Community Dynamics of Freshwater Picocyanobacteria and Development and Application of HIP 1 PCR (Cyanobacterial Typing Technique).

Katie Johanna Harper

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Abstract

The main aim of this study was to further our understanding of the diversity of a freshwater picocyanobacterial community. The HIP 1 PCR typing technique was developed and applied to 506 isolates of picocyanobacteria from a field study on Esthwaite Water in 2000. This study has demonstrated that genetic diversity existed within the picocyanobacteria characterised morphologically by rod shaped cells and high phycocyanin pigment content. Picocyanobacteria of this morphology represented a significant proportion of the picocyanobacterial community within the lake in 2000, which was typical of summer conditions for that lake. Twenty-one HIP 1 types were defined; ninety isolates were not assigned to a type as they generated HIP 1 PCR products which were insufficiently similar to less than five other isolates. The diversity of HIP 1 PCR products generated from isolates within the types ranged from homogeneous complex patterns to similarity of one key PCR product and heterogeneity of minor products. It is likely that on further isolation and testing of isolates within the more heterogeneous types further splitting of these types will be appropriate.

The HIP 1 types were each re-isolated on at least two sampling occasions (one month apart). Some types were isolated from sites spanning the sampling period demonstrating that there was some stability within the HIP 1 types within the picocyanobacterial community within Esthwaite Water over the summer of 2000. Some types appeared to be isolated more successfully at different times of the sampling period suggesting a temporal shift/changes of HIP 1 types in the picocyanobacterial community between June and October in Esthwaite Water. It was possible to demonstrate that three HIP 1 types were isolated at statistically different success rates from some spatial or temporal locations.

It was concluded that picocyanobacteria are considerably more diverse than indicated by gross morphology. Picocyanobacteria of different genotypes may play a range of functional roles within the freshwater environment. The HIP 1 PCR typing technique may allow us to get a handle on the diversity of this group in order to investigate this further.

Within this study the HIP 1 PCR typing technique was developed for application to field study. Several methods of DNA extraction were compared; the Dynabeads DNA Direct System 1 was selected for use in the field study as it produced the most reliable template for PCR at the lowest processing time and cost. The discrimination, specificity and reproducibility for the technique were investigated and the technique continued to show potential for application to field studies. A method was devised for assessing the similarity of PCR products amplified by HIP 1 PCR for application to the field study isolates. This method was demonstrated as sufficient to appropriately group the PCR products from four different templates of cyanobacterial DNA separated repeatedly on different electrophoresis gels. On application to the field isolates this method assisted with the grouping of isolates into HIP 1 types, however the final user interpretation of the PCR products was also necessary. It is also expected that on further isolation/analysis of these types further definition of HIP 1 genotypes will be appropriate.

The HIP 1 PCR technique has been demonstrated to be a useful tool for assessing diversity within ecological studies. The major advantages of the technique compared to others are the discrimination, cost, speed and simplicity of the technique and capacity

for analysis of large numbers of isolates. The major limitation of the technique is the requirement for isolation of cyanobacteria.

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I made it!

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Chapter 1 General Introduction

1.1 The importance of freshwater

Water is essential to all life. The distribution of water on Earth is shown in Table 1.1. Water is continuously moving between each component of the hydrological cycle supporting life in different ways during the process. Lakes and rivers, although comprising only 0.01% (Press *et al.* 1994) of water on the planet are vital to the survival of mankind; provide drinking water, food supply, transport systems and a recreational resource. Increasingly they are also being valued for their ecological importance (Moss 1988). The Water Framework Directive (Directive 2000/60/EC) is European legislation, which for the first time has set ecological targets for surface water. It also recognises the importance of water in all components of the hydrological cycle and will encourage management of water on a catchment scale. European and national designations under the Habitats Directive (Directive 92/43/EEC) and Wildlife and Countryside Act 1981 have also been applied to lakes and rivers in the UK setting targets for environmental improvements and protection. A full list and details of the features of interest is available on the Joint Nature Conservation Committee website (www.jncc.gov.uk).

Table 1.1 Distribution of water on Earth

Location of water on Earth	**Percentage of total water**
Oceans	97.3
Glaciers and polar ice	2.1
Underground aquifers	0.6
Lakes and rivers	0.01
Atmosphere	0.001
Biosphere	4×10^{-5}

N. B. Source: (Press *et al.* 1994)

1.2 The formation of lakes

Many lakes were formed by tectonic movement, either by the separation of continental plates of the Earth's crust or knock-on effects in adjacent land areas. These processes were responsible for the formation of some of the Earth's largest and most dramatic lakes, such as those of the Rift Valley and adjacent areas (East Africa) and Lake Baikal in the USSR (Press *et al.* 1994). Volcanic activity can dam river flows or leave craters within which lakes can form (Press *et al.* 1994). However, glacial activity has been responsible for the formation of most of the lakes on the planet. Ice movement scraped out hollows with debris plucked from the land beneath and rock fragments ripped free by freeze-thaw action. Debris was dumped during the retreat of glaciers formed plugs or natural weirs which now retain water (Fryer 1991).

1.3 Factors influencing lake ecology

Lake ecology is a function of hydrological, physical and chemical characteristics of each lake. The hydrology of lakes is related to water inputs from precipitation, streams and groundwater. Water outputs from lakes include evaporation, out-flowing streams and groundwater. The surrounding catchment characteristics (climate, topography, soils, geology, land-use etc) and lake bathymetry interact to impact on lake level and residence time (Winter 2004). Physical characteristics include; water temperature and density, particularly where changes in density within the water column lead to thermal stratification, and light penetration. Sub-surface light intensity is impacted by reflection (which alters with sun position and wind induced waves) and absorption by water molecules and suspended particles (including inorganic particles and plankton) (Reynolds 2004) and dissolved substances (Moss 1988). The climatic zone and

geomorphology of each lake basin control these factors. Lake water chemistry is related to the geology and land use/cover of catchment of each lake. Water chemistry is also affected by the ecology and chemical and biochemical processes which occur within the lake itself. Lakes are classified by nutrient levels and corresponding density of planktonic community as these characterize lakes which function in different ecological ways. Lakes classified on these characteristics are termed as having different trophic status. Table 1.2 shows the categories of trophic status (oligo-, meso-, eu- and hyper- trophic) based on mean annual phosphorus concentrations, mean annual chlorophyll and maximum chlorophyll concentrations, mean and maximum secchi depth (as an indicator of light penetration) and oxygen saturation.

Table 1.2 Summary of physical and chemical characteristics of lakes

Component	Trophic status				
	Ultra-oligotrophic	**Oligotrophic**	**Mesotrophic**	**Eutrophic**	**Hypertrophic**
TP_a	<4	4-10	10-35	35-100	>100
Chl_m	<1	1-2.5	2.5-8	8-25	>25
Chl_{max}	<2.5	2.5-8	8-25	25-75	>75
Sec_m	>12	12-6	6-3	3-1.5	<1.5
Sec_{min}	>6	6-3	3-1.5	1.5-0.7	<0.7
O_{sat}	90%	80%	90-40%	40-0%	10-0%

TP_a, annual mean total phosphorus ($\mu g\ l^{-1}$); Chl_m, annual mean chlorophyll ($\mu g\ l^{-1}$); Chl_{max}, annual maximum chlorophyll ($\mu g\ l^{-1}$); Sec_m, annual average secchi disc transparency (m); Sec_{min} minimum secchi disc transparency (m); O_{sat} percentage oxygen saturation. Reproduced from Padisak (2004).

These parameters are all interlinked with the density of algae (measured by chlorophyll concentration) dependent on nutrient availability (phosphorus concentration), often a prime cause of low light penetration and reduced oxygen saturation (due to bacterial breakdown of cells). However measurement of these parameters (phosphorus, chlorophyll, transparency and oxygen saturation) on a single time occasion could not be used to classify a lake into a trophic status. These parameters can overlap between the classifications of trophic status, thus mean annual

and maximum values are important. Different ecological communities develop in lakes of different trophic status. The diversity of the phytoplankton to the type of fish, which are the predators at the top of the food chain can characterise the communities within lakes of different trophic status. The designation of standing waters of European importance under the Habitats Directive specifies lakes of particular trophic status as providing habitats of specific importance (e.g. Ullswater is designated as a mesotrophic lake, and Wastwater as an oligotrophic lake). Although it is the whole lake that is designated, the notifications reference in particular the typical macrophytes (aquatic vascular plants) found within the littoral zones of these lakes (www.jncc.gov.uk).

1.4 Lake habitats

Lakes contain a variety of habitats for a diverse range of organisms. Figure 1.1 summarises these as the open water of pelagic zone, the shoreline or littoral zone, the benthic zone and bathypelagic zones and the abyssal zone and the key gradients which operate between each (Reynolds 2004). The bathymetry of each lake determines the respective significance of each zone and inhabiting food chains that are shown in Fig 1.2.

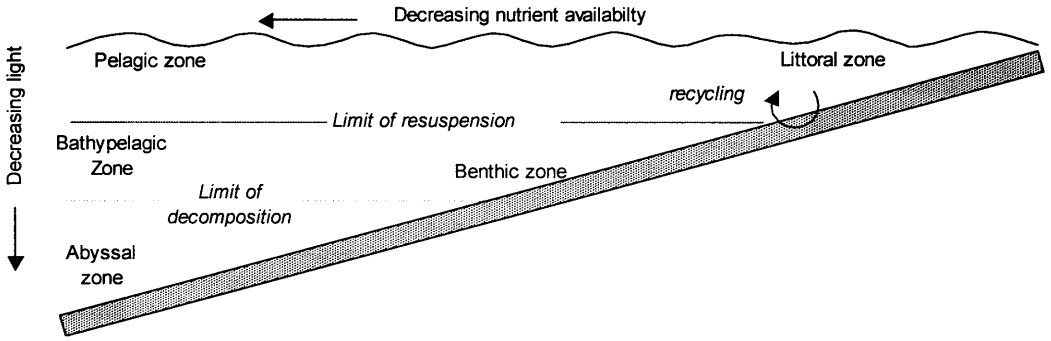


Figure 1.1 Diagrammatic section through a (very large) lake.
N. B The main sub-habitats (zones) and the main gradients of increasing limitation of primary production are shown. Reproduced from Reynolds (2004).

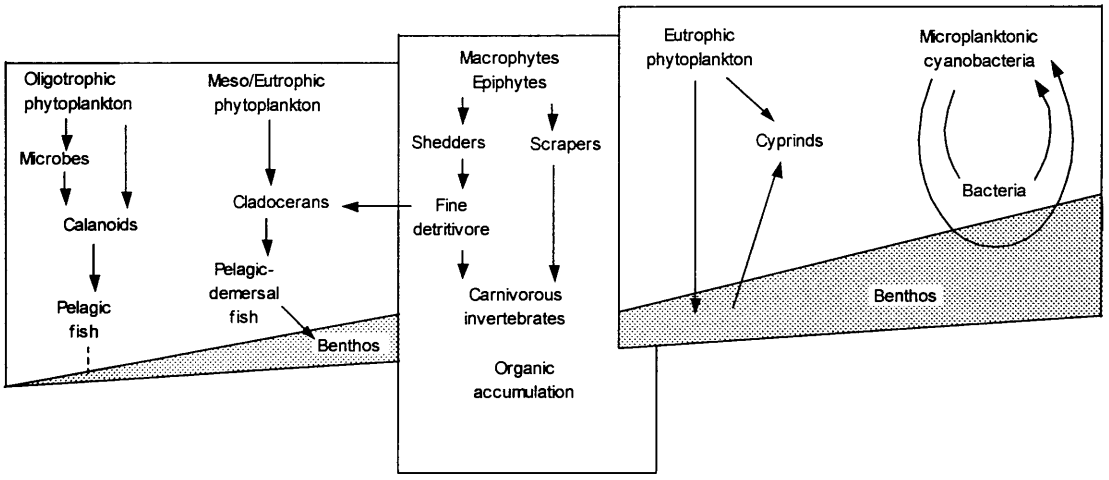


Figure 1.2 Stylised limnetic food chains, broadly segregated by habitats.
N. B The left hand box represents open-water, pelagic, self-sustaining trophic pathways, sub-divided to separate deep-column oligotrophic systems from more eutrophic or benthos-sustaining linkages. The right hand box accommodates two other food chains, the degraded alga-benthos-cyprinid pathway of small, enriched waters and the shallow lake dominated by *Oscillatoriales* between them is a macrophyte – based web which represents the sort of small water system and an important interface with open water in larger lakes. Reproduced from Reynolds (2004).

1.5 Phytoplankton

The phytoplankton of the pelagic zone does in itself contain a diverse spectrum of organisms. These are often classified on a size-structured basis as summarised in Table 1.3.

Table 1.3 Size classes of phytoplankton

Name	Lower limit	Upper limit	Examples
Picoplankton	0.2 μm	2 μm	*Synechococcus, Nanochloris, Chlorella*
Nanoplankton	2 μm	20 μm	*Rhodomonas,* many *Chlorococcales,* small *Chrysophytes*
Microplankton	20 μm	200 μm	*Asterionella, Ceratium, Sphaerocytis, Snowella*, filamentous cyanobacteria
Mesoplankton	200 μm	2 mm	*Gleotrichia, Aphanizomenon* clusters
Macroplankton	2 mm	2 cm	Extremely large M*icrocystis* colonies

N. B. Note that categories are also applicable to heterotrophic organisms. Viruses are included in femtoplankton (< 0.2 μm). (Reproduced from Padisak (2004))

Many years work has been dedicated to understanding the diversity and functioning of the phytoplankton community (reviewed by Reynolds (1984)). The amalgamation of much of this work and the brain power of around 30 ecologists culminated in the agreement of a model of seasonal succession in the phytoplankton – the PEG (Plankton Ecology Group) model (Sommer *et al.* 1986). This model formalised the synthesis of many of the proposed and well-understood hypotheses for the seasonal patterns within the plankton observed in many lakes. In summary nutrient availability and increasing light at the end of winter enable the growth of diatoms and cryptophytes and chlorophytes that form the spring bloom. This ends as nutrients, particularly silica, become limiting, the water column begins to stratify, and there is strong grazing pressure on these plankton from zooplankton such as Daphnia. A "clear-water phase" follows the spring bloom, where the abundance of phytoplankton is much less significant. As spring progresses into early summer, mixing of the water

column decreases and thermal stratification becomes more stable. Diatoms and small chlorophytes that characterise the spring bloom, but have escaped grazing by zooplankton, are sedimented as in general they are unable to regulate their positioning the water column. At this point the dominant nitrogen species generally alters in the water column. Ammonium is recycled from the breakdown of the algal cells of the spring bloom and any nitrate remaining after uptake from the spring bloom diminishes due to denitrification if the hypolimnion and/or sediment surface have become anoxic. Herbivorous zooplankton become food limited and are also controlled by predation from fish. Summer phytoplankton growth then increases, with a species richness and functional diversification of small 'undergrowth' species and inedible 'canopy' species. Nutrient depletion and grazing controls the growth of many algae and there is a shift towards those which can utilise phosphorus in the hypolimnion and fix nitrogen. There is a change in the herbivorous community to smaller species and rotifers which are less vulnerable to fish predation. Mixing of the water column results in replenishment of nutrients, but also circulates algae away from photosynthetically active radiation for long periods. Diatoms and small phytoplankton increase in numbers and provide a food source for zooplankton. As day length reduces, phytoplankton can no longer reproduce efficiently and decline to the winter minimum, followed by a decline in the grazing community. Viral lysis and other pathogens can also affect plankton community succession. For instance populations of *Ceratium* have been observed to crash suddenly as a result of fungal pathogens. Viruses are known to have similar affects on phytoplankton populations (Furhman 1990; Willhelm *et al.* 1999), although most work has been performed on marine environments. More recently studies have also been initiated in freshwater environments (Dorigo *et al.* 2004). On comparing viral abundance in marine and

freshwater environments, Maranger *et al.,* (1995) found a higher abundance in freshwater and associated this with higher bacterial numbers, but also the relative importance of cyanobacteria in these environments.

