Molecular Ecology of Cellulose-Degrading Microorganisms in Freshwater Lakes

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by Alexandre Barretto de Menezes

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Abbreviations

BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumin
CBD	carbohydrate binding domain
cDNA	complementary deoxyribonucleic acid
CFU	colony forming units
CMC	carboxymethylcellulose
C(t)	qPCR threshold cycle
CTAB	Cetyl Trimethyl Ammonium Bromide
DGGE	denaturing gradient gel electrophoresis
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNS	dinitrosalicylic acid
DOC	dissolved organic carbon
DOM	dissolved organic matter
dNTPs	deoxynucleotide triphosphates
EDTA	ethylenediamine tetra acetic acid
EW	Esthwaite Water
FISH	fluorescence *in situ* hybridization
GC	guanine and cytosine content of DNA
gyrB	DNA gyrase B gene
IPTG	isopropyl β-D-thiogalactopyranoside
ITS	internal transcribed spacer
LB	Lauria-Bertami
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pH	percentage hydrogen ion concentration
PP	Priest Pot
qPCR	quantitative polymerase chain reaction
rDNA	ribosomal DNA (rRNA gene)
RDP	ribosomal database project
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction

SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
SIP	stable isotope probing
SSU	small ribosomal subunit (16S or 18S ribosomal subunits)
SRB	sulfate reducing bacteria
TAE	Tris acetate EDTA buffer
Taq	DNA polymerase enzyme from *Thermus aquaticus*
TE	Tris-EDTA buffer
T_m	DNA melting temperature
UV	ultra violet
v/v	volume/volume
X-gal	5-bromo-4chloro-3indolyl-β-D-galactoside

Units

°C: degrees Celcius

G: 1 x gravitational force

kDa: kilo daltons

Kg: kilo gram

g: gram

Kb: kilo base pairs

Bp: base pairs

mg: milligram

µg: microgram

ng: nanogram

m: metre

cm: centimetre

nm: nanometer

L: litre

ml: millilitre

µl: microlitre

M: moles per litre

mM: millimoles per litre

h: hours

min: minutes

s: seconds

kV: kilo volts

U: unit of enzyme activity

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"In framing an ideal we may assume what we wish, but should avoid impossibilities"

Aristotle

Abstract

Cellulose accounts for almost 50% of the CO_2 fixed annually in the biosphere, and its degradation is a key step in carbon cycling. However, the ecology of cellulose degradation in the environment is poorly understood, and this is particularly true for aquatic habitats. The aim of this work was to assess the relative contribution of aerobic and anaerobic components of lake cellulolytic communities, and study their distribution in cotton baits, sediment and lakewater in two lakes of contrasting trophic status.

Micromonosporas are actinomycetes known to include cellulolytic strains and to be present in aquatic environments. Their presence in lakes has sometimes been attributed to terrestrial run-off of spores and their status as indigenous aquatic microorganisms is not established. A total of 72 *Micromonospora* isolates were obtained from cotton baits, sediments and lakewater from Esthwaite Water and Priest Pot, Cumbria, UK. A subset of strains was incubated with cotton in unsupplemented lakewater and all were capable of attacking cellulose to some extent as determined by Scanning Electron Microscopy. Filter paper degrading abilities and endoglucanase activity of all strains were determined, and phylogeny obtained by DNA Gyrase B gene sequencing. 16S ribosomal RNA gene sequencing was also applied but found to be insufficiently discriminatory within the genus *Micromonospora*. Most strains were capable of attacking cellulose but the group of highly cellulolytic isolates were equated with *M. chalcea.* A significant number of isolates (16) formed a cluster with no named species and could represent a novel centre of variation within the genus. The growth status of *Micromonospora* spp. was determined for cotton baits, sediments and lakewater using a combination of alkaline, antibiotic and heat treatments that allowed the detection of *Micromonospora* CFUs arising specifically from hyphal fragments. The presence of hyphae was established for cotton baits placed close to the lake surface, but evidence for their presence in deeper baits and in the sediments, although not in lakewater Itself, was also obtained

The relative abundance of *Micromonospora* spp. was determined by quantitative PCR, and compared to that of members of the *Clostridium* III group, which are well known anaerobic cellulose degraders occurring in freshwater sediments. In addition, the presence and abundance of *Fibrobacter* spp. and the anaerobic fungi (*Neocallimastigales*), both of which are important cellulose degraders in the rumen, was determined. The phylogenetic diversity of the anaerobic cellulose degraders investigated here was also examined by the construction of environmental SSU rRNA gene clone libraries. *Micromonospora* spp. and *Clostridium* III abundances reached 20-40% in some samples, but their numbers were not consistently high and generally low in the surface baits and in lakewater (≤ 1%). An inverse relationship existed between the two groups at depth. Whereas *Micromonospora* abundances were highest in baits above the sediments and an order or magnitude lower in the sediments themselves, the opposite distribution was observed for *Clostridium* III. Overall, *Micromonospora* abundances were higher in Esthwaite Water, whereas *Clostridium* III was more abundant in Priest Pot. This pattern is consistent with their contrasting oxygen relationships, whereas the low abundances of *Micromonospora* in the surface baits are probably due to their inability to compete with fast growing aerobic microorganisms. Fibrobacters and the anaerobic fungi were only present as trace populations in baits and sediments, and were not detected in lakewater. The phylogeny of *Clostridium* III sequences obtained from the lakes showed that they are distinct from those of rumen clostridia, whereas the *Fibrobacter* sequences formed a distinct freshwater cluster. The precise identity of the anaerobic fungi detected was unclear due to lack of sequence information for the group in SSU rRNA gene databases, but they were clearly distinct with some similarity to *Cyllamyces aberensis*, a recently described species in the *Neocallimastigales*.

In conclusion, *Micromonospora* spp. are indigenous to aquatic environments and together with *Clostridium* III represent significant components of the microbial cellulolytic community in lakes. Free living members of the *Fibrobacteres* and the *Neocallimastigales* occur in lakes and can therefore no longer be referred to as microorganisms that are found exclusively in the gut. Substrate and oxygen availability as well as the presence of competing microorganisms are significant determinants of the ecology of the lake cellulolytic microbial community, and evidence has been obtained for a distinct microaerophilic environment above lake sediments which appears to be well suited to *Micromonospora* growth.

Chapter 1 - Introduction

1.1. Overview

Energy flow is the underlying process driving the interactions that determine ecosystem function. All the energy required in the majority of ecosystems originates from photosynthesising organisms that fix CO_2 from the atmosphere, using the energy from light to extract electrons from water, and generating oxygen and biomass in the process. This biomass must be broken down and assimilated by other organisms if the energy stored in its chemical structure is to be released to rest of the community. Some components of plant biomass are readily utilizable, such as starch and simple sugars, but large proportions are of a recalcitrant nature (McCarthy, 1987).

Cellulose degradation is therefore a key aspect of ecosystem functioning and carbon cycling, and is mostly driven by microorganisms, which have the necessary physiological potential for cellulose breakdown. The ecology of cellulose degradation is not well understood, nor is the role played by the different cellulolytic species. There has been substantial research on the biochemistry of cellulose degradation and biotechnological applications of cellulolytic enzymes, but in the environment, physical and biological factors can have a marked influence in the microbial composition and activity, and organisms producing the most efficient cellulase systems are not necessarily the most relevant in a given cellulose-rich ecosystem. Factors such as nutrient availability, moisture content and oxygen status play an important role in determining the relative importance of the different microbial groups in cellulose breakdown (Bernard *et al.*, 2007).

Many microorganisms are known to be able to degrade cellulose, and the cellulolytic enzyme systems are also very diverse; this ability is found amongst members of several groups of fungi, bacteria and protozoa (Ljungdahl & Eriksson, 1985). Amongst the bacteria, one group that contains many cellulolytic species is the actinomycetes, which are usually considered soil organisms despite being also found in aquatic and marine environments. Of the actinomycetes, the micromonosporas provide an interesting target for research, as the ecological role of this group has been little studied, and are usually abundant in soils and can be found in freshwater sediments; the genus *Micromonospora* is known to contain members that are capable of cellulose breakdown. (de Menezes *et al.*, 2008).

Recent advances in molecular biological techniques have allowed a great improvement in the understanding of microbial diversity, and many new taxa are now known to exist despite having never been isolated and cultivated (DeLong & Pace, 2001). These new and powerful techniques have directed microbiological research towards accumulating greater and greater amounts of sequence data, but understanding the ecological function of different groups of organisms is still a very important task, and one which benefits from traditional microbiological approaches as well as from the use of cutting edge molecular methodologies.

In this project, traditional and molecular biological techniques were used to study cellulose degradation by the micromonosporas, their diversity and growth in freshwater lakes, and to compare their relative abundance with that of other cellulose degrading microorganisms, namely the cellulolytic clostridia, *Fibrobacter spp.* and anaerobic fungi.

1.2. Aquatic ecosystems

1.2.1 Biogeochemical cycles

1.2.1.1 Primary productivity

Unlike terrestrial environments where higher plants are the major primary producers, in most aquatic ecosystems microorganisms dominate carbon fixation. The most important mode of autotrophy is oxygenic photosynthesis (Paerl, 2007), but additional CO_2 is fixed by anoxigenic photosynthesis and chemolithotrophy (Paerl, 2007). Photosynthesis is restricted to the upper parts of the water column where light penetrates, and where a great diversity of plants, eukaryotic microalgae and cyanobacteria are responsible for the bulk of primary production. In addition to these chlorophyll a-containing organisms, some bacterial groups are able to perform oxygenic photosynthesis, but these groups have different photosynthetic pigments, the best known of which is bacteriochlorophyll a (Masin *et al.*, 2008; Salka *et al.*, 2008).

Anoxygenic photosynthesis is restricted to areas devoid of O_2 where light is available, such as in near surface anoxic sediments and laminated microbial mats, and is performed by specialised bacterial groups that use H_2S or organic electron donors as a source of electrons and protons (Paerl, 2007). This is considered to have been the prevalent form of aquatic photosynthesis before the rise of

cyanobacteria and consequent high levels of atmospheric O_2 (Paerl, 2007). Total primary production can vary hugely between different locations as a factor of nutrient availability, temperature and mixing of the water column. Very productive ecosystems are known as eutrophic or hypereutrophic and are often a result of human interference through pollution and enhanced nutrient loading. Excessive primary production is often associated with microalgal blooms and degradation of this biomass leads to oxygen depletion as the organic carbon sediments and is decomposed by heterotrophic microorganisms. The consequent onset of hypoxia is not unusual in many temperate lakes, but in extreme cases in which there is excessive nutrient loading, this can lead to mortality of the local fauna and flora (Paerl, 2007).

1.2.1.2. Microbial secondary productivity

Microorganisms also play an important role in aquatic secondary productivity. It is recognised that bacteria consume a substantial fraction of the carbon fixed by phytoplankton (20 – 40%) (Cole *et al.*, 1988; White *et al.*, 1991), and constitute a significant proportion of the biomass in a given habitat, especially in unproductive ecosystems where microbial respiration can exceed phytoplankton net production (delGiorgio *et al.*, 1997). Bacteria are responsible for converting dissolved organic matter (DOM) produced by phytoplankton into biomass, and its consumption by microzooplankton (ciliates and flagellates) is an important pathway for the transfer of DOM to higher trophic levels in the food web (Sherr & Sherr, 1987). It is estimated that the dissolved organic carbon (DOC) pool in the planet is equal in size to the total amount of carbon in the atmosphere (Cottrell & Kirchman, 2000). The DOC pool consists of a variety of compounds, such as amino acids, sugars and biopolymers, which vary in the speed at which they are utilized. Low molecular-weight compounds are used very rapidly by bacteria and consequently maintained at low concentrations in the environment (Chin-Leo, 2007). Low molecular weight components of DOC include amino acids, sugars, dimethylsulfoniopropionate (DMSP) and methanethiol. DMSP is converted by bacteria to dimethylsulfide (DMS) (Cole & Pace, 1995), which has a role in cloud formation and is an important source of sulfur in the atmosphere. Another important low molecular weight component of DOC is glycolate, released by phytoplankton during the photorespiration of diatoms (Hobbie, 1988) and which can be found in high concentrations where diatom numbers are high (Chin-Leo, 2007).

Higher molecular weight components of DOM, for example proteins and polysaccharides, are also important in the carbon flow and can support a large proportion of the bacterial population (Rosenstock & Simon, 2001).

The ability to assimilate DOM and its constituents will vary according to the different bacterial groups that comprise the aquatic microbial community, but little is known about the contribution of individual groups. Most of the research on bacterial metabolism in aquatic ecosystems has been concentrated on using methods that measure bulk community activity, such as amino acid uptake or oxygen consumption. Molecular characterization of the aquatic microbial community has shown that it is much more diverse than previously recognised, with many new taxa exclusive to freshwaters (Glockner *et al.*, 2000). A great challenge now exists in order to link phylogeny to function in these newly described groups.

1.2.1.3. Decomposition

A significant proportion of organic carbon is of a particulate recalcitrant nature and is not recycled by secondary producers in the microbial loop, sinking to the anaerobic layers and sediments of lakes where it undergoes anaerobic decomposition to two main end products: carbon dioxide (CO_2) and methane (CH_4). Anaerobic decomposition is only possible the complementary metabolism of four main groups of microorganisms: hydrolytic bacteria, fermenters, acetogenic bacteria and the methanogens (Boone *et al.*, 1993) (Fig 1.1.).

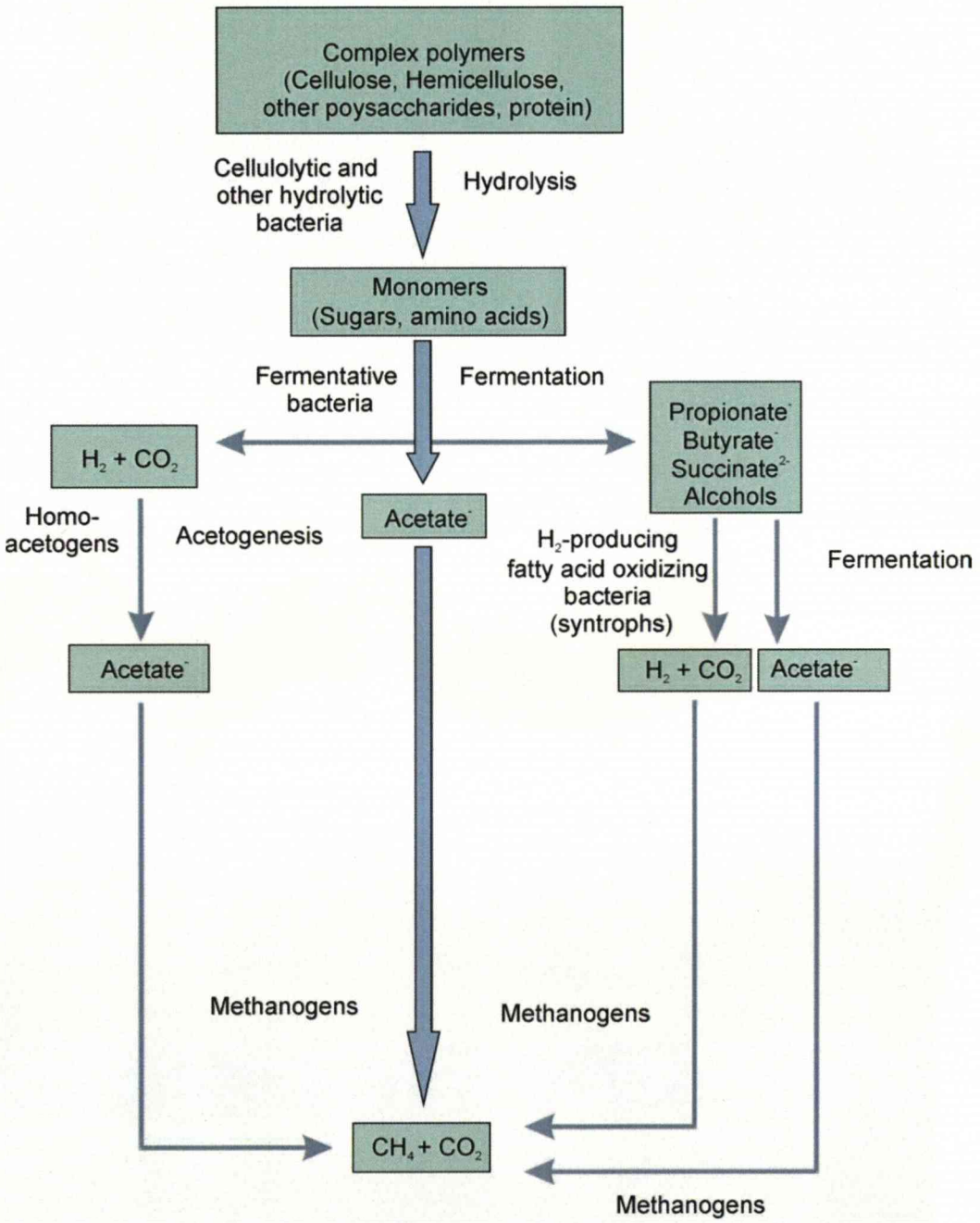


Figure 1.1. Diagram of anoxic decomposition.

Diagram based on Madigan *et al.* (2006).

The hydrolytic bacteria solubilise polymeric substrates, such as cellulose, xylans and pectin, with the production of monomers, such as sugars and amino acids; these are fermented to volatile organic acids (e.g. formic, acetic, propionic and butyric acids) and CO_2. A second group of fermenters, the proton-reducing acetogens, oxidise these organic acids (other than acetate) to acetate and H_2 or formate. The products of acetogenesis are the substrate for methanogenesis, which can proceed in two different ways: autotrophic methanogenesis, in which CO_2 is fixed with H_2 as an electron donor, and acetoclastic methanogenesis, in which acetate is consumed with water, producing methane and CO_2 (Boone *et al.*, 1993). All known methanogens are strictly anaerobic *Archaea*, such as *Methanosarcina*, *Methanococcus*, and *Methanothrix*; the association of these archaea with the acetogens is considered syntrophic, as not only the products of acetogenesis are needed for methane production, but also the removal of H_2 is necessary to make acetogenesis energetically viable (Boone *et al.*, 1993).

1.2.1.4. Methanotrophy

Methane is volatile and will move up to oxic waters where it is oxidised by methanotrophic bacteria, although approximately 90% escapes biological utilization and accumulates in the atmosphere where it can be photochemically oxidised. Methanotrophs are a unique group of bacteria in their ability to use methane as a sole carbon source (Hanson & Hanson, 1996) The methane monooxygenases, which catalyse the oxidation of methane, are remarkable in their lack of substrate specificity and will metabolise a number of compounds, including xenobiotic chemicals (Dalton & Stirling, 1982; Fox *et al.*, 1990). The methanotrophs are broadly divided into three main groups (types I, II and X) based on their metabolism and phylogeny (Hanson & Hanson, 1996). Methane and ammonia oxidisers have a number of metabolic similarities, and both groups can metabolise methane and ammonia, but to a lesser extent than the main substrate that defines each functional group.

1.2.1.5. The nitrogen cycle

Nitrogen is one of the essential elements needed for biological activity, and is often a limiting factor for biological activity in aquatic ecosystems. The key steps in the

nitrogen cycle are nitrogen fixation, denitrification, nitrification and anammox (Ward, 2007) (Fig 1.2.).

Nitrogen enters aquatic systems from the surrounding landscape, industrial processes and dry atmospheric deposition (Kelso *et al.*, 1999); nitrate is soluble and will wash in from the surrounding landscape, triggering biological activity. In certain environments, such as oligotrophic ocean basins, organic nitrogen sources are few and nitrogen becomes the limiting factor for biological activity, and nitrogen fixation is therefore important (Ward, 2007). In freshwater lakes, nitrogen fixation is normally associated with enhanced microbial growth triggered by increased phosphate loading that depletes the water column of available nitrogen. Recent studies however, have demonstrated nitrogen fixation occurring in large oligotrophic lakes (MacGregor *et al.*, 2001).

Nitrogen fixation is performed exclusively by prokaryotes, and is the enzymatic reduction of nitrogen gas to ammonium (Capone, 2001; Capone *et al.*, 2005). Nitrogen fixation is carried out by a phylogenetically diverse group of bacteria and Archaea, but the complex suite of enzymes involved are highly conserved (Zehr *et al.*, 2003). Recent advances in molecular biological methods have allowed the detection of a diversity of nitrogen fixing genes, with the implication that many more microbial groups participate in this process in addition to the major established species (Falcon *et al.*, 2002a; Falcon *et al.*, 2002b).

Another important step in the nitrogen cycle is nitrification, the oxidation of ammonium to nitrite and then to nitrate. This two-step process is traditionally attributed to small, phylogenetically defined groups of bacteria. Ammonium oxidising bacteria (AOB) are strictly autotrophic organisms, whereas nitrite oxidising bacteria (NOB) are generally autotrophic, but growth of some strains is enhanced by the uptake of small organic acids (Ward, 2007). Recently, molecular evidence has shown that members of the *Crenarchaeota* are involved in nitrification in marine environments (Schleper *et al.*, 2005; Venter *et al.*, 2004), and autotrophic archaeal nitrifiers have recently been cultivated (Konneke *et al.*, 2005), indicating that major groups involved in nitrification are still to be described in detail.

The nitrate produced by nitrification is very water soluble, and therefore tends to wash into the aquatic ecosystems where it triggers microbial growth as it is a readily utilizable nitrogen source.

Denitirification is an anaerobic respiratory process in which nitrate is used as an electron acceptor, with the production of N_2 or N_2O. The ability to perform denitrification is widespread in the microbial world, and has been described for many bacteria, and also in some Archaea and fungi (Zumft, 1997). Denitrification is an alternative respiratory mechanism, and most denitrifiers also respire using oxygen as an electron acceptor. Nitrate can also be reduced to ammonium during nitrate fermentation, and this process can exceed denitrification in some systems, e.g. in marine sediments, and unlike denitrification, does not lead to a loss of fixed nitrogen from the system.

During decomposition of compounds containing organic nitrogen (i.e. amino acids and nucleotides) ammonia is produced. In aerobic environments such as soils, ammonia is quickly recycled and converted into amino acids, but under anoxic conditions ammonia is very stable and tends to accumulate; in aquatic sediments it is the predominant form of nitrogen. Ammonia can be catabolised anaerobically in a process called anammox, in which it is oxidised with nitrite as an electron acceptor, releasing N_2 back to the atmosphere. Few organisms are known to perform annamox, and the main organism characterised for this type of metabolism are members of the *Planctomycetales* (Jetten *et al.*, 2003).

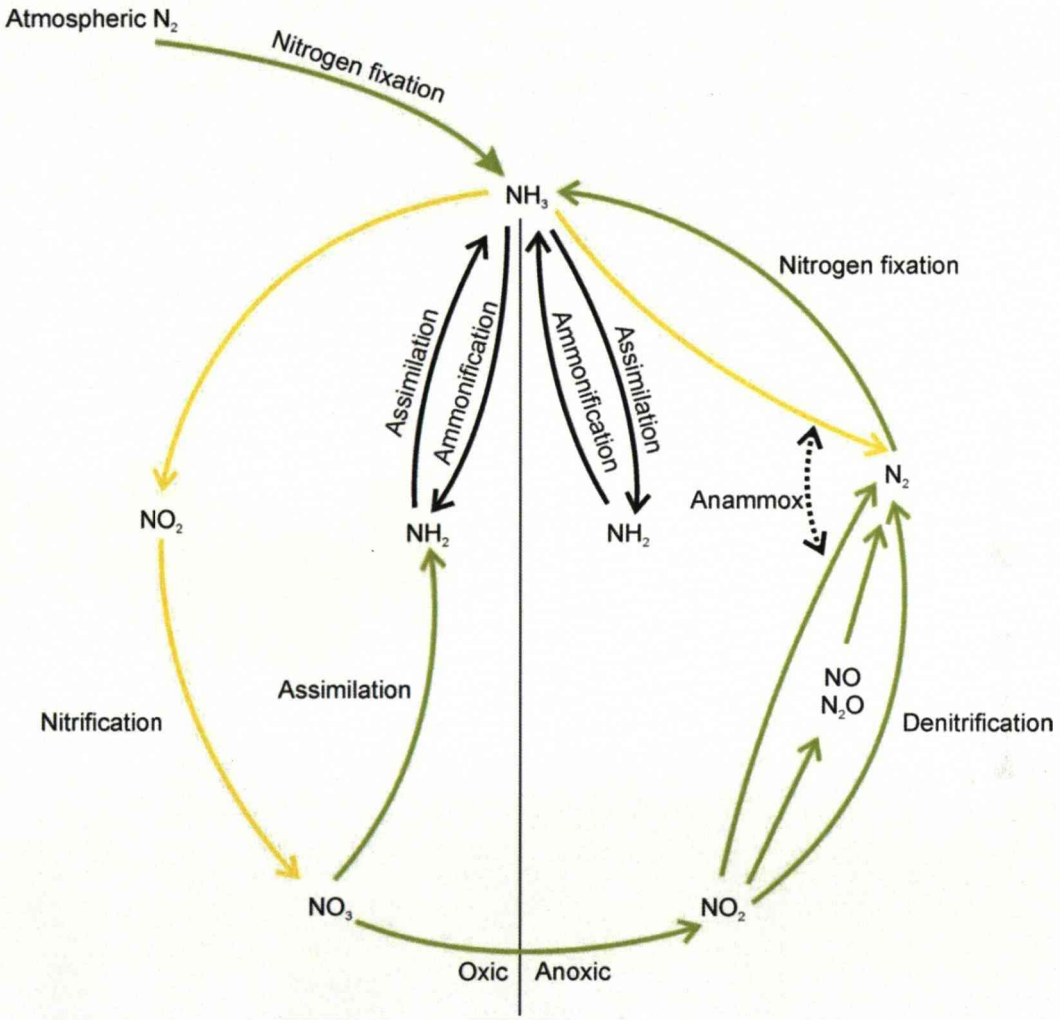


Figure 1.2. The nitrogen cycle.

Yellow and green arrows represent oxidation and reduction reactions respectively. Diagram based on Madigan *et al.*(2006).

1.2.1.6. The sulphur cycle

Sulphur consists of about 1% of the dry mass of organisms; it has structural and enzymatic functions and can also act as an electron donor or receptor in microbial metabolism (Jorgensen & Postgate, 1982). Sulphur exists in several oxidation states in nature; sulfide is the most reduced form, sulfate is the most oxidised. The main steps in sulphur cycling are: hydrogen sulfide and sulfate reduction, sulfide and elemental sulfur oxidation and reduction, and the cycling of organic sulphur compounds (Fig 1.3.).

Sulfate reduction associated with organic matter decomposition is a major mineralization pathway in anoxic environments, especially marine and freshwater sediments (Hines, 2007). Anaerobic sulfate reducing bacteria (SRB), are a phylogenetically and physiologically distinct group of bacteria consisting of several genera of relatively recent description (Mori *et al.*, 2003), of which the metabolisms are still being described. Although SRB are defined on the basis of their ability to reduce sulphur, these bacteria have a diverse metabolism, which includes the reduction of iron, manganese and oxygen, organic fermentations and utilization of various intermediate redox states of sulphur (Lie *et al.*, 1998; Lovley & Phillips, 1994). In their classical role, SRB reduce sulfate to hydrogen sulfide, providing sulfide for oxidation by sulphur-oxidising bacteria when it reaches the interface between the oxic and anoxic zones.

SRB compete with methanogens for hydrogen (Martens & Berner, 1974), and high sulfate concentrations will favour the former. Marine sediments or saline lakes are richer in sulfate than those of freshwaters, and sulfate reduction often predominates over methane production; in freshwaters on the other hand, methanogenesis usually predominates (Schink, 1997).

Biological sulfide oxidation in the presence of oxygen is performed by a diverse group of organisms, including colourless sulphur bacteria, some purple sulphur bacteria and SRB; under anoxic conditions, nitrate-respiring chemolithotrophs perform sulfide oxidation. During the oxidation of sulfide to sulfate, a variety of intermediates are formed (with valencies ranging from -1 to +5). Some of those intermediates will react chemically with organic matter forming organic sulphur compounds, but many will be either reduced or oxidised by SRB, or fermented (Hines, 2007).

The sulfide that is not used by bacteria is quickly oxidised chemically by either O_2 or Fe^{2+} in the oxic water column. When sulfide combines with iron it forms insoluble FeS and FeS_2, which gives a characteristic black colour to sediments (Vaughan & Lennie, 1991).

Anoxic oxidation of sulfide is also possible, if light is available, by the action of phototrophic sulphur bacteria (purple sulphur bacteria and green sulphur bacteria) (Visscher & Taylor, 1993). Elemental sulphur is produced by sulfide oxidation and this is further oxidised by sulphur-oxidising bacteria to sulfate and protons. Elemental sulphur can also be reduced by different groups of bacteria, such as hyperthermophilic *Archaea* (Ma *et al.*, 1994).

The sulphur cycle includes organic compounds as well as the inorganic forms discussed above. Dimethyl sulfide (DMS) is one of the most significant organic sulphur compounds in nature and it is produced mainly in marine environments as a degradation product of dimethylsulfoniopropionate (DMSP), an osmoregulatory compound found in marine algae. When DMS is released in the atmosphere, it is photo-oxidised into methane sulfonate, sulphur dioxide and sulfate, whereas when it is released in anoxic environments it can be utilised by microorganisms, and participate in methanogenesis or act as an electron acceptor for photosynthetic purple bacteria, or an electron donor for some chemolithotrophic organisms. In the latter two cases, DMSP will yield dimethyl sulfoxide, which can in turn be used as an electron acceptor for anaerobic respiration, which results in DMS (Burkill *et al.*, 2002; Kirst, 1996). Although DMS production is well documented in marine environments, it is potentially important in freshwaters where its production and degradation products can affect the odour of drinking water (Ginzburg *et al.*, 1998).

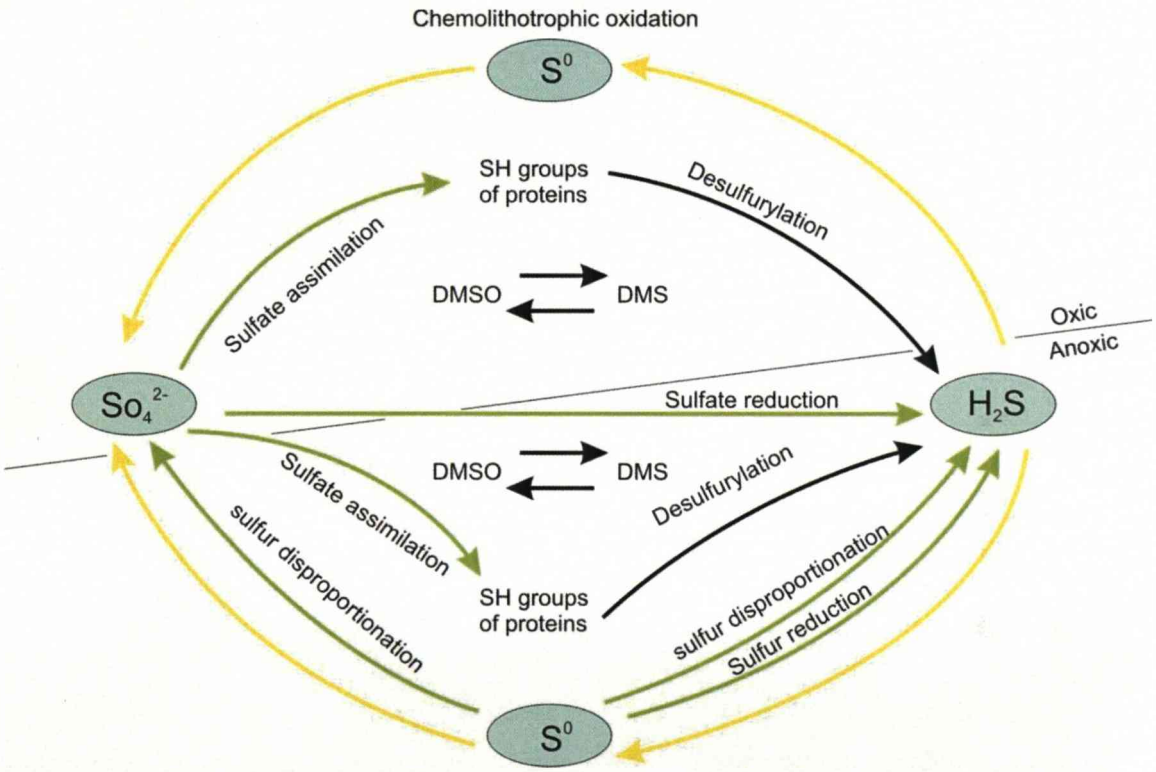


Figure 1.3. The sulphur cycle.

Yellow and green arrows represent oxidation and reduction reactions, respectively. Diagram based on Madigan *et al.* (2006).

1.2.1.7. The iron cycle

In the past, iron reduction was considered a minor hydrogen sink during fermentation, but it is now recognised that most biological iron reduction is catalysed by microorganisms that use ferric iron as a final electron acceptor during anaerobic respiration. Bacteria able to perform this type of metabolism are called Fe^{3+} Reducing Bacteria, or FeRB, and these organisms couple ferric iron reduction with oxidation of hydrogen or a short chain fatty acid, such as acetate. Most FeRB organisms are *Bacteria*, but this metabolism has also been demonstrated for *Archaea*. Iron reducing bacteria have a cosmopolitan distribution and are able to use a range of alternative terminal electron acceptors (Caccavo *et al.*, 1992; Caccavo *et al.*, 1994; Lloyd *et al.*, 2002).

Ferric iron (Fe^{3+}) reduction is common in anoxic sediments, and the ferrous iron produced is oxidised by iron bacteria in the oxic zone when water moves up from lake sediments by water column mixing. Ferrous iron is spontaneously oxidised at neutral pH in oxic conditions, so this biological iron oxidation is usually limited to the anoxic/oxic interface or to acidic waters where ferrous iron is stable. The oxidised iron then precipitates back to the sediment, where it can form ferric hydroxide deposits, or it complexes with organic compounds and remains soluble. Although iron oxides are insoluble, many iron reducing bacteria, such as *Shewanella* spp. and *Geobacter* spp., can attach and transfer electrons to these minerals (Cummings, 2007). A summarised description of the biogeochemical cycles in a lake through a seasonal cycle is presented in Fig 1.4.

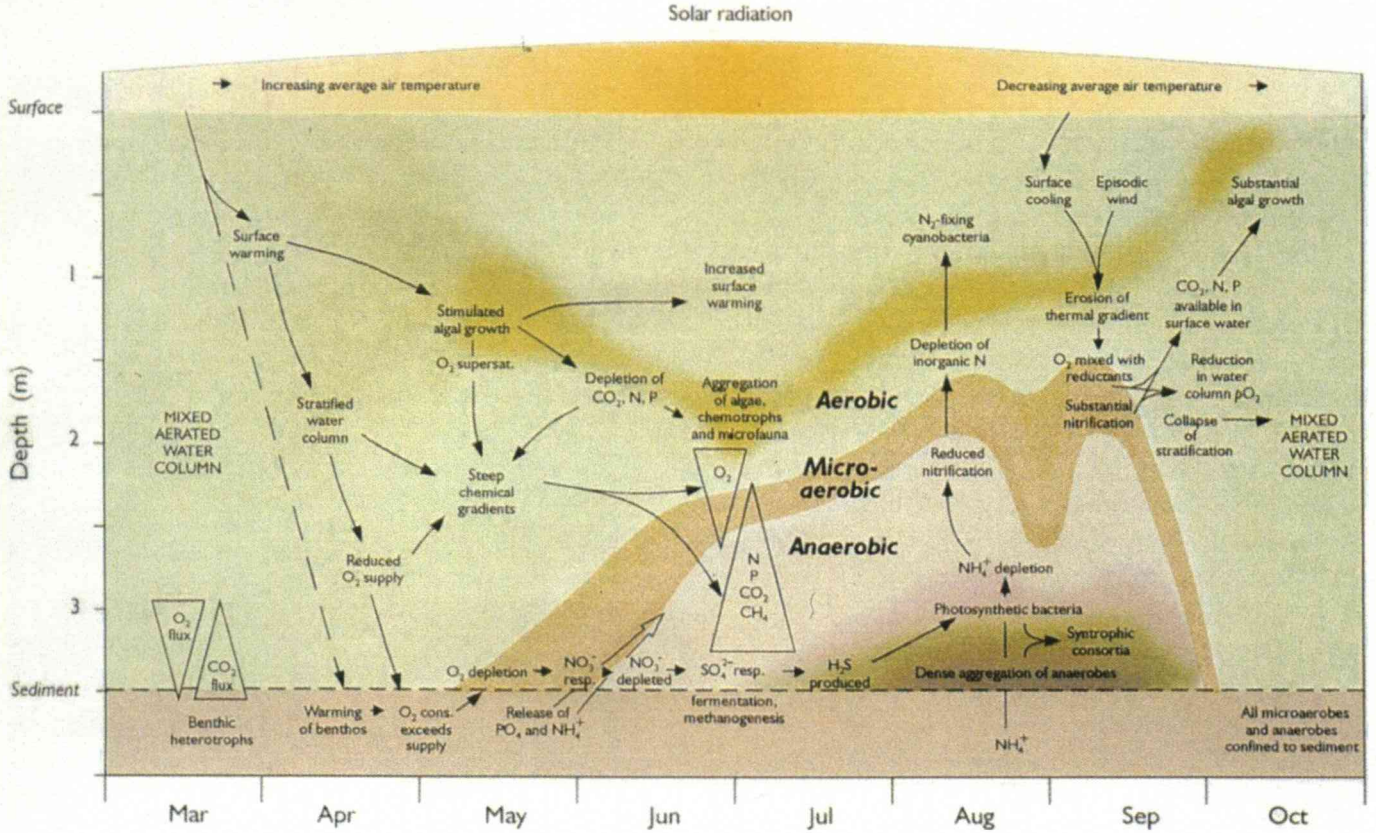


Figure 1.4. Schematic representation of biogeochemical changes in a hyperetrophic lake through a seasonal cycle.

From Finlay *et al.*(1997).

1.3. Freshwater microbial communities

1.3.1. Indigenous freshwater bacteria

With the use of traditional culture-dependent methods, planktonic freshwater bacteria were long considered similar to those inhabiting terrestrial or soil ecosystems (Zwart *et al.*, 2002). The advent of molecular techniques and their application to environmental samples allowed the detection of a much greater diversity of microorganisms in most ecosystems studied, and groups that were indigenous to freshwater environments became apparent (Glockner *et al.*, 2000). By comparing several 16S rRNA sequences available in databases available at the time, Zwart *et al.* (2002) found 14 clusters containing only sequences from water columns of rivers, lakes and ponds, and another 12 clusters that contained sequences from habitats that are close to freshwater, such as estuaries and flooded soils, but no "marine" and "soil" sequences. Four clusters contained freshwater and marine derived sequences, showing a common aquatic trait. Some of these aquatic clusters contained sequences from freshwater and coastal environments, but not from the open ocean. Of the 34 clusters described in this manuscript, only 11 had cultured representatives, and very little was known about the physiology and possible ecological role of these newly detected groups. Three of the clusters were considered to deserve particular attention by the authors: they belonged to the *Actinobacteria*, the *Verrucomicrobia* and the *Cytophaga-Flavobacterium-Bacteroides* cluster (CFB). These three clusters contained many taxa indigenous to freshwater, but no cultivated examples at the time. In addition to these, there were other major clusters belonging to the *β-Proteobacteria* and *α-Proteobacteria*. In that study (Zwart *et al.*, 2002) there was a clear separation between marine and freshwater clusters, and the authors suggested that successful transition between low and high salinity environments is relatively limited, and might be restricted to specific groups that show higher toleration to a wider range of salinities (for example, sequences clustering with the *Brevundimonas*, *Rhodoferax* and *Synochococcus* genera).

In one study, uncultured *Actinobacteria* were found to be a substantial component of the total bacterioplankton biomass (Glockner *et al.*, 2000), although most species constituted less than 1% of the bacterial numbers (Curtis *et al.*, 2002). It is thought that eukaryotic grazers selectively prey on dividing and metabolically active bacteria, and that the less abundant bacteria often show the highest cell-specific activities (Jurgens *et al.*, 1999). The high abundance of some groups, such as the freshwater *Actinobacteria* mentioned above, could be due to resistance to grazing (Hahn *et al.*,

2003; Pernthaler *et al.*, 2001), and the ecological importance of the less abundant groups could be proportionally greater due to higher metabolic rates. De Wever *et al.* (2005) demonstrated that bacterioplankton was spatially segregated in a large, tropical and permanently stratified lake (Lake Tanganika, East Africa), and that a major feature in structuring the bacterial community was the presence of the deep anoxic zones. Despite the prevalence of oxygen concentrations as a major factor in structuring the microbial population, a correlation between nutrient availability and microbial taxa was also observed, and groups mostly associated with the nutrient rich hypolimnion (below the thermocline) were observed in the epilimnion (above the thermocline) in areas where nutrient concentrations were higher. In other studies, the incubation of epilimnetic water with the addition of nutrients allowed the detection of bacterial groups normally associated with deep hypolimnetic waters (Gasol *et al.*, 2002; Lindstrom *et al.*, 2004). Some of the major freshwater bacterial groups as defined by Zwart *et al.* (2002) were not detected in this lake, and the groups detected in the hypolimnion, which included δ-*proteobacteria*, *Nitrospirae*, green nonsulfur bacteria and *Firmicutes*, were more closely related to genotypes isolated from other aquatic environments such as groundwater and acid hot springs.

1.3.2. Bacterioplankton community structure

Factors controlling the typical freshwater bacterioplankton community structure were investigated by Lindstrom *et al.* (2004), who found that pH, temperature and lakewater retention time had a significant effect on the community, even at the broad taxonomic levels investigated. Lakewater retention time had a significant effect in determining whether the lake bacterial community was of a typical freshwater nature, or had a strong terrestrial influence such as in those lakes that had short retention times. The position of the lake in the drainage system might also have an effect, as the authors provide evidence that headwater lakes contained a higher abundance of taxa with stronger soil/riverine associations.

Recently, efforts have been directed at using new methods to cultivate those taxa detected only by molecular methods. One such method is the high-throughput Micro-drop technique, in which bacterial cells are separated and encapsulated in gel microdroplets, followed by cultivation in microtiter plates. This technique allows a much higher number of pure cultures to be obtained when compared to the conventional most probable number dilutions (Bruns *et al.*, 2003). High throughput

dilution to extinction is another method that has recently allowed the cultivation of new, previously uncultivated bacterial taxa. In this way a number of isolates of typical freshwater bacterial groups with no previously known cultured representatives have recently been obtained. Gich *et al.* (2005) reported the cultivation of several strains of the typical freshwater bacteria described by Zwart *et al.* (2002), including 61 isolates that were most closely related to previously uncultured aquatic bacteria, 23 of which could be the same species as those of environmental clones based on 16S rRNA sequence similarities. Three of the isolated strains contained bacteriochlorophyll a, the first time such metabolism was described in freshwater planktonic bacteria.

In another study, Hahn *et al.* (2003) used filtration and careful acclimatization to isolate several ultramicrobacterial strains belonging to the previously uncultured freshwater actinobacteria, and reported that one of these strains was unusually resistant to predation by the flagellate *Ochromonas* sp. With the cultivation of these previously uncultured freshwater bacteria, the physiology and ecological role played by these microorganisms is beginning to be understood.

1.3.3. Organic particle associated bacteria

Aquatic microbiologists now realize that organic particles present in the water column constitute a separate ecological niche, potentially harbouring a distinct microbial community (Delong *et al.*, 1993). These particles comprise detritus washed in from the surrounding terrain, such as plant matter, anthropogenic wastes, decaying macrophytes and senescent algal blooms. Amorphous, mucilaginous aggregates can also form as a result of the coalescence of loose particles, transparent exopolymer particles (TEPs), and colloidal polysaccharides into larger pellets; these aggregates are termed marine or lake snow, and comprise a large reservoir of detritus (Alldredge & Silver, 1988).

Aggregates are often rich in organic matter, allowing a rich and active community to develop (Delong *et al.*, 1993; Paerl, 1973; Smith *et al.*, 1992). The high levels of heterotrophic microbial activity in aggregates cause the development within the aggregate of localised low oxygen or anoxic environments (Delong *et al.*, 1993), where microaerophilic and facultative or strict anaerobic bacteria grow. The close association of aerobes, microaerophilic and anaerobic bacteria creates ideal conditions for biogeochemical transformations to occur. Nutrient transformations

undetectable or insignificant in ambient waters can be detected in the aggregate (Paerl & Pinckney, 1996). Biogeochemical processes associated with aggregates include organic matter decomposition (Delong *et al.*, 1993; Smith *et al.*, 1992), nitirification (Ward, 2007), nitrogen fixation, denitrification, methanogenesis and methane oxidation (Paerl *et al.*, 1987; Paerl & Prufert, 1987; Paerl & Pinckney, 1996). Detritus is also a significant source of nutrients such as phosphate, iron, manganese and zinc (Paerl *et al.*, 1975). The transitional nature of organic aggregates, the fact that they sink and the steep oxygen gradients within are believed to benefit "opportunistic" microorganisms, which can grow quickly when resources are available, but would otherwise be rare in the bacterioplankton community (Pinhassi & Berman, 2003). Microorganisms capable of facultative anaerobic metabolism can be quite common in the bacterioplankton (Alonso & Pernthaler, 2005; Riemann & Azam, 2002), and this can perhaps be explained by the fact that those microorganisms colonising aggregates or sinking senescent algal bloom material will enter anoxic zones as they sink towards the bottom of the lakes. In contrast to the opportunistic bacteria that colonise organic aggregates, typical planktonic species do not seem to respond well to enhanced substrate levels (Straskrabova, 1983). In one study, (Schweitzer *et al.*, 2001), the main groups of *Betaproteobacteria* found in organic aggregates from Lake Constance, Germany, were more closely related to those found in activated sludge than to typical freshwater strains.

1.3.4. Phytoplankton - associated bacteria

Phytoplankton cells are also associated with bacteria, and there is evidence of specificity between the identity of the microalgae and the associated bacterial community, although other studies suggest that the main selection factor is the composition of the excreted carbohydrates and not the species of algae (Sapp *et al.*, 2007). Initial research effort was targeted at detecting antagonistic associations between bacteria and cyanobacteria, but in general these studies failed to detect antagonism (Caldwell, 1977; Lange, 1971), although more recent research has found evidence that certain actinobacteria and bacilli can attack and lyse cyanobacterial cells (Shi *et al.*, 2006; van Hannen *et al.*, 1999). Mutualisitic and symbiotic associations between microalgae and bacteria have being described, for example between heterocystous nitrogen-fixing cyanobacteria and heterotrophic bacteria (Paerl & Pinckney, 1996; Stevenson & Waterbury, 2006). These

associations may be a result of consortial exchange of metabolites and growth factors, with the cyanobacterial component providing fixed organic carbon, whereas the bacteria could be supplying the cyanobacteria with vitamins and chelators (Lange, 1971). In addition, organic nitrogen is often a limiting nutrient in pelagic (open water) environments, especially in oligotrophic lakes and in the open ocean, and bacteria could have a role in the recycling of oxidised nitrogen released by the algae, limiting the loss of organic nitrogen from the symbiotic system (Paerl & Pinckney, 1996).

1.3.5. Microbial community dynamics

Major factors regulating bacterial population size and composition are predation, viral lysis, resource availability and competition with other microorganisms. Ultraviolet radiation damage to bacterial cells might also inhibit growth in the surface of water bodies (Arrieta *et al.*, 2000; Herndl *et al.*, 1993). Many pelagic environments are deficient in nutrients, and bacteria that are able to incorporate different substrates should have a competitive advantage in these environments; however, lack of resources is not considered a major cause of bacterial mortality in aquatic plankton (Pernthaler & Amann, 2005).

Grazing of bacteria by heterotrophic nanoflagellates is often considered the dominant source of bacterial mortality in aquatic systems, but recently viruses have been recognised to have a major impact in microbial ecology. Viruses are the most abundant biological entities in the environment, and this is also true of freshwater ecosystems (Miki & Jacquet, 2008). Viruses have a dramatic effect on the microbial community in two major ways: by removing specific microbial taxa and by releasing dissolved organic matter from cells following lysis. Viral-mediated lysis may account for 5 to 50% of daily bacterial mortality in aquatic ecosystems, and there is evidence that their importance is greater in more productive ecosystems, and it can be as important as grazing pressure in bacterial mortality (Jacquet *et al.*, 2005). Lysis of bacterial cells due to viral infection is responsible for a significant release of dissolved organic matter in the environment, which is then taken up by the remaining live bacteria, enhancing microbial activity (Fuhrman, 1999; Suttle, 2007). Viral infection is specific to particular bacterial taxa, with the consequence that microbial community composition can be altered. Viral infection can also allow the coexistence of bacterial species competing for the same resources (Thingstad &

Lignell, 1997; Thingstad, 2000). Viruses can have other important impacts on microbial communities, such as inducing phenotypical changes (Weinbauer, 2004), and as vectors for horizontal gene transfer (Jiang & Paul, 1998).

Grazing by heterotrophic nanoflagellates and ciliates on bacteria has been extensively studied (Sherr *et al.*, 1992). Grazing by these protists is selective based on size and cell morphology, and intense grazing pressure can have a profound effect on bacterial community structure. Bacterial cells of 1 to 3 μm are preferably grazed, and the removal of this size class benefits microorganisms of a larger or smaller cell size. As a consequence, ecosystems in which grazing pressure is high, harbour an abundance of extremely small bacteria (Pernthaler *et al.*, 1996), and may also contain accumulations of filamentous or colonial bacteria (Jurgens & Stolpe, 1995), some of which will produce a matrix of exopolymers that protect against grazing (Hahn *et al.*, 2004). Changes in bacterial community composition in response to natural or induced microzooplankton blooms have been documented (Pernthaler *et al.*, 2004; Simek *et al.*, 1997; Simek *et al.*, 2001).

1.4. Cellulose degradation

1.4.1. Cellulose structure

Cellulose is a homopolymer of glucose of highly variable size, ranging from 500 to 14000 glucose moieties joined by β–1,4 linkages with additional hydrogen bonds between the C–3 hydroxyl group of one glucose residue and the ring of the other glucose moiety (Beguin & Aubert, 1994). Two consecutive glucose molecules form cellobiose, which is the repeating unit of cellulose, as each glucose molecule is rotated 180° in relation to its neighbour (Fig 1.5. A). Hydrogen bonding and Van der Waals forces cause the cellulose molecules to arrange in a parallel orientation and form microfibrils which are packed into cellulose fibres. The crystallinity of native cellulose varies from ca. 60% to 90% and the arrangements of the cellulose molecules are not homogeneous, with highly crystalline domains interspersed with less crystalline, amorphous domains (Fig 1.5. B) (Leschine, 1995). This heterogeneity is heightened by various microfibril surface irregularities such as twists, voids, pits and capillaries that contribute to an increased surface area. Native cellulose exists in nature in four different crystalline forms defined by x–ray crystallography, the most important of which is Cellulose I.

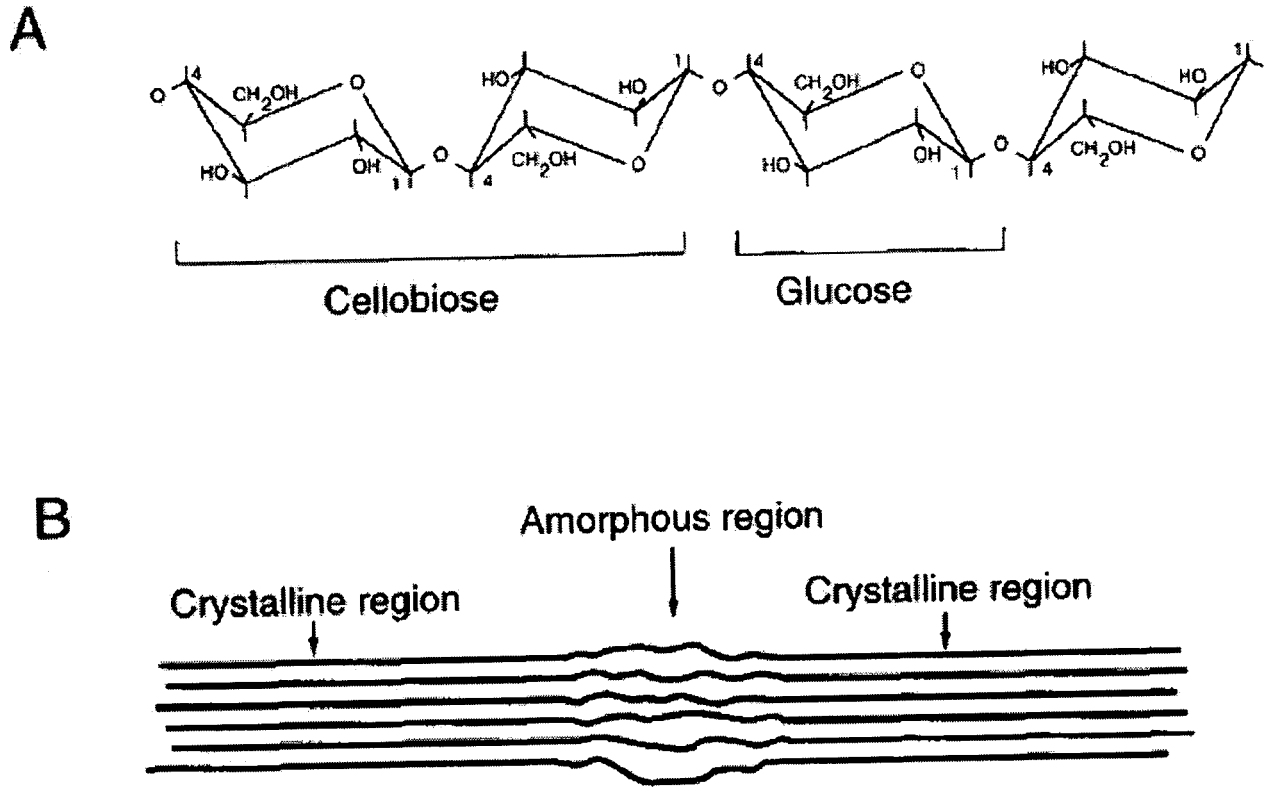


Figure 1.5. A, cellobiose and glucose molecular structure; B, diagram of cellulose fibre disposition.

From Beguin *et al.* (1994).

In plant tissues, cellulose is embedded in a matrix composed of various amounts of other compounds such as lignin, hemicelluloses, waxes, pectins and proteins. The predominance of cellulose in a particular plant tissue and its crystalline form varies amongst different plant species, tissues and growth stages; plant secondary cells walls for example tend to have a higher percentage of cellulose and a higher crystallinity than primary cell walls (Leschine, 1995). Cotton is composed of approximately 90% highly crystalline cellulose (McCarthy, 1987) and is considered its most pure natural form of cellulose. Lignin, a complex aromatic polymer, is the most recalcitrant component of plant material and is its degradation is generally the rate–limiting component in the degradation of lignocellulose. Hemicelluloses are variable linear or branched heteropolymers of β–1,4 linked sugar residues, the most common of which are xylans, mannans and galactans that take their name from the sugar forming the polymer backbone, i.e. D–xylose, D–mannose and D–galactose. Like lignin, hemicelluloses surround cellulose microfibrils and have to be degraded at least in part if cellulose itself is to be broken down.

1.4.2. Cellulases – overview

The complex nature of natural cellulose substrates, with the presence of several structural compounds in addition to cellulose, ensures that the enzyme systems deployed by microorganisms to utilize cellulose are also complex. Most cellulolytic organisms produce multiple cellulases with different functions, and in the natural environment synergy may occur between the different cellulose degrading organisms and their enzyme systems, leading to enhanced degradation. Many of these enzymes are also active on hemicelluloses and some enzymes are produced specifically to attack those compounds.

Cellulases are part of a large group of enzymes termed glycoside hydrolases which includes other non-cellulolytic enzymes that have in common the ability to cleave glycosidic bonds in oligo– and polysaccharides (Henrissat, 1998). Most cellulases have a multi-domain architecture consisting of a catalytic and a carbohydrate–binding domain (CBD), linked by a flexible peptide. These two domains have distinct structure and function. When the amino acid sequence and the structure of the catalytic and carbohydrate–binding domains from many different organisms were analysed separately, it was observed that they fall into distinct families that did not necessarily reflect enzyme function, i.e. endo– and exoglucanases were found in the

same families. This meant that details of the 3D structure of the enzyme determined its function, not the overall folding of the protein (Henrissat, 1994). When the amino acid sequence of more glycoside hydrolase catalytic domains had been determined, it was observed that sometimes enzymes that were not involved in cellulose degradation, such as xylanases or mannases, would group with cellulases in the same family. It is also striking that one organism can produce cellulases of different families and that cellulases of the same family are found in distantly related organisms, probably due to horizontal gene transfer (Quillet *et al.*, 1995). Cellulose–binding domains also fall into discrete families (Henrissat *et al.*, 1989) but these do not necessarily reflect the families of the catalytic domains with which they are associated. Indeed, multiple combinations are possible between catalytic and carbohydrate binding domains of different families (Gilkes *et al.*, 1991). Carbohydrate binding domains are also found in non cellulolytic enzymes that degrade other polysaccharides, such as xylanase and arabinofuranosidase (Kellett *et al.*, 1990).

The 3D structure of cellulases confirmed that different families of the catalytic site of glycoside hydrolases had different folding patterns and revealed that the main difference between endo– and exoglucanases was the level of accessibility of the catalytic site to the cellulose strand. For example, within one family, one exoglucanase from the fungus *Trichoderma reesei* and an endoglucanase from an actinomycete, *Thermomonospora fusca*, had a similar β/α barrel structure. In the *T. reesei* exoglucanase, the active site has a tunnel shape through which the cellulose strand must enter, while the endoglucanase contained an active site that was much more open and could therefore bind at random locations in the cellulose chain. Similar findings in other glycoside hydrolase families support this explanation (Juy *et al.*, 1992). The molecular structures of the carbohydrate binding domains have not been studied as extensively as the catalytic sites, but it is thought that the their binding properties are due at least in part to aromatic aminoacids present in their hydrophobic surface (Shoseyov *et al.*, 2006) (Fig 1.6.), such as tyrosine residues in one CBD from *T. reesei* (Johansson *et al.*, 1989).

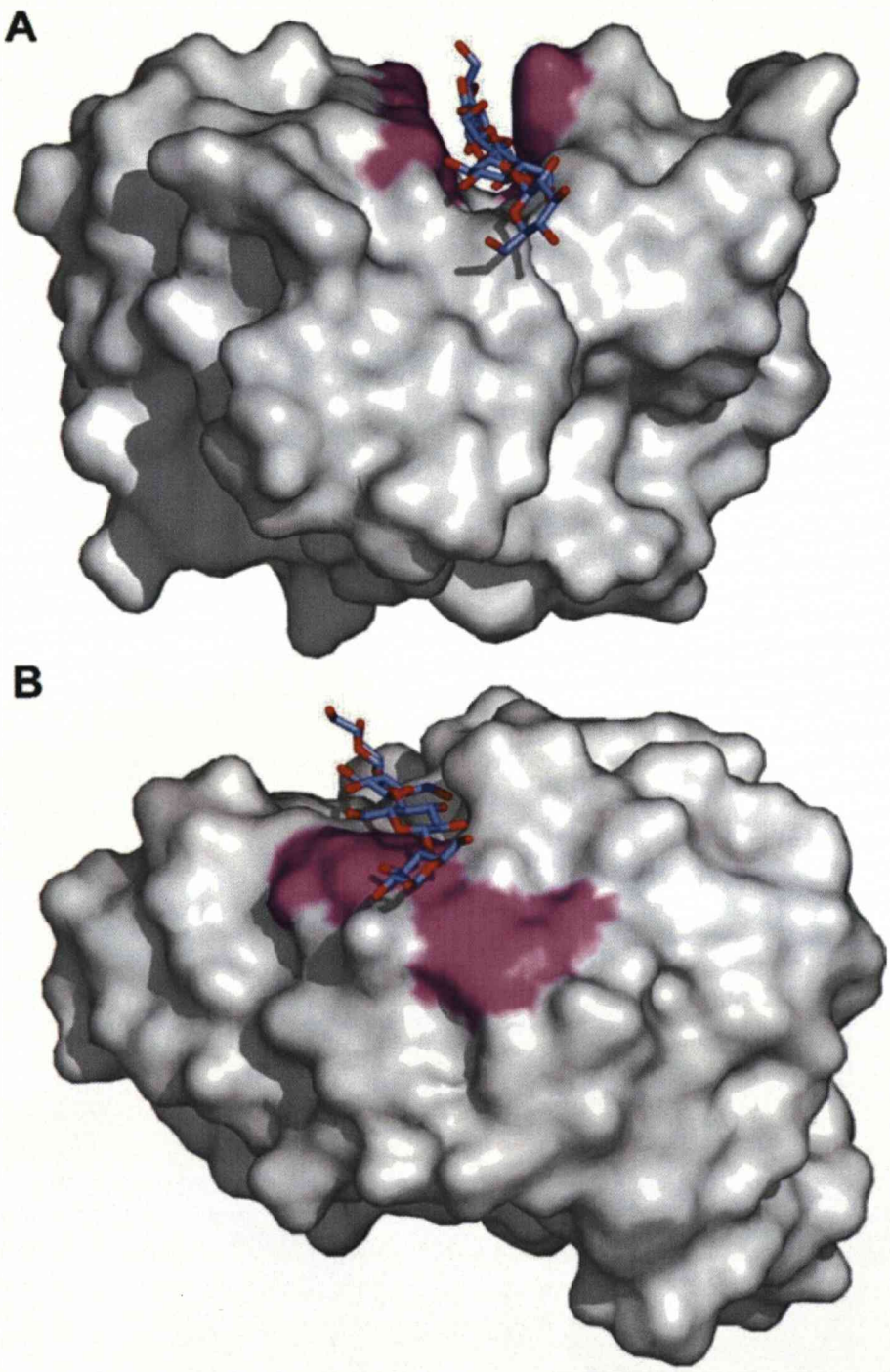


Figure 1.6. Two different carbohydrate binding domains from *Cellulomonas* spp.

A and B represent the carbohydrate binding domain attached to a cellopentaose (A) or cellotetraose (B) chain in the deep groove containing aromatic amino acids (in magenta). From Boraston *et al.* (2004).

1.4.3. Cellulosome

To increase breakdown efficiency, anaerobic cellulolytic bacteria deploy high molecular weight complexes known as cellulosomes (Fig 1.7. B) which position the cells in the hydrolysis site, minimizing the distance that breakdown products need to diffuse and thus increasing uptake efficiency. The cellulosome is composed not only of catalytic domains but also scaffolding proteins anchoring the complex to the cell walls. The cellulosome is also thought to allow concerted enzyme activity and optimum synergy between the different cellulolytic enzymes present in the complex.

1.4.4. Free cellulase systems

The described cellulases produced by most aerobic cellulolytic microorganisms are of a free, non–complexed nature. Filamentous fungi and actinomycetes have the ability to penetrate cellulose substrates with hyphal extensions and thus limit the problems related to diffusion of enzymes and hydrolysis products of cellulose; free enzymes are thus suitable for the efficient breakdown of cellulosic particles. The free cellulase system has been particularly well described for *Trichoderma. reesei*, on which models of degradation are based (Wood & Mccrae, 1972). Three major types of enzymes are identified in this model: endoglucanases, exoglucanases and β–glucosidases. It is thought that endoglucanases randomly attack β-1,4 bonds in amorphous sites in the cellulose polysaccharide chain producing oligosaccharides of various lengths with reducing and non–reducing ends susceptible to exoglucanase (cellobiohydrolase) action. Exoglucanases are considered the main depolymerisation enzymes and β–glucosidases, which are more cell–associated, facilitate the conversion of cellobiose and small oligosaccharides to glucose (Leschine, 1995; Lynd *et al.*, 2002) (Fig 1.7. A).

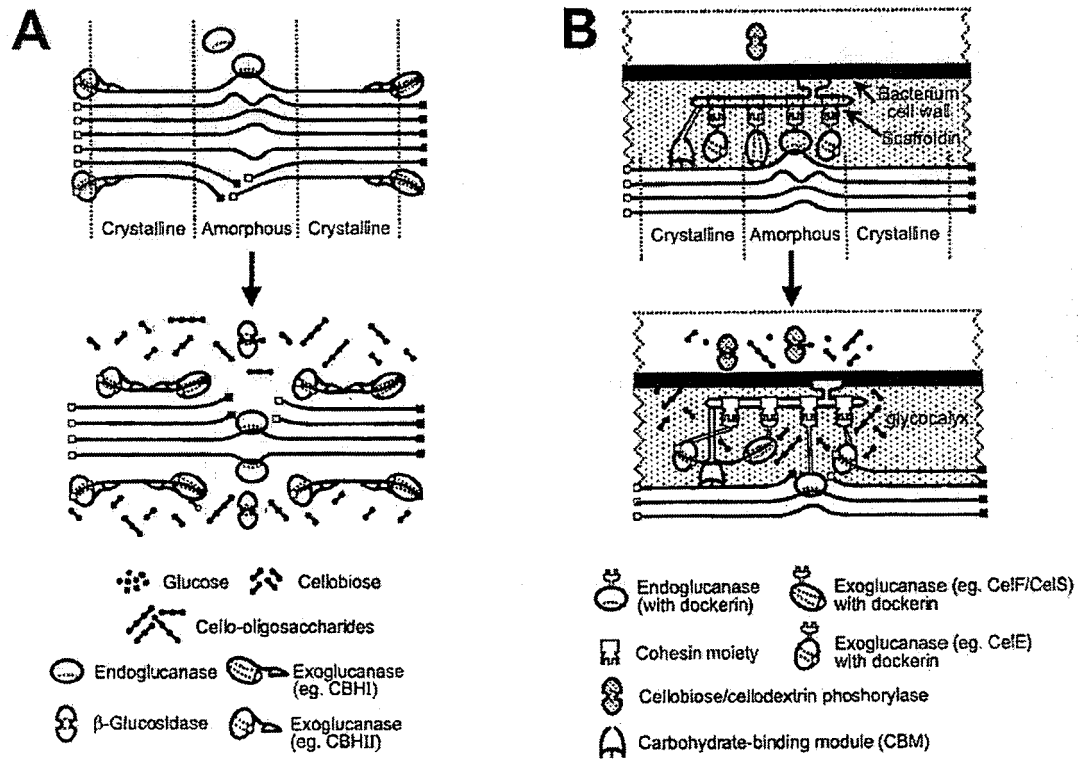


Figure 1.7. Models of cellulose degradation.

A, fungal model of free cellulase action; B, Cellulosome model of action. From Lynd *et al.* (2002).

1.4.5. Mode of action of cellulases

Due to the insoluble nature of cellulose, adsorption of cellulase to the cellulose strands is the first step to initiate cleavage. The adsorption of cellulases is provided by the CBD and it has been established that when this domain is removed from the enzyme, the cellulase loses much of its activity on cellulose, whereas its activity on soluble oligomers is retained (Gilkes *et al.*, 1988; Tomme *et al.*, 1988). As well as being responsible for the binding of the enzyme, the CBD is also capable of releasing small particles from cellulose fibres and it has been theorized that this domain increases the activity of the enzyme by promoting the dispersion of complex cellulosic substrates and releasing embedded substances. The binding of cellulase to cellulose has also been shown to be almost irreversible, which provides biotechnological applications for the carbohydrate–binding domains such as immobilizing enzymes or proteins fused with a carbohydrate–binding domain into a cellulose column (Ong *et al.*, 1989).

Cellulases hydrolyse cellulose by connecting the β–D–glucosyl residues of cellulose, usually through the action of two amino acids of the protein performing one or two nucleophile substitutions at the anomeric centre. There are two mechanisms known by which this hydrolysis take place, the retaining and the inverting mechanisms, which are differentiated on the basis of the retention or inversion of the enzyme anomeric configuration (Koshland, 1953).

A characteristic feature of cellulase systems is synergism, in which the collective activity of the different enzymes is higher than the sum of individual activities. Recombinant DNA technology has allowed the production of pure cellulases, improving the understanding of the role of individual enzymes (Mansfield & Meder, 2003). It has been shown that the action of different enzymes results in contrasting morphological alterations of the cellulosic substrate and the release of distinct hydrolysis products. In *Cellulomonas fimi* for example, endoglucanases were shown to increase relative cellulose crystallinity, indicating that these enzymes are more active against amorphous regions, whereas the two different exoglucanases present in the system could either increase or decrease crystallinity. The combined effect of all the enzymes did not alter the overall cellulose molecular structure, due to each enzyme imparting different modifications on different regions of the substrate (Mansfield & Meder, 2003). These data essentially reinforced the cellulose degradation model initially proposed by Wood and McCrae in which different cellulases act synergistically for the effective depolymerisation of cellulose (1972).

1.4.6. Regulation of cellulase synthesis

Cellulase production is considered inducible and often the highest levels of activity are observed when cellulolytic microorganisms are grown on cellulose (Ryu & Mandels, 1980; Wood, 1985). The insoluble nature of cellulose prevents it from inducing cellulase gene expression directly. This led to the generally accepted view that a low level of constitutively secreted cellulase hydrolyses cellulose to soluble sugars that induce cellulase production (Beguin & Aubert, 1994). Cellobiose can act as an inducer of cellulase systems in some organisms, but in others it has limited effect (Bhat *et al.*, 1993; Reese *et al.*, 1969). Cellulase systems are also known to be repressed by carbon catabolite repression, in which the end product of hydrolysis forms a complex with a cellular protein that interacts with the cellulase gene at the transcription level. In *T. reesei,* however some authors suggest that glucose represses cellulase secretion but not its synthesis (Bhat & Bhat, 1997). A putative regulatory protein that binds to a 14–bp palindromic sequence located upstream from endoglucanase genes in *Thermobifida* (*Thermomonospora*) *fusca* has been reported (Wilson, 1992). The different enzymes forming the cellulolytic system of an organism may be regulated co-ordinately as with *T. reesei,* but this is not always the case and in many organisms the level of transcription as well as the type of cellulase gene transcribed varies considerably according to the carbon source used for growth in many organisms (Beguin & Aubert, 1994).

1.4.7. Cellulase nomenclature

Traditionally cellulases were classified according to the producing organism and its mode of action, *i.e.* whether it was an endo–, exo– or β–glucosidase. However the main system of classification in current use is based on amino acid sequence similarity of glycosyl hydrolases proposed by Claeyssens & Henrissat (1992). This system of classification, it is argued, better reflects structural features and offers more mechanistic and evolutionary information on the different genes involved but does not take into account enzyme function. This system accommodates the 11 groups of cellulases previously described (Henrissat *et al.*, 1989; Henrissat & Bairoch, 1993) based on hydrophobic cluster analysis of its catalytic domains but has expanded and includes a total of 113 families so far (CAZy website: http://www.cazy.org/). For practical terms, however, classes of cellulases are often still referred to as endocellulases, exocellulases and β–glucosidases.

1.5. Identity and ecology of cellulose degrading organisms

The ecology of cellulose degradation is greatly influenced by environmental conditions, especially the oxygen tension and nutrient status, water content, temperature, pH, and also the nature of the cellulosic substrate. Cellulolysis occurs in terrestrial, aquatic and marine environments and is carried out by organisms of a wide range of taxonomic groups within fungi, bacteria and to a much lesser extent, protozoa; however, the number of organisms capable of depolymerising crystalline cellulose is relatively few ((Ljungdahl & Eriksson, 1985). In terrestrial habitats, a large proportion of the cellulose available is highly lignified and thus difficult to degrade; this recalcitrance together with low moisture content and nutrient patchiness of soils favours fungi and actinomycete degraders that, due to their hyphal growth, have greater physical access to the cellulose in the interior of stems, leaves and roots, and are able to overcome the heterogeneous nature of soils. There are several groups of fungi able to degrade cellulose, broadly included in three heterogeneous groups: the white–rot, brown–rot and soft–rot fungi plus anaerobic chytrid fungi. The white–rot fungi are considered to be the only microorganisms able to degrade all the components of wood comprehensibly and produce extracellular phenol–oxidases and peroxidases that improve the efficiency of the cellulose and lignin degrading enzyme systems (Ljungdahl & Eriksson, 1985; Lynd *et al.*, 2002). Brown–rot fungi are unable to degrade lignin and lack the production of β–1,4 exoglucanases but degrade cellulose and other wood polysaccharides aided through the secretion of hydrogen peroxide. Like brown–rot fungi, soft–rot fungi are generally incapable of attacking lignin and include species that are both very efficient cellulose degraders such as *T. reesei* and others that can only utilize more disordered, amorphous cellulose (Ljungdahl & Eriksson, 1985). The anaerobic chytrid fungus *Neocallimastix* is a very efficient degrader of cellulose and inhabits the rumen of mammalian herbivores. Actinomycetes also play an important role in the degradation of cellulose in terrestrial environments but their importance seems to be greater in specialized niches such as compost heaps, where ammonification increases the pH and favours their growth. There are several groups of actinomycetes capable of cellulolytic activity. The best known are members of the genera *Streptomyces, Thermomobifida* and *Micromonospora*. Other bacteria such as members of the genus *Bacillus*, *Pseudomonas* and *Cytophaga* are also known to be active against cellulose in terrestrial environments but are generally thought to play a minor role compared to that of the aerobic fungi and actinomycetes.

Aquatic habitats are generally well - mixed environments. Therefore movement and nutrient patchiness are not serious constraints to bacteria like they can be in soils. Aquatic plant biomass on the other hand is generally of much lower lignin content, favouring bacteria which are regarded as the most important aquatic cellulose degraders (Lynd *et al.*, 2002). Aquatic hyphomycete fungi such as *Alastopora*, *Articulospora* and *Cylindrocarpon* are considered by some as more important in the degradation of plant matter in streams than bacteria (Gulis & Suberkropp, 2003). However, physical conditions in a stream are different from those in a lake and in addition these studies have not focused on cellulose itself but on gross terrestrial plant matter. Most actinomycetes are considered to be soil organisms, but studies have demonstrated that some could be active in freshwater (Johnston & Cross, 1976; Warnecke *et al.*, 2005; Wohl & McArthur, 2001) and marine environments (Jensen *et al.*, 2005), and their slow growth on isolation plates might have led these organisms to be overlooked in plant decomposition studies (Wohl & McArthur, 1998). Das *et al.* (2007) used DGGE to study the diversity of fungi, bacteria and actinomycetes in decomposing leaves in streams and despite observing a greater fungal biomass, also observed a significant diversity of actinomycetes. Lower oxygen tensions in aquatic environments will act as a deterrent to fungi (Bilby, 2003) and in stratified lakes anaerobic zones develop, favouring the growth of bacteria such as clostridia. It is also possible that anaerobic fungi are active in these environments and thus also participate in the degradation of cellulose.

One important aspect of cellulose degradation in the natural environment is the interaction between different cellulolytic organisms attacking the same substrate. Because of the complex nature of cellulosic substrates, different organisms can attack different constituents of the plant material and the combined degradation resulting from a community of cellulolytic organisms is typically much greater than that achieved by one species alone (Hu & vanBruggen, 1997). However, Mille–Lindblom (2003), also described mutually antagonistic activities between fungi and bacteria in decomposing aquatic plant litter.

1.5.1. Clostridia - overview

The genus *Clostridium* was traditionally defined as non-sulfate reducing, spore forming anaerobic Gram-positive organisms with low GC genomic content. However, with the application of 16S rRNA gene sequencing, it was clear that the genus encompassed a huge diversity of organisms and that its status needed to be

re-assessed. Today, not only the genus *Clostridium* has been split into different genera, but also into different families (Wiegel, 2006). The taxonomic structure of *Clostridiaceae* species is still being elucidated, and as a consequence the different species traditionally grouped into the *Clostridium* genus are referred to belonging to 11 different *Clostridium* clusters (Collins *et al.*, 1994). The clostridial cluster I is now regarded as the *Clostridium* sensu *stricto*, it is the cluster with the largest number of species, and it exhibits relatively high levels of intracluster similarity, despite great phenotypical and physiological diversity (Collins *et al.*, 1994; Stackebrandt *et al.*, 1999). Two species that were previously considered Gram-negative (*Acetivibrio cellulolyticus* and *Bacteroides cellulosolvens*) were found to belong to clostridial cluster III, their cell walls now considered transitional in nature, with features typical of both Gram-negative and Gram-positive cell walls (Lin *et al.*, 1994a).

Many of the clostridia are found in soils, sediments, the rumen and decaying wood; others are pathogenic and some like *C. difficile*, *C. tetani* and *C. perfringens* can cause serious diseases that are often fatal.

Most members of the *Clostridiaceae* are mesophilic and grow best at neutral pH, but within the group there is a wide range of metabolisms, including psychrophilic (Alam *et al.*, 2006) to thermophilic, moderate halophilic (Brisbarre *et al.*, 2003; Oren & Oremland, 2000) and moderately acidophilic (Kuhner *et al.*, 2000) to alkaliphilic (Li *et al.*, 1993).

Some species can grow under microaerophilic conditions (*C. haemolyticum* and *C. novyi* type A), whereas others will be killed if exposed to oxygen for a few minutes (*C. novyi* type B) (Wiegel, 2006).

A wide range of carbon sources can be used by the clostridia; the genus includes saccharolytic, proteolytic (Hutson *et al.*, 1993), lipolytic (Cirne *et al.*, 2006; Gonzalez & Sierra, 1961), purynolitic (Durre *et al.*, 1981) species, organic acid utilizers (Seeliger *et al.*, 2002; van der Wielen *et al.*, 2002) and C-1 compound utilizers (Adamse & Velzeboer, 1982; Roske *et al.*, 2008) and many clostridia can fix nitrogen (Kuhner *et al.*, 2000; Line & Loutit, 1973).

Saccharolytic *Clostridium* species often produce lactate as a fermentation product, whereas some other members of the genus produce butyric acid as well as acetic acid, acetone, ethanol, propanol or butanol. Lactate produced by some species of clostridia is in turn fermented into propionic acid by other members of the genus (Wiegel, 2006).

1.5.1.1. Taxonomy of cellulolytic clostridia

Saccharolytic clostridia can degrade a variety of compounds, including starch, chitin, xylans and cellulose, but not lignin. Lactate is frequently the fermentation product of these species (Wiegel, 2006).

