Distributions of model microorganisms along an estuarine gradient

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Acknowledgments

Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged. It has not been submitted for a degree in any other University.

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Date…………..

Abstract

Microbial ecology is the younger sub-discipline of ecology, but its scope is clearly immense. Microorganisms' specificities represent a major hurdle to the application of traditional ecological approaches. Recently, the opportunity to apply ecological principles to microorganisms was suggested. It is assumed that existing principles might apply, but that they are probably also driven by specific principles.

Microorganisms have varying metabolic abilities and are classified as generalists or specialists. We hypothesised that contrasting metabolic properties may drive genotypic distribution. Model generalist and specialist genera were selected and distribution patterns along the salinity gradient of the River Colne estuary studied.

Two models were studied in detail, *Desulfobulbu*s a versatile sulphate-reducing bacterial (SRB) genus and *Methanosaeta*, a strict aceticlastic methanogenic archaea (MA). Isolation of *Methanosaeta* was attempted in order to link phenotypes to genotypic distribution. Two other models were also studied: *Methanosarcina* the most versatile MA genus and *Desulfobacter* a metabolically restricted SRB genus. Denaturing gel gradient electrophoresis (DGGE) and clone libraries analyses were used to determine genotypic distribution patterns.

Methanosaeta have proven to be highly recalcitrant to isolation. Adjustments to commonly used anaerobic culturing methods allowed the obtention of *Methanosaeta* colonies. In contrast to previous studies, colonies were successfully transferred into liquid medium, and growth of pure clonal cultures confirmed.

Desulfobulbus genotypic distribution pattern was previously shown to be sequential along the estuary. Contrastingly, *Methanosaeta* genotypic distribution pattern was found to be monotonic. Furthermore, active genotypes distribution was also found monotonic, with an apparent general increase in activity with decreasing salinity.

Distribution patterns of the four different genera confirmed this trend. The generalists were both shown to have sequential distribution patterns. Contrastingly, the specialists were both shown to have monotonic distribution patterns.

These results confirm the hypothesis that genotypic distribution patterns microbial communities structure are strongly driven by microorganisms' metabolic properties and adaptative potential.

Abstract

Abbreviations

AOB	Ammonia-Oxidising Bacteria
Bp	Base pairs
C	Celsius
CCA	Canonical Correspondence Analysis
cm	Centimetre
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
FISH	Fluorescent *In Situ* Hybridisation
g	Grams
km	Kilometre
M	Molar
MA	Methanogenic Archaea
mM	Millimolar
NTA	Nitrilotriacetic acid
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
PEG 6000	Polyethylene Glycol
ppm	Parts per million
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
RT	Reverse Transcriptase
SEM	Standard Error of the Mean

SRB   Sulphate-Reducing Bacteria

TAE   Tris-Acetate-EDTA; 1x Tris-acetate (40 mM), EDTA (1 mM)

T-RFLP   Terminal Restriction Fragment Length Polymorphism

TTGE   Temperature Gradient Gel Analysis

UV   Ultra-violet

Chapter 1 Introduction

1.1 Microbial ecology early history

1.1.1. Microbiology history and development of microbial ecology

Microorganisms were one of the last life forms discovered by mankind, with the first observation of "animalcules" under the microscope in the 17th century by Antonie van Leuwenhooek. Until the 19th century, bacteriology focused mainly on human needs (medicine, food safety and hygiene for example), with famous works by Pasteur and Koch. Studies on the natural world were mainly represented by Ferdinand Cohn attempts to describe and classify bacteria according to the Linnean taxonomy (Baker *et al.*, 1999).

In the late 19th century, Winogradsky and Beijerinck built the basis of what would be called environmental microbiology (Baker *et al.*, 1999). Beijerinck first discovered the viruses and used enrichment cultures that allowed him to describe the variety of microbes' metabolic properties. Winogradsky proved the existence of chemolithoautotrophy, the ability to derive energy from inorganic compounds without aerobic respiration or photosynthesis, and thus the crucial role of chemolithoautotrophs in geochemical processes.

1.1.2. The discovery of new microbial metabolisms

During most of the 20th century, culture-dependent methods have dominated, as the only available tools to gain insights into microbial activities. New metabolisms, unique to the microbial world were discovered and explained. Winogradsky isolated

the first nitrifying and nitrogen fixing bacteria. Barker showed evidence for the existence of a methanogenic pathway (Barker *et al.*, 1940). Other examples include acetogenesis, a pathway producing acetate from carbon dioxide and dihydrogen was also discovered (Adamse, 1980), and it was shown that iron or manganese and other metals and radionuclides can be used as electron acceptors by some bacteria (Lloyd and Lovley, 2001, Lovley and Phillips, 1988) (Table 1).

General type	Carbon source	Energy source	Electron donor	Electron acceptor
Heterotroph	Organic C	Organic C		
Aerobes			Organic C	O_2
Denitrifiers			Organic C	NO_3^-
Mn reducers			Organic C	Mn (IV)
Fe reducers			Organic C	Fe (III)
SRBs			Organic C	$SO_4^=$
Sulfur reducers			Organic C	S°
Methanogens			Organic C/H_2	CO_2
Syntrophs			Organic C	Organic C
Acetogens			Organic C/H_2	CO_2
Fermentors			Organic C	Organic C
Phototroph	CO_2	Light		
Cyanobacteria			H_2O	
Photosynthetic bacteria			S compounds, H_2, Organic C	
Lithotroph	CO_2/organic C	Inorganics		
H_2 oxidizers			H_2	O_2, NO_3^-, Mn (IV), Fe(III), $SO_4^=$, CO_2
Fe oxidizers			Fe (II)	O_2, NO_3^-
S oxidizers			H_2S, S°, $S_2O_3^=$	O_2, NO_3^-
N oxidizers			NH_3, NO_2^-	O_2
CH_4 oxidizers			CH_4	O_2

Table 1: The main microbial metabolisms occurring in sediments (Nealson, 1997)

All these metabolisms were shown to be of crucial importance in elemental (nitrogen, sulphur or metal) cycles (Figure 1).
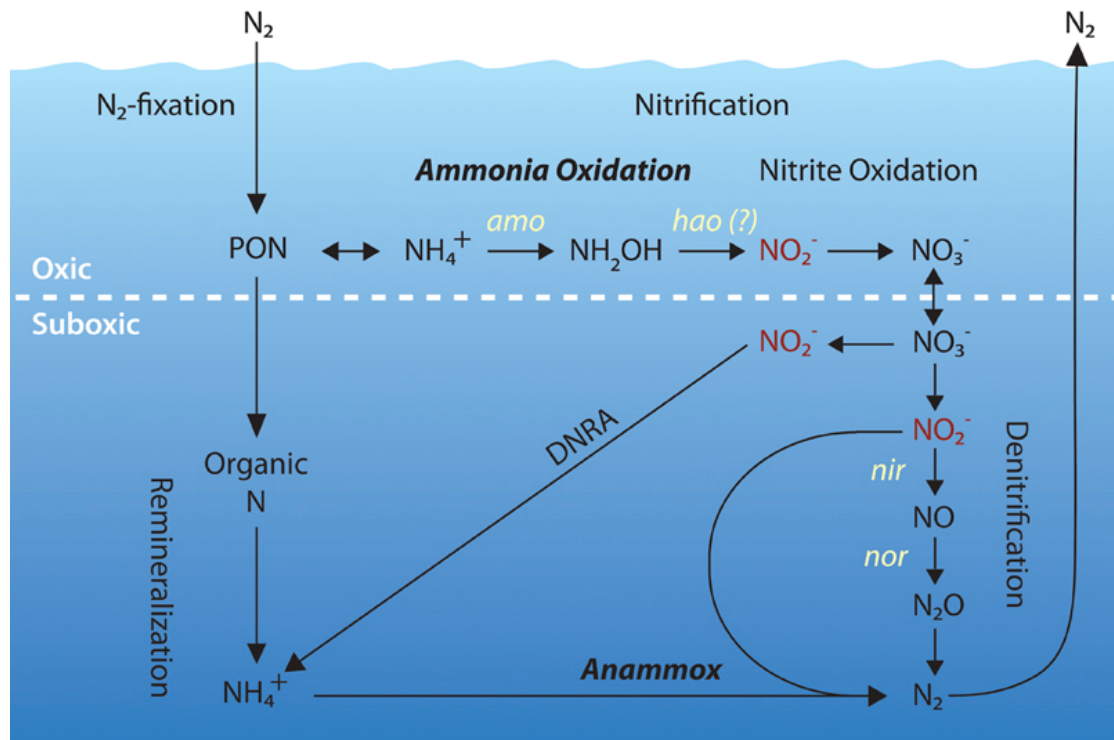
Figure 1: Oceanic nitrogen cycle and new microbial players (Francis *et al.*, 2007)

Microbial metabolic versatility is further increased by syntrophic mechanisms. Interspecies hydrogen transfer is a very important mechanism, allowing for example rumen microflora to coexist. It was shown that, in natural environments, microorganisms (sometimes methanogens) can consume methane through a syntrophic interaction with sulphate or nitrate reducers (Nauhaus *et al.*, 2002) or dissimilatory metal reducers (Beal *et al.*, 2009).

1.1.3. Examples of ecological principles illustrated by microorganisms

In 1934, Baas Becking postulated that all kind microbial populations had the potential to thrive in most environments but that local population patterns were shaped by environmental factors. This view suggesting a general occurrence of niche differentiation was summed by the sentence "everything is everywhere, the

environment selects" (de Wit and Bouvier, 2006).The same year, Gause conducted predator-prey experiments with cultures of *Paramecium*, yeast and bacteria that produced the first experimental evidences for the competitive exclusion principle and the niche concept (Gause, 1934).

Microbial cultures were also used in long-term evolutionary studies, as allowed by their short generation times. Adaptative radiation was shown for *Pseudomonas fluorescens* cultures that evolved distinct subpopulations adapted to niches in the culturing system (Rainey and Travisano, 1998). In even longer term (30,000 generations), it was shown that *Escherichia coli* cultures diverge from the parent strains with selection of mutations leading to a better fitness to the culturing conditions (Barrick *et al.*, 2009, Cooper and Lenski, 2010). The studies on small subunit ribosomal RNA led to the separation of the prokaryotic world into two distinct kingdoms, Archaea and Bacteria, and paved the way for molecular microbial ecology (Woese *et al.*, 1990).

1.2. Recent advances and drift from "traditional ecology"

1.2.1. The revolution of molecular ecology

In the recent years, microbial ecology developed as a new rapidly expanding field of science. This development was made possible by increasing developments of molecular biology methods that allowed indirect tracking of microorganisms and their genes in their environments.

1.2.1.1. Basic methods

The development of the Polymerase Chain Reaction (PCR) (Saiki *et al.*, 1985) was the cornerstone to introduce molecular methods in microbial ecology as it allowed amplification of target genes from environmental DNA. It was quickly assumed that culture-independent methods would help to uncover everything that could not be seen using culture-dependent methods. Next to the PCR, molecular cloning (Marx, 1976) and DNA sequencing (Sanger and Coulson, 1975, Sanger and Coulson, 1978, Sanger *et al.*, 1977) represents the other most used method in modern molecular microbial ecology.

1.2.1.2. Fingerprinting methods

A primary goal for microbial ecologists was to assess microbial diversity in environmental samples. To achieve this goal a suite of fingerprinting tools have been and are still being developed. Denaturing Gradient Gel Analysis (DGGE) and Temperature Gradient Gel Analysis (TGGE) are methods based on variable melting points of PCR amplified fragments on gels along gradients (Muyzer, 1999, Muyzer *et al.*, 1993, Muyzer and Smalla, 1998). DGGE is probably the most broadly used method in molecular microbial ecology (Figure 2a). It has been used on a wide diversity of sample type including food samples (Ercolini *et al.*, 2004, Fontana *et al.*, 2005), dental plaque (Fujimoto *et al.*, 2003), insects symbionts (Reeson *et al.*, 2003), contaminated lands (Stephen *et al.*, 1999) intestinal microbiota (Lubbs *et al.*, 2009) or deep-sea hydrothermal vent (Muyzer *et al.*, 1995).
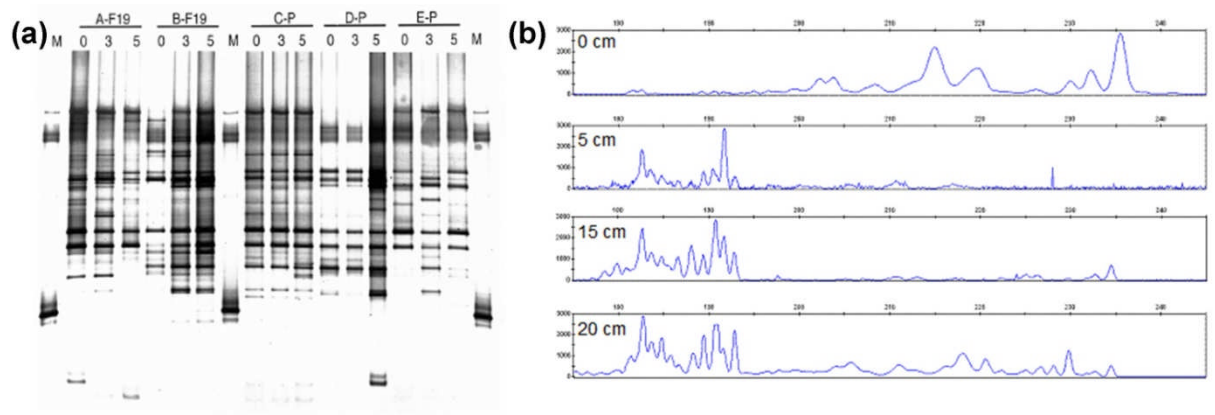
Figure 2: Examples of fingerprinting methods, (a) DGGE, (b) Capillary Electrophoresis SSCP (CE-SSCP)

Amplified Fragment Length Polymorphism (AFLP) (Vos *et al.*, 1995) and microsatellites are based on asymmetric PCR amplifications, they were designed for macroorganisms in the first instance (Vos *et al.*, 1995). rDNA internal spacer analysis (RISA) is based on the amplification of the region between the 16S and the 23S of the ribosomal operon (Garcia-Martinez *et al.*, 1999). Terminal Restriction Fragment Length Analysis (RFLP or T-RFLP) uses various combinations of restriction enzymes to obtain different fingerprints, the terminal 5' end can be labelled with fluorescence prior to digestion (Liu *et al.*, 1997, Saiki *et al.*, 1985). Single Stranded DNA Conformation Polymorphism (SSCP) is based on different electrophoretic properties of single stranded DNA, it gets increasingly used with sequencers capillary electrophoresis (Figure 2b) (Zinger *et al.*, 2007).

1.2.1.3. Phylogenetic methods

Direct sequencing and phylogenetic analyses are the other methods used in nucleic acids' based microbial ecology. Clone libraries are often constructed, by cloning

specific PCR products into bacterial plasmid vectors, the easier way to ensure reading of a single sequence each time. Metagenomics approaches have more recently been developed (Gabor *et al.*, 2007, Streit and Schmitz, 2004). They are based on total DNA amplification, cloning and genome reconstruction attempts. Environmental genome shotgun is another similar approach (Venter *et al.*, 2004). Several molecular databases have been set up such as the ARB-SILVA database (Pruesse *et al.*, 2007), GenBank (Karsch-Mizrachi and Ouellette, 2001) EMBL (Stoesser *et al.*, 1997). Phylogenetic affiliation softwares are also developed such as DOTUR (Schloss and Handelsman, 2005) and MOTHUR (Schloss *et al.*, 2009).

More recently, new technologies such as pyrosequencing and Illumina sequencing have been developed. Such methods generate large datasets of tens of thousands of DNA reads in one reaction (Table 2) at reduced costs (Elahi and Ronaghi, 2004).

Platform	Million base pairs per run	Cost per base (US¢)	Average read length (base pairs)
Dye-terminator (ABI 3730xl)	0.07	0.1	700
454–Roche pyrosequencing (GS FLX titanium)	400	0.003	400
Illumina sequencing (GAii)	2,000	0.0007	35

Table 2: Comparison of the cost and throughput of sequencing technologies (Hugenholtz and Tyson, 2008)

This technology has already permitted very extensive characterisation of environmental samples, and developments are on the way to increase read lengths and sequences number. With such methods, the new frontier will become the correct use and interpretation of exponentially growing molecular data sets.

1.2.1.4. Function and activity based methods

It has been argued that nucleic acids analyses are insufficient to represent significant (metabolically active) microbial communities (Dumont and Murrell, 2005, Radajewski *et al.*, 2000). It was often shown that marginally significant microorganisms can be over represented with such methods. The methods described in 1.2.1. can be targeted at RNA after cDNA production through reverse transcriptase enzymes. Metabolically active microorganisms should be better represented through RNA-based analyses as they should produce more RNAs. Real Time or Quantitative PCR (qPCR) has been developed as a way to evaluate relative quantities of genetic content in a given sample (Dong *et al.*, 2009). Multiplex assays can be performed to evaluate quantities of different taxa in a given sample (Fierer *et al.*, 2005, Kirs and Smith, 2007). Stable Isotope Probing (SIP), the use of labelled compounds (^{13}C- or ^{15}N-), has been developed in order to discriminate microorganisms that actually use or consume specific compounds from the rest of the community (Dumont and Murrell, 2005, Neufeld *et al.*, 2007, Radajewski *et al.*, 2000). SIP studies have allowed the determination of active methanotrophs in a variety of ecosystems (Cebron *et al.*, 2007, Hutchens *et al.*, 2004, Neufeld *et al.*, 2008, Radajewski *et al.*, 2002), and to assess microbial communities involved in different important biogeochemical processes (Bass *et al.*, 2007, Kunapuli *et al.*, 2007, Uhlik *et al.*,

2009, Wawrik *et al.*, 2009). Recently, mathematical models were suggested to couple traditional fingerprintings to functional activities (Dumont *et al.*, 2009). Transcriptomics (Guimil *et al.*, 2005, Poretsky *et al.*, 2009) and proteomics (Graham *et al.*, 2007, Keller and Hettich, 2009, VanBogelen, 2003) approaches have also been used. In parallel to metagenomics, metaproteomics are developing (Maron *et al.*, 2007, VerBerkmoes *et al.*, 2009).

1.2.2. Limits of molecular assessment in an ecological context

A direct comparison between the ecological sciences and molecular microbial ecology highlights the biggest limit of these studies. If a plant ecologist was to apply the same approach, he would grind and mix the different plants he wants to study, pool them and try to reassign taxonomic affiliations from molecular data. Of course, this would not happen, as direct taxonomic assignment is easily possible. Thus, fundamental questions arise about how to conduct accurate and ecologically significant studies on microorganisms. Some methodologies have been developed to try to detect and discriminate different types of microorganisms in environmental samples. Fluorescent In Situ Hybridisation (FISH) can be used to illuminate targeted groups under the microscope. A lot of methods based on FISH have been developed in order to target functional genes or to increase number of targets observed simultaneously (Behrens *et al.*, 2008, Coleman *et al.*, 2007, Sunamura and Maruyama, 2006, Tujula *et al.*, 2006). Recently, Raman microscopy (Patzold *et al.*, 2008, Schuster *et al.*, 2000), NanoSims (Behrens *et al.*, 2008, Clode *et al.*, 2007) have also been presented as ways to investigate microbial communities more directly. These approaches can be used to assess very small microbial communities

but would be too time consuming for applications on large communities that are aimed to be studied by ecological science.

Another commonly recognised limit is that molecular microbial ecology can hardly claim to provide an exact representation of the phenotypes present in the environment studied. Precise genotypic or phenotypic assignments are strongly dependent on method reliability and the existence of a reliable database for comparison. While molecular data accumulates, it is inevitable that errors are accumulating too (Ashelford *et al.*, 2005, Ashelford *et al.*, 2006). Emerging high-throughput sequencing methods are showing that detectable microbial diversity is even greater than previously thought. However, when dealing with such large datasets, data analyses methods become the bottleneck of studies, and there are growing concerns that diversity could be overestimated by methods artifacts (Kunin *et al.*, 2010). Thus, increasing the accuracy of nucleic acids datasets is required by development of powerful bioinformatics tools (Gomez-Alvarez *et al.*, 2009, Quince *et al.*, 2009, Reeder and Knight, 2009).

1.3. Integrating microbial ecology into general ecology?

1.3.1. Environmental microbiology or microbial ecology?

It is interesting that these two terms have been used to describe similar studies. Out of the five specialised journals in the field, three contains the term ecology and two the term environmental. However, just by looking at their respective scopes, it is clear that they share similar and overlapping interests.

"*Environmental Microbiology* is devoted to the study of microbial processes in the environment, microbial communities and microbial interactions" (http://onlinelibrary.wiley.com/journal/10.1111/(ISSN)1462-2920).

"*Applied and Environmental Microbiology* (AEM) publishes a substantial share of the most significant current research in the areas of biotechnology, microbial ecology, food microbiology, and industrial microbiology" (http://aem.asm.org/).

"*Microbial Ecology* coverage includes the ecology of microorganisms in natural and engineered environments; genomic, metagenomic microbial processes and interactions in extreme or unusual environments; microbial population and community ecology, and more" (http://www.springer.com/life+sciences/ microbiology /journal/248).

"*The International Society for Microbial Ecology Journal* seeks to promote diverse and integrated areas of microbial ecology spanning the breadth of microbial life, including bacteria, archaea, microbial eukaryotes, and viruses" (http://www.nature.com/ismej.html).

"*FEMS Microbiology Ecology* contains Research Articles and MiniReviews on fundamental aspects of the ecology of microorganisms in natural soil, aquatic and atmospheric habitats, including extreme environments, and in artificial or managed environments" (http://www.fems-microbiology.org/website/nl/page20.asp).

Scientists themselves seem confused about choosing one of these terms to qualify their research. The apparent consensus is that these terms are synonymous and cover one unique discipline. However, there is a clear differentiation between environmental sciences and ecological sciences. The first are rather multidisciplinary

and focus on the observation, quantification and management of the environment. The second are focused on principles, theories and understanding of ecosystems and organisms interactions. It is likely that the terms environmental microbiology and microbial ecology were primarily chosen to achieve a similar distinction between a descriptive and a theoretical science.

If we choose to use this distinction, it appears that a vast majority of studies would be qualified as environmental microbiology and only a minor part would be qualified as microbial ecology. Of course, this is only a semantic issue and it is not really a big deal, but it is a good illustration of the questions that "microbial ecology" needs to answer.

1.3.2. General principles in ecology

Although some theories concerning the natural world organisation can be found in ancient Greek times, the first step in a coherent ecological science might be with Linnaeus' taxonomic nomenclature (Miller, 2005) in the 18th century. The development of ecological theories only happened in the late 19th century, derived from some famous observations: Darwin's finches (Webster, 1965) and Clements' vegetation climaxs (Cook, 1996) for example.