1.6 Cyanobacteria – ecological importance and nuisance

Cyanobacteria play a number of key roles in the planktonic communities in lakes. They have been on Earth for at least 2.8×10^9 years (Whitton *et al.* 2000). They are prokaryotic and photosynthetic organisms. They are unique amongst the bacteria as they are able to synthesize chlorophyll *a* and use water as an electron donor during photosynthesis, which results in the evolution of oxygen. Cyanobacteria are thought to have been responsible for the transition of the Earth's atmosphere from its primordial anaerobic state to its present aerobic condition (Fogg, 1973).

Cyanobacteria are important constituents of virtually all aquatic systems on Earth (Hyenstrand *et al.* 1998). They have become best known in the non-scientific media for forming dense blooms and producing toxins in freshwater under eutrophic conditions (Crawford *et al.* 2000; Oliver *et al.* 2000). However they also exist in almost all other environments including the most extreme - from hot springs to hyper saline pools, and the polar regions to the tropics (Oren 2000; Vincent 2000). Some cyanobacteria genera can tolerate low oxygen concentrations (Stal 2000), exposure to heavy metals and free sulphide (Padan *et al.* 1982), and high UV-B and UV-C radiation (Castenholz *et al.* 2000) and can survive periods in the dark in anaerobic conditions (Doolittle *et al.* 1974). Cyanobacteria have been found to tolerate temperatures up to 73°C (Ward *et al.* 2000), and are still viable after freezing (Vincent 2000) and desiccation (Pentecost *et al.* 2000). The highest abundances and diversity of cyanobacteria are found at alkaline pH (Whitton *et al.* 2000), however some

picocyanobacteria are found at alkalinity as low as pH 4 (Steinberg *et al.* 1998; Whitton 2000).

Cyanobacteria play a significant role as carbon and nitrogen 'fixers' in marine ecosystems where they often dominate the plankton (Paerl 2000). The role played by cyanobacteria in freshwaters is complex due to the diversity of the group and variety of ecological adaptations possessed.

1.7 Ecophysiological diversity of cyanobacteria

In a review of the factors determining cyanobacterial success in freshwater, Hyenstrand *et al.* (1998) stated that single factor relationships have failed to explain the success of cyanobacteria as they represent such a diverse range of organisms. Thus he suggested that they should be split into at least three sub-groups: i) those not capable of buoyancy regulation and fixing molecular nitrogen, ii) those capable of buoyancy without the ability to fix nitrogen and iii) those capable of both buoyancy regulation and nitrogen fixation. Hyenstrand *et al.* (1998) also concluded that to understand cyanobacterial development and population dynamics parts of nine single factor theories proposed in the literature (summarised in Table 1.4) are required.

Table 1.4 Factors proposed to influence cyanobacterial success.

Hypothesis name	Details	Authors
TN/TP	Cyanobacteria can be favoured by a low ratio of nitrogen to total phosphorus	Schindler (1977), Smith (1983)
Low Light	Cyanobacteria can have competitive advantages at low light intensities	Mur *et al.* (1978) Zevenboom *et al.*(1980)
High pH/low CO_2	Cyanobacteria can out-compete other algae in water with a high pH or low CO_2 content	King (1970), Shapiro (1973; 1984; 1990)
Buoyancy	Cyanobacteria can gain a competitive advantage by being able to regulate their buoyancy and thus position in the water column.	Reviewed by Reynolds *et al.* (1987)
Elevated water temperature	Cyanobacteria can be favoured by high water temperature.	Reviewed by Robarts *et al.* (1987)
Zooplankton grazing	Cyanobacterial success can be due to resistance to grazing.	Reviewed by Heaney (1987)
Storage strategy	Cyanobacteria that migrate from the sediment can bring an internal phosphorus reserve from the bottom and thereby gain competitive advantage.	Pettersson *et al.* (1993)
Inorganic nitrogen	Ammonium-nitrogen can favour the development of non-nitrogen-fixing cyanobacteria, whereas nitrate-nitrogen favours eukaryotic phytoplankton. Nitrogen scarcity favours the development of nitrogen-fixing cyanobacteria.	Blomqvist *et al.* (1994)
Trace element	Cyanobacteria (generally nitrogen-fixing) can have higher requirements for trace elements compared with eukaryotic algae.	Reuter *et al.* (1987)

Reproduced from (Hyenstrand *et al.* 1998).

It is evident from reviewing the literature of cyanobacterial field observations and laboratory studies that the ecological roles and requirements of this group have changed substantially since their initial evolution. The group is now composed of such different organisms that each must be studied within the field and laboratory to understand the parameters involved in their population dynamics. These single parameter theories (Table 1.4) probably explain the success of particular genera or species of cyanobacteria but are inadequate for generalising about cyanobacteria as a group as they are so diverse and have such different roles.

The factors involved in promoting the success of cyanobacteria in different growth phases e.g. the initiation, development of the population, and prolonged success and domination of filamentous and colonial cyanobacteria also appear to be different. Those factors, which stimulate the initiation of cyanobacterial growth, are still poorly understood. As water temperature increases during spring cyanobacteria appear in the water column either arising from low numbers which over winter in the water column or from the sediment. After thermal stratification has developed, filamentous and colonial cyanobacteria have the advantage over most eukaryotic forms of algae due to their ability to acquire nitrogen. Large filamentous and colonial cyanobacteria are actually less efficient at assimilating both ammonium and nitrate in comparison with eukaryotic phytoplankton. However cyanobacteria appear to adopt one of three strategies to obtain nitrogen, which enables them to out-compete other phytoplankton. Most obviously cyanobacteria such as *Aphanizomenon*, *Anabaena* and *Nostoc* are able to fix molecular nitrogen. Secondly, genera such as *Microcystis* (Ganf 1975) and *Anabaena* (Reynolds *et al.* 1982) contain gas vacuoles, have the ability to regulate buoyancy to migrate from the epilimnion to the hypolimnion to obtain ammonium. A third group of cyanobacteria such as *Oscillatoria* (now renamed *Planktothrix* (Suda *et al.* 2002)) are able to produce metalimnetic populations that have access to ammonium which is rapidly recycled by heterotrophic bacteria in this deep layer. However light and temperature also play a fundamental role in the productivity and success of cyanobacterial populations at different depths. Walsby and co-workers have demonstrated that *Planktothrix* populations are controlled by light and temperature rather than nutrient loading (Walsby *et al.* 2001; Davies *et al.* 2003). Buoyancy still provides an advantage, as cyanobacteria capable of moving upwards can regain a position in the water column where light is available after a deep mixing

event. However, other factors such as chytrid parasitism can also increase the speed at which populations diminish.

Cyanobacteria also have the capacity for storage of nutrients. Once a high density of cyanobacterial cells has accumulated, they have other adaptations along side those that enable them to cope with nutrient depletion to allow them to persist under the abiotic conditions that develop. High photosynthetic activity results in an increase in pH as the assimilation of nitrate and phosphate reduces the concentration of hydrogen ions in the water column (Stumm *et al.* 1996). Cyanobacteria have a high pH optimum and tolerance compared to other phytoplankton. In Blelham Tarn in the English Lake District, Reynolds (1986) recorded a halt in the growth of most phytoplankton species at pH 9.5-10, only *Anabaena* and *Microcystis* continued to grow at pH> 10. Diatoms were particularly intolerant of high pH, *Asterionella* was sensitive to pH >7.5, whilst the growth of autotrophic flagellates such as *Dinobryon*, *Mallamonas* and *Uroglena* terminated abruptly at pH 8.8.

1.7.1 Cyanobacterial morphological diversity

Compared with other prokaryotic organisms, there is great morphological diversity amongst the cyanobacteria. The most well known cyanobacteria are formed from aggregations of cells in either filaments or spherical or amorphous colonies that may also group together to form larger colonies. Cyanobacteria which form filaments differ in the morphology of individual cells, the level of branching which occurs (non or true or false branching), the presence of a sheath or mucous, the presence of akinetes and heterocysts. Filaments can also be straight or helical to varying degrees. Spherical and amorphous colonies can be composed of cells with different morphologies; they can be tightly or loosely aggregated in an organised or haphazard manner. The simplest

forms of cyanobacteria are found as unicells (*Chroococcus*). Unicells vary in size from 0.4 μm to >50 μm in diameter and differ in the number of planes of division.

1.7.2 Classification of cyanobacteria

Simple cyanobacteria were first described by Linneaus (1753) and were subsequently classified by phycologists under the botanical code between 1886 and 1892 (Whitton *et al.* 2000). This description and classification system continued into the 20th Century and is still used today. Discriminatory properties used to classify genera and species being either structural, or ecological based on features determined in the field. 'Types' were represented by herbarium specimens or descriptions and illustrations; under the Botanical Code cultures were not recognised as valid 'type material' (Rippka *et al.* 1979). In comparison to other prokaryotes, cyanobacteria do show a high degree of morphological diversity, and lend themselves to some extent to characterization in this way. The best known flora is that of Geitler (1932), which is particularly comprehensive although written in German. Drouet (1968, 1973 and 1978) recognised that previous descriptions of cyanobacteria may have represented phenotypic variations of the same species. However, he did not subject live strains to variations in environmental conditions and had no genetic information (Castenholz 1992). Many figures within the Fritsch Collection (a constantly updated collection of over half a million published illustrations and taxonomic entries on fresh- and brackish-water algae from worldwide distributions) demonstrate the morphological variability between isolates of cyanobacteria assigned to the same genera. It is clear now, that many cyanobacteria, which look morphologically similar, are genetically and ecologically diverse. On the other hand cyanobacteria can demonstrate pleiomorphy. Hence cyanobacteria that are genetically very similar can appear morphologically

distinct due to different environmental conditions. Grouping cyanobacteria based on morphological traits can therefore be misleading. The 'Rippka-Stanier' system in 1979 brought cyanobacteria into the Bacteriological Code. This Code is based on the properties of cultures, such as ultra-structure, biochemistry, cell morphology, molecular G+C ratios and genome size. This system uses different differentiation criteria and provides useful information about many strains of cyanobacteria. However, the requirement for each strain to be in culture so the properties can be determined makes it unwieldy for general application to the identification of cyanobacteria in ecological studies. Many cyanobacteria are also difficult to isolate and culture, particularly axenically (Rippka 1988). Axenic cultures are required to ensure the properties observed are those of the cyanobacterium rather than other contaminating bacteria. Thus this system is impossible to apply routinely within all field studies and to all cyanobacteria. Recently there has been a call to integrate cyanobacteria more fully into the Bacteriological code to reduce the confusion caused by the current situation of inclusion in both that code and the Botanical code (Oren 2004). The ultimate goal of this work is to achieve a consensus nomenclature that is acceptable both to bacteriologists and to botanists, anticipating the future implementation of a universal 'Biocode' that would regulate the nomenclature of all organisms living on Earth.

1.7.3 The ecology of freshwater picocyanobacteria

Picocyanobacteria have been discovered relatively recently by Sieburth (1978) in marine waters. These cyanobacteria are unicellular belonging to the Order *Chroococcales*. They range in size from 0.2 to 2.0 μm in diameter thus fitting the pico size range (Table 1.3). Since their discovery a substantial amount of knowledge

concerning the ecophysiology and controlling factors of marine picocyanobacteria has been gathered (Stockner *et al.* 1986; Hagstrom *et al.* 1988; Stockner 1988; Kuuppo-Leinikki *et al.* 1994). Cell concentrations range from 10^2 to 10^5 cells ml^{-1} in oceans (Waterbury *et al.* 1979; Chen *et al.* 1993), where they can contribute up to 25% of photosynthetic carbon fixation in oligotrophic oceans (Waterbury *et al.* 1986) and account for 64% of total photosynthesis in the North Pacific Ocean (Iturriaga *et al.* 1986). The role of picocyanobacteria in freshwaters remains ambiguous compared with the larger colonial forms of cyanobacteria. Picocyanobacteria have been studied less rigorously than larger cyanobacteria due to their more recent discovery, ease of overlooking them due to their small size and because the issues of identification and discrimination are compounded in this group due to apparent lack of distinguishing morphological features. As the focus of this thesis is freshwater ecology, the following literature review concerns the ecophysiology of freshwater picocyanobacteria, but it is supplemented with information about marine picocyanobacteria where it adds value.

1.7.4 Influence of physico-chemical factors

Studies on picocyanobacteria in freshwater have proposed a number of hypothesis to explain their abundance, contribution to the planktonic biomass and production. Some of these are summarised in Table 1.5.

Table 1.5 Relationships proposed between nutrient status and picocyanobacterial success.

