Application of molecular biological techniques to study autotrophic ammonia-oxidising bacteria in freshwater lakes.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

by

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Contents.

Abbreviations

ANOVA	analysis of variance
bp	base pair
CFU	colony forming unit
CTC	5-cyano-2, 3-ditoyl tetrazolium chloride
(d)ATP	(deoxy) adenosine triphosphate
(d)CTP	(deoxy) cytidine triphosphate
(d)GTP	(deoxy) guanosine triphosphate
(d)TTP	(deoxy) thymidine triphosphate
DAPI	4',6'-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediamine tetra acetic acid
FBA	Freshwater Biological Association
FCM	flow cytometry
FITC	fluorescein isothiocyanate
g	1x gravitational force
IFE	Institute of Freshwater Ecology
kb	kilobase
KDa	kilodalton
LB	Luria-Bertiani
LSU	large ribosomal subunit
min	minutes
M	moles per litre
MFF	membrane-filtered formaldehyde
MPN	most probable number
mRNA	messenger ribonucleic acid
MSD	minimum significant difference
OD_n	optical density at n nanometres
OPD	oligonucleotide probe database
p	probability

PCR	polymerase chain reaction
rDNA	aka. genes encoding rRNA
RDP	Ribosomal Database Project
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
rrn	genes encoding ribosomal RNAs
rRNA	ribosomal ribonucleic acid
s	seconds
SDS	sodium dodecyl sulphate
SSPE	salt-sodium phosphate-EDTA
SSU	small ribosomal subunit
T_m	reversible melting temperature
Taq	*Thermus aquaticus*
UV	ultra-violet
v/v	volume / volume
w/v	weight / volume
X-gal	5-bromo-4chloro-3indolyl-β-D-galactoside

Standard abbreviations for bases

A	adenosine	**W**	A or T
C	cytosine	**K**	G or T
G	guanine	**M**	C or A
T	thymine	**B**	not A
U	uracil	**D**	not C
Y	C or T pyrimidine	**H**	not G
R	A or G purine	**V**	not T
S	C or G	**N**	unknown

Inter-conversions of mass and molarity for nucleic acids assume a molecular mass of 330 grams per nucleotide.

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Abstract.

Autotrophic ammonia-oxidising bacteria are largely responsible for the oxidation of ammonia to nitrite and are important in the environmental cycling of nitrogen. Analysis of population structure and diversity of nitrifying bacteria in nature is limited due to the difficulty in isolating pure cultures of these organisms. 16S rRNA gene sequences selected from ammonia-oxidising bacteria have enabled PCR amplification primers and probes of differing degrees of specificity to be designed. The 16S rDNA was isolated from Buttermere, an oligotrophic freshwater lake, and Windermere, a mesotrophic/ eutrophic freshwater lake in the English Lake District.

The 16S rRNA genes were amplified in a nested-PCR reaction and the products confirmed by hybridisation to an ammonia-oxidiser specific oligonucleotide probe. The ubiquity of *Nitrosospira* spp. was demonstrated in Buttermere and Windermere. In contrast, *Nitrosomonas* spp. were directly detected in both sediment and lakewater from Buttermere during the summer months and periodically throughout the year in the North Basin of Windermere.

Phylogenetic delineation of the 16S rRNA gene sequences confirmed the fidelity of the nitrosomonad DNA and identified two groups homologous to either *Nitrosomonas europaea* or *Nitrosomonas eutropha* which predominated in the littoral and profundal sediments respectively. Direct detection of nitrosospira but not nitrosomonad DNA has been reported in studies of other freshwater lakes. Population diversity was observed both spatially and temporally.

Culture techniques complemented 16S rDNA analysis to elucidate the community diversity of ammonia-oxidising bacteria in freshwater environments. Populations of ammonia-oxidising bacteria exhibiting tolerance to high ammonium concentrations were identified. The metabolically active populations were investigated by *in situ* hybridisation and flow cytometry. Further analysis may elucidate the significance of these populations in the nitrification of freshwater lakes.

Chapter 1. Introduction.

1.1. Overview.

Nitrification is an integral part of the nitrogen cycle, which is of fundamental importance to all ecosystems (Kuenen & Robertson, 1994). The reaction has been accepted as a microbiological process since the late 19th Century (Macdonald, 1986). Nitrifying bacteria have been isolated from diverse environments, and are ubiquitous in soils, sewage disposal systems, marine and fresh waters (Watson *et al.*, 1989; Koops & Möller, 1991; Hiorns *et al.*, 1995; Hovanec & DeLong, 1996).

The principal organisms involved are Gram-negative, obligate chemoautotrophs, that utilise carbon dioxide as the sole source of carbon, and derive energy from the oxidation of inorganic forms of nitrogen (Prosser, 1989; Watson *et al.*, 1989). The autotrophic physiology and consequent low growth rates of these organisms, have caused them to be intractible to conventional methods of isolation and phenotypic characterisation (Watson *et al.*, 1981; Koops & Moller, 1991). In that respect, culture-independent methods have provided invaluable tools in the ecological study of nitrification (Hiorns *et al.*, 1995; Ward *et al.*, 1997).

1.2. Nitrification as a bacteriological process.

Nitrification describes the oxidation of ammonia to nitrate, via nitrite, and provides a link between the reduced and oxidised components of the nitrogen cycle (**Fig. 1.1**) (Bock *et al.*, 1989). Chemoautotrophic bacteria are primarily responsible for the reaction (Hall, 1986). Nitrification by heterotrophic bacteria is considered to be significant only in certain environments that are unfavourable for the growth of

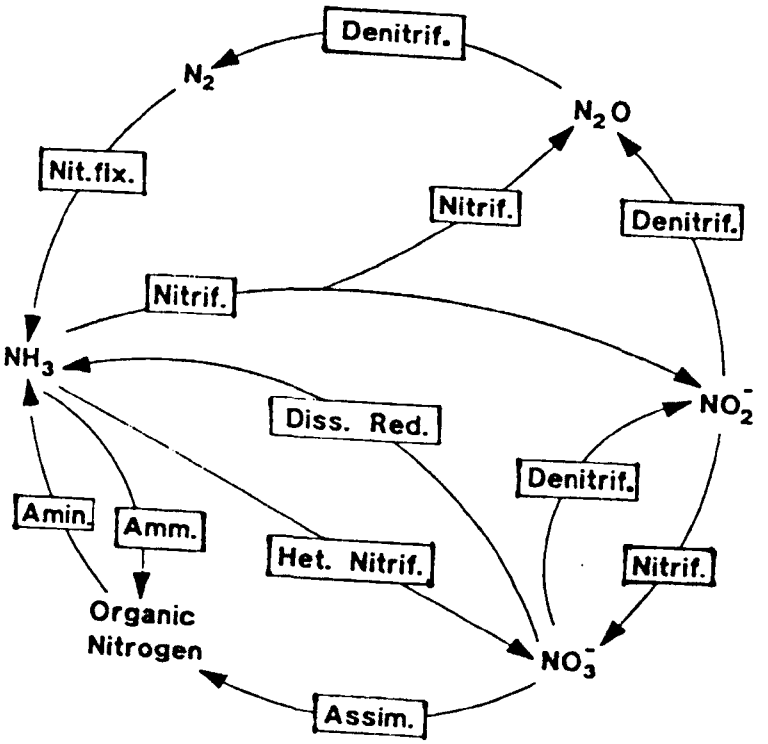


Fig. 1.1 The nitrogen cycle. From Underhill, 1990.

Abbreviations are as follows:

Amm. = ammonification; Assim = assimilation; .Amin. = amination; Denif. = denitrification; Nit. fix. = nitrogen fixation; Diss. red. = dissimilatory nitrate reduction; Het. nitrif. = heterotrophic nitrification; Nitrif. = heterotrophic and autotrophic nitrification.

autotrophs, such as the acidic soils of coniferous forests. (Focht & Verstraete, 1977; Killham, 1986; Frijlink *et al.*, 1992).

Early knowledge of nitrifying bacteria resulted chiefly from classical isolation work, performed largely by Winogradsky. By the turn of the century, it became accepted that nitrification proceeded in two steps, mediated by two specialised groups of microorganisms. The first step is the oxidation of ammonia to nitrite, the second is the oxidation of nitrite to nitrate. The generic names of these organisms reflect this distinction; the prefix 'Nitroso-' denotes ammonia-oxidisers whereas, the nitrite-oxidisers have the prefix 'Nitro-.'

1.3. Taxonomy of Nitrosobacteria.

Ammonia-oxidising bacteria are composed of two phylogenetically distinct lineages based on 16S rRNA sequence information (Head *et al.*, 1993); the gamma (γ) subdivision of the Proteobacteria contains *Nitrosococcus oceanus* and *Nitrosococcus halophilus* (Woese *et al.*, 1985; Koops *et al.*, 1990) (**Fig. 1.2**); the beta (β) subdivision of the Proteobacteria includes representatives of all of the other described genera of ammonia-oxidisers (**Fig. 1.3**) (Woese *et al.*, 1984, 1985; Stackebrandt *et al.*, 1988; Head *et al.*, 1993; Teske *et al.*, 1994; Utåker *et al.*, 1995). The ammonia-oxidisers in the beta subdivision form two deep branches; *Nitrosococcus mobilis*, *Nitrosomonas europaea*, and *Nitrosomonas eutropha* and a second branch comprising the genera '*Nitrosolobus*', '*Nitrosovibrio*' and *Nitrosospira* (Head *et al.*, 1993).

Classification was primarily based on morphological characteristics, such as cell size, shape, motility and the arrangement of intracytoplasmic membranes (Woese, 1984, 1985; Bock, 1986; Watson *et al.*, 1989; Koops *et al.*, 1991). Investigations

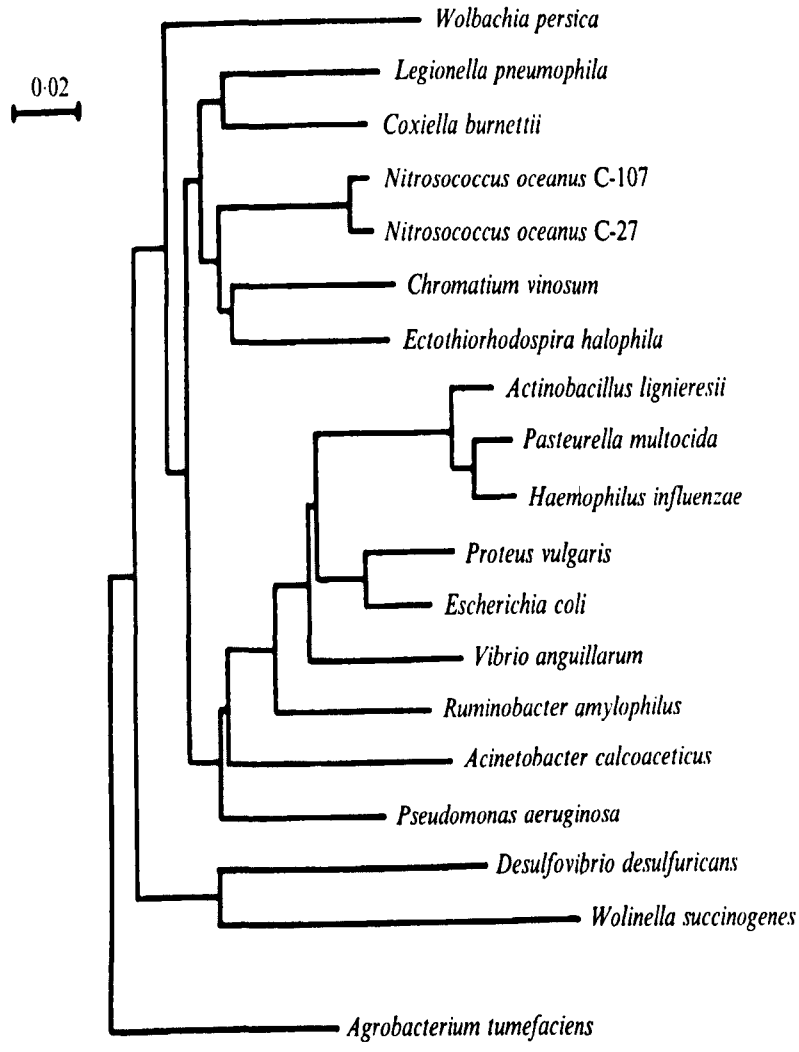


Fig. 1.2 Phylogenetic tree of representatives of the Proteobacteria showing the position of *Nitrosococcus oceanus* within the gamma-subdivision. The tree was generated using the method of Saitou & Nei (1987). *Agrobacterium tumefaciens* (alpha) was used as an outgroup. The scale bar indicates 0.02 substitutions per nucleotide position. From Head *et al.*, 1993.

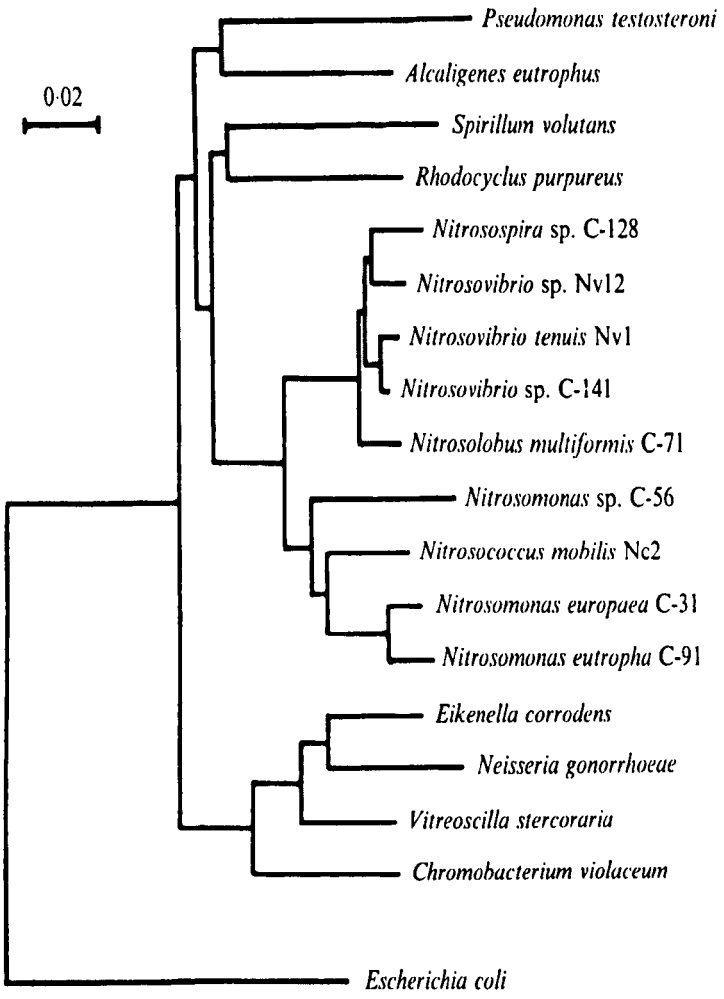


Fig. 1.3 Phylogenetic tree of autotrophic ammonia-oxidising bacteria from the beta-Proteobacteria derived from the neighbour joining method of Saitou & Nei (1987). *Escherichia coli* (gamma) used as the outgroup. The scale bar indicates 0.02 substitutions per nucleotide position. From Head *et al.*, 1993.

based on DNA/DNA hybridisation, mol % G +C and other chemotaxonomic markers allowed further differentiation into genera and species (Dodson *et al.*, 1983; Wood, 1986; Koops *et al.*, 1990, 1991). The DNA base composition among the genera of ammonia-oxidising bacteria varies within the limited range of 45-56% G+C (Koops & Harms, 1985). Taxonomic analysis of the genus *Nitrosomonas* demonstrated eight new species, including *Nitrosomonas marina*, distinguished from one another on the basis of DNA hybridisation values (Koops *et al.*, 1991). Two groups of *Nitrosospira* species were identified on the basis of differences in mol % G+C of the DNA (Koops & Harms, 1985). DNA hybridisation data allowed a further categorisation of the genera *Nitrosospira* into five species (Koops & Harms, 1985).

While groups of strains that could form the basis of species have been readily recognised using these approaches, they have been of limited utility for the resolution of bacterial genera. DNA pairing values between species were often very low and difficult to resolve (Huss *et al.*, 1983, Koops & Harms, 1985; Koops *et al.*, 1991). The application of cellular lipid profiles in discriminating between genera has also been indeterminative, because the genera are closely related and exhibit similar lipid profiles (Blumer *et al.*, 1969; Vestal & White, 1989). Differences observed in the lipid patterns seemed to be related more closely to habitat rather than genus identification (Blumer *et al.*, 1969).

Serotyping has provided a degree of clarification of the interrelationship between strains of both ammonia- and nitrite-oxidising bacteria (Belser & Schmidt, 1978; Josserand & Cleyet-Marel, 1979; Smorczowski & Schmidt, 1991). Evidence from serological studies using immunofluorescence indicated that isolates from seawater could be assigned to one of two serogroups of the marine ammonia-

oxidisers, *Nitrosomonas* spp. and *Nitrosococcus oceanus* (Ward & Carlucci, 1985). Bacteria that reacted with the antisera employed could be identified and enumerated from various marine and estuarine environments on this basis (Ward *et al.*, 1982; Ward, 1984; Ward & Carlucci, 1985). At least four unique serotypes among nitrite-oxidisers of identical morphology have been identified (Josserand & Cleyet-Marel, 1979). Fliermans *et al.* (1974) demonstrated that *Nitrobacter* isolates comprised two serotypes, whilst Stanley & Schmidt (1981) showed that strains of this genus were composed of several serotypes. Antisera produced against ammonia-oxidising strains had negligible cross-reactions with nitrite-oxidising strains or marine heterotrophs (Ward & Carlucci, 1985).

Comparative analyses of complete or partial 16S rRNA sequences has delineated further the phylogenetic relationships among ammonia-oxidising bacteria (Head *et al.*, 1993; McCaig *et al.*, 1994; Teske *et al.*, 1994; Utåker *et al.*, 1995; Stephen *et al.*, 1996). Sequence analysis of 16S rRNA of *Nitrosococcus oceanus* and *Nitrosococcus mobilis* suggested that the two species were not phylogenetically related (Woese *et al.*, 1984). Head *et al.* (1993) proposed that morphological differences in '*Nitrosovibrio*', '*Nitrosolobus*' and *Nitrosospira* were at species level and based on the high 16S rDNA sequence homology exhibited, they were reclassified in a single genus, for which the name *Nitrosospira* had precedence.

1.4. Growth & physiology.

Ammonia-oxidising bacteria are obligate chemoautotrophs, deriving energy and reducing power from the oxidation of ammonia to nitrite. Although strains are able to assimilate organic compounds, they remain reliant on lithotrophy, utilising

carbon dioxide as the main carbon source (Krummel & Harms, 1982). However, the uptake of organic compounds occurs more readily in the presence of inorganic energy sources (Clark & Schmidt, 1967; Williams & Watson, 1968; Kelly, 1971; Martiny & Koops, 1982). Many strains contain urease, enabling urea to be utilised as a substrate (Martikainen, 1985b; Allison & Prosser, 1991). Krummel & Harms (1982) demonstrated inhibition of nitrite formation in *Nitrosococcus mobilis* due to organic compounds. Williams & Watson (1968) used ^{14}C- labelled glucose, glutamate, pyruvate and methionine which were incorporated into the cellular material of resting cells of *Nitrosococcus oceanus* at insufficient levels to provide the major carbon and energy requirement of the cell. Therefore, strains of ammonia-oxidisers appear to have permeability barriers restricting the flow of organic molecules into the cell.

The ammonia-oxidising bacteria are strictly aerobic, but can be capable of growth under low oxygen tension. Bodelier *et al.* (1996) described specific adaptations to low-oxygen or anoxic situations by the indigenous ammonia-oxidising populations colonising the root zone of the macrophyte *Glyceria maxima.* Optimum growth temperature in liquid culture is between 25 and 30°C (Loveless & Painter, 1968), at pH 7.8 to 8.0 (Focht & Verstraete, 1977) and at ammonia concentrations in the range 2 to 10 mM (Watson *et al.*, 1981). In many natural environments, sub-optimal conditions are usually found. Autotrophic nitrification and energy transduction have been demonstrated in acid soils (DeBoer *et al.*, 1988, 1989). However, reports that *Nitrosomonas europaea* does not regulate intracellular pH indicated that this species was not adapted to be active at low pH (Kumar & Nicholas, 1982). Frijlink *et al.* (1992) demonstrated that energy transduction may occur in *Nitrosomonas europaea* at pH 5.0 when hydroxylamine replaces ammonia as an

energy source. Allison & Prosser (1993) maintained ammonia-oxidising activity of *Nitrosomonas europaea* biofilms at pH 5.4 within continuous flow vermiculite columns, whilst in liquid batch culture, *Nitrosomonas europaea* demonstrated optimum growth at pH 7.0 (Allison & Prosser 1993). Both high light intensity and high oxygen concentrations also inhibit growth (Hooper & Terry, 1973, 1974; Alleman *et al.*, 1987). The reported generation times in liquid media vary from 8h (*Nitrosomonas* spp.) to 60h (*Nitrosospira* spp.) (Bock, 1986). Strains are difficult to culture on solid media due to the small size of the colonies and require extremely long incubation periods, during which concomitant growth of heterotrophs often occurs (Focht & Verstraete, 1977).

The emphasis on *Nitrosomonas* spp., especially *Nitrosomonas europaea,* as the major ammonia-oxidisers in environments may be partially a result of culture bias. *Nitrosomonas europaea* has been demonstrated to have higher growth rates in enrichment culture and pure culture than other, possibly more ecologically significant nitrifiers (Belser & Schmidt, 1978; Wood, 1986). Belser and Schmidt (1978) observed selectivity among the different genera of ammonia-oxidisers, with *Nitrosomonas* spp. generally dominant over *Nitrosospira* spp., possibly due to a faster growth rate.

Strains of ammonia-oxidising bacteria have very similar physiologies, but vary in both cell shape and size (Koops & Möller, 1991). They are motile with polar flagella and are rich in cytochromes. Some strains possess carboxysomes that contain RuBisCo (Harms *et al.*, 1976; Wullenheber *et al.*, 1977). Glycogen inclusion bodies have also been observed in some species (Watson *et al.*, 1981).

The cells of *Nitrosomonas* spp. are ellipsoidal or rod shaped and occur singly or more rarely in chains (Watson *et al.*, 1981). The cells have extensive

intracytoplasmic membranes in the form of flattened vesicles located in the peripheral regions of the cytoplasm (Watson *et al.*, 1981). Occasionally, the intracytoplasmic membranes may intrude into the inner regions of the cytoplasm. The marine strains have an additional cell wall layer (Watson & Remsen, 1970).

The cells of *Nitrosococcus* spp. are spherical and occur singly, in pairs or as tetrads. They have intracytoplasmic membranes arranged centrally, peripherally or randomly in the cytoplasm. The genus *Nitrosococcus* contains at least three species: *Nitrosococcus nitrosus* (Buchanan, 1925); *Nitrosococcus oceanus* (Watson, 1965); *Nitrosococcus mobilis* (Koops *et al.*, 1976). Only *Nitrosococcus oceanus* and *Nitrosococcus mobilis* are currently available in laboratory culture. Two strains similar in shape and size to the cells of *Nitrosococcus nitrosus* have been isolated from terrestrial and industrial sewage environments (Koops & Harms, 1985). If observations on these two isolates are verified, it should be possible to describe a neotype strain of *Nitrosococcus nitrosus* which will facilitate some reorganisation of this genus.

The cells of the *Nitrosospira* genus are tightly wound spirals. '*Nitrosolobus*' spp. contain pleomorphic, lobate cells with invaginations of the cytomembranes that partially compartmentalise the cell and division is by constriction. '*Nitrosovibrio*' cells are slender rods, but spherical forms 1.0-1.2 μm in diameter are observed during stationary phase. They lack extensive intracytoplasmic membranes and carboxysomes found in most other ammonia-oxidising bacteria.

1.5. Biochemistry.

The oxidation of ammonia to nitrite occurs in two stages. The first is the endergonic formation of hydroxylamine and is catalysed by a membrane-bound ammonia monooxygenase enzyme (AMO) for which ammonia, rather than ammonium ions serves as the substrate (Suzuki *et al.*, 1974; Wood, 1988; Bedard & Knowles, 1989). The reaction requires molecular oxygen and reducing power and has a redox potential of +900 mV at pH 7.0 (Hooper, 1969; Suzuki *et al.*, 1974).

$$NH_3 + O_2 + 2H^+ + 2e^- \longrightarrow NH_2OH + H_2O$$

The second step is the oxidation of hydroxylamine to nitrite by the periplasmic enzyme hydroxylamine oxidoreductase (HAO) and releases four electrons (Andersson & Hooper, 1983; Stuven *et al.*, 1992). The reaction has a redox potential of +60mV (Wood, 1988).

$$NH_2OH + H_2O \longrightarrow NO_2^- + 5H^+ + 4e^-$$

The acceptor for the electrons is the tetraheme cytochrome C_{554} (Hooper, 1989). Photoinhibition may occur due to the photo-oxidation of cytochrome C. Two of the electrons pass down via an oxidative electron transport chain involving flavoproteins and cytochrome aa_3 terminal oxidase, and ATP formation occurs (Hyman & Wood, 1983; DiSpirito *et al.*, 1985). The other two electrons are required as a source of reductant for AMO during steady state ammonia oxidation (Hooper & Terry, 1973; 1974; DiSpirito *et al.*, 1985; Wood, 1988). The electron donor for AMO *in vivo* is unknown. Suzuki & Kwok (1981) demonstrated a stimulation in ammonia-oxidiser activity by the addition of cytochrome C_{554} indicating that cytochrome C_{554} can act as an indirect electron donor for AMO. A close coupling of NH_2OH and NH_4^+

oxidation has been found in resting cells, spheroplasts and cell-free extracts of *Nitrosomonas europaea.*

1.5.1. Ammonia monooxygenase.

The physiology of AMO and HAO have been extensively documented (Wood, 1986; Bedard & Knowles, 1989). Knowledge of the enzymes has been based on whole cell studies and isolated proteins (Hooper *et al.*, 1978; Hyman *et al.*, 1988; Hyman & Arp, 1992; Ensign *et al.*, 1993). AMO is a membrane-bound multi-subunit enzyme, responsible for the conversion of ammonia to hydroxylamine (Hyman & Arp, 1992). AMO has a broad substrate specificity and appears to be capable of oxidising a number of low molecular weight organic compounds (Rasche *et al.*, 1990; Vannelli *et al.*, 1990; Juliette *et al.*, 1993a,b). Alternative substrates for AMO such as sulphur, methane, ethylene and halogenated hydrocarbons can compete for the flux of oxygen and, consequently, inhibit the oxidation of ammonia by interacting with the active sites (Hyman & Wood, 1993; Juliette *et al.*, 1993a,b; Keener & Arp, 1994). AMO is also inhibited by metal chelators such as allylthiourea (Hooper & Terry, 1973; Ginestet *et al.*, 1998), or acetylene, which acts as a 'suicide' substrate (Hyman *et al.*, 1988). Exposure to visible light can inactivate AMO and therefore ammonia oxidation (Shears & Wood, 1985). A 27-30 KDa membrane-associated protein, which contains the active binding site of AMO, and a second protein of 38-43 KDa have been isolated as probable AMO subunits, AmoA, and AmoB from *Nitrosomonas europaea* (Hyman & Arp, 1992). The genes encoding AMO are designated *amoA* and *amoB* (McTavish *et al.*, 1993; Bergmann & Hooper, 1994). The *amoA* gene exists in two copies in the genome of *Nitrosomonas europaea* but three copies in *Nitrosospira multiformis*

(Norton *et al.*, 1996). Comparative sequencing of the major part of the *amoA* gene from *Nitrosospira multiformis* indicated that this functional gene has strong potential for the differentiation of ammonia-oxidisers (Rotthauwe *et al.*, 1995, 1997).

There has been particular interest in the similarity between AMO and genes encoding the particulate membrane-bound methane monooxygenase (pMMO) of methane-oxidising bacteria (Sayavedra-Soto *et al.*, 1994; Holmes *et al.*, 1995a,b; Semrau *et al.*, 1995). Both AMO and pMMO are copper-containing enzymes that are associated with intracytoplasmic membrane systems and they share similar inhibitors, substrates and sensitivity to chelating agents (Bedard & Knowles, 1989; Dalton & Leak, 1985; Holmes *et al.*, 1995a,b). Methanotrophs are capable of oxidising ammonia, but are unable to derive energy from the oxidation of ammonium ions (Dalton, 1977; Wood, 1988). *Nitrosomonas* spp. can oxidise methane, but at a lower rate than methanotrophs. Studies undertaken by Ward *et al.* (1989) on *Nitrosococcus oceanus* and *Nitrosomonas europaea* demonstrated the possibility of two analogous pathways for methane and ammonia oxidation, which share a common initial enzyme and similar capabilities for the oxidation of both methane and ammonia. The pMMO subunits are encoded by the genes designated *pmoA* and *pmoB* in analogy to the AMO encoding genes (Semrau *et al.*,1995). Sequence similarities between *amoA* and the homologous stretches of the *pmoA* gene of methanotrophs provided strong evidence of an evolutionary relationship (Holmes *et al.*, 1995a,b; Semrau *et al.*, 1995). A soluble form of methane monooxygenase (sMMO) has a very different active site to pMMO and requires NADH as the electron donor (Dalton, 1981; Semrau *et al.*, 1995).

The gene for HAO (*hao*) encodes a unique protein with no significant sequence similarity to other known proteins, and is present in three copies (McTavish

et al., 1993; Sayavedra-Soto *et al.*, 1994). The regulation of gene expression has, as yet, not been reported, but the promoter for the *hao* gene has been analysed (Sayavedra-Soto *et al.*, 1994). A recent study demonstrated the induction of AMO mRNA using $(NH_4)_2SO_4$ in concentrations as low as 0.013 mM, but the experiments could not distinguish between NH_3 and NH_4^+ as inducers (Sayavedra-Soto *et al.*, 1994).

1.5.2. Application of AMO in the study of ammonia-oxidising bacteria.

Functional probes can yield information regarding the distribution and expression of genes involved in certain biochemical pathways. AMO is an enzyme unique to ammonia-oxidising bacteria, and the functional gene of AMO is a promising specific target sequence to detect ammonia-oxidising bacteria in environmental samples by PCR amplification (Rotthauwe *et al.*, 1997). Oligonucleotide primers based on the published *amoA* sequence from *Nitrosomonas europaea* have been applied to amplify and partially sequence DNA from pure cultures of ammonia-oxidising bacteria and natural populations of unfiltered seawater samples (Sinigalliano *et al.*, 1995). Other partial *amoA* sequences from *Nitrosolobus multiformis* and *Nitrosospira* sp. (NpAV) have also been submitted to the GenBank database (Klotz & Norton, 1994, 1995). The two sequences demonstrated significant differences to that of the previously published *Nitrosomonas europaea* sequence (McTavish *et al.*, 1993; Klotz & Norton, 1995; Norton *et al.*, 1996). PCR amplification of the *amoA* gene from ammonia-oxidiser DNA derived from rice roots, activated sludge, freshwater and enrichment cultures have been used to construct gene libraries (Rotthauwe *et al.*, 1997). The direct detection of ammonia-oxidising bacteria has also been demonstrated

by PCR amplification of the *amoA* gene from soils treated with pig manure (Hastings *et al.*, 1997).

Other studies have revealed that the particulate methane monooxygenase (pMMO) and AMO are evolutionary related enzymes despite differing physiological roles (Holmes *et al.*, 1995b). Genes encoding pMMO and AMO share high sequence identity. When degenerate oligonucleotide primers were applied to both methanotrophs and ammonia-oxidising bacteria, a subsequent analysis of the amino acid sequences obtained revealed a strong conservation of both primary and secondary structure (Holmes *et al.*, 1995b). *Nitrosococcus oceanus amoA* showed a higher degree of homology to *pmoA* sequences from other members of the γ-Proteobacteria than to *amoA* sequences (Holmes *et al.*, 1995b). Thus, *amoA* might not be appropriate for examining phylogenetic relationships, but it may be a good target sequence for specifically detecting chemolithotrophic ammonia-oxidising bacteria from the environment under stringent conditions.

1.6. Ecological and economic importance of nitrification.

Nitrifying bacteria are central to the global nitrogen cycle, and have had a significant impact on pollution and the fertility of agricultural soils (Focht & Verstraete, 1977; Keeney, 1982). The oxidation of nitrogen fertilisers to nitrite and nitrate in agricultural systems has been considered detrimental due to ensuing losses of nitrogen by denitrification, or leaching of water-soluble anions (Keeney, 1982; Little, 1997). Whilst ammonium is adsorbed to the cationic components of soil, nitrite and nitrate are mobile and are leached out of the soil matrix (Gasser, 1982). Approximately one-third of nitrogen fertiliser used in the UK is lost due to leaching

and denitrification (Macdonald, 1986). This has led to eutrophication of lakes and rivers from contaminated run-off waters (Alexander, 1977; Paerl, 1982), resulting in algal or phytoplanktonic blooms which deoxygenate the system (Wilkinson & Greene, 1982).

Nitrate can also enter aquifers and accumulate to toxic concentrations in water supplies (Painter, 1986). The quantity of nitrate in drinking water has been a concern for public health following the production of nitrosamines from nitrite, and also the occurrence of methaemoglobinaemia in infants and farm animals. Consequently, regulations regarding water quality have been strengthened and the application of synthetic nitrification inhibitors such as nitrapyrin or sulphur-containing amino acids *e.g.* methionine and cysteine have been an attractive proposition to minimise nitrogen loss (Belser & Schmidt, 1981; Keeney, 1986).

In soil and aquatic environments, nitrates are substrates for denitrification, which, together with nitrification, result in the production of nitric and nitrous oxides (Tortosa *et al.*, 1990; Stuven *et al.*, 1992; Anderson *et al.*, 1993; Bock *et al.*, 1995). Production of these gases affects the Earth's atmosphere and controls the concentration of ozone (Dickinson & Cicerone, 1986; Hooper, 1989). Evidence has suggested that autotrophic nitrifiers are the primary producers of nitric and nitrous oxide in aerobic soils, whereas denitrifiers such as *Pseudomonas* spp. or *Alcaligenes* spp. are responsible for the majority of the nitric and nitrous oxide emissions from anaerobic soils (Anderson *et al.*, 1993). The formation of these gases has also contributed to the biodegradation of building materials (Kaltwasser, 1976; Bock & Sand, 1993). Ammonia-oxidising bacteria have been isolated from the pores of

sandstone where they oxidise ammonia scavenged from the atmosphere (Meincke *et al.*, 1989; Baumgärtner *et al.*, 1991; Bock & Sand, 1993).

Nitrifying bacteria have also been applied to ameliorate damage to the environment by reducing the ammonia content of wastewater in sewage treatment works prior to discharge into aquatic environments, (Painter, 1986; Sanden *et al.*, 1996; Wagner *et al.*, 1996). Frequent process failures do occur due to the sensitivity of the bacteria to pollutants and fluctuations in pH and temperature (Amann *et al.*, 1998; Schramm *et al.*, 1998). Turnover can be low due to their low specific growth rates. Attention has also been focused on the possibility of exploiting nitrifying bacteria in the bioremediation of contaminated soils due to the broad substrate range of AMO (Vannelli *et al.*, 1990; Vannelli & Hooper, 1993). Improved process control will depend on a better understanding of the physiology and biochemistry of the microorganisms involved in the nitrification process.

1.6.1. Nitrification in freshwater.

Lake environments are diverse in nutrient status and vary from oligotrophic to eutrophic depending on the catchment area and the morphometry of the lake basin (Odum, 1971). Lakes in temperate zones, such as the English Lake District, demonstrate an annual cycle of stratification and mixing (Hall, 1986; Jones, 1987). In winter and spring, the water column is isothermal and well oxygenated, but during the summer months an increase in solar radiation on the surface waters causes a gradient of temperature and oxygen. The lake becomes stratified and distinct layers are established (**Fig. 1.4**).

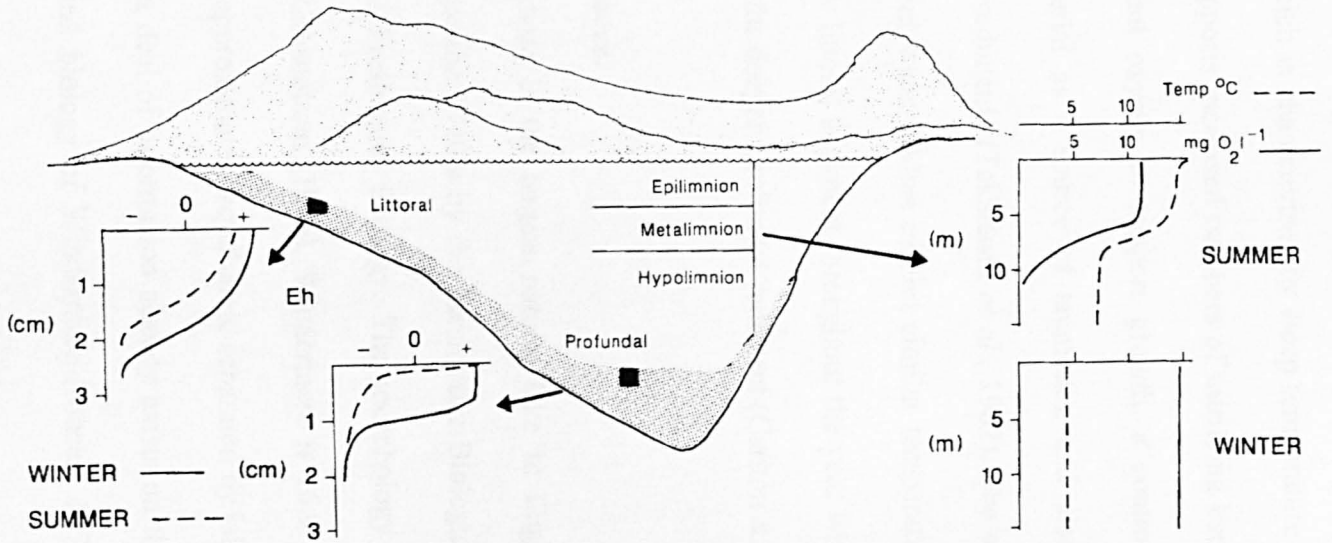


Fig. 1.4 Idealised stratified lake. Modified from Hall, 1986.

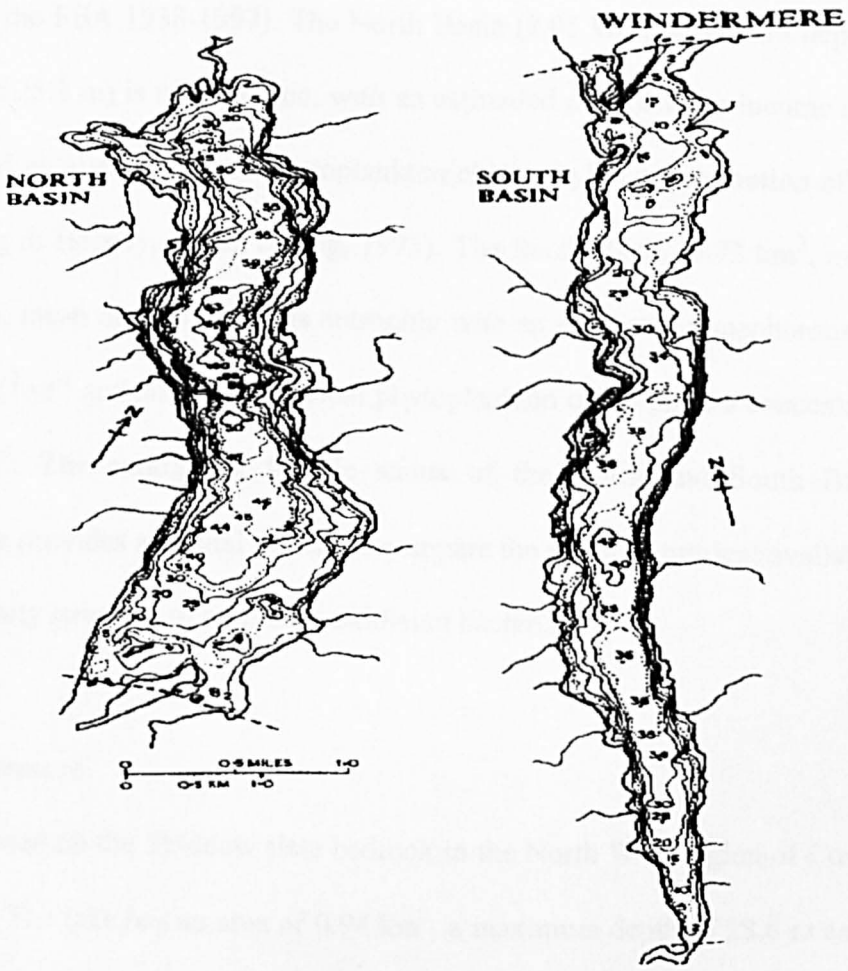
The warm, well mixed surface water region is known as the epilimnion and often supports algal planktonic blooms, whilst the deeper water (hypolimnion) remains cool and is not extensively mixed. Separating these two extremes is the metalimnion which is characterised by steep temperature and oxygen gradients. The metalimnion supports increased numbers of nitrifying bacteria at the oxycline where there is sufficient oxygen to support growth, a continual supply of sedimenting particulate material as a source of ammonia, and also the upward diffusion of ammonia from sediments (Takahashi *et al.*, 1982). The surface sediments in contact with the different water bodies exhibit similar temperature cycles. Oxygen diffuses into the shallow littoral sediment throughout the year whilst seasonal deoxygenation cycles occur in the deeper, profundal sediment (Carlton &Wetzel, 1985).

1.6.2. Windermere.

Windermere is the largest natural lake in England and has been studied extensively since the 1940's by the Freshwater Biological Association, and latterly, the Institute of Freshwater Ecology. The morphology and bathymetry have been described by Ramsbottom (1976). Windermere is 16.9 km long and comprises two lake basins of approximately equal area, separated by islands and a shallow sill (**Fig. 1.5.A**). A great deal of information already exists on the chemistry, sedimentology, hydrography and biology of Windermere (Macan, 1970; Lund, 1972; Pennington, 1981).

Sediment records show that both basins have experienced increased rates of nutrient loading due to a continued increase in the human population and input into the catchment, for example, an increased use of fertilisers (Pennington, 1978; Annual

A Windermere



B Buttermere

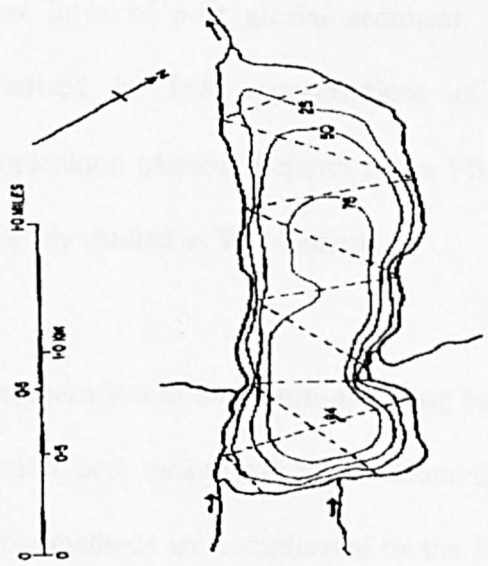


Fig. 1.5 Windermere (**A**), depth contours in metres; Buttermere (**B**), depth contours in feet. From Ramsbottom (1976).

Reports of the FBA 1938-1997). The North Basin (8.05 km^2, maximum depth 64 m, mean depth 25.1 m) is mesotrophic, with an estimated phosphorous income of 0.28 g m^{-2} yr^{-1}, and an average annual phytoplankton chlorophyll a concentration of 5.1 mg m^{-3} (Talling & Heaney, 1988; Talling, 1993). The South Basin (6.72 km^2, maximum depth 42 m, mean depth 16.8 m) is eutrophic with an estimated phosphorous income of 0.91 g m^{-2} yr^{-1} and an average annual phytoplankton chlorophyll a concentration of 8.0 mg m^{-3}. The contrasting trophic status of the North and South Basins of Windermere provides a natural system to compare the effect of nutrient availability on the community structure of ammonia-oxidising bacteria.

1.6.3. Buttermere.

Situated on the Skiddaw slate bedrock in the North West region of Cumbria is Buttermere. The lake has an area of 0.94 km^2, a maximum depth of 28.6 m and mean depth 16.6m (**Fig. 1.5.B**). The littoral zone is generally stony and steeply shelving with a black topmost layer of post glacial sediment. The lake is described as oligotrophic, characterised by low concentrations of nutrients and restricted development of phytoplankton (Annual Reports of the FBA 1938-1997). Buttermere has not been as extensively studied as Windermere.

1.7. Detection and enumeration of ammonia-oxidising bacteria.

The identification and quantification of ammonia-oxidising bacteria is a diffficult task. Available methods are complicated by the fact that nitrifiers have low rates of energy procurement and comprise only a nominal fraction of the viable cells

in sludges, sediments and soils. Both traditional culture based methods and molecular techniques are currently employed, and these are discussed below.

1.7.1. Most Probable Number (MPN).

The MPN technique employs successive, replicative dilutions of the sample in appropriate media, until an extinction point where no growth occurs is reached (Alexander & Clark, 1965). The degree of dilution of the sample required, and the number of positive tubes at different dilutions, are used to determine the MPN of viable organisms present in the original sample by reference to probability tables. Based on the Poisson distribution; a high degree of replication provides statistically significant enumeration of culturable organisms (Roszak & Colwell, 1987).

The MPN procedure is a traditional approach to isolating or detecting nitrifier populations in soil (Belser & Schmidt, 1978; Martikainen, 1985a), rivers (Curtis *et al.*, 1975), freshwater lake sediment (Smorczewski & Schmidt, 1991; Stehr *et al.*, 1995), and swine waste (StArnaud *et al.*, 1991). In one MPN estimate of a silt loam soil suspension, preliminary microscopic examination of all positive tubes revealed only *Nitrosospira* strains in the most dilute tubes (10^{-5}) but in the lower dilutions (10^{-2}, 10^{3}) *Nitrosomonas* strains were predominant (Belser & Schmidt, 1978). Therefore, medium selectivity can facilitate the growth rate of certain isolates and allow those strains to predominate over others (Belser & Schmidt, 1978; Both *et al.*, 1990). Difficulties also arise in obtaining pure cultures from the lower dilution tubes due to the association of contaminants that may out-compete the ammonia-oxidisers (Belser & Schmidt, 1978).

1.7.2. Dilution plate method.

Plating techniques can be extremely tedious to use for nitrifying bacteria due to the small size of colonies, long incubation periods and the concomitant growth of contaminants. Visualisation and counting of ammonia-oxidiser colonies may be facilitated by a clear zone formed in solidified calcium carbonate mixture due to acid production by the colony and identified microscopically (Winogradsky & Winogradsky, 1933; Soriano & Walker, 1968).

The reliability of culture-dependent methods for enumeration and identification of bacteria has inherent shortcomings. There is a general acceptance that, due to culture biases, there can be a gross underestimation and misrepresentation of the proportions of viable nitrifying bacteria and the significance of the population in the environment (Belser, 1979; Brock, 1987). Serial dilutions allow the isolation of both the dominant and fastest growing organisms in the sample (Watson *et al.*, 1981). Recovery of viable colonies cannot be guaranteed and there may be nitrifier populations that are environmentally viable but non-culturable (VBNC). Methods for direct counting that do not rely on the culturability of bacteria may be more quantitative than viable counting procedures (Fry, 1990).

1.7.3. Fluorescent antibody technique.

Immunoassays using fluorescent antibodies to visually tag nitrifying cells have the potential for direct enumeration and identification of the different species that contribute to nitrification in a particular environment (Rennie & Schmidt, 1977; Volsch *et al.*, 1990). A positive reaction is dependent on the presence of the appropriate antigen and relies on the initial pure cultures of bacteria that are used as

the antigens. Fliermans *et al.* (1974) observed serological diversity amongst strains of *Nitrobacter*. Belser & Schmidt (1978) first applied the method to detect ammonia-oxidisers and documented low cross-reactivities. Several studies have been documented on marine ammonia-oxidisers (Ward & Perry, 1980; Ward & Carlucci, 1985; Zaccone *et al.*, 1996). The procedure has also been applied to determine the biomass of *Nitrosomonas europaea* and *Nitrobacter agilis* cells immobilised in a gel bead (Hunik *et al.*, 1992).

The first application of an enzyme-labelled monoclonal antibody immunoassay with ammonia-oxidising bacteria has been described (Sanden *et al.*, 1994). A competitive enzyme-linked immunosorbent assay (ELISA) was used in the investigations to estimate pure culture densities of *Nitrosomonas* and *Nitrobacter* species with a lower limit of detection of 10^7 cells ml^{-1}. The method has been applied to enumerate *Nitrobacter* spp. in the influent of a waste water treatment plant (Sanden *et al.*, 1996). A less sensitive polyclonal antibody enzyme immunoassay for the detection of *Nitrosomonas europaea* against a range of pure cultures has been described (Saraswat *et al.*, 1994). Smorczewski and Schmidt (1991) applied a sophisticated approach whereby MPN tubes were screened with polyclonal species-specific antisera and the diversity of the cultures was determined by serological reactivity. The study revealed previously unknown strains of nitrifiers and documented the persistence of nitrifiers in anoxic sediments. Zaccone *et al.* (1996) demonstrated the presence of different serotypes in the medium used for MPN counts. Ward & Carlucci (1985) reported that the *Nitrosococcus* serum cross-reacted with other strains of ammonia-oxidising bacteria due to common surface antigens. Abeliovich (1987) described cross-reactions with nitrifying bacteria but not with

heterotrophic or coliform isolates. The selectivity of antibodies may present a problem as it relies on the preparation of fluorescent antibodies for all known strains of nitrifiers that reflect the composition of natural populations.

1.7.4. Nucleic acid-based detection of ammonia-oxidising bacteria.

Classic culture techniques misrepresent microbial biomass and have proved inadequate in the study of bacterial diversity in the environment (Liesack *et al.*, 1991; Wagner *et al.*, 1993; Reeves *et al.*, 1995; Kampfer *et al.*, 1996). The advent of the polymerase chain reaction (PCR) and other molecular techniques has enabled investigations into the biodiversity of bacterial communities where culturability compromises laboratory study (Saiki *et al.*, 1988; Pickup, 1991; Steffan & Atlas, 1991; Ward *et al.*, 1992; Jensen *et al.*, 1993; Muyzer & Ramsing, 1995). Microorganisms have been detected with oligonucleotide probes labelled with fluorescent antibody, radioactivity or dioxygenin reporter molecules that can hybridise to specific targets within the bacterial cell (Giovannoni *et al.*, 1988a; Stahl *et al.*, 1995; Trebessius *et al.*, 1994; Amann *et al.*, 1997).

The Oligonucleotide Probe Database (OPD) has centralised information related to the design, nomenclature and use of oligonucleotide probes and PCR primers (Alm *et al.*, 1996). The continually expanding OPD encompasses a large proportion of rRNA genes and accommodates probes that target other gene families. Analysis of RNA and DNA with PCR primers, oligonucleotide probes and nucleic acid polymorphisms offers potential species-specific detection and quantification.

1.7.5. Polymerase chain reaction (PCR).

PCR has been a useful diagnostic tool in the detection of ammonia-oxidising bacteria (McCaig *et al.*, 1994; Nejidat, 1994; Degrange & Bardin, 1995; Hiorns *et al.*, 1995; Hastings *et al.*, 1997; Ward *et al.*, 1997). The technique is highly sensitive and specific for the target genes when used in conjunction with confirmatory oligonucleotide probing. Conserved regions that flank variable portions of the genome can be used as primer-binding sites for the amplification and sequencing of variable regions from a wide variety of species. Highly conserved regions can also be used as targets for universal probes to discriminate bacterial phyla, whereas the variable regions can be used to design genus- and species-specific oligonucleotide probes (Manz *et al.*, 1993).

The phylogeny of nitrifiers makes them amenable to primer and probe development based on ribosomal RNA sequences. Permutations of PCR primers and probes based on 16S rRNA; 16S-23S rDNA spacer region and functional genes *e.g.* from AMO sequences have been developed and applied for the detection and characterisation of isolates and natural communities (McCaig *et al.*, 1994; Sinigalliano *et al.*, 1995; Hiorns *et al.*, 1995; Gurtler & Stanisich, 1996; Mobarry *et al.*, 1996; Hastings *et al.*, 1997; Kowalchuk *et al.*, 1997; Ward *et al.*, 1997). Direct amplification of nitrifier DNA extracted from an Antarctic lake verified the presence of unculturable nitrifiers (Voytek & Ward, 1995). *Nitrosospira* spp. were detected in a eutrophic freshwater lake by PCR amplification and confirmatory probing of the 16S rDNA (Hiorns *et al.*, 1995). Mobarry *et al.* (1996) characterised nitrifying bacteria associated with freshwater and seawater aquarium biofilters using a hierarchal set of 16S rRNA nucleic probes and showed that nitrifying populations grew in closely

associated aggregates. Hastings *et al.* (1997) applied high stringency PCR to detect *amoA* and nitrifier 16S rRNA genes in cultivated soil plots treated with swine manure. Juretschko *et al.* (1998) amplified *amoA* genes from ammonia-oxidiser DNA extracted from activated sludge and demonstrated the presence of *Nitrosococcus mobilis* and *Nitrosomonas europaea.*

1.7.5.1. DNA polymorphisms.

PCR has been used successfully to examine polymorphisms within complex bacterial populations (Brunk *et al.*, 1996). Ammonia-oxidising bacteria may be identified by comparison of nucleic acid bands of environmental isolates with those of known laboratory strains. Digestion of DNA with selected restriction enzymes of fragments is termed restriction fragment length polymorphism (RFLP). A variation of this technique is fluorescent-PCR-restriction fragment length polymorphism (flu-RFLP) whereby, the 5' end of the oligonucleotide primers are labelled with a fluorescent dye. The technique has been successfully applied to profile bacterial mercury resistance in the environment (Bruce, 1997). Information from banding patterns can be obtained using the DICE coefficient to calculate the percentage similarity between sequences and generate a dendrogram (Dice, 1945). Amplification with primers of a random sequence and analysis of bands produced is termed random amplified polymorphic DNA (RAPD) (Welsh & McClelland, 1990; Williams *et al.*, 1990).

