Microbial Community Structure & Function in Estuarine Sediments

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Summary

Microorganisms are ubiquitous, abundant and hugely phylogenetically diverse, showing a wide variety of metabolisms. The catabolic energy and carbon yielding activities of microorganisms play important catalytic roles in biogeochemical cycles which are ultimately fundamental to life on earth. Thus, understanding environmental microbial diversity and how it relates to ecosystem function and how this may change with global change, is a current major challenge of environmental microbiology. It is important to understand microbial transformations in estuarine and coastal sediments because they are key ecosystem components; being important sites for the mineralization of photic zone biomass. Estuarine sediment from the mouth of the Colne estuary was sampled and used to set up slurry microcosms for 16S rRNA archael and bacterial denaturing gradient gel electrophoresis (DGGE) analysis. Geochemical activity measurements and stable isotope probing experiments were carried out to investigate the response of microbial community composition and diversity to phytodetritus (PD) loading and across different redox phases typical of estuarine sediments. The addition of PD resulted in significantly different bacterial communities while the archaeal communities were not significantly different from the control. The increase in bacterial phylotypes was dominated by fermenting Alteromonadales and the versatile Shewanella genus. Stable isotope probing showed ¹³C-glucose utilization by Alteromonadales and Vibrio confirmed stimulation of these fermenting groups in the dysaerobic phase. ¹³C acetate incorporation by phylotypes similar to Firmicutes during the sulphate reduction phase demonstrated how functional groups not previously found to be important at the Brightlingsea site of the Colne estuary, may play an important role in anaerobic carbon mineralization at this site. Thermoplasmatales and MBG-D like phylotypes incorporated ¹³C-acetate suggesting heterotrophic metabolism and the methylated compound utilising Methanosarcinales and the predominantly H₂/CO₂ utilsing Methanomicrobiales also incorporated ¹³C-acetate or its degradation products. This research demonstrates how fermenting sedimentary estuarine microbial communities respond to organic matter loading in estuarine sediments and the prevailing active and dominant groups in the sulphate reduction phase.

This dissertation is the result of my own investigation and where appropriate explicit acknowledgement (with references) has been made to other sources used.

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1.Introduction

1.1. Microbes, the tree of life, and diversity

Microorganisms (microbes) are present in huge numbers on earth, they are ubiquitous with the majority of the earth's prokaryotic organisms occurring in the open ocean, in soil, and in oceanic and terrestrial subsurfaces, with a total estimate of 4-6 x 10³⁰ cells (Whitman et al., 1998). These microbes possess enormous metabolic and physiological versatility being essential to virtually all biogeochemical cycling processes on earth (Prosser, 2007). Our understanding of the microbial world has seen huge changes over the last half century, largely due to the revolution in molecular genetic techniques and the concept that biochemical sequences could be used to describe microbial phylogeny (Zuckerkand and Pauling, 1964). This led to comparative analyses of the small subunit rRNA molecule, which provides the most reliable view of early evolutionary events due to its ubiquity and slow rate of evolution, and has enabled the construction of a molecular rRNA sequence based phylogenetic tree of life (Woese and Fox, 1977) which in turn led to the articulation of the 3 domains of life (figure 1.1) (Woese et al., 1990). This turned the 5-Kingdom model of the classification of life (Whittaker, 1969) on its head and provides convincing evidence that there are in fact three main groups or divisions of organisms; Eukarya, Bacteria and Archaea (Pace, 2006). The term microbe often includes eukaryotic microorganisms which are taxonomically fundamentally different to prokaryotic microbes in that they contain nuclear membranes and other subcellular components. In this thesis the term microbe refers to the prokaryotic domains of Bacteria and Archaea.



It has been a known discrepancy for a long time in microbiology that not all microbes in a sample are recovered through cultivation and in fact it is estimated that >99% of microorganisms observed in nature are not readily cultivated using standard techniques (Anman *et al.*, 1995). Since the major advancement in our understanding of the evolution of life and the development of methods to assess the diversity of mixed natural populations (Stahl *et al.*, 1985), there has been an explosion of molecular genetic data which has discovered many unexpected evolutionary lineages and has shown that the main diversity of life is microbial (Pace *et al.*, 1997). There has been a huge increase in the number of major microbial phyla since 1987 when we knew of about 11/12 major phyla, to 60-80 major phyla in 2006 with many more new classification groups on the family, genus and species levels (DeLong, 2006).

So how do we define and measure microbial diversity? "In its broadest meaning, measuring biodiversity consists of characterising the number, composition and variation in taxonomic or functional units over a wide range of biological organisations from genes to communities" Zinger *et al.*, 2011. Diversity is described as a comparative science referring to spatially or temporally organised unit (Magurran, 2004) and different levels of diversity can be thought of: alpha diversity (the diversity within one location or sample with species richness often being measure); beta diversity (differences in diversity or community composition between two or more locations or samples); gamma diversity (regional diversity usually measured by species richness) (Whittaker, 1972). As well as species richness i.e number of different species there are in a community, the relative abundance of each species is also an important parameter of diversity, and diversity indices take both these parameters into account such as the Shannon diversity index (Shannon, 1948). Clearly there are challenges with these definitions when looking at microbial communities, firstly the challenge of defining a microbial species concept and secondly the challenge of accurately estimating the prevalence of individual species.

There has been much debate on the species concept for microbes since the explosion of genomic data (Achtman and Wagner, 2008). The biological species concept is a genetic definition which is applied to sexually reproducing eukaryotes and cannot be applied to asexual prokaryotes and one alternative to this has been the 'ecological species' concept where bacteria have 'ecotypes' that are adapted to discrete niches where genetically and ecologically distinct species can arise (Cohan, 2002) but this theory is limited in the context of continuous environmental variables and horizontal gene transfer. The microbial species concept is currently defined by a pragmatic and polyphasic approach that is based on clear rules for both genotype and phenotypic pathways (Vandamine *et al.*, 1996) and the prevailing species definition is "a category that circumscribes a (preferably) genomically coherent group of individual

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isolates/strains sharing a high degree of similarities in (many) independent features, comparatively tested under highly standardized conditions" (Gevers *et al.*, 2005). In practice, degree of similarity is assessed in molecular terms and where strains show a degree of phenotype consistency: "a prokaryotic species is considered to be a group of strains (including the type strain) that show phenotypic consistency, 70% of DNA-DNA binding and over 97% of 16S ribosomal RNA (rRNA) gene-sequence identity" (Gevers *et al.*, 2005).

The newly appreciated importance of lateral gene transfer (LGT) and homologous recombination has posed challenges for the species concept (Achtman and Wagner, 2008). Genomics tells us that bacteria often acquire novel genes from outside the ancestral populations by LGT and Coleman et al., 2006, concluded that genetic variability in the cyanobacteria *Prochlorococcus* between phenotypically distinct strains occurs mostly in genomic islands with the 16S rRNA sequences differing by <1%, also showing that genes conferring a highly complex adaptation can be acquired in one event and clearly conceptualising species by vertical gene transfer alone is not ecologically relevant in some cases. In fact Young et al., (2006) describe the bacterial genome in two parts, the core genome and the accessory genome. The realisation that not all bacteria are asexual and lacking recombination but rather that in some homologous recombination is so frequent that it out performs mutations as a source of strain to strain sequence differences (Doolittle and Papke, 2006), has led to the 'species genome' concept (Lan et al., 2000) and the more recent 'pangenome' concept (Fraser-Liggett, 2005) "denoting the total number of genes found in at least one of the strains of a species" (Doolittle and Papke, 2006). Doubt over the validity of any species concept for prokaryotes has been aired "it might not be possible to delineate groups within a continuous spectrum of genotypic variation: that is, clustering might not occur" Gever et al., (2005). For now though, the pragmatic species definition serves a purpose and with the known limited resolution of 16S rRNA gene sequencing, environmental microbial phylogenetic diversity can still be assessed through the analysis of gene sequences encoding 16S rRNA isolated from environmental samples (Garcia-Salamanca et al., 2013; Rappe and Giovannoni, 2003) and the phylogenetic units are termed operational taxonomic units (OTUs) or phylotypes (Schloss and Handelsman, 2005). Levels of similarities of phylotypes can then be compared to estimate evolutionary relatedness of phylotypes (Pace, 2006).

1.2. Functional diversity

Microbial communities, function and biogeochemical cycles

Understanding and quantifying microbial biodiversity remains an exciting and significant challenge in microbiology; 'who's there, what are they doing and how does this relate to ecosystem processes?' (DeLong, 2009).

Understanding the phenotypic traits, biochemistry and function of the morass of microbial diversity is a big challenge. Through the work of dedicated microbiologists, many species and strains have been characterised with respect to phenotype and metabolic capabilities, but the unknown is still large and the question of functionality *in situ* often still remains. We do know that microorganisms have important functional roles within ecosystems, being involved with the carbon cycle and key nutrient cycles such as the cycling of nitrogen (table 1.1), and

Element	Functional Group		
Carbon	autotrophs, heterotrophs, methanotrophs,		
	methylotrophs, methanogens		
Hydrogen	H ₂ oxidisers, H ₂ producers, fermenters.		
Nitrogen	N ₂ fixers, dentrifiers, nitrifiers.		
Sulphur	Sulphur oxidisers, sulphate reducers.		
Iron	Fe ²⁺ oxidisers, Fe ³⁺ reducers.		

Table 1.1:Functional groupsof microbes inrelation toelement cycles.

ultimately they sustain higher life forms on earth. The total amount of prokaryotic carbon on earth is estimated to be 60-100% of that of plants, and about 10 fold more nitrogen (N) and phosphorous (P), representing the largest pool of these nutrients in living organisms (Whitman *et al.*, 1998).

Biogeochemical cycles link microbes in nature where the product of one organism is the substrate for another with all nutrient cycles linking to the carbon cycle in some way. Fundamental to understanding the roles that microbes play in biogeochemical cycling is the notion that "microbial ecology is chemistry" (Robertsom *et al.*, 2005) where microbial cells catalyse energy and carbon yielding reactions through the production of enzymes, which is mediated by transcription and translation of the DNA: the central dogma. The complex interactions that comprise biogeochemical cycles are often considered in terms of the interactions of functional groups such as methanogens producing methane, which is in turn utilised by methanotrophs (Prosser, 2012). Carbon is cycled through Earth's major carbon reserves: the atmosphere, the land, the oceans, freshwater, sediment and rocks, and biomass. There is a large amount of carbon in dead organic matter and the most important contribution of CO2 to the atmosphere is by microbial decomposition of organic matter by chemoorganotrophs. Organic matter in marine sediments is derived from several sources: (1) pelagic organic carbon that reaches the sediment before it is degraded, including that produced by autotrophs (2) benthic organism detritus (3) external organic carbon, for example, run off from agricultural land. In shallow coastal systems a large fraction (50%) of the organic carbon input reaches the seafloor where it drives diagenesis (Help et al., 1995). The quality of organic carbon is an important factor influencing the degradation rates and incorporation efficiency, with structures such as monomeric sugars being easily remineralised, while more complex structures like cellulose are more refractory. The utilisation of organic matter in marine sediments feeds back organic matter otherwise locked in the long rock cycle back to higher trophic levels and near-surface sediment predators such as, nematodes, ciliates and nanoflagellates feed on microbes (Epstein, 1997). This has been termed the microbial loop in planktonic food webs (Azam et al., 1983).



Figure 1.2: Possible fates of **o**rganic matter including important degradation pathways in anoxic sediments (Jørgensen, 2000).

Organic matter is degraded via microbes using respiratory and fermentative metabolisms where respiration in produces more ATP through glycolysis, the electron transport chain and oxidative phosphorylation, while the energy yield from fermentation through glycolysis and substrate level phosphorylation is much lower, but sufficient for growth of the organisms (Jørgensen, 2000). Oxidation-reduction reactions determines the redox couple that is energetically more favourable and pathways with a high-energy gain are preferentially used over pathways with a lower gain, and in marine sediments the availability of electron acceptors is limited which results in a zonation of the degradation pathways (figure 1.2). Thus, in water-logged sediments with a relatively high organic carbon input, oxygen becomes depleted and anaerobic conditions invariably arise where the anaerobic degradation of organic inputs from the water column produces CO_2 and CH_4 as the terminal output. The most important reduction processes in the biodegradation of organic matter are considered to be oxygen, NO₃, manganese (IV), iron (III), sulphate and CO₂, with the final step producing methane. The various processes involved in the biodegradation of organic matter involve complex communities of microbes and there is much to be resolved regarding the succession of microbes in these processes. The metabolic pathway and microbe involved are affected by: (1) the quality and degradability of the organic carbon (2) the availability of terminal electron acceptors (TAEs). These are major factors governing the type of microbes found and they can be grouped into functional categories according to metabolic pathways: biopolymer hyrdrolysing, primary fermenters, secondary fermenters, nitrate reducers, metal reducers, sulphate reducers and methanogens. Sulphate reduction tends to dominate in marine sediments while in freshwater sediments sulphate is limiting, so methanogenesis dominates as the terminal outputharper. In coastal marine sediments, sulphate reduction accounts for up to 50% of organic matter degradation and fluctuating redox conditions in tidal zones mean alternative TEAs become transiently available. Fermentation while not as energetically favourable may be responsible for a large fraction of the dissolve inorganic carbon (DIC) from organic rich sediments near shore sediments (Abell et al., 2009). In dynamic coastal marine sediments, spatial heterogeneity caused by bioturbation and micro-niches means that the vertical pattern of degradation pathways is not always clear and several processes may occur at the same depth (Harper et al., 1999).

Each functional group involved in the degradation of organic matter depends on some of the others to provide suitable substrates for that particular metabolism, for example the primary fermenters produce short chain fatty acids (SCFAs) such as lactate which is in turn utilised by the anaerobic respirers such as the sulphate reducers, and acetate is produced which is then utilised by iron (III) reducers (Lovley, 2006). Syntrophy is very important in the anaerobic breakdown of organic matter where two microbes co-operate to obtain a favourable amount of free energy released (Gibbs free energy, ΔG^0) from an otherwise energetically unfavourable reaction for each. For example, secondary fermenting organisms utilise primary fermentation products which is kept energetically favourable through 'inter-species hydrogen transfer' by anaerobic respirers such as methanogens and sulphate reducers (Stams and Plugge, 2009). The steps that occur after polymer hydrolysis in the breakdown of organic matter in anaerobic environments have been termed 'intermediary ecosystem metabolism' (figure 1.3) (Drake *et al.*, 2009) where syntrophic fatty and aromatic acid metabolism accounts for much of the carbon flux in methanogenic environments (McInerney *et al.*, 2009). Our knowledge of intermediary ecosystem metabolism is limited due to the challenges of ascertaining information on the *in situ* occurrence and activity of the key players (McInerney *et al.*, 2009).

So what do we know about the identity and function of these functional groups that are involved in the biodegradation of organic matter in aquatic sediments? Fermenting microbial communities are important in the first steps of organic matter degradation and studies of anoxic tidal sediments have revealed that bacterial communities in the sediment column are dominated by fermentative bacteria (Köpke *et al.*, 2005; Wilms *et al.*, 2006) as well as being typically found also in other anoxic sediments (Schink, 2002). While the terminal oxidation processes of sulphate reduction and methanogenesis can be studied using functional genes and vertical profiles of sulphate and methane, because of the high diversity of fermenting microorganisms and fermentative pathways, no such key genes are available for this group and are therefore poorly understood (Graue *et al.*, 2012b)

Sedimentary nitrate in estuaries is quickly turned over and becomes depleted when it is no longer recharged by transport of nitrate from the water column and sediments are extremely important processors of nitrate from the water column but only when covered by the tide (Robinson *et al.*, 1998). Nitrate is often limiting at the marine sites of estuaries although hypernutrified estuaries show a high turnover of nitrate where it is constantly re-supplied, for example, the Colne estuary shows strong gradients of nitrate and ammonium from the estuary head to the mouth where 20 to 25% of the total N load entering the estuary is removed by denitrification (Dong *et al.*, 2000). Nitrate reduction is mediated by a diverse polyphyletic group of bacteria (Zumft, 1997) thought to be due to the fact that each bacterial species may participate in only one step of the denitrification process (Burgin and Hamilton, 2007). Genes encoding key enzymes involved in nitrate reduction have been used as molecular markers and in studies on nitrate reducing diversity in soil ecosystems have shown sequences related to

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Actinobacteria, and Alpha-, Beta-, Gammaproteobacteria (Philipot et al., 2002). In the Colne estuary, Smith et al., 2007 showed using membrane bound nitrate reductase narG markers and periplasmic nitrite reductase nrfA (used for nitrate ammonification), that the major clones detected were related to *Deltaproteobacteria*. Smith and colleagues also found that the nitrate and nitrite reductase gene copy numbers declined significantly from the estuary head to the mouth reflecting the nitrate and ammonium gradients in the estuary,

The reduction of iron (III) and manganese (IV) by microbes is environmentally significant in a variety of aquatic sediments where they have been estimated to oxidise anywhere between 10% and 100% of the organic matter in aquatic sediments and submerged soils (Lovley, 2006). Most microbes that can reduce iron (III) can also reduce manganese (IV) although in



Figure 1.3: Intermediary metabolism and major terminal output pathways in anoxic sediments a. in the presence of sulphate, sulphate reducing bacteria consume fermentation products b. in the absence of sulphate, hydrogen and acetate are consumed by methanogens (Muyzer and Stams, 2008).

general manganese oxides are found in relatively low concentrations in marine sediments when compared to ferric iron and sulphate, but like iron, reduced manganese can be reoxidised when diffusing upwards to the oxic zone of the sediment. Thus, iron (III) and manganese (IV) reduction is generally not the predominant terminal electron accepting process in marine estuarine sediments because of the low abundance of the amorphous iron and managanese oxide minerals that are readily utilised by bacteria. However, these energy yielding processes do occur in the suboxic zones and the

oxidised minerals become more transiently available in anaerobic sediments during reoxidation events such as low tides and burrowing by invertebrates such as Arenicola marina in marine mudflats. Microbes known to utilise dissimilatory iron (III) and manganese (IV) reducion pathways to conserve energy and support growth are phylognetically and morphologically diverse, mostly growing by oxidising organic compounds or hydrogen (Lovley, 2006). These include Geobacteraceae of the Deltaproteobacteria, Geothrix of the Acidobacterium and the Gammaproteobacteria Ferrimomas, Aeromonas, and Shewanella, for example, Rosselló-Mora et al., 1994 isolated a facultative Fe (III) reducing strain closely related to Shewanella alga (Gammaproteobacteria) from marsh sediment. Woodshole, USA. The Gammaproteobacteria involved in iron (III) and manganese (IV) reduction incompletely oxidise small organic acids and can use a variety of electron acceptors such as nitrate and fumarate (Lovley, 2006). However, the complex communities involved in dissimilative iron (III) and manganese (IV) reduction do remain poorly understood (Cummings et al., 2013).

Terminal processes of sulphate reduction and methanogenesis in organic matter degradation in marine environments are well studied (for example, Nedwell *at al.*, 2004; Purdy *et al.*, 2002; Kondo *et al.*, 2007) when compared to fermentation processes (Graue *et al.*, 2012b).

Dissimilative sulphate reduction for energy purposes is performed by the sulphate reducing bacteria and produces H₂S which is excreted from the cell. The reduction of SO_4^{2-} requires 8 electrons and the investment of 1 ATP to activate SO_4^{2-} which is relatively chemically stable. This is mediated by ATP sulfurylase and produces adenosine phosphosulfate. This is then reduced to sulfite SO₃²⁻ by APS reductase releasing AMP. The SO_3^{2-} is then reduced to H_2S by sulfite reductase. The electron transport required for the reduction of sulphate is started by periplasmic hydrogenase which reduces H_2 from the environment or H_2 from the conversion of organic acids to pyruvate. The electrons are then carried by cytochrome c3 and transferred across the membrane by a protein complex. This sets up a proton motive force which drives the ATP synthesis. Some sulphate reducers also obtain electrons from the oxidation of acetate to CO₂ through the acetyl-CoA pathway employing carbon monoxide dehydrogenase. Electron donors include H₂, short chain fatty acids including acetate, lactate and propionate, ethanol and other alcohols, long chain fatty acids and various alcohols. A few sulphate reducing bacteria can grow autotrophically using the acetyl-CoA pathway for the incorporation of CO₂ into the cell material and the various sulphate reducing reactions and their free enthalpy yield is shown in table 1.2.

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The sulphate-reducing bacteria have been termed a phenotypic group being found in several different phylogenetic lines (Pfennig & Biebel, 1986). In coastal habitats the most commonly found sulphate-reducing bacteria (SRB) belong to the *Deltaproteobacteria*, with members of the *Desulfobacteraceae* and *Desulfobulbaceae* being reported as the most dominant SRB. *Clostidia* members of the *Firmicutes* and some *Euryarchaeota* and *Crenarchaeota* (of the class *Thermoprotei*) are also known to be sulphate reducers (Muyzer and Stams, 2008). A range of electron donors are found in dissimilatory sulphate reduction metabolism with the commonly cited being acetate and hydrogen.

Equation	$\Delta G^{\circ'}$ (kJ/reaction)*
Sulphate-reducing reactions	
$4 H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4 H_2O$	-151.9
Acetate ⁻ + SO ₄ ²⁻ \rightarrow 2 HCO ₃ ⁻ + HS ⁻	-47.6
Propionate ⁻ + 0.75 SO ₄ ² → Acetate ⁻ + HCO ₃ ⁻ + 0.75 HS ⁻ + 0.25 H ⁺	-37.7
Butyrate ⁻⁺ + 0.5 SO ₄ ²⁻ \rightarrow 2 Acetate ⁻ + 0.5 HS ⁻ + 0.5 H ⁺	-27.8
Lactate ⁻⁺ + 0.5 SO ₄ ²⁻ \rightarrow Acetate ⁻⁺ HCO ₃ ⁻⁺ + 0.5 HS ⁻	-80.2
Acetogenic reactions	
Propionate [−] + 3 H_2O → Acetate [−] + HCO ₃ [−] + H ⁺ + 3 H_2	+76.1
Butyrate ⁻ + 2 $H_2O \rightarrow$ 2 Acetate ⁻ + H ⁺ + 2 H_2	+48.3
Lactate ⁻ + 2 H ₂ O \rightarrow Acetate ⁻ + HCO ₃ ⁻ + H ⁺ + 2 H ₂	-4.2
Methanogenic reactions	
$4 H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3 H_2O$	-135.6
$Acetate^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31.0
Homoacetogenic reactions	
$4 H_2 + 2 HCO_3^- + H^+ \rightarrow Acetate^- + 4 H_2O$	-104.6
Lactate ⁻ → 1.5 Acetate ⁻ + 0.5 H ⁺	-56.5

Table 1.2. Sulphate-reducing,methanogenic andacetogenic reactions(Muyzer and Stams,2008).

