Heat resistant thermophilic endospores in cold estuarine sediments

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Abstract

Microbial biogeography explores the spatial and temporal distribution of microorganisms at multiple scales and is influenced by environmental selection and passive dispersal. Understanding the relative contribution of these factors can be challenging as their effects can be difficult to differentiate. Dormant thermophilic endospores in cold sediments offer a natural model for studies focusing on passive dispersal. Understanding distributions of these endospores is not confounded by the influence of environmental selection; rather their occurrence is due exclusively to passive transport. Sediment heating experiments were designed to investigate the dispersal histories of various thermophilic spore-forming *Firmicutes* in the River Tyne, a tidal estuary in North East England linking inland tributaries with the North Sea. Microcosm incubations at 50-80°C were monitored for sulfate reduction and enriched bacterial populations were characterised using denaturing gradient gel electrophoresis, functional gene clone libraries and high-throughput sequencing. The distribution of thermophilic endospores among different locations along the estuary was spatially variable, indicating that dispersal vectors originating in both warm terrestrial and marine habitats contribute to microbial diversity in estuarine and marine environments. In addition to their persistence in cold sediments, some endospores displayed a remarkable heat-resistance surviving multiple rounds of autoclaving. These extremely heat-resistant endospores are genetically similar to those detected in deep subsurface environments, including geothermal groundwater investigated from a nearby terrestrial borehole drilled to >1800 m depth with bottom temperatures in excess of 70°C. The ability of these endospores to survive extreme temperatures whilst in a dormant state may enable them to withstand adverse conditions for long periods of time and then germinate in response to changing surroundings. This was investigated further in the context of seawater injection during secondary oil recovery, where cold seawater is injected into hot oil reservoirs, resulting in a cooler reservoir temperature near the injection well bore. Microcosm experiments designed to simulate this showed that cooling triggered the germination of endospores of sulfate-reducing *Desulfotomaculum* leading to the onset of souring in this model system. The results presented here, indicate that bacterial endospores are transported between terrestrial and marine, surface and subsurface environments. Their survival and distribution therefore has

relevance to understanding deep biosphere processes, and factors shaping microbial diversity in the marine environment.

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Chapter 1.

Introduction

1.1 Thermophilic Endospores in Cold Sediments

The detection of anaerobic thermophilic endospore-forming bacteria in cold sediments was first reported in heated sediment incubations from Aarhus Bay, Denmark (Isaksen *et al*., 1994). The *in situ* temperature of Aarhus Bay sediments ranges from 0-15°C, yet when incubated at 60°C thermophilic sulfate reduction was detected. Enumeration of viable thermophilic sulfate reducing bacteria (SRB) by most probable number (MPN) indicated the presence of $2.8 \cdot 10^4$ cells g^{-1} in the cold surface sediment. The thermophilic sulfate-reducing strain P60 was isolated from enrichments at 60°C, and based its physiological characteristics identified as *Desulfotomaculum kuznetsovii.* The temperature range for growth of strain P60 (52-69°C) was significantly higher than would ever be encountered *in situ*, thus it must have been delivered to the sediments from an external source. *Desulfotomaculum arcticum* strain 15^T, a moderate thermophile capable of growth between 26 and 46.5°C with optimum growth at 44°C, was later isolated from permanently cold fjord sediment off the west coast of Svalbard where the temperature never exceeds 4°C (Vandieken *et al*., 2006). Sediments from Smeerenburgenfjorden in the north-west of Svalbard have since been shown to host a diverse community of spore-forming thermophilic bacteria including sulfate-reducing *Desulfotomaculum* and putative fermentative *Firmicutes* capable of organic matter mineralisation when incubated at 50°C (Hubert *et al*., 2009; 2010). Combining spore abundance estimates with sedimentation rate, revealed a constant delivery of spores of thermophilic SRB at exceeding a rate of 10^8 spores m^{-2} y^{-1} to the permanently cold sediments of Svalbard (Hubert *et al*., 2009). Further investigation of Aarhus Bay sediments incubated between 46 and 69°C revealed 23 species level phylotypes of endospore forming sulfate-reducing *Desulfotomaculum* (de Rezende *et al*., 2013). Three of the *Desulfotomaculum* phylotypes detected in Aarhus Bay were highly similar to those detected in Svalbard sediments (Hubert *et al*., 2009; 2010) despite the two locations being 3000 km apart. This indicated the long-distance passive dispersal of endospores of thermophilic bacteria in the ocean from a source common to both locations.

Endospores of thermophilic bacteria are conspicuous in cold sediments and are inactive *in situ* making them useful model organisms for studying microbial biogeography (de Rezende *et al*., 2013; Müller *et al*., 2014).

1.2 Microbial biogeography

Biogeography explores the spatial and temporal distribution of organisms over multiple scales (Martiny *et al*., 2006; Lomolino *et al*., 2010). Microbial biogeography seeks to understand, what microorganisms live where, at what abundance and why. To answer these questions, biogeographers must understand the underlying mechanisms that cause differences in community composition (Lindström and Langenheder, 2012). A review by Hanson *et al*., (2012) identified four fundamental processes that underlie microbial biogeographic patterns – selection, drift, dispersal and mutation. Each of these processes has an effect on the distance-decay relationship, which models the decrease in community compositional similarity between two locations with increasing spatial distance (Figure 1.1). Selection occurs as a result of the influence exerted by environmental factors such as temperature, pH, salinity and other physical and chemical characteristics. Genetic drift occurs as a result of chance demographic events such taxa reproduction and deaths. Both processes result in a decrease in the similarity between two communities with distance (Hanson *et al*., 2012). In contrast, dispersal will weaken the distance-decay relationship. The strength of this effect may vary between communities as microorganisms can have different dispersal capabilities (Figure 1.1) (Foissner, 2006; Martiny, 2015). Taxa that are better at dispersing will show lower rates of distance-decay over a given landscape (Nekola and White, 1999). This includes abundant taxa, which have a greater chance of long-distance dispersal owing to population size (Martiny *et al*., 2006; Martiny, 2015). Additionally, deterministic traits such as spore formation, may increase the probability of long distance dispersal (Eisenlord *et al*., 2012; Hanson *et al*., 2012). This trait, amongst other factors, is thought to contribute to the widespread distribution of endospore-forming *Firmicutes* in the environment (Beuche *et al*., 2013). Microbial community composition at a given location therefore depends on a combination of factors, however the influences contributed by these different factors on the compositional similarity between locations can be difficult to disentangle from one another.

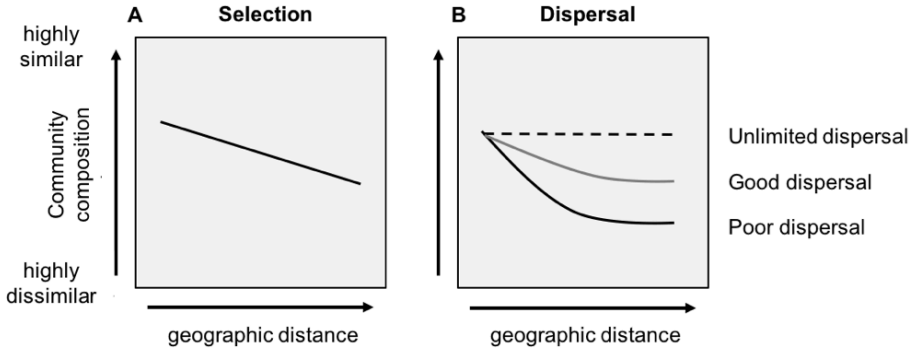

Figure 1.1: The effect of selection and dispersal on the community composition similarity between locations with increasing geographic distance. Selection will decrease the similarity between two communities with distance, if environmental conditions differ along a spatial gradient (**A**) and dispersal will increase the similarity between two locations (**B**). Unlimited dispersal (dashed line) would enable a microorganism to disperse globally, a good disperser (grey line) has increased probability of dispersing between geographically distant locations compared to a microorganism with a poor dispersal ability (black line). Adapted from Hanson *et al.*, (2012), *Nature Reviews Microbiology*.

1.2.1 Passive dispersal

The active dispersal of microorganisms (self-propulsion) is spatially constrained (Martiny *et al*., 2006, Fierer 2008), generally occurring on a minute scale e.g. from particle to particle (Lindström and Langenheder, 2012). Active dispersal therefore does not have a significant effect of microbial composition between distant locations. By contrast, passive dispersal has the potential to transport microorganisms over great distances, and even globally. The passive dispersal of cells may occur in the atmosphere, in ocean currents and on or in mobile macroorganisms (Fierer, 2008; Hervàs *et al*., 2009, Galand *et al*., 2010; Lennon and Jones, 2011). The global connectivity of these transport mechanisms, in addition to large population sizes and small cell size, suggest that microorganisms have the potential for unlimited dispersal and consequently taxa may be ubiquitous in the environment (Finlay, 2002; Fenchel and Finlay, 2004; Martiny *et al*., 2006). While microorganisms may have the ability to disperse across the globe, the extent to which geographic barriers contribute to changes in community composition by limiting dispersal is debated (Martiny *et al*., 2006; Eisenlord *et al*., 2012; Martiny, 2015). A study conducted in the English Channel showed that a large proportion of microbial lineages found in the International Census of Marine Microbes (ICoMM) dataset, which included samples from multiple distinct marine locations, could be detected in a single site in the English Channel with deep sequencing methods (ca. 10 million 16S rRNA V6 reads in the

deeply-sequenced English Channel site) (Gibbons *et al*., 2013). This discovery suggests that in any given location, all microbial diversity may be present. This would mean that the differences observed in communities between different locations reflect changes in relative abundance combined with the technical limitations of the survey method, rather than presence or absence (Gibbons *et al*., 2013). In addition, it suggests that dispersal limitation does not affect microorganisms and that biogeographers have simply not had the detection tools to exhaustively sample the full microbial diversity at a given location. On the other hand, there is evidence that microorganisms do display complex biogeographic patterns and can be dispersal limited despite high dispersal rates (Foisnner, 2006; Schauer *et al*., 2010; Eisenlord *et al*., 2012; Martiny *et al*. 2015). This is discussed in the marine context in the next section.

1.2.2 Dispersal limitation

Oceans are regionally formed water masses with different temperature and salinity characteristics, resulting in vertical and horizontal variation (Galand *et al*., 2010; Lovejoy and Potvin, 2011). This variation is reflected in the distribution of marine microorganisms (Lovejoy and Potvin, 2011). Functional groups of marine microorganisms in the water column can be vertically stratified with depth, and are often associated with the availability of light, oxygen and nutrients (Stevens and Ulloa, 2008; Bryant *et al*., 2012). On a global scale, water masses are considered to be a key factor controlling marine microbial biogeography as they act as physical barriers limiting dispersal, thus impacting upon the diversity of microorganisms in the ocean. This has been demonstrated by the presence of distinct communities in different water masses (Galand *et al*., 2010; Agogue *et al*., 2011; Monier *et al*., 2013; Hamdan *et al*., 2013) and bipolar species that are not detected at intervening latitudes (Sul *et al*., 2013). The influence of dispersal limitation can be difficult to distinguish from the selective factors that act within different water masses i.e. the distinct environmental conditions. Nevertheless, dispersal limitation in the ocean appears to play an important role distinct from and prior to environmental selection influencing microbial community composition (Sul *et al*., 2013). By tracking the dispersal of thermophilic endospores, which are inactive in the cold ocean and thus are not subject to environmental selection, Müller *et al*., (2014) were able to demonstrate dispersal limitation as a result different water masses, and how they

connect with global circulation. Additionally, by focusing on endospores, this study could show that dispersal limitation affects microorganisms thought to have greater dispersal ability, owing to the ability to persist in a dormant state.

Distributions of bacterial taxa in the seabed indicate that microorganisms from the overlying water column are deposited in surface sediments (Hamdan *et al*., 2013). Walsh *et al*., (2015) investigated the relationship between communities in the water column and the seabed by incorporating deep subseafloor sediment (defined as ≥ 1.5 metres below seafloor (mbsf)) in addition to shallow (surface) sediment and water column samples. The study demonstrated that communities in the deep subseafloor sediment were derived from a subset of the community from shallow seafloor sediment, which were recruited from the water column. Abundant taxa in the subseafloor could be detected at much lower relative abundance in the seawater, whereas taxa that were abundant in the seawater were generally not detected in the subseafloor. This is likely due to the different conditions experienced in the water column in comparison with the subseafloor, where sediments are characterised by anoxic conditions with low energy availability (Jørgensen and D'Hondt, 2006; Jørgensen and Marshall, 2015). This discovery demonstrates how microbes associated with the sedimentary environment may be transported in the water column as components of the rare biosphere, and could colonise the seafloor at a location distant from where they originated.

1.3 Dormancy and the Microbial Seed Bank

The microbial diversity of an ecosystem is comprised of both abundant and rare taxa. Rare taxa exist at low relative abundance, but collectively account for a large proportion of species richness (Pedrós-Alió, 2006; 2012). Rare organisms can have important ecological functions and are important for maintaining microbial diversity (Lynch and Neufield *et al*., 2015). All rare taxa must grow at some point in time, somewhere, and can become abundant in response to temporal and/or spatial changes in environmental conditions (Pedrós-Alió, 2012). As discussed above, certain rare taxa in seawater subsequently become dominant upon deposition and burial in seabed sediments (Walsh *et al*., 2015). This has also been observed in terrestrial settings, where rare taxa in upslope environments, subsequently became abundant taxa in a downstream lake (Crump *et al*., 2012). Many rare taxa are not actively growing or are dormant. Dormant microorganisms

enter a state of reduced metabolic activity as a strategy for maintaining microbial diversity for long-term survival (Lennon and Jones, 2011). In some cases, entering dormancy results in morphological differentiation, such as endospore formation. Dormant endospores are considered metabolically inert and can remain dormant for thousands (de Rezende *et al*., 2013), and possibly even millions of years (Cano and Borucki, 1995; Vreeland, 2000). Dormant microbial populations may persist at undetectable cell densities (Locey, 2010), together with other low abundance microbial populations, creating a rare biosphere of phylogenetic diversity (Sogin *et al*., 2006).

These dormant microorganisms contribute to a microbial seed bank of taxa that are able to persist during unfavourable environmental conditions (Lennon and Jones, 2011). Microorganisms in the seed bank are capable of responding to environmental change, and may be recruited from the rare biosphere and become active if favourable conditions are encountered (Jones and Lennon, 2010). This has important implications for the ecological processes which influence microbial diversity, such as succession and recovery following disturbance events, where dormant organisms may be recruited from the persistent seed bank (Fierer and Lennon, 2011; Caporaso *et al*., 2012; Gibbons *et al*., 2013). While many rare organisms in the environment are inactive or dormant, some may be actively growing, yet still rare. A study of anaerobic phototrophic bacteria inhabiting the meromictic Lake Cadagno found that although *Chromatium okenii* represented <1% of the total cell number, it contributed to approximately 40% to the total carbon uptake and 70% to the inorganic carbon (Musat *et al*., 2008). Similarly, Pester *et al*., (2010) found a *Desulfosporosinus* species to constitute only 0.006% of the total microbial community while still contributing an important biogeochemical process that diverts the carbon flow in peatlands from methane to CO_2. Despite these and other examples of biogeochemical activity by numerically rare microbial taxa, it is likely that a large proportion of the rare biosphere is made up of non-growing cells (Pedrós-Alió, 2012), and endospores are considered in more detail below.

1.3.1 Bacterial endospores

Endospores can be formed by certain bacteria within classes *Bacilli* and *Clostridia*, of the phylum *Firmicutes*. *Bacilli* are typically considered aerobic and *Clostridia* anaerobic, but there are also a number of anaerobic and facultatively

anaerobic *Bacilli* reported (Tang *et al*., 2009; Coorevits *et al*., 2012; Lin *et al*., 2014). The majority of studies on sporulation and germination have focused on the model organism *Bacillus subtilis*, a fast-growing aerobe. Studies that have investigated *Clostridium* spp., typically *C. difficile*, *C. acetobutylicum* or *C. perfringens*, have indicated that the basic morphological changes during spore formation are conserved between *Clostridium* and *Bacillus*, but genetic initiation, regulation and germination mechanisms are considerably different (Paredes-Sabja *et al*., 2010; Vecchia *et al*., 2014; Al-Hinai *et al*., 2015). Sporulation occurs in response to unfavourable environmental conditions, such as nutrient deprivation. In their dormant state, endospores are resistant to multiple forms of stress including desiccation, irradiation, and extreme heat (Nicholson *et al*., 2000). Spore resistance is achieved by condensation of the chromosome and dehydration of the spore core (Vecchia *et al*., 2014). The degree of heat resistance is attributed to a variety of factors that include: (i) the optimal growth temperature of the strain, (ii) the temperature at which sporulation occurred, (iii) the extent of spore core mineralisation with dipicolinic acid (DPA) and divalent cations, (iv) the protection of spore DNA by α/β type small acid soluble spore proteins, and (v) the core water content of the spore (Nicholson, 2000; Melly *et al*., 2002; Atrih and Foster, 2002; Setlow, 2006; Coleman *et al*., 2007; Setlow, 2014). Heat-resistance is lost when the spore core is rehydrated and DPA is released (Setlow, 2014).

For dormancy to be a successful survival strategy, spores must continually monitor their environment, enabling them to respond to specific nutrients, leading to germination and outgrowth to form a new vegetative cell (Atrih and Foster, 2002; Paredes-Sabja *et al*., 2011; McKenney *et al*., 2013). Endospore-forming *Firmicutes* are specifically adapted to grow quickly in response to favourable environmental conditions, and their frequent isolation from subsurface environments has been attributed to their fast growth response in enrichment cultures (Parkes *et al*., 2014).

1.3.2 Endospores in the subseafloor sediments

Parkes *et al*., (2014) recently estimated that there are $5.39 \cdot 10^{29}$ cells in the marine deep biosphere. This followed previous estimates of $2.9 \cdot 10^{29}$ cells (Kallmeyer *et al*., 2012) and $3.5 \cdot 10^{30}$ cells (Whitman *et al*., 1998). Cell abundance estimates are adjusted as an increasing number of deep biosphere samples and settings are explored. Whitman (1998) produced estimates from ocean margin

regions with high organic productivity and high sedimentation rates. Kallmeyer (2012) updated this global estimate, accounting for Pacific gyre regions with extremely low cell abundance and low sedimentation rates. Parkes *et al*., (2014) further included more deep ocean sediments with elevated prokaryotic activities and populations including subsurface gas hydrate formations and oil and gas reservoirs, accounting for the increased cell abundance compared to the estimate by Kallmeyer *et al*., (2012). Even taking the lowest estimate, these numbers show that marine sediments are a major microbial habitat that extends deep below the seafloor, with microbial abundance roughly equal to the scale of the seawater ($1.2 \cdot 10^{29}$), soil ($2.6 \cdot 10^{29}$) and the lower boundary estimate of the terrestrial subsurface ($2.5 \cdot 10^{29}$ to $25 \cdot 10^{29}$) global microbiomes (Whitman *et al*., 1998).

Spore formation is considered to be an important strategy for long-term survival in subseafloor sedimentary habitats (Parkes *et al*., 2014), and offers one explanation for how microorganisms deal with low energy fluxes in the deep subsurface. The contribution of endospores to the total microbial community might increase estimates of deep marine biosphere cell abundance, as endospores are generally impermeable to DNA stains and may not have been accounted for (Jørgensen, 2012; Lomstein *et al*., 2012). Although there is evidence that some endospores may be stained with acridine orange (Fichtel *et al*., 2008; Parkes *et al*., 2014). DPA is universally present in bacterial endospores and can be used as a marker to determine endospore abundance (Fichtel *et al*., 2007; 2008; Langerhuus *et al*., 2012). DPA extraction from sediment cores from North Sea tidal flats showed that the relative contribution of endospores to the total microbial community increased with depth, accounting for <1% of the total microbial community in the upper 50 cm of the sediment and increasing to 10% in deeper layers of the sediment (Fichtel *et al*., 2008). Estimates of endospore abundance in sediments off the coast of Peru, using DPA in addition to a muramic acid based method, indicated that they may be as abundant as vegetative cells in deep subsurface sediments with abundances of up to 10^7 endospores per cm^3 reported (Lomstein *et al*., 2012). For spore-formation to be a successful long-term survival strategy, conditions suitable for germination and growth would ultimately need to be encountered. In sediments where available energy decreases slowly and steadily with time, endospore formation may be a dead-end strategy, especially when

taking into account the energy required for germination (Jørgensen, 2012; Hoehler and Jørgensen, 2013). In the case of thermophilic endospores in subseafloor sediments, conditions suitable for growth may be encountered in warmer sediments encountered at depth, resulting in germination post burial (Hubert *et al*., 2010).

While these studies point towards the quantitative significance of endospores in sedimentary habitats, a recent study of subseafloor sediment metagenomes estimated that putative endospore formers accounted for <10% of the population, based on low frequencies of endospore-specific genes (Kawai *et al*., 2015). The discrepancy between these different studies may be the result of low DNA extraction efficiency from endospores in the sediments analysed with metagenomics, owing to the difficulty in extracting DNA from endospores using standard procedures. This would result in an underestimation of spore-specific genes in the metagenomes. In addition to this, the authors suggested that discrepancies could arise based on assumptions of the DPA content per spore. DPA cell content is used to calculate abundance estimates, but it can vary considerably between species, thus influencing DPA-based estimations of total endospore abundance. Another explanation for the discrepancy could be if sporulation genes in subsurface microorganisms are distinct from currently well known endospore specific genes (Kawai *et al*., 2015).

1.4 Thermophilic spore-forming *Desulfotomaculum*

The genus *Desulfotomaculum* was created to describe Gram-positive and obligately anaerobic sulfate-reducing bacteria that form heat-resistant endospores (Campbell and Postgate, 1965). *Desulfotomaculum* are members of the family *Peptococcaceae*, order *Clostridiales*, class *Clostridia* and phylum *Firmicutes* (Stackebrandt, *et al*., 1997). Spore-forming sulfate-reducing bacteria additionally include the genera *Desulfovirgula* (Kaksonen *et al*., 2007a), *Desulfurispora* (Kaksonen *et al*., 2007b), *Desulfosporosinus* (Stackebrandt *et al*., 1997), *Desulfosporomusa* (Sass *et al*., 2004) and the candidate species *Desulforudis audaxviator* (Chivian *et al*., 2008). *Desulfotomaculum* spp. have been detected in both freshwater and marine sediments and are of ecological significance in both surface and subsurface environments (Nakagawa *et al*., 2002; Detmers *et al*.,

2004; Moser *et al*., 2005; Kaksonen *et al*., 2006; Haouari *et al*., 2008; Basso *et al*., 2009; Guan *et al*., 2013; Watanabe *et al*., 2013).

Firmicutes often dominate bacterial communities from deep subsurface sediments (Fry *et al*., 2008; Edwards *et al*., 2010). The presence of sulfate-reducing *Desulfotomaculum* in these environments has been attributed to their adaptation to extreme conditions, through sporulation and the ability to grow at high temperature (Aüllo *et al*., 2013). In addition, *Desulfotomaculum* spp. are metabolically diverse, and can utilise a wide range of substrates, including acetate and hydrogen, both of which are thought to be important substrates for life in the deep subsurface (Wellsbury *et al*., 1997; Aüllo *et al*., 2013; Roussel *et al*., 2015). Sulfate-reduction is a significant process in marine sedimentary environments (Jørgensen, 1982). In addition to active psychrophilic and/or mesophilic communities in marine surface sediments (Robador *et al*., 2015), some cold and temperate marine sediments host thermophilic spore-forming *Desulfotomaculum* spp. as explained above (section 1.1). Unable to grow at *in situ* temperatures, they remain dormant and are not influenced by selective pressures. This makes them ideal model organisms for studying dispersal, without the confounding influence of selection. To use these model organisms for biogeographic studies, it is important to consider their source habitat in order to identify possible dispersal vectors and determine their dispersal histories.

1.4.1 Habitats of thermophilic endospore-forming bacteria

In cold Arctic sediments thermophilic sulfate reduction occurred optimally (T_{opt}) at 56°C, and was detected between 41 and 62°C, representing the minimum (T_{min}) and maximum (T_{max}) growth temperature for thermophilic SRB in those sediments (Hubert *et al*., 2009). The T_{min} is significantly higher than the annual *in situ* temperature (-2 to +4°C), indicating that the source must be an external habitat, with thermal conditions between the T_{min} and T_{max}. Given a flux of up to 10^8 thermophilic spores m^{-2} y^{-1} to cold Arctic sediments, the warm anoxic source habitat must be associated with an efflux mechanism, and have sufficient magnitude to support these population sizes (Hubert *et al*., 2009). Close relatives to the thermophiles enriched in Arctic sediments were detected in deep oil reservoir and mid-ocean ridge habitats, in keeping with the warm anoxic source habitat criteria. Advective flow of geofluids from these habitats could expel thermophiles

from subsurface into the cold ocean, explaining their occurrence and observation in cold surface sediments (Hubert *et al*., 2009).

1.4.1.1 Petroleum reservoirs

Despite harsh environmental conditions, petroleum reservoirs are active deep biosphere ecosystems (Head *et al*., 2003; Parkes and Sass, 2009) and have been shown to harbour elevated cell counts – up to 10^7 cells g^{-1} – at the oil-water transition zone (Bennett *et al*., 2013). *Firmicutes* are a frequently detected bacterial phylum in high temperature (>50°C) subsurface oil reservoirs (Hubert *et al*., 2012) and are detected in both pristine and water-flooded reservoirs (Frank *et al*., 2015). Thermophilic fermentative spore-forming *Firmicutes* isolated from oil-field formation waters include members of the genera *Thermoanaerobacter* and *Caldanaerobacter* (Cayol *et al*., 1995; Fardeau *et al*., 2000; 2004). Three thermophilic species of sulfate-reducing *Desulfotomaculum* spp. have been isolated from oil fields, *D. kuznetsovii* (Nazina *et al*., 1989), *D. salinum* (Nazina and Rozanova, 1978), and *D. thermocisternum* (Nilsen *et al*., 1996), in addition to the mesophilic species *D. halophilum* (Tardy-Jacquenod *et al*., 1998).

Seabed pockmarks and seeps result in the release of hydrocarbon fluids from subsurface oil reservoirs (Judd and Hovland, 2007). These natural seeps may transport thermophilic microorganisms adapted to hot petroleum reservoirs, out of the subsurface and into the cold ocean, where they are then transported in ocean currents (Figure 1.2) (Hubert and Judd, 2010). This process can explain the presence of thermophilic spore-forming bacteria detected in cold sediments that are close relatives of bacteria detected in oil reservoirs and oil production facilities (Hubert *et al*., 2009; de Rezende *et al*., 2013). Two *Desulfotomaculum* phylotypes detected in heated Aarhus Bay sediments shared greatest identity with bacteria originating in the Dan and Halfdan oilfields (Gittel *et al*., 2009), located 200 km west of Denmark, suggesting oil reservoirs in the region could be a source of thermophilic SRB in Aarhus Bay sediments (de Rezende *et al*., 2013). Seabed pockmarks and seeps are widespread in the ocean floor and could contribute to the dispersal of thermophilic organisms from the subsurface to the cold ocean on a global scale.

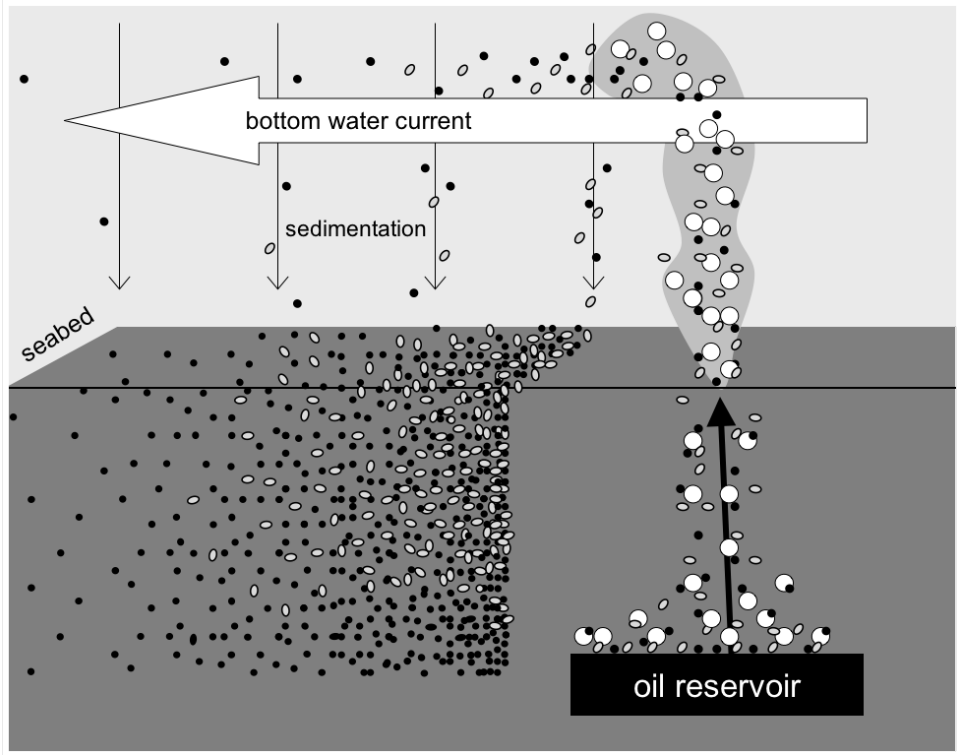

Figure 1.2: Dispersal of subsurface microorganisms from petroleum reservoirs. Leaking geofluids seep upwards (white circles) transporting microbial cells (grey ovals) and endospores (black circles) up into the ocean, where they may be dispersed in ocean currents. Microbial cells and endospores are then deposited in marine sediments potentially many kilometres away from the original source. The longevity of endospores may result in dispersal further from the source than vegetative cells, and their persistence deeper sediments layers. Adapted from Hubert and Judd (2010), *Handbook of Hydrocarbon and Lipid Microbiology*.

1.4.1.2 Mid-ocean ridges

A survey of endospore forming *Firmicutes* in >80 cold marine sediments from around the globe found that following experimental heating to 50°C, 44.5% of the thermophilic phylotypes identified (defined at 97% sequence similarity), were also detected in hydrothermally influenced sediments of the Guaymas Basin (43-150°C, Gulf of California) (Müller *et al*., 2014). The results from this study are consistent with hydrothermal fields as a source of thermophilic spore-formers in the cold ocean. The hydrologically active igneous ocean crust is the largest aquifer on Earth, representing approximately 2% of the global ocean's fluid volume (Edwards *et al*., 2011). The ocean circulates through this aquifer as fluids are exchanged with the overlying water column through hydrothermal circulation, mainly along ridge flanks which occur kilometres away from spreading centres at mid-ocean ridges (Edwards *et al*., 2011). High temperature microbial habitats persist in the oceanic crust beneath the flanks of mid-ocean ridge systems (Cowen

et al., 2003; 2004; Ehrhardt *et al*., 2007) with cell densities in the range of 10^5 ml^{-1} reported within the igneous crust (Salas *et al*., 2015). Fluid-flow at vent sites is characterised by recharging seawater and underlying hydrothermal fluids, presenting physiological challenges for microorganisms, in particular, high temperature (Schrenk *et al*., 2010; Yanagawa *et al*., 2014). Temperature gradients in the subsurface and at vent chimneys encompass hyperthermophilic, thermophilic and mesophilic growth from the hot interior to the interface with cold seawater (Teske 2008; Martin 2008; Teske *et al*., 2014). High temperature vent fluids tend to be dominated by archaeal lineages with maximum growth temperatures as high as 121°C reported (Kashefi and Lovely, 2003; Ehrhardt *et al*., 2007). Diffuse fluids vented at off-axis ridge flanks may be much cooler (ca. 10-90°C) with high concentrations of H_2, CH_4 and other low molecular mass hydrocarbons (Martin, 2008; Wankel *et al*., 2011). Advective fluid-flow at these sites may transport microorganisms from within this deep biosphere to the surface and into the water column via hydrothermal plumes or diffusive water flow (Huber *et al*., 2006; Orcutt *et al*., 2011). These fluids can harbour greater concentrations of microorganisms than background seawater (Summit and Baross, 1998; Lam *et al*., 2004; Huber *et al*., 2006), indicative of the dispersal of microorganisms from the subsurface. Hyperthermophile cell densities of at least 10^6 ml^{-1} were identified within hydrothermal plume of Macdonald Seamount, a submarine volcano in Polynesia (Huber *et al*., 1990) and up to 10^5 cells ml^{-1} were detected in warm hydrothermal fluids from an outcrop on the eastern flank of the Juan de Fuca Ridge, from which anaerobic thermophilic and hyperthermophilic enrichment cultures were obtained (Huber *et al*., 2006). These enrichment cultures included a gram-positive anaerobic thermophile that shared high sequence identity with *Caloranaerobacter azorenensis*, a fermentative member of the *Firmicutes* isolated from deep sea hydrothermal vent (Wery *et al*., 2001). The gram-positive thermophile was also closely related to a thermophilic spore-former (phylotype A) enriched from cold Arctic sediments (Hubert *et al*., 2009). The detection of closely related thermophilic microorganisms in both hydrothermal fluids and cold Arctic sediments supports the theory that thermophilic microorganisms expelled into the ocean at venting sites are dispersed by seawater currents, to be deposited on the seafloor at a distant location.

Anaerobic fermentation of peptides and sugars is the predominant metabolism of heterotrophic thermophilic *Firmicutes* found at hydrothermal vents and in addition to *Caloranaerobacter* spp., the genera *Caminicella*, *Tepidibacter*, *Caldanaerobacter* and *Thermosediminibacter* have also been detected (Teske, 2009). Sulfate-reducing *Desulfotomaculum* detected at vent sites include *Desulfotomaculum tongense*, which was isolated from a hydrothermal vent sediment collected from the Tofua Arc in the Tonga Trench (Cha *et al*., 2013). Bacteria closely related to *Desulfotomaculum* have been detected in deep-sea hydrothermal vent sulfide samples collected from the East Pacific, and South Atlantic (Jiang *et al*., 2015) and from vent chimneys and microbial mat samples from the Lost City hydrothermal field (Brazelton *et al*., 2006; Gerasimchuk *et al*., 2010).

1.4.1.3 Terrestrial thermal environments

In addition to marine habitats, terrestrial industrial environments may add to the flux of thermophilic endospores detected in cold marine sediments, such as wastewater treatment plants and coal-fired power stations, which were identified as possible local sources of endospores of SRB in cold Aarhus Bay sediments (Isaksen *et al*., 1994). Sulfate-reducing *Desulfotomaculum* have been detected in industrially heated environments such as *D. carboxydivorans* which was isolated from an anaerobic bioreactor treating paper mill waste (Parshina *et al*., 2005) and strains of *D. kuznetsovii* and *D. geothermicum* have been detected in cooling towers at petroleum refineries (Anandkumar *et al*., 2008; 2009). Compost piles can also generate significant amounts of heat reaching temperatures of 70°C and higher (Canganella and Wiegal, 2014), accordingly the thermophilic *Desulfotomaculum thermosapovorans* was isolated from compost enrichments at 55°C (Fardeau *et al*., 1995).

Thermophilic spore-formers are also detected in naturally thermal terrestrial environments, such as solfataric fields and hot springs, which form where volcanic activity heats surface waters and soils. These terrestrial volcanic environments can reach temperatures in excess of 100°C, and are host to diverse thermophilic and hyperthermophilic microorganisms (Fardeau *et al*, 2010; Bouanane-Darenfed *et al*., 2013; Chernyh *et al*., 2015). In non-volcanic, geologically old or stable environments, such as the UK, high temperatures occur where water at depth is

heated by the normal crustal gradient, which is typically around 2.6°C 100 m^{-1} in the UK (Busby *et al*., 2010). Heat is also generated and maintained by the decay of radioisotopes of uranium, thorium and potassium within the Earth's crust which can result in elevated geothermal gradients (McCay *et al*., 2014). Thermophilic sulfate-reducing *Desulfotomaculum* spp. isolated from naturally heated terrestrial thermal environments include; *D. solfataricum*, isolated from the sediments of a hot solfataric pool in Iceland (Goorissen *et al*., 2003), *D. thermosubterranean*, isolated from an underground mine in a geothermally active region of Japan (Kaksonen *et al*., 2006) and *D. geothermicum* strains which have been isolated from deep geothermal groundwaters in both France and Germany (Daumas *et al*., 1988; Sass and Cypionka, 2004). Other thermophilic members of the *Firmicutes* isolated from terrestrial thermal environments include *Caldinitratiruptor*, *Thermovenabulum*, and *Caldicoprobacter* (Fardeau *et al*., 2010; Ogg *et al*., 2010; Zarvarzina *et al*., 2012; Bouanane-Darenfed *et al*., 2013).

1.5 Estuaries

Estuaries occur at the transition between the terrestrial and marine biospheres. They are defined by the mixing of fresh- and saline water and provide a link for dispersal between terrestrial and marine habitats. Riverine flow transports sediment particles into the estuary from the erosion of riverbanks and catchment run-off. Catchment run-off is influenced by the catchment characteristics such as the local topography, geology, land use, soil and vegetation type. Sediment particles suspended in riverine flow may be deposited on the riverbed, or be transported offshore and be deposited on the seafloor. Tidal currents result in significant sediment loads being transported into estuaries from the marine environment. As a result of both fresh riverine outflow and saline tidal inflow estuarine sediments are sourced from both terrestrial and marine environments. The physical activity resulting in the entrainment and deposition of sediment particles in estuaries, similarly acts upon microorganisms attached to sediment particles and those suspended freely in the water column, resulting in a mixed estuarine microbial community.

