THE UNIVERSITY OF HULL

A multi-disciplinary approach to the characterization of waterlogged burial environments: Assessing the potential for the *in situ* preservation of organic archaeological remains

being a thesis submitted for the Degree of Doctor of Philosophy

in the University of Hull

by

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October 2007

Dedication

I would like to dedicate this thesis to my parents and my sister Rosario for giving me support and encouragement, even during difficult times in Spain.

Summary of thesis submitted for the degree of Doctor of Philosophy

by

Isabel Douterelo Soler

on

A multi-disciplinary approach to the characterization of waterlogged burial environments. Assessing the potential for the *in situ* preservation of organic archaeological remains

The aim of this study was to characterise waterlogged burial environments and to assess their potential for the *in situ* preservation of organic archaeological remains. To characterise these environments, environmental parameters were monitored through the soil profile and integrated with a study of the composition and activity of the microbial community.

Soil cores were taken from two wetland sites located in the Humberhead Levels in Yorkshire: Hatfield Moor and Sutton Common. Cores were subsampled at depth intervals down to 100 cm depth, to allow for the examination of the vertical distributions of the variables being studied. Redox potential, water level variation and other physico-chemical parameters were measured down the soil profile. Bacterial abundance was determined by direct counts; activity was assayed by extracellular enzyme activity and leucine assimilation. The physiological profile of the microbial community was analysed using BIOLOG and the bacterial community structure was examined by PCR-DGGE.

Redox potential readings were positive above the water table and negative below. The bacterial abundance and activity were greatest at the soil surface and, in general, decreased with depth. BIOLOG showed both depth variation and between site-variation in microbial physiological profile. DGGE gels presented a different bacterial community structure with depth and between-sites.

The results from monitoring of redox potential combined with water table height and determination of bacterial abundance and activity allowed the recognition of stratigraphic horizon where there was less potential for microbial degradation of organic archaeological artefacts. The information from BIOLOG and DGGE holds the potential for the development of a more subtle understanding of between-depth and between-site differences in the degradation process.

The physico-chemical and the conventional and molecular microbiological results presented in this thesis have shown that microbial activity is implicated as a key factor that could lead to compromised *in situ* preservation conditions at the sites studied.

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Acknowledgements

I would like to thank to my supervisors, Dr. Malcolm Lillie, Dr. Ray Goulder and Dr. Robert Smith for their support and guidance throughout this research.

I would also like to express my gratitude to Dr. Malcolm Lillie who was instrumental in generating the funding for this research, and English Heritage (EH) and the (former) Countryside Agency (CA) and the University of Hull for providing sponsorship. In particular Ian Panter (EH) and Stuart Pasley (CA) are thanked for assisting in the generation of funding. In addition, Ian Panter is thanked for his initial guidance during the development of this project. Further thanks are extended to the Department of Geography at the University of Hull.

I am especially grateful to Javier Montero Pau for his help in the laboratory and for his support and friendship. Thanks to Dr. Thorid Zierold for helping me with the cloning and sequencing analysis and to Dr. Helen Wilcock and Dr. Zahra Mahmoud for their invaluable help with the problems that I encountered with PCR. Dr. James Cheetham for his advice with the microbial techniques and to Dr. David Lunt for his help and advice with the molecular work.

Warmest thanks are expressed to my friends, including Dr. Thomas Breithaupt and Gabi Breithaupt (-my family in Hull), Dr. Africa Gómez, Sergi Campillo, Dr. Nora Bermingham, Dr. Chiara Zennaro, Maria Elena Mannarelli, Dr. Richard Case; all of whom provided continual support and encouragement during the difficult times in both the laboratory and in my personal life in Hull.

Finally I would like to express my gratitude to my friend Begoña Souto, my family, (especially my Mum and Dad) and to my husband Leonardo Manjarres, without whose understanding and support this work would not have been completed.

Chapter 1 Introduction

1.1 Introduction

The world's wetlands contain organic material that is fundamental to our understanding of past human-landscape, and socio-cultural interactions. Waterlogged deposits have the potential to expand our understanding of the past in a way that is seldom possible from the excavation of 'dryland' sites alone (e.g. Lillie and Ellis 2007). It is well established that wetlands preserve organic archaeological remains because they tend to be highly reducing environments, within which many of the biochemical reactions involved in the decomposition process are suppressed (Caple, 1996; Corfield, 1996; Raiswell, 2001; Van de Noort *et al.*, 2001). However, the world's wetlands are an extremely fragile and threatened resource, and recent research has shown that wetland losses are sufficiently serious to cause considerable concern about their sustainability (e.g. Mitsch and Gosslink, 1993; Coles and Olivier, 2001; Lillie and Ellis, 2007).

The significance of the culture-historic component of wetlands has been recognised in the recent past with the funding of four major surveys of England's Wetlands by English Heritage (e.g. Coles and Coles, 1986; Hall and Coles, 1994; Middleton and Wells, 1990; Ellis *et al.*, 2001). Despite these regional surveys, the ongoing discovery of new archaeological sites in wetlands generally coincides with a lack of funds for excavation, and a lack of finance and space for the adequate preservation, conservation and storage of the archive from excavations. Consequently, in 1991, a document entitled 'Planning and Policy Guideline-Archaeology and Planning' (PPG16) from the Department of the Environment, established that preservation *in*

situ was the preferred option for dealing with archaeology affected by development, and by implication, the majority of waterlogged archaeological remains in England. Therefore, it is essential that archaeological scientists gain a better understanding of the burial environment in which archaeological remains are preserved, in order to improve the management and preservation of these sites (Powell *et al.*, 2001).

The research described in this thesis aims to study a number of environmental variables in two wetland sites located within the Humber Wetlands region of England (i.e. Hatfield Moor and Sutton Common), as defined by the English Heritage funded Humber Wetlands Survey (e.g. Ellis *et al.*, 2001). Both Hatfield Moor and Sutton Common contain sensitive, waterlogged archaeological remains. The environmental status of these remains is assessed using a number of different parameters; with a focus on the quantitative and qualitative changes in the activity and composition of microbial communities within the soils obtained from the two sites.

This study primarily focuses on bacteria as these are primarily responsible for organic decomposition under oxygen-limited conditions such as those encountered in waterlogged deposits (Caple, 1994). To characterise these environments, different environmental variables were monitored through the soil profile. Two of the key variables influencing organic decay are the height of the water table (i.e. level of saturation) and associated redox potential values (Eh) (Caple 1996, Cheetham 2004). Variation in water table height is important because the presence of water-saturated conditions help to maintain an anaerobic environment in the soil. This leads to the inhibition of the aerobic microorganisms responsible for organic decay to grow (i.e. bacteria and fungi); thereby allowing the long term preservation of important

archaeological remains (Corfield, 1996; Björdal and Nilsson, 2002). Redox potential provides an indirect measure of the oxidation/reduction potential of the burial environment, and allows insights into the potential for the *in situ* preservation of archaeological remains.

1.2 Archaeology and the wetland context

Wetlands can be defined as land where an excess of water determines the nature of the soil and its fauna and flora (Cowardin *et al.*, 1979; Mitsch and Gosselink, 1993). The unique ecological value that wetlands possess is closely linked to their transitional state between terrestrial and aquatic systems. Wetlands tend to have high primary productivity levels (Nicholas, 2007:46), and it is precisely this productivity which made wetlands attractive to past human groups, resulting in the deposition of cultural remains within them.

Unfortunately, from an archaeological perspective, the term 'wetland' can be somewhat confusing. Numerous archaeological sites in Europe are in areas which were formally wetlands, but which have been drained in the historical past, and as a consequence, these would not necessarily be recognized as wetland areas in the present. A useful example of a drained wetland landscape is the Fens, located in Eastern England; this is currently an area where arable farming regimes dominate the landscape, but where archaeology survives below the water table due to the existence of waterlogged conditions in the past (Corfield, 1996; Pryor, 2007).

In general, under waterlogged conditions, oxygen exclusion affords a degree of protection from degradation of the organic archaeological component, because the

activity of the aerobic microorganisms involved in biodegradation is inhibited, and as a consequence, the decay process is slowed down (Caple and Dungworth, 1997). Freeman *et al.* (2001, 2004) have proposed that the low levels of biodegradation in peatlands are largely due to an oxygen limitation effect on phenol oxidase activity. Limiting phenol oxidase activity allows for the accumulation of phenolic compounds which inhibit the activities of the extracellular hydrolytic enzymes that are involved in the biodegradation process. The extracellular hydrolytic enzymes are produced by microorganisms for use in the breakdown of high-molecular-weight molecules outside the microbial cell. This process yields molecules with low molecular weight such us oligosaccharides, oligopeptides and amino acids, which are available for assimilation by heterotrophic bacteria (Chróst, 1991).

Deposits that contain an archaeological component are important because they are excellent sources of information about ancient human settlement, cultural development, and the interaction between people and the environment (Coles and Coles, 1986). A significant proportion of the archaeological remains found in wetlands is organic material such as wood, animal and plant fibres, seeds, leather and bog bodies (e.g. Gumbley *et al.*, 2005).

In the United Kingdom there are many significant examples of organic remains preserved in waterlogged environments. Examples of the more significant finds include the earliest dated prehistoric trackway in Europe, i.e. the Sweet Track. This is a Neolithic timber trackway which crosses part of the Somerset Levels, a low lying marsh from which peat has been extracted since the Roman period (Coles and Coles, 1986). From the perspective of *in situ* preservation this particular trackway has added

significance, as it has been partly preserved within the Shapwick Heath Nature Reserve (Brunning *et al.*, 2000). Similarly, at Flag Fen in Cambridgeshire, wooden timbers of a trackway and a platform of late Bronze Age were discovered in 1982 (Pryor, 1992). A concentration of buried posts and planks was found to extend over a hectare, and was stratified beneath deposits of peat and river alluvium (Caple, 1996). At Sutton Common, excavations of an Iron Age enclosure and its entrance annex have provided evidence of a range of construction features; these included ramparts, a palisade, hut platforms and waterlogged organic remains in ditches, including a possible wheel and a ladder (Van de Noort *et al.*, 2001). The above examples not only reinforce the significance of the waterlogged environment in which they were found, but they also highlight the important contribution that the English Heritage-funded Wetlands Surveys have made to our understanding of the waterlogged archaeological resources in England's wetlands.

Perhaps amongst the most spectacular examples of organic archaeological preservation are bog bodies such as Lindow Man, a prehistoric human of Iron Age date (2 BC-AD 119) (Turner, 1995). Lindow Man was excavated in 1984 from a peat bog (Lindow Moss) in Cheshire (Connolly, 1985). The archaeologists that interpreted the evidence from Lindow Man have suggested that he was killed as a result of a Celtic sacrifice because of the manner in which he died (triple death). This consisted of strangulation (with his throat cut from one end to the other), bludgeoning of the skull and oral ingestion of a poisonous plant (Parker Pearson, 1986; Turner, 1995).

Other examples of good preservation, specific to the study region of the Humber Wetlands, occur at Scaftworth near Bawtry, where, during excavations in 1983, the

remains of two Roman roads were found. The first road (approximately dated to 70 AD), was constructed of wood (alder, willow and poplar) and turves. A second road, consisting of alignments of oak posts with a gravel core, replaced the first one, probably in the third century AD. In recent years these roads have suffered from ongoing deterioration due to a lowering of water tables and ploughing practices. In the same region, the foundations of a Roman bridge at Rossington (which consists of groups of oak posts [Van de Noort *et al.*, 1997]) have been found. These sites, including Lindow Man, provide further insights into Iron Age ritual practices and Roman construction techniques that are unlikely to have been preserved in dryland contexts.

The Hasholme Boat (Figure 1.1) is a log boat from the fourth century BC, which was discovered in 1984 at Hasholme in the Foulness Valley (East Yorkshire). The substantial structure of the boat, which is almost 13 m long and 1.4 m wide, was made from a single oak log. The boat probably carried a maximum crew of eighteen rowers and two oarsmen. This vessel is currently being preserved in the Hull and East Riding Museum using polyethylene glycol (PEG) impregnation. This is a process whereby the water in the timbers is slowly replaced by PEG (McGrail and Millet, 1985, 1986; Millet and McGrail, 1987). At the present time, however, this preserving technique is not in use because of its excessive cost. Other preserved wooden remains are those of the sixteenth century Rose and Globe theatres. Both structures were sited at Bankside in South London, on the Thames floodplain. They were excavated in the 1980's. The Rose and Globe theatres have been preserved by maintaining the anaerobic nature of the waterlogged deposits in which they were buried. The soil environment is currently monitored at both sites in order to determine whether burial conditions promote the

preservation of the organic remains within (Caple, 1996; Gaimester, 1996; Corfield; 1998).

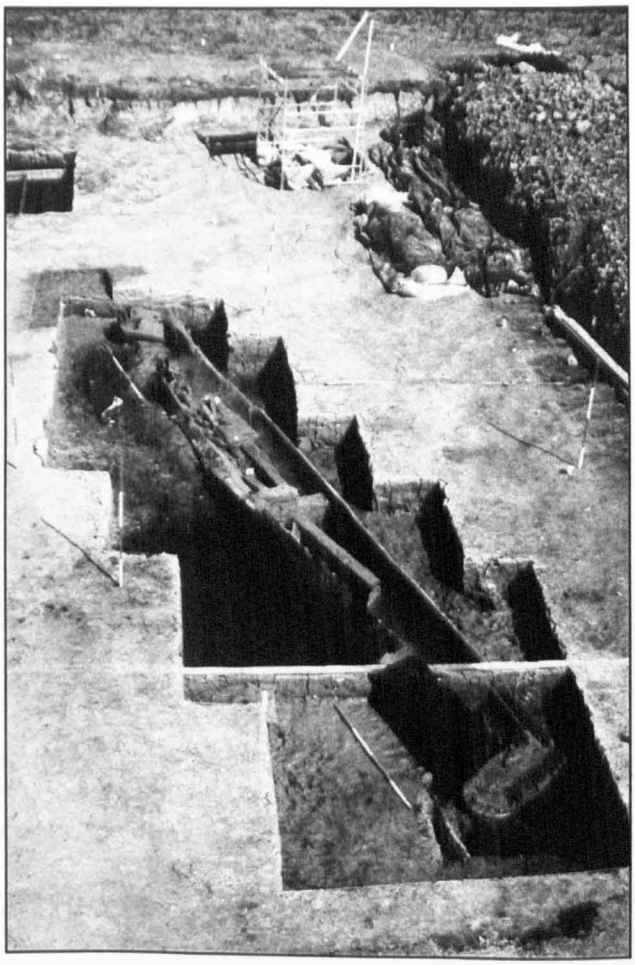


Figure 1.1: The Hasholme Boat (750-390 BC) which was discovered during drainage works near Holme-on-Spalding Moor (East Yorkshire). The boat is 13 m long and 1.4 m wide.

There are also important examples of organic archaeological remains in continental Europe; for example at Nidam Mose in Denmark. This archaeological site is rich in Iron Age artefacts which were probably deposited as ritual offerings. The artefacts discovered here included broken pieces of wooden boats and diverse weapons (Gregory *et al.*, 2001). Also in Denmark, research on the bog body of Tollund Man has shown that he lived during the fourth century BC, during the Scandinavian/Pre-Roman, Iron Age. The body and face of Tollund Man are very well-preserved. He was

found in 1950, naked apart from his cap and belt and with a rope around his neck, which is probably indicative of hanging. Researchers believe that Tollund Man was either a rich man who had been ritually sacrificed or a criminal who was hanged (www.tollundman.dk, 2004; Glob, 1969). Although his body is housed at the Silkeborg Museum in Denmark, the preservation techniques employed during the 1950's were crude in application. As a consequence, only the head is well-preserved today (Omar *et al.*, 1989).

On a worldwide scale, there are many other examples of organic archaeological remains which are preserved *in situ.* At the archaeological site of Ohalo II, which is situated on the edge of the Sea of Galilee in Israel; archaeologists discovered a variety of plant materials, organic traces of Stone Age huts or shelters in which people lived, and evidence of bedding. The site itself has been dated to approximately 21,000 BC. The bedding consists of bunches of stems and leaves of the halophyte grass *Puccinellia convolute*. Previous research has identified the bedding to be the oldest on record (Hole, 2004; Nadel *et al.*, 2004).

In South America, the Monte Verde wetland in Chile is considered to be the oldest archaeological site in the Americas (Late Pleistocene). It is the source of exceptionally well-preserved organic materials due to the reducing environment, and the protection offered by a peat layer and a silica gel-rich substrate. The site contains a rich array of plant remains, bones of extinct animals, and diverse artefacts of wood and stone (Tuross and Dillehay, 1995).

Wetlands are also important indicators of environmental change because, through the analysis of pollen, macro-flora fossils and invertebrate remains, they allow detailed reconstruction of past events (Cole, 2001; Hope *et al.*, 2007; Marchant, 2007). It is acknowledged that wetlands are the largest reservoir of sequestrated atmospheric carbon dioxide (CO_2) at a global level. However, if wetlands continue to be destroyed, it is possible that the stored CO_2 could be released into the atmosphere, thereby contributing to global warming and climate change. Furthermore, the removal of these 'reservoirs' reduces global storage capacities for atmospheric CO_2, compounding the impacts of these factors (Freeman *et al.*, 2004; Panter, 2007). Therefore, in respect of the reasons outlined above, it is important to preserve wetlands in their current state, and in particular, the archaeological remains located within them for future generations. However, British wetlands have been reduced to less than 1 % of the total area of the country (Van de Noort *et al.*, 2002). This percentage is very low when it is compared with the 16 % of wetland cover that existed at the beginning of the nineteenth century (Evesham, 1995).

At the global level, although Mitsch and Gosslink (1993:35-9) report that the reduction of wetlands is difficult to quantify, historically as much as 90 % of wetlands in New Zealand and 60 % of wetlands in China have been lost (*cf.* Lillie and Ellis, 2007).

In order to prevent this scenario from occurring in northwest Europe, there are several potentially useful approaches that might be applied to the preservation of wetland sites and the archaeological remains within them; these include:

1. *Maintenance of an elevated water table.* By keeping wetlands permanently wet and not subject to extremes of seasonal fluctuation or water level management regimes, it is possible to avoid the exposure of archaeological resources to the oxygenated conditions that favour microbial degradation (Björdal and Nilsson, 2002). In addition, a high water table excludes the activity of burrowing animals such as moles and rabbits and limits the growth of plant and tree roots (Van de Noort *et al.*, 2002). An example of this approach is the Sweet Track which is preserved *in situ* in a nature reserve. The water table in the reserve is maintained at a higher level than that of the surrounding land in order to obtain a positive water balance (Brunning *et al.*, 2000). At Hatfield Moor, English Nature [now Natural England] is maintaining a high water table by a system of pumped recharging. This measure has directly benefited the archaeological remains and the palaeoenvironmental resources in the area (Van de Noort, 2001a).

2. *Maintenance of low fertilizer and contaminant levels.* Agricultural and industrial chemicals and domestic sewage are significant pollutants to wetland sites (Cole, 1995). Impacts caused by contaminants and fertilizers can influence the chemical properties of the soil and groundwater (e.g. by changing the pH or the redox potential) (Banwart, 1998). Inputs of fertilizer may lead to eutrophication (excessive nutrient supply), a change in the vegetation and increased microbial activity. For example, the eutrophication of water in a ditch flowing through the Fens has altered the vegetation which runs adjacent to the ditch and also caused some decay of the peat at the ditch edges (Cole, 1995). At Flag Fen, sewage sludge applied to adjacent land has been

identified as a possible source of contamination, which may well affect the integrity of the archaeological remains within the site (Powell, 1999), although in some areas of the site the channelling of nutrient rich water through reed beds reduces nutrient inputs (Pryor 1992).

3. *Control of livestock and the use of farm vehicles.* High livestock density is liable to lead to the damage of archaeological sites by poaching or creating erosion scars. Farm vehicles also damage archaeological sites through the creation of wheel ruts and soil compaction. This is an important problem on waterlogged soils and might be solved by the use of alternative routes or by the use of lighter vehicles (Russell, 2003). At Sutton Common, cattle grazing has caused damage to the earthworks of the small Iron Age enclosure (Van de Noort, 2004a). Potential solutions to this problem include the relocation of feeding and watering troughs in order to persuade animals to gather in less sensitive areas, and the temporary exclusion of livestock from damaged areas.

The main factor preventing the application of the above approaches to the preservation of the archaeological remains tends to be a lack of agreement between the statutory bodies and the farmer/landowner. For example, at Sutton Common, prior to the site being purchased by the Carstairs Countryside Trust, inherent problems existed. These problems concerned the ability of the landowner to plough and drain a Scheduled Ancient Monument (SAM) and a Site of Special Scientific Interest (SSSI), with only limited financial penalty, thus causing significant damage to the archaeological site (Pearson and Sydes, 1997).

1.3 Characteristics of waterlogged environments

In a waterlogged environment the groundwater is at the soil/sediment surface or above, or within the soil root zone, during all or part of the growing season. The soil is frequently saturated for prolonged periods and this leads to the development of anaerobic chemical environments. There are many different types of anoxic waterlogged environments, including salt marsh, marine sediments, freshwater and estuarine sediments, paddy fields, mangrove swamps and peatlands (Caple, 1996).

Waterlogged burial environments are created by two different processes. The first process is known as 'terrestrialisation'. This occurs when open water ecosystems are colonised by emergent aquatic plants (e.g. the reed *Phragmites australis*). The plant litter produced accumulates at the bottom of the water column, where low oxygen content inhibits decay, and the water body eventually becomes completely filled with peat. The land subsequently becomes dry enough to support colonization by marshland and terrestrial plants (Caple and Dungworth, 1997). The Vale of Pickering in North Yorkshire is one of many examples of a lowland deposit formed within a post-glacial lake which has undergone terrestrialisation (Caple, 1996).

The second process, 'paludification', takes place when existing aerobic soils are inundated with water, leading to the death of existing plants and colonisation by plants that are resistant to waterlogged conditions (Caple, 1996). During the last post-glacial period, paludification resulted in the development of the raised mires in areas such as the Humberhead Levels, the Forth Valley and the North Solway shore (Dinnin, 1997; Crone and Clark, 2007:17).

Both of the sites which are being investigated as part of this thesis are located in the areas of peatland in Eastern England that are known locally as the Humberhead Levels. The Hatfield Moor site comprises of a peat containing partially decomposed plant remains, which has accumulated over time to form a raised mire system. The peat at Sutton Common has developed in the ditch of an Iron Age enclosure, as the rate of organic material production exceeded the rate of decomposition. The low levels of organic decomposition at both sites are probably due to the presence of acidic and anaerobic conditions. Within this environment there is an abundance of phenolic compounds which are enzyme inhibitors (Pind *et al.*, 1994; Freeman *et al.*, 1995; Freeman *et al.*, 2004). As a consequence of this, the nature of the peat not only provides a good record of the plants and animals that inhabited the archaeological sites, but also provides insights into the development of mire and ditch sequences in response to environmental change, both at the site specific level and also over time (Dinnin, 1997).

In wetlands, the soil itself may change with depth from aerobic and oxidizing conditions at the surface to anaerobic and reducing conditions at greater depths (Figure 1.2). When a soil becomes waterlogged there are slower gaseous exchanges between the soil and air, changes in microbial populations, and changes in redox potential (Eh) and pH values (McLatchey and Reddy, 1998). In the top few centimetres of the soil oxidizing conditions are often present. Oxygen (O_2), nitrate (NO_3^-), sulphate (SO_4^{2-}), ferric ions (Fe^{3+}), carbon dioxide (CO_2), phosphate (PO_4^{3-}) and ammonia (NH_4^+) may readily be detected (Gregory, 1998).

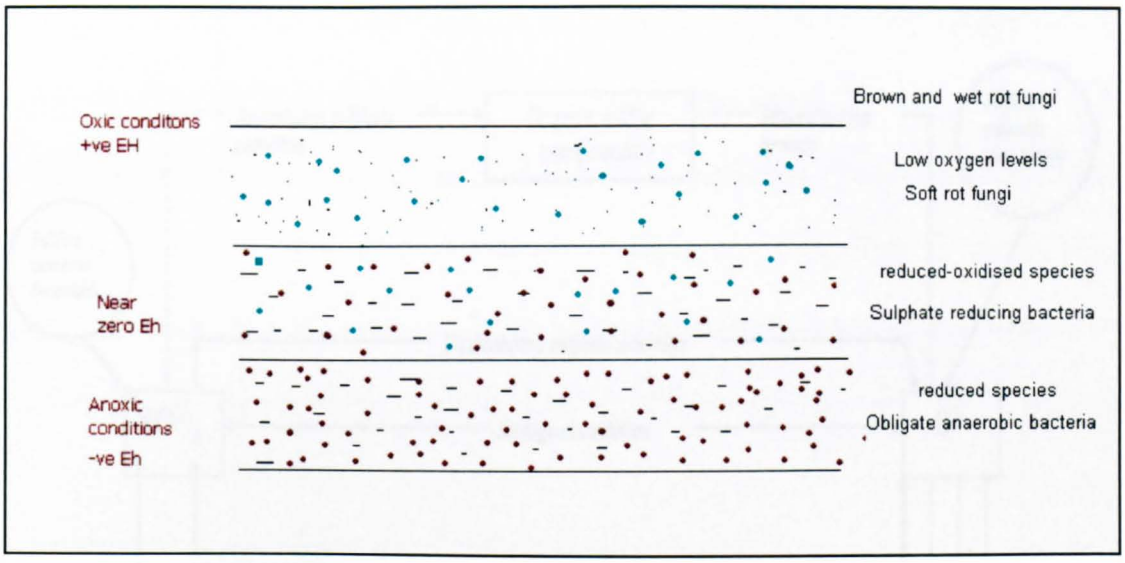


Figure 1.2: Soil zonation within anoxic deposits (Caple, 1993).

The topsoil is usually alkaline to slightly acidic and has a positive redox potential. However, the activity of aerobic and facultative anaerobic microorganisms in soil decreases the redox potential by the consumption of O_2 and NO_3^-, reducing ferric oxide/ferric hydroxide (Fe_2O_3/ $Fe(OH)_3$) and producing NH_4^+ (Kimura, 1984). With progression down the soil profile it is likely that the environment will become anoxic. Such soils are highly reducing and are characterized by the presence of ion species in reduced form and high organic matter content depending on the context (Corfield, 1996).

Under anoxic waterlogged conditions a number of changes in soil biology and chemistry take place. There is an increase in the concentration of methane (CH_4) and hydrogen (H_2) due to the activity of anaerobic bacteria. Sulphur (S^{2-}) and hydrogen sulfide (H_2S) increase as a result of the metabolic activity of sulphate-reducing bacteria (e.g. *Desulfovibrio desulfuricans*) (Figure 1.3) (Madigan *et al.*, 2006).

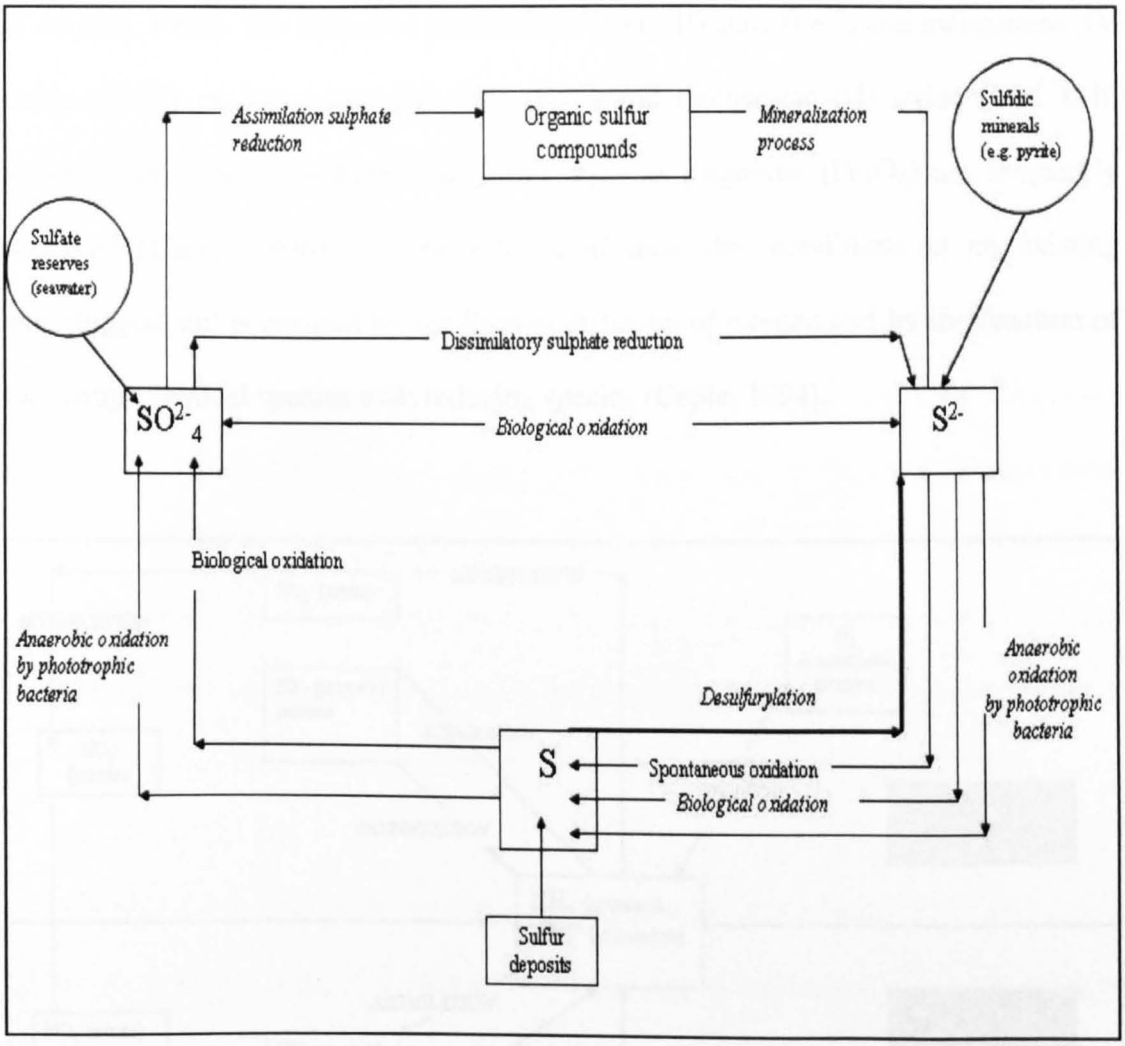


Figure 1.3: The sulphur cycle (adapted from Robertson and Kuenen, 1985).

Sulphur ions (S^{2-}) may be converted to sulphur dioxide (SO_2) and hydrogen sulphide (H_2S). H_2S can react with available ferrous ions (Fe^{2+}) in order to produce framboidal pyrites (FeS_2) which are frequently found in association with waterlogged archaeological wood (Corfield, 1996).

In anaerobic environments the denitrification process take place (Figure 1.4). Nitrate (NO_3^-) is reduced to nitrite (NO_2) and atmospheric nitrogen (N_2) by anaerobic bacteria such as *Thiobacillus dinitrificans*. NO_2 can also be transformed into ammonia (NH_4^+) by strict anaerobic denitrifying bacteria (e.g. *Pseudomonas fluorescens*) (Reddy and

D'Angelo, 1994). The microbial reduction of iron (III) state (Fe^{3+}) and manganese (IV) oxide (Mn^{4+}) produces iron (II) state (Fe^{2+}) and manganese (II) oxide (Mn^{2+}). In waterlogged deposits, reduced salt forms such as magnetite (Fe_3O_4) are frequently observed (Caple, 1996). The persistence of anaerobic conditions in an existing waterlogged soil is ensured by the limited diffusion of oxygen and by the reaction of oxidising chemical species with reducing species (Caple, 1994).

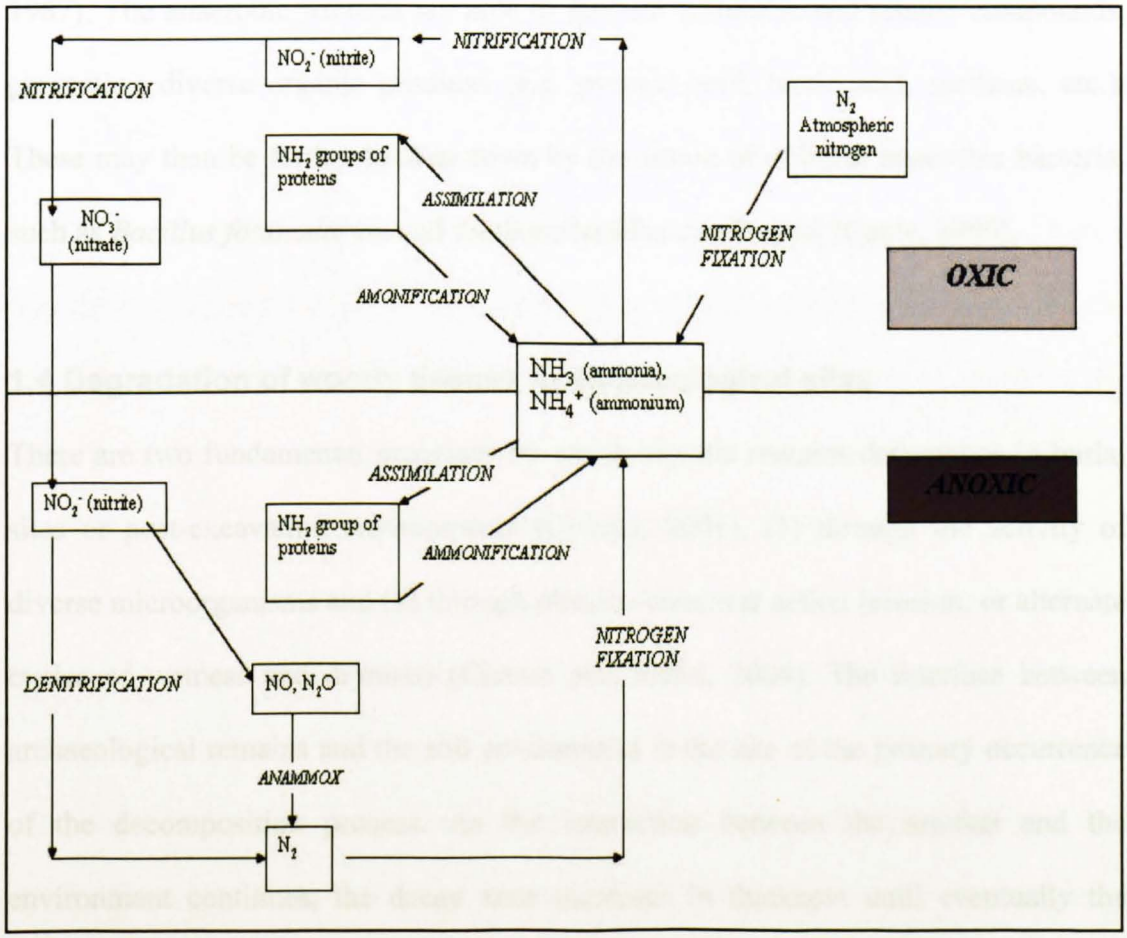


Figure 1.4: Nitrogen cycle (modified from Madigan *et al.*, 2006).

The reduced metal ions produced in reduced environments may act as either inhibitors or activators of enzymes (Pulford and Tabatabai, 1988). In anoxic environments chemical equilibria control the pH; with the CO_2 equilibrium being dominant above

pH 5.5 (i.e. $HCO_3^- \leftrightarrow CO_2 + OH^-$ and $HCO_3^- \leftrightarrow CO_3^{2-} + H^+$) and the NH_3 equilibrium dominant under low redox potential values (i.e. $NH_4^+ \leftrightarrow NH_3 + H^+$) (Caple, 1993).

In anaerobic strata, aerobic microorganisms such as basidiomycete fungi are inactive and anaerobic bacteria are the main inhabitants and the principal agents of microbial decomposition (Blanchette *et al.*, 1994). However, some soft rot fungi are occasionally detected under low oxygen conditions (Caple, 1993; Daniel and Nilsson, 1987). The anaerobic bacteria are able to ferment cellulosic and related compounds, generating diverse organic products (e.g. pyruvic acid, lactic acid, methane, etc.). These may then be further broken down by the action of obligate anaerobic bacteria, such as *Bacillus fossicularum* and *Methanobacillus omelianskii* (Caple, 1996).

1.4 Degradation of woody tissues in archaeological sites

There are two fundamental processes by which organic remains decompose in burial sites or post-excavation environments (Cronyn, 2001); (1) through the activity of diverse microorganisms and (2) through physico-chemical action (erosion, or alternate cycles of wetness and dryness) (Ciantar and Malia, 2004). The interface between archaeological remains and the soil environment is the site of the primary occurrence of the decomposition process. As the interaction between the artefact and the environment continues, the decay zone increases in thickness until eventually the process ends when the organic archaeological remains have been completely mineralised and are in equilibrium with the burial environment (Caple, 2001).

Archaeological wooden artefacts form the bulk of organic materials found in wet research excavations (Cronyn, 2001). The extent of preservation of archaeological

wood depends upon the specific characteristics of the burial environment. Wood is preserved when it is buried in an environment that limits microbial activity, for example, cold, dark and anaerobic conditions promote slower rates of degradation (Kim *et al.*, 1996; Blanchette, 2000; Kim and Singh, 2000; Powell *et al.*, 2001). However, even in the most extreme conditions, which are highly unfavourable to microbial activity, some physical and chemical modification of wood can take place (Blanchette, 2000).

There are several major environmental factors that influence the microbial decomposition of organic materials in soil; these include moisture, oxygen, pH, nutrients, temperature and clay content (Corfield, 1996). The process of deterioration also depends upon the depth at which organic artefacts are buried, as oxygen tends to decrease with depth (depending on the depositional sequence and stratigraphy of the soil). Nevertheless the decay of wooden artefacts can still take place even in deep deposits where oxygen is severely limited (Björdal *et al.*, 2000).

In order to understand the degradation of wood it is important to be familiar with the principal components of woody tissues. At the biochemical level the major components of woody tissues are cellulose, hemicellulose and lignin (Blanchette, 2000) (Table 1.1). There are a limited range of microorganisms which are capable of breaking down cellulose and hemicellulose. Lignin, however, is a phenolic component of wood with a complex, irregular pattern of linkages which even fewer microorganisms can effectively degrade. Although recently excavated wet archaeological wood can look well-preserved, as soon as the moisture content of the

wood is decreased the cells collapse as the degraded cellulose shrinks and the wood crumbles (Cronyn, 2001).

Table 1.1: Chemical components of plant cell walls (Bidlack *et al.*, 1991).

Wall components	Primary	Secondary
Polysaccharides	**90%**	**65-85%**
Cellulose	30	50-80
Hemicellulose	30	5-30
Pectin	30	--------
Proteins	**10%**	--------
Lignin	--------	**15-35%**

Fungi are the principal microorganisms responsible for the decay of wood under aerobic conditions, because they are the principal producers of the hydrolytic extracellular enzymes (e.g. cellulase, lignocellulase and lactase) that break down the cellulose and lignin components of the plant cell wall (Björdal *et al.*, 1999).

The two major groups of decay-causing fungi taxonomically belong to the subdivision Basiodiomycota (i.e. Brown- and White-rot fungi). These fungi require high levels of oxygen to decay wood and can cause total destruction of the structure of wood in a short period of time (Björdal *et al.*, 1999). White-rot fungi such as *Coriolus versicolor* are able to degrade all cell wall components (Figure. 1.5) and can even metabolize lignin. These fungi degrade the middle lamella (-the layer that makes up the outer wall of the cell and which is shared by adjacent cells) and successively destroy the wood tissue, leading to the complete destruction of wooden archaeological artefacts (Björdal and Nilsson, 2002).

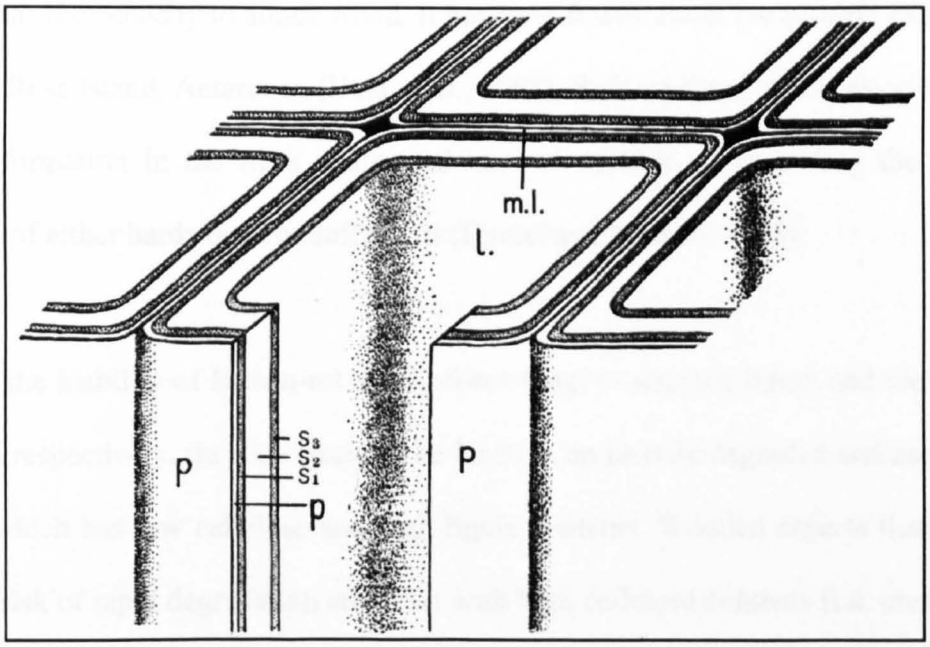


Figure 1.5: Diagrammatic representation of the cell wall of a woody plant (Jane, 1970). **P = primary wall**: wall that is produced by the cell while growing. It consists of a skeleton of cellulose microfibrils embedded in a matrix composed of pectin, hemicellulose and glycoproteins. **S1, S2, S3 = successive layers of the secondary wall:** part of the wall that is produced after cell growth is completed. It is made of cellulose, hemicellulose and lignin. **m.l. = middle lamella:** first layer formed during cell division. It makes up the outer wall of the cell and is shared by adjacent cells. It is composed of protein and pectic compounds. **l = lumen** of the cell.

Brown-rot fungi, such as *Serpula lacrymans*, break-down cellulose rapidly during the initial phase of wood colonization and cause significant damage in wooden building components in temperate regions around the world (Kauserud *et al.*, 2004). Although Brown-rot fungi are specialists in degrading cellulose, they are not able to degrade lignin in archaeological wood (Björdal and Nilsson, 2002).

Other fungi that degrade wood are Soft-rot fungi which belong to the ascomycete and *fungi imperfecti* groups. These degrade wood that has excessive moisture content and are active at low oxygen concentrations (Björdal *et al.*, 1999; Blanchette, 2000;

Björdal and Nilsson, 2002; Caple, 2004). *Cladosporium* is a genus of Soft-rot fungi which has the capacity to attack wood. It has been found inside the historic expedition huts of Ross Island, Antarctica (Held *et al.*, 2005). Soft-rot fungi attack wood through cavity formation in the fibre walls, and are not capable of degrading the middle lamella of either hardwoods or softwoods (Daniel and Nilsson, 1988).

Due to the inability of Brown-rot and Soft-rot fungi to degrade lignin and the middle lamella respectively, they are likely to be inactive on heavily degraded archaeological wood which has low cellulose and high lignin contents. Wooden objects that have a higher risk of rapid degradation are those with high cellulose contents (i.e. moderately or superficially degraded artefacts) (Björdal and Nilsson, 2002).

In waterlogged environments the lack of oxygen inhibits basidiomycete growth and metabolism (Björdal *et al.*, 2000), although Soft-rot fungi are occasionally detected in environments with low oxygen content (Caple, 1996). In water-saturated conditions bacteria are the main agents of microbial decomposition. They are responsible for the degradation of archaeological wood (Daniel and Nilsson, 1987; Blanchette and Hoffmann, 1994; Kim *et al.*, 1996; Björdal, 1999; Björdal 2000; Blanchette, 2000; Kim and Singh, 2000; Helms *et al.*, 2001; Powell *et al.*, 2001; Gregory *et al.*, 2002; Caple, 2004; Helms *et al.*, 2004). Previous research has identified the presence of specific genera of bacteria (e.g. *Bacillus*, *Pseudomonas* and *Clostridium*) in waterlogged wood (Boutelje and Goransson, 1972; Mouzouras *et al.*, 1987; Young, 1988). However, the process of wood degradation by bacteria is slow compared to aerobic fungal degradation, and depends upon environmental and stratigraphical

factors, in addition to age and wood species (Björdal *et al.*, 1999; Björdal and Nilsson., 2002).

Distinct patterns of attack on wood, and categories of bacterial decay, have been described in previous literature (e.g. Daniel *et al.*, 1987; Singh and Butcher, 1991). There are four different functional categories of bacteria that degrade wood. These are based on the specific morphological features of attack.

1. *Tunnelling bacteria* excavate tunnels through the secondary cell wall and penetrate the middle lamella which contains lignin. Examples include *Homalium foetium* and *Alstonia scholaris* (Daniel, 1994).

2. *Erosion bacteria* grow inside the lumen of wood (xylem) cells and are attached to the cell wall. They degrade secondary wall layers and deplete cellulose and hemicellulose from the wood. The eroded zones become filled with bacterial cells whilst the undegraded lignin wall remains. This results in a porous, lignin-rich residue (Blanchette, 2000; Schmitt *et al.*, 2005). Both tunnelling and erosion bacteria produce extracellular secretions in the form of vesicles that contain cellulolytic and hemicellulolytic enzymes, which increase the rate of wood decay (Daniel, 1994).

3. *Cavitation bacteria* cause irregular cavities in the secondary cell wall leaving only the residual wall intact.

4. *Bacterial scavengers* are found in wood that has already been degraded by the above processes. They utilize partially-degraded cell wall components, particularly simple sugars (Kim and Singh, 1994; Blanchette, 2000).

The main degraders of archaeological waterlogged wood are erosion bacteria, because they can tolerate oxygen-limited conditions (Blanchette *et al.*, 1990; Donaldson and Singh, 1990; Kim *et al.*, 1996; Björdal *et al.*, 1999; Björdal and Nilsson, 2002; Schmitt *et al.*, 2005). Soft-rot fungal and tunnelling bacterial decay are less frequently observed under oxygen-limited conditions (Björdal *et al.*, 1999).

1.5 Preservation *in situ* of organic archaeological remains and the conservation of wetland habitats

Archaeological sites and artefacts, once excavated, should be preserved and conserved for posterity; and also because improved techniques in the future may permit better analysis and understanding of the archaeological evidence (Corfield, 1996). However, excavation destroys the long term equilibrium that has evolved between the artefact and its burial environment, with the deterioration process starting as soon as the archaeological material is uncovered or excavated (Ciantar and Malia, 2004).

As such, there is an increasing need to preserve artefacts within archaeological sites *in situ* because excavation, storage and conservation is costly; particularly in the case of large wooden objects like trackways, shipwrecks and the timbers from house foundations (Björdal and Nilsson, 2002). The most frequent methods used to preserve wooden artefacts, such as ship hulls and timbers, are based on storing the remains in freshwater tanks, or by wrapping in polyethylene or multi-layered barrier foil (Jones *et*

al., 1986; Pitman *et al.*, 1993). However, these methods fail to prevent the growth of wood-degrading microorganisms, including Soft-rot and White-rot fungi (Jones *et al.*, 1986; Mouzouras, 1987). It is therefore imperative for researchers to understand whether the archaeological material will be better preserved, either undisturbed or after reburial.

Preserving archaeological sites *in situ* involves two different approaches:

1) Leaving suspected archaeological sites undisturbed so that the artefacts (albeit unknown) are preserved in their original undisturbed state.
2) Reburial of artefacts that have been dug up (and recorded) during excavation.

The active management of archaeological sites, with the aim of preserving archaeological remains *in situ*, has increased in recent years. In 1991, the Planning and Policy Guideline-Archaeology and Planning (PPG16) issued by the Department of the Environment, established that preservation *in situ* is the preferred option for dealing with archaeological remains. National archaeology policies also support the *in situ* preservation of sites that are not in danger of destruction by development, or at risk from other factors (Powell *et al.*, 2001).

The principal aim of *in situ* preservation is to create a constant burial environment that is sustainable through time (Van de Noort *et al.*, 2001). Most organic and inorganic materials survive well in anaerobic conditions (e.g. waterlogged sediments), or in desiccating environments (where air circulation leads to the removal of moisture), such as in the Egyptian tombs (Kim and Singh, 2000). In damp but aerated soils, aerobic conditions favour the decay of organic matter and the oxidizing of metals. In

addition, acidic soils can preserve some organic materials, because fungal and bacterial growth may be inhibited by the acidity of the burial environment (Caple, 2004; McCaffrey *et al.*, 2005).

The traditional method for reburial in wetlands is to cover the remains with sand bags and sediment, usually taken from the upper layers of the site from where the artefacts were excavated, or from the surrounding area (Waddell, 1994; Pournou *et al.*, 1999). With this approach the assumption is made that the original conditions of preservation will be re-established in the environment unaided (Caple, 1993). However, it has been shown that anaerobic conditions are not always re-established following reburial and that microorganisms may attack the re-buried artefacts (Jordan, 2001). For example, at Sutton Common waterlogged conditions were not re-established after excavation, and as a consequence, most of the wooden artefacts from the Iron Age were found to be in a highly degraded state (Caple, 2004).

To date there are relatively few archaeological sites where preservation *in situ* has involved the reburial of the artefacts to be preserved, with new and/or additional material. Examples include sites such as the Rose Theatre, where washed sand has been used for some reburial work. The sand is quite inert, which not only provides physical and thermal protection, but more importantly the use of sand does not introduce contamination into the burial medium (Caple, 2004). Although this example highlights one of the possible approaches to *in situ* burial, little information exists on the longer term sustainability of the *in situ* preservation of organic remains.

There is a range of threats that can affect waterlogged contexts containing archaeological remains and bring about subsequent damage to reburied artefacts. These can be of natural origin, such as climate change, or they can be anthropogenic, such as water abstraction or peat extraction (Coles, 1995; Corfield, 1996). The main causes of the destruction of archaeology in England's wetlands are: drainage to convert land to pasture or fen to arable land; water abstraction for agricultural, industrial or domestic use; peat extraction (for fuel and horticulture); gravel extraction and quarrying; peat wastage and erosion; and urban and industrial development (Coles, 1995; Van de Noort *et al.*, 2002). Drainage and peat extraction promote the access of oxygen into the burial environment, leading to the decay of any organic artefacts preserved *in situ* and to substantial loss of archaeological value (Nicholas, 2001; Van de Noort *et al.*, 2002). During drainage there is a shift from anaerobic to aerobic conditions, a change that potentially allows for the colonization of wooden artefacts by wood-rot fungi and aerobic wood-decay bacteria (Björdal and Nilsson, 2002). It has been suggested that, when there is no possibility of controlling the water level, excavation may be the only appropriate course of action available in order to prevent the archaeological remains from completely deteriorating (Gumbley *et al.*, 2005).

English Heritage recognizes the importance of wetlands from an archaeological perspective. Over the past thirty years it has funded survey and research in the four main lowland wetland areas of England (as highlighted in Section 1.1). The aim of these projects was to identify the archaeological potential and significance of each area, and subsequently to develop a management strategy for conservation and protection of the archaeological resource.

In 2001, English Heritage commissioned a report from the University of Exeter, entitled 'Monuments at Risk in England's Wetlands' (MAREW). This report collected data on the destruction of wetlands in England and assessed potential losses to the archaeological resources on a regional basis. The report also considered the impact upon wetlands of hydrological change, peat extraction and urban and industrial expansion (Van de Noort *et al.*, 2002). The MAREW report demonstrated that an estimated 10,450 wetland monuments have been destroyed or damaged over the past fifty years. This figure represents 78 % of the total identifiable resource (Van de Noort *et al.*, 2002). However, despite these potential losses, the extent of wetland areas with archaeological potential is still substantial and archaeologically important. For this reason it is essential to establish effective strategies for preservation, before these surviving wetlands are lost.

English Heritage considers that the most effective way to preserve England's wetland archaeology is to control the hydrological regime and protect the entire wetland resource, rather than to rely upon the preservation of 'monument islands' (*cf.* Darvill and Fulton, 1998). In order to obtain hydrological control of a wetland archaeological site, a large area of land around the site requires appropriate management (Coles, 1995). Although, even where geo-hydrological analysis is undertaken, the numerous variables in terms of inputs and outputs from a wetland can make effective management difficult at best (*cf.* Darvill and Fulton, 1998).

To preserve the archaeology of wetlands, the adoption of several management strategies is fundamental. These include undertaking more research on the preservation of archaeological remains *in situ*, protecting wetlands themselves and

their hydrological conditions, promoting co-operation with landholders and nature conservation organizations (i.e. The Environmental Agency, Natural England, DEFRA, RSPB and others), educating the community and communicating the importance of wetlands in general (Panter, 2007).

Although the above aspects are of considerable importance, without a fundamental knowledge of wetland complexity, in terms of hydrology and the burial environment, all other aspects of conservation are of secondary significance to the long term sustainability of wetlands.

1.6 Monitoring of wetlands

Several authors have advocated monitoring of the burial environment in order to protect organic archaeological remains *in situ* (Coles, 1990; Caple, 1993; Waddell, 1994; Riess and Daniel, 1997; Gregory, 1998; Kars, 1998; Pournou *et al.*, 1999; Caple and Hovmand, 2001; Coles, 2001;Van de Noort *et al.*, 2001). Monitoring of physico-chemical variables, biological processes and the condition of the archaeological remains themselves, is essential for the implementation of a management programme (Kenward and Hall, 2000; Gumbley *et al.*, 2005). Regular and ongoing monitoring of water level and quality, redox potential, pH and temperature is considered to be essential for successful *in situ* conservation. Monitoring of the conditions in burial environments at archaeological sites is being undertaken worldwide and should provide a greater understanding regarding the factors that influence the biodegradation of wooden artefacts (e.g. Gregory, 1998; Jordan, 2000; Caple and Hovmand, 2001; Hogan *et al.*, 2001; Powell *et al.*, 2001; Gregory *et al.*, 2002).

A preservation strategy that relies simply on maintaining the original conditions of the wetland landscape will be insufficient. Active measures for protection may well be needed. Monitoring programmes must be undertaken with regularity, and as the above discussion has shown, every wetland archaeological context has site-specific characteristics which need to be taken into account. Non-destructive procedures and cost-effective methods must be employed in the monitoring process in order to estimate the degree of degradation over time (Kars, 1998). To be effective, a monitoring programme must extend over the whole year in order to give information about patterns that are related to seasonal fluctuations of the water table (i.e. wetting and drying) (*cf.* Cheetham, 2004); and at least a one-year baseline dataset needs to be established before any potential impacts occur at the site being studied. Any conservation programme may need periodic revision. This would be based on the results of the monitoring and assessment of the site, which would identify any fluctuations in the burial environment (Gumbley *et al.*, 2005).

In 1993, English Heritage commissioned a study of the techniques that are used to protect and manage wetlands (Van de Noort and Davies, 1993). This survey showed that active management is necessary to preserve wetland archaeological sites, and that this management should integrate archaeological and nature conservation interests. English Heritage is continuing to work on the identification of the most suitable parameters that can be used in the assessment and monitoring of waterlogged archaeological sites, and by extension, those that are relevant to conservation *in situ* (Corfield, 1996). For example, researchers at Durham University (Caple, 1994; Caple and Dungworth, 1997) have studied chemical balances in anoxic waterlogged burial environments. The work undertaken included the monitoring of pH, redox potential

and concentration of diverse ionised chemical species, in some of the most important waterlogged archaeological deposits in England. The sites investigated included Flag Fen (Cambridgeshire Fens), where a Bronze Age platform and post alignment is preserved *in situ*, the Neolithic Sweet Track located in the Somerset Levels, and the Mesolithic archaeological site of Star Carr in the Vale of Pickering (Corfield, 1996). As mentioned in Section 1.5, Monuments at Risk in England's Wetlands Project (MAREW) created a point of reference that is useful for monitoring future changes at wetland archaeological sites.

A number of key parameters have been proposed for monitoring waterlogged archaeological sites *in situ.* The main physico-chemical factors which need to be considered in monitoring waterlogged burial environments that contain archaeological remains are as follows:

1. *Level of the water table and its annual fluctuation.* Study of the hydrological regime is useful in establishing the water balance, and the balance between oxidation and reduction conditions at a site. The water table should not fall below the stratum at which the archaeological remains are located (Gumbley *et al.*, 2005). A drop in the water table may permit the entry of air and/or oxygen rich rain water, or of water containing corrosive substances. All these factors are likely to promote the rapid decay of archaeological remains (Kenward *et al.*, 2000; Björdal and Nilsson, 2002). A high water table favours the maintenance of saturated conditions, helping to prevent the decay of artefacts by aerobic microorganisms (especially fungi) (Powell *et al.*, 2001). As noted in Section 1.3, waterlogged conditions might impede extracellular enzyme

activities by causing changes in the microbial community in soil, deceasing the synthesis of enzymes and increasing the concentrations of phenolic compounds and inhibitory metal ions such us Fe^{2+} (Freeman *et al.*, 1996; Kang and Freeman, 1999).

2. *Levels of contaminants in water and soil.* Water pollution can cause damage to wetlands. Pollution can arise from the application of pesticides and fertilizers (synthetic compounds or manure and slurries) to the catchments of wetlands, from drainage of pollutants and toxic material onto wetlands, and from accidental oil spills (Cole, 1995; DEFRA, 2002). An influx of water rich in nitrates and phosphates to a wetland may cause alterations to the burial conditions (Corfield, 2006). The proximity of industrial areas, waste facilities and heavy road traffic might affect wetland soils. The soil or the soil water can become contaminated with toxic pollutants (including heavy metals, dioxins, Polychlorinated Biphenyls (PCBs) and Polycyclic Aromatic Hydrocarbons (PAHs) which may percolate into adjacent areas (Hulme *et al.*, 2002).

3. *Redox potential.* Eh is a measure of the potential for electrochemical activity, and characterises the oxidation-reduction state of the soil (Powell *et al.*, 2001). Redox potential is measured by taking into account the potential for chemical reactions involving the transfer of electrons that take place in an aqueous environment (e.g. $Cu \rightarrow Cu^{2+}+2e^{-}$). In oxidizing conditions this reaction proceeds, thereby generating an electrical potential, but in reducing conditions there is an excess of electrons and this reaction may not take place. As a consequence, most waterlogged environments have low or negative redox

potential, indicating reducing conditions (Caple, 1994; Caple, 2004). In waterlogged environments it is suggested that redox potential is the principal variable that controls the survival of waterlogged wood (Cronyn, 2001). Research at a number of archaeological sites has shown that the redox potential for anaerobic waterlogged environments, that best preserve archaeological organic remains, is <–100 mV (Caple 1994; Corfield, 1996; Caple and Dungworth, 1997).

4. *The pH value*. Soil pH affects the availability of nutrients and thus influences the composition and diversity of the microbial community. pH also influences internal nutrient transformations and release within wetlands, including microbial-mediated inorganic nutrient transformations, chemical precipitation-complexation reactions, adsorption reactions and chemical solubility (Reddy and D'Angelo, 1994). Although high inorganic nutrient levels are usually associated with neutral pH, most bogs contain low nutrient contents and acid pH values (Caple and Dungworth, 1997). Decomposition is slower in acid soils when compared to neutral soils, due to reduced microbial activity (Corfield, 1996). It has also been shown previously that pH affects the preservation of archaeological remains, as sediments with a low pH tend to contain preserved archaeological wood (Powell *et al.*, 2001). Low pH values suppress the activity of the extracellular enzymes which break down cellulose and other polymers (Sinsabaugh *et al.*, 1991; Kang and Freeman, 1999; Dick *et al.*, 2000), and which are responsible for the process of microbial decay (Caple, 2004). Clays act as cation exchangers, contributing to a buffering

effect that maintains a higher pH, thus causing an increase in hydrolytic attack by extracellular enzymes on organic archaeological materials (Caple, 1994).

5. *Temperature*. The temperature of the soil strongly influences the physical, chemical and microbiological processes that occur within it. Soil temperature directly affects extracellular enzyme activity by modifying enzyme kinetics and promoting microbial proliferation and enzyme production (Kang and Freeman, 1999). Low temperatures limit extracellular enzyme activity, thereby limiting the decomposition of organic matter and promoting organic material accumulation in soil. Hopkins (1996) has recently shown that in upland soils where the average temperature during most of the year is close to 0 °C, there is virtually no organic decomposition.

6. *Soil moisture and organic matter content*. Soil organic matter is an important determinant of soil fertility and plant growth, its water-holding capacity and also microbiological activity. Organic matter stabilizes and binds soil particles together, maintaining its structure and reducing the negative effects of pollutants, whilst increasing the rate of water infiltration and reducing run-off. It also acts as a cation exchange reservoir, maintaining the pH and cationic equilibrium in the soil. The loss of soil organic matter can have significant consequences as it lead to soil erosion, a loss of fertility, compaction and general land degradation (USDA, 1996; Caple, 2004). The monitoring of soil moisture above and below the water table can provide insights into the movement of water through the soil profile. The content of water in soil affects the availability of water to microorganisms, soil aeration, the nature and

amount of soluble materials, osmotic pressure and the pH of the soil solution (Paul and Clark, 1996). The slow diffusion of oxygen through the water component of a waterlogged soil is the reason why oxygenation and biological activity are limited (Hopkins, 1996).

Along with monitoring of the physico-chemical variables highlighted above, the composition and activity of the microbial community through the soil profile will be assessed in order to understand the potential for the preservation of organic artefacts at archaeological sites.

1.7 Microbial communities in soil

The microorganisms that live in soils include viruses, bacteria, fungi, algae and protozoa. Soil microorganisms contribute to the cycling of organic carbon through compounds, and they play central roles in biogeochemical processes (Madigan *et al.*, 2006). Soil microorganisms influence above-ground communities by contributing to plant and animal nutrition and health, soil fertility and structure (Kirk *et al.*, 2004).

The relatively high concentration of organic matter in top-soils favours the growth of heterotrophic microorganisms which use organic carbon as their principal carbon source. The abundance of heterotrophic microorganisms in soil varies according to soil type and condition. For example, in the first 10 cm of a podsol approximately 9.8 x 10^6 bacterial colony forming units per gram wet weight of soil (excluding actinomycetes) have been recorded (Hopkins, 1996). Torsvik *et al.* (1990) estimated that in 1 g of soil obtained from the first 10 cm of a forest soil in Norway, it was possible to find 4000 different bacterial 'genomic units'. The biochemical versatility

of microorganisms provides soils with the capability to degrade both natural organic and xenobiotic compounds (-the latter of which are very difficult to degrade [i.e. complex hydrocarbons such as herbicides 2, 4-D and 2, 4, 5-T]) (Kästner, 2000).

Microbial populations vary with depth, soil type, from site to site and within a site. Soil is a very heterogeneous environment containing many micro-habitats where microorganisms can grow. As a result, bacteria tend to form aggregates in soil, known as 'hot spots' (Pepper *et al.*, 2000; Kirk *et al.*, 2004). Bacteria are not uniformly distributed through the soil profile. It is generally observed that bacterial distribution depends on the soil organic matter content and in many contexts their abundance decreases with depth (Wood, 1995). In addition, microbial activity may decrease with depth in response to limited oxygen availability and lower temperatures (Hopkins, 1996; Pepper *et al.*, 2000). In regularly waterlogged soils, the changing conditions favour the development of facultative anaerobic microorganisms that can live under both aerobic and anaerobic conditions (Caple, 2004).

Bacteria are the predominant microorganisms in a soil and because of their functional diversity they are involved in virtually every metabolic process (e.g. nutrient cycling, organic matter formation and decomposition, soil structure and plant growth promotion [Kennedy, 1999]). Bacteria such as actinomycetes are especially active in the decomposition of organic materials in soil, including plant remains such as cellulose and lignin, which as mentioned above, are the main components of wood (Heuer *et al.*, 1997).

In light of this discussion it can be concluded that any organic archaeological elements which are preserved in waterlogged burial environments depend upon the maintenance of equilibrium with the system. In particular, the maintenance of saturated conditions which inhibit the activities of microbial communities within the soil is essential for long term *in situ* preservation.

1.8 Aims and objectives

The main aims and objectives of the work described in this thesis are:

1) To undertake a two-year monitoring programme at two wetland sites in the North of England. The sites studied are an Iron Age site at Sutton Common where organic archaeological remains are preserved, and a 'natural' environment at Hatfield Moor where there is significant potential for *in situ* organic preservation (which is primarily due to the maintenance of a high water table under an active management regime).

2) To provide information on the burial environment at Sutton Common and to compare this with data collected in an earlier monitoring programme which was undertaken during 2003-2004 (Cheetham, 2004).

3) To characterize the genetic fingerprint of the bacterial community in the soil profile at different depths and throughout the seasons, by various molecular techniques (-these techniques will be discussed in detail in Chapter 5).

4) To assess correlations between measured environmental parameters and the nature of the microbial community in order to understand the processes involved in the preservation of organic archaeological material at the sites studied. Particular attention is placed upon the height of the water table and redox potential values through the soil profile; and upon changes in the activity, physiological profile and composition of the microbial community.

It is anticipated that the information obtained from this thesis will increase our understanding of the biological and physico-chemical processes related to the preservation of organic archaeological remains in waterlogged soils. Furthermore, this research will provide insights into the most appropriate and convenient environmental variables with which to monitor the *in situ* preservation of organic artefacts.

1.9 Structure of the thesis

Chapter 1 (the current chapter) outlines the importance of wetlands in terms of the *in situ* preservation of organic archaeological artefacts (in particular wood). It also highlights the key environmental variables which are responsible for organic decay, and the different ways in which these variables can be monitored and assessed.

Chapter 2 describes the sampling locations at Hatfield Moor and Sutton Common, taking into account their geography, history, archaeology, management and previous research.

Chapter 3 highlights the various field measurements employed to characterize the burial environments of the two sites chosen for study.

Chapter 4 describes the conventional microbiological techniques used to determine the abundance, activity and physiological profile of the microbial community in the burial environment at both sampling sites.

Chapter 5 introduces the molecular genomic techniques used to characterize the bacterial communities in the soils studied.

Chapter 6 presents the physico-chemical results obtained from the monitoring of Hatfield Moor and Sutton Common. This chapter includes a discussion of the main findings.

Chapter 7 presents the results obtained from the conventional microbial methods applied in the studied soil samples, together with a discussion of the most important findings.

Chapter 8 presents the results obtained from the molecular genomic approach used to study the activity of the bacterial communities through the soil profile. The results from the cloning and sequencing of some of the bacteria present in the soil samples are also included in this chapter.

Chapter 9 discusses the findings produced from the different approaches used in the previous three chapters.

Chapter 10, the final chapter, presents the key conclusions which have been obtained during the study and considers future research directions on the basis of the results obtained from this study.

1.10 Summary

This chapter explains the importance of wetland environments for the *in situ* preservation of organic archaeological remains. The biological and physico-chemical characteristics of the chosen sites are outlined, along with the environmental factors that are responsible for the decay of organic material. It also highlights the need for a sustained environmental monitoring program where there is the potential for archaeological remains to exist. Chapter 2 will provide a detailed overview of the sites being studied, put these into the regional context and consider them in relation to their current wetland archaeology and *in situ* preservation characteristics.

Chapter 2 Wetland archaeological sites selected for study

2.1 The environmental context of the Humber Wetlands and the Humberhead Levels

2.1.1 Introduction

The sampling sites used in this study are a location on the edge of Hatfield Moor which is within a natural palaeochannel feature, and the Iron Age site of Sutton Common; both of which are located in the Humberhead Levels. The Humberhead Levels form part of a more extensive low-lying area of wetlands in and around the Humber Estuary, of approximately 330,000 ha, that include parts of Yorkshire, Lincolnshire and Nottinghamshire (Figure 2.1) (Van de Noort and Ellis, 1993; Smith, 2002). The Humberhead Levels, as defined by the Humber Wetlands Survey, are limited to the north by the rivers Aire and Ouse, to the east by the River Trent and the Isle of Axholme, and to the south and west by the Pennines (Ellis, 1997).

In the Humberhead Levels there are three main wetland types; the first of these are the remaining (cut-over) raised mires of Thorne and Hatfield Moors. The second type of wetland is known as Marshland (which is formed and dominated by estuarine accretion). The third type of wetland comprises the river floodplains of the Aire, Went, Don, Torne and Idle, and their tributaries (Etté and Van de Noort, 1997).

Within and adjacent to these wetland areas, most of the Humberhead Levels has been altered by activities such as land drainage for reclamation and agriculture, mining (i.e. coal), peat extraction and industrial activities. However, in the east of the region the land is mostly dedicated to arable farming. Doncaster, Selby and Goole are the most

significant urban centres in the region. There are also many villages and farmsteads. Apart from the urban areas most of the land in the Humberhead Levels is currently used for agriculture. The principal arable crops are sugar beet, potato, oilseed rape, peas and beans. Linseed is the primary crop grown in the southern part of the Levels (Middleton, 1997).

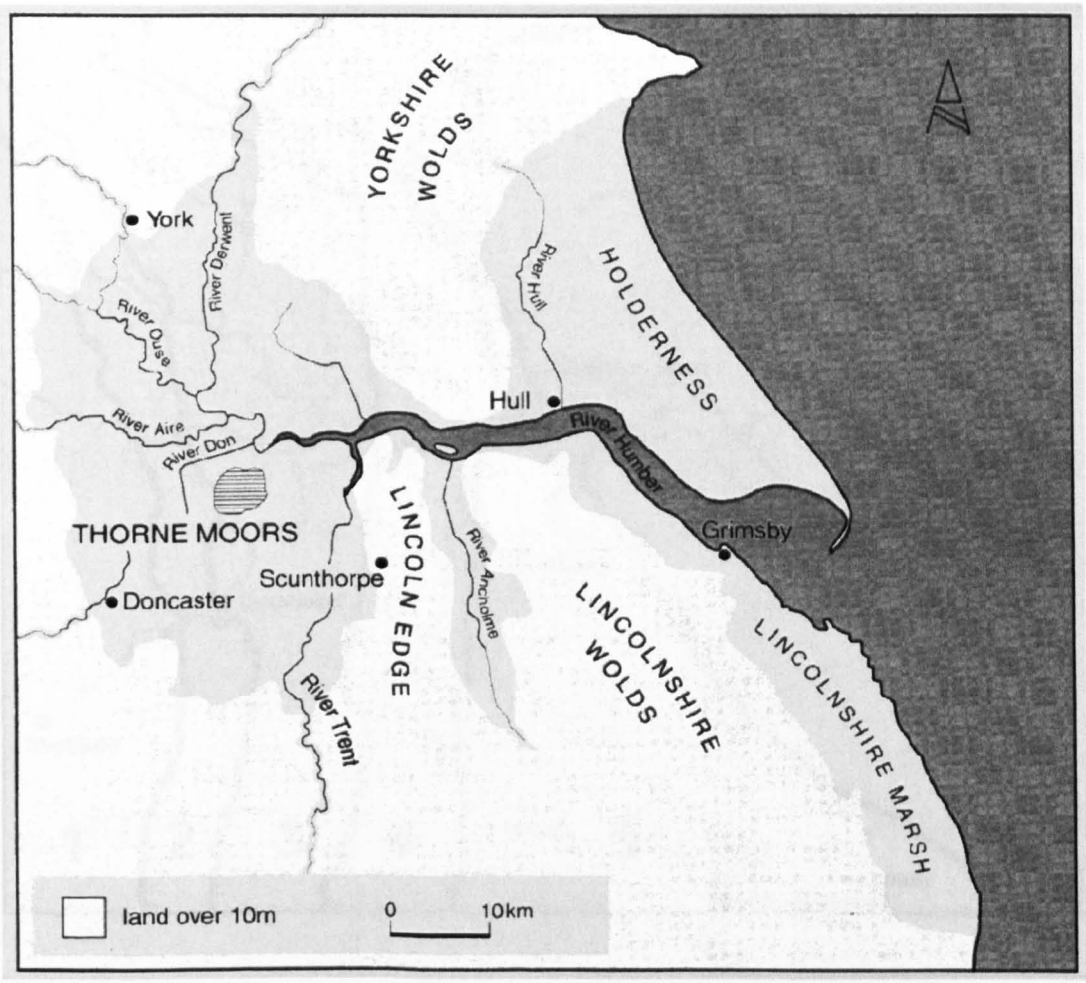


Figure 2.1: Map showing the location of the Humberhead Levels (simplified after Catt [1990] and Gaunt [1994], *after* Van de Noort 2004b).

2.1.2 Geology and landscape

The bedrock underlying most of the Humberhead Levels consists of Triassic rocks; Sherwood Sandstone and soft Mercia Mudstone (Figure 2.2). These rocks are very easily eroded; a factor that has contributed to the low lying situation of the Levels.

The Sherwood Sandstone occupies the central parts of the Humberhead Levels and the Vale of York. Further east, Mercia Mudstone overlies the Sherwood Sandstone (Van de Noort, 2004b). The bedrock underneath much of this region is below Ordnance Datum (OD). Most of the Triassic rocks are beneath different types of superficial Quaternary deposits.

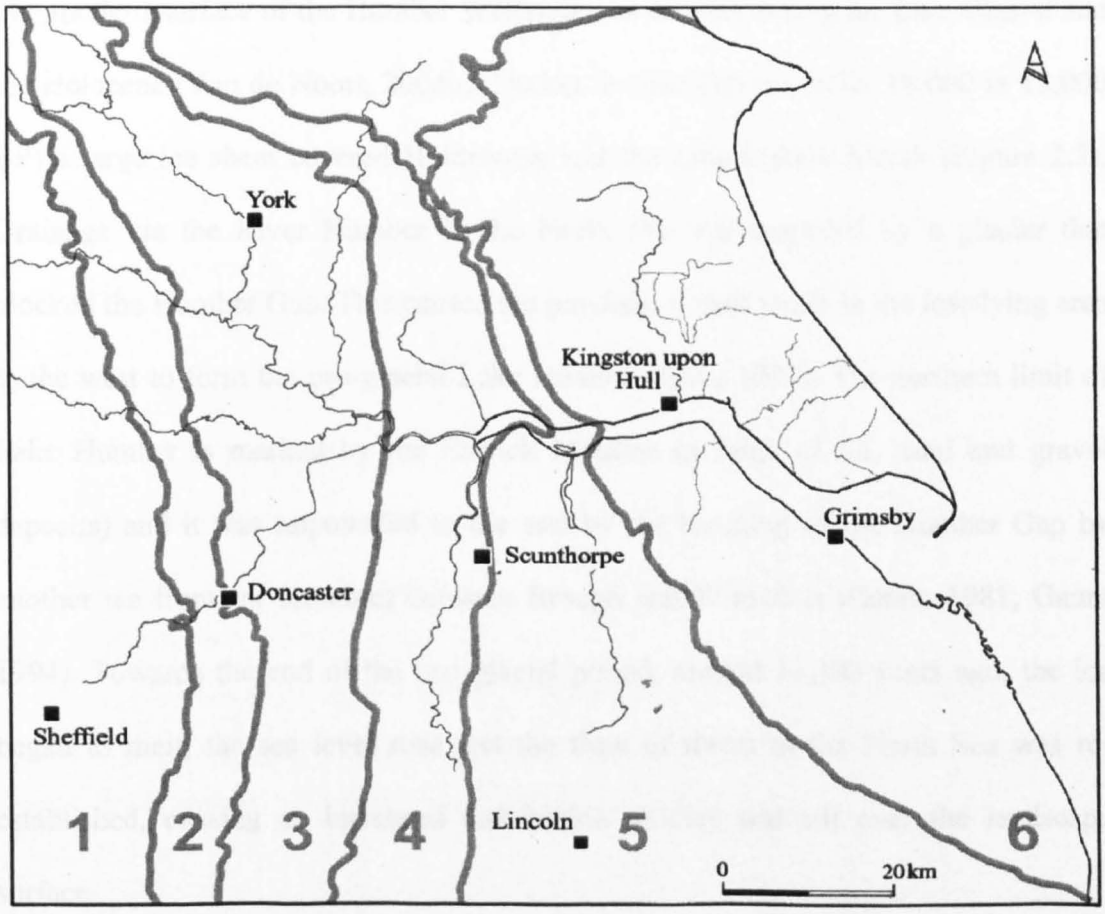


Figure 2.2: Map of the Humber Wetlands, showing the geology (simplified after Catt [1990] and Gaunt [1994] - *from* Van de Noort 2004b). **1**: Carboniferous Coal Measures; **2**: Permian Upper Magnesium Limestone and Upper Permian Marl; **3**: Triassic Sherwood Sandstone; **4**: Triassic Mercia Mudstone; **5**: Jurassic marls, limestones, sandstones and clays; **6**: Cretaceus Chalk (Van de Noort, 2004b).

The western boundary of the Humberhead Levels is formed by resistant Permian rocks (i.e. Lower and Upper Magnesium Limestone). The southern limit of the Levels merges with the Trent and Belvoir Vales. This area includes a low ridge of Triassic

rocks, which owes much of its existence to resistant gypsifeorus silstones, known as 'skerries'. The Humberhead Levels are bounded to the east by the Yorkshire Wolds and the Northern Lincolnshire Edge. To the north, they merge gradually with the Vale of York (Gaunt, 1994).

The modern surface of the Humber Wetlands was created during the Late Glacial and the Holocene (Van de Noort, 2004b). During the late Devensian (*c.* 18,000 to 13,000 BP) a large ice sheet covered Holderness and the Lincolnshire Marsh (Figure 2.3). Drainage via the River Humber to the North Sea was impeded by a glacier that blocked the Humber Gap. This caused the ponding of melt water in the low-lying area to the west to form the pro-glacial Lake Humber (Ellis, 1997). The northern limit of Lake Humber is marked by the Escrick Moraine (a ridge of till, sand and gravel deposits) and it was impounded to the east by the blocking of the Humber Gap by another ice front (or moraine) between Brough and Winterton (Gaunt, 1981; Gaunt 1994). Towards the end of the last glacial period, around 11,300 years ago, the ice began to melt, the sea level rose and the flow of rivers to the North Sea was re-established, causing an increased distribution of clay and silt over the landscape surface.

At the beginning of the subsequent Post Glacial period, the Holocene (or Flandrian), sea level was approximately 18 m below OD, which allowed for the incision of the rivers through the emergent lake plain (lake and associated deposits) to their base level at around that depth (Ellis, 1997). The continuing melting of the ice and subsequent sea level rise caused the process of river aggradation (i.e. the deposition of

alluvium in the river). The alluvial sediments were carried downstream by the rivers and upstream by the incoming tides of the Humber estuary (Ellis, 1997).

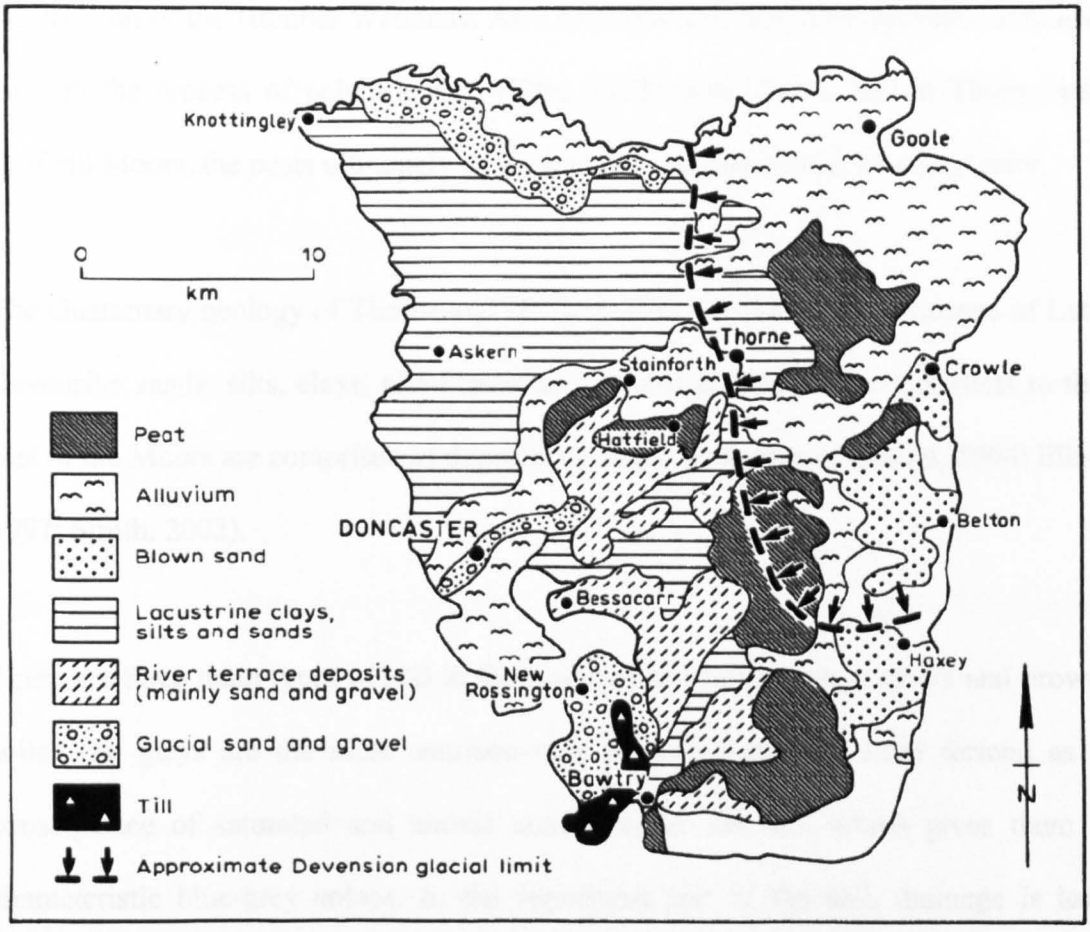


Figure 2.3: Quaternary geology of the Humberhead Levels (based on Institute of Geological Sciences, 1977 [from Van de Noort and Ellis, 1997]).

During the Holocene, sea level rise was nor constant. Fluctuations occurred, allowing for stand-still episodes, or slightly lowered phases, which allowed vegetation to colonise the poorly drained rivers and estuarine margins, promoting favourable conditions for the formation of peat (Ellis, 1997:8). By approximately 3,500 BP the river channels were mostly infilled, rising sea levels pushed the saline limits of the Humber Estuary further inland and the floodwaters tended to extend away from the channel margins, resulting in overbank alluviation (known locally as flood warp). Lake Humber had been filled with sediment (-mainly clay) creating gleyed soils,

which were locally overlain by peat as paludification progressed. Continuing sea level rise, combined with the restricted flow of the rivers in the area, ultimately resulted in the creation of the Humber Wetlands. As a consequence, peat development continued through the process of paludification (Ellis, 1997; Cole, 2001), and at Thorne and Hatfield Moors, the peats ultimately developed into extensive areas of raised mire.

The Quaternary geology of Thorne and Hatfield Moors is primarily composed of Late Devensian sands, silts, clays, and Flandrian peat and alluvium. The sediments to the east of the Moors are comprised of deposits of Older River Gravel (Gaunt, 1994; Ellis, 1997; Smith, 2002).

There are three main types of soil in the Humberhead Levels; gleys, peats and brown soils. The gleys are the most common type of soil; they have been formed as a consequence of saturated and anoxic conditions in the soil, which gives them a characteristic blue-grey colour. In the uppermost part of the soil, drainage is less restricted and the gleys (surface water gleys) acquire yellow, brown and orange colours due to the process of iron oxidation. Peat soils have developed at the interface between the water table and the land surface, where vegetation decay is inhibited. Brown soils are associated with less restricted drainage. These soils are located in the slightly higher marginal lands of the region (Ellis, 1997).

2.1.3 Regional vegetation history and environmental change

There is only limited palaeoecological evidence for the Late Glacial period in the Humberhead Levels. As a consequence, the information for this period has generally been extrapolated from an adjacent region (i.e. a palaeoenvironmental sequence from

the Vale of York) (Dinnin, 1997). The palaeoenvironmental sequence from this region showed three different periods or pollen sub-zones:

1) 13000-12000 BP; *Betula* woodland and herbaceous flora and grass dominated the landscape.
2) 12000-11000 BP; Late Glacial *Betula* woodland, and *Gramineae* and *Cyperaceae* remains are well represented.
3) 11000-10200 BP; the retreat of woodland and the expansion of ground vegetation indicative of the presence of tundra conditions (Dinnin, 1997).

The Holocene period is characterised by the absence of chronostratigraphic information for the Vale of York. As a consequence, the major bio-stratigraphic zones are based on correlations with dated material from other parts of the area (Dinnin, 1997). These are as follows:

1) 10200-9500 BP; *Betula* woodland dominated the area. *Pinus* and *Salix* were also in existence.
2) 9500-9000 BP; soils matured and trees spread from their glacial refuges. The *Betula* woodland was invaded by *Corylus*, *Pinus* and *Ulmus*.
3) 9000-6300 BP; a regional differentiation of forest mosaic occurred during this period, with pollen records of *Ulmus*, *Pinus*, *Quercus* and *Corylus*.
4) 6300-5200 BP; this period was characterised by a rise in *Alnus* and an increase in *Tilia*. *Tilia* is one of the most important arboreal taxa in the mid-Holocene within the area (Dinnin, 1997).

5) 5200-2600 BP; the decline in *Ulmus* is the first evidence for the human modification of Britain's primary forest by early agricultural practices. It is used to define the first period of the Neolithic (Godwin, 1975). There are also records of agricultural species such as *Plantago lanceolata* and *Plantago major* (Dinnin, 1977).