The methods have been used to study a range of organisms including *Salmonella choleraesius*, *Cryptosporidium parvum*, *Pseudomonas syringae*, and

Vibrio vulnificans (Hoi *et al.*, 1997; Manceau & Horvais, 1997; Shah & Romick, 1997; Shianna *et al.*, 1998). Studies of the genetic diversity of culturable aquatic denitrifying bacteria based on the variability of the rRNA genes have also been measured using RFLP analysis (Ward, 1995). RAPD analysis of the intergenic spacer region of the 16S-23S rRNA genes has been applied to identify natural populations of *Nitrobacter* species (Navarro *et al.*, 1992), and ammonia-oxidising bacteria (Aakra *et al.*, 1999).

A recent method based on PCR for the study of bacterial communities has been described as single strand conformation polymorphism (SSCP) (Lee *et al.*, 1995). The technique has primarily been applied to detect novel polymorphism and mutations in human genes (Orita *et al.*, 1989). The method is based on the folded structure of single stranded DNA determined by intramolecular interaction and nucleotide sequence. The DNA fragments can then be separated by differences in electrophoretic mobility related to the shape of the DNA. The bands can then be compared using a laser beam densitometer and their occurrence and intensity analysed statistically.

1.7.5.2. Template bias and PCR drift.

Potential biases may be introduced which may ultimately affect the sequences obtained. The biases depend on the protocols which have been adopted for the sample handling, selective lysis of bacterial cells, extraction of nucleic acids from environmental samples, specificity of PCR amplification and selective cloning (Rainey *et al.*, 1994; Rochelle *et al.*, 1994). The principal approaches are:

1. The separation and isolation of bacterial cells from soil particles and subsequent lysis of the cells followed by nucleic acid extraction.

2. Lysis of the cells within the soil matrix followed by isolation of the nucleic acids.

Various methods have been applied to isolate cells from the soil matrix. Each method has a differing degree of efficiency and therefore the cells isolated may not represent the *in situ* population (Steffan & Atlas, 1991).

A mixture of homologous genes present in the template DNA extracted from natural communities may amplify during the PCR reaction at different relative frequencies to produce a skewed distribution of amplicon products (Suzuki & Giovannoni, 1996). Various factors may be responsible for this PCR drift. Selective priming or secondary structures may favour PCR amplification of certain sequences. The G+C content of the template DNA varies in microbial populations and can influence gene amplification by PCR (Reysenbach *et al.*, 1992; Dutton *et al.*, 1993). The introduction of acetamide into the reaction mixtures has been suggested to reduce the melting point of the template hybrids, enabling high G+C genes to compete more effectively (Reysenbach *et al.*, 1992). Universal primers used for the amplification of rRNA genes may have different binding energies resulting from primer degeneracy which may influence the formation of primer-template hybrids. However, Suzuki & Giovannoni (1996) suggested that the amplification of larger products better reflects the proportions of starting templates in the final amplicon mixture. In addition, homologues may not be equally accessible to primer hybridisation following denaturation and primer-template hybrids may be formed with unequal efficiency. The effect of PCR drift may be negated by altering the number of cycles of replication and

pooling the products of multiple PCR reactions prior to cloning. In addition, the copy number of rRNA genes in bacteria is variable and may affect PCR amplification of the target genes (Farrelly *et al.*, 1995).

1.7.5.3. Chimeric products.

A potential drawback associated with PCR based methods is the creation of recombinant or chimeric products (Liesack *et al.*, 1991; Kopczynski *et al.*, 1994; Robison-Cox *et al.*, 1995; Wang & Wang, 1997). Recombinant amplification products can be produced *in vitro* when amplifying heterogenous genetic material such as RNA viruses, multigene families or repetitive sequences (Saiki *et al.*, 1988; Meyerhans *et al.*, 1990). Inclusion of such sequences in phylogenies could cause significant errors as the amplified product may not represent a sequence that actually exists in a single continous stretch of DNA within any organism.

Analysis of predicted secondary structure for mismatches in conserved helices can be applied to detect chimeric sequences. Nearest-neighbor methods can also be employed to search for chimeric sequences whereby the query sequence is split into two sub-sequences and compared with a database of related sequences. A chimera is suspected if the phylogenetic affiliation of the parts is inconsistent with the affiliation of the sequence as a whole.

Current nearest neighbor methods include CHECK_CHIMERA which defines sequence similarity by the number of common oligonucleotides of length *k* shared by a sequence (*k*-tuple matching) (Maidak *et al.*, 1994). A recent study suggested that *k*-tuple based matching methods can be more sensitive than alignment based methods

when there is significant parental sequence similarity (Komatsoulis & Waterman, 1997). The aligned similarity method of Robison-Cox *et al.* (1995) computes similarity by counting the number of aligned matched bases in two disjoint sequence domains by using the RDP universal multiple sequence alignment. Both methods have inherent disadvantages such as the inability to properly penalise insertions and deletions. A recent method, described by Komatsoulis & Waterman (1997), called chimeric alignment, complements the previous methods and applies the Genetics Computer Group suite of programmes (Genetics Computer Group, 1994).

1.7.5.4. *Taq* DNA polymerase.

Recombination frequency can vary as a function of using different polymerases. *In vitro* recombination occurs when DNA polymerases pause or prematurely dissociate from the template strand and incompletely extend a segment of DNA. The occurrence of premature termination may be reduced in the presence of sufficient dNTP substrates (Innis *et al.*, 1988). Partially amplified sequence can act as a primer during subsequent amplification cycles. If the partially amplified segment hybridises to an alternative form of the template strand then, subsequent PCR products will be recombinants of the two original template sequences. Recombination may be enhanced by short extension cycles or long amplifications.

The incorporation of incorrect bases during amplification can have deleterious effects on subsequent sequence analysis. Biases may also be introduced by the preparation of *Taq* polymerase used. PCR error rates have been reported to be between 0.02% to 0.3% (Dunning *et al.*, 1988; Bej *et al.*, 1991). Giovannoni (1991)

considered *Taq* reading errors to be insignificant in phylogenetic analysis when comparing rRNA sequence differences between species.

1.7.5.5. Contamination.

One of the main problems associated with PCR is the amplification of unknown and unrelated genes that contain the same primer sites as the target gene. At low stringency, primers may anneal to sites that contain mismatches in nucleotide sequence. 'Touchdown' PCR and 'hot start' methods of PCR amplification increase the stringency, and therefore can minimise these problems. In addition, the generation of false positives by the inadvertent amplification of contaminating DNA will grossly affect the population of amplicons obtained (Kwok & Higuchi, 1989; Rochelle *et al.*, 1994). Humic acids, phenolic compounds and chelating agents can inhibit molecular reactions such as restriction enzyme digestion and PCR amplification (Tsai & Olson, 1991; Tebbe & Vahjen, 1993). Any DNA not associated with contaminants would be expected to offer a preferential template for PCR. Various protocols and kits are commercially available to purify nucleic acids extracted from environmental samples.

Contaminated laboratory surfaces represent one of the potential sources of exogenous DNA. The application of UV irradiation has provided one tool for decontaminating DNA in reagent solutions and laboratory equipment, but has met with mixed reviews (Meier *et al.*, 1993; Dwyer & Saksena, 1992). Despite the various precautionary treatments, meticulous attention to experimental technique remains the priority in preventing contamination.

1.7.6. Flow cytometry and *in situ* hybridisation.

The application of flow cytometry for the rapid automated enumeration of environmental bacterial samples has been comprehensively reviewed by Porter *et al.* (1997). The basic principle of flow cytometry presents individual cells (2,000-10,000 cells s^{-1}) in a thin stream of fluid (sheath fluid) (at approximately 10 m s^{-1}) into a focused light beam within a sensing region (Shapiro, 1995). The cell or particle scatters the beam of light. The extent of scattering is detected and depends upon the size and granularity of the particle or cell. The fluorescence emitted from stained cells can be recorded. There is an extensive range of fluorescent agents that are specific for different cell structures or are dependent on cellular activity. When the stream of cells passes continuously through the detector, a range of light intensities is emitted which are distributed into appropiate channels as single events, and particles fulfilling certain criteria can be collected. Flow cytometry facilitates high speed analysis and the ability to effect cell sorting, which enables the rapid enumeration and viability of different bacterial populations found in environmental samples (*e.g.* Amann *et al.*, 1990ab; 1997; Deere *et al.*, 1996; Simon *et al.*, 1995; Wallner *et al.*, 1995; 1996; 1997). Problems may occur when a number of bacteria pass through the flow cytometer without being detected. In complex communities, an accurate discrimination of cells from background debris may be severely reduced due to overlapping fluorescence and light scattering characteristics.

The application of fluorescently-labelled rRNA-targeted oligonucleotides derived from databases has been well documented (DeLong *et al.*, 1989; Manz *et al.*, 1994; Amann *et al.*, 1995, 1996, 1997; Weiss *et al.*, 1996). A number of fluorescent nucleic acid stains are currently available *e.g.* FITC, SYTOX Green, each with

differing degrees of efficiency (Roth *et al.*, 1997; Lebaron *et al.*, 1998). The technique permits analysis of selected organisms with the minimal disruption of the natural state and circumvents the need to culture the microorganisms (Amann *et al.*, 1991, 1995; Hahn *et al.*, 1992; Spring *et al.*, 1992). The combination of rRNA-targeted hybridisation probes and immuno probes for flow cytometry has enabled the highly specific automated identification of bacteria (Wallner *et al.*, 1996). 16S rRNA probes have been applied to biofilm studies (Amann *et al.*, 1992, 1997; Manz *et al.*, 1993; Schramm *et al.*, 1996). Ramsing *et al.* (1996) applied *in situ* hybridisation to examine the distribution of bacterial populations in a stratified fjord.

Quantification may be obtained visually with confocal (scanning) laser microscopy and image analysis using a camera linked to appropriate computer software (Caldwell *et al.*, 1992; Poulson *et al.*, 1993; Bloem *et al.*, 1995). The technique can have a restricted sensitivity due to the low ribosome numbers present in prokaryotic cells (Silcock *et al.*, 1992). Schonhuber *et al.* (1997) increased sensitivity of whole cell hybridisations by the combination of horseradish peroxidase-labeled oligonucleotides and tyramide signal amplification. The advent of *in situ* PCR may also enhance sensitivity by allowing the identification of single prokaryotic cells within complex mixtures (Hodson *et al.*, 1995; Tani *et al.*, 1998), but this has as yet to be unequivocally and reproducibly applied to bacteria.

1.7.7. Density Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE).

Density gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are powerful, convenient tools for analysing the sequence diversity of complex natural microbial populations (Myers *et al.*, 1985; Muyzer *et al.*, 1993; Wawer & Muyzer, 1995; Ferris & Ward, 1997; Heuer *et al.*, 1997; Muyzer & Smalla, 1998). The techniques provide information on sequence variation in a mixture of PCR fragments (Nubel *et al.*, 1996). One of the PCR primers includes a GC-rich sequence (GC clamp) on the 5' end that imparts melting stability to the PCR products in a denaturing gradient gel. The resulting mixture of PCR fragments of identical length are separated into discrete bands based upon differential mobility during electrophoresis through an acrylamide gel matrix that contains an increasing linear gradient of denaturant achieved chemically *e.g.* urea and formamide (DGGE), or physically by temperature (TGGE). Individual double-stranded DNA molecules denature along their length adjacent to the GC-clamp according to their melting characteristics which are based on their sequences. This partial denaturation causes migration to essentially halt at unique positions forming discrete bands in the gel that can be probed or excised for sequencing (Muyzer & de Waal, 1994; Muyzer *et al.*, 1995a; Felske *et al.*, 1996). In addition, information can be obtained through analysis of banding patterns, as each band may represent a different genotype (Muyzer *et al.*, 1993, 1995b; Heuer *et al.*, 1997; Kowalchuk *et al.*, 1997). Differences in the composition of microbial communities have been inferred in a number of studies (Wawer & Muyzer, 1995; Teske *et al.*, 1996; Kowalchuk *et al.*, 1997; Speksnijder *et al.*, 1998; Stephen *et al.*, 1998).

1.7.8. Oligonucleotide microchips.

Oligonucleotide microchips were originally developed for rapid sequence analysis of genomic DNA by immobilising oligonucleotide probes within a polyacrylamide gel matrix bound to the surface of a glass slide (Yershov *et al.*, 1996). The method has as yet not been adapted for large scale sequencing projects due to inefficient determination of mismatched duplexes, tandem repeats in the DNA or secondary structures within single stranded DNA (Yershov *et al.*, 1996). However, the technique has proven to be excellent for the sequence analysis of mutations and gene polymorphisms. Guschin *et al.* (1997) adapted the technique for microbial ecology studies. A collection of small subunit (SSU) rRNA-targeted DNA probes facilitated enumeration and analysis of the microbial population by a single hybridisation to the microchip.

1.8. Measurement of Bacterial Phylogenetic Relationships.

1.8.1. Ribosomal RNA (rRNA) as a molecular chronometer.

Woese (1987) comprehensively described the criteria adopted for phylogenetic analyses. Generally, a molecule whose sequence changes randomly in time can be considered a chronometer (Woese, 1987). To be useful the molecule needs to behave in a clock-like manner, whereby changes in sequences occur randomly and the rate of change must be commensurate with the spectrum of evolutionary distance being measured. In addition, the molecule needs to be large enough to provide an adequate amount of information. For these reasons, studies have generally focused on the large ribosomal RNA molecules (16S and 23S in bacteria) rather than the smaller 5S

molecule. **Fig. 1.6** presents the eubacterial 16S rRNA secondary structure showing the universal, semi-conserved and variable regions. These regions facilitate the design of genus- and species-specific oligonucleotide primers and probes.

Currently, rRNAs are regarded as the most useful chronometers, as they occur in all organisms and fulfill the criteria described above. In addition, rRNAs can be sequenced directly by means of the enzyme reverse transcriptase (Lane *et al.*, 1985). The ease with which DNA can now be manipulated has led to the increased use of macromolecular sequence comparisons to define phylogenetic relationships further enhancing the field of bacterial taxonomy.

1.8.2.Oligonucleotide Cataloging.

Prior to the advent of nucleic acid sequencing as a routine procedure, species were characterised by the analysis of partial sequences (Fox *et al.*, 1977). Short oligonucleotides were generated from complete 16S rRNAs by digestion with ribonuclease T_1 which cleaves at G residues. The collection of sequence fragments from a given rRNA gene constitutes a catalogue. Comparison of the catalogues permitted phylogenetic groupings to be determined by the calculation of binary association coefficients (S_{AB} values) (Fox *et al.*, 1977; Woese & Fox, 1977). The cataloging approach failed to define the branching orders of bacterial phyla and of rapidly evolving lines of descent. Full sequencing of 16S rRNA has now completely replaced the earlier cataloging approach and generally supported it.

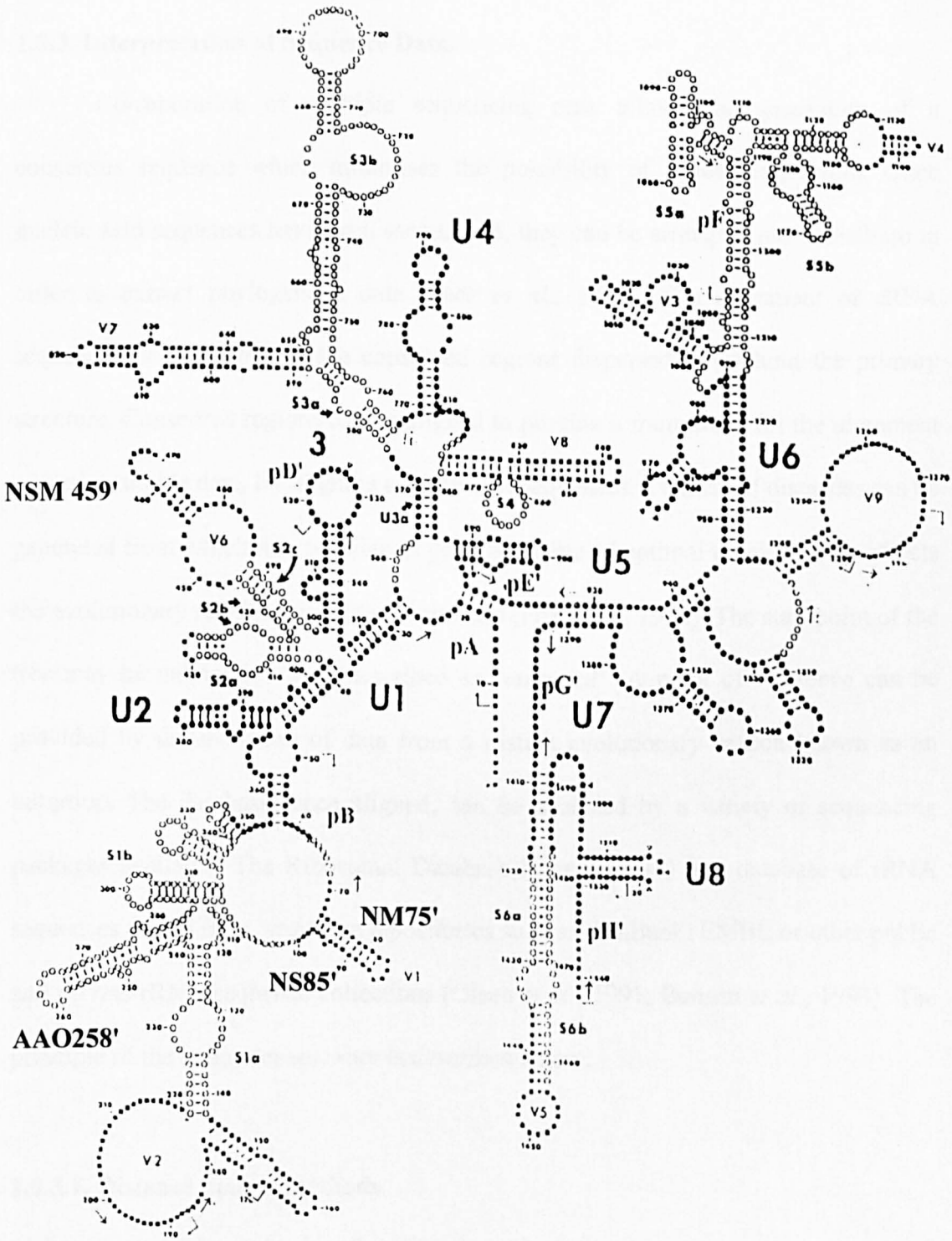


Fig. 1.6 Eubacterial (*E.coli*) 16S rRNA secondary structure model proposed by Woese *et al.*(1987), showing regions where oligonucleotide probes and PCR primers used in this work are targeted. (Table 2.5 describes primer and probe sequences and target specificity).

Universal: U; Semi-conserved: S and variable: V regions of SSU rRNAs.

1.8.3. Interpretation of Sequence Data.

Corroboration of multiple sequencing runs allows the generation of a consensus sequence which minimises the possibility of sequencing errors. Once nucleic acid sequences have been ascertained, they can be arranged into a database in order to extract phylogenetic data (Pace *et al.*, 1986). The alignment of rRNA sequences is facilitated by the conserved regions dispersed throughout the primary structure. Conserved regions can be aligned to provide a framework for the alignment of more variable data. By aligning a number of sequences, a matrix of distances can be generated from which it is possible to generate a tree of optimal topology that reflects the evolutionary relationship of the sequences (Fox *et al.*, 1992). The start point of the tree may be unknown and is described as 'unrooted'. A point of reference can be provided by the inclusion of data from a distant evolutionary branch known as an outgroup. The database, once aligned, can be analysed by a variety of sequencing packages available. The Ribosomal Database Project (RDP) is a database of rRNA sequences drawn from sequence repositories such as GenBank, EMBL or other public and private rRNA sequence collections (Olsen *et al.*, 1991; Benson *et al.*, 1993). The principle of the computer software is described below.

1.8.3.1. Distance matrix methods.

A comparison of pairs of sequences can be utilised to determine the fraction of positions in which the two sequences differ (Fitch & Margoliash, 1967; Felsenstein, 1993). The evolutionary genetic distances between organisms are then assumed to conform to an optimal tree topology. The method is susceptible to an underestimation of the true evolutionary distance between sequences due to multiple events occurring

at different rates. The proper correction for multiple changes has been the greatest restriction in tree construction.

1.8.3.2. Maximum Parsimony Analysis.

Maximum parsimony analysis assumes the correct phylogenetic tree to be that which requires the smallest number of mutational changes to have occurred in an ancestral sequence (Archie & Felsenstein, 1993). The nucleotide positions are considered independently, and the branch points found in the tree correspond to specific events in the evolution of an ancestral sequence (Felsenstein, 1993). Parsimony analysis tends to be restricted in considering rapidly evolving lineages and rapidly changing positions.

Both parsimony and distance matrix treeing consider all possible tree branching arrangements and the problem of finding the correct tree is computationally intense (Fitch & Margoliash, 1967; Felsenstein, 1993).

1.8.3.3. Cluster Analysis.

Cluster analysis groups together sequences on the basis of similarity (Olsen *et al.*, 1986). Pairs of nucleotides may be scored for identity yielding the 'percent similarity' between the molecules. An index may be derived of the percentage similarity which can provide an empirical insight between close relatives of the studied organisms. Cluster analysis can be sensitive to rapidly evolving lineages.

Improvements in sequence analysis will result from empirical approaches. Utilising a large enough sequence database, specific analyses can be ascertained that

correct more accurately for multiple changes at sites and utilise only the relevant positions in the molecule appropriate to the phylogenetic range being measured.

1.9. Application of 16S rRNA gene sequences in microbial ecology.

The accumulative database of 16S rRNA sequences has facilitated a greater resolution of microbial taxonomy than was previously obtained from DNA pairing or rRNA cataloguing (Stackebrandt & Goodfellow, 1991; Ward *et al.*, 1987, 1990). The bacterial community structure in activated sludge from a waste water treatment plant has been investigated by rRNA sequence analysis (Snaidr *et al.*, 1997; Glöckner *et al.*, 1998). Studies using 16S rRNA analysis have revealed uncultured novel morphotypes of a hot spring cyanobacterial mat and demonstrated a seasonal dominance of cyanobacterial and green non-sulphur bacterium-like populations (Britschgi & Giovannoni, 1991; Weller *et al.*, 1991; Fuhrman *et al.*, 1993; Ferris *et al.*, 1996, 1997). Novel bacterial groups of marine picoplankton have also been described (Giovannoni *et al.*, 1988b, 1990b; Schmidt *et al.*, 1991; Glöckner *et al.*, 1996; Suzuki *et al.*, 1997). Delong (1992) retrieved 16S rDNA sequences of archael origin from the Atlantic and Pacific Oceans. Molecular and microscopic identification of bacterial populations in multispecies biofilms has also been demonstrated (Amann *et al.*, 1992; Poulsen *et al.*, 1993). Other studies have demonstrated diversity in subtropical Australian soils (Liesack *et al.*, 1992), a stratified Fjord in Denmark (Ramsing *et al.*, 1996), and the pelagic zone of a high mountain lake (Alfreider *et al.*, 1996).

The publication of the rRNA sequences from culture collection strains of ammonia-oxidising bacteria by Head *et al.* (1993) provided information for the design

of specific PCR primers and oligonucleotide probes which have been used to study populations of ammonia-oxidising bacteria in the environment (McCaig *et al.*, 1994; Hiorns *et al.*, 1995; Voytek & Ward, 1995; Hovanec & Delong, 1996; Mobarry *et al.*, 1996; Pommerening-Röser *et al.*, 1996; Kowalchuk *et al.*, 1997). In the first of such studies, McCaig *et al.* (1994) applied PCR primers designed to recover 16S rRNA sequences from β-Proteobacterium ammonia-oxidisers and detected novel *Nitrosomonas*-like sequences in marine surface-water enrichment cultures. Voytek & Ward (1995) applied specific PCR amplification and oligonucleotide probes to infer the presence of β-Proteobacterium ammonia-oxidisers in saltwater samples. A detailed study of a eutrophic lake demonstrated the occurrence of *Nitrosospira* spp. in freshwater, activated sludge and soil (Hiorns *et al.*, 1995). A recent study by Kowalchuk *et al.* (1997) applied 16S rRNA targeted primers specific for all β-Proteobacterium ammonia-oxidisers to DNA from sand dune soil samples and demonstrated the presence of different *Nitrosospira* populations located in acid and alkali soils.

In situ analysis via whole-cell hybridisation using fluorescent 16S rRNA targeted probes has also been applied to identify and enumerate β-subgroup ammonia-oxidising bacteria in activated sludge and a trickling filter biofilm (Wagner *et al.*, 1995). However, a potential drawback to the use of PCR primers that target 16S rRNA genes is the possibility of cross-reactions with bacteria from other phylogenetic and physiological groups, especially when used with environmental samples which contain complex microbial gene pools (Mobarry *et al.*, 1996; Pommerening-Röser *et al.*, 1996; Stephen *et al.*, 1996).

Comparative 16S rRNA sequence analysis has facilitated a greater delinearisation of ammonia-oxidising bacterial taxonomy (Head *et al.*, 1993; Teske *et al.*, 1994; Utåker *et al.*, 1995). The identification of the *in situ* β-proteobacterium ammonia-oxidiser populations by 16S rRNA sequencing, facilitates comparisons between different studies and habitats and reveals a better understanding of ammonia-oxidiser sequence diversity present in the environment (Pommerening-Röser *et al.*, 1996; Stephen *et al.*, 1996, 1998; Speksnijder *et al.*, 1998; Ward *et al.*, 1997).

1.10. Aims of this project.

- To apply a combination of culture, direct microscopic observation and molecular techniques to compare the ecology of ammonia-oxidising bacteria present in Buttermere (an oligotrophic freshwater lake) and Windermere (a mesotropic/eutrophic freshwater lake), to previous data obtained from a eutrophic lake, within the English Lake District.

- To study changes in community structure as influenced by seasonal stratification and to compare ammonia-oxidiser diversity in sediment and water column.

- To examine the potential of *in situ* rRNA and flow cytometry approaches to study populations of ammonia-oxidising bacteria in freshwater lakes.

- To obtain 16S rDNA sequence information directly from amplified ammonia-oxidiser 16S rDNA from environmental samples for the design of primers and probes.

- To undertake a phylogenetic analysis to determine the relative occurrence and distribution of genotypes between sites and to examine the possibility of RFLP analysis as a rapid method of detecting genotypes.

Chapter 2. Materials and methods.

2.1. Chemicals and reagents.

Chemicals and reagents used throughout were of Analar grade (or equivalent) and were obtained from BDH (BDH, Gillingham, Dorset), Fisons (Fisons Scientific Equipment, Loughborough) or Sigma (Sigma Chemical Company Ltd., Poole, Dorset), unless otherwise stated.

2.2. Bacterial strains.

Type strains of bacteria used in this thesis were obtained either from the American Type Culture Collection (ATCC), the National Centre for Industrial and Marine Bacteria (NCIMB) or the School of Biological Sciences departmental culture collection. The ammonia-oxidising bacteria strains are detailed in **Table 2.1**. Other bacterial cultures that were also used are listed in **Table 2.2**.

2.3. Medium for ammonia-oxidising bacteria (Watson, S.W. and Mandel, M. 1971).

Nutrient	mg l^{-1}	final concentration
$(NH_4)_2SO_4$	1650	12.5 mM
$MgSO_4.7H_2O$	200	0.8 mM
$CaCl_2.H_2O$	20	0.16 mM
K_2HPO_4	87	0.5 mM
Trace metal solutions*	100 µl l^{-1}	
Iron/EDTA solution**	1 ml l^{-1}	
Phenol red	1 ml l^{-1} of a 0.5% (w/v) solution	

Species name	Source	Accession Numbers
Nitrosomonas europaea	Soil (USA)	C-31[1]; Nm50[2]; 25978[4];11850[5]
Nitrosomonas eutropha	Sewage (USA)	C-91; Nm57
Nitrosomonas sp.	Seawater (Gulf of Maine)	C-56; Nm63
Nitrosospira multiformis	Soil (Surinam)	C-71; Nl13; 25196; 11849
Nitrosospira tenuis	Soil (USA)	Nv1
Nitrosospira sp.	[unknown]	Nv12
Nitrosospira sp,	Soil (England)	Nv141[3]
Nitrosospira sp.	Soil (Crete)	C-128; Nsp 4; 25971
Nitrosococcus mobilis	North Sea (Germany)	Nc2
Nitrosococcus oceanus	Oceanic	C-107 Nc10; 19707; 11848
Nitrosococcus oceanus	Oceanic	C-27; Nc9

[1] Watson strains (from Dr. S. Watson, University of Texas).
[2] Koops & Harms, (1985) or Koops *et al.* (1991), except #141[3].
[3] Walker strain (Rothamsted).
[4] American Type Culture Collection accession number.
[5] National Collection of Industrial and Marine Bacteria accession number.

Table 2.1 Autotrophic ammonia-oxidising bacteria strains used in this study.

Species	Source
Escherichia coli JM109	School of Biological Sciences culture collection (241)
Pseudomonas aeruginosa	School of Biological Sciences culture collection (92)
Pseudomonas fluorescens	School of Biological Sciences culture collection (94)
Staphylococcus aureus	NCIMB 13062
Bacillus subtilis	NCIMB 3610[1]
Listeria monocytogenes	ATCC 15313[2]

[1] National Collection of Industrial and Marine Bacteria accession number.
[2] American Type Culture Collection accession number.

Table 2.2 Heterotrophic eubacterial strains used in this study

*Trace metal solution (to make 1 litre):

$MnCl_2.4H_2O$, 200 mg; $Na_2Mo_4.2H_2O$, 100 mg; $ZnSO_4.7H_2O$, 100 mg; $CuSO_4.5H_2O$, 20 mg; $CoCl_2.6H_2O$, 2 mg.

**Iron/EDTA solution. $FeSO_4.7H_2O$, 450 mg; EDTA, 250 mg; in 100 ml water.

The pH was adjusted to 6.8 prior to autoclaving (pH 7.5-7.8 following sterilisation). When the phenol red indicator turned yellow, 5% Na_2CO_3 (w/v) was added until pink colour was restored.

Three variations of the medium of Watson and Mandel (1971) were routinely employed for the isolation and maintenance of environmental isolates of ammonia-oxidising bacteria. The concentration of $(NH_4)_2SO_4$ was varied as follows:

Watson medium A 12.5 mM $(NH_4)_2SO_4$ per litre.

Watson medium B 3.0 mM $(NH_4)_2SO_4$ per litre.

Watson medium C 0.67 mM $(NH_4)_2SO_4$ per litre.

2.4. Cultivation of ammonia-oxidising bacteria.

Freeze-dried type strains of ammonia-oxidisers obtained either from ATCC or NCIMB were resuscitated according to the supplier's instructions. For subculture of laboratory strains received as biomass in liquid medium, 150 µl of mid-exponential phase culture was inoculated into 150 ml of medium in a 250 ml conical flask. All flasks were incubated in the dark at 30°C with gentle shaking (100 rpm). The cultures were examined periodically for the loss of pink colouration (phenol red) due to nitrite production. On the medium attaining a yellow colour, filter sterilised Na_2CO_3 (5% w/v) was added to neutralise the medium until the pink colour returned. The cultures

were further incubated until a subsequent colour change in the medium occurred. The bacteria were then either subcultured or harvested. For large scale nucleic acid preparations, the culture volumes were scaled up to 1 or 2 litres of medium per strain.

Cultures were checked for contamination prior to subculturing by inoculation into 1/4 strength Nutrient Broth (LabM, Amersham) and examined microscopically either by phase contrast (x40) or by fluorescent excitation of 4'6'-diamidino-2-phenylindole (DAPI) using Apo Oel 100/1.32 objective and x10 eyepiece on a Leitz Orthoplan microscope. Full protocols for fluorescence microscopy are given in section 2.9.

2.5. Nitrite and nitrate measurements.

Nitrate and nitrite in culture media were examined routinely using Dipstix (Quantifix, Camlab). The sensitivity limit for nitrate and nitrite was 10 mg l^{-1} and 1 mg l^{-1} respectively.

2.6. Enrichment Cultures.

Sediment (1% w/v) or lakewater (1% v/v) were inoculated into 200 ml of the three types of Watson medium as described in section 2.3. In addition, enrichment cultures containing the inhibitor allylthiourea (5 mg l^{-1} final concentration) were performed in parallel. The cultures were incubated in the dark at 30°C with gentle shaking (100 rpm). Cultures were examined at 5 day intervals and nitrate/nitrite production monitored as described in section 2.5. In addition, the pH was checked by aseptically removing 5 ml into a sterile Universal bottle. If required, the subsamples were frozen at -20°C for PCR analysis (see section 2.20).

2.7. Medium for heterotrophic bacteria.

Type strains were maintained on Nutrient agar plates (LabM, Amersham). Liquid cultures were grown in appropriate volumes of Nutrient Broth (LabM, Amersham) and incubated at 37°C overnight.

2.8. Most probable number counts (MPN).

The method described by Alexander (1982) was used. Serial dilutions were performed as five-fold replicates in test tubes under aseptic conditions using the appropriate medium. The pH value of the growth medium was adjusted to pH 8.0 prior to inoculation. All sediment and lakewater samples were blended for 1 min in a polytron blender (Kinematica, Switzerland), to dissociate whole cells from solid particle matrices. Tubes were incubated in the dark at 30°C for 6 weeks before examination. Nitrite and nitrate production was determined as described in section 2.5. Positive scores were evaluated and analysis performed using statistical tables from Alexander & Clark (1965).

2.9. Direct counting by epifluorescence microscopy.

The method described by Fry (1990) was used. All solutions were filter-sterilised through 45 mm diameter 0.22 μm pore size membrane filter (Schleicher & Schuell). Appropriate sample dilutions were fixed by the addition of membrane-filtered formaldehyde (MFF) to a final concentration of 2% (v/v). DAPI (100 μg ml^{-1} final concentration) was added and the samples incubated in the dark at 30°C. After incubation, samples were individually filtered onto a black 0.2 μm pore size polycarbonate filter (25 mm diameter, Nucleopore Corp.). Triplicate filters were

mounted in low fluorescence immersion oil on a microscope slide. A second drop of oil was applied and a coverslip pressed in place. Stained cells were immediately viewed on a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Germany), using appropriate optical filters.

Cell size was estimated using a micrometer eyepiece. A 12 x 12 eyepiece graticule was calibrated (12 edges = 100 μm) and used to count the number of units of area that contained a sum of 500 organisms. Counts were made in accordance with the recommendations of Fry (1990). Samples were suitably diluted such that 50 randomly chosen microscope fields of view equivalent to 500 bacteria were scanned. No correction of counts for particulate obstruction were performed.

2.10. Total respiring counts.

The fluorescent formazan derivative 5-cyano-2,3-ditoyl tetrazolium chloride (CTC) was used according to the method of Rodriguez *et al.* (1992). Samples were serially diluted and CTC (3.5 mM final concentration) was added. The samples were subsequently incubated at 20°C for 4 h. Following incubation, the samples were filtered on to black 0.2 μm pore size polycarbonate filters as described in section 2.9. Counts were determined immediately by epifluorescence microscopy. Controls were performed by the addition of membrane-filtered formaldehyde (MFF, 4% final concentration) prior to incubation.

2.11. Colony-forming units (CFU).

Colony-forming units (CFU) were determined following an appropriate dilution of samples in PBS. Spread plates were prepared in triplicate using Nutrient agar (LabM, Amersham). Colonies were counted after overnight incubation at 37°C.

2.12. Sampling of Sediments.

Sediment cores were taken from the profundal sediments of Buttermere and the North and South Basins of Windermere, Cumbria, UK. Sample sites were all proximal of 25 m from a fixed marker point. The sampling regime and location of sampling sites for Buttermere and Windermere are presented in **Tables 2.3 & 2.4**. All sediments were sampled using a modified Jenkin corer as described by Ohnstadt & Jones (1982). Prior to sampling, coring devices were cleaned with absolute ethanol. Recovery of all cores from site to laboratory was completed within 1 h and the cores were structurally undisturbed. The Windermere sediment cores were aseptically sectioned into the upper layer (0-0.5 cm) and lower layer (0.5-3.0 cm). Only the upper layer (0-0.5 cm) was sectioned in the Buttermere sediment cores. Samples of approximately 5 g wet weight were taken from the interior of the core and placed in sterile Universal tubes. The samples were held on ice until they were processed within 16 h of the core being taken. The water content of the samples was determined by drying weighed samples in triplicate at 105°C until a constant mass was attained.

2.13. Temperature and oxygen profiles.

The temperature and oxygen profiles of the lakes under examination were monitored with a combined oxygen electrode and thermistor (Model 57, Yellow

A

Buttermere Sampling Sites	
Littoral sediment	(10m depth) 0-0.5 cm layer
Profundal Sediment	(25m depth) 0-0.5 cm layer
Lakewater	(14m depth) 60 litres concentrated to 1 litre by tangential flow filtration

B

Windermere Sampling Sites	
North Basin profundal sediment	(60m depth) 0-0.5 cm layer
North Basin profundal Sediment	(60m depth) 0.5-3.0 cm layer
North Basin lakewater	(20m depth) 60 litres concentrated to 1 litre
South Basin profundal sediment	(40m depth) 0-0.5 cm layer
South Basin profundal Sediment	(40m depth) 0.5-3.0 cm layer
South Basin lakewater	(20m depth) 60 litres concentrated to 1 litre by tangential flow filtration

Table 2.3 Location of sampling sites for Buttermere (**A**), and Windermere (**B**).

Buttermere 1995-1996		Windermere 1996-1997	
July	28/7/95	March	26/3/96
August	30/8/95	April	23/4/96
September	30/9/95	May	21/5/96
November	27/11/95	June	18/6/96
March	31/3/96	August	13/8/96
May	22/5/96	September	24/9/96
		November	19/11/96
		January	27/1/97

Table 2.4 Seasonal sampling programme for Buttermere and Windermere.

Springs Instruments, Yellow Springs, Ohio). From a fixed sampling point, the probe was calibrated and lowered to the sediment surface and allowed to equilibrate. Readings were taken at metre intervals upwards from the sediment surface.

2.14. Tangential flow filtration of lakewater.

A weighted hose lowered to the desired depth, (*i.e.* at the thermocline) was connected to a peristaltic pump (13 1itres min^{-1} capacity, Millipore) and 60 litres of water was collected into containers which had been cleaned using absolute ethanol. The sample was then concentrated using a Pellicon™ tangential flow filtration apparatus (Millipore), using three 0.45 μm pore size Durapore cassettes (5 sq. ft. membrane each) according to the manufacturers' instructions. The final volume of the sample was 1 litre. The apparatus was washed between each sample with two 500 ml aliquots of sterile distilled water.

2.15. Cellular dissociation from sediment.

Whole cell dissociation from sediment was optimised by using a range of extractants. Following the appropriate dilution, the optimised technique using 0.1% Triton-X-100 and blended for 1 min in a polytron blender (Kinematica, Switzerland) (PTA 20S head) was applied, prior to either *in situ* hybridisation analysis, flow cytometry or DNA extraction. The extractants tested were: sterile distilled water (SDW); 0.1% Triton-X-100; 1.0% Tween 80; 0.1% sodium cholate; 0.2% Calgon; quarter strength Ringer's solution; phosphate buffered saline pH 7.3; 50 mM Tris buffer pH 7.6. Triplicate 1g sediment samples in sterile universal containers were shaken with 10 ml extractant for 5 min (100 rpm) at room temperature. Samples were

allowed to stand for 30 s prior to reading the optical density at 550 nm (OD_{550}) after appropriate dilution. Cell dispersal was also maximised by a range of treatments including shaking at 4°C for 4 h, shaking at 4°C for 4 h with 0.2 g chelex (sodium salt) both with and without 0.2 g sterile glass beads (1.5-2.0 mm, Hopkin & Williams, Chadwell Heath, Essex); blending using a polytron blender for 3 x 30 s (Kinematica, Switzerland) (PTA 20S head). Dispersal was quantified by OD_{550} measurements.

2.16. Bacterial DNA isolation from culture.

2.16.1. Mini-preparation.

Cells were pelleted at 12,000 rpm (Sorvall SS 34 rotor) and washed twice with TE (10 mM Tris.HCL pH 8.0, 1 mM EDTA pH 8.0) prior to resuspension in 100 µl TE, 1% (v/v) Tween 80. The suspension was heated in boiling water and then frozen in liquid nitrogen. The freeze-thaw cycle was repeated twice followed by protein extraction using an equal volume of phenol-chloroform-isoamyl-alcohol (25:24:1 vol/vol/vol pH 8.0). Nucleic acids were precipitated by the addition of 0.1 volume of 3M sodium acetate pH 5.0 and 2 volumes of absolute ethanol, and incubated for at least 1 h at -20°C. The DNA was pelleted by microcentrifugation (13,000 rpm for 10 min, MSE Microcentaur) and resuspended in 20 µl of Hypersolv water (BDH).

2.16.2. Ultrasonication.

Pelleted cells were washed in PBS and finally resuspended in 1 ml of PBS. Ultrasonication was performed for a 5 s period at 18 µm amplitude peak-to-peak. Lysates were extracted with an equal volume of phenol-chloroform-isoamyl-alcohol (25:24:1 vol/vol/vol pH 8.0). Nucleic acids were precipitated with 0.1 volume 3 M

sodium acetate pH 5.0 and 2 volumes absolute ethanol and incubated overnight (or a minimum of 2 h) at -20°C. After centrifugation, (13,000 rpm for 10 min; MSE Microcentaur), the nucleic acid pellets were resuspended in 20 µl Hypersolv water (BDH).

2.16.3. Boiling method of Holmes & Quigley (1981).

Pelleted cells were resuspended in 100µl of STET buffer (8% sucrose (w/v); 5% Triton-X-100 (v/v); 50 mM EDTA; 50 mM Tris.Cl pH 8.0). Following the addition of 7 µl of lysozyme solution (10 mg ml^{-1} in 10 mM Tris.Cl pH 8.0), the lysate was boiled for 40 s and then centrifuged (10 min at 13,000 rpm, MSE Microcentaur). The pellet was retained and nucleic acids precipitated by the addition of 0.1 volume of 3M sodium acetate (pH 5.0) and 2 volumes of absolute ethanol, and stored at -20°C for at least 2 h. Following centrifugation at 13,000 rpm for 15 min, the DNA was resuspended in 20 µl of Hypersolv water (BDH).

2.17. Extraction of environmental DNA from sediment.

The protocol used was as described by Bruce *et al.* (1992), which is an adaptation of the method of Selenska and Klingmuller (1991). Following sampling, sediment was stored until required at -70°C, as recommended by Rochelle *et al.* (1994). Samples were subdivided in order to reduce possible biases introduced by the DNA extraction method employed.

Four g (wet weight) of sediment were suspended in 7 ml extraction buffer (1% w/v SDS, 0.12M Na_2HPO_4, pH 8.0, lysozyme 5 mg ml^{-1}). After incubation for 1 h with shaking (100 rpm) at 70°C, solids were removed by centrifugation (10 min at

2,800g, 4°C). The supernatant was held at 4°C whilst the pelleted material was resuspended and extracted twice. Supernatants were pooled and centrifuged (at 8,000 g, 30 min, 4°C). Polyethylene glycol 6000 (final concentration 15% w/v) and 0.1 volume 5 M NaCl solution were added. After precipitation at 4°C overnight and centrifugation for 30 min at 5,000 g, the brown pellet was resuspended in TE pH 8.0 (10 mM Tris.HCL pH 8.0, 1 mM EDTA pH 8.0). The nucleic acids were purified by caesium chloride density centrifugation (see section 2.19).

2.17.1.Bead Beating.

One gram wet weight samples of soil or sediment were resuspended in 0.5 ml 0.12 M Na_2HPO_4, (pH 8.0) containing SDS (1% w/v) to which 0.5 g of glass beads (0.17-0.18 mm diameter) and 0.5 ml of phenol-chloroform-isoamyl alcohol (25:24:1 vol/vol/vol pH 8.0) were added. Lysis of the cells was achieved by bead beating for two 1 min periods at 1000 rpm (Bosch, West Germany). Following centrifugation 13,000 rpm for 10 min (Sorvall SS-34 rotor), the aqueous supernatant was removed and the pellet re-extracted. The procedure was repeated three times and the lysates pooled. Polyethylene glycol 6000 (15% w/v final concentration) and 0.1 volume 5 M NaCl solution were added. After precipitation at room temperature for a minimum of 2 h, the lysates were centrifuged 13,000 rpm for 10 min (Sorvall SS-34 rotor). The nucleic acid pellet was washed twice in 80% ethanol and dried in a vacuum dessicator. The DNA was resuspended in 20 µl of Hypersolv water (BDH).

2.18. Extraction of DNA from tangential flow filtered lakewater.

A modification of the method described by Schmidt *et al.* (1991) was applied. Following concentration of lakewater to a final volume of 1 litre by tangential flow filtration, cells were centrifuged at 13,000 rpm for 30 min (Sorvall SS-34 rotor) and resuspended in 4.5 ml of lysis solution (40 mM EDTA, 0.75 M Sucrose, 50 mM Tris-HCL pH 8.3). After the addition of lysozyme (Boehringer Mannheim) to a final concentration of 1 mg ml^{-1}, the mixture was incubated at 37°C for 30 min. Following the addition of proteinase K (0.5% w/v final concentration) and SDS (0.5% w/v final concentration), the lysates were incubated at 37°C for 2 h. Hexadecylmethyl ammonium bromide (final concentration 1% w/v) and 0.7 M NaCl were then added and the lysates incubated at 65°C for 20 min. The cell lysates were pelleted by centrifugation at 13,000 rpm for 10 min (Sorvall SS-34 rotor) and resuspended in TE buffer pH 8.0 (10 mM Tris.HCL pH 8.0, 1 mM EDTA pH 8.0). The nucleic acids were purified by caesium chloride ultracentrifugation as described in section 2.19.

2.19. Purification of DNA by caesium chloride density dependent gradient ultracentrifugation.

Ethidium bromide (400 µl of 10 mg ml^{-1} solution), and caesium chloride (1.075 g ml^{-1} final concentration) were added to produce solutions of 37% (w/v) glucose equivalent density. The lysates were transferred to an ultracentrifugation tube (Ultra crimp, DuPont) and centrifuged in a fixed angle rotor (18 h at 55,000 rpm 18°C LKB RP 55T rotor). Bands of DNA could be visualised at 260 nm with a UV transilluminator and transferred to sterile Eppendorf tubes. Removal of ethidium bromide was achieved by the addition of an equal volume of NaCl/water-saturated

isopropanol, and gentle mixing, followed by microcentrifugation at 13,000 rpm for 10 min (Sorvall SS-34 rotor). The aqueous (top) layer was retained and contaminating protein removed with an equal volume of phenol-chloroform. The DNA was dialysed twice against 1 litre TE pH 8.0 (10 mM Tris.HCl pH 8.0, 1 mM EDTA pH 8.0) overnight at 4°C. Nucleic acids were precipitated by the addition of 0.1 volume of 3 M sodium acetate pH 5.0 and 2 volumes of absolute ethanol at -20°C for 1 h. DNA was pelleted by microcentrifugation at 13,000 rpm for 10 min (Sorvall SS-34 rotor). Pellets were washed with 70% ethanol and vacuum-dried before resuspending in 100 µl Hypersolv water (BDH). DNA was quantitatively and qualitatively assessed by agarose gel electrophoresis (see section 2.21) and by UV spectroscopy at 260 nm and 280 nm. If required, the DNA was further purified using Centricon™ centrifugal microconcentrators C-100 (Amicon, Inc., MA) according to the manufacturers' instructions.

2.20. Polymerase Chain Reaction (PCR) amplification.

PCR primers used were either synthesised at the School of Biological Sciences, University of Liverpool, Liverpool (ABI 392 oligonucleotide synthesiser) or commercially synthesised by Perkin Elmer. Details of oligonucleotides are provided in **Table 2.5**. DNA template samples were serially diluted ten-fold to determine the optimal sample concentration for amplification. A 100 µl reaction mix was prepared comprising the following: 100 mM of deoxynucleotide mix (Pharmacia Biotech), 80 µl sterile Hypersolv™ water (BDH), 10 pM each of forward and reverse primer, 10 µl 10x manufacturer supplied buffer and 2U Super Taq polymerase (HT Biotechnologies). A 0.5-4 µl aliquot of template (equivalent to 4-10 ng environmental

Oligonucleotide	Sequence (5'-3')	Target of Primer	PCR Annealing Temp. (°C)
pA[1]	AGA GTT TGA TCC TGG CTC AG	16S rRNA gene of eubacteria (8-28)*	55
pB[1]	TAA CAC ATG CAA GTC GAA CG	16S rRNA gene of eubacteria (50-70)*	55
pC[1]	CTA CGG GAG GCA GCA GTG GG	16S rRNA gene of eubacteria (341-361)*	55
pC*[1]	CCC ACT GCT GCC TCC CGT AG	16S rRNA gene of eubacteria (361-341)*	55
pD[1]	CAG CAG CCG CGG TAA TAC	16S rRNA gene of eubacteria (518-536)*	55
pD*[1]	GTA TTA CCG CGG CTG CTG	16S rRNA gene of eubacteria (536-518)*	55
pE*[1]	CCG TCA ATT CCT TTG AGT TT	16S rRNA gene of eubacteria (928-908)*	55
pF*[1]	ACG AGC TGA CGA CAG CCA TG	16S rRNA gene of eubacteria (1073-1053)*	55
pG*[1]	ACG GGC GGT GTG TAC	16S rRNA gene of eubacteria (1407-1392)*	55
pH*[1]	AAG GAG GTG ATC CAG CCG CA	16S rRNA gene of eubacteria (1542-1522)*	55
EUB338[2]	GCT GCC TCC CGT AGG AGT	16S rRNA gene of eubacteria (338-355)*	(50)$^{\xi}$

* Numberings list corresponding target positions in the *E.coli* 16S rRNA (Edwards *et al.*, 1989).
( )$^{\xi}$ Oligonucleotide hybridisation temperature.
[1] Edwards *et al.* (1989). [2] Amann *et al.* (1990).

Table 2.5i Oligonucleotides used in this study for the analysis of indigenous bacterial populations by PCR.

Oligonucleotide	Sequence (5'-3')	Target of primer	PCR Annealing Temp. (°C)
Nm75[3]	CGG CAG CGG GGG CTT GGG CC	16S rRNA gene of *Nitrosomonas europaea* lineage (75-95)*	63
Nm75•[3]	GGC CGA AGC CCC CGC TGC CG	16S rRNA gene of *Nitrosomonas europaea* lineage (75-55)*	(45)[ξ]
Nm1007•[3]	TCTAATGGAGACATAAGAGTACCCG	16S rRNA gene of *Nitrosomonas europaea* lineage (1007-981)*	63
Ns85[3]	GGG GG CAA CCC TGG TGG CGA	16S rRNA gene of *Nitrosospira* species (85-105)*	62
Ns85•[3]	TCG CCA CCA GGG TTG CCC CC	16S rRNA gene of *Nitrosospira* species (105-85)*	(45)[ξ]
Ns1009•[3]	GCA CAC CCA CCT CTC AGC GG	16S rRNA gene of *Nitrosospira* species (1009-989)*	62
AAO258•[3]	GCC TTG GTA AGC CTT TAC C	16S rRNA gene of β-subgroup ammonia-oxidisers (258-238)*	(55)[ξ]
Nlm459•[3]	TAG TCA CGG GTA TTA ACC GT	16S rRNA gene of *Nitrosospira multiformis* (459-439)*	(45)[ξ]
NmoCL7_439[5]	CTCTTTCTTTCCGACTAA	16S rRNA gene of *Nitrosomonas europaea* lineage (439-457)	(44)[ξ]
CTO189fAB[4]	CCGCCGCGCCGGCGGGCGGGGCGGGGG CACGGGGGGAGRAAAGCAGGGGATCG	16S rRNA gene of β-subgroup ammonia-oxidisers (189-241)*	57
CTO189fC-GC[4]	CGCCCGCCGCGCGGCGGGCGGGGCGGGG GCACGGGGGGAGGAAAGTAGGGGATCG	16S rRNA gene of β-subgroup ammonia-oxidisers (189-244)*	57
CTOr•[4]	CTAGCYTTGTAGTTTCAAACGC	16S rRNA gene of β-subgroup ammonia-oxidisers (654-634)*	57

* Numberings list corresponding target positions in the E.coli 16S rRNA gene (Edwards *et al.*, 1989).
( )[ξ] Oligonucleotide hybridisation temperature.
[3] Hiorns *et al.* (1995). [4] Kowalchuk *et al.* (1997) [GC clamp underlined]. [5]Stephen *et al.* (1998).

Table 2.5ii Oligonucleotides used in this study for the analysis of ammonia-oxidiser bacterial populations.

DNA or 1 ng control DNA) was added to 96 µl of the reaction mix in a thin-walled PCR reaction tube and overlaid with 2-3 drops of sterile mineral oil to prevent evaporation during amplification.