Cellulolytic clostridia are found in families I, III, IV, and XIV as defined by Collins *et al.* (1994). Cellulolytic members of cluster I clostridia are known to be inhabitants of anaerobic digesters, soils, sediments and the rumen (Godon *et al.*, 1997; Wiegel, 2006). A well known example of cellulose degrading cluster I clostridia is *C. cellulovorans,* upon which some models of cellulosome action are based (Doi *et al.*, 1994).

Cluster III clostridia contained 8 species when it was defined by Collins *et al.* (1994), and these were all cellulolytic. New species have been added to this group; one new strain was recently isolated from a soda lake and is a cellulolytic alkaliphilile (Zvereva *et al.*, 2006). In the RDP database (http://rdp.cme.msu.edu/hierarchy/hb_intro.jsp), species belonging to the cluster III of clostridia are classified as members of the *Ruminococcaceae* (including Acetivibrio spp.) and most species are listed within "*Rumicoccocaceae incertae sedis*". Some of the best known cellulose degrading clostridia belong to this group, such as *C. cellulolyticum*, *C. cellobioparum* and *C. thermocellum.*

Cluster IV is phylogenetically close to cluster III and includes strains from different genera i.e. *Clostridium*, *Eubacterium* and *Ruminococcus.* Many species are mesophilic and cellulolytic, and some that do not degrade cellulose attack other polysaccharides (Rainey & Janssen, 1995). Cluster XIV on the other hand was defined by Collins *et al.* (1994) as a loose association of three species of three different genera (*Clostridium innocum*, *Eubacterium biforme*, and *Streptococcus pleomorphus*), and its members varied significantly in cellular morphology, spore formation and DNA base composition. For this reason it was split into two groups, cluster XIVa and XIVb. In the RDP database, sequences of the *Clostridium* cluster XIVa are classified as members of the *Lachnospiraceae* family, mostly within the "Lachnospiraceae incertae sedis" or "unclassified *Lachnospiraceae*". *Clostridium* cluster XIVb is also mostly found within the "unclassified *Lachnospiraceae*". Cellulolytic members of *Clostridium* cluster XIVa include *C. saccharolyticum*, isolated from sewage sludge (Murray *et al.*, 1982) , and *C. polysaccharolyticum*, isolated from sheep rumen (van Gylswyk, 1980).

Weber *et al.* (2001) analysed the microbial community composition of decomposing rice straw and found that *Clostridium* was the dominant genus colonising the substrate, especially clusters I, III, IV and XIVa. Cluster I was the most abundant of the four clusters according to FISH (fluorescence in-situ hybridization) counts, corresponding to 24% of the total bacterial community, followed by clusters XIVa, III and IV, with 16, 6 and 2% respectively. In a separate study, cluster III clostridia were the most abundant of the these clusters in anoxic landfill sites (Van Dyke & McCarthy).

The clostridial cellulolytic system is very efficient and for this reason it has been well described. It was by studying clostridial cellulases that the cellulosome was first described (Lamed *et al.*, 1983). It is considered an extracellular organelle and its quaternary structure confers stability in the environment (Beguin & Alzari, 1998). *Clostridium thermocellum*, which belongs to the clostridial cluster III, was the first organism to have its cellulosome described in detail (Shoham *et al.*, 1999). It has a molecular weight of 2×10^6 to 6×10^6 Da, a diameter of 18nm and contains 14 to 50 polypeptides ranging from 37 to 210 kDa in size. It contains a great variety of β-glycanases: 21 endoglucanases, three exoglucanses, two β-glucosidases, six xylanases, two lichenases, a chitinase and one putative xylan esterase, and it is possible that more β-glycanases are still to be described for this species (Beguin & Alzari, 1998; Zverlov *et al.*, 2002).

The disposition of the cellulosomes may vary in the cell: Felix & Ljungdahl (1993) and Hon-ami et *al.* (1986) found that cellulosomes were packed into larger aggregates (polycellulosomes), forming protuberances outside the cells containing several hundred cellulosomes (Lamed & Bayer, 1986); these protuberances congregate into "contact corridors", concentrating between the substrate and the cell, and are visible under the light microscope. When *C. thermocellum* is grown under cellulase-repressing conditions, these aggregates do not form, whereas when the soluble substrate cellobiose is added as the growth substrate, the cellulosomes are packed into discrete exocellular structures; the profile of cellulosomal components themselves are known to be affected by the growth conditions (Bayer *et al.*, 1985). In *C. cellulovorans*, cellulosomes were not formed when cultures were grown on cellobiose and the cellulosomal constituents were found to be dispersed in the medium (Matano *et al.*, 1994).

The catalytic components of the *C. thermocellum* cellulosome have specialised dockerin domains that have affinity to a large, non-catalytic scaffolding component

called CipA (cellulosome-integrating protein). This is a multimodular polypeptide with nine highly conserved domains that act as receptors for the dockerin domains of the catalytic sites. The *C. thermocellun* cellulases do not have individual CBDs, and instead the enzymes rely on the CBD of CipA for attachment to the substrate (Beguin & Lemaire, 1996). Similar arrangements can be seen in other clostridial cellulosomes (Doi *et al.*, 2003) (Fig 1.8.).

The cellulosome is thought to anchor the bacterial cell to the cellulose chain, and another dockerin domain on the carboxy-terminus of CipA attaches the cellulosome to an outer polysaccharide layer of the cell envelope, but not to the peptidoglycan layer (Leibovitz *et al.*, 1997; Lemaire *et al.*, 1995; Salamitou *et al.*, 1994). This dockerin domain has very little sequence similarity to those that link CipA to the catalytic sites, and consequently the two are termed type II and type I dockerin domains respectively.

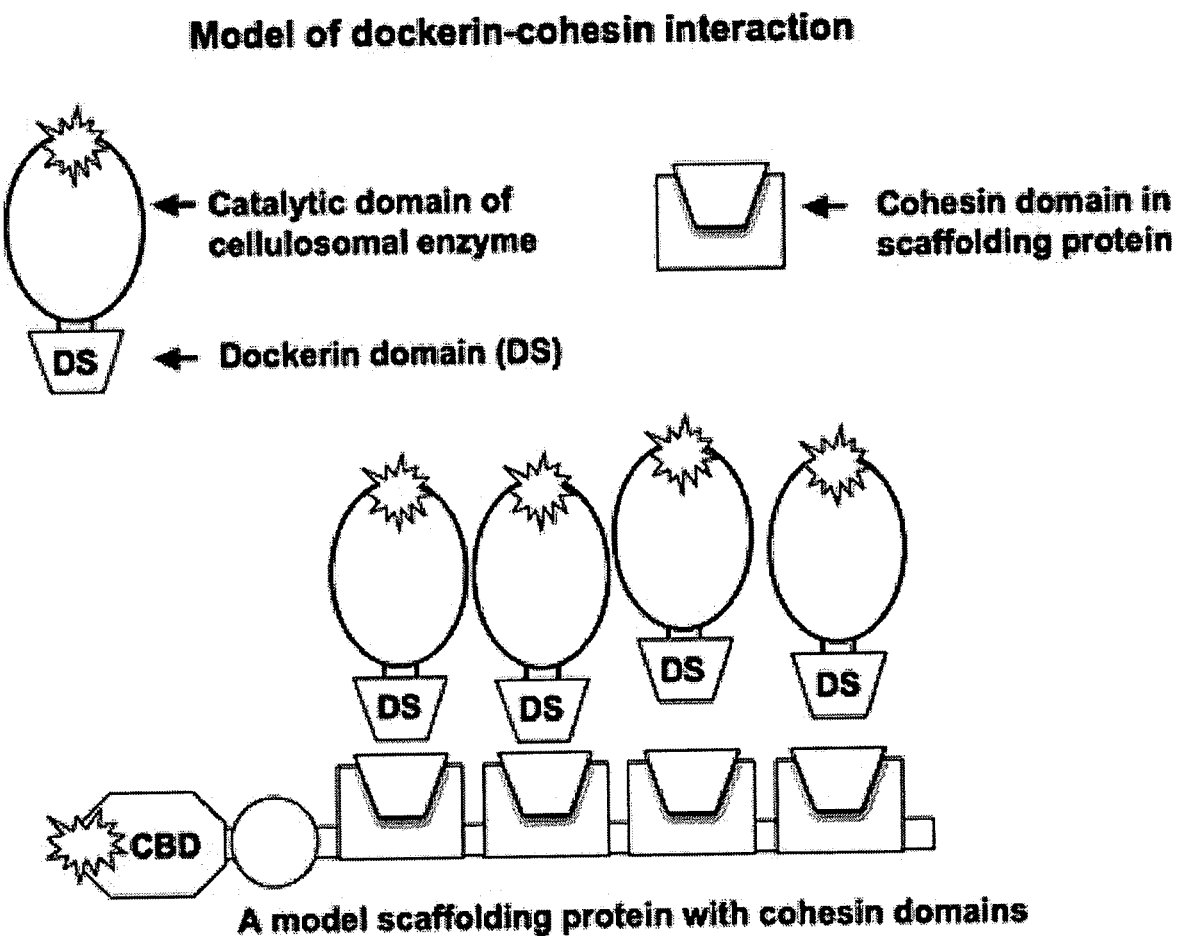


Figure 1.8. Schematic representation of scaffolding components of the *C. cellulovorans* cellulosome.

CBD, carbohydrate binding domain. From Doi *et al.* (2003).

1.5.2. *Fibrobacter*

Fibrobacteres is a phylum of bacteria with only two described species, both of which are gut commensals in herbivore animals and form an important component of the rumen cellulose-degrading community. *Fibrobacter succinogenes* was first isolated in 1947 by Hungate *et al.* (1947), and was initially classified within the genus *Bacteroides*. Few morphological and physiological traits could be used to uniquely define the genus, but all strains isolated were cellulolytic and produced succinate as a major fermentation product. The *Fibrobacteres* are strict anaerobes, do not form spores, stain Gram-negative and their cells are rod or coccobacilli shaped, but become *lemon* shaped or appear pleomorphic in older cultures. They do not posses flagella, but are thought to be able to migrate on solid substrates by gliding motility (Hungate, 1950). The genus currently comprise a second species *F. intestinalis*, but phylogenetic analysis showed a great genetic diversity between this species and *F. succinogenes*, which would support their division into separate genera. However, phenotypical basis for this division is lacking and currently both species remain classified within the genus (Lin & Stahl, 1995).

The two species of *Fibrobacter* are thought to have different ecological roles, *F. succinogenes* is a common bacterial species in the rumen, whereas *F. intestinalis* is more typically associated with the caecum of monogastric animals, such as rats and marsupials, although it has been isolated from bovine sources (Amann *et al.*, 1992). Within *F. succinogenes*, different strains are found which also differ in their ecological role, such as which animal species they inhabit and their diet; one of these strains is mostly associated with non-ruminant animals, as is *F. intestinalis* (Shinkai *et al.*, 2007). *Fibrobacteres* have also been found in the guts of higher (wood eating) termites, where they comprise one of the two major cellulolytic groups together with *Treponema* species (spirochaetes) (Warnecke *et al.*, 2007). Phylogenetic analysis showed that these recently described termite-gut fibrobacters are only distantly related to *F. succinogenes* and *F. intestinalis*, and were classified within 3 new candidate orders within the *Fibrobacteres* phylum (Warnecke *et al.*, 2007).

Recently, molecular evidence has challenged the notion that *Fibrobacter* species are strictly gut bacteria. Culture-independent analysis has obtained evidence for their presence in other anaerobic environments, such as landfills (McDonald *et al.*, 2008), and an acid impacted lake (Percent *et al.*, 2008), and in one study, a cellulase gene was cloned from DNA from soda lake sediment, and this gene had its

closest relative in a *Fibrobacter succinogenes* endoglucanase (Grant *et al.*, 2004). Many of these new environmental clones were quite divergent from the described gut *Fibrobacteres* and introduced new centres of variation within the genus (McDonald *et al.*, 2008).

Due to the importance of these organisms in the nutrition of livestock animals, the cellulolytic system of the *Fibrobacteres* is relatively well characterised. Cultured *F. succinogenes* strains produce at least 33 glycoside hydrolases that may be involved in cellulose degradation (Qi *et al.*, 2007), and some of the cellulolytic enzymes have been purified and characterized (Huang & Forsberg, 1988; Malburg *et al.*, 1997; Mcgavin & Forsberg, 1988; Miron & Benghedalia, 1993a; Miron & Benghedalia, 1993b). As with other cellulolytic enzyme systems, synergy has been documented for the different cellulose degrading enzymes of *Fibrobacter* (Qi *et al.*, 2007) and it has been shown that degradation of crystalline cellulose is very low when disrupted cell extracts or dead cells are used in the assays, whereas living cells can grow well on this substrate (Bera-Maillet *et al.*, 2004). It has been proposed that, in pure cultures, disruption of the crystalline structure of cellulose and not the hydrolysis of the glucan strands by endoglucanases, is the growth limiting factor when cultures are grown on crystalline cellulose (Huang & Forsberg, 1990). The cellulase enzyme systems in these organisms seem to be constitutively produced, although cellobiose and glucose seem to repress cellulolysis, as observed by a lag in the digestion of cellulose. The cellulolytic enzymes seem to be distributed in patches in the cell surface of these organisms, resembling the polycellulosomes of clostridia (Huang & Forsberg, 1990).

1.5.3. Neocallimastigales

The *Neocallimastigales* comprise the only known group of true anaerobic fungi, and as with *Fibrobacter*, are considered to exclusively inhabit the guts of mammalian herbivores. Interest in this group has grown significantly since their first description by Orpin *et al.* (1975) due to the discovery that they comprise an important component of the rumen cellulolytic community, and that they produce some of the most active cellulases described to date (Wood *et al.*, 1986a). They have been isolated from a variety of animals, both ruminant and hind-gut fermenting herbivores (Trinci *et al.*, 1994), including bovines, ovines, equines, elephants, marsupials and even rodents (Teunissen & Dencamp, 1993). The colonisation of the gut by

anaerobic fungi requires a high fibre diet and a capacious organ for fermentative digestion (Heath, 1988a).

The anaerobic fungi belong to the phylum *Chytridiomycota*, one of the four phyla of true fungi, and are the first and only fungal inhabitants of mammalian intestines described to date. Initially, *Neocallimastix* was thought to be a flagellate, as the first description of the genus involved its zoosporic stages (Orpin, 1975); subsequently, its vegetative hyphal stage was described, and two more species of presumed flagellates were shown to be chytrid fungi, *Pyromyces communis* and *Sphaeromonas communis* (Orpin, 1976; Orpin, 1977). Since their discovery, the group has grown to include more genera, *Orpinomyces*, *Anaeromyces*, *Caecomyces* (former *Sphaeromonas*) and the more recently described *Cyllamyces* (Ozkose *et al.*, 2001), and all are strongly cellulolytic.

The anaerobic fungi exhibit a life cycle involving an alternation of motile and sessile stages, both of which are asexual (Fig 1.9.). Although a sexual stage is still to be fully described for any of these organisms, haploid sporangia have been described in pure cultures of *Neocallimastix* spp (Teunissen & Dencamp, 1993). Zoosporogenesis in monocentric species can be triggered rapidly by soluble components of the host's diet, and this feature is considered an important adaptation for the rapid colonization of incoming forage (Heath, 1988b).

The strict anaerobic nature of members of the *Neocallimastigales* presented a problem to researchers trying to understand how it could be transmitted from one animal to another. Possible survival structures consisting of diploid melanised sporangia have been found in pure cultures of some species, and the presence of these in faecal smears support the hypothesis that this could be the strategy for transmission of these organisms (Wubah *et al.*, 1991).

Despite the general ignorance of the life cycle and biology of anaerobic fungi, significant research has been conducted on their cellulolytic system. Wood *et al.* (1986b) compared the extracellular cellulolytic enzyme complex produced by a strain of *Neocallimastix frontallis* to that of *Trichoderma reesei*, traditionally considered one of the most active cellulose degrading organisms, and demonstrated that the anaerobic fungus was much superior in solubilising crystalline cellulose when culture filtrates were obtained from a co-culture with a methanogen that removes hydrogen produced during fermentation. In these conditions, culture filtrates could solubilise up to 98% cotton cellulose in 72 hours (4 g in 800 ml

cultures), compared to 16% when grown axenically. More research into the cellulolytic system of the anaerobic fungi revealed that, unlike in aerobic fungi, a large proportion of the cellulase activity produced by these organisms was aggregated into large complexes similar to those of the clostridial cellulosome (Ali *et al.*, 1995; Teunissen *et al.*, 1992; Wilson & Wood, 1992a; Wilson & Wood, 1992b). In the same study, it was demonstrated that the majority of the cellulases produced by this organism do not have carbohydrate-binding domains, and it was postulated that like in the *C. thermocellum* cellulosome, those enzymes would be associated with another protein that posses the CBD and mediated the affinity of the whole complex to the substrate. Dijkerman *et al.* (1997) also found that the cellulase activity of a different *Pyromyces* strain resided within the high molecular mass complex, which was able to completely hydrolyse Avicel (microcystalline cellulose that has been partially hydrolysed with acid to produce a highly crystalline form of cellulose), whereas lower molecular weight fractions had very limited impact on this substrate. Upon purification and SDS-PAGE analysis of the high molecular mass complex, at least ten endoglucanases and one β-glucosidase bands were observed. A set of up to ten other polypeptides was observed in the partially purified complex, and these had no endoglucanase or β-glucosidase activities; it is possible that some of these could have exoglucanase activity or a scaffolding function, which was not determined by the authors (Dijkerman *et al.*, 1997). Ljungdahl *et al.* (2008) described the cellulosome of an *Orpinomyces* sp. showing that the organization of cellulases in a high molecular mass complex is widespread within the *Neocallimastigales*, and also that each catalytic domain found in the system had a non-catalytic domain attaching it to the cellulosome. The authors also described how the fungal cellulosomes arrange themselves into polycellulosomes of approximately 100 million Da, and contain ca. 20 hydrolytic enzymes.

Anaerobic fungal glycoside hydrolase genes are more homologous to bacterial sequences than to those of aerobic fungi, and it has been suggested that those genes were transferred from bacteria to fungi via horizontal gene transfer. The rumen environment can potentially favour HGT due to the high microbial activity, high concentration of bacteriophages and close proximity of organisms attaching to substrate particles. Of the bacterial glycoside hydrolase gene sequences analysed, those of *Fibrobacter succinogenes* were considered to be the closest to the anaerobic fungal sequences (Garcia-Vallve *et al.*, 2000).

The high activity of anaerobic fungal cellulases ensured that these enzymes have been studied for their potential biotechnological application, and reports of recombinant anaerobic fungal cellulases have been published (Li *et al.*, 2002; Liu *et al.*, 2005; Liu *et al.*, 2008), as well as studies on their applicability in commercial conversion of biomass and improved livestock nutrition (Cheng *et al.*, 1999; Selinger *et al.*, 1996).

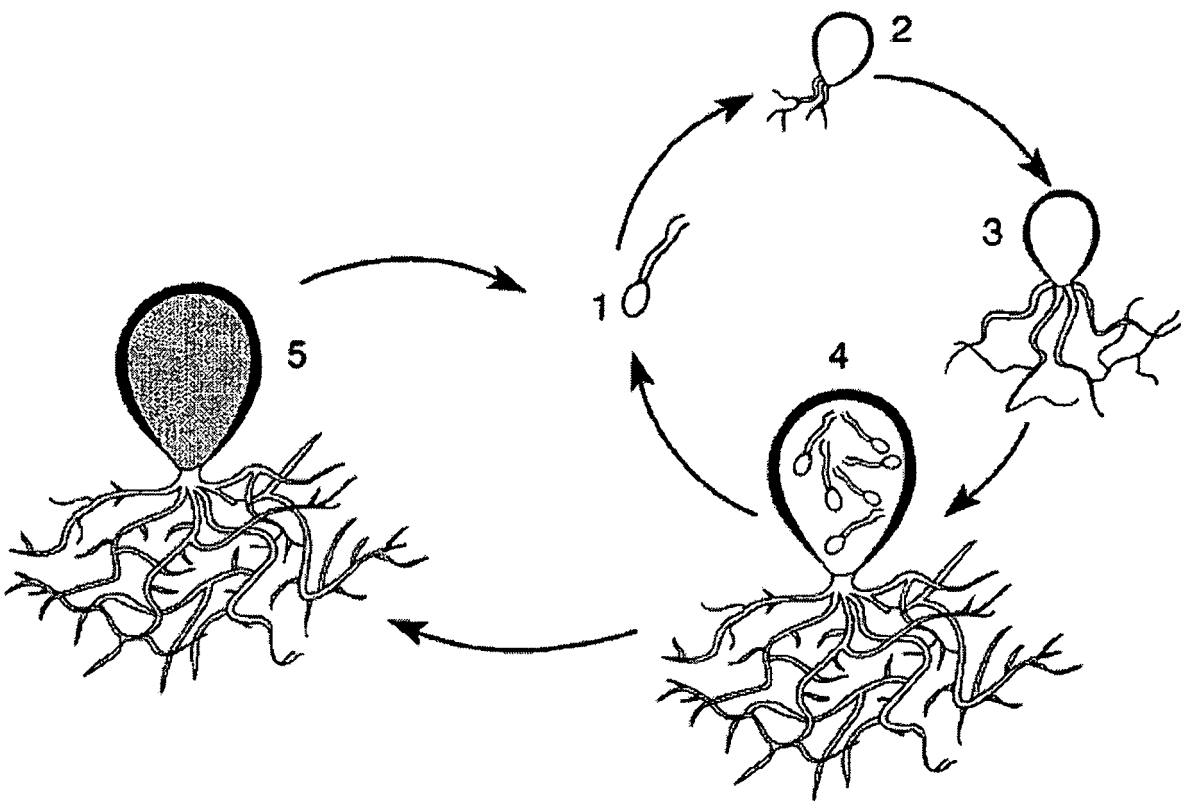


Figure 1.9. Anaerobic fungi life cycle.

1, free swimming zoospores; 2, zoospores loose flagella and germinate upon attachment to plant substrate; 3 mature thallus; 4 zoospore production; 5 survival stage. From Teunissen and Dencamp (1993).

1.5.4. Actinobacteria - overview

The *Actinobacteria* are one of the major groups within the domain *Bacteria*, and comprise 5 subclasses and 14 suborders (Ventura *et al.*, 2007). They are Gram-positive bacteria with a high GC content (ranging from 51 to 70%, with the exception of one obligate pathogenic species). A wide variety of morphologies and lifestyles can be found in the phylum, and a great metabolic diversity; the best known *Actinobacteria* are the members of the order *Actinomycetales*, the often hyphal organisms common in soil such as the genera *Micromonospora*, *Streptomyces* and *Nocardia*. With the recent advances in culture independent microbial ecological techniques, many new, uncultured taxa have been found, and the *Actinobacteria* is now known to be a major component of freshwater bacterioplankton, and is also found in marine environments where it can be locally significant, such as in the sponge microflora (Cassler *et al.*, 2008). The *Actinobacteria* group also contains important pathogenic species, such as *Mycobacterium tuberculosis, M. leprae* and *Corynebacterium diphtheriae*.

Actinomycete is the common name given to members of the order *Actinomycetales*. One of the defining characteristics of the actinomycetes is the complex morphology and life cycle, which in many ways show similarities with that of filamentous fungi. Many actinomycetes can grow in a filamentous mycelial form, and in addition produce spores, either borne singly, in clusters, or within highly differentiated sporangia. Until the 1950s, actinomycetes were considered to have affinities with both fungi and bacteria, but closer examination of their fine structure and chemical composition demonstrated their unequivocal prokaryotic cell biology. Actinomycetes have many attributes that have ensured the group receives considerable attention and is studied as a distinct group from other bacteria. Pathogenesis in humans, animal and crop plants, and most importantly the production of a great diversity of secondary metabolites with antibiotic activity, demonstrate the huge importance of these organisms. The phylogenetic structure of the actinobacteria is shown in Fig

1.10.

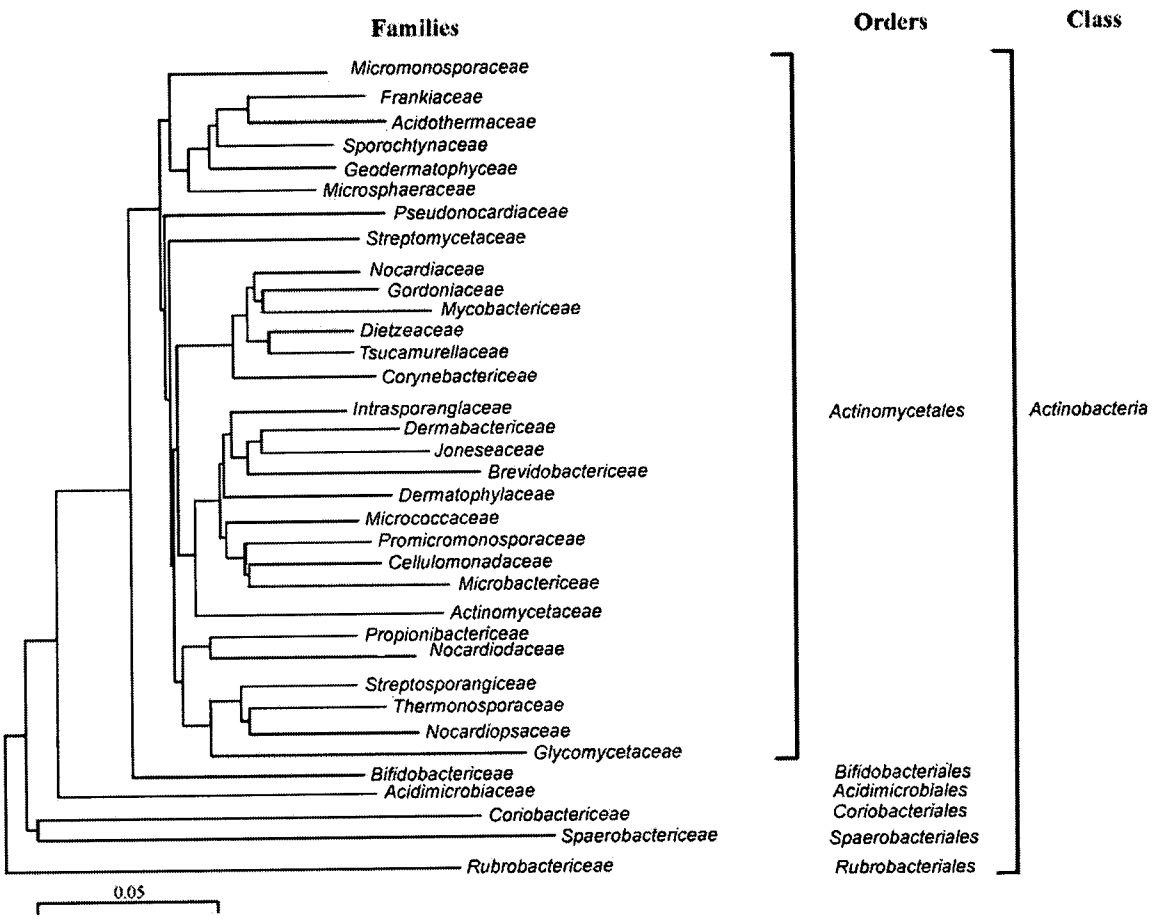


Figure 1.10. Actinobacterial 16S rRNA phylogenetic tree.

Scale bar represents 5 nucleotides. From Ventura *et al.* (2007).

1.5.4.1. Actinomycete morphology

A remarkable morphological diversity is found within the actinomycetes; some exhibit a rod or coccoid morphology, such as *Cellulomonas* spp., whereas others have attained a considerable morphological complexity for prokaryotes, with the development of filamentous hyphae that can differentiate into specialised structures. Most actinomycetes grow by apical hyphal extension, which is usually monopodial; the hyphae can be extensive or fragment into short filaments, such as in *Nocardia* spp., or rods (*Rhodococcus* spp.). Fragmentation of hyphae is usually the result of septation and disarticulation and is influenced by environmental factors, often occurring at the end of the growth cycle and allowing the dispersion of propagules. Species that undergo hyphal fragmentation as a dispersion strategy typically do not sporulate, or do so in limited amounts (Locci, 1983). Many more actinomycetes are spore formers and produce non-fragmenting mycelia that develop from the growth extension of a single propagule, the whole colony remaining in continuity (Locci, 1983).

Two forms of hyphae can be distinguished in actinomycetes: aerial and substrate hyphae. The substrate hyphae represent the active, vegetative part of the mycelium, whereas the aerial hyphae develop later during colony growth as vertical filaments arising above the substrate. The aerial and substrate hyphae are different in their morphology, structure and ontogeny (Ensign, 1978; Kalakoutskii & Agre, 1976), and the aerial hyphae is generally considered an adaptation to dispersion in terrestrial environments as, unlike substrate hyphae, it is hydrophobic (Higgins & Silvey, 1966), and often differentiates to spores or sporangia. Hyphae will aggregate in some species of actinomycetes to form more complex structures such as pseudoparenchymatous sclerotia, which contain substantial quantities of storage material and presumably have a survival function (Ganju & Iyengar, 1974; Lecheval *et al.*, 1973). Actinomycete cells may contain a variety of inclusions depending on the organism, their age and growth condition. Polyphosphate granules and lipid globules are a very characteristic cytoplasmic inclusions, and polysaccharide storage granules are also found (Locci, 1983).

Actinomycete spores are formed from pre-existing hyphal elements that go through a process of differentiation, septation and disarticulation. In some cases, the new cell wall is formed between the cytoplasmic membrane and the wall of the parent hypha, but never wholly within the cytoplasm, as it is with true endospores produced by *Bacillus* spp. and *Clostridium* spp. Actinomycete spores can be borne singly or in

chains of 2 to more than 50, and in some cases are enclosed in a spore vesicle (Cross, 1970). Some actinomycetes such as *Actinoplanes* spp. produce motile spores, but the majority are non-motile. Upon maturation, spore walls thicken to about 30 to 50 nm in diameter, conferring some resistance to heat and dehydration, but compared to true endospores, actinomycete spores are relatively unspecialised compartments of hyphae (Locci, 1983). Actinomycete spores are generally ovoid or spherical in shape, but in the genus *Micromonospora* they have warts or spines on the surface. Other genera such as *Streptomyces* may also have spore surface ornamentation, but in this case due to the presence of a fibrous sheath (Williams *et al.*, 1972).

1.5.4.2. Actinomycete genome organization

The huge importance of some members of the *Actinomycetales* in industry and medicine ensured that the genome sequences for some strains are now available. Of the mycelial actinobacteria genera, *Streptomyces* in particular has received most attention, but also strains of *Frankia*, *Thermobifida* and *Salinispora* have had their genomes sequenced. The comparison of the genomes of the first three of the listed genera has shown a similarity in organization congruent with the shared hyphal organization (Ventura *et al.*, 2007). The streptomycete chromosome is very large for a bacterium (8 to 10 Mb), being twice as large as that of *E. coli*, and harbouring more genes than *Saccharomyces cerevisae*, whereas *Frankia* chromosomes vary significantly in size (5.5 to 9 Mb) and that of *Thermobifida fusca* is only 3.64 Mb. The only sequenced *Salinispora* genome contains approximately 5 Mb of DNA (Udwary *et al.*, 2007). The streptomycete chromosome, like that of some *Micromonospora*, *Actinoplanes* and *Nocardia*, is linear (Redenbach *et al.*, 2000), which is not the case with the genome-sequenced strains of *Salinispora*, *Frankia* and *Thermobifida* (Udwary et al., 2007; Ventura et al., 2007). It is though that linearization of the chromosome occurred via a single cross-over recombination event between an originally circular chromosome and a linear plasmid (Chen, 1996; Volff & Altenbuchner, 1998). Some interesting discoveries from the comparative analysis of actinobacterial genomes include: the widely conserved bacterial gene *ftsA,* involved in cell division, is absent in most genomes in this group, as are *minC* and *minE*, which have a role in choosing the cell division site in unicellular bacteria (Ventura *et al.*, 2007). The streptomycete genome is rich in secondary metabolite genes and encodes a large number of predicted secreted proteins, including in the case of *S. coelicolor,* 147 hydrolases, of which 7 are cellulases and 5 are chitinases (Bentley *et al.*, 2002). The sequenced *T. fusca* genome encodes 45 hydrolases, 6 of which are

cellulases (Bentley *et al.*, 2002; Omura *et al.*, 2001; Ventura *et al.*, 2007). Udwari *et al.* (2007) showed that the genome of *Salinispora* contains even more gene clusters coding for secondary metabolites (approx. 10% of the total genome) than those of sequenced *Streptomyces* strains.

When comparing the genomes of all *Actinobacteria* available at the time, Ventura *et al.* (2007) found 123 core genes that were shared between all the genomes and could be used as phylogenetic markers. The relatively low number of core genes demonstrates the large phylogenetic distances within the *Actinobacteria*.

1.5.4.3. Actinomycete cellulases

Cellulolytic actinomycetes produce a free cellulase system, although a high molecular weight xylanolytic complex has been described in streptomycetes (Jiang *et al.*, 2004; van Zyl, 1985). One common characteristic of actinomycete cellulases is optimal activity at neutral or slightly alkaline pH, distinguishing them from fungal cellulases which often perform better under more acidic conditions (McCarthy, 1987).

Cellulases produced by some actinomycetes, especially *Thermobifida* (*Thermomonospora*) *spp*, *Streptomyces spp.* and *Microbiospora spp*, have been purified, characterized and in some cases the genes cloned and sequenced, to reveal a multiplicity of enzymes (Wilson, 1992). Six excreted cellulases have been identified and characterised biochemically in *T. fusca*. Four of these cellulases were endoglucanases (glycoside hydrolase families 5, 6 and 9) and two exocellulases (glycoside hydrolase families 5 and 6) (Collmer & Wilson, 1983; Ghangas *et al.*, 1989; Hu & Wilson, 1988; Irwin *et al.*, 2000; Zhang *et al.*, 1995). An intracellular β-glucosidase (glycoside hydrolase family 1) and a number of other hydrolytic enzymes specific for other polysaccharides have been characterized. Recently, the genome of *T. fusca* was sequenced and probed for the presence glycoside hydrolases. Hydrolytic enzymes (45) were found in the genome, of which 36 were glycoside hydrolases; these were distributed across 22 different glycoside hydrolase families, with six different enzymes in family 13 alone. The sequence analysis also allowed the authors to conclude that 16 of these enzymes were secreted based on the presence of signal peptides, the majority of which (13) have a twin-arginine transport (TAT) signal in their N terminus. This transport system is characterised by the ability to transport fully folded proteins and could play an important role in the secretion of cellulases in *T. fusca*. Another 28 putative glycoside hydrolases were identified in the *T. fusca* genome, and also 4 coding sequences that possessed

CBDs with no identifiable catalytic site. The authors predict that these could have a scaffolding function, facilitating hydrolysis of plant cell walls.

In a previous study, a particular nucleotide sequence consisting of a 14 bp inverted repeat was identified that appears to be a general regulatory sequence for actinomycetes; a protein encoded by a gene termed *celR* was identified as the regulator for induction of cellulase synthesis. CelR acts by binding to the 14 bp inverted repeat sequence upstream of the cellulase genes. The binding of this protein to the regulatory sequence is thought to block the activity of a repressor and is thus a negative control system. Cellobiose released from cellulose was found to be the true inducer of cellulase synthesis rather than cellulose itself, whereas glucose represses this cellulolytic system (Wilson, 1992).

In the *T. fusca* genome, this regulatory sequence is found at five other points associated with the sequence of various genes, including a protein involved with the ABC disaccharide transport cassette and a membrane protein of the major facilitator family, which may be involved in the transport of cellulolysis products into the cell; these authors also discovered another sequence distributed at 10 different points within the genome that could also function in the regulation of cellulase synthesis in this organism. An extensive set of transporters for carbohydrate uptake were also found, and many of the genes encoding these transporters are localised near glycoside hydrolase genes in the genome. These consisted of cellobiose/cellotriose, maltose and xylobiose ABC transport systems.

1.5.4.4. *Micromonospora*

1.5.4.4.1. Overview

The genus *Micromonospora* (Ørskov 1923) comprises finally branching (average diameter of hyphae is 0.5μm), slow–growing actinomycetes that lack aerial hyphae and produce a large variety of secondary metabolites, including many antibiotics. *Micromonospora* spp. produce heat resistant, non–motile spores that are single and derived from the sub–division of the sheathless hyphae and are thus not true endospores (Kawamoto, 1984). The spore (0.75–1.5μm in diameter) has spiny projections, a stratified wall and is much thickened compared to hyphae, conferring upon it a much greater resistance to heat, desiccation and exposure to solvents (Kawamoto, 1984). The genus is also characterized by the actinomycete type II cell wall (Kawamoto *et al.*, 1981), containing glycine and meso–diaminopimelic acid and is generally resistant to lysozyme attack. Micromonosporas are able to degrade

several complex recalcitrant materials such as cellulose and chitin and most strains are strongly proteolytic (Jendrossek *et al.*, 1997; Kawamoto, 1984; Wohl & McArthur, 2001). Most strains have optimum growth at neutral or slightly alkaline pH and are sensitive to acidic conditions (Kawamoto, 1984); there is evidence that they can grow in microaerophilic conditions (Goodfellow & Williams, 1983; Watson & Williams, 1974). On isolation agar plates, *Micromonospora* colonies are often pale orange or yellow, becoming red, brown, blue green or purple as the colonies age. After prolonged growth, hyphal lysis is extensive leaving very large masses of spores, and giving the colony a mucoid appearance.

1.5.4.4.2. Classification

Micromonosporas belong to the family *Micromonosporaceae*, which includes, according to Vobis (2006), *Actinoplanes*, *Dactylosporangium*, *Ampullariella*, *Pilimelia*, *Catellatospora,* and *Glycomyces*. The traditional features used to define the family is the common cell wall chemotype containing *meso*- and/or 3-hydroxy diaminopimelic acid and glycine in combination with xylose and arabinose as the characteristic sugars in the hydrolysates of the whole organism. Some members of this family (*Actinoplanes*, *Dactylosporangium*, *Ampullariella*, *Pilimelia*) produce sporangia and motile spores, whereas the others produce non-motile spores that can be arranged in chains or borne singly. Most members of the family are associated with decaying plant matter and aquatic ecosystems (Vobis, 2006). Except for the micromonosporas, these organisms are relatively difficult to isolate from the environment, requiring specific enrichment methods (Vobis, 2006). Many more new genera can be found in this family in the ribosomal database project II, some of which are well described, such as *Salinispora,* others remain poorly described, such as *Asanoa*, *Virgisporangium* and *Amorphosporangium*.

Kawamoto (1984) describes the existence of 8 *Micromonospora* species, with another 5 possible species still disputed taxonomically at the time. More recently, Kasai *et al.* (2000) used DNA gyrase *B* gene sequences to determine the phylogeny and DNA relatedness of previously recognized species and determined a total of 14 species. Many new species are being described as more extreme environments such as the Antarctic and mangrove swamps are probed for these organisms and Thawai *et al.* (2008) presents a phylogenetic tree containing 33 described species. The genus is represented in the ribosomal database project II with 486 16S ribosomal RNA gene sequences.

1.5.4.4.3. *Micromonospora* ecology

Micromonosporas are found in terrestrial, aquatic and marine environments but are particularly abundant in soils and freshwater sediments. Members of this genus are considered to be particularly suited to an aquatic lifestyle due to the absence of aerial hyphae and the production of hydrophilic spores; in some studies the micromonosporas were found to be the most common actinomycetes isolated from lake sediments (Cross, 1981). Johnston and Cross (1976) found numbers of *Micromonospora* ranging from 10 to 240 x 10^4 CFU per gram of dry weight of mud in lake sediments from the English Lake District. *Micromonospora* spores can remain viable for at least 100 years in lake sediments (Cross, 1981), which could explain their recovery from these environments. Johnston and Cross (1976) used sonication to fragment actinomycete hyphae in mud samples and differentiate CFU counts arising from hyphae from those arising from spores. Their data suggested that *Micromonospora* hyphae were present in lake sediments whereas *Streptomyces* and nocardioform actinomycetes were thought to be present mainly as dormant spores and hyphal fragments (Johnston & Cross, 1976). Despite this, doubt still remains on the importance of micromonosporas in the ecology of lake sediments, as lakes tend to stratify in the summer months, leading to lower oxygen tensions in the sediments precisely when most of the biological activity occurs.

1.5.4.4.4. Cellulases

Most *Micromonospora* strains are capable of degrading cellulose to some extent (Kawamoto, 1984), conferring upon these organisms a potentially important role in recycling particulate organic matter in freshwater environments. Despite the general acknowledgement that most strains of *Micromonospora* are able to degrade cellulose, only 6 studies characterizing their cellulolytic activity have been published (Chowdhury *et al.*, 1991; Gallagher *et al.*, 1996; Malfait *et al.*, 1984; Sandrak, 1977; Singal & Soloveva, 1974; van Zyl, 1985) and these studies were limited to determining overall activity of a single or a small number of strains. It is assumed that the *Micromonospora* cellulolytic system resembles those of other cellulolytic actinomycetes. Gallagher *et al.* (1996) investigated the production of cellulases and β–glucosidases by *Micromonospora chalcea* grown in cellulose–containing media and observed that the production of extracellular β–glucosidases lags behind cellulase production, suggesting a deficiency in that enzyme, whereas the levels of total cellulase produced were comparatively high. Contrary to what is described for many actinomycetes, especially *Streptomyces spp.*, the authors found only trace amounts of cell– associated β–glucosidase activity, and in this regard, *M. chalcea*

was considered to resemble the fungal cellulolytic system. In another study, carboxymethylcellulase (CMCase) and β-glucosidase activity were found to be mostly cell bound in the exponential growth phase of *M. chalcae* when grown on CMC, and cellobiose was confirmed to be the product of the hydrolysis of the extracellular cellulases. Cellobiose was found to be an inducer of cellulose synthesis, and glucose a repressor, although glucose concentrations had no effect in cultures growing on cellobiose or CMC (Malfait *et al.*, 1984). Van Zyl (1985) confirmed the intracellular nature of the β–glucosidases produced by the micromonosporas, but considered the cellulase activity to be mainly extracellular during the exponential growth phase. Chowdhury *et al.* (1991) compared cellulose production of a *Micromonospora* strain when grown with different substrates and found that when grown in cultures supplemented with xylan, the highest cellulose yields were obtained, whereas Avicel cellulose and cotton were poor substrates for cellulase production (Chowdhury *et al.*, 1991). However, the authors performed the assays on cultures that were only 24 h old, which is probably very premature for these slow growing actinomycetes.

Micromonospora is recorded in the Carbohydrate-Active Enzyme website (www.CAZy.com) as having glycoside hydrolases of 3 different families (6, 25, 33), but only one of these is a cellulase, an endoglucanase of the glycoside hydrolase family 6, named mcenA. The gene coding for this enzyme (*mcenA*) has been cloned and sequenced and its product deduced (Lin *et al.*, 1994b). McenA has 457 aminoacids and its molecular weight is 46742 Da; it contains a putative signal peptide at its N-terminus and a catalytic domain connected by a linker region to a cellulose binding domain. Enzymes from GH family 6 have the enverting mechanism of hydrolysis and are widely believed to act processively from the non-reducing ends of the cellulose chain to generate cellobiose.

1.5.4.4.5. Biotechnology

Over 300 different antibiotics have been described to be produced by members of the the genus *Micromonospora* (Vobis, 2006) including aminoglycosides (gentamicin, kanamycin), macrolides (megalomicin, erythromycin B), ansamysins (halomicins), everninomicins, actinomycins, and other less well characterized antibiotics (Wagman, 1980). Of all these antibiotics, gentamicin is the one that has most successfully been produced in a commercial scale. The search for antibiotics or other potentially useful secondary metabolites from members of the *Micromonosporaceae* had slowed in the 1980s, however, with the emergence of

many antibiotic-resistant pathogens, such as MRSA, there has been renewed effort, and the family was once again a target for biodiscovery research. The initial research effort into drug discovery had been directed at soil micromonosporas, and for this reason renewed effort has been put into isolating and screening metabolites from marine members of the genus and other closely related species. This has led to the isolation of many new strains of *Micromonospora* and other actinomycetes from marine sediments (Bredholdt *et al.*, 2007; Magarvey *et al.*, 2004), and the description of one obligate marine actinomycete genus, *Salinispora*. This research effort has also led to the description of new methods for the rapid identification and taxonomic screening of isolates (Maldonado *et al.*, 2008; Qiu *et al.*, 2008; Zhao *et al.*, 2004).

Since its discovery, *Salinispora* has received a large amount of attention; its genome was sequenced and several potentially useful compounds that it produces has been described, including Salinisporamide A, which is thought to have powerful properties against cancerous cells (Feling *et al.*, 2003).

1.6. Microbial molecular ecology

1.6.1. Overview

Molecular phylogeny showed that the prokaryotes were not a uniform group or organisms of low diversity. Firstly, they could be divided on two fundamentally different groups based on 16S rRNA sequence divergence: the *Eubacteria* and *Archaea* domains (Balch *et al.*, 1977). As more sequences were made available, it became clear that most of the genetic and metabolic diversity of life is prokaryotic (Oren, 2004), and that the majority of that diversity corresponds to uncultivated organisms; some studies suggest that only 1, 0.1% or even less of all the microbial species has been properly characterized through cultivation (DeLong & Pace, 2001; Torsvik *et al.*, 1998). Due to the discovery of this huge diversity of microorganisms, microbial ecology was also transformed. Even relatively simple systems, such as activated sludge reactors, needed a reassessment of their microbial community structure (Bond *et al.*, 1995).

One of the biggest impacts of molecular biology on the understanding of microbial ecology stems from the fact that no living cell is needed to characterize an organism in terms of its phylotypes (Hugenholtz & Pace, 1996), and as long as good quality DNA is made available to be used as a template for molecular studies, the

presence, diversity and abundance of different microbial taxa can be assessed without the need to isolate the organisms themselves.

1.6.2. Molecular ecological methods

PCR (polymerase chain reaction) allows the selective amplification of small amounts of DNA based on specific nucleotide sequences. Prior to its development, sequence analysis was very slow and time consuming due to the low amounts of DNA available to study. The amplification provided with PCR is the basis of many of the methods that are now standard in molecular biology. Its development was possible with the recombinant production of thermostable DNA polymerases that could withstand temperatures needed for the denaturing and separation of the double stranded DNA molecules. These polymerases were developed from organisms inhabiting hot thermal springs where temperatures are often near boiling point.

PCR can be used to detect the presence of organisms based on the use of specific primers, usually targeting the 16S rRNA gene, and the products of the amplification can then be cloned and sequenced allowing the diversity and phylogeny of uncultured microorganisms to be elucidated. PCR is a very efficient technique and will amplify minute concentrations of DNA template. It is therefore very important to prevent any contamination with foreign DNA, especially when using universal primers to amplify environmental DNA, as it is often impossible to know in advance which organisms are present.

Inherent problems exist when using PCR, especially when applying it to a mixed template DNA. Firstly, DNA polymerases are not error proof, and can add point mutations to the sequence; different enzymes have different misincorporation rates, and efficient enzymes such as the commonly used *Thermus aquaticus* DNA polymerase do not have a 3' to 5' proofreading function and are therefore less reliable. Despite such errors, the degree of misincorporation is relatively small compared to the natural variability of 16S rRNA sequences between species (Stackebrandt & Goebel, 1994). Chimeric PCR products are another well known problem, caused when the incomplete amplification of a gene that subsequently anneals to a homologous gene fragment and is then fully extended, generating a sequence that consists of two different templates that is therefore artificial. This problem is particularly serious for environmental DNA amplification and is enhanced in samples containing a large proportion of sheared DNA and highly similar

sequences (Wang & Wang, 1997). A number of software packages are available to determine if a sequence is chimeric, such as CHIMERA CHECK, available in the RDP website (http://rdp8.cme.msu.edu/cgis/chimera.cgi?su=SSU), but sometimes a phylogenetic analysis on the opposite ends of a sequence is the best way to determine if a sequence is chimeric (Roling, 2005).

Another problem when applying PCR to DNA extracted directly from the environment is bias due to the fact that PCR will not amplify individual components of a mixed DNA template equally. For example DNA with low GC content denatures more easily and is preferably amplified. In the case of Gram-positive organisms, molecules bound to DNA that are not removed during the extraction can lead to the formation of loops in the template that interfere with its elongation (Reysenbach *et al.*, 1992). Likewise, it is known that sequences flanking the region to be amplified can inhibit amplification (Hansen *et al.*, 1998). When using universal primers that amplify different taxa, amplification efficiency will vary amongst the different templates, and even when using degenerate primers, biases can still occur, as the physical properties of the primers are not always identical (Roling, 2005). Template modifications such as methylation (Stackebrandt, 1997) and template concentration (Chandler *et al.*, 1997) also affect amplification efficiency. The final proportion of genes amplified from a mixed community using degenerate primers will often be different to the original composition; the longer the number of cycles, the more the proportion of each gene being amplified changes from the initial proportion to a 1:1 proportion with all the other templates. This is due to the fact that as the amount of template increases, template re-annealing strongly outcompetes primer annealing, and amplification stops, with the final concentration of each template remaining similar at a saturation point (Suzuki & Giovannoni, 1996).

Due to PCR bias, clone libraries produced from environmental DNA amplified with general primers are unlikely to be a true representation of the proportions of each organism in nature. Great care must be taken when interpreting data from experimental procedures that include a PCR step(s).

PCR is a very sensitive technique, and nucleic acids must be properly purified in order to amplify properly. Two main approaches are used when extracting DNA from environmental samples: lysis of cells within the environmental matrix (soil, sediment, wood etc...) or removing cells from the matrix, followed by nucleic acid extraction. The first approach gives the highest extraction efficiency, but it increases the possibility of co-extraction of large amounts of other compounds other than nucleic

acids that can interfere with downstream PCR amplification, such as humic acids (Tebbe & Vahjen, 1993).

A crucial step in nucleic acid extraction is cell lysis, which is often achieved with a combination of treatments using chemical, enzymatic and mechanic disruption of cell walls, such as lysozyme, SDS and bead beating treatments. Bead beating is often employed for the mechanical disruption of cells, and is a very efficient strategy to increase DNA/RNA yields, but results in the shearing of nucleic acids, which can compromise further processing.

Bias can also exist when extracting DNA from a mixed community, as the cells of some organisms might be more susceptible to lysis than others; Gram-positive cell walls can be significantly harder to lyse than Gram-negative, and treatments have being designed for specific groups (Crameri *et al.*, 1983; Makinde & Gyles, 1998; Tavares & Sellstedt, 2000). A large number of publications detail the particulars of environmental DNA/RNA extraction methods for particular environments or specific downstream applications. For relevant examples, see Griffiths *et al.* (2000), Rivera *et al.* (2003), Lemarchand *et al.* (2005), Carrig *et al.*(2007), Lakay *et al.* (2007) and Mitchell *et al.* (2008).

1.6.3. Molecular microbial ecology methods

A range of molecular tools in addition to simple PCR amplification followed by clone library construction is available to the microbial ecologist. These include DNA/RNA probe hybridization, community fingerprinting, metagenomics (metagenomic library construction, pyrosequencing of total community DNA/cDNA), stable isotope probing (SIP) and quantitative PCR.

DNA/RNA probe hybridization relies on the Watson-Crick complementary base pairing between two nucleotide strands of identical or similar sequences. A variety of nucleotide probing methods are available, and in essence, these methods rely on the use of a labelled single stranded nucleic acid probe that allows the detection of the complimentary sequence in single stranded nucleic acid molecules that have been immobilized in a solid matrix such as a nylon membrane. The different nucleotide probing methods have different targets (i.e., genomic, environmental DNA or RNA, colony hybridization), specificities, and in which type of probe (large fragment probes, oligonucleotide or polynucleotide probes (Heuer *et al.*, 1999; Ludwig *et al.*, 1994)) and the labels used. The nucleotide sequence is crucial in

determining the specificity of the probe, and many computer packages are now available in order to check the specificity of a nucleotide sequence (Altschul *et al.*, 1990; Loy *et al.*, 2008). The choice of labels for probes has increased as these techniques became more popular, and in addition to the radioisotopes traditionally used, a range of fluorescent, chemiluminescent and colorimetric molecules have been successfully applied in oligonucleotide probing.

Two methods based on nucleotide acid hybridization that have been applied with great success in microbial ecology are FISH (fluorescence in-situ hybridization) and microarray analysis (oligonucleotide arrays). FISH involves the in situ microscopic observation of cells containing the target DNA to which the taxon specific probes containing a fluorescent label have hybridised. Microarrays are usually developed by attaching oligonucleotides, cDNA or PCR fragments to a slide (e.g. a DNA chip), to which the sample DNA is exposed. Fluorescence in spots in the slides containing specific probes will be indicative of hybridization and therefore of the presence of target DNA in the sample. Microarrays can also be used to detect the expression of genes in the environment by designing probes based on reverse-transcribed mRNA of specific functional genes. Many reviews are available describing the detail and applications of FISH (Amann *et al.*, 1995; Delong *et al.*, 1989; Ouverney & Fuhrman, 1999; Pernthaler *et al.*, 2002; Wagner *et al.*, 2003), and microarrays (Guschin *et al.*, 1997; Liu & Zhu, 2005; Voordouw *et al.*, 1991).

Community fingerprinting is often utilized when a relatively fast and broad characterization of a community is necessary, especially when changes in microbial composition is the main objective of the study. Different approaches are used to fingerprint communities: differential melting of GC rich strands of amplified DNA on chemical or temperature gradient gels (denaturing gradient gel electrophoresis, DGGE (Muyzer *et al.*, 1993) and temperature gradient gel electrophoresis,TGGE (Rosenbaum & Riesner, 1987)), differential mobility of single stranded DNA due to secondary structure formation on non-denaturing gels (single stranded conformation analysis, SSCP (Lee *et al.*, 1996; Schwieger & Tebbe, 1998)), and by restriction fragment digestion of 16S rRNA gene amplified with universal primers (terminal restriction fragment length polymorphism, T-RFLP (AvanissAghajani *et al.*, 1996; Liu *et al.*, 1997)). For a recent review on microbial community fingerprinting methods, see Nocker *et al.* (2007).

Stable Isotope Probing (SIP) is a relatively new technique that allows a link to be established between function and identity in a community of microorganisms. It

works by supplying a substrate labelled with a heavy isotope label, usually ^{13}C, which is taken up by the active organisms in that environment and is used in the production of cellular components, including nucleic acids, which are used as biomarkers. The labelled DNA is then separated by centrifugation. The technique was first developed by Radajewski *et al.* (2000) for methylotrophs but has been applied to other groups (Dumont & Murrell, 2005), including cellulose degraders (Haichar *et al.*, 2007).

With more powerful sequencing techniques, studies on microbial diversity have entered a new stage. Metagenomics is the term used for the study of the whole DNA extracted from the environment, the environmental "genome". In order to capture meaningful sequence information, cloned fragments have to be relatively large. This has been accomplished with the use of bacterial artificial chromosomes (BACs) or plasmid-derived Cosmids and Fosmids, which allow the cloning of fragments of up to 120 and 50 kb respectively (Xu, 2006). The analysis of the metagenomic clone library involves either the sequencing of clones to generate large amounts of data for bioinformatic analysis, or through functional screening of the clones for the expression of a desired gene product (Schmeisser *et al.*, 2007). After the publication of the Sargasso Sea metagenome (Venter *et al.*, 2004), the potential for gene discovery via metagenomics was realised, and many metagenomic studies have been published since, including some focusing on cellulase discovery (Feng *et al.*, 2007; Pang *et al.*, 2009; Voget *et al.*, 2006; Warnecke *et al.*, 2007).

With the commercial availability of high-throughput pyrosequencing technologies, such as 454 sequencing (454 life sciences), which uses nano-technology to perform massive parallel sequencing reactions by synthesis on a solid support, the coverage of metagenomic studies has increased dramatically, and this is accomplished without the need to perform *in vivo* cloning of DNA fragments. Given the enormous diversity of microbes found in natural environments (recent estimates suggest for example that 10g of soil could harbour 10^7 distinct bacterial taxa, which would yield the equivalent of 1000 human genomes in total bacterial genome sequences (Curtis & Sloan, 2004; Gans *et al.*, 2005)), the ability to generate a a very large number of sequence reads rapidly and cheaply offers great opportunities for research into natural microbial diversity. For a review of advanced sequencing technologies, see Hall *et al.* (2007).

1.6.3.1. Real-Time Quantitative PCR

Real-Time Quantitative PCR is a molecular technique that allows the quantification of gene copy numbers within a sample. It can be applied to environmental samples in which gene copy numbers can be used as a proxy for the abundances of selected groups of microorganisms. As discussed above, PCR amplification will often result in a change of the initial proportion of genes in the sample due to the competition of template for primers after a significant number of cycles. Quantitative PCR attempts to solve this problem by following the amplification in "real time", i.e., every cycle of amplification of a specific gene is tracked with the use of a fluorochrome molecule that emits fluorescence when a sequence is amplified. A variety of technologies can be used to this end but the technique was originally created based on 5'– nuclease assays developed by Livak *et al.* (1995) in which the 5' to 3' exonuclease activity of *Taq* DNA polymerase leads to the production of fluorochrome molecules during the extension step of PCR, Briefly, oligonucleotide probes with a sequence complementary to a region between the amplification fluorochrome-labelled primers emit fluorescence at both the 5' and 3' end. In the original state, the fluorescence from the 5' end is quenched by the reporter dye on the 3' end but during the PCR amplification, *Taq* polymerase removes the 5' reporter dye from the template bound probe and the cleaved reporter dye accumulates in proportion to the initial copy number of target genes in the template DNA.

SYBR green based chemistry is a more commonly used for quantitative PCR assays. This dye binds to double stranded DNA and emits fluorescence only when bound. The amount of PCR product accumulating in the reaction mix can be tracked by the increased fluorescence of the bound SYBR green. One disadvantage of this method is that fluorescence will be emitted for any double stranded DNA, including non-specific products and primer dimers, which could lead to an overestimation of amplification, particularly when quantifying genes that are present in low copy numbers (Hein *et al.*, 2001). The formation of primer dimers and secondary structure must be minimised when designing primers for use with SYBR green, and the formation of these during the assay can be monitored by the addition of a melting curve step at the end of the amplification step of a qPCR cycle (Ririe *et al.*, 1997). The melting profile of primer dimers and non-specific products differs from that of the target gene and the presence of these can be monitored (Smith, 2005).

In order to calculate gene copy numbers, the threshold cycle (Ct) must be determined for the unknown sample and for a range of known standards. The Ct

corresponds to the point at which the fluorescence from the accumulated amplicons exceeds the background fluorescence (Fig 1.11.). Once the fluorescence from the amplification of the target genes exceeds the background, the exponential accumulation of amplicons can be measured, and gene copies can be quantified. A standard curve made from dilutions of a known number of gene copies is prepared with either DNA/cDNA, linearised plasmid DNA or PCR product of the target gene (Suzuki *et al.*, 2000). The slope of the calibration curves is a measure of the amplification efficiency of each primer used. Unless a calibration curve is to be used for every primer in every run, the efficiencies of different primers should be similar.

This method quantifies gene copy numbers, and a calibration step in which numbers of target genes per cell are determined is therefore required; a database exists where the numbers of rRNA operons per cells are listed (Klappenbach *et al.*, 2001). By modifying the assay and reverse transcribing mRNA followed by PCR quantification of cDNA, it would enable the measurement of metabolic activity by expression of metabolic genes (Skovhus *et al.*, 2004). Ribosomal RNA can also be used to calculate abundances once it has been reverse-transcribed to cDNA and a higher signal can be obtained as each cell has multiple copies of ribosomes, although the numbers may not reflect cell numbers accurately.

Quantitative PCR will allow the quantification of absolute gene copy numbers in a sample, but often when applying this technique to measure microbial abundances in environmental samples, the relative quantification of one organism compared to the total community is a more appropriate approach. Gene copy numbers in a sample will be influenced by the efficiency of the DNA/RNA extraction, and these may vary between different samples. The presences of inhibitory compounds in the nucleic acid extract, variations in the performance of the primers and the equipment used, and experimental errors related to inaccurate pippeting and template degradation might still influence the final gene abundance, and successive runs might produce different absolute gene copy numbers. By measuring the relative abundance of the target gene to the total microbial community copy number, these inconsistencies are taken into account and reproducibility of the results is increased (Smith, 2005). In particular, this is useful to indicate if organisms whose DNA has been detected by PCR are present as a significant component of the indigenous community.

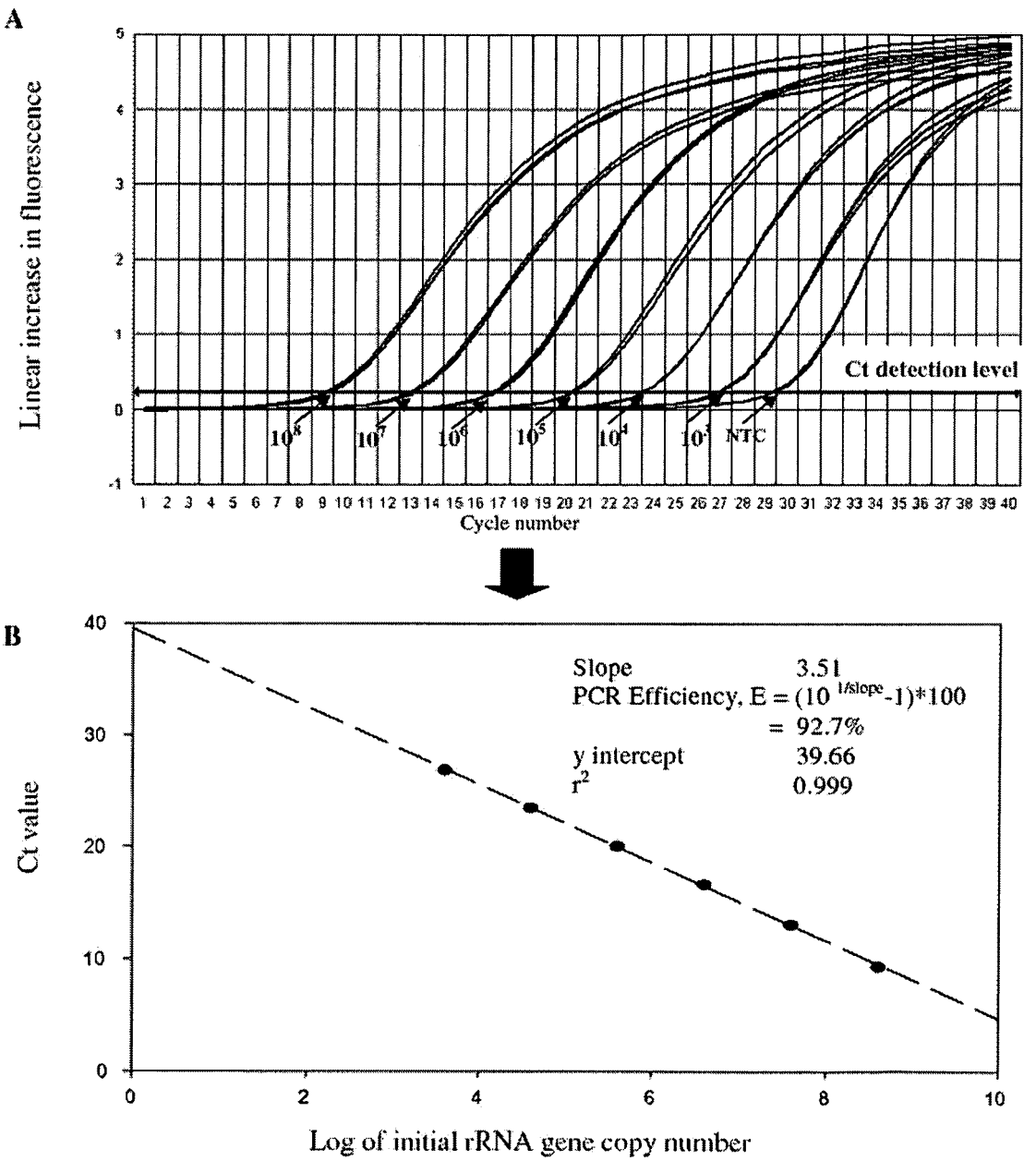


Figure 1.11. qPCR calibration amplification plot and calibration curve.

A, amplification plot of quantitative PCR calibration curve; B, linear regression of C(t) versus log of gene copy numbers. From Smith *et al.* (2006).

1.7. Molecular phylogenetics

1.7.1. Overview

The use of molecular markers has become standard in microbial taxonomy and phylogeny, and 16S rRNA gene sequences in particular are almost a universal marker for bacterial classification (Ludwig *et al.*, 1998); the number of sequences available in public databases such as GenBank and EMBL has grown exponentially for the past two decades, GenBank in particular doubles in size every 18 months, containing as of February 2008 82 million sequence records for example.

The understanding of microbial phylogeny has indeed been greatly improved by the use of molecular methods; previously the use of morphological and physiological criteria led many researchers to conclude that the prokaryotes were low in diversity when compared to higher organisms (Hugenholtz & Pace, 1996). Fox *et al.* (1977) were the first to suggest that the small subunit ribosomal RNA (SSU rRNA) gene should be used as a marker of bacterial evolution and phylogeny; many reasons led to this choice: it is a gene that is unlikely to be laterally transferred, it contains fast and slowly evolving portions, it has a universally conserved structure, it is ancient and essential, it interacts with many other co-evolved cellular RNAs and proteins and its sequence length is long enough to provide meaningful phylogenetic information, whilst being small enough for its analysis to be feasible. By analysing this gene, Balch *et al.* (1977) demonstrated that the prokaryotes were actually formed of two very distinct groups of organisms, the *Eubacteria* and the *Archaea*. Many other revelations led to the refinement of bacterial classification, and the demonstration that many established bacterial groups contained distantly related organisms, such as the inclusion of *Thermoactinomyces* within the actinomycetes, when this genus is actually more closely related to the *Bacillus* and a member of the low GC branch of the Gram-positive bacteria (Park *et al.*, 1993). The universal use of SSU rRNA genes for the phylogeny not only of prokaryotes but also eukaryotes led many to believe that finally, as Charles Darwin had hoped, a tree of life could be constructed linking every organism on earth (Fig 1.12.). The prokaryotes however, display many peculiarities that challenge the idea that gene phylogeny could necessarily be equated with organismal phylogeny. Genetic material frequently moves between different species of microorganisms through lateral gene transfer (LGT), which is considered one of the main means by which the bacterial genome evolves. *E. coli*, for example, is thought to have acquired all the genes that determine the phenotypical characteristics distinguishing it from *Salmonella enterica*

from laterally transferred genes, which comprise 18% of all of its open reading frames (Brown & Doolittle, 1997). However, it is clear that LGT occurs much more frequently with some genes that encode non-essential functions, such as antibiotic resistance or the use of unusual substrates, but more rarely with "core" genes that code for essential cell machinery such as nucleic acid replication. Still, it is possible that LGT will occur even with SSU rRNA genes, and the validity of the tree of life remains in question (Doolittle, 1999). Different markers often produce different phylogenies and genome-genome analysis shows that at deep phylogenetic ranks (such as when separating Eubacteria, Archaea and Eukarya), a clear cut organismal hierarchy may simply not be a biological reality, or cannot be determined with the methods currently available. Indeed, when analysing replication, transcription and translational genes, *Archaea* and *Eukarya* are considered sister groups, whereas many if not most eukaryotic cytosolic metabolism enzymes are more closely related to eubacterial genes (Brown & Doolittle, 1997).

Alternative molecular markers are not always expected to agree in every detail (Ludwig, 2001), but the phylogenetic patterns observable when analysing their sequences are still informative. By choosing the correct marker, different levels of resolution can be elucidated, i.e., species, genus or division levels. Many other genes are available to study bacterial phylogeny, for example the genes encoding 23S rRNA, elongation factor Tu (*tuf*), heat shock proteins (*hsp60*, *hsp70*), DNA gyrase B (*gyrB*) and *recA* genes (Ludwig & Schleifer, 2005). Some of these genes (for example *gyrB*) do not support established bacterial taxa at high ranking levels (phyla or divisions), but can be useful when determining species or genus identity (Ludwig, 2001).

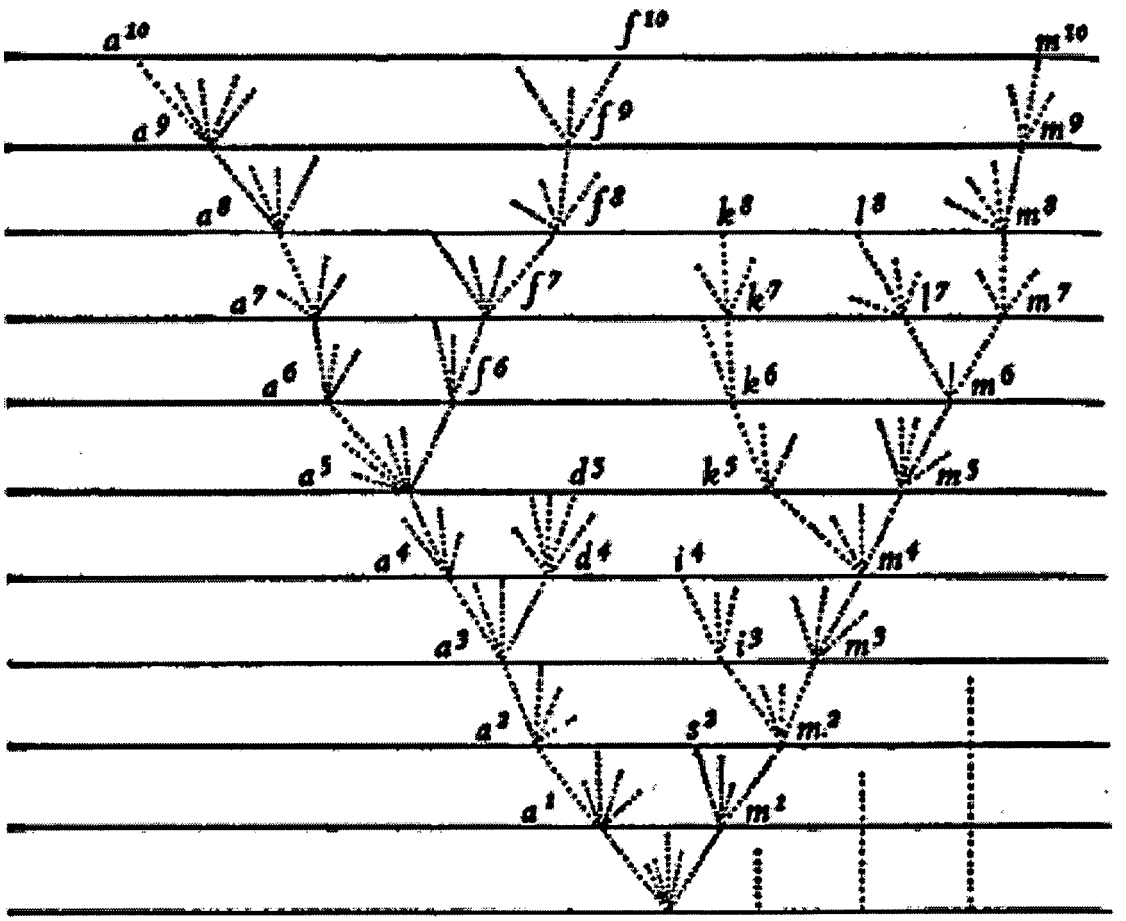


Figure 1.12. Charles Darwin's tree of life.

From Darwin (1859).

1.7.2. Bioinformatics

Comparative sequence analysis is the most important tool for phylogenetics today. It relies on the use of sequence changes to infer phylogenetic distances between organisms. A reasonable amount of conservation is required for a sequence to be a reliable measure of relatedness, and the amount of information that can be used from a gene will also depend on the number of characters (nucleotides or amino acids) and the number of potential character states per site (Ludwig *et al.*, 1998). Thus, in fast evolving genes, phylogenetic signals will quickly disappear, but when the sequence is very similar or identical between two genes, no phylogenetic relationship can be inferred. When considering that microorganisms have been evolving for 3-4 billion years, the amount of information available per evolutionary event is quite limited, and often sequences or positions within sequences that are functionally important will not evolve independently and will not yield valuable evolutionary information. Ludwig *et al.* (1998) calculated that the resolution of the analysis of a pair of most diverged 23S rRNA sequences is of 0.5 - 0.7 million years.

Sequence analysis relies on the correct alignment of homologous gene sequences, which can be assisted by specialized computer programmes such as CLUSTAL (Thompson *et al.*, 1994), but often requires manual checking and optimization, especially in more variable regions. The secondary structure, such as helical elements in the 16S rRNA, are features that can be checked to help to improve primary structure alignment (Ludwig & Schleifer, 1994). In the case of 16S rRNA, extensive databases with pre-aligned sequences are available (RDP database (Maidak *et al.*, 1996), ARB (Ludwig *et al.*, 2004)) which can be used as a template when new sequences are obtained and greatly reduce the analysis effort.

1.7.3. Phylogenetic trees

Phylogenetic distances are usually expressed in the form of trees. The relationship between organisms is expressed by the branching pattern, and the level of evolutionary change usually by branch length. There are several treeing methods, but most are based on three basic approaches: distance matrix, maximum parsimony and maximum likelihood methods (Ludwig *et al.*, 1998). Distance matrix methods work by measuring the level of difference between sequences, which is used to calculate the phylogenetic distance between those sequences. Due to the limitations of present day sequences in providing evolutionary information, a number

of evolutionary models have being devised to improve the accuracy of the analysis. One such method is the Jukes Cantor model (Jukes, 1969), which corrects for multiple base changes that may result in "false identities". The maximum parsimony method is based on the assumption that preservation is more likely than change, and the final tree is the one that requires the minimum number of sequence changes. The maximum likelihood method is the most computational intensive method, and uses sophisticated evolutionary models to calculate the tree that most likely explains the current sequences. Comparatively, this method utilizes the largest amount of information that can be extracted from the sequences, but due to its computational requirements, a limited amount of statistical confidence tests can be applied (Ludwig *et al.*, 1998).

Phylogenetic trees are not absolute realities, and a number of factors will limit the robustness of their topologies. False identities can exist when multiple mutations result in a particular residue mutating to the original sequence from which it had previously diverged. This phenomenon is called plesiomorphy and is particularly relevant in variable regions; when comparing highly similar sequences, the variable regions are often the only source of variations and reconstructing evolutionary relationships is sometimes impossible. Equally, different rates of evolutionary change between different organisms lead to skewed tree topologies, such as long branch attraction (Ludwig *et al.*, 1998). Tree topology will also vary even when the same set of sequences are analysed multiple times. Most treeing methods first build a tree with three sequences, so that only one topology is possible, and then add the remaining sequences. The orders with which organisms are added to a tree will influence its final topology, and for this reason, several runs should be performed with re-sampling of the sequences. This process is called bootstrapping and is routinely used to calculate the robustness of different branches within a tree; the final tree is presented with the bootstrap values for each node representing the percentage of trees that support that particular branching pattern.

1.8. Aims

The aims of this study are to determine the presence, diversity, cellulolytic activity and relative importance of *Micromonospora spp.* in two lakes of contrasting trophic status in the English Lake District. Micromonosporas are ubiquitous and abundant soil actinomycetes that although slow growing, are often capable of attacking biopolymers such as cellulose and chitin. There is some old evidence that they grow, i.e. occur as hyphae, in lake sediments, and the objective here is to demonstrate that micromonosporas colonise and degrade cellulose in situ by using baiting techniques in combination with electron microscopy, activity assays, and the development of methods for determining phylogeny (based on genes encoding 16S rRNA and DNA gyrase B). The abundance relative to other cellulolytic microorganisms in the lake (qPCR of the reverse transcribed RNA) will also be determined.

The specific hypotheses to be addressed are:

- Do micromonosporas contribute significantly to cellulose degradation in aquatic environments? How are they distributed in the water column and sediment and is it possible to prove the presence of their hyphae?
- What is the molecular diversity of cellulose–degrading *Micromonospora* spp. in the lakes?
- What is the comparative cellulose degradation activity of *Micromonospora* isolates from the lakes and does it correlate with phylogeny and species taxonomy?
- What is the relative abundance and distribution of micromonosporas and other cellulolytic microorganisms (clostridia, *Fibrobacter* and anaerobic fungi) in the lakes?

Chapter 2 - General methods

2.1. Field sampling

Cellulose baits were used to target cellulose degrading organisms in the lakes. These consisted of cotton strings that were de-waxed by repeated chloroform-ethanol wash in a Soxhlet apparatus (Wood, 1988) (Fig 2.1.). The baits were placed in nylon mesh bags that were attached to nylon ropes at intervals corresponding to the required depths. The ropes were placed in the lakes by attaching one end to bricks and the other to a buoy. The samples taken in October and November 2005 and in August, September and October 2006 were placed at five depths both in Esthwaite Water (3.0, 7.0, 11.5, 13.5 metres and sediment surface) and Priest Pot (0.5, 1.0, 2.0, 3.0 metres and sediment surface). The cotton baits removed in November 2005 were left in the lakes for 1 and 3 months, with one cotton bait sample at each depth. The samples taken in the summer of 2006 were left in the lakes for 6 (August), 12 (September) and 4 weeks (October) and each depth was represented by three cotton baits that were placed in separate nylon bags.

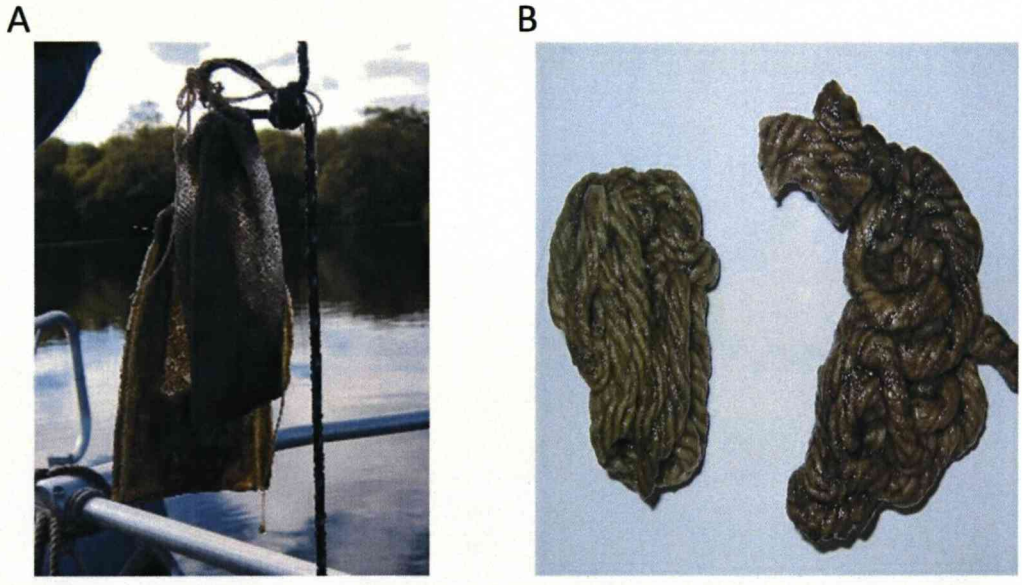


Figure 2.1.Cotton baits.