The peats of Thorne and Hatfield Moors have provided the main record of changes during the later prehistoric and historic periods. Two forest clearance episodes were assigned to the Iron Age and Romano-British periods. The latter period was followed by a decline in agricultural activity and woodland regeneration. The regional pollen zones constructed by Smith (1985) represent a series of forest and woodland clearance episodes from the Bronze Age to the Early Medieval period:

1) 4300-3600 BP; there is evidence of a dense forest dominated by *Quercus*, *Alnus* and *Corylus* trees, and lesser amounts of *Pinus* and *Tilia*.
2) 3600-2300 BP; during the Iron Age there was a sudden decrease in *Pinus* and *Tilia*, a spatially discontinuous decrease in *Ulmus* and a reduction in *Quercus*. There was an increase in the pollen and spores of agricultural taxa (*Plantago lanceolata*, *Pteridium* and *Urtica*).
3) 2335-1445 BP; the period between the Iron Age and the Roman period reflects the development of raised mire vegetation on Thorne and Hatfield Moors, with records of *Calluna* and *Cyperaceae* present. Buckland (1979) considered that at the end of the Roman period most of the forest in the area had been cleared for agriculture practices. This notion is evident by the presence of a mixed

farming economy, with records of *Gramineae* and arable cultivation plants such as *Secale cereale*.

4) The Post-Roman period (cal AD 440-680 to cal AD 1020-1270) showed evidence of agricultural decline and woodland regeneration. This was marked by the increase of *Quercus, Alnus*, *Fraxinus* and *Corylus* (Smith, 1985; Dinnin, 1997).

Throughout the Medieval period, large areas of the forest were destroyed. However, some areas of forest were preserved due to mire formation (Van de Noort and Ellis, 1999; Smith, 2002). The extension of the mire and the destruction of the forest were probably still taking place in the sixteenth and early seventeenth centuries (Smith, 2002).

2.1.4 Flora and fauna

At present, the Humber Wetlands is primarily flat and extensively farmed, mainly with 'industrialised' cropping systems. Root crops such as sugar beet (*Beta vulgaris*) and oilseed (*Brassica napus oleifera*), and cereal crops including wheat (*Triticum* spp.) and barley (*Hordeum vulgare)* dominate the landscape, along with mixed livestock farming. Woodlands are limited to the higher sandy ridges to the north and south, where relic birch (*Betula pendula*), oak (*Quercus robur*), and woodland coniferous plantations to the north and south of the area are present (Middleton, 1997).

There are pastures around Fishlake and Sykhouse which contain hedge rows, small ponds, drainage ditches, old field trees and parklands. These provide breeding habitats for amphibians such as the common frog (*Rana temporaria*), common toad (*Bufo*

bufo), and feeding habitats for the grass snake (*Natrix natrix*) (Sunter, 2006). In addition, there are alluvial flood meadows knows as Ings. The Ings around Goole and the Derwent Valley contain wetland vegetation. They are important habitats for wintering and migrating birds. The raised mires and peat deposits around Thorne, Hatfield, Crowle and Goole are also important in ecological and historical terms (Midldleton, 1997; DEFRA, 2005). There are small areas of heathland around Selby and the rivers Torne and Idle. Heathland are important habitats for reptiles such as the adder (*Vipera verus*) and common lizard (*Lacerta vivipara*).

2.1.5 Archaeology

2.1.5.1 The Humber Wetlands

In 1992, English Heritage commissioned the Humber Wetlands Project to investigate the palaeoenvironmental and archaeological resources within the Humber Wetlands (Van de Noort *et al.*, 1993). At present, a significant proportion of the Humber Wetlands region has been surveyed. In addition, there are approximately thirty sites which have been excavated. The archaeological sites in the Humber wetlands are abundant, especially in low lying, former wetland, areas. These sites range in date from the Early Mesolithic to the Middle Ages (Van de Noort, 2001b).

An example of one of these sites, located in an alluvial floodplain setting, is Stone Carr, which is a Late Mesolithic flint production site in the Hull Valley. During recent excavations, 780 worked flints (including a knife, a microlith and a pick) and 16 cores were discovered (Lillie, 2001). This site is one of a number of locations that demonstrate a close correlation between hunter-gatherer activity sites and the rivers of

the region; highlighting the rich resource base in riparian settings that was being exploited throughout the earlier Holocene

Two trackways, and a number of fish traps, of Bronze Age date, have also been found on the Melton foreshore, in the inter-tidal area of the Humber. Parts of the two trackways have recently been excavated, and the construction of these trackways has been shown to consist of hurdle panels of woven hazel rods. It is probable that these trackways were used to access the fertile saltmarsh for pasture use (Thomas, 2001).

Perhaps the most famous finds from the Humber are those of the fragmented remains of four Bronze Age boats, which were discovered by Ted Wright and his brother, at North Ferriby, in the clays of the Humber foreshore (McGrail and Millet 1985; Wright and Switsur, 1993; Thomas, 2001; Wright *et al.*, 2001; Chapman and Chapman 2005; Lillie, 2005). In addition, other prehistoric finds have also been recovered in this area; these include fragments of two ash (*Fraxinus*) paddles, possible boat components, wood chips and a forked timber (Chapman and Chapman, 2005).

A henge at Easington, on the east coast (Holderness region), dating from the Late Neolithic or Early Bronze Age, was discovered in 1998 and excavated by archaeologists from the Humber Wetlands Project. The henge included an outer and inner circular ditch-and-bank. The outer ditch and bank was approximately 30 m in diameter. Although the henge had at least one entrance, the destruction by erosion of part of the monument did not allow for the further identification of other structures. In the inner ditch, an *in situ* cremation was found; this was dated to approximately 2500 BC (Van de Noort, 2001c). The Easington henge is effectively a terrestrial monument,

but it now lies in a coastal position and experiences the vagaries of tidal inundation. In addition to this find, two Bronze Age barrows were also discovered on the beach at Easington; both of these structures would have originally been inland from the Bronze Age coast. These were excavated by the East Riding of Yorkshire Archaeological Society.

In the Early Iron Age, at Sutton Common, near Askern in the Humberhead Levels there appears to have been a high status settlement or refuge (Van de Noort and Fenwick, 1997). This site combines aspects of both wetland and dryland archaeology. In addition, near Kelk in the Hull Valley, an Iron Age enclosure has recently been excavated. This site was located on the banks of the River Hull, and provided a variety of finds including cups, moulds and a crucible (Fletcher, 2001). The function of this site has been interpreted as representing a metal working/production site.

There are ninety sites identified as scheduled monuments in the region; and of these over fifteen prehistoric records exist. However, none of these are considered to be settlements (the two Iron Age enclosures at Sutton Common await publication [as of October 2007]). The majority of the other sites are burial monuments, such as the Bronze Age barrow cemetery at Butterbump House, Willoughby in the Lincolnshire Marsh, which is located adjacent to a wetland, and the barrow cemetery, a dryland site, on Skipwith Common in the Vale of York (Van de Noort and Davies, 1993).

Monuments of the Roman period have also been recovered; including a possible villa in Wadworth Carrs, a Roman military fortress at Rossington, and a fort at Roall, all dryland sites located close to wetland areas. Small-scale excavations in the area have

also identified Roman pottery (Magilton, 1977). On a different scale, one of the largest excavated concentrations of Roman pottery kilns (ovens) in Britain has been discovered close to Doncaster. The location of these kilns is probably due to good water transport routes in the area; afforded by the rivers Torne and Done (Van de Noort and Fenwick, 1997). At Scaftworth, the remains of a Roman bridge and a road were also excavated (Van de Noort, 2001d). The road and bridge cross the floodplain of the Idle at this location, and well preserved waterlogged timbers relating to the two phases of road construction were recovered during excavations by the Humber Wetlands Survey (Van de Noort *et al.* 1997).

Archaeological evidence for human activity in the Early Middle Ages is limited (Buckland *et al.*, 1989). Although there is a lack of any notable remains from the Middle Ages, such as the remains of buildings or deserted villages (Van de Noort and Fenwick, 1997), a Late Anglo-Saxon salt production site has been previously excavated at Marsh Chapel in the Lincolnshire Marsh (Fenwick, 2001). This site would have originally functioned within the inter-tidal zone of the east coast during the period of use.

2.1.5.2 The Humberhead Levels

The area to the south of the rivers Aire, Ouse and Humber, west of the River Trent along a line drawn north and south of the Isle of Axholme, and bounded to the south and west by the 10 m OD contour line, has been defined as the Humberhead Levels by the Humber Wetland Project (*cf.* Etté and Van de Noort, 1997:2).

A number of the sites from this region, e.g. Sutton Common, Scaftworth and Rossington have been mentioned above. However, *c.* 44 key sites were studied as part of the Humber Wetlands Survey of this region (Van de Noort and Ellis, 1997). Significant sites include Misterton Carr, which again reinforced the exploitation of floodplain wetlands in the region. The Humber Wetlands Survey investigated or identified *c.* 113 sites or finds concentrations in this region.

Significant biases in the distribution of sites were attributed to the variability in overbank flooding deposits, or deliberate warping endeavours undertaken in the more recent historical period (*cf.* Lillie, 1997:193). In addition, the raised mires of Thorne and Hatfield Moors were considered to hold the potential both to mask significant areas of the landscape, and to have the potential to contain important wetland sites.

This latter observation was confirmed by the discovery of a trackway and platform at Hatfield Moor; located at Lindholme Island (discussed in detail below in section 2.2.4) (Chapman and Gearey, 2006a and b). This trackway is *c.* 50 m in length, terminating at a platform structure, and has been dated to the later Neolithic period. The discovery and investigation of this site lends weight to earlier assertions of a trackway or crossing point of Bronze Age date investigated by Buckland (1979) in Thorne Moors, and when considered alongside the evidence for bog bodies from the Moors, it is perhaps tempting to suggest that the extremely limited archive of finds from these wetlands represents only a small proportion of the possible actual number of sites that could have existed, but which have been rendered 'invisible' due to the active exploitation of the Moors for peat, both historically, and in recent decades through mechanical extraction processes.

As one of the primary intentions of the Humber Wetland Project was to establish a management programme which would protect archaeological sites and provide additional sources of research (Van de Noort, 2001b), the study of Sutton Common, undertaken here, adds considerable information that can enhance developing management strategies for this region. However, it should be noted that, at present, the strategies employed towards the protection of England's wetlands remain poorly defined and lack clear focus (*cf.* Van de Noort *et al.*, 2002). This situation has resulted in a lack of additional, targeted research in the Humber Wetlands as a whole, and in part, reflects a reluctance on the part of heritage managers to undertake research into wetland sites, due to the inherent costs involved.

In addition, the current (PPG16) approach to preservation *in situ* has changed modern approaches to the archaeological record, and as a consequence the present research has the potential to inform policy towards the preservation of waterlogged archaeological remains *in situ*. The archaeology of the Moors is considered in more detail below (Section 2.2.4).

2.1.6 Threats and damage affecting the natural resources and archaeology in the Humberhead Levels (and Humber Wetlands)

The Humberhead Levels has suffered considerable environmental damage over recent centuries. Land drainage, conversion of pasture into arable land, and peat extraction are the greatest threats affecting the area.

Land has been drained in the Humber Levels for agricultural improvement, which has led to a reduction in the wetland acreage (Van de Noort and Davies, 1993). Land

drainage can be achieved by increasing the water run-off from the soil surface (Thomasson, 1975). Increased run-off can be undertaken by using several different methods, such as the straightening of rivers, the construction of cut-off channels and the construction of retention reservoirs (washlands) (Van de Noort and Davies, 1993; Van de Noort *et al.*, 2001).

The large scale drainage of land for agricultural improvement in the Humber Levels commenced in the first half of the seventeenth century and was undertaken by the engineer Cornelius Vermuyden. This drainage led to the diversion of the rivers Don, Went and Idle, and subsequently converted waste land into pasture (Dinnin, 1997). The embankment of these three principal rivers also contributed to a change in the hydrological regime of the area. The main purpose of this drainage was to increase the quality of the land for agricultural use.

Alongside this drainage, the practice of deliberately flooding large areas of embanked land to add fertile sediment to it (-termed 'warping'), raised the level of the land. Warping consists of the repeated flooding of embanked land with tidal waters to leave a layer of sediment behind. The embanked area was dissected by a series of drains to ensure an even flow into the compartment, and water movement was controlled by a series of sluices, which kept the water in the compartment as the tide receded (Lillie, 1997). The fine silts and clays that were deposited were extremely fertile and directly contributed to the richness of the arable land in this region. The principal area of the Humberhead Levels that was associated with warping is located on the western, north and eastern sides of the Moors, and it has been recorded that *c.* 1220 acres of Hatfield Moor were warped by 1882 (Lillie, 1997:196).

The richness of the land in the Humberhead Levels has further contributed to the demise of the wetland resource, as water abstractions for agricultural and domestic use via licensed boreholes installed in the field contribute to the removal of water from the system. This can cause severe problems in the Humber lowlands due to the lowering of the regional water tables (Van de Noort and Ellis, 1999; Van de Noort *et al.*, 2002). The lowering of the water table not only affects the environmental nature of wetlands, but also influences the preservation of archaeological and palaeoenvironmental remains by introducing oxygen in the system and increasing biological activity (Van de Noort and Davies, 1993; Panter, 2007). The conversion of pastures into arable land is also an important threat to the archaeological resource, particularly in respect of deep ploughing regimes (Van de Noort and Davies, 1993; Van de Noort *et al.*, 2002).

The main cause of damage to Thorne and Hatfield Moors is peat extraction. Although this has occurred from the Roman period onwards, it has been especially pertinent from the 1960's to the present day (Van de Noort and Fenwick, 1997). The mechanization of peat cutting for horticultural use after 1960 was the main cause of damage to the archaeological resource of the moors. Previous research has shown that although archaeological remains dated from the Early Bronze Age to the Early Iron Age have been discovered in areas of peat extraction, the majority of these have been recovered in a damaged condition (Van de Noort and Davies, 1993).

The extraction of sand and gravel is considered to be another causal factor which damages the archaeological record in the Humber Wetlands. Within Humberside, during 1984, there were over twelve sand and gravel sites either in operation or with planning permission granted (Van de Noort and Davies, 1993), and the exploitation of

aggregates from the regions river systems is changing the nature and function of the lowland floodplain wetlands of the region.

Erosion is another source of damage affecting the Humber Wetlands. Along the Holderness coastline, the low boulder clay cliffs are suffering from tidal erosion caused by the North Sea. Near Easington, Bronze Age barrows and Roman ditches have been lost due to sea erosion (Sheppard, 1912). With the aim of protecting the Holderness coastline, barriers have been constructed along the coast in order to reduce sea erosion over the next hundred years (Van de Noort and Davies, 1993). The damage produced by erosion also takes place in the Humber Estuary, caused in part by the shifting deep water shipping channel which is unconstrained and prone to realignment. The process of erosion has contributed to the unearthing the North Ferriby boats (Wright, 1976) and the timber structures at Melton (Crowther, 1987).

The ongoing urban and industrial development in the Humber Levels is also affecting the environmental resources of the region (Van de Noort and Ellis, 1999; Van de Noort *et al*, 2002). In 1993, there were twenty locations of industrial development, the majority of which were located within or around the estuary frontage (Van de Noort and Davies, 1993).

2.2 Thorne and Hatfield Moors

2.2.1 Introduction

Thorne and Hatfield Moors are located in South Yorkshire and are included in the Humberhead Levels region (as discussed above; Figure 2.4 below). In the past, they were the most extensive areas of raised mire in lowland Britain (Eversham *et al.*,

1995). They currently form the Humberhead Peatlands National Nature Reserve and when combined they still cover an extensive area totalling 2449.2 ha.

Both Thorne and Hatfield Moors have been classified as Sites of Special Scientific Interest (SSSI) under the Wildlife and Countryside Act (1981) and as Special Protection Areas under EC legislation, qualifying as Wetlands of International Importance under the terms of the RAMSAR Convention (1971). In 1985, part of Thorne Moor was designated as a National Nature Reserve (NNR).

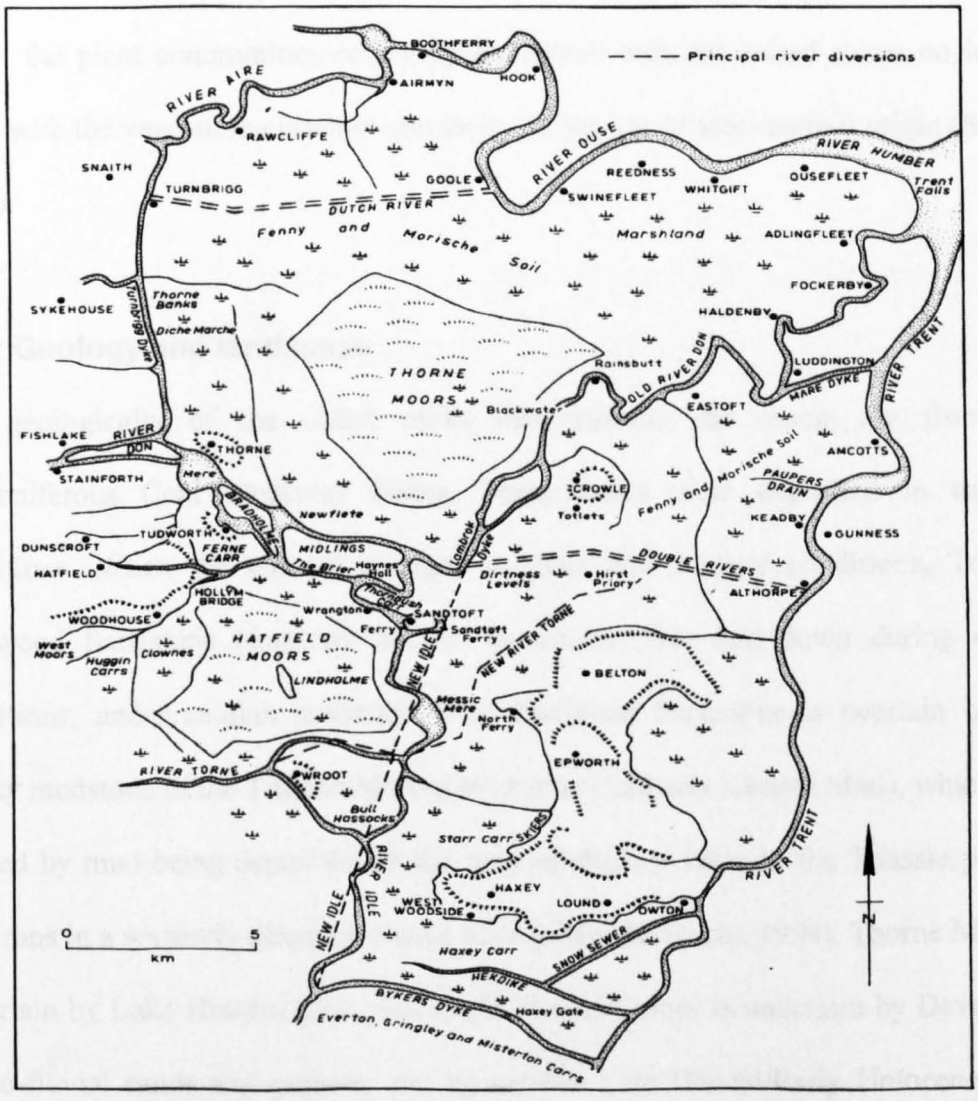


Figure 2.4: Location map of Thorne and Hatfield Moors (from Van de Noort and Ellis, 1997).

Thorne and Hatfield Moors have previously been identified as one of the most important Holocene sites in Britain. As such, they are deemed to be of international importance (Smith, 1985; Buckland and Dinnin, 1997; Dinnin, 1997; Whitehouse *et al.*, 1997; Whitehouse *et al.*, 2001; Boswijk and Whitehouse, 2002; Buckland and Smith, 2003). The moors contain significant sedimentary features, such as a buried prehistoric forest, a late glacial dune system and a terminal Devesian moraine (Smith, 1985). They also contain important fossil insect species; and have excellent preservation of records of mire development, regional landscape change and Holocene climate (Dinnin *et al.*, 1997). Although the moors still have significant vegetation cover, the plant communities originally associated with the raised mires no longer exist, with the vegetation currently consisting of species of semi-natural origin (Smith, 2002).

2.2.2 Geology and landscape

The geologically of the oldest rocks that underlie the moors are from the Carboniferous Coal Measures Series. These rocks were deposited in tropical conditions. When the climate changed to drier and warmer conditions, Triassic Sherwood Sandstone (formerly Bunter Sandstone) was laid down during desert conditions, under aeolian processes. The Sherwood Sandstone is overlain by the chalky mudstone of the Triassic Mercia Mudstone (formerly Keuper Marl), which was formed by mud being deposited on the beds of shallow lakes in the Triassic period. This runs in a northerly direction across Thorne Moors (Gaunt, 1994). Thorne Moor is underlain by Lake Humber clay silts, while Hatfield Moor is underlain by Devensian glacio-fluvial sands and gravels, and an aeolian Late Glacial/Early Holocene sand dune system (Gaunt, 1994).

Radiocarbon dating has indicated that peat development began on both moors approximately 4500 years ago (Smith, 1985). Before this period, the moors were covered by woodland (Boswijk and Whitehouse, 2002). Raised mire development began through the process of paludification and increased in height due to rising Holocene sea levels. The associated impeded movement of water resulted in the isolation of the vegetation from the groundwater (Dinnin, 1997). The system was exclusively fed by rain water, favouring the creation of ombrotrophic conditions. The accumulation of dead plant material further isolated these raised bogs (Chapman and Gearey, 2003). The Moors consist of the remnants of what was once an extensive complex of raised mires and other wetlands, and are the two largest surviving examples of lowland raised mire peatland in England (Dinnin 1997).

The development of the two moors is markedly different. Thorne Moor was covered by undisturbed mixed forest before the accumulation of peat. Subsequently, the mire developed through the succession of minerotrophic fen (that received groundwater and/or surface flow from the surrounding watershed), mesotrophic fen (which developed under the influence of groundwater), ombrotrophic fen (not influenced by groundwater and receiving only atmospheric nutrient inputs) and oligotrophic (nutrient-poor) bog. Hatfield Moor was initially covered by pine forest and heath. Afterwards, mesotrophic and then ombrotrophic peat developed over the original surface as the preceding heathland became increasingly waterlogged (Smith, 1985; Dinnin, 1997; Van de Noort and Ellis, 1997; Kellogg *et al.*, 2003).

2.2.3 Flora and fauna

More than four thousand species of plants and animals, including twenty-five of Britain's rarest species, have been recorded on the moors. Bog habitats contain a characteristic flora, such as common cotton grass (*Eriophorum angustifolium*), mosses (*Sphagnum* spp.), cranberry (*Vaccinium oxycoccos*) and bog-rosemary (*Andromeda glaucophylla*). The drier areas of the peatland contain bracken (*Pteridum aquilinum*), heather (*Calluna vulgaris*), common reed (*Phragmites australis*) and birch (*Betula pendula*). Marsh orchid (*Dactylorhiza praetermissa*), twayblade (*Listera ovata*), greater yellow rattle (*Rhinanthus serotinus*) and bird's foot trefoil (*Lotus corniculatus*) can be found.

There are large areas of peat on Thorne and Hatfield Moors that are without vegetation, following extensive peat-cutting activities. However, the south-western part of Hatfield Moor contains relict mire plant and insect communities; while Lindholme 'Island' in the centre of the bog supports calcareous grassland and woodland (Whitehouse, 2004). The moors also provide habitats for a wide range of uncommon invertebrates. Despite the damage caused by the peat extraction in the area, the moors have the largest concentration of Red Data Book and nationally notable invertebrate species at a single location. The moors are the only site in Britain where the Mire Pill Beetle (*Curimopsis nigrita*) is found; and the only site in Northern England which supports the Giant Raft spider (*Dolomedes fimbriatus*). Water beetles (such as *Hydroporus rufifrons* and *Lymnebius nitidus*), wolf spiders (*Lycosa* spp.), dragonflies (*Libellula fulva*), craneflies (*Phalacrocera replicata*), hairy canary flies (*Phaonia jaroschewskii*) and large head butterflies (*Coenonympha tullia*) are also in abundance.

The diversity of habitats that exist within Thorne and Hatfield Moors contributes to their ornithological interest. There are different species of breeding birds such as the common teal (*Anas crecca crecca*), common snipe (*Gallinago galinago*) and tree pipit (*Anthus trivialis*); while the population of nightjar (*Cuprinulgus europaeus*) has international importance. Wintering birds include the hen harrier (*Circus cianeus*) and merlin (*Falco columbaris*). Reptiles such as adder (*Vipera verus*), grass snake and common lizard (*Lacerta vivipara*) are also present. Some mammals can also be found on the moors; these include roe deer (*Capreolus capreolus*), fox (*Vulpex vulpex*) and brown hare (*Lepus capensis*).

2.2.4 The archaeology of Thorne and Hatfield Moors

As mentioned above, archaeological finds at Thorne and Hatfield Moors include a wooden trackway which is dated to the Bronze Age. It is constructed of oak timbers of various sizes and was possibly used as a temporary crossing place (Buckland, 1979). There are also records of five well-preserved human bodies from Hatfield Chase and Thorne Waste (Dinnin *et al.*, 1997). In addition, a series of prehistoric finds (e.g. Neolithic flint flakes, and Mesolithic and Neolithic axes), lithic scatters and worked timbers have also been found in the area (Dinnin, 1997; Van de Noort and Ellis, 1997; Van de Noort and Fenwick 1997; Van de Noort, 2001a).

On Hatfield Moor, adjacent to the Island of Lindholme, there are important records of Neolithic and Bronze Age artefacts being found (Eversham *et al.*, 1995). In addition, there is also possible Mesolithic evidence of fire use, due to the discovery of charred wood burial remains of pine and oak on the underlying sands and gravels (Eversham *et al.*, 1995).

The most recent find on Hatfield Moor was in October 2004. A 50 m trackway which was made of pine logs was found in the centre of the moor (Figure 2.5). This was dated to the Late Neolithic (2900-2500 BC) period (Chapman and Gearey, 2006a and b). Wood samples were extracted from this trackway during November 2004 and subjected to microbial assessment (as described in Chapter 4). Unfortunately, some sections of the trackway have been exposed and damaged by peat extraction.

Figure 2.5: Nora Bermingham and Ben Gearey during excavations in November 2004 at the northern end of the trackway.

This type of trackway is known as a 'corduroy' trackway. The timbers were laid transversely to the direction of movement on pairs of longitudinal rails. The Hatfield trackway is the earliest example of its kind in Britain and is the only prehistoric wooden trackway with both ends complete (Chapman and Gearey, 2006a and b).

The function of the trackway had not been established at the time of writing. This is primarily due to the lack of archaeological finds on the site. However, there are different hypotheses regarding its function. Research has suggested that the trackway was either constructed as a means of crossing the wetland, or to access the pool located there during Neolithic times, for hunting or fowling (Chapman and Gearey, 2006a and b).

In addition, the wooden platform at the end of this trackway has been interpreted by Chapman and Gearey (2006a and b) as being a possible ceremonial structure, with the trackway potentially represented a narrowing route to access the platform. A combination of these hypotheses could also account for the sites function. The results from the assessment in 2004 showed that the wood from the trackway was poorly preserved, with no suitable areas for long term *in situ* preservation (*ibid.* 2004).

2.2.5 Peat extraction and drainage

As has been previously highlighted, the most important threat to Thorne and Hatfield Moors was peat cutting and extraction. In the fourteenth century, peat was cut by local people and used as fuel and horse and cattle litter. During this period, peat-cutting was undertaken by hand digging on a small scale. However, in the 1880s, commercial peat cutting started in response to the growing need for animal bedding. The methods employed to cut the peat were such that big areas were left undrained between the cutting periods, allowing plants and animals to re-colonise the area (Eversham, 1991).

By the 1980s, peat extraction had grown into a large industry (Eversham, 1991). Modern peat cutting for horticultural use was both extensive and mechanised (i.e. the

peat milling method). In 1990, peat extraction at Thorne and Hatfield Moors covered a total area of 1600 ha and 1200 ha, respectively. The peat milling method involved the removal of a superficial layer of peat (surface stripping) from the top 4-5 cm, over a vast area of land. Subsequently, the peat was dried and stored in large heaps. To facilitate this process, large drainage ditches were cut to lower the water table and allow the surface to dry. As a consequence of this process, and despite the status of Thorne and Hatfield Moors as Sites of Special Scientific Interest (SSSI), the moors suffered from severe desiccation. Furthermore, the extraction of peat in this area was extremely damaging to both the archaeological and palaeoenvironmental resources (Van de Noort *et al.*, 1993; Van de Noort and Ellis, 1997).

Approximately 1.0-1.5 m thickness of peat remains in the southern part of the moors, with only 0.5-1.0 m left in the remaining area. This figure contrasts with information from historical records which show original depths of at least 4.5 m; indicating that a significant portion of the historic resource has been lost (Whitehouse, 2004).

Peat extraction ceased on Thorne and Hatfield Moors in 2004 when an agreement was reached between the Scotts Peat Company and English Nature (now Natural England) (who bought the rights to the land). The main objectives of English Nature are to conserve the flora and fauna in the area, and protect the remaining archaeological and biological record within the surviving peat deposits (Eversham *et al.*, 1994).

2.2.6 Conservation and management

The environmental importance of Hatfield Moors is primarily due to the presence of a high percentage (88 %) of raised bog that, although degraded, is still thought to be

capable of natural regeneration (Natura, 2000). Actions in support of this natural regeneration include the cessation of peat extraction, the maintenance of a high water table by a system of pumped re-charging, and the maintenance of good quality water with low oxygen and nutrients. These measures have directly benefited the archaeological and palaeoenvironmental resource, even though the preservation of these was not considered in the management plan for the area (Van de Noort, 2001a).

The sampling point at Hatfield Moors (Figure 2.6) is located on the surface of a paleochannel feature, to the north of a drainage ditch, within which English Nature has kept an artificially elevated water table (Figure 2.7). This was undertaken in an attempt to generate an area of enhanced biodiversity as part of the Countryside Agency's 'Value in Wetness' initiative. The initiative was originally designed to identify new, economically viable and environmental sustainable approaches for a better management of water resources and land within the area.

Figure 2.6: Monitoring point at Hatfield Moor showing piezometers and redox probes *in situ*.

One of the main objectives of the Value in Wetness initiative was to control water level fluctuations in order to favour an environment which will allow the potential preservation of archaeological remains (The Countryside Agency, 2005). Since the amalgamation of a number of agencies under the banner of Natural England the management of the Value in Wetness initiative has become moribund, and is no longer employed as a strategy for sustainable land management in the Humberhead Levels.

Figure 2.7: Hatfield Moors sampling location showing an artificially elevated water table.

2.3 Sutton Common

2.3.1 Introduction

Sutton Common comprises three fields encompassing an approximate area of 170 ha. Located 9 km north of Doncaster, near the village of Askern, South Yorkshire, this is an Iron Age archaeological site, with two 'enigmatic' enclosures (Figures 2.8 and 2.9). The large enclosure is situated on the right bank of a former river or stream and the smaller enclosure is sited on the opposite bank. Both enclosures were linked by what appears to be a ceremonial walkway and were divided by a palaeochannel (known as Hampole Beck). Previous research has suggested that these enclosures were used as a 'marsh fort', which used the surrounding marsh land for defensive purposes (Van de Noort, 2004a).

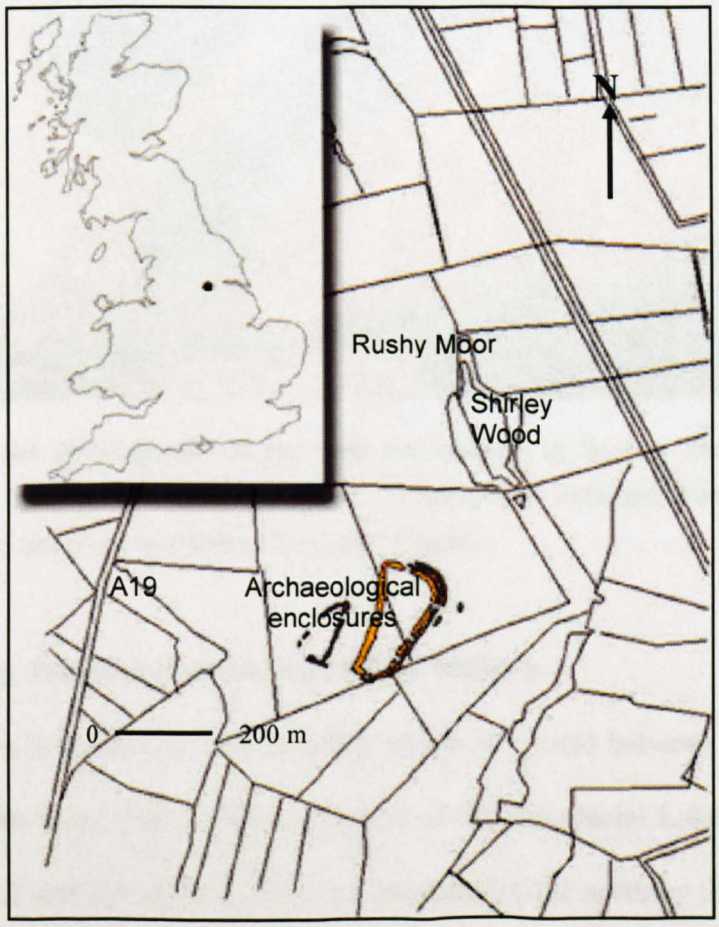


Figure 2.8: Location of Sutton Common and archaeological enclosures (Cheetham, 2004).

Sutton Common contains a Scheduled Ancient Monument (SAM) and a Site of Special Scientific Interest (SSSI). The SSSI consists of Shirley Pool which is an area of open water bordered by trees and Rushy Moor which is an area of wet bog and alder woodland. The bog and the open water area were probably formed by Medieval and later peat extraction (Pearson and Sydes, 1995).

Figure 2.9: Aerial photograph of the two enclosures at Sutton Common and the palaeochannel known as Hampole Beck. (Photograph obtained from © the Sutton Common Project, and the universities of Hull and Exeter).

2.3.2 Geology, landscape and vegetation history

Sutton Common is situated on part of a bog which is located between the Magnesium Limestone to the west, and the silts and clays of the pro-glacial Lake Humber to the east (Figures. 2.2 and 2.3 above). The site is bounded to the north by the River Aire, to the east by the River Trent, and to the south and west by the Pennines (Gaunt, 1994; Pearson and Sydes, 1995). Within this area, a palaeochannel, the Hampole Beck runs

along the ridge of the Upper Magnesium Limestone; thereafter meandering between the archaeological enclosures of Sutton Common and continuing to run in a northerly direction past the site (Head *et al.*, 1997). Calcareous conditions exist at Sutton Common due to the location of the site between Magnesium Limestone to the west and Upper Permian Marl to the east (Pearson and Sydes, 1995).

At Sutton Common, peat formation began between *c.* 5320 and *c.* 3990 cal BC in the palaeochannel feature and the associated enclosures. Peat deposition occurred until the Mid-Late Medieval period (Lillie, 1997). A basal peat sample obtained from the east of Shirley Wood produced pollen assemblage which was datable to the Late Bronze Age/Iron Age. The pollen sample contained *Graminae* (45 %), *Cyperaceae* (26.5 %), *Salix* (8 %) and *Betula* (8 %). There was low arboreal pollen consisting of *Quercus*, *Alnus*, *Fraxinus*, *Ulmus* and *Myriophyllum* as trace components. A high representation of aquatic plants was also detected; this included *Sparganium* (8 %), *Typha latifolia* (4 %) and *Achillea* (2 %). The palynological assemblage indicates the presence of locally open conditions during this period. Iron Age anthropogenic forest clearance episodes have been detected through the region, with the high levels of *Graminae* and *Plantago* reflecting a pastoralism-dominated mixed farming economy (Lillie, 1997).

2.3.3 Flora and fauna

The sampling location at Sutton Common is in pasture land which is grazed by cattle. The vegetation is dominated by species such as ray grass (*Lolium perenne*), stinging nettle (*Urtica eolica*), greater plantain (*Plantago major*) and common bent. In addition, it is possible to find a calciphile flora with species such us parnassus grass (*Parnassia*

palustris), black bog-rush (*Schoenus nigricans*), blunt flowered rush (*Juncus subnodulosus*) and common reed (*Phragmites australis*).

Due to grassland and livestock dominating the area, no significant fauna is present at this location. However, one endangered species, endogenous dipteran (*Stenomicra cogani*) has been recorded. Additionally, the area is well known for its dragonfly populations (Meade, 1991; Pearson and Sydes, 1995).

2.3.4 Archaeology

Sutton Common is an important Iron Age archaeological site, comprising two distinct enclosures (Figure 2.10). The largest enclosure measures approximately 250 m in length by 130 m in width, and the smallest enclosure measures approximately 150 m in length by 80 m in width (and is more triangular shaped in appearance [Cheetham, 2004]). Both enclosures are banked and ditched (Pearson and Sydes, 1995; Van de Noort *et al.*, 2001), and within the largest of the enclosures, early occupational remains from the Neolithic and Early Bronze Age have been discovered. Excavations undertaken in 2004 have shown that Sutton Common was re-used either as a cemetery, or for the deposition of cremated human remains; probably at the end of the Iron Age or the beginning of the Roman period (Van de Noort, 2004a).

The importance of Sutton Common is due to the survival of wood and other organic remains below the depth of ploughing (Figure 2.11) (Pearson and Sydes, 1995). Although both enclosures survived as earthworks until 1980, the larger enclosure was bulldozed and subsequently drained for agricultural improvement. The extensive drainage of the area significantly lowered the water table thereafter (*ibid.* 1995).

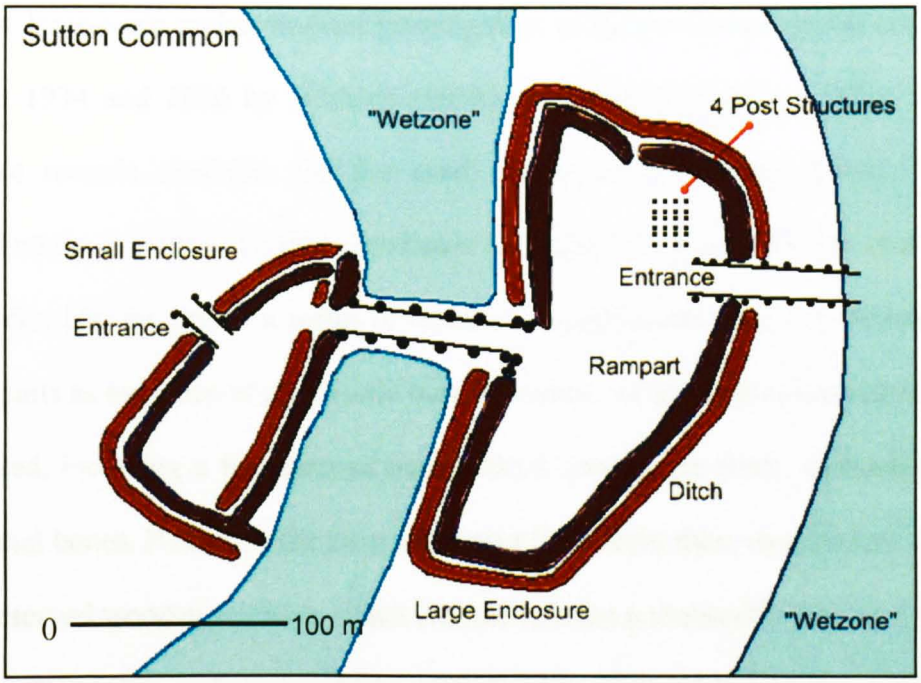


Figure 2.10: Plan of the enclosures (obtained from the University of Exeter). The dark brown line represents the rampart and the light brown area shows the ditches. The blue 'wetzone' shows which areas are likely to have been waterlogged in order to ensure the defensive nature of the enclosure system.

Figure 2.11: Part of the excavated palisade from the larger enclosure at Sutton Common (1999). The timbers are oak and have a 'pencil-point' which was driven into the sands and clays. (Photograph obtained from © the Sutton Common Project, and the universities of Hull and Exeter).

The first large-scale archaeological investigation at Sutton Common was undertaken between 1934 and 1936 by Whiting (1938). These excavations provided the only extensive records available for the small enclosure (Cheetham, 2004). Whiting established the presence of a timber palisade and ramp which predated the multivallate earthworks. He interpreted a series of circular and sub-rectangular depressions along the ramparts as evidence of prehistoric huts. A number of archaeological remains were discovered, including a fragment of human skull, prehistoric flints, carbonised grain and animal bones. However, the most important finds from these excavations were the well-preserved wooden artefacts which included planks a timber-lined pit and a wheel. Unfortunately, none of these artefacts survive today (Pearson and Sydes, 1995). As a result of Whiting's work the enclosures were recognised as a nationally-important monument.

Between 1987 and 1988, English Heritage funded evaluation studies of both enclosures (Sydes and Symonds, 1987; Adams *et al.*, 1988; Pearson and Sydes, 1995). In 1990, working in conjunction with English Nature, English Heritage undertook a detailed survey of the complete common (Pearson and Sydes, 1996). This survey provided the foundations for understanding the topography and hydrology of the site. It showed that the peat was suffering severe desiccation, the groundwater levels were low, and that the buried organic remains in both enclosures were unstable (which was probably due to an increase in bacterial activity that was associated with the excavation in 1987).

In 1993, a further project (Pearson and Merrony, 1993) assessed degradation rates in the small enclosure and discovered that the wooden artefacts had decayed even further,

despite the presence of water at the base of the excavated ditch. In 1994, although English Nature raised the water levels in the SSSI, Sutton Common was excluded from this due to doubts regarding the state and degree of desiccation of the archaeological remains (Pearson and Sydes, 1995).

English Heritage also funded excavations in 1998 and 1999. These excavations were undertaken by the University of Hull, and showed that only the basal 20 cm of the ditch deposits were waterlogged in the small enclosure. As a consequence, this proved to be the only location on the site where the potential existed for the continual preservation of organic archaeological remains (Van de Noort, 2004a).

During the summers of 2002 and 2003, further excavations of the large enclosure were undertaken due to the rapid desiccation of the organic archaeological remains within (Cheetham, 2004; Van de Noort, 2004a). The material found during these excavations was mainly the severely degraded ends of wooden posts and stakes. In contrast to this, some wooden posts were preserved in the fills of ditches along the eastern and southern edge of the larger enclosure. They consisted of vertical stakes which were interwoven by rods. The better preservation of these remains is due to their location below the level of the water table, where they were not affected by seasonal fluctuations in water level (Cheetham, 2004; Van de Noort, 2004a).

The excavations of 2003 and 2004 highlighted a number of important finds. These included flints and pottery fragments from the Iron Age, three fragments of quern stones (possibly with a ritual or structural function), a bone weaving comb, a glass bead from the Late Iron Age-Early Roman period and a gold strip associated with a

Roman cemetery. In addition, a number of human remains were found, including two skulls and seven deposited cremation remains (Van de Noort, 2004a).

2.3.5 Drainage activities

In 1982 Sutton Common was drained and converted to arable land. The lowered groundwater levels subsequently affected the preservation of the SSSI and the SAM (Pearson and Sydes, 1995; Cheetham, 2004; Van de Noort, 2004a). Between 1980 and 1997 the land has supported many different types of arable crops. This cultivation caused considerable damage to the archaeological remains beneath due to root penetration and ploughing activities.

As a consequence of the actions highlighted above, English Heritage acquired the ownership of 33 ha of Sutton Common in 1997. As part of the remediation scheme associated with preserving the archaeological remains, English Heritage subsequently blocked the drains on site, which promoted an increase in the level of the water table. Also in 1997, 25 ha of adjacent land, including part of the Shirley Pool SSSI, were acquired by the Carstairs Countryside Trust (CCT) with help from the Heritage Lottery Fund, English Heritage and several other organisations (Van de Noort, 2001b).

From 1997 onwards, the land management regime has changed. This has involved the transformation of arable land to pasture and the establishment of a water table management programme (Van de Noort, 2004a). Since 2001, English Nature and English Heritage have negotiated with the landholder and other interested parties in an effort to find solutions to the on-going problems of drainage and cultivation on the site.

The monitoring point at Sutton Common (Figure 2.12) is located roughly central to the ditch associated with the smaller enclosure (annex) at this site. As this location has been inferred as being one of the main locations where the potential for the preservation of waterlogged material *in situ* occurs.

Figure 2.12: Location of monitoring point in the small enclosure ditch

The monitoring point is set up in a similar way to that at Hatfield for continuity (Figure 2.13).

Figure 2.13: Sutton Common monitoring point.

2.4 Summary

This chapter has outlined the study region, and the location of the sites which are used as the basis for the research presented in this thesis (i.e. the Humberhead Levels). The two sampling locations at Hatfield Moor and Sutton Common are also discussed in greater detail to provide a background to their importance to the current study, and they are set into their regional and local context. An overview of the geology, flora and fauna, and previous archaeological investigations are also included in this overview.

Chapter 3 will outline the methods used in the field for monitoring the *in situ* soil status at Hatfield Moor and Sutton Common.

Chapter 3 Field Methods

3.1 Introduction

The first stage in the monitoring of the burial environments studied here, has involved the measurement of the parameters in soil that have the potential to influence the preservation of organic archaeological remains (Caple, 1996; Corfield, 1996, 2007; Caple and Dungworth, 1997, 1998).

The monitoring of the water tables at Hatfield Moor and Sutton Common is of fundamental importance in order to obtain a greater understanding of the impacts that changes in hydrology may have upon the burial environment. Variation in the dynamics of the ground water at Sutton Common has previously been studied by James Cheetham, who installed a network of sixty piezometers across the site in order to obtain high resolution data on intra- and inter-annual variation in water table height (Cheetham, 2004).

The current research aims to study the hydrology of the ditch within the small enclosure at Sutton Common, in order to facilitate comparison between the results from this enclosure, which represents an anthropogenically-modified archaeological context, against those obtained from the Hatfield Moor monitoring location, which represents a 'natural' depositional context. This approach facilitates comparison between a recognized archaeological site, which has recently been managed in terms of its water levels, but which was drained for arable agriculture until relatively recently, against Hatfield Moor which already possesses a stable (managed) elevated water table, that can provide good conditions for the preservation of organic remains.

The key difference between these locations is the context at Hatfield Moor, which whilst under arable, is not a location where significant anthropogenic modification has occurred in the past.

The measurement of the level of water in a soil is influenced by the dynamic characteristics of the ground water (Welch and Thomas, 1996). Previous research by Cheetham (2004) has demonstrated that piezometers can be used successfully to monitor changes in the water table. This is possible even though there are stratigraphic differences throughout the profile of the soil, and even though the constrained/unconstrained nature of the landscape will exert an influence at the wider catchment level on the hydrology of a site within the catchment (Welch and Thomas, 1996; Chapman and Cheetham, 2002; Lillie, 2007).

Water table variation and redox potential are considered to be two of the more important parameters to study when attempting to characterize waterlogged burial environments; and as a consequence, the potential preservation of organic artefacts (Caple and Dungworth, 1998; Van Bochove *et al.*, 2002; Holden *et al.*, 2006). Water levels are important, as it is generally accepted that saturation of an organic deposit excludes oxygen and inhibits microbial activity to the point where *in situ* preservation is to be anticipated (Chapman and Cheetham, 2002).

Redox potential is a measure of the potential for electrochemical activity, which characterizes the oxidation-reduction state of the soil. It is a quick and effective way of distinguishing between anoxic and aerobic conditions in the soil profile (Caple and Dungworth, 1997; Blackwell *et al.*, 2004; Gumbley *et al.*, 2005). Despite inherent

limitations, it is generally accepted that redox potential provides a proxy measure of the potential for the preservation/degradation of organic material in a burial environment (Vorenhout *et al.*, 2004). Previous research has highlighted that anaerobic sites present low or negative redox potentials (<+200 mV), while aerobic soils have redox potentials of >+400 mV (Table 3.1) (Patrick and Mahaptra, 1968; Caple and Dungworth, 1997; Powell *et al.*, 2001). However, the boundaries that exist between good and poorly preserving environments are ill-defined at present.

Table 3.1: Redox potential ranges (Patrick and Mahaptra, 1968).

Oxidised soils	=	>+400 mV
Moderately reduced	=	+200 to +400 mV
Reduced	=	–100 to +200 mV
Highly reduced	=	–300 to –100 mV

To study the redox potential of the burial environment, *in situ* platinum tipped, copper redox probes were permanently installed at different depths in the soil at Hatfield Moor and Sutton Common. The use of *in situ* probes has proven to be a more accurate technique with which to measure the redox potential of the soil, when compared to the use of redox measurement of ground water (Caple and Dungworth, 1998; Cheetham, 2004).

The measurement of soil redox potential has been successfully carried out at a number of archaeological sites (Caple, 1996; Caple and Dungworth 1997; Hogan *et al.*, 2001; Cheetham, 2004). The redox potential of the soil surrounding the Sweet Track in the Somerset Levels has been measured using *in situ* redox probes. The results to date indicate that the site conditions are anaerobic (Brunning, 2000). Research undertaken by Durham University has demonstrated the effectiveness of using redox potential, pH and conductivity as useful tools for characterizing waterlogged burial environments

that contain organic archaeological artefacts (Caple *et al.*, 1996). In addition, Blackwell *et al.* (2004) have successfully used redox probes and piezometers located at different depths within the soil profile in order to monitor changes in estuarine and coastal sediments which encompass a number of archaeological sites on the south coast of England.

Total values of Carbon (C), Nitrogen (N), Phosphorus (P), Sulphur (S), Sodium (Na), Potassium (K), Magnesium (Mg), Calcium (Ca), Manganese (Mn), Cobalt (Co), Nickel (Ni), Zinc (Zn), Iron (Fe) and Molybdenum (M) were measured in the soil samples obtained from Hatfield Moor and Sutton Common. This sampling methodology was undertaken due to the proven metabolic uptake of a number of elements by bacterial activity in the process of organic decay (Corfield, 1996). As a consequence, the monitoring of such elements is important in assessing the preservation potential of archaeological sites (Gumbley, 2005). A range of the above chemical traces are used in the calculations of nutrient availability undertaken in Chapter 6.

In addition, chemical changes to the soil, caused by the inclusion of fertilizers and/or pesticides, may alter the redox potential of the sediment and impact upon archaeological preservation (Caple, 1996). Therefore, it has been noted that data on the chemistry of the soil would be useful when determining its preservation potential (Holden *et al.*, 2006), providing further justification for the study of the chemical status of the soils investigated.

Temperature and pH will also be used in the current study in order to characterize the soil profile at both sampling locations. Although different types of electrodes can be used to measure soil temperature and pH, the most widely used electrode is the glass-tipped SenTix 21. The pH of the soil is best measured *in situ* in order to avoid any potential changes in sediment which may occur during transport to the laboratory (Gumbley *et al.*, 2005).

3.2 Monitoring location and sampling strategy

The sampling location at Hatfield Moor was situated on the edge of the moor, in an area of managed farmland, where a high water table is maintained by a farmer. The farmer manages this land as a part of the (then) Countryside Agency's 'Value in Wetness' initiative (Pasley, 2007). This location enables the study of the conditions occurring in a soil with a high potential to preserve archaeological remains *in situ,* thereby enabling a comparison with the conditions occurring at Sutton Common; where it has been suggested that the organic archaeological materials which are present may be at risk of decay (Cheetham, 2004). The information obtained from this comparison will enhance our understanding of how the environmental conditions at Sutton Common may be influencing the potential for the preservation of the buried archaeological remains.

The sampling location at Sutton Common was situated in the east ditch of the small Iron Age archaeological enclosure (which is described above in Section 2.3). Previous research by Parker Pearson and Sydes (1995) has identified the existence of wooden archaeological remains within this context. This monitoring point is also located near to one of Cheetham's piezometer points (Cheetham 2004).

At each sampling location, a monitoring point comprising three piezometers and sixteen redox probes was installed between February-April 2004 (Figures 3.1 and 3.2). The piezometers were placed at depths of 50 cm, 100 cm and 150 cm to allow the monitoring of water table variation throughout the soil profile.

Figure 3.1: Photograph showing the monitoring point (100 cm x 50 cm) at Hatfield Moor, including three piezometers and redox probes at different depths.

A cluster of sixteen redox probes was installed in the soil to measure the redox potential at depths which mirror the piezometers (with the exception of the shallow redox cluster). Redox probes were inserted to depths of 10 cm, 50 cm, 100 cm and 150 cm. The 0.10 m cluster acted as a control for the lower redox clusters.

Temperature and pH values were also recorded from the same depth intervals that the redox probes were situated. This was undertaken in order to contrast the samples obtained from the aerobic surface environments to those occurring at depth within the soil profile.

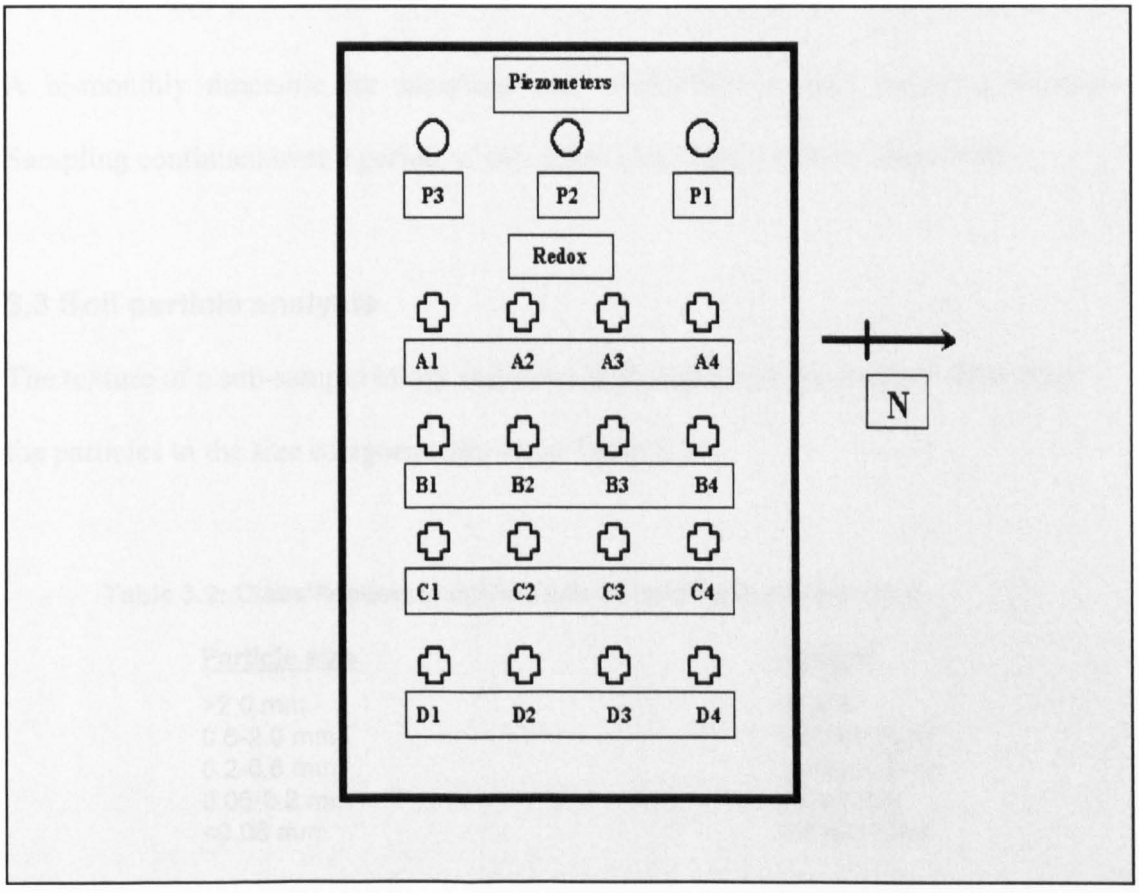


Figure 3.2: Diagram showing the monitoring network at Hatfield Moor, including position of piezometers and redox probes in the soil.

For Figure 3.2, piezometer depths in relation to the redox clusters are:

P1: piezometer at 150 cm
P2: piezometer at 100 cm
P3: piezometer at 50 cm

A1-A4: redox probes at 150 cm
B1-B4: redox probes at 100 cm
C1-C4: redox probes at 50 cm
D1-D4: redox probes at 10 cm

Four redox probes were installed at each depth to ensure that any outliers or corrupted probes would not reduce the reliability of the results. Significant outliers were rejected, and the means of the remaining three probes are used in the discussions below.

A bi-monthly timetable for sampling was established at each sampling location. Sampling continued over a period of two years from April 2004 to April 2006.

3.3 Soil particle analysis

The texture of a sub-sample of dry soil from each depth was analysed by allocating the particles to the size categories shown in Table 3.2.

Table 3.2: Classification of soil texture according to particle size.

Particle size	Texture
>2.0 mm	Gravel
0.6-2.0 mm	Coarse Sand
0.2-0.6 mm	Medium Sand
0.06-0.2 mm	Fine Sand
<0.06 mm	Silt and Clay

The soil analysis was carried out using oven dried soil with any stones and/or macroscopic plant material removed. 30 g of oven dried soil were weighed into a pre-weighed 500 ml glass beaker. 100 ml of 6 % hydrogen peroxide (H_2O_2) was added. The mixture was heated on a hotplate in a fume cupboard and stirred gently with a glass rod. When the initial effervescence ceased, a further 25 ml of H_2O_2 was added in order to complete the removal of organic matter. When the effervescence had finally ceased, the contents of the beaker were dried overnight in an oven at 105 °C, cooled in a desiccator and subsequently weighed.

To disperse any aggregates before sieving, 100 ml of Calgon (sodium hexametaphosphate) were added to each of the beakers. The beakers were then warmed on a hotplate for 10 min and stirred gently with a glass rod. The sample was then cooled. 50 ml of deionised water were used to wash the sediment into a 200 ml beaker. The contents of the beaker were then agitated in an ultrasonic bath for 10 min. The mixture was sieved through a 0.06 mm wet sieve. Deionised water was used to ensure that all the sediment was washed from the beaker. The sediment that passed through the sieve was transferred to a 500 ml sedimentation tube using a plastic funnel. The sediment that was retained by the sieve was transferred into an evaporating basin and dried in an oven at 105 °C.

The dried sediment was lightly ground using a pestle and mortar, and then sieved using different mesh sizes, i.e. 0.2 mm, 0.6 mm and 2.0 mm. The residue retained on each sieve was dried, cooled and weighed. The following quantities were determined:

Weight of organic-free soil = Wp
Weight of gravel on 2 mm sieve = Ws
Weight of gravel free soil = (Wp–Ws) = Wg
Weight of coarse sand (0.6 mm sieve residue)
Weight of medium sand (0.2 mm sieve residue)
Weight of fine sand (0.06mm sieve residue)

The sedimentation of particles was undertaken using Stoke's Law, at a temperature of 20 °C (Day, 1965). The sedimentation tubes that contained the 0.6 mm fraction of the sediment were immersed overnight in a water bath at approximately 20 °C.

500 ml of pure water were added to the sedimentation tubes. The stem of a 25 ml glass volumetric pipette was marked at exactly 9.0 cm from its tip. The suspensions were vigorously stirred for 30 seconds in order to ensure an even distribution of sediment particles throughout the cylinder. Care was taken to avoid the introduction of air bubbles into the suspensions. After the temperatures of the suspensions were taken, they were then stirred again for a further 30 seconds. At precise time intervals (as shown in Table 3.3 below) a sample from a depth of 9.0 cm below the water surface was taken using the 25 ml glass volumetric pipette. Approximately 20 seconds before the time of sampling, the pipette was slowly lowered to the required depth. The sample was obtained at the exact time shown in order to minimize disturbance in the water column.

Table 3.3: Time intervals used for sampling the sediment.

Setting time	Equivalent diameter (mm)
4 min 48 seconds	0.02
1 hour	0.006
8 hours	0.002

The quantity of material within these sizes limits can be calculated as follows:

$$(\%\ \text{material} - \text{the equivalent diameter})\ d = \frac{(Wd \times V^1 - Wcal)}{V} \times \frac{100}{Wg}$$

Where: Wd = weight of material in pipette at time equivalent to effective diameter d

V^1 = sedimentation tube

V = volume of pipette

Wcal = weight of Calgon added to sedimentation tube (4 g)

Wg = total weight of gravel-free soil

The sample was subsequently transferred from the pipette to a pre-weighed crucible. Any residue remaining in the pipette was rinsed into the crucible using pure water. The sample was evaporated to dryness in an oven overnight at 105 °C, cooled and subsequently weighed.

3.4 Moisture and organic matter content in soil

Two sub-samples of about 10 g fresh weight from each soil sample were placed in pre-weighed evaporating dishes. The dishes were re-weighed. The soil was subsequently dried in an oven at 80 °C for 24 hours. After 24 hours, the sample was placed in a desiccator until cooled and then re-weighed. To determine the percentage of organic matter the dry samples were ignited in a muffle furnace at 475 °C for 4 hours (*cf.* Burdon *et al.* 2005). The samples were then cooled in a desiccator and re-weighed.

The following calculations were used to measure the moisture and organic matter content of the samples:

Moisture content:
% of fresh weight = ([mass fresh soil- mass dry soil]/mass fresh soil) x 100
Organic matter content:
% of dry weight = ([mass dry soil- mass of ash after ignition]/ mass dry soil) x 100

3.5 Determining P, S, Na, K, Mg, Ca, Mn, Co, Ni, Zn, Fe, Mo, C, H and N.

All soil sub-samples were oven dried overnight at 100 °C. They were subsequently grained before analysis.

Total P, S Na, K, Mg, Ca, Mn, Co, Ni, Zn, Fe and Mo in the soil sub-samples were analysed using a Perkin Elmer Optical Emission Spectrometer Optima 5300 DV (Chemistry Department, University of Hull). The samples were analysed by Bob Knight in the Chemistry Department, University of Hull.

Prior to the introduction of the samples into the optical emission spectrometer, 0.5 g of soil was dissolved in 10 ml of aqua regia (3 HCl+1 HNO_3) within special vessels. The digestion solution was heated in a CEM MARS (microwave assisted reaction system) for 20 minutes at 170 °C.

C, H and N were measured by combustion analysis using a Carlo Erba 1108 Elemental Analyser (Chemistry Department, University of Hull). The samples were analysed by Carol Kennedy in the Chemistry Department, University of Hull.

3.6 Water levels

Each piezometer consisted of a PVC tube of 19 mm internal diameter, which is open to the atmosphere at its top, but which is capped to prevent inputs of water from external sources such as rainfall. The bottom section (the buried end) of the tube has a 300 mm length tip, consisting of a perforated PVC tube containing a filter membrane, which is designed to avoid contamination from the surrounding soil whilst allowing water to enter the tube (Figure 3.3[1] below).

At both sampling locations three piezometers were installed in different boreholes at depths of 50 cm, 100 cm and 150 cm. Installation involved coring to the required depth using an Eijelkamp hand auger of 30 mm diameter with a screw tip, obtained

from Van Walt Ltd, UK. The polypropylene tubes (piezometers) were pushed down into these holes to the base. The top of the tubes was cut in a way that ensured a short protrusion of tube above the ground surface in order to facilitate measurement (Figure 3.3[2] below). All piezometers were capped to prevent contamination of the piezometer pipe.

Water levels were measured using an acoustic dipper (Van Walt Ltd, UK), which consisted of an electrical sensor attached to a plastic measuring tape. The sensor was lowered into the piezometer tube, until it made contact with the water when an audible alert sounded (Figure 3.4 below).

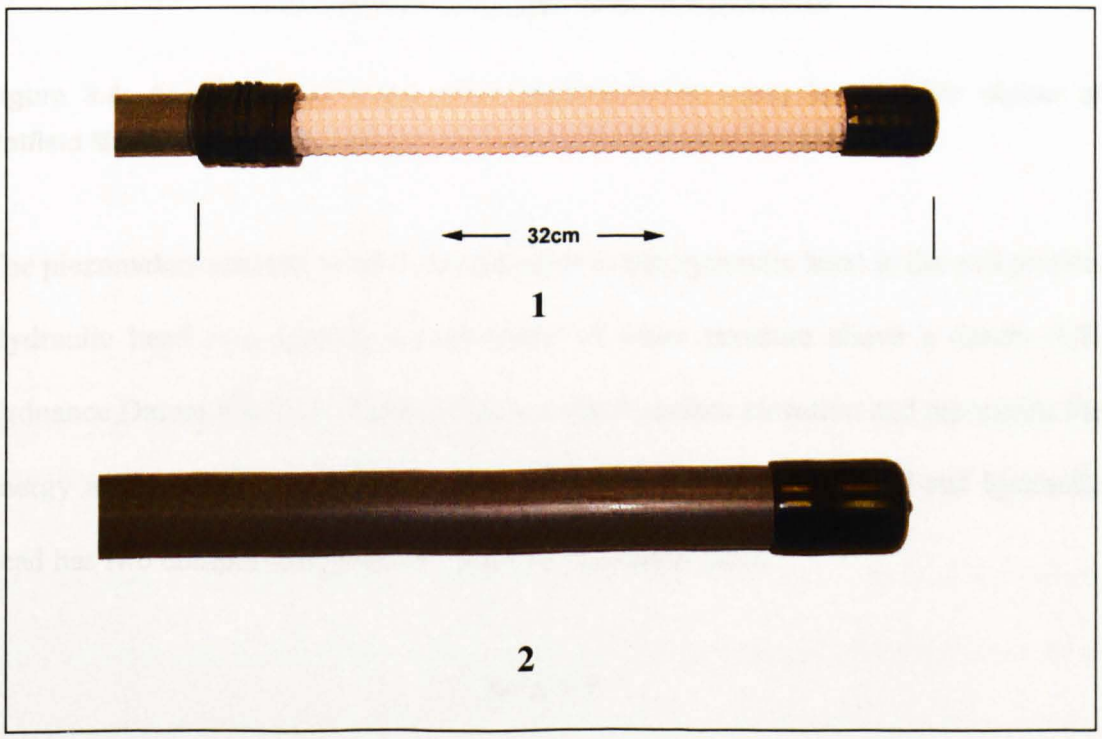


Figure 3.3: An example of a piezometer used during the hydrological monitoring. (1) Bottom section (buried end) of the tube, consisting of a perforated PVC tube containing a filter membrane. (2) Top section (open to the atmosphere) which is capped.

Figure 3.4: Acquisition of water level measurements using an acoustic dipper at Hatfield Moor

The piezometers allowed for the measurement of the hydraulic head in the soil profile. Hydraulic head is a specific measurement of water pressure above a datum (UK Ordnance Datum [OD]). It is measured as a water surface elevation and represents the energy at the bottom of the piezometer (Freeze and Cherry, 1979). Total hydraulic head has two components; pressure head and elevation head:

$$h = z + \Psi$$

Where: h = total hydraulic head (metres of water)

z = elevation head (metres of water)

Ψ = pressure head (metres of water)

Hydraulic measurements were related to OD (Figure 3.5). When a ground water aquifer is unconfined, i.e. it is open to receive water from the surface and is not below a confining layer, the water table is free to vary in height, depending on the water recharge/discharge rate. Under this condition, because the top of the aquifer is the water table, the aquifer is also called a water table aquifer (Freeze and Cherry, 1979).

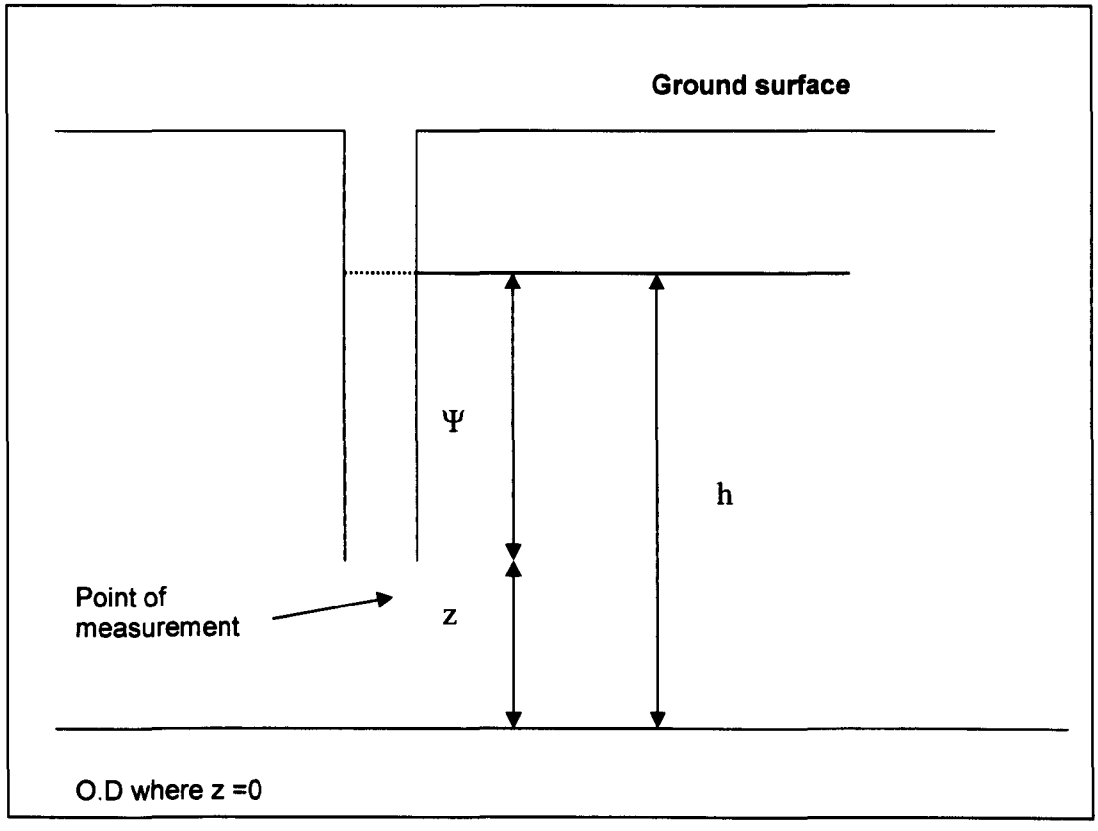


Figure 3.5: Hydraulic head measured by piezometers. h = total hydraulic head, z = elevation head, Ψ = pressure head (Freeze and Cherry, 1979).

At both sampling sites, the unconfined aquifers were monitored. As a consequence, the measurements from the piezometers represented the depth at which the water table was located (*cf.* Cheetham, 2004).

The piezometers were installed in a cluster at different depths in order to identify the flow of water within the vertical plane. The water movement would be upwards if the

readings of the piezometers at greater depths give higher readings that the shallower ones. The contrary situation will indicate downwards movement. (*cf.* Cheetham, 2004, 45).

3.7 Redox potential

The redox probes were constructed according to the design of Faulkner *et al.* (1989). The redox probes consist of a tip of platinum wire welded to a copper wire at its terminal (Figure 3.6). The terminal was sealed with waterproof epoxy resin and isolated from the burial environment by heat-shrink plastic tubing, which provides durability for long-term *in situ* studies (Jacobs and Gilliam, 1983). The copper terminal wire that is exposed above the ground surface was additionally sealed to prevent water penetration, along with the wire between the plastic sleeve and the copper wire itself. The probes used were constructed by Hunter's Dale, Berkshire (Figure 3.6), to a 5 % tolerance; and were developed through the work which was carried out at the Royal Holloway Institute for Environmental Research (Hogan *et al.*, 2001).

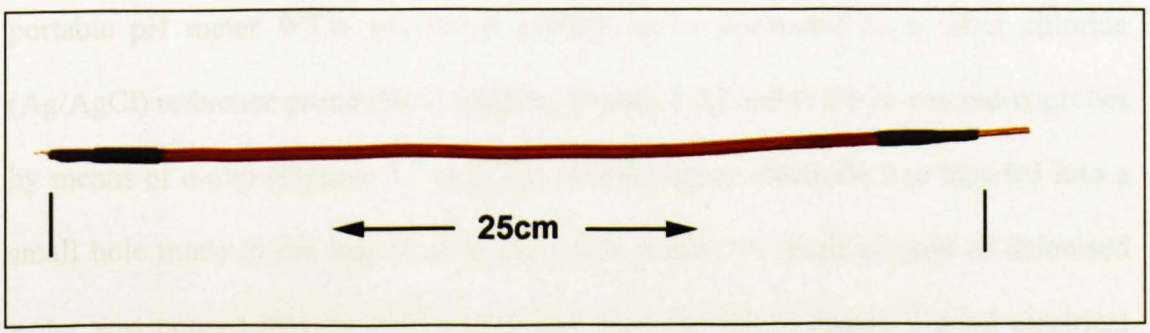


Figure 3.6: Redox probe consists of a tip of platinum wire welded to a copper wire at its terminal.

The probes' tolerance is a measure of the reliability of their output. To test the redox probes, they were immersed in a redox buffer solution (10.211 g of potassium hydrogen phthalate in 1 litre of deionised water, previously saturated with quinhydrone [potential +218 mV]). Any probes that gave a reading outside the range of ± 5 % of +218 mV were rejected (*cf.* Cheetham, 2004).

The redox probes were inserted into the soil by first making a hole to the required depth using a metal auger and then pushing the probe in until good contact was established with the surrounding soil at the required depth. The borehole was sealed at the surface by compaction of the soil around the probe.

The redox probes were installed close to the piezometers (within 30-50 cm) in order to enable comparison of the oxidation-reduction state of the soil with its hydrological condition (Figures 3.1 and 3.2).

The redox potential was measured as the voltage between the redox probes in the studied soil and a standard reference electrode. Redox readings were obtained using a portable pH meter WTW pH-340-A pH/mV meter connected to a silver chloride (Ag/AgCl) reference probe (BDH Gelplas, Dorset, UK) and to the *in situ* redox probes by means of a clip (Figures 3.7 and 3.8). The reference electrode was inserted into a small hole made in the soil close to the redox probes. A small amount of deionised water was poured into the hole and mixed with the soil to ensure a good electrical contact. The potential obtained between the reference electrode and the platinum tip is recorded as a redox potential reading.

The pH of the soil surface was measured using the WTW pH 340 pH/mV meter (WTW, Germany) with a temperature-compensated pH electrode (WTW SenTix 21 combination electrode/TFK325/HC temperature sensor). The pH was measured because pH has a direct influence on redox measurements and is required for the adjustment of the redox readings to a standard pH (as outlined below). pH is also

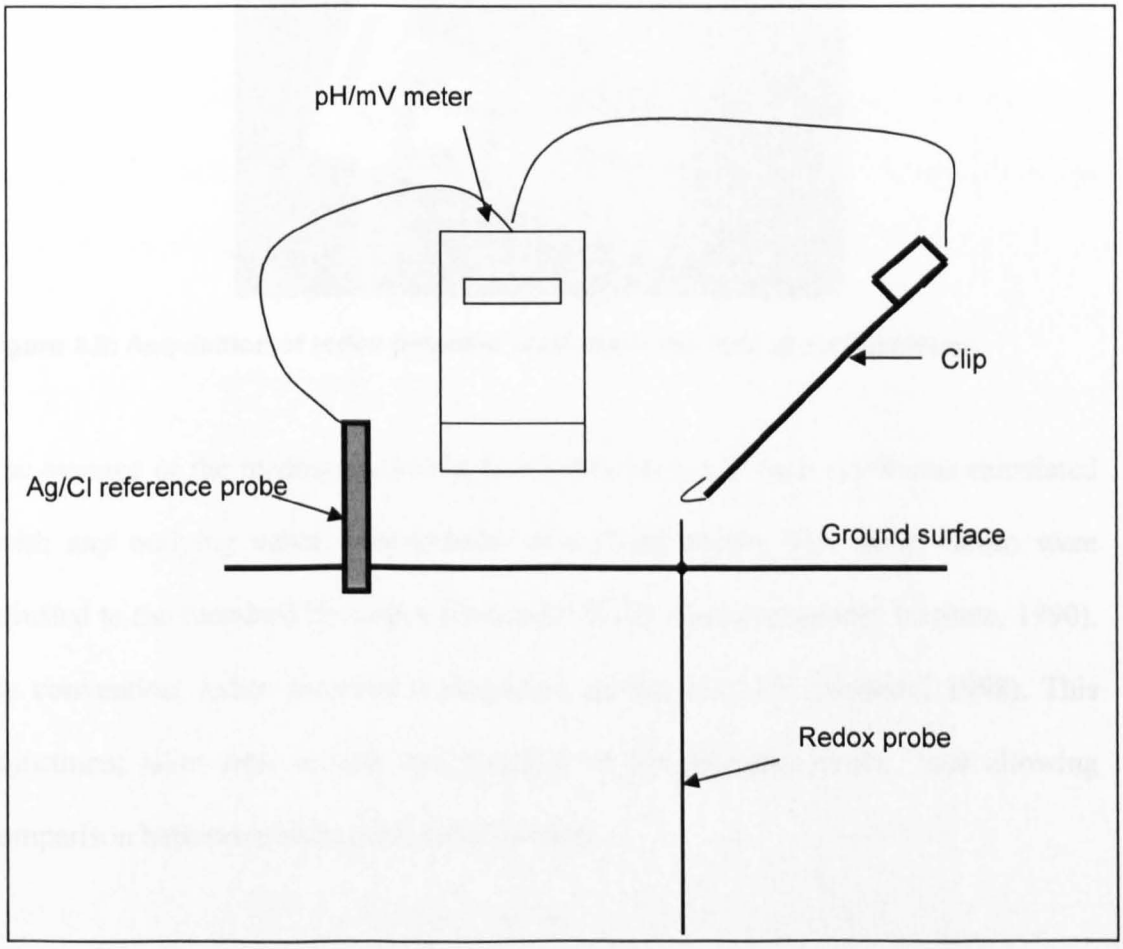


Figure 3.7: Diagram showing the equipment used to obtain of redox readings in the field.

Figure 3.8: Acquisition of redox potential readings in the field at Hatfield Moor.

The average of the readings from the four redox probes at each depth was calculated (with any outlying value disregarded), as outlined above. The mean values were adjusted to the Standard Hydrogen Electrode (SHE) (British Standard Institute, 1990). By convention, redox potential is measured against the SHE (Howard, 1998). This adjustment takes into account the potential of the reference probe, thus allowing comparison between results from different sites.

A silver chloride (Ag/AgCl) reference probe was employed. This has a potential value of +222 mV. Therefore, a value of 222 mV is added to the readings obtained from each of the redox probes.

Because pH affects redox potential, it is necessary to adjust the measured values to pH 7 by a factor of +59 mV per pH unit (Bohn, 1971; British Standard Institute, 1990).

Under acidic conditions, for each pH unit below pH 7 a value of 59 mV was subtracted from the recorded redox potential; under alkaline conditions, for each pH unit above pH 7, a value of 59 mV was added to the recorded value.

3.8 Soil sampling and sample preservation

Soil samples were recovered from diverse depths using a (Eijkelkamp) hand auger. The auger head is 20 cm in length and collects approximately 450 cm^3 of soil, from which sub-samples of 100-200 g were taken (Figure 3.9). Samples were obtained throughout the soil profile at 10 cm, 30 cm, 50 cm, 70 cm and 100 cm depths. Below this depth saturation restricts the recovery of the sediment.

Figure 3.9: A soil sample within the auger head.

The soil sub-samples were placed in ziplock plastic bags and sealed to prevent drying-out (*cf.* Pett-Ridge and Firestone 2005). A second plastic bag encapsulated each original bag. Whilst no standard sampling methodology exists, it is generally acknowledged that some potential for minor contamination may occur during the

retrieval of samples (RTDF 1999). The samples were stored in the dark and transported within 4 hours of collection to the laboratory and stored at 4 °C (again in the dark) until microbial analysis was undertaken. This minimized any potential changes in the microbial communities in the soil samples. For the molecular genomic work, *c.* 50 g sub-samples of soil were sealed in small plastic bags and were preserved at –20 °C for future analysis.

3.9 pH and temperature

As outlined in Section 3.4, pH was measured using a portable pH/mV-meter WTW pH 330, with a temperature compensated pH electrode (WTW SenTix 21 combination electrode/TFK325/HC temperature sensor) (Figure 3.10). A soil sub-sample from each depth was diluted 1:5 (v/v) with deionised water. The mixture was vigorously mixed by hand and the pH probe was inserted into the slurry. A pH reading was subsequently taken.

Figure 3.10: Equipment used for the measurement of temperature and pH in the field.

Soil temperature was recorded for each soil sample using the temperature probe on the pH meter (Figure 3.10). The temperature was obtained by inserting the probe into the soil sample contained in the hand auger at each sampling depth.

3.10 Summary

This chapter has described the techniques and methods used to monitor the burial environments as defined by this study. Particular emphasis has been placed on the methods used for measuring water table levels and redox potential, as these factors provide a strong indication as to the potential for the *in situ* preservation of organic archaeological remains within the ground.

The following two chapters will describe the conventional microbial and molecular genomic methods used in this study in order to characterize the microbial communities present in the soil profiles at Hatfield Moor and Sutton Common.

Chapter 4 Conventional microbial methods

4.1 Introduction

The role of bacteria in the decay of organic archaeological remains has been widely studied (e.g. Blanchette *et al.*, 1991; Blanchette and Hoffmann, 1994; Blanchette, 1995, Powell *et al.*, 2001), and several studies of ancient waterlogged woods have shown that bacteria are responsible for wood deterioration under anaerobic conditions (Donaldson and Singh, 1990; Hedges, 1990; Kim 1990; Blanchette *et al.*, 1991; Blanchette and Hoffmann, 1994; Blanchette, 1995; Powell *et al.*, 2001). This information has been reported in a number of conference proceedings, for example, in the Proceedings of the International Council of Museums-Committee for Conservation (ICOM-CC) and the Working Group on Wet Organic Archaeological Materials (WOAM).

To date, techniques based on optical and electron microscopy have provided information on the pattern of cell wall deterioration of wood as caused by bacteria and fungi (Blanchette *et al.*, 1990; Blanchette and Hoffmann, 1994; Nilsson, 1999). Electron microscopy has shown that rod-shaped bacteria are able to degrade a range of woods from different tree species (Daniel, 1994), and Björdal *et al.* (1999) have previously identified the major forms of microbial decay, from different samples of waterlogged archaeological wood, using this technique. Subsequent research by Powell *et al.* (2001) has investigated the decay of buried wood at a Bronze Age archaeological site using light and electron microscopy. The results obtained confirmed the presence of bacteria under near anaerobic conditions.

However, by contrast studies based on the application of microbial culturing techniques have had only limited success in identifying the microorganisms responsible for the decay of organic archaeological remains (Cronyn, 2001). In order to address this imbalance, the current study will employ a number of conventional microbiological approaches. These techniques are based on measuring microbial biomass, bacterial abundance, and the metabolic activities of microbial communities (i.e. organic-substrate uptake rates and/or extracellular enzyme activities). Complimentary to these techniques, scanning electron microscopy (SEM) will be undertaken in order to assess the pattern of microbial decay within archaeological wood samples obtained from both of the sites studied.

4.2 Preparation of soil slurries

Prior to conventional microbiological analysis, the soil samples obtained from the two sampling locations at Hatfield Moor and Sutton Common were homogenised into a slurry. 5.0 g wet weight of each soil sample were weighed and made up to 30 ml using 0.2 μm filtered, sterile, pure water. The slurry then transferred into a sterile polythene bag and homogenised in a stomacher (Colworth Lab Blender 400, A.J. Seward Ltd, London), for 5 minutes.

The contents of the polythene bag were then transferred into a 50 ml sterile glass beaker. 20 ml of 0.2 μm filtered, sterile, pure water were used to rinse the remaining slurry from the bag. This produced a 50 ml of slurry with soil concentration 100 g wet weight l^{-1}.

The slurries were used to determine:

1) Bacterial abundance by direct counting using epifluorescence microscopy
2) Extracellular enzyme activities
3) Heterotrophic microbial activity by determining ^{14}C-leucine assimilation rate
4) Microbial community function through use of Biolog Ecomicroplates.

4.3 Conventional microbiological techniques

This section outlines the different types of conventional microbiological approaches used during the current study, i.e. bacterial abundance, extracellular enzyme activity, ^{14}C-leucine assimilation and microbial community function (as discussed below in Sections 4.3.1-4.3.4 respectively).

4.3.1 Bacterial abundance

Microbial abundance can be investigated by culturing on nutrient agar plates (Barken, 1997; Johnsen *et al.*, 2001). However, since only a limited range of microorganisms can be cultured on agar plates, no more than 10 % of the overall soil microbial community can be isolated and/or counted with this method. This is due to the inherent difficulties associated with imitating *in situ* environmental conditions within a laboratory culture medium (Torsvik *et al.*, 1996; Muyzer and Small, 1998; Heuer *et al.*, 2001).

The application of direct microbial counts by fluorescence microscopy can produce between 100 to 1000 times more microbes than those obtained by plate counting. This approach is considered to be one of the best methods for the enumeration of total

bacteria in environmental samples (Bowden, 1977; Fry, 1988; Herbert, 1990; Johnsen *et al.*, 2001). Direct counts of cells are usually based on the staining of the sample containing the microbial population with a suitable fluorescent dye, and using an epifluorescence microscope to visualize them.

The soil samples investigated in the current study were stained with acridine orange (3, 6-bis [dimethylamonio] acridinium chloride). This compound binds to RNA and DNA. It has a maximum fluorescence at an excitation wavelength of 470 nm. Previous research has suggested that acridine-orange-stained single stranded nucleic acids emit an orange-red fluorescence, while double stranded nucleic acids tend to emit a green fluorescence (Kepner and Pratt, 1994).