Thermal cycling was performed in a Perkin Elmer Centus 480 thermal cycler using the cycling parameters according to the melting temperature (Tm) of the primers. The reaction parameters using universal primers were 26 cycles consisting of 94°C 1 min, 55°C 2 min, 72°C 2 min, after 26 cycles a final elongation step of 10 min at 72°C. Reaction tubes were held at 0°C upon completion.

Amplifications using *Nitrosospira*-specific primer pair Ns85 and Ns1009• and *Nitrosomonas*-specific primer pair Nm75 and Nm1007• were annealed at 62°C and 63°C respectively. PCR amplification using these genus and species-specific primers was routinely performed on templates produced by amplification with universal PCR primers pA and pH•. Products were suitably diluted (1-10% (v/v) in Hypersolv water BDH) to prevent interference due to primers from the initial PCR reaction. Primers used in the second amplification annealed to sites internal to those of the primary reaction, and this is termed 'nested' PCR. This increased the sensitivity of diagnostic primers for the detection of environmental template DNA.

To enhance product yield for environmental samples, a 'hot start' PCR protocol was adopted. Template DNA was added to a standard PCR reaction mix giving a total volume of 100 µl and comprising 10 pM of each primer, 10 mM of each deoxynucleoside triphosphate (Pharmacia), 10x manufacturer supplied PCR buffer and sterile Hypersolv water (BDH). The reaction was heated to 95°C for 6 min to denature the DNA and the temperature held at 80°C for the addition of 2U Super Taq

polymerase (HT Biotechnologies) and 10x PCR reaction buffer (HT Biotechnologies) through the overlaying mineral oil. Cycling parameters were as described above.

PCR amplification for DGGE and TGGE used primers designed by Kowalchuk *et al.* (1997). An equimolar mixture containing three forward primers, each with a GC-clamp, and a single reverse primer containing a single ambiguous base were synthesised separately. Approximately 10-100 ng of environmental DNA or 10 ng of cloned DNA were used in a 50 µl PCR reaction mixture containing: 2U of Expand (Boehringer Mannheim), 10x manufacturer supplied buffer, 10 pM of each primer, 10 mM of each deoxynucleoside triphosphate (Pharmacia), and sterile Hypersolv water (BDH). All reaction mixtures were overlaid with an equal volume of mineral oil. Cycling parameters were as follows: 60 s at 94°C, followed by 35 cycles of 30 s at 94°C, 60 s at 57°C, and 40 s at 72°C, followed by a 10 min final extension at 72°C.

2.21. Agarose gel electrophoresis

Agarose gels (0.8-1.0% w/v) were prepared in 1x TAE buffer (2 ml 50x solution TAE: 0.5 M Tris.HCl, pH 7.6; 0.05 M EDTA; 57.1 ml glacial acetic acid) and 2 µl ethidium bromide (10 mg ml^{-1}) added to 100 ml molten agarose (Seakem ME, FMC Bioproducts) prior to pouring the gel. Nucleic acid samples (1-15 µl) were mixed with 4 µl tracker dye (50% (v/v) glycerol, 50% (v/v) TE buffer pH 7.6, 0.05% (w/v) bromophenol blue) prior to loading onto the gel. Nucleic acids were separated by electrophoresis at 90 V cm^{-1} for 1 h and visualised on a UV transilluminator at 320 nm. For the resolution of smaller fragments (< 1kb), 2% (w/v) 1x TAE gels were used and electrophoresis performed at 60 V cm^{-1} for at least 2 h.

2.22. Recovery of DNA from agarose gels.

The required DNA product was carefully excised from the gel and placed in an Eppendorf tube. Extraction and purification of the DNA was performed using Qiaprep spin columns (Qiagen) in accordance with the manufacturers' instructions.

2.23. *In Vitro* DNA manipulations.

Ligation and restriction of DNA were performed using enzymes supplied by either Boehringer-Mannheim or Promega in accordance with the manufacturers' instructions. Electrophoretic assessment of restriction products was in conjunction with the appropriate molecular weight markers (Boehringer-Mannheim) prepared with the equivalent volumes of restriction buffer. Quantification of ethidium bromide-stained DNA bands was performed using a Molecular Dynamics Computing Densitometer and Image Quant version 3.0 (Molecular Dynamics, Inc., Sunnyvale, California).

2.24. Density gradient gel electrophoresis (DGGE).

The DGGE was performed by the method of Muyzer *et al.* (1995a) with a D-Gene system (Bio-Rad Laboratories) and performed at the University of Aberdeen with the assistance of Dr. J. Stephen. Polyacrylamide gradient gels (8% polyacrylamide; 1.5 mm thick; 0.5x TAE; 37:1 acrylamide-bisacrylamide; 38-50% denaturant; 200 x 200 mm) were poured with the aid of a gradient maker (CBS, Del Mar, California) and an Econo-pump (Bio-Rad Laboratories) at a speed of 5 ml min^{-1} (40 ml gradient volume). Denaturing acrylamide (100%) was defined as 7 M urea with 40% formamide (Myers *et al*, 1987). Gels were poured from bottom to top. If

required, a 10 ml stacking gel containing no denaturants was added on top prior to polymerization. Gels were run for either 6.5 h at 200 V or for 16 h at 85 V in 0.5x TAE buffer at a constant temperature of 60°C. The gels were stained with Hypersolv water (BDH) containing 0.5 mg l^{-1} of ethidium bromide and destained twice in 0.5x TAE buffer prior to UV transillumination. Gel images were stored using the Phosphoimager System (Molecular Dynamics). Quantification of ethidium bromide-stained bands was performed with the ImageQuant, version 3.3 (Molecular Dynamics).

2.25. Temperature gradient gel electrophoresis (TGGE).

Polyacrylamide gels (6%) were cast as described for DGGE in section 2.24 but without the denaturants. Gels were run at 43-60°C for 16 h (ramp rate 1.25°C h^{-1}) in 1.25x TAE buffer at 200 V for 15 min followed by 100 V for 16 h using a D-Gene system (Bio-Rad laboratories). Gels were stained using Cyber Green (Flowgen) (final concentration 1x) and scanned using the phosphoimager (Molecular Dynamics).

2.26. Immobilisation of nucleic acids.

2.26.1.Southern transfer of DNA

DNA samples were transferred from agarose gels to Positive ™ (Appligene) nylon membrane by capillary action according to the manufacturers' recommendations : (" Membrane Transfer and Detection Methods", Hybond ™ -N^+ :Protocols for nucleic acid blotting and hybridisation", Amersham), and those of Sambrook *et al.* (1989). In all experiments 0.4 M NaOH was used for alkaline transfer of nucleic acids.

2.26.2. Slot blotting of DNA using a vacuum manifold.

DNA was applied to positively charged nylon membrane (Positive™ Appligene) with a Minifold II manifold (Schleicher & Schuell) which had a sample footprint of 6 mm^2 . DNA samples (50 µl maximum volume) were heated to 95°C, chilled on ice and one volume of ice cold 20x SSC (3 M NaCl; 0.3 M sodium citrate, pH 8.0) added. Samples were stored on ice until required. The samples were applied to the manifold following the manufacturers' instructions (see section 2.25.1) and the slots washed twice with 200 µl 20x SSC (3 M NaCl, 0.3 M sodium citrate pH 8.0) under vacuum. Nucleic acids were fixed on to the membrane by air drying for 1 h and heat-fixed at 80°C for 1 h. If required, membranes were wrapped in cling film and stored at 4°C.

2.27. Oligonucleotide probe labelling

Oligonucleotide probes were quantified by UV spectroscopy (at 260 nm and 280 nm) and diluted to a final concentration of 10 pM μl^{-1}. Probes were end-labelled with γ- ^{32}P-dATP (ICN Supplies) using, 10 pM oligonucleotide, 1 µl 10x kinase buffer (Tris.HCl, 50 mM; $MgCl_2$, 10 mM; EDTA, 0.1 mM; dithiothreitol, 5 mM; spermidine, 0.1 mM; pH 8.2), 6 µl sterile Hypersolv water (BDH), 1 µl T4 phage polynucleotide kinase, (Boehringer-Mannheim) and 1 µl γ- ^{32}P-dATP (370 Kbq) were mixed and incubated at 37°C for 1 h. Probe labelling efficiency was assessed by applying aliquots (1 µl of a solution containing 10% (v/v) reaction mix and sterile Hypersolv water BDH) spotted on to duplicate squares (1 cm approximately) of DE81 paper (Whatman). Upon drying, 50% of the membrane samples were rinsed in 300 ml ice cold 0.5 M Na_2HPO_4 for 2 min. The membranes were transferred to a beaker

containing a fresh solution of 0.5 M Na_2HPO_4 and the process repeated twice before briefly rinsing the filter in 70% (v/v) ethanol and air drying. Subsequently, the membranes were inserted in scintillation counter vials and radioactivity quantified by counting Cerenkov radiation using the ^3H channel of a scintillation counter (Beckman LS 1801). Comparison of the radioactivity present on the unwashed and washed filters (which retained only labelled oligonucleotide probe) revealed the percentage labelling efficiency. Labelling efficiency in excess of 20% was deemed satisfactory.

2.27.1. Oligonucleotide probing solutions.

10% Blocking reagent solution:

Maleic acid (0.1 M) and NaCl (0.15 M) were dissolved in 500 ml DEPC-treated water and adjusted to pH 8.0 by the addition of NaOH. Blocking reagent (final concentration 50 g 500 ml^{-1}, Boehringer Mannheim) was dissolved in this solution prior to autoclaving. The solution was stored at 4°C prior to use at 2% (w/v) in prehybridisation solution.

Prehybridisation solution:

Blocking reagent (2% w/v); SSPE, 5x (Briefly, to 800 ml distilled H_2O was added NaCl, 45.83 g; $Na_2HPO_4 . H_2O$, 27.6 g; EDTA, 7.4 g; adjusted to pH 7.4 and made up to 1 litre), deionised formamide, 20 % (v/v); SDS, 0.02% (w/v); N-laurylsarcosine, 0.1% (w/v) were prepared in sterile deionised water. Sufficient solution was used to cover the membranes and prehybridisation was for 1 h at 45°C.

Hybridisation /wash solution:

SSPE, 5x (Briefly, to 800 ml distilled H_2O was added NaCl, 45.83 g; $NaH_2PO_4.H_2O$, 27.6 g; EDTA, 7.4 g; adjusted to pH 7.4 and made up to 1 litre); deionised formamide, 20% (v/v); SDS, 0.02% (w/v); N-laurylsarcosine, 0.1% (w/v) were prepared in sterile deionised water. Blocking reagent was not included in hybridisation or wash solutions, as this may inhibit probe annealing, particularly when probes are less than 100 nucleotides in length, (Sambrook *et al.*, 1989). Hybridisations containing 10 pmol. of labelled probe were carried out overnight at the specific annealing temperature inside a thermostatically controlled oven (Hybaid). The membranes were washed three times for 5 min in fresh wash solution at the same temperature. The filters were double wrapped in cling film and X-ray film (Fuji-film X-O-graphic) exposed for both two and seven days at -70°C.

2.27.2. Removal of probes from DNA on nylon membranes

The probes were eluted from the membranes by immersion in boiling 0.1% SDS solution. The membranes were agitated and allowed to cool to approximately 40°C. This was repeated, if required, for probes with a strong binding efficiency. The membranes were then rinsed in 0.1x SSC. The efficiency of probe removal was confirmed by either Geiger counts or re-exposure to new X-ray film. Stripped membranes were wrapped in cling film and stored at -20°C until required.

2.28. Scanning Densitometry

Images generated from X-ray films were quantified using Molecular Dynamics Computing Densitometer and Image Quant version 3.0 (molecular Dynamics). Mean

signal (pixel) intensity above background and signal volume above background were determined and calibrated against DNA of a known concentration.

2.29. *In Situ* probing.

2.29.1. Fixation.

Cells from overnight mid-exponential phase cultures were harvested by centrifugation (13,000 rpm for 30 min, Sorvall SS-34 rotor) and washed twice in PBS. The pellet was resuspended in 0.25 ml PBS and 0.75 ml fixative (pH 7.2) and incubated at 4°C overnight. Fixative comprised: 44.5 ml sterile distilled water heated to 60 °C, 5 ml 10 X PBS, 1 drop (from a pasteur pipette) 10 M NaOH and 2 g paraformaldehyde. After cooling on ice, the fixative pH was adjusted to pH 7.2 and filtered through a 0.45 μm filter. After fixation, the cells were washed with 1 ml PBS and stored in a 1:1 mixture of PBS and absolute ethanol at -20°C until required.

2.29.2. Liquid hybridisation.

An appropriate volume of fixed cells was added to a sterile Eppendorf tube and centrifuged (13,000 rpm for 5 min Sorvall SS-34 rotor). Hybridisation buffer (38 μl) was added to the cell pellet and vortexed. (Hybridisation buffer comprised: 50% formamide (v/v); 20% sterile distilled water (v/v); NaCl 4.5M; 10% SDS; Tris-HCl (pH 7.2) 200mM). Fluorescently labelled 5'-fluorescein isothiocyanate (FITC) oligonucleotide probe 100 ng μl^{-1} was added to the cell suspension and the tube incubated at 37°C overnight. Controls were 5' FITC-labelled EUB338 (a domain eubacteria rRNA complementary probe) and EUB338* (a non-complementary probe) (Amman *et al.*, 1990). An additional negative control in the absence of

oligonucleotide probe was included to evaluate autofluoresence. Following incubation, the cells were pelleted by centrifugation at 13,000 rpm for 5 min (Sorvall SS-34 rotor), resuspended in 0.5 ml hybridisation buffer and incubated at room temperature for 15 min. This washing procedure was repeated twice. Finally, the pellet was washed in 1 ml sterile Hypersolv water and pelleted. The supernatant was removed and the pellet resuspended in 20-30 µl of water. The suspension was then transferred to a gelatin-coated microscope slide and analysed on a fluorescence microscope equipped with the appropriate filters.

2.30. Flow Cytometry.

Lakewater samples and MPN enrichments from profundal sediment and lakewater obtained from Windermere were fixed in paraformaldehyde as described in section 2.29.1. Fluorescently labelled 5'-fluorescein isothiocyanate (FITC) oligonucleotide probes (100 ng μl^{-1} final concentration) were added to the fixed cells and hybridisation performed as described in section 2.29.2. Samples were analysed cytometrically using a FACStar Plus flow cytometer (Becton Dickinson, Oxford, UK) located at the Institute of Freshwater Ecology, Windermere, UK and operated by Dr. R.W. Pickup. The flow cytometer was set to measure two parameters, forward light scatter (FSC) and fluorescence at 525 nm. The laser power at 488 nm was set at 0.2 W and all recordings were taken on a log scale. The photomultiplier voltages were set at 600 V for FSC and at 520-530 nm for the fluorescence detector. The cytometer was aligned with 0.5 µm diameter Fluoresbrite YG fluorescent latex beads (Polysciences Inc., USA). The nozzle diameter was 70 µm and for each sample, 5000 events were recorded. The FACStar Plus computing package was used to determine the proportion

of cells that were fluorescently labelled and displayed the data as single dot plots. Unlabelled samples were used as negative controls to determine the baseline fluorescence of non-labelled cells.

2.31. Cloning of PCR Products.

Prior to cloning, amplicons were quantified using a low DNA mass ladder (GibCo) and the optimum amount of product was ligated into the PGEM-T vector (Promega) in accordance to the manufacturers' instructions. Recombinant plasmids were used to transform 50 µl of high efficiency competent cells (Promega). White tranformants were screened using selective LB agar (LabM, Amersham) containing X-Gal (50 mg ml^{-1} final concentration, Boehringer-Mannheim), and ampicillin (50 µg ml^{-1} final concentration). Briefly, for 1 litre LB agar, 15 g agar (LabM, Amersham), 10 g bacto tryptone (LabM, Amersham), 5 g bacto yeast extract (LabM, Amersham), and 10 g NaCl were dissolved in 950 ml distilled water, and the pH adjusted to 7.0 with 5 M NaOH. The final volume was adjusted to 1 litre with distilled water and the medium sterilised by autoclaving.

2.32. Plasmid isolation.

Plasmid DNA was extracted from an overnight culture grown in LB broth supplemented with ampicillin (50 µg ml^{-1} final concentration) using the plasmid midi kit (Qiagen) in accordance with the manufacturer's instructions. Plasmid DNA was routinely resuspended in 100 µl of Hypersolv water (BDH).

2.33. DNA Sequencing.

Clone banks obtained were screened for insert-carrying strains by radioisotopically labelled DNA hybridisation and plasmid miniprep restriction analysis. Clones selected for sequencing were grown overnight in 10 ml LB broth (containing 50 $\mu g\ ml^{-1}$ ampicillin final concentration). The plasmid was extracted using Qiagen plasmid midi prep kit (Qiagen). Electrophoresis and reading of the sequences were performed using the School of Biological Sciences sequencing facility (an automated laser flourescence ABI 373A DNA sequencer).

2.33. Data analysis.

2.33.1. Analysis of sequence data.

Sequences were analysed using the Genetics Computer Group (GCG) suite of programs (Devereux *et al*, 1984) either running on the SEQNET facility at Daresbury, UK or on the UNIX computer at the University of Liverpool. 16S rRNA sequences derived from environmental samples were aligned by eye in conjunction with representative sequences deposited in the National Science Foundation Ribosomal Database Project (RDP) (Larsen *et al.*, 1993) and the Genbank database (Benson *et al.*, 1993). Data analysis and manipulation were performed using the Genetic Data Environment (GDE) software running on a SPARC 10 workstation. The application of a 'Mask' allowed the alignment of unambiguous sequences for comparison. Phylogenetic analyses of distance corrections were achieved using Phylogeny Inference Programs (PHYLIP 3.4) (Felsenstein, 1993). Phenograms illustrated in this thesis were generated using the Jukes-Cantor (1969) correction in the DNADIST

program from PHYLIP 3.4. Phylogenetic trees were constructed from the computed distance values using the neighbor joining method (Saitou & Nei, 1987). The robustness of the inferred phylogeny was determined by bootstrap analysis consisting of 100 resamplings of the data performed using SEQBOOT (PHYLIP 3.4). A consensus phenogram was generated using neighbor joining and the program CONSENSE (PHYLIP 3.4). The topologies of phenograms were corroborated by maximum parsimony analysis using PAUP version 3.0 (Swofford, 1991)

2.34.2. Analysis of RFLP data.

RFLP data were analysed using the DICE coefficient of variation (Dice, 1945). The percentage similarity between two clones under examination was calculated using the formula:

% similarity=100x (2x number of indistinguishable bands/ total number of bands)

A distance matrix of similarity coefficients was generated.

2.34.3 Statistical Analysis.

Analysis of variance (ANOVA) was performed with MINITAB 8.21 (Minitab Inc., State College, PA, USA), using the Minitab macros recommended by Fry (1993). In order to attain the ANOVA assumptions of homogeneity of variance and normality errors, $\log_{10}$ X data transformation was required. Minimum significant differences were calculated using the Tukey-Kramer method (Fry, 1993).

Chapter 3. Analysis and enumeration of freshwater ammonia-oxidising bacteria by culture-based methods.

3.1. Introduction.

Early studies on the occurrence and significance of ammonia-oxidising bacterial populations in the environment have been dependent upon the isolation of pure cultures (Belser, 1979; Smorczewski & Schmidt, 1991). Whilst conventional methods for the enumeration and identification of bacteria can provide important ecological information, there may be difficulties in determining whether isolates present are dominant species *in situ* (Belser & Schmidt, 1978). Enrichment cultures can be used to concentrate initial inocula to enable the development and optimisation of novel molecular techniques. However, the fraction of culturable cells from surface soils and sediments may not be representive of the total bacterial community present, due to biases introduced by media selectivity, but the most probable number technique (MPN), in appropriate media, can provide a statistically significant enumeration of culturable ammonia-oxidising bacteria (Roszak & Colwell, 1987).

The North and South Basins of Windermere are mesotrophic and eutrophic respectively and Buttermere, although not extensively studied, has been described as oligotrophic (Dr. R.W. Pickup, pers. comm.). This thesis was based on a seasonal study of a number of spatially distinct sites located at Buttermere and Windermere as previously described in section 2.12. In this chapter, conventional culture methods are applied to analyse and enumerate ammonia-oxidising bacterial communities derived from both freshwater lakes. Physical data obtained from Buttermere and Windermere are also presented.

3.2. Results.

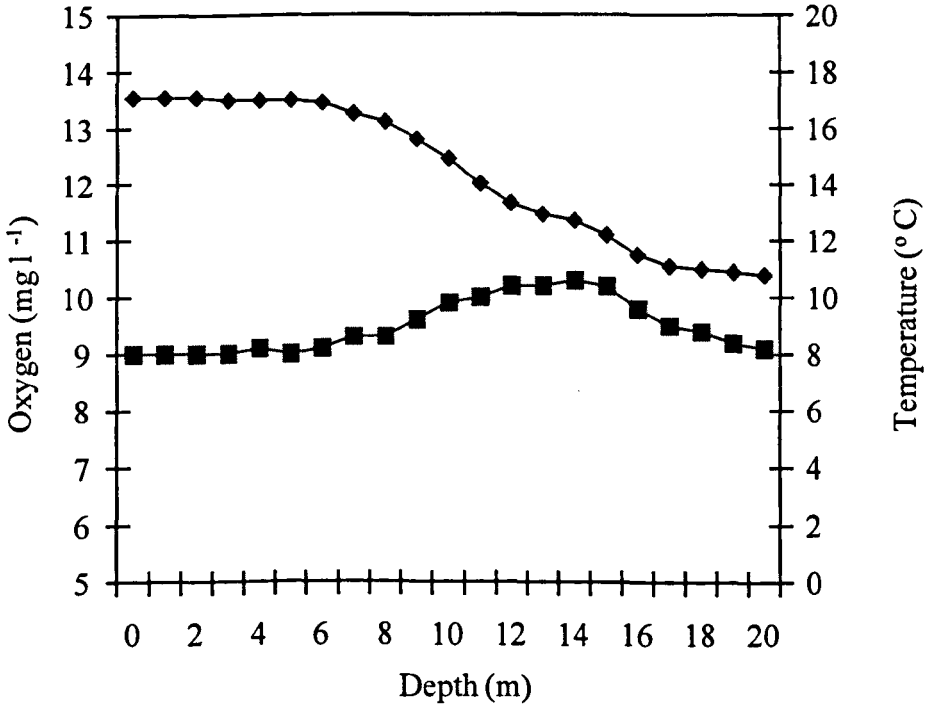
3.2.1. Temperature and oxygen profiles of Buttermere 1995-1996.

The temperature and oxygen concentrations were recorded on the same day as the sediment and lakewater samples were collected from Buttermere. The water column profiles are presented in **Figs. 3.1 (A-F)**. The thermocline was established by July and therefore demonstrated that stratification had occurred. The production of the thermocline facilitates the gradient of oxygen to develop. Both the temperature and oxygen gradients were maintained throughout the summer months. In September, the temperatures change and the lake becomes isothermal. Consequently, the oxycline breaks down and circulation is maintained throughout the winter months.

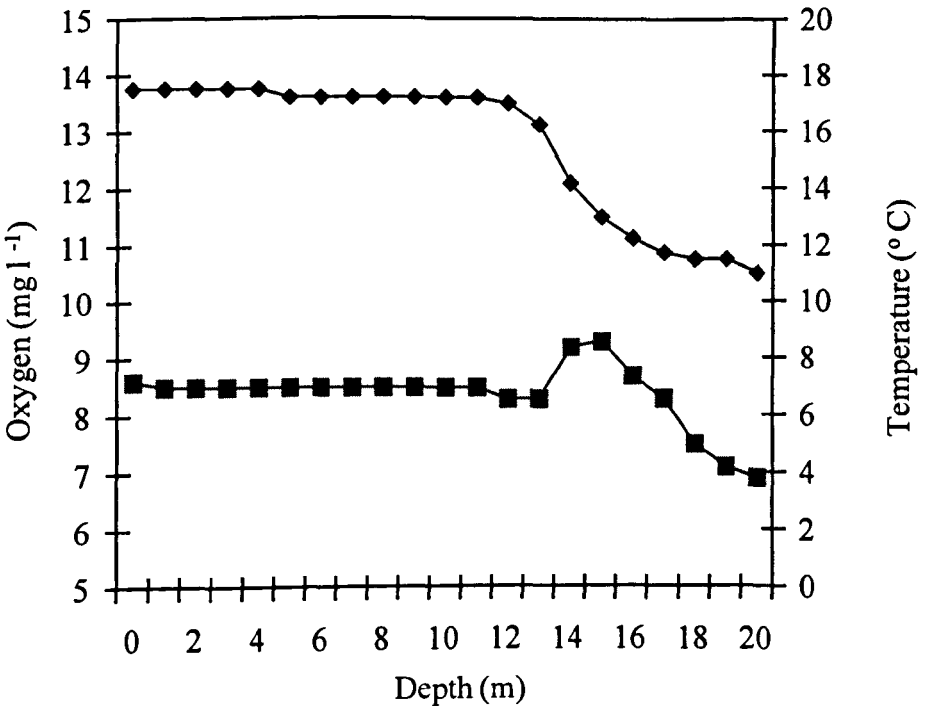
3.2.2. Temperature and oxygen profiles of Windermere 1996-1997.

Temperature and oxygen concentrations were monitored at regular intervals throughout the year by the Institute of Freshwater Ecology. The water column profiles corresponding to the same day that the sediment and lakewater samples were collected from Windermere are presented in **Figs. 3.2 (A-H)**. Stratification of the lake was established by June (1996) and was maintained throughout the summer months. By September, the lake became isothermal, and circulation continued during the winter months. The trends shown in the graphs suggest that the temperature and oxygen concentrations in the North and South basins were similar.

A

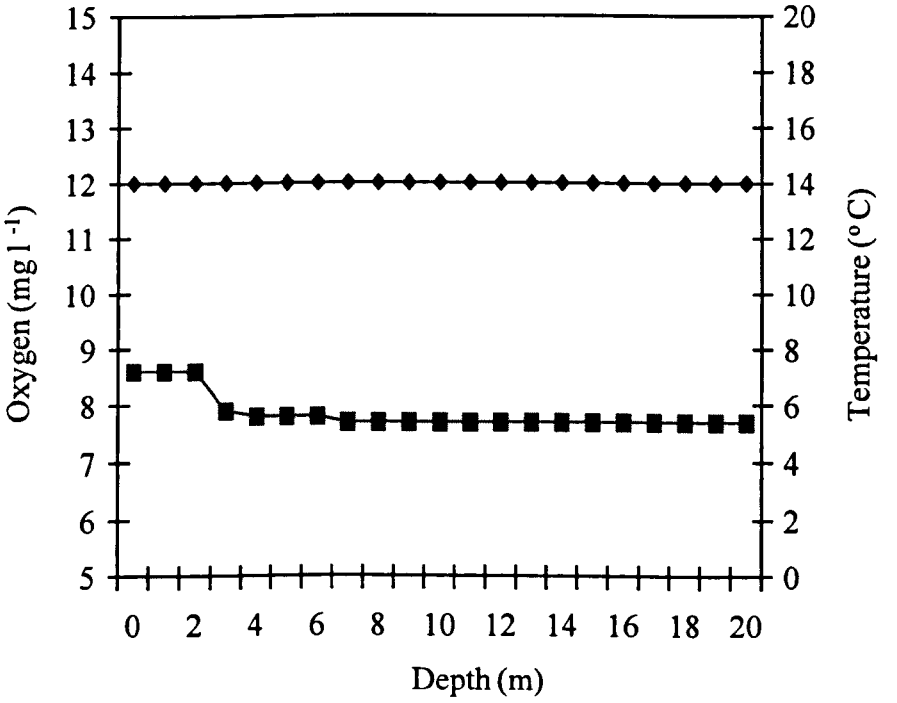


B

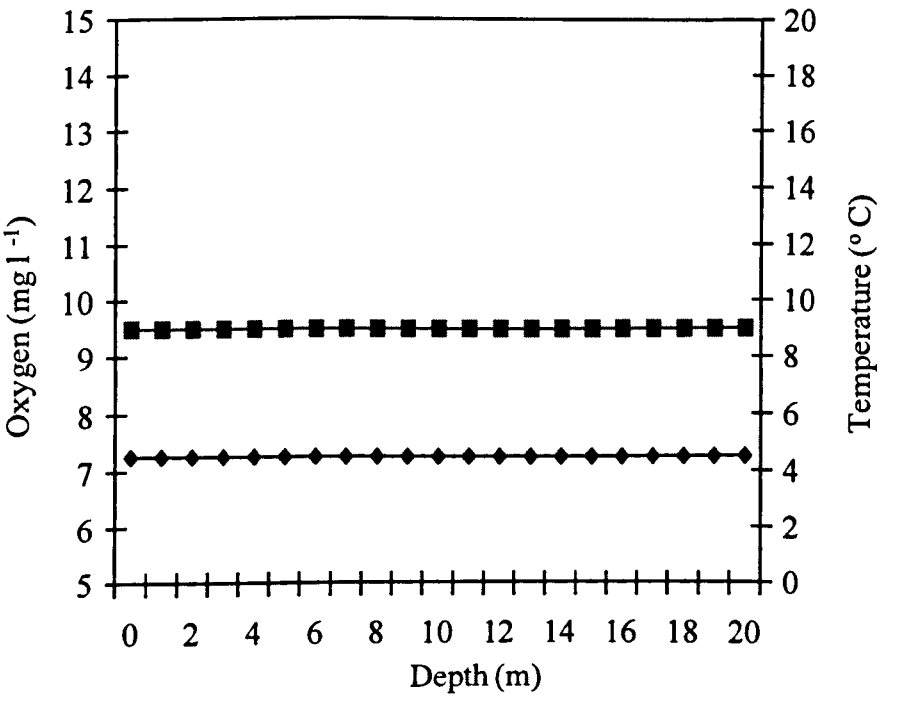


Figs. 3.1 (A-F) Temperature and oxygen profiles in Buttermere. July 1995 (**A**). August 1995 (**B**). Temperature (° C) —◆— Oxygen (mg l^{-1}) —■— .

C

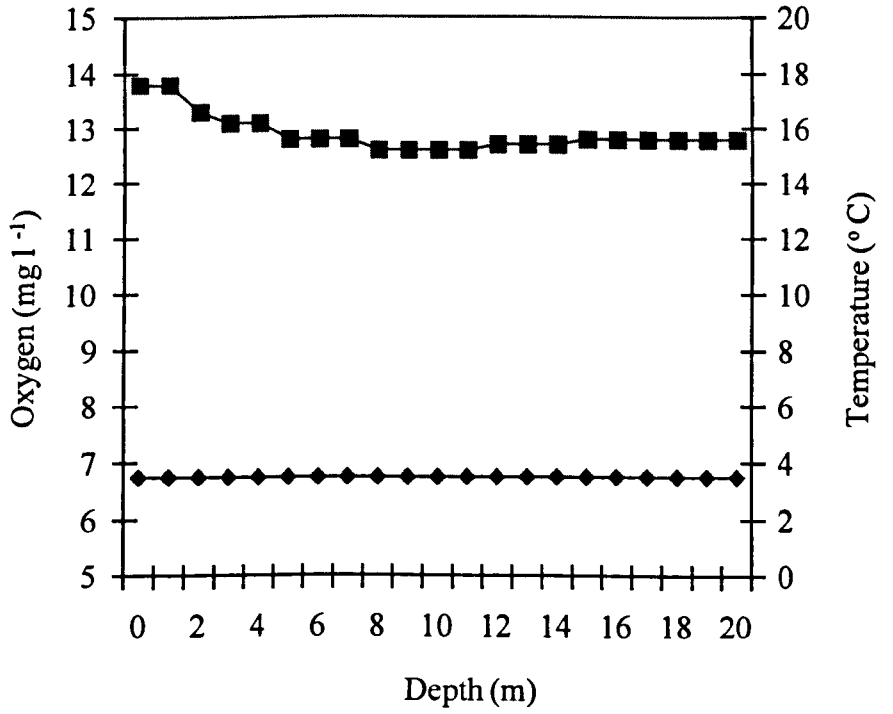


D

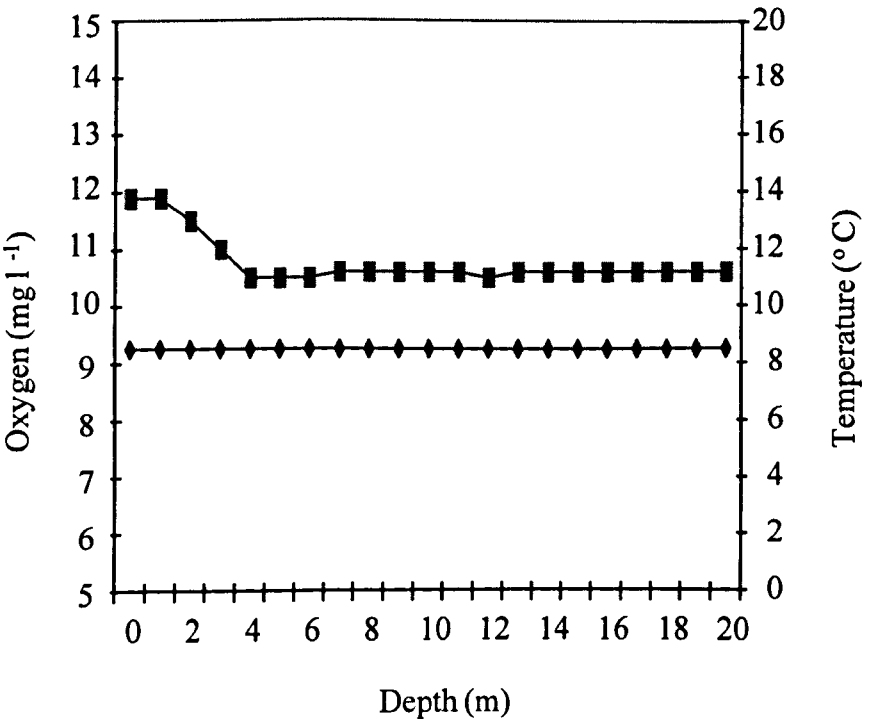


Figs. 3.1 (A-F) Temperature and oxygen profiles in Buttermere. September 1995 (**C**). November 1995 (**D**). Temperature (°C)—◆— Oxygen (mg l^{-1})—■—.

E

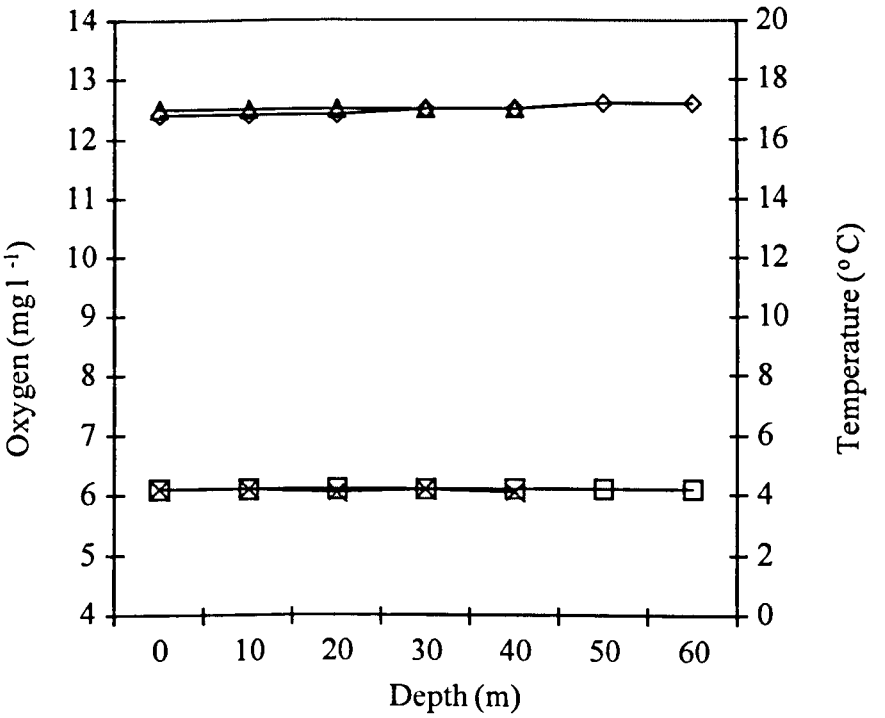


F

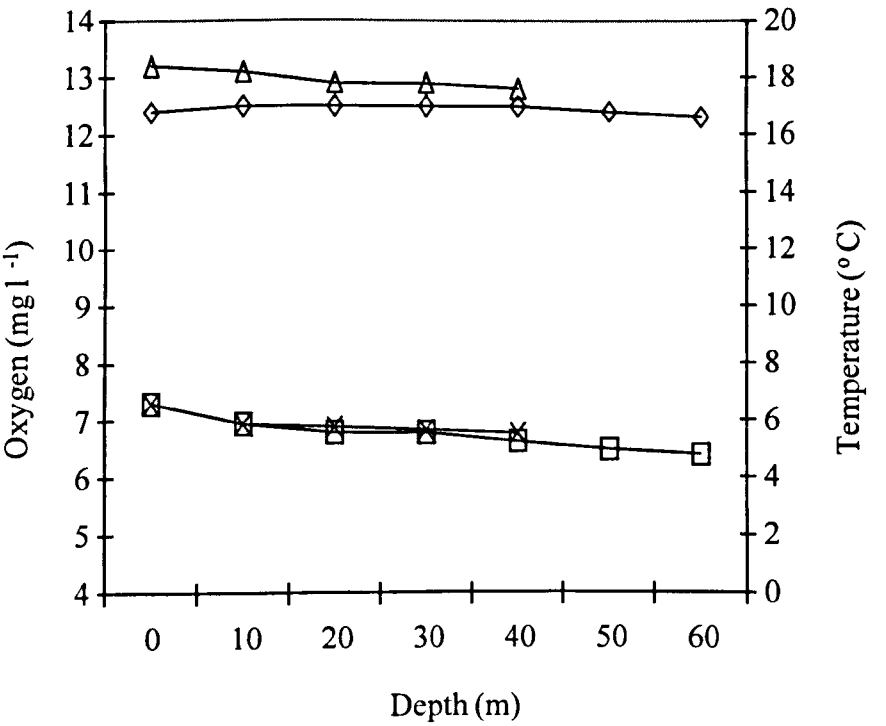


Figs. 3.1 (A-F) Temperature and oxygen profiles in Buttermere. March 1996 (E). May 1996 (F). Temperature (°C)—◆— Oxygen (mg l^{-1})—■— .

A

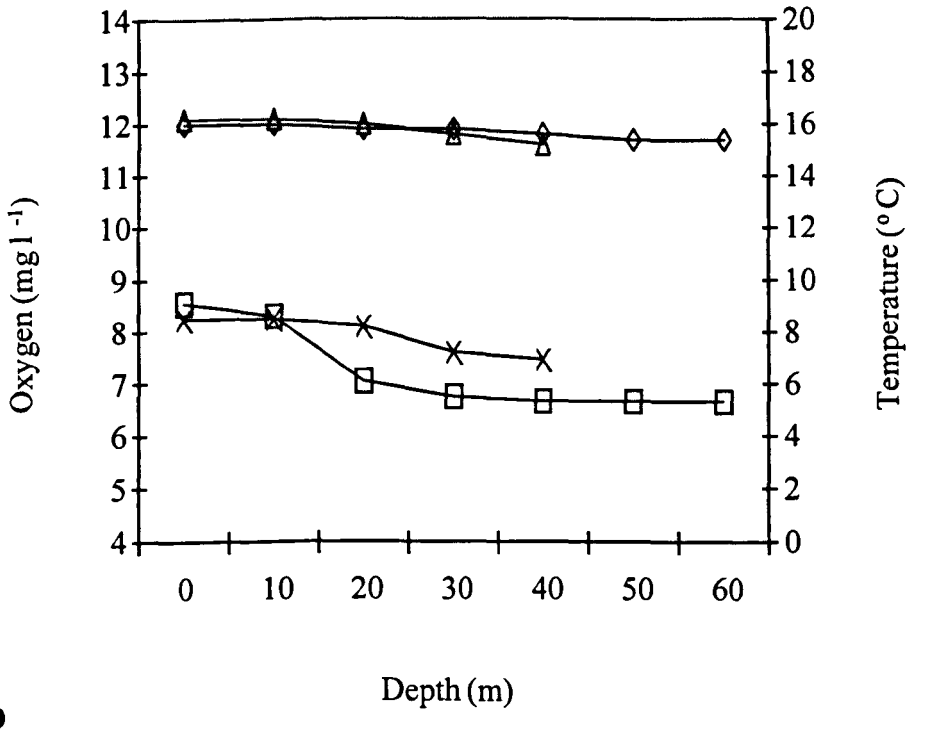


B



Figs. 3.2 (A-H) Temperature and oxygen profiles in Windermere. March **(A)**, April **(B)**.
Temperature: North Basin —□— South Basin —×—
Oxygen: North Basin —◇— South Basin —△—

C



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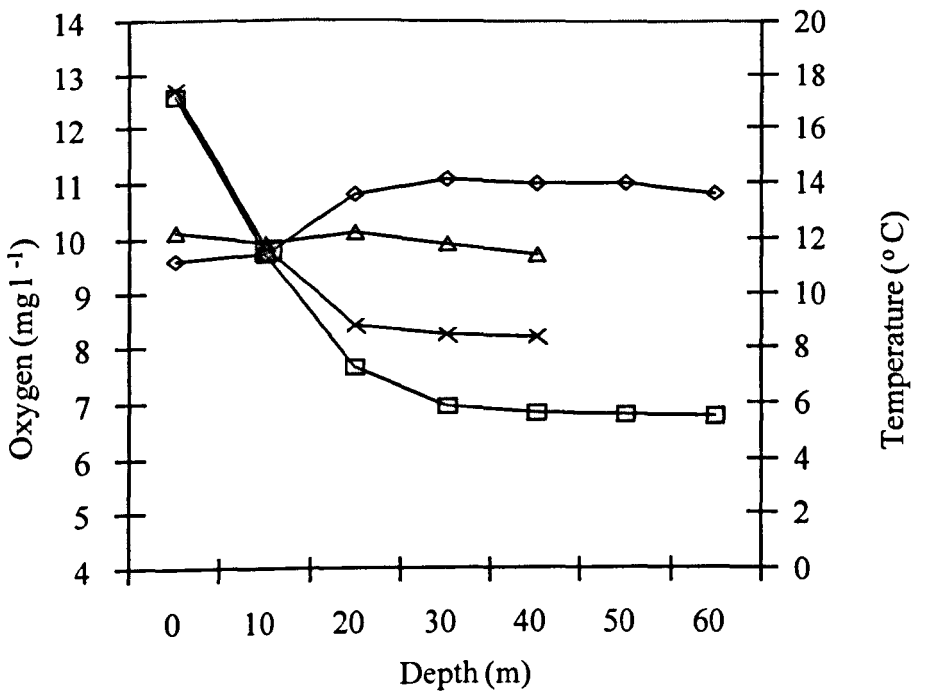
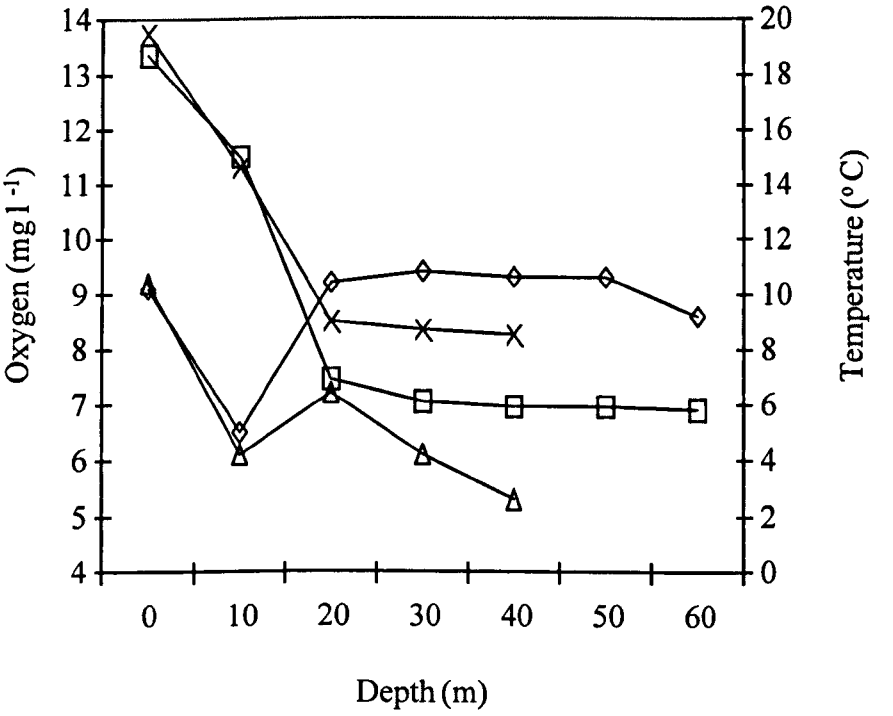
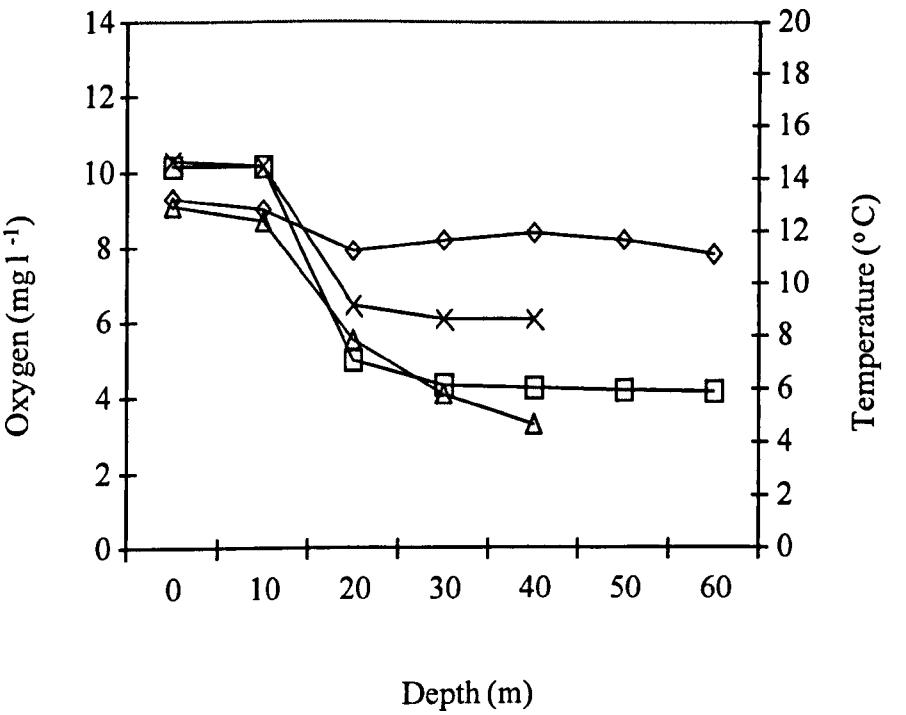


Figs. 3.2 (A-H) Temperature and oxygen profiles in Windermere for May (**C**), June (**D**).
Temperature: North Basin —□— South Basin —×—
Oxygen: North Basin —◇— South Basin —△— .

E

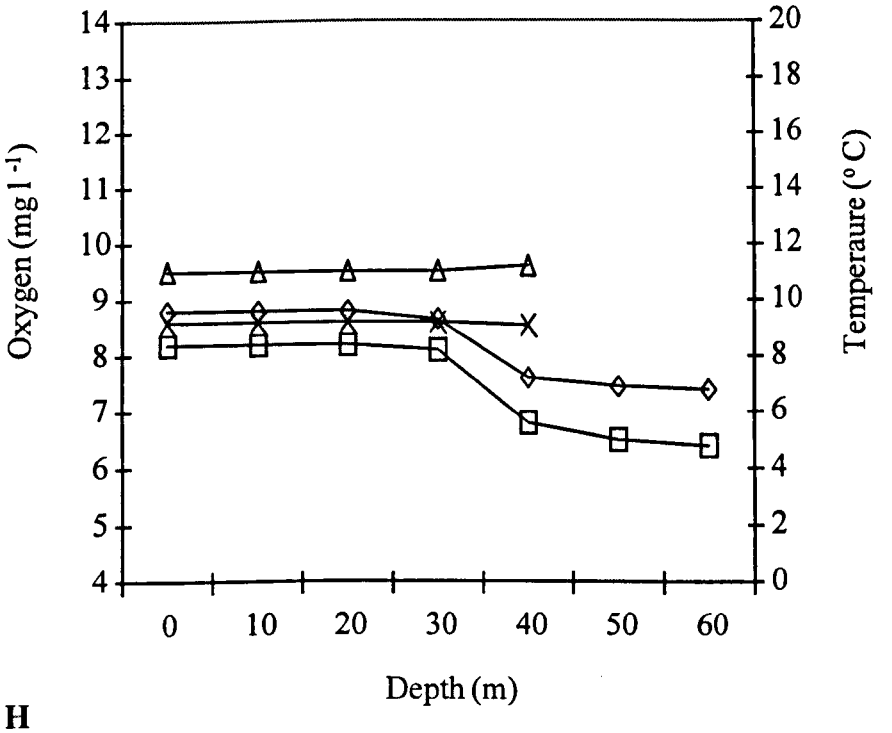


F

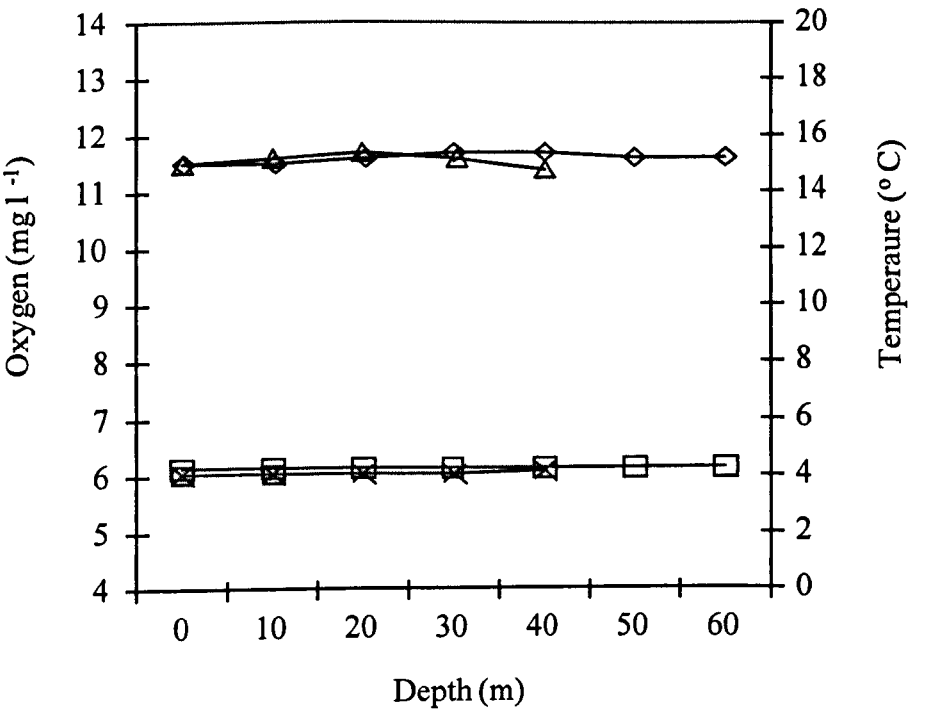


Figs. 3.2 (A-H) Temperature and oxygen profiles in Windermere. August (**E**), September (**F**). Temperature: North Basin —□— South Basin —×— Oxygen: North Basin —◇— South Basin —△— .

G



H



Figs. 3.2 (A-H) Temperature and oxygen profiles in Windermere. November (**G**), January (**H**). Temperature: North Basin —□— South Basin —×— Oxygen: North Basin —◇— South Basin —△— .

3.3. Enumeration of ammonia-oxidising bacteria by MPN.

The bacterial counts of ammonia-oxidising bacteria derived from Windermere, obtained by MPN methods are presented in **Figs. 3.3 (A-C)**. In addition, MPN assays of sediment and lakewater samples from Buttermere obtained in March and May were also performed for comparison and are presented in **Table 3.1**. The MPN assays performed on samples derived from Windermere were also used for flow cytometric analysis (see Chapter 6).

There is no significant difference (at $p = 0.05$) in the numbers of ammonia-oxidising bacteria in the upper and lower layers of profundal sediment derived from the North Basin. However, there is a significant difference (at $p = 0.05$) in the numbers of ammonia-oxidising bacteria between the upper and lower layers of profundal sediment derived from the South Basin. The counts for ammonia-oxidising bacteria for all the profundal sediment samples derived from Windermere, with the exception of samples taken in May, June and January (upper layer) and June (lower layer), were higher for the South Basin compared to the North Basin. South Basin sediment counts ranged between $2.5x10^2$-$2.4x10^6$ g^{-1} dry weight and the counts for the North Basin ranged between $3.5x10^2$-$1.0x10^4$ g^{-1} dry weight throughout the year. The data suggest there is a significant difference between basins, in the numbers of ammonia-oxidising bacteria present in the sediment (at $p = 0.05$). Differences between basins were observed in the upper oxic layer of sediment in April prior to stratification, whereby the South Basin was >100 cells g^{-1} dry weight greater than the North Basin.