1.5.1 Microbial distribution in estuarine sediments

Estuaries are complex ecosystems, characterised by changing physical and chemical conditions both laterally and with depth. Microorganisms may be advected into estuaries from riverine flow, surface run-off, groundwater seepage, and tidal currents, resulting in a mixed estuarine community of soil, sediment and marine derived species adapted to the estuarine environment (Crump *et al*., 2004). Terrestrial habitats in the upper reaches of estuarine environments influence the community composition of downstream habitats through downslope dispersal of microorganisms (Crump *et al*., 2012). The mixing of freshwater and seawater results in physical and chemical gradients in salinity, nutrient input and organic matter composition (Crump *et al*., 2004). These changes are reflected in the microbial community composition, which results from the gradual mixing of freshwater and seawater communities, in addition to the inactivation of some microbial groups resulting from physiological stress (Bouvier *et al*., 2002). Microbial salinity tolerance in particular contributes significantly to compositional changes in estuarine microbial communities (Telesh and Khlebovich, 2010). A study of the Choptank River in the USA demonstrated that the distribution α- and β-*proteobacteria* along the salinity gradient overlapped, but that α-*proteobacteria* were generally found in lower reaches of the estuary with higher salinities and β-*proteobacteria* in upstream reaches of the estuary with lower salinities (Bouvier *et al*., 2002). Sulfate reduction can be the dominant process in estuaries receiving input of sulfate from tidal currents. A study of the Colne estuary, UK, detected an SRB community of *Desulfobacter*, *Desulfobacterium*, *Desulfobulbus* and *Desulfovibrionaceae*, and demonstrated that their distributions were linked to their metabolic flexibility (Purdy *et al*., 2002a). A study of the same estuary, identified methanogenesis to be an important process in the estuary, and found it to be predominant in upstream sites where sulfate-rich tidal currents had less influence (Purdy *et al.*, 2002b).

In addition to lateral gradients, estuarine microbial populations are depth distributed related to the availability of electron acceptors and donors. Oxygen is rapidly depleted in surface estuarine sediment, resulting in an anoxic subsurface characterised by microorganisms that utilise nitrate, manganese, iron, sulfate and carbon dioxide as terminal electron acceptors (Köpke *et al*., 2005; Wilms *et al*.,

2006; O'Sullivan *et al*., 2013). An investigation of tidal flat sediments, which form in intertidal regions from the deposition of sediment from both riverine and tidal flow, showed that the microbial community shifted from one dominated by *Proteobacteria* in near surface sediments, to one dominated by *Firmicutes* in sulfidic layers at depth (Köpke *et al*., 2005). A study of sediments from the same region demonstrated that depth distributed bacterial communities were affected by the availability of carbon sources, while archaeal communities correlated with methane and sulfate concentrations (Wilms *et al*., 2006).

1.6 Objectives and Outline of this Thesis

Thermophilic endospores in cold sediments can be used as models for investigating the dispersal of marine microorganisms (de Rezende *et al*., 2013; Müller *et al*., 2014). Work presented in this thesis aims to use these model organisms to reveal dispersal vectors that deliver microorganisms to estuarine environments, which are influenced by both terrestrial and marine biosphere inputs. Chapter 3 explores this by identifying changes in the community composition of thermophilic spore-forming bacteria along an estuarine gradient to reveal distribution patterns indicative of different dispersal histories. Chapter 4 investigates the survival physiology of thermophilic endospores which demonstrate extreme heat-resistance when exposed to high temperature. Geothermal groundwater was investigated as a potential local source habitat of thermophilic microorganisms, and a comparison of the microbial community in the groundwater and estuarine sediment enrichments is presented. Chapter 5[*] applies the sediment heating research approach in an industrially relevant context by testing the hypothesis that the germination of thermophilic endospores can be induced by cooling from hot to warm temperature, simulating the situation in petroleum reservoirs that receive seawater injection during secondary oil recovery. Chapter 6 discusses the activity of extremely thermophilic *Firmicutes* enriched in sediments incubated at >80°C. The results presented throughout are summarised and discussed in Chapter 7.

[*] [Chapter 5 includes data contributed by Dr. Angela Sherry, *Newcastle University*. Contributions are acknowledged within].

Chapter 2.

Materials and Methods

2.1 The Tyne estuary

The River Tyne is a tidal estuary in northeast England, its two principal tributaries, the South Tyne and the North Tyne, have a combined catchment area of ca. 2000 km^2). The tidal limit of the estuary is located approximately 32 km from the ocean at Tynemouth. It is estimated that 250,000 tonnes of sediment that accumulates annually in the Tyne estuary is of marine origin and 350,000 tonnes is of terrestrial origin (Hall, 1967). The River Tyne discharges into the North Sea at Tynemouth, where freshwater run-off mixes with North Atlantic water. Deep North Sea waters consist of water of Atlantic origin while shallower regions are influenced by the mixing of freshwater discharge. Freshwater discharge from rivers into the North Sea is in the order of 300 km^3 y^{-1} (OSPAR Commission, 2000). Estuaries along the east-coast of the UK (including the Tyne, Tees, Humber, Thames and Forth) have a combined total catchment of 115,500 km^2 with an annual freshwater run-off of 48 km^3. River discharges combined with tidal action result in intensive sediment transport.

2.1.1 Sampling locations

Sediment was collected from six stations (Figure 2.1), where the “F”, “B” and “M” notation indicate Freshwater, Brackish and Marine, respectively. Estuarine sediment was sampled from Ovingham (station F1; 54°57'56"N, 1°52'10"W), Newburn (station B2; 54°58'47"N, 1°44'35"W), Scotswood (station B3; 54°57'51"N, 1°40'60"W), Byker (station B4; 54°58'22"N, 1°35'38"W) and Jarrow (station B5; 54°59'27"N, 1°28'35"W) (Figure 2.1). Marine sediment was sampled from the North Sea ca. 175 km off the English north-east coast where the River Tyne discharges (station M6; 55°05'13"N, 1°15'09"W). Station F1 at Ovingham is upstream of the tidal limit of the estuary, where the river tree-lined and backed with agricultural land. Stations B2-B4 are within the tidal range, where the river is channelised and flows through the urbanised city of Newcastle upon Tyne. Sediment was collected from 20-30 cm depth and stored in sealed containers at 4°C. Geothermal water was sampled from a borehole located within the Tyne

catchment (GB; 54°58'23"N, 1°37'34"W) (Figure 2.1). Additional details on the geothermal groundwater are provided in section 4.3.5.

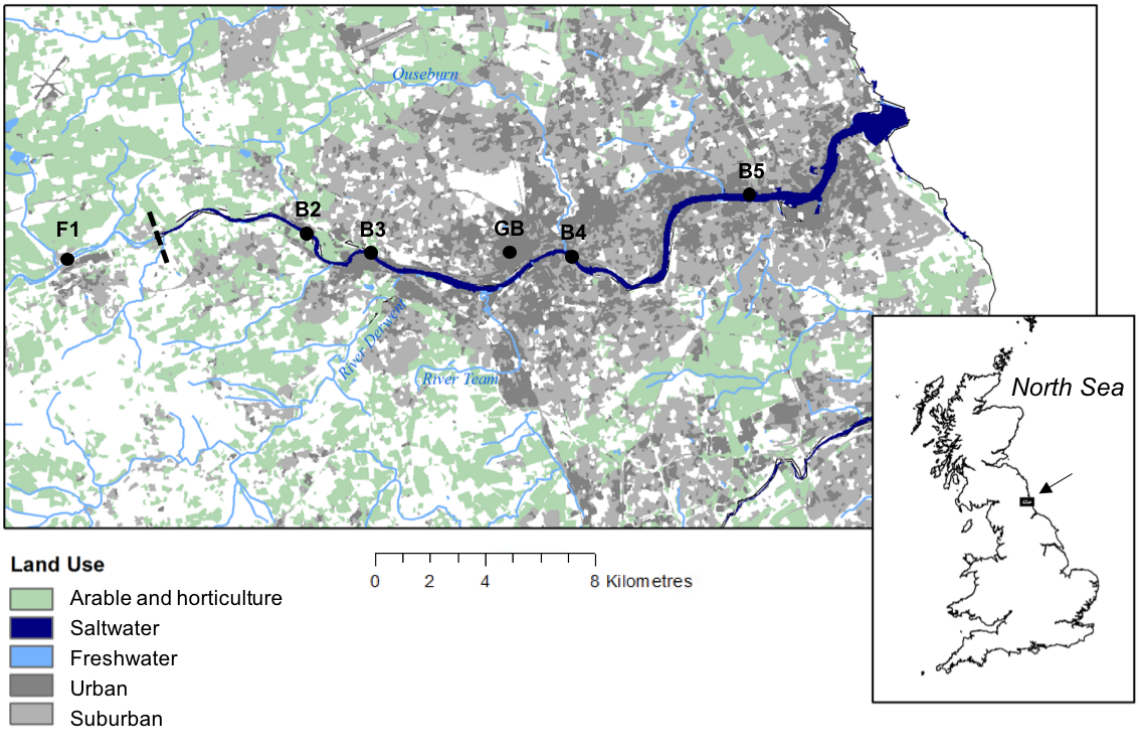

Figure 2.1: The River Tyne, UK. The River Tyne is a tidal estuary in the northeast of England, highlighted on the UK inset map with a box and arrow. Sediment was collected from six stations; Ovingham (station F1), Newburn (station B2), Scotswood (station B3), Byker (station B4) and Jarrow (station B5). Station M6 was located in the North Sea, approximately 175 km off the coast. The "F", "B" and "M" notation indicate Freshwater, Brackish and Marine, respectively. The tidal limit of the estuary is located between station F1 and B2, indicated by the dashed line. GB marks the location of the Geothermal Borehole from which geothermal water was sampled. Land use data was accessed from the EDINA Environment Digimap Service (Land Cover Map, 2007).

2.2 Sediment slurry incubations

2.2.1 Preparation of microcosms

Sediment heating experiments were used to enrich thermophilic endospores in cold sediments. Sediment heating enables spore-forming taxa to be identified following germination and growth, as the extraction of DNA directly from endospores can be difficult owing to their resilience to standard protocols used to extract DNA from vegetative cells (Wunderlin *et al*., 2013). Sediments were mixed with either brackish or marine basal medium prepared according to Widdel and Bak (1992) (Table 2.1 and 2.2) with the concentration of sulfate adjusted to 20 mM. Organic substrates were added to the medium from sterile stock solutions. The

final assembled medium was dispensed anaerobically into Wheaton glass serum bottles (60 or 100 ml, Sigma-Aldrich, UK). Sediment was added to each serum bottle under a constant flow of N_2 to give a final ratio of 2:1 (v/w) and the bottles were sealed using butyl rubber stoppers and aluminium crimps.

Table 2.1: Preparation of basal media from Widdel and Bak, (1992). *Na_2SO_4 was altered from 4 g to 2.84 g to give a concentration of 20 mM in the final medium.

	Brackish medium	Marine medium
Distilled water	1.0 litre	1.0 litre
NaCl	7.0 g	20.0 g
$MgCl_2 \cdot 6H_2O$	1.2 g	3.0 g
$CaCl_2 \cdot 2H_2O$	0.1 g	0.15 g
NH_4Cl	0.25 g	0.25 g
KH_2PO_4	0.2 g	0.2 g
KCl	0.5 g	0.5 g
Na_2SO_4*	2.84 g	2.84 g

Table 2.2: Assembling the final medium. Aliquots from stock solutions were aseptically added to autoclaved basal medium under a flow of N_2/CO_2. The pH was adjusted to 7.0 – 7.3 by adding 1 M H_2SO_4 or 1 M Na_2CO_3.

	Per litre basal medium
Non-chelated trace element solution	1.0 ml
Selenite-tungstate solution	1.0 ml
Vitamin solution	1.0 ml
Vitamin B_{12}	1.0 ml
$NaHCO_3$ solution (1.0 M)	30.0 ml
Na_2S solution (0.6 M)	2.5 ml

2.2.2 Analysis of sulfate

Sediment microcosms were subsampled by removing 1.5-2 ml of homogenised slurry from the microcosms using a N_2 flushed syringe. Aliquots of sediment slurry were centrifuged (13,000 g, 5 minutes, Hettich Mikro 200). The supernatant was used for sulfate analysis and the sediment pellet was stored at -20°C for DNA extraction. Sulfate was analysed by ion chromatography using a

Dionex ICS-1000 with an AS40 auto-sampler. The column for measuring sulfate and other inorganic anions was an IonPac AS14A analytical column with the flow rate set to 1ml min^{-1}. The eluent was 8.0 mM Na_2CO_3 / 1.0 mM $NaHCO_3$ solution and the injection loop was 25 μl. Chromatograms were visualised using Chromeleon Dionex software and peak areas calibrated using standard sulfate solutions.

2.2.3 Analysis of volatile fatty acids (VFA)

VFA (butyrate, propionate and acetate) were analysed by ion chromatography using a Dionex ICS-1000 with an AS40 auto-sampler. The column for measuring organic acids was an IonPac ICE-AS1, 4x250 mM analytical column with the flow rate set to 0.16 ml/min. The eluent was a 1.0 mM heptafluorobutryic acid solution, and the cation regenerant solution used for the AMMS-ICE II Supressor was 5 mM tetrabutylammonium hydroxide. The injection loop was 10 μl. Chromatograms were visualised using Chromeleon Dionex software and peak areas calibrated using standard solutions.

2.2.4 Analysis of sulfide

Sulfide was measured in enrichments with little to no sediment particles. The concentration of sulfide in culture medium was determined spectrophotometrically as a colloidal solution of copper sulfide as described by (Cord-Ruwisch, 1985). A 50 mM HCl 5 mM $CuSO_4$ solution was prepared and 4 ml added to a polypropylene cuvette, 100 μl culture medium was aseptically removed with an N_2 flushed syringe and added to the copper sulfide solution. The sulfide concentration was measured at 480 nm wavelength and mM concentrations determined using standard solutions prepared with sodium sulfide nonahydrate.

2.3 Microbial community analysis

2.3.1 DNA extraction

Sediment microcosms were subsampled by removing 1.5-2 ml of homogenised slurry from the microcosms using a N_2 flushed syringe. Aliquots of sediment slurry were centrifuged, the supernatant was used for sulfate and organic acid analysis (described in 2.2.2 and 2.2.3). and sediment pellet used for DNA extraction. DNA was extracted using the PowerSoil DNA isolation Kit (MoBio

Laboratories, Inc) following the manufacturer's protocol, except for the elution step (step 20), which was modified by eluting DNA with 50 µl instead of 100 µl of elution buffer (solution C6) and leaving to elute for 30 minutes instead of centrifuging immediately (step 21). A procedural blank was prepared with every set of DNA extractions performed, where all of the steps were carried out but no sample was added. Extracted DNA was used as a template for PCR amplification.

2.3.2 Polymerase chain reaction (PCR) amplification

For PCR amplification each sample contained 1.0 µl of DNA template and 49 µl of master mix (1.0 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 1.0 µl of 10 mM dNTPs (Thermo Scientific Fermentas Ltd, UK), 1.6 µl of 50 mM $MgCl_2$, 5.0 µl of 10x NH_4^+ buffer, 0.2 µl of *Taq* DNA polymerase (Biotaq, Bioline Reagents Ltd, UK) and 39.2 µl of sterile water. A negative control containing master mix only with no added DNA template was included to detect contamination of the reagents or contamination from the PCR preparation. In addition, the DNA extraction procedural blank (section 2.3.1) was used as a DNA template to demonstrate that no contamination occurred during the DNA extraction process. DNA from a positive control sample, known to contain the amplicon of interest, was used as a template to verify negative amplification results. Specific cycling conditions are given in the subsequent sections according to the analytical strategy that was chosen. PCR was carried out using an automated thermal cycler Techne TC-512. PCR products were visualised by electrophoresis on a 1% agarose gel.

2.3.3 Amplification of 16S rRNA genes

The most widely used gene for inferring phylogenetic relationships among prokaryotes is the 16S ribosomal RNA (rRNA) gene. This genetic marker is used for a number of reasons including (1) it is ubiquity amongst prokaryotes, (2) it is functionally constant (3) it is long enough (ca. 1500 bp) to provide sufficient sequence information for defining phylogenetic relationships and (4) the presence of highly conserved regions in addition to variable regions allow primers to be designed for multiple scales of phylogenetic resolution from domain- to species-level (Head *et al*., 1998; Janda and Abbott 2007). Coverage of primers used throughout are detailed in Appendix C, Table C.1.

2.3.3.1 Denaturing gradient gel electrophoresis (DGGE) targeting **Desulfotomaculum** *spp.*

DGGE separates gene amplicons of the same length, but with different base-pair sequences, electrophoretically based on their differential mobility in a denaturing gradient polyacrylamide gel (Muyzer *et al*., 1993). DGGE was carried out following two-step nested PCR. The initial amplification used primer pair DEM116f (5'-GTA ACG CGT GGA TAA CCT-3') and DEM1164r (5'-CCT TCC TCC GTT TTG TCA-3) that targets the 16S rRNA gene of *Desulfotomaculum* spp. (positions 116-1164) (Stubner and Meuser, 2000). The PCR programme comprised an initial denaturation step at 94°C for 3 minutes followed by 20 cycles of denaturation (94°C, 1 min), annealing (60°C, 1 min) and extension (72°C, 1 min), then final annealing (60°C, 1 min) and extension (72°C, 7 min) steps. The PCR product was used as a template in a second round of PCR amplification using universal bacterial primers 341f (5'-GCC TAC GGG AGG CAG CAG-3') (Muyzer *et al.*, 1993) and 907r (5'-CCG TCA ATT CMT TTG AGT TT-3') (Muyzer *et al.*, 2004), targeting the V3-V5 region of the 16S rRNA gene (positions 341-907). A 39 nucleotide GC-clamp was added to the 5' end of the primer 341f (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG). PCR cycling conditions were an initial denaturation cycle (94°C, 5 min) followed by 1 cycle of annealing (65°C, 1min) and extension (72°C, 3min), 19 touchdown cycles of denaturation (94°C, 1min), with the annealing temperature decreased by 1°C every second cycle (64°C-55°C, 1 min) and extension (72°C, 3min), followed by 5 cycles denaturation (94°C, 1min) annealing (55°C, 1min) and extension (72°C, 3min) and a final extension (72°C, 10 min). PCR products were loaded on a 6% polyacrylamide gel with a 50–75% urea and formamide denaturing gradient (100% denaturant was 80 ml formamide with 84 g urea made up to 200 ml with distilled water). Gels were cast using an INGENYphorU electrophoresis cassette and run at 60°C in Tris-acetate-EDTA buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.3) for 16 hours at 100V, then stained in a 1:200 dilution of SYBR-Gold (Life Technologies Ltd., Paisley, UK) for 1 h. The acrylamide gel was visualised on a UV transilluminator (Ultra-Violet Products Ltd, Cambridge, UK). Distinctive bands were excised with a sterile scalpel blade and stored in 100 µl sterile distilled water at 5°C for 24 h. DNA eluted from the excised bands was re-amplified using universal

bacterial rRNA gene primers 341f and 907r and Sanger dideoxy sequencing was performed using primer 341f at Geneius Labs (Cramlington, UK). 16S rRNA gene sequences were trimmed and edited in BioEdit Sequence Alignment Editor version 7.0.9.0 (Hall, 1999) and resulted in 16S rRNA gene sequences of 400-500 bp.

2.3.3.2 16S rRNA gene clone libraries

Cloning of the 16S rRNA was used to retrieve longer sequences to obtain a finer genetic resolution. Near full-length 16S rRNA gene amplicons (positions 8-1542) were obtained by PCR with forward primer pA (5'-AGA GTT TGA TCC TGG CTC A-3') and reverse primer pH (5'-AAG GAG GTG ATC CAG CCG CA-3') (Edwards *et al*., 1989). PCR components were as described in section 2.6. The PCR cycling conditions were an initial denaturation cycle with 3 min at 95°C, followed by 30 cycles consisting of denaturation (1 min at 95°C), annealing (1 min at 55°C), extension (1 min at 72°C), and final extension of 10 min at 72°C. The 16S rRNA PCR products were visualised and purified by agarose gel electrophoresis using crystal violet and cloned using TOPO XL PCR Cloning Kit according to the manufacturers instructions (Invitrogen, Paisley, UK), Clones were screened to determine insert size using PCR with the vector-specific primers pUCF (5'-GTT TTC CCA GTC ACG AC-3') and M13R (5'-CAG GAA ACA GCT ATG AC-3'). PCR cycling conditions were 10 min at 95°C, followed by 30 cycles consisting of denaturation (1 min at 95°C), annealing (1 min at 57°C), extension (1 min at 72°C), and a final extension at 72°C for 10 min. The insert size (~1.5 kb) was determined by electrophoresis with a 1% agarose gel. Cloned inserts of the correct size were purified using ExoSAP-IT (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. To retrieve near-full length 16S rRNA sequences Sanger dideoxy sequencing was performed with primers pA and pH in addition to the internal primers pC (5'-CCG TCA ATT CMT TTG AGT TT-3'), pF (5'-ACG AGC TGA CGA CAG CCA TG-3') (Edwards *et al*., 1989) at Geneius Labs (Cramlington, UK). Sequences were trimmed and consensus alignments constructed in BioEdit Sequence Alignment Editor version 7.0.9.0 (Hall, 1999).

2.3.3.3 Ion Torrent sequencing

The advent of high-throughput sequencing methods enables rapid and relatively low-cost sequencing of microbial communities retrieving millions of

sequences per run (Loman *et al*., 2012). 16S rRNA gene amplicons were sequenced using the Ion Torrent Personal Genome Machine (PGM) (Life Technologies). Ion Torrent determines sequence information by measuring changes in pH resulting from the release of a hydrogen ion when a nucleotide is incorporated into the sequence. Extracted DNA was used as a template for PCR amplification using Golay barcoded fusion primers (synthesised at Thermo Fisher Scientific, Inc.) that amplify the V4-V5 region (position 515-926) of the 16S rRNA gene with forward primer V4F (5'-GTG NCA GCM GCC GCG GTA A-3') and V5R (5'-CCG YCA ATT YMT TTR AGT TT-3'). The PCR protocol was denaturation at 95°C for 4 minutes followed by 25 cycles consisting of denaturation (1 min, 95°C), annealing (45 s, 55°C) and extension (1 min, 72°C) and a final extension for 10 minutes at 72°C. In instances where amplicons from triplicate microcosms were pooled, PCR products from individual microcosm DNA extracts were first quantified using the Quant-iT Picogreen dsDNA Assay kit (Invitrogen, Life Technologies, Inc.) with the Tecan Infinite 200 PRO. Triplicate amplicons were then pooled in equimolar concentrations prior to purification using Agencourt AMPure XP paramagnetic beads (Beckman Coulter, Inc.). Following purifications, amplicons were quantified using the Qubit 3.0 Fluorometer and Qubit dsDNA High Sensitivity Assay kit (Life Technologies, Inc.) to enable pooling of equimolar amounts of each amplicon, at 100 pM each, for sequencing.

Pooled amplicons were submitted for sequencing using an in-house Ion Torrent PGM (School of Civil Engineering and Geosciences, Newcastle University) and standard Ion Torrent sequencing procedures (Life Technologies, Inc.). The Quantitative Insights Into Microbial Ecology (QIIME) software package (version 1.7.0) was used to process raw sequence data (Caporaso *et al.*, 2010b). Sequences were assigned to samples based on their barcodes and simultaneously filtered to remove poor quality reads (those with a quality value of <20 were discarded). Organisation of good quality reads into operational taxonomic units (OTUs) was performed using UClust (Edgar, 2010), with an OTU threshold defined at 0.97 (97% sequence identity). Clustering of OTUs was first performed open reference against the Greengenes 16S rRNA database (DeSantis *et al.*, 2006) and then de novo. Taxonomy was assigned using RDP Classifier (Wang *et al.*, 2007) and sequences aligned using PyNAST (Caporaso *et al.*, 2010a). Chimeric sequences were identified with ChimeraSlayer (Haas *et al.*, 2011) and removed

from subsequent analysis. A final OTU table was generated by rarefying all libraries in the dataset to the smallest library. QIIME scripts used throughout are detailed in Appendix B.

2.3.4 Amplification of dsrAB *genes*

The *dsr* gene encodes for dissimilatory sulfite reductase, a key enzyme for sulfate reduction, which catalyses the reduction of sulfite to sulfide (Wagner *et al*., 1998). The *dsr* gene was amplified using primers DSR1F (5'-ACS CAC TGG AAG CAC G-3') and DSR4R (5'-GTG TAG CAG TTA CCG CA-3') resulting in a 1.9 kb gene amplicon (Wagner *et al*., 1998). The cycling conditions for primer pair DSR1F/DSR4R were according to Guan *et al*., (2013) starting with 5 min at 95°C, followed by 35 cycles consisting of denaturation (45 s at 94°C), annealing (45 s at 55°C), extension (90 s at 72°C), and a final extension at 72°C for 10 min. The *dsrAB* cloned insert was sequenced with primers DSR1F and DSR3R (5'-GAA GAA SAT GWA CGG GTT) (Wagner *et al*., 1998). Sequences were trimmed and consensus alignments constructed in BioEdit Sequence Alignment Editor version 7.0.9.0 (Hall, 1999), resulting in *dsrA* sequences of 800-900 bp.

Chapter 3.

The distribution of thermophilic *Firmicutes* along an estuarine gradient reveals multiple dispersal histories for endospores in estuarine and marine sediments

3.1 Abstract

Thermophilic endospores are found in cold and temperate sediments where they are unable to grow and thus persist in a dormant state for long periods of time. Their inability to grow in these environments effectively removes the confounding influence of environmental selection, which contributes to changes in microbial community composition. To understand their biogeography, spores can therefore be treated as tracer particles, and their spatial distribution used as an indicator of their dispersal history. To test the hypothesis that tidal currents deliver marine-derived thermophilic endospores to estuarine sediments, the distribution of thermophilic endospore populations in sediments of the River Tyne, a tidal estuary in northeast England connected to the North Sea, was investigated. The distribution pattern of 80 OTUs corresponding to thermophilic *Firmicutes* was determined, and indicated that 78% of thermophilic spore-formers detected in estuarine sediments from the River Tyne were terrestrially derived, and the remaining 22% were of marine origin. In addition, 70% of OTUs from terrestrially derived thermophilic spore-formers were detected in low relative abundance in sediment from the North Sea indicating that endospores from terrestrial environments are delivered to the open ocean. Different dispersal histories revealed within the estuary suggest that the diversity of thermophilic endospores in estuarine sediments is the result of bi-directional currents, terrestrial run-off, groundwater seepage, and effluent from industrial activities which may all operate as vectors for the passive dispersal of microorganisms.

3.2 Introduction

Large-scale bacterial dispersal is facilitated by the movement of ocean currents and air currents (Hervàs *et al.*, 2009; Galand *et al.*, 2010; Yamaguchi *et al.*, 2012; Müller *et al.*, 2014). Bacteria in transit are eventually transported to locations different from where they last grew or were active, potentially far away from that original source. If the new environment is favourable, cells in transit may have the opportunity to grow and successfully colonise. Without appropriate growth conditions, bacteria will only persist in their new location if their loss is balanced by influx of new cells (Pedrós-Alió, 2012), in which case they may form part of a microbial seed bank of low abundance taxa (Lennon and Jones, 2011; Gibbons *et al.*, 2013). Bacteria able to form endospores remain dormant during unfavourable conditions and can persist in the environment for long periods of time – characteristics that are particularly relevant for the microbial seed bank. Several studies have shown this seed bank to include inactive spores of thermophilic bacteria (Bartholomew and Paik, 1966; Isaksen *et al.*, 1994; Dobbs and Selph, 1997; Hubert *et al.*, 2009; de Rezende *et al.*, 2013; Müller *et al.*, 2014) that constitute a non-indigenous, low abundance, dormant component of microbial communities in cold sediments. As spores, these microorganisms are a particularly useful marker of dispersal, as spore formation not only facilitates their dispersal across large distances and through hostile surroundings but also means that once deposited in an environment unsuitable for their growth they remain viable and leave a record of their distribution. As such they can be used to investigate the passive dispersal of bacteria in the marine environment in general (de Rezende *et al.*, 2013; Müller *et al.*, 2014).

Past investigations of the diversity and abundance of thermophilic endospores in cold environments have focused on marine sediments. These studies have revealed that significant numbers thermophilic endospores of sulfate-reducing bacteria (SRB) are deposited in cold marine surface sediments (up to 10^8 spores m^{-2} y^{-1}) (Hubert *et al*., 2009; de Rezende *et al*., 2013). These thermophilic endospores appear to derive from warm subsurface environments that must be delivering microorganisms into the cold ocean (Hubert *et al*., 2009). Once in the water column, microorganisms may be transported in ocean currents and seed marine sediments below (Müller *et al.*, 2014; Walsh *et al*., 2015).

Estuaries are dynamic ecosystems that form the transition between the terrestrial and marine biospheres along a terrestrial-freshwater-marine continuum. As tidally influenced environments estuarine sediments could be a sink for marine derived thermophilic endospores dispersed via ocean currents. This would result in a gradient in the distribution of thermophilic *Firmicutes* in estuarine sediments, from marine to freshwater, as an effect of ocean currents seeding the tidally-influenced reaches of the estuary. To test this hypothesis, the distribution of thermophilic endospores within the River Tyne, a tidal estuary adjoining the North Sea, was investigated. Different sediments were incubated under sulfate-reducing conditions and at high temperature, with the resulting microbial communities analysed using 16S rRNA gene amplicon libraries. The distribution of thermophilic *Firmicutes* and in particular sulfate-reducing *Desulfotomaculum* spp. were tracked along the estuarine gradient, suggesting newly considered dispersal histories involving different warm source environments.

3.3 Methods

3.3.1 Study sites

Sediment was sampled from 20-40 cm depth from five locations within the estuary (Figure 2.1); stations F1, B2, B3, B4 and B5, as well as from one location in the North Sea, station M6. Station F1 is upstream of the tidal limit of the estuary, while the other four estuarine locations are within the tidal range. Sediment descriptions were determined by visual inspection (Table 3.1).

3.3.2 Sediment slurry incubations at elevated temperature

Estuarine sediments were mixed with brackish basal medium prepared according to Widdel and Bak (1992) (section 2.2.1) with the concentration of sulfate adjusted to 20 mM. The medium was amended with tryptic soy broth (TSB) in a final concentration of 3 g L^{-1}, glucose at a final concentration of 3 mM, and the organic acids acetate, propionate, butyrate and lactate also at a final concentration of 3 mM each. Sediment collected from the North Sea was mixed with marine basal medium (Widdel and Bak, 1992) (section 2.2.1) with the same levels of sulfate and organic substrates as indicated above. Microcosms were always prepared in triplicate from all sample locations. Microcosms were pasteurised for 1 hour at 80°C to kill vegetative cells, then incubated at 50°C for 8 days.

3.3.3 Monitoring of time course incubations

Sediment microcosms were subsampled daily by removing 2 ml of homogenised slurry from the microcosms using a N_2 flushed syringe. Aliquots of sediment slurry were centrifuged (13,000 g, 5 minutes, Hettich Mikro 200). The supernatant was used for sulfate analysis (section 2.2.2) and the sediment pellet was stored at -20°C for DNA extraction (section 2.3.1).

3.3.4 Analysis of 16S rRNA amplicon libraries

16S rRNA gene amplicon libraries were sequenced using the Ion Torrent platform and analysed using QIIME (section 2.3.3.3). Sulfate concentrations (Figure 3.1) and DGGE profiles (Appendix A, Figure A.1) were highly reproducible, therefore DNA extracts from triplicate time points were pooled prior to purification on amplicon libraries as described in section 2.3.3.3. Each library was rarefied to the smallest library in the dataset (12,852 reads) and used to determine distribution patterns within the dataset. Rarefaction curves from all stations indicated that this was a sufficient sampling depth (Appendix A; Figure A.2A). OTUs were defined at 97% similarity. In certain instances, if an OTU of interest was absent in the rarefied OTU table, the unrarefied OTU table was checked to confirm the absence of that OTU from the larger full amplicon library (mean, median and maximum library sizes were 25,729, 23,647 and 91,369 reads, respectively; Appendix A, Table A1). Representative sequences of *Desulfotomaculum* OTUs detected at ≥0.05% relative abundance in the rarefied libraries were extracted, and closest sequence matches within the Genbank database identified with the Basic Local Alignment Search Tool (BLAST) using the blast (blastn) suite at the National Center for Biotechnology Information (NCBI) (Altschul *et al.*, 1990). Four of these OTUs (*Desulfotomaculum* spp. G, R, F and AA) were named based on closely related (99% sequence identity) *Desulfotomaculum* phylotypes previously identified in sediment heating experiments from Svalbard (Hubert *et al*., 2010) and Aarhus Bay, Denmark (de Rezende *et al.*, 2013).

3.3.5 MPN quantification of endospores of thermophilic sulfate-reducing bacteria

Endospores of thermophilic sulfate-reducing bacteria in sediments from stations F1, B3 and M6 were enumerated using a three-tube MPN approach.

Brackish medium was prepared as described in section 2.2.1 and amended with the organic acids butyrate, propionate lactate and acetate, to final concentrations of 3 mM each. Medium was then dispensed into Hungate tubes under a constant flow of N_2. A 1:10 dilution (w/v) of sediment and medium was pasteurised for 1 hour at 80°C after which the 10^{-1} dilution of sediment was serially diluted (1 ml into 9 ml medium) up to 10^{-7}. Inoculated Hungate tubes plus nine sterile blanks, containing only medium and substrates, were incubated at 50°C for three months, after which time the concentration of sulfide was determined spectrophotometrically as a colloidal solution of copper sulfide as described by Cord-Ruwisch, (1985) (section 2.2.4). Hungate tubes that showed sulfide production were scored positive for growth.

3.3.6 Total organic carbon (TOC)

Sediment TOC was determined according to ISO 10694, (1995). The TOC of triplicate samples was analysed using a LECO CS230 carbon analyser (LECO Instrument UK, Ltd), and reported as a percentage of total mass.

3.4 Results

3.4.1 Endospores of thermophilic SRB in temperate estuarine sediments

Sulfate reduction in pre-pasteurised microcosms was detected within 24 to 48 hours of incubation at 50°C for sediments from every location, both tidally influenced (Figure 3.1B-F) and upstream of the tidal limit (Figure 3.1A). This revealed the presence of viable thermophilic spore-forming SRB in all reaches of the estuary. Sulfate consumption was rapid at all locations and was completely reduced to 0 mM within 120 h of incubation at 50°C.

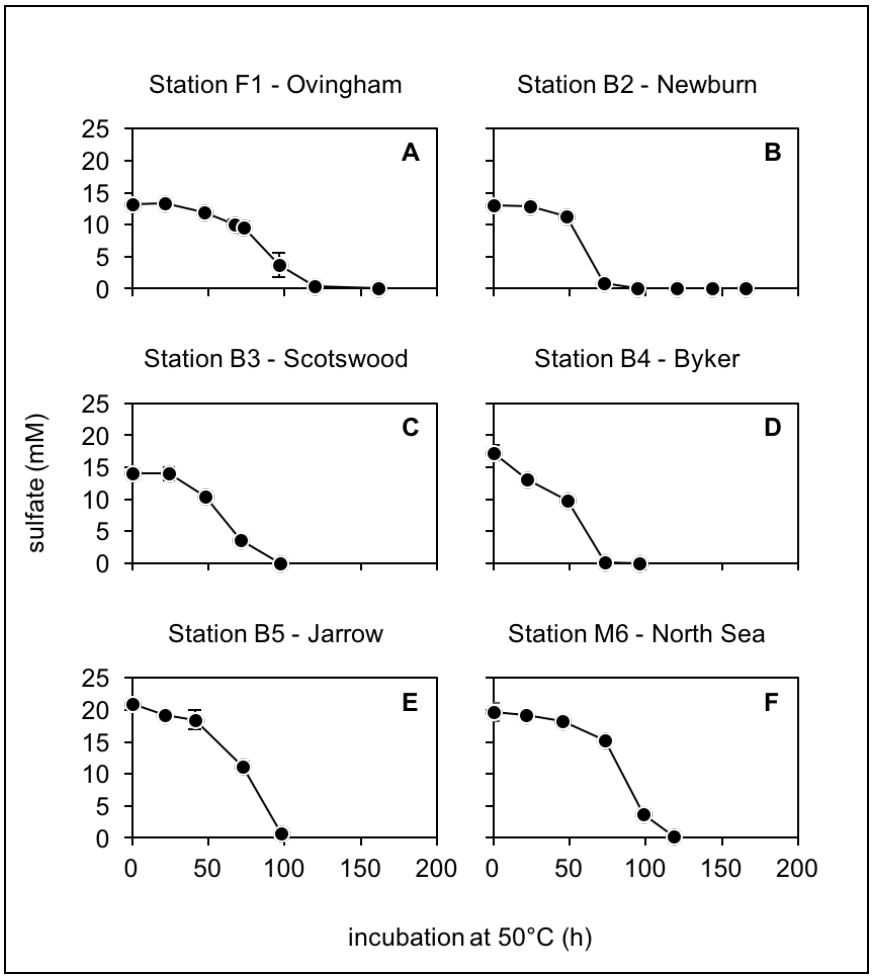

Figure 3.1: Sulfate reduction in pasteurised sediment microcosms (1 hour at 80°C) incubated at 50°C. Sediment from freshwater (station F1: **A**) and brackish (stations B2-B5: **B-E**) reaches of the estuary and one marine site from the adjacent North Sea (station M6: **F**) were incubated. The "F", "B" and "M" notation indicate Freshwater, Brackish and Marine, respectively. Sediment microcosms were prepared in triplicate and amended with organic substrates. Sulfate concentration was measured daily with bars showing standard error among triplicate bottles. In many cases, the error bars are smaller than the size of the sulfate symbols.