Methanogenesis is a unique terminal energy yielding process exclusive to *Archaea* that occurs via two main pathways: CO_2 reduction or the formation of methane from methylated compounds (table 1.2) with the methanogenic archaea displaying heterotrophic metabolism and autotrophy via the acetyl-Co-A pathway. Methanogens commonly detected in marine, salt-marsh, river, estuarine and tidal flat sediments belong to *Methanosarcinales* and *Methanomicrobiales* (Purdy et al., 2002; Parkes *et al.*, 2012). The distribution and prevalence of methanogens is thought to be largely restricted by the presence of other alternative electron acceptors that allow for more thermodynamically and kinetically favourable metabolisms such as sulphate reduction that compete for the major substrates of methanogenesis: H_2/CO_2 and acetate. Thus, methanogenic community structure is thought to be related to the availability of 'competitive substrates', for example, Purdy *et al.*, (2002) compared a predominantly freshwater sediment site of the Cole estuary with a marine site with phylotypes closely

related to the specialist obligate acetate-utilising *Methanosaeta concili* found at the sulphate limited freshwater site, and the more generalist *Methanogenium* being found at both sites of the estuary. Parkes *et al.*, (2012) found a close relationship between the depth distribution of methanogenic substrate utilisation and methanogens that can utilise these compounds in a salt-marsh creek in Dorset, UK where methlyamineutilising *Methanosarcinales* were dominant in the near surface sulphate reduction sediments with *Methanosaeta* being dominant in the deeper sediment layers. Noncompetitive substrates such as methanol and trimethylamine utilised by many *Methanosarcinales* spp. are important substrates for methanogenesis especially in sulphate containing anoxic sediments (Oremland and Polcin, 1982).

1.3. Microbial ecology and global change

A key question of environmental microbiology is whether microbes show similar patterns predicted by ecological theory for macroorganisms. We know that microbial diversity may be important to ecosystem functioning (Brussaard et al., 1997) but testing this with the fundamental parameters used in ecological theory faces challenges when describing microbes due to their smaller size, faster growth, greater dispersal and asexuality. Phylotypes can be described as having a niche, which is a particular set of environmental conditions and resources that they exploit. Measuring species richness is at the heart of much ecological theory and the aforementioned difficulties in describing species and the fluid nature of bacterial genomes means we are a long way from a coherent body of ecological theory for bacterial communities (Prosser et al., 2007). Developing microbial ecological theory is however very valuable so that predictions can be made in practical scenarios and some studies have shown that existing ecological theory can be applied to microbial systems, for example, using species abundance curves to estimate diversity such as Curtis et al., (2002) who used log-normal relationship abundance curves to estimate theoretical species richness on small and large scales. Although, this use of classical ecological theory is limited by the size of clone libraries as curves of in excess of one million clones are required to cover 80% of bacterial species within a 1g soil sample (Gans et al., 2005).



Figure 1.4. Aquatic microbial communities showing biotic and abiotic factors (adapted from Prosser *et al.*, 2007).

Other important areas of ecological theory to investigate include how microbial communities vary over spatial scales, temporal scales and other factors such as competition, diversity-energy relationships, variable activity and behaviour (Prosser *et al.*, 2007) (figure 1.4). Considering spatial scales is important as microbial cells are rarely uniformly distributed in the environment and microenvironments form within niches which determine the availability of resources to microbial cells. Temporal scales are important in understanding microbial communities as microbes have the potential for rapid growth and short generation times, although the maximum is not reached in environmental conditions where resources are limited but under favourable conditions this can lead to variable patterns of microbial diversity over different temporal scales, and evolution in microorganisms can occur rapidly with the convergence of ecological and evolutionary time scales (Prosser *et al.*, 2007). Succession of a community also happens over time and where organic matter is being degraded, changes in the composition of the resource caused by the microbial metabolism changes the community over time and space.

Gaining insights into microbial community composition and function is important in terms of global change scenarios, for example, it has been reported that ocean algae face an uncertain future due to global warming (Behrenfeld, 2011) indicating that primary productivity is likely to be affected by global warming which may impact on microbial community diversity and function (Nogales *et al.*, 2011). Older studies assume

that with higher biodiversity there is a concurrent increased resistance and recovery to perturbations (Loreau, 2001) but the general patterns underlying mechanisms controlling biodiversity and function in microbial communities are still to be elucidated and even though microbes play a key role in ecosystem functioning, the factors regulating diversity and particularly the relationship between diversity and resource availability/productivity (diversity-energy relationships) have rarely been investigated (Langenheder and Prosser, 2008). Edmonds et al., 2008 used terminal restriction fragment length polymorphism analysis and functional gene clone libraries for sulphate reducing bacteria and methanogens (dsrA, mcrA) to investigate the effect of increased high molecular weight carbon resource on coastal tidal creek sediments. They found no difference in community composition between amended and unamended treatments and no relationship between community richness and evenness with resource level (Edmonds et al., 2008). Langenheder and Prosser, (2008) used RNA stable isotope probing to investigate how the diversity and community structure of a heterotrophic benzoate soil degrading bacterial community is influenced by resource concentration. They found that the composition of the benoate degrading community changed with benzoate concentration with a decrease in taxa evenness at higher concentrations. There were generalists active at all resource concentrations and specialists active at one particular concentration or low and high concentrations. Smith, (2007), reviewed microbial diversity-productivity relationships in aquatic systems and concluded that patterns were strikingly similar to those found for macroorganisms. Bienhold et al., (2012) investigated the relationship between a natural phytodetritus gradient on the diversity and activity of bacterial communities in Arctic deep sea sediments. They found richness increased with sedimentary chlorophyll pigment content in oligotrophic sites, levelling off at mesophilic sites, and the dominant tax showed strong positive or negative relationships with the phytodetritus input.

1.4. Estuarine sediments

Estuaries are dynamic and complex environments being in a near constant state of flux with temperate estuaries and coastal margins including mud flats and saltmarshes which are differentially flooded depending on the tides. Large amounts of particulate organic matter (POM) is carried into estuaries which serves as food reservoirs for micro and macro fauna and providing fertile substrate for the flora making saltmarshes amongst the most productive primary producers in the world (Whittaker, 1975). Estuaries provide important ecosystem services: they are important fish nurseries with many being of high commercial value (Nybakken, 2000); migratory birds benefit from invertebrates and safety and they act as sinks for pollutants such as heavy metals. Threats to estuarine systems include hypernutrification caused by nitrate and

phosphate enrichment from agricultural and sewage run-off potentially causing eutrophication, and heavy metal contamination (Bryan and Langston, 1992). Eutrophication can have cascading effects for the estuarine food chain, for example, Mann (2000) described how nitrate and phosphate levels in the Baltic sea which had risen 2-3 times that of the levels in the 1950s due to agricultural run off and sewage caused higher primary production and a 60% increase in oxygen consumption and the water below the halocline becoming anoxic causing the amphipod *Pontoporeacia* population to crash as well as it's predators, the large isopod *Soduria entomon* and small fish *Lumpensus* and their cod predator's population also to crash. So clearly understanding microbial communities that underpin estuarine ecosystems and play important roles in biogeochemical cycling and how they may respond to perturbation is important.

1.5. Aims of Study

It is important to understand microbial transformations in estuarine and coastal sediments because they are key ecosystem components; being important sites for the mineralization of photic zone biomass (Jørgensen, 1982). This research aimed to assess estuarine sedimentary bacterial and archaeal community composition, diversity and function in relation to the biodegradation of organic matter in temperate estuarine sediment:

- Comparisons of the bacterial and archaeal community composition, diversity and function were made between redox phases, and between different levels of phytodetritus loading and raised temperature.
- Stable isotope labelling experiments were conducted to provide more specific information about microbial carbon transformations in temperate estuarine sediment.

Redox phases that are typically present in the depth profiles of marine and estuarine sediments were experimentally simulated over time (figure 1.5). Microcosms were set up and microbial function was assessed through the inference of activity by:

- Geochemical measurements
- DNA stable isotope probing (SIP) experiments
- Changes in community composition (assessed through denaturing gradient gel electrophoresis (DGGE))
- Published knowledge of the physiology and ecology of identified community members.

The study site was the Colne estuary, a small hypernutrified and turbid estuary on the east coast of the UK entering the North Sea at Brightlingsea. This thesis describes the methodology used for the research, with the third chapter focusing on the SIP approach and optimisation, the fourth chapter outlining the geochemistry of the experiments, the fifth chapter outlining the community compositions, diversity, stable isotope probing and community comparison analyses, with the fifth chapter discussing the results.



Figure 1.5: Redox depth pattern common in marine and estuarine sediments (not always clearly zoned) and experimentally simulated over time. The length of time depends on experimental conditions. Depth at which redox zonations occur and the degree of zonation varies with site. Measurements at the Brightlingsea sample site of the Colne estuary measured sulphate reduction to be occurring at 30cm sediment depth and methanogenesis was measured to be occurring at 43cm depth (O'sullivan *et al.*, 2013).

2. Methodology

2.1. Colne estuary microcosms



Site description and sampling

The study site was The Colne Estuary situated on the east coast of England entering the North Sea at Brightlingsea in Essex. It is a relatively small macrotidal estuary with a catchment of 500 km² of which the River Colne drains 300 km² much of which is arable land (Kondo *et al.*, 2007). It is a turbid and muddy estuary, and there is a sewage treatment work serving the town of Colchester (figure 2.1) which discharges into the estuary (Thornton *et al.*, 2002). The estuary is hypernutrified with a pronounced gradient of inorganic nutrients including NO₃⁻ and PO₄³⁻, and organic carbon inversely related to the salinity gradient (Thornton *et al.*, 2002). Dong *et al.*, 2002 showed that the salinity at the top of the estuary near Colchester was variable in the range of 2 to 17 ‰, in the middle region near Alresford Creek it was less variable in the range of 20 to 32 ‰ and at the estuary mouth near Brightlingsea the salinity was more stable in the range of 28 to 34‰. Sediment characterisation by Dong *et al.*, 2002 showed that sediments at Hythe and Alresford were fine silt while at Brightlingsea the sediment was largely clay with a very thin layer of muddy sand on the surface.

Estuarine sediment cores were collected in October 2005 and February 2006. Cores with a diameter of 6 cm (figure 2.1) were taken at low tide from three sites of the Colne estuary: an open mud creek at the estuary mouth (BR; 51°47.920'N, 001°01.075'E), a mid-estuarine creek [Alresford (AR); 51°50.716'N, 000°58.912'E] and a salt marsh at

the estuary head [Hythe (HY); 51°52.687'N,000°56.011'E] (figure 2.2). 10 cm diameter and 30–60 cm long sediment cores were collected at each site and at each sampling time; cores were sealed with rubber bungs and transported to the laboratory on ice and stored at 10 °C in a cold temperature room until processing.

2.1.a. Preliminary experiments

Experiments were set up in February 2006 to test two main areas (1) whether active populations in the sulphate reduction zone along an estuarine salinity gradient were the same and how that compared to the total community (2) whether the response to organic loading of the active and total populations were the same. These experiments were set up using sediment from Brightlingsea (salinity of 530 mM), Alresford (salinity of 500 mM) and Hythe (salinity of 160 mM). Syringes and anaerobic bags were used for the sub-slurries and once significant sulphate reduction had established in the slurries stable isotope probing experiments were set up using some kind of catalytic effect in the slurry. The experiments were terminated as the ¹³C-acetate label was too dilute. Thus modifications to the experimental set-up for the stable isotope probing experiments were outlined in the next section.

2.1.b. Brightlingsea experiments

Master slurries

475 cm³ of sediment from the top 0-20 cm of cores was added to 1500 cm³ of oxic mineral salts medium (Wellsbury *et al.*, 1994) with a salinity of 530 mM. 15 ml of 0.5 M Na₂HPO₄ was added to the slurries to buffer the system. Five slurries were made in total with a control and varying concentrations of phytodetritus (PD) and an elevated temperature of 18°C (table 2.1). Slurries were contained in modified 2-L bottles with a three-way stopcock at the base for slurry sampling and foam bungs at the top opening during the aerobic phase and screw caps in the dysaerobic and anaerobic phases with three-way stopcocks for headspace sampling (figure 2.2). The vessels were repressurised with oxygen free nitrogen when headspace samples and sediment slurry samples were taken. These were incubated in the dark at the corresponding temperatures on orbital shakers.

Phytodetritus concentration (mg/ml)	Incubation temperature (°C)
A = 0.2 mg/ml	10
B = 1 mg/ml	10
C = 5 mg/ml	10
D = 5 mg/ml	18
E = 0 mg/ml	10

 Table 2.1. Brightlingsea slurry treatments.

Slurries were connected to an air supply with a flow rate of approximately 100 ml/minute to establish aerobic conditions. Oxygen measurements were taken every 24 hours using a Rank Bros Ltd oxygen electrode. The system was calibrated using slurry medium for 100 % saturation and sodium dithionite for 0% saturation. Sediment samples were taken for pore water analysis and headspace samples were taken during the dysaerobic and anaerobic phases. The master slurries were sub-sampled for stable isotope probing (SIP) experiments in the geochemical phases simulating the vertical zonation of the degradation pathways: aerobic-100% oxygen; dysaerobic 1% and 10% oxygen (Raiswell and Canfield, 1998); sulphate reduction, a significant decrease in sulphate with time; a significant decrease in methane with time.1 ml of slurry was taken for estimating microbial numbers and immediately fixed in 9ml of filter sterilised 2% w/v formaldehyde of salinity 530 mM NaCl to match the slurry medium.



Figure 2.3. Brightlingsea master slurries (blue text shows phytodetritus loading and incubation temperature)

Sub-slurry experiments

Sub-slurries were set up for all treatments in the aerobic and dysaerobic phases to serve as a comparison between the different PD treatments. The sub-slurry experiments were carried out using a modified approach to ensure anaerobic conditions following the challenges encountered in the preliminary experiments. Therefore different air-tight vessels were used rather than syringes. For each incubation phase 3 sub-slurries were made by aseptically transferring sediment slurry from the corresponding master slurry, two sub-slurries with c.165 ml of master slurry in 250ml vessels and one sub-slurry with 60ml in a 100ml vessel (figure 2.3). For all experiments ¹³C-label was added to one of the 250ml vessels, ¹²C-label to the other 250ml vessel and no substrate was added to the 100ml vessel control. For the aerobic and dysaerobic slurries 100 µM (final concentration) of fully ¹³C-labelled glucose was added at the start of the incubation and then a further 100 µM ¹³C-labelled after 24 and 48 hours of incubations, and the same protocol was carried out for the ¹²C-label. Fully ¹³C-labelled acetate was added to the anaerobic (sulphate reduction and methanogenesis phase) incubations at final concentrations comparable to that in the master slurry which was 14 mM for both slurries in the sulphate reduction phase, and 1 mM for the 10 °C slurry and 200 µM in the 18 °C (exactly the same final concentration of ¹²C-acetate was added to the ¹²C control incubations).

The aerobic phase vessels were closed from the environment with autoclaved foam bungs and the dysaerobic and anaerobic experiments were sealed with srew-caps and 3 way valves for sampling which was done in the a laminar flow cabinet. Samples for pore water analysis, DNA fractionation and molecular biology analysis were taken from each experiments at time zero and after 6, 12, 24 and 48 hours except the 12 hour sample was not taken for the anaerobic experiments. 20 ml of slurry were taken for DNA analysis and frozen in liquid nitrogen and then stored at -80 °C for storage until processing.



Figure 2.3. Outline of stable isotope sub-slurry set-up.

Porewater analysis

5ml sediment slurry was sampled from the master and sub-slurries for pore water analysis. The slurry was centrifuged for 15 minutes at 2300 *g* in a Hettich Rotanta 460T centrifuge at 10 °C, the supernatant was retrieved and the pellet was saved and stored at -20 °C as back up molecular genetics samples. Porewater was diluted by a factor of 4 and nitrate, phosphate, sulphate, chloride and short chain fatty acid (SCFA) concentrations were determined using an ICS-2000 ion chromatography system with an AS50 autosampler (Dionex UK Ltd) fitted with two lonpac AS15 columns in series. Determination of species was carried out using an anion self-regenerating suppressor (ASRS-ULRA II 4-mm) in combination with a DS6 heated conductivity cell (Dionex UK Ltd). Concentrations were calculated using standard calibration curves and reproducibility was good with σ between 0.5 and 5%. Some of the pore water samples were analysed collaboratively with Joachim Rinna, postdoctoral research associate, School of Earth and Ocean Sciences, Cardiff University.

Headspace analysis

Headspace samples were taken a gas tight syringe and the slurries were overpressurised to 100 mbar. Samples were injected directly into the valve injection system and analysed using an Arnel Modified Model 2101 Natural Gas Analyzer (NGA) Natural Gas Analyser two channels: channel A detected hydrocarbons and carbon dioxide with helium as the carrier gas and two detectors in series used for compound detection: a thermal conductivity detector followed by a flame ionisation detector; channel B detected hydrogen with nitrogen as the carrier gas and one thermal conductivity detector for calibration curves of three gas standards with different concentrations. Reproducibility for hydrocarbons was $1\sigma = 2\%$ and for H₂ and CO₂ $1\sigma = 2-5\%$.

DNA extraction

Sediment slurry DNA was extracted from 5 x 1g sediment slurry (master slurry, ¹³Cenriched sediment ¹²C-substrate control and no substrate control slurries) using the FastDNA SPIN Kit for Soil (MP Biomedicals) with modifications as described by Webster *et al.*, (2003). Genomic DNA was extracted from pure cultures of *Bacillus subtilus*, *Bacillus licheniformis* and *Pseudomonas putida* strain 3 using the FastDNA SPIN kit (MP Biomedicals). Extracted DNA was visualised using agarose gel electrophoresis and the DNA was quantified against Hyperladder I DNA marker (Bioline) using the Gene Genius Bio Imaging System (Syngene).

CsCl density gradient ultracentrifugation

The optimisation of the CsCI density gradient centrifugation and DNA fractionation is outlined in chapter 3. 4 μ g of extracted DNA of both the ¹³C- and ¹²C- label were separated in a CsCI solution of final average density of 1.755 g/ml which was decanted into a Quick–Seal pollyallomer ultracentrifuge tube (~13.5 ml; 16 × 76 mm; Beckman Coulter UK). DNA fractions were resolved following ultracentrifugation at 177 000 g using a VTi 65.1 vertical-tube rotor (Beckman Coulter UK) for 44 h at 20 °C (Radajewski et al., 2000). DNA fractions were collected in a drop-wise fashion and the DNA was recovered by precipitation with 1 μ l glycogen and PEG solution following the protocol of Neufeld *et al.*, (2007).

16S rRNA gene PCR-denaturing gradient gel electrophoresis (DGGE) analysis

Bacterial 16S rRNA genes were amplified directly from DNA extracted from sediment slurry and from the recovered DNA from the CsCl gradient fractions of the ¹³C-substrate gradients and the ¹²C substrate gradients with the primer set 357FGC-518R (Muyzer *et al.*, 1993) (table 2.2). 50 μ l PCR reactions contained 1.5 mM MgCl₂, 200 μ m each dNTP (Promega), 200 nM each primer, 10 μ g bovine serum albumin (BSA) (Promega), 1 μ l DNA template and 2 units *Taq* DNA polymerase (Promega), in 1 x PCR buffer (Promega), Bacterial DGGE PCR products were generated with the following programme: were generated with the following programme: 95 °C for 5 min; 10 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for

1 min; 26 cycles of 92 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min; followed by 10min at 72 °C (O'Sullivan *et al.*, 2008).

The archaeal 16S rRNA genes were amplified from the same samples using a nested PCR approach with the first round with archaeal primer sets 109F and 958R (GrossKopf *et al.*, 1998; DeLong, 1992) using the same PCR reagents as the bacterial PCR described above with the archaeal 16S rRNA gene PCR products being generated with the following programme: 98 °C for 30 s; 5 cycles of 92 °C for 45 s; 45 °C for 45 s; 72 °C for 45 s; followed by 5 minutes at 72 °C. This was followed by a dilution of 1 in 20 with sterile nuclease-free molecular-grade water (Severn Biotech Ltd.) and another round of PCR using SAfGC-PARCH519R (Øvreås *et al.*, 1997) (table 2.2) and the same PCR reagents as previously described except the BSA was omitte and the volume made up with sterile nuclease-free molecular-grade water (Severn Biotech Ltd.). Archaeal DGGE PCR products were generated with the following programme: 95 °C for 5 min; 5 cycles of 94 °C for 30 s, 53.5 °C for 30 s and 72 °C for 1 min; 30 cycles of 92 °C for 30 s, 53.5 °C for 30 s and 72 °C for 1 min; 4*l*, 2008).

PCR products were then checked using agarose gel electrophoresis and ladder and then *c*.100 ng of each PCR product was separated using denaturing gradient gel of urea and formamide and movement of the DNA fragments through the gradient by electrophoresis; denaturing gradient gel electrophoresis (DGGE) as done by Webster *et al.*, 2003. 8% polyacrylamide gels with a 30-60% denaturing gradient (100% denaturant was equivalent to 7M urea and 40% (w/v) formamyde) were cast 1-mm-thick in 16 x 16 cm glass plates being poured with a 50-mL volume Gradient Mixer (Fisher Scientific, Loughborough, UK) and prepared with 1x TAE buffer (pH 8; 40mM Tris base, 20mM acetic acid, 1mM EDTA). A stacking gel free of denaturant was poured on top of the resolving gel, allowing the focusing of the DNA into a sharp band before it enters the resolving gel, ensuring reproducibility. Electrophoresis was at 200 V for 5 h (with an initial 10 min at 80 V) at 60 °C in 1 x TAE buffer. Polyacrylamide gels were stained with SYBR® Gold nucleic acid gel stain (Molecular Probes) for 30 min and viewed under UV. Gel images were captured with a Gene Genius Bio Imaging System (Syngene).

PCR primer	Sequence (5'-3')	Reference
357F-GC	CGCCCGCCGCGCGCGGGG CGGGGCGGGGGGCACGGGGG CCTACGGGAGGCAGCAG	Muyzer <i>et al</i> . (1993)
518R	ATTACCGCGGCTGCTGG	
SAf ^a		
SA1t	CGCCCGCCGCGCGCGGGG	Øvreas <i>et al.</i> (1997)
	CGGGGCGGGGGGGCACGGGGGGG	
0.4.05		NICOI <i>et al</i> . (2003)
SAZI		
109F		GrossKonf et al. 1998
958B	YCCGGCGTTGAMTCC	Del ong 1992
357F-GC-M13B	CAGGAAACAGCTATGAC	O'Sullivan <i>et al.</i> , 2008
	GGGCGGGGCGGGGGGCACGGGGGG	
	CCTACGGGAGGCAGCAG	
518R-AT-M13F	GTAAAACGACGGCCAG	
	ΤΑΑΑΤΑΑΑΑΤΑΑΑΑΑΤGΤΑΑΑΑΑΑΑ	
	TTACCGCGGCTGCTGG	
SAf-GC-M13R⁵:		O'Sullivan <i>et al</i> ., 2008
SAaf-GC-M13R	CAGGAAACAGCTATGAC	
	GGGCGGGGGGGGGGGCACGGGGGG	
	CCTACGGGGCGCAGCAGG	
SAbf-GC-M13R	CAGGAAACAGCIAIGAC	
	GGGCGGGGCGGGGGGCACGGGGGG	
SANT CO MIND		
SACI-GC-IVITSR		
PARCH519r-AT	GTAAAACGACGGCCAG	
M13F		TOPO TA cloning®
	TTACCGCGGCKGCTG	(Invitrogen)

Table 2.2. PCR primers utilised in this research. ^a Primer SAf is a mixture of SA1f and SA2f in a 2:1 molar ratio (Nicol *et al.*, 2003). ^b Primer SAf-GC-M13R is a mixture of SAaf-GC-M13R, SAbf-GC-M13R and SAcf-GCM13R in a 1:1:1 molar ratio (O'Sullivan *et al.*, 2008).