Measure of Picocyanobacterial success	Effect	Nutrient parameter	References
Abundance	increases with	increasing nutrient concentrations	Hawley *et al.* (1991); Stockner (1991); Schweizer *et al.* (1992); Takamura *et al.* (1994)
Abundance	generally low in	lakes of high trophic status	Voros *et al.* (1998); Ball (1999)
Abundance	occasionally high in	lakes of high trophic status e.g. eutrophic or hyper-eutrophic status.	Wehr *et al.* (1987); Sondergaard (1991); Carrick *et al.* (1997)
Community dominance	decreases	with increasing trophic status and/or chlorophyll a concentration	Stockner (1988); Capblancq (1990); Takamura *et al.* (1994); Voros *et al.* (1998); Ball (1999)
Community dominance	increases with	ratio of total nitrogen to total phosphorus	Takamura *et al* (1994)
Community dominance	concentrations	of total nitrogen	Wehr (1989)
Production	no change	along trophic gradient	Capblancq (1990)

N. B Where Abundance = numbers of cells per ml, Community dominance = proportion of the biomass within the plankton composed of picocyanobacteria, Production = carbon fixation.

A number of studies have suggested a positive correlation between picocyanobacterial abundance/production and trophy, thus implying that picocyanobacterial abundance and production is higher in more productive lakes, with higher nutrient concentrations (Hawley *et al.* 1991; Stockner 1991; Schweizer *et al.* 1992; Takamura *et al.* 1994). Picocyanobacteria, and other autotrophic picoplankton (APP), are often found to be the dominant photosynthetic organisms in oligotrophic lakes (Stockner 1988; Capblancq 1990). Possible reasons for the relative success of picocyanobacteria in environments with low nutrient availability include their high surface area to volume

ratio, low growth requirements inherent for organisms of such small size (Wehr 1990; Raven 1998), and storage of nutrients. Voros *et al.* (1998) in a study of 32 Hungarian lakes observed that in lakes with less than 10 μg total chlorophyll *a* l^{-1} the contribution of picocyanobacteria to the total chlorophyll *a* concentration was more than 70%, and, conversely when total chlorophyll *a* exceeded 100 μg l^{-1}, the proportion contributed by the picocyanobacterial fraction did not exceed 10%.

Occasionally picocyanobacteria can dominate, or form significant populations in eutrophic and hypereutrophic waters (Wehr *et al.* 1987; Sondergaard 1991). Carrick and Schelske (1997) reported that picocyanobacteria contributed an average of 30% towards phytoplankton biomass in the hypereutrophic Lake Apopka (USA), and abundance exceeded 10^7 cells per ml^{-1}. Other studies have reported episodic midsummer picocyanobacterial blooms occurring in eutrophic lakes (Cronberg *et al.* 1981; Bailey-Watts *et al.* 1991). However, most studies agree that the contribution of picocyanobacteria in terms of biomass decreases with trophy (Stockner *et al.* 1988; Capblancq 1990; Sondergaard 1991; Takamura *et al.* 1994; Ball 1999).

It has been hypothesized that the ratios of nutrients, rather than trophy play an important role in controlling the contribution of picophytoplankton (all chlorophyll *a* 0.2-2 μm) to phytoplankton biomass with evidence that it is positively correlated to an increasing ratio of total nitrogen (TN) to total phosphorus (TP) (Takamura *et al.* 1994). The TN-TP ratio typically decreases with increasing trophy. Thus oligotrophic lakes have high TN-TP ratios (Downing *et al.* 1992). Wehr (1989), found that, by experimentally increasing TN:TP ratios in Lake Calder, the dominance of picoplankton was not favoured. He suggested that actual concentrations of specific nutrients were more responsible for limiting the abundance of different size classes of algae. The chlorophyll *a* concentration of the micro- and nano- plankton increased

after N additions (2.5 to 50 μM), and P additions (1 to 20 μM) whilst the picoplanktonic fraction did not respond to P additions, and responded only slightly to N additions. It has been reported from several studies that picocyanobacteria are not affected by phosphorus limitation as they have rapid and superior rates of uptake compared to other algae (Suttle *et al.* 1988; Wehr 1993; Mastala *et al.* 1996), however, this fast uptake rate may be a sign of nutrient limitation. The induction of a high affinity uptake system may occur at low nutrient concentrations ensuring the competitive advantage above other organisms in the uptake of phosphorus when available only in low concentrations. This may explain why they compete successfully in environments with low phosphorus availability. In common with many cyanobacteria, picocyanobacteria also have the ability to store phosphorus intracellularly in the form of polyphosphate granules (Kulaev *et al.* 2000). Picocyanobacteria can scavenge phosphate in low concentrations and then mediate growth rate and uptake dependent on internal stores (Wagner *et al.* 1995; Ritchie *et al.* 2001). The high contribution of picoplankton to production in waters with a low trophic status may be a consequence of higher phosphorus limitation of autotrophic nanoplankton (ANP) and larger algae compared to picocyanobacteria. Pinel-Alloul *et al.* (1996) found that total phosphorus concentrations of >20-30 μg l^{-1} acted as a threshold, below which APP were dominant and above which, ANP were able to out-compete APP. Episodic midsummer picocyanobacterial blooms in eutrophic waters may also be a result of very low biologically available phosphorus concentrations due to uptake by algal blooms (Wehr 1989) which becomes limiting to larger algae.

Most of our current knowledge about the nitrogen sources used by picocyanobacteria has emerged from the studies of marine systems. Marine picocyanobacteria can utilize ammonium, urea and nitrate as nitrogen sources, although the first two are generally

preferred (Glibert *et al.* 1990), and an strain without the ability to utilise nitrate has been isolated (Moore *et al.* 2002). Some freshwater isolates have been demonstrated to lack the ability to utilise nitrate (Probyn *et al.* 1985; Miller *et al.* 2001). Berman and Chava (1999) observed that a freshwater isolate of *Synechococcus* from Lake Kinneret grew markedly better on urea compared to ammonia or nitrate. Pinel-Alloul (1996) also found a negative correlation between nitrate and freshwater APP. Neilson and Larsson (1980) found that marine picoplankton have the capacity to use a range of organic nitrogen sources and that preference may be species or even strain specific.

Picocyanobacteria appear not to use cyanophycin (a polypeptide synthesized by many cyanobacteria when protein synthesis is curtailed and nitrogen still remains available) as an insoluble nitrogen reserve (Allen 1984; Carr 1988). They can, however, use photosynthetic pigments including; phycocyanin (Antia *et al.* 1977) or phycoerythrin (Wyman *et al.* 1985) as a source of nitrogen in times of nitrogen limitation, which results in an alteration of cell colour (Allen *et al.* 1969).

In 1969, Wyatt *et al.* discovered that a chroococcoid cyanobacterium (*Gleocapsa* sp. later re-designated as *Gleothece* (Rippka *et al.* 1979)) had the ability to fix atmospheric nitrogen without heterocysts. Since then, other unicellular cyanobacteria from marine, brackish and freshwater environments have been reported as possessing this ability (Kallas *et al.* 1985; Roger 1985; Reddy *et al.* 1993; Leon *et al.* 1996). The separation of nitrogen fixation from the presence of oxygen is managed temporally within these cells (e.g. at night when respiration utilises oxygen and generates carbon dioxide (Bergman *et al.* 1997)). However, although some were originally classified as *Synechococcus* or *Synechocystis* (Waterbury *et al.* 1989), only one N_2-fixing isolate (PCC 7335) remains assigned to *Synechococcus* and two marine isolates remain assigned to *Synechocystis* (Bergman *et al.* 1997). The rest have been reclassified as

Cyanothece or *Gleothece* which are > 2 μm in diameter. Some freshwater picocyanobacteria have been shown to lack the nifH gene which encodes for a subunit of nitrogenase required to fix nitrogen (Postius *et al.* 2001). As previously mentioned, freshwater picocyanobacteria are also more often limited by nitrogen than phosphorus (Wehr 1989; Suttle *et al.* 1991; Wehr 1991) indicating that they can not fix nitrogen, at least within the environmental conditions of those studies.

Light intensity, and quality, are known to affect picocyanobacterial production (Stockner *et al.* 2000). Picocyanobacteria have been found at a variety of depths and light irradiance values (Fahnenstiel *et al.* 1992; Nagata *et al.* 1994; Callieri *et al.* 1995). Laboratory experiments have shown that a marine *Synechococcus* will grow under irradiances of up to 2000 μmol photon $m^{-2}s^{-1}$ but photosynthetic capacity, and hence growth rate, is saturated at 200 μmol photon $m^{-2}s^{-1}$ (Kana *et al.* 1987b). Pick (1991) found an inverse relationship between light attenuation and freshwater picocyanobacterial abundance in lakes of varying trophy, so a higher abundance was found in lakes with higher penetration of light to greater depths. Wehr (1993) demonstrated that picocyanobacteria can out-compete larger algae under conditions of low phosphorus availability and low light intensity. These two conditions are also characteristic of many of the marine environments where picocyanobacteria are successful (Glover *et al.* 1985). Picocyanobacteria also have the ability to withstand periods of darkness and have low light optima (Waterbury *et al.* 1986) and can thus survive circulation below the euphotic zone (where there is sufficient light for net photosynthesis to occur). Some picocyanobacteria alter their pigment composition in response to light intensity, typically increasing accessory pigment concentration relative to chlorophyll *a* (Falkowski *et al.* 1991). Therefore it appears that some picocyanobacteria are euryphotic and can adapt to different light intensities, whereas

others without the ability to adapt are only able to survive under particular light intensities (Stockner *et al.* 2000).

The quality of light influences the pigment type of the dominant picocyanobacteria. The penetration of different wavelengths of light is reduced by absorption and scattering within the water column. Although other matter such as dissolved yellow organic substances (Gelbstoff) and particles such as inorganic and organic detritus contribute to the attenuation of light, the quantity of algal cells/chlorophyll *a* in the water column is a major factor involved in light attenuation in freshwaters. Consequently the underwater light spectrum alters along the trophic scale with blue and green light penetrating well through oligotrophic waters and red light dominating the underwater light in eutrophic waters (Moss 1988). Picocyanobacteria, with the photosynthetic pigment phycoerythrin, are selected for by green light and hence dominate in oligotrophic waters, whereas those which only contain phycocyanin are favoured by red light and dominate in eutrophic waters (Callieri *et al.* 1996; Voros *et al.* 1998; Ball 1999). Postius *et al.* (1998) observed differential light adaptation of two genetically different isolates of phycoerythrin rich picocyanobacteria; one was isolated during spring and was able to alter its pigment composition to protect it from high light intensity, whilst the growth of an autumnal isolate, without this ability, was inhibited under high light intensities.

1.7.5 Influence of predation

Protozoa are recognised as the most important consumers of microbial production in fresh and marine waters (Porter *et al.* 1985). The significance of protozoan predation specifically on picocyanobacteria is also becoming increasingly clear (Stockner *et al.* 1986; Stockner *et al.* 1988). Protozoa are often grouped into flagellates, ciliates and

amoebae, however only flagellates and ciliates are of major importance in pelagic environments (Laybourn-Parry 1994).

Flagellates as small as 1μm in diameter can be found in marine environments, but in freshwater most fall into the 'nano' or 'micro' size categories and are referred to as nanoflagellates or microflagellates (Porter *et al.* 1985; Weisse 1990). Many flagellates are non-pigmented and, although they were initially thought to be osmotrophic, existing on dissolved organic compounds (DOC) which diffuses into the cells (Beers 1982), research has now firmly established heterotrophy of particulate matter as their main mode of nutrition (Porter *et al.* 1985). Heterotrophic flagellates have been found to be the principal predators of picoplankton in both marine (Perkins *et al.* 1981) and freshwater ecosystems (Boraas *et al.* 1985; Caron *et al.* 1985a; Fahnenstiel *et al.* 1986). Pernathaler *et al.* (1996) used fluorescently labelled prey to show that picocyanobacteria could constitute 15.9% (on average) of the total carbon uptake of heterotrophic nanoflagellates even though heterotrophic bacterial biomass was one order of magnitude greater than the picocyanobacterial biomass. This demonstrates that heterotrophic nanoflagellates can select for picocyanobacteria and that high turnover rates of picocyanobacterial biomass may be required to maintain standing stocks.

Many photoautotrophic flagellates are taxonomically, and structurally, related to heterotrophic flagellates, so it has been hypothesized that they may also be able to utilise the heterotrophic mode of nutrition (mixotrophy). Evidence for mixotrophic nutrition has been observed among a diverse range of pigmented flagellates including; *Ochromonas* (Fenchal 1982), *Cryptomonas borealis* (Wawrik 1970), *Cyathomonas truncata* (Schuster 1968), *Chrysochromulina* sp. (Jones *et al.* 1993), *Dinobryon* sp. (Jones *et al.* 1994) and a number of photoautotrophic dinoflagellates (Schnepf *et al.*

1992; Jacobson 1999). A more extensive study on the importance of, and factors that regulate mixotrophy in *Dinobryon* sp. has been performed (Bird *et al.* 1986; 1987) with the conclusion that mixotrophy could be important in the energy flow processes of lakes.

In freshwaters, ciliates generally fall into the 'micro' size range (Porter *et al.* 1985). They are being increasingly recognised as important predators of picocyanobacteria. For example in Lake Kinneret (Israel), where ciliates form a greater biomass than flagellates, ingestion of picocyanobacteria has been observed in 80% of ciliates in the epilimnion, which are responsible for grazing 4-9.5% of picocyanobacterial production per day. The growth rate of the ciliate *Colpoda* sp. was ten times higher on a diet which included picocyanobacteria than in its absence (Sherr *et al.* 1991). However, diet supplementation with heterotrophic bacteria and other small algae has been shown to be necessary to sustain the ciliate populations (Hadas *et al.* 1998). Laboratory experiments have also demonstrated that ciliates prefer, and select for, picocyanobacteria in preference to heterotrophic bacteria (Simek *et al.* 1995). Although most of the examples above indicate that protozoa prey on picocyanobacteria, the level of predation depends upon the environment studied. Jurgens *et al.* (2000) indicated that prokaryotic APP could resist grazing by protozoa in hypertrophic environments and increased to elevated levels at the expense of heterotrophic bacteria. Features which have been demonstrated to enable APP to resist grazing include slightly larger cell size and hydrophobicity of the cell wall (Monger *et al.* 1999). Alternatively they could be ingested, but resist digestion through the cell wall properties.

Rotifers and immature copepod nauplii have been shown to be responsible for significant grazing of picoplankton in marine environments (Roff *et al.* 1995). In

freshwater, ingestion of picocyanobacteria by rotifers and copepods has been observed (in Lake Ontario, Canada); however non-digested cells were identified in a copepod faecal pellet suggesting that they are resistant to digestion by these zooplankton (Caron *et al.* 1985). In 1991 (Fahnenstiel) demonstrated that although there was a slight increase in picocyanobacterial losses when *Daphnia* were more abundant, they never accounted for more than 21% of total grazing, and protozoa were always the dominant picocyanobacterial grazers.