Ammonia-oxidiser counts derived from lakewater were low (<100 cells ml^{-1}) throughout the year, with the exception of a seasonal peak in August (950 cells ml^{-1})

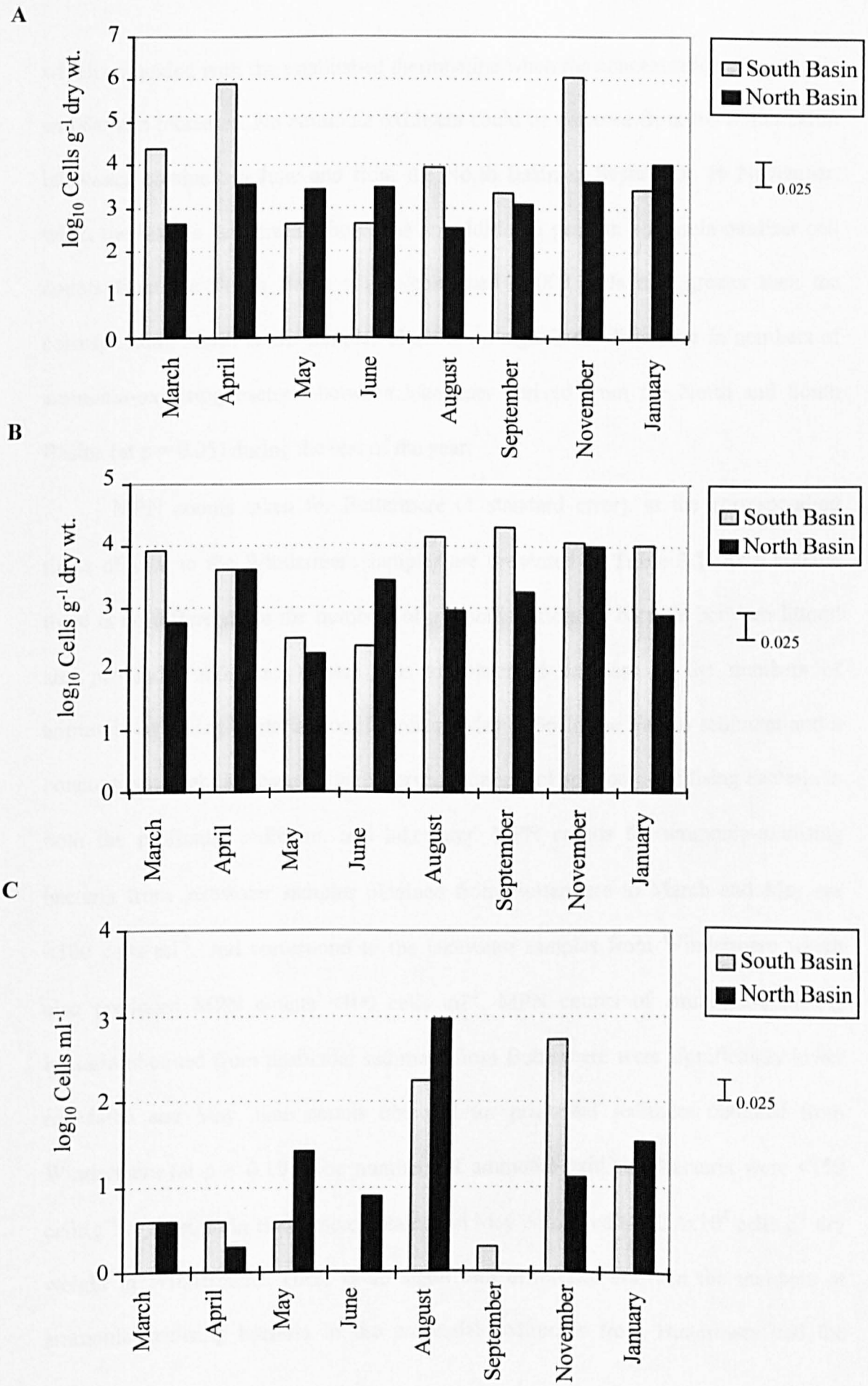


Fig. 3.3 MPN counts of ammonia-oxidising bacteria in Windermere. Profundal sediment: 0-0.5 cm (**A**), 0.5-3.0 cm (**B**), lakewater (**C**). All standard errors are within the bars indicated.

which coincided with the established thermocline when the concentration of ammonia and oxygen increases. No ammonia oxidisers could be detected from the South Basin lakewater obtained in June and from the North Basin in September. In November, when the lake is isothermal, there was an additional peak in ammonia-oxidiser cell counts from the South Basin which contained <1000 cells ml^{-1} greater than the corresponding North Basin sample. There is no significant difference in numbers of ammonia-oxidising bacteria between lakewater derived from the North and South Basins (at $p = 0.05$) during the rest of the year.

MPN counts taken for Buttermere (± standard error), at the corresponding times of year to the Windermere samples are presented in **Table 3.1**. At $p = 0.05$, there is no difference in the numbers of ammonia-oxidising bacteria between littoral and profundal sediments. There was an observed decrease in the numbers of ammonia-oxidising bacteria from March to May 1996 in the littoral sediment and a concomitant slight increase in the observed numbers of ammonia-oxidising bacteria in both the profundal sediment, and lakewater. MPN counts for ammonia-oxidising bacteria from lakewater samples obtained from Buttermere in March and May are <100 cells ml^{-1}, and correspond to the lakewater samples from Windermere which also produced MPN counts <100 cells ml^{-1}. MPN counts of ammonia-oxidising bacteria obtained from profundal sediment from Buttermere were significantly lower in March and May, than counts obtained for profundal sediment obtained from Windermere (at $p = 0.10$). The numbers of ammonia-oxidising bacteria were <150 cells g^{-1} dry weight in Buttermere obtained in May compared to $<2.4 \times 10^4$ cells g^{-1} dry weight in Windermere. There is no significant difference between the numbers of ammonia-oxidising bacteria in the profundal sediments from Buttermere and the

profundal sediments from Windermere (at p = 0.05). In order to determine whether the numbers of ammonia-oxidising bacteria are independent of sediment location, a greater number of samples need to be analysed. The error rate for both Windermere and Buttermere counts within 95% confidence limits is variable and demonstrates the imprecise nature of MPN estimates. The inconsistency in standard errors for all the MPN counts suggests that reproducible results are difficult to obtain by this method.

	Sample	**MPN count** (cells g^{-1} dry weight or cells ml^{-1})	
March 1996	littoral sediment (10m depth)	1700.0	(± 10.62)
	Profundal sediment (25m depth)	53.2	(± 1.0)
	Lakewater (14m depth)	4.0	(± 0.12)
May 1996	littoral sediment (10m depth)	28.3	(± 0.72)
	Profundal sediment (25m depth)	116.0	(± 0.43)
	Lakewater (14m depth)	14.0	(± 0.43)

Table 3.1. MPN counts of nitrifying bacteria obtained from sediment and lakewater from Buttermere, March 1996 and May 1996 (± standard error).

3.4. Enrichment culture of ammonia-oxidising bacteria.

Enrichment cultures were performed on all environmental samples collected from both Windermere and Buttermere. Media containing three different concentrations of ammonium sulphate were employed (medium A 12.5 mM; medium B 3.0 mM; medium C 0.67 mM). The *in vitro* oxidation of ammonia to nitrite and nitrate was monitored at five day intervals over an incubation period of 40 days as described in section 2.5. Data for sediment and lakewater enrichment cultures derived from Buttermere corresponding to July, September and November are presented in **Figs. 3.4-3.6**. Data for sediment and lakewater enrichment cultures derived from Windermere corresponding to March and May are presented in **Figs. 3.7-3.10**. All enrichment cultures were analysed further, for the detection of ammonia-oxidiser 16S rDNA, by PCR amplification and oligonucleotide probing, as described in Chapter 4.

3.4.1 *In vitro* autotrophic ammonia-oxidation in Buttermere samples.

Littoral sediment sampled in July demonstrated nitrate (500 mg l^{-1}) and nitrite (40 mg l^{-1}) production in media A and B after 40 days incubation. Lower nitrate and nitrite concentrations were attained in the profundal sediment for media A and C compared to medium B. The trends in the graphs suggest the nitrate and nitrite values produced by nitrifiers derived from both the littoral sediment and the profundal sediment were similar in medium B. Both littoral and profundal sediments demonstrated poor growth in medium C, where the ammonia concentration was the lowest. Data from lakewater samples was different from that observed for the corresponding sediment samples. After 40 days incubation, no detectable nitrate or nitrite was observed in medium A, whereas nitrate (500 mg l^{-1}) and nitrite production

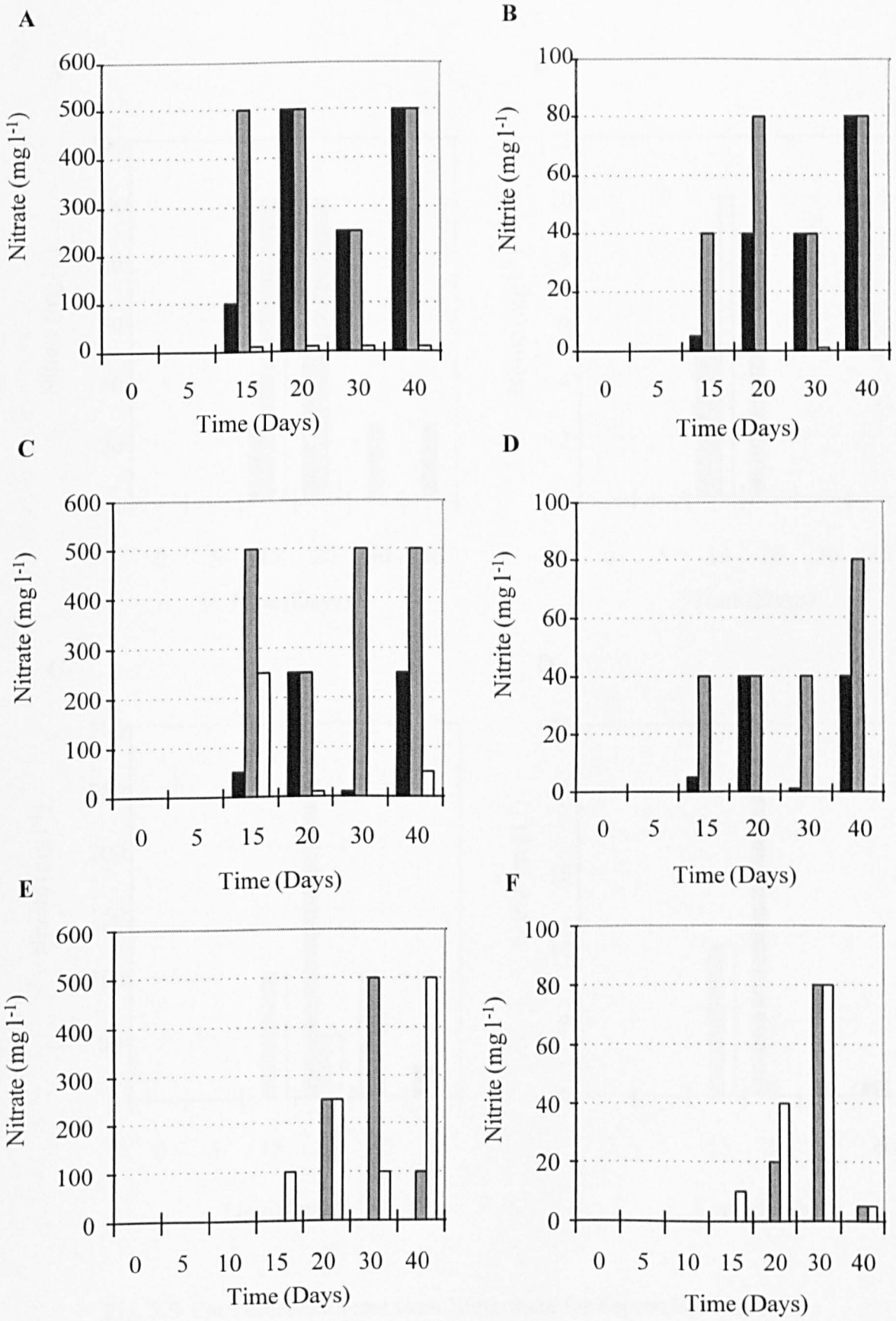


Fig. 3.4 Enrichment cultures from Buttermere for July 1996. littoral sediment nitrate (**A**), nitrite (**B**); profundal sediment nitrate (**C**), nitrite (**D**); lakewater nitrate (**E**), nitrite (**F**). High ■ Medium ▒ Low □ .

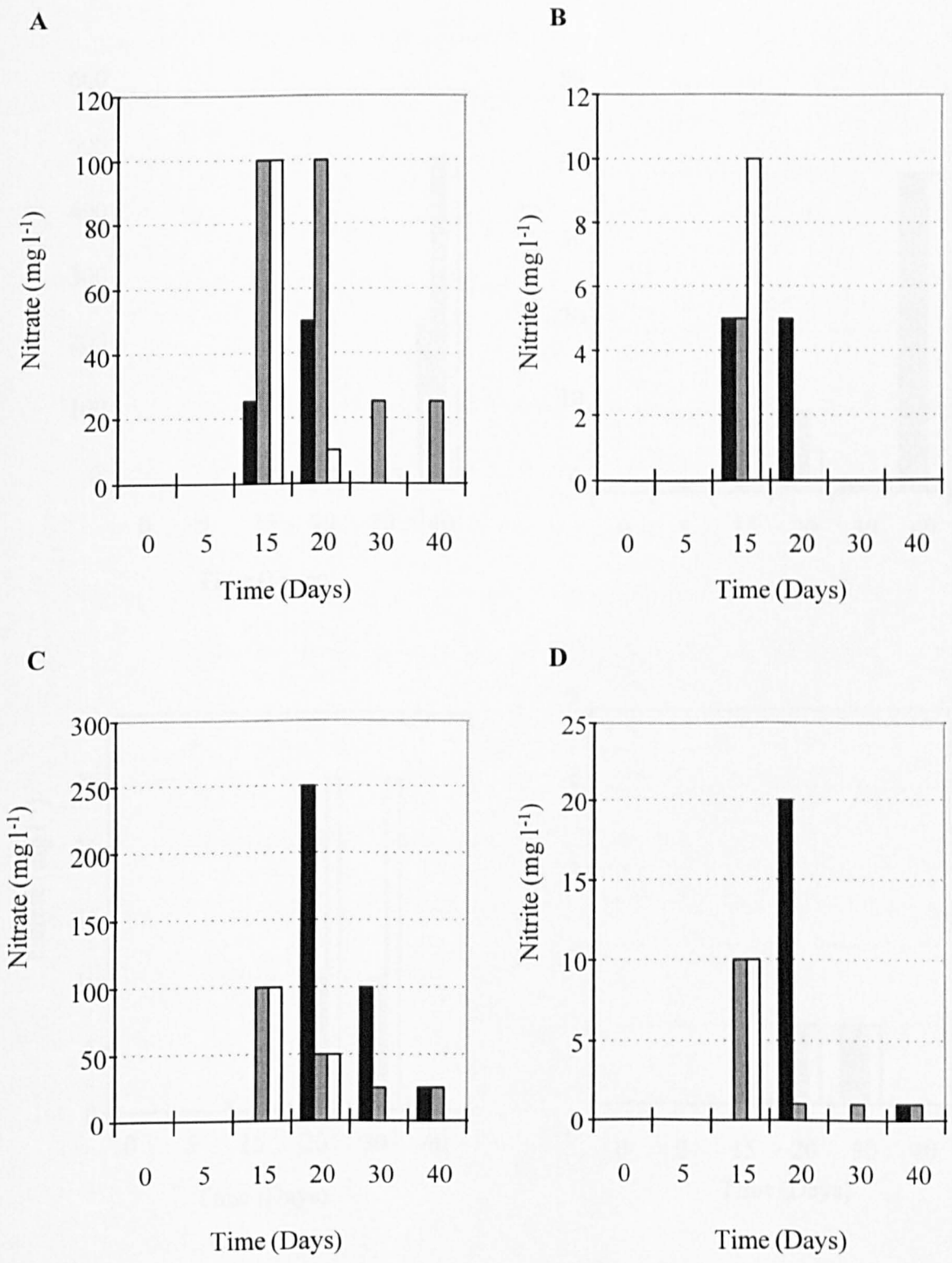


Fig. 3.5 Enrichment cultures from Buttermere for September 1996. littoral sediment nitrate (**A**) nitrite (**B**), profundal sediment nitrate (**C**) nitrite (**D**). High ■ Medium ▒ Low □ .

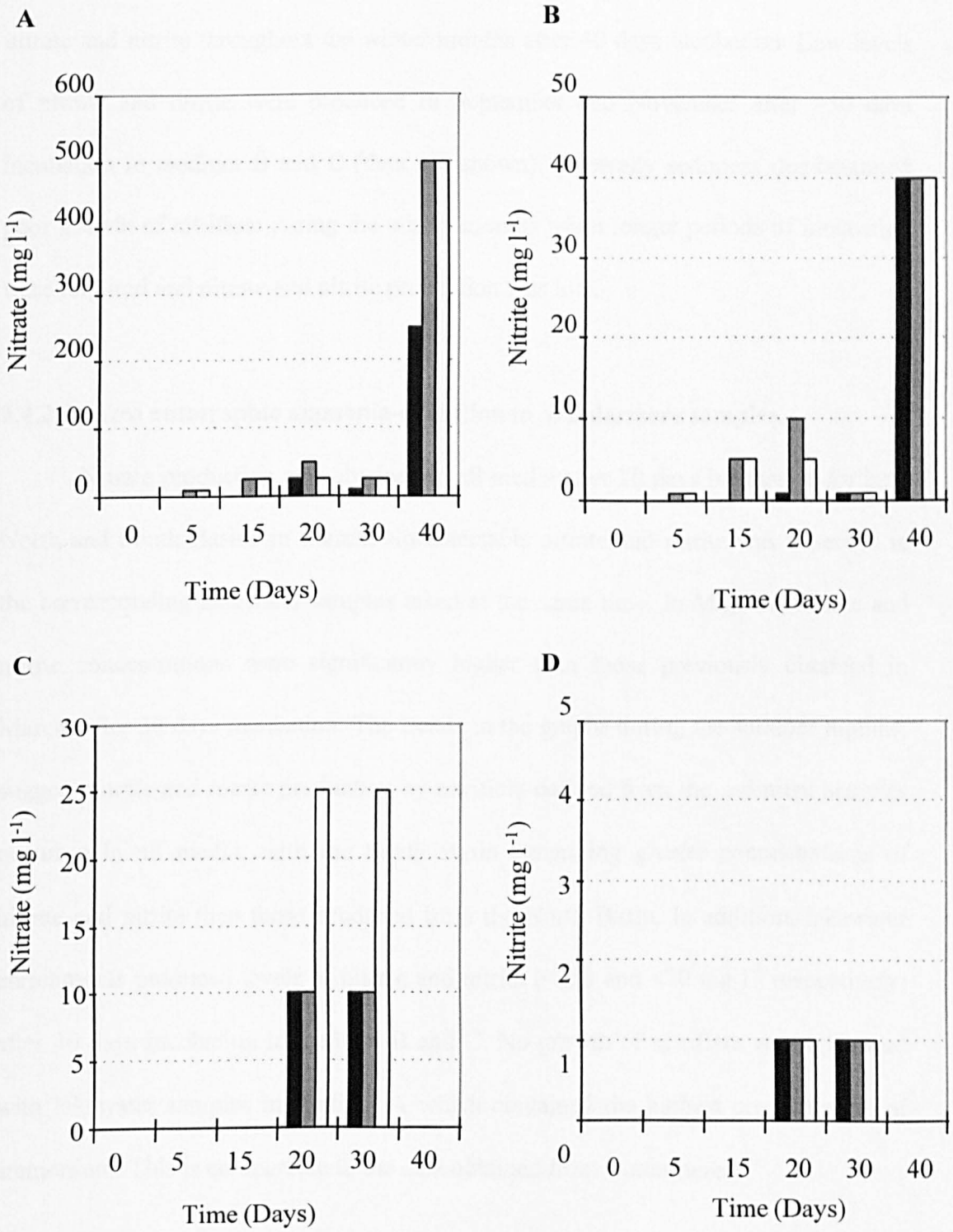


Fig. 3.6 Enrichment cultures from Buttermere for November 1996. littoral sediment nitrate (**A**) nitrite (**B**), profundal sediment nitrate (**C**) nitrite (**D**). High ▮ Medium ▯ Low □ .

(40 mg l^{-1}) were obtained in medium B and C. Lakewater failed to produce detectable nitrate and nitrite throughout the winter months after 40 days incubation. Low levels of nitrate and nitrite were produced in September and November after >50 days incubation in medium B and C (data not shown). Generally sediment demonstrated poor growth of nitrifiers during the winter months when longer periods of incubation were required and nitrate and nitrite production was low.

3.4.2 *In vitro* autotrophic ammonia-oxidation in Windermere samples.

Nitrate production was obtained in all media after 20 days incubation for both North and South Basins in March. No detectable nitrate and nitrite was observed in the corresponding lakewater samples taken at the same time. In May, the nitrate and nitrite concentrations were significantly higher than those previously obtained in March, after 30 days incubation. The trends in the graphs during the summer months, suggest nitrate and nitrite production by nitrifiers derived from the sediment samples occurred in all media, with the South Basin generating greater concentrations of nitrate and nitrite than those produced from the North Basin. In addition, lakewater enrichments produced levels of nitrate and nitrite (<250 and <20 mg l^{-1} respectively) after 30 days incubation in medium B and C. No growth of nitrifiers was evidenced with lakewater samples in medium A which contained the highest concentration of ammonium. This is comparable to the data obtained from Buttermere.

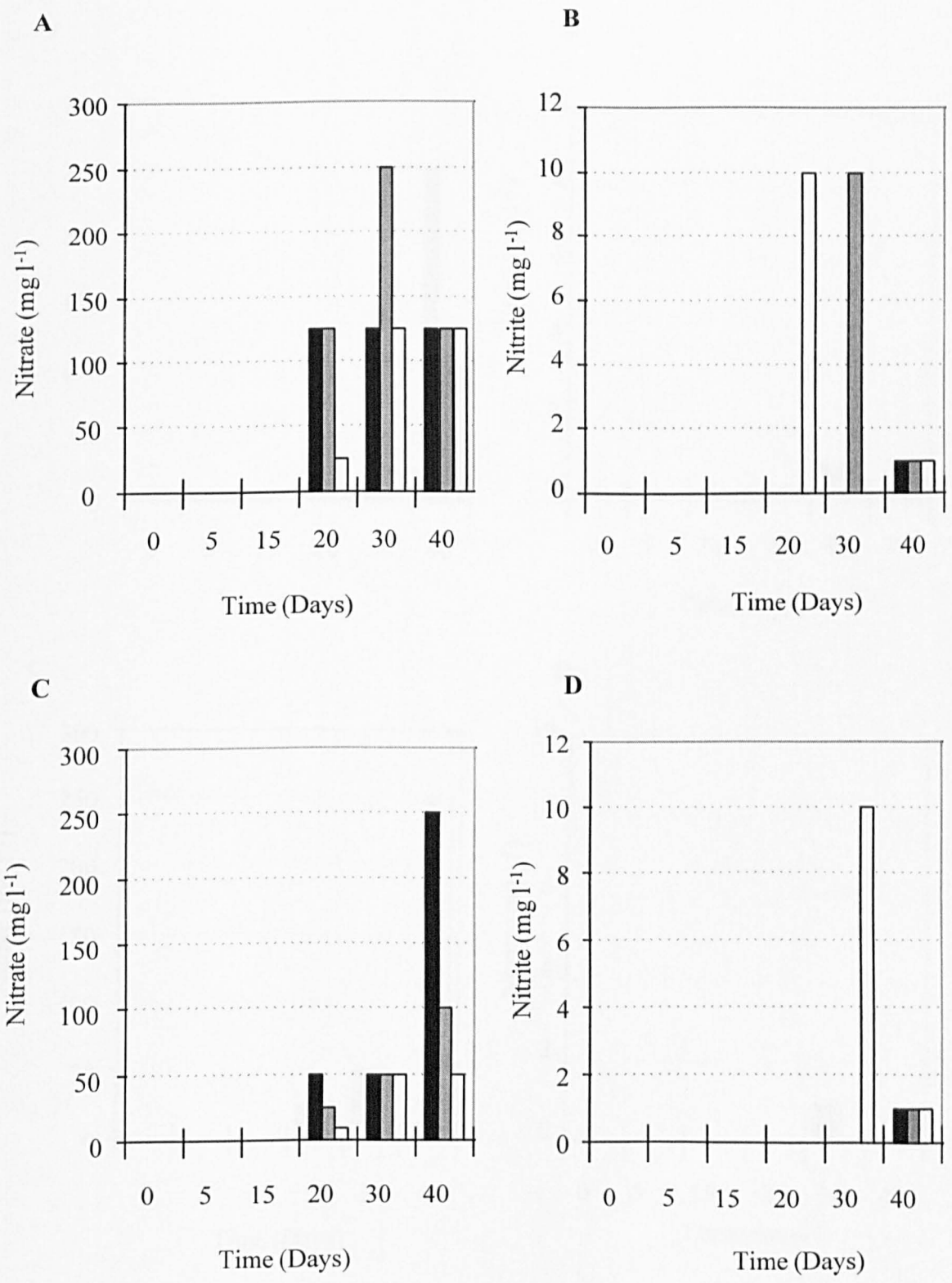


Fig. 3.7 Enrichment cultures from North Basin Windermere, March 1996. Profundal sediment (0-0.5 cm) nitrate (**A**) nitrite (**B**); profundal sediment (0.5-3.0 cm) nitrate (**C**) nitrite (**D**).
High ■ Medium ▒ Low □ .

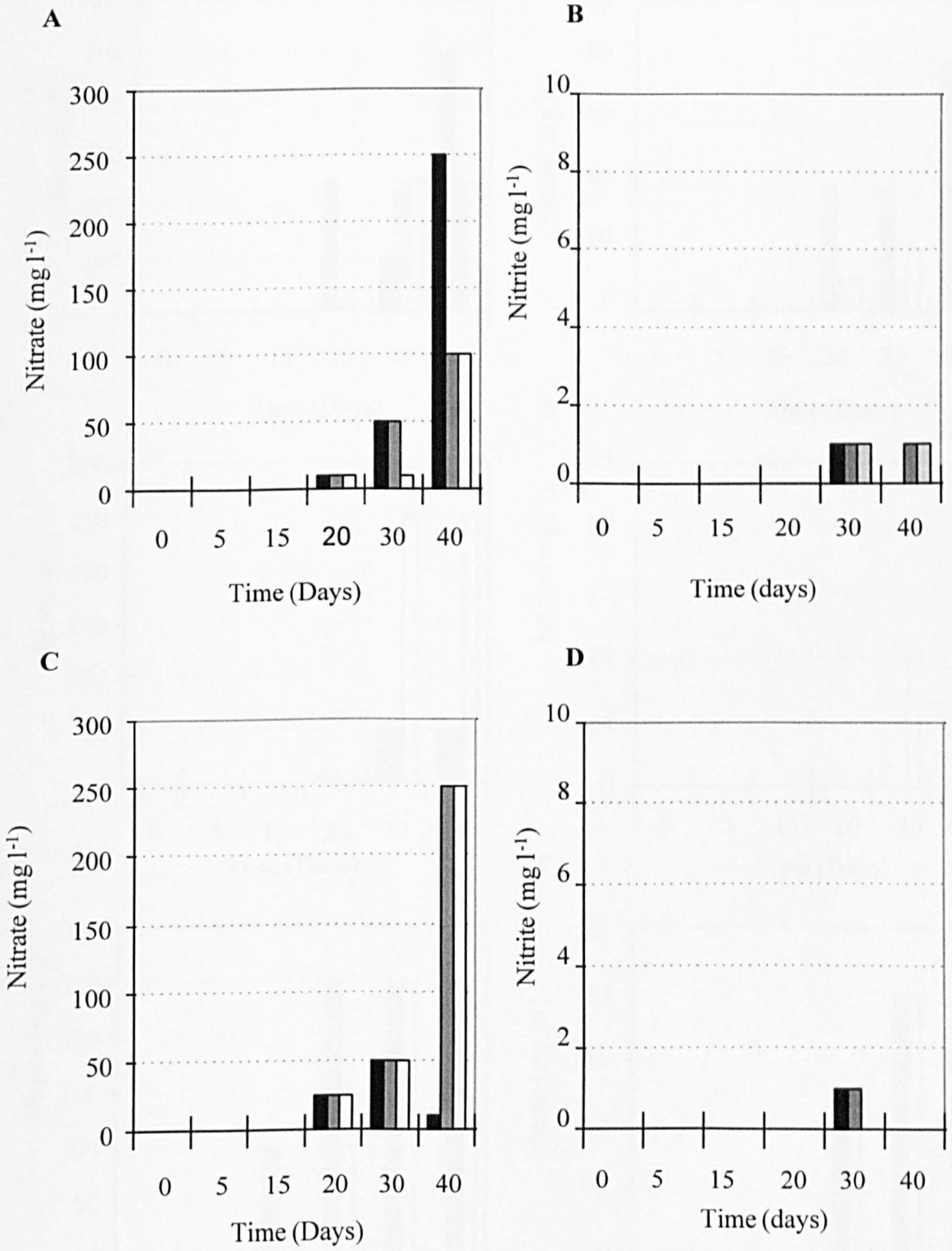


Fig. 3.8 Enrichment cultures from South Basin Windermere, March 1996. Profundal sediment (0-0.5 cm) nitrate (**A**) nitrite (**B**); profundal sediment (0.5-3.0 cm) nitrate (**C**) nitrite (**D**).
High ■ Medium ▒ Low □ .

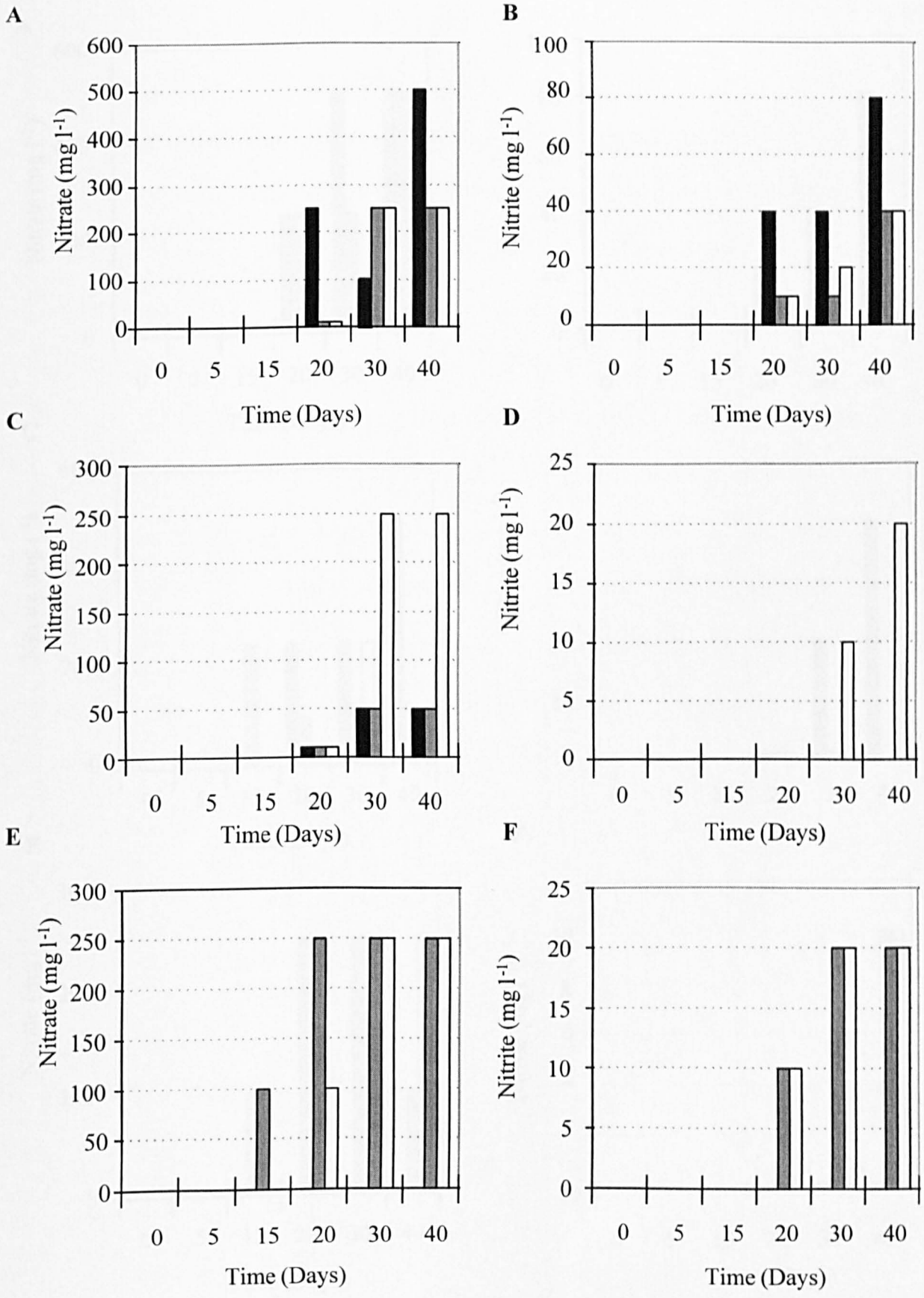


Fig. 3.9 Enrichment cultures for North Basin Windermere, May 1996. Profundal sediment 0-0.5cm nitrate (**A**), nitrite (**B**), profundal sediment 0.5-3.0cm nitrate (**C**) nitrite (**D**), lakewater nitrate (**E**) nitrite (**F**). High ■ Medium ▒ Low □ .

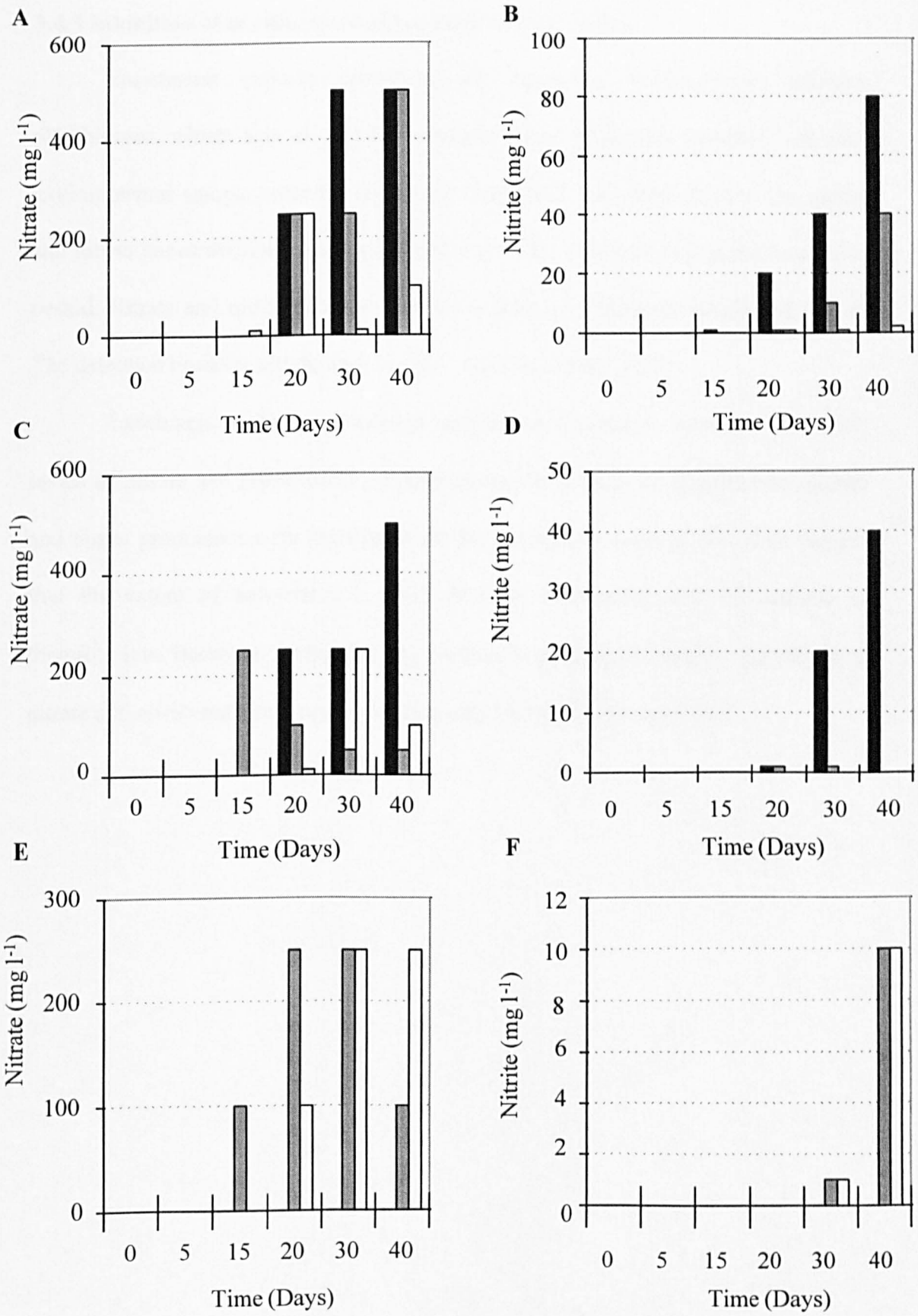


Fig. 3.10 Enrichment cultures for South Basin Windermere May 1996. Profundal sediment 0-0.5cm nitrate (A), nitrite (B), profundal sediment 0.5-3.0cm nitrate (C) nitrite (D), lakewater nitrate (E) nitrite (F). High ■ Medium ▒ Low □ .

3.4.3 Inhibition of *in vitro* autotrophic ammonia oxidation.

Enrichment cultures containing the ammonia monoxygenase inhibitor allylthiourea which acts as a metal chelator were performed routinely on each environmental sample collected from both Buttermere and Windermere. The nitrate and nitrite concentrations were monitored at five day intervals during the incubation period. Nitrate and nitrite production was not detected during the incubation period. The detection limit for nitrate was 10 mg l^{-1} and for nitrite 1 mg l^{-1}.

Enrichment cultures containing allylthiourea failed to produce detectable levels of nitrate and nitrite (data not shown). In the absence of allylthiourea, nitrate and nitrite production were detected in all the enrichment cultures. The data suggest that the extent of heterotrophic nitrification in Buttermere and Windermere is therefore low. However, during the long incubation period (five weeks), the effects of nitrate and nitrite reduction or assimilation may become more significant.

3.5. Discussion.

An attempt was made in this study to correlate relative activities with ammonia-oxidiser numbers during the year. A seasonal increase in MPN counts of ammonia-oxidising bacteria for sediment and lakewater during the summer months would be expected to coincide with increases in nitrate and nitrite production for sediment and lakewater enrichment cultures. However, population levels of ammonia-oxidisers in Windermere measured by MPN determinations decreased from March to May, whereas the corresponding nitrate and nitrite production in enrichment cultures increased.

Previous studies of nitrification activities in soils and sediments using traditional techniques demonstrate a diversity of results (Belser & Schmidt, 1978; Martikainen, 1984; Smorczewski & Schmidt, 1991). The interpretation of data from culture-based studies to assess nitrification is difficult due to inherent statistical inaccuracies and media selectivity (Belser, 1979; Hall, 1986). The MPN method is particularly useful in determining low cell numbers in water samples (*i.e.* >1 viable cell ml^{-1}). However, MPN estimates can be imprecise and are frequently higher than plate count methods. These errors may be reduced by increasing the number of tubes inoculated at each dilution, or by narrowing the dilution ratio *e.g.* the use of a two-fold dilution series gives greater precision than tenfold dilutions (Alexander & Clark, 1965). The mechanics of counting usually necessitate tenfold dilution series, especially if population sizes are unknown.

Profundal sediments can become anoxic rapidly with depth and the degree with which this can happen may increase during the summer due to stratification of the water column causing the waters overlying the sediments to become anoxic

(Brandl *et al.*, 1993). Microprofiles of oxygen concentration gradients within sediments have been demonstrated (Carlton & Wetzel, 1985). The range in depth of the sediment sections taken in this study (0-0.5 cm and 0.5-3.0 cm) may be too great to demonstrate the change in oxygen concentration. Sediment sections that are <0.5 cm may be more informative but the least practical. There may also be a transfer of oxygen to deeper sediments by the action of invertebrates, but at the present time, the extent of oxygen transfer by this action is unknown. The oxygen concentration in the profundal sediments affects the *in situ* activity of the ammonia-oxidising bacteria present.

The complexities that exist between populations of bacteria in the environment enable only tentative conclusions to be made from this study. Evidence from the *in vitro* inhibition studies suggests that heterotrophic nitrification in Buttermere and Windermere is low. Data from this study suggest that autotrophic ammonia-oxidation was the dominant mediator of nitrification in both the sediment and lakewater for Buttermere and Windermere. Heterotrophic organisms, including denitrifying bacteria, have been frequently isolated from lakewater and sediments (Gode & Overbeck, 1972; Jones, 1987). Heterotrophic nitrification has been considered to be significant under certain environmental conditions such as acid coniferous soils (Killham, 1986). Evidence for this was derived from the use of selective physiological inhibitors of autotrophic ammonia-oxidiation that do not affect heterotrophic nitrification (Hooper & Terry, 1973; Powell & Prosser, 1986; 1992; Ginestet *et al.*, 1998). The use of molecular techniques has identified specific groups of ammonia-oxidisers present in acid soils (Stephen *et al.*, 1996, 1998; Kowalchuk *et al.*, 1997). The attachment of cells to soil particle matrices facilitates nitrification at low pH

values (Powell & Prosser, 1992). The effect of pH alone may not be sufficient to select for nitrification, which was dominantly heterotrophic. The diverse populations of ammonia-oxidisers that co-exist in multiple niches with other organisms compete for substrate availability. Therefore, complex interactions occur between heterotrophic organisms and ammonia-oxidisers, and the individual environmental parameters, which would contribute to affect the rates of nitrification produced.

Direct comparisons of the ammonia-oxidiser populations between lakes of different nutrient status was difficult to ascertain. Generally, the amount of nitrogen as either ammonium or nitrate is greater throughout the year in Windermere compared to Buttermere (Pickup, pers. comm.). In addition, the concentration of inorganic ions such as calcium, magnesium, potassium and phosphorous is greater throughout the year in Windermere compared to Buttermere (Pickup, pers. comm.). Inter-site differences between MPN counts from profundal sediments derived from Windermere and Buttermere (at $p = 0.20$) may be due to the variation in the nutrient status of the sediment. However, no significant differences were observed in the numbers of ammonia-oxidising bacteria present in lakewater from both Windermere and Buttermere (at $p = 0.20$). Reasoner & Geldrech (1985) demonstrated that bacteria from low nutrient environments were isolated with a greater efficiency upon dilute media. Fry (1990) demonstrated the growth of oligotrophic bacteria on medium containing only agar with impurities as carbon sources. Although the North and South Basins of Windermere are of different nutrient status, the MPN assays demonstrated no significant intra-site differences between the two basins throughout the year, with the exception of two peaks in the South Basin upper layer (0-0.5cm) in April and November (at $p = 0.20$).

Comparisons with other published reports using culture-based techniques can be inconclusive (Smorczewski & Schmidt, 1991; Hastings, 1996). MPN counts obtained from profundal sediment and lakewater derived from Esthwaite were not significantly different (at $p = 0.20$) to MPN counts obtained by this study for Buttermere and Windermere (Hastings, 1996). Any direct conclusions from these studies must be made with caution, as different media were employed which would consequently create different culture biases. The high sensitivity of ammonia-oxidisers derived from Windermere and Buttermere lakewater to medium A containing a high ammonia concentration was also demonstrated in the studies undertaken on Esthwaite (Hastings, 1996). In addition, the ammonia-oxidisers in lakewater from Buttermere, Windermere and Esthwaite could nitrify effectively in medium C containing a low ammonia concentrations. In contrast to this, profundal sediments from Buttermere, Windermere and Esthwaite were tolerant to medium A and unable to nitrify in medium C that contained low concentrations of ammonium.

Hall (1986) demonstrated that the isolation of ammonia-oxidising bacteria from lakewater requires culture media containing low ammonium salt concentrations. Suwa *et al.* (1994) isolated strains of ammonia-oxidising bacteria from activated sludge that were sensitive to high ammonium concentrations whilst other strains were tolerant. Ammonium sensitivity has also been demonstrated for *Nitrosomonas* (Stehr *et al.*, 1995) and for freshwater ammonia-oxidisers (Hastings, 1996). It is possible that sub-populations of autotrophic ammonia-oxidisers exist in sediments that are more tolerant to increased ammonium present in the lakewater. The numbers of these organisms could therefore increase or decrease in accordance to variations in ammonium concentrations throughout the year. It is possible there may be a species

shift that relates to the effects of the ammonium concentrations in the three media. This is suggested by the presence of ammonia-oxidisers in lakewater enrichments obtained in the summer and a subsequent decrease in ammonia-oxidisers from enrichments taken during winter months from Buttermere and Windermere. In addition, long incubation periods were required for Buttermere samples (up to 40 days) until detectable levels of nitrate and nitrite were achieved. Hastings (1996) demonstrated that prolonged enrichment of cultures enabled *Nitrosomonas* spp. to be dominant over *Nitrosospira* spp. *in vitro*.

The community structure of ammonia-oxidising bacteria was not determined by the culture-based methods performed in this study. The MPN procedure masks genus and species composition of nitrifier populations. The data obtained from *in vitro* ammonia-oxidation studies indicate there may be different populations of ammonia-oxidisers present in the sediments and lakewater. The application of 16S rDNA extracted directly from environmental samples attempts to address the population diversity of ammonia-oxidising bacteria present in freshwater lakes and is presented in Chapters 4 and 5.

3.6. Conclusions.

- Inter-lake differences were observed in the numbers of ammonia-oxidising bacteria present in profundal sediment, but the numbers of ammonia-oxidising bacteria present in lakewater for Buttermere and Windermere were comparable.

- MPN counts suggest the numbers of ammonia-oxidising bacteria present in sediment and lakewater from the North and South Basins of Windermere were similar.

- MPN counts of ammonia-oxidising bacteria were lower in all lakewater samples compared to sediment samples.

- The extent of heterotrophic nitrification was low and suggests that autotrophic nitrification is the main process in Windermere and Buttermere.

- Populations of ammonia-oxidising bacteria present in lakewater are less tolerant to high ammonium concentrations compared to populations of ammonia-oxidising bacteria present in sediment. Thus, in sediment and lakewater, different communities of ammonia-oxidising bacteria may be selected.

Chapter 4. Detection and identification of autotrophic ammonia-oxidising bacteria by PCR amplification of the 16S rRNA gene.

4.1. Introduction.

PCR-based technology has become a useful investigative tool in determining evolutionary relationships of unculturable organisms through rDNA gene analysis and circumvents the requirement for culture (Amman *et al.*, 1991; Gordon & Giovannoni, 1996). Molecular techniques have been particularly useful in the study of ammonia-oxidising bacteria which are notoriously difficult to isolate from pure cultures (Head *et al.*, 1993; Kowalchuk *et al.*, 1997; Ward *et al.*, 1997). PCR amplification of DNA extracted from soils and sediments is often inhibited by substances such as humic acids, although modifications of published protocols facilitate the preparation of PCR quality nucleic acids from environmental samples (Tsai & Olsen, 1992; Tebbe & Vahjen, 1993; Young *et al.*, 1995; Jackson *et al.*, 1997).

The increasing numbers of 16S rRNA sequences from ammonia-oxidising bacteria deposited into the databases have enabled a range of oligonucleotide PCR primers and probes to be designed with varying degrees of specifity (McCaig *et al.*, 1994; Mobarry *et al.*, 1996; Pommerening-Röser *et al.*, 1996; Kowalchuk *et al.*, 1997; Ward *et al.*, 1997). The combination of oligonucleotide PCR primers and probes facilitates the target of either individual species or genera present in the environment (Hiorns *et al.*, 1995; Wagner *et al.*, 1995; Hovanec & Delong, 1996; Hastings *et al.*, 1997; Kowalchuk *et al.*, 1997). This chapter describes the isolation and PCR amplification of 16S rDNA from sediment and lakewater to identify the occurrence and distribution of ammonia-oxidising bacteria in freshwater lakes.

4.2. Results

4.2.1. Evaluation of diagnostic oligonucleotide probe specificity.

The specificity of the oligonucleotide probes used in this study was investigated for their application in analysing ammonia-oxidiser communities. Design and optimisation of these diagnostic oligonucleotide probes has been demonstrated previously (Head *et al.*, 1993; Hiorns *et al.*, 1995). Database searches were undertaken using the BLAST program (National Centre for Biotechnology Information, Washington); FASTA (GCG suite of programs, Wisconsin) and CHECK_PROBE program supported by the Ribosome Database Project (Urbana, Illinois).

The data indicated that target sequences for the oligonucleotide PCR primers; Nm75, Nm1009*, Ns85, and Ns1007* and the oligonucleotide probe, AAO258, were complementary to a number of non-target organisms (1bp mismatch) *e.g.* the Proteobacteria β-sub group genus *Azoarcus*. In addition, the oligonucleotide primers (Nm75 and Nm1009*) under high stringency, failed to recognise certain target *Nitrosomonas* sequences *e.g. Nm. ureae*. The specificity of these oligonucleotide primers to ammonia-oxidiser sequences is increased considerably when the genus-specific *Nitrosospira* (Ns85/1009*) or *Nitrosomonas* spp. (Nm75/1007*) primer pairs are used in conjunction with the confirmatory oligonucleotide probe AAO258 under high stringency. There are no published non-target sequences recognised when these oligonucleotide primers and probe combinations are applied. Despite the limitations of the oligonucleotide primers and probes, they provide useful diagnostic tools for the identification of ammonia-oxidiser genera and species. Since the primary aim of this study was a comparative analysis of the population diversity of ammonia-oxidising

bacteria between different freshwater environments, the same molecular tools were employed to those used in previous studies (Hiorns *et al.*, 1995; Hastings *et al.*, 1997).

Eubacterial primers pB/pF* were used to amplify DNA extracted from laboratory strains of eubacteria and ammonia-oxidising bacteria (approximately 1.0 kb of the eubacterial 16S rRNA gene (Edwards *et al.*, 1989). Successful amplification was achieved, and the products obtained were separated by agarose gel electrophoresis (**Fig. 4.1**). The amplification products were attached to a nylon membrane by Southern blotting as described in section 2.26.1. Hybridisation with a range of eubacterial, genus and species-specific oligonucleotide probes was performed under the stringent conditions detailed in section 2.27. The results are shown in **Fig. 4.2 (A-E)**. **Table 4.1** summarises the hybridisation specifities obtained from the autoradiographs.

The oligonucleotide pD* (536-518) (Edwards *et al.*, 1989) has been used in this study as a sequencing primer. The oligonucleotide targets a conserved region of the 16S rRNA gene and therefore provides a suitable positive control for the presence of 16S rRNA sequences. Hybridisation to the internal probe pD* was achieved with all of the laboratory strains under investigation and confirmed that the amplified DNA fragment originated from the 16S rRNA gene. Differentiation of ammonia-oxidising bacteria from other eubacterial strains was achieved using the oligonucleotide AAO258. Although this probe has been reported to be specific for all terrestrial and freshwater ammonia-oxidising bacterial 16S rRNA genes (Hiorns *et al.*, 1995), it is now known not to be the case (Stephens *et al.*, 1996; Utåker *et al.*, 1998). The target sequence recognises the majority of published 16S rDNA sequences from the β-subgroup ammonia-oxidisers with an exact match. However, in a few cases there are one or two base mismatches and therefore, under stringent conditions these sequences

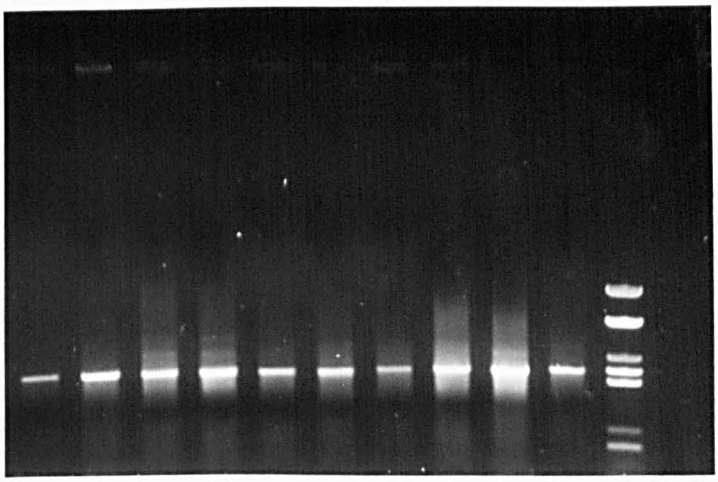


Fig. 4.1 Agarose gel electrophoresis of Eubacterial 16S rDNA PCR products (pB & pF') from nucleic acids directly extracted from pure cultures.