A goal of traditional ecology is to explain and predict the occurrence of any given organisms in any given spatial and temporal unit. Thus, traditional ecology defines ecological units in a hierarchy, ranging from ecosystem, sets of coherent spatial and temporal communities, to biomes (Figure 3) which are larger units mainly defined by dominant vegetation (Woodruff, 2001)
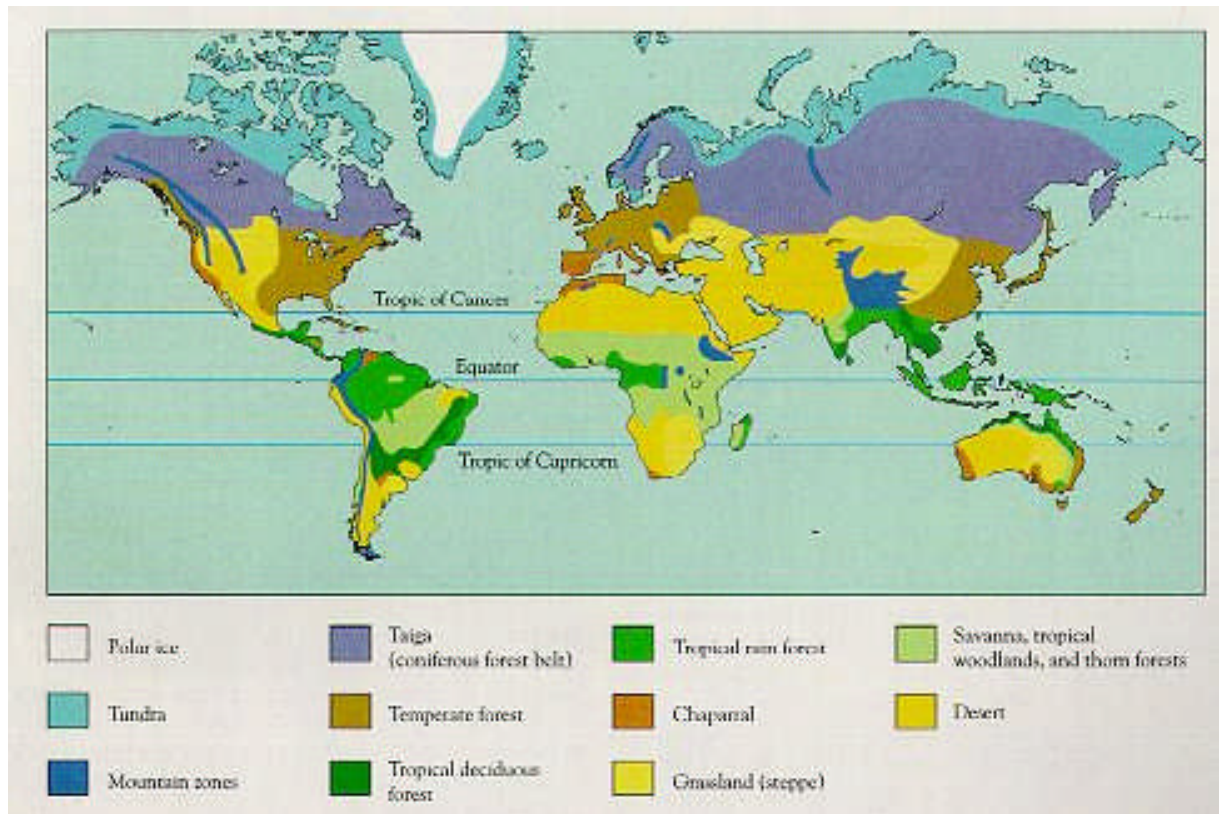
Figure 3: Major biomes on Earth

The ecological niche is a fundamental concept in ecology. It is defined as "the set of biotic and abiotic conditions in which a species is able to persist and maintain stable population sizes" (Wiens, 2004). This is further divided between fundamental niche (set of environmental conditions allowing a species peristence) and the realised niche (fundamental niche restricted by ecological factors) (Wiens, 2004). Species harbour a number of traits and biological specificities that define their niches (McGill *et al.*, 2006).

Ecology and evolution are strongly connected, as evolutionary events are the basis of ecological organisation. Patterns of coevolution, when the evolution of a species is directed by the evolution of other species, have been often decribed (Bergelson *et al.*, 2001, Gilbert, 1971).

Biodiversity is probably the most familiar ecological concept for a general audience. It is basically defined as the life forms numbers in any given ecological unit. Several hypotheses have been developed that suggest a positive effect of biodiversity on natural processes (ecosystems' productivity or stability) on Earth. As a consequence, biodiversity has become a growing concern in public policy around the world. Authors have suggested that biodiversity implies functional redundancies in ecosystems. This redundancy was proposed as a "biological insurance" in case of drastic environmental perturbations (Yachi and Loreau, 1999). Food web models suggest that multiple species play similar roles, and can thus be replaced, which avoids breaks in trophic chains and the extinction of all species that depend on a given species.

Biogeography is the study of species distribution according to geography and environmental variations. It was shown that generally, biodiversity increases gradually from the Poles to the Tropics (Figure 4) (Diniz-Filho *et al.*, 2007, Fine and Ree, 2006, Hillebrand, 2004, Jablonski *et al.*, 2006).
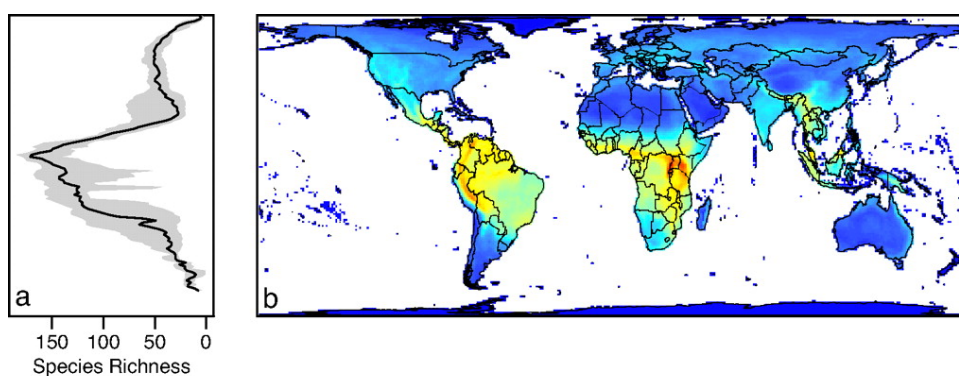
Figure 4: Latitudinal gradient in mammals biodiversity, mean species richness (a) and geographic richness (b) from low (blue) to high (red) (Davies *et al.*, 2008).

Landscape ecology studies biogeographical patterns at very large scales and attempts to integrate smaller biogeographic patterns (Pickett and Cadenasso, 1995).

1.3.3. Microorganisms versus macroorganisms

Ecologists have long been focusing only on larger organisms, for practical and methodological reasons. Metazoan ecology has thus led to the development of numerous hypotheses and theories on the "natural world" functioning, from local to global scale. A lot of these have impacted on general audiences and public policies. Demonstrations of anthropogenic impacts on ecosystems have been released at an increasing pace. Conservation and restoration policies have led to a significant number of successes.

If the immense diversity of macroorganisms has not been a hurdle for ecological sciences, one might think that it should not be one for microorganisms. Actually, the real limit with microorganisms relies in the prefix "micro". In the vast majority of cases, we are not able to see and thus directly assess microorganisms. The only examples of visible microorganisms are still multi-cellular assemblages such as biofilms, microbial mats and fruiting bodies of fungi or amoeba. Only one bacterial type, sulphide-oxidisers such as *Thiomargarita namibiensis*, can develop multicellular forms of few centimetres (Schulz and Jorgensen, 2001). Due to this scale limit, any insight into microbial contribution to ecosystems will always be speculative to some extent.

Thus, for decades, ecological models have ignored or left aside microbial contribution to ecosystems. Actually, depending on study scale it can be perfectly acceptable to not take them in account. For example, in landscape ecology, vegetation is the major variable studied, and arguably sufficient to support proposed models (Pickett and Cadenasso, 1995). At the ecosystem scale, microorganisms

influence has been clearly shown to be crucial. This fact has gradually led to the acceptance of microbial ecology as an important subdivision of ecological sciences.

It now appears that microbial ecology has really drifted from "traditional" ecology to form a separate scientific community. Links between the two communities are sparse, and collaborations appear surprisingly limited. However, as they possess different expertise, it would be expected that reciprocal collaborations should help rapid progresses in general ecological knowledge.

Unfortunately, methodological issues made difficult to clarify the situation. Molecular studies on whole microbial communities, even with high resolution, can always carry some biases and are difficult to interpret. In comparison to macroecology, sample size and numbers play a crucial role, and will often be seen as either too big or too limited (Curtis *et al.*, 2002, Hughes *et al.*, 2001). On the other hand, studies on more specific microbial groups will often be seen as too specific to be generalised to general communities. Also, functional role in the ecosystem can hardly be extrapolated from commonly used molecular methods.

1.3.4. A need to fully integrate microbial ecology to general ecology

An extremely limited proportion of microbial ecology studies have been truly ecologically driven (Prosser *et al.*, 2007). This should not be felt as a criticism, as the microbial world has been discovered and explored far more recently than the natural ecosystems studied by traditional ecology. During the last 10 years, a growing interest has been expressed by several researchers in the search for application of ecological theory in microbial ecology (Cebron *et al.*, 2007). The search for environmentally driven distribution patterns and biogeography was suggested to test

the primary assumption by Baas-Becking: "Everything is everywhere, but the environment selects" (Fuhrman *et al.*, 2008, Martiny *et al.*, 2006, Ramette and Tiedje, 2007). Another field of research consists in the testing of the neutral theory originally developed for plants, which states that biogeography is mostly shaped by an equilibrium between immigration rates and mortality of species in any given environment (Hubbell, 2006). Generalisation of this theory is defended by some authors (Sloan *et al.*, 2006). It is further argued that, due to microorganisms' unlimited dispersal potential, it would support the concept of "microbial cosmopolitanism" (Fenchel and Finlay, 2004) and that any distribution pattern would only be due to insufficient sampling (Curtis *et al.*, 2002, Fenchel and Finlay, 2004, Finlay and Clarke, 1999).

1.4. Distribution patterns and biogeography in the microbial world

1.4.1. Marine microbial ecology

Biogeography is generally thought to occur at global scale in the oceans. Zwirglmaier *et al* (2008) showed that major clades of the marine cyanobacteria *Prochlorococcus* and *Synecochoccus* are distributed according to 4 major domains (polar, coastal boundary, trade winds and westerly winds). These domains can be superimposed on climatic zones, thus indicating a crucial role for environmental variables in controlling cyanobacterial spatial partitioning. Paleologic studies on diatoms showed that communities have slowly evolved over the last 1.5 million years, and that geographic isolation can not be maintained for long periods. It suggests that dispersal is not limited and local environmental factors are the main

drivers of diatom community structure (Cermeno and Falkowski, 2009). Follows *et al.* (2007) suggested a model compiling randomly assigned traits, phytoplankton types and environmental variables. Communities' structure and biogeography generated by this model was consistent with the ones observed in the oceans, and physiological properties were also convergent. This model has the power to explore relations between planktonic ecosystems, biogeochemical cycles and climate change, and be tested with the data gathered through oceanic expeditions.

It is well documented that macroorganism diversity increases with latitude from the poles to the Tropics and Equator, although it is unclear whether it is due mainly to productivity, temperature or historical factors (Diniz-Filho *et al.*, 2007, Fine and Ree, 2006, Hillebrand, 2004, Jablonski *et al.*, 2006). Several reports argued that such gradients are weaker or absent in the case of microorganisms (Fenchel and Finlay, 2004, Finlay and Clarke, 1999). However, two studies showed that latitudinal diversity gradients can be detected among marine microorganisms. A clone library based study showed that most detected bacterioplankton genotypes were geographically restricted and that few of them were cosmopolitan. Genotypic richness was also shown to increase with decreasing latitude (Pommier *et al.*, 2007). Fingerprints (ARISA) of bacterioplankton communities confirmed a significant gradient. Diversity was also found to be strongly correlated to temperature, whereas other variables (productivity and salinity) show weaker correlations. It suggests that metabolic kinetics have strong influence on bacterioplankton diversity (Fuhrman *et al.*, 2008). A bacterioplankton clade named MGB/SAR324 was shown to occur only in tropical and subtropical waters, which makes it the first tropical restricted microbial species and genus level phylotype reported (Brown and Donachie, 2007). Pyrosequencing and sequencing applied to three different arctic water basins

revealed a bacterial biogeography, indicating that water masses dynamics can act as barriers to dispersal (Galand *et al.*, 2009). A molecular marker study on three protozoan species suggested that most protozoan types were cosmopolitan, and only a few of them were restricted to specific geographic locations (Bass *et al.*, 2007). Stratified water columns have been shown to lead to biogeographic patterns. *Prochlorococcus* can be divided in two types, low light (LL) and high light (HL) adapted. In the north-eastern Atlantic Ocean, these types were found to be restricted to their respective ideal niche, LL in the bottom of the water column and HL on the top (West and Scanlan, 1999). However in the Red Sea, HL types were found throughout the water column, co-existing with LL (Gaston and Spicer, 2001). It has been shown that microbial seasonal blooms occur in different marine environments. Less is known about the exact microbial successions throughout the year. Recently, it was shown in the Pacific Ocean coast that *Synechococcus* clades harbour a temporal variation. Two dominant clades showed a seasonal cycle, with one becoming more dominant in early spring and early summer, whereas two less abundant clades were absent in spring, but appear in late summer and winter. These temporal changes are thought to be due to seasonal changes in the water masses (Dong *et al.*, 2009). A temporal and sediment depth variation in bacterial diversity was shown in subtidal sands of the German Wadden Sea. Operational taxonomic units (OTU) numbers increased with depth, and up to 47% of OTUs were replaced over two years. Where the depth variation can be explained by lower disturbance, water movements and environmental factors, temporal variation can not be explained by such factors, showing a probable influence of stochastic events or undetermined environmental factors (Boer *et al.*, 2009).

1.4.2. Freshwater microbial ecology

Freshwater systems, and particularly lakes, are probably the ecosystems in which the search for ecological principles has been most focused. Trophic chains have been studied extensively and general principles seem to have reached a consensus. The microbial loop is a famous model, defining a chain from phytoplankton to zooplankton and bigger grazing protists (Amblard *et al.*, 1995).

Various abiotic and biotic factors have been classified as bottom-up- resources factors-, or top-down- predation, viruses -, a direct application of macroorganism ecology to microbial communities. Several studies reported distribution patterns that correlated with these factors (Bettarel *et al.*, 2004, Kent *et al.*, 2006).

Lake trophic status was also often suggested as a driver of microbial community composition (Ryszard *et al.*, 2009). These observations clearly indicate that some biogeography is occurring in freshwater environments. Recently, biogeography was shown for sulphate-reducers in river floodplains (Miletto *et al.*, 2008). A distribution pattern, influenced mostly by human activities was observed for the genus *Campylobater* in coastal watersheds (Vereen *et al.*, 2007). In contrast, it appears that, due to the complex trophic chains and the small size of lakes compared to oceans, it is difficult to determine whether dispersal and immigration are as important as in oceans, it appears that it has some importance in diatom biogeography at least (Vyverman *et al.*, 2007). A study on salt lakes in China revealed general archaeal and bacterial biogeography. Based on statistics, the authors suggested that bacterial, but not archaeal populations were linked to geographic distance, which appears very unlikely and questioned the reasoning for such a distinction (Pagaling *et al.*, 2009). Biogeography was claimed in arctic lakes, however similarity in bacterioplankton

were measured as 78% between connected lakes and 67% in non-connected ones, a difference that does not appear so striking (Crump *et al.*, 2007). It was found that mountain lakes from Sierra Nevada had significantly different bacterial composition, and that composition similarity increases with decreasing distance between lakes (Reche *et al.*, 2005).

Microbial seasonal successions are well documented in lakes. In addition it has been shown that it is strongly correlated to food web characteristics, which results in shifted sequences (Briand *et al.*, 2008, Casamayor *et al.*, 2000). Seasonal bacterioplankton shifts were also observed in large arctic rivers, in correlation with hydrology and seasonal shifts in biogeochemistry (Crump *et al.*, 2009).

1.4.3. Terrestrial microbial ecology

Soil microbial ecology has been extensively studied as an emerging factor in agronomy and to try to better understand ecosystems functioning. It has been suggested that soil food webs can shape microbial communities structure (Kaspari and Yanoviak, 2009). Temporal variation were shown and suggested to have reciprocal links with metazoans (Schmidt *et al.*, 2007). Nematodes and vegetation were suggested as factors shaping general microbial biogeography at large and fine scale in chalk grasslands soil (Yergeau *et al.*, 2010). Methanotrophs in landfill cover soil were not found to be distributed according to abiotic variables, but strong temporal changes were observed in the composition of communities (Kumaresan *et al.*, 2009). High troughput sequencing analyses revealed a potential effect of oak rhizospheres on the microbial communities in temperate forests' soils (Uroz *et al.*, 2010). The symbiotic nitrogen-fixing bacteria Frankia was found to be rather

cosmopolitan in a temperate rainy forest, despite some differences between few trees' rhizosphere (Kennedy *et al.*, 2010). Microbial distribution patterns were found to be mainly influenced by land use history in a study encompassing numerous soils in California (Drenovsky *et al.*, 2010). Afforestation was shown to severly alter soil microbial communities and general biogeochemical processes in Uruguayan grasslands (Berthrong *et al.*, 2009).

It was suggested that the frequently occurring genus *Pseudomonas* shows high endemicity among distant soils (Berthrong *et al.*, 2009). "Island biogeography" was found in 4 soils for which increasing geographic distance resulted in increasing microbial communities' differences (Fulthorpe *et al.*, 2008). pH was suggested as the major predictor of soil microorganisms diversity, with neutral soils harbouring higher diversity (Fierer and Jackson, 2006, Lauber *et al.*, 2009). Temperature was found to to shape patterns of AOB in several north American soils (Fierer *et al.*, 2009).Microscale biogeography was shown to occur separating diverse level of soil aggregation (Mummey *et al.*, 2006). A precipitation gradient was found to have no effect on soil microbial distribution, whereas dominant climate and vegetation cover were the main drivers of microbial communities composition (Angel *et al.*, 2010).

In France a consortium has been formed in order to assess microbial diversity and biogeography across all the country, and link the findings with measurements of biotic and abiot factors (Ranjard *et al.*, 2010).

1.4.4. Other environments

A recurrent theme in microbial ecology is the search for "extreme microorganisms". It may be more precise to talk about microorganisms living in extreme environments

from a human point of view. Maybe one of the most famous examples of microbial distributions constrained by local environmental factors is the cyanobacterial successions around hot springs (Allewalt *et al.*, 2006, Ruff-Roberts *et al.*, 1994). Niche adaptation was also observed in other microbial groups co-occuring in these mats (Allewalt *et al.*, 2006). It was further shown that *Sulfolubus* populations have evolved separately in geographically separated hot springs across the world, indicating that unlimited dispersal did not apply to them and that allopatric speciation had occurred (Papke *et al.*, 2003, Whitaker *et al.*, 2003). Furthermore allopatric speciation was shown to occur among thermophiles between different hot springs in Yellowstone or in Costa Rica (Finsinger *et al.*, 2008, Kozubal *et al.*, 2008, Takacs-Vesbach *et al.*, 2008).

Studies on polar deserts showed the existence of relatively diversified and highly specialised microbial communities, with the notable absence of Archaea. No apparent distribution pattern or changes in diversity was found, probably because the extreme environmental conditions dominate other variables (Pointing *et al.*, 2009). Complex and highly stratified microbial communities were shown in a deep hypersaline basin in the Mediteranean Sea. Sulphate-reducers and sulphur-oxidisers were shown to be dominant in chemocline and methanogens were dominant in deeper layers (Borin *et al.*, 2009). Microbial eukaryotes were shown to be distributed acoording to large scale patterns in abyssal floor (Scheckenbach *et al.*). In alpine tundra soils, it was suggested that microbial populations' distribution are, similarly to plants, strongly influenced by snow cover (Zinger *et al.*, 2009).

1.5. Hypotheses on functional and genetic diversity role

One of the advantages of macroorganisms over microorganisms for ecological studies is obviously their greater size, and the possibility for researchers to manipulate them. Several ecological theories or principles are thus derived from manipulated ecosystem studies. From a decade-long experiment on various plant assemblages, diversity was suggested to have a positive effect on productivity, stability (Reich *et al.*, 2001), CO_2 sequestration (Reich *et al.*, 2001) and biological invasions (Kennedy *et al.*, 2002). A somewhat contrasting view is that functional diversity rather than genotypic diversity is the key to these processes (Johnson *et al.*, 2008). Similar assemblages face several hurdles to be applied with microorganisms. It appears very hard to mimic the immense microbial diversity of most studied environments. It is also difficult to maintain stable mixed communities for a long time without one component becoming better adapted to the experimental conditions. Last, but not least, external contamination occurs very easily and is very hard to contain and monitor. An experimental denitrifying bacterial assemblage was devised and results indicated a limited effect of species diversity on productivity, and that functional diversity was the main driver of the assemblages' functioning (Salles *et al.*, 2009). Mesocosm experiments have been used to assess soil microbial diversity potential effect on resilience to environmental disturbances. Microbial communities resilience did not appear to be affected by diversity *per se*, but more by the absence of key functional groups (Wertz *et al.*, 2007).

Functional redundancy, the possibility that organisms sharing similar metabolisms play the role of a so called "biological insurance" in ecosystems, has been suggested, but also highly questioned by macroorganisms ecologists. This term has also been

used by microbiologists to qualify the existence of multiple copies of gene leading to the same metabolic properties, widespread in the microbial world. Several reports have argued that functional redundancy exists in microbial ecology. (Caron and Countway, 2009) argue that the protistan rare biosphere does not represent inactive or moribund taxa, but that these taxa can become dominant with major environmental changes. It appears that the rare microbial biosphere in the Arctic Ocean has a biogeography, and that it is mostly parallel to the one exhibited by dominant taxa (Galand *et al.*, 2009). This supports the idea that rare species are subject to ecological drivers and that these organisms might be a biological insurance for function.

1.6. Context of this work

1.6.1. Estuarine microbiology

Estuaries are dominated by two contrasting water fluxes, river current and tidal input. This results in spatial and temporal variability of the estuary hydrography. There are daily marked variations in water level, which leads to the river bed sides to be alternatively exposed and covered by water. These variations are strongly influenced by seasonal variation in tidal forces and punctuated by floods or lack of water.

Being at the interface between rivers and seas, the estuarine environment is a mixture of freshwater and marine environments. As a result, marine and freshwater flora and fauna collide and coexist along estuaries. Salinity gradients are observed as marine water influence decreases when going upstream (Telesh and Khlebovich, 2010). Such salinity gradients affect directly the chemical processes, resulting in unique

geochemical properties (Turner, 2003). Salinity gradients are more stable in sediments, where many of the biogeochemical processes occur.

