based on the Zhou et al. (1996) method for recovery of DNA from soils. First 2.5 g of sediment was weighed under aseptic conditions and 6.75 ml of extraction buffer (100 mM Tris-Hydrochloric acid (HCl) [pH 8.0], 100 mM sodium Ethylenediaminetetraacetic acid (EDTA) [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% w/v cetyltrimethylammonium bromide (CTAB) and 50 µl of Proteinase K (10

mg/ml)) was added. Samples were horizontally shaken at 225 rpm at 37°C for 30 min. 750 µl of 20% w/v SDS was added and samples were incubated in a 65°C water bath for 2 h with gentle inversion every 20 min. Samples were centrifuged at 4000 x *g* for 10 min at RT and the supernatant removed and kept. The sediment pellet was extracted twice more by addition of 2.25 ml of extraction buffer and 250 µl of 20% w/v SDS and mixed by vortexing. This was followed by a 10 min incubation at 65°C and centrifugation at 4000x *g* for 10 min at RT. The supernatants from all 3 extractions were combined and purified using phenol-chloroform (1:1). Isopropanol was used to precipitate DNA from the top aqueous phase of the phenol-chloroform mixture and DNA was pelleted by centrifugation at 20,000 x *g* for 15 min at 4°C. The pellet was washed with ice cold 70% w/v ethanol and resuspended in sterile DEPC treated water (Fisher Scientific). To purify the DNA, the spin columns used in the PowerSoil protocol were used on the resuspended DNA.

2.2.2.4 Sharma Protocol

The Sharma et al. (2007) was also based on the Zhou et al. (1996). However, this method was reduced to a smaller scale and contained a single step purification using Q-Sepharose (GE Healthcare). 0.5 g of wet sediment was weighed out aseptically and 1.3 ml of extraction buffer (100 mM Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% w/v CTAB) and 13 µl of Proteinase K (10 mg/ml) were added. The samples were incubated at 37°C for 45 min with horizontal shaking. 160 µl of 20% w/v SDS was added and vortexed. A second incubation at 60°C for 2 h with mixing every 15 min followed. The samples were centrifuged at 5000 x *g* for 10 min at RT and the supernatant transferred to a new microfuge tube. The sediment pellet was extracted three times more by addition of 400 µl of extraction buffer and 60 µl of 20% w/v SDS and incubation for 15 min at 60°C. Samples were mixed every 5 min during the incubation. These were centrifuged as before and the four supernatants were pooled. The pooled supernatant was purified using chloroform-isoamyl alcohol (24:1) in equal volumes to the supernatant. The

aqueous phase was retained and DNA was precipitated from it using 0.6 volumes of isopropanol. Precipitated DNA was pelleted by centrifugation at 20,000 x *g* for 15 min at 4^{o}C and washed with ice cold 70% w/v ethanol. After removal and drying of the ethanol, the pellet was resuspended in 1 ml Tris-EDTA buffer (10 mM Tris, 1 mM EDTA; TE) [pH 8.0].

The purification step began with the washing and equilibration of Q-Sepharose with 10 mM potassium phosphate buffer [pH 7.2]. 150 µl aliquots of Q-Sepharose with 10 mM potassium phosphate buffer [pH 7.2] were placed in microfuge tubes and centrifuged for 1 minute at 650 x *g* at RT to pack the Q-Sepharose. DNA in 500 µl TE buffer [pH 8.0] was added to each microfuge tube containing Q-Sepharose, inverted slowly for 15 min, and incubated at room temperature for 5 min. Genomic DNA was retained in the buffer (Sharma et al., 2007). The DNA was separated from the Q-Sepharose mixture by centrifugation at 1000 x *g* for 1 min at RT. The supernatant containing the DNA was retained.

2.2.2.5 Peršoh Protocol

This protocol was unique in that it utilised aluminium sulphate to chelate humic acid contaminants and could simultaneously extract both DNA and RNA from the same sediment sample (Peršoh et al., 2008). A shortened version of the protocol was used to predict the amount of aluminium sulphate needed to chelate the humic acids in the sediments. 100 µl of 1 M Tris- HCl [pH 5.5] and 800 mg of 0.5 mm diameter sterile glass beads were added to five 0.5 g replicates of the 0-5 cm section of the Portishead core. Different amounts of aluminium sulphate were added to each sample (50 µl, 100 µl, 150 µl, 200 µl and 250 µl) and the final volume of liquid brought up to 1 ml with sterile water (Fisher Scientific). The cells were lysed in the FastPrep Instrument (MP Biomedicals) for 1 min at 5.5 m/s. The pH of the solution was adjusted to pH 8.0 using 4 M NaOH and mixed for second time in the FastPrep Instrument (MP Biomedicals) for 15 s at 5.5 m/s. Finally, the samples were centrifuged for 1 min at 11,000 x *g* at RT. The sample with the volume of aluminium sulphate that produced a clear supernatant indicated the volume of aluminium sulphate to be used. In this case it was 50 µl.

The amounts of each chemical added were calculated using the equations in Peršoh et al. (2008) The main protocol used 0.5 g sediment samples, weighed using aseptic techniques. 100 µl of 1 M Tris-HCl buffer [pH 5.5], 850 µl of sterile distilled water and 50 µl of aluminium sulphate (Fisher Scientific), which was determined previously, was added to the sediment. This was shaken in the FastPrep Instrument (MP Biomedicals) for 15 s at 4.0 m/s. 16.7 µl of 4 M sodium hydroxide and 383 µl of 100 mM Tris- HCl [pH 8.0] was added to the pellet. The mixture was shaken again in the FastPrep Instrument (MP Biomedicals) for 15 s at 4.0 m/s. The sample was adjusted to over pH 8 using stepwise additions of 10 µl of 4 M NaOH. Samples were centrifuged for 2 min at 3500 x *g* at RT. The supernatant was measured, noted and discarded. 100 mM Tris-HCl [pH 8.0], 325 µl of extraction buffer [pH 8.0] (4 M LiCl, 100 mM Tris-HCl and 120 mM EDTA), 325 µl of 10% SDS [pH 8.0] and beads from the FastDNA lysing tubes were also added to the pellet. Samples were shaken in the FastPrep Instrument (MP Biomedicals) for 30 s at 4.0 m/s, and incubated on ice for 5 min to prevent overheating. Two further rounds of shaking at 5.5 m/s for 30 s, with 5 min of incubation on ice in between were performed. Samples were centrifuged for 1 min at 11,000 x *g* at RT, and 750 µl of the supernatant was treated with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1). This was incubated on ice for 5 min with vigorous shaking every minute. Samples were centrifuged for 15 min at 16,000 x *g* at RT and the supernatant removed to a clean 2 ml microfuge tube. Two purification steps of equal volumes of chloroform:isoamyl alcohol (24:1) to supernatant and centrifugation for 15 min at 16,000 x *g* at RT followed. The supernatant was mixed with 0.1 volumes of 5 M NaCl and 0.7 volumes of isopropanol. The precipitating mixture was left overnight at RT. This incubation was followed by 1 h at 18,000 x *g* at RT. Isopropanol was removed completely; the pellet resuspended in 50 µl of DEPC treated sterile water (Fisher Scientific), and the DNA solution was divided into two aliquots, one treated with DNAse (Promega) and the other with RNAse A (Promega). DNA was precipitated with isopropanol (as above) after the enzyme treatment.

2.2.2.6 Combination of FastDNA and PowerSoil Protocols

To combine the high yields of DNA extracted with the FastDNA protocol and the high purification of the PowerSoil protocol, the two protocols were merged for use on the same set of sediments. The FastDNA protocol was performed on the Penarth sediments in its entirety. The final eluted DNA was subsequently passed through the purification steps of the PowerSoil protocol which used silica spin filters.

2.2.3 DNA Analysis

DNA extracted by the previous techniques was quantified and the purity of the DNA was assessed. All DNA quantification values were adjusted to the unit µg/g of sediment, for further comparison.

2.2.3.1 Agarose Gel Electrophoresis

Presence of DNA after successful extraction was detected by 1.2% w/v agarose gel electrophoresis and stained, in gel, using SYBR Safe DNA (Invitrogen) and included the molecular weight marker Hyperladder I (Bioline). The samples were loaded with 0.5% bromophenol blue loading dye and run at 90 V for 35 mins in 1x Tris-acetate EDTA (TAE) buffer [pH 8.0]. DNA was visualised using the Gene Genius Bio Imaging System (Syngene). For DNA quantification using gel electrophoresis, a 1.2% agarose gel with no stain was used. 0.05% bromophenol blue loading dye was used to minimise the interference of the coloured dye during quantification. The gel was post-stained with 0.5 µg/ml of ethidium bromide in 1x TAE buffer [pH 8.0] for 45 min with gentle mixing. The DNA was quantified using GeneTools software (SynGene) by comparison with DNA quantification marker Hyperladder I (Bioline).

2.2.3.2 DNA Quantification and Purity Using NanoDrop

NanoDrop based DNA estimation used the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies), according to manufacturer's instructions. The NanoDrop was blanked with TE buffer [pH 8.0] or sterile

water at the start of the quantification and at subsequent intervals during use. The absorbance ratio $A_{260/280}$ indicated the level of protein and the $A_{260/230}$ ratio indicated the amount of organic substances in the sample. These values were used to estimate DNA purity in the extracted sample. Absorbance ratios of 1.5 or above were considered pure as suggested by Peršoh et al.(2008) and Bachoon et al. (2001).

2.2.3.3 DNA Quantification Using Quanti-iT Assay Protocol

The Quanti-iT™ Broad Range Assay protocol (Invitrogen) utilised fluorescent dyes to quantify DNA. The assay was conducted according to manufacturer's protocol. The Qubit® Fluorometer (Invitrogen) was used and calibrated at the beginning of each run use using the DNA standard solutions (0 – 100 ng/µl).

2.2.3.4 16S rRNA gene PCR

All PCRs were performed under aseptic conditions using autoclaved plasticware, UV irradiated pipettes and nuclease free, molecular grade water (Severn Biotech). Molecular grade water was used as a negative control in all PCR amplifications. The concentrations of the PCR reactions were as follows: 1 µl extracted DNA samples (0.1-75.5 ng/µl), 1x reaction buffer (Promega), 0.25 mM dNTPs (Promega), 0.25 mM $MgCl_2$, 0.2 mg/ml bovine serum albumin (BSA; Promega), 0.2 pmol/µl of forward and reverse primers (MWG Biotech), 2.5 U *Taq* DNA polymerase (Promega) and molecular grade water (Severn Biotech Ltd.) up to total volume of 50 µl. The protocol used was 95^{o}C for 2 min followed by 36 cycles of 94^{o}C for 30 s, 52^{o}C for 30 s, 72^{o}C for 90 s plus 1 second per cycle and a final extension step of 72^{o}C for 5 min (Webster et al., 2006). All amplifications were carried out in a DNA Engine Dyad Thermal Cycler (MJ Research). The PCR product was viewed using agarose gel electrophoresis, as described in **Section 2.2.3.1**.

A 16S rRNA gene PCR assay was used to estimate the purity of the extracted DNA. The more contaminated the DNA sample with organic substances and proteins that can act as PCR inhibitors, then the more dilution required to amplify the DNA. Extracted DNA was used undiluted and at 1:10 and 1:50 dilutions. The primers used were 27F (Lane, 1991) and

907R (Muyzer and Smalla, 1998) (see Table 2.2 for list of primers and sequences). A second 16S rRNA gene PCR assay was performed using the primer set 27F and 1492R (Lane, 1991; Table 2.2). The reaction mix and protocol were as above (DeLong, 1992; Webster et al., 2003). These primers amplify a larger portion of the 16S rRNA gene and so this assay tests the quality of the extracted DNA: shearing or damage to DNA would affect amplification yield.

2.2.3.5 PCR-DGGE

In preparation for DGGE, the bacterial primers 357FGC and 518R were used (Muyzer et al., 1993; Table 2.2). The PCR reaction concentrations are stated in **Section 2.2.3.4**. The thermocycler protocol differed for this reaction: 95^{o}C for 5 min, 10 cycles of 94^{o}C for 30 s, 55^{o}C for 30 s and 72^{o}C for 1 min. This was followed by 25 cycles of 92^{o}C for 30 s, 52^{o}C for 30 s and 72^{o}C for 1 min and a final extension step of 72^{o}C for 10 min (Muyzer et al., 1993). For analysis of the *Chloroflexi* community, the bacterial primer 27F and *Chloroflexi* specific primer 941R (Gich et al., 2002) were used in the first round of a nested PCR followed by the 357FGC and 518R primers (Table 2.2). The protocol follows that given in **Section 2.2.3.4**. DNA was visualised as described in **Section 2.2.3.1**.

The DGGE protocol was previously described (Muyzer et al., 1993; Webster et al., 2006). Briefly, a double denaturant gradient of 30% to 60% w/v of urea and formamide and an acrylamide gradient of 6% to 12% w/v of 40% w/v acrylamide was poured using 50 ml volume Gradient Mixer (Fisher Scientific) to create a 1 mm thick gel. Samples were run on the gel in a tank of 1x TAE buffer at 60^{o}C, at 80 V for 10 min then for 290 min at 200 V (5 hours in total). Every DGGE gel was run with a standard marker made by amplifying the 16S rRNA gene of six bacterial type strains with the Muyzer et al. (1993) primers. DGGE gels were stained with SYBR Gold DNA stain (Invitrogen) for 30 mins and viewed using the Gene Genius Bio Imaging System (Syngene). Bands of interest were removed using a sterilised scalpel under UV light and stored individually at -20^{o}C.

2.2.3.6 DNA Sequencing

Bands containing DNA fragments separated by DGGE were extracted and prepared for sequencing by the Molecular Biology Support Unit of Cardiff University. DNA was eluted from the bands as described by O'Sullivan et al. (2008). The eluted DNA was amplified with sequencing primers as described in **Section 2.2.3.4**. The 518R primer used contains a M13F sequencing primer at the 5' end joined by an AT linker region to allow sequencing immediately after DGGE isolation (Table 2.2). The DNA product was viewed and quantified as described in **Section 2.2.3.1**.

2.2.4 Bioinformatics and Statistical Analysis

2.2.4.1 Statistical Analysis of DNA Quantification

All DNA quantifications of the same sample (Portishead 0-5 cm), extracted by each method, were compared for significant difference using a One-way ANOVA and Tukey's Method in the R statistical package (Ihaka and Gentleman, 1996).

2.2.4.2 Statistical Analysis of DGGE

The ability to extract DNA from the bacterial community without biases was analysed using DGGE (Section 2.2.3.5). The 0-5 cm of the Portishead core and 0-2 cm of the Penarth core were extracted with each method and run on the same DGGE gel for comparison. The banding pattern and therefore number of phylotypes detected in each sample was analysed using GelCompar II software (version 6.5; Applied Maths). Cluster analysis was performed on the profiles using Pearson correlation coefficient and UPGMA.

Table 2.2 – 16S rRNA gene primers used in this work with sequence and reference

Primer	Target Taxon	Sequence (5'-3')	Reference
27F	*Bacteria*	AGA GTT TGA TCM TGG CTC AG[a]	Lane, 1991
1492R	*Bacteria*	GGT TAC CTT GTT ACG ACT T	Lane, 1991
907R	*Bacteria*	CCG TCA ATT CMT TTG AGT TT	Muyzer and Smalla, 1998
941R	*Chloroflexi*	AAA CCA CAC GCT CCG CT	Gich et al., 2002
357FGC[b]	*Bacteria*	CCT ACG GGA GGC AGC AG	Muyzer et al., 1993
518R	*Prokaryotes*	ATT ACC GCG GCT GCT GG	Muyzer et al., 1993
518R-AT-M13F[c]	*Bacteria*	GTA AAA CGA CGG CCA GTA AAT AAA ATA AAA ATG TAA AAA AA	O'Sullivan et al., 2008

[a] Key: M = A or C
[b] Primer has GC clamp attached at 5' end – CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG (Muyzer et al., 1993).
[c] Primer has AT linker region followed by the M13F primer for sequencing the whole of the DGGE product (O'Sullivan et al. 2008).

2.3 Results

2.3.1 DNA Quantification

2.3.1.1 DNA Yields for Extraction Protocols

DNA concentrations were estimated using the Quanti-iT™ Broad Range Assay protocol (Invitrogen), as summarised in Figure 2.2 and Table 2.3. Figure 2.2 allows comparison of the DNA yields of each protocol across the two sites (Portishead and Penarth) and with sediment depth. There was a trend of higher amounts of DNA at the shallowest depths across the three sites. The Penarth core contained the highest DNA concentration.

As expected, the four bead beating protocols gave the overall highest DNA yields and the two chemical lysis protocols gave the lowest DNA yields. The FastDNA spin protocol for soil was the most consistently and significantly (using ANOVA and Tukey's method; P value = 0.00) high yielding method even at deeper sediment depths which would endorse its use for a range of sediment types (Figure 2.2; Table 2.3). The Peršoh protocol gave the second highest yields (Figure 2.2). It gave a higher average yield of DNA than the FastDNA protocol at the lower Penarth sample (26-28 cm;10.6 µg/g of sediment compared to 9.7 µg/g of sediment). However, the yields from the Peršoh protocol were variable (Figure 2.2), as reflected by the larger error bars for these samples. The PowerSoil protocol again gave variable yields, which appeared dependent on sample depth. The extensive purification steps in this protocol appeared to limit the extraction efficiency. The chemical based Sharma and Luna protocols were the lowest yielding methods (Figure 2; Table 2.3). The FastDNA protocol and Luna protocol had the most significant difference using Tukey's method (P value = 0.00)

2.3.1.2 Comparison of DNA quantification methods

The Quanti-iT™ Broad Range Assay protocol (Invitrogen), NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies) were compared (Figure 2.3) to determine the most appropriate method for DNA quantification with

Table 2.3 - Summary of the results of DNA analysis for extraction methods used in this study

Extraction Method	Average Preparation Time	Average DNA Yield Penarth 0-2 cm (µg/g of sediment)[a]	Average DNA Yield Penarth 26-28 cm (µg/g of sediment)[a]	Average $A_{260/280}$[b]	Average $A_{260/230}$[b]	Minimum Dilution Needed for PCR Amplification	Amplifiable by 27F-1492R PCR
FastDNA	2 h	19.7	9.7	1.58	0.11	1/10	Yes
PowerSoil	2 h	10.1	5.5	1.59	1.10	No dilution	Yes
Peršoh	3 days	15.7	10.6	1.53	1.08	1/10	Yes
Sharma	8 h	9.3	4.7	1.29	1.23	No dilution	Yes
Luna	8 h	1.3	0.1	1.42	0.79	1/10	Yes
Combination of FastDNA & PowerSoil	3 h	8.7	5.8	1.82	1.83	No dilution	Yes

[a] This is an average of 3 biological replicates of the 0-2 cm and 26-26 cm Penarth samples, respectively, per extraction method.
[b] This is an average of the 6 Penarth samples, at all depths, per extraction method.

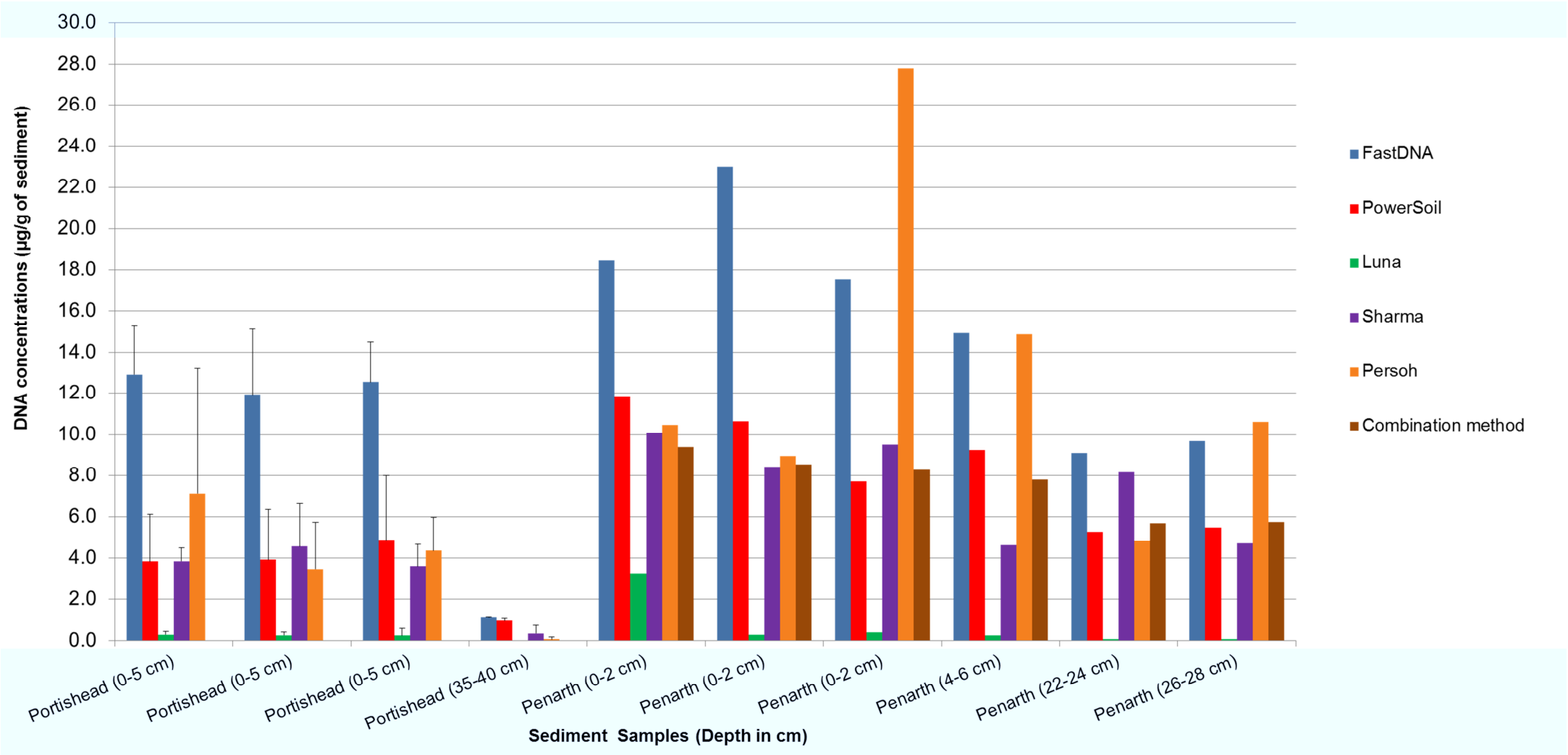


Figure 2.2 – Comparison of DNA concentrations for each method by sediment sampling site. Depths of sample are noted in brackets. DNA concentrations were averages and quantified using the Quanti-iT™ Broad Range Assay kit (Invitrogen). Triplicates of Portishead (0-5 cm) and Penarth (0-2 cm) are given to indicate the reproducibility of the method. Error bars were calculated using standard deviation of triplicate extractions of the 0-5 cm Portishead sample. Error bars for Penarth data are not shown as standard deviation could not be calculated with a single data set.

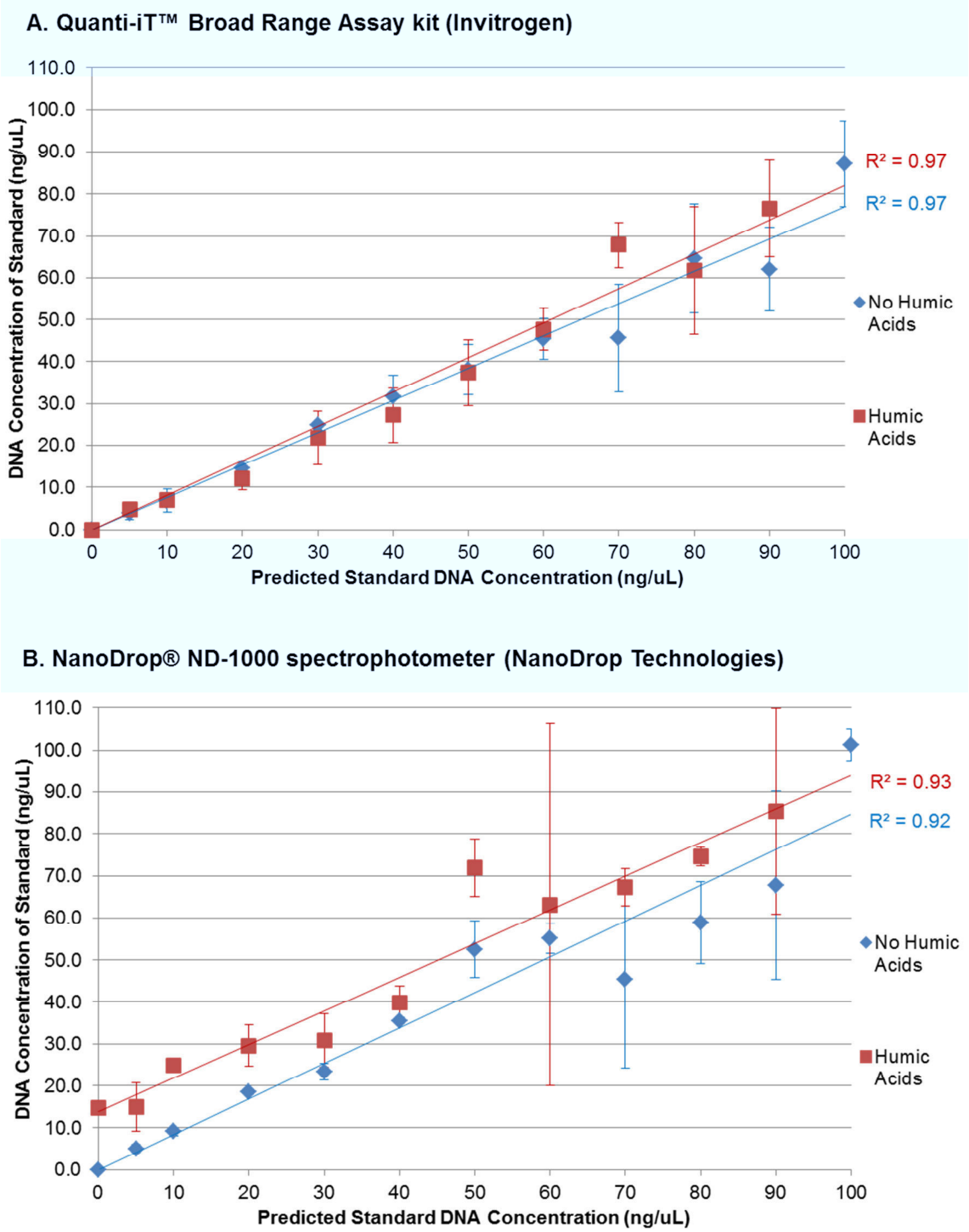


Figure 2.3 – Dilutions of 100 ng/µl DNA stock diluted with either sterile water or sediment extract containing humic acid substances. Concentrations were measured using: A, Quanti-iT™ Broad Range Assay (Invitrogen) and; B, NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies). Measurements were performed with 3 technical replicated to produce error bars from standard deviation.

sediments. Two standard curves from 0 – 100 ng/µl of DNA were made by diluting a stock solution of 100 ng/µl DNA with either sterile water or filtered (0.2 µm) sediment extract containing humic acids. Overall the Quant-it method gave the higher R^2 values of 0.97 for both standard curves (Figure 2.3). This indicated that the humic acids did not significantly quench the fluorescence. The NanoDrop method had lower R^2 values than the Quant-it, and the standard curve with humic acids gave a slightly lower R^2 value than without (R^2= 0.93 and 0.92, respectively). This suggested that the presence of humic acids affected the NanoDrop's function and so would not be ideal for sediment extracts. Also, the larger error bars of the NanoDrop standard curve indicated variability even when humic acids were not present.

2.3.2 DNA Purity

2.3.2.1 16S rRNA Gene PCR

The only methods that gave PCR amplifiable DNA without dilution were the PowerSoil, Sharma and the combination protocols (Table 2.3); DNA extracted by other methods was not PCR amplifiable without a dilution of at least 1:10. For samples containing higher concentrations of the more humics, a dilution of 1:50 could not be amplified (results not shown). All of the methods gave PCR amplifiable DNA with 27F-907R.The 27F-1492R primer set was used to test the integrity of the DNA after each method by amplification of a much larger region of the gene. All of the methods gave DNA amplifiable by 27F-1492R meaning that the DNA integrity was maintained (Table 2.3).

2.3.2.2 Absorbance Ratios $A_{260/280}$ and $A_{260/230}$ Ratios

Only the combination of the FastDNA and PowerSoil protocols gave an average value over the threshold of 1.7 for $A_{260/280}$ and 1.5 for $A_{260/230}$ (Table 2.3). All of the methods could adequately remove proteins from the extract but not to the standards stated previously (Peršoh et al., 2008). The $A_{260/230}$ values indicating presence of humic acids etc. were all below the recommended value of 1.5 (Bachoon et al., 2001; Table 2.3). Aside from the combination protocol, the highest value was from the Sharma protocol (1.23).

The lowest was from the FastDNA protocol, 0.11, indicating a poor ability to remove humic acids.

2.3.3 Analysis of Bacterial Communities by DGGE and Cluster Analysis

Potential biases in PCR of the 16S rRNA gene sequences from DNA extracted using the various protocols in this study, were investigated and compared by DGGE bacterial and *Chloroflexi* community profiling. The similarity in DGGE band patterns indicated very similar bacterial community profiles (Figure 2.4). Pearson cluster analysis was performed on the band pattern of each sample to statistically compare extraction methods, creating a UPGMA tree (Figure 2.5). There was 83% and 85% overall similarity between the methods for the Portishead and Penarth samples, respectively. This suggested a great deal of shared community diversity was extracted by all methods. The Sharma and Peršoh protocol profiles for Portishead 0-5 cm branched together with 95% similarity, away from the other methods. This was most likely due to two dominant bands.

As the *Chloroflexi* phylum of *Bacteria* will be a focus of following investigations, the extracted *Chloroflexi* community was also investigated using DGGE (Figure 2.6). Cluster analysis of a *Chloroflexi* DGGE indicated a similarity between all extractions of 86% and 88% for the Portishead and Penarth communities respectively (Figure 2.6). This suggested a high degree of similarity between the *Chloroflexi* communities extracted.

However, caution must be taken with the interpretation of cluster analysis of both DGGE and RISA profiles. Slight misalignment of the gel lanes by the software may introduce false microdiversity between samples (e.g. Figure 2.5A & 2.6A), which may indicate that the community profiles were more similar than suggested by cluster analysis. Though attempts were made to avoid this by normalising each profile to a standard banding pattern using a DNA ladder.

2.3.4 Combination of the FastDNA and PowerSoil protocols

A combination of the FastDNA and PowerSoil protocols was tested to utilise the high DNA extraction ability of the former and the efficient purification of

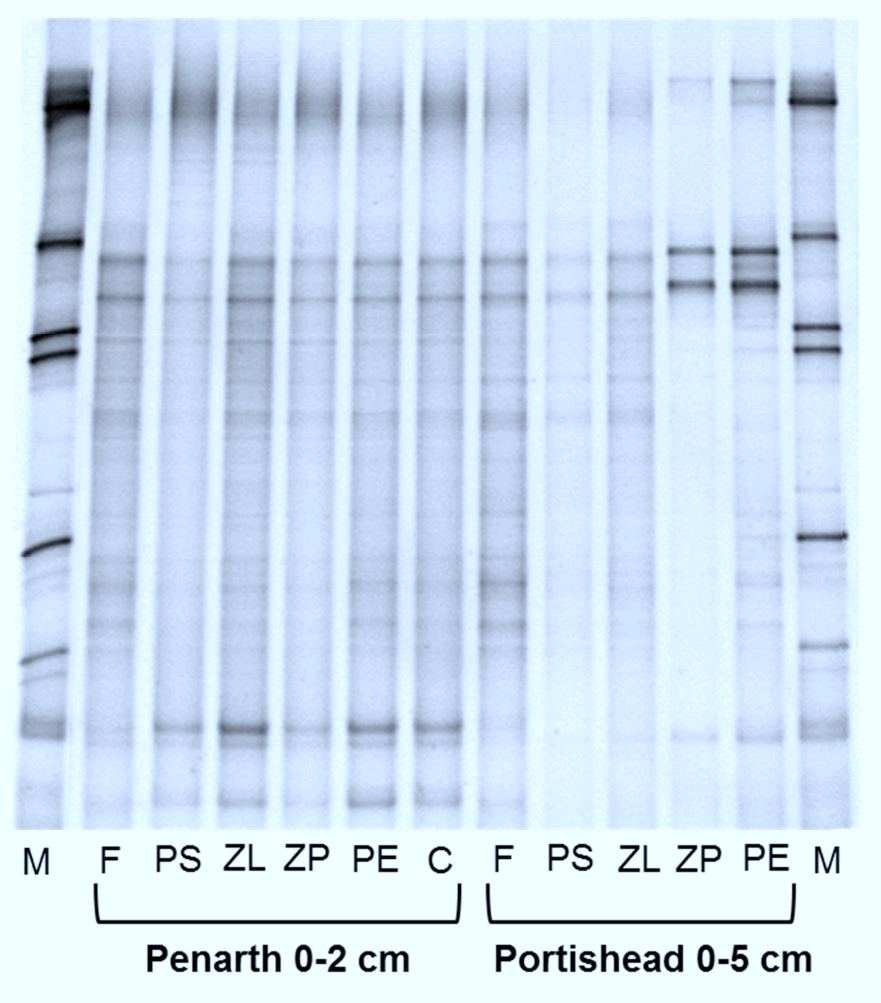


Figure 2.4 – PCR-DGGE of bacterial 16S rRNA gene fragments amplified from Penarth 0-2 cm and Portishead 0-5 cm samples. The same sample extracted by each method was run on the same gel for further comparison and cluster analysis. M – marker, F – FastDNA, PS – PowerSoil, ZL – Luna, ZP – Sharma, PE – Peršoh and C – combination of FastDNA and PowerSoil protocols.

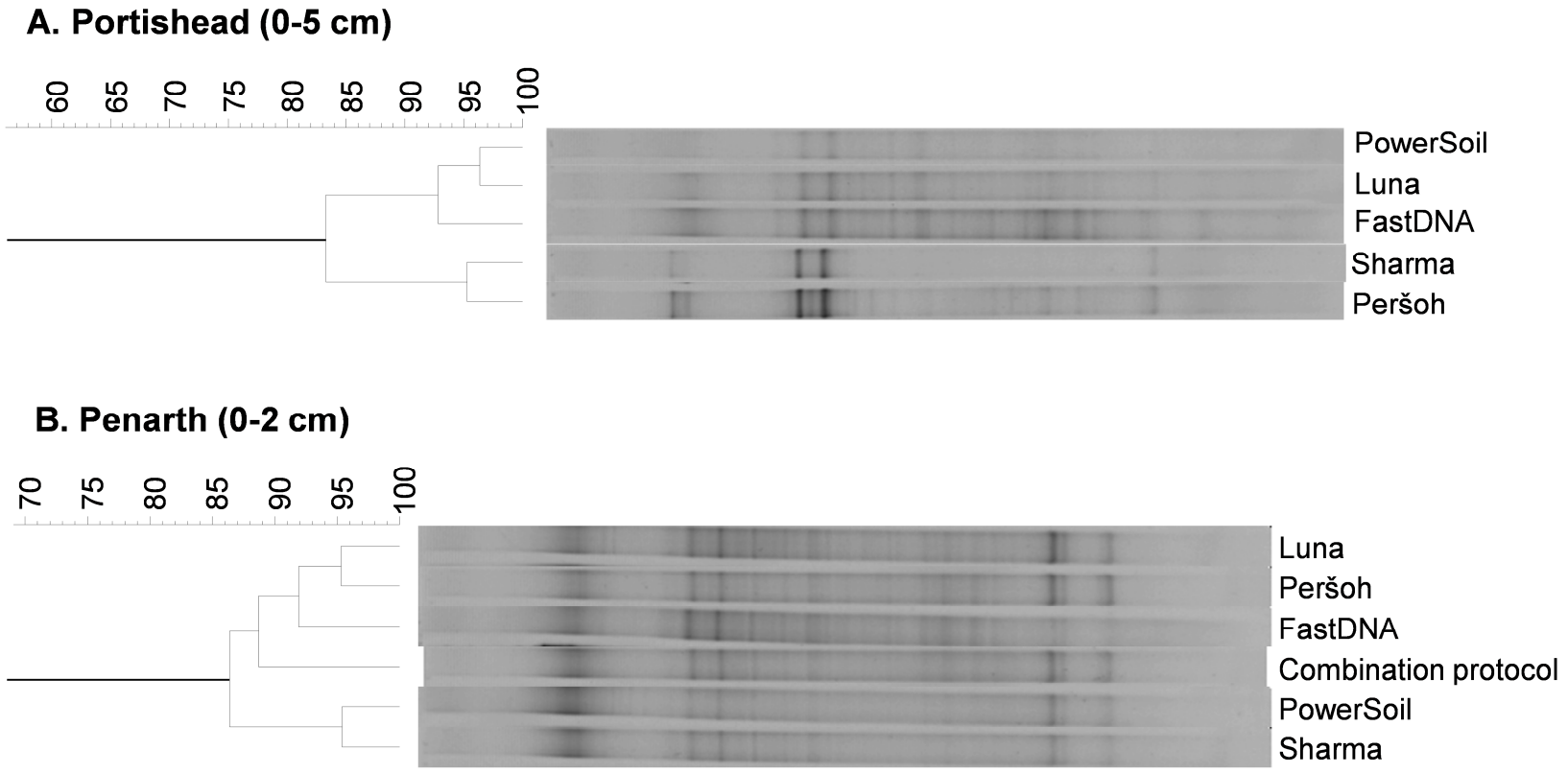


Figure 2.5 - Cluster analysis of DGGE profiles of the bacterial community at: A, Portishead (0-5 cm) and; B, Penarth (0-2 cm) sites (shown in Fig 2.4). Each sample was extracted with each extraction technique and the amplified 16S rRNA gene DNA run on the same DGGE gel for clustering comparison. Pearson correlation and UPGMA were performed using GelCompar II software (version 6.5; Applied Maths). Percentage similarity scale bar shown. UPGMA trees are rooted with DGGE ladder profile (not shown).

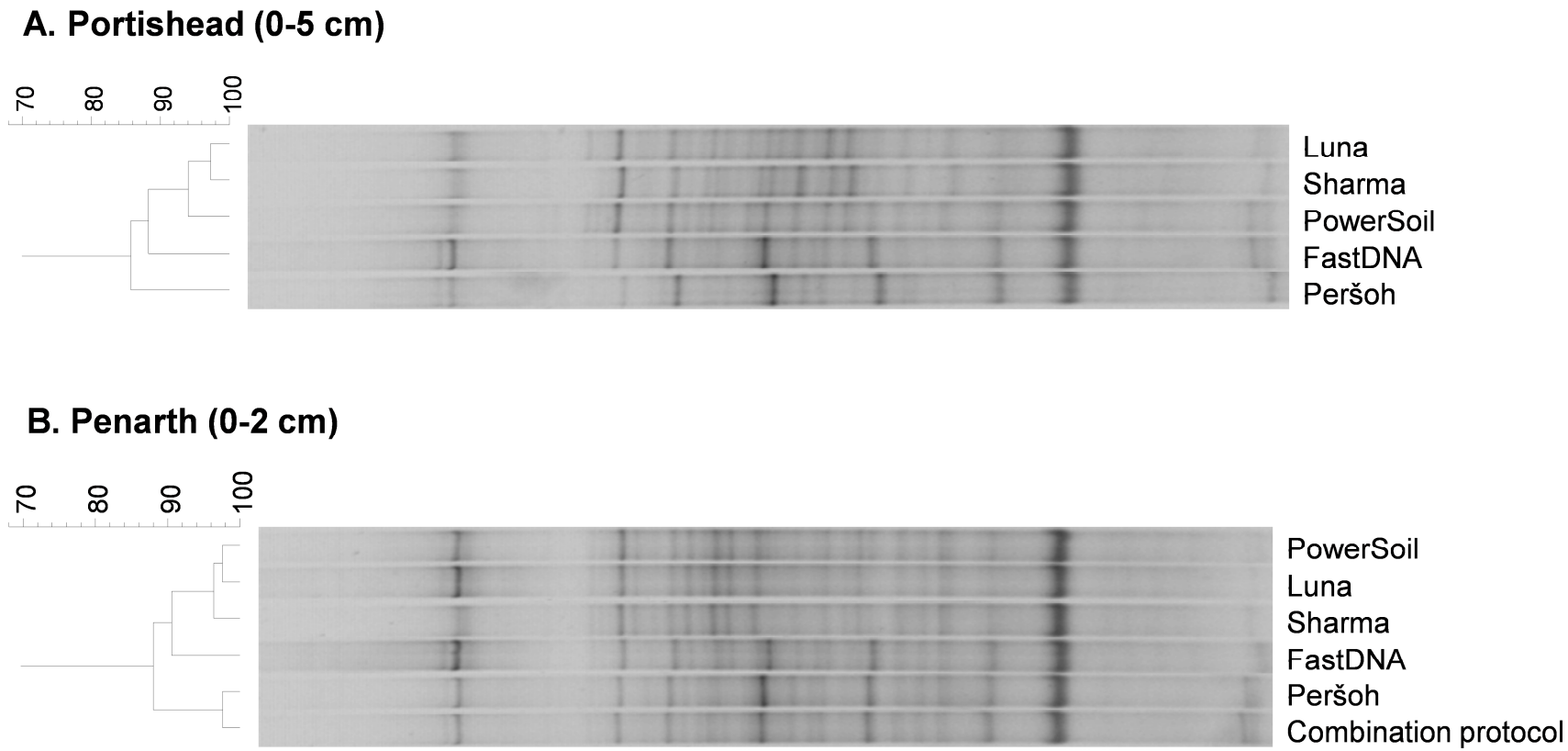


Figure 2.6 - Cluster analysis of DGGE profiles of the *Chloroflexi* community at: A, Portishead (0-5 cm) and; B, Penarth (0-2 cm) sites. Each sample was extracted with each extraction technique and the amplified 16S rRNA gene DNA was run on the same DGGE gel for clustering comparison. Pearson correlation and UPGMA were performed using GelCompar II software (version 6.5; Applied Maths). Percentage similarity scale bar shown. UPGMA trees are rooted with DGGE ladder profile (not shown).

the latter. The combination method performed less well than expected, with the DNA yield much below that of the FastDNA and PowerSoil protocols (Figure 2.7; Table 2.3). However, the average absorbance ratios for the combined method were $A_{260/280}$ = 1.82 and $A_{260/230}$ = 1.83 which were much higher than both of the separate protocols and the absorbance ratios minimum limits. Also, DNA could be amplified by PCR without prior dilution. This indicated that the extra purification steps in the PowerSoil method sacrificed DNA yield for purity.

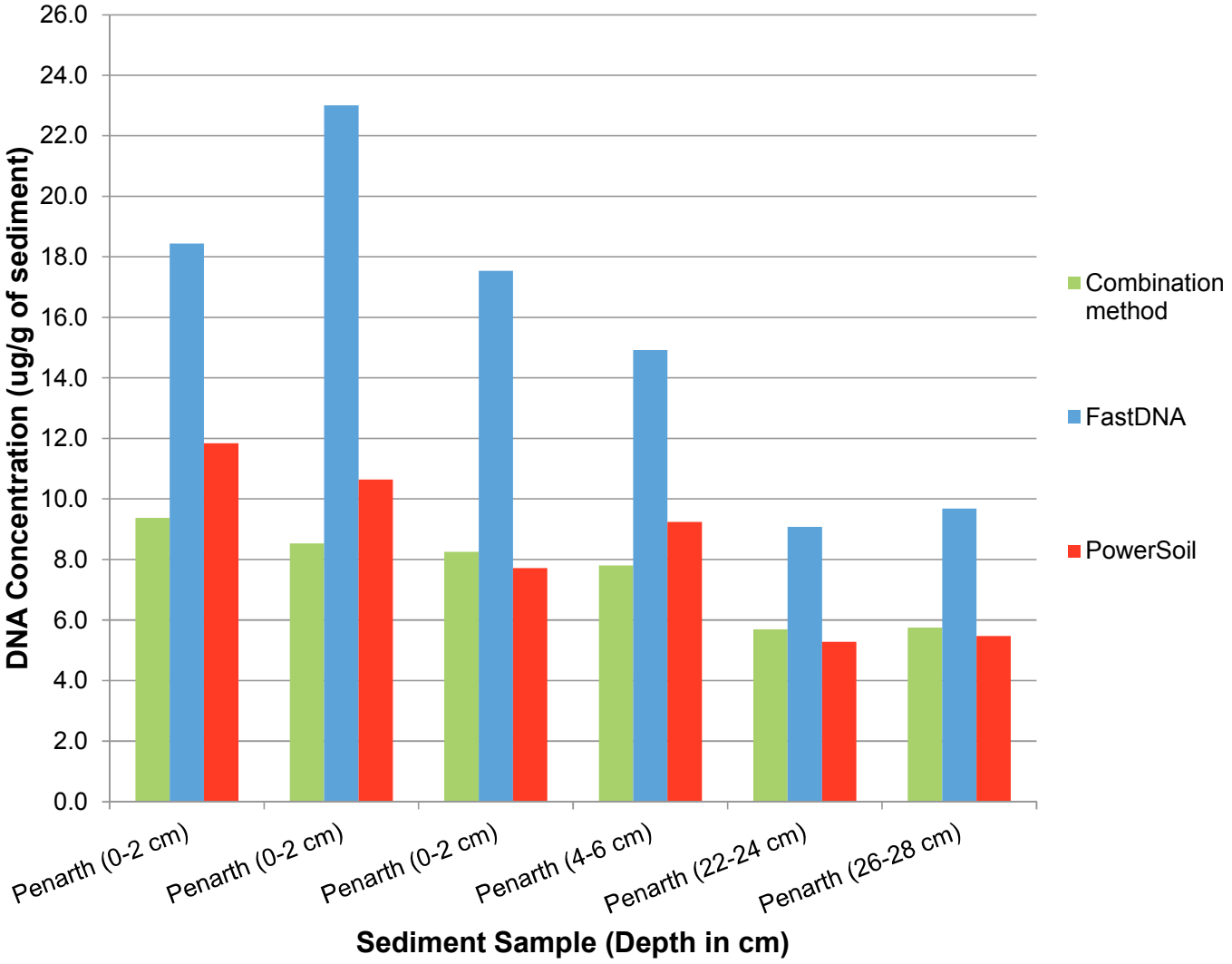


Figure 2.7 - Comparison of DNA yields from Penarth sediments (depth below surface given in bracket) extracted by the FastDNA and PowerSoil protocols and the combination method. DNA concentrations are averages and were quantified using the Quanti-iT™ Broad Range Assay protocol (Invitrogen). Triplicates of Penarth (0-2 cm) are given as a test for reproducibility of the method.

2.4 Discussion

As it has been estimated that only 1% of the prokaryotic community in an environment could be cultured successfully, it was not surprising that cultivation-independent methods have become vital in the quest to describe microbial community diversity (Torsvik et al., 1990). This was particularly true for low prokaryotic biomass sediments as it has been estimated that approximately 0.001–0.1% of microbes could be grown from marine environments (Amann et al., 1995; Kallmeyer and Smith, 2009; Kogure et al., 1979). The crucial step in cultivation-independent analysis is the DNA extraction method, as this initial step determines the amount and quality of the DNA to be analysed (Amann et al., 1995). Others have observed that the yield and purity of DNA extracted from environmental samples was greatly influenced by the type of extraction used (Feinstein et al., 2009). Furthermore, the type of DNA extraction technique has been found to affect the composition of the extracted bacterial community (Carrigg et al., 2007).

Three different methods for DNA quantification were compared for quantification of the extracted DNA. Agarose gel electrophoresis was discounted from further analysis as this method gave inaccurate estimates of DNA concentration due to its relative insensitivity. The Quanti-iT™ Broad Range Assay (Invitrogen) and NanoDrop methods were compared using standard curves made with and without humic acids from sediment extract (Figure 2.3). The NanoDrop method was found to overestimate DNA concentration in humic containing samples making it unsuitable for sediment extracts. The Quanti-iT™ Broad Range Assay (Invitrogen) used fluorescent dyes to specifically detect dsDNA. Therefore DNA concentrations could be reliably estimated even in the presence of humic acids. Though previous research (Bachoon et al., 2001; Zipper et al., 2004; Zipper et al., 2003) has shown that humic acids have a quenching effect on fluorescent dyes, Figure 2.3A indicated that the protocol was not affected by quenching.

The optimised FastDNA spin protocol for soil consistently gave the largest average yield across all sediments types and depths by comparison with all other methods (Table 2.3). The results obtained in this study indicated that

bead beating was the most effective method to lyse cells, which is consistent with other studies (Howeler et al., 2003). The bead beating methods used (FastDNA, PowerSoil and Peršoh protocols) gave the highest average DNA yields. Of the chemical lysis methods, the Luna protocol resulted in the lowest DNA yields. One disadvantage of bead beating techniques was the possible shearing of DNA molecules which could bias the sample since smaller genes would be over represented (Hazen et al., 2012). However, this does not appear to be an issue with the bead beating protocols used here as all samples were amplifiable using 27F-1492R primers to amplify almost the entire 16S rRNA gene, the focus of the community analysis to be used in this study.

All methods produced PCR amplifiable DNA, indicating that they removed humic acids and other PCR inhibitors from the samples. Humic acids have been shown to greatly inhibit downstream processes such as PCR (Bachoon et al., 2001), necessitating more dilution of the DNA for PCR amplification. The PowerSoil protocol consistently produced DNA pure enough for PCR without any dilution of the DNA (Table 2.3). Although the FastDNA protocol did not provide DNA that could be PCR amplified without dilution, it did so consistently at 1:10 dilution. The dilution of DNA to enable PCR amplification has been a common feature of DNA extracted from sediments due to humic acid contamination, and maintains the integrity of the sample by limiting freeze –thawing (Rochelle et al., 1992; Webster et al., 2003).

Cluster analysis of DGGE banding patterns indicated high overall similarity of community profiles from all DNA extracts; however, DGGE profiles have been difficult to analyse due to gel variability (Heuer and Smalla, 1997; Muyzer and Smalla, 1998). The Pearson similarity values were therefore an estimate of the bacterial community similarity. Since the *Chloroflexi* sediment community was to be the focus of further investigation in this work, it was important to identify the most appropriate DNA extraction method for analysis of this community too. Cluster analysis indicated a high degree of similarity between the *Chloroflexi* communities with all methods used (Figure 2.6).

As for ease of use, the FastDNA and PowerSoil protocols were the quickest and easiest methods taking only 2 hours to complete (Table 2.3). This was useful for creating a standardised sediment extraction protocol in the lab, which is lacking at this time. The FastDNA protocol also allows extraction from 50 ml of sediment per extraction tube. This would be very useful in the future for processing high volumes of sediment in a short time and for extraction from low biomass sediment samples. The Peršoh protocol allowed the extraction of RNA at the same time as DNA and so could be beneficial for future RNA work. However it also took the longest preparation time of 3 days and so would be inappropriate when working with a high volume of samples.

It was anticipated that a combination of the two best methods (FastDNA and PowerSoil) would give the highest yields and DNA purity in extracts. Though this combination produced extremely pure DNA ($A_{260/280}$ = 1.82 and $A_{260/230}$ = 1.83), the multiple purification steps reduced the DNA yield, to approximately half the yield from the FastDNA protocol (Table 2.3; Figure 2.2). Also, the cost of using two separate protocols for a single extraction would be prohibitive for this study with a large number of samples. Therefore it was concluded that the FastDNA protocol was the best method for use in this investigation.

Chapter 3 – Profiling of Bacteria, Archaea and Chloroflexi Communities along a Geomorphological Gradient in Severn Estuary Sediment

3.1 Introduction

3.1.1 Prokaryotes in Estuarine and Tidal Sediment

Oxygen is depleted quickly in shallow sediment, and so alternative electron acceptors are consumed in order of decreasing energy production (Figure 1.1; Canfield et al., 2005; Froelich et al., 1979). Therefore anaerobic geochemical processes such as sulphate reduction, methanogenesis and denitrification are often dominant in coastal and estuarine sediment (Dong et al., 2002; O'Sullivan et al., 2013; Wellsbury et al., 1996). Approximately 60% of total organic matter degradation was accounted for by sulphate reduction in estuarine sediment (Purdy et al., 2002a; Wellsbury et al., 1996).

As prokaryotic communities were responsible for many of these anaerobic processes, it was unsurprising that prokaryotic diversity studies of coastal and estuarine sediment have focused on microbes that are able to utilise alternative electron acceptors, such as the *Deltaproteobacteria*. The bacterial community can be influenced by the estuary's dynamic factors such as lithology, geochemistry, electron acceptor/ donor availability and carbon source (O'Sullivan et al., 2013; Wilms et al., 2006b). As these properties vary greatly between sites in an estuary and with depth, the prokaryotic communities often shift in relative abundance of key taxa (Köpke et al., 2005; Wilms et al., 2006a). *Proteobacteria* of the alpha, gamma and delta classes have been shown to be the dominant bacterial community members in surface sediment, with the *Alphaproteobacteria* and *Deltaproteobacteria* being the most abundant in estuarine sediment (Köpke et al., 2005; O'Sullivan et al., 2013; Rappé et al., 1997; Wilms et al., 2006b). This was linked to the ability of the *Proteobacteria* classes to utilise a wide range of organic carbon compounds as electron donors, and a variety of electron acceptors, such as sulphate in the case of *Deltaproteobacteria*. Other commonly detected taxa in surface sediment include *Actinobacteria,*

Firmicutes, Bacteroidetes and *Gemmatimonadetes* (Durbin and Teske, 2011; Sun et al., 2012; Wilms et al., 2006b). The *Chloroflexi* subdivision *Dehalococcoidia* has adapted to anaerobic sediment environments by utilising chlorinated compounds in the process of dehalorespiration, which has made this subdivision a target of much research (Futagami et al., 2008). Dehalogenating communities related to *Chloroflexi* have been previously detected in anaerobic estuarine sediment (Ahn et al., 2008).

The high relative abundance of *Chloroflexi* in deep subsurface sediment has been widely recognised (Biddle et al., 2008; Durbin and Teske, 2011; Parkes et al., 2005). The subdivisions *Anaerolineae, Dehalococcoidia* and Subdivision IV were commonly found in sediment and often stratified with depth (Blazejak and Schippers, 2010; Coolen et al., 2002; Inagaki et al., 2006; Inagaki et al., 2003). The presence and distribution of *Chloroflexi* subdivisions in surface sediment has been less widely investigated. Previous cultivation-based analyses of surface sediment have been unable to detect *Chloroflexi* due to the difficulties in culturing these species (Adrian, 2009; Köpke et al., 2005; Wilms et al., 2006a). Using clone libraries of German Wadden Sea sediment, a significant shift in abundance from *Proteobacteria* to *Chloroflexi,* 2 m below the surface, was discovered that had not be seen in coastal sediment before (Parkes et al., 2005; Tamura et al., 2004; Wilms et al., 2006a). *Dehalococcoidia* have been detected in intertidal sediment, from approximately 2 cm below the surface, establishing a community that increased with depth (Wasmund et al., 2014a; Wilms et al., 2006a).

3.1.2 Prokaryotic Microbial Communities in Severn Estuary Sediment

The Severn Estuary has been the subject of intense research due to its unique physical features, such as its hyper-tidal and resultant turbid nature (Kirby, 2010; Manning et al., 2010), which were thought to directly affect the microbial communities in estuarine sediment. The upper layers of sediment can be substantially disturbed and mixed, and the effect of the resultant oxygen penetration on prokaryotic communities is likely to be significant, but as yet has not been systematically investigated (Wellsbury et al., 1996). The

estuary receives nutrients from both land and water, creating intense primary production and heterotrophic activity (Joint, 1984; Joint and Pomroy, 1981; Morris, 1984). These chemical inputs could have a profound effect on the prokaryotic metabolic processes as seen by Engelen and Cypionka (2009).

The first in investigations into the microbial populations of Severn Estuary were by the Sabrina project, hosted by the University of Bristol (Dineley and Smith, 1975). This was partly focused on the influence of the physical and chemical aspects of primary production and phytoplankton in the water column (Joint, 1984; Joint and Pomroy, 1981). Another part of the study focused on analysis of so called 'bacterial pollution' by *Enterococci* bacteria, demonstrating how the estuary's physical properties may affect bathing waters around the Bristol Channel (Anson and Ware, 1974; Gao et al., 2013a; Kay et al., 2005; Ware et al., 1972).

Joint and Pomroy (1982) later analysed activity of *Bacteria* in the water column. They found the highest numbers of bacteria (2.2×10^5 cells/ml) and activities were in the estuary itself and this related to high turbidity. In fact, all of the aforementioned research focuses on the unusually high turbidity of the estuary and the consequences on microbial communities in the water column. The first examination of Severn Estuary intertidal sediment prokaryotic communities was by Wellsbury et al. (1996) from Aust Warth, using molecular, rather than cultivation-based methods. Acridine orange direct cell counts (AODC) showed that prokaryote numbers were consistently high (~10^9 cells/ml) in the top 8 cm of sediment that had been mixed, but then dropped substantially with increasing depth. Sulphate reduction was found to be the predominant process linked to prokaryotic activity occurring in the estuary sediment samples. Webster et al. (2010) used a range of DNA based analysis of both the 16S ribosomal RNA (rRNA) gene and the functional genes for dissimilatory sulphite reduction to investigate the activity of prokaryotes in different geochemical zones in tidal flat sediment from the Severn Estuary at Portishead. The extent of the anaerobic biogeochemical zones in intertidal Severn Estuary sediment were defined by changes in sediment pore water (Webster et al., 2010). The results highlighted that

many uncultured prokaryotes may have very important ecological roles in the tidal sediment. *Gammaproteobacteria* and *Marine Group 1 Archaea* proliferated in aerobic and dysaerobic zones, whereas *Epsilonproteobacteria* and *Deltaproteobacteria* were more dominant in anaerobic zones; however, surprisingly, no gene sequences related to known sulphate reducing bacteria (SRB) were detected.

3.1.3 Aims

The aim of this study was to create an in-depth quantitative representation of prokaryotic communities in Severn Estuary sediment to identify significant community fluxes in relation to geochemical and geophysical sediment parameters. Cultivation-independent based profiling methods were used to investigate the patterns of the *Bacteria* and *Chloroflexi* community structure across a gradient from intertidal sediment, exposed to oxygen and light, to deep water sediment, with high sediment turbidity in the middle of the Severn Estuary channel. To further analyse *Chloroflexi* subdivision composition, novel quantitative PCR (qPCR) and ribosomal intergenic spacer analysis (RISA) assays were developed specifically for the *Chloroflexi* subdivisions *Anaerolineae/Caldilineae* and *Dehalococcoidia*.

3.2 Materials & Methods

3.2.1 Pure Cultures

Three *Chloroflexi* strains were used as positive controls from the microbial culture collection the German Collection of Microorganisms and Cell Cultures at The Leibniz Institute (DSMZ). *Anaerolineae thermophila* UNI-1 (DSM 14523) and *Caldilinea aerophila* STL-6-01 (DSM 14535) were grown under anaerobic conditions at 55^{o}C in Medium 1004 (DSMZ). It was not possible to grow *Chloroflexus aurantiacus* J-10-fl (DSM 635) due to insufficient equipment. DNA was extracted from the three pure cultures using the FastDNA Spin for Soil Kit (MP Biomedicals; **Section 2.2.2.1**). *Dehalococcoides ethenogenes* 195 DNA was kindly provided by Prof. Stephen Zinder (Cornell University, NY). *Clostridia* species*, Pseudomonas putida* PP3*,* acetogen species DNA and *Methanococcoides methylutens* were provided by Dr. Louise O'Sullivan, Nicholas Passiotis and Dr. Gordon Webster, respectively.

3.2.2 Sediment Sampling and Description of Cores

Sediment cores were taken in the Severn Estuary, UK (Figure 3.1) during neap tide on 24th August 2011. A marine multi-corer (Duncan & Associates, Cumbria, UK) with 4 corer tubes, each 500mm in length, was deployed mid-channel using a hydraulic A-frame with electrohydraulic spencer carter winch system from the research vessel R.V. Guiding Light. Cores were taken at three sampling sites with different geomorphology and sedimentology. The first site was an intertidal mud flat (Wentloodge Levels, 51 30.33N, 3 2.17W). 18 cm of sediment was recovered from a water depth of 8.3 m. From 0-6 cbsf was a mixture of brown mud and fine sand and was presumed to be an aerobic zone. A colour change to grey occurred from 6-8 cbsf indicating a suboxic zone. The rest of the core (8-18 cbsf) was black in colour suggesting the beginning of the reduced zones. The second core was from shallow waters with a water depth at the time of 9.7 m (Peterstone Flats, 51 29.66N, 3 1.17W). Total core recovered was 20 cm. Here, the brown aerobic zone was less than 1 cbsf followed by a grey, suboxic zone up to 6 cbsf. From 6-

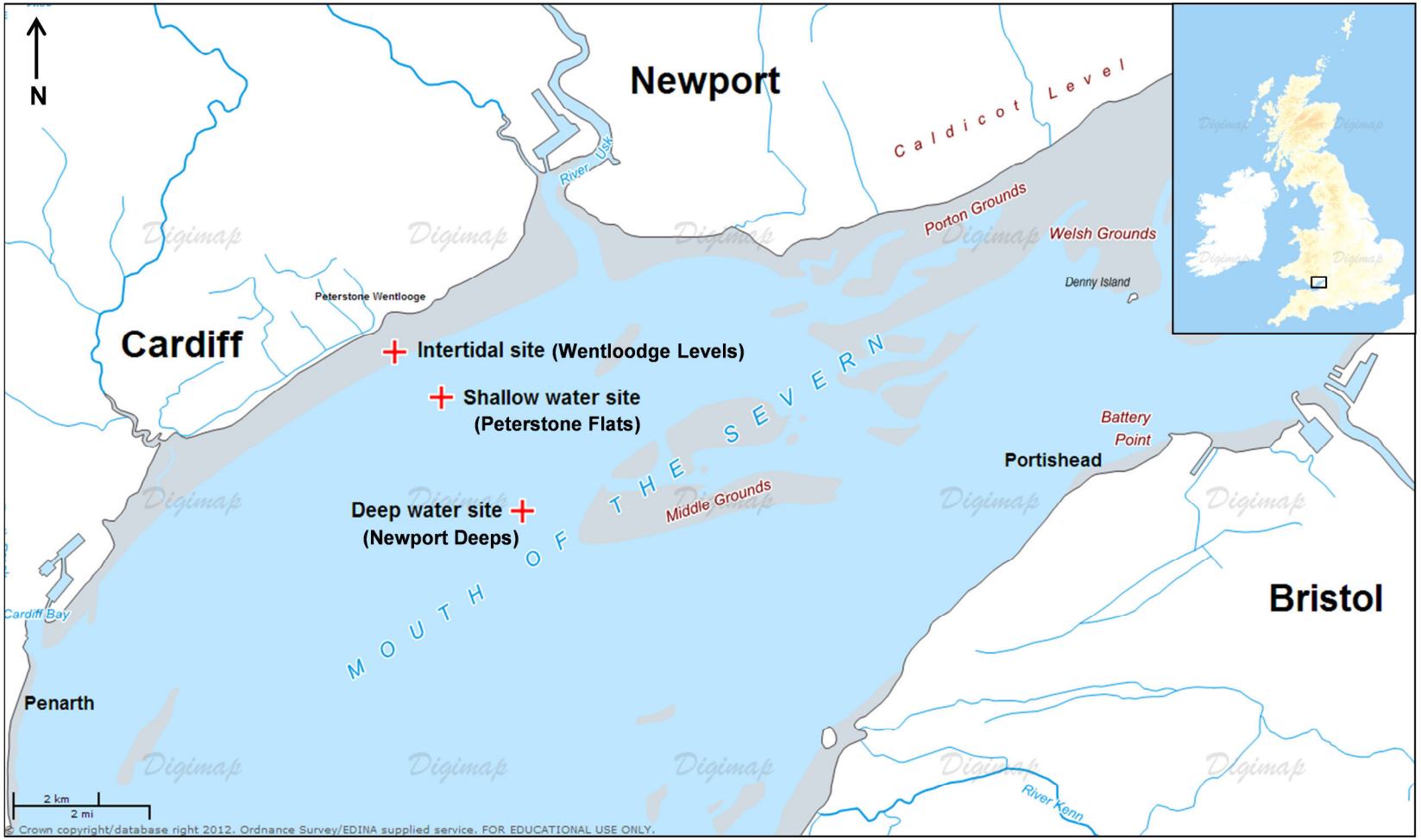


Figure 3.1 - Map of the Severn Estuary, UK with sampling sites indicated with red crosses and name of the site. Grey colouring indicates foreshore areas. Scale 1 : 160 000 (PDF map), OS Strategi, Ordnance Survey UK, updated January 2011. Map created June 2013 using EDINA Digimap Ordnance Survey Service, http://edina.ac.uk/digimap.