Lanes:

1. *Pseudomonas fluorescens*
2. *Pseudomonas aeruginosa*
3. *Staphylococcus aureus*
4. *Escherichia coli*
5. *Nitrosomonas europaea* Nm50
6. *Nitrosomonas eutropha* Nm57
7. *Nitosococcus mobilis* Nc2
8. *Nitrosospira* sp. NV141
9. *Nitrosospira multiformis* Nl13
10. *Nitrosospira* sp. Nsp22

M MBI molecular weight marker, 21

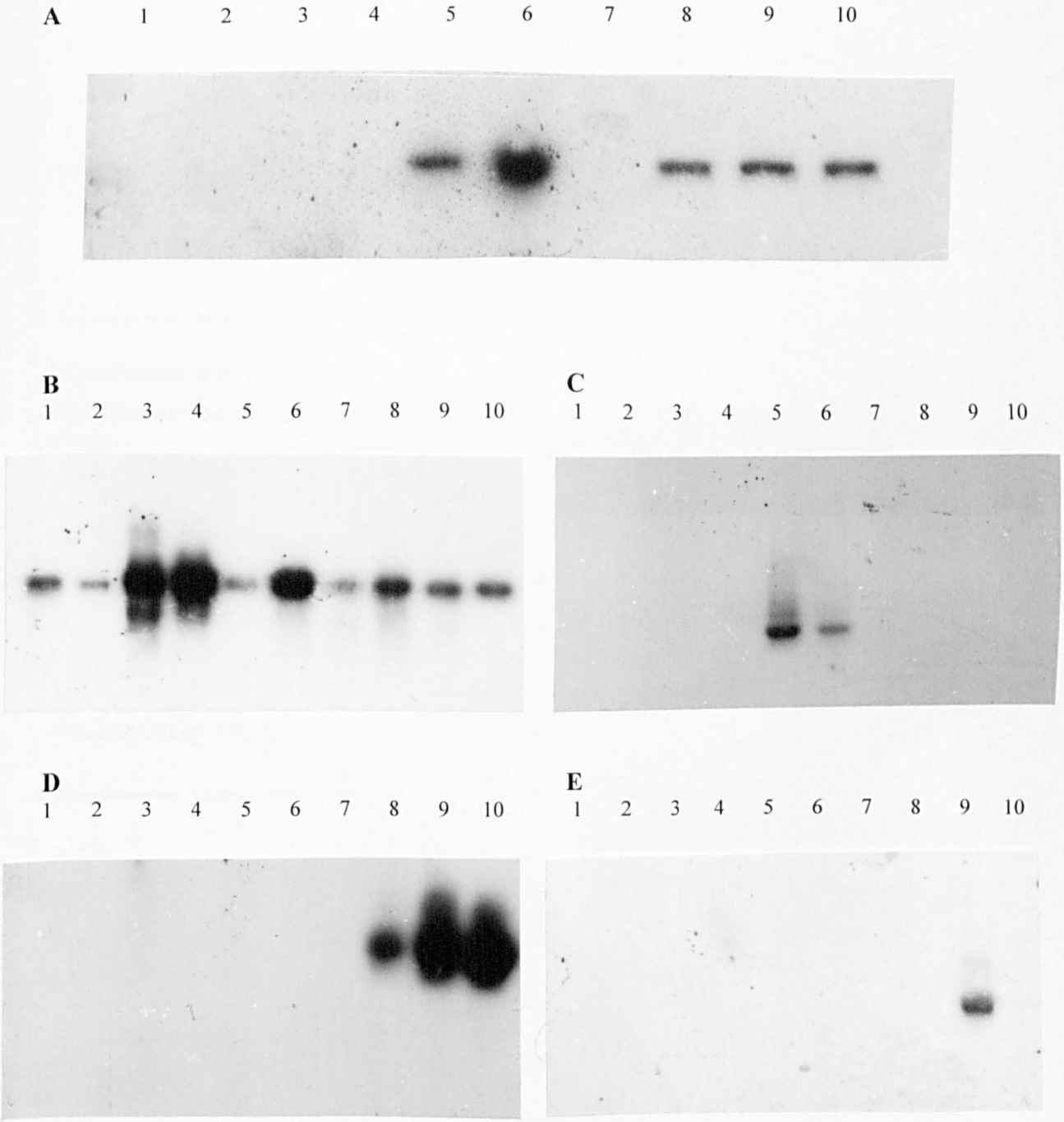


Fig. 4.2 Southern blot of Eubacterial 16S rDNA PCR products (pB & pF•) from nucleic acids directly extracted from pure cultures and hybridised with oligonucleotide AAO258 (**A**), pD• (**B**), Nm75 (**C**), Ns85 (**D**) and Nlm459• (**E**).

Lanes:

1. *Pseudomonas fluorescens*
2. *Pseudomonas aeruginosa*
3. *Staphylococcus aureus*
4. *Escherichia coli*
5. *Nitrosomonas europaea* Nm50
6. *Nitrosomonas eutropha* Nm57
7. *Nitosococcus mobilis* Nc2
8. *Nitrosospira* sp. NV141
9. *Nitrosospira multiformis* Nl13
10. *Nitrosospira* sp. Nsp22

Strains	Oligonucleotide Probes				
	pD	Ns85	Nm75	AAO258	Nsm459
Pseudomonas fluorescens	+	-	-	-	-
Pseudomonas aeruginosa	+	-	-	-	-
Staphylococcus aureus	+	-	-	-	-
Escherichia coli	+	-	-	-	-
Nitrosomonas europaea	+	-	+	+	-
Nitrosomonas eutropha	+	-	+	+	-
Nitrosococcus mobilis	+	-	-	-	-
Nitrosopira sp. (Nv141)	+	+	-	+	-
Nitrosospira multiformis	+	+	-	+	+
Nitrosospira sp. (Nsp22)	+	+	-	+	-

Table 4.1. Summary of the qualitative evaluation of the oligonucleotide probe specificities. Probes were applied to 16S rDNA amplified by PCR. (+) hybridisation signal obtained (-) no hybridisation signal obtained.

will not be recovered. The probe AAO258 hybridised to 16S rDNA from all of the terrestrial ammonia-oxidiser strains used in this study *i.e. Nitrosomonas* spp. and *Nitrosospira* spp. No cross-reactions were observed and no binding was apparent between the oligonucleotide probe AAO258 and the 16S rDNA of *Nitrosococcus mobilis* NC2, despite only a single nucleotide pair mismatch. Probe Nm75 and Ns85 differentiated terrestrial *Nitrosospira* spp. from the *Nitrosomonas* spp. (*Nm. europaea & Nm. eutropha*) respectively (Hiorns *et al.*, 1995). These oligonucleotides were used to discriminate the genus *Nitrosospira* from the *Nm. europaea-eutropha* lineage. No cross-reactions were observed between the two probes and non-target 16S rDNA used in this investigation. Oligonucleotide Nsm459* has been reported to be specific for the 16S rDNA of *Ns. multiformis* (Hiorns *et al.*, 1995). Hybridisation occurred only with the 16S rDNA corresponding to *Ns. multiformis*. No cross-reactions with 16S rDNA from the other eubacterial strains were observed.

4.3. Extraction of DNA from sediment and lakewater samples.

The sampling of sediments and lakewater has been previously described in section 2.12. Sediment cores were obtained using a Jenkin corer and were taken from littoral and profundal sediments in Buttermere periodically throughout the year. Samples of approximately 8g wet weight were removed from the upper aerobic layer (0-0.5 cm) of the core. Profundal sediments were also taken from Windermere North Basin (60 m depth) and South Basin (40 m depth) periodically throughout the year. Windermere cores were sectioned into two layers, upper (0-0.5 cm) and lower (0.5-3.0 cm).

Lakewater (60 litres) pumped from the thermocline (Buttermere (14 m), Windermere (20 m North and South Basins) was tangentially flow-filtered and concentrated to 1litre. Total community DNA was extracted directly from all sediment samples by the method of Bruce *et al.* (1992) and from lakewater samples by the method of Schmidt *et al.* (1991). The extracted DNA was diluted to act as template for a nested PCR amplification reaction. Where sample dilution failed to negate the inhibitory effect of contaminating substances, an additional treatment utilising Centricon™ columns was employed. Such treatment improved sample quality without a significant loss of DNA yield and facilitated successful PCR amplification where necessary.

4.3.1. Eubacterial PCR amplification of 16S rDNA from sediment and lakewater.

Direct PCR amplification, utilising genus-specific ammonia-oxidiser oligonucleotide primers failed to generate products from environmental DNA in a single step reaction. Therefore, a nested PCR technique was employed for all environmentally derived DNA samples. The nucleic acids were subjected to PCR utilising eubacterial 16S rDNA primers pB/pF*, (Edwards *et al.* 1989) and amplification of a region approximately 1.0 kb of the eubacterial 16S rRNA gene was performed. Successful primary amplification was achieved in all samples from Buttermere and Windermere (data not shown).

4.3.2. PCR amplification of *Nitrosospira* 16S rDNA from Buttermere.

Amplicons obtained from the initial eubacterial PCR amplification were diluted appropriately and used as template DNA for the nested PCR reaction with

Nitrosospira-specific primers. Amplification of a 0.93 kb region of the 16S rRNA gene was achieved in all sediment and lakewater samples using the *Nitrosospira* specific primer pair Ns85/Ns1009* (Head *et al.*, 1993) (**Figs. 4.3A & 4.4**). Hybridisation of the amplification products to the internal oligonucleotide probe AAO258 confirmed the validity of the *Nitrosospira* 16S rDNA (**Figs. 4.3B & 4.5**). Therefore, *Nitrosospira* spp. were detected in Buttermere in both sediment and lakewater throughout the year (1995-1996). In addition, the *Nitrosospira* 16S rDNA amplification products were hybridised to the species specific probe Nsm459* in an attempt to demonstrate the presence or absence of *Ns. multiformis* in Buttermere (data not shown). The signals obtained were weak in comparison to hybridisation signals obtained previously using AAO258. The strongest signals were obtained from profundal sediment and lakewater samples. The presence of *Ns. multiformis* was only detected in May and November in the profundal sediment and November, March and May in the lakewater (data not shown). Weak signals were also obtained for the littoral sediment obtained in November when hybridised to Nsm459* (data not shown).

4.3.3. PCR amplification of *Nitrosomonas* 16S rDNA from Buttermere.

Amplicons obtained from the initial eubacterial PCR amplification were diluted appropriately and used as template DNA for the nested PCR reaction with the *Nitrosomonas*-specific primer pair Nm75/Nm1007*. Template DNA extracted from littoral sediment obtained in July and September and profundal sediment obtained in July and August produced amplification products of the correct size (0.9 kb) (**Figs. 4.6A & 4.7A**). Lakewater samples obtained in August also produced amplification

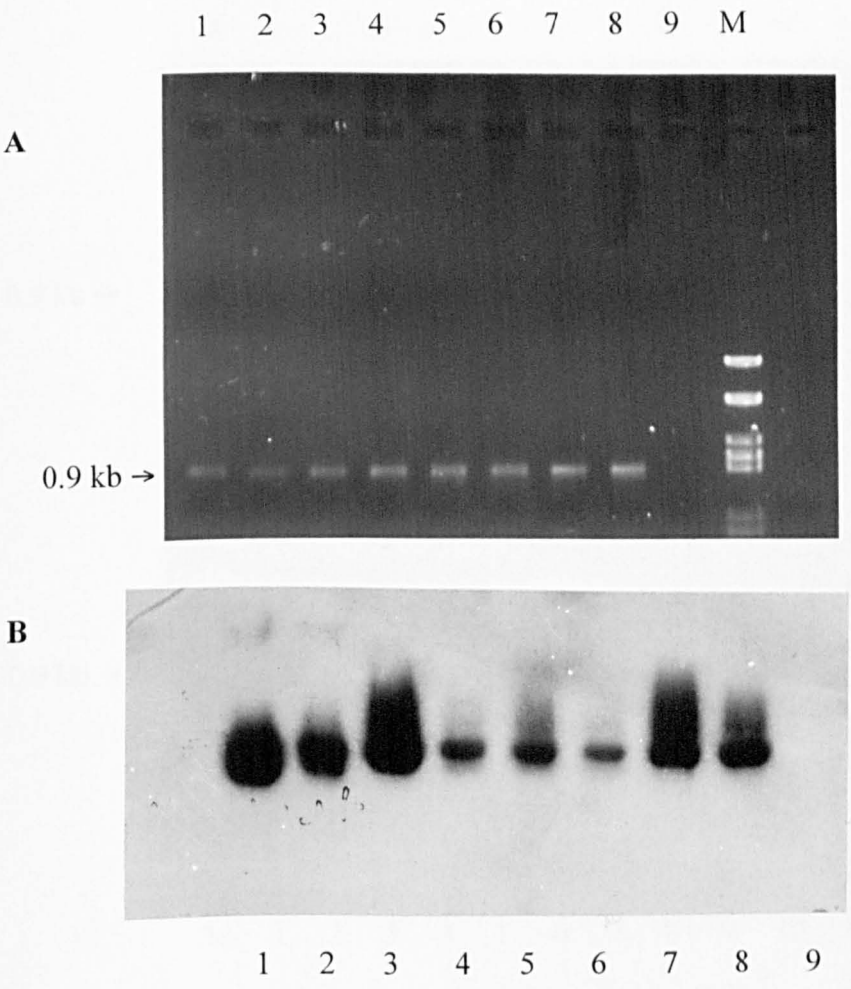


Fig. 4.3 Agarose gel electrophoresis of *Nitrosospira*-specific amplification products from littoral sediment (lanes **A**1-6), Southern blot of *Nitrosospira*-specific amplification products from littoral sediment hybridised with oligonucleotide AAO258 (lanes **B**1-6).

Lanes:

1. July 1995
2. August 1995
3. September 1995
4. November 1995
5. March 1996
6. May 1996
7. *Nitrosospira* sp. NV141
8. *Nitrosospira multiformis* Nl13
9. *Nitrosomonas europaea* Nm50

M MBI molecular weight marker, 21

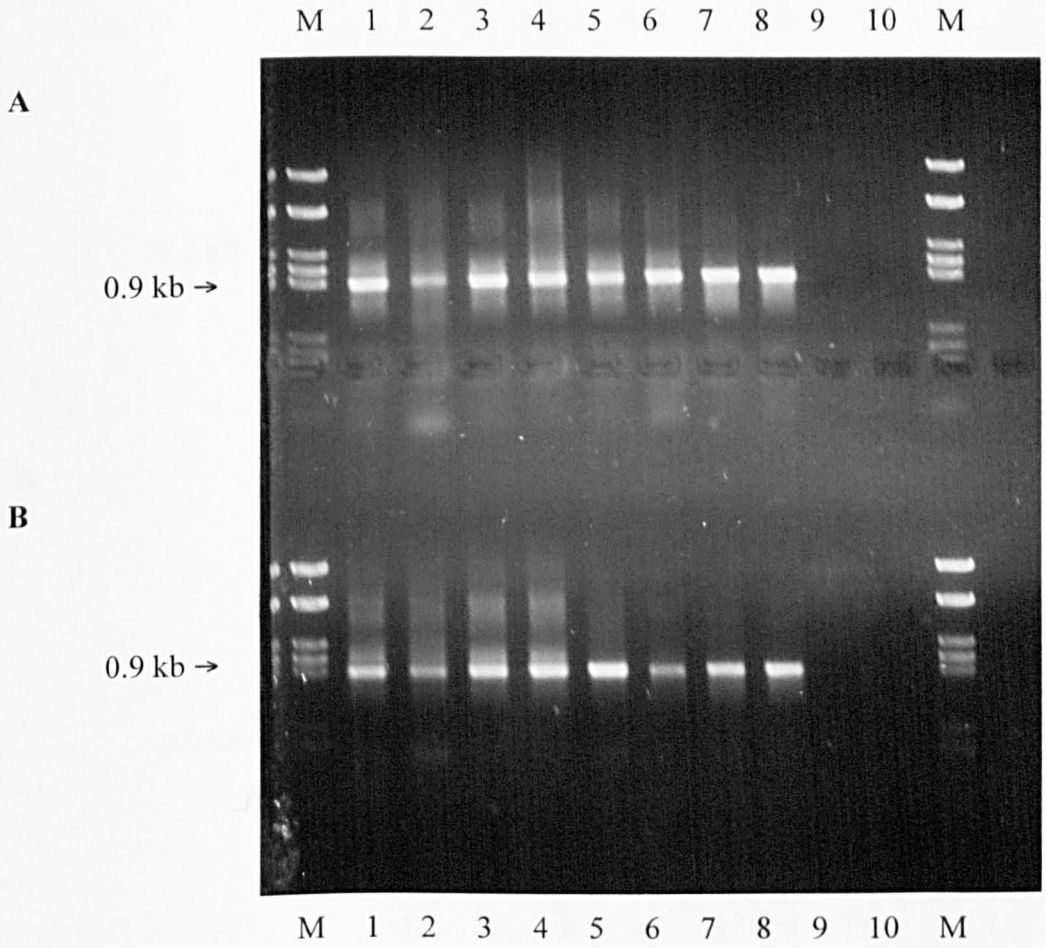


Fig. 4.4 Agarose gel electrophoresis of *Nitrosospira*-specific amplification products derived from lakewater (14m depth) (lanes **A**1-6), profundal sediment (lanes **B**1-6).

Lanes:

1. July 1995
2. August 1995
3. September 1995
4. November 1995
5. March 1996
6. May 1996
7. *Nitrosospira* sp. NV141
8. *Nitrosospira multiformis* Nl13
9. *Nitrosomonas europaea* Nm50
10. sterile distilled water

M MBI molecular weight marker, 21

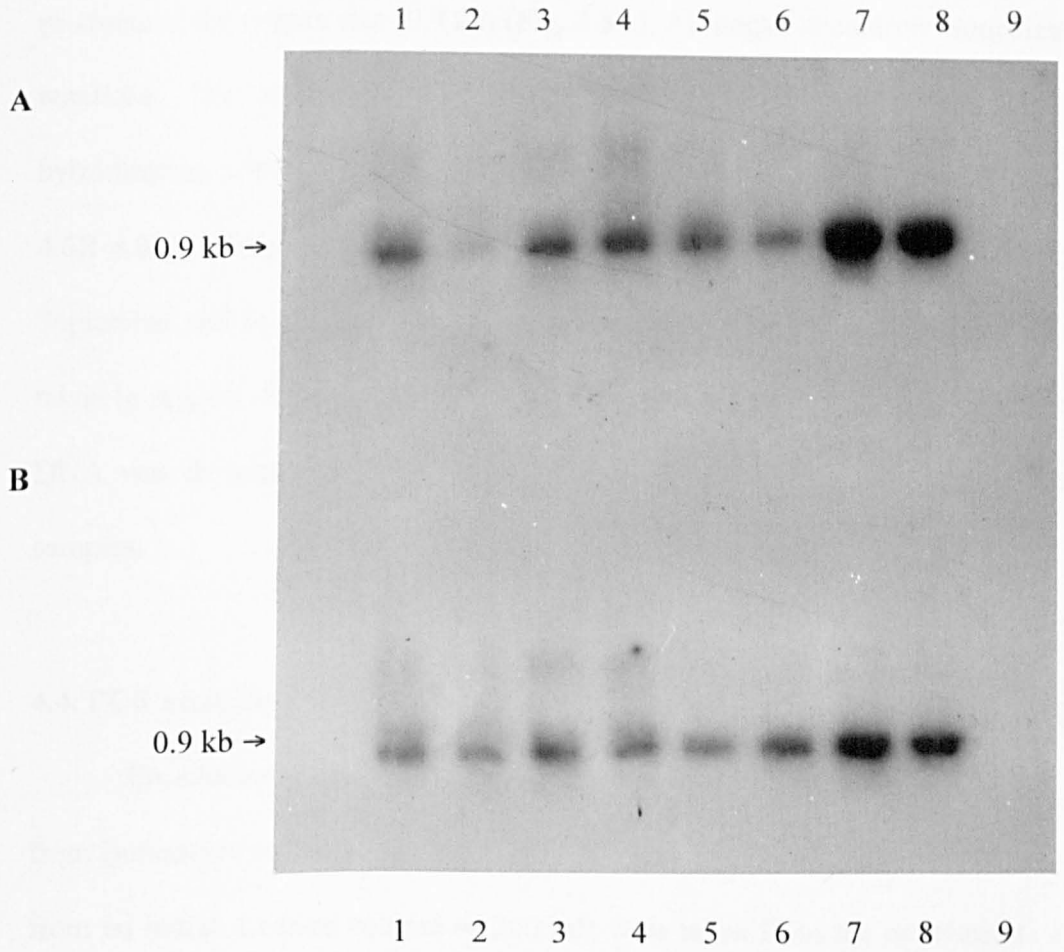


Fig. 4.5 Southern blot of the gel shown overleaf (Fig. 4.4). *Nitrosospira*-specific amplification products derived from lakewater (14m depth) (lanes **A**1-6), profundal sediment (25m depth) (lanes **B**1-6) hybridised with oligonucleotide AAO258..

Lanes:

1. July 1995
2. August 1995
3. September 1995
4. November 1995
5. March 1996
6. May 1996
7. *Nitrosospira* sp. NV141
8. *Nitrosospira multiformis* Nl13
9. *Nitrosomonas europaea* Nm50

products of the correct size (0.9 kb) (**Fig. 4.8A**). All negative controls were clear in all reactions. The amplicons were confirmed as ammonia-oxidiser *rrn* genes by hybridisation with the radioactively labelled oligonucleotide probe AAO258 (**Figs. 4.6B-4.8B**). Nitrosomonad DNA was detected in littoral sediment in July and September and in profundal sediment in August and September. Lakewater samples taken in August demonstrated the presence of nitrosomonad DNA. No nitrosomonad DNA was detected during the winter months from either sediments or lakewater samples.

4.4. PCR analysis of laboratory enrichment cultures derived from Buttermere.

Enrichment cultures were performed on sediment and lakewater samples taken from Buttermere as described in section 2.6. Following inoculation, sub-samples (1 ml from an initial medium volume of 200 ml) were taken from the enrichments at five day intervals. This was performed for a total period of forty days incubation. During the incubation period, the nitrate and nitrite concentrations were monitored and the pH was adjusted to 8.0 as required. Cells were pelleted and the DNA was extracted for a PCR analysis as described in section 2.16. Since the presence of *Nitrosospira* 16S rDNA had been previously demonstrated with all the environmental samples collected (section 4.3.2), the main objective of this work was the demonstration of *Nitrosomonas* spp. in enrichment cultures. *Nitrosomonas* spp. could be present in Buttermere throughout the year but in low numbers that were below the detection limits for direct methods of DNA extraction and amplification. Therefore, an attempt was made using enrichment cultures to increase the numbers of *Nitrosomonads* to detectable levels. **Table 4.2** summarises the results obtained.

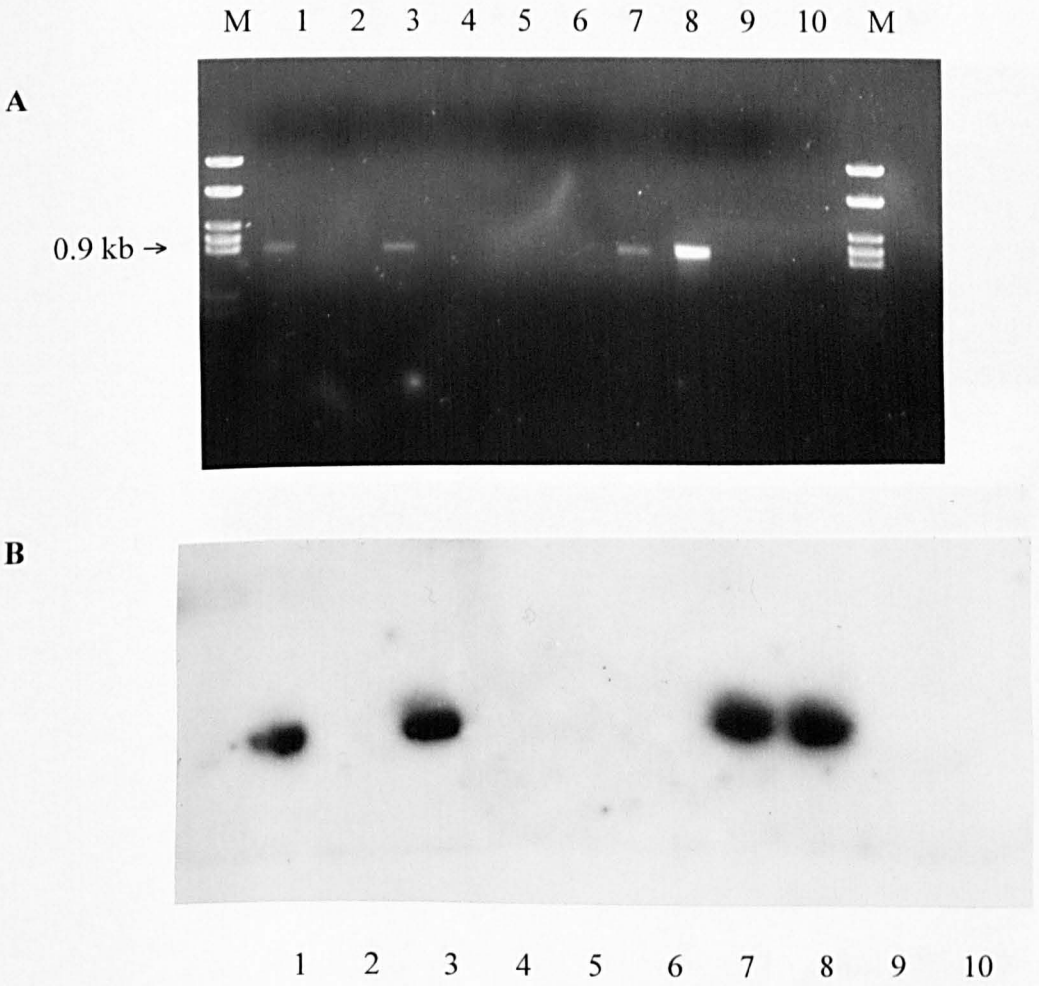


Fig. 4.6 Agarose gel electrophoresis of *Nitrosomonas*-specific amplification products derived from littoral sediment (lanes **A**1-6), Southern blot of *Nitrosomonas*-specific amplification products derived from littoral sediment hybridised with oligonucleotide AAO258 (lanes **B**1-6).

Lanes:

1. July 1995
2. August 1995
3. September 1995
4. November 1995
5. March 1996
6. May 1996
7. *Nitrosomonas europaea* Nm50
8. *Nitrosomonas eutropha* Nm57
9. *Nitrosospira* sp. NV141
10. sterile distilled water

M MBI molecular weight marker, 21

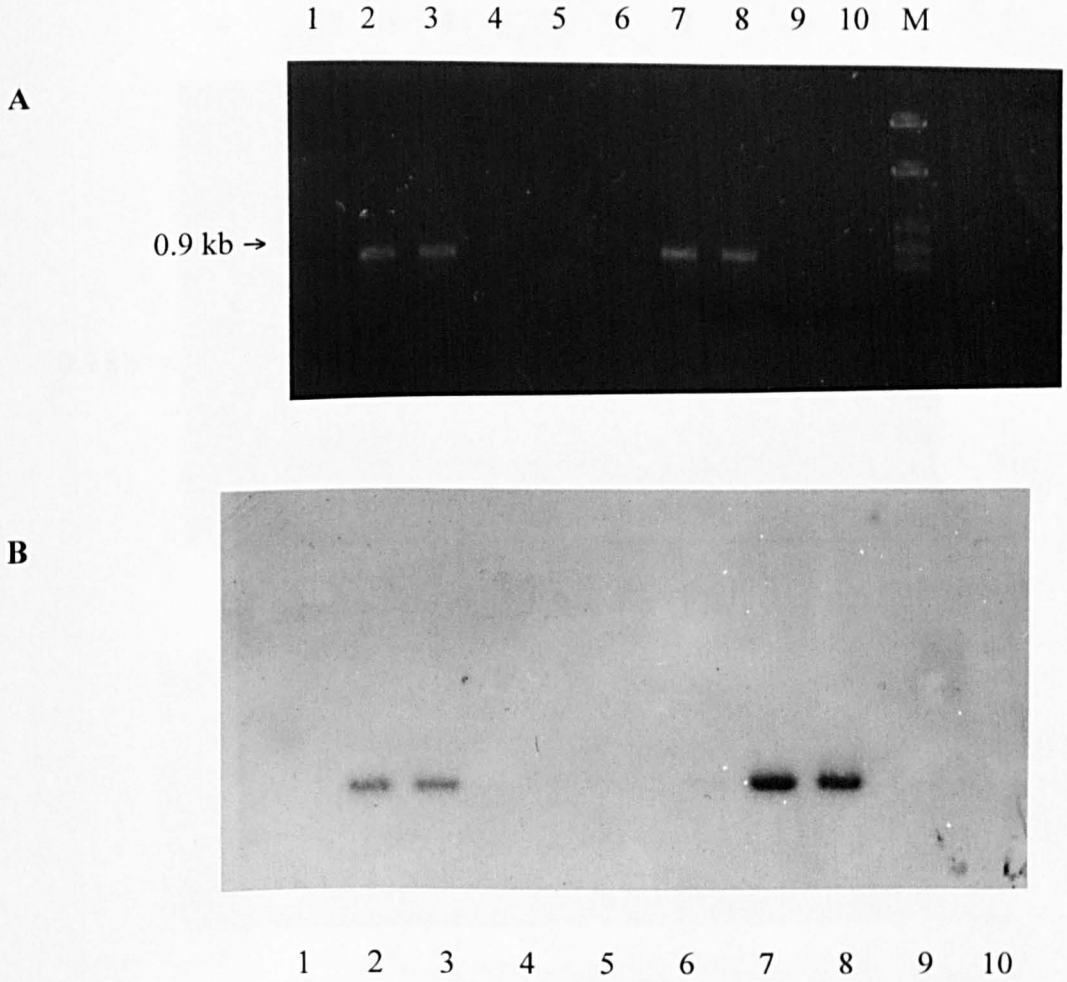


Fig. 4.7 Agarose gel electrophoresis of *Nitrosomonas*-specific amplification products derived from profundal sediment (lanes **A**1-6), Southern blot of *Nitrosomonas*-specific amplification products derived from profundal sediment hybridised with oligonucleotide AAO258 (lanes **B**1-6).

Lanes:

1. July 1995
2. August 1995
3. September 1995
4. November 1995
5. March 1996
6. May 1996
7. *Nitrosomonas europaea* Nm50
8. *Nitrosomonas eutropha* Nm57
9. *Nitrosospira* sp. NV141
10. sterile distilled water

M MBI molecular weight marker, 21

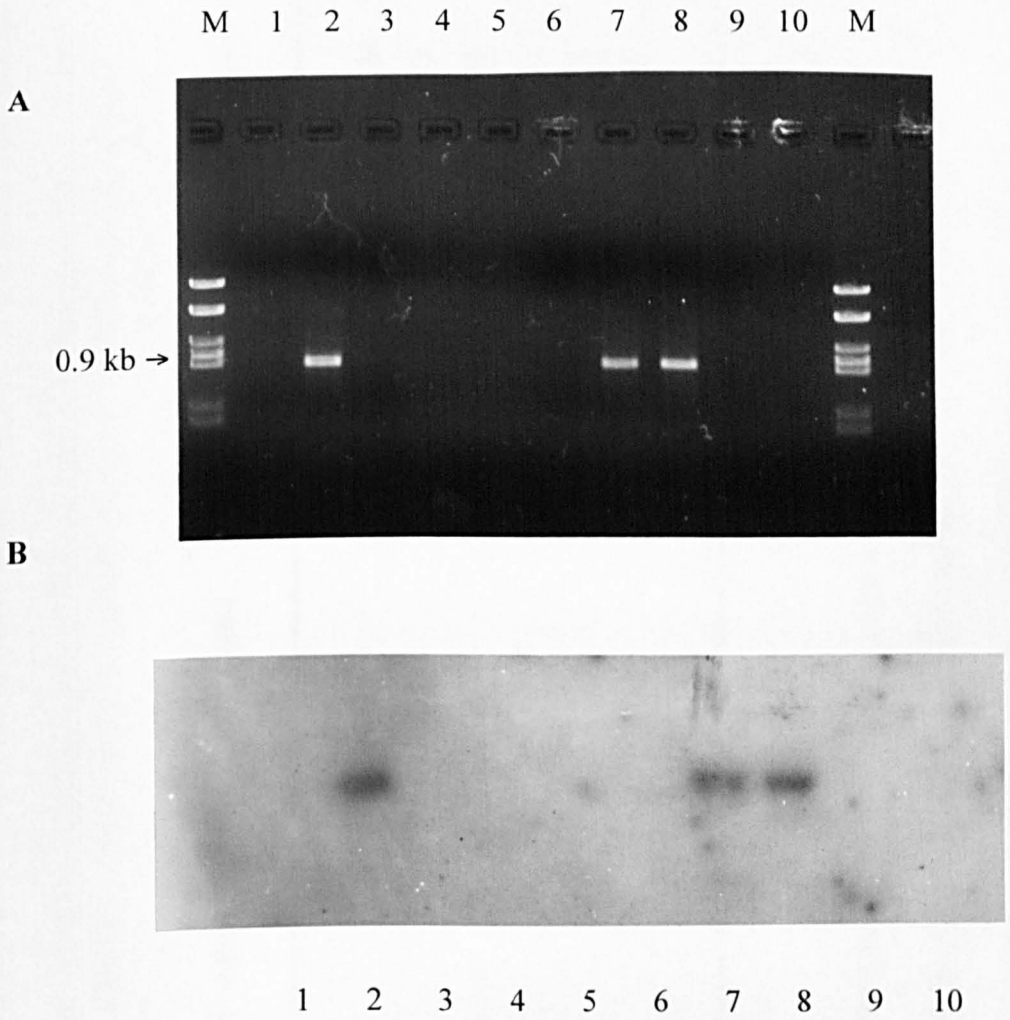


Fig. 4.8 Agarose gel electrophoresis of *Nitrosomonas*-specific amplification products derived from lakewater (14m depth) (lanes **A**1-6), Southern blot of *Nitrosomonas*-specific amplification products derived from lakewater (14m depth) hybridised with oligonucleotide AAO258 (lanes **B**1-6).

Lanes:

1. July 1995
2. August 1995
3. September 1995
4. November 1995
5. March 1996
6. May 1996
7. *Nitrosomonas europaea* Nm50
8. *Nitrosomonas eutropha* Nm57
9. *Nitrosospira* sp. NV141
10. sterile distilled water

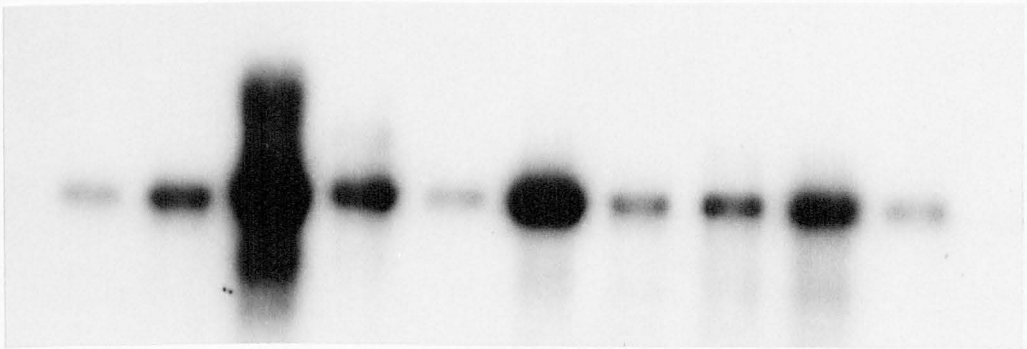
M MBI molecular weight marker, 21

Samples	*Nitrosospira*-specific PCR amplification of samples			*Nitrosomonas* spp.-specific PCR amplification of samples			Hybridisation of *Nitrosospira* 16S rDNA to Nsm459* probe			*Nitrosomonas* spp.-specific PCR amplification of enrichment cultures		
	Littoral	Profundal	Lakewater	Littoral	Profundal	Lakewater	Littoral	Profundal	Lakewater	Littoral	Profundal	Lakewater
July	+	+	+	+	-	-	-	-	-	ND	+M	+LM
August	+	+	+	-	+	+	-	-	-	+H	ND	ND
September	+	+	+	+	+	-	-	-	-	ND	ND	+M
November	+	+	+	-	-	-	*	*	*	+HM	+HM	+L
March	+	+	+	-	-	-	-	-	*	ND	ND	ND
May	+	+	+	-	-	-	-	*	*	ND	ND	ND

Table 4.2. Summary of the 16S rDNA PCR amplifications and oligonucleotide probings obtained from Buttermere sediment and lakewater samples and also enrichment cultures of sediment and lakewater samples.

(+) PCR amplification product obtained, (-) no PCR amplification product or hybridisation signal obtained (*) hybridisation signal obtained. (H: medium A containing high ammonium concentrations, M: medium B containing intermediate ammonium concentrations and L: medium C containing low ammonium concentrations. ND are samples not determined).

Nitrosomonas-specific PCR amplification products were generated from enrichment cultures inoculated with samples that had previously failed to generate *Nitrosomonas* amplification products by direct extraction of DNA and PCR amplification. All ten of the *Nitrosomonas* PCR amplification products obtained from lakewater and sediment enrtichment cultures hybridised to the oligonucleotide probe AAO28 with varying degrees of signal intensity (**Fig. 4.9**). Therefore, all of the PCR amplificaation products obtained were confirmed as ammonia-oxidiser 16S rDNA. The strongest hybridisation signals were obtained from an enrichment grown in high ammonium concentration (medium A) derived from a littoral sample taken in August and profundal sediment taken in November. The weakest hybridisation signals were obtained from enrichments derived from sediment and lakewater samples taken in November and grown in medium B. Since the presence of nitrosomonads was previously demonstrated directly from littoral sediment obtained in July and September, profundal sediment obtained in August and September, and lakewater obtained in August, no PCR amplifications were performed on enrichments derived from these samples (**Table 4.2**). In addition, no data were obtained for March and May samples as there was either no initial *in vitro* growth or the enrichments had ceased nitrifying during the incubation period (**Table 4.2**). *Nitrosomonas* spp. derived from sediment grew in high, medium and low ammonium concentration (media A, B & C). No nitrosomonad DNA from sediment was isolated from the low ammonium concentrations (medium C). Nitrosomonad DNA derived from the lakewater was isolated from enrichments grown in medium ammonium concentration (medium B) and low ammonium concentrations (medium C). All lakewater enrichments failed to nitrify in high ammonium concentrations (medium A).



1 2 3 4 5 6 7 8 9 10

Fig. 4.9 Southern blot of *Nitrosomonas*-specific PCR products from nucleic acids directly extracted from enrichment cultures derived from Buttermere and hybridised with oligonucleotide probe AAO258. (H) medium A containing high ammonium concentration, (M) medium B containing medium ammonium concentration, (L) medium C containing low ammonium concentration.

Lanes:				
	1.	littoral sediment,	November 1995	(M)
	2.	littoral sediment,	November 1995	(H)
	3.	littoral sediment,	August 1995	(H)
	4.	profundal sediment,	July 1995	(M)
	5.	profundal sediment,	November 1995	(M)
	6.	profundal sediment,	November 1995	(H)
	7.	lakewater (14m depth),	July 1995	(L)
	8.	lakewater (14m depth),	July 1995	(M)
	9.	lakewater (14m deth),	November 1995	(L)
	10.	lakewater (14m depth),	November 1995	(M)

4.5. PCR amplification of ammonia-oxidiser 16S rDNA from Windermere.

DNA extracted from profundal sediments and lakewater taken from the North and South Basins of Windermere was also subjected to a nested PCR amplification regime. Visible *Nitrosospira* amplification products corresponding to 0.93 kb were detected in all samples when the *Nitrosospira*-specific primer pair (Ns85/Ns1009*) was employed. The amplicons were confirmed by the hybridisation to the oligonucleotide probe AAO258. Data for profundal sediment are presented in **Figs. 4.10** and **4.11**, by way of an example. In addition, *Nitrosomonas* amplification products were also obtained in a number of samples derived from the North Basin. No *Nitrosomonas* products were obtained from samples derived from the South Basin throughout the year. **Table 4.3** summarises the PCR amplification results obtained.

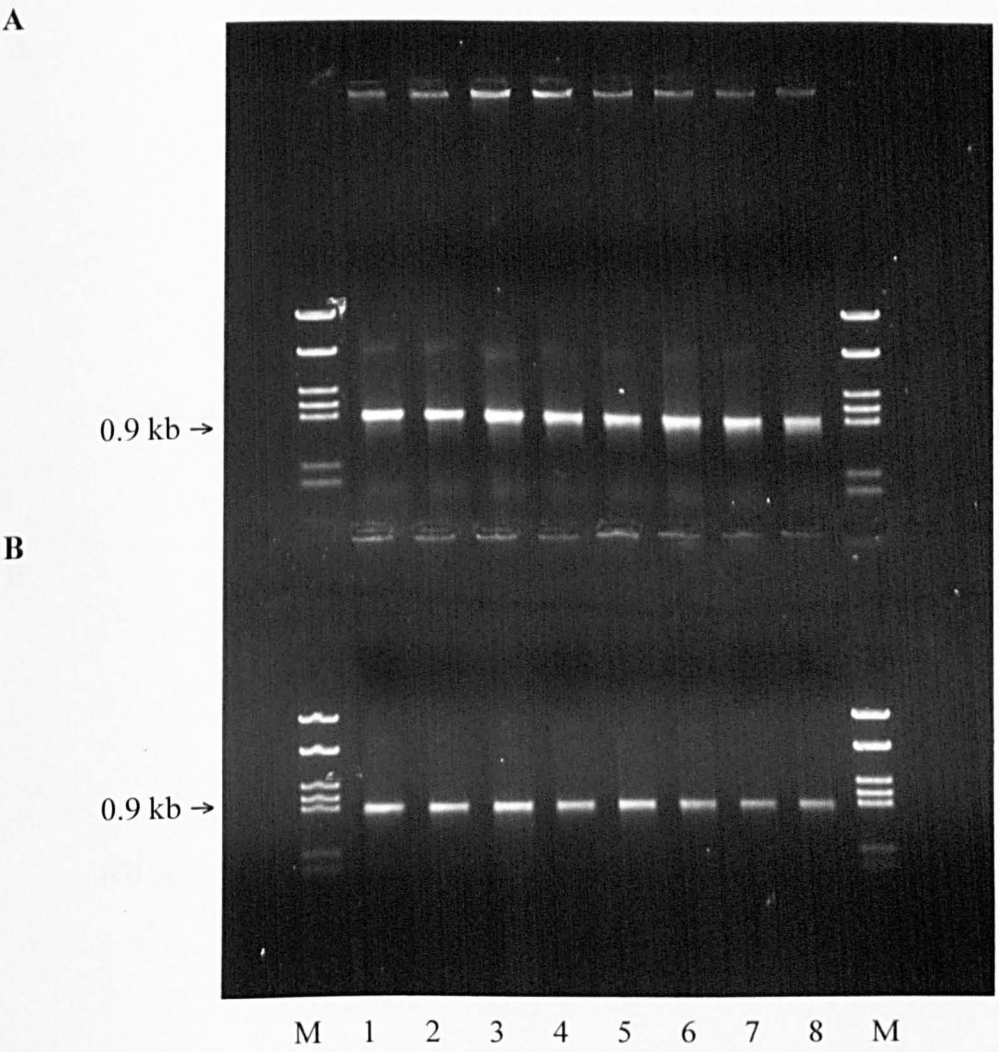


Fig. 4.10 Agarose gel electrophoresis of *Nitrosospira*-specific amplification products derived from North Basin profundal sediment (0-0.5cm) (lanes **A**1-8), South Basin profundal sediment (0-0.5cm) (lanes **B**1-8).

Lanes:

1. March 1996
2. April 1996
3. May 1996
4. June 1996
5. August 1996
6. September 1996
7. November 1996
8. January 1997

M MBI molecular weight marker, 21

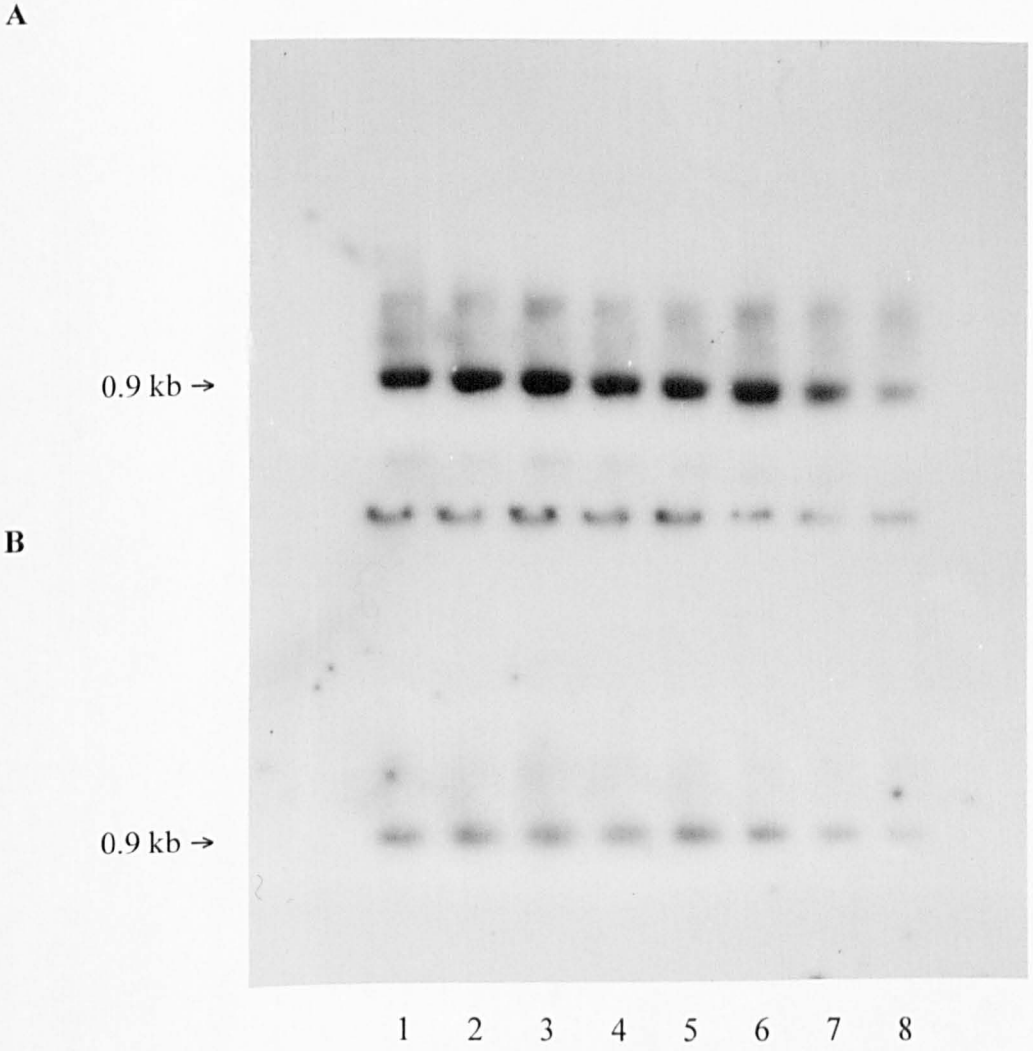


Fig. 4.11 Southern blot of *Nitrosospira*-specific amplification products derived from North Basin profundal sediment (0-0.5cm) (lanes **A**1-8), South Basin profundal sediment (0-0.5cm) (lanes **B**1-8) hybridised to oligonucleotide AAO258.

Lanes:

1. March 1996
2. April 1996
3. May 1996
4. June 1996
5. August 1996
6. September 1996
7. November 1996
8. January 1997

Samples	*Nitrosospira*-specific PCR amplification						*Nitrosomonas* spp. PCR amplification					
	North Basin			South Basin			North Basin			South Basin		
	upper	lower	lakewater	upper	lower	lakewater	upper	lower	lakewater	upper	lower	lakewater
March	+	+	+	+	+	+	+	+	+	-	-	-
April	+	+	+	+	+	+	-	-	+	-	-	-
May	+	+	+	+	+	+	-	-	+	-	-	-
June	+	+	+	+	+	+	ND	ND	ND	ND	ND	ND
August	+	+	+	+	+	+	-	-	+	-	-	-
September	+	+	+	+	+	+	+	+	+	-	-	-
November	+	+	+	+	+	+	-	-	-	-	-	-
January	+	+	+	+	+	+	ND	ND	ND	ND	ND	ND

Table 4.3 Summary of the PCR amplification results obtained from Windermere.

(+) PCR product of the predicted size obtained and hybridised to the oligonucleotide probe AAO258.

(-) no PCR product or hybridisation signal (AAO258) obtained. (ND) not determined.

(upper denotes upper layer (0-0.5cm) of profundal sediment, lower denotes lower layer (0.5-3.0cm) of profundal sediment).

4.6. Discussion.

The direct recovery and PCR amplification of bacterial 16S rDNA from diverse environments has been described in a number of reports (Ward *et al.*, 1997; Hiorns *et al.*, 1995; Tsai & Olson, 1992). All the environmental sample DNAs studied here amplified directly with the eubacterial primers. The presence of autotrophic ammonia-oxidising bacteria in sediment and lakewater was detected by nested PCR amplification and subsequent oligonucleotide probing. Whilst direct amplification of ammonia-oxidiser 16S rDNA from the environment without a nested approach has been demonstrated (*e.g.* Stephens *et al.*, 1996) there is a posssibility that only the most abundant organisms will be amplified. Therefore, the failure of the genus-specific ammonia-oxidising primers to amplify DNA without the prerequisite of a nested PCR reaction, suggests that the ammonia-oxidiser target DNA was present in low abundance, rather than inhibition of the polymerase by contaminants.

The oligonucleotides used in this study were not specific when applied individually, as primer design was based on a limited number of laboratory cultures which may not be representative of the diversity of bacteria in the environment (Head *et al.*, 1993). Sequence analysis of published terrestrial *Nitrosospira* spp. demonstrated that all the sequences contained the *Nitrosospira* primer target sites (Ns85/Ns1009*). In addition, the *Nitrosospira* primer sequences failed to recognise the 16S rDNA sequences from *Nitrosomonas* spp. The *Nitrosomonas* primers (Nm75/Nm1007*) are highly specific for *Nm. europaea* and *Nm. eutropha* but the other β-subgroup ammonia-oxidisers 16S rDNA sequences are not recognised. Both the *Nitrosospira* and *Nitrosomonas* spp. primer sequences are homologous to 16S rDNA sequences from a number of non-ammonia-oxidising bacteria such as *Azoarcus*

and *Acinetobacter* spp. even under stringent conditions. The oligonucleotide probe AAO258 was designed to recognise 16S rDNA sequences from all terrestrial ammonia-oxidising bacteria β-Proteobacteria (Hiorns *et al.*, 1995). Indeed, the probe sequence is contained in all of the sequences from published terrestrial ammonia-oxidising bacteria with either a complete match, or in a few cases, one or two mismatches. In addition, the target site for AAO258 has also been found in a number of soil sequences from *Nitrosospira* clusters (Stephens *et al.*, 1996). Encouragingly, the marine strains and marine *Nitrosospira*-like sequences and marine *Nitrosomonas*-like sequences derived from soil samples did not contain the target site for AAO258 (Stephens *et al.*, 1996). However, database searches have demonstrated the presence of AAO258 probe sequence in a number of non-target organisms, but when the probe is performed in combination with ammonia-oxidiser specific primers under stringent conditions, a high degree of specificity is achieved and no non-target sequences are recognised (Hiorns *et al.*, 1995; Hastings *et al.*, 1997; this study). In addition, two *Nitrosospira* spp. sequences isolated from soil by Utåker *et al.* (1995) and a third sequence isolated from the soil sample pH4.2A/23 by Stephen *et al.* (1996) contain one and two mismatches respectively, and therefore, will not be recognised by AAO258 under stringent conditions.

Whilst the use of other published PCR primers may detect ammonia-oxidisers that the oligonucleotides used here failed to recognise, they also amplify DNA from a number of non-target organisms (McCaig *et al.*, 1994; Voytek & Ward, 1995; Ward *et al.*, 1997). The amplification of functional genes such as the ammonia monoxygenase gene could confirm the presence of species that could not be detected by primers

targeting the 16S rRNA gene (Norton *et al.*, 1996; Rotthauwe *et al.*, 1997; Hastings *et al.*, 1997).

Members of the genus *Nitrosospira* were found to be distributed ubiquitously in both Buttermere and Windermere based on the detection of *Nitrosospira* 16S rDNA. This further confirms a number of reports that suggest the prevalence of *Nitrosospira* spp. in the environment and that *Nitrosospira* spp. are more abundant than *Nitrosomonas* spp. (Belser 1979, Bock *et al.*, 1986; Hiorns *et al.*, 1995; Stephen *et al.*, 1996). The data from the species-specific hybridisation using the oligonucleotide Nsm459* demonstrated the presence of *Ns. multiformis* in both sediments and lakewater from Buttermere during the period September to May. Whether this species is a dominant member of the ammonia-oxidiser population in Buttermere could not be determined from this study as other species-specific oligonucleotide probes were not applied.

Previous culture-based studies suggested that the members of the genus *Nitrosomonas* were the predominant mediators of nitrification in natural environments due to the frequency of isolation of *Nitrosomonas* spp compared to the genus *Nitrosospira*. McCaig *et al.* (1994) demonstrated the dominance of *Nitrosomonas* spp. by PCR amplification of 16S rDNA from enrichment cultures. Ward *et al.* (1997) detected *Nm. europaea* in marine enrichments derived from the Belauer See, which is of low salinity and also in a hypersaline pond, but they were not detected in a freshwater lake.