Many of the known chemolithotrophs have been shown to inhabit estuarine sediments: Methanogenic Archaea (MA) (Purdy *et al.*, 2002) sulphate-reducing bacteria (SRB) (Nedwell *et al.*, 2004), ammonia-oxidisers (Abell *et al.*, 2010, Mosier and Francis, 2008), denitrifiers (Dong *et al.*, 2009), dissimilatory iron-reducers (Cebron *et al.*, 2007).

1.6.2. The Colne estuary

The Colne is a small river in Essex United Kingdom. It is not the tributary of any other river. The estuary (Figure 5) starts at East Hill Bridge weir in Colchester, and is around 11 kilometres long.
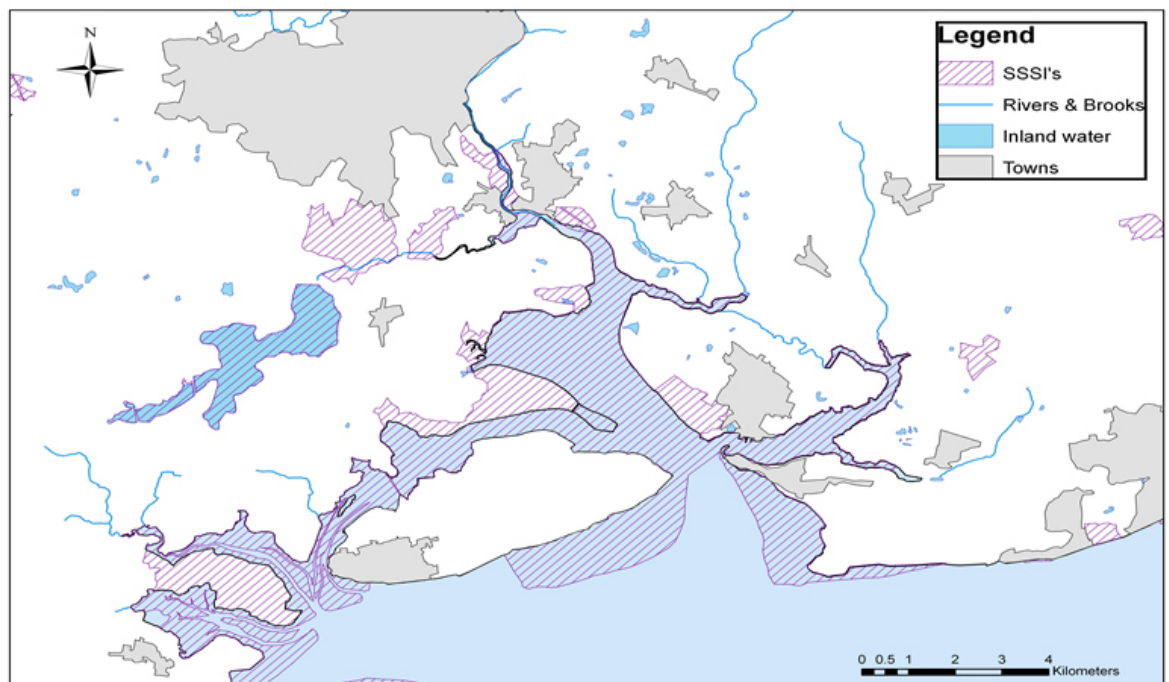
Figure 5: The Colne estuary map (www.colne-estuary.org).

At the estuary mouth, Colne point, large saltmarshes surround the river. They constitute a nature reserve due to their geomorphological features and the diverse flora and fauna they harbour (Figure 6).

Figure 6: Aerial view of Colne point and its surroundings saltmarshes (www.users.zetnet.co.uk/rjseago/cp.jpg).

1.6.3. Microbial activities on the Colne estuary

Microbial communities, especially chemolithtrophs inhabiting the sediments, have been subject to numerous studies. The euryarchaeal communities were investigated by clone libraries in two contrasting sites, the marine mouth and the freshwater part (Purdy *et al.*, 2002). Clones related to clades performing the three methanogenic

pathways (Figure 7) were retrieved in the freshwater sediment. *Methanosaeta* appeared more abundant in freshwater sediments.

(1) $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$

(2) $CH_3COO^- + H^+ \rightarrow CH_4 + 2H_2O$

(3) $4R\text{-}CH_3 + 2H_2O \rightarrow 4RH + 3CH_4 + CO_2$
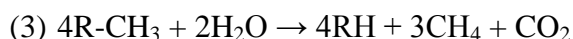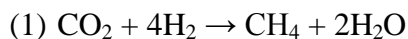
Figure 7: The three methanogenic pathways general equations; (1) carbon dioxide pathway, (2) aceticlastic pathway, (3) C3-methyl pathway (Ferry, 2010)

Some genera were restricted to the marine site (*Metanoculleus* and *Methanococcoides*) and to the freshwater site (*Methanosarcina* and *Methanocorpusculum*). This suggests that a biogeographic signal among methanogens probably exists along the Colne estuary.

Sediment slurry microcosms were used to investigate the role of different MA or SRB in the consumption of several key environmental molecules (Purdy *et al.*, 2003). Acetate consumption in freshwater sediments was mediated by *Desulfobacter* under high sulphate concentration and Methanosarcinales under low sulphate. Acetate was also consumed by *Desulfobacter* in the marine sediments. Methanosarcinales growth in marine slurries was enhanced by the addition of trimethylamines, a substrate for a non-aceticlastic methanogenic pathway. *Desulfobulbus* was linked to sulphate-independent dihydrogen consumption in freshwater sediments. These findings indicate that microbial biogeography along the Colne estuary is probably driven by the salinity gradient.

The genotypic distribution of *Desulfobulbus*, a versatile SRB, was further investigated along 11 sites uncovering the Colne estuary salinity gradient. A

sequential distribution pattern was observed, with marine, brackish and freshwater genotypes. This distribution pattern was significantly correlated to the environmental variables, chloride, sulphate and nitrate. The pattern presents similarities with estuarine macroorganisms distribution, indicative of classic niche separation (Carbonero *et al.*, 2010, Diniz-Filho *et al.*, 2007). *Desulfobulbus* is thus considered as a model microbial generalist, in this study distribution of a model microbial specialist, *Methanosaeta*, will be studied in the same estuary in order to provide a direct comparison. Two other models, the specialist *Desulfobacter* and the generalist *Methanosarcina* will also be investigated.

1.6.4. The principal model, *Methanosaeta*

Methanosaeta are methanogenic archaea (MA). They have an elongated rod shape and form multicellular filaments that aggregate in sheaths at very high cell densities (Figure 8). They are cited as Gram-variable, as Gram staining does not give consistent results (Patel, 2001). *Methanosaeta* were firstly named *Methanothrix*, and their first representative *Methanosaeta concilii* (formerly *Methanothrix soehngenii*) was isolated from a mesophilic sewage digester (Huser *et al.*, 1982). A thermophilic species, *Methanosaeta thermophila* was isolated from an anaerobic sludge digestor (Kamagata *et al.*, 1992, Kamagata and Mikami, 1991). Only recently, a third species *Methanosaeta harundinacea* was isolated, again from a sludge digestor (Allewalt *et al.*, 2006).
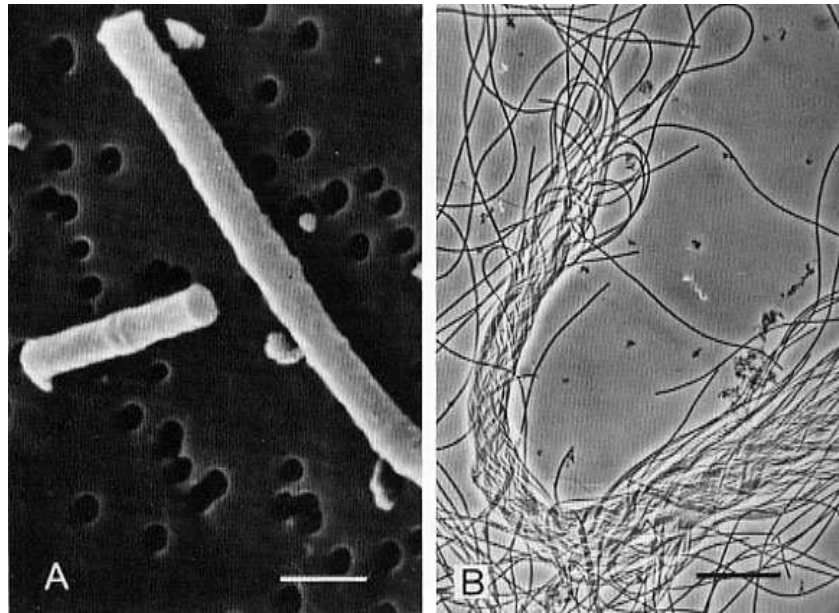
Figure 8: *Methanosaeta concilii* A: electronic microscopy picture, B: filaments and sheath under light microscopy (Patel, 2001).

Despite a limited strain collection, *Methanosaeta* are known to be very abundant in many environments such as sludge digestors, rice paddies, freshwater sediments (Mizukami *et al.*, 2006, Purdy *et al.*, 2003, Rocheleau *et al.*, 1999, Zheng and Raskin, 2000). It has even been suggested that they might produce two thirds of the global biogenic methane emissions on Earth (Bass *et al.*, 2007).

To date, *Methanosaeta* have been shown to only use the aceticlastic methanogenic pathway. Only one other methanogen genus, the closely related *Methanosarcina* can also use this pathway. Furthermore, *Methanosaeta* appear to use acetate as their unique carbon source, electron acceptor and electron donor (Patel, 2001). Thus they can be classified as an extreme metabolic specialist. *Methanosaeta* was also shown to be able to grow on low acetate concentrations (<200 μM), and be outcompeted by other acetate users when acetate concentration increased (Min and Zinder, 1989, Zinder *et al.*, 1984). They are extremely sensitive to oxygen and may be the strictest anaerobes on Earth (Steinhaus *et al.*, 2007).

Methanosaeta have been detected in anaerobic sludge digesters (Zheng and Raskin, 2000), rice paddies (Mizukami *et al.*, 2006), freshwater and marine environments (Parkes *et al.*, 2007, Penning *et al.*, 2006, Purdy *et al.*, 2003) and flooded soils (Janssen, 2003). This genus was also found to be an endosymbiont of *Metopus* sp., an anaerobic ciliate (Narayanan *et al.*, 2009). To aid isolation, an enrichment culture method was developed, based on indirect feeding by acetone-consuming bacteria present in the environment (Janssen, 2003).

1.6.5. The other models, *Desulfobulbus*, *Desulfobacter*, *Methanosarcina*

1.6.5.1. *Desulfobulbus*

Desulfobulbus are rod-shaped Gram negative sulphate-reducing bacteria (SRB) (Kuever, 2005) (Figure 9). *Desulfobulbus* were primarily defined by their use of propionate, with the first isolate being *Desulfobulbus propionicus* (Widdel and Pfennig, 1982). *Desulfobulbus rhabdoformis*, a species isolated from a water-oil separation system, was shown to be able to consume malate and fumarate (Lien *et al.*, 1998). *Desulfobulbus mediteranneus*, isolated from deep sea sediments, was shown to use a variety of fatty acids and saccharides (Sass *et al.*, 2002). *Desulfobulbus japonicus*, isolated from an estuary was shown to be able to use thiosulphate as well as sulphate as an electron acceptor (Suzuki *et al.*, 2007).
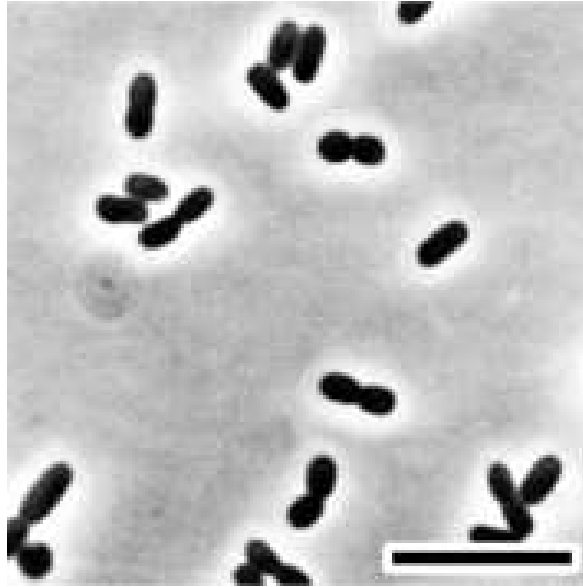
Figure 9: Light microscopy of *Desulfobulbus japonicus* (Suzuki *et al.* 2007)

Overall, *Desulfobulbus* were shown to be very flexible metabolically, using propionate and various fatty acids, sugars, alcohols, amino acids as carbon sources, all sulphur oxyanions and nitrate as electron acceptors; they are also able to ferment some fatty acids and ethanol (Kuever, 2005).

Desulfobulbus were detected in freshwater and marine environments (Laanbroek and Pfennig, 1981), in the human digestive tract (Gibson *et al.*, 1988), anaerobic sludge digesters (Raskin *et al.*, 1995), rhizosphere (Finlay and Clarke, 1999, Hines *et al.*, 1999).

1.6.5.2. *Desulfobacter*

Desulfobacter are Gram negative rod-shaped SRB (Kuever, 2005). *Desulfobacter postgatei*, the type species was described as using only acetate in conjunction with sulphate reduction (Widdel and Pfennig, 1981). Three species, *Desulfobacter hydrogenophilus*, *D. latus* and *D. curvatus* were further described with similarly

restricted metabolisms (Widdel, 1987). An halotolerant species, *Desulfobacter halotolerans* was isolated from the Great Salt Lake (Brandt and Ingvorsen, 1997). *Desulfobacter vibrioformis* was the only species described able to use sulphite or thiosulphate as an alternative to sulphate (Lien and Beeder, 1997). *Desulfobacter psychrotolerans*, a phychrotolerant species, was isolated from the North Sea (Tarpgaard *et al.*, 2006).

Desulfobacter were detected in freshwater and marine environments (Laanbroek and Pfennig, 1981), in the human digestive tract (Gibson *et al.*, 1988), anaerobic sludge digesters (Raskin *et al.*, 1995) flooded soils (Miletto *et al.*, 2008), and may be the dominant acetate consumer in high sulphate anaerobic systems (Purdy *et al.*, 2003, Purdy *et al.*, 2001).

1.6.5.3. *Methanosarcina*

Methanosarcina are MA with typical sarcina morphology, which form aggregates (Figure 10). They are the only MA genus that can use all three methanogenic pathways, but not all species can use all these pathways (Boone, 2001). *Methanosarcina acetivorans*, isolated from marine sediments, can use acetate and C_1-methyl compounds (Sowers *et al.*, 1984). *Methanosarcina semesiae*, isolated from mangrove sediments, can only use C1 methyl compounds including dimethylsulphide (Lyimo *et al.*, 2000). *Methanosarcina lacustris*, isolated from the anoxic zone of a lake, can use C_1-methyl compounds and dihydogen and carbon dioxide (Simankova *et al.*, 2001). *Methanosarcina baltica*, isolated from Baltic Sea sediments, can use acetate and C_1-methyl compounds (Singh *et al.*, 2005, von Klein *et al.*, 2002).

Figure 10: *Methanosarcina acetivorans* aggregates

(https://www.broadinstitute.org/annotation/microbes/methanosarcina/background.ht

ml)

Methanosarcina have been extensively studied in their metabolism. Three species genomes are available, *M. barkeri*, *M. acetivorans* and *M. mazei* (Maeder *et al.*, 2006).

Methanosarcina were detected in sludge digesters (Diaz *et al.*, 2003), arctic wetlands (Hoj *et al.*, 2005), lacustrine sediments (Ward and Frea, 1980) and the rumen (Jarvis *et al.*, 2000). *Methanosarcina* usually outcompete *Methanosaeta* for acetate under relatively high (>200 μM) acetate concentration (Janssen, 2003).

1.6.6. Experimental design

We have chosen a median approach in an attempt to overcome, at least partly, these issues. The goal of our studies was to evaluate the influence of microbial metabolic properties in their distribution according to environmental variables. We have chosen

to focus on a binomial classification in the microbial world, which separates metabolic specialists and generalists. Metabolic specialists have restricted requirements mostly in their carbon sources and electron donors, and electron acceptors in the case of chemolithotrophs. Conversely, metabolic generalists are able to use a wider range of molecules, and to switch metabolism relatively quickly. We decided to work at the genus level, as this level usually groups metabolically consistent individuals (Cohan, 2006). We have further selected model genera, generalists and specialists, which we expect can sufficiently represent general trends (see chapter 1.6). We assessed environmental factors effects on models distribution through sediment sampling along the salinity gradient of an estuary in Eastern England (the Colne estuary, Essex). Our model genera are anaerobic terminal oxidisers of organic matter, SRB and MA, representing two of the three kingdoms of life.

Through this experimental design, we hoped to determine the models genotypic and phenotypic distribution patterns. We further expected to determine if metabolic properties play a role in these distribution patterns. From previous similar studies we could expect either cosmopolitan (Carney *et al.*, 2004) or biogeographic (Hawkins and Purdy, 2007) distributions. Because our models have highly contrasting metabolic properties, we further expected that different distribution patterns may be explained by these properties. In theory, we would thus expect specialist's ecotypes to marginally adapt to environmental variability and be rather cosmopolitan. Conversely, generalist ecotypes would be expected to adapt to local environments where they are best fitted. This would imply a crucial role of metabolic abilities in ecological niche adaptation and speciation, two fundamental concepts in "traditional" ecology.

Chapter 2 Material and methods

2.1. Sampling

2.1.1. Sediment sampling and conditioning

Triplicates sediment cores were taken at low tide from 11 sites (numbered from 1 (marine) to 11 (freshwater)) along the River Colne, in September 2004 and September 2007 (Figure 11). Sediment samples (0-5 cm depth horizon) were centrifuged at 4000 g for 3 minutes to collect pore water. The sediment was then quickly washed in 120 mM sodium phosphate, pH 8.0, to remove extracellular DNA (Tsai and Olson, 1991), then centrifuged at 4000 g for 3 minutes and the supernatant discarded. The washed sediment was immediately divided into three roughly equal subsamples, placed into foil packets and stored frozen at -20°C, in order to avoid ribosomal RNA degradation.
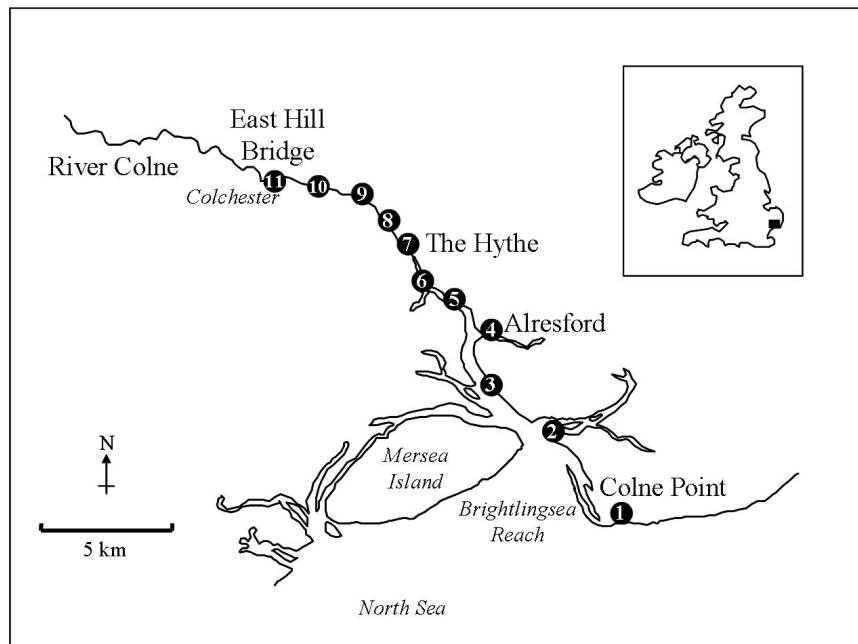
Figure 11: Map of the Colne estuary, Essex, UK showing the 11 sampling sites along the full extent of the estuary (Hawkins and Purdy, 2007).

2.1.2. Pore water analysis

Porewater sulphate, chloride and nitrate concentrations in the 2004 sediment samples were measured by ion chromatography, using a Dionex DX-500 HPIC fitted with an AS14 sparator column (Dionex Corp., California, USA) as reported in Hawkins and Purdy (2007) (Figure 12).
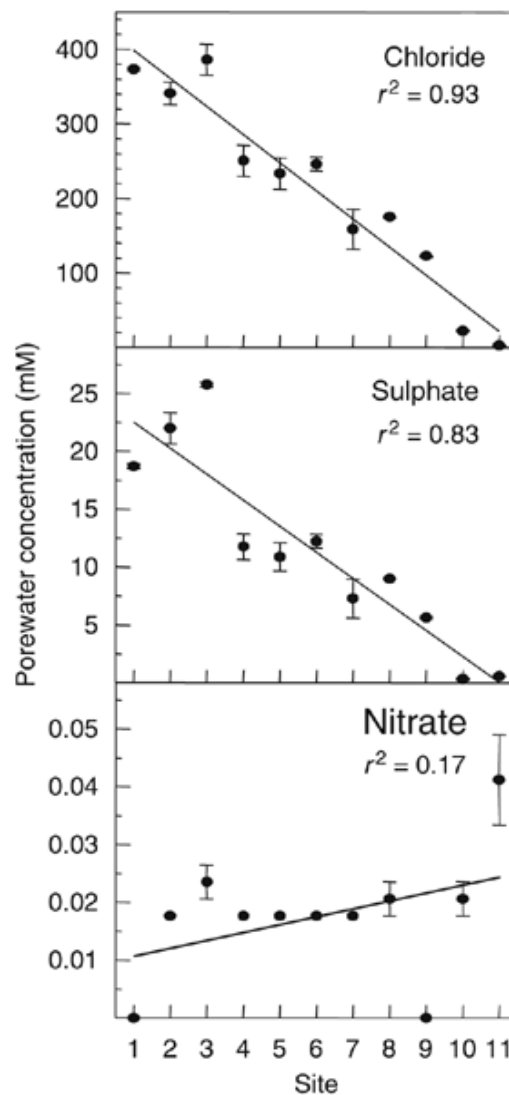
Figure 12: Porewater concentrations (mM) of chloride (salinity); sulphate and nitrate from the 11 sampled sites along the Colne. Error bars represent the SE of the mean (SEM, $n=3$).

A coherent decreasing gradient was shown for chloride and sulphate. Acetate concentrations in sites 1 and 11 have been measured previously (da Silva, 2004) and range from 19-26 µM at Site 1 and 19-100 µM at Site 11.