20 cbsf were black reduction zones. The third core was taken from deep waters (~15 m at time of sampling) with detectable fluid mud pools and strong tidal currents, increasing sediment turbidity (Newport Deeps, 51 28.39N, 2 59.31W). This core was 34 cm in length and consisted entirely of liquid brown mud and sand bodies between 14-22 cbsf. On the same day as collection, cores were transported to the laboratory and frozen at -20^{o}C prior to sub-sampling. Each core was divided into 2 cm depth increments using a hydraulic core extruder (Duncan & Associates, Cumbria, UK) on the following day. First sediment samples for geochemical analysis were taken using sterile syringes with the tips removed. For ion chromatography, 6 cm^3 of sediment was taken per depth slice centrifuged (15 m at 2300 xg) and the supernatant diluted 1/10 for pore water analysis. 4 cm^3 of sediment was taken per depth slice for gas chromatography. Samples of 10-15 g of the remaining core slice were then taken using a sterilised stainless steel spatula and stored in sterile 50 ml Falcon tubes at -20^{o}C for DNA extraction. Geochemical analysis began immediately whereas samples were stored at -20^{o}C for 3 months before DNA extraction and analysis.

3.2.3 Geochemical Analysis of Sediment Cores

Geochemical analysis of each core segment was performed by Shaun Thomas (Cardiff University). Sediment slurry headspace gases (methane) were analysed by a natural gas analyser as described by Webster et al. (2010). Briefly, 4 cm^3 of sediment was mixed with 20 ml 10% (w/v) KCl in gas-tight serum bottles, incubated overnight and headspace gas analysed with gas chromatography the following day. An Arnel Clarus 500 Natural Gas Analyser (Perkin Elmer) with a flame ionization detector and a thermal conductivity detector was used. Anions (sulphate, nitrate and chloride) from sediment slurry pore waters were determined by ion chromatography with the ICS-2000 (Dionex UK Ltd) ion chromatography system with an AS50 autosampler (Dionex UK Ltd) fitted with two Ionpac AS15 columns and an anion self-regenerating suppressor (ASRS-ULTRA II 4-mm) in combination with a DS6 heated conductivity cell (Dionex UK Ltd) as described previously (Webster et al., 2009; Webster et al., 2010). Cations (ammonium) were

analysed using a DX-120 (Dionex UK Ltd) ion chromatography system with an AS40 autosampler (Dionex UK Ltd) fitted with an Ionpac CS16 and a cation self-regenerating suppressor (CSRS-300 4mm) in combination with a DS4-1 heated conductivity cell (Dionex UK Ltd), and using 25mM methanesulphonic acid as an eluent.

3.2.4 DNA Extraction

Three sediment samples weighing 0.5 g were taken from each 2 cm depth under aseptic conditions in a lamina flow cabinet with a sterile stainless steel spatula. An amended version of the FastDNA Spin for Soil Kit (MP Biomedicals) protocol (Webster et al., 2003), described in **Section 2.2.2.1**, was used for DNA extraction. Triplicate depth extractions were pooled and stored at -20^{o}C. There were 9, 10 and 17 pooled depth samples from the intertidal, shallow water and deep water sites, respectively. The concentration range of extracted DNA was 0.31-1.79 ng/ µl.

3.2.5 PCR and Community Profiling Methods
3.2.5.1 Denaturing Gradient Gel Electrophoresis

Bacterial and *Chloroflexi* DGGE depth profiles for each sampling site were produced as described in **Section 2.2.3.5**. Bands were excised and the DNA sequenced for further phylogenetic analysis as described in **Section**s **2.2.3.6**.

3.2.5.2 Ribosomal Intergenic Spacer Analysis (RISA)

Bacterial (primers 1406F and 23SR) and *Chloroflexi* (primers 941F and 23SR) targeted PCRs were used to amplify the intergenic spacer region between the 16S and 23S rRNA genes (Table 3.1; Borneman and Triplett, 1997). All PCR reactions were performed under aseptic conditions using autoclaved plastic ware, UV irradiated pipettes and nuclease free molecular grade water (Severn Biotech). Molecular grade water was used as a negative control in all PCR amplifications. The protocol was 95^{o}C for 5 min, 35 cycles of 95^{o}C for 30 seconds, 55^{o}C for 30 seconds and 72^{o}C for 30 seconds and a final step of 72^{o}C for 5 min. The reactions were 1 µl of 1:10

Table 3.1 - Primers used throughout experiments with sequences, target, protocol and references.

Primer	Approach	Target	Sequence (5' – 3')	Reference
27F[a]	PCR-DGGE	*Bacteria*	AGA GTT TGA TCM TGG CTC AG[b]	Lane, 1991
1492R	PCR	*Bacteria*	GGT TAC CTT GTT ACG ACT T	Eden et al., 1991
357F[c]	PCR-DGGE	*Bacteria*	CCT ACG GGA GGC AGC AG	Muyzer et al., 1993
518R	PCR-DGGE	*Prokaryotes*	ATT ACC GCG GCT GCT GG	Muyzer et al., 1993
941R	PCR-DGGE	*Chloroflexi*	AAA CCA CAC GCT CCG CT	Gich et al., 2002
518R-AT-M13F	Sequencing primer	*Bacteria*	GTA AAA CGA CGG CCA GTA AAT AAA ATA AAA ATG TAA AAA AA	O'Sullivan et al., 2008
1406F	RISA	*Bacteria*	TGY ACA CAC CGC CCG T	Borneman and Triplett, 1997
23SR	RISA	*Bacteria*	GGG TTB CCC CAT TCR G	Borneman and Triplett, 1997
M13F	Clone library	-	TGT AAA ACG ACG GCC AGT	TOPO TA cloning® (Invitrogen)
M13R	Clone library	-	CAG GAA ACA GCT ATG AC	TOPO TA cloning® (Invitrogen)

[a] rRNA gene primer designations are in accordance with the established standard (Lane, 1991), reflecting the position of the primers' 3'-ends with respect to *the Escherichia coli, Anaerolineae thermophila* UNI-1 (AP012029) (AN1018F - AN1290R) and the *Dehalococcoides mccartyi* 195 (CP000027) (DHC663F - DHC1128R) 16S rRNA gene.

[b] Key: M = A or C, S = C or G, H = A or C or T, B = C or G or T, W = A or T, Y = C or T, R = A or G, D = A or G or T

[c] This primer has GC clamp attached at 5' end – CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG (Muyzer and Smalla, 1998)

518F	q-PCR	*Bacteria*	CCA GCA GCC GCG GTA AT	Muyzer et al., 2003
907R	q-PCR	*Bacteria*	CCG TCA ATT CMT TTG AGT TT	Muyzer and Smalla, 1998
S-D-Arch-0025-a-S-17 F	q-PCR	*Archaea*	CTG GTT GAT CCT GCC AG	Vetriani et al., 1999
S-D-Arch-0344-a-A-20 R	q-PCR	*Archaea*	TCG CGC CTG CTG CGC CCC GT	Vetriani et al., 1999
941F	q-PCR/ RISA	*Chloroflexi*	AGC GGA GCG TGT GGT TT	Gich et al., 2002
1340R	q-PCR	*Chloroflexi*	CGC GGT TAC TAG CAA C	Gich et al., 2002
AN1018F	q-PCR	*Anaerolineae/ Caldilineae*	TCG GGG AGC BTR CAC AGG TG	This study
AN1290R	q-PCR	*Anaerolineae/ Caldilineae*	GCG GTT ACT AGC AAC TCC RK	This study
DHC663F	q-PCR	*Dehalococcoidia*	GRR AGG GTC GAT ACT CCC	This study
DHC1128R	q-PCR	*Dehalococcoidia*	GGG AGG CAG CAG CAA GGA	This study

diluted extracted DNA (0.31-1.79 ng/ µl), 1x reaction buffer (PCR Biosystems), 0.25 mM of each dNTP (PCR Biosystems), 2.5 mM $MgCl_2$, 0.5 mg/ml bovine serum albumin (BSA), 400 nM of primers, 10 U *Taq* DNA polymerase (PCR Biosystems) and sterile molecular grade water (Severn Biotech Ltd.) to a total volume of 50 µl. All amplifications were carried out in a DNA Engine Dyad Thermal Cycler (MJ Research). PCR product was detected by 1.2% agarose gel electrophoresis and viewed using SYBR Safe DNA gel stain (Invitrogen).

Following the manufacturer's instructions, the PCR amplified DNA (1 µl) was separated on the Agilent 2100 Bioanalyzer (Agilent Technologies Inc,) using the DNA 7500 microfluidics kit (100 – 7500 base pairs (bp) size range) (Agilent Technologies Inc.). A molecular weight ladder (50 – 10,380 bp) was run as standard with each chip and upper (10,380 bp) and lower (50 bp) marker DNA was added to each sample for the standardisation of the protocol. Both these reagents were included in the 7500 microfluidics kit. Individual bacterial diversity profiles for each sample depth and site were generated.

3.2.6 Quantitative PCR

3.2.6.1 Anaerolineae/Caldilineae *and* Dehalococcoidia *Primer Design and Testing*

16S rRNA gene primers were designed to target the *Anaerolineae* (including the closely related *Caldilineae,* which together make Subdivision I) and *Dehalococcoidia Chloroflexi* subdivisions for use with PCR and qPCR. A database of 16S rRNA gene sequences was compiled for both *Anaerolineae/Caldilineae* and *Dehalococcoidia* which included type strains and marine environmental clones retrieved from the GenBank database using BLAST (Altschul et al., 1990). Primrose software (Ashelford et al., 2002) was used to design targeted oligonucleotides for use as potential primers from the 16S rRNA gene databases. Prospective taxon-specific oligonucleotides were filtered based on length (18-20 bp), number of degenerate bases (maximum of 2 per primer), GC content at 3'-end and

melting temperature (highest possible melting temperatures required). Specificity of primers was theoretically tested using Probe Match (Ribosomal Database Project; Cole et al., 2009) and the National Centre for Biotechnology Information (NCBI) software BLASTN (Altschul et al., 1990). On the basis of these selection criteria, primers AN1018F, AN1290R, DHC663F and DHC1128R (Table 3.1) were chosen for experimental testing. rRNA gene primer designations were in accordance with the established standard (Lane, 1991), reflecting the position of the primers' 3'-ends with respect to *Anaerolineae thermophila* UNI-1 (AP012029) 16S rRNA gene for AN1018F and AN1290R, and with respect to the *Dehalococcoides mccartyi* 195 (CP000027) 16S rRNA gene for DHC663F and DHC1128R.

Gradient PCRs with a selection of pure culture DNA **(see Section 3.2.1)** were utilised to test the specificity of the *Anaerolineae/Caldilineae* and *Dehalococcoidia* primers experimentally. The *Chloroflexi* primers 941F and 1340R (Gich et al., 2002) were also tested with gradient PCRs in this way as DGGEs with these primers showed non-specific amplification of *Firmicutes* **(see Section 3.3.1).** The highest possible annealing temperature that amplified target DNA well but excluded all non-target DNA was chosen for use in PCRs and qPCRs.

3.2.6.2 PCR Protocols for Chloroflexi, Anaerolineae/Caldilineae *and* Dehalococcoidia *16S rRNA Gene Amplification*

A standard 16S rRNA bacterial PCR protocol was adapted for use with the *Chloroflexi, Anaerolineae/Caldilineae* and *Dehalococcoidia* 16S rRNA gene primers. All PCR reactions were performed under aseptic conditions using autoclaved plastic ware, UV irradiated pipettes and nuclease free molecular grade water (Severn Biotech). Molecular grade water was used as a negative control in all PCR amplifications. The concentrations of the PCR reactions were as follows: 1 µl extracted DNA samples (0.31-1.79 ng/ µl), 1x reaction buffer (PCR Biosystems), 0.25 mM dNTPs (PCR Biosystems), 2.5 mM $MgCl_2$, 0.2 mg/ml bovine serum albumin (BSA), 0.2 pmol/µl of forward and reverse primers, 2.5 U *Taq* DNA polymerase (PCR Biosystems) and molecular grade water (Severn Biotech Ltd.) up to total volume of 50 µl. The

protocol used was 95^{o}C for 2 min followed by 36 cycles of 94^{o}C for 30 s, annealing (see below) for 30 s, 72^{o}C for 90 s plus 1 second per cycle and a final extension step of 72^{o}C for 5 min (Webster et al., 2006). From gradient PCRs **(Section 3.2.6.1)** the optimum annealing temperature of each primer set were found to be 58^{o}C, 62^{o}C and 55^{o}C for the *Chloroflexi* (941F – 1340R)*, Anaerolineae/Caldilineae* (AN1018F – AN1290R) and *Dehalococcoidia* (DHC663F – DHC1128R), respectively (Table 3.1). All amplifications were carried out in a DNA Engine Dyad Thermal Cycler (MJ Research). PCR product was detected by 1.2% agarose gel electrophoresis and viewed using SYBR Safe DNA gel stain (Invitrogen).

3.2.6.3 Construction of 16S rRNA Gene Libraries from Sediment DNA Using Chloroflexi*,* Anaerolineae/Caldilineae *and* Dehalococcoidia *targeted PCR Primers*

Analysis of the newly designed primers included empirical testing of specificity. For this, clone libraries of 16S rRNA gene PCR products amplified from sediment sample DNA were constructed, and screened by DNA sequencing. Sediment DNA from 4 samples from the intertidal (0-2 cbsf and 16-14 cbsf), shallow water (8-10 cbsf) and deep water (16-18 cbsf) sites were PCR amplified with the *Chloroflexi*, *Anaerolineae/Caldilineae* and *Dehalococcoidia* primer pairs **(see Section 3.2.6.2)**. Two samples from the upper and lower depths of the intertidal core were chosen to represent the separate biogeochemical zones in the core. One sample from the middle of each of the shallow and deep water cores were chosen to represent the whole core as very little change with depth was seen in at these site by DGGE analysis.

Each sample was amplified individually 5 times, pooled, cleaned using Wizard PCR Preps DNA Purification System (Promega, according to the manufacturer's instructions) and quantified using the Quanti-iT™ Broad Range Assay kit and Qubit® Fluorometer (Invitrogen). PCR products were cloned using the pGEM-T Easy Vector System I (Promega) following manufacturer's instructions. Optimised vector ratios of 3:1 (insert:vector) were calculated for use in the ligation and incubated overnight at 4^{o}C

(Webster et al., 2006). *Escherichia coli* JM109 High Efficiency Competent Cells (Promega) were transformed with ligated plasmid vector using the heat shock method. Transformed cells were plated on to a selection media (agar and Lysogeny Broth (LB) with 100 mg/ml of Ampicillin, 40 ug/ml of X-gal and 100 mM of IPTG), sealed and grown over night at 37^{o}C. Remaining cells were stored in 20% w/v sterilised glycerol at -80^{o}C. White colonies with successfully transformed plasmids were picked, grown overnight at 37^{o}C and stored at -80^{o}C in liquid LB with ampicillin (100 µg/ml) and 10% w/v glycerol. Stored clones were screened using PCR with M13F and M13R primers. Clones with inserts (20 per sample, per primer set) were randomly chosen and sequenced by the dideoxy chain termination method (Sanger et al., 1977) on the Applied Biosystems ABI 3730XL platform (Life Technologies) by MWG Eurofins Genomics.

3.2.6.4 Quantitative PCR (qPCR) Protocols

qPCR was used to quantify 16S rRNA gene copy numbers for *Bacteria, Archaea, Chloroflexi, Anaerolineae/Caldilineae* and *Dehalococcoidia*. All plastic wear was autoclaved and/or UV irradiated before use and sterile molecular grade water (Severn Biotech Ltd.) was used for all dilutions and in the reaction. All reactions were prepared in a microflow cabinet. Primers used in each assay were listed in Table 3.1. Sybr Green (Maxima Sybr Green Lo Rox qPCR Master Mix (x2), Fermentas) chemistry was used and reactions run on the MX3000P, Agilent Technologies UK Ltd. for all assays. The qPCR reaction for all assays was 1x Master Mix, 1 µL of 1 in 10 diluted DNA (0.31-1.79 ng/ µl) and 0.4 pmol/µL of each primer, in a total volume of 20 µL with sterile water (Severn Biotech Ltd.). The PCR cycle conditions were as follows: 95^{o}C for 7 min, 40 cycles of 95^{o}C for 30 seconds, 52^{o}C for 30 seconds and 72^{o}C for 1 min, followed by a melting curve of 55-95^{o}C (Wilms et al., 2007). Data acquisition occurred after the elongation at 72^{o}C for 1 min. The annealing temperatures for each reaction were 52^{o}C for *Bacteria* and *Archaea* and 58^{o}C, 62^{o}C and 55^{o}C for *Chloroflexi, Anaerolineae/Caldilineae* and *Dehalococcoidia,* respectively. All samples were measured in triplicate in each assay using optical grade plastic ware.

All qPCR reactions were run with a standard curve, in triplicate, for absolute quantification of the 16S rRNA gene in the original sediment sample. The 16S rRNA genes of bacterial type strains relevant to each targeted assay were amplified using the primers 27F and 1492R (Table 3.1) and purified using Wizard PCR Preps DNA Purification System (Promega). This was to attain as large a proportion of the 16S rRNA gene as possible. The genomic DNA (gDNA) used for the standard curve for *Bacteria, Chloroflexi* and *Anaerolineae/Caldilineae* assays was *Anaerolineae thermophila* UNI-1 (DSM 14523). *Dehalococcoides mccartyi* 195 gDNA was used in the standard curve for the *Dehalococcoidia* targeted assay and finally *Methanococcoides methylutens* (DSM 2657) gDNA was used for the *Archaea* specific assay. The standard curve was a serial dilution (10^{-1} to 10^{-8}) of the purified PCR product diluted from and freshly for each reaction. Results were rejected if either the R^2 value of the standard curve was <0.95, or the efficiency value of the standard curve was not in the range 95%-105%. For full calculations of 16S rRNA gene copy number from qPCR data see Appendix 1.

3.2.7 Bioinformatic and Statistical Analysis

3.2.7.1 Phylogenetic Analysis of DGGE and Clone Library Sequences

Phylogenetic trees were created using MEGA5 (Kumar et al., 2008). Sequences were analysed and used to construct phylogenetic trees to assess taxonomy as described in **Section 2.2.4.3**. A new web application BlastCat was created specifically for this project as described in **Section 3.2.7.3**, to BLASTN batches of sequences and manipulate the output from the NCBI site before analysis and creation of phylogenetic trees. Taxonomy of clones was deduced from these phylogenetic trees.

3.2.7.2 Statistical Analysis of DGGE and RISA Profiles

Each DGGE and RISA site profile was examined for similarities with depth by cluster analysis using Pearson correlation coefficient and UPGMA. Dendrograms and percentage similarity scores were created using the GelCompar II software (version 6.5; Applied Maths). Individual phylotypes from the DGGE profiles were analysed using Pearson's correlation and

NMDS to identify which phylotypes and geochemistry were influencing the DGGE profiles. Briefly, phylotypes (or unique bands) were identified and the percentage intensity of the band in each lane or sample was calculated using ImageJ 1.47u (Abràmoff et al., 2004). Pearson's correlation compared percentage band intensity with depth in Microsoft Excel 2010. P values for each correlation coefficient were determined by comparison to critical values for correlation coefficients at P = 0.05 (Fry and Iles, 1994). NMDS was performed to analyse patterns and similarities in the phylotypes and the potential effects of sediment geochemistry on the depth profile. NMDS plots were created using the R statistical package (Ihaka and Gentleman, 1996). The Bray-Curtis calculation of dissimilarity (Bray and Curtis, 1957) was used to make a matrix of dissimilarities for the NMDS. R scripts are included in Appendix 2. Samples at each site were analysed for significant differences between depths using a single factor ANOVA in Microsoft Excel 2010.

3.2.7.3 Design and Application of BlastCat, a Web Tool for the Efficient Analysis of Many Sequences

The process of using BLAST to search the GenBank nucleotide database and identify close matches to DGGE and clone library gene sequences against the (via NCBI), choosing relevant clones to include in phylogenetic trees and collating data on these clones, was a slow and arduous task. Therefore, a web application, called BlastCat, was developed in this study (with the assistance of Gareth Rees, a Web Developer) to make the process more efficient. The application was written in Ruby with a web interface using HTML. It provided remote access to the NCBI server to simultaneously input multiple gene sequences into a BLASTN search, with the choice of either including or excluding "environmental and clone sequences" in the search. The output of subsequent GenBank database matches ("hits") was customised to list the accession number, sequence name, source (publications), E value and percentage match/coverage. Any sequence hit without a published source was excluded from the list. The database hits obtained in this way can then be edited and exported into a CSV file, along

with a FASTA file of sequences for use in phylogenetic analysis. For BlastCat open source code see Appendix 3.

3.3. Results

3.3.1 DGGE Community Profiles and Phylogenetic Analysis of Severn Estuary Sediment Prokaryotic Communities

DGGE depth profiles of *Bacteria* and *Chloroflexi* communities were generated for each of the three Severn Estuary sites (Figures 3.2 and 3.3). All DGGE profiles showed a high number of bands representing an abundant and diverse community. Each site had a different community profile, the intertidal site being the most complex with a changing depth profile (Figures 3.2A and 3.3A). Both the bacterial and *Chloroflexi* profiles revealed a change in community structure at approximately 4-8 cbsf at the intertidal site, characterised by the appearance or disappearance of phylotypes. Conversely, the profiles of the shallow and deep water sites were constant with depth for both *Bacteria* and *Chloroflexi* (Figures 3.2B & C and 3.3B & C).

Sequenced DGGE bands were used to construct phylogenetic trees for the *Bacteria* (45 sequences from all three sites) and *Chloroflexi* (58 sequences from all three sites) communities (Figures 3.4 and 3.5; Tables 3.2 and 3.3). The bacterial communities at all sites shared the taxa: *Proteobacteria* (*Alphaproteobacteria* and *Gammaproteobacteria*) and *Firmicutes (Clostridia* and *Bacilli*). Some interesting points appeared when relating specific bands to depths. At the intertidal site, a chloroplast related phylotype, likely a diatom was prominent from 0 – 6 cm but then decreased in intensity and throughout the profile at the deep water site (Table 3.2). Similarly, the *Actinobacteria* were only detected from 0-8 cbsf at the intertidal site and at all depths at the deep water site.

The *Chloroflexi* community showed a very high level of diversity with sequences in the phylogenetic tree clustering away from type strains. The intertidal and shallow water sites both had communities comprised of a number of *Anaerolineae* and *Dehalococcoidia*. In addition, Subdivision IV and *Thermomicrobia* were detected at the deep water site at all depths. *Dehalococcoidia* appear at the intertidal site after 4 cbsf whereas they

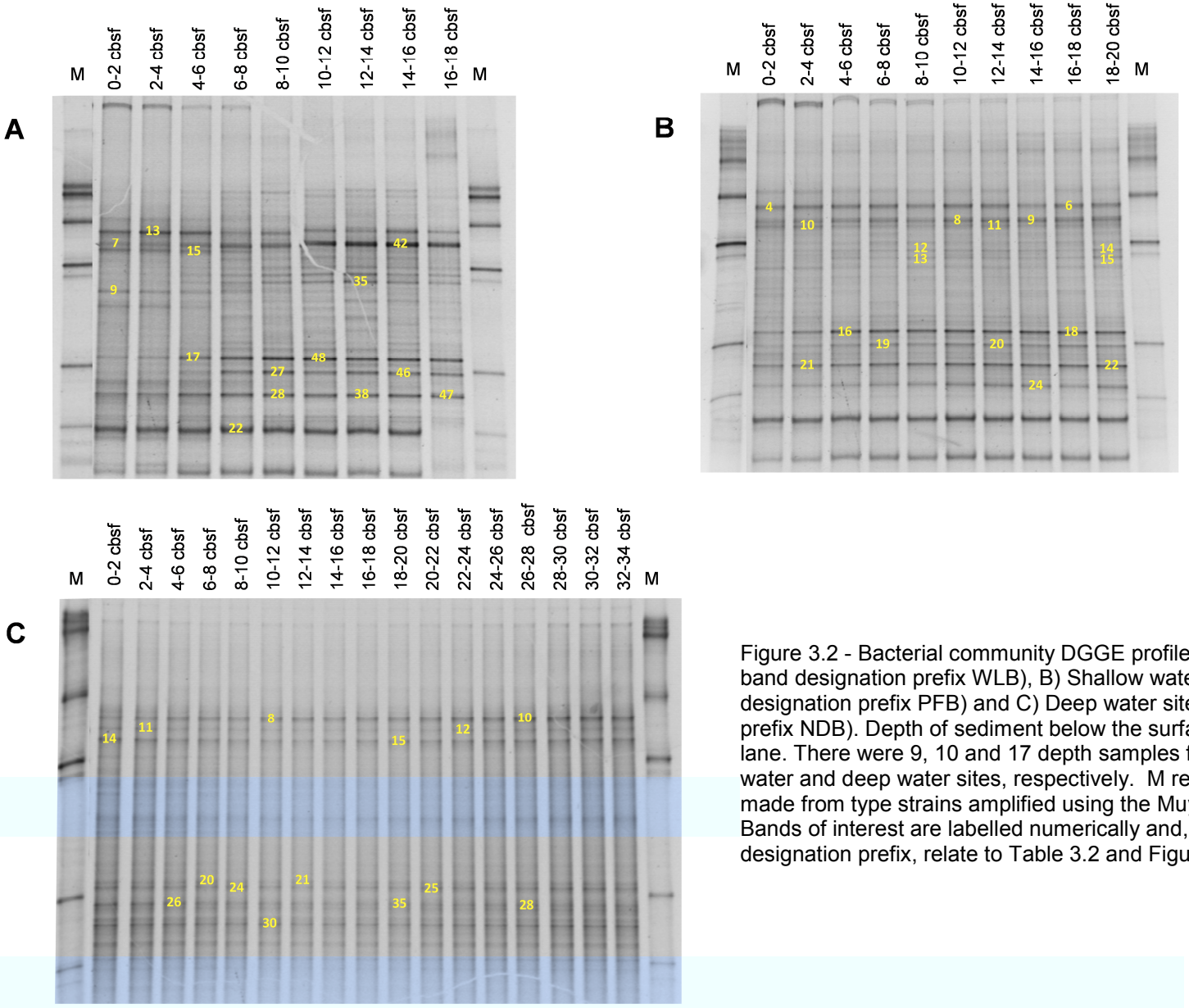


Figure 3.2 - Bacterial community DGGE profiles of A) Intertidal site (WL; band designation prefix WLB), B) Shallow water site (PF; band designation prefix PFB) and C) Deep water site (ND; band designation prefix NDB). Depth of sediment below the surface is given above each lane. There were 9, 10 and 17 depth samples from the intertidal, shallow water and deep water sites, respectively. M represents marker lanes made from type strains amplified using the Muyzer et al (1993) primers. Bands of interest are labelled numerically and, together with band designation prefix, relate to Table 3.2 and Figure 3.4.

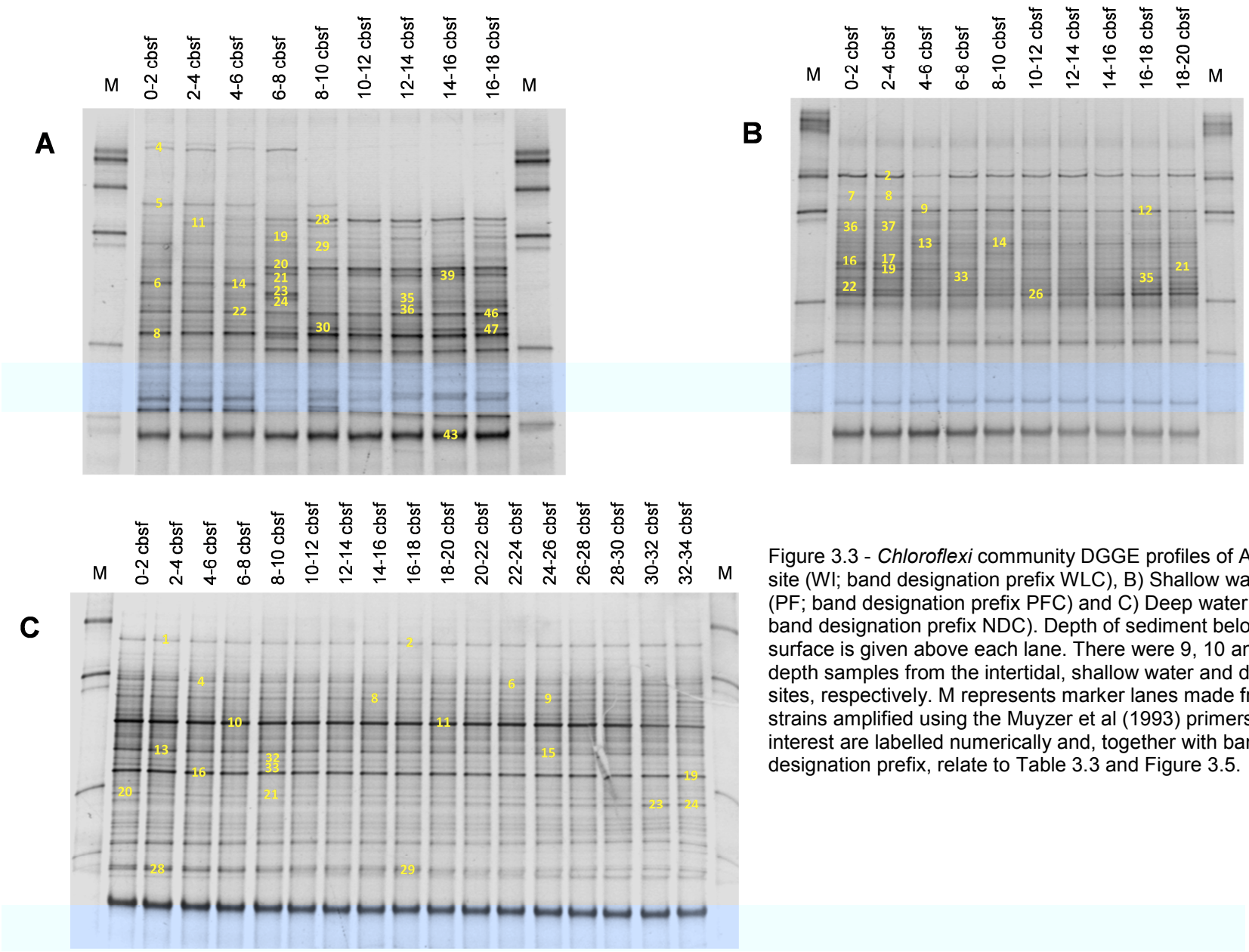


Figure 3.3 - *Chloroflexi* community DGGE profiles of A) Intertidal site (WI; band designation prefix WLC), B) Shallow water site (PF; band designation prefix PFC) and C) Deep water site (ND; band designation prefix NDC). Depth of sediment below the surface is given above each lane. There were 9, 10 and 17 depth samples from the intertidal, shallow water and deep water sites, respectively. M represents marker lanes made from type strains amplified using the Muyzer et al (1993) primers. Bands of interest are labelled numerically and, together with band designation prefix, relate to Table 3.3 and Figure 3.5.

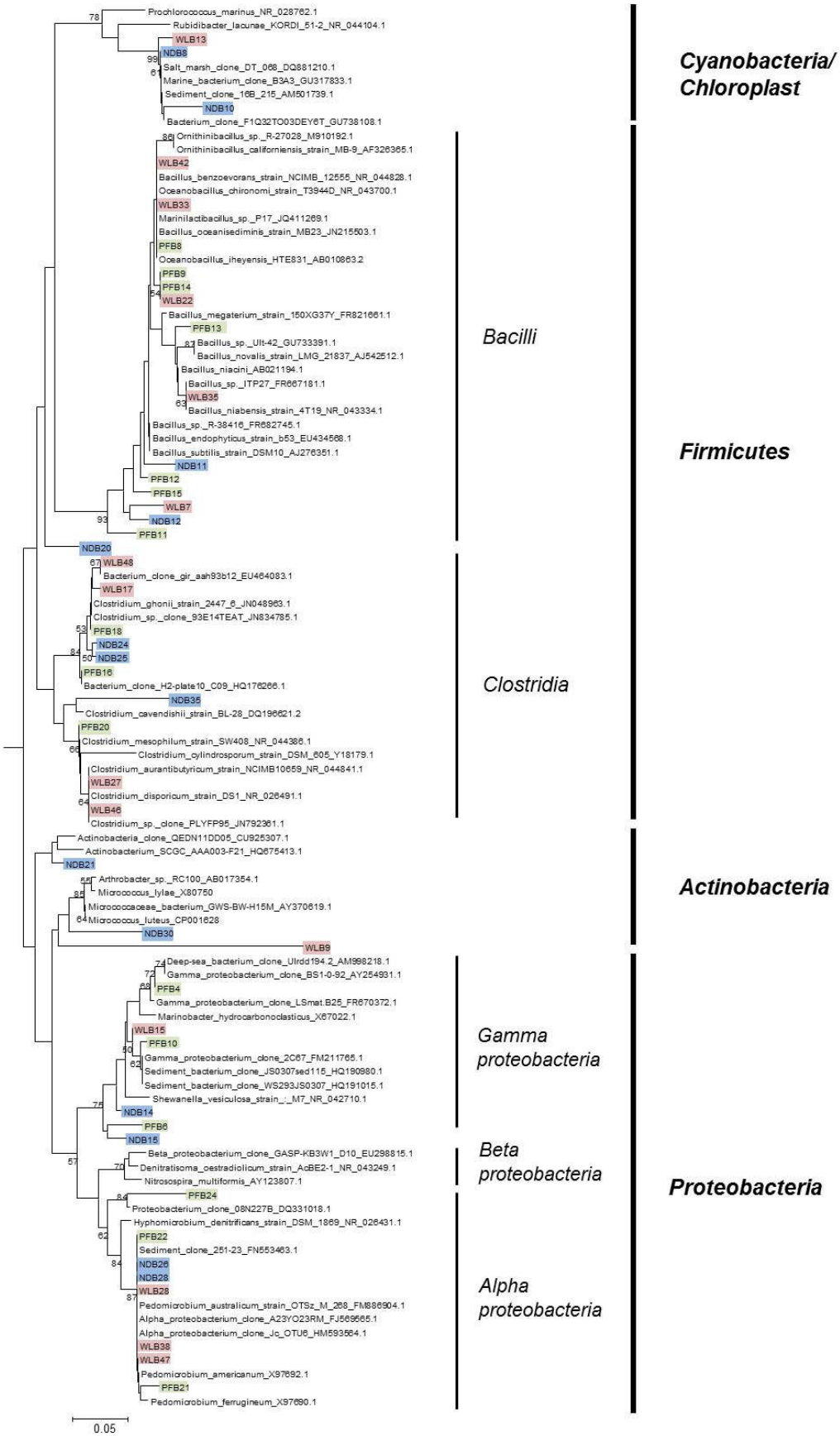


Figure 3.4 - Phylogenetic tree of bacterial sequences from DGGE bands. DGGE fragment designations (Table 3.2) are colour coded according to sites. Red – intertidal (WL); green - shallow water (PF); and blue – deep water site (ND). An out-group of *Thermotoga maritima* (M21774) was used. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

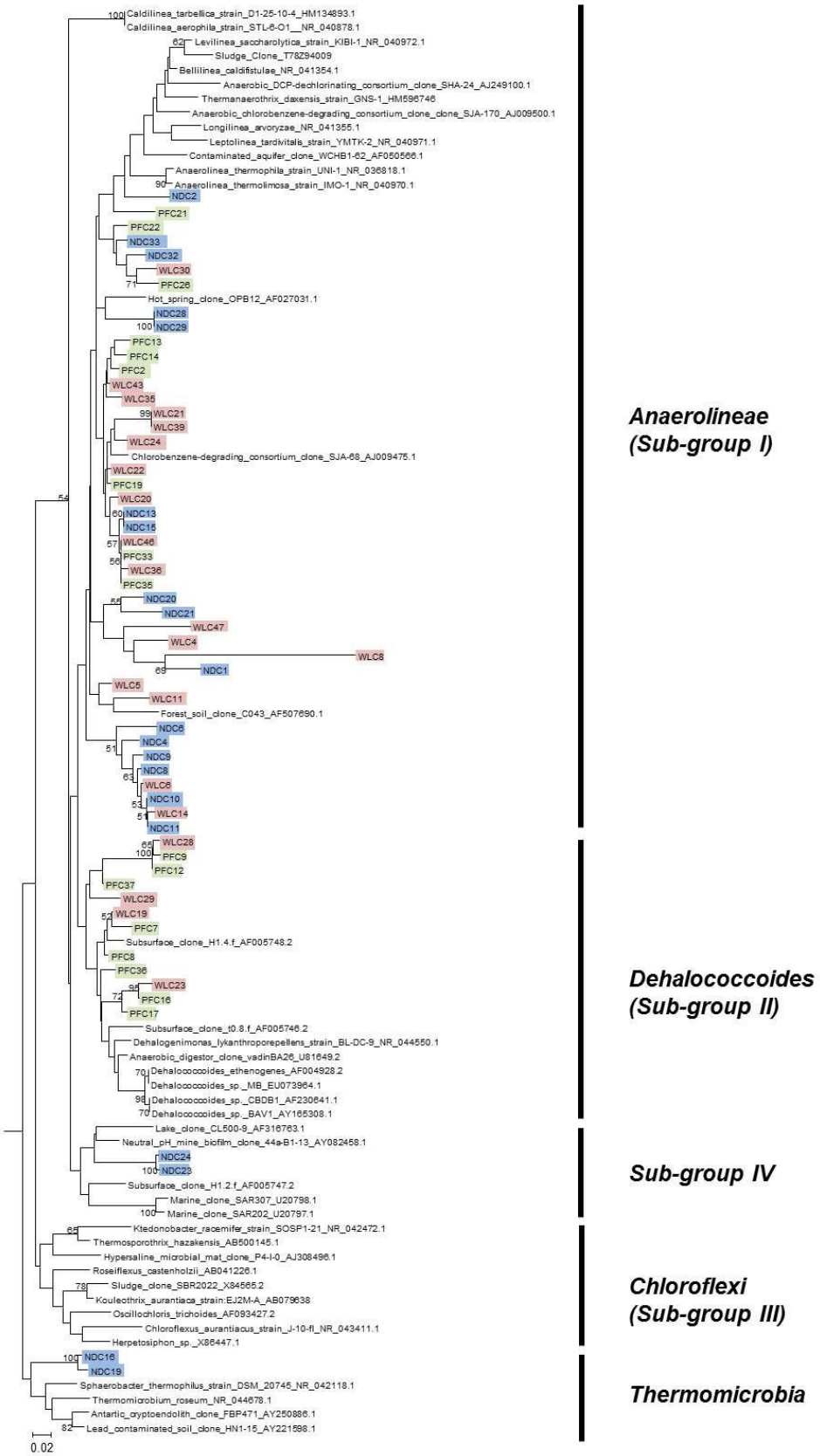


Figure 3.5 - Phylogenetic tree of *Chloroflexi* sequences from DGGE bands. DGGE fragment designations (Table 3.3) are colour coded according to sites. Red – intertidal (WL); green - shallow water (PF); and blue – deep water site (ND).An out-group of *Thermus aquaticus* (NR_025900) was used. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

Table 3.2 - Bands extracted from bacterial DGGE profiles of the intertidal, shallow water and deep water sites and their closest BLAST match.

Sampling Site	Band Identifier	Depth of Band (cbsf)	Closest Match by BLASTN Search (Accession Number)	Sequence Identity (%; 161bp)	Phylogenetic Affiliation of Band	Environment of Closest Match
Intertidal Site	WLB7	0-2	*Ornithinibacillus bavariensis* strain WSBC 24001 (NR_044923)	93	*Firmicutes*	Pasteurised milk
	WLB9	0-2	Clone GASP-KB3W1_D10 (EU298815)	77	*Actinobacteria*	Agricultural soil, Kansas. USA
	WLB13	2-4	Clone 16B_215 (AM501739)	98	*Chloroplast*	Lagoon sediment, Venice, Italy
	WLB15	4-6	Clone JS0307sed115 (HQ190980)	98	*Proteobacteria*	Intertidal sediment, German Wadden Sea
	WLB17	4-6	*Clostridium ghonii* strain 2447_6 (JN048963)	99	*Firmicutes*	Mountainous spring water
	WLB22	6-8	*Marinilactibacillus* sp. P17 (JQ411269)	97	*Firmicutes*	Sub-surface sediment, Juan de Fuca Ridge, Northeast Pacific
	WLB27	8-10	*Clostridium disporicum* strain DS1 (NR_026491)	99	*Firmicutes*	Rat cecum
	WLB28	8-10	*Alphaproteobacteria* clone A23YO23RM (FJ569565)	99	*Proteobacteria*	Alpine tundra soil, French southwestern Alps
	WLB35	12-14	*Oceanobacillus chironomi* strain T3944D (NR_043700)	99	*Firmicutes*	Chironomid egg mass
	WLB38	12-14	*Pedomicrobium australicum* strain OTSz_M_268 (FM886904)	99	*Proteobacteria*	Biofilm, Odertal National Park, Germany
	WLB42	14-16	*Oceanobacillus chironomi* strain T3944D (NR_043700)	99	*Firmicutes*	Chironomid egg mass
	WLB46	14-16	*Clostridium disporicum* strain DS1 (NR_026491)	99	*Firmicutes*	Rat cecum
	WLB47	16-18	*Pedomicrobium australicum* strain OTSz_M_268 (FM886904)	99	*Proteobacteria*	Biofilm, Odertal National Park

Sampling Site	Band Identifier	Depth of Band (cbsf)	Closest Match by BLASTN Search (Accession Number)	Sequence Identity (%; 161bp)	Phylogenetic Affiliation of Band	Environment of Closest Match
Shallow Water Site	WLB48	8-10	Clone gir_aah93b12 (EU464083)	100	*Firmicutes*	Mammalian gut
	PFB4	0-2	*Gammaproteobacterium* clone BS1-0-92 (AY254931)	99	*Proteobacteria*	Tidal sediment, Ganghwa Island, Republic of Korea
	PFB6	16-18	*Gammaproteobacterium* clone BS1-0-92 (AY254931)	95	*Proteobacteria*	Tidal sediment, Ganghwa Island, Korea
	PFB8	10-12	*Bacillus benzoevorans* strain NCIMB 12555 (NR_044828)	97	*Firmicutes*	Pasteurized soil
	PFB9	14-16	*Bacillus benzoevorans* strain NCIMB 12555 (NR_044828)	99	*Firmicutes*	Pasteurized soil
	PFB10	2-4	Sediment bacterium clone WS293JS0307 (HQ191015)	97	*Proteobacteria*	Intertidal sediment, German Wadden Sea
	PFB11	12-14	*Bacillus* sp. Ult-42 (GU733391)	93	*Firmicutes*	Fertilized soil, Uppsala, Sweden
	PFB12	8-10	*Bacillus oceanisediminis* strain MB23 (JN215503)	95	*Firmicutes*	Salt effected area of Eastern U.P
	PFB13	8-10	*Bacillus niacini* strain IFO15566 (NR_024695)	96	*Firmicutes*	Soil
	PFB14	18-20	*Marinilactibacillus* sp. P17 (JQ411269)	97	*Firmicutes*	Sub-surface sediment, Juan de Fuca Ridge, Northeast Pacific
	PFB15	18-20	*Bacillus* sp. ITP27 (FR667181)	95	*Firmicutes*	Agricultural Mediterranean soil
	PFB16	4-6	Bacterium clone H2-plate10_C09 (HQ176266)	100	*Firmicutes*	Human intestine
	PFB18	16-18	*Clostridium* sp. clone 93E14TEAT (JN834785)	100	*Firmicutes*	Cow teat skin
	PFB19	6-8	*Clostridium* sp. clone PLYFP95 (JN792361)	100	*Firmicutes*	Porpoise faecal material

Sampling Site	Band Identifier	Depth of Band (cbsf)	Closest Match by BLASTN Search (Accession Number)	Sequence Identity (%; 161bp)	Phylogenetic Affiliation of Band	Environment of Closest Match
Deep Water Site	PFB20	12-14	*Clostridium amylolyticum* strain SW408 (NR_044386)	100	*Firmicutes*	Upflow anaerobic sludge blanket reactor
	PFB21	2-4	*Pedomicrobium australicum* strain IFAM ST1306 (NR_026337)	98	*Proteobacteria*	Fresh water reservoir, Australia
	PFB22	18-20	Sediment clone 251-23 (FN553463)	99	*Proteobacteria*	Surface sediment, Mid Atlantic Ridge
	PFB24	14-16	*Proteobacterium* clone 08N227B (DQ331018)	95	*Proteobacteria*	Hypersaline microbial mat, Guerrero Negro, Mexico
	NDB8	10-12	*Cyanobacterium* clone DT_068 (DQ881210)	100	*Chloroplast*	Coastal ocean water, Sapelo Island, Georgia, USA
	NDB10	26-28	Bacterium clone F1Q32TO03DEY6T (GU738108)	98	*Chloroplast*	Water treatment plant, Seoul, South Korea
	NDB11	2-4	*Bacillus* sp. R-38416 (FR682745)	97	*Firmicutes*	Princess Elisabeth Station, East Antarctica
	NDB12	22-24	*Bacillus endophyticus* strain b53 (EU434568)	91	*Firmicutes*	Waste water treatment plant, Hebei Province, China
	NDB14	0-2	*Gammaproteobacterium* clone BS1-0-92 (AY254931)	98	*Proteobacteria*	Tidal sediment, Ganghwa Island, Republic of Korea
	NDB15	18-20	*Gammaproteobacterium* clone LSmat.B25 (FR670372)	92	*Proteobacteria*	Lucky strike hydrothermal vent, Mid Atlantic Ridge
	NDB20	6-8	*Actinobacteria* clone QEDN11DD05 (CU925307)	95	*Actinobacteria*	Anaerobic sludge digester
	NDB21	12-14	*Actinobacterium* SCGC AAA003-F21 (HQ675413)	97	*Actinobacteria*	Subtropical ocean gyre, South Atlantic
	NDB24	8-10	*Clostridium* sp. clone PLYFP95 (JN792361)	99	*Firmicutes*	Porpoise faecal material
	NDB25	20-22	*Clostridium* sp. clone PLYFP95 (JN792361)	99	*Firmicutes*	Porpoise faecal material

Sampling Site	Band Identifier	Depth of Band (cbsf)	Closest Match by BLASTN Search (Accession Number)	Sequence Identity (%; 161bp)	Phylogenetic Affiliation of Band	Environment of Closest Match
	NDB26	4-6	*Alphaproteobacterium* clone Jc_OTU6 (HM593564)	99	*Proteobacteria*	Community associated with *Terpios hoshinota* marine sponge
	NDB28	26-28	*Alphaproteobacterium* clone Jc_OTU6 (HM593564)	99	*Proteobacteria*	Community associated with *Terpios hoshinota* marine sponge
	NDB30	10-12	*Micrococcaceae* bacterium GWS-BW-H15M (AY370619)	90	*Actinobacteria*	Tidal flat sediment, German Wadden Sea
	NDB35	18-20	*Clostridium cavendishii* strain BL-28 (DQ196621)	87	*Firmicutes*	Contaminated ground water

Table 3.3 **-** Bands extracted from *Chloroflexi* DGGE profiles of the intertidal, shallow water and deep water sites and their closest BLAST match

Sampling Site	Band Identifier	Depth of Band (cbsf)	Closest Match by BLASTN Search (Accession Number)	Sequence Identity (%; 584 bp)	Phylogenetic Affiliation of Band	Environment of Closest Match
Intertidal Site	WLC4	0-2	*Chloroflexi* clone QEEB1BH07 (CU918316)	92	*Anaerolineae*	Anaerobic sludge digester
	WLC5	0-2	*Anaerolineae* sp. clone ZZ-S9A4 (EF613453)	96	*Anaerolineae*	Sand reactor column
	WLC6	0-2	*Chloroflexi* clone: K103 (AB116399)	99	*Anaerolineae*	Coastal marine sediment, Sanriku coast, Japan
	WLC8	0-2	*Chloroflexi* bacterium clone RUGL6-422 (GQ366653)	86	*Anaerolineae*	Soil from Roopkund Glacier, Himalayan mountain ranges, India
	WLC11	2-4	*Chloroflexi* clone: IODP1324B11H3.11, (AB448918)	96	*Anaerolineae*	Deep subseafloor sediment, Ursa Basin, Gulf of Mexico
	WLC14	4-6	*Chloroflexi* clone: K103 (AB116399)	99	*Anaerolineae*	Coastal marine sediment, Sanriku coast, Japan
	WLC19	6-8	Bacterium clone 30-B02 (AJ867602)	99	*Dehalococcoidia*	Subsurface sediment, Peru Margin
	WLC20	6-8	*Chloroflexaceae* clone BS1-0-122 (AY304374)	97	*Anaerolineae*	Tidal sediment, Ganghwa Island, Republic of Korea
	WLC21	6-8	*Chloroflexi* clone F08I1_INITIAL (GQ242335)	100	*Anaerolineae*	Tidal freshwater sediment, Altamaha River, Georgia, USA
	WLC22	6-8	Bacterium clone H05_S02A (AM911615)	98	*Anaerolineae*	Community associated with *Lophelia pertusa* coral
	WLC23	6-8	*Chloroflexi* clone: IODP1319B11.1 (AB433043)	99	*Dehalococcoidia*	Deep subseafloor sediment, Ursa Basin, Gulf of Mexico

Sampling Site	Band Identifier	Depth of Band (cbsf)	Closest Match by BLASTN Search (Accession Number)	Sequence Identity (%; 584 bp)	Phylogenetic Affiliation of Band	Environment of Closest Match
	WLC24	6-8	*Chloroflexi* bacterium clone: GH-54 (AB293361)	96	*Anaerolineae*	Anoxic rice field soil
	WLC28	8-10	*Chloroflexi* clone RAT24_23 (GU236098)	95	*Dehalococcoidia*	Contaminated soil, Ronneburg, Germany
	WLC29	8-10	Bacterium clone CFXSte7 (GU971207)	99	*Dehalococcoidia*	Marine sponge, New Zealand
	WLC30	8-10	*Thermomicrobia* clone GoM156_Bac65 (FN421261)	100	*Anaerolineae*	Marine sediment, Gulf of Mexico
	WLC35	12-14	*Chloroflexi* clone: IODP1319B11.21 (AB433048)	96	*Anaerolineae*	Deep subseafloor sediment, Ursa Basin, Gulf of Mexico
	WLC36	12-14	Bacterium clone H05_S02A (AM911615)	99	*Anaerolineae*	Community associated with *Lophelia pertusa* coral
	WLC39	14-16	*Chloroflexi* clone F08I1_INITIAL (GQ242335)	100	*Anaerolineae*	Tidal freshwater sediment, Altamaha River, Georgia, USA
	WLC43	14-16	Bacterium clone: AHH20B_102 (AB588701)	99	*Anaerolineae*	Freshwater sediment, Kanto Plain, Japan
	WLC46	16-18	*Chloroflexaceae* clone BS1-0-122 (AY304374)	100	*Anaerolineae*	Tidal sediment, Ganghwa Island, Republic of Korea
	WLC47	16-18	*Chloroflexi* clone: IODP1319B109.3 (AB433061)	90	*Anaerolineae*	Deep subseafloor sediment, Ursa Basin, Gulf of Mexico
Shallow Water Site	PFC2	2-4	Bacterium clone: AHH20B_116 (AB588711)	97	*Anaerolineae*	Freshwater sediment, Kanto Plain, Japan
	PFC7	0-2	*Chloroflexi* clone LCA-5B (JF305755)	96	*Dehalococcoidia*	Sand tailings, Mildred Lake Settling Basin, Alberta, Canada

Sampling Site	Band Identifier	Depth of Band (cbsf)	Closest Match by BLASTN Search (Accession Number)	Sequence Identity (%; 584 bp)	Phylogenetic Affiliation of Band	Environment of Closest Match
	PFC8	2-4	Bacterium clone 30-B02 (AJ867602)	98	*Dehalococcoidia*	Subsurface sediment, Peru Margin
	PFC9	4-6	Bacterium clone PTA-08 (EU826728)	95	*Dehalococcoidia*	RDX contaminated aquifer sediment,
	PFC12	16-18	*Chloroflexi* clone RAT24_23 (GU236098)	95	*Dehalococcoidia*	Contaminated soil, Ronneburg, Germany
	PFC13	4-6	*Chloroflexi* clone: IODP1324B2H5.22 (AB448868)	95	*Anaerolineae*	Deep subseafloor sediment, Ursa Basin, Gulf of Mexico
	PFC14	8-10	*Chloroflexi* clone: IODP1324B2H5.22 (AB448868)	98	*Anaerolineae*	Deep subseafloor sediment, Ursa Basin, Gulf of Mexico
	PFC16	0-2	Bacterium clone MB-A2-101 (AY093456)	99	*Dehalococcoidia*	Marine deep-subsurface sediment, Nankai Trough, Japan
	PFC17	2-4	Bacterium clone:OHKB2.14 (AB094801)	98	*Dehalococcoidia*	Subseafloor sediment, Sea of Okhotsk, Japan
	PFC19	2-4	Bacterium clone: AHH20B_116 (AB588711)	100	*Anaerolineae*	Freshwater sediment, Kanto Plain, Japan
	PFC21	18-20	*Chloroflexi* clone: IODP1324B2H2.54 (AB448847)	95	*Anaerolineae*	Deep subseafloor sediment, Ursa Basin, Gulf of Mexico
	PFC22	0-2	Bacterium clone AMSMV-10-B36 (HQ588506)	98	*Anaerolineae*	Mud volcano sediment, Mediterranean Sea
	PFC26	10-12	Bacterium clone AMSMV-10-B29 (HQ588504)	91	*Anaerolineae*	Mud volcano sediment, Mediterranean Sea
	PFC33	6-8	Bacterium clone H05_S02A (AM911615)	100	*Anaerolineae*	Community associated with *Lophelia pertusa* coral

Sampling Site	Band Identifier	Depth of Band (cbsf)	Closest Match by BLASTN Search (Accession Number)	Sequence Identity (%; 584 bp)	Phylogenetic Affiliation of Band	Environment of Closest Match
Deep Water Site	PFC35	16-18	Bacterium clone H05_S02A (AM911615)	100	*Anaerolineae*	Community associated with *Lophelia pertusa* coral
	PFC36	0-2	*Chloroflexi* clone: IODP1324B53X3.4 (AB448928)	96	*Dehalococcoidia*	Deep subseafloor sediment, Ursa Basin, Gulf of Mexico
	PFC37	2-4	*Chloroflexi* clone slm_bac_1101 (HQ711385)	95	*Dehalococcoidia*	Biofilms on methane bearing marine sediment
	NDC1	2-4	Bacterium clone: AHH20B_102 (AB588701)	94	*Anaerolineae*	Freshwater sediment, Kanto Plain, Japan
	NDC2	8-10	Bacterium clone P9X2b3A11 (EU491096)	98	*Anaerolineae*	Seafloor lavas, Loi'hi Seamount, Hawaii
	NDC4	4-6	Bacterium clone 2FSeds_D02 (GQ412930)	96	*Anaerolineae*	Marine sediment, Bolinao, Philippines
	NDC6	22-24	Bacterium clone 1NSeds_E08 (GQ412852)	100	*Anaerolineae*	Marine sediment, Bolinao, Philippines
	NDC8	14-16	Bacterium clone 34MIC074 (JF341316)	99	*Anaerolineae*	Concrete sewer biofilm
	NDC9	24-26	Bacterium clone F2_130X (GQ263006)	99	*Anaerolineae*	Simulated low-level radioactive waste site
	NDC10	6-8	Bacterium clone sediment_deep17 (GQ259281)	100	*Anaerolineae*	Deep sediment, Svalbard, Arctic Ocean
	NDC11	18-20	Bacterium clone sediment_deep17 (GQ259281)	100	*Anaerolineae*	Deep sediment, Svalbard, Arctic Ocean
	NDC13	2-4	Bacterium clone H05_S02A (AM911615)	95	*Anaerolineae*	Community associated with *Lophelia pertusa* coral

Sampling Site	Band Identifier	Depth of Band (cbsf)	Closest Match by BLASTN Search (Accession Number)	Sequence Identity (%; 584 bp)	Phylogenetic Affiliation of Band	Environment of Closest Match
	NDC15	24-26	*Chloroflexaceae* clone BS1-0-122 (AY304374)	99	*Anaerolineae*	Tidal sediment, Ganghwa Island, Republic of Korea
	NDC16	4-6	*Chloroflexi* clone Alchichica_AL67_2_1B_187 (JN825472)	94	*Thermomicrobia*	Alkaline lake sediment, Alchichica, Mexico
	NDC19	32-34	*Chloroflexi* clone Alchichica_AL67_2_1B_187 (JN825472)	91	*Thermomicrobia*	Alkaline lake sediment, Alchichica, Mexico
	NDC20	0-2	*Chloroflexi* bacterium clone LC1-24 (DQ289898)	96	*Anaerolineae*	Permeable shelf sediment, South Atlantic Bight
	NDC21	8-10	*Chloroflexaceae* clone BS1-0-122 (AY304374)	95	*Anaerolineae*	Tidal sediment, Ganghwa Island, Republic of Korea
	NDC23	30-32	*Chloroflexi* clone SHBH1146 (GQ350778)	99	*Subdivision IV*	Oxygen minimum zone, Saanich Inlet, Canada
	NDC24	32-34	*Chloroflexi* clone SHBH1146 (GQ350778)	99	*Subdivision IV*	Oxygen minimum zone, Saanich Inlet, Canada
	NDC28	2-4	*Chloroflexi* clone MS-A135 (FJ949426)	96	*Anaerolineae*	Coastal marine sediment, Mallorca, Spain
	NDC29	16-18	*Chloroflexi* clone MS-A135 (FJ949426)	97	*Anaerolineae*	Coastal marine sediment, Mallorca, Spain
	NDC32	8-10	Bacterium clone B050B06 (FJ455904)	99	*Anaerolineae*	Continental margin sediment, Santa Barbara Basin, California
	NDC33	8-10	*Chloroflexi* clone: K54 (AB116396)	96	*Anaerolineae*	Coastal marine sediment, Sanriku coast, Japan

appear immediately from 0-2 cbsf at the shallow water site. Though PCR-DGGE was not quantitative, the *Anaerolineae* appeared to be very abundant all sites, with most sequences being attributed to this subdivision.

During analysis of *Chloroflexi* sequences, a small number of *Firmicutes* related sequences were found. This was also reported in a study with the same primer (941R) by Yamada et al. (2007b). This was due to a high sequence similarity of *Firmicutes* and *Chloroflexi* in the V6 region of the 16S rRNA gene that the primers target. The DGGE image may therefore overestimate the abundance of *Chloroflexi* and so more specific techniques were required for quantification.

3.3.2 RISA Community Profiles

RISA profiling was performed on all sediment depths at the three sites. This enabled comparison of community diversity between as well as within the sites using cluster analysis (Figure 3.6). There was more similarity in the bacterial community within the sites than between the sites as the samples from each site clustered together. The only exception being the intertidal site 6-8 cbsf sample, which clustered with the shallow water site 0-4 cbsf sample with 95% similarity. There was some depth profiling at the intertidal and shallow water sites. The intertidal samples 0-6 cbsf clustered together with 96.5%, away from all other samples. The top and bottom two samples of the shallow water site clustered together separately from other shallow water samples. Whereas deep water site samples showed no difference with depth and a similarity of 98.5% between all samples.