1.8 Morphological diversity of picocyanobacteria

1.8.1 Size

Sieburth (1978) first recognized 'picoplankton' as ranging between 0.2 and 2 μm in size. Most studies use this size range to define picocyanobacteria. Methods used to sample and enumerate picocyanobacterial cells however often involve a pre-filtration step to segregate the 'pico' particles. The pore size of the filters used for pre-filtration varies between studies as shown in Table 1.6. Komarek (1999) suggested that picocyanobacteria range from 2-3 μm in diameter, but proposed that cells larger than 3 μm are also taxonomically related to those beneath that arbitrary cut-off imposed in many studies.

Table 1.6 Pore size of filters used to count and isolate picocyanobacteria

Pore size used for pre-filtration	Studies
2 μm	Ball (199); Takamura *et al* (1994); Malinsky-Rushanksy *et al* (1991); Voros (1998)
3 μm	Happey-Wood (1990); Hawley *et al* (1991); Craig (1984) Fahnenstiel *et al.* (1986); Nagata *et al.* (1996).
5 μm	Carrick *et al* (1997)

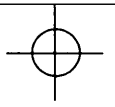
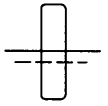
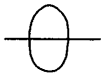
1.8.2 Cell Shape

Picocyanobacteria range from spherical, to long rods. They can also be ovoid to varying extents (see Table 1.7).

1.8.3 Cell aggregation

Many single-celled picocyanobacteria produce extra-cellular slime. Some colonial cyanobacteria are aggregates of pico-sized cells stuck together with extra-cellular slime. Whether the same species or strains can be found as single cells and aggregates is unclear. Naegli (1849) (referenced within (Komarek 1999), suggested that the genus *Aphanothece* (roughly aggregated pico-sized rod shaped cells) might be combined with *Synechococcus*.

Table 1.7 Intergeneric characters in unicellular, solitary living cyanobacteria (Komarek 1999)

Genus (Number of species)	Shape of cells	Type of cell division	Thylakoid pattern	Type of nucleoids	Type of binary fission	Involution cells	Type of S-layer	GC (% mol)	Cell dimensions (μm)
Synechocystis (21)	Spherical 					-	P6	30-48	0.7-15 diameter
Cyanobium (13)	Oval to short rod-like 	 Symmetric		Band like	Pinching 	Irregular	(p4?)	(36) 49.1-71	0.4-4.5x0.2-3
Synechococcus (24)	Cylindrical 		 Parietal			Filamentous	P4	47-56	1.2-28 x 1-6
Cyanobacterium (8)	Cylindrical to widely oval 	Symmetric or asymmetric	Length - wise 	 Irreg. Granular to net like	 Cleavage	Irregular to short filamentous		39-41	3.4-12x2-12
Cyanothece (6)	Cylindrical to widely oval 	 Symmetric	 Radial infrath. spaces*	 Net like		Irregular	P2	41	7-70x7-52

N.B Radial infrath. spaces = radial position of thylakoids with tendancy to form intrathlylakoidal spaces.

1.8.4 Differentiation using morphological characteristics

Initial attempts at classification of single-celled Cyanobacteria by Nagaeli in 1849 (Komarek 1999) split picocyanobacteria into two genera; *Synechococcus* – rods, and *Synechocystis* – cocci. The cell shape is a result of the number of planes of division rods are formed from a single plane, where as spherical cells are formed from two or more planes of division. The differentiation of picocyanobacteria in most field studies is often still limited to this gross cell morphology. This has resulted in the common perception that picocyanobacteria and in particular rod shaped or *Synechococcus*-type picocyanobacteria are ubiquitous in both fresh and marine open waters (Fogg 1995). Similarities have been drawn between the morphology of picocyanobacteria isolated from different environments in different decades. For instance Kennaway (1989) (PhD thesis referenced within Fogg (1995)) isolated a *Synechococcus* from a Welsh lake which was 'similar' to the marine Type I described by Johnson and Sieburth in 1979. Rippka *et al.* (1979) presented a simple table for the differentiation of strains of picocyanobacteria dependent on the presence/absence of thylakoids, method of reproduction (division in one or two planes, or cell budding) and presence/absence of a sheath (see Table 1.8).

Table 1.8 Classification of picocyanobacteria based on morphological characteristics

Reproduction by binary fission	Thylakoids absent	Division in one plane	Division in two or three planes
		Sheath present ***Gleobacter***	
	Thylakoids present	Sheath present ***Gleothece***	Sheath present
		Sheath absent ***Synechococcus***	Sheath absent ***Synechocystis***
Reproduction by budding	Thylakoids present	***Chamaesiphon***	

Reproduced from Rippka *et al.* 1979

Rippka *et al.* (1979) also recognised that *Synechococcus* show a very wide span of mean DNA base composition (Stanier *et al.* 1971; Herdman *et al.* 1979) and suggested that 'this character in conjunction with the phenetic properties may eventually make possible the recognition of additional genera; but to propose a generic split without taking base compositional data into account appears unwise'. More recently Komarek (1999) has combined literature on the classification of picocyanobacteria in a review of the intergeneric characters of cyanobacteria living in solitary cells. He differentiated 6 genera with gross and micro-morphological criteria and the percentage of G and C nucleotides (Table 1.7). An examination of the gross morphological criteria (cell dimensions which can be measured under light microscopy) demonstrates that picocyanobacteria not in culture cannot be differentiated with confidence even into just 5 genera. Figure 1.3 shows how the length of picocyanobacteria belonging to different genera overlap. It is also evident that with the range of dimensions possible, the shape of the cells will vary considerably, and may also overlap between genera.

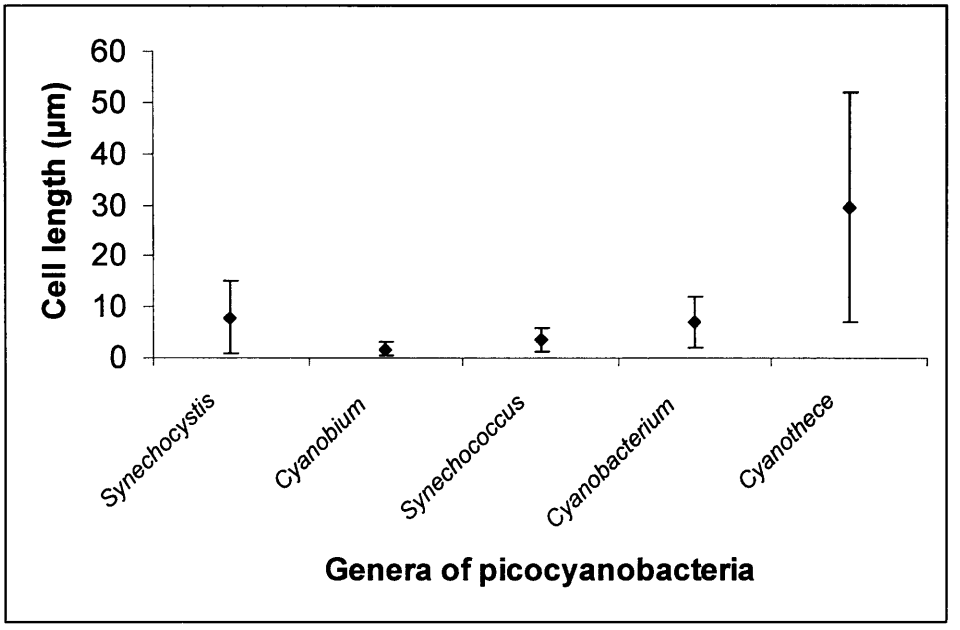


Figure 1.3 Ranges of cell lengths of picocyanobacteria belonging to different genera according to Komarek (1999).

S-layer proteins form the outermost cell envelope component of a broad spectrum of bacteria and archaea. S-layers are composed of a single protein or glycoprotein species (Mw 40-200 kDa) and exhibit either oblique (p1, p2), square (p4) or hexagonal (p3, p6) lattice symmetry with unit cell dimensions in the range of 3 to 30 nm (Sletyr *et al.* 1983). Observation of S-layer protein structure by electron microscopy can be used to differentiate between strains but will not alone provide an identity for a particular strain or species. This means that in order to differentiate picocyanobacteria by morphological characteristics the isolates must:

- be in culture (type of cell division)
- and/or observations using electron microscopy (e.g. type of nucleoids, arrangement of thylakoids, type of binary fission and type of S layer)
- plus methods not based on morphology (G+C % molecular) are required.

This classification matrix and classification based on this type of criteria therefore although have the potential for the discrimination of a small number of

picocyanobacterial isolates, it is too expensive in terms of cost and time, and requires a level of technical competency too high to apply routinely to large field studies.

1.9 Biochemical differences

Rippka *et al.* (1979) presented a table of the biochemical properties and physiological capabilities of *Synechoccocus* strains (Table 1.9). This demonstrates aptly that even within a small number of isolates (28), admittedly from a wide range of locations, a vast array of growth requirements and physiological capabilities are evident.

Table 1.9 Biochemical properties and physiological capabilities of *Synechococcus* strains

Mean DNA base composition (mol % GC)	PCC no.*	Facultative heterotroph, using: Glucose	Fructose	Sucrose	Glycerol	Synthesis of nitrogenase in anaerobiosis	Synthesis of C-PE Ψ	Marine #	Thermophile ϕ	Vitamin B12 requirement	Cell width > 3μm
↑	7202	-	-	-	-	-	-	-	-	-	-
39-43	7418	-	-	-	-	ND	-	+	-	+[s]	+
↓	7424	-	-	-	-	+	+	-	-	-	+
	7502	-	-	-	-	-	-	-	-	-	-
	7511	+	-	+	-	-	-	-	-	-	-
↑	6301	-	-	-	-	-	-	-	-	-	-
	6311	-	-	-	-	-	-	-	-	-	-
	6908	-	-	-	-	-	-	-	-	-	-
	6312	-	-	-	-	-	-	-	-	-	-
	6715	-	-	-	-	ND	-	-	+	-	-
	6716	-	-	-	-	ND	-	-	+	-	-
47-56	6717	-	-	-	-	ND	-	-	+	-	-
	6910	-	-	-	-	-	-	-	-	-	-
	7002	(+)	-	-	+	-	-	-	-	+°	-
	73109	(+)	(+)	-	+	-	-	-	-	+°	-
↓	7003	(+)	-	-	+	-	-	+	-	+°	-
	7117	-	-	-	-	-	-	-	-	-	-
	7335	-	+	-	-	+	-	+	-	-	-
	7425	-	-	-	-	+	+	-	-	-	+
↑	6307	-	-	-	-	-	-	-	-	-	-
	6603	-	-	-	-	-	-	-	-	-	-
	6710	-	-	-	-	-	-	-	-	-	-
66-71	6713	-	-	-	-	-	-	-	-	-	-
	6904	-	-	-	-	-	-	-	-	-	-
	6907	-	-	-	-	-	-	-	-	-	-
	6911	-	-	-	-	-	-	-	-	-	-
↓	7001	-	-	-	-	-	-	-	-	-	-
	7009	-	-	-	-	-	-	-	-	+[s]	-

N. B ND; Not determined; (+), weak growth; * strains bracketed together are probably independent isolates of the same species; Ψ C-PE = C-phycoerythrin; # Requirement for high concentrations of NA+, Mg2+, and Ca2+; ϕ Maxiumum growth temperature 53°C; °, Obligate requirement for B_{12}, [s] vitamin B_{12} stimulates growth but not an obligate requirement.

Pigment composition has been used as a characteristic to assess the diversity of populations of picocyanobacteria in marine environments (Wood *et al.* 1998; 1999). Picocyanobacteria with different pigment types in freshwater have been shown to dominate in different light and nutrient conditions (Pick 1991; Callieri *et al.* 1996; Postius *et al.* 1998; Voros *et al.* 1998). In general picocyanobacteria can be characterised as phycocyanin rich (PC rich), which auto-fluoresce red using epifluorescent microscopy, or phycoerythrin rich (PE rich), which auto-fluoresce orange using epifluorescent microscopy (Postius *et al.* 1999). It has however been demonstrated that pigment composition may vary with environmental conditions (Downes *et al.* 1998). As with morphology, biochemical properties and physiological characteristics of picocyanobacteria maybe unstable and vary with conditions. Thus discrimination of cyanobacteria using these features must be performed with caution as isolates, which appear to have different properties may actually be exhibiting pleiomorphy and be genetically similar.

1.10 Molecular diversity

Genetic techniques are increasingly being used to analyse the diversity and phylogeny of cyanobacteria. New molecular data confirm that 'modern' cyanobacteria constitute a phylogenetically coherent group, representing one of ten or more well defined phyla within the Domain Bacteria. The rRNA sequence diversity has demonstrated that of the five orders distinguished by Rippka (1979) only the Orders Nostocales and Stigonematales form a consensus group. The others; Orders Pleurocapsales, Chroococcales, and Oscillatoriales do not all group together. Individual taxa are grouped throughout the tree, indicating that the latter orders do not represent coherent

evolutionary lineages. In particular the genus *Synechococcus* appear to be clearly polyphyletic falling into at least five discrete groups (Preisig 1999). This throws into question the validity of much of the classification of cyanobacteria using morphological characteristics proposed to date.

1.11 Why identify?

So far I have discussed conventional methods for the classification of organisms and highlighted that genetic techniques can be used to assist with phylogenetic studies, where, the purpose is to differentiate between organisms and understand evolutionary lineages. From these systems have arisen terms used to label organisms based on how similar they are to each other for example an "isolate", a "strain" or a "species". A definition of each of these follows:

- An "isolate" is a population of microbial cells in pure culture derived from a single colony or cell (Struelens *et al.* 1996).
- A bacterial "species" can be defined as a group of isolates showing maximal similarity in all ascertainable phenotypic and genotypic characters and thus to show "overall similarity" (Pitt 1994)
- A "strain" is an isolate or group of isolates that exhibit phenotypic or genotypic traits which are distinctive from those of other isolates of the same species (Struelens *et al.* 1996).