The abundance of bacteria in a soil was determined by direct counting after staining with acridine orange (Francisco *et al.*, 1973). A 1g l^{-1} soil suspension was obtained by diluting the 100 g wet weight l^{-1} slurry with 0.2 μm filtered sterile water. Sub-samples (10 ml) of this soil suspension were fixed with 0.5 ml of filtered neutral formaldehyde (final concentration 2 % w/v). The sub-samples were then stored for up to two weeks at 4 °C.

The 1g l^{-1} suspensions were then further diluted (x 10) with sterile 0.2 μm filtered pure water. Acridine orange solution (0.1 ml, 1g l^{-1}) was added to 10 ml sub-samples of the 0.1 g l^{-1} soil suspension. The suspensions were left in the dark for 10 minutes in order to allow for staining of the bacteria. Two replicate controls of 10 ml of 0.2 μm filtered sterile water were also stained with acridine orange.

Polycarbonate membrane filters of 0.2 μm pore size (Nuclepore-Whatman) were dyed black, using irgalan black (0.2 % in 2 % acetic acid) solution, for 10 minutes. This was undertaken in order to create a contrast between the membrane and fluorescent bacteria during the subsequent counting process.

After staining with acridine orange, 1 ml aliquots of the 0.1 g l^{-1} soil suspension were filtered through the black 0.2 μm polycarbonate membranes by gentle suction, provided by a hand-operated vacuum pump. Each membrane was rinsed with 5 ml of 0.2 μm filtered, sterile, pure water. It was subsequently removed from the filter holder and placed face up on a glass slide smeared with immersion oil (Olympus, Japan), to ensure its adherence to the surface of the glass.

An additional drop of non-fluorescent immersion oil was added to the filter surface. A Nikon Alphashot epifluorescence microscope was used, with blue light illumination, to count the bacteria at 1250 x magnification. The bacteria fluoresced against a black background. For each preparation, bacteria were counted in 30 eyepiece squares of 0.084 mm side. Three replicates were used from each soil slurry.

The concentration of bacteria in the 0.1 g l^{-1} soil suspension was calculated as follows:

$$= \frac{N}{F} \times \frac{A}{S^2} \times \frac{1}{V} \times 1.05^{*} \text{ cells ml}^{-1}$$

Where: N = number of bacteria counted – mean count in 30 control fields.

F = number of eyepiece graticule squares counted (30)

A = area of filter (mm^2)

S = length of side of graticule square (0.084 mm)

V = volume of suspension filtered (1 ml)

* To compensate for the dilution by formalin

The abundance of bacteria in the soil sample was calculated as follows:

Bacterial abundance = concentration in the 0.1 g l^{-1} soil suspension (cells ml^{-1}) x 1000 x 10 cells g^{-1} wet weight of soil

4.3.2 Extracellular enzyme activities

Enzyme activities are commonly used in soil studies as they measure the 'potential' activity of microbial communities in a soil (Burns, 1982; Nannipieri *et al.*, 1990). Enzymes act as bio-indicators in order to assess soil quality because of their rapid response to environmental change (Bandick and Dick, 1999). Extracellular enzymes play an important role within soil as they are involved in the cycling of nutrients, reflect the microbial activity of soil, and act as indicators of soil change (Burns, 1982). Extracellular enzymes mediate in the degradation of organic matter and are able to regenerate inorganic nutrients from organic compounds (Sinsabaugh *et al.*, 1991; Tabatai, 1994; Kang and Freeman, 1998; McLatchey and Reddy, 1998; Dick *et al.*, 2000; Nannipieri *et al.*, 2002).

The major extracellular soil enzymes include carbohydrases, esterases, proteases, amidases and oxidoreductases. The carbohydrases are involved in the breakdown of polysaccharides such us cellulose. The carbohydrase β-D glucosidase is involved in the final step of cellulose degradation. This enzyme hydrolyses cellobiose, which is contained in cellulose, and provides an indication of oligosaccharide release for assimilation (Acosta Martinez *et al.*, 1999; Bandick and Dick, 1999; McLatchey and

Reddy, 1998). The esterase phosphatase is vital in the mineralization of phosphate from organic sources (McLatchey and Reddy, 1998; Wright and Reddy, 2001). Proteases and amidases degrade proteins and are involved in the mineralization of organic nitrogen (Tabatabai *et al.*, 2002). The oxidoreductase ligninase is able to degrade lignin (-a complex polymer of aromatic nuclei which is normally extremely resistant to chemical and microbial degradation) (Wood, 1995).

Research into the impact on extracellular enzyme activity in soils from different land management practices is widespread; examples include irrigated or dryland water management (Pulford and Tabatabai, 1988; Acosta Martinez *et al.*, 2003), tillage regime (Kandeler *et al.*, 1999; Acosta Martinez *et al.*, 2003) and lime application to agricultural soil (Acosta Martinez and Tabatabai, 2000). Extracellular enzyme activities have also been used to explore soil pollution (Trasar-Cepeda *et al.*, 2000; Avidano *et al.*, 2005) and characterize biological processes in wetlands (such as organic matter decomposition and nutrient release) (Freeman *et al.*, 1995; McLatchey and Reddy, 1998; Kang and Freeman, 1999; Wright and Reddy, 2001).

Freeman *et al.* (1995) have demonstrated the importance of applying fluorogenic substrates to environments which contain low levels of extracellular enzyme activity. Fluorogenic methylumbelliferyl (MUF) substrates (i.e. MUF β-D-glucosidase, MUF phosphatase and MUF sulphate) were used to study depth-dependent variation in enzyme activities in a riparian wetland. Fluorogenic substrates provide greater sensitivity, when compared to chromogenic substrates, as they avoid the interference caused by coloured phenolic compounds (Freeman *et al.*, 1995; Niemi *et al.*, 2005).

The current study will assay phosphatase, β-glucosidase and aminopeptidase activities. These assays were based on the hydrolysis of a bound target substrate and its 'reporter' molecule (tag). Two fluorescent reporter molecules have been used during the current research; 4-methylumbelliferone (MUF) and 7 amino-4-methylcoumarin (MCA) (Sigma Chemical Co., Poole, UK). The non-fluorescent substrates labelled with these tags were subsequently added to the soil slurries (Nannipieri *et al.*, 1990). The fluorescence released was then quantified using a fluorometer.

Assays of extracellular-enzyme activities were carried out the day after sampling. The procedure was based on Hoppe (1993). β-glucosidase, phosphatase, and leucine aminopeptidase activities were measured using the fluorogenic substrates 4-methylumbelliferyl-ß-D-glucopyranoside, 4-methylumbelliferyl-phosphate and l–leucine-7-methyl-4-amidocoumarin (Sigma Chemical Co., Poole, UK), respectively.

Stock solutions of the three substrates were prepared at a concentration of 5 mmol l^{-1} in 0.2 μm filtered, sterile, pure water, after dissolution in 40 % (v/v) methanol (Goulder, 1990). For each of the enzyme assays, three replicate sub-samples (5.76 ml) of each slurry sample (1 g wet wt l^{-1}) were used, i.e. 3 different substrates with 3 replicates of each for each soil sub-sample. An additional slurry sub-sample of 1 g l^{-1} was also prepared for use as a control. This was undertaken by boiling the sample for 5 minutes and then cooling prior to use, in order to destroy enzyme activity. Enzyme substrate (0.24 ml) was added to each sub-sample and to the blanks to give a final concentration of 200 μmol l^{-1}.

The sub-samples were subsequently incubated in darkness for 5 hours at 10 °C. After incubation, the sub-samples were centrifuged at 2225 RCF for 5 minutes. A 5 ml aliquot was then added to 0.4 ml of pH 10 borate buffer solution (BDH, Dorset, UK). The fluorescence intensity was measured using a fluorometer (Turner Designs Model 10 Series fluorometer, Steptec Instrument Services, Bedfordshire, UK), which was fitted with an excitation filter 10-069 and an emission filter combination 10-059 and 10-061. The fluorescence reading of the blank was subtracted to allow for fluorescent impurities and/or non enzymatic hydrolysis.

To determine the concentration of the post-incubation fluorescent products (4-methylumbelliferone [MUF] and methyl-4-amidocoumarin [MCA]), straight-line calibration graphs for MUF and MCA were prepared. Buffered standard MUF solution was made up in 0.2 μm filtered, sterile, pure water, at concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5 μmol l^{-1}. In addition, buffered standard MCA solution was made up at 0.25, 0.5, 1.0 and 2.0 μmol l^{-1}. The fluorescence intensity was measured and the calibration graphs were plotted. From the calibration graphs, the concentration of product equivalent to 1 relative fluorescent unit was determined.

The extracellular enzyme activity in 1.0 g l^{-1} soil suspension was calculated as follows:

$$= \frac{M}{T} \times MCU \text{ nmol } l^{-1} h^{-1}$$

Where: M = relative fluorescence of post-incubation sub-samples – relative fluorescence of blank

MCU = concentration of product equivalent to 1 relative fluorescent unit (nmol l^{-1})

T = Incubation time (h)

Since the concentration of soil suspension used was 1.0 g l^{-1} wet weight, the enzyme activity in the suspension (nmol l^{-1} h^{-1}) was numerically equal to the activity per gram wet weight of soil (nmol h^{-1} g^{-1} wet weight).

The model-substrate concentration used in the assays represented a saturation concentration. As a consequence, the rate of substrate hydrolysis measured approximated to V_{max} (Brown and Goulder, 1996).

4.3.3 ^{14}C-leucine assimilation

Amino acids and proteins are the most important forms of organic nitrogen in soils, and are a readily available source of C and N for soil microorganisms (Vinolas *et al.*, 2001). The incorporation rate of the radiolabelled amino acid ^{14}C-leucine into bacterial proteins enables the evaluation of bacterial production. The method involves the addition of radiolabelled leucine to a soil suspension and the subsequent measurement of accumulated radioactivity over a defined period of time (Kirchman *et al.*, 1985).

A number of previous studies have employed the use of this technique in soils. For instance, Griffith *et al.* (2001) have used ^{14}C-labelled leucine to study the relationship between biodiversity and ecosystem functioning in arable soils. In addition, Ranneklev and Bääth (2001) measured rates of leucine assimilation in order to study the temperature-driven adaptation of the bacterial community in a peat soil. Leucine assimilation was also used in studying the activity of topsoil bacterial assemblages at different temperatures (Diaz-Raviña *et al.*, 1994).

The current study will evaluate the effect that nutrient enrichment (N and P) has upon ^{14}C-leucine assimilation in soil suspensions from different depths. This technique will be employed in order to assess the impacts of the contamination of arable land from fertilizer inputs, which is considered to increase the rate of deterioration of buried artefacts in wetland archaeological sites (Van de Noort *et al.*, 2001). A further impact from this is that, as Gumbley *et al.* (2005) have suggested, nutrient over-enrichment can promote plant growth. This can lead to a lowering of the water table, cause physical damage due to the penetration of plant roots into the archaeological deposits, and promote microbial growth.

Measurements of ^{14}C-leucine assimilation under anaerobic conditions on a number of soil suspensions, which were obtained from beneath the water table at Hatfield Moor and Sutton Common, were analysed during November of 2005. This method provided additional information about the activity of anaerobic bacteria at these two sites.

^{14}C-leucine solution (0.1 ml; c. 0.1μCi, L-[U-^{14}C] leucine) (Amersham Pharmacia, Biotech Ltd, UK) was added to 10 ml of each of 3 replicates of 1.0 g l^{-1} soil suspension, in sterile universal bottles and to a blank with formalin added at a final concentration of 2 % (w/v) (Goulder, 1991). The final concentration of leucine was 32 nmol l^{-1} (Tulonen, 1993).

The universal bottles were incubated in darkness at 10 °C for 5 hours. At the end of the incubation period, 2 ml sub-samples of the contents of each bottle were concentrated on 2.5 cm diameter cellulose acetate membrane filter (0.2 μm pore size) (Sartorius, Germany). The filters were washed through with 5 ml of 0.2 μm filtered,

sterile, pure water and were then transferred to scintillation vials which contained 10 ml of scintillation fluid (Filtron-X, National Diagnostics, USA). This dissolved the filters and the radioactivity in the vials was assayed by liquid scintillation counting (Tri-Carb 2100TR Liquid Scintillation Analyser, Canberra Packard, UK).

The radioactivity of the ^{14}C-leucine solution that had been added to the incubation was determined by adding 10 µl of the ^{14}C-leucine solution to the scintillation vials containing 10 ml of scintillation fluid. Three replicates were used.

The rate of leucine assimilation (V) was calculated using the following equation:

$$V = f\,(A/T)\ \text{nmol}\ l^{-1}\ h^{-1}$$

Where: f = the fraction of ^{14}C-leucine supplied, which was taken up by bacteria during the incubation. This was calculated as follows:

$$f = \frac{([\text{mean CPM samples}] - [\text{CPM blank}]) \times 5}{\text{Radioactivity added (CPM)} \times 10}$$

A = concentration of the added substrate (nmol l^{-1})

T = time of incubation (h)

Since the concentration of soil suspension used was 1.0 g l^{-1} wet weight, the ^{14}C-leucine assimilation rate in the suspension (nmol l^{-1} h^{-1}) was numerically equal to the assimilation per gram wet weight of soil (nmol h^{-1} g^{-1} wet weight).

4.3.3.1 The effect of inorganic nutrients on ^{14}C-leucine assimilation

Soil samples collected at 10 cm and 100 cm depths from Sutton Common in November 2005, were used to explore the effect of phosphate and nitrogen enrichment on ^{14}C-leucine assimilation.

To explore the effect of enrichment with phosphate, two concentrations of sodium dihydrogen orthophosphate (NaH_2PO_4 $2H_2O$) were added to two different 10 ml samples of 100 g l^{-1} soil slurry, in sterile universal bottles, to give final phosphorus concentrations of 50 μg P g^{-1} wet weight soil and 500 μg P g^{-1} wet weight soil.

To assess the effect of nitrogen enrichment, two concentrations of ammonium chloride (NH_4Cl) were added to two different 10 ml samples of 100 g l^{-1} soil slurry, in sterile universal bottles. The final concentrations of nitrogen in the universal bottles were 22.4 μg N g^{-1} wet weight soil and 224 μg N g^{-1} wet weight soil. Six 10 ml replicates of 100 g l^{-1} soil slurry were also prepared without added phosphorous or nitrogen, to act as controls.

The above slurry samples were incubated for 24 hours in darkness at 10 °C. These 100 g l^{-1} soil suspensions were then diluted with 0.2 μm filtered sterile pure water to give a concentration of 1.0 g l^{-1}. Subsequently, the ^{14}C-leucine assimilation assay was undertaken on the soil slurries (as described in Section 4.3.3).

4.3.3.2. Assays under anaerobic conditions

Fresh soil samples obtained from Hatfield Moor and Sutton Common, at depths of 50 cm, 70 cm and 100 cm in November 2005, were rapidly removed from the corer head

and were placed into sterile polythene bags. The soil samples were then sealed within an anaerobic jar that contained an anaerobic generation packet Anaero Gen™ (Oxoid Ltd, UK) in order to create an anaerobic environment.

The soil samples were transported to the laboratory within 5 hours of collection. The anaerobic jars were opened and one soil sub-sample was taken from each jar by inserting a 50 ml sterile universal bottle into the anaerobic soil contained within the polythene bag. Each soil sub-sample was rapidly removed and placed into a 500 ml sterile conical flask with nitrogen gas (N_2) passing though in order to maintain the anaerobic conditions.

The weight of the soil sub-sample was estimated by inserting a sterile universal bottle of known weight into a soil sample within the polythene bag under aerobic conditions. The bottle and sample were subsequently weighed. The difference between the weight of the bottle containing the soil sample and the weight of the empty bottle equalled the weight of the soil.

100 ml of anaerobic sterile Ringer solution (Oxoid, UK) was added to the conical flasks that contained the anaerobic soil sub-samples to give a 10 g l^{-1} soil solution. The anaerobic Ringer solution was prepared a day in advance of the on-site soil sampling. The solution was stored in Winchester flasks and kept overnight with N_2 bubbling through it to generate anaerobic conditions.

Three 10 ml replicates of each anaerobic soil suspension were transferred to sterile universal bottles which were adapted for anaerobic assays (Figure 4.1). Each bottle

contained two plastic tubes; one tube carried N_2 into the soil suspension, whilst the other tube acted as an exhaust. N_2 was bubbled through the bottles for 10 minutes. 0.1 ml of ^{14}C-leucine solution were then added into the universal bottles and the tubes of each bottle were then sealed with clamps. This was followed by incubation in darkness for 5 hours at 10 °C.

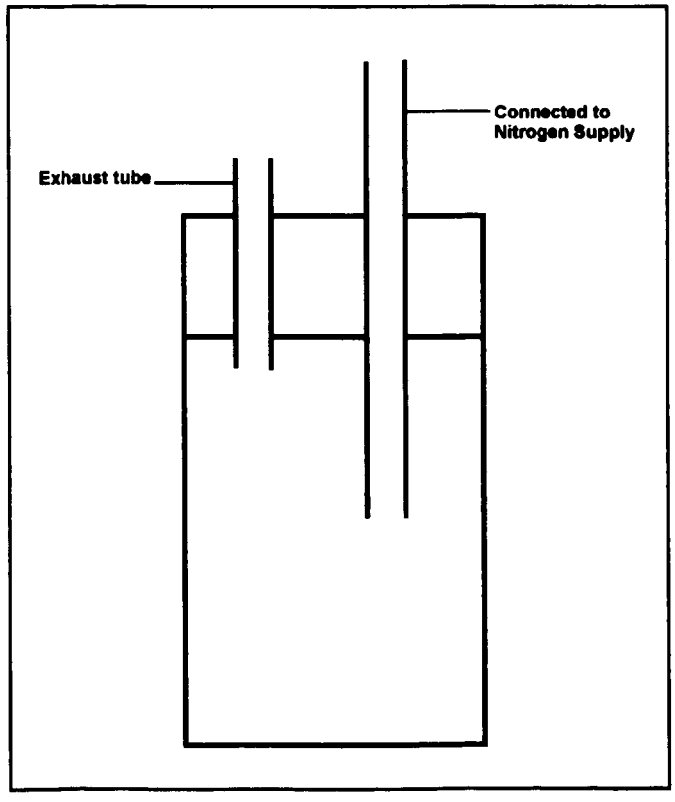


Figure 4.1: Diagram of a universal bottle, which was adapted for anaerobic assay.

At the end of the incubation, the same procedure, as described in Section 4.3.3, was followed. For comparative purposes, aerobic assays were also carried out using the same soil samples.

4.3.4 The use of Biolog Ecomicroplates

Changes in microbial communities within environmental samples have also been monitored by using a Biolog system (Biolog Inc, Hayward, California, USA) (Garland

and Mills, 1991). The Biolog system assesses the physiological profile of the microbial communities within a sample and characterizes them using a pattern of substrate utilization in 96 well-microplates (Figure 4.2) (Garland and Mills, 1991; Fließbach and Mäder, 1996; Di Giovanni *et al.*, 1999; El Fantroussi *et al.*, 1999).

A bacterial cell suspension is used to inoculate the wells of the microplate in which the wells contain different carbon sources, nutrients and a tetrazolium dye. The wells per plate contain 3 replicates of 31 different environmentally important carbon sources and 1 control well per replicate (Table 4.1) (Kirk *et al.*, 2004). The microplates are incubated and are monitored periodically.

The growth of aerobic, heterotrophic microorganisms in the wells is indicated by the oxidation of the substrate with the concomitant reduction of the tetrazolium dye. This reaction produces colour development which can be quantified colorimetrically (Figure 4.2). The technique provides a metabolic fingerprint of the specific microbial population or community in the sample (Smalla *et al.*, 1998; Widner *et al.*, 2001).

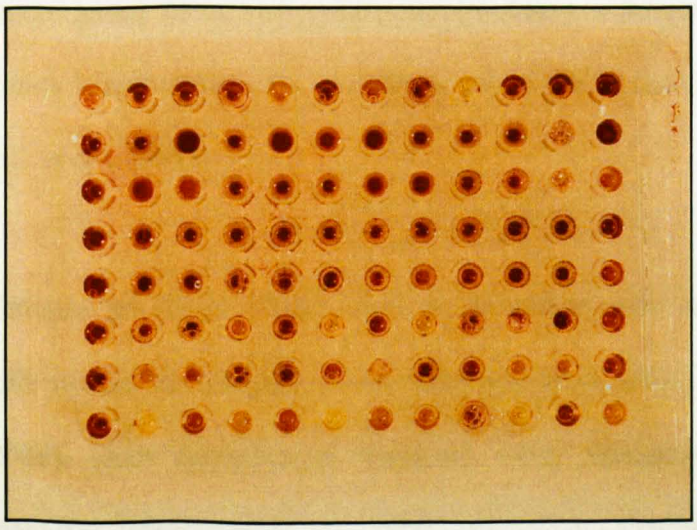


Figure 4.2: The wells of a Biolog plate showing colour development after 5 days of incubation at 10 °C.

Table 4.1: Carbon substrates used in Biolog Ecomicroplates. Assignment to biochemical categories follows that of Insam (1977).

Carbohydrates	**Polymers**
β-methyl-D glucoside	Tween 40
D-xylose	Tween 80
i-Erythritol	Glycogen
D-Mannitol	α-cyclodextrin
N-acetyl D-glucosamine	**Phenolic acids**
D-cellobiose	2-Hydroxy Benzoic acid
α-D lactose	4-Hydroxy Benzoic acid
Carboxilic acids	**Phosphorylated chemicals**
D-galactonic acid γ lactone	Glucose-1-phosphate
D-galacturonic acid	D,L-α-glycerol phosphate
γ-hydroxibutiric acid	**Amines**
D-glucosaminic acid	Phenylethylamine
Itaconic acid	Putrescine
α-ketobutyric acid	**Esters**
D-Malic Acid	Piruvic acid methyl ester
Amino acids	
L-arginine	
L-asparagine	
L-phenylalanine	
L-Serine	
L-threonine	
Glyciyl-L-glutamic acid	

The results obtained using these microplates are primarily a reflection of bacterial metabolism, as many fungal species are not able to reduce the tetrazolium dye (Kirk *et al.*, 2004; Avidano *et al.*, 2005).

The Biolog technique has been widely used in diverse studies of soil microbial communities. Soils studied include plant cover (Grayston *et al.*, 2001; Ritz *et al.*, 2004, Singh *et al.*, 2006), plant rhizospheres (Garland, 1996; Grayston *et al.*, 1998), herbicide treatment (el Fantroussi *et al.*, 1999), pollution (Avidano *et al.*, 2005;

Knight *et al.*, 1997), composting processes (Laine *et al.*, 1997), and aquifers (Röling *et al.*, 2000).

Although the Biolog system allows a rapid and simple study of the functional diversity of soils, it does have some inherent problems. It is culture dependent (Haack *et al.*, 1995; Garland and Mills, 1991), favours fast growing microorganisms (Yao *et al.*, 2000); and it may be difficult to obtain reproducible results due to the different densities of replicate inocula and changes to the microbial community during the inoculation process (Garland 1996; Insam, 1997; Singh *et al.*, 2006). Because of these problems, the data obtained from Biolog microplates are perhaps best considered as relevant to the functional diversity of cultivable and fast growing microorganisms (Smalla *et al.*, 1998).

A 1.0 g l^{-1} soil suspension was obtained by diluting the 100 g wet weight suspension using 0.2 μm filtered, sterile water. 150 μl of 1.0 g l^{-1} soil suspension were added to each well of the Biolog Ecomicroplates. Soil samples obtained at different depths from Hatfield Moor and Sutton Common, in February 2005, June 2005, October 2005 and April 2006, were used for this assay. During each sampling month, 10 microplates were used, one for each different depth of the soil profile at both sampling sites. The microplates were incubated in the dark at 10 °C for 5 days. The optical density in the wells was read every 24 hours using a MRX II Microplate Reader (Dynex Technologies, USA), which was set at a wavelength 590 nm.

The data from each microplate were processed as follows:

1) The colour development in the control well was subtracted from the absorbance reading in all other wells
2) Values for substrates with no colour development (i.e. negative values), were set to 0
3) Each microplate was analysed based on its Average Well Colour Development (AWCD) (Garland, 1996). Single time readings were normalized by dividing for the AWCD of the microplate in order to account for possible differences in the inoculation density between samples

   AWCD = (sum of OD_i)/ 31

   Where: OD_i = Optical density at 590 nm from each well

After normalization of the Biolog dataset, principal component analysis (PCA) was used for further data investigation. PCA is a statistical technique which allows the characterization of multivariate data by a smaller number of new variables, i.e. the first principal component (PC1) is a linear combination of the original variables that represents the greatest spread observed in the data. PC1 by definition explains the highest percentage of total variance in the data. Due to the unbiased nature of the analysis, univariate statistical procedures such us plots, correlations, t-tests and ANOVA techniques can subsequently be applied to the principal component values in order to describe the relationships under study (Selvin, 1995). Community Analysis Package, version 2.0 (Pisces Conservation Ltd, Lymington, UK), was used for this analysis.

4.4 Scanning electron microscopy

For scanning electron microscopy (SEM) studies, oak wood samples were obtained from the Hatfield Moor trackway in November 2004. Further wood samples were also obtained from Hatfield Moor at a depth 150 cm in October 2005 and Sutton Common at a depth 60 cm in October 2005.

Wood sections of approximately 3 mm thickness were cut from the wood samples using a razor blade. The sections were fixed at room temperature in 5 % v/v glutaraldehyde in 0.1 mol l^{-1} cacodylate buffer for 4 days. The fixed samples were subsequently washed in cacodylate buffer solution on three further occasions. The sections were then dehydrated in increasing strengths of ethanol (30-100 %), 15 minutes in each of 10 % steps. After dehydratation, the samples were immersed in HMDS (hexamethyldisilazane) and then left to air dry overnight. The dried sections were mounted on aluminium stubs and gold coated in a Nanotech SEMPREP 2 Sputter Coater.

The prepared samples were analysed using a Cambridge Instruments S360 Scanning Electron Microscope, which was fitted with an in-house Digital Image Capture System at an accelerating voltage of 20 kV.

4.5 Summary

This chapter has described the conventional techniques that were used to study microbial communities in the soil. These techniques measure bacterial abundance microbial metabolic activity by extracellular enzyme activity; and ^{14}C-leucine assimilation rates through the soil profile.

The physiological profile of the microbial community using Biolog Ecomicroplates has also been applied over a seasonal timescale in order to characterize the soil microbial communities at both sampling locations.

In addition, methods for SEM observations made on buried wood samples from the prehistoric trackway at Hatfield Moor (Section 2.2.4), and the two study sites, are also described.

Finally, Chapter 5 will outline the molecular techniques that were employed in the current research in order to characterize the bacterial communities.

Chapter 5 Molecular genomic methods

5.1 Introduction

The majority of previous research into soil microbial communities has been based on culture-dependent methods and isolation techniques (Wagner *et al.*, 1993; Amman *et al.*, 1995; Torsvik *et al.*, 1996; Hugenholtz *et al.*, 1998). However, culture-dependent methods do not accurately reflect the bacterial community structure, because growth media are selective for certain groups of bacteria and it is not possible to design culture conditions suitable for all members of the bacterial community (Von Wintzingerode *et al.*, 1997; Theron and Cloete, 2000; Prosser, 2002). In addition, it is accepted that culture methods recover less than 1 % of the total bacteria present in environmental samples (Borneman *et al.*, 1996; Torsvik *et al.*, 1998; Schabereiter-Gurtner *et al.*, 2001; Nannipieri *et al.*, 2003). Nevertheless, the appropriate culture techniques can be used to complement molecular studies in order to give a fuller representation of the microbial community in an environmental sample.

The advent of molecular genomic techniques has enabled the characterization of natural microbial communities without the need to culture microorganisms (Borneman *et al.*, 1996; Clement *et al.*, 1998; Engelen *et al.*, 1998; Dunbar *et al.*, 1999; Tiedje *et al.*, 1999; Dobrovol'skaya *et al.*, 2000; Felske *et al.*, 2000; Ranjard *et al.*, 2000). The use of these molecular techniques allows the characterization of those components of the soil microbial community that currently cannot be cultured, and as a consequence, demonstrates the presence of a huge diversity of microorganisms in soil (Torsvik *et al.*, 1990; Borneman *et al.*, 1996; McCaig *et al.*, 1998). Previous research by Torsvik *et al.*

(1990) has estimated that in 1 g of soil it is possible to find 4000 different bacterial 'genomic units'.

A useful molecular approach, which characterizes microbial community composition and diversity in soils, is the analysis of 16s ribosomal RNA (rRNA) genes (Amman *et al.*, 1995; Kuske *et al.*, 1997; Head *et al.*, 1998; Hugenholtz *et al.*, 1998; Felske *et al.*, 1999; McCaig *et al.*, 1999; O'Donnell and Görres, 1999; Rondon *et al.*, 1999; Kozdrój and van Elsas, 2001). During evolution, different regions of the ribosome gene mutated at different rates. As a consequence, some regions of this gene are highly conserved and similar between organisms. However, other regions are variable even between closely related species. This variability allows for the distinction between different groups (Prosser, 2002).

The rRNA genes are conserved across all phylogenetic domains (i.e. *Bacteria*, *Eucarya* and *Archaea*). This allows the identification of 'universal' fragments of the 16s rRNA sequence. It is possible to analyse signature sequence motifs at various taxonomic levels for use in evolutionary-based identification (Head *et al.*, 1998; Theron and Cloete, 2000; Kirk *et al.*, 2004). During recent years a considerable and expanding database of 16s rRNA gene sequences for bacteria has been developed (Nannipieri *et al.*, 2003). This database is a useful tool in the identification of DNA sequences recovered from environmental samples.

The molecular genomic analysis of environmental samples frequently includes the extraction and purification of bacterial DNA. This procedure is followed by the polymerase chain reaction (PCR) amplification of the 16s RNA genes using specific

primers (Rondon *et al.*, 1999; Prosser, 2002). The resulting PCR product may be analysed in several ways. The main techniques include:

1) Terminal restriction fragment length polymorphism (T-RFLP) (Bruce, 1997; Clement *et al.*, 1998; Osborn *et al.*, 2000)
2) Single strand conformational polymorphism (SSCP) (Schweiger and Tebbe, 1998; Stach *et al.*, 2001)
3) Amplified ribosomal DNA restriction analysis (ARDRA) (Porteous *et al.*, 1994; Heyndrickx *et al.*, 1996; Hoppe-Seyler *et al.*, 2003)
4) Percentage gas chromatography (GC) content (Nüsslein and Tiedje, 1998)
5) Fluorescence *in situ* hybridisation (FISH) (Amman *et al.*, 1990)
6) Intergenic transcriber spacer (ITS) (Johnson *et al.*, 2003)
7) Denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.*, 1993; Teske *et al.*, 1996; Gelsomino *et al.*, 1999; Griffiths *et al.*, 2003a and b)
8) Temperature gradient gel electrophoresis (TGGE) (Heuer *et al.*, 1997; Muyzer and Smalla, 1998; Felske *et al.*, 1999; Smit *et al.*, 1999)

The techniques highlighted above offer a rapid, cost effective and reproducible way of characterizing microbial communities, and of studying associations between community structure and soil habitat (Johnson *et al.*, 2003). In light of this information, several molecular techniques will be used in the current study in order to understand changes in the composition of the microbial communities present within the soil profiles of Hatfield Moor and Sutton Common.

An integrated approach employing biochemical, physiological and molecular methods is important in ecological studies of complex microbial communities (such as those

present in soil), as the use of a single method will provide only limited data (Singh *et al.*, 2006). Figure 5.1 highlights the molecular approaches used in this research.

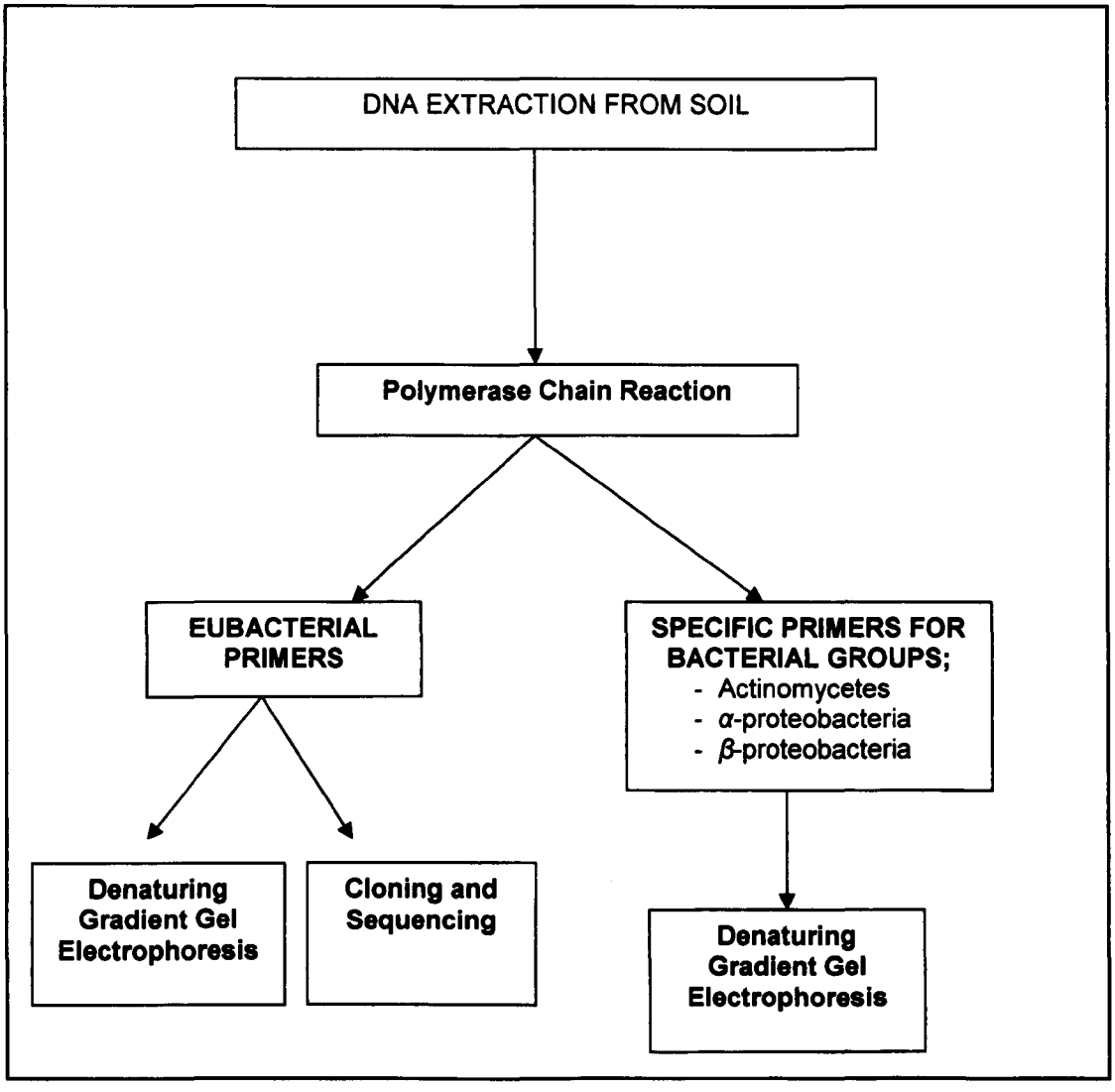


Figure 5.1: The molecular genomic techniques used in the current study.

Prior to molecular analysis of the environmental samples it is necessary to extract and purify the DNA. This procedure is followed by the polymerase chain reaction (PCR) amplification of the DNA by using both general primers for bacteria (Eubacteria), and specific primers for three of the most common groups of bacteria found in soil (α-proteobacteria, β-proteobacteria and actinomycetes). The PCR products were

subsequently separated by denaturing gradient gel electrophoresis (DGGE) which allowed the separation of the dominant bacterial taxa throughout the soil profile.

In order to obtain detailed information concerning the bacterial species present at different depths within the soil profile at Hatfield Moor and Sutton Common, a number of samples were collected from three depths (10 cm, 30 cm and 50 cm) in December 2005. All of the samples were subsequently cloned and sequenced.

5.2 DNA extraction and purification

5.2.1 Introduction

Various methods are available for extracting and purifying nucleic acids from environmental samples. These are commonly based on the chemical and/or physical disruption of cells (Hugenholtz and Pace, 1996; Theron and Cloete, 2000). One of the most employed methods to extract DNA from soil is the bead-beating method. This procedure mechanically lyses the cells in the presence of phenol or sodium dodecylsulphate (SDS) (Duarte *et al.*, 1998). An alternative method is cell lysis facilitated by the heating of the soil sample in SDS followed by treatment with enzymes (Macrae, 2000).

Different DNA extraction methods are liable to result in different fingerprints from the same microbial community (Kozdrózj and van Elsas, 2000; Martin-Laurent *et al.*, 2001; Ranjard *et al.*, 2003; Crecchio *et al.*, 2004). As a consequence, the efficiency of the extraction method needs to be considered prior to use. Insufficient or preferential disruption of cells during DNA extraction can misrepresent the structure of the microbial community; for example, bacterial spores are more resistant to lysis than

vegetative cells and Gram-negative cells are easier to lyse than Gram-positive cells. DNA recovery may also be reduced by the degradation or the adsorption of nucleic acids by soil materials such as clay particles (Wintzingerode *et al.*, 1997; Head *et al.*, 1998; Theron and Cloete, 2000). Previous research has shown that a combination of different extraction methods, such as lysis with detergents, bead-beating, or cycles of freezing and drying, can yield DNA from approximately 96 % of cells in a soil sample (including bacterial endospores) (Moré *et al.*, 1994).

One of the most important processes involved in the extraction of DNA from soil is the separation of the DNA from humic substances, organic matter, clay and metals. All these materials can interfere with the PCR amplification of the DNA by inhibiting the *Taq* polymerase (Bachoon *et al.*, 2001; Niemi *et al.*, 2001; Kirk *et al.*, 2004). In addition, co-extracted humic substances can interfere with the fluorometric quantification of the DNA with PicoGreen reagent or Hoechst dye (Bachoon *et al.*, 2001). These humic and other contaminating substances can mostly be removed from the extracted soil DNA by using commercial extraction kits which are widely available. Niemi *et al.* (2001) have previously reported that DNA extraction using the Soil DNA Isolation Kit (Mo Bio Laboratories Inc, California, USA) yielded good quality DNA, which can give consistent and clear banding in denaturing gradient gel electrophoresis (DGGE) gels. In light of this information, the Soil DNA Isolation Kit will be used in the current study in order to obtain clean DNA which is ready for PCR.

Two different DNA extraction methods were used in the current study; the bead-beating procedure which was followed by phenol: chloroform: isoamyl alcohol extraction and DNA extraction using the Ultraclean™ Soil DNA Isolation Sample Kit

(Sections 5.2.2 and 5.2.3 below). The absence of PCR inhibitors within the different DNA samples was subsequently assessed.

5.2.2 Bead-beating and phenol: chloroform: isoamyl alcohol extraction of DNA from soil

0.5 g of sterile glass beads (0.17-0.18 mm diameter, B. Braun Biotech International, Germany) were placed into a 2 ml plastic screw-top microcentrifuge tube. 1 g of soil sample was added to the microcentrifuge tube. 750 µl of 0.12 mol l^{-1} sodium phosphate buffer (0.03 mol l^{-1} Na_2HPO_4, 0.09 mol l^{-1} NaH_2PO_4, adjusted to pH 8 with NaOH) and 500 µl phenol: chloroform: isoamyl alcohol (25:24:1 v/v/v, Sigma) were subsequently added. The samples were then placed in a Mini-BeadBeater-1 (Biospec Products, USA) and treated for 2 minutes at 1600 beats per minute (bpm).

The samples were centrifuged in a microcentrifuge IEC Micromax (Thermo Electron Corporation, Beverly, USA) for 5 minutes at 10,000 Rotational Centrifugal Force (RCF). The top aqueous layer, which contained the DNA, was removed and stored on ice in a fresh 2 ml microcentrifuge tube. The dark brown phenol: chloroform layer was pipetted off and discarded. A further 500 µl of sodium phosphate buffer and 500 µl of phenol: chloroform: isoamyl alcohol was added to the residue. The bead-beating and centrifugation procedure were repeated. The aqueous layer was removed and pooled with the aqueous layer which was obtained from the first bead-beating procedure.

1 ml of phenol: chloroform: isoamyl alcohol was added to the pooled aqueous layers. The sample was then shaken thoroughly to mix the layers and centrifuged for 5

minutes at 10,000 RCF. The washing and centrifugation process was performed until no protein interface was observed between the aqueous and phenol: chloroform layer.

To precipitate the DNA, one tenth volume of 5 mol l^{-1} NaCl solution (typically 100 µl) and an equal volume of 30 % (w/v) polyethylene glycol 8000 (PEG) were added to the sample. This was stored at room temperature for 2 hours. The sample was centrifuged for 10 minutes at 10,000 RCF. The aqueous fraction was removed and the nucleic acid pellet was then washed by adding 100 µl of 80 % ethanol, followed by centrifugation for 2 minutes at 10,000 RCF. This washing process was repeated. After the final ethanol wash, the contents of the tube were left to air-dry overnight, until the ethanol had evaporated and the pellet was completely dry. The DNA pellet was re-suspended in 100 µl of molecular biology grade water.

A sample (5 µl) of the DNA solution was used to check that there was adequate DNA product. This was undertaken using horizontal agarose gel electrophoresis (as described in Section 5.3 below). The remaining DNA was stored at –20 °C prior to its characterization.

5.2.3 Extraction of DNA from soil using the Ultraclean™ Soil DNA Isolation Sample Kit

The Ultraclean™ Soil DNA Isolation Sample Kit (Mobio, UK) was used on the soil samples which contained potential contaminants, i.e. those samples where it was not possible to extract a good yield of DNA by the bead-beating procedure. 1 g of soil sample was added to a 2 ml bead solution tube (containing beads) and vortexed to mix for 5 seconds. The solution tube was provided by the kit manufacturer. Next, 60 µl of

solution S1 (containing sodium dodecyl sulphate [SDS]) was added and the tube was vortexed for 5 seconds. Then, 200 µl of Inhibitor Removal Solution (IRS) was added to the tubes in order to precipitate the humic acids. The tubes were then secured horizontally in a MoBio Vortex Adapter VX100 (Mobio, UK) and vortexed at maximum speed for 10 minutes.

The tubes were then centrifuged in a Sigma 1-15 microcentrifuge (Sigma) at 10,000 RCF for 30 seconds and the supernatant was transferred to a clean microcentrifuge tube (which was provided by the kit manufacturer). 250 µl of solution S2, which contained a protein precipitation reagent, was added to the supernatant. The mixture was vortexed for 5 seconds. The tubes were incubated at 4 °C for 5 minutes and subsequently were centrifuged for 1 minute at 10,000 RCF. 450 µl of the supernatant were then transferred to a clean microcentrifuge tube and 900 µl of solution S3 (a DNA binding salt solution) were added. The tubes were again vortexed for 5 seconds and 700 µl of the solution was loaded onto a spin filter (which was provided by the kit manufacturer) and centrifuged at 10,000 RCF for 1 minute. The flow through was discarded and the remaining supernatant was added to a further spin filter and centrifuged at 10,000 RCF for 1 minute.

300 µl of solution S4 (an ethanol based wash solution) were added to the tubes which contained the spin filter with the DNA attached. The mix was centrifuged for 30 seconds at 10,000 RCF. The flow through was discarded and the tubes were subsequently centrifuged for 1 minute. The spin filter was then placed in a new clean tube and 50 µl of solution S5 (an elution buffer) were added to the centre of each filter.

The tubes were centrifuged for 30 seconds at 10,000 RCF and the spin filters were discarded. The DNA contained in the tube was stored at –20 °C.

5.2.4 DNA extraction from defined bacterial cultures

In order to obtain DNA markers for denaturing gradient gel electrophoresis (DGGE) analysis (as described in Section 5.5 below), DNA was extracted from liquid cultures of *Actinomadura atramentaria* NCIMB 12575, *Agromonas oligotrophica* NCIMB 12151 and *Burkholderia cepacea* NCIMB 12618. The same methodology was also used to extract DNA from liquid cultures of *Escherichia coli* and *Bacillus sp.* from the Department of Biological Sciences collection, at the University of Hull.

1.0 ml of the bacterial cultures were added to sterile Eppendorf tubes and centrifuged at 10,000 RCF in a microcentrifuge IEC Micromax (Thermo Electron Corporation, Beverly, USA) for 20 minutes to pellet the cells. The cells were resuspended and washed twice with sterile phosphate buffer solution (PBS) and centrifuged between each wash. After the second wash, the cells were resuspended in 200 µl of PBS, to obtain a dense suspension. 50 µl of the sample were transferred to 0.2 ml PCR tubes and put into a PCR machine (PHC-3 DNA Thermal Cycler) where they were treated at 99 °C for 15 minutes and 4 °C for 10 minutes. The tubes were subsequently centrifuged at 10,000 RCF in a microcentrifuge for 5 minutes. The top 30 µl of supernatant were removed; this solution contained the extracted DNA.

Using 5 µl of the DNA solution, agarose gel electrophoresis was undertaken to check that there was sufficient extracted DNA in the samples, (as described in Section 5.3). The extracted DNA was stored at –20 °C before further use.

5.2.5 Purification of DNA

DNA purification eliminated any contaminants still present in the samples (such us humic acids and other organic substances) which can interfere with the PCR amplification process (Zhou *et al.*, 1996). This purification method was used on the DNA extractions which were obtained by using the bead-beating and phenol: chloroform: isoamyl alcohol method (as described in Section 5.2.2). Purification was undertaken using the Wizard® DNA Clean-Up System (Promega, Madison, USA).

1 ml of Wizard® DNA Clean-Up Resin and 50 µl of DNA solution which was obtained from the bead-beating extraction procedure were added to a 1.5 ml microcentrifuge tube. The solution was mixed by gently inverting the tube several times. The contents were pipetted into a 3 cm^3 syringe barrel and transfered slowly into a Wizard® Minicolumn (provided by the kit manufacturer) by using a syringe plunger. To wash the column, 2 ml of 80 % isopropanol was pipetted into the syringe; the solution was gently pushed through the Minicolumn using the syringe plunger. The syringe barrel was removed and the Minicolumn was transferred to a 1.5 ml microcentrifuge tube. The solution was centrifuged for 2 minutes at 10,000 RCF in a microcentrifuge IEC Micromax (Thermo Electron Corporation, USA) in order to dry the resin.

After centrifugation the Minicolumn was transferred to a fresh 1.5 ml microcentrifuge tube, to which 50 µl of pre-warmed PCR water (65-70 °C) were added and left for 1 minute. The Minicolumn was then centrifuged for 20 seconds at 10,000 RCF. The Minicolumn was then discarded and the aqueous solution of purified DNA was stored in the microcentrifuge tube at –20 °C.

Using 5 µl of the DNA solution, agarose gel electrophoresis was undertaken in order to check that there was adequate DNA product (as described in Section 5.3 below).

5.3 Separation of DNA using agarose gel electrophoresis

Horizontal submerged agarose gel electrophoresis was used to check for the presence of DNA, both after the extraction and purification process, and after the PCR amplification procedure.

A 10 % agarose gel was prepared by boiling 0.3 g of agarose (Sigma) in 30 ml of running buffer Tris- Borate-EDTA (0.5 x TBE). Once cool, the agarose was poured into the gel casting tray, where it was left to set at room temperature. 5 µl of DNA solution and 1 µl of 6 x loading buffer IV (40 % [w/v] sucrose, 0.25 % [w/v] bromophenol blue in molecular biology grade water [BDH] [Sambrook *et al.*, 1989]) were added into each well. 6 µl of a purple ladder size-marker (PCR 100 base pair [bp] low ladder [Sigma-Aldrich, UK]) were added into the first well in order to give a reference to the size of the DNA fragments after electrophoresis. A voltage of 70 mV was applied for approximately 1 hour, until the DNA had migrated sufficiently through the gel.

After electrophoresis the bands of DNA were observed by soaking the gel for 15 minutes in 1 mg l^{-1}ethidium bromide, which binds to DNA by intercalation between the paired nucleotides of the double helix. Gels were visualised under UV radiation (302 nm) and recorded using the Molecular Analyst software in conjunction with the Gel Doc 1000 gel documentation system (Bio-Rad Laboratories Ltd, California, USA).

5.4 Polymerase chain reaction

5.4.1 Introduction

Polymerase chain reaction (PCR) is a molecular technique which may be used to amplify specific nucleic acid sequences obtained from the total DNA extracted from an environmental sample.

The target sequence for amplification is defined by two primers (oligonucleotide sequences) which flank the target sequence and that anneal to the complementary DNA of the target sequence (Figure 5.2). The PCR employs cycles of DNA denaturation (DNA separation into single strands), annealing of primers to the target DNA and extension of the primers across the target sequence. This three stage cyclic procedure results in more copies of the target DNA.

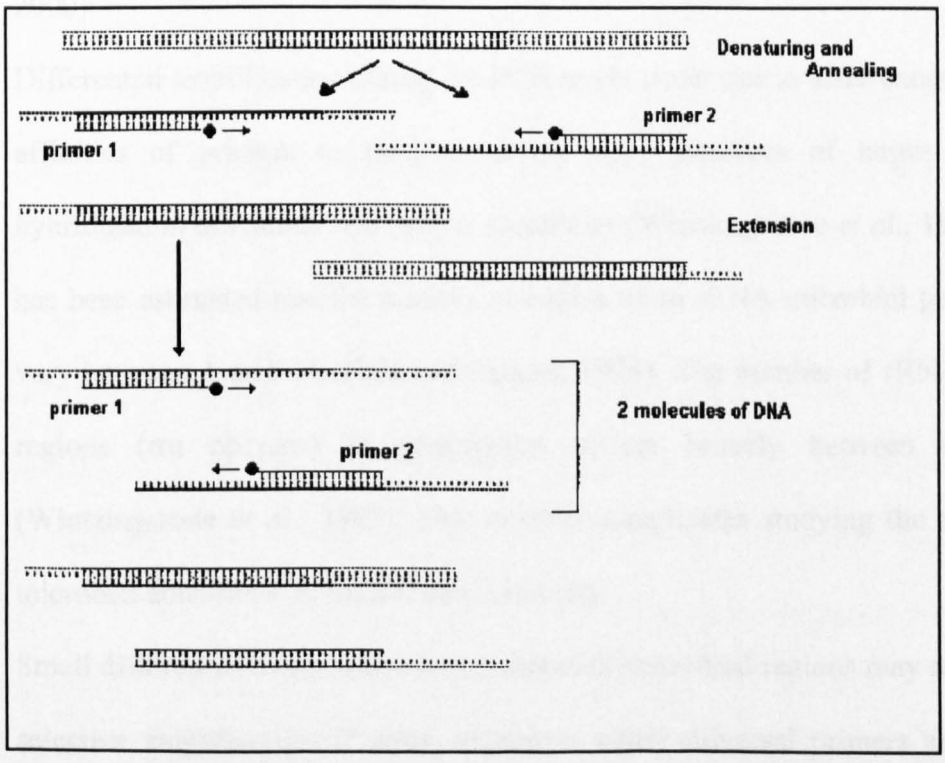


Figure 5.2: The methodology behind PCR (modified from Hugenholzt and Pace, 1996).

One must however be aware of several potential problems that exist during the PCR amplification procedure. These are as follows:

1) The PCR might be inhibited by substances which are co-extracted with the DNA in the initial extraction step (e.g. humic acids) (Zhou *et al.*, 1996). However, this problem may be reduced by including additives into the PCR reaction mix (e.g. bovine serum albumin [BSA] and T4 gene 32 protein [Kreader, 1996]).
2) The inefficiency of the PCR might be caused by the addition of too little template DNA. When very low DNA concentrations are used, for example when the template DNA solution is diluted to minimise the effects of the humic acids, the PCR reaction can suffer random fluctuations in priming efficiency (Wintzingerode *et al.*, 1997; O'Donell and Görres, 1999; Macrae, 2000).
3) Differential amplification during the PCR might occur due to differences in the affinities of primers to template DNA, copy numbers of target genes, hybridization efficiency and primer specificity (Wintzingerode *et al.*, 1997). It has been estimated that the number of copies of an rRNA microbial gene can vary between 1 and 14 (Cole and Girons, 1994). The number of rRNA gene regions (rrn operons) in prokaryotes differs broadly between species (Wintzingerode *et al.*, 1997). This number complicates studying the relative microbial abundance of natural environments.
4) Small differences in the sequence of universal conserved regions may result in selective amplification of some sequences when universal primers are used (Head *et al.*, 1998). More abundant sequences are also preferentially amplified (Ward *et al.*, 1992) and high % G+C templates are discriminated due to a

lower efficiency of strand separation during the denaturing step (Reysenbach *et al.*, 1992; Wintzingerode *et al.*, 1997).

5) The PCR amplification of DNA might produce the formation of artefactual PCR products (such as chimeric products and mutations). Chimeric products are formed when fragments from two different sequences are fused during the amplification process. Mutations can be of two types; deletions due to DNA stable secondary structure and point mutants due to mis-incorporation by DNA polymerases (Liesack *et al.*, 1991; Wintzingerode *et al.*, 1997). Wang and Wang (1996) have previously demonstrated that up to 30 % of PCR products which were generated during the co-amplification of similar templates were chimeric. Furthermore, Liesack *et al.* (1991) have identified that when DNA is extracted by bead-beating, small fragments might be formed and during PCR these may produce chimeric hybrids.
6) PCR analysis tends to be limited to the more numerically abundant populations which are targeted by the primer used. As a consequence, DGGE community profiles are a representation of the most abundant members of the bacterial community (Forney *et al.*, 2004).

The most common problem with PCR in this research was contamination of the PCR mix. Contamination by DNA that contained the specific sequence of the PCR reaction resulted in amplification in negative controls where DNA was not added and in co-amplification in test reactions. Non specific DNA can be introduced into PCR reactions when previous PCR products are unintentionally transferred to fresh reactions, or by contaminated reagents. Wintzingerode *et al.* (1997) have demonstrated that the amplification of ribosomal RNA genes using universal bacterial

primers is extremely sensitive to contamination by bacterial DNA. Many authors have also detected DNA contamination in commercial *Taq* DNA polymerase (Böttger, 1990; Rand and Houck, 1990; Schimdt *et al.*, 1991; Meier *et al.*, 1993; Hughes *et al.*, 1994; Maiwald *et al.*, 1994; Niederhauser *et al.*, 1994; Millar *et al.*, 2000; Kawai *et al.*, 2002). Of several de-contamination procedures suggested, the most effective method for the de-contamination of PCR products is by ultra-violet (UV) treatment (Sarkar and Sommer, 1993; Niederhauser *et al.*, 1994).

5.4.2 The application of general primers for eubacteria

To avoid contamination problems, all PCR set up was carried out in an environment that was free of contamination by PCR products; i.e. the Ancient DNA Laboratory in the Department of Biological Sciences at the University of Hull. The preparation of the PCR reactions was done in a safety cabinet Class I with UV radiation and laminar flow (Microflow®, Safety Cabinet, UK). All the materials employed for PCR (i.e. pipettes, pipette tips, gloves, PCR tubes, etc.) were sterilised by applying 30 minutes of UV radiation. A kit of pipettes solely for preparation of PCR reactions was used. In addition, all the reagents (primers, PCR buffer, PCR water, $MgCl_2$ and *Taq* polymerase) were dispensed in small quantities, sufficient only for one day's work.

To characterize the bacterial community in the soil samples the 16S rRNA gene was amplified by PCR using general primers for Eubacteria (Table 5.1). For PCR reactions a master mixture was prepared (Table 5.2).

From this PCR mixture 49 µl were added to 1 µl of template DNA (diluted with sterile water 1:10 or 1:100) in 0.2 ml sterile microcentrifuge tubes. A negative control, of

molecular grade sterile water, and a positive control containing *E. coli* DNA were also included.

Table 5.1: General eubacterial PCR primers.

Primer	16S rDNA target (position in *E. coli*)	Sequence
GM5F	41-357	5'-CCTACGGGAGGCAGCAG-3'
DS907R	907-928	5'-CCCCGTCAATTCMTTTGAGTTT-3'
GC-clamp	Attached to the 5' end of GM5F	5'-CGCCCGCCGCGCGCGGCGG GCGGGGCGGGGGCACGGGGGG-3'

Table 5.2: Composition of the PCR master mixture.

Reagent	Volume added to PCR mixture per sample
10 x PCR reaction buffer 100 mmol l^{-1} Tris-HCl, pH 8.8, 500 mmol l^{-1} potassium chloride [KCl], 1 % [v/v] Triton X-100)	5 µl
$MgCl_2$ (25 mmol l^{-1})	2 µl
Forward primer at 20 pmol μl^{-1}	1 µl
Reverse primer at 20 pmol μl^{-1}	1 µl
dNTPs (125 µmol l^{-1} of dATP, dCTP, dGTP, dTTP; Ultrapure dNTPs,)	1 µl
***Taq* DNA Polymerase** [5 U μl^{-1}]	0.25 µl
Molecular biology grade water	38.75 µl
Total	**49 µl**

To increase the number of single-base changes that could be distinguished by DGGE, a 30 to 50 base guanine/cytosine-rich sequence (GC-clamp) was added onto the 5′ end of one of the PCR primers (Table 5.1). The GC-clamp acts as a high melting domain that prevents the two DNA strands from completely disassociating (Sheffield *et al.*, 1989; Muyzer *et al.*, 1993).

The PCR tubes were placed in a PCR machine (PHC-3 DNA Thermal Cycler) for approximately 3 hours. Two alternative amplification programmes were used.

1) Programme 1: amplification was performed by the use of 30 cycles of 5 minutes of denaturation at 94 °C, 1 minute at 65 °C for primer annealing and 1 minute at 72 °C for primer extension.

2) Programme 2 used a touchdown PCR technique (Don *et al.*, 1991; Teske *et al.*, 1996). Touchdown PCR is a method in which the amplification of non specific sequences by degenerate primers is avoided (Don *et al.*, 1991; van Elsas and Wolters, 1995). The annealing temperature was initially set at 65 °C, and lowered by 1 °C, every second cycle down to 55 °C, at which temperature 12 additional cycles were carried out. Denaturation was at 94 °C, for 1 minute. Extension was at 72 °C for 1 minute.

5 µl aliquots of the PCR products were analysed by agarose gel electrophoresis (3 % [w/v]). This was followed by ethidium bromide staining (as previously shown in Section 5.3). A PCR ladder with a DNA range between 100-1000 bp (Sigma-Aldrich, UK) was used to quantify the size of the amplified DNA fragments.

5.4.3 The application of specific primers for α-proteobacteria, β-proteobacteria and actinomycetes

The application of eubacterial primers for DGGE results in a complex pattern of bands, showing only the most dominant species. Soil microbial communities are too complex to be understood as a whole by a fingerprint technique (Ovreas and Torsvik, 1998; Boon *et al.*, 2002). For this reason, the use of primers for specific bacterial groups is useful to study defined components; e.g. α-proteobacteria, β-proteobacteria and

actinomycetes, within a complex microbial community (Heuer *et al.*, 1997; Gomes *et al.*, 2001; Boon *et al.*, 2002; Gelsomino and Cacco, 2006).

The α and β-proteobacteria, and actinomycetes are usually well represented in soil (Rondon *et al.*, 1999). Actinomycetes are involved in nutrient cycling, decomposition of diverse organic compounds, formation of humus, and in the biological control of plant pathogens, insects and weeds (Heuer *et al.*, 1997). They are phylogenetically defined as taxa within the high G+C subdivision of Gram positive bacteria (Embley *et al.*, 1994) and they comprise more than 30 % of the total microbial population in soil (Kennedy, 1999). The α-proteobacteria group contain most of the Agrobacteria subdivisions, including most of the methane oxidizing bacteria (Bowman, 2000). The *β*-subdivision of the proteobacteria comprises ammonia-oxidizing bacteria such as *Nitrosomonas* spp., *Nitrosospira* spp. and *Nitrosococcus* spp. (Head *et al.*, 1998; Prosser, 2002). Ammonia oxidizers grow slowly under laboratory conditions and their isolation and identification is difficult (Prosser, 2002).

Primers specific for α- and β-proteobacteria and actinomycetes were used. The choice of selective primers for the initial amplification of 16S rDNA from α- and β-proteobacteria and actinomycete 16S rDNA fragments are based on research undertaken by Gomes *et al.* (2001) (Table 5.3). The products served as template DNA in a second PCR using general eubacteria specific primers. In the second PCR, the GC-clamp was used in order to allow subsequent DGGE analysis of the PCR products.

The initial amplification of group-specific 16S rDNA fragments was carried out using a nested PCR technique with the primer systems F203*α*/R1492, F948*β*/R1492 and

F243HGC/R1492 for α- and β-proteobacteria and actinomycetes, respectively. For each PCR reaction, a master mixture was prepared. Aliquots of 49 µl of this mixture were distributed into PCR tubes containing the template DNA from the different soil samples. The template DNA was substituted with sterile destilled-deionized H_2O in the negative control.

The running conditions in the initial PCR amplification of α- and β-proteobacteria were as follows: 5 minutes at 94 °C, followed by 25 cycles consisting of 1 minute at 94 °C, 1 minute at 56 °C (for α-proteobacteria)/61 °C (for β-proteobacteria) and 2 minutes at 72 °C. Cycling was followed by final primer extension at 72 °C for 10 minutes and cooling to 4 °C.

Initial PCR amplification of actinomycetes was undertaken following the protocol of Heuer *et al.* (1997). The primer pair 1378R and 243HGF were used in the amplification process (Table 5.3). After 5 minutes of initial denaturation at 94 °C, amplification was by 35 cycles of 1 minute of denaturation at 94 °C, 1 minute at 63 °C for primer annealing and 2 minutes at 72 °C for primer extension, followed by a final primer extension at 72 °C for 10 minutes and cooling to 4 °C.

Table 5.3: Primers used for selective amplification of 16S rDNA fragments.

Primer	16S rDNA target (position in *E. coli*)	Sequence
F203α	α-Proteobacteria (174–203)	5'-CCGCATACGCCCTACGGGGGAAAGA TTTAT-3'
F948β	β-Proteobacteria (931–948)	5'-CGCACAAGCGGTGGATGA-3'
F243HGC	Actinomycetes (226–243)	5'-GGATGAGCCCGCGGCCTA-3'
R1492	Bacteria (1492–1513)	5'-TACGG(C/T)TACCTTGTTACGACTT-3'
F984GC	Bacteria (968–984)	5'-gc-AACGCGAAGAACCTTAC
R1378	Bacteria (1378–1401)	5'-CGGTGTGTACAAGGCCCGGGAACG-3'
GC-clamp	Attached at 5' end of F984	5'-GCCCGGGGCGCGCCCCGGGCGGGGC GGGGGCACGGGGGG-3'

5µl aliquots of the products of the initial PCR were checked for successful DNA amplification (Figure 5.3) by electrophoresis on 3 % (w/v) agarose gels, followed by ethidium bromide staining (as previously described in Section 5.3).

The products of the initial PCRs, diluted 1:10, were used as template DNA for a second PCR using general eubacteria-specific primers (F984GC/R1378). The procedure was as follows: 5 minutes at 94 °C followed by 20 thermal cycles consisting of 1 minute at 94 °C, 1 minute at 53 °C and 2 minutes at 72 °C, followed by a final extension step at 72 °C for 10 minutes and cooling to 4 °C.

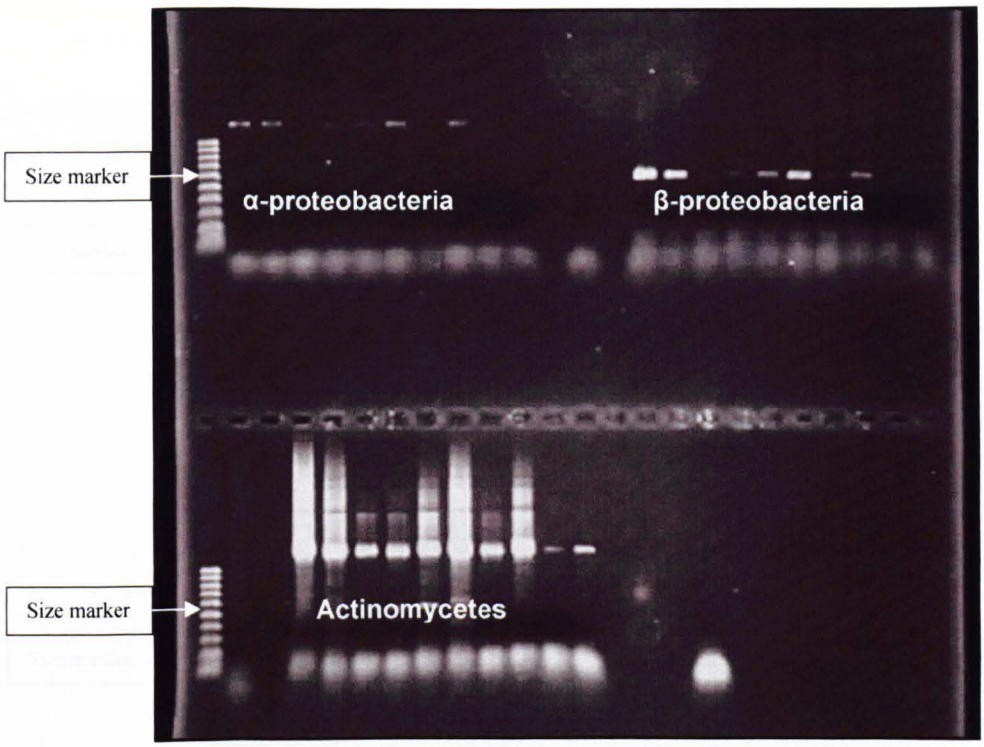


Figure 5.3: Agarose gel to check PCR products from DNA samples from Hatfield Moor and Sutton Common, August 2004 (using specific primers, α- and β-proteobacteria, and actinomycetes).

5 µl aliquots of the PCR products were checked for successful DNA amplification (Figure 5.4) by electrophoresis on 3 % (w/v) agarose gels followed by ethidium bromide staining (as described in section 5.3).

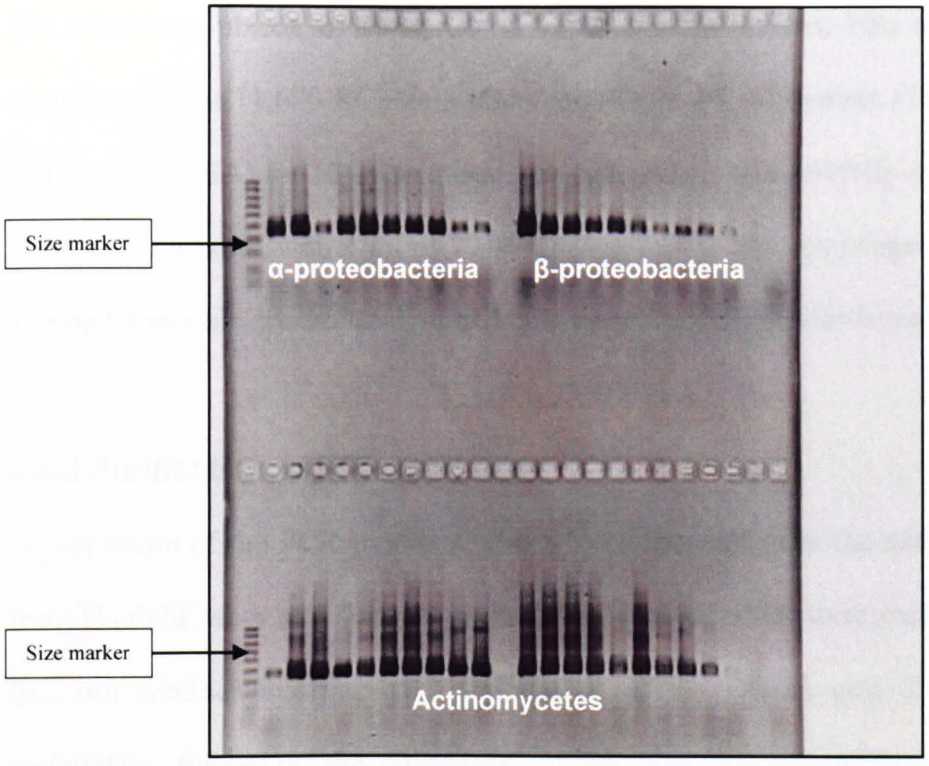


Figure 5.4: Agarose gel to check nested PCR products with eubacteria primers using the GC-clamp (obtained using PCR products from Hatfield Moor and Sutton Common, August 2004).

5.4.4 Concentration of PCR products

The majority of the PCR products from a single PCR reaction did not contain the required amount of amplified DNA to be used for DGGE analysis. At least 100 ng of DNA was needed to load each well of a DGGE gel. Therefore, the products of at least 3 PCR reactions were combined and then concentrated by precipitation in ethanol.

The volume of the combined amplified DNA solution was measured and the salt concentration was adjusted by adding a 1/10 volume of sodium acetate, pH 5.2 (to give a final concentration of 0.3 mol l^{-1}) in a microcentrifuge tube. The mixture was mixed well; 2-2.5 volumes of cold 100 % ethanol (calculated after salt addition) was subsequently added; the mixture was inverted several times.

The tubes were placed on ice at –20 °C for at least 20 minutes. This was followed by centrifugation at 11,600 RCF in a microcentrifuge IEC Micromax (Thermo Electron Corporation, USA) for 10-15 minutes. The supernatant was carefully decanted and the DNA pellet washed with 1 ml of 70 % ethanol, mixed and centrifuged for 5 seconds. The pellet was air-dried overnight and then dissolved in pure sterile water.

5.4.5 Purification of PCR products

A proportion of the PCR products which were obtained from the soil samples taken from Hatfield Moor and Sutton Common in December 2004 were purified in order to find out whether this improved the quality of the DGGE gels. Purification was undertaken following the QIAquick Purification Microcentrifuge and Vacuum Protocol (Quiagen Ltd, UK). To 40 µl of PCR reaction product, 200 µl of Buffer PBI (provided by the kit manufacturer) were added. The solution was mixed thoroughly. Each sample was placed in a 2 ml QIAquick collection tube (Quiagen Ltd, UK) and was centrifuged for 60 seconds in a microcentrifuge (IEC Micromax Thermo Electron Corporation, USA).

The flow-through liquid was discarded and the QIAquick column was washed using 0.75 ml of buffer PE (provided by the kit manufacturer) and centrifuged for 60 seconds. The flow-through was again discarded and the QIAquick column was centrifuged for 1 minute. Each QIAquick column was transferred to a clean 1.5 ml microcentrifuge tube and the DNA was eluted from it by the addition of 50 µl of buffer EB (10 mmol l^{-1} Tris-Cl [pH 8.5]) (provided by the kit manufacturer). The tube was centrifuged for 1 minute. The purified DNA remained in the buffer solution for subsequent analyses.

5.4.6 Quantification of DNA from PCR products

A fresh aqueous working solution of PicoGreen Reagent (Invitrogen™, UK) was prepared on the day of the assay by making a 1:200 dilution of the concentrated dye in 10 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA [pH 7.5] [TE]). The working solution was kept in the dark as the PicoGreen Reagent is susceptible to photodegradation.

A DNA calibration graph of concentration range 1 ng ml^{-1} to 1 µg ml^{-1} was prepared using a stock solution of Lambda DNA (100 µg ml^{-1}) (which was provided by the kit manufacturer). The test DNA solutions were diluted with 1 x TE buffer to a final volume of 100 µl in microcentrifuge tubes. The contents were mixed and transferred to a 96 well microplate Microlon® (Greiner Bio One, Germany) where 100 µl of PicoGreen working solution were added to each well. The microplate was incubated in the dark at room temperature for 2-5 minutes. Fluorescence was then measured, at excitation wavelength 480 nm and emission wavelength 520 nm, in a PolarStart Optima microplate reader (BMG Labtech GmbH, Germany).

5.5 Denaturing gradient gel electrophoresis

5.5.1 Introduction

DGGE is a technique that has been used to characterize bacterial communities in a variety of different soil types. These include soils which are contaminated by metals (Ellis *et al.*, 2003; Renella *et al.*, 2005), oil spillage (MacNaughton *et al.*, 1999), grassland soils (McCaig *et al.*, 2001; Griffiths *et al.*, 2003a and b; Rizt *et al.*, 2004), agricultural soils (Øvreas *et al.*, 1998; Smit *et al.*, 2001; Sun *et al.*, 2004) and compost (Kowalchuk *et al.*, 1999; Ros *et al.*, 2006). DGGE has also been employed to

characterize bacteria that cause deterioration of monuments (Rölleke *et al.*, 1996; 1998; 1999).

One of the most useful techniques available for the characterization of microbial communities in soil is community fingerprinting by denaturing gradient gel electrophoresis (DGGE). This technique allows the simultaneous analysis of multiple samples, and so facilitates the study of spatial and temporal variation in the composition of soil microbial communities (Muyzer and Smalla, 1998). Sequence differences in bacterial 16S rDNA genes allow their separation along a denaturing gradient. DGGE is performed using a polyacrilamide gel that contains a linear gradient of denaturant (urea and deionised formamide) concentration. As individual double stranded DNA fragments migrate through the polyacrilamide gel they become progressively denatured. This partial 'melting' cause the DNA molecules to halt at a specific point in the gel (Figure 5.5). Minor variation in DNA sequences alter the DNA melting characteristics, and as a consequence, modify its electrophoretic mobility. This behaviour causes DNA fragments with a similar size, but different base composition, to migrate to different positions in the gel in order to produce a profile of different bands (Muyzer *et al.*, 1993).

DGGE is relatively rapid and simple to perform (Muyzer and Smalla, 1998; Heuer *et al.*, 2001; Kirk *et al.*, 2004). Several samples can be analysed simultaneously in the same gel, which allows comparison of different microbial communities (Head *et al.*, 1998; Muyzer, 1999). Bands on the gel can subsequently be characterized by hybridization using specific probes, or by DNA extraction and sequencing (Muyzer and Smalla, 1998). In addition, DGGE allows temporal monitoring of microbial

communities by the comparison of band patterns from samples taken at different times from the same site (Piñar *et al.*, 2001).

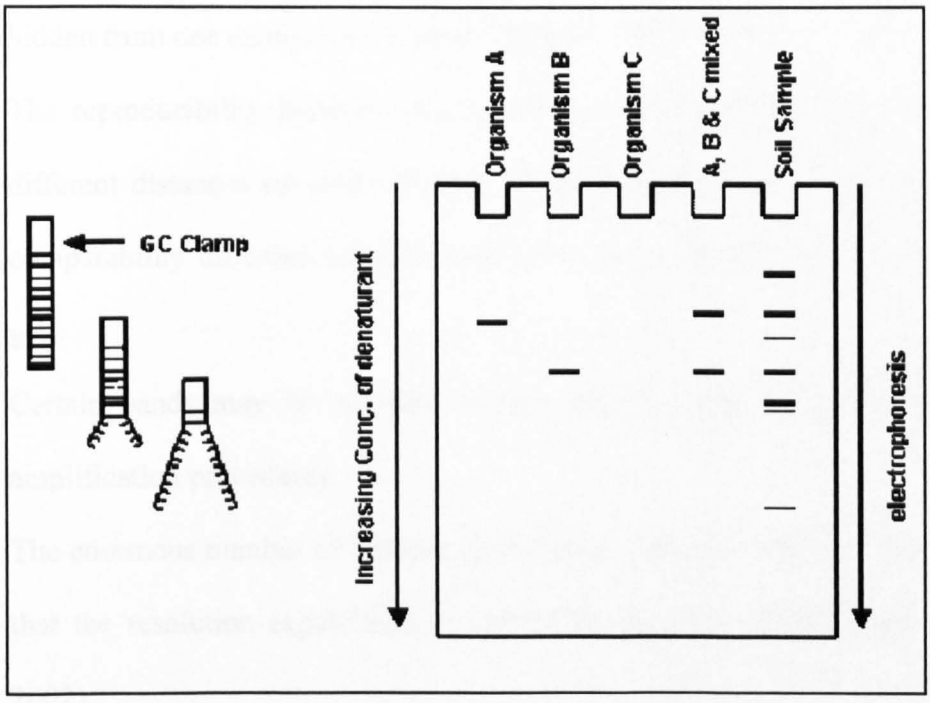


Figure 5.5: Schematic representation of denaturing gradient gel electrophoresis (based on Muyzer *et al.*, 1993).

Despite the many advantages of using DGGE to study microbial community diversity and structure, this technique does have a number of limitations. These are highlighted below:

1) The separation of multiple bands amplified from all members of a highly diverse bacterial community is not possible. Only the dominant (i.e. most abundant) species are shown (Heuer *et al.*, 2001).
2) Single bacteria species may contribute to more than one band on a DGGE gel if multiple copies of an operon are present in a single species, having slightly different sequences (Muyzer and Small, 1998; Heuer *et al.*, 2001).

3) One single band on a gel might not correspond to a particular bacterial species, because DNA fragments from different species may be different but have similar electrophoretic mobility, thus bands from more than one species can be hidden from one another (Muyzer and Smalla, 1998; Heuer *et al.*, 2001).
4) The reproducibility between gels is low, similar fragments tend to migrate different distances on different gels (Wintzingerode *et al.*, 1997); hence for comparability different samples need to be run in parallel lanes on the same gel.
5) Certain bands may be chimeric (which can be produced during the PCR amplification procedure).
6) The enormous number of different genomes in soils may yield so many bands that the resolution capabilities of DGGE is exceeded (Torsvik and Ovreas, 2002).

Although there are a number of limitations in using DGGE (which have been highlighted above), other molecular biological techniques also suffer from their own problems and are not necessarily better than DGGE in separating the components of complex microbial communities. As a consequence, this study will employ the use of DGGE in the separation of the DNA obtained from complex microbial communities within the soil samples; whilst at the same time being acutely aware of the problems associated with using the technique.

5.5.2 Procedure for DGGE

DGGE was carried out using the DCODE Universal Mutation Detection System (Bio-Rad Laboratories Ltd, California, USA) following the manufacturer's instructions and

protocols. The procedure for DGGE analysis was undertaken following the methodology of Muyzer *et al.* (1993).

5.5.2.1 Preparation of gel plates

The glass plates and accessories were thoroughly cleaned prior to use. The plate spacers and comb were washed in distilled water and subsequently cleaned with 70 % ethanol. The gel was assembled using a 16 cm x 16 cm gel sandwich. The sandwich was assembled by fitting the 20 cm x 16 cm glass plate on the top of the 20 cm x 18 cm glass plate. The plates were separated from each other by spacers located at the indent edge on the left and the right sides of the plates. The plates were held together by clamps at both sides and were aligned using an alignment card and casting stand. The sandwich was fixed on to the casting stand with a sponge seal (Figure 5.6).

5.5.2.2 Gel pouring

Gel pouring was carried out using the Model 475 Gradient Delivery System (Bio-Rad Laboratories Ltd, California, USA) according to the manufacturer's instructions (Figure 5.7). Two 7.5 % polyacrylamide gel solutions were used for casting the DGGE which had denaturant gradients of 30 % and 60 %. The composition of the gel solutions are described in Table 5.4.

The gel solutions were filtered through 0.45 μm cellulose nitrate membrane filters (Whatman, UK) prior to the gel casting. To cast the gel, 175 μl of 10 % (w/v) ammonium persulphate solution (Sigma, UK) and 17.5 μl of N, N, N', N'-tetramethylethylene-diamine (TEMED) (Sigma, UK) were added to 17.5 ml of each denaturing solution (which were contained in sterile universal tubes). The tubes were

capped and inverted several times in order to mix the solutions. All of the solution for each tube was subsequently drawn into a 30 ml syringe, one syringe for each solution. Both the syringes were attached to the gradient delivery system syringe holder (Figure 5.7). The gel solutions were delivered into the casting tray by a Y-tube which connected the syringes to the top of the sandwich. Slow rotation of the cam wheel forced the gel solutions into the casting tray.

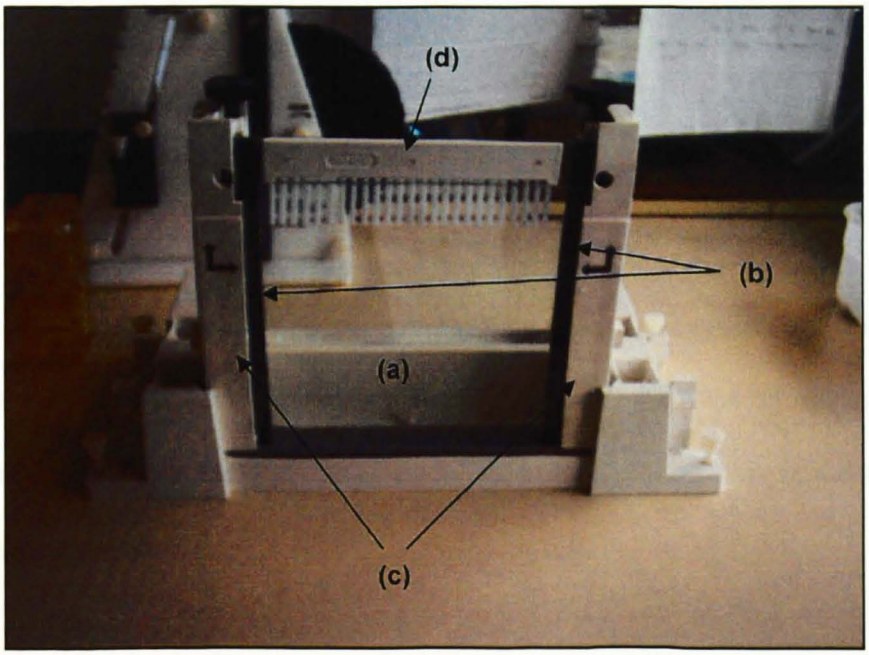


Figure 5.6: The denaturing gradient gel assembly unit. This unit contained the gel which was sandwiched between 2 glass plates (a), separated by spacers (b) and held together by two clamps (c). The gel comb (d) is used to make the wells into which the samples are loaded.

Table 5.4: Content of the 7.5 % polyacrylamide gel solutions used in preparation of the 30 % and 60 % solutions for DGGE.

Reagent	Quantity for 30% solution	Quantity for 60% solution
40% Acrylamide/Bisacrylamide	18.75 ml	18.75 ml
50 x TAE Buffer	2 ml	2 ml
Formamide	12 ml	24 ml
Urea	12.6 g	25.2 g
Molecular biology grade water	100 ml	100 ml

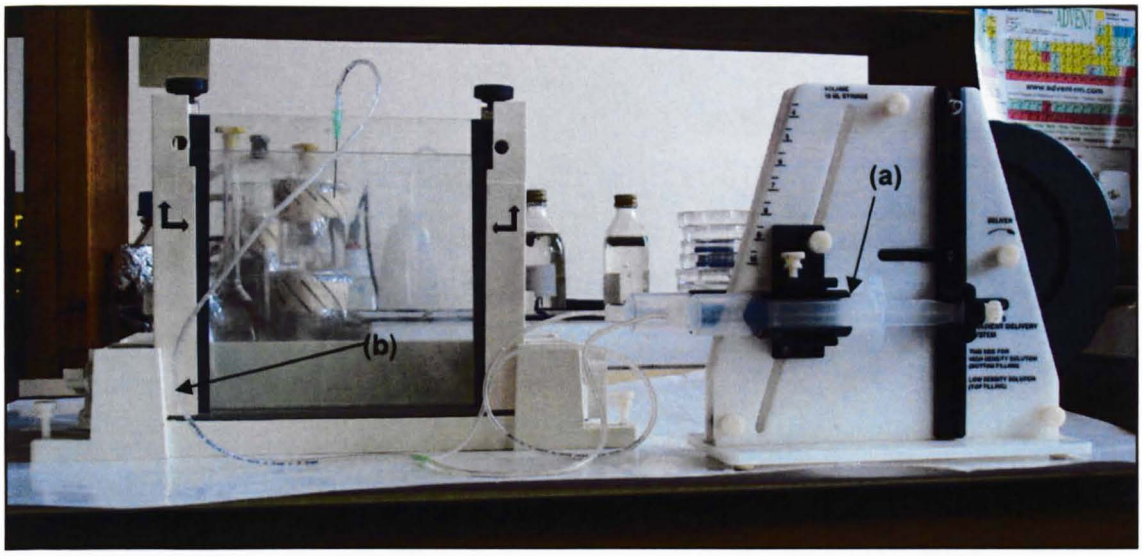


Figure 5.7: Cam operated gradient delivery system mixes high and low density solutions to create the denaturing gradient. Syringes (a) used to deliver the gel to the casting tray through the Y-shaped tubes (b).

When the gel solution was completely poured, a 16 or 32 well comb was placed in the top of the gel to form the loading wells. The gel was allowed to polymerize for approximately 1 hour, prior to use in DGGE. Once set, the comb was removed and the gel was used immediately.

5.5.2.3 Gel loading and running

1 x TAE buffer was poured into the buffer tank and pre-heated to 60 °C (Figure 5.8). The glass sandwich containing the polymerized gel was loaded into the buffer tank. The wells of the gel were washed with a 19" gauge needle and syringe containing 1 x TAE buffer prior to loading the sample, with the aim of eliminating residual polyacrylamide.

100 ng of test DNA were loaded into each well and DGGE was conducted at a constant voltage of 200 mV in a Bio-Rad™ DCODE Universal Mutation Detection System (Bio-Rad Laboratories Ltd, California, USA) for 5 hours.