The *Chloroflexi* RISA cluster analysis indicated an overall similarity of 64% between all three sites (Figure 3.6B). This suggested more sample to sample variation and therefore depth variation within the *Chloroflexi* community. In fact the *Chloroflexi* cluster analysis had a distinct depth profile in both the intertidal and shallow water sites. The upper 0-8 cbsf sediment from the intertidal site distinctly clustered together with 94% similarity with the middle 4-16 cbsf shallow water samples. These were separated from the rest of the

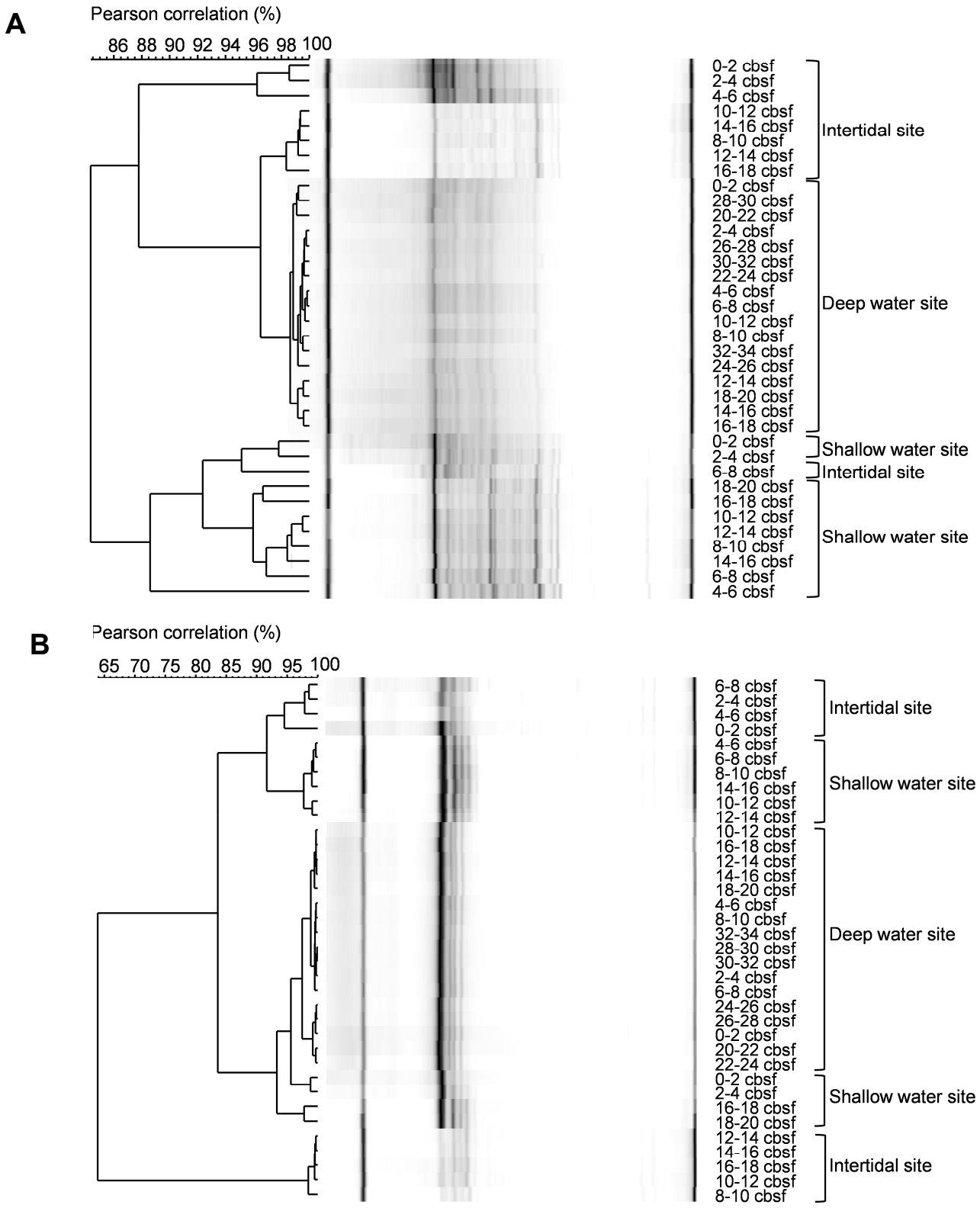


Figure 3.6 - Cluster analysis of A, *Bacteria* and B, *Chloroflexi* RISA community profiles from all three sites. There were 9, 10 and 17 depth samples from the intertidal, shallow water and deep water sites, respectively. Cluster analysis was performed using Pearson correlation coefficient and UPGMA and the dendrogram created with the GelCompar II software (version 6.5; Applied Maths). Scale of percentage similarity is given next to each dendrogram and depth of sediment is given next to each sample lane. Note the difference in scale for each dendrogram.

intertidal and shallow water site samples. The lower intertidal site samples 8-18 cbsf had 98% similarity to each other but 64% similarity to all other sites. The shallow water samples 0-2 cbsf and 16-20 cbsf again together separately, away from other shallow water samples. Again, the deep water site samples show no difference with depth, with a similarity of 97% between all deep water samples.

As discussed in **Section 2.3.3,** caution must be taken with the interpretation of cluster analysis of both DGGE and RISA profiles as slight misalignment of the gel lanes by the software may introduce false microdiversity between samples. Though attempts were made to avoid this by normalising each profile to a standard banding pattern using a DNA ladder.

3.3.3 Statistical Comparison of Bacterial Community Profiles with Depth

Cluster analysis compared bacterial and *Chloroflexi* DGGE band patterns with depth at each site as this method couldn't be applied between gels and therefore sites (Figure 3.7). Cluster analysis supports the previous description of the sites. The intertidal site had the lowest overall similarity between samples for both *Bacteria* and *Chloroflexi* (75% and 84% respectively; Figure 3.7A and 3.7D). A change within both communities at 4-6 cbsf at the intertidal site was also suggested by the branching pattern of the cluster analysis. The bacterial and *Chloroflexi* communities at the shallow water site and deep water site had much higher overall similarity, suggesting a greater diversity with depth within the intertidal community (Figure 3.7B, C, E, and F).

NMDS plots were created to assess the relatedness between depths and the effect of measured sediment geochemistry on the depth profile (Figure 3.8). It should be recognised that these communities may be influenced by geochemical concentrations, not measured in this experiment (e.g. dissolved organic carbon or oxygen). The intertidal site NMDS again indicated that the bacterial community was separated at 6 cbsf but also that 16-18 cbsf was also separate (Figure 3.8A). Depths 0-6 cbsf appeared to be related to sulphate levels whereas 16-18 cbsf was influenced by ammonium

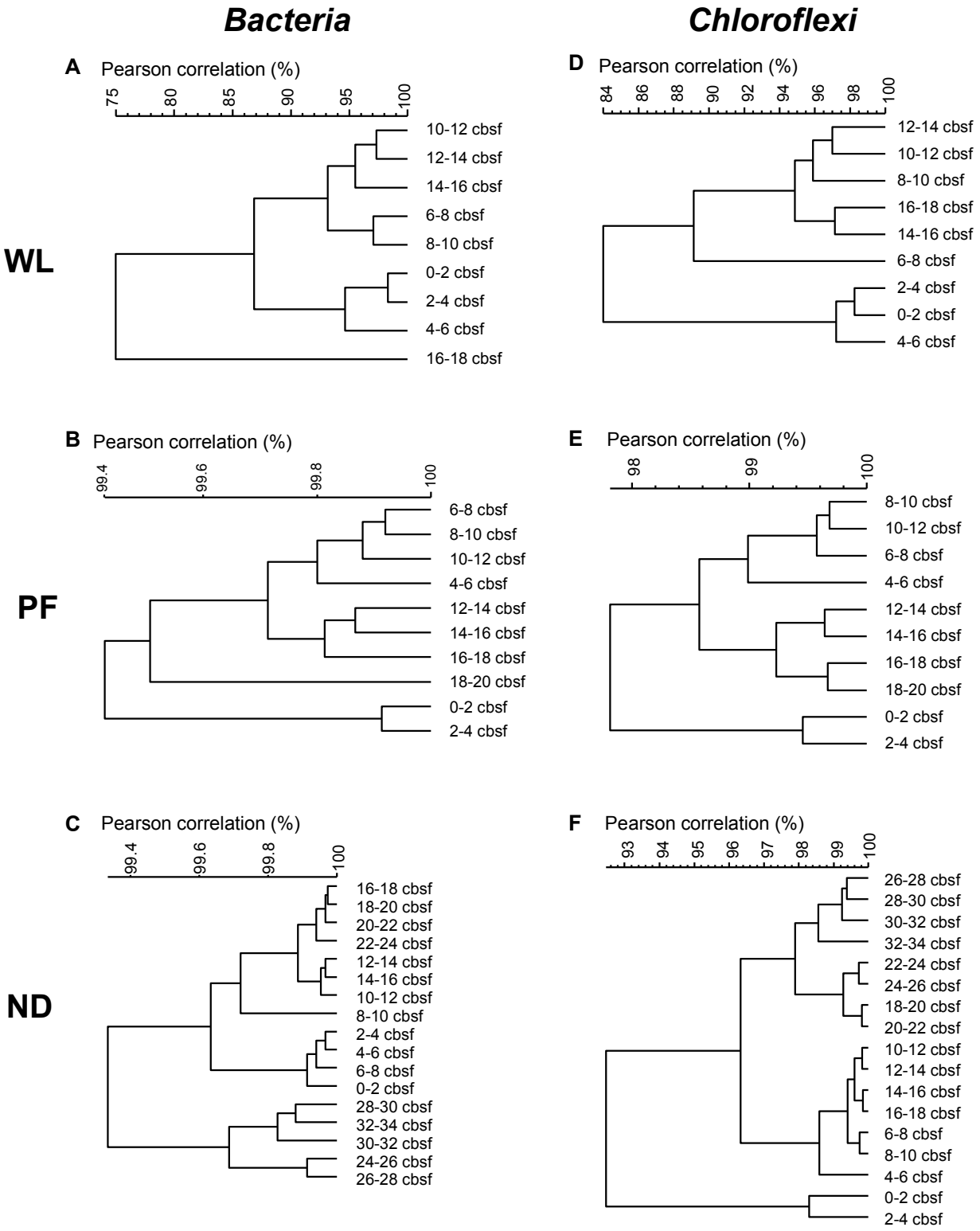


Figure 3.7 - Cluster analysis dendrograms of bacterial and *Chloroflexi* DGGE community profiles. A, Intertidal site *Bacteria* (WL); B, Shallow water site *Bacteria* (PF); C, Deep water site *Bacteria* (ND); D, Intertidal site *Chloroflexi* (WL); E, Shallow water site *Chloroflexi* (PF); and F, Deep water site *Chloroflexi* (ND). There were 9, 10 and 17 depth samples from the intertidal, shallow water and deep water sites, respectively. Cluster analysis dendrograms using Pearson correlation coefficient and UPGMA were constructed with the GelCompar II software (version 6.5; Applied Maths). Scales of percentage similarity are given next to each dendrogram and depth of sediment is given next to branch. Note the difference in scales for each dendrogram, particularly for the intertidal site (WL).

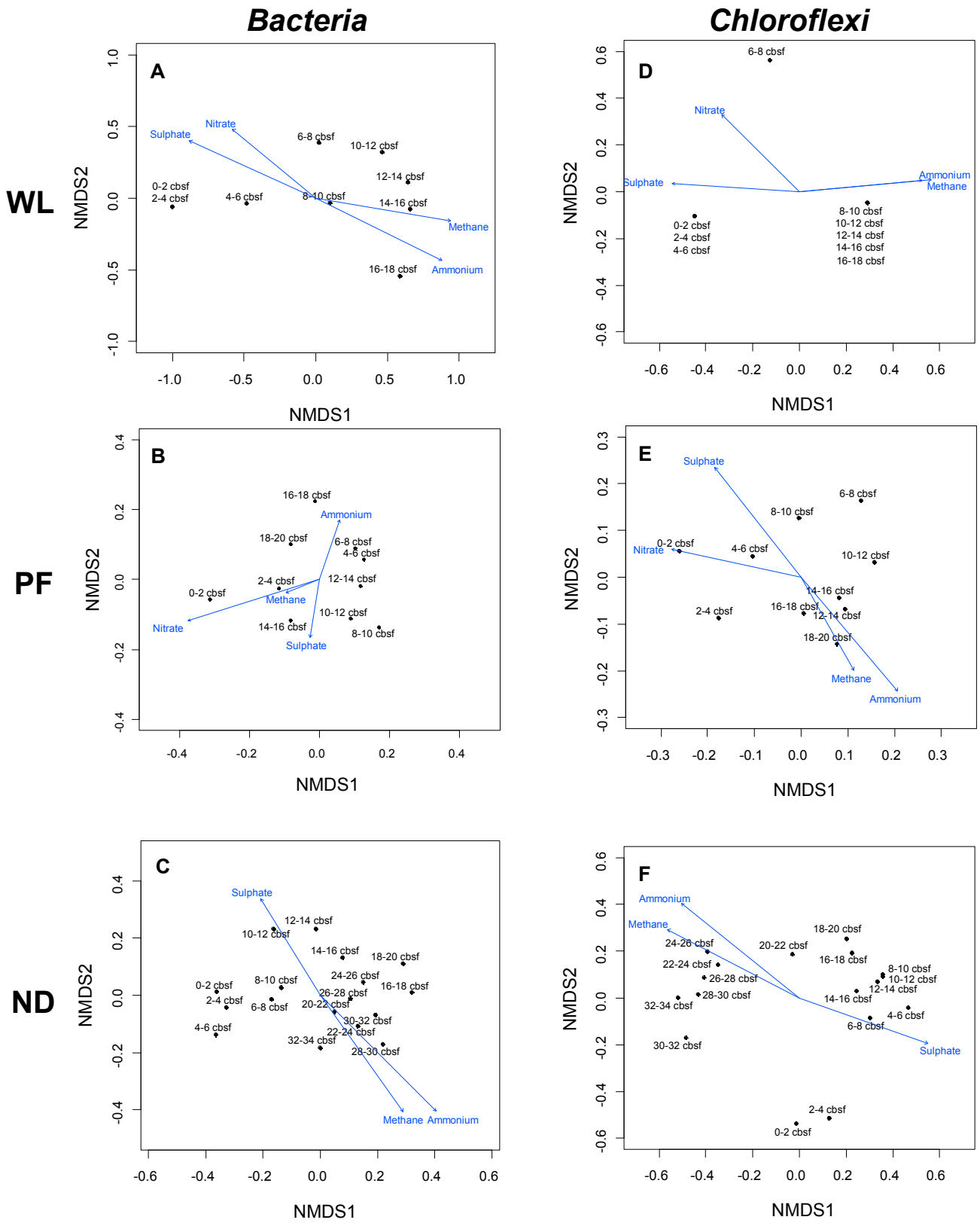


Figure 3.8 - NMDS analysis of bacterial and Chloroflexi DGGE community profiles. A, Intertidal site *Bacteria* (WL); B, Shallow water site *Bacteria* (PF); C, Deep water site *Bacteria* (ND); D, Intertidal site *Chloroflexi* (WL); E, Shallow water site *Chloroflexi* (PF); and F, Deep water site *Chloroflexi* (ND). There were 9, 10 and 17 depth samples from the intertidal, shallow water and deep water sites, respectively and sample depth is given next to each sample point. Blue arrows are sample vectors, representing geochemistry at each site. NMDS was performed using the R statistical software (Ihaka and Gentleman, 1996).

concentrations. The *Chloroflexi* intertidal site NMDS displays a larger distinction between the upper and lower sediment depths with three distinct groups of samples (Figure 3.8D). Again depths 0-6 cbsf appeared to be related to sulphate levels whereas ammonium concentrations influenced 8-18 cbsf. However ANOVA of each site indicated no significant difference between the means by depth for *Bacteria* and *Chloroflexi* (P value <<0.05 and P value = 1, respectively).

The shallow water NMDS indicated the top 0-4 cbsf were influenced by nitrate whereas the deepest samples were influenced by ammonium and sulphate (Figure 3.8B). The *Chloroflexi* community appeared to be drawn apart by nitrate in the shallow sediment and methane and ammonium in the deeper sediment (Figure 3.8E). This was a very similar pattern to the intertidal site but less clear. The deep water site *Bacteria* NMDS appeared more mixed than the previous sites, but a connection between surface sediment and sulphate and deeper sediment and methane and ammonium could be seen (Figure 3.8C). The *Chloroflexi* NMDS showed a similar pattern except samples 0-4 cbsf were entirely withdrawn from all other samples (Figure 3.8F). This suggested that the *Chloroflexi* in surface sediment were under the influence of unknown geochemical variables, not measured in this experiment (e.g. dissolved organic carbon or oxygen). However ANOVA of each site indicated no significant difference between the means by depth for *Bacteria* and *Chloroflexi* at shallow and deep water sites (P value = 1 for all).

Pearson's correlation was utilised to compare phylotype presence/absence with depth and geochemistry in both bacterial and *Chloroflexi* DGGEs (Table 3.4). At the intertidal site, correlations indicated *Actinobacteria*, *Alphaproteobacteria* and *Gammaproteobacteria* phylotypes declined significantly with depth. *Firmicutes* of the classes *Bacilli* and *Clostridia* were positively correlated with depth. In the *Chloroflexi* profile *Anaerolineae* were found to be significantly positively correlated with depth. Conversely, the *Dehalococcoidia* were found to not be significantly correlated with depth. No phylotypes tested were significantly correlated with depth in the shallow water bacterial profile. Of the *Chloroflexi* profile only *Dehalococcoidia* were

Table 3.4 - Correlation of phylotypes from bacterial and *Chloroflexi* DGGEs with sediment depth and geochemical measurements at each site. Pearson's correlation was performed using Microsoft Excel 2010. P values were derived using a correlation coefficients table (Fry and Iles, 1994). Not all bands were sequenced from DGGE gels thus this is not a true representation of the whole bacterial community.

Phylotype	**Depth**			**Sulphate**			**Nitrate**			**Ammonium**			**Methane**		
	I[a]	S	D	I	S	D	I	S	D	I	S	D	I	S	D
Actinobacteria	-0.91*	-	-0.66*	0.95*	-	0.56*	0.57	-	-	-0.91*	-	-0.61*	-0.94*	-	-0.52*
Cyanobacteria/ Chloroplast	-0.85*	-	0.84*	0.77*	-	-0.58*	0.77*	-	-	-0.89*	-	0.78*	-0.79*	-	0.62*
Alphaproteobacteria	-0.94*	-0.01	-0.29	0.93*	0.09	0.13	0.64	-0.45	-	-0.95*	-0.06	-0.34	-0.87*	0.00	-0.29
Gammaproteobacteria	-0.84*	-0.25	0.50*	0.87*	0.20	-0.37	0.60	-0.43	-	-0.89*	-0.30	0.50*	-0.92*	-0.24	0.56*
Bacilli	0.68*	-0.44	0.22	-0.70*	0.50	-0.23	-0.79*	0.41	-	0.73*	-0.44	0.17	0.69	-0.22	0.16
Clostridia	0.75*	0.59	0.65*	-0.71*	-0.61	-0.33	-0.58*	-0.82*	-	0.73*	0.54	0.59*	-0.72*	0.35	0.47
Anaerolineae	0.84*	-0.18	0.17	-0.82*	-0.63	-0.13	-0.87*	-0.04	-	0.80*	-0.18	0.24	0.74*	0.02	0.25
Dehalococcoidia	0.31	-0.66*	-	-0.37*	0.06	-	-0.20	0.01	-	0.30	-0.67*	-	0.44	-0.45	-
Subgroup IV	-	-	0.46	-	-	-0.23	-	-	-	-	-	0.39	-	-	0.29
Thermomicrobia	-	-	0.64*	-	-	-0.76*	-	-	-	-	-	-0.62*	-	-	0.68*

[a] I – Intertidal site (WL); S – Shallow water site (PF); and D – Deep water site (ND)
* Indicates P value was significant at <0.05 and also highlighted in green

found to be negatively and significantly ($P < 0.05$) correlated with depth. At the deep water site, A*ctinobacteria* were found to be significantly increasing whereas *Cyanobacteria/*chloroplasts, *Gammaproteobacteria* and *Clostridia* were significantly increasing with depth. Of the three *Chloroflexi* subdivisions detected at the deep water site, only the *Thermomicrobia* increased significantly with depth.

Geochemical correlations gave some evidence toward what microbial processes could be occurring at each site (Table 3.4). The strongest significant correlations with geochemistry were seen at the intertidal site. There was very little correlation between any of the phylotypes and decreasing sulphate concentrations at either the shallow or deep water sites. However, the *Bacilli, Clostridia, Anaerolineae* and *Dehalococcoidia* appeared to significantly increase as sulphate decreased. Nitrate decreased and ammonium increased, respectively, with depth at each site. Whereas in the surface sediment *Alphaproteobacteria* and *Gammaproteobacteria*, which include known ammonia oxidisers, significantly decreased as ammonium increased. Methane concentrations were very low and stable at both the intertidal and deep water sites. However a peak was seen at 15 cbsf at the shallow water site, though none of the phylotypes analysed correlated with methane.

As noted in Table 3.4, not all DGGE bands were extracted and sequenced, therefore, the correlation of bacterial phylotypes with geochemical variables must be treated with caution. There were other geochemical factors contributing to this environment (e.g. dissolved organic carbon and oxygen) that were not measured and also other, un-sequenced, bacteria were likely to be involved in geochemical processes in the sediment community. Thus this analysis is not a true representation of the whole prokaryotic community.

3.3.4. Specificity of Chloroflexi *and Subdivision Targeted qPCR Primers*

The *Chloroflexi* targeted 16S rRNA gene primers 941F and 1340R (Table 3.1; Gich et al., 2002) selected for q-PCR, were previously shown to amplify *Clostridia* DNA. A gradient PCR ($50^{o}C$-$60^{o}C$) with a range of bacterial DNA templates, including *A. thermophila,* and *Clostridia* strains AS4C and AS17,

was performed to optimise conditions for more targeted amplification of *Chloroflexi*. Results (not shown) indicated *Clostridia* DNA was amplified at temperatures below 59^{o}C. Through amplification of a range of DNA, including various *Chloroflexi* species, *Clostridia* and *Pseudomonas putida* at 60^{o}C*,* only *Chloroflexi* and sediment DNA were amplifiable.

The specificity of *Anaerolineae/Caldilineae* and *Dehalococcoidia* targeted primers was tested in a similar way to the *Chloroflexi* targeted primers. Gradient PCRs (50^{o}C-65^{o}C) were performed with both primer sets to target *Chloroflexi* DNA, non-target *Chloroflexi* DNA and *Clostridia.* The highest possible annealing temperature was chosen that excluded non-target DNA (63^{o}C for *Anaerolineae/Caldilineae* primers and 55^{o}C for *Dehalococcoidia*). The primers were tested at these temperatures with target, non-target and sediment DNA. Clone libraries of specific sediment depths were constructed using the *Chloroflexi*, *Anaerolineae/Caldilineae* and *Dehalococcoidia* primers to verify specificity. The total number of sequenced clones for each primer set were 78 for *Chloroflexi*, 83 for *Anaerolineae/Caldilineae* and 79 for *Dehalococcoidia*. *Chloroflexi* primers detected *Anaerolineae*, *Caldilineae*, *Dehalococcoidia*, and *Thermomicrobia* (Figure 3.9). The *Anaerolineae/Caldilineae* primers showed good specificity (total 88%) for the *Anaerolineae* (53%) and *Caldilineae* (35%; Figure 3.9). However *Dehalococcoidia*, Subdivision IV and one unidentifiable clone were amplified, suggesting these primers were not truly specific to Subdivision I, which is unsurprising due to the sequence similarity within these subdivisions. The specificity of these primers could be improved with the use of additional, recently published sequences to eliminate the other *Chloroflexi* subdivisions. 93% of the sequences retrieved using the *Dehalococcoidia* primers were *Dehalococcoidia* (Figure 3.9) indicating excellent specificity. Other subdivisions detected were *Anaerolineae, Thermomicrobia* and *Acetobacteria*.

3.3.5 Quantification of Prokaryotic Community at Intertidal Site by qPCR

Bacterial cell counts were estimated using qPCR for each sediment depth at each site (Figure 3.10). The general trend was decreasing with depth..

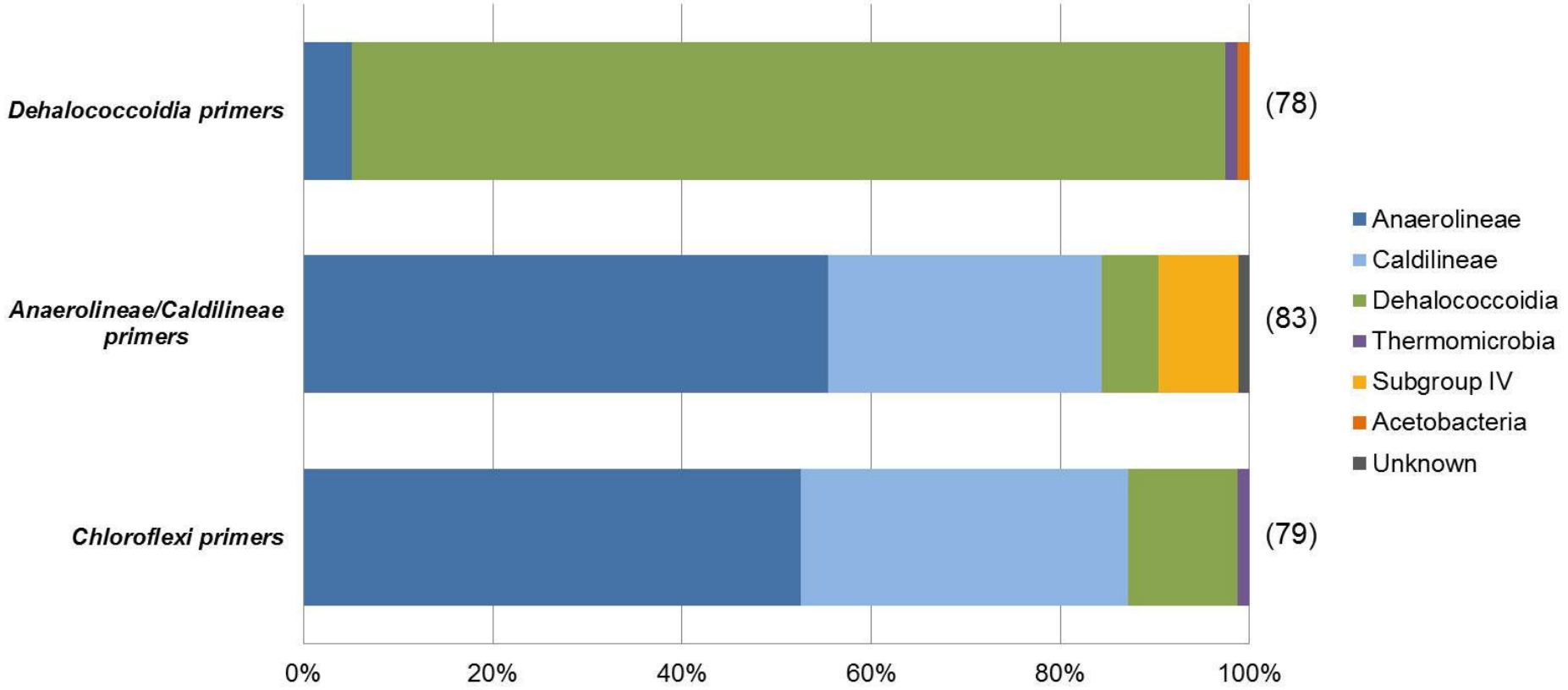


Figure 3.9 - Specificity of the three tested primer sets, *Chloroflexi* (Gich et al., 2002), *Anaerolineae/Caldilineae* (this study) and *Dehalococcoidia* (this study). Percentages represent clone library sequences produced from each primer set using relevant Severn Estuary sediment samples. Total numbers of clones sequenced are given in brackets from clone libraries constructed from 4 samples from the intertidal (0-2 cbsf and 16-14 cbsf), shallow water (8-10 cbsf) and deep water (16-18 cbsf) sites.

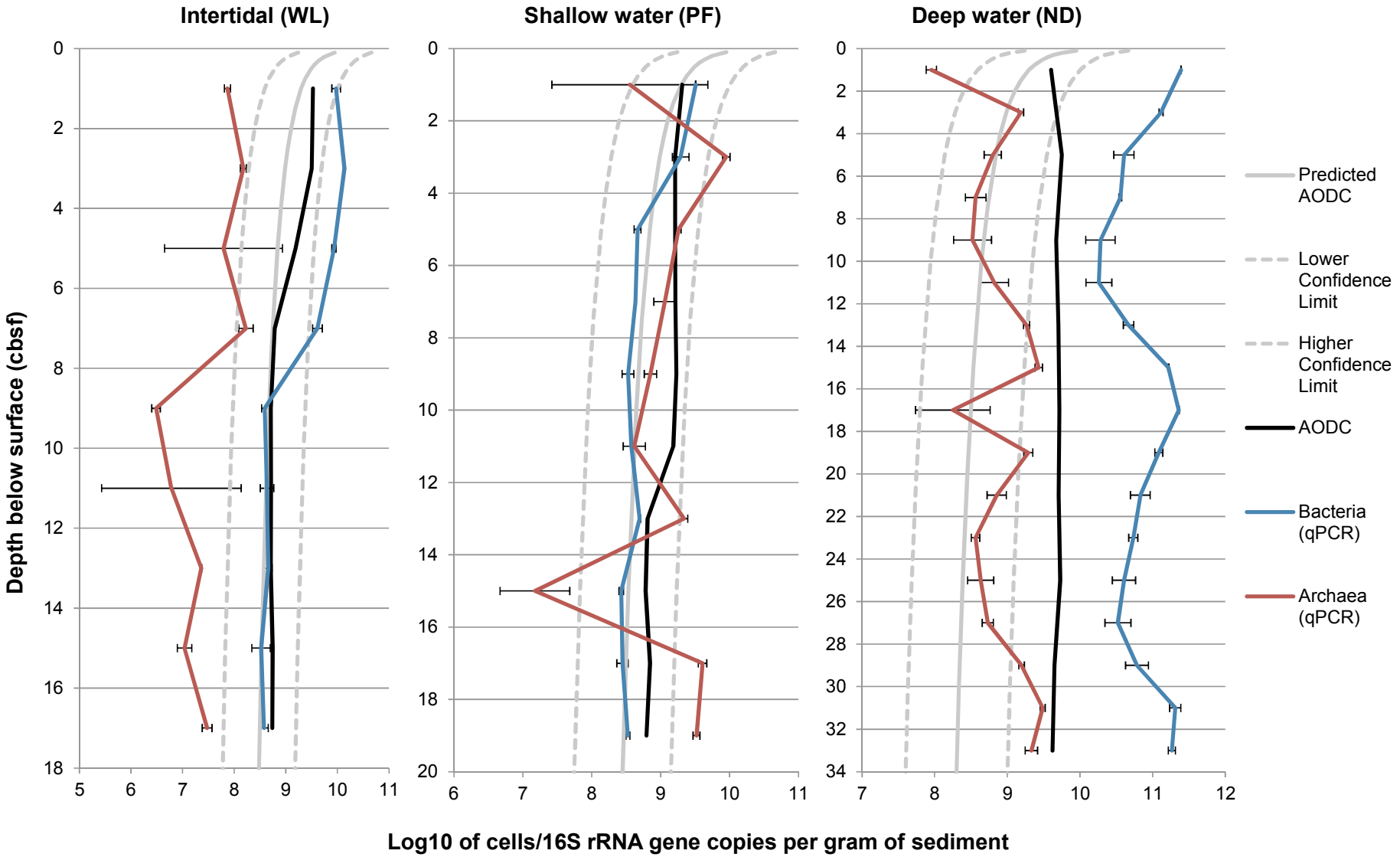


Figure 3.10 - Depth profiles of *Bacteria, Archaea* and Total Prokaryote cell numbers by AODC and qPCR at the three sampling sites: Intertidal site, Shallow water site and Deep water site. There were 9, 10 and 17 depth samples from the intertidal, shallow water and deep water sites, respectively. Predicted AODC and 95% confidence limits from marine sediments general model are shown in grey from (Parkes et al., 2000). Standard error bars were calculated using standard deviation of 3 technical replicates.

Bacterial qPCR counts at the intertidal site began at ~10^9-10^{10} cells/g of sediment from 0-8 cbsf which decreased to ~10^8 cells/g of sediment from 8-18 cbsf (Figure 3.10). The *Bacteria* were estimated to be at least one order of magnitude larger than *Archaea,* reaching up to two orders of magnitude from 0-6 cbsf. Archaeal qPCR counts were more variable than the bacterial, dropping from 10^7-10^8 cells/g of sediment to 10^6-10^7 cells/g of sediment after 8 cbsf. The q-PCR data followed the AODC (provided by Shaun Thomas, Cardiff University). AODC estimated ~10^9 cells/g of sediment for 0-8 cbsf which then decreased to ~10^8 cells/g of sediment from 8-18 cbsf. This trend was higher than predicted AODC up to 8 cbsf and followed it exactly from 8-18 cbsf. The AODC values were approximately an order of magnitude lower than the bacterial qPCR values from 0-8 cbsf.

qPCR indicated that the *Chloroflexi* community was a notable portion of the overall bacterial community at the intertidal site at approximately 10^7-10^8 cells/g of sediment (Figure 3.11). The percentage of *Chloroflexi* increased with sediment depth, between 0 and 6 cbsf to account for 2% of the total bacterial community, which increased to 8-13% from 6-18 cbsf. This was opposite to the trend in the *Bacteria* which saw a drop in bacterial cell counts after 6 cbsf, suggesting that the *Chloroflexi* community numbers remained constant despite drop in overall bacterial numbers. The highest percentage of *Chloroflexi* was detected between 8-12 cbsf. The *Anaerolineae/Caldilineae* were the largest subdivision of the *Chloroflexi* with ~10^7-10^8 cells/g of sediment, mirroring the total *Chloroflexi* trend (Figure 3.11). The *Dehalococcoidia* were detected at much lower numbers than the *Anaerolineae/Caldilineae*, ranging from ~10^5-10^6 cells/g of sediment.

3.3.6 Quantification of Prokaryotic Community at Shallow Water Site by qPCR

Archaea numbers were over all higher than *Bacteria* (Figure 3.10). Bacterial qPCR counts were ~10^9 cells/g of sediment from 0-4 cbsf, decreasing slightly to 10^8 cells/g of sediment from 6-20 cbsf. Again there was an overall decreasing trend with depth. The *Archaea* qPCR counts were more variable with depth. After peaking at 2-4 cbsf at 10^9 cells/g of sediment, there was a

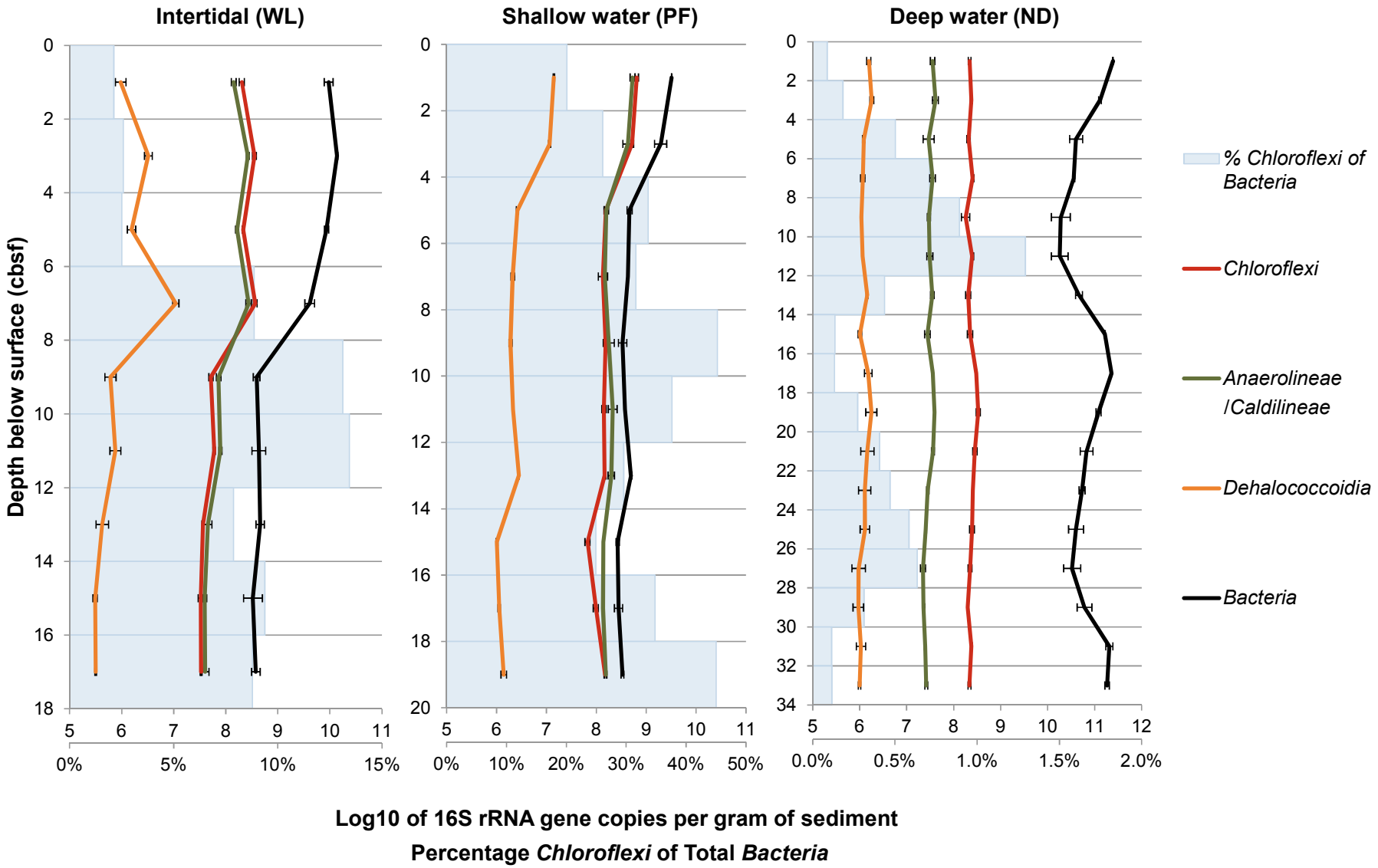


Figure 3.11 - Depth profiles of *Bacteria, Chloroflexi, Anaerolineae/Caldilineae* and *Dehalococcoidia* cell numbers by qPCR at the three sampling sites: Intertidal site, Shallow water site and Deep water site. Percentage *Chloroflexi* of total *Bacteria* with depth is shown with grey bars. There were 9, 10 and 17 depth samples from the intertidal, shallow water and deep water sites, respectively. Standard error bars were calculated using standard deviation of 3 technical replicates. Clone libraries indicated that 88% of sequences amplified by the *Anaerolineae/Caldilineae* primers were related to Subdivision I and so the primers were not as specific as *in vivo* as expected.

steady decline to 10^8 cells/g of sediment. At 12-14 cbsf there was another small peak of 10^9 cells/g of sediment and then a large drop to 10^7 cells/g of sediment from 14-16 cbsf, with counts returning to 10^9 cells/g of sediment from 16-20 cbsf. From 0-12 cbsf the AODC values remained at 10^9 cells/g of sediment and then decreased slightly to 10^8 cells/g of sediment after 12 cbsf. The AODC values corresponded well with the bacterial and Archaeal qPCR counts.

The *Chloroflexi* were found to be a major proportion of the bacterial community at the shallow water site with counts in the order of 10^8 cells/g of sediment (Figure 3.11). The *Chloroflexi* community accounted for 20-45% of the bacterial counts which was the highest percentage of all three sites. This was reminiscent to previously described deep subsurface sediment such as the Pacific Peru margin (Webster et al., 2006), The Gulf of Mexico (Nunoura et al., 2009) and South Pacific gyres (Durbin and Teske, 2011). The percentage of *Chloroflexi* fluctuated throughout the depth profile, with a general increasing trend with depth. The highest percentages (45%) were detected at 8-10 cbsf and 18-20 cbsf. The lowest percentages (20%) were found at 0-2 cbsf and 14-16 cbsf. Again, the *Anaerolineae/Caldilineae* were the most abundant subdivision with approximately 10^8 cells/g of sediment which mirrored the total number of *Chloroflexi.* The *Dehalococcoidia* were detected at approximately 10^6 cells/g of sediment, much lower than that of the *Anaerolineae/Caldilineae* (Figure 3.11).

3.3.7 Quantification of Prokaryotic Community at Deep Water Site by qPCR

The deep water sites gave the highest bacterial qPCR count of all three sites which was approximately an order of magnitude larger than the AODC values at some depths (Figure 3.10). *Bacteria* numbers appear to fluctuate between 10^{10}-10^{11} cells/g of sediment throughout the core, with no obvious decline with depth. There was a peak of 10^{11} cells/g of sediment between 14-16 cbsf and 30-34 cbsf. *Archaea* cell numbers were approximately an order of magnitude lower at ~10^9 cells/g of sediment and a drop between 16-18 cbsf to 10^8 cells/g of sediment. This corresponds to the peak in *Bacteria* at

the same depth. AODC counts were also high (~10^9-10^{10} cells), well above the predicted AODC values, supporting the qPCR results.

Although total *Chloroflexi* numbers (10^8 cells/g of sediment) were equal to those at the intertidal and shallow water sites, the percentage of *Chloroflexi* of total bacterial community was far lower at the deep water site (Figure 3.11). This coincides with the extremely high bacterial counts at this site (Figure 3.10). The range of percentages was 0.1-1.29%, with the highest proportion found at 10-12 cbsf. The peaks in *Chloroflexi* percentages corresponded to falls in the bacterial community, again suggesting that though the bacterial numbers change, the *Chloroflexi* number remain constant. This was seen in the general trend lines of the *Chloroflexi* and *Anaerolineae/Caldilineae* and *Dehalococcoidia* subdivisions. The *Anaerolineae/Caldilineae* were again in greater abundance than the *Dehalococcoidia* (10^7 and 10^6 cells/g of sediment respectively). However, the *Anaerolineae/Caldilineae* numbers do not dictate the numbers of total *Chloroflexi* as in the previous sites, indicating that other undetected subdivisions were present, accounting for 10-18% of the total *Chloroflexi* community.

3.4 Discussion

3.4.1 Development of Chloroflexi *and Subdivision Targeted PCR Assays*

Novel subdivision targeted approaches to better describe the *Chloroflexi* phylum in environmental samples were successfully developed, which have allowed detailed subdivision composition and abundance analysis. Though the *Chloroflexi* phylum is extremely large, it is poorly described, with few cultured representatives and even less is known about the biochemistry, physiology and ecology of the phylum. Previous work with *Chloroflexi* in sediment has resulted from either the use of general bacterial primers that only detect *Chloroflexi* when abundant in the microbial community or have focused exclusively on individual subdivisions, primarily the *Dehalococcoidia* (Biddle et al., 2008; Durbin and Teske, 2011; Wasmund et al., 2014a; Wilms et al., 2006a), which has known dehalogenation properties, has been extensively investigated for biotechnologically uses (Ahn et al., 2007; Ho and Liu, 2011; Lee et al., 2011). Blazejak and Schippers (2010) used qPCR primers to target *Anaerolineae, Caldilineae* and JS1, and Yoon et al. (2010) solely targeted the *Caldilineae* using qPCR in activated sludge samples. Wasmund et al. (2014a) went further by designing *Dehalococcoidia* specific primers and implementing them with 454 pyosequencing on a variety of marine surface sediments. They found a great diversity and difference in distribution with depth in the *Dehalococcoidia* using this method, which implicated a much greater metabolic capability of the *Dehalococcoidia*, relating to sediment geochemistry, than previously realised. Therefore, a direct approach to target as many subdivisions as possible, such as the qPCR and RISA assays developed in this work, were important to elucidate the rare biosphere inhabited by these subdivisions.

3.4.2 Prokaryotic Communities Vary Across a Geomorphological Gradient in Severn Estuary Sediment

Due to the hyper-tidal nature of the Severn Estuary, each site was physically manipulated by the turbidity of the water. The intertidal site was not only open to air and light when the tide goes out but the force of the tide will churn

the top sediment or removes them completely. Wellsbury et al. (1996) found that the top 8 cm of tidal sediment in the Severn Estuary was turned over to create a uniform bacterial community and porosity. Similarly at the deep water site up to 1 m of sediment can be disturbed as the Newport Deeps were known for high sediment turbidity, fluid mud deposits and strong currents (Kirby, 2010). In comparison, the shallow water site remains little disturbed as it was not subject to strong currents. These physical influences in turn affect the geochemistry and oxygenation of the sediment. The depth of oxygen penetration varies at each site, as described in the changing sediment colour of the cores **(Section 3.2.2)**. This oxygen gradient dictates the dominant geochemical processes that can occur in each zone (Böttcher et al., 2000; Wilms et al., 2006a).

High diversity was detected at all sites, as can be seen in the great number of phylotypes in the DGGE gels, however the intertidal site had the most complex depth profile of the three sites. *Bacteria* dominate *Archaea* throughout the core, which has been previously described in other near surface coastal and marine sediment (Knittel et al., 2003; Schippers et al., 2010; Schippers and Neretin, 2006). qPCR indicated high numbers of *Bacteria* in the first 8 cbsf which suggested a highly active aerobic community. The number of estimated *Bacteria* from 0-8 cbsf correlates well with data from Wellsbury et al. (1996) who used AODC to count microbes from intertidal sediment at Aust Warth in the Severn Estuary. They found that numbers of microbes were high at the surface and dropped exponentially with depth.

Proteobacteria and *Firmicutes* appear to be the predominant phyla in the intertidal sediment. *Proteobacteria* were commonly found to be the dominant phylum in shallow and tidal sediment and work by Webster et al.(2010) indicated the prevalence of *Gammaproteobacteria* in Severn Estuary tidal sediment (Kim et al., 2008; Parkes et al., 2005; Wilms et al., 2006a). *Gammaproteobacteria* and *Deltaproteobacteria* were often found to dominate in tidal and shallow sediment due to their roles in sulphur and nitrogen cycling. However, this study suggested that *Alphaproteobacteria* were more

common in this estuarine sample, which was consistent with previous estuarine environments (Durbin and Teske, 2011; Sun et al., 2012). In this study, a number of clones were detected belonging to the *Alphaproteobacteria, Actinobacteria* and *Clostridia* from sources other than marine sediment e.g. soils and sewage. The presence of these phylotypes suggested the drainage of sewage and terrestrial water bodies into the estuary heavily contribute to the bacterial community in these surface estuarine sediment. Surprisingly, no *Deltaproteobacteria* were detected even though previous research on the Severn Estuary point to sulphate reduction being the main method of organic matter degradation in estuarine sediment (Purdy et al., 2002; Wellsbury et al., 1996). Previously, Webster et al. (2010) were only able to detect sulphate reducing bacteria (SRB) using specialist primers in nonenriched slurries from the Severn Estuary.

A community shift was highlighted at 6 cbsf, by *Bacteria* and *Chloroflexi* DGGE, RISA and qPCR results and supported by geochemical data. Low levels of ammonium and high levels of nitrate were found between 0-8 cbsf indicating aerobic ammonium oxidation was possibly occurring. Classes that included known with aerobic and photosynthetic metabolisms (i.e. *Actinobacteria, Cyanobacteria/*Chloroplast and *Alphaproteobacteria*) were negatively correlated with depth, appearing to swap with *Firmicutes* that were positively correlated with depth. The *Alphaproteobacteria* were significantly associated with ammonium levels and DGGE indicated one phylotype was related to *Pedomicrobium australicum* (order *Rhizobiales,* class A*lphaproteobacteria*), which was associated with manganese oxidation, iron fixation and nitrogen fixing in the rhizosphere (Braun et al., 2009). This further suggests that oxygen dependent biogeochemical processes were occurring in these sediment and the particular importance of nitrogen cycling. Furthermore oxygen penetration appeared to be the cause of the community striation seen at this site. Photosynthetic *Cyanobacteria/*chloroplasts (Hess, 2011) were relatively abundant from 0-6 cbsf but not below this, possibly indicating transfer of diatom related organism from the water column. The strictly anaerobic *Dehalococcoidia* were only detected below 6 cbsf indicating a change to anaerobic conditions (Kube et al., 2005), also reflecting as

sulphate concentration which steadily decline below 6-8 cbsf indicating the potential start of the sulphate reduction zone.

Surprisingly, *Archaea* were present in numbers approximately an order of magnitude higher than *Bacteria* at the shallow water site (Figure 3.10). This was unusual for coastal and marine sediment but not in deep subsurface sediment and so the question remains, why were the *Archaea* so abundant in this sediment (Knittel et al., 2003; Schippers et al., 2010; Schippers and Neretin, 2006)? Methanogenesis is a common process in coastal sediment and so seems a likely explanation (Purdy et al., 2003; Wilms et al., 2007). A peak in methane concentration and the dramatic decrease in hydrogen and carbon dioxide at 14-16 cbsf supported this (unpublished data from Shaun Thomas, Cardiff University). Corresponding peaks of formate between 10-20 cbsf suggested bicarbonate methanogenesis processes (Thiele and Zeikus, 1988).

The deep water bacterial and *Chloroflexi* profiles showed very little change with depth as seen at the shallow water site which was supported by single factor ANOVA analysis. In fact, DGGE and RISA cluster analysis gave extremely high over-all similarity percentages suggesting that the entire sediment core was homogenously mixed. The highest number of phylotypes was found at this site indicating a great diversity accompanying the extreme sediment disturbance. This was also seen by Wellsbury et al. (1996). The same phyla as the intertidal site were found here but with very different, mixed correlations with depth. This again indicated a homogenous community in comparison to the stratification of the intertidal site. *Cyanobacteria*/chloroplast diatom-related sequences were detected throughout the core and were significantly increasing with depth, which indicated mixing of the water communities with sediment. As with the previous sites a number of phylotypes were from soil and faecal matter from external inputs to the estuary.

The highest total prokaryote numbers were found at the deep water site with no decrease with depth. Other hyper-nutrified estuaries have been shown to also have high cell counts in the order of 10^{10} cells/g of sediment due to input

of nutrients from the land leading to a highly productive bacterial community (O'Sullivan et al., 2013; Ogilvie et al., 1997). Geochemical data (Shaun Thomas, Cardiff University) indicated constant supplies of substrates such as acetate, formate, lactate, sulphate and ammonium suggesting a sustained active population of prokaryotes. Previous work by Yallop et al. (1994) with Severn Estuary sediment indicated that the turbidity of the sediment in the estuary prevented the dominance of anaerobic processes leading to a range of aerobic geochemistry prevailing.

The cells numbers detected by qPCR at the deep water site were up to an order of magnitude larger than the estimated AODC values. At the intertidal and shallow water sites however, the qPCR and AODC values correlate far better than at the deep water site apart from the aerobic section of the intertidal site. This discrepancy could be explained by the targets of each method. AODC will target both living and dead cells and so would be expected to overestimate prokaryotic cell numbers. However qPCR targets the 16S rRNA gene, of which prokaryotes can have a greatly varying copy number. The calculations of copy number using qPCR must estimate the average number of 16S genes per genome for *Bacteria* and *Archaea* and so can lead to error in the calculation. Many prokaryotes naturally have more than one genome per cell including *Proteobacteria, Cyanobacteria* and methanogenic *Archaea,* often as a mechanism of defence against environmental pressures such as radiation and heat (Comai, 2005; Griese et al., 2011; Hildenbrand et al., 2011). Actively growing communities, similar to those seen in the deep water site and 0-6 cbsf at the intertidal site were known to up regulate their ploidy to up to 40 copies during exponential phase (Pecoraro et al., 2011). Thus, qPCR detection would vastly overestimate gene copy number in these highly active environments.

3.4.3 Chloroflexi *Subdivisions were Widespread in Severn Estuary Sediment*

Chloroflexi subdivisions were directly analysed in Severn Estuary sediment at geomorphologically different sites and with depth. The *Chloroflexi* were commonly found in deep subsurface sediment, often as a dominant bacterial phylum (Fry et al., 2008; Inagaki et al., 2003). However, the distribution of

Chloroflexi is poorly characterised in tidal and estuarine environments. *Chloroflexi* were present at all three geomorphologically different sites but at varying abundances. The percentage of *Chloroflexi* of the bacterial community was lowest (1.3%) at the deep water site, though qPCR counts of *Chloroflexi* were equal to the shallow water site as the bacterial community was particularly large. Interestingly, the shallow water site had the highest percentage of *Chloroflexi,* up to 45% of the bacterial community, and significant increase of *Dehalococcoidia* with depth. This coincided with the high relative abundance of *Archaea* and so with its anoxic geochemistry, points towards the shallow water site being most like a deep subsurface environment where *Archaea* were often dominant and *Chloroflexi* can account for up to 80% of the bacterial community (or 13% on average) (Fry et al., 2008; Inagaki et al., 2006; Teske and Sørensen, 2008). The sequences retrieved from these sites appear to be previously undescribed with little similarity to the cultured representatives in each subdivision. There was much intra-clade diversity, with many different phylotypes in each group with little relation to depth or geochemistry. This was commonly seen in the *Chloroflexi* in deep subsurface sediment (Fry et al., 2008). However, this makes predictions of *Chloroflexi* metabolism very difficult. Further geochemical studies and functional gene assays similar to those performed in the deep subsurface by Orsi et al. (2013) are needed to broaden our knowledge of the *Chloroflexi*.

At both the intertidal and shallow water sites, only the *Anaerolineae* and *Dehalococcoidia* subdivisions were detected by DGGE and qPCR. *Anaerolineae* appear to be the most dominant subdivision at these sites with numbers resembling those of the whole *Chloroflexi* community. *Anaerolineae* were the most commonly detected as the most abundant subdivision in surface sediment with *Dehalococcoidia* and Subdivision IV substituting after the oxycline (Durbin and Teske, 2011). They were found to constitute 91% of a clone library of sediment from 4 cbsf in the Peru Margin (Blazejak and Schippers, 2010). Though little was known of the role of *Anaerolineae* in biogeochemical cycles, cultivated species indicate aerobic respiration and anaerobic fermentative growth with a range of organic compounds (Grégoire

et al., 2011; Kale et al., 2013; Sekiguchi et al., 2003). This has been illustrated recently by Hug et al. (2013) who analysed the metagenome of an *Anaerolineae* species from sediment. They found that the single species was facultatively anaerobic with an extensive range of genes for sugar metabolism to scavenge from organic cell debris. Thus the potential metabolic range of the *Anaerolineae* could flourish in these dynamic sediment, with the influx of nutrients from both land and marine sources and with independence of geochemical parameters such as sulphate (Wilms et al., 2006b). Also, certain isolates have been shown to grow more efficiently in a syntrophic association with hydrogenotrophic methanogens (Sekiguchi et al., 2003; Yamada et al., 2007a; Yamada et al., 2006). As methanogenesis was common in estuarine and tidal sediment, often in syntrophic relationships with *Bacteria,* it was possible that the abundance of *Anaerolineae* and *Archaea* (especially at the shallow water site) were involved in syntrophic growth (Gray et al., 2011; Tischer et al., 2013; Wilms et al., 2006b).

Dehalococcoidia were detectable by qPCR (at approximately 10^5-10^6 cells/g of sediment) at all three sites, including the deep water site, which was highly perturbed and likely to be an aerobic or dysaerobic environment. As all known cultured *Dehalococcoidia* strains were strictly anaerobic and often found to be the dominant subdivision in the anaerobic deep subsurface, this was an unforeseen result (Ahn et al., 2007; He et al., 2005; Maymo-Gatell et al., 1999). In DGGE profiles, *Dehalococcoidia* were only detected at the intertidal and shallow water sites, and only below the expected oxycline. It is possible that greater specificity of the qPCR method with targeted primers potentially unique species, tolerant to oxygen have been detected. The *Chloroflexi* phylogenetic tree (Figure 3.5) indicated previously undescribed diversity, quite unlike the type strains, which supports this observation. Or the primers amplified extracellular, inactive DNA that had been transferred up from lower depths. Another explanation maybe that the deep water environment contains anaerobic pockets more suited to *Dehalococcoidia* growth (Carreira et al., 2013). This has been noted in river bed sediment with high organic carbon and small grain size which allows the co-occurrence of

anaerobic and aerobic vinyl chloride biodegradation, the former by *Dehalococcoides* species (Atashgahi et al., 2013).

The deep water site contained the most *Chloroflexi* diversity. qPCR results indicated *Anaerolineae* were less abundant at this site and 10-18% of the *Chloroflexi* were from another subdivision, with *Thermomicrobia* and Subdivision IV also detected by DGGE and all but Subdivision IV by the clone library. Due to the turbulent nature and therefore deep oxygen penetration of the deep water site it was likely to be at least partially aerobic and receive a constant influx of nutrients and substrates. This would possibly allow more subdivisions to utilise these nutrients and thrive in this environment. Whereas *Anaerolineae, Dehalococcoidia* and Subdivision IV were commonly found in marine environments, the *Thermomicrobia* usually inhabit moderate to hyperthermophilic aerobic habitats, such as thermal soils and hot springs (Botero et al., 2004). However, *Thermomicrobia* have recently been detected in coastal sediment in Xiangshan Bay, China, indicating a role in coastal surface sediment (Qiu et al., 2013). Sorokin et al. (2012) recently described a nitrite-oxidizing bacteria *Nitrolancetus hollandicus*, a member of the *Thermomicrobia*, which could indicate a role in nitrification in these deep water sediment. Hug et al. (2013) found the potential for nitrification and nitrate respiration in the *Chloroflexi* metagenome of aquifer sediment, emphasising the potential role of *Chloroflexi* in the nitrogen cycle.

3.4.4. Conclusions and Further Analysis

This study has taken a novel route in investigating the prokaryotic communities in dynamic Severn Estuary sediment, especially the *Chloroflexi* phylum and its subgroups. The geomorphology of the sediment appeared to directly affect the geochemistry and prokaryotic communities that inhabit it. In this case the hyper-tidal nature of the estuary and an oxygen penetration of the sediment appear to be the principal factors. From these influences it was postulated that nitrogen cycling and methanogenesis may be the primary processes occurring in these sediment, possibly relating to the *Thermomicrobia* and *Anaerolineae* subdivisions. It has often been found that

the geochemical environment of a habitat will greatly reflect on the prokaryotic communities (Huber et al., 2007). However, it was impossible to infer functionality to the prokaryotic communities using just 16S rRNA gene analysis as this holds no real functional evidence. Therefore greater emphasis on functional gene and RNA analysis would be beneficial.

Novel molecular analysis of the *Chloroflexi* subdivisions has led to the conclusion that the *Anaerolineae* were the most prevalent subdivision in this estuarine surface sediment with a possible role in the scavenging of detritus. This research has an impact on the study of *Chloroflexi* communities with a number of new and targeted methods attempted for the first time (e.g. *Chloroflexi* targeted RISA and subdivision targeted qPCR). With these new molecular tools the knowledge of the distribution, function and diversity of the subdivisions can be expanded.

Chapter 4 – The Influence of Sediment Depth and Geomorphology on Severn Estuary Prokaryotic and *Chloroflexi* Community Diversity

4.1 Introduction

4.1.1 Next Generation Sequencing and Sediment Microbial Ecology

The development of next generation sequencing (NGS) methods in the early 2000s led to the replacement of cloning and Sanger sequencing in microbial ecology research (Brown et al., 2009). The advantage over more traditional methods such as cloning and profiling are many and especially beneficial in microbial ecology research (Liu et al., 2012). For example, until recently, the most widely used NGS method with microbial ecologists has been pyrosequencing by 454 Life Sciences and Roche Diagnostics, which detects the incorporation of individual bases by the release of inorganic pyrophosphate, which produces light pulses in association with an enzyme system (Margulies et al., 2005; Roh et al., 2010). Though this method will soon be discontinued and new technologies will replace it. One of the benefits of NGS is long read length with Illumina paired end sequencing reaching 600 bp in length and the new PacBio® RS II P5-C3 chemistry achieving reads of 8500 bp on average (PacBio® RS II Brochure, http://files.pacb.com/pdf/PacBio_RS_II_Brochure.pdf, accessed August 2014). Other advantages include running of high throughput parallel samples using MID (multiplex identifier) tagged primers, greater depth of coverage (up to 1 million reads in one run) and less biases introduced than cloning in *Escherichia coli* (Liu et al., 2012; Roh et al., 2010; Shokralla et al., 2012). In fact, several comparisons between clone library Sanger sequencing and pyrosequencing have found that much greater diversity was detected using the latter method (Biddle et al., 2008; Hamdan et al., 2012; Kim et al., 2008; Quince et al., 2008). Furthermore, pyrosequencing has allowed microbial ecologists access to the ‘rare biosphere’, those organisms in low abundance in a given environment. It is believed that this pool of unknown and uncharacterised diversity may harbour unique metabolisms with the potential

to buffer communities and/or become abundant under fluctuations or extreme environmental or anthropogenic changes (Brown et al., 2009; Sogin et al., 2006).

There are limitations to the pyrosequencing method, for example general prokaryotic primers may not target all taxa in an environment (Soergel et al., 2012; Teske and Sørensen, 2008; Wang and Qian, 2009) and homopolymers of six or more bases create artefacts in pyrosequencing libraries that could be construed as novel taxa (Kunin et al., 2010). These limitations are being addressed by improving primer design (Pinto and Raskin, 2012) and bioinformatics software to target sequences with artefacts or chimeras (Haas et al., 2011; Quince et al., 2008). A further limitation that must be considered by the researcher is the amount of sequencing effort to apply to an environmental sample to detect as much of the diversity as possible. Quince et al. (2008) used a statistical method to estimate that diverse environments such as the deep ocean and soils would need hundreds of times greater number of samples than currently processed to achieve 90% of the taxonomic diversity.

A range of approaches utilising pyrosequencing are currently in use in the microbial ecology field. Amplicon sequencing using 16S ribosomal RNA (rRNA) gene targeted primers as a DNA “barcode” to specifically analyse the taxonomic diversity of a prokaryotic community has become widely popular. This method has been applied to soils (Roesch et al., 2007), tidal flat sediment (Kim et al., 2008) and underwater freshwater springs (Ionescu et al., 2012) to name just a few. Amplicon sequencing has also been adapted for use with functional genes to analyse the metabolic potential in a community (Lüke and Frenzel, 2011; Sun et al., 2011). A metagenomic approach (i.e. shotgun sequencing of whole community DNA) has been largely successful in identifying functional aspects of environmental communities (Biddle et al., 2008; Edwards et al., 2006; Varin et al., 2012). This has been particularly useful in geochemically interesting environments such as sediment where the role of prokaryotes in global geochemical cycles is still being unravelled. Finally metatranscriptomic approaches or RNA-seq,

which sequence cDNA libraries made from total or mRNA from a prokaryotic community, are now being used (DeLong, 2009; Frias-Lopez et al., 2008; Poretsky et al., 2009). These methods allow the targeting of active communities only, which in ancient environments such as deep subsurface sediment, can be important, where dead cells or ancient extracellular DNA may impact the sequencing profile (Mills et al., 2012; Newberry et al., 2004).

Research into marine sediment prokaryotic communities has benefitted from NGS technologies. Sediment environments are some of the most populated and diverse environments for prokaryotic communities, with estimated cell numbers in the order of 2.9×10^{29} (Kallmeyer et al., 2012) to 3.5×10^{30} cells (Whitman et al., 1998). Huge sequencing efforts must be made to describe the as yet unknown diversity particularly in the deep subsurface biosphere (Biddle et al., 2008; Biddle et al., 2011; Huber et al., 2007; Jørgensen et al., 2013; Wang et al., 2011). Many of these have taken a metagenomic sequencing approach to try to elucidate rare communities, missed by conventional cloning. The most commonly detected bacterial phyla were the *Proteobacteria, Chloroflexi, Planctomycetes* and the JS1 clade, within the *Atribacteria* (Durbin and Teske, 2011; Jørgensen et al., 2013; Rinke et al., 2013). Common Archaeal lineages include the Deep Sea Archaeal Group (DSAG), Marine Group I (MG-I) the Miscellaneous Crenarchaeotic Group (MCG), and the South African Goldmine Euryarchaeotal Group (SAGMEG) (Fry et al., 2008; Jørgensen et al., 2013; Teske and Sørensen, 2008). Orsi et al. (2013) successfully used a metatranscriptomic approach with messenger RNA (mRNA) to describe potential microbial metabolism in the deep biosphere. The *Chloroflexi* phylum was in very low abundance in Orsi et al.'s work, whereas amplicon sequencing at this site indicated *Chloroflexi* as a dominant group (Biddle et al., 2008), indicating organisms with lower expression levels may be missed with mRNA based techniques.

The work described in this chapter focuses on bacterial, archaeal and *Chloroflexi* communities in Severn Estuary surface sediment detected by pyrosequencing. Previous research using profiling methods and clone libraries has focused on the communities responsible for the dominant

biogeochemical processes occurring in marine sediments e.g. sulphate reduction, methanogenesis, nitrification, denitrification and ANAMMOX. Much of this work has fixated on the effects of salinity gradients on the activity and structure of the prokaryotic community. The analysis of both sulphate reducing bacteria (SRB) and their functional genes in estuarine sediments has been widely discussed, particularly in the Colne Estuary, UK (Nedwell et al., 2004; O'Sullivan et al., 2013; Purdy et al., 2002). Less focus has been placed on whole community analysis of estuarine sediments. Webster et al. (2010) and Wellsbury et al. (1996) investigated whole prokaryotic communities in Severn Estuary sediments and a number of groups have analysed Pearl River Estuary sediment (Sun et al., 2012). There have been few deep sequencing studies on shallow and intertidal sediment. Pyrosequencing has supported previous investigations using clone libraries into estuarine communities by identifying *Proteobacteria* as the dominant phylum in surface intertidal and estuarine sediment, with the classes *Deltaproteobacteria* and *Gammaproteobacteria* being the most abundant (Kim et al., 2008; Lee et al., 2011; Sun et al., 2013; Wang et al., 2013). Similarly, *Alphaproteobacteria* were found to be seasonally dominant in English Channel water column (Gilbert et al., 2009). Other common phyla include *Firmicutes, Cyanobacteria, Bacteroidetes* and *Acidobacteria* (Kim et al., 2008; Sun et al., 2013).