MPNs were calculated for thermophilic sulfate-reducing endospores for one freshwater, one brackish and one marine sediment (stations F1, B3 and M6)

incubated at 50°C. Stations B3 and M6 both had an abundance of $4.3 \cdot 10^3$ spores g^{-1} sediment. MPN values from station F1 indicated a low abundance of thermophilic endospores of SRB with just $3.6 \cdot 10^0$ spores g^{-1} detected. Sediment incubations from station F1 had a long lag-phase (Figure 3.1A), which could be the result of the low spore numbers, as indicated by MPN. Station F1 contained sandy sediment and had the lowest measured TOC (0.19%) of all of the sediments (Table 3.1). Sediment texture has been shown to affect endospore abundance in marine sediments, with significantly lower numbers found in sandy sediments than in black mud layers with higher TOC (Fichtel *et al.*, 2008). The study included both autochthonous and allochthonous endospores, but it was suggested that endospores delivered from an external source may be preferentially captured in muddy sediments with small pore space and lower hydraulic conductivity. This may in part explain the lower abundance of endospores being retained in the sandy low-TOC sediments at station F1, as compared to other stations consisting of muddy sediments with higher TOC (Table 3.1).

Table 3.1: Station descriptions. ND denotes not determined.

Station	F1	B2	B3	B4	B5	M6
Location	Ovingham 54°57'56"N 1°52'10"W	Newburn 54°58'47"N 1°44'35"W	Scotswood 54°57'51"N 1°40'60"W	Byker 54°58'22"N 1°35'38"W	Jarrow 54°59'27"N 1°28'35"W	North Sea 55°05'13"N 1°15'09"W
TOC	0.19	7.03	6.42	6.42	2.42	4.27
Sediment Description	Sandy	Black mud	Black mud	Black mud	Sandy mud	Sandy mud
MPN spores g^{-1}	$3.6 \cdot 10^0$	ND	$4.3 \cdot 10^3$	ND	ND	$4.3 \cdot 10^3$

3.4.2 Microbial community composition in heated sediments under sulfate-reducing conditions

Amplicon libraries targeting the V4-V5 region of the 16S rRNA gene were constructed using DNA extracted from pasteurised sediment microcosm incubations at 50°C. Amplicon libraries for each location correspond to the times where sulfate concentrations are plotted in Figure 3.1A-F, i.e., for non-incubated sediments (0 h) and every subsequent 24 h of incubation at 50°C. Comparison of 0 h and 24 h amplicon libraries show a clear shift in the microbial community

structure. *Proteobacteria* (represented by the classes *Delta-, Gamma-, Alpha-, Beta-* and *Epsilon-* in rank order), *Bacteroidetes* and *Chloroflexi* were the predominant phyla before incubation (43% ± 2.8, 20% ± 3.7 and 8% ± 1.2, respectively). Following pasteurisation and 24 h incubation at 50°C, *Proteobacteria*, *Bacteroidetes* and *Chloroflexi* decreased in relative abundance and were replaced by *Firmicutes* as the most dominant phylum. The relative abundance of *Firmicutes* increased from 3% ± 0.8 at 0 h to 73% ± 8.0 at 24 h, consistent with endospore germination and growth in the high temperature incubations. The majority of enriched *Firmicutes* belong to the order *Clostridiales* (66% ± 7.8), with the most abundant families across all sampling sites being *Clostridiaceae, Peptococcaceae, Peptostreptococcaceae* and *Gracilibacteraceae*, with relative proportions differing at each site (Figure 3.2). Within the estuary (stations F1-B5) the families *Tissierellaceae*, *Ruminococcaceae* and *Symbiobacteriaceae* also increased in relative abundance when incubated at 50°C, but these families were not prevalent in heated marine sediment from the North Sea (all <1.3%).

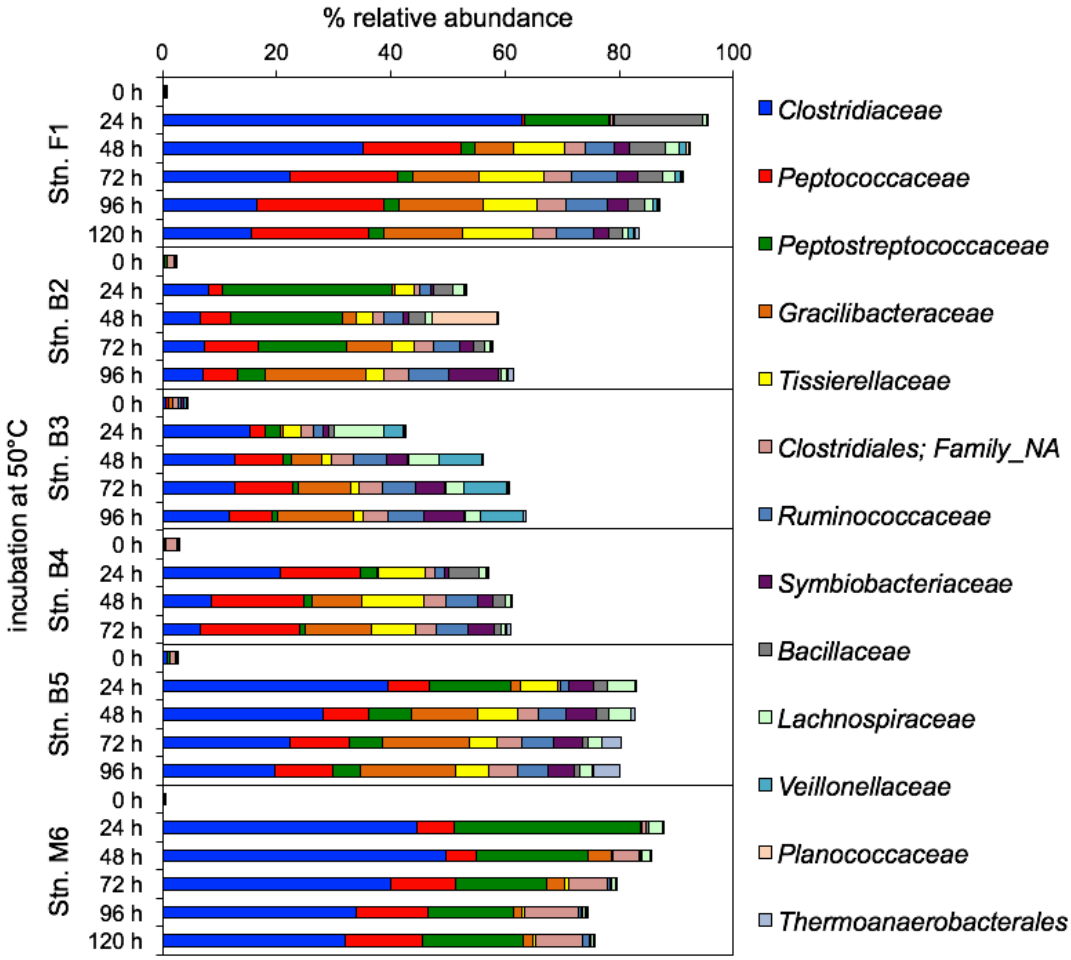

Figure 3.2: Families within the *Firmicutes* detected in 16S rRNA amplicon libraries from stations F1-M6 with a relative abundance of ≥3% in at least one library. Relative abundance was calculated as a percentage of 12,852 reads. Family_'NA' denotes Family_'Not Assignable'

A total of 133 OTUs that increased by ≥0.5% relative abundance upon incubation at 50°C were identified, all belonging to the *Firmicutes* and predominantly to the class *Clostridia* (11 OTUs belonged to the class *Bacilli*). To investigate the potential dispersal vectors for thermophilic endospores within the estuary, OTUs that were detected at ≥0.5% in three or more libraries were selected for additional analysis. Based on their distribution between sites these OTUs were assigned to having either a marine, terrestrial, or cosmopolitan dispersal pattern. This resulted in a total of 80 OTUs with 59 being identified as having a terrestrial distribution, 18 a marine distribution, and 3 a cosmopolitan distribution. Of the 59 terrestrial OTUs, 20 were not detected in marine sediments (station M6). Five were detected at >0.5% relative abundance (belonging to the genera *Lutispora* and *Clostridium*) and the remaining 34 OTUs were detected at <0.5%. Marine OTUs

were detected in amplicon libraries from sites at variable distances into the estuary. They were most abundant at stations M6 and B5, decreasing in relative abundance upstream towards station F1. Eight of the 18 marine OTUs were not detected at station F1, three were detected at >0.05% relative abundance and none were detected at >0.5% relative abundance. OTUs were classified as cosmopolitan if they were (1) detected at every site and (2) were detected at ≥1% in a freshwater, brackish and marine sediment sample. Three OTUs met these criteria (belonging to the genera *Desulfotomaculum* and *Tepidibacter*).

3.4.3 Diversity of thermophilic Desulfotomaculum *spp. within the Tyne estuary and North Sea sediments*

Within the *Firmicutes* the only known sulfate-reducing genus identified in these heated sediment incubations was *Desulfotomaculum*, detected at every location. To investigate the distribution of different *Desulfotomaculum* spp. along the estuarine gradient, *Desulfotomaculum* OTUs with greater than 0.5% abundance in any given library were extracted and grouped based on their distribution. By this approach a total of 11 *Desulfotomaculum* OTUs were retrieved from pasteurised sediment incubations, six of which were designated as terrestrial, three as marine and two as cosmopolitan. Two representative examples from each of these groupings were selected and are shown in Figure 3.3A-F. Representative 16S rRNA sequences of all 11 *Desulfotomaculum* OTUs were compared with closest relatives identified in BLAST searches, which are shown in Figure 3.4 with detail of the stations that all 11 *Desulfotomaculum* OTUs were detected in.

Five *Desulfotomaculum* OTUs were detected in North Sea sediment (station M6), OTUs G, R, F, 5 and 6. By incubating North Sea sediment microcosms with marine basal medium this demonstrated that OTUs detected in marine sediment had a salinity tolerance that would allow growth in a marine environment. All five *Desulfotomaculum* OTUs detected in North Sea sediment incubations were also detected at the mouth of the Tyne estuary (station B5) using brackish medium and therefore are capable of growth at both brackish and marine salinities.

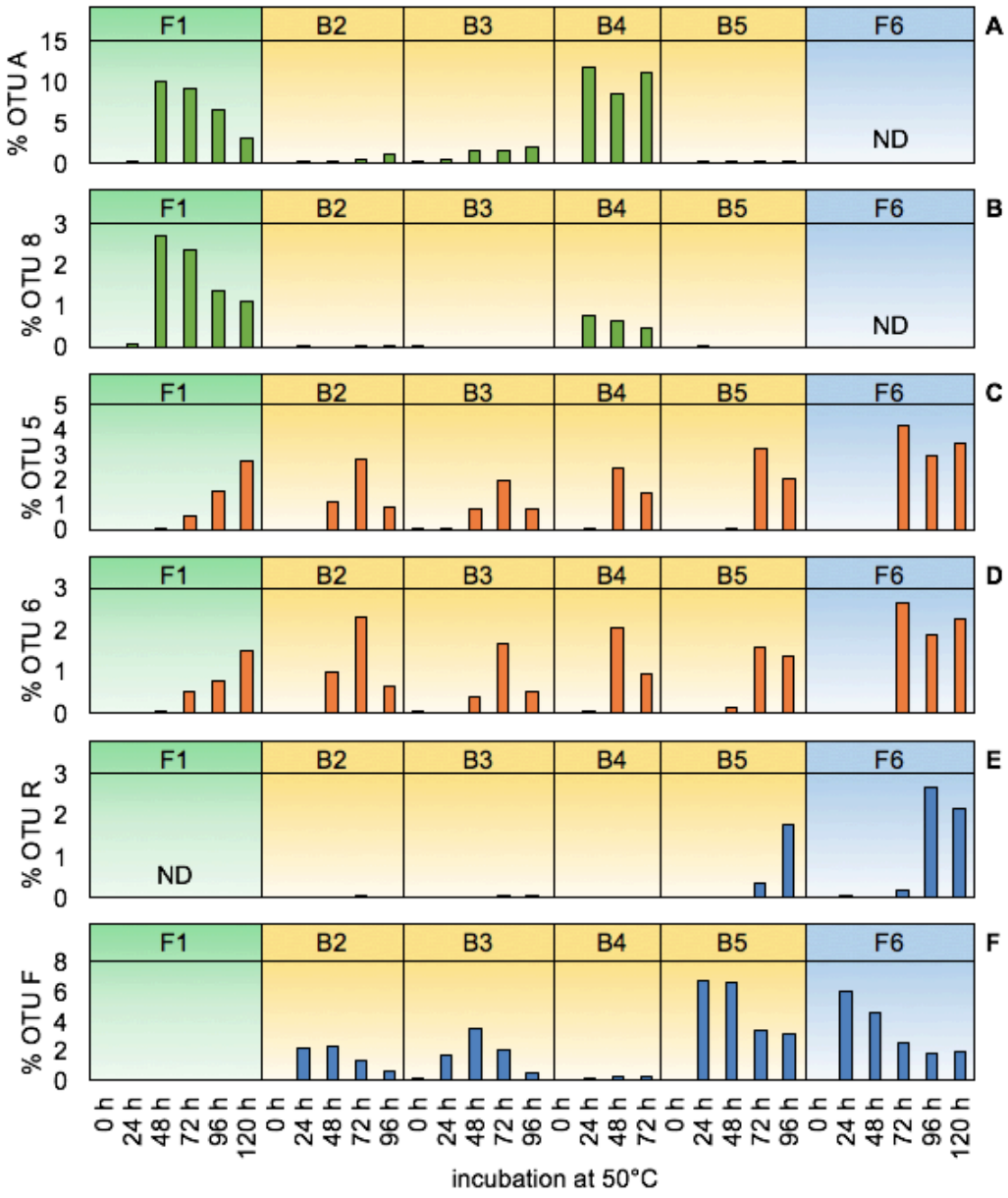

Figure 3.3: The distribution of six *Desulfotomaculum* OTUs along an estuarine gradient from freshwater (station F1, green) to brackish (stations B2-B5, yellow) and marine (station M6, blue). *Desulfotomaculum* OTUs that were detected at greater than 0.5% relative abundance in three or more libraries were designated as; terrestrial (**A-B**), cosmopolitan (**C-D**) or marine (**E-F**) based on their distribution pattern. The two most abundant OTUs from each of these groupings are shown as representative examples. Stations marked ND (not detected) indicate that OTU was absent from unrarefied amplicon libraries. All libraries were rarefied to 12,852 reads, on which relative abundance calculations (percentages) are based.

Desulfotomaculum OTUs R and G were not detected at greater than 0.03% abundance in libraries from sites upstream of Jarrow (station B5) (Figure 3.4) and were not detected at all at Ovingham, upstream of the tidal limit (station F1) as shown for OTU R in Figure 3.3E. The distribution of OTUs R and G therefore

suggests dispersal originating from a marine source. The closest cultured relative of *Desulfotomaculum* OTU R is *Desulfotomaculum geothermicum* strain DSM 3669 (92% identity), isolated from geothermal groundwater (Daumas *et al.*, 1988). *Desulfotomaculum* OTU R also shared close identity with uncultured *Desulfotomaculum* spp. from production water from the Halfdan oil field in the North Sea as well as *Desulfotomaculum* spp. detected in a Western Siberian oil well (Figure 3.4). *Desulfotomaculum* OTU G is most closely related to representatives from the genera *Desulfotomaculum* and *Sporotomaculum* (Figure 3.4), sharing 95% identity with *Desulfotomaculum sapomandens* strain DSM 3223 and *Desulfotomaculum thermosapovorans* strain DSM 6562, and 94% identity with *Sporotomaculum syntrophicum* strain FB and *Sporotomaculum hydroxybenzoicum* strain BT.

Desulfotomaculum OTU F was enriched in sediment microcosms from stations M6 to B2 (Figure 3.3F). In unrarefied libraries OTU F was detected in three libraries from station F1 at 0 h (5/17366 reads) 24 h (1/23209 reads) and 48 h incubation (1/12852 reads). *Desulfotomaculum* OTU F was most closely related to *Desulfotomaculum peckii* strain LINDBHT1 (96% sequence identity) isolated from abbatoir wastewaters (Jabari *et al.*, 2013) and *Desulfotomaculum halophilum* strain SEBR 3139 (92% sequence identity) isolated from an oilfield (Tardy-Jacquenod *et al.*, 1998). Uncultured *Desulfotomaculum* spp. related to OTU F were detected in deep sea hydrothermal environments (JX183068 and FJ792442, Figure 3.4). *Desulfotomaculum* OTUs G, R and F were also closely related to *Desulfotomaculum* phylotypes detected in heated marine sediments from Svalbard (Hubert *et al*., 2010) the Baltic Sea (de Rezende *et al*., 2013) and coastal marine sediment (Ji *et al*., 2012) (Figure 3.4)

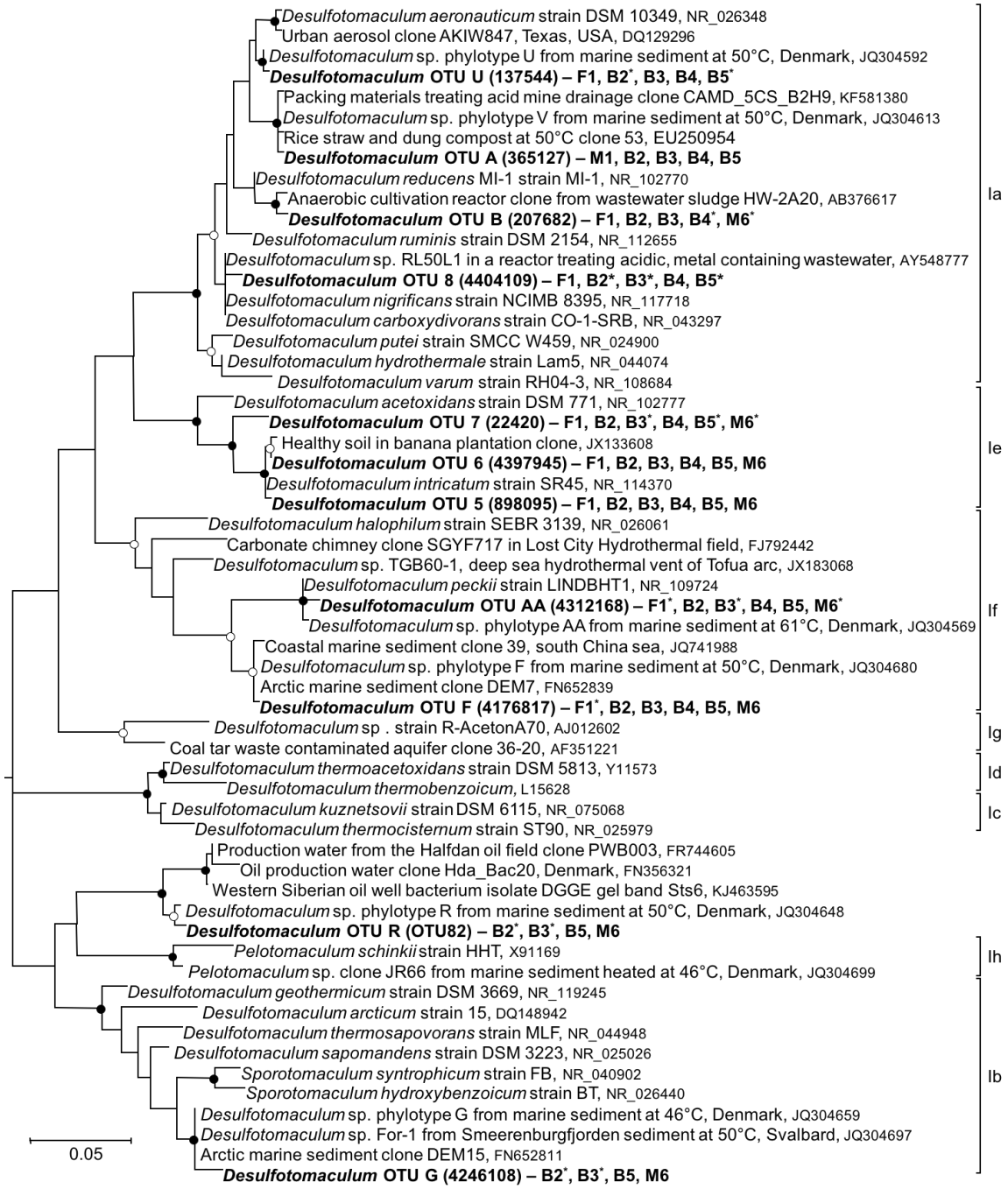

Figure 3.4: 16S rRNA tree of *Desulfotomaculum* spp. with gene sequences derived from heated sediments in this study shown in bold. Only *Desulfotomaculum* OTUs that made up ≥0.5% of any given library are shown. The stations which OTUs were detected at are indicated in the tree (F1, B2-B5 and F6), with asterisks (*) denoting detection of the OTU at ≤ 0.03 % at that location. Closely related sequences were retrieved from Genbank and included in the phylogenetic analysis, performed using MEGA 5.2 by Maximum Likelihood with a total of 372 nucleotide positions. *Desulfotomaculum* subclusters are according to Imachi *et al*., (2006). Filled and open circles at branching nodes indicate bootstrap support values of >90% and 70 to 90% respectively (1000 resamplings). The out-group was *Helicobacteraceae* clone DVBSD (accession# KF463694).

Seven *Desulfotomaculum* OTUs were detected upstream of the tidal limit at Ovingham (station F1). *Desulfotomaculum* OTU A was the most enriched OTU in

amplicon libraries from estuary locations (station F1-B5) and was detected at its highest relative abundance (and greater than any of the other *Desulfotomaculum* OTUs) at stations F1 and B4. *Desulfotomaculum* OTU 8 displayed the same distribution pattern as *Desulfotomaculum* OTU A (Figure 3.3). OTU A was absent from station M6 in unrarefied libraries (21471, 18809, 27313, 25877, 29856 and 23904 reads at 0, 24, 46, 72, 96 and 120 h incubation respectively). OTU 8 was present as a single read from station M6 at 120 h incubation. High abundance of *Desulfotomaculum* OTUs A and 8 upstream of the tidal limit at station F1 implies an input of these OTUs either at or upstream of station F1. Both OTUs were most closely related to *Desulfotomaculum nigrificans* strain NCIMB 8395 detected in soils and compost heaps (Campbell and Postgate, 1965) and *Desulfotomaculum carboxydivorans* strain CO-1-SRB isolated from paper mill effluent (Parshina *et al.*, 2005) (Figure 3.4). Sequences related to these *Desulfotomaculum* OTUs were also detected in culture-independent evaluations of warm terrestrial environments such as compost and metal rich waters treating acid mine drainage (Figure 3.4).

Cosmopolitan *Desulfotomaculum* OTUs 5 and 6 were detected in sediments from every location, including upstream of the tidal limit and in marine sediment (Figure 3.3C and D). Both OTUs were most closely related to *Desulfotomaculum intricatum* strain SR45 (99 and 100% sequence identity for OTUs 5 and 6, respectively), isolated from sediment of a freshwater lake in Japan (Watanabe *et al.*, 2013). The dispersal of microorganisms upstream, past the tidal limit, could occur during storm events with high winds and high river levels resulting in the formation of wind-blown aerosols (Aller *et al*., 2005; Crump *et al*., 2012). However, this dispersal mechanism is likely to be limited in magnitude as compared to the downstream dispersal of microorganisms in riverine flow. It is therefore most likely that the cosmopolitan OTUs are terrestrially-derived and are delivered marine sediments by river discharge into the North Sea.

3.5 Discussion

Temperate estuarine sediments harbour a diversity of thermophilic endospores that are dormant under *in situ* conditions (0-15°C) yet remain viable and capable of germination and growth when incubated at 50°C (Figure 3.1). When incubated at 50°C the prevailing community structure in the sediments rapidly changes to one dominated by thermophilic spore formers including *Desulfotomaculum* spp. capable of sulfate reduction (Figures 3.1 and 3.2). Amplicon libraries also reveal an increased abundance of putative thermophilic fermentative bacteria that are capable of utilising more complex organic substrates, in most cases preceding the enrichment of *Desulfotomaculum* and the detection of sulfate reduction. This suggests that in addition to the small organic acids present in the medium, fermentation products also fuel the sulfate reduction as 50°C incubations proceed, as shown in Figure 3.1, and demonstrated in prior studies (Hubert *et al.*, 2010).

Thermophilic *Firmicutes* enriched in sediment incubations from the River Tyne estuary appear to represent a combination of freshwater and marine populations transported into the estuary possibly in a variety of ways. 80 OTUs of thermophilic *Firmicutes* were detected at ≥0.5% relative abundance in three or more libraries. The majority of thermophilic spore-formers analysed (59 out of 80 OTUs) had a distribution pattern indicative of dispersal from a terrestrial source (e.g. Figure 3.3A and B). Of the terrestrial OTUs, 20 were absent from amplicon libraries from marine sediment (station M6), 34 were detected at low relative abundance and five were detected at >0.5%, suggesting that endospores from terrestrially derived sources contribute to thermophilic endospore populations in marine sediments. In addition, the three cosmopolitan OTUs were detected at >1% relative abundance in freshwater, brackish and marine stations and are likely dispersed from upstream environments by riverine flow. OTUs that were prevalent in marine sediments were also enriched in estuarine sediments, primarily near the mouth of the estuary (station M5) and to a lesser extent at upstream locations, showing that they may be dispersed kilometres into the estuary (Figure 3.3E-F). This distribution is consistent with tidal currents seeding estuarine sediments with marine derived thermophilic endospores.

Industrial discharges into the Tyne estuary are regulated by trade effluent consents to protect the environment (Environment Agency, 2013). Discharged fluids may still contain microorganisms, including endospores, resulting in their delivery to the estuary from an industrial source. Endospores of *Desulfotomaculum* detected in the estuary were related to bacteria that had been detected in paper mill wastewater, compost, domestic wastewater sludge and metal rich mine waters (Figure 3.4). Within the catchment there are a number of potential sources of thermophilic bacterial endospores, including discharge from industries. Hot industrial effluents are cooled before being discharged into the estuary and could carry thermophilic endospores e.g. hot waters from a woodchip factory are cooled to 25°C before discharging near Hexham, upstream of station F1 and paper mill effluent is discharged into the River South Tyne, also upstream of station F1. Sewage treatment works located near station B5 also discharge treated domestic sewage into the River Tyne. Historically, water discharge from coal and metal mines and their spoil heaps were major sources of pollution into the estuary. Water discharges from mine adits and the erosion of spoil heap material still pollute the River Tyne today, resulting in metal rich sediments throughout the estuary (Tyne Rivers Trust, 2013). Mine water treatment schemes are in place within the estuary, such as reed beds that co-treat mine water and sewage effluent (Johnson, 2004; The Coal Authority, 2016), the treated water is then discharged into the River Team, which joins the River Tyne between stations B3 and B4 (Figure 2.1). *Desulfotomaculum* OTUs with the greatest abundance at stations F1 and B4 were closely related to uncultured bacteria detected in packing materials treating acid mine drainage and metal-rich wastewater (Figure 3.4). The closely related *Desulfotomaculum nigrificans* was originally isolated from a freshwater habitat in the Netherlands (Campbell and Postgate, 1965) and has also been isolated from metal mine tailings and mine pit water in India (Natarajan, 2008). *Desulfotomaculum reducens*, closely related to the terrestrially derived *Desulfotomaculum* OTUs U and B, was also originally isolated from sediment contaminated with metals (Tebo and Obraztsova, 1998) and, interestingly, has also been shown to have the unique capability of reducing metals in its sporulated state (Junier *et al.*, 2009).

Desulfotomaculum OTU A was detected in greatest relative abundance in amplicon libraries from upstream estuarine sediments (Figure 3.3) and is closely

related to a bacterium detected in compost (Figure 3.4). A large portion of the Tyne catchment is used for agriculture and as a result the River Tyne receives significant loads of agricultural run-off including soil, fertilisers and manure. Microorganisms in compost degrade and utilise organic compounds, and the heat generated through this metabolism can maintain the temperatures up to 70°C (Maeda *et al*., 2010). The mature product is returned to the land as organic fertiliser, which may thus contain spores of thermophilic bacteria, that are subsequently washed into the river catchment. *Desulfotomaculum* OTU A and 8 both increased in relative abundance at station B4 (Figure 3.3A and B) coinciding with a lower relative abundance of OTU F at this site compared to other estuary locations (Figure 3.3F). Site B4 is at the confluence of the River Tyne and the Ouseburn river (Figure 2.1) and it is possible that this tributary delivers a fresh supply of terrestrially-derived OTU A and OTU 8 endospores to the estuary, such that they out-compete OTU F when sediment from this particular location is incubated at 50°C. In addition to the Ouseburn, two other tributaries, the River Team and River Derwent, both join the River Tyne slightly upstream of station B4 but downstream of station B3 (Figure 2.1) and could similarly transport a supply of terrestrially-derived endospores resulting in the distribution pattern observed for *Desulfotomaculum* OTUs A and 8 (Figure 3.3A and B).

Seabed fluid flow from petroleum reservoirs may be a source of thermophiles into the cold ocean (Hubert *et al.*, 2009). Studies from Aarhus Bay sediments detected thermophiles at depths corresponding to approximately 4500 years of sedimentation, preceding human activity in the North Sea and thus indicating long-term dispersal by natural forces (de Rezende *et al.*, 2013). However, industrial activity in the North Sea may also contribute to the dispersal of thermophilic endospores from the subsurface. Produced water is one of the largest waste products routinely discharged into seawater, at offshore oil and gas platforms (Yeung *et al.*, 2011). This discharge can transport significant numbers of microorganisms from the subsurface into the ocean, with an estimated 3 to 16 kg of cells day^{-1} expelled with production fluids from high temperature (70-110°C) North Sea oil reservoirs (Parkes and Sass, 2009). In recent decades the amount of produced water discharged into the North Sea has significantly increased owing to extension of offshore activities. The maturation of many producing oil fields in this region is resulting in larger volumes of water both being injected for secondary

oil recovery and co-produced during oil extraction (OSPAR Commission, 2000). Produced water discharge could be an anthropogenic large-scale dispersal vector for transporting subsurface bacteria into the ocean, since many North Sea oil fields are hot and anoxic and could harbour thermophiles as a deep biosphere habitat. *Desulfotomaculum* OTU R was detected in greatest relative abundance in North Sea (station M6) sediments and was closely related to 16S rRNA genes cloned from produced water from the Halfdan oil field in the North Sea (Figure 3.4; Gittel *et al.* (2009)). Hot North Sea oil fields are therefore a likely source of thermophilic *Desulfotomaculum* spp. in North Sea marine and tidal sediments, and could be where *Desulfotomaculum* OTU R detected in this study originates.

A study conducted assessing the impact on the bacterial community near a discharge site at a Canadian offshore oil platform in the northwest Atlantic Ocean found that near-platform sediments (250 m) were impacted by the discharge but that more distant sediments and the surrounding seawater had a stable bacterial community (Yeung *et al.*, 2011). In a later study of the same platform, a *Thermoanaerobacter* sp. detected in the produced water was also detected up to 1 km from the oil platform using q-PCR (Yeung *et al.*, 2015). It is not clear whether the methods used in these studies are capable of extracting DNA from spores, and it is possible that beyond 1 km only spores are further dispersed and thus no longer detectable by routine molecular methods. Thermophilic endospores of *Desulfotomaculum* were detected in seawater in Aarhus Bay but only following incubation at a suitable growth temperature (de Rezende *et al.*, 2013). Thermophilic endospores detected in the present study and previous studies (Hubert *et al.*, 2009; de Rezende *et al.*, 2013), are not readily detected in sediments without incubation at high temperature. Sediment heating experiments that promote endospore germination may therefore reveal a greater impact area from produced waters in the marine environment, and could contribute to studies assessing changes in the microbial community as an indicator of pollution. The use of microbial indicators for pollution is already widely applied in water quality assessments, in particular for the detection of pathogens (Tallon *et al.*, 2005). Microbial indicators used for water quality also rely on endospore germination for detection e.g. the spore-former *Clostridium perfringens*, a moderate thermophile, is used as an indicator of faecal pollution and its detection relies on culture-based

methods, that allow for germination and growth before enumeration (Vierheilig *et al*., 2013).

Thermophilic microorganisms may be used as part of petroleum exploration strategies as indicators of hydrocarbon seeps (Hubert and Judd, 2010). Thermophilic spore-forming bacteria represent particularly good targets for these kind of exploration strategies as they are conspicuous in cold sediments, and readily detected when induced to grow in high temperature experiments. The detection of thermophilic endospores associated with industrial activities in this study, presents the possible application of using spore-formers as indicators of pollution from multiple industrial processes that discharge effluent into the surface waters. Endospore longevity in the environment could mean they could be used as indicators of long-term or past activities.

The results presented here show that the distribution of spore-forming thermophiles can be used to reveal insights into the passive transport of microorganisms in estuarine environments. The distribution of thermophilic *Firmicutes* in the River Tyne indicates that while tidal currents do seed estuarine sediments with marine microorganisms a greater number of OTUs detected are apparently associated with warm terrestrial environments. This suggests that thermophilic endospores are delivered to estuarine sediments via terrestrial run-off, groundwater seepage, and effluent from industrial activities. Some endospores were distributed indicative of dispersal from a terrestrial source were also detected in marine sediments indicating that freshwater discharge into the ocean contributes to marine populations of thermophilic endospores. Terrestrially-derived spores delivered to the ocean and subsequently transported in ocean currents could therefore colonise a warm marine environment with suitable growth conditions.

Chapter 4.

Extremely heat-resistant thermophilic endospores in temperate estuarine sediments

4.1 Abstract

Cold and temperate sediments harbour diverse populations of spore-forming thermophilic bacteria that become active at high temperature. Estuarine sediments pasteurised at 80°C for one hour prior to being heated to 50-70°C revealed distinct populations of thermophilic sulfate-reducing *Desulfotomaculum* spp. and putative thermophilic fermentative bacteria, enriched dependent incubation temperature. More extreme pasteurisation through pre-autoclaving the same sediments prior to similar 50-70°C incubations selected for extremely heat-resistant phylotypes that were not otherwise detected in sediment incubations of the same temperature. Sulfate was consumed in microcosms autoclaved for 20 minutes at up to 140°C, and in microcosms autoclaved for 8 hours at 121°C, significantly extending the known limits to survival for bacterial spores exposed to extreme high temperature for a prolonged period. Analysis of 16S rRNA and *dsrAB* gene sequences showed that close relatives to the extremely heat-resistant phylotypes were detected in thermal environments, including oil reservoirs, hot springs and geothermal groundwater. Consistent with this, two partial 16S rRNA gene sequences retrieved from a nearby hot geothermal groundwater from a sedimentary aquifer at 1800 m depth were identical to those enriched in autoclaved sediment slurry microcosms. The detection of identical 16S rRNA gene sequence fragments in geographically proximal surface and subsurface environments indicates that microorganisms dwelling in the terrestrial deep biosphere may be transported to the surface, e.g., in groundwater up-flow that could be encountered in local mining operations or naturally occurring saline springs.

4.2 Introduction

Members of the classes *Clostridia* and *Bacilli* belonging to the phylum *Firmicutes* are able to form endospores, a dormant resting state that is resistant to heat, irradiation, desiccation and nutrient limitation (Nicholson *et al*., 2000). An endospore's heat-resistance is generally ≥40°C higher than the maximum growth temperature (T_{max}) of its corresponding vegetative form (Warth, 1978). In keeping with this, the most heat-resistant endospores known belong to strains of thermophilic bacteria. Extreme heat-resistance, here defined as survival at temperatures above standard pasteurisation protocols of 80-95°C, was first reported for two strains of *Desulfotomaculum* spp. isolated from oil field waters in the North Sea buried down to 4000 mbsf (Rosnes *et al*., 1991a). Both strains survived 20-minute exposure to 131°C. Goorissen (2002) later reported that sterilisation procedures of longer than two hours at 120°C were insufficient to kill spores of *Desulfotomaculum kuznetsovii*, originally isolated from underground thermal mineral waters 3000 m depth (Nazina *et al*., 1989). *D. kuznetsovii* currently holds the record for greatest heat-resistance, surviving 15 minutes at 140°C (Goorissen, 2002; O'Sullivan *et al*., 2015). *Desulfotomaculum geothermicum* B2T also originated from the deep subsurface and was isolated from pore water brines in sandstone at 1060 m depth (Sass and Cypionka, 2004), and was recently shown to survive triple-autoclaving at 121°C (O'Sullivan *et al*., 2015). In addition to *Desulfotomaculum* spp., *Thermoanaerobacter siderophilus* and *Thermovenabulum ferriorganovorum*, anaerobic thermophilic Fe(III) reducing species isolated from a terrestrial hydrothermal vent on the Kamchatka peninsula in Russia, both survived 90-minute exposure at 121°C (Slobodkin *et al*., 1999; Zarvarzina *et al*., 2002). In contrast, endospores of *Desulfotomaculum thermosapovorans* and *Desulfotomaculum acetoxidans* isolated from enrichments on rice hulls and piggery waste, respectively (Fardeau *et al*., 1995; Widdel and Pfenning, 1977) do not survive triple-autoclaving at 121°C (O'Sullivan *et al*., 2015), demonstrating that extreme heat-resistance is not shared by all spore-formers.