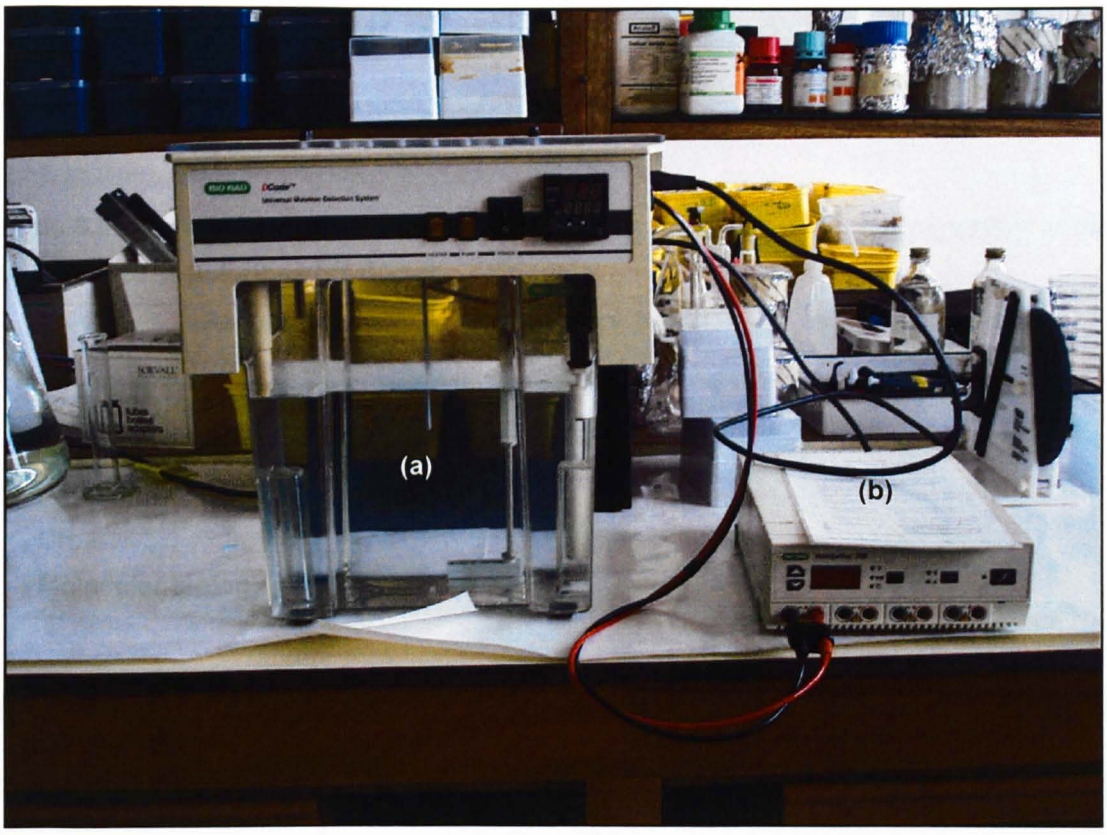


Figure 5.8: The DGGE electrophoresis system. The DCODE electrophoresis tank with the gel assembly in position and the temperature control unit in place (a). The electrical controller (b) is adjacent to the tank.

Following electrophoresis, the gel was separated from the plates, washed in deionised water and then stained in SyberGreen II (Sigma-Aldrich, UK) for 45 minutes. The gel was washed with deionised water, visualised under UV radiation and recorded using the Gel Documentation and Analysis Gene Genius System (Syngene, Cambridge, UK).

5.5.3 DGGE with specific primers

PCR-amplified 16S rDNA fragments were separated using the DCODE Universal Mutation Detection System (as previously described in Section 5.5.2). 15 µl of PCR product were loaded onto 6 % (w/v) polyacrylamide gel (acrylamide: *N*, *N'*-methylenebisacrylamide, w/w, 37:5:1) containing a denaturing gradient ranging from

40-58 %. The polyacrylamide gel was prepared by using the reagents highlighted in Table 5.5 below.

Table 5.5: Content of the 7.5 % polyacrylamide gel solutions used in preparation of the 40 % and 58 % solutions for DGGE.

Reagent	Quantity for 40 % solution	Quantity for 58 % solution
40 % Acrylamide/Bisacrylamide	15 ml	15 ml
50 x TAE Buffer	2 ml	2 ml
Formamide	16 ml	23.2 ml
Urea	16.8 g	24.36 g
Molecular biology grade water	100 ml	100 ml

Electrophoresis was undertaken using the Model 475 Gradient Delivery System which contained 1 x TAE buffer solution (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.3). Electrophoresis was performed at 60 °C, at a constant voltage of 100 V for 7 hours. Following electrophoresis the gel was analysed as described in Section 5.5.2.3.

5.5.4 Analysis of the gel

The DGGE gels were analysed using the Gel Documentation and Analysis Gene Genius System (Syngene, Cambridge, UK) software. The similarities between lanes in the DGGE gels were calculated by taking into account the position and the presence or absences of bands.

A binary matrix was constructed for each gel which represents the presence (1) or absence (0) of bands in each lane within a DGGE gel. The matrices were analysed statistically using unweighted pair-wise grouping, with mathematical averaging (UPGMA) clustering method, using the Jaccard coefficient to create a distance matrix

(Schafer and Muyzer, 2001). UPGMA uses pair-wise similarities to produce a dendrogram which represents these distances in diagrammatic form. The results were also analysed using principal component analysis. Both analyses were carrying out using the software Community Analysis Package, version 2.0 (Pisces Conservation Ltd, Lymington, UK).

5.6 Cloning and sequencing

5.6.1 Introduction

Cloning is a method that has been applied worldwide to analyse bacterial diversity in soils. It has been used to study diversity in agricultural soils (Borneman *et al.*, 1996), forest soils (McVeigh *et al.*, 1996), arid soils (Kuske *et al.*, 1997; Dunbar *et al.*, 1999) and grassland soils (Felske *et al.*, 1998; McCaig *et al.*, 2001). The technique has also been used to characterize bacterial, fungal and archaeal communities in deteriorating historic wall paintings (Piñar *et al.*, 2001; Gorbushina *et al.*, 2004) and to identify bacteria within archaeological wood (Helms *et al.*, 2004).

In this research cloning of 16S rDNA fragments from three selected samples was applied in order to complement and also compare the results obtained with those from DGGE. The creation of 16S rRNA clone libraries and their subsequent sequencing allowed the recognition of constituents of soil bacterial communities at a species level that was not possible by DGGE.

Phylotypes from soil samples were obtained by amplifying rRNA genes from the total community DNA with specific primers. The amplified DNA is subsequently cloned using standard cloning methods (Giovannoni *et al.*, 1990; Hugenholtz and Pace, 1996).

The analysis and screening of clone libraries has been facilitated by the application of DNA sequencing systems (Vainio *et al.*, 1997; Head *et al.*, 1998). The sequences obtained can be compared with published sequences to identify the species of bacteria present in a sample, thus allowing the identification of individuals from the sample (Theron and Cloete, 2000).

Cloning consists of the transfer of a DNA fragment with the gene of interest (-in the current research this was bacterial 16S rDNA) into a self-replicating genetic element (cloning vector) such as a bacterial plasmid (Figure 5.9). Plasmids are extra-chromosomal, double-stranded circular molecules of DNA that are found in bacteria (Snustad *et al.*, 1997). The DNA fragment with the gene of interest is isolated from the chromosomal DNA by using restriction enzymes and then inserted in a plasmid that has been cut with the same restriction enzymes; this step in the cloning process is known as ligation (Madigan *et al.*, 2006). The bacterial plasmid with the insert of interest is transferred to a foreign host cell (*Escherichia coli*) and these cells are then grown in culture (Snustad *et al.*, 1997; Madigan *et al.*, 2006).

To allow identification of colonies of cells into which the DNA of interest has been transferred, the vector includes selectable antibiotic-resistance markers which only allow the growth of those cells that have been transfected with the vector. In addition, the cloning vector (plasmid) contains a functional fragment of the lacZ operon; this is the *Escherichia coli* gene that encodes the enzyme β-galactosidase (Figure 5.9). Those fragments cloned into the vector disrupt the lacZ-gene and inhibit β-galactosidase activity. Therefore, if DNA is successfully ligated into the plasmid, the host bacterium grows to form a white colony. White colonies indicate that the DNA has been inserted

into the vector and the lacZ-gene has been disrupted. By contrast, blue colonies are clones with the plasmid (which conferred resistance to ampiciline), but without insert. As a consequence, the lacZ-gene has not been disrupted and is able to transform X-Gal (present in the culture medium). Xgal is cleaved by β-galactosidase giving a compound that when oxidised is blue in colour.

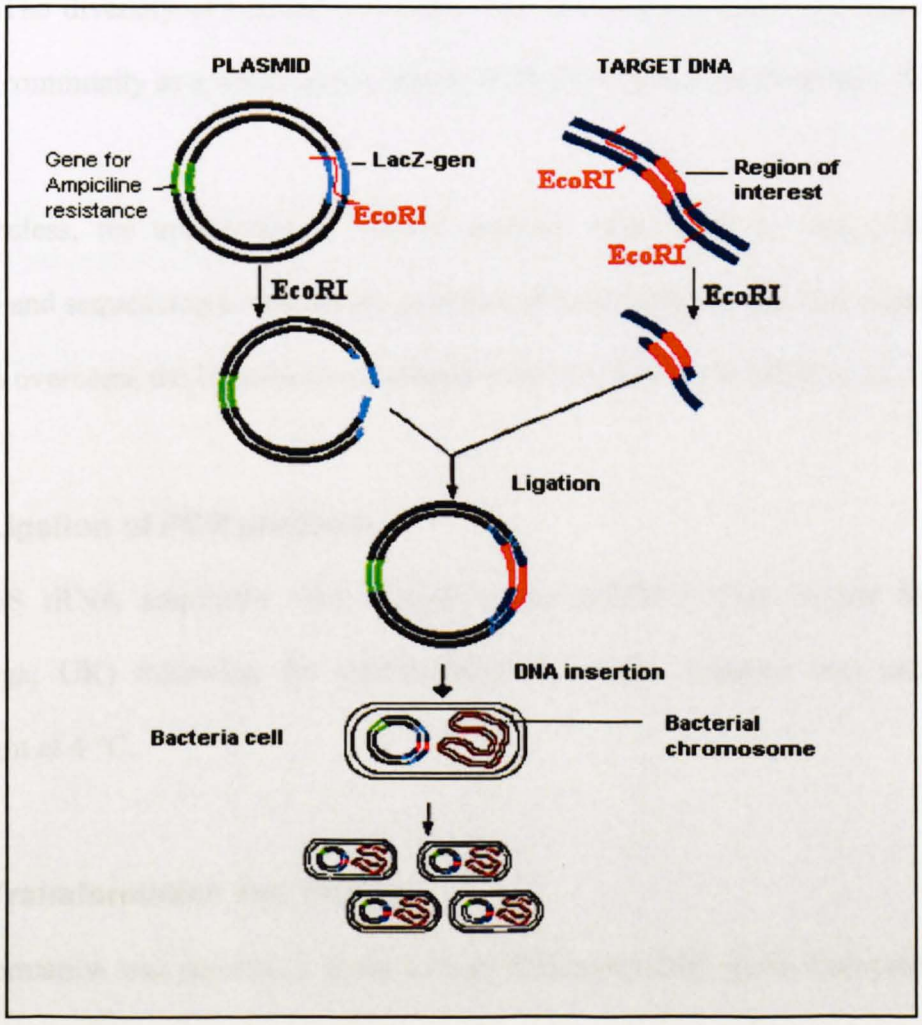


Figure 5.9: Diagram showing stages in the cloning process. A DNA fragment with the gene of interest is introduced into a bacterial plasmid and transferred to a foreign host cell. (source: http//www.accessexcellence.org/AB/GG/plasmid.html).

Despite the advantages of using cloning and sequencing techniques in order to study microbial community diversity and structure, they do have a number of limitations:

1) The process is time consuming and expensive.
2) It is necessary to sample a large number of clones in order to obtain an adequate representation of a microbial community.
3) The diversity of bacteria identified may not be an accurate reflection of the community as a whole due to bias in PCR (Embly and Stackebrandt, 1966).

Nevertheless, the application of DGGE analysis when used in conjunction with cloning and sequencing combines the potential of both methods, and as a consequence, helps to overcome the limitations associated with each technique (Piñar *et al.*, 2001).

5.6.2 Ligation of PCR products

The 16S rRNA amplicons were ligated in the pGEM-T Easy Vector System I (Promega, UK) following the manufacturer's protocol. Ligation was undertaken overnight at 4 °C.

5.6.3 Transformation and cloning

Transformation was performed using Library Efficiency DH5 alpha Competent Cells (Invitrogen, UK). The transformation procedure was undertaken following the manufacturer's protocol, using 10 µl of the ligation reaction and 100 µl of heat competent cells. The products of the transformation reaction were plated on 1 x LB agar plates with ampiciline (100 µg ml^{-1}) (Sigma, UK) which had previously been treated with 0.1 mmol l^{-1} X-Gal and 1 mmol l^{-1} IPTG (Isopropyl- D-Thiogalactopyranoside) (Invitrogen, UK) for blue-white colony detection.

5.6.4 Screening for insert size by PCR

16 white colonies from each soil sample were picked from the agar plates using sterile pipette tips and were transferred to fresh 1 x LB agar plates containing ampiciline (100 μg ml^{-1}) (Sigma, UK). These were then incubated overnight at 37 °C. Subsequently, the same pipette tip was inserted into a 0.2 ml PCR tube containing the PCR reaction mixture. This process was repeated for all the colonies.

PCR reactions were performed using vector-specific primers:

- M13 forward (5'-CGCCAGGGTTTTCCCAGTCACGAC-3')
- M13 reverse (5'-AGCGGATAACAATTTCACACAGGA-3').

Amplification was performed in a total volume of 10 μl, comprising 1 x NH_4 buffer (Bioline, UK), 0.2 mmol l^{-1} of each dNTP, 1.5 mmol l^{-1} magnesium chloride, 0.2 mmol l^{-1} of each primer (forward and reverse), 0.03 μl *Taq* polymerase (Bioline) and 1 μl template DNA (average concentration 1.5±0.8 μg/ml).

The reaction mix was subjected to the following cycles; 4 minutes initial denaturation at 94 °C, followed by 39 cycles of 15 seconds of denaturation at 94 °C. This was followed by a primer-specific annealing temperature (ranging from 50 °C to 67 °C) for 20 seconds and 72 °C for 40 seconds; concluding with 10 minutes final extension at 72 °C.

The success of the PCR amplification was checked by running the product on agarose gel electrophoresis (3 % [w/v]) (Section 5.3).

5.6.5 Sequencing

16S rDNA sequences were determined in order to characterize the diversity and community structure of the bacterial community. The PCR products were cleaned before sequencing using ExoSAP-IT (USB, USA). 0.8 µl of ExoSAP-IT and 2 µl of PCR product were added to a microcentrifuge tube. The mixture was incubated for 15 minutes at 37 °C, followed 15 minutes at 80 °C and was then stored at 4 °C.

The sequencing reaction mixture was prepared using 8.7 µl of PCR water, 0.5 µl of primer (forward or reverse), 8 µl of Quickstart solution and 2 µl of cleaned PCR product. The sequencing reactions were undertaken separately for both primers (forward and reverse). 8.5 µl of the sequencing reaction mixture and 1.4 µl of each EXOSAP mixture from the initial step were added to each well of a 96-well microstartplate (Beckman Coulter Inc, USA). The sequencing PCR was performed on a tetrad PCR machine MJ Research PTC 225 (MJ Research, USA) using the following programme: 30 cycles at 96 °C for 20 seconds, 50 °C for 20 seconds, 60 °C for 4 minutes and 10 minutes at 4 °C.

A fresh stop solution was prepared mixing together 2 µl of 3 mmol l^{-1} sodium acetate (pH 5.2) (Sigma, UK), 2 µl of 100 mmol l^{-1} Na_2 EDTA (pH 8) and 1 µl of 20 mg/ml glycogen for each PCR sequencing reaction. 5 µl of stop solution were added to each sequencing reaction into the 96-well microstarplate (Costar Co, Massachusetts, USA). The plate was vortexed for 20-30 seconds. 60 µl of ice-cold 95 % ethanol were added to each well and mixed thoroughly by vortexing for 1 minute. The microplate was centrifuged at 1200 RCF for 30 minutes at 4 °C in an Eppendorf Centrifuge 5810R (Eppendorf, Germany). The supernatant was removed by ensuring that the plate was

kept in an inverted position. Subsequently, 200 µl of 70 % ethanol was added to each well of the microplate and it was re-centrifuged and the supernatant poured off. This step was repeated.

The DNA samples in the wells of the microplate were dried using a Vacuum Model PV100 Red-Evac (Hoefer Scientific Instruments, USA) and re-suspended in 40 µl of Sample Loading Solution (Beckman, USA). Finally, 1 drop of mineral oil was overlain onto each sample.

A buffer microplate was prepared by filling the wells of another microplate with separation buffer (Beckman, USA). The DNA samples were sequenced in a Beckman CEQ8000 capillary sequencer (Beckman, USA) following the manufacturer's instructions.

The sequences produced were analysed and assembled using the Codon Code Aligner software (Codon Code Corporation, USA). The sequences were identified online using the Basic Local Aligment Search Tool (BLAST) (Altschul *et al.*, 1997) (www.ncib.nlm.nih.gov/BLAST). The BLAST search tool allows comparisons to be made between the sequences produced using the methodology highlighted above, to a database of sequences obtained from the Ribosomal Database Project II (www.rdp.cme.msu.edu).

5.7 Summary

This chapter describes the molecular techniques which have been used in order to characterize bacterial communities in the soil samples obtained from Hatfield Moor

and Sutton Common. The methodology involved the extraction of total bacterial DNA from the soil samples, amplification of target DNA by PCR with primers general for bacteria and with specific primers for three different bacterial groups, followed by separation using DGGE analysis. In addition, cloning and sequencing were applied to DNA from selected samples in order to identify the presence of specific bacterial species.

The next three chapters will present the results obtained from the application of the different methods outlined in Chapters 3-5 (Field methods [Chapter 3], Conventional microbial methods [Chapter 4] and Molecular genomic methods [Chapter 5]).

Chapter 6 Physico-chemical results

6.1 Introduction

This chapter presents the results obtained from the analysis of soil cores from both sampling sites from April 2004 to April 2006, which were obtained by hand auguring. The analysis was undertaken in order to understand how the various soil components might affect the movement of water within the soil profile, and the influence that this may have upon the redox potential, temperature and other relevant variables that can influence the *in situ* preservation of organic material at the locations investigated.

An initial description of the soil profiles is presented in Section 6.2. The results from the analysis of soil texture by particle size analysis are outlined in Section 6.3. Organic matter and soil moisture content are considered in Sections 6.4 and 6.5 respectively. The results of analysis for carbon (C), nitrogen (N), phosphorous (P) and sulphur (S) for October 2005, February 2006 and April 2006 are presented in Section 6.6. (These data are presented in their entirety in Appendix 1).

Finally, the data that describe the depth of the water table, redox potential, pH and temperature in the soils from April 2004 to April 2006 for Hatfield Moor, and from February 2004 to April 2006 for Sutton Common, are presented in Sections 6.7 to 6.10. The data are presented in a graphical format, summarized, and assessed in terms of their site specific characteristics and the influence that each variable may have upon the preservation of the buried archaeological resource.

6.2 Description of soil profiles

The descriptions of the soil profiles in Tables 6.1 and 6.2 are based on cores obtained from Hatfield Moor and Sutton Common in April 2004.

Table 6.1: The soil profile at Hatfield Moor.

Depth	Soil description
0-30 cm	Topsoil medium brown colour, clay, silt and moderately organic, increasingly organic below 20 cm.
30-45 cm	Frequent wood compacted in an organic matrix (wood values 80-90 %) and distinct to compact alluvium.
45-100 cm	Blue-grey Fe^{+} mottled clay-silt alluvium, frequent fibrous material and twigs enter sequence below 45 cm depth; below 70 cm the alluvium is increasingly organic.
100-150 cm	Humified peat with occasional alluvium below 100 cm. Frequent macrofossils.
150 cm	Too wet to obtain sample below this depth.

Table 6.2: The soil profile at Sutton Common.

Depth	Soil description
0-13 cm	Dark grey-brown silt with occasional clay, distinct at 13 cm to light grey-brown Fe^{+} mottled fine sands and silt.
13-70 cm	Grades to humified organics with frequent fine sands and silt.
70-105 cm	Below 70 cm very silty and wet with frequent wood.
105 cm	Below this depth blue-grey clays and medium sands dominate.

It is apparent from the above descriptions (Tables 6.1 and 6.2) that below the modern plough soil, Hatfield Moor contains a peat filled palaeochannel sequence with fluctuations in flow regime during infilling being represented by the organic alluvial unit located between 45 and 100 cm depth. It is anticipated that this alluvial material

will result in some restriction to the downward movement of water through the profile at this location.

At Sutton Common, the sequence recovered represents the in-filling of an Iron Age ditch located within the small enclosure (or annex) to this site. The main organic deposition is located between 13-105 cm depth, with intercalated fine sands and silts, comprising varying degrees of organic/wood content to the base of the sequence, between 70-105 cm depth. Below 105 cm depth, the sequence comprises clays and medium sands of late-glacial (Lake Humber) derivation.

6.3 Particle size analysis

Table 6.3 outlines the results of the particle size analysis obtained from two soil profiles taken from Hatfield Moor and Sutton Common in October 2005. Soil texture was determined using the US soil texture triangle (Saxton *et al.*, 1986). In the soil profile from Hatfield Moor, between 10 cm and 70 cm depth, the sediment is classified as a silt loam. Below this depth the material is a sandy loam. At Sutton Common the soil texture changed through the soil profile, from a silt loam (10-50 cm depth), to sandy loam (50-70 cm depth) and back to a silt loam below 100 cm.

Both soil profiles, in general, had a high percentage of silt, followed by sand and clays. However, the soil samples obtained from Hatfield Moor contain more silt and clay at 10 cm, 50 cm and 70 cm depths than the equivalent samples from Sutton Common. This silt-clay material reflects the alluvial nature of the depositional environment within the soil profile at Hatfield Moor.

Table 6.3: Particle size analyses of soil cores from Hatfield Moor and Sutton Common, October 2005.

Hatfield 10 cm		
% Gravel		No
% Sand	% coarse sand	8.81
	% medium sand	10.42
	% fine sand	18.22
% Silt	% coarse silt	59.52
	% medium silt	0.94
	% fine silt	0.42
% Clay		1.67
Hatfield 30 cm		
% Gravel		No
% Sand	% coarse sand	16.20
	% medium sand	12.64
	% fine sand	11.22
% Silt	% coarse silt	55.22
	% medium silt	0.50
	% fine silt	2.12
% Clay		2.10
Hatfield 50 cm		
% Gravel		No
% Sand	% coarse sand	11.17
	% medium sand	4.63
	% fine sand	3.67
% Silt	% coarse silt	75.92
	% medium silt	0.82
	% fine silt	0.78
% Clay		3.01
Hatfield 70 cm		
% Gravel		No
% Sand	% coarse sand	32.06
	% medium sand	7.61
	% fine sand	7.88
% Silt	% coarse silt	49.31
	% medium silt	0.60
	% fine silt	0.40
% Clay		2.13
Hatfield 100 cm		
% Gravel		No
Sand	% coarse sand	34.40
	% medium sand	9.39
	% fine sand	10.91
% Silt	% coarse silt	42.65
	% medium silt	0.42
	% fine silt	0.32
% Clay		1.92

Sutton Common 10 cm		
% Gravel		No
% Sand	% coarse sand	0.03
	% medium sand	6.10
	% fine sand	37.16
% Silt	% coarse silt	56.03
	% medium silt	0.14
	% fine silt	0.08
% Clay		0.45
Sutton Common 30 cm		
% Gravel		No
% Sand	% coarse sand	0.15
	% medium sand	5.72
	% fine sand	33.65
% Silt	% coarse silt	59.88
	% medium silt	0.07
	% fine silt	0.02
% Clay		0.52
Sutton Common 50 cm		
% Gravel		No
% Sand	% coarse sand	0.65
	% medium sand	10.70
	% fine sand	51.66
% Silt	% coarse silt	36.50
	% medium silt	0.15
	% fine silt	0.03
% Clay		0.30
Sutton Common 70 cm		
% Gravel		No
% Sand	% coarse sand	No
	% medium sand	14.10
	% fine sand	58.10
% Silt	% coarse silt	27.40
	% medium silt	0.04
	% fine silt	0.02
% Clay		0.34
Sutton Common 100 cm		
% Gravel		No
Sand	% coarse sand	No
	% medium sand	17.67
	% fine sand	31.45
% Silt	% coarse silt	50.25
	% medium silt	0.10
	% fine silt	0.06
% Clay		0.46

6.4 Organic matter content

At Hatfield Moor the highest levels of organic matter content were at 30 cm, 70 cm and 100 cm depths (Figure 6.1). The degree of variation between each sampling event was greater at 100 cm depth than at lesser depths, reflecting the intercalated character of the lower deposits as flow regimes fluctuated between cessation of flow (peat formation) and increasing flow (alluvium). At 50 cm depth, the lower concentration of organic matter reflects the highly alluvial nature of the sediment at this depth, as an alluvial unit occurs at 45-100 cm depth in the profile at this location (Table 6.1).

Organic matter content at Sutton Common (Figure 6.1) was higher at 50 cm and 70 cm depths than at the other depths within the soil profile, reflecting the organic nature of the infill sequence between 13 cm and 70 cm in the ditch sequence (Table 6.2). Also values tended to fluctuate more at 50 cm and 70 cm depths due to the mixed nature of the sediments. Values were more stable at 10 cm, 30 cm and 100 cm depths where the values range between 0 to 30 %, and are associated with higher mineral contents; the upper part of the organic ditch fill is visibly drying out. Organic matter concentrations provide a substrate for microbial activity, and it is apparent from the variability in evidence at Sutton Common that a considerable degree of heterogeneity in organic matter contents will have the potential to influence microbial community diversity and function throughout the soil profile.

6.5 Soil moisture content

The moisture content at Hatfield Moor (Figure. 6.2) generally increased with depth, with the exception of April 2004, when the moisture content at 30 cm depth increased to 74.3 %. The highest value recorded was at 100 cm depth (maximum 84.4 %) in

June 2004. The saturated conditions at this depth were associated with high peat content. The minimum moisture content (27.5 %) was observed in August 2005 at 10 cm depth.

The moisture content at Sutton Common fluctuated widely, with peaks at 50 cm and 70 cm depths (Figure. 6.2). The highest values were recorded at 70 cm depth (maximum 80.5 %) in February 2004. The minimum moisture content (21.0%) was observed in February at 70 cm depth. Moisture content data at Sutton Common are clearly influenced by the variability in organic matter content, as peaty soils tend to store water (e.g. Dubbin 2000), although the inorganic fraction still retains a measure of water storage capacity (compare Figures 6.1 and 6.2 for Sutton Common).

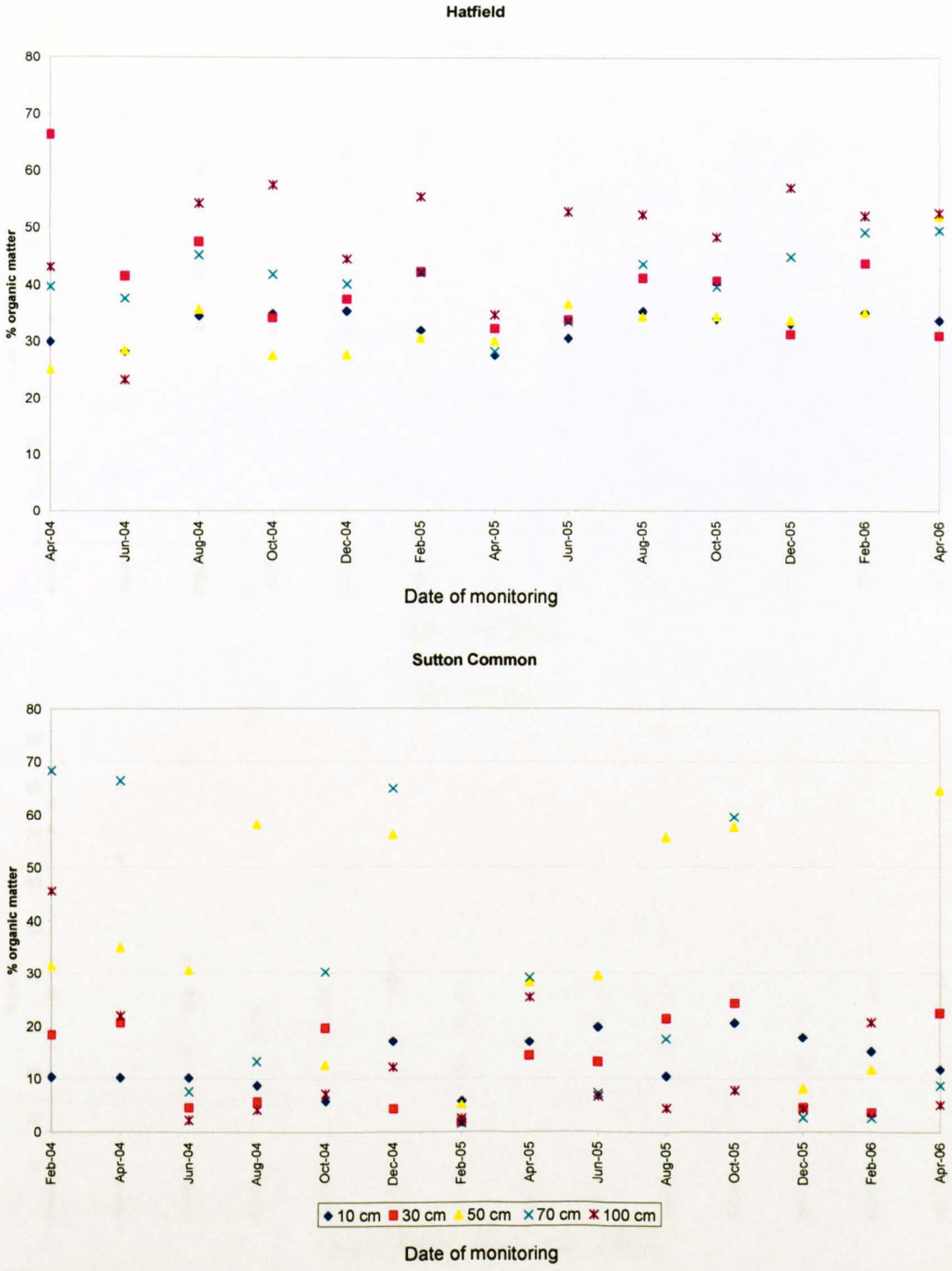


Figure 6.1: Variation in organic matter content with depth at Hatfield Moor and Sutton Common, 2004-2006.

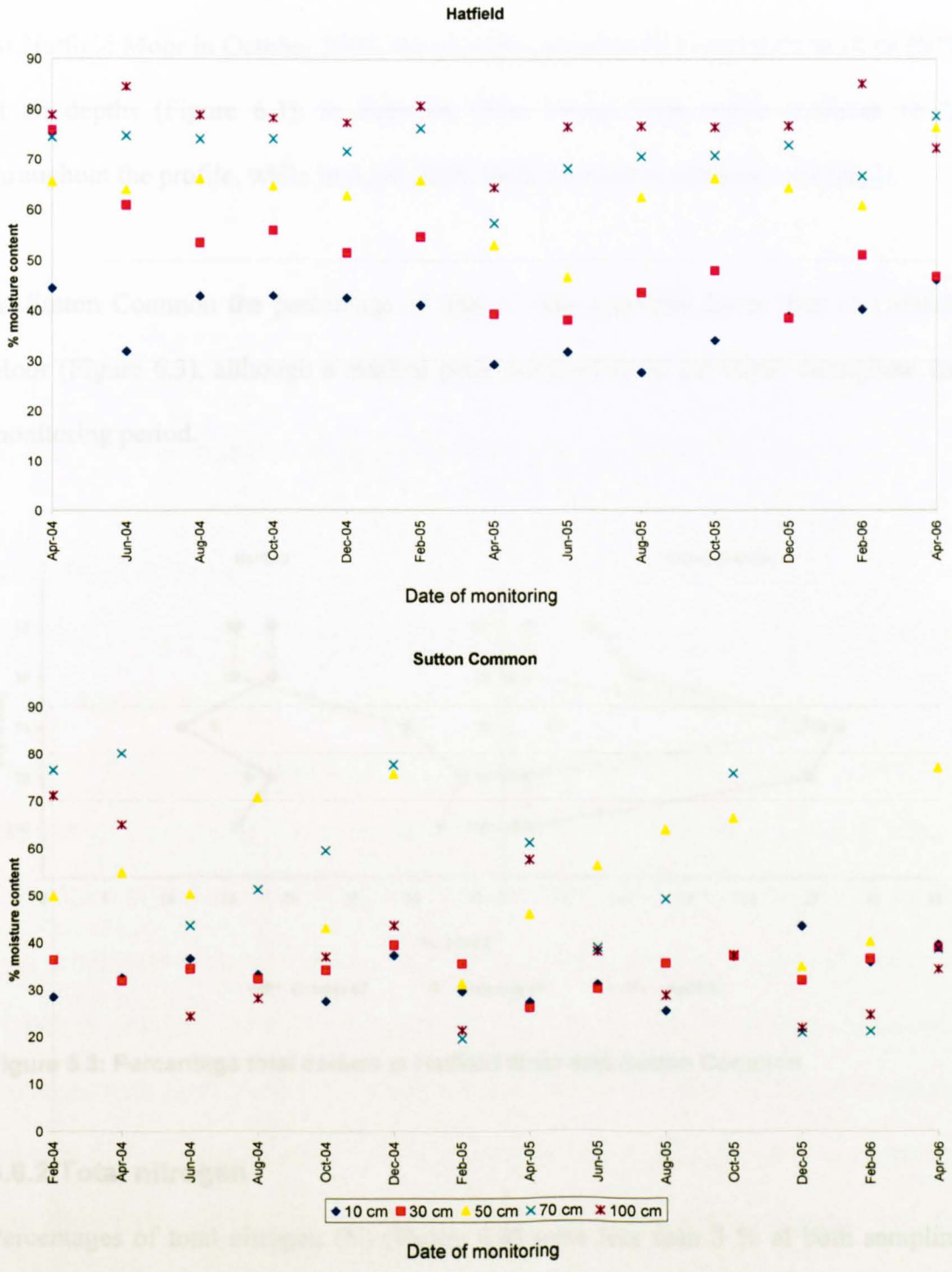


Figure 6.2: Variation in soil moisture content with depth at Hatfield Moor and Sutton Common, 2004-2006.

6.6 Soil chemical analysis

6.6.1 Total carbon

At Hatfield Moor in October 2005, the percentage carbon (C) ranged from 10 to 20 % at all depths (Figure 6.3); in February 2006 values were stable at about 15 % throughout the profile, while in April 2006, values tended to increase with depth.

At Sutton Common the percentage of total C was generally lower than at Hatfield Moor (Figure 6.3), although a marked peak occurred at 50 cm depth throughout the monitoring period.

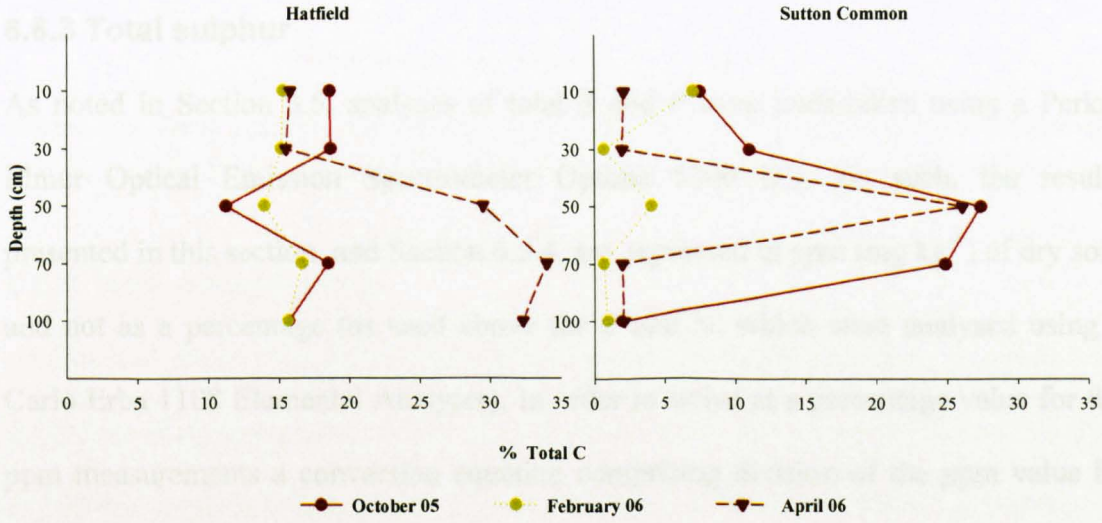


Figure 6.3: Percentage total carbon at Hatfield Moor and Sutton Common

6.6.2 Total nitrogen

Percentages of total nitrogen (N) (Figure 6.4) were less than 3 % at both sampling sites. At Hatfield Moor total N was more stable, between 0.5 and 2 %. At Sutton Common there was a clear peak at 50 cm in October of 2005, and also in April of 2006. In February 2006 this peak was minimal, with the maximum percentages occurring at 10 cm depth during this monitoring period.

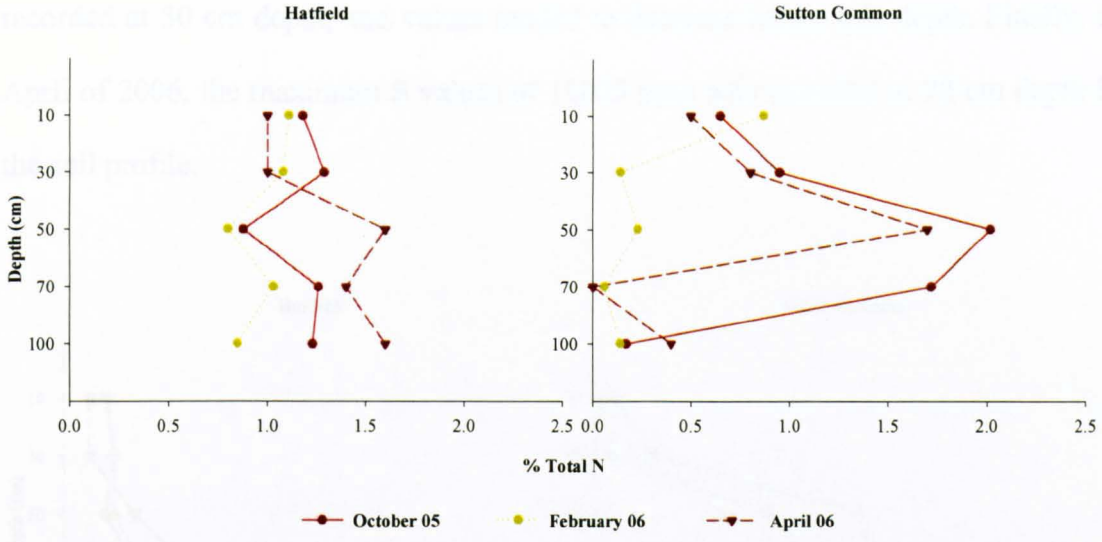


Figure 6.4: Percentage total nitrogen at Hatfield Moor and Sutton Common.

6.6.3 Total sulphur

As noted in Section 3.5, analyses of total S and P were undertaken using a Perkin Elmer Optical Emission Spectrometer Optima 5300 DV. As such, the results presented in this section, and Section 6.5.4, are expressed in ppm (mg kg^{-1}) of dry soil, and not as a percentage (as used above for C and N, which were analysed using a Carlo Erba 1108 Elemental Analyser). In order to arrive at a percentage value for the ppm measurements a conversion equation comprising division of the ppm value by 10,000 is required. At Hatfield Moor (Figure 6.5) the total S content consistently increased with depth, reaching maximum values at 100 cm depth. At Sutton Common, the total S concentration did not vary consistently with depth, but the S concentration was always considerably less at 10 cm and 30 cm depths when compared with the deeper soil horizons.

During October of 2005, at Sutton Common, the maximum S value of 24131 ppm was recorded at 100 cm depth. In February 2006 the maximum value of 6519 ppm was

recorded at 50 cm depth, and values tended to decrease below this depth. Finally, in April of 2006, the maximum S values of 11805 ppm was recorded at 70 cm depth in the soil profile.

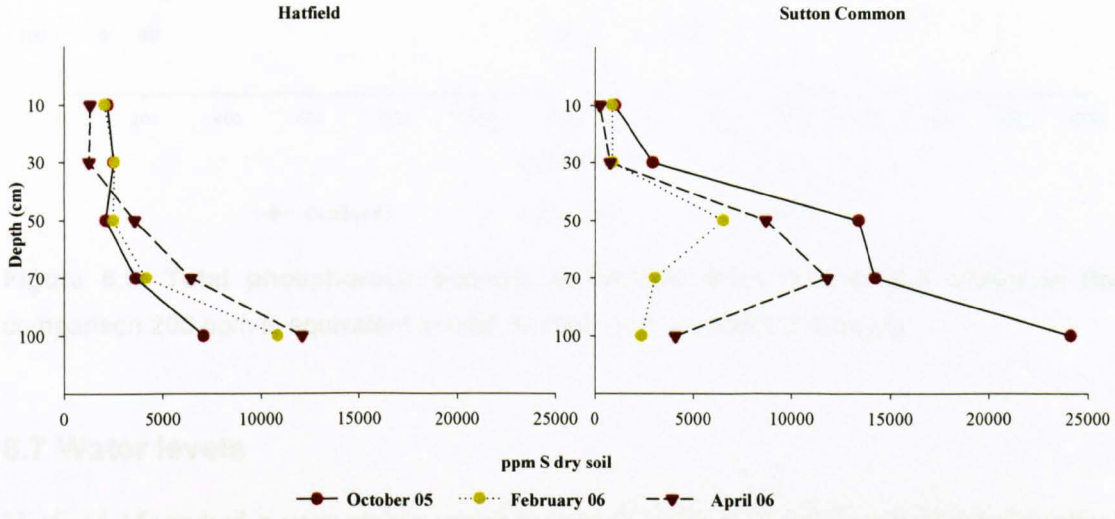


Figure 6.5: Total sulphur expressed in ppm (mg kg^{-1}) of dry soil at Hatfield Moor and Sutton Common (for comparison 5000 ppm is equivalent to 0.5 % sulphur content in the soil).

6.6.4 Total phosphorous

At Hatfield Moor (Figure 6.6) total phosphorous (P) levels tended to decrease with depth, reaching a minimum of 187 ppm at 50 cm depth in October 2005 and 101 ppm at 100 cm depth in February and 222 ppm in April of 2006. At Sutton Common, total P also tended to decrease with depth. During October 2005, the maximum content of 668 ppm was reached at 50 cm depth, decreasing to a minimum of 45 ppm at 100 cm depth. In February 2006 higher values of between 914 and 943 ppm were obtained in the top 30 cm of the soil profile, and a minimum of 237 ppm was reached at 50 cm depth. In April 2006, total P values were at a maximum of 530 ppm at 30 cm depth and decreased to a minimum of 113 ppm at 100 cm depth.

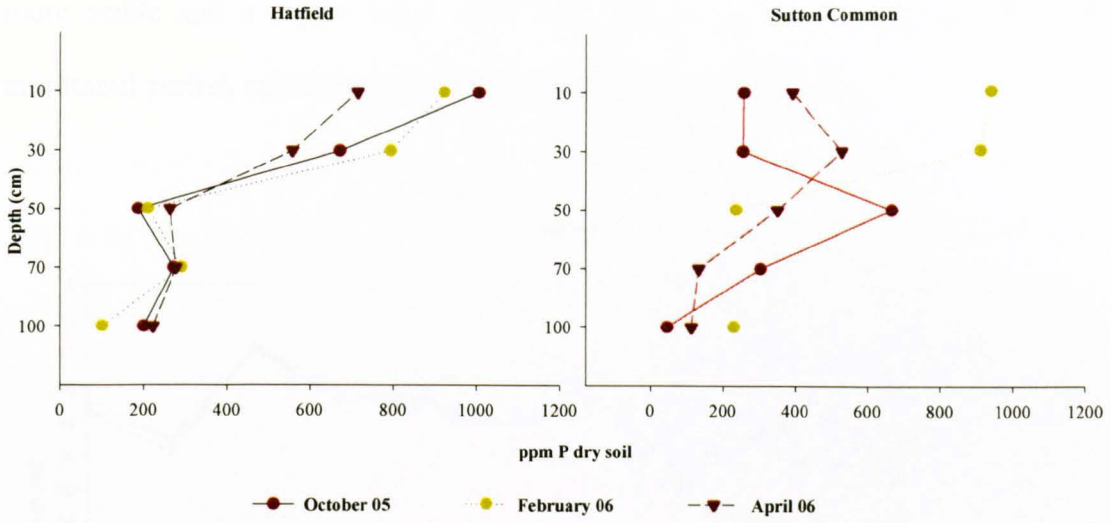


Figure 6.6: Total phosphorous content at Hatfield Moor and Sutton Common (for comparison 200 ppm is equivalent to 0.02 % phosphorus content in the soil).

6.7 Water levels

Hatfield Moor had a very stable water regime (Figure 6.7), with only limited vertical variation over the entire monitoring period. No clear seasonal pattern occurred, but in general the water table tended to be higher in 2004 than in 2005. A summer fall in water level in 2005 was more pronounced than in the summer of 2004. The depth of the water table ranged between approximately 20 cm and 60 cm depth below the soil surface; with the highest water level being recorded in August 2004 (20 cm depth) and the lowest in June and October 2005 (55 cm depth).

At Sutton Common the water table was also generally higher in 2004 than in 2005 (Figure 6.7). In 2004 the water table was always above 80 cm depth, being above 50 cm depth in August and October. In contrast, water levels below 80 cm depth were recorded in 2005, and in August and December of this year the water table fell to below 150 cm depth throughout the ditch sequences. In general, Hatfield Moor had a

more stable and a higher water table than Sutton Common throughout the entire monitored period, reflecting the active management at this location.

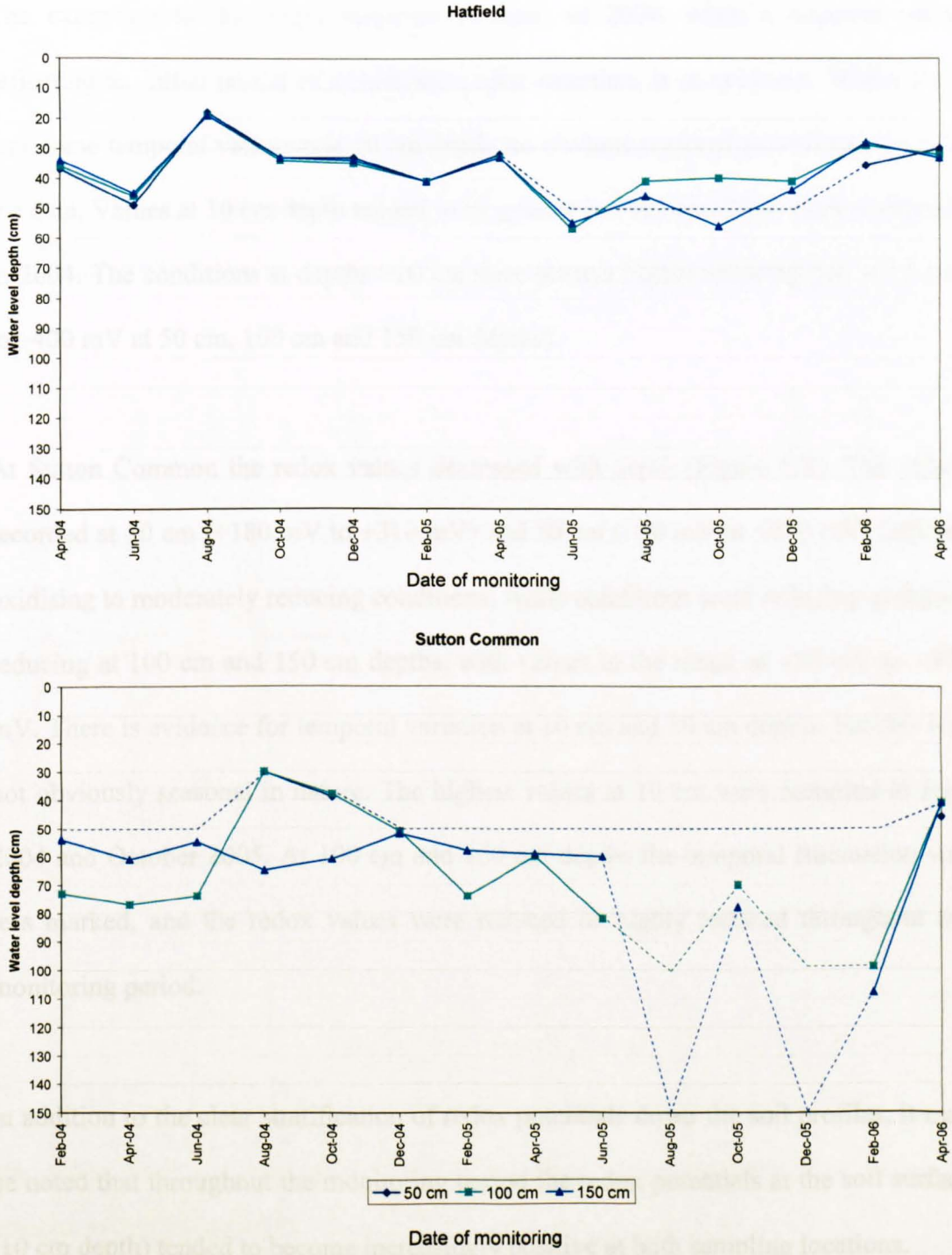


Figure 6.7: Water levels recorded in the three piezometers at Hatfield Moor and Sutton Common, 2004-2006. Dotted lines indicate base of piezometers due to lack of water table data for these monitoring points i.e the piezometers were dry.

6.8 Redox potential

At Hatfield Moor, redox potential decreased markedly with depth over the monitoring period (Figure 6.8). Positive values were only recorded at 10 cm (+0 mV to +300 mV). The exception to this trend occurred in April of 2004, when a negative value, reflecting an initial period of stabilisation after insertion, is in evidence. Whilst there was some temporal variation at 10 cm depth, no obvious seasonal patterning occurs in the data. Values at 10 cm depth tended to be greater in 2005 and 2006 when compared to 2004. The conditions at depths >10 cm were always highly reducing (i.e. –100 mV to –400 mV at 50 cm, 100 cm and 150 cm depths).

At Sutton Common the redox values decreased with depth (Figure 6.8). The values recorded at 10 cm (+180 mV to +310 mV) and 50 cm (–50 mV to +240 mV) indicate oxidising to moderately reducing conditions, while conditions were reducing to highly reducing at 100 cm and 150 cm depths, with values in the range of –30 mV to –300 mV. There is evidence for temporal variation at 10 cm and 50 cm depths, but this was not obviously seasonal in nature. The highest values at 10 cm were recorded in June 2004 and October 2005. At 100 cm and 150 cm depths the temporal fluctuation was less marked, and the redox values were reduced or highly reduced throughout the monitoring period.

In addition to the clear stratification of redox potentials down the soil profiles, it may be noted that throughout the monitoring period the redox potentials at the soil surface (10 cm depth) tended to become increasingly positive at both sampling locations.

Figure 6.8: Redox potential at Hatfield Moor and Sutton Common, 2004-2006. Values are adjusted to the SHE and pH 7.

6.9 pH

At Hatfield Moor, over the duration of the monitoring programme, the pH tended to decrease with increasing depth (Figure 6.9), although the range of values was mostly restricted to between pH 5 and 7.

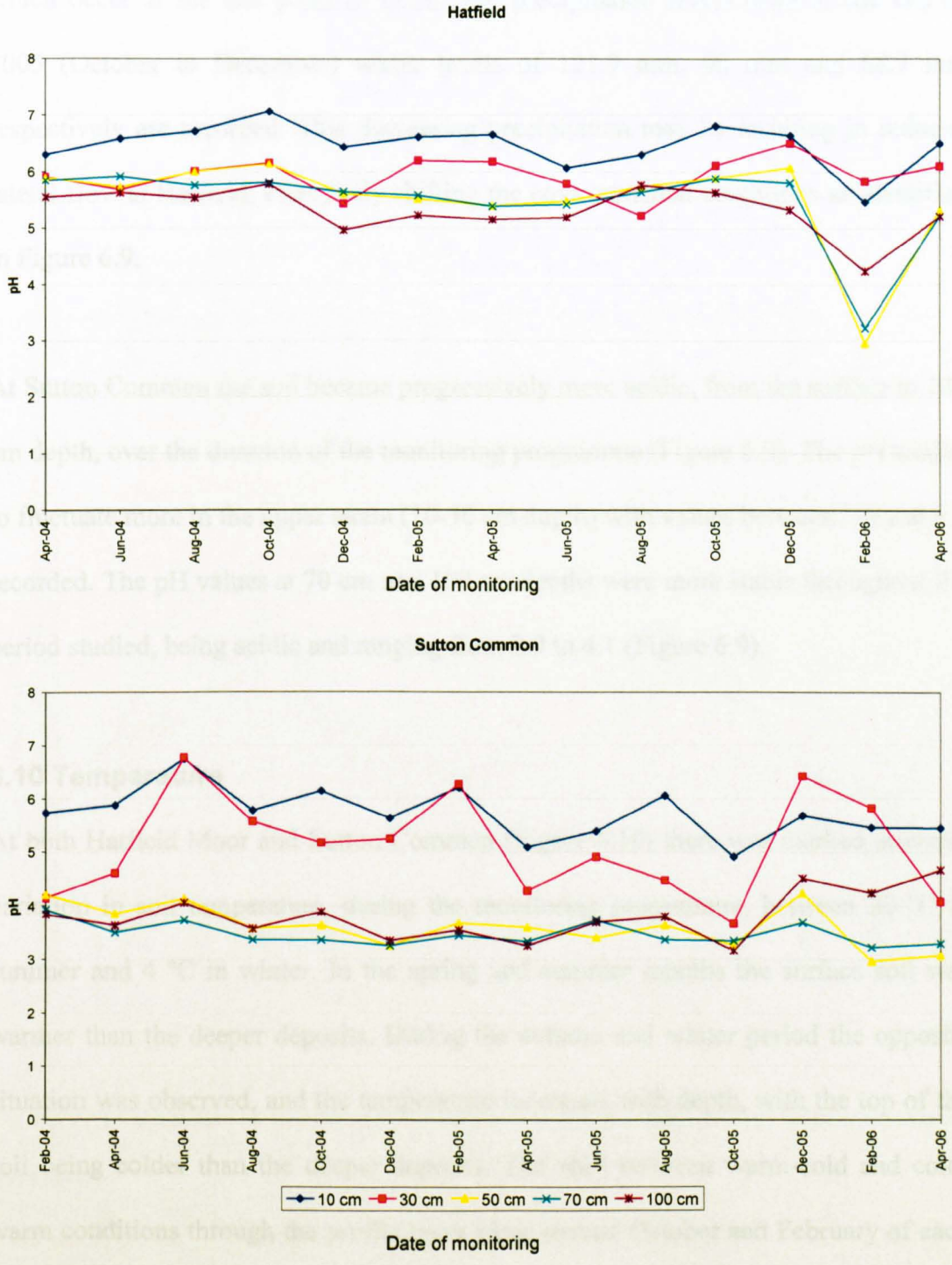


Figure 6.9: Variation in pH with depth at Hatfield Moor and Sutton Common, 2004-2006.

As can be seen in February 2006 (Figure 6.9), at Hatfield Moor, there was a shift in pH at all depths, towards more acid conditions, with the pH being <6 at 10 cm depth and <3 at 100 cm depth. This may reflect the low rainfall measurements recorded across January to April of 2006 (35 mm, 56.3 mm, 93.5 mm and 46 mm respectively) which occur at the end point of decreasing precipitation levels towards the end of 2005 (October to December) where levels of 121.9 mm, 90 mm and 68.7 mm respectively are recorded. This decreasing precipitation may be resulting in reduced lateral flow at Hatfield, effectively shifting the environmental conditions as identified in Figure 6.9.

At Sutton Common the soil became progressively more acidic, from the surface to 100 cm depth, over the duration of the monitoring programme (Figure 6.9). The pH tended to fluctuate more in the upper strata (10-30 cm depth) with values between 3.6 and 6.9 recorded. The pH values at 70 cm and 100 cm depths were more stable throughout the period studied, being acidic and ranging from 2.9 to 4.1 (Figure 6.9).

6.10 Temperature

At both Hatfield Moor and Sutton Common (Figure 6.10) there was marked seasonal variation in soil temperature, during the monitoring programme, between 20 °C in summer and 4 °C in winter. In the spring and summer months the surface soil was warmer than the deeper deposits. During the autumn and winter period the opposite situation was observed, and the temperature increased with depth, with the top of the soil being colder than the deeper deposits. The shift between warm-cold and cold-warm conditions through the profile takes place around October and February of each year.

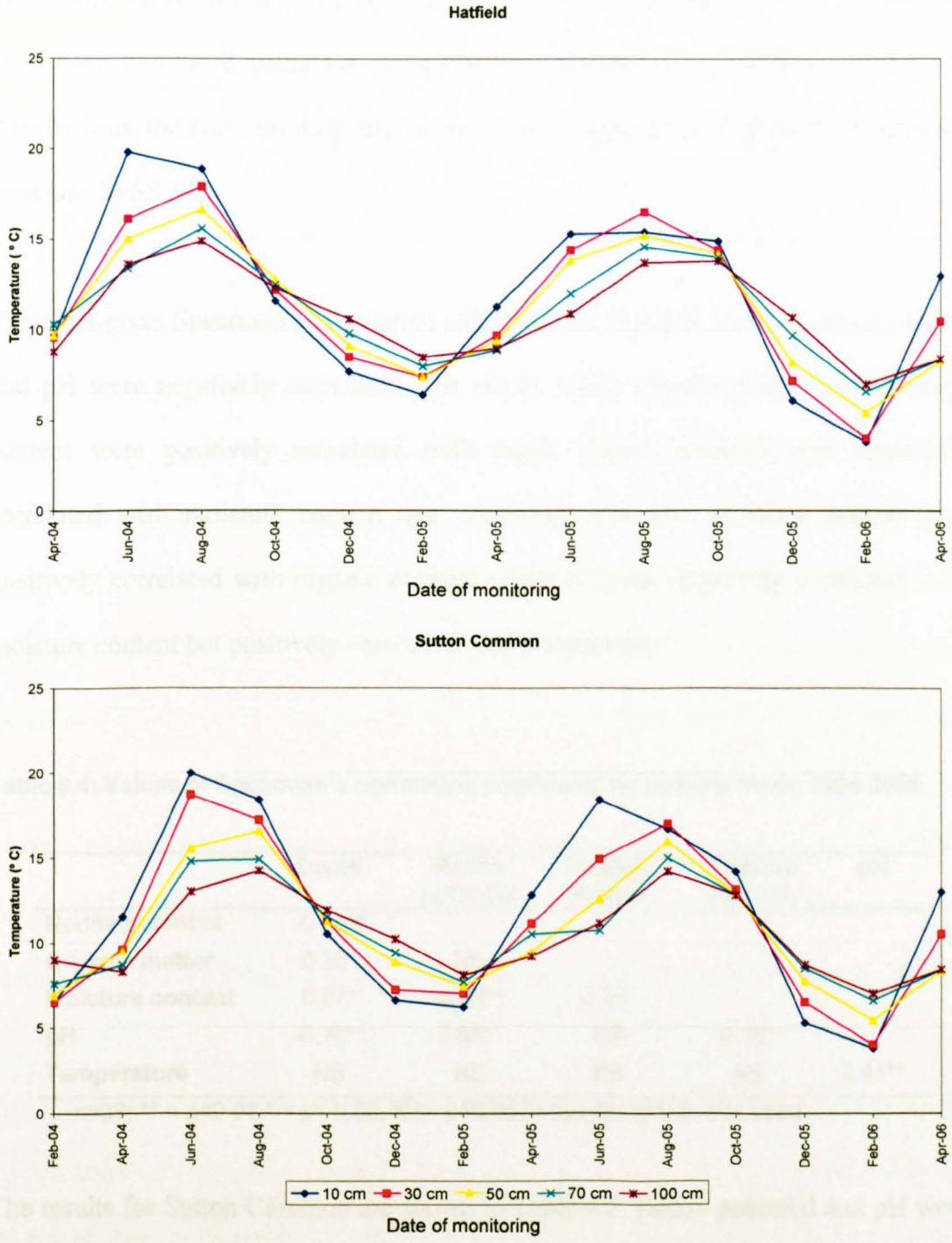


Figure 6.10: Variation in temperature with depth at Hatfield Moor and Sutton Common, 2004-2006.

6.11 Relationships between physico-chemical variables

To explore the relationships between physico-chemical variables, the results obtained have been correlated using the non-parametric Spearman's correlation coefficient. Results from the two sampling sites were analysed separately. The software package used was SPSS 14.0.

Table 6.4 gives Spearman's correlation values (r_s) for Hatfield Moor. Redox potential and pH were negatively correlated with depth, while organic matter and moisture content were positively correlated with depth. Redox potential was negatively correlated with moisture content and positively with pH. Moisture content was positively correlated with organic content; whilst pH was negatively correlated with moisture content but positively correlated with temperature.

Table 6.4: Values of Spearman's correlation coefficient for Hatfield Moor, 2004-2006.

	Depth	Redox potential	Organic matter	Moisture content	pH
Redox potential	-0.75**				
Organic matter	0.36*	NS			
Moisture content	0.87*	-0.60**	0.38*		
pH	-0.75**	0.65**	NS	-0.58**	
Temperature	NS	NS	NS	NS	0.41**

n=39; ** = p<0.01,* = p< 0.05, NS= p>0.05; a two-tailed test was used.

The results for Sutton Common are shown in Table 6.5. Redox potential and pH were again negatively correlated with depth. Redox potential was positively correlated with pH. Moisture content was positively correlated with organic matter content and negatively correlated with pH.

Table 6.5: Values of Spearman's correlation coefficient for Sutton Common, 2004-2006.

	Depth	Redox potential	Organic matter	Moisture content	pH
Redox potential	-0.91**				
Organic matter	NS	NS			
Moisture content	NS	NS	0.79**		
pH	-0.63**	0.55**	NS	-0.53**	
Temperature	NS	NS	NS	NS	NS

n=39; ** = p<0.01,* = p< 0.05, NS= p>0.05; a two tailed test was used.

6.12 Discussion of physico-chemical results

At both sampling sites it was observed that soil samples with a higher organic matter content (Figure 6.1) also had higher moisture contents (Figure 6.2). Statistically significant correlations between organic matter and soil moisture content have confirmed this observation (Tables 6.4 and 6.5). This relationship probably occurs due to the fact that organic humus can hold approximately five times more water than clays, as the organic matter content enhances water holding capacity by increasing the porosity of the soil structure (Dubbin, 2000). Organic-rich soils such as peat can impede water movement and encourage the formation of anaerobic conditions within the burial environment, thereby favouring *in situ* preservation of organic archaeological remains (Singer and Munns, 1986; Powell, 2001; Holden *et al.*, 2006).

At Hatfield Moor, organic matter and moisture contents characterize the depositional environments in the palaeochannel sequences, and the prevailing conditions at this site; where managed water levels result in near complete saturation of the soil profile (especially below *c.* 30 cm depth), appear to be producing excellent conditions for *in situ* preservation. The organic composition of the channel sequence at Hatfield Moor reflects the variability in flow regimes, wherein the channel is periodically abandoned, promoting peat growth below 100 cm depth and above 45 cm depth; and re-activated,

as attested by the introduction of minerogenic clay-silts between 45-100 cm depths. This alluvial unit could be linked to increased arable cultivation in the catchment, and concomitant increases in run-off.

In contrast, at Sutton Common the moisture and organic matter content tended to fluctuate, especially at 50 cm and 70 cm depths, but were generally more constant above and below these depths. This again reflects the contrasting nature of the two sites as the sequences at Sutton Common represent the in-filling of an Iron Age ditch feature, which has a more varied depositional history due to anthropogenic influences, than are in evidence at Hatfield Moor. The fact that the organic part of the infill sequence in the ditch occurs from 13 cm to 70 cm depth, and that drying-out and humification is evident in the upper part of the organic sequence results in a more minerogenic content at 30 cm depth. In addition, it was apparent during the coring programme that lenses of mineral-rich material were intercalated with the organics from 13-70 cm depth. This material appears to represent natural weathering of bank material, and has a clear influence on the percentages of organic matter in evidence through the profile (especially at 50 and 70 cm depths).

Percentage of total carbon (C) content fluctuated in parallel with the percentage of organic matter (Figure 6.3), because the greater part of the soil C is found in organic matter and in carbonate minerals (Allison *et al.*, 1965). At Hatfield Moor, organic matter and percentage C increased with depth in April 2006, but these levels were lower in October 2005 and February 2006, probably due to the variability in organic content at depth. At Sutton Common organic matter and percentage of total C were

greatest at 50 cm depth. The relationship between organic matter and percentage C is assessed below in Chapter 7.

In general, soil nitrogen (N) is mainly organic, with as much as 90 % of terrestrial N occurring as soil organic matter (Pulford, 1991). A significant fraction of N in soil is associated with humic colloids, clays or microbial biomass (Mulvaney and Khan, 2001). Percentage of total N was generally higher at Hatfield Moor than at Sutton Common, especially at depth. In addition, at Hatfield Moor percentage of total N increased slightly in October 2005 and April 2006. However, at Sutton Common, the percentage of total N did not follow a clear trend, although high concentrations of total N were observed at 50 cm depth. This variability in concentration highlights the considerable heterogeneity occurring in the depositional sequences at these sites, both through the soil profile and over time, although there is a general correlation of N concentration with organic matter content (Figures 6.1 and 6.4).

Although sulphur occurs in many forms in soils (e.g. sulphide, sulphate and organic forms), most of the sulphur is present in organics (Bardesly and Lancaster, 1965). At both sampling sites, the total content of S (Figure 6.5) tended to increase with depth, possibly reflecting its translocation through the profile. At Hatfield Moor this increase ranges from *c.* 0.1 % at the soil surface to >1 % at the base of the profile. At Sutton Common there are very low levels at the surface (>0.1 %) but these increase somewhat towards the base, where maximum levels of *c.* 2.5 % were recorded for October 2005. This is possibly associated with an increase in organic matter, which subsequently increases organic S throughout the soil profile at both sites. However, the increases at Sutton Common may be influenced by the grazing of cattle, as

improving the S status of grassland through fertilizers has been shown to have a beneficial effect on animal health and growth (Wang *et al.* 2001).

The content of total phosphorus (Figure 6.6) tended to decrease with depth at both sampling sites. In most soils, 50 to 75 % of the P is inorganic, although this percentage can vary widely. Both organic and inorganic forms of P are important sources of P for bacteria; however, their availability is primarily controlled by soil characteristics (Schulte and Kelling, 1996). It is suggested that the limited concentration of potential nutrients at these depths is also responsible for limiting bacterial activity (see Chapter 7).

At Hatfield Moor during the three studied months, and at Sutton Common during February and April of 2006, total P decreased with depth (Figure 6.6). This decrease in P with depth might indicate that very little phosphorus moves through the subsoil (Schulte and Kelling, 1996). In acidic soils, inorganic P is adsorbed to iron and aluminium oxides and clay minerals; and in neutral and calcareous soils P precipitates as calcium phosphate minerals and/or is absorbed by clays (Schulte and Kelling, 1996). This limits P leaching and leads to accumulation of P at the soil surface. This pattern was also observed by Blume *et al.* (2002) who identified that the levels of P decline with depth, being elevated in the summer as a consequence of fertilizer applications. At Hatfield Moor, an arable site, the P concentrations are consistently higher than those in evidence at the pasture site of Sutton Common, thereby supporting the observations made by Blume *et al.* (2002).

At Hatfield Moor, the water was at its highest level during August 2004 (Figure 6.7). The data demonstrate that the water table at this location was not influenced by increased abstraction and reductions in rainfall during the summer months. During 2004, Hatfield Moor did not exhibit clear seasonal variation, and it appears that the water table was mainly controlled by ground water, as there is no indication of variation due to changes in precipitation. However, during the summer of 2005 the water level experienced a slight decrease, which while limited, is clearly more pronounced than in the summer of 2004. This fluctuation is again related to regional water tables, and probably occurs because the water table was influenced by the lack of precipitation and associated increases in abstraction occurring during this season (2005).

The sampling site at Hatfield Moor is located within a palaeochannel feature in which the water table is artificially maintained at a high level by the landowner as part of a Countryside Stewardship agreement with the former Countryside Agency, entitled the 'Value in Wetness' initiative (Chapter 2, Section 2.6). The results obtained (Figure 6.7) confirm the successful maintenance of the water table at a high level, i.e. at above 50 cm depth below the soil surface, for most of the monitoring period. The artificial manipulation of the ground water level at Hatfield Moor was clearly producing good levels of saturation, and as a consequence they were masking any significant natural seasonal variation. The continued presence of a high water table creates a saturated environment, leading to the stratification of the redox conditions through the soil profile. For this reason, Hatfield Moor is a site with high potential for *in situ* preservation, as a direct result of the maintenance of a high water table under an active management regime.

At Sutton Common, previous research has shown significant variability in the physical composition of the deposits in and around the site (Van de Noort *et al.*, 2001). Lillie and Schofield (2002) excavated sixty-six boreholes across the palaeochannel and adjacent areas at Sutton Common. Their results indicated that the channel infill sequences comprise silts and clays intercalated with sand and clay-silt horizons, with woody detritus throughout. In addition, they observed that there was a general trend towards the formation of more organic-rich and peaty deposits towards the upper part of the infill sequences (Lillie, 2007). It has been suggested that this complexity in the depositional sequences at Sutton Common will probably influence future conservation management, including the raising of the water table at the site (Lillie, 2007).

Prior to the current study, Cheetham (2004) observed some seasonal fluctuation in water level in the study area at Sutton Common, and noted a significant fall in water level during the summer. He argued that this variability was a reflection of the substantial dependence at this site, of the water table, on precipitation inputs (Cheetham, 2004). The significant lowering of water level in 2005 was probably a reflection of catchment-wide reductions in water availability (Figure 6.7). It has been noted elsewhere that effective management depends on an appreciation of the source of water supply, and that significant variability in water content in the capillary zone can result in considerable levels of desiccation and shrinkage depending on the nature of the sediments at the site being studied (Corfield 1998).

The results which have been obtained at Hatfield Moor and Sutton Common demonstrate that, in general, an important association occurs between water table variation and redox potentials (Figures 6.7 and 6.8). This is because redox potential

generally responds to levels of saturation; thus, when water levels decrease, oxidising conditions are established within the soil (Hogan *et al.*, 2001). However, at Sutton Common (Figure 6.7), at a time when the water is fully removed from the ditch sequences (August and December of 2005), no corresponding reaction occurs in the redox potentials at this location. There is clearly a 'lag' effect in the redox reactions, and despite the absence of a water table, the removal of saturation is not necessarily correlated. The fact that the ditch sequences contain a high proportion of silts and clays may be resulting in the retention of water within the soil profile.

Throughout the monitoring period surface redox potentials at Sutton Common (Figure 6.8) become increasingly positive, suggesting increasingly compromised (drier) conditions in the upper part of the ditch at this location. This drying out is likely to affect the activity of aerobic bacteria, which are likely to become more active with the establishment of oxidising conditions in the upper part of the ditch sequence.

To date, studies that have investigated the nature of the degradation processes impacting upon organic archaeological remains (mainly wood) in burial environments (Caple and Dungworth, 1997; Caple *et al.*, 1997; Hogan *et al.*, 2001; Chapman and Cheetham, 2002; Cheetham, 2004; Corfield, 2007), have shown that changes in the degree of saturation and redox potentials can have a significant influence upon the potential for *in situ* preservation.

The results from both sites demonstrate how the combination of water level measurement and assessment of redox potentials can indicate where, in the soil profile, burial conditions are oxidizing or reducing. In particular, environments with low

redox potentials have been associated with good preservation of archaeological wood (Caple *et al.*, 1996). An examination of the data recovered from a range of archaeological burial environments, where organic archaeological materials have been preserved, suggests that redox potentials should be maintained at between –100 mV and –400 mV for optimal preservation conditions *in situ* (Caple and Dungworth, 1997; Caple, 1996; French, 2004). Conversely, the results obtained by Smith (2005), suggest that the decay of archaeological wood is mainly a result of microbial activity, and is not necessarily dependent on levels of saturation, as the composition of the microbial community in a soil will influence the ability of these communities to degrade, irrespective of changes in the level of saturation and/or redox potential.

There was a strong negative correlation between pH and depth at both sampling sites (Tables 6.4 and 6.5). Seasonality, the time since last rainfall event and the quantity of fertilizer in the soil can affect soil pH (Caple, 2004). The trend in pH (Figure 6.9) was towards greater acidity with depth, which was probably associated with increases in peat/organic matter content and leaching through the soil profile. Some soils with a high organic content become more acidic through the accumulation of organic acids and the residues generated by microbial metabolic activity (Jordan, 2001). However, there was no significant correlation between pH and soil organic matter at either site (Tables 6.4 and 6.5). The upper strata of the soil profile at both locations (10 cm and 30 cm depths) were most alkaline, probably because of the presence of fertilizers in cropping and improved grassland regimes.

It has been suggested that high acidity inhibits decomposition in peatlands (Farrish and Grigal, 1988; Fisher *et al.*, 1998) and that acidic soils have lower microbial

biomass (Wardle, 1992) and activity (Bååth, 1998; Andersson and Nilsson, 2001). Moreover, Bååth and Arnebrant (1994) suggest that different bacterial communities develop at different soil pH values, and that the optimum pH for growth of a bacterial community is related to the soil pH at which it is found. From an archaeological perspective, several studies have shown that most organic remains are better preserved in acidic environments (Corfield *et al.*, 1996; Lillie and Smith, 2007). In addition, Hogan *et al.*, (2001) have suggested that organic archaeological remains require a neutral pH to remains stable, and if the pH falls below 4 or rises above 8, rapid deterioration can occur.

Seasonal fluctuation in temperature was substantial at both sampling sites (Figure 6.10). Since variation in soil temperature is affected by atmospheric circulations and sun radiation, during the spring and summer the temperature decreases with increasing depth. As a consequence, the top of the soil (10 cm depth) was warmer than the base of the soil studied. However, during the autumn and winter the opposite effect was detected; as the temperature increased with depth, with the top of the soil (10 cm depth) being colder than the deeper deposits (Brady, 1984). The influence of seasonal variability in temperature through the profile, in relation to microbial activity, is considered below in Chapter 7.

6.13 Summary

This chapter has presented the results of the physico-chemical analysis of the soil profiles at Hatfield Moor and Sutton Common throughout the monitoring period. The results outline the nature of the soil profiles, with a description of the stratigraphy at each site and an assessment of soil texture using particle size analysis. Soil analysis,

for C, N, P and S were described for three of the sampling months. In addition, this chapter has included data relating to soil composition, organic matter and moisture content, water table levels, redox potential, pH and temperature. This has been undertaken in order to provide an overview of the *in situ* conditions prevalent at each site and to facilitate an assessment of the potential for the preservation of waterlogged archaeological remains.

The results have demonstrated clear differences in the physico-chemical variables studied, both with depth, time of year and between sampling sites, especially in relation to water table levels and redox potentials.

The next chapters (Chapters 7 and 8) will present the results obtained from the application of conventional microbial techniques and molecular genomic methods. These are used in order to study microbial abundance and activity, and to characterize the bacterial communities in evidence through the depositional sequences at both sites.

Chapter 7 Conventional microbiological results

7.1 Introduction

This chapter presents the results obtained from counts of total bacteria (Section 7.2), three different extracellular enzyme activities (Section 7.3), and ^{14}C-leucine assimilation (Section 7.4). These were assayed in order to assess bacterial abundance and activity respectively, through the soil profiles at Hatfield Moor and Sutton Common. The analyses were undertaken throughout a two-year monitoring period which began in April 2004 and finished in April 2006. In addition, a number of soil and wood samples from the Hatfield Trackway, exhibiting different states of degradation, were included in the assays (Section 7.6).

In order to test whether the addition of nutrients (i.e. phosphates and nitrates) into the soil caused an increase in bacterial activity, an experiment using two soil samples from Sutton Common (the first obtained near the surface [10 cm depth] and the second taken from the deeper sediments [100 cm depth]) was carried out (Section 7.4.3).

Although the conventional microbial methods used in Sections 7.2 and 7.3 were applied under aerobic conditions, the results from the bacterial counts demonstrated the presence of bacteria in the deepest horizons in the soil profile from both Hatfield Moor and Sutton Common. In light of these results, a further experiment was undertaken in order to investigate the potential for bacterial processes under anaerobic conditions in three different soil samples obtained from each of the study sites (Section 7.4.4).

The results generated from the physiological profiling of the microbial community at Hatfield Moor and Sutton Common are also described in this chapter (Section 7.5). Samples were obtained throughout the soil profile, and during different seasons, for comparative purposes.

Section 7.7 presents a number of scanning electron microscopy (SEM) micrographs from various archaeological wood samples obtained from the Hatfield Trackway, and several samples of wood collected at different depths through the soil profile from the ditch at Sutton Common.

In order to investigate the correlations between the different microbial variables and five selected physico-chemical variables, the data were analysed using the 2-tailed Spearman's correlation coefficient (Section 7.8). To compare the readings obtained for each microbial variable from the two sampling sites, a 2 tailed Mann-Whitney U test was used (Section 7.9). In addition, the results obtained from principal component analysis (PCA) of the microbiological and physico-chemical variables are shown in Section 7.10.

The penultimate section (Section 7.11) provides a discussion of the main findings that have been identified from the conventional microbiological techniques used throughout this chapter.

7.2 Bacterial abundance by acridine orange direct counts.

The range of bacterial abundance at Hatfield Moor was 0.8-10.9 x 10^9 cells g^{-1} fresh weight (Figure 7.1). Bacterial abundance tended to be greater towards the soil surface.

Peaks in abundance were, however, sometimes recorded in the deeper strata (e.g. at 50 cm depth in June and August 2004, and at 70 cm depth in April and August 2005). There appeared to be marked seasonal variations in the surface soil; with bacterial abundance being lower in the autumn and winter, when compared to the spring/summer months. However this variation was not seen in the deeper strata of the soil. At Hatfield Moor the maximum number of cells was obtained during August 2004 at 10 cm depth (10.9×10^9 g^{-1} wet weight) and the minimum number of cells in December 2004 at 100 cm depth (0.8×10^9 g^{-1} wet weight).

The abundance of bacteria in the soil at Sutton Common ranged from 0.9-11.4 x 10^9 cells g^{-1} fresh weight (Figure 7.1), and was similar to that at Hatfield Moor. Abundance was generally greatest in the surface soil (at 10 cm depth) and decreased with depth, although there were sometimes marked increases in abundance in the deeper strata (e.g. at 50 cm depth in August 2004, and at 70 cm depth in April 2005). Abundance in the soil surface tended to be greater in the spring and summer months (e.g. June and August 2004).

Comparisons between sampling months for each site were made using the non-parametric Kruskal-Wallis test. All of the data obtained through the soil profile at Hatfield Moor were analysed, and significant temporal differences (chi-squared with five degrees of freedom = 21.17, p = 0.001) were detected. October and December displayed the lowest mean ranking in the analysis. There was also significant temporal variation at Sutton Common (chi-squared with five degrees of freedom = 20.80, p = 0.001), with October and December displaying the lowest levels of abundance.

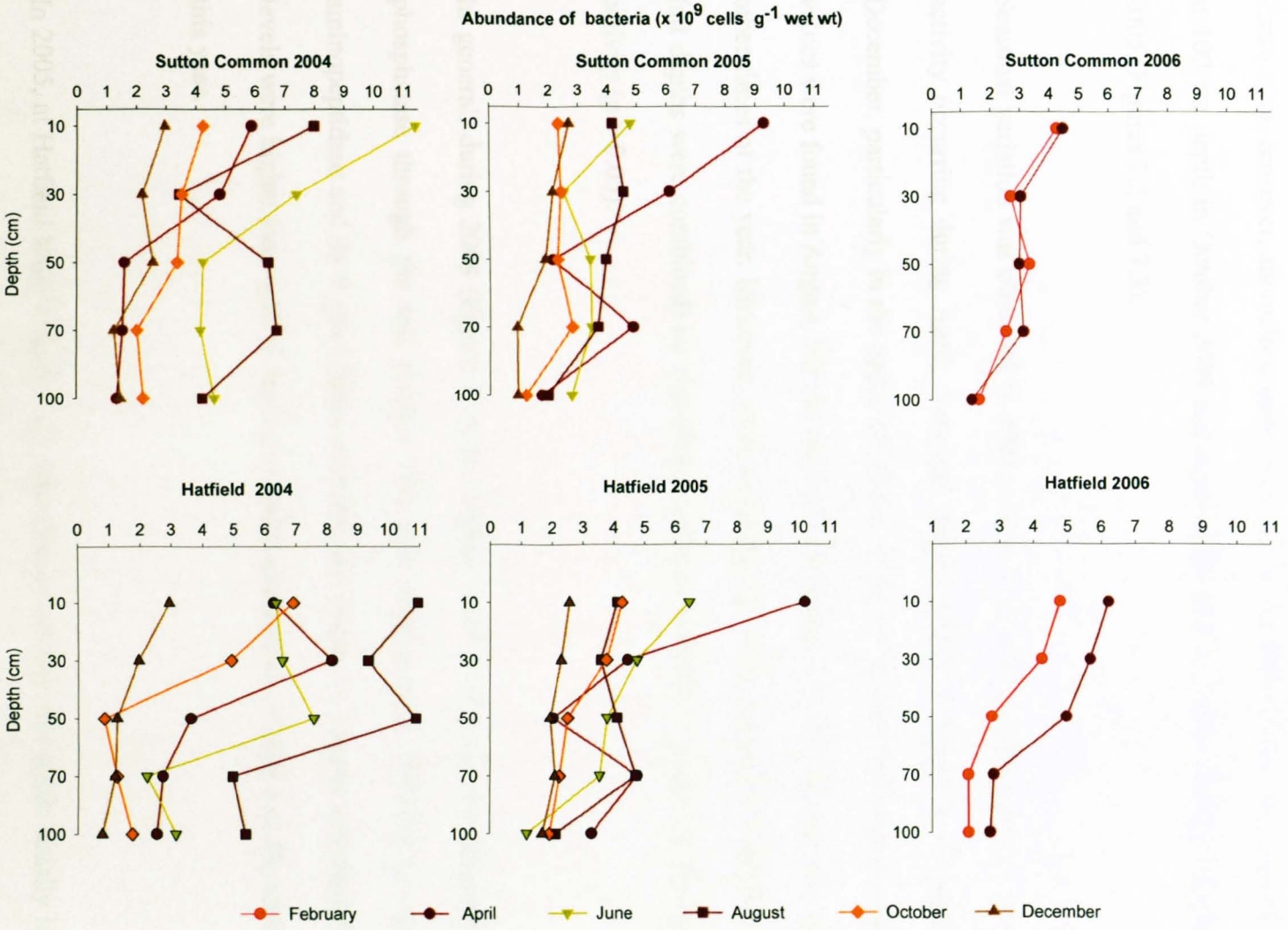


Figure 7.1: Variation in bacterial abundance with depth at Hatfield Moor and Sutton Common, 2004-2006.

7.3 Extracellular enzyme activity

7.3.1 Extracellular enzyme activity at Hatfield Moor

Enzyme activity tended to decrease with depth at Hatfield Moor (Figures 7.2-7.4). There were, however, anomalous peaks recorded in the deeper strata; e.g. phosphatase at 100 cm depth in October 2004 and April 2005, and at 70 cm depth in December 2005 (Figures 7.2 and 7.3).

Seasonal variation was observed in 2004 (Figure 7.2), with generally greater enzyme activity occurring during April, June and August when compared to October and December, particularly in the upper 10-30 cm of the soil profile. In 2005, the highest values were found in August, but the values in December were not notably less than at other times of the year. However, when analysing all of the data from Hatfield Moor (all depths were combined) no significant temporal variation was shown by enzyme activity ($p > 0.05$).

In general, during 2004 (Figure 7.2), the highest level of activity was displayed by phosphatase through the soil profile. This was subsequently followed by leucine aminopeptidase and by β-glucosidase respectively. However, leucine aminopeptidase levels were higher than both phosphatase and β-glucosidase in June and December of this year.

In 2005, at Hatfield Moor (Figure 7.3), phosphatase activity was again usually higher than aminopeptidase and β-glucosidase, but there were a number of exceptions; e.g. at 10 cm depth in August and December leucine aminopeptidase had the highest levels of activity.

At Hatfield Moor, during February of 2006 (Figure 7.4), generally, phosphatase was the most active enzyme throughout the soil profile, followed by leucine aminopeptidase and β-glucosidase. However, in April, at 10 cm depth β-glucosidase was the most active enzyme, but below this depth phosphatase activity dominates, with this subsequently being followed by leucine aminopeptidase and β-glucosidase.

The Kruskal-Wallis test showed a highly significant difference (chi-square with two degrees of freedom = 28.69, p = 0.00) among the three extracellular enzymes assayed. The mean rank of phosphatase was higher than both leucine aminopeptidase and β-glucosidase respectively.