Sun et al (2013) compared six estuaries in Australia using 454 pyrosequencing of the 16S rRNA gene with varying levels of urbanisation and therefore anthropogenic contamination. They reported a core set of four bacterial classes that were shared at all sites, regardless of contamination level; which comprised 13 operational taxonomic units (OTUS) of *Deltaproteobacteria, Gammaproteobacteria, Alphaproteobacteria* and *Acidobacteria*. 454 pyrosequencing of the 16S rRNA gene was also used to detect four known *Archaea* phyla in lower abundances than *Bacteria* from surface tidal flat sediments of Dongmak, Ganghwa Island, Korea, where methanogens such as the *Methanomicrobia* were common (Kim et al., 2008).

The apparent ubiquity of environmental clones related to *Chloroflexi* phylum of *Bacteria* in terrestrial and marine ecosystems hints at an important role for the diverse phylum (Hugenholtz et al., 1998). Though few members of the phylum have been cultured, therefore the full metabolic potential of the *Chloroflexi* and their role in these environments is largely unclear (Costello and Schmidt, 2006; Durbin and Teske, 2011). A *Chloroflexi* targeted pyrosequencing approach has not been reported to date. However, *Chloroflexi* have been regularly detected, at substantial levels, in surface sediment, not just in deep subsurface biosphere (Kim et al., 2008; Sun et al., 2013; Wilms et al., 2006a). It appeared that of the *Chloroflexi* subdivision *Anaerolineae* (including *Caldilineae*) has been the most prevalent in surface sediment as these organisms have been frequently detected in tidal flat and estuarine sediment with no evidence of other subdivisions (Kim et al., 2008; Lee et al., 2011; Wang et al., 2013). It has been noted that *Anaerolineae* and *Caldilineae* have been often abundant in methane seeps and in methanogenesis zones and so may have an indirect role in methanogenesis (Hamdan et al., 2012; Yamada et al., 2007). Also, *Anaerolineae* and *Caldilineae* were found by Sun et al. (2013) to be significantly correlated with higher contamination concentrations and metal tolerance. Wasmund et al. (2014a) were able to detect *Dehalococcoidia* in tidal surface sediment with a *Dehalococcoidia* targeted pyrosequencing method although at very low abundances. Also, Wasmund et al. (2014b) and Kaster et al. (2014) used single cell genome analysis to predict the function of *Dehalococcoidia* in shallow surface sediments (10 cbsf) from Aarhus Bay, Denmark and deep subsurface sediments form the Peru Margin. Though the groups found evidence of great metabolic diversity, such as beta-oxidation and oxidation of aromatic compound genes, no evidence of reductive dehalogenase genes was detected. These findings suggest that *Chloroflexi* sub-groups could play important roles in surface sediment geochemistry and pollutant remediation.

4.1.2 Aims

Using pyrosequencing with *Bacteria, Archaea* and *Chloroflexi* specific primers, this work sought to describe prokaryotic community compositions across an estuarine gradient of three geomorphologically different sites in the

Severn Estuary. This was the first time the *Chloroflexi* phylum, in any environment, has been directly targeted for in depth sequencing using these methods.

4.2 Materials and Methods

4.2.1 Sediment Sampling and Extraction

DNA from three cores at three geomorphologically different Severn Estuary sites was extracted as described in **Sections 3.2.2 and 3.2.4**. For 16S rRNA gene 454 pyrosequencing, three samples were used from the intertidal site (0-2 cbsf, aerobic zone; 6-8 cbsf intermediate or suboxic zone and 16-18 cbsf anaerobic zone; WL). Two samples were sequenced from both the shallow water (0-2 cbsf and 18-20 cbsf; PF) and deep water (0-2 cbsf and 32-34 cbsf; ND) sites to cover the top and bottom halves of the core.

4.2.2 Community analysis

For preliminary community profiling and quantification of these samples see **Chapter 3**.

4.2.2 Sample Preparation and Sequencing with Roche 454 GS Flx Jnr

Extracted DNA for each sample was diluted by 1 in 10 with sterile Tris-acetate-EDTA (TAE) buffer [pH 8.0]. DNA concentration was measured using Quanti-iT™ Broad Range Assay kit and Qubit® Fluorometer (Invitrogen) according to the manufacturer's instructions and were in the range of 0.31-1.79 ng/ µl. Absorbance ratios of 260/280 to determine purity of samples were measured using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies).

Pyrosequencing was performed by Research and Testing Laboratory, Lubbock, Texas, using a Roche 454 GS FLX Titanium system. The company performed the triplicate PCRs to add the MID tag and 454 Life Sciences A and B adaptor sequences using bacterial, archaeal and *Chloroflexi* specific primers (Table 4.1; Sun et al., 2011). Each sample had a unique MID tag for distinction in downstream processing (Hamady et al., 2008). Due to the expected higher diversity of *Bacteria* in the samples, compared with the *Chloroflexi* and *Archaea*, the *Bacteria* were sequenced at 10,000 reads per sample, whereas *Chloroflexi* and *Archaea* were

Table 4.1- Primers used in pyrosequencing experiments by Research and Testing, Lubbock, Texas with sequences, target, protocol and references.

Primer	Target Organism	16S rRNA Gene Region[a]	Sequence (5' – 3')[b]	Reference
357F	*Bacteria*	V3 - V5[a]	CCT ACG GGA GGC AGC AG	Muyzer et al., 1993
907R			CCG TCA ATT CMT TTG AGT TT	Muyzer and Smalla 1998
341F	*Archaea*	V3 - V5	CCC TAC GGG GYG CAS CAG	Øvreås et al., 1997
958R			YCC GGC GTT GAM TCC AAT T	Delong, 1992
941F	*Chloroflexi*	V6 - V7	AGC GGA GCG TGT GGT TT	Gich et al., 2002
1340R			CGC GGT TAC TAG CAA C	Gich et al., 2002

[a] V, variable region.
[b] Key: M = A or C, S = C or G, Y = C or T

sequenced at 3,000 reads per sample. A total of 85,009 *Bacteria,* 52,016 *Archaea* and 41,765 *Chloroflexi* sequences were obtained with all samples tested. The average read length for the *Bacteria, Chloroflexi* and *Archaea* libraries was 539 bp, 420 bp and 579 bp respectively.

4.2.3 Data Analysis using QIIME

All analysis of sequencing data was performed in QIIME v1.6.0 (Caporaso et al., 2010b). The raw Sff files were used to create fasta and quality files (Appendix 4). Software program Acacia (Bragg et al., 2012) was used for initial quality checking of the sequencing data. This software corrected pyrosequencing errors such as homopolymers and denoised the sequences. Further quality control was performed with the *split_libraries.py* command in QIIME. This removed sequences that were outside of expected sequence lengths for each primer set. MID tags and primer sequences were also trimmed from each sequence. Any sequences that had a mismatched or did not have a MID tag or correct primer were removed. Chimeras were detected and removed using the USEARCH61 algorithm in the QIIME pipeline (Edgar, 2010). Data was normalised by randomly subsampling each sample to the lowest number of sequences in each library, given in Table 4.2, before further processing and statistical analysis. OTUs were picked using the UCLUST algorithm (Edgar, 2010), which generates sequence clusters based on percentage identity and a representative sequence chosen for each OTU based on the most abundant sequence method. BLAST (Altschul et al., 1990) and the most recent Greengenes database (May 2013; DeSantis et al., 2006) were used to assign taxonomy to each OTU at a cut-off of 97% similarity before the OTU table was made. Singletons (OTUs represented by a single sequence) and any nonspecific sequences (e.g. sequences other than *Chloroflexi* in the *Chloroflexi* library) were filtered from the OTU table, although a comparative table with descriptions and diversity estimates with singletons has been included. Sequences were aligned using PyNAST (Caporaso et al., 2010a) to create a phylogenetic tree. Core OTUs for each community were calculated using QIIME and restricted to OTUs only present

Table 4.2 - Descriptive statistics and diversity estimates for *Bacteria, Chloroflexi* and *Archaea* in Severn Estuary sediments.

Sampling Site[a]	Depth (cbsf)	No. of Sequences[bc]			No. of OTUs[d] per Sample[e]			Chao1			Shannon Index			Simpson's Index of Diversity[f]		
		Bacteria	*Archaea*	*Chloroflexi*	*Bacteria*	*Archaea*	*Chloroflexi*	*Bacteria*	*Archaea*	*Chloroflexi*	*Bacteria*	*Archaea*	*Chloroflexi*	*Bacteria*	*Archaea*	*Chloroflexi*
Intertidal (WL)	0 - 2	11 671	15 630	1932	459	96	203	598	139	240	5.30	1.58	4.12	0.99	0.49	0.96
	6 - 8	5793	2185	1665	385	309	236	423	342	259	5.00	5.00	4.37	0.98	0.98	0.97
	16 - 18	2273	5680	1955	255	146	179	282	178	199	4.46	3.63	4.11	0.96	0.93	0.96
Shallow Water (PF)	0 - 2	27 058	1821	2341	467	221	247	704	278	289	5.11	3.30	4.54	0.98	0.82	0.97
	18 - 20	14 088	1725	6880	301	175	206	386	231	304	4.35	2.77	4.22	0.96	0.74	0.97
Deep Water (ND)	0 - 2	3941	6167	2266	477	62	166	560	85	198	5.50	1.31	4.09	0.99	0.55	0.97
	32 - 34	5156	4447	1974	466	69	191	536	96	223	5.42	1.35	4.16	0.99	0.52	0.97

[a] A total of 7 samples from the three sites were used in pyrosequencing of the *Bacteria, Archaea* and *Chloroflexi* libraries.
[b] Number of sequences after quality control steps and before normalisation by subsampling to lowest number of sequences. This was the lowest sequence count in each library.
[c] Total number of sequences for *Bacteria, Archaea* and *Chloroflexi:* 69, 980, 37,655 and 19,013 respectively.
[d] OTU: Operational taxonomic unit.
[e] Total number of OTUs for *Bacteria, Archaea* and *Chloroflexi:* 2,810, 1,348 and 1,428 respectively.
[f] Simpson's Index of Diversity (1-D) was used.

in all of samples. All figures were either outputs from QIIME or created in Microsoft Excel or the R statistical package (Ihaka and Gentleman, 1996). For full QIIME pipeline scripts see Appendix 5 and for OTUs tables see Appendix 6.

4.2.4 Statistical Analysis of Sequencing Profiles

Data was normalised by randomly subsampling each sample to the lowest number of sequences in each library before statistical analysis. Alpha rarefaction was performed to test the sequencing coverage for each sample. A range of alpha diversity metrics were calculated using QIIME to describe the community diversity and structure. The metrics included Chao1, Shannon and Simpson. Chao1 was used to estimate the richness of the community by predicting the total number of unique OTUs in each sample and thus the percentage coverage of the sequence effort (Chao, 1984). This was a non-parametric method that utilised the ratio of singletons to doubletons to estimate richness. The non-parametric Shannon Index was also calculated to estimate species richness based on the difficulty in predicting the identity of the next OTU (Chao and Shen, 2003). A value of 0 indicated every OTU in the sample was the same, whereas values >4 indicated that OTUs are evenly distributed in the sample (Chao and Shen, 2003). Simpson's Index of Diversity was used to estimate the community diversity, based on abundance, by estimating the chance of two random sequences originating from the same OTU (Simpson, 1949). The 1-D value was used which indicated the probability of the sequence not being from the same OTU, therefore the closer the value to was to 1, the more likely the sequences were different. The Chao1 and Shannon indexes were weighted towards rare species whereas the Simpsons index was heavily influenced by the most abundant species. Thus, the combination of these was considered to provide good overall description of each community. Correlation of the Shannon and Simpson's indices was performed using Pearson's Correlation in Microsoft Excel to verify the relationship between the two indices.

Further statistical analysis was performed to analyse the significance of both specific taxa and OTUs in each site and depth. Bacterial, archaeal and

Chloroflexi OTUs at each site and depth were compared in the R statistical software package (Ihaka and Gentleman, 1996). NMDS with geochemical concentrations and heatmaps with dendrograms for both OTUs and samples were created for the *Bacteria, Archaea* and *Chloroflexi*. The Bray-Curtis calculation of dissimilarity (Bray and Curtis, 1957) was used to make a matrix of dissimilarities for the NMDS. Heatmaps and clustering dendrograms were made using Euclidean distances (Mardia et al., 1979) and complete hierarchical clustering (Sørensen, 1948). For full R scripts see Appendix 2.

The statistics software package STAMP (Statistical Analysis of Metagenomic Profiles; Parks and Beiko, 2010) was used to identify specific bacterial, archaeal and *Chloroflexi* taxa that indicated a significant change at various levels, including between all sites, combinations of sites or between two individual samples. Each depth in each site was compared, as were specifically selected pairs, chosen by NMDS analysis, using G-test (with Yates') and Fishers' exact test at P value < 0.05 with 95% confidence limits and Bonferroni correction (Abdi, 2007; Fisher, 1922; Rivals et al., 2007; Yates, 1934). The length of the sequences after quality control meant that analysis of the community was only possible to the Family taxa level.

4.3 Results

4.3.1 Sequencing Library Descriptive Statistics and Diversity Estimates

Quality control of the raw sequences led to an expected reduction in the total number of sequences analysed. The *Bacteria* library decreased by 18% (85,009 to 69,980 sequences), the *Archaea* by 28% (52,016 to 37,655 sequences) and the *Chloroflexi* by 55% (41,765 to 19,013 sequences; Table 4.2). The percentage coverage of each community was estimated using Chao1 values (Chao, 1984), calculated in QIIME. The coverage of the bacterial, archaeal and *Chloroflexi* communities ranged from 66 – 91%, 69 – 90% and 68% - 91%, respectively (Table 4.2). Alpha rarefaction was also performed to indicate coverage of each community (Figure 4.1). The *Bacteria* rarefaction curves for the intertidal site 6-8 cbsf and 16-18 cbsf and shallow water site 18-20 cbsf samples indicated good community coverage (Figure 4.1A). The *Archaea* rarefaction graph indicated a good overall coverage of the community with the intertidal 6-8 cbsf and shallow water 0-2 cbsf samples potentially needing a greater sequencing depth (Figure 4.1B). The *Chloroflexi* community at the intertidal 6-8 cbsf and shallow water samples appeared to be the most under sampled (Figure 4.1C).

The number of bacterial OTUs was higher than the total archaeal OTUs, which indicted greater richness in the bacterial community (Table 4.2). As expected, the number of OTUs decreased with depth at both the intertidal and shallow water sites, which indicated a decrease in community diversity. This could be linked to a switch in the community to specific anaerobic metabolisms with depth, as alternative electron acceptors were required. Whereas the number of OTUs at the deep water site did not change greatly, this indicated a more homogenous community with little effect from depth, related to the constant disturbance of these sediments. Interestingly, at the intertidal site 6-8 cbsf, the number of OTUs did increase in both the *Archaea*

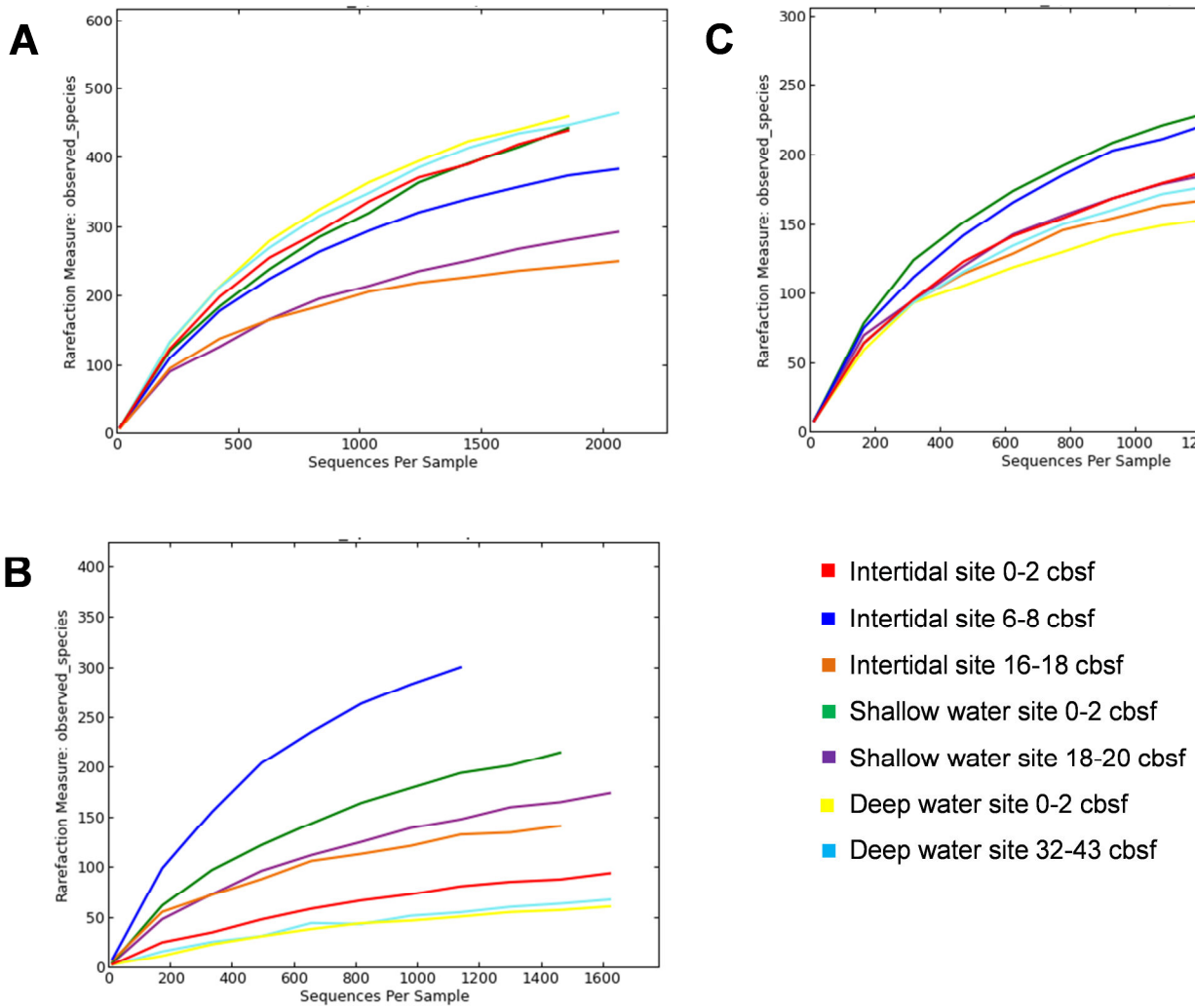


Figure 4.1 - Alpha rarefaction graphs using observed species method indicating the coverage level of 454 pyrosequencing of the 16S rRNA gene for each Severn Estuary sediment sample with singletons removed. A, *Bacteria*, B, *Archaea* and, C, *Chloroflexi*. A colour key indicates the individually sequenced sample in each graph. A total of 7 samples from the three sites were used in pyrosequencing of *the Bacteria, Archaea* and *Chloroflexi* libraries.

and *Chloroflexi,* which indicated a change in the sediment habitat at this depth that favoured these communities. The Chao1 estimates of richness (Chao, 1984) emulate the pattern of OTUs with depth. Singletons were removed before continuing with OTU taxonomic assignment to limit pyrosequencing noise. 1205 singletons (30% of OTUs), 593 (30% of OTUs) and 547 (28% of OTUs) were removed from the bacterial, archaeal and *Chloroflexi* libraries respectively. The number of OTUs increased dramatically with the inclusion of singletons and so also increased the Chao1 estimate of richness (Table 4.3). However, the pattern of decreasing number of OTUs with depth remained the same as previously described without singletons (Table 4.2). The comparisons described indicated that the removal of singletons led to little effect in the richness and diversity of the community and greatly improved the community coverage (Table 4.3).

The Shannon Index (Chao and Shen, 2003) and Simpson's Index of Diversity (Simpson, 1949) were calculated using QIIME to estimate richness and diversity of the communities at each site and with depth (Table 4.2). As expected, the two indices for the *Bacteria* and *Archaea* were significantly negatively correlated with depth (P value <0.05), which was previously indicated by OTU numbers (Table 4.2). However, there was no significant correlation in the richness and diversity estimates with site or depth for the *Chloroflexi*. Both metrics indicated high richness and diversity in all *Bacteria* and *Chloroflexi* samples with Shannon Index values over 4 and Simpson's Index of Diversity consistently over 0.96 (**Section 4.2.3**; Table 4.2). There was particularly high diversity in the *Bacteria* and *Chloroflexi* at 0-2 cbsf of the intertidal and shallow water sites and also both samples from the deep water site. The *Archaea* metrics indicated a more complicated community structure across the three sites and with depth. High richness and diversity was found in the *Archaea* at all sites except intertidal site 0-2 cbsf and at deep water site at both depths, which were notably low values (Table 4.2).

Table 4.3 **–** Descriptive statistics and diversity estimates for each sample for *Bacteria, Chloroflexi* and *Archaea* with singletons

Sampling Site[a]	Depth (cbsf)	No. of Sequences[bc]			No. of OTUs[d] per Sample[e]			Chao1			Shannon Index			Simpson's Index of Diversity[f]		
		Bacteria	*Archaea*	*Chloroflexi*	*Bacteria*	*Archaea*	*Chloroflexi*	*Bacteria*	*Archaea*	*Chloroflexi*	*Bacteria*	*Archaea*	*Chloroflexi*	*Bacteria*	*Archaea*	*Chloroflexi*
Intertidal (WL)	0 - 2	11 671	15 630	1932	686	132	268	1401	279	429	5.63	1.73	4.29	0.99	0.51	0.96
	6 - 8	5793	2185	1665	589	771	377	944	2044	634	5.33	5.89	4.72	0.98	0.99	0.97
	16-18	2273	5680	1955	317	257	218	452	640	292	4.58	3.93	4.21	0.96	0.94	0.96
Shallow Water (PF)	0 - 2	27 058	1821	2341	683	358	367	1628	757	661	5.45	3.71	4.83	0.98	0.85	0.98
	18-20	14 088	1725	6880	376	253	275	620	506	608	4.49	3.03	4.40	0.96	0.76	0.97
Deep Water (ND)	0 - 2	3941	6167	2266	694	78	218	1219	138	441	5.80	1.37	4.27	0.99	0.55	0.97
	32-34	5156	4447	1974	670	92	252	1099	178	406	5.72	1.44	4.32	0.99	0.53	0.97

[a] A total of 7 samples from the three sites were used in pyrosequencing of the *Bacteria, Archaea* and *Chloroflexi* libraries.
[b] Number of sequences after quality control steps and before subsampling to lowest sampling level. This was the lowest sequence count in each set.
[c] Total number of sequences for *Bacteria, Archaea* and *Chloroflexi:* 69, 980, 37,655 and 19,013 respectively.
[d] OTU: Operational taxonomic unit. The given number of OTUs includes singletons.
[e] Total number of OTUs for *Bacteria, Archaea* and *Chloroflexi:* 4,015, 1,941 and 1,975 respectively.
[f] Simpson's Index of Diversity (1-D) was used.

4.3.2 Analysis of the Bacterial Severn Estuary Community with Depth and Sediment Geochemistry

The proportion of each phylum appeared to be stable at the deep water site whereas changes with depth were seen at the intertidal and shallow water sites (Figure 4.2). Analysis of bacterial phyla using ANOVA in STAMP (Parks and Beiko, 2010) indicated no statistical difference between each site except for the phylum, *Verrucomicrobia,* which were found to be significantly (P value <<0.05) higher in proportion in the deep water samples. However, the *Verrucomicrobia* represented <1% of the total *Bacteria* community, which was consistent with the overall similarity in the abundance of phyla across all sites shown in Figure 4.2.

The *Proteobacteria, Chloroflexi, Firmicutes, Actinobacteria, Acidobacteria* and *Bacteroidetes* were common to each site and were the most abundant phyla, as they collectively constituted over 90% of the community (Figure 4.2). The *Proteobacteria* were the dominant phylum at all sites and depths from 34% of the community at the intertidal site 6-8 cbsf to 55% at the intertidal site 0-2 cbsf, though appeared to decrease in abundance with depth (Figure 4.2). Interestingly, *Chloroflexi* equalled the *Proteobacteria* at intertidal 6-8 cbsf at 34% of the community (for further analysis of the *Chloroflexi* community see **Section 4.3.3**). *Firmicutes* increased in abundance with depth at the intertidal site and the shallow water site whereas the *Actinobacteria* were more variable across the sites and depths (Figure 4.2). The *Bacteroidetes* decreased with depth, at the presumed beginning of anaerobic zones (Figure 4.2).

A core community, consisting of OTUs present at all sites and depths (i.e. in all samples) was computed using QIIME (Table 4.4; Figure 4.3). A total of 31 OTUs comprised the *Bacteria* core community from the most abundant phyla *Proteobacteria, Chloroflexi, Firmicutes, Actinobacteria* and *Bacteroidetes* (Table 4.4). The total number of sequences from these OTUs constituted 34% of the total bacterial community across all 7 samples (Table 4.4). The percentage of the total community at each site and depth that the core OTUs represented was calculated.

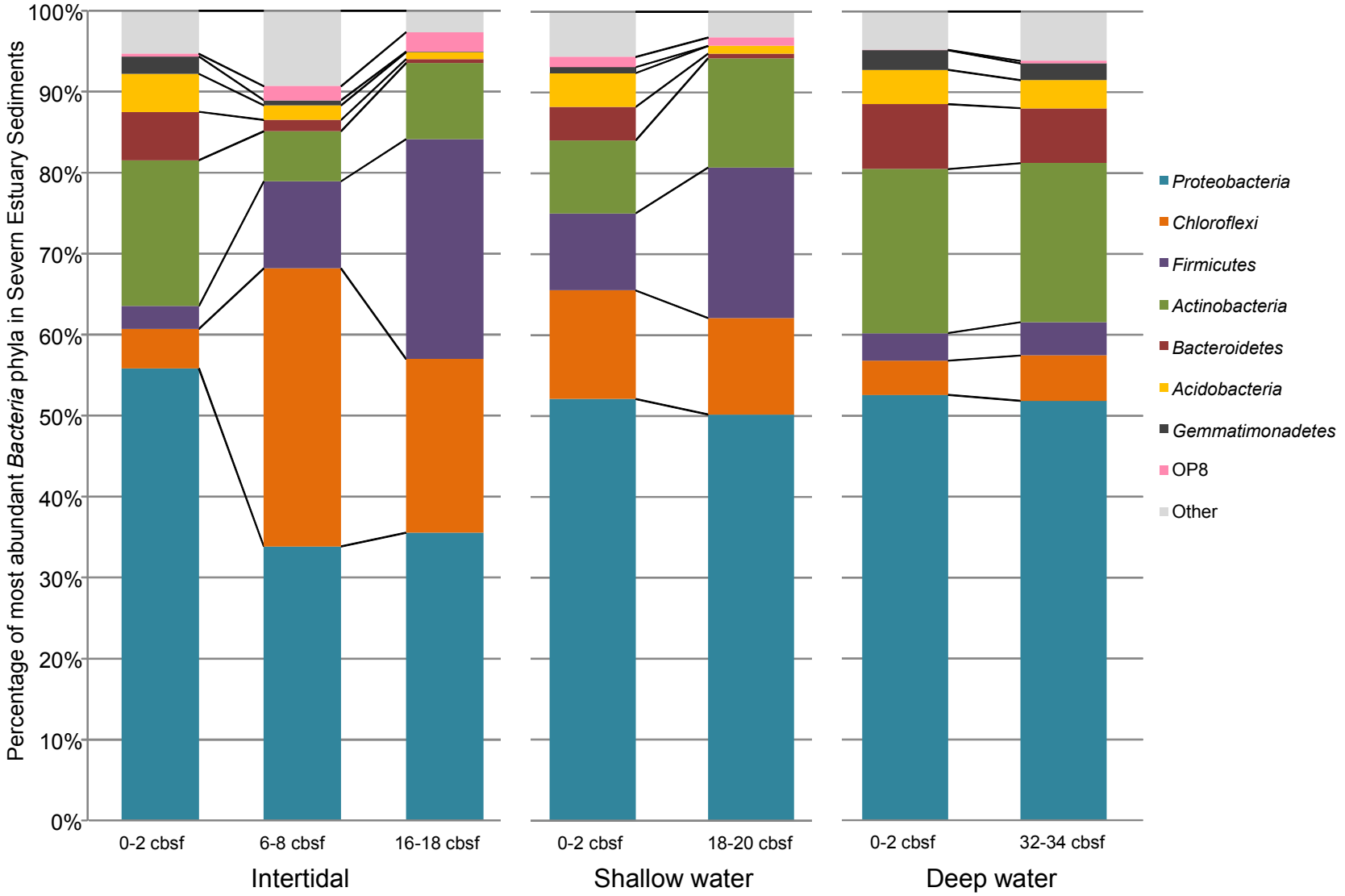


Figure 4.2 - Phylogenetic assignments (to phyla) of all bacterial 16S rRNA gene barcodes for sediments at each Severn Estuary site and depth. The percentage of the total number of sequences of the most abundant Bacteria phyla is given. The total number of sequences used was 2046, 2068, 2211, 2057, 2198, 2056 and 2069 respectively. A total of 7 samples from the three sites were used in pyrosequencing of the *Bacteria, Archaea* and *Chloroflexi* libraries. Series lines are given to highlight trends in phylum changes.

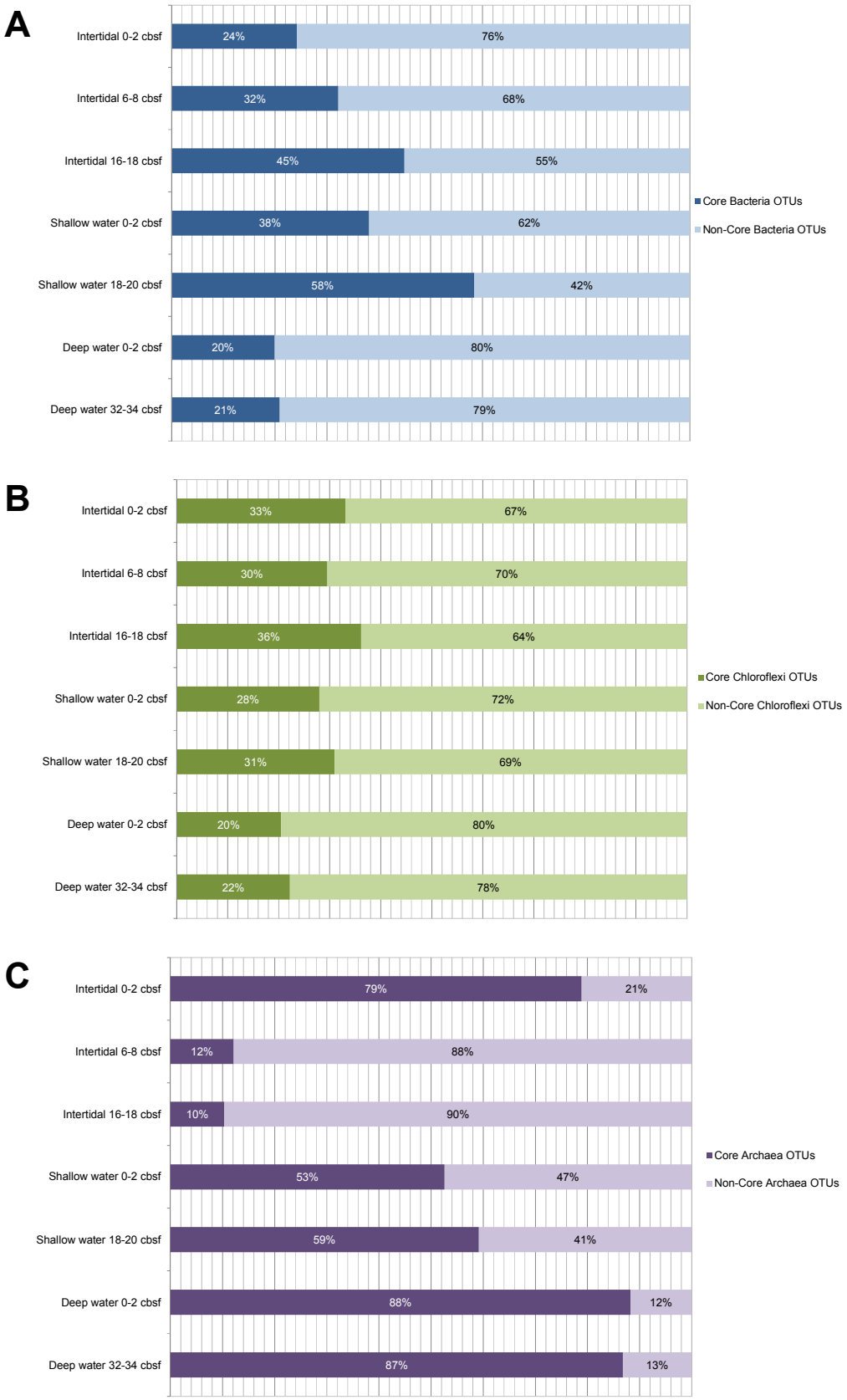


Figure 4.3 - Distribution of core OTUs at each Severn Estuary sample site and sediment depth for A, *Bacteria*, B, *Chloroflexi*, and C, *Archaea*. Core OTUs were defined as OTUs shared across sites and depths. The number of core OTUs in the *Bacteria*, *Chloroflexi* and *Archaea* was 31, 15 and 3, and accounted for 34%, 29% and 58% of the total sequences community in all samples, respectively. The number of non-core OTUs was 2,810, 1,428 and 1,348, for *Bacteria*, *Chloroflexi* and *Archaea*, respectively. OTUs were derived from 97% 16S rRNA gene sequence similarity binning in QIIME. For individual OTU numbers and taxonomy see Tables 4.4, 4.5 and 4.6. A total of 7 samples from the three sites were used in pyrosequencing of the *Bacteria, Archaea* and *Chloroflexi* libraries.

Table 4.4 **-** Core community OTUs with taxonomy for the *Bacteria* community across all three Severn Estuary sites and depths.

Taxonomy					OTU Number[a]	Percentage of Total *Bacteria* Community (%)
Phylum	Class	Order	Family	Genus/Species		
Actinobacteria	*Acidimicrobiia*	*Acidimicrobiales*	koll13		1018	0.20
Actinobacteria	*Acidimicrobiia*	*Acidimicrobiales*	koll13		2417	0.43
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Streptomycetaceae*		2119	0.16
Actinobacteria	*Thermoleophilia*	*Gaiellales*			881	1.50
Actinobacteria	*Thermoleophilia*	*Solirubrobacterales*			1245	0.40
Actinobacteria	*Thermoleophilia*	*Gaiellales*	*Gaiellaceae*		1472	0.37
Bacteroidetes	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*		1340	0.37
Chloroflexi	*Anaerolineae*	S0208			747	0.87
Chloroflexi	*Anaerolineae*	SB-34			1099	0.40
Firmicutes	*Clostridia*	*Clostridiales*	*Peptostreptococcaceae*		365	0.44
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*		514	1.38
Firmicutes	*Clostridia*	*Clostridiales*	*Peptostreptococcaceae*		2083	1.15
Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus longiquaesitum*	671	2.05
Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	1100	0.58
Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus muralis*	1559	0.34
Firmicutes	*Bacilli*	*Bacillales*	*Planococcaceae*	*Sporosarcina*	2104	0.52
Firmicutes	*Bacilli*	*Turicibacterales*	*Turicibacteraceae*	*Turicibacter*	2301	0.56
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*		72	0.74
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*		141	0.66
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*		556	0.31
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*		694	0.38
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Hyphomicrobium*	716	0.17

[a] OTU number relates to OTUs in Figure 4.7.

Taxonomy					OTU Number[a]	Percentage of Total
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*		789	2.58
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*		1112	0.33
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Hyphomicrobium*	1730	9.91
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*		2020	0.30
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Rhodobiaceae*		2180	0.61
Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*	*Desulfobacteraceae*		101	0.92
Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*	*Desulfobacteraceae*	*Desulfococcus*	1828	0.82
Proteobacteria	*Gammaproteobacteria*	*Chromatiales*			564	1.68
Proteobacteria	*Gammaproteobacteria*	*Xanthomonadales*			739	3.30
					Total	34%

The high abundance of OTUs related to the *Hyphomicrobium* indicated in denitrification and nitrogen fixation (Kloos et al., 1995; Martineau et al., 2013; Sakairi et al., 1996; Satoh et al., 2006) indicated that these processes could be an important, if not dominant, metabolic process in these Severn Estuary sediments.

Gammaproteobacteria were the most abundant *Proteobacteria* class at the intertidal 0-2 cbsf and deep water sites (Figure 4.4). The class significantly decreased with depth at all sites and were the only statistically significant class to change in abundance at the deep water site (Class level; Figures 4.4 and 4.5A-D). The orders *Xanthomonadales* and *Chromatiales* were the most abundant in the potentially oxygenated sediment at the intertidal site (0-2 cbsf) and throughout the deep water site core and both contributed two of the most abundant OTUs to the bacterial core community (OTU739, 3.30% and OTU564, 1.68%, respectively; Table 4.4). However, below the class taxonomic level, no further significant changes in abundances were seen. The abundance of *Xanthomonadales* and *Chromatiales* in the oxygenated surface sediments indicated that these orders were utilising aerobic metabolism, though no further taxonomic information was available to define the exact metabolism (Imhoff, 2005; Saddler, 2005). OTU564 was found to be related (97% sequence similarity) to the uncultivated environmental clone 2C67, which was isolated from sediments from the Bizerte Lagoon, Tunisia, in a study to analyse the effects of heavy metal and hydrocarbon pollutants on the bacterial community (Ben Said et al., 2010). The *Gammaproteobacteria,* including clone 2C67, were higher in abundance at the more polluted lagoon site and so it was hypothesized that the *Chromatiales* OTU564 was involved in pollutant adaption in these Severn Estuary sediments.

The *Deltaproteobacteria* significantly decreased with depth at the intertidal and shallow water sites, typified by a significant decrease in the *Desulfobacteraceae* and *Desulfobulbaceae* families (Figure 4.4 and 4.5B & C). The decrease in abundance of these SRB coincides with the

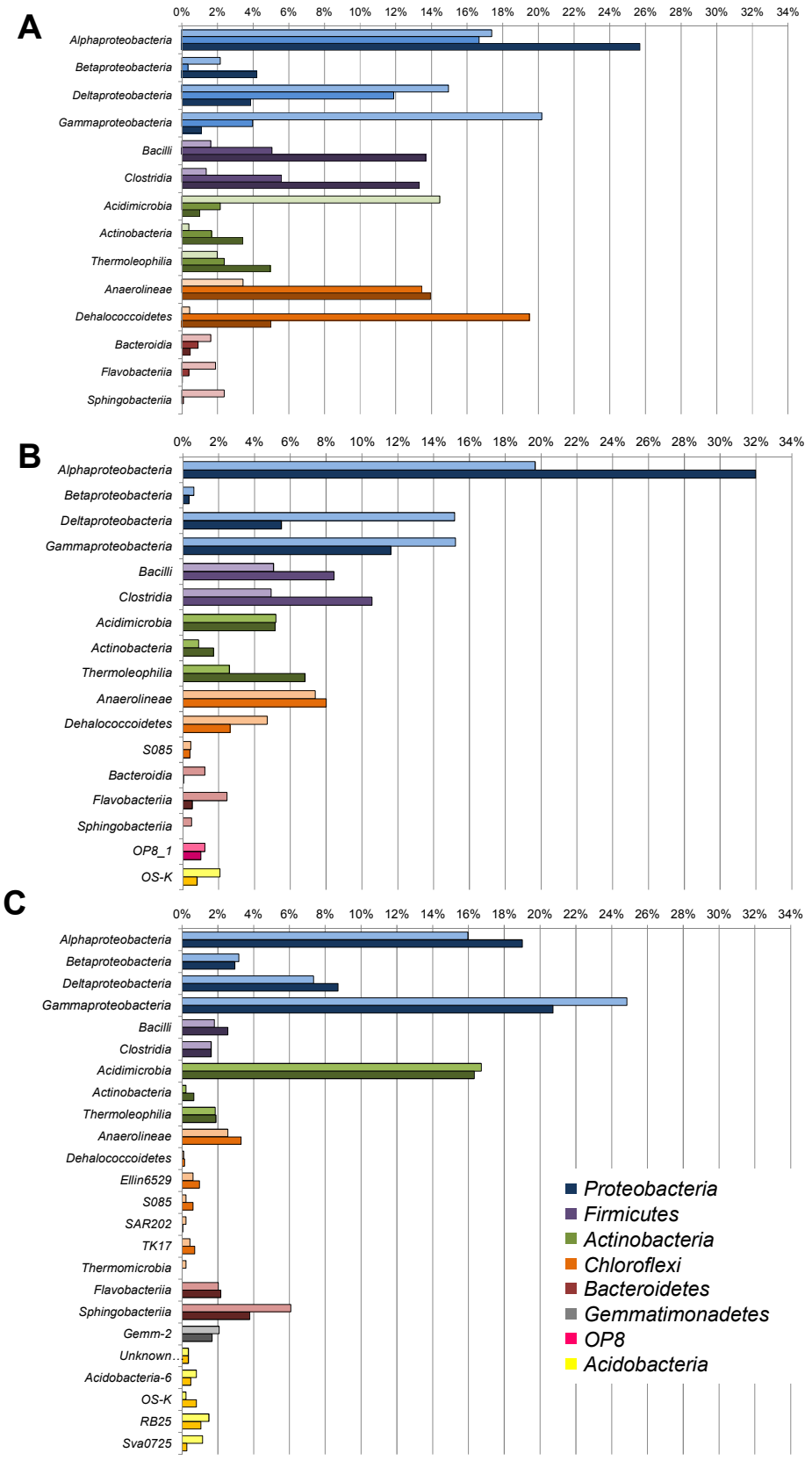


Figure 4.4 - Percentage of the most abundant class level 16S rRNA gene sequences of the total number of *Bacteria* for the Severn Estuary sediment sites A, intertidal (0-2, 6-8 and 16-18 cbsf), B, shallow water (0-2 and 18-20 cbsf) and, C, deep water (0-2 and 32-34 cbsf). The taxa were derived from BLASTs of all bacterial 16S rRNA gene barcode sequences by 454 pyrosequencing. The total number of sequences used were 2046, 2068, and 2211 for the intertidal depths (0-2 cbsf, 6-8 cbsf and 16-18 cbsf, respectively), 2057, and 2198 for the shallow water depths (0-2 and 18-20 cbsf, respectively), 2056, and 2069 for the deep water depths (0-2 and 32-34 cbsf, respectively). Classes are colour coded according to phylum and each depth is represented by a shade of the colour (lightest being the surface sample and the darkest being the deepest sample in each case).

Class

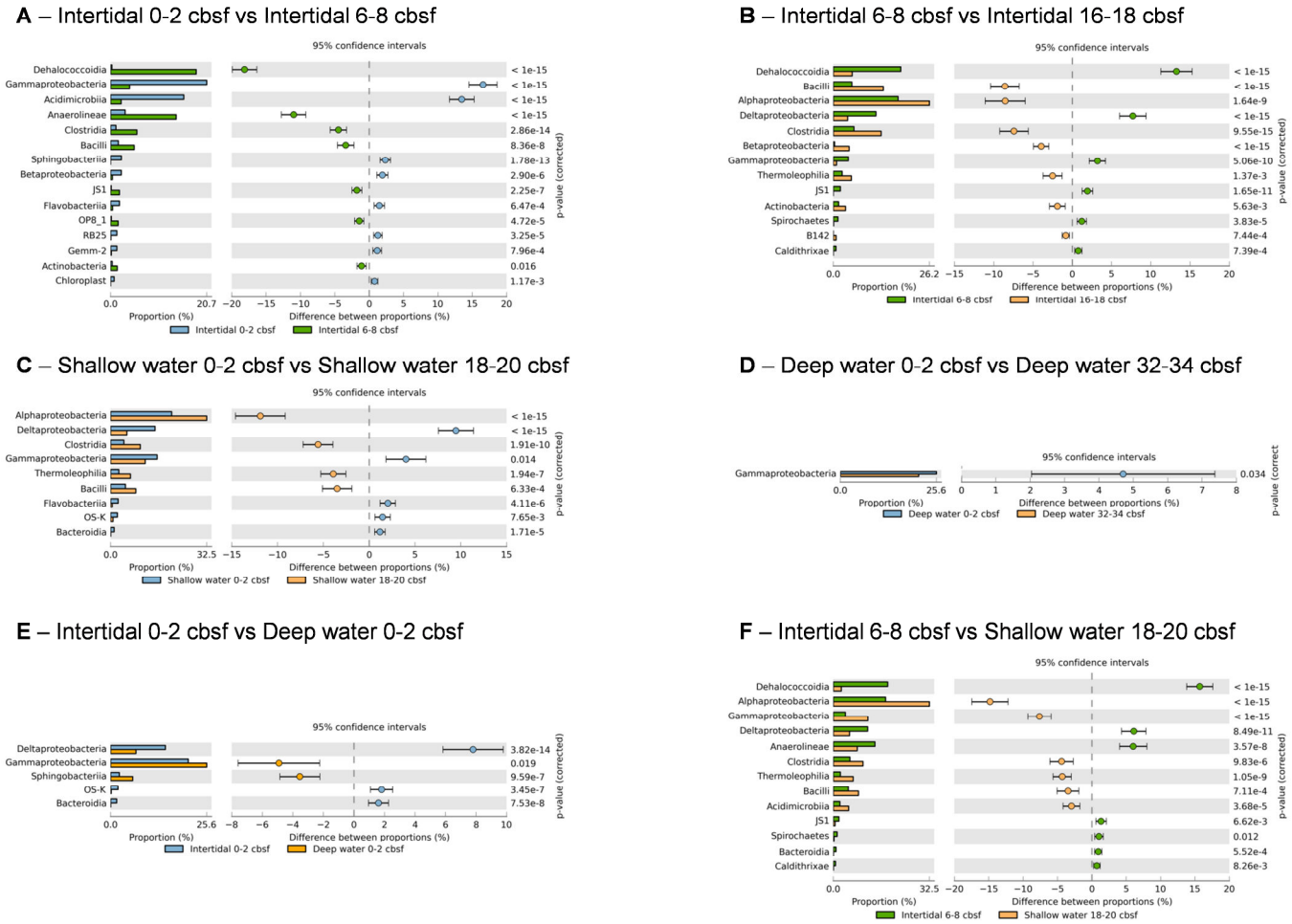


Figure 4.5 **-** Statistical comparisons of the abundance of *Bacteria* taxonomic classes in two depth samples from 454 pyrosequencing of the 16S rRNA gene from Severn Estuary sediment. A, intertidal 0-2 cbsf vs intertidal 6-8 cbsf, B, intertidal cbsf vs Intertidal 16-18 cbsf, C, shallow water 0-2 cbsf vs shallow water 18-20 cbsf, D, deep water 0-2 cbsf vs deep water 32-34 cbsf, E, intertidal 0-2 cbsf vs deep water 0-2 cbsf and, F, intertidal 6-8 cbsf vs shallow water 18-20 cbsf. The comparisons E and F were chosen based on groupings of these samples in NMDS (Figure 4.7). Samples were compared using G-test (with Yates') and Fishers' at P value <0.05 with 95% confidence limits and Bonferroni correction (Abdi, 2007; Fisher, 1922; Rivals et al., 2007; Yates, 1934). Only significant (P value <0.05) differences in abundances are shown. Classes are listed in order of descending effect size. Samples tested and colour key are given below each chart. 95% confidence intervals, of the range of effect sizes that have a specified probability of being compatible with the observed data, calculated using asymptotic with continuity correction differences in proportion, are indicated by bars. All statistical analysis and diagrams performed and made using STAMP v2.0.0 (Parks and Beiko, 2010).

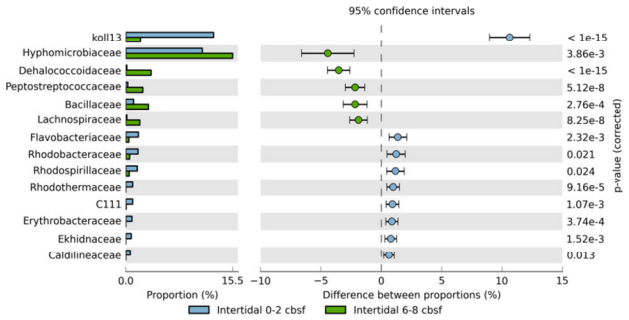
decrease in sulphate concentrations with depth at the intertidal and shallow water sites (Shaun Thomas, Cardiff University; data not shown). This correlation indicated a decrease in sulphate reduction with depth as the electron acceptor was depleted and so the SRB were constrained to the upper surface sediment.

The *Clostridia* and *Bacilli* significantly increased in abundance at both the intertidal and shallow water sites (Figure 4.4 and 4.5A, B & C). The *Clostridia* families, *Peptostreptococcaceae, Lachnospiraceae, Clostridiaceae* and *Ruminococcaceae*, and the *Bacilli* families, *Bacillaceae, Oxalobacteraceae* and *Alicyclobacillaceae,* all significantly increased in abundance with depth (Figure 4.6A-C). *Bacteria* core community OTUs related to *Peptostreptococcaceae, Lachnospiraceae,* and *Bacillaceae* constituted large percentages of the total *Bacteria* community (Table 4.4). The largest percentage of these was OTU671, which contributed to 2.05% of the total *Bacteria* community and was found to share >97% sequence similarity with *Bacillus longiquaesitum,* an unpublished species, cultivated from soil (Table 4.4). The other most abundant *Bacilli* and *Clostridia* OTUs from the core community were found to be related to uncultivated clones from paddy soils and human and animal faecal material (Ley et al., 2008; Watanabe et al., 2011). From this information, it is difficult to interpret what role the *Firmicutes* were playing in these deeper sediments, but these environments indicated anaerobic metabolism and particularly the paddy soil clone indicated a relationship with methanogenic *Archaea* that assimilated carbon from easily decomposable organic matter (Watanabe et al., 2011). Possibly, the readily available organic matter in the Severn Estuary sediments promoted growth of the *Firmicutes* that could assimilate the available carbon, with depth.

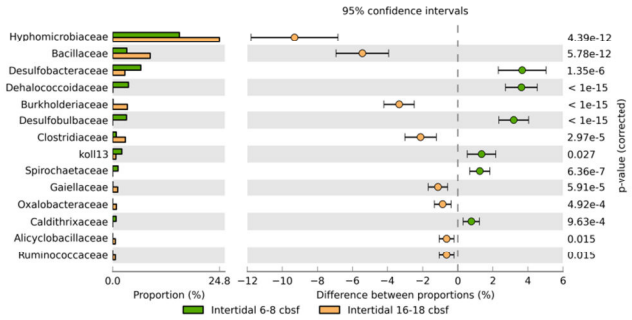
The class *Acidimicrobiia* (phylum *Actinobacteria*) was an abundant community member at all sites but particularly at the intertidal 0-2 cbsf and at both deep water depths (Figure 4.4). At the lower intertidal and shallow water depths, the class *Thermoleophilia* was the dominant group with a

Family

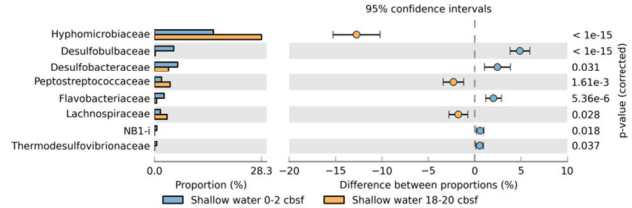
A – Intertidal 0-2 cbsf vs Intertidal 6-8 cbsf



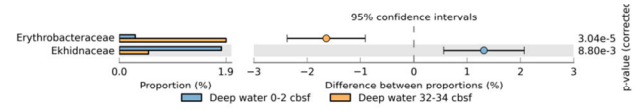
B – Intertidal 6-8 cbsf vs Intertidal 16-18 cbsf



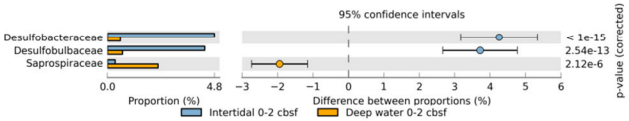
C – Shallow water 0-2 cbsf vs Shallow water 18-20 cbsf



D – Deep water 0-2 cbsf vs Deep water 32-34 cbsf



E – Intertidal 0-2 cbsf vs Deep water 0-2 cbsf



F – Intertidal 6-8 cbsf vs Shallow water 18-20 cbsf

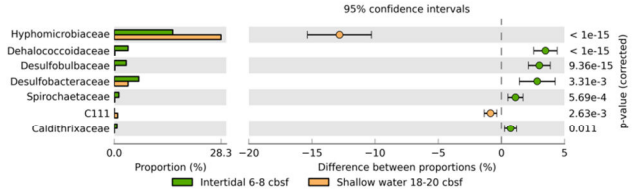


Figure 4.6 **-** Statistical comparisons of the abundance of *Bacteria* taxonomic families in two depth samples from 454 pyrosequencing of the 16S rRNA gene from Severn Estuary sediment. A, intertidal 0-2 cbsf vs intertidal 6-8 cbsf, B, intertidal cbsf vs Intertidal 16-18 cbsf, C, shallow water 0-2 cbsf vs shallow water 18-20 cbsf, D, deep water 0-2 cbsf vs deep water 32-34 cbsf, E, intertidal 0-2 cbsf vs deep water 0-2 cbsf and, F, intertidal 6-8 cbsf vs shallow water 18-20 cbsf. The comparisons E and F were chosen based on groupings of these samples in NMDS (Figure 4.7). Samples were compared using G-test (with Yates') and Fishers' at P value <0.05 with 95% confidence limits and Bonferroni correction (Abdi, 2007; Fisher, 1922; Rivals et al., 2007; Yates, 1934). Only significant (P value <0.05) differences in abundances are shown. Families are listed in order of descending effect size. Samples tested and colour key are given below each chart. 95% confidence intervals, of the range of effect sizes that have a specified probability of being compatible with the observed data, calculated using asymptotic with continuity correction differences in proportion, are indicated by bars. All statistical analysis and diagrams performed and made using STAMP v2.0.0 (Parks and Beiko, 2010)

large proportion of *Actinobacteria* (Figure 4.4).The *Acidimicrobiia* family related to the uncultivated environmental clone koll13 decreased with sediment depth at the intertidal site and was a core community OTU (OTU1018 and OTU2417; Table 4.4 & Figure 4.6A & B). The environmental clone koll13 was isolated from a nitrogen-removing biofilm from a waste water treatment filter (AJ224541, J. R. van,der,Meer, C. Werlen, and H. Siegrist, unpublished results). When the koll13 clone sequence was queried through BLAST, many of the related environmental clone sequences (>97% sequence similarity) were from sediment and intertidal sources. The presence of koll13 at the intertidal 0-2 cbsf and at both deep water depths indicated that nitrogen cycle processes were important in these Severn Estuary sediments, as previously indicated by the prevalence of the *Hyphomicrobium,* and organisms related to koll13 play important roles in surface sediment habitats.

Analysis of abundance of individual OTUs was assessed using NMDS and heatmaps. Bacterial OTUs from all sites were plotted as individual samples on an NMDS with geochemical concentrations as vectors, indicating correlation (Figure 4.7A). Four individual groups were found using this method, though these were not found to be statistically significant by ANOVA in STAMP (Parks and Beiko, 2010). The 0-2 cbsf intertidal sample was grouped with both deep water site depths. This group appeared not to be influenced by the geochemical concentrations analysed but as these were surface samples it is likely that oxygen penetration had an influencing role in these sediments. The second group consisted of the intertidal 6-8 cbsf and the 18-20 cbsf shallow water samples, which appeared to correlate closely with increased methane concentrations with depth. Finally, the intertidal 16-18 cbsf sample and shallow water 0-2 cbsf sample were separated into two separate groups, the former associated with increasing ammonium concentrations and the latter with decreasing sulphate, nitrate and chloride concentrations. Not all of the samples appeared strongly correlated with the geochemical vectors,

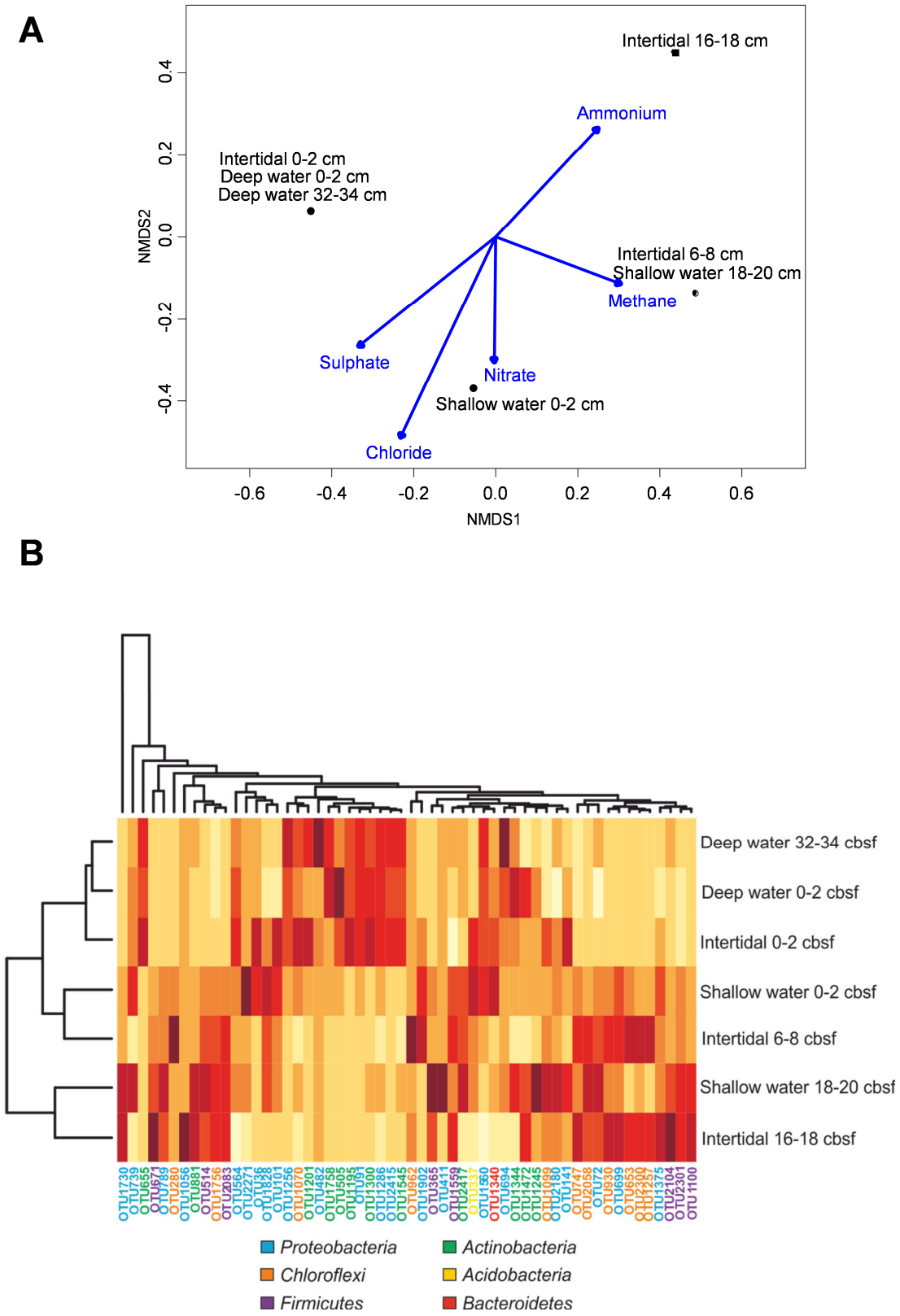


Figure 4.7 - Statistical analysis of specific *Bacteria* OTUs from 454 pyrosequencing of all Severn Estuary sediment samples using the R statistical package (Ihaka and Gentleman, 1996). A, NMDS plot with samples represented as black circles and labelled and geochemical vectors as blue arrows. A total of 7 samples from the three sites were used in pyrosequencing of the *Bacteria, Archaea* and *Chloroflexi* libraries. B, Heatmap demonstrating the prevalence of the 50 most abundant OTUS based on the number of sequences across each site and depth. UPGMA clustering dendrograms are given for both the sample and OTUS. The colour gradient indicates abundance with dark red being the most and pale yellow being the least abundant. The OTU number are given next to each row and these are coloured, as indicated in the key, by phyla, discerned from BLAST results.

which indicated that other geochemical or geomorphological factors were important influencing factors in these Severn Estuary sediments.

The NMDS groupings informed specific statistical comparisons between the intertidal 0-2 cbsf and deep water 0-2 cbsf samples and the intertidal 6-8 cbsf and shallow water 18-20 cbsf (Figures 4.5E & F and 4.6E & F). The main difference between the intertidal 0-2 cbsf and deep water 0-2 cbsf samples was the significantly higher abundance of *Deltaproteobacteria* in the intertidal site and the higher abundances of *Bacteroidetes* in the deep water site (Figures 4.5E & 4.6E). As *Deltaproteobacteria* are anaerobic and the *Bacteroidetes* aerobic, this finding indicated that anaerobic metabolisms such as sulphate reduction dominate much higher in the intertidal sediments, within 2 centimetres, due to the lack of sediment mixing. There were greater differences between the intertidal 6-8 cbsf and shallow water 18-20 cbsf, typified by the unusually high abundance of *Chloroflexi,* mainly the *Dehalococcoidia* and to a lesser extent the *Anaerolineae* at the intertidal site and the greater abundance of *Hyphomicrobiaceae* at the shallow water site (Figures 4.5F & 4.6F).

Heatmaps showing the top 50 abundant bacterial OTUs in each sample were clustered by UPGMA (Figure 4.7B). A cluster containing the 0-2 cbsf intertidal and both deep water samples was detected in the heatmap, as previously indicated by the NMDS (Figure 4.7A). However the remaining groups differed from those previously seen. A second branch consisted of shallow 0-2 cbsf and intertidal 6-8 cbsf. The third consisted of shallow 18-20 cbsf and intertidal 16-18 cbsf, which appeared to be associated with depth and potential geochemistry. A number of abundant OTUs were shared within the first group that were assigned to the *Actinobacteria* environmental clone, koll13 (Figure 4.7B). The second group shared OTUs associated with the *Desulfobulbaceae*. The final group consisting of the deeper samples shared mainly *Firmicutes* OTUs from both a selection of *Bacilli* and *Peptostreptococcaceae. Hyphomicrobiaceae* and

Anaerolineae OTUs were also shared between the deeper samples at the intertidal and shallow water sites.