Bacteria form endospores in response to environmental stress, enabling them to escape unfavourable conditions both spatially and temporally e.g. during periods of nutrient limitation (Nicholson *et al*., 2000). Bacterial endospores can remain dormant and viable for thousands (Rothfuss *et al*., 1996; de Rezende *et al*.,

2013) and possibly millions of years (Cano and Borucki, 1995) and make up an increasingly significant component of total cell counts with sediment depth (Fichtel *et al*., 2007; 2008; Langerhuus *et al*., 2012; Lomstein *et al*., 2012). Endospore formation may contribute to long-term survival strategies in subsurface sedimentary habitats (Kawai *et al*., 2015). The formation of extremely heat-resistant endospores may offer a survival advantage if spores are exposed to short periods of high temperature in the environment, such as during dispersal in the deep biosphere through hot hydrothermal plumes or within ridge flank crustal fluids (O'Sullivan *et al*., 2015). In some instances, short exposure to high temperature may even serve to activate spores of thermophilic bacteria, with maximal percent germination of thermophilic *Moorella thermoacetica* spores observed in cultures that were first heated to 100°C for up to 90 minutes (Byrer *et al*., 2000).

The survival physiology of thermophilic endospores detected in cold estuarine sediments was investigated. To select for extremely heat-resistant endospores sediments from the Tyne estuary were autoclaved at 121-140°C prior to experimental incubations at 50-80°C. Autoclaving was performed for up to 8 hours to see if extremely heat-resistant species could survive prolonged exposure to extreme high-temperature. Sulfate concentration was monitored in subsequent incubations, with decreases indicating germination and growth of spore-forming SRB. The surviving sulfate-reducing microbial community was analysed using *Desulfotomaculum*-specific DGGE and cloning and sequencing of the *dsrA* gene, encoding one of the major subunits of the dissimilatory sulfite reductase, a key enzyme for sulfate reduction (Wagner *et al*., 1998). To look beyond only SRB, whole microbial community analysis was performed by Ion Torrent sequencing using universal primers targeting the V4-V5 region of the 16S rRNA gene. Moreover, near full-length 16S rRNA sequences from extremely heat-resistant *Desulfotomaculum* spp. were retrieved from 16S rRNA gene amplicons generated using universal primers encompassing the V1-V9 regions of the gene. Extremely heat-resistant endospore-forming bacteria were enriched from cold (2-22°C) estuarine sediments. Their growth temperature (50-70°C) indicates that they are advected into the estuary from an external warm source habitat. The microbial community in geothermal groundwater retrieved from a sedimentary aquifer underlying the Tyne catchment was also examined as a potential warm-source environment.

4.3 Methods

4.3.1 Sulfate-reducing high temperature enrichments

Anoxic slurries of estuarine sediment from station B3 at Scotswood (Figure 2.1) were prepared under sulfate-reducing conditions as described in section 2.2.1. The medium was amended with tryptic soy broth (3 g L^{-1}) as well as glucose, butyrate, propionate, lactate, and acetate (3 mM each). Sediment microcosms were subject to different heat pre-treatments prior to incubation at 50, 60, 70 and 80°C. Heat-treatment was either pasteurisation for 1 h at 80°C, or autoclaving at 121°C, 130°C or 140°C either once or three times in succession. Where multiple autoclave cycles were performed, after the first cycle of autoclaving, the autoclave cooled to ca. 79°C before immediately commencing the next cycle of autoclaving. Microcosms were prepared and incubated in triplicate for all experimental conditions.

4.3.2 Sulfate measurements

Microcosms were sampled at 0 h (i.e. before pasteurisation or autoclaving) and then periodically during incubation at 50, 60, 70 and 80°C. Sediment slurry sub-samples were centrifuged, the supernatant was used to determine the concentration of sulfate in the enrichment culture (section 2.2.2), the sediment pellet was used for DNA extraction (section 2.3.1).

4.3.3 Microbial community analysis of heated estuarine sediments

Aliquots of DNA were used for PCR-DGGE targeting *Desulfotomaculum* spp. (section 2.3.3.1), 16S rRNA and *dsrAB* gene cloning (sections 2.3.3.2 and 2.3.4) and 16S rRNA gene amplicon sequencing using the Ion Torrent platform (section 2.3.3.3). Amplicon libraries from pasteurised incubations and autoclaved incubations were constructed from separate ion torrent sequencing runs. Amplicon libraries from pasteurised sediment incubations were rarefied to 12852 reads and libraries from autoclaved sediment incubations to 6030 reads. Rarefaction curves from both analyses indicated that this was a sufficient sampling depth for the respective datasets (Appendix A, Figures A.2A and B)

4.3.4 Enumeration of thermophilic endospores by MPN assays

Endospores of thermophilic sulfate-reducing bacteria in sediments were enumerated using a three-tube most probable number (MPN) approach, which enables quantification of a specific sub-group of endospores present in the sediment based on cultivation conditions. The presence of solid particles has been reported to stimulate bacterial enrichment (Vester and Ingvorsen, 1998). Sterile sediment was therefore used in the growth medium for MPN enumeration. To prepare the sterile sediment medium, sediment from station B3 was mixed in a 1:3 ratio (w/v) with brackish basal medium (section 2.2.1). The sediment slurry was autoclaved at 137°C for 20 minutes, then degassed and incubated at the temperature which would be used for MPN enumeration of SRB (50°C or 70°C) for 72 hours, then autoclaved a second time at 137°C for 20 minutes and incubated a second time at 50°C or 70°C for 120 hours, then autoclaved a final time at 137°C for 20 minutes. This triple-autoclaving tyndallization approach was adopted to promote germination of any heat-resistant endospores during the 72- and 120-hour incubation periods between autoclave cycles. Vegetative cells from heat-resistant endospores ultimately being targeted by the MPNs are presumed to germinate such that they are killed by the subsequent autoclaving of the sterile sediment medium. $FeCl_2$ was added to the final sterile sediment medium to a concentration of 0.1 mM to assist in positive scoring of growth by the visual formation of a black precipitate of FeS. For a reducing agent in this medium, Na_2S was substituted with $Na_2S_2O_4$ to avoid formation of FeS upon $FeCl_2$ addition (Brandt *et al*., 2001; Yu *et al*., 2010). Butyrate, propionate, lactate and acetate were all added to the medium from sterile stock solutions to a final concentration of 3 mM each. Sterile sediment medium was then dispensed into Hungate tubes under a flow of N_2/CO_2. A 1:10 dilution of sediment from Scotswood at station B3 to sterile sediment medium (v/v) was serially diluted (1 ml into 9 ml medium) up to 10^{-7}. Sterile blanks containing sterile sediment medium only were also prepared, so that 15 sterile blanks were incubated at 50°C, and 19 sterile blanks were incubated at 70°C. In addition to scoring tubes positive following precipitation of FeS, sulfate was measured by ion chromatography (section 2.2.2) to confirm sulfate reduction by the activity of SRB.

4.3.5 Geothermal groundwater

Geothermal water was sampled from a terrestrial subsurface borehole situated in the city centre of Newcastle upon Tyne, and within the Tyne estuary catchment (Figure 2.1 GB; 54°58'23"N, 1°37'34"W). The borehole was drilled to 1,821 m depth targeting a deep sedimentary aquifer (Younger *et al*., 2015). In most of the UK the geothermal gradient is 2.6°C 100 m^{-1} (Adams *et al*., 2010), however the geothermal gradient at this drill site is 3.5°C 100 m^{-1} resulting in groundwater temperatures above 70°C at the bottom of the borehole (BritGeothermal, 2016). The source of heat originates from Weardale granite, west-southwest of Newcastle upon Tyne, where radiogenic isotope decay leads to heat generation (Manning *et al*., 2007; Younger and Manning, 2010). The warmed groundwater migrates eastward along a fault zone, the Ninety Fathom Stublick fault, recharging Carboniferous Fell sandstone beneath the city of Newcastle upon Tyne (BritGeothermal, 2016).

4.3.6 Microbial community analysis of geothermal water

A water sample was retrieved from the geothermal borehole at 1500 m depth, at a water temperature of ca. 65°C. DNA was extracted from the geothermal water by centrifuging 2 mL aliquots for 5 minutes (10,000 g, Hettich Mikro 200). The supernatant was discarded and aliquot centrifugation repeated until a pellet was visible in the centrifuge tube. DNA was extracted from the pellet following the same procedure as for sediment extractions (section 2.3.1). For DGGE analysis, 16S rRNA genes were amplified directly using primer pair 341f-gc / 907r. Touchdown PCR conditions were initial denaturation (95°C, 5 min), annealing (65°C, 1 min) and extension (72°C, 3 min) followed by 19 touchdown cycles; denaturation (94°C 1 min), annealing temperature was decreased by 1°C every second cycle (64°C-55°C, 1 min) and extension (72°C, 3 min) and a final extension cycle (72°C, 10 min) followed by 5 cycles denaturation (94°C, 1 min) annealing (55°C, 1 min) and extension (72°C, 3 min) and extension (72°C, 10 min). The PCR product was visualised by DGGE distinctive bands were excised with a sterile scalpel blade and stored in PCR grade water overnight at 5°C before re-amplification and Sanger dideoxy sequencing (section 2.3.3.1).

To assess the viability of SRB in the geothermal water, 100 ml was passed through a Sterivex filter unit (Merck Millipore Ltd., Hertfordshire, UK) with a 0.22 μm

polyethersulfone gamma irradiated membrane. After filtration the filter unit was stored at 5°C. The filter was used to inoculate a microcosm containing sterile sediment medium prepared as described above (section 4.3.2) amended with butyrate, propionate, lactate, and acetate (3 mM each). In addition, a medium-only microcosm was prepared to serve as a blank control. Microcosms were incubated at 70°C and subsamples were taken at regular intervals to monitor the concentration of sulfate by ion chromatography (section 2.2.2).

4.3.7 Isolation of pure cultures

Sediment from the highest positive MPN dilution was transferred into fresh brackish medium (prepared as described section 2.2.1), amended with 10 mM lactate and incubated at 70°C. When sulfide production was detected (section 2.2.4) a subsample was transferred into fresh medium and this was repeated until no sediment particles remained in the culture. Single colonies were isolated using the agar shake technique (Parkes *et al*., 2009). Single colonies that were picked were transferred to fresh brackish medium containing 10 mM lactate. Growth was monitored by the production of H_2S (section 2.2.4) and the purity of cultures checked by microscopy and 16S rRNA PCR-DGGE (described in 4.3.6).

4.4 Results

4.4.1 Extremely heat-resistant endospores of Desulfotomaculum *enriched from cold estuarine sediments*

Anoxic sediment microcosms were either pasteurised (1 h at 80°C) or autoclaved (20 minutes at 121°C, 3x in succession) before incubation at 50, 60, 70 and 80°C. Rapid sulfate removal occurred in microcosms incubated at 50, 60 and 70°C, following either heat pre-treatment (Figures 3.1A, B and C), revealing the presence of extremely heat-resistant SRB in cold estuarine sediments from the River Tyne. No sulfate reduction was detected during incubation at 80°C (Figure 4.1D). Comparing pasteurised and autoclaved sediment incubations at the same temperature showed that the lag phase prior to observing sulfate reduction was greater in autoclaved microcosms incubated at 50 and 60°C (Figure 4.1A and B). Sulfate reduction commenced after a 24 h in both pasteurised and autoclaved sediment incubations at 70°C (Figure 4.1C).

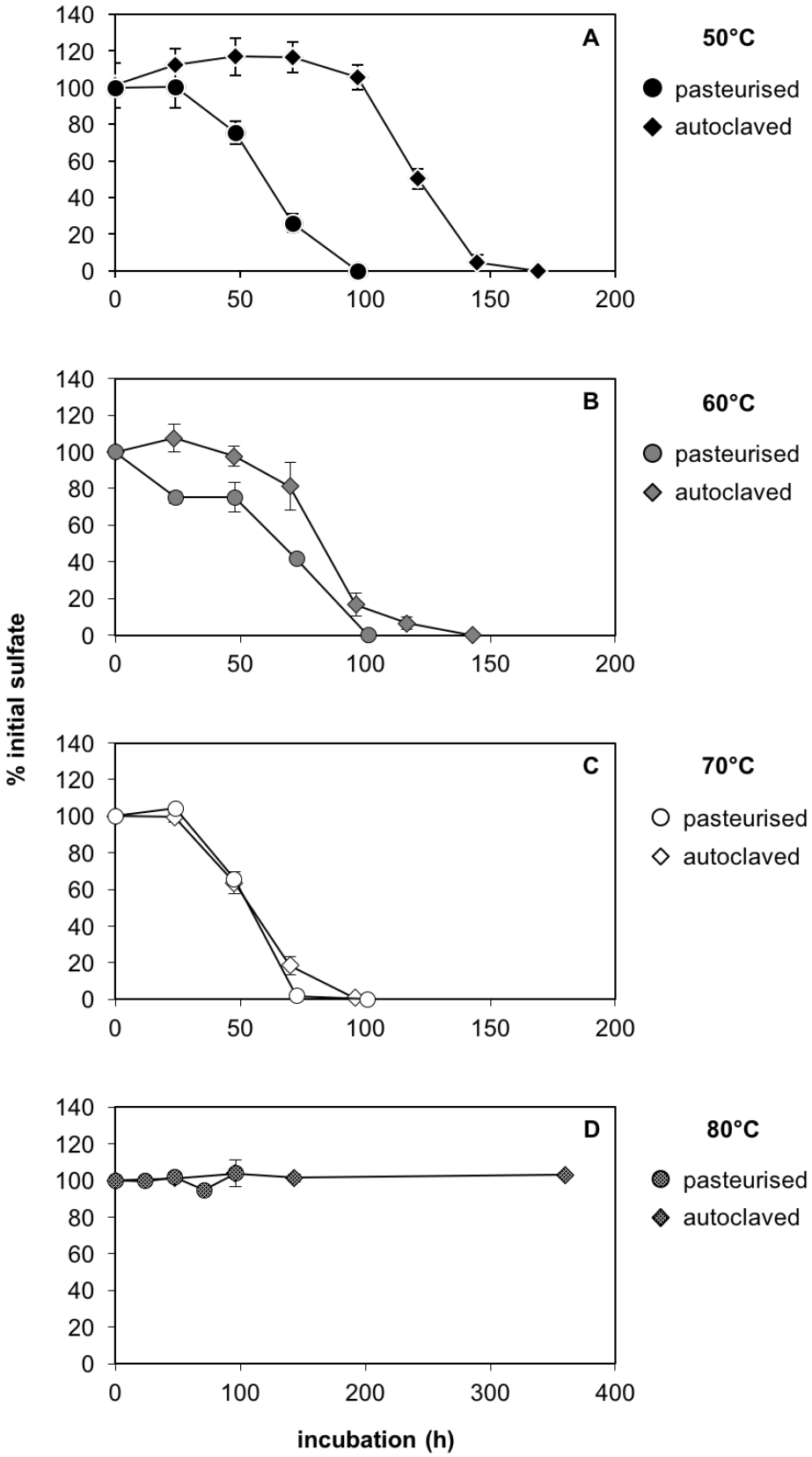

Figure 4.1 Sulfate reduction in microcosms that were either pasteurised (1 h at 80°C; circles) or triple-autoclaved (20 minutes at 121°C, 3x in succession; diamonds), then incubated at 50°C (**A**), 60°C (**B**), 70°C (**C**) and 80°C (**D**). All microcosms were prepared in triplicate and amended with organic substrates. Error bars show standard error, and in some instances are smaller than the sulfate symbols.

Desulfotomaculum spp. were not detected by PCR with *Desulfotomaculum* specific primers immediately before or after the heat-treatment (0 h incubation). Following incubation at 50, 60 or 70°C for 24-96 h *Desulfotomaculum* 16S rRNA genes could be amplified by PCR suggesting an enrichment of *Desulfotomaculum* spp. with incubation. Analysis of *Desulfotomaculum* 16S rRNA gene fragments by DGGE showed a shift in the dominant *Desulfotomaculum* spp. enriched as the temperature increased (Figure 4.2). In addition, different *Desulfotomaculum* spp. were enriched in pasteurised sediments incubated at 50 and 60°C compared to autoclaved sediments incubated at the same temperature (Figure 4.2).

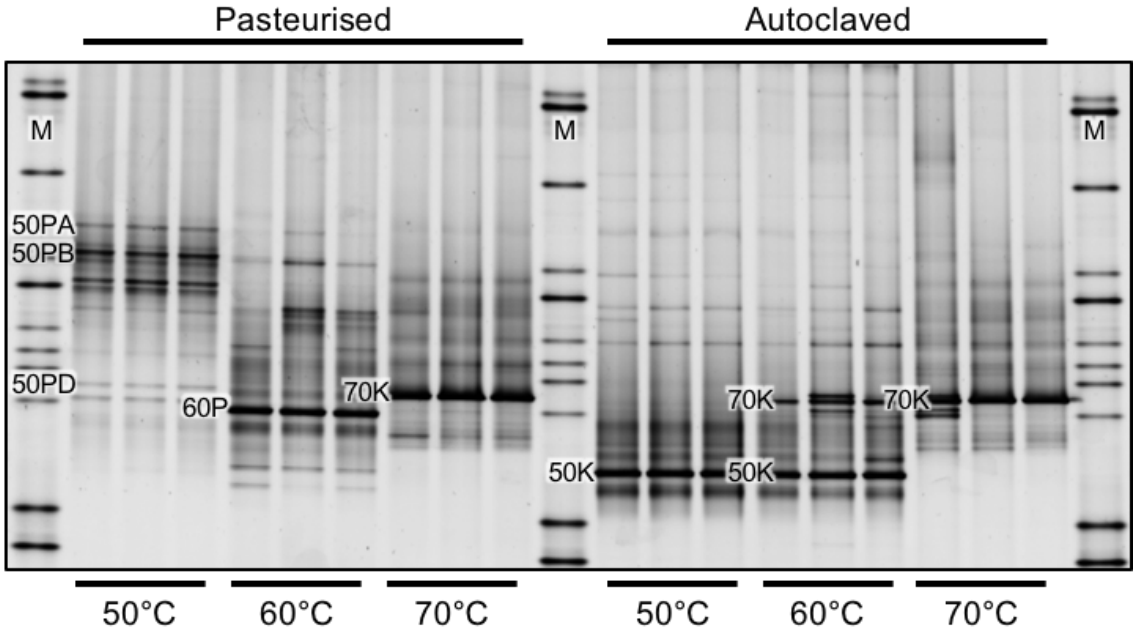

Figure 4.2: DGGE profile of *Desulfotomaculum* spp. enriched in sediment incubations at 50, 60 and 70°C. DNA was extracted after 72 h incubation from pasteurised sediments (1 hour at 80°C) and 96 h incubation from autoclaved sediments (20 minutes at 121°C, 3x in succession). Labelled bands were excised and sequenced. M denotes a marker lane.

The presence of different *Desulfotomaculum* spp. in autoclaved sediment incubations at 50 and 60°C appears to be the result of the autoclave treatment eliminating competition from faster growing *Desulfotomaculum* spp. present in the pasteurised sediments (Figure 4.1A and B). *Desulfotomaculum* sp. 70K was enriched in both pasteurised and autoclaved microcosms incubated at 70°C, autoclaving did not appear to affect its ability to grow, hence it is labelled “70K” on both DGGE gels shown (Figure 4.1C). Selected distinct DGGE bands were excised and sequenced (Table 4.1) where the “P” and “K” notation indicate Pasteurised and Killed respectively (since autoclaved sediment slurry microcosms were initially

intended to be “killed controls”). The extremely heat-resistant *Desulfotomaculum* sp. 50K shared 97% sequence identity with *Desulfotomaculum geothermicum* strain DSM 3669 (NR_119245), isolated from anoxic geothermal groundwater from 2500 m depth with an *in situ* temperature of ca. 58°C (Daumas *et al*., 1988). The second extremely heat-resistant SRB, *Desulfotomaculum* sp. 70K, shared 98% sequence identity with *Desulfotomaculum thermocisternum* DSM 10259 (NR_117746) isolated from a North Sea oil field water sample originating 2.6 km below the seafloor with *in situ* temperatures up to 90°C (Nilsen *et al*., 1996).

dsrAB clone libraries were constructed from microcosms triple-autoclaved at 121°C and incubated either at 50°C or 70°C, to target the two extremely heat-resistant SRB revealed by DGGE, *Desulfotomaculum* sp. 50K and 70K (Figure 4.2). The sequences of 26 cloned *dsrA* amplicons were retrieved (13 from the 50°C incubation and 13 from the 70°C incubation). Phylogenetic analysis showed that all *dsrA* sequences from both incubations were affiliated with the genus *Desulfotomaculum* (FN666333, AF074396, AF273031). In sediments incubated at 50°C, all *dsrA* amplicons (13/13) were most closely related (99% sequence identity, max. sequence length 902 bp) to a *Desulfotomaculum* clone from a geothermal hot spring in Tunisia that reaches temperatures up to 73°C (Sayeh *et al*., 2010). The next closest relative (84% sequence identity) was *Desulfotomaculum geothermicum* (Daumas *et al*., 1988). Two clusters of *dsrA* sequences were retrieved from the 70°C clone libraries (9/13 clones and 4/13 clones), and shared 89% sequence identity with each other across an 882 bp fragment. A sequence identity of <90% over the 1.9 kb fragment of the *dsrAB* gene will likely represent two different species, and correspond to <99% 16S rRNA gene sequence identity (Müller *et al*., 2014). The two shorter (882 bp *dsrA* gene fragments) sequenced from 70°C clone libraries might therefore come from two different *Desulfotomaculum* spp., even though there was only one obvious band resulting from 16S DGGE analysis for this experiment. The larger of the two groups of *dsrA* amplicons (9/13) shared 99% identity with *Desulfotomaculum thermocisternum* (Nilsen *et al*., 1996), the same species that was identified as a close relative to SRB in the 70°C incubations based on comparisons of the 16S rRNA gene (discussed above). The remaining *dsrA* amplicons (4/13) shared 97% identity with *Desulfotomaculum kuznetsovvi* DSM 6115 isolated from thermal mineral waters at 68°C from 2.8-32.5 km depth (Nazina *et al*., 1989). Both *D.*

thermocisternum and *D. thermocisternum* group within the *Desulfotomaculum* subcluster Ic (Stackebrandt *et al*., 1997; Imachi *et al*., 2006).

4.4.2 Effect of increased autoclave temperature on the selection for extremely heat-resistant endospores of Desulfotomaculum

Microcosms were autoclaved for 20 minutes at 130°C and 140°C either once or three times in succession before incubation at 50°C and 70°C. Sulfate reduction occurred in microcosms both single- and triple-autoclaved at 130°C during subsequent incubation at both 50 and 70°C (Figure 4.3A and C). Close relatives to the SRB identified in these experiments by *Desulfotomaculum*-specific DGGE are given in Table 4.1. Sulfate measurements from microcosms incubated at 50°C were less reproducible among replicates following autoclaving at 130°C (Figure 4.3A). Analysis of 16S rRNA amplicons showed that different *Desulfotomaculum* spp. were enriched in these different replicates following the same length of incubation (Figure 4.3B), explaining the variability of sulfate measurements between replicates. *Desulfotomaculum* sp. 50K was only detected in two of the triplicate microcosms subject to a single cycle of autoclaving at 130°C, indicating that autoclaving likely eliminated this organism in one of the triplicate bottles. Following three cycles of autoclaving at 130°C *Desulfotomaculum* sp. 50K was not detected. Instead, triple-autoclaving at 130°C selected for two additional *Desulfotomaculum* spp. (50K16 and 50K19) that were not detected in incubations at the same temperature after lower temperature heat pre-treatments (i.e. pasteurisation at 80°C or autoclaving at 121°C). Phylotype 50K16 was most closely related (98%) to *Desulfotomaculum salinum* strain 435 (NR_115338) isolated from western Siberian oil and gas fields (Nazina *et al*., 2005). Phylotype 50K19 was most closely related to spore-forming *Pelotomaculum propionicicum* strain MGP (NR_041000) (96%) affiliated with subcluster Ih of *Desulfotomaculum* cluster I (Imachi *et al*., 2007). *Pelotomaculum propionicum* strain MGP oxidises propionate in syntrophy with methanogens but does not have the ability to reduce sulfate. *Desulfotomaculum* sp. 70K was enriched in all incubations at 70°C following autoclaving at 121°C and 130°C (Figure 4.3D) and sulfate reduction was highly reproducible among replicates (Figure 4.3C).

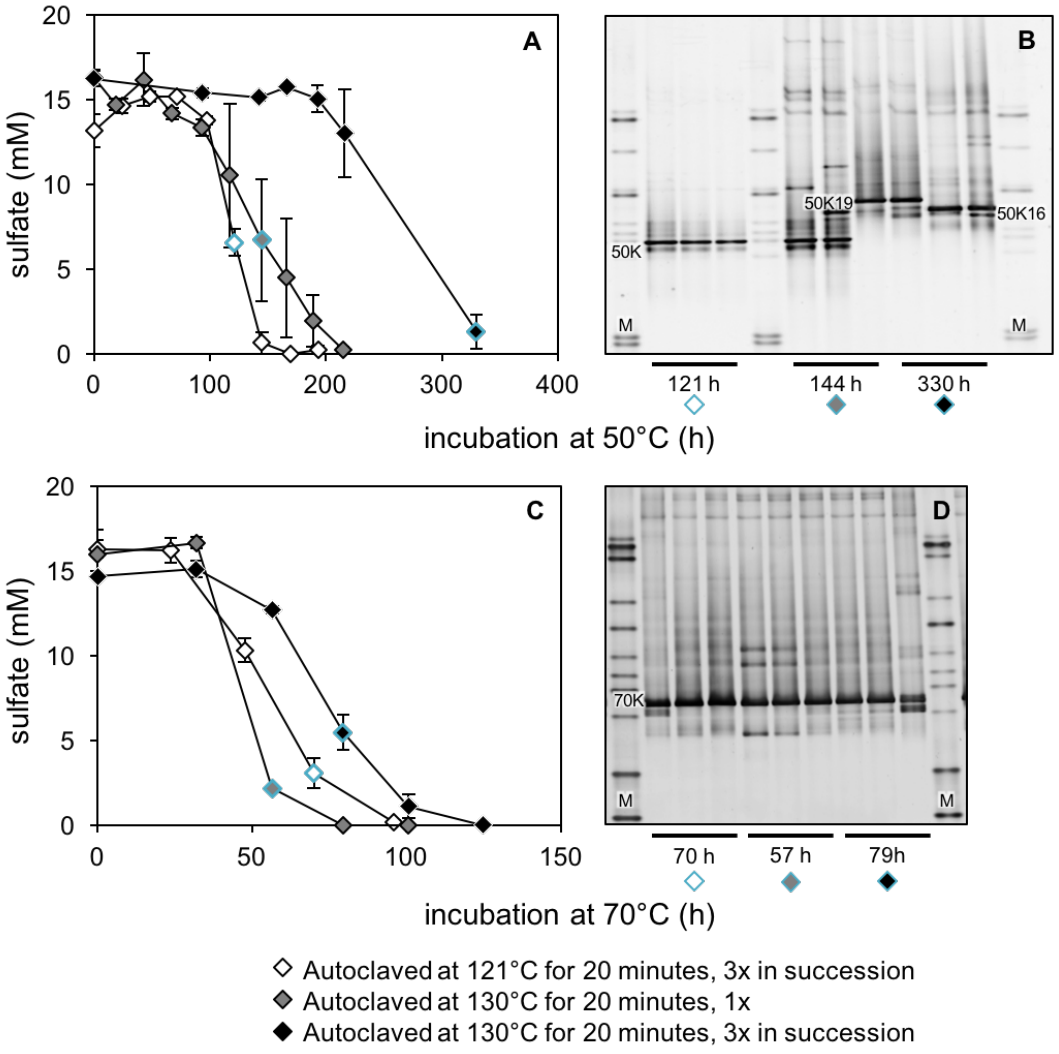

Figure 4.3: Sulfate reduction in microcosms autoclaved at 121°C and 130°C either singularly or 3x in succession. Microcosms were incubated at either 50°C (**A**) or 70°C (**C**). All conditions were carried out in triplicate and error bars show standard error. DGGE targeting *Desulfotomaculum* spp. was carried out on DNA extracts from times points highlighted in blue, corresponding to the phase of active sulfate reduction (**C** and **D**). Marker lanes are marked with a 'M'.

Microcosms subjected to a single autoclave cycle at 140°C were monitored for two-weeks at 50 and 70°C, during which time no sulfate reduction could be observed at either temperature, initially suggesting that spores of *Desulfotomaculum* spp. 50K, 50K19, 50K16 and 70K did not survive exposure to 140°C for 20 minutes. After two-weeks, microcosms continued to incubate but were not monitored regularly. A subsample taken after 674 days' incubation revealed that sulfate had been completely consumed in the microcosms incubated at 50°C. Extraction of DNA at this point revealed a *Desulfotomaculum* sp. that had not previously been detected in autoclaved sediment incubations, and presumably was responsible for sulfate reduction occurring following an initial lag-phase longer

than the two-week observation period. The 16S rRNA gene sequence retrieved shared 99% sequence identity with *Desulfotomaculum luciae* strain DSM 12396 (NR_117590) isolated from a geothermal hot spring (Liu *et al*., 1997). Sulfate had not been reduced in the 70°C microcosms after 156 days' incubation.

4.4.3 Prolonged exposure to extreme high temperature

The survival of *Desulfotomaculum* sp. 50K in response to longer high temperature treatments was tested by autoclaving microcosms for 10, 20, 30, 60, 119, 240 and 480 minutes at 121°C. Sulfate reduction was detected following all autoclave treatments in microcosms subsequently incubated at 50°C (Figure 4.4A). *Desulfotomaculum* sp. 50K was detected in all incubations (Figure 4.4B), demonstrating endospore survival by this organism during prolonged exposure to extreme high temperature. Sulfate reduction commenced rapidly in microcosms autoclaved at 121°C for up to 2 hours, and sulfate was completely consumed within 120 hours of 50°C incubation (Figure 4.4A). When the autoclaving was increased to four hours, sulfate reduction was not detected until after 144 h incubation at 50°C had elapsed, indicating that a reduced number of spores of *Desulfotomaculum* sp. 50K survived the longer autoclave treatment. Sulfate reduction was only detected in two out of three of the replicate microcosms autoclaved for 8 hours and incubated at 50°C for two weeks.

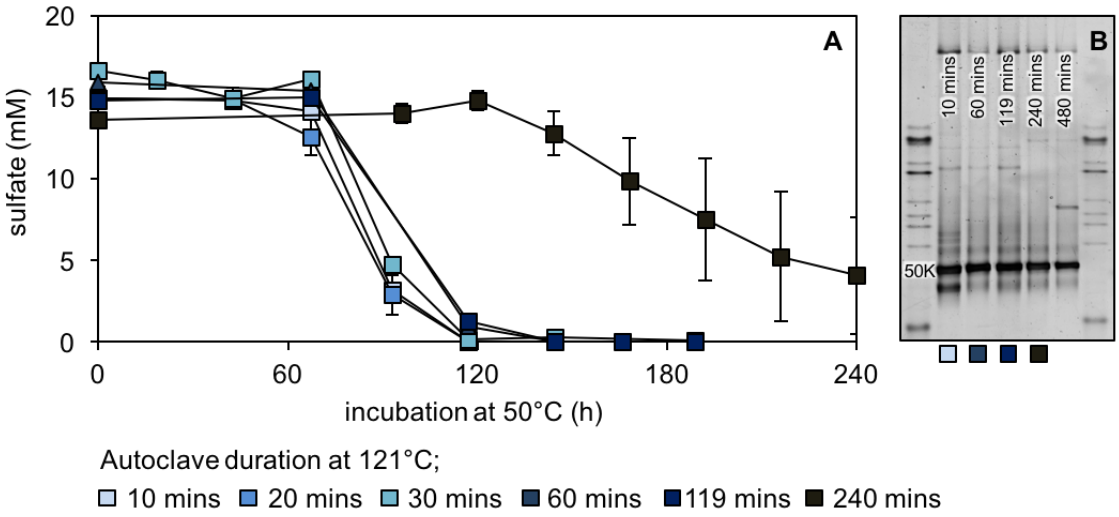

Figure 4.4: Sulfate reduction in microcosms incubated at 50°C following 10, 20, 30, 60, 119 and 240 minutes autoclaving at 121°C (**A**). *Desulfotomaculum* specific DGGE shows the same *Desulfotomaculum* sp. was enriched in all 50°C incubations (**B**), including after 480 minutes of autoclaving (sulfate reduction for this experiment is not shown in A).

4.4.4 Abundance of extremely heat-resistant endospores in estuarine sediment

Thermophilic endospores of sulfate-reducing bacteria present in the sediment were enumerated by a MPN technique. All dilutions incubated at 50°C (up to 10^{-7}) plus 15/15 sterile sediment medium-only blanks were positive for growth. As such the abundance of thermophilic endospores in sediments incubated at 50°C could not be determined. However, this indicated a remarkable survival of extremely heat-resistant SRB that withstood three rounds of autoclaving at 137°C, with 72- and 120-hour incubation periods at 50°C in between autoclave cycles. This tyndallisation with autoclaving approach was chosen to eliminate heat-resistant endospores by allowing them to germinate during incubation periods between autoclave cycles for subsequent killing of vegetative cells during autoclaving. The survival of endospores of sulfate-reducing bacteria despite these measures, indicates that the incubation period may not have been sufficiently long enough to allow for the germination of endospores with lag-phases greater than 120 hours; e.g., *Desulfotomaculum* sp. 50K16 and 50K19 were detected in 50°C incubations following a lag period of 180 h (Figure 4.3).

However, the tyndallization with autoclaving approach was successful for the 70°C MPN test. All of the blank controls (19/19) incubated at 70°C showed no sulfate reduction following 28 days' incubation, indicating that spore germination at

70°C occurs within 120 h (consistent with observations reported above in Figure 4.1C; regardless of initial pasteurisation or autoclaving treatments the lag phase was <48 h). MPN estimates showed the presence of $7.5 \cdot 10^2$ endospores per cm^3 in incubations at 70°C. MPN estimates at 70°C from both upstream and downstream of this site, indicated the presence of $7.3 \cdot 10^0$ endospores per cm^3 in sediments from station F1 and $4.3 \cdot 10^1$ endospores per cm^3. *Desulfotomaculum* sp. 70K was successfully purified following serial transfers from the highest positive dilution (10^{-4}). The isolated *Desulfotomaculum* sp. 70K strain reduced sulfate with lactate between 55 and 70°C, but showed no growth in tests at lower (50°C) or higher (75°C) temperatures.

Table 4.1: *Desulfotomaculum* spp. 16S rRNA sequences detected by DGGE. DGGE fragment lengths are approximately 440 bp.

Sequence ID	Inc. Temp	Treatment	Closest cultured relative (accession)	% identity	Isolation source	Reference
Desulfotomaculum sp. 50PA	50°C	Pasteurised	*Desulfotomaculum defluvii* strain A5LFS102 (NR_132388)	96	Subsurface sample of landfill	Krishnamurthi *et al*., (2013)
Desulfotomaculum sp. 50PB	50°C	Pasteurised	*Desulfotomaculum reducens* MI-1 strain MI-1 (NR_102770)	98	Sediment contaminated with Cr(VI) and other heavy metals from San Francisco Bay estuary, California.	Tebo *et al*., (1998)
Desulfotomaculum sp. 50PD	50°C	Pasteurised	*Desulfotomaculum thermosaporvorans* strain DSM 6562 (NR_119247)	97	Mixed compost containing rice hulls and peanut shells	Fardeau *et a*l., (1995)
Desulfotomaculum sp. 60P	60°C	Pasteurised	*Desulfotomaculum geothermicum* strain DSM 3669 (NR_119245)	96	Geothermal groundwater	Daumas et al., (1988)
Desulfotomaculum sp. 50K	50 and 60°C	Autoclaved 3x at 121°C	*Desulfotomaculum geothermicum* strain DSM 3669 (NR_119245)	95	Geothermal groundwater	Daumas *et al*., (1988)
Desulfotomaculum sp. 70K	60 and 70°C	Pasteurised or Autoclaved 3x at 121°C	*Desulfotomaculum thermocisternum* DSM 10259 (NR_117746)	98	Hot North Sea oil reservoir	Nilsen *et al*., (1996)
Desulfotomaculum sp. 50K16	50°C	Autoclaved 130°C	*Desulfotomaculum salinum* strain 435 (NR_115338)	98	Western Siberian oil and gas fields	Nazina *et al*., (2005)
Desulfotomaculum sp. 50K19	50°C	Autoclaved 130°C	*Pelotomaculum propionicicum* strain MGP (NR_041000)	96	Methanogenic sludge treating artificial wastewater	Imachi *et al*., (2007)
Desulfotomaculum sp. 140	50°C	Autoclaved 130°C	*Desulfotomaculum luciae* strain DSM 12396 (NR_117590)	99	Geothermal hot spring, St. Lucia	Liu *et al*., (1997)

4.4.5 *Multiple genera of thermophilic* Firmicutes *can form extremely heat-resistant endospores*

Analysis of microbial communities in heated estuarine sediments by DGGE and *dsrA* clone libraries revealed diverse *Desulfotomaculum* spp. with varying heat-resistance properties. In addition, microbial community analysis was conducted using universal primers that target both bacteria and archaea, encompassing microbial diversity beyond only SRB. This was approached using two methods of analysis; (1) full length 16S rRNA gene amplicon clone libraries were constructed and (2) 16S rRNA gene amplicon libraries were sequenced using the Ion Torrent platform. The construction of clone libraries allowed longer fragments of the 16S rRNA genes to be sequenced (mean amplicon length 675 bp) enabling near full-length gene sequences for extremely heat-resistant *Desulfotomaculum* spp. (ca. 1500 bp) to be reconstructed. The high-throughput sequencing approach gave shorter 16S rRNA gene fragments (mean amplicon length 304 bp ± 106 from the V4/V5 region) with the advantage of much greater sequencing depth (minimum of 6030 reads per sample) compared to the clone library approach (maximum of 29 sequences retrieved per sample).