As many bands as possible, and those of particular interest were excised under UV using a sterile scalpel, leaving the edges of the bands to minimise cross contamination between bands. Bands were stored in UV treated PCR tubes and frozen at -20 °C if the DNA was not extracted straight away. DNA was then extracted from the gel bands by washing with molecular grade water, drying in a laminar flow hood for, re-suspending in 10 µl sterile nuclease-free molecular-grade water (Severn Biotech Ltd.) and mashing with a pipette to aid liberation of DNA. Samples were then frozen and thawed before used as PCR templates. 1 µl of the supernatant was then used as template DNA for re-amplification of the DGGE bands with modified linker-PCR bacterial (357F-GC-M13R and 518R-AT-M13F) and archaeal (SAf_GC-M13R and PARCH519r-AT-M13F) primer sets (O'Sullivan *et al.*, 2008). PCR reactions contained the same reagents (with BSA) as the initial amplifications with the PCR-DGGE primers, and the re-amplified DGGE

PCR products were generated with the same programmes as the initial bacterial and archaeal DGGE PCR products.

Sequencing and phylogenetic analysis of excised DGGE bands

Re-amplified PCR products were sequenced using an ABI 3130x1 Genetic Analyzer (Applied Biosystems) with primer M13F (thanks to Steve Hope for performing the sequence reactions). Sequences were reverse transcribed, visually inspected and edited using FinchTV 1.4.0 (Geospiza Inc). Partial bacterial 16S rRNA gene sequences were subjected to a NCBI BLASTN (http://blast.ncbi.nlm.nih.gov) search to identify sequences with highest similarity. Closest cultured representatives were found by excluding uncultured/environmental sequences in the search parameters. Phylotypes were defined as the same species by similarity values of \geq 97%, \geq 95% were the same genus and \geq 80% the same phylum. While these definitions do not constitute a rigorously validated hierarchy of taxonomy (Schloss and Handelsman, 2005) and the realisation that 16S rRNA is of limited use when considering other parameters of microbial genomes such as lateral gene transfer (Achtman and Wagner, 2008), it is a nonetheless useful approach when assessing environmental microbial community structure with 16S rRNA sequence fragments.

Groups of sequences were compared with published environmental sequences using tree-building methods where possible as another way of assessing the composition of the communities. Groups of sequences were saved as alignments using Clustalx. These were then aligned in Mega 5.05 using MUSCLE, uneven ends were trimmed and duplicate sequences were removed by computing pairwise distances. The reliability of the alignment was checked by determining the average distance under the p-distance model and if the average distance was more than 0.33 the algnment was not used to estimate a tree. This ensured that a minimum of 66% sequence identity was upheld to ensure an alignment accuracy of about 50% (Kumar and Filipski, 2006). The suitability of the data for a neighbour-joining tree was assessed by determining the average Jukes-Cantor distance and only if <1.0 was a neighbour-joining tree constructed as otherwise the data is not suitable for that method (Hall, 2011). Neighbour-joining trees were estimated using the Jukes-Cantor model with bootstrapping test of phylogeny with 1000 bootstraps.

DGGE profile analysis using Phoretix 1D (Totallab)

DGGE profile patterns for the amplified archaeal and bacterial 16S rRNA genes were analyses and compared using Phoretix 1D (Totallab). Background was subtracted using the rolling ball technique and band detection was carried out with the following parameters: Minimum slope of 100, noise reduction of 9 and a percentage max peak of 8. Rf calibration was performed to standardise positions on the gels using the marker lanes. At this stage the number of bands detected through the designated parameters were collated for each gel. Lanes were then matched between groups of gels to be analysed and the dendrogram built using the Unweighted Pair Group Mehod with Arithmetic mean (UPGMA) using the highest average similarity between two entries or clusters to determine which to merge, and the Dice cooefficient, which has been shown to produce better dendrograms for RTLP-type-experiments. (Rogers et al., 2006).

Statistical analysis

Bacterial and archaeal band numbers between each treatment and between each defined incubation redox phase were compared using two-tailed t-tests performed on Minitab 15 and those of probability ≤0.05 were accepted as significantly different. The data were first tested for suitability for parametric testing by assessing whether the data were normally distributed using an Fmax test. In cases where the data were not normally distributed a Mann-Whitney U test was performed on Minitab 15. Regression analyses were also performed on Minitab 15.

3. Stable Isotope Probing

3.1. Stable isotope labelling Studies

The use of stable isotopes in microbial ecology stems from the 'you are what you eat' concept where the natural isotopic composition of carbon in consumers can be used to identify the origin of organic matter (Boschker and Middelburg, 2002). For example, Norman *et al.*, 1995 used stable isotope ratios to trace carbon phytoplankton to bacteria during a diatom bloom where the diatom carbon was preferentially used over the background more refractory dissolved organic carbon (DOC). This concept has been developed further combining stable isotope and biomarker analysis allowing carbon cycling to be investigated in more detail, identifying carbon cycling at the molecular level, providing a more thorough understanding of microbial processing in complex systemsby linking carbon specifically to microbes. For example in furthering our understanding of the mechanisms of, and the identity of those involved in, the anaerobic oxidation of methane (for example, Hinrichs *et al*, 1999; Kellermann *et al.*, 2012).

Stable isotope probing (SIP) is a culture-independent technique that enables direct linking of microbial populations to specific biogeochemical processes by ¹³C-labelling of biomarkers (Boschker *et al.*, 1998) and their subsequent extraction and analysis. This involves exposing an environmental sample to stable isotope-enriched substrates and subsequently analysing the labelled biomarkers for enrichment and identity information. Phospholipid fatty acids (PLFA) have been important biomarkers for SIP studies over the last decade, particularly studies investigating methanotrophy in soils (e.g. Bodelier *et al.*, 2012; Shrestha *et al.*, 2008). Nucleic acid SIP experiments using ¹³C compounds and retrieving labelled nuclei acids through fractionation has become a widely used tool in microbial ecology to understand complex communities important in carbon cycling (e.g. Webster *et al.*, 2010; Miyatake *et al.*, 2013) and more recent microbial cell specific stable isotope probing techniques have been applied to complex communities, for example, Mayali *et al.*, (2013), applied Chip-SIP, 'a microarrays and NanoSIMS based approach, to investigate taxon-specific C/N relative use efficiency for amino acids in an estuarine community'.

PLFA-Stable Isotope Probing (PLFA-SIP)

Incorporation of label into PLFAs is analysed by a combination of gas chromatography and isotope ratio mass spectrometry (GC-c-IRMS), which produces a PLFA profile (figure 3.1). These profiles are then compared with profiles of cultured organisms, which may provide taxonomic information on the labelled organisms.



Figure 3.1: DNA-based stable isotope probing (SIP) and ¹³C-phospolipid fatty acids (PLFA) analyses (Dumont & Murrell, 2005)

The first major SIP study was conducted by Boschker *et al.*, 1998, in which they exposed two communities with ¹³C-labels. A sulphate-reducing community from the Tamar estuary was exposed to ¹³C -acetate in the range of the *in situ* pore water acetate concentration (100 μ M final concentration) and PLFAs were then analysed for labelling. It was found that four PLFAs had incorporated most of the label (figure 3.2).



Figure 3.2: Percentage of added ¹³C-labelled acetate incorporated into PLFAs (Boschker et al., 1998).

Molybdate inhibition experiments provided evidence that the label had been incorporated by sulphate reducers and the profile matched up with that of the Grampositive *Desulfotomaculum acetoxidans*. This was interesting as acetate-utilizing isolates from anaerobic marine sediments had previously mostly belonged to the Gram-

negative genus *Desulfobacter*, underlining the importance of using cultivation independent methods like SIP.



Figure 3.3: Amounts of ¹³C-methane incorporated into PLFAs (Boschker *et al.*, 1998).

Boschker *et al.*, 1998, also demonstrated that PLFA-SIP can be used on freshwater sediments to label methanotrophs. There are clear differences in the PLFA compositions of type I methane oxidizing bacteria, which have predominantly 16-carbon mono-unsaturated PLFAs and type II, which contain 18-carbon mono-unsaturated PLFAs. After exposing the freshwater sediment to labelling with ¹³C-methane, 16-carbon PLFAs showed clear enrichment (figure 3.3) which suggested that type I methanotrophs dominated methane oxidation and high label incorporation into 16:1 compared to 14:0 and 16:0 suggested the dominant methanotrophs belonged to *Methylobacter* and *Methylomicrobium*. Bull *et al.*, (2000), conducted ¹³C-methane labelling experiments on soil from a temperate forest and showed, through analysis of labelled PLFA, the presence of a new methanotroph.

DNA-Stable Isotope Probing (DNA-SIP)

The rationale behind the DNA-SIP approach is based on the experiments that proved semi-conservative replication of DNA, where it was shown that DNA labelled with ¹⁵N could be separated from DNA containing ¹⁴N by density gradient centrifugation (Meselson & Stahl, 1958 cited in Dumont & Murrell, 2005). DNA in environmental samples which becomes ¹³C labelled when incubated with ¹³C -labelled substrates is extracted and subjected to caesium chloride density gradient centrifugation which separates the 'heavy' ¹³C DNA from the 'light' ¹²C DNA. These form two distinct bands when stained and illuminated under ultraviolet light (figure 3.4). These two fractions of DNA are retrieved from the gradient with a needle and syringe or by fractionation and the DNA from the heavy fraction can then be used as a template in PCR using general primer sets that amplify 16S rRNA genes and primers that amplify functional genes, for example, dissimilatory sulphate reductase primers. The analysis of the PCR products enables the identification of the microorganisms that have assimilated the ¹³C-labelled substrate to be revealed (figure 3.1).



Figure 3.4: Equilibrium centrifugation of isotopically labelled DNA in CsCl/ethidium bromide density gradients (Radajewski *et al.*, 2000)

The first major DNA-SIP study was done by Radajewski *et al.*, (2000), who conducted microcosm experiments to investigate methanol-utilizing microorganisms in soil. Oak forest soil was incubated with ¹³C-labelled methanol and enriched and depleted DNA was separated using density gradient centrifugation. The enriched DNA was amplified using 16SrRNA primer sets and amplification products were cloned and clone libraries were constructed. The authors screened the libraries by digestion with restriction endonucleases and complete sequences were obtained for each unique restriction pattern. Phylogenetic analyses showed that bacteria within two distinct lineages of the *Alphaproteobacteria* and *Acidobacterium* ad assimilated ¹³C –methanol.

The major advantage of DNA-SIP is the potential for high taxonomic resolution using a range of molecular tools: denaturing gradient gel electrophoresis (DGGE), the construction of clone libraries, DNA sequencing, microarrays to identify which amplified genes are the most abundant and metagenomic approached such as the construction of metagenomic libraries (for example, Schloss & Handelsman, 2003). The disadvantage of DNA-SIP is that is the least sensitive SIP approach because, unlike PLFA- and RNA-SIP, incorporation of the labelled substrate requires cell division and DNA synthesis, so longer incubations are required.

RNA-Stable Isotope Probing (RNA-SIP)

The approach of RNA-SIP is similar to that of DNA-SIP and was first demonstrated by Manefield *et al.*, (2002), who investigated an industrial bioreactor community responsible for the bioremediation of phenol-contaminated wastewater. The degradation of 500 μ g ¹³C-phenol mL⁻¹ was monitored over 8 hours and incorporation into RNA and DNA was measured using isotope ratio mass spectrometry (IRMS) which showed that the incorporation of label into RNA exceeded that of DNA.

RNA samples taken throughout the SIP were subjected to caesium trifluoroacetate density gradient centrifugation to separate ¹³C -labelled RNA from ¹²C-unlabelled RNA. Labelled RNA was retrieved from the gradient, reverse transcribed and amplified for DGGE analysis. The DGGE gel showed that after 8 hours there was a change in the number and intensities of bands and the five most prominent bands were excised, re-amplified, cloned and sequenced, and it was found that the organism that dominated the acquisition of carbon from the labelled phenol was a member of the *Thauera* genus of the *Betaproteobacteria*. The importance of culture-independent functional approaches is highlighted in the study conducted by Manefield *et al.*, (2002), as previous work using culture dependent and molecular methods had found that *Pseudomonad* species and species belonging to the *Gammaproteobacteria* and *Cytophaga-Flavobacterium* groups dominated such reactor communities.

Stable Isotope Probing: insights into microbial *in situ* physiology: labelling with multi-carbon compounds

Widening the scope of stable isotope probing studies to include labelling with multicarbon compounds has been an important step in understanding microbially mediated anaerobic carbon transformations. It is important to understand these transformations as they are key ecosystem components; estuarine and coastal sediments are important sites for the mineralization of photic zone biomass (Jørgensen, 1982). The low weight molecular intermediates, such as acetate and propionate, produced by fermentative bacteria, in 'intermediary metabolism' and then the mineralization of these by terminal oxidisers can be investigated through SIP using ¹³C labelled intermediate compounds such as acetate.

Boschker *et al.*, (2001) conducted a PLFA-SIP study to investigate the degradation of ¹³C-labelled propionate and acetate in anoxic brackish sediments. It was found that acetate and propionate were predominantly consumed by different, specialised groups of sulphate-reducing bacteria: for ¹³C -propionate there was no resemblance to known strains while the ¹³C -acetate labelled PLFA profile was similar to the acetate consuming sulphate-reducers, *Desulfomaculum acetoxidans* and *Desulfofrigus* spp.

Leuders *et al.*, (2004), conducted the first anoxic soil SIP study, investigating propionate degradation in flooded soil using RNA-SIP. Due to thermodynamic constraints, propionate oxidation is carried out via a 'syntrophic' partnership between, propionate-fermenting, proton-reducing bacteria and hydrogen-consuming methanogens. It was found, after 7 weeks of incubation that ¹³C-labelled RNA belonged to *Syntrophobacter*

spp, *Smithelia* spp and the novel *Pelotomaculum* spp. While ¹³C-labelled archaeal RNA belonged to members of the Rice Cluster I, *Methanobacterium* and *Methanosarcina* spp.

Webster *et al.*, (2006) conducted the first comparative DNA- and PLFA-SIP study on marine anaerobic sediment slurries, using a range of multi-carbon ¹³C-labelled compounds: glucose, acetate and pyruvate, and providing the first indication of the metabolism of JS1, a candidate division originally found in deep subsurface sediments. It was found that in the glucose-labelling experiments that there was widespread incorporation into PLFA and DNA with the identity of the major glucose utilizers remaining unclear. The ¹³C-labelled PLFA profiles in the acetate and pyruvate labelling experiments were similar, with diagnostic PLFA (i15:0, a15:0 & 15:1w6) possibly indicating the presence of *Desulfococcus* or *Desulfosarcina* and sequences related to these were found in the ¹³C -acetate DNA gene library. DGGE analysis of the ¹³C –acetate labelled DNA showed that *Firmicutes* and JS1 were actively assimilating acetate. JS1 was also present in the ¹³C -glucose labelled DNA, showing that JS1 metabolizes acetate and glucose (or metabolites).

Recent studies have used ¹³C-labelled macromolecular substances with DNA SIP approaches to study the degradation of organic matter, for example, Gihring *et al.*, (2009) added ¹³C-enriched, heat-killed *Spirulina* cells to intact Gulf of Mexico sediment core incubations with particular interest in the fermenting communities. Graue *et al.*, (2013) also used a ¹³C-enriched *Spirulina* SIP approach to study decomposition in anoxic tidal flat sediments.

3.2. Stable Isotope Probing optimisation for Colne estuary microcosms

DNA-SIP was applied to the Brightlingsea site Colne estuary sediment microcosms as outlined in chapter 2. The protocol used was largely based on Neufeld *et al.*, (2007) rather than the original DNA-SIP protocol (Radajewski *et al.*, 2000). This method was chosen as compared to the visualisation of DNA bands with ethidium bromide (EtBr) and their recovery with a syringe and needle, the fractionation based approach is reported to provide a more objective recovery of all visible and invisible DNA from across the entire gradient. Neufeld *et al.*, (2007), published a detailed protocol for DNA-SIP in which two CsCI density gradient centrifugation approaches were detailed. One approach involves the addition of EtBr for visualization of the two DNA bands and
collection of these fractions with a needle from the side of the centrifuge tube. This method is advantageous as it can be seen immediately after centrifugation whether the gradient has formed. Also, the gradient does not move a great distance through the centrifuge tube upon fraction collection reducing the risk of contamination from other fractions. Disadvantages of this approach are as follows: detail of the gradient is lost as not many fractions are collected; involves handling of EtBr in high concentrations which is mutagenic and thought to be carcinogenic. The other approach detailed in this protocol is the centrifugation of DNA in a solution of CsCl of higher concentration without EtBr and the collection of fractions in a dropwise fashion. The advantages of this method are: the whole gradient is collected in a sequential manner (important in accessing extent of ¹²C DNA smearing into ¹³C band); no EtBr is required making handling safer and quicker. The disadvantage of this approach is that the DNA bands cannot be visualised, although it is often only the ¹²C band that can be seen after SIP on a complex environmental community.

The procedure was optimised through several steps:

- Reference ¹³C-enriched DNA and DNA with natural ratios of ¹²C and ¹³C DNA was harvested through the cultivation of bacteria on fully ¹³C-labelled glucose and unlabelled glucose for use in 'marker' gradients (Webster *et al.*, 2006).
- Gradient fractionation methods using a peristaltic pump or the drop-wise fractionation (Neufeld *et al.*, 2007) were compared for the gradients with the best linearity in CsCl density fractionations.
- Fast spinning conditions, used by Webster *et al.*, (2006) were compared to slower spinning conditions used by Neufeld *et al.*, (2007) to provide maximum separation of ¹²C- and ¹³C-labelled DNA.
- A marker gradient with visualisation of bands using EtBr was fractionated and analysed using DNA quantification and DGGE to use as a reference for DGGE patterns of fractionated gradients of gradients with no EtBr.

Pure cultures for CsCl gradient marker DNA

Three species of gram-positive bacteria from the *Bacillus* genus were grown on unlabelled glucose for the unlabelled DNA: *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus globigii*. The selection of species from the *Bacillus* genus for unlabelled DNA was two-fold: they are relatively easy to grow; they have relatively low guanosine and cytosine content in their DNA (*Bacillus* is a genus in the *Firmicutes*, of which all genera

have genomes with relatively low G+C content (Wiegel *et al.*, 1996)). *Bacillus licheniformis* extracted DNA was used as marker DNA in the control gradients and the GC content of genomes of *B.licheniformis* stains are reported to range between 45.6 to 46.1% (www.ncbi.nlm.nih.gov/genome). A gram-negative bacterium, *Pseudomonas putida*, strain 3 (PP3) was grown on ¹³C-glucose for the ¹³C-labelled DNA. This species was selected as it has a higher GC content and is therefore easier to distinguish from Bacillus species on DGGE gels; the GC content of the genomes of *Pseudomonas putida* strains have been reported as ranging from 61.4 to 62.6% (www.ncbi.nlm.nih.gov/genome).

All *Bacillus* species and PP3 were grown on solid agar medium at 30 °C and then transferred to 20 ml liquid cultures (liquid culture 1), incubated at 30 °C for 5 days, 0.1 ml was then transferred to fresh liquid medium (liquid culture 2), incubated at 30 °C for 2 days and finally 0.1ml was transferred to 50 ml volumes of media and incubated at 30 °C for a further 3 days. Three 1.7 ml samples of each culture at liquid culture stages 1 and 2 and all of the cultures at liquid culture 3 stage, were spun down, supernatant removed and frozen.

All *Bacillus* species were grown on nutrient agar (Oxoid) for solid cultures and nutrient broth (Oxoid) for liquid culture. The medium used to grow PP3 was modified standard basal salts A (SBS) (Slater *et al.*, 1979), with 1.5 % purified agar for solid medium and 0.1% (w/v) unlabelled glucose (Fisher) as the carbon source in the solid medium and liquid culture 1. The carbon source in liquid cultures 2 and 3 was fully labelled ¹³C-glucose (Cambridge Isotope Laboratories) 0.1 % (w/v). DNA from two samples of each species for each liquid culture were intially extracted using chelex. This DNA was then amplified with bacterial DGGE primers as described in chapter 2. DNA for CsCI density gradient centrifugation was extracted from pure culture *Bacillus subtilis*, *Bacillus licheniformis* and PPS using Fast DNA[®] Kit (QBiogene). The DNA yield from 17 ml of *Bacillus subtilis* PP3 pure culture was 23 µg. DNA extraction yield from 3.4 ml of *Bacillus licheniformis* and 3.4 ml PP3 was 33 µg and 30 µg, respectively. The second round of extractions (*B.licheniformis* and PP3) yielded more DNA because a new Fast DNA[®] Kit (QBiogene) was used.

Gradient Fractionation

The two methods of gradient fraction collection of using a peristaltic pump or dropwise fractionation of CsCl gradients were compared using a gradient solution with no ethidium bromide (EtBr) dye, a gradient solution with 1 μ l EtBr and a solution with 120 μ l EtBr. The drop-wise method was found to be more linear than using a pump with r² values of greater than 0.93 using the drop-wise fractionation method (table 3.1). Therefore this approach was applied to all gradients.

Method	No EtBr	1 μl EtBr	120 μl EtBr
Peristaltic Pump	0.50	0.84	0.86
Drop-wise	0.96	0.97	0.93
fractionation			

Table 3.1: R² values of linear plots of CsCl gradients of 3 concentrations of ethidium bromide fractionated using the drop-wise fraction collection method and the peristaltic pump.

Visualisation of marker gradients with different spin lengths

Fast spinning conditions, used by Webster *et al.*, (2006) were compared to slower spinning conditions used by Neufeld *et al.*, 2007 to ascertain how this would affect the resolution of the DNA fractions. Four gradients, each with 4 μ g *Bacillus licheniformis* DNA and 4 μ g PP3 DNA were set up. Two were spun under the fast separation conditions (265 000 *g* (55 000 r.p.m.) for 16 h) (figure 3) and two were spun under slow conditions 177 000 g for 36 h (figure 3.6).



Figure 3.6 Comparison of ultracentrifugation speed and time a. 265 000 *g* for 16 h b. 177 000 g for 36 h.

The bands representing the ¹³C-DNA and the ¹²C-DNA were separated further with slower and longer spinning times and this was reflected in clearer separation of the PP3 and *B.licheniformis* bands with the DGGE analysis. Therefore the slower and longer spin time was selected for future analyses.

Comparison of different CsCl final average densities and optimisation of centrifugation conditions

To ensure maximum separation of ¹²C and ¹³C DNA while covering both fractions in the gradient, comparisons of different average final densities of the CsCl gradient medium with replicates to were carried out to ensure that the set-up would reliably separate the isotopically distinct nucleic acid species (figure 3.7).