The detection of *Nitrosomonas* spp. has been reported from a variety of nutrient rich environments (Hovanec *et al.*, 1996; Mobarry *et al.*, 1996; Hastings *et al.*, 1997). Purified DNA samples extracted from concentrated water derived from an

oxidation pond amplified *Nitrosomonas* spp. and the identity was confirmed by Southern blot hybridisation (Nejidat *et al.*, 1994). *Nitrosomonas* spp. were also shown to be predominant in close association with *Nitrobacter* spp. in a biofilm reactor (Mobarry *et al.*, 1996). The presence of *Nm. europaea* was reported to high levels in seawater aquaria, but only two samples demonstrated the presence of β-subdivision ammonia-oxidisers in a nitrifying freshwater aquarium (Hovanec & DeLong, 1996). Ward *et al.* (1997) detected nitrosomonad DNA from a marine environment in the Pluβsee to a depth of 25 m. Despite using *Nitrosomonas* spp.-specific primer pairs a study by Hiorns *et al.* (1995) failed to amplify *Nitrosomonas* 16S rDNA from a eutrophic freshwater lake. Pommerening-Röser *et al.* (1996) suggested that representatives of the *Nm. europaea*-lineage have been isolated only from brackish waters and sewage disposal plants rather than freshwater (Koops *et al.*, 1991). However, the work described in this chapter has been the first to demonstrate the presence of members of the *Nm. europaea-eutropha* lineage directly by amplification of 16S rDNA extracted from sediment and lakewater derived from freshwater lakes, without any prior enrichment.

The direct approach of PCR amplification and confirmatory hybridisations circumvents any bias incurred by the use of culture media. The direct amplification of *Nitrosomonas* spp. in Buttermere was only achieved during the summer months (July to September). This seasonal occurrence corresponds to a slight increase in ammonium concentration from 5 $\mu g\ l^{-1}$ (January) to 12 $\mu g\ l^{-1}$ (July) at 5 m depth (Pickup, pers. comm.). It is possible that there is an increase in *Nitrosomonas* cell numbers in Buttermere to detectable levels corresponding to the increase in ammonia concentrations in the summer months. This corroborates data obtained by Hiorns *et al.*

(1995) and Hastings *et al.* (1997), who suggested that nitrosomonads are enriched in nutrient-rich situations such as in soils amended with pig slurry. Ward *et al.* (1997) reported that oxygen concentration, temperature and inorganic nutrient concentrations across the oxic/anoxic interface of the Plußsee, a eutrophic environment, may affect the distribution of ammonia-oxidisers.

The data obtained from the PCR amplifications derived from the enrichment cultures corroborated the presence of nitrosomonad DNA from Buttermere sediment and lakewater, and demonstrated the presence of nitrosomonad DNA, which had previously failed to amplify directly. The period of enrichment had probably increased the copy number of target molecules to that which could be detected by PCR. This is comparable to data obtained from Esthwaite, whereby *Nitrosospira* DNA was detected directly by PCR amplification, but nitrosomonad DNA was only detectable after a period of laboratory enrichment (Hiorns *et al.*, 1995). It is possible that *Nitrosomonas* spp. are present throughout the year in Buttermere, but in relatively low numbers in comparison to the *Nitrosospira* spp. The *Nitrosomonas* cell numbers may increase during the summer months with the establishment of the thermocline and the subsequent increase in ammonium concentrations.

The occurrence of *Nitrosomonas* spp. was also demonstrated by amplification of 16S rDNA in the North Basin of Windermere in lakewater from March to September, and in the profundal sediments in March and September only. No nitrosomonad DNA was detected directly in the South Basin, but it is possible that *Nitrosomonas* spp. were present below detectable limits. However, *Nitrosomonas* spp. were detected in MPN enrichment cultures from Windermere by flow cytometry (see Chapter 6). There appears to be no significant difference in ammonium concentrations

between Buttermere and the North Basin of Windermere. The ammonium concentrations between basins of Windermere are significantly different with a rapid increase in the South Basin from 7-60 μg l^{-1} from January to July, and this may influence the presence of *Nitrosomonas* spp.

The differences observed in the distribution of ammonia-oxidising bacteria in the two freshwater lakes may be attributed to the formation of biofilms, which may provide an advantage in the environment when substrate supply is intermittent. Batchelor *et al.* (1997) demonstrated that starved biofilms of *Nm. europaea* resuscitated more rapidly than free cell suspensions, as ammonium can be released from soil particles after ammonium supply has been halted. These effects may be attributed to the production of extracellular polymeric substances (EPS) and N-acyl homoserine lactones which are features of a number of Gram negative bacteria, including ammonia-oxidiser biofilms (Allison & Prosser, 1993; Batchelor *et al.*, 1997). *Nitrosomonas* spp. present in Buttermere and Windermere may have adapted to survive fluctuations in ammonia concentrations by being present in biofilms.

The possibility of sub-populations of ammonia-oxidisers that inhabit lakewater that are sensitive to ammonium concentrations has previously been reported (Suwa *et al.*, 1994, 1997; Stehr *et al.*, 1995; Hastings *et al.*, 1997). Culture-based studies presented in Chapter 3 indicated that ammonia-oxidiser populations present in lakewater were less tolerant to high ammonium concentrations compared to sediment populations. The *Nitrosomonas* spp. present in the two freshwater lakes may have adapted to low ammonium concentrations present whereas *Nitrosospira* spp. may be able to respond to fluctuations in ammonium concentrations by hydrolysing other available nitrogen sources *e.g.* urea. De Boer *et al.* (1989) isolated a urease-positive

Nitrosospira strain from an acid heath soil and suggested urea hydrolysis as a growth mechanism in acid soils.

4.7. Conclusions.

- The combination of *Nitrosospira*-specific primers or *Nitrosomonas* spp. primers with the oligonucleotide probe AAO258 under stringent conditions is highly specific for terrestrial β-Proteobacteria ammonia-oxidisers.

- *Nitrosospira* spp. were detected by nested PCR amplification of 16S rDNA in Buttermere and Windermere and they were found to be distributed ubiquitously.

- *Nitrosomonas* 16S rDNA was detected directly by nested PCR amplification of 16S rDNA in profundal sediment and lakewater obtained from Buttermere during the summer months.

- *Nitrosomonas* 16S rDNA was detected directly by nested PCR amplification of 16S rDNA in profundal sediment and lakewater obtained from the North Basin of Windermere but not from the South Basin.

- The detection of *Nitrosomonas* 16S rDNA from enrichment cultures by nested PCR amplification of 16S rDNA suggest the presence in low abundance of *Nitrosomonas* spp. throughout the year in Buttermere.

Chapter 5. Genetic diversity of ammonia-oxidising bacterial 16S rRNA sequences recovered from Buttermere.

5.1. Introduction.

PCR and sequence analysis of ribosomal genes provides a basis for the identification of bacterial genotypes. Phylogenetic studies based on 16S rRNA gene sequences of ammonia-oxidising bacteria within the β-Proteobacteria have demonstrated a close relationship between strains (Head *et al.*, 1993; Teske *et al.*, 1994; Pommerening-Röser *et al.*, 1996; Utåker *et al.*, 1995). The determination of detailed phylogenetic information has been difficult due to the high sequence homology between isolates of ammonia-oxidising bacteria (Head *et al.*, 1993; Teske *et al.*, 1994; Utaker *et al.*, 1995). However, several studies have applied 16S rDNA sequence information to assess the diversity and distribution of ammonia-oxidisers in natural environments (McCaig *et al.*, 1994; Stephen *et al.*, 1996; Kowalchuk *et al.*, 1997).

An alternative phylogenetic tool is PCR coupled with RFLP analysis which has previously been applied to characterise a variety of natural populations of bacteria (e.g. Bruce *et al.*, 1992; Navarro *et al.*, 1992; Osborn *et al.*, 1993; Hoi *et al.*, 1997). The community structure of ammonia-oxidising bacteria within an oligotrophic lake has not been documented. This chapter describes the development of a PCR-RFLP analysis based on sequence information obtained, to investigate the diversity of 16S rRNA genes from ammonia-oxidising bacteria derived from Buttermere. The technique permits the differentiation within a phylogenetic lineage *i.e.* between *Nm. europaea* and *Nm. eutropha* spp. on the basis of specific restriction sites and provides

a more targeted approach to sequence analysis. The restriction profiles obtained are compared to the sequence data to enable a detailed phylogenetic analysis of the clones under investigation.

Density gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) may also provide information about sequence variation in a mixture of PCR fragments based on denaturant concentration (DGGE) or temperature (TGGE) (Muyzer *et al.*, 1993; Heuer *et al.*, 1997). This chapter describes the application of DGGE and the development of TGGE to analyse the genetic diversity of ammonia-oxidisers within Buttermere. Both techniques facilitate the identification of dominant community members and evaluate sequence types either by sequencing of the excised bands or hybridisation to oligonucleotide probes.

5.2. Results.

5.2.1. PCR amplification and cloning of the 16S rRNA genes from Buttermere.

PCR amplification of heterogeneous template DNA can lead to biases due to products generated from initial rounds of PCR amplification having exponential kinetic behaviour and thus dominating the final amplicon products. To reduce this potential bias, the products from three separate reactions were pooled prior to cloning. As described in Chapter 4, 0.9 kb fragments of *Nitrosospira* and *Nm. europaea-eutropha* 16S rDNA amplification products were obtained from sediment and lakewater taken from Buttermere.

Both *Nitrosospira* spp. and *Nm. europaea-eutropha* PCR products were ligated into PGEM-T cloning vector with high transformation efficiencies (<5.86 x 10^9 CFU μg^{-1}). The control reactions supplied by the manufacturer also performed to a satisfactory efficiency. In total, 96 clones were obtained and screened for the presence of inserts of the correct size (0.9 kb) by plasmid mini-preparation, restriction endonuclease digestion using the *NcoI* and *PstI* restriction sites present in the PGEM-T cloning vector (**Fig. 5.1**) and hybridisation to the ammonia-oxidiser specific probe AA0258. All 96 clones were identified to contain inserts of the correct size and hybridised to the oligonucleotide probe AAO258 (data not shown). This further confirmed the common occurrence of the AAO258 target sequence present in most β-Proteobacteria. All clones were placed in storage in glycerol broths at -70°C until required for subsequent RFLP and sequence analysis.

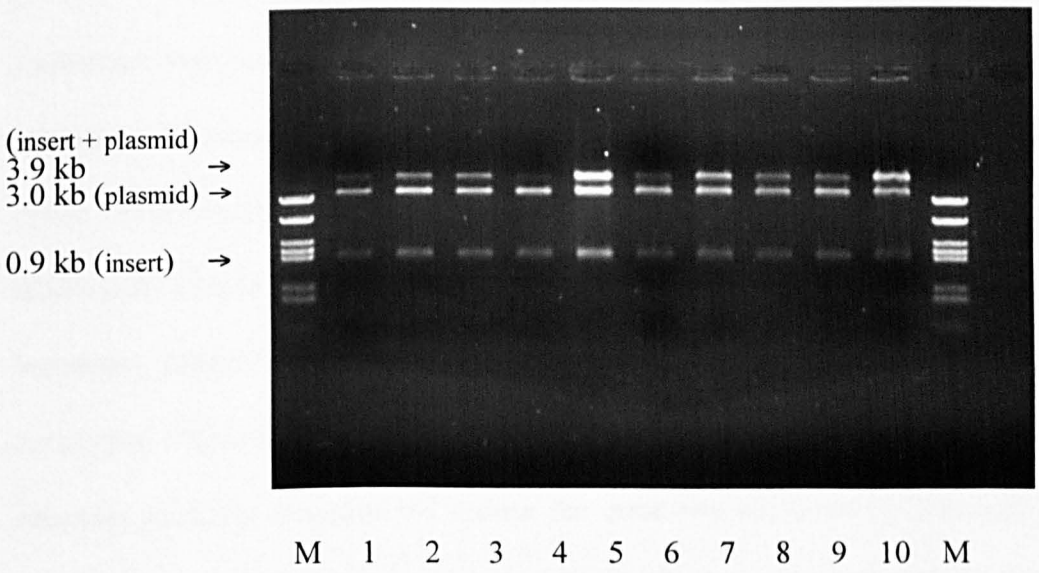


Fig. 5.1 Agarose gel electrophoresis of a selection of plasmid clones digested with the restriction enzymes *NcoI* and *PstI* (Boehringer Mannheim).

Lanes:

1. clone LV3A
2. clone LV3B
3. clone LV3C
4. cloneLV2A
5. clone LV3M
6. clone LV3H
7. clone LV3I
8. clone LV3J
9. clone LV3K
10. clone LV3L

M MBI molecular weight marker, 21

5.3. Ammonia-oxidising bacteria 16S rRNA sequence diversity.

The 96 clones obtained from Buttermere comprised, 73 *Nm. europaea-eutropha*-like inserts and 23 clones that contained *Nitrosospira*-like inserts. Since previous studies have failed to directly amplify nitrosomonad 16S rDNA from freshwater environments, the main focus of this work was to analyse the clones containing *Nitrosomonas* homologues obtained from sediment and lakewater. Twenty seven *Nitrosomonas* sequence homologues of 345 bp of the V3 region of the 16S rRNA gene were isolated from cloned DNA extracted from both profundal and littoral sediments derived from Buttermere. Due to time constraints, none of the clones containing *Nitrosomonas* homologues isolated from lakewater was analysed. To maintain sequence integrity and reduce the error rate attributed to *Taq* polymerase reading errors, the clones were sequenced in both directions and corroborated by a third analysis using different primers.

Data obtained from unrestricted cluster type searches (*i.e.* FASTA & BLAST) of GenBank confirmed that fourteen clones derived from littoral sediment (designated L) were >95% homologous to previously described 16S rDNA from the *Nm. europaea* type strain. In addition, thirteen clones derived from profundal sediment (designated P) were >95% homologous to previously described 16S rDNA from the *Nm. eutropha* type strain. In both cases the next 10 matches were sequences of ammonia-oxidising bacteria. Although a greater number of sequences would be required, the data suggested that the nitrosomonad population at each site was specific, with either *Nm. europaea* or *Nm. eutropha* predominating. The data further corroborated the specificity of the combination of primers with the oligonucleotide probe AAO258 in the identification of ammonia-oxidiser 16S rDNA, as non-target sequences

demonstrated <85% homology to the twenty seven *Nitrosomonas* sequences obtained from Buttermere.

Phylogenetic trees constructed by the analysis of alignments of unambiguous sequences generated by DNA distance methods (Jukes-Cantor, Kimura-2-Parameter and Maximum Liklihood) produced trees with similar topologies and bootstrap values. In **Fig 5.2** the phylogenetic tree generated by the Jukes-Cantor DNA distance method is presented. The topology of the trees was confirmed using Maximum Parsimony analysis of the bootstrapped sequence data. The branching order of the trees demonstrated that the *Nitrosomonas*-like sequences derived from Buttermere formed two clusters related to the location of the sediment from which the sequence had been obtained. Sequences (345bp) derived from the littoral sediment (designated L) grouped with *Nm. europaea* type strain, and sequences (345bp) derived from profundal sediment (designated P) grouped with *Nm. eutropha*. The relatively high bootstrap values confirmed the distinction between the two groups. Bootstrap values within each group were low due to high sequence homology between the isolates and the relatively short length of sequence used in the analysis. Consequently, there is low level sequence variation within each group and the relative branching positions of the sequences are not significant.

In order to analyse in more detail the relationships between the clones and the published sequences of ammonia-oxidising bacteria, two clones (designated PV_3 and LV_3 from profundal and littoral sediments respectively) were selected from each cluster and analysed further using a longer stretch of 16S rDNA sequence (701bp). The phenograms generated using different DNA distance methods were all analogous to those previously obtained from the shorter sequences (345bp) with comparable

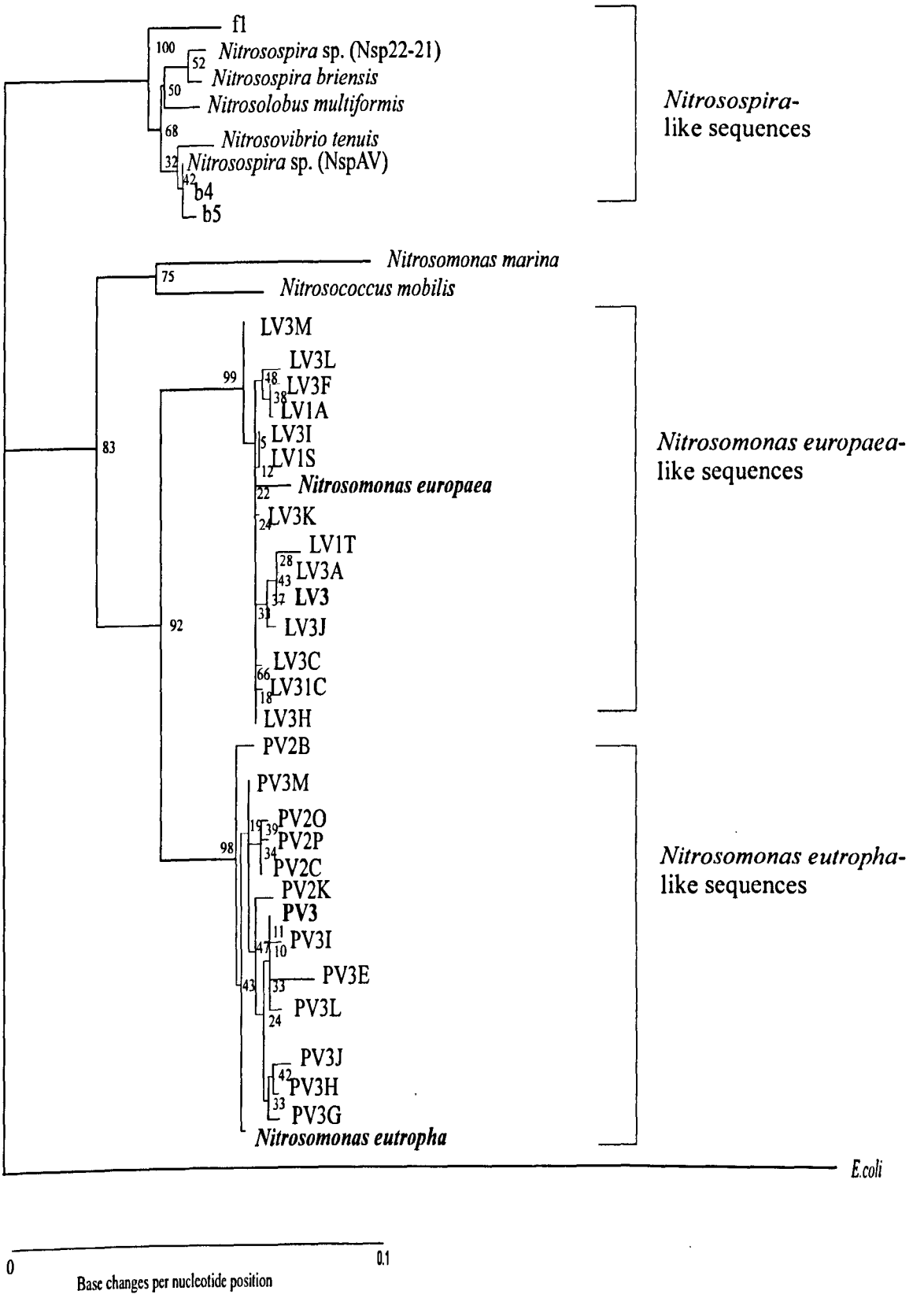


Fig. 5.2. Phylogenetic relationship of ammonia-oxidising bacteria- like 16S rDNA gene sequences isolated from Buttermere sediments.

Reference strains were obtained either from GenBank or The Ribosomal Database Project (Larsen *et al.*, 1993). Analysis based on 345 base positions. Bootstrap values were derived from 100 analyses. PV3: profundal sediment (September), PV2: profundal sediment (August), LV1: littoral sediment (July), LV3: littoral sediment (September). LV3 & PV3 (bold): clones that were sequenced further (Fig. 5.3.) b4 & b5: *Nitrosospira* sequences from littoral sediment (September), f1: *Nitrosospira* sequences from lakewater (November).

bootstrap values. In **Fig. 5.3** the phylogenetic tree constructed using parsimony analysis of the bootstrapped sequence data is presented. The topology of the tree is similar to that produced from the shorter sequences (**Fig. 5.2**). The grouping of the two *Nitrosomonas* clusters with each sediment type was corroborated by the high bootstrap values obtained (100%) for both *Nm. europaea* and *Nm. eutropha* from the analysis of the longer sequences. This study is the first reported recovery of *Nm. europaea* and *Nm. eutropha* sequence homologues directly from a freshwater lake.

In addition to the *Nm. europaea* and *Nm. eutropha* sequences obtained, the *Nitrosospira* PCR amplification products obtained from Buttermere (see section 4.3.2) were also cloned and sequenced. This was done in collaboration with Dr. J. Rodrigeuz. Analysis of partial sequences from three of the clones, two derived from littoral sediment (designated b4 and b5) sampled in September and the third from lakewater sampled in November (designated f1), revealed >95% homology to previously published *Nitrosospira* sequences (**Fig. 5.2**). Again the phylogenetic tree demonstrates low bootstrap values within the *Nitrosospira* cluster due to the short sequence lengths and the small number of clones analysed. Further work would be to sequence a greater number of the *Nitrosospira* clones and to analyse longer stretches of sequences.

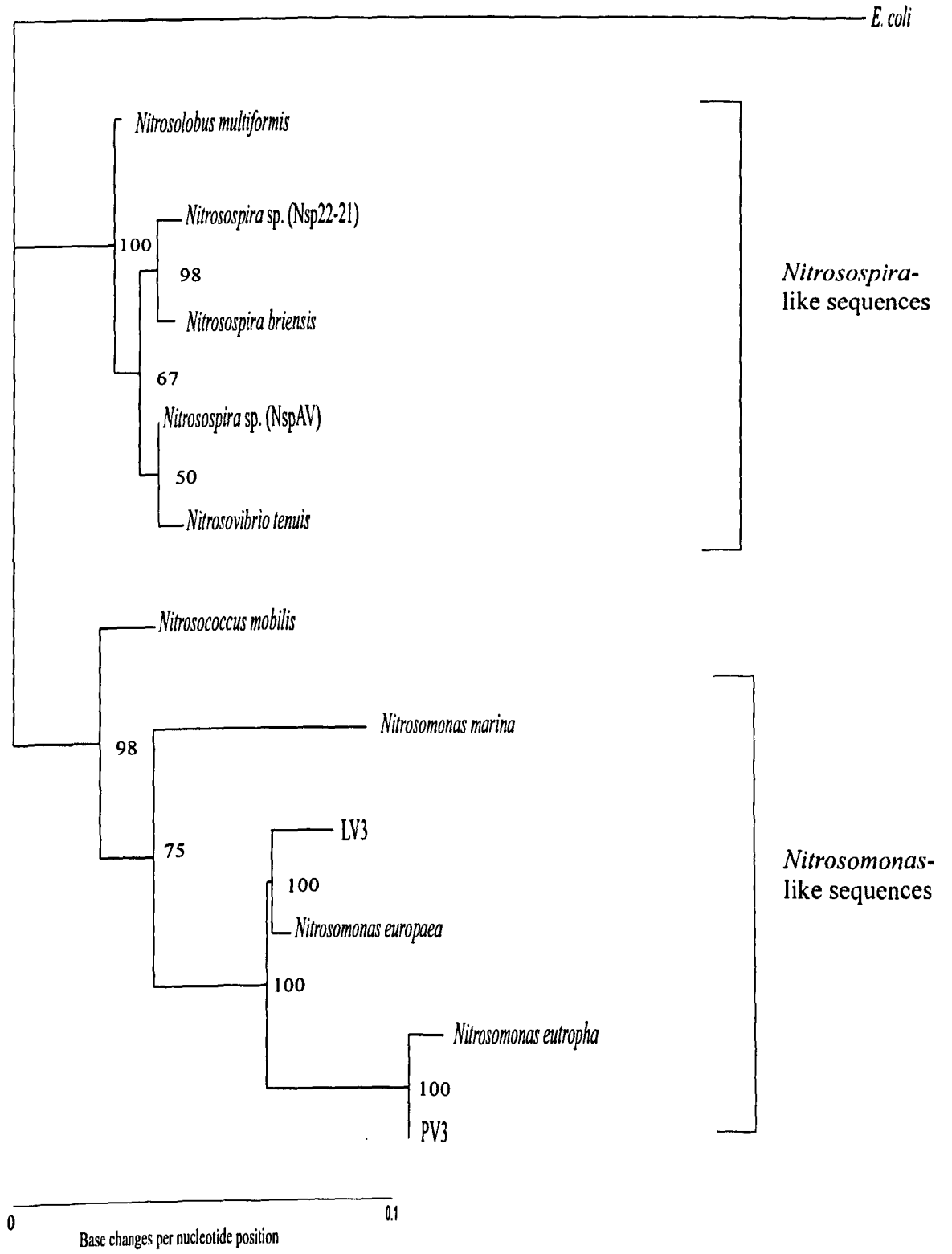


Fig. 5.3. Phylogenetic relationship of ammonia-oxidising bacteria- like 16S rDNA gene sequences isolated from Buttermere sediments.

Reference strains were obtained either from GenBank or The Ribosomal Database Project (Larsen *et al.*, 1993). Analysis based on 701 bp. Bootstrap values were derived from 100 analyses.

5.3.1. Restriction analysis 16S rRNA genes of ammonia-oxidising bacteria.

Analysis of suitable restriction sites within a 0.93 kb region of the 16S rRNA gene from selected strains of ammonia-oxidising bacteria was undertaken using the MAPSORT program in the GCG package. The restriction profiles for published ammonia-oxidising bacteria 16S rRNA sequences are presented in **Fig. 5.4**. The data obtained demonstrated that a single restriction site consisting of a single base change at position 213 bp (*E. coli* 16S rRNA gene numbering, Edwards *et al.*, 1989) could differentiate between two *Nitrosomonas* spp.; *Nm. europaea* and *Nm. eutropha*. Two possible restriction enzymes that cut at this particular site are the six base cutters *EagI* and *EaeI*. In addition, *EaeI* also discriminates the *Nitrosospira* group from the *Nm.europaea-Nm.eutropha* lineage. The restriction enzymes *HaeIII* and *HinfI* can also be applied to differentiate *Nm. europaea* from *Nm. eutropha*. *HinfI* (like *EaeI*) also differentiates the *Nitrosospira* group from the *Nm. europaea-eutropha* lineage. A combination of the three restriction enzymes *HaeIII*, *RsaI* and *TaqI* revealed RFLP patterns that delineated further the six *Nitrosospira* sequences examined from each other, and from the two *Nitrosomonas* spp. (*Nm. europaea* and *Nm. eutropha*) (**Fig. 5.4**).

A preliminary analysis of the 0.93 kb region of the 16S rDNA from two laboratory cultures of *Nitrosomonas* spp. (*Nm. europaea* and *Nm. eutropha*) and from two laboratory cultures of *Nitrosospira* spp. (Nv141 and Nsp22) was performed. The restriction enzymes *EaeI*, *HinfI*, *TaqI* and *HaeIII* were applied and the restriction profiles demonstrated the same patterns as those depicted in the MAPSORT program (**Fig. 5.5**). The RFLP patterns generated using either of two restriction enzymes *HaeIII* or *TaqI* could differentiate the two *Nitrosospira* spp. (**Fig. 5.5**). This

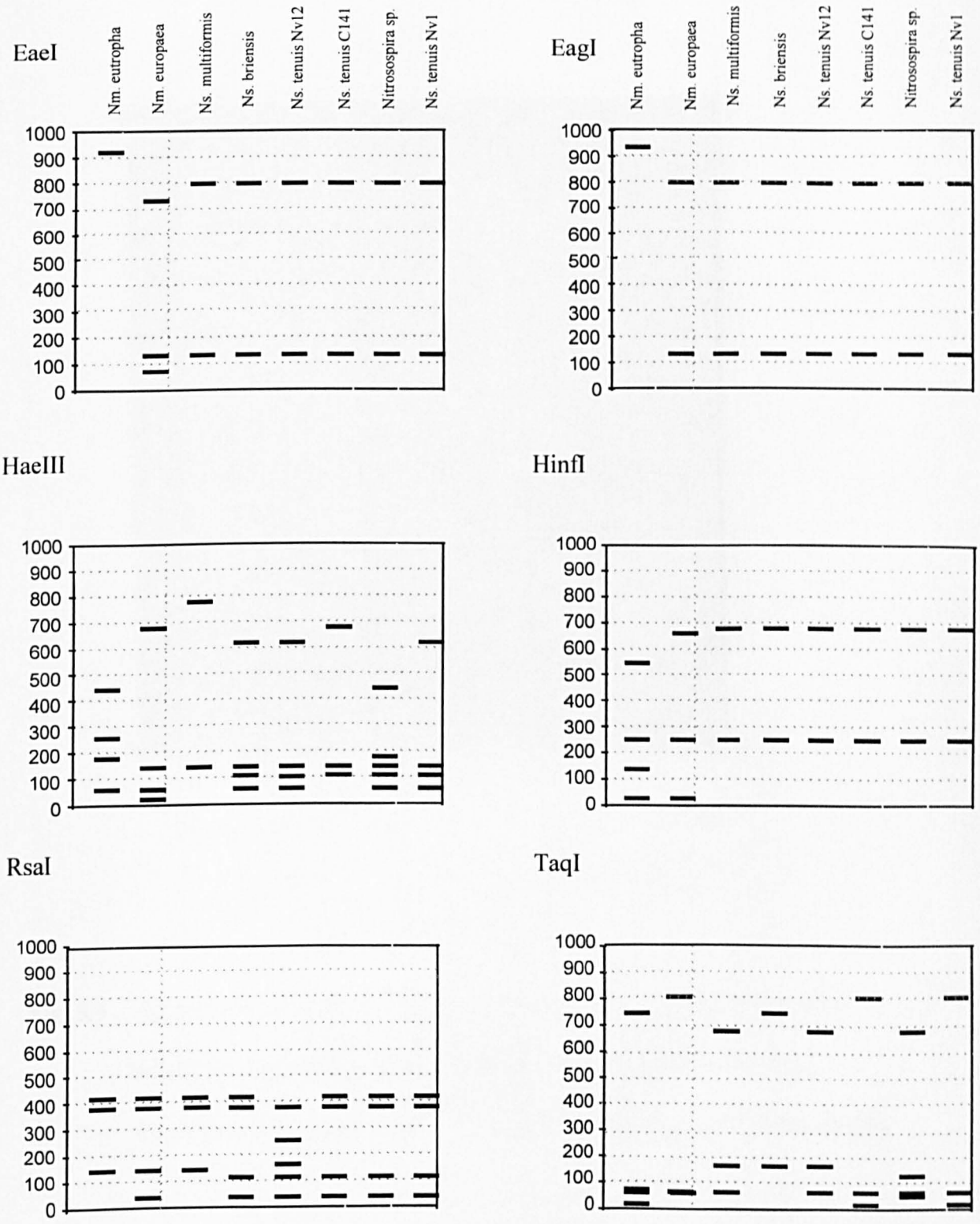


Fig. 5.4. Schematic diagram of the RFLP patterns produced for published ammonia-oxidiser 16S rRNA sequences with six restriction enzymes.

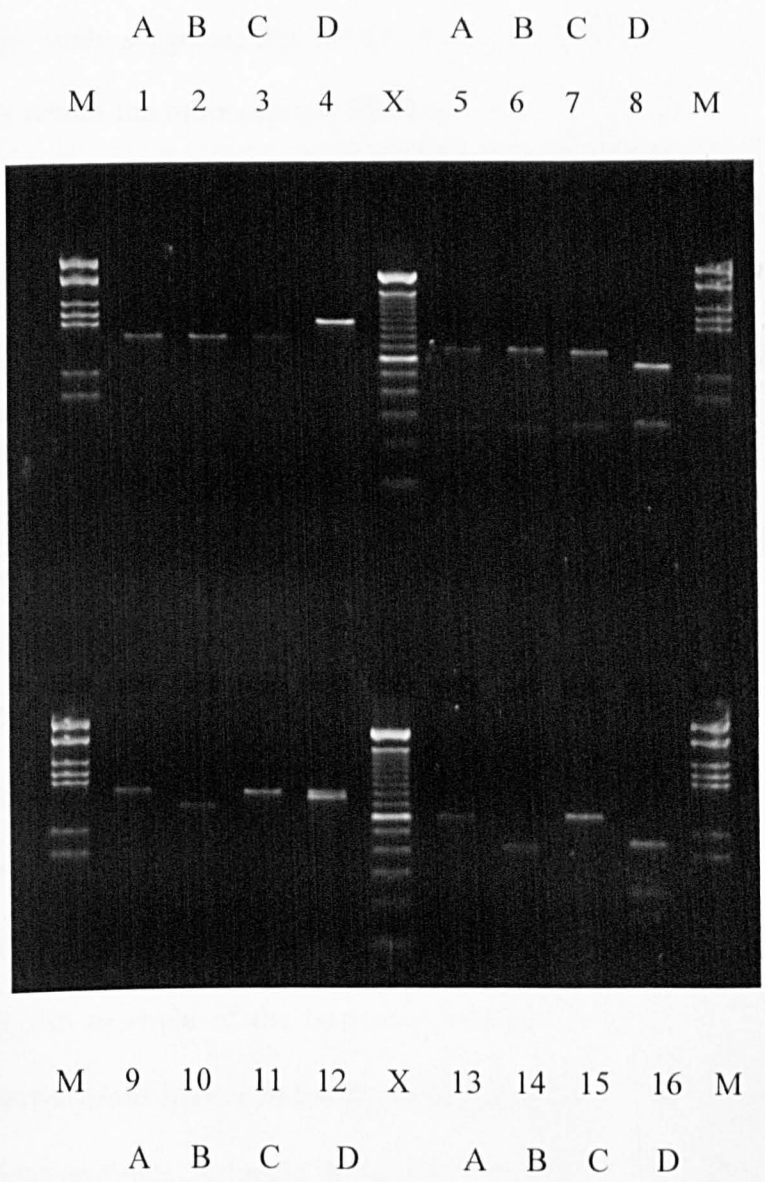


Fig. 5.5 Agarose gel electrophoresis demonstrating the restriction patterns of a selection of ammonia-oxidiser 16S rDNA sequences digested with *EagI* (lanes 1-4), *HinfI* (lanes 5-8), *TaqI* (lanes 9-12), *HaeIII* (lanes 13-16).

Lanes A: *Nitrosospira* spp. (NV141), lanes B: *Nitrosospira* sp. (Nsp.22), lanes C: *Nitrosomonas europaea*, lanes D: *Nitrosomonas eutropha.* M: MBI molecular weight marker, 21. X: 100bp ladder GibCo.

preliminary study suggested that the RFLP analysis had the potential to discriminate genotypes within the nitrosospiras. However, since the main focus of this work was the *Nitrosomonas*-like clones, the RFLP analysis to differentiate genotypes of nitrosospiras was not investigated further. When the restriction enzymes *EaeI*, *HinfI* and *HaeIII* were applied to the 16S rDNA from the *Nitrosomonas* spp., the two strains (*Nm. europaea* and *Nm. eutropha*) could be identified on the basis of the RFLP patterns generated (**Fig. 5.5**).

Following the RFLP analysis of laboratory strains, the PCR-RFLP system of classification was applied to the *Nitrosomonas*-like clones of 16S rDNA PCR-amplified from Buttermere littoral and profundal sediments. The PCR-RFLP analysis developed for this study used the enzymes *HinfI*, *EagI*, and *HaeIII*. Initially, the restriction analysis was undertaken on clones whose sequences had previously been identified to ensure that the distinction between *Nm. europaea* and *Nm. eutropha* was consistent. An example of the restriction profiles obtained from four clones (two clones derived from littoral sediment, designated LV_3L and LV_3K, and two clones derived from profundal sediment designated PV_3 and PV_2P) are presented in **Fig 5.6**. The three restriction enzymes *HaeIII*, *HinfI* and *EaeI* were applied. Both the clones (LV_3L and LV_3K) have restriction patterns which are consistent with *Nm. europaea* sequences, whereas the clones PV_3 and PV_2P demonstrated restriction patterns consistent with *Nm. eutropha* sequences.

Subsequent restriction analysis was then undertaken on 19 clones selected randomly prior to sequencing. These data are presented in the form of a summarised schematic diagram (**Fig. 5.7**). The *Nitrosomonas* clones segregated into two clusters that could be equated with *Nm. europaea* and *Nm. eutropha*. The distribution of

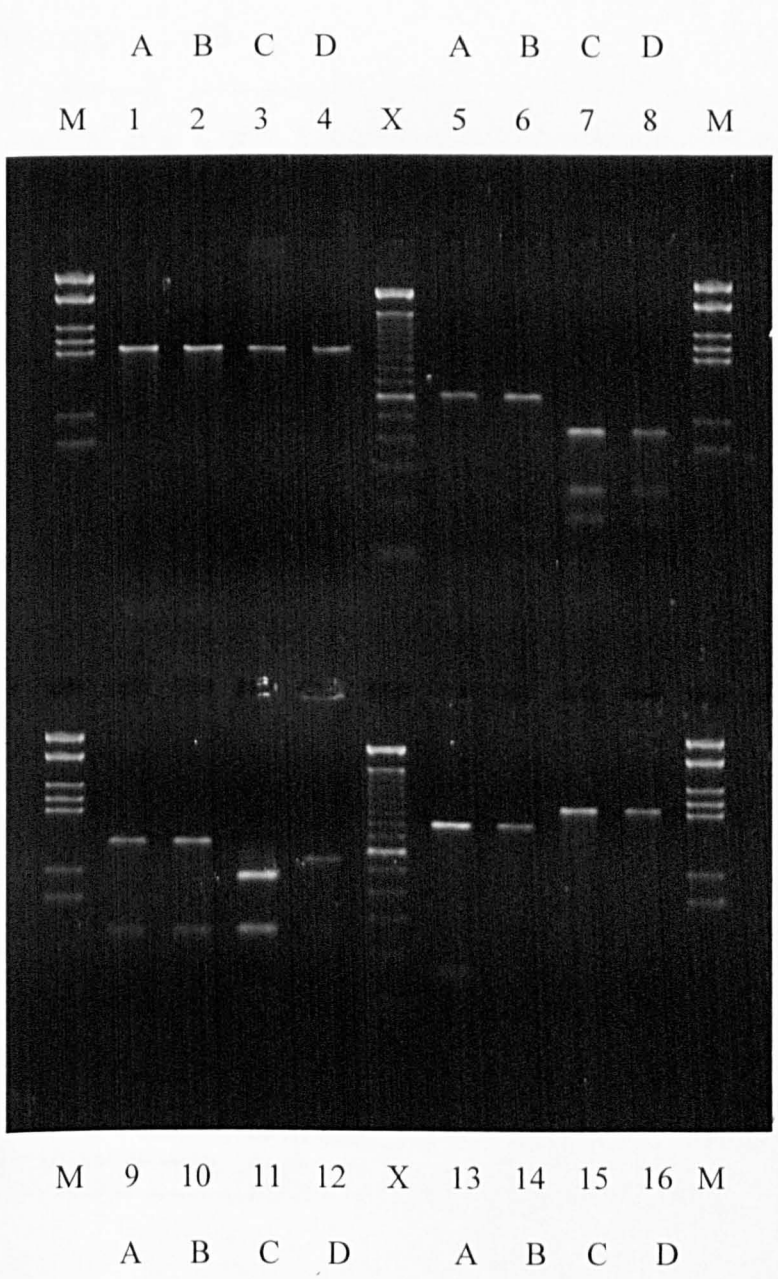


Fig. 5.6. Agarose gel electrophoresis demonstrating the restriction patterns of cloned 16S rDNA from Buttermere digested with *HaeIII* (lanes 5-8), *HinfI* (lanes 9-12), *EagI* (lanes 13-16), uncut DNA (lanes 1-4).

Lanes A: clone LV3K, lanes B: clone LV3, lanes C: clone PV2P, lanes D: clone PV3K. M MBI molecular weight marker, 21. X 100bp ladder GibCo.

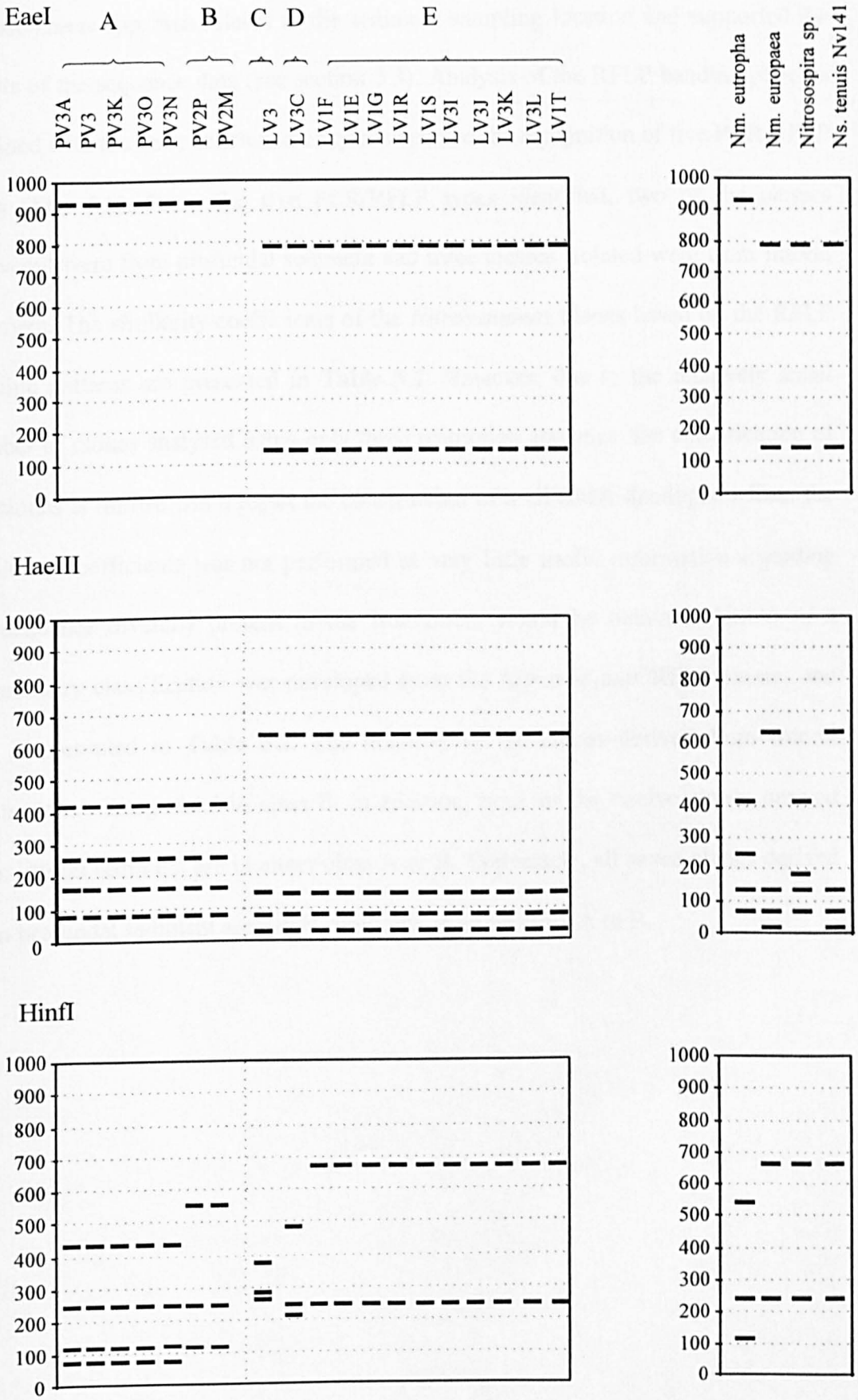


Fig. 5.7. Schematic diagram of the RFLP patterns produced from cloned ammonia-oxidiser 16S rDNA sequences from Buttermere.

Reference RFLP patterns for published ammonia-oxidiser 16S rDNA sequences are also presented.

Nitrosomonas spp. was related to the sediment sampling location and supported the results of the sequence data (see section 5.3). Analysis of the RFLP banding patterns obtained with the three restriction enzymes enabled the recognition of five PCR/RFLP types (**Fig. 5.6**). From the five PCR/RFLP types identified, two of the classes recovered were from profundal sediment and three classes isolated were from littoral sediment. The similarity coefficients of the *Nitrosomonas* clones based on the RFLP banding patterns are presented in **Table 5.1**. However, due to the relatively small number of clones analysed using only three restriction enzymes, the classification of the clones is limited. As a result the construction of a UPGMA dendogram from the similarity coefficients was not performed as very little useful information regarding the sequence diversity present in the Buttermere would be obtained. However, a rudimentary classification was developed from the *Nitrosomonas* RFLP classes and this is presented in **Table 5.2**. The majority of the clones derived from littoral sediment are categorised in class E. In addition, none of the twelve clones derived from littoral sediment are in either class A or B. Conversely, all seven clones derived from profundal sediment are categorised only in either class A or B.

	A	B	C	D	E
A		**82.4**	**11.1**	**22.4**	**23.5**
B	14/17		**11.8**	**23.5**	**25.0**
C	2/18	2/17		**66.7**	**70.6**
D	4/18	4/17	12/18		**82.4**
E	4/17	4/16	12/17	14/17	

Table 5.1 Classification and derivation of similarity coefficients[a] of *Nitrosomonas* clones based on RFLP band patterns.

Lower half: number of restriction fragments shared by pairs of isolates (before slash) and the total number of fragments observed for the two isolates in each pair (after slash). Upper half (bold): genetic distances (s, substitutions per site) for each pairwise strain comparison.

[a] Similarity coefficients were calculated by using the formula $s = 100\ (n_{xy}/n_x+n_y)$, where n_{xy} is the number of bands shared between a pair of isolates (x and y), and n_x and n_y are the number of bands strains x and y, respectively.

		Number of clones within each PCR/RFLP class				
Sediment	**Total**	**A**	**B**	**C**	**D**	**E**
Profundal	7	5	2	-	-	-
Littoral	12	-	-	1	1	10

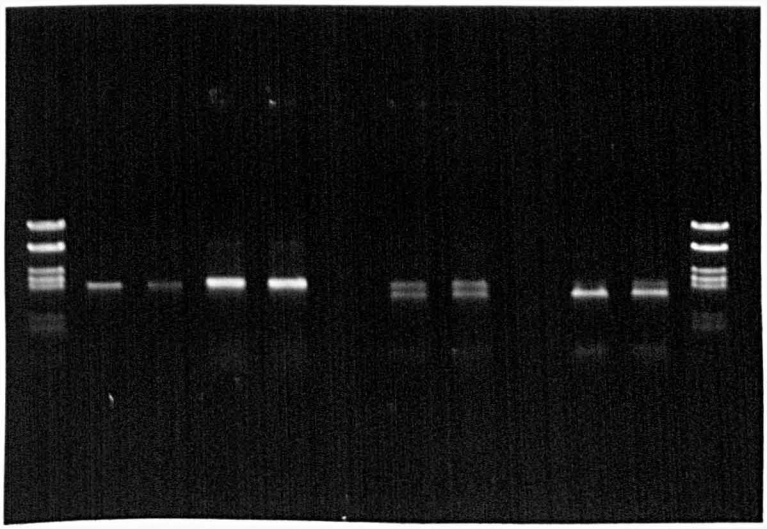
Table 5.2 Distribution of *Nitrosomonas* RFLP classes present in sediment.

5.3.2. Quantification of *Nitrosomonas* spp. derived from sediment.

The quantification of the 16S rDNA amplified from *Nm. europaea* and *Nm eutropha* from sediment and lakewater would provide information as to the relative predominance of each species in each environment. The restriction enzyme *EagI* differentiates *Nm. europaea* and *Nm. eutropha* on the basis of a single restriction site as demonstrated in section 5.3.1 *Nm. eutropha* does not contain the restriction site for *EagI* and therefore remains uncut whereas *Nm. europaea* is cut into two fragments (794 bp and 138 bp). On this basis, *EagI* was applied to the *Nitrosomonas* spp. PCR amplification products obtained in this study in an attempt to quantify the ratio of the two *Nitrosomonas* spp. in sediment.

Quantification was performed in a preliminary analysis using known concentrations of template DNA from *Nm. europaea* and *Nm. eutropha* mixed together in different ratios and digested with the restriction enzyme *EagI*. In **Fig. 5.8** the relative intensity of the banding pattern obtained in relation to the ratio of DNA digested and stained with ethidium bromide is presented. The digested products were probed with 10pmol. of the oligonucleotide probe AAO258. Replicate blots were quantified using a scanning densitometer (Molecular Dynamics phosphoimager) and the image analysis was carried out by ImageQuant version 3.0. The intensity of the signal also correlated with the increase in DNA concentration loaded. **Table 5.3** presents the data obtained from the scanning densitometry analysis.

When mixed at a 1:1 ratio, the two DNA templates produced bands of equivalent signal intensity. When mixed at a ratio of 3:1 (*Nm. europaea*: *Nm. eutropha*), the observed intensity of each band increased to a value within 25% of the predicted values. When the template DNA was mixed in a 3:1 ratio (*Nm. eutropha* :



M 1 2 3 4 5 6 7 8 M

Fig. 5.8. Agarose gel electrophoresis of mixtures of *Nitrosomonas* spp. digested with *EaeI*.

Lane 1: uncut *Nitrosomonas europaea* DNA, lane 2: uncut *Nitrosomonas eutropha* DNA, lane 3 & 4 *Nitrosomonas europaea* : *Nitrosomonas eutropha* 1:3, lane 5 & 6 *Nitrosomonas europaea* : *Nitrosomonas eutropha* 1:1, lane 7 & 8 *Nitrosomonas europaea* : *Nitrosomonas eutropha* 3:1. M: MBI molecular weight marker, 21.

DNA	Mean Pixel Intensity (A)	Mean Pixel Intensity (B)
Nm.europaea (uncut)	4.4	2.8
Nm.eutropha (uncut)	3.8	4.4
Nm.europaea (3X)	10.0 (± 2.305)	8.1 (± 1.272)
Nm. eutropha (1X)	3.3 (± 0.035)	4.2 (± 0.775)
Nm.europaea (1X)	3.8 (± 0.378)	3.0 (± 0.373)
Nm. eutropha (1X)	4.1 (± 0.551)	3.5 (± 1.427)
Nm.europaea (1X)	4.3 (± 0.190)	4.1 (± 0.355)
Nm. eutropha (3X)	11.5 (± 1.006)	12.0 (± 0.743)

Table 5.3. Mean pixel intensity produced by scanning densitometry analysis of duplicate autoradiographs (A & B) after overnight hybridisation at 55°C with 10 pmol. oligonucleotide probe AAO258. DNA blotted in duplicate series on each membrane and arithmetic means are shown (± standard deviations).

Nm. europaea), the observed band intensity increased to within 10% of the expected value. The data were reproducible as analysis of duplicates produced relative band intensities approximate to the expected values (**Table 5.3**).

Following this preliminary analysis, an attempt was made to quantify the relative ratio of *Nm. europaea* and *Nm. eutropha* from nested PCR-amplified DNA extracted from the littoral and profundal sediments sampled from Buttermere. The 16S rDNA was digested with the restriction enzymes *HaeIII*, *HinfI* and *EaeI* and the RFLP patterns produced are presented in **Fig. 5.9**. The RFLP patterns obtained from both littoral sediments was inconclusive. However, following digestion, the 16S rDNA derived from profundal sediment sampled in August and September produced *Nm. eutropha* RFLP patterns; and that of littoral sediment sampled in July produced *Nm. europaea* RFLP patterns (**Fig. 5.9**). This suggested that *Nm. eutropha* was predominant over *Nm. europaea* in profundal sediment and conversely that *Nm. europaea* was predominant over *Nm. eutropha* in littoral sediment. This corroborated the sequence data and the previous RFLP data obtained from the *Nitrosomonas* clones (section 5.3).

Both the profundal sediment samples which were classified as *Nm. eutropha* (**Fig. 5.9.**, lanes 1 & 2, B-D) and the RFLP patterns for the littoral sediment sample sampled in August and classified as *Nm. europaea* were unequivocal. However, the RFLP patterns for the littoral sediment samples sampled in September could not be definitely assigned to *Nm. europaea* and suggested that either a partial digestion of the DNA had occurred (**Fig. 5.9.**, lanes 3 B-D) or that *Nm. eutropha* 16S rDNA was also present. The five bands (630, 430, 260, 170 and 70bp) produced following restriction digestion with *HaeIII* (**Fig. 5.9.**, lanes 3 B) are consistent with the RFLP pattern

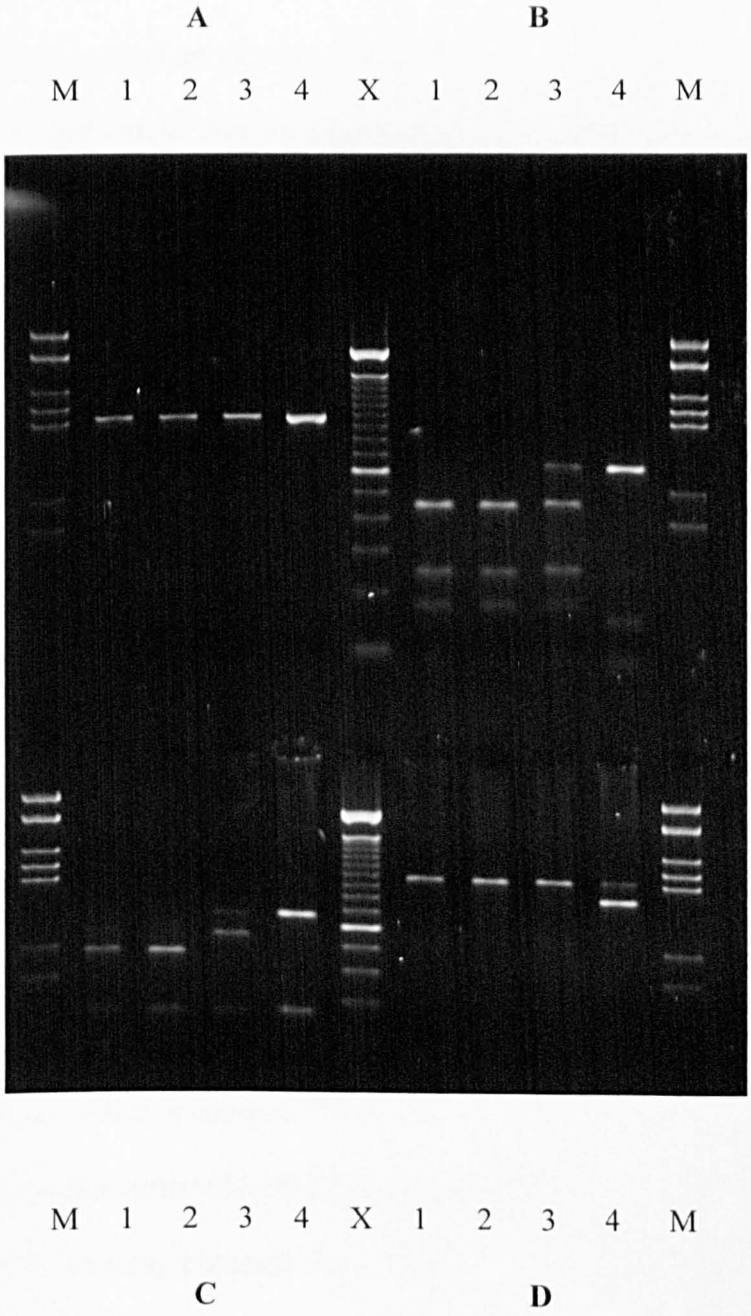


Fig. 5.9. Agarose gel electrophoresis of *Nitrosomonas* PCR amplification products from Buttermere digested with *HaeIII*, *HinfI* and *EaeI*.