16S rRNA gene libraries sequenced by Ion Torrent were constructed from DNA extracts from pasteurised and autoclaved sediments incubated at 50, 60, and 70°C (Figure 4.5A and B). Pasteurised enrichments at all temperatures showed an increase in the relative abundance of *Clostridiales* from 5% at 0 h incubation to 73 ± 3% after 72 h incubation. The majority of OTUs in pre-pasteurised sediments after high temperature incubation were affliated to the genera *Symbiobacterium, Desulfotomaculum, Lutispora, Caloramator* and *Caldicoprobacter* (Figure 4.5A). *Symbiobacterium, Desulfotomaculum* and *Caloramator* were detected at all temperatures, while *Lutispora* was only detected in incubations 50 and 60°C. *Caldicoprobacter*, despite being detected in all incubations, was most abundant at 70°C. Autoclaving sediments prior to incubation resulted in a different community in subsequent 50-70°C incubations (Figure 4.5B). The most abundant OTUs were affiliated to the genera *Desulfotomaculum*, *Thermoanaerobacter*, *Gelria*, *Tepidanaerobacter* and *Moorella*. Interestingly, other than *Desulfotomaculum* spp., genera that were abundant in pasteurised sediment incubations (Figure 4.5A) were not detected after autoclaving (Figure 4.5B). Extreme heat-resistance has not

previously been reported for *Tepidanaerobacter* or *Gelria* spp., which were detected in microcosms autoclaved at 121°C and 130°C prior to incubation at 50, 60 and 70°C.

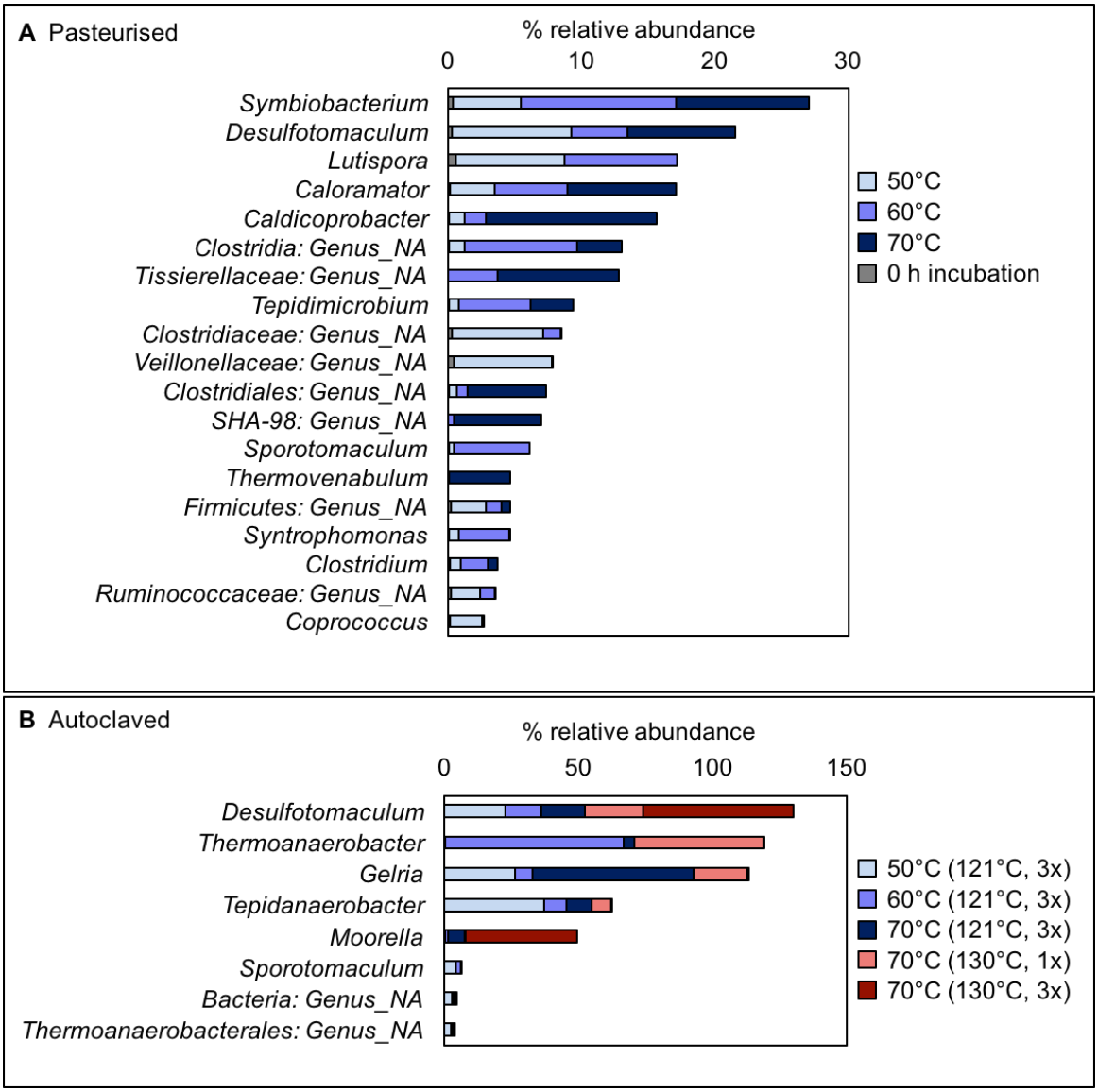

Figure 4.5: Genera detected at ≥2% relative abundance in 16S rRNA amplicon libraries generated by Ion Torrent, from sediments that were either pasteurised (**A**) or autoclaved (**B**) and incubated at 50, 60 and 70°C. Genus_'NA' denotes Genus_'Not Assignable'.

Different OTUs within the genera *Desulfotomaculum, Gelria* and *Tepidanaerobacter* were enriched depending on the incubation temperature (Figure 4.6). *Thermoanaerobacter* spp. was only detected in 60 and 70°C incubations, and *Moorella* only became abundant in 70°C incubations following triple-autoclaving at 130°C (Figure 4.5B). *Moorella* and *Desulfotomaculum* were the only OTUs detected at >1% relative abundance in the 70°C incubation triple-autoclaved at 130°C making them the most heat-resistant spore-formers detected

in these experiments. 16S rRNA gene sequence comparisons showed that heat-resistant OTUs were closely related to bacteria from hot environments including oil reservoirs, geothermal groundwater, hot springs and bioreactors (Figure 4.6).

16S rRNA gene clone libraries were constructed from microcosms incubated at 50°C and 70°C for 96 h following triple-autoclaving at 121°C. All 16S rRNA gene amplicons sequenced from both samples (26 and 29 respectively) were affiliated within the *Firmicutes* (Table 4.2). The majority of cloned 16S rRNA inserts (22/26) sequenced from 50°C incubations were classified as *Tepidanaerobacter* and shared 95% sequence identity with *Tepidanaerobacter syntrophicus* a thermophilic anaerobic syntrophic alcohol- and lactate-degrading bacterium (Sekiguchi *et al*., 1996). Two sequenced clone inserts shared greatest identity (99 and 95%) with *Gelria glutamica* strain TGO a thermophilic anaerobic syntrophic, glutamate-degrading bacterium (Plugge *et al*., 2002). A near full-length sequence was retrieved for *Desulfotomaculum* sp. 50K, which shared greatest sequence identity (95%) with *Desulfotomaculum* sp. MP104 PS13, detected in fracture water from deep crystalline rock (according to its Genbank entry). Its closest cultured relative is *Desulfotomaculum geothermicum* strain DSM 3669, isolated from geothermal groundwater with H_2 (Daumas *et al*., 1988). 16S rRNA gene sequences related to *Gelria*, *Desulfotomaculum*, *Thermosediminibacter* and the candidate order SHA-98 were retrieved from clone libraries of autoclaved sediments incubated at 70°C. *Gelria* amplicons shared 94% sequence identity with *Gelria glutamica* strain TGO (Plugge *et al*., 2002). Amplicons affiliated with the Order *Thermoanaerobacterales* shared 94% sequence identity with *Thermosediminibacter oceani* strain DSM 16646 a thermophilic barophilic anaerobic chemoorganotroph isolated from subsurface marine sediments (Lee *et al*., 2006), and 96% identity with clones from a H_2 producing bioreactor seeded with groundwater (Baito *et al*., 2015). Amplicons classified as candidate order SHA-98 had no closely cultured relatives but shared 99% sequence identity an uncultured bacterium from a methanol fed thermophilic bioreactor that produced methane in co-culture with methanogens (Roest *et al*., 2004). Four near full length 16S rRNA gene sequences affiliated within the *Desulfotomaculum* were retrieved. The four sequences shared between 92 and 97% identity with each other, and shared greatest identity (92-99%) with *Desulfotomaculum salinum* strain 435 isolated from a mixture of formation and condensation water extracted together

with gas at the Igrim gas condensate field in western Siberia (Nazina *et al*., 2005). *Desulfotomaculum thermocisternum, Desulfotomaculum solfataricum*, and *Desulfotomaculum kuznetsovii* are all also closely related. It is possible that the four *Desulfotomaculum* sequences may represent multiple 16S rRNA operons from the same species; the presence of multiple divergent 16S rRNA genes is a common characteristic of *Desulfotomaculum* spp. (Tourova *et* al., 2001). Closely related *Desulfotomaculum kuznetsovii* DSM 6115 and *Desulfotomaculum* sp. C1A60 both contain three copies of the 16S rRNA gene with up to 8.3% sequence divergence (Visser *et al*., 2013; O'Sullivan *et al*., 2015). Most of the sequence variation is observed at both the 5' and the 3' termini of the 16S rRNA genes (Tourova *et al*., 2001; Visser *et al*., 2015), and is thus not always identifiable in shorter sequences from within the 16S rRNA gene, including the V4-V5 region targeted by the Ion Torrent sequencing approach used in this study. Several instances of multiple 16S rRNA gene copies within the genus *Desulfotomaculum* are reported, with *D. intricatum* strain SR45 having the most 16S rRNA operons with at least 13 copies (Watanabe *et al*., 2013); *D. acetoxidans* has 10 copies (Spring *et al*., 2009) and *D. reducens* has eight copies (Junier *et al*., 2010).

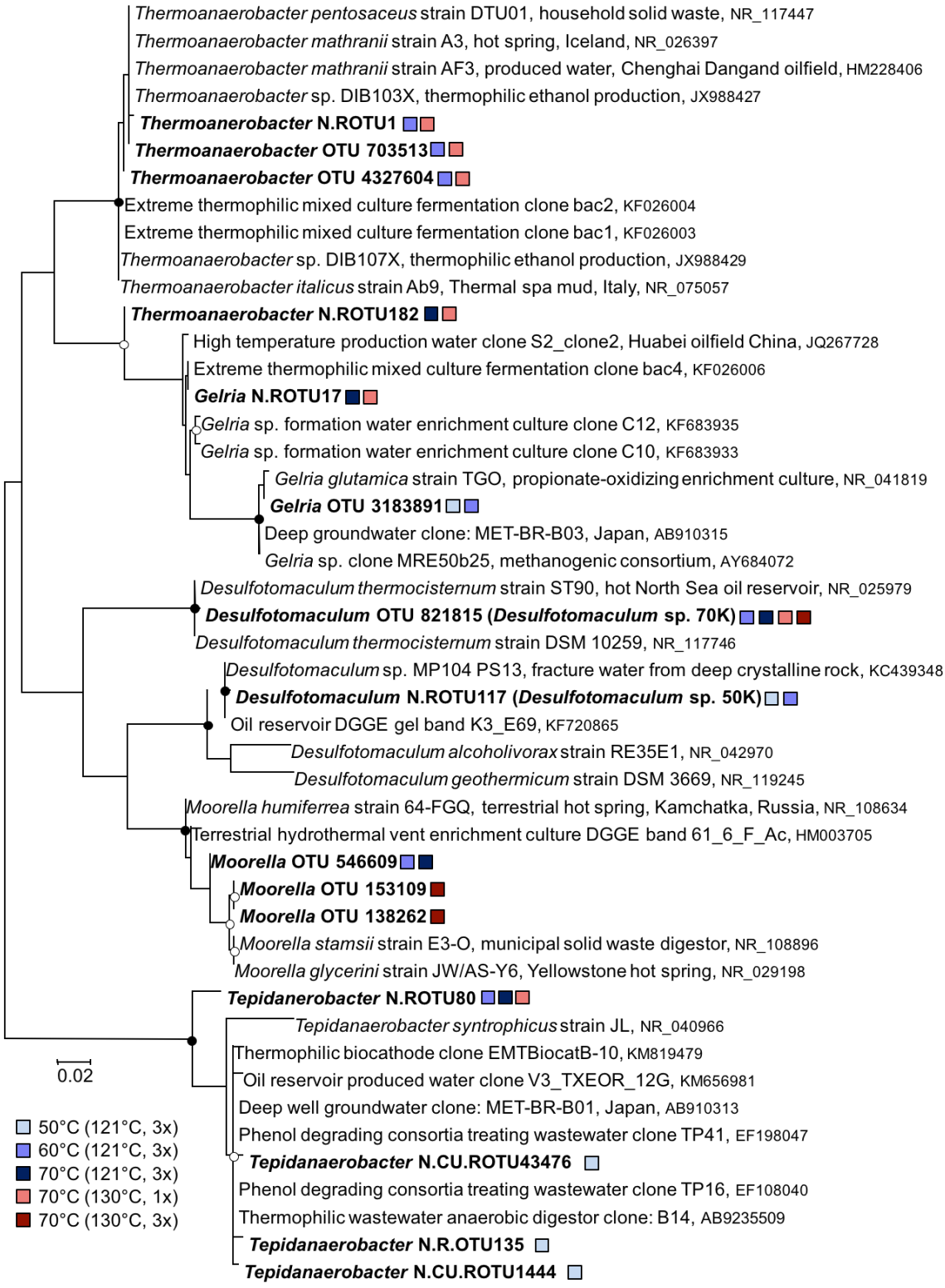

Figure 4.6: Phylogenetic analysis of the V4-V5 region of the 16S rRNA gene retrieved by Ion Torrent sequencing. The analysis was performed in MEGA 5.2. (Tamura *et al*., 2011) by Maximum Likelihood based on the Tamura-Nei model (Tamura and Nei, 1993). The analysis was performed with 345 base positions. Filled and open squares at the branching notes indicate bootstrap support values of >90% and 70 to 90%, respectively (1000 resamplings).

4.4.6 Thermophilic Firmicutes *in geothermal groundwater*

Geothermal water retrieved from 1500 m depth in the terrestrial subsurface was highly saline with 134.80 g L^{-1} chloride, 53.81 mg L^{-1} sodium and 18.03 g L^{-1} calcium. Sulfate was measured at 0.93 g L^{-1} (9.7 mM). Sulfate reduction was detected in a filter incubation of 100 ml geothermal groundwater incubated at 70°C for one month (Figure 4.7), showing that SRB in the geothermal water were viable.

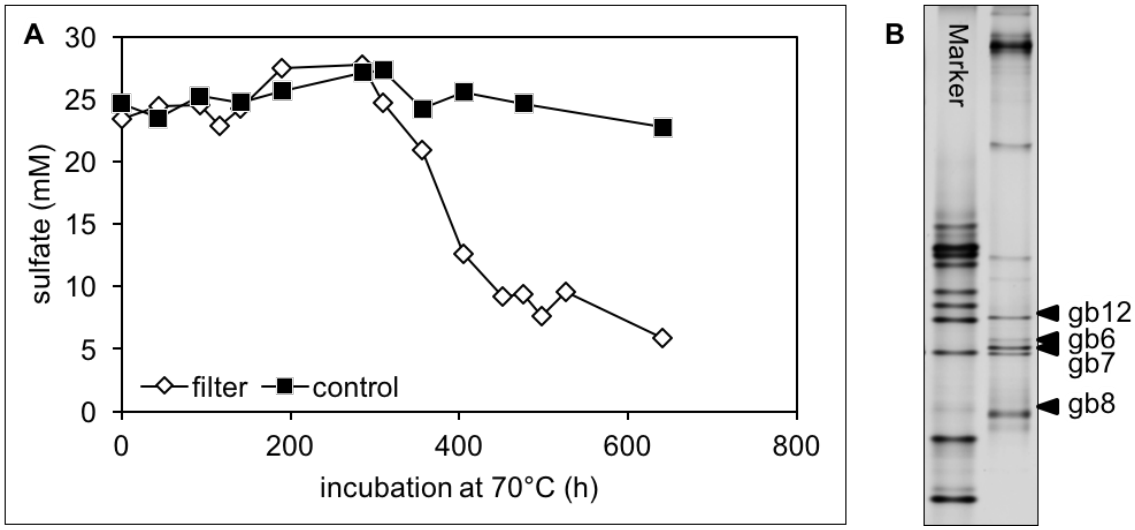

Figure 4.7: Filter incubation of geothermal water at 70°C (**A**), amended with butyrate, propionate, lactate and acetate (3 mM each). Sulfate reduction was detected in the microcosm inoculated with a filter membrane that had 100 ml of geothermal water was passed through (white diamonds). No sulfate reduction was detected in the control microcosm that was not inoculated (black squares). DNA extracted from the geothermal water inoculum was analysed by PCR-DGGE with universal bacterial primers (**B**). Labelled bands were excised and sequenced.

Four 16S rRNA gene sequences were retrieved by PCR-DGGE using universal bacterial primers from the geothermal water used to inoculate the filter incubation (Figure 4.7). All four sequences were most closely related to genera within the *Firmicutes* (Figure 4.8). Two of the four sequences (DGGE bands gb7 and gb8) shared 100% identity with 16S rRNA sequences from amplicon libraries of autoclaved Tyne estuary sediments incubated at 70°C. These were DGGE band gb7, which is identical (across 349 bp) to *Desulfotomaculum* sp. 70K, and DGGE band gb8, which is identical (across 487 bp) to a *Thermoanaerobacterales* cloned sequence (Figure 4.8). The closest cultured relatives (93% identity) to the latter are *Thermosediminibacter litoriperuensis* and *Thermosediminibacter oceani*, both thermophiles isolated from the Peru margin (Lee *et al*., 2006). Greater identity (96%) was shared with clones from a bioreactor seeded with deep groundwater (AB910319 and AB910313, Baito *et al*., 2015). DGGE from the filter incubation

indicated that *Desulfotomaculum* sp. 70K was present in the enrichment culture, based on the migration of DGGE bands. DGGE bands gb12 and gb6 were not similar to any sequences detected in Tyne sediment enrichments. These two groundwater sequences had neither cultured nor uncultured relatives with sequence homology greater than 92% identity (Figure 4.8). The nearest relatives to DGGE band gb12 were sulfate-reducing bacteria detected in subsurface geothermal environments (EU730988 and AB518055) as well as *Candidatus Desulforudis audaxviator* strain MP104C, inferred from its genome to be a sporulating, sulfate-reducing, chemoautotrophic thermophile isolated from 2.8-km depth in a South African gold mine (Chivian *et al*., 2008). The most closely related sequence to DGGE band gb6 (91% identity) was an uncultured organism detected in a hexadecane-degrading consortium cultured at 55°C from samples from a disposal plant of the Shengli oil field, China (Cheng *et al*., 2013).

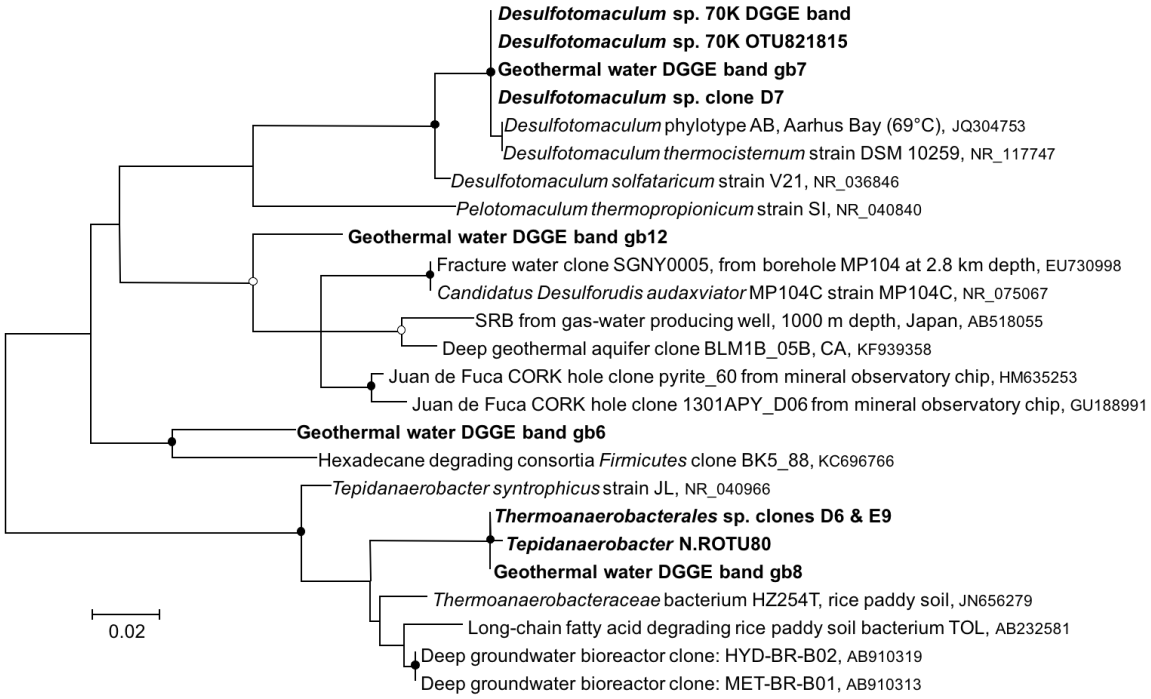

Figure 4.8: Phylogenetic tree of 16S rRNA amplicons constructed using Maximum Likelihood with Tamura Nei methods (Tamura and Nei, 1993). Sequences from this study are in shown in bold; and include all four sequences retrieved from the geothermal water sample (Geothermal water DGGE bands 6,7,8 and 12), in addition to closely related sequences from autoclaved Tyne estuary sediment incubations retrieved from *Desulfotomaculum*-specific DGGE, clone- and ion torrent amplicon libraries. The analysis was performed in MEGA 5.2. (Tamura *et al*., 2011) with 289 bp. Filled and open squares at the branching notes indicate bootstrap support values of >90% and 70 to 90%, respectively (1000 resamplings).

Table 4.2: 16S rRNA sequences detected in sediments incubated at 50 and 70°C following triple-autoclaving at 121°C. 16S rRNA sequences were retrieved from clone libraries using universal bacterial primers. Average fragment lengths were 675 bp, excluding *Desulfotomaculum* spp. for which longer sequences were retrieved.

Sequence ID (number of clones or sequence length)	**Inc. Temp**	**Closest cultured relative (accession), maximum % identity**	**Isolation source**	**Closest relative (accession), maximum % identity**
Tepidanaerobacter (22/26)	50°C	*Tepidanaerobacter syntrophicus* strain JL (NR_040966), 94%	Thermophilic digested sludge	Thermophilic biocathode clone EMTBiocatB-10, (KM819479), 99%
Desulfotomaculum sp. 50K (1503 bp)	50°C	*Desulfotomaculum geothermicum* strain DSM 3669 (NR_119245), 92%	Geothermal groundwater	*Desulfotomaculum* sp. MP104 PS13, fracture water from deep crystalline rock (KC439348), 95%
50K clone C3/H11 (2/26)	50°C	*Thermosediminibacter oceani* strain DSM 16646 (NR_074461), 93%	Subsurface marine sediment	Thermophilic biocathode clone EMTBiocatB-10, (KM819479), 95%
50K clone G3 (1/26)	50°C	*Gelria glutamica* strain TGO (NR_041819), 99%	Propionate-oxidising enrichment culture	Deep groundwater clone: MET-BR-B03, Japan (AB910315), 99%
Gelria (12/29)	70°C	*Gelria glutamica* strain TGO (NR_041819), 94%	Propionate-oxidising enrichment culture	Extreme thermophilic mixed culture fermentation clone bac4 (KF026006), 100%
Candidate order SHA-98 (5/29)	70°C	*Calditerricola satsumensis* JCM 14719 (NR_112611), 88%	High temperature compost	Methanol fed-thermophilic bioreactor clone D2 (AY526501), 99%
Desulfotomaculum sp. clone D7 (1404 bp)	70°C	*Desulfotomaculum solfataricum* strain V21 (NR_036846), 92%	Hot solfataric fields in Krafla region of Iceland	*Desulfotomaculum salinum* strain 781 (AY918123), 92%
Desulfotomaculum sp. clone G5, (1390 bp)	70°C	*Desulfotomaculum salinum* strain 435 (NR_115338), 96%	Western Siberian gas field	*Desulfotomaculum salinum* strain 781 (AY918123), 96%

Sequence ID (number of clones or sequence length)	**Inc. Temp**	**Closest cultured relative (accession), maximum % identity**	**Isolation source**	**Closest relative (accession), maximum % identity**
Desulfotomaculum sp. clone E3, (1394 bp)	70°C	*Desulfotomaculum salinum* strain 435 (NR_115338), 97%	Western Siberian gas field	*Desulfotomaculum solfataricum* strain V21 (NR_036846), 97%
Desulfotomaculum sp. clone C12, (1440 bp)	70°C	*Desulfotomaculum salinum* strain 435, (NR_115338), 99%	Western Siberian gas field	*Desulfotomaculum salinum* strain 781 (AY918123), 99%
Thermoanerobacterales sp. clones (2/29)	70°C	*Thermosediminibacter oceani* strain DSM 16646 (NR_074461), 94%	Subsurface marine sediment	Deep groundwater clone: MET-BR-B01 (AB901303) 96%
70K clone E11 (1/29)	70°C	*Moorella humiferrea* strain 64-FGQ (NR_108634), 93%	Terrestrial hot spring	Extreme thermophilic mixed culture fermentation clone bac4 (KF026006), 97%
70K clone F4 (1/29)	70°C	*Gelria glutamica* strain TGO (NR_041819), 90%	Propionate-oxidising enrichment culture	Anaerobic thermophilic bioreactor clone D2 (AY526501), 94%

4.5 Discussion

The presence of extremely heat-resistant endospores in cold estuarine sediments that are genetically closely related to deep-biosphere dwelling taxa can be explained by dispersal from the subsurface via geofluid transport vectors. Thermophilic endospores in marine sediments have been proposed to originate from petroleum reservoirs and hydrothermally influenced oceanic crust, either associated with sufficient fluid flow to expel endospores out of these subsurface biomes and into the ocean (Hubert *et al*., 2009; 2010). The results presented here suggest that in addition to the aforementioned possibilities, microorganisms from the terrestrial subsurface may be transported via the migration of groundwater to surface environments. *Desulfotomaculum* and *Thermoanaerobacterales* 16S rRNA gene fragments (349 and 487 bp, respectively) detected in geothermal water from a sandstone aquifer at 1500 m depth and 65°C, shared 100% identity with extremely heat-resistant thermophilic bacteria enriched in pre-autoclaved Tyne estuary sediment incubated at 70°C. In northern England, saline groundwater discharges naturally at the surface at saline springs (Younger *et al*., 2015). Many natural saline springs documented in the region (Tyneside and Durham) have dried up as a result of mining. For example, former springs at Birtley and Saltwell, both located on the eastern side of the River Team (a tributary of the River Tyne, Figure 2.1) ceased to flow as a result of mining in the region (Banks *et al*., 2006). Saline groundwater was subsequently encountered in deep mine workings, and tepid saline waters sampled from the Slitt vein within Cambokeels Mine at Eastgate, south of Newcastle upon Tyne, were shown to derive from depth within the Weardale Granite (Manning and Strutt *et al*., 1990), the source of the geothermally heated groundwater in this study. As mining in the region ceased, and many mines are no longer pumped, saline groundwater rises through previously worked strata and commonly decants in disused mines (Adams and Younger *et al*., 2001; Yu *et al*., 2006). Mine waters enter the Tyne catchment along the River South Tyne, Derwent, Team and Ouseburn tributaries (Entec UK Limited, 2011), and in addition to any naturally occurring saline springs, could result in the passive transport of deep subsurface microorganisms to surface estuarine sediments.

The incubation temperature (70°C) of the microcosms from which the identical *Desulfotomaculum* and *Thermoanaerobacterales* sequences were

retrieved is similar to the temperature of the geothermal water from the depth the sample was retrieved (ca. 65°C at 1500 m depth). This further supports that geothermal groundwater could be a source of thermophilic endospores in the Tyne estuary. In addition, both 16S rRNA and *dsrA* genes of *Desulfotomaculum* detected in the pre-autoclaved incubation at 70°C were closely related to isolates from subsurface environments (Nilsen *et al*., 1996; Nazina *et al*., 1989; 2005) and the *Thermoanaerobacterales* 16S rRNA gene sequence shared greatest identity with uncultured bacterial sequences from an H_2 and CH_4 producing bioreactor seeded with groundwater from a well drilled to 1489 m depth in Japan (Baito *et al*., 2015).

MPN estimates indicated the presence of 7.5×10^2 g^{-1} endospores of SRB from estuarine sediments incubated at 70°C. This number is much lower than previous estimates of endospore abundance in marine sediments incubated at the lower temperature of 50°C where up to 10^5 thermophilic spores of SRB have been detected per cm^3 (Hubert *et al*., 2009; de Rezende *et al*., 2013). If endospores are being dispersed to the Tyne estuary via rising groundwater as suggested, this fluid flow mechanism may deliver fewer spores to the surface than marine sources associated with the active efflux of geofluids, either due to the flow dynamics or due to these SRB being present at lower abundance in the source geothermal groundwater. MPN incubations at 70°C from sediments upstream (station F1) indicated that *Desulfotomaculum* sp. 70K was present upstream of the tidal limit (in low abundance) consistent with dispersal from a terrestrial source. The low abundance in sediments from station F1 could be related to the sediment texture, as discussed in section 3.4.1. Alternatively, if saline groundwater is the source of these spores, then the higher abundances detected at station B3 may result from an efflux site (natural or a disused former mine site) near that station. Low numbers of *Desulfotomaculum* sp. 70K were also detected in marine sediments (station M6; $4.3 \cdot 10^1$ cm^3) and could indicate an decreasing abundance gradient with increasing distance from the terrestrial source.

In addition to the two geothermal groundwater phylotypes, multiple extremely heat-resistant thermophilic *Desulfotomaculum*, *Thermoanaerobacter*, *Tepidanaerobacter*, *Gelria* and *Moorella* spp. were detected in pre-autoclaved Tyne sediment enrichments at 50-70°C. Different OTUs were selected depending both on heat pre-treatment and subsequent incubation temperature. Autoclaving

sediments revealed phylotypes that were not otherwise detected in pasteurised sediments of the same temperature (Figure 4.2 and 4.5), and at hotter autoclave temperature additional phylotypes were revealed (Figure 4.3B). Extremely heat-resistant endospores detected in autoclaved enrichments were able to survive temperatures significantly above the reported maximum growth temperature (T_{max}) for closely related isolates. Sulfate reduction was detected in cultures of the isolated strain *Desulfotomaculum* sp. 70K between 55-70°C, but not at 50°C or 75°C. This strain was enriched in microcosms that had been triple-autoclaved at 130°C, showing that its spores can survive temperatures ca. 60°C higher than the maximum growth temperature of its vegetative cells.

Desulfotomaculum 16S rRNA and *dsrA* gene sequences from the pre-autoclaved incubation at 50°C were most closely related to uncultured *Desulfotomaculum* spp. from hot springs and deep fracture fluids (FN666233 and KC439348), and the cultured *Desulfotomaculum geothermicum* (Daumas *et al*., 1988; Sass and Cypionka, 2004). Spores of *Desulfotomaculum* have been shown to survive short exposures (multiple 20 minute cycles) of high temperature (Rosnes *et al*., 1991b; O'Sullivan *et al*., 2015; and this study). *Desulfotomaculum* sp. 50K spores still germinated after 8 hours autoclaving at 121°C, showing that endospores may be able to survive significantly longer periods of extreme high temperature. Microorganisms in thermal vent structures may be exposed to temperatures above their T_{max} (Pagé *et al*., 2008). For endospores transported in crustal fluids and hydrothermal systems, extreme heat-resistance may enable dispersal in hot fluids (>100°C) which might otherwise inactivate vegetative thermophiles or spores that are less heat-resistant. Spore survival in hot zones may only occur if a given mass of crustal fluid only gets heated to extreme high temperatures during short transit periods along a thermal gradient, or in an axial hydrothermal plume.

A recent study with spores of *Thermoanaerobacter siderophilus*, which has a T_{max} of 78°C, suggested that spores could survive entry into the Earth's atmosphere within an artificial meteorite that reached temperatures high enough to melt the surface of the basalt (Slobodkin *et al*., 2015). Extremely heat-resistant endospores that survive autoclaving may therefore be good candidates for further lithopanspermia studies.

Endospore survival at high temperature may also have relevance to the temperatures required for deep burial sterilisation that has been observed in subsurface petroleum reservoirs. Deep burial sterilisation or 'palaeopasteurisation' is thought to occur when oil reservoirs are subject to heating in excess of 80-90°C during sediment burial. This has been inferred from observations that oil biodegradation as a result of anaerobic hydrocarbon-degrading microbial communities is not observed in reservoir formations that have been buried to depths that are warmer than these temperatures, even if those reservoirs are subsequently uplifted to shallower, cooler depths (Wilhelms *et al*., 2001). The temperature for sterilisation may be considerably higher for extremely heat resistant endospores inhabiting the subsurface, though it is unclear whether or not the heat-resistant organisms discovered in River Tyne sediments are capable of anaerobic hydrocarbon degradation. Extremely heat resistant endospores that are not oil-degraders may survive deep burial sterilisation, while having no effect on the oil quality in petroleum reservoirs. However, oil reservoirs that have been subject to deep burial sterilisation have experienced high temperatures for many millions of years (Wilhelms *et al*., 2001). As such, although the survival of endospores may be greater than that of other organisms, this prolonged heat exposure may kill extremely heat-resistant endospores as well.

These results highlight the ability of bacterial endospores to survive temperatures much hotter than their T_{max} for vegetative growth. This may influence fluid transport pathways by which they can be dispersed in the deep hot biosphere, in addition to certain environments that they may be able to persist in that might otherwise be considered too hot based on evaluations based only on T_{opt} and T_{max}.

Chapter 5.

Temperature shifts simulating seawater injection for secondary oil recovery trigger the germination of endospore-forming *Firmicutes*

5.1 Abstract

Sulfate-reducing bacteria (SRB) belonging to the genus *Desulfotomaculum* have been implicated in reservoir souring problems at offshore oil fields. Members of this genus are also endospore-formers, which may confer a survival advantage in the warm deep biosphere. Spores are known to germinate in response to changes in temperature, such as the cooling that happens when cold seawater is injected into hot subsurface reservoirs as part of engineering strategies for oil recovery. To investigate this, endospores of thermophilic SRB were subjected to temperature shifts similar to those encountered near the injection wellbore region in warm oil reservoirs, in two-phase sediment heating experiments. Different sediments known to harbour endospores of thermophilic bacteria were initially incubated at high temperature (90 or 80°C) prior to cooling by between 10 and 40°C to simulate seawater injection. Sulfate reduction was only observed during the second phase of incubation, at the cooler temperatures of 70, 60 and 50°C. Sulfate reduction did not occur when microcosms were maintained at ≥80°C. Sulfate reduction coincided with enrichment in *Desulfotomaculum* spp. that were not detected in amplicon libraries corresponding to the initial 80-90°C incubation. Both prolonged exposure to high temperature for 463 days, as well as incubation in the presence of crude oil led to the enrichment of a specific sulfate reducer closely related to *Desulfotomaculum* sp. previously discovered at North Sea oil production platforms. Putative fermentative endospore-forming bacteria were also detected which may contribute to reservoir souring by generating fermentation products that serve as electron donors for SRB. The survival and enrichment of certain strains under these conditions suggests adaptation to hot conditions such as those in North Sea oil reservoirs and other deep biosphere environments.