Figure 3.7. Comparison of final average density of CsCl gradient medium with the two areas of the two stable carbon isotope species marked (determined from previous gradients run under the same conditions the DNA was quantified against Hyperladder I DNA marker (Bioline) using the Gene Genius Bio Imaging System (Syngene).

A final average density of the CsCl gradient medium was set at 1.755 g/ml to ensure coverage of the isotopically distinct nucleic acid. Several solutions of CsCl densities were brought to the following temperatures:10, 25, 30 and 37 °C, to assess if this had any affect on density of the solutions and there was none.

Marker gradients run with EtBr and without EtBr

A comparison of the marker gradients with EtBr (figure 3.8) and without EtBr (figure 3.9) was made using DGGE analysis of recovered DNA from the fractions and the linearity of density values for each fraction was checked and both were linear across the desired range. Both showed good separation of the isotopically distinct nucleic acid species but there seemed to be smearing of the ¹³C PP3 DNA into the ¹²C fraction or some possible contamination of the *B.licheniforms* DNA as the marker looks different to that of previous gels and the DGGE analysis of the gradient with EtBr.

Similar concentrations of ¹³C DNA were retrieved from the CsCl gradients with EtBr and without EtBr but less was ¹²C DNA was retrieved from the gradient with no EtBr (figures 3.8 and 3.9).



Figure 3.8. 4 μg PP3 and Bacillus licheniformis DNA CsCl gradient with EtBr



Figure 3.9. 4 μg PP3 and Bacillus licheniformis DNA CsCl gradient without EtBr

3.3. Conclusion

Experimental conditions for the DNA fractionation were developed to optimise the separation of ¹³C labelled and unlabelled DNA. The results of these optimisations, along with the fractionation analysis of DNA from the 'control' slurries ensured the effective interpretation of the DNA stable isotope probing experiments for the Brightlingsea microcosms.

Visualisation of marker DNA separated with slower centrifugation conditions confirmed that this set-up effectively separated labelled and unlabelled DNA. And the analysis of the marker gradients with no EtBr and separated with slower centrifugation conditions confirmed the effective separation of DNA with no EtBr. Finally the identification of the optimal final average density of CsCl ensured all fractionated DNA would be recovered.

4. Geochemistry

4.1. Master Slurry Redox phases

Aerobic phase

The master slurries for all treatments were subsampled for the aerobic phase stable isotope probing experiments after 3 days of incubation (figures 4.1, 4.2 & 4.3). In the control and low PD slurries there was 100% oxygen saturation suggesting fully aerobic conditions. In both the high PD slurries the oxygen was measured at around 20% saturation (figure 4.1) suggesting a higher oxygen demand caused by the addition of a larger concentration of PD and the higher incubation temperature of slurry D.

Treaturent	Aerobic phase	Dysaerobic phase
Treatment	SO ₄ ²⁻ (µM hour ⁻¹)	SO ₄ ²⁻ (µM hour ⁻¹)
E (Control)	30.42	12.50
A (0.2 mg/ml)	5.42	9.58
B (1.0 mg/ml)	42.92	5.42
C (5.0 mg/ml)	25.42	-27.08
D (5.0 mg/ml)		-109.58

Table 4.1. Rate of sulphateaccumulation/removal in theaerobic and dysaerobicphases of the master slurries.

At the time of the aerobic phase subsampling the sulphate was steadily increasing in the control and low and medium PD and high PD 10 °C (slurry C) slurries with the medium PD slurry (slurry B) showing the fastest rate of increase (table 4.1), likely due to the reduced sulphur compounds from the anoxic sections of the cores being reoxidised by the oxygen from the air pump in all the slurries. The oxygen demand in the high PD 18 °C slurry (slurry D) was so much higher that the sulphate had reached a lower maximum concentration of 16.2 mM at the time of the aerobic phase subsampling before starting to decrease (figure 4.1.5).

The short chain fatty acid (SCFA) measurements showed that lactate was present in the low and medium PD slurries (figure 4.1.3) suggesting fermentation in these slurries. In slurry C acetate had increased from 0 to 5.9 mM and propionate to 270 μ M. At this time in slurry D, propionate had increased from 0.2 mM to 2.4 mM and acetate had increased from 5.7 mM at the start of the incubation to 6.2 mM (figure 4.1.5). The appearance of these SCFAs was indicative of secondary fermentation.



Figure 4.1. Sediment master slurry pore water short chain fatty acid and sulphate concentrations 1. Control 2. 0.2 mgml⁻¹ PD 3. 1.0 mgml⁻¹ 4. 5.0 mgml⁻¹ 5. 5.0 mgml⁻¹ 18 °C. Markers at the tops indicate when the sub-slurries were taken for the stable isotope probing experiments at each redox phase and red bar = aerobic phase sampling, blue bar = dysaerobic sampling, green bar = sulphate reduction and grey bar = methanogenesis sampling.

Dysaerobic phase

The master slurries for all treatments were subsampled for the dysaerobic phase stable isotope probing experiments after 8 days of incubation. In both the control, low and medium PD slurries, the sulphate was measured to be increasing still but at slower rates (table 4.1). In both the high PD slurries the sulphate had started to be removed from the systems with a higher rate of removal of 109.58 μ M SO₄⁻² hour⁻¹ in slurry D compared to 27.08 μ M SO₄⁻² hour⁻¹ in the slurry C (figure 4.1. 4 & 5). This suggested sulphate reduction in both of these slurries at the time of the dysaerobic phase subsampling.

The SFCAs lactate and formate peaked in the control slurry shortly after the dysaerobic phase subsampling while in the low PD slurries these peaked earlier, and the formate increased to 48.83 μ M in the slurry A and 34.31 μ M in slurry B (figure 4.1.2 & 3). At the time of the dysaerobic phase subsampling, the propionate had reached a maximum of 2.28 mM in the slurry C while in slurry D the propionate had been removed at a rate of 14.17 μ M hour⁻¹ to 0.06 mM (figure 4.1. 4 & 5). In slurry D between days 3 and 5 of the incubation there was a very large removal of sulphate at 136.25 µM SO₄-² hour⁻¹ and measured decrease in propionate at a rate of 47.92 μ M hour⁻¹ (figure 4.1.5). Microbially mediated sulphate reduction utilisation of this propionate as an electron donor would account for approximately 35.94 µM SO₄⁻² hour⁻¹ according to the stoichiometry for propionate sulphate reduction (Muyzer and Stams, 2008). This suggested that the remaining 100.31 µM SO4-2 hour-1 sulphate reduction was via different sulphate reduction pathways such as hydrogen and acetate oxidation. Acetate was likely being produced through the incomplete degradation of organic compounds to acetate via sulphate reduction, fermentation and acetogenic reactions. It is interesting that the acetate started to be removed from the system at a rate of 18.75 µM hour⁻¹ when the propionate was measured as being near to zero at 5 days (figure 4.1.5). This suggested a range of sulphate reduction metabolisms were being employed by microorganisms during the dysaerobic phase of the high PD 18 °C slurry.

At the time of the dysaerobic phase subsampling in the high PD slurries, the phosphate was measured to be increasing in both slurries (figure 4.2) whilst at a faster rate of 7.92 μ M hour⁻¹ in slurry D than 2.63 μ M hour⁻¹ in slurry C.



Figure 4.2. Sediment master slurry pore water phosphate and nitrate concentration 1. 5.0 mgml⁻¹ 2. 5.0 mgml⁻¹ 18 °C. Markers at the tops indicate when the sub-slurries were taken for the stable isotope probing experiments at each redox phase and red bar = aerobic phase sampling, blue bar = dysaerobic sampling, green bar = sulphate reduction and grey bar = methanogenesis sampling.

Sulphate reduction phase (SRP)

The control and low PD slurries did not reach the sulphate reduction phase (SRP) during the course of the incubations and so were not subsampled for sulphate reduction SIP experiments. The high PD slurries were both subsampled for sulphate reduction experiments after 20 days of incubation when sustained sulphate removal was measured. The rate of sulphate reduction in slurry C leading up to 20 days, between days 5 and 17, was 27.5 μ M hour⁻¹ and acetate was being removed at 6.67 μ M hour⁻¹. Sulphate reduction in slurry D had established after 5 hours of incubation due to the high BOD from the added phytodetritus and elevated temperature of 18 °C. The sulphate had been completely removed after 9 days (figure 4.1.5). 10 mM sulphate was added to re-establish sulphate reduction. The rate of sulphate reduction when slurry D was subsampled for the SRP labelling experiment was 47.5 μ M hour⁻¹.

Acetate was completely depleted in slurry D shortly after subsampling for the sulphate reduction labelling experiments at 25 days of incubation (figure 4.1.5). There was a concomitant reduction in the rate of sulphate reduction to 5 μ M hour¹between 24 and 54 days until sulphate became limiting. Net methanogenesis started around 54 days (figure 4.3.5), at which point acetate and propionate started to build up in the slurry after 54 days of incubation.

Headspace CO₂

 CO_2 was measured to increase in all slurries except slurry D where CO_2 was reducing in the first 30 days of incubation (figure 4.3). With increasing concentration of added phytodetritus there was an increase in the rate of CO_2 production (first 3 measurments of CO_2 headspace) (table 4.2) and a significantly higher maximum concentration of CO_2 in slurry C signifying a larger mineralisation of the higher PD to CO_2 (figure 4.3). The measured depletion of CO_2 in slurry D reflects the more anaerobic conditions reached more quickly due to the higher incubation temperature and the CO_2 potentially being used for methanogenesis metabolism as methane was measured to be increasing (figure 4.3.5).

Treatment	CO ₂ (μmoles L ⁻¹ sediment hour ⁻¹)
E (Control)	2.50
A (0.2 mg/ml)	3.33
B (1.0 mg/ml)	3.75
C (5.0 mg/ml)	17.08
D (5.0 mg/ml)	-2.92

 Table 4.2. Rate of CO₂ production in the

 aerobic and dysaerobic phases of the master

 slurries.

Methanogenesis phase

Methanogenesis had established in slurry C from 11 to 57 hours with a rate of 0.42 mmoles μ mole L⁻¹ sediment hour⁻¹ which then increased to 2.50 mmoles μ mole L⁻¹ sediment hour from 57 to 67 hours (figure 4.3.4). Acetate concentration was c.6 mM and CO₂ *c*.9 mmoles L⁻¹ sediment. The increase in acetate during methanogenesis between 23 and 74 days (figure 4.3.4) suggests other methanogenesis pathways such as hydrogenotrophic methanogenesis (although the CO₂ wasn't decreasing), or the use of other C1 compounds rather than acetoclastic methanogenesis alone.

Methanogenesis established in slurry D after 53 hours at a rate of 0.83 mmoles μ mole L⁻¹ sediment hour⁻¹ when the sulphate was limiting at <2 mM (figure 4.3.5). Acetate was completely depleted and CO₂ was relatively constant around 4 mmoles L⁻¹ sediment. The complete depletion of acetate and the steady levels of CO₂ in slurry D suggest methanogenesis from other C1 compounds such as methanol and methylamines.



Figure 4.3. Sediment master slurry headspace CO_2 and methane concentrations, and prokaryotic cell numbers indicated by leftmost grey bar 1. Control 2. 0.2 mgml⁻¹ PD 3. 1.0 mgml⁻¹ 4. 5.0 mgml⁻¹ 5. 5.0 mgml⁻¹ 18 °C. Markers at the tops indicate when the sub-slurries were taken for the stable isotope probing experiments at each redox phase and red bar = aerobic phase sampling, blue bar = dysaerobic sampling, green bar = sulphate reduction and grey bar = methanogenesis sampling.



Figure 4.3. Continued.

4.2. Stable Isotope Probing sub-slurry geochemistry

Dysaerobic phase

As in the high PD master slurries, sulphate reduction had established in the dysaerobic phase sub-slurries during the glucose labelling experiments (figure 4.4). The ¹³C-glucose and ¹²C-glucose showed similar patterns in the 5 mg/ml 10 °C slurry (C) and the 5 mg/ml 18 °C slurry (D) with the rate of sulphate reduction and acetate removal being much larger in slurry D with the elevated temperature.



Figure 4.4. Sulphate, acetate, formate and lactate porewater concentrations in the 5 mg/ml 10 °C slurry (C) incubated with 1. ¹²C-glucose 2. ¹³C-glucose and the 5 mg/ml 18 °C slurry (D) incubated with 3. ¹²C-glucose 4. ¹³C-glucose.

Sulphate reduction phase (SRP)

Figure 4.5 shows the sulphate and acetate in the sub-slurries during the SRP labelling probing experiment. There was net sulphate reduction and acetate utilisation in both the ¹²C and ¹³C labelled acetate addition incubations for slurry D. The rate of ¹³C acetate utilisation was 2.6 mM hour ⁻¹ between 0 and 6 hours and then 0.21 mM hour ⁻¹ between 6 and 48 hours with a rate of sulphate removal of 0.20 mM hour ⁻¹. This was very comparable to the ¹²C acetate incubation where the rate of ¹²C acetate utilisation was

2.63 mM hour ⁻¹ between 0 and 6 hours, 0.23 mM hour⁻¹ between 6 and 48 hours and a rate of sulphate removal of 0.20 mM hour ⁻¹(table 4.8). The very large removal of acetate in the first 6 hours after the addition of the label suggested a physicochemical sorption process such as adsorption to a mineral component of the sediment. The relatively small amount of sulphate removal *c*.30 μ M hour⁻¹ in comparison to the initial change in acetate concentration in the first 6 hours, compared to the remainder of the incubations where changes in sulphate and acetate concentrations after 6 hours were almost equal, suggests that this initial change in acetate concentration in solution was rather due to a physicochemical process than microbially mediated and was likely related to the elevated temperature of slurry D as this large removal of the acetate label was not seen in slurry C which was incubated at the lower temperature of 10 ^oC. Elevated temperature is known to affect sorption kinetics, sometimes causing faster rates of sorption of organic compounds (Hulscher and Cornelissen, 1996).

There was clear stimulation of acetate utilisation and sulphate reduction in the slurries where labels were added as the rate of acetate removal in the control slurry was 19.2 μ M hour⁻¹ and 60 μ M SO₄²⁻ hour⁻¹ and the rate of acetate removal and sulphate reduction in the master slurry at the time of subsampling for the SRP experiment was 3.4 μ M hour⁻¹ and 7.2 μ M hour⁻¹ respectively. At time zero of the SRP incubation the concentration of acetate in the control slurry was similar to the master slurry *c*.1 mM, however the rate of acetate utilisation was stimulated in the control, possibly due to greater physical mixing of the subsample slurry.



Figure 4.5. Sulphate and acetate in the SIP sub-slurries 1.1^{2} C-acetate 5 mg/ml 10 0 C (C) 2. 13 C-acetate 5 mg/ml 10 0 C (C), 3, 12 C-acetate 5 mg/ml 18 0 C (D), 4, 13 C-acetate 5 mg/ml 18 0 C (D).

Time	¹³ C A	Acetate	¹² C A	Acetate	Co	ontrol	Maste	er slurry
	Ac (mM hour ⁻¹)	SO4 ²⁻ (mM hour ⁻¹)	Ac (mM hour ⁻¹)	SO42- (mM hour -1)	Ac (µM hour ⁻¹)	SO4 ²⁻ (µM hour ⁻¹)	Ac (µM hour ⁻¹)	SO42- (µM hour -1)
0 - 6 hours	2.6	0.03	2.63	0.05				
6 - 48 hours	0.21	0.2	0.23	0.2				
0 - 48 hours					19.11	0.05		
17 - 24 days							3.4	7.23

Table 4.3. Rate of acetate utilisation and sulphate removal during the SRP-SIP experiment and the master slurry in the 5 mg/ml 18 °C slurry (D).

5. Colne estuary sediment microcosm microbial communities

5.1. Glucose incubations

Temporal changes in microbial community structure of sediment slurries as assessed by 16S rRNA gene PCR-DGGE

Bacterial community

The DGGE profiles of the bacterial 16S rRNA genes of the DNA from the ¹³C –glucose incubations showed that the loading of phytodetritus resulted in different community profiles and dominant populations in the slurries compared to the control (figures 5.2a-5.6a). The control was a relatively simple profile throughout the incubation with one or two bands dominating. Excision, sequencing of these bands and BLAST analysis showed that the dominant members were similar to a *Vibrio lentus* like *Gammaproteobacteria* and an *Epsilonproteobacteria* sequence fragment similar to the chemolithotrophic *Sulforovum lithotrophicum* (table 5.1). The profiles of the control slurry during the dysaerobic phase were very similar to those of the aerobic phase except for the disappearance of band Eada7, a *Vibrio* like *Gammaproteobacterium*, and the appearance of Eada11, an *Arcobacter* like *Epsilonproteobacterium* which appeared after 48 hours of dysaerobic incubation, otherwise there with little change over the course of the incubation (figure 5.2a).



Figure 5.2. PCR-DGGE analysis of a. bacterial and b. archaeal 16S rRNA genes from the total DNA extracted from all time points of the control slurry E during the aerobic and dysaerobic phase incubated with ¹³C-glucose. Lanes show incubation, A=aerobic, DA=dysaerobic and time of incubation in hours. RedLanes marked M, DGGE marker. Red bands show excised bands Eab 2 to Edab 11.

	DGGE Band	Sediment slurry	Nearest match by BLASTN search (accession number)	% sequence similarity (alignment length, bp)	Phylogenetic affiliation	Isolation environment of nearest sequence match
	Eab2	Slurry Eaerobic phase	Uncultured epsilongrafeabacterium clone SE7R35LBbA36 (FR797966)	96 (134)	Epsilonproteobactera	Hydrothermal
	Eab7	Slurry Eaerobic phase	Uncultured bacterium clone My46-460 (GQ866074)	96 (159)	Gammaproteobacteria	Norway lobster gut bacterial community
	Eab10	Slurry Eaerobic phase	Vibrio sp. FALF307 (EU655386)	94 (159)	Gammaproteobacteria	Bacterioplankton, Sapelo Island, Georgia,
	Edab11	Slurry E dysaerobic phase	Uncultured Epsilonproteobacterium clone ML0409-14 (JN653192)	93 (135)	Epsilonproteobactera	Bacterial Communities Associated with the Ctenophores <i>Mnemiopsis le klyi</i> and <i>Beroe</i> ovata
Ĵ						
	Bab4	Slurry Baerobic phase	Uncultured Pseudoaiteromonas sp. clone DP11 (JQ965713)	99% (160)	Gammaproteobacteria	Mangrove rhizospheres of Devipattinam, Tamil Nadu, India
	Bab8	Slurry Baerobic phase	Colwellia sp. WH009076c (JF820680)	95 (160)	Gammaproteobacteria	Sponge-specific microbial communities in sponges in irish waters
	Bab12	Slurry Baerobic phase	Uncultured bacterium clone V-S-45 (JN018716)	95 (155)	Gammaproteobacteria	Deepwater Horizon oil spill
	Bab14	Slurry Baerobic phase	Pseudoalteromonas sp. SB-E1 (AB457051)	95 (158)	Gammaproteobacteria	Fish farm sediments
	Bdab15	Slurry B dysaerobic phase	Pseudoalteromonas mariniglutinosa strain FV3 (JQ867499)	95 160	Gammaproteobacteria	Biofilms and epibiotic microbial assemblages on barnacles
	Bdab19	Slurry B dysaerobic phase	Vibrio sp. J2132c (JF820750)	95 (159)	Gammaproteobacteria	Sponge-specific microbial communities in sponges in irish waters
	Bdab28	Slurry B dysaerobic phase	Pseudoalteromonas citrea strain G29q-5a (AF261051)	95 (160)	Gammaproteobacteria	Danish coastal waters

Table 5.1. Bacterial 16S rRNA gene sequence matches to excised DGGE bands from the control sediment slurry E and slurry B (1 mg/ml phytodetritus) incubated with ¹³C-glucose for 3 days.

The low PD and medium PD slurry showed relatively similar profiles with more complex communities compared to the control (figures 5.3a and 5.4a). Slurry A profiles were similar throughout the course of the aerobic and dysaerobic incubations, and they were relatively similar with some bands becoming more intense, for example, Ada14 which was 97% similar to a *Sulforovum lithotrophicum*-like *Epsilonproteobacterium* (table 5.2). Excision, sequencing and BLAST analysis of selected bands from the aerobic incubation showed the dominant members of the communities in slurry A were all similar to *Alteromonadaceae* like sequences with two *Shewanella*-like DNA sequence fragments, one *Pseudoalteromonas*- like fragment and a fragment 95% similar to *Saccharophagus degradans* (table 5.2). Excision and sequencing of selected bands from the dysaerobic phase showed similarities with *Shewanella, Colwellia*-like *Gammaproteobacteria* and the aforementioned *Epsilonproteobacterium* like sequence (table 5.2).



Figure 5.3. PCR-DGGE analysis of a. bacterial and b. archaeal 16S rRNA genes from the total DNA extracted from all time points of the 0.2 mg/ml 10^oC slurry (A) during the aerobic and dysaerobic phase incubated with ¹³C-glucose. Lanes show incubation, A=aerobic, DA=dysaerobic and time of incubation in hours. Lanes marked M, DGGE marker. Blue markers represent bands excised and red markers represent those successfully sequenced.

DGGE Band	Sediment slurry	Nearest match by BLASTN search (accession number)	% sequence similarity (alignment length, bp)	Phylogenetic affiliation	Isolation environment of nearest sequence match
Aab4	Slurry A aerobic phase	Uncultured Pseudoalteromonas sp. clone CT_008 (DQ880984)	96 (159)	Gammaproteobacteria	Coastal seawater, Sapelo Island, Georgia.
Aab5	Slurry A aerobic phase	Shewanella sp. 53S-26 (GU371694)	96 (159)	Gammaproteobacteria	Shenzhen coastal waters, China
Aab11	Slurry A aerobic phase	Gammaproteobacterium UMB11F (AF505741)	97 (155)	Gammaproteobacteria	Biofilm forming isolate, Boston Harbor, MA
Aab13	Slurry A aerobic phase	Shewanella sp. GCDN4_III (JQ072066)	96 (159)	Gammaproteobacteria	sea surrace microlayer and underlying water, ia de Aveiro estuary, Portugal
Aab14	Slurry A aerobic phase	Saccharophagus degradans 2-40 strain 2-40 (NR_041705)	95 (159)	Gammaproteobacteria	Proposal of <i>Saccharophagus</i> genus for strain 2-40
Adab2	Slurry A dysaerobic phase	Uncultured bacterium clone RS1B2E08 (HM226653)	95 (160)	Gammaproteobacteria	Microbial diversity of a Brazilian coastal region influenced by an upwelling system and anthropogenic activity
Adab5	Slurry A dysaerobic phase	Uncultured bacterium clone:OHKB4.37 (AB094844)	96 (160)	Gammaproteobacteria	Coastal subseafloor sediments from the sea of okhotsk
Adab6	Slurry A dysaerobic phase	Pseudoalteromonas sp. J2134c (JF820738)	100 (160)	Gammaproteobacteria	Sponge microbial communities, Irish waters
Adab7	Slurry A dysaerobic phase	Bacterium fme09x22p174 (JQ657555)	97 (160)	Gammaproteobacteria	Near-shore coastal fog, south- facing shore of South port Island , ME, USA
Adab8	Slurry A dysaerobic phase	Shewanella marisflavi strain SW-117 (NR_025793)	96 (160)	Gammaproteobacteria	Seawater, Yellow Sea, Korea
Adab9	Slurry A dysaerobic phase	Uncultured Pseudoalteromonas sp. clone DP11 (JQ965713)	98 (160)	Gammaproteobacteria	Avicennia marina rhizosphere from mangroves of Devipattinam, Tamil Nadu , India
Adab14	Slurry A dysaerobic phase	Uncultured Epsilonproteobacterium clone ES09-P-48 (JQ712487)	97 (135)	Epsilonproteobacteria	Hydrothermal vents of the Juan de Fuca Ridge
Adab15	Slurry A dysaerobic phase	Uncultured Colwellia sp. clone OTU_C18_WB_110 (JF928822)	96 (160)	Gammaproteobacteria	Microplastics in coastal marine sediments
Adab17	Slurry A dysaerobic phase	Shewanella sp. enrichment culture clone KWE30-43 (JQ670719)	96 (160)	Gammaproteobacteria	Seaweed bed associated with Marine Hot Springs on East Coast of Kalianda Island, Indonesia

Table 5.2. Bacterial 16S rRNA gene sequence matches to excised DGGE bands from slurry A (0.2 mg/ml phytodetritus) incubated with ¹³C-glucose for 3 days.