Lane 1: DNA taken from profundal sediment in September, lane 2: DNA taken from profundal sediment in August, lane 3: DNA taken from littoral sediment in September, lane 4 : DNA taken from littoral sediment in July. A: uncut DNA, B: digested with *HaeIII*, C: digested with *HinfI*, D: digested with *EaeI*. M: MBI molecular weight marker, 21. X: 100bp DNA mass ladder, GibCo.

obtained from a mixture of *Nm. eutropha* and *Nm. europaea*. Restriction digestion with *HaeIII* and unique *Nm. europaea* DNA produces four bands (620, 150, 90 and 20bp) (**Fig. 5.9.** lane 4, B; **Fig. 5.6.** lanes 1 & 2, B). Furthermore, the PCR-amplified DNA from littoral sediment sampled in September also exhibited a unique RFLP pattern when digested with *HinfI* (**Fig. 5.9.** lane 3, C). Again a partial digest may have occurred, but the RFLP pattern suggests that the DNA may be a mixture of *Nm. europaea* and *Nm. eutropha* (RFLP class B, section 5.2.3). Three bands were visible on the gel (two bands of 690 bp & 240 bp) consistent with *Nm. europaea* RFLP pattern and a third band (550 bp) consistent with *Nm. eutropha* (RFLP class B) RFLP pattern.

In addition, the RFLP pattern observed with the PCR-amplified DNA from littoral sediment obtained in September following digestion with *EaeI*, produced a faint band of the expected size (794 bp). An uncut band suggested a partial digest may have occurred. In addition a fraction of the DNA may have contained no *EaeI* restriction site which is consistent with *Nm. eutropha* DNA (**Fig. 5.9**, lane 3, D). The RFLP pattern demonstrated by this DNA does not corroborate either the sequence data or the RFLP patterns obtained from unique nitrosomonad DNA. When the PCR-amplified DNA from littoral sediment obtained in August was digested with *EaeI*, a band of approximately 790 bp was generated but also a faint band representing uncut DNA was produced. Again this suggests that a partial digest had occurred. Although a small fraction of *Nm. eutropha* DNA may also be present in this sample, the RFLP patterns obtained using the other two restriction enzymes (*HaeIII* and *HinfI*) are consistent with that of unique *Nm. europaea* DNA. Therefore data depend on the fact that all the digests have proceeded to completion. Although quantification of the

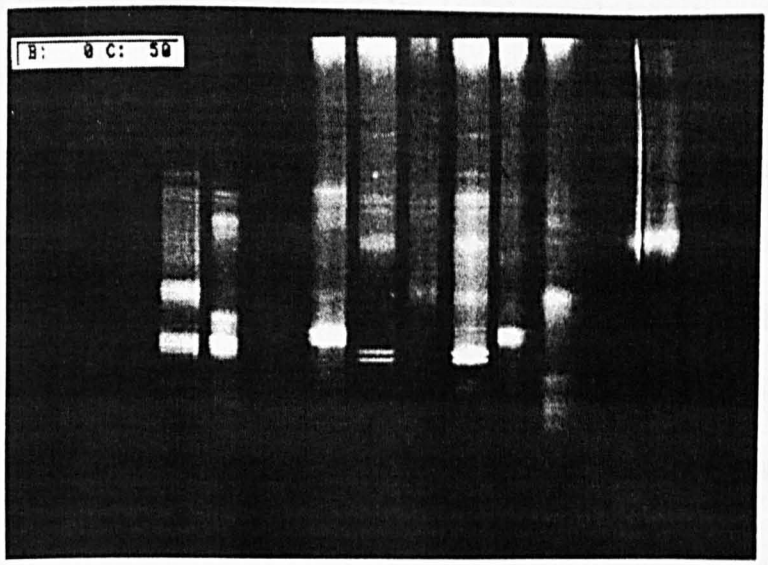
bands by scanning densitometry was inconclusive, the mean signal intensity obtained for the digested products was quantified as <25% of the uncut DNA for the minor bands obtained.

5.4. Genetic Diversity of ammonia-oxidiser bacteria from Buttermere determined by DGGE & TGGE.

The spatial and temporal diversity of ammonia-oxidising bacteria present in Buttermere were investigated by DGGE and TGGE analysis. The community structure of ammonia-oxidising bacteria was analysed either by sequencing of the individual bands or by electroblotting and hybridisation with a specific *Nitrosomonas* spp. oligonucleotide probe NmoCl7_439 designed by Stephen *et al.* (1998).

Degenerate primers designed by Kowalchuk *et al.* (1997) which incorporated a 5' GC clamp were used to amplify a 465 bp region spanning the V2 & V3 variable domains on the 16S rRNA gene. The primers were designed to recover 16S rDNA sequences from the β-sub-group ammonia-oxidising bacteria. The primers were applied to amplify 16S rDNA sequences from the DNA extracted from Buttermere samples. The products were analysed by DGGE using the method described by Muyzer *et al.* (1993) in collaboration with Dr. J. Stephen at the University of Aberdeen. The DNA electrophoresis profiles revealed that many of the 16S rDNA sequences could be separated and co-migrated with a number of ammonia-oxidiser like sequences isolated from gene libraries constructed from cloned DNA samples derived from sand dunes (Stephen *et al.*, 1996). The electrophoretic profiles for Buttermere are presented in **Fig. 5.10A**. A schematic diagram of the banding patterns obtained in (**Fig. 5.10A**) is also presented in (**Fig. 5.10B**).

A



B

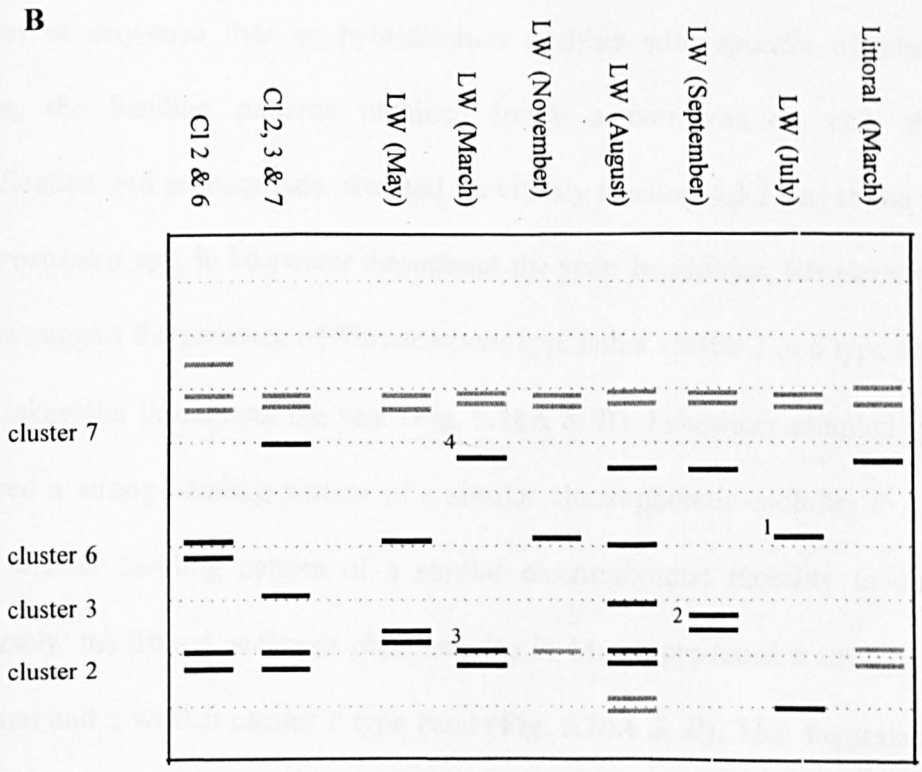


Fig. 5.10. DGGE of 16S rDNA from Buttermere samples with representative clones (**A**). Schematic diagram of the DGGE bands obtained and showing the bands which were excised for sequence analysis (**B**).

LW denotes lakewater samples and littoral denotes littoral sediment sample. Bands marked grey were faint on the DGGE gel. Cluster associations (Stephen *et al.* 1996) are as follows:
Nitrosospira spp., (Cluster 2); *Ns. tenuis* & *Ns. briensis* lineage, (Cluster 3);
Nm. ureae & *Nm. marina*, (Cluster 6); *Nm. europaea* & *Nm. eutropha* lineage, (Cluster 7).

The greatest number of bands and therefore probably the greatest sequence diversity was produced from lakewater (14m depth) sampled in August. The bands obtained correspond to the ammonia-oxidiser sequence clusters as described in Stephen *et al.* (1996). A total of five bands of equivalent electrophoretic mobility to the bands corresponding to sequences from clusters 7, 6, 3 and 2 were observed **(Fig. 5.10A & B)**. Lakewater obtained in November produced the lowest number of bands with one band of equivalent electrophoretic mobility to the band corresponding to sequences from *Nitrosomonas* spp. present in cluster 6. Possible *Nitrosospira* spp. either cluster 2 or 3 type sequences were observed in lakewater from Buttermere throughout the year **(Fig. 5.10A & B)**. Although this is not unequivocal without the support of sequence data or hybridisation analysis with specific oligonucleotide probes, the banding patterns obtained imply a corroboration with the PCR amplification and probing data obtained previously (section 4.3.2.) as to the ubiquity of *Nitrosospira* spp. in lakewater throughout the year. In addition, the electrophoretic profiles suggest the presence of *Nitrosomonas* spp. either cluster 7 or 6 type sequences in the lakewater throughout the year **(Fig. 5.10A & B)**. Lakewater sampled in March produced a strong banding pattern of a similar electrophoretic mobility to cluster 2 and a weaker banding pattern of a similar electrophoretic mobility to cluster 7. Conversely, the littoral sediment obtained also in March produced a strong cluster 7 type band and a weaker cluster 2 type band **(Fig. 5.10A & B)**. This suggests that the relative numbers of different ammonia-oxidising bacteria genotypes varied between the sediment and the lakewater in response to time.

The number of bands generated by DGGE or TGGE may not accurately reflect the number of sequences present in a mixture of PCR products. Identity of sequences

cannot be reliably inferred on the basis of co-migration. Additional data were required to assign bands to recognised sequence clusters. Four bands (1-4 **Fig. 5.10B**) from the DGGE gels of the Buttermere samples were excised and re-amplified for sequencing. Analysis of the sequences recovered by FASTA searches of GenBank revealed that the four excised bands could be identified and were related to β-subdivision ammonia-oxidiser 16S rDNA (>93% homology). This gave additional support to the data obtained by Kowalchuk *et al.* (1997) as to the specificity of the CTO primer pair for ammonia-oxidiser sequences. The sequences recovered had a high degree of homology to the sequences of the reference clones from the clusters defined by Stephen *et al.* (1996), which demonstrated relative mobilities to bands from PCR-amplified DNA from the environment. Band 3 demonstrated 95% sequence homology to cluster 2 *Nitrosospira* spp. The sequence obtained from band 2 had 99% homology to cluster 3 and band 1 had 93% homology to cluster 6, (*Nm. ureae*). A strong similarity was produced between the recovered band 4 and the corresponding region in cluster 7; *Nm. europaea*-lineage and exhibited 97% and 95% homology to *Nm. europaea* and *Nm. eutropha* respectively. Co-migration of bands does not neccessarily indicate sequence identity using DGGE, as heterogeneous sequences can exhibit equivalent electrophoretic mobilities depending on the conditions applied during electrophoresis. However, all four bands sequenced here confirmed very strong homology between the co-migrating bands.

The development of TGGE was performed to analyse the environmental DNA samples derived from Buttermere. A temperature gradient between 43-60°C produced banding patterns showing a good separation of sequences (**Fig 5.11.**). In **Fig. 5.11.**, lanes 1 & 2 show the electrophoretic mobilities of 16S rDNAs amplified from

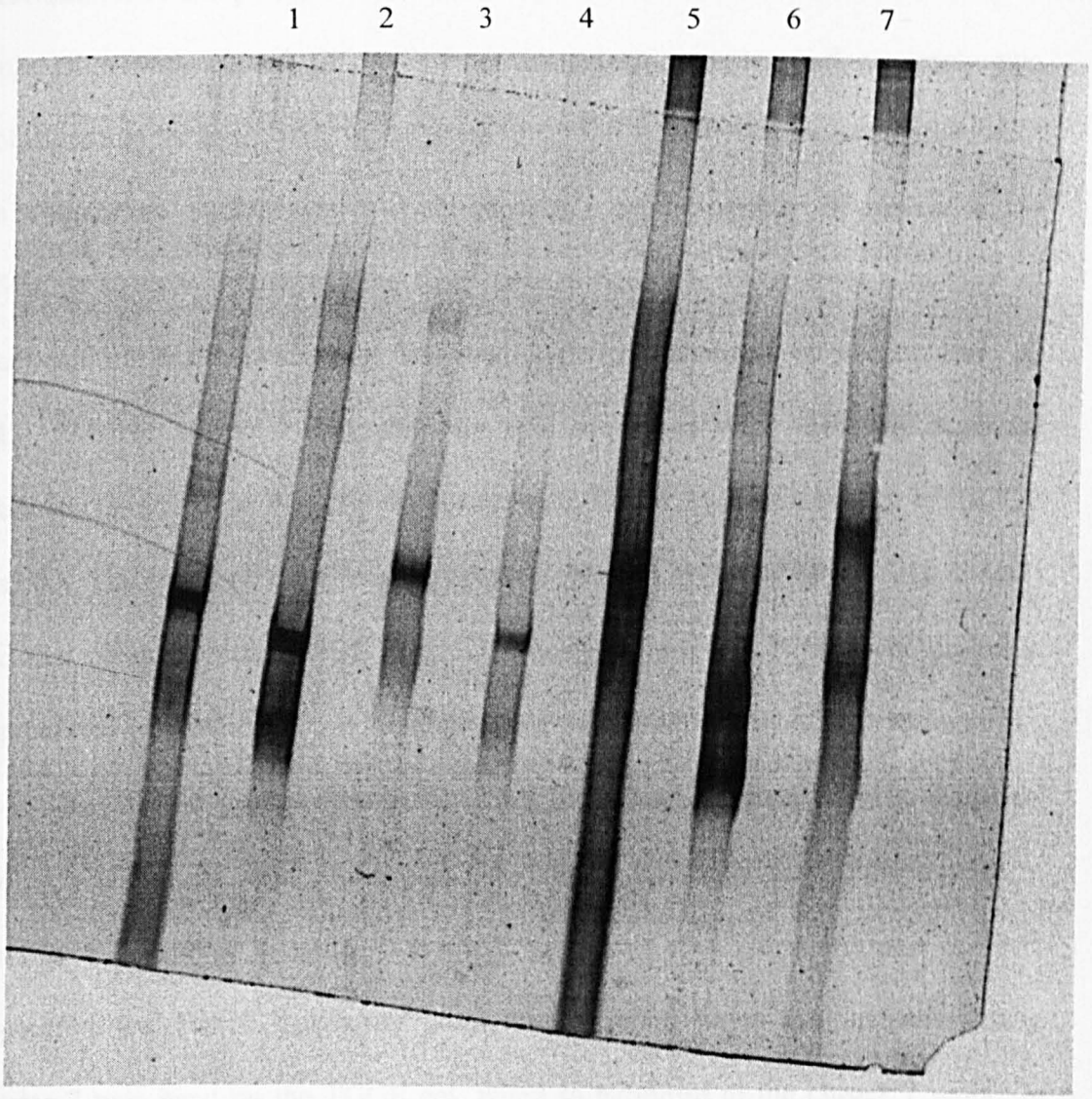
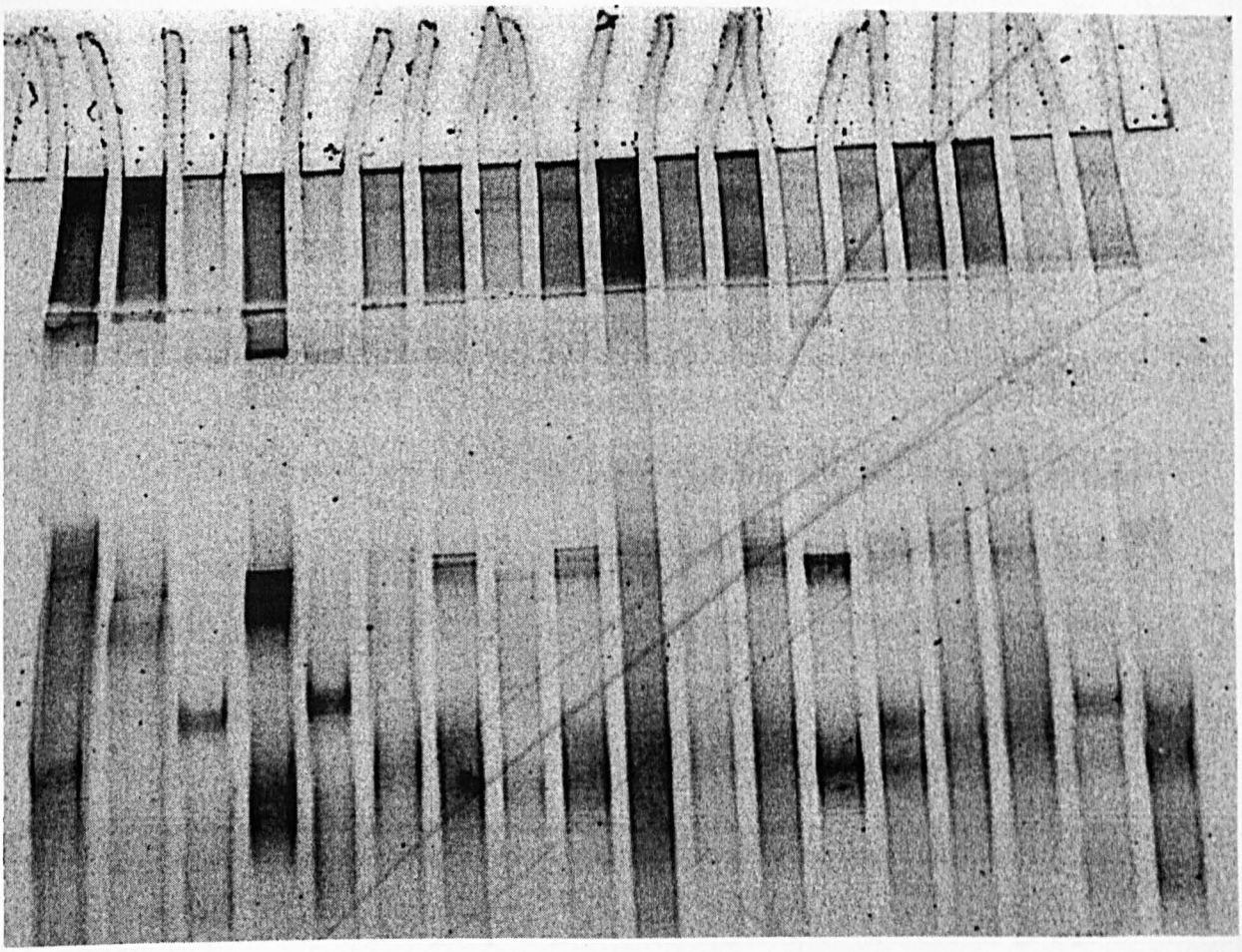


Fig. 5.11 Development of TGGE using 16S rDNA from cloned sand dune DNA (Stephen *et al.*, 1996). Includes two 16S rDNA samples obtained from Buttermere for comparison.

(lane 1: lakewater obtained in November; lane 2: profundal sediment obtained in May: lane 3: *Nitrosomonas* spp. (cluster 6); lane 4: *Nitrosospira* spp. (cluster 2); lane5: *Nitrosospira* spp. (cluster 3); lane 6: *Nitrosospira* spp. (cluster 4); lane 7: *Nitrosomonas* spp. (cluster 7).

lakewater DNA sampled in November and profundal sediment obtained in May in conjunction with cloned DNA. Both environmental samples had electrophoretic mobilities similar to cluster 2 type (*Nitrosospira* spp.). Following the determination of the temperature gradient which would generate a good separation of sequences, the TGGE analysis was then applied to PCR-amplified 16S rDNA from a number of lakewater and sediment samples. The banding patterns obtained are presented in **Fig. 5.12.** Possible cluster 7 *Nitrosomonas* spp. sequences were observed from the lakewater sampled in August, September and March as demonstrated by DGGE analysis. The electrophoretic banding profiles obtained by TGGE (**Figs. 5.11 & 5.12**) and that obtained using DGGE (**Fig. 5.10**) using the same PCR products, produced comparable separation patterns, demonstrating the reproducibility of both techniques.

The TGGE gel was electroblotted and probed using a cluster 7 (*Nitrosomonas* spp.)-specific probe (data not shown). The bands which hybridised corresponded to samples of lakewater taken in August and September and littoral sediment taken in September and March. Lakewater taken in November, which demonstrated a faint cluster 7 type band on the TGGE gel, failed to hybridise to the cluster 7 probe. In addition, the TGGE banding profile of the DNA taken from the littoral sediment in July, failed to produce clear banding patterns. The electrophoretic profiles obtained from both DGGE and TGGE revealed more than one band from a unique starting template (cloned DNA *i.e.* clusters 2, 3, 4, 6 & 7). This may be due to the formation of heteroduplexes between different sequence types (Muyzer *et al.*, 1993). In addition, the forward primer consists of three different sequences which may cause primer ambiguities and be a source of the double bands observed in the electrophoretic patterns (**Fig. 5.11 & 5.12**).



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Fig. 5.12 TGGE of 16S rDNA samples obtained from Buttermere samples with representative cloned sand dune DNA (Stephen *et al.*, 1996) for comparison.

(lane 1:*Nitrosospira* spp., (cluster 2); lane 2: *Nitrosomonas* spp., (cluster 6); lane 3: *Nitrosospira* spp., (cluster 3); lane 4: *Nitrosomonas* spp., (cluster 7); lane 5: *Nitrosospira* spp., (cluster 4); lane 6: lakewater (July); lane 7: lakewater (August), lane 8: lakewater (November), lane 9: lakewater (March), lane 10: littoral (July), lane 11: littoral (August), lane 12: littoral (September), lane 13; littoral (November), lane 14; littoral (March), lane 15: profundal (August), lane 16: profundal (September), lane 17: profundal (November), lane 18: profundal (May).

5.5. Discussion.

Nitrosomonas spp. have been identified from a variety of environments and enrichment cultures (Nejidat *et al.*, 1994; Hiorns *et al.*, 1995). Prior to this study, *Nm. europaea* and *Nm. eutropha* have not been directly detected from any freshwater lakes and in particular from an oligotrophic freshwater lake. The V3 region of the 16S rRNA gene was selected for phylogenetic analysis as it is a highly variable region which may provide sufficient information on the phylogenetic relationships of the *Nitrosomonas* clones obtained in this study. The *Nitrosomonas* PCR amplification products generated from the two littoral and two profundal sediment samples were confirmed by sequence analysis.

Phylogenetic analysis of the 16S rDNA sequences suggests the presence of two lineages dependent on the sediment location. One group of sequence homologues derived from littoral sediment grouped with *Nm. europaea* and the second group of sequence homologues derived from profundal sediment clustered with *Nm. eutropha*. The data suggest that the genotypes are location-specific and have adapted to the conditions present at each niche. The high bootstrap values of 92% (based on 345 bp) supported the position of the two groups. The bootstrap value increased to 100% based on the longer sequence of 701 bp. Since the clones have not been sequenced along their full length, the degree of sequence divergence and branching order within each group is difficult to ascertain on the basis of the partial sequences. None of the partial sequences analysed was 100% homologous to any of the published sequences from cultured ammonia-oxidising bacteria. This suggests that, not surprisingly, the limited number of published sequences from cultured ammonia-oxidisers may not

represent the β-subdivision ammonia-oxidisers present in Buttermere. Similar findings were also previously demonstrated in soil (Stephen *et al.*, 1996).

Previously published molecular and culture-based investigations suggested that laboratory culture favoured *Nitrosomonas* spp. over *Nitrosospira* spp. (Belser, 1978; McCaig *et al.*, 1994; Hiorns *et al.*, 1995). McCaig *et al.* (1994) obtained partial sequences from marine sediment enrichments and revealed new lineages related to *Nitrosomonas* rather than *Nitrosospira* spp. However, in the study described here, a seasonal occurrence of *Nitrosomonas* spp. was demonstrated by direct PCR amplification. In addition, due to the high specificity of the combination of oligonucleotide primers and probe, new lineages of *Nitrosomonas* sequences could not be identified. The study also could not provide information on the contribution of the *Nitrosomonas* species on nitrification in Buttermere and the recovery of 16S rRNA gene sequences provides no indication of *in situ* cellular activity.

The RFLP analysis provided a robust strategy for screening the clones prior to sequencing and confirmed the sequence divergence obtained. This study is the first to demonstrate that single unique nitrosomonad DNA could be differentiated into either *Nm. europaea* or *Nm. eutropha* on the basis of a single restriction site. RFLP analysis using cloned *Nitrosomonas* DNA corroborated the sequence data as to the ecological site-specific dominance of either *Nm. europaea* or *Nm. eutropha*. Furthermore, the *Nitrosomonas* clones could be separated into five RFLP classes consisting of *Nm. eutropha* in class A & B and *Nm. europaea* in classes C-E. In addition, *Nitrosospira* spp. could also be discriminated using three restriction enzymes. Other studies have applied PCR/RFLP analysis to analyse the sequence diversity of bacterial populations in the environment (Bruce *et al.*, 1992; Navarro *et al.*, 1992; Osborn *et al.*, 1993;

Ward, 1995; Bruce, 1997). Ward (1995) identified intra- and inter-site diversity of sequences from isolates of aquatic denitrifying bacteria by RFLP analysis. In a similar study, Navarro *et al.* (1992) characterised populations of *Nitrobacter* from soils and a freshwater lake. The study applied PCR/RFLP analysis to DNA sequences from the intergenic spacer region (IGS) and suggested that the diversity of *Nitrobacter* populations that coexisted in the same niches were genetically divergent. In a recent study, the IGS region of ammonia-oxidising bacteria was determined and applied in a phylogenetic analysis of twelve isolates (Aakra *et al.*, 1999). The study applied ribotyping of the 16S and 23S rDNA from the twelve isolates of ammonia-oxidising bacteria and revealed the presence of only one *rrn* operon per genome compared to 5-10 copies of the rRNA genes per genome in most bacteria (Aakra *et al.*, 1999). It has been suggested that the presence of only one set of rRNA genes is related to the slow growth demonstrated by ammonia-oxidising bacteria (Aakra *et al.*, 1999).

Quantification of the ratio of *Nm. europaea* and *Nm. eutropha* was successful, however, when applied to mixtures of amplicons derived from environmental samples quantification was inconclusive. A number of biases are introduced by the DNA extraction, PCR amplification and restriction enzyme digestion. The minor bands observed from the littoral sediment samples which corresponded to RFLP patterns consistent with *Nm. eutropha* may be due to the presence of less-dominant *Nm. eutropha* derivatives within the littoral sediment samples, that were not recovered by the cloning and sequence analysis. In addition the bands may be a result of partial digestion by the restriction enzymes. Therefore, RFLP analysis performed on mixtures of PCR products taken from the environment should be approached with caution. It is possible that cloning and sequence analysis may misrepresent the sequence diversity

present in Buttermere due to sub-optimal cloning procedures, and less dominant gene sequences present in low numbers may evade isolation in a mixture of PCR amplicons. However, analysis of a statistically significant number of clones and longer sequences would reduce the bias and therefore less dominant genotypes present in populations may be identified.

A qualitative assessment of the presence or absence of amplicons throughout a sampling year may be indicative of the changing population structure of ammonia-oxidisng bacteria within the environment. The high degree of specificity of the *Nitrosomonas* primers (Nm75 and Nm1007*) used in this study has been discussed previously in Chapter 4. Other *Nitrosomonas* species in addition to *Nm. europaea* and *Nm. eutropha* may also be present in Buttermere. Therefore, oligonucleotide primers designed by Kowalchuk *et al.* (1997) were also applied to the same template DNA derived from Buttermere in conjunction with DGGE and TGGE analysis to assess the presence and absence of ammonia-oxidising bacteria that were not detected using the Hiorns *et al.* (1995) primers.

The application of PCR-TGGE or -DGGE analysis facilitates comparison of the microbial community structure of environmental samples (Muyzer *et al.*, 1995a; Teske *et al.*, 1996; Heuer *et al.*, 1997; Ferris *et al.*, 1997). Both techniques facilitate the detection of predominant populations which are PCR amplifiable and, as bands can be sequenced directly, the biases incurred from cloning can be eliminated. The sequences obtained from all the excised bands from the DGGE gels were closely related to the β-subdivision ammonia-oxidising bacteria from the representative clones that demonstrated equivalent mobilities. The data presented in this study support the evidence obtained by Kowalchuk *et al.* (1997) as to the specificity of the

CTO primer pair. Sequence data from the excised bands confirmed the presence of both *Nitrosomonas* and *Nitrosospira* spp. present in lakewater. The sequence obtained from band 4 was 93% homologous to *Nm. ureae* and suggests the presence of other *Nitrosomonas* spp., in addition to *Nm. europaea* and *Nm. eutropha*, in Buttermere. Speksnijder *et al.* (1998) also reported the recovery of sequences related to *Nm. ureae* from freshwater samples.

Differences in the ammonia-oxidising bacterial populations were demonstrated both spatially and temporally. The DGGE patterns obtained for lakewater taken in March demonstrated a weak cluster 7 type band corresponding to the *Nm. europaea-eutropha* lineage. In addition, a strong band corresponding to *Nitrosospira* spp. was also obtained. By comparison, the electrophoretic profile obtained from the littoral sediment taken in March, demonstrated a strong cluster 7 type band corresponding to the *Nm. europaea-eutropha* lineage. A weak band corresponding to *Nitrosospira* spp. was also obtained. Kowalchuk *et al.* (1997) and Stephen *et al.* (1998) demonstrated differences in the sequences from DGGE bands obtained from soils that differed either in pH or salinity. This adds support to the evidence that sub-populations of ammonia-oxidising bacteria have adapted to tolerate the conditions that are present at individual niches.

Other studies have applied DGGE or TGGE to demonstrate species diversity in the environment (Muyzer *et al.*, 1993; 1994; Wawer & Muyzer, 1995; Ferris *et al.*, 1996). Nübel *et al.* (1996) detected sequence heterogeneities in 16S rRNA genes from individual strains of *Paenibacillus polymyxa* by TGGE analysis. In another study, the microbial mat community of Octopus Spring, Yellowstone National Park was analysed (Ferris *et al.*, 1996). DGGE and sequence analysis identified a spatial

heterogeneity of cyanobacterial populations between sites of different temperature ranges and suggested that adaptation to temperature had occurred among the cyanobacteria that were phylogenetically very similar (Ferris *et al.*, 1996). Both DGGE and TGGE offer the potential to analyse the diversity of bacterial populations present in different environments.

5.6. Conclusions.

- Sequence analysis of *Nitrosomonas* 16S rDNA from ammonia-oxidising bacteria present in sediment identified two lineages that related to sediment location; *Nm. eutropha* predominated in profundal sediment whilst *Nm. europaea* predominated in littoral sediment.

- PCR/ RFLP analysis of the *Nitrosomonas* clones confirmed the results of the sequence data and also identified five RFLP classes. PCR/ RFLP analysis offers the potential for identifying dominant genotypes in environmental samples.

- DGGE and TGGE analysis revealed a spatial and temporal diversity of 16S rDNA sequences from ammonia-oxidising bacterial populations present in Buttermere. Sequence analysis and oligonucleotide probing identified the presence of *Nitrosomonas* and *Nitrosospira* spp. in sediments and lakewater and confirmed the banding patterns obtained.

Chapter 6. Analysis of ammonia-oxidising bacteria by fluorescent *in situ* oligonucleotide probing and flow cytometry.

6.1. Introduction

Detection of active bacteria *in situ* would be ecologically informative in community analysis. A number of fluorescent dyes are available for measuring cell viability *e.g.* rhodamine 123 (Rh-123) (Porter *et al.*, 1996). Epifluorescence microscopy using nucleic acid (*e.g.* 4',6'-diamidino-2-phenylindole (DAPI)) or protein stains, permits a quantitative estimation of the size of bacterial populations (Fry, 1990).

Whole cell hybridisation using fluorescent 16S rRNA oligonucleotide probes provides a discriminatory detection system by targeting individual microorganisms (Manz *et al.*, 1993; Poulsen *et al.*, 1993; Amann *et al.*, 1995; Schramm *et al.*, 1996). Such probes have been applied to study a range of environments such as eutrophic freshwater lakes, activated sludges and biofilms (Amann *et al.*, 1992; 1998; Manz *et al*, 1993; Wagner *et al.*, 1993). In oligotrophic systems, only a small proportion of the bacterial populations have been detected using fluorescent eubacterial probes due to low cell wall permeability or low cellular ribosome content which may limit detection sensitivity (Amann *et al.*, 1995; Alfreider *et al.*, 1996). Attempts to increase signal intensity by the application of multiple probes has been demonstrated with samples from coastal waters (Manz *et al.*, 1993). When linked to confocal microscopy, image analysis and flow cytometric techniques, precise spatial information and quantification of cell numbers may be obtained without the extraction of ribosomal nucleic acid (Wagner *et al.*, 1995; Mobarry *et al.*, 1996).

Flow cytometry enables detection and quantification of single cells which can be sorted and subsequently cultivated (Porter *et al.*, 1993; 1996; Wallner *et al.*, 1995). Under optimum conditions, flow cytometry can rapidly analyse > 2000 cells sec^{-1} (Shapiro, 1995; Davey & Kell, 1996). Flow cytometry has predominantly been applied to enumerate microbial populations in aquatic environments (Porter *et al.*, 1997). Physical and chemical properties of individual cells can be measured on the basis of fluorescence emitted from specifically bound dyes and by light scatter. In this chapter, the application of flow cytometry in conjunction with fluorescent probes to assess the *in situ* activity of ammonia-oxidising bacteria in Windermere is described.

Flow cytometry requires cells to be delivered separately into the sensing region, therefore, any particulate matter presents difficulties due to cell clumping or the attachment of cells to aggregates (Wallner *et al.*, 1995). Particulate matter may also mask the signals obtained from individual cells. Standard procedures such as sonication or homogenisation of samples in a high speed blender have been employed to dissociate bacteria from solid particles and the addition of surfactants also increases desorption of cells from particulate matter and prevents reaggregation (Fry, 1990; Porter, 1996). The efficiency of bacterial cell removal was assessed and the optimum procedure was adopted for all the samples used in the study.

6.2. Results

6.2.1. Evaluation of whole-cell extraction methods from sediment.

A preliminary study was performed to optimise cell dissociation of *E. coli* from solid particles prior to analysis by microscopy and flow cytometry. A selection of extractants and extraction techniques were assessed following a review of the literature. Cell dispersal was recorded by the optical density at 550 nm and all cell suspensions were diluted ten-fold prior to analysis. The data are presented in **Fig. 6.1**.

The most effective extractants were Triton, Calgon and distilled water, which gave significantly greater dispersal than all of the other solutions ($p< 0.05$). Dispersal was also significantly affected by the extraction methods tested ($p< 0.05$). Cell dispersal from soil particles after gentle agitation for up to 30 mins was ineffective in all of the extractants tested (OD_{550} <0.104 ± 0.037). The addition of Chelex and glass beads improved cell dispersion and the results varied depending on extractant (between OD_{550} 0.361 ±. 0.05 for Ringer's solution and OD_{550} 1.643 ± 0.067 for Calgon). The results of blending the soil particles at full speed using a Polytron blender and the application of sonication gave the greatest cell dispersal and were uniformly effective with all buffers (OD_{550} ≤1.0) (with the exception of Ringer's solution). The use of blending was selected for the studies as it greatly reduced the number of large aggregates and this would be a prerequisite for the application of flow cytometry. Triton was finally selected as the extractant as it was consistently effective in cell dispersal in most treatments, particularly in conjunction with blending (and was not significantly lower than Calgon $p = 0.05$). Since Triton is a mild detergent, it may also facilitate cell permeabilisation and enhance entrance of the probes into the cells.

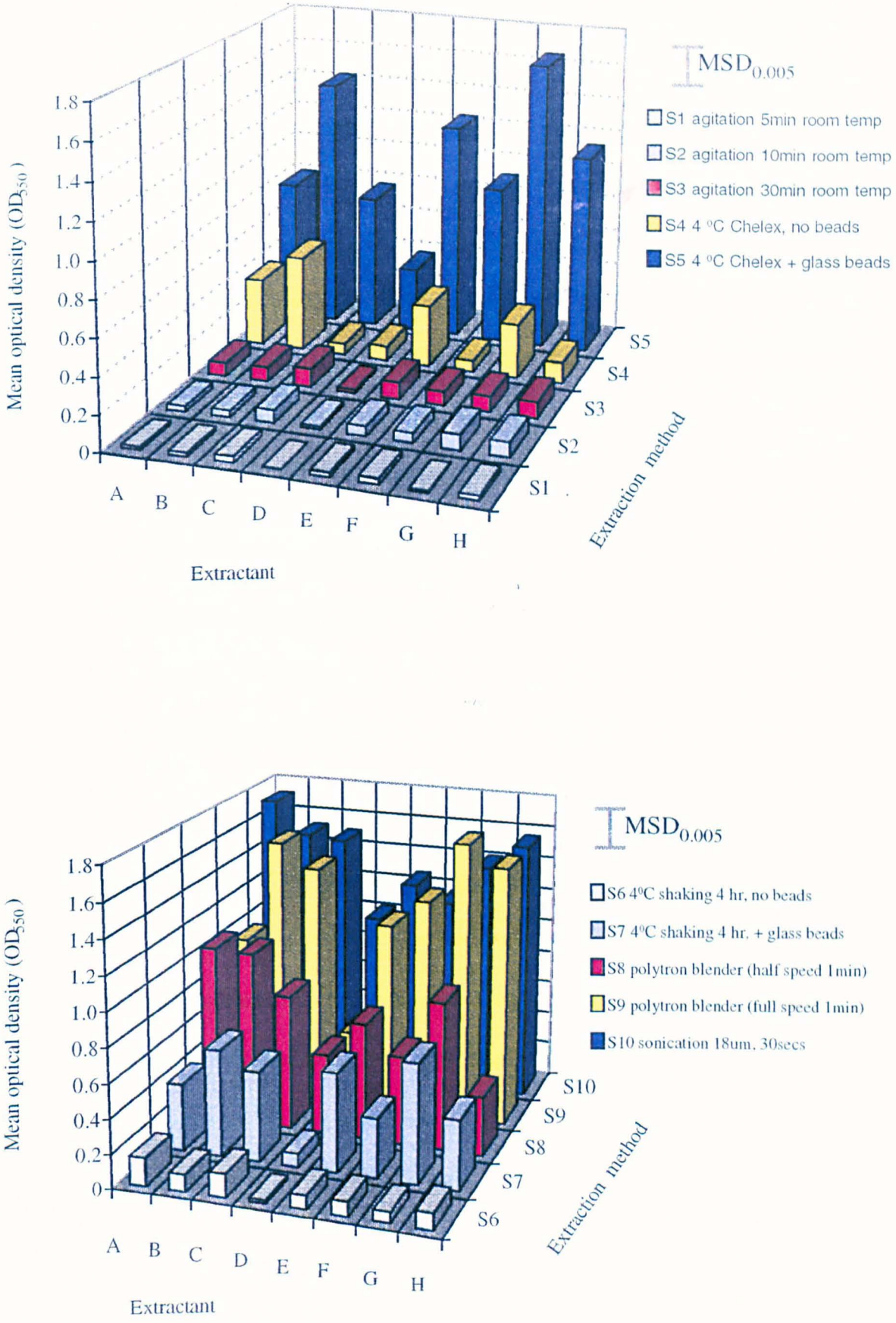


Fig. 6.1 Bar charts of cell dispersal using different extractants and extraction methods against mean optical density at 550 nm (OD_{550}). MSD: minimum significant difference.

Extractants as follows: sterile distilled water (A), Triton (B), Tris buffer (C), Ringer's solution (D),phosphate buffered saline (E), Tween 80 (F), Calgon (G), sodium cholate (H).

6.2.2. Efficiency of bacterial cell recovery from soil.

The numbers and metabolic activities of the bacterial populations present in soil after pretreatment by blending with Triton were examined by the application of fluorogenic dyes and microscopy in accordance with the recommendation of Fry (1990). DAPI was the fluorochrome used to enumerate 'total' bacterial populations. The number of metabolically active bacteria present after pretreatment was assessed by the application of CTC fluorogenic viability dye. Negative controls performed by the addition of formamide (2% final concentration) gave no visible fluorescence (data not shown).

Mid-exponential phase cultures of *E. coli* were inoculated into sterilised soil at a level of 1.5×10^8 CFU g^{-1} dry weight soil and blended in the Polytron blender for 60s in Triton. Triplicate CFU counts were taken before and after treatment. The mean recovery of cells after blending was 3.9×10^7 ($\pm$ 1.93) cfu g^{-1} dry weight soil compared to the mean pretreatment count of 5.0×10^6 ($\pm$ 1.43) cfu g^{-1} dry weight. Recovery efficiency was 17-41% greater by the application of blending, determined by CFU counts. Total bacterial counts were determined by staining with DAPI and filtering the soil suspension on to triplicate 0.2 μm pore diameter black membranes. At least 500 cells in different fields of view were enumerated. The mean recovery efficiency detemined by DAPI staining was 2.3×10^7 ($\pm$ 0.23) cells g^{-1} dry weight for blended compared to 4.8×10^6 ($\pm$ 2.01) cells g^{-1} dry wt. unblended soil. Repeated samples gave recovery efficiencies 14-18% greater by the application of blending, as determined by DAPI staining. Bacterial cell counts of the uninoculated soil blended were 6.5×10^3 cfu g^{-1} dry weight and 6.8×10^3 cfu g^{-1} dry weight unblended of indigenous bacteria.

Analysis of the viability of soil bacterial populations after treatment by blending was performed by staining the bacterial cells with the viability dye 5-cyano-2,3-ditoyl tetrazolium chloride (CTC). The reduction of tetrazolium indicates metabolic activity as it requires the presence of active enzymes involved in oxidative electron transport. The resolution of active bacteria *in situ* could be distinguished with minimal non-specific staining. All controls behaved accordingly, with no visible fluorescence obtained following the addition of formamide (data not shown). The mean recovery of viable cells prior to treatment was 7.4×10^5 ($\pm$ 2.95) cells g^{-1} dry weight soil. After treatment by blending the mean recovery of viable cells was 1.22×10^7 ($\pm$ 0.54) cells g^{-1} dry wt. soil. 15% of the recovered cells were viable prior to treatment compared to 52% viable cells recovered following blending. The number of viable cells recovered increased by 37% following treatment. A significant difference was observed between CTC microscopy and viable plate counts. The total number of CTC reducing cells was lower than the culturable fraction of the soil by three to six fold.

6.3. Detection of ammonia-oxidising bacteria using fluorescence *in situ* hybridisation.

FITC labelled probes that targeted the small-subunit 16S rRNA molecule were performed on laboratory cultures to assess the probe specificity. Optimisation with respect to time, hybridisation temperature, probe concentration and fixative concentration for cell permeabilisation had been previously determined (Hastings, 1996). The FITC labelled eubacterial probe Eub338 (Amann *et al.*, 1990), the *Nm. europaea-eutropha* probe (Nm75) and the *Nitrosospira* probe (Ns85) (Hiorns *et al.*, 1995) were applied. Probe specificities of Nm75 and Ns85 have been determined previously (Chapter 4). Late-exponential phase cells of *Nm. europaea* and *Ns. multiformis* were fixed in paraformaldehyde and hybridised with Eub338, Nm75 and Ns85. Samples were examined by fluorescence microscopy and results recorded photographically (**Fig. 6.2** and **Fig. 6.3A & B**).

Discrimination between the microbial cells was demonstrated. Oligonucleotide Nm75 hybridised specifically to *Nm. europaea* cells and Ns85 hybridised specifically to *Ns. multiformis* cells. Both probes demonstrated no non-specific binding. The eubacterial probe Eub338 hybridised to the strains of bacteria examined (*i.e. Ns. multiformis, Nm. europaea* and *E. coli*). Probe Eub338* hybridising to rDNA only, and control preparations without the fluorescent probes were used as negative controls and little or no fluorescence was observed (data not shown).

Following the assessment of probe specificity on laboratory cultures, the FITC-labelled probes were used to analyse ammonia-oxidiser populations present in sediment and lakewater samples derived from Windermere. Sediment and lakewater samples were blended in Triton and all cell suspensions were fixed in

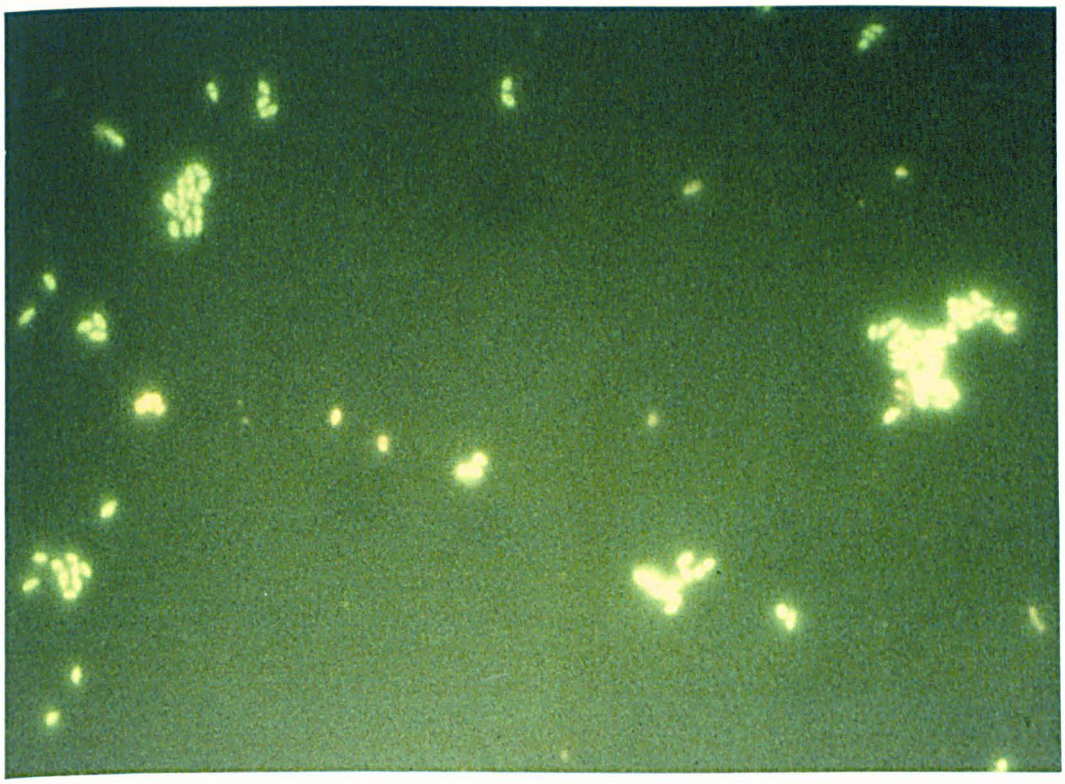


Fig. 6.2. Laboratory culture of *Nitrosomonas europaea* hybridised with FITC-labelled eubacteria oligonucleotide EUB338.

A Ns85

B Nm75

Fig. 6.3. Laboratory culture of *Nitrosospira multiformis* hybridised with FITC-labelled oligonucleotide Ns85 (**A**). Laboratory culture of *Nitrosomonas europaea* hybridised with FITC labelled oligonucleotide Nm75 (**B**).

paraformaldehyde. The particulate nature of the sediment made probe hybridisation difficult. Microscopic analysis of sediment and lakewater samples hybridised with Nm75 and Ns85 was inconclusive due to low cell numbers, high levels of background fluorescence and cells being masked by particulate matter (data not shown). Therefore, flow cytometry was applied to analyse the lakewater samples from Windermere in an attempt to circumvent problems of high background fluorescence and cells masked by particulate matter. The enrichment cultures of sediment and lakewater samples from Windermere would increase cell numbers, and therefore the hybridisation signals. However, *in situ* hybridisation analysis was not performed due to time constraints and the fact that these enrichments had been previously analysed by PCR amplification.

6.4. Analysis of Windermere lakewater by flow cytometry.

Flow cytometry was performed on lakewater samples obtained from Windermere to facilitate a rapid analysis of the predominant ammonia-oxidiser species present. The control oligonucleotide probes used for flow cytometry were those previously described for *in situ* hybridisation analysis (section 6.3) i.e. 5' end labelled with FITC of complementary sequence to EUB338 designated EUB338• (Manz *et al.*, 1993). This probe was applied to all lakewater samples and failed to give a significant fluorescent signal and demonstrated little non-specific binding. In addition, lakewater samples with no probe were also included as controls for background fluorescence. The background levels of fluorescence obtained were 5.5% (South Basin) and 5.0% (North Basin) of the total cells tested.

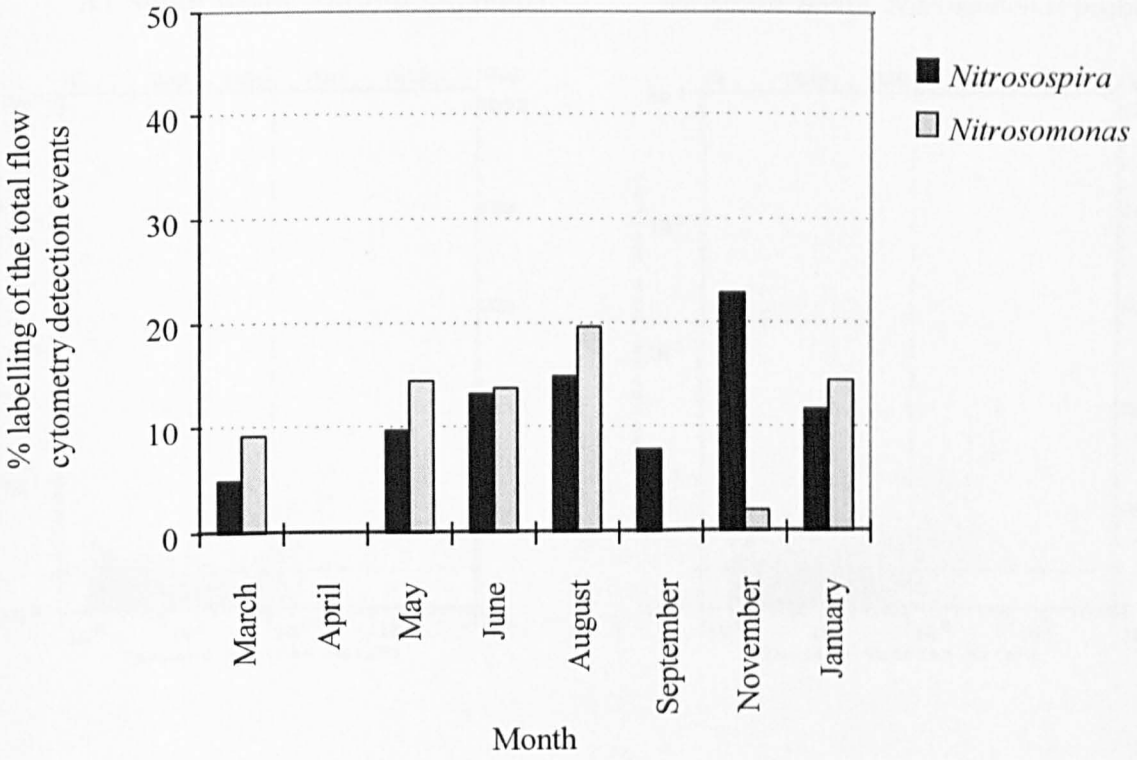
Concentrated lakewater samples (1litre) taken from Windermere South Basin in August and November were centrifuged and the cell pellets resuspended in a small volume of sterile Hypersolv water (BDH). A preliminary analysis of 'total' bacterial counts was achieved by staining the cell suspensions with DAPI and filtering the lakewater suspensions from the North and South Basins on to triplicate 0.2 μm pore diameter black polycarbonate membranes. The mean total count for the South Basin in November was 5.9×10^6 cells ml^{-1} (± 1.85) and the mean total count in August was 5.9×10^7 cells ml^{-1} (± 3.07). The mean total count for the North Basin in November was 5.85×10^6 cells ml^{-1} (± 1.53) and the mean total count in August was 5.1×10^7 cells ml^{-1} (± 2.64). Thus, a ten-fold increase in total cell counts in both basins during the summer was observed. In addition, viable counts were undertaken by staining the lake water suspension with CTC. The mean viable count for the South Basin in November was 3.3×10^6 cells ml^{-1} (± 1.78) and the mean viable count in August was 1.6×10^7 cells ml^{-1} (± 1.46). A 21% increase of viable cell numbers occurred between November and August in the South Basin. The mean viable count for the North Basin in November was 5.5×10^6 cells ml^{-1} (± 1.25) and the mean viable count in August was 1.36×10^7 cells ml^{-1} (± 2.93). Thus, a ten-fold increase in viable cell counts in both basins during the summer was observed.