5.2 Introduction

H_2S production by sulfate-reducing microorganisms (SRM) can cause problems in oil reservoirs and oil production facilities, such as reservoir souring and microbiologically influenced corrosion (Gittel *et al.*, 2009; Kaster *et al.*, 2009; Hubert and Voordouw, 2007; Gittel, 2011). Consequences of reservoir souring include lowered production efficiency, health and safety issues due to the presence of toxic and flammable H_2S gas and decreased value of the crude oil. Reservoir souring often occurs in response to changes to subsurface ecosystems, e.g., induced by seawater injection during secondary oil recovery. The introduction of cold fluid into a hot formation cools the region near the injection well, resulting in a more favourable environment for SRM activity. This 'Thermal Viability Shell' (TVS) is the zone in the reservoir that permits the activity of SRM that were apparently otherwise inactive at the formation temperature (Eden *et al.*, 1993). Injected seawater is often deaerated and naturally contains high concentrations of sulfate (approximately 28 mM in seawater), hence providing a suitable electron acceptor for SRM. This sulfate, together with organic compounds derived from oil as electron donors, facilitates reservoir souring, as well as SRM activity in flow-lines and top-side infrastructure (Sunde and Torsvik, 2005; Gittel, 2011). Continued water flooding sustains a mixing zone within the formation water in which growth conditions can be met, as the TVS matures and creates a spatially enlarging thermal gradient between injection and production wells (Eden *et al.*, 1993).

Firmicutes are a frequently detected bacterial phylum in high temperature (>50°C) subsurface oil reservoirs (Hubert *et al.*, 2012), and may be an important component of both pristine and water-injected reservoirs (Frank *et al.*, 2015). Thermophilic *Firmicutes* detected in oil reservoirs comprise of bacteria with fermentative, sulfate-reducing and syntrophic metabolisms (Magot, 2005; Shestakova *et al.*, 2011). Four sulfate-reducing *Desulfotomaculum* spp. have been isolated from different oil field environments: *D. kuznetsovii* (Nazina et al., 1989), *D. salinum* (Nazina and Rozanova, 1978), *D. halophilum* (Tardy-Jacquenod *et al.*, 1998) and *D. thermocisternum* (Nilsen *et al.*, 1996). This genus has been widely detected in oil fields both in the North Sea (Rosnes *et al.*, 1991b; Christensen *et al.*, 1992; Leu *et al.*, 1998; Gittel *et al.*, 2009) and other parts of the world (Liu *et al.*, 2008; Lan *et al.*, 2011; Guan *et al.*, 2013; Guan *et al.*, 2014).

The indigenous nature of microorganisms detected in oil field environments is sometimes questioned, and is generally ruled out in cases where the reservoir temperature is significantly higher than corresponding maximal growth or activity temperatures determined for closely related pure cultures in instances where culture-independent reservoir characterisation methods are used (Magot, 2005; Dahle *et al.*, 2008). Seawater is known to harbour thermophilic microorganisms (de Rezende *et al.*, 2013; Müller *et al.*, 2014) and has been proposed as a vector for inoculating exogenous SRM into reservoirs during water flooding for secondary oil recovery (Stetter *et al.*, 1993). Observations of oil reservoirs that are initially sweet but that sour following seawater injection indicate that SRM could be introduced during drilling or seawater injection. On the other hand, SRM originally present *in situ* must have either been deposited with the original sediment and survive over geological time (Hubert *et al*. 2010), or possibly have migrated into the reservoir through subsurface faults and fissures (Parkes and Maxwell, 1993). In the reservoir, if conditions are unsuitable for their activity, certain SRM may be present in a dormant state. Thermophilic endospores can survive exposure to extreme high temperature (Chapter 4), and may be able to persist at temperatures higher than their T_{max} for long periods of time. Dormant endospores remain viable and can germinate and become active if conditions change to become more favourable, for *Desulfotomaculum* spores in oil reservoirs, secondary oil recovery may represent such a change.

Current souring mitigation strategies include the addition of biocides or nitrate to the injection water (Gittel *et al*., 2009). Nitrate injection reduces the production of H_2S by altering the reservoir community to one dominated by nitrate-reducing, sulfide-oxidising bacteria, which reduce sulfide by oxidising it sulfate, or organotrophic nitrate-reducing bacteria which compete with SRB for electron donors (Hubert and Voordouw, 2007). This chapter presents the results of an investigation of the effect of injected seawater temperature on oil-field souring. To test the hypothesis that altered conditions in oil reservoirs during water flooding activate dormant *Desulfotomaculum* endospores, leading to reservoir souring reactions, sediments from three locations known to harbour thermophilic endospores were incubated in two phases. An initial high temperature was chosen mimic ambient conditions in hot reservoir formations, and this was followed by a subsequent lower temperature to simulate cooling encountered as a result of water

injection. Over the production life of an oil reservoir the TVS will grow, resulting in a thermal gradient that stretches from the injection to the production well (Eden *et al*., 1993). This experimental model was therefore used to test a range of temperature scenarios, to simulate different zones within such thermal gradients. The results show that the sediment source chosen, incubation temperature shift regime, and incubation time all affect endospore selection, germination and enrichment. Furthermore, it is shown that crude oil may select for specific *Desulfotomaculum* spp. within this setup. These observations will help to develop better prediction and mitigation strategies for managing oil reservoir souring.

5.3 Materials and Methods

5.3.1 Preparation of microcosms

Seawater medium was prepared as described by Widdel and Bak (1992) with the sulfate concentration adjusted to 20 mM (section 2.2.1). The medium was either (1) unamended, (2) amended with a mixture of complex substrates, or (3) amended with a mixture of simple substrates. The complex substrate mixture contained tryptic soy broth (TSB) in a final concentration of 3 g L^{-1}, glucose at a final concentration of 3 mM, and the organic acids acetate, propionate, butyrate and lactate also at a final concentration of 3 mM each. TSB contained casein peptone (1.7 g/L) dipotassium hydrogen phosphate (0.25 g/L), glucose (0.25 g/L), sodium chloride (0.5 g/L) and soya peptone (0.3 g/L). The simple substrate mixture contained only 1 mM final concentration of the organic acids formate, acetate, propionate, butyrate, lactate and succinate, as well as 1 mM ethanol. Substrates were added to autoclaved media aseptically from sterile stock solutions stored under N_2 headspace. Medium was then dispensed anaerobically into Wheaton glass serum bottles (100 ml, Sigma-Aldrich, UK). Surface sediments from the River Tyne estuary (United Kingdom; 54°57'51"N, 1°40'60"W), Aarhus Bay (Denmark; 56°06'20"N, 10°27'48"E) and Smeerenburgfjorden in the Arctic (Svalbard; 79°56'N, 11°05'E) were used as inocula of thermophilic endospores in these experiments. Sediment was added to serum bottles under a constant flow of N_2 to a final sediment-to-medium ratio of 1:2 (w/v) and bottles were sealed using butyl stoppers and aluminium crimps to maintain the resulting sediment slurries under anoxic conditions.

A series of experiments were designed to simulate temperature shifts possibly encountered in offshore oil reservoirs during seawater injection for secondary oil recovery. Microcosms were first incubated at 90 or 80°C to mimic hot reservoir conditions, then the temperature was lowered to simulate the cooling effect of seawater injection and the development of a TVS. Combinations of different temperatures, incubation times and substrate amendments were tested, as outlined in Table 5.1. All experiments conditions were conducted using triplicate sediment slurries.

Table 5.1: Experimental conditions used in this study.

Sediment inoculum	Phase 1 incubation	Phase 2 incubation	Amendments
River Tyne* Aarhus Bay* Svalbard*	80°C for 138 h	60°C	complex substrates simple substrates no amendment
River Tyne**	80°C for 138 h	60°C	complex substrates complex substrates with 300 mg crude oil
River Tyne	80°C for 279 h	70°C 60°C 50°C	complex substrates no amendment
River Tyne	90°C for 279 h	80°C 70°C 60°C 50°C	complex substrates no amendment
River Tyne	80°C for 463 days	70°C 60°C 50°C	complex substrates no amendment

*Experiments performed by Dr. Angela Sherry.
**Microcosms were prepared by Dr. Ana Suárez-Suarez and analysed by Guillermo Cueto (M.Sc.) under the supervision of Dr. Angela Sherry.

5.3.2 Sulfate and Organic Acid measurements

Sediment microcosms were subsampled at regular intervals by removing 1.5 ml of homogenised slurry using a N_2 flushed syringe. Aliquots of sediment slurry were centrifuged (13,000 g, 5 minutes, Hettich Mikro 200). The supernatant was used for determination of sulfate (section 2.2.2) and organic acid concentrations (section 2.2.3) and the sediment pellet was stored at -20°C for DNA extraction (section 2.3.1). Sulfate reduction rates were calculated by applying a linear regression to the sulfate consumption data for each individual replicate and averaged. Statistical comparisons (Two-Sample t-Test) were calculated using Minitab 17.1.0.

5.3.3 16S rRNA amplicon libraries

16S rRNA amplicon libraries were prepared and sequenced using the Ion Torrent platform as outlined in section 2.3.3.3. Based on highly reproducible sulfate reduction profiles (Figure 5.1), PCR products derived from a common sub-

sampling time from triplicate microcosms were pooled prior to clean-up and then subjected to Ion Torrent sequencing resulting in a single pooled amplicon library. To verify the suitability of this approach, amplicon libraries from triplicate microcosms were also assessed as individual replicates (from respective sediment slurries) in addition to being analysed as a pooled sample. Comparing OTUs (defined at 97% sequence identity) identified in libraries generated from individual triplicate DNA extracts with OTUs in the pooled sample library (where PCR amplicons were combined prior to sequencing) indicated that the pooled library was a good representation of the microbial community at the tested sampling points (Appendix A, Table A2). Amplicon libraries were constructed from separate sequencing runs, libraries from experiments performed by Dr. Angela Sherry (indicated in Table 5.1) were rarefied to 9159 reads (mean, median and maximum library sizes were 23538, 23076 and 38602 reads, respectively). Amplicon libraries from the second sequencing run were rarefied to the same size (mean, median and maximum library sizes were 18382, 18543 and 26083 reads, respectively). Representative OTU sequences were extracted and closest sequence matches identified within the Genbank database (Altschul *et al*., 1990).

5.4 Results

5.4.1 Sulfate reduction in response to simulated reservoir cooling

The germination of sulfate-reducing bacterial endospores in response to changing temperature was investigated in high temperature sediment incubation experiments. Initial experiments used sediments from Svalbard, Aarhus Bay and the River Tyne, all known to harbour thermophilic endospores (Hubert *et al.*, 2009; de Rezende *et al.*, 2013; O'Sullivan *et al.*, 2015; respectively) as sources of endospores. These microcosms were incubated at 80°C for 138 h to mimic a high temperature oil reservoir prior to seawater injection. During the 80°C phase sulfate reduction was not observed (Figure 5.1A-F). Monitoring concentrations of organic acids showed an increase in acetate and propionate at 80°C in River Tyne sediment (Figure 5.1A, C and E) whereas no such changes were observed during the 80°C phase in both Aarhus Bay and Svalbard sediment incubations. After six days (138 h) the temperature was lowered from 80°C to 60°C. Sulfate reduction commenced in microcosms inoculated with River Tyne sediment and Aarhus Bay sediment following the temperature downshift to 60°C under all experimental conditions (Figure 5.1A-F), but not in any of the incubations with sediment from Svalbard (Figure 5.2).

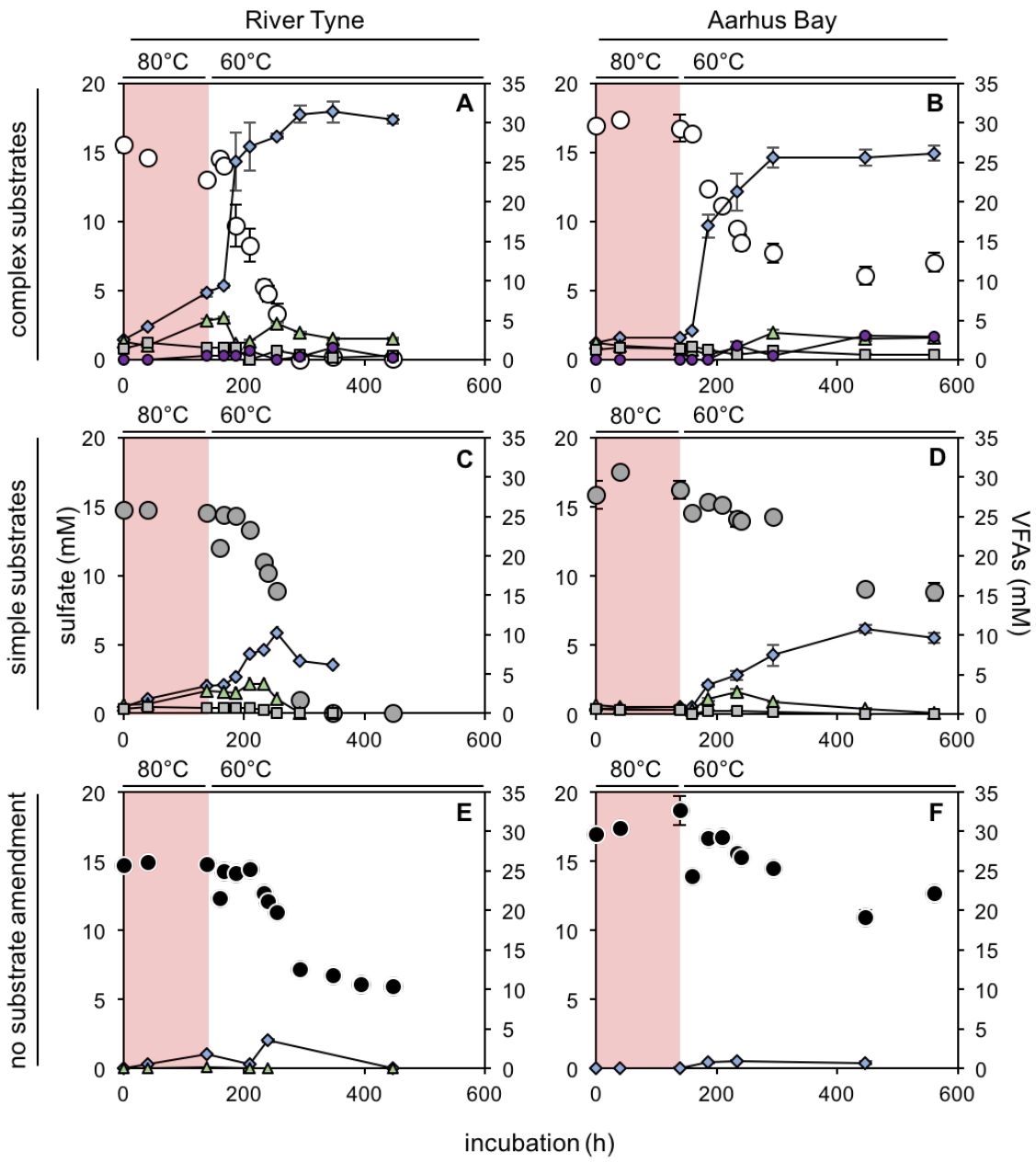

Figure 5.1: River Tyne (left: **A**, **C**, **E**) and Aarhus Bay (right: **B**, **D**, **F**) sediment slurries incubated at 80°C for 138 h (red shaded area) then incubated at 60°C. Sulfate reduction at 60°C in the presence of complex substrates (white circles; **A** & **B**), simple substrates (grey circles; **C** & **D**) or without substrate amendment (black circles; **E** & **F**) is shown. Acetate (blue diamonds), propionate (green triangles) butyrate (grey squares) and isovalerate (purple circles) were monitored over the course of the incubation. Acetate production was observed during the 80°C phase in all River Tyne sediment microcosms (**A**, **C**, **E**), propionate was also produced during the 80°C phase in amended River Tyne microcosms (**A**, **C**). Similar increases in organic acids were not observed in Aarhus Bay sediment microcosms incubated at 80°C (**B**, **D**, **F**). Error bars show standard error determined from triplicate incubations, in many cases error bars are smaller than sulfate and VFA symbols.

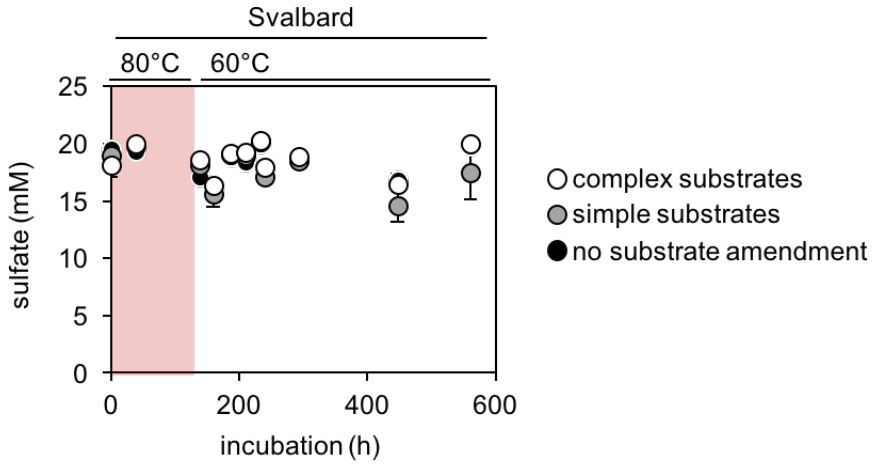

Figure 5.2: Sulfate concentration in Arctic (Svalbard) sediment microcosms incubated at 80°C for 138 h (red shaded area) then incubated at 60°C. Regardless of whether microcosms were amended with complex substrates (white circles), or simple substrates (grey circles) or left unamended (black circles) sulfate reduction was not observed. Concentrations of VFA did not increase during the incubation (data not shown). Triplicate microcosms were incubated for all substrate amendments; error bars show standard error and are visible when the standard error is larger than the size of the symbols.

As sulfate reduction proceeds in River Tyne and Aarhus Bay microcosms, butyrate and propionate are consumed as acetate is produced (Figure 5.1A-D). The greatest production of acetate was observed in microcosms amended with complex substrates, consistent with the presence of fermentable compounds (glucose, casein and tryptone) and higher concentrations of organic acids (3 mM of four different compounds, as compared to 1 mM each of seven organic compounds in the simple substrate mixture). Isovalerate production was only detected in microcosms amended with complex substrates. Small amounts of isobutyrate were detected in Aarhus Bay sediment amended with complex substrates (<300 μM). Sulfate was fully consumed in substrate-amended River Tyne sediment microcosms (both complex and simple; Figure 1A & C), whereas in unamended microcosms only ca. 10 mM sulfate was consumed after which the process apparently became substrate limited. Sulfate was not fully consumed in any of the Aarhus Bay sediment microcosm experiments, with an average sulfate removal of 9.9, 7.0 and 4.3 mM with complex substrates, simple substrates and no external substrate amendment, respectively (Figure 5.1B. D and E).

Amplicon libraries were generated prior to incubation (0 h), at the end of the 80°C exposure (138 h incubation), and at different points throughout the sulfate reduction phase at 60°C. For Svalbard sediment, where no sulfate reduction was

observed, libraries were only constructed at 0 h, 138 h and 447 h (the end of the monitored incubation period). River Tyne and Aarhus Bay sediments both experienced an enrichment of *Firmicutes* during incubation at 80°C (Figure 5.3A; see also Chapter 6). Sulfate reduction following the temperature downshift to 60°C corresponded to the emergence of *Desulfotomaculum* spp. in amplicon libraries corresponding to 166 h and 233 h in the overall incubation (i.e., 28 h and 95 h after the temperature downshift to 60°C) in River Tyne sediment incubations, and 186 h (48 h after downshift to 60°C) in Aarhus Bay sediment libraries (Figure 5.3B). *Desulfotomaculum* spp. were not detected in Svalbard sediment incubation following the temperature downshift, indicating that *Desulfotomaculum* endospores known to be present in the sediment (Hubert *et al*., 2009) did not survive the 138 h incubation period at 80°C.

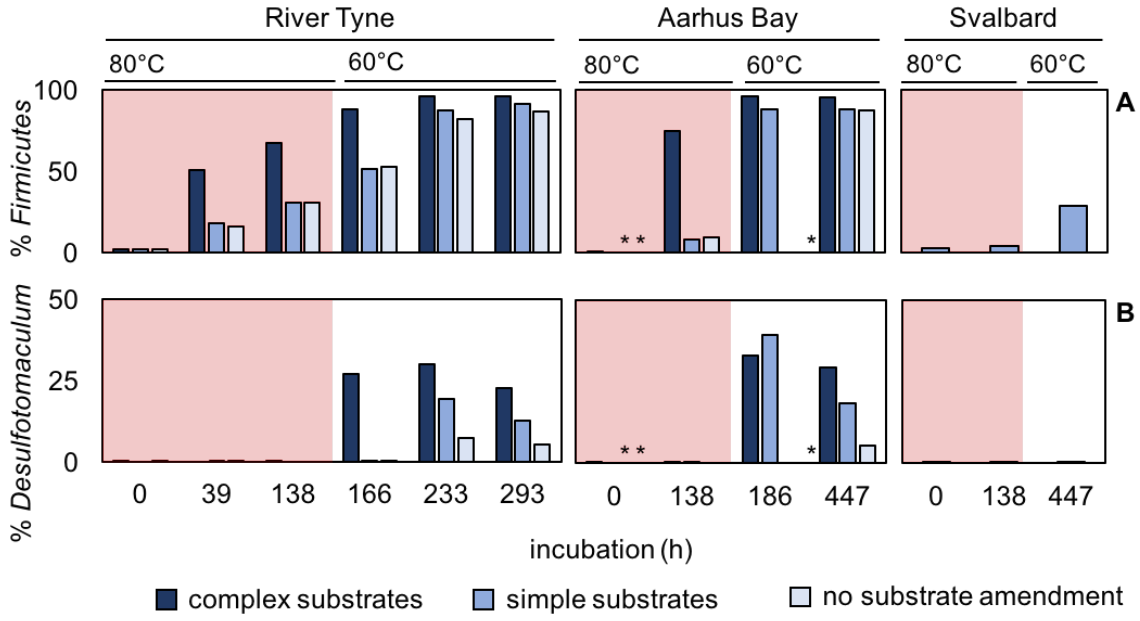

Figure 5.3: Relative abundance of *Firmicutes* (**A**) and *Desulfotomaculum* (**B**) in 16S rRNA amplicon libraries of sediment from the River Tyne, Aarhus Bay and Svalbard in microcosms incubated at 80°C for 138 h (red shared area) and incubated at 60°C. Columns marked with a * denote an absent data point not a zero value.

Tyne sediments were selected for further investigation where the initial hot incubation period was increased to 279 h (11.5 days) and microcosms were incubated at either 90 or 80°C. Microcosms were then cooled to 80, 70, 60 or 50°C, to simulate a wider range of TVS scenarios that are likely to occur between injection and production wells in different oil field settings. For these tests, microcosms were

either amended with complex substrates or not substrate amended (the simple substrates were omitted). Sulfate reduction again commenced when microcosms entered the second phase of incubation and the temperature was reduced to either 70, 60 or 50°C (Figure 5.4A-D). Sulfate reduction did not occur in the second phase of incubation when the temperature was reduced from 90°C to 80°C (Figure 5.4A). In microcosms amended with complex substrates (Figure 5.4A and C), regardless of whether experiments began at 90°C or 80°C, the subsequent sulfate reduction rate following the downshift to 70, 60 or 50°C was not significantly different (Two-Sample T-Test, p = 0.595, 0.205, 0.156 for microcosms incubated at 70, 60 and 50°C respectively). The rate of sulfate reduction decreased in amended microcosms between 358 h and 448 h of the overall incubation as sulfate was depleted (79 h and 169 h after the temperature downshift). In unamended microcosms the rate of sulfate reduction was greater in the first 79 h of the second phase of incubation (Figure 5.4B and D, 279-358 h) in microcosms pre-incubated at 90°C compared microcosms pre-incubated at 80°C, and was significantly higher in microcosms subsequently downshifted to 60 and 50°C (Two-Sample T-Test, p = 0.067, 0.008, 0.002 for microcosms incubated at 70, 60 and 50°C respectively).

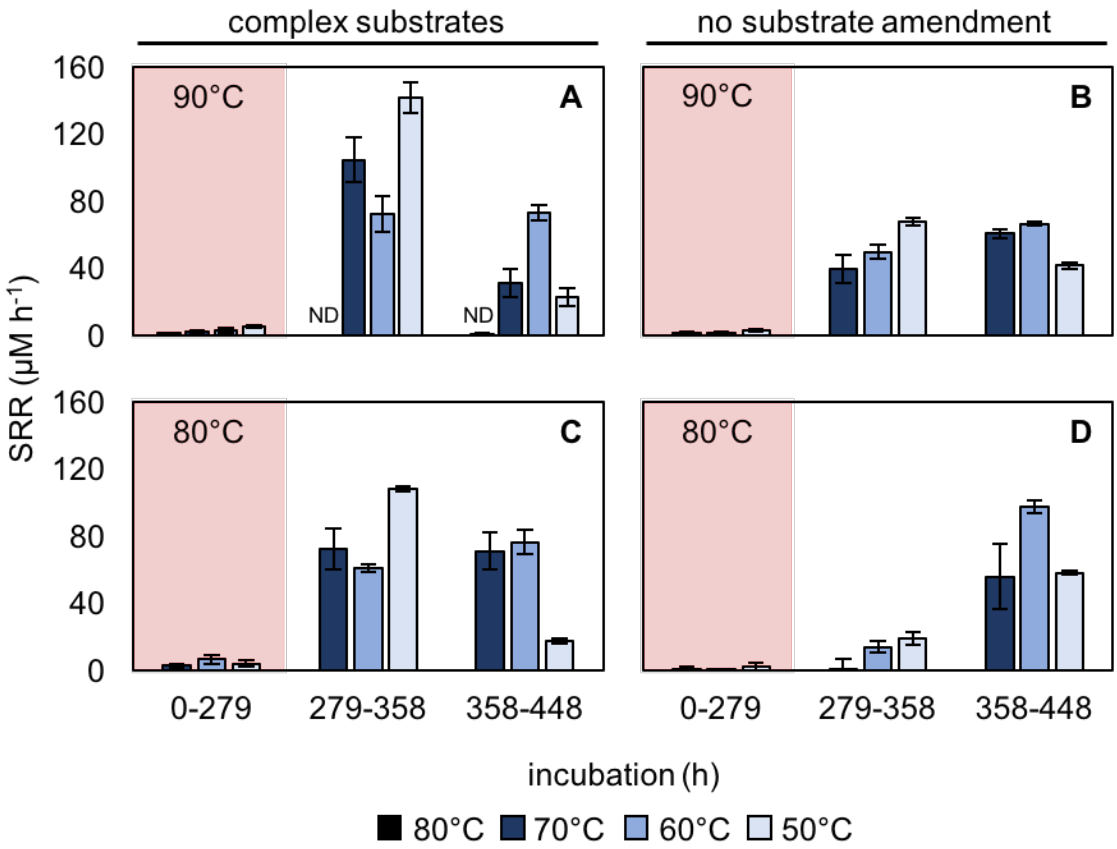

Figure 5.4: Sulfate reduction rates (SRR) in microcosms incubated at 80, 70, 60 and 50°C following incubation at 90°C (**A**-**B**) or 80°C (**C**-**D**) for 279 h. Microcosms were either amended with organic substrates (**A** & **C**) or were unamended (**B** & **D**). Sulfate reduction was detected in all microcosms reduced to 70, 60 and 50°C. Sulfate reduction was not observed following the temperature downshift from 90 to 80°C, ND denotes 'not detected' (A). Error bars show standard error.

Amplicon libraries constructed at 309 h and 358 h (30 h and 79 h into the second-phase of incubation at 70, 60 and 50°C) showed an enrichment of *Desulfotomaculum* following the temperature downshift from initial 90°C or 80°C conditions (Figure 5.5A and B). Comparing *Desulfotomaculum* OTUs enriched following the temperature downshift showed that the same SRB were enriched regardless of whether the first-phase of incubation was at 90 or 80°C, though they were enriched to different relative abundances (Figure 5.5A and B). However, different *Desulfotomaculum* spp. were enriched depending on whether the second temperature was 70, 60 or 50°C, reflecting a scenario where multiple *Desulfotomaculum* could germinate and occupy different zones within TVS gradients.

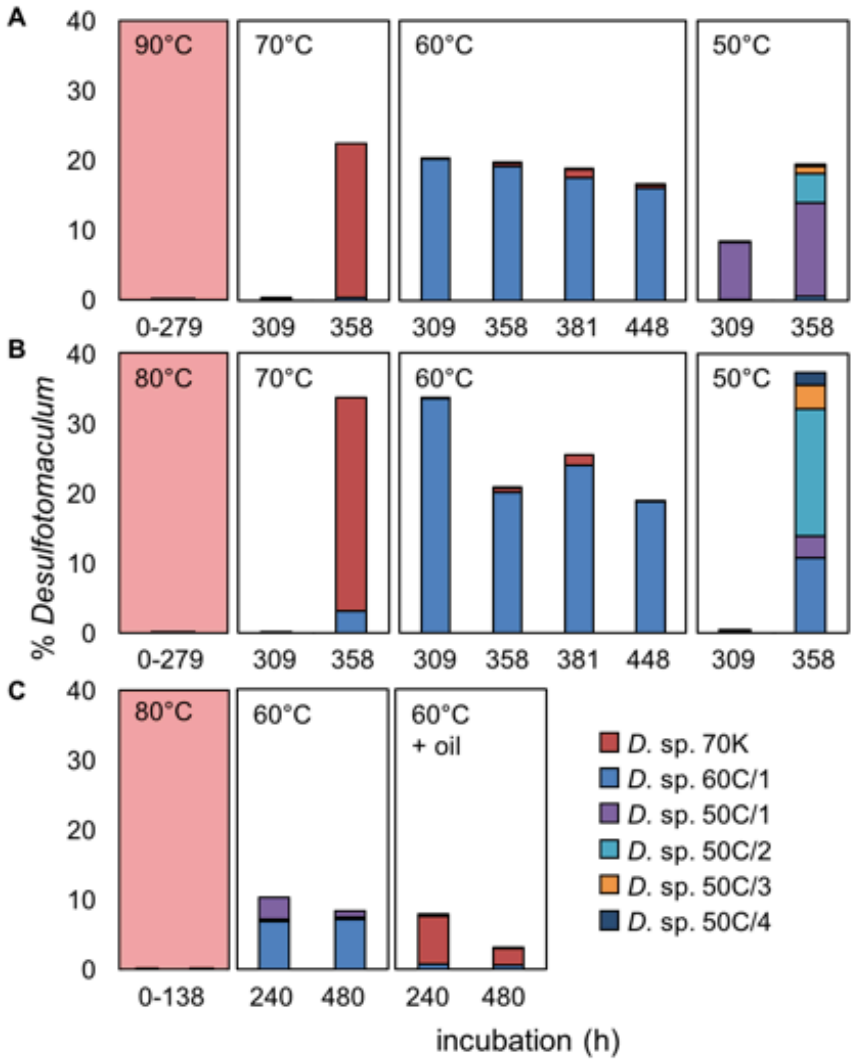

Figure 5.5: Sulfate-reducing *Desulfotomaculum* OTUs detected at >1% relative abundance in 16S rRNA gene amplicon libraries from microcosms incubated at; 90°C for 279 h then downshifted to 70, 60 and 50°C (**A**); microcosms incubated at 80°C for 279 h then downshifted to 70, 60 and 50°C (**B**); and microcosms incubated for 138 h then downshifted to 60°C either with or without oil (**C**). All microcosms were amended with complex substrates.

Crude oil components can potentially be inhibitory to microbial growth. To investigate the effect of oil on thermophilic SRB, an additional set of triplicates amended with 300 mg/L crude oil were prepared. Oil-amended microcosms inoculated with River Tyne sediment were incubated 80°C for 138 h then downshifted to 60°C. Following the temperature downshift to 60°C, *Desulfotomaculum* spp. were detected in amplicon libraries, consistent with the results observed in equivalent no-oil experiments (Figure 5.5C). Comparing *Desulfotomaculum* OTUs showed that different *Desulfotomaculum* spp. were enriched in the presence of oil. *Desulfotomaculum* sp. 70K (detected in autoclaved

sediments; Chapter 4) was the predominant OTU enriched in oil-amended incubations at 60°C (Figure 5.5C), whereas it was not detected in incubations at the same temperature without oil. The *Desulfotomaculum* spp. that were abundant in microcosms without oil (60C/1 and 50C/1) were only detected at a maximum 1.2% and 0.28% relative abundance in oil-amended microcosms, respectively. *Desulfotomaculum* sp. 70K is closely related to isolates previously detected subsurface environments, whereas *Desulfotomaculum* spp. 50C/1 and 60C/1 are not (Table 5.2).

Desulfotomaculum spores indigenous to the deep subsurface will of course be exposed to high temperatures for much longer periods of time than the initial six- to 12-day exposure used for experiments described above (Figures 5.1-5.5). To determine whether *Desulfotomaculum* endospores can survive prolonged exposure to high temperature, microcosms inoculated with River Tyne sediment were incubated at 80°C for 463 days. No sulfate reduction was observed during this period. Following a decrease in temperature to 70, 60 or 50°C microcosms were monitored for a further 70 days. No sulfate reduction was observed in microcosms incubated at 50°C (Figure 5.6C), suggesting that no sulfate-reducing endospores capable of growth at 50°C survived the prolonged 463-day incubation at 80°C. However, at 70°C sulfate reduction commenced rapidly and reproducibly following a five-day lag phase (Figure 5.6A) while at 60°C the lag phase varied between triplicates (7, 10 and 21 days) but sulfate was still eventually completely consumed (Figure 5.6C). Amplicon libraries generated from the 60 and 70°C incubations showed that the same *Desulfotomaculum* OTU was predominant at both temperatures (Figure 5.6D and E). This OTU was not detected at >1% relative abundance in the shorter high temperature exposure experiments (Figures 5.1-5.5). In addition, *Desulfotomaculum* sp. 70K, the OTU that was enriched in the oil-amended incubations (Figure 5.5C), was detected in microcosms incubated at 70°C for 144 h (Figure 5.7D). No other *Desulfotomaculum* OTUs that were detected in microcosms following the shorter 279 h incubation at 80 and 90°C (Figure 5.5A and B) were detected following the extended 463-day heating period at 80°C.

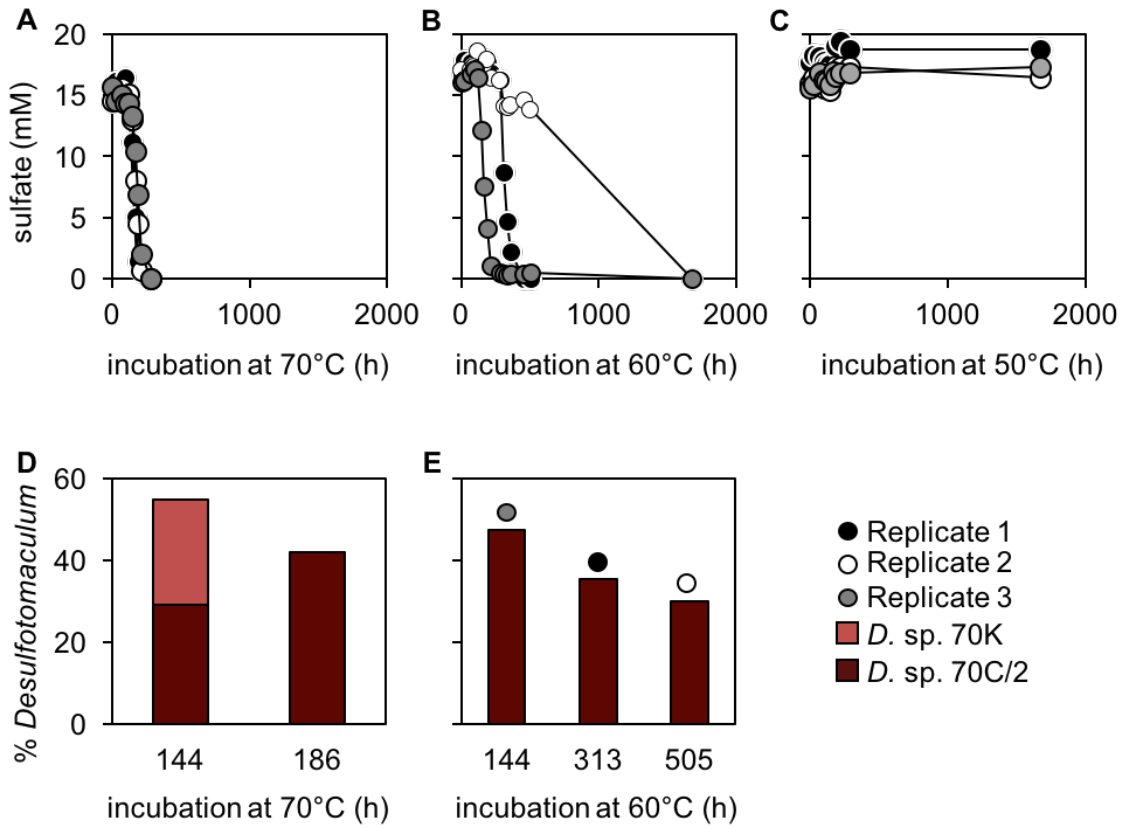

Figure 5.6: Sulfate reduction in microcosms amended with complex organic substrates and incubated at 70°C (**A**), 60°C (**B**) and 50°C (**C**) following 463 days incubation at 80°C. *Desulfotomaculum* spp. were detected in amplicon libraries from incubations at 70°C (**D**) and 60°C (**E**). All libraries were rarefied to 9,159 reads, on which relative abundance calculations (percentages) are based. DNA extractions from 144 h and 186 h incubation at 70°C were pooled prior to sequencing as sulfate reduction was highly reproducible in replicate microcosms (A). Amplicon libraries from microcosms incubated at 60°C were constructed from individual DNA extractions at 114 h, 313 h and 505 h incubation, given that the lag phase varied between replicates (B). No sulfate reduction was detected in microcosms incubated at 50°C (C) therefore no sequencing was performed.