A, bags containing baits attached to ropes; B, colonised cotton baits recovered from Priest Pot.

For the experiment to detect the presence of hyphae in the lakes (November 2007), the cotton baits were placed at 6 depths in Esthwaite Water only; the depths

analysed were those indicated for the previous samplings, with inclusion of a surface sample. Three baits were placed at each depth and left in the lakes for a 5 week period.

A similar sampling arrangement was used for the molecular biological quantification of cellulolytic organisms in the lakes, with baits in both Esthwaite Water and Priest Pot following the arrangement indicated above, including a surface cotton bait. There were three baits at each depth, one of which was used for the extraction of nucleic acids, and the remaining two for the quantification of *Micromonospora* CFUs on isolation plates. Each depth also contained a 0.5 g nylon rope sample to be used as a control for surface attachment of microorganisms. Water and sediment samples were collected using the Freedinger apparatus and Jenkin corer (Ohnstad, 1982), respectively (Fig 2.2.).

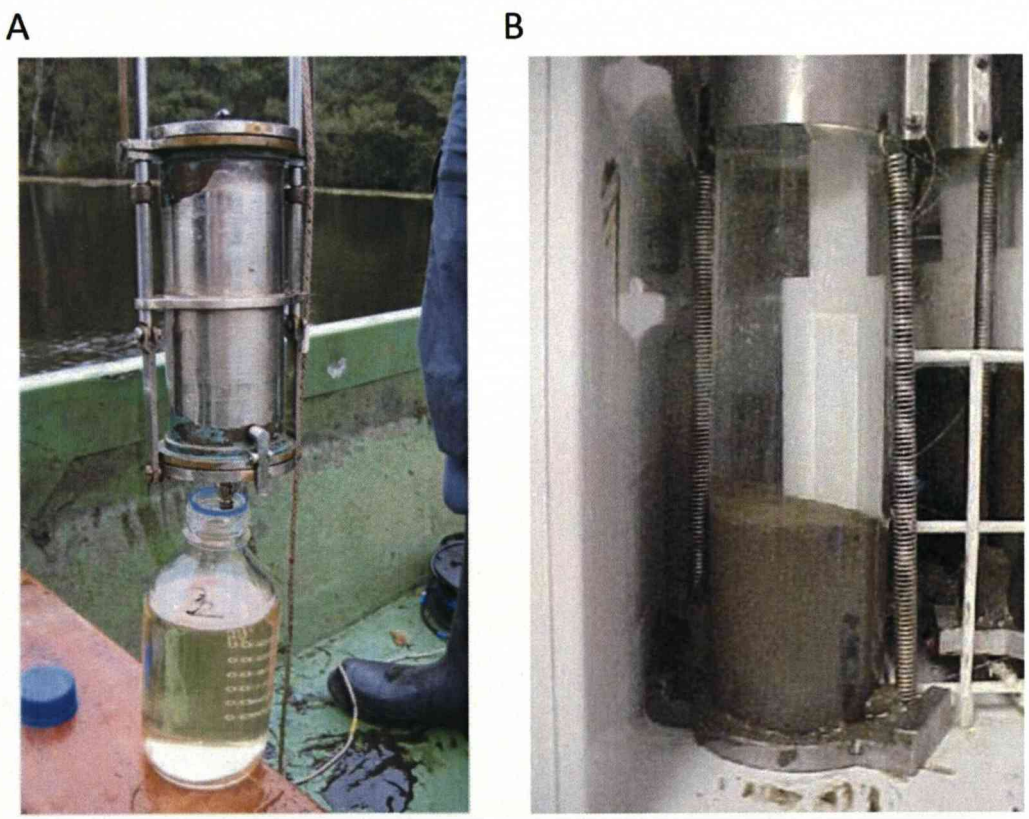


Figure 2.2. Sampling instrumentation.

A, Freedinger apparatus; B, Jenkin sediment corer.

2.2. Isolation of microorganisms

2.2.1. *Micromonospora*

Micromonospora strains were isolated by vigorous physical shaking of the recovered baits in sterile phosphate buffer (10 ml added to each 0.5 g bait) followed by heat treatment of the suspension at 65 °C for 10 min. The suspension was then plated in triplicate on M3 agar plates (McCarthy & Williams, 1990; Rowbotham & Cross, 1977). Sediment samples (20 ml wet volume) were serially diluted in sterile buffer before heat treatment and each dilution (150 µl) samples spread onto M3 agar plates in duplicate or triplicate. Lakewater samples (20 – 40 ml) were heated at 65° C for 10 min and passed through membranes (0.2 µm pore diameter) (Millipore), which were then placed face down on M3 agar plates, incubated at 30°C and removed after 24 h. The M3 plates were incubated for a further 2 weeks, *Micromonospora* colonies were enumerated and representative morphotypes were isolated and subcultured. *Micromonospora* colonies were identified by their appearance (small to medium sized orange, red, brown or black colonies, finely branching substrate hyphae <0.5 µm in diameter and little or no aerial hyphae) verified by direct observation of colonies under a light microscope fitted with a x32 long working distance objective lens. *Micromonospora* strains and isolates were routinely cultivated on Oatmeal agar (Shirling & Gottlieb, 1966) plus 0.2% yeast extract with incubation at 30° C. Cultures were stored as suspensions of hyphae and spores in 12.5% (v/v) glycerol at -80° C.

2.2.2. *Streptomyces*

Grey-green actinomycete colonies containing well developed aerial hyphae with a velvety or powdery appearance, displaying chains of spores and substrate hyphae of 0.5-2 µm in diameter were tentatively ascribed to the genus *Streptomyces*. A small number (10) of representatives of this group was isolated as described for *Micromonospora* sp. (2.21).

2.3. Determination of the presence of hyphae in colonised cellulose baits, and samples of water and sediment.

The determination of the presence of hyphae was based on the isolation method of Wakisaka *et al.* (1982). Extracts (4.5 ml) obtained from cellulose baits (section 2.2.) were added to 0.5 ml 0.1M NaOH on ice, followed after 5 min by neutralization with

0.5 ml 0.1M HCl. A 150 µl aliquot was plated on M3 agar containing 10 µg ml^{-1} tunicamycin, in triplicate. Heat treatment (65°C for 10 min) was used to destroy hyphae in the remaining aliquot (2 ml), 150 µl of which was also spread in triplicate on M3 agar plates containing tunicamycin, as were 10^{-1} dilutions. The plates were incubated at 30°C for 15 days before identifying and counting *Micromonospora* colonies. Sediment samples were initially diluted 10x in phosphate buffered saline (PBS, Sigma, St. Louis, Missouri, USA) and processed as described above for cellulose baits. Water column samples were treated similarly but without dilution, and aliquots filtered through 0.2 µm pore diameter membranes which were placed face down on tunicamycin-containing M3 agar, removed after 24h, and the plates returned to the incubator for up to 30 days.

2.4. Chlorophyll a measurements

Lakewater samples (100-200 ml) were filtered through GFF glass fibre membranes (Whatman, Maidstone, UK) and frozen at -20°C until required. Chlorophyll a was extracted by the addition of 5 ml of 90% acetone to the filters contained in 15 ml acetone-resistant centrifuge tubes. Samples were kept on ice and in the dark and were sonicated for 30 s at amplitude setting of 6 µm, followed by centrifugation for 5 min at 4380 g. Absorbances were measured in an Ultrospec 2000 spectrophotometers (Pharmacia Biotech) at 750, 664, 647 and 630 nm after blanking with 90% acetone. To calculate chlorophyll a concentrations, the equation used was obtained from Jeffrey and Humphrey (1975):

$$[\text{chlorophyll a}] = \left(11.85 \times (\text{abs } 664\text{ nm} - \text{abs } 750\text{ nm})\right) - (1.54 \times (\text{abs } 647\text{ nm} - \text{abs } 750\text{ nm} - 0.08 \times \text{abs } 630\text{ nm} - \text{abs } 750\text{ nm}$$

2.5. Scanning Electron Microscopy

Cotton bait samples colonised with *Micromonospora* strains were dehydrated in ethanol, critical point dried and coated with 60% Au/Pd in a Polaron E5100 Sputter Coater. Degraded cotton samples were examined using a Phillips XL 30 Scanning Electron Microscope with an accelerating voltage of 10 to 15 kV.

2.6. Growth media

M3 agar (Rowbotham & Cross, 1977): KH_2PO_4 (137 µM), Na_2HPO_4 (209 µM), KNO_3 (2 mM), NaCl (10.36 mM), $MgSO_4.7H_2O$ (769 µM), $CaCO_3$ (400 µM), Sodium propionate (0.2 g L^{-1}), $FeSO_4.7H_2O$ (1.389 µM), $ZnSO_4.7H_2O$ (1.216 µM), $MnSO_4$ (274 nM), cyclohexamide (5 mg L^{-1}), aneurine hydrochloride (0.4 mg L^{-1}), agar (18 g L^{-1}).

Minimal salts medium (McCarthy *et al.*, 1985): $(NH_4)_2SO_4$ (28.57 mM), $MgCl_2.6H_2O$ (1.887 mM), $CaCl_2$ (204 µM), yeast extract (2 g L^{-1}) and agar (18 g L^{-1}) were added to 0.1 M pH 8 phosphate buffer.

Oatmeal agar medium (Shirling & Gottlieb, 1966): 20 g of oatmeal was boiled in 1 litre of tapwater for 20 min and filtered through lint. The filtrate was restored back to 1 litre with the addition of 2 g of yeast extract, 18 g of agar and 50 mg of cyclohexamide, and the pH adjusted to 7.2.

LB Luria-Bertani (LB) (Merck, New Jersey, USA): yeast extract (5 g L^{-1}), casein peptone (10 g L^{-1}), NaCl (357 mM), with or without agar (15 g L^{-1}).

Yeast extract- malt extract (YEME): glucose (55.5 mM), yeast extract (3 g L^{-1}), malt extract (3 g L^{-1}), peptone (5 g L^{-1}), with of without agar (18 g L^{-1}).

2.7. Phosphate buffers

pH	1 M K_2HPO_4 (ml)	1M KH_2PO_4 (ml)
6.0	13.2	86.8
7.0	61.5	38.5
8.0	94.0	6.0

Table 2.1. Phosphate buffer composition (1M).

Phosphate buffers were produced for endoglucanase assays and for adding to the minimal salts media by the preparation of 1 M K_2HPO_4 and 1 M KH_2PO_4 which were added together at different volumes to achieve the required pH as shown in the table above and diluted appropriatelly.

2.8. Anaerobic incubation of *Micromonospora* strains

Micromonospora lake strains were grown on oatmeal plates and placed in anaerobic jars with the addition of AnaeroGEM™ (Oxoid, Cambridge, UK) to remove oxygen from the interior of the jar, and with the anaerobic indicator BR 55 (Oxoid) to monitor anoxic conditions.

2.9. Cellulose degradation by *Micromonospora* isolates

2.9.1. Cellulose agar

Cellulose agar plates were produced by adding a thin layer of M3 agar containing 5 g L^{-1} of ball milled filter paper (Whatman, Maidstone, UK) to plates already containing a thick layer of standard M3 or M3 supplemented with 1 g L^{-1} yeast extract. Low viscosity CMC (Sigma, St. Louis, Missouri, USA) (0.1%) was also added to the cellulose top layer to aid in keeping the cellulose fibres in suspension.

2.9.2. Degradation assays

2.9.2.1. Visual inspection of filter paper degration

Micromonospora strains were incubated in test tubes containing filter paper strips and M3 plus 0.4% yeast extract to a depth of ca. 20% of the tube. Degradation was assessed by visual examination after one month incubation at 30°C.

2.9.2.2. Scanning Electron Microscopy (SEM)

A selection of 17 strains were incubated at 30°C in 20 ml universal bottles containing 4 ml of sterile lakewater with dewaxed cotton (0.2 g), the degradation of which was examined by scanning electron microscopy.

2.9.3. Endoglucanase activity, extracellular protein and biomass measurements

Endoglucanase production was determined by growing *Micromonospora* isolates in minimal salts medium (McCarthy *et al.*, 1985) supplemented with 1% fibrous filter paper powder (Whatman®) and then measuring reducing sugars released from carboxymethylcellulose (CMC) following incubation with culture supernatants. The enzyme assays were modified from McCarthy *et al.* (1985). One ml of culture

supernatant, diluted appropriately in 0.1 M potassium phosphate buffer (pH 7) was added to 2 ml of 5% (w/v) low viscosity CMC (Sigma, St. Louis, Missouri, USA) dissolved in the same buffer and incubated for 1 h at 50°C with shaking at 170 rpm. Substrate and enzyme controls were used for every sample. Reducing sugars were determined by the dinitrosalicylic acid method (DNS) (Miller, 1959). Enzyme activity was expressed as units (U), corresponding to 1 µmol of glucose equivalents released per minute.

A total of 72 *Micromonospora* strains were cultured in minimal salts medium supplemented with 1% filter paper powder and incubated with shaking (75 rpm) at 30°C, in 250 ml conical flasks containing 40 ml of medium. After 17 days, cultures were harvested for determination of endoglucanase activity, supernatant protein concentration and total biomass (estimated as cellular protein).

2.9.3.1. DNS assay

The dinitrosalicylic acid reagent was prepared following the protocol published by Miller (1959) and comprised: 1% dinitrosalicylic acid, 0.2% phenol, 0.05% sodium sulfite, 1% sodium hydroxide. The assay was performed by adding 1 part of sample to one part of DNS reagent and heating to 85°C for 15 min followed by cooling in running tap water. Absorbances were measured at 575 nm. Glucose calibration curves were produced by dissolving glucose in 0.1 M pH 8 phosphate buffer at concentrations ranging from 0.025 to 1.5 mg L^{-1}.

2.9.3.2. Protein assays

Protein was measured in the culture supernatant and biomass extracts using the Bradford method (Bradford, 1976), the reagent comprised of methanol (20 ml), brilliant blue G (0.02 g), orthophosphate acid (20 ml) and distilled water (160 ml).
Two variations of the Bradford assay were used, the micro- and the macro assay, which are suitable for measuring high or low protein concentrations, respectively. The macroassay was used to measure protein concentrations in the culture supernatants and it involved adding 800 µl of sample to 200 µl of Bradford reagent, followed by a 15 min incubation and measurement of the A_{600}.

The microassay was used to quantify total biomass protein in the cultures: the culture was centrifuged and cell pellets resuspended in 1M NaOH and boiled for 20 min; cell debris was removed by centrifugation and 20µl of the resulting supernatant was added to 300 µl of Bradford reagent, followed by a 15 min incubation and measurement of the A_{600}.Tests were performed to show that the presence of 1M

NaOH in the 20 µl sample from boiled cell pellets did not interfere with the linearity of the Bradford reaction.

2.9.3.3. Trichloroacetic acid (TCA) precipitation of proteins

Proteins were precipitated using the TCA method:

Equal volumes of TCA (100%) were added to protein samples, incubated on ice for 30 min, followed by centrifugation at 12.000 G for 15 min. The resulting pellet was washed 3 times with a solution containing 95% ethanol and 5% 0.5 M Tris-HCl at pH 6.8 and air dried.

2.10. DNA extraction

2.10.1. Pure cultures

Micromonospora strains were grown in yeast extract – malt extract broth at 30 °C with shaking for 10 days. The biomass was harvested by centrifugation and total DNA extracted using a bead beating DNA extraction kit (Mo Bio UltraClean™) according to the manufacturer's instructions.

2.10.2. DNA/RNA extraction method

This method was published by Griffiths *et al.* (2000) and comprises the use of CTAB (Cetyl Trimethyl Ammonium Bromide)/Phenol/Chloroform extraction with bead beating, and PEG precipitation of the extracted nucleic acids. 0.5 ml of 5% CTAB/phosphate buffer (120 mM, pH 8) plus 0.5 ml phenol/chloroform/isoamylalcohol (25:24:1) were added to 0.5 g (dry weight) of sample in a bead beating tube (Qbiogene, Mesa California, USA) and shaken for 30 s in a FastPrep™ FP120 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 5.5 m s^{-1}, followed by centrifugation for 5 min at 14,500 G. The resulting top layer was harvested and placed in a clean eppendorf tube to which an equal volume of chloroform/isoamylalcohol (24:1) was added, followed by centrifugation for 5 min at 14,000 rpm. The top layer was extracted and placed in a clean eppendorf tube and a volume of PEG (30% polyethylene glycol 6000/1.6 M NaCl) equivalent to twice that of the sample was added and left either at room temperature for 2 h or overnight at 4°C. The DNA/RNA was harvested following centrifugation at 16,000 G

for 15 min and the resulting nucleic acid pellet washed with 70% ice cold ethanol and air dried. All the glassware used was washed with chloroform and the distilled water used was treated with diethyl pyrocarbonate (1 ml L^{-1} added with mixing until fully dissolved) to destroy RNAses.

For the extraction of nucleic acids from lakewater, samples were filtered through 0.2 µm pore size polycarbonate membranes (Millipore, Billerica, Massachusetts, USA) and these membranes were then added to the bead-beating tubes; the phenol caused the membrane to dissolve and did not interfere with the extraction.

2.10.3. NanoDrop quantification of nucleic acid

DNA and RNA extracts were quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA).

2.10.4. Picogreen quantification of nucleic acid

Double-stranded DNA was quantified for qPCR using Quanti-iT™ Picogreen® dsDNA reagent (Invitrogen, Paisley UK). A calibration curve was produced using phage lambda DNA diluted from 1 ng to 1 µg ml^{-1} in TE buffer in triplicate in a final volume of 200 µl with the Quanti-iTTM PicoGreen Reagent. The reactions were incubated for 5 min at room temperature in the dark. Sample fluorescence was measured using a microplate reader (Victor 3™, 1420 multilabel counter, Perkin Elmer™) and standard fluorescein wavelengths (excitation at 480 nm, emission at 520 nm).

2.11. PCR amplification

2.11.1 End point PCR

2.11.1.1. Direct PCR amplification

PCR amplification was performed using a PTC-200 DNA engine gradient cycler (MJ Research Ramsey, Minnesota, USA) or a Techne TC-312 cycler (Burlington, New Jersey, USA) or a G-storm™ (Gene Technologies Corporation, Braintree, Essex, UK). The Taq polymerases used in this study were either the BioTaq produced by Bioline (London, UK) or SuperTaq (HT-Biotech, Cambridge, UK). Standard amplification was carried out with an initial denaturing step for 2.5 min at 94°C,

followed by 30-35 amplification cycles of 1 min at 94°C, 1 min annealing step at the appropriate annealing temperature (Table 2.3.), and 1-1.5 min extension at 72°C. The amplification cycle was followed by a final extension step for 10 min at 72°C, and PCR products were stored in the PCR machine at 4°C until removed and processed. Each PCR reaction (50µl) contained 1µl of each primer solution (25 µM each), 1 µl of dNTP solution (each nucleotide at 10 nM), 5 µl of 10x PCR buffer (HT-Biotech or Bioline), 2.5 µl of Mg_2Cl (10 mM), 1 µl of SuperTaq or BioTaq, 1 or 2 µl of sample DNA at 50 ng μl^{-1}, 25µl of UV-sterilised 2.6% dimethylsulfoxide (DMSO) stock solution and distilled water to 50 µl. The primers used for PCR in this study and their specifications are presented in Table 2.3.

2.11.1.2. Nested PCR

Nested PCR involves the use of general primers for an initial round of amplification, followed by a second round using specific primers that should target a region within the sequence of the amplified PCR product. In this study, pA/pH general primers (Edwards *et al.*, 1989) were used to amplify the 16S rRNA gene followed by the amplification of *Fibrobacter* specific 16S rRNA using Fib1F and FibAR primers (McDonald *et al.*, 2008); Ns1-eukaryotic (White, 1990) and Univ-1390-universal (Zheng *et al.*, 1996) used to amplify the 18S rRNA followed by the specific detection of anaerobic fungi using Neocal-forward and Neocal-reverse primers (Lockhart *et al.*, 2006).

In order to increase amplicon length to 350 bp for phylogenetic analysis of the anaerobic fungi, nested PCR was performed again using a set of primers (NS-1 eukaryotic (White, 1990) and JB 205 (Tuckwell *et al.*, 2005)) to amplify a region of eukaryotic DNA including the 18S rRNA, ITS 1, 5S rRNA, ITS 2 and the 5' end of the 28S rRNA gene, followed by specific anaerobic fungal amplification of 18S rRNA gene and the 5' end of the ITS region using primers Neocal-forward (Lockhart *et al.*, 2006) and ANNR (Denman & McSweeney, 2006).

2.12. Quantitative PCR

2.12.1. Removal of DNA from RNA samples

DNA was removed from nucleic acid extracts used in cDNA synthesis for the qPCR assays by the use of Turbo DNA-free™ kit (Applied Biosystems, Carlsbad, California, USA). A volume of 0.5 µl. of 10xTurbo DNA-free buffer in addition to 1 µl

of DNAse was added to 45µl of nucleic acid sample and incubated at 37°C for 20 min. This was followed by the addition of 5µl of DNAse de-activation reagent and incubation at room temperature for 2 min with occasional mixing. The mixture was then centrifuged at 10.000 G for 1.5 min for the removal of the DNAase de-activation reagent.

2.12.2. Reverse transcription

Nucleic acid extracts were treated for the removal of DNA, and the presence of remaining DNA was monitored by qPCR. RNA was reverse transcribed using bioscript (Bioline, Taunton, MA, USA) RT-PCR kit: 0.01-0.5 µg of RNA was added to 0.2 µg of random hexamers plus and the volume made up to 12 µl with nuclease free water, followed by incubation at 70°C for 5 min. After incubation, 1 µl of 40 nM nucleotide solution containing 10 nM each of adenine, thymine, cytosine and guanine was added, as was 4.5 µl of 5x reaction buffer and nuclease-free water to a final volume of 19.75 µl, followed by the addition of 0.25 µl of bioscript containing the reverse-transcriptase at 200 units μl^{-1} and incubation at 37°C for 1 h.The reaction was stopped by heating at 70°C for 10 min.

2.12.3. Quantitative PCR analysis

Quantitative PCR method development was performed in the MJ mini™ system (Bio-Rad, Hercules, California, USA), whereas sample analysis was performed in the 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, California, USA), using SensiMix*Plus* SYBR kit (Quantace, London, UK), which contained a reaction buffer, heat activated Taq DNA polymerase, dNTPs, 6 nM $MgCl_2$, internal reference dye, stabilisers and SYBR® Green I. The amplification cycle consisted of a 10 min activation step at 95°C, followed by 35-40 cycles starting at 95°C for 15 s, 30 s at the appropriate primer annealing temperature, and 5-10 s (1 s for every 66 bp of to be amplified) at 72°C for extension. Measurement of the SYBR fluorescence was performed at the end of every extension step.

A melting curve from 55 to 95°C with readings taken at 0.5 °C increments held for 1 s was added to the end of the amplification cycle to determine the melting profile of the PCR products generated.

Each well on the multiwell tray of strip contained 12.5 µl of Sensimix qPCR reagent mix, 1-2 µl of DNA/cDNA template (<100 ng), 1µl of each primer solution (final concentration 400 nM) and distilled water to a final volume of 25 µl. Strips or trays were briefly centrifuged prior to analysis to collect the contents in the bottom of the well. The amplification products of qPCR analysis from natural samples were run on 1 or 2% agarose gels to confirm the presence of a single band in each sample.

2.12.4. Quantitative PCR calibrations

Primers pA and pH were used to amplify almost full length 16S rRNA sequences of *Micromonospora* lake strain 13, *Clostridium cellulolyticum* and *Fibrobacter succinogenes,* as wells as primer NS1-Eukariotic (Edwards *et al.*, 1989) and Univ-1390-universal (Zheng *et al.*, 1996) to amplify the 18S rRNA gene of *Neocallimastix frontallis*. These were cloned in Promega pGEM® -T Easy plasmids, which were purified using QIAprep miniprep plasmid purification kit and linearised using *ndEI* (*Micromonospora* lake strain 13, *C. cellulolyticum* and *F. succinogenes*) and *psTI* (*N. frontallis*) restriction enzymes. The control clones containing *C. cellulolyticum, F. succinogenes* and *N. frontallis* were provided by Dr. James McDonald. The concentration of DNA in the plasmid extract was calculated using PicoGreen and the copy number of plasmids and the SSU rRNA insert it contained was calculated using the following formula:

$$\left(\frac{X \frac{g}{\mu l} DNA}{[plasmid\ length\ in\ base\ pairs\ x\ 660]} \right) x\ 6.022\ x\ 10^{23} = Y \frac{molecules}{\mu l}$$

which was obtained from the QuantiFast™ SYBR® Green PCR Handbook. Dilutions of the plasmid extracts were made in order to obtain 5 concentrations ranging from 1x 10^8 to 1 x 10^3 SSU rRNA copies per µl. These were used to obtain calibration curves for every primer used in every run performed. Each concentration of plasmid was measured in triplicate in the calibration curves.

2.13. Electrophoresis

Electrophoresis was performed at constant voltage of 100V for 1 h, and the DNA was visualised on an ultraviolet transilluminator at a wavelength of 254 nm. DNA ladders (Hyperladder I or V, Bioline, London, UK) were run on each gel to enable

estimation of PCR product sizes and quantities (Fig 2.3.). Agarose gels were prepared by adding 1% agarose to TAE buffer and heating in a microwave oven until all agarose had melted. Once the agarose had cooled to approximately 50°C, ethidium bromide was added (0.5 μg ml^{-1}).TAE was made as a 50x stock (242 g Tris base, 57.1 ml glacial acetic acid, and 100 ml 0.5 M EDTA L^{-1}.

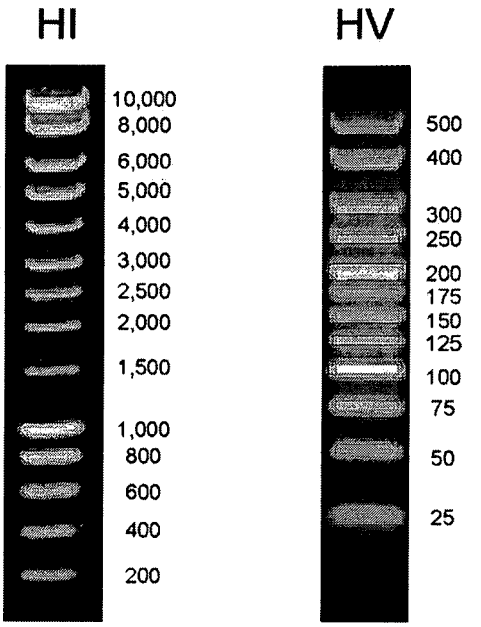


Figure 2.3. Molecular sizes of bands in DNA ladders.

Molecular sizes are in base pairs. HI, hyperladder I, HV, hyperladder V. Images obtained from Bioline.

2.14. Cloning PCR products

Gel-extracted PCR products were cloned using Promega pGEM® -T Easy vectors, which comprised pGEM plasmids cut with *EcoR* V and to which 3' thymidine was added to both ends, to increase ligation efficiency. According to the manufacturer's manual, the plasmids were 3015 bp in size and contained the α-peptide coding region of β-galactosidase, which allowed the identification of clones containing inserts by insertional inactivation followed by colour development of the clones when growing the cells in plates containing X-gal and IPTG. Successful cloning of an insert prevents the expression of β-galacosidase by interrupting the *lac*Z gene, which allows differentiation of blue colonies lacking insert (the production of β-

galactosidase allows the metabolism of x-gal, producing a blue colour).The plasmid contains multiple restriction sites, including *Eco*R I, used in this study to liberate inserts. This plasmid also contains an ampicillin resistance gene to enable the selection of cells that were successfully transformed. Briefly, the ligation involved adding 5µl of 2X ligation buffer, 1 µl of solution containing vector, 3 µl of the PCR products, and 1 µl of T4 phage DNA ligase, followed by incubation at room temperature for 1 h. The ligated plasmids were cloned into competent cells by transferring 50 µl of competent cells to the tubes containing the ligated plasmids and heat shocking the cells for 45-50 s at 42°C, with the immediate return of the tubes to ice for 2 min. Subsequently, 950 µl of LB broth was added to each tube and incubated for 2 h at 37°C with shaking at 150 rpm. Cells were plated in triplicate (10, 100 µl and re-suspended cell pellets) on LB agar plates containing ampicillin (100 ug ml^{-1}), and in which 100 µl of 100 mM IPTG and 20 µl of 50 mg ml^{-1} X-Gal had been incorporated. The IPTG was added to promote the expression of β-galactosidase, whereas the metabolism of X-Gal will result in the blue colouring of colonies containing plasmids devoid of inserts. The plates were left growing overnight at 37°C and white colonies were picked after leaving the plates at 4°C for 2 h to enhance the blue colour of colonies of cells lacking inserts. Cells from 4 white colonies in each cloning reaction were inoculated into LB broth containing ampicillin and grown at 37°C with shaking (300 rpm), and plasmids were extracted (section 2.15.).

2.14.1. Production of high transformation efficiency competent cells

Single *E. coli* colonies from LB plates containing 20 mM $MgSO_4$ were inoculated into 20 ml of TYM broth (2% bacto tryptone, 0.5% yeast extract, 0.1M NaCl, 10 mM $MgSO_4$) in 250 ml conical flasks. The cells were grown until mid-log phase (OD_{600} 0.2-0.8) then poured into 2 L flasks containing 100 ml of TYM broth and incubated with agitation at 37°C. At OD_{600} 0.6, the flask was cooled in cold water and shaken gently, followed by centrifugation of the cells for 15 min at 3,000 G. The cell pellet was re-suspended gently in 100 ml of cold Tfb I buffer which contained 30 mM KOAc, 50 nM $MnCl_2$, 100 mM KCL, 10 mM $CaCl_2$ and glycerol (final concentration 15%) and centrifuged again for 8 min at 3,000 g. The resulting cell pellet was re-suspended gently in 20 ml of cold Tfb II buffer which contained 10 mM pH 7 Na-MOPS, 75 mM $CaCl_2$, 10 mM KCl and glycerol (final concentration 15% v/v), and the resulting cell suspension was aliquoted (200 ml) into pre-chilled eppendorf tubes

and frozen by immersion in liquid nitrogen. The cells were stored at -80°C until needed.

2.15. Plasmid extraction and purification

Plasmids were extracted from transformed cells using QIAprep® miniprep (Qiagen, Crawley, UK), which provided highly pure plasmid DNA that could be sent directly for sequencing without further processing.

2.16. RFLP

The *gyr*B genes of 67 *Micromonospora* strains were amplified using primers UP1TL and UP2rTL (Kasai *et al.*, 2000) and were concentrated by ammonium acetate precipitation and incubated with *Bst*N I restriction enzyme overnight at 60°C. The products of restriction were run on a 2% agarose gel for 2 h and examined in a transilluminator. Since the *gyrB* gene of examples of each RFLP type were sequenced, a simulation of the RFLP was performed online at the New England Biosciences website (http://tools.neb.com/NEBcutter2/index.php) and details of band sizes could be verified. This check allowed the resolution of similar RFLP patterns that were difficult to differentiate in the gels.

Two restriction digest enzymes were used in this study: *Hae* II and *Bst*N I

Hae II digests were performed in a volume of 20 µl, with the addition of 2 µl of NEB 4 buffer, 20 µg of BSA, 20 units of enzyme and 384-5000 ng of template; reactions were run overnight at 37°C.

*Bst*N I digests were performed in a volume of 20 µl, with the addition of 2 µl of NEB 2 buffer, 20 µg of BSA, 20 units of enzyme and 384-5000 ng of template; reactions were run overnight at 60°C.

2.17. Precipitation of PCR products

The PCR precipitation methods used in this study were taken from Sambrook and Russell (2001).

Sodium acetate DNA precipitation method: 45 µl of PCR products was added to 4.5 µl 3 M sodium acetate and 90 µl of 100% ethanol at -20°C for 1 h, after which the DNA was recovered by centrifugation at 12.000 G for 15 min and air dried after washing with 70% ethanol.

Ammonium acetate DNA precipitation method: for a given volume of PCR products, a ¼ volume of 10 M ammonium acetate and 2x volume of 100% ethanol at -20°C were added and frozen in liquid nitrogen for 2 min, followed by centrifugation at 13.000 rpm for 30 min. The resulting pellet was washed twice with 70% acetone and air dried.

2.18. DNA sequencing

Plasmids and PCR products in suspension (30 µl of 100 ng μl^{-1} plasmid DNA or 50 ng μl^{-1} amplified DNA) in eppendorf tubes were sent to MWG Biotech (Ebersberg, Germany) or GATC (Konstanz, Germany) or Macrogen (Seoul, South Korea) for sequencing.

2.19. Sequence analysis

2.19.1. Contig assembly

Forward and reverse *gyrB*, 16S rRNA and 18S rRNA-ITS I gene sequences were assembled together into contigs using the Pregap4 and Gap4 programs of the Staden package (Staden, 1996), and accuracy of the assembled traces was manually inspected.

2.19.2. *Micromonospora* 16S rRNA and *gyrB* gene phylogenetic analysis

For the *gyrB* phylogenetic analysis, the positions corresponding to primer sequences, gap columns, and hypervariable bases were removed, leaving a final alignment of 1151 bp covering the region 314 to 1486 of the *gyrB* sequence of *Escherichia coli* K-12. The optimised alignment was exported and a maximum-likelihood tree was constructed using PhyML (v 2.4.4) (Guindon *et al.*, 2005), run as a standalone package with 100 bootstrap samplings, the HKY substitution model,

empirical base frequency estimates, fixed Ts/tv ratios (4.00), fixed proportion of invariable sites (p-inv = 0.00) and 4 substitution rate categories with fixed gamma distribution (alpha = 2.00). Phylip DNAPARS was run in ARB in order to calculate a maximum-parsimony tree with vertical gaps compression, random sequence order and 100 bootstrap samplings. Tree topology and bootstrap support for branching were compared between the two trees.

2.19.3. *Clostridium* III, *Fibrobacter* and anaerobic fungi environmental clones phylogenetic analysis

Environmental clone sequences were uploaded into the SILVA aligner (http://www.arb-silva.de/) (*Clostridium* III and anaerobic fungi), or the Greengenes aligner (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) (*Fibrobacter*), with the addition of the 5 (*Clostridium* III and *Fibrobacter* and anaerobic fungi 18S-ITS trees) or 15 (anaerobic fungi 18S trees) closest matches present in the database. Identical or chimaeric sequences downloaded from the databases were removed based on Pintail analysis built into the online Silva aligner and Greengenes database.

For the *Clostridium* III and *Fibrobacter* tree outgroups, it was necessary to download and align specific sequences (*Actinoplanes* spp. or *Bacteroides fragillis*) in Greengenes, and import these into the *Clostridium* III Arb and *Fibrobacter* database where they were re-aligned using the Arb aligner using pre-aligned *Clostridium* III and *Fibrobacter* sequences as a reference. The outgroup for the anaerobic fungi was present in the initial custom Arb database as the 15 closest matches in the databases included sequences from organisms from a different phylum (Zygomycota).

Neighbour Joining trees were produced in Arb, with 1000 bootstraps and Olsen (*Clostridium* III, *Fibrobacter*) or Felsenstein (anaerobic fungi 18S rRNA) substitution model corrections. The susbstitution model used is detected automatically by Arb and the choice is based primarily on type of nucleic acid, sequence length and GC content. Phyml trees were produced by exporting the alignment to PhyMl online (http://www.atgc-montpellier.fr/phyml/) and using PhyML (v 3) (Guindon *et al.*, 2005), run as a standalone package with 100 bootstrap samplings, the HKY substitution model, empirical base frequency estimates, fixed Ts/tv ratios (4.00), fixed proportion of invariable sites (p-inv = 0.00) and 4 substitution rate categories with fixed gamma distribution (alpha = 2.00).

2.20. Strains

Group	Strain	Origin and reference number
Bacteria	*Bacillus subtilis*	UoL 52
	Clostridium acetobutylicum	DSMZ 792^{T}
	Clostridium cellulolyticum	DSMZ 5812
	Clostdidium leptum	DSMZ 753
	Clostridium propionicum	DSMZ 1682
	Escherichia coli	UoL 220
	Fibrobacter succinogenes	RRI S85
	Micromonospora chalcea	NCIMB 12879[I]
	Pseudomonas putida	UoL 186
Fungi	*Aspergillus nidulans*	G 00
	Fusarium fujikuroi	IMI 58289
	Neocallimastix frontallis	UoL M62704
	Neurospora crassa	UoL
	Sacharomyces cerevisiae	UoL A9/2

Table 2.2. Strains used in this study.

Strains were used as negative or positive controls for PCR amplifications, qPCR calibration curve, endoglucanase activity measurement or phylogenetic analysis. DNA was extracted from the strains by boiling cells for 5 min followed by centrifugation to remove cell debris. UoL, University of Liverpool culture collection; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen; NCIMB, National Collection of Industrial and Marine Bacteria; RRI, Rowett Research Institute; IMI, Commonwealth Mycological Institute, Kew, UK; G, Glasgow Collection. T, type strain.

2.21. PCR and qPCR primers used in this study.

Table 2.3. Sequence and properties of primers used in this study.

Target	Name	Sequence (5'-3')	Annealing temperature	Application	Reference
Bacterial 16S rRNA	pA	AGAGTTTGATCCTGGCTCAG	55°C	PCR	Edwards *et al.* (1989)
	pH	AAGGAGGTGATCCAGCCGCA			
	1369F	CGGTGAATACGTTCYCGG	60°C	qPCR	Susuki *et al.*(2000)
	Prok 1429R	GGWTACCTTGTTACGACTT			
Bacterial *gyrB* gene	UP1TL	CAYGCNGGNGGNAARTTYGA	59°C	PCR	Kasai *et al.* (2000)
	UP2rTL	TCNACRTCNGCRTCNGTCAT			
Eukaryotic 18S	NS1-eukaryotic	AAGGAGGTGATCCAGCCGCA	50°C	PCR	White *et al.* (1990)
	Univ-1390-universal	CGGTGAATACGTTCYCGG	50°C		Zheng *et al.*(1996)
Eukaryotic 28S	JB205	TCCTCCGCTTATTAATATGC	55°C	PCR	Tuckwell *et al.* (2005)
Micromonospora spp. 16S rRNA	M1C	TTACCTGGGTTTGACATGG	60°C	PCR/qPCR	This study
	M2D	ACATCGAACGAGGGTTGC			

Clostridium cluster III 16S rRNA	Clost3F (S-*-Cther-0650-a-S-23)	TCTTGAGTGYYGGAGAGGAAAGC		PCR/qPCR	Van Dyke and McCarthy (2002)
	Clost3R (S-*-Cther—1352-a-A-19)	GRCAGTATDCTGACCTRCC		PCR	
	829C3R	GGATACTTATTGTGTTWACTMCGG	60°C	qPCR	This study
	Clost3RF	GGYAGGTCAGHATACTGY		qPCR	
	1423C3R	TAYYGACTTCGGGTGTTGC		qPCR	
Fibrobacter spp. 16S rRNA	Fib1F	CCGKSCCAACGSSCGG	60°C	PCR	McDonald *et al.* (2008)
	Fib2AR	ATCTCTTCGCYGCGGCGWTYCC			
	FibroQ153F	CCGKSCCAACGSSCGGHTAA		qPCR	
	FibroQ238R	CSCCWACTRGYTAATCRGAC			
Neocallimastigales 18S rRNA	Neocal-forward (Chyt-719-F)	GCACTTCATTGTGTGTACTG	60°C/58°C	PCR	Lockhart *et al.* (2006)
	Neocal-reverse (Chyt-1553-R)	GGATGAAACTCGTTGACTTC	60°C	PCR/qPCR	
	NeoQF	CTTAGAGGGACTATAGATTTTTAATC	60°C	qPCR	This study
Neocallimastigales ITSI region	ANNF	GAGGAAGTAAAAGTCGTAACAAGGTTTC	60°C	qPCR	Denman *et al.*(2006)
	ANNR	CAAATTCACAAAGGGTAGGATGATT	60/58°C	PCR/qPCR	

Chapter 3 - The ecology and cellulolytic activity of micromonosporas in lakes

3.1. Overview

Micromonosporas are known to occur in lakes, especially in the sediments where they can be relatively abundant (Cross, 1981). Evidence for growth in lake sediments has been reported in the past (Johnston & Cross, 1976), but due to the generalised view that actinomycetes are soil organisms, their status as indigenous freshwater bacteria is not established. Micromonosporas are also well known degraders of polymeric substances including cellulose (Kawamoto, 1984) but their role in cellulose degradation in the environment has never been assessed, and their cellulolytic system is not well characterised. Against this background, this chapter focuses on the isolation of micromonosporas from colonised cellulose baits placed in two lakes of the English Lake District, and on the cellulose degradation abilities of these isolates. The growth form of these organisms in the colonised baits (i.e. spores or hyphae) is also investigated.

3.2. Sampling Sites

The sampling sites chosen were two lakes of contrasting trophic status in the English Lake District, Esthwaite Water and Priest Pot. The former is 15 m in depth, 113.3 ha in area and eutrophic (average chlorophyll a concentration 54 μg L^{-1}) (George *et al.*, 2000). Priest Pot is 3.5 m deep, 1 ha in area and is hypereutrophic (chlorophyll a concentration reaching 800 μg L^{-1}) (Finlay, 2000). Stratification and the formation of an anaerobic zone occur in the summer months in both lakes but is more persistent in Priest Pot, which is sheltered and has steep oxygen and temperature gradients.

A significant amount of microbiological research has been carried out in Esthwaite Water and Priest Pot, making these sites ideal for this project. These two lakes were once a single, larger water body, formed when glaciers covering the region started to retreat at the end of the last glacial period, and separated sometime between the 17^{th} and 19^{th} centuries as sediment accumulated in the region connecting the two lakes (Finlay, 2000). The perimeter of Priest Pot itself is formed mostly of woodland,

whereas Esthwaite Water is mostly surrounded by pasture where sheep can periodically graze. A trout farm is also located within Esthwaite Water.

The changes in the general characteristics of the terrain have been recorded in detail through the centuries, and show that Priest Pot is decreasing in area, and that the woodland around it is becoming more dense. No major stream enters Priest Pot and the majority of the sediment deposited in this pond originated from the immediate surroundings. Drainage ditches cut through the field grazed by sheep and ensure an ample supply of nutrients into Priest Pot, leading to very high productivity levels. Esthwaite, being a larger lake, is not as productive as Priest Pot, but is still classified as eutrophic (George *et al.*, 2000).

A number of investigations have been performed on the physical and biological properties of both lakes and relevant examples include studies on viruses (Goddard *et al.*, 2005), protozoans (Finlay & Esteban, 1998), phytoplankton and zooplankton (Madgwick *et al.*, 2006; Talling, 2003), physical parameters and biogeochemistry (Casper *et al.*, 2000; Folkard *et al.*, 2007; George *et al.*, 2000; Suggett *et al.*, 2006), and microbial communities (Edwards *et al.*, 2001; Goddard *et al.*, 2005; Miskin *et al.*, 1999), including ammonia oxidising bacteria (Whitby *et al.*, 2001).

3.3. Isolation of *Micromonospora* strains

3.3.1. Cellulose baits

Cellulose baits consisting of dewaxed cotton strings were used to to isolate cellulolytic micromonosporas present in the lakes. The cotton was dewaxed in order to prevent interference of waxes in the selection of exclusively cellulose degrading organisms.

A total of 3 sampling trips were undertaken in order to isolate micromonosporas.

3.3.2. Isolation media

It is standard practice to use low nutrient media to select for micromonosporas from environmental samples. Minimal medium M3 (Rowbotham & Cross, 1977) is often used to select for actinomycetes, and this medium was compared to standard LB and half strength tryptone soy agar for the growth of selected *Micromonospora*

laboratory strains. It was observed that micromonosporas would show visible growth on M3 agar after 6 days in some cases, whereas no colonies were formed on LB and very limited growth was visible in the half strength tryptone soy agar plates even after 10 days of incubation. *Micromonospora* growth on M3 agar plates was therefore considered good and rapid, as two or more weeks after inoculation are often cited as the necessary incubation period before visual inspection of growth (Johnston & Cross, 1976; Kawamoto, 1984; McCarthy & Williams, 1990; Vobis, 2006).

3.3.3. First isolation experiment

The aim of this first sampling experiment was to determine if micromonosporas could be recovered from baits, sediment and water samples from Esthwaite Water and Priest Pot on M3 agar plates in combination with heat treatment. Due to the production of heat resistant spores, the recovery of *Micromonospora* spores from natural samples is straightforward with the use of a heat treatment that removes Gram-negative contaminants, although the same treatment can potentially remove *Micromonospora* hyphal fragments from the sample. Application of a treatment regime of 70°C for 10 minutes has been used in previous studies to isolate micromonosporas (Rowbotham & Cross, 1977; Sandrak, 1977) and should allow the recovery of *Micromonospora* spores, but it was decided to test whether a more gentle treatment could still be effective in removing unwanted bacteria whilst enabling the recovery of *Micromonospora* hyphal fragments. A treatment of 55°C for 6 minutes was therefore also applied to cotton bait extracts as a starting point for the development of a milder isolation method.

Samples were taken from 3 different depths (surface, middle and bottom of water column) from 1 and 2 month old baits from both lakes (section 2.1.). Weather conditions were good, but the area had received considerable amounts of precipitation in the previous two weeks; water levels were very high and consequently the lakes had received considerable amounts of terrestrial wash-in.

Samples were maintained cold on ice and subsequently in a temperature controlled room overnight (4°C), followed by isolation experiments. Extracts were produced from the baits by shaking in sterile phosphate-buffered saline for 10 min at 14000 rpm; two separate extract aliquots were subjected to either the stringent (70°C x 10

min) or the gentle (55°C x 6 min) heat treatment, and 150 µl of these extracts were plated on M3 agar, and incubated at 30°C. Growth of *Micromonospora* colonies was monitored over a 12 day period.

It was clear after three days incubation that the gentle heat treatment was not effective, and the plates were covered with a mixed bacterial biofilm. Serial dilutions produced cleaner plates but the recovery of actinomycetes in these plates was very low. When extracts heated at 70°C for 10 min were re-examined, very few bacteria other than actinomycetes or bacilli were observed, and a significantly higher recovery of *Micromonospora* colonies was obtained at the same dilution.

3.3.4. Optimization of heat treatments

Although 55°C for 6 min was not effective for *Micromonospora* isolation, it was possible that their hyphae were more heat resistant to heat than most of the competing bacteria on isolation plates, and the effects of varying heat treatments on the hyphae, spores and on the competitors was studied in order to optimise the isolation method. The aim was to lower the temperature used in order to increase chances of hyphal recovery and to minimise any effects that heat might have on spore germination; it was also critical to understand the sensitivity of spores and hyphae to the treatment used. The effect of a 65°C heat treatment for increasing lengths of time was determined for a pure culture at different growth stages, from a young, largely spore-free culture to a mature culture with a very high concentration of spores. (Fig 3.1.).

The results show that treating 1 and 2 day old cultures for 5 minutes at 65°C produced a sharp decrease in the number of colonies obtained, followed by a small increase due perhaps to spore heat activation (Hoskisson *et al.*, 2000) and stabilization at 10, 15 and 30 minutes. This was probably caused by the selective removal of hyphae at 5 min. Subsequently, when the culture was 4 and 5 days old, at 5 minutes the same dramatic decrease was observed, but was followed by another significant decrease to 10 minutes, followed by stabilization. This longer CFU number decrease in older cultures might be due to increased resistance to heat in older hyphal fragments, or lack of heat resistance in immature spores.

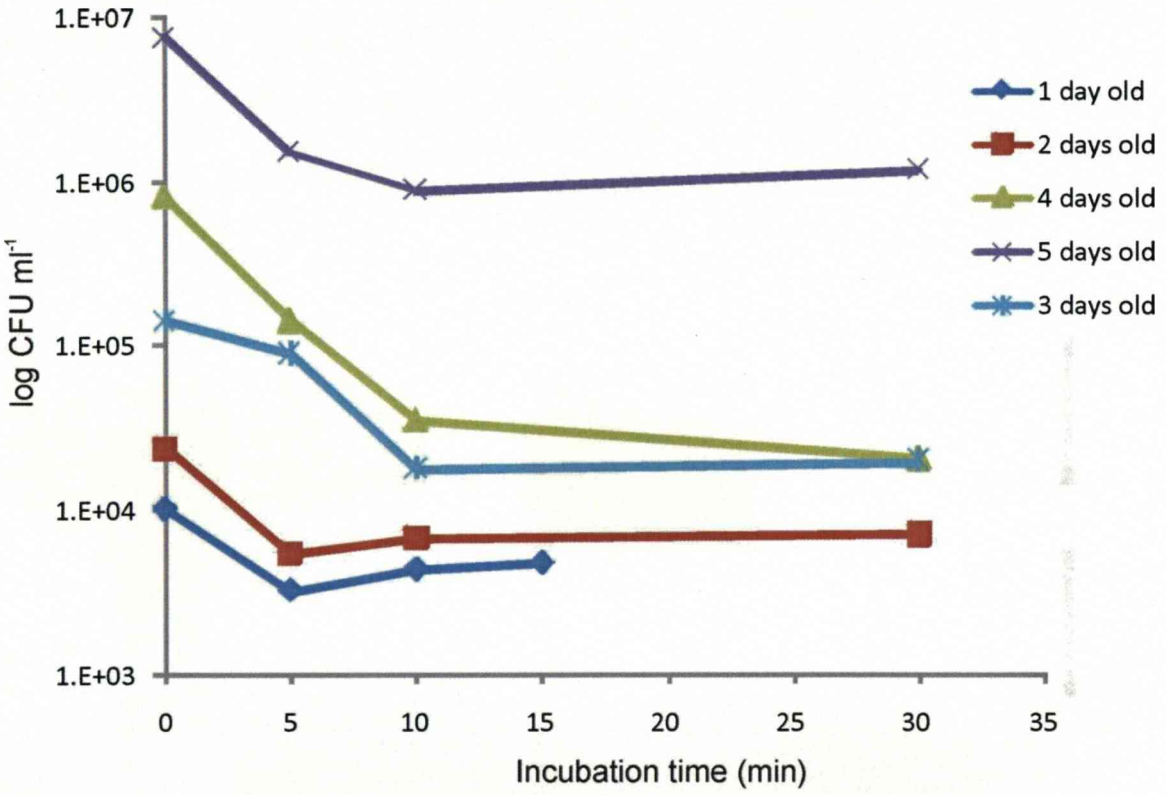


Figure 3.1. Effect of heat treatment on CFU recovery at different growth stages of lake strain N° 14.

Suspensions were prepared from the same culture at different ages and treated at 65°C for 0, 5, 10, 15 and 30 minutes. (Note: the value at 0 min, 5 days old is an estimate based on the previous day's proportions as colony numbers were too high to be counted on the plates). Cell suspensions were spread on M3 agar and incubated at 30°C for 16 days prior to determination of CFU numbers.

A second experiment was aimed at selecting for both hyphae and spores from a mixed community by decreasing the heat treatment time. Extracts were produced as described above from two separate cotton baits incubated in a lakewater microcosm (20 L carboy filled with lakewater and kept at room temperature with occasional mixing) in the laboratory for one month, and aliquots were plated in duplicate after being subjected to heat treatment at 65°C for 0, 1, 3 and 10 min. The plates that received unheated extracts contained no *Micromonospora* colonies at all, and were covered with film of mixed bacterial growth (Fig 3.2.). After one minute, the amount of general bacterial growth on the plates decreased and was separated into individual colonies, but still covering most of the plate surface. After 3 min, unwanted non-actinomycete colonies were still in significant numbers, but much reduced in size, and *Micromonospora* recovery was significantly increased. At 10 min, very few non-actinomycete colonies were present, and *Micromonospora* numbers were higher than at 3 min for one of the baits (B), but lower in the other bait.

The decrease in *Micromonospora* numbers from 3 to 10 min in bait A may have been caused by selective elimination of hyphae, which suggests that the application of a heat treatment regime of 65°C for 3 min may be adequate for the recovery of both *Micromonospora* hyphae and spores. However, bait B did not give the same result. From the previous experiment, it was known that most or all the hyphae were destroyed at 5 min. at 65°C, and it was concluded that attempting to select for both hyphae and spores using this short heat treatment could potentially introduce errors; in addition plates for both baits were significantly covered with Gram-negative bacterial contaminants at 3 min.

Heat treating the extracts at 65°C for 10 min. produced clean agar plates from which the isolation of *Micromonospora* would be straightforward, whereas the survival of spores observed at 15 and 30 min in Fig 3.1. showed that this heat treatment regime was not affecting spore survival. This was chosen as the standard heat treatment to be used in subsequent experiments.

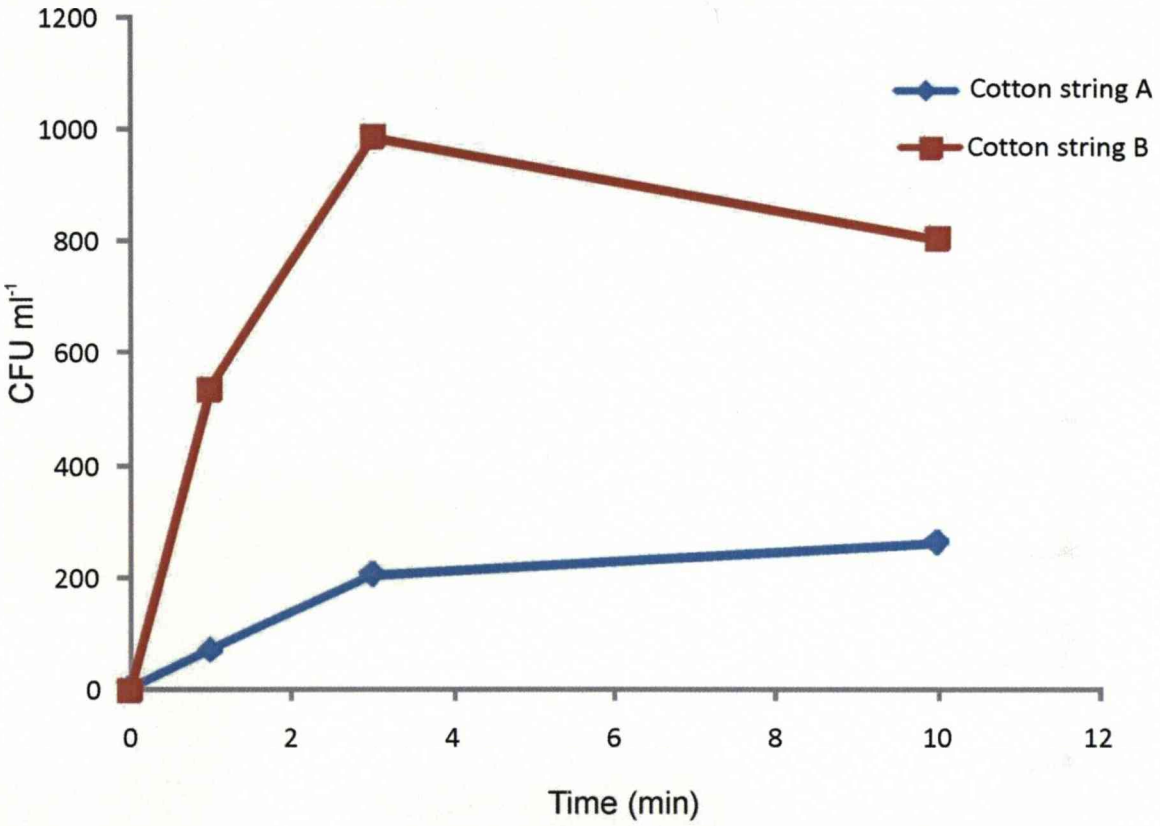


Figure 3.2. Determination of the effects of heat treatment on *Micromonospora* recovery from cotton baits.

Numbers were obtained on isolation agar plates from two cotton baits incubated in a lakewater microcosm and treated at 65°C for different periods of time (averages of two determinations). Bait extracts were spread on M3 agar and incubated for 13 days at 30°C.

3.3.5. *Micromonospora* isolates

There were three rounds of *Micromonospora* isolation. The first 23 strains were isolated from the plates obtained from the first field experiment described above. Twelve days after inoculation, *Micromonospora* colonies were tentatively identified based on morphological features (section 2.2.1.). The isolation was carried out with the intention of obtaining a wide range of morphologies; a plate with a high number of isolates was initially scanned for morphotypes, which were subcultured, and new strains were isolated from successive plates only if they had a different morphology. A few other actinomycete strains were also isolated and tentatively identified as *Streptomyces spp.*

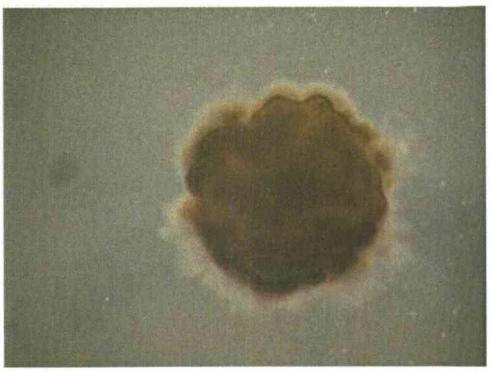
Colonies were subcultured on fresh M3 agar plates and the morphology was again checked when the colonies were well developed. Several strains were discarded because their morphology was very similar to other isolates, or their identification as a *Micromonospora* strain was dubious, i.e. due to the presence of aerial hyphae not observed in the original colony.

A second round of isolations was carried out in November 2005, using 1 month-old cotton baits recovered from Esthwaite Water and Priest Pot. Again, isolates were chosen on the basis of morphology. This experiment was mainly designed to study the distribution of micromonosporas in the baits, water column and sediments, and only four isolates were retained in the culture collection. A third round of isolation was carried out in August 2006 in order to increase coverage of the diversity of *Micromonospora* strains from the lakes. The isolation of strains was carried out using the same criteria as described above.

A total of 72 isolates representing diverse morphologies constituted the culture collection. In Fig 3.3., examples of the morphology of different *Micromonospora* isolates are presented, highlighting the features that were used as criteria to identify and differentiate the colonies.

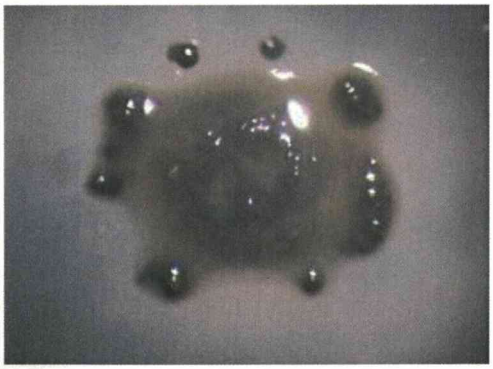
Figure 3.3. Examples of *Micromonospora* morphologies observed in the isolated strains, and the morphological features used to differentiate strains.

Strain 55: Typical *Micromonospora* morphology: brown, leathery surface colonies with a concentration of spores on the surface (approx. 2 mm in diameter).



Strain 70: Medium sized brown colony with a sub-surface spore mass covering most of the colony area (approx. 1.5 mm in diameter).

Strain 66: Colony showing a less common morphology, white or pale pink hyphae forming a sparse network. Spore mass scattered in clumps in the central colony area (approx. 1.5 mm in diameter).



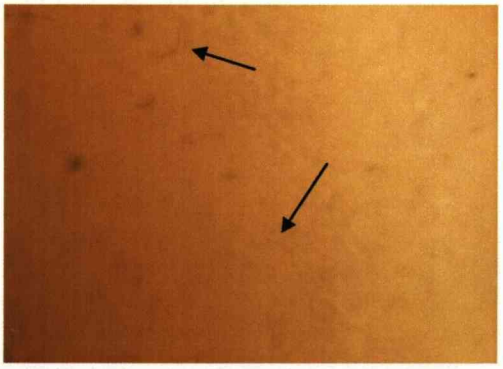
Strain 67: *Micromonospora sp.* producing a large spore mass that has a mucoid appearance, readily dispersible. Hyphal network not spreading beyond the area covered by spore mass (approx. 2 mm in diameter).

Fig 3.3. continued.

Strain 84: Light brown, leathery colony with no distinct spore mass on surface. Hyphal network only just visible at the edges of the colony (approx. 2 mm in diameter).

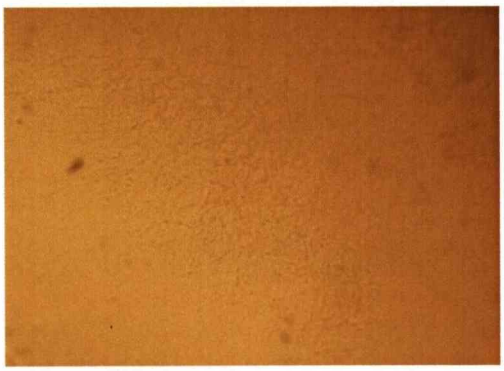
Strain 66: Small colony with a dry mass of spores forming a raised structure above the agar surface (approx. 1 mm in diameter).

Strain 72: Unusual morphology. Light pink/brown colony, spore mass forming concentric rings on the surface, hyphae finely branching but forming a rather sparse network (approx. 2 mm in diameter).

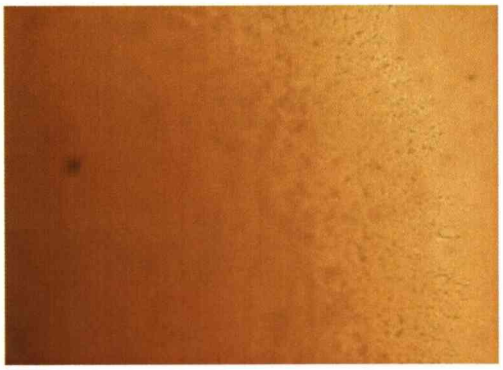


Unidentified colony on isolation plate showing the presence of short sterile aerial hyphae (arrows). 320X magnification.

Fig 3.3. continued.



Close up of the edge of an unidentified colony showing a relatively long and sparse extension of the hyphal network beyond the centre of the colony. 320X magnification.



Close up of the edge of an identified colony showing a compact and relatively short extension of hyphal network beyond the main colony centre. 320X magnification.

3.4. Distribution and growth of micromonosporas in lakes

3.4.1. Overview

The distribution of *Micromonospora* strains in the two lakes was analysed using the same cotton bait system as described for strain isolation (section 2.1. and 2.2.). Specific questions to be addressed in these experiments were: Is there any relationship between water depth and the numbers of micromonosporas recovered from baits and water samples? Is there evidence that they have colonised the cellulose baits? Is there a difference in the size of the *Micromonospora* population between the eutrophic lake Esthwaite Water and the hypereutrophic Priest Pot? Are there marked differences in *Micromonospora* numbers at different times of the year?

Using a combination of heat treatments and antibiotics, a method was also developed to definitively differentiate *Micromonospora* colonies that had arisen from hyphae and spores in the colonised baits, lakewater and sediment samples.

Numbers of *Micromonospora* were determined for the baits from November 2005 and through the summer of 2006. In November 2005, baits that had been in the lakes for 1 and 3 months were removed and processed as described previously (section 2.1 and 2.2). These baits were part of a different study and only a sample of varying string lengths was used for the isolation. For this reason the bait length was measured and numbers adjusted accordingly to make the data comparable between the baits, and the numbers obtained on this occasion are not directly comparable to those of subsequent samplings, in which measurements were made per gram of bait placed in the lakes. In June 2006, two sets of cotton baits were placed in the lakes, one removed after 6 weeks and the other after 12 weeks. Another set of ropes was placed in the lakes in August and removed after 12 weeks. The baits were placed in triplicate in the lakes, and three replicate isolation series were produced per bait in the laboratory. Samples were also taken from the water column at three different depths and from the sediments in August and October (section 2.2.). Numbers of *Streptomyces* spp. as defined in the methods section 2.2 were also determined as these organisms are considered more terrestrial in contrast to micromonosporas and could offer an interesting comparison with the latter.

3.4.2. Sampling

Temperature and dissolved oxygen profiles show that except for November 2005, there was a marked stratification of the water column, as would be expected for the summer season (Fig 3.4. and 3.5.). Priest Pot was generally cooler than Esthwaite Water, as it is sheltered and surrounded by dense woodland. Dissolved oxygen profiles show that Esthwaite Water was still stratified in November 2005, despite temperatures remaining very stable throughout the water column. Except for Priest Pot in November 2005, dissolved oxygen concentrations were always low at and just above the sediments of both lakes (Fig. 3.5.).

Visual inspection of recovered baits (Table 3.1.) showed that those placed near the surface were often more colonised with a biofilm than those placed in the deeper reaches of the lakes. Despite the presence of a thick microbial biofilm, the baits in the upper water column were often not as physically degraded as those placed near or at the sediment surface. The baits recovered from the sediment surface would often seem devoid of a biofilm, being white in colour or light brown/black due to the presence of silt, however they were often significantly degraded as verified by their lack of physical integrity. The baits placed in the middle of the water column were often the least degraded, especially in Esthwaite Water. As expected, baits that were submerged in the lakes for the longest periods of time were more colonised and degraded. Levels of degradation and colonisation were generally similar for both lakes.

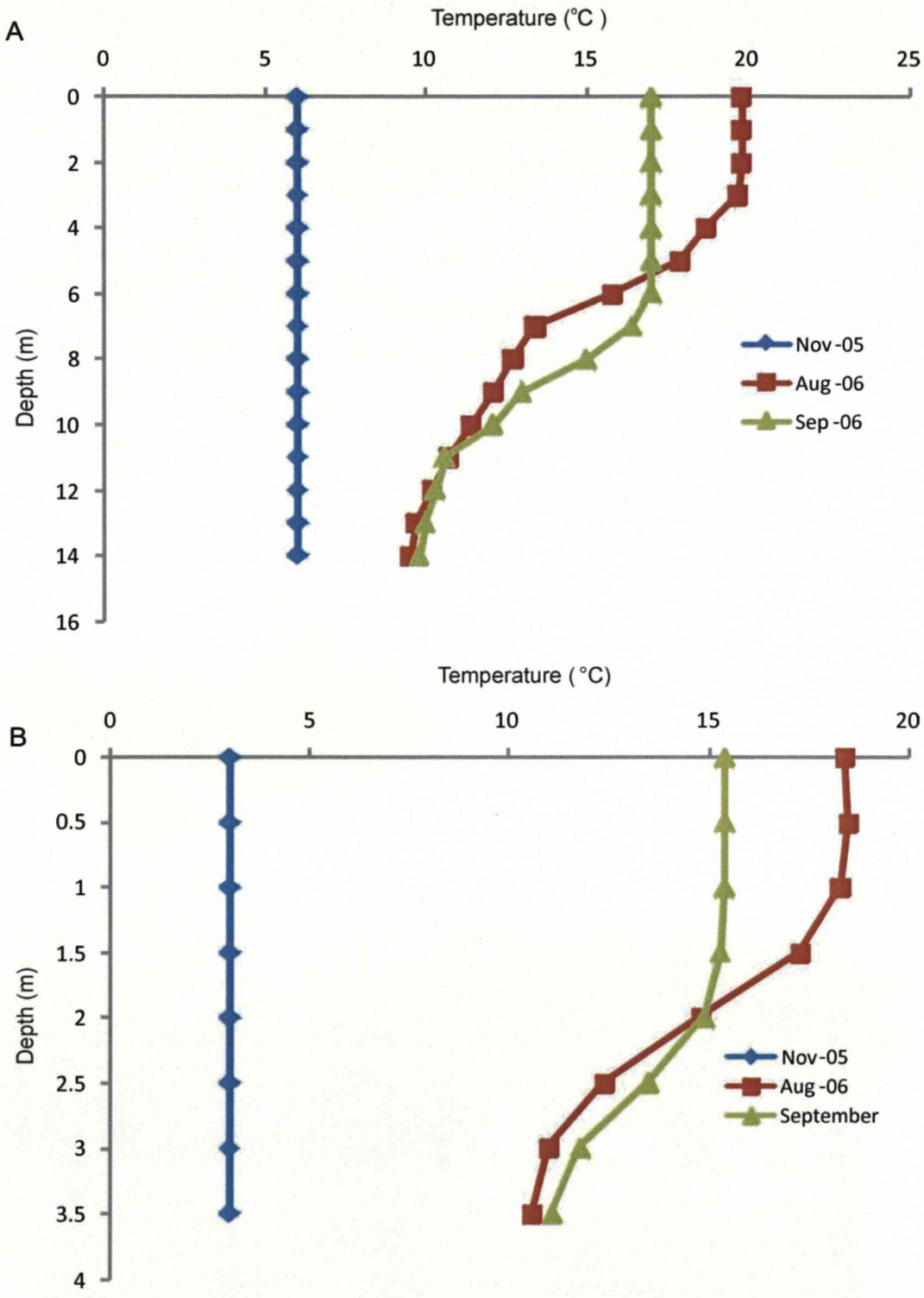


Figure 3.4. Temperature profiles in Esthwaite Water and Priest Pot during the 2005- 2006 samplings.

A, Esthwaite Water; B, Priest Pot.

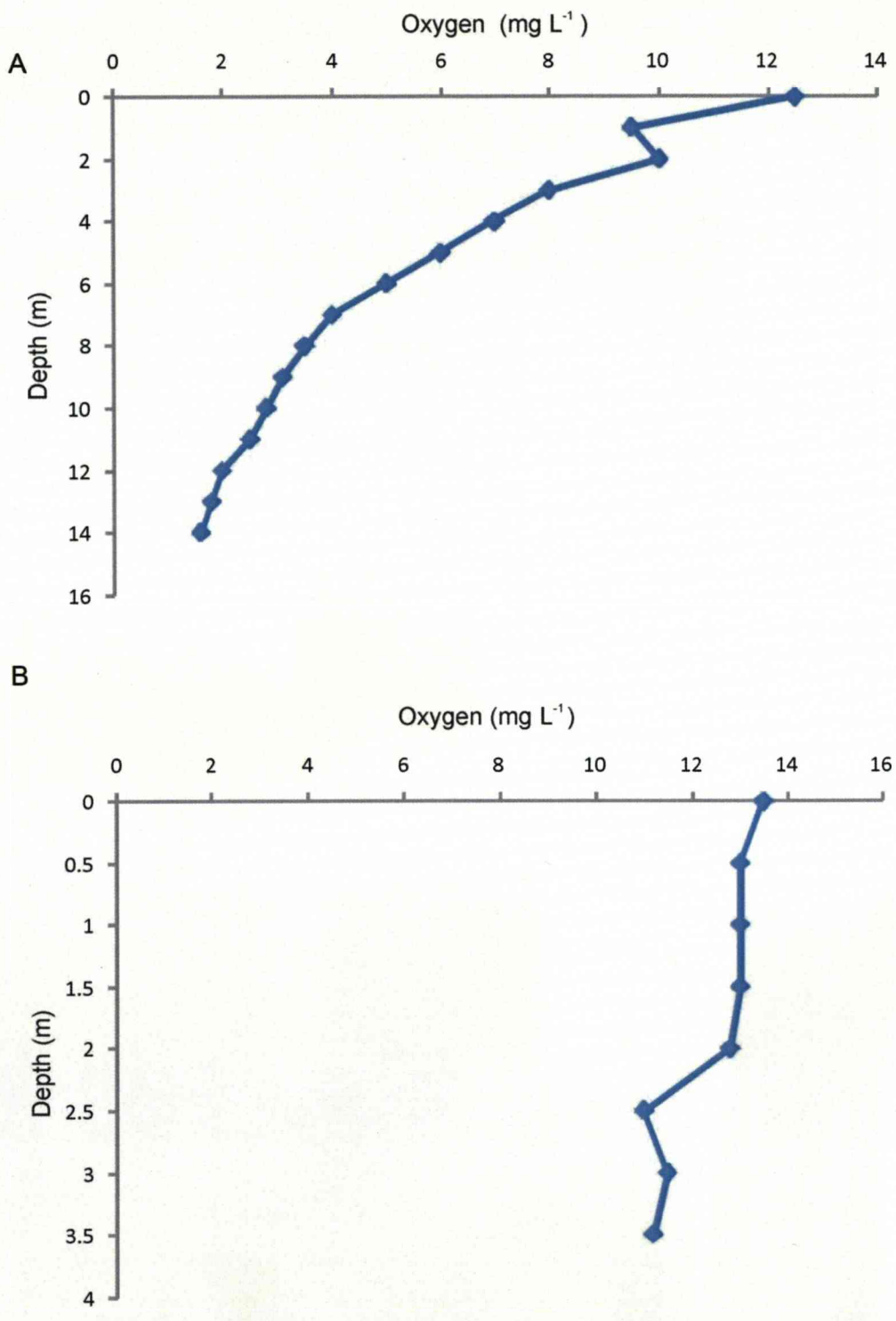


Figure 3.5. Dissolved oxygen profiles in November 2005.

A, Esthwaite Water; B, Priest Pot (B).

Esthwaite Water		
Depth (m)	colonisation	degradation
Nov-05 1 month		
3	+	+/-
7	+	+
11	+/-	+/-
14	-	+
15	-	+
Nov-05 3 month		
3	+	+/-
7	+	+/-
11	+/-	+
14	-	+
15	-	++
Aug-06		
3	+/-	++
7	+/-	++
11	-	+/-
14	-	+/-
15	-	+/-
Sept-06		
3	++	+
7	++	+/-
11	+/-	+/-
14	-	+/-
15	+/-	++
Oct-06		
3	+	+/-
7	+	+/-
11	++	+/-
14	-	+/-
15	-	+/-

Priest Pot		
Depth (m)	colonisation	degradation
Nov-05 1 month		
0.5	+	+/-
1.5	+	+/-
2	+/-	+/-
3	+/-	+
3.5	+/-	+
Nov-05 3 month		
0.5	+	+/-
1.5	+	+/-
2	+/-	+/-
3	-	+
3.5	-	++
Aug-06		
0.5	++	+/-
1.5	+	+/-
2	+/-	+/-
3	-	+/-
3.5	-	++
Sept-06		
0.5	++	+
1.5	+	+/-
2	+/-	+/-
3	+/-	+/-
3.5	-	++
Oct-06		
0.5	+	+/-
1.5	+	+/-
2	+/-	+/-
3	+/-	+/-
3.5	+/-	+/-

Table 3.1. Levels of colonisation in the form of biofilm and physical degradation of baits retrieved from the lakes.

+/-, low or no visible colonisation or degradation; +, colonisation or degradation visually obvious; ++, colonisation or degradation extensive.

3.4.3. *Micromonospora* and *Streptomyces* depth profiles

Depth profiles of *Micromonospora* and *Streptomyces* numbers were similar in each lake in November 2005 (Fig 3.6. and 3.7.), but this was not reproduced in the summer 2006 samples, except in the August-October baits. Depth profiles of either *Micromonospora* spp. or *Streptomyces* spp. were not similar when comparing one lake with the other, although there were exceptions, such as in June-September samples. One distinctive feature for the profiles of both organisms analysed was that in the three month old samples, the *Micromonospora* numbers were significantly higher in the bait placed above the sediment in both lakes.

In the summer of 2006 samples, *Micromonospora* and *Streptomyces* numbers increased in the surface baits with residence time, as was the case with the samples recovered from Priest Pot in November 2005. The baits placed deep in the lakes on the other hand did not show a distinctive pattern, and sometimes yielded higher numbers than those at the surface; likewise baits submerged for longer periods did not always yield higher numbers of *Micromonospora* and *Streptomyces* colonies (Fig 3.8. and 3.9.). The absence of an increase of *Micromonospora* numbers with bait residence time may have occurred due to the onset of stratification of the water column and formation of an oxycline below which oxygen concentrations may remain below that required for the growth of these microorganisms. Stratification may be temporary, and growth and sporulation could occur whenever oxygen concentrations increase due to mixing of the water column, but frequent interruption of growth due to periodical anoxia could prevent a constant increase in the size of the *Micromonospora* population in the deep baits, contrasting with the observations on samples taken closer to the surface.

As explained above, the low numbers observed in November 2005 cannot be directly compared to the numbers obtained in the summer 2006 samples; in relative terms, the numbers of *Micromonospora* were lower than those of *Streptomyces* on this occasion, but the numbers were generally low for both groups of organisms. In one set of samples (June-August baits), numbers of *Streptomyces* were much lower than those of *Micromonospora* in both lakes, which could indicate that, despite the coincidence in their depth profiles observed in November 2005, the dynamics of micromonosporas in these lakes differs to that of streptomycetes.