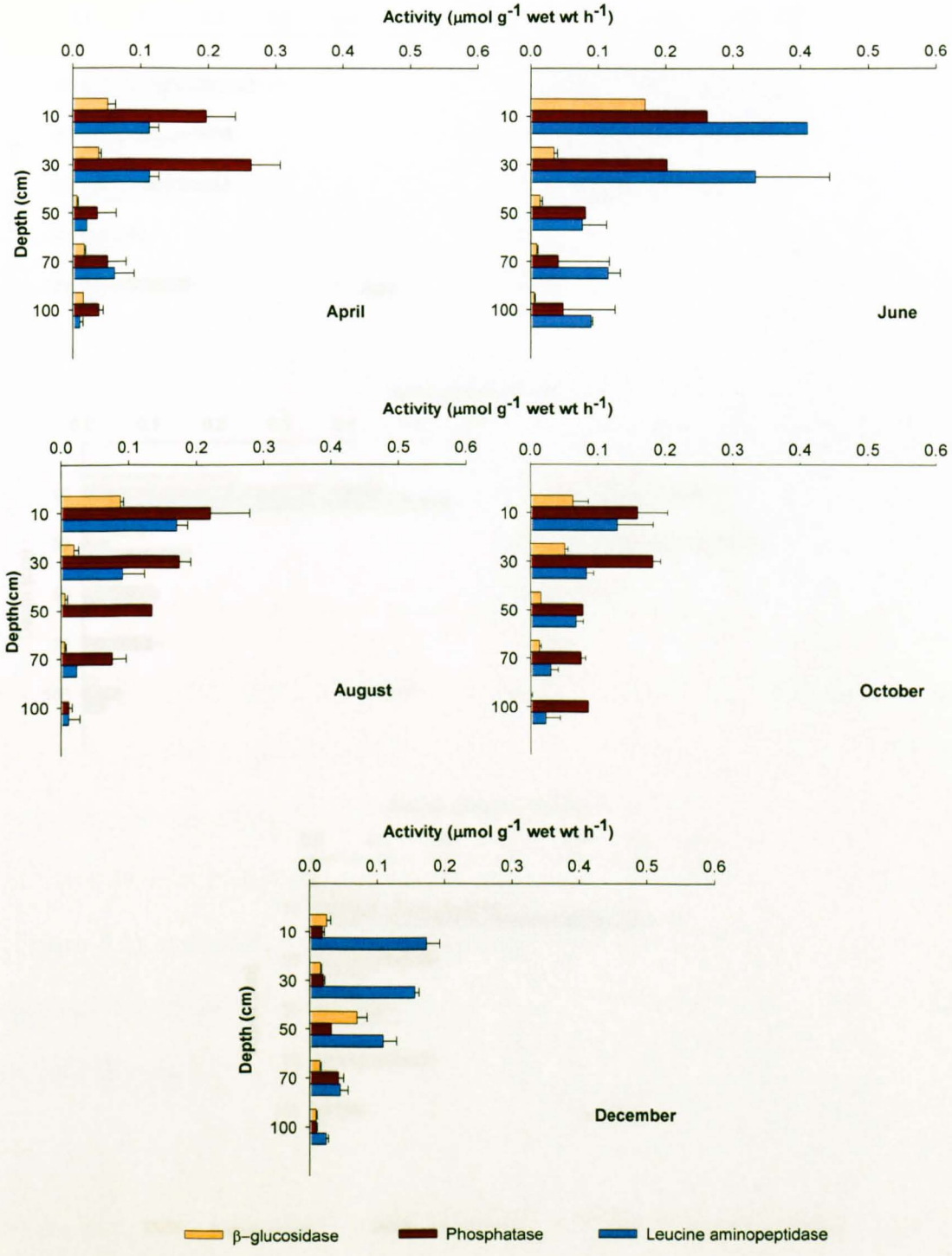


Figure 7.2: Extracellular enzyme activity at depth intervals at Hatfield Moor, 2004. Error bars indicate standard deviation.

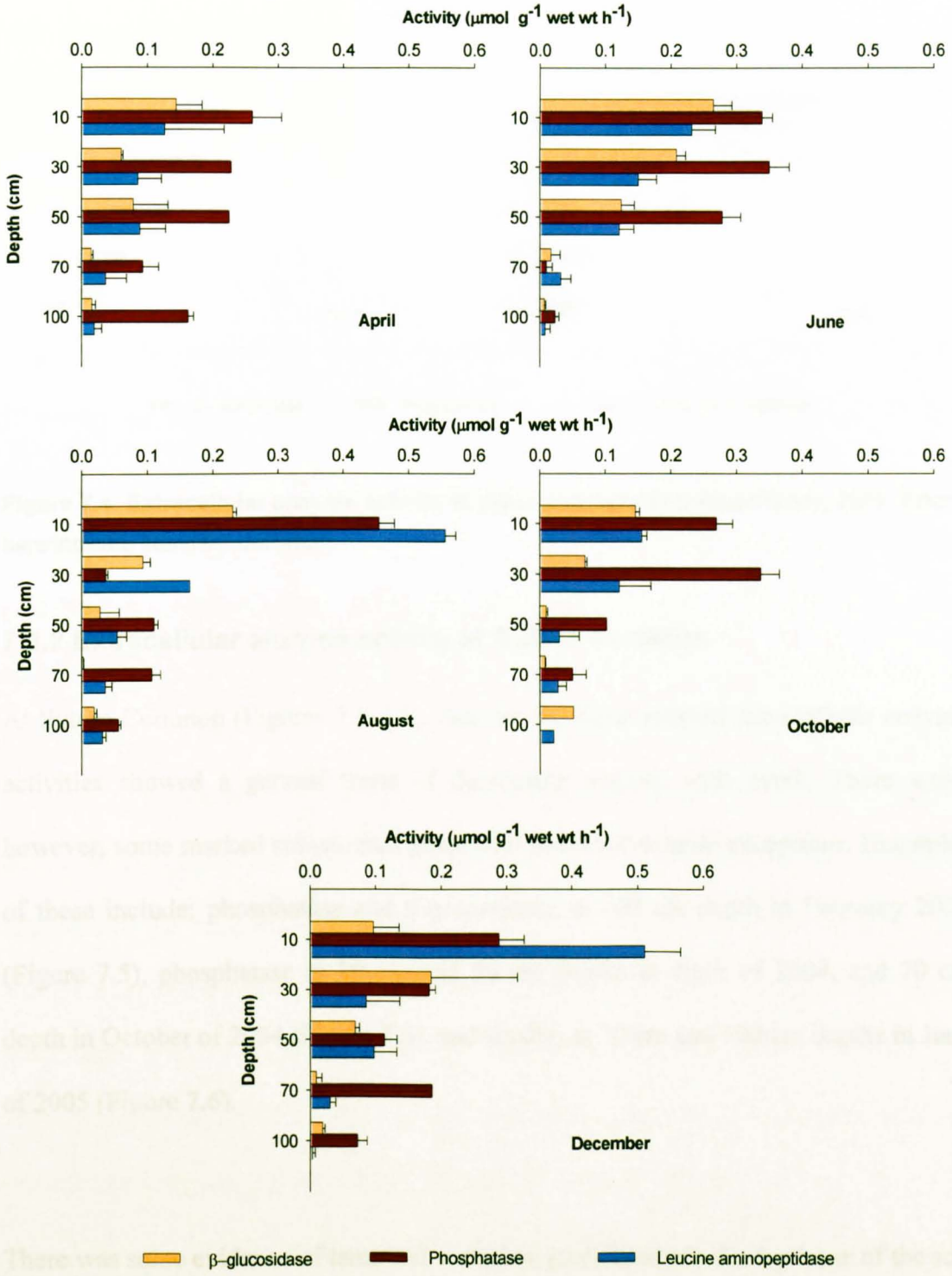


Figure 7.3: Extracellular enzyme activity at depth intervals at Hatfield Moor, 2005. Error bars indicate standard deviation.

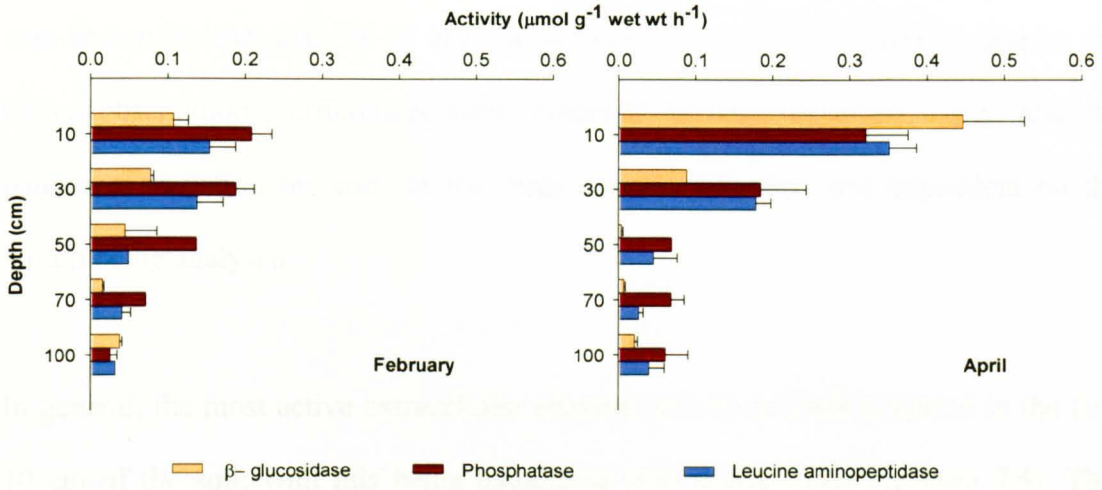


Figure 7.4: Extracellular enzyme activity at depth intervals at Hatfield Moor, 2006. Error bars indicate standard deviation.

7.3.2 Extracellular enzyme activity at Sutton Common

At Sutton Common (Figures 7.5-7.7), data for the three assayed extracellular enzyme activities showed a general trend of decreasing activity with depth. There were, however, some marked sub-surface peaks that provided notable exceptions. Examples of these include; phosphatase and β-glucosidase at 100 cm depth in February 2004 (Figure 7.5), phosphatase at 30 cm and 50 cm depths in April of 2004, and 70 cm depth in October of 2004 (Figure 7.5), and finally, at 70 cm and 100 cm depths in June of 2005 (Figure 7.6).

There was some evidence of temporal variation, particularly in the top layer of the soil, but no obvious seasonal patterning is in evidence. Thus in 2004 the highest activities were recorded in April-August and the lowest in October and December (Figure 7.5), while in 2005 the highest values were in June and August, and the lowest in April and October (Figure 7.6). When all of the data from Sutton Common was statistically

analysed (combining all data from different depths), no significant temporal variation was shown by the analysis of enzyme activities ($p > 0.05$). However, despite the above observations, differences were observed between sampling visits, and the pattern of variation for each of the three enzyme activities was dependent on the month being analysed.

In general, the most active extracellular enzyme activity in 2004 occurred in the first 10 cm of the soil; with this being associated with β-glucosidase (Figure 7.5). This activity was followed during the majority of the analysed months by leucine aminopeptidase and phosphatase. However, in August of 2004 leucine aminopeptidase presented the highest levels of activity, with β-glucosidase being the least active enzyme through the profile (except at 10 cm depth). Below 10 cm depth the activity of each of the three enzymes varied depending on the month being analysed, for example in April 2004 (Figure 7.5), leucine aminopeptidase presented low levels of activity above 70 cm depth, but from 70 cm depth through to the base of the soil profile leucine was the most active enzyme. In June 2004 there was a gradual decrease with depth for all of the three assayed extracellular enzymes (with the exception of high β-glucosidase activity at 10 cm depth). In October and December of 2004 very low activity was recorded for all three of the extracellular enzymes at all depths, with the recorded activity ranging from 0 to 0.15 μmol g^{-1} fresh weight h^{-1}.

In 2005 (Figure 7.6) phosphatase and leucine aminopeptidase were generally the most active enzymes. In June, a marked increase in phosphatase activity was detected, reaching a maximum at 70 cm depth. During this month, the levels of activity at 100 cm depth were reduced to levels not dissimilar to those in evidence at 30 cm and 50

cm depths. In August and December high levels of leucine aminopeptidase activity occurred at 10 cm depth.

Statistical difference between extracellular enzyme activities were analysed using a Kruskal-Wallis test. The test showed highly significant differences (chi-square with two degrees of freedom = 7.91, p = 0.019) among the three extracellular enzymes assayed. Phosphatase was the enzyme with the highest mean ranking, followed by leucine aminopeptidase and β-glucosidase.

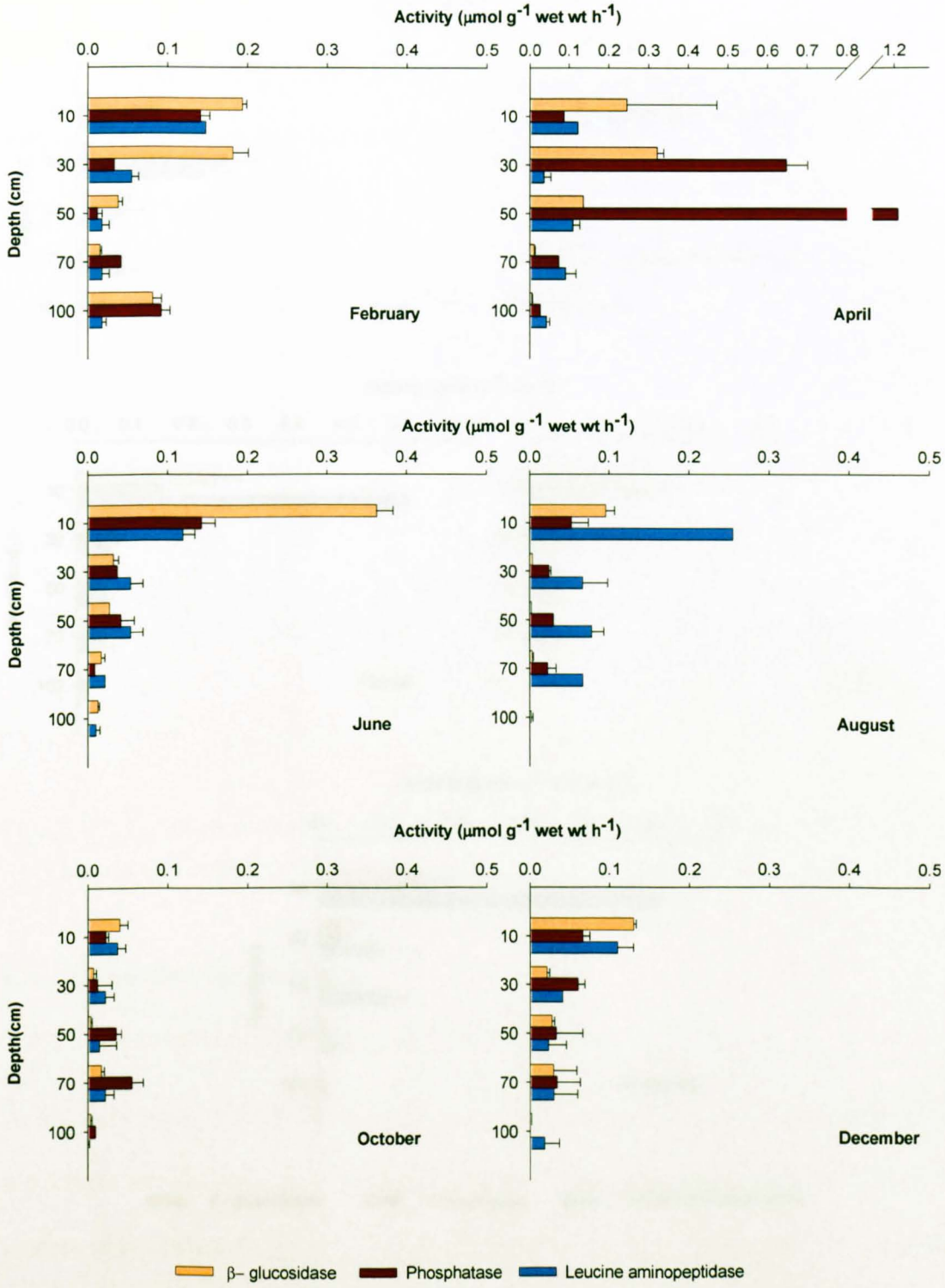


Figure 7.5: Extracellular enzyme activity at depth intervals at Sutton Common, 2004. Note that a different horizontal scale is use for April due to high levels of activity. Error bars indicate standard deviation.

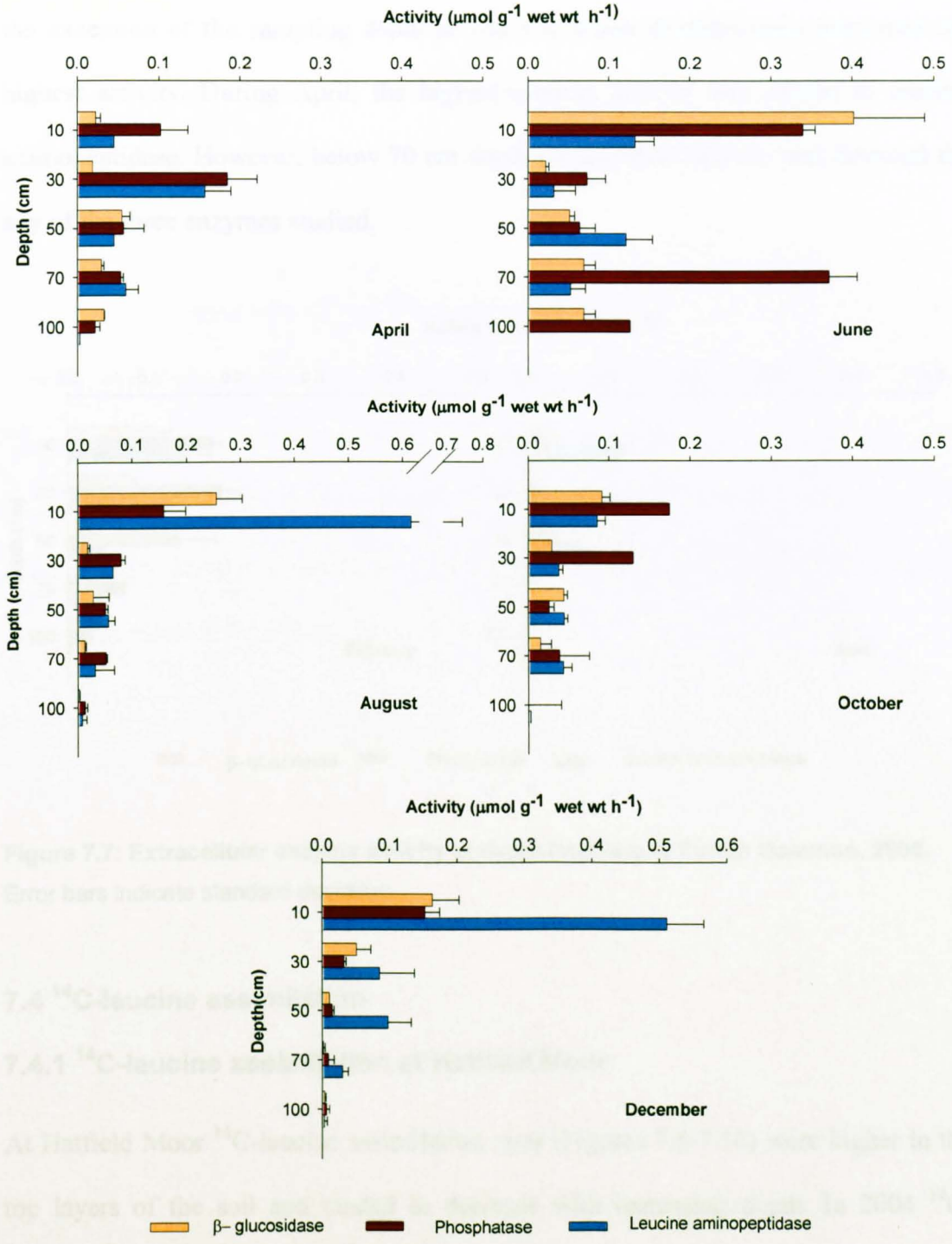


Figure 7.6: Extracellular enzyme activity at depth intervals at Sutton Common, 2005. Note that a different horizontal scale is used for August and December due to high levels of activity. Error bars indicate standard deviation.

In February 2006 (Figure 7.7) phosphatase exhibited the highest enzyme activity, with the exception of the sampling depth at 100 cm, where β-glucosidase displayed the highest activity. During April, the highest enzyme activity was related to leucine aminopeptidase. However, below 70 cm depth no important activity was detected for any of the three enzymes studied.

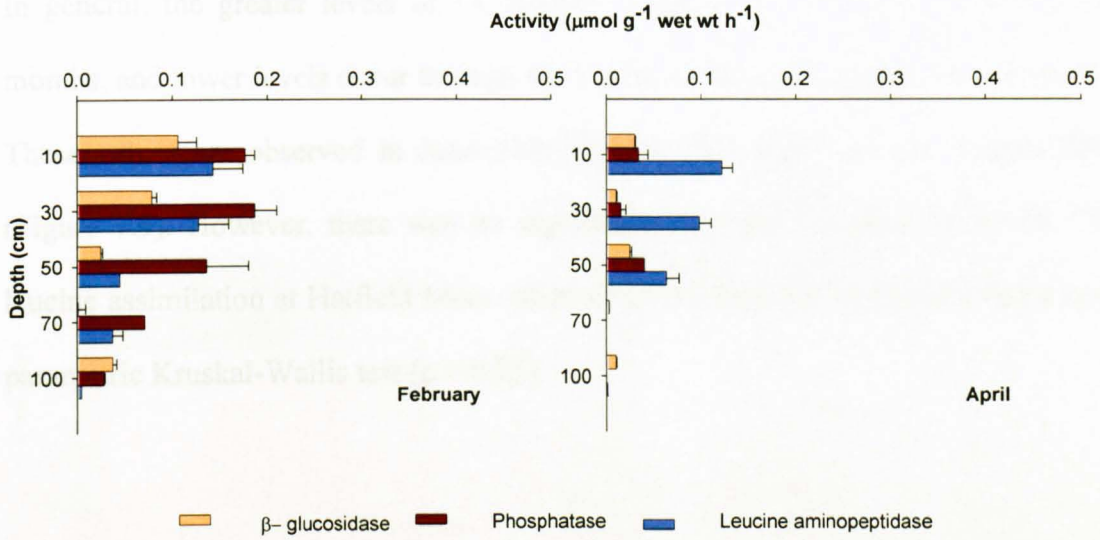


Figure 7.7: Extracellular enzyme activity at depth intervals at Sutton Common, 2006. Error bars indicate standard deviation.

7.4 ^{14}C-leucine assimilation

7.4.1 ^{14}C-leucine assimilation at Hatfield Moor

At Hatfield Moor ^{14}C-leucine assimilation rates (Figures 7.8-7.10) were higher in the top layers of the soil and tended to decrease with increasing depth. In 2004 ^{14}C-leucine assimilation rates only showed appreciable values at 10 cm and 30 cm depths (Figure 7.8). Below 50 cm depth little activity was detected, with the exception of June at 100 cm depth.

The highest ^{14}C-leucine assimilation rate in 2004 was observed in June at 10 cm depth (24.2 nmol g^{-1} h^{-1}). It appears that June favours more assimilation through the profile than the other monitored months. The ^{14}C-leucine uptake rates throughout the soil profile were higher in 2005 than in 2004.

In general, the greater levels of ^{14}C-leucine assimilation occur during the warmer months, and lower levels occur through the colder months, throughout the soil profile. These values are observed in June 2004 (Figure 7.8), and June and August 2005 (Figure 7.9). However, there was no significant temporal variation shown by ^{14}C-leucine assimilation at Hatfield Moor when all of the data was analysed using a non-parametric Kruskal-Wallis test ($p > 0.05$).

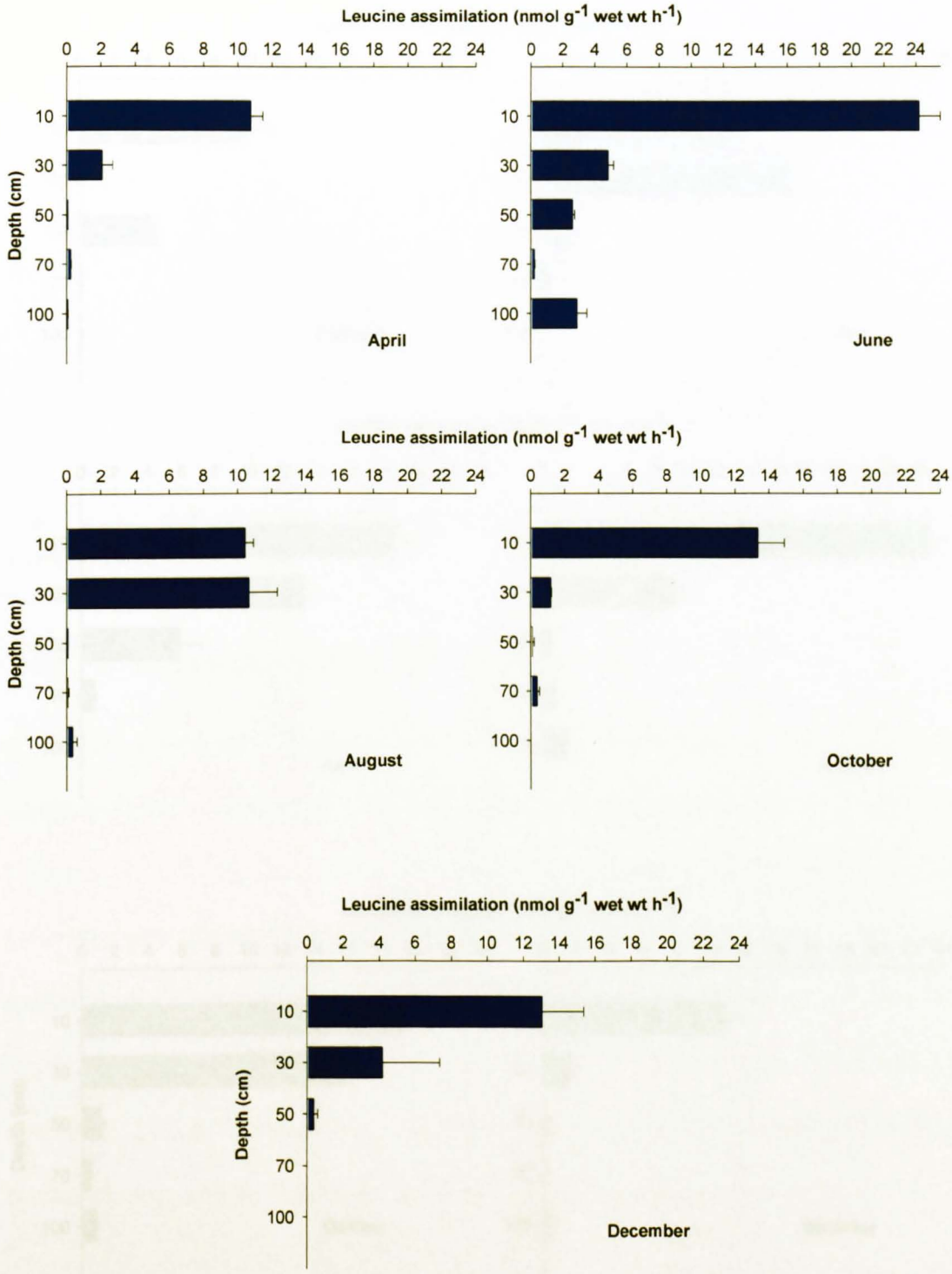


Figure 7.8: ^{14}C-leucine assimilation at depth intervals at Hatfield Moor, 2004. Error bars indicate standard deviation.

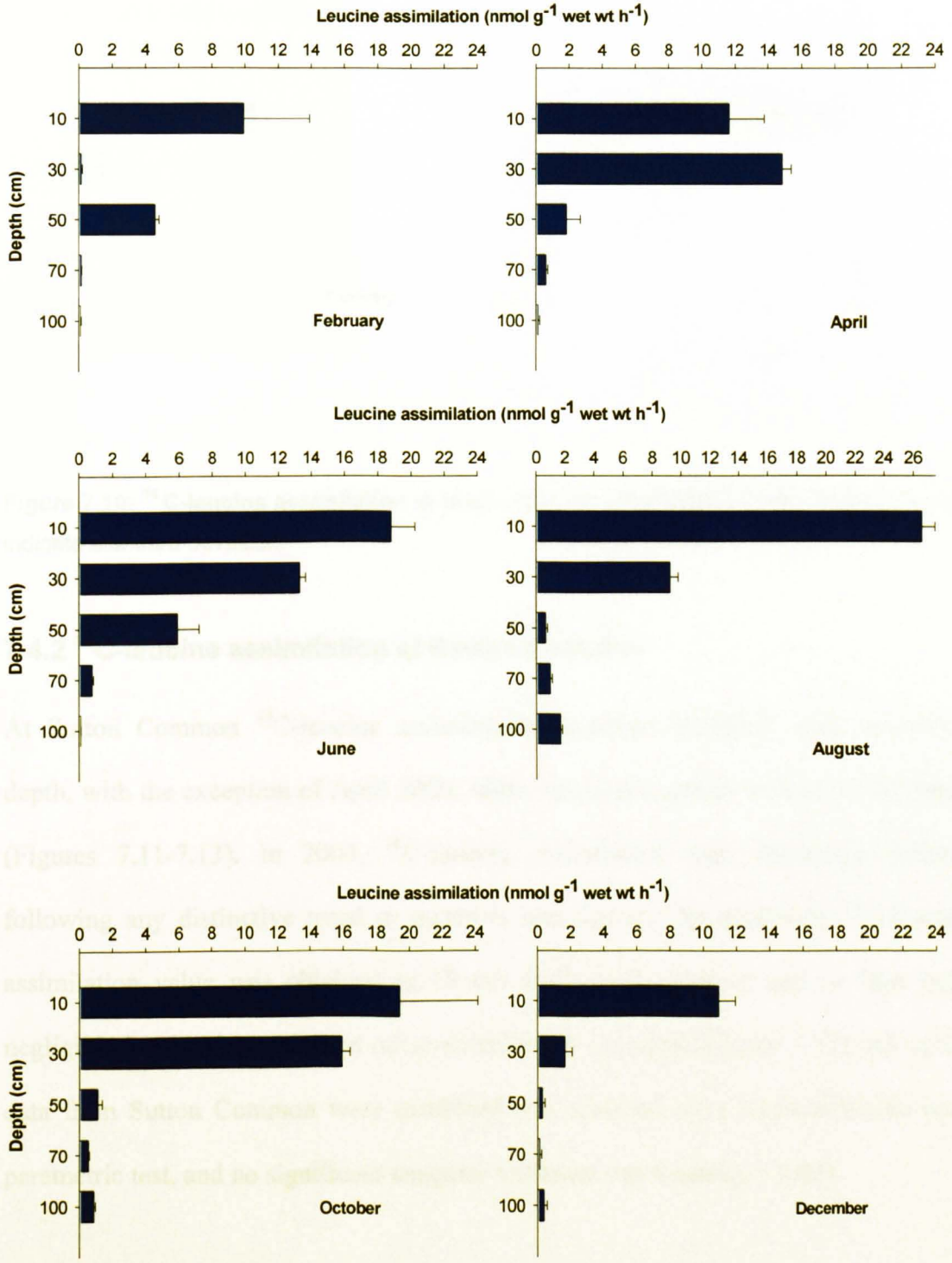


Figure 7.9: ^{14}C-leucine assimilation at depth intervals at Hatfield Moor, 2005. Note that a different horizontal scale is used for August. Error bars indicate standard deviation.

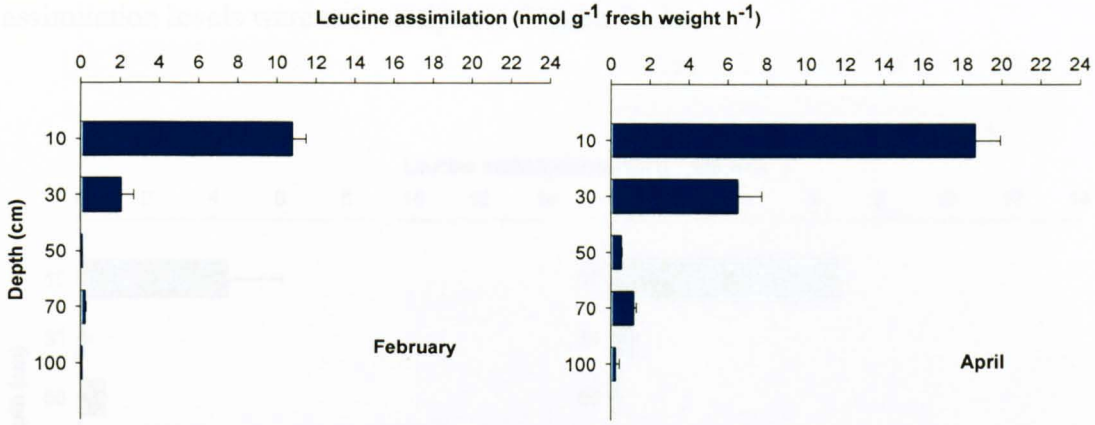


Figure 7.10: ^{14}C-leucine assimilation at depth intervals at Hatfield Moor, 2006. Error bars indicate standard deviation.

7.4.2 ^{14}C-leucine assimilation at Sutton Common

At Sutton Common ^{14}C-leucine assimilation decreased markedly with increasing depth, with the exception of April 2005, when maximum activity was at 30 cm depth (Figures 7.11-7.13). In 2004, ^{14}C-leucine assimilation rates fluctuated without following any distinctive trend or apparent seasonality. The maximum ^{14}C-leucine assimilation value was obtained at 10 cm depth in December, and in June only negligible levels of assimilation occurred below 30 cm depth (Figure 7.11). All of the data from Sutton Common were combined and analysed by a Kruskal-Wallis non-parametric test, and no significant temporal variation was found ($p > 0.05$).

However, in 2005, higher assimilation rates were detected in April, August and December, in the uppermost layers of the soil, and in general, lower assimilation rates occurred in February and October (Figure 7.12). In 2006 the levels of ^{14}C-leucine

assimilation were only notable at 10 cm depth, and below 30 cm depth the ^{14}C-leucine assimilation levels were extremely low (Figure 7.13).

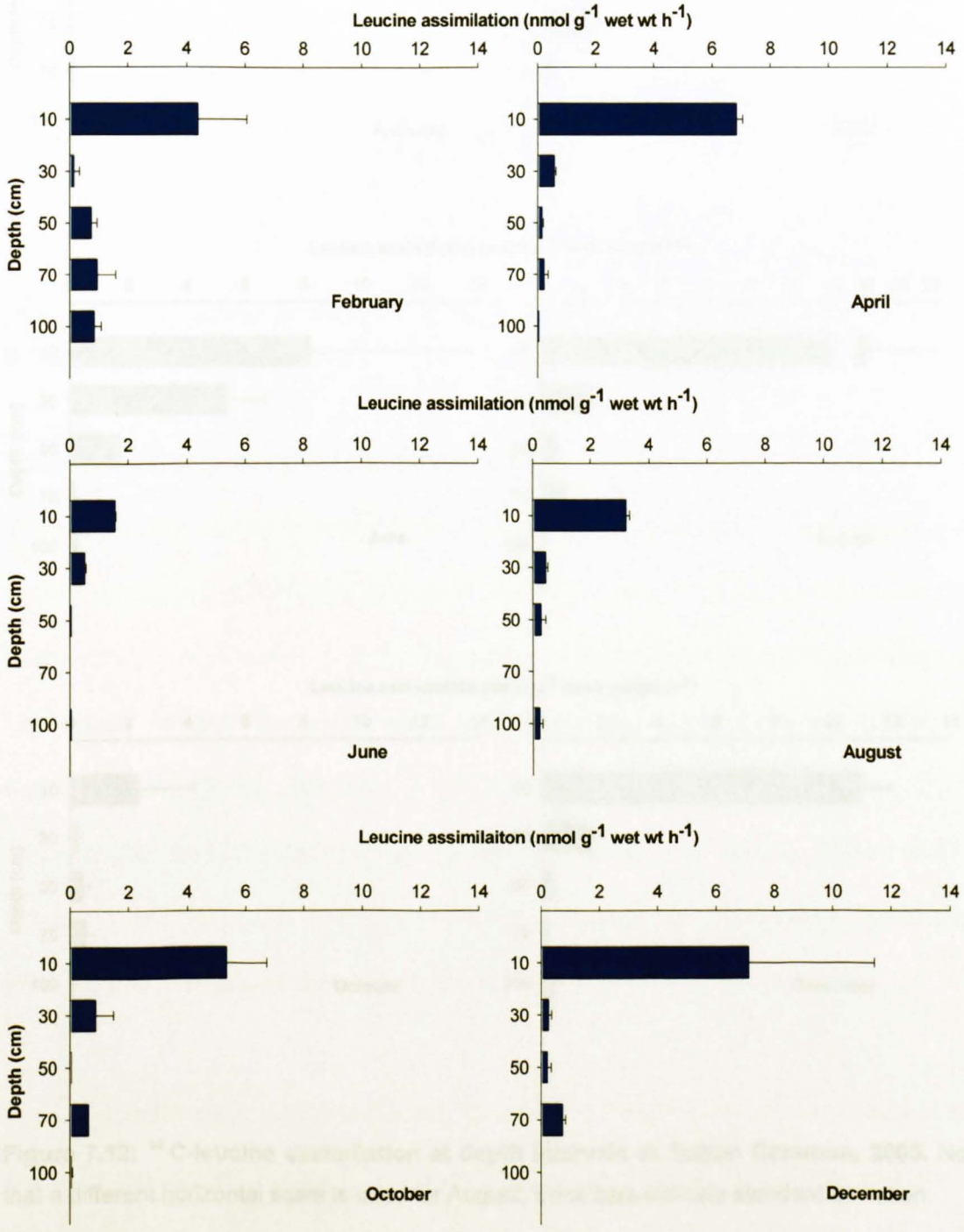


Figure 7.11: ^{14}C-leucine assimilation at depth intervals at Sutton Common, 2004. Error bars indicate standard deviation.

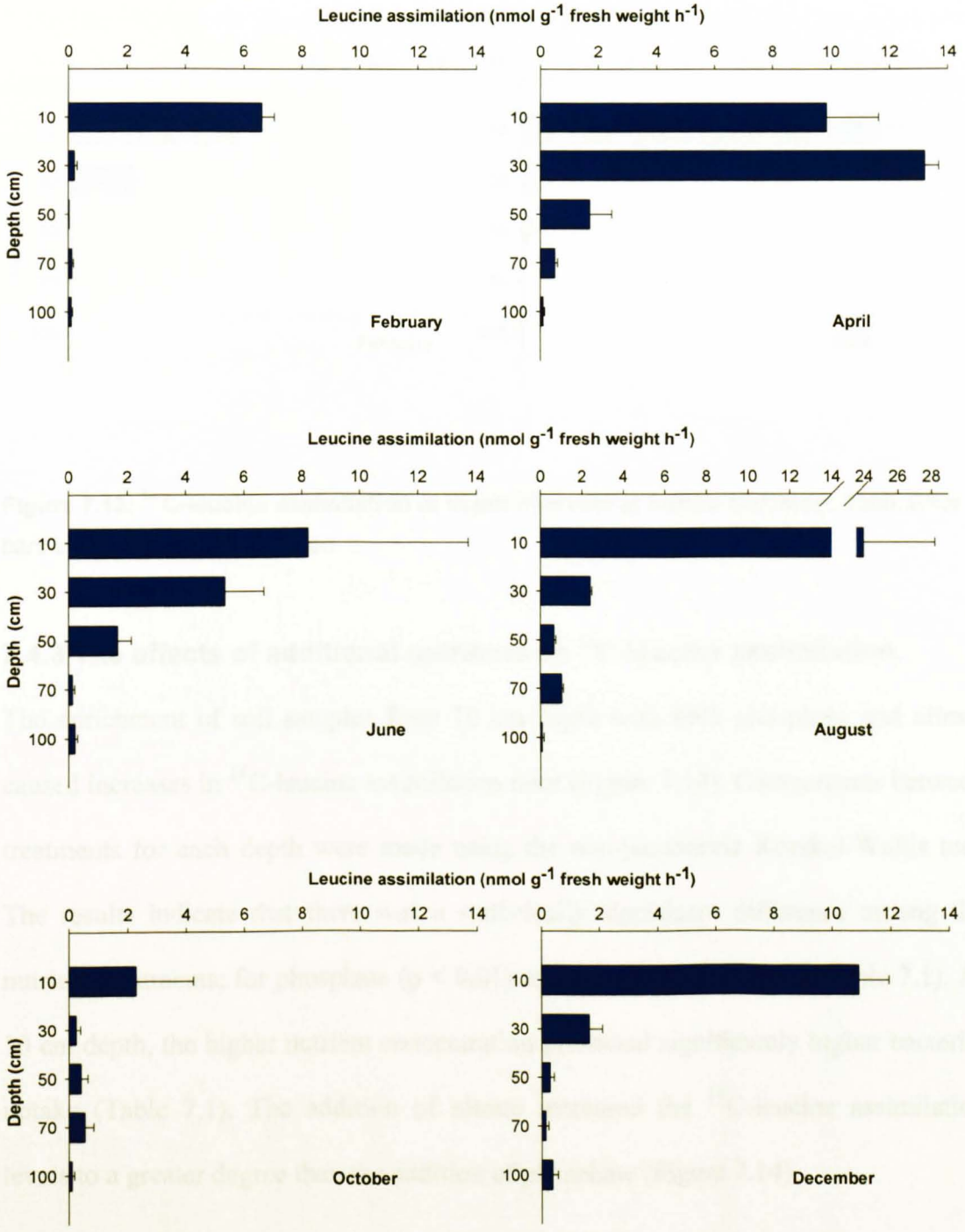


Figure 7.12: ^{14}C-leucine assimilation at depth intervals at Sutton Common, 2005. Note that a different horizontal scale is used for August. Error bars indicate standard deviation.

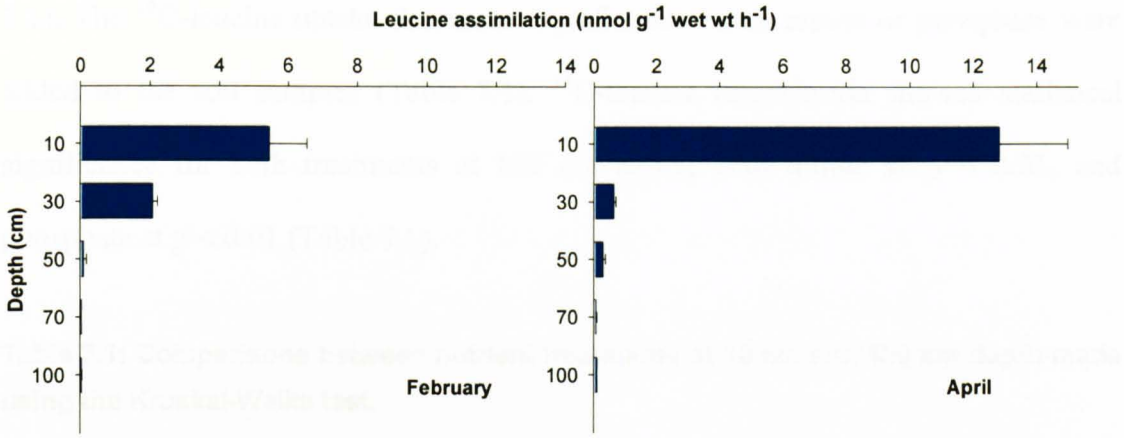


Figure 7.13: ^{14}C-leucine assimilation at depth intervals at Sutton Common, 2006. Error bars indicate standard deviation.

7.4.3 The effects of additional nutrients on ^{14}C-leucine assimilation.

The enrichment of soil samples from 10 cm depth with both phosphate and nitrate caused increases in ^{14}C-leucine assimilation rates (Figure 7.14). Comparisons between treatments for each depth were made using the non-parametric Kruskal-Wallis test. The results indicate that there was a statistically significant difference among the nutrient treatments; for phosphate ($p < 0.01$) and for nitrate ($p < 0.01$) (Table 7.1). At 10 cm depth, the higher nutrient concentration promoted significantly higher bacterial uptake (Table 7.1). The addition of nitrate increased the ^{14}C-leucine assimilation levels to a greater degree than the addition of phosphate (Figure 7.14).

The ^{14}C-leucine assimilation in the soil from 100 cm depth was much less than in soil from 10 cm depth (Figure 7.14). Furthermore, at 100 cm depth the addition of phosphate or nitrate did not promote greater ^{14}C-leucine assimilation (Figure 7.14). Higher levels of ^{14}C-leucine assimilation were detected in the control sample (without added nutrients), when compared to the samples where nutrients were added (Table

7.1). The ^{14}C-leucine uptake decreased significantly when nitrate or phosphate were added to the soil samples (Table 7.1). ^{14}C-leucine assimilation showed statistical significance for both treatments at 100 cm depth, with nitrate at $p < 0.01$, and phosphate at $p < 0.01$ (Table 7.1).

Table 7.1: Comparisons between nutrient treatments at 10 cm and 100 cm depth made using the Kruskal-Wallis test.

	Mean rank 10 cm		Mean rank 100 cm
Phosphate		**Phosphate**	
n = 18	**C** 3.67	n = 18	**C** 14.17
χ^2 13.205	N_1 10.00	χ^2 12.53	P_1 10.83
p 0.001*	N_2 14.83	**p 0.002***	P_2 3.50
Nitrate		**Nitrate**	
n = 18	**C** 3.50	n = 18	**C** 15.50
χ^2 12.78	N_1 10.67	χ^2 14.74	**P1** 3.67
p 0.002*	N_2 14.33	**p 0.001***	**P2** 9.33

The number of degrees of freedom for each test is 2.* $p<0.05$.
C: Control; P1: 50 µg P g^{-1} wet wt soil; P2: 500 µg P g^{-1} wet wt soil;
N1: 22.4 µg N g^{-1} wet wt soil; N2: 224 µg N g^{-1} wet wt soil.

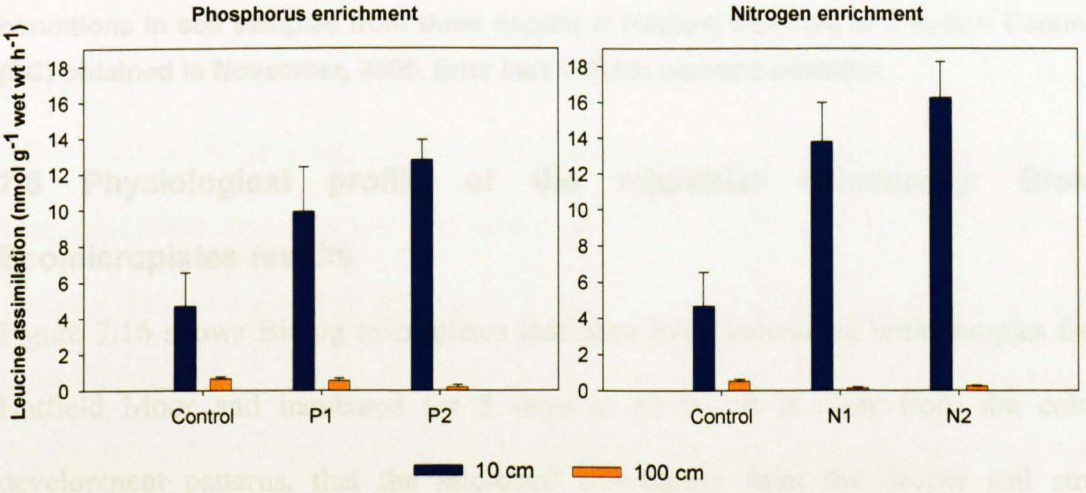


Figure 7.14: Change in ^{14}C-leucine assimilation in response to nutrient addition in soil samples from Sutton Common, November, 2005 (10 cm and 100 cm depth). P1: 50 µg P g^{-1} wet weight soil; P2: 500 µg P g^{-1} wet weight soil; N1: 22.4 µg N g^{-1} wet weight soil; N2: 224 µg N g^{-1} wet weight soil. Error bars indicate standard deviation.

7.4.4 ^{14}C-leucine assimilation under anaerobic conditions

The mean ^{14}C-leucine assimilation rates were always higher under aerobic conditions than under anaerobic conditions (Figure 7.15), and the difference was least marked in the deeper soil (100 cm depth). At Hatfield Moor the difference was greatest in the soil from 50 cm depth, while at Sutton Common, the greatest difference was in soil from 70 cm depth.

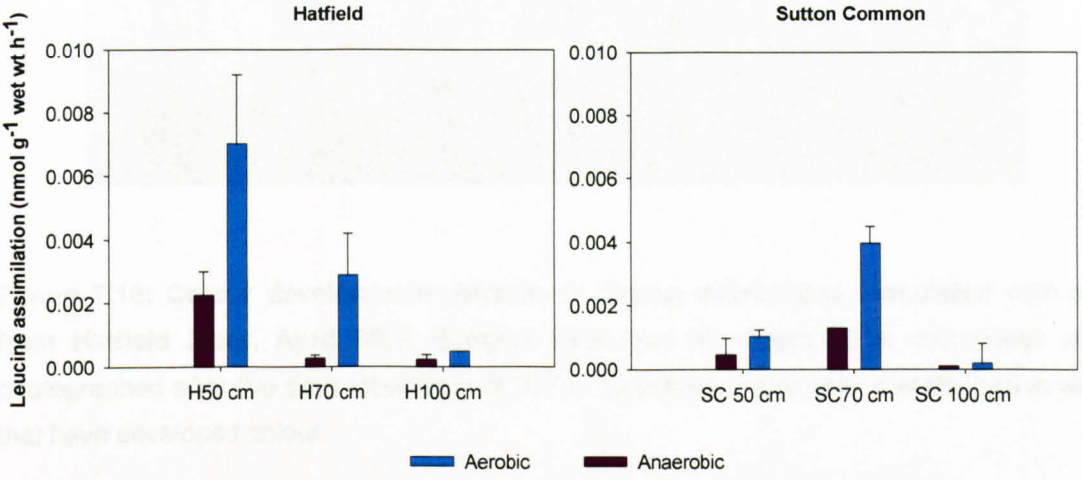


Figure 7.15: ^{14}C-labelled leucine assimilation rates under aerobic and anaerobic conditions in soil samples from three depths at Hatfield Moor (H) and Sutton Common (SC) obtained in November, 2005. Error bars indicate standard deviation.

7.5 Physiological profile of the microbial community: Biolog Ecomicroplates results

Figure 7.16 shows Biolog microplates that have been inoculated with samples from Hatfield Moor and incubated for 5 days at 10 °C. It is clear from the colour development patterns, that the microbial community from the deeper soil strata metabolize fewer carbon sources.

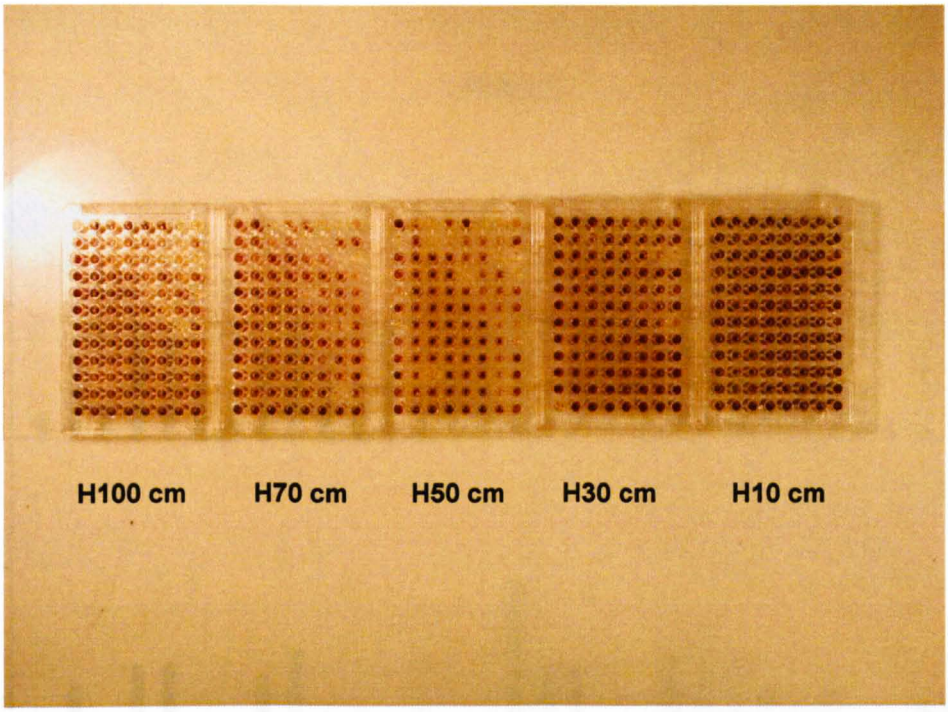


Figure 7.16: Colour development patterns in Biolog microplates inoculated with soil from Hatfield Moor, April 2005. Samples were from five depths. The microplates were photographed after five days incubation at 10 ° C. Substrates have been metabolized in wells that have developed colour.

7.5.1 February 2005

At Hatfield Moor the microbial communities from all depths were able to metabolise all or most of the substrates provided (Figure 7.17). However, the number of carbon sources utilized showed a tendency to decrease with depth. In contrast, the microbial communities from Sutton Common were unable to utilize all of the available carbon sources. At Sutton Common the communities, from 10 cm, 30 cm, 50 cm and 100 cm depths, were able to use 15-25 of the 31 substrates provided, but the community from 70 cm utilized only two substrates (D-galacturonic acid and Tween 80) (Figure 7.18).

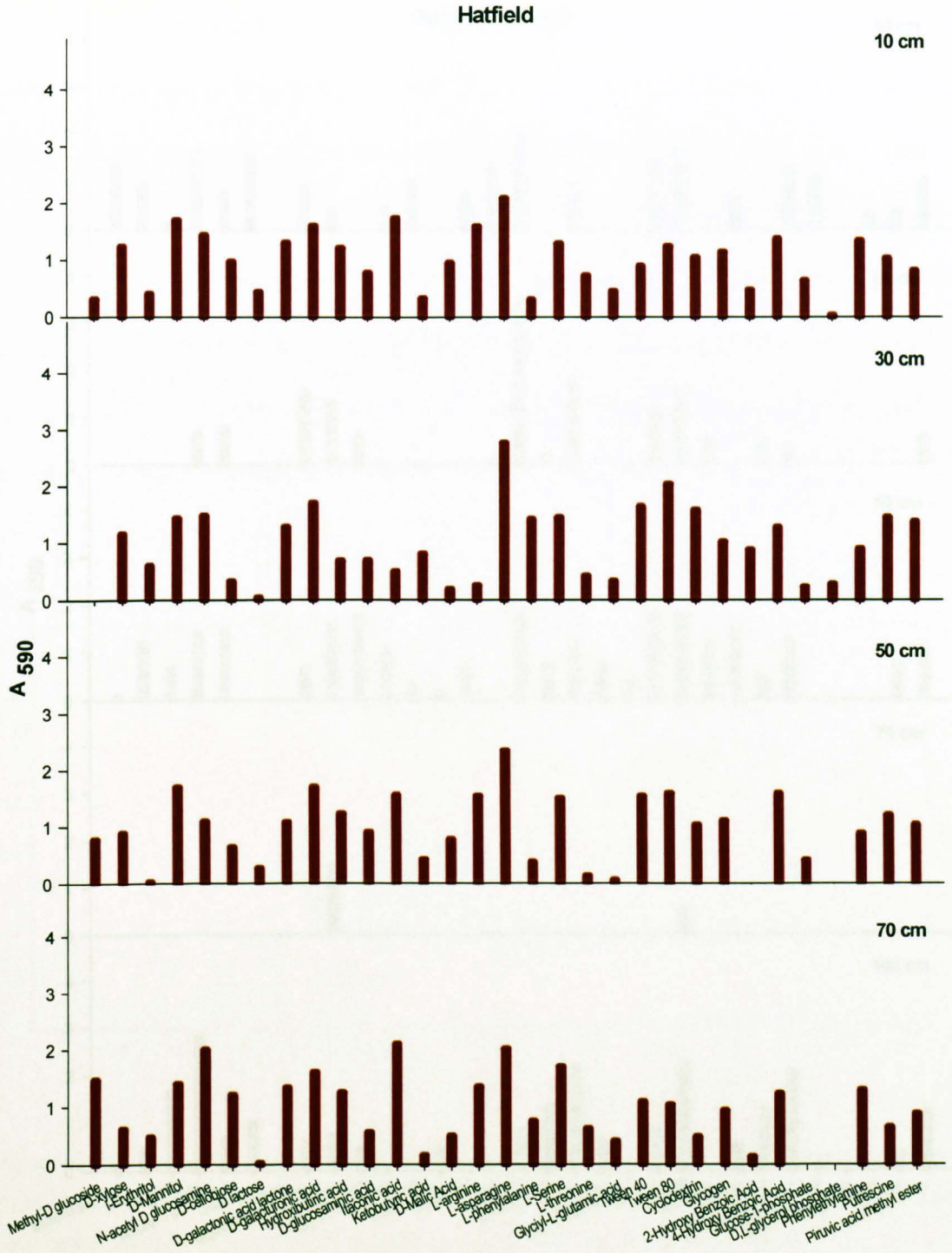


Figure 7.17: Carbon source utilization by the soil microbial community from Hatfield Moor February 2005, after 5 days of incubation at 10 °C. Data were normalized as described in Section 4.3.4. No microplate results were obtained for 100 cm depth.

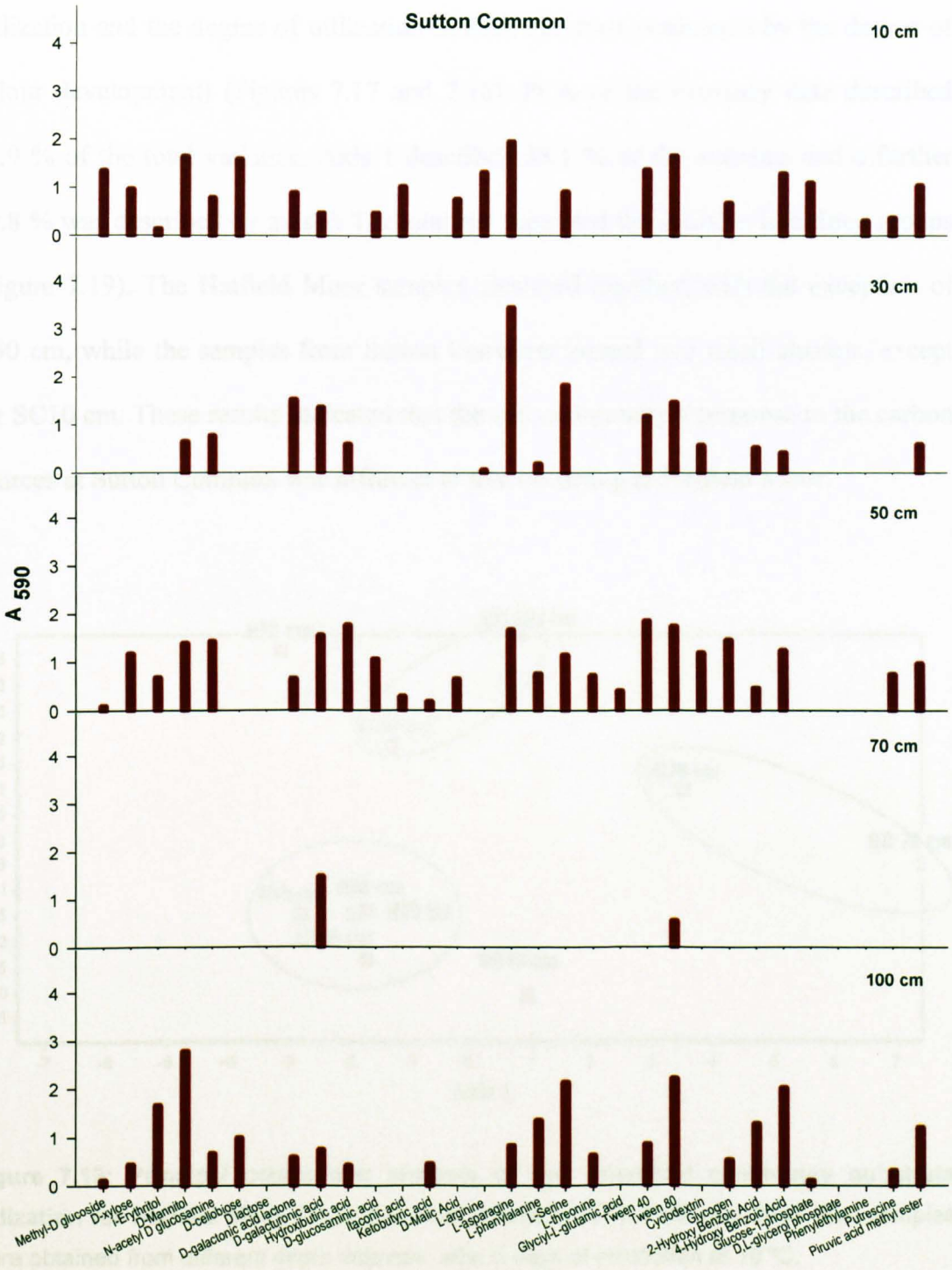


Figure 7.18: Carbon source utilization by the soil microbial community from Sutton Common February 2005, after 5 days of incubation at 10 °C. Data were normalized as described in Section 4.3.4.

Principal component analysis (PCA) was carried out using the data on substrate utilization and the degree of utilization of each substrate (estimated by the degree of colour development) (Figures 7.17 and 7.18). PCA of the February data described 54.9 % of the total variance. Axis 1 described 35.1 % of the variance and a further 19.8 % was described by axis 2. The analysis separated the samples into three groups (Figure 7.19). The Hatfield Moor samples clustered together, with the exception of H30 cm, while the samples from Sutton Common formed two small clusters, except for SC10 cm. These results indicated that the soil community's response to the carbon sources at Sutton Common was different to that occurring at Hatfield Moor.

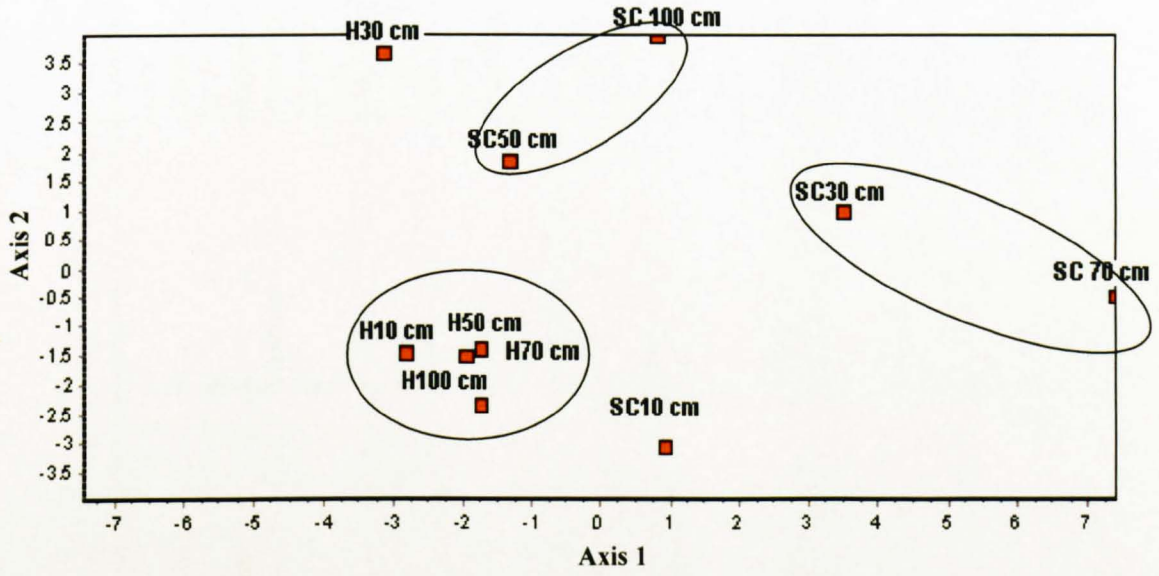


Figure 7.19: Principal component analysis of soil microbial community substrate utilization for Hatfield Moor (H) and Sutton Common (SC), February 2005. Soil samples were obtained from different depth intervals, after 5 days of incubation at 10 °C.

7.5.2 June 2005

At Hatfield Moor in June 2005 there was a tendency for both the number of carbon sources, and the extent of their utilization, to decrease with depth (Figure 7.20). The microbial communities from 10 cm, 30 cm and 50 cm were able to metabolise all or

most (30-31) of the carbon sources. The communities from 70 cm and 100 cm, however, were largely only able to metabolize sugars and aminoacids.

At Sutton Common in June 2005 most substrates were utilized by the microbial community from most depths (Figure 7.21). A minor exception was that only 25 out of 31 substrates were used by the community from 70 cm depth when contrasted with the utilisation of 30-31 substrates by the samples from the remaining depths. The capacity of the microbial communities to use the different carbon sources decreased with increasing depth (Figure 7.21).

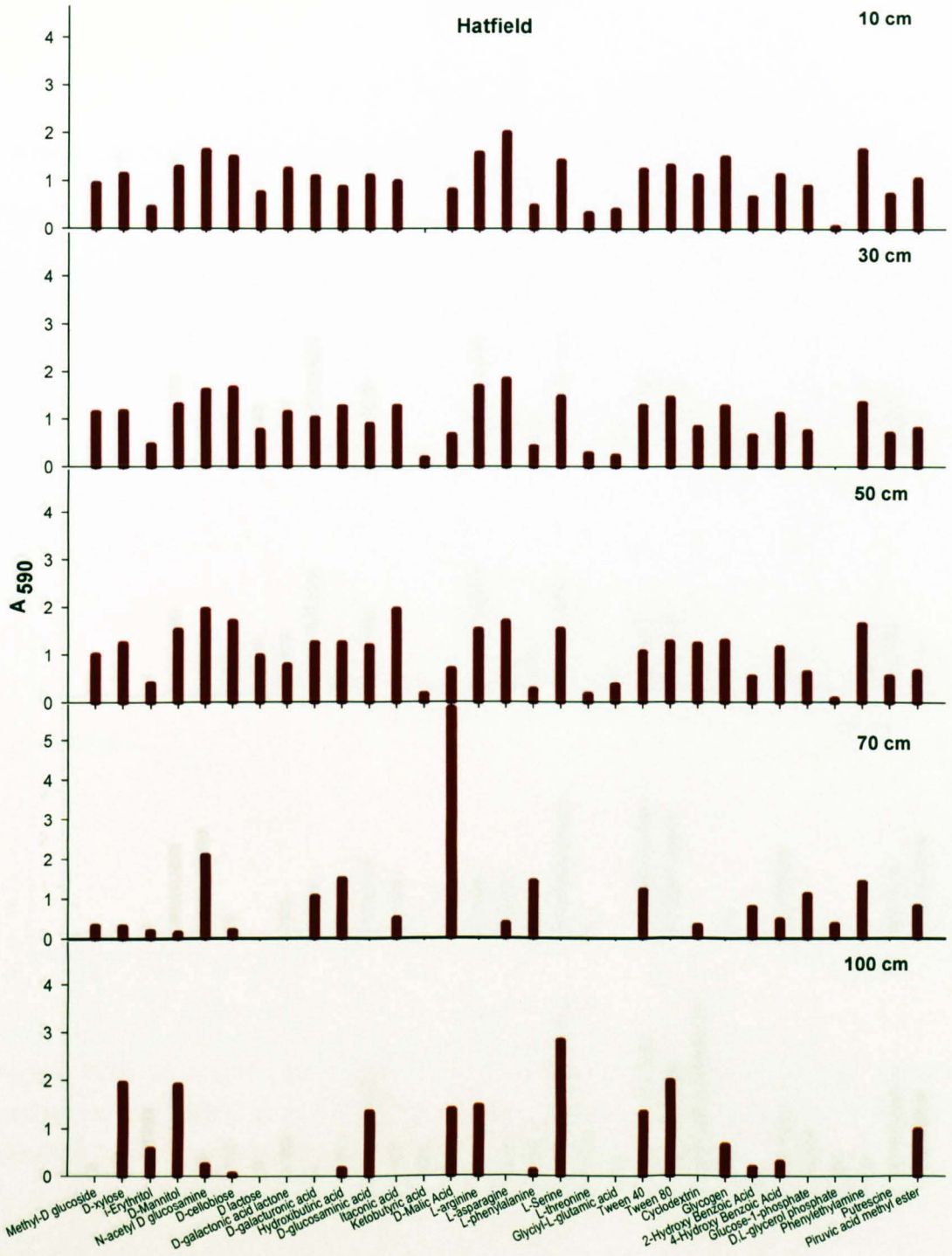


Figure 7.20: Carbon source utilization by the soil microbial community from Hatfield Moor, June 2005, after 5 days of incubation at 10 °C. Data were normalized as described in Section 4.3.4. Note that a different horizontal scale is used for 70 cm.

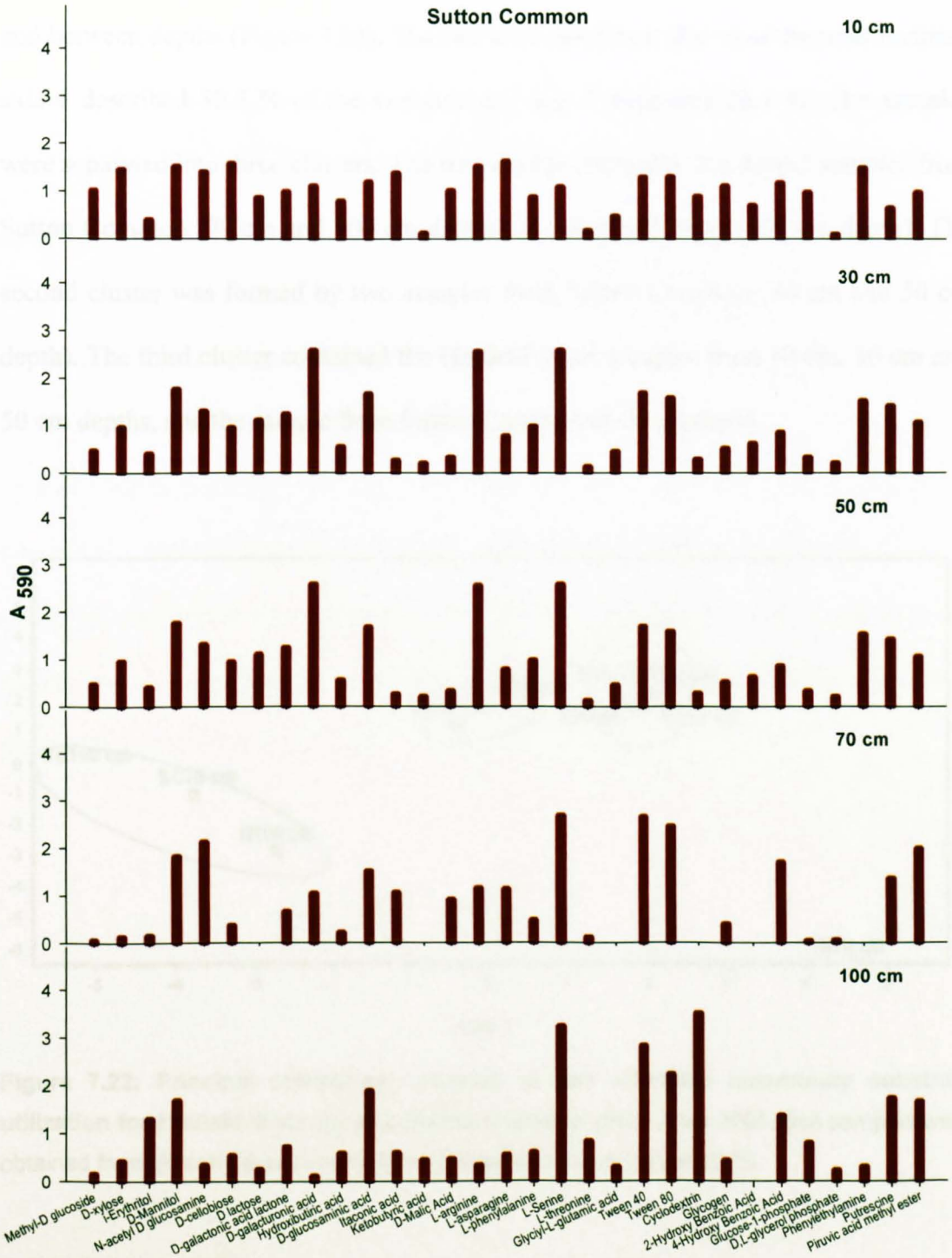


Figure 7.21: Carbon source utilization by the soil microbial community from Sutton Common, June 2005, after 5 days of incubation at 10 °C. Data were normalized as described in Section 4.3.4

Principal component analysis for June 2005 showed differences, both between sites and between depths (Figure 7.22). The two axes described 58.6 % of the total variance; axis 1 described 32.5 % of the variance and axis 2 described 26.1 %. The samples were separated into three clusters. The first cluster comprised the deeper samples from Sutton Common (70 cm and 100 cm depths) and Hatfield Moor (100 cm depth). The second cluster was formed by two samples from Sutton Common (30 cm and 50 cm depth). The third cluster contained the Hatfield Moor samples from 10 cm, 30 cm and 50 cm depths, and the sample from Sutton Common at 10 cm depth.

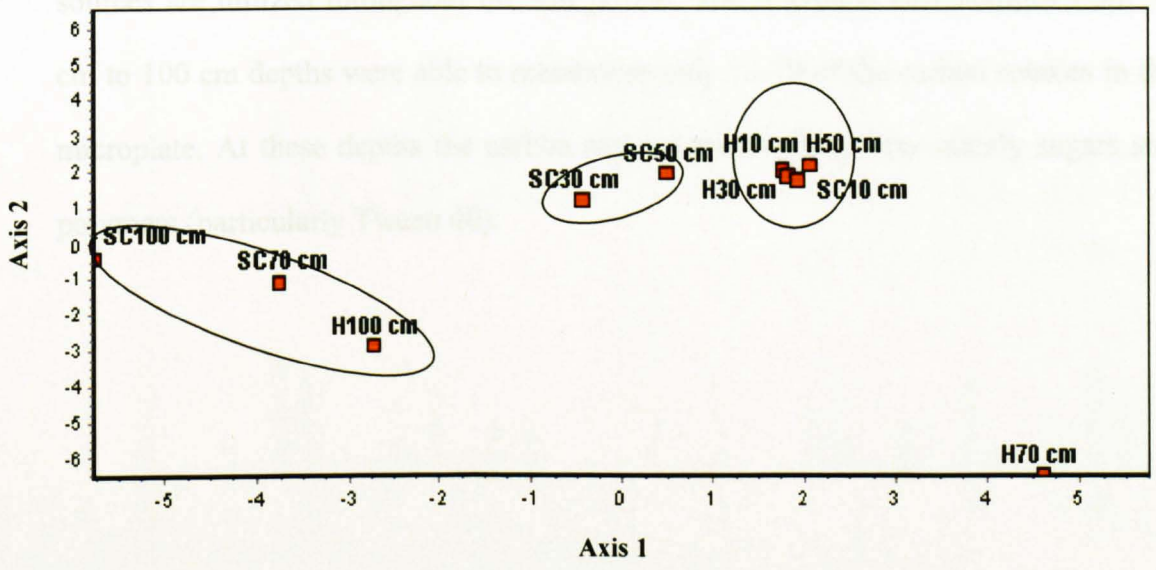


Figure 7.22: Principal component analysis of soil microbial community substrate utilization for Hatfield Moor (H) and Sutton Common (SC), June 2005. Soil samples were obtained from different depth intervals, after 5 days of incubation at 10 °C.

7.5.3 October 2005

At Hatfield Moor in October of 2005, in general, a decrease in the number of carbon sources metabolised occurs with increasing depth, with the exception to this trend being observed at H70 cm (Figure 7.23). Throughout the soil profile the greater number of substrates metabolised were aminoacids (L-arginine, L-asparagine and L-

serine), followed by sugars (D-manitol and N- acetylglucosamine), and then the carboxylic acid, D-galacturonic, and the amine, phenylethylamine. At 100 cm depth the microbial community in the microplate was able to metabolise fewer carbon sources (23); for example, it was not able to metabolise sugars such as Methyl-D-glucosidase, carboxylic acids such as ketobutiric acid, or the polymer cyclodextrin.

At Sutton Common, in October of 2005 the number of carbon sources that the bacterial communities were able to use decreased with increasing depth (Figure 7.24). In addition, when compared with Hatfield Moor, a more limited range of carbon sources are utilized throughout the soil profile. The microbial communities from 50 cm to 100 cm depths were able to metabolise only 13-19 of the carbon sources in the microplate. At these depths the carbon sources metabolised were mainly sugars and polymers (particularly Tween 40).

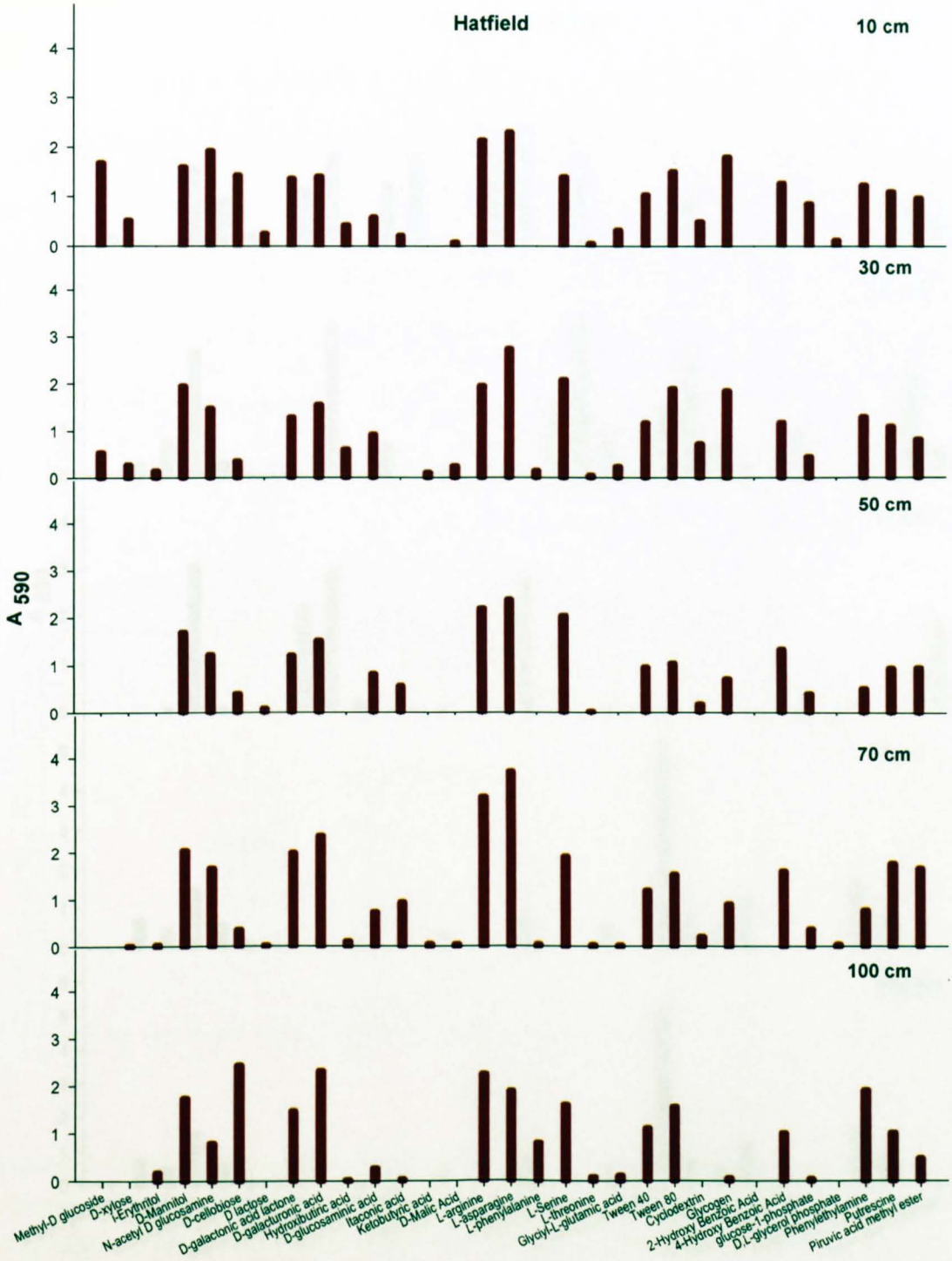


Figure 7.23: Carbon source utilization by the soil microbial community from Hatfield Moor, October 2005, after 5 days of incubation at 10 °C. Data were normalized as described in Section 4.3.4

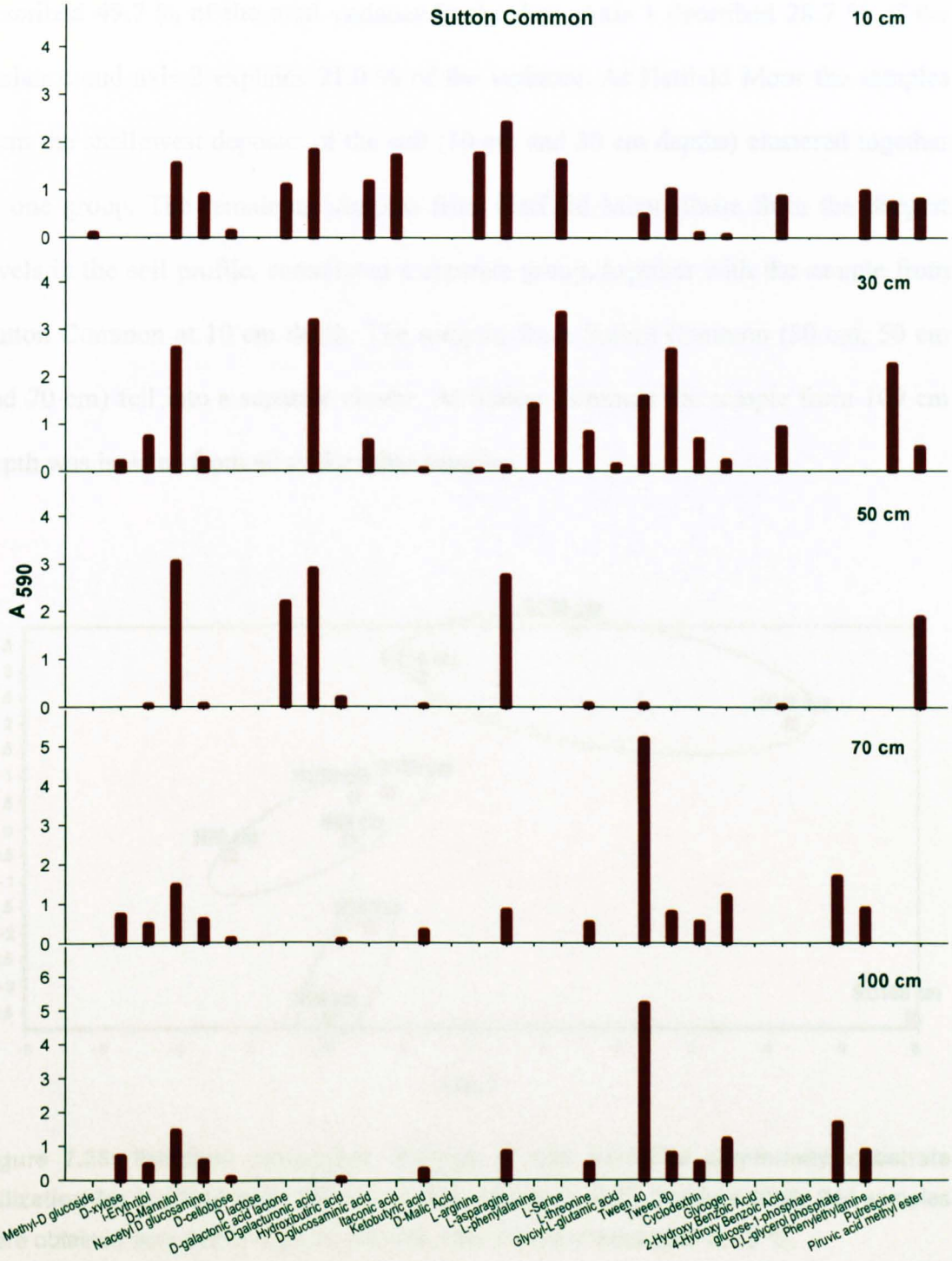


Figure 7.24: Carbon source utilization by the soil microbial community from Sutton Common, October 2005, after 5 days of incubation at 10 °C. Data were normalized as described in Section 4.3.4. Note that a different horizontal scale is used for 70 cm and 100 cm.

The two axes of the principal component analysis in October 2005 (Figure 7.25) described 49.7 % of the total variance in the data. Axis 1 described 28.7 % of the variance and axis 2 explains 21.0 % of the variance. At Hatfield Moor the samples from the shallowest deposits of the soil (10 cm and 30 cm depths) clustered together in one group. The remaining samples from Hatfield Moor, those from the deepest levels in the soil profile, constituted a separate group, together with the sample from Sutton Common at 10 cm depth. The samples from Sutton Common (30 cm, 50 cm and 70 cm) fell into a separate cluster. At Sutton Common the sample from 100 cm depth was isolated from all of the other samples.