2.2. *Methanosaeta* enrichment cultures

2.2.1. Anaerobic media preparation and techniques

Liquid media were set up in Hungate tubes or serum bottles, sealed with butyl rubber stoppers. Transfers were performed using sterile syringes and needles.

A gassing station system was used to maintain anaerobic headspaces into culture vessel. Oxygen-free nitrogen (OFN) or carbon dioxide/nitrogen (20%/80%) were used to flushed the media. When media needed to be manipulated without seals, manipulations were performed in an anaerobic cabinet under an atmosphere of carbon dioxide/dihydrogen/nitrogen (20%/10%70/%).

2.2.2. Other chemicals and solutions

Reducing agents were used in order to maintain highly reduced media. Sodium sulphide (Na_2S, 0.2M) was prepared by washing briefly 4 g of crystals with water. Crystals were then dissolved in 100 mL of oxygen-free distilled water under OFN and dispensed in aliquots into serum bottles. Titanium(III) citrate (1% v/v) was prepared by adding 5 mL of a titanium(III) chloride (15% w/v) to 50 mL of a sodium citrate (0.2 M) solution, the subsequent solution was neutralised by a saturated sodium carbonate solution (Zehnder and Wuhrmann, 1976). Acetone stock solution

(2 M) was filter-sterilised using Millex[®]-LG sterilizing filter unit (Millipore, Ireland). Vancomycin (6 mM), kanamycin (50 mg/mL), erythromycin (200 mg/mL), gentamicin (10 mg/mL) and streptomycin (50 mg/mL) stock solutions were filter-sterilised using Minisart® single use filter units (Sartorius, UK). Vitamin solution SL-7 and trace elements solution were prepared as recommended by the DSMZ resource.

2.2.3. *Methanosaeta* enrichment method

Five cubic centimetres of sediments were sampled using cut out syringes with subaseals. Sediments were diluted into 50 mL of enrichment media in the anaerobic cabinet.

The method described by Janssen (2010) was followed. Enrichment medium (Methanosaeta medium with acetone (10 mM) replacing acetate, see Table 3 for composition) was set up. Sediments diluted in the medium were further serially diluted by 100, 10,000 and 1 million. Several transfers in fresh enrichment medium (20% inoculum) were performed. When *Methanosaeta* development was confirmed, transfers (20% inoculum) in *Methanosaeta* medium containing acetate and vancomycin were performed. One or two further transfers into acetate/vancomycin media were performed in order to eliminate the acetone-using bacteria in the cultures.

For 1 liter

KH_2PO_4	0.300	g
NaCl	0.600	g
$MgCl_2$ x 6 H_2O	0.100	g
$CaCl_2$ x 2 H_2O	0.080	g
Trace element solution (see below)	10.000	ml
Vitamin solution	10.000	ml
NH_4Cl	1.000	g
Resazurin	Few drops	
($KHCO_3$)	2.000	g
Sodium sulphate (trace sulphur)	0.050	g
acetate	6.8	g
Distilled water	1000.000	ml

Adjust final pH to 6.8.
Gas phase: 80% N_2 + 20% CO_2. Sterilize the vitamins (by filtration).
Add Sterile $KHCO_3$, Vitamins solution, trace elements (see below) and 10 mL of titanium citrate (or 5 mL sodium sulphide).

Trace elements:

FeCitrate	5	g
$MnCl_2$ x 4 H_2O	0.100	g
$CoCl_2$ x 6 H_2O	0.024	g
$CaCl_2$ x 2 H_2O	0.100	g
$ZnCl_2$	0.100	g
$CuCl_2$ x 2 H_2O	0.025	g
H_3BO_3	0.010	g
Na_2MoO_4 x 2 H_2O	0.024	g
NaCl	1.000	g
$NiCl_2$ x 6 H_2O	0.120	g
Na_2SeO_3 x 5 H_2O	0.026	g
Distilled water	1000.000	ml

First dissolve Fecitrate in 200 ml of distilled water and adjust pH to 6.5 with KOH, then dissolve mineral salts.

Table 3: *Methanosaeta* medium composition and protocol

2.2.4. *Methanosaeta* development check methods

2.2.4.1. Fluorescent *in situ* Hybridisation

Methanosaeta growth was checked by Fluorescent *in situ* Hybridisation (FISH) using bacteria (EUB338 (Amann *et al.*, 1990)), archaea (p915 (Amann *et al.*, 1990)) and *Methanosaeta* (MX825) specific probes, labelled either with Cy3 or fluorescine. FISH was performed using previously described protocols (Amann *et al.*, 1990), including fixation on specific microscope slides, dehydration by ethanol series (50%, 80%, 96% (v/v)), *in situ* hybridisation and observation through an epifluorescence microscope (Axioskop40, Zeiss) equipped with a camera and an image software analysis (OpenLab 4.0.2, Improvision).

2.2.4.2. Gas chromatography

Methane production in the vessel headspaces was measured by gas chromatography using a Flame Ionisation Detector Agilent 6890N, POROPAK column (Phase separation LTD).

2.2.4.3. PCR amplification

PCR using protocols and primers (bacterial, archaeal and *Methanosaeta*-specific) described in section 2.5 have been attempted on cell suspensions from cultures, or DNA extracted using the DNeasy Blood & Tissue Kit

(Quiagen, UK). Sequencing was used to assess phylogenetic affiliation and clonality.

2.3. *Methanosaeta* isolation

2.3.1. Solid media

Solid media were set up with agar (0.5%), and gellan gum (1%). Semi-solid media (0.1% gellan gum) were also prepared. Gellan gum was added slowly to the mineral solution before autoclaving. The solution was then cooled under OFN or N_2/CO_2, and poured into Petri dishes or anaerobic vessels in the anaerobic cabinet.

2.3.2. Purity controls

Transfers in rich (glucose-yeast-peptone) medium were performed as purity controls, to detect the presence of non-aceticlastic MA in the cultures or any other microorganisms. PCR amplification using protocols and primers specific for Bacteria was used to check potential bacterial contamination.

2.4. Nucleic acids extraction

2.4.1. Separate extraction of DNA and RNA

DNA and RNA were extracted separately directly from sediment samples using the hydroxyapatite spin-column method (Purdy, 2005, Purdy *et al.*, 1996). DNA was

extracted from the 33 sites sampled in 2004 by Hawkins (2008). DNA and RNA were extracted from the 33 sites sampled in 2007

2.4.2. PEG 6000 purification

Some DNA and RNA extracts were cleaned up by precipitation in 1 volume of 30% w/v PEG6000 and 0.1 volume of 5M NaCl, a method that was reported to eliminate more environmental contaminants than the ethanol precipitation carried out in the DNA and RNA extraction protocol (Purdy, 2005, Selenska and Klingmuller, 1991).

2.4.3. DNA and RNA analyses

2.4.3.1. Spectrophotometry analyses

DNA extractions yield were evaluated by introducing 1 µL of extract on a specially designed spectrophotometer (Nanodrop, Thermo Scientific, UK).

2.4.3.2. Agarose gel electrophoresis

Gel electrophoresis was used to check the quality of separated nucleic acids extractions. Agarose (1%) gels were prepared with 1x TAE buffer (40 mM Tris-HCl acetate pH 7.5, 1 mM EDTA pH 8.0). The DNA intercalatant ethidium bromide (1 µg/mL) was added directly in the gel. 5 µL of nucleic acids and Hyperladder I

(Bioline, London, UK) were run alongside at 100 V.cm^{-1} for 20 to 40 minutes. Gels were visualised and photographed under a UV trans-illuminator (Geneflow Ltd, UK).

2.5. Nucleic acids amplification

2.5.1. Genera-specific primers

2.5.1.1. *Methanosaeta*

A new *Methanosaeta*-specific 16S rRNA gene-targeted reverse primer (Mst 746r, see Table 3) was designed using ARB (Ludwig *et al.*, 2004). Its specificity was tested by amplification on DNA extracted (Blood and tissue kit, Invitrogen) from the three *Methanosaeta* strains that are available in the DSM culture collection (*M. concilli* DSM6752; *M. thermophila* DSM3870; *M. harundinaceae* DSM17206) as well as against 30 negative controls including other MA (*Methanosarcina* spp.), other euryarchaea isolated from the Colne estuary (Nedwell *et al.*, 2004) and bacteria.

2.5.1.2. *Desulfobulbus* and *Desulfobacter*

Specific primers for *Desulfobulbus* (Dbb121f/Dbb1237r) and *Desulfobacter* (DSB127/DSB1273) (Table 3) were designed and checked for specificity previously (Daly *et al.*, 2000). Annealing temperature for Dbb121f/Dbb1237r was altered to 59°C as per Hawkins and Purdy (2007).

2.5.1.3. *Methanosarcina*

A new set of primers specific for *Methanosarcina* 16S rRNA gene (Msc214f and Msc613r, see Table 4) was designed using the web-based primer design software Primer3 (http://frodo.wi.mit.edu/primer3/), and assessed for genus specificity using Thermophyl (http://www2.warwick.ac.uk/fac/sci/bio/research/thermophyl, a perl script programme by Oakley). Specificity was checked by amplification on *Methanosarcina mazei* and *Methanosarcina acetivorans* pure cultures DNA (extracted with a Blood and Tissue DNA kit, Invitrogen) and on several other methanogens, archaeal and bacterial negative controls.

2.5.2. PCR protocols and nested approaches

2.5.2.1. PCR protocols

All PCR amplifications were performed using a Mastercycler® or MastercyclerPro® (Eppendorf, UK). Wide range primers for Archaea (1Af/1404r), Euryarchaea (1Af/1100r) and Bacteria (8f/1541r) were used in addition to the genera-specific primers described earlier (Table 3). Generally, programs used were a 96°C hot start, 2 min at 96°C, 10 cycles of denaturation (96°C for 1 min), annealing (annealing temperature for 1 min) and elongation (72°C for 2 min 30), then 20 cycles of denaturation (96°C for 30 s), annealing (annealing temperature for 30 s) and elongation (72°C for 2 min) and finally 7 minutes of elongation at 72°C.

Primer pair	Sequence 5'-3'	Annealing temp (°C)	Product size (bp)	Reference
Archaea				
1Af	TCYGKTTGATCCYGSCRGAG			
-1404r	CGGTGTGTGCAAGGRGC	53	1404	(Munson *et al.*, 1997)
-1100r	TCTCGCTCGTTGCCTGACT	50	1100	
Methanosarcina				
Msc214f	TCTGCGGCCTATCAGGTAGT	56	400	This study
Msc613r	GGAACCGGGAGAGGTAAGAG			
Methanosaeta				(Munson *et al.*, 1997)
1Af	TCYGKTTGATCCYGSCRGAG	67	746	
Mst746r	GTCCCTTGCCGTCAGGTC			(Carbonero *et al.*, 2010)
Bacteria				
8f	AGAGTTTGATCCTGGCTCAG	50	1531	(Embley, 1991)
1541r	AAGGAGGTGATCCAGCCGCA			
Desulfobulbus				
DBB121	CGCGTAGATAACCTGTCYTCATG	59	1120	(Daly *et al.*, 2000)
DBB1237	GTAGKACGTGTGTAGCCCTGGTC			(Diniz-Filho *et al.*, 2007)
Desulfobacter				
DSB127	GATAATCTGCCTTCAAGCCTGG	60	1150	(Daly *et al.*, 2000)
DSB1273	CYYYYYOCRRAGTCGSTGCCCT			
DGGE Archaea				
PA340f-GC	CGCCCGCCGCGCGCGGCGGGCG GGGCGGGGGCACGGGGGGCCCT ACGGGGYGCASCAG	53.5	200	
PA519r	TTACCGCGGCKGCTG			(Ovreas *et al.*, 1997)
DGGE Bacteria				
Prba340f-GC	CGCCCGCCGCGCGCGGCGGGCG GGGCGGGGGCACGGGGGGACTC CTACGGGAGGCAGCAG	60	200	
Prun518r	ATTACCGCGGCTGCTGG			

Table 4: List of PCR primers and annealing temperatures used in these studies

For the Archaea and the DGGE pimer pairs, a touchdown was added in the first 10 cycles, with annealing temperature decreasing by 1°C each step, to reach the annealing temperature stated in Table 3.

PCR amplifications were performed with GoFlexiTaq (Promega) as per manufacturer reccomandations, with 0.2 pmol of each primer. Templates were added at a 1/50 ratio in the PCR reactions.

2.5.2.2. Nested approaches

In order to obtain specific products for *Methanosarcina* and *Methanosaeta*, a nested approach was used by amplifying extracted nucleic acid as template for the general archaeal primers (1Af/1404r). Then this PCR product was used as template for amplification with the euryarchaeal (1Af/1100r) primers and similarly with either the *Methanosarcina* (Msc214f /Msc613r) or *Methanosaeta* (1Af/Mst746r) specific primers, and finally the Archaea DGGE primers (PA340f-GC/PA519r).

A similar approach, as used by Hawkins *et al.* (2007), was used for both *Desulfobulbus* and *Desulfobacter*. Here, the bacterial (8f/1541r) then either the *Desulfobulbus* (Dbb121f/Dbb1237r) or *Desulfobacter* (DSB127/DSB1273) specific primers and finally the Bacteria DGGE primers (Prba340f-GC/Prun518r) were sequentially amplified.

2.5.3. Amplification from RNA

2.5.3.1. Removal of DNA of RNA extracts

Remanent DNA in the RNA extracts was digested using DNaseQ (Invitrogen, UK). Digestion was carried out in thermocyclers, with one hour of digestion at 37°C, then the enzyme was inactivated by incubation at 65°C for 10 minutes.

2.5.3.2. Reverse transcriptase PCR protocols

Reverse transcriptase PCR (RT-PCR) was performed using the reverse primer needed for the subsequent PCR. Superscript reverse transcriptase II (Invitrogen, UK) was used to obtain cDNA from the RNA extracts, as per manufacturer's recommendations. cDNAs obtained were then used as templates for PCR following protocols and nested approaches described in chapter 2.4.2. DNAse-treated RNA from each individual sample was used as a negative control to ensure that no amplifiable DNA was present in the RNA.

2.6. DGGE analyses

2.6.1. DGGE protocol

The final PCR products (20 µL) were analysed on 8% (w/v) acrylamide/bis-acrylamide (37.5 : 1; 40% w/v) gels with a 20-60% denaturant gradient according to the method described by Schafer & Muyzer (2001). The HyperLadderI (Proline, UK)

was used as reference in all gels. DGGE were run for 16 hours at 75 V and 60°C. DGGE gels were stained with ethidium bromide, then visualised by UV transillumination.

2.6.2. Sequencing of excised bands

Roughly half of the bands from each gel were excised and re-amplified using the DGGE primers for sequence analysis (Schafer and Muyzer, 2001) to assess the specificity of this DGGE approach.

2.7. Clone libraries

2.7.1. Cloning strategy

Clone libraries were constructed with *Methanosaeta* PCR products from 6 sites (1, 2, 3, 4, 7 and 11), using pGEM-T (Promega) or TOPO 2.1 cloning kits (Invitrogen) following manufacturer's recommendations.

2.7.2. Sequencing

Plasmid mini preps (Qiagen) of overnight cultures were sequenced at the sequencing facilities of the National History Museum (London), using the vector-based M13f and M13r primers.

2.7.3. Phylogenetic analyses

2.7.3.1. OTU affiliation

Clone sequences were assembled in SeqMan (Lasergene). The software DOTUR (Schloss and Handelsman, 2005) was used to assign individual sequences to Operational Taxonomic Units (OTUs) according to sequences similarities.

2.7.3.2. Phylogenetic trees

Sequences were aligned using the SILVA (Pruesse *et al.*, 2007) or Greengenes (DeSantis *et al.*, 2006) aligner and analysed in ARB (Ludwig *et al.*, 2004). Phylogenetic trees were drawn using Neighbour-Joining or Maximum Likelihood methods.

2.8. Statistical analyses

2.8.1. DGGE gels clustering analyses

Gel images were analysed in the software package GELCOMPARII (Applied Maths) using Hyperladder I (Bioline, UK) as a reference to compare band migration across samples and gels. Both relative band intensities of selected bands and a Pearson analysis of the whole profile of each lane were determined. Cluster analyses in GELCOMPARII were performed with both the Jaccard correlation analysis (based on

band presence/absence similarity matrixes) and the Pearson correlation analysis (based on the intensity curve along each sample lane).

2.8.2. Phylogeny versus sampling site

Statistical analyses from the UniFrac software (Lozupone *et al.*, 2006) were used to test whether the recovered *Methanosaeta* phylogeny was significantly clustered by site and the degree of similarity between sites. Shannon diversity indexes and their derived evenness, were calculated as described previously (Shannon, 1948).

2.8.3. Distribution patterns correlation with environmental variables

2.8.3.1. Mantel and partial Mantel test

Dissimilarity matrixes were obtained from Pearson and Jaccard analyses in GELCOMPARII, and calculated from band intensities values in R. Environmental variables (geographic distance, chloride and sulphate) were also converted into dissimilarity matrixes. In addition, Mantel and partial Mantel tests (Mantel and Fleiss, 1980), which test the correlation between two matrixes, were performed using these different genotype and environmental similarity matrices.

2.8.3.2. Canonical Correspondence analyses (CCA)

Canonical Correspondence Analyses (CCA) are widely used to assess the effect of an environmental gradient on the distribution of organisms, that allow multiple

regression with multiple environmental variables (Peres-Neto *et al.*, 2006). CCAs were performed in R using the vegan library.

Chapter 3 Results: Improving the isolation of anaerobes on solid media: The example of the fastidious *Methanosaeta*

This work has been published in Journal of Microbiological Methods (Carbonero *et al.*, 2010).

3.1. Introduction

Methanosaeta spp. are obligate acetoclastic methanogens that are ubiquitous in low sulphate environments (Purdy *et al.*, 2002) and play a key role in global natural methane emissions, producing up to two thirds of biogenic methane emissions (Bass *et al.*, 2007). Isolation of axenic *Methanosaeta* is a difficult task (Allewalt *et al.*, 2006, Kamagata *et al.*, 1992, Patel and Sprott, 1990) primarily because transferable colonies do not grow on solid media (Kamagata and Mikami, 1991, Mizukami *et al.*, 2006). Janssen described a selective enrichment and purification method using indirect feeding that helps bypass some of these issues (Janssen, 2003). However, neither Janssen's method nor existing dilution to extinction appears to be able to achieve clonal isolation and thus valid physiological studies on *Methanosaeta* are scarce. Our goal was to rapidly isolate clonal *Methanosaeta* strains from a UK estuary to initiate ecological studies on this taxon.

3.2. Results

Enrichment cultures from estuarine sediments were set up following Janssen's method (Janssen, 2003). DSM medium 334 was used with important changes, replacing both cysteine-HCl and sulphide with 10 mL per liter of a 1.25% (w/v) Titanium (III) citrate solution as a reductant (Zehnder and Wuhrmann, 1976). Gellan gum was chosen as a solidifying agent as it has been used previously to obtain colonies of recalcitrant microorganisms (Hara *et al.*, 2009).

To determine the viability of this method, solid (1% w/v gellan gum) and semi solid (0.1% w/v) media were inoculated with *M. concilii* (DSM 6752). Colonies formed in the semi solid media after only a few days rather than months as reported previously (Mizukami *et al.*, 2006). Furthermore, very small (less than 1 mm diameter) low convex, entire, white/cream colonies were observed on solid plates after one month's incubation (Figure 13a). Colonies were transferred into titanium (III) citrate-reduced liquid medium where they grew successfully.
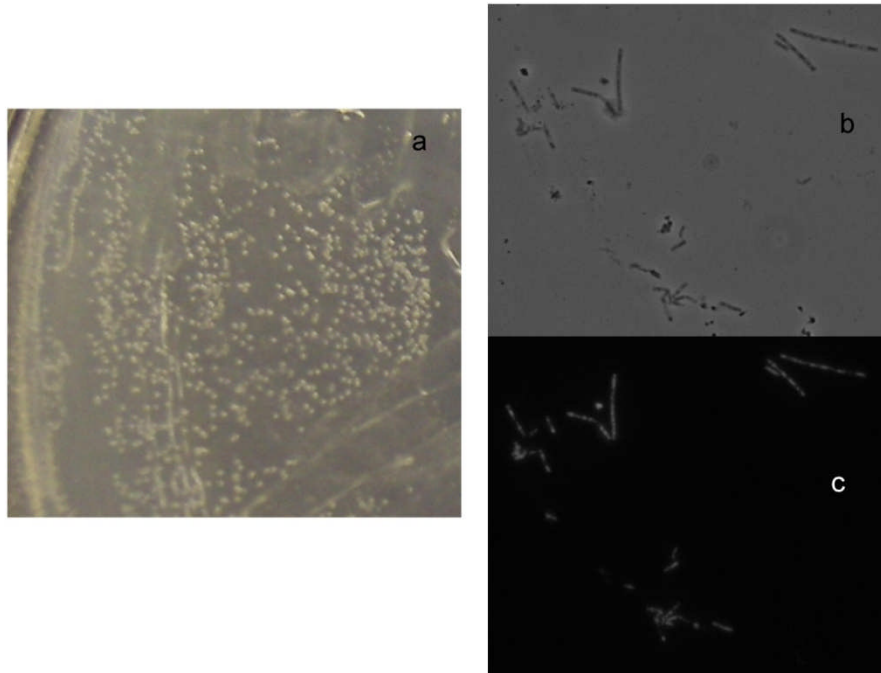
Figure 13: (a) *Methanosaeta concilii* (DSM 6752) colonies obtained on gellan gum solidified plates of titanium (III) citrate-reduced DSM 334 medium. (b) Phase contrast and (c) FISH images of an estuarine isolate culture using a Cy3-labelled Archaea probe (p915 (Amann *et al.*, 1990)).