There is much discussion over what these terms really mean, in particular the definition of a "species" has been highly contentious in relation to asexual organisms (Dijkshoorn *et al.* 2000) which although do not form from the genetic code passed from two parents, but instead may undergo drastic decreases in genomic information

in response to stress or increases for strengthening and DNA maintenance purposes (Kovacs *et al.* 1999). The species concept has also been discussed specifically in relation to cyanobacteria (Hoffman 1988; Castenholz 1992). It is important to understand what these terms mean in relation to the study of evolutionary lineages so different studies can compare "like" with "like" and naming of isolates is consistent. However, in order to study the ecological role of organisms although it is essential that organisms can be identified and discriminated, it is not necessary to know how they are evolutionarily related to others. This work is aimed at developing a technique, which can discriminate between picocyanobacteria using genetic information for the purpose of improving our understanding of the ecological role(s) of the group. The term clone, however is important as it defines isolated that are extremely similar to each other, and would be expected to produce the same HIP 1 PCR products.

1.12 Genotyping

Genotyping is based on the premise that the genomes of different organisms are different. DNA contains the code that ultimately describes why each organism functions and looks like it does. Thus the DNA of a bacterium contains significantly different information from that of a giraffe. DNA also contains a large quantity of non-coding information. In organisms that reproduce by sexual reproduction the genomic DNA is a mixture of DNA provided by each parent. In organisms that reproduce asexually the DNA of each progeny is a copy of that belonging to its parent. However, differences between the DNA of individuals can arise through various mechanisms, e.g. recombination, and can involve inversions, or translocations, insertions or deletions of DNA, and mis-incorporation of bases by DNA polymerases can also occur (Arbeit et al. 1990). Viruses can also promote genetic alterations

through transduction (Furhman 1990). Differences in DNA composition are exploited by genotyping. Genotypic classifications, therefore, are based on relatively stable and uniform molecular targets within each individual. Problems with discrimination (many look the same/appear to behave the same but are very different) and reproducibility (due to plasticity of these characteristics) and the long processing time associated with many techniques based on phenotypic characteristics (Kolbert et al. 1999) has lead to the development of numerous DNA-based methods for the analysis of heterotrophic bacteria (reviews include (Busch et al. 1999; Soll 2000; van Belkum et al. 2001). These techniques have been applied to heterotrophic bacteria for a wide range of purposes such as clinical practice (Maslow et al. 1993; van Belkrum 1994; Grundmann et al. 2001), the tracing of sources of food poisoning (Dombek et al. 2000; Guillaume-Gentil et al. 2002), and the study of diversity of natural populations of bacteria (Gray et al. 1999). Many of these techniques have also been applied to study the diversity of cyanobacteria, (examples of some of these studies are summarised in Table 1.10). These techniques differ in the target DNA analysed, the specificity in terms of which organisms they are applicable to, and reproducibility of the results. The complexity of the methodology and results, the time required and the cost also vary between the techniques considerably. All of these factors are important considerations in choosing the most appropriate technique for the type of study proposed. Table 1.10 compares the complexity of set up, procedure, results and analysis of results, plus the cost of setting up and performing the technique, the time required and level of discrimination, and reproducibility each technique affords for a range of genotyping techniques that have been applied to cyanobacteria. Each of the techniques was ranked for each of the criteria based on the specifics of each technique; see Table 1.11 for examples of justifications for the rankings.

Table 1.10 Comparison of molecular techniques with potential to analyse the diversity of cyanobacteria.

Molecular Technique	Method	Complexity of: Set up*	Procedure*	Results*	Analysis*	Set up Cost*	Processing cost*	Time*	Discrimination*	Reproducibility*	Cyanobacteria applied to:	References
Restriction Fragment Length Polymorphisms (RLFPs).	Chromosomal DNA is cut with rare-cutting restriction endonucleases and separated with pulse-field gel electrophoresis or Southern hybridisation is used to locate bands.	H	M	H	H	H	M	H	H	H	*Synechococcus* *Synechocystis* *Anabaena* and *Nostoc*	Chen *et al.* (1993); Churin *et al.* (1995); Plazinski *et al.*(1990); Lotti *et al*(1996)
RFLPs of Single gene/smaller area of DNA	Polymerase chain reaction (PCR) step followed by restriction. Often PCR products are separated by gel electrophoresis prior to restriction and afterwards. Alternatively restriction of genome is followed by Southern hybridisation step.	L	M	M	M	M	M	M	V	H	*Microcystis*. *Microcystis*, *Anabaena* and *Nostoc* isolates – No. of cyanobacterial genus and species. *Synechococcus* Marine *Synechococcus*	Otsuka *et al* (1999); Neilan *et al.* (1995); Bolch *et al.* (1996) phycocyanin gene locus. Lu *et al.* (1997); RFLP of 16-23S ribosomal DNA spacer region Ernst *et al.* (1995); *psbA* Neilan (2002); phycocyanin gene and 16S-23S rRNA internal transcribed spacer.
Ribosomal DNA/RNA (rDNA/rRNA) sequencing	PCR amplification which may need to be proceeded by primer development and DNA sequencing.	M	M	H	H	M	M	M	L	H	*Nodularia* Cyanobacteria *Planktothrix (Oscillatoria)* sp.	Barker *et al.* (1999); Iteman *et al.* (2000); Chen (2000)
Other gene sequencing	PCR amplification which may need to be proceeded by primer development and sequencing.	M	M	H	H	M	M	M	V	H	*Anabaena Azollae* *Nodularia*	Jackman *et al.* (1995); Nif loci Barker *et al.* (1999) for three non-coding areas of the genome

Molecular Technique	Method	Complexity of:									Cyanobacteria applied to:	References
		Set up*	Procedure*	Results*	Analysis*	Set up Cost*	Processing cost*	Time*	Discrimination*	Reproducibility*		
Denaturing gradient Gel Electrophoresis (DGGE)/ Temperature Gradient Gel Electrophoresis (TGGE)	PCR amplification directly from environmental sample. Separation of fragments by DGGE or TGGE.	H	H	M	M	M	M	M	V	H	Hot spring communities and microbial mats which included *Synechoccocus.*	Ferris *et al* (1996); Ferris *et al.* (1997); Nubel *et a.l* (1997)
Single Nucleotide Polymorphism genotyping by Single Nucleotide polymorphism extension (SNuPE)	PCR amplification of specific location from genomic DNA followed by single base extension labelling PCR amplification of the amplicon.	M	L	M	L	M	M	L	M	H	*Nodularia* from the Baltic Sea	Batley *et al.* (2003)
Allele targeted PCR	DNA sequencing followed by primer design to specific allele sequences. PCR to amplify from homologous sequence applied to isolates. DNA separation by gel electrophoresis.	M	M	L	L	M	L	M	V	H	*Nodularia* from the Baltic Sea	Hayes *et al.*(1997)

Molecular Technique	Method	Complexity of:									Cyanobacteria applied to:	References
		Set up*	Procedure*	Results*	Analysis*	Set up Cost*	Processing cost*	Time*	Discrimination*	Reproducibility*		
Targeted Repetitive PCR	PCR amplification using primers based on repetitive sequence. DNA separation by gel electrophoresis.	L	L	M	M	L	L	L	H	M	HIP1, STRRs and LTRRs are repetitive sequences which have been targeted in cyanobacteria	(Mazel *et al.* 1990; Jackman *et al.* 1995; Rouhiainen *et al.* 1995; Rasmussen *et al.* 1998)Mazel *et al.* (1990); Jackman *et al.* (1995); Rassmussen *et al.* (1998)
Non-specific RAPD	PCR amplification using arbitrary primers. DNA separation by gel electrophoresis.	L	L	M	M	L	L	L	H	L	*Anabaena* and *Microcystis*	(See also review below) Eskew *et al.* (1993); Neilan (1995)
Optimum rank for technique applicable for field studies	-	L	L	L	L	L	L	L	M/ H	H	-	-

N.B.* H = High, M= Moderate, L= Low, V= Variable. Assigned on comparative basis between techniques see Table 1.11 for further definitions.

Table 1.11 Examples of criteria used to assign ranks to genotyping techniques in Table 1.10.

	High (H)	**Moderate (M)**	**Low (L)**	**Variable (V)**
Complexity of set up	Much preparatory work required to set up systems e.g. requires temperature or denaturation gradient gels	Some pre-application work required	Minimal preparation – can just about hit the road running	-
Complexity of procedure	Requires a number of steps and/or complex electrophoresis	Requires at least 2 steps or can be fully automated	One-step technique	-
Complexity of results	High level of data yielded e.g. provides DNA sequence data	Results provide information on specific loci or a low level of information about locations dispersed through out the genome	Information yielded is about the presence of specific nucleotides in a small range of locations	-
Complexity of analysis	Requires sophisticated software to analyse results from a single genome	Requires sophisticated software to compare results from a number of isolates	Can be compared rapidly by eye	-
Cost – set up	Requires some state of the art equipment or a number of different pieces of equipment	Requires equipment of moderate cost	Requires only a small amount of equipment of minimal cost	-
Cost - processing	Consumables are expensive	Consumables are of moderate cost /required in moderate quantities	Consumables are inexpensive	-
Time (includes all preparation and analysis of results)	One isolate can be analysed over a number of days	20- 100 isolates can be analysed at once in a small number of days	>100 isolates can be analysed in a small number of days	-
Discrimination	One isolate can be distinguished from any other	Unlikely to distinguish between isolates of the same species but can differentiate between species	Isolates of the same species can be distinguished	Depends on cyanobacteria analysed
Reproducibility	Few factors can influence the results	Factors can affect the results at a number of stages but good laboratory practice and optimization/negative controls should minimise problems	It is difficult to ensure results are accurate as for example technique is non specific	-

It is evident from Table 1.10 that each technique has different attributes and limitations, which affect suitability for application to different types of study. Table 1.10 also shows how the best technique for application to field studies would compare. Some aspects of the techniques are however more important than others, with set up cost being variable dependent on equipment already available. Processing cost (cost for analysis per sample) and time affecting the number of isolates that can be analysed, and discrimination and reproducibility affecting the quality of results. This comparison demonstrates that whilst there is such a broad spectrum of techniques available to analyse genetic differences between organisms, the suitability of most of them in terms of application to field studies is limited. On one end of the spectrum are techniques which are complex, time consuming and expensive such as genome mapping and gene sequencing. These techniques are not applicable to a large number of isolates. Although the automation of sequencing has reduced the complexity and cost of this technique, and increased access to it recently, prior to the initiation of this study it was still not deemed to be appropriate on the field scale as the results remained complicated to analyse restricting application to small numbers of isolates (Muyzer 1999). The level of discrimination of sequencing can also be limited due to the highly conserved nature of some gene loci, which may mask diversity in microbes. Sequencing of rDNA loci is frequently used to assess the relationships between organisms. However it can be problematic as organisms currently recognised as different species can have almost identical 16S rDNA sequences (Fox *et al.* 1992), whilst significant differences have also been found between operons in a single bacterial clone (Amann *et al.* 2000). Ernst (2003) demonstrated that picocyanobacterial isolates with different morphological (e.g. S-layer type) and growth characteristics had very few differences in ITS-1 sequences. Thus phenotypic

diversification can occur at a higher frequency than the fixation of mutations in the non-coding sequence of the ribosomal operon.

Many techniques applied to analyse diversity require some prior knowledge of the organisms. Most techniques require a PCR step which means a primer complementary to a genomic sequence is required (e.g. techniques focused on a specific DNA locus described in Table 1.10). DNA sequence information is useful for generating these primers, but some developmental work may also be needed to ensure it is complementary to the isolates in question, and to optimise PCR conditions (Saiki 1989; Meunier *et al.* 1993). Genotyping techniques based on Single Nucleotide Polymorphisms (SNPs) have recently been developed (Syvanen 2001). Combined with fluorescent technology and capillary electrophoresis this technique can be employed to analyse the diversity of high numbers of isolates. Batley *et al.* (2003) analysed SNPs of three genetic locations within 500 filaments of *Nodularia* from the Baltic Sea. This technique has the advantage of not requiring a time-consuming gel electrophoresis step, and providing a definitive genotype for each isolate. Disadvantages include requiring the knowledge of SNP locations and some optimisation of the reactions to ensure the appropriate products are generated and contamination is minimized at each stage. The information yielded is also quite low as is restricted to the presence of a specific nucleotide at each location tested. It will be a useful technique to aid our understanding of genetic exchange. However, for application to studies to analyse ecological roles this type of technique may need to be applied to a number of loci to differentiate genetic types sufficiently. This is due to the fact that minimal variations in genotypes (i.e. three) can be observed at each loci. Akin with the problems associated with sequencing and resolution between phenotypically different types a high level of genetic and ecological diversity may be

hidden variation within the rest of the genome. Some loci may also be found to be more polymorphic than others as well which would aid in the discrimination and understanding of genotypes. At the other end of the scale are techniques that are non-specific and based on targeting random DNA sequences (Eskew *et al.* 1993; Neilan 1995; Mileham 1997). These techniques are useful where there is no prior knowledge of the organisms DNA sequences. However, when analysing non-axenic isolates, or cultures contaminated with free DNA (e.g. from environmental samples) randomly generated sequences will also target sequences from the contaminating DNA, as they are not specific to a single group of organisms (Mileham 1997; Soll 2000). This will have a severely detrimental affect on the reproducibility of the technique.

Techniques based on separation of PCR products by DGGE and TGGE are relatively recent in development (Ferris *et al.* 1996; Muyzer 1999). They require specialised equipment and a high level of testing before they can be used for routine application to samples or isolates. Problems associated with co-migration of DNA fragments and limited detection of rare community members affect the reproducibility and effectiveness of these techniques (Ferris *et al.* 1996; Muyzer 1999).

A number of different repetitive sequences have been used as targets to analyse the diversity of heterotrophic bacteria and cyanobacteria (Mazel *et al.* 1990; Versalovic *et al.* 1991; Gillings *et al.* 1997; Smith *et al.* 1998). Genotyping based on repetitive sequences is a one step technique; therefore it is quick, and relatively cheap making it suitable for the analysis of large numbers of isolates (Dombek *et al.* 2000; Zheng *et al.* 2002). Once the presence of a repetitive sequence has been commonly established in a group of organisms, primers can be developed which target this sequence. This technique can then be applied to most environmental isolates without considerable further development. Primers anneal to repetitive sequences, and DNA fragments are

amplified if the sequences are located closely enough together to support efficient amplification between neighbouring primer binding sites (Busch *et al.* 1999). The complexity of the results depends on the frequency and distribution of the repetitive sequence in the DNA of the target organism. Genotyping based on targeting repetitive sequences shows potential for suitability to application to field studies due to the limited work required to set up the procedure, ease of use and low cost.