The bands excised from slurry B profiles were all similar to the *Gammaproteobacteria* with three *Pseudoalteromonas* like sequence fragments and one *Colwellia* like sequence fragment (table 5.1). Band Bda15 was 95% similar to a *Pseudoalteromonas mariniglutinosa* which was also similar to Ba6 from the aerobic incubation. There were several other *Pseudoalteromonas* like sequences in the dysaerobic phase, as well as a *Vibrio* like sequence (table 4.1).



Figure 5.4. PCR-DGGE analysis of a. bacterial and b. archaeal 16S rRNA genes from the total DNA extracted from all time points of the 1.0 mg/ml 10^oC slurry (B) during the aerobic and dysaerobic phase incubated with ¹³C-glucose. Lanes show incubation, A=aerobic, DA=dysaerobic and time of incubation in hours. Lanes marked M, DGGE marker. Blue markers represent bands excised and red markers represent those successfully sequenced.

The bacterial DGGE profiles of slurry C were relatively similar to the lower phytodetritus loading slurry profiles with a sequence fragment 99% similar to a *Sulforovum lithotrophicum*-like *Epsilonproteobacteria* and other sequence fragments similar to the *Gammaproteobacteria*; *Vibrio* and *Pseudoalteromonas* (table 5.3). However, after 24 hours of incubation the profiles changed and many of the bands became more intense, for example, Cab9 and Cab14 which were 95% similar to a *Shewanella*-like sequence (table 5.3). There was also an appearance of some new bands such as Cab12, Cab15 and Cab16 that were all similar to *Vibrio*-like sequence fragments with Cab16 being 95% similar to a *Photobacterium* like sequence fragment (table 5.3). Band Cab7 appeared after 24 hours of incubation time and after excision and sequencing was shown to be 97% similar to a *Shewanella* sp. (table 5.3). The profiles of the dysaerobic phase for slurry C were very similar to the aerobic phase with the appearance of band Cada17 (figure 5.5a) which was 100% similar to a *Bacteriodetes* like sequence.



Figure 5.5. PCR DGGE analysis of a. bacterial and b. archaeal 16S rRNA genes from the total DNA extracted from all time points of the 5.0 mg/ml 10^oC slurry (C) during the aerobic and dysaerobic phase incubated with ¹³C-glucose. Lanes show incubation, A=aerobic, DA=dysaerobic and time of incubation in hours. Lanes marked M, DGGE marker. Blue markers represent bands excised and red markers represent those successfully sequenced.

DGGE Band	Sediment slurry	Nearest match by BLASTN search (accession number)	% sequence similarity (alignment	Phylogenetic affiliation	Isolation en vironment of nearest sequence match
Cab2	Slurry C aerobic phase	Uncultured Epsilonproteobacterium clone ES09-P-48 (JQ712487)	length, bp) 99 (135)	Ep silon proteo bacte ra	Hydrohermal vents of the Juan de Fuca Ridge
Cab3	Slurry C aerobic phase	Uncultured Vibrio sp. clone V2_D03_025 (EU167177)	98 (160)	Gammaproteobacteria	Bacterioplankton, Sapelo Island, Georgia,
Cab4	Slurry C aerobic phase	Pseudoalteromonas sp. NO3 (FJ600315)	100 (160)	Gammaproteobacteria	lsolated from the sea squirt <i>Halocynthia</i> <i>rorentzi</i>
Cab7	Slurry C aerobic phase	Shewanella sp. J2011s (JF820760)	97 (159)	Gammaproteobacteria	Sponge microbial communities, Irish waters
Cab10	Slurry C aerobic phase	<i>Vibrio</i> sp. S2895 (FJ457480)	99 (160)	Gammaproteobacteria	Global sampling of ocean surface waters and surface swabs of marine organisms
Cab11	Slurry C aerobic phase	Pseudoalteromonas sp. BCw096 (FJ889582)	100 (160)	Gammaproteobacteria	Bacteria isolated from Arctic seawater
Cab14	Slurry C aerobic phase	Shewanella sp. Sw66 (HE81823)	95 (159)	Gammaproteobacteria	Atlanto-Mediterranean marine sponges of the family <i>Irciniidae</i>
Cab16	Slurry C aerobic phase	Uncultured Photobacterium sp. clone O-A11 (HQ897490)	95 (160)	Gammaproteobacteria	Intestinal microbiota of Atlantic salmon
Cdab17	Slurry C dysærobic phase	Uncultured <i>Bacteroidetes</i> bacterium clone OMEGA_pl_cont_6_F08 (EU052254)	100 (155)	Bacteroidetes	Microbial fuel cells supplied with marine plankton
Cdab19	Slurry C dysærobic phase	Vibrio sp. FALF307 (EU655386)	100 (160)	Gammaproteobacteria	Coastal bacterioplankton
Cdab20	Slurry C dysærobic phase	Pseudoalteromonas sp. NO3 (FJ600315)	99 (160)	Gammaproteobacteria	lsolated from the sea squirt <i>Halocynthia</i> <i>rorentzi.</i>
Cdab22	Slurry C dysærobic phase	<i>Shewanella</i> sp. MB4 (EU147246)	96 (160)	Gammaproteobacteria	Characterization of aerobic and anaerobic heavy metal resistance of <i>Shewanella</i> -like isolates from marine sediments
Cdab24	Slurry C dysærobic phase	<i>Pseudoatteromonas mariniglutinosa</i> strain FV3 (JQ867499)	95 (160)	Gammaproteobacteria	Biofilms and epibiotic microbial assemblages on barnacles
Cdab25	Slurry C dysærobic phase	Vibrio litoralis strain MANO22P (DQ097524)	96 (160)	Gammaproteobacteria	Yellow Sea tidal flat in Korea
Cdab28	Slurry C dysærobic phase	Vibrio sp. SC25 (FN433055)	100 (160)	Gammaproteobacteria	Marine biofilms
Cdab31	Slurry C dysærobic phase	Pseudoalteromonas sp. BCw096 (FJ889582)	99 (160)	Gammaproteobacteria	Bacteria isolated from Arctic seawater
Cdab32	Slurry C dysærobic phase	<i>Vibrio</i> sp. S2895 (FJ457480)	98 (160)	Gammaproteobacteria	Global sampling of ocean surface waters and surface swabs of marine organisms
Cdab33	Slurry C dysærobic phase	Pseudoalteromonas sp. Pa93 (HE818258)	95 (160)	Gammaproteobacteria	Atlanto-Mediterranean marine sponges of the family <i>Irciniidae</i>

Table 5.3. Bacterial 16S rRNA gene sequence matches to excised DGGE bands from slurry C (5.0 mg/ml phytodetritus) incubated with ¹³C-glucose for 3 days.

The bacterial 16S rRNA gene DGGE profiles of the aerobic incubation of slurry D showed relatively simple profiles for the first 12 hours of the incubation after which some bands became more intense and several new bands appeared (figure 5.6a). The bands excised from the profiles of the first 12 hours of the incubation showed highest similarities to *Pseudoalteromonas* and *Shewanella* like sequences (table 5.4). Most of the bands that appeared after 24 hours of incubation were similar to *Vibrio* like

sequences for example, Dab10, and one band (Dab17) was similar to a *Pseudoalteromonas* like sequence (table 5.4). There was a clear appearance of band Dab20 after 24 hours of incubation which was 100% similar to a *Bacteriodetes* like sequence like that identified in the dysaerobic incubation of slurry C (table 5.3). Band Dab22 which also appeared after 48 hours of incubation showed 90% similarity to a *Firmicutes* like sequence fragment. The profiles from the dysaerobic incubation for slurry D were very similar to the aerobic profiles and to the dysaerobic incubation profiles of slurry C with the appearance of two new bands, Dada38 which was 94% similar to an *Arcobacter* like sequence fragment and Dada40 which was 97% similar to a *Lutibacter* like fragment (table 5.4).



Figure 5.6. PCR DGGE analysis of a. bacterial and b. archaeal 16S rRNA genes from the total DNA extracted from all time points of the 5.0 mg/ml 18^oC slurry (D) during the aerobic and dysaerobic phase incubated with ¹³C-glucose. Lanes show incubation, A=aerobic, DA=dysaerobic and time of incubation in hours. Lanes marked M, DGGE marker. Blue markers represent bands excised and red markers represent those successfully sequenced.

DGGE Band	Nearest match by BLASTN search (accession number)	% sequence similarity (alignment length, bp)	Phylogenetic affiliation	Isolation environment of nearest sequence match
Dab1 & Ddab29	Pseudoalteromonas mariniglutinosa strain FV3 (JQ867499)	97 (160)	Gammaproteobacteria	Biofilms and epibiotic microbial assemblages on barnacles
Dab3	Pseudoalteromonas sp. J2042s (JF820749)	100 (160)	Gammaproteobacteria	Sponge microbial communities, Irish waters
Dab4	Shewanella sp. 53S-2 (GU371694)	96 (160)	Gammaproteobacteria	Shenzhen coastal waters, China
Dab5	Pseudoalteromonas citrea strain G29q-5a (JN68179)	97 (160)	Gammaproteobacteria	Danish coastal waters
Dab6	Uncultured Bacteroidetes bacterium clone OMEGA_pl_cont_6_F08	96 (156)	Bacteriodetes	Microbial fuel cells supplied with marine plankton
Dab10	Uncultured Vibrio sp. clone V2_D03_025 (EU167177)	99 (160)	Gammaproteobacteria	Bacterioplankton, Sapelo Island, Georgia,
Dab11	Uncultured proteobacterium clone LON07S3 (JQ218616)	100 (160)	Gammaproteobacteria (99% similar to Vibrio lentus)	Symbiotic Associations in the Brown Alga Saccharina japonica
Dab12 & Ddab31	Vibrio sp. S2895 (FJ457480)	99 (160)	Gammaproteobacteria	Global sampling of ocean surface waters and surface swabs of marine organisms
Dab17	Pseudoalteromonas sp. PaH3.10 (GQ406774)	95 (160)	Gammaproteobacteria	Diseased gorgonian coral
Dab19	Vibrio sp. S1126 (FJ457368)	95 (160)	Gammaproteobacteria	Global sampling of ocean surface waters and surface swabs of marine organisms
Dab20	Uncultured Bacteroidetes bacterium clone FII-OX064 (JQ579713)	100 (155)	Bacteriodetes	Marine oil-polluted sediments Galicia, Spain
Dadb22	Bacterium enrichment culture clone AOM-SR-B4 (HQ405663)	90 (134)	Firmicutes	Enrichment of a microbial community performing anaerobic oxidation of methane in a continuous high-pressure bioreactor
Dab23	Shewanella sp. 12La (JN040592)	100 (160)	Gammaproteobacteria	Coastal marine sponges Amphilectus fucorum and Eurypon major
Ddab32	Vibrio lentus strain Sat201 (AY292936)	97 (160)	Gammaproteobacteria	Squid/fish light organs
Ddab36	Pseudoalteromonas sp. BSi20493 (EF639350)	95 (160)	Gammaproteobacteria	Arctic sea ice, Canada Basin
Ddab37	Pseudoalteromonas sp. H18 (AY277272)	95 (160)	Gammaproteobacteria	Orkney Isles and the Firth of Forth
Ddab38	Uncultured Arcobacter sp. clone S30 (JQ861842)	95 (135)	Epsilonproteobacteria	Environmentally significant and contaminating bacteria
Ddab40	Lutibacter sp. MA-My1 (HM234096)	97 (155)	Bacteriodetes	Tidal flat sediment
Ddab41	Vibrio sp. J2132c (JF820750)	96 (160)	Gammaproteobacteria	Sponge microbial communities, Irish waters

Table 5.4. Bacterial 16S rRNA gene sequence matches to excised DGGE bands from slurry D (5.0 mg/ml phytodetritus 18 ^oC) incubated with ¹³C-glucose for 3 days.

DGGE Band	Sediment slurry	Nearest match by BLASTN search (accession number)	% sequence similarity (alignment length, bp)	Phylogenetic affiliation	Isolation environment of nearest sequence match
Aada3	Slurry A Aerobic phase	Uncultured archaeon clone MC10B_B02 (HM019067)	95 (118)	Thermoplasmatales	Mangrove sediments, southeastern Brazil
Aada4	Slurry A Aerobic phase	Uncultured Thermoplasmatales archaeon clone GoM87_Arch341 (FN421133)	95 (118)	Thermoplasmatales	Gulf of Mexico cold-seep sediments
Bada6	Slurry B Aerobic phase	Uncultured archaeon clone MNO302arcC9 (GU996759)	100 (118)	Thermoplasmatales	Methanogenic crude oil degrading microccsms inoculated with sediment from the River Tvne, Newcastle, UK
Bada3	Slurry B Aerobic phase	Uncultured archaeon clone GP-BJ92 (EF639459)	95 (118)	Thermoplasmatales	Lake Pontchartrain basin
Bada9	Slurry B Aerobic phase	Uncultured archaeon clone Kazan-2A-47/BC19-2A-47 (AY59	91 (115)	MG1	Mediterranean cold seep
Cad6	Slurry C Aerobic phase	Uncultured archaeon Arc.148 (AF005763)	92 (116)	Crenarchaeota	Deep Subsurface Paleosol Stratified communities of
Cad9	Slurry C Aerobic phase	Uncultured crenarchaeote clone BS1-1-38 (AY396673)	94 (116)	MCG	archaea in the sediments of Jiulong River Estuary, China
Cad8	Slurry C Aerobic phase	Uncultured archaeon clone R-UA-42 (JQ258636)	94 (115)	Crenarchaeota	Tropical West Coast of India
Cad3	Slurry C Aerobic phase	Uncultured archaeon isolate H2 oxic incubation Lilliput fluids (324 ROV 6-8) (1/53), clone 62.63_c13 (HE579831)	97 (118)	Euryarchaoeta	Low-temperature hydrothermal venting sites on the southern Mid-Atlantic Ridge
Cad34	Slurry C Dysaerobic phase	Uncultured archaeon clone R-UA-81 (JQ258674)	98 (113)	MCG	Tropical West Coast of India
Dada13	Slurry D aerobic phase	Uncultured archaeon clone ArchControl-11-66 (JF728006)	91 (119)	Crenarchaeota	Lake Kinneret, Isræl
Dada16	Slurry D aerobic phase	Uncultured <i>crenarchaeote</i> partial 16S rRNA gene, clone MOBA58 (AM942162)	90 (114)	MCG	Gassy Subsurface Sediments of Marennes- Oleron Bay (France)
Dada4	Slurry D aerobic phase	Uncultured archaeon clone TVVP2-38 (GQ410821)	91 (117)	MBG-D	Deep subsurface sediments from the tropical Western pacific
Dada17	Slurry D aerobic phase	Uncultured archaeon clone MC10A_F06 (HM019056)	92 (116)	MBG-D	Mangrove sediments, southeastern Brazil
Dada26 and 29	Slurry D aerobic/dysaerobic phase	Uncultured <i>Methanococcoides</i> sp. clone LF_16SA_enr_anoxic_H8 (JX015362)	93 (114)	Methanococcoides	Meromictic saline Lake Faro (Messina-Italy)
Dada35	Slurry D dysaerobic phase	Uncultured archaeon clone MKCST-C5 (DQ363807)	93 (115)	Crenarchaeota	Mangrove soil, Hainan Island, China.

Table 5.6. Archaeal 16S rRNA gene sequence matches to excised DGGE bands from all treatments incubated with ¹³C-glucose for 3 days.

Archaeal community

The DGGE profiles of the archaeal 16S rRNA genes amplified from the aerobic and dysaerobic ¹³C-glucose addition incubations are shown in figures 5.2b to 5.6b. The sequences obtained from the bands excised were generally not very clear in comparison to the bacterial 16S rRNA gene sequence fragments obtained from the bacterial community, possibly due to low numbers of archaeal cells. Consequently many of the similarity values were low and only those above 90% are presented (table 5.6).

The DGGE profiles of the archaeal 16S rRNA genes amplified from the aerobic and dysaerobic ¹³C-glucose addition incubations from slurry A were fairly similar to the control slurry and there was also little change in the profiles throughout the course of the two incubations. Of many bands excised only a couple gave high similarity scores with two sequence fragments being 95% similar to *Thermoplasmatales* like sequences (table 5.6). Slurry B showed many not so well defined bands compared with the other incubations but again there was high similarity throughout the incubations and between the aerobic and dysaerobic incubations with the only noticeable change being the

disappearance of band Bada1 (figure 5.4b). There were several *Thermoplasmatales* like sequence fragments with Bada6 being 100% similar to a *Thermoplasmatales* like sequence and there were several *Crenarchaeote* like sequences which showed low similarity values such as Bada9 which was 91% similar to a MG1 like *Crenarchaeote* sequence (table 5.6).

The profiles of slurry C during the aerobic phase were fairly similar throughout the incubation with the appearance and fading of a few bands, for example, Cada17, and some bands became more intense during the course of the incubation, for example, Cada25. The dysaerobic phase profiles for slurry C showed the appearance of some more intense bands, for example, Cada25, and more variation during the course of the incubation, for example, the appearance of Cada28 and Cada44 and 45. In comparison with the control and low phytodetritus loading slurries, there were more clearly defined bands suggesting more archaeal populations had established. Unfortunately it was difficult to ascertain the identity of many of these bands and resulted in low sequence similarities but of those that were analysed (bands 1-10 and bands 30 and 34) there were 7 Crenarchaeote like sequences with Cada9 94% similar to a MCG like sequence, and there was one clear Euryarchaeote, Thermoplasmatales like sequence amplified from bands Cad3. The profiles of slurry D were quite faint for the first 12 hours of the aerobic incubation and then after 24 hours some bands became more intense, for example, Dada14 and Dada16 which was 90% similar to an MCG like Crenarchaeote sequence. After 72 hours the profile looked guite different with several new intense bands, for example Dada24, Dada25 and Dada26, the latter being 93% similar to a Methanococcoides like Euryarchaeote sequence. There were several sequences identified that were similar to Crenarchaeote like sequences but these had relatively low similarity scores (table 5.6).

DNA fractionation

The total DNA extracted from slurry B after 72 hours of incubation with ¹³C-glucose was centrifuged and fractionated to retrieve the ¹³C labelled DNA and the natural abundance unlabelled DNA fractions as described in chapter 3. The gradient fractions did not cover the full range of fractions as determined in the calibration of the technique but covered more ¹³C enriched fractions and less natural abundance fractions (figure 5.7). The DNA recovered from the gradients was not visible on the gel dock imaging system, however, the imaging of the bacterial 16S rRNA gene PCR products of the fractions showed more intense bands in the ¹³C enriched fractions compared to the natural abundance fractions (figure 5.7).

The DGGE analysis of the PCR products showed that the same community was present in the ¹³C enriched and natural abundance fractions and the profiles were very similar to the bacterial 16S rRNA profiles of the unfractionated bacterial 16S rRNA gene PCR-DGGE profiles which looked remarkably similar throughout the aerobic and dysaerobic incubations (figure 5.4) suggesting that the same community of *Bacteria* was present throughout the incubation with ¹³C-glucose. Excision of bands and sequencing showed that the bands in the same positions as the unfractionated profiles had highest similarities to the same BLAST sequences as the bands from the fractionated profiles, for example, B1 and Bda15 (figures 5.10 and figure 5.4a respectively) were both similar to *Pseudomonas marinigulosa*. The similarity between the DGGE patterns of the ¹²C-DNA fractions, the mixed region and the ¹³C-DNA fractions suggest that all the *Bacteria* identified in the labelled fractions of the dysaerobic phase of slurry B (table 5.7) were able to utilise ¹³C-glucose and/or its degradation products.



Figure 5.7. PCR-DGGE analysis of bacterial 16S rRNA genes amplified from all fractions of the CsCl ultracentrifugation analysis of the DNA from the 1 mg/ml phytodetritus slurry (B) after 72 hours of incubation with ¹³C-glucose. Lanes marked 1-18 represent the ¹³C enriched fractions, lane 19-21, the 'mixed' fractions and lanes 22 to 24 the ¹²C fractions.

DGGE Band	Sediment slurry	Nearest match by BLASTN search (accession number)	% sequence similarity (alignment length, bp)	Phylogenetic affiliation	Isolation environment of nearest sequence match
Bdal1	B Dysaerobic phase	Pseudoalteromonas mariniglutinosa strain FV3 (JQ867499)	96 (160)	Gamma proteobacteria	Biofilms and epibiotic microbial assemblages on barnacles
Bdal5	B Dysaerobic phase	Pseudoalteromonas sp. SB-E1 (AB457051)	96 (158)	Gamma proteobacteria	Fish farm sediments
Bdal7	B Dysaerobic phase	Uncultured bacterium clone Mn13T4_6-H6 (JQ863501)	95 (160)	Gamma proteobacteria	Coastal marine surface sediment
Bdal11	B Dysaerobic phase	Vibrio sp. TD29 (DQ978247)	95 (160)	Gamma proteobacteria	Oyster aquaculture

Table 5.7. Bacterial 16S rRNA gene sequence matches to excised DGGE bands from the ¹³C enriched fractions of the 1 mg/ml phytodetritus slurry (B) after 72 hours of incubation with ¹³C-glucose.

5.2. Acetate incubations

Temporal changes in microbial community structure of sediment slurries as assessed by 16S rRNA gene PCR-DGGE

Sulphate reduction phase

The bacterial 16SrRNA PCR-DGGE analysis of the slurry C SRP labelling incubations showed that the bacterial community was similar throughout the duration of the incubation (figure 5.10a) and thus the bacterial community hadn't significantly changed in response to the addition of the acetate label in terms of species richness. Excision and sequencing of bands showed sequence fragments similar to the *Bacteriodetes, Firmicutes, Shewanella* and *Pseudoalteromonas* (table 5.9) with the *Gammaproteobacteria* dominating (figure 5.9).



Figure 5.9: Proportions of the major bacterial phyla shown to be present in the sulphate reduction phase of the ¹³C-acetate incubation of slurry C.