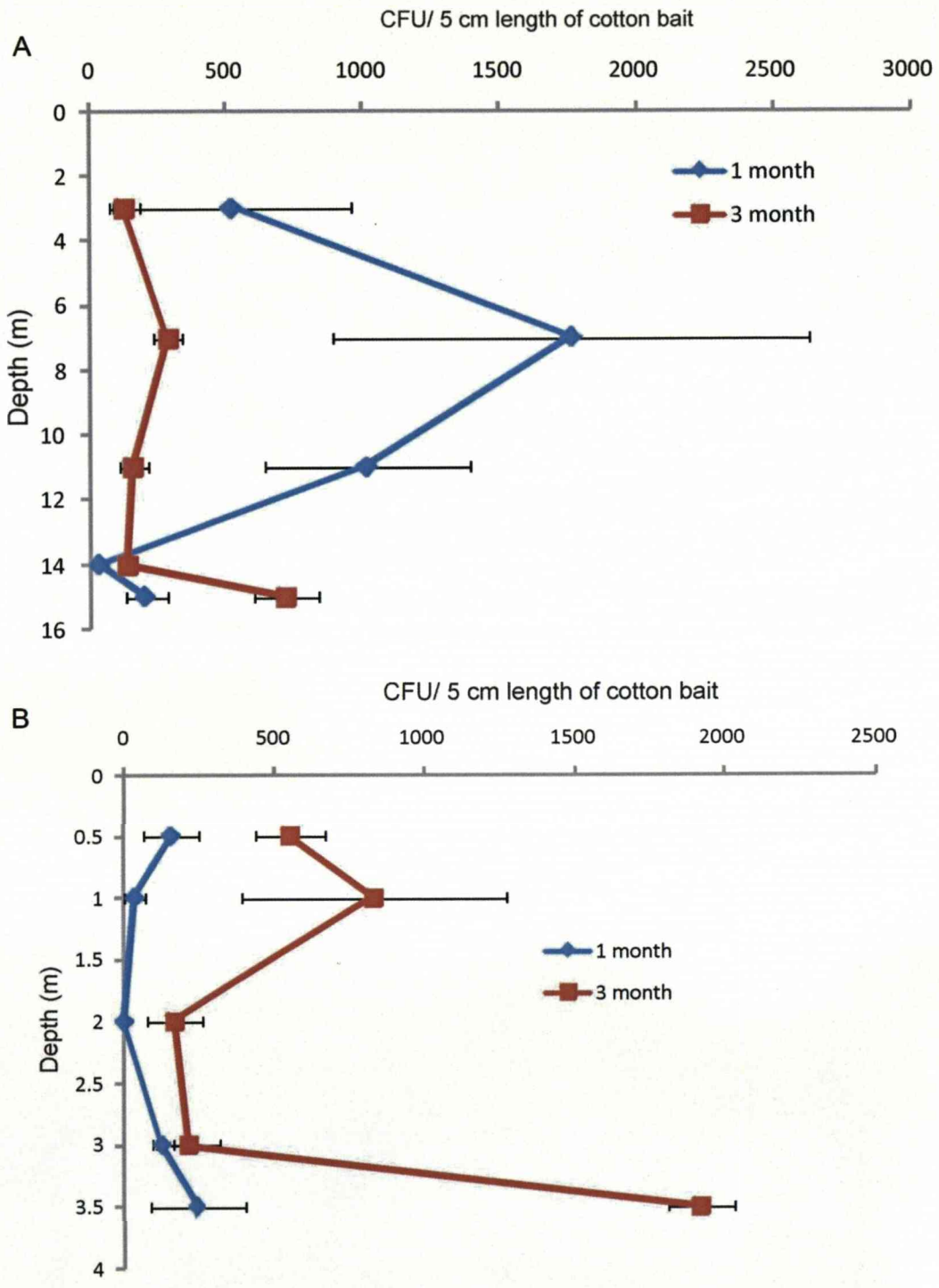


Figure 3.6. Depth profile of *Micromonospora* CFU numbers in baits recovered from the lakes in November 2005.

Numbers were obtained from 1 month and 3 month old cellulose bait recovered from Esthwaite Water (A) and Priest Pot (B) in November 2005. Cotton bait extracts were spread on M3 agar and incubated at 30°C for 14 days prior to CFU number determination (averages of 3 determinations, bars are standard errors).

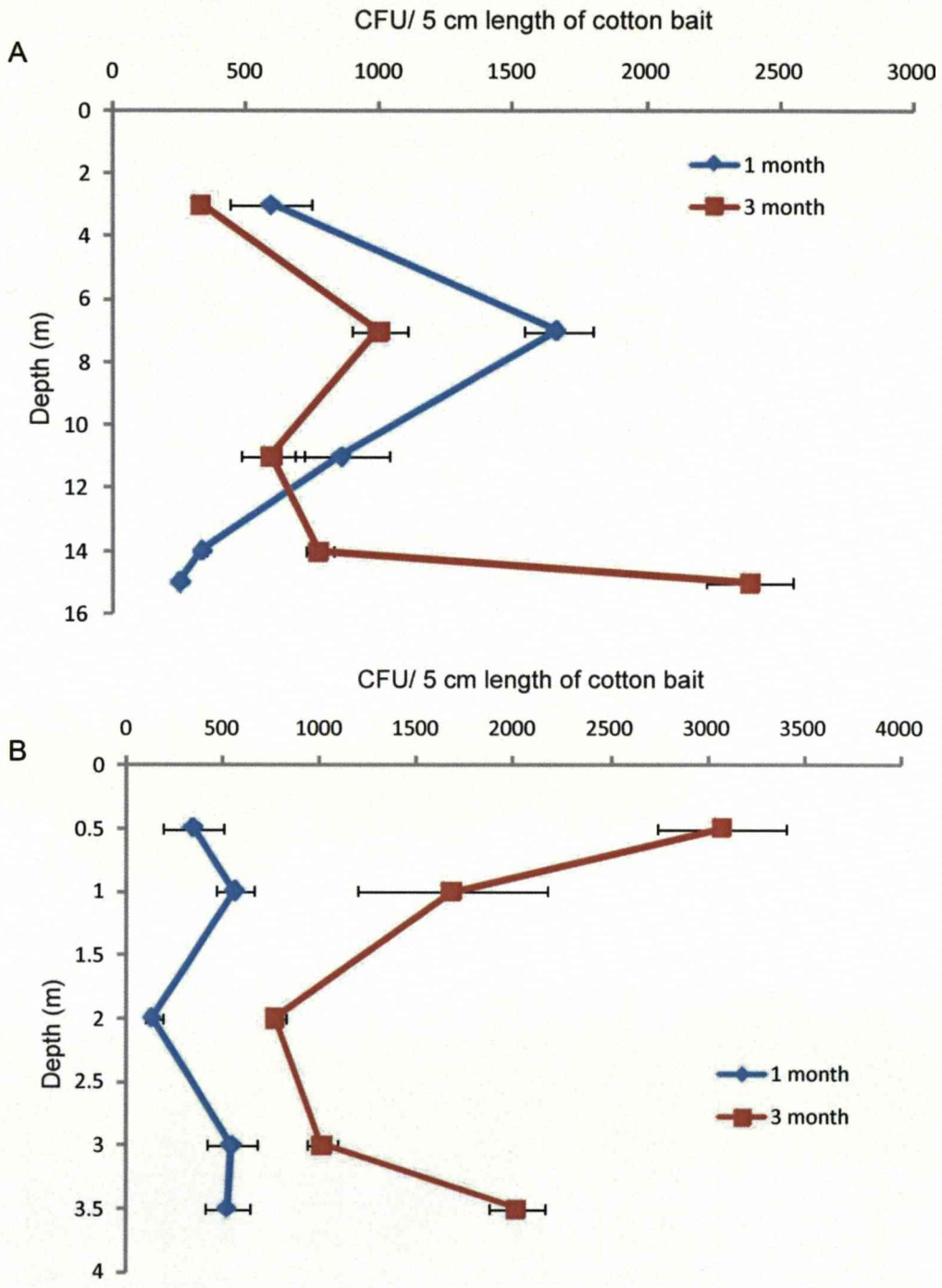


Figure 3.7. Depth profile of *Streptomyces* CFU numbers in baits recovered from the lakes in November 2005.

Numbers were obtained from 1 month and 3 month old cellulose baits recovered from Esthwaite Water (A) and Priest Pot (B) in November 2005. Cotton bait extracts were spread on M3 agar and incubated at 30°C for 14 days prior to CFU number determination (averages of 3 determinations, error bars are standard errors).

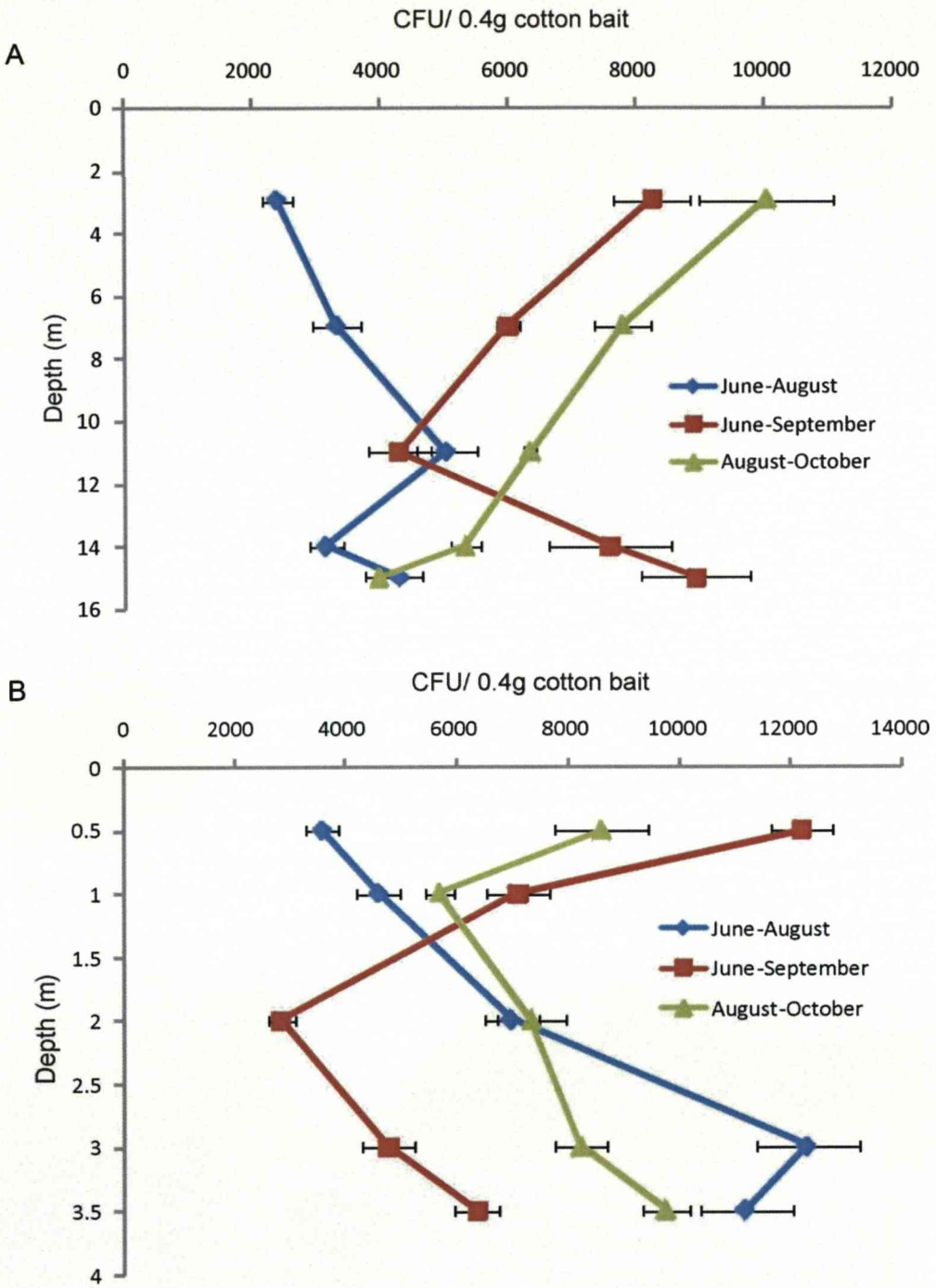


Figure 3.8. Depth profile of *Micromonospora* CFU numbers in the summer of 2006.

Numbers were obtained from baits placed in Esthwaite Water (A) and Priest Pot (B). (averages of 9 determinations, error bars are standard errors).

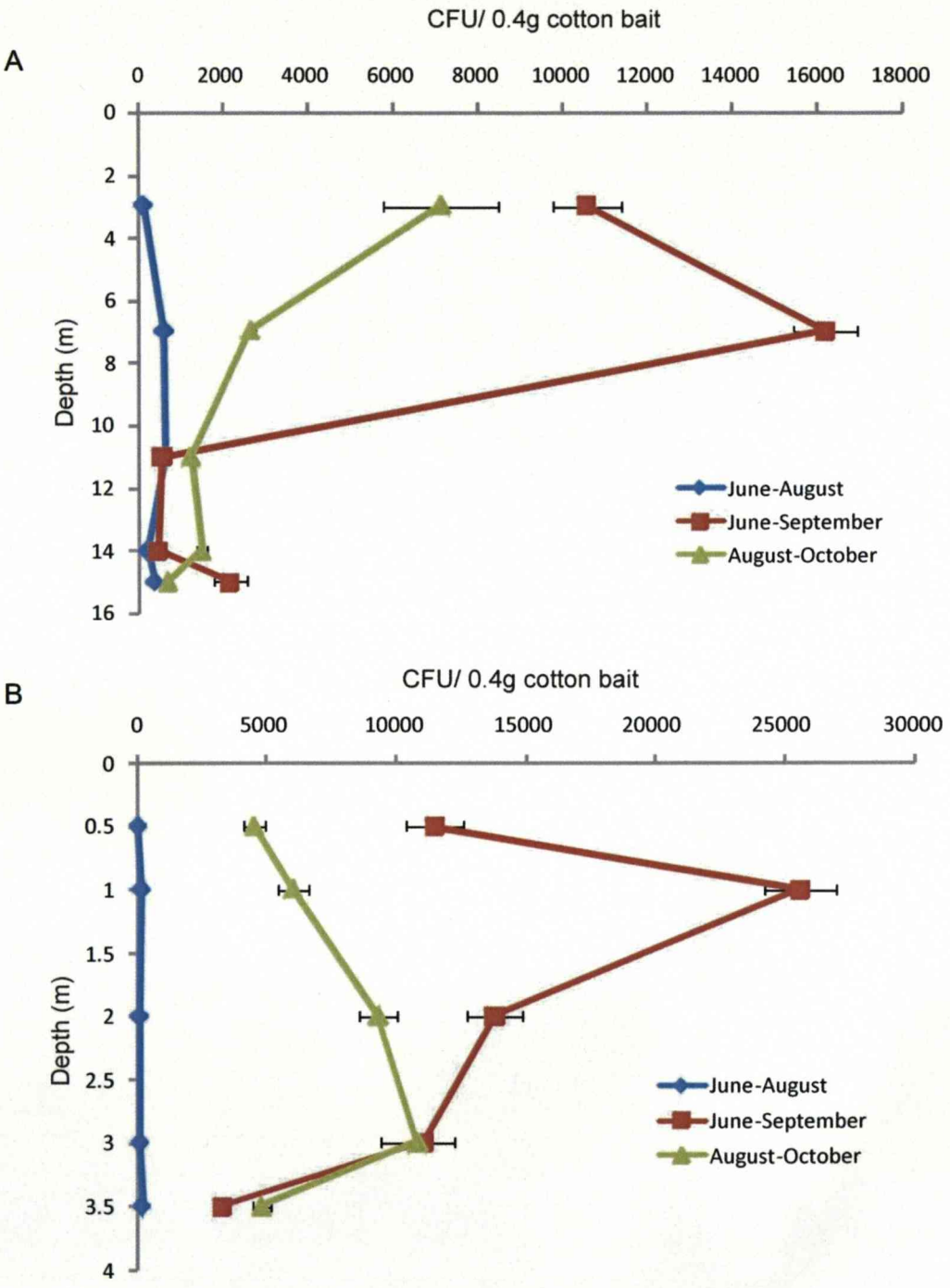


Figure 3.9. Depth profile of *Streptomyces* CFU numbers in the summer of 2006.

Numbers were obtained from baits placed in Esthwaite Water (A) and Priest Pot (B). (averages of 9 determinations, error bars are standard errors).

Micromonospora numbers in lakewater were much higher in Priest Pot than in Esthwaite Water in August in surface waters and were also higher in the deeper samples in both lakes (Table 3.2. A). This scenario changed significantly in October when the values for both lakes were quite similar. *Streptomyces* numbers on the other hand were very low in August and increased 10 – 40-fold in October (Table 3.2. B). In August, the numbers of streptomycetes were too low to allow any pattern to be determined but in October values were higher in deeper samples. The large increase in *Streptomyces* numbers might have caused an artefact on the isolation plates since streptomycetes are faster-growing and could out-compete *Micromonospora* colonies, decreasing overall numbers of the latter. This would explain the observed reverse trend in comparative numbers of these two genera. The same situation occurred in the sediment samples in October, in which the numbers of *Micromonospora* spp. seemed to decrease compared to the numbers obtained in August whereas those of *Streptomyces* spp. increased. Despite this, it is clear that whereas *Micromonospora* numbers are similar in the sediments of both lakes, *Streptomyces* spp. numbers are significantly (6 – 10 times) higher in Esthwaite Water than in Priest Pot, both in August and October (Table 3.2. A and 3.2. B).

In summary, it was possible to establish that *Micromonospora* is easily isolated from the cotton baits, and that their numbers vary seasonally. Depth profiles of *Micromonospora* CFU numbers do not necessarily follow those of *Streptomyces* spp., which were more variable. The number of *Micromonospora* CFU recovered from the baits often increased with bait residence time, although in deep samples that was not always the case, possibly due to interruption of growth caused by periodical anoxia.

A

Priest Pot		
Depth (m)	August	October
0.5	3.9×10^3	6.1×10^3
2.5	1.3×10^4	4.9×10^4
Sediment	2.4×10^5	1.5×10^5

Esthwaite Water		
Depth (m)	August	October
3	1.2×10^3	
7		7.8×10^3
11	3×10^3	5.7×10^3
Sediment	2×10^5	8.4×10^4

B

Priest Pot		
Depth (m)	August	October
0.5	1.1×10^2	2.9×10^3
2.5	8.3×10^1	2.3×10^3
Sediment	6.2×10^3	5.6×10^3

Esthwaite Water		
Depth (m)	August	October
3	1.4×10^2	
7		1×10^3
11	1.2×10^2	1.9×10^3
Sediment	1.7×10^4	3.2×10^4

Table 3.2. Numbers of micromonosporas and streptomycetes in the water column and sediment.

Numbers of micromonosporas (A) and streptomycetes (B) in the water column (CFU L^{-1}) and in the sediment (CFU g^{-1} wet sediment) of Priest Pot and Esthwaite Water. Numbers shown are averages of two or three determinations.

3.5. Determination of the presence of *Micromonospora* hyphae in the lakes.

3.5.1. Method development

The methods used to determine *Micromonospora* numbers in the lakes selected for spores only, and the numbers obtained were not necessarily representative of an active community indigenous to the lake ecosystem. It was therefore important to determine if vegetative cells, i.e. hyphae, were also present in the lakes, and if so, their distribution. Johnston *et al.* (1976) used sonication to demonstrate that culture suspensions of *Micromonospora*, *Nocardia* and *Streptomyces* yielded increased CFU numbers due to the fragmentation of the mycelium into progressively smaller units, with numbers peaking just before a lethal effect became apparent and the numbers decreased dramatically. With this method, the presence of mycelium in lake muds could be demonstrated for *micromonosporas* but not for *streptomycetes* or nocardiae. Sonication was used here in an attempt to demonstrate the presence of hyphae in the two lakes studied. Fig 3.10. shows the results of sonicating a 3 day old cell suspension consisting mainly of hyphae for 10 seconds at 3 different amplitudes. Even at the lowest amplitude tested, the increase in the CFU numbers was significant, and 10s at amplitude of 3.5 was chosen as a potential test treatment to be applied to environmental samples.

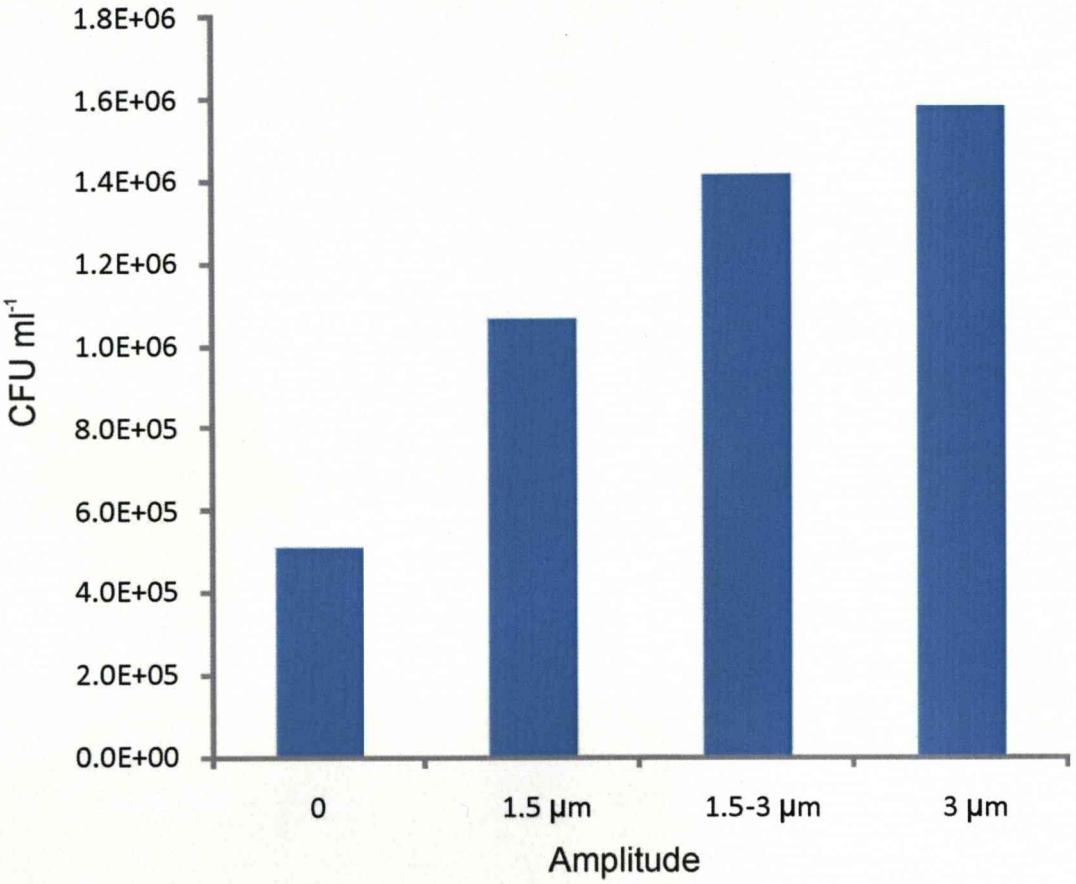


Figure 3.10. Fragmentation of *Micromonospora* hyphae by the application of sonication.

Numbers of colony forming units were obtained from a cell suspension of a 3 day old *Micromonospora* culture sonicated for 10 seconds at different intensities. Cultures were incubated at 30°C on M3 agar for 14 days.

An alternative approach was also considered to differentiate colonies arising from hyphal fragments and spores. Wakisaka *et al.*(1982) described a selective isolation method for micromonosporas that does not require the use of heat treatment but uses a combination of NaOH to decrease contamination by Gram-negative organisms, and the antibiotic tunicamycin, which prevents the growth of most Gram-positive organisms, but not micromonosporas. This isolation method does not therefore eliminate the hyphae from environmental samples, but by subsequently using heat treatment to remove the hyphae, it should be possible to determine whether the sample contained only spores. The results (Fig 3.11.) show that sonication did not increase the numbers of CFUs, as would be expected if hyphae were present in the samples, rather it resulted in a decrease. Upon heat treatment, CFU numbers were halved, suggesting selective elimination of heat sensitive units, most likely hyphae. The detection of a drop in the number of CFU's following a heat treatment as an indication of the presence of *Micromonospora* hyphae is therefore possible, whereas sonication did not produce the expected results, and would at least require further optimization in order to produce reliable data. The effects of tunicamycin concentrations and NaOH treatment on *Micromonospora* strains were studied in detail by Wakisaka *et al.* (1982), and a selection of the strains isolated in this study were grown in Tunicamycin containing plates (10 μg ml^{-1}) and found to develop normally.

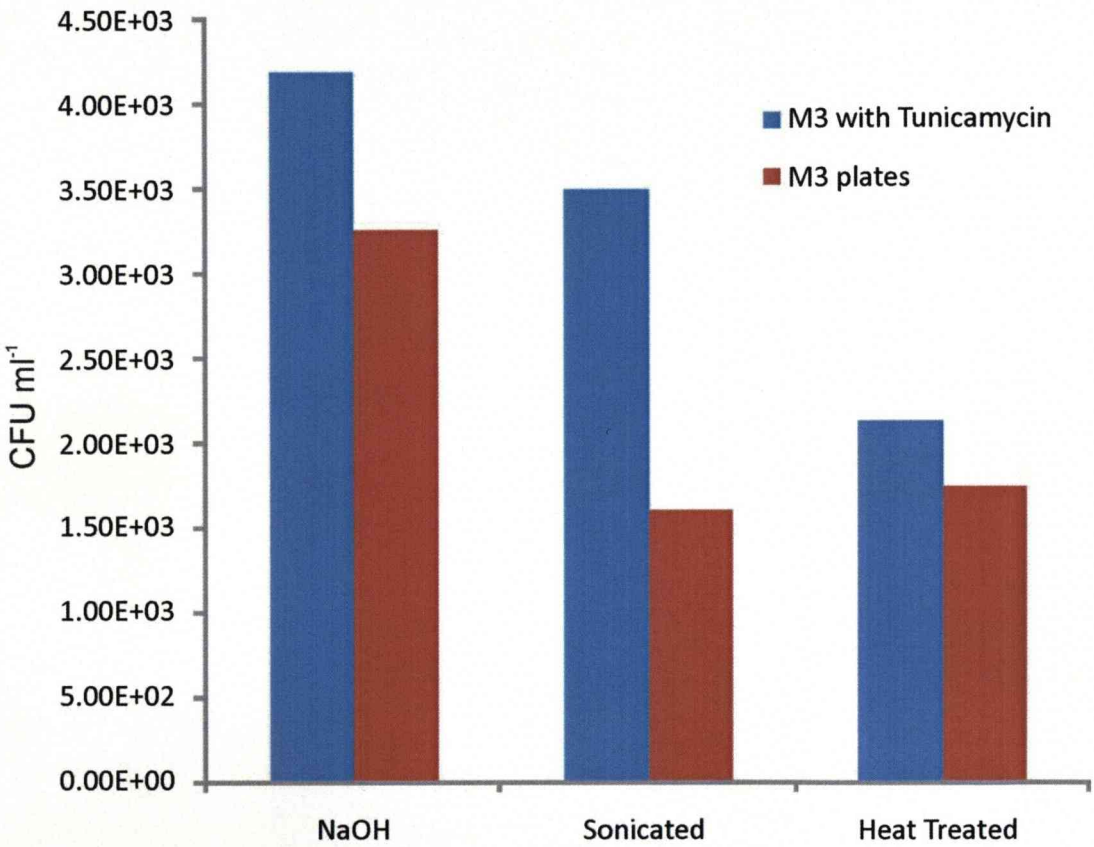


Figure 3.11. Effects of NaOH, sonication and heat treatment on the number of *Micromonospora* CFU numbers from colonised bait extracts.

Micromonospora colony forming units obtained from colonised baits (Priest Pot, 1 m deep October 2006) treated with 0.01M NaOH (section 2.3.) alone or followed by sonication (10 s at amplitude of 3.5 µm) or heat treatment (65°C for 10 min), and isolated on M3 with or without tunicamycin (10 µg ml^{-1}) (averages of two determinations). Agar plates were incubated at 30°C for 15 days.

In order to ensure that a decrease in CFU numbers following heat treatment was not caused by progressive elimination of spores, the effect of the chosen heat treatment on *Micromonospora* spores was investigated. Evidence had already being gathered for one strain (N° 16) suggesting that *Micromonospora* spores were not destroyed by heat treatment at 65°C for 10 min (Fig 3.1.), but in order to confirm this finding, a similar experiment was performed for two more lake isolates. Suspensions were produced from sporulating agar slopes (21 days old), in order to minimise the number of hyphal fragments present. Fig 3.12. shows the results of applying the heat treatment to spore suspensions of *Micromonospora* isolates 14 and 36 for 0 to 30 min. Strain 14 shows a slight (not significant) increase in numbers up to 10 min, followed by a small decrease to 15 and then to 30 min; CFU counts from strain 36 on the other hand show ca. 30% decline in numbers until 10 min, followed by a more gentle decline to 15 min and stabilization at 30 min. The pattern observed for strain 14 is expected, and the increase in numbers at 10 min is probably due to heat activation of spores, as has been described previously (Hoskisson *et al.*, 2000); the pattern displayed by isolate 36 is best explained by the presence of significant numbers of hyphal fragments in the original spore suspension, despite great care being taken to prevent any disruption of colonies in the agar surface. Mycelial fragmentation is well documented for actinomycetes (Locci, 1983), and it is possible that significant amount of such fragments were mixed within the spore mass, as these themselves originated from hyphae. The decline observed for isolate 36 from 10 to 15 min mirrors that observed with isolate 14, and could reflect the fact that spore germination was increased at 10 min due to heat activation.

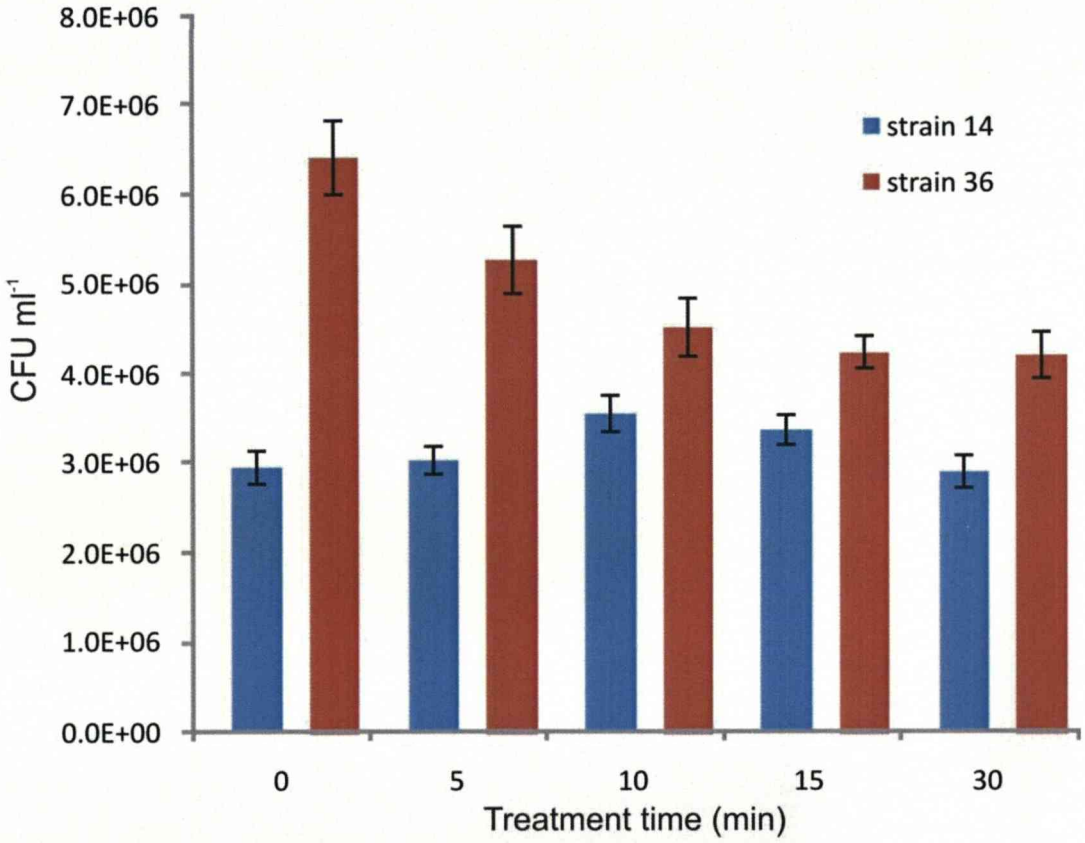


Figure 3.12. Effects of heat treatment on spore germination.

Number of colony forming units obtained from suspensions of two *Micromonospora* isolates treated at 65°C for different lengths of time (averages of 4 determinations). Colonies were inoculated on M3 agar and incubated for 15 days prior to determining CFU numbers.

3.5.2. Detection of hyphae in lakes

The combination of NaOH, tunicamycin and heat treatments was applied to determine the presence and relative distribution of *Micromonospora* hyphae in baits at different depths, and also in the water column and sediment samples.

Baits were placed in Esthwaite Water in October 2007 following a similar arrangement as described previously, but with an additional sample at the surface (section 2.1.), and were removed after 5 weeks. Samples were also taken from the water column (surface, 7 m and 14 m deep), and sediments (surface and 10 cm deep sediment). The water column at the time of sampling was mixed, with the temperature remaining at ca. 8 °C; dissolved oxygen however decreased sharply in the final 1.0 m of the water column (Fig 3.13.).

The reducing power of lake sediments probably prevented a significant rise in oxygen tensions in the deepest layers of the water column after the disruption of the summer thermocline. The relative numbers of CFU obtained from cotton baits before and after heat treatment are shown in Fig 3.14. A. There was a decrease in numbers after heat treatment for all of the bait samples, but this was more significant for samples closer to the surface than to those deeper in the water column (paired students t test values: surface $p = 0.048$, 3 m $p = 0.023$, 7 m $p = 0.01$, 11 m $p = 0.267$, 14 m $p = 0.122$, sediment $p = 0.410$). Fig 3.14. B shows the results for water column samples and surface and deep sediment samples, with statistically significant decreases observed for both sediment samples, but not for the lakewater samples. A decrease in numbers after heat treatment indicates the presence of hyphae in the baits, particularly closer to the surface, as would be expected at the generally higher dissolved oxygen tensions and temperatures observed there. Lower in the water column, mycelial growth could occur occasionally when dissolved oxygen tension increases due to physical mixing of the water column, but if the water column stratified again and the thermocline returned, growth could cease and sporulation could occur, making the relative numbers of hyphae decrease. In the water column, hyphal fragment numbers were very low, with only one, statistically non-significant ($p = 0.371$) decrease in CFU numbers observed for surface waters. Deeper in the water column, after heat treatment CFU numbers increased (paired t test significance values for water at 7 m and 14 m: $p = 0.008$ and 0.012 respectively); this could be due to the absence of hyphae combined with an increased germination of spores caused by heat activation, as was shown for one strain in the laboratory.

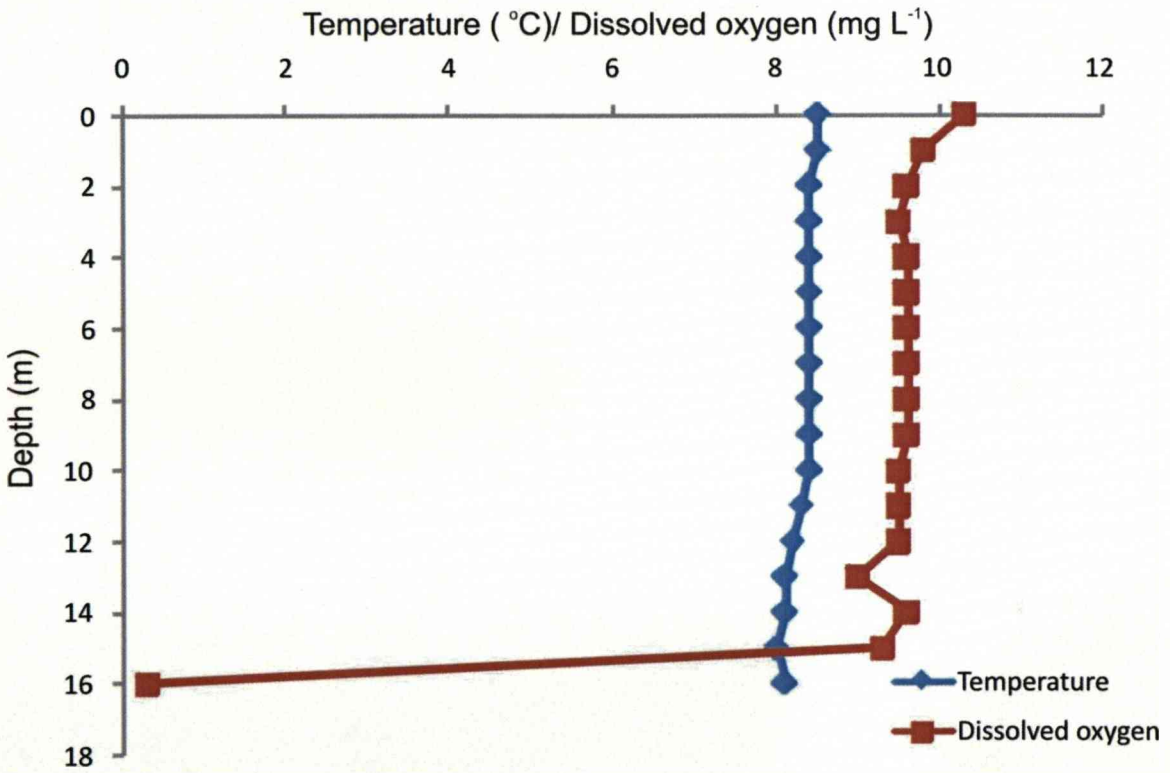


Figure 3.13. Temperature and dissolved oxygen profiles in the water column of Esthwaite Water in November 2007.

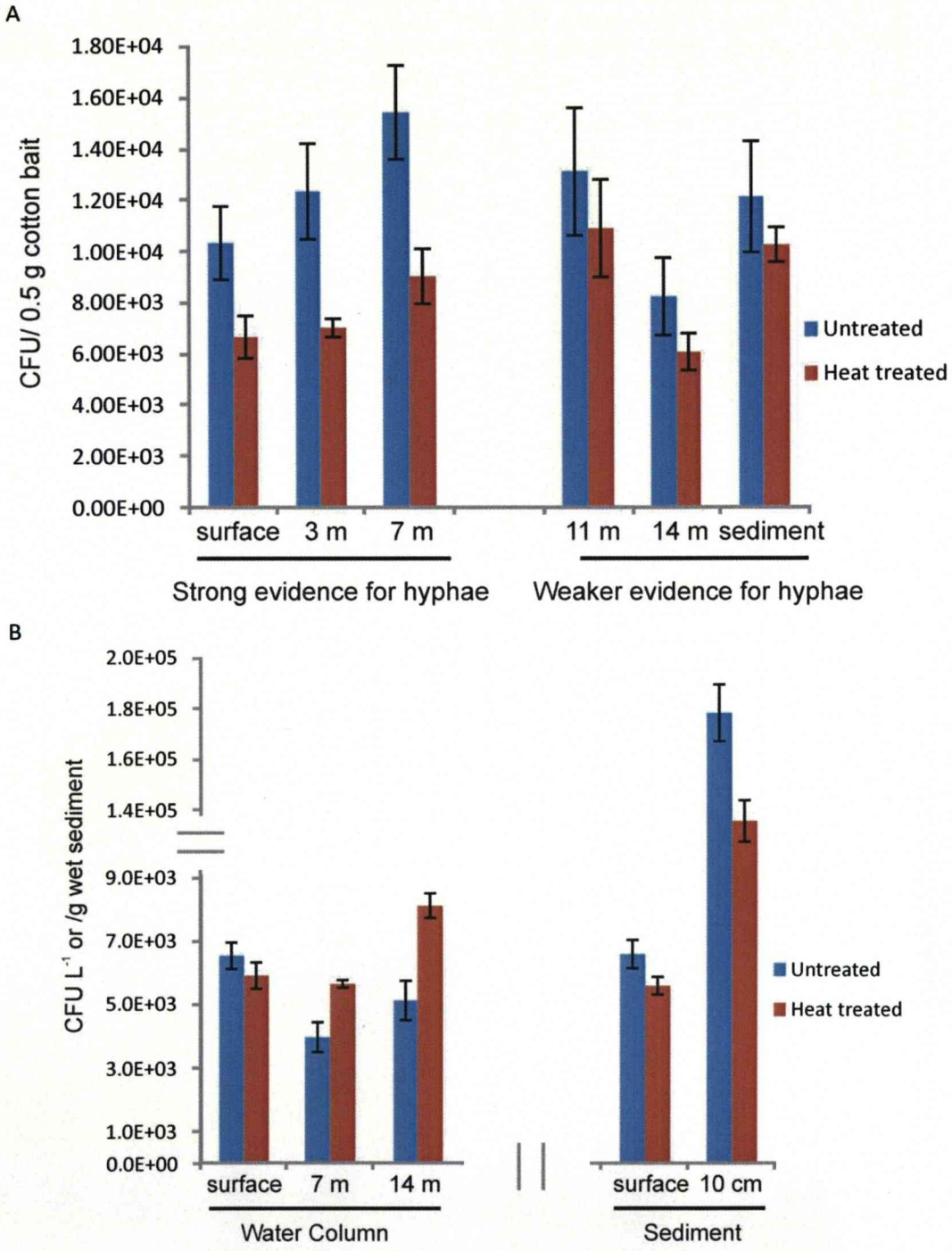


Figure 3.14. Evidence for hyphae in baits, lakewater and sediments.

Presence of *Micromonospora* hyphae was determined by the decrease in CFU numbers between untreated and heat treated samples in cellulose baits (A) and water column and sediments (B). Extracts were plated in M3 agar supplemented with tunicamycin (10 µg ml^{-1}) and incubated at 30°C for 18 days.

Non-heat treated lakewater samples also had more contamination problems with Gram-negative organisms, and this may have artificially lowered the numbers of *Micromonospora* colonies on these plates.

In the surface sediment sample, presence of hyphae was detected, as had been previously for other lakes in the lake district (Johnston & Cross, 1976) and was therefore expected, although the decrease in CFU numbers following heat treatment was marginally above significance threshold (paired student's t test $p = 0.051$). In the deep (10 cm) sediment sample, evidence for hyphae was also detected, although significance test showed that this was also marginally above significance threshold (paired student's t test $p = 0.053$). The presence of hyphae in the deep sediment sample is an unexpected result as oxygen tensions within the sediment remain very low, and should only allow the growth of organisms capable of anaerobic metabolism, which is not the case for documented *Micromonospora* species, with only one (dubious) exception (Hungate, 1946). Possible explanations are that the heat treatment is removing spores from these samples, and that there are facultative anaerobic, tunicamycin resistant actinomycetes distinct but morphologically similar to micromonosporas in the sediment that do not produce heat resistant spores. The possibility that heat treatment removes spores from sediment samples can only occur if there are of two classes of *Micromonospora* spores, heat resistant and heat sensitive. This could occur if different species produced spores with significantly different heat tolerances, however the treatment applied here was mild, and is a standard procedure used to select for *Micromonospora* spores (McCarthy & Williams, 1990) and spores of tested lake isolates could tolerate up to at least two hours at 65°C (data not shown). However, spores present at 10 cm sediment samples could be many years old, and have lost some of their heat tolerance properties. It is possible that, since the plates contained significant numbers of colonies (250-300), some small colonies counted as *Micromonospora* were in reality colonies of other actinomycetes that had not fully developed. Wakisaka *et al.* (1982) demonstrated that micromonosporas were the only tunicamycin resistant species in a list of Gram-positive organisms, including *Nocardia* spp. and *Streptomyces* spp., but their analysis did not include other members of the *Micromonosporaceae*. However, most members of the micromonosporaceae other than micromonosporas are difficult to isolate and are unlikely to have been recovered in the isolation plates used in this study. The presence of *Micromonospora* mycelium in deep sediments is possibly due to several

scenarios: bioturbation by sediment fauna could lead to mixing of the sediment layers and allow material from the surface to enter the deeper zones; nematodes, molluscs, gastrotrichs, rotifers, annelids have been shown to be present in the nearby Priest Pot (Finlay, 2000), and are likely to also occur in Esthwaite Water. Seepage of hyphal fragments from the surface sediment down to the deeper zones is another possible explanation for the detection of mycelium in these environments. A further possibility is that the mycelium can remain dormant but viable for years in this cold, anoxic environment, a possibility heightened by evidence that micromonosporas can grow under microaerophilic conditions (Goodfellow & Williams, 1983; Watson & Williams, 1974).

3.6. Cellulose degradation by *Micromonospora* spp.

3.6.1. Degradative assays

The genus *Micromonospora* is known to contain cellulose degraders (Kawamoto, 1984), but relatively little attention has been given to characterisation of their activity. Cellulose degradation by the *Micromonospora* isolates was studied by four different approaches: cellulose clearing in agar plates; filter paper and cotton string degradative assays; scanning electron microscopy of colonised cotton strings and enzymatic assays for endoglucanase.

3.6.1.1. Cellulose plates

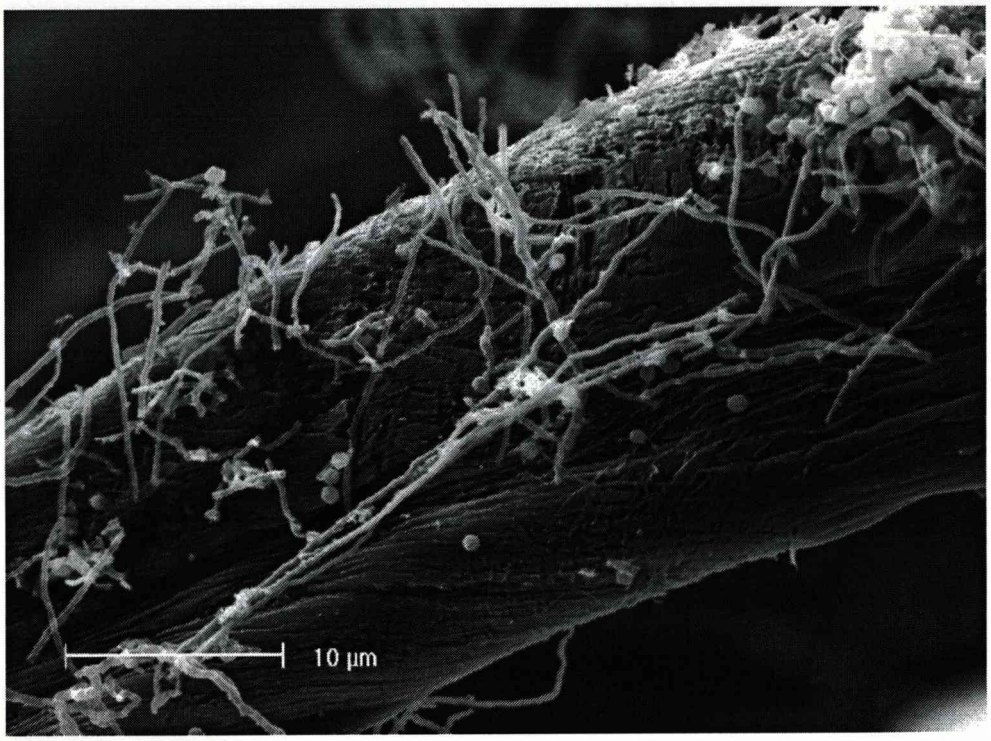
Cellulose-containing agar plates were prepared by adding ball-milled filter paper to M3 agar plates. However, none of the isolates produced zones of clearing even after 6 weeks incubation, which was considered unusual as a number of micromonosporas are known to be able to degrade cellulose. This could have been due to the filter paper powder being of insufficiently fine particle size, making degradation difficult to detect by clearing zones, or due to the cellulases remaining bound to the cell surface. This method for detecting cellulose degrading abilities was abandoned.

3.6.1.2. SEM analysis

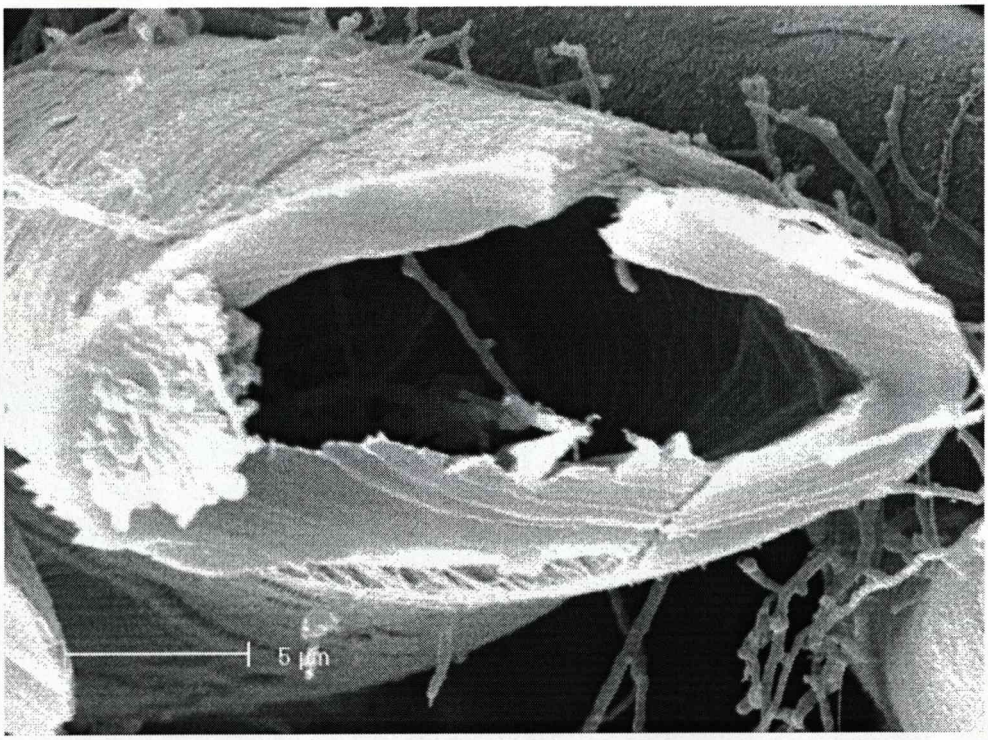
A selection of 28 strains were incubated at 30°C in 20 ml universal bottles containing 4 ml of sterile lakewater with dewaxed cotton (0.2 g), and the levels of colonisation and degradation examined visually after 8 weeks. Although some isolates grew relatively well and there was evidence for cellulose degradation, this was not easy to record unambiguously, as cotton strings were still superficially intact. This could be

due to the fact that the vials were not shaken, and no extra nutrient was added. A subset of 6 well colonised cotton strings incubated in lakewater were examined by SEM to determine the physical patterns of cellulose degradation. This revealed that all of the strains were able to attack cotton, to different extents. In Fig 3.15. B, F and M, it is evident that *Micromonospora* hyphae frequently penetrate the cotton fibres, and degrade from within, producing a hollow degradation pattern. Often, the fibres were completely degraded, with the hyphae remaining in place (Fig 3.15. C, G and K). Degradation on the external fibre surface was also common (Fig 3.15. A, H, L and M), resulting in long grooves running the length of the fibre.

Figure 3.15. Scanning electron microscopy of cellulose baits colonised by different *Micromonospora* isolates.

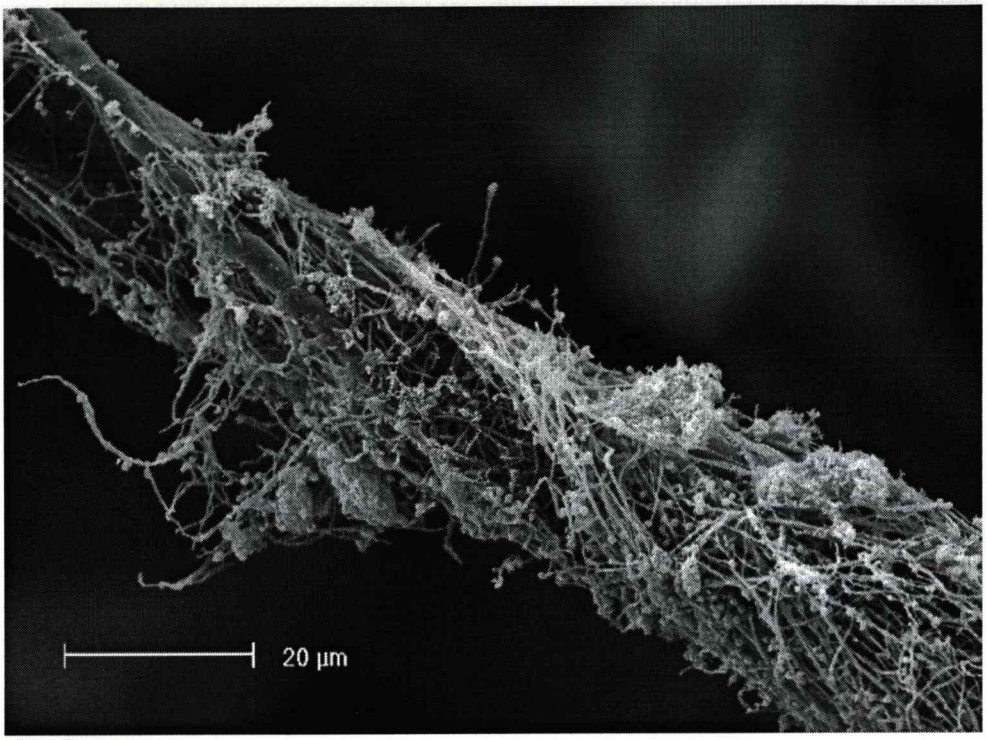


A) Strain 3: Hyphae surrounding the cellulose fibre and emerging from within. Superficial degradation evident.

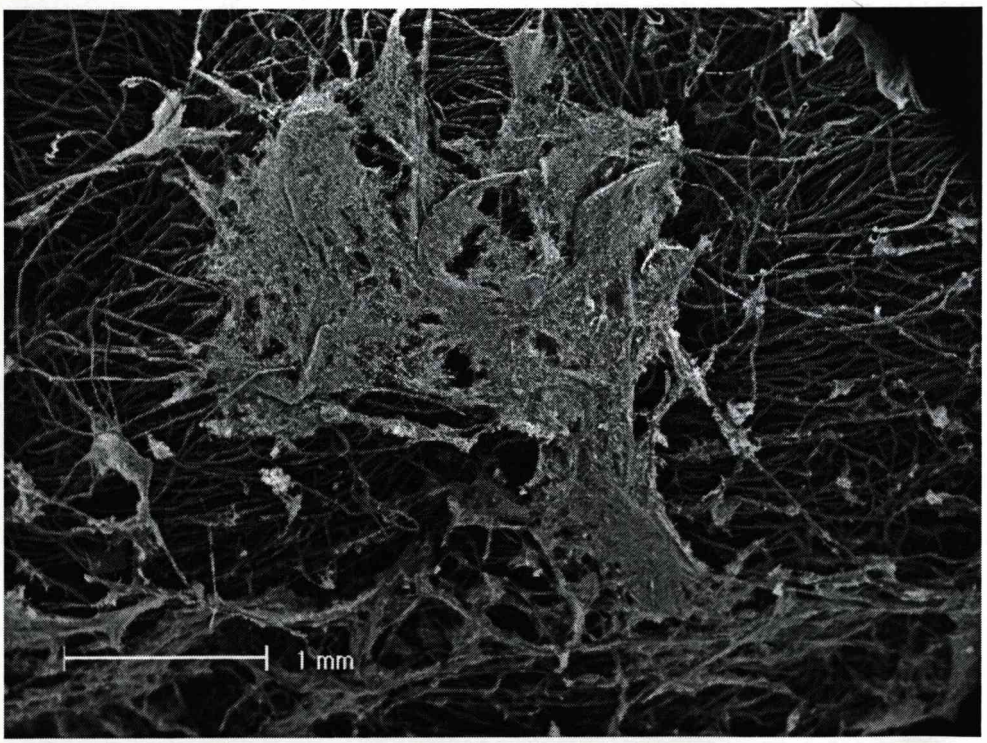


B) Strain3: Hyphae within the cellulose fibre, demonstrating the physical advantage that mycelial organisms have in attacking a crystalline substrate when compared to unicellular bacteria.

Figure 3.15. continued

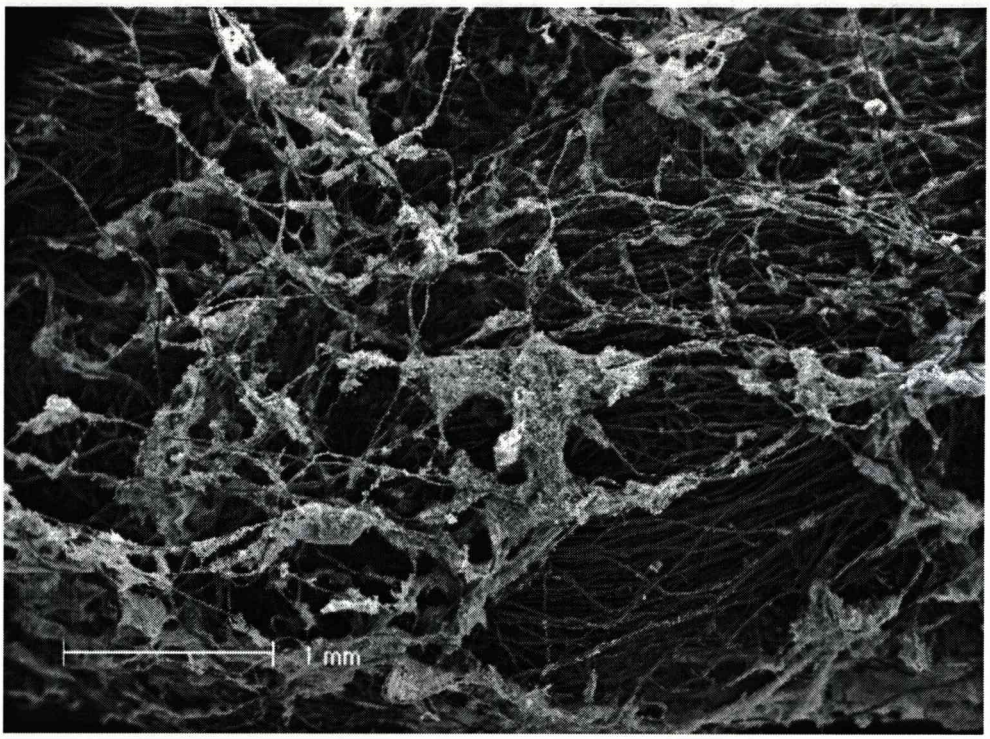


C) Strain 17 (subsequently lost from culture collection): Cotton fibre is significantly degraded, and largely replaced by hyphae

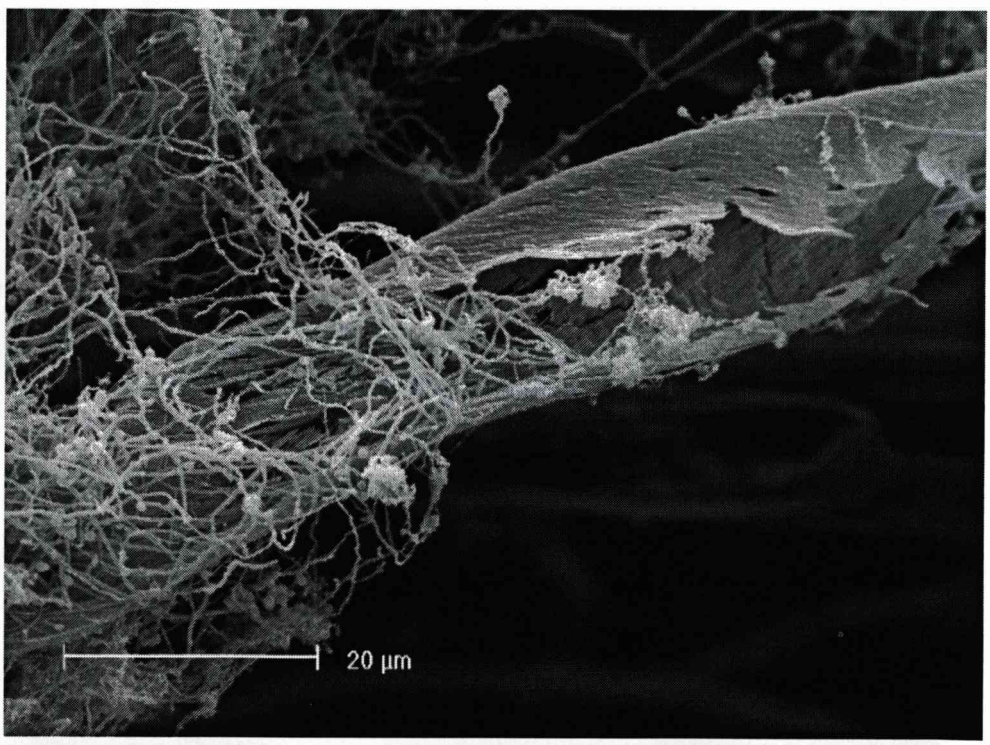


D) Strain 17: Overview of cotton string showing patches of dense colonisation by the test isolate. Here, most of the colonisation is restricted to the outer area of the string.

Figure 3.15. continued

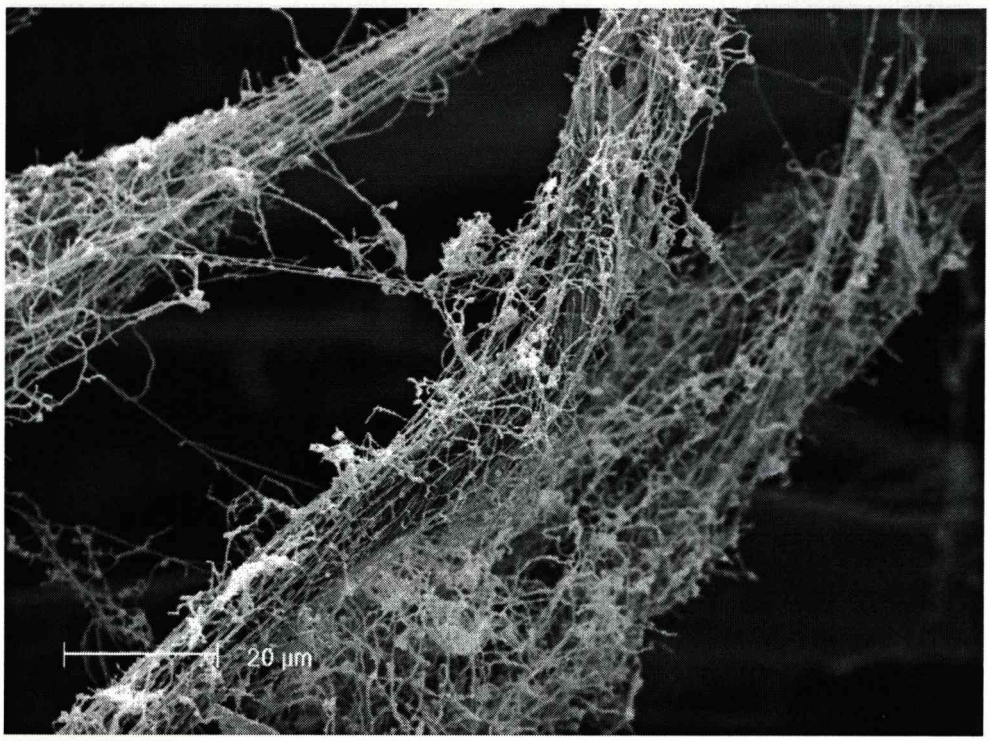


E) Strain 16: image of cotton string showing a more extensive level of colonisation than strain 17. Colonisation restricted to external areas of string.

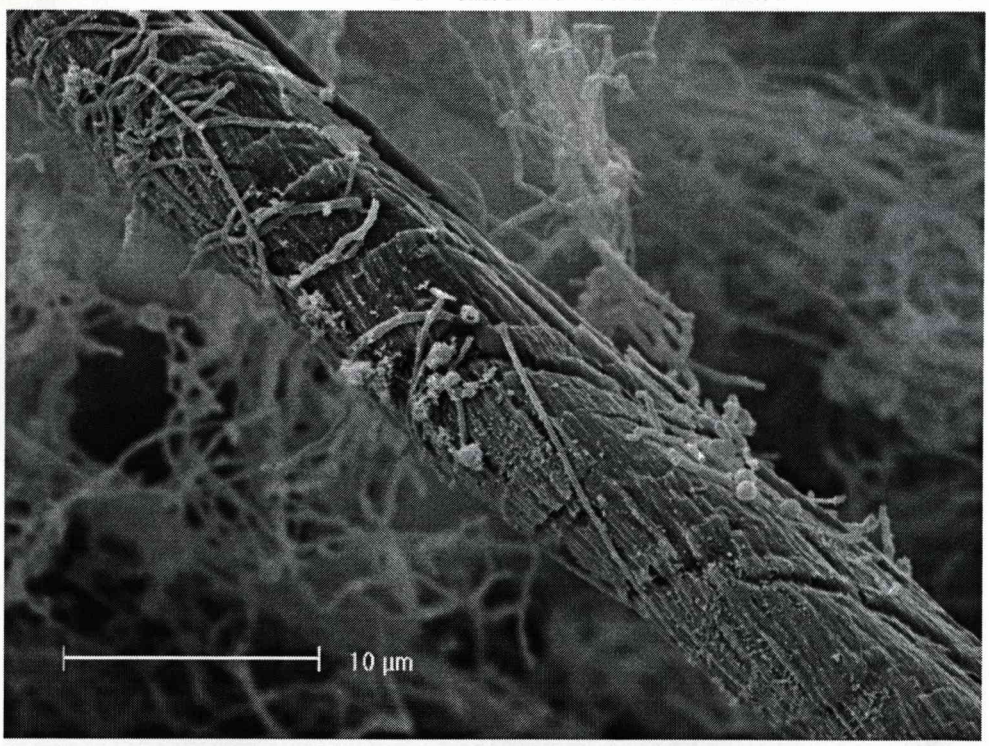


F) Strain 16: Significantly degraded cotton fibre, with a hollow interior caused by hyphal degradation from within.

Figure 3.15. continued

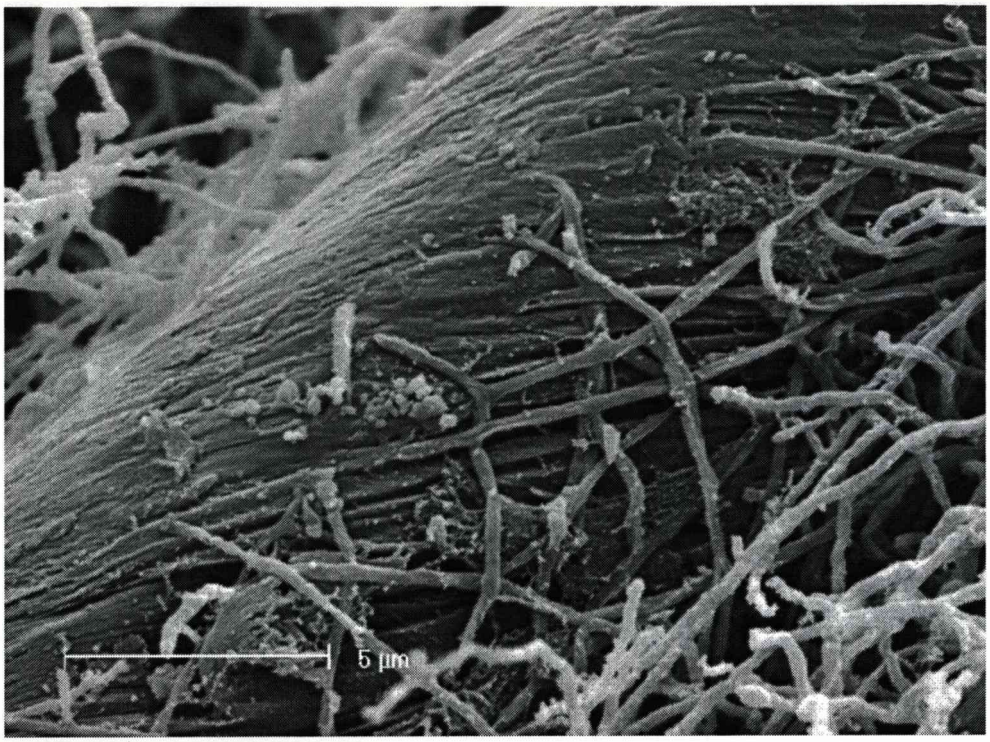


G) Strain 11: Significantly colonised cotton fibres, hyphae almost completely replacing the string.

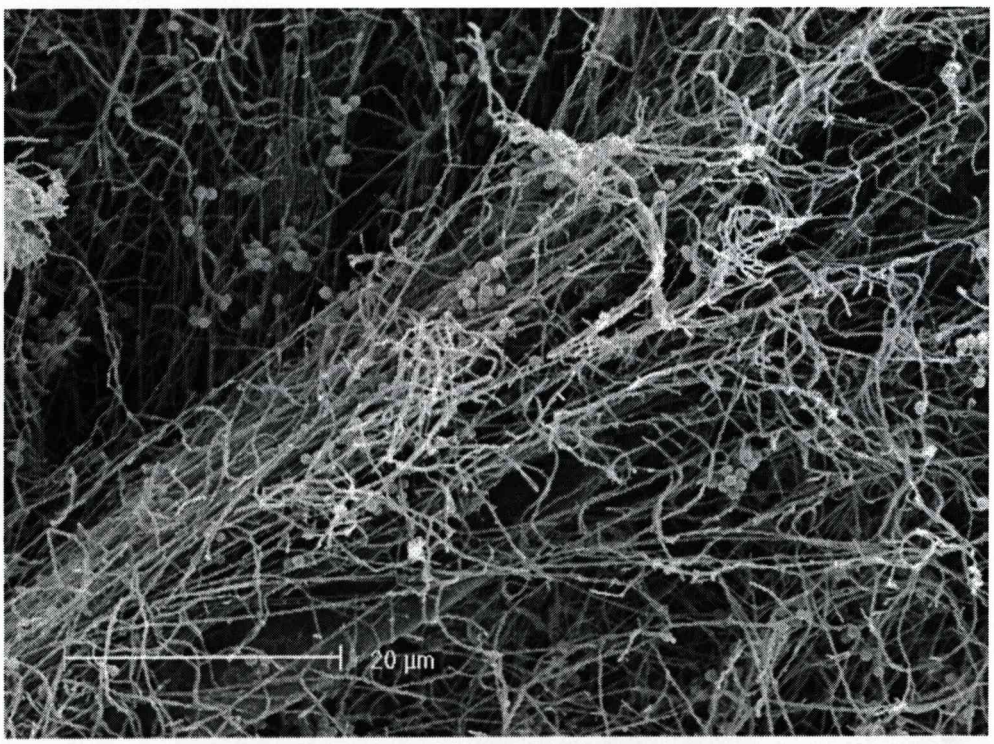


H) Strain 11: Close up of colonised cotton fibre showing superficial degradation and a close association between hyphae and substrate.

Figure 3.15. continued

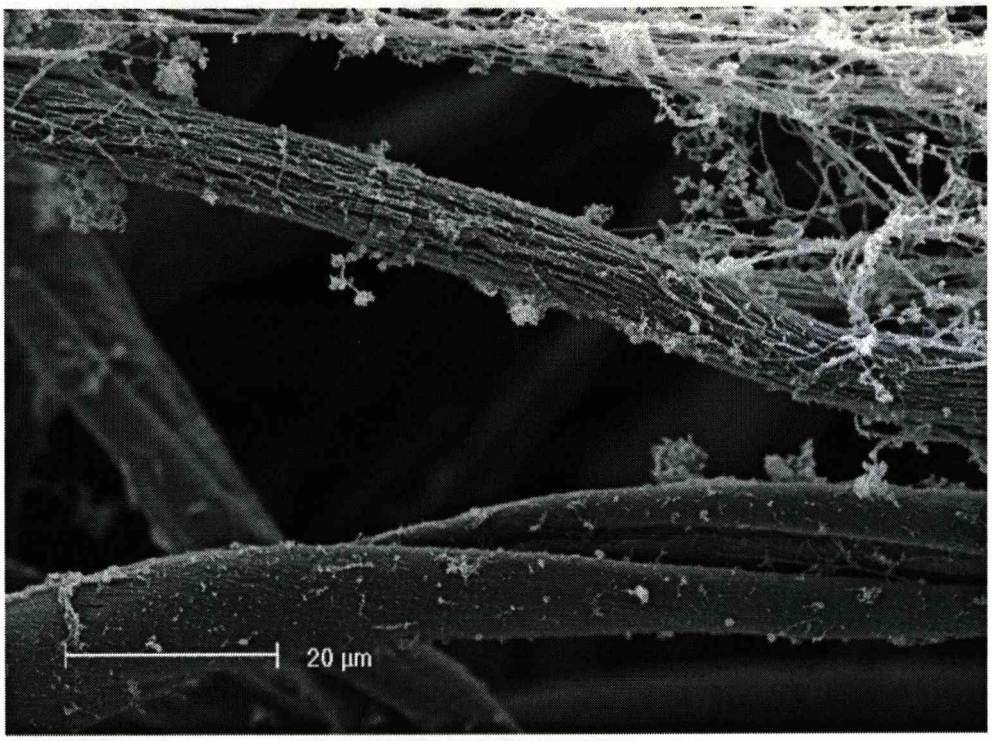


J) Strain 9: Close up of colonised cotton fibre showing hyphae penetrating the cellulosic substrate.

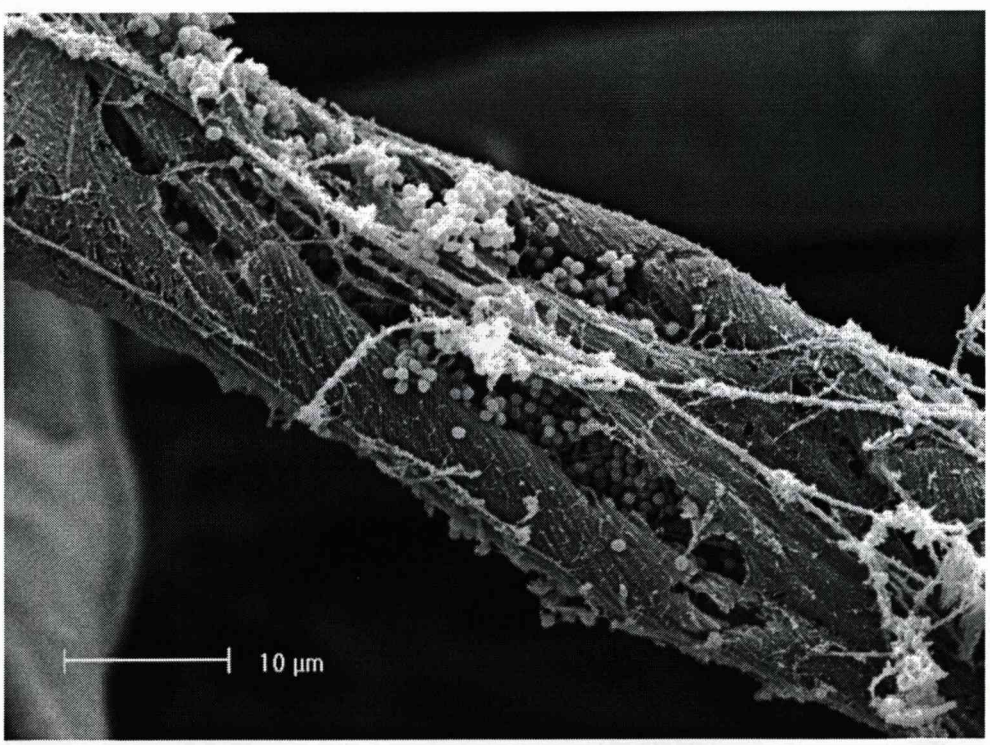


K) Strain 9: Dense concentration of hyphae, significant cotton degradation, strands replaced by hyphae, with typical single *Micromonospora* spores evident.

Figure 3.15. continued



L) Strain 22: Image showing three stages of degradation: bottom of picture shows an undegraded fibre, in the middle a fibre showing some colonisation and superficial degradation; top, hyphae remain in place where a cotton fibre existed before being completely degraded.



M) Strain 22: Image showing cotton fibre with a small amount of external colonisation, but a great number of spores within, indicating significant colonisation of the fibre beneath the surface.

3.6.1.3. Comparative cellulose degradation by *Micromonospora* strains

Although SEM showed that all of these isolates were able to attack cellulose to some extent in unsupplemented lakewater, it was not possible to assess the relative activity of the different strains. A comparative degradative assay was designed to determine the relative abilities of each strain to attack filter paper and cotton.

In order to prevent nutrient limitation, yeast extract was added to M3 broth used in the subsequent degradative assays. Strains were inoculated into 30 ml test tubes containing 10 ml of M3 plus 0.4 g litre^{-1} yeast extract, and a strip of filter paper (less crystalline and therefore easier to degrade than cotton). The cultures were incubated at 30°C and were not shaken.

In addition to the filter paper assay, six of the fastest growing strains were also inoculated in 200 ml conical flasks containing 40 ml of M3 plus 0.4% yeast extract and a cotton string, and were incubated at 30°C with shaking at 170 rpm. Degradation of the filter paper strips was visually checked at weekly intervals up to eight weeks, and test tubes were manually shaken to determine if the filter paper was losing its integrity. Growth was usually concentrated in the filter paper strip, demonstrating the tendency of micromonosporas to attach to and grow on insoluble organic substrates. Degradation was evident after 3 weeks for some strains, and paper strips were losing their integrity at the liquid/ air interface, demonstrated by folding or rupture of the filter paper.

After 8 weeks of incubation, some strains had degraded the filter paper extensively, and most of the paper in the liquid/ air interface had been replaced by *Micromonospora* hyphae. Upon manual agitation of test tubes, the filter paper strip would lose its integrity, except those parts that had remained dry, or were located deep in the test tube. Other strains had only softened the filter paper at the well colonised liquid/ air interface, and some had apparently caused no damage at all. Examples of filter paper degradation patterns are presented in Fig 3.16.

Cotton strings incubated with shaking were significantly degraded by some of the strains (isolates 3, 14 and 16), and eventually the cotton string completely lost its integrity, whereas the other three (isolates 11, 9 and 39) had no disruptive effect, despite reasonable amounts of growth and the fact that two of these strains (11 and 9) had been confirmed as cellulose degraders by SEM.

Figure 3.16. Filter paper degradation by different strains of *Micromonospora.*

The test tubes contained filter paper incubated with 6 different *Micromonospora* strains. Different levels of degradation can be observed. Photograph taken on the 30th day of cultivation.

3.7. Endoglucanase activity

Although the degradative assays were useful at demonstrating comparative levels of cellulose degradation between the different isolates, enzymatic assays were required to obtain a quantitative measure of activity. Since cellulose is an insoluble and recalcitrant substrate, obtaining cellulase activity measurements on crystalline cellulose is often impractical, and a soluble proxy, carboxymethylcellulose (CMC) is often used. This method relies on the production of reducing sugars generated from progressive cleavage of long CMC chains into smaller oligosaccharides, and is a measure of endoglucanase activity and not of the total cellulose degradation ability. Most organisms capable of degrading crystalline cellulose will secrete a cellulolytic enzyme system consisting of both endo- and exoglucanase activities, but some organisms that produce endoglucanases may not produce significant levels of exoglucanases; in this study, the degradative assays performed previously with the lake isolates demonstrated that some produce a complete cellulolytic system.