4.3.3 Analysis of the Chloroflexi Severn Estuary Community with Depth and Sediment Geochemistry

The *Chloroflexi* phylum of *Bacteria* was targeted for pyrosequencing using a specific primer set (Table 4.1; Gich et al., 2002). Seven classes were detected including the sub-groups *Anaerolineae, Dehalococcoidia,* Sub-group IV/SAR202, *Thermomicrobia* and *Ktedonobacteria* **(Table 1.2)**. Also detected were two clades related to the uncultivated environmental clones Ellin6529 and TK17. However, the bacterial primers detected four *Chloroflexi* classes and the uncultivated clone group S085 (Figure 4.4). The *Anaerolineae* were dominant in both primer sets however the percentage of the *Chloroflexi* community rose from 41-68% with the bacterial primers to 67-98% with the *Chloroflexi* primers. Thus the *Dehalococcoidia*, which were more abundant than *Anaerolineae* at intertidal site depth 6-8 cbsf, shrank to less than half that of the *Anaerolineae* in the *Chloroflexi* primer targeted community (Figure 4.4).

A core *Chloroflexi* community of shared OTUs, across all sites and depths, was computed using QIIME. The core OTUs composed 29% of the total *Chloroflexi* community at all sites and depths (Table 4.5). This consisted of mainly *Anaerolineae* OTUs related to uncultivated environmental clones with one *Dehalococcoidia*: FS117-23B-02 (Table 4.5). Clone FS117-23B-02 was isolated from old oceanic crust fluids from the Juan de Fuca Ridge, Pacific Ocean, where *Chloroflexi* were found to dominate bacterial communities in samples with a large surficial sediment influence (Huber et al., 2006). The abundance and presence of the sequences related to FS117-23B-02 in the core community indicated that this *Dehalococcoidia* clade have an important and widespread role in surface sediments, and, according to Huber et al. (2006), could be related to anaerobic, thermophilic metabolisms. The percentage of the core community OTUs of the total *Chloroflexi* community varied, with no obvious pattern related to depth (Figure 4.3B). The core community percentages were lower than the

Table 4.5 - Core community OTUs with taxonomy for the *Chloroflexi* community across all three Severn Estuary sites.

Taxonomy		**OTU Number**[a]	**Percentage of Total *Chloroflexi* Community (%)**
Class	Order		
Anaerolineae	DRC31	385	3.22
Anaerolineae	GCA004	275	0.63
Anaerolineae	GCA004	521	0.35
Anaerolineae	GCA004	1218	9.21
Anaerolineae	MSB-1E9	979	0.48
Anaerolineae	OPB11	610	1.61
Anaerolineae	OPB11	1222	4.26
Anaerolineae	S0208	79	0.68
Anaerolineae	S0208	145	5.54
Anaerolineae	S0208	470	0.29
Anaerolineae	S0208	1084	0.46
Anaerolineae	S0208	1233	0.62
Anaerolineae	SB-34	613	0.77
Anaerolineae	SHA-20	493	0.38
Dehalococcoidia	FS117-23B-02	617	0.28
		Total	29%

[a] OTU number related to OTUs in Figure 4.12.

Bacteria at all depths, which indicated a large amount of diversity in the *Chloroflexi* community even though the *Anaerolineae* dominated the community (Figure 4.3B & 4.8). This indicated that there was a great amount of diversity within the *Anaerolineae* itself, much of which is currently uncultivated.

The *Anaerolineae* were by far the most abundant sub-group ranging from 67% to 98% of the *Chloroflexi* community (Figure 4.8). The highest proportion of *Anaerolineae* was found at the intertidal site at 0-2 cbsf (97%) and the deep water sites at 0-2 cbsf and 32-34 cbsf (96% and 98%, respectively). The *Anaerolineae* significantly decreased in abundance at the intertidal site at 6-8 cbsf and were replaced by *Dehalococcoidia* (33%; Class level, Figure 4.9A & B). The *Anaerolineae* community mainly consisted of sequences related to uncultured environmental clones, although the aerobic family *Caldilineaeceae* was detected at all sites and significantly increased in abundance at the oxygenated intertidal 0-2 cbsf (Figures 4.10 & 4.11A). Many of the *Anaerolineae* uncultivated groups were common to all sites at variable amounts, possibly linked to site geomorphology and geochemistry (Figure 4.10).

Uncultivated group GCA004 was one of the most dominant *Anaerolineae* clades, which accounted for 8-28% of the whole *Chloroflexi* community and was the largest single *Chloroflexi* core OTU (OTU1218) at 9.21% of the total *Chloroflexi* community (Figure 4.10; Table 4.4). GCA004 is an order level taxonomic group, consisting entirely of uncultivated environmental clones from mud volcano sediment (AY592319, S. K. Heijs, A. M. Laverman and L. J. Forney, unpublished results), hydrocarbon and methane seeps associated with anaerobic oxidation of methane (AOM; Beal et al., 2009), and marine sediments (GQ246423, Y. Zhao, unpublished results). The top BLAST hits for OTU1218 (99% sequence similarity) were environmental clones from marine sediment habitats including methane and hydrocarbon seeps, unfortunately, none of these sequences had published references and so further information could not be gained. Though, the connection with methane and

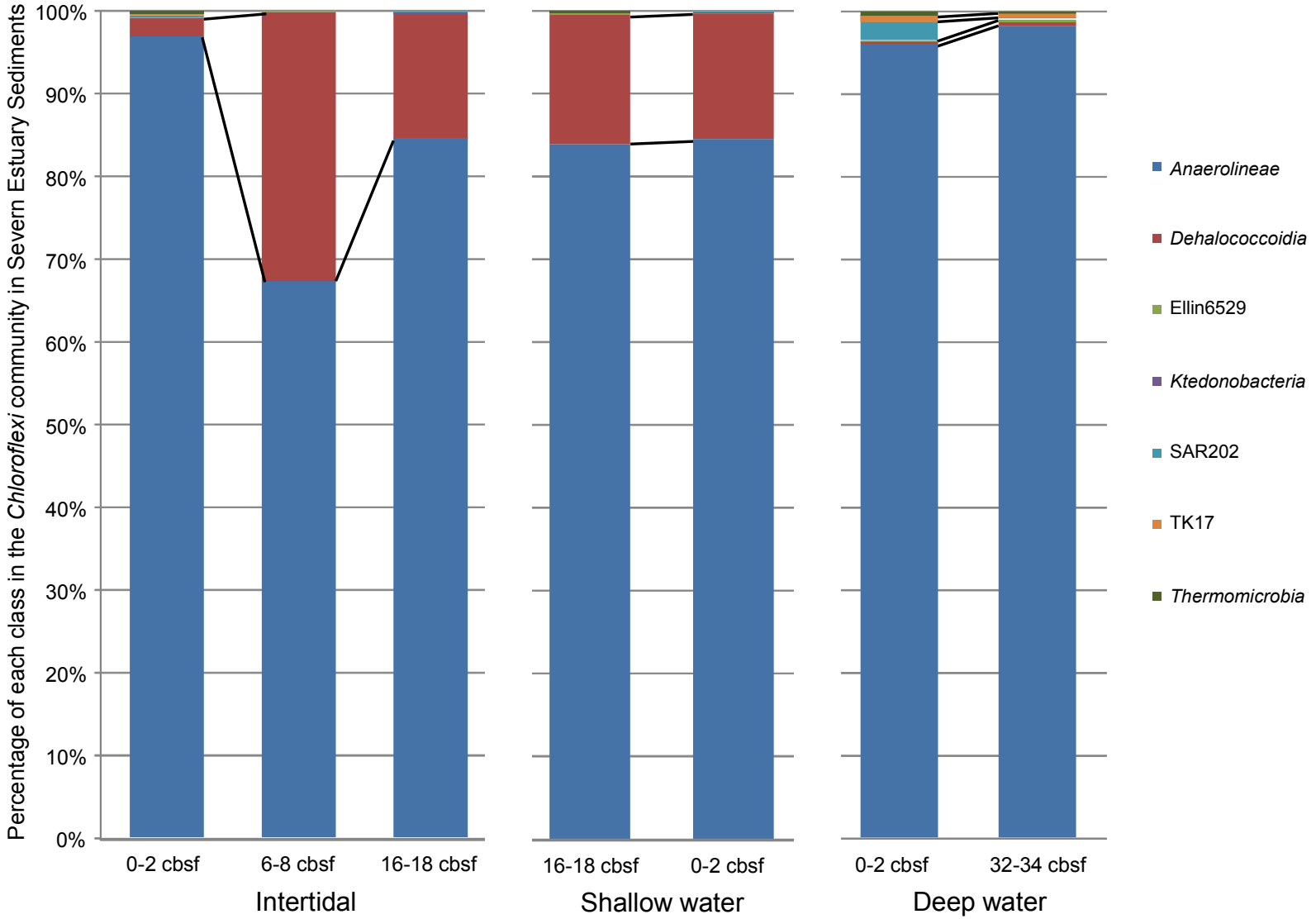


Figure 4.8 - Phylogenetic association at class level based on BLAST results of all *Chloroflexi* 454 sequences at each Severn Estuary site and depth. The percentage of the total number of sequences of the most abundant Chloroflexi class is given. The total number of sequences used were 1561, 1497, 1549, 1477, 1516, 1549 and 1567 respectively. A total of 7 samples from the three sites were used in pyrosequencing of the *Bacteria, Archaea* and *Chloroflexi* libraries. Series lines are given to highlight trends in class changes.

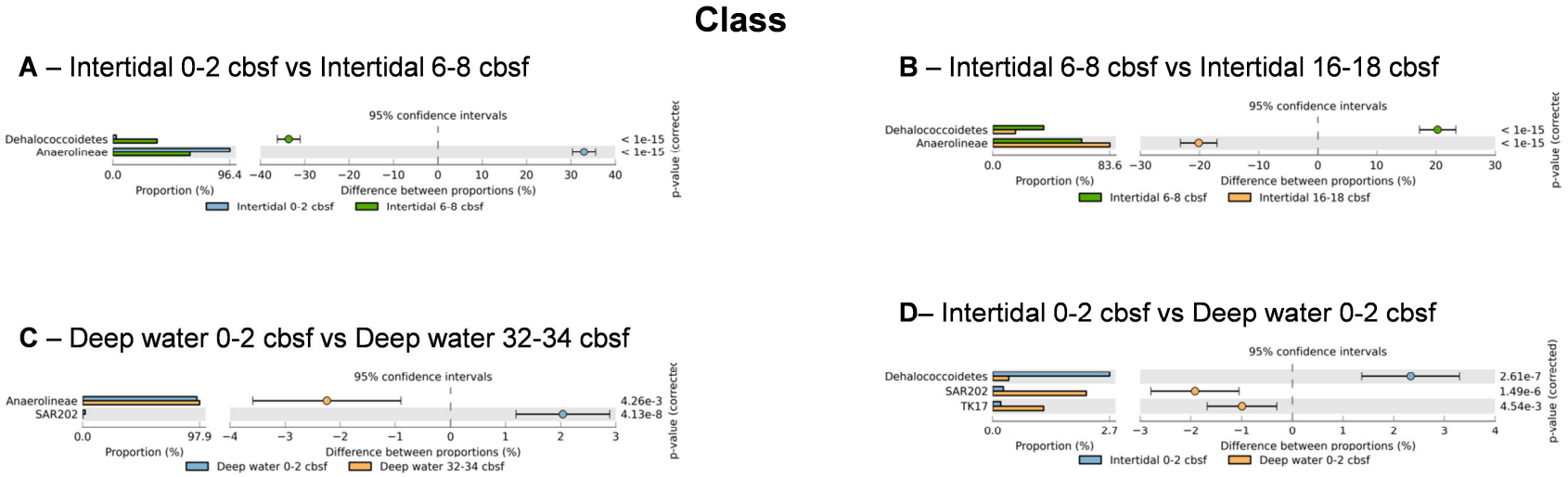


Figure 4.9 **-** Statistical comparisons of the abundance of *Chloroflexi* taxonomic classes in two depth samples from 454 pyrosequencing of the 16S rRNA gene from Severn Estuary sediment. A, intertidal 0-2 cbsf vs intertidal 6-8 cbsf, B, intertidal cbsf vs Intertidal 16-18 cbsf, C, deep water 0-2 cbsf vs deep water 32-34 cbsf, and, D, intertidal 0-2 cbsf vs deep water 0-2 cbsf. No comparison between shallow water sites is given because there was no significant difference at class level. The comparisons E and F were chosen based on groupings of these samples in NMDS (Figure 4.12). Samples were compared using G-test (with Yates') and Fishers' at P value <0.05 with 95% confidence limits and Bonferroni correction (Abdi, 2007; Fisher, 1922; Rivals et al., 2007; Yates, 1934). Only significant (P value <0.05) differences in abundances are shown. Classes are listed in order of descending effect size. Samples tested and colour key are given below each chart. 95% confidence intervals, of the range of effect sizes that have a specified probability of being compatible with the observed data, calculated using asymptotic with continuity correction differences in proportion, are indicated by bars. All statistical analysis and diagrams performed and made using STAMP v2.0.0 (Parks and Beiko, 2010).

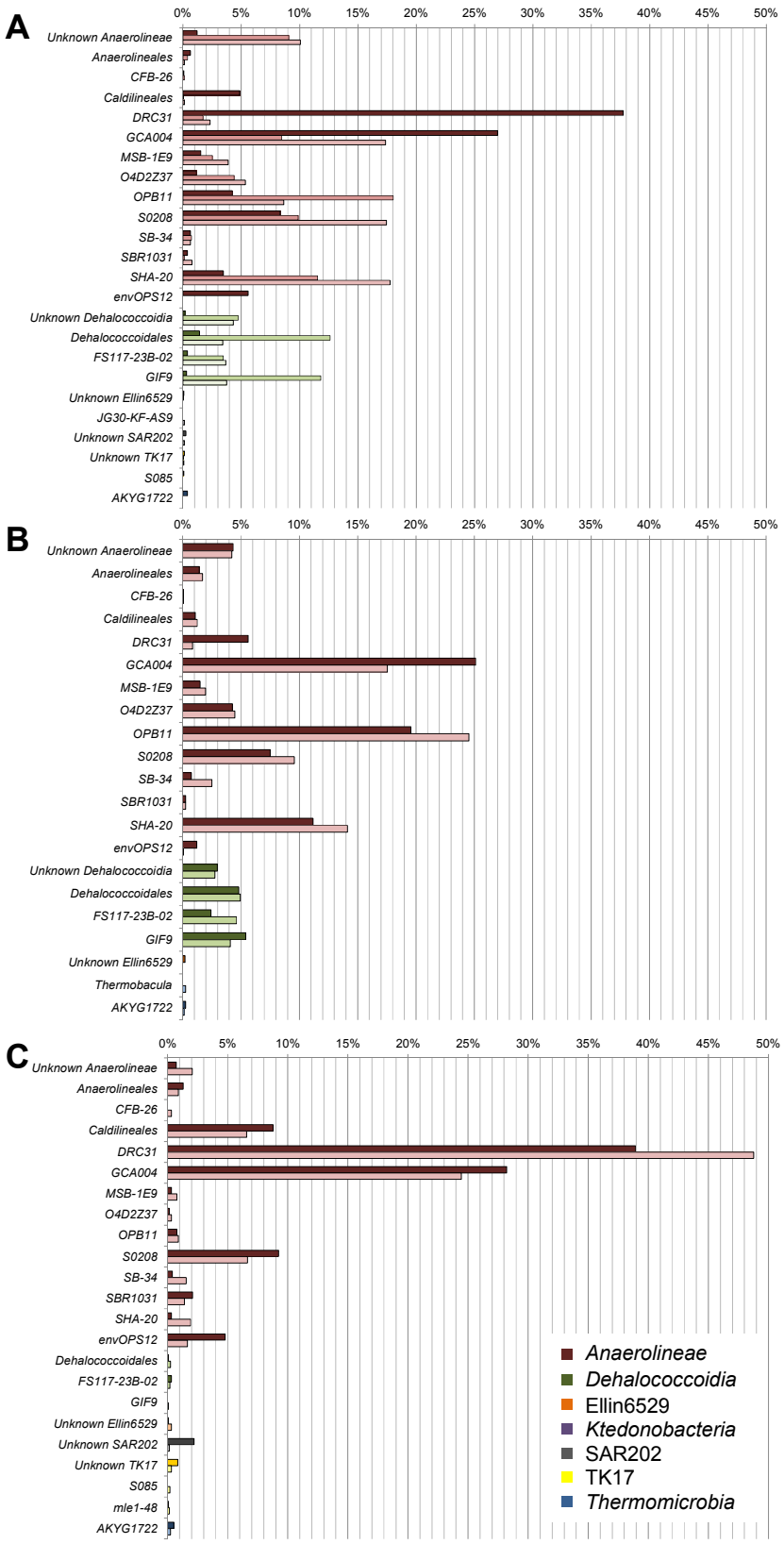


Figure 4.10 - Percentage of the most abundant order level 16S rRNA gene sequences of the total number of *Chloroflexi* for the Severn Estuary sediment sites A) the intertidal (0-2, 6-8 and 16-18 cbsf), B) the shallow water (0-2 and 18-20 cbsf) and C) the deep water (0-2 and 32-34 cbsf). The taxa were derived from BLASTs of all *Chloroflexi* 16S rRNA gene barcode sequences by 454 pyrosequencing. The total number of sequences used were 2046, 2068, and 2211 for the intertidal depths (0-2 cbsf, 6-8 cbsf and 16-18 cbsf, respectively), 2057, and 2198 for the shallow water depths (0-2 and 18-20 cbsf, respectively), 2056, and 2069 for the deep water depths (0-2 and 32-34 cbsf, respectively). Classes are colour coded according to phylum and each depth is represented by a shade of the colour (darkest being the surface sample and the lightest being the deepest sample in each case).

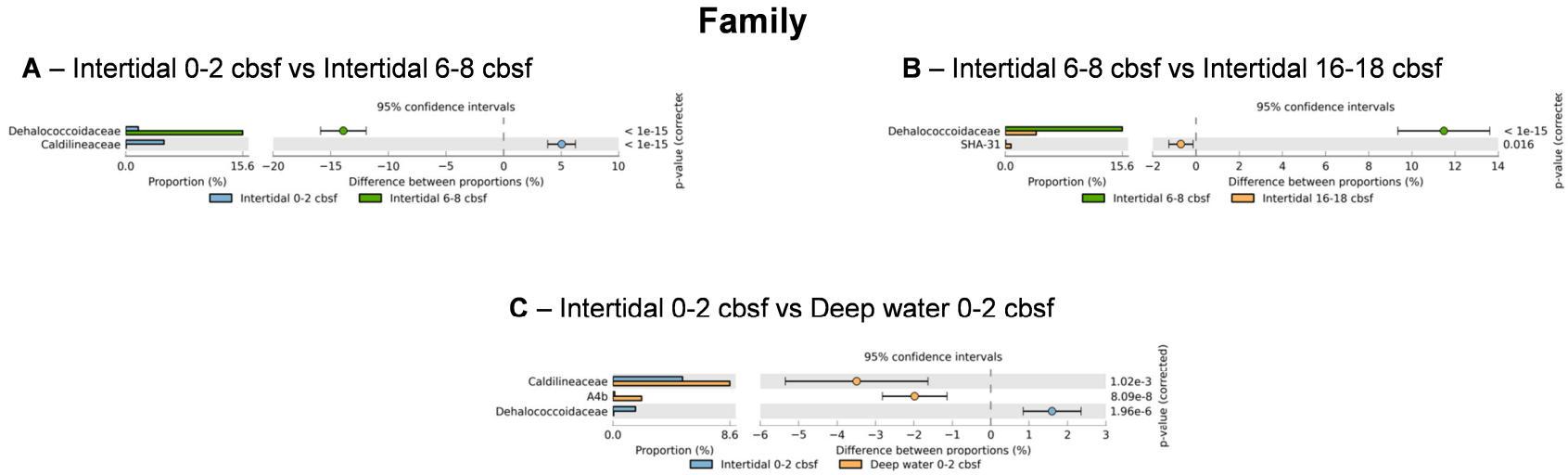


Figure 4.11 **-** Statistical comparisons of the abundance of *Chloroflexi* taxonomic families in two depth samples from 454 pyrosequencing of the 16S rRNA gene from Severn Estuary sediment. A, intertidal 0-2 cbsf vs intertidal 6-8 cbsf, B, intertidal cbsf vs Intertidal 16-18 cbsf, and, C, intertidal 0-2 cbsf vs deep water 0-2 cbsf. No comparisons between shallow water and deep water sites are given because there was no significant difference at class level. The comparisons C and D were chosen based on groupings of these samples in NMDS (Figure 4.12). Samples were compared using G-test (with Yates') and Fishers' at P value <0.05 with 95% confidence limits and Bonferroni correction (Abdi, 2007; Fisher, 1922; Rivals et al., 2007; Yates, 1934). Only significant (P value <0.05) differences in abundances are shown. Families are listed in order of descending effect size. Samples tested and colour key are given below each chart. 95% confidence intervals, of the range of effect sizes that have a specified probability of being compatible with the observed data, calculated using asymptotic with continuity correction differences in proportion, are indicated by bars. All statistical analysis and diagrams performed and made using STAMP v2.0.0 (Parks and Beiko, 2010).

hydrocarbon laden environments indicated that GCS004 was most likely contributing to the anaerobic terminal organic degradation of carbon in the organic rich sediments of the Severn Estuary.

The clade DRC31 accounted for 37% of the *Chloroflexi* community at the intertidal site at 0-2 cbsf and for 38% and 49% at both depths at the deep water site, respectively (Figure 4.10A & C). The core OTU clone, OTU385, was also related (97% sequence similarity) to DRC31 and made up 3.22% of the total *Chloroflexi* community (Table 4.4). As for GCA004, DRC31 is an order level taxonomic group, consisting entirely of uncultivated environmental clones from a variety of anaerobic environments, including digester sludge (Riviere et al., 2009), soils (DQ811864; B. Yan, and K. Hong, unpublished results) and marine mud volcano clones related to the anaerobic oxidation of methane (AOM)(Heijs et al., 2007). OTU385, for example, shared 99% sequence similarity with the environmental clone OrigSedB11, which was isolated from marine methane-seep sediment, Eel River Basin, California (Beal et al., 2009). Interestingly, the OrigSedB11 was part of a bacterial community capable of coupling the reduction of manganese and iron to the oxidation of methane (Beal et al., 2009). Though the prediction of physiology from 16S rRNA gene DNA is uncertain, the similarity of the *Chloroflexi* OTUs with a number of environmental clones involved in AOM indicated the involvement of *Anaerolineae* in the AOM process, which is novel for the class. The archaeal groups ANME, which are capable of AOM, were detected at the intertidal site at the lowest depths and at the deepest shallow water site samples **(Section 4.3.4)** and these require indirect bacterial partners to reduce the electron acceptor (Niemann et al., 2006; Orphan et al., 2001). Also, DRC31 were in highest abundances in aerobic or anoxic sediment and so not suitable for AOM, so it was hypothesized that the *Anaerolineae* were involved in the reduction of the electron acceptor step for the AOM process.

Conversely, OPB11, S0208 and SHA-20 clades were detected in high abundances at the deeper intertidal samples and both shallow water

samples, though were not significantly different (Figure 4.10A & B). These sediment were likely to maintain anoxic or dysaerobic geochemistry. Again, these clades were an order level taxonomic group, consisting entirely of uncultivated environmental clones. OPB11 is made up of a large number of clones isolated from hypersaline microbial mats (Ley et al., 2006); S0298 is dominated by soil clones (Elshahed et al., 2008), and SHA-20 consists of clones originally isolated from mesophilic mangrove and mud volcano sediments (EF061971, X. Zhang and J. Xu, unpublished results; AY592333, S. K. Heijs, A. M. Laverman and L. J. Forney, unpublished results). All of these clades were included in the *Chloroflexi* core community OTUs (Table 4.4). S0208 had the most core OTUs, with OTU145 being the largest abundance (5.54%; Table 4.4). OTU145 shared 99% sequence similarity with two clones: the first an unpublished sequence from siliciclastic sediment from a sea grass bed and the second, D221W_H73, isolated from a coastal site in the South China sea, with low salinity and high total organic carbon (TOC) and total organic nitrogen (TON) due to influence from coastal freshwaters (Du et al., 2011). OPB11 also contributed to the *Chloroflexi* core community with OTU1222 constituting 4.26% of the total community (Table 4.4). The highest sequence similarity to OTU1222 (97%) were all from the same unpublished experiment, which investigated carbon cycling at cold seep sediments. As with GCS004, these related sequences indicated that the OPB11 and S0208 were involved with anaerobic terminal organic degradation of carbon in the organic rich sediments of the Severn Estuary. This concurred with research which found that *Anaerolineae* may have an indirect role in methanogenesis by providing hydrogen substrates (Hamdan et al., 2012; Yamada et al., 2007) and work by Kindaichi et al. (2012), which hypothesized that *Anaerolineae* have broad metabolic capability to scavenge organic compounds and decaying cell debris to survive in mixed aerobic and anaerobic niches such as sediments.

The *Dehalococcoidia* sub-group constituted a significant proportion of the *Chloroflexi* community at the lowest intertidal depths (6-8 and 16-18 cbsf) and throughout the shallow water core (15-16%; Figure 4.8). The

Dehalococcoidia significantly increased in abundance at the intertidal intermediary layer (6-8 cbsf; Figures 4.5A & B and 4.9A & B), which was also where the *Chloroflexi* rose to the most abundant phylum, indicating a very important role. The *Dehalococcoidia* consisted of members of the family *Dehalococcoidaceae* and sequences related to the uncultivated clone GIF9 (Family level, Figure 4.11A & B). However, the *Dehalococcoidaceae,* to which the cultured representatives of the dehalorespiring *Dehalococcoidia* belong, was the only family to significantly increase in abundance (Figure 4.11A & B; Löffler et al., 2013). The uncultivated GIF9 clone was originally isolated from a reactor system for dechlorinating polluted groundwater (Alfreider et al., 2002). The significant increase in the *Dehalococcoidia* could indicate that dehalogenation was occurring at the intertidal 6-8 cbsf and as the *Chloroflexi* phylum also increased rapidly, this indicated that dehalogenation was a major method of alternative anaerobic metabolism in Severn Estuary intertidal sediments. Interestingly, the JS1 clade, within the *Atribacteria* (Rinke et al., 2013), which was often associated with *Chloroflexi* in the deep subsurface (Webster et al., 2004), also increased in abundance significantly at the intertidal site 6-8 cbsf (Figure 4.5B), which indicated a possible relationship between the two.

The SAR202 were found at the intertidal and deep water sites and constituted 2% of the *Chloroflexi* community in the surface deep water sediment (0-2 cbsf), which was a significantly higher abundance (Figure 4.9C). The SAR202 usually dwell in the water column, which suggested that these bacteria were mixed into these sediments by tidal forces (Morris et al., 2004). All other subdivisions were below 1% of the total *Chloroflexi* community (Figure 4.8). *Ktedonobacteria* were detected at just the intertidal site at 16-18 cbsf and the *Thermomicrobia* were detected at all sites and depths except the bottom depths of the intertidal site, none of which were in statistically significant amounts (Figures 4.8 & 4.10). Of the two clone groups, Ellin6529 was detected in all surface (0-2 cbsf) samples at all three sites as well at 32-34 cbsf at the deep water site and 6-8 cbsf at the intertidal site (Figures 4.8 & 4.10). TK17 related sequences were

found at all depths at the intertidal and deep water sites but not in the shallow water sediment (Figures 4.8 and 4.10A & C).

NMDS and heatmaps were again used to assess the impact of individual OTUs in the Chloroflexi *community.* As previously stated, the NMDS for all three communities were identical, which suggested that the *Chloroflexi* followed a similar pattern of distribution in relation to sediment geochemistry and geomorphology to the *Bacteria* (Figure 4.12A; **Section 4.3.1**). The UPGMA clustering of samples within the heatmap revealed two main groups, one, like the *Bacteria,* consisted of intertidal 0-2 cbsf and both deep water site depths (Figure 4.12B). All other samples were in the second group and appeared to be clustering partially by site. The first group, with the intertidal 0-2 cbsf and both deep water site depths, saw most of the OTUs shared between the deep water sites. These were mainly *Anaerolineae* OTUs of various clonal groups. One OTU was shared by all three samples in large amounts and was related to DRC31, an environmental clone from methane seep sediment (Beal et al., 2009). Again, in the second group, a number of *Anaerolineae* OTUs were shared between all samples. Only the two intertidal samples shared *Dehalococcoidia* OTUs. These however were from an unknown source. The shallow water sites grouped together by UPGMA and were also found to not be significantly different to each other, based on sequence abundances in STAMP. A comparison between intertidal 0-2 cbsf and deep water 0-2 cbsf was performed due to the similarity indicated in the UPGMA clustering and NMDS (Figure 4.9D & 4.11E). Between these samples, *Anaerolineae* was not significant, presumably due to similar abundances (Figure 4.8) but *Dehalococcoidia* was significant at the intertidal site and the clone groups SAR202 and TK17 were significant at the deep water site (Figures 4.9D & 4.11E).

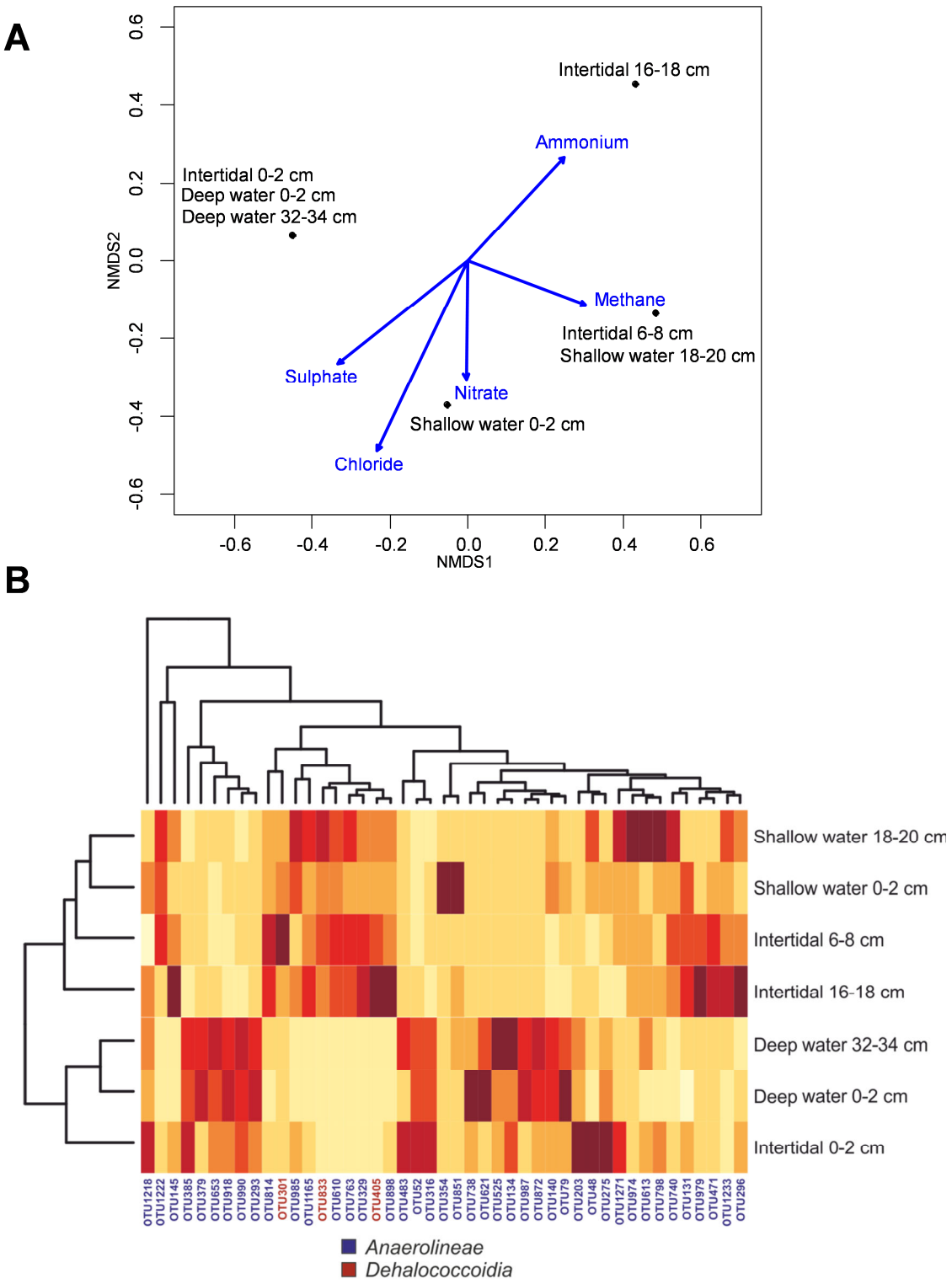


Figure 4.12 - Statistical analysis of specific *Chloroflexi* OTUs from 454 pyrosequencing of all Severn Estuary sediment samples using the R statistical package (Ihaka and Gentleman, 1996). A, NMDS plot with samples represented as black circles and labelled and geochemical vectors as blue arrows. A total of 7 samples from the three sites were used in pyrosequencing of the *Bacteria, Archaea* and *Chloroflexi* libraries. B, Heatmap demonstrating the prevalence of the 50 most abundant OTUS based on the number of sequences across each site and depth. UPGMA clustering dendrograms are given for both the sample and OTUS. The colour gradient indicates abundance with dark red being the most and pale yellow being the least abundant. The OTU number are given next to each row and these are coloured, as indicated in the key, by phyla, discerned from BLAST results.

4.3.4 Analysis of the Archaeal Severn Estuary Community with Depth and Sediment Geochemistry

The archaeal diversity of Severn Estuary sediments was assessed using well established archaeal specific PCR primers. Three archaeal phyla, the *Crenarchaeota, Thaumarchaeota* and *Euryarchaeota,* were detected at each site and depth in varying amounts, relating to the geochemistry and geomorphology of each site and depth. At phylum level there was found to be no significant difference between the archaeal communities at each site by ANOVA in STAMP (Parks and Beiko, 2010).

This was explained by the generally high numbers of *Thaumarchaeota* across the three sites and the relatively few phyla (Figure 4.13). The *Thaumarchaeota* was the most abundant phylum in five of the seven samples, composing 97% of the archaeal community at the intertidal site at 0-2 cbsf and 99% of the community at both deep water site depths (Figure 4.13). The *Crenarchaeota* replaced the *Thaumarchaeota* as the most dominant phylum at the bottom intertidal depths, constituting 48-65% of the archaeal community. The *Euryarchaeota* varied across the sites and depths; less than 1% of the community at the intertidal surface sediment and deep water site but rose to 11-31% at the shallow water site and deepest intertidal site sediment.

The core community of *Archaea,* shared across the three sites and all depths, consisted of only 3 OTUs but these constituted 58% of the total archaeal community in all samples (Table 4.6). Furthermore, the core community percentage at each depth indicated that these 3 OTUs dominated the intertidal 0-2 cbsf, the shallow water site and the deep water site (Figure 4.3). The very small number of shared OTUs but large community coverage indicated low diversity at these sites and depths, which was also indicated by alpha diversity indices (Table 4.2). At the deeper intertidal depths, however, the percentage of the core OTUs of the whole archaeal community dropped to 12% and 15%, respectively, which indicated a much higher amount of diversity, characterised by a

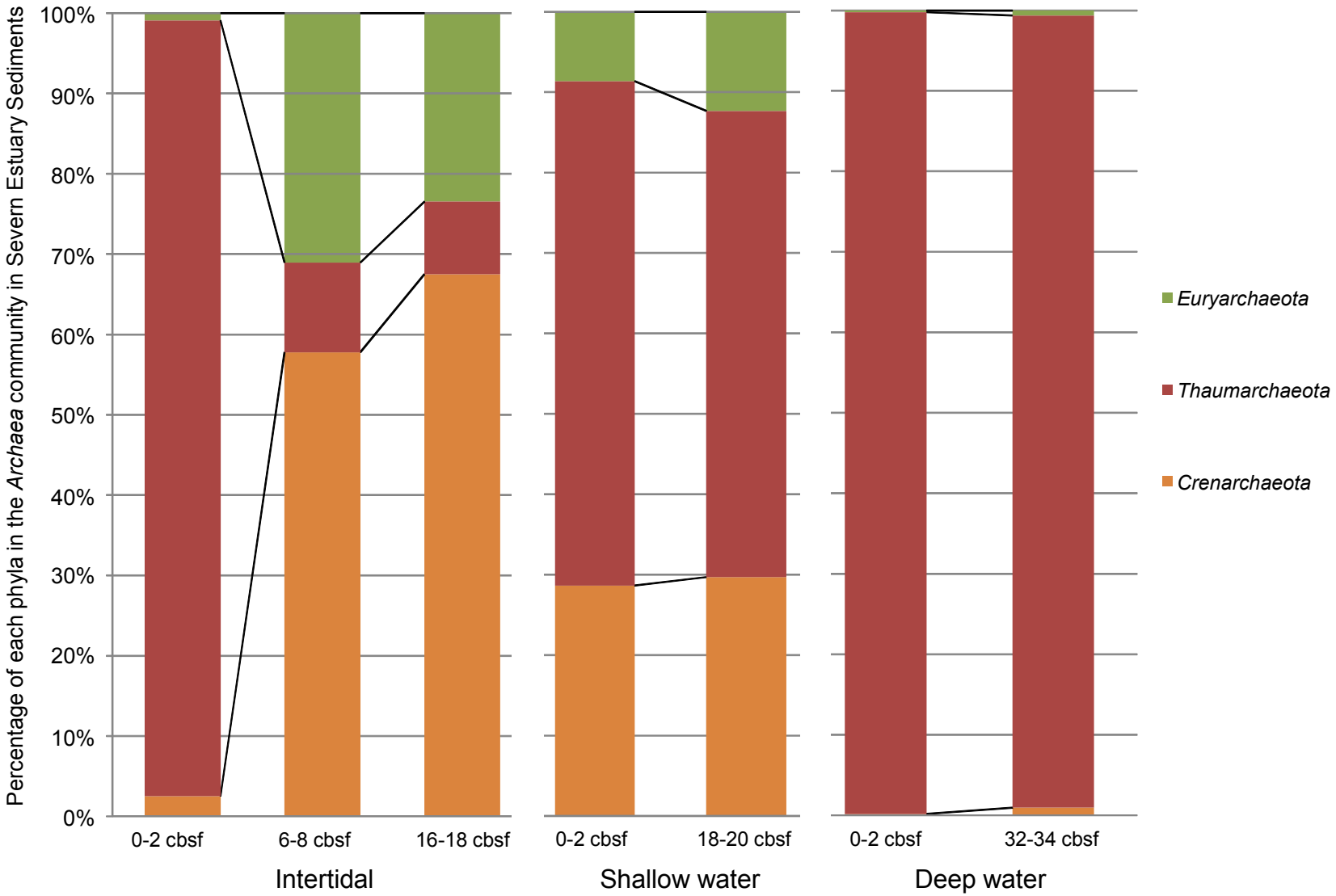


Figure 4.13 **-** Phylogenetic association, at phylum level, based on BLAST results of all archaeal 454 sequences at each Severn Estuary site and depth. The percentage of the total number of sequences of the Archaea phyla is given. The total number of sequences used were 1670, 1244, 1595, 1569, 1625, 1690 and 1683 respectively. A total of 7 samples from the three sites were used in pyrosequencing of the *Bacteria, Archaea* and *Chloroflexi* libraries. Series lines are given to highlight trends in phylum changes.

Table 4.6 **-** Core community OTUs with taxonomy for the *Archaea* community across all three Severn Estuary sites.

Taxonomy					OTU Number[a]	Percentage of Total *Bacteria* Community (%)
Phylum	Class	Order	Family	Genus/Species		
Thaumarchaeota	Marine Group 1.1a	*Cenarchaeales*	*Cenarchaeaceae*	*Nitrosopumilus*	427	44.33
Thaumarchaeota	Marine Group 1.1a	*Cenarchaeales*	*Cenarchaeaceae*	*Nitrosopumilus*	428	10.13
Crenarchaeota	MCG	B10			1134	3.07
					Total	58%

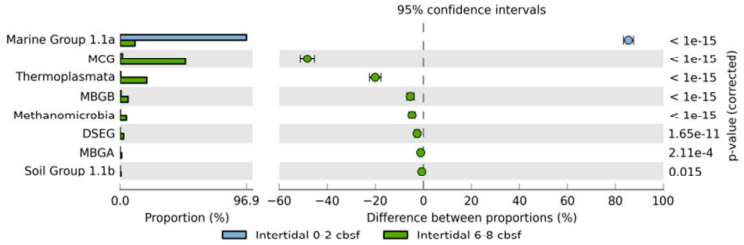
[a] OTU number related to OTUs in Figure 4.17.

statistically significant increase in the class MCG (phylum *Crenarchaeota;* Figure 4.14A & B). At the depths where the *Thaumarchaeota* dominated, the 'Marine' Group 1.1a was the most dominant class of the archaeal community (57% and 99% of *Archaea;* Figures 4.14A & D and 4.15A & C) and even the lower taxonomic order *Nitrosopumilales* composed 57-99% of the total *Archaea* community. The 'Marine' Group 1.1a showed little diversity in its lower taxonomic levels, as the family *Cenarchaeaceae* was by far the most significantly abundant family in intertidal surface and deep water sediments (Figure 4.16A & D). Two of the archaeal core OTUs (OTU427 and OTU428) were *Nitrosopumilus,* accounting for 44.33% and 10.13% of the total archaeal community, respectively (Table 4.6). The largest, OTU427, shared 99% sequence similarity with the environmental clone E09-BR2-1, which was isolated from Brightlingsea, in the Colne Estuary UK. This was a high salinity marine sediment, dominated entirely by *Thaumarchaeota*, as were these Severn Estuary sediments, which emphasized the general importance of the *Thaumarchaeota* in estuarine sediments (G. Webster, L. A. O'Sullivan, Y. Meng, A. S. Williams, A. M. Sass, A. J. Watkins, R. J. Parkes & A. J. Weightman, unpublished results). The genus consists of aerobic, chemolithoautotrophic, ammonia oxidisers, involved in nitrification which indicated that this process was occurring in these Severn Estuary sediments (Brochier-Armanet et al., 2008; Könneke et al., 2005), which was previously indicated by the abundance of *Hyphomicrobium* in the bacterial community at depth rather that surface sediments **(Section 4.3.2)**.

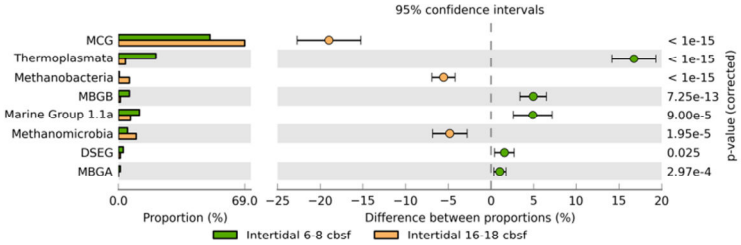
The *Nitrososphaerales* order of the 'Soil' Group 1.1b (also a member of the *Thaumarchaeota)* was also detected in much smaller proportions at all sites except the deep water site and the 'Soil' Group 1.1b significantly increased in abundance with depth at the intertidal site (Figures 4.14A & 4.15A). The *Nitrososphaerales* is also capable of ammonia oxidation (Tourna et al., 2011). Together the presence of these multiple nitrogen cycling pathways in such large abundances suggested that nitrogen based metabolisms were important in prokaryotic communities in Severn Estuary sediments at all depths investigated. In the intertidal 6-8 cbsf and 16-18 cbsf sediment, the

Class

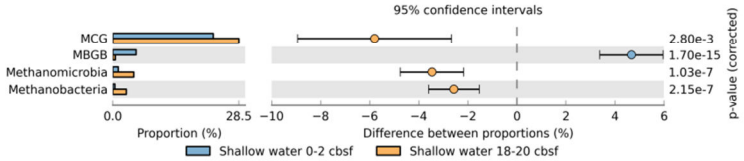
A – Intertidal 0-2 cbsf vs Intertidal 6-8 cbsf



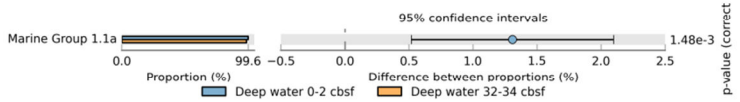
B – Intertidal 6-8 cbsf vs Intertidal 16-18 cbsf



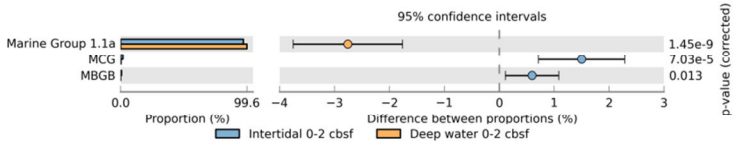
C – Shallow water 0-2 cbsf vs Shallow water 18-20 cbsf



D – Deep water 0-2 cbsf vs Deep water 32-34 cbsf



E – Intertidal 0-2 cbsf vs Deep water 0-2 cbsf



F – Intertidal 6-8 cbsf vs Shallow water 18-20 cbsf

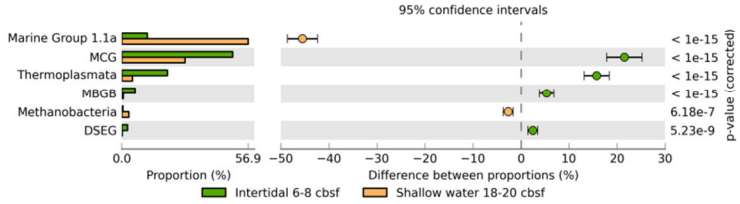


Figure 4.14 - Statistical comparisons of the abundance of *Archaea* taxonomic classes in two depth samples from 454 pyrosequencing of the 16S rRNA gene from Severn Estuary sediment. A, intertidal 0-2 cbsf vs intertidal 6-8 cbsf, B, intertidal cbsf vs Intertidal 16-18 cbsf, C, shallow water 0-2 cbsf vs shallow water 18-20 cbsf, D, deep water 0-2 cbsf vs deep water 32-34 cbsf, E, intertidal 0-2 cbsf vs deep water 0-2 cbsf and, F, intertidal 6-8 cbsf vs shallow water 18-20 cbsf. The comparisons E and F were chosen based on groupings of these samples in NMDS (Figure 4.17). Samples were compared using G-test (with Yates') and Fishers' at P value <0.05 with 95% confidence limits and Bonferroni correction (Abdi, 2007; Fisher, 1922; Rivals et al., 2007; Yates, 1934). Only significant (P value <0.05) differences in abundances are shown. Classes are listed in order of descending effect size. Samples tested and colour key are given below each chart. 95% confidence intervals, of the range of effect sizes that have a specified probability of being compatible with the observed data, calculated using asymptotic with continuity correction differences in proportion, are indicated by bars. All statistical analysis and diagrams performed and made using STAMP v2.0.0 (Parks and Beiko, 2010).

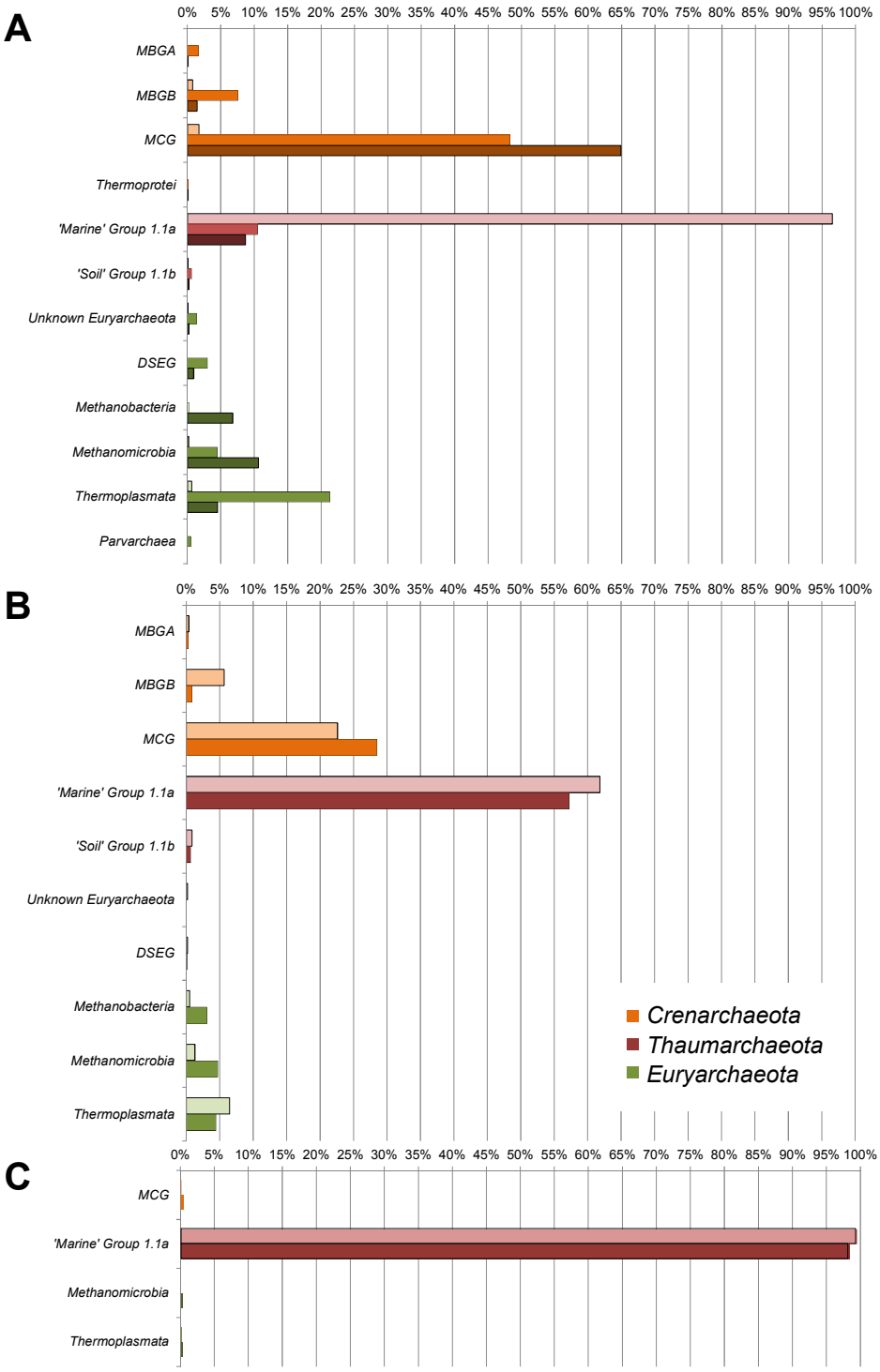
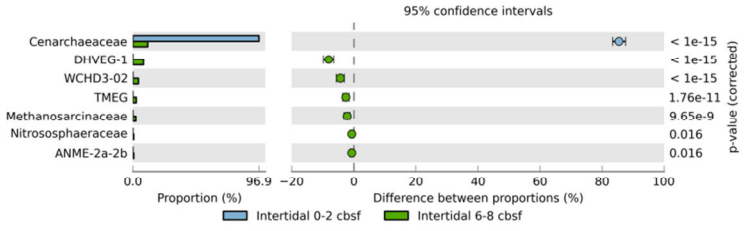


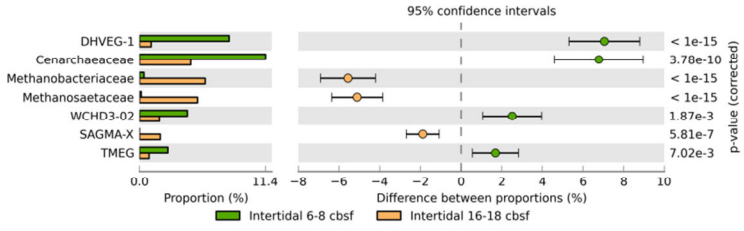
Figure 4.15 - Percentage of the most abundant class level 16S rRNA gene sequences of the total number of *Archaea* for the Severn Estuary sediment sites A) the intertidal (0-2, 6-8 and 16-18 cbsf), B) the shallow water (0-2 and 18-20 cbsf) and C) the deep water (0-2 and 32-34 cbsf). The taxa were derived from BLASTs of all archaeal 16S rRNA gene barcode sequences by 454 pyrosequencing. The total number of sequences used were 1670, 1244, and 1595 for the intertidal depths (0-2 cbsf, 6-8 cbsf and 16-18 cbsf, respectively), 1569, and 1625 for the shallow water depths (0-2 and 18-20 cbsf, respectively),1690, and 1683 for the deep water depths (0-2 and 32-34 cbsf, respectively). Classes are colour coded according to phylum and each depth is represented by a shade of the colour (lightest being the surface sample and the darkest being the deepest sample in each case).

Family

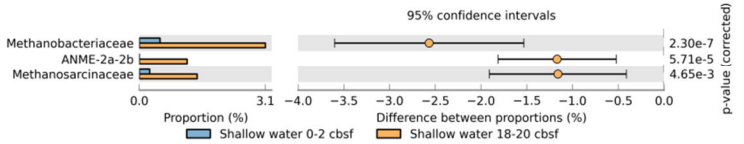
A – Intertidal 0-2 cbsf vs Intertidal 6-8 cbsf



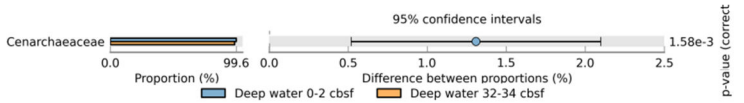
B – Intertidal 6-8 cbsf vs Intertidal 16-18 cbsf



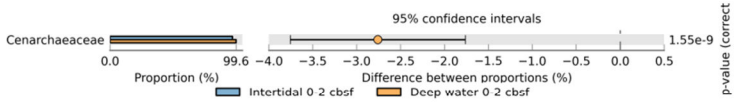
C – Shallow water 0-2 cbsf vs Shallow water 18-20 cbsf



D – Deep water 0-2 cbsf vs Deep water 32-34 cbsf



E – Intertidal 0-2 cbsf vs Deep water 0-2 cbsf



F – Intertidal 6-8 cbsf vs Shallow water 18-20 cbsf

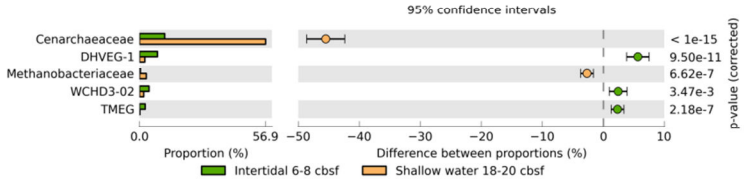


Figure 4.16 - Statistical comparisons of the abundance of *Archaea* taxonomic family in two depth samples from 454 pyrosequencing of the 16S rRNA gene from Severn Estuary sediment. A, intertidal 0-2 cbsf vs intertidal 6-8 cbsf, B, intertidal cbsf vs Intertidal 16-18 cbsf, C, shallow water 0-2 cbsf vs shallow water 18-20 cbsf, D, deep water 0-2 cbsf vs deep water 32-34 cbsf, E, intertidal 0-2 cbsf vs deep water 0-2 cbsf and, F, intertidal 6-8 cbsf vs shallow water 18-20 cbsf. The comparisons E and F were chosen based on groupings of these samples in NMDS (Figure 4.17). Samples were compared using G-test (with Yates') and Fishers' at P value <0.05 with 95% confidence limits and Bonferroni correction (Abdi, 2007; Fisher, 1922; Rivals et al., 2007; Yates, 1934). Only significant (P value <0.05) differences in abundances are shown. Families are listed in order of descending effect size. Samples tested and colour key are given below each chart. 95% confidence intervals, of the range of effect sizes that have a specified probability of being compatible with the observed data, calculated using asymptotic with continuity correction differences in proportion, are indicated by bars. All statistical analysis and diagrams performed and made using STAMP v2.0.0 (Parks and Beiko, 2010).

Crenarchaeota were the dominant phyla and MCG became the dominant class representing 48% and 65% of the community respectively (Figure 4.15A & C). The MCG significantly increased in abundance with depth at both the intertidal and shallow water sites (Figure 4.14A-C). Most of the MCG related sequences could not be assigned below the class level. The only core *Archaea* OTU (OTU1134, 3.07% of the total archaeal community) which was not *Thaumarchaeota* was related to the MCG order level environmental clone group B10 (Table 4.6). OTU1134 shared 100% sequence similarity with an uncultivated clone from Hythe, in the Colne Estuary, UK (Webster et al., unpublished results). The Hythe sediments, in comparison to Brightlingsea, were low salinity sediments dominated by methanogens and MCG with depth. This link suggested that MCG are more abundant at greater depths in estuarine sediments and are associated with methanogenic processes. MCG are a clonal group with little description of their biogeochemical or metabolic potential.

Whereas the *Euryarchaeota* comprised <1% of the archaeal community at the intertidal surface and deep water site sediment, they constituted 8-30% in the other sediment (Figure 4.13). The greatest proportion (30%) appeared at the intertidal site at 6-8 cbsf (Figure 4.13). The *Euryarchaeota* were not a part of the archaeal core community which suggested that the specific metabolic process associated with the *Euryarchaeota,* such as methanogenesis and AOM, were isolated to the deeper, anaerobic niches. Class dominance in the deepest intertidal and both shallow water sediment varied between the *Thermoplasmata* (order E2) and the *Methanomicrobia* (Figure 4.14A & B). The *Methanomicrobia* and the families *Methanosarcinaceae* and *Methanobacteriaceae* significantly increased in abundance with depth and both the intertidal and shallow water sites (Figures 4.14 A-C & 4.16A-C). *Methanomicrobia* consisted of several different orders including the *Methanocellales, Methanomicrobiales* and *Methanosarcinales*. These, and the *Methanobacteria,* which were also detected in low amounts, were all methanogenic *Archaea* indicating methanogenesis was occurring at depth in this sediment. As previously described, the abundance of SRB decreased with depth at these sites

(Section 4.3.2) and so sulphate reduction appeared to be replaced with methanogenesis. The class *Thermoplasmata,* which significantly increased in abundance at intertidal 6-8 cbsf (Figure 4.14A & B), as did the *Euryarchaeota* phylum, mainly consisted of clone groups such as the TMEG and Marine group II (Figure 4.16), which were commonly thermophilic, acidophilic organisms although methanogenic *Thermoplasmata* have recently been described (Iino et al., 2013; Poulsen et al., 2013).

The NMDS (Figure 4.17A) performed on archaeal OTUs was very similar to those for the *Bacteria* (Figure 4.5A) and *Chloroflexi* (Figure 4.9A), which, again, indicated a shared community structure in response to the geochemical conditions. NMDS and a heatmap of the most abundant OTUs in the archaeal community revealed three clusters of samples (Figure 4.17B). The first group, like the *Bacteria* and *Chloroflexi,* consisted of both deep water samples and the surface intertidal site sample. The second contained the remaining intertidal samples and the third grouping was shallow water sites. These clusters were similar to the smaller *Chloroflexi* community but different to the *Bacteria.* In the first group, there were very few very abundant OTUs that were shared between all samples (Figure 4.17B). Remarkably, all of these shared OTUs related to the same clone from deep marine sediment and belonging to the genus *Nitrosopumilus*. The second group, which was the remaining intertidal samples, shared some OTUs with the shallow water sediment. These were mainly MCG in the shallow water sediment but *Thermoplasmata, Methanomicrobia* and *Methanobacteria* were all detected in the intertidal sediment. The third group also shared OTUs with group one related to *Nitrosopumilus* (Figure 4.17B).

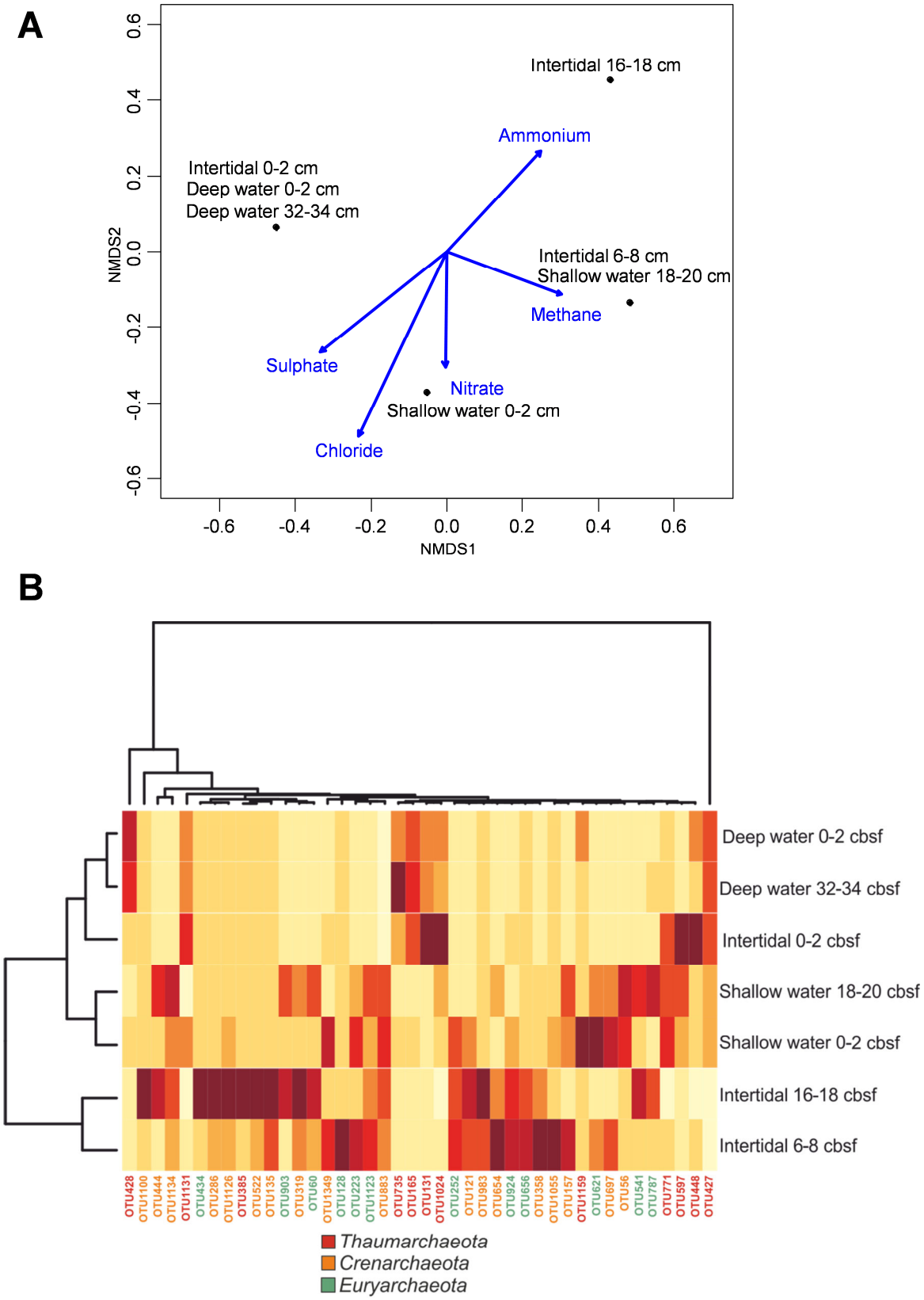


Figure 4.17 - Statistical analysis of specific *Archaea* OTUs from 454 pyrosequencing of all Severn Estuary sediment samples using the R statistical package (Ihaka and Gentleman, 1996). A, NMDS plot with samples represented as black circles and labelled and geochemical vectors as blue arrows. A total of 7 samples from the three sites were used in pyrosequencing of the *Bacteria, Archaea* and *Chloroflexi* libraries. B, Heatmap demonstrating the prevalence of the 50 most abundant OTUS based on the number of sequences across each site and depth. UPGMA clustering dendrograms are given for both the sample and OTUS. The colour gradient indicates abundance with dark red being the most and pale yellow being the least abundant. The OTU number is given next to each row and these are coloured, as indicated in the key, by phyla, discerned from BLAST results.