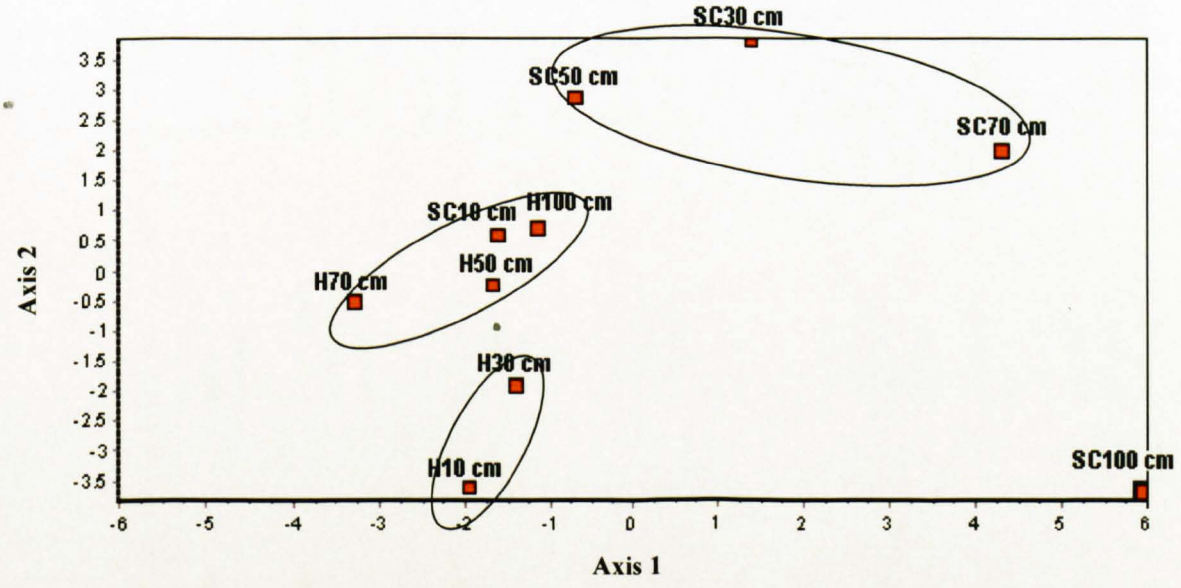


Figure 7.25: Principal component analysis of soil microbial community substrate utilization for Hatfield Moor (H) and Sutton Common (SC), October 2005. Soil samples were obtained from different depth intervals, after 5 days of incubation at 10 °C.

7.5.4 April 2006

At Hatfield Moor in April of 2006 the number of carbon sources that the bacterial communities were able to utilize remains relatively constant with depth. The communities throughout the soil profile were able to use 29-30 of the 31 carbon

substrates provided. The carbon sources used through the soil profile were primarily the sugars D-manitol and N-acetyl D-glucosamine, the aminoacids L-arginine, L-asparagine and L-serine and the amine phenylthylamine.

In contrast, the microbial communities from Sutton Common were unable to utilize all the available carbon sources at all depths. Very low numbers of carbon sources were used at 70 cm depth (2 carbon sources), with 12 used at 30 cm depth and 17 carbon sources used at 100 cm depth. The microbial community at 70 cm depth was only able to metabolise the polymer Tween 80 and the carboxylic acid D-galacturonic acid.

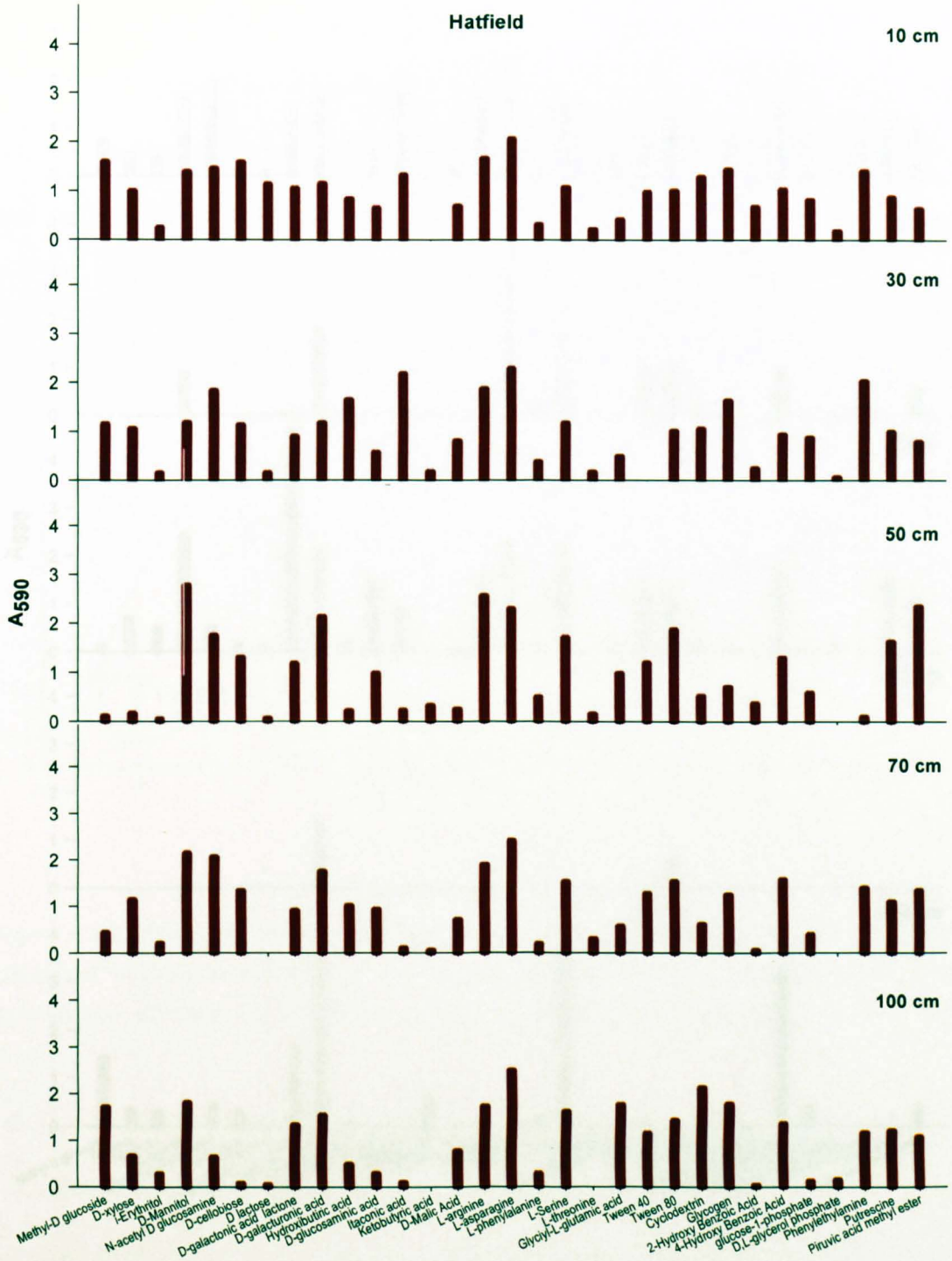


Figure 7.26: Carbon source utilization by the soil microbial community from Hatfield Moor, April 2006, after 5 days of incubation at 10 °C. Data were normalized as described in Section 4.3.4.

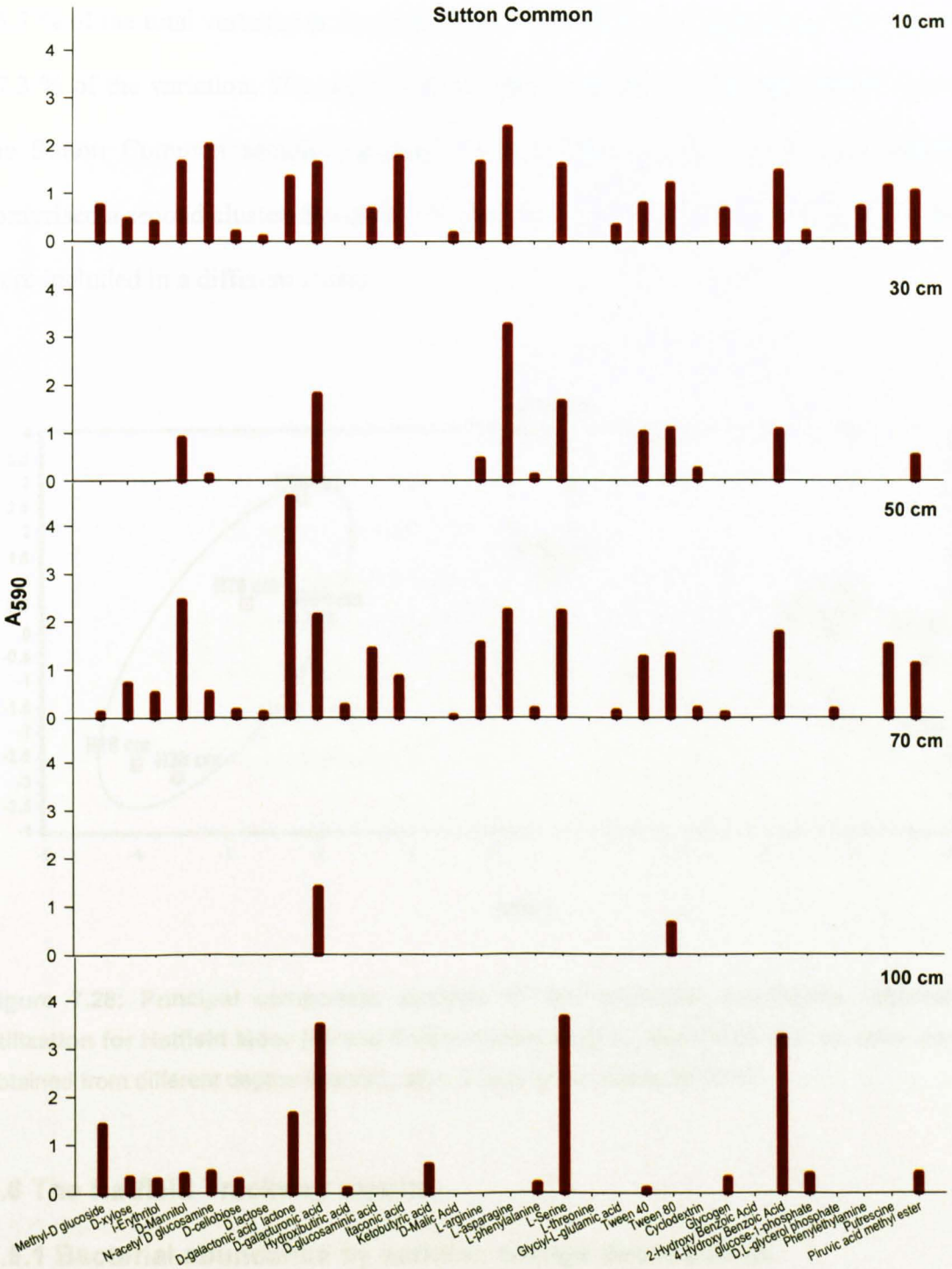


Figure 7.27: Carbon source utilization by the soil microbial community from Sutton Common, April 2006, after 5 days of incubation at 10 °C. Data were normalized as described in Section 4.3.4

The principal component analysis of community substrate utilization for April 2006 divided the samples into three main clusters (Figure 7.28). The two axes described

55.7 % of the total variance in the data. Axis 1 described 38.4 % and axis 2 explained 17.3 % of the variation. The samples from Hatfield Moor fall into one cluster, while the Sutton Common samples obtained from 30 cm, 70 cm and 100 cm depths comprised a second cluster. Samples from Sutton Common at 10 cm and 50 cm depths were included in a different cluster.

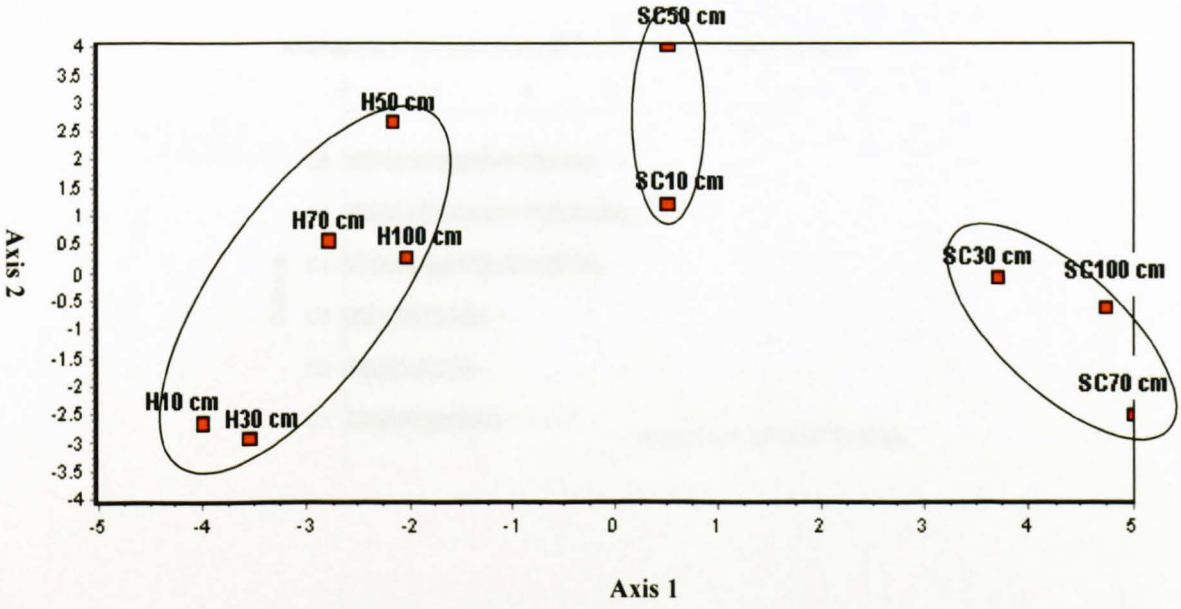


Figure 7.28: Principal component analysis of soil microbial community substrate utilization for Hatfield Moor (H) and Sutton Common (SC), April 2006. Soil samples were obtained from different depths intervals, after 5 days of incubation at 10 °C.

7.6 The Hatfield Trackway results

7.6.1 Bacterial abundance by acridine orange direct counts

Bacterial abundance in two soil cores obtained from sampling locations adjacent to the trackway was greatest at the surface (10 cm depth) (Figure 7.29). In contrast, bacterial abundance was lower in the wood samples obtained from Cuttings 1-3 than in samples from Cuttings 4-6 (Figure 7.29).

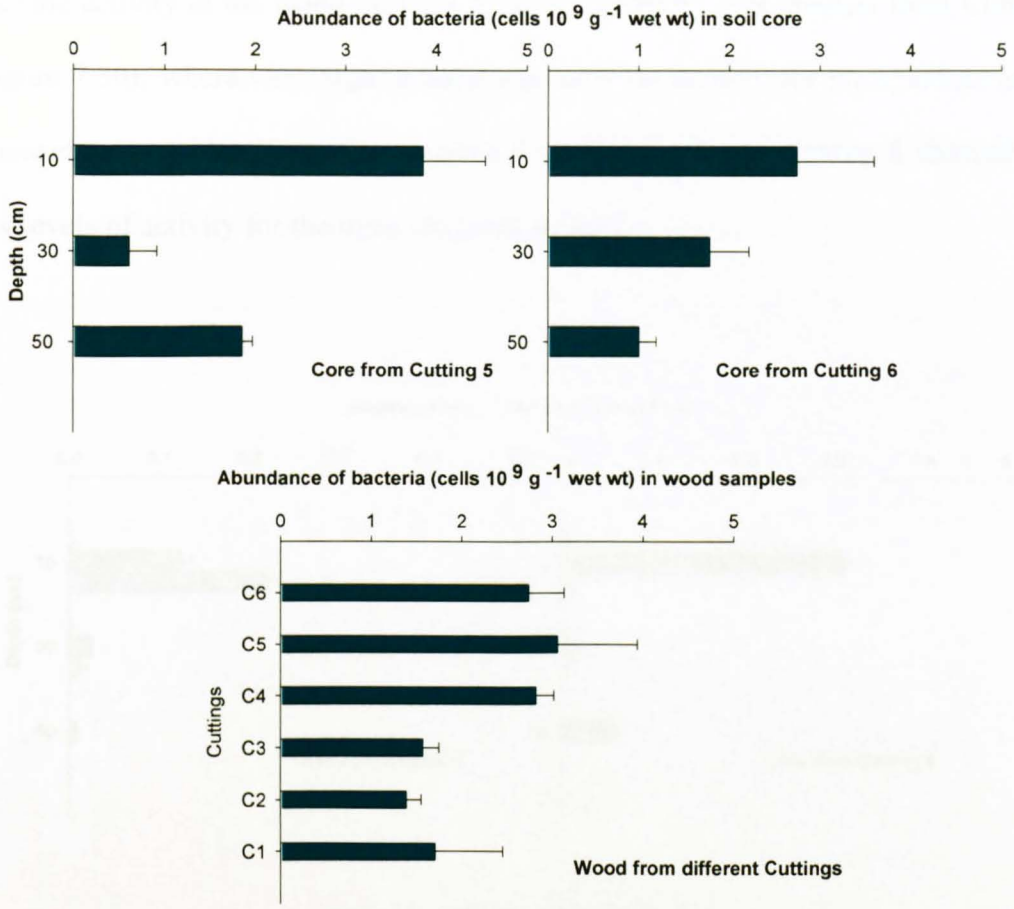


Figure 7.29: Abundance of directly counted bacteria in soil cores from adjacent to the Hatfield Trackway (Cuttings 5 and 6), and in wood samples from the trackway, November 2005. The nomenclature of sample location (Cuttings 1-6) follows Chapman and Gearey (2006).

7.6.2 Extracellular enzyme activity

In general, the three extracellular enzyme activities obtained from both soil profiles (Cuttings 5 and 6) decreased with depth. Little extracellular enzyme activity was detected below 10 cm depth in the cores from adjacent to the trackway (Figure 7.30). β-glucosidase activity was very low throughout the soil profiles. Phosphatase generally had the highest activity through the soil profile, except at 10 cm depth in the core from Cutting 5, where leucine aminopeptidase activity was the highest.

Enzyme activity in the wood samples from the trackway was greatest from Cutting 6 (Figure 7.30), where very high extracellular enzyme activity for phosphatase and β-glucosidase was identified. The samples from Cutting 1 and Cutting 5 showed very low levels of activity for the three enzymes studied.

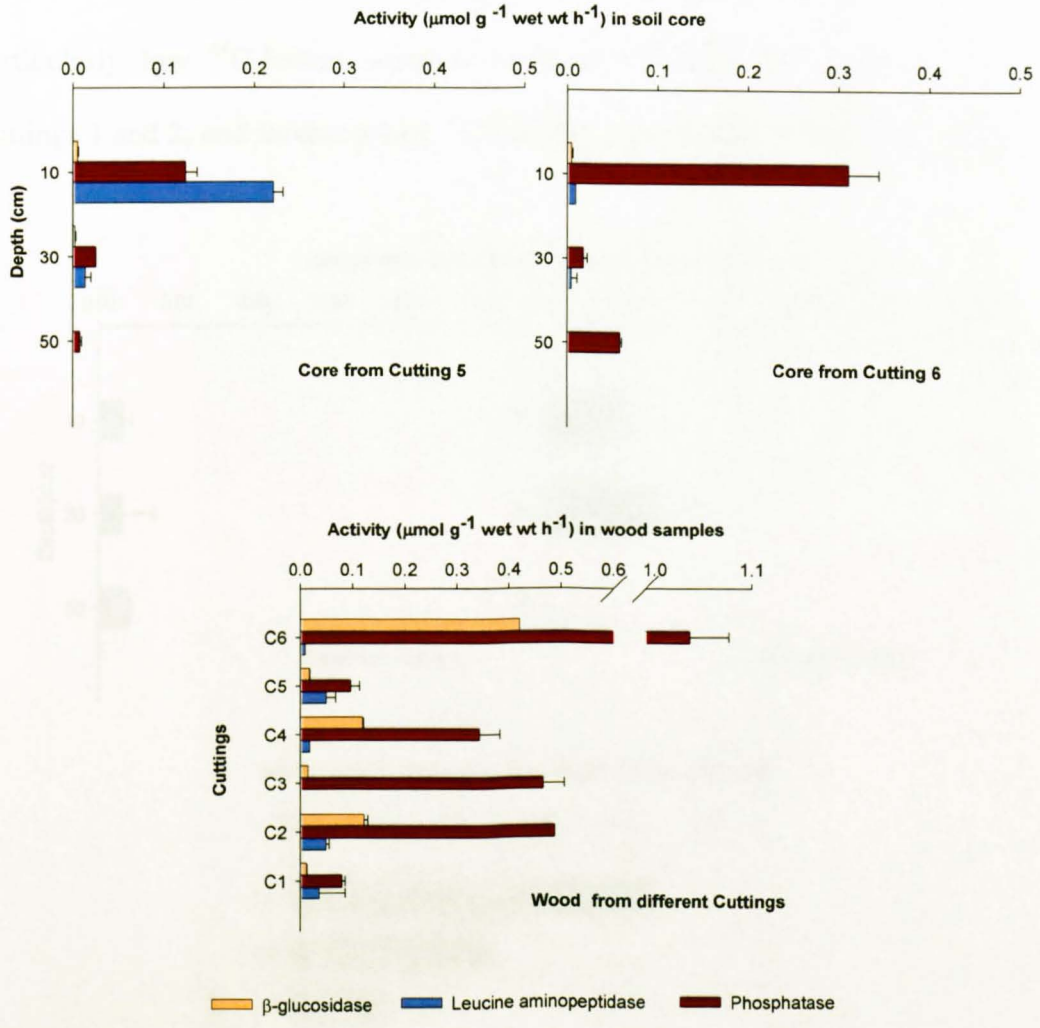


Figure 7.30: Extracellular enzyme activities in soil cores from adjacent to the Hatfield Trackway and in wood samples from the trackway, November 2005. The nomenclature of the sample location (Cuttings 1-6) follows Chapman and Gearey (2006). Note that a different horizontal scale is used for activity in wood samples.

7.6.3 ^{14}C-leucine assimilation

^{14}C-leucine uptake was low at all depths in the core adjacent to Cutting 5 (Figure 7.31). ^{14}C-leucine uptake in the core adjacent to Cutting 6 was greatest at 30 cm depth and not measurable at 50 cm depth, while leucine uptakes in the wood samples from different cuttings along the trackway's length were greatest in Cutting 6. These levels accord well with the value obtained for the soil sample at the same cutting. Particularly low ^{14}C-leucine uptakes were observed in the wood samples from Cuttings 1 and 2; and an absence of ^{14}C-leucine assimilation occurred at Cutting 3.

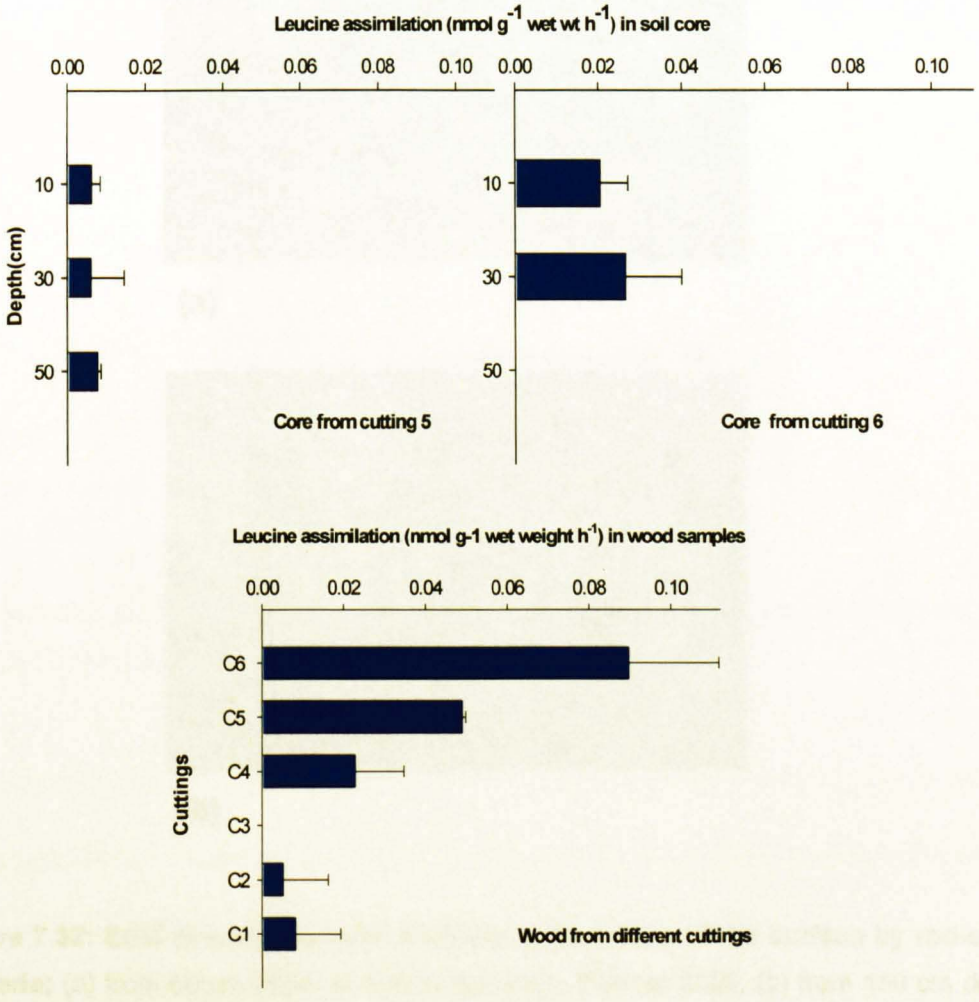
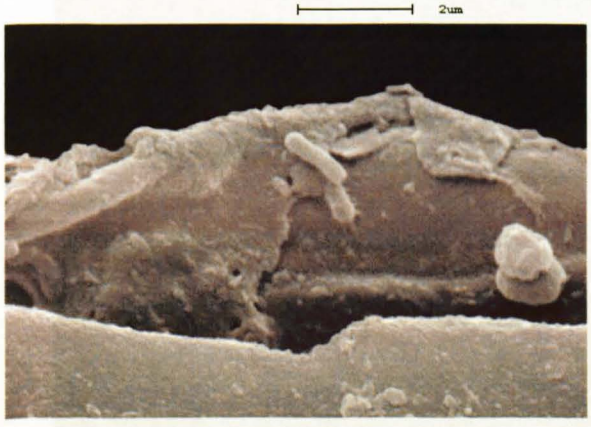


Figure 7.31: ^{14}C-leucine assimilation in soils cores from adjacent to the Hatfield Trackway and in wood samples from the trackway, November 2005. The nomenclature of the sample location (Cuttings 1-6) follows Chapman and Gearey (2006).

7.7 Scanning electron microscopy analysis

The figures below (Figures 7.32 and 7.33), show micrographs of wood samples observed by scanning electron microscopy (SEM). The samples are of wood remains from Hatfield Moor and Sutton Common found at different depths during the coring programme; and from a wood sample recovered from the Neolithic trackway excavated at Hatfield Moor during November 2005.



(a)

(b)

Figure 7.32: SEM of wood remains showing colonization of the surface by rod-shaped bacteria; (a) from 60 cm depth at Sutton Common, October 2005, (b) from 150 cm depth at Hatfield Moor, October 2005.

All of the samples were characterized by the presence of 2-3 rod-shaped bacteria on the wood surface. It is suggested that these are tunnelling or erosion bacteria, but this would need further analysis for confirmation.

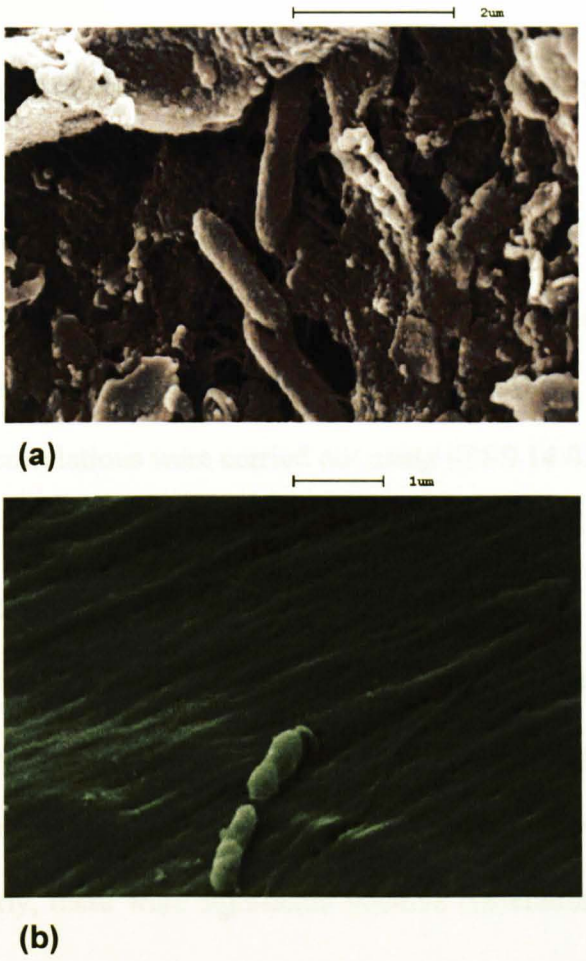


Figure 7.33: SEM micrographs of wood from Hatfield Moor. (a) from 150 cm depth, October 2005, (b) from the Neolithic trackway, November 2005.

Figures 7.32 and 7.33 also highlight fragmentary structures within the wood samples. Although these structures are difficult to determine, they do indicate that the physical composition of the majority of the wood samples (i.e. the primary and secondary cell walls, and the cell lumen) were heavily degraded. This can be seen by the holes in the cell walls, which were associated with bacterial attack. An amorphous residue was also evident in samples, and it is notable that this was probably the by-product of

bacterial degradation. It was not possible to identify the extent of degradation on the wood sample obtained from the Hatfield Trackway, but the presence of bacteria might suggest that decay processes are implicated.

7.8 Relationships between microbial variables and physico-chemical variables

To identify the relationship between microbial variables and 5 of the physico-chemical variables, the results obtained have been correlated using the non-parametric Spearman's correlation coefficient. Results from both sampling sites were analysed independently. The correlations were carried out using SPSS 14.0.

7.8.1 Hatfield Moor

The results from Hatfield Moor are shown in Table 7.2. Depth was negatively correlated with all of the microbial variables. There was a strong positive correlation between bacterial abundance, the three extracellular enzyme activities and ^{14}C-leucine assimilation. Similarly, there were significant positive correlations between the three enzyme activities.

The physico-chemical variables that most strongly correlated with the microbial variables were pH and moisture content. pH was positively correlated with all of the microbial variables, while moisture content was negatively correlated with all of them. Soil organic matter content was negatively correlated with ^{14}C-leucine assimilation, β-glucosidase and aminopeptidase activities. Soil temperature only showed a significant correlation with bacterial abundance and phosphatase (Table 7.2).

Table 7.2: Values of Spearman's correlation coefficient of physico-chemical and microbial variables at Hatfield Moor, 2004-2006.

	Depth	Bacterial abundance	Leucine assimilation	Phosphatase	β-Glucosidase	Aminopeptidase	Organic matter	Moisture content	pH
Bacterial abundance	-0.58**								
Leucine assimilation	-0.76**	0.52**							
Phosphatase	-0.56**	0.39**	0.54**						
β-Glucosidase	-0.72**	0.26**	0.69**	0.57**					
Aminopeptidase	-0.70**	0.31**	0.73**	0.55**	0.71**				
Organic matter	0.33**	NS	-0.25*	NS	-0.41**	-0.28*			
Moisture content	0.85**	-0.40**	-0.71**	-0.55**	-0.80**	-0.65**	0.50**		
pH	-0.70**	0.43**	0.52**	0.42**	0.46**	0.48**	NS	-0.52**	
Temperature	NS	0.36**	NS	0.24*	NS	NS	0.30*	NS	0.29*

n=65; **= $p < 0.01$,*= $p < 0.05$, NS= $p > 0.05$; a two tailed test was used.

7.8.2 Sutton Common

The results from Sutton Common are shown in Table 7.3. All of the microbial variables have shown a negative correlation with depth. Bacterial abundance was positively correlated only with ^{14}C-leucine assimilation and aminopeptidase activity. The extracellular enzyme activities were positively correlated between each other and with ^{14}C-leucine assimilation.

Together with sampling depth, pH was the only physico-chemical variable correlated with all of the other microbial variables, showing a positive correlation. Organic matter was positively correlated with the three extracellular enzyme activities. However, moisture content did not show a significant correlation with any of the microbial variables analysed. Soil temperature was positively correlated with bacterial abundance and ^{14}C-leucine assimilation.

Table 7.3: Values of Spearman's correlation coefficient of physico-chemical and microbial variables at Sutton Common, 2004-2006.

	Depth	Bacterial abundance	Leucine assimilation	Phosphatase	β-Glucosidase	Aminopeptidase	Organic matter	Moisture content	pH
Bacterial abundance	-0.43**								
Leucine assimilation	-0.77**	0.26*							
Phosphatase	-0.55**	NS	0.55**						
β-Glucosidase	-0.58**	NS	0.59**	0.75**					
Aminopeptidase	-0.65**	0.26*	0.62**	0.70**	0.64**				
Organic matter	NS	NS	NS	0.364**	0.27*	0.25*			
Moisture content	NS	NS	NS	NS	NS	NS	0.73**		
pH	-0.67**	0.28*	0.28*	0.25*	0.42**	0.41**	-0.36**	-0.49**	
Temperature	NS	0.35**	0.35**	NS	NS	NS	NS	NS	NS

n=65; **= $p < 0.01$,*= $p < 0.05$, NS= $p > 0.05$; a two tailed test was used.

7.9 Comparison of microbial variables between Hatfield Moor and Sutton Common using the Mann-Whitney non parametric test

Data from both sampling sites, obtained throughout the monitoring period, and through the soil profile, were compared using the non-parametric Mann-Whitney (U) test. The results are shown in Table 7.4. Significance differences between sites were found for phosphatase, aminopeptidase and ^{14}C-leucine assimilation. Phosphatase and ^{14}C-leucine assimilation was higher at Hatfield Moor, however, in contrast aminopeptidase activity were higher at Sutton Common. Bacterial abundance and β-glucosidase did not vary significantly between the two sites.

Table 7.4: Mann-Whitney (U) non-parametric test between samples obtained from Hatfield Moor and Sutton Common using 5 microbial variables.

	Bacterial abundance	Phosphatase	β-Glucosidase	Aminopeptid-ase	Leucine assimilation
Hatfield Moor					
Mean rank	68.24	79.22	68.48	46.51	73.38
Sum of ranks	4435.50	5149.00	4451.50	3023.00	4770.00
n	65	65	65	65	65
Sutton Common					
Mean rank	62.76	51.78	62.52	84.49	57.62
Sum of ranks	4079.50	3366.00	4063.50	5492.00	3745.00
n	65	65	65	65	65
Mann-Whitney U	4079	1221	4063	878	3745
p (2 tailed)	NS	0.00**	NS	0.00**	0.017*

p values are from the 2-tailed Mann-Whitney U test, ** = $p < 0.01$, * = $p < 0.05$, NS = $p > 0.05$.

The non-parametric Mann-Whitney (U) test was applied to the data from both sites in order to investigate the differences in microbial variables at each depth as analysed through the soil profile. The results are shown in Table 7.5. As can be seen, there was no significant difference ($p > 0.05$) between bacterial abundance at both sampling sites or at each of the analysed depths. However, significant differences between sites were shown by phosphatase at most depths (the exception being 70 cm depth). β-

glucosidase and leucine aminopeptidase were significantly different between the two sites at 30 cm depth. ^{14}C-leucine assimilation shows significant differences between sites at 10 cm and 30 cm depths.

Table 7.5: Mann-Whitney (U) non-parametric test between Hatfield Moor and Sutton Common, using bacterial abundance and activity at different depths.

	Bacterial abundance	Phosphatase	β-Glucosidase	Aminopepti-dase	Leucine assimilation
10 cm					
Mean rank (H/SC)	14.35/12.65	17.23/9.77	13.62/13.38	15.65/11.35	18.35/8.65
Mann-Whitney U	73.50	36.00	83.00	56.5	21.5
p	NS	**0.013***	NS	NS	**0.001****
30 cm					
Mean rank (H/SC)	16.15/10.85	16.58/10.42	17.23/9.77	17.96/9.04	17.27/9.73
Mann-Whitney U	50.00	44.50	36.00	26.5	35.50
p	NS	**0.040***	**0.013***	**0.003****	**0.012***
50 cm					
Mean rank (H/SC)	13.85/13.15	17.15/9.85	13.88/13.12	13.81/13.19	13/13
Mann-Whitney U	80.00	37.00	79.50	80.50	62.50
p	NS	**0.015***	NS	NS	NS
70 cm					
Mean rank (H/SC)	12.92/14.08	16.08/10.92	11.96/15.04	14.08/12.92	15.00/12.00
Mann-Whitney U	77	51	64.50	77.00	65.00
p	NS	NS	NS	NS	NS
100 cm					
Mean rank (H/SC)	13.92/13.08	17.77/9.23	14.62/12.38	16.42/10.58	15.81/11.19
Mann-Whitney U	79.00	29.00	70.00	46.50	54.50
p	NS	**0.004****	NS	NS	NS

n=13, i.e. the number of data collected from each depth, p values are from the 2-tailed Mann-Whitney U test, ** = $p < 0.01$, * = $p < 0.05$, NS = $p > 0.05$.

7.10 Discussion

The bacterial abundance at both sampling sites and throughout the monitoring period (April 2004-April 2006), had a significant tendency to decrease with increasing depth (Tables 7.2 and 7.3), albeit with some variation in evidence (Figure 7.1). This pattern could be explained by the fact that the uppermost deposits of the soil exhibit greater levels of oxygen content, and nutrients, when compared to the deepest sediments, thereby allowing for an increase in microbial abundance.

Cheetham (2004) obtained similar results for the surface sediments at Sutton Common from different monitoring points across the area of the scheduled monument. Previous studies have also shown greater microbial abundance in the shallower horizons of a diverse range of soil types (Dodds *et al.*, 1996; Fierer *et al.*, 2003; Kieft *et al.*, 1998; Taylor *et al.*, 2002).

The presence of bacteria in the deeper strata, at low concentrations, at both sampling sites might be explained by the development of an anaerobic community at these depths. Ekuland *et al.* (2001) found that in forest soils, bacteria tended to increase in abundance in the presence of peat and black bog as a consequence of the presence of partially anaerobic conditions.

Throughout the monitoring period (April 2004-April 2006), it was possible to detect a significant degree of seasonal variation in the bacterial counts ($p = 0.001$, Kruskal-Wallis test), particularly in the first 10 cm of the soil profile (Figure 7.1). At all depths, a greater abundance of bacteria cells was generally detected during the spring and summer, and lower counts were recorded in the autumn and winter. Similar fluctuations in microbial biomass were obtained by Bardgett *et al.* (1999), in English grasslands.

Hatfield Moor presented higher numbers of bacteria than Sutton Common, particularly in the top 50 cm of the soil profile, and also during the spring and summer months (Figure 7.1). However, no statistically significant difference between sites at this depth (50 cm) was found (Table 7.5). This result may highlight a difference in the effects of water table management between the two sampling locations (i.e. the water

table at Hatfield Moor was higher than the water table at Sutton Common, particularly during the warmer months of the year), or variation in the composition of the soil between the sites, at this depth. In particular, the peaks of abundance at 50 cm and 70 cm depths at Sutton Common and 50 cm depth at Hatfield Moor (Figure 7.1) might be associated with fluctuations in the percentage of organic matter with depth (Cheetham, 2004; Nsabimana *et al.*, 2004; Diepening *et al.*, 2006). Nevertheless, no statistically significant correlation was found between soil organic matter content and bacterial abundance at Sutton Common (Table 7.3). In the deeper soil horizons, the numbers of bacteria cells tended to be equally low at both sampling sites.

The three extracellular enzymes studied; β-glucosidase, phosphatase and leucine aminopeptidase, showed a significant trend for decreasing activity with depth throughout the monitoring period at Hatfield Moor and Sutton Common (Tables 7.2 and 7.3); although there was again variation between enzyme activity at the different depths studied. These results are generally in agreement with other studies in which enzyme activity decreased as soil depth increased (Federle *et al.*, 1986; Eivazi and Tabatai 1990; Aon and Colaneri, 2001; Griffiths *et al.*, 2003; Niemi *et al.*, 2005). Comparable results were also obtained by Cheetham (2004) when studying the same extracellular enzymes assayed in this research, from Sutton Common.

The results obtained at both sampling sites demonstrated that very low bacterial activity occurs in the deepest layers of the soil profile, when compared to the upper horizons. However, as has been suggested in previous chapters, despite low levels of microbial activity, the degradation of archaeological wood still occurs in environments where oxygen supply is limited (Jordan, 2001).

Freeman *et al.* (1995) have used fluorogenic substrates for measuring enzyme activity in peatlands. These researchers found that the highest levels of activity occur above 10 cm depth in the soil profile. This observation reinforces the results from the current research, in that the uppermost sampling point in the soil profiles studied was the same level at which the highest bacterial counts and activity occurred. Another factor, apart from oxygen availability, that could explain the high activity found in this uppermost layer of the soil was the more neutral pH in evidence when compared with the deepest soil horizons (Section 6.9). More neutral pH levels increase exudation from plants and favour soil fertility and bacterial growth at this depth (Grayston, 2004). In the current research, at both sampling sites, pH was positively correlated with all of the microbial variables analysed (Tables 7.2 and 7.3). An additional reason that might explain high bacteria abundance and activity, especially in the first 10 cm of the soil profile, is the tendency of organic nutrients to accumulate and fluctuate in the root zone (Grayston *et al.*, 1998; Lahav *et al.*, 2001).

Kang *et al.* (1998) applied enzymatic analysis to the study of the decomposition process in wetlands. They found lower decomposition rates (i.e. less enzyme activity) under constant flood conditions; relating the decrease in enzyme activity to two seperate processes. The first process identified was a decrease in the synthesis of enzymes due to changes in the microbial population, and the second process was the inhibition of enzyme activity due to the accumulation of metals or phenolic compounds under reduced conditions (a factor that has been previously suggested by other researchers) (e.g. Pulford and Tabatai, 1988; Freeman *et al.*, 1996).

The inhibitory effect of phenolic compounds on enzyme activity has been well documented (e.g. Biek 1963). Freeman *et al.* (1995, 2001, and 2004) studied the regulatory role of the enzyme phenol oxidase upon decomposition in peatlands through β-glucosidase, phosphatase and sulphatase activity, measured using methylumbelliferyl substrates. They found that phenol oxidase activity was inhibited by the lack of oxygen, thereby allowing for the accumulation of phenolic compounds that inhibited other enzyme activities. As a consequence of this process, hydrolytic enzymes (β–glucosidase, phosphatase, sulphatase, etc.), with no oxygen requirement, are limited in peatlands.

Following on from the observations above, it is apparent that a decrease in water table levels might increase oxygen levels and subsequently cause a decrease in the concentration of phenolic compounds, as well as the removal of inhibitory metal ions. This would consequently increase hydrolytic activity (Freeman *et al.*, 1996; Kang and Freeman, 1999; Freeman *et al.*, 2001; Freeman, 2004). At Hatfield Moor, soil moisture content was negatively correlated with all of the microbial variables that were analysed statistically, while at Sutton Common no significant correlations were found between moisture content and any other microbial variable (Tables 7.2 and 7.3).

The accumulation of phenolic compounds might explain the decrease in enzyme activity found at Hatfield Moor and Sutton Common, below 50 cm depth, as both soil profiles had significant peat contents in their deeper horizons (Section 6.2 above). However, the significant decline in the water table recorded at Sutton Common during August 2005 and December 2005 (Figure 6.7) was not associated with a general elevation in enzyme activity for these months in the results of the present study. This

was probably associated with a lack of general stimulation of microbial metabolism due to the inherently low microbial abundance (Figure 7.6).

Only phosphatase activity increased at 70 cm and 100 cm depths in August and December of 2005 at Sutton Common, when compared to the other months studied (Figure 7.6). An explanation for this result might be that the soil water availability increases the diffusion of soluble substrates and extracellular enzymes, thereby favouring microbial metabolism (Griffin, 1981). This may be the cause of the greater enzyme activity recorded at Hatfield Moor, when compared to Sutton Common (Section 7.3, above). Niemi *et al.* (2005) also observed that with warm and wet conditions enzyme activities such as β-glucosidase, increased in grassland soils.

Several authors (Pulford and Tabatai, 1988; Freeman *et al.*, 1996; Kang and Freeman, 1999) have observed that phosphatase activity increases following a fall in the water table. Similar results were obtained from the current study for this enzyme (Section 7.3). Previous authors have attributed this increased activity to a reduction in the concentration of inhibitors, such as reduced iron and phenolic compounds, together with an increase in soil temperature. However, by contrast, Pulford and Tabatabai (1988) found that increases in alkaline phosphatase and phosphodiesterase activity which occurred in certain soils were probably due to the activation of these enzymes by the metal ions being released, or an increase in the concentration of the enzymes due to microbial adaptation to the reduced conditions.

Another important result obtained from the extracellular enzyme data in the present research was the presence of a degree of seasonal variation, particularly in the upper

sediments of the soil. In the surface soil extracellular activity was often low during the autumn and winter, possibly due to lower temperatures which might lead to lower potential for enzyme activity. This suggestion is supported by the results obtained in this study, i.e. from the statistical analysis, where temperature showed a strong positive correlation with bacterial abundance and phosphatase activity at Hatfield Moor (Table 7.2), and with bacterial abundance and ^{14}C-leucine assimilation at Sutton Common (Table 7.3). In accordance with these results, Blume *et al.* (2002) found a strong relationship between temperature and microbial activity, detecting that enzyme activities increased with summer temperatures, especially at the soil surface. This is comparable to the results obtained by other researchers, who have found that in a range of different soil types, higher microbial biomass and activity is observed in the spring and summer months (Rastin *et al.*, 1988; Buchanan and King, 1992; Kaiser and Heinemeyer, 1993, Bardgett *et al.*, 1997; Hogan *et al.*, 2001; Niemi *et al.*, 2005).

At Hatfield Moor, β-glucosidase was the least active of the enzymes studied throughout the monitoring period (Figures 7.2-7.4), particularly below 10 cm depth. This may be because β-glucosidase activity was mainly originating from release by proliferating microorganisms, rather than being an accumulated enzyme fraction in clays or organic matter (Turner *et al.*, 2002). A positive, strong correlation was found between bacterial abundance and β-glucosidase at Hatfield Moor (Table 7.2), but no significant correlation was obtained at Sutton Common (Table 7.3). In addition, Cheetham (2004) also found that β-glucosidase was the least active of the enzymes that he assayed at Sutton Common, thereby suggesting that this enzyme was produced only by active cells that were metabolising in the presence of a suitable organic substrate. It has also been suggested that this enzyme is the least susceptible to form

complexes with clay and organic matter, and it is therefore less likely to be retained within the soil environment in the long term (Turner *et al.*, 2002).

At Sutton Common β-glucosidase was generally the most active enzyme in the upper 10 cm of the soil profile (Figures 7.4-7.6), but it subsequently decreased with depth. McLatchey and Reddy (1999), found that β-glucosidase activity decreases when soil conditions become more reduced, and that this was associated with a decrease in microbial biomass and mineralization rates. It is known that β-glucosidase is involved in the final step of cellulose degradation (Acosta-Martinez *et al.*, 2003) and is induced by the presence of suitable substrates (products of cellulose breakdown) including cellulose, glucose and their metabolites (Turner *et al.*, 2002). Therefore, the high levels of β-glucosidase activity at Sutton Common, in the top 10 cm of the soil profile, could be related to an increase in organic remains of vegetal origin, such as plant residues, or to differences in livestock density. The livestock at Sutton Common might increase soil nutrient availability and hence influence the soil microbial community (Grayston *et al.*, 2001; Kohler *et al.*, 2005). Similar results were also obtained by Cheetham (2004) at Sutton Common, where it was suggested that the impact of cattle grazing resulted in an increase in extracellular enzyme activity.

Extracellular enzyme activity appears to be greater in soils that have high clay contents because the enzyme becomes embedded within soil clay-particles. In addition, extracellular enzymes can be adsorbed onto soil colloidal organic matter (Burns, 1982; Haynes and Tregurtha 1999; Ostle, 2002). These complexes can retain a proportion of the original activity, and therefore represent extracellular activity that can be long-lived, but unrelated to the actual microbial biomass within the soil (Burns, 1983). It

has been calculated that free extracellular enzymes can maintain their activity and stability for up to one year in the soil (Kiss *et al.*, 1975). Overall, the Hatfield Moor soil profile exhibited a greater percentage of clay than Sutton Common, at all depths (Table 6.3). Consequently, active enzymes associated with clay particles might make an important contribution to total enzyme activity in the soil from Hatfield Moor.

Several authors have found a strong positive relationship between the organic matter content of soils and enzyme activities (e.g. Elvazi and Tabatai, 1990; Degens *et al.*, 2000; Niemi *et al.*, 2005). At Hatfield Moor a negative correlation was found between soil organic matter content and β-glucosidase and aminopeptidase (Table 7.2). However, at Sutton Common the three extracellular enzymes were positively correlated with the organic matter (Table 7.3). Since the current study was based on peaty soils, the percentage of organic matter was greater at specific depths; for example at Hatfield Moor organic content increased with depth, reaching a maximum at 100 cm depth, while at Sutton Common the organic content was higher at 50 cm and 70 cm depths in the soil profile. However, as has previously been suggested by Cheetham (2004), it is possible that the presence of bacteria, and their metabolic activity, at Sutton Common is not dependent on the amount of organic matter in the soil.

At Hatfield Moor, phosphatase displayed the highest enzyme activity on most sampling days (Figures 7.2-7.4). At Sutton Common, below 10 cm depth, phosphatase was the most active enzyme throughout the monitoring period (Figures 7.5-7.7). The phosphatases are inducible enzymes and the extent of their excretion by microorganisms is determined by their requirement for orthophosphate (Acosta-

Martinez and Tabatabai, 2000). It is probable that the high activity of this enzyme in the soils at both sampling sites indicated a high demand for orthophosphate. In contrast, however, a lower level of biological activity in soil would lead to a reduction in the demand for phosphate, thereby allowing phosphate to accumulate (Freeman *et al.*, 1997; Sinsabaugh *et al.*, 2005).

In general, the synthesis of many enzymes (e.g. phosphatase and aminopeptidase) indicates the availability of inorganic nutrients. For example, with a low availability of N and P the microorganisms in the soil expend more energy producing extracellular enzymes in order to obtain N and P from organic sources. However, the accumulation of excess products of enzyme hydrolysis may cause competitive inhibition of enzyme activity (Sinsabaugh *et al.*, 2005). As a consequence, it is possible to relate the results of the current research to nutrient availability at the sites studied. In wetlands, nutrients can easily be transferred through the movement of water. Therefore mineralization may be less important for supplying nutrients to wetland microorganisms than is usually the case in upland mineral soils.

Leucine aminopeptidase is an enzyme involved in the breakdown of proteins and polypeptides to oligopeptides and aminoacids, which are made available to heterotrophic bacteria (Ainsworth and Goulder, 2000). The decrease in the activity of this enzyme with depth at both sites indicates a drop in the number of heterotrophic bacteria in the deepest sediments. At both sampling sites positive correlations were observed between bacterial abundance and aminopeptidase activity (Tables 7.2 and 7.3). The relative increase in leucine aminopeptidase with depth might also be explained by the fact that aminopeptidase activity is induced by low nitrogen

concentrations, and is not competitively inhibited by carbon content (Chróst, 1991). As a consequence, it is probable that with increasing depth at both sampling sites aminopeptidase activity was induced by low levels of available nitrogen.

^{14}C-leucine assimilation rates decreased down the soil profile at both sampling locations throughout the monitoring period (see Figures 7.8 to 7.13), indicating a decrease in the rate of total bacterial production with depth (Kirchman *et al.*, 1985). The values obtained were higher in the uppermost layers of the soil, decreasing notably below 30 cm depth at Hatfield Moor (Figures 7.8 to 7.10), and below 10 cm depth at Sutton Common (Figures 7.11 to 7.13).

At both Hatfield Moor and Sutton Common seasonal variation was detected in the first 10 cm of the soil profile; there was a significant increase in assimilation within the uppermost layers of the soil (10-30 cm depth) during this period, especially in the spring and summer months. Similar results were obtained at Sutton Common by Cheetham (2004), with values for ^{14}C-leucine assimilation being considerably higher in the surface deposits across all sampling locations.

The results from the ^{14}C-leucine assimilation experiment, where phosphate and nitrate were added into two different soil samples from Sutton Common, showed that at 10 cm depth, an increase in ^{14}C-leucine assimilation was associated with an increase in nutrients (Figure 7.14). The results obtained suggest that P and N were probably limiting nutritional factors at this depth.

Previous researchers have studied the influence of nutrients on microbial biomass and activity; for example Bardget and Leemans (1995) found that soil microbial biomass decreased in an upland grassland soil when fertilizer inputs were reduced; Boyer and Groffman (1996) have suggested that organic N was the principal factor affecting microbial processes in forest and agricultural soils; and Griffiths *et al.* (2001) found that microbial activity was limited by N in arable soil.

In contrast, the results from 100 cm depth showed more ^{14}C-leucine assimilation in the control sample than in the samples with nutrients added (Figure 7.14). This might indicate an inhibitory effect; with the microorganisms being adapted to oligotrophic conditions and as a consequence, the addition of nutrients could be toxic for them (Steffensen and Alexander, 1995).

It has been suggested elsewhere that the abundance of microorganisms in the deeper strata of soils is limited by low nutrient availability (Krumholz, 2000). Research on peat bogs has suggested that the preservation of bog bodies in these environments is perhaps a result of insufficient nutrients rather than low oxygen levels, as was previously considered (Painter, 1995; Cronyn, 2001). Caple (1996) proposed that the activity of anaerobic microorganisms can become inhibited with the depletion of suitable nutrients and through the build up of toxic metabolic products. Despite these previous studies, the results from the current research have indicated that nutrient availability was not a limiting factor for the microbial populations in the deeper layers of the soil profile.

The experiment that compared ^{14}C-leucine assimilation rates under aerobic and anaerobic conditions gave higher rates of assimilation in aerobic conditions (Figure 7.15). In the soil samples obtained from the deeper layers (100 cm depth) the difference in assimilation was very low. This result confirms that the routine measurements made under aerobic conditions were producing meaningful results, as the presence of oxygen was not inhibiting an obligate-anaerobic community in the deeper layers of the soil.

In the present research, microbial metabolic diversity, as indicated by the number of carbon substrates that could be oxidised in the Biolog assays, declined with depth at both sites, particularly at Sutton Common (Figures 7.18, 7.21 7.24 and 7.27). In general, the majority of the 31 substrates were used by microbes in the shallow sediments. In the deeper sediments fewer substrates were utilized. Griffiths *et al.* (2003) studying the influence of depth on bacterial community structure in a grassland upland soil, also observed that the highest levels of substrate utilization occurred in the top 5 cm of the soil, and that a decreasing capacity for substrate utilization occurred down the soil profile. This probably indicates that bacteria; 1) were not able to use the substrates, 2) were present in lower numbers at these depths, 3) the cells failed to maintain viability (Haack *et al.*, 1995).

Previous studies on field soils have shown differences in soil microbial communities using substrate utilization patterns. These studies have allowed the recognition of different soil communities, mainly according to soil type (Buyer and Drinkwater, 1997; Smalla *et al.*, 1998; Widmer *et al.*, 2001; Girvan *et al.*, 2003; Crecchio *et al.*, 2004).

Principal component analysis has revealed differential utilization of carbon sources between Hatfield Moor and Sutton Common, probably reflecting differences in microbial community composition (Figures 7.19, 7.22, 7.25 and 7.28). Also, different utilization of carbon sources between locations suggests varying availability of these carbon sources at the sites (Grayston *et al.*, 2001). The greater diversity of carbon compounds utilized at Hatfield Moor suggests that there were more readily-available carbon sources at this site than at Sutton Common. Another explanation for the higher diversity of response at Hatfield Moor might be that the metabolic diversity in the community at Hatfield Moor was greater than at Sutton Common. It is not known if this greater metabolic diversity was due to few taxonomic groups, or whether it was related to many specialist groups of microorganisms.

Heuer and Smalla (1997) have proposed that only a limited number of species are able to utilize the Biolog substrates due to competition between species for the carbon sources. As a consequence, only a limited number of species contribute to colour development. However, it is unlikely that the microorganisms in a single well are reduced to a single isolate, and that the wide group of substrates in the microplate results in a variety of selection pressures, which might allow non-cultivable species to survive and contribute to colour development (Smalla *et al.*, 1998; Preston *et al.*, 2002).

When analysing the results from the Biolog microplates it is necessary to take into account the fact that patterns of substrate utilization might only indicate functioning of the cultivable fraction of the microbial community that was inoculated into the microplates (Zack *et al.*, 1994; Smalla *et al.*, 1998; Widmer *et al.*, 2001; McCaig *et al.*,

2001; Crecchio *et al.*, 2004). In addition, utilization may be dependent on cell growth and division in the substrate provided (Garland and Mills, 1991; Haack *et al.*, 1995). Several authors suggest that only fast growing bacteria, such as γ-proteobacteria, adapted to high substrate concentrations, become dominant in the Biolog wells (Heuer and Smalla, 1997; Konopka *et al.*, 1998; Smalla *et al.*, 1998). Due to the incubation period necessary to obtain a colour response in the plates, it is inevitable that growth occurs in the plates (Garland and Mills, 1991; Haack *et al.*, 1995).

In order to avoid the problem of the influences imposed by inoculum cell density on the rate of colour development, the method of calculation from Garland and Mills (1991) was employed in the current study (Section 4.7). In addition, the Biolog plates were read after no further colour development occurred (5 days), as proposed by Griffiths *et al.* (2003). As such, it is unlikely that the large differences in substrate utilization patterns between sampling sites were due to differences in inoculum density.

The results of this study have shown that the pattern of substrate utilization persisted through time. Both Hatfield Moor and Sutton Common show similar patterns of response in different months.

The soil microbial communities from both sites utilized a number of carbohydrates (N-acetyl D glucosamine, D-cellobiose and D-manitol), amino acids (L-asparagine, L-arginine and L-serine) and the amine phenylethylamine, to a greater extent than other carbon sources, throughout the soil profiles. This suggests that fast-growing microbes were responsible for the utilization of the more easily available substrates,

carbohydrates, aminoacids and amines and that these played an important role in the community physiological profiles.

The Biolog technique has been shown to be a useful approach for comparing the microbial communities present throughout the soil profile at the sites studied, and has provided useful information relating to the functional ability of the microbial communities. The decrease in extracellular enzyme activity with depth, as demonstrated in this chapter, was related to the lower overall metabolic activity of the bacterial community; an observation which is in accordance with the results shown by the Biolog microplates. In addition, the statistical analyses employed in this study have demonstrated significant differences between sites for some of the microbial variables analysed; such as phosphatase, aminopeptidase activities and ^{14}C-leucine assimilation (Tables 7.4 and 7.5). These results were in accordance with those from the PCA of the Biolog microplates showing a differential utilization of carbon sources between Hatfield Moor and Sutton Common. The results obtained are probably reflecting differences in microbial community composition and metabolism.

It is possible then to conclude that the Biolog assays, when used in conjunction with enzyme activity and ^{14}C-leucine assimilation, yielded useful information regarding the metabolic diversity of the microbial communities in the soils being studied.

The analysis of soil and wood samples from the Neolithic Trackway from Hatfield Moors showed higher bacterial abundance, extracellular enzyme activity and ^{14}C-leucine assimilation in those samples of wood that were considered by Chapman and

Gearey (2006a and b), to be showing the greatest signs of degradation (Figures 7.29 to 7.31).

At the Hatfield trackway, low extracellular enzyme activity was associated with the better preservation of particular wood samples, such as those from Cutting 1 (Figure 7.30). High levels of phosphatase were observed in most of these wood samples; possibly due to the microorganisms producing more extracellular phosphatase. The high levels of β-glucosidase in the samples associated with Cutting 6 can be related to an increase in wood degradation (Figure 7.30).

^{14}C-leucine assimilation was higher in wood from Cuttings 4-6, but very low in wood from Cuttings 1-3 (Figure 7.31). These results parallel those obtained from the bacterial counts and enzyme activity. The conclusion is that the more abundant and active communities of bacteria were associated with the worst preserved wood samples obtained from along the trackway's length (Figure 7.31).

Scanning electron microscopy (SEM) has shown that rod-shaped bacteria, about 1-5 μm in length and 0.5-1.0 μm in breadth, occurred in all of the samples analysed (Figures 7.32-7.33). On the basis of previous SEM studies (Daniel and Nilsson, 1986, 1997: Kim and Singh, 2000), it is likely that these microbes were erosion bacteria, but further analysis would be necessary to confirm this assertion. Erosion bacteria appear to be common in wood exposed to wet and oxygen poor conditions and they are considered to be a cosmopolitan organism (Björdal *et al.*, 1999; Björdal and Nilsson, 2002; Gregory *et al.*, 2002).

Björdal *et al.* (2002) have shown that the process of wood degradation by erosion bacteria is so slow that it may be prolonged for hundreds of years. Erosion bacteria start to degrade wood from the surface, they enter the wood tissue through the rays and pits; and from there spread out into the fibres through the pit openings, and colonise cell lumens (Björdal and Nilsson, 2001). Erosion bacteria, however, are not able to degrade highly lignified material. This favours the survival of the burial wooden cultural heritage (Björdal and Nilsson, 2001). The SEM results have also shown that the wood samples obtained from both study sites have been subjected to extensive bacterial attack, which has significantly degraded the physical structure of the wood.

7.11 Summary

This chapter has presented data from the application of conventional microbial methods that have been undertaken on soil samples from Hatfield Moor and Sutton Common, and from wood samples from the prehistoric trackway excavated at Hatfield Moor.

The results obtained for bacterial abundance, extracellular enzyme activities and ^{14}C-leucine assimilation have been presented and discussed, and general patterns in the data have been identified for both sampling sites. Physiological profiles of the bacterial community at both sampling sites through the soil profile have also been presented in this chapter.

Finally, this chapter has also included a discussion section which is based on the key results of this stage of the analysis; taking into account the results obtained using the

conventional microbial methods, and assessing the significance of the variability in evidence.

The next chapter (Chapter 8) presents the results obtained from the molecular techniques applied to soil samples in order to characterize the bacterial community through the soil profiles at both sampling sites.

Chapter 8 Molecular genomic analysis

8.1 Introduction

In the current study an approach that combines conventional microbial methods and 16S rDNA gene-based molecular analysis of soil-community DNA was employed in order to characterise the microbial communities throughout the soil profiles.

Bacterial compositional variation in the soil profiles at Hatfield Moor and Sutton Common was analysed using the molecular fingerprint technique; denaturing-gradient gel electrophoresis (DGGE). The results obtained from this method are presented in this chapter. In addition, shifts in the community structure of selective bacterial groups of ecological importance in soil (e.g. α-proteobacteria, β-proteobacteria and actinomycetes), were studied by DGGE using amplified 16S rDNA.

Finally, DNA samples from soil collected from Hatfield Moor in December 2005 from depths of 10 cm, 30 cm and 50 cm were cloned and sequenced. The results obtained are also described in this chapter.

8.2 DNA extraction results

Two different DNA extraction methods were employed in this stage of the analysis. Firstly, the bead-beating method for cellular lysis was employed. This yielded DNA with a high content of humic acids (Figure 8.1) from all the soil samples. These acids were co-extracted with the target DNA, and as a consequence, caused PCR inhibition problems. The presence of these humic acids was shown by the persistence in the DNA extractions of brown or red coloured compounds. The cleaning of these DNA

extractions with Wizard Columns (Promega, UK) was essential for all samples after the extraction of DNA by the bead-beating method. Unfortunately, even after the Wizard columns were used, the extracted DNA was not completely free of humic substances. As a consequence, dilution of the extracted DNA after purification was required in order to avoid inhibition of the PCR. However, dilution of the DNA leads to very weak PCR products, and did not always resolve the PCR inhibition problem. This was especially so with the deeper soil samples which contained low quantities of DNA. In general therefore, it was difficult to obtain amplified DNA following the use of the bead-beating method (Figure 8.1).

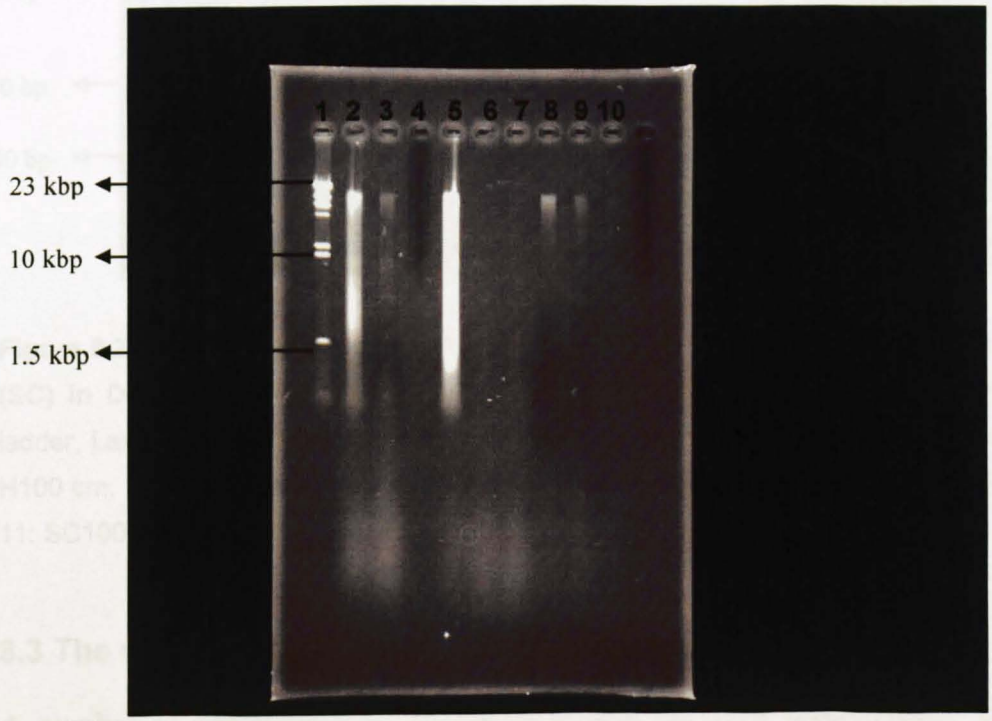


Figure 8.1: DNA extraction of soil samples from Sutton Common in February 2004, extracted by the bead-beating method. Lane 1: 250 ng of Lambda DNA Hind III marker, Lane 2: sample from 20 cm depth, Lane 3: 40 cm, Lane 4: 150 cm, Lane 5: 20 cm, Lane 6: 58 cm, Lane 7: 80 cm, Lane 8: 90 cm, Lane 9: 100 cm, Lane 10: 150 cm.

The second DNA extraction method used was the commercially available MO BIO Kit UltraClean™ Soil DNA Isolation Kit (MO BIO Laboratories, USA). This

procedure included a DNA purification step and yielded good quality DNA (Figure 8.2). The extracted DNA was largely free of humic acids, thereby avoiding problems with PCR inhibition. Other advantages of this method were that it yielded a greater quantity of DNA when compared to the bead-beating method, and that enough DNA was extracted for use in the PCR amplification even from the deepest soil deposits studied.

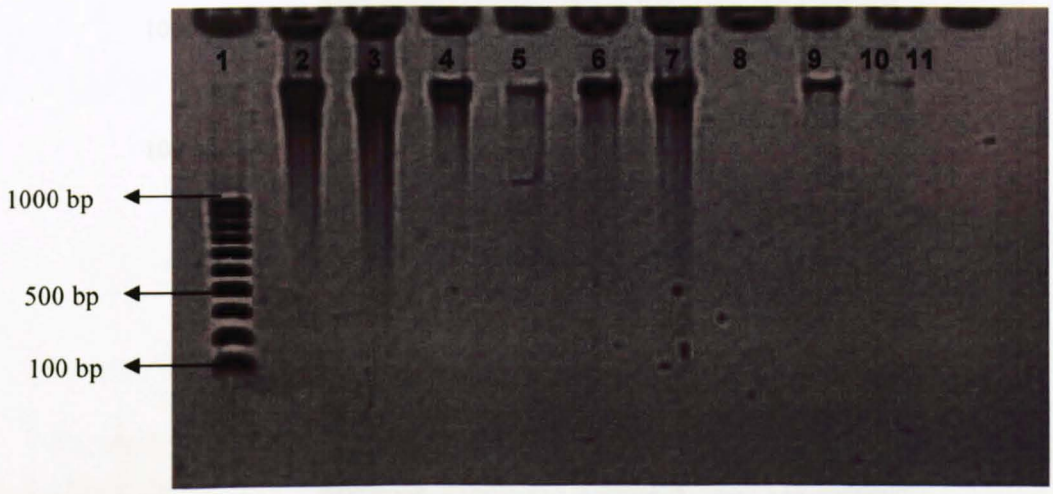


Figure 8.2: DNA extraction of soil samples from Hatfield Moor (H) and Sutton Common (SC) in December 2004, extracted by the MOBIO Isolation Kit. Lane 1: 100 bp DNA ladder, Lane 2: H10 cm depth, Lane 2: H30 cm, Lane 3: H50 cm, Lane 4: H70 cm, Lane 5: H100 cm, Lane 7: SC10 cm, Lane 8: SC30 cm, Lane 9: SC50 cm, Lane 10: SC70 cm, Lane 11: SC100 cm.

8.3 The effects of PCR programmes and conditions

A number of problems were encountered with the PCR amplification undertaken during this study. The first problem, which has been outlined above, was the inhibition of the PCR, which was caused by humic acids co-extracted during the bead-beating DNA extraction method. This problem was solved by using the MO BIO extraction Kit, as explained in Section 8.2.

However, despite the above, the most important problem with the PCR performance was the presence of false-positives in amplifications of negative controls (Figure 8.3). This contamination problem was solved by working in a clean space free of PCR products and using a UV cabinet (as explained in Section 5.4.2).

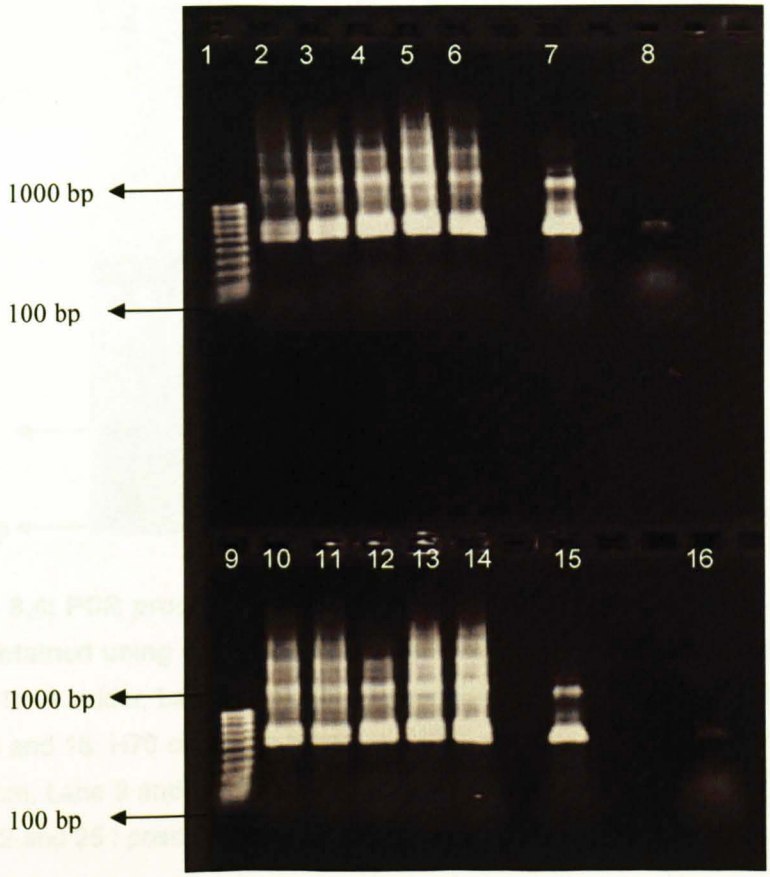


Figure 8.3: PCR amplification of soil samples from Hatfield Moor in April 2004. Lane 1 and 9: 100 bp DNA ladder, Lane 2 and 10: samples from10 cm depth, Lane 3 and 11: 30 cm, Lane 4 and 12: 50 cm, Lane 5 and 13: 70 cm, Lane 6 and 14: 100 cm, Lane 7, 8, 15 and 16 show false-positives.

Another problem that was frequently encountered in the PCR amplification procedure was the appearance of spurious smaller bands in the agarose gel (Figure 8.4). This problem is usually due to a phenomenon called mispriming. Mispriming is the result

of a primer binding to an unintended DNA template, resulting in the amplification of sequences internal or external to the target template (Don *et al.*, 1991).

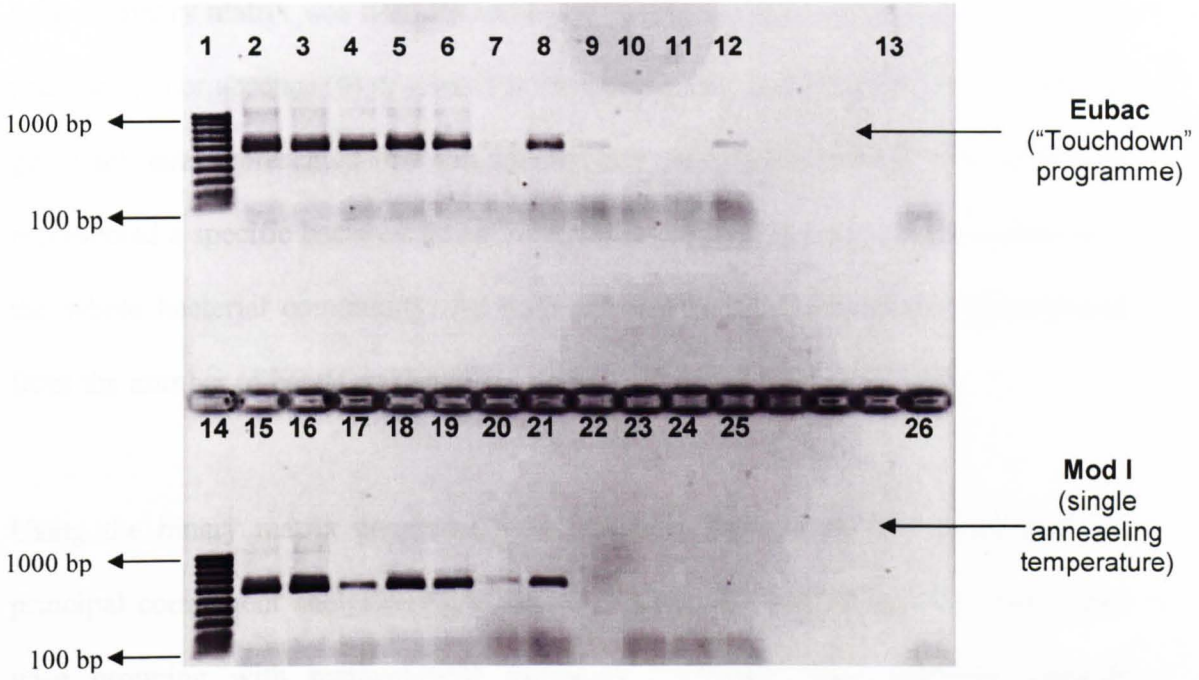


Figure 8.4: PCR products of soil samples from Hatfield Moor (H) and Sutton Common (SC) obtained using primers specific for eubacteria in October 2005. Lane 1 and 14: 100 bp low DNA ladder, Lane 2 and 15: H10 cm, Lane 3 and 16: H30 cm, Lane 4 and 17: H50 cm, Lane 5 and 18: H70 cm, Lane 6 and 19: H100 cm, Lane 7 and 20: SC10 cm, Lane 8 and 21: SC30 cm, Lane 9 and 22: SC50 cm, Lane 10 and 23: SC70 cm, Lane 11 and 24: SC100 cm, Lane 12 and 25 : positive control (*E. coli*), Lane 13 and 26: negative control.

To avoid the mispriming problem, two different PCR programmes were used; both with general bacterial primers. One was based on the 'touchdown' PCR (EUBAC) programme (Section 5.4.2), while the other used only one annealing temperature (Mod 1) (Section 5.4.2). The results presented in Figure 8.4 demonstrate how the use of the simple PCR programme, with only one annealing temperature, yielded better PCR products which were free of 'fuzzy' bands on the gel.

8.4 Analysis of DGGE profiles for eubacteria

To analyse the DGGE gels, the analysis system Gene Genius (Syngene, Cambridge, UK) was used in order to determine the presence or absence of bands on the DGGE gels. A binary matrix was used (Schafer and Muyzer, 2001), which was based on the presence (1) or absence (0) of a band at the same distance along each lane of a DGGE gel. Each lane represented one soil sample, and each band within a lane potentially represented a specific bacterial taxon, which was probably an important component of the whole bacterial community. As such, the taxonomic richness can be estimated from the number of bands on the gel.

Using the binary matrix generated, two statistical approaches were employed; (1) principal component analysis (PCA) and (2) cluster analysis using unweighted pair-wise grouping with mathematical averaging (UPGMA) (and utilizing Jaccards coefficient). The Community Analysis Package Version 2.0 (Pisces Conservation Ltd, Lymington, UK) was used for these analyses.

8.4.1 Analysis of DGGE profiles for Hatfield Moor and Sutton Common during four different months in 2004

The genetic fingerprint of the bacterial community for 2004 (Figures 8.5-8.8), has highlighted the presence of a number of strong dominating bands appearing across all of the samples analysed. Together with these bands, a greater number of fainter but well-resolved bands appeared in the profiles. All of these bands were considered when the clustering method was applied.

Table 8.1 shows the number of bands from each soil sample analysed during April, August, October and December of 2004. In general, a greater number of bands were observed at Hatfield Moor than at Sutton Common. At Hatfield Moor, the number of bands fluctuated with depth, with no clear variation of patterning in evidence (-with the exception of December where the number of bands decreased with increasing depth).

At Sutton Common (with the exception of the April samples), there were fewer bands at 100 cm depth, thereby indicating lowered species richness in the deepest deposits.

Table 8.1: Values are the number of bands on the DGGE gels per sample for the soil eubacteria community in 2004.

	Site							
	Hatfield				Sutton Common			
Soil depth (cm)	April	August	October	December	April	August	October	December
10	9	16	14	16	7	10	10	9
30	11	16	18	15	13	*	*	5
50	12	14	13	10	17	12	7	5
70	15	19	15	9	9	12	10	8
100	9	19	13	9	10	2	6	4
Average	11.2	16.8	14.6	11.8	11.2	9	8.25	6.2

* Indicates no bands on the gel

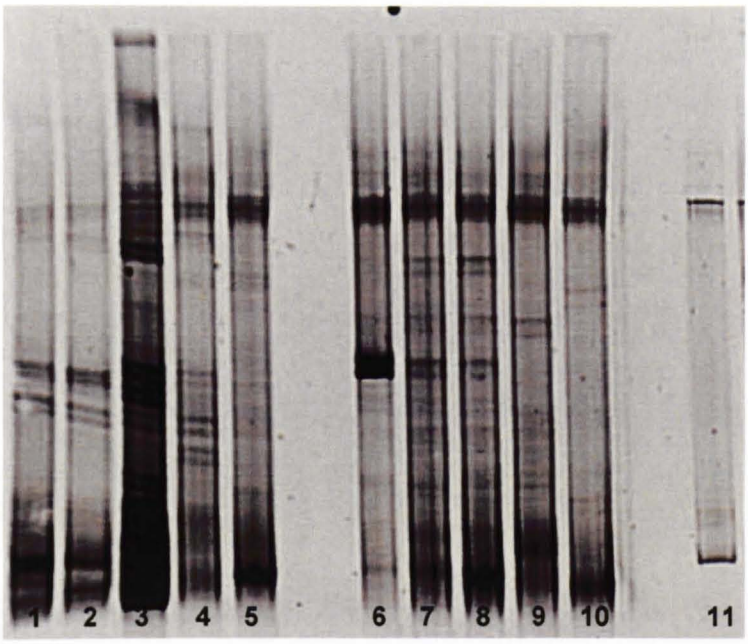


Figure 8.5: DGGE profiles for eubacteria, April 2004 at Hatfield Moor (H) and Sutton Common (SC). Lane 1: SC100 cm, Lane 2: SC70 cm, Lane 3: SC50 cm, Lane 4: SC30 cm, Lane 5: SC10 cm, Lane 6: H100 cm, Lane 7: H70 cm, Lane 8: H50 cm, Lane 9: H30 cm, Lane 10: H10 cm, Lane 11: positive control (*E. coli*).

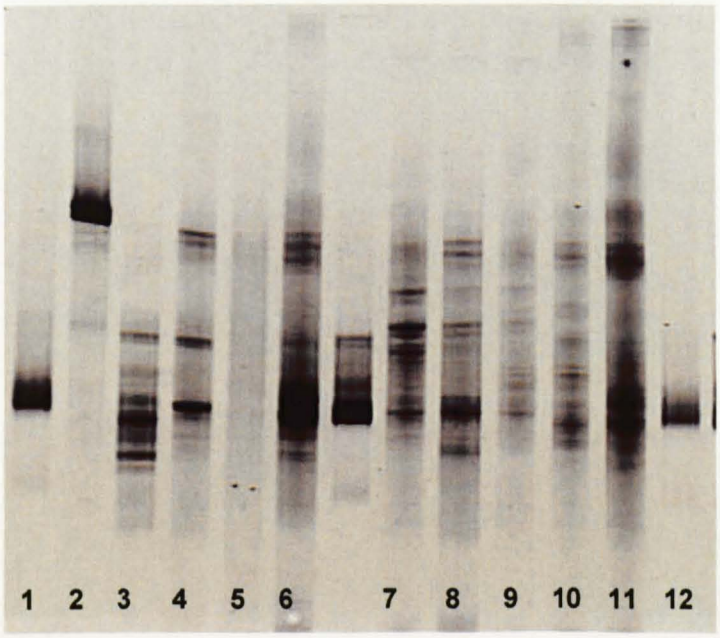


Figure 8.6: DGGE profiles for eubacteria, August 2004 at Hatfield Moor (H) and Sutton Common (SC). Lane 1: positive control (*E. coli*), Lane 2: SC100 cm, Lane 3: SC70 cm, Lane 4: SC50 cm, Lane 5: SC30 cm, Lane 6: SC10 cm, Lane 7: H100 cm, Lane 8: H70 cm, Lane 9: H50 cm, Lane 10: H30 cm, Lane 11: H10 cm, Lane 12: positive control (*E. coli*).

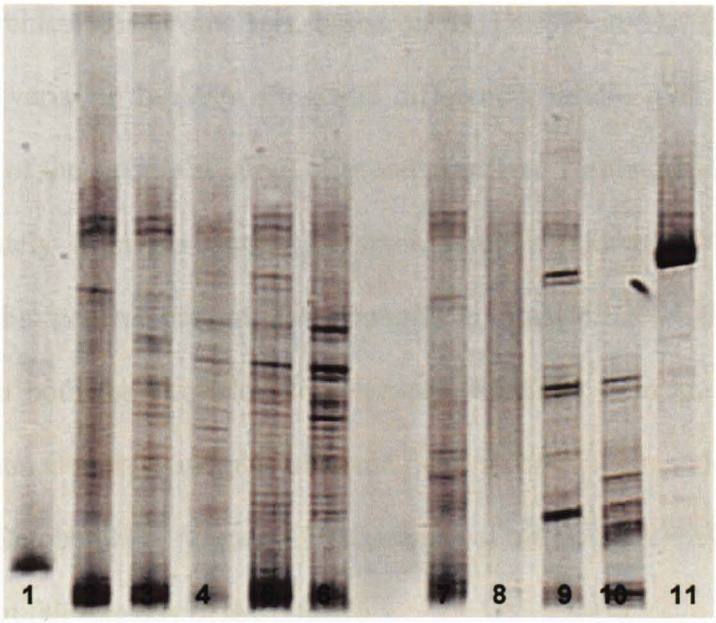


Figure 8.7: DGGE profiles for eubacteria, October 2004 at Hatfield Moor (H) and Sutton Common (SC). Lane 1: positive control (*E. coli)*, Lane 2: H10 cm, Lane 3: H30 cm, Lane 4: H50 cm, Lane 5: H70 cm, Lane 6: H100 cm, Lane 7: SC10 cm, Lane 8: SC30 cm, Lane 9: SC50 cm, Lane 10: SC70 cm, Lane 11: SC 100 cm.