The protocols tested here were based on Bachmann & McCarthy.(1989) and McCarthy *et al.*(1985), but a series of experiments were required to determine the optimal conditions for the CMCase assay.

3.7.1. Endoglucanase assay optimization

Culture supernatants were incubated with CMC and phosphate buffer, and reducing sugars produced from cleavage of the CMC chains were measured using the dinitrosalicylic acid method (DNS) (Miller, 1959). Xylanase activity was also determined initially to help determine whether the strains were secreting any polysaccharide hydrolases, and xylan replaced CMC in these assays. Strain 14, which was shown by degradative assays to be an active cellulose degrader, was used for the optimization of growth and cellulase assay conditions. The strain was inoculated into each of three 2.5 litre flasks containing 250 ml of mineral salts medium, supplemented with 0.2% low viscosity CMC, 0.2% xylan or 1% filter paper powder, and incubated at 30°C with shaking (section 2.9.3.)

The strain grew well under these conditions and significant growth was observed after 48 h. Initial tests showed high levels of xylanase in the culture supernatants, but very low levels of CMCase, with readings only just above the detection limit of the DNS method for reducing sugar measurements.

Initial levels of endoglucanase activities were very low, remaining below 30 U litre^{-1} even after running the assay overnight (data not shown). Despite the low values obtained, it was possible to determine that the enzyme activity was linear for ca. 2 h, and subsequent assays were measured after 1 h of reaction time.

Fig 3.17. shows the evolution of CMCase and xylanase in the culture supernatants of the three cultures tested. CMCase activity was low (10-20 U) for all the cultures but the highest values were obtained in the culture containing filter paper. Those culture containing filter paper and xylan had much higher levels of xylanase activity. It is not known whether this was caused by co-expression of xylanases, an unlikely alternative as the culture growing on xylan did not produce appreciable amounts of CMCases, or by hydrolytic action of the cellulases (polysaccharide hydrolases) on xylan. However, of the three growth substrates tested, milled filter paper was best for the production of CMCases, and was chosen for subsequent experiments.

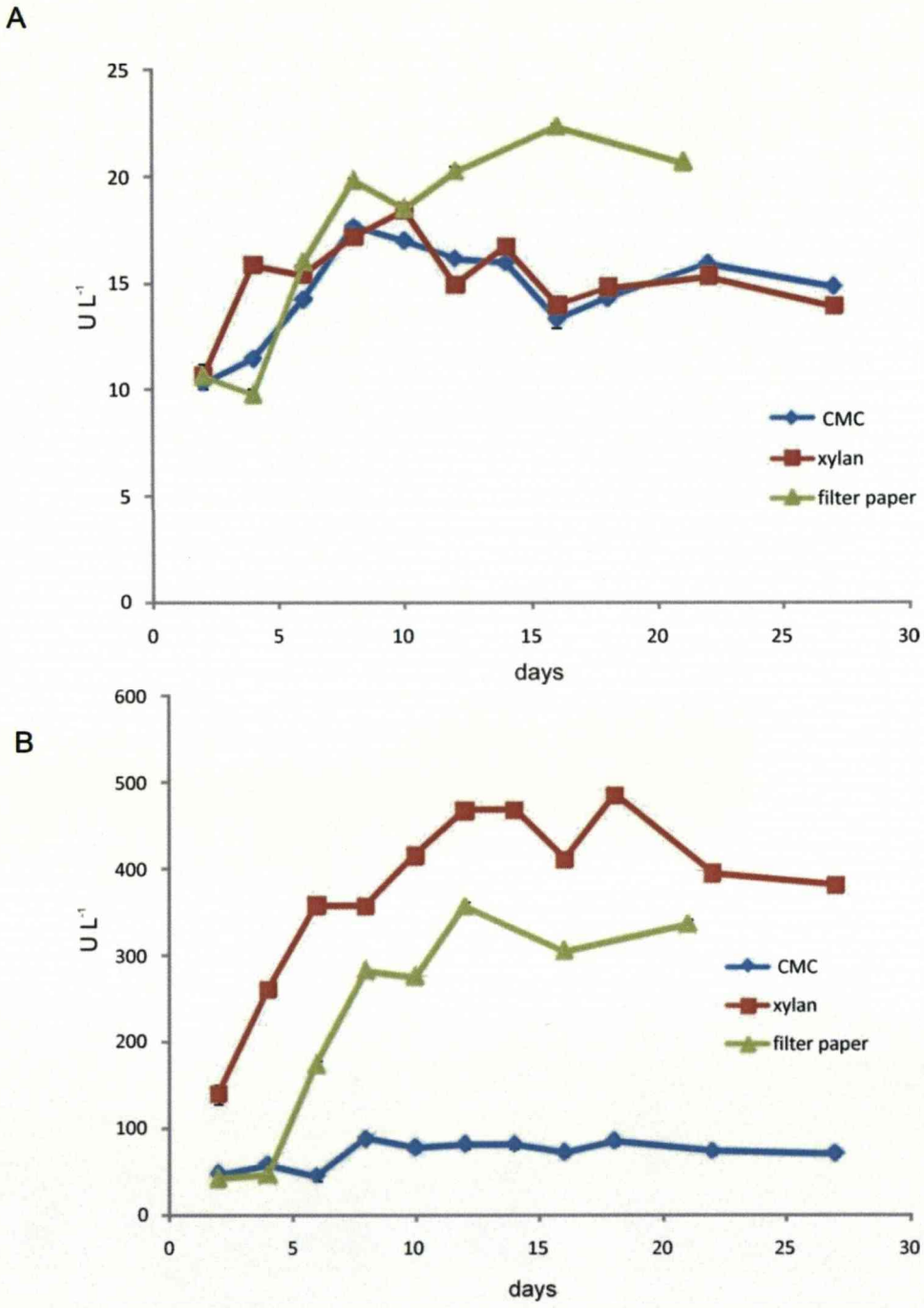


Figure 3.17. CMCase and xylanase activity in the culture supernatants of *Micromonospora* strain 14.

A and B are CMCase and xylanase activity respectively. Cultures were grown with 0.2% low viscosity CMC (LV CMC), xylan or 1% filter paper, incubated at 30°C with shaking. Cellulase assays were performed at 50°C for 1 h (averages of 9 determinations).

Strain 14 was considered one of the most active cellulose degrading strains in the collection obtained from the lakes, and this low CMCase activity was unexpected. Several attempts were made to determine if the procedure used to measure CMCase was failing to detect the cellulase activity produced by this strain. One hypothesis raised was that the endoglucanases produced were mainly cell-bound, and therefore not present in the assays which were performed using the culture supernatant. Sonication, glass bead-beating, buffer extractions and combinations of these were used to extract proteins from cell pellets. Values obtained for CMCase were not increased by with the use of these extraction methods, but values for xylanase were lower for the pellet proteins than for the supernatant. It was likely therefore that a significant proportion of the hydrolytic enzyme system for this strain was indeed in the supernatant; precipitation of the supernatant proteins (section 2.9.3.3.) followed by endoglucanase assay was also attempted, but yielded no increase in the level of activity measured.

The low CMCase activity measured could be an indication that this strain did not produce significant amounts of endoglucanases, or it could be due to inappropriate enzyme assay conditions. A positive control was used to determine whether the assays were failing to detect the presence of endoglucanases, and dilutions of commercially available cellulases from *T. reesei* replaced culture supernatant in a test assay.

A solution containing 0.9 mg ml^{-1} *T. reesei* cellulases (6 U mg^{-1}) was diluted 10 and 100-fold in phosphate buffer and incubated at 37°C for 1 h, and the activities compared. The results (data not shown) showed that the activity levels detected did not decrease in proportion to the dilutions used, an indication that the amount of substrate used was limiting. A second experiment was designed to determine the effects of increasing the amount of substrate on the level of CMCase activity detected from the supernatant of *Micromonospora* strain 14. Fig 3.18. shows that levels of activity of 5-fold diluted culture supernatant increased significantly when the amount of substrate was doubled. It was therefore necessary to increase the amount of CMC added to the assays in order to determine the point at which the concentration was no longer limiting the reaction.

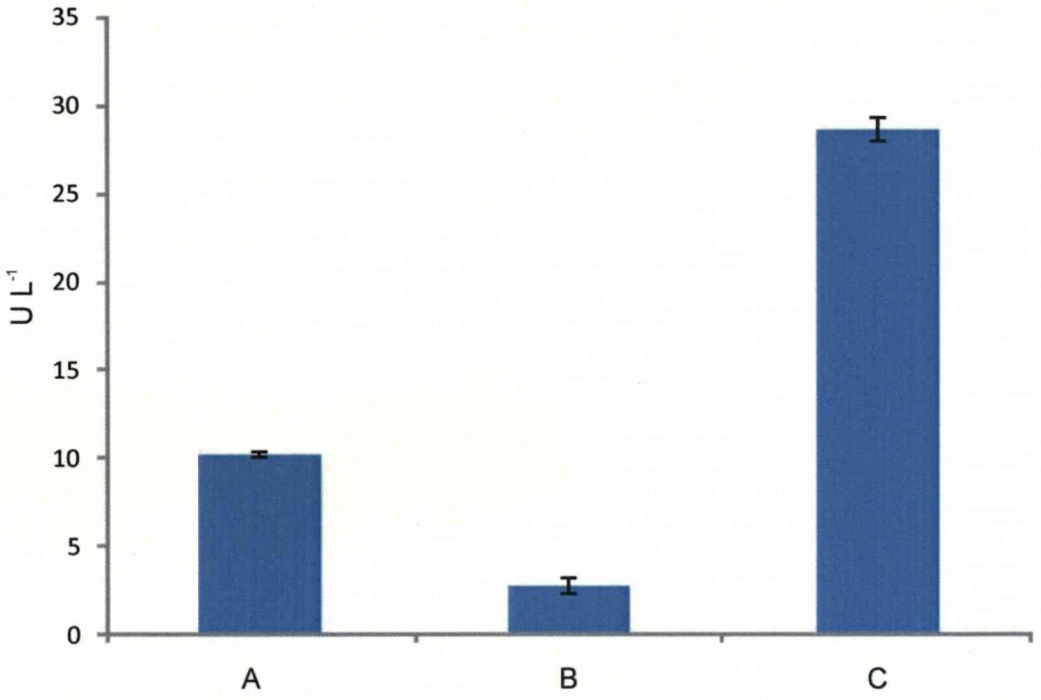


Figure 3.18. CMCase activity of culture supernatant of *Micromonospora* strain 14 cultivated on filter paper powder.

A, undiluted supernatant preparations; B, 5-fold dilution; C, 5-fold dilution of culture supernatant with double the amount of substrate. The cellulase assays were performed using CMC as substrate (averages of 9 determinations), at 50°C for 1 h.

3.7.2. Determination of substrate saturation

CMC is a viscous substance that does not solubilise immediately, and for practical reasons was added to the assay already dissolved in phosphate buffer; the amount added to the assay was limited as greatly increasing the volume of dissolved CMC would lead to a dilution of the enzyme, potentially compromising the determination. It was therefore necessary to increase the concentration of dissolved CMC added to the assay; however, increasing the concentration of CMC increased the viscosity of the solution, making highly concentrated solutions increasingly difficult to obtain. The dissolved CMC stock concentration was increased from 2.5% to 5%, and an experiment was designed to determine how much of this stock was needed in order to reach substrate saturation levels. In a series of conical flasks, increasing amounts of 5% CMC were added to 15-fold diluted culture supernatant, with decreasing amounts of phosphate buffer added to keep the volume constant. Fig 3.19. shows that at 2 ml of 5% CMC was added to the 3 ml assay, substrate saturation was finally obtained. Subsequently, the optimal pH, temperature, and kinetics of the cellulase activity in the culture supernatants were determined. Fig 3.20. A and B show that CMCase activity was highest at pH 7 and at 50°C, compared to pH 6 and 8, and 40 and 60°C. This optimal range of pH and temperature is common amongst mesophilic actinomycete cellulases.

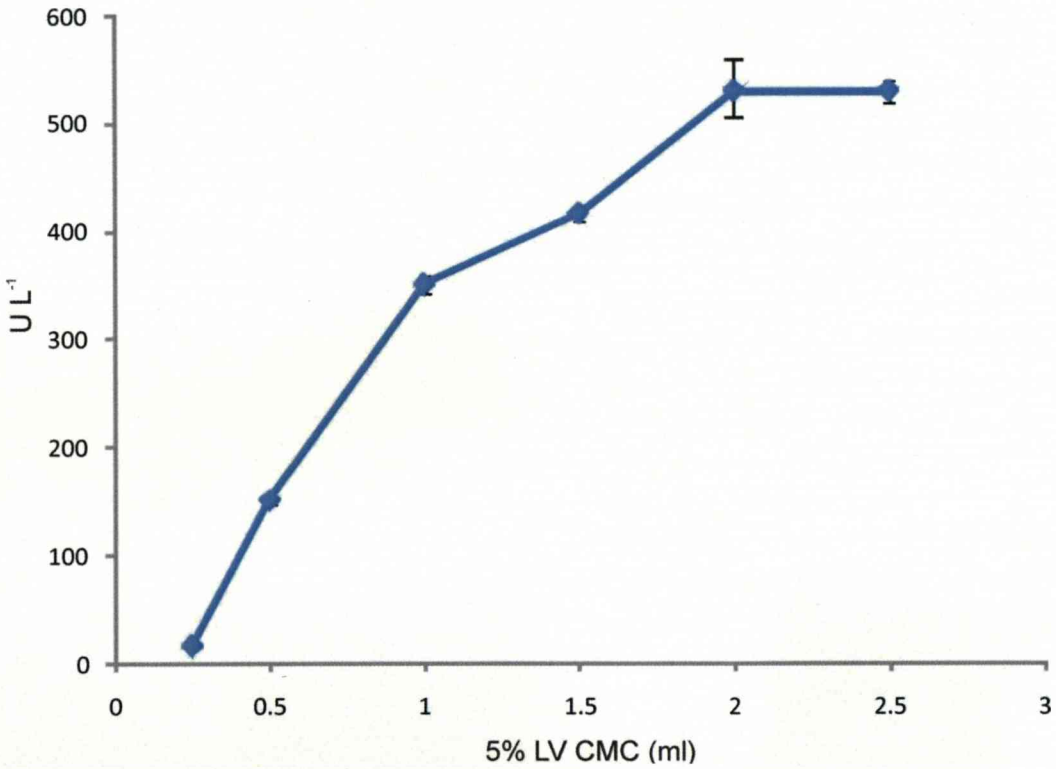


Figure 3.19. Determination of substrate saturation for endoglucanase assays.

Units of CMCase activity were measured in *Micromonospora* strain 14 culture supernatant with increasing amounts of 5% low viscosity CMC added to the enzyme assays (final volume 3 ml; averages of 9 determinations). Assays were performed at 50°C for 1 h.

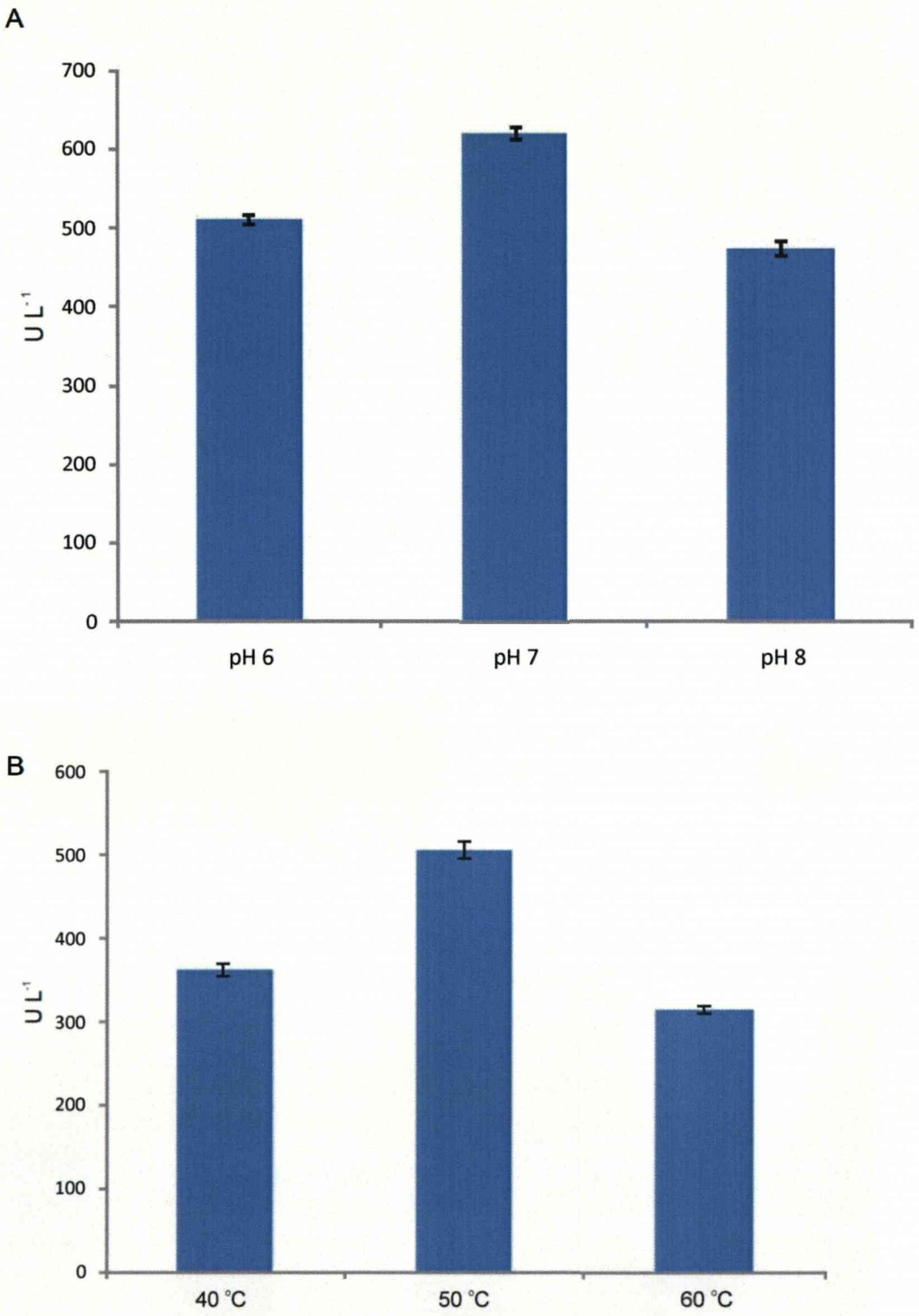


Figure 3.20. Optimal pH and temperature of *Micromonospora* strain 14 endoglucanases.

CMCase activity was determined in culture supernatant of *Micromonospora* strain 14, measured at different pH (A) and temperatures (B) values (averages of 9 determinations). The culture was grown at 30°C with shaking, assays performed for 1 h.

3.7.3. Determination of cellulase kinetics and production in pure cultures

As all previous determinations had been performed under substrate limitation, the rates of enzyme activity were re-assessed and determinations made every 15 min over a period of 2 h, using supernatants diluted 15, 20 and 30 -fold to ensure that substrate limitation did not interfere with the results. Fig 3.21. shows that the release of reducing sugars was approximately linear for 100 min. It was therefore decided to take measurements after 1 h of incubation in order to ensure linearity, but after a sufficient time to ensure that values are significantly above the limit of detection.

Levels of CMCase activity as well as supernatant protein were also monitored for 30 days in a culture of *Micromonospora* strain 14; this was done in order to determine how many days of growth were necessary before cultures could be used in a comparative assay. The evolution of enzyme activity in the culture (Fig 3.22.) shows that the production of CMCases rapidly accelerates between days 8 and 12 and then stabilized; protein concentrations in the supernatant followed a similar pattern as the CMCase activity.

The production of endoglucanases in cultures supplemented with CMC was revaluated, as previously the assays used to measure the activity were not optimised, and only low viscosity CMC had been tested. Minimal salts medium was prepared with 0.2% medium and high viscosity CMC, as well as 1% milled filter paper. The results (data not shown) showed that only in cultures supplemented with milled filter paper were the levels of CMCase activity significantly high, and in both medium and low viscosity CMC the values were barely above the detection limit of the method. The absence of cellulase production on CMC could be due to repression, as the CMC would be degraded more quickly to soluble sugars by any constitutively produced endoglucanases. The higher growth rates achieved when growing on CMC could lead to growth rate mediated repression (Wood *et al.*, 1984), or alternatively, the oligomers produced by the hydrolysis of CMC could act as repressors of cellulase synthesis (Beguin & Aubert, 1994).

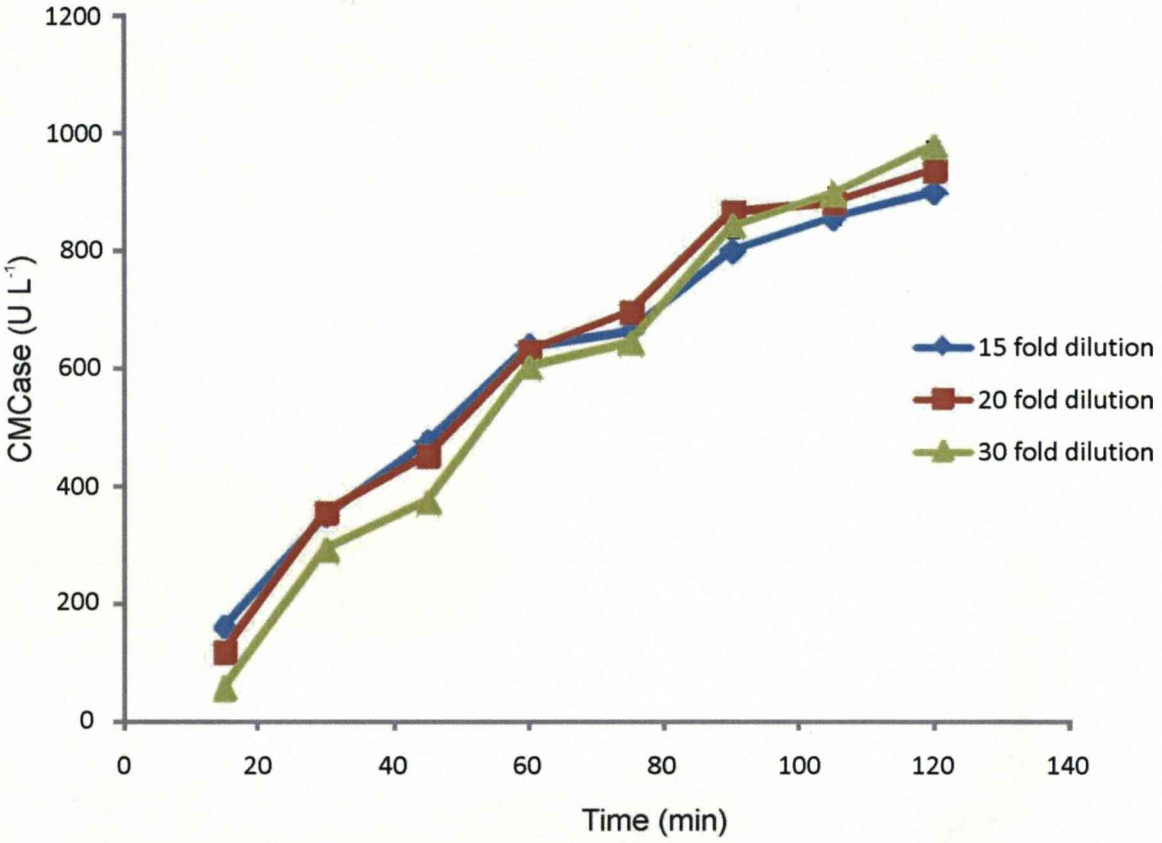


Figure 3.21. The effect of incubation time on the production of endoglucanase activity.

CMCase activity was measured over 120 minutes in culture supernatants of strain 14 at three different dilutions (averages of 9 determinations). Assays were performed at 50°C.

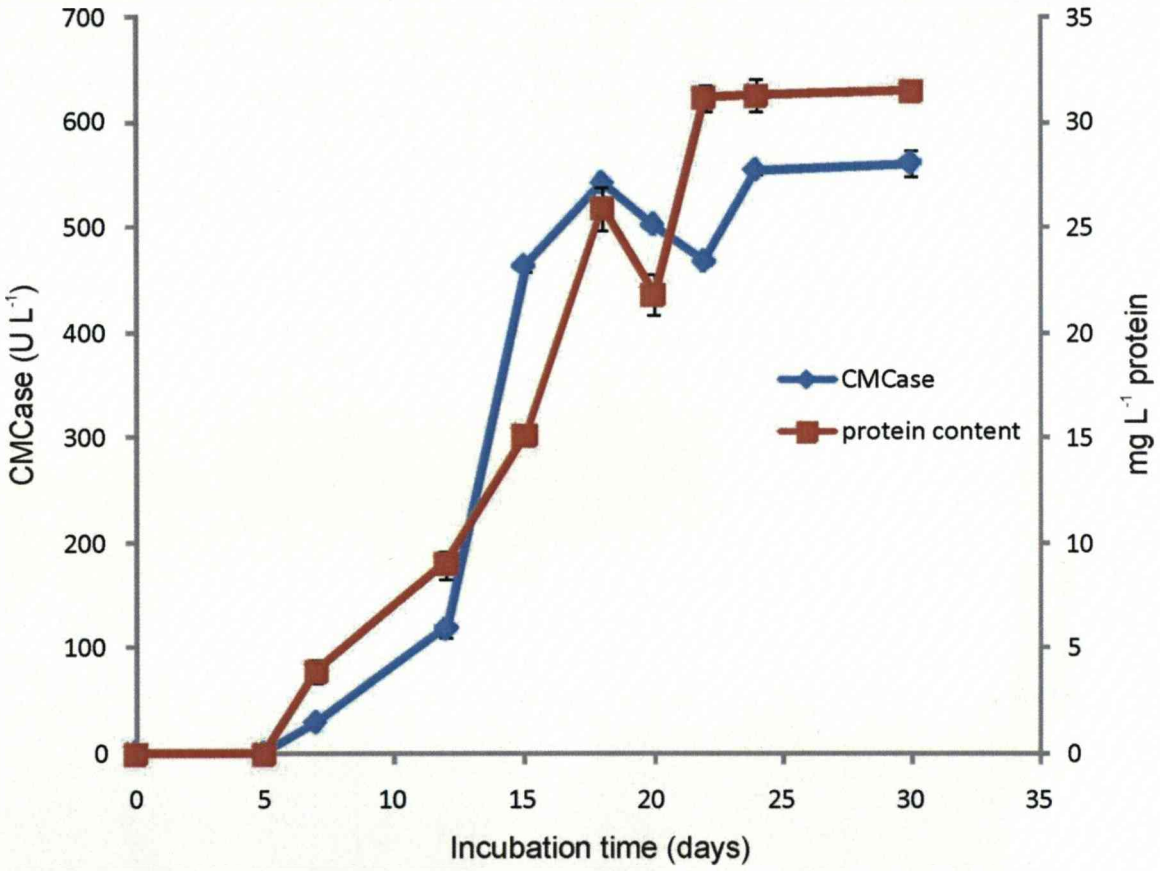


Figure 3.22. *Micromonospora* endoglucanase production and supernatant protein content during growth.

CMCase activity and supernatant protein concentration in culture of strain 14 grown over 30 days on milled filter paper. CMCase assays were performed at 50°C for 1 h (averages of 9 determinations); protein content measured using the method of Bradford (1976) (section 2.9.3.2.). Averages of 3 determinations.

3.7.4. Endoglucanase activity on filter paper

The activity of *Micromonospora* strain 14 cellulases on filter paper was also measured using the reducing sugar assay (Fig. 3.23.), and it was found to be very low under the conditions of the assay (1 h at 50°C). Cellulase activity on filter paper was only 3 U litre^{-1} compared to ca. 500 U litre $^{-1}$ of CMCase activity. This is not an unusual result as filter paper is significantly more recalcitrant than CMC; it is possible that with optimization of assay conditions higher levels of activity could be determined, however, activity of *Micromonospora* against filter paper had already been determined with the degradative assays discussed above, and it was not considered relevant for the present study to devote a significant amount of time optimizing assay conditions to determine cellulase activity on filter paper. The cellulase activities observed here are lower than these observed by Chowdury *et al* (1991), who measured values of CMCase and Avicelase of 90,000 and 2,800 U L^{-1} for a *Micromonospora* isolate growing on xylan, but are significantly higher than those reported by Malfait *et al.* (1984), who measured no more than 1.2 U L^{-1} of CMCase activity on cultures supplemented with 1% CMC after 5 days. The values observed by Chowdury *et al.* (1991) seem excessive, as 1 mg of commercial *T. reesei* cellulases (the most active commercial cellulases available) produce 6 µmol from crystalline cellulose after one hour of incubation (data not shown), which would suggest 28 g of cellulases in a litre of *Micromonospora* culture, assuming that their cellulases had the same specific activity as those of commercial *T. reesei* cellulases. Gallagher *et al.* (1996) determined the activity of *M. chalcea* cellulases on filter paper and reported 185 U litre^{-1} after 8 days; this is a more reasonable value than that described by Chowdury *et al.* (Chowdhury *et al.*, 1991) although still very high, as it would imply production of 11 mmol of glucose equivalents after 1 h of incubation, which would be equivalent to 1.8 g of cellulases of similar specific activity as *T. reesei* cellulases in one litre of culture supernatant. *T. reesei* has been used as a model organism to study cellulose degradation (Wood, 1985); its cellulolytic system is considered highly active and it is one of the major sources of commercial cellulases (Nieves *et al.*, 1998). This comparison exposes the difficulties, or indeed impossibility in comparing cellulase activity measurements between different laboratories.

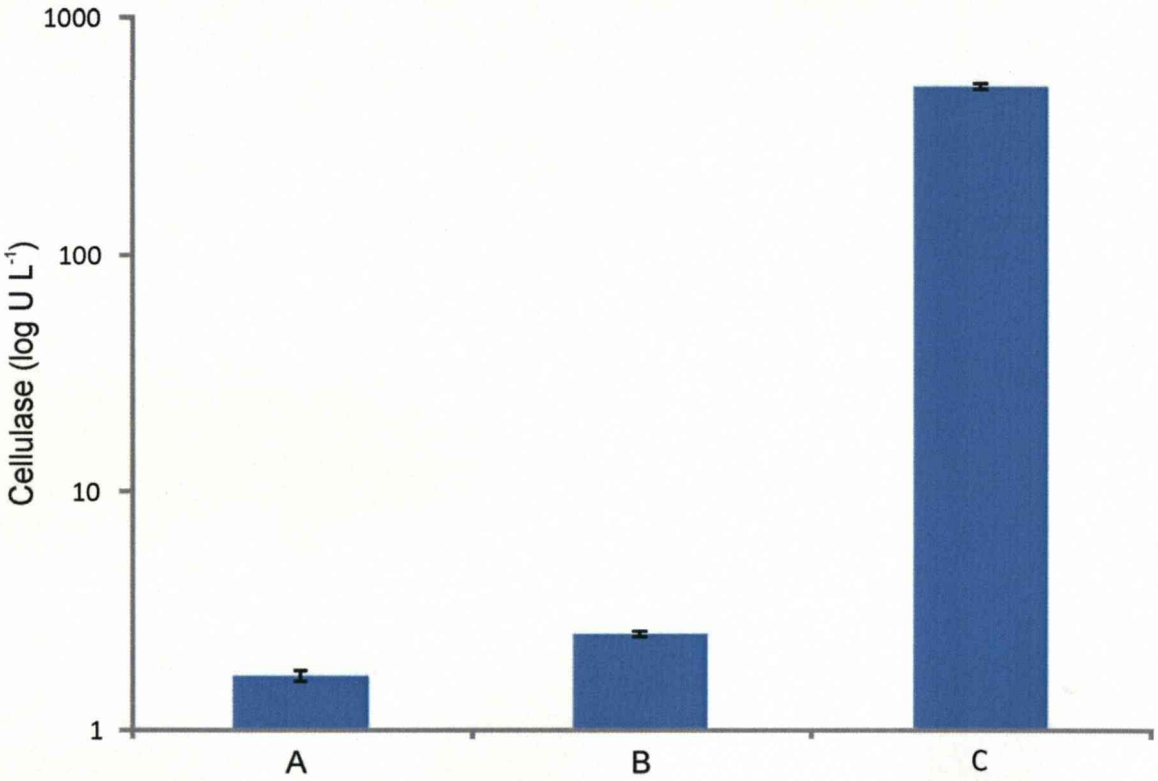


Figure 3.23. Determination of *Micromonospora* cellulase activity on filter paper.

Cellulase activity of *Micromonospora* strain 14 (20 day old culture) on filter paper was determined after 60 min (A) and 120 min (B) of incubation at 50°C; C, CMCase activity determined after 1 h at 50°C.

3.7.5. Comparative endoglucanase activity of *Micromonospora* strains

Strains were grown in 250 ml conical flasks filled with 40 ml of mineral salts medium supplemented with 1% filter paper, and cultures were harvested after 17 days of growth, and both CMCase activity and cell pellet protein determined. The length of time the cultures were grown before harvesting was set at 17 days based on Fig 3.22. This could have been slightly too long for some fast growing strains that might have already reached stationary phase when activities were determined, however other strains were slower growers and others never produced significant amounts of biomass. The time of harvest was set to allow for the detection of activities in slow growing strains.

Results for the CMCase activity, as well as the filter paper degradation abilities and cell pellet protein are presented in Table 3.3. CMCase activity varied significantly between the isolates, with the distinction between strains producing high and medium activities difficult to determine within the spectrum. However, an arbitrary line was drawn below 240 U L^{-1}, as all 8 strains with activities > 240 U $litre^{-1}$ were also highly active against filter paper, and it is therefore reasonable to classify these strains as highly cellulolytic. It was decided to consider the 17 strains producing between 42 U L^{-1} and 169 U L^{-1} as strains with medium levels of activity, as some of these strains degraded filter paper significantly, and most to some extent, whereas the remaining 48 strains producing less than 42 U L^{-1} were generally poor at degrading filter paper. It can also be seen in Table 3.3. that the highly active strains did not produce significantly higher amounts of cell pellet protein compared to the strains producing low levels of cellulase activity, which shows that these strains were not simply better at growing in the conditions used, but that they produced a relatively more active cellulolytic system. Strains 77, 3 and 96 did not produce significant amounts of endoglucanase activity but degraded the filter paper to a great extent, an indication that these strains may produce more exoglucanase activity or have a better substrate binding affinity. A similar explanation is offered for strains 60 and 48, which produced negligible values for endoglucanase activity but were able to attack filter paper to some extent. This illustrates the limitations of endoglucanase assays on culture supernatants and CMC as an indicator of cellulose degrading ability.

Table 3.3. Filter paper degradation, CMCase activity and biomass protein values for *Micromonospora* lake isolates.

strain	Filter paper degradation	CMCase U L^{-1}	cell pellet protein mg L^{-1}
70	+++	807.3 ± 6.1	16.8 ± 4.9
55	+++	763.1 ±10.6	48.5 ± 1.6
64	+++	736.7 ± 7.0	44.3 ± 0.2
14	+++	592.2 ± 3.3	46.8 ± 1.6
8	+++	416.7 ± 3.6	44.2 ± 1.8
M. chalcea NCIMB 12879^{T}.	+++	340.1 ± 4.2	53.5 ± 2.2
98	+++	306.9 ± 3.0	43.1 ± 4.1
45	+++	242.2 ±1.5	54.5 ± 2.3
67	++	169.4 ± 1.2	57.6 ± 1.7
36	+++	153.5 ± 1.6	29.9 ± 0.9
13	+	138.0 ± 0.7	25.9 ± 1.5
77	+++	119.0 ± 1.3	61.3 ± 1.5
56	+	108.1 ± 1.8	58.8 ± 2.6
68	±	105.9 ± 1.4	19.7 ± 0.7
11	+	85.5 ± 0.6	28.5 ± 2.3
66	+	85.0 ± 1.6	42.0 ± 0.9
49	+/-	72.3 ± 1.9	60.8 ± 1.8
3	+++	67.8 ± 0.6	22.8 ± 0.8
71	+/-	67.2 ± 2.4	41.7 ± 0.7
46	+	65.6 ± 0.7	48.5 ± 1.5
16	+	60.9 ± 0.8	31.7 ± 1.4
62	+	56.2 ± 0.9	43.5 ± 2.0
86	+/-	49.2 ± 0.5	43.4 ± 1.6

96	+++	47.4 ± 1.9	26.5 ± 1.1
39	+/-	44.2 ± 0.7	39.2 ± 1.7
84	+	42.4 ± 0.3	62.8 ± 3.2
61	+	29.5 ± 0.4	48.0 ± 2.8
73	+	23.7 ± 0.1	26.8 ± 2.9
69	+/-	21.5 ± 1.0	16.9 ± 0.1
93	-	20.2 ± 0.8	19.3 ± 0.9
44	+/-	18.5 ± 0.7	44.4 ± 1.7
89	+	18.1 ± 0.4	39.2 ± 1.3
59	+/-	17.7 ± 0.3	57.8 ± 2.7
87	+/-	14.9 ± 0.5	47.2 ± 0.7
53	+	14.3 ± 0.9	51.8 ± 3.6
92	-	12.9 ± 0.8	33.5 ± 1.4
90	+/-	10.5 ± 0.2	52.5 ± 2.1
63	-	7.4 ± 0.2	28.2 ± 1.9
58	+	6.4 ± 0.4	27.3 ± 0.7
79	+	6.0 ± 0.7	50.0 ± 0.6
94	+/-	4.5 ± 0.1	32.5 ± 2.3
74	-	3.8 ± 0.6	22.3 ± 0.4
82	+/-	3.8 ± 0.4	27.1 ± 1.3
88	+/-	3.3 ± 0.2	20.3 ± 1.5
15	+/-	2.9 ± 0.2	45.9 ± 1.0
20	+/-	1.6 ± 0.1	19.4 ± 0.6
24	+/-	1.4 ± 0.1	2.4 ± 0.6
60	++	1.4 ± 0.1	29.7 ± 1.6
48	++	1.3 ± 0.2	28.6 ± 1.0

81	+	1.2 ± 0.1	26.3 ± 1.0
72	+/-	1.2 ± 0.1	13.8 ± 1.3
80	-	1.2 ± 0.2	25.9 ± 0.9
41	+/-	1.2 ± 0.2	33.0 ± 2.1
22	+/-	1.0 ± 0.1	39.5 ± 1.6
42	+/-	1.0 ± 0.1	13.5 ± 0.5
83	+/-	1.0 ± 0.1	3.1 ± 0.2
47	+/-	1.0 ± 0.1	28.5 ± 1.6
19	+/-	0.9 ± 0.1	26.9 ± 0.8
37	+/-	0.8 ± 0.1	35.4 ± 1.1
99	+	0.8 ± 0.1	19.3 ± 1.4
25	+	0.8 ± 0.0	19.9 ± 0.4
78	-	0.7 ± 0.1	26.5 ± 2.2
26	+/-	0.7 ± 0.1	34.0 ± 0.5
18	+	0.7 ± 0.1	5.9 ± 1.6
97	+	0.7 ± 0.1	2.4 ± 0.4
75	+	0.5 ± 0.2	33.4 ± 1.9
85	+/-	0.3 ± 0.1	22.9 ± 0.8
57	+	0.2 ± 0.1	5.1 ± 1.4
65	+	0.0 ± 0.1	15.5 ± 0.8
76	+/-	0.0 ± 0.1	4.8 ± 0.1
95	+/-	0.0 ± 0.1	2.1 ± 0.2
91	+/-	0.0 ± 0.1	10.9 ± 1.0
9	++	0.0 ± 0.1	2.4 ± 0.7

+++, filter paper lost its integrity completely; ++ , filter paper was easily disrupted when flasks were shaken; +, some softening of the filter paper strip; +/-, some degradation might have occurred but couldn't be easily demonstrated through visual inspection; -,paper strip intact.

3.8. Discussion

There are few studies published on the ecology of *Micromonospora* in lakes to which the data presented here can be compared. Most of the information on the occurrence of *Micromonospora* spp. in aquatic ecosystems is covered in a review by Cross (1981) and Johnston & Cross (1976) together with Kawamoto *et al.* (1984) present further data on the growth and persistence of micromonosporas in lakes. Their presence along with other actinomycetes and their interaction with fungi in submerged macrophytes and decomposing leaf matter has been investigated in some studies (Mille-Lindblom & Tranvik, 2003; Wohl & McArthur, 1998; Wohl & McArthur, 2001), whereas others focused on their diversity and isolation from habitats including marine sediments and Antarctic rocks (Hirsch *et al.*, 2004; Jensen *et al.*, 2005; Jiang & Xu, 1996; Mincer *et al.*, 2002). None of these studies addressed their role in the environment.

The results presented in this chapter show that *Micromonospora* strains are common in the two lakes studied, that they colonise submerged cellulosic substrates, and that they are actively growing in these environments. Their distribution in the lakes shows that growth is more significant in the upper water column, as it was possible to demonstrate by the strong evidence for the presence of hyphae there (Fig 3.14.), and also by the increasing numbers of spores isolated from baits that had been submerged for longer periods of time.

Micromonospora strains were found at every isolation attempt; on one occasion (Nov. 2005) their numbers were relatively low. No clear distinction could be made between *Micromonospora* and *Streptomyces* numbers in the baits placed at or near the surface of both lakes, although on one occasion the baits were virtually free of *Streptomyces* in both lakes, whereas *Micromonospora* numbers were high. Numbers of *Micromonospora* in the baits placed near or in the sediment were frequently significantly higher than those of *Streptomyces* spp., as was the *case for* the water column and sediments. It is possible that *Streptomyces* spp. are also growing in the biofilm present in the baits closer to the surface, as these environments are rich in organic matter that these organisms can exploit, but the lower numbers in the water column and sediments, as well as in the baits placed close to the sediment indicate that this organism is not adapted to growing in the deeper layers of the lake.

There was no strong evidence for hyphae in the water column, and most of the micromonosporas there seemed to be present as spores; *Micromonospora* grows by hyphal extension, and it seems reasonable to expect that it grows much more extensively when attached to organic particles and solid substrates, which would explain the lack of evidence for hyphae in the water column.

The size of the population obtained varied considerably between the different samplings, perhaps as a result of episodic wash–in of spores from surrounding fields, as described by Cross (1981). The consistency in the shape of the depth profiles of *Micromonospora* numbers in the two lakes can be interpreted as an indication that seasonal factors are controlling the population dynamics, as the two lakes are quite different in physical attributes; Esthwaite Water is a large eutrophic lake surrounded by pasture whereas Priest Pot is small, hypereutrophic and surrounded by woodland. When compared to the literature, the numbers of *Micromonospora* obtained in lake muds in this study are of a similar order of magnitude ($8x10^4$ to 2.5×10^5 g^{-1} of wet sediment) as those obtained by other authors for lakes in North America (Trout Lake, Wisconsin) and the English Lake District (Blelham Tarn) several decades ago (Cross, 1981), showing the ubiquity of members of the genus in these environments; numbers obtained for water column here were generally one order of magnitude lower than those described in those studies.

The baits near the surface always had thicker biofilms compared to other depths, probably due to higher oxygen tensions and availability of light to promote the growth of photosynthetic bacteria and eukaryotes. The thick biofilm and higher oxygen tensions should favour *Micromonospora* growth and explain the high numbers of these organisms found in the baits, as these are aerobic organisms and capable of utilizing a variety of organic compounds.

Likewise, sedimentation of colonized particulate matter to the lake bottom would lead to high numbers of micromonosporas in lake sediments despite lower oxygen tensions. Bursts of *Micromonospora* growth in lake sediments are also possible when oxygen levels increase, such as in the autumn, and hyphae from these organisms have been found in lake sediments as was demonstrated here and previously (Johnston & Cross, 1976). The growth of micromonosporas in the sediments should stall with the onset of stratification and the decrease in dissolved oxygen concentrations to anoxic levels. This explains the fact that numbers of

micromonosporas isolated from baits placed near or in the sediment did not always increase in the baits that were submerged for longer periods of time. However, in the deep water column, nutrient and organic matter concentrations tend to be higher, and the large numbers of *Micromonospora* spores that settle above the sediment provide a source of germination points to develop when oxygen concentrations permit, making these organisms potentially important in the ecology of the sediment environment.

The baits placed in the sediment were frequently the most degraded compared to all others. This observation is corroborated in an separate study (Dr. Robert G. Lockhart, unpublished), which investigated cellulose degradation rates in the lakes and showed that cotton submerged in or near the sediments underwent the highest weight loss, although only in the summer months was the difference between sediment and shallower samples significant. In some cases in the winter months, weight loss was greatest in the cotton baits placed closer to the surface of the lakes. The high cellulose degradation rates near the sediment is probably due primarily to the action of anaerobic cellulose degraders, for example clostridia (Leschine, 1995).

The method used to detect hyphae in environmental samples is robust and can be used in conjunction with other methods, such as the sonication procedure described previously, and molecular biological tools, to show if micromonosporas are active in a particular environment. Although one set of results showed unexpected results, these do not invalidate the method, and reasonable explanations can be found to explain the presence of hyphal fragments in a relatively deep sediment sample.

In this study, it was not the intention to compare isolates arising from the two different lakes, or from different parts of each lake, and isolation of *Micromonospora* strains was based on morphology only. The isolation of *Micromonospora* strains reported here showed that the community present in the lakes is morphologically diverse, and the collection of isolates includes members that are capable of relatively high activity against cellulose and others that have only moderate or low activity; it was found that even when strains were confirmed as able to degrade cellulose by SEM, they were not necessarily able to disrupt the integrity of filter paper or cotton, or to release significant amounts of endoglucanase into the culture supernatant, even when growth was considerable. Whether these strains are able to compete for cellulose with *M. chalcea* strains in the natural environment cannot be determined, as other metabolic factors may confer an advantage to those strains

that produce less endoglucanases under laboratory conditions. In any case, particulate organic matter and sedimented biomass contains many other carbon sources to support the growth of micromonosporas in general. Cotton was used here to try to ensure that truly cellulolytic strains could be enriched. The cellulolytic system produced by *Micromonospora* isolate number 14 was not induced by CMC in this study, although Malfait *et al.*(1984) reported that 1% CMC was a good inducer of CMCase activity for *M. chalcea*. This discrepancy may have occurred due to the fact that in this study only 0.2% of CMC was added to the medium, although 0.2% xylan was quite sufficient to induce the secretion of xylanases in the same strain. In this study, cellulases were measured in the culture supernatant, but it is possible that a significant proportion of the hydrolytic system for these strains is cell bound, as concluded by Malfaiet *et al.* (1984). Other researchers regarded the *Micromonospora* cellulase system as a true free cellulase system (van Zyl, 1985), however this has not been analysed in any detail. Here xylanase activity was indeed detected in the cell pellet, and it is not unreasonable that in the early stages of growth a significant proportion of the cellulases are cell bound. Comparing the endoglucanase activity obtained in this study with that available in the literature is problematic as the cellulolytic system produced by *Micromonospora* strains has not been studied in detail and often the identity of the species used in not clear.

The degree of variation in cellulose degradation abilities was greater than expected, as many strains were very poor at attacking the substrate; the great number of strains that were poor at degrading cellulose is not an indication that these form the majority of the micromonosporas in the lakes as isolates were selected based on differing morphologies and not on abundance. Some of the highly active strains had high growth rates and produced significant amounts of spores, however their relative abundance in the lakes is unknown. Clearly, there are micromonosporas in the lakes that will colonise crystalline cellulose, without necessarily using it as a carbon source.

Despite more information still being required to determine the relative importance of micromonosporas in relation to other cellulose degrading microorganisms, the high numbers of micromonosporas recovered, the fact that the majority of these strains could attack cellulose, some to a great extent, and the detection of hyphae in surface sediments where organic matter accumulates, all indicate that the micromonosporas potentially have a significant role in the depolymerisation of cellulosic material in the two lakes studied.

Chapter 4 - Molecular diversity and phylogeny of *Micromonospora* lake isolates

4.1. Overview

A collection of 72 *Micromonospora* isolates was obtained from baits, lakewater and sediments from Esthwaite Water and Priest Pot. The isolates displayed a broad range of morphologies and considerable variability in their ability to degrade cellulose (Chapter III). The molecular diversity of the isolates was investigated in order to determine how diverse the community was, and what if any was the relationship between phylogeny and cellulose degrading abilities. The 16S rRNA gene was sequenced for 20 isolates, and phylogenetic trees were constructed, confirming the genus affiliation of the isolates and displaying some clustering which suggested that cellulose degradation abilities were not randomly distributed. However this gene was not very informative and the branching patterns had low bootstrap support. The DNA gyrase B gene was used to determine the phylogeny of the genus, with sequencing preceded by RFLP screening. The resulting phylogenetic trees were very robust with high bootstrap support, and the clustering of highly cellulolytic species into a single taxon was confirmed.

4.2. DNA extractions and PCR amplification

DNA extraction and PCR amplification of *Micromonospora* 16S rDNA was not straightforward due to the high DNA GC content, thick Gram positive nature of the cell walls, and presence of nucleases and PCR inhibitors in the cells, a problem typical of actinomycetes (Makinde & Gyles, 1998). In addition, micromonosporas have been reported to be resistant to lysozyme (Kawamoto, 1984). Boiled cells provided DNA of poor quality that had undergone extensive degradation, and PCR amplification was irreproducible and often failed to work. Phenol-chloroform based extraction methods and soil DNA extraction kits with ribolysing produced better results but still often failed to yield DNA of good quality. Non-specific amplification bands were also frequently observed.

In order to improve PCR amplifications of the 16S rRNA gene, the annealing temperature was increased from 55°C to 59°C, dimethyl sulfoxide (DMSO) was added to the PCR mix, and different sources of Taq polymerase were used,

resulting in a decrease in the occurrence of non-specific amplification, however yields remained low. PCR amplification improved dramatically when DNA was extracted from younger cultures that had not sporulated, and yields were significantly improved. It is possible that the spores contained PCR inhibitors or that they were physically resistant to lysis. Alternatively, cultures that had undergone extensive sporulation may have contained mainly senescent or lysed cells, and the DNA present in those cultures had degraded significantly. *Micromonospora* spp. are well known producers of a diverse array of secondary metabolites (Kawamoto, 1984), and cultures that had sporulated may have contained significant amounts of these compounds.

4.3. *Micromonospora* Phylogeny.

4.3.1. 16S rRNA gene phylogeny

The 16S rRNA genes of a total of 19 isolates were amplified, cloned, sequenced, aligned, and phylogenetic trees constructed using the neighbour adjoining method (sections 2.14., 2.15., 2.18. and 2.19.) (Fig 4.1.). It is clear from the phylogenetic trees produced that the 16S rRNA gene had poor resolving power for *Micromonospora* species phylogeny (Fig 4.1.). Once the branches with low bootstrap support (<75%) were collapsed, the resulting tree topologies were flat. It was possible to observe clustering of strains that produce high levels of endoglucanase activity, however the bootstrap support for these clusters was not very high (<70%). The trees obtained were not satisfactory and could not be relied upon for a rigorous analysis of the molecular diversity and phylogeny amongst the *Micromonospora* isolates obtained. This is due to insufficient variation in the 16S rRNA gene sequences within *Micromonospora* (>96% identity in the region amplified by universal primers pA and pH (Edwards *et al.*, 1989)).

A different phylogenetic marker was therefore required in order to determine the phylogeny of these strains.

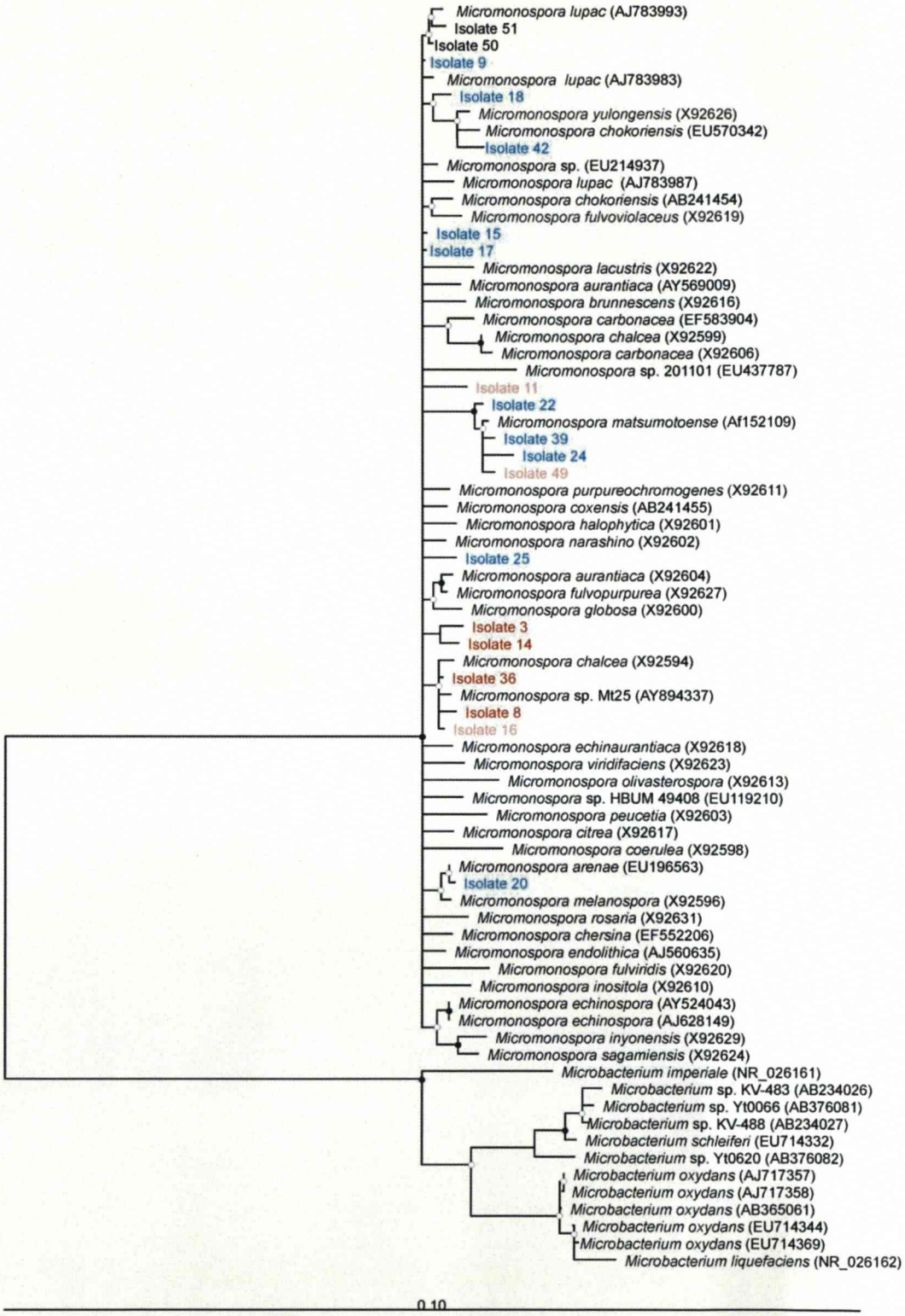


Figure 4.1. *Micromonospora* 16S rRNA phylogenetic trees.

Neighbour joining phylogenetic tree constructed with 16S rRNA gene sequences from 19 *Micromonospora* isolates and cultured representatives from the databases. The neighbour joining tree was constructed using the felsenstein evolutionary model for phylogeny correction with 1000 bootstraps. *Microbacterium* spp. were used as the outgroup. Branches with less than 75% bootstrap support were collapsed and the remaining bootstraps are represented by the circles in the branch nodes: filled circles, >95%; open circles, 75 to 95%. Colours indicate level of cellulose degradation: red, high activity; pink, medium activity; blue, low activity (see Table 3.3.). Isolates 50 and 51 were lost from the culture collection and their cellulose degradation ability is unknown. Scale represents 0.1 substitutions per nucleotide position; accession numbers are in brackets.

4.3.2. DNA gyrase B gene phylogenetic analysis

4.3.2.1. RFLP

Previous studies had already demonstrated that the 16S rRNA gene is not a good phylogenetic marker for *Micromonospora* species (Kasai *et al.*, 2000). The DNA gyrase B gene is one alternative marker that has been successfully applied to study bacterial phylogeny in a number of genera including *Mycobacterium*, *Gordonia*, *Acinetobacter* and *Pseudomonas* (Niemann *et al.*, 2000; Shen *et al.*, 2006; Yamamoto & Harayama, 1995; Yamamoto & Harayama, 1996). The gene encodes the beta subunit of DNA gyrase, a type II DNA topoisomerase, and is present in a single copy only in all prokaryotes and is unlikely to be laterally transferred (Shen *et al.*, 2006). Although an essential functional gene, there is enough variation that it can be exploited in taxonomy, and a database of DNA gyrase B gene sequences exists (http://www.mbio.co.jp).

PCR amplification of the *gyrB* gene was more difficult than 16S rRNA gene (Fig 4.1.), and amplified DNA yields were poor. Two different degenerate primers were tested for gyrB gene amplification, GYRB1 and GYRB2 (Shen *et al.*, 2006), and UP1TL and UP2rTL (Kasai *et al.*, 2000). GYRB1 and GYRB2 gave poor yields from DNA extracts of *Micromonospora* lake isolates, whereas UP1TL UP2rTL performed reasonably well after the annealing temperature was decreased from the 63°C used previously (Kasai *et al.*, 2000) to 59°C. Since the template DNA was from pure cultures, the decreased primer specificity caused by lowering the annealing temperature was not a potential problem, as long as the amplification products were confirmed as the *gyrB* gene. This was inferred from the observation that the product size did not change with the decrease in PCR annealing temperatures.

Initially, the DNA gyrase B gene of 66 isolates (PCR amplification of 6 isolates failed to produce enough DNA to visualize the RFLP patterns on agarose gels) was amplified using UP1TL and UP2rTL, 8 of which were cloned, sequenced and the contigs assembled as described in the section 2.19.1. Prior to determining the *gyrB* gene sequences of the remainder, it was decided to screen amplified *gyrB* gene fragments by RFLP analysis, so that representatives could be selected for sequencing.

The 8 *gyrB* gene sequences obtained were used in online restriction digest simulations in the New England Biosciences website (http://tools.neb.com/NEBcutter2/index.php) to determine which restriction enzymes

had potential for determining the phylogenetic diversity of the isolates. An enzyme was required that cut the amplified *gyrB* gene fragments at least once to produce fragments of different sizes to yield restriction patterns that allowed the separation of strains into groups.

Two potentially useful enzymes were tested in the laboratory. *Hae* II produced good results initially (Fig 4.2. A), and a number of restriction profiles identified potential groups from which representatives could be selected for cloning. However, when the *gyrB* gene from more strains was digested with this enzyme, there were a number of cases where no restriction of the gene was obtained (Fig 4.2. B, strains 60-62 and 66). This proved to be the most common outcome of the restriction with *Hae* II, and 13 of the 32 tested isolates had no restriction sites for this enzyme.

The second restriction enzyme tested was *Bst*N I, and the banding patterns observed were indeed sufficient for the separation of the isolates into seven different groups. These groups formed the basis for the selection of representative strains for sequencing. Once the new sequences were available, the online digestion was performed again in the New England Biosciences website to determine the sizes of the fragments produced; this analysis allowed the resolution of two new RFLP groups that were not previously distinguishable, as some of the fragments generated were very small and could not be visualised on the gels. A total of nine groups were identified when all of the available sequences were submitted to the online restriction analysis (Fig 4.3.; Table 4.1.).

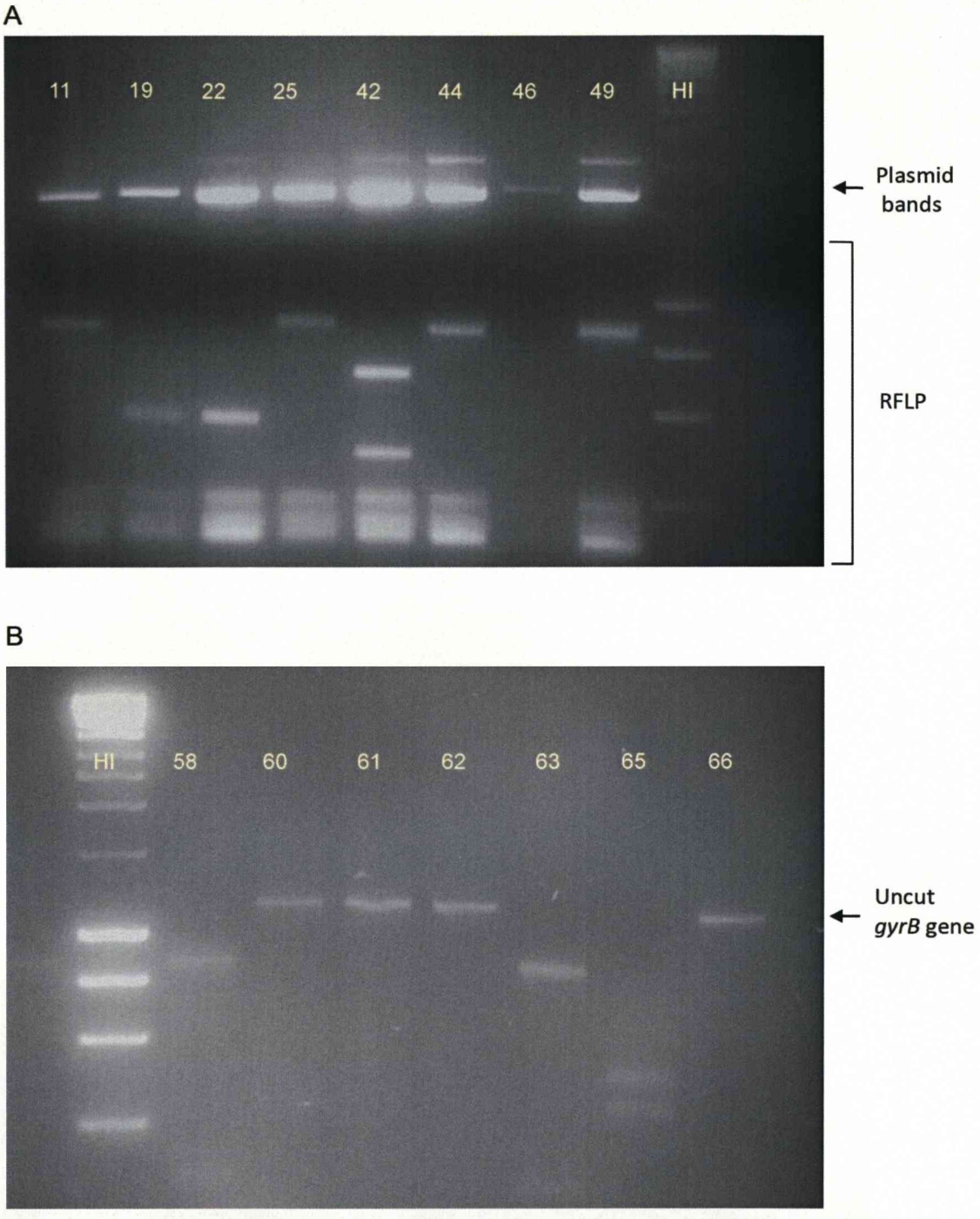


Figure 4.2. RFLP banding profiles produced from amplified *gyrB* gene products incubated with *Hae* II restriction enzyme.

A, restriction digests obtained with the combination of *Eco*R I and *Hae* II incubated with plasmids containing g*yrB* gene inserts at 37°C overnight (2% agarose gel); B, RFLP profile obtained from concentrated PCR products. Strain numbers are indicated. HI, hyperladder I. Uncut control not shown.

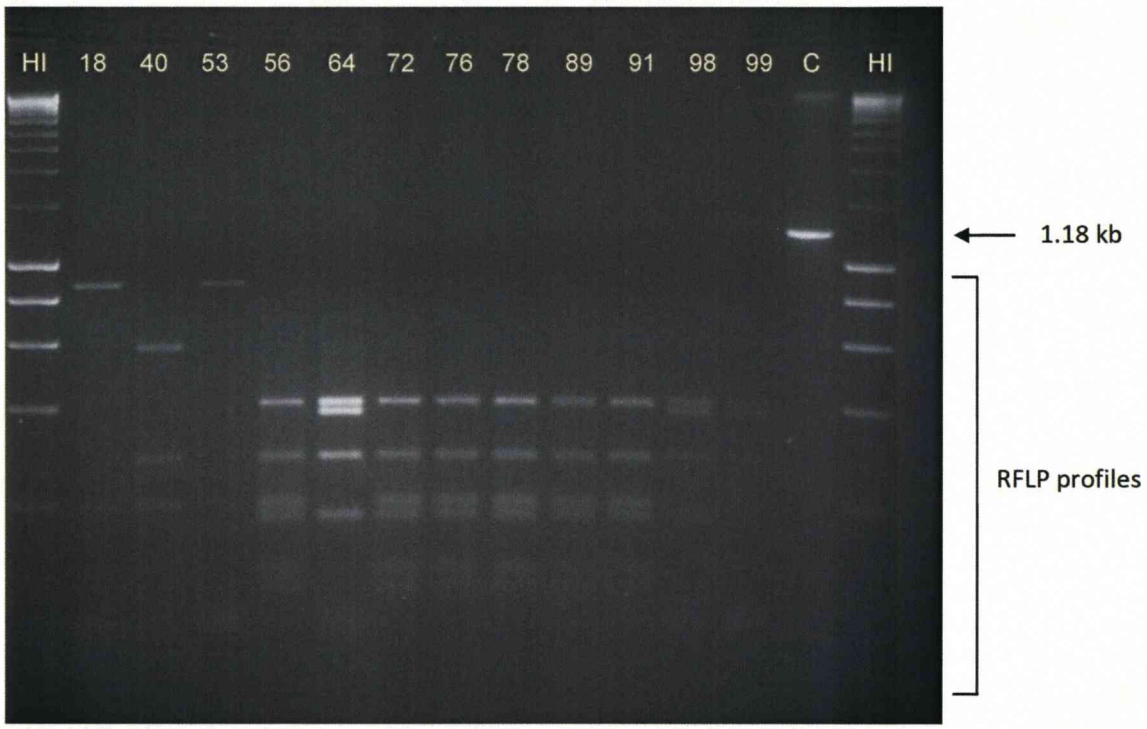


Figure 4.3. RFLP banding profiles produced from amplified *gyrB* gene products incubated with *Bst*N I restriction enzyme.

RFLP profile obtained from concentrated *gyrB* gene amplification products digested with *Bst*N I at 60°C overnight. Strain numbers are indicated. HI, hyperladder I. C, uncut *gyrB* control.

RFLP groups								
1A	1B	1C	1D	2	2B	3A	3B	4
399	399	399	399	573	573	827	827	653
353	254	353	254	254	254	192	273	353
254	192	184	192	192	192	81	80	174
174	174	174	174	81	161	80		
	161	70	81	80				
			80					
Total size								
1180	1180	1180	1180	1180	1180	1180	1180	1180

Table 4.1. Sizes of *gyrB* restriction fragments incubated with *Bst*N I.

Size expressed in base pairs.

Most of the strains belonged to four main RFLP groups (Table 4.2.). Their identity was compared to the data on cellulolytic activity (Table 3.3.). All strains that were very active against CMC and filter paper clustered in a single group (1A) that contained a total of 13 strains. RFLP types 2/2B included 28 strains that produced intermediate or low levels of CMCase activity; group 2B included one strain that could only be differentiated from RFLP type 2 through the online simulation of the *Bst*N I digestion, and it is therefore possible that more strains belong to this group. The same problem exists between groups 3A and 3B, with a total of 11 members, of which 5 of the 6 sequenced strains were of type 3A; all the isolates from these two groups had poor activity against CMC. The fourth main group was RFLP type 1D which included 9 isolates with intermediate or low activity against CMC. Three other groups comprised only one (1C) or two (4 and 1B) strains, which were all poor producers of endoglucanase activity (Table 4.2.).

FLP groups				
1A	1B	1C	1D	2
		Strain number		
99,98,73,70 64,55,45,36 16,14,13,8,3	83,78	48	91,89,86 76,74,72 57,56,11	94,93,92,90,88 84,82,81,80,77 75,71,69,68,66 63,62,61,60,59 58,49,46,44,41 39,24
RFLP groups				
2B	3A	3B	4	Unassigned
		Strain number		
25a	53,47,42,37 22,19,18 15,9,95	85	65,20	97,96,87 79,67,26

Table 4.2. RFLP profiles of each *Micromonospora* lake isolate produced from *gyrB* with *Bst*N I restriction enzyme.

Unassigned strains are those for which there was insufficient amplified DNA to produce clear RFLP patterns. For strain details and endoglucanase activity see Table 3.3.

4.3.2.2. Phylogenetic trees

Phylogenetic trees constructed with the *gyrB* sequences (sections 2.14., 2.15., 2.18. and 2.19.) (Fig 4.4.) revealed a robust clustering pattern, with a remarkable consistency between the trees obtained with different clustering methods, and with high bootstrap support values throughout. This clustering was also generally consistent with the RFLP groups, but there were exceptions. RFLP group 1A isolates all clustered within one branch; this cluster included two *M. chalcea* sequences, one obtained from the database and another produced here from the cultured type strain. Branch lengths within this cluster were very small indicating that the strains are very closely related. The strains did however exhibit morphological and physiological variations, indicating that these were not simply re-isolations of same strain. A second main cluster containing 16 strains was delineated, but it did not include any named species from the database, and therefore could represent a novel centre of variation within *Micromonospora*; this group is divided into two main branches corresponding to RFLP types 1D and 2. Other clusters of strains were related to *M. coerulea*, *M. purpureochromogenes* (both associated with RFLP profiles 3, 3A and 3B) and *M. aurantiaca* (strain 48, RFLP type 1C). Strain N° 42 was not closely associated with any named species or any other isolate from the lake samples.

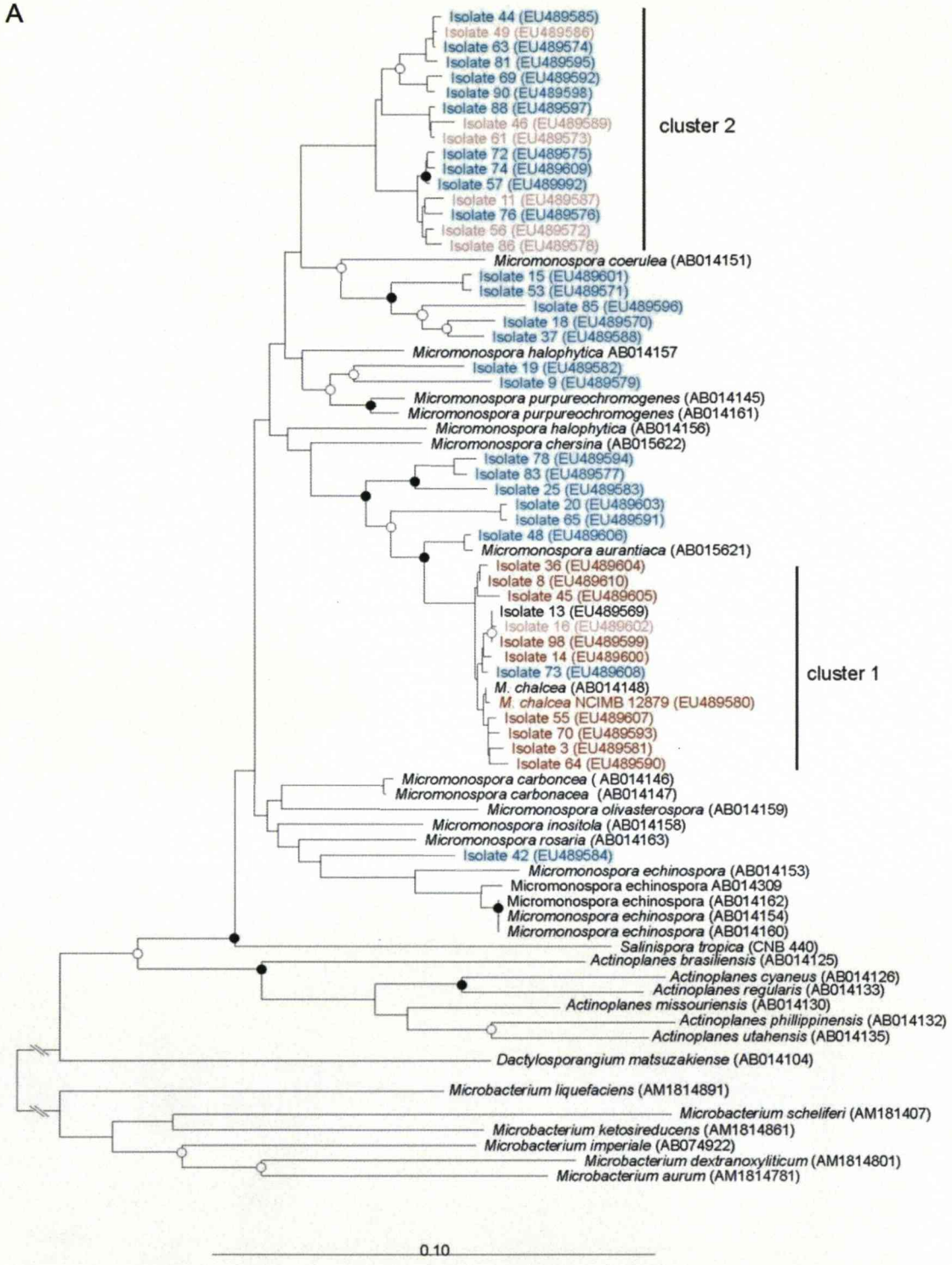
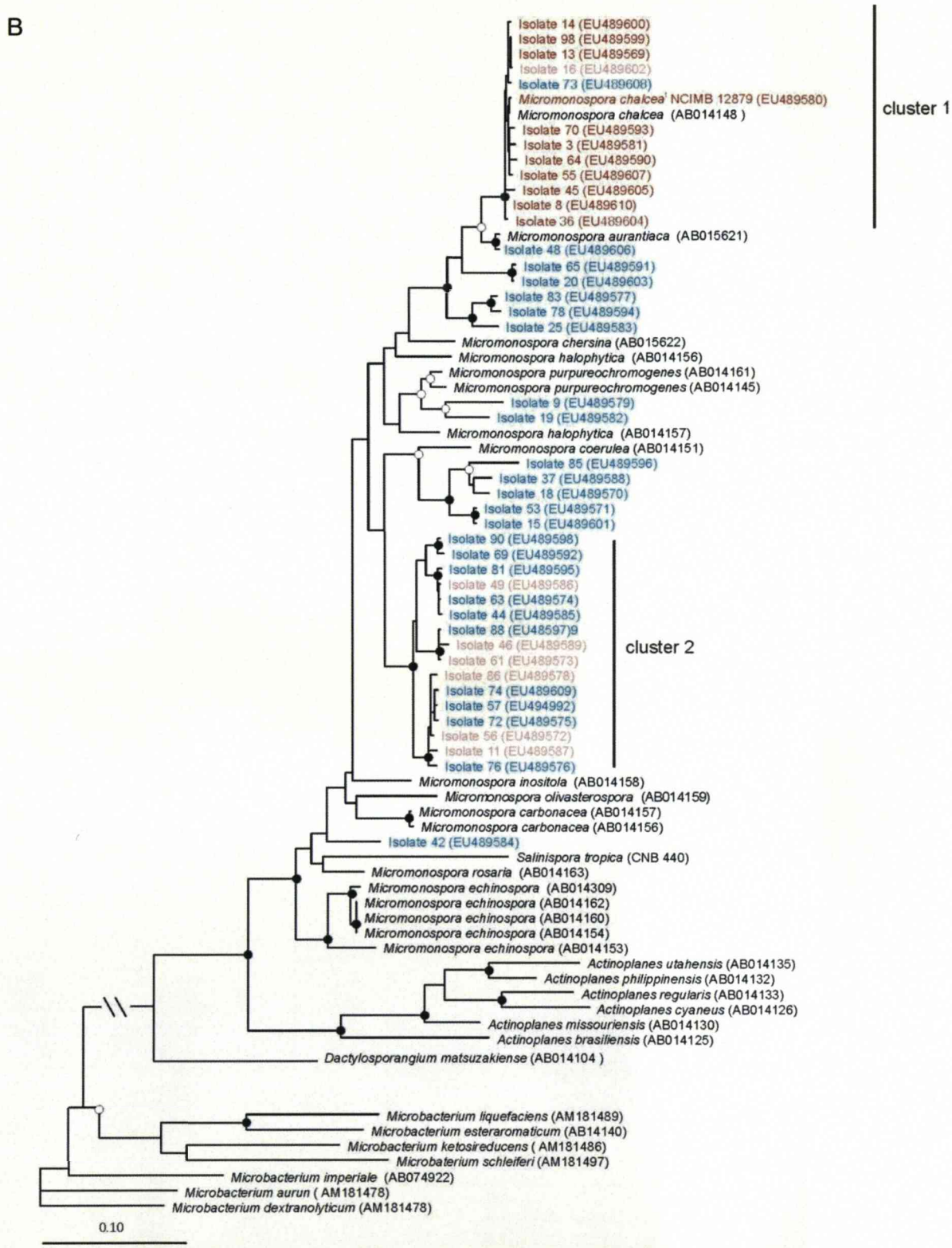


Figure 4.4. *Micromonospora gyrB* gene phylogenetic trees.

A and B (overleaf): phylogenetic tree obtained using the neighbour joining and maximum likelihood methods respectively.