Subsequently, enrichment cultures from estuarine sediments derived from the application of Janssen's method were inoculated onto 1% (w/v) gellan gum solidified titanium citrate-reduced DSM 334 plates. Small white/cream colonies were again obtained, identified as *Methanosaeta* by broad-range 16S rRNA gene PCR amplification and sequencing and successfully transferred into liquid culture. Seven isolates, shown to be clonal by 16S rRNA gene sequence analysis, were obtained and growth was confirmed by methane production (4 to 7 ppm per week) measured by gas chromatography and by Fluorescent *In Situ* Hybridisation (FISH) microscopy (Figure 13b). Growth was extremely slow in the titanium citrate-reduced liquid

media. Good growth was finally obtained once the cultures were transferred into sulphide-reduced liquid medium. Strains from colonies and liquid transfers were PCR-amplified using 1Af (TCYGKTTGATCCYGSCRGAG) and Mst 746r (GTCCCTTGCCGTCAGGTC, designed to specifically amplified *Methanosaeta spp.* sequences, this study) primers and phylogenetic affiliation with *Methanosaeta* confirmed (Figure 14), although, interestingly, our strains form a separate cluster with *M. concilii* as their closest relative. Purity controls using rich Glucose-Yeast-Peptone media under anaerobic and aerobic conditions were also performed and no growth was detected.
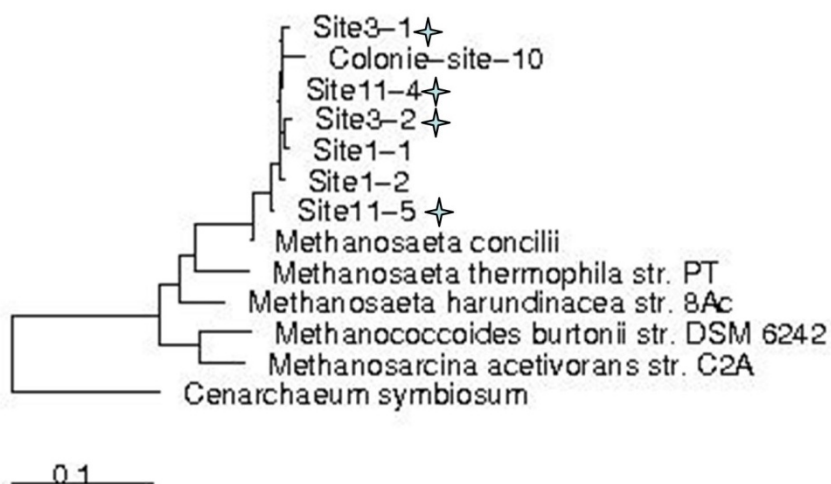
Figure 14: Neighbor-joining Phylogenetic tree, based on ca. 700 bp of 16S rRNA gene sequence, showing the affiliation of several estuarine isolates (colonies or liquid transfers). Stars indicate clonal cultures according to methods described in the text.

This highlights the variety of cultivation requirements needed to achieve a complete isolation process from environmental samples and the need to empirically test the effect specific media components have on growth. Characterisation of selected isolates is ongoing.

Bacterial contamination was often observed on gellan gum plates. This was easily contained by the use of a suite of antibiotics (gentamicin (35 μg/mL), streptomycin (18 μg/mL), kanamycin (1 μg/mL) and erythromycin (2 μg/mL) in addition to the vancomycin (60 μM) suggested by Janssen). These concentrations are reported to be non-inhibitory to acetoclastic methanogens (Sanz *et al.*, 1996). In retrospect, a simple improvement to the method would be to utilise this antibiotic mixture in the last transfer steps of Janssen's enrichment method. Growth appeared to be inhibited in the first colony transfers attempted. A possible explanation for this was the presence of the iron-chelating agent nitrilotriacetic acid (NTA) in the trace elements solution used in the DSM medium 334, which can be toxic to *Methanosaeta* (Janssen, personal communication). NTA was successfully replaced by citrate in the trace elements solution.

3.1. Discussion

Previous attempts to isolate *Methanosaeta* on gellan gum-solidified plates have been unsuccessful (Kamagata, personal communication). Therefore, it appears that a specific factor or combination of factors is required to obtain *Methanosaeta* colonies. In this case, the use of the traditional reductant titanium citrate might be important as it is a less toxic reducing agent than sulphide for many anaerobic organisms (Brauer

et al., 2006). It should also be noted that NTA, even in trace amounts, may have inhibited previous attempts to isolate *Methanosaeta* strains.

Our experiences with *Methanosaeta* allow us to make some general comments that should help with the isolation of recalcitrant anaerobes. Various combinations of solidifying (high quality agar, agarose or gellan gum) and reducing agents other than sulphur-based reductants should be tried. The use of carefully chosen antibiotics can be extremely useful, although we recognise that the selection of antibiotics is much easier when attempting to isolate Archaea. If an isolate shows only limited growth then all medium components should be carefully checked and potentially inhibitory compounds replaced if possible. Figure 15 summarises the differences between the "traditional" approach and the one we recommend; and highlights the time potentially gained by applying this systematic approach. For anaerobes that will not grow on any kind of solid medium then isolation may still be achieved using semi-solid media, while obtaining single colonies ready for further transfer will require some trial work. We have shown here that even organisms that are renowned for their resistance to culturing can be grown with a suitably determined and systematic approach, and in this case, it is possible to reduce the whole process from sampling to isolation and characterisation from several years to around one year. We expect that this advice will be of use to researchers that have forsaken isolation because of the time that it requires to be successful.
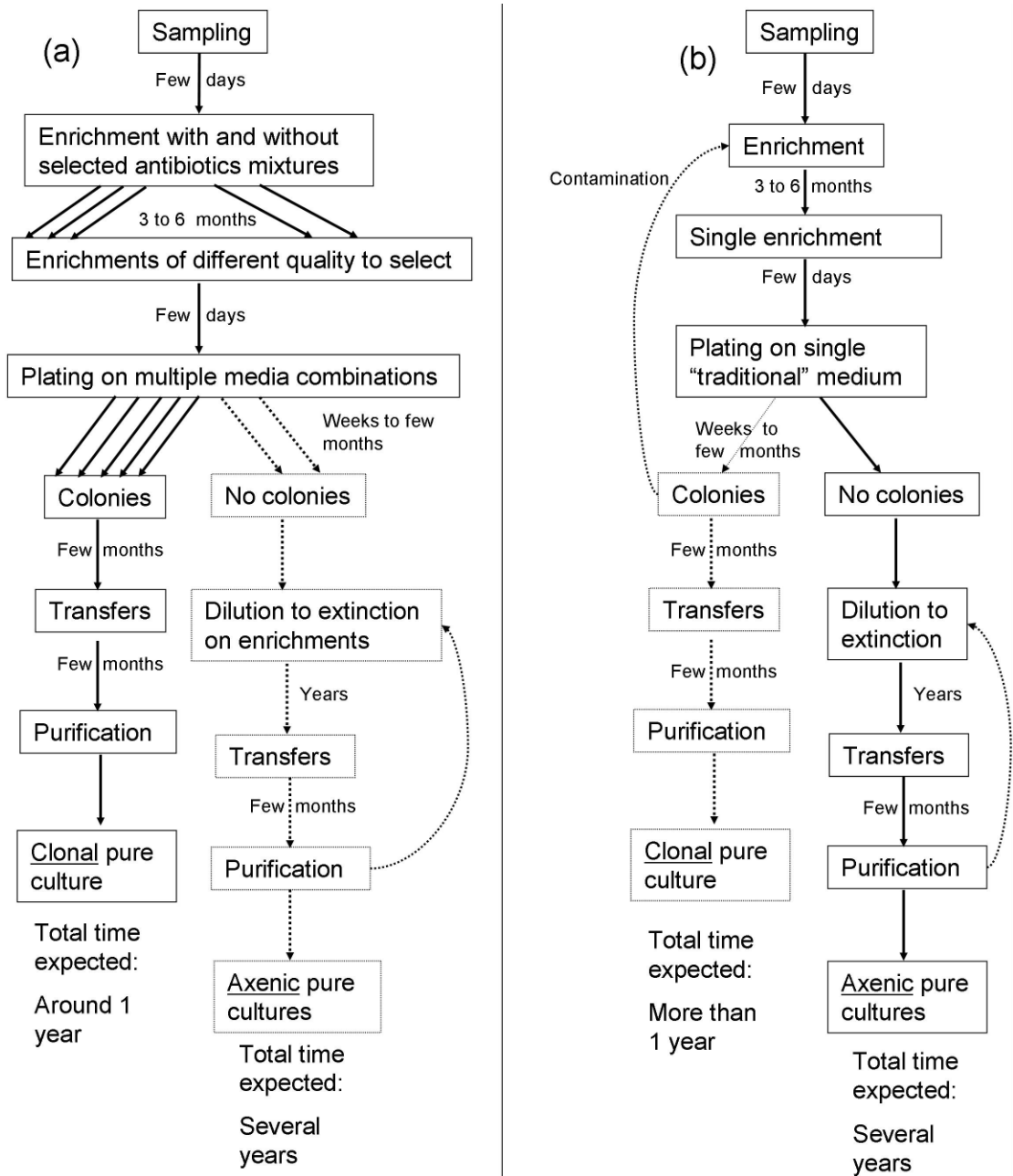
Figure 15: Flow chart comparing anaerobic isolation timelines between (a) the approach detailed here and (b) a traditional approach.

Chapter 4 Results: Genotypic distribution of *Methanosaeta*, a specialist model microorganism, along an estuary: When biogeography does not apply?

This work has been submitted for publication.

4.1. Introduction

The importance of ecological theory in microbial ecology has recently been highlighted (Cebron *et al.*, 2007, Kassen and Rainey, 2004). It is argued that the application of established ecological theories to microbiological systems will facilitate the organisation and interpretation of the ever-growing molecular data (Strous, 2007) with the ultimate aim being the ability to predict the structure and function of microbial communities. For example, ecological models suggested that cycling the use of antimicrobial drugs would not reduce antimicrobial resistance (Bergstrom *et al.*, 2004). Yet, the application of such theories in microbial ecology is uncommon, mostly because of the differences in approaches to studying microorganisms and macroorganisms (Cebron *et al.*, 2007). Thus, new approaches are needed to overcome the commonly recognised great complexity of microbial communities (Curtis *et al.*, 2002, Hughes *et al.*, 2001).

Determining patterns of distribution and biodiversity is one of ecology's primary goals (Lubchenco, 1991), but effective studies on microbial distribution are relatively rare. Biogeography at large scales has been shown for some microbial groups (Whitaker *et al.*, 2003) and is thought to be generally applicable to

microorganisms (Fenchel, 2003). Recently, more studies have shown biogeographic signatures in various environments (Angel *et al.*, 2010, Berthrong *et al.*, 2009, Cermeno and Falkowski, 2009, Desnues *et al.*, 2008, Follows *et al.*, 2007, Takacs-Vesbach *et al.*, 2008) and links with environmental variables such as pH (Fierer and Jackson, 2006, Lauber *et al.*, 2009) or salinity (Crump *et al.*, 2004) were suggested. Gradients have always been of particular interest in population and distribution ecology (Doebeli and Dieckmann, 2003), as they allow an indirect assessment of the environmental factors effects on populations' structure and function.

Studies in microbial ecology are often very broad in their approach (Fierer and Jackson, 2006) and, as a consequence, can fail to reach clear conclusions about whether ideas drawn from ecology are relevant to microbes (Cebron *et al.*, 2007). Therefore, in order to simplify the analysis of a microbial community a traditional ecological approach, focusing on a clearly defined component of a total community, was applied. This work is focused on the important process of anaerobic terminal oxidisation of organic matter in sediments along an estuarine salinity gradient using indigenous sulphate-reducing bacterial (SRB) and methanogenic archaeal (MA) genera. Genera were selected that represent contrasting functional groups, in this case metabolic specialists and generalists. The general hypothesis is that ecologically relevant differences exist in genotypic and phenotypic distributions between specialists and generalists.

Initially two model genera were chosen, for which the distinction specialist/generalist is unambiguous. These two model genera, both of which are ubiquitous in estuarine sediments (Purdy *et al.*, 2002, Purdy *et al.*, 2001), are *Desulfobulbus* (SRB), as the model generalist and *Methanosaeta*, as the model specialist. *Desulfobulbus* are able to use a wide range of carbon sources during

sulphate reduction (Laanbroek *et al.*, 1982, Laanbroek and Pfennig, 1981, Sass *et al.*, 2002), and are also able to use alternative electron acceptors (nitrate and nitrite) or fermentation to gain energy (Widdel and Pfennig, 1982). *Methanosaeta* are considered to be obligate acetoclastic MA that account for a major part of the global biogenic methane emissions (Bass *et al.*, 2007). All 3 isolated *Methanosaeta*, *M. concilii* (Patel and Sprott, 1990), *M. thermophila* (Kamagata *et al.*, 1992) and *M. harundinacea* (Allewalt *et al.*, 2006) use acetate as their sole electron acceptor, electron donor and carbon source and there is no indication that they might use alternative substrates or metabolic pathways (Patel, 2001, Patel and Sprott, 1990).

Genotypes of the generalist *Desulfobulbus* have been shown to be sequentially distributed along an estuarine gradient (Carbonero *et al.*, 2010, Diniz-Filho *et al.*, 2007) in a pattern similar to macrofauna indicative of classic niche separation driven by gross environmental factors (Gaston and Spicer, 2001, Spooner, 1947). *Methanosaeta* are a significant part of the Colne estuary MA community (Purdy *et al.*, 2002). Given that *Methanosaeta* have very specific metabolic requirements, it is hypothesised that their distribution should be more limited and less structured than *Desulfobulbus*. We hypothesised that, in contrast to *Desulfobulbus*, *Methanosaeta* distribution is not constrained by general environmental factors such as salinity, but possibly by more specific factors such as the availability of acetate above its low threshold concentration (Westermann *et al.*, 1989). Thus, it is expected that *Methanosaeta* populations should be less structured than *Desulfobulbus* and that genotypic specialisation will be marginal, leading to a weaker biogeographic pattern. If there are contrasting distribution patterns between these two model genera then these may be explained by their contrasting metabolic properties, and parallels might be drawn with general ecological theories.

The aim of this study was to determine *Methanosaeta* genotypic distribution along the Colne estuary by analysing DNA to determine the presence of *Methanosaeta* and RNA to determine whether they were active. The influence of some major environmental variables on *Methanosaeta* distribution was statistically assessed. The *Methanosaeta* genotypic distribution patterns that were detected were then compared to those for the model generalist, *Desulfobulbus* (Carbonero *et al.*, 2010, Diniz-Filho *et al.*, 2007) and differences between these patterns discussed, with reference to macroecology theories.

4.2. Results

4.2.1 Nucleic acids extraction and *Methanosaeta*-specific PCR

DNA and RNA were successfully extracted from all 33 samples sampled either in 2004 and 2007.

Methanosaeta-specific primer specificity was confirmed as amplification was only obtained from *Methanosaeta* cultures at the annealing temperature of 67°C (Carbonero *et al.*, 2010). Fragments of around 700 basepairs in size were obtained from DNA after the semi-nested rounds of PCR and from cDNA obtained by reverse transcription.

4.2.2. DNA-DGGE analysis of *Methanosaeta* along the Colne estuary

Seven bands appearing in the DGGE at more than one site were analysed for variation in bands intensity. These seven bands represent all the clearly visible bands

in most of the samples. The mean corrected intensities for each band were fairly constant at all sites along the estuary, but corrected intensities were variable between the different replicates within each site which explains the relatively large standard error of the mean (SEM) that can be seen in Figure 16. Sequences from excised DGGE bands were all closely related to previously sequenced *Methanosaeta* isolates and clones.
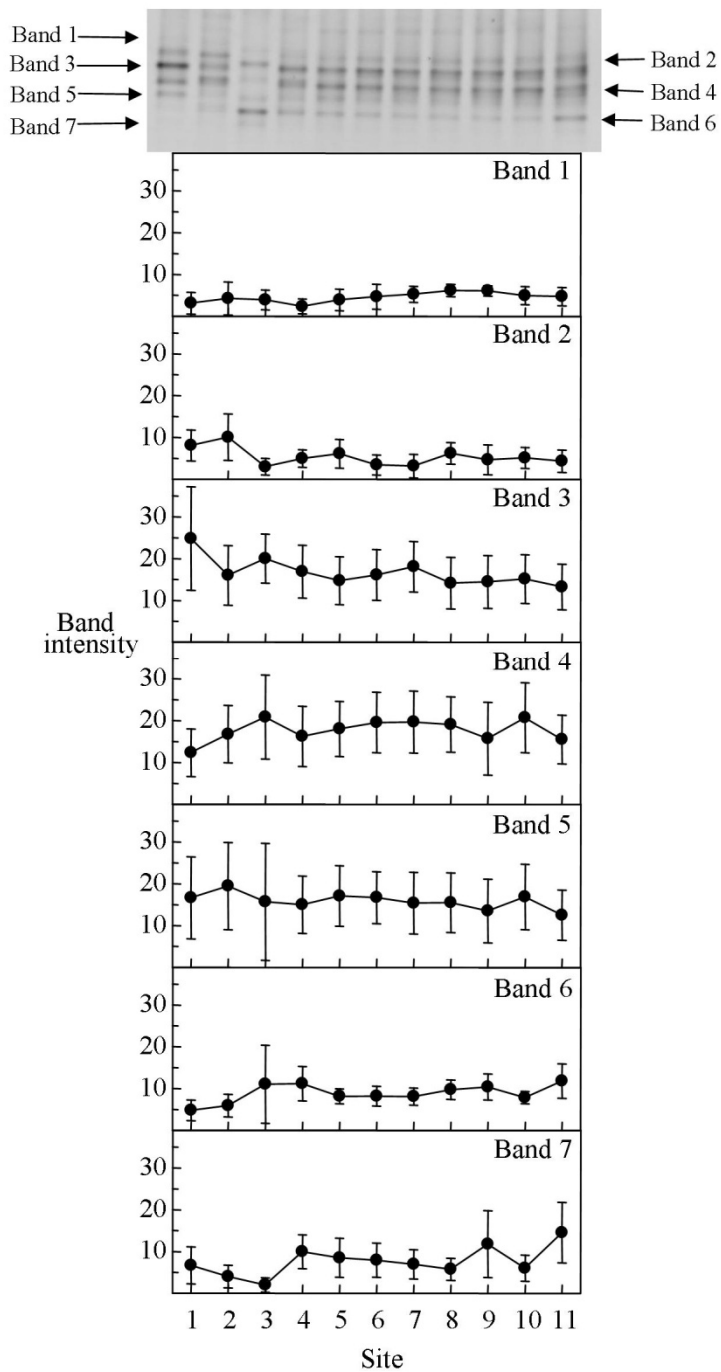
Figure 16: DGGE analysis of *Methanosaeta* genotypes along the estuary indicating the environmental ranges of 7 bands detected at multiple sites. Band intensity was determined using GelComparII (Applied Maths, USA) and corrected using the method of Dunbar (2002) to account for loading differences. Error bars represent the SEM (n=3). DGGE gel shown was run with a pooled sample of all replicates.

Replicated DGGE analyses indicated that no clear distribution of *Methanosaeta* genotypes occurs along the estuary (Figure 17C). A Jaccard (band presence/absence) cluster analysis of a DGGE of the pooled replicates, based on the 7 ubiquitous bands, showed high similarity between sites (>59%) and no clear clustering of sites from the same regions of the estuary (Figure 17A). A Pearson analysis, which compares the complete profile and not just selected bands gives similar level of overall similarity (>65%) and while there is some clustering of sites (sites 4-10 cluster closely) it is not consistent across the estuary (Figure 17B). Cluster analyses on the 3 replicated DGGE gave similar results, but despite the consistency of the DGGE analysis it was difficult to compare across different gels as data from individual gels tended to cluster together. This pooled DGGE tends to show an increase in band intensities with decreasing salinity. The corrected relative total band intensities confirmed this trend (Figure 17D), with the two marine sites (1-2) at less than 50% of the maximal band intensity observed, 7 brackish sites (3-9) at between 50 to 60% and the two freshwater sites 75 to 100%.
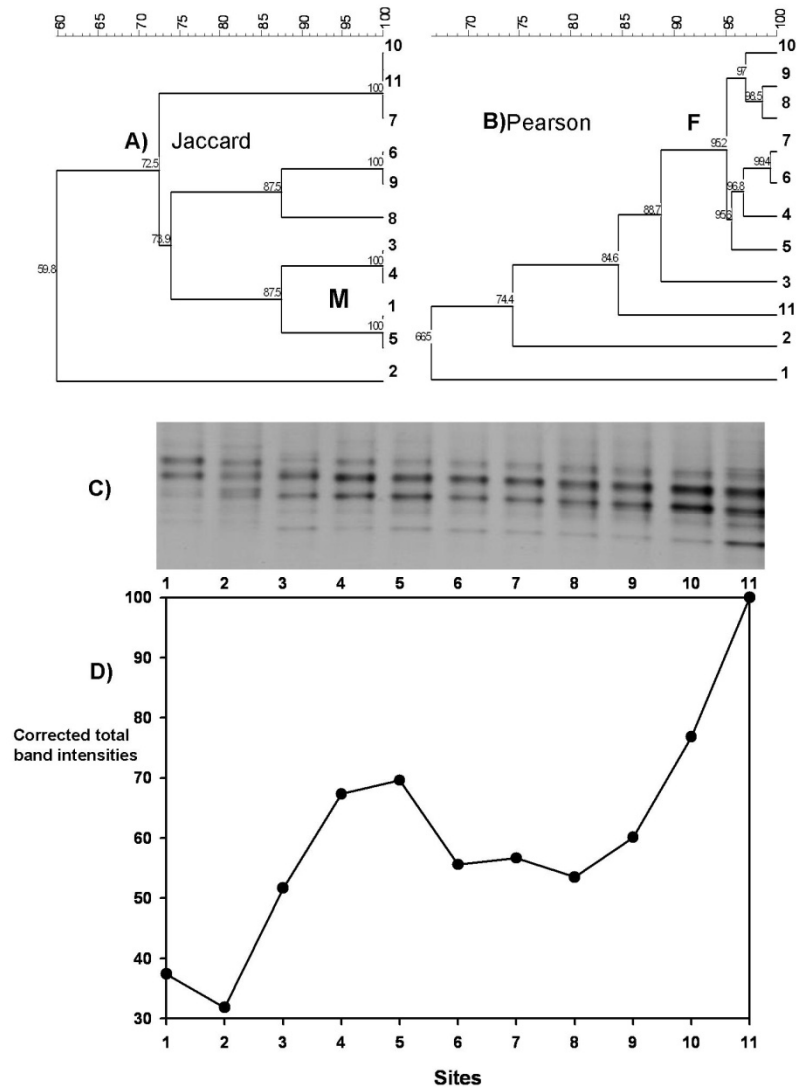
Figure 17: *Methanosaeta* DNA-DGGE band patterns from a pooled sample from each site. A. Jaccard analysis of the 7 ubiquitous bands. B. Pearson analysis on complete profile of each lane. C. DGGE profile of pooled samples from each site. D. Variable corrected total band intensity at each site. Clustering between sites does not support a biogeographical distribution of the *Methanosaeta* genotypes order. Numbers correspond to sites affiliation (see Figure 1) in all figures.

The overall consistency of DGGE profiles from replicated DNA extractions and comparable clustering suggests that the potential biases from the multi-step analysis used here is at least consistent from sample to sample.