1.13 Repetitive sequences

Repeated sequences are present in the genomes of all organisms (Lupski *et al.* 1992). Families of short interspersed repetitive elements have been found to exist in Bacteria. These include the repetitive extragenic palindromic (REP) elements, enterobacterial repetitive intergenic consensus (ERIC) sequences and the BOX element (Mazel *et al.* 1990; Versalovic *et al.* 1991; Gillings *et al.* 1997). Consensus primers to each of these elements have been used in polymerase chain reactions designed to amplify regions between neighbouring repetitive elements. This has resulted in unambiguous DNA fingerprints which enable the differentiation of bacterial species and closely related strains (Versalovic *et al.* 1991). Sequence data only describes REP and ERIC sequences in gram negative enteric bacteria and closely related phyla and the BOX element in gram positive *Streptococcus pneumoniae.* However, Gillings and Holley (1997), amplified PCR products from other organisms including fungi and plants using a primer based on ERIC sequences. Rasmussen and Svenning (1998) also produced PCR products from ERIC primers using axenic cyanobacterial templates. The use of ERIC primers to produce fingerprints of cyanobacteria for routine discrimination would, however, be inconvenient because axenic cultures are difficult to obtain and contamination would result in poor reproducibility.

In bacteria the most commonly repeated DNA sequences are derived from the large family of transposable elements or bacteriophage. Generally there are 2 to 40 copies of these repeated sequences per genome, and they are 500 to 10,000 bp in size (Kleckner 1981). Other repeated DNA sequences found in bacteria are smaller and often more highly repetitive. Examples include an 11 bp sequence involved in uptake of DNA by *Haemophilus influenzae* and the 33 bp REP sequence described for *E. coli* (Gilson *et al.* 1984). The number of copies of these short sequences varies from 500 to 1000 per genome. The involvement of REP sequences in biological phenomena as diverse as DNA gyrase binding and transcript stability has been proposed, but the precise functions of these sequences are still controversial (Stern *et al.* 1984; Mazel *et al.* 1990).

Other groups of repetitive sequences have been identified in cyanobacteria. Short tandemly repeated repetitive sequences (STRRs) have been found in heterocystous cyanobacteria (Mazel *et al.* 1990) and have been used to fingerprint cyanobacteria isolated from a Finnish lake (Rouhiainen *et al.* 1995). However, so many sequences were present in the DNA of the isolates tested, that a two staged approach of RFLP of the genome, followed by Southern hybrization with the repetitive sequence used as the probe. Long tandemly repeated repetitive sequences (37 bp) have also been identified in *Anabaena* PCC 7120 in low copy number, and other heterocystous and non heterocystous cyanobacteria (Masephol *et al.* 1996). Two other sequences REP-A and REP B have been described in *Microcystis* sp., but they appear to be absent from *Synechococcus* PCC 7942 and *Synechocystis* PCC 6803 (Asayama *et al.* 1996).

HIP 1 is a repetitive sequence found in genomic cyanobacterial DNA. It is an octameric sequence 5' GCGATCGC 3' which has been designated the highly iterated palidrome 1 (HIP 1) (Gupta *et al.* 1993). It was first identified at the junctions of a

gene (the *smtB* repressor) deletion event in *Synechococcus* PCC 6301 (Gupta *et al.* 1993), subsequently it was observed that this sequence occurred many times in the *smt* region. Analysis of cyanobacterial database entries showed that the HIP 1 sequence was in fact over-represented in the genomic DNA of many cyanobacteria (Robinson *et al.* 1995). Analysis of sequences from those organisms in the Genbank (and EMBL) release 74.0 suggested that HIP 1 may be abundant in all cyanobacterial genomes, but not other micro-organisms (Gupta *et al.* 1993). To confirm the significance of the HIP 1 sequence Robinson *et al.* (2000) assessed the frequency of occurrence of all possible 256 octameric palindromes. HIP 1 was singly over-represented in several strains, and constituted 2.5% of novel sequences obtained from clones selected at random from a library prepared from *Synechococcus* PCC 6301. This equates to a site occurring every 320 nucleotides within this organism. Evidence has shown that HIP 1 is absent from some cyanobacteria. No HIP 1 sequences have been identified in database sequences of a number of marine *Synechococcus* isolates, and HIP 1 PCR of *Calothrix* D253 produced no amplification products (Robinson *et al.* 1995). Robinson *et al.* (1997) demonstrated that HIP 1 is not restricted to particular clades of cyanobacteria by comparing the presence of HIP 1 sequences to phylogenetic clades produced using 16S rRNA. It occurs polyphyletically with those species without HIP 1 interspersed amongst those that possess the sequence. This poses questions about its origin, means of propagation, and function.

1.14 The HIP 1 PCR typing technique

The potential for the use of the HIP 1 sequence as a primer for the PCR to generate strain specific fingerprints of cyanobacteria was first demonstrated by Robinson *et al.* (1995). A smear of DNA was obtained from several strains of *Synechococcus* and

Synechocystis, but the PCR products from some isolates of *Nostoc* were less intense, suggesting that HIP 1 was less abundant in these strains. Characteristic banding patterns were also apparent. Smith *et al.* (1998) developed the technique of HIP 1 PCR further. Two bases were added to the 3' end of the HIP 1 sequence, increasing the length of the primer and thus specificity of the technique. Amplification was reduced to sites where the HIP 1 sequence was followed by the two additional bases. Smith *et al.* (1998) applied HIP 1 PCR to isolates of cyanobacteria representative of ten different genera which all yielded distinctive HIP 1 PCR products. They also applied HIP 1 PCR to a number of isolates of two species of cyanobacteria and found that HIP 1 PCR could provide resolution beneath the species level. Zheng *et al.* (2002) applied HIP 1 PCR to Cycad cyanobionts, and Orcutt *et al.* (2002) applied it to *Trichodesmium* spp. to analyse diversity. Comte *et al.* (2004) examined 32 strains of cyanobacteria, representative of 11 genera. They found the results to correlate with interspecies distinctions between *Planktothrix argardhii* and *P. rubescens* (both formerly *Oscillatoria*) and intraspecific diversity of *Microcystis areuginosa*, thus they confirmed the usefulness of this technique for rapid genotyping and verification of presumably identical strains from different culture collections. They also noted its cost-effectiveness compared to techniques which required expensive restriction enzymes. The successful application of HIP 1 PCR to cyanobacteria from such different genera and from completely different environments suggests that it may be a suitable technique for analysing the diversity of cyanobacteria in a vast range of field studies.

1.15 Aims of the study

The overall aim of this work was to investigate diversity within the picocyanobacterial community. Three objectives were proposed to meet this aim:

- Firstly to sample a larger number of picocyanobacterial isolates than previous studies
- Secondly to gain an overview of the dynamism of the diversity of a picocyanobacterial community in one habitat.

In order to achieve these objectives a field study was undertaken within an otherwise well characterised lake in the English Lake District (Chapter 2). The HIP 1 PCR typing technique was chosen as it appeared to have the potential to provide a useful insight into the diversity of picocyanobacteria from Esthwaite Water. However, before application of this technique to a field study, it required further optimimzation and development. This formed a third key objective of this study (Chapter 3). The application of the HIP 1 PCR typing technique to the isolates from the field study is described in Chapter 4.

Chapter 2 Field Study to investigate the spatio-temporal distribution of picocyanobacteria of Esthwaite Water

2.1 Introduction

2.1.1 The ecological field study

The role of picocyanobacteria in freshwaters requires further investigation as discussed in Chapter 1. The factors, which control the abundance, productivity and contribution to the phytoplankton community in terms of biomass or carbon fixation, are still uncertain. Due to their morphological homogeneity the diversity of the group beyond gross cell morphology (rods or spheres) has infrequently been studied in large scale field investigations. It has become apparent that different genera of filamentous and colonial cyanobacteria are controlled by different abiotic and biotic factors. This is almost certainly the case for picocyanobacteria as well. Some work has been performed on marine picocyanobacteria (Campbell *et al.* 1983; Waterbury *et al.* 1986; Campbell 1988; Glibert *et al.* 1990), and some work has been performed on small numbers of isolates from the freshwater environment (Ernst 1991; Ernst *et al.* 1995; Postius *et al.* 1999; Crosbie *et al.* 2003; Ernst *et al.* 2003). In the absence of an understanding of the diversity within the picocyanobacterial community, any ecophysiological diversity within the community of picocyanobacteria is also poorly understood, but may be fundamental to the role picocyanobacteria play in freshwater planktonic communities.

The purpose of this field study is to initiate the investigation of the diversity of picocyanobacteria of an otherwise relatively well studied freshwater lake in the UK.

2.1.2 Esthwaite Water – the study site

Esthwaite Water is a lake of approximately 1 km^2, it has a mean depth 6.42 m and maximum depth of 15.5 m (Ramsbottom 1976). It is located in the English Lake District (latitude 54°22'N, longitude 0° 49'W, Fig 2.2) to the west of Lake Windermere.

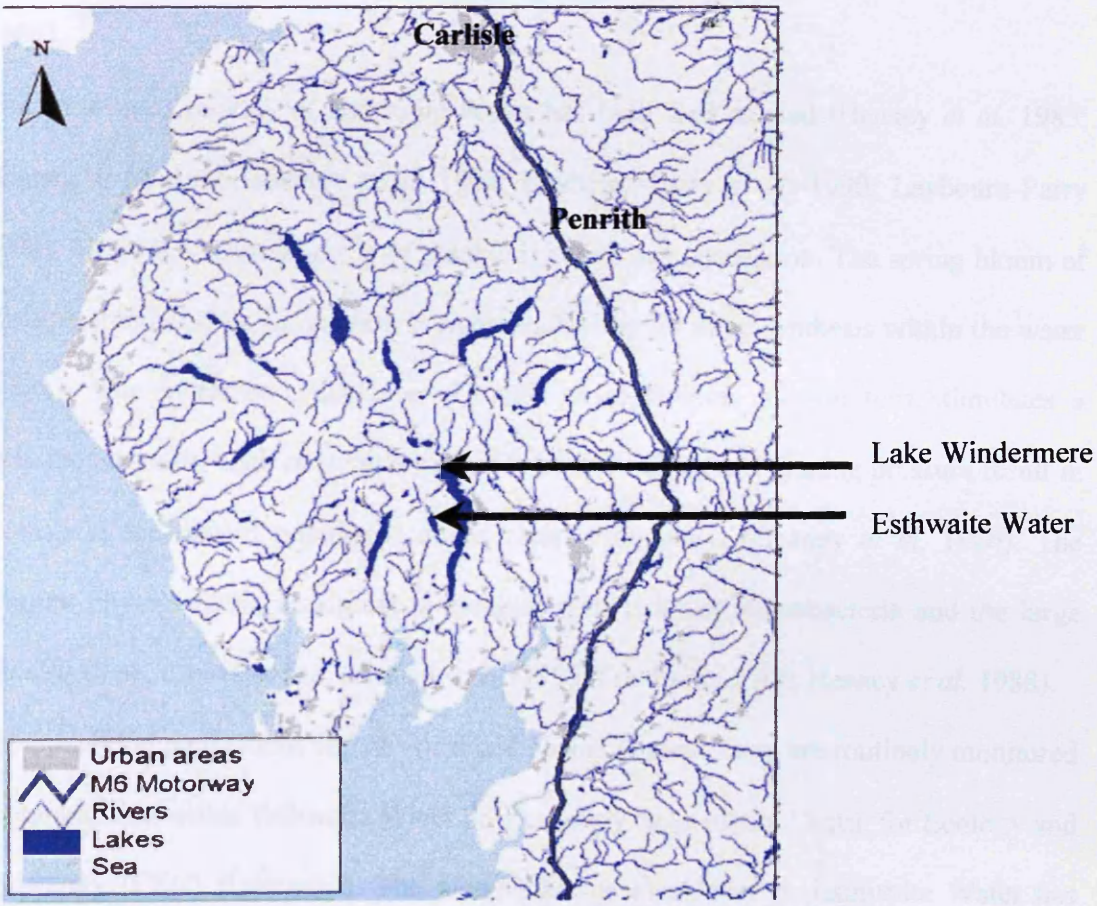


Figure 2.1 Location of Esthwaite Water in the English Lake District

The alluvial soils from the shallow lake basin provide a rich allochthonous supply of nutrients, furthermore in the early 1960s, lake productivity increased due to inputs of N

and P from inflows, particularly the major river Black Beck, which carried some sewage effluent. A more rigid phosphate stripping system was implemented in 1986 to sewage from Hawkshead, the largest village in the basin, and consequently the nutrient input to the lake has since decreased (Fryer 1991).

Esthwaite Water is monomictic, stratifying annually from May/June to September/October depending on prevailing meteorological conditions (Heaney *et al.* 1986).

The freshwater ecology of Esthwaite Water has been well studied (Heaney *et al.* 1985; Heaney *et al.* 1986; Heaney *et al.* 1988; Laybourn-Parry *et al.* 1990; Laybourn-Parry 1994). There is a seasonal cycle of plankton growth and succession. The spring bloom of diatoms is induced by an increase in light availability for photosynthesis within the water column. The increased abundance of small phytoplankton cells in turn stimulates a zooplankton peak. Lack of turbulence, a depletion of silica and grazing pressure result in a crash of the diatom population and a clear water phase (Heaney *et al.* 1986). The summer phytoplankton maximum is dominated by colonial cyanobacteria and the large dinoflagellate, *Ceratium* spp. (Heaney *et al.* 1980; Frempong 1981; Heaney *et al.* 1988).

Phytoplankton populations and physical and chemical parameters are routinely monitored at one location within Esthwaite Water on a monthly basis by the Centre for Ecology and Hydrology (CEH) (Lancaster). The picoplanktonic abundance in Esthwaite Water has been studied by Hawley et al. in 1991 and Ball in 1999. They both sampled a single depth, at the routine monitoring station used by CEH (*Windermere)* in Esthwaite Water, and compared abundances to those in other lakes in the English Lake District. These studies indicated that cell densities can reach a summer maximum of 3.42×10^3 to 3.5×10^4 cells ml^{-1} in Esthwaite Water (Hawley *et al.* 1991; Ball 1999).

2.2 Aims

The aims of the field work were :

- to gather data on the biotic and abiotic characteristics of the sampling locations to proivde a context within which the diversity of picocyanobacteria could be assessed.
- to collect the isolates to analyse to enable the advancement of our understanding of picocyanobacterial diversity.