B
Isolate 14 (EU489600)
Isolate 98 (EU489599)
Isolate 13 (EU489569)
Isolate 16 (EU489602)
Isolate 73 (EU489608)
Micromonospora chalcea' NCIMB 12879 (EU489580)
Micromonospora chalcea (AB014148)
Isolate 70 (EU489593)
Isolate 3 (EU489581)
Isolate 64 (EU489590)
Isolate 55 (EU489607)
Isolate 45 (EU489605)
Isolate 8 (EU489610)
Isolate 36 (EU489604)
cluster 1
Micromonospora aurantiaca (AB015621)
Isolate 48 (EU489606)
Isolate 65 (EU489591)
Isolate 20 (EU489603)
Isolate 83 (EU489577)
Isolate 78 (EU489594)
Isolate 25 (EU489583)
Micromonospora chersina (AB015622)
Micromonospora halophytica (AB014156)
Micromonospora purpureochromogenes (AB014161)
Micromonospora purpureochromogenes (AB014145)
Isolate 9 (EU489579)
Isolate 19 (EU489582)
Micromonospora halophytica (AB014157)
Micromonospora coerulea (AB014151)
Isolate 85 (EU489596)
Isolate 37 (EU489588)
Isolate 18 (EU489570)
Isolate 53 (EU489571)
Isolate 15 (EU489601)
Isolate 90 (EU489598)
Isolate 69 (EU489592)
Isolate 81 (EU489595)
Isolate 49 (EU489586)
Isolate 63 (EU489574)
Isolate 44 (EU489585)
Isolate 88 (EU48597)9
Isolate 46 (EU489589)
Isolate 61 (EU489573)
Isolate 86 (EU489578)
Isolate 74 (EU489609)
Isolate 57 (EU494992)
Isolate 72 (EU489575)
Isolate 56 (EU489572)
Isolate 11 (EU489587)
Isolate 76 (EU489576)
cluster 2
Micromonospora inositola (AB014158)
Micromonospora olivasterospora (AB014159)
Micromonospora carbonacea (AB014157)
Micromonospora carbonacea (AB014156)
Isolate 42 (EU489584)
Salinispora tropica (CNB 440)
Micromonospora rosaria (AB014163)
Micromonospora echinospora (AB014309)
Micromonospora echinospora (AB014162)
Micromonospora echinospora (AB014160)
Micromonospora echinospora (AB014154)
Micromonospora echinospora (AB014153)
Actinoplanes utahensis (AB014135)
Actinoplanes philippinensis (AB014132)
Actinoplanes regularis (AB014133)
Actinoplanes cyaneus (AB014126)
Actinoplanes missouriensis (AB014130)
Actinoplanes brasiliensis (AB014125)
Dactylosporangium matsuzakiense (AB014104)
Microbacterium liquefaciens (AM181489)
Microbacterium esteraromaticum (AB14140)
Microbacterium ketosireducens (AM181486)
Microbaterium schleiferi (AM181497)
Microbacterium imperiale (AB074922)
Microbacterium aurun (AM181478)
Microbacterium dextranolyticum (AM181478)
0.10

Phylogenetic trees include *Micromonospora* isolates, named strains and other members of the *Micromonosporaceae*. Colours indicate level of cellulose degradation (Table 3.3.): red, high activity; pink, medium activity; blue, low activity (Table 3.3.). Both trees were produced with 100 bootstraps, and bootstrap values are represented by the circles in the branch nodes: filled circles, >95%; open circles, 75 to 95%. The trees were rooted with members of the genus *Mycobacterium*; sequence accession numbers are in parentheses at the end of each branch. The scale bar represents 0.1 substitution per nucleotide position.

4.4. Discussion

The 16S rRNA gene is the gold standard of bacterial taxonomy and phylogeny (Stackebrandt & Goebel, 1994), and there are many features that make this an ideal gene for bacterial phylogenetics, as discussed previously (section 1.5.1.). However, while an ideal gene for studying deep branches in the bacterial and archaeal lineages it is much less informative at species level. 16S rRNA genes evolve slowly, and in some cases there is insufficient divergence to produce an accurate phylogeny at the species level. Nucleotide substitutions in the variable regions of the 16S rRNA gene may cause errors in the phylogenetic analysis of closely related strains, and the removal of the variable regions often improves the congruence of phylogenies produced with the 16S rRNA gene and other phylogenetic markers such as the DNA gyrase *B* gene (Yamamoto & Harayama, 1995). Some housekeeping protein encoding genes such as *gyrB*, whilst retaining enough conservation to be used as a molecular marker, evolve faster than the 16S rRNA gene and can be more informative for resolving species and genera, although unreliable at deeper branches, for example at the phylum level (Ludwig & Schleifer, 1999).

DNA-DNA hybridization studies are often used when it is necessary to study relationships between closely related strains or species, however this method suffers from a number of limitations and results are often inconsistent (Coenye *et al.*, 2005). It has become customary to use housekeeping genes to complement bacterial phylogenies at the genus and species levels. These genes are widespread in prokaryotes, and should not be susceptible to frequent horizontal gene transfer events if they are to be used in phylogenetic studies (Ludwig, 2001).

The gyrB gene has been successfully applied to study the phylogeny of closely related bacterial species and strains. Yamamoto *et al.* (1995) pioneered the use of the *gyrB* gene for bacterial taxonomy, and applied it to study the phylogenetic structure of members of the genus *Pseudomonas*. The DNA gyrase B gene is now a common alternative to the 16S rRNA gene and DNA-DNA hybridization studies when resolving the finer detail of prokaryote phylogeny. Other genes have also been used to elucidate the phylogeny of actinomycetes, such as the *rpoB* gene (Kim *et al.*, 2003) which encodes for the beta subunit of RNA polymerase.

In the particular case of *Micromonospora*, Kasai *et al.* (2000) found that 16S rRNA phylogenetic trees did not always agree with other taxonomic characteristics used to

classify members of the genus, and more specifically did not agree with DNA-DNA relatedness studies, whereas those trees obtained with from *gyrB* gene sequences did.

Here, some congruence was observed between trees obtained with 16S rRNA and *gyrB* genes; strains belonging to cluster 1 in the *gyrB* gene trees are also clustered, albeit with low boostrap support, in the neighbour joining 16S rRNA trees, but not in the tree produced with the treepuzzle method (Fig 4.1.). It cannot be determined whether the other clusters observed in the trees obtained from *gyrB* gene sequences, such as cluster 2, also occur in the 16S rRNA trees as these contain fewer strains that do not entirely coincide with those used to construct the *gyrB* gene trees. Despite the low bootstrap support, the clustering observed in the 16S rRNA trees of highly cellulolytic strains gives further support to the phylogenetic structure observed in the trees constructed with the *gyrB* gene sequences.

Kasai *et al* (2000) did not observe significant congruence between 16S rRNA and *gyrB* gene phylogenetic trees for micromonosporas, even when removing variable regions. They concluded that selective pressure might have influenced the 16S rRNA phylogeny within the *Micromonospora* genus, since some of the strains isolated were producers of aminoglycoside antibiotics, which are known to bind to the A site in the 16S rRNA subunit (Fourmy *et al.*, 1996), although the authors did not offer an explanation of how this selective pressure might take place.

The clustering observed in the trees produced here (Fig 4.4.) is sometimes congruent with that produced by Kasai *et al.* (2000); for example *M. purpureochromogenes* is closely associated with one *M. halophytica* strain, whereas the other *M. halophytica* strain branches closely to *M. chersina*. *Micromonospora chalcea* branches closely to *M. aurantiaca* in both studies, as do *M. rosaria* and *M. echinospora* strains; the position of *M. inositola* relative to the other species is similar in both studies; branching order however was not identical in both studies. One interesting result from this analysis was the clustering of *Salinispora tropica* amongst the *Micromonospora* strains, and not as a related group outside the *Micromonospora* radiation. This is a newly described member of the *Micromonosporaceae* family that has an obligate marine lifestyle, contrary to previous *Micromonospora* strains isolated from marine environments (Mincer *et al.*, 2002). The branch joining to *Salinispora* is quite lengthy, indicating that it is significantly differentiated from the typical "micromonosporas"; however the

clustering pattern suggests that it evolved from an ancestral *Micromonospora* that adapted to the marine environment and not from a common ancestor of the two groups, as such an evolutionary pattern would have been evidenced in *Salinispora* branching as a sister group to *Micromonospora*, and not within it. Conversely, the tree topology obtained could be explained if micromonosporas evolved from *Salinispora*.

The phylogenetic tree presented by Thawai *et al.* (2008) using 16S rRNA sequences showed some similarities with the trees produced with *gyrB* gene sequences discussed above, such as the clustering of *M. chalcea* and *M. aurantiaca*, but there were few other similarities, and a comprehensive analysis is made difficult as the species that the author included in his studies do not coincide entirely with the studies using *gyrB* to determined *Micromonospora* phylogeny (Kasai *et al.*, 2000). Thawai *et al.* (2008) also included *Salinispora arenicola* in this tree, and it branched as a sister group to the micromonosporas, albeit with low bootstrap support; bootstrap support for most of the *Micromonospora* branches within this tree is also low.

Cluster 2 was the largest cluster in the trees constructed with *gyrB* gene sequences (Fig 4.4.), and this cluster corresponded to the RFLP patterns 2 and 1D, which together comprised almost 50% of all the isolates. No named *Micromonospora* strain clustered within this group and this could represent a new centre of variation within the genus. It would not be unexpected if these strains correspond to one or more new *Micromonospora* species for which *gyrB* gene sequences have not been produced. Although this cluster contained the largest number of isolates, this is not evidence that they are the most abundant micromonosporas in the lakes, as isolates were selected on differences in morphology in order to ensure that diversity was properly represented. Rather, it suggests that this cluster contains a significant proportion of the diversity of *Micromonospora* strains in the lakes.

Most new species of *Micromonospora* have been described on the basis of 16S rRNA gene sequence similarities, DNA-DNA hybridization studies, and basic biochemical characterizations, with 19 new species of the genus described in this way over the last 5 years (Ara & Kudo, 2007a; Hirsch *et al.*, 2004; Kroppenstedt *et al.*, 2005; Thawai *et al.*, 2005a; Thawai *et al.*, 2005b; Thawai *et al.*, 2007; Thawai *et al.*, 2008; Trujillo *et al.*, 2005; Trujillo *et al.*, 2006; Trujillo *et al.*, 2007). None of these studies used the *gyrB* gene sequence analysis, or that of any other housekeeping

gene, to give further support to the taxonomic status of these newly described species. In this study, it was not intended to perform extensive phenotypic analysis of isolates in order to determine whether they comprised new species or were strains of species already described. The *gyrB* gene sequence analysis alone is not enough to determine whether groups of isolates constitute a distinct species, and in order to properly determine the species status of these strains it would be necessary to perform phenotypic tests, and possibly DNA-DNA hybridization studies. In addition, the *gyrB* gene sequences for the new species of *Micromonospora* that have been recently described are not available, and it would have been necessary to determine these sequences if the novelty of the groups revealed here (e.g. cluster 2, Fig 4.4.) were to be confirmed.

Few studies have focused on the cellulose degrading abilities of *Micromonospora* and there has been no previous attempt to relate phylogeny to the cellulolytic ability of different species and strains. Cellulose degrading abilities of other microorganisms, such as clostridia, and aerobic and anaerobic fungi have been extensively studied, however it is rarely reported how cellulose degrading abilities are distributed in the different species or strains of a given taxon known to contain cellulolytic members, with a few exceptions (Duncan *et al.*, 2008; Ibrahim *et al.*, 1982; Ren *et al.*, 2007; Schulz & Thormann, 2005).

In conclusion, it is clear that within the isolates obtained in these study, high levels of cellulose degradation ability under optimal growing conditions is restricted to a specific group of strains that equate or are closely related with *M. chalcea*. Cellulose degradation ability, although widespread, is not uniform within the genus. Further research is needed to determine if the differences observed in the cellulose degradation abilities of the isolates are due to quantitative or qualitative differences in their cellulolytic systems, and whether they are relevant to their ability to compete for cellulose in the natural environment.

Chapter 5 - Molecular ecology of *Micromonospora*, *Clostridium* cluster III, Anaerobic Fungi and *Fibrobacter* in freshwater lakes

5.1. Overview

Previous results in this study (Chapter III) have shown the presence and distribution of *Micromonospora* in the lakes with the use of microbiological methods, and demonstrated the presence of hyphae. The cellulose degradation abilities and phylogeny of the *Micromonospora* isolates was also determined, with the demonstration that at least one group of isolates closely related to *M. chalcea,* had a significant ability to degrade cellulose. In order to determine the importance of *Micromonospora* spp. in the lakes, it was necessary to study their abundance relative to other cellulolytic microorganisms. Specific quantitative PCR primers were designed, or obtained from the literature, targeting the 16S rRNA gene of *Micromonospora, Clostridium* cluster III, anaerobic fungi and *Fibrobacter spp.* and were used to quantify these organisms in cellulose baits, as well as in the sediments and water column of Esthwaite Water and Priest Pot. DNA and RNA were extracted and the presence of the target groups was detected by direct and nested PCR, and their numbers quantified by quantitative-PCR. The PCR products from the amplification of DNA from the lakes were also used to obtain clones of *Clostridium* cluster III, *Fibrobacter* and anaerobic fungi, and their phylogenies determined.

5.2. *Micromonospora* quantitative PCR primer design

Oligonucleotide primers specific to the 16S rRNA gene of actinomycetes or the Actinobacteria have been developed previously (Heuer *et al.*, 1997; Stach *et al.*, 2003) and also for selected actinomycete genera such as *Streptomyces* spp. (Kumar *et al.*, 2007), *Actinopolyspora* and *Streptomonospora* spp (Zhi *et al.*, 2007); recently specific PCR primers were designed for members of the genus *Micromonospora* (Qiu *et al.*, 2008), however these primers were not applied to nucleic acids extracted from the environment, and specifically there are no published primers designed for quantifying these organism by qPCR.

For the purpose of molecular quantification of *Micromonospora* numbers in the lakes, it was initially considered to target the *gyrB* gene for qPCR analysis as this

gene allowed the discrimination of individual clusters within the *Micromonospora* phylogenetic tree, including the highly cellulolytic strains. However this was not taken forward for practical reasons: previous work in the laboratory had shown that by using reverse transcribed RNA as a template, it was possible to get a higher signal (16S rRNA copy numbers) than targeting the 16S rRNA gene directly in the extracted DNA fraction. The very large number of 16S rRNA gene sequences in the databases makes it a good target gene for primer design when working with mixed communities, particularly as it is possible to check for potential mismatches with sequences of non-target organisms. In the case of the *gyrB* gene, there are much fewer sequences in the databases, making specificity checks impractical; in addition the gene would have to be quantified in the DNA fraction, which would potentially result in a lower signal than can be achieved with 16S rRNA. A further reason for not using the *gyrB* gene is that a general qPCR primer would have to be designed for this gene in order to determine relative abundances, which would require considerable effort, whereas general qPCR primers targeting the 16S rRNA gene are available (Suzuki *et al.*, 2000). It was therefore decided to design *Micromonospora* specific primers for 16S rRNA only.

The primers were designed specifically for quantitative PCR analysis and in order to be efficient, the following criteria were applied from the QuantiFast™ SYBR® Green PCR Handbook:

- Between 18 and 30 bp in length.
- GC content between 40 and 60%.
- T_m of each primer with similar values.
- The amplified PCR products no longer than 200 bp in length.
- Complementarity of 2 or more bases at the 3' end avoided in order to minimise primer dimer formation.
- Mismatches of 3 bp between the 3' end and the target avoided.
- Runs of 3 guanines or cytosines at the 3' end avoided, as should a 3' end thymine as these increase tolerance to a mismatch.
- Complementary sequences within a primer and between primer pairs should also be avoided in order to minimize primer dimer and secondary structure formation.

The initial choice of potential sequences for primers was made using the alignments in ARB (Ludwig *et al.*, 2004). A database containing pre-aligned bacterial 16S rRNA

sequences was obtained from SILVA (http://www.arb-silva.de) and imported into ARB with the addition of the 19 *Micromonospora* 16S rRNA sequences obtained from the lake isolates (Chapter IV). Strings of sequence that highlighted only *Micromonospora* sequences were selected and tested for specificity using the probe match tool of the RDP database website: http://rdp.cme.msu.edu/probematch/search.jsp.

Potential sequences identified in ARB for primer development were obtained and tested in the laboratory using DNA from *Micromonospora* pure cultures. Several forward and reverse primers were tested in order to obtain good amplification efficiency, low ammouts of primer dimer formation, calibration curves with high R^2 and an annealing temperature close to 60°C. Primers M1C and M2D were finally chosen for qPCR quantification of *Micromonospora.*

5.2.1. *Micromonospora* M1C M2D primer specificity analysis

The specificity of the modified primers M1C and M2D was evaluated using Probe Match (Cole *et al.*, 2009) (Table 5.1.) and probeCheck (Loy *et al.*, 2008). In addition, probeCheck also showed that primer M1C had additional matches with members of some newly described species of the *Micromonosporaceae*: *Solwaraspora* sp. (Maldonado *et al.*, 2009) and *Luedemannella* sp. (Ara & Kudo, 2007c), whereas primer M2D also had matches with, *Solwaraspora* sp., *Krasilnikova cinnamoneum* (Ara & Kudo, 2007b) and *Couchioplanes* sp. (Tamura *et al.*, 1994), and in addition the coryneform actinobacteria *Agrococcus* sp. (Groth *et al.*, 1996) and *Ornithinimicrobium* sp. (Groth *et al.*, 2001). The reason for this discrepancy between the two search engines is difficult to explain as both perform searches in the same databases.

Although each primer had matches and 1 bp mismatches with non-*Micromonosporaceae* strains, the combination of M1C and M2D resulted in matches with only *Micromonospora* and a smaller number of other *Micromonosporaceae* strains: 13 strains of *Actinoplanes* (7 of which were uncultured), one uncultured *Dactylosporangium* and two *Polymorphospora* strains. There were also 10 matches within the unclassified and uncultured *Micromonosporaceae*. Other than members of the *Micromonosporaceae,* the database search showed that the only other groups that could potentially be amplified by these primers were members of the

Nocardioidaceae and the *Pseudonocardiaceae,* as there were a significant number of 1 bp mismatches for both forward and reverse primers. However it was hoped that the specificity of the primers would be enhanced by the buffers used in the qPCR survey, as this is a feature of commercial qPCR buffering systems. Although these are not the target of the study, some members of *Pseudonocardiaceae* are also saprophytic (Henssen, 1984) and could therefore have a similar ecological role as *Micromonospora* spp. within these environments.

Further tests were done in the laboratory to determine the suitability of this primer pair to quantify *Micromonospora* spp. in environmental samples. M1C and M2D were used to amplify DNA extracted from pure cultures of *Escherichia coli*, *Bacillus* sp., *Pseudomonas* sp. and six different strains of *Streptomyces* spp. isolated from the lake samples (Fig 5.1.), and no amplification products were detected.

Table 5.1. Specificity analysis of primers M1C and M2D using Probe Match.

	Organism	M1C		M2D	
		Match	1 bp mismatch	match	1 bp mismatch
Micromonosporaceae	*Micromonospora* spp.	585/765	601/765	556/765	591/765
	Asanoa spp.			7/16	7/16
	Catenuloplanes spp.		3/9		3/9
	Cattelatospora spp.	11/47	17/47		25/47
	Longispora spp.		2/4		2/4
	Pilimelia spp.		¾		3/4
	Verrucosispora spp.		21/24		
	Dactylosporangium spp.	7/68	27/68	3/68	23/68
	Spiriliplanes spp.			1/1	1/1
	Polymorphospora spp.	2/2		2/2	2/2
	Virgisporangium spp.	12/18	12/18		
	Actinoplanes spp.	23/265	132/265	112/265	138/265
	unclassified *Micromonosporaceae*	53/402	171/402	52/402	152/402
Actinobacteria	*Acidimicrobiaceae*		45/373		
	Actinomycenae				1/796
	Actinosynnemataceae				166/233
	Bifidobacteriales		25/2868		
	Catenulisporaceae				22/108
	Coriobactericeae		2/2445		
	Corynebacterinae		2511/9698		4/9698
	Micrococcinae		1/12682	1/12682	5204/12682
	Frankineae		3/738	4/692	234/692
	Lentisphaerae		10/258		
	Propionibactericeae	638/1257	693/1257		
	Nocardioidaceae	1/1720	36/1720		214/1720
	unclassified propionibacterinae		5/140		
	Pseudonocardiaceae		316/985		106/985
	unclassified Pseudonocardinae				20/33
	Rubrobacterinae	1/1934	172/2611		1/2611
	Streptosporanginae			1/1693	205/1693
	unclassified actinobacteria	83/3950	881/3950		6/3950
	unclassified actinobacteridae	1/65	4/65		
	unclassified actinomycetales	39/4451	243/4451	1/4451	93/4451

Other Bacteria	Acidobacteria		1/27308	4/27308
	Aquificaceae		42/12148	
	Bacteroidetes	0/78826	3/78826	
	Burkholderiales			
	Chlamydiae		47/538	
	Chloroflexi	78/5754	823/5754	
	Chloroplast		42/4490	
	Nitrospira	76/2004	131/2004	
	Thermodesulfobactericeae		7/119	
	Defrribacteres		36/440	
	Fibrobacteres	1/364	233/328	
	Firmicutes	1/170839	206/170839	4/170839
	Spirochaetes	523/4152	1043/4152	
	Planctomycetes	9/6566	263/6566	
	unclassified bacteria	53/30558	780/30558	7/30558
	Rhizobiales			
	OP11		2/142	
	OP10			11/394
	WS3		1/223	
	Proteobacteria	12/248347	146/248347	2/248347
	Gemmatimonadaceae		26/1763	1/1763
	Anaeroplasmataceae		1/109	
	Verrucomicrobiota	5/7070	78/7070	

Columns show the number of matches within the total number of sequences available in the RDP II database for each group. The 1 bp mismatch column shows added numbers of matches and 1 bp mismatches for each group.

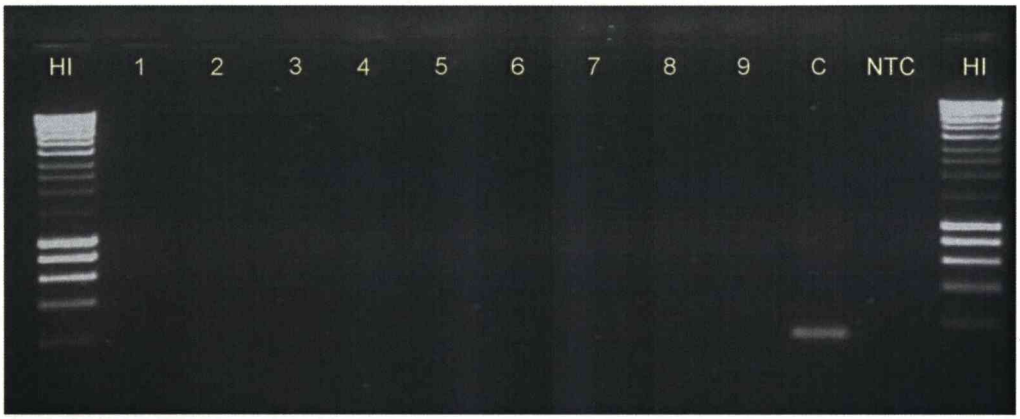


Figure 5.1. PCR specificity analysis of the M1C and M2D primers.

PCR performed with annealing temperature of 60°C. 1, *E. coli*; 2, *Pseudomonas* sp; 3, *Bacillus subtilis*.; 4, actinomycete lake isolate N° 1; 5, actinomycete lake isolate N° 4; 6, actinomycete lake isolate N° 5; 7, actinomycete lake isolate N° 6; 8, actinomycete lake isolate N° 7; actinomycete lake isolate N° 30; C, *Micromonospora* lake isolate N° 13; NTC, no template control. HI, hyperladder I.

5.2.2. Application of primers on test environmental samples

Following the specificity test on pure-cultures, the primers were tested on nucleic acid extracts obtained from environmental samples. DNA and RNA were extracted from baits recovered from the lakes in November 2007, and stored at - 80°C. DNA was also extracted from cotton baits recovered from the lakes in July 2005 as part of a separate study and stored at - 80°C (the RNA extracts from these two samples were not used as it was significantly degraded). The RNA extracts were reverse transcribed as described in the methods section (2.12.2.). The results (Table 5.2.) show that the numbers and relative abundance of *Micromonospora* were generally low, with the proportion of *Micromonospora* in the general bacterial cDNA remaining below 0.1% for all samples. As these DNA/RNA samples had been extracted from cotton baits that had been frozen for several months or a few years and could be degraded, fresh extracts were also obtained from local soils (at a site in the University of Liverpool and another close to a pond in Cheshire). The 16S rRNA was reverse transcribed and numbers of *Micromonospora* and general bacteria obtained by qPCR; numbers of *Micromonospora* in the soil samples were also determined on isolation plates using the methods described in section 2.2.2. The results obtained (Table 5.3.) show that the proportion of *Micromonospora* relative to total bacteria in the soils was low (< 1%), but higher in the University soil samples compared to the pond sample.

Sample	*Micromonospora*	Std error (%)	r^2	General bacteria primers	Std error (%)	r^2	*Micromonospora* abundance (%)
1	1.42E+04	14.51	0.99	4.01E+07	6.75	0.99	0.0354
2	1.54E+05	13.65	0.99	2.11E+08	8.37	0.99	0.0729
3	5.24E+01	11.49	0.99	6.72E+07	9.28	0.99	0.0001
4	5.01E-01	1.10	0.99	4.46E+03	1.53	0.99	0.0112
5	5.45E+00	15.36	0.99	2.78E+06	7.09	0.99	0.0002

Table 5.2. Results of the first test using M1C and M2D primers to quantify *Micromonospora* 16S rRNA copies in environmental DNA samples.

Copy numbers of *Micromonospora* and general bacterial 16S rRNA were obtained from reverse transcribed 16S rRNA extracted from cotton baits and measured by qPCR. 1, Esthwaite Water surface bait, November 2007; 2, Esthwaite Water sediment bait November 2007; 3, Esthwaite Water 3 m deep bait, July 2005; 4, Esthwaite Water sediment bait, July 2005; 5, Priest Pot 0.5 m deep bait, July 2005.

Sample	*Micromonospora* 16S rRNA copies	Std error (%)	General bacteria 16S rRNA copies	Std error (%)	*Micromonospora* abundance (%)	*Micromonospora* in isolation plates (CFU g^{-1})
University	5.59E+05	0.81	1.77E+08	1.21	0.32	6.27E+05
Wet soil	2.46E+03	3.03	1.21E+07	2.31	0.02	1.05E+05

Table 5.3. Numbers of *Micromonospora* isolated from soil compared to 16S rRNA copy numbers obtained from qPCR analysis.

Micromonospora CFU numbers were obtained in M3 agar plates; *Micromonospora* 16S rRNA was quantified using M1C and M2D primers. The r^2 of all the qPCR calibration curves are >0.99. Standard errors are given as percentages of the mean.

Micromonospora CFU numbers per gram of soil were higher in the University sample than the pond sample, correlating with their relative abundance to total bacteria in the same samples determined by qPCR (0.32 and 0.02%) (Table 5.3.). It is not possible to directly compare the data obtained from the qPCR with that obtained with isolation plates, as one method shows the relative quantification and the other the number of spores present in the sample, however it does demonstrate that a relatively high number of *Micromonospora* spores isolated in plates correspond to trace abundances on the qPCR for these soil samples.

As all the primer specificity tests showed specific amplification of *Micromonospora* spp., in addition to the fact that the *Micromonospora* abundances obtained in test samples were not unreasonable and were very low (Table 5.2. and 5.3.), primers M1C and M2D were regarded as appropriate for use in the quantification of *Micromonospora* in the lakes.

5.2.3. Comparison of general and specific 16S rRNA primers on the quantification of *Micromonospora* in pure cultures.

In addition to the specificity tests discussed above, an experiment was also used to determine if 16S rRNA gene copy numbers of *Micromonospora* quantified using specific primers agreed with the values obtained using general bacterial primers. As DNA had been extracted from pure cultures and both specific and general primers targeted the 16S rRNA gene, copy numbers obtained with both primers should be similar.

DNA and RNA were extracted from cotton bait (see section 2.10.2.) colonised with a pure culture of *Micromonospora* strain 13, and 16S rRNA copies quantified both in the DNA and reverse-transcribed 16S rRNA fractions (Table 5.4.) using both M1C and M2D primers and 1369F and 1492R for general bacteria (Suzuki *et al.*, 2000) in the same analysis.

Micromonospora specific primers consistently overestimated gene copy number, although the discrepancy was sometimes smaller when using cDNA rather than DNA samples. Diluting the nucleic acids also decreased the discrepancy, but it was never completely eliminated and numbers of *Micromonospora* quantified with M1C and M2D were at least ca. 30% higher than the numbers observed using 1369F and

1492R. Nucleic acids were re-extracted from a separate bait colonised with the same strain (bait b) and submitted to the same analysis in order to check if the overestimation was due to the quality of the original sample. The analysis revealed no improvement in the agreement between the numbers obtained using the two primer pairs (Table 5.4.).

A different general bacterial reverse primer was used to replace 1492R as it was suspected that the low T_m (53°C) of this primer could be contributing to the discrepancies observed. Primer RTU8 (Ott *et al.*, 2004)), with a T_m of 63.4°C was tested in combination with primer 1369F, however the results showed that M1C and M2D were still overestimating *Micromonospora* 16S rRNA gene copy numbers (Table 5.5.).

The reason for this discrepancy is unknown, but it could be due to the physical properties of the target DNA. Calibration curves were produced using linearised primers containing 16S rRNA gene inserts of known amounts, whereas the target was either genomic DNA or reverse transcribed RNA from *Micromonospora* strain 13, both of which have a high GC content. The interaction between the two primer sets with linearised primers, genomic *Micromonospora* DNA or cDNA may not occur in the same manner (i.e. calibration curves produced with linearised plasmids containing 16S rRNA inserts may have different slopes to calibration curves using genomic DNA or cDNA), and the primers designed specifically for *Micromonospora* spp. may amplify genomic *Micromonospora* DNA more easily than the general bacterial primers.

It was not known whether the same problems would apply to environmental samples; it is unlikely that this limitation of the gene copy quantification using qPCR could be easily resolved, and it was decided to use primers M1C and M2D, albeit recognising the possibility that *Micromonospora* abundances may be overestimated.

Sample	M1C-M2D			1369F-1492R			M1C-M2D/ 1369F-1492R
	Average copy numbers	% std error	r^2	Average copy numbers	% std error	r^2	
Bait A DNA	3.56E+07	6.3	0.99	5.42E+06	11.9	0.99	6.57
Bait A cDNA	1.64E+06	2.1	0.99	1.05E+06	9.2	0.99	1.56
Bait A DNA x 10^{-1}	4.69E+06	7.8	0.99	9.83E+05	6.1	0.99	4.78
Bait A DNA x 10^{-2}	1.27E+06	35.5	0.99	4.01E+05	13.9	0.99	3.16
Bait B DNA	4.86E+07	8.8	0.99	3.60E+06	5.5	0.99	13.49
Bait B cDNA	3.98E+06	0.5	0.99	6.74E+05	5.4	0.99	5.90
Bait B DNA x10^{-1}	1.32E+07	17.3	1.00	7.52E+06	5.1	1.00	1.76
Bait B cDNA x10^{-1}	8.46E+05	12.0	1.00	6.06E+05	15.8	1.00	1.4
Bait B DNA x 10^{-4}	2.16E+04	14.5	1.00	1.69E+04	19.4	0.98	1.28
Bait B cDNA x10^{-2}	1.02E+05	3.5	1.00	4.10E+04	17.3	0.98	2.49
Bait B cDNA	7.29E+06	20.7	0.99	1.89E+05	18.8	0.98	38.56
Spore cDNA	2.28E+06	2.9	0.99	4.12E+04	15.9	0.98	5.55

Table 5.4. Copy numbers of *Micromonospora* 16S rRNA genes obtained with M1C-M2D and general bacterial primers.

The r^2 values provided are from the calibration curve used in each analysis. Standard errors are given as percentages of the mean. Bait A, first DNA/RNA extraction, Bait B, DNA/RNA extraction from a second bait colonised with the same *Micromonospora* strain (Isolate 13). Spore cDNA refers to reverse transcribed 16S rRNA from a pure culture colonising bait (bait B) extracted after sporulation.

sample	M1C-M2D			1369F-RTU8			M1C-M2D/ 1369F-RTU8R
	average copy numbers	Std. error (%)	r^2	average copy numbers	Std. error (%)	r^2	
CDNA strain 13	2.52E+06	5.21	0.994	4.93E+05	48.88	1	5.11

Table 5.5. Analysis showing the discrepancies in 16S rRNA copy numbers between the *Micromonospora* specific and an alternative reverse general bacterial primer pair to 1492R.

16S rRNA gene copy numbers from pure cultures (*Micromonospora* isolate 13) were obtained with primers M1C and M2D and 1369F and RTU8. The rRNA was extracted from cultures of the same species (lake isolate N° 13) before and after significant sporulation had occurred. The r^2 are from the calibration curve used in each analysis.

5.3. *Clostridium* cluster III primer design

Cellulolytic clostridium species are likely to play a significant role in cellulose degradation in the anaerobic zones of lakes (Leschine, 1995). The clostridia include some of the most active cellulose degraders known, and their polysaccharide-degrading activities in anaerobic sediments have been documented on only a few occasions (Chin *et al.*, 1998; Uz *et al.*, 2007), as well as their presence in other anaerobic environments rich in cellulose, such as landfills (Van Dyke & McCarthy, 2002). Four major taxonomic groups within clostridia are known to contain cellulolytic species, comprising clostridial clusters I, III, IV and XIV as defined by Collins *et al.* (1994). However, cluster III is the only clostridial group in which all named, cultivated species are cellulolytic. As the phylogenetic diversity within the genus is enormous, comprising organisms that are now known to belong to different families, primer design for cellulolytic clostridia needs to be focused on subgroups within clostridia, and cluster III was chosen as the target for this study.

The clostridial qPCR primer design was performed in an Arb database downloaded from SILVA, which contained sequences from all the cultured *Clostridium* III species as defined by Collins *et al.* (1994) in addition to strains isolated more recently but with known affiliations to cluster III clostridia (Akasaka *et al.*, 2003; Chin *et al.*, 1999; Kato *et al.*, 2005; Kjeldsen *et al.*, 2007; Lin *et al.*, 1994a; Rainey & Stackebrandt, 1993).

Potential sequences that were specific to *Clostridium* III were identified in the regions flanking Clost3F and Clost3R and new primers were designed: primer 829C3R was designed 184 bp upstream of Clost3F with which it was to be used as a reverse primer. Primer 1423C3R was designed 54 bp upstream of Clost3R and was to be used as a reverse primer in combination with the reverse complemented version of the former, named Clost3RF (which was modified with the removal of one base in the end to increase the T_m and the GC content of the primer) (Fig 5.2.).

Name	Sequence (5' to 3')	*E. coli* numbering	T_m (°C)	Secondary structure	Dimers
Clos3F	TCTTGAGTGYYGGAGAGGAAAGC	650-672	65.7	Very weak	No
Clost3R	GRCAGTATDCTGACCTRCC	1352-1370	53.2	weak	No
829C3R	GGATACTTATTGTGTTWACTMCGG	856-879	59.4	None	No
Clost3RF	GGYAGGTCAGHATACTGY	1352-1369	44.8	Weak	No
1423C3R	TAYYGACTTCGGGTGTTGC	1423-1441	61.1	Weak	No

Table 5.6. *Clostridium* cluster III 16S rRNA gene qPCR primer sequences tested in this study.

Primer specifications were obtained from manufacturer (Sigma). Primer secondary structure and dimer formation are predicted. Primer Clost3F was obtained from Van Dyke & McCarthy (2002) and the others were developed in this study (section 2.20.). The GC content of all primers was between 38 and 48% and their length 18-24 bp.

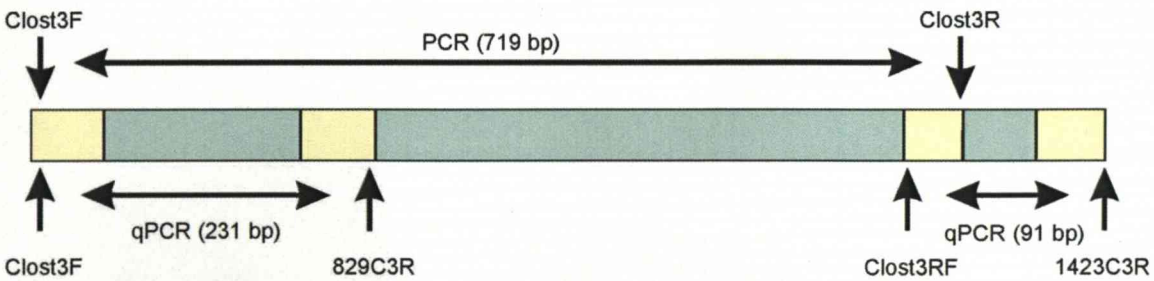


Figure 5.2. Diagram showing the position of *Clostridium* III PCR and qPCR primers in the 16S rRNA gene.

In yellow are the primer regions. Primer position in the *E. coli* numbering system and other general features are listed in Table 5.6.

The specificity of each primer was evaluated using Probe Match and probeCheck prior to laboratory tests. All sequences matching Clost3F primer were *Clostridium* cluster III strains except for: *C. sporogenes* (*Clostridium* cluster I), 8 *Selenomonas* sp. oral clones (from family *Veillonellaceae* within the Clostridiales), and a number of sequences of uncultured strains, most of which had affiliations with the *Clostridiaceae*. Except for *C. sporogenes*, there were no matches with any cultured strain that did not belong to *Clostridium* cluster III. The 16S rRNA sequence of the *C. sporogenes* strain that was matched by the primers was checked for identity in BLAST and many of its closest matches were *Clostridium* cluster III strains (*C. stercorarium* and *C. thermocellum*), which indicates that this strain was probably a *Clostridium* III strain that had been misidentified. The details of matches and 1 bp mismatches wth primer Clost3F are shown in Table 5.7.

Primer 829C3R was also degenerate and had four different forms; all the named species that were full matches with this primer belonged to the *Clostridium* cluster III group, except the same dubious *C. sporogenes* mentioned above, and *Papillibacter cinnamivorans,* which is closely related to rumen clostridia and ruminococci (Defnoun *et al.*, 2000). There were also a significant number of full matches that were sequences from uncultured *Clostridiaceae* bacteria or unassigned uncultured strains. The details of matches with primer 829C3R can also be seen in Table 5.7.

Although there was a significant number of non-*Clostridium* cluster III close matches (1 bp mismatch) for primer 829C3R, the combination with Clost3F should provide good selectivity for amplification of *Clostridium* III strains. In addition most of the 1bp matches were from groups of organisms of rumen or oral origin that were clearly closely related to *Clostridium*. In addition to these, there were a significant number of 1 bp mismatches for both primers within the Bacteroidetes group which corresponded to the genus *Megamonas* (Shah & Collins, 1982). The taxonomic status of this genus is disputed and some authors argue it should be placed within the Clostridiales (*Acidaminococcacea* family) (Morotomi *et al.*, 2007).

Clost3RF was not as specific as Clost3F (Table 5.8.), having a greater number of matches within *Clostridium* cluster III (*Ruminococcocaceae*), and within other clostridia, as well as a greater number of 1 bp mismatches with non-clostridial groups, such as the Cyanobacteria and Proteobacteria. Primer 1423C3R was generally more specific than its forward counterpart, except for the presence of a significant number of 1 bp mismatches within the Bacillales (Table 5.8.).

Table 5.7. Sequences matching primers Clost3F and 829C3R in the databases as determined by Probe Match.

	Organism	Clost3F		829C3R	
		match	1 bp mismatch	Match	1 bp mismatch
Clostridium III and related organisms	*Ruminococcaceae*	320/25045	804/25045	515/25045	4801/25045
	Ruminococcus spp.		1/2420	2/2420	766/2420
	Acetanaerobacterium spp.	1/43	1/43		8/43
	Acetivibrio spp.	38/110	65/110	25/110	60/110
	Faecalibacterium spp.		02/6838		19/6838
	Ruminococcaceae Incertae Sedis	148/1064	249/1064	214/1064	715/1064
	Papillibacter spp.		1/1329	67/1329	647/1329
	Sporobacter spp.		3/473		83/473
	Anaerotruncus spp.			1/241	68/241
	unclassified *Ruminococcaceae*	133/10256	482/10256	206/10256	2414/10256
Veillonellaceae	*Veillonellaceae*	34/5156	695/5156	2/5156	934/5156)
	Centipeda spp.	1/7	1/7		2/7
	Selenomonas spp.	1/160	58/160		
	Dialister spp.			2/1221	557/1221
	Megasphaera spp.				228/624
	unclassified *Veillonellaceae*	32/1005	151/1005		37/1005
Other clostridia	*Clostridiaceae* I		6/5682		92/5682
	Lachnospiraceae		1949/45773		8/45773
	Peptococcaceae			20/1339	231/1339
	Peptostreptococcaceae		02/3353		
	Syntrophomonadaceae		1/207		
	Eubacteriaceae				8/452
	Gracilibacteraceae				2/95
	Incertae Sedis XII				1/478
	Incertae Sedis XIII		17/716		1/940
	Incertae Sedis XVI				5/6
	Thermoanaerobacterales				90/793
	unclassified Clostridiales	21/4019	288/4019	8/4019	126/4019
	unclassified Clostridia		8/2834	1/2834	48/2834
	Unclassified Firmicutes		2/1303	3/1303	5/1303
Other Bacteria	Deltaproteobacteria	18/20346	49/20346		
	unclassified Proteobacteria		1/5273		
	Acidobacteria		4/27308		
	Bacteroidetes		122/73464		136/73464
	Actinobacteria				2/57091

Other Bacteria	Chloroflexi				1/5754
	Alphaproteobacteria				1/7068
	Gammaproteobacteria				3/111810
	Fibrobacteres				1/366
	Verrucomicrobia				1/7707
	BRC1				2/92
	OP10				1/394
	unclassified Bacteria	5/30558	31/30558	13/30558)	74/30558

Data in columns show the number of matches within the total number of sequences available in the RDP II database for each group. The 1 bp mismatch column shows added numbers of matches and 1 bp mismatches for each group.

Table 5.8. Sequences matching primers Clost3RF and 1423RF in the databases as determined by Probe Match.

	Organism	Clost3RF		1423C3R	
		match	1 bp mismatch	Match	1 bp mismatch
Clostridium III and related organisms	*Ruminococcus* spp.		3/2420	1/2420	112/2420
	Acetivibrio spp.	31/110	47/110	10/110	32/110
	Faecalibacterium spp.	3/6838	6/6838		1/6838
	Fastidiosipila spp.	8/41	8/41		2/41
	Papillibacter spp.	2/1329	5/1329		5/1329
	Acetanaerobacterium spp.			1/43	6/1943
	Ethanoligenens spp.				1/1998
	Anaerotruncus spp.			1/241	9/241
	Ruminococcaceae Incertae Sedis	136/1064	154/1064	96/1064	130/1064
	unclassified Ruminococcaceae	787/10256	878/10256	104/10256	521/10256
Other clostridia	*Clostridium* I	1/5682	2/5682		50/5682
	Lachnospiraceae	1/45773	369/45773		15/45773
	Peptococcaceae	1/1288	587/1288		2/1288
	Eubacteriaceae		1/452		117/452
	Gracilibacteraceae		1/95		
	Heliobacteriaceae		2/31		24/31
	Veillonellaceae		1735/5156		13/5156
	Incertae Sedis XIII		1/940		13/153
	Syntrophomonadaceae				1/207
	Incertae Sedis XI				1/1669
	unclassified Clostridiales	505/4019	610/4019	1/4019	34/4019
	Thermoanaerobacterales			1/793	13/793
	unclassified clostridia	1/2834	96/2834	2/2834	79/2834
Other Firmicutes	*Halanaerobiaceae*		204/358		
	Erysipelotrichaceae		02/6071		7/6071
	Lactobacillales				32/30686
	Streptococcaceae	1/12030	3/12030		3/12030
	Bacillaceae		25/18164		
	Incertae Sedis XII		343/595		
	unclassified Bacillales		1/695		
	Bacillales	1/33253	369/33253	4/33253	2212/33253
	unclassified Firmicutes	2/1303	17/1303		7/1303

Other bacteria	Cyanobacteria	2/19857	4385/19857	
	Gammaproteobacteria	1/103205	822/103205	
	Deinococci		18/1341	
	Chrysiogenetes		2/4	
	Chloroflexi		54/5754	17/5754
	Nitrospira		1/2004	1/2004
	Alphaproteobacteria		12/64494	
	Betaproteobacteria		1534/47833	1/47833
	Deltaproteobacteria		4/20346	
	Epsilonproteobacteria			
	unclassified Proteobacteria	2/5273		
	Planctomycetes		80/6566	
	Chlamydiae		4/538	
	Acidobacteria		1/27308	2/27308
	Bacteroidetes		145/73464	1/73464
	OP10		1/394	
	TM7		11/1045	
	Tenericutes		1/2466	
	Actinobacteria			118/57091
	Verrucomicrobia			1/7070
	unclassified Bacteria		161/30558	20/30558
Archaea	Euryarchaeota		1/23248	

Data in columns show the number of matches within the total number of sequences available in the RDP II database for each group. The 1 bp mismatch column shows added numbers of matches and 1 bp mismatches for each group.

5.3.1. Performance of *Clostridium* III specific primers in qPCR

The primers discussed above (Clost3F-829C3R and Clost3RF-1423R) were obtained and tested for their suitability for qPCR amplification; both primer combinations produced good calibration curves ($r^2 > 0.99$), but the melting profile of the amplification products showed that primer pair Clost3F-829R had better physical properties, with the production of a sharp single peak corresponding to the main amplification. Primers Clost3RF-1423R also produced good calibration curves and their amplification products had a jagged melting profile with slightly more primer dimer formation than with primers Clost3F-829C3R. However, both the jagged melting profile and the primer dimer formation were not significantly higher than that observed for published qPCR primers.

The primer pairs Clost3F-829C3R and Clost3RF-1423C3R were tested on DNA and reverse-transcribed RNA samples extracted from cotton baits placed in the sediment of Esthwaite Water and recovered in Nov. 07 (Table 5.9.); neither primer pair gave consistently higher numbers than the other, as primer pair Clost3RF-1423R produced higher numbers for the DNA fraction but not for the RNA fraction. Relative abundances were low, from 0.14% to 0.5% for Clost3RF-1423R and Clost3R-829C3R in the RNA (cDNA) fractions respectively. Although these numbers were relatively low, they were not unrealistic and both primer pairs produced similar values.

Both *Clostridium* cluster III primer pairs designed here produced similar numbers of *Clostridium* III 16S rRNA copies from natural samples, however two factors led to the preference of Clost3R-829C3R for further tests: the melting profile of the real-time PCR amplification products were slightly better and with less primer dimer formation for Clost3F-829RF. In addition, the T_m of primer, Clost3RF was significantly lower than that of its pair (Table 5.6.).

sample	Clost3F-829R			Clost3RF-1423R			1369F-1492R	
	Gene Copies	% total bacteria	r^2	Gene copies	% total bacteria	r^2	Gene copies	r^2
Sediment bait DNA	1.51E+00	0.00	0.99	1.96E+01	0.01	0.99	1.40E+05	0.99
Sediment bait cDNA	1.59E+03	0.51	0.99	4.37E+02	0.14	0.99	3.14E+05	0.99

Table 5.9. Comparison of *Clostridium* cluster III qPCR primers.

Numbers and relative quantity of *Clostridium* cluster III 16S rRNA genes was quantified in DNA and SSU RNA extracted from baits recovered from the sediment of Esthwaite Water in November 2007 and quantified with primers Clost3F-829R and Clost3RF-1423R. Averages of two determinations.

5.3.2. Laboratory specificity tests of *Clostridium* III qPCR primers

Laboratory tests were then designed to check for the specificity of the Clost3F-829C3R primer pair and four species of *Clostridium* representing clusters I, III, IV and XIV were obtained and the DNA extracted (section 2.20.); these were used for PCR amplification using different annealing temperatures from 55°C to 62°C, which showed that the primer pair Clost3F-829C3R only amplified the 16S rRNA gene from DNA extracted from *Clostridium* cluster III (*C. cellulolyticum*) at 58°C and 62°C; weak amplification of *C. acetobutylicum* (*Clostridium* cluster I) was observed at 55°C (Fig 5.3.), however this was significantly lower than the temperature used in the qPCR amplification tests (60°C).

The specificity of Clost3F and 829C3R in amplifying only *Clostridium cellulolyticum* 16S rRNA gene in conventional PCR amplification combined with the knowledge that qPCR reagents are designed to enhance the specificity of the primers gave support to the use of this primer pair for further studies of *Clostridium* cluster III populations in the environment.

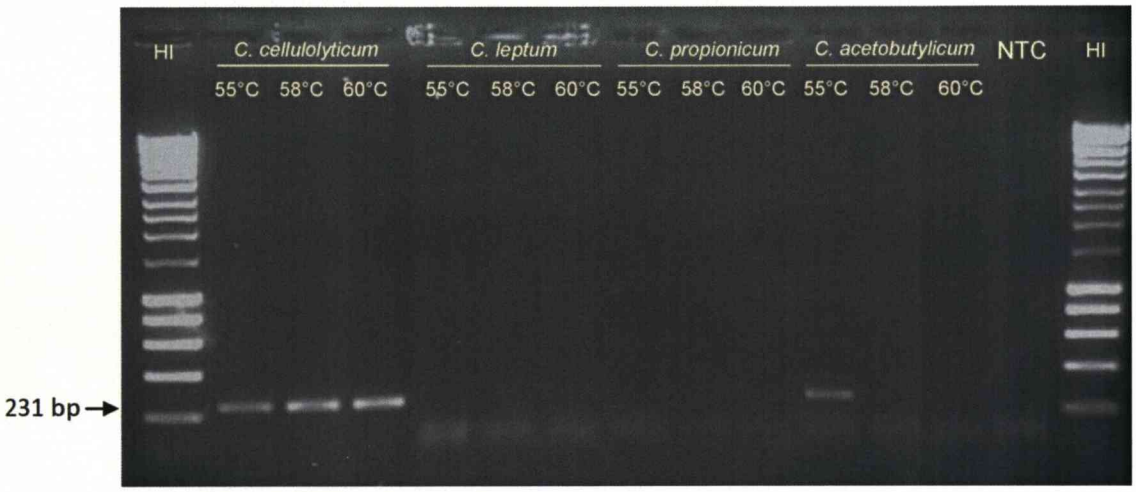


Figure 5.3. PCR specificity analysis of primers Clost3F and 829C3R.

1% agarose gel showing the PCR products obtained with primers Clost3F and 829C3R using genomic DNA from four clostridial species representing clusters III (*C. cellulolyticum*), IV (*C. leptum*), XIV (*C. propionicum*) and I (*C. acetobutylicum*). Annealing temperatures used are indicated. NTC, no template control, HI, hyperladder I.

5.4. Anaerobic fungi qPCR primer design

The anaerobic fungi are a monophyletic group (the *Neocallimastigales*) and are well known cellulose degraders in the rumen environment, however their presence has been recently documented in anaerobic landfill sites (Lockhart *et al.*, 2006). In a separate project in this laboratory, the 18S rRNA gene of these organisms has also been amplified from Esthwaite Water and Priest Pot samples (Dr. Robert Lockhart, personal communication).

Quantitative-PCR primers specific for the anaerobic fungi had already been designed (Denman & McSweeney, 2006), however, these targeted the ITS 1 region between 18S and 5S rRNA genes and could not be used in this study as it was intended to target the 18S rRNA itself (i.e. cDNA) in order to obtain higher signal than is normally obtained when targeting the DNA fraction. Primers specific for the anaerobic fungi 18S rRNA gene for conventional PCR amplification already exist (Lockhart *et al.*, 2006), and sequences flanking these primers were analysed for potential application as specific primers in qPCR analysis.

Sequences covering the 18S rRNA region of anaerobic fungi were obtained from Genbank and aligned using Clustal W2 (Larkin *et al.*, 2007) and imported into ARB. A sequence that was observed 72 bases downstream of the published reverse primer Neocal-reverse and named NeoQF (Table 5.10.) was identified that provided the necessary specificity and fulfilled the requirements for application in qPCR. Specificity analysis showed that this primer only matched four strains of *Neocallimastix* and *Cyllamyces aberensis* in the SILVA 18S rRNA database, however few Neocallimastigales 18S rRNA gene sequences covering the region of interest are available in the databases (4 *Neocallimastix* and 1 *Cyllamyces* sequence), as most researchers focus on the ITS 1 region for phylogenetic analysis of the anaerobic fungi. The lack of more matches of other genera of the anaerobic fungi is probably due to the unavailability of sequences covering the region of the 18S rRNA targeted by the primer. No 18S rRNA sequences covering the full gene were available for *Piromyces*, *Anaeromyces* and *Orpinomyces* or *Caeocomyces*, and the position of the sequence identified as a potential primer is 142 bp upstream of the nearest sequenced positions in any of the available sequences for members of these genera.

primer	Sequence (5'-3')	bp	T_m (°C)	GC%	Secondary structure	Dimer
AnFF	GAGGAAGTAAAAGTCGTAACAAGGTTTC	28	64.8	39	weak	none
AnFR	CAAATTCACAAAGGGTAGGATGATT	25	64.7	36	weak	none
NeoQF	CTTAGAGGGACTATAGATTTTTAATC	26	55.9	31	weak	none
Neocal-reverse	GGATGAAACTCGTTGACTTC	20	58.7	45	weak	none

Table 5.10. *Neocallimastigales* 18S rRNA gene qPCR primer sequences tested in this study.

The primer specifications were obtained from manufacturer (Sigma). Primer secondary structure and dimer formation are predicted. AnFF and AnFR were obtained from Denman *et al.* (2006), Neocal-reverse from Lockhart *et al.* (2006) and NeoQF developed in this study (section 2.20.).

The matches obtained for the published anaerobic fungi PCR primer Neocal-reverse are exactly the same as those obtained with the primer discussed here, named NeoQF. The 18S rRNA gene of the anaerobic fungi is known to be highly conserved (Brookman *et al.*, 2000), and it is therefore reasonable to expect that the primer NeoQF would also match the sequences of the other genera of anaerobic fungi in this order. The 20 available uncultured anaerobic fungal 18S rRNA sequences were found to have a matching sequence for NeoQF.

Primer NeoQF was to be used in combination with Neocal-reverse, and both were obtained for laboratory tests. The melt curve qPCR amplification products showed the presence of a single peak, and small amounts of primer dimer; calibration curves produced using this primer pair and *Neocallimastix frontallis* 18S rRNA gene inserts in linearised primers were also good ($r^2 > 0.99$).

In order to test the primer pair specificity in the laboratory, DNA from *Saccharomyces* sp., *Fusarium* sp., *Neurospora* sp. and *Aspergillus* sp. (see section 2.20.) were obtained and used as template for conventional PCR amplification (Fig 5.4.), showing that amplification under these conditions was specific to *Neocallimastix frontallis*. NeoQF and Neocal-reverse were used to quantify 18S rRNA gene copies in test DNA/RNA extracts from a cotton bait recovered from the sediment in Esthwaite Water and also from rumen fluid (Table 5.11.), showing that the expected recovery from the rumen was obtained, in contrast to evidence for the presence of at most trace numbers of genes in lake sediment cotton bait. Surprisingly, copy numbers were 2 fold higher for DNA than RNA; this may have occurred due to the degradation of the RNA fraction, as the sample had to be re-extracted due to impurities that could interfere with the PCR reaction.

The numbers of anaerobic fungi obtained with qPCR are not expressed here as relative abundances: comparing anaerobic fungal abundances with that of total eukaryotic 18S rRNA genes is not sensible, i.e., eukaryotic 18S rRNA numbers would include abundances of animals and plants as well as that of protozoans and microalgae and would not reflect the relative importance of the target organisms in the microbial community. The numbers of anaerobic fungi can be compared to those of total bacteria and to those of the other cellulolytic organisms targeted in this study, but cannot be reported as a proportion of total microbial populations as they belong to different phylogenetic kingdoms.

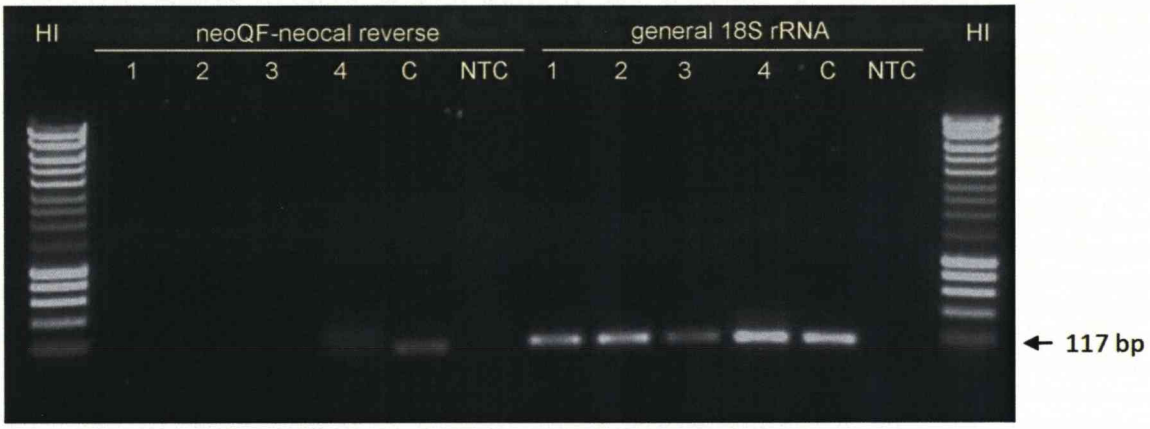


Figure 5.4. PCR specificity analysis of primers NeoQF and Neocal-reverse.

1% agarose gel showing the PCR products obtained with primers NeoQF-neocal reverse and the general primers NS1-eukaryotic and Univ-1390-universal. DNA from *Neocallimastix frontalis* and from four species of fungi were used as positive and negative controls respectively. 1, *Saccharomyces* cerevisiae.; 2, *Fusarium* sp.; 3, *Neurospora crassa*.; 4, *Aspergillus nidulans*.; C, *N. frontalis*; NTC, no template control; HI, hyperladder I. Annealing temperature: 58°C.

.

Name	Average N° of 18S rRNA copies	standard error %
Sediment bait DNA	0.00E+00	0
Sediment bait CDNA	2.48E+00	34.86
Rumen DNA	1.32E+05	10.50
Rumen CDNA	5.65E+04	12.43

Table 5.11. Results from first test application of the NeoQF-neocal reverse primers.

Anaerobic fungal 18S rRNA copy numbers were obtained in the DNA and RNA fractions extracted from baits recovered from the sediment in Esthwaite Water and from the rumen. Rumen nucleic acid extract was provided by Dr. James McDonald. R^2 of calibration curve: 0.994; standard error are given as percentages of the mean.

5.5. Field work and nucleic acid extractions

5.5.1. Physical and biological conditions in the lakes

When the cotton baits were placed in Esthwaite Water, the water column was mixed and the oxygen concentrations remained relatively high even in the deeper sections of the lake (Fig 5.5. A); when the baits were recovered 5 weeks later, the water column was distinctly stratified, with very little dissolved oxygen below 13 m (Fig 5.5. B). The temperatures increased significantly in the upper water column but changed little in the deep parts of the lakes. No physical data were available for Priest Pot, as the temperature and oxygen probe malfunctioned later in the day when sampling in Priest Pot on the 03/06/08; in addition, the samples placed in Priest Pot were vandalised. Despite the absence of data, it is reasonable to assume that Priest Pot was also stratified at the time that the baits were recovered, as previous work in this laboratory had shown that this lake tends to stratify earlier than Esthwaite Water.

Chlorophyll a concentrations throughout the water column are remarkably different between Esthwaite Water and Priest Pot (Fig 5.6.). In the former, chlorophyll a is highest near the surface, being close to zero at 13 m, and concentrations were always lower than 10 μg L^{-1}; in Priest Pot, concentrations are similar to one another from the surface down to 2 m and remained close to 10 μg L^{-1}, but increased dramatically at 3 and 3.5 m to circa 270 μg L^{-1}.

Samples were frozen and stored at -80°C and DNA/RNA extractions commenced two days after sampling. The presence of distinct RNA bands in most samples shows that the samples were in good condition (data not shown). Some degradation of the RNA occurred in the sediment samples, as these were taken to the laboratory frozen in 10 ml plastic flasks, making the removal of material for the extraction difficult; some of the sediment thawed which may have caused degradation of the RNA.

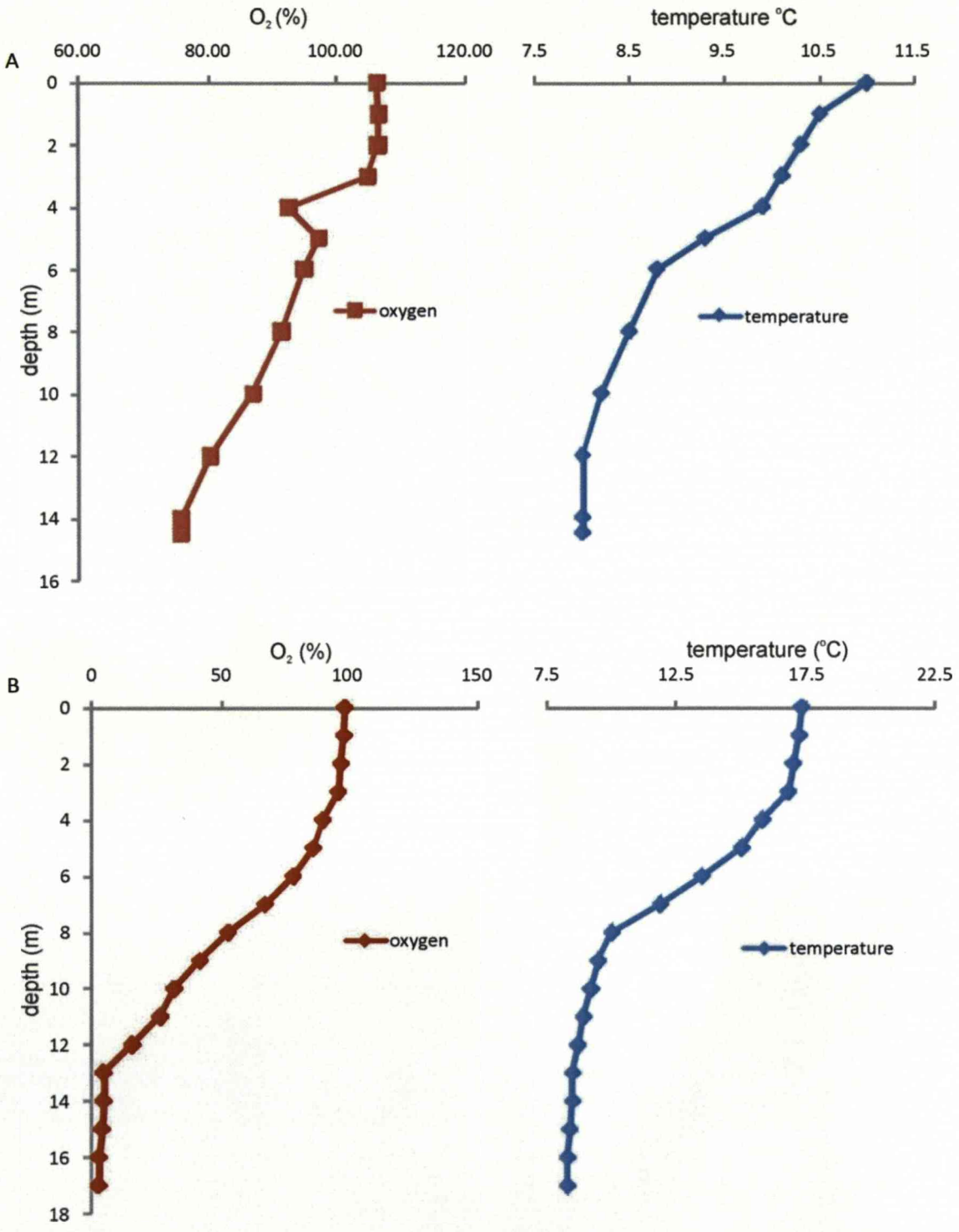


Figure 5.5. Dissolved oxygen and temperature depth profiles from Esthwaite Water.

Data obtained from the constant monitoring station on the 29/04/08 (A) and the 03/06/08 (B).

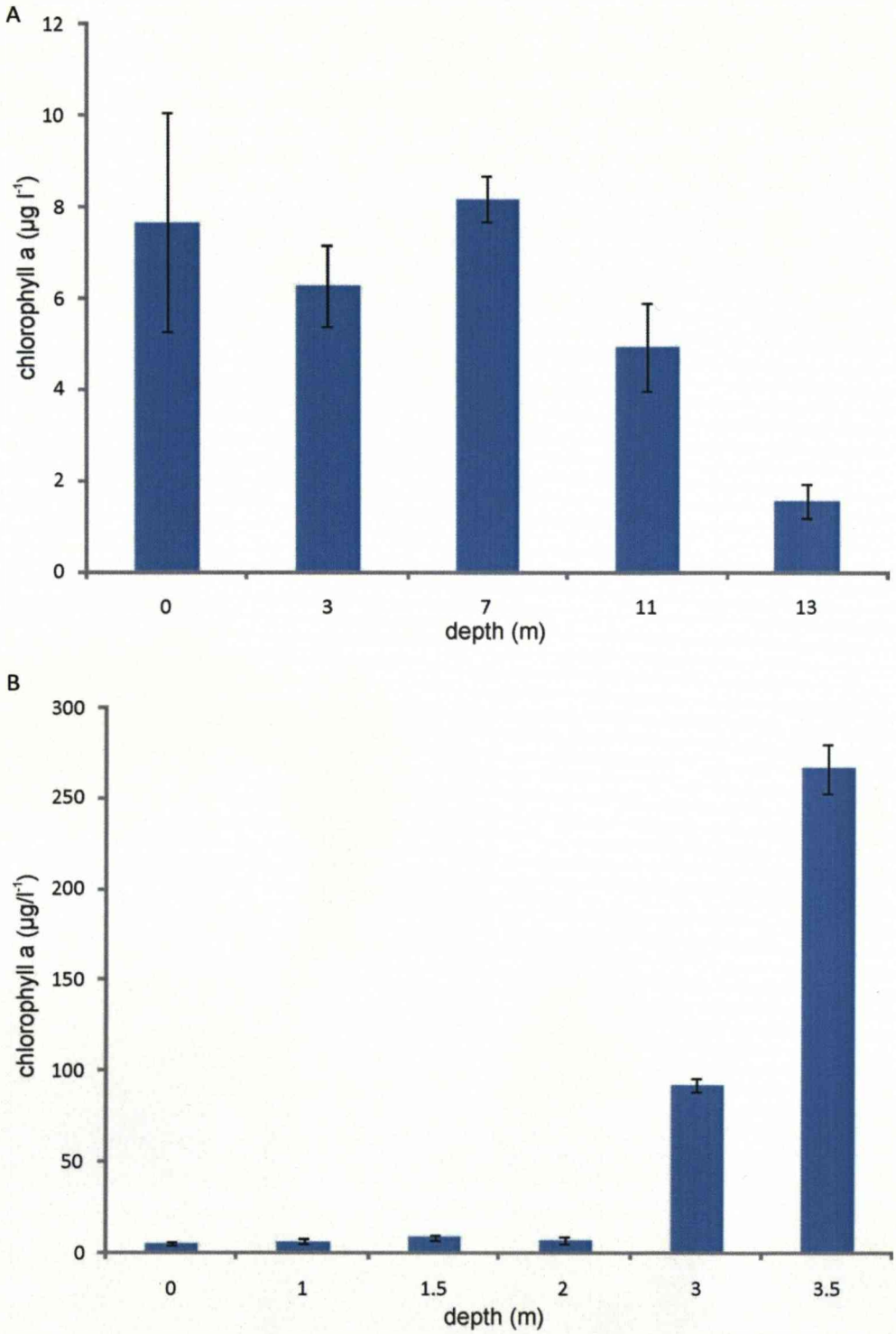


Figure 5.6. Chlorophyll a concentration in Esthwaite Water and Priest Pot on the 03/06/08.

A, Esthwaite Water; B, Priest Pot. Error bars represent standard errors.

5.5.2. Molecular detection of *Micromonospora* spp., *Clostridium* cluster III, anaerobic fungi and *Fibrobacter* spp. in the lakes.

Specific primers were *used to detect* the p*resence of Micromonospora* spp. *Clostridium* clu*ster III,* anaerobic fungi and *Fibrobacter* spp. in the cotton baits, sediment and water column of Esthwaite Water and Priest Pot. The primers used are presented in Table 5.12. *Micromonospora* and *Clostridium* cluster III could be detected by direct PCR amplification of DNA extracts, whereas *Fibrobacter* and anaerobic fungi could only be detected through nested PCR amplification of DNA.

The results show the presence of *Micromonospora* in cotton baits, water and sediment but the brightest bands were from the cotton baits and deep water samples (Fig 5.7., Table 5.13.). DNA/RNA extracts from the sediments had suffered the greatest degradation, and the bands were less bright possibly due to the presence of PCR inhibitors in the organic matter rich sediment. Both in Esthwaite Water and Priest Pot, amplified DNA bands for *Micromonospora* were brighter in the deeper bait and water samples. No band was observed in the sample extracted from the nylon rope, indicating that it was barely, if at all, colonised by *Micromonospora* (Fig 5.23., Table 5.13.).

Clostridium cluster III was only detected in Esthwaite Water as faint bands in the sediment cotton bait and in the deep sediment sample; in Priest Pot the amplified DNA bands were significantly brighter than those in Esthwaite Water, and were mainly found in the deeper cotton bait samples and in both sediment samples; in the water column only the deepest sample showed the presence of *Clostridium* III (Fig. 5.8., Table 5.13.).

Anaerobic fungi were present in both lakes but they could only be detected with nested-PCR. In Esthwaite Water, the group was present in the two shallowest cotton bait samples and in the deep sediment sample; in Priest Pot their amplified DNA was detected in only one (2 m deep) bait sample and in both top and deep sediment samples (Fig 5.9., Table 5.13.).

Fibrobacter was only detected by nested PCR in the Priest Pot cotton baits at 1, 2 and 3 m depth. (Fig 5.10. Table 5.13.).

Target	Name	Sequence	Ref.
Eubacterial 16S rRNA	pA	AGAGTTTGATCCTGGCTCAG	Edwards *et al.* (1989)
	pH	AAGGAGGTGATCCAGCCGCA	
	1369F	CGGTGAATACGTTCYCGG	Susuki *et al.* (2000)
	Prok 1429R	GGWTACCTTGTTACGACTT	
Eukaryotic 18S rRNA	NS1-eukaryotic	CCAGTAGTCATATGCTTGTC	White *et al.* (1990)
	Univ-1390-universal	GACGGGCGGTGTGTACAA	Zheng *et al.* (1996)
Micromonospora spp.	M1C	TTACCTGGGTTTGACATGG	This study
	M2D	ACATCGAACGAGGGTTGC	
Clostridium cluster III	Clost3F	TCTTGAGTGYYGGAGAGGAAAGC	Van Dyke and McCarthy (2002)
	Clost3R	GRCAGTATDCTGACCTRCC	
	829C3R	GGATACTTATTGTGTTWACTMCGG	This study
	Clost3RF	GGYAGGTCAGHATACTGY	
	1423C3R	TAYYGACTTCGGGTGTTGC	
Fibrobacter spp.	Fib1F	CCGKSCCAACGSSCGG	McDonald *et al.* (2008)
	Fib2AR	ATCTCTTCGCYGCGGCGWTYCC	
	FibroQ153F	CCGKSCCAACGSSCGGHTAA	
	FibroQ238R	CSCCWACTRGYTAATCRGAC	
Anaerobic fungi 18S rRNA	Chyt-719-F	GCACTTCATTGTGTGTACTG	Lockhart *et al.* (2006)
	Chyt-1553-R	GGATGAAACTCGTTGACTTC	
	NeoQF	CTTAGAGGGACTATAGATTTTTAATC	This study
	ANNR	CAAATTCACAAAGGGTAGGATGATT	Denman *et al* 2006.

Table 5.12. Primers used for the molecular detection of target microbial groups in Esthwaite Water and Priest Pot.

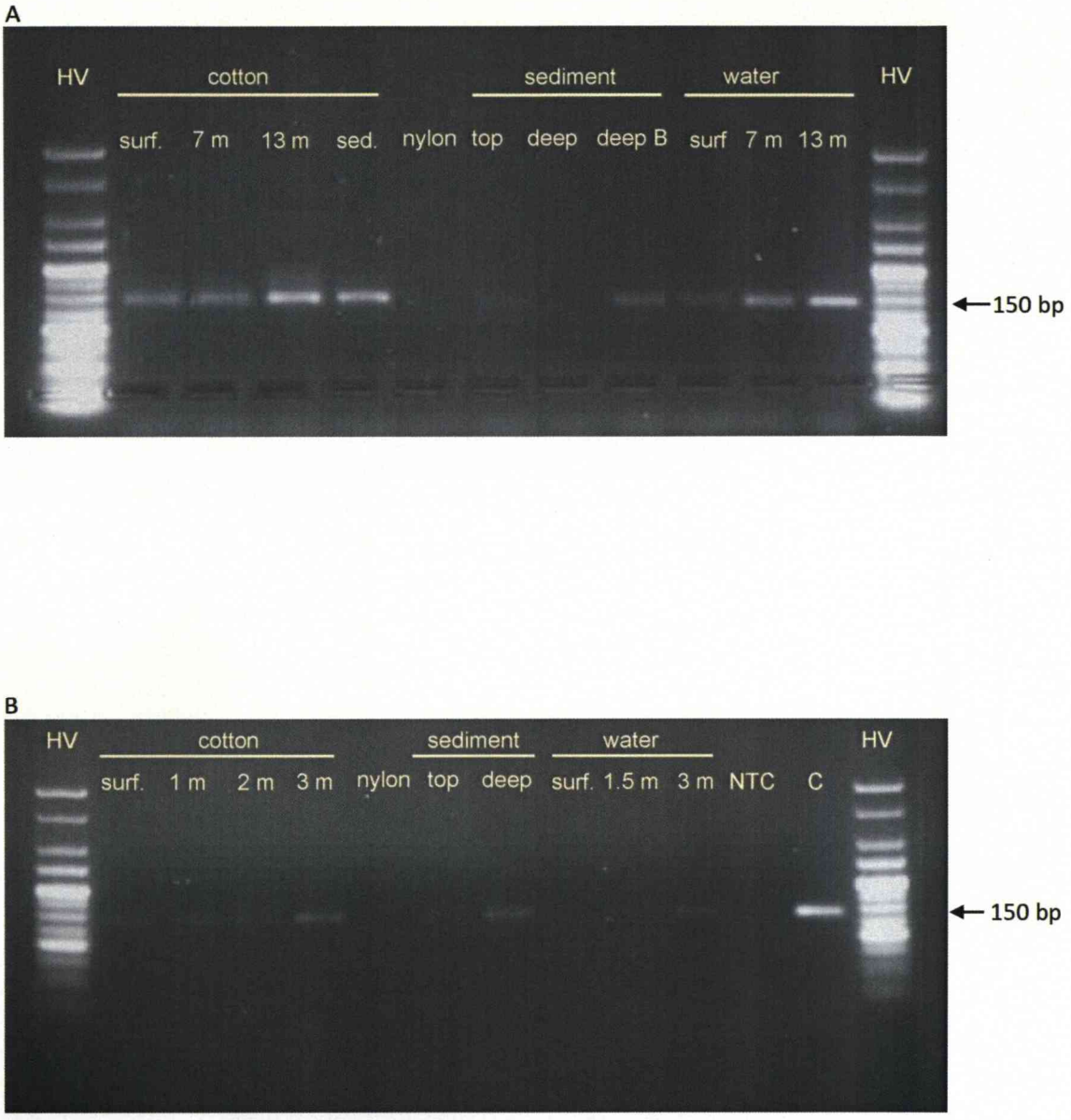


Figure 5.7. PCR detection of *Micromonospora* in Esthwaite Water and Priest Pot.

A, Esthwaite Water; B, Priest Pot. Agarose gel (2%) showing the products of direct amplification with the M1C and M2D primers using as template DNA extracted from baits, sediment and water. NTC, no template control; C, positive control (*Micromonospora* lake isolate N°13 16S rRNA clone); HV, hyperladder V.

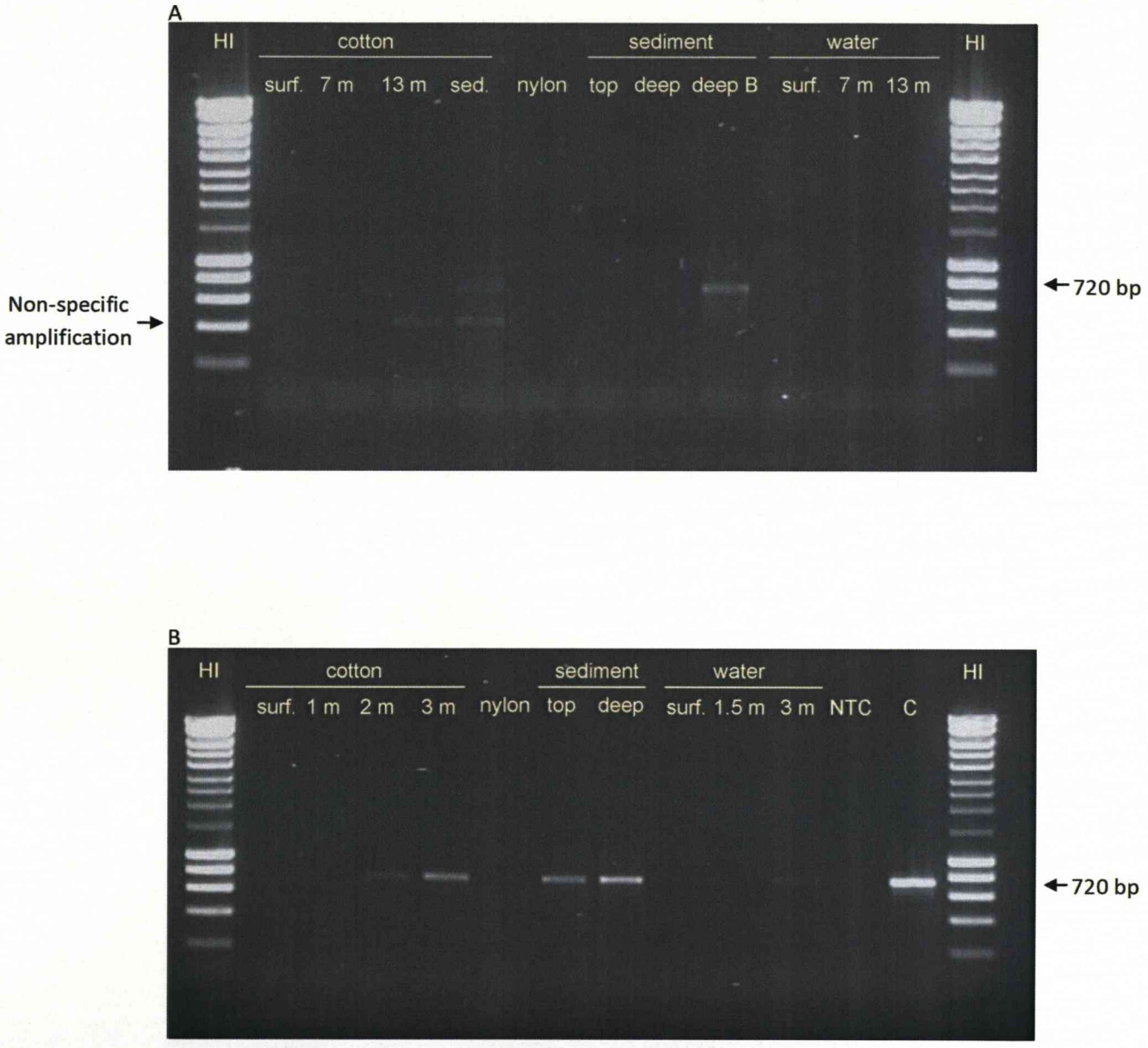


Figure 5.8. PCR detection of *Clostridium* III in Esthwaite Water and Priest Pot.