4.4 Discussion

4.4.1 Prokaryotic Communities across Different Severn Estuary Sites were Highly Similar

A core community (OTUs shared across all sites and depths) of prokaryotes was discovered to be present across the surface sediment of all three geomorphologically different sites in the Severn Estuary. For *Bacteria*, this core set consisted of five phyla, *Proteobacteria, Chloroflexi, Firmicutes, Actinobacteria* and *Bacteroidetes* which made up over 90% of the bacterial community. The *Archaea* core community was much less diverse than the *Bacteria* and *Chloroflexi,* consisting mainly of the *Thaumarchaeota.* Statistically, the prokaryotic community structure and abundances at all three sites were not significantly different from each other at the phylum level. These findings compare well with the previous profiling studies described in **Chapter 3**, which, for the *Bacteria* community, also indicated that *Proteobacteria, Chloroflexi, Firmicutes, Actinobacteria* and *Bacteroidetes* were common to all sites **(Section 3.3.1)**. Similarly, *Proteobacteria* were found to frequently dominate estuarine sediment communities at the Colne Estuary (O'Sullivan et al., 2013), Pearl River Estuary (Sun et al., 2012) and the intertidal sediments at Portishead, Severn Estuary (Webster et al., 2010). Webster et al. (2010) found a dominance of *Crenarchaeota* in Severn Estuary sediment, not the abundance of *Thaumarchaeota* found in this study. However, the investigation by Webster utilised SIP and DGGE whereas this study delved much deeper to give a broader description of the prokaryotic communities in the Severn Estuary sediments. Previous investigations into estuarine prokaryotic communities found links between salinity, geochemical gradients from head to mouth of the estuary, and shifts in the microbial communities in these sediments (Bernhard and Bollmann, 2010; Crump et al., 2004). This study limited these effects by utilising transect across the estuary with the same salinity at all sites, which would explain the general similarity in the communities in comparison to previous studies (Dong et al., 2009; O'Sullivan et al., 2013; Purdy et al., 2002).

4.4.2 Sediment Depth and Geochemistry Influence Bacterial Community Structure

It transpired that sediment depth was the greatest influence on Severn Estuary Prokaryotic communities across the site transect. *Proteobacteria* were replaced by *Firmicutes* and *Chloroflexi* as depth increased and within the *Proteobacteria* depths changes were noted. The *Gammaproteobacteria* and to some extent the *Deltaproteobacteria* also decreased and were replaced by *Alphaproteobacteria.* The change appeared to be largely due to the switch from aerobic to anaerobic sediment and therefore also linked to the unique geochemical processes that occur in these environments. These exact changes from *Proteobacteria* to *Firmicutes* and *Chloroflexi* were also described by Wilms et al. (2006b) in tidal flat sediments, over 220 cm, including the shift from *Gammaproteobacteria* in the upper sandy sediments.

A potentially important finding from this study was that in each of the main prokaryotic communities, one family dominated, which appeared related to depth. For the *Bacteria* this was the *Hyphomicrobiaceae*, which increased significantly with depth at each site to a maximum of 28% of the entire bacterial community at 18-20 cbsf at the shallow water site. The genus *Hyphomicrobium* was the most common, which mainly consists of metabolically diverse, common in soils, aquatic habitats and sewage plants (Sakairi et al., 1996; Satoh et al., 2006). Many of these strains possess denitrification, nitrogen fixation and also metal oxidisation (such as manganese) capabilities (Fesefeldt et al., 1998; Martineau et al., 2013; Stein et al., 2001). As yet no evidence of a role nitrification has been found (Fesefeldt et al., 1998). Previous work, discussed in **Chapter 3**, suggested that nitrification and denitrification were occurring in the intertidal and deep water sediment. *Alphaproteobacteria* profiles were found to be significantly negatively correlated with ammonium in at the intertidal site **(Table 3.4)**. Also, geochemical profiles (Shaun Thomas, Cardiff University, results not shown) indicated a decrease in nitrate and an increase in ammonium with depth at the intertidal site, and the same pattern for ammonium at the deep water site. *Hyphomicrobiaceae* species*,* related to denitrifying strains, have previously been detected in surface marine sediments (Mills et al., 2008) and

deep sea sediments (Teske et al., 2002). Mills et al., (2008) hypothesised that the metabolically diverse *Hyphomicrobiaceae* would exploit the abundance of nutrients from multiple sources in coastal sediment to become a significant contributor to geochemical processes. This could be the reason for the sheer abundance of the *Hyphomicrobiaceae* in these organic rich Severn Estuary sediments.

4.4.3 The Chloroflexi were an Important Community Member in Surface Sediment

Pyrosequencing analysis showed that *Chloroflexi* constituted a substantial proportion of the bacterial community at all sites and increased with depth. This coincides with previous descriptions of *Chloroflexi* communities in surface sediments (Kim et al., 2008; Webster et al., 2007; Wilms et al., 2006a). Very few of the *Chloroflexi* OTUs could be assigned to cultured representatives; far more were related to uncultivated phylotypes, and, therefore, prediction of functionality was difficult. The best described were the *Dehalococcoidia* that have been characterised particularly in terms of their role in dehalorespiration, members of the class discussed further below.

The *Anaerolineae* were the most abundant class, constituting over 90% of the *Chloroflexi* at the intertidal and shallow water sites. This was also seen previously in the *Chloroflexi* targeting DGGEs and *Anaerolineae* specific qPCRs **(Sections 3.3.1 & 3.3.5 – 3.3.7)**. The qPCR estimated the *Anaerolineae* to be in almost equal numbers to the total *Chloroflexi.* As previously discussed, *Anaerolineae* were the most commonly detected *Chloroflexi* subdivision in surface sediment (Blazejak and Schippers, 2010; Durbin and Teske, 2011). This appeared to be due to their large metabolic range including aerobic and anaerobic growth and the presence of a multitude of sugar metabolism genes, enabling them to scavenge cell debris (Hug et al., 2013; Sekiguchi et al., 2003). By analysing specific OTUs from uncultivated order level taxonomic groups, such as GCA004 and DRC31 (Section 4.3.3), further insights into the potential role for the *Anaerolineae* in surface estuarine sediments was revealed. Comparisons of these OTUs with uncultivated clones, the environment, geochemistry and community

processes that the clones were isolated from gave further insights. Notably, the sequence similarity of multiple *Anaerolineae* OTUs with uncultivated clones from methane seeps, related to AOM processes suggested the involvement of *Anaerolineae* in the reduction of the electron acceptor step for the AOM process, which is novel for the subdivision. Though previous studies have indicated syntrophic associations with hydrogenotrophic methanogens (Sekiguchi et al., 2003; Yamada et al., 2007; Yamada et al., 2006). The recently cultivated strain *Pelolinea submarina* was isolated from deep subseafloor sediments in Japan and was found to be an anaerobic fermentative organism, capable of utilising a range of carbohydrate compounds in a methanogenic community, which lends weight to this hypothesis (Imachi et al., 2014).

The most notable feature of the *Chloroflexi* community was that at the intertidal site, at 6-8 cbsf, the *Chloroflexi* became the joint largest phylum and the *Dehalococcoidia* was the dominant subdivision for the first time. This dominance was also found in the qPCR assays **(Figure 3.11)**. In a survey of *Dehalococcoidia* sequences with depth in Wadden Sea tidal flat sediments, Wasmund et al. (2014a) found that *Dehalococcoidia* were established in low numbers at 2 cbsf and increased with depth to a peak at 8 cbsf, which directly mirrors the results in this study. In longer Baffin Bay cores, the *Dehalococcoidia* increased or maintained numbers before decreasing at the deepest sediment depths (Wasmund et al., 2014a). The sudden peak in *Dehalococcoidia* abundance, in the intertidal 6-8 cbsf sediment, indicated an abrupt change in metabolism, which promoted growth. Looking more closely at this sub-group indicated that the *Dehalococcoidales* order, which includes all known cultured representatives such as *Dehalococcoides mccartyi,* and also the uncultivated environmental clone group GIF9, were the main constituents. Both groups were associated with dehalorespiration as GIF9 was isolated from dechlorinating ground waters associated with aquifer sediment (Alfreider et al., 2002). This was a strong indication that dehalorespiration was occurring in Severn Estuary sediment. However, it was not clear why this was so abundant at this site and depth. Dehalogenating *Dehalococcoidia* communities have been previously

described in estuarine and coastal sediment, always increasing in numbers with depth as oxygen was depleted (Ahn et al., 2008; Wilms et al., 2006a).

4.4.4 The Archaeal Community in Severn Estuary Sediments were Dominated by Ammonia Oxidizing Species

The *Archaea* were much less diverse than the *Bacteria* due to the extreme dominance of just one family, the *Cenarchaeaceae,* in all but two samples. The Marine Group 1.1a, which *Cenarchaeaceae* belongs to, has been frequently reported in marine surface sediment and the water column (Agogué et al., 2008; Erguder et al., 2009); the *Thaumarchaeota* are thought to be one of the most abundant phyla of *Archaea* on the planet and have a major role in the global nitrogen cycle (Park et al., 2012b; Spang et al., 2010). The *Cenarchaeaceae* were further dominated by the genus *Nitrosopumilus,* which consists of aerobic, chemolithoautotrophic, ammonia oxidisers, involved in nitrification (Brochier-Armanet et al., 2008; Könneke et al., 2005). Ammonia oxidizing *Archaea* (AOA) have been found to dominate Ammonia oxidizing *Bacteria* (AOB) in marine sediment, which was also the case in these intertidal and deep water sediment (Park et al., 2012a; Park et al., 2008; Zheng et al., 2014). AOB such as *Nitrosomonadales* were present but were <1% of the bacterial community. It was proposed that this dominance was due to reduced oxygen levels and high ammonia concentrations inhibiting AOB growth (Park et al., 2010; Suwa et al., 1994). The presence of *Cenarchaeaceae* involved in ammonia oxidation (nitrification) and *Hyphomicrobium* involved in denitrification and nitrogen fixation suggested that many elements of the nitrogen cycle were occurring in Severn Estuary intertidal sediment. Ammonia oxidation was predicted in the surface (0-2 cbsf) sediment with a swift change to denitrification by *Bacteria* occurring in deeper anaerobic sediment. Indeed, previous studies have highlighted the importance of marine sediment in the coupling of these processes as well as the production of greenhouse gases like nitrous oxide (Dalsgaard et al., 2005; Park et al., 2010; Suwa et al., 1994). This indicated a very important role of Severn Estuary sediment in the global nitrogen cycle. Estuaries act as an important sink for nitrogen compounds from agricultural

and industrial waste that would otherwise cause eutrophication in estuarine habitats (Dong et al., 2009; Mosier and Francis, 2008).

4.4.5 Comparison of Chloroflexi Community from Bacteria and Gich Primers

Chloroflexi were specifically targeted using the Gich et al. (2002) PCR primers and were also detected with the bacterial PCR primers 357F and 907R (Table 4.1). Subsequent analysis of the *Chloroflexi* community OTUs detected with these different PCR primer pairs indicated that the bacterial pair revealed greater diversity within the phylum. Another difference was in the proportions of each subdivision. The distribution of the subdivision was very similar to the *Chloroflexi* primer and bacterial primer profiles except that the *Dehalococcoidia* were detected in lower abundances by the *Chloroflexi* PCR primers.

The main difference in the PCR primer sets was the targeted variable region of the 16S rRNA gene. The bacterial PCR primers target the V3-V5 region, which has a better coverage in sequence databases than the V6 region targeted by the *Chloroflexi* PCR primers (Cai et al., 2013; Pinto and Raskin, 2012). Thus the *Chloroflexi* primers may be limited to the number of database matches and, as *Anaerolineae* account for approximately 70% of the *Chloroflexi* in the RDP database (Yamada and Sekiguchi, 2009), this would explain the difference in the proportions of *Anaerolineae* detected with the *Chloroflexi* primers. The ability of the 16S rRNA gene variable regions to give consistent phylogenetic assignment has been widely analysed (Cai et al., 2013; Morales et al., 2009; Morales and Holben, 2009; Wang et al., 2007). Morales et al. (2009) and Wang et al. (2007) tested the reliability of the V4-V5 regions for the use in NGS methods and found it to consistently describe the phylogeny of bacterial soil communities. Also, experiments by Claesson et al. (2010) indicated that Illumina sequencing of gut microbiota with V6 targeted PCR primers consistently assigned less sequences to the genus level than using V4 primers and Vasileiadis et al. (2012) found that the V6 regions was the least informative when compared with other variable regions for the description of soil bacterial diversity. These findings suggested that the bacterial primers used in this work, targeting the V3-V5

regions, gave a more reliable description of the *Chloroflexi* community than the Gich et al. (2002) primers that targeted the V6 region.

4.4.6 Conclusions

In conclusion, prokaryotic communities at three geomorphologically different sites across the Severn Estuary were found to be highly similar to each other, with the greatest changes occurring with depth. Interestingly, single groups such as the *Cenarchaeaceae* dominated at surface and potentially oxygenated site, leading to a less diverse community, particularly in the *Archaea.* The novel use of *Chloroflexi* targeted primers indicated an abundant community, with the *Anaerolineae* dominating the *Chloroflexi* and indicated in important roles in estuarine sediments.

Chapter 5 – The Impact of Temperature on Prokaryotic Community Activity in Severn Estuary Sediments

5.1 Introduction

5.1.1 Temperature and Prokaryotic Communities

Temperature is an important factor influencing the structure and activities of microbial communities. Microorganisms can survive at the extremes of temperature, the lowest recorded being -15^{o}C by *Planococcus halocryophilus* OR1 (Mykytczuk et al., 2013), whereas *Methanopyrus* strain 116 can survive at up to 122^{o}C (Takai et al., 2008). It is also thought that temperature is one of the main limiting factors on the depths at which microorganisms can penetrate the deep subsurface (Whitman et al., 1998). Due to the high impact of temperature on prokaryotic communities, its influence on specific taxa and pure cultures has been widely analysed, though less is known about the effects of temperature on whole communities and their biogeochemistry (Hall et al., 2008; Rivkin et al., 1996). Soil and hot springs have become target environments due to their role in climate change and natural temperature gradients, respectively (Miller et al., 2009; Wang et al., 2013b). Coastal and intertidal sediments are especially susceptible to changes in temperature due to their shallow or tidal natures (Day et al., 2008; Ser et al., 1999; Vieira et al., 2013). To attempt to understand the effect of temperature on these unique prokaryotic communities more work is needed (Day et al., 2008).

The effects of temperature on sediments can be analysed from a variety of aspects including seasonal variation, climate change, extreme natural gradients and *in vitro* incubation experiments. This study concentrated on the effects of experimental temperature gradients and seasonal variation on marine sediments. It is known that seasonal temperature changes (-0.3 - 40^{o}C; Finke and Jørgensen, 2008) regulate both prokaryotic taxonomy and their metabolic processes in marine sediments (Hall et al., 2008). The dominant phyla in both *Bacteria* and *Archaea* were found to alter over steep temperature gradients. The common bacterial phyla such as *Proteobacteria*

and *Firmicutes* switched to thermophilic species at high temperatures in geothermal systems (38 - 87^{o}C; Cole et al., 2013; Spear et al., 2005). Some studies found a negative relationship between increasing temperature and species richness but also an increase in novel taxonomic groups (38 - 87^{o}C; Cole et al., 2013; Miller et al., 2009), which indicated a selection of specialist, uncultivated, thermophilic organisms by temperature. The types of metabolism utilised by sediment prokaryotic communities and the rates at which these processes are performed are both affected by temperature (Finke and Jørgensen, 2008). Seasonal temperature increases have led to increases in metabolic rate of microbial activity and to the selection of thermophilically adapted microorganisms and vice versa (Castro et al., 2010; Kuffner et al., 2012; Westrich and Berner, 1988). Thermophilic *Archaea* lineages involved in methanogenesis and ammonia oxidation have been shown to increase in sediments with seasonal temperature rises (Nozhevnikova et al., 1997; Weston and Joye, 2005; Wu et al., 2013). Increasing rates of denitrification, methanogenesis, ANAMMOX, ammonia oxidation, CO_2 production and sulphate reduction with increasing temperature have been recorded in marine sediments (Arnosti et al., 1998; He et al., 2012; Kallmeyer and Boetius, 2004; Weston and Joye, 2005). Interestingly, a temperature window of 25-46^{o}C for metabolic processes in surface sediments has been suggested through a collection of studies (Conrad et al., 2009; Finke and Jørgensen, 2008; Nozhevnikova et al., 2007; Weston and Joye, 2005). These studies focused on the temperature driven decoupling of the use of fermentation products by anaerobic respiration, such as sulphate reduction (Finke and Jørgensen, 2008), or methanogenesis (Conrad et al., 2009; Nozhevnikova et al., 2007; Weston and Joye, 2005). This was characterised by the switch from hydrogenotrophic and acetoclastic methanogenesis to exclusively hydrogenotrophic methanogenesis above 40^{o}C (Conrad et al., 2009; Nozhevnikova et al., 2007).

5.1.2 Marine Sediment Prokaryotic Communities Role in Climate Change

The effect of temperature has particular significance as we strive to understand the potential impact of global warming on our planet and, though this study was over a much greater temperature range than those predicted

by climate models; it does provide insight into the response of prokaryotes within that window. Seas around the UK have increased in temperature by 0.2 - 0.6^{o}C since the 1980s (MCCIP, 2008) and are predicted to increase by a further 1.5 - 4^{o}C by the end of the 21st century depending on location and emission levels (UK Climate Projections science report: Marine and coastal projections, MCCIP, June 2009). Marine sediments, particularly coastal and intertidal zones, are extremely susceptible to the effects of temperature and climate change. Coastal sediments account for 55% of global sediment organic matter oxidation and so temperature effects on microbial communities involved in organic matter turnover could have important subsequent implications on global geochemical cycles and climate change (Weston and Joye, 2005). Marine sediment prokaryotes have both positive and negative implications for climate change particularly in the control of greenhouse gases. For example, archaeal methanogenesis produces methane but the process of anaerobic oxidation of methane (AOM) by ANME can alleviate methane emissions (Biddle et al., 2012; Kallmeyer and Boetius, 2004).

5.1.3 The HERMIONE Project

The EC funded Hotspot Ecosystem Research and Man's Impact on European Seas (HERMIONE) consortium project (including Cardiff University) aimed to study how climate change and human activities were affecting marine ecosystems in Europe. The project investigated the response of prokaryotic communities from marine and mud volcano sediments to temperature changes with respect to biodiversity, methane and CO_2 fluxes (HERMIONE Report, 2011). As part of the project, a series of amended and unamended marine sediment slurries were incubated on a temperature gradient (1 - 80^{o}C): organic substrates (including acetate, methylamine and hydrogen) and extra CO_2 were added to "amended" slurries to mimic increased nutrients due to eutrophication and increased atmospheric CO_2 levels. "Unamended" slurries had no additions, to investigate the effect on anaerobic prokaryotic processes without influence from other prokaryotes (syntrophs). Methanogenesis and CO_2 formation increased considerably within environmentally relevant temperature ranges,

but sulphate reduction remained largely unaffected by increasing temperature. It was also found that these processes, especially methanogenesis, were heavily influenced by substrate supply. In amended slurries, *Bacteria* responded to increased temperature much more quickly than *Archaea* and a range of phylotypes associated with the above metabolisms were detected. Crucially, a critical temperature window at approximately 43^{o}C was indicated, in which the dominant geochemical process switched from chemoorganotrophic at lower temperatures to chemolithotrophic and hydrogenotrophic processes at higher temperatures (E.G. Roussel, B.A. Cragg, G. Webster, X. Tang, R. Gorra, H. Sass, A.J. Weightman and R.J. Parkes, unpublished results).

5.1.4 Aims

Three research questions were addressed in this work. Firstly, how do intertidal prokaryotic communities respond to extreme temperature increase with time? More specifically, changes in the *Chloroflexi* community were examined, many species of which are known thermophiles. Secondly, do the prokaryotic community changes influence sediment geochemistry through changing anaerobic metabolisms? Finally, what is the potential impact on the intertidal and estuarine environment? These questions were investigated using molecular methods to analyse the unamended sediment gradient subjected to an extreme temperature gradient as part of the HERMIONE project.

5.2 Materials and Methods

5.2.1 Sediment Sampling, Slurry Preparation and Incubation

Sediment cores were taken at low tide from tidal flats in the Severn Estuary at Woodhill Bay, Portishead, UK (51°29'31.66" N, 2°46'27.95" W) in June 2009 and February 2010. The cores ranged from 49-58 cm in length and were sealed and immediately processed back in the laboratory. 500 ml of the lower part of the sediment cores (below 30 cm) were slurried with 1500 ml of artificial sea water in a 2 litre screw cap bottle with a gas headspace of N_2:CO_2 (80:20, v:v; Webster et al., 2010). A three-way stopcock was fitted to the bottom of the vessel for further sampling of the slurry. The slurry was pre-incubated at the approximate *in situ* temperature of 10^{o}C in the dark on an orbital shaker (100 r.p.m). After 26 days the sulphate concentration had reached a steady state of ~2.3 mM and was ready for further sampling.

"Unamended" sediment slurry (described in **Section 5.1.3**) was distributed in 20 ml volume headspace vials (with 10 ml of slurry) and 100 ml serum vials (with 50 ml of slurry) in an anaerobic chamber (model 1024, Forma Scientific). In order to stimulate sulphate removal and other sequential terminal oxidizing processes, stoichiometric amounts of H_2 were added to the vials. The vials were incubated upside down on a temperature gradient system of 1-80^{o}C for 100 days (Parkes et al., 2007). Replicates of vials were destructively sampled at Day 15, Day 35, Day 62 and Day 100 for analysis. A Day 0 reference sample was also retained for comparative analysis to the *in situ* sediment.

5.2.2 Geochemical Measurements

Concentrations of methane, hydrogen, CO_2, acetate, formate, sulphate and phosphate were measured at each sampling point. Sediment slurry headspace gases (methane, hydrogen and CO_2) were analysed by a natural gas analyser (PerkinElmer Clarus 500; Webster et al., 2009; Webster et al., 2010). Anion and volatile fatty acids (acetate, formate, sulphate and phosphate) from sediment slurry pore waters were determined by ion chromatography (Dionex ICS-2000 and DX-120, Camberley UK; Webster et al., 2009; Webster et al., 2010). Head space gases were analysed by Dr

Gordon Webster and Dr Barry Cragg and pore waters were examined by Dr Erwan Roussel.

5.2.3 DNA Extraction

DNA was extracted from all temperature samples for Day 15 and Day 100 and Day 0. Five target samples from Day15, Day 35 and Day 62 at temperatures 25^{o}C, 35^{o}C, 38^{o}C, 46^{o}C and 66^{o}C were extracted for use in 454 pyrosequencing (total of 16 samples for *Bacteria* and *Archaea* and 11 for *Chloroflexi)*. DNA was extracted using the FastDNA Spin Kit for Soil protocol, amended by Webster et al. (2003). The DNA concentrations of the extractions used in PCR were in the range of 1.35-12.2 ng/ µl. For full DNA extraction protocol see **Section 2.2.2.1**.

5.2.4 Community profiling with 16S rRNA Gene PCR-DGGE and 16S - 23S rRNA Gene RISA

Both bacterial and archaeal communities were analysed by DGGE, but only *Bacteria* were analysed using 16S – 23S rRNA gene intergenic spacer region RISA barcoding. Nearly complete temperature ranges were analysed by 16S rRNA gene PCR-DGGE for Day 15 and Day 100 as comparison of the beginning and end time points. For bacterial DGGE and RISA protocols and statistical analysis see **Section 3.2.5**. Day 0 was included in all analysis as a comparison, except *Archaea* DGGE, which was performed by Gordon Webster, Cardiff University. *Archaea* DGGE followed the same protocol as bacterial DGGE except for the initial PCR amplification method. The archaeal community was amplified with nested PCR of the 16S rRNA gene (Webster et al., 2006). The first round used primers 109F (ACK GCT CAG TAA CAC GT; Großkopf et al., 1998a) and 958R (YCC GGC GTT GAM TCC AAT T; DeLong, 1992). The second round used the SAf (2:1 ratio of SAf1 and SAf2; Nicol et al., 2003) and PARCH519R (TTA CCG CGG CKG CTG; Øvreås et al., 1997) primers. The first round PCR was performed as described by Newberry et al. (2004), and the second round as described by Nicol et al. (2003). All Pearson's Cluster analysis of DGGE and RISA profiles was performed using the GelCompar II software (version 6.5; Applied Maths).

5.2.5 Quantitative PCR of Bacteria, Archaea and Chloroflexi Sub-groups

The *Bacteria, Archaea* and *Chloroflexi* communities including sub-groups were quantified using qPCR. Samples from temperatures 1°C, 8 °C, 15 °C, 22 °C, 28 °C, 35 °C, 42 °C, 49 °C, 55 °C, 62 °C, 69 °C and 75 °C at both Days 15 and 100 were utilised in qPCR with three technical replicates for each sample for statistical analysis (see **Section 3.2.6**).

5.2.6 Sample Preparation and 454 16S rRNA Gene Barcode Sequencing

Sixteen samples were sequenced for both *Bacteria* and *Archaea* and 11 for *Chloroflexi* (Table 5.1). For sample preparation, MID tag 454 pyrosequencing of the 16S rRNA gene was performed by Research and Testing, Lubbock, Texas and sequencing data analysis was done in house using QIIME v1.6.0 (Caporaso et al., 2010b); see **Sections 4.2.2 and 4.2.3** and **Table 4.1**. A total of 85,009 *Bacteria,* 52,016 *Archaea* and 41,765 *Chloroflexi* sequences were obtained. Full description of statistical analysis of the pyrosequencing data is given in **Section 4.2.4**, briefly, Pearson correlation of diversity statistics with temperature, sampling day and geochemical variables were performed in Excel 2010. ANOVA with Bonferroni corrections of the each taxa with temperature and sampling day were performed in STAMP (Parks and Beiko, 2013). The R package vegan was used to perform NMDS of OTUs and create heatmaps with UPGMA clustering of both OTUs and sample (Ihaka and Gentleman, 1996; Oksanen et al., 2013)

Table 5.1 - Community diversity analysis of sediment slurry DNA samples by pyrosequencing in this study. Total of 16 samples for *Bacteria* and *Archaea* and 11 for *Chloroflexi.*

Sampling Day	Temperature (°C)					16S rRNA Gene Barcode[a]		
						Bacteria **(357F-907R)**	***Archaea*** **(341F-958R)**	***Chloroflexi*** **(941F-1340R)**
0			10			✓	✓	✓
15	25	35	38	46	66	✓	✓	✓
35	25	35	38	46	66	✓	✓	×
100	25	35	38	46	66	✓	✓	✓

[a] Designations of PCR primer pairs used to generate fragments for 454 pyrosequencing shown in parentheses.

5.3 Results

5.3.1 Comparison of the Prokaryotic Community in Sediment Slurries used for Temperature Gradient Experiment with In Situ Intertidal Sediment Communities

It was important to determine whether the sediment slurries used in this study were representative of the *in situ* sediment prokaryotic communities from the Severn Estuary. This was done by comparison of the Day 0 reference sample (intertidal sediment from Portishead, Severn Estuary) and the Severn Estuary intertidal sediment samples (Wentloodge Levels), analysed in **Chapters 3 and 4**. The Day 0 and intertidal site 454 pyrosequencing samples were analysed together using QIIME to create a biom table for direct comparison. Comparisons at phylum and OTU level of the bacterial and archaeal communities from Day 0 reference sample and the intertidal site samples by ANOVA with Bonferroni correction in STAMP (Parks and Beiko, 2010) indicated no significant differences between the sediment slurry and *in situ* communities. A core community of 33 and 16 *Bacteria* and *Archaea* OTUs, respectively, shared across the four samples was computed (Tables 5.2 & 5.3). The core OTUs accounted for 27% and 44% of the total bacterial and archaeal sequences in all four samples, respectively, which indicated a high degree of similarity between the slurried and *in situ* sediments. Slurried sediments were used to create a homogenous prokaryotic community so that multiple replicates could be used in the thermal gradient, for sacrifice at multiple time points. Slurrying of sediments disrupts the original stratifications in the sediment, destroying geochemical redox zones that some prokaryotes rely on, such as methanogenic and dissimilatory sulphate reducing populations (Cappenberg, 1974; Hall et al., 1972). However, this work indicated that the prokaryotic communities from the reference sample and the intertidal site were extremely similar; sharing a number of core OTUs and so the slurry was a reasonable representative sample for *in situ* sediments.

Table 5.2 – Core community OTUs with taxonomy for the *Bacteria* community in the reference sample (Day 0) and all Severn Estuary intertidal site samples using in Chapters 3 & 4.

Phylum	Class	Order	Family	Genus	OTU Number	Percentage Abundance of Total Community
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*			224	0.73
Proteobacteria	*Gammaproteobacteria*	*Chromatiales*			584	1.08
Proteobacteria	*Gammaproteobacteria*	*Thiotrichales*	*Piscirickettsiaceae*		1029	1.45
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobacteraceae*	*Desulfococcus*	1091	0.46
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobacteraceae*	*Desulfococcus*	1092	0.22
Proteobacteria	*Deltaproteobacteria*	*Desulfobacterales*	*Desulfobacteraceae*		1204	0.11
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Hyphomicrobium*	1219	0.05
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*		1241	0.29
Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*			1243	0.57
Proteobacteria	*Alphaproteobacteria*				1281	0.16
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Hyphomicrobium*	1382	8.65
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*		1524	2.00
Proteobacteria	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophobacteraceae*		1569	0.02
Proteobacteria	*Deltaproteobacteria*	*Desulfarculales*	*Desulfarculaceae*		1576	0.73
OP8	OP8_1	HMMVPog-54			244	0.40
OP8	OP8_1				1497	0.59
Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	292	0.24
Firmicutes	*Bacilli*	*Bacillales*			1242	2.11

Phylum	Class	Order	Family	Genus	OTU Number	Percentage Abundance of Total Community
Firmicutes	*Bacilli*	*Bacillales*	*Planococcaceae*		1378	0.25
Chloroflexi	*Anaerolineae*	SHA-20			42	1.11
Chloroflexi	*Anaerolineae*	GCA004			43	0.22
Chloroflexi	*Anaerolineae*	S0208			285	1.48
Chloroflexi	*Anaerolineae*	MSB-1E9			546	0.08
Chloroflexi	*Anaerolineae*	SB-34			590	0.69
Bacteroidetes	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*		1023	0.23
Actinobacteria	*Acidimicrobiia*	*Acidimicrobiales*	koll13		544	0.48
Actinobacteria	*Acidimicrobiia*	*Acidimicrobiales*	EB1017		578	0.12
Actinobacteria	*Acidimicrobiia*	*Acidimicrobiales*	koll13		611	0.18
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Nocardiaceae*	*Rhodococcus*	630	0.11
Actinobacteria	*Thermoleophilia*	*Gaiellales*	*Gaiellaceae*		698	0.27
Actinobacteria	*Thermoleophilia*	*Gaiellales*			853	1.31
Actinobacteria	*Acidimicrobiia*	*Acidimicrobiales*	koll13		1171	0.27
Actinobacteria	*Actinobacteria*	WCHB1-81	At425_EubF1		1314	0.33
					Total	27%

Table 5.3 - Core community OTUs with taxonomy for the *Archaea* community in the reference sample (Day 0) and all Severn Estuary intertidal site samples using in Chapters 3 & 4.

Phylum	Class	Order	Family	Genus	OTU Number	Percentage Abundance of Total Community
Crenarchaeota	MBGB				73	1.59
Crenarchaeota	MCG				82	0.68
Crenarchaeota	MCG				352	0.32
Crenarchaeota	MBGB				387	0.86
Crenarchaeota	MCG	B10			458	0.29
Crenarchaeota	MCG				472	0.75
Crenarchaeota	*Thaumarchaeota*	*Cenarchaeales*	*Cenarchaeaceae*	*Nitrosopumilus*	494	4.50
Crenarchaeota	MCG	pGrfC26			529	1.85
Crenarchaeota	MCG				734	0.19
Crenarchaeota	MCG				811	0.58
Crenarchaeota	MCG	B10			873	0.12
Crenarchaeota	MBGB				1024	0.14
Crenarchaeota	*Thaumarchaeota*	*Cenarchaeales*	*Cenarchaeaceae*	*Nitrosopumilus*	1123	23.76
Crenarchaeota	MCG	B10			1142	4.39
Euryarchaeota	*Methanomicrobia*	*Methanosarcinales*	*Methanosarcinaceae*	*Methanosarcina*	61	1.02
Euryarchaeota	*Methanomicrobia*	*Methanosarcinales*	ANME-2a-2b		716	0.41
Crenarchaeota	MBGB				73	1.59
Crenarchaeota	MCG				82	0.68

Phylum	Class	Order	Family	Genus	OTU Number	Percentage Abundance of Total Community
Crenarchaeota	MCG				352	0.32
					Total	44%

5.3.2 Community Profiling and qPCR of Prokaryotic Communities along the Temperature Gradient

Cluster analysis of bacterial DGGE community profiles appeared to indicate a change at temperatures above the 35^{o}C at both Day 15 and Day 100, typified by a decrease in the number of phylotypes (bands; Figure 5.1), which suggested a less diverse community was selected for by increasing temperature. RISA bacterial community analysis supported the results obtained by DGGE profiling (Figure 5.2). Changes in the *Archaea* community DGGE were more complex than the *Bacteria* (Figures 5.1 & 5.3). The most distinct change in the archaeal community was seen at the very high temperatures (65^{o}C, 75 ^{o}C and 79 ^{o}C; Figure 5.3A), which appeared to be linked to a decrease in the number of phylotypes. However, though efforts were made to regulate the concentration of PCR product loaded onto the gel (approx. 200 ng/µl), the inherent decrease in cell numbers and therefore DNA as temperature increased (Figure 5.4) may have influenced the cluster analysis of the community. What appears to be a decrease in bands at higher temperatures could actually be less DNA available in the PCR.

qPCR to quantify *Bacteria* and *Archaea* in the sediment slurry communities indicated an overall decrease in cell numbers as temperature increased (Figure 5.4A & B). At Day 15, the number of cells fell from approximately 10^{7} and 10^{6} cells per ml of slurry for *Bacteria* and *Archaea,* respectively, to approximately 10^{5} cells per ml of slurry for both as temperature increased. There was a slight increase in both *Bacteria* and *Archaea* cells at approximately 59^{o}C, but at temperatures above this cell numbers dropped further (Figure 5.4A). At Day 100 cell numbers were similar to Day 15 at low temperature except for a slight increase in both *Bacteria* and *Archaea* cells at approximately 15^{o}C, which indicated that cells were still growing after 100 days (Figure 5.4B). At temperatures above 15^{o}C cell numbers decreased steadily to a final drop at 79^{o}C to approximately 10^{3} cells per ml of slurry for *Bacteria* and *Archaea,* respectively (Figure 5.4A & B).

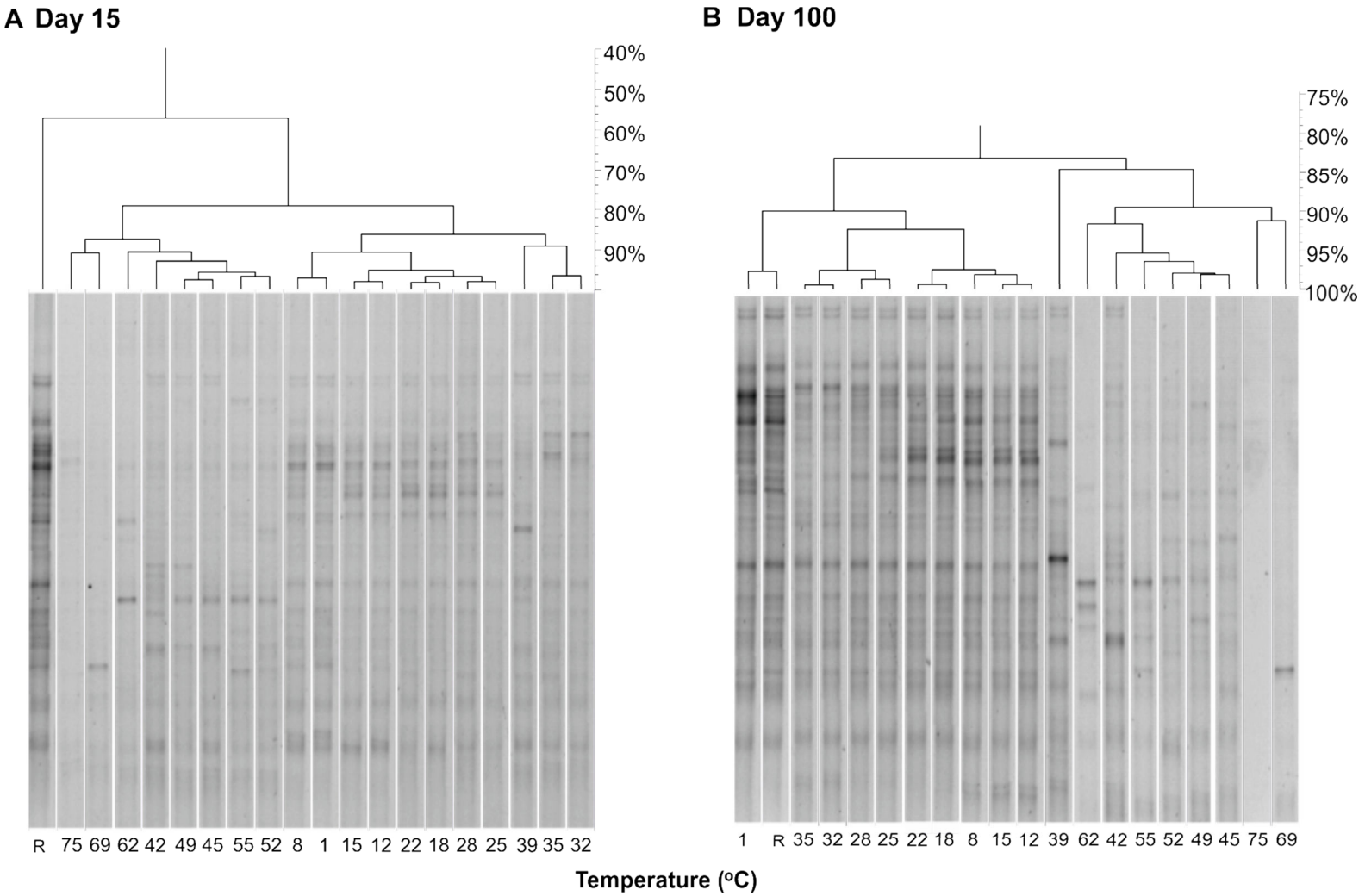


Figure 5.1 - 16S rRNA gene DGGE community profiles of bacterial sediment slurry communities incubated at a range of temperatures at, A, Day 15 and, B, Day 100. Temperature of sample is given below each lane and a reference sample (R), which was Day 0, representing the original community. Cluster analysis dendrograms using Pearson correlation coefficient and UPGMA are shown above the DGGE lanes. Scales of percentage similarity are given next to each dendrogram and depth of sediment is given next to branch. Cluster analysis was performed with the GelCompar II software (version 6.5; Applied Maths).

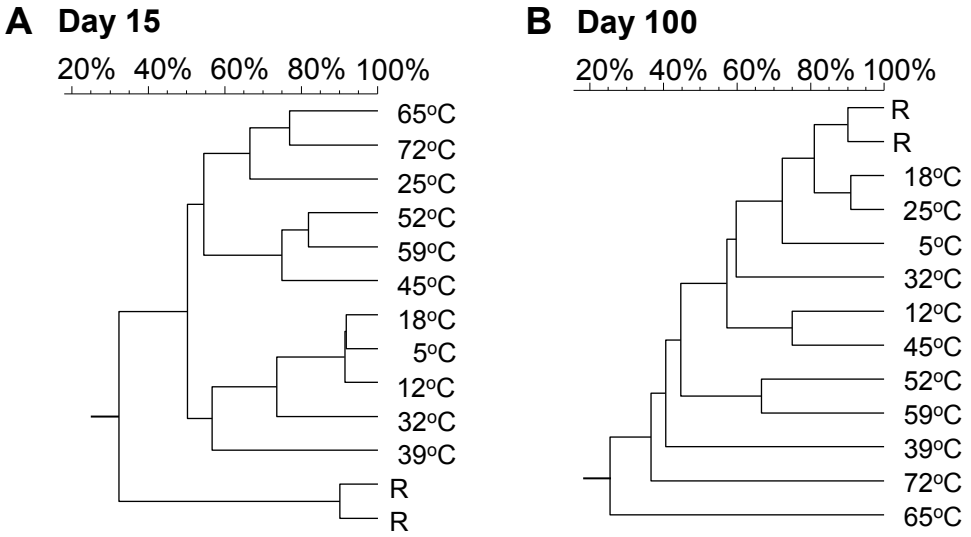


Figure 5.2 - Cluster analysis using DICE of *Bacteria* RISA profiles from sediment slurry at selected temperatures at, A, Day 15 and, B, Day 100. Temperature of sample is given next to each branch and a reference sample (R), which was Day 0, representing the original community. Scales of percentage similarity are given next to each dendrogram and depth of sediment is given next to branch. Cluster analysis was performed with the GelCompar II software (version 6.5; Applied Maths).

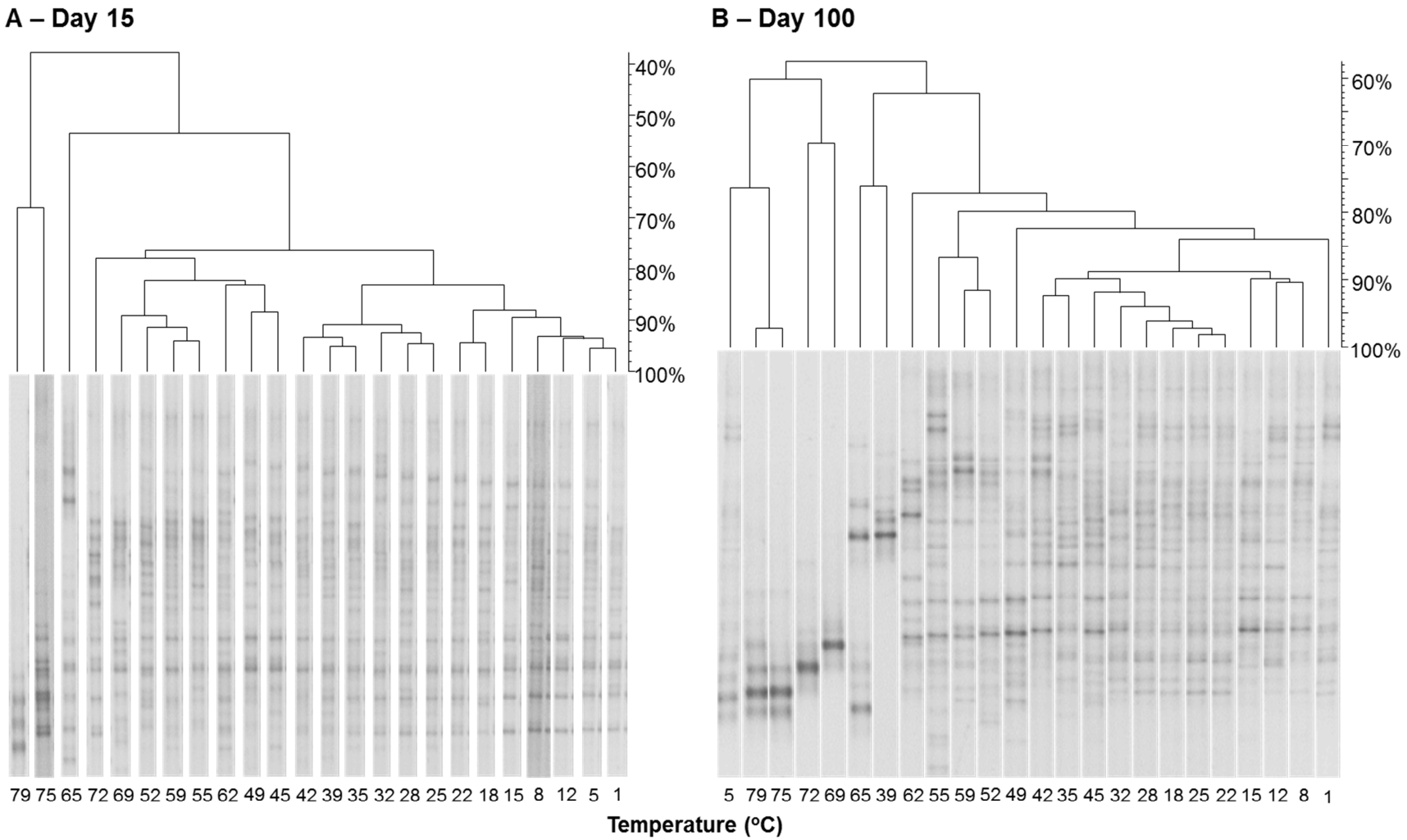


Figure 5.3 - 16S rRNA gene DGGE community profiles of archaeal sediment slurry communities incubated at, A, Day 15 and, B, Day 100. Temperature of sample is given below each lane. Cluster analysis dendrograms using Pearson correlation coefficient and UPGMA are shown above the DGGE lanes. Scales of percentage similarity are given next to each dendrogram and depth of sediment is given next to branch. Cluster analysis was performed with the GelCompar II software (version 6.5; Applied Maths). *Archaea* DGGEs by Gordon Webster (Cardiff University).

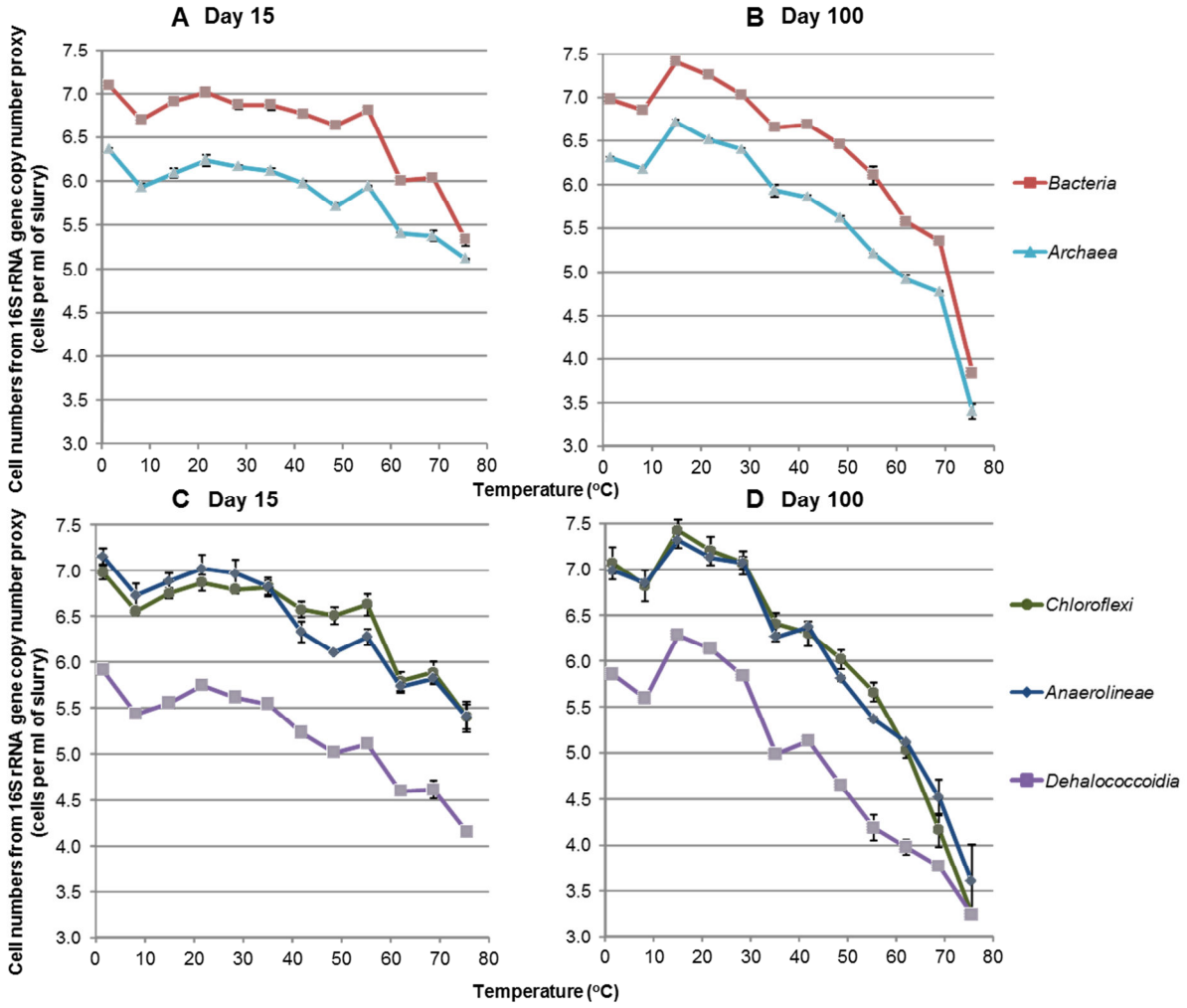


Figure 5.4 - Quantification of sediment slurry communities along the temperature gradient using 16S rRNA gene qPCR. A, Day 15 *Bacteria* and *Archaea,* B, Day 100 *Bacteria* and *Archaea,* C, Day 15 *Chloroflexi, Anaerolineae/Caldilineae* and *Dehalococcoidia*, and D, Day 100 *Chloroflexi, Anaerolineae* and *Dehalococcoidia.* The sample temperatures from Day 15 and Day 100 measured were 1°C, 8°C, 15 °C, 22°C, 28°C, 35°C, 42°C, 49°C, 55°C, 62 °C, 69°C and 75°C. Standard deviations are indicated by error bars at each data point from triplicate technical replicates. Clone libraries indicated that 88% of sequences amplified by the *Anaerolineae/Caldilineae* primers were related to Subdivision I and so the primers were not as specific as *in vivo* as expected.

Numbers of *Chloroflexi* decreased as temperature increased, mirroring the bacterial cell numbers (Figure 5.4C &D). The high abundance of *Chloroflexi* compared to total *Bacteria* suggested that the *Chloroflexi* were a substantial component of the bacterial community. *Anaerolineae/Caldilineae* were the most abundant sub-group detected, mirroring the numbers of *Chloroflexi* closely, which was expected from previous analysis of the *Chloroflexi* in Severn Estuary sediments **(Chapters 3 & 4)**. *Dehalococcoidia* were detected in much lower numbers (approximately 10^6 – 10^4 and 10^6 - 10^3 cells per ml of slurry at Day 15 and Day 100, respectively), which suggested the *Dehalococcoidia* were less well suited to the slurry sediment environment than the diverse *Anaerolineae*.

5.3.3 Analysis of the Prokaryotic Sediment Slurry Communities by 16S rRNA Gene Pyrosequencing over Temperature Gradient

5.3.3.1 Comparison of the Relationship between Diversity Estimates and Temperature in Bacteria, Archaea and Chloroflexi Sediment Slurry Communities

Following quality assessment of the raw sequence data (see **Section 5.2.6**) totals of 12,266 *Bacteria,* 70,281 *Archaea* and 24,731 *Chloroflexi* 16S rRNA gene barcode sequences were submitted for bioinformatic analysis (Table 5.4), which were sub-sampled to give 2919, 1617, and 1300 sequences, respectively. Coverage of each library was estimated from the number of OTUS and the Chao1 value. The *Bacteria* coverage ranged from 75% - 86%, *Archaea* coverage from 49% - 80% and *Chloroflexi* from 62% - 82%, which indicated the *Bacteria* were better described that *Archaea*. Pearson correlation between diversity indices (Chao1, Shannon's Index and Simpson's Index of Diversity) and sampling day and temperature were performed (Table 5.5). There was no significant correlation between the diversity indices and sampling day. However, there were significant negative correlations between the Chao1 and Shannon indices and temperature for each library indicating a loss of diversity and richness as temperature increased. Only the Simpson's Index of Diversity for *Archaea* and temperature were significantly negatively correlated. This pattern of loss of.

Table 5.4 - OTU diversity estimates for Bacteria, Chloroflexi and Archaea in sediment slurry samples from temperature gradient.

Day	Temperature (°C)[a]	No. of sequences[b]			No. of OTUs[c] per sample			Chao1			Shannon Index			Simpson's Index of Diversity[d]		
		Bacteria	*Archaea*	*Chloroflexi*[e]	*Bacteria*	*Archaea*	*Chloroflexi*	*Bacteria*	*Archaea*	*Chloroflexi*	*Bacteria*	*Archaea*	*Chloroflexi*	*Bacteria*	*Archaea*	*Chloroflexi*
0	-	14759	3717	2407	461	348	194	551	609	303	5.07	4.81	4.02	0.98	0.98	0.96
15	25	6174	5226	2404	438	277	212	544	433	297	4.83	4.37	4.13	0.97	0.97	0.96
	35	5654	3742	2595	433	318	185	548	566	258	5.04	4.37	4.01	0.98	0.95	0.96
	38	7333	2139	2629	380	303	167	443	464	234	4.62	4.51	4.05	0.97	0.97	0.96
	46	10267	8593	1752	365	234	138	464	477	185	4.48	3.72	3.97	0.97	0.91	0.96
	66	3080	6505	2873	384	155	101	458	220	136	4.73	3.35	3.43	0.97	0.92	0.94
35	25	3595	2322	ND	451	321	ND	565	519	ND	4.76	4.41	ND	0.97	0.95	ND
	35	12292	4320	ND	444	262	ND	576	457	ND	4.98	4.42	ND	0.98	0.97	ND
	38	8030	2986	ND	396	257	ND	470	457	ND	4.75	4.39	ND	0.98	0.97	ND
	46	11899	3190	ND	367	326	ND	475	529	ND	4.62	4.76	ND	0.97	0.98	ND
	66	17669	1617	ND	281	226	ND	323	327	ND	4.26	4.01	ND	0.96	0.95	ND
62	25	5210	4117	1908	496	266	180	630	423	278	5.07	4.26	4.00	0.98	0.96	0.96
	35	8781	4719	1954	454	323	195	556	550	316	5.10	4.58	3.99	0.98	0.97	0.96
	38	8946	2799	1300	398	278	184	533	490	241	4.68	4.55	4.29	0.97	0.98	0.97
	46	2919	6984	2620	289	221	90	341	275	110	4.28	4.21	3.14	0.96	0.96	0.87
	66[f]	ND	7305	2289	ND	167	104	ND	260	145	ND	3.14	3.39	ND	0.86	0.93

[a] The total number of samples for *Bacteria* and *Archaea* was 16 and the total for *Chloroflexi* was 11.
[b] Number of sequences after quality control steps and before normalisation by subsampling to lowest number of sequences. Total numbers of sequences for *Bacteria, Archaea* and *Chloroflexi* were 12,266, 70,281, and 24,731, respectively. Total numbers of OTUs for *Bacteria, Archaea* and *Chloroflexi* were, 6037, 4282 and 1750, respectively.
[c] OTU: Operational taxonomic unit at 97% sequence similarity to reference sequence by UCLUST Edgar, R.C. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*. 26:2460-2461.
[d] Simpson's Index of Diversity (1-D) was used.
[e] ND, not submitted for sequencing
[f] It was not possible to sequence Bacteria from sample Day 62 Temperature 66°C sufficiently and so it was excluded from analysis.

Table 5.5 - Pearson correlation coefficients for the relationship between diversity indices (Table 5.4), temperature and day of sampling.

		Pearson Correlation Coefficient[a]		
		Chao1	Shannon Index	Simpson's Index of Diversity
Bacteria	Temperature	-0.76 *	-0.62 *	-0.25
	Day	0.03	-0.08	-0.05
Chloroflexi	Temperature	-0.83 *	-0.72 *	-0.46
	Day	-0.18	-0.26	-0.32
Archaea	Temperature	-0.71 *	-0.72 *	-0.62 *
	Day	-0.29	-0.10	-0.08

[a] * indicates Pearson's correlation coefficient is significant at $p < 0.05$.

diversity as temperature increased was also observed by analysis of DGGE and RISA profiles and suggested a less diverse, more temperature resilient community at higher temperatures. Alpha rarefaction was performed in QIIME for each of the five temperatures and Day 0 (Figure 5.5). In all three communities, the higher temperatures (46^{o}C and 66^{o}C) were less diverse and better sampled than Day 0 and the lower temperatures (25^{o}C, 35^{o}C and 38^{o}C). Again, this pointed to the greater OTU diversity or in this case observed species in the lower temperatures compared to the higher temperatures.

5.3.3.2 Analysis of the Effects of Temperature and Incubation Time on Bacteria, Archaea and Chloroflexi Sediment Slurry Communities

NMDS of the *Bacteria* community indicated close association between each temperature, with especially small standard deviation ellipses at 25^{o}C and 35^{o}C, with very little influence from incubation time (Figure 5.6A). Higher temperature samples (38-66^{o}C) were influenced by the geochemical concentrations in the slurry sediment, the extreme being 66^{o}C samples, which clustered away from all other temperature samples and were mostly influenced by the acetate and CO_2 concentrations. The 38^{o}C group appeared to be influenced by formate, sulphate and methane concentrations. This indicated that as temperature increased, and diversity decreased, the bacterial community became more restricted to specific, perhaps thermophilic, metabolic processes as seen in previous studies (Cole et al., 2013; Finke and Jørgensen, 2008). Correlations between higher temperatures and acetate and CO_2 concentrations were identified, which indicated that as the substrates hydrogen and CO_2 increased with temperature, from other temperature dependent reactions, acetogenesis was occurring.

The *Chloroflexi* community NMDS showed a very similar pattern to the *Bacteria* community NMDS (Figure 5.7A). Lower temperatures (25^{o}C, 35 ^{o}C and 38 ^{o}C) grouped closer to Day 0, while the 46 ^{o}C and 66 ^{o}C samples were spread further apart. The decreasing methane and sulphate concentrations appeared to have some influence the 38^{o}C group. Increasing concentrations

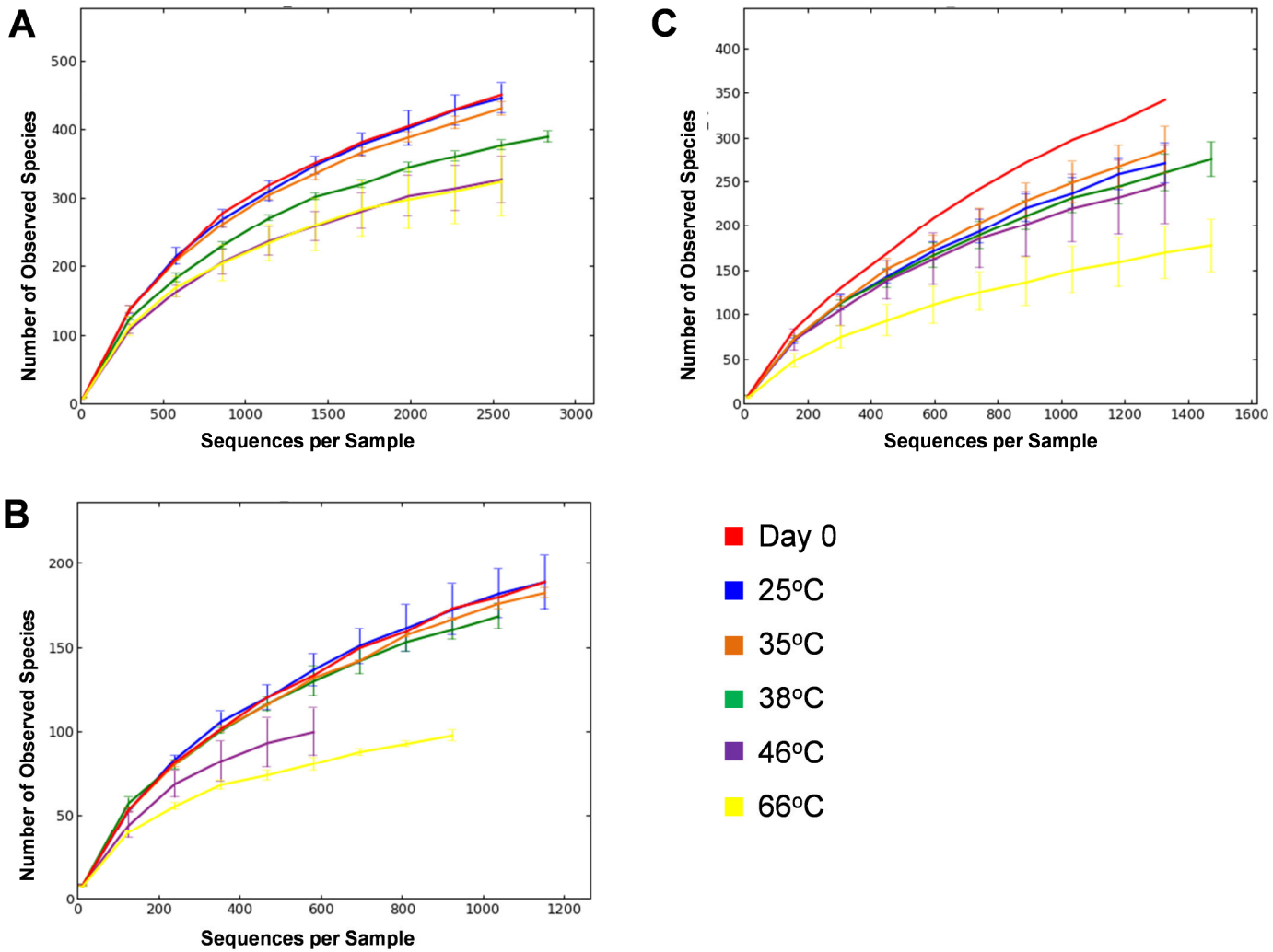


Figure 5.5 - Alpha rarefaction graphs using observed species method of 16S rRNA gene sequences from each temperature and Day 0 over a temperature gradient with singletons removed and created in QIIME (Caporaso et al., 2010b). A, *Bacteria*, B, *Archaea* and C, *Chloroflexi.* Trend lines are colour coded by sample. The number of samples in the categories Day 0, 25°C, 35°C, 38°C, 46°C and 66°C were 1, 3, 3, 3, 3, and 3, respectively.