As this study aimed to provide an overview of the diversity of picocyanobacteria within one habitat, the fieldwork was designed to provide picocyanobacterial isolates from the Esthwaite Water at monthly intervals over five months over the summer of 2000. However in an attempt to assess the diversity of a broad sample of the picocyanobacterial community, on each sampling occasion three sites were sampled at two metre depth intervals.

Although Esthwaite is a well-studied lake, the abiotic conditions vary slightly each year and have an impact on the planktonic community. Thus, so the diversity could be set into context with the typical conditions for the lake, and habitat parameters in other environments, abiotic parameters and some of the major groups of the planktonic community were also monitored at each spatio-temporal location.

2.3 Methods

2.3.1 Fieldwork – the sites

Fieldwork was carried out at monthly intervals between June and October 2000 (June 21st, July 26th, August 30th, September 27th and October 26th) to cover the period of the summer phytoplankton maximum. Three sites were sampled in Esthwaite Water to obtain an indication of the spatial variation within the lake, rather than to determine the characteristics of a single site. Figure 2.1 shows the location of the three study sites (labelled A, B and C), chosen because they occupy deep basins within the lake. Site A is located in the most northern deep basin close to the major inflow of river water (Black Beck), and a number of smaller inflowing rivers. Site A is approximately 15 m deep, and is the site used for long-term monitoring of the chemical, physical and biological parameters by CEH, Windermere (1945 to present). Site B is located in a basin approximately 10 m deep, quite close to the western banks of Esthwaite Water. It is located away from river inflows but can be affected by runoff from the land surface. Site C is located towards the south of the lake and is situated in a basin approximately 12.5 m deep. Site C is in close proximity to three enclosures used for fish farming. Inputs to this site include fish food and faeces. In addition boats and an artificial diffuser are also responsible for inducing increased water movements at this site.

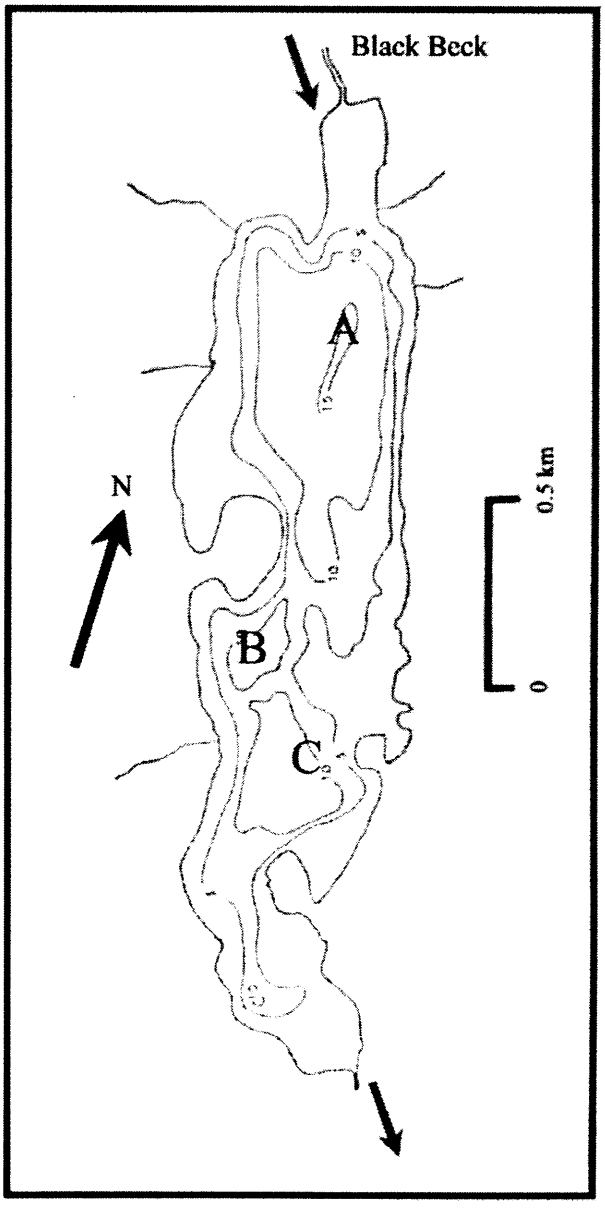


Figure 2.2 Study sites in Esthwaite Water
N. B Site A: SD 360 972, Site B: SD 359 962 and Site C: SD 363 959. Contours indicate depth of lake in metres.

2.3.2 Field work - on site procedures

To ascertain the abiotic characteristics of the sampling sites the light (photosynthetically available radiation (PAR), 400-700 nm), temperature and dissolved oxygen concentration were measured using underwater probes (LI-COR Quantum/Radiometer/Photometer, Model LI-189", Clandon YSI combined temperature and dissolved oxygen probe, Model 57) at 2 m depth intervals throughout the water column at each site. The depth of the thermocline (depth at which stratification occurs) was calculated as the interval of maximal temperature change. The euphotic zone, is the layer of water within which the light availability is suitable for a net energy gain from photosynthesis for autotrophic organisms. The depth this zone extends to is considered to be equivalent to the depth at which light is attenuated to 1% of surface light. The measured sub-surface light intensity was converted to percentage of surface irradiance and an exponential curve plotted through the data. The equation of the line was rearranged to calculate the depth to which 1% surface irradiance penetrated.

Water was collected from 2 m depth intervals using a Friedinger sampling device at each site for the following requirements:

Abiotic:

- The pH was measured using a portable pH meter (Radiometer, RIIM001) as soon as it was brought to the surface of the lake.
- One 50 ml sample was collected in a sterile tube for nutrient analysis.

Biotic:

- Two 50 ml samples were fixed with phosphate buffered glutaraldehyde (1% v/v final concentration) for the enumeration of planktonic organisms.
- A 1litre sample was collected for the isolation of picocyanobacteria (see Chapter 5).

2.3.3 Laboratory procedures on sampling day

The 50 ml aliquots collected for nutrient analysis were filtered through 0.2 µm filters (Whatman Puradisc 25^{TM}) to remove biologically active constituents within 3 hours of collecting the samples. All water samples were then stored at 4 ^{o}C prior to subsequent analysis.

2.3.4 Laboratory procedures after sampling day

2.3.4.1 Nutrient analysis

Samples were analysed for nutrients using colorimetric assays within one month of the sampling day by Janice Drinkall at Lancaster University. Three sub-samples were analysed for the concentration of soluble reactive phosphorus (SRP) using the Ploen method, nitrate (total oxidised nitrogen) by copper-hydrazine reduction (Downes 1978) and ammonium by formation of indophenol blue (Mackereth *et al.* 1978).

2.3.4.2 Enumeration of planktonic organisms <100 µm diameter

An appropriate volume of each sample (dependent on the concentration of cells present) was stained with 4,6-Diamidinophenylindole (DAPI) at a final concentration of 0.0001%

(v/v) for 10 minutes (Porter *et al.* 1980). Each sample was then filtered onto a 0.2 μm pore size, black polycarbonate track etched screen membrane (Poretics) under minimal vacuum (<100 mm Hg vacuum). To enumerate heterotrophic bacteria and picoplankton a pre-filtration step through a 3.0 μm filter was performed (following the method of Hawley *et al.* (1991) after their review of the previously used techniques). Organisms were counted within a Whipple grid, or field of view, on a Leitz Laborlux S fluorescent microscope illuminated with a 100 Watt mercury lamp (final magnification x1250). The appropriate bandpass (BP) or longpass (LP) emission (barrier) filters capable of selectively isolating fluorescence emission were used to excite the cells so they could be distinguished using the characteristics summarised in Table 2.1, by employing previously published keys (Prescott 1978; Porter *et al.* 1980; Tikkanen 1986) and through knowledge gained during an algal identification field course, Kindrogan, Scotland, 2000.

The number of cells per Whipple grid, or field of view, was converted to cells per ml of lake water (n) using the equation of (Jones 1979) (see below).

$$n = \frac{\bar{y}.A.d}{a.v}$$

$\bar{y}$ = mean count per Whipple grid or field of view

A = effective filtration area (μm^2)

a = Whipple grid or field of view area (μm^2)

v = volume of sample filtered (ml)

d = dilution factor.

Table 2.1 Fluorescence characteristics of planktonic organisms enumerated by epifluorescence microscopy

Group	Excitation	Fluorescence characteristics	Cell characteristics
Picocyanobacteria	Blue	Red (Chlorophyll)	Whole cell fluoresces. Single cells. 0.2-3 μm diameter, spherical (coccoid) to rod shaped
	Green	Red (Phycocyanin)	
		Orange (Phycoerythrin)	
Filamentous and colonial cyanobacteria	Blue	Red (Chlorophyll)	Whole cell fluoresces. Single cells > 3 μm, or filaments or colonies*
	Green	Red (Phycocyanin)	
		Orange (Phycoerythrin)	
Chlorophytes	Blue	Red (Chlorophyll)	Chloroplasts fluoresce. Single cells, colonies or filaments*
Diatoms	Blue	Red (Chlorophyll)	Chloroplasts fluoresce. Single cells, colonies or filaments*
	UV	Blue	Nucleus fluoresces
Autotrophic flagellates	Blue	Red (Chlorophyll)	Chloroplasts fluoresces
	UV	Blue	Nucleus fluoresces
Heterotrophic flagellates	Blue	Yellow	Whole cell fluoreces
	UV	Blue	Nucleus fluoresces
Ciliates	Blue	Yellow	Whole cell fluoresces
	UV	Blue	Nuclei fluoresce (> 1 present)
Heterotrophic bacteria	UV	Blue	Whole cell fluoresces

* Cells in filaments were counted directly, or estimated by multiplying the length of filament by the average cell length (determined from100 cells).
Filter sets: blue excitation: BP 350-460 nm, suppression LP 515 nm; green excitation: BP 530-650 nm, suppression LP 580 nm; and UV excitation: BP 340-380 and suppression LP 430 nm.

2.3.4.3 Enumeration of planktonic organisms > 100 μm diameter.

The concentrations of larger organisms e.g. dinoflagellates and rotifers were determined in the 50 ml fixed samples after concentrating the sample to 5 ml by settling for 48 hours followed by removal of the upper 45 ml. Cells were counted in 5 μl drops on a slide using light microscopy (x100). The number of organisms in each drop was converted to number of organisms per ml of lake water (n) using the following equation:

$$n = \bar{y} \times c$$

$\bar{y}$ = mean number of individuals per 5 μl

c = concentration factor.

The "spent" 45 ml was examined to ensure the concentrating step had been successful. No large organisms were observed in this fraction.

2.3.4.4 Isolation of picocyanobacteria

Picocyanobacteria were isolated from the 2 litre water samples collected during field sampling from each spatio-temporal sampling location. The water was pre-filtered under minimal vacuum (<100mm Hg vacuum) through 47 mm, 3 μm cellulose nitrate membrane filters (Whatman) to remove detritus and larger biota. Particles between 3 μm and 0.2 μm were concentrated onto 0.2 μm, cellulose nitrate membrane filters (Whatman). These filters were inverted onto BG11 solidified with 1.5% agarose (Rippka *et al.* 1979). The plates were inverted and incubated for 6 weeks at 20°C ± 2 °C with a 12:8 hour light:dark cycle. Light was provided using 65 Watt fluorescent tubes.

Picocyanobacteria and other photo-autotrophic micro-organisms produced colonies between the agarose and filter paper. Green colonies were isolated (50 from each spatio-temporal location) using standard microbiological aseptic techniques to liquid BG11 in 96 well plates and incubated under the temperature and light conditions outlined above. Each isolate was observed using epifluorescence microscopy to determine whether they were picocyanobacteria (Table 2.1). The morphology and pigment type of each isolate was recorded format outlined in Table 2.2.

Table 2.2 Record of details about picocyanobacterial isolates

Isolate Reference			
Isolation Details	Date	Site	Depth
Picocyanobacterial			
	PE rich	PC rich	
Rod			
Sphere			

2.4 Results

2.4.1 Spatio-temporal variation in physico-chemical factors

2.4.1.1 Thermal stratification

The water column at all three study sites in Esthwaite Water was thermally stratified by the end of June and remained stratified until after the end of September when isothermal conditions returned (Fig 2.3). This is shown by the thermocline, marked as a red dashed line on Fig 2.3, which is also shown on all further figures. During the stratified period, the contours appear to show a three-layered structure to the water column; a warm surface layer (red contours > 18 °C), separated from cool deep water (blue contours < 14 °C) by water of intermediate temperature (green contours approx. 14-17 °C) (Fig 2.4). These layers could represent the epi-, meta- and hypo-limnion typically recorded in temperate stratified lakes (Moss 1988; Fryer 1991). The epilimnion is the upper, wind-mixed layer of a thermally stratified lake. This water is turbulently mixed throughout at least some portion of the day and because of its exposure, can freely exchange dissolved gases (such as O_2 and CO_2) with the atmosphere. The metalimnion is the middle or transitional zone between the well-mixed epilimnion and the colder hypolimnion layers in a stratified lake. This layer contains the thermocline, but is loosely defined depending on the shape of the temperature profile. The hypolimnion is the bottom, and most dense layer of a stratified lake. It is typically the coldest layer in the summer and warmest in the winter (where lakes stratify during winter). It is isolated from wind mixing and typically too dark for most organisms to accomplish a net gain from photosynthesis. Mixing within this layer occurs through movement induced by internal seiches (rocking of the thermocline so that

the hypolimnion at one end of the lake is deeper than the other, which then rocks to become the deeper end). Internal seiches on a lake the size of Esthwaite could influence thermocline depth by a two metres. Movement of the epilimnion over the hypolimnion as the surface water moves in the direction of the wind, and lower water returns in the opposite direction can also set currents of movement up within the hypolimnion and induce a small amount of mixing between the layers. Both of these types of movement are wind induced (Fryer 1991).

The depth of the thermocline varied between sites at the end of June; it was 3 m at Site C, 4 m at Site B and 5m at Site A. At Sites B and C, the depth of the thermocline was above the metalimnetic layer, but increased in depth as more of the surface water warmed. At Site A the thermocline was consistently beneath the warm surface water and some of the water of moderate temperature within the metalimnion. Thus there may have been several thermally stratified layers including the thermocline. The thermocline is a stable (primary) stratification which persists for a number of months. Those layers above the thermocline are characteristic of secondary stratified layering which form for short periods, either diurnally or for a few days or weeks, mixing together during moderate to high wind events.