Table 5.2: *Desulfotomaculum* spp. detected in this study.

OTU ID	Temp. detected °C	Closest isolated relative (accession number)	% I.D.	Isolation Source	Temp. range °C (T_{opt})
D. sp. 70K	70, 60	*Desulfotomaculum thermocisternum* strain ST90: DSM 10259 (NR_025979)	99	Hot North Sea Oil Reservoir (Nilsen *et al.*, 1996)	41-75 (62)
D. sp. 70C/2	70, 60	*Desulfotomaculum kuznetsovii* strain 17: DSM 6115 (NR_115129)	99	Thermal water sample from a spontaneous effusion from a rift in the Sukhumsk deposit, Russia (Nazina *et al*., 1989)	50-85 (60-65)
D. sp. 60C/1	70, 60, 50	*Desulfotomaculum peckii* strain LINDBHT1 (NR_109724)	99	Anaerobic filter treating abbatoir wastewaters (Jabari *et al.*, 2013)	50-65 (55-60)
D. sp. 50C/1	50	*Desulfotomaculum carboxydivorans* strain CO-1-SRB (NR_074579)	99	Sludge in an anaerobic bioreactor treating paper mill wastewater (Parshina *et al.*, 2005)	30-68 (55)
D. sp. 50C/2	50	*Desulfotomaculum intricatum* strain SR45 (NR_114380)	96	Freshwater Lake sediment (Watanabe *et al.*, 2013)	28-58 (42-45)
D. sp. 50C/3	50	*Desulfotomaculum intricatum* strain SR45 (NR_114379)	99	Freshwater Lake sediment (Watanabe *et al.*, 2013)	28-58 (42-45)
D. sp. 50C/4	50	*Desulfotomaculum intricatum* strain SR45 (NR_114378)	97	Freshwater Lake sediment (Watanabe *et al.*, 2013)	28-58 (42-45)

5.4.2 *Thermophilic fermentative* Firmicutes *enriched in high temperature incubations*

Following the temperature downshift multiple putative fermenters belonging to the *Firmicutes* were enriched in consort with sulfate-reducing *Desulfotomaculum* spp. (Figure 5.7). The genera *Thermosediminibacter*, *Caldicoprobacter*, *Caldinitratiruptor*, *Symbiobacterium* and *Thermoanaerobacter* were the most abundant putative thermophilic fermenters detected. Differences in community composition were observed depending on whether the first phase of incubation was at 90 or 80°C, in addition to whether the subsequent incubation was at 70, 60 or 50°C. *Thermosediminibacter*, *Caldicoprobacter* and *Caldinitratiruptor* were also detected during the first phase of incubation at 80 and 90°C and may have been active at these high temperatures (Figure 5.3A; discussed in Chapter 6). End products of fermentation with glucose from isolates of these genera include acetate, lactate, ethanol, H_2 and CO_2, all of which may be utilised by *Desulfotomaculum* spp. (Fardeau *et al*., 2000; Bouanane-Darenfed *et al*., 2013)

An enrichment of OTUs belonging to the *Bacillales* was observed following the temperature downshift in all microcosms pre-incubated at 90°C (Figure 5.7A-C). This was not observed in microcosms downshifted from 80°C (Figure 5.7D-E). One explanation for *Bacillales* OTUs only being enriched following the 90°C incubation may be that they were able to grow to abundance by filling a niche left behind by a less heat-resistant OTU inactivated by the 90°C incubation period. Alternatively, the heating at 90°C may have activated *Bacillales* endospores in the sediment, which subsequently displayed greater growth when the temperature is downshifted in the second phase of incubation. This has been observed amongst other *Firmicutes*, where a heat activation step is required to achieve maximal percentage germination (Byrer *et al*., 2000; Setlow, 2013; 2014). The same OTU of *Geobacillus* was enriched in microcosms incubated at 70 and 60°C (Figure 5.7A and B) and was closely related to the anaerobic *Geobacillus thermoglucosidans* (99% sequences identity, Coorevits *et al*., 2012). Microcosms incubated at 50°C were dominated by two *Bacillus* OTUs (Figure 5.7 C) that were most closely related to *Bacillus thermoamylovarans* (99% sequence identity), a facultative anaerobe capable of carbohydrate fermentation (Combet-Blanc *et al.*, 1995) and *Bacillus*

polygoni (97% sequence identity) an aerobic alkaliphile which hydrolyses casein (Aino *et al.*, 2008).

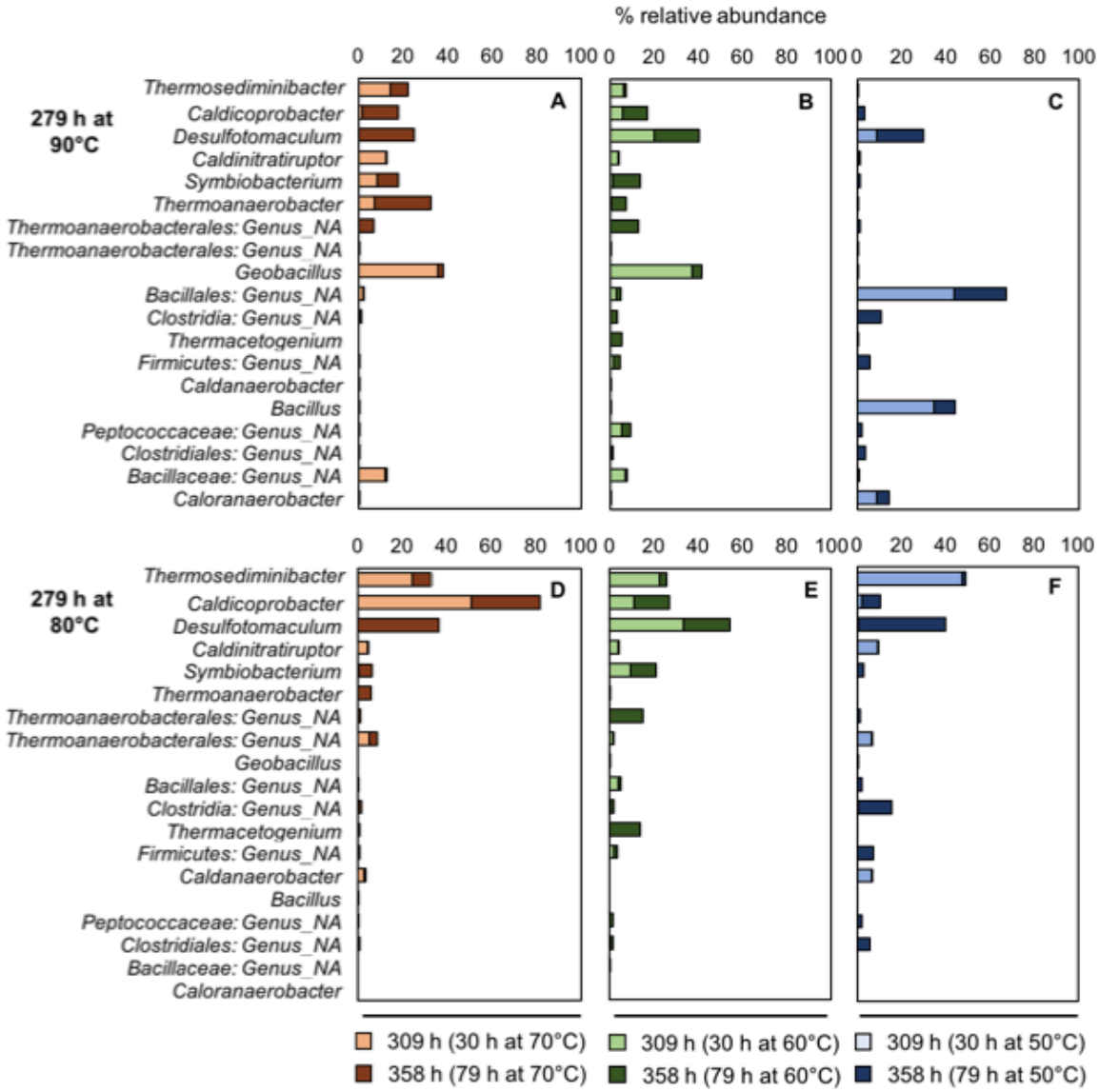

Figure 5.7: Genera belonging to the *Firmicutes* detected at greater than 3% relative abundance in any one amplicon library from microcosms incubated at 90°C for 279 h then reduced to 70°C (**A**), 60°C (**B**) or 50°C (**C**) and microcosms incubated at 80°C for 279 h then reduced to 70°C (**A**), 60°C (**B**) and 50°C (**F**). All microcosms were amended with a complex substrate mixture. Genus_'NA' denotes Genus_'Not Assignable'.

5.5 Discussion

5.5.1 Endospore-forming bacteria and reservoir souring

A consortium of hydrolytic, fermentative and sulfate-reducing endospore-forming bacteria were enriched in sediment microcosms subject to two-phase incubation simulating changing conditions in oil reservoirs during seawater injection (Figure 5.7 A-F). The enrichment of sulfate-reducing *Desulfotomaculum* spp. after the temperature downshift in the microcosms offers a parallel scenario for considering *Desulfotomaculum* endospore germination in response to cooling that occurs in reservoirs when seawater is injected. Temperature incubations conducted at 70-50°C resulted in different *Desulfotomaculum* OTUs being enriched, modelling roles reservoir-dwelling sulfate reducers with different temperature optima could play within distinct zones of a dynamic TVS. The metabolic activity of microorganisms in oil reservoirs will largely depend on the prevailing conditions including temperature and the availability of electron donors and acceptors. In North Sea formation water, aliphatic carboxylic acids are the most abundant organic substrates; acetic acid is found at concentrations up to 20 mM, while other short chain aliphatic carboxylic acids are typically at lower concentrations (Barth and Riis, 1992; Nilsen *et al.*, 1996; Gittel *et al.*, 2009). These are potential electron donors for sulfate reduction; while more complex compounds such as glucose may not be naturally present in the reservoir, they are sometimes found in viscosity promoters injected into oil reservoirs or can be produced by hydrolytic bacteria under anaerobic conditions (Rosnes *et al.*, 1991c).

In water-injected oil reservoirs both electron donors and electron acceptors suitable for SRM activity may be abundant, yet typically only a fraction of the sulfate gets consumed, one explanation for this may be the hostile conditions in the reservoir (Sunde and Torsvik, 2005; Vance and Thrasher, 2005). A microorganism's ability to grow in a subsurface oil reservoir will not only be affected by temperature, they will face multiple physicochemical pressures (Lin *et al.*, 2014a). Some endospores have characteristics which could support their survival in a deep hot oil reservoir. In this study seven *Desulfotomaculum* spp. were shown to be capable of surviving for 11.5 days at 90 and 80°C, but only two *Desulfotomaculum* OTUs were able to survive and be enriched after incubation at 80°C for 463 days. Interestingly, these two OTUs, *Desulfotomaculum* spp. 70K and

70C/2, were most closely related to *Desulfotomaculum* spp. previously isolated from the deep subsurface, indicating that they may be adapted to such conditions enabling them to survive in the hot subsurface for potentially long periods of time. In addition to surviving 463 days at 80°C, *Desulfotomaculum* sp. 70K was previously demonstrated to be resistant to extreme high temperature (autoclaving at 130°C; Figure 4.3) possibly a characteristic of endospores from deep environments (discussed in Chapter 4). *Desulfotomaculum* sp. 70C/2 was most closely related to *Desulfotomaculum kuznetsovii*, which was originally isolated from thermal mineral waters (Nazina *et al.*, 1989), and has also been isolated from two well head samples from non-water flooded oil fields in the Paris Basin (Magot *et al.*, 2000).

Desulfotomaculum OTUs detected in incubations at 50 and 60°C following 279 h incubation at 80 and 90°C, were most closely related to SRB that had previously been isolated in cooler surface environments (Table 5.2); accordingly, these were not enriched following longer high temperature exposure and apparently did not survive prolonged heating. However, these organisms were apparently still more heat-resistant than thermophilic endospores known to be present in Arctic sediments, which were not detected in incubations following 279 h at 80°C (Figure 5.2).

Despite differences thermal tolerance, all of the *Desulfotomaculum* spp. detected in this study may be present in seawater (i.e., they are enriched from cold estuarine and marine sediment), and if they were to be introduced with injected seawater into a reservoir where a TVS has developed, they could nevertheless potentially rapidly colonise different zones of a water flooded reservoir. However, even when all growth requirements are met (temperature, electron donor, electron acceptor and nutrients), the presence of oil itself may limit bacterial growth. In experiments incubated with and without crude oil, growth of *Desulfotomaculum* spp. 60C/1 and 50C/1 was apparently inhibited by adding oil to the sediment incubations (Figure 5.5C) and neither OTU was detected following the 463-day incubation at 80°C (Figure 5.6D and E). In contrast, the presence of crude oil did not inhibit the growth of *Desulfotomaculum* sp. 70K (Figure 5.6C), the same OTU which survived 463 days of incubation at 80°C (Figure 5.6D), indicating that this OTU has multiple characteristics that would enable its survival in the deep subsurface. Survival during long-term high temperature exposure and the ability to

grow in the presence of crude oil suggest that this species could represent spores able to survive in reservoir conditions prior to seawater injection.

Microbial sulfate reduction can occur at temperatures >90°C. Thermophilic archaeal *Archaeoglobus* spp. isolated from a North Sea oil reservoir are able to reduce sulfate at temperatures up to 94°C with an optimum around 80°C (Stetter *et al.*, 1993). The highest recorded growth temperature for SRB is reported for *Desulfotomaculum kuznetsovii* strain 6115 with growth recorded at 85°C (Nazina *et al.*, 1989), even though this strain's temperature optimum is 60-65°C. *Desulfotomaculum* sp. 70C/2 was closely related to *D. kuznetsovii* sp. strain 6115 (99% sequence identity), but no sulfate reduction was observed during incubation at 80°C, indicating different temperature physiology between the two. *Desulfotomaculum* sp. 70C/2 was nevertheless able to survive the high temperature and grow in subsequent 70°C incubations. No other *Desulfotomaculum* spp. have been reported to grow at >80°C. This is consistent with the observations in this study, where no sulfate reduction was observed in microcosms incubated at 80°C (Figure 5.1A-F) including in microcosms downshifted from 90°C to 80°C (Figure 5.4A). In terms of souring mitigation strategies, this has important implications for the injection of seawater in secondary oil recovery. If the temperature of the reservoir can be maintained at ≥80°C, possibly by heating seawater prior to its injection, this may limit the development of a TVS. By maintaining the reservoir temperature, endospores either injected with the seawater or present in the reservoir, would not have the opportunity to germinate and contribute to reservoir souring. In addition to inhibiting endospore germination, prevention of TVS development should similarly reduce the activity of non-endospore forming mesophilic and thermophilic SRM.

The results presented here support the hypothesis that *Desulfotomaculum* germination is triggered by temperature shifts in oil fields caused by cold fluid introduction into hot formations, which can lead to reservoir souring. Endospore-forming bacteria are physiologically and metabolically diverse and are able to survive long periods in unfavourable conditions, aiding their colonisation and establishment across a wide range of environments. Some species detected in this study have characteristics that would make them well adapted to life in hot oil reservoirs. Seawater injection provides suitable growth conditions for a wide range

Desulfotomaculum that would be capable of exploiting different zones of the TVS. Formation waters contain substrates suitable for growth, and additionally thermophilic fermenters can contribute to the substrate pool. However, the results here also suggest that the presence of oil can have an inhibitory effect on some species that may be inoculated into the reservoir via injected seawater, and that they may not be able to grow in parts of the reservoir where conditions are otherwise favourable.

Chapter 6.

Extremely thermophilic *Firmicutes* in temperate estuarine sediments

6.1 Abstract

Acetate production was stimulated in surface estuarine sediments incubated at 80 and 90°C for ~12 days, indicating the presence of an extremely thermophilic microbial population in these temperate sediments. 16S rRNA amplicon libraries showed an enrichment in *Thermosediminibacter* and *Caldinitratiruptor* spp. in incubations at 90°C, possibly representing novel hyperthermophilic strains of these genera, which have not previously been shown to grow above 76°C. Extremely thermophilic phylotypes of *Caldicoprobacter* and *Thermoanaeromonas* were also detected in high temperature incubations at 80°C, but they were not detected at 90°C. Greater concentrations of acetate were detected in microcosms amended with a complex substrate mixture, compared to microcosms that did not receive any additional substrate amendment. Consistent with this, extremely heat-resistant genera were detected in greater relative abundance in amplicon libraries from microcosms amended with the complex substrate mixture. The results suggest that extremely thermophilic fermentative bacteria could contribute to biological acetate production in deep hot sediments.

6.2 Introduction

Microbial life can be found in many extreme environments on Earth. These environments – considered 'extreme' to those accustomed to life at ambient conditions – host extremophiles which thrive under such conditions. Extremophilic microorganisms include halophiles, piezophiles, acido- and alkaliphiles, psychro- and thermophiles, which are adapted to extremes of salinity, pressure, pH and temperature respectively (e.g. Conner and Benison, 2013; Chernyh *et al*., 2015; González-Toril *et al*., 2015).

Thermophiles may be classified as moderate thermophiles (T_{opt} 40-60°C), extreme thermophiles (T_{opt} 60-80°C) and hyperthermophiles (T_{opt} >80°C) (Bonch-Osmolovskaya and Atomi, 2015). Hyperthermophilic microorganisms are mainly represented by archaeal lineages (Stetter, 2013), and are found in deep ocean and terrestrial hydrothermal environments, in hot hydrothermal fluids, vent chimneys and surrounding thermally-heated sediments (Stetter *et al*., 1993; Ehrhardt *et al*., 2007; Chernyh *et al*., 2015). The highest reported growth temperature for life on Earth was recorded at 121°C for an archaeal strain closely related to *Pyrodictium occultum* (Kashefi and Lovely, 2003). The strain was isolated from a black smoker from a hydrothermal vent field of the Juan de Fuca Ridge (with temperatures up to 300°C) and demonstrated growth at extreme high temperature when incubated with Fe(III) and formate. In addition to hyperthermophilic archaea, hyperthermophilic bacteria have been isolated from thermal environments. Anaerobic heterotrophic *Thermotoga petrolphilia* and *Thermotoga napthophila* were isolated from production fluid from the Kubiki oil reservoir in Niigata, Japan, and are capable of growing in the temperatures ranges 47-88°C and 48-86°C, respectively (Takahata *et al*., 2001). Both species displayed optimal growth at 80°C, on the border between 'extreme thermophiles' and 'hyperthermophiles' based on the above definitions. Members of the same genus, *Thermotoga maritima* and *Thermotoga neapolitana*, also grow optimally at 80°C and were isolated from geothermally heated marine sediments (Huber *et al*., 1986; Belkin, 1986; Jannasch *et al*., 1988). *Aquifex pyrophilus*, a chemolithoautotroph, was also isolated from hot marine sediments and displayed growth between 67 and 95°C with a T_{opt} at 85°C (Huber *et al*., 1992). Thermal springs are also host to hyperthermophilic bacteria, and the sulfate-reducing chemolithoautotrophic *Thermodesulfobacterium*

geofontis strain OPF15^T was recently isolated from thermal springs within Yellowstone National Park (Hamilton-Brehm *et al*., 2013).

The study of hyperthermophilic microorganisms is of scientific interest for numerous reasons. Discovering the upper temperature limits for life is of broad general interest as well as being inherent to understanding microbial processes in present-day thermal environments, including possibly determining the depths to which life exists in the subsurface. Microbial processes in thermal environments also have relevance for understanding the evolution of life on early Earth and may offer analogues for understanding life on other planets (Stetter, 1996; Walter *et al*., 1998; Nisbet and Sleep, 2001; Bonch-Osmolovskaya, 2010). In addition, the study of thermophiles and hyperthermophiles can have biotechnological applications, as their enzymes (thermozymes) are active and stable at high temperature (Dalmaso *et al*., 2015; Elleuche *et al*., 2015).

Microcosms inoculated with Tyne estuary and Aarhus Bay sediment were incubated at 80°C and 90°C for up to 279 h as part of an investigation into the germination of thermophilic endospores in response to changing temperature (Chapter 5). Monitoring concentrations of VFA during the high temperature incubations and the generation of 16S rRNA gene amplicon libraries indicated the presence of active bacterial communities at these high temperatures.

6.3 Methods

6.3.1 Monitoring sediment incubations at 80 and 90°C

Microcosm incubation experiments with sediment from the River Tyne and Aarhus Bay were amended with either a complex or simple mixture of organic substrates, or were left unamended. Microcosm preparation was otherwise as described in section 5.3.1. Organic acid analysis was performed at sub-sampling time points during incubation at 80 and 90°C (see section 5.3.2) and DNA extracts from the same time points were used to construct 16S rRNA amplicon libraries (see section 5.3.3).

6.4 Results

6.4.1 Acetate production in estuarine sediment at high temperature

Estuarine sediments from the River Tyne and marine sediments from Aarhus Bay were heated to 80°C for 138 h. During incubation at 80°C sulfate reduction was not observed (Chapter 5; Figure 5.1). Monitoring concentrations of organic acids showed an increase in acetate at 80°C in microcosms inoculated with River Tyne sediment, at the same time the relative abundance of *Clostridia* increased in 16S rRNA amplicon libraries (Figures 6.1A-C). The relative abundance of *Clostridia* increased from 2.4% at 0 h incubation to 48.2%, 29.5% and 28.96% at 138 h incubation in microcosms amended with complex organic substrates, simple organic substrates and unamended microcosms, respectively (Figure 6.1A-C). Acetate production was greatest in microcosms amended with complex substrates (Figure 6.1A) suggesting that some of the acetate produced came from the breakdown of glucose, casein and/or peptone, in addition to acetate production stimulated by the organic acids amendment. The production of acetate in unamended River Tyne sediment microcosms incubated at 80°C, indicates the presence of degradable organic substrates present in the sediment inoculum (Figure 6.1C).

Small concentrations of acetate were detected in Aarhus Bay sediment microcosms amended with complex organic substrates (<500 μm) (Figure 6.1D). No acetate production was detected in Aarhus Bay sediment microcosms that were amended with simple substrate or that were not unamended (Figure 6.1E and F). The relative abundance of *Clostridia* in amplicon libraries from Aarhus Bay sediment incubations at 0 h and 138 h incubation did not indicate an enrichment of this group with incubation at 80°C. *Clostridia* was detected at 1.04% relative abundance at 0 h incubation and 4.36 ± 0.75% at 138 h incubation. Analysis of Aarhus Bay amplicon libraries did show an increase in relative abundance of *Bacilli* in microcosms incubated at 80°C for 138 h and amended with the complex substrate amendment, increasing from 0.04% at 0 h to 68.28% (Figure 6.1D). This was not observed in Aarhus Bay sediment microcosms at the same temperature without the complex organic substrate amendment (Figure 6.1E and F). Sediments from Svalbard incubated under the same conditions (138 h at 80°C; Chapter 5) showed no increase in acetate and no enrichment of the classes *Clostridia* or *Bacilli*

(relative abundance of 1.75 and 0.69% at 0 h incubation and 3.06 and 0.43% at 138 h incubation for *Clostridia* and *Bacilli*, respectively).

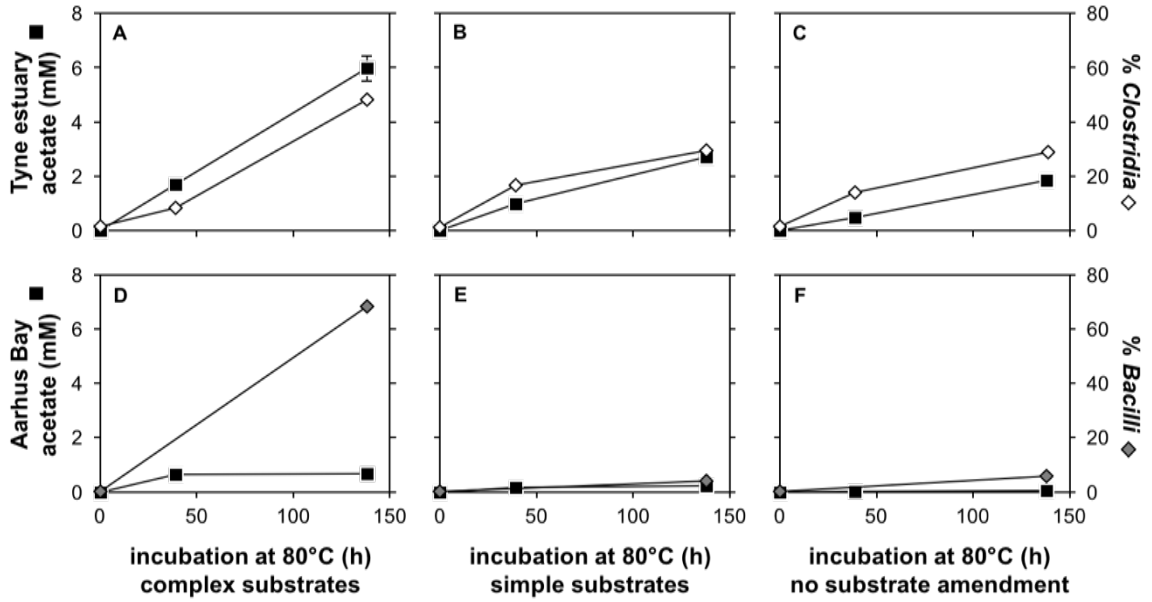

Figure 6.1: Production of acetate in Tyne estuary sediment incubated at 80°C for 138 h was monitored in microcosms amended with complex substrates (**A**), simple substrates (**B**) or not amended with substrates (**C**). Acetate concentrations were also monitored incubations at 80°C with Aarhus Bay sediment amended with complex substrates (**D**), simple substrates (**E**) or not amended with substrates (**F**). The concentration of acetate at 0 h was subtracted from acetate measurements to show the amount of acetate produced. Acetate was measured in triplicate microcosms with error bars showing standard error among triplicate bottles. In many cases the error bars are smaller than the size of the acetate symbols. The relative abundance of *Clostridia* in amplicon libraries from Tyne estuary incubations is indicated on the secondary y-axis (A-C) and *Bacilli* is indicated for Aarhus Bay incubations (D-F). Relative abundances were calculated as a percentage of 9159 reads.

Analysis of amplicon libraries from Tyne estuary sediment incubations showed that 10 OTUs from within the *Clostridia* were present at ≥1% relative abundance following 138 h incubation at 80°C (Figure 6.2A). These OTUs were members of the genera *Thermosediminibacter* and *Thermoanaeromonas* of the order *Thermoanaerobacterales* and of the genera *Caldinitratiruptor* and *Caldicoprobacter* within the order *Clostridiales* (Figure 6.2A). The greatest enrichment was observed in microcosms amended with the complex substrate mixture, consistent with the greatest production of acetate in complex substrate amended microcosms (Figure 6.1A). *Geobacillus* of the order *Bacillales* was also detected in complex substrate amended microcosms increasing to 9.2% relative abundance at 138 h incubation (Figure 6.2A). Amplicon libraries from Aarhus Bay sediments showed an increase in *Bacillales* spp. in microcosms amended with complex organic substrates (Figure 6.2B).

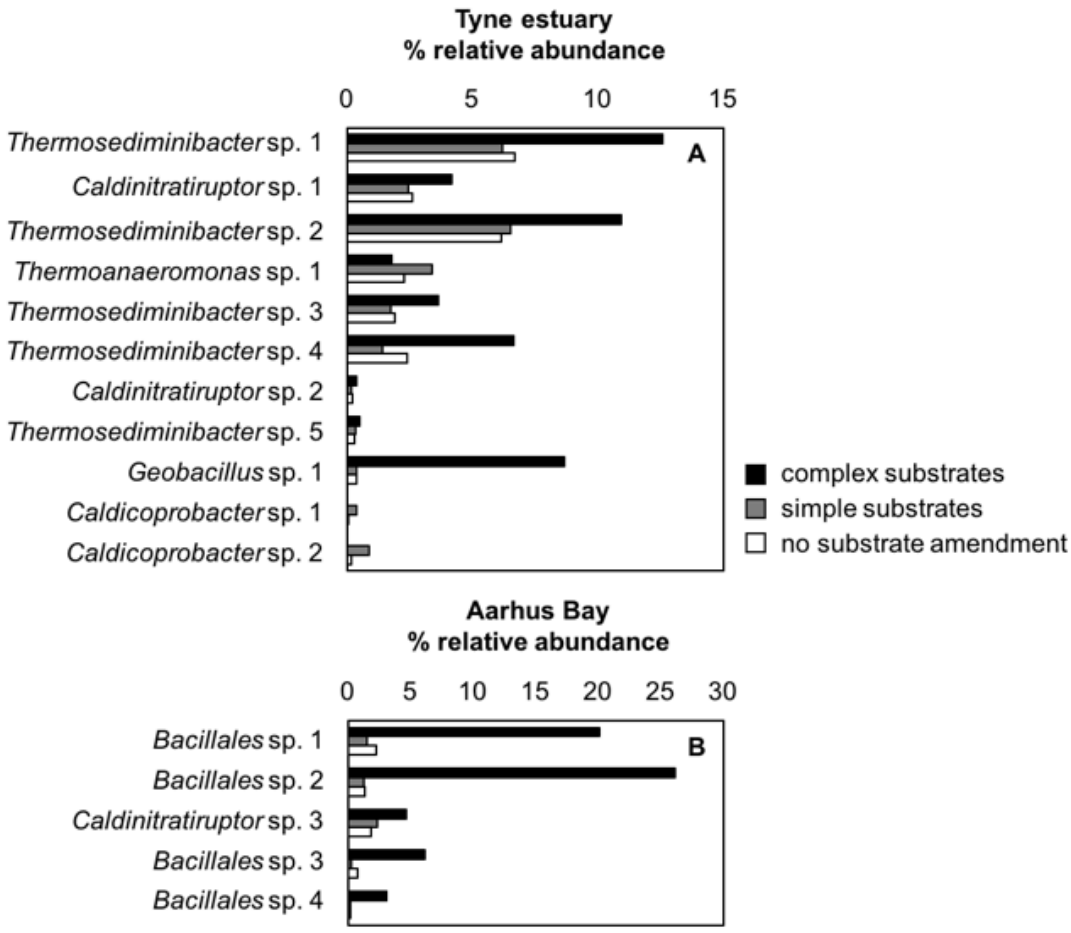

Figure 6.2: OTUs within the classes *Clostridia* and *Bacilli* that represented ≥1% relative abundance in of 16S rRNA amplicon libraries following 138 h from incubation of Tyne estuary sediment at 80°C (**A**) and Aarhus Bay marine sediment at 80°C (**B**). Microcosms were either amended with complex substrates (black bars), simple substrates (grey bars) or did not have any substrate addition (white bars). Relative abundances were calculated as a percentage of 9159 reads.

Microcosms prepared with only Tyne estuary sediment were also incubated for twice as long (279 h) at 80°C and also at 90°C. Acetate production was detected under all conditions (Figure 6.3A-C).

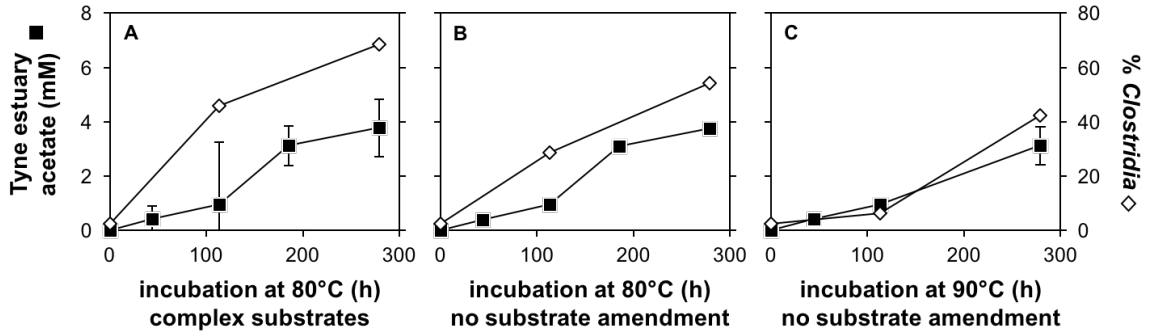

Figure 6.3: Production of acetate in Tyne estuary sediment incubated for 279 h at 80°C and amended with complex substrates (**A**), incubated at 80°C with no substrate amendment (**B**) and incubated at 90°C with no substrate amendment (**C**). The concentration of acetate at 0 h incubation was subtracted from acetate measurements to show the amount of acetate produced. Acetate was measured in triplicate microcosms with error bars showing standard error among triplicate bottles. In many cases the error bars are smaller than the size of the acetate symbols. The relative abundance of *Clostridia* in amplicon libraries is indicated on the secondary y-axis. Relative abundances were calculated as a percentage of 9159 reads.

16S rRNA gene amplicon libraries from the extended incubations at 80°C again showed an enrichment of *Thermosediminibacter*, *Thermoanaeromonas*, *Caldinitratiruptor* and *Caldicoprobacter* spp. (Figure 6.4A-B). Enriched genera increased in relative abundance with incubation time at 80°C and 90°C, (Figure 6.4A-D) as the concentration of acetate increased (Figure 6.3A-C). *Thermosediminibacter* and *Caldinitratiruptor* spp. were detected in incubations at 90°C, but *Thermoanaeromonas* and *Caldicoprobacter* spp. were not (Figure 6.4C-D). In addition to changes in the microbial community as a result of incubation temperature, differences in community composition were observed as a function of substrate amendment. In unamended microcosms incubated at 90°C, there was a longer lag phase before *Thermosediminibacter* spp. were detected in amplicon libraries (Figure 6.4D) compared to its detection in 80°C incubations, consistent with the slower production of acetate at the higher temperature (Figure 6.3C). In addition, the *Thermosediminibacter* spp. that were abundant in unamended microcosms at 90°C (Figure 6.4B) did not appear to be enriched in microcosms at the same temperature amended with complex organic substrates (Figure 6.4D). Instead, *Caldinitratiruptor* was the only genus enriched in the complex substrate-amended microcosms at 90°C (Figure 6.4D).

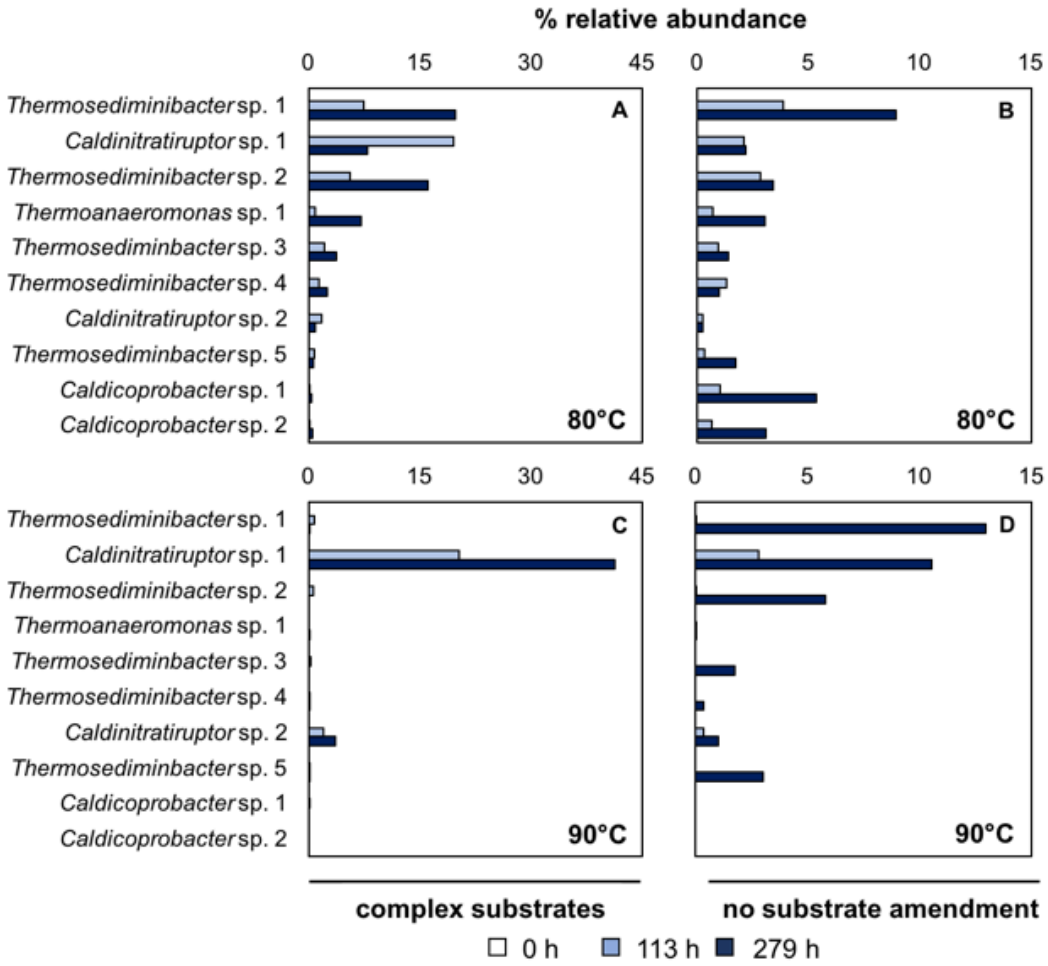

Figure 6.4: Comparison of ten OTUs from within the class *Clostridia* that represented ≥1% relative abundance in 16S rRNA amplicon libraries from incubations of Tyne estuary sediment at 80°C (**A-B**) and 90°C (**C-D**) for 279 h. Microcosms were either amended with complex substrates (A and C) or were not amended with substrates (B and D). Relative abundances were calculated as a percentage of 9159 reads.