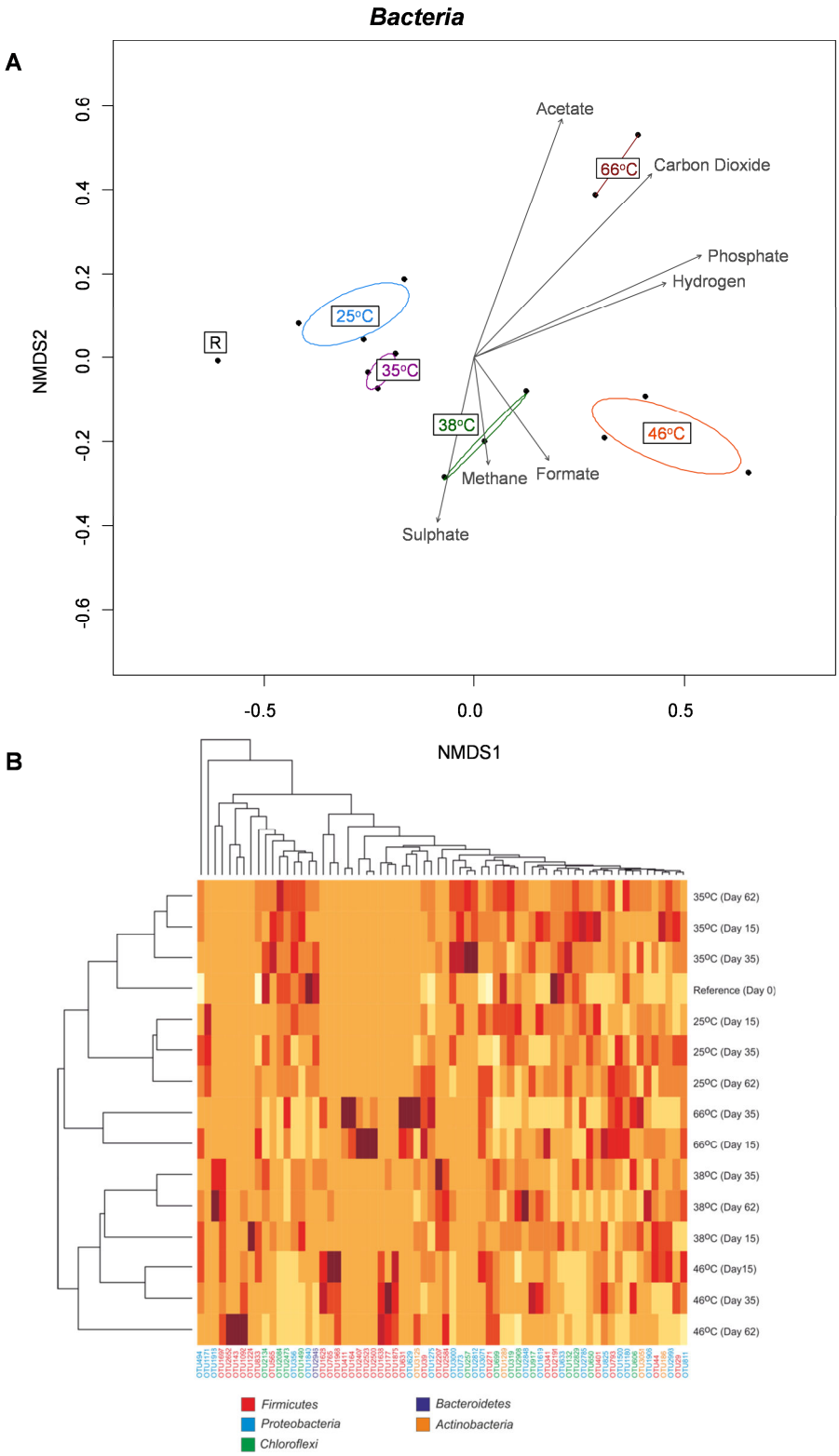


Figure 5.6 - Statistical analysis of *Bacteria* 16S rRNA gene OTUs from 454 pyrosequencing of sediment slurry on a temperature gradient, using the R statistical package (Ihaka and Gentleman, 1996). A, NMDS analysis of *Bacteria* OTUs. Samples are labelled by temperature with colour coded standard deviation of the samples given by the ellipse. R represents Day 0 as a reference sample. Geochemical vectors are represented by the grey arrows and labelled accordingly. The total number of samples for *Bacteria* was 16. B, Heatmap demonstrating the prevalence of the 100 most abundant OTUS based on the number of sequences across each day and temperature. UPGMA clustering dendrograms are given for both the sample and OTUS. The colour gradient indicates abundance with dark red being the most and pale yellow being the least abundant. The sample name is given next to each row, colour coded by phylum, is given below each column.

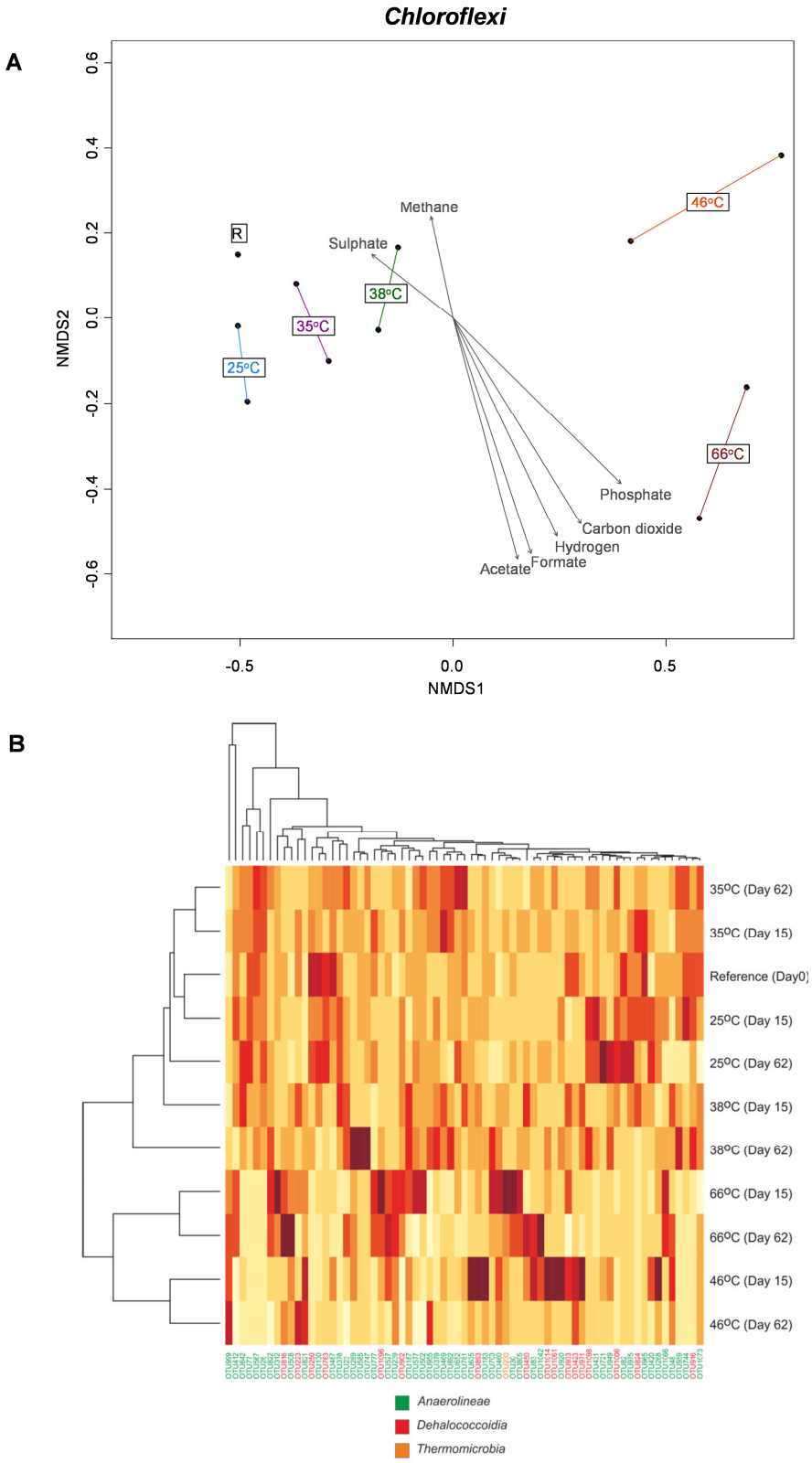


Figure 5.7 - Statistical analysis of *Chloroflexi* 16S rRNA gene OTUs from 454 pyrosequencing of sediment slurry on a temperature gradient, using the R statistical package (Ihaka and Gentleman, 1996). A, NMDS analysis of *Chloroflexi* OTUs. Samples are labelled by temperature with colour coded standard deviation of the samples given by the ellipse. R represents Day 0 as a reference sample. Geochemical vectors are represented by the grey arrows and labelled accordingly. The total number of samples for *Chloroflexi* was 11. B, Heatmap demonstrating the prevalence of the 50 most abundant OTUS based on the number of sequences across each day and temperature. UPGMA clustering dendrograms are given for both the sample and OTUS. The colour gradient indicates abundance with dark red being the most and pale yellow being the least abundant. The sample name is given next to each row and the OTU, colour coded by class, is given below each column.

of CO_2 appeared to have an influence on the 66°C group. The limited influence of the geochemical concentrations in the sediment slurry on the *Chloroflexi* suggested that other geochemical variables not measured here (e.g. dissolved organic carbon) were responsible for the *Chloroflexi* community distribution or that temperature was in fact the largest influence on this community.

The *Archaea* NMDS appeared to show the same pattern as the *Bacteria* groupings but on a more extreme scale (Figure 5.8A). The lower temperatures (25°C, 35°C and 38°C) were very tightly clustered with Day 0 with some influence from decreasing methane and sulphate concentrations (see magnification box in Figure 5.8A). However, the higher temperatures (46°C and 66°C) were spread apart on a larger scale with very large standard deviations, which indicated a much larger heterogeneity in these higher temperatures, compared to the lower temperature samples. The geochemical vectors appeared to have little influence on these samples except for one of the 66°C samples, which clustered with increasing acetate, hydrogen and CO_2 concentrations. It is possible that due to the peak activity of methanogenesis occurring at 46°C, methanogenic substrates such as acetate, hydrogen and CO_2 were able to build up as temperature increased and methanogenesis rates dropped.

Heatmaps with UPGMA clustering revealed patterns of abundance within individual OTUs in each community. The samples clustered based on temperature and Day 0 with the 25°C and 35°C groups within the *Bacteria* and *Chloroflexi* (Figure 5.6B & 5.7B). A number of the most abundant OTUs within the *Bacteria* were present at all temperatures including *Proteobacteria, Chloroflexi, Firmicutes* and *Actinobacteria* OTUs. A group of *Chloroflexi* and *Proteobacteria* OTUs typified the 35°C and the initial community (Day 0) samples. These OTUs belonged to *Anaerolineae* (MSB-1E9 and SHA-20) and *Dehalococcoidia* (GIF9) clonal groups, *Thiotrichales* of the *Gammaproteobacteria* and *Desulfobacterales* of the *Deltaproteobacteria.* The *Archaea* were clustered in a complicated and mixed pattern, not entirely dependent on temperature or day of sampling, which indicated a complex

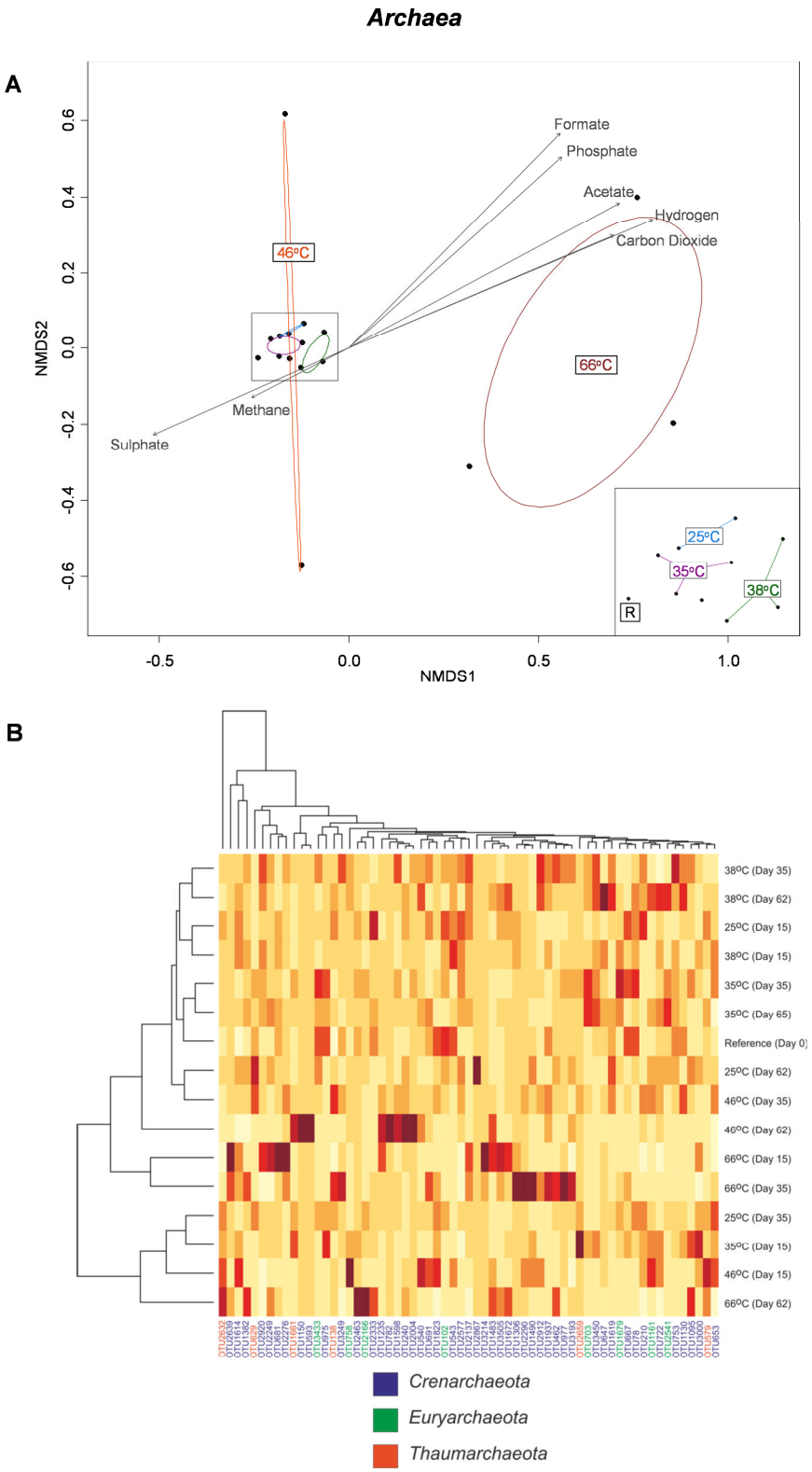


Figure 5.8 - Statistical analysis of *Archaea* 16S rRNA gene OTUs from 454 pyrosequencing of sediment slurry on a temperature gradient, using the R statistical package (Ihaka and Gentleman, 1996). A, NMDS analysis of *Archaea* OTUs. Samples are labelled by temperature with colour coded standard deviation of the samples given by the ellipse. R represents Day 0 as a reference sample. Geochemical vectors are represented by the grey arrows and labelled accordingly. The detail of the sample cluster is magnified in the bottom right box. The total number of samples for *Archaea* was 16. B, Heatmap demonstrating the prevalence of the 50 most abundant OTUS based on the number of sequences across each day and temperature. UPGMA clustering dendrograms are given for both the sample and OTUS. The colour gradient indicates abundance with dark red being the most and pale yellow being the least abundant. The sample name is given next to each row, colour coded by phylum, is given below each column.

response to the temperature incubation (Figure 5.8B). For example all three 25^{o}C samples were split between 38^{o}C, 46^{o}C and 66^{o}C groups. The most abundant OTUs belonged to the MCG of the *Crenarchaeota*. The most abundant groups in each temperature sample were not shared equally in that temperature group i.e. there was more sample to sample variation and again a more complex community response.

5.3.3.3 Changes in the Bacteria Community from Sediment Slurry over an Increasing Temperature Gradient and Time Scale

The *Proteobacteria, Firmicutes, Chloroflexi, Actinobacteria, Bacteroidetes, Acidobacteria* and OP8 were the most abundant phyla, together constituting more than 90% of the bacterial community (Figure 5.9). *Proteobacteria* and *Chloroflexi* were the most abundant at lower temperatures but decreased significantly as *Firmicutes* proliferated to a peak at 46^{o}C (80% of the total *Bacteria* community; Figures 5.9 & 5.10). *Bacteroidetes, Acidobacteria* and OP8 decreased as temperature increased whereas *Actinobacteria* slightly increased. A one tailed ANOVA was used to compare the significance of each taxonomic group with temperature. Of these, the *Proteobacteria, Firmicutes, Bacteroidetes* and *Chloroflexi* were all statistically significant ($P < 0.05$) and will be further discussed (Figure 5.10).

5.3.3.3.1 The Firmicutes Response to Temperature

The *Firmicutes* significantly increased at higher temperatures, constituting 55-65% of the bacterial community at 46^{o}C at all-time points (Figures 5.9 & 5.10B). The *Bacilli* decreased as temperature increased whereas the *Clostridia* became the dominant class with a significant increase in abundance ($P < 0.05$; Figure 5.11). At the order level, two distinct communities of *Clostridia* emerged. *Clostridiales* and uncultivated environmental clone group BSA2B-08 (>97% sequence similarity between clones), which was originally identified in thermophilic anaerobic waste reactors (AB175380; Y.Tang, T.Shigematsu, S. Morimura, and K. Kida, unpublished results) were detected at significantly higher levels at 46^{o}C,

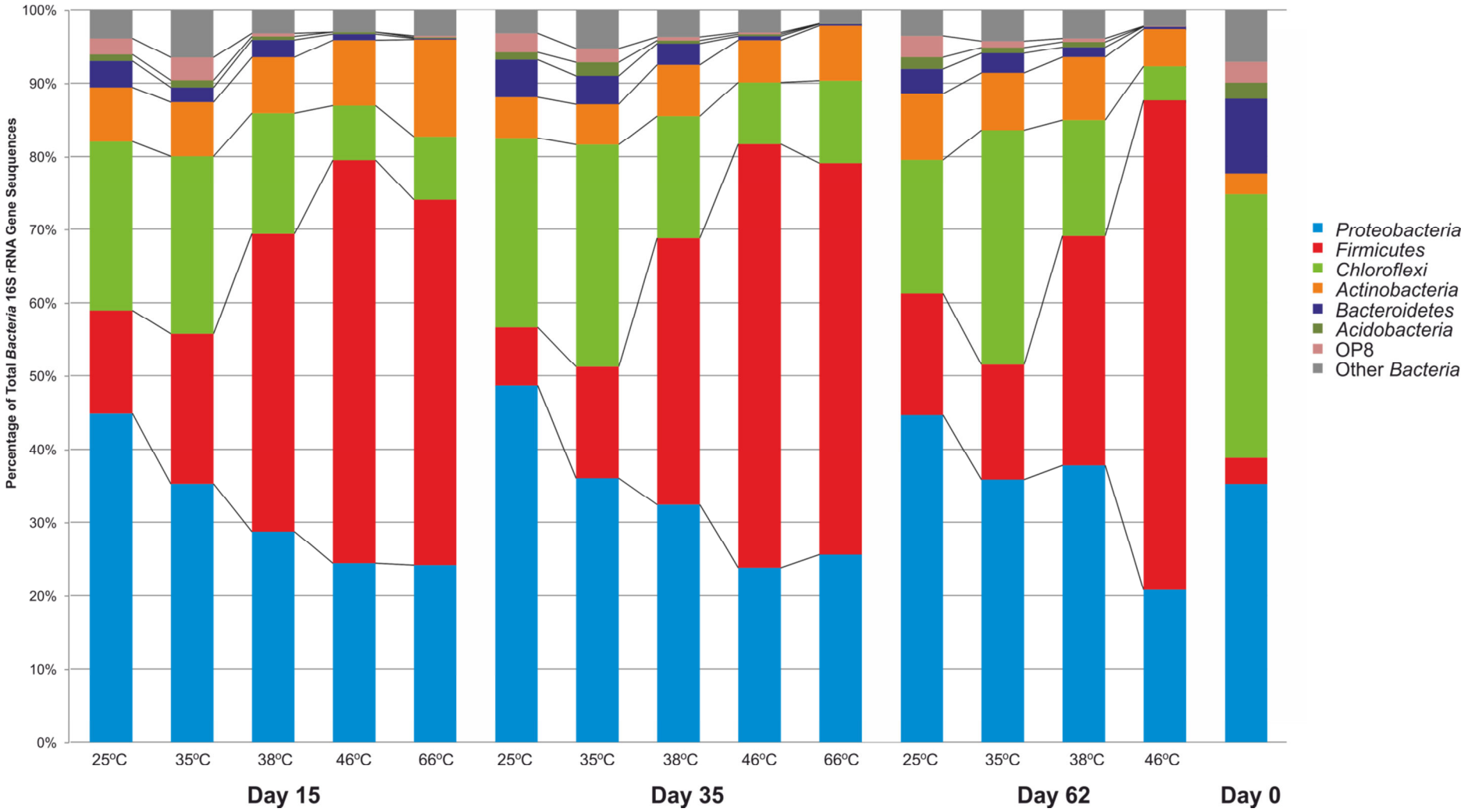


Figure 5.9 - Phylogenetic association of all bacterial 454 sequences from sediment slurry at each temperature and time point. The 7 most abundant phyla are shown with series lines to indicate trends. Phylogenetic designation was by based on BLAST. The total number of sequences used was 25°C – 7860, 35°C – 7743, 38°C – 8165, 46°C – 8197, 66°C - 5492 and Day 0 - 2437. The total number of samples for *Bacteria* was 16.

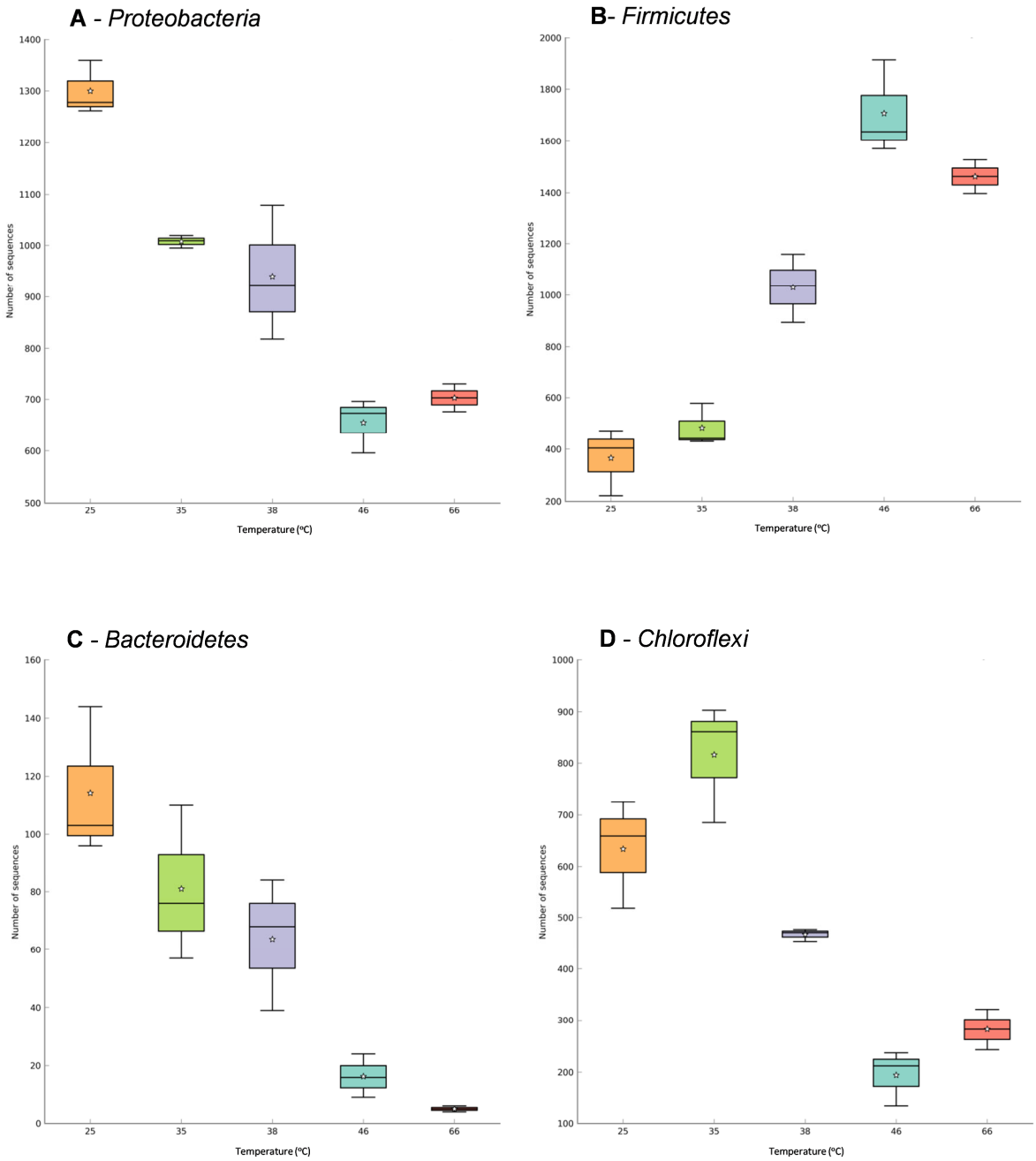


Figure 5.10 - Abundance profiles of statistically significant *Bacteria* phyla in sediment slurries incubated at selected temperatures over all sampling days. A, *Proteobacteria,* B, *Firmicutes,* C, *Bacteroidetes* and, D, *Chloroflexi.* Samples were compared using an ANOVA with a Tukey-Kramer Post-Hoc test using STAMP v2.0.0 (Parks and Beiko, 2010). Only statistically significant changes in abundance with temperature are shown (P value << 0.05). There was a total number of 15 samples analysed, 3 for each temperature. Day 0 was omitted from this analysis as there was only one sample and so could not perform ANOVA. The stars indicate the average sequence number for each temperature group, the line is the median and the top and bottom edges of the box are the 75th and 25th percentile, respectively. The whiskers indicate the maximum and minimum values.

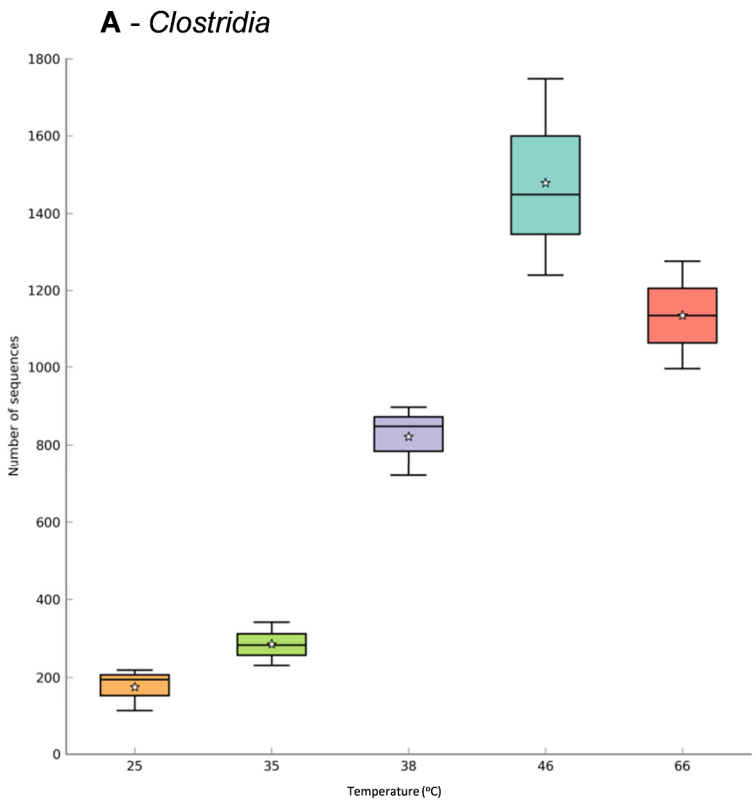


Figure 5.11 - Abundance profiles of statistically significant *Firmicutes,* class *Clostridia* in sediment slurries incubated at selected temperatures over all sampling days. Samples were compared using an ANOVA with a Tukey-Kramer Post-Hoc test using STAMP v2.0.0 (Parks and Beiko, 2010). Only statistically significant changes in abundance with temperature are shown (P value << 0.05). There was a total number of 15 samples analysed, 3 for each temperature. Day 0 was omitted from this analysis as there was only one sample and so could not perform ANOVA. The stars indicate the average sequence number for each temperature group, the line is the median and the top and bottom edges of the box are the 75th and 25th percentile, respectively. The whiskers indicate the maximum and minimum values.

whereas *Thermoanaerobacterales* were significantly abundant at 66^{o}C (Figure 5.12).

Two genera were found to increase significantly in abundance as temperature increased: the *Sporotomaculum* and *Tepidimicrobium* (Figure 5.13). The *Sporotomaculum* genus, which showed a peak at 46^{o}C, indicated a rise in the production of spore-forming bacteria, resilient to the increase in temperature (Figure 5.13A; Qiu et al., 2003). The *Tepidimicrobium* genus, which was most abundant at 66^{o}C, is characterised by two type strains, both anaerobic, moderately thermophilic organisms (Niu et al., 2009; Slobodkin et al., 2006). *Tepidimicrobium xylanilyticum* is a fermentative organism which utilises a number of carbohydrates and had the higher growth temperature range (25-67^{o}C) of the two type strains and was spore-forming (Niu et al., 2009), which further indicated that spore-forming bacteria were more abundant at higher temperatures.

Desulfotomaculum genus sequences (family *Peptococcaceae,* order *Clostridiales*) were also found at high abundances at 46-66^{o}C, and contributed two of the most abundant OTUs at these temperatures (OTU1968 at 46^{o}C, and, OTU2407 and OTU2500 at 66^{o}C; Figure 5.6B). The *Desulfotomaculum* are also thermophilic and spore-forming and are unusual in the sulphate reducing bacteria in that they are able to grow autotrophically (Madigan et al., 2010), utilising hydrogen and sulphate as electron donor and acceptor, respectively, and CO_2 as a carbon source (Aullo et al., 2013). Due to this metabolic versatility and resilience, it is believed that the *Desulfotomaculum* may play an important role in lithoautotrophic communities in the deep subsurface environment (Aullo et al., 2013; Hubert et al., 2010). OTU1968 shared 99% sequence similarity with uncultivated environmental clones from two investigations into endospore community activation by rising temperature in Artic and Aarhus Bay surface sediments (de Rezende et al., 2013; Hubert et al., 2010), which indicated that OTU1968 was part of a wider *Desulfotomaculum* community, commonly found in marine sediments. OTU2407 and OTU2500 shared 99% and 100% sequence similarity, respectively, with cultivated species such as

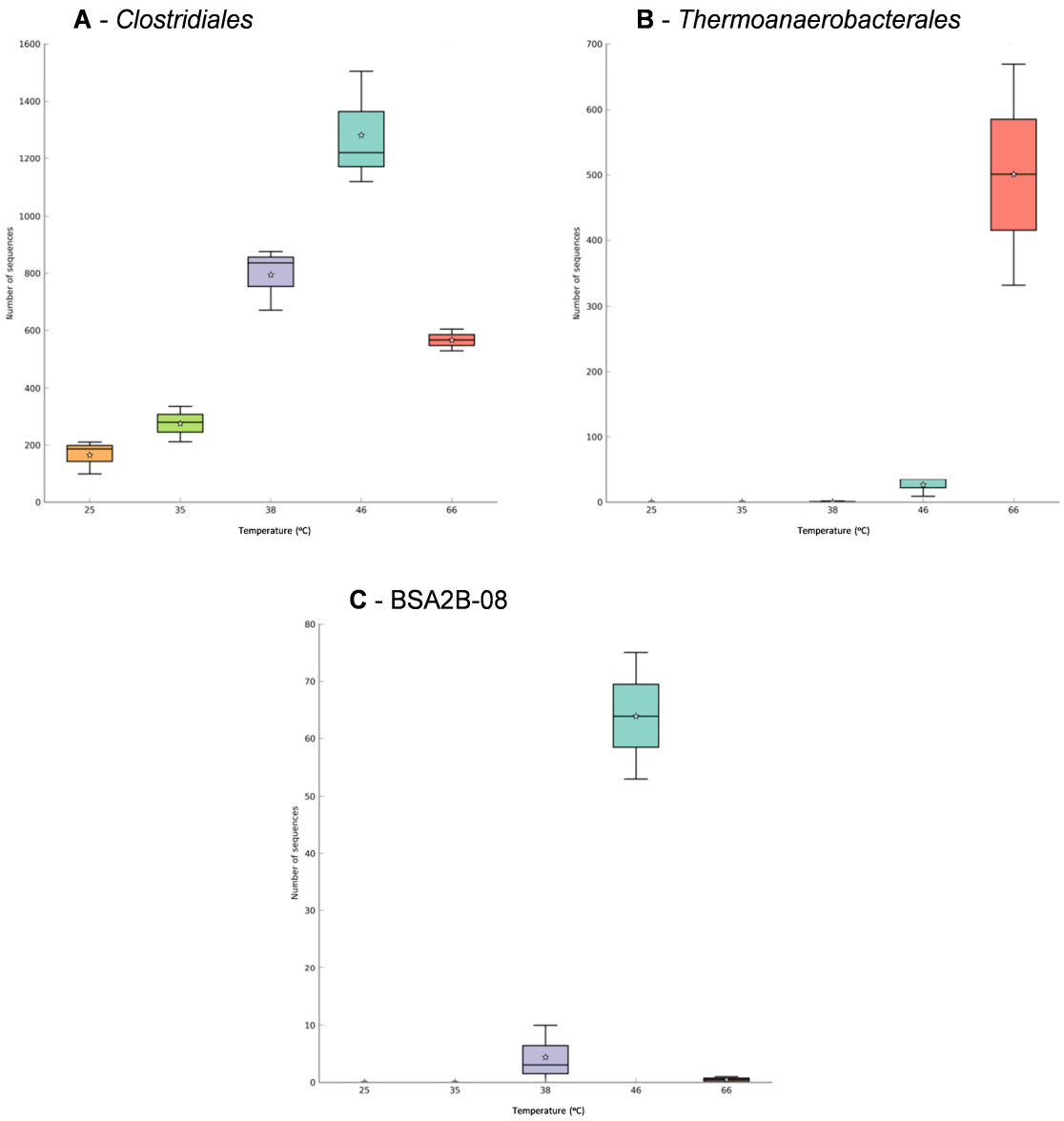


Figure 5.12 - Abundance profiles of statistically significant *Clostridia* order level taxa in sediment slurries incubated at selected temperatures over all sampling days. A, *Clostridiales,* B, *Thermoanaerobacterales* and, C, the uncultivated clone group BSA2B-08. Samples were compared using an ANOVA with a Tukey-Kramer Post-Hoc test using STAMP v2.0.0 (Parks and Beiko, 2010). Only statistically significant changes in abundance with temperature are shown (P value << 0.05). There was a total number of 15 samples analysed, 3 for each temperature. Day 0 was omitted from this analysis as there was only one sample and so could not perform ANOVA. The stars indicate the average sequence number for each temperature group, the line is the median and the top and bottom edges of the box are the 75th and 25th percentile, respectively. The whiskers indicate the maximum and minimum values.

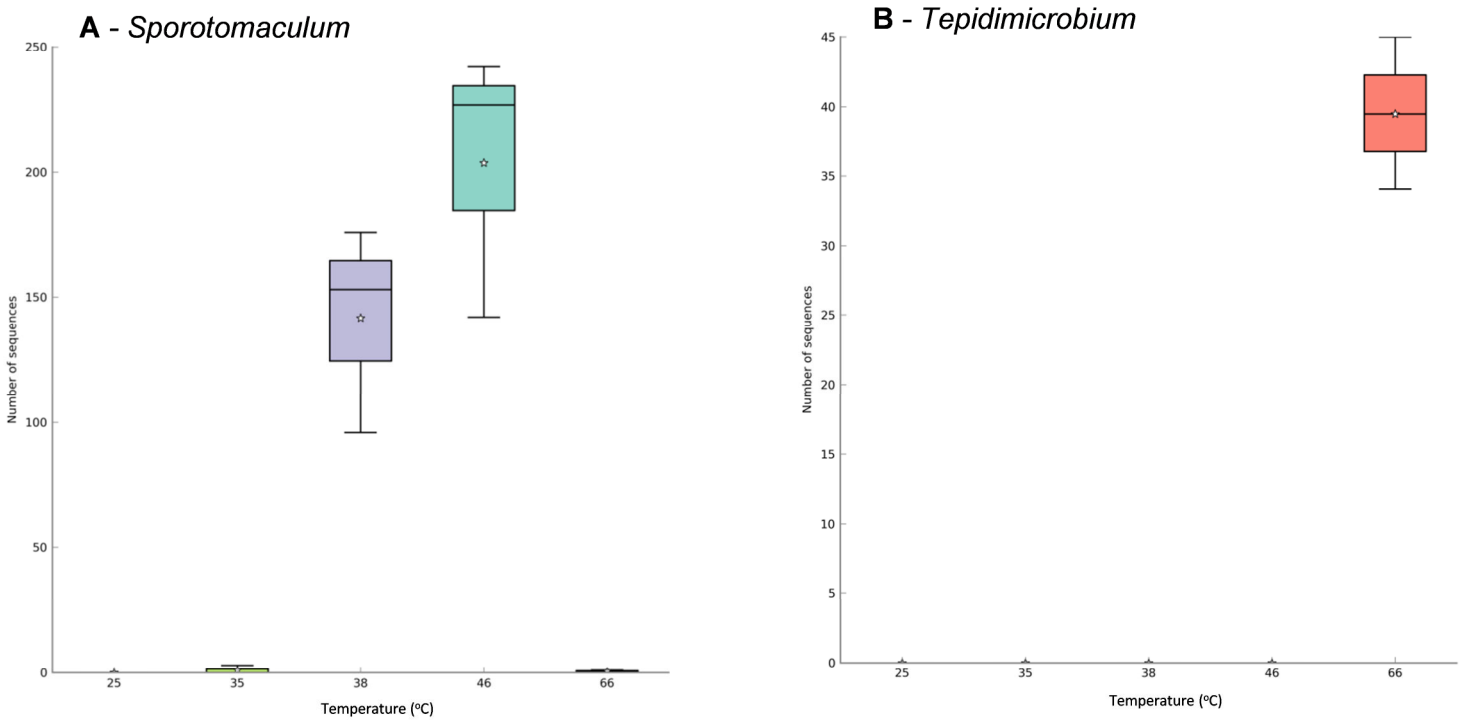


Figure 5.13 - Abundance profile of the statistically significant *Clostridia* genus, A, *Sporotomaculum* and B, *Tepidimicrobium* in sediment slurries incubated at selected temperatures over all sampling days. Samples were compared using an ANOVA with a Tukey-Kramer Post-Hoc test using STAMP v2.0.0 (Parks and Beiko, 2010). Only statistically significant changes in abundance with temperature are shown (P value << 0.05). There was a total number of 15 samples analysed, 3 for each temperature. Day 0 was omitted from this analysis as there was only one sample and so could not perform ANOVA. The stars indicate the average sequence number for each temperature group, the line is the median and the top and bottom edges of the box are the 75th and 25th percentile, respectively. The whiskers indicate the maximum and minimum values.

Desulfotomaculum australicum AB33 (Love et al., 1993; Patel et al., 1992) and *Desulfotomaculum solfataricum* V21 (Goorissen et al., 2003). The abundance of the *Desulfotomaculum* at temperatures above 46^{o}C indicated a shift from heterotrophic sulphate reduction by *Deltaproteobacteria* (see **Section 5.3.3.3.2**), to autotrophic sulphate reduction by the *Desulfotomaculum*, which was in line with other findings (Roussel, E.G., et al., unpublished results).

5.3.3.3.2 The Proteobacteria Response to Temperature

ANOVA analysis showed the *Deltaproteobacteria* decreased significantly in abundance; this was the only *Proteobacteria* class to change significantly in abundance with temperature (Figure 5.14). The *Deltaproteobacteria* decreased from approximately 50% of the *Proteobacteria* community at Day 0 and at 25^{o}C to less than 13% at 66^{o}C and as such were significantly negatively correlated with temperature (Table 5.6). The *Desulfobulbaceae* were abundant at lower temperatures (14-20% of the bacterial community at 25^{o}C), but decreased to <0.01% of the bacterial community at higher temperatures*. Desulfobulbaceae* are heterotrophic and utilise complex organic carbon molecules as electron donors for sulphate reduction (Sass et al., 2002; Sorokin et al., 2011). The most abundant *Proteobacteria* OTU at 25^{o}C was OTU1171, which was related to *Desulfobulbaceae* (Figure 5.6B), shared 99% sequence similarity with *Desulfotalea* sp. SFA4, isolated from an intertidal flat from the North Sea (H. Ruetters, H. Sass, H. Cypionka, and J. Rullkoetter, unpublished results). The type strain of the genus, *Desulfotalea psychrophila,* is a psychrophilic organism, isolated from marine sediments with *in situ* temperatures permanently below 0^{o}C (Rabus et al., 2004). As previously discussed in **Section 5.3.3.3.1**, the abundance of *Desulfobulbaceae* and the subsequent increase in abundance of *Desulfotomaculum* indicated a shift from heterotrophic to autotrophic sulphate reduction as temperatures increased. Here, the *Desulfobulbaceae* OTUs were closely associated with low temperatures, indicating a strong influence of temperature on the sulphate reducing bacterial community in the sediment slurry.

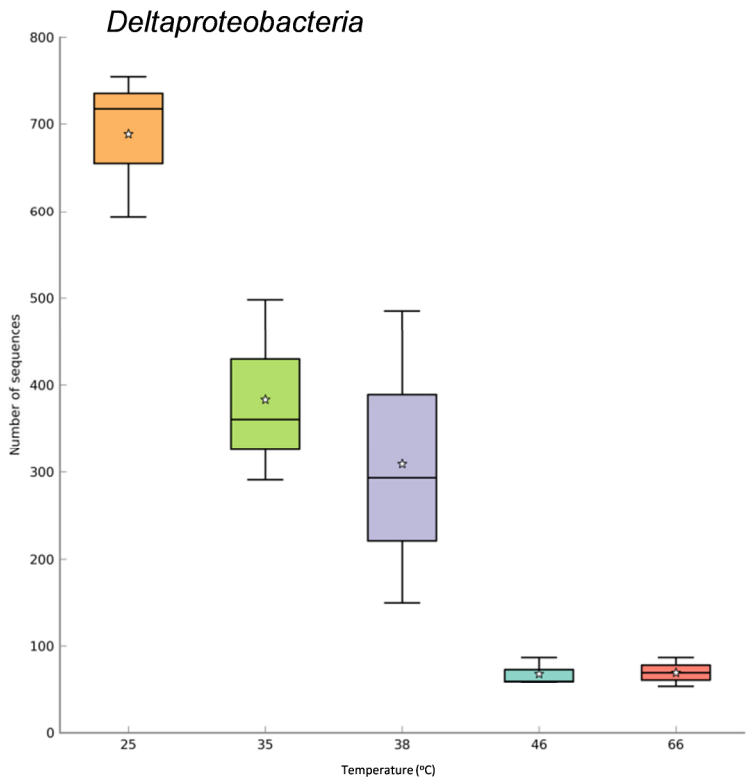


Figure 5.14 - Abundance profiles of statistically significant *Proteobacteria* class level *Deltaproteobacteria* in sediment slurries incubated at selected temperatures over all sampling days. Samples were compared using an ANOVA with a Tukey-Kramer Post-Hoc test using STAMP v2.0.0 (Parks and Beiko, 2010). Only statistically significant changes in abundance with temperature are shown (P value << 0.05). There was a total number of 15 samples analysed, 3 for each temperature. Day 0 was omitted from this analysis as there was only one sample and so could not perform ANOVA. The stars indicate the average sequence number for each temperature group, the line is the median and the top and bottom edges of the box are the 75th and 25th percentile, respectively. The whiskers indicate the maximum and minimum values.

Table 5.6 - Pearson correlation coefficients for the relationships between *Bacteria* phyla, temperature and geochemistry. Pearson's correlation was performed using Microsoft Excel 2010. Only statistically significant correlations (P value <0.05) are shown. P values were derived using a correlation coefficients table (Fry and Iles, 1994).

Phylum	Class	Temperature	Methane	Acetate	Sulphate	Hydrogen	Carbon Dioxide
Proteobacteria	-	-0.67	-	-	-	-	-
Proteobacteria	*Deltaproteobacteria*	-0.67	-	-	-	-	-
Chloroflexi	-	-0.75	-	-	-	-0.56	-
Chloroflexi	*Anaerolineae*	-	-0.70	-	-	-	-
Chloroflexi	*Ktedonobacteria*	-	-	0.87	-	-	-
Chloroflexi	S085	-	-0.60	-	-	-	-
Firmicutes	-	0.79	-	-	-	0.59	-
Firmicutes	*Clostridia*	-	0.71	-	-	-	-
Actinobacteria	-	0.62	-	-	-	0.53	-
Actinobacteria	MB-A2-108	-	-	-	-0.54	-	-0.60
Acidobacteria	-	-0.82	-	-	-	-0.75	-0.55
Acidobacteria	*Acidobacteria-6*	-	-0.62	-	-	-	-
Acidobacteria	*Acidobacteriia*	-	-	0.99	-0.52	-	0.52
Acidobacteria	Sva0725	-	-0.52	0.56	-	-	-
Bacteroidetes	-	-0.90	-	-	-	-0.67	-0.73
Bacteroidetes	*Sphingobacteriiia*	-	-	0.85	-0.54	-	0.55
OP8	-	-0.76	-	-	-	-0.65	-0.57
OP8	OP8-1	-	-0.64	-	-	-	-
Cyanobacteria	-	0.58	-	0.96	-	-	0.56
Caldithrix	-	-0.75	-	-	-	-	-0.55
Chlorobi	-	-0.61	-	-	-	-	-

5.3.3.3.3 The Bacteroidetes Response to Temperature

The *Bacteroidetes* decreased significantly with temperature by ANOVA and were significantly negatively correlated with temperature (Figure 5.9 & 5.10C& Table 5.6). The class *Flavobacteriia* and order *Flavobacteriales* decreased with temperature, mirroring the pattern of the phylum overall. The *Flavobacteria* are a group of environmental bacteria often found in seawater, sediments and soils and the known cultivated strains, including the type strain *Flavobacterium aquatile,* are either psychrophilic, psychrotolerant or mesophilic and so would not be expected to thrive at higher temperatures (Sheu et al., 2013).

5.3.3.3.4 Other Phyla of Interest and Response to an Increase in Temperature

The candidate phylum OP8 (now known as *Aminicenantes;* Rinke et al. 2013) phylotypes were also found to decrease significantly (P value << 0.05) in abundance as temperature increased, and was significantly negatively correlated with temperature, hydrogen and CO_2 (Table 5.6). As all OTUs were related to environmental clones, further speculation on the role of OP8 was limited. The uncultivated clone class level group OP8-1 was, however, found to be significantly, negatively correlated with methane (Table 5.6). The environmental clones that constitute OP8-1 were originally isolated from a range of different environments, including mangrove soil, contaminated soils, marine sediments, and microbial mats. The most abundant OP8-1 OTU (OTU562) at 25^{o}C, and gave top BLAST matches (97% sequence similarity) to uncultivated environmental clones from marine surface sediments, including clone MD2896-B22, originally isolated from surface sediments of the South China Sea (Li and Wang, 2013). The OP8 OTUs found in this work are related to mesophilic sediment isolates, which indicated that the OP8 community did not have sufficient resilience to cope with the extreme temperature change.

The *Actinobacteria* increased and were significantly positively correlated with temperature (Figure 5.9 & Table 5.6). However, the phylum was not found to change significantly. The moderately thermophilic *Thermoleophilia* class

increased rapidly from 20% of the *Actinobacteria* community to more than 50% at 66^{o}C. The most abundant *Thermoleophilia* OTU (OTU3126) was from the order *Gaiellales* and shared 99% sequence similarity to two heterotrophic uncultivated clones isolated from the surface of high alkaline microbialites and were thought to be involved with carbonate precipitation by the degradation of complex organic compounds (Figure 5.6B; Couradeau et al., 2011; Lopez-Garcia et al., 2005).

5.3.3.4 Changes in the Chloroflexi Community from Sediment Slurry over an Increasing Temperature Gradient and Time Scale

The *Chloroflexi* phylum was found to decrease significantly in abundance as temperature increased from approximately 20% at 25^{o}C to <10% at 66^{o}C of the total bacterial community (Figures 5.9 & 5.10D). The *Chloroflexi* community were targeted using specific PCR primers, which detected *Anaerolineae, Dehalococcoidia, Thermomicrobia, Ktedonobacteria* and a number of groups of uncultivated environmental clones, such as Ellin6529, S085, Gitt-GS-136, SHA-26 and Tk10 (Figure 5.15). The *Anaerolineae* dominated at each temperature with the *Dehalococcoidia* second in abundance, together they constituted >95% of the *Chloroflexi* community (Figure 5.15). A small number of *Thermomicrobia* and *Ktedonobacteria* sequences were detected at 66^{o}C (Figure 5.15). Below the class level of taxonomy, many of the *Chloroflexi* identified were related to groups of uncultivated environmental clones for which there is no biochemical, physiological or ecological information. Some *Chloroflexi* were assigned to cultured groups at the family level (*Anaerolinaceae, Caldilineaceae, Dehalococcoidaceae* and *Ktedonobacteraceae*), but could not be assigned to genus, which indicated relatives of these cultured organisms were present in Severn Estuary sediments but the majority of the *Chloroflexi* were of a previously undescribed, high diversity.

Pyrosequencing and qPCR gave conflicting figures of the abundance of the *Chloroflexi* phylum of the total *Bacteria*. Whereas pyrosequencing indicated that the *Chloroflexi* were approximately 5-35% of the total community (Figure 5.9), qPCR indicated *Chloroflexi* were 18-100% of the total *Bacteria* (Figure

5.4). The ability of the *Chloroflexi* primers (Gich et al. 2002) to non-specifically amplify *Firmicutes* has been previously described **(Sections 3.3.1 & 3.4.1)**; therefore it is possible that as *Firmicutes* increased with temperature, the amount of non-specific amplification increased too. Also, calculations of copy number using qPCR estimate the average number of 16S genes per genome for *Bacteria* and *Archaea* and so can lead to error in the calculation **(Section 3.4.2)**. Actively growing communities, such as the *Firmicutes* at increased temperatures (indicated by qPCR; Figure 5.4A & B), have been known to up regulate their ploidy to >40 copies during exponential phase (Pecoraro et al. 2011), which would further bias the qPCR counts of *Chloroflexi*.

5.3.3.4.1 The Anaerolineae Response to Temperature

Anaerolineae sequence numbers were found to decrease as temperature increased, to a minimum at 46^{o}C, and then rise again at 66^{o}C, which mirrored the *Chloroflexi* (Figures 5.10D, 5.16A & 5.17A). No other class level group were found to change significantly by ANOVA. The increased abundance at 66^{o}C indicated a selection of *Anaerolineae* adapted to higher temperatures, possibly linked to the growth of a thermophilic community. The *Anaerolineae* type strains are all thermophilic and so the presence of new thermophilic communities would be unsurprising (Sekiguchi et al., 2003; Yamada et al., 2006). There appeared to be two communities of *Anaerolineae.* The first peaked in abundance at 35^{o}C, then decreased as temperature increased and consisted of the most abundant uncultivated clone groups SHA-20, OPB11 and O4D2737 (Figures 5.16A, 5.17B & 5.18A & B), which indicated a mesophilic community. The SHA-20 uncultivated environmental clone group consists of a small number of clones originally isolated from mesophilic mangrove and mud volcano sediments (EF061971, X. Zhang and J. Xu, unpublished results; AY592333, S. K. Heijs, A. M. Laverman and L. J. Forney, unpublished results), whereas OPB11 and O4D2737 are made up of clones isolated from mesophilic hypersaline microbial mats (Ley et al., 2006). This suggested that the *Anaerolineae*

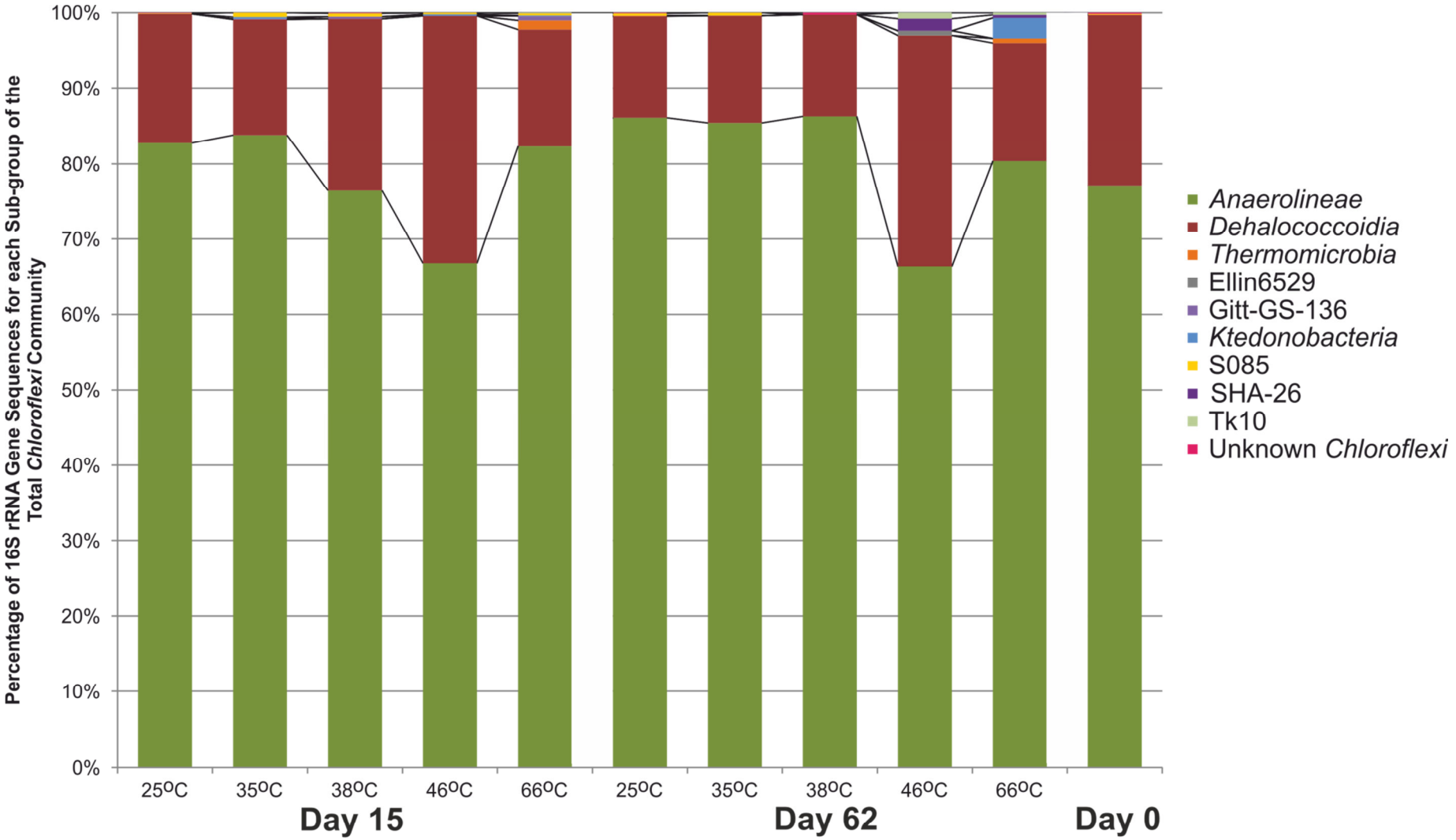


Figure 5.15 - Phylogenetic association based on BLAST results of all *Chloroflexi* 454 sequences at each temperature forma library amplified with *Chloroflexi* specific primers. The percentage of the total number of *Chloroflexi* sequences of each class is given. The total number of sequences for each temperature used was 25°C – 2474, 35°C – 2472, 38°C – 2277, 46°C – 1565, 66°C - 2094 and Day 0 - 1211. A total of 11 samples were sequenced for *Chloroflexi.* Series lines are given to indicate trends between classes.

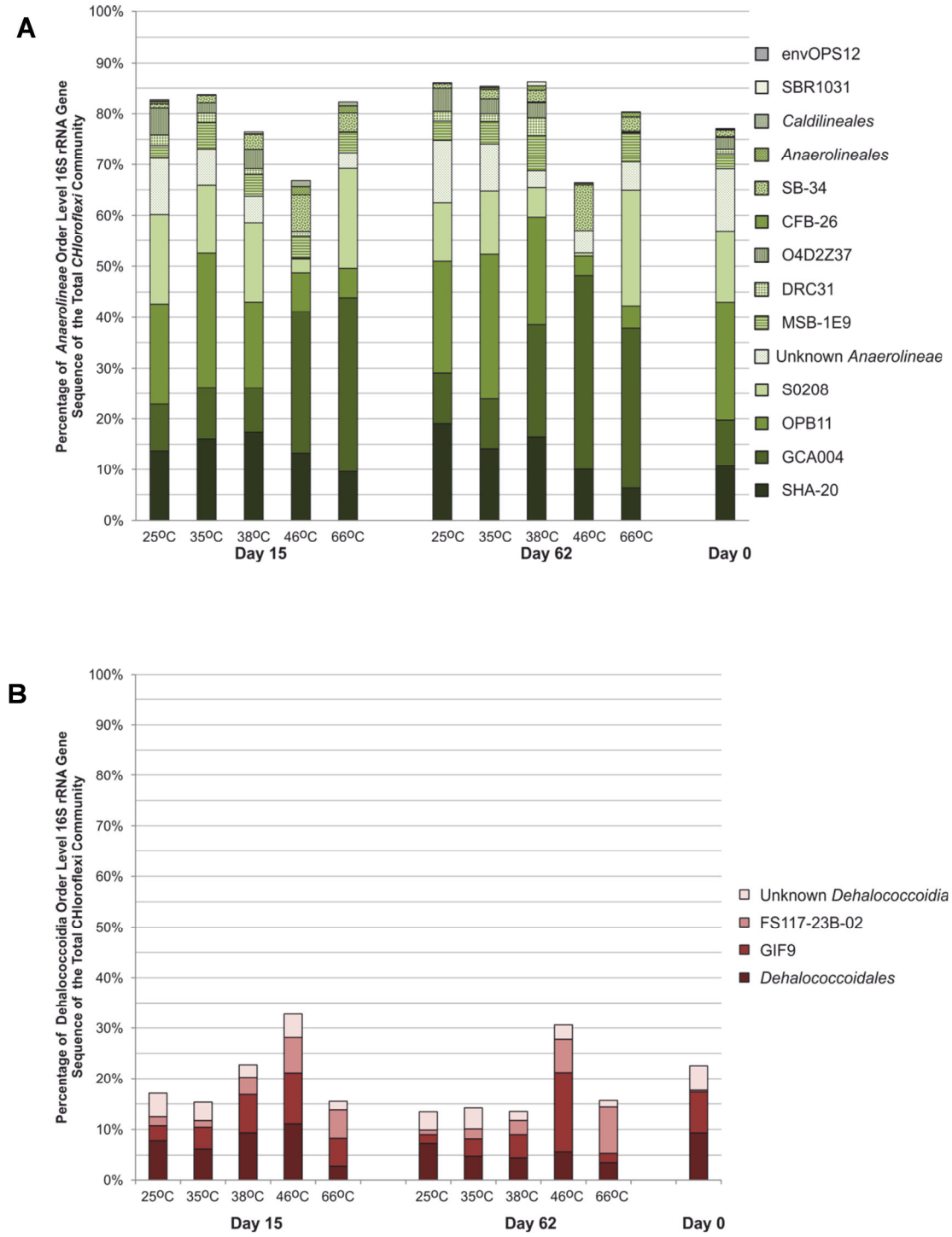


Figure 5.16 - The percentage of 16S rRNA gene sequences of each, A, *Anaerolineae* and, B, *Dehalococcoidia* order of the total *Chloroflexi* community in sediment slurries incubated on a temperature gradient and sampled at Day 0 (reference), Day 15 and Day 62. A total of 11 samples were sequenced for the *Chloroflexi* library.

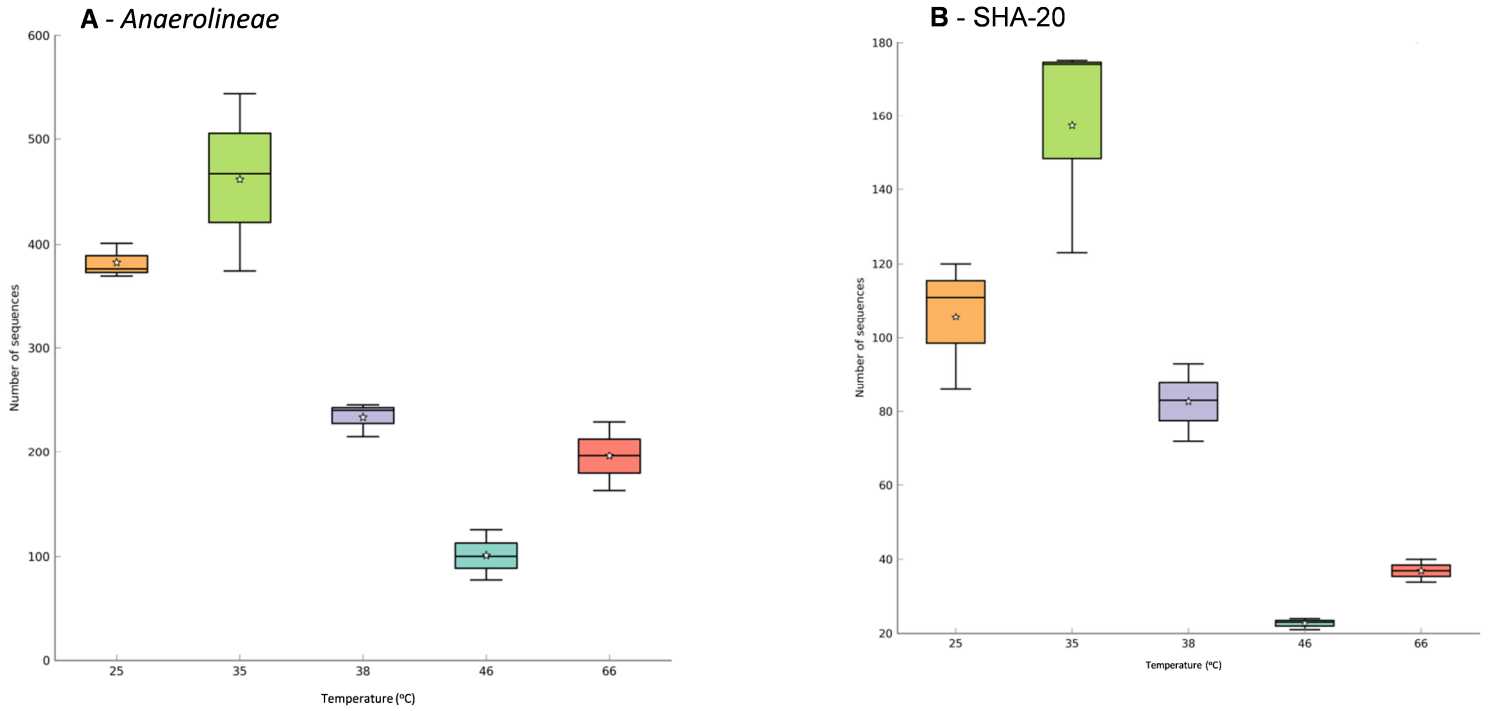


Figure 5.17 - Abundance profiles of statistically significant *Chloroflexi,* A, class *Anaerolineae* and, B, the uncultivated clone order-level taxa SHA-20, in sediment slurries incubated at selected temperatures over all sampling days, from the bacterial sequencing data set. Samples were compared using an ANOVA with a Tukey-Kramer Post-Hoc test using STAMP v2.0.0 (Parks and Beiko, 2010). Only statistically significant changes in abundance with temperature are shown (P value << 0.05). There was a total number of 15 samples analysed, 3 for each temperature. Day 0 was omitted from this analysis as there was only one sample and so could not perform ANOVA. The stars indicate the average sequence number for each temperature group, the line is the median and the top and bottom edges of the box are the 75th and 25th percentile, respectively. The whiskers indicate the maximum and minimum values.