The archaeal 16SrRNA PCR-DGGE analysis of all the time points showed that there was some variation in the archaeal communities in the ¹³C-acetate SRP incubation of

slurry C (figure 5.12b). Six *Crenarchaeote* like sequences were identified with some similar to MCG like sequences and three *Euryarchaoete* like sequences, including *Thermoplasmatales* and *Methanococcoides* like sequences (table 5.9). The *Crenarchaeota* dominated and *Methanomicobiales* like populations dominates the *Euryarchaote* like phylotypes (figure 5.11). The identified *Crenarchaeote* like sequences seemed to become less dominant in the later time points of the incubation, for example, CSRa10 and CSRa13, while the *Euryarchaeote* bands were present throughout the incubation and became more dominant in the later stages, for example, CSRa21 which was 100% similar to a *Methanococcoides* like sequence.



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Figure 5.10. PCR-DGGE analysis of a. bacterial 16S rRNA genes and b. archaeal 16S rRNA genes amplified from the total DNA extracted from all time point of slurry C (5mg/ml phytodetritus) during the sulphate reduction phase.

DGGE Band	Nearest match by BLASTN search (accession number)	% sequence similarity (alignment length, bp)	Phylogenetic affiliation	Isolation environment of nearest sequence match
Csrb1	Uncultured Bacteroidetes bacterium clone OMEGA_pl_cont_6_F08 (EU052254)	99 (155)	Bacteriodetes	Microbial fuel cells supplied with marine plankton
Csrb3	Uncultured bacterium clone: 560D_B7 (AB598237)	100 (135)	Firmicutes	Continuous-flow bioreactor, Shimokita Peninsula subseafloor sediment
Csrb4	Shewanella sp. J2011s (JF820760)	97 (160)	Gammaproteobacteria	Sponge microbial communities, Irish waters
Csrb5	Uncultured Pseudoatteromonas sp. clone DP11 (JQ965713)	100 (160)	Gammaproteobacteria	Mangrove plant Avicennia marina rhizosphere, nam, Tamil Nadu, India
Csrb10	Pseudoalteromonas elyakovii strain BSi20460 (DQ665793)	95 (160)	Gammaproteobacteria	Arctic sea ice, Canada Basin
Csra2	Uncultured Crenarchaeote clone MOBKM43T9549 (AM942172)	95 (116)	Crenarchaeota (MCG)	Gassy Subsurface Sediments of Marennes- Oleron Bay France)
Csra6	Methanococcoides sp. MO-MCD (AB598271)	100 (117)	Euryarchaeota (Methanococcoides)	Continuous-flow bioreactor, Shimokita Peninsula subseafloor sediment
Csra8	Uncultured archaeon clone MKCST-C5 (DQ363807)	95 (116)	Crenarchaeota	Mangrove soil
Csra10	Uncultured archaeon clone Kazan-2A-24/BC19-2A-24 (AY592001)	95 (116)	Crenarchaeota	Mediterranean cold seep
Csra13	Uncultured archaeon clone BD72AR33 (GU363084)	95 (116)	Crenarchaeota	Qiongdongnan basin, South China Sea
Csra14	Uncultured archaeon isolate SB-24a1C2 (AF354132)	97 (117)	Euryarchaeota (Methanococcoides)	Californian continental margin
Csra15	Uncultured archaeon clone PM-154-76-M13R (JQ925289)	97 (116)	Euryarchaeota (Thermoplasmatales)	Cold seep sediments
Csra17	Uncultured archaeon clone R-UA-121 (JQ258714) (AB301861=published)	95 (116)	Crenarchaeota (MCG)	Tropical West Coast of India

Table 5.9. Bacterial and archaeal 16S rRNA gene sequence matches to excised DGGE bands from the sulphate reduction phase of slurry C incubated with ¹³C-acetate for 2 days.





Methanogenic phase: the DGGE profiles of the bacterial 16S rRNA genes of slurries C and D were fairly similar throughout the methanogenic incubations (figure 5.15a) with one of the identified bands present in all time points of both incubations being 99% similar to a *Fusobacteria* like sequence (table 5.10). Also present in slurry C was a *Dethiosulfatibacter* like *Firmicutes* sequence and an *Epsilonproteobacterium* like sequence. Some of the bands became more intense in the higher temperature slurry D incubation and some new bands appeared such as Dm13, Dm14, and Dm15 which were similar to *Bateriodetes* and *Firmicutes* like sequences (table 5.10). *Firmicutes* like phylotypes dominated the methanogenic phase incubations of slurries C and D (figure 5.16).

The DGGE profiles of the archaeal 16S rRNA genes of slurries C and D showed similar intense bands marked as cluster1 (C1) in figure 5.12b. One of the bands from this cluster and Cma10 with a much higher melting domain position were shown to be similar to the methanogenic *Methanococcoides* and *Methanomicrobiales* like sequences (table 5.10) suggesting that this cluster was representative of methanogenic populations. There were other bands which appeared and disappeared higher up on the gradient throughout the later stages of the slurry C incubation and throughout the slurry D incubation which were similar to a *Methanococcoides* like sequence, and a MCG like *Crenarchaeote* sequence. The methanogenic phase incubations were dominated by *Euryarchaeote* like sequences with a high proportion of *Methanomicrobiales* like phylotypes (figure 5.14).



Figure 5.12. PCR-DGGE analysis of a. bacterial 16S rRNA genes and b. archaeal 16S rRNA genes amplified from the total DNA extracted from all time point of slurry C (5mg/ml phytodetritus 10^oC) and slurry D (5mg/ml phytodetritus 18^oC) during the methanogenic phase.

DGGE Band	Nearest match by BLASTN search (accession number)	% sequence similarity (alignment length, bp)	Phylogenetic affiliation	Isolation environment of nearest sequence match
Cma7	Uncultured <i>Methanococcoides</i> sp. isolate DGGE gel band 5.1 (JN387607)	95 (117)	Euryarcheota	Hydrothermal system of Deception Island, Antarctica
Cma10	Methanomicrobiales archaeon 'SBAK-CO2-reducing Enrichment-4' (DQ280485)	95 (117)	Euryarcheota	Marine sediments from Skan Bay, Alaska.
Cma17	Uncultured archaeon clone SMTZArch6 (EU681930)	95 (117)	Euryarcheota	Shallow sediments of the Pearl River Estuary, Southern China
Dma22	Uncultured archaeon clone E505-A1-89 (HQ214605)	97 (116)	Crenarchaeota (MCG)	Marine sediments, South China Sea
Dma25	<i>Methanococcoides methylutens</i> type strain DSM2657T (FR733669)	95 (116)	Euryarcheota	Sequencing Orphan Species: filling the gaps in the 16S rRNA gene sequence database.
Dma38	Uncultured archaeon clone 21A176 (EU179130)	100 (97)	Euryarchaeota	Deep Eastern Mediterranean Sea
Dma38 Cm2 & Dm9	Uncultured archaeon clone 21A176 (EU179130) Uncultured bacterium clone 44H9 (JF827409)	100 (97) 99 (139)	Euryarchaeota Fusobacteria	Deep Eastern Mediterranean Sea Bacterial communities in the live Pacific oyster (<i>Crassostrea gigas</i>)
Dma38 Cm2 & Dm9 Cm5	Uncultured archaeon clone 21A176 (EU179130) Uncultured bacterium clone 44H9 (JF827409) Dethiosulfatibacter sp. enrichment culture clone MB2_7 (AM933654)	100 (97) 99 (139) 100 (135)	Euryarchaeota Fusobacteria Firmicutes (Clostridia)	Deep Eastern Mediterranean Sea Bacterial communities in the live Pacific oyster (<i>Crassostrea gigas</i>) Enrichment of a dioxin- dehalogenating <i>Dehalococcoides</i> species in two-liquid phase cultures
Dma38 Cm2 & Dm9 Cm5 Cm6	Uncultured archaeon clone 21A176 (EU179130) Uncultured bacterium clone 44H9 (JF827409) Dethiosulfatibacter sp. enrichment culture clone MB2_7 (AM933654) Uncultured epsilon proteobacterium clone ES08-G-34 (JQ712465)	100 (97) 99 (139) 100 (135) 100 (135)	Euryarchaeota Fusobacteria Firmicutes (Clostridia) Epsilonproteoba cteria	Deep Eastern Mediterranean Sea Bacterial communities in the live Pacific oyster (<i>Crassostrea gigas</i>) Enrichment of a dioxin- dehalogenating <i>Dehalococcoides</i> species in two-liquid phase cultures Hydrothermal vents of the Juan de Fuca Ridge
Dma38 Cm2 & Dm9 Cm5 Cm6 Dm13	Uncultured archaeon clone 21A176 (EU179130) Uncultured bacterium clone 44H9 (JF827409) Dethiosulfatibacter sp. enrichment culture clone MB2_7 (AM933654) Uncultured epsilon proteobacterium clone ES08-G-34 (JQ712465) Uncultured Bacteroidetes bacterium clone 70mos_0s_C2 (GQ261807)	100 (97) 99 (139) 100 (135) 100 (135) 95 (156)	Euryarchaeota Fusobacteria Firmicutes (Clostridia) Epsilonproteoba cteria Bacteriodetes	Deep Eastern Mediterranean Sea Bacterial communities in the live Pacific oyster (<i>Crassostrea gigas</i>) Enrichment of a dioxin- dehalogenating <i>Dehalococcoides</i> species in two-liquid phase cultures Hydrothermal vents of the Juan de Fuca Ridge Localized organic loading in the deep sea
Dma38 Cm2 & Dm9 Cm5 Cm6 Dm13 Dm14	Uncultured archaeon clone 21A176 (EU179130) Uncultured bacterium clone 44H9 (JF827409) Dethiosulfatibacter sp. enrichment culture clone MB2_7 (AM933654) Uncultured epsilon proteobacterium clone ES08-G-34 (JQ712465) Uncultured Bacteroidetes bacterium clone 70mos_0s_C2 (GQ261807) Uncultured Gram-positive bacterium clone GoM GC234 (AY211678)	100 (97) 99 (139) 100 (135) 100 (135) 95 (156) 100 (135)	Euryarchaeota Fusobacteria Firmicutes (Clostridia) Epsilonproteoba cteria Bacteriodetes Firmicutes (Clostridia)	Deep Eastern Mediterranean Sea Bacterial communities in the live Pacific oyster (<i>Crassostrea gigas</i>) Enrichment of a dioxin- dehalogenating <i>Dehalococcoides</i> species in two-liquid phase cultures Hydrothermal vents of the Juan de Fuca Ridge Localized organic loading in the deep sea Gas hydrate mounds in the Gulf of Mexico

Table 5.10. Bacterial and archaeal 16S rRNA gene sequence matches to excised DGGE bands from the methanogenic phase of slurries C and D incubated with ¹³C-acetate for 3 days.



Figure 5.13. Proportions of the major bacterial phyla shown to be present in the methanogenic phase of the ¹³C-acetate incubation of slurries C and D.



Figure 5.14 Proportions of the major archaeal phyla shown to be present in the methanogenic phase of the ¹³C-acetate incubation of slurries C and D.

DNA fractionation

Bacterial community

The PCR-DGGE analysis of the bacterial 16S rRNA genes from the total DNA extracted from all the slurry treatments and sampling time points of the SRP incubation of slurry D showed considerably similar profiles with a few new bands for example DSb1 and the cluster DSb3 (figure 5.15). The profiles of the bacterial communities from the slurries stimulated with a relatively large concentration of acetate and concomitant rates of utilisation were remarkably similar to the profile of the control slurry at 48 hours which had no addition of substrate. Some bands of the dominant members of the community became rather more dominant over the incubation such as DSb2.





The fractionated DNA from the 24 hour sampling point of the incubation with ¹³C-acetate and ¹²C-acetate was fractionated using CsCl gradients and ultracentrifugation to separate ¹³C-enriched and natural abundance DNA and all fractions were collected. Both the gradients were linear and covered the density range that the approach had been optimised for (chapter 3). The DNA recovered from the gradient fractions of the ¹³C-acetate slurry DNA was visible across the gradient but the DNA could not be quantified using the imaging system. The DNA recovered from the ¹²C-acetate slurry gradient was not visible but bacterial and archaeal 16S rRNA genes were able to be amplified from both the ¹²C-acetate and ¹³C-acetate gradients.



Figure 5.16. PCR-DGGE analysis of bacterial 16S rRNA genes amplified from all fractions of the CsCl ultracentrifugation analysis of the DNA from the 5 mg/ml phytodetritus slurry at 18 ^oC (D) after 24 hours of incubation with ¹³C-acetate. Lanes marked 1-14 represent the ¹³C-enriched fractions, lane 16-17, the 'mixed' fractions and lanes 18 to 23 the ¹²C fractions

PCR-DGGE analysis of the bacterial 16S rRNA genes showed that several members of the bacterial community were able to utilize ¹³C-acetate and/or its degradation products. There were bands present in the fractions experimentally shown to contain ¹³C labelled DNA, 'mixed' and unlabelled DNA (figure 5.16). The bands found in the labelled fractions were in similar positions to some bands in the unlabelled fractions but were much fainter. This labelling was confirmed by the ¹²C-acetate slurry DNA gradient which showed profiles in the fractions containing DNA with the natural abundances of stable carbon isotopes and the lighter end of the 'mixed' profiles but no profiles in the fractions where labelled DNA is expected (figure 5.17). Bands that were able to be excised and sequenced from the profiles showed similarities with *Bacteriodetes, Firmicutes* and *Gammaproteobacteria* (table 5.11) with the *Firmicutes* being the

dominant phylum of bacteria able to utilize ³C-acetate and/or its degradation products (figure 5.18).

DGGE Band	Nearest match by BLASTN search (accession number)	% sequence similarity (alignment length, bp)	Phylogenetic affiliation	Isolation environment of nearest sequence match
DSRIb3	Cytophaga sp. N05VI (AJ786088)	96 (154)	Bacteriodetes	Coastal subsurface sediments, The Wadden Sea
DSRIb4	Uncultured Firmicutes bacterium clone A12-45 (JN371667)	89 (135)	Firmicutes	Microbial community composition following Microcystis blooms
DSRIb5	Sulfurospirillum sp. enrichment culture clone SGLB4-52 (JQ256485)	86 (134)	Firmicutes	Oil reservoir fluids
DSRIb7	Lutibacter litoralis strain CL-TF09 (NR_043301)	90 (160)	Bacteriodetes	Tidal flat sediment, Ganghwa, Korea
DSRIb6	Pseudoalteromonas carrageenovora strain ZR027 (JX173519)	90 (160)	Gammaproteobacteria	Seaweeds in Qingdao
DSRIb10	Dethiosulfatibacter aminovorans strain C/G2 (= JCM 13356, = NBRC 101112, = DSM 17477) (NR_041309)	99 (134)	Firmicutes	Coastal marine sediment

Table 5.11. Bacterial 16S rRNA gene sequence matches to excised DGGE bands from the sulphate reduction phase ultracentrifugation fractions of slurry D DNA from the¹³C-acetate incubation after 24 hours.



Figure 5.17. PCR-DGGE analysis of bacterial 16S rRNA genes amplified from all fractions of the CsCl ultracentrifugation analysis of the DNA from the 5 mg/ml phytodetritus slurry at 18 ^oC (D) after 24 hours of incubation with ¹²C-acetate. Lanes marked 1-14 represent the ¹³C-enriched fractions, lane 15-17, the 'mixed' fractions and lanes 18 to 24 the ¹²C fractions.



Figure 5.18. Proportions of the major bacterial phyla shown to have utilised ¹³C-acetate and/or its degradation products after 24 hours of incubation of slurry D.

Archaeal community

The PCR-DGGE analysis of the archaeal 16S rRNA genes from the total DNA extracted from the slurry D ¹²C-acetate and ¹³C-acetate incubations and all sampling time points of the SRP incubations (figure 5.19) showed that, with the exception of the ¹²C-acetate slurry at 6 hours, the archaeal communities looked remarkably similar between and throughout the incubations, and between the control and the other incubations suggesting that the addition of the relatively large amount of acetate did not significantly alter the total archaeal community in terms of the species richness. There was however some variation in some faint bands that were clearly not dominant populations within the community.



Figure 5.19. PCR-DGGE analysis of archaeal16S rRNA genes amplified from the total DNA extracted from all time points of the ¹²C and ¹³C-acetate incubations and the control incubation of slurry D during the sulphate reduction phase.


Figure 5.20. PCR-DGGE analysis of archaeal 16S rRNA genes amplified from all fractions of the CsCl ultracentrifugation analysis of the DNA from the 5 mg/ml phytodetritus slurry at 18 ^oC (D) after 24 hours of incubation with ¹³C-acetate. Lanes marked 1-14 represent the ¹³C-enriched fractions, lane 16-17, the 'mixed' fractions and lanes 18 to 23 the ¹²C fractions.

The analysis of fractions using PCR-DGGE analysis of the archaeal 16S rRNA genes showed some bands appearing in the ¹²C-DNA fractions and the ¹³C-DNA fractions and some that were only present in the ¹³C-DNA fractions (figure 5.23). Thus, there was a subset of a relatively complex community able to utilise the ¹³C-acetate and/or its degradation products that were not present in the ¹²C fractions, for example bands DSRIa1 and DSRIa26 (figure 5.20) which were both found to be similar to Thermoplasmatales like sequences (table 5.12). A selection of these bands were excised and sequenced and were mainly Euryarchaeote sequences belonging to the Thermoplasmatales and MBG-D, the methanogenic Methanomicrobiales and Methanosarcinales with one sequence being 100% similar to an ANME-1 sequence (table 5.12). There was also one Crenarchaeote MG1 like sequence which had incorporated the ¹³C-acetate and/or its degradation products. As before, the ¹²C-acetate slurry DNA fractionation provided further evidence that the 'heavy' fractions were indeed ¹³C labelled DNA as the ¹³C-enriched fractions were empty in the ¹²C-acetate gradient except for lanes 9 and 12 which were possibly contaminated as the lanes between and 13 after were blank (figure 5.21). There were profiles in the 'lighter' end of the 'mixed'



M 1 2 2 4 5 6 7 9 0 10 11 12 13 14 15 16 17 18 19 20 2			
M 1 2 3 4 3 6 7 6 9 10 11 12 10 10 17 10 13 20 2	1 22 23 24	- +	- + M
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Figure 5.21. PCR-DGGE analysis of archaeal 16S rRNA genes amplified from all fractions of the CsCl ultracentrifugation analysis of the DNA from the 5 mg/ml phytodetritus slurry at 18 $^{\circ}$ C (D) after 24 hours of incubation with 12 C-acetate.

DGGE band	¹² C/ ¹³ C DNA	Closest match by Blastn (Accession number)	% Sequence ID (alignment length) of most similar band	Phylogenetic affiliation	Environmental location of sequence match
DSRIa1	¹³ C	Uncultured archaeon clone MC10+_H01 (HM019181.1)	99 (118)	Thermoplasmales	Tropical mangrove sediments (pristine and anthropized)
DSRIa6	¹³ C	Uncultured Thermoplasmatales archaeon clone GoM GC234 045A (AY211725)	99 (118)	Thermoplasmales	Sediments associated with surface-breaching gas hydrate mounds in the Gulf of Mexico
DSRIa12	¹³ C	Uncultured Euryarchaeote clone Arch4486_093 (JF937744)	93 (118)	MBGD	Guaymas Basin hydrothermal sediments
DSRIa11	¹³ C	Uncultured archaeon clone SBAK-shallow-42 (DQ640173)	99 (118)	MBGD	Marine sediments from Skan Bay, Alaska
DSRIa2	¹³ C and ¹² C	Uncultured archaeon clone MNO302arcC9 (GU996759)	100 (118)	Methanomicrobiales	Methanogenic crude oil degrading microcosms (sediment from River Tyne)
DSRIa14	¹³ C	Uncultured Euryarchaeote clone ClipA 5 (HQ692040)	98 (116)	MBGD	unique stratified lagoon, the Clipperton atoll (N Pacific)
DSRIa43	¹³ C	Uncultured Euryarchaeote clone Arch4486_080 (JE937739)	91 (116)	MBGD	Guaymas Basin hydrothermal sediments
DSRIa47	¹³ C	Uncultured archaeon clone MC10B_B02 (HM019067)	95 (118)	Euryarchaeota	Tropical mangrove sediments (pristine and anthropized)
DSRIa26	¹³ C	Uncultured archaeon clone TWP4-10 (GQ410890)	95 (117)	Thermoplasmatales	Deep subsurface sediments from the tropical Western pacific
DSRIa44	¹³ C	Uncultured archaeon clone Ar25-51 (AY192633)	96 (118)	MG1	Marine sponges from Korea
DSRIa32, 72	¹³ C and ¹² C	Uncultured archaeon clone K2NMV-30-AB (FJ712396)	100 (113)	ANME-1	East Mediterranean Sea Kazan mud volcano
DSRIa74	¹² C	Uncultured archaeon clone AMSMV-25-A21 (HQ588680)	99 (113)	Euryarchaeota	Sediment vertical profile of a deep-sea mud
DSRIa23	¹³ C	Methanospirillum hungatei gene (AB517987)	92 (118	Methanomicrobiales	Puddly soil
DSRIa67	¹² C	Uncultured Methanococcoides sp. isolate DGGE gel band 5.1 (JN387607.1)	92 (115)	Methanosarcinales	Archaeal hyperthermophiles from hydrothermal system of Deceptio Island, Antarctica
DSRIa53, 70, 71	¹³ C	Methanococcoides sp. MO-MCD gene (AB598271)	99 (116)	Methanosarcinales	Continuous-flow bioreactor, Shimokita Peninsula subseafloor sediment
DSRIa20, 62, 69	¹³ C and ¹² C	Methanomicrobiales archaeon 'SBAK-CO2-reducing Enrichment-4' (DQ280485)	100 (117)	Methanomicrobiales	Marine sediments from Skan Bay, Alaska.
DSRIa18	¹³ C	Methanogenic archaeon enrichment culture clone NapK- 0_20-enr36 (HM004947)	100 (118)	Methanosarcinales	Napoli mud volcano, Eastern Mediterranean Sea

Table 5.11. Archaeal 16S rRNA gene sequence matches to excised DGGE bands from the sulphate reduction phase ultracentrifugation fractions of slurry D DNA from the¹³C-acetate incubation after 24 hours.

Diversity of archaea that utilised ¹³C-acetate and/or its degradation products

The fractionation of the DNA, sequencing and BLAST analysis of the retrieved sequence showed that the largest proportion of phylotypes found to have incorporated the ¹³C-acetate and/or its degradation products were similar to *Methanomicrobiales* and *Methanosarcinales* like *Methanobacteria* sequences (figure 5.22) A large proportion of the sequences were similar to *Thermoplasmatales*/MBG-D like sequences and the phylogenetic analysis showed that the *Crenarchaeote* sequence did indeed group with MG1 like sequences (figure 5.23).



Figure 5.22. Proportions of the major archaeal phyla shown to have utilised ¹³C-acetate and/or its degradation products after 24 hours of incubation of slurry D.



Figure 5.23. Neighbour joining phylogenetic tree showing the diversity of *Archaea* 16S rRNA gene DGGE sequence fragments from the ¹³C-DNA fractions extracted from the sulphate reduction phase of Colne estuary sediment slurry D which was treated with 5 mg/ml phytodetritus and incubated at 18^oC with ¹³C-acetate for 24 hours. Bootstrap support values over 40% (1000 replicates) are shown.