4.2.3. RNA-DGGE analysis of *Methanosaeta* along the Colne estuary

RNA-DGGE profiles were similar to those detected in the DNA-DGGE analysis, with a large variability in band intensities (Figure 18C). As with the DNA-based analysis cluster analyses showed quite high similarities for all profiles. As with the DNA-based analysis, a pooled DGGE was performed and a Jaccard clustering analysis, based on the 7 ubiquitous bands, is shown in Figure 18A. The lowest similarity values were in the range of those observed for DNA (62.9%) and there is some clustering by region of the estuary (sites 9-11 cluster together and close to site 7) it is not absolutely consistent as Site 8 cluster closely to site 4 and within what could be defined as a marine/brackish cluster. The Pearson analysis also divides into 2 distinct clusters, but has less site-based consistency than the Jaccard analysis with sites 5, 10 and 11 forming one cluster (Figure 18B). Furthermore, there are inconsistencies between the two analyses. While sites 7 and 9 cluster with sites 10 and 11 in a "freshwater" group using a Jaccard analysis these two sites are in a "marine/brackish" group using a Pearson analysis. This would suggest that there is only limited evidence for a biogeographic signal in these samples. This pooled DGGE also tends to show an increase in band intensities with decreasing salinity. The corrected relative total band intensities confirmed this trend (Figure 18D).
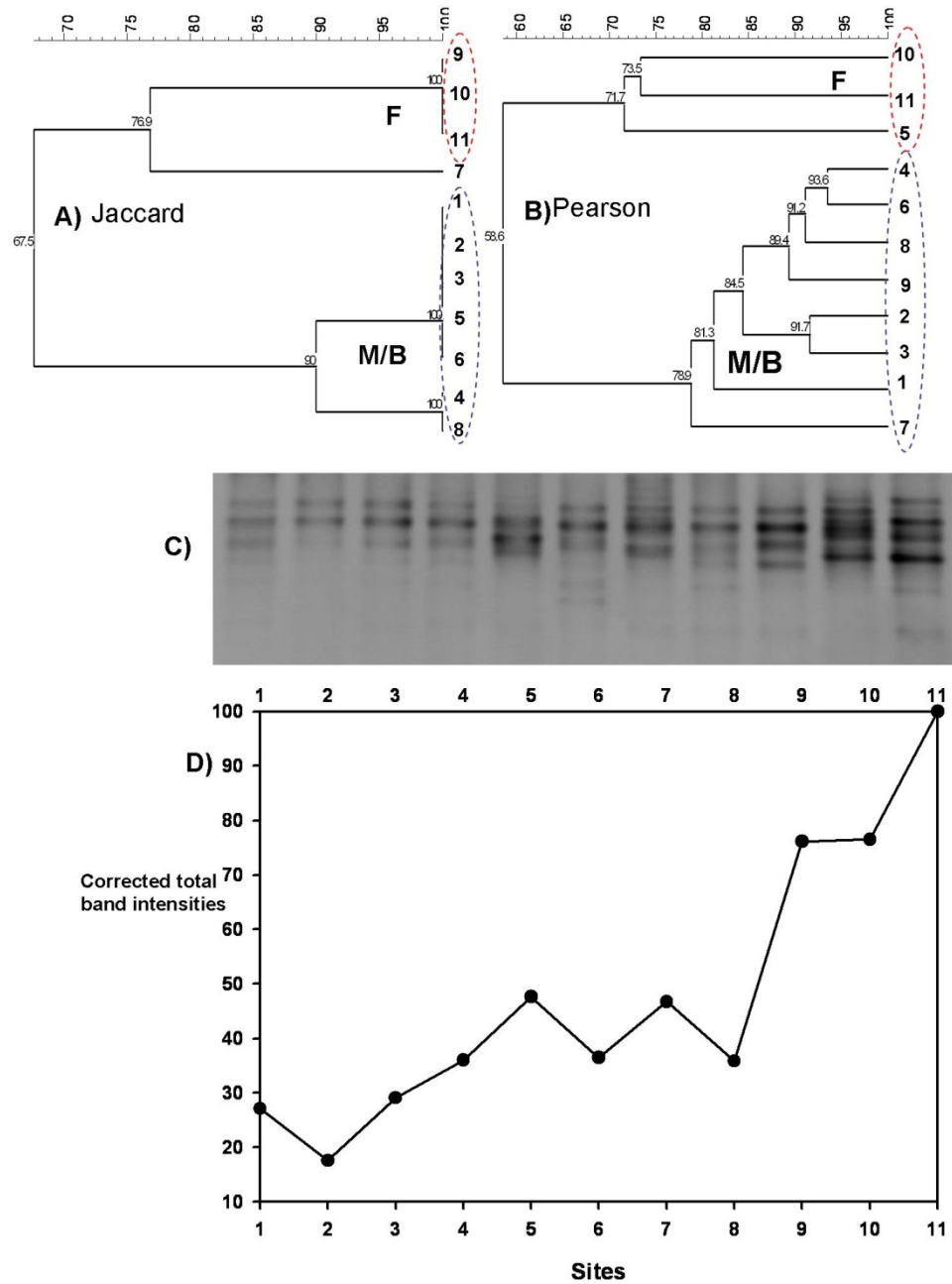
Figure 18: *Methanosaeta* RNA-DGGE band patterns from a pooled sample from each site. A. Jaccard analysis of the 7 ubiquitous bands. B. Pearson analysis on complete profile of each lane. C. DGGE profile of pooled samples from each site. D. Variable corrected total band intensity at each site. Weak clustering between marine (M) and brackish/freshwater sites (B/F) is apparent.

In this case the only distinction is between marine and brackish sites (1-8) at less than 50% of the maximal band intensities observed and the freshwater sites (9-11) between 75 to 100%.

4.2.4. Analysis of clone libraries

In total, 239 good quality clone sequences were obtained and 197 of these sequences clustered closely with sequences from *Methanosaeta* isolates. Sequences have been deposited into Genbank, accession numbers GU332303-GU332499. However, the sequences were quite dissimilar to *M. concilii* (<97% similarity), the only previously described mesophilic *Methanosaeta* isolate (Patel and Sprott, 1990). Twelve OTUs were defined in DOTUR using a 3% sequence dissimilarity cut-off (Figure 19). There was no clear distribution of these OTUs according to site, although there is less diversity detected at sites 1 and 3. UniFrac analyses show no significant correlation between genotypes and sites, either by testing sites individually or by pairs (for all analyses corrected $p>0.15$). In addition, distinction between more general environmental divisions, e.g. marine, freshwater and brackish sites also did not support linking genotypes to a particular environment. Cluster analysis using UniFrac showed a limited dissimilarity (<40%) between each sites and no clear pattern of similarity between any sites (Figure 19).
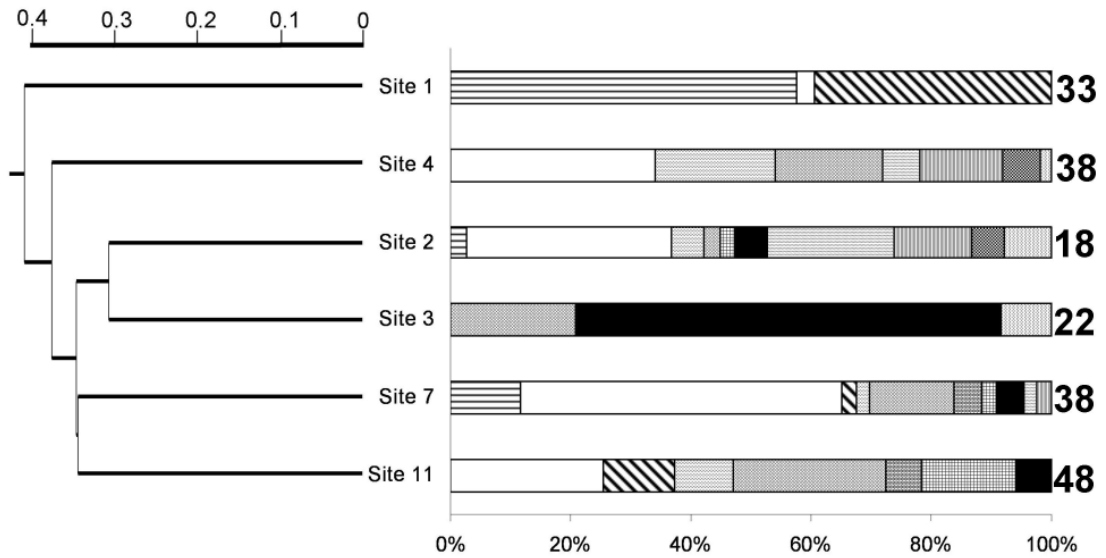
Figure 19: Distribution and relative abundance of *Methanosaeta* OTUs as defined by DOTUR according to the site. Number of clones obtained from each site is on the right of bar chart. A cluster analysis (UniFrac) is shown on the left; a value of 0 means that the environments are perfectly identical in their genotypes content and 1 means that environments are totally dissimilar. All sites show a limited dissimilarity (between 0.3 and 0.4), and clustering that would not support the presence of a biogeographical signal in the clone libraries.

Diversity indexes were calculated from the *Methanosaeta* clone library and, for comparison, indexes were also calculated from a clone library based on the *dsrB* functional gene for *Desulfobulbus*, obtained from the same sediments DNA extracts (Carbonero *et al.*, 2010). The values obtained are shown in Figure 20. For the two genera, the Shannon index was variable, but *Desulfobulbus* showed greater variation and the lowest diversity values (<0.2) at sites 2 and 4. Evenness was also highly variable for *Desulfobulbus*, following the Shannon index trends. In contrast, *Methanosaeta* evenness was stable and always high (>0.8).
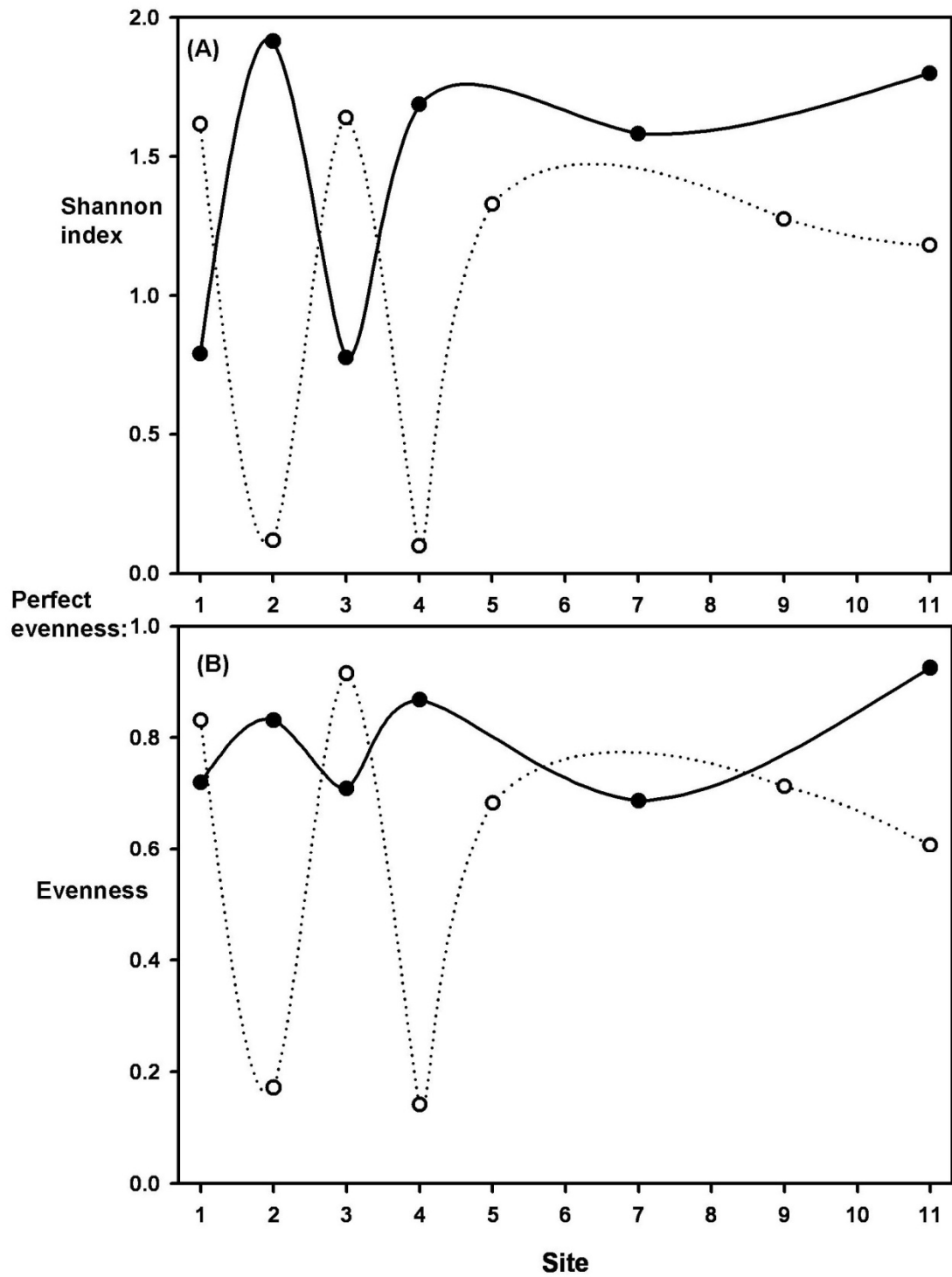
Figure 20: Shannon (A) and evenness (B) indexes calculated from the *Methanosaeta* clone libraries (black circles, solid line) and *Desulfobulbu*s *dsrB* clone libraries (white circles, dotted line)

4.2.5. Correlations between genotypic distribution patterns and environmental variables

Environmental variables (chloride and sulphate) for these sediment samples have been reported previously (Carbonero *et al.*, 2010, Diniz-Filho *et al.*, 2007) and have been used here.

Unconstrained CCA did not indicate any clear patterns of distribution of *Methanosaeta* in the estuary (Figure 21). There is an indication of a marine cluster (sites 1 and 2) in the DNA-based analysis, but this cluster is not apparent in the RNA analysis. Eigen values for variation are very low in DNA-based analysis (Fig 21A) and low in the RNA-based analysis (Fig 21B) indicating a poor correlation between environmental variables and genotypic dissimilarity matrixes of obvious geographic clusters in the CA plot suggests that the spatial variation is not related to geography.

Mantel and partial Mantel tests were generally not significant for the DNA patterns, but Mantel tests were significant for all variables and all genotypic matrixes for RNA data. However, the partial mantel tests do not show clearly which environmental factors are relevant to these significant differences. In total these analyses show that no significant correlation was clearly supported between genotypes and environment.

A. *Methanosaeta-DNA*

		Band intensities	Pearson	Jaccard
Mantel	Distance (D)	0.119	0.144	0.139
	Chloride (C)	0.263	0.316	0.183
	Sulphate (S)	0.317	0.291	0.159
Partial Mantel	D/C	0.011	0.001	0.148
	D/S	0.02	0.087	0.327
	C/D	0.913	0.935	0.647
	C/S	0.285	0.325	0.803
	S/D	0.902	0.794	0.32
	S/C	0.686	0.488	0.1

B. *Methanosaeta-RNA*

		Band intensities	Pearson	Jaccard
Mantel	Distance (D)	0.007	0.024	0.001
	Chloride (C)	0.008	0.012	0.001
	Sulphate (S)	0.001	0.013	0.003
Partial Mantel	D/C	0.062	0.836	0.447
	D/S	0.134	0.494	0.253
	C/D	0.705	0.03	0.021
	C/S	0.927	0.284	0.325
	S/D	0.107	0.028	0.022
	S/C	0.006	0.194	0.089

Figure 21: A. Unconstrained CCA plot of DNA-DGGE bands shows a weak clustering of sites 1 and 2. Axes shown account for only 4.2% of the detected variance in these samples. Results of Mantel and partial mantel tests do not indicate any strong support of spatial variation in clustering. B. Unconstrained CCA plot of RNA-DGGE bands shows no clear geographic clustering of any sites. Axes shown account for only 21.3% of the detected variance in these samples. Results of Mantel tests do indicate some support for spatial variation, but partial Mantel tests do not support such clustering.

4.3 Discussion

Three rounds of semi-nested PCR were needed to amplify *Methanosaeta* from all extracted DNA samples. Although this data is not quantitative, this would suggest that *Methanosaeta* DNA was present in quite low proportions in the sediments, as the specific primer designed to target this genus gave good amplification from pure cultures. Interestingly, amplification of *Methanosaeta* sequences from RNA was easier. Only a one-step PCR, using the *Methanosaeta*-specific primer set 1Af and Mst746 was required after reverse-transcription of *Methanosaeta* RNA using Mst746r at all sites. Defining how DNA and RNA from a specific genus is related in environmental samples is complex, but the relative ease with which reverse-transcribed RNA was amplified would suggest that *Methanosaeta* RNA was present in reasonable quantities in the sediments, perhaps indicating that although the population was small, hence the difficulty in amplifying *Methanosaeta* 16S rRNA genes directly from DNA, it was active.

The multi-step PCR approach that is used in this work could introduce biases into these studies that could make interpretation of the ecological relevance of the data presented difficult. Previous studies have required the use of nested PCR approaches to successfully amplify 16S rRNA gene sequences from estuarine methanogens (Munson et al 1997; Purdy et al 2002), while a previous study by Hawkins and Purdy (2007) used a three-step nested PCR to analyse the *Desulfobulbus* community along the Colne. The distribution patterns seen with *Desulfobulbus* using a three-step DGGE analysis have been supported by a direct PCR analysis of *Desulfobulbus dsrB* (Oakley et al 2010). The similarity of the DNA-DGGE and RNA-DGGE in this work would suggest that no substantial additional

biases have been introduced in the DNA-based analysis by the four-step PCR approach.

This study can give a representation of *Methanosaeta* genetic diversity and its potential variation along the Colne estuary, even if it is not possible to determine how representative this data is of the actual community. From the different DGGE patterns and clone libraries it seems that *Methanosaeta* genotypic diversity is quite high all along the estuary. This is confirmed by the Shannon index values, which are consistently above 0 (Figure 20), the minimal value, indicative of an undiversified community. In contrast, *Desulfobulbus dsrB* analysis has a low Shannon index (<0.2) at two sites. This high genotypic diversity in *Methanosaeta* is intriguing and was unexpected given that estuarine sediments would not seem to be an ideal place for *Methanosaeta* to thrive. In the environment *Methanosaeta* face competition from acetate-utilising SRBs that reduce *in situ* acetate concentrations below the threshold *Methanosaeta* require to grow where sulphate is freely available (Chidthaisong and Conrad, 2000, Jetten *et al.*, 1990) and from *Methanosarcina* which can outgrow *Methanosaeta* at high acetate concentrations (Westermann *et al.*, 1989). Therefore, the *in situ* concentration of acetate would appear to be the critical factor in the success of *Methanosaeta*. In marine and brackish regions of estuaries competition for acetate from SRB reduces acetoclastic methanogenesis effectively to zero, but acetate availability may vary on a microscale. Thus, it could be hypothesised that the diversity reflects differences in acetate affinity and threshold concentration among *Methanosaeta* strains and this genotypic diversity would be a driver for the community stability.

Previous studies on *Desulfobulbus*, using either 16S rRNA or functional genes, showed a clear sequential distribution pattern along the estuary. This data

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from *Desulfobulbus* shows that nested 16S rRNA gene sequence DGGE analysis can reveal genotypic distributions, despite potential amplification biases. In comparison to *Desulfobulbus* distribution patterns (Carbonero *et al.*, 2010, Diniz-Filho *et al.*, 2007), *Methanosaeta* genotypes do not appear to have restricted distributions. Relative band intensities did not show significant variations between sites, with variability found between replicates from individual sites. No significant clustering was supported by analysis of DGGE gels, although the freshwater sites 9, 10 and 11 cluster together in Figure 18B, but only in the RNA-based DGGE. There is no supporting evidence from such a distribution from the individual bands (Figure 17), and therefore may reflect the marked increase in band intensity at sites 9-11 rather than a differential distribution of genotypes. UniFrac analyses of the clone sequence data did not allow the rejection of the null hypothesis that sequence phylogeny is related to sampling site. Therefore UniFrac does not support a differential genotype distribution pattern and there is no evidence that any detected genotype was restricted to any particular part of the estuary. However, some dissimilarity in clone libraries from the different sites was detected and variability in sites OTU composition can be seen in Figure 19. This variability, which does not seem so important on the DGGE pattern, could be explained by the limited scale of the clone libraries from each site, a perennial problem in microbial ecology. Emerging methods of high throughput sequencing could help to characterise genotype distribution patterns in extensive detail. Therefore the *Methanosaeta* distribution pattern can be described as monotonic and contrasts markedly with the one observed for *Desulfobulbus* (Carbonero *et al.*, 2010, Diniz-Filho *et al.*, 2007). Evenness of the *Desulfobulbus* community varies over a wide range (0.2-0.9) which is consistent with unevenly distributed genotypes producing a sequential distribution pattern (Figure

20). In contrast evenness of the *Methanosaeta* community varies much less (0.7-0.95) and is always high confirming the monotonic *Methanosaeta* distribution pattern (Figure 20). Thus, we can conclude that *Methanosaeta* genotypes are more evenly distributed than *Desulfobulbus* genotypes. The absence of statistical support for environmental variables effecting *Methanosaeta* populations is somewhat surprising as even small differences in salinity tolerances between genotypes could have produced a weak biogeographic signal.

If gross environmental factors do not affect *Methanosaeta* genotype distribution then competition could produce a geographic distribution pattern. However, this potential intra-genus competition is not detected with the methods used here, and even with very sensitive methods, it would be very challenging to detect it. The detected *Methanosaeta* genotypes would experience classical competition from SRB, but there is no evidence that any of the different genotypes exhibit a different sensitivity to this competition that produces a differential distribution.

Methanosaeta distribution patterns derived from RNA and DNA could have showed some differences, specifically that a sequential distribution pattern could have been seen in an RNA (activity-based) analysis in contrast to a monotonic DNA (presence-based) pattern. This would have suggested that while the overall distribution of genotypes was monotonic the activity of these genotypes differed in different sites. However, our data does not show any difference in distribution between DNA- and RNA-based analyses. The remarkable similarity of RNA and DNA DGGE shows that, at least for *Methanosaeta*, all detected genotypes have some activity in the sediments from all along the estuary.

It is well known that in estuarine environments, SRB dominate organic matter terminal oxidation in marine-dominated sediments and MA in freshwater sediments (Purdy *et al.*, 2003, Senior *et al.*, 1982). Here, this trend appears to be confirmed both from DNA and RNA analyses showing increasing band intensities at the freshwater end of the estuary, which suggests that *Methanosaeta* numbers and activity (determined by increasing ease of detection) increases with decreasing salinity. Although given the analysis methods used this cannot be considered quantitative data, the *Methanosaeta* genotypic distribution could be further described as monotically decreasing with increasing salinity. The slight differences in trends between RNA and DNA patterns might suggests that *Methanosaeta* populations could be classified as rare at the marine end, present in the brackish regions and abundant in the freshwater end of the estuary, with active populations limited to the more freshwater regions.