All cell suspensions from lakewater samples were fixed overnight in paraformaldehyde and hybridised against *Nm. europaea-eutropha* and *Nitrosospira* specific oligonucleotide probes. The percentage of fluorescent cells labelled was counted using a flow cytometer (Beckton Dickinson FACStar Plus, Becton Dickinson, Oxford, UK). The percentage increase in cells labelled above the background fluorescence with the probe control EUB338* and that of the natural background from

unlabelled samples was determined. Where no increase above background levels were observed, the result was plotted as zero. However, where non-specific binding occurred the baseline level was set to the highest value obtained for Eub338* or the unlabelled sample. The data obtained are summarised in **Fig. 6.4**. Example flow cytometric (FCM) dot plot quadrants corresponding to 5000 event counts sample for unamended lakewater obtained in August are presented in **Fig. 6.5**. Both **Figs 6.4 & 6.5** show the percentage increase in fluorescence obtained above the controls.

Complete discrimination between the labelled and unlabelled populations by FCM analysis of the lakewater samples was difficult to achieve. However, it was possible to determine the percentage of target organisms in the cell suspensions that was significantly higher than background populations using the controls as stated. FCM analysis of the South Basin demonstrated that the cells binding the *Nitrosospira* and *Nm. europaea-eutropha* probes fluctuated throughout the year (**Fig. 6.4**). The fluorescent signal obtained from all samples gave signals approximately <25% of the total sample fluorescent signal. Control samples for the FISH procedure maintained an approximately constant low autofluorescence level during the experiment. No cells were detected using the *Nitrosospira* probe and the *Nm. europaea-eutropha* probe during the early summer months in the North Basin and in April in the South Basin. No cells were detected in June using the *Nitrosospira* probe and in September using the *Nm. europaea-eutropha* probe in the North Basin. The number of cells labelled with the *Nitrosospira* and *Nm. europaea-eutropha* probe for the South Basin increased from May to August with an additional peak in *Nitrosospira* fluorescent signal in November. The fluorescent signal obtained using the *Nitrosomonas* probe in the North Basin increased to detectable levels in June and August, fluctuating during

A South Basin



B North Basin

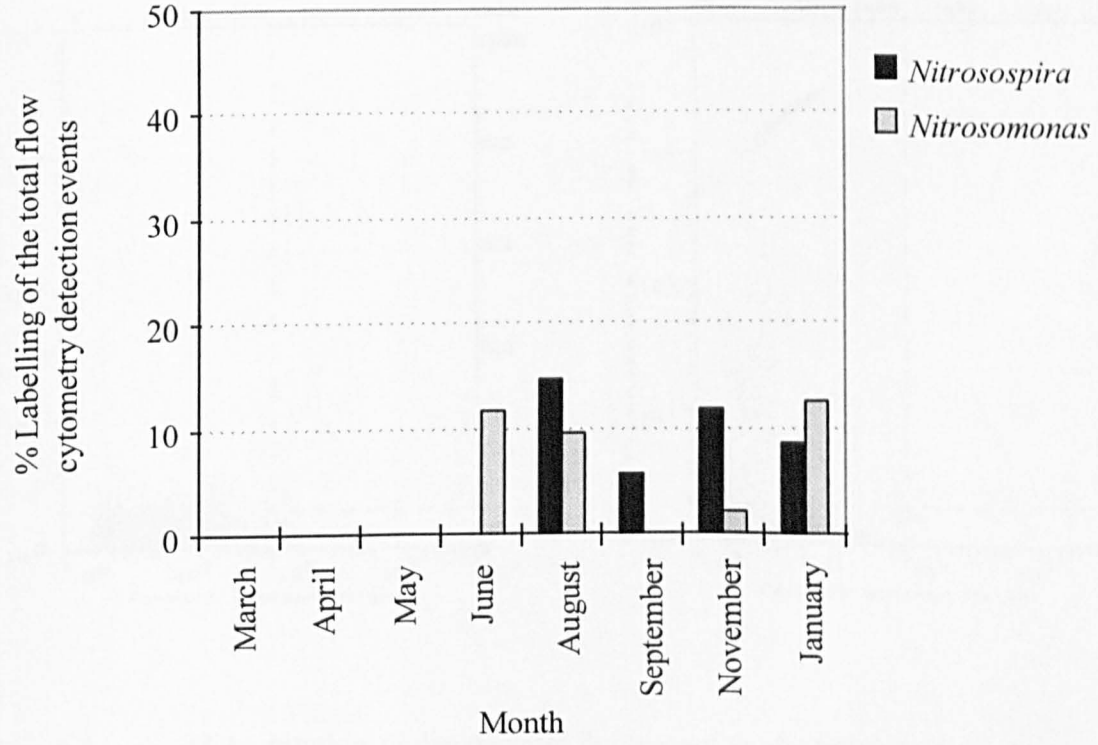
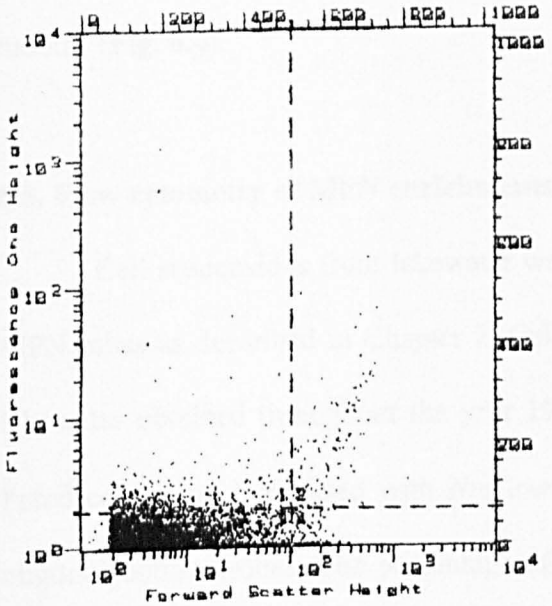
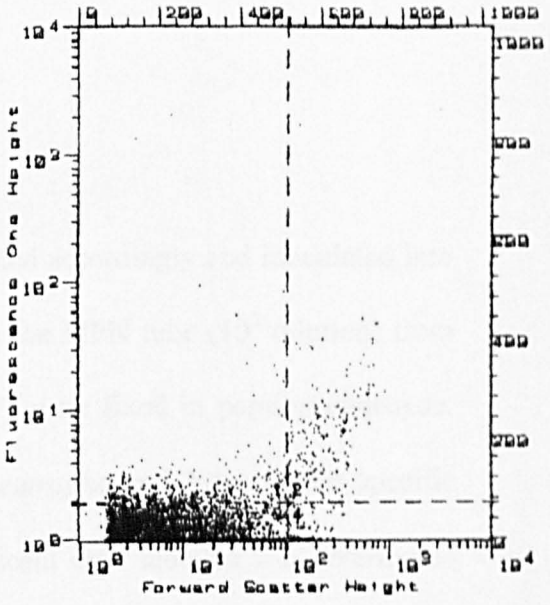


Fig. 6.4. Bar charts of percentage cells labelled of the total cell population in Windermere lakewater South Basin (A), North Basin (B). Cells labelled with *Nitrosospira*-specific probe ■ , *Nitrosomonas*-specific probe □ .

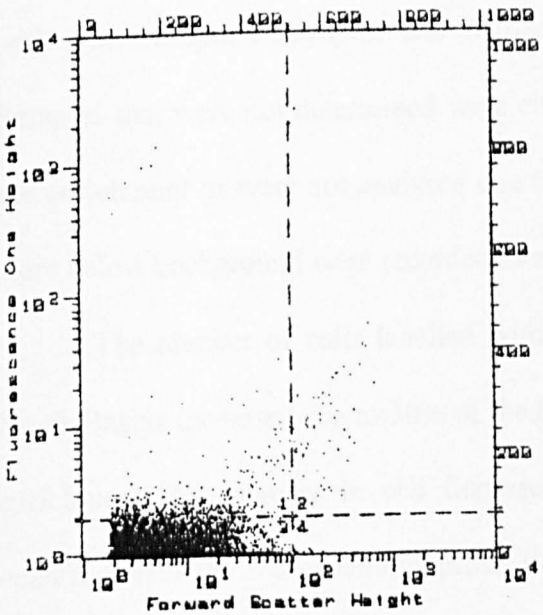
A1 South Basin. *Nitrosospira* probe.



A2 South Basin. *Nitrosomonas* probe.



B1 North Basin. *Nitrosospira* probe.



B2 North Basin. *Nitrosomonas* probe.

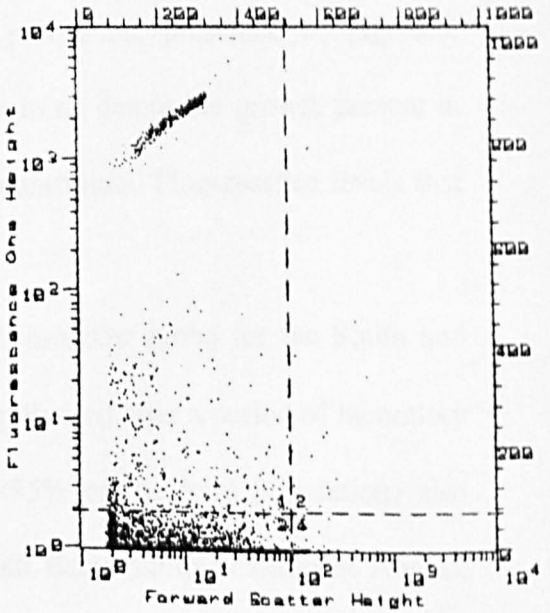


Fig. 6.5 FCM dot plots to demonstrate fluorescent *in situ* hybridisation of ammonia-oxidising bacteria from Windermere lakewater taken in August.

Cells labelled with *Nitrosospira* spp. probe (**1**), *Nitrosomonas* spp. probe (**2**), South Basin (**A**), North Basin (**B**).

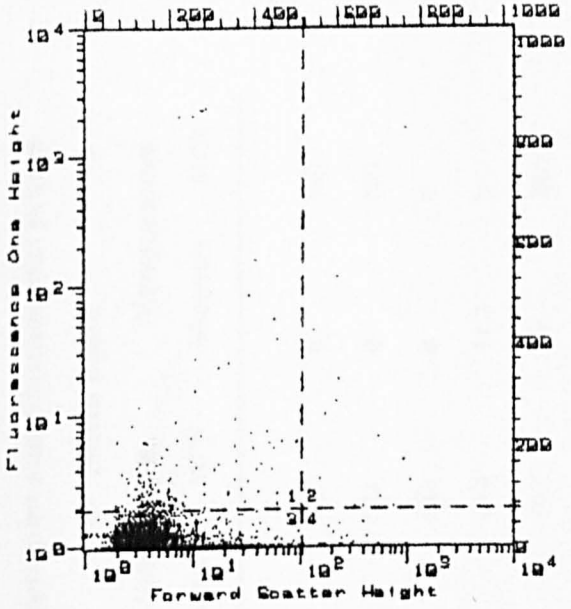
the winter months. The number of *Nitrosospira* cells labelled in the North Basin increased to detectable levels in August and was maintained throughout the winter months (**Fig. 6.4**).

6.5. Flow cytometry of MPN enrichments.

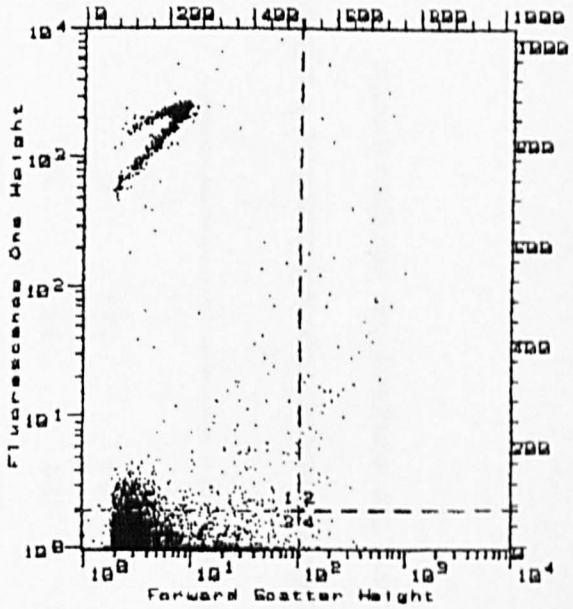
Cell suspensions from lakewater were diluted accordingly and inoculated into MPN tubes as described in Chapter 2. Cells from the MPN tube (10^2 dilution) from lakewater obtained throughout the year 1996/1997 were fixed in paraformaldehyde. Fixed cells were hybridised with *Nm. europaea-eutropha* and *Nitrosospira* specific oligonucleotide probes. The percentage of fluorescent cells labelled was determined using flow cytometry. Data on the percentage of cells labelled above background fluorescence obtained with EUB338* and the unlabelled samples are presented in **Table 6.1**. Example FCM dot plots of lakewater enrichments taken in August labelled with *Nm. europaea-eutropha* and *Nitrosospira* probes are presented in **Fig. 6.6**. Samples that were not determined were either due to no detectable growth present in the enrichment or were not analysed due to time constraints. Fluorescence levels that were below background were recorded as zero.

The number of cells labelled with the *Nitrosospira* probe for the South and North Basins increased (up to 30% of the total population) after a period of laboratory enrichment. An increase in cell fluorescence (<35% of the total population) also occurred with the *Nitrosomonas* probe with South Basin samples taken in August, November and January. An increase in the percentage of *Nitrosomonas* cells labelled (<25% of the total population) in the North Basin enrichments, only occurred with samples taken in August and November. The enrichments demonstrated a greater

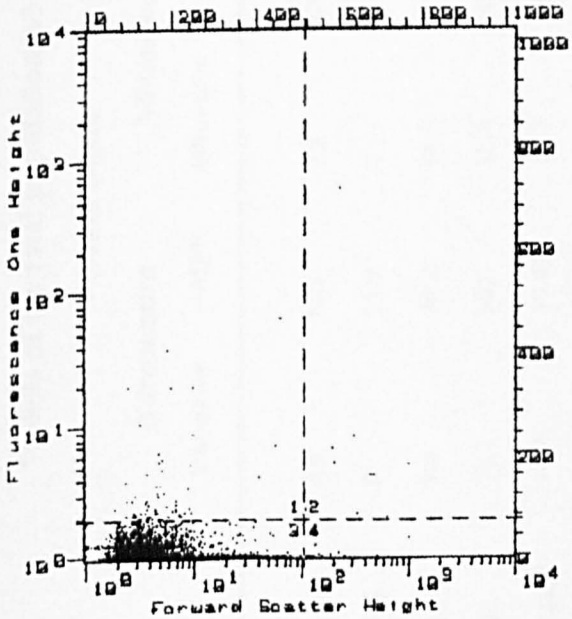
A1 South Basin. *Nitrosospira* probe.



A2 South Basin. *Nitrosomonas* probe.



B1 North Basin. *Nitrosospira* probe.



B2 North Basin. *Nitrosomonas* probe.

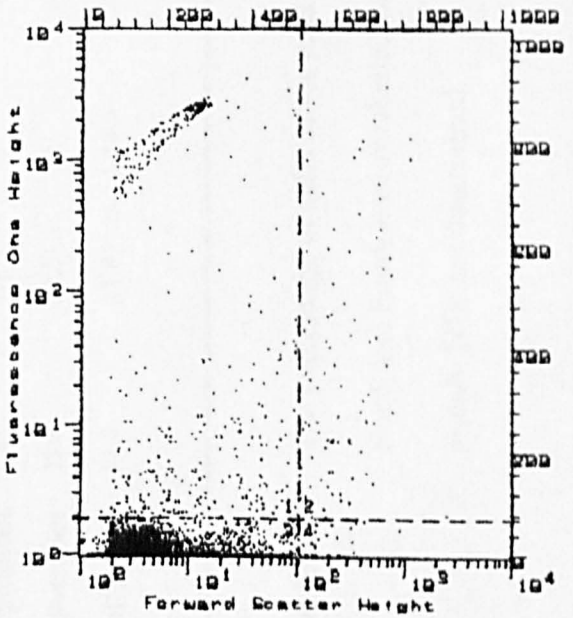


Fig. 6.6 FCM dot plots to demonstrate fluorescent *in situ* hybridisation of ammonia-oxidising bacteria from Windermere lakewater MPN enrichments taken in August.

Cells labelled with *Nitrosospira* spp. probe (**1**), *Nitrosomonas* spp. probe (**2**), South Basin (**A**), North Basin (**B**).

	% cells of the total population labelled with *Nitrosospira* and *Nitrosomonas* probes							
	South Basin				North Basin			
	Nitrosospira		*Nitrosomonas*		*Nitrosospira*		*Nitrosomonas*	
	lakewater	MPN	lakewater	MPN	lakewater	MPN	lakewater	MPN
March	4.9	ND	9.2	ND	0	ND	0	ND
April	0	11.9	0	0	0	ND	0	ND
May	9.6	30.2	14.5	0	0	ND	0	0
June	13.1	ND	13.8	ND	0	17.4	11.7	0
August	14.9	19.43	19.7	34.5	14.2	19.7	9.6	24.5
September	7.8	7.1	0	0	5.8	ND	0	ND
November	22.8	22.3	2	34.6	11.9	17.9	2.1	25.6
January	11.6	15.8	14.5	27.4	8.6	22.4	12.6	0

Table 6.1. The percentage of cells of the total population in lakewater and MPN enrichments from Windermere North and South Basins labelled with *Nitrosospira* and *Nm.* europaea/ *Nm. eutropha* specific probes. ND: not determined.

number of *Nitrosospira* cells labelled than *Nitrosomonas* in both Basins with the exception of samples taken in August, November and January from the South Basin and samples taken in August and November from the North Basin. In enrichment cultures taken from the North Basin in May, June and January, no *Nitrosomonas* cells were detected. Conversely, the enrichments taken in August and November demonstrated a high proportion of *Nitrosomonas* cells. This is compared to the enrichments taken from the South Basin where a high number of *Nitrosomonas* cells were labelled with the probe in both August, November and January.

6.6. Discussion.

One basic problem in the microbial analysis of sediments by flow cytometry (FCM) and microscopy is the removal of bacterial cells from particulate matter, without causing damage to the cell and also to facilitate individual delivery into the sensing region of the flow cytometer. The procedure used in this study attempted to optimise the extraction of cells from Windermere sediments. The cell recoveries presented in this study were low, with 3% of the cell population recovered prior to treatment which increased to 27% following blending, demonstrated a degree of dispersal by pretreating the soil prior to further analysis. Other workers have had comparable recovery efficiencies to that demonstrated by this study, with recovery efficiencies between 10-30% of the indigenous population of soil by a repeated process of blending, sonication and centrifugation (Bakken, 1985). The use of extraction procedures may introduce biases by the selection of easily removable cells that are subsequently labelled. Physical dispersal methods may damage cells and lead to inherent bias (MacDonald, 1986). Due to time constraints, the effect of pretreating sediment on the dispersal of ammonia-oxidiser cells was not investigated by this study. Further investigation is required to determine whether the extraction method would favour the removal of different species or genera. However, this is probably unlikely since the cell envelope composition and architecture for both *Nitrosomonas* and *Nitrosospira* are similar.

Analysis of cell viability for ammonia-oxidising bacteria is difficult due to the difficulty in culturing the organisms on to solid media. Fluorescent viability probes exist for a range of cellular functions which aim to reflect cell viability (Diaper & Edwards, 1994; Mcfeters *et al.*, 1995; Porter *et al.*, 1996). The use of fluorescent

viability probes were applied in this study in an attempt to capture the viable fraction of lakewater. The viable counts of *E. coli* cells used in this investigation as determined by CTC were significantly lower (between 3-6 fold) than the plate counts. Distinguishing between definite bacterial cells from the background particulate matter was difficult. The underestimation obtained by CTC counts may be due to high levels of background fluorescence and soil particles obscuring cells, although the counts presented in this study were of objects of approximately the correct size which fluoresced brightly. The reduction of tetrazolium should indicate the presence of active enzymes involved in oxidative electron transport. However, tetrazolium salts can also be reduced by obligately anaerobic fermentative bacteria that lack cytochromes which may also lead to inherent inaccuracies in cell counts by compensating for the underestimations (Oren, 1987). Cell viability can be further complicated by dormancy. This was demonstrated in the non-sporulating Gram positive bacterium *Micrococcus luteus* under starvation conditions, but was reversible by the addition of nutrients (Kaprelyants & Kell, 1992).

The *in situ* identification of microorganisms with rRNA targeted probes has facilitated detection of specific populations and microscopic evaluation provides information on cell morphology, floc structures and spatial distribution of cells (Manz *et al.*, 1993; Amann *et al.*, 1990ab, 1991). Quantitative microscopy, even in combination with image analysis can be time consuming and labour intensive. In contrast, flow cytometry (FCM) can provide a means for rapid analysis of microbial communities present in the environment (Wallner *et al.*, 1995; Porter *et al.*, 1997)

Despite optimum conditions, ammonia-oxidising bacteria grow to low cell densities (Belser, 1979; Bock *et al.*, 1986). Ward & Perry (1980) reported the

detection of *Nm. europaea* at levels <10 cells ml^{-1} using fluorescently labelled antibodies and microscopy. Sub-optimum oligotrophic environments such as Buttermere and the North Basin of Windermere may contain inactive ammonia-oxidiser bacteria with too few ribosomes to permit sufficient probe binding. Wallner *et al.* (1995) studied activated sludge using a combination of eubacterial, γ and β-Proteobacterial oligonucleotide probes and reported bright hybridisation signals due to the high cellular ribosome content of the organisms present in the nutrient-rich environment. However, Wallner *et al.* (1995) also reported that 20-30% of cells could not be identified due either to low ribosome content or the cells were impermeable to the probes. The data presented in this study demonstrated that the number of ammonia oxidiser cells present in lakewater was low and often below detectable levels. However, after a period of laboratory enrichment, the numbers increased in many samples to detectable levels, particularly in August for the South Basin and November for both Basins. The increased cell fluorescence observed at these times of year correspond to an increase in the nitrate and nitrite production detected in the enrichment cultures (see Chapter 3). MPN counts of the ammonia-oxidising bacteria were in the range of 1-100 $x10^4$ cells ml^{-1}.

Signal intensity has been demonstrated to be related to the physiological status of the cells (DeLong *et al.*, 1989). Porter *et al.* (1995) demonstrated the variability of fluorescent probes in the assessment of starved *E. coli* cells. Cells that grow at lower rates have fewer 16S rRNA target sites and a reduced fluorescent signal. The technique is complicated by the low rRNA levels present in ammonia-oxidising bacteria and the sub-optimal growth conditions. Wallner *et al.* (1993) reported that a probe concentration of 0.01 ng μl^{-1} limited fluorescence due to insufficient probe,

while concentrations of 20 ng μl^{-1} led to non-specific binding. An alternative method developed is the use of multiple probes (Amann *et al.*, 1990ab). To date, only modest increases in signal:noise ratio have been achieved using multiple probes to different sites in the ribosome (Lee *et al.*, 1993; Trebessius *et al.*, 1994).

The FISH method may introduce bias due to hybridisation effiency of the target to the probe binding site. The results presented here demonstrated that the FISH method was specific in detecting laboratory cultures of ammonia-oxidising bacteria. However, the detection of the genera, *Nitrosospira* and *Nitrosomonas* in environmental samples was problematical. Flow cytometric analysis demonstrated a greater number of *Nitrosomonas* cells labelled from lakewater obtained from the South Basin in June and january compared to the North Basin at the same times of year. In addition, *Nitrosomonas* cells failed to label in the enrichment cultures inoculated from lakewater taken from the North Basin in May, June and January despite the fact that the *Nitrosomonas* cells could be detected from the corresponding samples taken in May. The flow cytometry data presented here was inconclusive. It is possible that *Nitrosomonas* cells were present in the lakewater, but failed to grow in the cultures, or the percentage of cells labelled from the lakewater samples was a result of non-specific binding. FCM analysis of the lakewater samples for the South Basin corroborated the PCR data (Chapter 4) as to the ubiquity of *Nitrosospira* spp. However, FCM analysis of the lakewater samples from both Basins contradicted the data obtained previously by PCR (Chapter 4). For example *Nitrosomonas* spp. were detected from samples taken in June to January in the North Basin but throughout the year in the South Basin.

The observed high percentage of cells labelled with the fluorescent probes (35%) may be due to non-specific binding rather than an increase in ammonia-oxidiser cell numbers. However, the use of EUB338* control probe in this work confirmed that the level of non-specific binding was low. The discrimination of target and non-target cells has been reported with FCM of mixed populations of cultured cells (Amann *et al.*, 1990). Discrimination of target cells from high background fluorescence in many environmental samples is difficult. FISH has been applied to identify microbial populations present in activated sludge that were present in different proportions using culture-based techniques (Manz *et al.*, 1993; Wagner *et al.*, 1993). Therefore, FISH and FCM may overcome a number of the problems encountered when using culture-based techniques.

The application of immunological methods offers the potential for reduced background fluorescence. A combination of antibodies labelled with different fluorochromes targeting separate antigens may remove high background events and increase sensitivity (Page *et al.*, 1991). Background levels have also been reduced by the combination of labelling with a monoclonal antibody with the non-specific DNA stain propidium iodide (Page *et al.*, 1991). *In situ* PCR combined with flow cytometry may also facilitate an increase in sensitivity by the amplification of the DNA present in the cell (Hodson *et al.*, 1995; Porter *et al.*, 1997), although the application of *in situ* PCR in prokaryotic studies has led to concern over the compromise between access of the PCR reagents into the cell and limiting product diffusion. FCM and FISH will continue to be useful in environmental microbiology as instrument design, image analysis and data proccessing are constantly improved.

6.7. Conclusions

• Total cell numbers in lakewater from Windermere, as assessed by DAPI, increased ten-fold from November to August. The metabolically active bacterial populations present in lakewater from Windermere were $<2.0 \times 10^7$ cell ml^{-1} as assessed by CTC viability dye. Cell dispersal was optimised by blending in Triton-X-100.

• FISH analysis of laboratory cultures of ammonia-oxidising bacteria demonstrated probe specificity. FCM analysis demonstrated that no cells from lakewater were labelled with either the *Nm. europaea-eutropha* probe or *Nitrosospira* probe in the North Basin from March to May. A greater number of cells from lakewater were labelled with the *Nm. europaea-eutropha* probe and *Nitrosospira* probe in the South Basin throughout the year (except in April).

• FCM analysis of lakewater MPN enrichments demonstrated <30% of the total population of cells were labelled with the *Nitrosospira* probe in both basins. No *Nm. europaea-eutropha* cells were detected throughout the year from lakewater MPN enrichments from the North Basin (except in August and November) or the South Basin (except in August, November and January).

Chapter 7. General Discussion.

Analysis of prokaryotic populations present in the environment are restricted by the limitations of the techniques employed (Amann *et al.*, 1998). Ammonia-oxidising bacteria have been isolated from a variety of natural environments, and studied using both traditional culture-based techniques, and molecular methods (Belser & Schmidt, 1978; Hiorns *et al.*, 1995; Ward, 1995; Wallner *et al.*, 1996). In this thesis, the community structure of ammonia-oxidising bacteria present in freshwater lakes was analysed by culture, molecular analyses and direct microscopic observation. More specifically, it was intended to identify individual species and compare sequences obtained, in order to analyse phylogenetic relationships.

Studies of nitrification activities of bacteria in soils and sediments using conventional media-based techniques have been well documented, but demonstrate a diversity of results (Belser & Schmidt, 1978; Martikainen, 1984; Hall, 1986; Smorczewski & Schmidt, 1991). Ammonia-oxidising bacterial populations present in the environment may be misrepresented as different media may select for different genera and species (Belser & Schmidt, 1978). Assessment of the growth and activity of ammonia-oxidising bacteria by the oxidation of ammonium, and subsequent nitrite production must be performed with caution. Preliminary culture studies performed in this work, demonstrated no correlation between nitrate and nitrite production with lakewater MPN counts taken from Windermere during the summer months. Although *in vitro* autotrophic ammonia-oxidation inhibition was demonstrated, enrichment cultures containing allylthiourea failed to produce detectable nitrate and nitrite production.

The direct extraction and amplification of nucleic acids provides the rapid analysis of ammonia-oxidising bacterial populations (Tsai & Olsen, 1992; Hiorns *et al.*, 1995; Ward *et al.*, 1997). PCR amplification and oligonucleotide probing of ammonia-oxidiser 16S rDNA found that members of the genus *Nitrosospira* were distributed ubiquitously in both Buttermere and Windermere. These observations corroborate other studies, and support the evidence that *Nitrosospira* spp. may have a greater involvement in the mediation of nitrification in the environment than was previously considered (Hiorns *et al.*, 1995; Stephen *et al.*, 1996).

The presence of *Nitrosomonas* 16S rDNA was demonstrated during the summer months in Buttermere and the North Basin of Windermere, without the prerequisite to culture. Hiorns *et al.* (1995) demonstrated the presence of *Nitrosomonas* spp. in Esthwaite Water only after a period of laboratory enrichment. The seasonal occurrence of nitrosomonad DNA presented in this study, coincided with an increase in ammonia and oxygen concentrations. These observations add support to evidence that nitrosomonads are enriched as nutrient concentration increases (Hiorns *et al.*, 1995; Hastings *et al.*, 1997). The possibility of sub-populations of ammonia-oxidising bacteria that exhibit ammonium sensitivity has previously been reported (Suwa *et al.*, 1994, 1997; Stehr *et al.*, 1995). The culture-based studies presented here indicated that spatially distinct populations of ammonia-oxidising bacteria sensitive to high ammonium levels were present in lakewater, whilst ammonium insensitive species were present in the sediment.

The phylogenetic relationships of ammonia-oxidising bacteria in Buttermere were investigated. Sequence analysis of the 16S rRNA genes revealed genetic diversity between spatially distinct sediment. *Nm. europaea*-like 16S rRNA sequences

were dominant in littoral sediment and *Nm. eutropha*-like 16S rRNA sequences were prevalent in profundal sediment. Restriction analysis of 16S rDNA confirmed this distinction and facilitated the development of a rapid screening method of clones for sequencing. Pommerening-Röser *et al.* (1996) suggested that the failure to directly detect *Nm. europaea* from Esthwaite Water by Hiorns *et al.* (1995) was not surprising since the organism was isolated from brackish waters and estuaries. Contrary to this, analysis of sequence and RFLP data presented in this study, demonstrated the presence of both *Nm. europaea* and *Nm. eutropha* in an oligotrophic lake.

The application of 16S rRNA technology for phylogenetic analysis is limited by the specificity of the primers employed in the PCR amplification. This has been a concern for molecular biologists as biases may be introduced. The problem has been highlighted in this study by the high degree of specificity of the *Nitrosomonas* primers (Nm75/ Nm1007*) which fail to recognise certain *Nitrosomonas* spp. This was highlighted by the application of the CTO primers designed by Kowalchuk *et al.* (1997), which have a broader specificity and encompass all published β subdivision ammonia-oxidisers. When these primers were applied to samples from Buttermere one of the sequences was closely related to *Nm. ureae*. The application of ammonia monoxygenase gene (AMO) as an alternative target gene for the identification and phylogenetic delineation of ammonia-oxidising species, may circumvent the limitations observed with 16S rRNA sequencing (Norton *et al.*, 1996; Rotthauwe *et al.*, 1997). Although the number of AMO sequences obtained is increasing, the paucity of the sequences deposited in the databases at present makes this approach difficult.

In situ hybridisation with fluorescently labelled oligonucleotide probes permits the examination of selected components of microbial consortia and provides semi-quantitative assessment of microbial activity (Amann *et al.*, 1995, 1997). The metabolic activity of ammonia-oxidising bacteria within Windermere was attempted by *in situ* hybridisation and flow cytometry. The oligonucleotide probes were specific when applied to laboratory cultures, but lakewater samples and enrichment cultures produced high levels of background fluorescence. Although the number of ammonia-oxidiser cells present in lakewater was low, Volsch *et al.* (1990) detected ammonia-oxidising bacteria that constituted <2% of the total bacterial population present in activated sludge. *In situ* PCR may increase sensitivity by increasing the target DNA present within the cells but further work would be required to determine the relative contributions that *Nitrosospira* and *Nitrosomonas* spp. have on nitrification in Buttermere and Windermere.

The analysis of RNAs extracted from sediment and lakewater would complement the work performed in this thesis. Sequence analysis derived from RNA rather than DNA templates would possibly be more representative of the activity of ammonia-oxidising bacteria *in situ* (Teske *et al.*, 1996). However, the extraction of RNA from sediment is complicated by inhibitory substances such as humic acids that are co-extracted with the RNA and cause degradation of the RNA (Tsai & Olsen, 1991). An accurate assessment of the community structure and activity of ammonia-oxidising bacteria in the environment will become more apparent, as techniques continue to develop.

Chapter 8. References.

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Appendix I- Nucleotide sequences of cloned 16S rDNA from Buttermere.

PV3G	----------	GGAAT--ACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
PV3H	----------	GGAAT--ACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
PV2C	----------	GGGATAACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
PV3M	----------	GGAATAACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
PV2B	----------	GGAATAACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
PV3E	----------	GGAATAACGC	ATCGAAAGAT	GTGCTAACAC	CGCATA--TCT
PV2O	----------	GGGATAACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
PV3I	----------	GGAATAACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
PV3L	----------	GGAATAACNC	ATCGAAANAT	GTGCTAATAC	CGCATA--TCT
PV3	GTGTCCTTAAGTGG	GGAATAACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
PV3K	----------	GGGATAACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
PV2P	----------	GGGATAACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
PV3J	----------	GGAATGACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
LV3C	----------	GGAATAACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
LV3	GTGTCCTTAAGTGG	GGAATAACGC	ATCGAAAGAC	GTGCTAATAC	CGCATA--TCT
LV1A	----------	GGAATAACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
LV3F	----------	GGAATAACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
LV3K	----------	GGAATAACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
LV3M	----------	GGAATAACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
LV3I	----------	GGAATAACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
LV1S	----------	GGAATAACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
LV3L	----------	GGAATAACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
LV3J	----------	GGAATAACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
LV1C	----------	GGAATAACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
LV1T	----------	GGAATAACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
LV3A	----------	GGAATAACGC	ATCGAAAGAC	GTGCTAATAC	CGCATA--TCT
LV3H	----------	GGAATAACGC	ATCGAAAGAT	GTGCTAATAC	CGCATA--TCT
b5	----------	GGGATAACGC	ACCGAAAGGT	GTGCTAATAC	CGCATAATCT
b4	----------	GGGATAACGC	ACCGAAAGGT	GTGCTAATAC	CGCATAATCT
f1	----------	GGGATAACGC	ACCGAAAGGT	GTGCTAATAC	CGCATAATCT
no1	----------	----------	----------	----------	----------
no2	----------	----------	----------	----------	----------
no3	----------	----------	----------	----------	----------
no4	----------	----------	----------	----------	----------

PV3G	CTCAGGAGGA	AAGCAGGGGA	TCGAAAGACC	TTGCGCTAAA	GGAGCGGCTG
PV3H	CTCAGGAGGA	AAGCAGGGGA	TCGAAAGACC	TTGCGCTAAA	GGAGCGGCTG
PV2C	CTCAGGAGGA	AAGCAGGGGA	TCGAAAGACC	TTGCGCTAAA	GGAGCGGCTG
PV3M	CTCAGGAGGA	AAGCAGGGGA	TCGAAAGACC	TTGCGCTAAA	GGAGCGGCTG
PV2B	CTCAGGAGGA	AAGCAGGGGA	TCGAAAGACC	TTGCGCTAAA	GGAGCGGCTG
PV3E	CTCAGGAGGA	AAGCAGGGGA	TCGAAAGACC	TTGCGCTAAA	GGAGCGGCTG
PV2O	CTCAGGAGGA	AAGCAGGGGA	TCGAAAGACC	TTGCGCTAAA	GGAGCGGCTG
PV3I	CTCAGGAGGA	AAGCAGGGGA	TCGAAAGACC	TTGCGCTAAA	GGAGCGGCTG
PV3L	CTCAGGAGGA	AAGCAGGGGA	TCGAAAGACC	TTGCGCTAAA	GGAGCGGCTG
PV3	CTCAGGAGGA	AAGCAGGGGA	TCGAAAGACC	TTGCGCTAAA	GGAGCGGCTG
PV3K	CTCAGGAGGA	AAGCAGGGGA	TCGAAAGACC	TTGCGCTAAA	GGAGCGGCTG
PV2P	CTCAGGAGGA	AAGCAGGGGA	TCGAAAG-CC	TTGCGCTAAA	GGAGCGGCTG
PV3J	CTCAGGAG-A	AAGCAGGGGA	TCGAAAGACC	TTGCGCTAAA	GGAGCGGCTG
LV3C	CTCAGGAGAA	AAGCA-GGGA	TCGCAAGACC	TTGCGCTAAA	GGAGCGGCCG
LV3	CTCAGGAGAA	AAGCA-GGGA	TCNCAAGACC	TTGCGCTAAA	GGAGCGGCCG
LV1A	CTCAGGAGAA	AAGCAGGGGA	TCGCAAGACC	TTGCGCTAAA	GGAGCGGCCG
LV3F	CTCAGGAGAA	AAGCAGGGGA	TCGCAAGACC	TTGCGCTAAA	GGAGCGGCCG
LV3K	CTCAGGAGAA	AAGCAGGGGA	TCGCAAGACC	TTGCGCTAAA	GGAGCGGCCG
LV3M	CTCAGGAGAA	AAGCAGGGGA	TCGCAAGACC	TTGCGCTAAA	GGAGCGGCCG
LV3I	CTCAGGAGAA	AAGCAGGGGA	TCGCAAGACC	TTGCGCTAAA	GGAGCGGCCG
LV1S	CTCAGGAGAA	AAGCAGGGGA	TCGCAAGACC	TTGCGCTAAA	GGAGCGGCCG
LV3L	CTCAGGAGAA	CAGCAGGGGA	TCGCAAGACC	TTGCGCTAAA	GGAGCGGCCG
LV3J	CTCAGGAGAA	AAGCAGGGGA	TCGCAAGACC	TTGCGCTAAA	GGAGCGGCCG
LV1C	CTCAGGAGAA	AAGCAGGGGA	TCGCAAGACC	TTGCGCTAAA	GGAGCGGCCG
LV1T	CTCAGGAGAA	AAGCAGGGGA	TCGCAAGACC	TTGCGCTAAA	GGAGCGGCCG
LV3A	CTCAGGAGAA	AAGCAGGGGA	TCGCAAGACC	TTGCGCTAAA	GGAGCGGCCG
LV3H	CTCAGGAGAA	AAGCA-GGGA	TCGCAAGACC	TTGCGCTAAA	GGAGCGGCCG
b5	CTACGGAGAA	AAGCAGGGGA	TCGCAAG-CC	TTGCGCTCTT	GGAGCGGCCG
b4	CTACGGAGAA	AAGCAGGGGA	TCGCAAGACC	TTGCGCTCTT	GGAGCGGCCG
f1	CTACGGAGAA	AAGCAGGGGA	TCGCAAG--CC	TCGCGCTGAT	GGAGCGGCCG
no1	----------	----------GGA	TCGAAAGACC	TTATGCTTTT	GGAGCGGCCG
no2	----------	----------GGA	TCGAAAGACC	TTGCGCTTTT	GGAGCGGCCG
no3	----------	----------GGA	TCGAAAGACC	TTGCGCTTTT	GGAGCGGCCG
no4	----------	----------GGA	TCGAAAGACC	TTGCGCTAAA	GGAGCGGCCG

PV3G	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
PV3H	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
PV2C	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
PV3M	ATGTCTGATT	ANCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
PV2B	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
PV3E	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
PV2O	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
PV3I	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
PV3L	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
PV3	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
PV3K	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
PV2P	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
PV3J	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
LV3C	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
LV3	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
LV1A	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
LV3F	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
LV3K	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
LV3M	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
LV3I	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
LV1S	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
LV3L	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
LV3J	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
LV1C	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
LV1T	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
LV3A	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
LV3H	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
b5	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
b4	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
fl	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA
no1	ATGTCTGATT	AGATAGTTGG	TGGGGTAATG	GCCTACCAAG	GTGACGATCA
no2	ATGTCTGATT	AGCTAGTTGG	TGAGGTAAAG	GCTTACCAAG	GCTTCGATCA
no3	ATGTCTGATT	AGCTAGTTGG	TGAGGTAAAG	GCTTACCAAG	GCTTCGATCA
no4	ATGTCTGATT	AGCTAGTTGG	TGGGGTAAAG	GCTTACCAAG	GCAACGATCA

PV3G	GTAGCTGGTC	TGAGAGGACG	ACCACCCACA	CTGGGACTGA	GACACGGCCC
PV3H	GTAGCTGGTC	TGAGAGGACG	ACCAGCCACA	CTGGGACTGA	GACACGGCCC
PV2C	GTAGCTGGTC	TGAGAGGACG	ACCAGCCACA	CTGGGACTGA	GACACGGCCC
PV3M	GTAGCTGGTC	TGAGAGGACG	ACCAGCCACA	CTGGGACTGA	GACACGGCCC
PV2B	GTAGCTGGTC	TGAGAGGACG	ACCAACCACA	CTGGGACTGA	GACACGGCCC
PV3E	GTAGCTGGTC	TGAGAGGACG	ACCAGCCACA	CTGGGACTGA	GACACGGCCC
PV2O	GTAGCTGGTC	TGAGAGGACG	ACCAGCCACA	CTGGGACTGA	GACACGGCCC
PV3I	GTAGCTGGTC	TGAGAGGACG	ACCAGCCACA	CTGGGACTGA	GACACGGCCC
PV3L	GTAGCTGGTC	TGAGAGGACG	ACCAGCCACA	CTGGGACTGA	GACACGGCCC
PV3	GTAGCTGGTC	TGAGAGGACG	ACCAGCCACA	CTGGGACTGA	GACACGGCCC
PV3K	GTAGCTGGTC	TGAGAGGACG	ACCAGCCACA	CTGGGACTGA	GACACGGCCC
PV2P	GTAGCTGGTC	TGAGAGGACG	ACCAGCCACA	CTGGGACTGA	GACACGGCCC
PV3J	GTAGCTGGTC	TGAGAGGACG	ACCAGCCACA	CTGGGACTGA	GACACGGCCC
LV3C	GTAGCTGGTC	TGAGAGGACG	GCCAACCACA	CTGGGACTGA	GACACGGCCC
LV3	GTAGCTGGTC	TGAGAGGACG	GCCAACCACA	CTGGGACTGA	GACACGGCCC
LV1A	GTAGCTGGTC	TGAGAGGACG	GCCAACCACA	CTGGGACTGA	GACACGGCCC
LV3F	GTAGCTGGTC	TGAGAGGACG	GCCAACCACA	CTGGGACTGA	GACACGGCCC
LV3K	GTAGCTGGTC	TGAGAGGACG	GCCAACCACA	CTGGGACTGA	GACACGGCCC
LV3M	GTAGCTGGTC	TGAGAGGACG	GCCAGCCACA	CTGGGACTGA	GACACGGCCC
LV3I	GTAGCTGGTC	TGAGAGGACG	GCCAACCACA	CTGGGACTGA	GACACGGCCC
LV1S	GTAGCTGGTC	TGAGAGGACG	GCCAACCACA	CTGGGACTGA	GACACGGCCC
LV3L	GTAGCTGGTC	TGAGAGGACG	GCCAACCACA	CTGGGACTGA	GACACGGCCC
LV3J	GTAGCTGGTC	TGAGAGGACG	GCCAACCACA	CTGGGACTGA	GACACGGCCC
LV1C	GTAGCTGGTC	TGAGAGGACG	GCCAACCACA	CTGGGACTGA	GACACGGCCC
LV1T	GTAGCTGGTC	TGAGAGGACG	GCCAACCACA	CTGGGACTGA	GACACGGCCC
LV3A	GTAGCTGGTC	TGAGAGGACG	GCCAACCACA	CTGGGACTGA	GACACGGCCC
LV3H	GTAGCTGGTC	TGAGAGGACG	GCCAACCACA	CTGGGACTGA	GACACGGCCC
b5	GTAGCTGGTC	TGAGAGGACG	GCCAGCCACA	CTGGGACTGA	GACACGGCCC
b4	GTAGCTGGTC	TGAGAGGACG	GCCAGCCACA	CTGGGACTGA	GACACGGCCC
fl	GTAGCTGGTC	TGAGAGGACG	GCCAGCCACA	CTGGGACTGA	GACACGGCCC
no1	GCAGTTGGTA	TGAGAGGACG	ACCAGCCACA	CTGGGACTGA	GACACGGCCC
no2	GTAGCTGGTC	TGAGAGGACG	ACCAGCCACA	CTGGGACTGA	GACACGGCCC
no3	GTAGTTGGTC	TGAGAGGACG	ACCAGCCACA	CTGGGACTGA	GACACGGCCC
no4	GTAGTTGGTC	TGAGAGGACG	ACCAGCCACA	CTGGGACTGA	GACACGGCCC

PV3G	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
PV3H	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
PV2C	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
PV3M	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
PV2B	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
PV3E	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
PV2O	AGACTCCTAC	--GGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
PV3I	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
PV3L	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
PV3	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
PV3K	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
PV2P	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
PV3J	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
LV3C	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
LV3	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
LV1A	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
LV3F	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
LV3K	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
LV3M	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
LV3I	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
LV1S	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
LV3L	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
LV3J	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
LV1C	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
LV1T	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
LV3A	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
LV3H	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
b5	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
b4	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
f1	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAC	GGGCGAAAGC
no1	AGACTCATAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAT	GGGCGAAAGC
no2	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAT	GGGGGCAACC
no3	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAT	GGGGGCAACC
no4	AGACTCCTAC	GGGAGGCAGC	AGTGGGGAAT	TTTGGACAAT	GGGCGCAACC

PV3G	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
PV3H	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
PV2C	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
PV3M	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
PV2B	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
PV3E	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
PV2O	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
PV3I	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
PV3L	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
PV3	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
PV3K	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
PV2P	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
PV3J	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
LV3C	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
LV3	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	-GCCTTCGGG	TTGTAAAGCT
LV1A	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
LV3F	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
LV3K	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAANCT
LV3M	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
LV3I	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
LV1S	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
LV3L	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
LV3J	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
LV1C	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
LV1T	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
LV3A	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	--GCCTTCGGG	TTGTAAAGCT
LV3H	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
b5	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
b4	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
f1	CTGATCCATC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
no1	CTGATCCAGC	ANTGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTNTAAAGTT
no2	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT
no3	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGTA
no4	CTGATCCAGC	CATGCCGCGT	GAGTGAAGAA	GGCCTTCGGG	TTGTAAAGCT

PV3G	CTTTTAGTCG	GTCATAGTAA	ATAGCTATGA	TTTATGACGG	TACCGACAGA	AAAA
PV3H	CTTTTAGTCG	GTCATAGTAA	ATAGCTATGA	TTTATGACGG	TACCGACAGA	AAAA
PV2C	CTTTTAGTCG	GTCATAGTAA	ATAGCTATGA	TTTATGACGG	TACCGACAGA	AAAA
PV3M	CTTTTAGTCG	GTCATAGTAA	ATAGCTATGA	TTTATGACGG	TACCGACAGA	AAAA
PV2B	CTTTTAGTCG	GTCATAGTAA	ATAGCTATGA	TTTATGACGG	TACCGACAGA	AAAA
PV3E	CTTTTAGTCG	GTCATAGTAA	ATAGCTATGA	TTTATGACGG	TACCGACAGA	AAAA
PV2O	CTTTTAGTCG	GTCATAGTAA	ATAGCTATGA	TTTATGACGG	TACCGACAGA	AAAA
PV3I	CTTTTAGTCG	GTCATAGTAA	ATAGCTATGA	TTTATGACGG	TACCGACAGA	AAAA
PV3L	CTTTTAGTCG	GTCATAGTAA	ATAGCTATGA	TTTATGACGG	TACCGACAGA	AAAA
PV3	CTTTTAGTCG	GTCATAGTAA	ATAGCTATGA	TTTATGACGG	TACCGACAGA	AAAA
PV3K	CTTTTAGTCG	GTCATAGTAA	ATAGCTATGA	TTTATGACGG	TACCGACAGA	AAAA
PV2P	CTTTTAGTCG	GTCATAGTAA	ATAGCTATGA	TTTATGACGG	TACCGACAGA	AAAA
PV3J	CTTTTAGTCG	GTCATAGTAA	ATAGCTATGA	TTTATGACGG	TACCGACAGA	AAAA
LV3C	CTTTTAGTCG	GTTGCAATGA	ATAACTATGA	TTTATGACGG	TACCGACAGA	AAAA
LV3	CTTTTAGTCG	GTTGCAATGA	ATAACTATGA	TTTATGACGG	TACCGACAGA	AAAA
LV1A	CTTTTAGTCG	GTTGCAATGA	ATAACTATGA	TTTATGACGG	TACCGACAGA	AAAA
LV3F	CTTTTAGTCG	GTTGCAATGA	ATAACTATGA	TTTATGACGG	TACCGACAGA	AAAA
LV3K	CTTTTAGTCG	GTTGCAATGA	ATAACTATGA	TTTATGACGG	TACCGACAGA	AAAA
LV3M	CTTTTAGTCG	GTTGCAATGA	ATAACTATGA	TTTATGACGG	TACCGACAGA	AAAA
LV3I	CTTTTAGTCG	GTTGCAATGA	ATAACTATGA	TTTATGACGG	TACCGACAGA	AAAA
LV1S	CTTTTAGTCG	GTTGCAATGA	ATAACTATGA	TTTATGACGG	TACCGACAGA	AAAA
LV3L	CTTTTAGTCG	GTTGCAATGA	ATAACTATGA	TTTATGACGG	TACCGACAGA	AAAA
LV3J	CTTTTAGTCG	GTTGCAATGA	ATAACTATGA	TTTATGACGG	TACCGACAGA	AAAA
LV1C	CTTTTAGTCG	-TTGCAATGA	ATAACTATGA	TTTATGACGG	TACCGACAGA	AAAA
LV1T	CTTTTAGTCG	ATTGCAATGA	ATAACTATGA	TTTATGACGG	TACCGACAGA	AAAA
LV3A	CTTTTAGTCG	GTTGCAATGA	ATAACTATGA	TTTATGACGG	TACCGACAGA	AAAA
LV3H	CTTTTAGTCG	GTTGCAATGA	ATAACTATGA	TTTATGACGG	TACCGACAGA	AAAA
b5	CTTTTAGTCG	GTCACGGTTA	ATAACTATGA	CTACTGACGG	TACCGACAGA	AGAA
b4	CTTTTAGTCG	GTCACGGTTA	ATAACTATGA	CTACTGACGG	TACCGACAGA	AGAA
f1	CTTTTAGTCG	GTCACGGTTA	ATACCCGTGA	CTACTGACGG	TACCGACAGA	AGAA

PV3	CACCGGCTAA	CTACGTGCCA	GCAGCCGCGG	TAATACGTAG	GGTGCGAGCG
LV3	CACCGGCTAA	CTACGTGCCA	GCAGCCGCGG	TAATACGTAG	GGTGCGAGCG
PV3	TTAATCGGAA	TTACTGGGCG	TAAAGGGTGC	GCAGGCGGCC	TTGTAAATCA
LV3	TTAATCGGAA	TTACTGGGCG	TAAAGGGTGC	GCAGGCGGCC	TTGCAAGTCA
PV3	GATGTGAAAG	CCCCGGGCTT	AACCTGGGTA	TTGCGTTTGA	AACTACAAAG
LV3	GATGTGAAAG	CCCCGGGCTT	AACCTGGGTA	TTGCGTTTGA	AACTACAAAG
PV3	CTAGAGTGCA	GCAGAGGGGA	GTGGAATTCC	ATGTGTAGCA	GTGAAATGCG
LV3	CTAGAGTGCA	GCAGA--GGGA	GTGGAATTCC	ATGTGTAGCA	GTGAAATGCG
PV3	TATAGATGTG	GAAGAACACC	GATGGCGAAG	GCAGCTCCCT	GGGTTGACAC
LV3	TACAGATGTG	GAAGAACACC	GATGGCGAAG	GCAGCTCCCT	GGGTTGACAC
PV3	TGACGCTCAT	GCACGAA--GC	GTGGGGAGCA	AACAGGATTA	GATACCCTGG
LV3	TGACGCTCAT	GCACGAAAGC	GTGGGGAGCA	AACAGGATTA	GATACCCTGG
PV3	TAGTCCACGC	CCTAAACTAT	GTCAACTAGT	TGTCGGATCTATTA	
LV3	TAGTCCACGC	CCTAAACGAT	GTTAACTAGT	TGTCGG--TCTATTC	