5.3 Microbial community composition and phytodetritus

Analysis of the banding patterns of the DGGE gels using Total lab Phoretix1D software to estimate dendrograms showed that the control slurry, with no added phytodetritus, had the most different banding pattern when compared with the other slurries (figure 5.24). This supported the findings of the qualitative analysis of the gels, that the addition of the phytodetritus altered the bacterial communities in the estuarine sediment slurries with the groupings of the profiles reflecting the broad taxonomic composition of the communities as identified through the BLAST analysis (figure 5.25) where in the control slurry the *Gammaproteobacteria* and *Epsilonproteobacteria* were found in equal proportions while in all the other slurry treatments the *Gammaproteobacteria* dominated. Within the *Gammaproteobacteria*, *Pseudoaltermonadaceae* dominated in the 0.2 and 1.0 mg/ml slurries while in the 5m/ml slurries the *Vibrionaceae* were found

in similar proportions to the *Gammaproteobacteria* and *Bacteriodetes* were also detected.

Colwellia were not detected in the DGGE profiles of the 5mg/ml (C & D) slurries but were recovered from the 0.2 (A) and 1.0 (B) mg/ml slurries. There were some similarities in the aerobic phase between the high and low phytodetritus loaded slurries, for example, bands Aa5 from slurry A and Dada4 from slurry D which both showed high similarity (96%) to *Shewanella sp.* (table 5.2 and 5.4). A *Gammaproteobacteria* related sequence relating to *Pseudoalteromonas mariniglutinosa* was found in the 1.0 mg/ml (B), and the 5.0 mg/ml (C and D) slurries.



Figure 5.24. Dendrogram of similarities of DGGE banding patterns of bacterial 16S rRNA gene analysis of all incubations. Ctrl = control, 5.0 = slurry C, 5.0 18 = slurry D, 1.0 = slurry B, 0.2 = slurry A. ab = aerobic phase, dab = dysaerobic phase, SRB = sulphate reduction phase, meth = methanogenic phase. Incubation time shown as 01=0 hours, 02=6 hours, 03=12hours, 04=24 hours, 05=48 hours, 06=72 hours, 07=0 hours, 08=6 hours, 09=12hours, 10=24 hours, 11=48 hours, 12=72 hours . Values on axes show average similarity.



Figure. 4.25 Phylogenetic distribution of phylotypes found from bacterial 16S rRNA gene DGGE analysis of 1) Control 10 $^{\circ}$ C 2) 0.2 mg/ml 10 $^{\circ}$ C PD (A) 3) 1.0 mg/ml 10 $^{\circ}$ C PD (B) 4) 5.0 mg/ml PD 10 $^{\circ}$ C (C) 5) 5.0 mg/ml PD 18 $^{\circ}$ C (D).

The analysis of the archaeal 16S rRNA gene banding patterns of the DGGE gels using Total lab Phoretix1D software to estimate dendrograms showed that for the aerobic and dysaerobic phases of the incubations, the control was more similar to the low phytodetritus loading slurries than they were to the high phytodetritus loading slurries suggesting there was a clear change in community structure with the higher phytodetritus loading which can clearly be seen in the profiles (figure 5.26). The loading of less phytodetritus did not effect such a deviation from the control in the archaeal community structure during the aerobic and dysaerobic phases as compared to the bacterial community. The archaeal communities were quite different during the anaerobic phases of the incubation being grouped away from the other phases. The number of archaeal bands detected did not change throughout the aerobic and dysaerobic incubations with all treatments and were higher in those treated with more phytodetritus. The number of bands decreased in the methanogenic phase of the incubation in a similar way to the number of bacterial bands and Chi² testing showed the distribution was shown to be significantly different in both the 5 mg/ml phytodetritus slurries across the incubation phases.



Figure 5.26. Dendrogram of similarities of DGGE banding patterns of archaeal 16S rRNA gene analysis of all incubations. Ctrl = control, 5.0 = slurry C, 5.0 18 = slurry D, 1.0 = slurry B, 0.2 = slurry A. ab = aerobic phase, dab = dysaerobic phase, SRB = sulphate reduction phase, meth = methanogenic phase. Incubation time shown as 01=0 hours, 02=6 hours, 03=12hours, 04=24 hours, 05=48 hours, 06=72 hours, 07=0 hours, 08=6 hours, 09=12hours, 10=24 hours, 11=48 hours, 12=72 hours . Values on axes show average similarity.



Figure 5.27. Number of bacterial bands in each treatment as defined by parameters in Phoretix 1D. blue=aerobic phase, red=dysaerobic phase. Error bars=standard deviation.



Figure 5.28. Number of bacterial bands in each treatment as defined by parameters in Phoretix 1D during the defined incubation stages. blue=5mg/ml PD 10 °C, red=5mg/ml PD 18 °C. Error bars=standard deviation.

The number of unique bacterial bands in each incubation as determined with fixed parameters in Phoretix1D (figure 5.27.) were shown through paired t-tests to be significantly different in all treatments compared to the controls with p values of 0 which reflects the findings of the dendrogram (figure 5.25). From the 1 mg/ml PD treatment to both the high PD treatments there were significant differences in bacterial band

numbers and the number of bacterial bands in the high PD treatments showed significant differences between the redox phases of the incubations with the methanogenic phase showing significantly lower bands than the dysaerobic and sulphate reduction phases for both (figure 5.28)

The number of unique archaeal bands in each incubation as determined with fixed parameters in Phoretix1D (figure 5.29.) showed no significant difference between treatments and the control.



Figure 5.29. Number of archaeal bands in each treatment as defined by parameters in Phoretix 1D. blue=aerobic phase, red=dysaerobic phase. Error bars=standard deviation.



Figure 5.30. Number of archaeal bands in each treatment as defined by parameters in Phoretix 1D. blue=5mg/ml PD 10 °C during the defined incubation phases, red=5mg/ml PD 18 °C. Error bars=standard deviation. There was a decrease in the number of archaeal bands in the high PD slurries in the sulphate reduction and methanaogenesis phases (figure 5.30) and was only significantly different between the high PD 18 $^{\circ}$ C sulphate reduction phase and the high PD 18 $^{\circ}$ C aerobic and dysaerobic phases, and between the high PD 10 $^{\circ}$ C slurry during the methanogenic and the aerobic and dysaerobic phases.

6. Discussion - Insights into diversity and function

6.1. Community structure and function

The addition of phytodetritus (PD) to marine estuarine sediment microcosms clearly stimulated microbial activity resulting in the medium and high phytodetritus loaded slurries having faster rates of oxygen depletion. Larger accumulation of short chain fatty acid fermentation products and larger CO₂ production rates in the medium and high phytodetritus slurries. In the high phytodetritus loaded microcosms sulphate reduction established and methanogenesis established even before sulphate reached limiting concentrations. The prevalence of highly reducing condition was almost immediately after the oxygen was depleted in the 5 mg/ml phytodetritus microcosm incubated at the higher temperature of 18 °C indicating the higher temperature caused the selection of a consortia with a higher temperature optimum capable of remineralising organic matter at a faster rate (Arnosti et al., 1998) and caused a faster rate of reaction of the exoenzymes acting on PD polymers (Mullholland and Gilbert, 2003). This rapid biogeochemical response to the addition of PD was similar to the response found by Gihring et al., 2009, who added ¹³C labelled Spirulina cells to coastal sediment incubations, and suggests that members of the sedimentary microbial community were ready to begin degrading organic matter that arrived in the sediment.

The significant change in the bacterial community structure with the addition of PD and the resultant significantly greater numbers of dominant bacterial phylotypes initially in the aerobic phase, were largely represented by fast growing generalist aerobic and hydrolysing/fermenting species involved in the involved in the initial degradation steps in the breakdown of PD macromolecule. The significant increases in bacterial phylotypes in the dysaerobic phases of the higher PD and higher temperature incubations reflects the larger availability of organic substrates allowing fast growing generalists to flourish further with the short chain fatty acids such as propionate and acetate accumulations suggesting a more complex fermenting community, and with the more heterogeneous redox conditions with depleted oxygen and possibly semi oxic microenvironments, anoxic adapted phylotypes such as *Bacteriodetes* were able to compete with the fast growing facultatively anaerobic generalists.

In all of the phytodetritus treatments in the aerobic and dysaerobic phases and the sulphate reduction phase, the *Gammaproteobacteria* were dominant which is consistent with this subphyla often being dominant in the surface of coastal and tidal sediments (Webster *et al.*, 2010) and the *Alteromonadales*, present in the low and high aerobic and dysaerobic phases but not the control show metabolic readiness and have a high activity of hydrolases and carry out mixed acid fermentation (Mikhailov *et al.*,

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2006). In the high PD slurries the obligatory anaerobic *Bacteriodetes* appeared and in the sulphate reduction and methanogenic phases the *Firmcutes* and *Fusobacteria* became important components of the community with no *Gammaproteobacteria* present in the methanogenic phase. The initial increase in dominant bacterial phylotypes with incubation time and then decrease in the methanogenic phase was likely due to the increase in phylotypes important in 'intermediary metabolism' such as *Shewanella* phylotypes utilising fermentation end products produced earlier in the incubation as they are known to utilise simple carbon compounds such as lactate rather than fermenting (Nealson and Scott, 2006). The significantly smaller number of bands in the high PD slurries incubated for the longest and progression into the methanogenic phase, suggested a much more specialised community in the highly reducing and high organic matter microcosms.

The lack of significant difference between archaeal community profiles and dominant phylotypes numbers between PD treatments and the control in the aerobic and dysaerobic phases suggest that the archaeal community was relatively stable in response to the input of organic matter although it is not known from these experiments what parts of the communities were active as there were no clear changes. So the role of the archaeal community in the first steps of organic matter breakdown in the system was not clear and requires further investigation. However the community profiles showing significantly different banding patterns and significantly less dominant phylotypes in the sulphate reduction and methanogenic phases showed that the archaeal community and the archaeal community changed from being dominated by the *Crenarchaeota* in the sulphate reduction phase to the *Euryarchaeota* in the methanogenic phase.

Potential activity of the microbial populations and communities was inferred through the geochemical measurements, changes in community composition and closest cultured representatives and environmental sequences, and specific activity was related to acetate and glucose utilisation from stable isotope probing analysis

Bacteria

No phytodetritus

The *Sulfurovum lithotrophicum* like sequence detected in the control, low, high PD and methanogenic phase microcosms, was likely responsible for the measured increased SO_4^{2-} in these slurries as Inagaki *et al.*, (2004) characterised *Sulfurovum lithotrophicum* from a hydrothermal system and found that almost all 7.5 mM thiosulfate was oxidized to 15 mM sulphate during cell growth, suggesting that sulphate was the end product of sulphur oxidation. The *Sulfurovum* genus is described as facultatively anaerobic chemolithoautotrophic sulphur- and thiosulfate-oxidizing gram-negative and mesophilic bacteria using oxygen and nitrate as the electron acceptor and CO_2 as the carbon source (Inagaki *et al.*, 2004). This suggests that such a species may be important in the sulphur cycling at the Brightlingsea site of the Colne estuary re-oxidising reduced sulphur species, however this would be unlikely at the more brackish sites such as Hythe, as *S.lithotrophicum* requires sea salts for growth (Inagaki *et al.*, 2004). Although the level of activity of *S.lithotrophicum* in the microcosms described here remains unclear.

The other two dominant populations within the control microcosm bacterial community were similar to *Vibrio lentus* like *Gammproteobacteria* sequence fragments. These were clearer after 24 hours incubation time in the aerobic phase of the control slurry and when there was a clear increase in lactate in the master slurry, and the oxygen was about to start becoming limiting. *Vibrio* are a diverse genus but in general they are facultatively aerobic and employ a fermentative metabolism readily incorporating glucose under anaerobic and aerobic conditions (Farmer and Hickman-Brennar, 2006) and *Vibrio lentus* type strains have all been found to ferment glucose without gas production and to utilise glucose as a sole carbon source (Macián *et al.*, 2001) suggesting that this population of *Vibrio* like phylotypes was fermenting the added ¹³C glucose label in the slurry producing lactate as the fermentation end product. Due to the facultative nature of *Vibrio* it has been suggested that they are perfectly adapted oxic-anoxic interfaces (Alonso and Pernthaler, 2005) which is supported by the appearance of these phylotypes in the control slurry when the oxygen was starting to become limiting.

The *Epsilonproteobacteria Arcobacter nitrofigilis* like sequence in the control bacterial community is known to be a nitrogen-fixing bacterium associated with the roots of the salt marsh plant *Spartina alterniflora* and in root associated sediment (McClung *et al.,* 1983) suggesting the population could be associated with organic matter from a nearby salt-marsh such as Colne Point salt marsh. These chemoorganophs are obligately

microaerophilic when using oxygen as the terminal electron acceptor, also being able to utilise asparate and fumarate under anaerobic conditions and they utilize organic acids and amino acids as carbon sources but not carbohydrates (McClung *et al.*, 1983) suggesting that if the population was active within the sediment slurry that they may have been utilising the organic acids produced from fermentation, although the band was faint suggesting it wasn't a dominant population. *Arcobacter nitrofigilis* would likely be restricted to the marine areas of the estuary as these require NaCl of approximately oceanic salinity (McClung *et al.*, 1983).

Low phytodetritus

The heterotrophic community in the estuarine sediment was stimulated by the addition of 0.2 mg/ml of PD with an increase in the diversity of the dominant bacterial phylotypes. This diversity was represented by Pseudoalteromonas, Shewanella and Colwellia phylotypes with one less Epsilonproteobacteria phylotype. This increase in these groups fits agrees with a previous study using ¹³C Spirulina in coastal sediment incubations which found that members of the Alteromonadales had incorporated the labelled carbon (Gihring et al., 2009). The appearance of this group in all slurries with added PD except the methanogenic phase suggest it is important in benthic organic matter degradation in estuarine and coastal sedimentary systems. Pseudoalteromonas are reported to be largely aerobic chemoheterotrophs (Bowman, 2007) using a respiratory type of metabolism with some facultative anaerobes (Mikhailov et al., 2006). The closest cultured representatives to some phylotypes were similar to Pseudoalteromonas piscicida and similar species such as Pseudoalteromonas espegiana, Pseudoalteromonas atlantica, P. issachenkonii, P. tetraodonis, P.carrageenovora, P.paragorgicola and another phylotype's closest cultured representative was Pseudoalteromonas elyakovii. This was interesting as these phylotypes have previously been grouped in different clades within the Pseudoalteromonas genus (Bowman, 2007) possibly demonstrating the unresolved phylogeny of the genus and the limitations of the short sequences described in this research. Pseudoalteromonas elyakovii is an aerobic chemorganotroph that does ferment glucose and hydrolyses starch (Sawabe et al., 2000) and Pseudoalteromonas atlantica is also reported to be strictly aerobic being able to hydrolyse several biopolymers including starch, agar and DNA, as well as being able to ferment glucose with most strains being able to utilise short chain fatty acids, starch and glucose as their sole carbon and energy source (Akagawa-Matsushita et al., 1992). Phylotypes similar to the aforementioned species were found in all the phases of treatments, including the sulphate reduction phase of the high PD 10 °C slurry and in this and the high PD slurries during the dysaerobic phases the conditions were anoxic, thus it is likely that the

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Pseudoalteromonas community was stimulated by the addition of PD as it was hydrolysing the biopolymers in the PD, aerobically respiring the monomers in the aerobic phase, and then fermenting in the dysaerobic and sulphate reduction phases.

The Saccharophagus degradans like sequence fragment in the low PD was possibly stimulated by the addition of PD and was utilising the complex polysaccharides in the PD as a carbon and energy source such as cellulose and/or agar. It is known to be an aerobic chemoorganotroph that is able to degrade at least 10 complex polysacharrides using them as sole carbon and energy sources (Ekborg 2005). It has been reported that *S.degradans* degrades agar nearly twice as quickly as *Pseudoalteromonas atlantica* (Whitehead *et al.*, 2001) suggesting that *S.degradans* would have outcompeted *P.atlantica* for this substrate in the low PD community although this doesn't seem likely as the biomass indicated by the band intensities was higher for *P.atlantica* suggesting they were possibly metabolising other biopolymers and/or glucose.

Colwellia like phylotypes also featured in the bacterial community in the low PD slurry, all strains are known to reduce nitrate to nitrite and degrade chitin and starch under aerobic conditions (Mikhailov et al., 2006) although these phylotypes seemed to become more prevalent in the community during the dysaerobic phase accounting for the significantly higher number of dominant bacterial phylotypes, suggesting they were either reducing nitrate (although NO_{3²⁻} was less than 10 μ M) or fermenting sugars. The Colwellia like phylotypes were similar to the obligate psychrophile Colwellia *psychrerythraea* which is capable of growth up to 10 °C and is known to be facultatively anaerobic being able to utilise electron acceptors other than oxygen. Many strains are able to hydrolyse urea, and ferment sugars including D-glucose with some strains to utilise glucose and some short chain fatty acids such as lactate (Bowman et al., 1998). One phylotype in the low PD slurry was found to be similar to *Colwellia rossensis* which is described as a facultatively anaerobic chemoheterotroph, being psychrophilic with an optimum growth temperature of 10 °C (the same as the incubation temperature) and able to ferment chitin, N-acetylglucosamine and D-glucose, able to utilise Nacetylglucosamine, D-fructose, D-glucose, glycerol, acetate and other compounds as sole carbon and energy sources (Bowman et al., 1998). Thus, it seems likely that the Colweillia members of the community were involved in the hydrolysis of biopolymers in the PD, the aerobic respiration of subsequent compounds and likely fermentation of sugars, including glucose as a *Colwellia* like phylotype was detected in the stable isotope probing analysis of the ¹³C fractions of the dysaerobic phase of the medium PD slurry. Similar *Colwellia* like phylotypes were found in the medium PD slurry but not in any of the incubation phases of the high PD slurries, suggesting that the genus cannot compete in more reducing conditions.

Shewanella

Shewanella like phylotypes were found in all slurry treatments and phases except for the methanogenesis phase. These are known to be facultative anaerobes that do not ferment but respire using oxygen and a wide variety of other electron acceptors including NO_3 , elemental sulphur and iron and manganese oxides, they are unable to utilise complex carbohydrates, rarely using glucose but rather simple carbon sources particularly lactate, formate and amino acids (Nealson and Scott, 2006) and it is thought possible that Shewanella species enter the environment as 'intermediates' during the biodegradation of organic compounds (Ivanova et al., 2003), seeming likely that Shewanellae are syntrophic partners of fermentation microbes during anaerobic metabolisms via the removal of fermentation end products (Nealson and Scott, 2006). Thus this group is potentially as important as methanogenesis and sulphate reduction in the biodegradation of organic matter in estuarine sediments. It is likely that Shewanella like phylotypes in the slurries were using simple carbon sources produced from the metabolism of other members of the bacterial community such as Colwellia that were producing fermentation products such as lactate and formate, that were measured in the porewater of the slurries, and possibly utilising amino acids from protein hydrolysis, as it is known that cold-adapted members of *Pseudoalteromonas*. Shewanella and Colwellia produce extracellular proteases (Yu et al., 2009). They were likely using oxygen as the electron acceptor in the aerobic phase contributing to the decrease in oxygen measured in the slurries, and possibly NO_{3} , elemental sulphur and iron and manganese oxides in the dysaerobic and anaerobic phases.

The activity of *Shewanella* like phylotypes may have contributed to the release of phosphate in the dysaerobic and anaerobic phases of both the high PD slurries as it is known that iron oxides strongly bind phosphate, with the microbial reduction of these oxides resulting in the release of phosphate (Nealson and Scott, 2006). This is supported by the phosphate measurements where in all slurries the phosphate initially decreased and then increase under more anaerobic conditions and then levelled out or decrease during the methanogenic phases of the high PD slurries where no *Shewanella* like phylotypes were detected. The concentrations of phosphate measured in the high PD 18 °C slurry were *c*.15x higher than the control, low and medium PD slurries with the high PD 10 °C being in between. This is reflective of the amount of organic matter added, and suggests that much of the measured phosphate was from the breakdown of phosphorus containing organic compounds such as proteins and phospholipids in the PD with the rate of enzymatic activity mediating these reactions being higher in the 18 °C slurry. At the pH measured at the beginning of the incubations, the released phosphorus likely formed complexes with metals in the sediment, subsequently being

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released due to microbial activity of phylotypes such as *Shewanella*. Also contributing to the PO_4^{3-} release in the 5 mg/ml phytodetritus slurries may have been the sulphate reduction which can cause PO_4^{3-} mobilization due to the formation of iron sulphides which have less anion binding sites than iron hydroxides (Smolders *et al.*, 2006). Due to their catabolic activities *Shewanella* are found at interfaces with sulphate reducers and aerobic heterotrophs, spanning a wide variety of ecological niches in layered communities (Nealson and Scott, 2006) which is reflected by their distribution in the communities of varying geochemical conditions in these slurries.

Sequence fragments similar to Shewanella marisflava, Shewanella affinis and S.algidipiscicola, and Shewanella violacea and Shewanella hanedai were detected in the community of the low and high PD slurries except in the methanogenesis phase. S.algidipiscicola isolated from marine fish of the Danish Baltic Sea have been described as aerobic mesophilic chemoheterotrophs able to reduce nitrate and utilise lactate, Nacetylglucosamine and malate as energy sources (Satomi et al., 2007). Phylogenetic analysis based on 16S rRNA gene sequences report 99% similarity between S.algidipiscicola with S.affinis but gyrB gene sequence analysis and DNA hybridisation resolve them as separate species (Satomi et al., 2007). S.affinis is facultatively anaerobic where anaerobic growth occurs by fermentation of D-glucose by anaerobic respiration of nitrate and is able to use glucose as a sole carbon source (Ivanova et al., 2004) although utilisation of glucose seems unlikely as Shewanellae were not detected in the stable isotope probing analysis of the glucose utilising community in the medium PD slurry. Shewanella marisflava is grouped further away from S.affinis and S.algidipiscicola and is reported to grow anaerobically, reduces nitrate to nitrite and is able to ferment some sugars including cellobiose and glucose (Yoon et al., 2004). Shewanella violacea is described as facultatively anaerobic with respiratory and fermentative type metabolisms, able to reduced nitrate and ferment glucose (Nogi et al., 1998) while Shewanella hanedai is described as non-fermentative, able to reduce nitrate and grow on acetate and propionate (Jenson et al., 1980) which were measured to increase in the high PD slurries. Thus all phylotypes detected were similar to known nitrate reducers but the capacity for metal reduction by such phylotypes is unclear. It seems that Shewanella like phylotypes were likely utilising any available nitrate keeping concentraions low (<10 µM in the low PD slurry and below detection limit in the high PD slurries) and in the high PD 10 °C slurry the nitrate increased during the methanogenic phase where there were no Shewanella phylotypes present. This increase in nitrate suggests that there was some anoxic ammonia oxidation (anammox) occurring in the annoxic microcosms with the autotrophic CO₂ fixing by anammox bacteria oxidising NO_2^- to NO_3^- , although Dong *et al.*, 2009 detected anammox only at the estuary head when assessing process rates along the nutrient gradient of the Colne estuary.