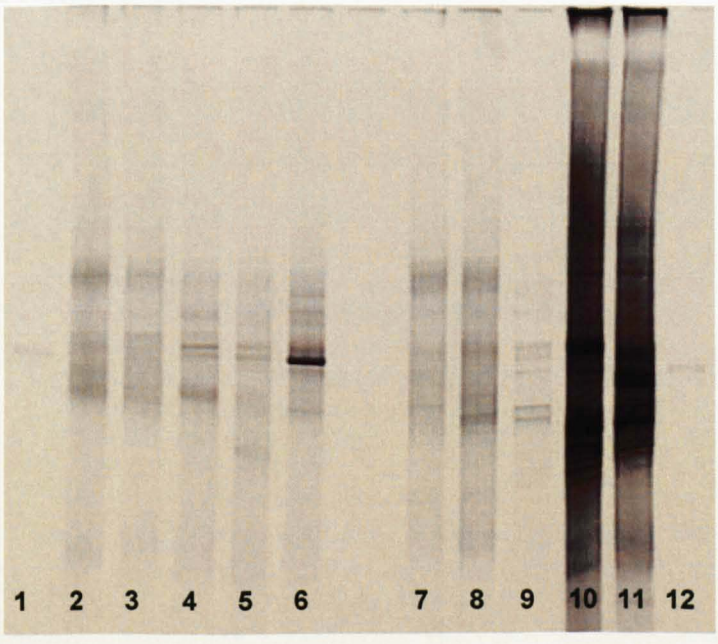


Figure 8.8: DGGE profiles for eubacteria, December 2004 at Hatfield Moor (H) and Sutton Common (SC). Lane 1: positive control (*E. coli*), Lane 2: SC100 cm, Lane 3: SC70 cm, Lane 4: SC50 cm, Lane 5: SC30 cm, Lane 6: SC10 cm, Lane 7: H100 cm, Lane 8: H70 cm, Lane 9: H50 cm, Lane 10: H30 cm, Lane 11: H10 cm, Lane 12: positive control (*E. coli).*

PCA and hierarchical cluster analysis, based on the DGGE profiles (Figures 8.9 and 8.10), detected variation between sites, and differences within each site (dependent upon the depth of the sample studied). The samples from Hatfield Moor tended to be grouped separately from the Sutton Common samples. However, there are some exceptions to this general pattern; for example, in April of 2004 (Figure 8.9) one cluster included both the Hatfield Moor samples obtained from 10 cm and 30 cm depths and Sutton Common samples obtained from 10 cm, 70 cm and 100 cm depths. In addition, in October 2004 (Figure 8.10), samples obtained from Hatfield Moor at 30 cm depth did not fall into any of the clusters.

Samples from Hatfield Moor show certain tendency to group together depending upon depth. For example in April 2004 and December 2004 the deepest samples clustered together and the surface samples fall apart into another cluster.

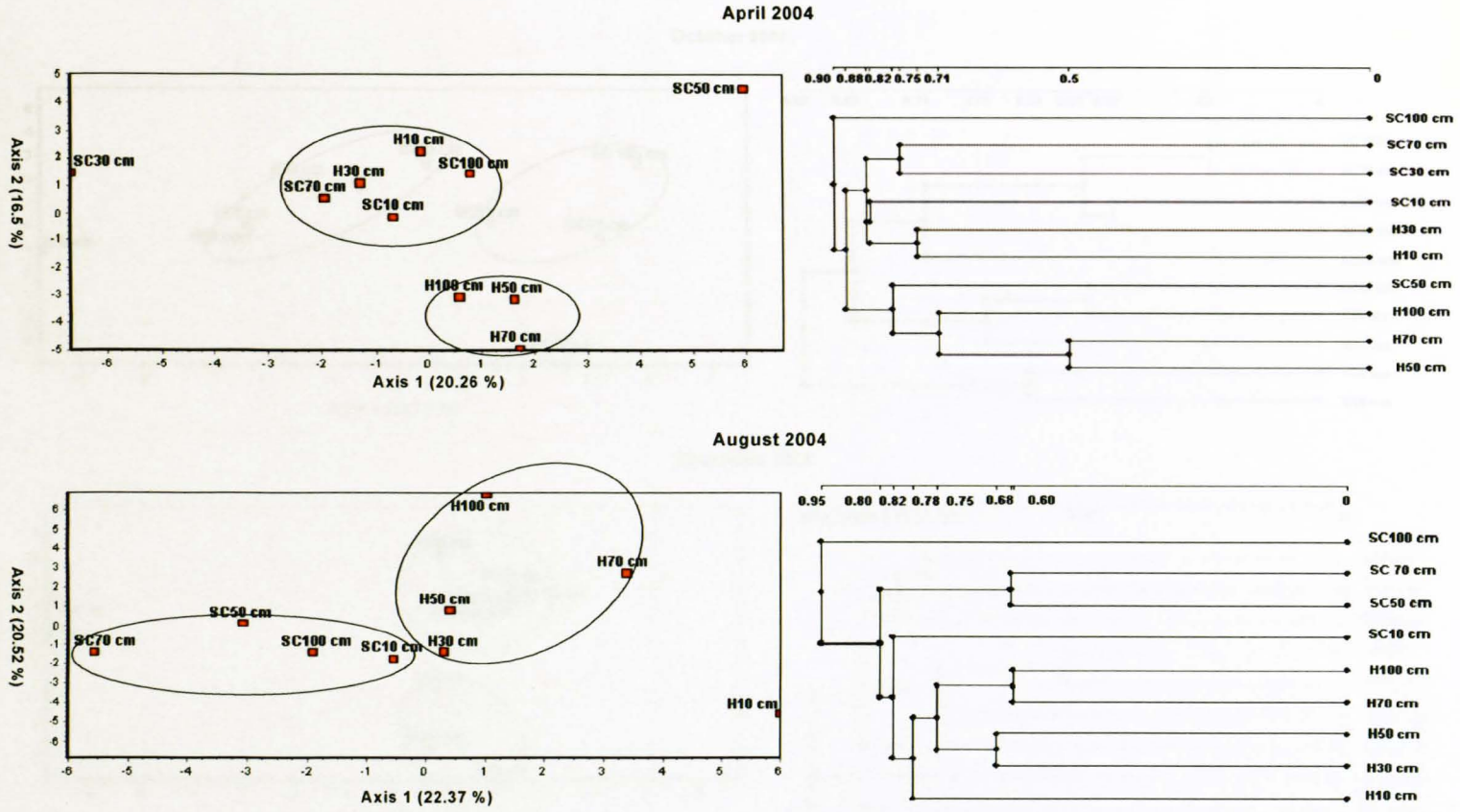


Figure 8.9: Analysis of the DGGE profiles for Hatfield Moor (H) and Sutton Common (SC) in April and August 2004 by PCA and cluster analysis. Percentages next to the axis title of the PCA plot illustrate the percentage of the total variation explained by each axis. The *x*-axis of the dendrogram shows the dissimilarity measure; the clusters become increasingly similar than their predecessors, resulting in a cross-over of branches.

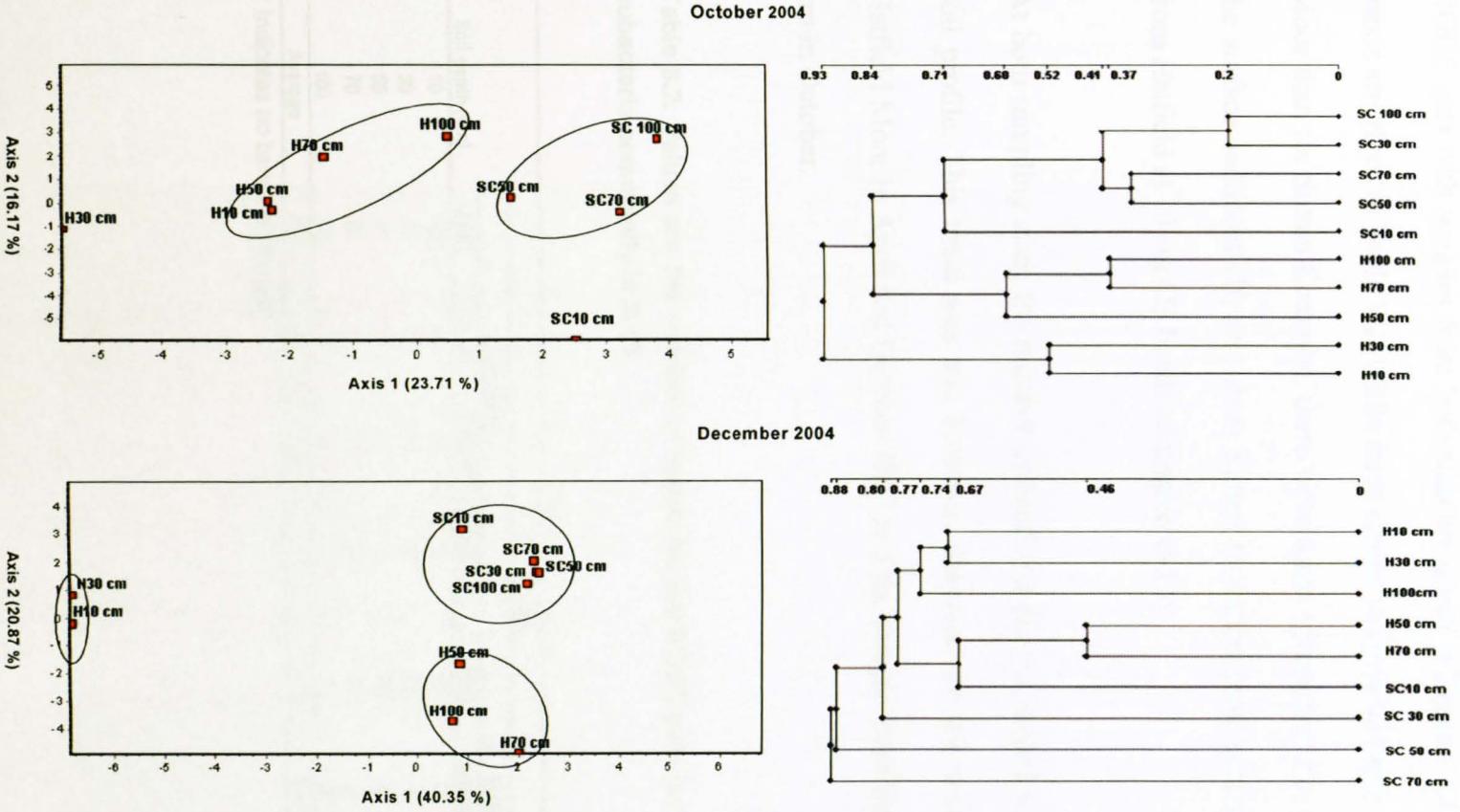


Figure 8.10: Analysis of the DGGE profiles for Hatfield Moor (H) and Sutton Common (SC) in October and December 2004 by PCA and cluster analysis. Percentages next to the axis title of the PCA plot illustrate the percentage of the total variation explained by each axis. The *x*-axis of the dendrogram shows the dissimilarity measure; the clusters become increasingly similar than their predecessors, resulting in a cross-over of branches.

8.4.2 Analysis of DGGE profiles for Hatfield Moor and Sutton Common during four different months in 2005

DGGE gels with samples from both sites are given in Figures 8.11-8.14. Numbers of bands are given in Table 8.2. While there tended to be more bands on gels for Hatfield Moor than for Sutton Common, there were some exceptions: e.g. in April 2005 when the surface sediment (10 cm) from Sutton Common gave notably more bands than from Hatfield at 10 cm (23 bands compared to 13).

At both sampling sites, the number of bands on the gel tended to decrease down the soil profile. This trend was not, however, observed for the samples obtained from Hatfield Moor in April and October 2005 and the sample from Sutton Common at 70 cm in October.

Table 8.2: Values are the number of bands on the DGGE gels per sample for the soil eubacteria community in 2005.

	Site							
	Hatfield				Sutton Common			
Soil depth (cm)	April	August	October	December	April	August	October	December
10	13	19	16	15	23	14	15	15
30	17	17	20	12	12	9	8	*
50	17	16	15	14	10	8	8	11
70	23	7	20	8	9	8	17	5
100	14	11	15	8	*	9	7	5
Average	16.8	14	17.2	11.4	13.5	9.6	11	9

* Indicates no bands on the gel

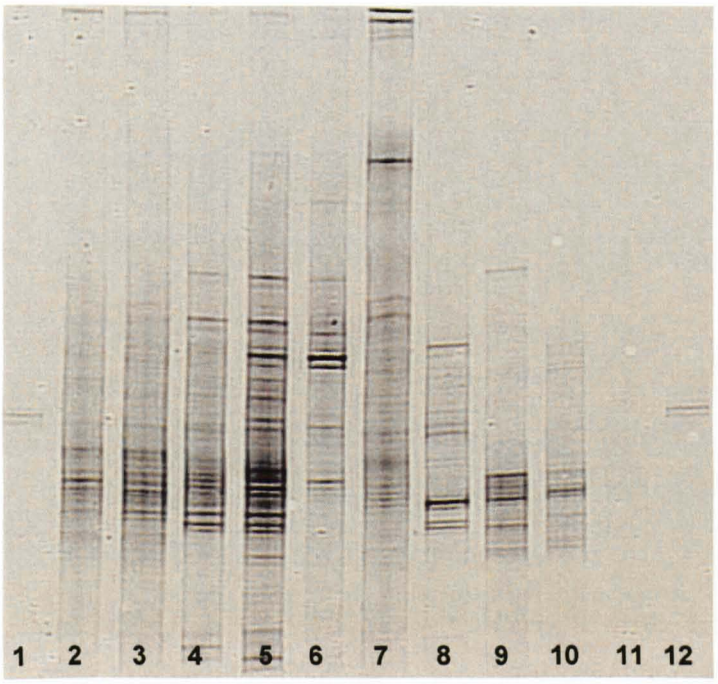


Figure 8.11: DGGE profiles for eubacteria, April 2005 at Hatfield Moor (H) and Sutton Common (SC). Lane 1: positive control (*Bacillus sp.*), Lane 2: H10 cm, Lane 3: H30 cm, Lane 4: H50 cm, Lane 5: H70 cm, Lane 6: H100 cm, Lane 7: SC10 cm, Lane 8: SC30 cm, Lane 9: SC50 cm, Lane 10: SC70 cm, Lane 11: SC100 cm, Lane 12: positive control (*Bacillus sp.).*

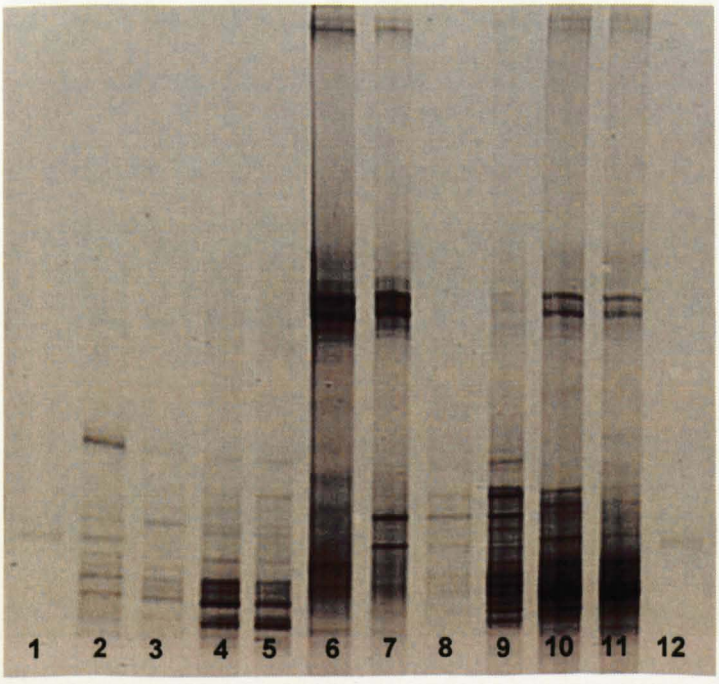


Figure 8.12: DGGE profiles for eubacteria, August 2005 at Hatfield Moor (H) and Sutton Common (SC). Lane 1: positive control (*E. coli)*, Lane 2: SC100 cm, Lane 3: SC70 cm, Lane 4: SC50 cm, Lane 5: SC30 cm, Lane 6: SC10 cm, Lane 7: H100 cm, Lane 8: H70 cm, Lane 9: H50 cm, Lane 10: H30 cm, Lane 11: H10 cm, Lane 12: positive control (*E. coli).*

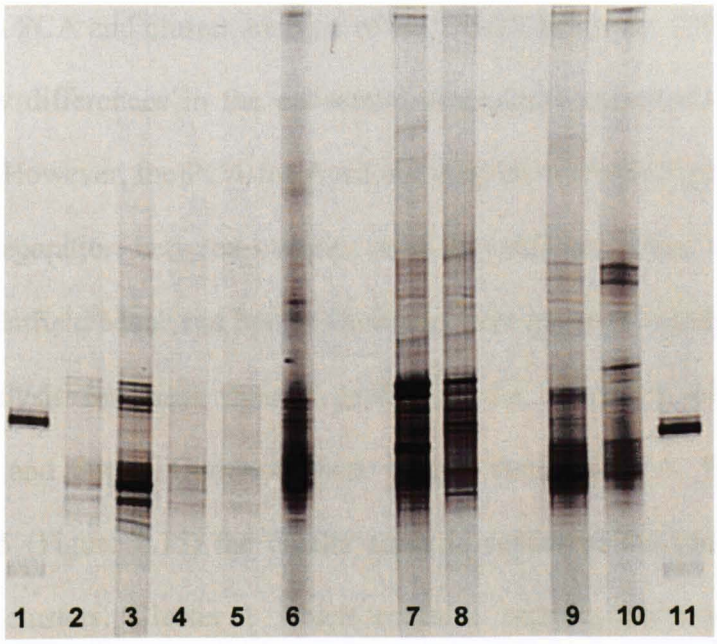


Figure 8.13: DGGE profiles for eubacteria, October 2005 at Hatfield Moor (H) and Sutton Common (SC). Lane 1: positive control (*E. coli*), Lane 2: SC100 cm, Lane 3: SC70 cm, Lane 4: SC50 cm, Lane 5: SC30 cm, Lane 6: SC10 cm, Lane 7: H100 cm, Lane 8: H70 cm, Lane 9: H30 cm, Lane 10: H10 cm, Lane 11: positive control (*E. coli).*

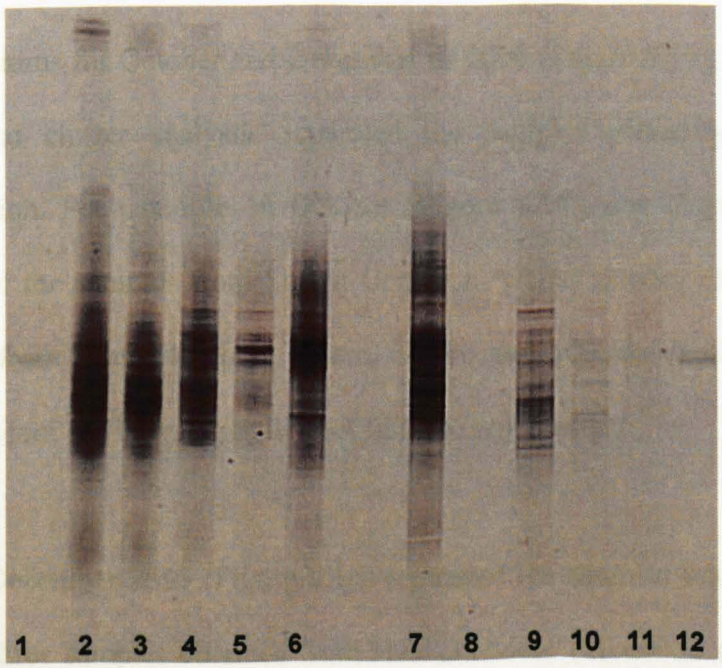


Figure 8.14: DGGE profiles for eubacteria, December 2005 at Hatfield Moor (H) and Sutton Common (SC). Lane 1: positive control *(E. coli)*, Lane 2: H10 cm, Lane 3: H30 cm, Lane 4: H50 cm, Lane 5: H70 cm, Lane 6: H100 cm, Lane 7: SC10 cm, Lane 8: SC30 cm, Lane 9: SC50 cm, Lane 10: SC70 cm, Lane 11: SC100 cm, Lane 12: positive control (*E. coli).*

In general, both PCA and cluster analysis of the DGGE bands for 2005 (Figures 8.15 and 8.16) show differences in the eubacteria community composition between the sampling sites. However, the PCA for April and August of 2005 (Figure 8.15) did not show a clear separation between samples from the different sites, as a mixture of samples from Hatfield Moor and Sutton Common were grouped together. Conversely, the cluster analysis for these months performed the separation of samples from Hatfield Moor and Sutton Common more clearly than the PCA. For example, in August of 2005 (Figure 8.15) the cluster analysis separated the samples into three main discrete clusters. Cluster 1 which contains samples obtained from Sutton Common at 10 cm, 70 cm and 100 cm depths, cluster 2 which contains samples from Hatfield Moor, and cluster 3 which contains samples from Hatfield Moor at 70 cm depth and Sutton Common from 30 cm and 50 cm depths.

The DGGE patterns for October and December of 2005 (Figure 8.16), when analyzed using PCA and cluster analysis, separated the samples primarily according to sampling location. For example, in October (Figure 8.16), one cluster of the PCA contained all of the samples from Sutton Common, whilst another cluster contained three samples from Hatfield Moor; 10 cm, 30 cm and 100 cm depths. The sample from Hatfield Moor at 70 cm depth did not fall into any cluster.

The PCA for December 2005 (Figure 8.16) separated the samples into three clusters. One included three samples obtained from Hatfield Moor (10 cm 30 cm and 50 cm), the second cluster included two samples from Hatfield Moor (70 cm and 100 cm) and Sutton Common 70 cm. The last cluster included two samples from Sutton Common (50 cm and 100 cm).

The analysis of the DGGE pattern for October of 2005 (Figure 8.16) did not separate the samples according to difference in depth. However, in December 2005 (Figure 8.16) the samples from closer depths obtained from Hatfield Moor tended to cluster together.

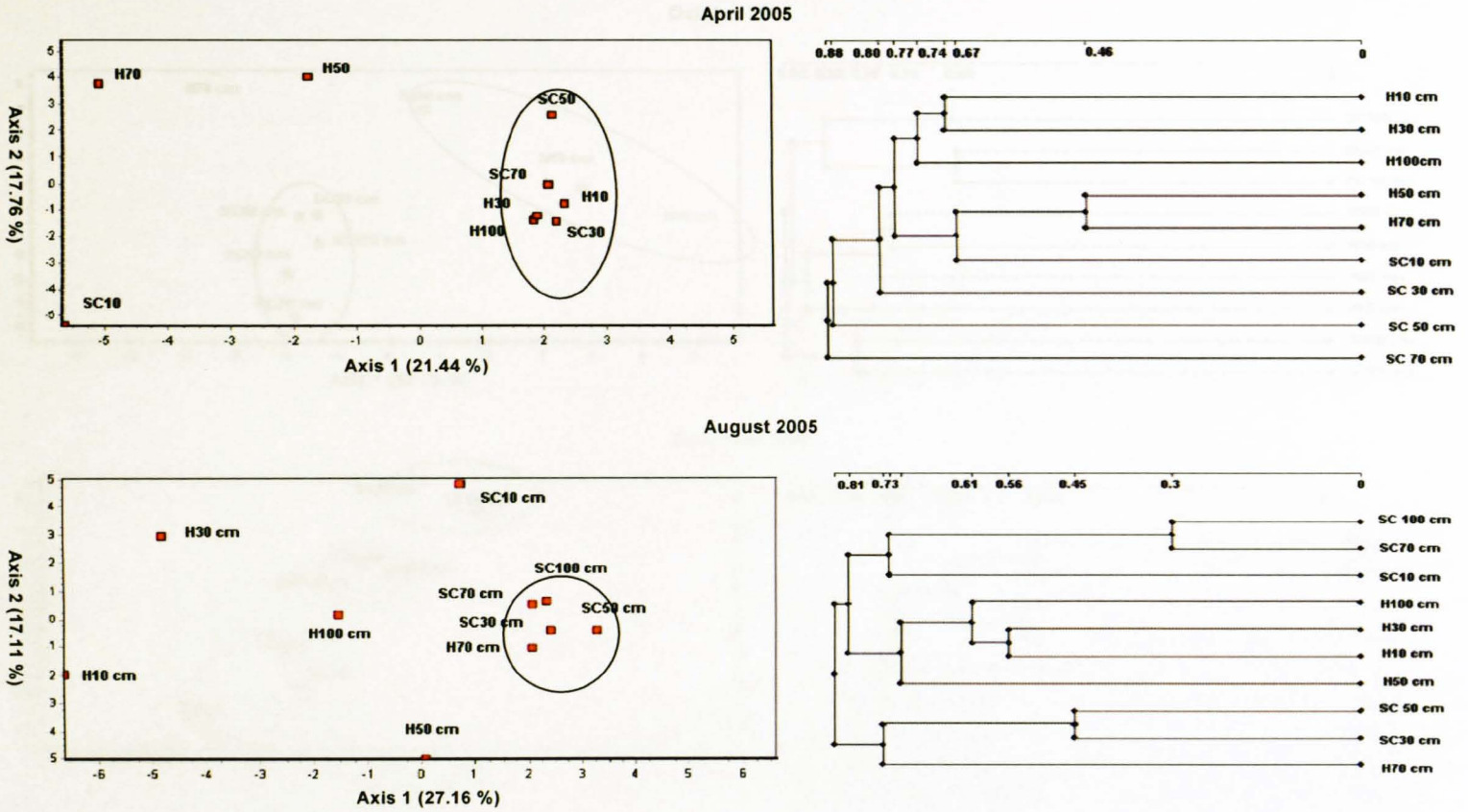


Figure 8.15: Analysis of the DGGE profile from Hatfield Moor and Sutton Common in April and August 2005 by PCA and cluster analysis. Percentages next to the axis title of the PCA plot illustrate the percentage of the total variation explained by each axis. The *x*-axis of the dendrogram explains the dissimilarity measure, the clusters become increasingly similar than their predecessors, resulting in a cross-over of branches.

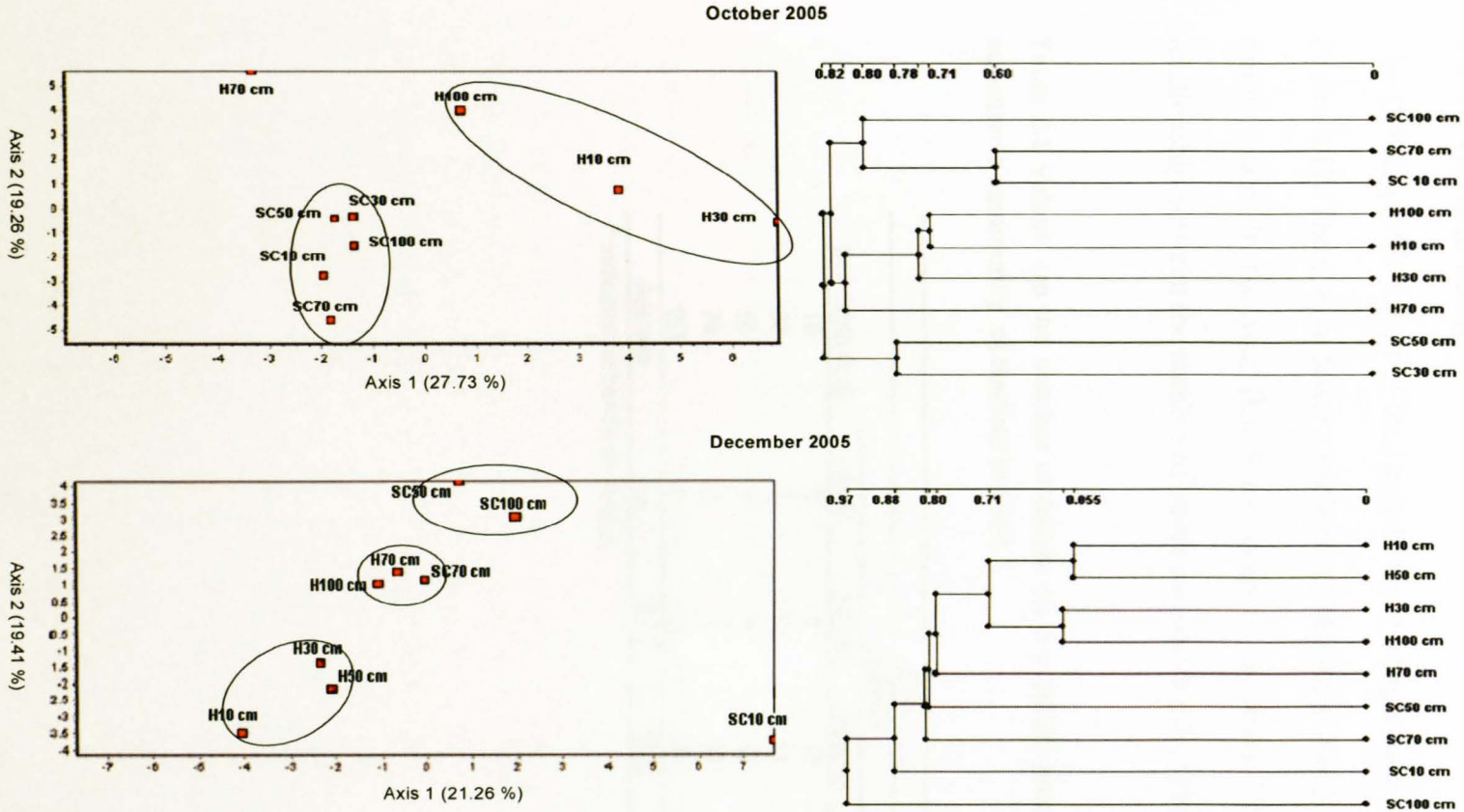


Figure 8.16: Analysis of the DGGE profile from Hatfield Moor and Sutton Common in October and December 2005 by PCA and cluster analysis. Percentages next to the axis title of the PCA plot illustrate the percentage of the total variation explained by each axis. The *x*-axis of the dendrogram explains the dissimilarity measure; the clusters become increasingly similar than their predecessors, resulting in a cross-over of branches.

8.4.3 Analysis of DGGE profiles for Hatfield Moor and Sutton Common in 2004

8.4.3.1 Hatfield Moor

The DGGE profile for all of the Hatfield Moor samples collected in 2004 is shown in Figure 8.17. There were fewer visible bands from the April samples (6-8) than from samples later in the year (i.e. 9-13 bands in October). There appeared to be no relationship between the number of bands and soil depth (Table 8.3).

Table 8.3 Values are the number of bands on the DGGE gels per sample for the soil eubacteria community at Hatfield in 2004.

	Site			
	Hatfield			
Soil depth (cm)	April	August	October	December
10	8	6	13	9
30	7	11	11	6
50	8	*	10	11
70	6	7	10	13
100	8	11	9	11
Average	7.4	8.75	10.6	10

* Indicates no bands on the gel

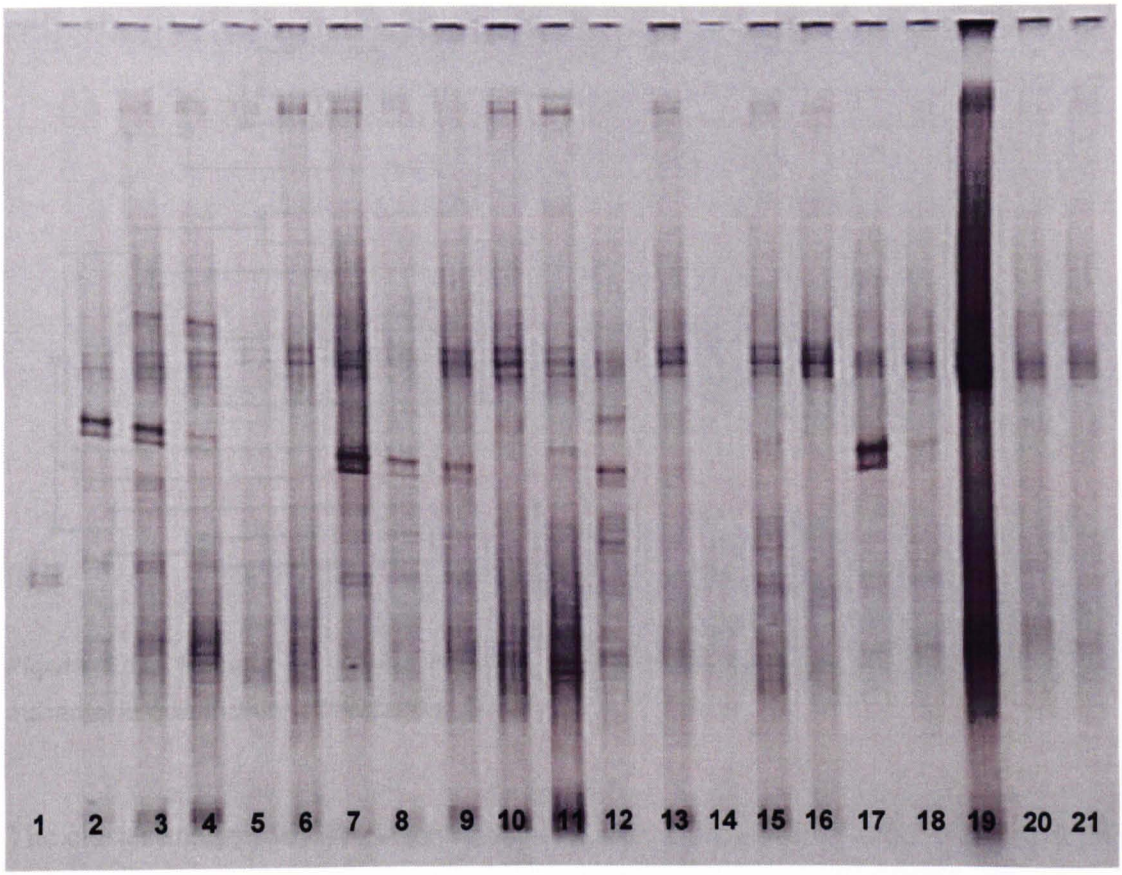


Figure 8.17: DGGE profile for the eubacteria community at Hatfield Moor in 2004. Lane 1: positive control (*Bacillus sp*), Lane 2: 10 cm December, Lane 3: 30 cm December, Lane 4: 50 cm December, Lane 5: 70 cm December, Lane 6: 100 cm December, Lane 7: 10 cm October, Lane 8: 30 cm October, Lane 9: 50 cm October, Lane 10: 70 cm October, Lane 11: 100 cm October, Lane 12: 10 cm August, Lane 13: 30 cm August, Lane 14: 50 cm August, Lane 15: 70 cm August, Lane 16: 100 cm August, Lane 17: 10 cm April, Lane 18: 30 cm April, Lane 19: 50 cm April, Lane 20: 70 cm April, Lane 21: 100 cm April

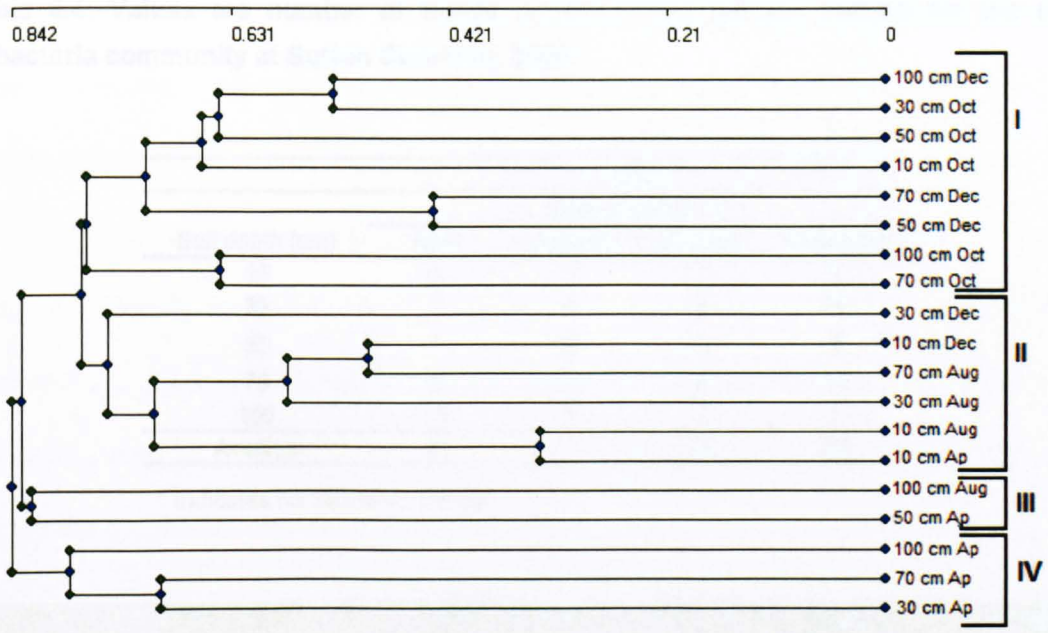


Figure 8.18: Results of cluster analysis using bands on the DGGE gel for the soil eubacteria community at Hatfield in 2004.

The cluster analysis divided the samples into 4 clusters (Figure 8.18). There was some tendency for samples to group according to sampling date; i.e. all the October samples are in cluster I together with three samples from December; three samples from August are in cluster II and three samples from April (the deepest samples) fall into cluster IV.

8.4.3.2 Sutton Common

Table 8.4 displays the number of bands obtained from the Sutton Common samples (Figure 8.19). The greatest number of bands was found at 100 cm depth in April (15 bands). The lowest band richness was found at 100 cm in December (2 bands). There was no obvious pattern in the number of bands with sampling date and/or depth, although the number of bands at 100 cm was less than at the other depths through the soil profile during all of the studied months, with the exception of April.

Table 8.4: Values are number of bands on the DGGE gel per sample for the soil eubacteria community at Sutton Common, 2004.

	Site			
	Sutton Common			
Soil depth (cm)	April	August	October	December
10	6	11	6	5
30	7	8	6	10
50	*	10	11	6
70	8	10	8	14
100	15	6	5	2
Average	9	9	7.2	7.4

* Indicates no bands on the gel

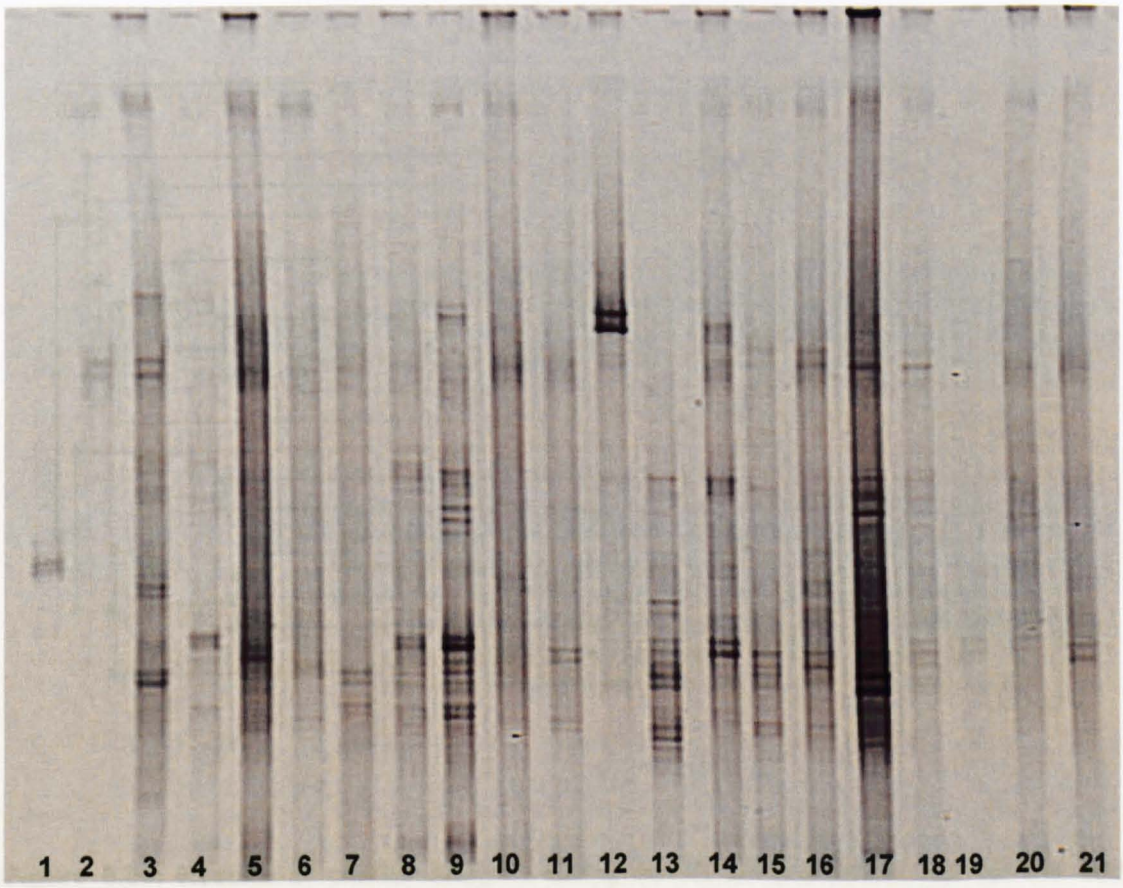


Fig 8.19: DGGE profile for the eubacteria community at Sutton Common in 2004. Lane 1: positive control (*Bacillus sp.*) Lane 2: 100 cm December, Lane 3: 70 cm December, Lane 4: 50 cm December, Lane 5: 30 cm December, Lane 6: 10 cm December, Lane 7: 100 cm October 2005, Lane 8: 70 cm October, Lane 9: 50 cm October, Lane 10: 30 cm October, Lane 11: 10 cm October, Lane 12: 100 cm August, Lane 13: 70 cm August, Lane 14: 50 cm August, Lane 15: 30 cm August, Lane 16: 10 cm August, Lane 17: 100 cm April, Lane 18: 70 cm April, Lane 19: 50 cm April, Lane 20: 30 cm April, Lane 21: 10 cm April.

Figure 8.20 shows the cluster analysis for Sutton Common in 2004. This analysis clearly separated samples according to sampling date. Two clusters can be distinguished; the first cluster included all of the October and December samples, the second cluster contained all April and August samples. The sub-clusters, within the principal clusters, tended to put together samples from either the surface or from the deeper soil depths. For example the samples obtained from December at 10 cm and 30 cm depths formed a sub-cluster in cluster I, the samples collected at 70 cm and 100 cm depths in October formed another sub-cluster, etc.

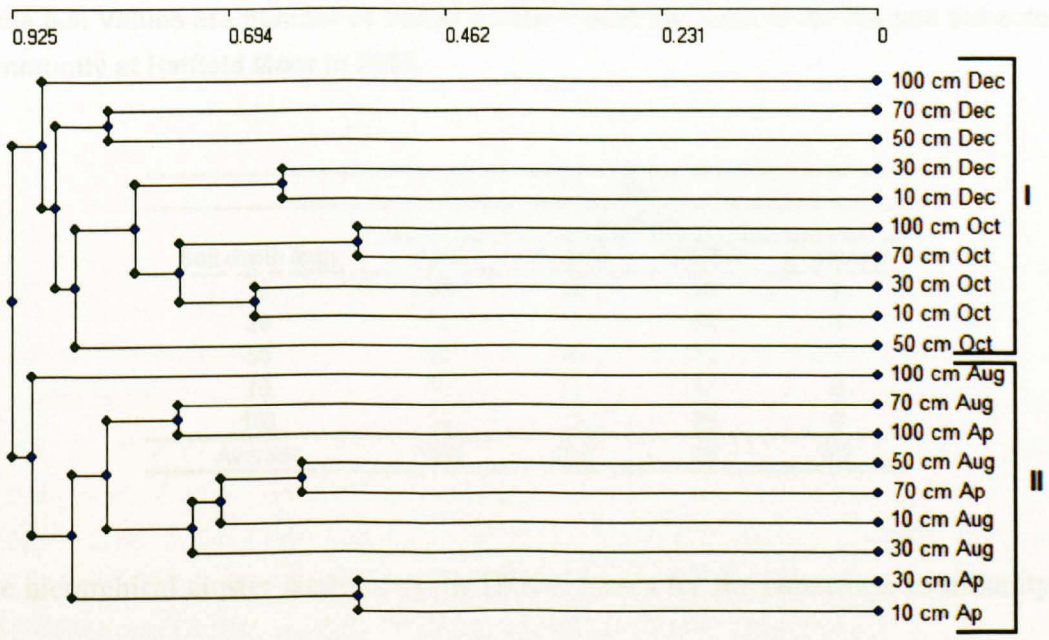


Figure 8.20: Results of cluster analysis using bands on the DGGE gel for the soil eubacteria community at Sutton Common in 2004.

8.4.4 Analysis of DGGE profiles for Hatfield Moor and Sutton Common 2005

8.4.4.1 Hatfield Moor

The number of bands for the eubacteria community from Hatfield Moor in 2005 (Figure 8.21 and Table 8.5), did not show an obvious pattern with depth. The greatest number of bands (22) was from 30 cm depth in October, and the lowest number of bands (3) occurred at 70 cm depth in August. In general, fewer bands were observed through the soil profile in December, and more bands were detected in October.

Table 8.5: Values are number of bands on the DGGE per sample for the soil eubacteria community at Hatfield Moor in 2005.

	Site			
	Hatfield			
Soil depth (cm)	April	August	October	December
10	16	15	14	9
30	11	17	22	8
50	13	15	11	7
70	16	3	17	8
100	12	12	16	9
Average	13.6	12.4	16	8.2

The hierarchical cluster analysis of the DGGE bands for the eubacteria community at Hatfield Moor in 2005 (Figure 8.22), tended to group the samples according to sampling date. Samples were divided into three clusters; cluster I is composed for all of the April samples, the deepest October samples and two August samples (10 cm and 30 cm depths). The December samples fell into cluster II (with the exception of 50 cm depth), together with the samples obtained from 100 cm depth for August, 10 cm and 30 cm depths for October. Cluster III is formed by three samples which were collected from 50 cm and 70 cm depths for August, and 50 cm depth for December.

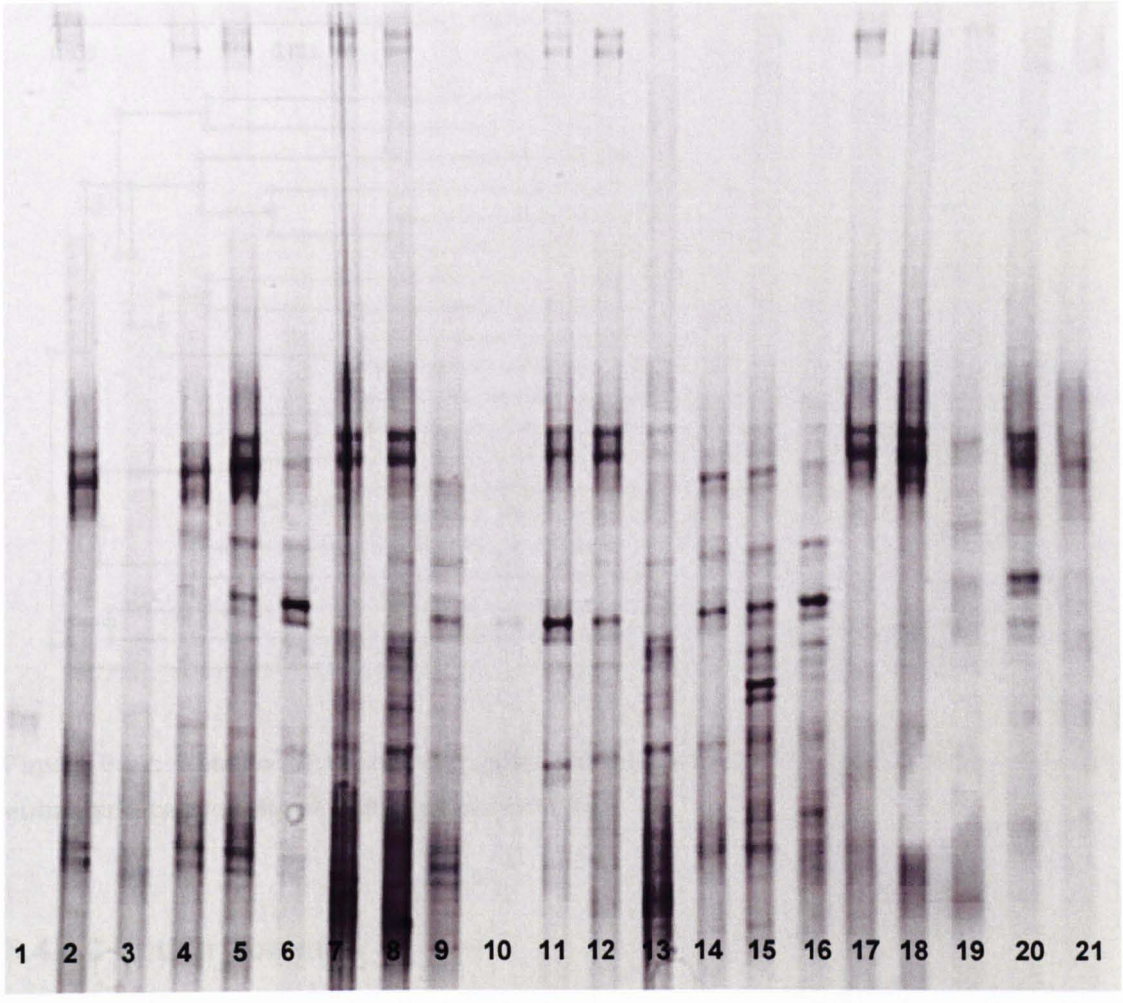


Figure 8.21: DGGE profile for the eubacteria community at Hatfield Moor in 2005. Lane 1: positive control (*Bacillus sp.*), Lane 2: 10 cm April, Lane 3: 30 cm April, Lane 4: 50 cm April, Lane 5: 70 cm April, Lane 6: 10 cm August, Lane 7: 30 cm August, Lane 8: 50 cm August, Lane 9: 70 cm August, Lane 10: 100 cm August, Lane 11: 10 cm August, Lane 12: 10 cm October, Lane 13: 30 cm October, Lane 14: 50 cm October, Lane 15: 70 cm October, Lane 16: 100 cm October, Lane 17: 10 cm December, Lane 18: 30 cm December, Lane 19: 50 cm December, Lane 20: 70 cm December, Lane 21: Hatfield 100 cm December.

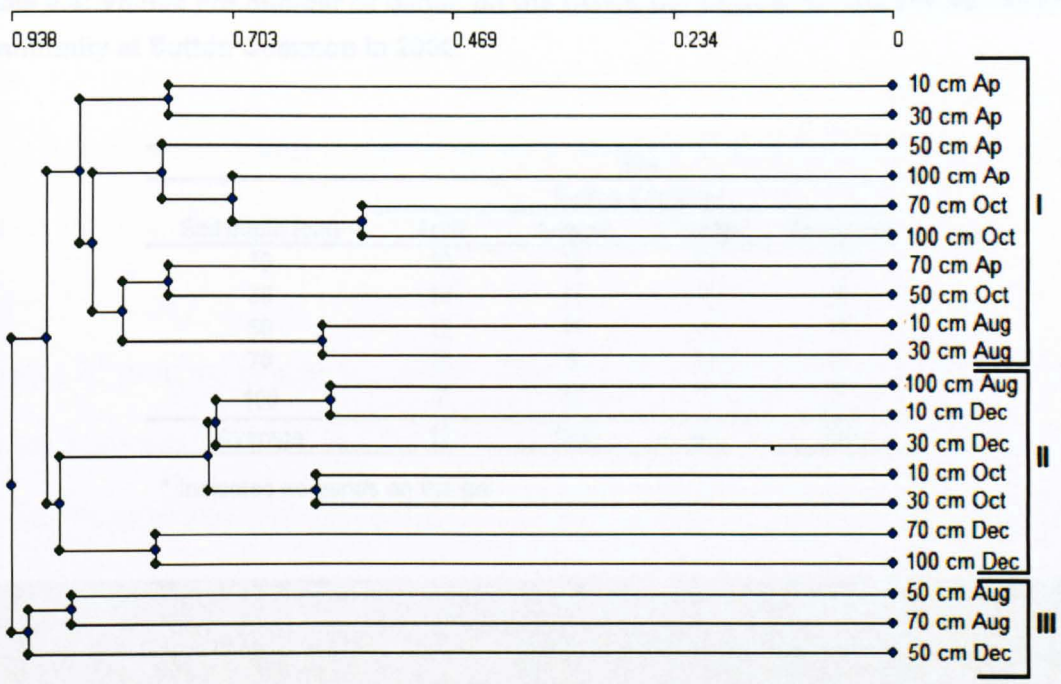


Figure 8.22: Results of cluster analysis using bands on the DGGE gel for the soil eubacteria community at Hatfield Moor in 2005.

8.4.4.2 Sutton Common

Table 8.6 shows the number of bands on the DGGE gel for Sutton Common 2005 (Figure 8.23). The band richness did not show an obvious pattern in relation to depth and/or sampling date.

The samples with the greater number of bands (18) were obtained from 10 cm depth for October, 50 cm depth for December and 70 cm depth for April. The least number of bands (6) was at 70 cm depth in August.

Table 8.6: Values are number of bands on the DGGE per sample for the soil eubacteria community at Sutton Common in 2005.

	Site			
	Sutton Common			
Soil depth (cm)	April	August	October	December
10	10	15	18	13
30	13	17	7	8
50	12	14	7	18
70	18	6	12	17
100	7	16	*	7
Average	12	13.6	11	12.6

* Indicates no bands on the gel

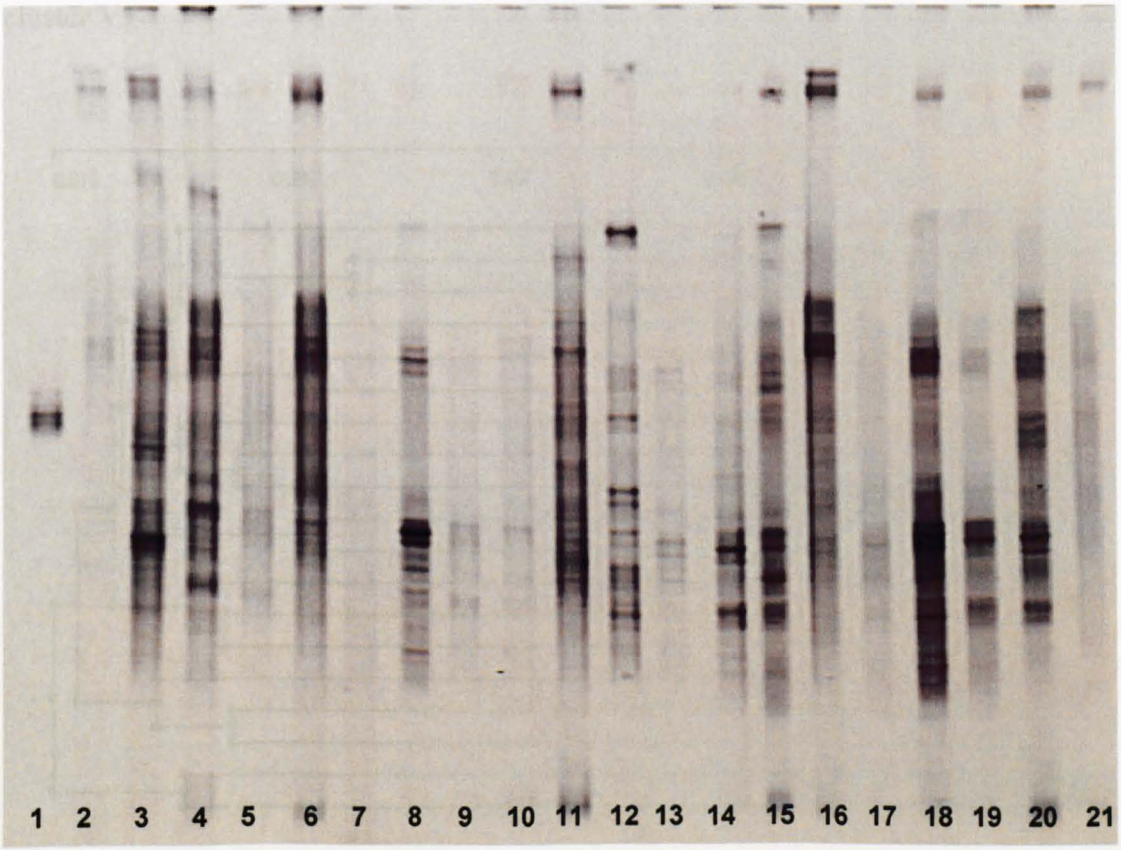


Figure 8.23: DGGE profile for the eubacteria community at Sutton Common in 2005. Lane 1: positive control (*Bacillus sp.*), Lane 2: 100 cm December, Lane 3: 70 cm December, Lane 4: 50 cm December, Lane 5: 30 cm December, Lane 6: 10 cm December, Lane 7: 100 cm October, Lane 8: 70 cm October, Lane 9: 50 cm October, Lane 10: 30 cm October, Lane 11: 10 cm October, Lane 12: 100 cm August, Lane 13: 70 cm August, Lane 14: 50 cm August, Lane 15: 30 cm August, Lane 16: 10 cm August, Lane 17: 100 cm April, Lane 18: 70 cm April, Lane 19: 50 cm April, Lane 20: 30 cm April, Lane 21: 10 cm April.

The cluster analysis (Figure 8.24) grouped the samples mainly according to sampling date. For example, cluster I included most of the December samples (with the exception of 30 cm depth); cluster II was composed of the April samples (10 cm to 70 cm depths) and 30 cm depth in December.

Cluster III grouped two of the August samples (30 cm and 50 cm depths) and the sample collected from 100 cm depth in April. The samples from October between 30 cm to 70 cm depths fell into cluster IV, whilst the deepest August samples fell into cluster V.

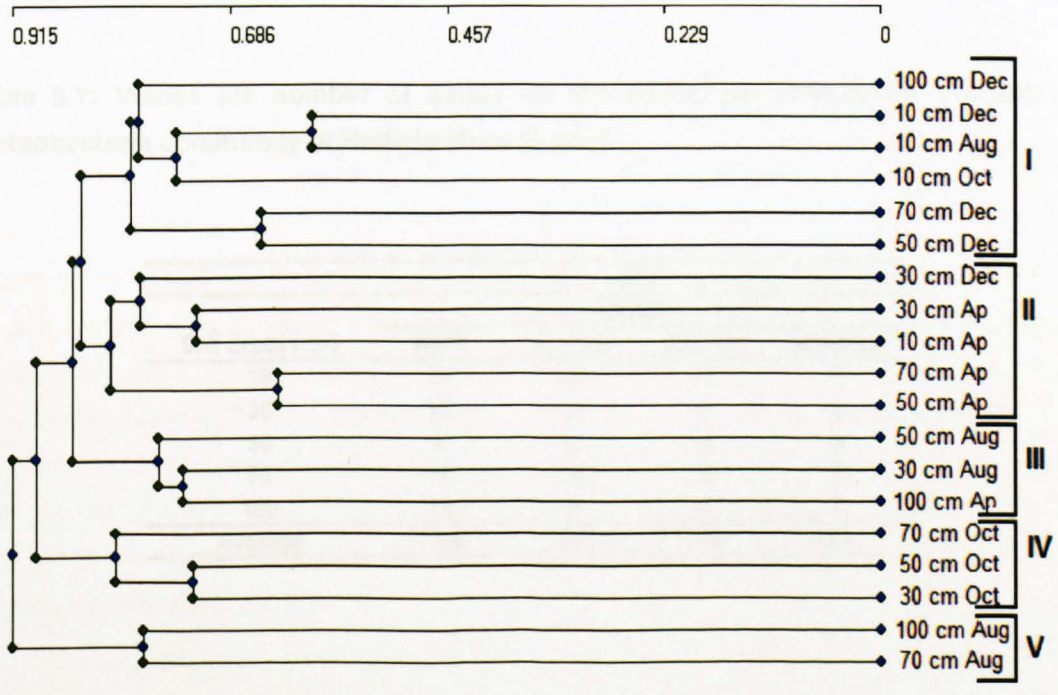


Figure 8.24: Results of cluster analysis using bands on the DGGE gel for the soil eubacteria community at Sutton Common in 2005.

8.5 Results of analysis of DGGE profiles for specific bacterial groups

8.5.1 Hatfield Moor 2004

8.5.1.1 α-proteobacteria

Table 8.7 shows band richness for the DGGE gel of the α-proteobacteria community at Hatfield Moor for 2004 (Figure 8.25). The highest number of bands (18) was found in the sample obtained from 30 cm depth for April and at 70 cm for October; the lowest number of bands (6) was detected at 50 cm depth in April. There was a general tendency for the number of bands in April, August and October to be greater at 10 cm and 70 cm depths. However, in December the number of bands decreases considerably below 10 cm depth.

Table 8.7: Values are number of bands on the DGGE per sample for the soil α-proteobacteria community at Hatfield Moor in 2004.

	Site			
	Hatfield			
Soil depth (cm)	April	August	October	December
10	15	14	16	15
30	18	13	9	9
50	6	14	14	8
70	15	14	18	8
100	15	9	11	7
Average	13.8	12.8	13.6	9.4

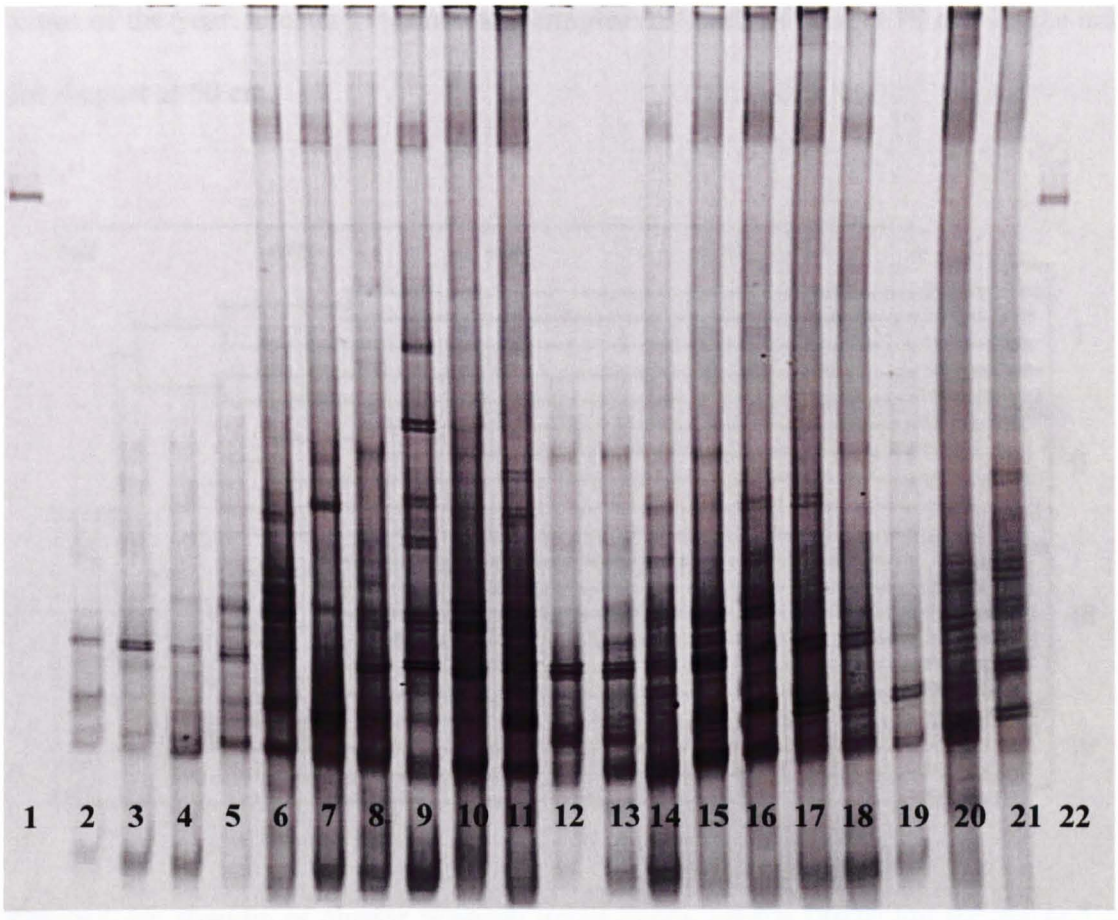


Figure 8.25: DGGE profile for the α-proteobacteria community at Hatfield Moor in 2004. Lane 1: positive control (*Agromonas oligotrophica);* Lane 2: 100 cm December, Lane 3: 70 cm December, Lane 4: 50 cm December, Lane 5: 30 cm December, Lane 6: 10 cm. Lane 7: 100 cm October, Lane 8: 70 cm October, Lane 9: 50 cm October, Lane 10: 30 cm October, Lane 11: 10 cm October, Lane 12: 100 cm August, Lane 13: 70 cm August, Lane 14: 50 cm August, Lane 15: 30 cm August, Lane 16: 10 cm August, Lane 17: 100 cm April, Lane 18: 70 cm April, Lane 19: 50 cm April, Lane 20: 30 cm April, Lane 21: 10 cm April, Lane 22: positive control (*Agromonas oligotrophica*).

The cluster analysis (Figure 8.26) for α-proteobacteria at Hatfield Moor in 2004 separated the samples into four clusters, but there was no clear pattern related to date or depth of sampling. For example, the December samples obtained below 10 cm depth and the sample from 100 cm depth in August were both grouped together (Cluster I). Clusters II and III included a mixture of samples from different depths and

times of the year. Cluster IV contained samples for April at 30 and 70 cm depths and for August at 50 cm.

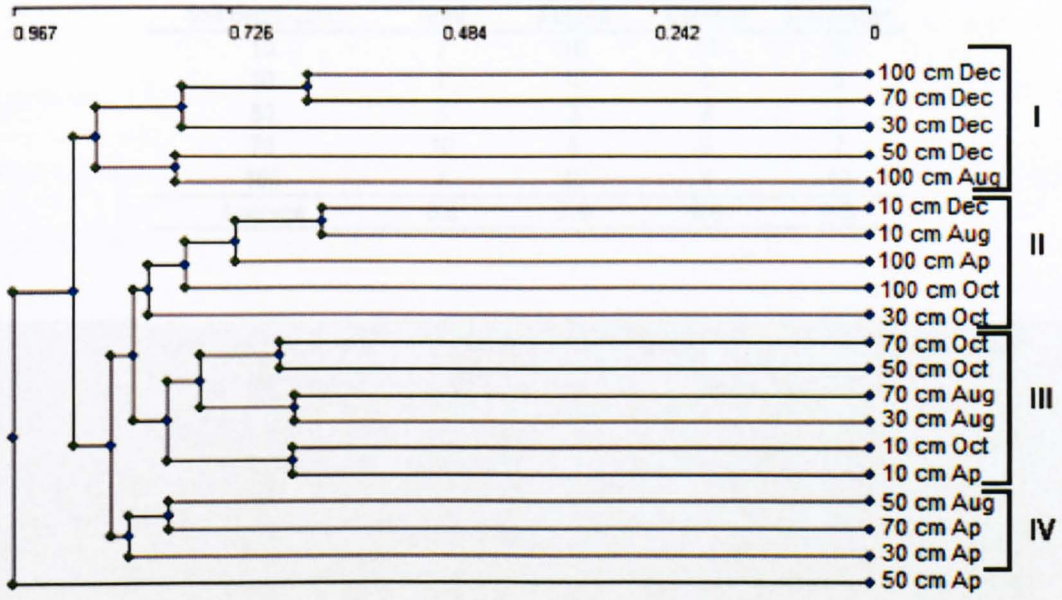


Figure 8.26: Results of cluster analysis using bands on the DGGE gel for the soil α-proteobacteria community at Hatfield Moor in 2004.

8.5.1.2 β-proteobacteria

The number of bands (Table 8.8) on the DGGE profiles for the β-proteobacteria community at Hatfield Moor in 2004 (Figure 8.27) was lowest (3) at 50 cm depth in August. The highest number of bands (10) was found for several samples, mostly from 10 cm depth.

Table 8.8: Values are number of bands on the DGGE per sample for the soil β-proteobacteria community at Hatfield Moor in 2004.

	Site			
	Hatfield			
Soil depth (cm)	April	August	October	December
10	7	10	10	10
30	7	10	5	9
50	6	3	6	3
70	10	5	9	7
100	4	10	4	10
Average	6.8	7.6	6.8	7.8

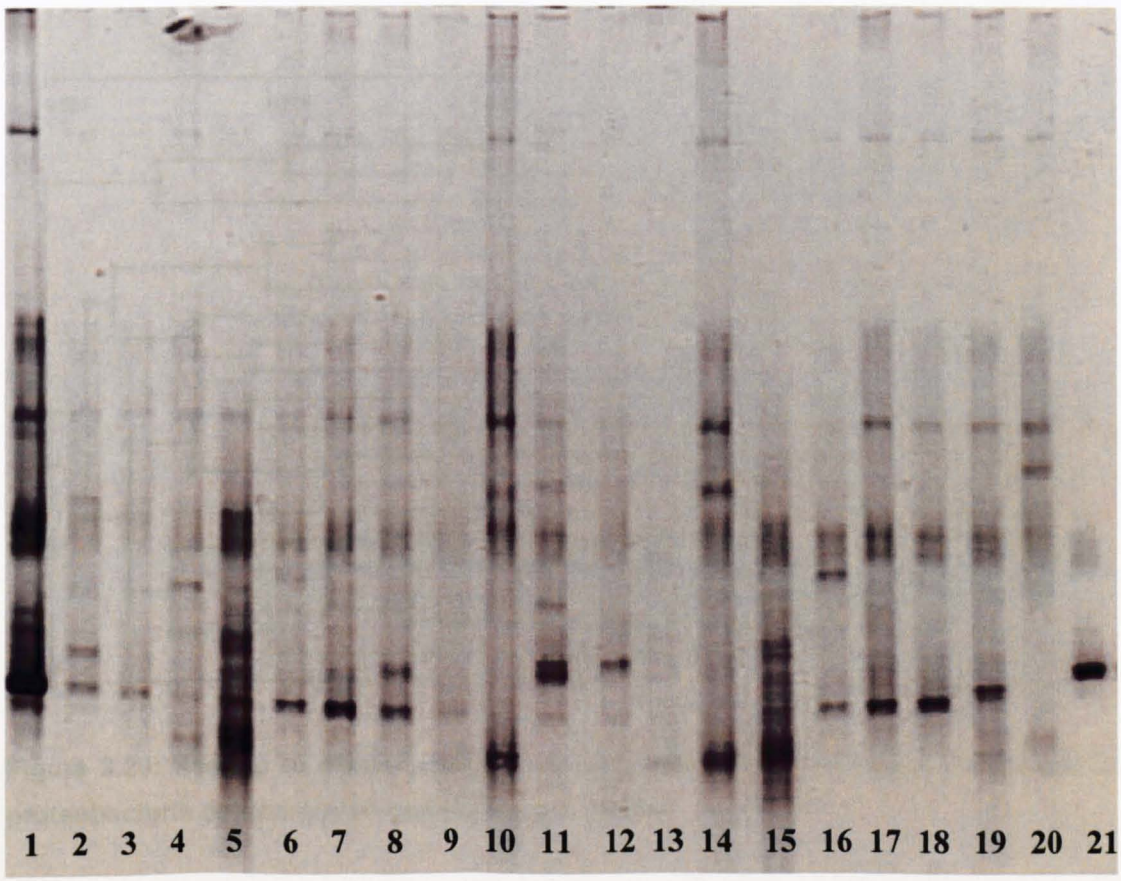


Figure 8.27: DGGE profile for the β-proteobacteria community at Hatfield Moor in 2004. Lane 1: 100 cm December, Lane 2: 70 cm December, Lane 3: 50 cm December, Lane 4: 30 cm December, Lane 5: 10 cm. Lane 6: 100 cm October, Lane 7: 70 cm October, Lane 8: 50 cm October, Lane 9: 30 cm October, Lane 10: 10 cm October, Lane 11: 100 cm August, Lane 12: 70 cm August, Lane 13: 50 cm August, Lane 14: 30 cm August, Lane 15: 10 cm August, Lane 16: 100 cm April, Lane 17: 70 cm April, Lane 18: 50 cm April, Lane 19: 30 cm April, Lane 20: 10 cm April, Lane 21: positive control (*Burkholderia cepacea*).

Cluster analysis for the β-proteobacteria community at Hatfield Moor in 2004 (Figure 8.28), divided the samples into 5 clusters which to some extent were related to sampling date. Cluster I contained December samples (obtained between 50 to 100 cm depths), and cluster II included a mix of samples from April, August, October and December. Cluster III comprised all the October samples except for the 100 cm sample. Cluster IV contained August samples (30, 70 and 100 cm depths) and cluster V contained April samples from 10 cm, 30 cm and 50 cm depth.

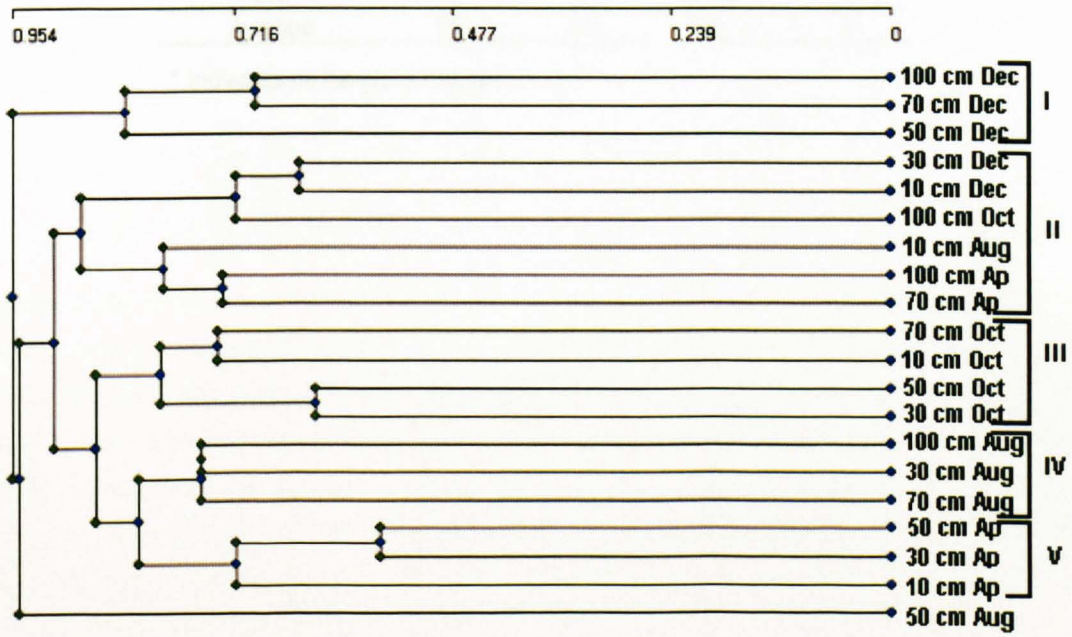


Figure 8.28: Results of cluster analysis using bands on the DGGE gel for the soil β-proteobacteria community at Hatfield Moor in 2004.

8.5.1.3 Actinomycetes

The highest band richness (12) for the actinomycete DGGE profiles (Figure 8.29 and Table 8.9) at Hatfield Moor in 2004 was from 70 cm depth in August. The lowest band number (3) was displayed in the sample collected at 100 cm depth in October. The number of bands was not obviously related to soil depth.

The average number of bands (9.4) was higher in April and August when compared with October and December (6-8).

Table 8.9: Values are number of bands on the DGGE per sample for the soil actinomycetes community at Hatfield Moor in 2004.

	Site			
	Hatfield			
Soil depth (cm)	April	August	October	December
10	9	9	9	8
30	9	9	4	*
50	10	10	8	11
70	9	12	6	9
100	10	7	3	4
Average	9.4	9.4	6	8

* Indicates no bands on the gel

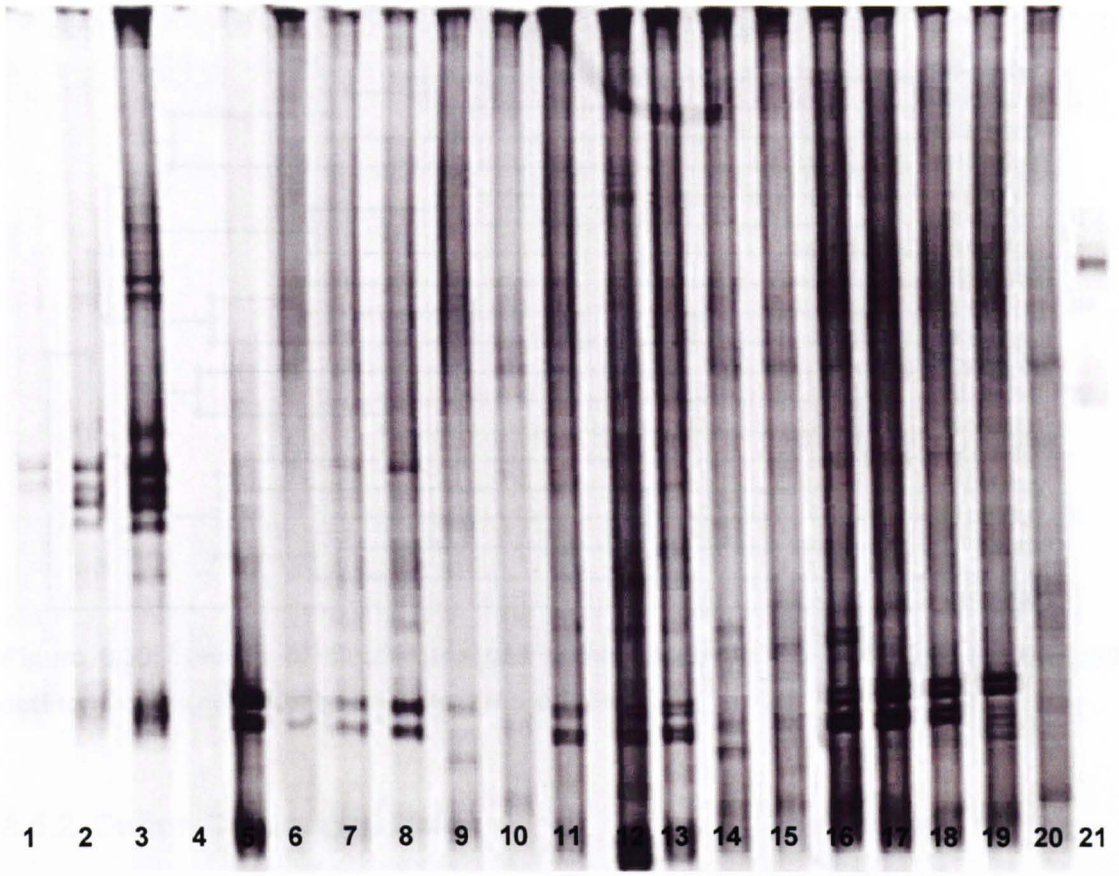


Figure 8.29: DGGE profile for the actinomycete community at Hatfield Moor in 2004. Lane 1: 100 cm December, Lane 2: 70 cm December, Lane 3: 50 cm December, Lane 4: 30 cm December, Lane 5: 10 cm. Lane 6: 100 cm October , Lane 7: 70 cm October, Lane 8: 50 cm October, Lane 9: 30 cm October, Lane 10: 10 cm October, Lane 11: 100 cm August, Lane 12: 70 cm August, Lane 13: 50 cm August, Lane 14: 30 cm August, Lane 15: 10 cm August, Lane 16: 100 cm April, Lane 17: 70 cm April, Lane 18: 50 cm April, Lane 19: 30 cm April, Lane 20: 10 cm April, Lane 21: positive control (*Actinomadura atramentaria*).

The cluster analysis of the actinomycete community at Hatfield Moor for 2004 (Figure 8.30) tended to group the samples according to time of year when sampling was undertaken. Cluster I included all of the December samples; Cluster II contained April samples (obtained between 30 cm and 70 cm depths). Cluster III had the samples from 10 cm depth in April and from 50 cm and 100 cm depth in August; cluster IV contained samples from October (50 cm, 70 cm and 100 cm depths); cluster V contained samples obtained from diverse depths and months (April, August and October). The 30 cm depth sample for October is an outlier in the cluster analysis.

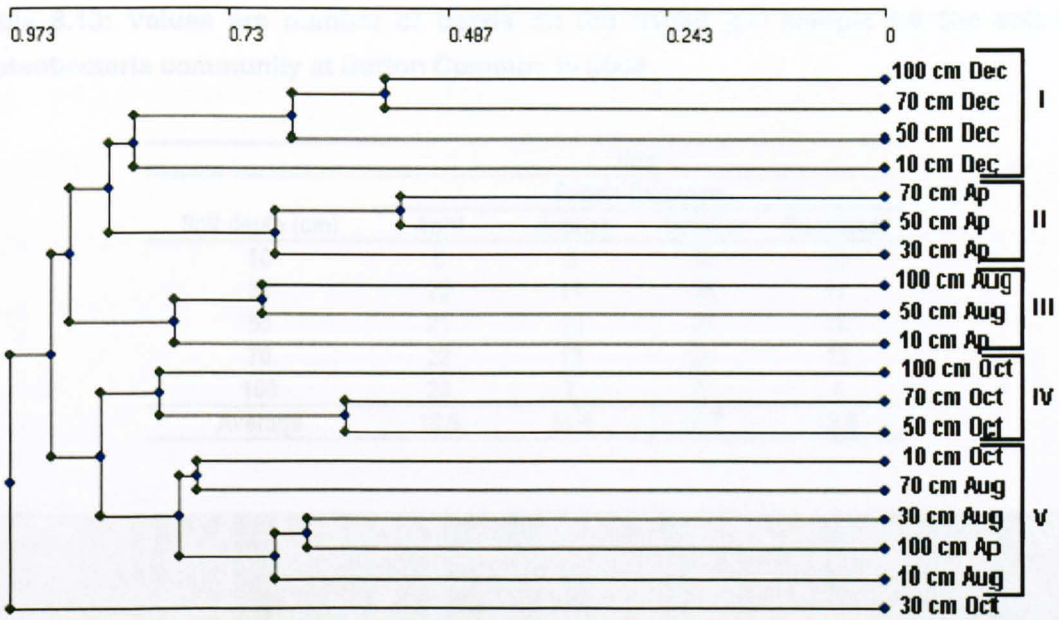


Figure 8.30: Results of cluster analysis using bands on the DGGE gel for the soil actinomycetes community at Hatfield Moor 2004.

8.5.2. Sutton Common in 2004

8.5.2.1 α-proteobacteria

The DGGE gel for the α-proteobacteria community at Sutton Common in 2004 (Figure 8.31), displayed the greatest band richness in October at 10 cm depth (32) (Table 8.10). Indeed the greatest band richness at all depths was in October. The lowest number of bands was found in the sample collected at 100 cm depth in December (4) (Table 8.10).

Table 8.10: Values are number of bands on the DGGE per sample for the soil α-proteobacteria community at Sutton Common in 2004.

	Site			
	Sutton Common			
Soil depth (cm)	April	August	October	December
10	6	8	32	20
30	22	11	28	17
50	21	19	24	14
70	22	13	26	13
100	23	7	21	4
Average	18.8	11.6	26.2	13.6

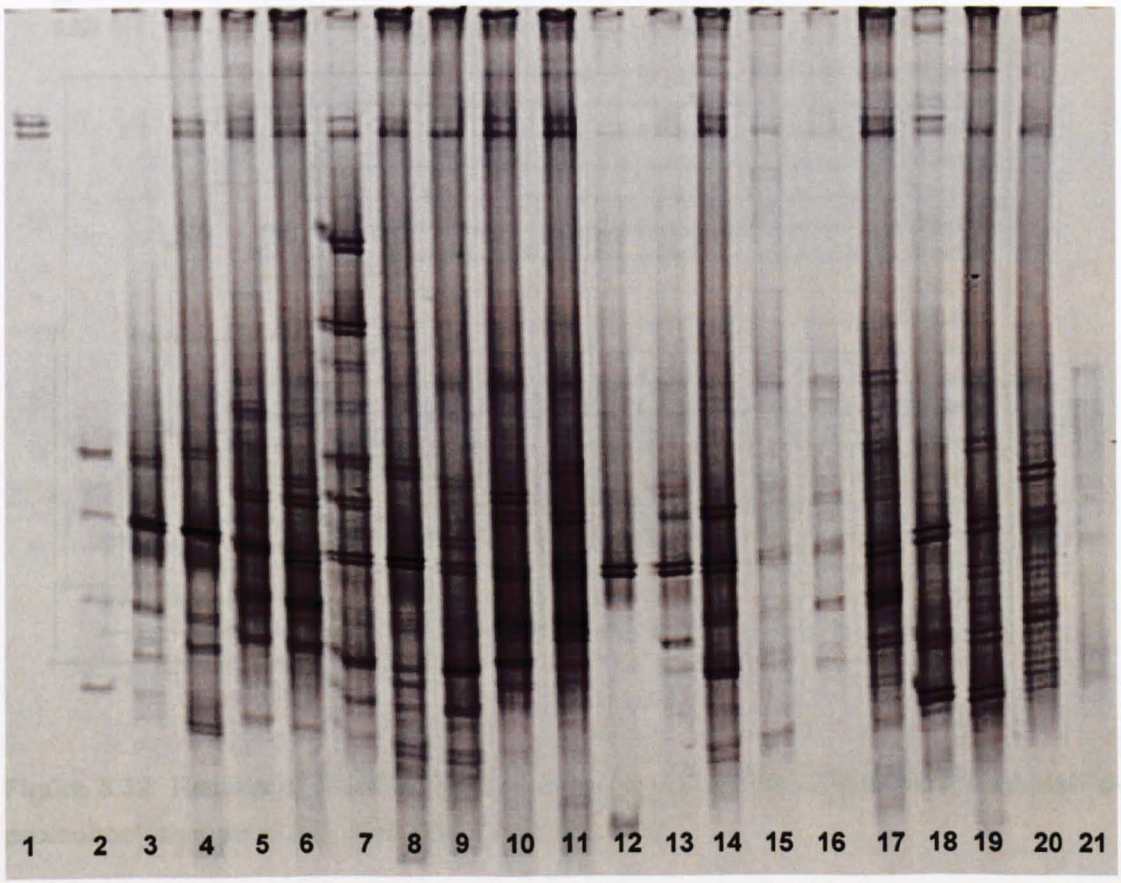


Figure 8.31: DGGE profile for the α-proteobacteria community at Sutton Common in 2004. Lane 1: positive control (*Actinomadura atramentaria*), Lane 2: 100 cm December, Lane 3: 70 cm December, Lane 4: 50 cm December, Lane 5: 30 cm December, Lane 6: 10 cm December, Lane 7: 100 cm October, Lane 8: 70 cm October, Lane 9: 50 cm October, Lane 10: 30 cm October, Lane 11: 10 cm October, Lane 12: 100 cm August, Lane 13: 70 cm August, Lane 14: 50 cm August, Lane 15: 30 cm August, Lane 16: 10 cm August, Lane 17: 100 cm April, Lane 18: 70 cm April, Lane 19: 50 cm April, Lane 20: 30 cm April, Lane 21:10 cm April

Cluster analysis for the soil α-proteobacteria community at Sutton Common, 2004 (Figure 8.31) tended to combine a number of samples from the same time of the year. For example, the October samples fell into cluster II (with the exception of the 100 cm depth sample), and the August samples were all in cluster IV. Nevertheless, samples from April and December were distributed across the dendrogram, in different clusters.