A, Esthwaite Water; B, Priest Pot. Agarose gel (1%) showing the products of direct amplification with the Clost3F and Clost3R primers and using as template DNA extracted from baits, sediment and water. NTC, no template control; C, positive control (*Clostridium cellulolyticum* 16S rRNA clone); HI, hyperladder I.

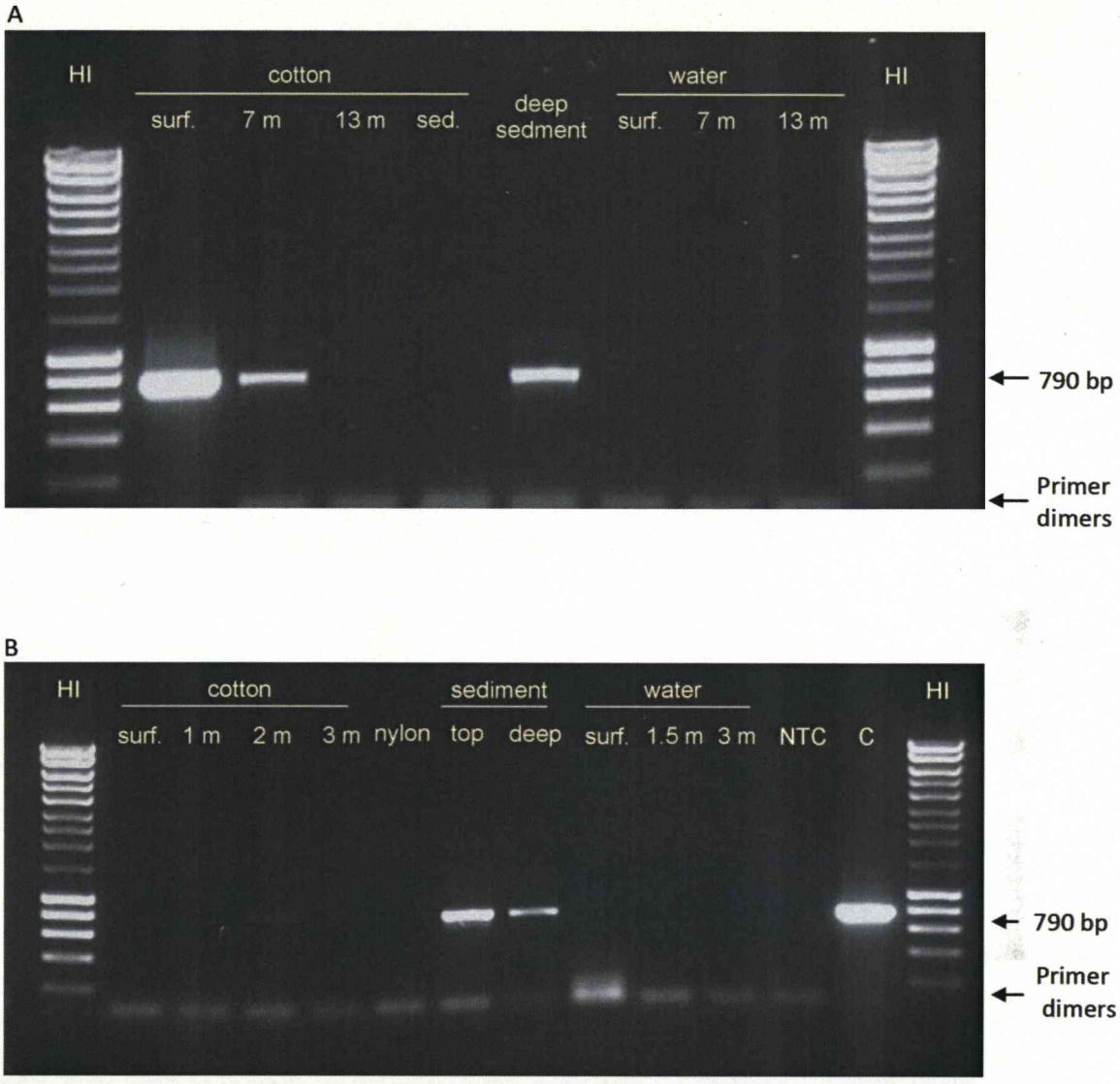


Figure 5.9. Nested PCR detection of anaerobic fungi in Esthwaite Water and Priest Pot.

A, Esthwaite Water; B, Priest Pot. Agarose gel (1%) showing the products of nested amplification products with the Neocal-foraward and Neocal-reverse primers and using as template DNA extracted from baits, sediment and water. The surface sediment sample in Esthwaite Water is not shown in this gel as no general 18S rRNA gene PCR products were obtained for this sample. NTC, no template control; C, positive control (*Neocallimastix frontalis*18S rRNA clone); HI, hyperladder I.

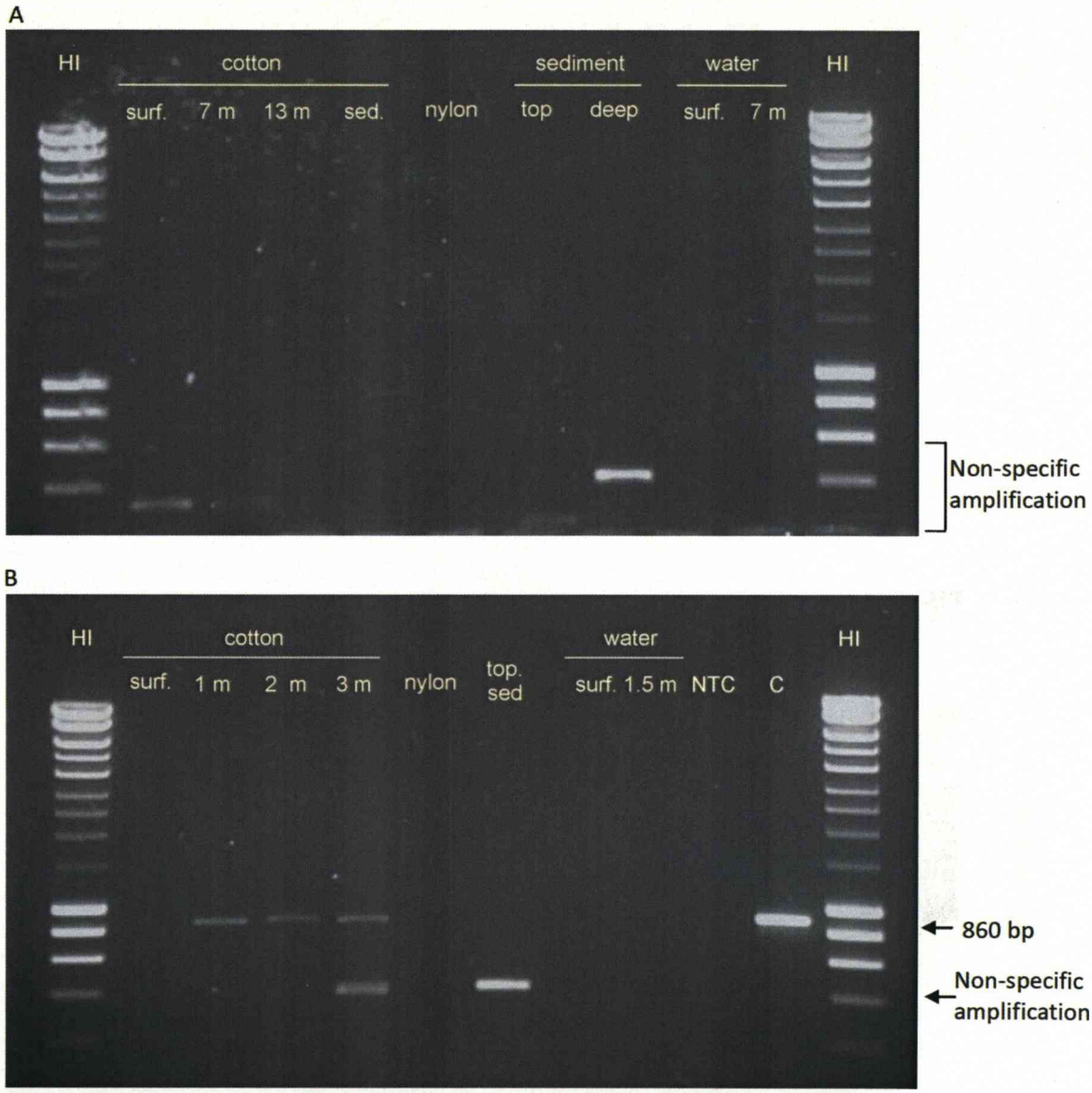


Figure 5.10. Nested PCR detection of *Fibrobacter* spp. in Esthwaite Water and Priest Pot.

A, Esthwaite Water; B, Priest Pot. Agarose gel (1%) showing the products of nested amplification products with the Fib1F and Fib2AR primers and using as template DNA extracted from baits, sediment and water from the lakes. No general 16S rRNA PCR products were obtained from the 13 m deep water sample from Esthwaite Water, in the deep sediment sample and 3 m deep water sample from Priest Pot. NTC, no template control; C, positive control (*Fibrobacter succinogenes 16* rRNA clone); HI, hyperladder I.

A

Esthwaite Water		Direct amplification		Nested amplification	
		Micromonospora	*Clostridium* III	Anaerobic fungi	*Fibrobacter*
Cotton	Surface	++	-	+++	-
	7m	++	-	+++	-
	13m	+++	-	+	-
	Sediment	+++	+	-	-
Nylon – 3m		-	-	-	-
Sediment	Surface	+	-	+++	-
	Deep	+	+	-	-
Water	Surface	-	-	-	-
	7m	++	-	-	-
	13m	+++	-	-	-

B

Priest Pot		Direct amplification		Nested amplification	
		Micromonospor a	*Clostridium* III	Anaerobic fungi	*Fibrobacter*
Cotton	Surface	+	-	-	-
	0.5m	+	-	-	+
	2m	+	+	+	+
	Sediment	+	++	-	+
Nylon – 0.5m		-	-	-	-
Sediment	Surface	+	+	+++	-
	Deep	+	+++	++	-
Water	Surface	-	-	-	-
	1.5m	-	-	-	-
	3m	+	++	-	-

Table 5.13. Summary of the results of the molecular detection of target groups in Esthwaite Water and Priest Pot.

A, Esthwaite Water; B, Priest Pot. +++, bright PCR band; ++ medium brightness PCR band; + faint PCR band; - no PCR band.

5.5.3. Abundances of *Micromonospora*, *Clostridium* cluster III, Anaerobic Fungi and *Fibrobacter* using quantitative-PCR

The abundances of the target groups were assessed for the same samples used for PCR/nested PCR detection but using the specific qPCR primers designed in section 5.4., except those used for *Fibrobacter* quantification (Fib1F and Fib2R) (McDonald *et al.*, 2008); the primer sequences are in Tables 5.1 and 5.6 and 5.12. The quantification was performed only for the reverse-transcribed rRNA fraction, as it was anticipated that this would increase the signal obtained (see section 5.2.). Sample analysis proceeded as predicted by the data in sections 5.2.-5.4. and the calibration curves had r^2 always equal to or above 0.99 (Table 5.14.). The relative numbers of *Micromonospora* were higher in Esthwaite Water compared to Priest Pot, and increased with depth for the cotton baits in both lakes, reaching 20% of the total bacterial community in the sediment cotton bait in Esthwaite Water. In the surface cotton bait of both lakes their numbers were ≤ 0.1%; in the sediments themselves the relative numbers were much lower than in the cotton baits, as was also the case in the water column (Fig 5.11.).

Micromonospora numbers on isolation plates followed a similar trend to those observed for the abundances of these organisms, with increasing values from the top to the bottom of both lakes for both the cotton baits and the lakewater samples (Fig 5.12.). The numbers obtained in the sediments were an order of magnitude higher than those observed in the cotton baits, but these values cannot be directly compared. Total numbers were similar for both lakes.

In contrast to *Micromonospora*, *Clostridium* cluster III numbers were generally higher in Priest Pot, and also increased with depth, never reaching relative abundances as high as those of *Micromonospora* in the cotton baits. However, in the surface sediment samples, relative abundances of 20 or 40% were recorded in Esthwaite Water and Priest Pot, respectively, and numbers were also high in the deeper (10 cm) sediment sample. *Clostridium* III numbers in the water column were very low in Esthwaite Water (> 0.1%), but reached circa 6% in the deepest water column sample in Priest Pot (Fig 5.13.). The significant increase in numbers of *Clostridium* III in sediments is congruent with their obligate anaerobic physiology, and this in itself provides validation for this qPCR system for measuring abundance of *Clostridium* III in environmental samples.

Fibrobacter numbers were significantly lower than both *Micromonospora* and *Clostridium* III, always < 0.03% of the bacterial population; *Fibrobacter* was restricted to cotton baits in the deeper sections of both lakes and the surface sediment sample in Esthwaite Water (Fig 5.14.). Anaerobic fungi were detected in only two samples from Esthwaite Water, the surface cotton bait and surface sediment sample and numbers were also extremely low (Fig 5.13.).

In all cases, the primers used in the qPCR analysis produced single bands when run on 1 % agarose gels (data not shown), showing that there was little or no non-specific amplification.

Although the results obtained with qPCR did not always match the location where detection with conventional PCR had been recorded, there was significant agreement between the two sets of data. For example, brighter bands of PCR amplified *Micromonospora* DNA were seen in Esthwaite Water compared to Priest Pot, and the opposite was observed for *Clostridium* III; these two groups could be detected without the need for nested PCR, whereas *Fibrobacter* and anaerobic fungi could only be detected after an initial round of amplification of general bacterial 16S or eukaryotic 18S rRNA genes. There was correlation in the detection of anaerobic fungi in that the sample from which the brightest PCR band was obtained (surface cotton bait in Esthwaite Water) was also the sample which gave the highest number in the qPCR quantification. Quantitative-PCR showed the presence of *Fibrobacter* in two samples from Esthwaite Water, whereas nested PCR had not indicated the presence of these organisms in this lake at all; in Priest Pot, nested PCR showed the presence of the group in three cotton bait samples whereas qPCR indicated its presence in only one, at 2 m.

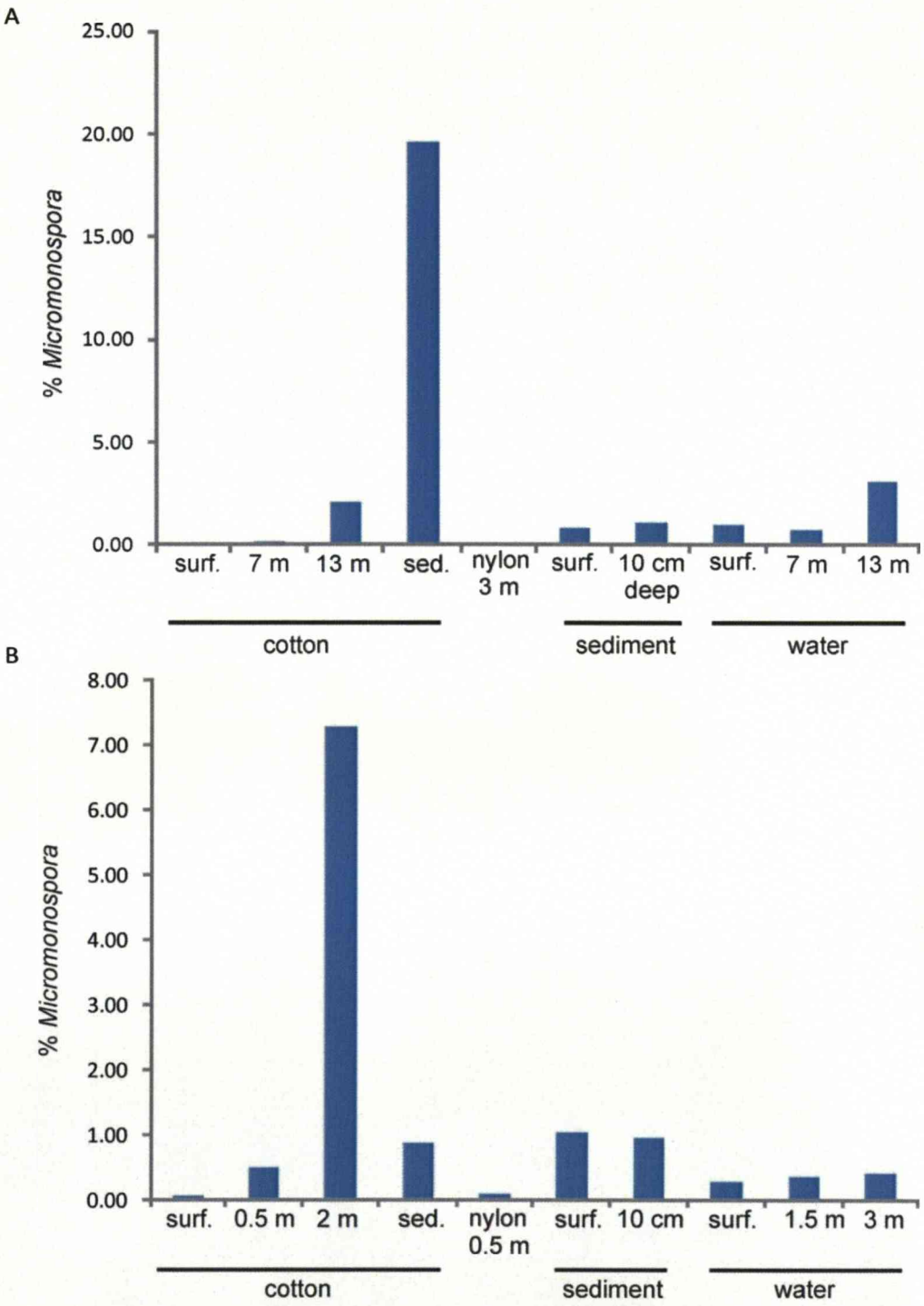


Figure 5.11. Relative abundance of *Micromonospora* spp. in Esthwaite Water and Priest Pot.

A, Esthwaite Water; B, Priest Pot. *Micromonospora* and total bacterial 16S rRNA numbers were determined using M1C and M2D and 1369F-1492R respectively and using cDNA as template. Surf., surface. $R^2 > 0.99$ for both primer sets.

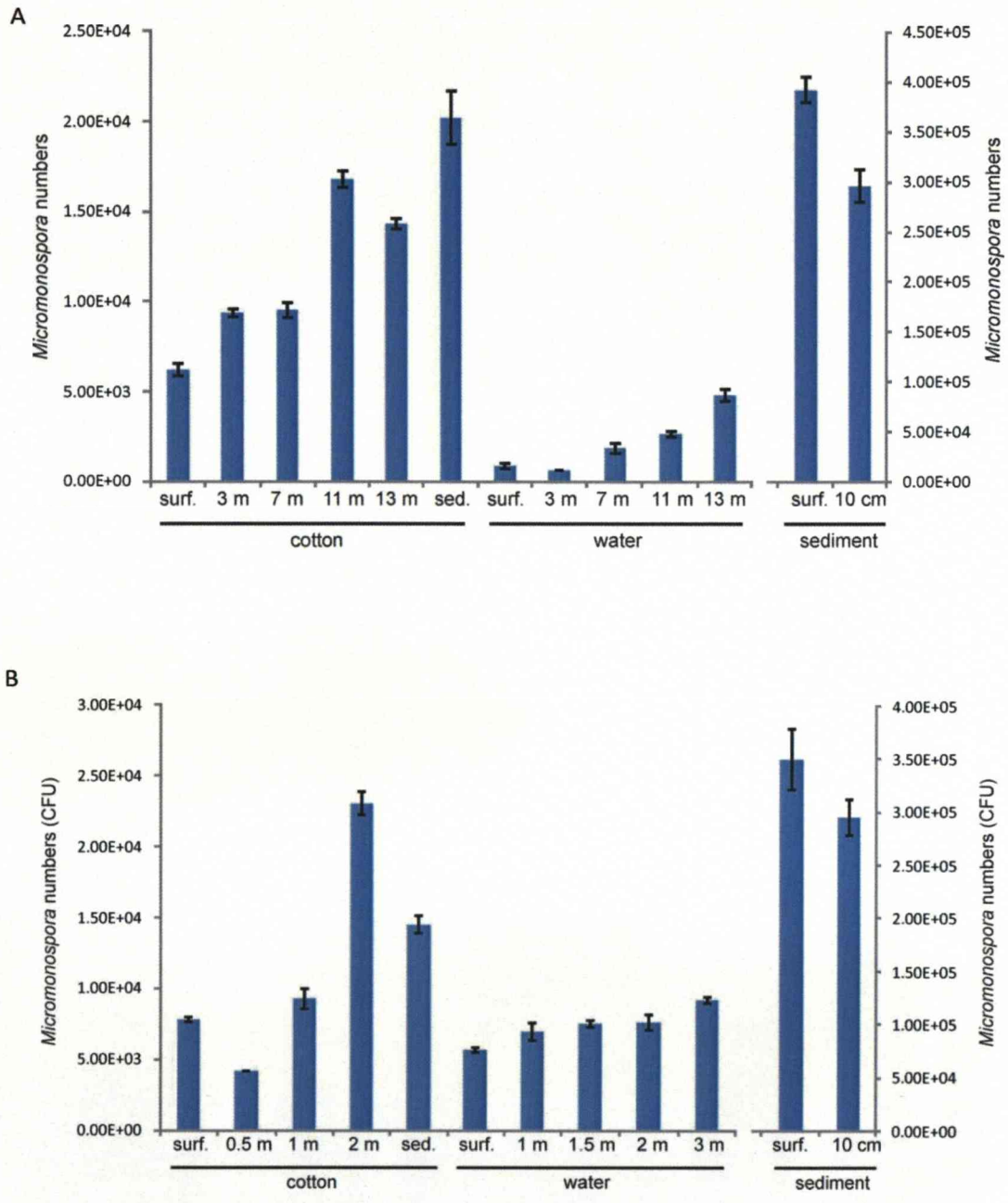


Figure 5.12. Numbers of *Micromonospora* spp. (CFU) in Esthwaite Water and Priest Pot.

A, Esthwaite Water; B Priest Pot. CFU numbers were obtained on M3 agar isolation plates from cotton baits (CFU g^{-1} bait), water column (CFU L^{-1}) and sediment (CFU g^{-1} wet sediment). Surf., surface. Error bars are standard errors.

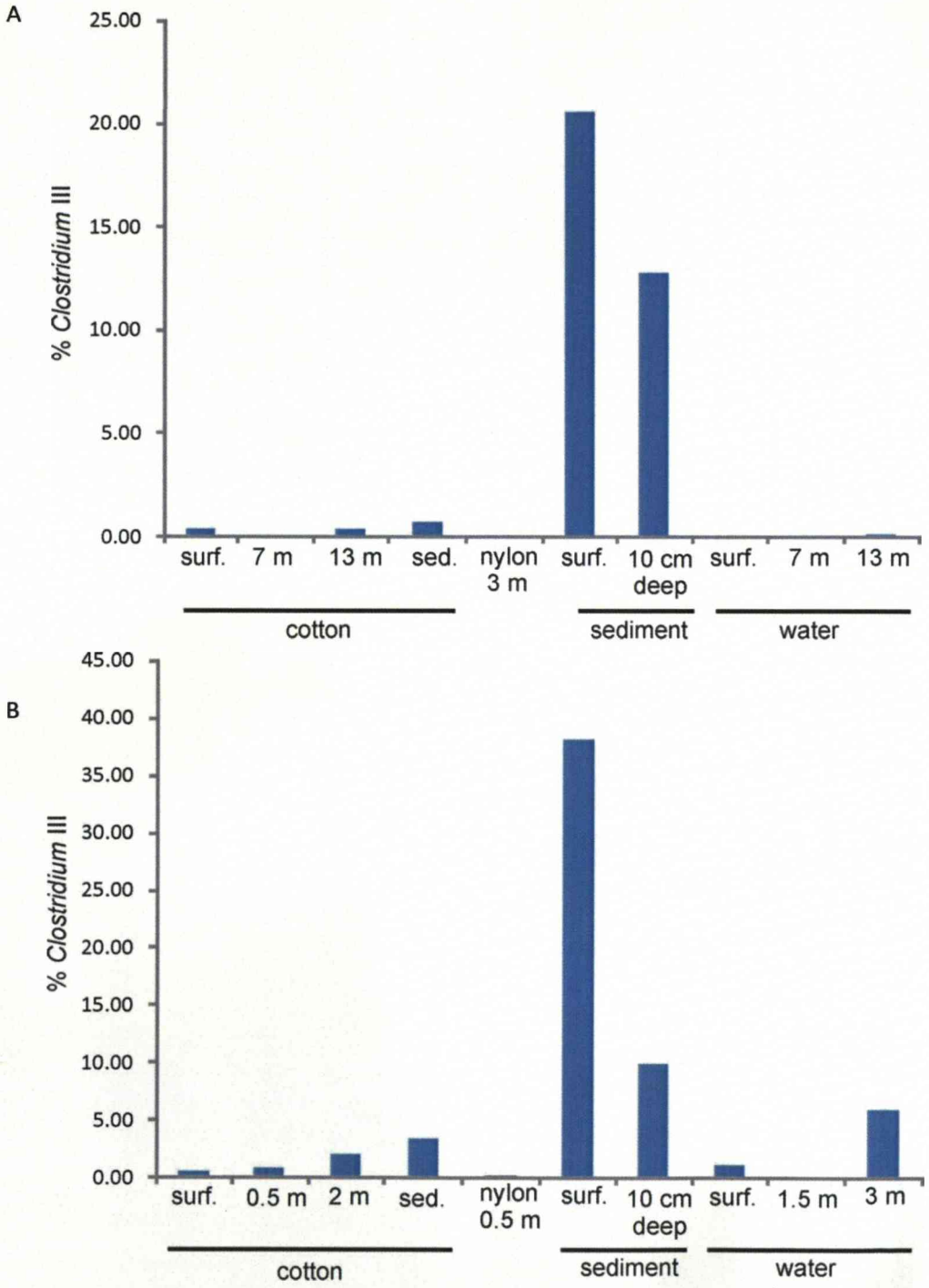


Figure 5.13. Relative abundance of *Clostridium* cluster III in Esthwaite Water and Priest Pot.

A, Esthwaite Water; B, Priest Pot. *Clostridium* III and total bacterial 16S rRNA numbers were determined using primers Clost3F and 829C3R and 1369F-1492R respectively and using cDNA as template. Surf., surface. $R^2 > 0.99$ for both primer sets.

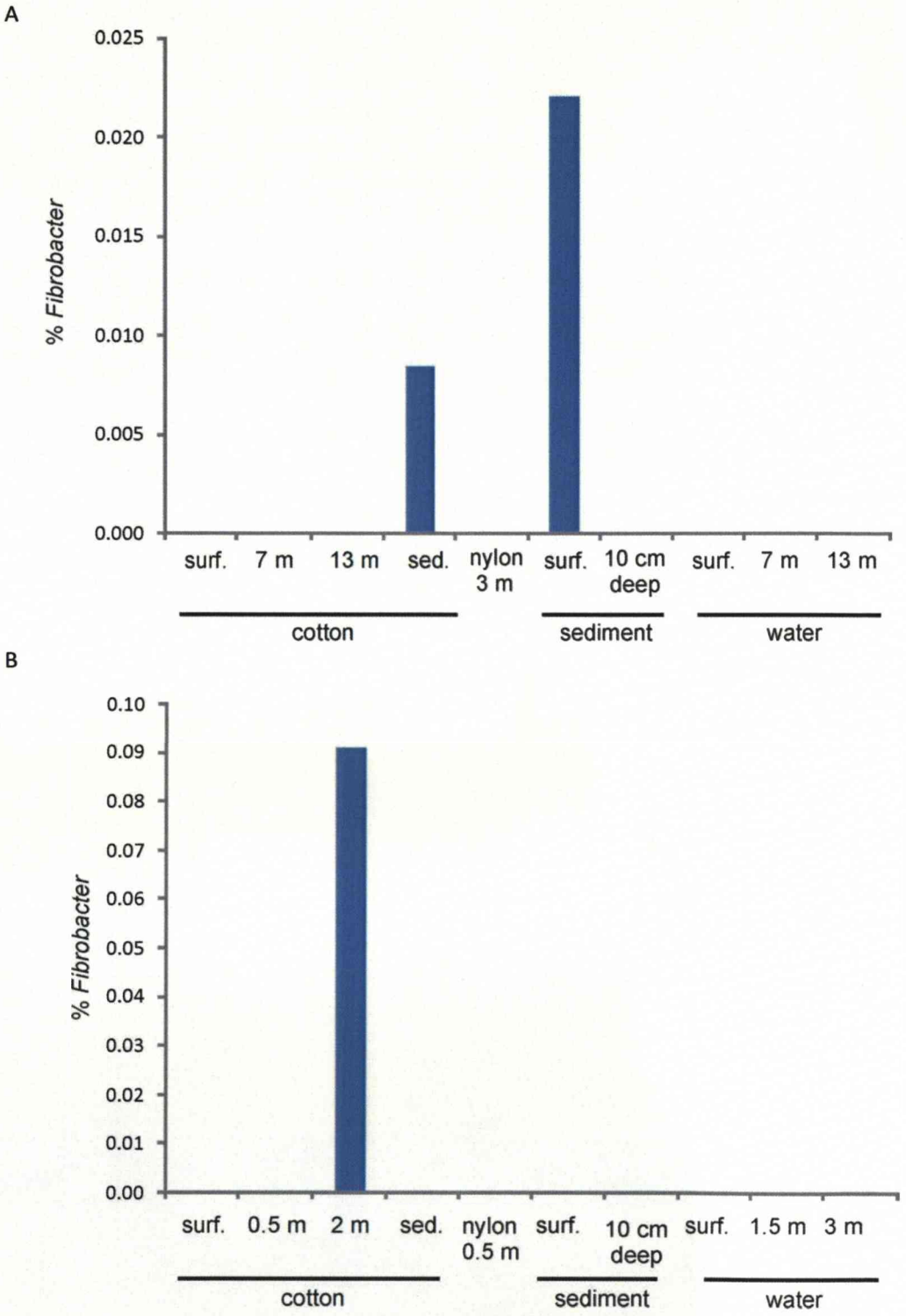


Figure 5.14. Relative abundance of *Fibrobacter* spp. in Esthwaite Water and Priest Pot.

A, Esthwaite Water; B, Priest Pot. *Fibrobacter* and total bacterial 16S rRNA numbers were determined using primers FibroQ153F and FibroQ238R and 1369F-1492R respectively and using cDNA as template. Surf., surface. $R^2 > 0.99$ for both primer sets.

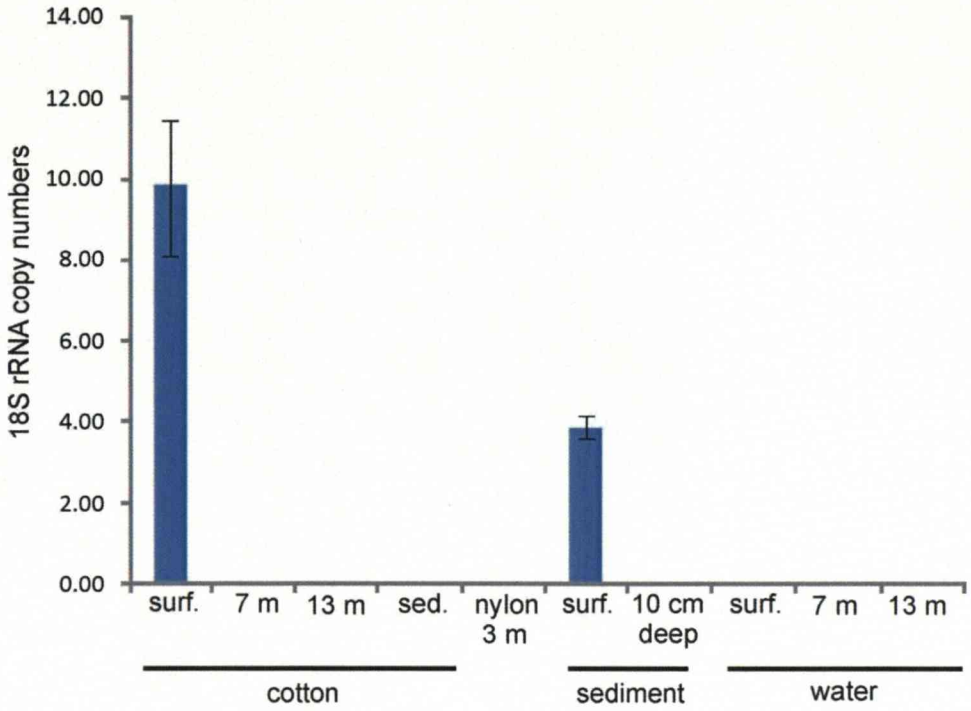


Figure 5.15. Total number of anaerobic fungi 18S rRNA copies in Esthwaite Water.

Total anaerobic fungi 18S rRNA copy numbers determined with NeoQF and necocal-reverse. Surf., surface. $R^2 > 0.99$. No anaerobic fungal 18S rRNA was detected in Priest Pot with this method.

Analysis	Specific primers		Universal primers	
	slope	r^2	slope	r^2
Micromonospora EW	-3.48	1.00	-3.30	1.00
Micromonospora PP	-3.48	1.00	-3.30	1.00
Clostridia EW	-4.14	0.99	-3.89	0.99
Clostridia PP	-4.17	1.00	-3.70	0.99
Fibrobacter EW	-3.57	1.00	-3.04	0.99
Fibrobacter PP	-3.62	1.00	-3.28	1.00
Anaerobic fungi EW	-3.42	1.00	-	-
Anaerobic fungi PP	-3.41	0.99	-	-

Table 5.14. Slope and goodness of fit of the calibration curves used in the relative quantification of target groups.

EW, Esthwaite Water; PP, Priest Pot.

5.6. Diversity of *Clostridium* III, *Fibrobacter* spp. and anaerobic fungi in the lakes

As all the target groups in this study were detected in the lakes, it was decided to determine their phylogeny and diversity. Little information is available in the literature about the diversity of *Clostridium* and *Fibrobacter* species in aquatic environments, and none is available for members of the anaerobic fungi group. Samples of amplified 16S rRNA genes from members of the *Clostridium* III group and *Fibrobacter* spp. were obtained from the molecular detection study described above (section 5.5.2.), whereas for the anaerobic fungi, a separate round of amplification was performed with primers targeting general eukaryotes at the 5' end of the 18S rRNA and the 5' end of the 28S rRNA gene (using primers NS-1 eukaryotic, (Table 5.12.) and JB 205 obtained from Tuckwell *et. al* (2005) - TCCTCCGCTTATTAATATGC), followed by specific amplification of the anaerobic fungal 18S rRNA gene and the 5' end of the ITS 1 region. This resulted in an extra 350 bp of sequence length in comparison to the sequence amplified initially, which was the same used in a previous study targeting the anaerobic fungi in landfills (Lockhart *et al.*, 2006). It was intended to use these sequences to classify the anaerobic fungal sequences to the genus level using a perl script programme described in Denman *et al.* (2006), however upon communication with the authors it was discovered that the program is no longer available, and in addition the fragment amplified in this study does not contain enough sequence length of the ITS 1 region to be used for classification, although it does offer additional sequence information to be used in the construction of phylogenetic trees.

The samples to be cloned were chosen solely on the quantity of PCR products obtained during the molecular detection study, and *Clostridium* III clones were obtained from the Priest Pot deep (10 cm) sediment sample, *Fibrobacter* from the 2 m deep cotton bait sample in Priest Pot, and the anaerobic fungi from the surface cotton bait sample in Esthwaite Water. The cloning, DNA sequencing and phylogenetic analysis of the amplified DNA was performed as described in sections 2.14., 2.18. and 2.19.

5.6.1. *Clostridium* III

The phylogenetic analysis confirmed the identity of the *Clostridium* III clones obtained in this study (Fig 5.16.), but did not show any particular clustering that could suggest a separate population in the lakes studied. With the exception of *Bacteroides cellulosolvens*, all the cultured strains of *Clostridium* III were found in two clusters with high bootstrap support, and one clone (C2) clustered within one containing amongst others *C. cellulolyticum*. Most of the other clones were located in clusters that had very low bootstrap support and which were collapsed in the final trees; their definitive position therefore is not known. Clone C7 clustered with uncultured strains obtained from environmental samples, such as anoxic rice paddy soils or contaminated groundwater. Clone C5 also clustered with uncultured strains, however this cluster was distinctly separate from the main group of cluster III sequences.

All of the *Clostridium* III sequences obtained from the databases and used to build the trees corresponded to the five closest matches to each clone as determined by the SILVA aligner software. All of the uncultured sequences were derived from environmental samples, many of which were cellulose-rich such as rice paddies and landfill bioreactors; interestingly none of the sequences were from the rumen, an environment inhabited by many strains considered to belong to or to be closely related to cluster III clostridia (i.e. *Ruminococcus* strains).

5.6.2. *Fibrobacter*

A distinctive cluster was obtained with the *Fibrobacter* clones when these were used to construct phylogenetic trees (Fig 5.17.), and these clones were distinct from those obtained from soil and sheep faeces collected in the lake's surrounding area in a separate study in this laboratory (James McDonald, personal communication). These clones appear to represent a population of indigenous freshwater *Fibrobacter* spp. and the nearest sequences from the databases to cluster with them are from other aquatic environments, such as acid impacted and cavewater lakes (McDonald *et al.* in press).

5.6.3. Anaerobic Fungi

The sequences from the anaerobic fungi clones were used to build two sets of trees: including only the 18S rRNA gene and corresponding to the sequences obtained previously in other environmental studies of these organisms in this laboratory (Lockhart *et al.*, 2006) (Fig. 5.18.) and another set including the ITS fragment of the sequence's 3' end (Fig 5.19.). The reason for the construction of two sets of trees is that phylogenetic treeing methods require the sequences to be of the same length, which would prevent the inclusion of the sequences from landfill clones (Lockhart *et al.*, 2006) when constructing trees with the full sequences from the clones.

Both sets of trees showed that the clones obtained in the lakes formed a single cluster, which included the cultured *Cyllamyces aberensis* sequence. Despite the low amount of variation (7 bp mismatches in the 18S only tree and 17 bp in the 18S – ITS1 tree that were consistently different between the *Cyllamyces* – freshwater clones and the *Neocallimastix frontallis* sequences), this clustering had high bootstrap support, particularly in the tree that included the partial ITS fragment. The phylogenetic trees produced with the 18S only sequences showed that the lake isolates did not cluster with the clones derived from landfill clones, which were more closely related to *Neocallimastix* spp. The genera to which these clones are likely to be derived from cannot be determined due to a lack of sequences from other genera of anaerobic fungi covering the same regions as the clones obtained here; the majority of the sequences in the databases overlap with the clones obtained here for 200 bp or less. The phylogenetic position of *C. aberensis* which clustered with the lake clones is not well known either as this is a recently described species whose position in the anaerobic fungal phylogeny has not been determined (Ozkose *et al.*, 2001).

A

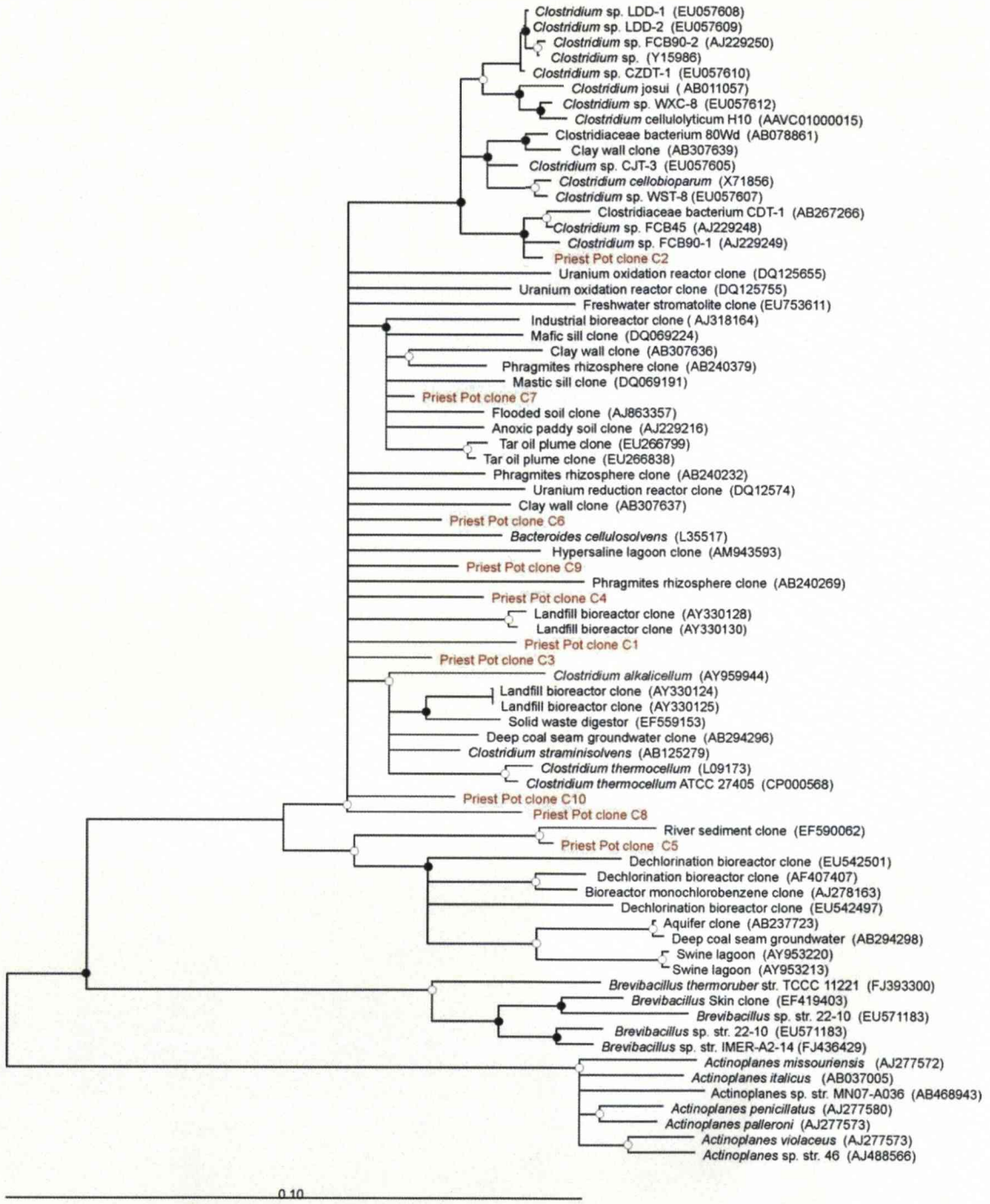


Figure 5.16. Phylogenetic trees of *Clostridium* III clones obtained from Esthwaite Water.

A, Neighbour Joining; B, maximum likelihood (overleaf).

B

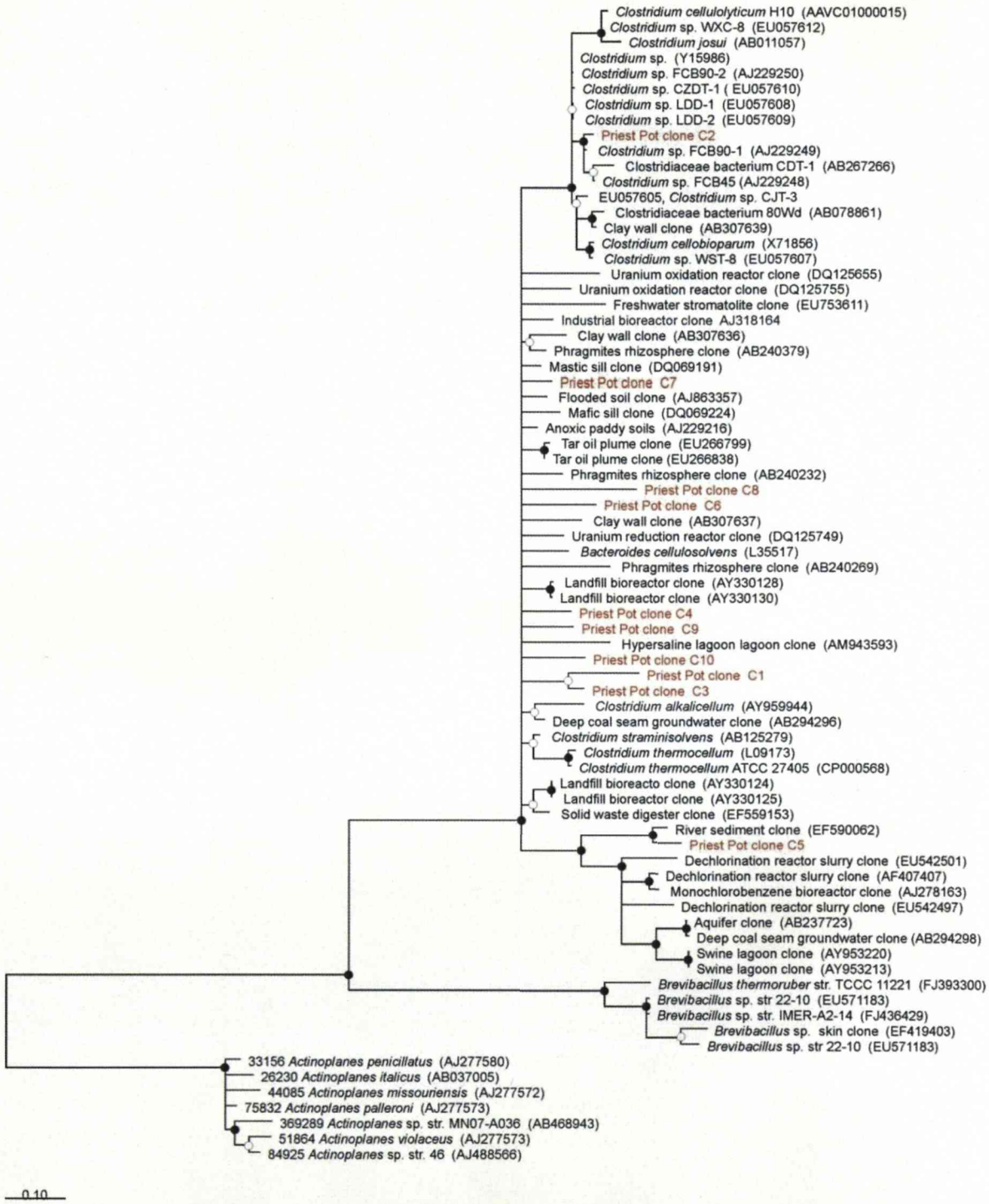

Clostridium cellulolyticum H10 (AAVC01000015)
Clostridium sp. WXC-8 (EU057612)
Clostridium josui (AB011057)
Clostridium sp. (Y15986)
Clostridium sp. FCB90-2 (AJ229250)
Clostridium sp. CZDT-1 (EU057610)
Clostridium sp. LDD-1 (EU057608)
Clostridium sp. LDD-2 (EU057609)
Priest Pot clone C2
Clostridium sp. FCB90-1 (AJ229249)
Clostridiaceae bacterium CDT-1 (AB267266)
Clostridium sp. FCB45 (AJ229248)
EU057605, *Clostridium* sp. CJT-3
Clostridiaceae bacterium 80Wd (AB078861)
Clay wall clone (AB307639)
Clostridium cellobioparum (X71856)
Clostridium sp. WST-8 (EU057607)
Uranium oxidation reactor clone (DQ125655)
Uranium oxidation reactor clone (DQ125755)
Freshwater stromatolite clone (EU753611)
Industrial bioreactor clone AJ318164
Clay wall clone (AB307636)
Phragmites rhizosphere clone (AB240379)
Mastic sill clone (DQ069191)
Priest Pot clone C7
Flooded soil clone (AJ863357)
Mafic sill clone (DQ069224)
Anoxic paddy soils (AJ229216)
Tar oil plume clone (EU266799)
Tar oil plume clone (EU266838)
Phragmites rhizosphere clone (AB240232)
Priest Pot clone C8
Priest Pot clone C6
Clay wall clone (AB307637)
Uranium reduction reactor clone (DQ125749)
Bacteroides cellulosolvens (L35517)
Phragmites rhizosphere clone (AB240269)
Landfill bioreactor clone (AY330128)
Landfill bioreactor clone (AY330130)
Priest Pot clone C4
Priest Pot clone C9
Hypersaline lagoon lagoon clone (AM943593)
Priest Pot clone C10
Priest Pot clone C1
Priest Pot clone C3
Clostridium alkalicellum (AY959944)
Deep coal seam groundwater clone (AB294296)
Clostridium straminisolvens (AB125279)
Clostridium thermocellum (L09173)
Clostridium thermocellum ATCC 27405 (CP000568)
Landfill bioreacto clone (AY330124)
Landfill bioreactor clone (AY330125)
Solid waste digester clone (EF559153)
River sediment clone (EF590062)
Priest Pot clone C5
Dechlorination reactor slurry clone (EU542501)
Dechlorination reactor slurry clone (AF407407)
Monochlorobenzene bioreactor clone (AJ278163)
Dechlorination reactor slurry clone (EU542497)
Aquifer clone (AB237723)
Deep coal seam groundwater clone (AB294298)
Swine lagoon clone (AY953220)
Swine lagoon clone (AY953213)
Brevibacillus thermoruber str. TCCC 11221 (FJ393300)
Brevibacillus sp. str 22-10 (EU571183)
Brevibacillus sp. str. IMER-A2-14 (FJ436429)
Brevibacillus sp. skin clone (EF419403)
Brevibacillus sp. str 22-10 (EU571183)
33156 *Actinoplanes penicillatus* (AJ277580)
26230 *Actinoplanes italicus* (AB037005)
44085 *Actinoplanes missouriensis* (AJ277572)
75832 *Actinoplanes palleroni* (AJ277573)
369289 *Actinoplanes* sp. str. MN07-A036 (AB468943)
51864 *Actinoplanes violaceus* (AJ277573)
84925 *Actinoplanes* sp. str. 46 (AJ488566)
0.10

Figure legend: phylogenetic trees of partial 16S rRNA gene sequences from *Clostridium* cluster III clones obtained from the Priest Pot 10 cm deep sediment sample in addition to sequences obtained from the SILVA database; A tree topology generated using the Neighbour Joining method with 1000 bootstraps; B, tree topology generated using the maximum likelihood method with 100 bootstraps. In red are the clones obtained in this study. Members of the *Brevibacillus* and *Actinoplanes* genera were also added. Open and filled circles represent nodes with 65 – 95% and > 95% bootstrap support, respectively. Accession numbers are shown in parenthesis; the scale bar represents 0.1 substitution per nucleotide position.

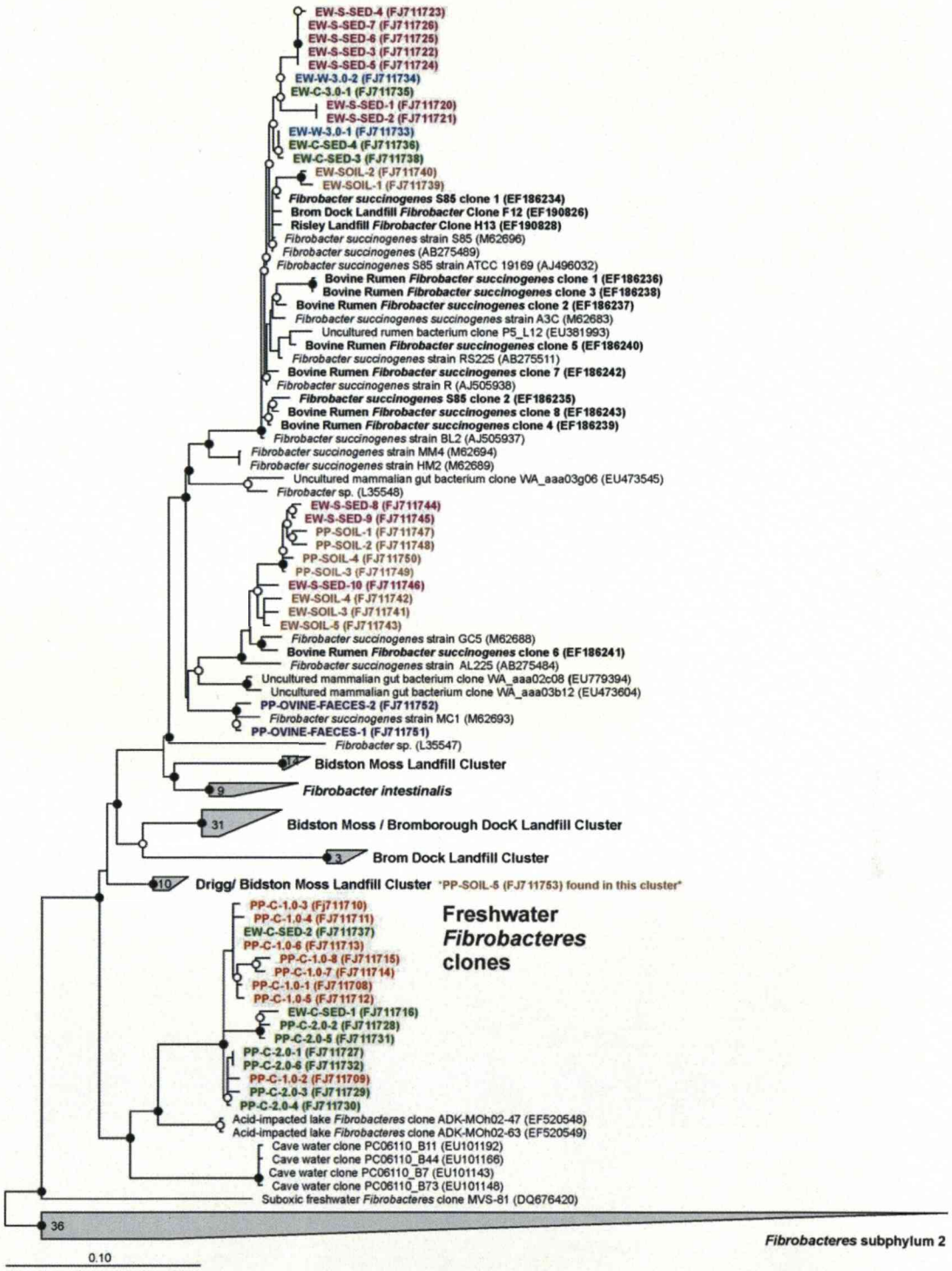


Figure 5.17. Phylogenetic tree of *Fibrobacter* clones obtained from Priest Pot.

Figure legend: maximum likelihood tree of partial 16S rRNA gene sequences from *Fibrobacter* clones obtained from the Priest Pot 2 m deep cotton bait sample in addition to sequences obtained from a separate study in this laboratory and others downloaded from the Greengenes database. In red are the clones obtained in this study; green, cotton bait clones except those from this study; blue, lakewater clones; purple, lake sediment clone, brown, soil clones, dark blue, ovine faeces clones. *Bacteroides fragillis* were used as the outgroup but removed from the figure due to the great extension of branch lengths. Open and filled circles represent nodes with 75-95% and 95% bootstrap support respectively. Sequence accession numbers are in parenthesis. Numbers shown on collapsed branches indicate the number of sequences within the branch.
The scale bar represents 0.1 substitution per nucleotide position. Figure modified from McDonald *et al.* (2009).

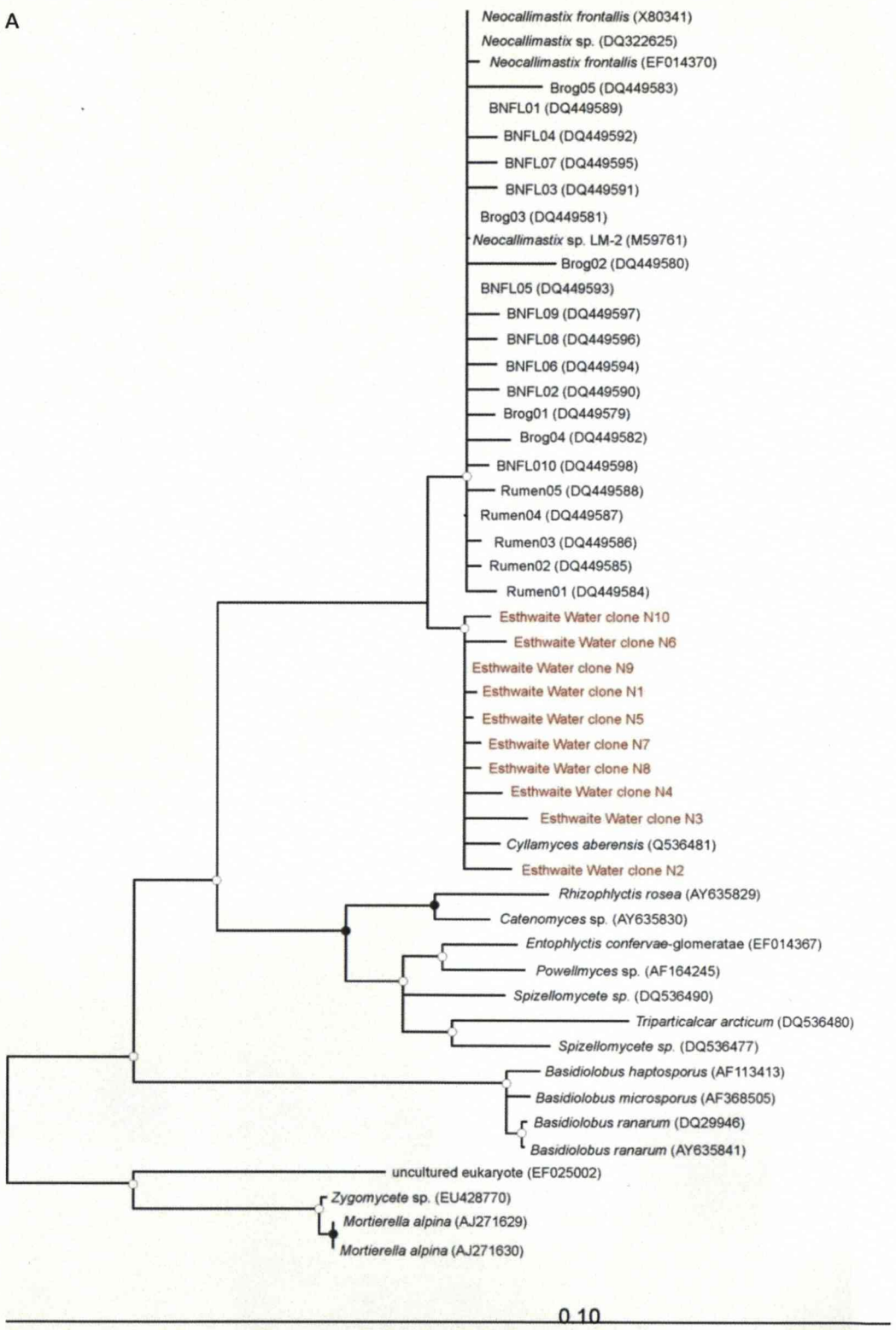


Figure 5.18. Phylogenetic trees of anaerobic fungi clones (partial 18S rRNA gene only) obtained from Esthwaite Water.

A, neighbour Joining; B, maximum likelihood methods (overleaf).

B

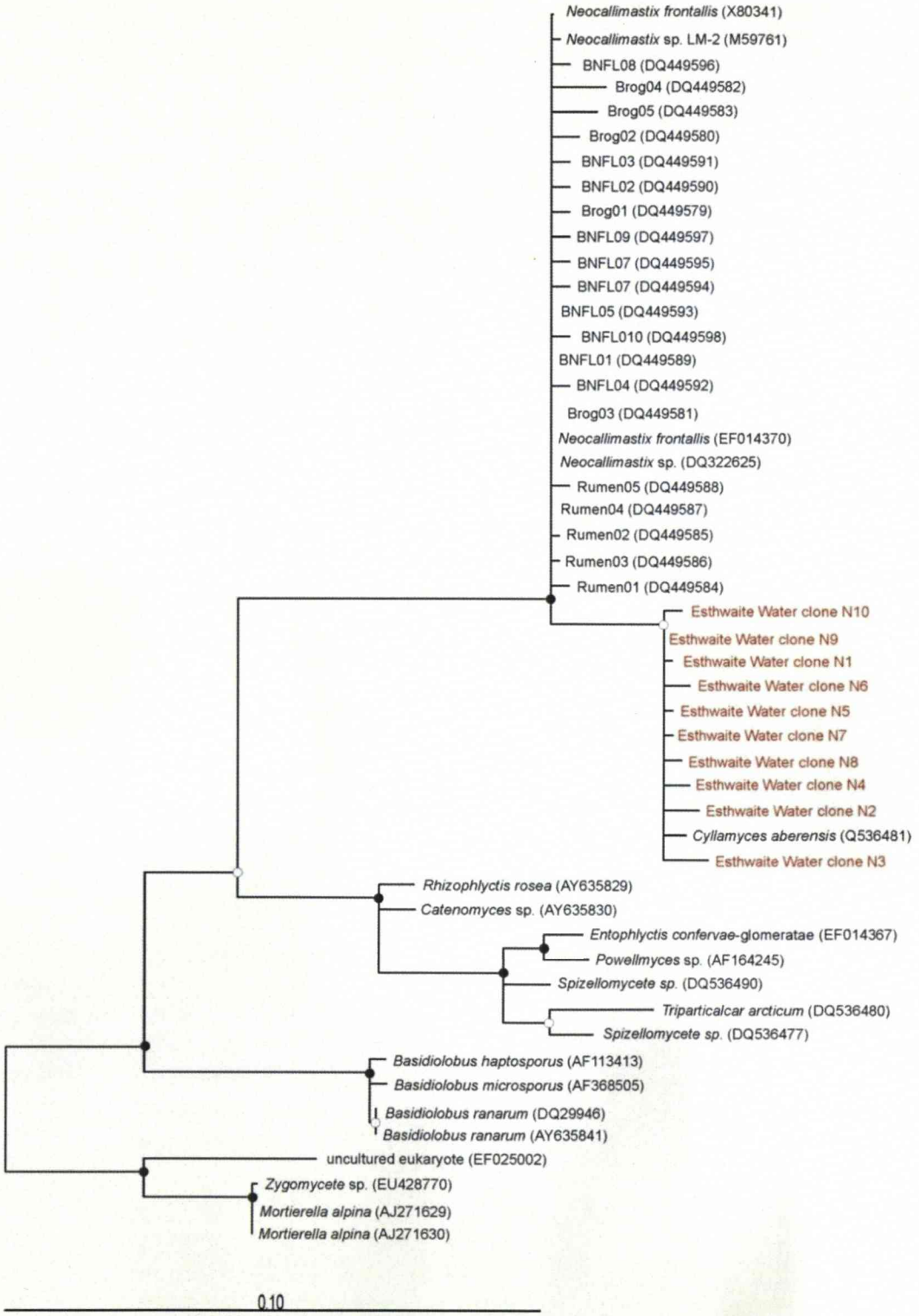

Neocallimastix frontallis (X80341)
Neocallimastix sp. LM-2 (M59761)
BNFL08 (DQ449596)
Brog04 (DQ449582)
Brog05 (DQ449583)
Brog02 (DQ449580)
BNFL03 (DQ449591)
BNFL02 (DQ449590)
Brog01 (DQ449579)
BNFL09 (DQ449597)
BNFL07 (DQ449595)
BNFL07 (DQ449594)
BNFL05 (DQ449593)
BNFL010 (DQ449598)
BNFL01 (DQ449589)
BNFL04 (DQ449592)
Brog03 (DQ449581)
Neocallimastix frontallis (EF014370)
Neocallimastix sp. (DQ322625)
Rumen05 (DQ449588)
Rumen04 (DQ449587)
Rumen02 (DQ449585)
Rumen03 (DQ449586)
Rumen01 (DQ449584)
Esthwaite Water clone N10
Esthwaite Water clone N9
Esthwaite Water clone N1
Esthwaite Water clone N6
Esthwaite Water clone N5
Esthwaite Water clone N7
Esthwaite Water clone N8
Esthwaite Water clone N4
Esthwaite Water clone N2
Cyllamyces aberensis (Q536481)
Esthwaite Water clone N3
Rhizophlyctis rosea (AY635829)
Catenomyces sp. (AY635830)
Entophlyctis confervae-glomeratae (EF014367)
Powellmyces sp. (AF164245)
Spizellomycete sp. (DQ536490)
Triparticalcar arcticum (DQ536480)
Spizellomycete sp. (DQ536477)
Basidiolobus haptosporus (AF113413)
Basidiolobus microsporus (AF368505)
Basidiolobus ranarum (DQ29946)
Basidiolobus ranarum (AY635841)
uncultured eukaryote (EF025002)
Zygomycete sp. (EU428770)
Mortierella alpina (AJ271629)
Mortierella alpina (AJ271630)
0.10

Figure legend: phylogenetic trees of partial 18S rRNA gene sequences (fragments amplified by the Neocal-forward and Neocal-reverse primers) from anaerobic fungi clones obtained from the Esthwaite Water surface sample in addition to sequences obtained from the SILVA database; A tree topology generated using the Neighbour Joining method with 1000 bootstraps; B, tree topology generated using the maximum likelihood method with 100 bootstraps. In red are the clones obtained in this study. Members of the family *Mortierellaceae* within the phylum *Zygomicota* were used as the outgroup. Open and filled circles represent nodes with 65 – 95% and > 95% bootstrap support, respectively. Accession numbers are shown in parenthesis; the scale bar represents 0.1 substitution per nucleotide position.

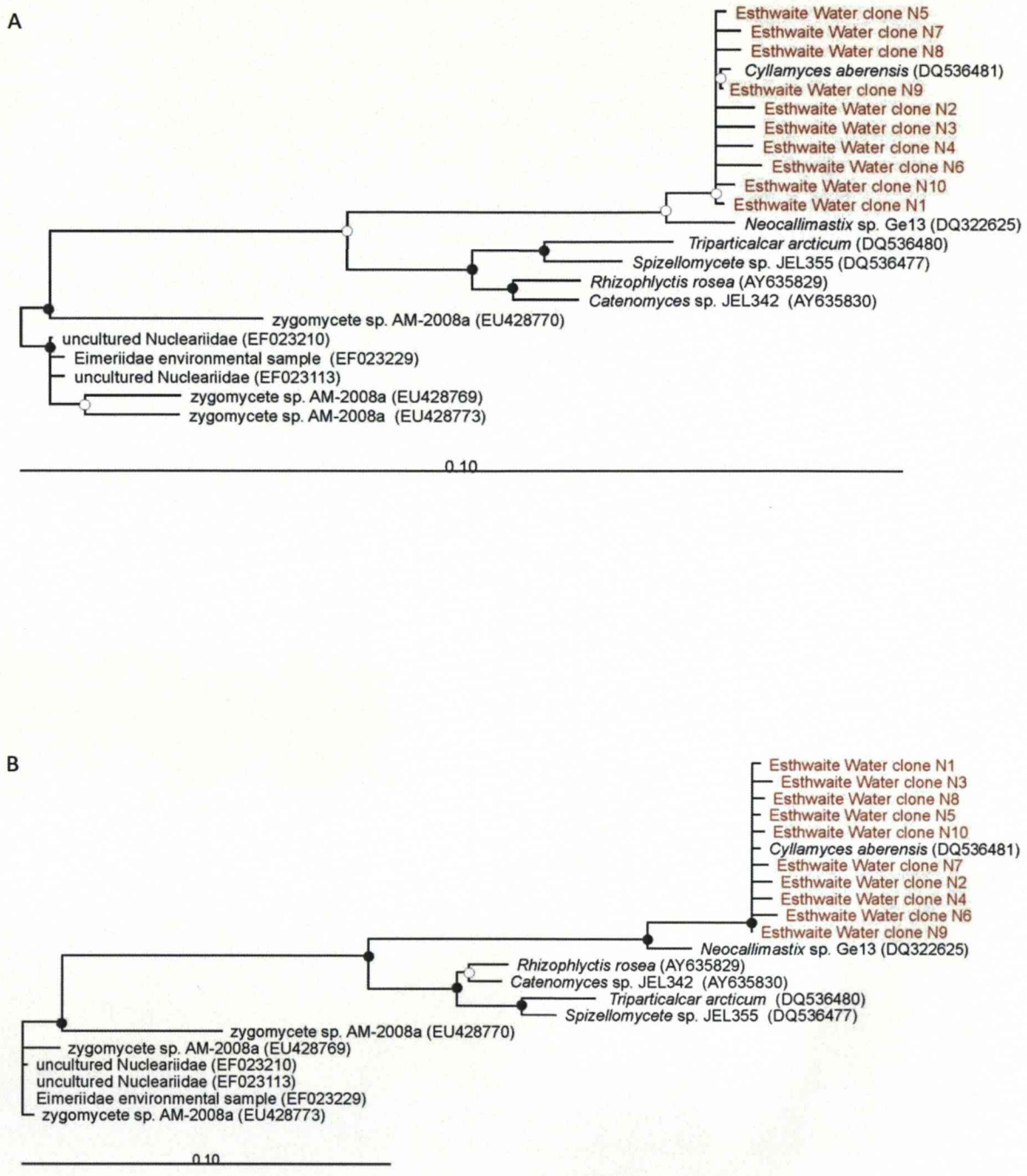


Figure 5.19. Phylogenetic trees of anaerobic fungi clones (partial 18S rRNA gene and 5' end of the ITS1 region) obtained from Esthwaite Water.

A, Neighbour Joining; B, maximum likelihood methods.

Figure legend: phylogenetic trees of partial 18S – ITS1 rRNA gene sequences (fragments amplified by the Neocal-forward and ANNFR primers) from anaerobic fungi clones obtained from the Esthwaite Water surface sample in addition to sequences obtained from the SILVA database; A tree topology generated using the Neighbour Joining method with 1000 bootstraps; B, tree topology generated using the maximum likelihood method with 100 bootstraps. In red are the clones obtained in this study. Members of the phylum Zygomycota that were the closest relatives to the chytrid fungi with sequences covering the same regions used in this study were used as the outgroup. Open and filled circles represent nodes with 65 – 95% and > 95% bootstrap support, respectively. Accession numbers are shown in parenthesis; the scale bar represents 0.1 substitution per nucleotide position.

5.7. Further analysis of *Micromonospora* and *Clostridium* III abundance data

As the abundances of *Micromonospora* and *Clostridium* III shown in section 5.5. were found to reach high values in some samples, the specificity of the primers was re-assessed in order to rule out the possibility that the numbers obtained were not due to non-specific amplification. PCR products amplified with primers M1C and M2D were cloned and their identity analysed, whereas the specificity of primers Clost3F and 829C3R was revaluated for qPCR, and *Clostridium* III abundances re-analysed using primers Clost3RF and 1423R.

5.7.1. Sequence analysis of *Micromonospora* PCR amplification products

PCR products amplified with M1C and M2D primers from DNA extracted from the 13 m Esthwaite Water cotton bait sample were cloned, and 10 of these sequenced. The clones were only 142-151 bp in length, and only a limited amount of sequence information can be obtained from a fragment of this size. The presence of a relatively small number of bp mismatches can significantly affect the list of matches obtained, however it was hoped that by analysing the closest matches to the clones in BLAST it would be possible to draw some conclusions.

BLAST analysis of the clones showed that 1 of the sequences matched with 100% similarity with the equivalent sequence of *M. chalcea,* whilst 3 others had matches with cultured and uncultured *Micromonosporaceae or* Actinomycetales although the similarity levels were low (< 95%). The remaining clones had matches with non-actinomycete sequences (mainly *Chloroflexy* spp.) as well as *Micromonospora* but with lower levels of similarity.

Upon closer inspection it was evident that BLAST was ignoring sections of the sequence which included at least one of the primers, and the closest matches were selected based on similarities with a section of the sequences near its 3' end that was found to be very variable.

When the results were filtered for sequences matching with 100% coverage of the clones (i.e., not excluding the 3' and 5' ends with the primer sequences), *Micromonospora* was closest match for 4 of the 6 clones that were not initially matched with the *Micromonosporaceae*. The two other clones whose affinities were less close to *Micromonospora* (M4 and M5) were found to have closer BLAST

matches with the *Propionibacterium/Nocardiodes* group, although *Micromonospora* was also found in the list of hits.

The possibility that the clones were chimeric was investigated using chimera_check (http://rdp8.cme.msu.edu/cgis/chimera.cgi), and this analysis showed similar results for the different clones, including clone M8, which is identical to the equivalent sequence in *M. chalcea*. The sequences were therefore not chimeric.

The level of sequence similarity between the clones and their nearest *Micromonospora* match in BLAST varied from 83-100%, which was a higher level of divergence than that found in sequences of the same region between cultured species of *Micromonospora* (92-100%). Similarity values as low as 83% were found between the *M. chalcea* sequence and sequences from members of the *Micromonosporaceae* other than *Micromonospora,* and also with uncultured strains within this family.

The relatively low levels of similarity of most clones with cultured *Micromonospora* species is not surprising as environmental clones often show greater diversity than cultured strains, however it is possible that some of the clones correspond to strains that are closely related to *Micromonospora*, but of different genera; one clone (M8) was almost certainly from a *Micromonospora* strain whereas two others (M4 and M5) may have come from more distantly related organisms, probably within the *Actinomycetales* as these had the lowest levels of similarity to cultured micromonosporas. The GC content of the clones was also in the range expected for actinomycetes (51-58%).

Although the real identity of the clones could not be determined with certainty, it is reasonable to conclude that the primers perform mostly as a *Micromonosporaceae* primers with a loose specificity centred on members of the genus *Micromonospora*.

5.7.2. Re-assessment of *Clostridium* III abundance

The specificity of primers Clost3F and 829C3R had been tested in the laboratory with the conventional PCR amplification of genomic DNA from pure cultures of three species of *Clostridium* belonging to clusters I, IV and XIV, in addition to cluster III, and it was found that no amplification occurred for any of the test strains at 58°C or

above (Fig 5.3.). The primers were considered therefore specific to *Clostridum* III at 60°C which was the temperature at which the qPCR analysis was carried out.

Due to the high abundances obtained, it was decided to test whether the primers chosen would amplify DNA from the control *Clostridium* species during qPCR. The results (data not shown) show that DNA from *C. acetobutylicum* was indeed amplified, but not the other two strains.

It is not known how well the amplification of cluster I clostridia occurred in the environmental samples, however it is possible that the data shown above (Fig 5.13.) represents the combination of the populations of *Clostridium* clusters I and III in the lakes.

It was therefore decided to test if the other primers designed for *Clostridium* cluster III (1423R in combination with Clost3RF) amplified the DNA from the three test *Clostridium* strains from the other clusters. The results (data not shown) showed that this primer combination did not amplify any of the control *Clostridium* DNA. As it had been previously shown that this primer pair performed relatively well in calibration curves, it was decided to re-analyse the lake samples with this primer combination.

The results (Fig 5.20. and Table 5.15.) show that *Clostridium* cluster III relative numbers analysed with primers Clost3RF and 1423C3R, with the exception of one sample (Priest Pot, sediment cotton sample), followed similar depth profile trends in both lakes, and the difference between the lakes was also similar. Total abundance obtained with primers Clost3RF and 1423C3R were in general higher and often double those obtained with Clost3F and 929C3R. It was not expected to obtain higher abundances of *Clostridium* III with primers Clost3RF and 1423C3R as these were shown not to amplify any of the control non cluster III clostridial test samples. The reasons for these higher abundances are not clear. However, as discussed in the primer design section (section 5.3.) primers Clost3RF and 1423C3R were not ideal for qPCR due to the large difference in T_m between the primers.