Medium phytodetritus

In the slurry with the medium 1mg/ml phytodetritus added, the spikes of organic acids were also earlier than in the control but the concentration wasn't as high suggesting that these were being respired/fermented to CO₂ as this was higher than in the control and low PD slurry with the maximum measured CO_2 release being c1.7 mmoles L⁻¹ sediment compared to just under 1 mmoles L⁻¹ sediment in both the control and low PD slurries. The number of bacterial phylotypes in the aerobic phase of this slurry increased in comparison to the low PD slurry but the diversity and the number of bands actually decreased in the dysaerobic phase of the incubation. The DGGE profiles of the dysaerobic phase of the medium PD slurry were similar to the low PD profiles, being grouped together in the profile analysis so it is likely that the diversity was lower due to something in the band excision, PCR and sequencing process rather than a representative insight into the diversity or that certain populations were more dominant at this particular PD concentration. The Pseudoalteromonas like sequence fragments including Pseudoalteromonas mariniglutinosa and a Vibrio and Colwellia like sequences that were shown to have utilised glucose and/or its degradation products suggested that all of the sequence types identified in the unfractionated DNA analysis were in fact utilising glucose and/or its degradation products after 72 hours of incubation. This fits with a previous study using ¹³C Spirulina in coastal sediment incubations which found that members of the Alteromonadales and Vibrio had incorporated the labelled carbon (Gihring et al., 2009) and were clearly involved in the initial stages of the PD decomposition in the medium PD slurry. The dominant populations with the most intense bands in the ¹³C fractions were similar to *Colwellia* and Pseudoalteromonas. The closest cultured representative Pseudoalteromonas nigrifaciens has been described as strictly aerobic and able to grow on lactate and glucose (Ivanova et al., 1996). It seems that a P. nigrifaciens like phylotype was capable of growing under oxygen limiting conditions, either growing on glucose or lactate produced by primary fermenters from the labelled glucose, and due to the lower measured lactate levels than in the low PD slurry but higher CO2 at 72 hours of incubation it could be that some were secondary fermenting labelled lactate or there were oxic microenvironments present. The Vibrio like phylotype from the ¹³C fractions was shown to be 95% similar to Vibrio gigantus which is reported to be facultatively anaerobic producing nitrite and uses glucose as its sole carbon source (Roux et al.,2005), thus suggesting that this phylotype was possibly metabolising glucose via a nitrate reduction pathway. The closest cultured representative to the Colwellia like band

was *C.pychrerythraea* and as previously mentioned this phylotype within the community would likely have been anerobically respiring glucose or fermenting glucose and/or lactate. It should be noted that with this particular fractionation analysis, the ¹²C substrate DNA was not analysed and so the deduction that these phylotypes were utilising glucose and/ or it's degradation products should be taken with caution and rather as an indication that these phylotypes were utilising glucose.

High phytodetritus

The analysis of the DGGE banding patterns of the bacterial community of the slurry incubated with the high amount of PD incubated at 10 °C showed that, with the exception of a few lanes the aerobic phase, community profiles clustered with the low PD aerobic phase profiles while the dysaerobic phase bacterial community profiles of the high PD 10 °C slurry mostly clustered with the high PD 18 °C slurry aerobic and dysaerobic bacterial community profiles (figure 5.7). Vibrio like phylotypes had clearly been stimulated by the addition of a larger amount of PD in both the high PD slurry communities while *Colwellia* were not detected. There was a large biological oxygen demand in the high PD slurries with the oxygen reaching zero saturation after 2 days in the 10 °C and 3 days in the 18 °C slurry with the oxygen spiking shortly after in both suggesting there was still oxygen in the system, compared to 100% oxygen saturation for 4 days in the control, low PD and medium PD slurries. Secondary fermentation products propionate and acetate being produced early on in the incubations with the appearance of acetate unlikely from acetogens as none were detected in the bacterial community. Thus, these slurries had moved into a more anaerobic environment where Vibrio were likely increased due to increased fermentation activities.

During the dysaerobic phase of the high PD 10 °C slurry a *Bacteriodetes* like phylotype appeared in the community. Sulphate reduction had established during this dysaerobic phase experiment and it is tempting to suggest that the appearance of the *Bacteriodetes* like phylotype was utilising sulphate as an electron acceptor under the anoxic conditions. However, the sequence fragment was 100% similar to the closest cultured representative *Cytophaga* sp which was isolated from The Wadden Sea and reported to not use anaerobic electron acceptors but rather employ a fermentative type metabolism having been shown to ferment glucose (Köpke *et al.*, 2005). Thus it seems likely that in the dysaerobic phase incubation of the *Cytophaga* like phylotype was utilising the added glucose label and/or the monomers from the hydrolysis of the PD biopolymers. The lack of detection of known sulphate reducing bacteria was possibly due to them being only a minor part of the community in comparison to fermentative members of the community that are known to be more abundant (Köpke *et al.*, 2005).

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The generality of the primer set used and the lack of sensitivity of DGGE may also have contributed and it would be interesting to investigate the sulphate reducing bacteria further with functional primers such as dissimilatory sulphate reductase (dsrA) gene sequences (Wagner *et al.*, 1998).

The *Cytophaga* sp like phylotype was also found in the bacterial community of the high PD 18 °C slurry also likely employing a fermentative type metabolism. Another *Bacteriodetes* like phylotype similar to a *Lutibacter* like species of the *Flavobacteriaceae* was present under reducing conditions and was possibly utilising a fermentative type metabolism. The *Lutibacter* genus was originally described as aerobic (Choi and Choi, 2006) but this description was amended to include facultatively anaerobic strains (Lee *et al.*, 2012). A recent stable isotope probing experiments in coastal sediment incubations using ¹³C labelled *Spirulina* cells provided further evidence that *Bacteriodetes* are able to break down high molecular weight organic compounds and are important in benthic matter degradation (Gihring *et al.*, 2009). Thus the *Bacteriodetes* may have been utilising complex organic compounds derived from the added PD.

High phytodetritus sulphate reduction phase

In the sulphate reduction phase, the *Firmicutes* like phylotype in the high PD 10°C slurry was similar to Clostridia like phylotypes with the closest cultured sequence (90% similar) to *Clostridiisalibacter paucivorans*, a strictly anaerobic gram-positive bacterium isolated from olive mill wastewater, found to be heterotrophic with a fermentative metabolism unable to use sugars as a carbon source and fermenting pyruvate, succinate, fumarate in the presence of yeast (Liebgott et al., 2008). The strain is reported to not use sulphate, nitrate or oxygen as electron acceptors and in the SRP of the high PD 10 °C slurry was possibly secondary fermenting other fermentation end products from other phylotypes such as Pseudoalteromonas spp. Although the 90% similarity to *Clostridiisalibacter paucivorans* and the 100% similarity to the uncultured clone present in a methanogenic bioreactor community cultivated from subseafloor sediments (Imachi et al., 2011) suggest a range of metabolism could be possible. The sulphate reduction phase stable isotope probing experiment showed that a Firmicutes like phylotype was 99% similar to Dethiosulfatibacter aminovorans was incorporating the ¹³C acetate label. Dethiosulfatibacter aminovorans have been characterised and use thiosulphate as an electron acceptor and amino acids but not acetate as carbon sources, thus it seems more likely that Dethiosulfatibacter aminovorans was utilising ¹³C labelled amino acids from senescing cells that had utilised the ¹³C acetate, as the sample was taken 24 hours into the incubation and a relatively large ¹³C acetate label

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was added. Phytoplankton are rich in proteins (Jørgensen, 2000) and this study indicated that *Dethiosulfatibacter aminovorans* play a role in the degradation of protein in estuarine sediments with a high organic loading, and Dethiosulfatibacter aminovorans like sulphate reducing bacteria are thought to play a role in amino acid oxidation in eutrophic marine sediments (Takii et al., 2007) which is supported by this study. Other authors have found Firmicutes in sulphur rich environments and/or involved in sulphur cycling in sediments (Boschker et al., 1998; Goorisen et al., 2003). Webster et al., (2006)b found uncultured members of the Firmicutes incorporating ¹³Cacetate in sulphate reducing marine sediment and Kondo et al., 2005 found Firmicutes clones in a *dsrB* library of sulphate reducing communities of organically enriched fish farm sediments. There are known sulphate reducing *Firmicutes* isolates such as Desulfotomaculum reducens (Junier et al., 2010), and similar clones to D.reducens and the distinct uncultivated groups Colne Group 1 and Colne Group 3 and have been detected at freshwater sites of the Colne estuary (Kondo et al., 2007). The rates of sulphate reduction in the high PD slurries were high at 0.2 mM SO₄²⁻ hour⁻¹which was very high compared to 13.1-57.8 nmol ml⁻¹ day⁻¹ at Alresford Creek (Kondo et al., 2007) and there were concomitant decreases in propionate and then increases in propionate (a known substrate for sulphate reduction) when sulphate was limiting (in the high PD 10 °C slurry), thus, dissimilatory sulphate reduction metabolism by the Firmicutes like phylotypes in these slurries seems likely. The faint labelling of the Firmicutes like phylotypes in the ¹³C acetate stable isotope probing indicate the use of other carbon sources and may have reflected the limiting sulphate concentrations in the high PD 18 ℃ slurry at that time point.

The *Shewanella* like phylotype was 97% similar to a strain isolated from sponge communities for Irish waters (Jackson *et al.*, 2012) which was in turn 98% similar to *Shewanella piezotolerans* which is facultatively anaerobic, with nitrate, fumarate, TMAO, DMSO and insoluble Fe(III) as terminal electron acceptors and hydrogen sulfide is produced from thiosulfate with acetate, lacate, glucose and propionate being used as carbon sources (Jackson and Kennedy 2007) suggesting a wide range of metabolisms this phylotype could have been engaged with. The sequence fragment was also 96% similar to *S.violacea,* a pschrophilic *Shewanella* species with optimal growth at 8 °C being facultatively anaerobic and chemoorganotrophic, with both respiratory and fermentative type metabolisms, fermenting glucose and reducing nitrate (Nogi *et al.*, 1998). Thus, considering the phenotypic metabolic characteristic of similar strains it seems likely that this phylotype was fermenting monomers and/or acetate and/or lactate or possibly employing a respiratory type metabolism reducing ferric iron.

High phytodetritus methanogenic phase

The bands indicated in most of the methanogenic incubations that the bacterial populations were much less dominant when compared to the archaeal populations, although some Clostridia like populations became more dominant in the high PD 18 °C slurry after 48 hours of incubations time. The closest cultivated representative to these phylotypes was *Clostridiisalibacter paucivorans* isolated from olivemill wastewater in Morrocco (Pierre-Pol Liebgott, 2008). C. paucivorans was described as a heterotroph utilising pyruvate, succinate and fumurate and various amino acids producing acetate, with a growth temperature range of 20 to 50 °C explaining why similar phylotpes were not present in the high PD 10 °C microcosm. Acetate was measured to be increasing during the methanogenic phase of the high PD 18 °C slurry which was possibly due to the activity of C. paucivorans like phylotypes fermenting organic acids and/or amino acids. The *Dethiosulfatibacter* like phylotype present in the high PD 10 °C was possibly using thiosulphate as an electron acceptor and possibly using amino acids hydrolised by fermenters from the added phytoplankton which is rich in proteins (Jørgensen, 2000). Dethiosulfatibacter aminovorans like sulphate reducing bacteria are thought to play a role in amino acid oxidation in eutrophic marine sediments which is supported by this study (Takii et al., 2007). The obligately anaerobic Fusobacteria like sequence was similar to Cetobacterium somerae which has been found to inhabit the intestinal tracts of fish and has been found in children's faeces (Tsuchiya et al., 2008) and freshwater strains of Ilyobacter insuetus specialised in the fermentation of hydroaromatic compounds (Brune et al., 2002). Thus it seems likely that such populations are adapted to highly anaerobic conditions where there is high supply of plentful supply of organic matter and they can outcompete other fermenters and anaerobic respirers possibly due to the ability to withstand the toxicity of some waste products such as H₂S in eutrophic conditions. The Cytophaga like Bacteriodetes phylotype was also likely fermenting with the nearest cultured representative being the like Cytophaga sp. seen in the other dysaerobic and anerobic phases of the high PD slurries. The most similar environmental sample was shown in deep sea sediment which had received a high organic loading input from a whale fall and the bacterial microbial community was primarily Bacteriodetes, Firmicutes and Epsilonproteobacteria were recovered compared to Gammaproteobacteria and Planctomycetes in the reference sediments (Goffredi and Orphan, 2010).

Archaea

Aerobic phases

As previously mentioned the archaeal phylotypes indicated by the DGGE banding pattern were of lower quality than the bacterial bands. However some bands were recovered which gave some insight into the archaeal phylotypes present. There were changes in the DGGE profiles between treatments demonstrating that some archaea were active under the prevailing conditions. *Thermoplasmatales* like sequences found in the low and medium PD slurries were likely stimulated by the addition of PD as none were present in the control slurry. *Thermoplasmatales* are thought to be a ubiquitous archaeal group in shallow sediments (Teske and Sorensen, 2008). The ¹³C acetate stable isotope probing experiment in the sulphate reduction phase of the high PD 18 ^oC slurry showed that *Thermoplasmatales* like sequences had incorporated the label indicating a heterotrophic metabolism in the group. This finding was similar to the results of Webster *et al.*, 2010 who showed that a MBG-D/*Thermoplasmatales* like clone had incorporated ¹³C-acetate and/or its degradation products during the sulphate reduction incubation of temperate estuarine sediment. It would be useful to further investigate the activity of the *Thermoplasmatales* and MBG-D group in the oxic zones of the sediments.

DGGE analysis showed that MG1 related sequences were present in the aerobic and likely the dysaerobic fractions of the medium PD slurry. The only cultured representative of the MG1 is an aerobic, chemolithoautotrophic ammonia-oxidizing archaeon (Konneke *et al.*, 2005) although carbon isotopic composition of MG1 lipids does show that some members of MG1 have a heterotrophic or mixotrophic metabolism (Ingalls *et al.*, 2006). In the aerobic phases of the incubations this may have been aerobically oxidising ammonia but was also found to have incorporated ¹³C acetate and/or its degradation products in the sulphate reduction stable isotope probing experiment so such a related phylotypes was either employing a heterotrophic metabolism or utilising recycled label as ¹³CO₂.

The miscellaneous Crenarchaeote group (MCG) were present in the aerobic and dysaerobic incubations. This group has a wide habitat range with a rapidly growing number of clones from different environments (Teske and Sorensen, 2008) and they are one of the predominant archaeal groups in 16S rRNA clone libraries from deep sea marine subsurface environments (Fry *et al.*, 2008). They have been found in coastal marine surface sediments (Roussel *et al.*, 2009) and shown through stable isotope probing to incorporate ¹³C acetate under anaerobic conditions in estuarine sediment incubations (Webster *et al.*, 2010). Clearly further investigation is required to elucidate the role of this group in marine and estuarine sediments.

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Anaerobic phases

The archaeal community in the high PD 18 °C slurry during the methanogenic phase was dominated by terminal-oxidizing methanogens, with the closest cultured representatives being Methanococcoides alaskense and Methanococcoides methylutens. Methanococcoides alaskense is known to utilise trimethylamine as a sole carbon and energy source and not acetate (Singh et al., 2005). Thus suggesting that Methanococcoides alaskense was utilising trimethylamine produced by other prokaryotes in the community from the breakdown of the phytodetritus and this phylotype was also found in the later stages of the aerobic incubation for the high PD 18 °C slurry which is supported by the geochemical data as by this stage of the incubation according to the master slurry as the oxygen was totally depleted, the sulphate was reaching limiting concentrations and acetate was depleted. Methanococcoides methylutens is known to produce methane from methylamines and methanol but not H-CO₂ or acetate (Sowers and Ferry, 1982). Thus it seems that these methanogens were playing an important role in the terminal oxidation of methylated organic compounds (e.g. choline). The other archaeal phylotypes were related to uncultivated groups of Archaea including the diverse MCG, Thermoplasmatales and one phylotype that was not easily affiliated with a group. The MCG group are highly diverse phylogenetically and in terms of their distribution being found in marine and continental habitats (Kubo et al., 2012) but they remain uncultivated so specific physiology is yet to be elucidated, although it is known that most members are heterotrophic (Biddle et al., 2006). It has recently been shown in diverse types of marine sediments and microbial mats that the MCG are unlikely to be methanotrophic but are likely to be globally important in sedimentary processes (Kubo et al., 2012). No MCG phylotypes were ¹³C labelled in the high PD 18 °C sulphate reduction phase incubated with ¹³C labelled acetate which is surprising as it has previously been shown that members of the MCG group utilized ¹³C-acetate in temperate estuarine sediments (Webster et al., 2010). This could be due to the band excision method not being exhaustive of all the diversity present and such sequences would perhaps have turned up in clone libraries and other molecular approaches to analyse the identity of phylotypes within the community. One environmental Thermoplasmatales like phylotype was detected in this slurry and it is difficult to infer the activity as there are few cultured representatives which include facultative anaerobes, thermoacidophiles, autotrophs and heterotrophs (Huber and Stetter, 2006). The appearance of Thermoplasmatales phylotypes in the labelled fractions of the ¹³C-acetate incubations does suggest that they were utilizing acetate, although after 24 hours of incubation it might be possible for them to be utilizing recycled ¹³C label such as CO₂. This requires

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further experiments to elucidate the metabolism of *Thermoplasmatales* in organically loaded estuarine sediments.

The archaeal community in the high PD 10 °C slurry during the sulphate reduction phase also showed phylotypes similar to *Methanococcoides alaskense* and a *Thermoplasmatales* like phylotype with more *Crenarchaeote* members including two MCG like *Crenarchaeotes*. The Euryarchaeota seemed to become more dominant in the high PD 18 °C which is interesting as *Crenarchaeota* have been reported to be more dominant in the glucose and acetate labelling experiments with temperate estuarine sediments (Webster *et al.*, 2010) which was the case with the glucose incubations in these experiments but not the acetate incubations at the higher temperature.

All the phylotypes shown to be labelled in the ¹³C-acetate sulphate reduction phase were Euryarchaeotes except for one MG1 like phylotype. It should be noted that methanogenesis in this master slurry had established and at the time of the 24 hours sampling point of the subslurry the sulphate was c.4mM, although there was clear sulphate reduction at this stage. It is interesting that there was an ANME-1 like sequence labelled at this stage of the incubation suggesting acetate methanogenesis was occurring with possible methane oxidation. However, there were no detected phylotypes similar to the Delatproteobacteria which are syntrophic with ANME-1 (Knittel and Boetius, 2009). Also, whether any of the carbon from the methane is utilised for anabolism is unclear, although it has recently been suggested that ANME-1 Archaea should be classified as methane-oxidizing chemoorganoautotrophs as they were shown, through stable isotope probing experiments, to utilise dissolved inorganic carbon rather than methane as a carbon source (Kellermann et al., 2012). It does seem possible that the ¹³C-acetate was recycled after 24 hours due to the labelling of Methanococcoides alaskense which utilises exclusively methylated compounds. As well as the *Methanosarcinales* like phylotypes, H₂/CO₂ utilising methanogens were also labelled with one phylotype similar to *Methanospirillum hungatei* which doesn't produce methane from acetate but acetate or yeast are required for growth (Eulers and Susuki, 2010). Hence, acetate may have been utilised for anabolism. It is interesting that the labelled methanogens were all non-acetate utilising groups as the substrate was provided in a relatively high concentration, although the sulphate had been very high and so non-competitive substrates would have been likely used at that point of the incubation, which may have resulted in those methanogenic populations remaining dominant, and previouse studies have found methylated-compound-utilising Methanocoiccoides clones only at the marine Colne Point site of the Colne estuary with the more specialist acetate-utilising Methanosaeta related clones at the freshwater sites (Purdy et al., 2002). The labelling of several MBGD and Thermoplasmatales like

phylotypes after 24 hours of incubation time suggests that these groups are heterotrophic although further experimentation is required after shorter incubation times to confirm this as, again, it is possible that they could have been utilising labelled metabolites.

6.2. Ecological theory and implications for global change

Clearly the addition of PD to Colne estuarine sediment slurries resulted in a change in the bacterial communities under all treatments, but only clear changes in the archaeal communities in the anaerobic phases. The initial fermentation processes involved in anaerobic organic matter degradation are poorly understood (Graue *et al.*, 2012) and this work gives some insight into how fermenting sedimentary communities respond to organic matter loading in estuarine sediments. The fast dominance of *Vibrio, Alteromonacaea* and *Colwellia* like member of the communities showed an opportunistic ecological strategy similar to the "feast or famine" concept proposed by Eulers *et al.*, (2000) for bacteriopankton assemblages. This also accords with the everything is everywhere hypothesis (Baas-Becking, 1934) on the biodiversity of microorganisms, the particular set of environmental parameters in the sediment incubations selected for the groups that were present which are important to understand in relation to the *in situ* environment and how the function could be affected in environmental change scenarios.

It was interesting that *Firmicutes* like phylotypes were stimulated and ¹³C-acetate labelled in the sulphate reduction phases as these have previously been cited at the more freshwater sites of the Colne estuary (Kondo *et al.*, 2007) demonstrating how microbial functional groups can change with organic loading.

The decrease in dominant species of both bacteria and archaea in the highly reducing methanogenic phases and the transformations they catabolise have consequences for the wider ecosystem, for example, the build-up of toxic H₂S from sulphate reduction. The release of phosphate in the high phytodetritus slurries indicated that internal eutrophication had occurred due to the high organic matter loading (Smolders *et al.*, 2006). This microcosm was a closed system and further investigation on the *in situ* effects of organic matter inputs to such estuarine sedimentary microbial community is required to incorporate other variables such as nutrient influxes and trophic interactions from higher organisms.

Clearly the dominant terminal output pathways in the more eutrophic slurries were sulphate reduction and methanogensis, and understanding the producing of the potent greenhouse gas methane which is clearly of environmental significance. Anthropogenic activities have been shown to increase the potential production of these gases in pristine systems (Sanders *et al.*, 2007) so understanding these potential changes *in situ* and how the microbial community may respond to reoxidation events in relation to the efficiency of organic matter degradation, nutrient cycles and terminal output processes requires further investigation.

6.3. Conclusions.

This work has provided some useful insight into temperate estuarine microbial community structure and function under organic loading scenarios. The Colne estuary is a hypernutrified turbid estuary with limited primary production, but it does face anthropogenic pressures from agriculture and sewage works so understanding the potential impact of organic loading on microbial communities and function is important. This work was limited by the extent of the stable isotope analysis and would benefit from the additional use of other molecular techniques such as cloning and metagenomics (e.g. Chen *et al.*, 2008) to enable deeper phylogenetic and functional analysis of the active phylotypes and diversity measurements, as DGGE is a very limited approach for estimating species richness (Bent *et al.*, 2007). Stable isotope experiments using a range of labelled substrates would also be interesting, for example, ¹³C labelled amino acids to look at protein cycling (e.g. Miyatake *et al.*, 2013) Further experiments looking at a larger range of organic loading and temperatures would further investigate microbial community changes with environmental parameters.

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