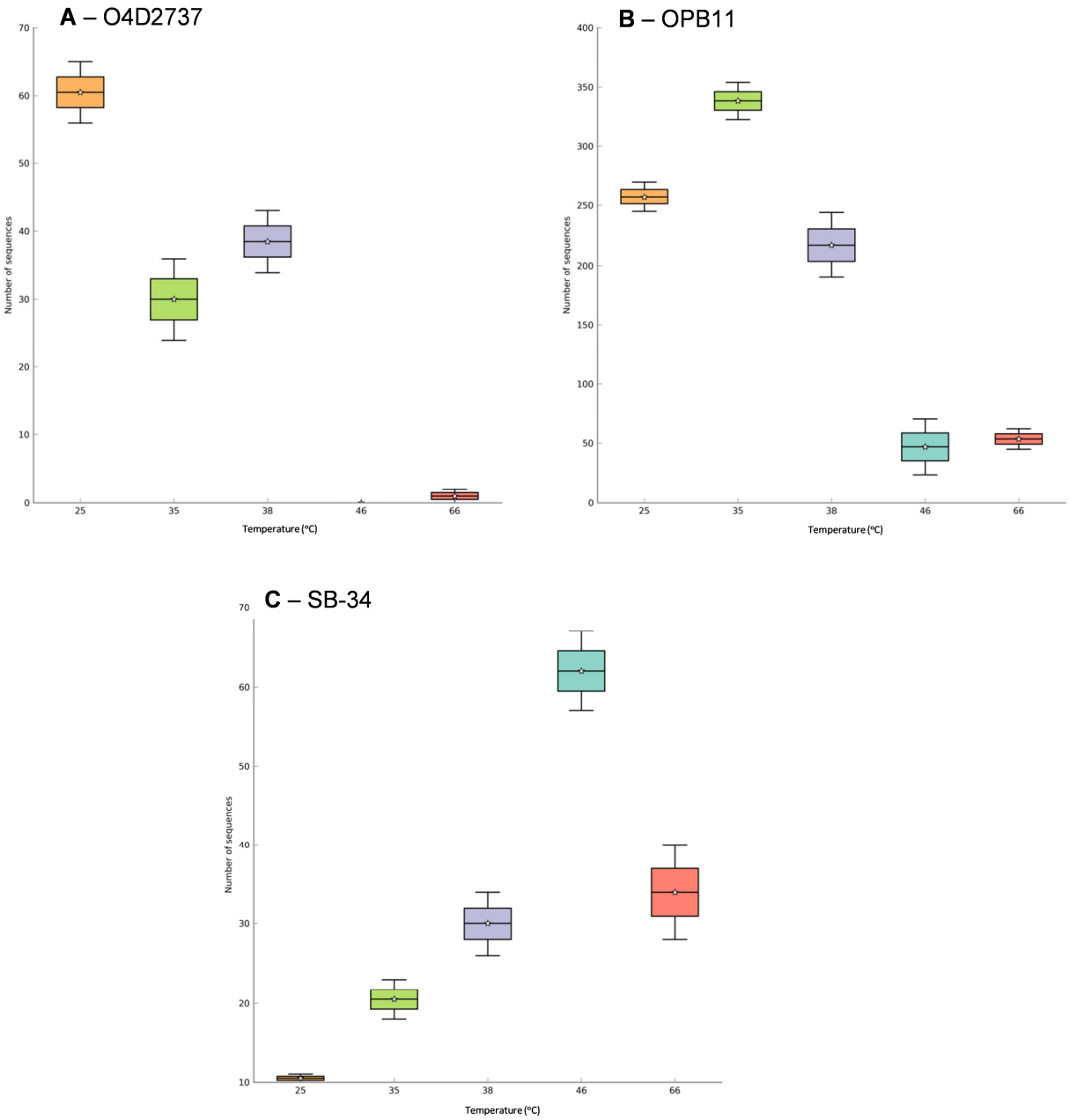


Figure 5.18 - Abundance profiles of statistically significant *Anaerolineae* order level taxa, A, O4D2737, B, OPB11, and, C, SB-34, in sediment slurries incubated at selected temperatures over all sampling days, from the *Chloroflexi* sequencing data set. These orders are groups consisting entirely of uncultivated environmental sequences. Samples were compared using an ANOVA with a Tukey-Kramer Post-Hoc test using STAMP v2.0.0 (Parks and Beiko, 2010). Only statistically significant changes in abundance with temperature are shown (P value << 0.05). There was a total number of 10 samples analysed, 2 for each temperature. Day 0 was omitted from this analysis as there was only one sample and so could not perform ANOVA. The stars indicate the average sequence number for each temperature group, the line is the median and the top and bottom edges of the box are the 75th and 25th percentile, respectively. The whiskers indicate the maximum and minimum values.

community enriched in the sediment slurries incubated at temperatures below 38^{o}C were mesophilic bacteria common to marine sediment environments.

The second community increased in abundance at temperatures >46^{o}C, indicating a thermotolerant/ thermophilic community, and included the uncultivated clone groups GCA004, S0208 and the significantly more abundant group SB-34 (Figures 5.16A & 5.18C). GCA004 is an order level taxonomic group, within the Greengenes database, consisting entirely of uncultivated environmental clones from mud volcano sediment (AY592319, S. K. Heijs, A. M. Laverman and L. J. Forney, unpublished results), hydrocarbon and methane seeps (Beal et al., 2009), and marine sediments GQ246423, Y. Zhao, unpublished results); S0208 consists of clones isolated from a variety of sources including mangrove and prairie soils (Youssef et al., 2009), hypersaline microbial mats (Isenbarger et al., 2008), ANNAMOX reactors (FJ710693, A. Terada, S. Lackner, A. Dechesne and B. F. Smets, unpublished results) and geothermal springs (Harris et al., 2004) and the group SB-34 is made of clones isolated from deep sea mud volcanos (AY592366, S. K. Heijs, A. M. Laverman and L. J. Forney, unpublished results) and surface and deep-sea marine sediment clones (Durbin and Teske, 2011).

The *Chloroflexi* community as a whole was dominated by *Anaerolineae* OTUs, with increasing numbers of very abundant OTUs at temperatures above 38^{o}C. Though the cell numbers from qPCR decreased, which indicated less diversity at the higher temperatures and a smaller, thermophilically resilient community (Figures 5.4C & D and 5.8B). At 66^{o}C, the abundant OTUs were mostly related to the GCA004 group and shared >99% sequence similarity with uncultivated environmental clones from deep sea mud volcanos and sub-tidal surface sediments (Acosta-Gonzalez et al., 2013).

5.3.3.4.2 The Dehalococcoidia Response to Temperature

In contrast to the *Anaerolineae*, the *Dehalococcoidia* abundance increased to a maximum at 46^{o}C (31-33% of the total *Chloroflexi* community), and

decreased in abundance at 66^{o}C (Figures 5.15 & 5.16B). The corresponding decrease in *Anaerolineae* at 46^{o}C may be coincidental or might indicate competition between these groups in marine sediments. The increase in *Dehalococcoidia* abundance was characterised by an increase in all of the four orders detected, particularly the uncultivated environmental clone groups GIF9 and FS117-23B-02 (Figure 5.16B). The uncultivated GIF9 clone was originally isolated from an *in situ* reactor system for dechlorinating polluted groundwater (Alfreider et al., 2002). Uncultivated clone FS117-23B-02 was isolated from old oceanic crust fluids from the Juan de Fuca Ridge, Pacific Ocean, where *Chloroflexi* were found to dominate bacterial communities in samples with a large surficial sediment influence (Huber et al., 2006). The largest number of the most abundant *Dehalococcoidia* OTUs were detected at 46^{o}C, and were related to GIF9 and FS117-23B-02 (Figure 5.8B). The GIF9 related OTUs OTU423, OTU893 and OTU223 (Figure 5.8B) shared 98%, 98% and 92% sequence similarity, respectively, with an uncultivated clone isolated from a terrestrial, methane emitting, mud volcano (Cheng et al., 2012). The clone SYNH02_ew01B-148 was isolated from a bubbling mud pool at 23.6^{o}C, rich in thermophilic prokaryotes, which suggested that the abundant GIF9 OTUs are themselves thermophiles, though their metabolism was unclear. The most abundant FS117-23B-02 OTU was OTU1061 (Figure 5.8B), which shared 98% sequence similarity with uncultivated clones isolated from coastal and lake surface sediments (Asami et al., 2005; Song et al., 2012), perhaps indicating that a group of *Dehalococcoidia,* common in surface sediments, were able to adapt to the increasing temperatures.

5.3.3.4.3 The Thermomicrobia Response to Temperature

Low abundances of *Thermomicrobia* sequences were detected at 66^{o}C and were also positively correlated with temperature (Table 5.7; Figure 5.15). All *Thermomicrobia* OTUs were related to the clone AKYG1722 which was also

Table 5.7 - Pearson correlation coefficients for the relationships between *Chloroflexi* classes, temperature and geochemistry. Pearson's correlation was performed using Microsoft Excel 2010. Only statistically significant correlations (P value <0.05) are shown. P values were derived using a correlation coefficients table (Fry and Iles, 1994).

Class	Temperature	Acetate	Formate	Hydrogen	Carbon Dioxide
Ktedonobacteria	-	0.99	0.99	0.80	0.68
Thermomicrobia	0.64	-	-	-	-

the single most abundant OTU related to *Thermomicrobia*, found at 66^{o}C (Figure 5.8B). The uncultivated clone AKYG1722 was originally isolated from farm soil, and has been assigned to the thermophilic *Sphaerobacter* genus (Hugenholtz and Stackebrandt, 2004; Tringe et al., 2005). These findings suggest that thermophilic *Thermomicrobia* were mostly active at temperatures above 66^{o}C in the sediment slurries. OTU230, detected at 66^{o}C (Figure 5.8B), shared the highest sequence similarity (96%), with uncultivated clones isolated from hypersaline microbial mats from a lake and salt marsh (Bachar et al., 2007; Eilmus et al., 2007). The relatively low sequence similarity and lack of closer matches indicated that OTU230 was only distantly related to *Thermomicrobia* and they represent a novel grouping.

5.3.3.5 Changes in the Archaea Community from Sediment Slurry over a Gradient and Time Scale

The *Crenarchaeota* were the most abundant *Archaea* phylum detected in thermal gradient subjected sediment slurries at all days and temperatures (52 – 98% of total *Archaea*; Figure 5.19). The *Thaumarchaeota* and *Euryarchaeota* were variable in their community composition with time and temperature; no pattern was evident at phylum level (Figure 5.19). The newly proposed *Parvarchaeota* phylum (Rinke et al., 2013) was also detected at the highest temperatures but only at very low levels. Analysis by ANOVA and Pearson correlation found that none of the phyla were significantly different in abundance from each other with temperature, and at the phylum level there was no significant correlation with temperature or sampling day, due to the very high abundance of *Crenarchaeota* in all samples.

5.3.3.5.1 The Crenarchaeota Response to Temperature

The *Crenarchaeota* showed increased abundance as temperature increase at Day 15 and Day 35 (Figure 5.19). The community was consistent at all days and temperatures, and was dominated by the class Miscellaneous

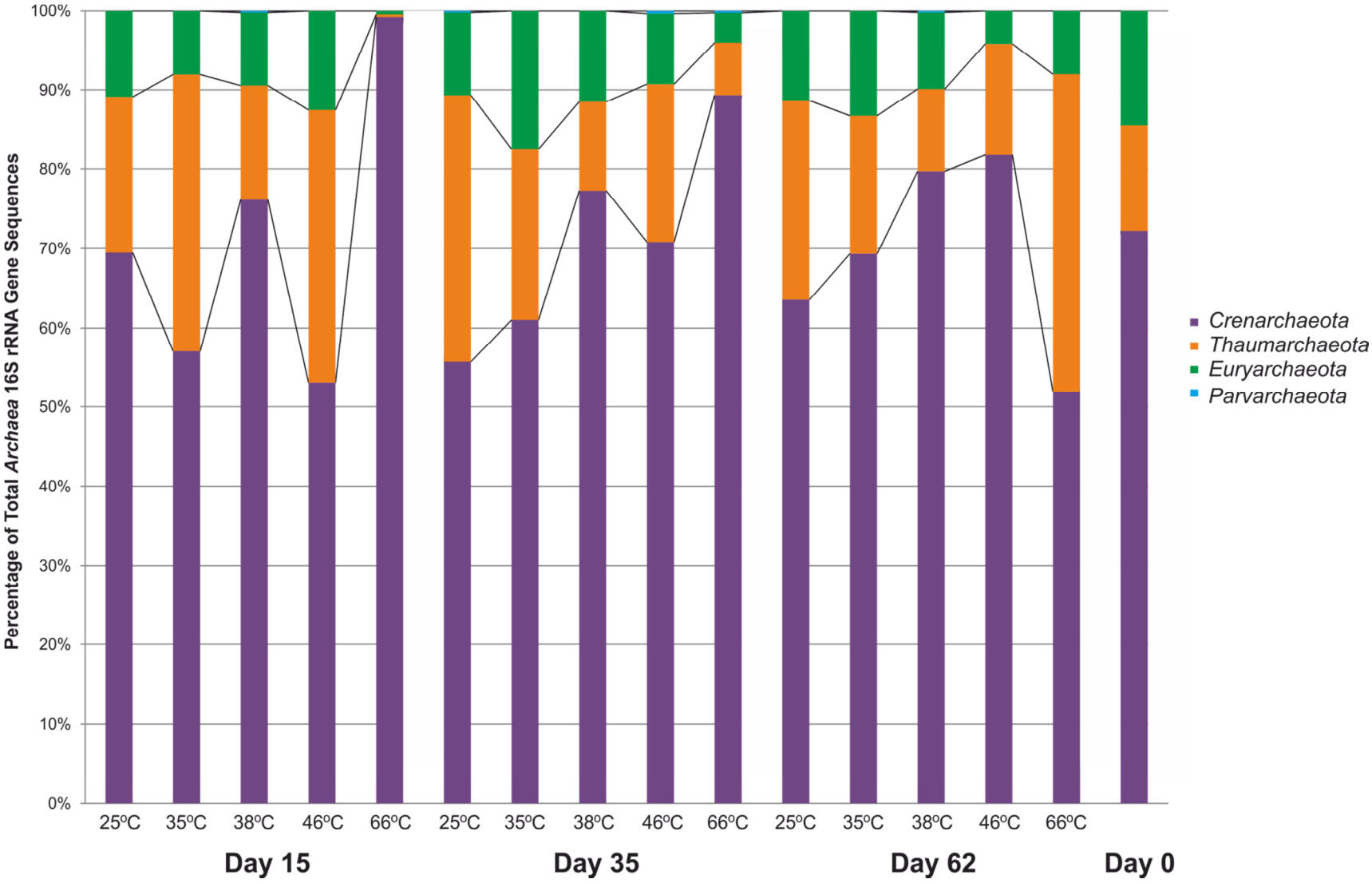


Figure 5.19 - Phylogenetic associations of all *Archaea* 454 sequences from sediment slurry at each temperature and time point. The 4 detected phyla are shown with series lines to indicate trends. Phylogenetic designation was by based on BLAST. The total number of sequences used was 25°C – 4368, 35°C – 4384, 38°C – 4488, 46°C – 4340, 66°C - 4637and Day 0 - 1358. A total of 16 samples were sequenced for the *Archaea* library.

Crenarchaeota Group (MCG), which ranged from 88 - 99% (of *Crenarchaeota*) and was consistently >45% of the total archaeal community. The dominance at all times and temperatures indicated an important role for the MCG in the sediment slurry incubations and in the original reference sample (Day 0). Nearly all of the most abundant archaeal OTUs belonged to the MCG, though different MCG OTUs were abundant at different temperatures, which indicated a great diversity within the MCG and an influence of temperature on the community (Figure 5.8B). OTU210 and OTU2867 were most abundant at 25^{o}C, whereas OTUs relating to the uncultivated environmental clone order level group pGrfC26 were abundant at higher temperatures, e.g. OTU2639 and OTU2276 were abundant at 66^{o}C (Figure 5.8B). OTU210 and OTU2867 shared 99% and 95% sequence similarity, respectively, with environmental clones from Arctic Ocean coastal sediments (DQ146758, P. E. Galand, C. Lovejoy and W. F. Vincent, unpublished results) and limestone sinkholes (FJ901719, J. W. Sahl, M. O. Gary, J. K. Harris and J. R. Spear, unpublished results), respectively. The clones related to OTU210 and OTU2867 suggested that the MCG community at 25^{o}C is common in marine sediments and an integral part of the original sediment community.

The abundant OTUs at 66^{o}C, OTU2639 and OTU2276 were from the group related to uncultivated clone pGrfC26, which was isolated from a temperature marsh environment and was also detected in freshwater lake sediments and rice paddy soils, and have not, until now, been identified in thermophilic environments (Großkopf et al., 1998b; Hershberger et al., 1996). The top BLAST matches (>98% sequence similarity) for OTU2639 and OTU2276 were uncultivated environmental clones isolated from coastal and estuarine sediments including an uncultivated clone from Hythe, in the Clone Estuary, UK (G. Webster, L. A. O'Sullivan, Y. Meng, A. S. Williams, A. M. Sass, A. J. Watkins, R. J. Parkes & A. J. Weightman, unpublished results). The Hythe sediments were low salinity sediments dominated by methanogens and MCG with depth. This link suggested that MCG are more abundant at greater depths in estuarine sediments and were associated with methanogenic processes. The MCG and the *Crenarchaeota* phylum were

found to be weakly, positively correlated with methane levels ($P < 0.05$; Table 5.8). MCG are a clonal group with little description of their biogeochemical or metabolic potential. However, they are commonly found in anaerobic, sulphate depleted, low energy environments, with little indication of involvement in sulphate reduction or methane oxidation (Kubo et al., 2012).

The class Marine Benthic Group B (MBGB) were a large part of the *Crenarchaeota* community that generally decreased in abundance as temperature increased, but showed an increase at the highest temperature (66^{o}C), and was again positively correlated with methane. The most abundant MBGB OTUs were detected only at 25^{o}C (OTU2333) and 66^{o}C (OTU681; Figure 5.8B). OTUs 681 and 2333 both shared 95% sequence similarity to uncultivated environmental clone T_36, which was originally isolated from marine sediment in the Okinawa trough (Hoshino and Inagaki, 2013).

5.3.3.5.2 The Thaumarchaeota Response to Temperature

The *Thaumarchaeota* were the second most abundant archaeal phylum in the temperature gradient sediments, of which the class Marine Group 1.1a were the predominant group, with peaks of 32-37% of the total archaeal community at 35-66^{o}C (Figure 5.19). However, at Days 15 and 35 at 66^{o}C, the Marine Group 1.1a decrease to <6% of the total archaeal community and the Soil Group 1.1b increased in abundance. There appeared to be little diversity within the Marine Group 1.1a and Soil Group 1.1a, as only two families, the *Cenarchaeaceae* and *Nitrososphaeraceae,* were present in these samples. *Nitrosopumilus* was the most dominant *Thaumarchaeota* genus at all temperatures and OTU2632, which was related to this genus, was the most abundant archaeal OTU in all samples (Figure 5.8B). OTU2632 shared 99% sequence similarity with the environmental clone E09-BR2-1, which was isolated from Brightlingsea, (Colne Estuary UK; G. Webster et al., unpublished results) and was also closely related to the most

Table 5.8 - Pearson correlation coefficients for the relationships between *Archaea* phyla, and geochemistry. There were no significant correlations with temperature. Pearson's correlation was performed using Microsoft Excel 2010. Only statistically significant correlations (P value <0.05) are shown. P values were derived using a correlation coefficients table (Fry and Iles, 1994).

Phylum	Class	Methane	Acetate	Formate	Sulphate	Hydrogen
Crenarchaeota	-	0.51	-	-	-	-
Crenarchaeota	Unknown *Crenarchaeota*	0.56	-	-	-	-
Crenarchaeota	*Aigarchaeota*	-	0.66	0.66	-	0.59
Crenarchaeota	MBGA	0.61	-	-	-	-
Crenarchaeota	MBGB	-0.61	-	-	-	-
Crenarchaeota	MCG	0.58	-	-	-	-
Crenarchaeota	*Thermoprotei*	-0.51	-	-	-	-
Euryarchaeota	-	-	-	-	-	-
Euryarchaeota	*Thermoplasmata*	-	0.69	0.67	-0.56	-
Parvarchaeota	-	-	-	-	-	-
Parvarchaeota	*Parvarchaea*	0.54	-	-	-	-

abundant OTU in the core archaeal community from other all Severn Estuary sediment sites (See **Section 4.3.4**). This was a high salinity marine sediment, dominated entirely by *Thaumarchaeota* (Webster, G., unpublished results).

5.3.3.5.3 The Euryarchaeota Response to Temperature

The *Euryarchaeota* appeared to be a highly dynamic group that changed markedly as temperature increased, with a peak of 13% of the *Archaea* community at 35^{o}C (Figure 5.19). The *Euryarchaeota* community was initially dominated by the *Thermoplasmata* in the reference sample (12% of *Archaea* community; Day 0) and maintained a steady presence at approximately 1-6% of the total archaeal community in all samples. *Thermoplasmata* were positively correlated with both acetate and formate, and negatively correlated with sulphate (Table 5.8). At least one of the most abundant *Euryarchaeota* OTUs at each temperature was related to the *Thermoplasmata* class level cultivated clone group E2. OTU102 was most abundant in the reference community and at 25^{o}C, and OTU3433 was most abundant at 46^{o}C. Both of these OTUs were related, at the family level, to *Methanomassiliicoccaceae,* to which the cultivated methanogenic species of *Thermoplasmata* belong (Figure 5.8B; Dridi et al., 2012; Iino et al., 2013). Both OTUs shared >99% sequence similarity to uncultivated environmental clones from marine sediment habitats, including a hypersaline mud volcano and methane seep sediment (Beal et al., 2009; Lazar et al., 2011), indicating that the *Thermoplasmata* community in the reference and unamended sediment slurries contributed to methane metabolism.

The distribution of sequences associated with known groups of methanogens in sediment slurries, with respect to incubation time and temperature, was complex. However, a pattern did emerge at the class level (Figure 5.20). Only the *Methanomicrobiales,* of the class *Methanomicrobia,* significantly decreased in abundance as temperature increased, with a slight re-emergence at 46^{o}C (Figure 5.21). Abundance of *Methanobacteriales* fluctuated, also with peaks at 46^{o}C, which coincided with the appearance of the most abundant OTU1161, related to *Methanobrevibacter,* a genus

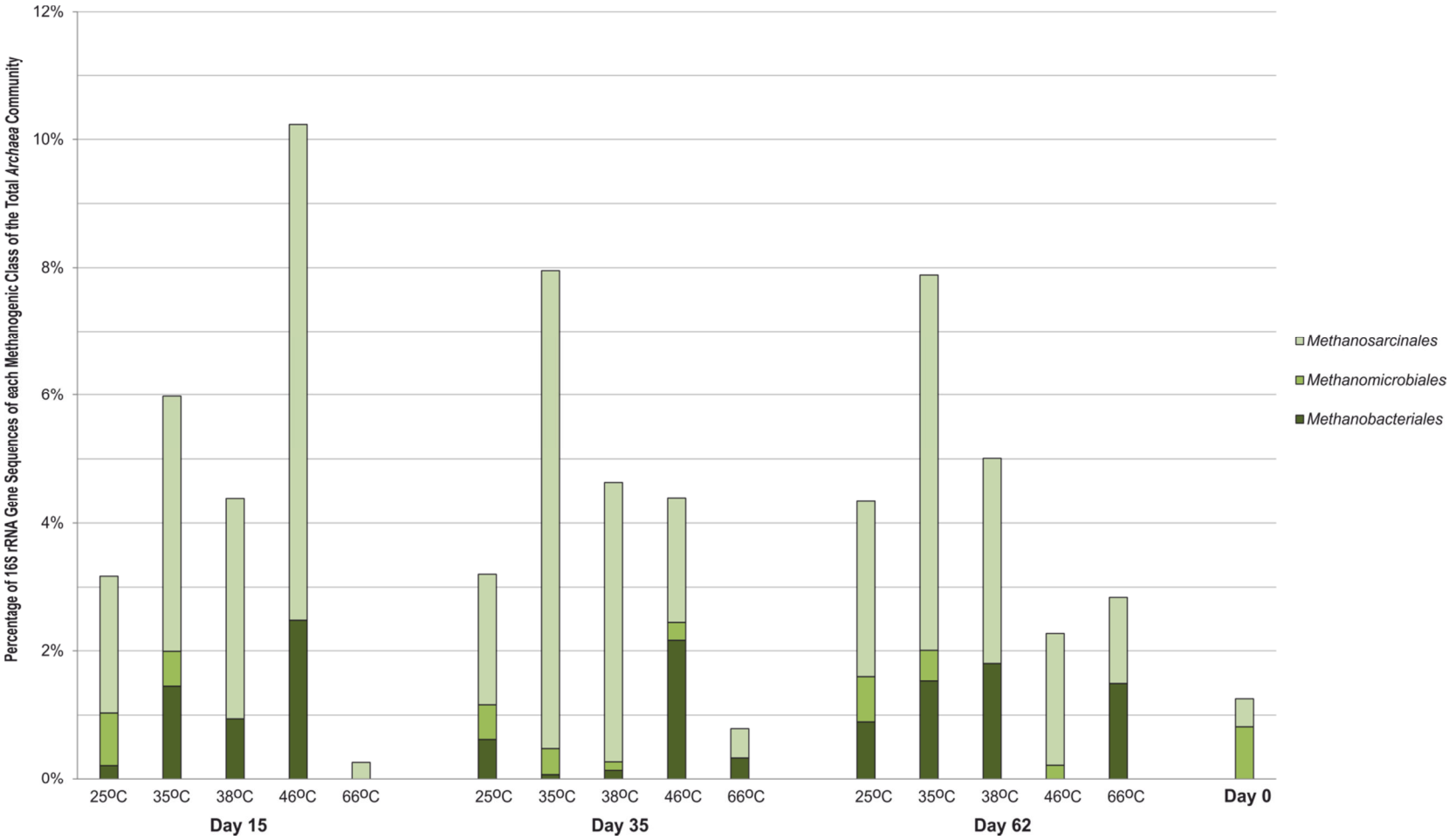


Figure 5.20 - The percentage 16S rRNA gene sequences of each methanogenic class of the *Euryarchaeota* of the total archaeal community in sediment slurries incubated on a temperature gradient and sampled on separate days.

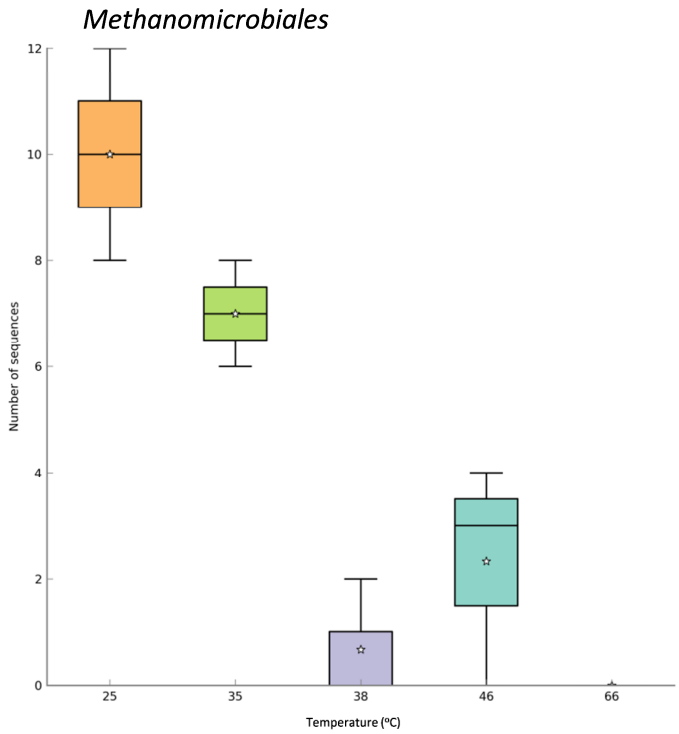


Figure 5.21 - Abundance profile of the statistically significant order *Methanomicrobiales* of the class *Methanomicrobia* in sediment slurries incubated at selected temperatures over all sampling days. Samples were compared using an ANOVA with a Tukey-Kramer Post-Hoc test using STAMP v2.0.0 (Parks and Beiko, 2010). Only statistically significant changes in abundance with temperature are shown (P value << 0.05). There was a total number of 15 samples analysed, 3 for each temperature. Day 0 was omitted from this analysis as there was only one sample and so could not perform ANOVA. The stars indicate the average sequence number for each temperature group, the line is the median and the top and bottom edges of the box are the 75th and 25th percentile, respectively. The whiskers indicate the maximum and minimum values.

restricted to chemolithotrophic growth with CO_2 and H_2 (Leadbetter and Breznak, 1996). The general decrease in *Methanomicrobiales* and *Methanobacteriales* with a small peak at 46^{o}C, together with the increased rate of methanogenesis at 35^{o}C (Roussel et al., unpublished results), indicated that the *Methanomicrobiales* and *Methanobacteriales* were contributing to methane production at lower temperatures (Figures 5.8B & 5.20).

The metabolically diverse *Methanosarcinales,* capable of methylotrophic, acetotrophic and hydrogenotrophic methanogenesis and AOM, were, generally, the most abundant with a peak at 35^{o}C, which coincided with a peak in methanogenesis rates (Figure 5.20; Roussel, E.G., et al., unpublished results). The most abundant archaeal OTUs at 35^{o}C and 46^{o}C were related to the *Methanosarcinales* (Figure 5.8B). OTU1679, related to the clade ANME 2a-2b, was abundant at 35^{o}C detected only in low numbers of sequences at other temperatures. OTU703 was also one of the most abundant archaeal OTUs at 35^{o}C and was related to the family *Methanosarcinaeceae* (Figure 5.8B). This OTU shared >99% sequence similarity with uncultivated environmental clones from habitats associated with AOM such as a mud volcano and a sulphate reducing bacteria enrichment colony (Nazina et al., 2013).

5.4 Discussion

5.4.1 Extreme Temperature Change Heavily Influence Severn Estuary Intertidal Sediment Prokaryotic Communities and their Biogeochemistry

5.4.1.1 The Bacteria Community

The response of intertidal sediment prokaryotic communities to extreme temperature increase with time was investigated in this work. Correlation between community composition, determined by 16S rRNA gene sequence analysis, and sediment slurry geochemistry were also explored. Through various statistical analyses, such as Pearson correlations, NMDS and ANOVA, it was found that temperature was a very important controlling factor of the prokaryotic communities in Severn Estuary intertidal sediment, especially the *Bacteria.* The influence of temperature on the community structure was much stronger than either incubation time or geochemistry. However, the effect on *Archaea* appeared to be more complex than on *Bacteria*.

The ways in which temperature affected each community varied. Firstly, through correlations between OTU richness and diversity indices a strong negative relationship between species richness, diversity and increasing temperature was found (Table 5.4). As qPCR indicated a shrinking community at higher temperatures, these results suggested that these smaller communities became dominated by fewer species in large, unequal abundances (Figure 5.4). This trend has been well documented in other related environmental niches such as sediments and hot springs (Cole et al., 2013; Miller et al., 2009; Wang et al., 2013b). Further to this, it appeared that higher temperatures selected for thermophilic and thermotolerant microorganisms, in a similar way to changes reported recently for other sediment communities. For example, Cole et al. (2013) found a switch from *Firmicutes, Proteobacteria* and *Bacteroidetes* to thermophilic phyla such as the *Aquificae* in hot spring sediments >80^{o}C. Biddle et al. (2012) reported the same pattern with increasing temperature gradients (with greater sediment depth) at the Guaymas Basin.

There was a significant increase in *Firmicutes* of which the *Sporotomaculum, Tepidimicrobium* and *Desulfotomaculum* were the most abundant genera (Figure 5.13). The prevalence of the spore-forming organisms indicated that one method the *Firmicutes* utilised to survive at higher temperatures was to sporulate, enabling them to survive long periods of extreme temperatures and lack of nutrients (Kaksonen et al., 2007; Kaksonen et al., 2008; Mohapatra and La Duc, 2013; Qiu et al., 2003). This would explain why *Sporotomaculum* were not detected at 66^{o}C after a large peak at 46^{o}C, as the DNA inside the spore would have been difficult to extract (Mohapatra and La Duc, 2013). Recent work has found endospores to be widespread in estuarine (O'Sullivan et al., 2014), marine (Muller et al., 2014) and deep subsurface sediments (D'Hondt et al., 2004; Lomstein et al., 2012). Endospores in sediments have been found in abundances equal to living cells (Lomstein et al., 2012). *Desulfotomaculum* spp. were hypothesised to be distributed from thermophilic environments like thermal springs to mesophilic sediments by ocean currents and were successfully cultured after autoclaving of 154^{o}C (O'Sullivan et al., 2014).

The *Desulfotomaculum* are thermophilic sulphate reducing bacteria, capable of utilising hydrogen as an electron donor, and were possibly taking advantage of the increase in sulphate concentrations that were detected at higher temperatures (Jackson and McInerney, 2000; Kaksonen et al., 2007; Kaksonen et al., 2008). However, a slight rise in methanogenic *Archaea* and the significant positive correlation between *Clostridia* and methane could indicate association with methanogens in interspecies hydrogen transfer. There is precedent within the *Sporotomaculum* for this behaviour in warm, methanogenic environments whereby the syntrophic *Bacteria* produce hydrogen that the methanogens can then utilise (Mizuno et al., 1998; Qiu et al., 2003).

The main route for anaerobic degradation of organic matter in estuarine sediments is by sulphate reduction (Purdy et al., 2002a; Wellsbury et al., 1996). The *Deltaproteobacteria* are known to have roles in sulphate reduction and were the only *Proteobacteria* class to decrease significantly

with increasing temperature. The *Desulfobulbaceae* were replaced by *Desulfobacteraceae, Desulfuromonadaceae, Desulfarculaceae* and *Syntrophobacteraceae* at higher temperatures. The significant loss in *Desulfobacterales* and *Desulfuromonadales* indicated the loss of heterotrophic sulphate reduction in the high temperature sediments (Leloup et al., 2006). As previously discussed in **Sections 5.3.3.3.1** and **5.3.3.3.2**, the abundance of *Desulfobulbaceae* and the subsequent increase in abundance of the *Firmicutes* genus *Desulfotomaculum* indicated a shift from heterotrophic to autotrophic sulphate reduction at the critical temperature window of 43^{o}C (Roussel et al., unpublished data; Aullo et al., 2013). This sensitivity of the sulphate reducing bacteria to increased temperature has been previously noted in marine sediments (Finke and Jørgensen, 2008; Weston and Joye, 2005) and in the Yellowstone geothermal ecosystem (Spear et al., 2005).

Other important processes in marine sediments include those involved in the nitrogen cycle, particularly aerobic nitrification and anaerobic denitrification (Dong et al., 2002; O'Sullivan et al., 2013). The prevalence of denitrifying and nitrifying microorganisms in Severn Estuary sediments, typified by the genus *Hyphomicrobium* of the *Alphaproteobacteria* and the *Nitrosopumilus* of the Marine Group 1.1a of the *Thaumarchaeota,* respectively, was previously described **(Section 4.4.2)**. *Hyphomicrobium* and *Nitrosopumilus* maintained a significant presence at all temperatures in this experiment. Again, the importance of the nitrogen cycle in Severn Estuary sediments has been demonstrated. Though previous work suggested that rates of processes such as nitrification, denitrification and ANAMMOX increase as sediment temperature increases, a minor decrease in these organisms with temperature was detected, possibly indicating an upper temperature limit had been reached (Cole et al., 2013; Wu et al., 2013).

5.4.1.2 The Chloroflexi Community

The *Chloroflexi* phylum constituted a substantial part of the total bacterial community, peaking at 29% at 35^{o}C. As previously seen in Severn Estuary intertidal sediment **(Sections 3.4.3 and 4.4.3)**, *Anaerolineae* OTUs were the

largest sub-group, followed by *Dehalococcoidia.* In other investigations of bacterial communities in sediment, the *Anaerolineae* are the most commonly detected sub-group, and with their large metabolic range they appear to be widely adaptable (Blazejak and Schippers, 2010; Hug et al., 2013; Sekiguchi et al., 2003). The *Anaerolineae* and *Dehalococcoidia* were characterised by uncultured, clonal groups such as SHA-20 and GIF9, respectively.

A thermophilic/ thermotolerant *Anaerolineae* community, which consisted of the uncultivated clone groups GCA004, S0208 and SB-34, were found to increase in abundance, past the critical temperature window of 43^{o}C (Figures 5.16A & 5.18C). These uncultivated groups and the most abundant *Anaerolineae* OTUs related to them were characterised by isolates from mud volcanos **(Section 5.3.3.4.1)**. The presence of mud volcano related communities was interesting but not surprising as mud volcanos are formed in geothermal environments from hot water mixing sediments and have a variable temperature, not unlike the sediment slurries analysed here (Sauter et al., 2006). Also, as mud volcanos are sources of methane discharge from deep sediments and there was a peak in methanogenesis rates at 35^{o}C; it is possible that the *Anaerolineae* OTUs were stimulated by the hydrogenotrophic methanogenic communities. *Anaerolineae* have been previously found to grown syntrophically with hydrogenotrophic methanogens (Sauter et al., 2006; Sekiguchi et al., 2003; Yamada et al., 2007a).

Though the *Chloroflexi* phylum as a whole was negatively correlated with temperature, NMDS and ANOVA of abundance changes with temperature indicated the possibility of a thermophilic *Chloroflexi* community arising at 66^{o}C (Figures 5.7, 5.17 & 5.18). The significant but slight rise in *Anaerolineae* OTUs (SHA-20, S0208 and GCA004) and the *Thermomicrobia* at 66^{o}C suggested that further investigation of temperatures above 66^{o}C is wholly warranted. The *Thermomicrobia, Ktedonobacteria* and the clonal group Gitt-GS-136 were also abundant at 66^{o}C. The *Thermomicrobia* were strongly positively correlated with temperature, and, as they are a mainly thermophilic group in this phylum, this would explain the increase in abundance at higher temperatures (Botero et al., 2004). The numbers of

Ktedonobacteria were not correlated with temperature but were strongly correlated with acetate, formate, hydrogen and CO_2, suggesting a geochemical influence. Those detected were mainly related to the cultured family *Ktedonobacteraceae* whose type strain, *Ktedonobacter racemifer,* is a mesophilic, spore-forming organism (Chang et al., 2011). There are two possible explanations for this increased abundance, either the *Ktedonobacteria* were stimulated by the geochemical changes noted above, or they have the ability to form structures such as spores, as seen in the *Firmicutes*, which enabled them to survive at high temperatures.

5.4.1.3 The Archaea Community

As previously stated, the *Archaea* appeared to have a more complicated reaction to the temperature change than the *Bacteria.* By analysis of Pearson correlation coefficients, none of the *Archaea* taxa were significantly correlated with temperature at any phylogenetic level (Table 5.8). By NMDS the Day 0 and temperatures <38^{o}C were extremely similar, and the 46^{o}C and 66^{o}C samples were unique in their great disparity to each other and all other temperatures (Figure 5.8A). The MCG dominated the community at every temperature with very little variation with respect to either temperature or incubation time. The prevalence of MCG at higher temperatures was also detected via DGGE in the amended sediment slurries (Roussel, E.G., et al., unpublished results). Though none of the MCG OTUs or groups was related to thermophilic environments or metabolic processes, the diversity of the MCG may account for the resilience of the MCG to the rise in temperature. Inagaki et al. (2003) detected MCG phylotypes from coastal subseafloor sediments which shared 90.1% similarity with an uncultivated clone from a hot spring in Yellowstone National Park and in their work on AOM communities at different temperatures, Biddle et al. (2012) found MCG at all temperatures in the range from 15^{o}C to 95^{o}C, which indicated a relationship with thermal environments. Aside from its phylogeny within the *Crenarchaeota* and global distribution in marine and land habitats, and dominance in deep sediment archaeal clone libraries, very little is known about the MCG, with no known cultured representatives or definitive functionality (Biddle et al., 2006; Inagaki et al., 2003; Kubo et al., 2012;

Parkes et al., 2005). It has been reported that they are abundant in anoxic and low nutrient environments, such as deep sediments, and are not associated directly with methanotrophy or sulphate reduction (Kubo et al., 2012). Biddle et al. (2006), reported that MCG dominated archaeal communities were heterotrophic and able to degrade fossilized organic carbon as a substrate. More recently, Lloyd et al. (2013) sequenced the genome of a single MCG cell and found the ability to degrade detrital proteins as an alternative carbon source in low nutrient sediments. Interestingly, the peptidase sequences from the MCG genome were found to share sequence similarity with thermophilic protein-degrading bacteria (Lloyd et al., 2013). Possibly in the anoxic, heated sediment slurries used for these experiments, with no nutrient input, this low energy adapted *Archaea* prevailed, utilising carbon sources that were unusable by other prokaryotes.

The genus *Nitrosopumilus* has been frequently reported in marine surface sediment and the water column (Agogué et al., 2008; Erguder et al., 2009) and have a major role in the aerobic, chemolithoautotrophic process of ammonia oxidation (Park et al., 2012b; Spang et al., 2010). Though as the sediment slurry incubations were anaerobic, it would be unlikely that ammonia oxidation was actually occurring. Instead, the prevalence of the *Nitrosopumilus* indicated a very important role in the original estuarine sediment *in situ* community, as was seen in all Severn Estuary sediment sites **(Sections 4.3.4 & 4.4.4)** and perhaps the cells or DNA were retained, at lower levels than those seen in **Chapter 4,** which indicated a resilient yet not active community.

Methanogens were the only archaeal group found to change significantly in abundance with temperature by ANOVA (Figure 5.20 & 5.21). By comparison with *Methanomicrobiales, Methanosarcinales* proliferated between 35^{o}C and 46^{o}C. As methane concentrations increased due to increased methanogenesis rates at this temperature window (Roussel et al., unpublished results), ANME2a-2b related OTUs become the most abundant archaea OTUs (Figure 5.8B; **Section 5.3.3.5.3**). These OTUs exploited this peak for the autotrophic AOM. After this point, sulphate levels reduced and

so the ANME2a-2b were inhibited by the loss of the electron acceptor (Knittel and Boetius, 2009; Ruff et al., 2013; Wegener et al., 2008).

Since CO_2 and H_2 concentrations increased with temperature, it was likely that the peak at 46^{o}C of the *Methanomicrobiales* and *Methanobacteriales* was due to these organisms utilising chemolithotrophic hydrogenotrophic methanogenesis, possibly syntrophically with the *Anaerolineae,* as previously described **(Section 5.3.3.4.1 and 5.4.1.2).** Roussel et al., (unpublished results) also saw a change in metabolic activity rates at the critical temperature window of 43^{o}C from chemoorganotrophic methanogenesis to chemolithotrophic methanogenesis. The *Methanosarcinales* and *Methanomicrobiales* utilise different methanogenesis pathways, the *Methanomicrobiales* only reduce CO_2 with either hydrogen or formate, whereas the more physiologically diverse *Methanosarcinales* are able to use all three pathways of methanogenesis (acetoclastic, methylotrophic and CO2 dependent; Ferry and Lessner, 2008). This suggested that as the geochemical environment changes with temperature, the pathway of methanogenesis was altered and so specific mesophilic and thermophilic communities proliferated (Nozhevnikova et al., 1997). Several studies have previously shown that methanogens can thrive in heated, thermal sediments, more so than sulphate reducers for example, with an optimum growth at 35-45^{o}C, (Finke and Jørgensen, 2008; Kelly and Chynoweth, 1981; Weston and Joye, 2005). Thus it is likely that the increase in production of methane at higher temperatures in this study and others (Roussel et al., unpublished results; Stadmark and Leonardson, 2007), was attributed to these more thermotolerant or thermophilic methanogens reliant on chemolithotrophic hydrogenotrophic methanogenesis.

5.4.2 Environmental Implications of Intertidal Communities Subject to Warming

Climate change is one of the largest and most important issues we face now and in the future. Understanding of flux of CO_2 and methane, the two of the most important greenhouse gases, between land, sea, sediment and air is vital. The abilities of biological reactions to both enhance and buffer the

exchange of these gases in the carbon cycle needs to be characterised (Heimann and Reichstein, 2008). There has been an abundance of soil related research, especially with respect to the methanogenic communities (Bardgett et al., 2008; Castro et al., 2010; Kuffner et al., 2012).

On the other hand, there has been little research into how whole sedimentary prokaryotic communities, especially those from coastal and intertidal areas, affect climate change and vice-versa (He et al., 2012). The effects of temperature on intertidal sediment prokaryotic community have been experimentally investigated and found to be a major influence on both the community structure and biogeochemistry. Schink (1998) previously noted that methane fluxes from ocean environments are “not as important as soils and tundra” because sulphate will be preferentially reduced in marine sediments. However, this work contradicts this statement. A significant increase in CO_2, methane and methanogens with temperature was found as was a significant decline in sulphate reducing bacteria. This increase in methanogenic *Archaea* with temperature has been widely recorded in other sediments (Finke and Jørgensen, 2008; Stadmark and Leonardson, 2007). Though the critical temperature change at 43^{o}C, indicated by this work and by Roussel et al. (unpublished results), is above the mesophilic range and so the metabolic and geochemical changes with temperature would be more subtle in sediment environments. As these were intertidal sediments, and so have the ability to exchange gases directly with the atmosphere, further work in this area is needed so that future studies may be able to incorporate prokaryotic communities in global climate projections.

Chapter 6 - General Discussion

The prokaryotic communities in estuarine sediments have been well documented but with a heavy focus on sulphate reducers, methanogens and the effects of salinity gradients. Very little work has been published on the prokaryotic communities, particularly *Archaea,* or the effect of geomorphology and physicality of dynamic sediments such as the Severn Estuary (Jiang et al., 2011; Sun et al., 2012; Webster et al., 2010; Wellsbury et al., 1996). The prokaryotic communities in the Severn Estuary, in particular, are poorly described (Webster et al., 2010).

The aim of this study was to characterise Severn Estuary prokaryotic communities and attempt to relate the community structure with biogeochemistry, geomorphology and physicality of the sediment to further the current knowledge of whole estuarine prokaryotic communities. Particular attention was paid to the *Chloroflexi*, a deeply branching but mostly uncharacterised phylum of *Bacteria;* with potentially important roles in global geochemical cycling (Hugenholtz and Stackebrandt, 2004; Sorokin et al., 2012). *Chloroflexi* are common in sediment environments, but many studies have concentrated on deep subsurface communities, whereas surface and coastal sediment communities have been neglected (Fry et al., 2008; Wilms et al., 2006a). The present study therefore shifts the focus from association of *Chloroflexi* with nutrient starved ecosystems, such as the deep subsurface, to more productive coastal sediments (Kawaichi et al., 2013; Sorokin et al., 2012). Cultivation-independent methods based on 16S rRNA gene analysis were utilised, and a secondary aim was the development of *Chloroflexi* specific PCR assays to better describe the community structure and potential function.

6.1 Comparison of Community Profiling Methods and Next Generation Sequencing for Analysis of Severn Estuary Sediment Prokaryotic Communities

In this study, comparisons of clone libraries and profiling methods (e.g. 16S rRNA gene denaturing gradient gel electrophoresis (DGGE) and ribosomal intergenic spacer analysis (RISA)) to 16S rRNA gene next generation sequencing (NGS) indicated consistency between results **(Sections 3.3.1, 3.3.2, & 4.3.2 – 4.3.4)**. Profiling methods overestimated the difference between sites whereas pyrosequencing emphasized the difference with depth; however, both methods identified the same abundant phyla. The drawbacks of DGGE have been described at length, including detection of organisms at <1% of the total community and the inability to cope with highly complex communities (Muyzer et al., 1993; Muyzer and Smalla, 1998). However, RISA and DGGE still have a place in preliminary analysis to make an informed choice of the most appropriate samples for further sequencing, avoiding unnecessary redundancy. NGS methods reliant on PCR have inherent biases linked to primer specificity (Soergel et al., 2012; Teske and Sørensen, 2008; Wang and Qian, 2009). Whereas the shotgun metagenomic approach avoids PCR bias and has been largely successful in identifying functional aspects of environmental prokaryotic communities (Biddle et al., 2008; Edwards et al., 2006; Varin et al., 2012). With the current accessibility, ever decreasing cost and vast improvement in the technology, it is foreseeable that 16S rRNA gene NGS will become, and perhaps already is, the standard method of community profiling in microbial ecology (Bartram et al., 2011).

6.2 Prokaryotic Communities in Severn Estuary Sediments are Strongly Influenced by the Dynamic Nature of the Estuary

This study investigated how Severn Estuary sediment geomorphology and geochemistry affected prokaryotic communities at three different sites. An emerging pattern was that the level of sediment turbidity at each site strongly influenced the Severn Estuary sediment communities. Wellsbury et al. (1996) speculated that huge turnover in Severn Estuary sediments would

homogenise prokaryotic communities and limit niche metabolic processes, such as sulphate reduction and methanogenesis. This study builds on this previous research and refines the types of changes in the community. As Wellsbury et al. (1996) predicted, there was more similarity in prokaryotic communities between each site than expected. The core phyla *Proteobacteria, Chloroflexi, Firmicutes, Actinobacteria, Bacteroidetes* and *Thaumarchaeota* made up over 90% of each sample at every site and depth (Figure 4.2). *Proteobacteria* were found to frequently dominate estuarine sediment communities at the Colne Estuary (O'Sullivan et al., 2013), Pearl River Estuary (Sun et al., 2012) and the intertidal sediments at Portishead, Severn Estuary (Webster et al., 2010), which corroborates these findings.

The samples most heavily affected by sediment turnover were the upper intertidal site and all deep water site samples, which reintroduced oxygenated water to deeper sediments with mixing. Cowan and Boynton (1996) found that Chesapeake Bay estuarine sites with high turbidity and suspended sediment, similar to these Severn Estuary sites, had highly oxygenated bottom waters and high level of dissolved oxygen in surface sediments. It was these samples that clustered together most consistently, indicating more similarity in these turbid sediments than the other sites and depths (Figures 4.7 & 4.17). These samples also had high cell counts via qPCR, which suggested highly active communities (Figure 3.10). Sediment turbidity has been indicated in promoting prokaryotic growth activities and homogeneity of communities by introduction of dissolved oxygen, fresh nutrients and organic materials into the surface sediments, which would corroborate the findings in this study (Bowman et al., 2003; Ciobanu et al., 2012; Wellsbury et al., 1996).

In the surface and disturbed deep water samples, the *Thaumarchaeota* dominated the *Archaea* as a single class, the Marine Group 1.1a, and the genus *Nitrosopumilus,* made up 86-96% of the *Archaea* in these samples (Figure 4.13), indicative of highly specialised community for ammonia oxidation. The abundance of *Nitrosopumilus* was the most obvious indication that nitrogen cycling was important in these sediments. The further discovery

of high densities of other prokaryotes reported to be involved in nitrification, denitrification, ANAMMOX and nitrogen fixation, suggests that a complete nitrogen cycle is occurring in Severn Estuary sediments. Previous studies have highlighted the importance of marine sediment, especially estuarine environments, in the coupling of nitrification-denitrification and ANAMMOX as a natural sink for nitrogen compounds, as well as the prospective production of greenhouse gases like nitrous oxide (Mosier and Francis, 2008; Nedwell, 1999; Nicholls and Trimmer, 2009). This study indicated that sediment physicality directly affected ammonia oxidising *Archaea*, and that this should be taken into account when estimating the impact of estuarine prokaryotic communities on the global nitrogen cycle.

6.3 The *Chloroflexi* Phylum is Abundant and Diverse in Severn Estuary Sediments

The diversity of *Chloroflexi* subdivisions was an important focus of this work. The successful development and use of novel phylum and subdivision specific 16S rRNA gene PCR assays in this study allowed a greater depth of insight into the *Chloroflexi* in estuarine sediments, where previous research has focused broadly on bacterial communities or at just the *Dehalococcoidia* (Ahn et al., 2007; Wasmund et al., 2014a; Wilms et al., 2006a). However, insufficient sequence data was available from DNA databases to design specific primers for subdivisions other than *Anaerolineae* and *Dehalococcoidia.*

It was concluded that the *Chloroflexi* were ubiquitous in estuarine sediment, occurring at high numbers, in all samples and as the most abundant phylum in the suboxic zone at the intertidal site (6-8 cbsf; Figure 4.3). This indicated the importance of this phylum in coastal and, more specifically, shallow surface sediments. Previous investigations into deep biosphere-related bacteria in tidal flat sediments also indicated a substantial *Chloroflexi* presence; however this occurred below 2 m (Wilms et al., 2006a; Wilms et al., 2006b), whereas *Chloroflexi* were found to be prevalent from surface sediments (0-2 cbsf) in the present study. Recent research into Wadden Sea tidal flats, focussed solely on the *Dehalococcoidia,* found they established in

low numbers at 2 cbsf and increased with depth to a peak at 8 cbsf (Wasmund et al., 2014a), which directly mirrored this study and suggested that *Dehalococcoidia* are important in surface sediments as well as the deep subsurface. The *Anaerolineae* were found to dominate the *Chloroflexi* community in all samples (Figure 4.8). This was surprising since the *Dehalococcoidia* and subdivision IV are generally identified as marine sediment organisms and *Dehalococcoidia* were the only subdivision detected in Wadden Sea tidal sediments (Wilms et al., 2006a).

The *Chloroflexi* phylotypes identified in this study were dissimilar from current cultured representatives indicating great intra-clade diversity. Together with the number of class level clonal groups detected, this indicated that our current knowledge of the breadth of the *Chloroflexi* phylum underestimates its full diversity (Rappé and Giovannoni, 2003; Teske, 2012). The phylum itself is constantly under review (Hugenholtz et al., 1998; Hugenholtz and Stackebrandt, 2004) and new classes added, such as the *Ardenticatena,* as recently as 2013 (Kawaichi et al., 2013). Due to the limitations in the knowledge of the metabolism of the largely uncultivated *Chloroflexi* classes, predictions of their metabolic and ecological activities are lacking. Recent studies on *Chloroflexi* have taken the new approach of utilising single cell sequencing and metagenomics, described by Rinke et al. (2013), to understand the evolutionary and functional diversity in environmental prokaryotic communities. For example, Hug et al. (2013) used metagenomic sequencing to postulate that the *Anaerolineae* are cell debris scavengers with a host of metabolic genes related to degradation of carbohydrate compounds, which was corroborated by the recent isolation of *Pelolinea submarina*, which utilised a large range of carbohydrates as substrates, from deep subseafloor sediments in Japan (Imachi et al., 2014). These findings suggest that the *Anaerolineae* were able to thrive in the organic rich sediments commonly found in estuarine and coastal sediments, and explains their abundance in Severn Estuary sediments.

6.4 Temperature Influences the Change to Thermally Adapted Organisms in Severn Estuary Sediments

In the temperature gradient experiment, an increase in temperature led to a decrease in species richness and diversity (Table 5.4) and thus the selection of thermotolerant *Bacteria* and *Archaea*, such as spore-forming *Firmicutes* (Figure 5.13). These spore-forming organisms are able to resist extreme temperatures and lack of nutrients (Kaksonen et al., 2008; Qiu et al., 2003). Recent work has found endospores to be widespread in estuarine (O'Sullivan et al., 2014), marine (Muller et al., 2014) and deep subsurface sediments (D'Hondt et al., 2004; Lomstein et al., 2012). Selection of thermophilic and thermotolerant organisms has been described previously in hot spring sediments and thermal Guaymas basin sediments (Biddle et al., 2012; Cole et al., 2013).

An important finding from the thermal gradient experiment was that a temperature window at approximately 43^{o}C, previously described by Roussel et al., (unpublished work) in amended slurried sediments. At temperatures above 43^{o}C, sulphate reduction and methanogenesis altered to hydrogenotrophic pathways. As previously discussed in **Sections 5.4.1.1**, the abundance of *Desulfobulbaceae* below 43^{o}C and the subsequent increase in abundance of the genus *Desulfotomaculum* indicated a shift from heterotrophic to autotrophic sulphate reduction (Aullo et al., 2013; Sass et al., 2002). A peak at 46^{o}C of the *Methanomicrobiales* and *Methanobacteriales* (Figure 5.20) was due to these organisms utilising chemolithotrophic hydrogenotrophic methanogenesis, as previously described in **Section 5.4.1.3.** Interestingly, a temperature window of 25-46^{o}C for metabolic processes in surface sediments has been suggested through a collection of studies (Conrad et al., 2009; Finke and Jørgensen, 2008; Nozhevnikova et al., 2007; Weston and Joye, 2005), which focused on the temperature driven decoupling of fermentation products by anaerobic respiration. This was characterised by the switch from hydrogenotrophic and acetoclastic methanogenesis to exclusively hydrogenotrophic methanogenesis above 40^{o}C (Conrad et al., 2009; Nozhevnikova et al.,

2007).This study corroborates these studies and refines the ecological changes over this critical temperature window.

6.5 Future Perspectives

To gain a broader knowledge of the Severn Estuary prokaryotic communities, a number of different sampling regimes could be implemented to create a collection of sediment from different environmental conditions. For example, samples could be collected over a seasonal timescale as prokaryotic communities have been shown to shift with seasonal changes (Finke and Jørgensen, 2008; Hall et al., 2008). To continue this study, which utilised a partial transect across the estuary, two different approaches could be used. Firstly, a nationwide sampling effort of estuary sediments would be beneficial, to ascertain the shared and diverse communities in the estuarine sediment environment. However, due to the similarity seen in this study with sequences from the Colne Estuary, it would perhaps be more beneficial to intensively sample one estuary, extending sampling to include salt marsh areas or even tributary rivers, which could indicate the influence of the surrounding land and rivers on the deposition of nutrients and microorganisms into the estuary. A number of organism usually found in soils were detected in this study and so the source of these could be traced. To do this intensive collection of metadata, including geochemistry, sedimentology, and metabolic rates would be crucial to understanding the active metabolisms occurring in estuarine sediment.

To extend this study a focus on analysis of ecosystem function, by a variety of methods, would be highly beneficial. During this study, a number of organisms involved in major geochemical cycles such as nitrification, denitrification, methanogenesis, sulphate reduction and dehalorespiration were identified. However one of the major limitations of community profiling of the 16S rRNA gene is that little functionality can be derived. To overcome this, the direct targeting of functional genes with PCR, qPCR and potentially next generation sequencing could be used (Lüke and Frenzel, 2011; Ritalahti et al., 2006). Also, the field of metagenomics and single cell genomics is continually advancing, enabling the full functional potential of a single

organism to be derived from its genome. This has already been utilised with the *Chloroflexi* with exciting results (Hug et al., 2013; Kaster et al., 2014; Wasmund et al., 2014b). However, even these state of the art methods are limited by the targeting of DNA, which has been shown to remain intact, outside of cells and within dead cells for an extremely long time (Corinaldesi et al., 2005). The targeting of messenger RNA (mRNA) through qPCR and RNA-seq would be a distinct advantage by increasing the sensitivity of these methods and thus in indicating not only the metabolically active communities but also any temporal or spatial shifts in activity with the sediment micro-habitats (Dumont et al., 2011; Lay et al., 2013; Shtarkman et al., 2013).

As previously discussed, the *Dehalococcoidia* are capable of the dehalorespiration of both pollutants and naturally occurring halogenated compounds (Adrian et al., 2007; Aulenta et al., 2004; Futagami et al., 2008; Hölscher et al., 2004). Their importance in the global halogen cycle in both coastal and surface marine sediments has also been discussed (Sections 1.5.1.2 & 1.5.2; Ahn et al., 2007; Futagami et al., 2009). Futagami et al. (2009) utilised primers targeting the reductive dehalogenase gene (*rdhA*) from *Dehalococcoides mccartyi* and measured dehalogenation activity to describe widespread dehalogenation in Pacific deep subsurface sediments (<358 mbsf). Attempts were made in this study to use the same primers with the same sediments and *D. mccartyi* 195 genomic DNA as positive controls. Despite repeated attempts at optimisation, *rdhA* genes could not be amplified from Severn Estuary sediments, despite working positive controls and the detection of 16S rRNA gene sequences related to the *Dehalococcoidia* but not closely to *D. mccartyi* 195 in the sediments. From the great sequence diversity seen within the *Chloroflexi* and the *Dehalococcoidia* specifically (Figure 3.5), it was proposed that as the Futagami primers were targeted to *D. mccartyi* 195, they were thus unable to match the full diversity of the *Dehalococcoidia.* A database of all *rdhA* genes found in the NCBI database was compiled to try to target as much *rdhA* sequence diversity as possible and produce new degenerate primers using the software program COnsensus-DEgenerate Hybrid Oligonucleotide Primers (CODEHOP; Santos and Ochman, 2004). This software is designed to produce universal

degenerate primers from multiple amino acid sequence alignments where protein sequences are particularly diverse and distant from one another, such as the *rdhA*. However, after creating and testing these, *rdhA* sequences were still not detected in Severn Estuary sediments.

On one hand, it could be ineffectual amplification methods, however, single cell genomics studies isolating *Dehalococcoidia* cells distantly related to *D. mccartyi* 195 found no evidence of *rdhA* genes (Hug et al., 2013; Kaster et al., 2014). Instead they found evidence of anaerobic lithotrophic or chemotrophic lifestyles. Further still, Lee et al. (2009) found that *D. mccartyi* 195 was capable of nitrogen fixation after a complete nitrogenase operon was discovered in its genome (Seshadri et al., 2005). Kaster et al. (2014) hypothesized that terrestrial *Dehalococcoides* species are highly niche adapted to dehalogenation, recently gaining *rdhA* genes from horizontal gene transfer as these genes are often found in high plasticity regions of their genome. This suggests that *Dehalococcoidia* in uncontaminated environments may survive on different metabolisms in the absence of high concentrations of halogenated compounds. Evidence in this study to support this includes the widespread detection of the clone GIF9 in all samples, a member of which was predicted to be reliant on fermentative metabolisms, most likely acetogenesis, from its single cell genome (Hug et al., 2013). The use of metagenomic and single cell genomics for the description of so-called “microbial dark matter”, described by Rinke et al. (2013), has already been successfully implemented for the study of *Chloroflexi* and promises great advances in the field.

Appendices

To view appendices see ORCA supplementary file

“AngharadWilliams_2015_Appendix_Supplementary_materials” or go to

https://angharadwilliams.co.uk/appendices/

Username: thesis

Password: Vc$3x

Appendix 1

Quantitative PCR calculations for the enumeration of *Bacteria, Archaea* and *Chloroflexi* 16S ribosomal RNA gene copy numbers in sediment.

Appendix 2

All R scripts used for statistical analysis throughout the thesis.

Appendix 3

Link to BlastCat open source code.

Appendix 4

Raw data files from 454 pyrosequencing for *Bacteria, Archaea* and *Chloroflexi*.

Appendix 5

Full QIIME pipeline and scripts for the analysis of 16S rRNA gene sequences from 454 pyrosequencing for Bacteria, Archaea and Chloroflexi.

Appendix 6

OTU Tables generated by QIIME for *Bacteria, Archaea* and *Chloroflexi* sequencing libraries.

Appendix 7

Conference posters and presentation abstracts presented during the PhD.

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