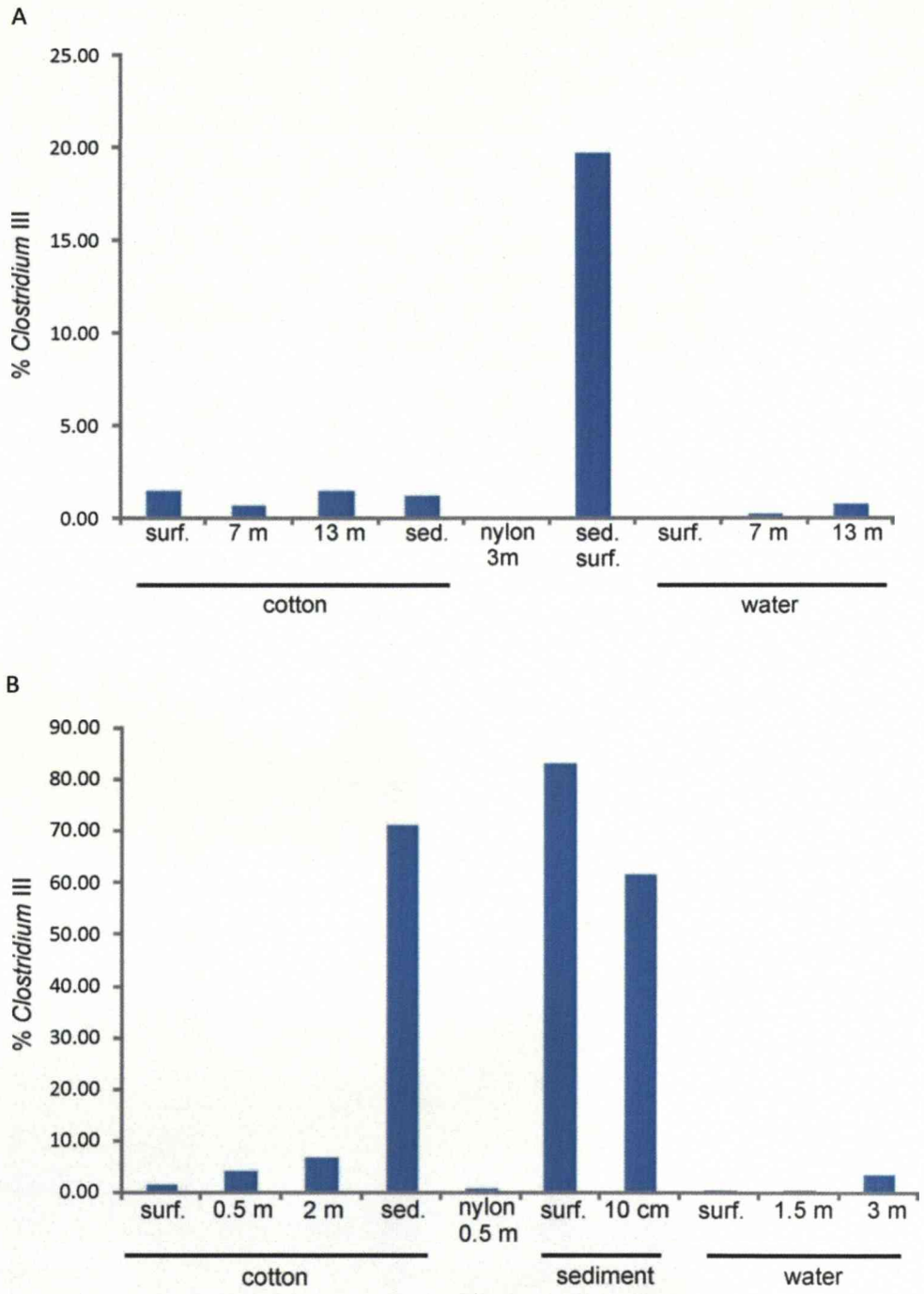


Figure 5.20. Relative abundance of *Clostridium* cluster III measured with primers Clost3RF and 1423C3R in Esthwaite Water and Priest Pot.

A, Esthwaite Water; B, Priest Pot. *Clostridium* III and total bacterial 16S rRNA numbers were determined using primers Clost3RF and 1423C3R and 1369F-1492R respectively and using cDNA as template. $R^2 > 0.99$ for both primer sets. The 10 cm sediment sample in Esthwaite Water showed very small numbers of total bacteria (242 copies) and of *Clostridium* III (169 copies), and was therefore removed from this analysis.

Analysis	Clost3RF-1423R		universal primers	
	slope	r^2	slope	r^2
Clostridia EW	-3.61	1.00	-3.30	0.99
Clostridia PP	-3.75	1.00	-3.34	0.99

Table 5.15. Slope and goodness of fit of the calibration curves used in the second relative quantification of *Clostridium* III.

EW, Esthwaite Water, PP, Priest Pot.

Despite the higher abundances obtained in this second attempt to quantify *Clostridium* III, the fact that general patterns observed were similar to those obtained in the previous analysis is an indication that the abundances obtained represent the population of *Clostridium* cluster III strains, although as both *Clostridium* I and III species are anaerobic saprophytic organisms it is acceptable that both would have a similar distribution in the lakes.

It is reasonable to assume therefore that *Clostridium* I may have contributed to the abundances observed, although the lack of amplification during conventional PCR suggests that this contribution was limited.

In a separate study carried out in this laboratory, *Clostridium* III strains were detected more frequently by conventional PCR than *Clostridium* cluster I in a seasonal study in both lakes (Robert Lockhart, personal communication), and it is therefore possible that even if primers did amplify *Clostridium* I strains, this may not have contributed significantly to the total abundances obtained.

5.8. Discussion

There are no published studies with which the molecular quantification of cellulolytic microorganisms in lakes presented in this study can be compared. In addition, molecular quantification of *Micromonospora* and *Clostridium* III has never been attempted, whereas *Fibrobacter* spp. and the anaerobic fungi have only been quantified in other environments i.e the rumen (Denman & McSweeney, 2006) and landfill (McDonald *et al.*, 2008). The anaerobic fungi have been detected outside of the mammalian gut (Lockhart *et al.*, 2006), but their presence in lakes has never been reported, and neither has that of *Fibrobacter* spp.

The use of qPCR for the quantification of microorganisms in natural populations has been accomplished previously, and in lakes. For example, there are studies in which sulfate reducing bacteria (Foti *et al.*, 2007), anoxygenic bacteria (Jiang *et al.*, 2009) and picocyanobacteria (Sanchez-Baracaldo *et al.*, 2008) have been targeted. A number of studies have targeted cellulolytic bacteria in the herbivore gut (Denman & McSweeney, 2006; Hastie *et al.*, 2008; Koike *et al.*, 2007; McSweeney & Denman, 2007; Mosoni *et al.*, 2007; Ozutsumi *et al.*, 2006; Shinkai & Kobayashi, 2007; Shinkai *et al.*, 2007; Stevenson & Weimer, 2007; Sung *et al.*, 2007; Tajima *et al.*, 2001; Wanapat & Cherdthong, 2009; Zhou *et al.*, 2007), however only one has quantified a group of cellulolytic microorganisms outside the rumen, in landfills (McDonald *et al.*, 2008).

This study therefore makes a significant contribution to the current knowledge of the abundance of cellulose degrading organisms in the environment, and to the understanding of microbial populations in lakes in general.

The accuracy of the qPCR method in determining gene copy numbers has been assessed in some studies by comparing numbers with microscopical cell or CFU counts. In some of these studies, the numbers obtained with qPCR agreed (Matsuda *et al.*, 2007; Morrison *et al.*, 2008; Rueckert *et al.*, 2007). However, variations between observed and expected numbers have also been reported, for example, Brinkman *et al.* (2003) used qPCR (TaqMan) to quantify known numbers of *Candida* spp. cells added to tap water and found that the method underestimated or overestimated the numbers by 50-200%. Similar discrepancies obtained using qPCR and other quantification methods have been reported (Kolb *et al.*, 2003; Lees *et al.*, 2002; Vaitomaa *et al.*, 2003). The reason for these discrepancies is often attributed to the efficiency of DNA extraction and presence of PCR inhibitors in the

template (Coyne *et al.*, 2005) and it has been established that the amplification efficiency of plasmids or DNA extracted from cultures is different from that of DNA extracted from the environment (Becker *et al.*, 2000). The nature of the template and its molecular association with the primers is variable, causing changes in primer efficiency. Here, a discrepancy was observed when using general bacterial and *Micromonospora* specific primers to quantify 16S rRNA gene copies in genomic DNA extracted from *Micromonospora* pure cultures. The cause for this discrepancy is likely to be due to the changes in amplification efficiency of the two primer pairs (general bacterial and *Micromonospora* specific) being different for the two templates (cloned 16S rRNa gene and *Micromonospora* genomic DNA).

A comparison of gene copy determination of the same template using different primers has not been found in the literature. Despite the overestimation of numbers when using primers M1C and M2D to quantify DNA from *Micromonospora* cultures, it is not known whether the same problem would have occurred when using DNA extracted from the environment, as the nature of the two templates is very different. M1C and M2D were shown to be specific to *Micromonospora* and related organisms and produced good calibration curves, and it was decided to retain them for the quantification of *Micromonospora* in the lakes.

The relative numbers obtained for *Micromonospora* and *Clostridium* cluster III in some samples greatly exceeded those obtained in preliminary tests during specific primer development (Tables 5.2., 5.3. and 5.9.). This could be due to the fact that the samples were frozen on-site and treated appropriately for molecular analysis, as most tests done previously were performed using a sample obtained in November 2007 during the experiment used to detect hyphae in the lakes and was not frozen on site; two other samples used were from July 2005, which had been kept at -80°C for three years prior to analysis and could have been degraded significantly at the time of analysis. All the analyses performed subsequent to the primer design were performed in samples obtained from one sampling date in the end of spring of 2008, and therefore at a time usually associated with high biological activity in the lakes studied (Finlay, 2000). The differences in abundance of *Micromonospora* and *Clostridium* III compared to the test samples could therefore be due to fluctuations in their population in the lakes. The fact that the abundances of *Micromonospora* and *Clostridium* cluster III were not uniformly high in all samples (many samples had abundances of < 1% for both groups), is evidence that spatial and temporal factors may explain the large difference in abundances observed between the test samples

and those obtained in June 2008. In the case of *Micromonospora*, the fact that the depth profile of their numbers from the cotton baits obtained in isolation plates was similar to the depth profile of the relative abundance of *Micromonospora* in the same samples is a further indication that the primers were amplifying the expected target organisms.

The abundance of each group analysed show a pattern of distribution that reflects the dissolved oxygen depth profiles as well as the organic loading of each lake. The *Micromonospora* group quantified in this study was more abundant in Esthwaite Water than in Priest Pot, which correlates with their aerobic physiology, as Priest Pot has a higher nutrient load which is associated with higher consumption of oxygen. The abundances of *Micromonospora* in the cotton baits show a steady increase towards the bottom of the lakes, being negligible in the surface but rising to 21% in the deepest cotton bait in Esthwaite Water and 7% in the second deepest bait in Priest Pot; this pattern could be explained by the fact that micromonosporas are slow growing organisms, and are outcompeted in the surface where the availability of light and higher temperatures allow the growth of a thick microbial biofilm. Although oxygen concentrations are lower towards the bottom of the lake, this is not necessarily an indication of its complete absence. Rather, oxygen will diffuse to the lower reaches of the lakes and that may be enough to sustain microbial growth (Fenchel & Finlay, 2008), and this flux of oxygen may be enough to support the growth of micromonosporas. In addition, periodic storms and strong winds can disrupt the oxycline and replenish the oxygen in the deep layers of the water column. There is evidence that *Micromonospora* spp. can grow under microaerophilic conditions (Vobis, 2006), and the low oxygen concentrations could therefore restrict the growth of other microorganisms more so than that of *Micromonospora*, but remain high enough to prevent significant growth of strict anaerobes. The closeness to the sediment can also boost the growth of the micromonosporas as sediment is a major source of both nutrients and *Micromonospora* spores (Cross, 1981). The fact that in Priest Pot the bait with highest abundance of *Micromonospora* was the second deepest one is further evidence for the role of oxygen in shaping the distribution of these organisms, as in Priest Pot with its high nutrient loading it is possible that oxygen unavailability would extend further above the sediment than in Esthwaite Water. *Micromonospora* abundances in the actual sediment were significantly lower when compared to those observed in the baits just above, being close to 1%, but not as low as those

observed in the surface bait samples. *Micromonospora* spores are numerous in the sediments (Cross, 1981), which explains their detection there, and their lower abundance may be due to the presence of a significant community of anaerobes in these environments; alternatively, the spores are more difficult to lyse than hyphae, and they may contain fewer ribosomes than the latter, causing the detection of a relative lower abundance. In the water column, *Micromonospora* abundance was around 1% or less, except in the 13 m deep sample at Esthwaite Water, where it was approx. 3%; this value is similar to the abundance observed for the bait at the same depth in Esthwaite Water and is not a surprising finding as *Micromonospora* spores are hydrophilic and are readily recovered from lakewater. The abundances in the water column never reach those for the deep cotton baits however, and in Priest Pot evidence can be seen for the bait acting as an enrichment for *Micromonospora* spp. as the abundances in the water column are < 1% between 1.5 and 3 m, whereas in the baits the numbers reaches 7% at 2 m. The numbers of micromonosporas counted in isolation plates correlate with the gene copy number abundances particularly in the increase in numbers in the baits with depth, being highest in the bait just above the sediment in Esthwaite Water, and second deepest in Priest Pot. The molecular abundance dataset is not directly comparable to CFU numbers, but the increasing recovery of *Micromonospora* towards the sediment of the lakes can be explained by the same factors as described above for the gene copy abundances, and therefore reinforces the findings.

In contrast to *Micromonospora, Clostridium* III abundances are higher in Priest Pot than in Esthwaite Water, which can again be explained by the lower dissolved oxygen concentrations in the smaller lake. *Clostridium* III numbers as measured with both primer sets used in this study show a similar pattern of increasing abundance towards the deeper parts of the lakes, both for the baits and for the water column samples. However the discussion here will be referring to the results obtained with the first primer set used, Clost3F and 829C3R. In both lakes, numbers of *Clostridium* III in the actual sediment are an order of magnitude higher than those for the baits and water column samples. The high abundance of *Clostridium* III in the sediment can be explained by the fact that whereas in the water column even at depth, oxygen may be available by diffusion from the shallower oxygenated waters, in the sediments the presence of soluble reduced compounds ensures that almost no dissolved oxygen is available.

The *Clostridium* III abundances were up to 4 fold higher in the sediment surface compared to the deep (10 cm) sediment sample. The presence of freshly settled biomass from the senescent algal blooms and from the surrounding field and woodlands provide the microbial community with fresh nutrients and carbon sources, which clostridia can readily utilize. Deeper in the sediment, the lower *Clostridium* III relative abundances may be related to the decrease in quantity and change in nature of the carbon sources available there compared to the sediment surface.

In Esthwaite lakewater, the abundances of *Clostridium* III were low in (< 0.01%), whereas in Priest Pot they reached values above 5% in the deepest sample. Water samples taken from the deeper layers of Priest Pot were thick with organic debris and the distinction between the water column and the sediment was not as clear as it was with Esthwaite Water; it is therefore not surprising that the abundance of *Clostridium* III was high in the deep water column of Priest Pot as this was of mixed sediment/water column in nature.

In the lakes studied here, *Clostridium* III spp. are significant members of the community in the strictly anaerobic sediments, however they were also detected in the baits and water samples taken from the shallower, more oxygenated zones of the lakes, although generally at low abundances (< 1.3%). The growth of anaerobes in aerobic environments is not unexpected as the presence of a microbial biofilm can lead to the development of microhabitats devoid of oxygen (Delong *et al.*, 1993), and in addition, members of the genus *Clostridium* are spore formers, and could therefore be detected in samples from oxygenated areas even if vegetative cells were not present.

The fact that samples that contained very high abundances of *Clostridium* III spp. (anaerobes) had low abundances of *Micromonospora* spp. (aerobes) is a further indication that the patterns observed here reflect real distributions of the organisms targeted. Abundances obtained with the qPCR method are affected by the physical properties of the primers used as well as those of the target DNA/cDNA being amplified, and it is possible that the real abundances for the groups studied here are somewhat different to those reported. However it is reasonable to consider that both *Clostridium* III and *Micromonospora* spp. form a significant part of the microbial community in those samples where their abundances were highest.

Fibrobacter and the anaerobic fungi were detected in the lakes, and this has not previously been reported. However, their numbers were very low and only trace populations were present. Both *Fibrobacter* and the anaerobic fungi were only detected in the cotton baits or in the sediments, but never in the water column, which suggest that these organisms are not simply washing in from the surrounding fields and are indigenous to this freshwater habitat, albeit at low numbers. Copy numbers of *Fibrobacter* were an order of magnitude higher than those of the anaerobic fungi, but in both cases the numbers were too low to be considered accurate. Surprisingly, the anaerobic fungi were more readily detectable in Esthwaite Water, where their 18S rRNA gene was amplified from the surface cotton bait samples as well as from the sediment. Although they were detected in Priest Pot through nested PCR, qPCR failed to detect any copies of the 18S rRNA of these organisms in this lake, whereas in Esthwaite Water the two shallowest samples showed the presence of circa 10 – 4 copies of its SSU rRNA gene. The presence of members of the *Neocallimastigales* in the surface bait samples is not evidence of washing in of external DNA and can be explained by the formation of anoxic microhabitats within the thick microbial biofilm found in these samples, as described above. Alternatively, there are members of the *Neocallimastigales* in the lakes that are aerobic. Cultured Members of the *Neocallimastigales* are strictly anaerobic, and the likelihood that aerobic members of this order exist will be greater if it can be demonstrated that anaerobic metabolism can evolve from ancestral aerobes in other groups of eukaryotes. Ongoing debate exists as to whether anaerobic metabolism in eukaryotes is a primitive condition derived from the common eukaryotic ancestor prior to the engulfment of the mitochondrion, or whether it is a derived condition in which organisms loose the mitochondrion as an adaptation for anaerobic metabolism (Embley, 2006). The anaerobic fungi contain hydrogenosomes, cytoplasmic organelles found also in other anaerobic eukaryotes, such as the trichomonads and anaerobic ciliates. These organelles allow the use of different terminal electron acceptors for anaerobic respiration, with the production of molecular hydrogen and ATP. Evidence has been obtained showing that the hydrogenosome has evolved repeatedly from mitochondria (Hackstein *et al.*, 2006). Phylogenetic analysis of proteins from the hydrogenosome of the anaerobic ciliate *Nyctotherus ovalis* showed that these are closely related to those of the mitochondrion of aerobic ciliates (Boxma *et al.*, 2005), and the organelle itself was considered an anaerobic mitochondrion. It is possible therefore that the

hydrogenosome evolved from mitochondria in an ancestral aerobic member of the *Neocallimastigales*, and that these are still present in the environment.

None of the organisms in this study were present in significant numbers in the nylon samples, which shows that the simple presence of a surface for attachment is not the sole function of the cotton baits, and that attachment to and degradation of the cellulose is providing a selective/enrichment effect.

The phylogeny of the clones of *Clostridium* III reveals that, although there was no clustering of the clones into a single, Priest Pot cluster, the closest relatives to all of the clones were other environmentally derived sequences. The phylogeny of *Clostridium* species is complex (Collins *et al.*, 1994; Stackebrandt *et al.*, 1999), and the cluster III species should belong to a separate family to *Clostridium* sensu stricto (Collins *et al.*, 1994); the sequences that are closest to the clones obtained here are classified in the RDP II database as unclassified *Ruminococcaceae*, but none of the clones had close matches with rumen-derived clostridia. The *Clostridium* III community in the Priest Pot sediment sampled here is diverse, as the different clones occupied different positions in the trees produced. However, they all seem to belong to distinct groups of *Clostridium* III present in anoxic aquatic environments such as rice paddies, deep groundwater and bioreactors; of particular note is the fact that most of the recently isolated strains that were closely related to the clones obtained in this study were either characterised as cellulolytic or were isolated from cellulose-containing environments such as landfills and cellulose degrading bioreactors (Akasaka *et al.*, 2003; Chin *et al.*, 1998; Chin *et al.*, 1999; Kato *et al.*, 2005; Monserrate *et al.*, 2001; Stackebrandt *et al.*, 1999).

The *Fibrobacter* clones on the other hand did show a distinct cluster of lake strains, with closest relatives being other uncultured *Fibrobacter* derived from aquatic sources such as acid mine drainage (Percent *et al.*, 2008), cavewater (Macalady *et al.*, 2008), and a suboxic freshwater pond (Briee *et al.*, 2007). These lake strains are distinct from the cultured rumen *Fibrobacter* and from the rumen-derived sequences, as well as from landfill and termite gut-derived sequences, suggesting that this is a distinct clade of freshwater fibrobacters.

This clade is currently undescribed in the literature, and more studies would be needed in order to determine their specific ecological role. However, the two currently described cultured species of *Fibrobacter* are cellulolytic, and in this study they were only detected in the cotton baits or in the sediments, which are rich with

decaying plant biomass. This indicates that these freshwater strains are likely to play a role in polysaccharide depolymerisation, as was suggested for the other fibrobacters detected in cellulose-rich landfills (McDonald *et al.*, 2008), and that the fibrobacters have a wider role in cellulose degradation than is currently recognised i.e., they are not solely herbivore gut bacteria.

The anaerobic fungi sequences represent a distinct cluster of strains, however their closest relative is *Cyllamyces aberensis*, a poorly characterised and recently described species, isolated from cattle faeces (Ozkose *et al.*, 2001). Of interest is the fact that the sequences obtained in this study clustered with strong bootstrap support separately from landfill derived sequences, showing that the anaerobic fungi found outside the rumen may represent a diverse group of organisms. As the full life cycle of these organisms is still poorly described, especially their sexual cycle, this environmental population could simply represent a distinct stage of the life cycle of the rumen anaerobic fungi. More research is needed in order to clarify their contribution to cellulose degradation outside of the rumen and whether some members of the group are aerobic.

This study has therefore shown that *Micromonospora* and *Clostridium* III are likely to play a important role in plant biomass degradation in the Esthwaite Water and Priest Pot, with small contributions from *Fibrobacter* and the anaerobic fungi; the availability of oxygen is a determinant in the distributions of these populations in the lakes, the populations of *Clostridium* III and *Fibrobacter* in the lakes are distinct from the rumen-derived strains, and the anaerobic fungi are able to grow in these lakes as demonstrated by their occurrence limited to cotton baits or sediment.

Finally the classification of micromonosporas as "soil" organisms and fibrobacters and anaerobic fungi as "gut" organisms is untenable. These groups have representatives that are indigenous to aquatic environments.

Chapter 6 - General Discussion

This study was designed to elucidate the role of cellulolytic organisms in the degradation of cellulose in lake environments. Very little information was previously available on the ecology of cellulolytic organisms, and their role in lakes had largely been ignored (de Menezes *et al.*, 2008).

It was known that fungi and actinomycetes play an important role in the degradation of lignocelluloses in terrestrial environments, but even in these environments the relative contribution of each group has not been assessed. It is generally assumed that cellulolytic organisms are present in lake sediments where anaerobic degradation will occur, and that the clostridia are contributing to this process, but studies determining their relative importance are non-existent.

Cellulose degrading communities have been studied in other environments such as landfills (Lockhart *et al.*, 2006; McDonald *et al.*, 2008; Van Dyke & McCarthy, 2002), but the only environment in which the cellulolytic community has been characterised in detail is the mammalian rumen, where the diversity and role of the different fibrolytic communities has been studied (Leschine, 1995; Russell & Rychlik, 2001; Shinkai & Kobayashi, 2007; Trinci *et al.*, 1994; Ziemer *et al.*, 2000). The attention devoted to rumen cellulolytic organisms is largely driven by the economic importance of livestock nutrition (Krause *et al.*, 2003), and little information obtained from the rumen communities can be used to understand cellulose degradation in the environment.

As an example of a cellulolytic organism that can be abundant in lakes, but whose ecology was largely unknown, this study initially focused on determining what role, if any, *Micromonospora* spp. had on cellulose degradation in lakes.

The isolation of *Micromonospora* strains from the lakes was relatively straightforward, and isolates were obtained from sediments, lakewater and cotton baits. Their abundances in the baits could be explained on the basis of dissolved oxygen concentrations and residence time in the lakes. The shape of the depth profile of CFU numbers was often similar for both lakes studied, an indication that seasonality and weather are major factors in structuring the populations of *Micromonospora* strains, as these lakes are very different in size and nutrient loading. Terrestrial run-off could be supplying the lakes with spores from the

surrounding fields, whereas low dissolved oxygen concentrations could limit their growth. Mixing of the water column may lead to occasional bursts of growth in the sediments during the summer months when a thermocline develops in both lakes.

The ease with which the micromonosporas were isolated provided some evidence of their presence and ubiquity in the lakes, but further studies were needed in order to confirm their growth in these environments. The production of spores that can survive in aquatic environments is a further reason why it is necessary to prove the growth status for *Micromonospora* in lakes. Using a combination of antibiotic, alkaline and heat treatments, it was possible to obtain differential CFU counts for *Micromonospora* hyphae and spores, and it was demonstrated that hyphae were indeed present in the lakes. The evidence for the presence of hyphae was strong for the baits and sediments, but not for the water column, suggesting that *Micromonospora* preferentially attaches and grows on solid organic substrates.

Micromonospora spp. are hyphal and their mode of growth is particularly suited for attaching to and penetrating biomass, as was observed in the scanning electron micrographs obtained in this study (Fig 3.15).

The SEM images of cotton strands cultivated with lake isolates showed that micromonosporas are indeed able to degrade cellulose, even when cultivated in pure crystalline cellulose (cotton) in unsupplemented lake water. The ability to degrade pure cellulose proves that *Micromonospora* spp. have the full complement of cellulose degrading enzymes, and are capable of "true" cellulolytic activity, as opposed to those organisms that are only able to attack amorphous cellulose.

True cellulolytic activity in unsupplemented lakewater is evidence that micromonosporas have a potential role in the degradation of cellulose in the lakes, as it can be argued that activity observed on artificial media in the laboratory is a poor indicator of *in situ* cellulolysis.

Having shown that some strains degraded cellulose to a significant extent, the activity of a large collection of lake isolates was analysed in order to determine whether significant activity was found across the genus, or whether it was limited to a particular set of strains. Activity was found to be variable within the culture collection, although most strains were able to attack filter paper to some extent. One set of strains was consistently superior in solubilising filter paper as well as in producing higher levels of endoglucanase activity. This high cellulolytic ability was

not simply to higher growth yields, as some strains grew well but were still unable to degrade cellulose to a significant extent. It is not known whether they would necessarily be superior cellulose degraders under in-situ conditions, where cellulose often occurs within a matrix of lignin, hemicelluloses, waxes and pectins. SEM analysis showed that all strains incubated with cotton were able to attack cotton at least superficially, but some of those strains (9, 16 and 22) produced small or undetectable amounts of endoglucanase, and were not able to significantly solubilise filter paper. It is possible that the strains identified as poor cellulose degraders when grown in supplemented media at high temperatures may be as efficient as the more active strains under natural conditions.

The phylogenetic structure of the lake isolates was characterised through the analysis of their *gyrB* gene by RFLP and sequencing, showing that the strains with high cellulose degrading abilities equated with the type species of the genus, *M. chalcea*. The few studies that have been published characterising cellulose degradation by *Micromonospora* strains have used this species (Gallagher *et al.*, 1996; Malfait *et al.*, 1984), or have not named the species (Chowdhury *et al.*, 1991). One study characterised the activity of *M. melanosporea* (van Zyl, 1985), but this is not a recognised *Micromonospora* species in Bergey's Manual of Systematic Bacteriology (Kawamoto, 1984), and its taxonomic affiliations are not clear. *Micromonospora chalcea* may therefore be the most cellulolytic species of the genus as a whole, and not only in the lakes studied here.

Other than *Micromonospora chalcea,* the strains obtained in this study were distributed across the phylogeny of the genus, showing that there was a significant diversity of micromonosporas in the lakes. A large number of strains formed a cluster that contained no named species, and this could represent a new centre of variation within the genus. This could simply be due to the size of the *gyrB* sequence databases, as a number of new *Micromonospora* isolates have been described in recent years, and this is a limitation of phylogenetic analysis on gene targets other than SSU rRNA sequences.

This study has proven that *Micromonospora* spp. grow in freshwater lakes, and that they are able to colonise and attack crystalline cellulose. They could play a role in cellulose degradation in lakes, however their importance in the microbial community was unknown. Quantitative PCR was used to determine whether the micromonosporas formed a significant component of the microbial community, and

in addition, it was decided to quantify the total and relative abundance of three other groups of organisms, *Clostridium* III, fibrobacters and the anaerobic fungi. *Clostridium* III is a cluster in the genus *Clostridium* (Collins *et al.*, 1994) whose characterised strains are all cellulolytic; its members are associated with the *Ruminococcaceae* family in the RDP II database (Cole *et al.*, 2009), however their taxonomic affiliations are not fully characterised. Clostridia are assumed to play a role in the anaerobic degradation of cellulose both in the rumen of herbivores and in the environment (Burrell *et al.*, 2004; Leschine, 1995; Li *et al.*, 2009; Stevenson & Weimer, 2007; Uyeno *et al.*, 2007; Van Dyke & McCarthy, 2002), but their abundance, distribution and importance in lakes is unknown. Fibrobacters and the anaerobic fungi are rumen organisms which have only recently been detected elsewhere i.e. in landfills. It is therefore important to determine if the extended ecological range for fibrobacters and anaerobic fungi encompasses freshwater lakes.

The quantitative-PCR data has shown that both *Micromonospora* and *Clostridium* III are important members of the microbial communities in lakes, and their numbers can be a significant proportion of the total bacterial abundance; fibrobacters and the anaerobic fungi also occur in lakes, but the qPCR data suggested that they were trace components of the microbial population.

The distribution and relative abundance of the target organisms was influenced by the concentration of dissolved oxygen in the water column and the presence of substrate and competing organisms. *Micromonospora* spp. were present as small populations in most samples (≤1%), but their abundance increased significantly towards the sediment of both lakes, which might be explained by the lack of competition from faster growing organisms that constituted thick biofilms in the shallow baits. Oxygen flux from the upper water column may have been enabled *Micromonospora* growth at depth despite low measured values for dissolved oxygen. The fact that their abundance was highest just above the sediment in both lakes, whilst being relatively low in the sediments themselves, fits the hypothesis that they are slow growing cellulolytic organisms that can thrive under microaerophilic conditions. In addition, the *Micromonospora* spp. abundances in baits close to the sediment will be promoted by the large number of spores serving as growth centres, and the higher concentration of nutrients present in the sediment. The data on presence of hyphae are not entirely congruent with the *Micromonospora* abundances obtained by qPCR. The former had shown strongest

evidence for hyphae on baits closer to the surface; however, that experiment was performed in the autumn whereas the qPCR quantification was obtained in the following spring. Seasonal variations could explain this discrepancy, and both methods were in agreement in showing that *Micromonospora* growth was more prolific in the baits than in the water column itself.

Clostridium III relative abundances was very high in the sediments, reaching 40% of the total bacteria in Priest Pot, but an order of magnitude lower in the baits just above the sediment and negligible in the shallower baits. This is a further indication that complete anaerobiosis is only achieved in the sediments themselves, whereas in the water column sufficient oxygen is present to enable the growth of some aerobes even after the onset of the thermocline. Accumulation of clostridial spores in the sediments may have contributed to the high abundances observed there, however spores should have contributed less to total abundances than active cells because the analysis was performed on cDNA from ribosomal RNA extractions.

Most of the target microorganisms were detected in higher abundances in the baits compared to those numbers observed in the water column of the same depth. Fibrobacters and the anaerobic fungi were only detected in the baits and in the sediments.

The phylogeny of the lake *Clostridium* III clones obtained in this study showed that these were distinct from rumen strains, however no freshwater specific *Clostridium* III cluster was delineated. The fibrobacters found in the lakes on the other hand did form a distinct freshwater cluster, together with other sequences obtained by Dr. James McDonald, and represent a currently unrecognised component of the free living aquatic microbial community (McDonald *et al.*, in press). As all cultured *Fibrobacter* strains are cellulolytic, this new group could also have a role in cellulose turnover in lakes; this possibility is strengthened by the detection of these organisms in the cellulose baits and the organic matter-rich sediment samples only.

In conclusion, this study has provided the first insights into the relative contribution of different cellulolytic groups to the lake microbial community, in which *Micromonospora* spp. and *Clostridium* III could be major players. The micromonosporas isolated from the lakes are able to degrade cellulose in lakewater and are likely to play a role in its degradation in those habitats. The rumen organisms, *Fibrobacter* spp. and the anaerobic fungi (*Neocallimastigales*) are present in the lakes, and the *Fibrobacter* sequences obtained form a lineage distinct

from those found in the rumen and distinct from other members of the *Fibrobacteres* phylum found in other "gut" environments i.e. termites (Warnecke *et al.*, 2007).

One final observation from this study is that cotton bait enrichments are an effective method for attracting cellulolytic organisms, and could be used as a tool in the search for new cellulases from the environment.

Further research

The data presented here have shown the contribution of four groups of cellulolytic organisms to the microbial community in the lakes but have raised many new questions. The relative abundance of each of the targeted groups should be followed through a seasonal cycle; *Fibrobacter* spp. and the anaerobic fungi were present only as trace populations but their abundance may be higher at other periods of the year. *In-situ* cellulolytic activity has still to be determined in order to prove that *Micromonospora* and *Clostridium* III are indeed taking part in this process. Other groups of cellulolytic microorganisms can also play a role in cellulose degradation in aquatic environments, such as *Cytophaga* spp. (Nilsson *et al.*, 2008) *Cellvibrio* spp. (Berg *et al.*, 1968) *Pseudomonas* spp., *Cellulomonas* spp. (Ljungdahl & Eriksson, 1985) *Xanthomonas* spp. (Fong & Tan, 2000) and the hyphomycete fungi (Gulis & Suberkropp, 2003). Although these groups are known to contain cellulolytic members and have being associated with aquatic environments, with the exception of the hyphomycete fungi they are currently not recognised as freshwater cellulose degrading microorganisms. Their contribution to cellulose turnover in aquatic environments has to be determined in order to obtain a more complete picture of cellulose degradation in lakes.

The discovery of significant and active populations of the "aerobic" *Micromonospora* spp. below the thermocline raises the question of which other organisms are active in this microaerophilic environment, and could this represent an unrecognised compartment of lakes with an ecology distinct from the aerobic shallow layers and the strictly anaerobic sediment. The application of powerful metagenomic techniques fuelled by developments in pyrosequencing, will enable the structures of these microbial communites to be fully determined.

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Appendix 1 – de Menezes, AB; Lockhart RJ; Cox, MJ; Allison, HE; McCarthy AJ. Cellulose degradation by micromonosporas recovered from freshwater lakes and classification of these actinomycetes by DNA gyrase B gene sequencing. *Applied and Environmental Microbiology* 74: 7080-7084

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Cellulose Degradation by Micromonosporas Recovered from Freshwater Lakes and Classification of These Actinomycetes by DNA Gyrase B Gene Sequencing[▽]

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A number of *Micromonospora* strains isolated from the water column, sediment, and cellulose baits placed in freshwater lakes were shown to be able to degrade cellulose in lake water without any addition of nutrients. A selective isolation method was also developed to demonstrate that CFU arose from both spores and hyphae that inhabit the lake environment. Gyrase B gene sequencing performed on the isolates identified a number of new centers of variation within *Micromonospora*, but the most actively cellulolytic strains were recovered in a single cluster that equated with the type species of the genus, *M. chalcea*.

Cellulose, the most abundant form of fixed carbon in the biosphere, is insoluble and its efficient degradation is largely restricted to those specific groups of microorganisms that are able to produce multiple cellulases (14). Many microorganisms can attack amorphous cellulose to some degree, but relatively few taxa can completely degrade highly crystalline cellulose, of which the best example is cotton. In terrestrial environments, cellulose tends to be highly lignified and more difficult to degrade, and both fungi (14) and actinomycetes (15) can obtain access to cellulose in woody tissue due to their hyphal growth form. In contrast, little is known about the contributions of different groups of microorganisms to cellulose degradation in aquatic ecosystems.

Micromonosporas are a group of actinomycetes that are usually present in large numbers in soil but are well adapted to water dispersal, as evidenced by the absence of aerial hyphae and production of hydrophilic spores. They can be readily recovered from aquatic environments, especially lake sediments (4), but it is important that their presence is not equated with activity, for micromonosporas produce enormous numbers of spores that can accumulate and remain viable in lakes. Although they grow slowly and can be easily missed or outcompeted in culture-based surveys (27), the ability to degrade complex polysaccharides such as cellulose and chitin is regarded as common among the micromonosporas (12). Against this background, the study reported here concentrates on determining the presence, growth, diversity, and cellulolytic ability of *Micromonospora* strains colonizing cellulose baits placed in freshwater lakes.

Micromonosporas were recovered from two lakes of contrasting trophic status in the English Lake District: Esthwaite Water and Priest Pot. The former is 15 m in depth, 113 ha in area, and eutrophic (average chlorophyll *a* concentration, 54 μg liter^{-1}) (5). Priest Pot is 3.5 m deep, 1 ha in area, and is hypereutrophic (average chlorophyll *a* concentration, 300 μg liter^{-1}) (3). Stratification leading to the formation of an anaerobic zone occurs in the summer months in both lakes but is more persistent in Priest Pot, which is sheltered and has steep oxygen and temperature gradients. Cotton was dewaxed by repeated chloroform-ethanol extraction (29) in a Soxhlet apparatus (Quickfit; SciLabware, Stone, England) prior to use as baits for the recovery of cellulolytic microorganisms from the lakes. The baits (0.5 g) were placed in nylon mesh bags, tethered to ropes at different depths, and recovered at intervals of up to 3 months. Water was also sampled at two depths from both lakes using a Niskin bottle, and loose surface sediment samples were taken with a Jenkins sediment corer (21).

Micromonospora strains were isolated by vigorous physical shaking of the recovered baits in 10 ml phosphate-buffered saline (Sigma) followed by heat treatment of the suspension at 65°C for 10 min prior to plating in triplicate on M3 agar (17, 22). Sediment samples (20 ml) were serially diluted in sterile buffer before the heat treatment and plating on M3, while lake water samples (20 to 40 ml) were membrane filtered (0.2 μm pore size; Millipore) after heat treatment, and the membranes placed face down on M3 agar plates, incubated at 30°C and removed after 24 h. The M3 plates were incubated for a further 2 weeks, *Micromonospora* colonies were enumerated, and representative morphotypes were isolated and subcultured. *Micromonospora* colonies were identified by their appearance (small orange, red, brown or black colonies, finely branching substrate hyphae of <0.5 μm in diameter, and few or no aerial hyphae) verified by direct observation of colonies under a light microscope fitted with a 32× long-working-distance objective lens (Leitz, Wetzlar, Germany). A total of 72 isolates were obtained in this way, and these were routinely cultivated on oatmeal agar (24) plus 0.2% yeast extract with incubation at 30°C. Cultures were stored as suspensions of hyphae and spores in 12.5% (vol/vol) glycerol at −80°C.

Micromonospora numbers recovered from cellulose baits

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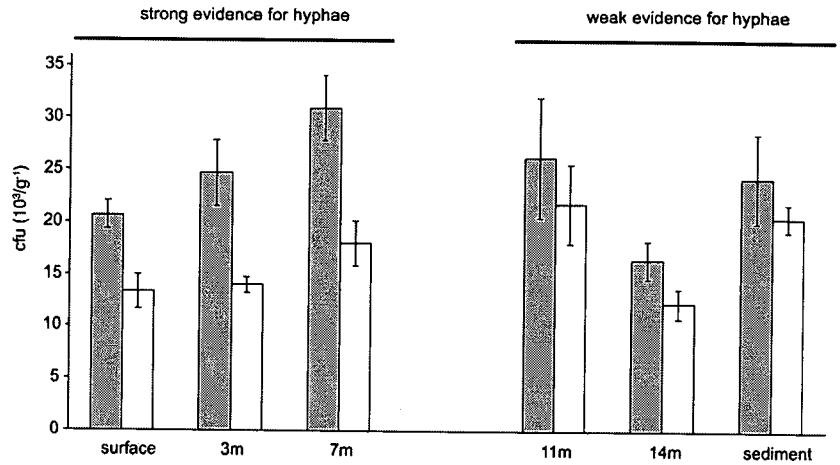

FIG. 1. Presence of hyphae in cellulose baits placed in Esthwaite Water. Gray and white columns represent numbers of micromonosporas recovered from the baits (10^3 CFU per g cellulose bait) before and after heat treatment, respectively. A decrease in CFU numbers after heat treatment is indicative of the presence of hyphae in the samples, which can be observed with strong confidence in the surface, 3-m, and 7-m samples. Baits placed deeper in the water column and sediment also showed evidence for the presence of hyphae, but this was not statistically significant.

varied between 2×10^3 and 1.2×10^4 CFU per bait (0.5 g cellulose). The numbers of micromonosporas recovered from the lake water itself were in the range of 1×10^3 to 1.3×10^4 CFU liter^{-1}, and those from sediment were in the range of 1.5×10^5 to 8.4×10^5 CFU g^{-1} wet weight. The numbers and distributions of micromonosporas were similar in each of the two lakes. In order to obtain evidence for the growth of micromonosporas in the lakes, a differential isolation method was developed based on that used by Wakisaka et al. (26), in which the antibiotic tunicamycin is incorporated in the M3 isolation agar at 10 μg ml^{-1} to inhibit the growth of gram-positive bacteria other than micromonosporas. This procedure enabled their isolation without the use of heat treatment, and by comparing CFU obtained, the relative numbers of micromonosporas from hyphal fragments and spores could be determined in any sample. Extracts were obtained from cellulose baits as described above, and 4.5 ml was added to 0.5 ml of 0.1 M NaOH on ice, neutralized with 0.5 ml of 0.1 M HCl after 5 min. This reduces the number of gram-negative bacteria (26). A 150-μl aliquot was plated on M3 agar containing 10 μg ml^{-1} tunicamycin. Heat treatment (65°C for 10 min) was used to destroy hyphae in the remaining aliquot (2 ml), 150 μl of which was also spread on M3 agar plates containing tunicamycin, as were 10^{-1} dilutions. The plates were incubated at 30°C for 15 days before identifying and counting the *Micromonospora* colonies. Sediment samples were diluted 10-fold in phosphate-buffered saline and processed as described above, and undiluted water column samples were filtered through 0.2-μm-pore-diameter membranes, which were placed face down on tunicamycin-containing M3 agar and removed after 24 h, after which the plates were returned to the incubator.

Strong evidence for the presence of *Micromonospora* hyphal fragments was obtained. The data in Fig. 1 show that the proportion of *Micromonospora* colony counts from hyphal fragments was higher in the baits closer to the surface and decreased with depth. Although there was evidence for hyphae in the baits suspended deep in the water column and at the sediment, the differences between total CFU numbers and those from *Micromonospora* spores were not statistically significant. Likewise, there was weak evidence for the presence of hyphae in surface waters themselves and in the sediment. In the deepest water column samples (7 and 14 m), micromonosporas were present only as spores (data not shown). This method is not ideal for quantifying absolute numbers of vegetative hyphae in the baits, but it certainly enables the conclusion that (i) micromonosporas will grow in lakes when particulate organic matter is available for attachment under aerobic conditions and (ii) the organisms are not simply present as spores washed in from the surrounding soil.

Scanning electron microscopy (SEM) was used to observe the extent of physical breakdown of cellulose by micromonosporas in lake water. A selection of 17 strains were incubated at 30°C in 20-ml universal bottles containing 4 ml of sterile lake water with dewaxed cotton (0.2 g), the degradation of which was examined by SEM. Samples were dehydrated in ethanol, critical point dried, and coated with 60% Au/Pd in a Polaron E5100 sputter coater. Degraded cotton samples were examined using a Phillips XL 30 scanning electron microscope with an accelerating voltage of 10 to 15 kV. SEM revealed different levels of degradation; colonized cellulose strands sometimes only showed superficial degradation (Fig. 2A). Often, however, the strands were penetrated and degraded from the interior, leaving an outside "shell" almost intact (Fig. 2B). Scanning electron micrographs of baits recovered from the lakes themselves revealed two main degradation patterns: hollow, similar to that observed above with *Micromonospora* pure cultures (Fig. 2C); and in the form of pits in the fiber surface, which appeared to be due to erosion by unicellular bacteria (Fig. 2D).

Endoglucanase production was determined by growing *Micromonospora* isolates in minimal salts medium (16) supplemented with 1% fibrous filter paper powder (Whatman, Maidstone, United Kingdom) and then measuring reducing sugars released from carboxymethylcellulose incubated with culture supernatants. The enzyme assay procedure was modified from

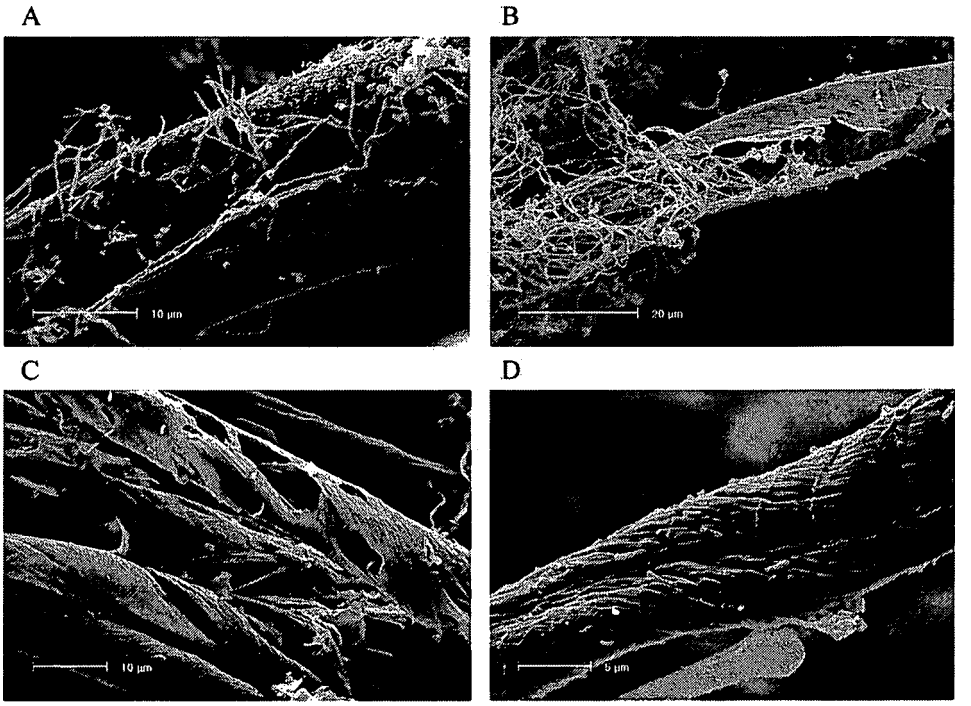

FIG. 2. Scanning electron micrographs of cellulose degradation patterns produced by *Micromonospora* strains (A and B) and by natural microbial populations in the lakes Priest Pot(C) and Esthwaite Water (D).

that described previously (16). One milliliter of culture supernatant, diluted appropriately in 0.1 M potassium phosphate buffer (pH 7), was added to 2 ml of 5% (wt/vol) low-viscosity carboxymethylcellulose (Sigma) dissolved in the same buffer and incubated for 1 h at 50°C with shaking at 170 rpm. Substrate and enzyme controls were used for every sample. Reducing sugars were determined by the dinitrosalicylic acid method (19). Enzyme activity was expressed as units, each unit corresponding to 1 μmol of glucose equivalents released per minute. Protein was measured in the culture supernatant and biomass extracts using the Bradford method (2). For the determination of biomass, the culture was centrifuged and cell pellets were resuspended in 1 M NaOH and boiled for 20 min. Cell debris was removed by centrifugation prior to determination of the protein concentration. In order to assess levels of activity on crystalline cellulose, the strains were incubated in test tubes containing filter paper strips and M3 plus 0.4% yeast extract to a depth of ca. 20% of the tube. Degradation was assessed by visual examination after 1 month at 30°C.

The best eight strains included the type strain of *Micromonospora chalcea* and produced very high levels of endoglucanase (240 to 800 U liter^{-1}), and all extensively degraded filter paper. When incubated in minimal salts medium supplemented with 0.4% yeast extract for 6 weeks at 30°C, these strains almost completely solubilized the cellulose baits (ca. 0.2 g). The levels of enzyme activity produced per unit of biomass (cell pellet protein) indicated that these highly cellulolytic strains were not simply those with the highest growth yields. A second group of 17 isolates produced intermediate levels of endoglucanase activity (42 to 169 U liter^{-1}), but only two of these strains degraded filter paper to any great extent, with the majority producing little or no evidence of degradation. The remaining 48 isolates were poor producers of endoglucanase activity, and none of these strains degraded filter paper to a significant extent.

The gyrase B gene was chosen as a phylogenetic marker for the isolates, as the 16S rRNA gene sequence does not resolve species structure in the genus *Micromonospora* (11), which was confirmed by our 16S rRNA gene sequence data for 20 of the isolates. Prior to sequencing, restriction fragment length polymorphism analysis of the amplified *gyrB* gene fragments (1.18 kbp) was performed in order to group the isolates and to select a subset of strains for sequence determination. The strains were grown in yeast extract-malt extract broth (10 g glucose, 3 g yeast extract, 3 g malt extract, 5 g peptone) at 30°C with shaking for 10 days. The biomass was harvested by centrifugation, and total DNA was extracted using a DNA extraction kit (Mo Bio UltraClean). PCR amplification of the *gyrB* gene was performed using *gyrB* universal bacterial primers UP1TL and UP2rTL (11). The amplification products from 67 of the 72 *Micromonospora* isolates were concentrated and partially purified by ethanol-sodium acetate precipitation (23) and incubated with BstNI restriction enzyme overnight at 60°C. The products were run on a 2% (wt/vol) agarose gel containing ethidium bromide (0.5 μg ml^{-1}) for 2 h and examined using a transilluminator. This enabled recognition of seven distinguishable restriction fragment length polymorphism patterns, and multiple examples of each were sequenced.

The amplification products were gel extracted using a QIAprep spin Miniprep kit and cloned using the Promega pGEM-T Easy vector plasmid cloning kit. The plasmids with inserts were extracted using the Eppendorf Perfectprep plas-

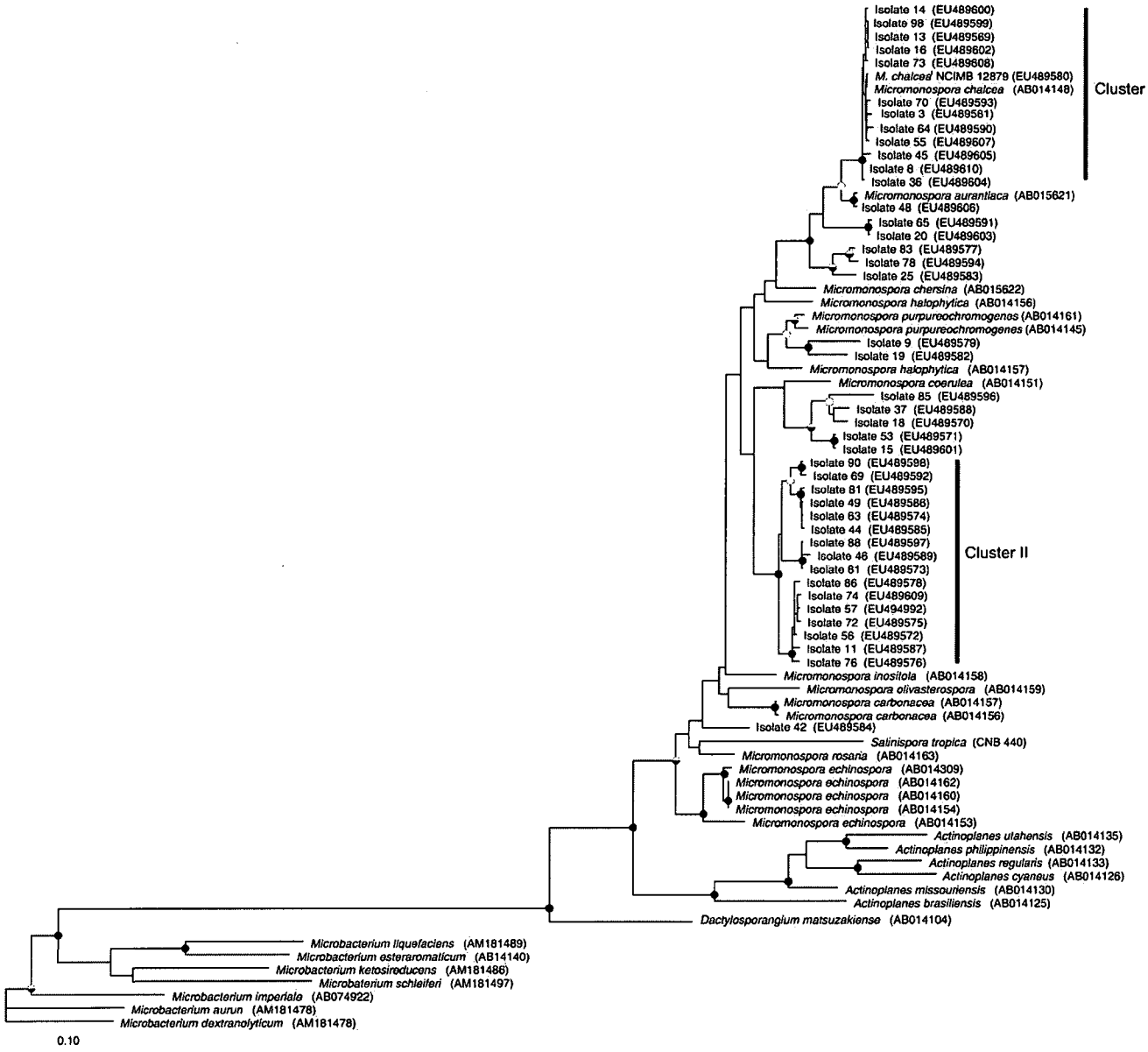

FIG. 3. Maximum likelihood tree constructed with *gyrB* gene sequences from *Micromonospora* isolates, named strains, and other members of the *Micromonosporaceae*. The tree topology generated by both maximum likelihood and maximum parsimony methods was strongly supported by bootstrap values which were similar for both methods. The bootstrap values are represented by the circles in the branch nodes: filled circles, bootstrap values of >95% for both methods; half-filled circles, bootstrap values of >95% by only one method; open circles, bootstrap values of 75 to 95% for both methods. The tree was rooted with *Microbacterium* spp. as the outgroup; sequence accession numbers are in parentheses at the end of each branch. The scale represents 0.1 substitution per nucleotide position.

mid isolation kit, and insert sequences were determined by GATC Biotech (Constance, Germany). Forward and reverse *gyrB* gene sequences of 42 *Micromonospora* strains and that of the type strain of *M. chalcea* NCIMB 12879 were assembled together into contigs (1,180 bp) using the Pregap4 and Gap4 programs of the Staden package (25), and accuracy of the assembled traces was manually checked. Sequences in GenBank that were closely related to experimental sequences were identified using BlastN (1), and these sequences were aligned using ARB software (beta v. 2003-08-22) (13). The positions corresponding to primer sequences, gap columns, and hypervariable bases were removed, leaving a final alignment of 1,151 bp covering the region 314 to 1486 of the *gyrB* sequence of *Escherichia coli* K-12. The optimized alignment was exported, and a maximum likelihood tree was constructed using PhyML (v. 2.4.4) (6), with 100 bootstrap samplings. Phylip DNAPARS was run in ARB in order to calculate a maximum parsimony tree with vertical gap compression, random sequence order, and 100 bootstrap samplings. Tree topologies and bootstrap support for branching were compared between the two trees

and found to be similar, and the maximum likelihood tree is presented in Fig. 3. All of those isolates that exhibited high cellulolytic activity were recovered in cluster I, as well as the two *gyrB M. chalcea* sequences: one from the type strain cultured in the laboratory and the other downloaded from the GenBank database. A second major cluster (II) containing 16 strains was delineated, but it did not include any named species from the database and may represent a novel center of variation within the genus. Other strains were related to *Micromonspora coerulea* and *Micromonospora purpureochromogenes*, whereas isolates 48, 65, 20, 83, 78, and 25 branched close to but not within the *Micromonospora aurantiaca* and *M. chalcea* clusters. *Micromonospora* strain 42 was not closely associated with any named species or any other lake isolate.

There are few studies on the ecology of micromonosporas in lakes to which the data presented here can be compared. Most of the information on the occurrence of *Micromonospora* spp. in aquatic habitats was published over 20 years ago (4, 10, 12). The presence of micromonosporas along with other actinomycetes and their interaction with fungi in submerged macrophytes and decomposing leaf matter have been investigated in some more recent studies (18, 27, 28), whereas others have focused on the diversity and isolation of members of the *Micromonosporaceae* from different habitats such as marine sediments and Antarctic rocks (7–9, 20). None of these studies have addressed the potential role of micromonosporas as degraders of particulate organic matter in the aquatic environment, and there have been no previous attempts to relate function to species identity.

The data presented here show that micromonosporas are part of the indigenous cellulolytic community in freshwater lakes. The importance of micromonosporas in cellulose degradation in lakes relative to other aerobic microorganisms remains to be determined. Recognition that micromonosporas can be truly aquatic actinomycetes of freshwater lakes may be added to the relatively recent classification of certain members of the *Micromonosporaceae* as marine microorganisms and the suggestion that they are the first actinomycetes to be identified as such (20).

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Appendix 2- McDonald, JE; de Menezes, AB; Allison, HE; McCarthy AJ. Molecular biological detection and quantification of novel *Fibrobacter* populations in freshwater lakes. *Applied and Environmental Microbiology*, in press.

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Molecular Biological Detection and Quantification of Novel *Fibrobacter* Populations in Freshwater Lakes[▽]

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PCR and quantitative PCR (qPCR) primers targeting the 16S rRNA gene were used to detect and quantify members of the genus *Fibrobacter* in lake water, sediment and colonized cotton taken from two freshwater lakes. Phylogenetic analysis identified two groups of sequences; those clustered with *Fibrobacter succinogenes*, the type species, and a defined cluster of clones loosely associated with several *Fibrobacter* sequences observed previously in clone libraries from freshwater environments. 16S rRNA gene sequences recovered in the same way from soil samples and ovine feces in the surrounding land were all *F. succinogenes* and did not include any from this group of the "freshwater" *Fibrobacteres*. In all cases, nested PCR was required to detect *Fibrobacter* 16S rRNA genes, and qPCR analysis of reverse transcribed bacterial community RNA confirmed their very low relative abundance on colonized cotton baits in the water column (at 0, 3, 7, 11, and 13 m) and on the sediment surface (<0.02% of total bacterial rRNA). However, in Esthwaite Water sediment itself, the relative abundance of fibrobacters was 2 orders of magnitude higher (ca. 1% of total bacterial rRNA). The presence of fibrobacters, including the cellulolytic rumen species *F. succinogenes*, on colonized cellulose samples and in lake sediment suggests that these organisms may contribute to the primary degradation of plant and algal biomass in freshwater lake ecosystems.

Cellulose is the most abundant organic polymer on Earth, and its decomposition under anoxic conditions in freshwater, marine, and estuarine sediments is an essential process in the global carbon cycle. The complete decomposition of lignocellulosic material under anaerobic conditions is mediated by a metabolically diverse microbial community comprised of several trophic groups of microorganisms (16). However, the composition of the anaerobic cellulolytic population in freshwater lakes is barely understood, and our knowledge of their identity is restricted to a small number of isolated strains belonging to the genus *Clostridium* (17, 25, 36).

Both molecular and culture-based studies have suggested that *Clostridium* spp. are likely to be the predominant cellulolytic microorganisms in anoxic environments, such as landfill sites (32) and freshwater sediments (17). Conversely, it is well established that cellulose hydrolysis in the herbivore gut is due largely to members of the bacterial genus *Fibrobacter* and the anaerobic fungal order *Neocallimastigales* (14, 19), and the distribution of both groups had been thought to be restricted entirely to the gut environment. Recently anaerobic fungi (*Neocallimastigales*) were detected in a cellulose-rich landfill site using molecular biological methods (20). The presence of *Fibrobacter* spp. was inferred from the analysis of DNA extracted from a number of municipal waste sites using genus-specific primers, with quantitative reverse transcriptase PCR (qRT-PCR) data demonstrating that they can comprise a significant proportion of the landfill microbial community (23). It is possible that *Fibrobacter* spp. occupy other terrestrial and aquatic habitats where cellulose is degraded under anoxic conditions, such as freshwater and marine sediments, anaerobic sludge, and waterlogged soils. Furthermore, since all cultured strains assigned to the genus *Fibrobacter* thus far are capable of cellulose hydrolysis (1, 24), their detection in these environments suggests a role for *Fibrobacter* spp. in cellulose hydrolysis beyond the herbivore gut.

The genus *Fibrobacter* contains two cultivated species, *F. succinogenes* and *F. intestinalis*, and these organisms have long been thought to exclusively occupy gut environments (24). Direct molecular ecological approaches have provided evidence for these and new lineages within *Fibrobacter*, but the taxonomic limits of the genus have been questioned (1). Recently *Fibrobacter*-related sequences were described for termite gut environments, and these sequences have been assigned to a subphylum of the *Fibrobacteres* (13). There are currently two subphyla in the *Fibrobacteres* lineage, and clone sequences detected in termite gut environments have been assigned to *Fibrobacteres* subphylum 2 (13), with representatives of the genus *Fibrobacter* classified as *Fibrobacteres* subphylum 1. The genus *Fibrobacter*, as currently defined, therefore encompasses only the two cultivated "gut" species *F. succinogenes* and *F. intestinalis* and those related sequences obtained from landfill sites (23).

A small number of sequences belonging to the *Fibrobacteres* phylum or described as *Fibrobacter* related have been reported in general bacterial 16S rRNA gene clone libraries generated from geographically and geochemically distinct freshwater lake samples (3, 10, 18, 22, 27). These data demonstrate that while novel *Fibrobacter*-related populations are present in freshwater lakes, their poor representation in clone libraries suggests a negligible contribution to the freshwater microbial community.

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TABLE 1. PCR primer sets targeting the 16S rRNA gene

Primer set	*E. coli* numbering	Sequence (5′–3′)[a]	Specificity	Annealing temp (°C)	Amplicon size (bp)	Reference
pA	8–28	AGAGTTTGATCCTGGCTCAG	General bacteria	55	~1,534	9
pH′	1522–1542	AAGGAGGTGATCCAGCCGCA	General bacteria			9
Fib1F	153–168	CCGKSCCAACGSSCGG	*Fibrobacter* genus	60	~855	23
Fib2AR	998–1017	ATCTCTCGCYGCGGCGWTYCC	*Fibrobacter* genus			23
1369F	1369–1386	CGGTGAATACGTTCYCGG	General bacteria	60[b]	~141	28
Prok 1492R	1492–1510	GGWTACCTTGTTACGACTT	General bacteria			28
FibroQ153F	153–172	CCGKSCCAACGSSCGGHTAA	*Fibrobacter* genus	60[b]	~104	23
FibroQ238R	238–257	CSCCWACTRGYTAATCRGAC	*Fibrobacter* genus			23

[a] Ambiguities: K = G or T; S = G or C; W = A or T; Y = C or T; H = A, C, or T; R = A or G.
[b] The QuantiFast SYBR green PCR assay (Qiagen) uses the same annealing temperature (60°C) for all primer sets.

However, *Fibrobacter* sequences are frequently underrepresented in general 16S rRNA gene and ribosomal intergenic spacer clone libraries generated from rumen samples, an environment where they are known to predominate (5, 15, 29–31, 34). Therefore, the importance of *Fibrobacter* spp. in aquatic ecosystems has yet to be properly addressed and may have been underestimated.

Here, genus-specific PCR primers targeting the 16S rRNA gene of *Fibrobacter* spp. were applied to DNA extracted from colonized cotton samples, membrane-filtered lake water, and sediment samples from two productive lakes in the English Lake District. The relative abundance of *Fibrobacter* spp. within the samples was determined by qRT-PCR, and this is important because simple PCR-based detection of fibrobacters in the freshwater lake environment could merely be indicative of runoff from land grazed by ruminant animals.

Freshwater lake samples were obtained from Esthwaite Water and Priest Pot, situated in the English Lake District, United Kingdom. Esthwaite Water is 2.4 km in length and ca. 16 m deep. It is regarded as one of the most productive lakes in the area and is eutrophic. Priest Pot is a small, hypereutrophic lake with a depth of around 3 m and 1 ha in area. Three types of sample were obtained from Esthwaite Water in November 2007 and June 2008. Dewaxed cotton string (35) in nylon mesh bags was suspended on a nylon rope at various depths within the water column (surface, 3 m, 7 m, 11 m, 13 m, and sediment of Esthwaite Water; surface, 0.5 m, 1 m, 2 m, and sediment of Priest Pot) (6). The cotton samples were retrieved after 1 month's residence within the lakes. A Freedinger apparatus was used to sample 1-liter volumes of water at depths equivalent to those of the cotton baits, and sediment samples were obtained using a Jenkin corer (26).

The oxygen concentration and temperature of the water column were measured using a YSI 85 dissolved-oxygen, conductivity, salinity, and temperature meter. Samples of waterlogged soil and ovine feces from the fields adjacent to the lakes were also obtained. Immediately after removal from the lake, colonized cotton and sediment samples were frozen on dry ice and stored at −80°C until use. Water samples were stored in an insulated bag for transport to the laboratory and immediately filtered through sterile Supor200 (0.2-μm pore diameter) membranes (PALL Life Sciences), which were subsequently stored at −80°C.

For DNA and RNA coextraction, an entire 0.2-μm (pore diameter) membrane filter (through which 1 liter of lake water was filtered) or 0.5 g of waterlogged soil, ovine feces, colonized cellulose, or lake sediment was subjected to the nucleic acid extraction method of Griffiths et al. (11). The method is based on the use of hexadecyltrimethylammonium bromide and phenol-chloroform-isoamyl alcohol extraction with mechanical bead beating. Extracted nucleic acid from each sample was resuspended in 50 μl nuclease-free water and divided into two 25-μl aliquots. One aliquot was treated with RNase A (Sigma) as described previously (11) for use as a community DNA sample. The second aliquot was DNase treated using a Turbo DNA-free kit (Ambion) for use as a community RNA sample. Extracted RNA (0.4 μg) was reverse transcribed using BioScript RNase H Low (Bioline), following the manufacturer's protocol. These processed DNA and cDNA samples were stored at −80°C until required.

Direct and nested PCRs were performed on community DNA for each environmental sample (50 ng DNA per PCR assay) using Fib1 and Fib2A as the primer set (Table 1) and Super*Taq* DNA polymerase as previously described (23). *Fibrobacter* spp. could only be detected in lake samples via nested PCR (Fig. 1) in which an initial round of amplification with the general bacterial primer set, pA and pH′, was followed by a second round of amplification with the *Fibrobacter* genus-specific primer set (Fib1 and Fib2A).

Fibrobacters were detected in water, colonized cotton, and sediment samples in the lower depths of Esthwaite Water and Priest Pot but were more readily detected in colonized cotton samples than in lake water, suggesting that they are members of the biofilm community on particulate organic matter in the water column (Fig. 1). Priest Pot has a depth of 3 m, and fibrobacters were detected only at depths of 1 m and 2 m and in sediment (3 m) colonized cotton samples but not from cotton baits moored at the surface and at a 0.5-m depth. *Fibrobacter* spp. were detected in Esthwaite Water only between a depth of 7 m and the sediment, where the percentage of oxygen saturation varied from 67% at 7 m to 3% in the sediment (Fig. 1). Their restriction to the more anoxic regions of the water column is consistent with the obligately anaerobic physiology of all known cultured members of this genus. Amplification products were cloned into *Escherichia coli* JM109 cells, and a total of 46 clones were sequenced by Macrogen Inc. (Seoul, South Korea) in both orientations to obtain coverage of the entire clone sequence. Analysis by both the RDP (4) and Pintail (2) chimera detection software packages demonstrated that none of the sequences were chimeric.

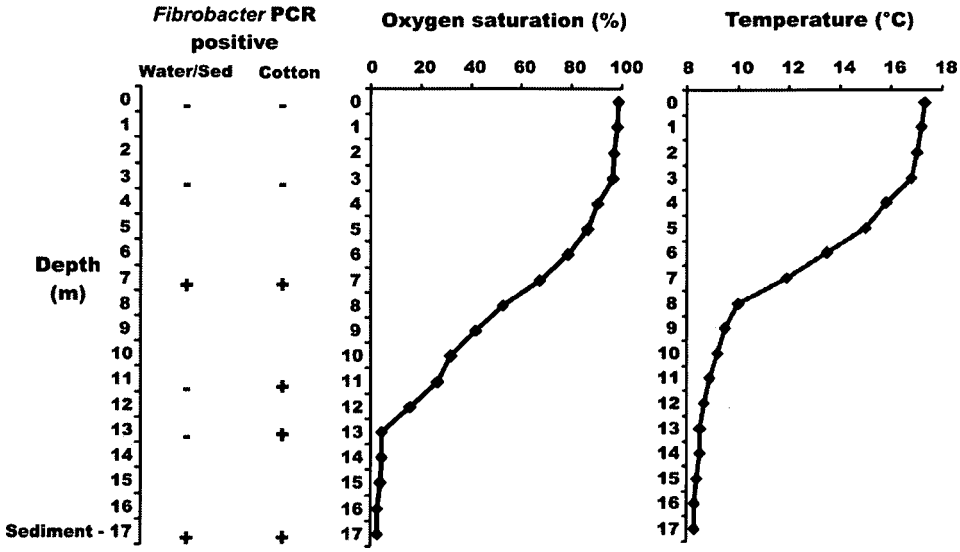

FIG. 1. Data on the nested PCR amplification of community DNA extracted from colonized cotton, membrane-filtered lake water, and sediment samples using the *Fibrobacter* genus-specific 16S rRNA gene primer set (Fib1F and Fib2AR). The 17-m-depth sample represents the lake sediment, and 0- to 13-m samples represent the water column. Percentage oxygen saturation and temperature readings of the water column and sediment of Esthwaite Water in the English Lake District were also measured. +, nested PCR amplification product obtained; −, no PCR amplification observed; Sed, lake sediment sample.

Phylogenetic analysis of *Fibrobacter* amplification products obtained using the Fib1 and Fib2A primer set demonstrated the presence of both *F. succinogenes*-like and novel *Fibrobacter* sequences in lake samples (Fig. 2). Of 19 clones obtained from colonized cotton samples from both Esthwaite Water and Priest Pot, only 3 clones were affiliated with the cultivated rumen species *F. succinogenes*, while the remaining 16 formed a distinct lineage with bootstrap support of >95% (Fig. 2). Six clone sequences from this cluster (PP-C-2.0-1 to PP-C-2.0-6) were obtained from reverse-transcribed community RNA extracted from colonized cotton at a depth of 2 m in the Priest Pot water column, demonstrating that these organisms were likely to be metabolically active on the colonized cellulosic material. These clones were related to but distinct from *Fibrobacteres* sequences obtained from geographically distinct freshwater environments in other studies (3, 27) (Fig. 2). The branching pattern within this freshwater *Fibrobacter* lineage was supported by high bootstrap values (>95%), suggesting that although this cluster represents a related group of organisms, there are also new taxa within the lineage (Fig. 2).

16S rRNA gene amplification products from ovine feces and soils from the adjacent fields in which sheep were grazing were included to provide evidence that the detection of fibrobacters was not due simply to runoff and/or fecal contamination from the surrounding land. All clone sequences obtained from ovine feces were restricted to a distinct lineage that contained *F. succinogenes* strain MC1, an isolate from the ovine rumen (1) (Fig. 2).

Nine of the 10 sequences obtained from soil samples clustered with the type strain *F. succinogenes*; the remaining clone was clustered with a group of sequences previously detected in landfill (Fig. 2).

Phylogenetic analysis of cloned *Fibrobacter* DNA amplification products from freshwater lakes has demonstrated that in addition to *F. succinogenes*-like sequences, novel *Fibrobacter* spp. are present. This confirms that fibrobacters truly inhabit lakes and are not present due to fecal runoff. Sequence analysis of *Fibrobacter*-specific amplification products from a range of samples has demonstrated a surprising diversity of *Fibrobacter* spp. The assignment of novel lake *Fibrobacter* clones to both new and existing lineages supports our suggestion that fibrobacters are widely distributed in terrestrial and aquatic ecosystems and are not restricted to the gut environment.

Reverse-transcribed community RNA, extracted from samples of six colonized cotton baits and one lake sediment core obtained in June 2008, was subjected to quantitative PCR in triplicate using the 1369F/Prok1492R (general bacterial) and FibroQ153F/FibroQ258R (*Fibrobacter* genus-specific) primer sets (Table 1), as described previously (23). Insufficient RNA could be extracted from lake water samples for qRT-PCR analysis. The relative abundance of *Fibrobacter* spp. in comparison to total bacterial RNA varied from 0.005 to 0.02% on colonized cotton samples throughout the water column of Esthwaite Water (at 0, 3, 7, 11, and 13 m) but ca. 1% in the sediment. The relative abundance of *Fibrobacter* spp. on colonized cotton samples in the water column increased with depth, but the absolute values for relative abundance were always very low (<0.02%). The *Fibrobacter* abundance value for sediment is comparable to quantitative-PCR-derived data for landfill sites in a previous study (23), suggesting that fibrobacters are members of the indigenous lake microbial community, particularly in the more anoxic regions of the lake ecosystem.

The failure to amplify 16S rRNA genes from fibrobacters other than *F. succinogenes* in ovine feces samples here, and previously in bovine rumen samples (23), suggests that this group exhibits restricted diversity in the herbivore gut and is perhaps strongly niche adapted compared to environments

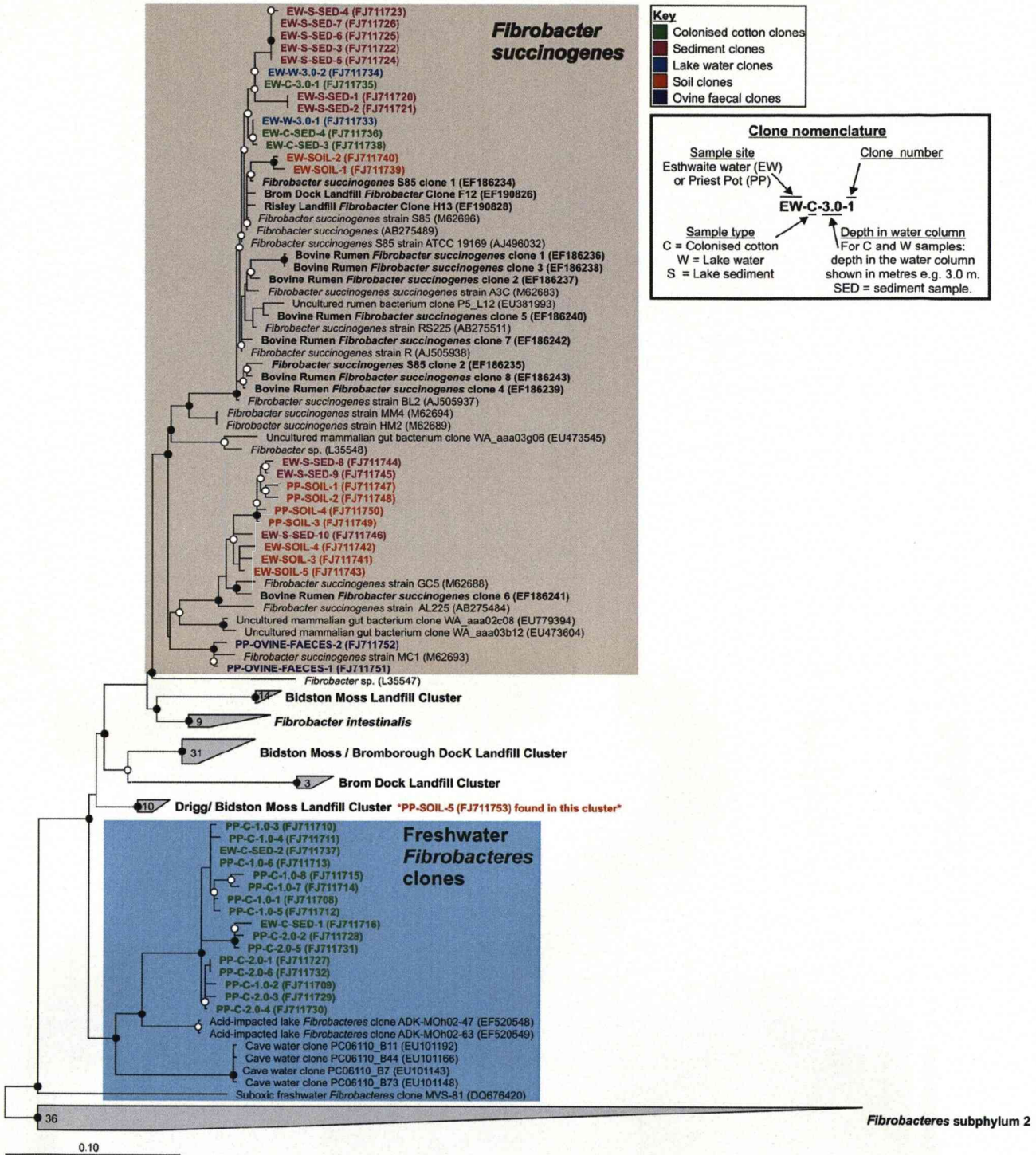

FIG. 2. Maximum-likelihood tree of 16S rRNA gene sequences amplified from lake water, sediment, and colonized cotton samples using the *Fibrobacter* genus-specific Fib1 and Fib2A PCR primer set. Contiguous sequences were aligned with their three nearest neighbor sequences from both cultured and uncultured isolates using the Greengenes NAST aligner (7, 8), in addition to a number of representatives from other bacterial phyla. The alignment was imported into the Arb software program (21) and manually optimized. A final alignment corresponding to *E. coli* 16S rRNA gene positions 153 to 1017 was exported from Arb, and a maximum-likelihood tree was constructed by PhyML online (12) using the HKY85 substitution model and the Shimodaira-Hasegawa (SH)-like aLRT branch support method. The tree was imported back into Arb, where non-*Fibrobacteres* sequences were omitted from the tree to better demonstrate the phylogeny of the *Fibrobacteres* phylum. *Fibrobacteres* subphylum 2 contains sequences drawn from previous studies of the termite gut (13, 33). Filled circles indicate those nodes at which a bootstrap value of >95% was observed, and unfilled circles denote nodes with bootstrap values between 75 and 95%. The accession number of each sequence is displayed in parentheses alongside the sequence. Numbers shown on collapsed branches indicate the number of sequences within the branch. Sequences shown in bold were amplified using the Fib1 and Fib2A primer set applied in this study. The scale bar represents 0.1 base substitution per nucleotide.

such as freshwater lakes and landfill sites. Consequently, fibrobacters in environments beyond the rumen may be a source of novel cellulases and hemicellulases, which are important in many industrial applications, for example, in second-generation biofuel production. Molecular biological approaches for the identification of novel cellulases have already revealed the presence of such enzymes in the termite gut (33), and metagenomic analyses of lake sediment and landfill samples could be similarly fruitful.

Nucleotide sequence accession numbers. The sequence data obtained in this work have been submitted to the GenBank database under accession numbers FJ711708 to FJ711753.

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