Five OTUs of *Thermosediminibacter* were detected in Tyne sediments incubated at 80 and 90°C (Figure 6.4A-D). *Thermosediminibacter* OTUs were closely related *Thermosediminibacter oceani* strain JW/IW-1228P and *Thermosediminibacter litoriperuensis* strain JW/YJL-1230-7/2. *Thermosediminibacter* sp.1, the most abundant of the five OTUs, shared 97% and 96% sequence identity with *T.oceani* and *T.litoriperuensis*, respectively (Figure 6.5). Both strains are anaerobic thermophiles, isolated from deep sea sediments from the Peru Margin (Lee *et al*., 2005). The T_{max} for both strains was 76°C, with optimum growth for *T.oceani* was observed at 68°C, and at 64°C for *T.litoriperuensis*. Spore formation was not reported for either strain. The next closest relative to *Thermosediminibacter* sp. 1 was *Thermovorax subterraneus* strain 70B (95% sequence identity), a spore-forming bacterium isolated from a

geothermally active underground mine in Japan (Mäkinen *et al*., 2009). The T_{max} of *T.subterraneus* is 81°C with optimum growth at 71°C. Both *Thermosedimibacter* spp. and *T.subterraneus* use thiosulfate as an electron acceptor, and can utilise amino acids and sugars with the main fermentation end product from glucose being acetate (Lee *et al*., 2009; Mäkinen *et al*., 2009).

Two OTUs of *Caldinitratiruptor* were enriched in River Tyne sediment incubations at 80 and 90°C, with the greatest enrichment (based on amplicon libraries) observed in microcosms incubated at 90°C (Figure 6.4C). Only one member of this genus has been described, *Caldinitratiruptor microaerophilus*, which shared 92% sequence identity with *Caldinitratiruptor* spp.1 and 2. *C. microaerophilus* a facultative microaerophilic anaerobic thermophile within the family *Symbiobacteraceae* and was isolated from a French hot spring (Fardeau *et al*., 2010). *Caldinitratiruptor* also uses glucose and amino acids as substrates, glucose is oxidised to acetate with nitrate as an electron acceptor. The reported T_{max} was 75°C. Spore formation was not reported for *Caldinitratiruptor microaerophilus* or related *Symbiobacterium* spp. (Rhee *et al*., 2002; Ueda *et al*., 2004; Fardeau *et al*., 2010).

Caldicoprobacter and *Thermoanaeromonas* spp. were detected in greatest relative abundance in amplicon libraries from enrichments at 80°C for 279 h (Figure 6.4A-B). They were not enriched in the same sediments incubated at 90°C (Figure 6.4C-D). *Thermoanaeromonas* sp.1 was most closely related to *Thermoanaeromonas toyohensis* (97% sequence identity), a spore-forming thiosulfate reducer isolated from a geothermal aquifer at 550 m depth in the Toyoha Mines in Japan (Mori *et al*., 2002). *T. toyohensis* has a reported T_{max} of 73°C, and can ferment sugars. Nitrate and nitrate reduction was also reported in lactate as an electron donor. Two OTUs of *Caldicoprobacter* were detected in 80°C enrichments at 279 h which shared 98-99% sequence identity with *Caldicoprobacter oshami*, a spore-forming thermophile with a T_{max} of 77°C (Yokoyama *et al*., 2010). *Caldicoprobacter guelmensis* (96% identity) has the highest recorded T_{max} among *Caldicoprobacter* isolates with growth observed of 85°C (Bouanane-Darenfed *et al*., 2013). The reported T_{max} for other cultured *Caldicoprobacter* spp. is <80°C (Figure 6.5), and endospore formation was not reported for *C.guelmensis*, *C. algeriensis* or *C. faecale* (Bouanane-Darenfed *et al*., 2011; 2013; Winter *et al*., 1987). *Caldicoprobacter* spp. can use xylan and glucose

as substrates, with acetate as produced as one of the main products of fermentation.

Aarhus Bay 16S rRNA amplicon libraries from microcosms incubated at 80°C for 138 h and amended with complex substrates showed an increase in relative abundance of *Bacillales* spp. compared at 0 h incubation (Figure 6.2B). The most abundant *Bacillales* OTU was most closely related to *Geobacillus thermoglucosidans* (97% sequence identity), an anaerobic thermophile with a T_{max} of <60°C (Suzuki *et al*., 1983; Coorevits *et al*., 2012). The maximum growth temperature for most members of the genus *Geobacillus* is <80°C, but a T_{max} of 80°C has been reported for *Geobacillus thermantarcticus* and *Geobacillus thermocatenulatus*, although these species are aerobic (Coorevits *et al*., 2012).

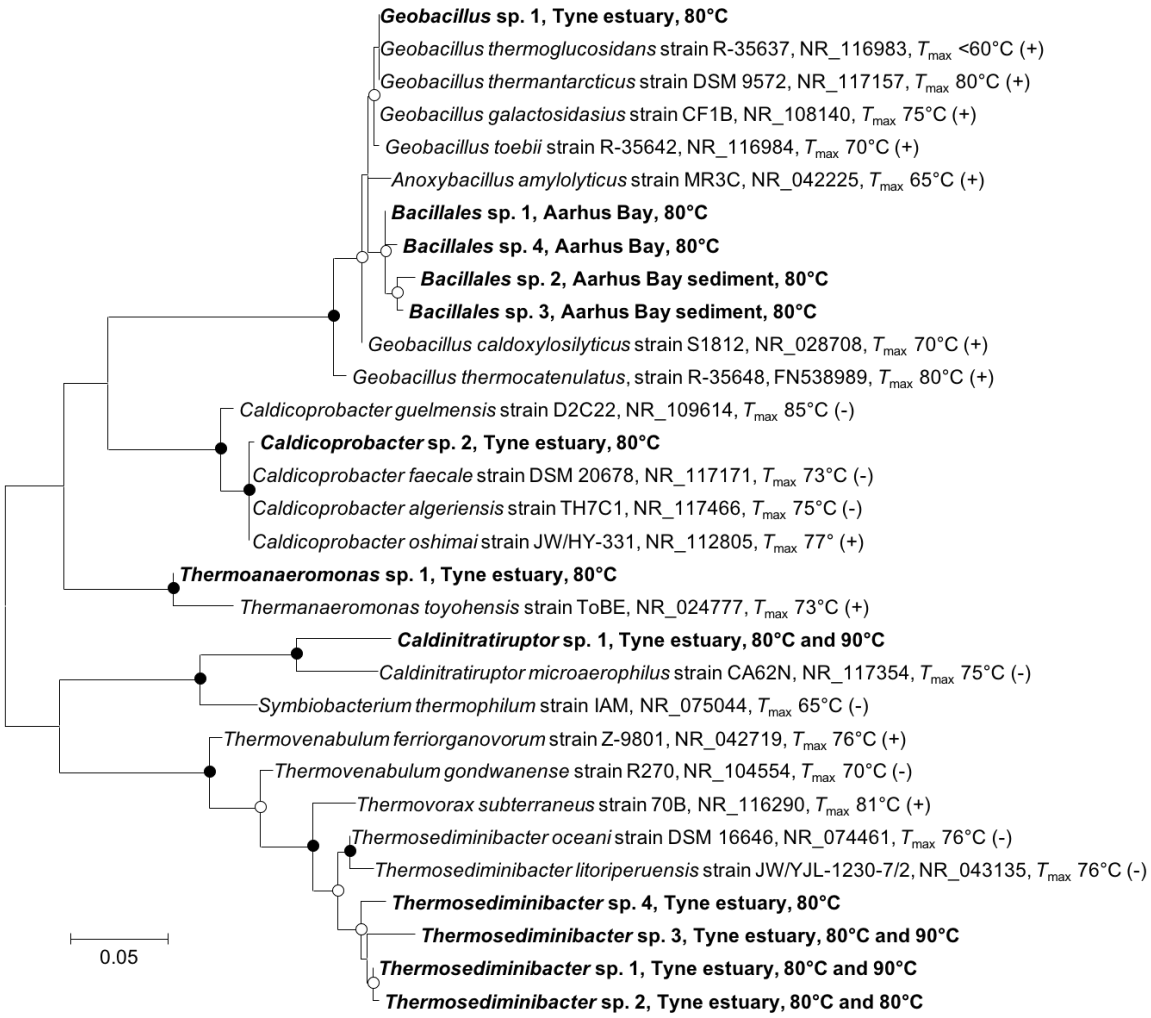

Figure 6.5: 16S rRNA tree of OTUs detected in Tyne sediment incubations at 80 and 90°C with gene sequences derived from this study shown in bold. Only sequences greater than 300 bp were included in the tree. Closely related sequences were retrieved from Genbank and included in the phylogenetic analysis, performed using MEGA 5.2 by Maximum Likelihood with a total of 332 nucleotide positions. Reported T_{max} for closely cultured isolates are shown in the tree, in addition to whether spore formation was reported (+) or not (-). Filled and open circles at branching nodes indicate bootstrap support values of >90% and 70 to 90% respectively (1000 resamplings).

6.5 Discussion

The results presented within indicate that biological acetate production was stimulated in estuarine sediments from the River Tyne when heated to 80 and 90°C. Acetate production occurred concurrently with an increase in relative abundance of *Clostridia*, indicating that acetate may be a metabolic product from the activity of the detected *Clostridia*. The reported metabolisms for all of the isolates closely related to the OTUs detected in amplicon libraries from this study are consistent with the production of acetate as a metabolic product.

Thermosediminibacter and *Caldinitratiruptor* were the only genera detected in incubations at 90°C. Both of these genera are only represented by 1-2 isolates, none of which have had growth at ≥80°C previously reported (Lee *et al*., 2006; Fardeau *et al*., 2010). The detection of *Thermosediminibacter* and *Caldinitratiruptor* spp. in Tyne sediments incubated at 90°C may therefore represent new strains or species within these genera. Growth at 90°C indicates that the species detected in the Tyne estuary are hyperthermophilic, however the T_{opt} was not determined and therefore it cannot be said whether their optimum growth is above 80°C, making them true hyperthermophiles. Spore-formation was not reported for *Thermosediminibacter* or *Caldinitratiruptor* isolates, although other close relatives to the *Thermosediminibacter* spp. (*Thermovorax subterraneus* and *Thermovenabulum ferriorganovorum*) could form spores (Figure 6.5) (Zarvarzina *et al*., 2002; Mäkinen *et al*., 2009). The OTUs detected in this study were enriched from cold sediments and spore formation would provide a mechanism for survival at cold temperatures. Nevertheless, non-spore forming thermophiles have been isolated from cold sediments, including *Thermosediminibacter* spp. which were isolated from sediments at 12°C (Lee *et al*., 2006) and non-spore forming hyperthermophiles have been detected in cold seawater (Stetter *et al*., 1993). From the results presented within it is unclear whether the *Thermosediminibacter* and *Caldinitratiruptor* spp. detected at 90°C are spore-formers.

Caldicoprobacter and *Thermoanaeromonas* spp. were detected in microcosms at 80°C, but not at 90°C. Based on these occurrence patterns, it is likely that both the *Caldicoprobacter* and *Thermoanaeromonas* spp. detected at 80°C are extreme thermophiles operating at or near their T_{max}. Consistent with this, *Caldicoprobacter* spp. increased from 12.42% relative abundance at 279 h

incubation at 80°C, to 51.10% in libraries from the same microcosms that had been reduced to 70°C for an additional 30 hours (Chapter 5; Figure 5.7). *Caldicoprobacter* spp. were also detected in amplicon libraries from sediments incubated at 70°C that first underwent heating at 90°C, indicating that although they did not grow at 90°C, they were still viable after the 279 h incubation period at 90°C. This suggests that *Caldicoprobacter* spp. were present in the sediment as spores. Spore formation was also reported for the close relative *Caldicoprobacter oshami* (Yokoyama *et al*., 2010). *Thermoanaeromonas* sp. 1 was also detected in incubations at 70°C that have previously been subject to 279 h incubation at 90°C indicating that it may also be a spore former, although it was detected at low relative abundance (<1%). This OTU was also detected in low relative abundance in the libraries reduced from 80°C (<3% relative abundance). Microorganisms may have alternative mechanisms for survival at high temperature e.g. *Thermovenabulum gondwanese* strain CA9F1, which was related to the *Thermosediminbacter* spp. detected in this study, was not shown to form spores, but has a complex multilayered cell wall structure, thought to aid in resistance to high temperatures (>80°C) encountered in the thermal hot springs from which it was isolated (Pradel *et al*., 2013).

It is uncertain whether *Bacillales* spp. enriched in Aarhus Bay sediment microcosms were active, as little to no acetate production was observed (Figure 6.1C-F). DNA is not easily extracted from endospores using standard extraction protocols, thus the detection of endospore-forming genera in considerable abundance in amplicon libraries from sediments incubated at 80°C suggests that spores in the sediment had germinated, making their DNA readily extractable. The significant enrichment of *Bacillales* in microcosms amended with complex organic substrates (68.3%) compared to microcosms incubated under the same conditions other than the substrate amendment (5.9% in unamended microcosms) suggests that the organic substrate amendment did enhance germination and/or growth of *Bacillales* spp. in heated Aarhus Bay marine sediments. Substrates in the complex substrate mixture may have activated germinant receptors in *Bacillales* spp. causing germination, thus making DNA extractable and amplifiable by PCR. This would result in their detection in amplicon libraries, but does not suggest that they were active at 80°C following germination. If *Bacillales* spp. were active, they may

have produced metabolic products, e.g., ethanol, that would not have been detected by the IC method used to monitor organic substrates in this study.

The biological production of acetate from buried organic matter at high temperature may be an important process for sustaining microbial communities in deeply buried sediments (Wellsbury *et al*., 1997; Parkes *et al*., 2007). Both acetate and H_2 were produced in coastal sediment incubations up to 90°C (Parkes *et al*., 2007) and acetate accumulation at depth was also demonstrated in pore waters from deep marine sediment cores (Wellsbury *et al*., 1997). The production of acetate in sediments from the Tyne estuary at 80 and 90°C suggests that hyperthermophilic and/or extremely thermophilic fermentative bacteria, like those detected in this study, could contribute to biological acetate production in deeply buried sediments. The geothermal gradient of the seabed is normally 2-4°C 100 m^{-1} (Jørgensen and Marshall, 2015), long-term survival may allow thermophilic and hyperthermophilic bacteria to reach warm deep sediments via sedimentation (Hubert *et al*., 2010). This would be more likely to occur in areas with a steep geothermal gradient and a high rate of sedimentation, otherwise the process would require endospores deposited at the surface to survive for millions of years before reaching warmer deep sediments. If endospores can successfully survive during burial, thermophiles in cold sediments could in this way become active members of the deep biosphere.

Chapter 7.

Conclusions and Perspective

7.1 Estuaries as dispersal vectors of thermophilic endospores

The presence of inactive anaerobic thermophilic endospores in cold sediments provides a natural model to study microbial dispersal, a fundamental process underlying microbial biogeographic patterns. Since their first discovery in cold Aarhus Bay sediments (Isaksen *et al*., 1994), thermophilic endospores have been shown to be both metabolically diverse (Hubert *et al*., 2010) and geographically widespread (de Rezende *et al*., 2013; Müller *et al*., 2014). Prior to the contribution presented here, studies had focused on marine sediments, and provided significant contributions towards understanding marine microbial biogeography. The work presented in this thesis focuses on the diversity and distribution of thermophilic endospores in estuarine sediments, at the interface of the terrestrial and marine biospheres. The detection of thermophilic spore-forming sulfate-reducing and fermentative bacteria upstream of the tidal limit in the River Tyne (station F1, Chapter 3) confirms that in addition to marine dispersal (Hubert *et al*., 2009; de Rezende *et al*., 2013; Müller *et al*., 2014) thermophilic endospores are dispersed in riverine flow, from terrestrial sources.

The dispersal histories and biogeography of over 100 different OTUs of thermophilic *Firmicutes* detected here within the Tyne estuary system, indicate that endospores are delivered to the estuary via multiple dispersal vectors, both marine (hydrocarbon seeps, production fluids discharge and diffuse hydrothermal flow) and terrestrial (compost, industrial discharges, mine water, and groundwater). Freshwater discharge from rivers into the North Sea is in the order of 300 $km^3\ y^{-1}$ (OSPAR Commission, 2000), and contributes $4 \cdot 10^{10}\ km^3\ y^{-1}$ to oceans globally (Johnson and Pruis, 2003). Significant numbers of thermophilic endospores transported in riverine flow could therefore be transported to marine environments, consistent with the detection of terrestrially-derived endospores in North Sea marine sediments (Chapter 3). Once in the ocean, terrestrially-sourced thermophilic endospores may be transported on multiple further journeys, thus far proposed for marine microorganisms. Thermophilic endospores in seawater may be deposited at the cold seafloor and reach warm deep sediments following deep burial, becoming active members of the deep biosphere (Hubert *et al*, 2010).

Alternatively, endospores could be delivered to a warm hydrothermal site where they could colonise vent chimneys or geothermally heated sediments, or get drawn down with seawater in hydrothermal circulation at ridge flanks inoculating the warm ocean crustal aquifer system. The annual flux from venting hydrothermal fluid is estimated to be 18% of the total riverine flow to oceans (Johnson and Pruis, 2003), possibly expelling microorganisms from the subsurface into the ocean if they can survive the transit within a very hot plume. Microorganisms expelled at vent sites, may be inoculated into oil reservoir environments during seawater flooding for secondary oil recovery. This was a mechanism hypothesised following the first discovery of hyperthermophilic archaea in oil fields, in samples of produced water from deep North Sea and Alaskan oil reservoirs, since these organisms were similar to those detected from a distant hydrothermal vent site based on the best similarity comparisons that could be made at the time (Stetter *et al*., 1993). Active oil reservoir communities may then be transported back to the ocean in discharged production water, and be dispersed to another location. Any one of these journeys mediated by ocean currents could potentially be taken upon by a terrestrially-derived endospore, discharged to the ocean in riverine flow. These transport routes highlight the potential power of dispersal, connecting terrestrial and marine, surface and subsurface environments.

The dispersal histories identified for thermophilic endospores detected in the River Tyne indicated that the majority originated from a terrestrial source, with current industrial discharges and historic mining activities implicated as vectors of terrestrial spores (Chapter 3 and 4). While terrestrial sources appear to be significant in the Tyne estuary, they may only constitute a substantial flux of thermophilic endospores in estuaries influenced by human activity. If the same lateral sampling strategy was applied to an estuary from a relatively pristine environment, without the industrial activity of the River Tyne, it is possible that the same predominance of apparently terrestrially-derived endospores would not be observed. Consequently, estuarine dispersal vectors may be most relevant in ocean regions connected to estuaries that flow through urbanised areas.

7.2 Thermophilic endospores as bioindicators

Microorganisms may be used as bioindicators for oil prospecting, as markers of undiscovered petroleum reservoirs (Hubert and Judd, 2010). Close

relatives to the many thermophilic phylotypes enriched from cold sediments were detected in petroleum reservoirs (this study; Hubert *et al*., 2009; 2010; de Rezende *et al*., 2013), and display multiple characteristics indicative of subsurface origins (Chapter 5), including in some cases extreme heat-resistance (Chapter 4). If these bacteria originate from deep hot oil reservoirs via the upward flow of petroleum fluids, then studies of these dormant endospores could be integrated into oil exploration strategies that rely on seabed hydrocarbon seep prospecting. Screening marine sediments for microorganisms thought to be otherwise indigenous to oil reservoirs could indicate the presence of hydrocarbon seepage from a subsurface petroleum reservoir (Hubert and Judd, 2010). Following the identification of a phylotype of interest, a quantitative screening method would be required to track the abundance towards the source, assuming that population size decreases in abundance with dispersal distance. Thermophilic endospores are conspicuous and persistent in cold sediments, making them ideal candidates for microbial source tracking. The quantification method would need to be species-specific, targeted at the phylotype of interest. As thermophilic endospores would not be readily detected in cold sediments by molecular methods independent of enrichment, a cultivation step may be required. Growth of thermophilic endospores in sediment enrichments can be rapid (Chapter 3, Figure 3.1) and the work presented here shows how enrichment conditions can be tailored towards a specific phylotype e.g. growth in the presence of oil (Chapter 5) or activity screening following varying degrees of heat-treatment (Chapter 4). Enrichments that are positive for growth under specific conditions could be screened relatively rapidly and inexpensively using high-throughput sequencing.

A challenging aspect of using thermophilic endospores as bioindicators of hydrocarbon seeps would be ensuring that the indicator organism chosen truly derived from a petroleum reservoir. The results presented within demonstrate that there may be multiple origins of thermophilic endospores in cold sediments, and that thermophilic endospores in marine sediments could have been delivered in riverine flow and not have come from a marine habitat at all (Chapter 3 and 4). In addition, the connectivity of distant source environments resulting from passive dispersal (as discussed above) could obscure a clear source signal, making one specific target source even more difficult constrain. Using endospores as bioindicators of undiscovered petroleum reservoirs may only be feasible in

environments that are not influenced by human industrial activity and that are distant from tapped reservoirs. Otherwise, even if a petroleum-reservoir dwelling organism was correctly identified, source-tracking may lead to the site where an active oil platform discharges production water. In regions where offshore oil platforms are in place, thermophilic endospores could instead be used as indicators of pollution from production water. The same issue arises – determining whether the organism is truly from oil reservoir production water – but knowing the source could provide the opportunity to first characterise the production water, or multiple production waters from oil reservoirs in the region, to screen for indicator organisms. This could be applied to various types of industrial pollution identified as possible dispersal vectors (Chapter 3). As with production waters, the effluent of interest could first be screened for an indicator organism. An additional challenge for quantitatively tracking thermophilic endospores may arise from changes in sediment type along a lateral gradient, which may affect the endospore retention and thus the *in situ* abundance of spores of interest (Chapter 3; Fichtel *et al*., 2008). Sediment properties would therefore have to be accounted for when determining abundance along a transect with heterogeneous sediment properties. The development of quantitative screening methods could also be used for tracking endospores from unknown sources, for new biogeography investigations.

7.3 Heat-resistant endospores

Autoclaving sediments from the Tyne estuary revealed the presence extremely heat-resistant endospore-forming *Firmicutes*, which were closely related to bacteria detected in the deep hot biosphere (Chapter 4). Sampling subsurface environments is often challenging and costly. If endospores detected in surface sediments are dispersing from the deep biosphere, strain isolation and genome studies could reveal insights into metabolic capabilities of deep biosphere organisms in easily accessed samples. In the case of extremely heat-resistant organisms, this could include studies of extreme physiology, and what determines extreme heat-resistance. Cultivation strategies generally detect the faster-growing abundant microbial populations. Autoclaving sediments prior to cultivation introduces a harsh selection step, enabling strain isolation of microorganisms that are not otherwise readily detected (Chapter 4). Different spore-forming phylotypes are enriched depending on incubation temperature and heat pre-treatment

(Chapters 4-6), suggesting that temperature optima and heat resistance may serve as indicators of different source environments where different conditions (e.g., local temperature) may influence endospore formation. Differences in endospore heat-resistance have been attributed to a number of factors (detailed in section 1.2.1), including sporulation conditions, which could have a significant effect (Condon *et al*.,1992; Melly *et al*., 2002). Melly *et al*., (2002) demonstrated that spores of *Bacillus subtilis* that were formed at higher temperatures were more heat resistant than those in clonal cultures that sporulated at lower temperatures. This increased resistance appeared to be achieved, at least in part, by a decrease in the core water content of spores formed at higher temperature. Cultivation conditions were otherwise identical and the different-temperature spores had equal amounts of DPA and small acid soluble proteins. Whether these kinds of observations would also be made in experiments with thermophiles including *Desulfotomaculum* spp. remains to be tested; could the heat-resistance of extremely thermophilic strains be changed by inducing sporulation at different points along their temperature activity range, e.g., T_{min} vs T_{max}. If so, differences in heat-resistance could be incorporated into biogeographic studies, as indicators of different microbial habitats of origin where sporulation conditions may differ.

The ability of endospores to survive at temperatures much greater than their T_{max} whilst in a dormant state (Chapter 4) enabled them to be used in a model system for studying reservoir souring, a troublesome and costly problem often encountered during secondary oil recovery (Chapter 5). Sulfate-reduction was not detected in experiments maintained at 80°C or higher, suggesting if the ambient reservoir temperature can be maintained during secondary oil recovery, souring could be prevented or reduced. As a consequence of these experiments, the pre-heating of injection water, before introduction into the formation, is being considered as part of a souring mitigation strategy. This has significant industrially relevant implications, as successful souring mitigation strategies can reduce operating costs and maintain the oil value through the prevention of H_2S production.

The study of out-of-place thermophilic endospores in temperate estuarine sediments has enabled multiple hypotheses to be explored in particular relating to microbial dispersal and extreme survival. This study has focused on thermophilic endospores, but the dispersal vectors identified are not exclusive to this group.

They likely transport microorganisms of all kinds thus influencing biogeographic patterns of many microbial populations.

Summary of findings

1. Thermophilic spore-forming SRB and putative thermophilic fermenters are present throughout the temperate River Tyne estuary, UK, including upstream of the tidal limit (Figures 3.1-3.3)
2. Different populations of thermophiles are detected depending on incubation temperature (50-90°C) and heating (pasteurisation and autoclaving) indicating multiple warm source environments with different environmental conditions (Figures 4.2, 5.5, and 6.4).
3. The identification of different dispersal histories adds to the known possibilities for dispersal vectors explaining biogeographic distributions of thermophilic endospores cold sediments (Chapters 3 and 4)
4. The presence of identical partial 16S rRNA sequences in geothermal groundwater with two extremely heat-resistant phylotypes suggests microorganisms in the terrestrial subsurface may be transported to the surface in migrating groundwater (Figure 4.8)
5. Extreme heat-resistance was demonstrated among the genera *Desulfotomaculum*, *Tepidanaerobacter*, *Moorella, Thermoanaerobacter* and *Gelria*, contributing towards the known diversity and physiology of extremely heat-resistant spore-formers (Figure 4.5B)
6. Extreme heat-resistance appears to be a characteristic of deep biosphere microorganisms (Chapter 4)
7. Autoclaving sediments reveals microbial diversity that may not be otherwise be detected in enrichment cultures (Figures 4.2 and 4.5)
8. Extremely thermophilic and hyperthermophilic fermentative species detected in estuarine sediments contribute to bacterial acetate production at high temperature (up to 90°C) (Figures 6.2 and 6.4).
9. Thermophilic endospores have been shown to survive 8 hours at 121°C and 463-days at 80°C, extending the known survival times at high temperature (Figures 4.4 and 5.6).
10. The survival physiology of endospores is industrially relevant in the context of reservoir souring. Temperature shifts trigger endospore

germination of SRB, which may subsequently contribute to souring (Figure 5.1).

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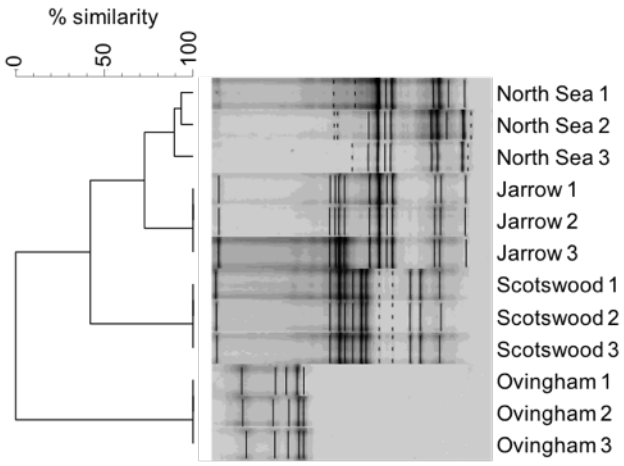
Appendix A

Sediments from the Tyne estuary (stations F1, B3, B5 and B6) were analysed by *Desulfotomaculum*-specific DGGE (section 2.3.3.1), following pasteurisation at 80°C (1 h) and incubation at 50°C (72 h), as described in section 3.3.1-3.3.3. Based on highly similar DGGE profiles (Figure A1), in addition to reproducible sulfate measurements (Figure 3.1), PCR products from replicate microcosms were pooled prior sequencing by Ion Torrent (section 2.3.3.3).



Site	1 (Ovingam 1–3)	3 (Scotswood 1-3)	5 (Jarrow 1-3)	6 (North Sea 1-3)
Average % similarity within replicates	100	100	100	90.79
S.E.	0.00	0.00	0.00	2.54

Figure A.1: *Desulfotomaculum*-specific DGGE following two-step nested PCR; DEM116f – 1164r (targeting Desulfotomaculum spp.) followed by 341f-gc – 907r (universal bacterial 16S rRNA primers). Sediments were pasteurised sediments (1 hour at 80°C) and incubated at 50°C. DNA was extracted after 72 hours' incubation. Average % similarity between triplicates was calculated in Bionumerics software package (Applied Maths, Austin, Texas, US). Band matching data was used to calculate Dice similarity indices.

Amplicon libraries generated from pasteurised Tyne estuary sediment incubations (Chapter 3 and 4) were rarefied to 12,852 reads. Rarefaction analysis indicated that the sampling depth was sufficient for representing and comparing microbial communities (Figure A.2A). Amplicon libraries generated from pre-autoclaved Tyne estuary sediments (Chapter 4) were rarefied to 6030 reads (Figure A2B). Autoclaving sediments prior to incubation resulted in reduced species richness compared to pasteurised sediment incubations conducted at the same temperature.

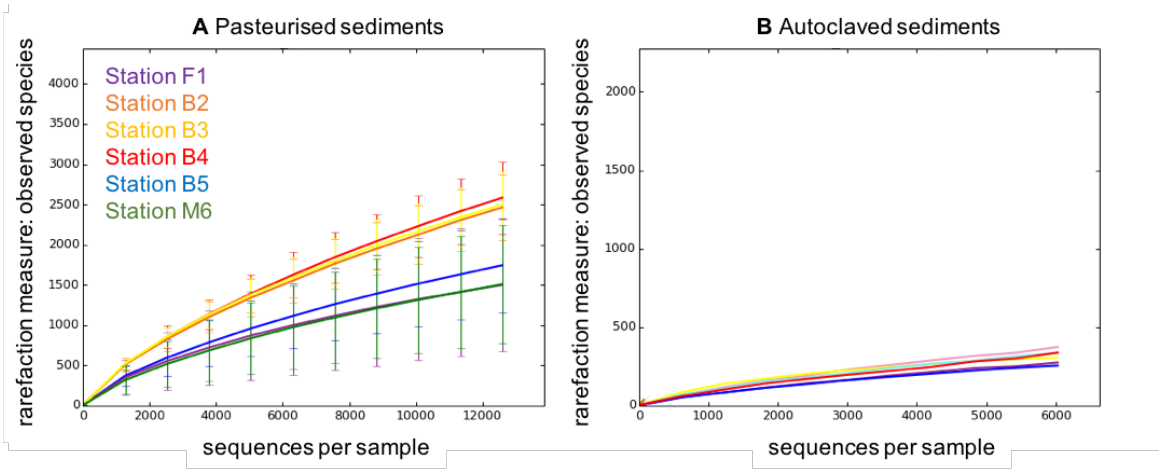


Figure A.2: Rarefaction analysis of amplicon libraries from pasteurised (**A**) and autoclaved (**B**) Tyne estuary sediments (Chapters 3 and 4). Libraries in A are grouped according to station; error bars show the standard error between different amplicon libraries from the same station. Pasteurised incubations at 50, 60 and 70°C (Chapter 4) are included within the group station B3. Rarefaction curves in B each represent a single amplicon library.

In Chapter 3, in instances where an OTU of interest was absent from the rarefied OTU table the unrarefied OTU table was checked. Unrarefied library sizes are shown in Table A.1.

Table A.1: Reads per library generated by Ion Torrent (Chapter 3), from sediment incubations at 50°C.

	0 h	**24 h**	**48 h**	**72 h**	**96 h**	**120 h**
Station F1	17336	23209	12852	27882	25141	22429
Station B2	20430	21280	92272	24064	19882	ND
Station B3	19288	28744	26135	29123	23358	ND
Station B4	21527	32954	21031	24505	ND	ND
Station B5	23125	29584	26441	12900	28458	ND
Station M6	21471	18809	27313	25977	29856	23904

Amplicon libraries in Chapter 5 were generated from individual triplicate DNA extracts in addition to pooled libraries (where PCR amplicons were combined prior to sequencing). Comparing shared phylotypes between single and pooled amplicon libraries indicate that the pooled library was a good representation of the microbial community at the tested sampling points (Table A2).

Table A.2 Libraries were first rarefied to 9159 reads then low abundance OTUs were filtered from the OTU table (OTUs observed less than 92 times). OTUs shared between pooled and single amplicon libraries were compared using the shared_phylotypes.py command in QIIME.

Sample ID	Total number of OTUs in library	Number of OTUs shared	% of OTUs represented
Tyne 116 h rep 1	171	146	85.38
Tyne 116 h rep 2	175	149	85.14
Tyne 116 h rep 3	142	131	92.25
Tyne 116 h POOL	*178*	*178*	*100.00*
Tyne 223 h rep 1	146	125	85.62
Tyne 223 h rep 2	167	119	71.26
Tyne 223 h rep 3	140	118	84.29
Tyne 223 h POOL	*158*	*158*	*100.00*

Appendix B

Amplicon libraries generated by Ion Torrent were analysed in QIIME (Caporaso *et al.*, 2010b). Raw sequence data was processed in QIIME 1.7.0 using a pipeline developed by Gregg Iceton. Core diversity analyses was conducted in QIIME 1.8.0. Analysis steps are outlined below.

(1) Convert the fastq file into fasta and qual files

convert_fastaqual_fastq.py

(2) Split the libraries according to the mapping file, remove sequences <100 bp

split_libraries.py

(3) Pick OTUs using open reference (first compare to Greengenes, then de novo). Align sequences, build tree, assign taxonomy, similarity 0.6.

parallel_pick_otus_uclust_ref.py

filter_fasta.py

pick_rep_set.py

pick_otus.py

pick_rep_set.py

make_otu_table.py

Assign taxonomy

parallel_assign_taxonomy_rdp.py

Add taxa to OTU table command

add_metadata.py

(4) Alignment, filter alignment, filter pynast failures from OTU table

parallel_align_seqs_pynast.py

filter_alignment.py

filter_otus_from_otu_table.py

(5) Identify and remove chimeras from fasta file and OTU table

parallel_identify_chimeric_seqs.py

filter_fasta.py

filter_otus_from_otu_table.py

(6) Make phylogenetic tree

make_phylogeny.py

(7) Core diversity analyses

core_diversity_analyes.py

Appendix C

Table C.1: Primer coverage was tested using the SILVA Test Prime tool (Klindworth *et al*., 2012). Coverage was calculated with 0 mismatches. Primer targets are shown in bold and additional coverage information is shown for comparison between primer pairs.

Primer Pair (Application)	**Domain**	**Phylum**	**Order**	**Class**	**Family**	**Genus**
DEM116F / DEM1164R (DGGE)	*Bacteria* – 0.1%	*Clostridia* – 0.4%	*Clostridia* – 0.4%	*Clostridiales* – 0.4%	*Peptococcaceae* – 20.9%	*Cryptanaerobacter* – 81.3% ***Desulfotomaculum* – 31.6%** *Desulfurispora* – 84.5% *Sporotomaculum* – 75% *Uncultured Peptococcaceae* – 12.7%
pA / pH (Cloning)	***Bacteria* – 22.6%**	*Firmicutes* – 31.0%	*Clostridia* – 32.4% *Bacilli* – 30.9%	*Clostridiales* – 32.4%	Peptococcaceae – 20.3%	*Desulfotomaculum* – 23.8%
V4F / V5R (Ion Torrent)	***Archaea* – 85.4%** ***Bacteria* – 88.0 %**	*Firmicutes* – 87.7%	*Clostridia* – 90.2% *Bacilli* – 83.7%	*Clostridiales* – 90.3%	*Peptococcaceae* – 92.2%	*Desulfotomaculum* – 93.1%