If we assume that marine and brackish sediments are not the ideal niche for *Methanosaeta*, because they are out-competed for acetate by SRB, it is possible to hypothesise that their actual habitable sites will be freshwater sediments. From this assumption, the monotonic pattern observed here for *Methanosaeta* could be explained by distinguishing between habitable sites and sink sites as described by Pulliam (1988). This model considers sites that can transiently support an organism as sinks, which may contain significant numbers of immigrant organisms. It is possible to consider a model where *Methanosaeta* are slowly washed away from their habitable (freshwater) sites to the sink (marine dominated) sites. This would result in decreasing activity and numbers with increasing salinity, but not necessarily in a diversity decrease. More quantitative data will be needed to support this model.

A common ecological theory that may be applicable is the distinction between r- and K-strategists, although this distinction should be considered with care (Reznick *et al.*, 2002). Briefly, r-strategists thrive better with abundant resources while K-strategists are more adapted to low resource conditions (Andrews and Harris, 1986). Specialists and generalists microorganisms have been thought to correspond respectively to r- and K-strategists in that a specialist will be able to use resources more efficiently. However in this estuarine ecosystem, this parallel is contradicted by the fact that the specialist acetoclastic *Methanosaeta* appears less efficient in acetate uptake compared to the only other acetoclastic methanogen, *Methanosarcina* (Janssen, 2003), which is a generalist. A parallel between r- and K-strategists and copiotrophs and oligotrophs microorganisms respectively has been suggested (Fierer *et al.*, 2007). This distinction between copiotrophs and oligotrophs appears more relevant in this case, with *Methanosaeta* being an oligotroph with a probably marginal contribution to ecosystem productivity in most regions of the estuary, whereas *Desulfobulbus* would be a copiotroph with selected populations able to account for a more significant part of the estuarine productivity. Subsequently, in this environment, *Methanosaeta* may be classified as a K-strategist and *Desulfobulbus* an r-strategist, but this pattern might be reverted in other environments such as rice paddies (Bass *et al.*, 2007). Assigning a microorganism to either r- or K-categories should be made with care for microbial genera, as their metabolic potential is strongly dependent of their surrounding environment.

From the present study and the *Desulfobulbus* studies, it is clear that *Methanosaeta* genotypes show a monotonic distribution pattern while *Desulfobulbus* genotypes show a sequential distribution apparently driven by the gross environmental factors. Thus, at least for these two models, the general theory that

ecologically relevant differences exist in genotypes between specialists and generalists seems to be confirmed. It appears that *Methanosaeta* distribution is not constrained by general environmental factors such as salinity but, perhaps, primarily by the availability of acetate. The salinity gradient appears to have very little, if any, effect on genotype qualitative distribution, but may affect their quantitative distribution. As shown here comparing model organisms using different metabolisms can illustrate general principles of microbial communities' distribution. As it stands, it is not possible to definitely prove that the contrasting distribution patterns are only driven by the generalist/specialist difference between the two genera. The subsequent question is whether these contrasting distribution patterns could be generalised to other microorganisms and other environments. While we might expect an estuarine salinity gradient to affect SRB it is less likely that such a gradient would affect MA. A next logical step would be to study genotypic distribution of specialists and generalists that use a similar metabolism. *Methanosarcina* is the only other known acetoclastic MA genus, but is also the most flexible MA, able to use all three methanogenic pathways (Maestrojuan and Boone, 1991), thus it can be studied as a generalist MA. Isolated *Desulfobacter* spp. utilise only sulphur oxyanions as electron acceptors and acetate is their preferred carbon and energy source (Kuever, 2005, Widdel, 1987, Widdel and Pfennig, 1981), and so can be studied as specialists SRB. Determining distribution differences or similarities among and within metabolic groups could be the cornerstone to strengthen this general theory in microbial ecology. Linking genotypes to phenotypes will be another important step. To date, it is still unknown if the distribution pattern seen for *Desulfobulbus* is due to strict adaptation to ecological niche, or competitive advantages, or other ecological forces. Based on the monotonic genotypic distribution and the metabolic specificity of

Methanosaeta, it would be expected that *Methanosaeta* isolates from the Colne estuary will have similar phenotypes. Characterisation of isolates from the estuarine sediments samples (Carbonero *et al.*, 2010) should provide answers to these studies as well as improving our understanding of this important genus.

Chapter 5 Results: Metabolic flexibility as a major predictor of microbial communities' structure and distributions

This work has been submitted for publication.

5.1. Introduction

There is currently an intense debate in determining to what extent ecological principles and patterns observed on macroorganisms may be directly applied to microorganisms (Fuhrman *et al.*, 2008, Martiny *et al.*, 2006). Studies showing that ecological principles do apply to microorganisms remain scarce, but are of crucial importance to fully exploit data collected by microbial ecologists (Cebron *et al.*, 2007). A primary assumption developed as early as 1934 by Baas-Becking was that distinct microbial communities were only shaped by the local environmental factors (de Wit and Bouvier, 2006). Recently, two studies showed that similar hot springs in remote places across the world harbour different thermophilic archaeal and bacterial assemblages (Papke *et al.*, 2003, Whitaker *et al.*, 2003). Numerous studies have shown the existence of biogeographic patterns for microorganisms (Berthrong *et al.*, 2009, Carbonero *et al.*, 2010, Cermeno and Falkowski, 2009, Desnues *et al.*, 2008, Follows *et al.*, 2007, Takacs-Vesbach *et al.*, 2008), which are often suggested to depend on biotic or abiotic factors (Amann and Ludwig, 2000, Crump *et al.*, 2004, Fierer and Jackson, 2006, Lauber *et al.*, 2009).

Several studies showed that sampling size (Curtis and Sloan, 2004), very high genetic diversity and difficulties in defining unequivocal taxonomic groups (Cohan, 2006) are among the numerous barriers that make the application of ecological theories to microorganisms difficult. Early ecological science faced similar hurdles and overcame these by a reductionist approach, using model species that were easier to monitor and thought to be a good representation of larger groups. This kind of approach has rarely been applied to microorganisms, mostly because of the difficulties in defining clearly homogeneous species. This issue can be avoided by studying higher taxonomic groups such as genera which are usually homogeneous functional and phylogenetic groups. A major distinction in microbial ecology is between generalists and specialists.

Microbial generalists are characterised by metabolic flexibility whereas specialists are far more restricted in their metabolic requirements. The aim of this study is to use different specialist and generalist models and determine whether differences in their distribution patterns along an environmental gradient are related to their generalist or specialist phenotype.

The environmental gradient to be studied is the salinity gradient in the sediments of the Colne estuary, Essex, UK. Four anaerobic terminal oxidisers genera were used as models, *Desulfobacter*, *Desulfobulbus*, *Methanosaeta* and *Methanosarcina*. All have been shown to be present in estuary sediments by previous studies (Nedwell *et al.*, 2004, Purdy *et al.*, 2002). *Desulfobulbus* and *Methanosarcina* are the model generalists. *Desulfobulbus spp.* are sulphate-reducing bacteria (SRB) able to gain energy from various electron acceptors, carbon sources and electron donors. *Methanosarcina* is the only methanogenic archaeal (MA) genus able to perform the three known methanogenic pathways. *Desulfobacter* and *Methanosaeta*

are the model specialists. *Desulfobacter* are SRB that only gain energy from oxidised inorganic sulphur compounds (sulphate, sulphite and thiosulphate) reduction and acetate consumption. *Methanosaeta* are the most restricted acetoclastic MA, using acetate as electron acceptor and donor as well as their sole carbon source.

To assess distribution patterns fingerprinting methods, in this case Denaturing Gradient Gel Electrophoresis (DGGE) will be used. The accuracy of such methods has been questioned, especially when studying whole communities (Bent and Forney, 2008). In this case, the analyses are limited to single genera and thus should produce a good representation of the actual distributions. We have shown for *Desulfobulbus* (Carbonero *et al.*, 2010, Diniz-Filho *et al.*, 2007) and for *Methanosaeta* (Chapter 4; Carbonero *et al*, submitted) that DGGE profiles produce comparable results to more extensive methods (clone libraries analyses, group specific qPCR assays). Thus we would argue that when targeting specific genera, specificity of PCR is the most important issue and that distribution patterns can be assessed sufficiently using traditional fingerprinting such as DGGE.

Previous studies have shown contrasting distribution patterns along this estuary for *Methanosaeta* and *Desulfobulbus* (see Chapter 4). A strong biogeographic pattern driven by gross environmental factors was shown for *Desulfobulbus* (Carbonero *et al.*, 2010, Diniz-Filho *et al.*, 2007). On the other hand, *Methanosaeta* was shown to be distributed monotonically and not be affected by these environmental factors. Thus, biogeography driven by environmental factors would appear to be dependent of the microorganisms' adaptative potential. Sympatric differentiation or niche differenciation would then only occur if individuals harbour sufficient metabolic flexibility. However, as *Methanosaeta* is an extreme specialist, it might only be a rare exception to widespread microbial biogeography.

The general hypothesis tested is that contrasting metabolic properties may influence the distribution of specific microbial communities. Confirmation of this hypothesis could support a more general theory in microbial ecology. Metabolic specialists and generalists might be distributed differently irrespective of their contrasting properties. Differences in distribution patterns could be strongly explained by these metabolic properties. Finally, the null hypothesis would be that metabolic properties do not define coherently contrasting patterns.

To test these hypotheses, distribution patterns along the Colne estuary for *Desulfobulbus*, *Methanosarcina* and *Desulfobacter* were investigated, and compared to the data previously obtained for *Methanosaeta* (Chapter 4). It is expected that *Desulfobulbus* distribution pattern will be similar to those previously determined (Carbonero *et al.*, 2010, Diniz-Filho *et al.*, 2007). The use of RNA-based fingerprints will also allow us to determine if active genotypes mirror the genotypes detected along the estuary (e.g. DNA-based). *Methanosarcina* represents an excellent comparison with *Methanosaeta*, being a very close phylogenetic relative that possess the highest metabolic flexibility of all the MA. It is expected to have a biogeographical pattern linked to the estuarine gradient as their diverse carbon source must be unevenly distributed along the gradient. *Desulfobacter*, being a little less specialised than *Methanosaeta*, appears more unpredictable. A biogeographic pattern linked to decreasing sulphate availability along the estuary, a monotonic pattern or an intermediate pattern could all be expected. It might thus be the key genus to confirm our hypothesis.

5.2. Results

5.2.1. Nucleic acids extraction and *Methanosarcina*-specific PCR

DNA and RNA were successfully extracted from all 33 samples either of 2003 and 2007, as described previously (Diniz-Filho *et al.*, 2007). Fragments of the correct sizes were obtained for *Desulfobulbus*, *Desulfobacter* and *Methanosaeta* specific primer pairs after their respective nested PCRs (see Section 2.5). *Methanosarcina*-specific primer pair (see Section 2.5) specificity was confirmed as amplification was only obtained from *Methanosarcina* cultures and not from other related MA and various bacterial controls (data not shown). Fragments of around 400 basepairs in size were obtained from estuarine sediment samples, and were successfully amplified for DGGE analyses.

5.2.2. DNA-DGGE analysis of the four models along the Colne estuary

Banding patterns, based on three DGGE profiles corresponding on the three replicates of all four genera are shown in Figure 24. Generalists (*Methanosarcina* and *Desulfobulbus*) specialists (*Methanosaeta* and *Desulfobacter*) DNA distribution patterns were highly dissimilar (Figure 22). Both *Methanosarcina* and *Desulfobulbus,* the generalist models showed sequential restricted patterns, with bands present between one to nine sites. For *Desulfobulbus*, bands which are clearly restricted to marine-brackish regions (bands 1 and 6) marine brackish (band 4), brackish-freshwater (bands 2and 3) and freshwater (band 5) regions are shown on Figure 22(I). For *Methanosarcina*, bands which are clearly restricted to marine (band

5), marine-brackish regions (bands 1 and 3), brackish-freshwater (bands 4 and 6) and freshwater (band 2) regions are shown on Figure 22(II).

Methanosaeta and *Desulfobacter*, the specialist models, showed monotonic patterns, with ubiquitous bands detected all along the estuary. Mean corrected values were relatively constant and somewhat variable between replicates.
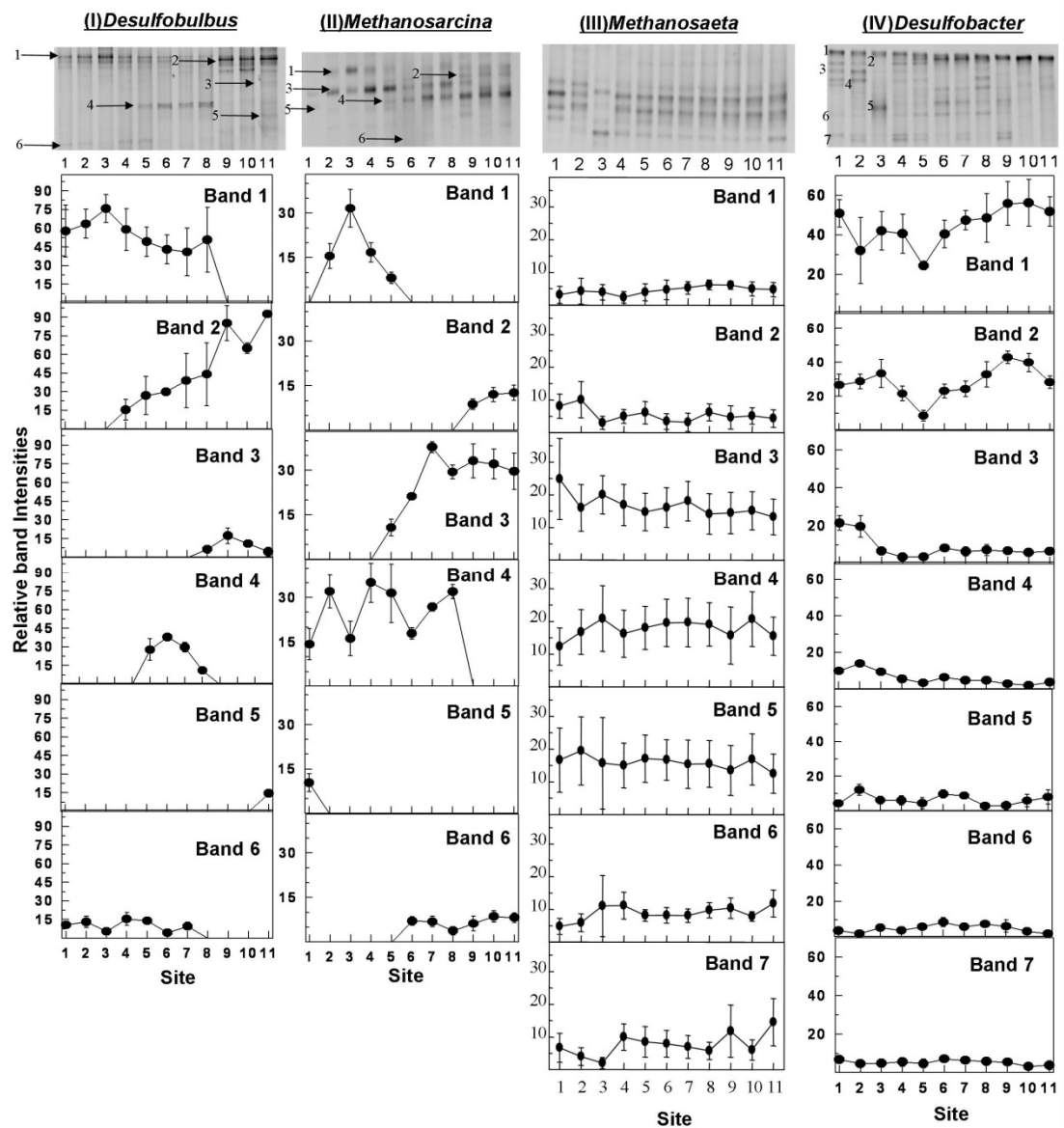
Figure 22: Corrected mean band intensities along the 11 sites (from the marine site 1 to the freshwater site 11) from replicated DNA-DGGE for: (I) *Desulfobulbus*, (II) *Methanosarcina*, (III) *Methanosaeta* and (IV) *Desulfobacter*. Band numbers are indicated on the left of gel images (see Chapter 4 for *Methanosaeta*).

Clustering analyses were based on DGGE profiles of pooled replicates (Figure 23). Specialist models, *Desulfobulbus* and *Methanosarcina*, have minimal similarities of 50% whereas generalist models, *Methanosaeta* and *Desulfobacter*, have much lower minimal similarities of 0.5%. Using both band presence/absence (Jaccard) and total profile (Pearson) analyses, strong clustering was supported for the generalist models. Marine, brackish and freshwater clusters were shown for *Desulfobulbus*; marine (site 1 only), two brackish and freshwater clusters for *Methanosarcina*. Clustering splitting marine (1-3) and freshwater (4-11) sites was less supported for both specialist models.
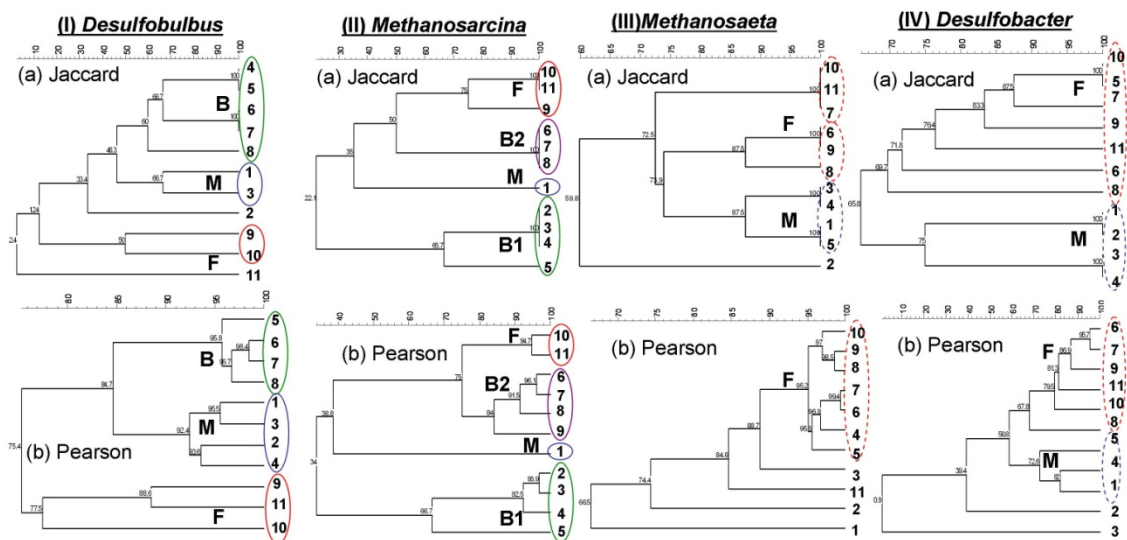
Figure 23: Cluster analyses of the DNA-DGGE profiles: (a) Jaccard and (b) Pearson for (I) *Desulfobulbus*, (II) *Methanosarcina*, (III) *Methanosaeta* and (IV) *Desulfobacter*. Marine (M), Brackish (B, B1, B2), and Freshwater (F) clusters are circled.

Sequences from excised DGGE bands were all closely related to previously sequenced *Methanosaeta*, *Methanosarcina*, *Desulfobulbus* or *Desulfobacter* isolates and clones in each case.

5.2.3. Correlations between genotypic distribution patterns and environmental variables

Results of unconstrained CCA are shown in Figure 24. CCA constrained by any of the 3 environmental variables were similar. Apparent strong clustering was shown by CCA for all genera. However, for the specialists *Methanosaeta* and *Desulfobacter*, very low eigen values were obtained, indicative of a poor correlation between genotypic dissimilarity matrixes and environmental variables. Eigen values were higher for generalists, indicating a better correlation between genotypic patterns and environmental variables.

(I) Desulfobulbus

(a) Unconstrained CCA

(b)

		BI	P	J
Mantel	D	0.001	0.001	0.001
	C	0.001	0.001	0.001
	S	0.002	0.002	0.001
Partial Mantel	D/C	0.006	0.206	0.045
	D/S	0.004	0.032	0.01
	C/D	0.418	0.028	0.051
	C/S	0.41	0.084	0.07
	S/D	0.048	0.22	0.404
	S/C	0.021	0.011	0.036

(II) Methanosarcina

(a) Unconstrained CCA

(b)

		BI	P	J
Mantel	D	0.002	0.001	0.002
	C	0.008	0.003	0.009
	S	0.002	0.001	0.007
Partial Mantel	D/C	0.015	0.025	0.004
	D/S	0.031	0.086	0.009
	C/D	0.919	0.635	0.976
	C/S	0.858	0.821	0.828
	S/D	0.273	0.143	0.613
	S/C	0.01	0.012	0.05

(III) Methanosaeta

(a) Unconstrained CCA

(b)

		BI	P	J
Mantel	D	0.119	0.144	0.139
	C	0.263	0.316	0.183
	S	0.317	0.291	0.159
Partial Mantel	D/C	0.011	0.001	0.148
	D/S	0.02	0.087	0.327
	C/D	0.913	0.935	0.647
	C/S	0.285	0.325	0.803
	S/D	0.902	0.794	0.32
	S/C	0.686	0.488	0.1

(IV) Desulfobacter

(a) Unconstrained CCA

(b)

		BI	P	J
Mantel	D	0.396	0.548	0.035
	C	0.512	0.602	0.169
	S	0.437	0.368	0.158
Partial Mantel	D/C	0.151	0.327	0.008
	D/S	0.335	0.732	0.045
	C/D	0.85	0.702	0.9
	C/S	0.812	0.995	0.512
	S/D	0.538	0.219	0.68
	S/C	0.23	0.009	0.312

Figure 24: (a) Canonical Correspondence Analysis and (b) Mantel tests correlating environmental variables (geographic distance (D), chloride (C) and sulphate (S)) and model specialists' genotypic distribution pattern (Band intensities (BI), Pearson (P) and Jaccard (J)). Marine (M), Brackish (B, B1, B2), and Freshwater (F) clusters are circled.

Similarly to the profile based clustering, marine, brackish (one for *Desulfobulbus*, two for *Methanosarcina*) and freshwater clusters were found for the generalist models. Interestingly, a similar marine/freshwater clustering was shown for *Methanosaeta*, but a brackish cluster was also found for *Desulfobacter*. As expected, sulphate and chloride concentrations were strongly correlated with geographical distance, and can not be separated. Clusters discriminated by genotypic based matrixes were strongly clustered in the CCA.

Mantel and partial Mantel test revealed a strong correlation between the geographic distance and generalist (*Desulfobulbus* and *Methanosarcina*) models' distribution pattern (p<0.05 for dissimilarity matrixes based on 3 methods) (Figure 26). Weaker correlations were shown with chloride and sulphate. Very limited significant correlations were found significant for *Desulfobacter*. More correlations were significant for *Methanosaeta*, but no coherent correlation was found when considering all methods.