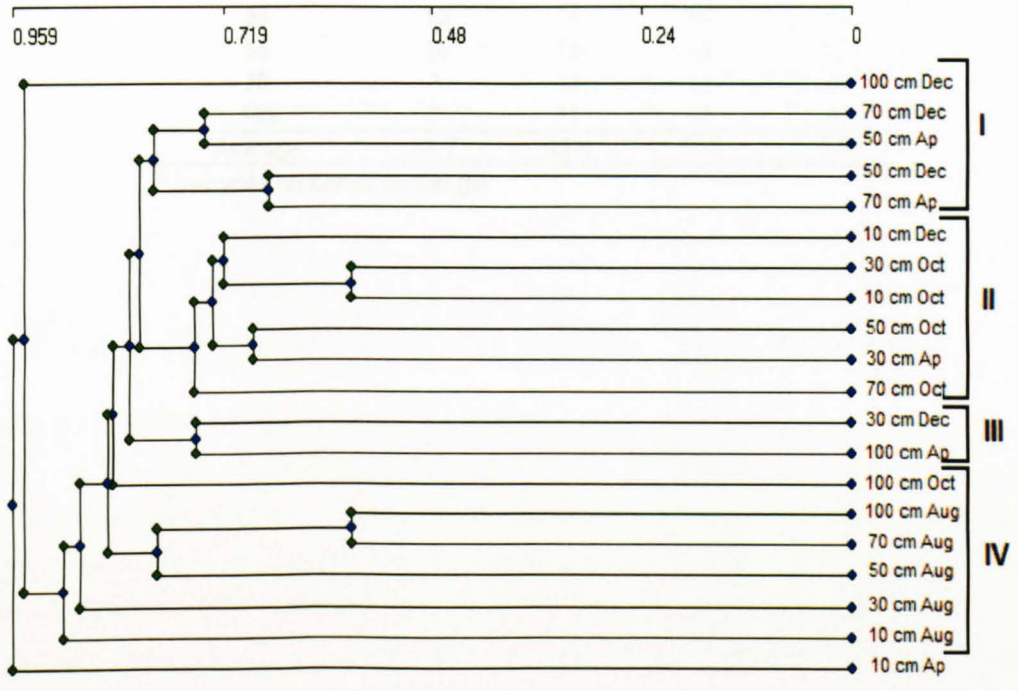


Figure 8.32: Results of cluster analysis using bands on the DGGE gel for the soil α-proteobacteria community at Sutton Common, 2004.

8.5.2.2 Actinomycetes

The highest band richness (Table 8.11) on the DGGE profiles for the actinomycete community at Sutton Common in 2004 (Figure 8.33) was recorded for August, at 10 cm depth (16). The lowest richness occurred in December (4 bands) at 100 cm depth.

The number of bands tended to decrease with depth, except for the April samples, since no bands were obtained for the deepest samples.

Table 8.11: Values are number of bands on the DGGE per sample for the soil actinomycete community at Sutton Common in 2004.

	Site			
	Sutton Common			
Soil depth (cm)	April	August	October	December
10	11	16	15	14
30	10	14	14	10
50	14	13	13	12
70	*	14	11	8
100	*	11	13	4
Average	11.7	13.6	13.2	9.6

* Indicates no bands on the gel

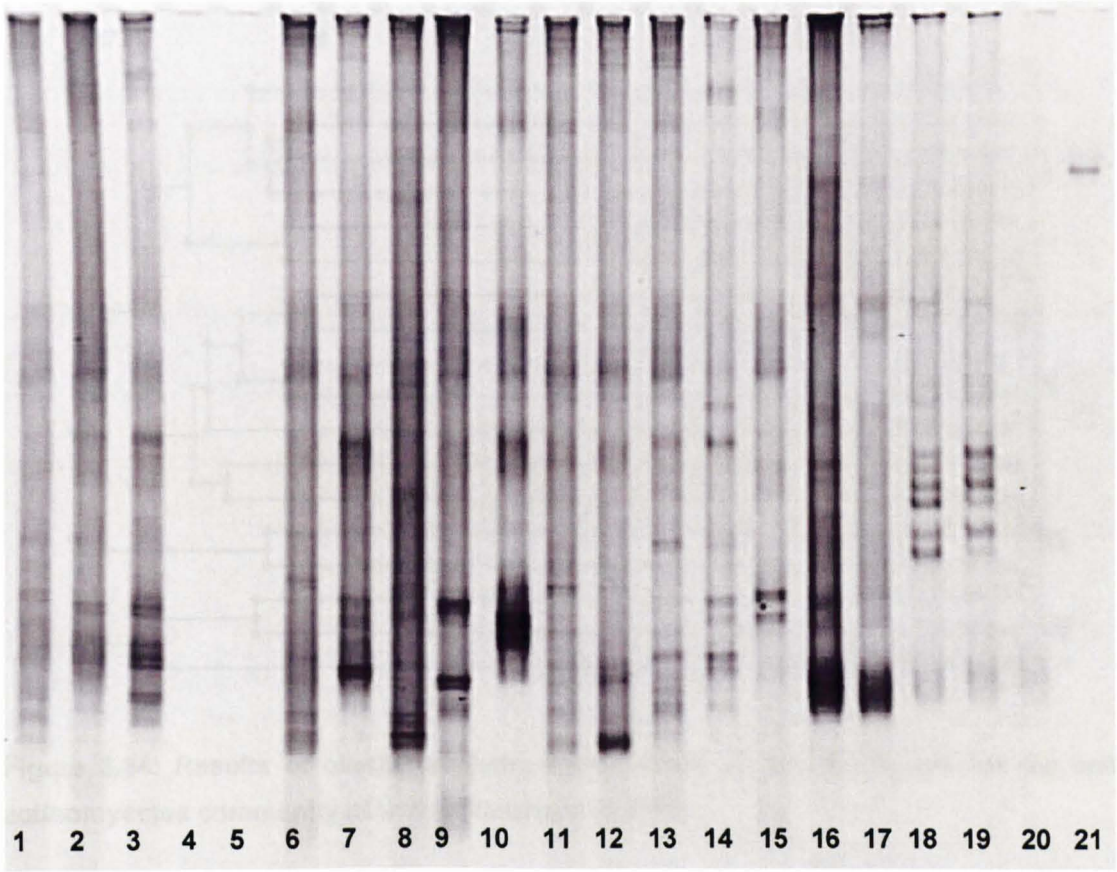


Figure 8.33: DGGE profile for the actinomycetes community at Sutton Common in 2004. Lane 1: 10 cm April, Lane 2: 30 cm April, Lane 3: 50 cm April, Lane 4: 70 cm April, Lane 5: 100 cm April, Lane 6: 10 cm August, Lane 7: 30 cm August, Lane 8: 50 cm August, Lane 9: 70 cm August, Lane 10: 100 cm August, Lane 11: 10 cm October, Lane 12: 30 cm October, Lane 13: 50 cm October, Lane 14: 70 cm October, Lane 15: 100 cm October, Lane 16: 10 cm December, Lane 17: 30 cm December, Lane 18: 50 cm December, Lane 19: 70 cm December, Lane 20: 100 cm December, Lane 21: positive control (*Actinomadura atramentaria*).

The cluster analysis (Figure 8.34) divided the samples into four clusters. Cluster I included samples obtained from April (at 10 cm depth), August (at 10 cm and 50 cm depths), and at 10 cm depth for October. Cluster II comprised different samples collected from April, August and October. December samples fell into clusters III and IV.

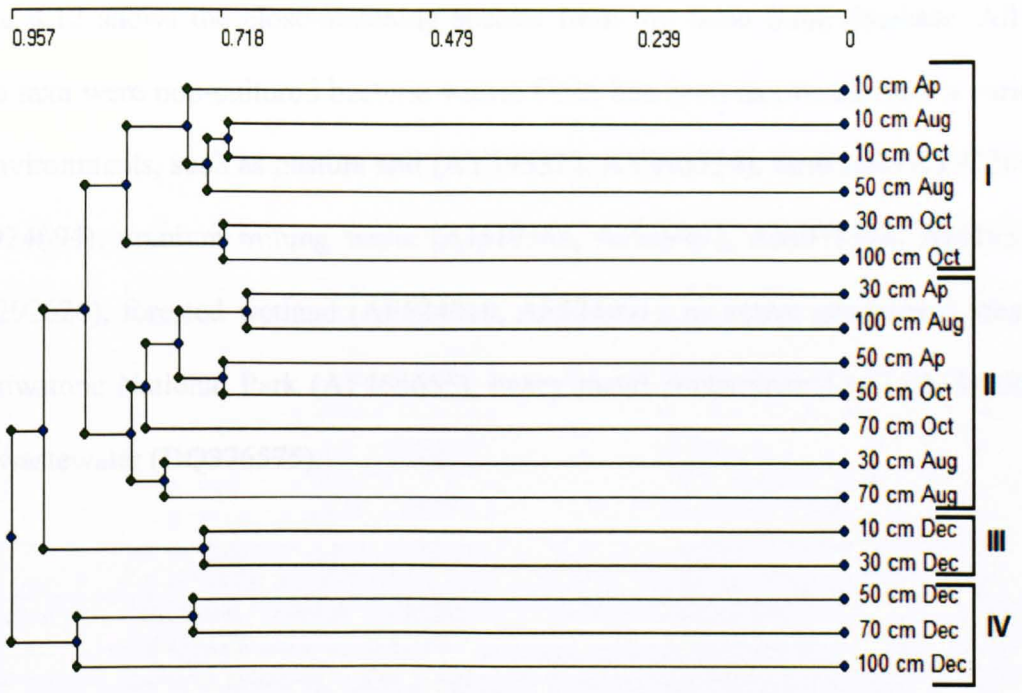


Figure 8.34: Results of cluster analysis using bands on the DGGE gel for the soil actinomycetes community at Sutton Common in 2004.

8.6 Results from cloning and sequencing using samples from Hatfield.

None of the sequences obtained were an exact match to sequences recorded in the Gene Bank database, although similarity values of 88 % and 99 % were obtained. As such, some of the bacteria from Hatfield Moor can be affiliated with known bacterial taxa (Table 8.12).

The results obtained from cloning and sequencing showed a change in bacterial phyla with increasing depth down the soil profile. The predominant phyla were Acidobacteria and Proteobacteria, mainly represented by members of the alpha subclass (α-Protobacteria). At 10 cm depth Acidobacteria and α-Proteobacteria were the principal phyla. At 30 cm depth β-Proteobacteria, Actinobacteria and Acidobacteria became important. Finally, at 50 cm depth, Spirochetes and Firmicutes were the main phyla in evidence.

Table 8.12 shows the close-matching species from the Gene Bank database. All of these taxa were non-cultured bacteria whose DNA has been recovered from a variety of environments, such as pasture soil (AY395322, AY395354), farm soil (AY922044, AY924694), uranium mining waste (AJ519382, AJ519401, AM071378, AJ519379, AM292626), forested wetland (AF524010, AF524001), an active geothermal area in Yellowstone National Park (AF465655), heavy metal contaminated soil (AJ581583) and wastewater (DQ376575).

Table 8.12: Matching of clones to the closest sequences of known phylogenetic affiliation.

Clon[1]	Identity[2]	Taxonomic group	Similarity[3]
H10 Clon 1	*Acidobacteria bacterium* AY9222041	Acidobacteria	0.933
H10 Clon 2	*Holophaga sp*. AJ519379	Acidobacteria	0.963
H10 Clon 4	*Bradyrhizobium sp.* AY141982	Alphaproteobacteria	0.922
H10 Clon 5	*Bacteroidetes bacterium* AY922149	Bacteroidetes	0.932
H10 Clon 6	*Bacterium sp.* DQ129290	Actinobacteria	0.983
H10 Clon 7	*Bacterium sp.* AJ863228	Genera_incertae_sedis	0.880
H10 Clon 8	*Chloroflexi bacterium* AY922044	Firmicutes	0.990
H10 Clon 9	*Holophaga sp*. AJ519379	Acidobacteria	0.963
H10 Clon 11	*Alpha proteobacterium* AJ518771	Alphaproteobacteria	0.947
H10 Clon 15	*Alpha proteobacterium* AJ581583	Alphaproteobacteria	0.943
H10 Clon 16	*Bacterium sp.* DQ 376575	Bacteroidetes	0.919
H30 Clon 17	*Chloroflexi bacterium* AY922047	Firmicutes	0.990
H30 Clon 18	*Actinobacterium sp*. AJ519401	Actinobacteria	0.958
H30 Clon 19	*Actinobacterium sp.* AY922024	Actinobacteria	0.983
H30 Clon 22	*Acidobacteria bacterium* EF111088	Acidobacteria	0.933
H30 Clon 23	*Beta proteobacterium* AY921694	Betaproteobacteria	0.985
H30 Clon 24	*Beta proteobacterium* AY395322	Betaproteobacteria	0.912
H30 Clon 25	*Beta proteobacterium* AY395322	Betaproteobacteria	0.904
H30 Clon 26	*Alpha proteobacterium* AJ536857	Alphaproteobacteria	0.960
H30 Clon 27	*Holophaga sp.* AJ519382	Acidobacteria	0.950
H30 Clon 28	*Acidobacteria bacterium* AM292623	Acidobacteria	0.974
H30 Clon 29	*Acidobacteriales bacterium* AY395450	Acidobacteria	0.955
H30 Clon 30	*Bacterium sp*. AJ863186	Actinobacteria	0.988
H50 Clon 33	*Acidobacteria bacterium* AY395354	Acidobacteria	0.894
H50 Clon 37	*Alpha proteobacterium* AF465655	Alphaproteobacteria	0.973
H50 Clon 39	*Spirochete sp.* AY605144	Spirochaetes	0.874
H50 Clon 40	*Spirochetec sp*. AY133081	Spirochaetes	0.895
H50 Clon 41	*Bacterium sp*. AF524001	Actinobacteria	0.873
H50 Clon 42	*Bacterium sp.* DQ088814	Firmicutes	0.792
H50 Clon 45	Low G+C Gram-positive bacterium DQ432326	Firmicutes	0.876
H50 Clon 46	*Bacterium sp.* AF524010	Spirochaetes	0.985
H50 Clon 47	Delta proteobacterium AM071378	Deltaproteobacteria	0.979

1 H: Hatfield Moor and the number on the right indicates the sampled depth.
2 Closest matches to band sequence obtained by comparison with RDP release 9.46. Capital letters and numbers indicate the GeneBank accession number.
3 Similarity calculated with the RDP similarity matrix facility between the database entry and band sequence.

The phylogenetic tree in Figure 8.35 shows a tendency for clones from similar depth and phyla to cluster together. For example, all the clones matching with Acidobacteria clustered together at the top of the phylogenetic tree. Clones obtained from 10 cm and

30 cm depths, which matched with proteobacteria, are clustered at the bottom of the tree.

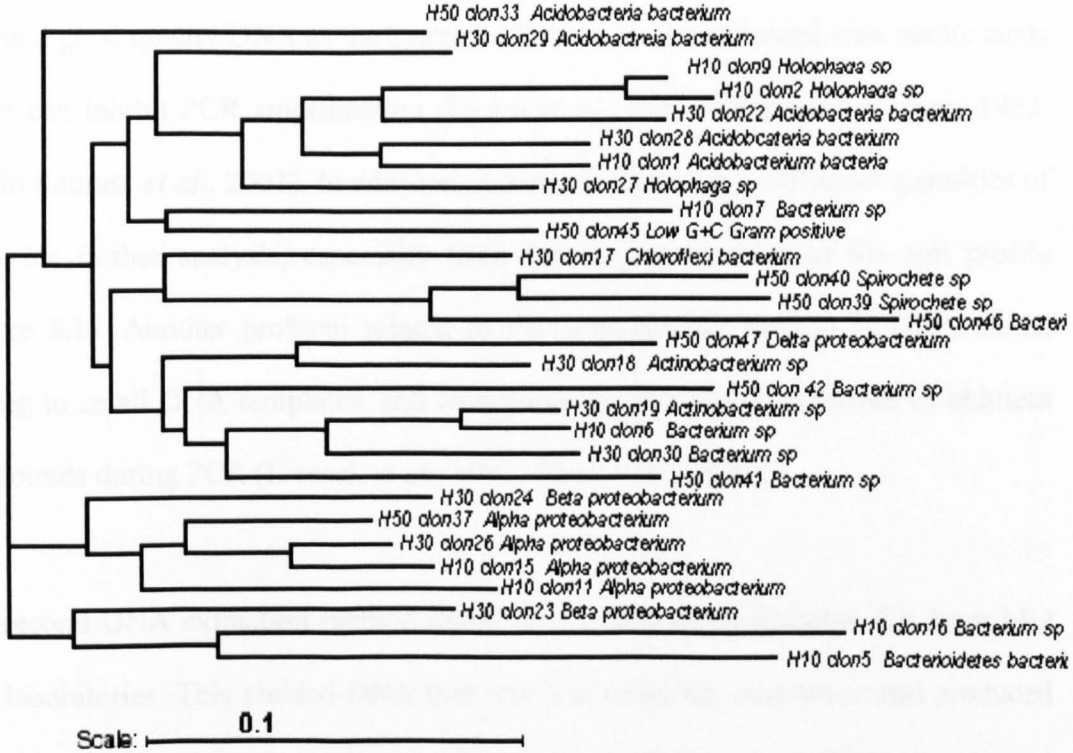


Figure 8.35: Phylogenetic tree using the Weighbor weighted neighbor-joining tree building algorithm of 16S rRNA gene sequences cloned directly from total soil DNA. H30 clon 25, H10 clon 8 and H10 clon were not included in the tree because share less than 200 comparable positions with the other sequences.

8.7 Discussion

The molecular techniques applied in this study provide a comprehensive overview of microbial diversity in the soils studied; thereby allowing for an assessment of the changes in bacterial community in relation to soil depth and time.

In order to obtain good quality DNA for further analysis, two different DNA extraction methods were evaluated in the present research. This was important because the DNA extraction method employed determines the yield of DNA per unit

soil sample and influence the composition of the bacterial community available for study (Duarte *et al.*, 1998; Krsek and Wellington 1999; Martin-Laurant *et al.*, 2001). The first DNA extraction method tested was the bead-beating method; this method did not yield good quality DNA as the extracted DNA was contaminated with humic acids which can inhibit PCR amplification (Picard *et al.*, 1992; Tebbe and Vahjen, 1993; Martin-Laurant *et al.*, 2001). In addition, this method yielded insufficient quantities of DNA for further analysis, especially from the deepest samples of the soil profile (Figure 8.1). Another problem related to the bead-beating method is DNA shear, leading to small DNA templates, and increasing the risk of the formation of chimera compounds during PCR (Liesack *et al.*, 1991; Zhou *et al.*, 1996).

The second DNA extraction method tested used a Soil DNA Isolation Kit from MO BIO laboratories. This yielded DNA that was free of humic substances and produced more DNA than the bead-beating method, even from the deepest soil samples. In agreement with these results, Niemi *et al.* (2001), when testing different methods to extract bacteria DNA from rhizosphere soil samples for PCR-DGGE, found that the Soil DNA Isolation Kit method led to more DGGE bands than other DNA extraction methods.

In the current research the use of eubacteria primers for PCR-DGGE permitted amplification of bacterial 16S rDNA from the total extracted soil DNA. However, this approach resulted in continued problems due to contamination in the negative control. It has been found that *Taq* polymerase is frequently contaminated with bacterial DNA (Böttger, 1990; Rand and Houck, 1990; Schmidt *et al.*, 1991; Rochelle *et al.*, 1992). Webster *et al.* (2003), found sequences of *Escherichia coli* in PCR negative controls

which were attributed to a batch of *Taq* DNA polymerase from the company Bioline. This *Taq* was used in the initial stages of the present study and caused the consequent problems of contamination. When Sigma Ready Mix was employed during the amplification procedure the contamination problems were greatly reduced.

Because small amounts of PCR product can potentially be carried over from one reaction to the next, and this can cause false-positive results (as has previously been outlined in Section 5.4.2), all of the PCR reactions were prepared in the Ancient DNA laboratory at the University of Hull, in order to avoid any risk of cross-contamination.

Two different PCR programs were tested for specificity in this research and the appearance of spurious smaller bands was reduced by running PCR cycles at a single annealing temperature and with low numbers of PCR cycles (Figure 8.4).

In general, research to date has focussed on the composition of microbial communities at the soil surface horizon, because this zone is assumed to be the most active. As a consequence, relatively fewer studies have considered the deeper horizons of the soil profile. Only a limited number of researchers, to date, have studied the subsurface soil layers in order to characterize microbial communities; these include Zvyaginstev (1994); Ekelund *et al.* (2001); Bundt *et al.* (2001) and Fierer *et al.* (2003).

DGGE analyses of eubacteria 16S rRNA has provided useful information about the dominant (most abundant) species within the bacterial community throughout the soil profile. This technique was employed to produce site-specific profiles of Hatfield Moor and Sutton Common. DGGE analysis of the total eubacteria community

produced complex band patterns, indicating the presence of a diverse bacterial microflora. This result accords well with previous findings in soil analysis (e.g. Torsvik *et al.*, 1990; Kuffner *et al.*, 2004).

When interpreting the data in the DGGE gels, several potential drawbacks need to be considered. Firstly, DGGE only reflects the composition of the predominant members of the bacterial community (e.g. Felske *et al.*, 1998; Nübel *et al.*, 1999). Other pitfalls during DNA extraction and PCR amplification might cause incorrect interpretation of the DGGE gels (as previously explained in Section 5.4.1) (Wintzingerode *et al.*, 1997). Another potential cause of low DGGE resolution is that communities with a large number of dominant organisms might generate an unresolved 'smear' in the gel due to the occurrence of 16S rDNA sequences with multiple melting domains (Myers *et al.*, 1988; Hedrick *et al.*, 2000; Wieland *et al.*, 2001). In addition, the assumption that one band equals one genome is not always valid, as one band might be composed of several species, or several bands can be generated from a single species, due to co-migration of bands in the gel (Van Hannen *et al.*, 1998; Sekiguchi *et al.*, 2001).

The DGGE profiles obtained in the present study generally varied with regard to the sampling site, because each site has different characteristics. There is a tendency for samples from one site throughout the soil profile to cluster together, as was observed, for example, with the August 2004 samples (Figure 8.9), October and December 2004 samples (Figure 8.10) and the October and December 2005 samples (Figure 8.16). Different soil types have specific microhabitats originated by the wide range of abiotic factors in each soil (Bossio *et al.*, 2005).

The sampling sites studied in the current study are under different land management regimes; Hatfield Moor has an agricultural regime in place, while Sutton Common is under pasture. In general, higher band richness and hence probably species richness was detected at Hatfield Moor when compared with Sutton Common, on the gels analysed (Tables 8.1 and 8.2). The DGGE analysis has therefore revealed differences in the microbial community structure in relation to soil management practice and soil type.

In agreement with the present study, Zhou *et al.* (2004), who studied microbial diversity and heterogeneity in sandy sub-surface soils in the USA, also found that samples from different geographical locations were well separated, while samples from the same location were grouped closely together. The conclusion that different soils types tend to support different bacterial communities is also consistent with the results of previous studies on several temperate agricultural soils. These studies found that soil type was the main factor in determining bacterial community structure (Gelsomino *et al.*, 1999; Buckley and Schmidt, 2001; Girvan *et al.*, 2003; Bossio *et al.*, 2005; Diepeningen *et al.*, 2006).

It should be noted that the transport of extracellular DNA through the soil profile, from the surface sediments to deeper horizons, might occur; and this extracellular DNA can persist in soil for extended periods of time (Paget *et al.*, 1998; Frostegård *et al.*, 1999 and Niemeyer and Gessler, 2002). This occurs because the DNA is protected from hydrolysis due to its absorption to soil components such as clays, sand, humic substances and polysaccharides (Alvarez *et al.*, 1988; Crecchio and Stotzky, 1988 and Pietramellara *et al.*, 2001). In addition to the transport of DNA through the soil

profile, vertical transport of microorganisms can also take place. Kieft and co-workers (1998) observed that in a grassland soil, the microbial communities in the shallower sub-surface strata were most likely influenced by transport of microorganisms from the surface. In addition, microorganisms in deeper soil horizons might be derived from the original depositional environment and persist in the soil through time (Kennedy *et al.*, 1994; Kieft *et al.*, 1998).

Another result, obtained from several of the DGGE gels in the current study, was a decrease in species richness with increasing depth. For example, this occurred in the following samples; December 2004 at Hatfield Moor, and October and December 2004 at Sutton Common for the eubacteria community (Table 8.1); August and December 2005 at Hatfield and all depths at Sutton Common in 2005 with the exception of samples from October (Table 8.2); in October and December at Hatfield Moor in 2004 for the α-proteobacteria community (Table 8.7) and in October of 2004 for the actinomycete community (Table 8.9). This situation was also in evidence during October and December of 2004, for the α-proteobacteria community at Sutton Common (Table 8.10), and in December for the actinomycete community (Table 8.11).

In agreement with the results outlined above, several other authors have found changes in microbial species richness, as a function of depth in different soil types (e.g. Henckel *et al.*, 2000; Fritze *et al.*, 2000; Ekelund *et al.*, 2001; Blume *et al.*, 2002; Fierer *et al.*, 2003; Buckley and Schmidt, 2003; Griffiths *et al.*, 2003; Agnelli *et al.*, 2004; Zhou *et al.*, 2004; Goberna *et al.*, 2005). However, the majority of these studies only considered the soil profile down to relatively shallow depths, such as 10 cm

(Buckley and Schmidt, 2003), or 20 cm depth (Griffiths *et al.*, 2003). It is likely that the greater species richness in the upper strata reflects greater taxonomic diversity associated with the rhizosphere (Smalla *et al.*, 2001; Griffiths *et al.*, 2003).

Conversely, Bund *et al.* (2001) did not find a depth gradient in species richness when analysing the bacterial communities in forest soils. In addition, the results obtained by Smith (2005), from the analysis of bacterial DGGE banding patterns from the Woldgate former landfill site (maximum depth studied 52 cm), and several archaeological sites in wetlands, showed the presence of similar bacterial populations at all depths, in different sediments and under differing physico-chemical conditions.

In the current research, several examples on DGGE gels did not show a gradient in species richness with depth. For example; the April, August and October samples at Hatfield Moor and the April and August samples at Sutton Common in 2004, for eubacteria (Table 8.1), the Hatfield Moor samples for eubacteria in 2004 (Table 8.3), and the Sutton Common samples for eubacteria in 2004 (Table 8.4), all fail to display a gradient with depth. In addition, the Hatfield Moor and Sutton Common samples for eubacteria in 2005 (Table 8.5 and Table 8.6) also fail to display any trends in reduced species abundance with depth. These results are supported by some previous studies, in which data obtained from 16S rDNA profiling has suggested that some soil bacterial communities are temporally and spatially dominated by a number of stable and ubiquitous organisms, and that many of these may be inactive (Torsvik *et al.*, 1996; Felske *et al.*, 1998). Gelsomino *et al.* (1999), using DGGE profiles of 16S rDNA (in silt loam soil), found a significant similarity between communities at various depths and over spatial and temporal gradients.

From the DGGE gels used to study the temporal dynamics of the microbial populations; e.g gels for eubacteria with 2004 and 2005 samples from Hatfield Moor and Sutton Common separately (Figures 8.17, 8.19, 8.21, and 8.23) and gels with specific primers (Figures 8.25, 8.27, 8.29, 8.31 and 8.33) change in the bacterial communities over time were identified. DGGE showed some bands that were present in all of the seasons studied, but some other bacterial species appeared only at specific times. These changes were also observed in the DGGE patterns of α- and β-proteobacteria and actinomycetes.

Seasonal dynamics of microbial populations have previously been studied by several researchers. For example, Smit *et al.* (2001) used DGGE to show differences in bacterial communities related to soil type and seasonal variation in silt loam. Considerable temporal variation by soil bacterial communities has also previously been observed in agricultural soils (Smalla *et al.*, 1998; Buckley *et al.*, 2003).

In contrast to the observations of the above authors, Gelsomino *et al.* (1999) used bacteria DGGE profiles from one plot over one year and found that seasonal fluctuation was small. This suggested that the soil bacterial community was dominated by a few stable microorganisms. Felske *et al.* (1998) also found little variation in DGGE bacterial patterns over time in a grassland soil in the Netherlands.

It has been suggested that banding patterns based on universal primers might give an inadequate image of the real species richness in natural samples (Kisand *et al.*, 2003; Gelsomino and Cacco, 2006), and that they may overlook relatively less abundant, but potentially very important species (Schmalenberger *et al.*, 2001; Boon *et al.*, 2002).

DGGE analysis of specific bacterial groups was used in the present study in order to explore the ecological importance of these groups through the soil profile. This approach has been advocated by a number of researchers (e.g. Brinkhoff and Muyzer, 1997; Heuer *et al.*, 1997; Gomes *et al.*, 2001; Gelsomino *et al.*, 2005). It is also used in the present study in order to increase understanding of the complex banding patterns obtained with the general eubacteria primers.

The use of specific primers confirmed the presence of temporal variation at both sampling sites (Figures 8.25, 8.27, 8.29, 8.31 and 8.33). This approach gave a different overview of the microbial communities at both locations. High band (species) richness of α-proteobacteria was found at both sites (Figs. 8.25 and 8.31) but especially at Sutton Common. There high species richness of actinomycetes was also found, mainly in the surface layer of the soil (Figure 8.33). Actinomycetes are widely distributed in soil; they are aerobic heterotrophs and live on decaying organic matter by decomposing a wide array of substrates, including cell wall polymers and litter, and they become dominant in the final stages of composting (Gelsomino *et al.*, 2006). β-proteobacteria showed low species richness at Hatfield Moor (Figure 8.27). Unfortunately, a DGGE gel for this group was not obtained from Sutton Common due to time constraints.

The results obtained suggest that the diversity of bacteria at Sutton Common is in fact higher than was observed using the general primers for eubacteria. However, it should be remembered that when specific primers are applied to DGGE, two successive PCR reactions are needed, and the possibility of greater bias due to preferential amplification is introduced into the analysis. As can be observed above (Figures 8.25,

8.27, 8.29, 8.31 and 8.33), the DGGE gels obtained using the specific primers presented a less clear band pattern that those obtained using general primers, and a dark background. A more thorough study of microbial diversity, employing these primers and with further development of methods is warranted.

Although none of the sequenced clones exactly match any of the sequences in the database, it is known that sequences which are cloned from environmental samples are seldom similar to the sequences of some cultured bacteria that are well represented in gene databases (McCaig *et al.*, 1999).

The predominant phyla in the cloned sequences were Acidobacteria, α-Proteobacteria and Actinobacteria. The Acidobacteria are a recently discovered linage that contains only a few representatives that have been cultured, to date. Previous studies have suggested that they are involved in methanol metabolism (Radajewski *et al.*, 2002). Dunbar *et al.* (1999) found in arid soils that approximately 50 % of clones belonged to this group. Smit *et al.* (2001) found that in a wheat field a large proportion of the sequences belonged to the Acidobacterium division. Other researchers have detected them in a range of soils (Kuske *et al.*, 1997; Hugenholzt *et al.*, 1998; Barns *et al.*, 1999; Dunbar *et al.*, 1999; Smit *et al.*, 2001). However, it appears that preferential development of these bacteria occurs in acidic environments (Hirashi *et al.*, 1995). In agreement with this observation, the cloned sample from Hatfield Moor at 30 cm, which showed higher Acidobacteria representatives, was an environment that was more acidic than, for example, that in evidence at 10 cm depth.

The Acidobacterium group and α-Proteobacteria, are able to grow aerobically and also by mixed fermentation. Fermentative growth occurs with a range of sugars, including cellulose; the use of cellulose however is not common among members of the division proteobacteria (Chin *et al.*, 1999).

The phylum proteobacteria is a varied group of bacteria, presenting significant metabolic and morphological diversity. It occurs in different environments, but more frequently in cultivated soils (Pereira *et al.*, 2006). The dominance of α-proteobacteria in soils has been observed previously. For example: in a clover pasture soil (Borneman *et al.*, 1996); acid forest soils (McCaig *et al.*, 1999; McCaig *et al.*, 2001); grassland soils (Felske *et al.*, 2000), and agricultural soils (Buckley and Schmidt, 2003). Zhou *et al.* (2004) studied microbial diversity in sandy sub-surface soils in the USA and demonstrated that most of the bacterial clones (*c.* 90 %) were affiliated with members of the Proteobacteria family. Buckley and Schmidt (2003) observed that α-Proteobacteria and β-Proteobacteria were significantly abundant from 0-10 cm depth in an agricultural soil.

In agreement with the results outlined above, the sample from Hatfield Moor at 10 cm depth presented more α-proteobacteria clones when compared with the other two analysed depths. This probably occurs because bacteria belonging to the α-proteobacteria class exhibit high growth rates in fertilized soils, where available nutrients are abundant, i.e. the top soil of Hatfield Moor (Smit *et al.*, 2001).

In previous archaeological studies, other authors such as Helms and Kilstrup (2001) also found that many of the bacteria inhabiting waterlogged wooden artefacts from the

Nydam Bog (an Iron Age site in Denmark), were Proteobacteria, mostly in the Rhyzobium-Agrobacterium subclass.

The Actinobacteria is a heterogeneous group of Gram positive bacteria which exhibit a wide range of degradative enzymatic activities (Goodfellow and Williams, 1983; Jaatinen *et al.*, 2007). They are able to degrade many resistant substances such as lignin, complex aromatics and humic acids. Acidophilic actinobacteria are implicated in the decomposition of organic matter below ground (Jaatinen *et al.*, 2007). In the current research, clones belonging to this phylum were more abundant at 30 cm depth; as a consequence, part of the enzymatic activity obtained for this depth at Hatfield Moor can be attributed to this group of bacteria.

The Actinobacteria have been isolated from, and detected in, a wide range of soils, and are generally considered to be important members of the soil microbial community (Stackebrandt *et al.*, 1997; Felske *et al.*, 2000). Some representatives of the Actinobacteria phylum produce slow-growth antibiotics commonly found in pasture and cultivated soils (Borneman *et al.*, 1996; Borneman and Triplett, 1997; McCaig *et al.*, 2001; Dunbar *et al.*, 2002).

Other divisions represented in the current study, included β-Proteobacteria, Firmicutes, Spirochaetes, Bacteroidetes and δ-proteobacteria. Some representatives of the Firmicutes phylum, such as bacteria of the genus Bacillus and Clostridium are not good in competition, and they prevail only in areas with a great quantity of available nutrients (low competition areas) (Pereira *et al.*, 2006). In the current research four representatives of this phylum were detected down the studied profile (Table 8.12),

probably indicating that nutrient availability was not a restrictive factor to bacterial growth in the soils studied.

Chouari *et al.* (2005) found that Bacteroidetes, Firmicutes, and the Delta-proteobacteria were the predominant phylogenetic groups in an anaerobic sludge digestor, where they were able to degrade a wide range of macromolecules such as cellulose, pectin, chitin, proteins and various xenobiotic compounds. In agreement with this study, the conditions at 50 cm in the soil from Hatfield Moor were anaerobic, as shown by the results obtained for redox potential at that depth in December 2005 (Figure 6.8).

Cultivation-independent 16S rRNA studies suggest that many species of Chloroflexi (Firmicutes) thrive in diverse aquatic habitats including freshwater lakes (Von Wintzingerode *et al.*, 1997; Hugenholzt *et al.*, 1998), and they were the dominant group in some grass land soils (Felske *et al.*, 1998). Chloroflexi and Acidobacteria have commonly been found in other soil clone libraries (Hugenholtz *et al.*, 1998; McCaig *et al.*, 2001; Smit *et al.*, 2001).

One particular cloned sequence matched a species from the δ-proteobacteria division. The sulphate reducing bacteria belong to this phylum (Hugenholtz *et al.*, 1998). This particular clone came from Hatfield Moor at 50 cm depth, where the conditions were anaerobic and probably favourable to the development of this group of bacteria.

The similarity values between some clones and their nearest phylogenetic neighbour were sometimes low, and many of the clones may represent uncharacterized genera.

Because these clones are different to the known sequences from cultivated bacteria, and because they branch deeply with the universal bacteria phylogenetic tree, it is not possible to draw conclusions about the detailed phenotypic properties of these microorganisms (Chandler *et al.*, 1997). However, the presumptive phylogenetic identification of the uncultured microorganisms has added significantly information to our knowledge of soil ecosystems.

8.8 Summary

This chapter has presented the results from molecular approaches based on DNA extraction from soil, PCR and DGGE using general eubacterial primers and specific primers for certain bacterial groups. In addition, the results from cloning and sequencing based on samples from three soil depths at Hatfield Moor have been presented.

The application of these molecular techniques has allowed for the characterization of the bacterial community down the soil profiles at both sampling sites, and demonstrated change in microbial community diversity over-time.

Chapter 9 will discuss and integrate the results from the current research, as derived from the integration of all of the monitoring and microbiological parameters investigated, and consider the results in relation to the specifics of site context and its relationship to *in situ* preservation studies.

Chapter 9 Discussion

9.1 Introduction

The multidisciplinary approach adopted during this study, which combined the measurement of a range of physico-chemical and microbiological parameters in the soil profile of two sites in the Humberhead Levels (Section 2.5.2), has facilitated the development of a holistic understanding of the processes taking place in the burial environment at the sites studied.

As has been previously outlined in Chapters 1 and 2, the world's wetlands contain organic material that is fundamental to our understanding of past human-landscape and socio-cultural interactions. In general, waterlogged deposits have the potential to expand our understanding of the past in a way that is seldom possible from the excavation of 'dryland' sites alone (e.g. Lillie and Ellis, 2007). As has been noted above, the world's wetlands are an extremely fragile and threatened resource. Globally, wetland losses are sufficiently serious to cause considerable concern in terms of the sustainability of this resource (e.g. Mitsch and Gosslink, 1993; Coles and Olivier, 2001; Lillie and Ellis, 2007). The situation is noticeable at the local level, in locations such as the Humberhead Levels, where impacts from peat extraction and drainage for agricultural purposes have significant impacts on the former raised mires of Thorne and Hatfield Moors (Section 2.2.5).

In light of the imminent threats to wetlands, recent research has begun to elucidate the factors in the burial environment that are responsible for the preservation or desiccation of the buried archaeo-environmental resource (Smith, 2005).

In order to ensure that a multi-disciplinary investigation of contrasting wetland contexts was possible within the confines of a PhD research framework, the current study has examined two locations with a proven potential to contain and preserve archaeo-environmental remains, Hatfield Moor (Section 2.2.4) and Sutton Common (Section 2.3). At the former location, a deep sequence of peats, representing a palaeochannel that would have flowed into the moors, provide a natural burial environment for comparative purposes. This location is contrasted against a site that is purely anthropogenic in derivation, represented by a ditch sequence located inside the smaller enclosure at the Iron Age site of Sutton Common.

The basic context of each location has been studied using the analysis of water tables, soil characteristics (redox, pH, temperature, moisture and organic matter content), particle size analysis and chemical composition (Total P, S Na, K, Mg, Ca, Mn, Co, Ni, Zn, Fe and Mo) (Chapter 3 and Appendix 1). The methodological approaches used in the conventional microbial and molecular genomic analysis of the soil bacterial communities have been outlined in Chapters 4 and 5.

Chapter 6 outlines the results obtained from the field monitoring at both sampling sites, while Chapter 7 presents and discusses the results obtained from the bacterial counts, microbial activity and physiological profiles, through the soil profile at both sites. Finally, Chapter 8 has analysed the results obtained from genetic fingerprinting by DGGE of the bacterial community, and from the cloning and sequencing of DNA from selected samples.

This discussion chapter integrates the results obtained from the different approaches outlined above in order to identify any general patterning in the data as revealed by the current study. This will allow for the recognition of the key factors responsible for wood degradation and *in situ* preservation, and a determination of the most useful parameters to study in order to shed light on the potential for the preservation of archaeological remains in wetland contexts.

9.2 Environmental factors influencing microbial abundance and activity and resultant biological decay

It is well known that abiotic conditions in soil, such as temperature, pH, nutrient levels, moisture and organic matter content influence microbial community structure (Fierer *et al.*, 2003a and b; Laiho, 2006). Different environmental factors, such as soil type, redox potential, pH, temperature, level of the water table and local hydrological regime, have been associated with the preservation of archaeological wood *in situ* (Caple and Dungworth, 1997; Corfield, 1996).

Water content and redox potential are considered to be important factors in structuring the manner in which microbial community composition varies with depth (Ekulend *et al.*, 2001; Fierer *et al.*, 2003a and b; Allison *et al.*, 2007). The results obtained from Hatfield Moor and Sutton Common have demonstrated an important association between water table variation and redox potentials (Figures 6.7 and 6.8). At Hatfield the high water table created a saturated environment, leading to the stratification of the redox conditions throughout the soil profile (Figures 6.8). However, at Sutton Common a "lag" effect in the redox reaction was observed, wherein despite a deficit of water, the removal of saturation was not automatically correlated to redox potential,

probably due to the retention of water within the silt-clay horizons at this site (Figures 6.7 and 6.8).

The monitoring of water table and redox potential in the current study has indicated those horizons where soil conditions were oxidising or reducing, and as a consequence, has functioned well as a useful tool in identifying the extent to which organic archaeological remains might be degraded when oxidising conditions are established in the burial environment (Caple, 1996). The results of a range of studies of different archaeological burial environments have led to the recommendation that the redox potential of soils must be maintained at between -100 and -400 mV, in order to ensure optimal preservation conditions for organic remains (Caple, 1996; Caple and Dungworth, 1997).

It is generally accepted that a high water table helps to prevent the *in situ* decay of archaeological artefacts as any reduction in water level has the potential to increase oxygen availability at depth (*cf.* Smith 2005). As a consequence of the removal of saturation, organic matter decomposition rates may be accelerated (Bergman *et al.*, 1999; Blodau *et al.*, 2004; Fenner *et al.*, 2005; Laiho, 2006). For example, this has previously been observed at the Sweet Track (Coles and Coles 1986), where, at the Shapwick Heath site, the water level has been maintained by pumping. As a result, the trackway appears to be in a good state of preservation and that the conditions are highly reduced (Holden *et al.*, 2006). In addition, at Wood Hall (North Yorkshire) medieval bridges over a moat were well preserved in saturated conditions (Holden *et al.*, 2006). At Sutton Common the best preserved remains were found at depth in the southern part of the large Iron Age enclosure. The good preservation of these remains

was due to their location, with the organic artefacts being permanently below the water table level. As such, these remains (at the time of study) were not affected by seasonal fluctuations in water level (Cheetham, 2004; Van de Noort, 2004a).

At Hatfield Moor, the water table has been artificially maintained at a high level as part of the 'Value in Wetness' initiative (Section 2.2.6); and the redox potentials obtained from below 10 cm in the soil profile have indicated reducing conditions throughout the current study (Figure 6.7). The physico-chemical variables studied at this site tended to be more stable than those in evidence at Sutton Common. In general, at Sutton Common, the water table was relatively high, but showed seasonal fluctuation during the monitored period (Figure 6.7) (as was also observed in previous studies [Van de Noort *et al.*, 2001; Cheetham, 2004]). In particular, there was a marked lowering of water levels in the Sutton Common ditch between June 2005 and April 2006. However, despite this observation, one of the main findings of this research is that bacterial abundance, activity and species richness was higher at the site with the higher water table (Hatfield Moor) throughout the study period. How this finding might affect the decay of archaeological remains is discussed below.

Soil moisture and organic matter content exhibit a strong positive correlation at both sampling sites (Tables 7.2 and 7.3). Both of these factors have been shown to influence the composition and distribution of microorganisms in the soil (e.g. Schimel *et al.*, 1999; Wilkinson *et al.*, 2002; Fierer *et al.*, 2003a and b). The results presented in Chapter 7 support these earlier observations as organic matter showed a positive correlation with the extracellular enzymes at Sutton Common (Table 7.3), while a negative correlation was detected for leucine assimilation and two extracellular

enzymes at Hatfield Moor (Table 7.2). The results suggest that a different chemical composition of soil organic matter might influence the activities of soil enzymes at both sites.

The results for Hatfield Moor probably indicate that the negative correlation between organic matter and microbial activity is not explained by organic matter content *per se*, as this factor was highly associated with moisture content; a factor that showed a strong negative correlation with all of the microbial variables at Hatfield Moor. Another possible explanation for the negative correlation between organic matter and microbial activity at Hatfield Moor may be that an important part of the enzymes were forming complexes with the organic matter, and as a consequence, extracellular enzyme activity was unrelated to the actual microbial biomass in the soil.

From an archaeological point of view, previous investigations have suggested that the pH levels at most sites where archaeological remains have been preserved were in the near neutral region of pH 6.0-7.2 (Caple, 1996; Welch and Thomas; 1996).

In the results obtained from the current study, at both sampling locations, the soil samples tended towards greater acidity with depth, probably because of the increase in peat content. Soil pH is considered to be an important factor affecting microbial community composition and diversity, and as a consequence pH can directly influence microbial decomposition (Hopkins, 1996; Powell *et al.*, 2001; Bååth and Anderson, 2003; Grayston *et al.*, 2003; White *et al.*, 2005; Allison *et al.*, 2007). Jordan *et al.* (2001) have previously noted that the bacteria which break-down the cellulose fraction of wood become inhibited below pH 3.

Changes in acidity and soil temperature are considered to have a 'slow down' effect on decomposition rates (Bridgham and Richardson, 1992; Bergman *et al.*, 1999). However, despite this observation, it is possible that many microorganisms inhabiting peatlands may be tolerant of acidic conditions to some degree, and as a consequence will be adapted to function at low pH levels (Williams and Crawford, 1983; Fisk *et al.*, 2003).

The results obtained in the current study have shown a strong positive correlation between pH and all of the microbial variables analysed (Tables 7.2 and 7.3). Decreases in bacterial numbers and rates of ^{14}C-leucine assimilation in soil have previously been related to increasing acidity, since bacteria are less adapted to acidic conditions (Bääth *et al.*, 1980; Matthies *et al.*, 1997). However, since the most acid soils studied in the current investigation had the highest organic matter content, it is difficult to separate the relative influence of pH *vs.* organic matter on reduced bacterial abundance (Bääth, 1998).

Several authors have suggested that litter type has a greater effect on aerobic decomposition in peatlands than the variation in environmental variables (Szumigalski and Bayley, 1996; Scheffer *et al.*, 2001). Overall, lower microbial biomass in subsurface peat can be expected since subsurface peat is highly decomposed and, as a consequence, is less energetically favourable for microbial utilization (Fisk *et al.*, 2003).

Seasonal fluctuations in temperature were also observed at both sampling locations (Figure 6.10). Although the observed temperatures increased with depth in the winter

months, in spring and summer temperatures tended to decrease with depth. Surface soil temperature varies according to air temperature and therefore exhibits more fluctuation than the subsoil (Brady, 1984), and the temperature of the soil has been shown to influence microbial activity (Williams and Crawford, 1983; Trevors, 1985; Hopkins, 1996). This situation has been observed in the present study, as temperature exhibits a strong positive correlation with bacterial abundance and phosphatase at Hatfield Moor (Table 7.3), and with bacterial abundance and ^{14}C-leucine assimilation at Sutton Common (Table 7.4).

It has been suggested that in peatlands, the biodegradation of organic compounds is often slow because one or more of the inorganic nutrients needed for microbial growth are only present in low concentrations. As a consequence, the preservation of organic archaeological remains in peatlands may be a result of insufficient nutrients as opposed to low oxygen levels, as previously thought (Painter, 1995; Cronyn, 2000). Steffensen and Alexander, (1995) proposed that the addition of inorganic nutrients to the soil may increase biodegradation. In the present study, the addition of nutrients into soil samples showed significant statistical differences in ^{14}C-leucine uptake rates between the controls and the nutrient-enriched samples (Table 7.1). Nutrient loading only affected the upper layers of the soil, thereby indicating that nutrient availability was not a limiting factor for the microbial populations in the deeper layers of the soil profile. Subsequently it is possible to conclude that decomposition was not restricted by nutrient availability in the deeper soil horizons at Sutton Common.

As previously suggested (Joergesen and Scheu, 1999; Feng *et al.*, 2003; Fierer *et al.*, 2003b; Allison *et al.*, 2007) depth has a strong impact on microbial community

composition. Bacterial abundance, extracellular enzyme activity and ^{14}C-leucine assimilation significantly decreased with increasing depth at both sampling sites. Depth was negatively correlated with all the microbial variables studied (Tables 7.2 and 7.3). However, the deepest sediments showed evidence for viable organisms and activity.

Bacterial abundance decreases with depth in the soil profiles obtained from Hatfield Moor and Sutton Common. This is in general agreement with the results obtained by other authors, in studies from different soils (Federle *et al.*, 1986; Kieft *et al.*, 1998; Ekulend *et al.*, 2001; Blume *et al.*, 2002; Taylor *et al.*, 2002; Fierer *et al.*, 2003b; Griffiths *et al.*, 2003a; Agnelli *et al.*, 2004; Allison *et al.*, 2007). The decrease in bacterial abundance with depth might be explained by the environmental conditions with depth, which become less favourable for microbial growth and activity, with low pH values, a lack of oxygen, cold temperatures and high levels of phenolic substances, etc. (Fisk *et al.*, 2003; Fenner *et el.*, 2005; Laiho, 2006). The present study corroborates the results obtained from previous studies for extracellular enzymes, together with ^{14}C-leucine assimilation (Federle *et al.*, 1986; Eivazi and Tabatai, 1990; Brake *et al.*, 1999; Aon and Colaneri, 2001; Niemi *et al.*, 2005), and confirms those obtained by Cheetham (2004) at Sutton Common. However, most of these studies only consider the soil profile to a limited depth, for example Griffiths *et al.* (2003a) studied the temporal and spatial variation of soil bacterial communities in a soil profile to a depth of 20 cm.

As has been previously shown in this study, the presence of bacteria and anaerobic activity were detected in the deepest deposits of the soils studied. This finding

probably indicates that even under anaerobic conditions, microbial activity can take place, as was previously suggested by Smith and Lillie (2007). On the other hand, it is known that wood degrading bacteria have a wide tolerance to different temperature, pH and oxygen conditions (Daniel and Nilsson, 1998); but it appears that in constrained environments bacteria degrade wood very slowly (Blanchette *et al.*, 1991; Blanchette, 1995). Low activity rates in the deeper horizons of the soil profile might indicate that most bacteria are inactive or have very slow metabolism rates (Hopkins, 1996; Welch and Thomas, 1996; D'Hondt *et al.*, 2002).

Conversely, in the surface horizons, under oxic conditions, more substrate is available for aerobic microorganisms to transfer into their biomass; they are also able to utilise energy more efficiently than anoxic organisms (Brake *et al.*, 1999). Dyckmans *et al.* (2006) observed, in a laboratory experiment, that higher soil microbial activity occurred under oxic conditions when compared with anoxic conditions, and most of the microbes that were able to survive under anoxic conditions became dormant. However further analysis will be necessary in order to understand to what extent this activity can affect organic archaeological remains. For example, it would be interesting to find out if the active bacteria evident in the lower levels of the soil profiles studied at both sites are able to degrade cellulose or lignin.

Depth of burial has been considered as an important factor affecting the decay of organic archaeological remains, as the decrease of decay with depth might be related to a reduction in oxygen availability, which influences the activity of erosion bacteria (Grinda, 1997; Björdal *et al.*, 2000; Hogan *et al.*, 2001; Cheetham *et al.*, 2004; Allison *et al.*, 2007). Research undertaken by Freeman *et al.* (1990; 1995; 2001; 2004) has

demonstrated that, in peatlands, oxygen availability limits phenol oxidase activity, thereby allowing for the accumulation of phenolic compounds that inhibit the activity of hydrolitic enzymes. However, Williams *et al.* (2000) have reported that phenol oxidase activity was regulated more by pH than by aeration of the peat, and when pH is favourable, phenol oxidase activity depends more upon the composition of the peat than on the water level. As a consequence, it is possible to assume that the role of phenol oxidase may vary according to peatlands type. This mechanism is important for the preservation of organic archaeological remains, since enzyme activity may possibly be repressed. Taking into account this research, it might be expected that if the water table falls, increased levels of oxygen will be available and a reduction in iron and phenolic compounds (enzyme activity inhibitors) could increase enzyme activities (Kang and Freeman, 1999; Fenner *et al.*, 2005).

Drainage generally increases the thickness of the aerobic layer and enhances substrate availability to heterotrophic bacteria in soil layers close to the surface (Jaatinen *et al.*, 2007). This model would explain the results obtained in the summer and autumn of 2005, when a decrease in water table level at Hatfield Moor and particularly at Sutton Common (Figure 6.7) was associated with an increment in ^{14}C-leucine assimilation rates, and to some extent to increased enzyme activities, particularly in the upper layers of the soil.

Another important observation revealed from the study of bacterial abundance, extracellular enzyme activity and ^{14}C-leucine assimilation in the current research, is the presence of seasonal variation, particularly in the upper sediments of the soil. Previous authors have obtained similar results in different soils (Rastin *et al.*, 1988;

Buchanan and King, 1992; Kaiser and Heinemeyer, 1993; Bardgett *et al.*, 1997; Hogan *et al.*, 2001; Niemi *et al.*, 2005). However, most of the results from these earlier studies are only related to depths above 50 cm in the soil profile. The higher temperatures observed in the soil profiles during the warmer months, especially in the upper layers of the soil, favour bacterial abundance and activity. This is supported by the strong positive correlations found between temperature and bacterial abundance at both sites, and with ^{14}C-leucine assimilation at Sutton Common and phosphatase at Hatfield Moor (Table 7.2 and Table 7.3).

The results of the current study highlight differences between both sampling locations in relation to bacterial abundance, activity and metabolism. Statistically significant differences were found between both sites in terms of ^{14}C-leucine assimilation, phosphatase and aminopeptidase activities (Table 7.4). These variations can be associated with different land management practices or soil types. The composition of bacterial communities has been shown to vary with land use type (Borneman and Triplett, 1997; Brake *et al.*, 1999; Nusslein and Teidje, 1999), temperature (Ward *et al.*, 1998; Trevors, 1985), nutrient status (Broughton and Gross, 2000), agricultural growing practises (McCaig *et al.*, 1999; Buckley and Schmidt, 2001), and other environmental variables.

It is possible to conclude from the present study that the combination of different environmental factors exerts an influence on the abundance, activity and metabolism of the microbial communities at each site. As a consequence, a range of different physico-chemical variables need to be considered when assessing the potential for the *in situ* preservation of archaeological remains.

9.3 Characterization and comparison of the microbial community structure at both sampling sites

One of the most important aims of this study was to investigate how microbial community diversity varies between natural and anthropogenic sites, with depth and over time. The molecular techniques have allowed for the characterization of the microbial community structure at both sampling sites, without the limitations inherent in traditional microbial studies. This has provided a more accurate view of diversity through the soil profile.

In general, the most significant results obtained from the analysis of DGGE gels from the bacterial communities at Hatfield Moor and Sutton Common are as follows:

1) The DGGE profiles obtained primarily varied with regard to sampling site; since each site has different environmental characteristics
2) There is a general trend for decreasing species richness (as indicated by the number of bands) with increasing depth
3) Shifts in the composition of the bacterial communities occur over time

DGGE analysis of the total eubacterial community produced a complex pattern of bands, indicating the presence of diverse bacterial communities in the soils studied. These results accord well with previous studies (e.g. Torsvik *et al.*, 1990; Kuffner *et al.*, 2004). Hatfield Moor had higher species richness than Sutton Common, where a limited number of dominant species were present throughout the majority of the study (Sections 8.4 and 8.5).

The DGGE analysis has also revealed differences in the microbial community structure at both sites, probably as a result of soil management practices and soil type. The sampling locations in the study are under different land management regimes; Hatfield Moor has an arable cropping regime in place, while Sutton Common is used for pasture. Previous authors have showed that land management determines microbial community structure in soil (e.g. McCaig *et al.*, 1999b; Webster *et al.*, 2002; Clegg *et al.*, 2003; Griffiths *et al.*, 2003a and b; Bossio *et al.*, 2005; Kennedy *et al.*, 2005). The results from the current research conform to those obtained from previous studies of shallow soil profiles under differing land use regimes.

Soil type is also an important determinant of the composition of microbial communities, as the combination of soil abiotic conditions such as soil texture and structure, organic matter, soil moisture, temperature, pH, nutrient availability, etc determine the habitable niches in soil (Fierer *et al.*, 2003a and b; Garbeva *et al.*, 2004; Diepening *et al.*, 2006). The results obtained from the DGGE gels in the present study were consistent with findings of previous studies on temperate agricultural soils. These have demonstrated that soil type is the main factor determining bacterial community structure (e.g. Gelsomino *et al.*, 1999; Buckley and Schmidt, 2001; Wieland *et al.*, 2001; Girvan *et al.*, 2003; Bossio *et al.*, 2005; Diepening *et al.*, 2006; Singh *et al.*, 2006).

Hatfield Moor has different and more stable environmental conditions when compared with Sutton Common. As a consequence, Hatfield Moor has high biological diversity and is probably more resilient to stresses as for example to changes in the height of the water table. High biological diversity in the soil might be explained by two main

factors; resource heterogeneity and spatial isolation (Zhou *et al.*, 2002; Horner-Devine *et al.*, 2004). Zhou *et al.* (2004) observed that, in sandy soils, the microbial community in the subsurface was much less diverse than at the surface, with this being mainly due to spatial isolation. They suggest that low moisture in the surface reduces the connectivity between soil particles, creating a high degree of spatial isolation. In contrast, in saturated soils the excess of water allows for a high level of connectivity. Consequently, there is an opportunity for bacterial competition due to movement of nutrients and microbes. Thus, some bacteria become dominant, which leads to a decrease in diversity and a more uniform community composition (Zhou *et al.*, 2002; Horner-Devine *et al.*, 2004). Nevertheless, this would have little effect on the bacterial community within a peat soil such as at Hatfield Moor, where high species richness and high substrate utilization diversity were detected throughout the soil profile.

Another important result obtained from the DGGE gels in this study was the decrease in species richness with increasing depth. In agreement with this result, several authors have also found significant shifts in microbial species richness as a function of depth in different soils (e.g. Henckel *et al.*, 2000; Fritze *et al.*, 2000; Ekelund *et al.*, 2001; Blume *et al.*, 2002; Fierer *et al.*, 2003b; Buckley and Schmidt, 2003; Griffiths *et al.*, 2003a; Agnelli *et al.*, 2004; Zhou *et al.*, 2004; Goberna *et al.*, 2005).

The composition of the bacterial community in the surface soil (adjacent to the rhizosphere) has been widely studied because it has higher activity than the subsurface soil horizons. As a consequence, only a limited range of studies have considered the subsurface horizons of the soil profile (1-2 metres depth) (e.g. Ekulend *et al.*, 2001;

Bundt *et al.*, 2001, Fierer *et al.*, 2003b; Agnelli *et al.*, 2004). Several authors have explained the greater number of bands in the upper part of the soil profile as being a result of the abundance of taxa associated with the rhizosphere (Smalla *et al.*, 2001; Griffiths *et al.*, 2003a). Agnelli *et al.* (2004) observed that in a forest soil, the DGGE analysis evidenced complex banding patterns for the upper horizons and less diversity in deeper horizons (to a depth of 150 cm). Griffiths *et al.* (2003) indicate that differences in genetic diversity through the soil profile can be explained by the fact that soils present greater resource heterogeneity in the upper layers and that this creates a variety of microhabitats allowing for the maintenance of high levels of microbial diversity.

The presence of temporal microbial community shifts was detected on the DGGE gels analysed in the current study (Sections 8.4 and 8.5). The seasonal dynamics of microbial populations have previously been observed by several studies in different soils (Gomes *et al.*, 2001; Smit *et al.*, 2001; Girvan *et al.*, 2004). Smit *et al.* (2001) found differences in soil type and seasonal variation on bacterial DGGE profiles in a silt loam soil. Considerable temporal variation in soil bacterial community structure has also been observed for agricultural soils (Smalla *et al.*, 1998; Buckley *et al.*, 2003). However, in contrast, Gelsomino *et al.* (1999) found that by using bacteria DGGE profiles seasonal fluctuations were small, thereby suggesting that the soil bacteria community is dominated by a few dominant and stable microorganisms. Felske *et al.* (1998) also discovered limited variation in DGGE bacterial patterns over time in a grassland soil in the Netherlands.

The predominant phyla in the cloned sequences were Acidobacteria, α-Protobacteria and Actinobacteria. The Acidobacteria are present in many soils (Boon *et al.*, 2001; Smit *et al.*, 2001) and are involved in methanol metabolism (Radajewski *et al.*, 2002). The dominance of Alpha-proteobacteria in other soils has been observed in acid forest soils (McCAig *et al.*, 1999; McCaig *et al.*, 2001); grassland soils (Felske *et al.*, 2000) and agricultural soils (Buckley and Schmidt, 2003). Helms *et al.* (2004) identified anaerobic bacteria from archaeological wood, of Iron Age date, by culturing and cloning. Most of the clones were affiliated to unidentified members of the α-proteobacteria phyla. The Actinobacteria have been isolated and detected from a wide range of soils, and are generally considered to be important members of the soil microbial community (Stackebrandt *et al.*, 1997; Felske *et al.*, 2000). Actinobacteria are able to degrade many resistant substances such as lignin, complex aromatics and humic acids; and some acidophilic actinobacteria are able to decompose acid litter above ground (Jaatinen *et al.*, 2007).

9.4 The potential for preservation *in situ* of organic archaeological remains at Hatfield Moor and Sutton Common

From an archaeological perspective, the fact that Hatfield Moor has presented more enzyme activity, greater bacterial abundance and species richness, etc, throughout the majority of the soil profile, when compared with Sutton Common, confirms the previous observations that both saturated conditions and a high water table are not enough to guarantee the preservation *in situ* of archaeological remains (Smith, 2005; Smith and Lillie, 2007).

Degens *et al.* (2001) have suggested that reductions in catabolic diversity and changes in soil properties due to land use could reduce the resistance of the microbial community to stress or disturbance (e.g. fluctuations in moisture due to changes in the water table). When applying this hypothesis to the results in the current research, the Hatfield Moor samples clearly present high catabolic diversity (richness in the number of substrates metabolised by the soil microbial community), and as such would be more resistant to stress or disturbance than the Sutton Common samples. Initially, Hatfield Moor (before management of the water table at levels close to the ground surface) probably presented high catabolic activity. Consequently, the fact that the water table was increased, did not greatly affect the microbial community, since high biodiversity provides new species to the ecosystem, thereby allowing for the maintenance of soil biological processes (Griffiths *et al.*, 2001). On the basis of the above observation it appears that biodiversity is of greater significance to the stability of ecosystems after a perturbation event. In contrast, Sutton Common, with lower catabolic and community diversity, would present less resistance to stress or disturbance in the archaeological sequences studied.

Changes in the moisture regime in the soil (i.e. levels of saturation) represent a physiological stress for the microbial community and can reduce soil microbial diversity in favour of those species that are best adapted to deal with the given stress (Atlas *et al.*, 1984b; Fierer *et al.*, 2003a). It is important to consider the fact that it may not be necessary for an archaeological deposit to be completely waterlogged in order for the contained organic materials to be preserved, as there are other factors that need to be considered (Smith, 2005; Holden *et al.*, 2006).

The reburial of archaeological remains in a terrestrial environment, beneath the water table, does not ensure that anaerobic conditions are going to be re-established; and as a consequence microbial activity can attack the wood (Riess and Daniel, 1997; Jordan, 2001). The level of the water table is an insufficient indicator of the state of preservation (Pollard, 1998; Kenward and Hall, 2000; Van de Noort *et al.*, 2001). In addition, a drop in the water table below the level of the organic deposits may not lead to the destruction of the anoxic conditions, as moisture can be held in the soil (e.g. the Sweet Track) (Holden *et al.*, 2006). The changes and interactions observed in the biotic and abiotic factors affecting decomposition, following a natural or artificial lowering of peatland water levels, are complex and have not been previously explored (Laiho, 2006). In the few field experiments where drainage-induced changes were studied, contradictory results were obtained; changes in decomposition rates can increase or decrease and even situations where no change occurred have been observed (Lieffers, 1988; Minkkinnen *et al.*, 1999; Domisch *et al.*, 2000; Laiho *et al.*, 2004). All of these different responses could be associated with peat type and environmental conditions (Laiho *et al.*, 2006).

Hogan *et al.* (2001) have suggested that the reburial of exposed organic archaeological remains in a similar, but stable, natural environment may provide long term preservation. Similarly, Welch and Thomas (1996) have proposed that preservation depends on the maintenance of stable chemical conditions in the burial environment. Holden *et al.* (2006) have suggested that permanent waterlogging could lead to good *in situ* preservation. Powell *et al.* (2001) recommended that a saturated burial environment would be the better option to preserve wooden Bronze Age timbers, since oxygen would be excluded from the system and decay by aerobic fungi would

be prevented. Nevertheless, despite all of the above observations, the findings from the current research have demonstrated that the waterlogged and stable natural environment at Hatfield Moor may actually present less potential for *in situ* preservation, because bacterial abundance, enzyme activity, catabolic activity and species richness were higher in this site type when compared with Sutton Common. Fisk *et al.* (2003) have also found that microbial activity was higher in sites with lower pH and higher water tables in northern peatland ecosystems.

As a consequence, it is possible to suggest that microbial decay may be the principal cause of degradation of archaeological wood in the soil environment. This observation has previously been suggested by a number of researchers (e.g. Blanchette *et al.*, 1990; Blanchette and Hoffman, 1994; Blanchete, 1995; Hopkins, 1996; Nilsson, 1999; Powell *et al.*, 2001; Björdal and Nilsson, 2002; Smith, 2005; Smith and Lillie, 2007). Smith (2005) monitored the degradation of fresh wood from the initial stages of burial over a two-year period under controlled conditions (lysimeters), and demonstrated that changes in the degree of saturation of the sediment, as suggested by different authors (Chapman and Cheetham 2002, Cheetham 2004, Hogan *et al.*, 2001), was not the principal variable responsible for degradation.

9.5 Summary

This chapter has presented a summary of the main findings obtained by using several different techniques adopted during this thesis. The techniques included the monitoring of the physico-chemical nature of the burial environments studied, and the study of bacterial communities through the soil profile from each of the sites by using conventional microbial techniques and a molecular fingerprint approach.

By applying this holistic approach, the research has monitored the physico-chemical and microbiological characteristics and function through two soil profiles at depth intervals of 10 cm to 100 cm. Therefore, this study has enhanced our understanding of microbial ecology through the soil profile and studied changes in the physico-chemical characteristics of soils as a function of depth.

The methodology used during this thesis has determined the main factors controlling microbial community structure, activity and function; which has taken place through the soil profile at both sampling locations. The results have been discussed in terms of the potential for the decay of organic archaeological remains in wetland contexts.

The main finding of the current research has been to demonstrate that microbial activity can be very high, even at depth, in saturated environments. Since current research suggests that this is the principal factor affecting the degradation of organic archaeological remains in wetlands, it is suggested that the level of the water table and the redox potential of the soil are insufficient indicators in determining the potential for *in situ* preservation. As a consequence, the analysis of microbial activity and composition in the soil profile are necessary in order to fully assess the potential for the *in situ* preservation of organic archaeological remains.

The final chapter of this thesis will consider the aims of the present research, provide an overview of the main findings and evaluate these in relation to the *in situ* preservation of archaeological remains in wetlands. Finally, suggestions for advancing the agenda will be offered in light of the observations presented above.

Chapter 10 Conclusions

10.1 Introduction

Different European directives, such as the Environmental Impact Assessment Directive and the English legislation, Planning and Policy Guidance Note 16 (PPG 16), propose that archaeological remains might be preserved in their 'natural' environment (i.e. *in situ*) until such a time that better analysis, excavation or storage can be achieved (Holden *et al.*, 2006).

The main aim of the current research was to enhance our understanding of the physico-chemical and microbiological processes related to the preservation of organic archaeological remains in waterlogged soils, and to provide insights into the most appropriate environmental parameters for the monitoring of archaeological sites in wetlands. The use of a multidisciplinary approach to characterise the soil profile has provided valuable information about the burial environment at both sampling sites.

The most important objectives of the research can be summarized as:

1. To assess the influence of environmental variables at both sampling sites, especially the height of the water table and redox potential (through the soil profile), upon changes in the activity, physiological profile and composition of the bacterial community related to the decomposition process.
2. To enhance current understanding of bacterial populations within the burial environment by using polymerase chain reaction-denaturant gradient gel electrophoresis (PCR-DGGE) on soil samples obtained at different depths and during seasons through the soil profile.

3. To identify the potential for preservation *in situ* of organic archaeological remains at both sites studied.

This chapter will review the key results from the various approaches applied in this study and assess the relationship between each of the areas of analysis. Finally, from the results obtained in the thesis, some recommendations for future work and management will be drawn.

10.2 Review of the main findings

10.2.1 Characterising the soil profile using physico-chemical and microbiological approaches

The current research has studied quantitative changes in a number of physico-chemical variables (as shown in Sections 3.3-3.9), and quantitative and qualitative changes in bacterial variables, through the soil profile, using a range of different techniques. The data generated during the monitoring process has allowed for an enhanced understanding of both the general processes taking place in the burial environment and the complex interactions between different variables through the soil profile.

The main observations obtained from the results of this study are summarised as:

1. Monitoring of the primary physico-chemical processes that take place within the burial environment was necessary in order to identify the potential for *in situ* preservation of organic archaeological remains in wetlands. On the contrary, the monitoring of a single variable (in particular the height of the

water table) is an insufficient indicator as to the state of preservation of organic remains.

2. Bacterial abundance, extracellular enzyme activity, ^{14}C-leucine assimilation and carbon substrate utilization generally decreased with increasing depth through the soil profile. This variation indicates where the bacterial potential for degradation was reduced, and vice versa.
3. In general, bacterial abundance, extracellular enzyme activity and carbon substrate utilization were lower at Sutton Common when compared with Hatfield Moor. This demonstrated metabolic and compositional differences in bacterial community structure between both sites.
4. The bacterial communities were characterized by DGGE; which allowed for the separation of the dominant bacterial taxa through the soil profile. DGGE not only showed a different pattern of banding at each sampling location; but in general fewer bands were present in the deeper layers of the soil which was in contrast to the increased number of bands in the upper soil horizons. These results indicate differences in bacterial community structure at each site and through the soil profile.

In summary, the current research has demonstrated that the bacterial community differs between sampling locations, in relation to soil depth, and also temporal changes. These results have facilitated an assessment of the fundamental physico-chemical and microbiological conditions that might favour or inhibit the decomposition of organic archaeological remains at the sites studied, and as a consequence provided direct insights into their *in situ* preservation at the locations investigated. The integration of standard approaches to the characterisation of the

burial environment alongside conventional microbiological methods and molecular techniques was fundamental to the realization of the above analysis.

10.2.2 Main factors affecting microbial communities in the studied soils

As noted above, the present study has employed a multidisciplinary monitoring programme which included physico-chemical soil assessment, conventional microbial techniques and molecular genomic analysis through the soil profile to a depth of 1 metre at both sampling sites. The results obtained from these three different approaches have characterised the patterns and processes responsible for bacterial decay in both burial environments.

The monitoring of soil redox potential combined with water table height has allowed for the successful characterisation of the burial environment in terms of presence or absence of anaerobic conditions. The results from the monitoring programme demonstrated a redox gradient through the soil profile, with conditions generally becoming more reduced with increased depth.

The results generated during this study have identified several factors responsible for bacterial abundance and activity. Depth is the most important environmental factor to consider when the *in situ* preservation of organic archaeological remains needs to be assessed. Depth has a strong impact on bacterial community composition and metabolism, since all of the microbial variables analysed in this study significantly decreased with depth. The influence of depth is explained by the presence of unfavourable or less favourable conditions (i.e. reducing environment, increasing

acidity, low temperature, etc.) for bacterial activity and growth; however no nutrient limitations were detected in the deepest layers of the soil.

One of the main physico-chemical parameters affecting the presence and abundance of microorganisms through the study was pH. The value of pH showed a statistically significant correlation with all of the microbial variables studied, and as a consequence, was one of the main factors influencing bacterial decay. The pH data were also significantly correlated with redox potential values and it is recognized that both can cause important changes in bacterial activity and metabolism.

One of the key findings of this research was that bacterial abundance, activity and species richness was higher at Hatfield Moor when compared with Sutton Common. The continual high water table at Hatfield Moor promoted more stable environmental conditions. This stability promotes high bacterial variability, and as a consequence, this might indicate a high potential for organic degradation (Daniel and Nilsson, 1987; Blanchette and Hoffmann, 1994; Kim *et al.*, 1996; Björdal, 1999; Björdal 2000; Blanchette, 2000; Kim and Singh, 2000; Helms *et al.*, 2001; Powell *et al.*, 2001; Gregory *et al.*, 2002; Caple, 2004; Helms *et al.*, 2004). At Sutton Common, the water table showed seasonal fluctuations during the monitored period, especially in the summer of 2005. This site, where the conditions are less stable, presented a low level of bacterial activity and diversity. Consequently, given the extensive literature relating to microbial decay cited above, it could be suggested that this site is more suitable for the continued preservation *in situ* of organic archaeological remains when compared to Hatfield Moor.

The soil moisture content results indicate that this factor was negatively correlated to the microbial variables studied at Hatfield Moor, while no significant influence was detected at Sutton Common. These findings support the view that a high water table might not always promote good preserving conditions.

The differences between the two sites are mainly explained by soil type and land management regimes. Soil type is an important factor in determining the composition of the microbial communities, as the combination of soil abiotic conditions (such as soil texture and structure, organic matter content, soil moisture content, temperature, pH, nutrient availability, etc.) all determine the habitable niches in soil. In addition, as noted above, the sampling sites in this study are under different land management regimes. Hatfield Moor has an agricultural regime in place with water levels continually kept high as part of the (then) Countryside Agency's 'Value in Wetness' initiative (-despite the demise of this initiative the current landowner continues this management regime). Until recently, Sutton Common has been under various arable regimes and subjected to heavy drainage until its purchase by the Carstairs Countryside Trust. It is currently under pasture.

In order to assess the potential for preservation of organic archaeological remains, the characterisation of the microbial communities, together with the other monitoring parameters, has provided an explanation for the patterning observed in the data generated during the current study.

10.2.3 Potential for *in situ* preservation of organic archaeological remains

From the results generated during the current study it is possible to conclude that microbial decay is the principal cause of the degradation of archaeological wood in the soil environment. However, this does not discount the potential decay of the wooden artefacts by fungal activity prior to burial and/or cellulose hydrolysis during burial. In relation to waterlogged burial environments in particular, bacteria are the main variable responsible for the decay of organic archaeological remains. As such, the present study has focussed on their analysis and characterisation.

The results obtained from this research have demonstrated that through the soil profile microbiological monitoring, which is based on the detection of the presence and activity of bacteria operating both under anaerobic and oxidising environments that have the potential for *in situ* preservation, is a fundamental tool for characterising waterlogged archaeological sites.

Perhaps counter intuitively, the results of the current research have shown that the waterlogged and stable 'natural' environment at Hatfield Moor might, in fact, present a lower potential for *in situ* preservation, when compared with the archaeological context at Sutton Common. This is due to the fact that bacterial abundance, enzyme and catabolic activity, and species richness were higher at Hatfield Moor.

However, it is recognised in this research that only one monitoring location was studied at each study site, and as a consequence the monitored parameters might not reflect the general conditions in the area as a whole. The monitoring of a wider area,

including more sampling locations, will better reflect the conditions in the burial environment; but this level of analysis was beyond the scope of the current study. In particular, the microbiological investigations undertaken during this study necessitated a considerable amount of laboratory time, both in the development of the techniques applied, and the analysis of multiple depths through the soil profiles at each of the study sites. Any future studies would need to account for the time needed to undertake this type of analysis during the design of the research programme.

Overall, the results obtained suggest that changes in microbial community structure and activity in response to environmental changes will be dependant on the nature of the depositional environment. However, the application of the monitoring techniques considered during this study to other sites containing wet-preserved archaeological remains would help to characterise the burial environment and facilitate an assessment of the potential for preservation of any contained archaeological remains.

10.3 Recommendations for future research and management

In view of the results presented above, the implementation of more intensive monitoring schemes for the future management and conservation of wetland archaeological sites is recommended. Investigating the following parameters associated with the burial environment is considered to be fundamental to any future monitoring programmes at locations/sites with the potential to contain waterlogged archaeological remains:

1. Water table (via piezometers)

2. Redox potential (via nests of *in situ* redox probes located throughout the archaeological layers and monitored at regular intervals)
3. pH and temperature (through the profile)
4. Percentage of organic matter and moisture content in the soil
5. Microbial activity (via enzyme activity or ^{14}C-leucine assimilation)
6. Physiological profile of the microbial community (via Biolog ecomicroplates)
7. DGGE profiling of the bacterial community at locations through the profile that conform to archaeological horizons or pre-determined sampling depths (- parameters such as redox potential, pH and temperature should all be monitored at corresponding depths).

In addition to an effective monitoring programme, further analysis will be necessary in order to determine the extent to which any microbial activity detected in the deeper layers of the soil can affect organic archaeological remains, and to evaluate the rate at which any decomposition might occur. Moreover, the improvement of the techniques employed in the anaerobic approach to the soil samples collected during this study should assist in determining the actual potential for degradation in the deepest layers.

Phylogenetic studies should be taken further in order to identify the precise composition of the bacterial community through the soil profile and at different sites in order to obtain a collection of sequence information that can be used for future research. This will help, for example, in the selection of specific microorganisms to function as bio-indicators of particular conditions in the soil that might benefit, or compromise, the decomposition process in the burial environment.

The results generated during this study demonstrate that, in terms of the management of sites with a high archaeological potential such as Sutton Common, flooding or re-wetting of the site in order to maintain a high water table might be an unhelpful approach to the preservation of the organic resource. This is contrary to previous research (e.g., Cheetham 2004, Van de Noort *et al.*, 2001), and following on from the observations made by Chris Caple (1996: 122), who states that: "if we are to preserve these deposits it is essential to maintain *all* elements (his emphasis) of this environment at the appropriate levels. The presence of water in such deposits is not sufficient to preserve the anoxic conditions and thus their archaeological contents", the current study has shown that over a decade later, these requirements are still essential if we are to manage *in situ* preservation in wetlands.

10.4 Summary

In summary, the physico-chemical and the conventional and molecular microbiological results presented in this thesis have shown that microbial activity was the principal factor affecting the degradation of organic archaeological remains in the sites studied.

It is also possible to conclude that the site hydrology and/or soil redox potential are insufficient indicators with which to measure the state of, and the potential for, *in situ* preservation at a specific site. As such, the application of a variety of microbiological techniques, in order to determine the levels of microbial activity and community composition within the burial environment, is clearly essential for the assessment of preservation potential in waterlogged burial environments.

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Appendix 1

This appendix contains the results from the soil analysis measured in ppm (mg kg^{-1}) of dry soil throughout the soil profile at both sampling sites in October 2005, February 2006 and April 2006. Table A1 presents the values obtained from Hatfield Moor and Table A2 presents the values from Sutton Common.

Table A1: Hatfield Moor soil analysis measured in ppm (mg kg^{-1}) of dry soil throughout the soil profile in October 2005, February 2006 and April 2006.

Oct-05												
Depth	**P**	**S**	**Na**	**K**	**Mg**	**Ca**	**Mn**	**Co**	**Ni**	**Zn**	**Fe**	**Mo**
10 cm	1005.4	2183.7	93.6	1818.4	4241.4	9596.1	1021.5	15.6	29.7	145.5	29665.5	0.3
30 cm	671.5	2497.1	117.6	1788.3	4126.1	9327.8	912.5	15.9	29.3	162.2	24307.2	0.8
50 cm	187.5	2101.5	164.2	1552.5	4761.6	3309.0	115.1	7.7	28.5	77.8	14897.0	0.8
70 cm	270.7	3631.7	219.2	1493.6	3787.6	4343.3	102.5	6.8	28.4	88.7	12703.3	1.0
100 cm	199.6	7066.8	205.0	1044.4	2431.4	3973.9	74.2	6.6	21.8	75.5	11758.2	1.3

Feb-06												
Depth	**P**	**S**	**Na**	**K**	**Mg**	**Ca**	**Mn**	**Co**	**Ni**	**Zn**	**Fe**	**Mo**
10 cm	922.6	2050.7	130.9	2111.9	3805.1	8374.0	917.0	14.6	27.8	136.0	27294.0	1.3
30 cm	794.4	2546.3	144.7	2230.5	4100.6	9574.1	697.5	13.2	27.0	148.3	23317.4	0.4
50 cm	209.5	2476.4	183.4	2251.2	4549.5	5392.7	136.7	9.1	28.9	97.7	14994.1	0.4
70 cm	290.4	4141.6	263.9	2212.5	4609.2	6631.9	125.9	11.0	34.1	107.0	13721.2	1.9
100 cm	101.4	10838.0	172.0	1060.0	1799.2	6010.4	119.8	9.8	17.7	121.8	12416.0	2.0

Apr-06												
Depth	**P**	**S**	**Na**	**K**	**Mg**	**Ca**	**Mn**	**Co**	**Ni**	**Zn**	**Fe**	**Mo**
10 cm	714.6	1334.3	121.0	3819.3	2915.5	5966.2	579.9	9.3	19.4	92.3	19854.5	0.0
30 cm	558.9	1278.5	148.3	3857.0	3150.9	5748.1	583.2	9.9	20.4	91.7	19821.5	-0.1
50 cm	263.5	3567.2	236.5	3508.3	2867.3	4952.6	90.8	5.5	22.8	56.6	10607.6	1.0
70 cm	277.3	6423.2	227.0	2560.0	2128.4	4398.5	65.4	5.6	19.0	56.7	11123.3	0.7
100 cm	222.2	12090.8	239.8	2385.5	1853.8	4185.3	95.9	10.3	22.4	74.3	15605.9	0.5

Table A2: Results from Sutton Common soil analysis measured in ppm (mg kg^{-1}) of dry soil throughout the soil profile in October 2005, February 2006 and April 2006.

Oct-05												
Depth	**P**	**S**	**Na**	**K**	**Mg**	**Ca**	**Mn**	**Co**	**Ni**	**Zn**	**Fe**	**Mo**
10 cm	258	997	54	225	414	3085	60	2.9	7.5	36.5	11489	1.481
30 cm	256	2940	34	368	328	1204	16	2.5	14.6	29.3	8172	2.086
50 cm	668	13384	51	449	162	1719	28	2.3	16.4	19.2	20174	4.853
70 cm	304	14245	56	289	117	1567	31	1.9	10.8	15.6	6846	4.242
100 cm	45	24131	30	586	852	260	307	11.5	22.3	96.1	21507	0.251

Feb-06												
Depth	**P**	**S**	**Na**	**K**	**Mg**	**Ca**	**Mn**	**Co**	**Ni**	**Zn**	**Fe**	**Mo**
10 cm	943	869	102	1414	483	2870	158	3.4	10.6	75.1	9829	1.448
30 cm	914	882	123	1392	451	1722	117	5.2	15.7	88.8	14025	2.968
50 cm	237	6519	63	717	402	381	32	3.5	6.3	16.0	17079	0.352
70 cm	0.95	3051	28	291	315	232	61	2.0	4.5	9.2	7408	0.379
100 cm	230	2364	76	2267	3855	1830	823	16.6	43.7	74.9	23358	0.214

Apr-06												
Depth	**P**	**S**	**Na**	**K**	**Mg**	**Ca**	**Mn**	**Co**	**Ni**	**Zn**	**Fe**	**Mo**
10 cm	393.2	253	80.5	1003	532	1508	94.0	2.99	9.95	45.10	9175	0.52
30 cm	530.8	772	69.2	814	549	1192	33.2	3.63	14.61	33.67	10427	1.80
50 cm	352.5	8684	90.7	1443	471	1335	23.9	1.95	13.50	15.98	11385	2.93
70 cm	133.5	11805	57.9	1441	612	214	172.4	7.21	13.75	40.74	12392	-0.19
100 cm	113.3	4098	53.7	1527	897	793	211.5	5.75	13.77	33.67	12915	-0.02