5.2.4. RNA-DGGE analysis of *Methanosaeta* and *Desulfobulbus* along the Colne estuary

RNA-DGGE for *Methanosaeta* was described in Chapter 4. Banding pattern analysis, cluster analysis and correlations with environmental variables for *Desulfobulbus* and *Methanosaeta* RNA-DGGE, as performed on DNA-DGGE, are shown in Figure 25. Similarly to the DNA-DGGE, marine-brackish (bands 1and 6), brackish (band 4), brackish-freshwater (bands 2 and 3), and freshwater (band 5), bands were found for *Desulfobulbus*. For *Methanosaeta*, bands were constantly detected all along the estuary, but most of them seem to show an increase in intensity with decreasing salinity.

Cluster analyses showed a well supported clustering for *Desulfobulbus*, with marine, brackish and freshwater clusters. Lower values separating the clusters were found with the Jaccard analysis (<25%) than with the Pearson analysis. As for DNA-based analysis, weak clustering was found for *Methanosaeta*, separating sites differently between marine-brackish and freshwater clusters.
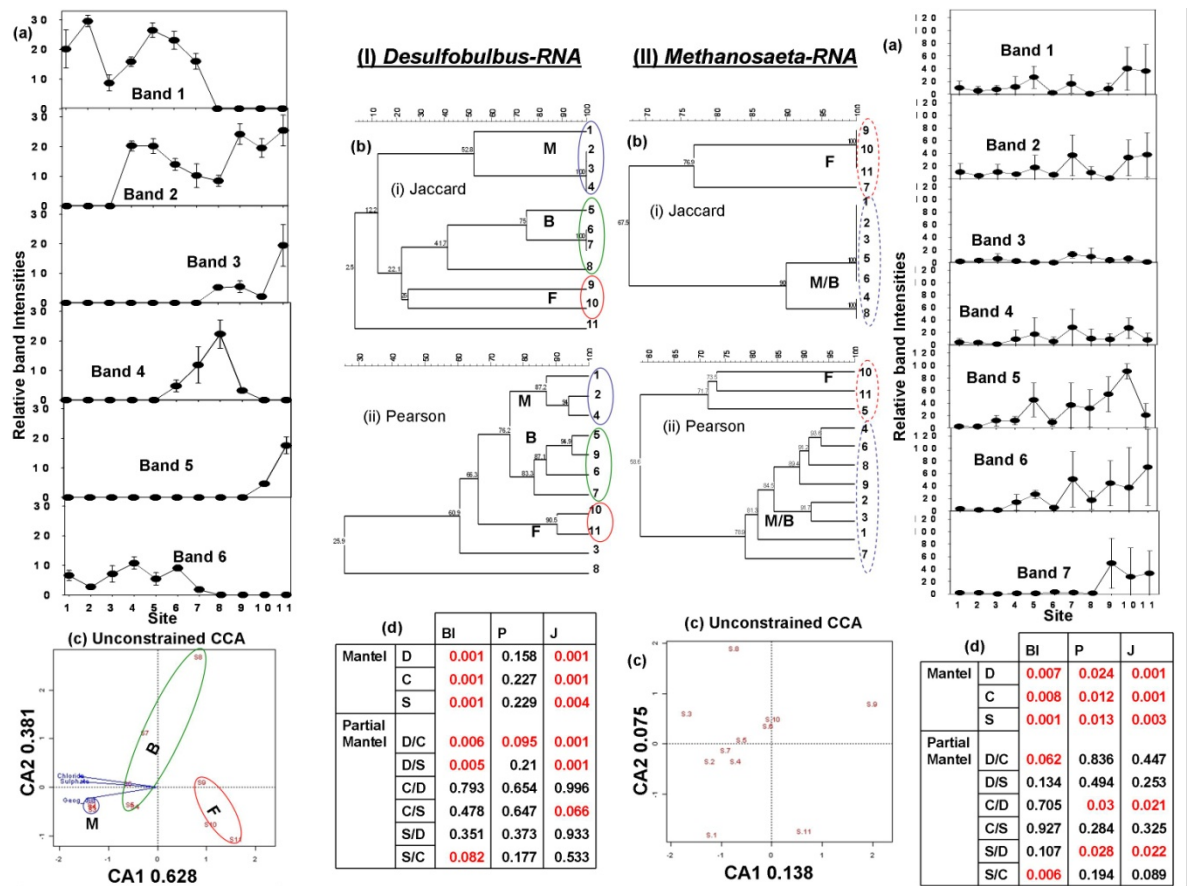
Figure 25: Analyses on RNA-DGGE for (I) *Desulfobulbus* and (II) *Methanosaeta*.

(a) Corrected mean band intensities along the 11 sites from replicated RNA-DGGE.

(b) Cluster analyses of the pooled RNA-DGGE profiles: (i) Jaccard and (ii) Pearson f

Marine (M), Brackish (B, B1, B2), Marine-Brackish (M/B) and Freshwater (F)

clusters are circled. (c) Canonical Correspondence Analysis and (d) Mantel tests

correlating environmental variables (geographic distance (D), chloride (C) and

sulphate (S)) and model specialists' genotypic distribution pattern (Band intensities

(BI), Pearson (P) and Jaccard (J)). Marine (M), Brackish (B), Marine-Brackish (M/B)

and Freshwater (F) clusters are circled.

Unconstrained and constrained CCAs showed strong clustering for *Desulfobulbus*

(eigen values >0.9) and no clustering for *Methanosaeta* (eigen values<0.5), Figure

25(a) shows unconstrained CCA for each model. Mantel and partial Mantel tests (Figure 25(b)) revealed a correlation between all the variables (geographic distance, chloride, sulphate) and *Desulfobulbus* distribution pattern ($p < 0.05$ for dissimilarity matrixes except for the Pearson), but only geographic distance appeared clearly correlated. Weaker correlations were shown with chloride and sulphate. All Mantel tests were significant for *Methanosaeta*, but no coherent correlation was found considering partial Mantel tests.

5.3. Discussion

The results of this study show a general difference in the four genera which were analysed for their genotypic distribution. Regardless of functional group (and kingdom), generalists genera are sequentially distributed, with specific genotypes restricted to specific regions of the estuary, whereas specialists are more evenly distributed and seem generally unaffected by gross environmental variables. They confirm the previous studies that were performed on *Desulfobulbus* (Carbonero *et al.*, 2010, Diniz-Filho *et al.*, 2007) and *Methanosaeta* (Chapter 4). The fact that such distinct genera are so similar in their distribution suggests that these contrasting patterns have biological and ecological meanings that may be generalised to all microorganisms. We suggest that increasing metabolic flexibility correlates with increasing adaptation potential, leading to greater niche specific differentiation and ultimately a clear biogeography. To our knowledge, such a binomial distinction has never been suggested, either for micro or macroorganisms. If supported by more data, this idea about how organisms are distributed according to their metabolic properties could provide predictive power to microbial ecology.

The genotypic distribution pattern of *Desulfobulbus* has been previously investigated in the same sediment samples (Hawkins and Purdy, 2007). In this study, DNA and RNA were extracted again, using the same method, and *Desulfobulbus* genotypes were amplified using the same nested PCR-DGGE approach. In addition to the previous study, distribution pattern of active genotypes was also assessed through an RNA-based DGGE analysis. Interestingly, the DGGE images were not a perfect match with the previous study. However the same number of bands was separated and revealed a similar sequential distribution pattern. Presumably, minor differences in the PCR-DGGE methodology and stochastic variations could explain the small differences between these two studies. Importantly, DNA and RNA based distribution patterns are very similar, as observed previously for *Methanosaeta* (Chapter 4). This implies that detected genotypes (DNA) are generally active (RNA), and thus DNA based patterns represent a satisfactory representation of metabolically significant populations. In both RNA and DNA analyses of *Desulfobulbus*, there are clearly coherent distributions of marine, brackish and freshwater types, as previously found.

The distribution of the generalist MA genus *Methanosarcina* was assessed for the first time in this study. It has a sequential distribution pattern, similarly to the generalist SRB *Desulfobulbus*, with genotypes restricted to marine, marine-brackish, brackish-freshwater and freshwater regions of the estuary. Characterised *Methanosarcina* isolates come from various environments, including marine environments, as illustrated by a marine-adapted *Methanosarcina acetivorans* strain (Sowers *et al.*, 1984). All *Methanosarcina* isolates were also shown to be able to use at least two of the methanogenic pathways, but with important differences in their substrate preference (Boone, 2001). In an estuary, acetate concentrations will be

higher in freshwater parts, and decrease due to consumption by SRB when sulphate is freely available. *Methanosarcina* are known to use C_1-methyl compounds that are more abundant in marine environments (Fitzsimons *et al.*, 2001). Thus, both the salinity gradient and the potential substrate variability along the gradient could be strong drivers of *Methanosarcina* distribution.

The monotonic pattern detected for *Methanosaeta* was described in detail previously (Chapter 4; Carbonero et al submitted). The partial correlation found with environmental factors on the RNA-based fingerprints is intriguing. It may be explained by the steady increase in band intensities that would be inversely correlated to the salinity gradient. This would fit with the assumption that decreasing sulphate concentrations, with decreasing competition for acetate by SRBs, favours *Methanosaeta* metabolic activities.

Desulfobacter distribution was also assessed for the first time. Some bands appear to fluctuate along the estuary, but no correlation was found with the environmental variables, although weak site-related clustering is observed in Figure 24.IV. Thus the only supportable conclusion is that all genotypes are found, to some extent, all along the estuary. *Desulfobacter* sp. sole electron acceptors are sulphur oxyanions, and they can use a very limited number of carbon sources. The alternative substrates to acetoclastic sulphate reduction appear to be of marginal importance in natural environments (Kuever, 2005). This implies that the *Methanosaeta* monotonic pattern is not exceptional due to their unique metabolic restrictions, and supports the primary assumption that an organism's monotonic distribution pattern is linked to how metabolically restricted the organism is.

Patterns observed for specialists are intriguing, as many factors other than gross estuarine environmental factors would be expected to play a role in shaping

distribution patterns. Arguably, the global microbial communities should change all along the estuary, as shown here at least for *Desulfobulbus* and *Methanosarcina*. Such patterns have been previously reported based on functional, not phylogenetic groups (Bass *et al.*, 2007, Bernhard *et al.*, 2005). Thus, competition on specialist individuals should also be variable and affect their distribution. It is possible that the methodology used here was not sensitive enough to detect such changes, which may be subtle, but preliminary pyrosequencing analyses showed similar distribution patterns for *Methanosaeta* (Oakley, unpublished). Thus, it would be hypothetised that all specialist genotypes observed here have very similar metabolic properties, and that genotypic diversity is maintained on a microscale at all sites. Sediments are clearly heterogeneous, any chemical measurements on a core sample are only an average of various local concentrations. Characterisation of estuarine isolates will be needed to confirm this hypothesis, and evaluate if niche-driven sympatric differentiation may occur to model specialists, but at a far smaller scale.

Generalists' biogeographical distribution patterns are more similar to what has been observed in macroorganismal ecosystems, such as the biogeographical signal observed with the genus *Gammarus* in estuaries (Spooner, 1947). Obviously, in this relatively small estuary, it is difficult to discriminate the distribution patterns drivers. All potential drivers -e.g. salinity gradient, geographic distance, varying competition- are inter-correlated and it is impossible to determine which one is crucial to the organisms. Determining such drivers is a difficult aim, as natural microbial communities and most importantly their interactions can hardly be entirely defined, and artificial communities based studies cannot really mimic this complexity. Again, characterisation of estuarine isolates may help to evaluate mainly the potential role of the chemical variables.

Studies in macroecology have defined specialists as species that are somewhat restricted to certain niches, and generalists as species that are more evenly distributed (Colles *et al.*, 2009, Julliard *et al.*, 2006). Thus, these definitions are based on the existing structure, but not on intrinsic properties of the individuals. As stated before, it is almost impossible to determine the exact structure of microbial communities, especially as they can be highly dynamic in time. Thus, the "ecological" definition of specialist/generalist was never used to describe microbial populations. In this estuarine ecosystem, the metabolic specialists would be classified as ecological generalists and visa versa. It is somewhat surprising that metabolic versatility can reduce a species' potential niches and that metabolic restriction result in larger niche occupancy. The most likely explanation is that environmental factors are not the only drivers of microbial communities' structure. The individuals' adaptative potential and competition must also play a crucial role in shaping these structures, although these two drivers are highly dependent on environmental factors. The SRBs (*Desulfobacter* and *Desulfobulbus*) are competing for electron acceptors, thus *Desulfobulbus* with various abilities (nitrate reduction in the freshwater part for example) can be selected by either of the three drivers (exclusive use of specific chemical, adaptation to certain conditions or competition advantage). On the other hand, *Desulfobacter* populations that use similar, but more limited metabolisms, seem to be largely unaffected by these drivers. Similarly, *Methanosarcina* might be selected thanks to their differences in methanogenic pathways, whereas *Methanosaeta* are also unaffected by these drivers.

In this study, the distinction generalist/specialist was clear cut, as the anaerobic models we have chosen have rather simple metabolisms. For different microbial guilds, especially aerobic ones, the line might be drawn on different scales,

separating more generalist/less generalist for example. Carney et al (2004) studied the effect of a plant diversity gradient on Ammonia Oxidising Bacteria (AOB) diversity and composition in Costa Rica. However, they did not find the gradient to have a significant effect on AOB communities, but that these communities were different among land use and nitrification potential rates. AOB are performing a restricted metabolic pathway, converting ammonium to nitrite, thus we would be tempted to classify them as specialist. Consequently, the absence of effect from the plant diversity gradient would be in support of the distribution pattern we have described. It is a very likely explanation that only drastic changes, such as anthropogenic impact, may shape patterns in such communities.

A subsequent question is why the specialists seem almost unaffected by competition. Cluster analyses have shown slight differences between the marine part and the freshwater end of the estuary. This pattern may be explained by a source/sink model (Pulliam, 1988). In this model, the source sites are the "ideal" niche for any given organism, and sink sites are places where individuals can transiently thrive. Thus, sink sites see constant immigration from source sites. We can hypothesise that *Methanosaeta* source site is the most freshwater sediments, as they can not compete with SRBs for acetate where sulphate is freely available. Conversely, *Desulfobacter* source site might be the most marine sediments, as they will face sulphate depletion in freshwater parts. Thus, specialists may have distribution patterns that exist in terms of population's size and metabolic activities. It has been shown that, SRBs dominate in marine-dominated sediments and MA dominate in freshwater-dominated sediments (Purdy *et al.*, 2003, Senior *et al.*, 1982). This trend was confirmed for *Methanosaeta* by assessing the global signal obtained by RNA-DGGE (RNA indicates that populations are metabolically active), which showed an important

increase and maximal values in band intensities for the three most freshwater sites (Carbonero, submitted).

This source/sink model seems to have some convergence with the neutral theory supported by several authors. It would appear that dominant specialist species (of sufficient numbers to appear on the DGGE) are all constantly migrating and maintain a rather stable community composition. On the other hand, sympatric differentiation and niche adaptation, mainly driven by environmental factors, seem to apply to generalist populations. Thus, these two views might not be exclusive. Attempts for generalising them to the whole microbial world might have been biased by methodology issues. For example, Ostman et al (2010) argued that microbial populations were generally invariant across several lakes. But it is likely that using a fingerprinting (T-RFLP) method on whole bacterial communities can lead to important biases due to PCR preferential amplification of the "dominant taxa". Trying to assess whole microbial communities ecologically, with very high diversity and population sizes, might be compared to an ecologist trying to assess an entire ecosystem, from the tiniest insects to the elephants and various plants, without seeing them and by pooling it in a small nucleic acid sample. However, rapidly evolving high-throughput sequencing methods will increase the ability of surveying wide communities.

For these four models, contrasting genotypic distribution patterns are unequivocal. Metabolic generalists generally appear to be subject to niche differenciation explained by gross environmental variable, where metabolic specialists' genotypic distribution are generally unaffected by these environmental variables. Metabolic flexibility of the model microorganisms appears to be a crucial driver of genotypic distribution and niche differentiation along this small-scale estuarine gradient. Thus,

generalisation of this theory to the whole microbial world may be possible on a variety of gradients.

Chapter 6 General discussion and conclusions

My PhD work had led to two distinct advances. First, I have made some crucial progress in the approach to isolate and culture *Methanosaeta*, which would have appeared unrealistic three years ago. Second, I have found solid and convergent evidence for contrasting distributions of metabolic specialists and generalists along environmental gradients.

6.1. An efficient approach for *Methanosaeta* isolation

Methanosaeta isolation had almost been forsaken by microbiologists, as too time consuming and uncertain. The approach usually applied would have been too long for my project, given that I had two years from sediment sampling to the end of the project. Physical isolation, by classic solid media means, had thus to be attempted. It has proved successful thanks to an empirical approach, of using alternative media agents. I do not think that the particular protocol I developed is a miracle solution to isolate recalcitrant anaerobes. Rather, I suppose that it is an empirical approach, of using extensive numbers of media components that should help to increase isolation successes (Carbonero *et al.*, 2010). To illustrate this, it was thought that the gelling agent itself could allow isolation on solid media. However, I was told by a *Methanosaeta* expert (Kamagata, personal communication) that he tried this gelling agent unsuccessfully.

Through these experimental adjustments, I managed to obtain a few pure isolates. Metabolic characterisation was obviously the principal goal of this side of the project. However, confirmation of purity, taxonomic affiliation and clonality happened too late to reasonably attempt characterisation. In addition, culture growth

and development have been disturbed by the methodological trials. Some isolates are thus available for further characterisation and comparison with the genotypic distribution patterns obtained.

6.2. Genotypic distribution of the specialist model *Methanosaeta*

Methanosaeta was the main model studied for my PhD project. Genotypic assessment of their distribution along the estuary was in total contrast with that of the previously studied *Desulfobulbus* (Carbonero *et al.*, 2010, Diniz-Filho *et al.*, 2007). From these previous studies, we could have expected a sequential biogeographic pattern driven by environmental factors, or at least a decrease in genotypic diversity with only salinity tolerant types at the estuary mouth. Surprisingly, the distribution pattern was monotonic, with constant genotypic diversity along the estuary. Active genotypes, as assessed by RNA, were shown to follow the same distribution pattern. It seems that the only pattern would be a quantitative one, with populations' sizes decreasing with salinity, in accordance with typical methanogenesis rates in estuaries. This pattern might be explained by *Methanosaeta* being an extreme metabolic specialist, having close to null adaptation potential. Thus, niche adaptation and/or speciation, the basis for biogeography, would be impossible, leading to an "everything is everywhere" situation. If not for large scale specialisation, the important genetic diversity must have a reason. Sediments are patchy and irregular environments, and acetate bioavailability must vary at microscales. Thus, it is possible that niche adaptation is occurring on centimetre or millimetre scale and very dynamically. These dynamics would obviously be very difficult to model or detect accurately. In addition, *Methanosaeta* distribution pattern might only have been an

exception to general biogeographic rules for microbial genera distribution. Only investigation of other models' distribution patterns, preferably whithin similar metabolic guilds, could allow us to propose a general hypothesis from these observations.

6.3. Contrasting distribution patterns of four models

Desulfobulbus has been the most extensively studied model along the estuary. Hawkins & Purdy (2007) showed the existence of a strong biogeographic pattern, with apparent seasonal shifts. In the frame of the project, this biogeography was confirmed by multiple extensive methodologies. Direct links with environmental variables were shown and suggested that sympatric differentiation had occurred. I repeated Hawkins & Purdy (2007) experimental approach, additionally analysing RNA in order to assess the distribution pattern of active genotypes. Despite fingerprinting images being different, a similar distribution pattern was obtained. In addition, the active genotypes distribution pattern was shown to reflect the presence-based distribution patterns almost perfectly, reinforcing its reliability. Strong links with environmental variables were also found, supporting the existence of types adapted to different parts of the estuary. *Desulfobacter* a specialist that belongs to the same guild as *Desulfobulbus* was shown to have a monotonic distribution pattern with constant genotypic diversity all along the estuary. Being less specialised than *Methanosaeta*, this gives convincing support to the theory that metabolic specialists might, in general, not adapt to niches on a large scale. *Methanosarcina* gives the opportunity of a very good comparison with *Methanosaeta*, being a closely related genus characterised by the wider metabolic versatility among methanogens. A

biogeographic pattern was found, similar to the one shown for *Desulfobulbus*. Correlations were found with geographic distance and salinity variables, and it is possible that salinity tolerance varies among *Methanosarcina* strains. It is also possible that substrates for the different methanogenic pathways could influence this pattern, as acetate is found in higher concentration in freshwater parts and C_1-methyl compounds are probably more typical of marine parts. The third pathway, use of dihydrogen and carbon dioxide could be favoured in parts where other substrates might be depleted.

6.4. Potential meaning of these findings in an ecological context

From all the results obtained during my PhD and other studies by Hawkins & Purdy (2007) and Oakley *et al* (2010), the emerging trend is that specialists and generalists do have contrasting distributions along environmental gradients. We still lack phenotypic data to directly prove that niche adaptation has occurred, and we have experienced that isolated strain might be marginally relevant to the genotypic studies, as some appear to be part of the rare types that we can not easily assign to any environment. We believe that the emerging distribution theory seen here has strong biological and ecological meaning. Niche adaptation and speciation are fundamental concepts in macroorganisms ecology, which have always been linked to adaptative strategy and fitness. In that light, it is arguable that generalist microorganisms possess greater adaptative potentials and more variable fitness than specialist ones. Extension of this theory thus appears reasonable, and there are some published datasets that can be explained by this theory. Parallels with macroorganisms are difficult to draw, as the specialist/generalist concept can hardly be applied to

metazoans. Resource needs and metabolic properties are not so important characteristics for marcroorganisms compared to microorganisms, and it was shown that macroorganisms distributions were shaped by a cohort of factors that influence a wide range of cellular activities.

To our knowledge, this is the first time that an ecological distribution theory is formulated by the study of and for microorganisms. This represents an important step in the attempts to build connections between microbial ecology and traditional ecology. It could provide a predictive power to microbial ecology, and help to elaborate more ecologically oriented studies on microorganisms

6.5. Perspectives

Available new *Methanosaeta* isolates will be characterised, and their genome might be sequenced in order to increase the knowledge of this genus.

To complete and support the major hypothesis, that links microorganisms' metabolic status and their ecological distribution, suggested by these results, several studies can be undertaken. Extensive characterisation of distribution patterns along the Colne estuary will be performed (Oakley *et al.*, unpublished) by high throughput sequencing methods. Similar studies as described here could be undertaken on tributaries of the Colne that are affected by the tide. Other estuaries of varying length, chemical gradient or latitude could be studied to compare distribution patterns and phylogenetic divergences of microbial populations. Other natural gradients could also be studied, with different model microorganisms being targeted.

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Appendix: Publications related to the PhD