2.4.1.2 Euphotic depth

Light was attenuated rapidly as it entered the water column at all three sampling sites in Esthwaite Water over the entire sampling period (Fig 2.4). Light attenuation appeared to be invariable at Sites A and B, with 60% of surface irradiance being attenuated within the first metre of the water. The depth of the euphotic zone was approximately 4 m at Sites A and B throughout the sampling period. Light attenuation was slightly lower at Site C at

the end of June and July, 60% of surface irradiance was attenuated within 1.5, and 2m, on these sampling dates respectively. Thus the depth of the euphotic zone was deeper at Site C at the end of June (4.5 m) and the end of July (4.8 m). By the end of August, the euphotic zone depth at Site C was also 4 m deep and it remained at that depth for the rest of the sampling period. It is interesting to note that the depth of the euphotic zone was always higher than the thermocline (red dashed line) at Site A (euphotic zone < epilimnion), whereas at Site B and C the euphotic zone extended below the thermocline at the end of June (euphotic zone > epilimnion). By the end of July the euphotic zone was above the thermocline at Sites B and C, but it appears that this transition occurred earlier at Site B than Site C. The significance of this is that where the euphotic zone < epilimnion cells are mixed through water that is too dark for net gain from photosynthesis to be achieved. Where euphotic zone is > epilimnion, cells in the epilimnion are constantly within the zone where net gain of carbon fixation from photosynthesis can exceed losses from respiration. This is a crude representation of the euphotic depth, which is sufficient for the scale at which this study has been performed. Other studies have calculated the euphotic depth more precisely (Walsby 1997).

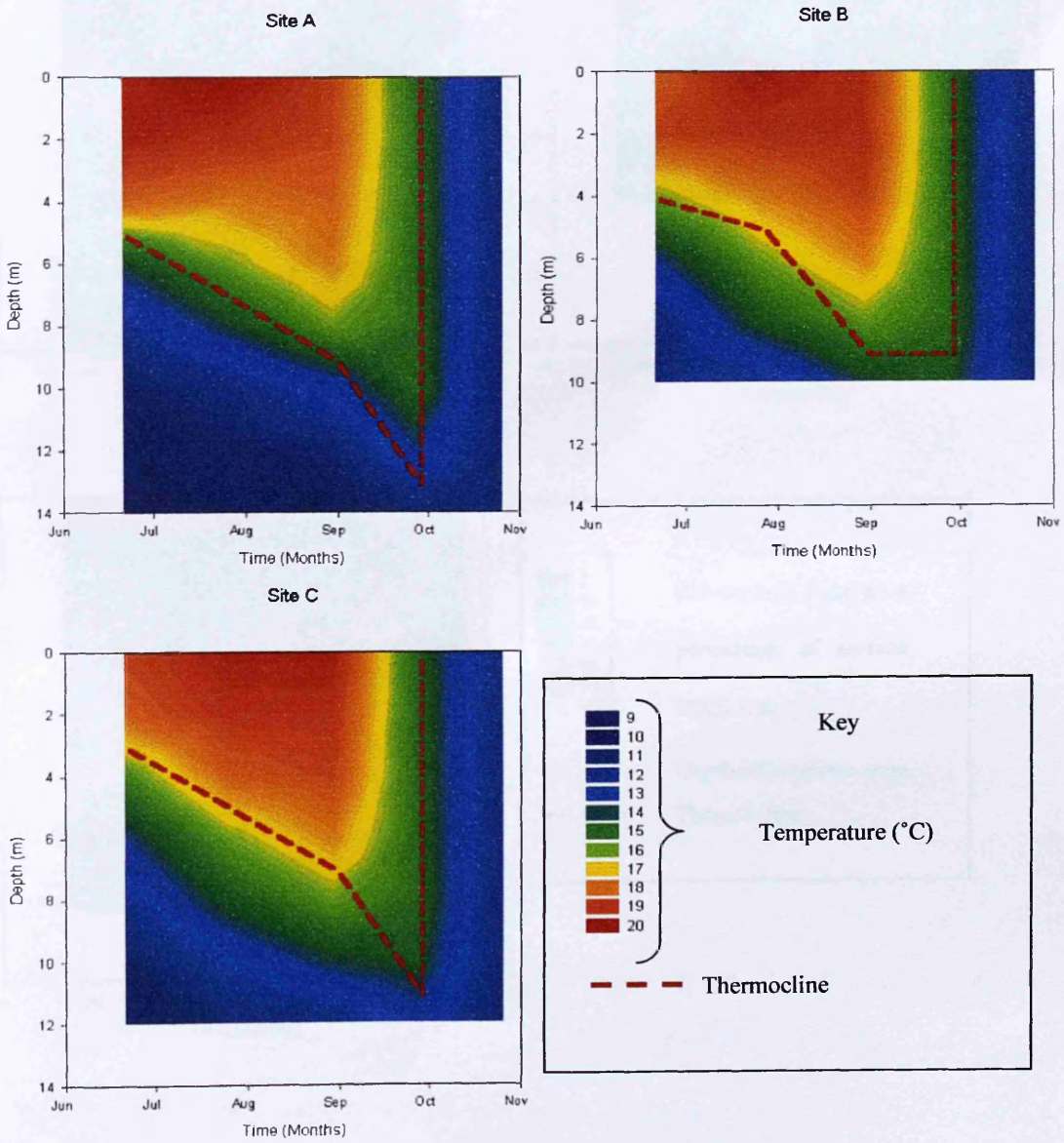


Figure 2.3 Variation of temperature, and depth of thermocline in Esthwaite Water, at the three sampling sites over sampling the period

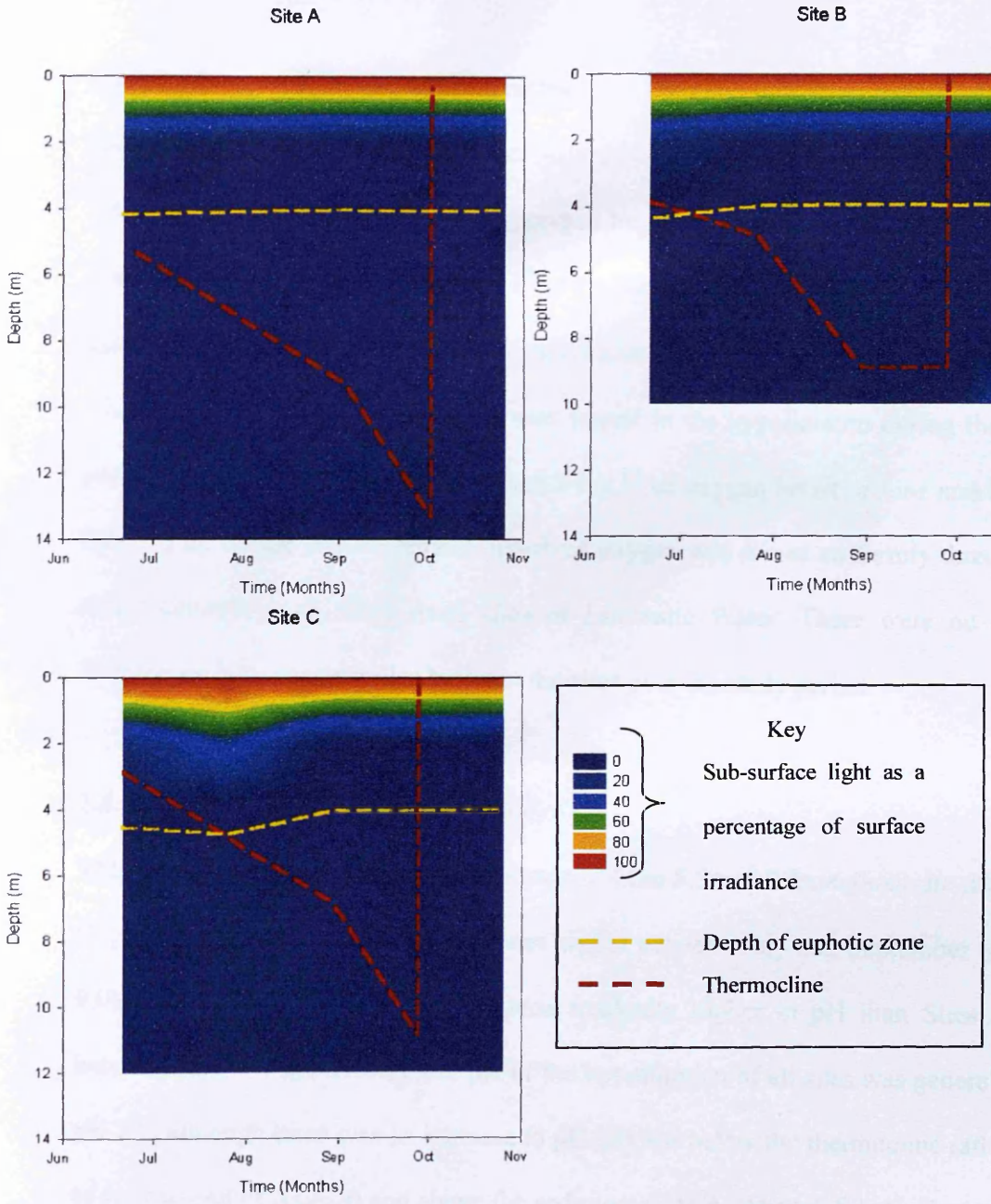


Figure 2.4 Variation in sub-surface light as a percentage of surface irradiance, and depth of euphotic zone in Esthwaite Water at the three sampling sites over the sampling period

2.4.1.3 Dissolved oxygen concentration

Dissolved oxygen was detected in the epilimnion of all three sites at the end of June (Fig 2.5). The concentration of dissolved oxygen began to increase in this water layer by the end of July, and peaked at 20-22 mg l^{-1} at the end of August. At the end of September the dissolved oxygen concentration of the epilimnion had started to decline.

The dissolved oxygen concentration was lowest in the hypolimnion during the stratified period, with a concentration of less than 2 mg l^{-1} of oxygen between June and September below 8 m. By the end of October dissolved oxygen was mixed uniformly throughout the water column at all three study sites in Esthwaite Water. There were no detectable differences in oxygen profiles between the sites over the study period.

2.4.1.4 pH of the water column

The pH of the water column at all sites ranged from 6.5 to 9.0 throughout the study period (Fig 2.6). The pH of the epilimnion was higher between July and September (pH 8.5 to 9.0). The epilimnion of Site A, became markedly higher in pH than Sites B and C between July and September. The pH of the hypolimnion of all sites was generally below pH 7.5, although there was an increase to pH 8.0 just below the thermocline (all sites end of June to end of August) and above the sediment (Site A, August, Site C, September Site B July and August). This meant the entire water column at Site B (only 10 m deep) was at pH 8.0 from the end of July to the end of August.

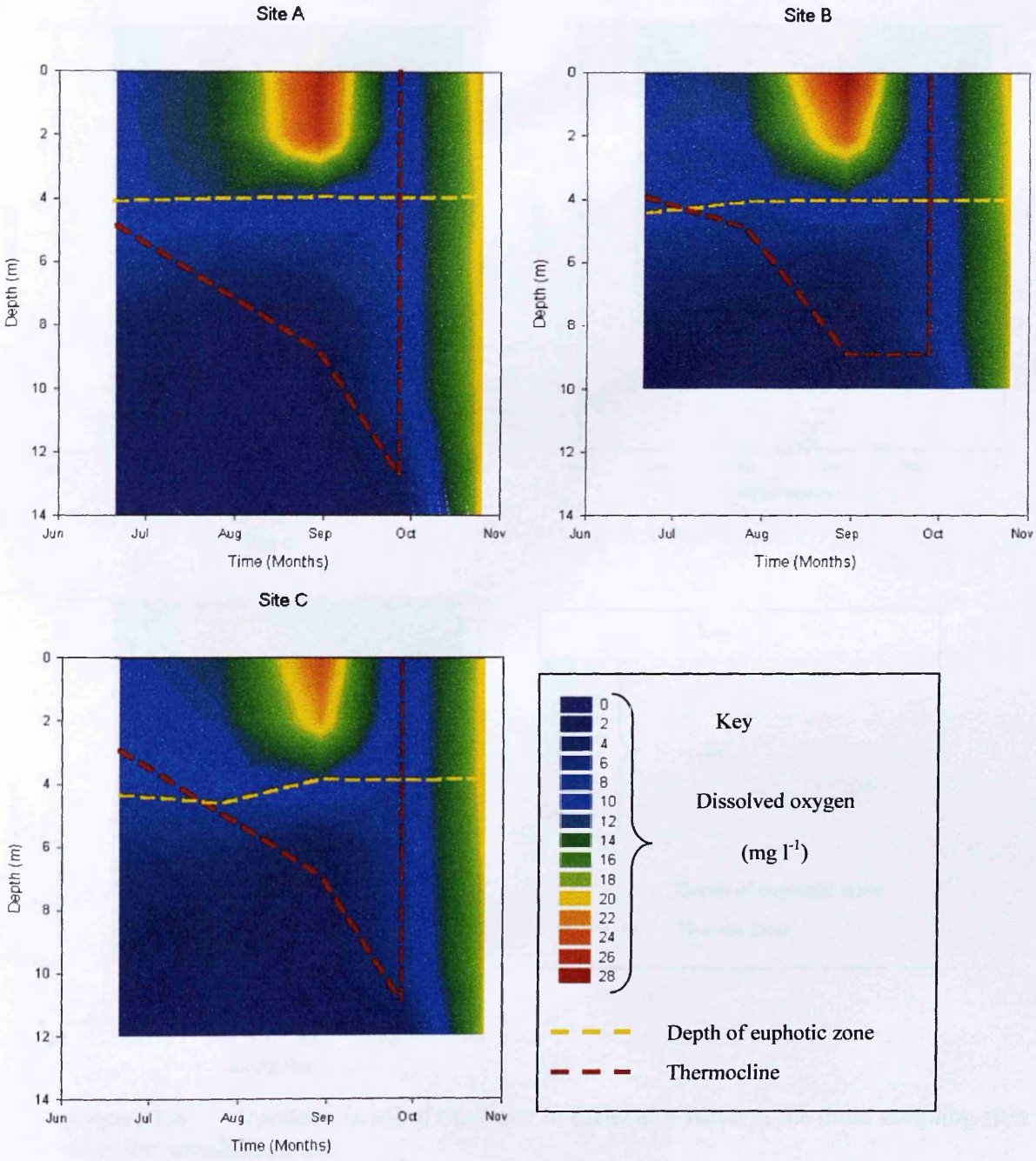


Figure 2.5 Variation in dissolved oxygen concentration in Esthwaite Water at the three sampling sites over the sampling period

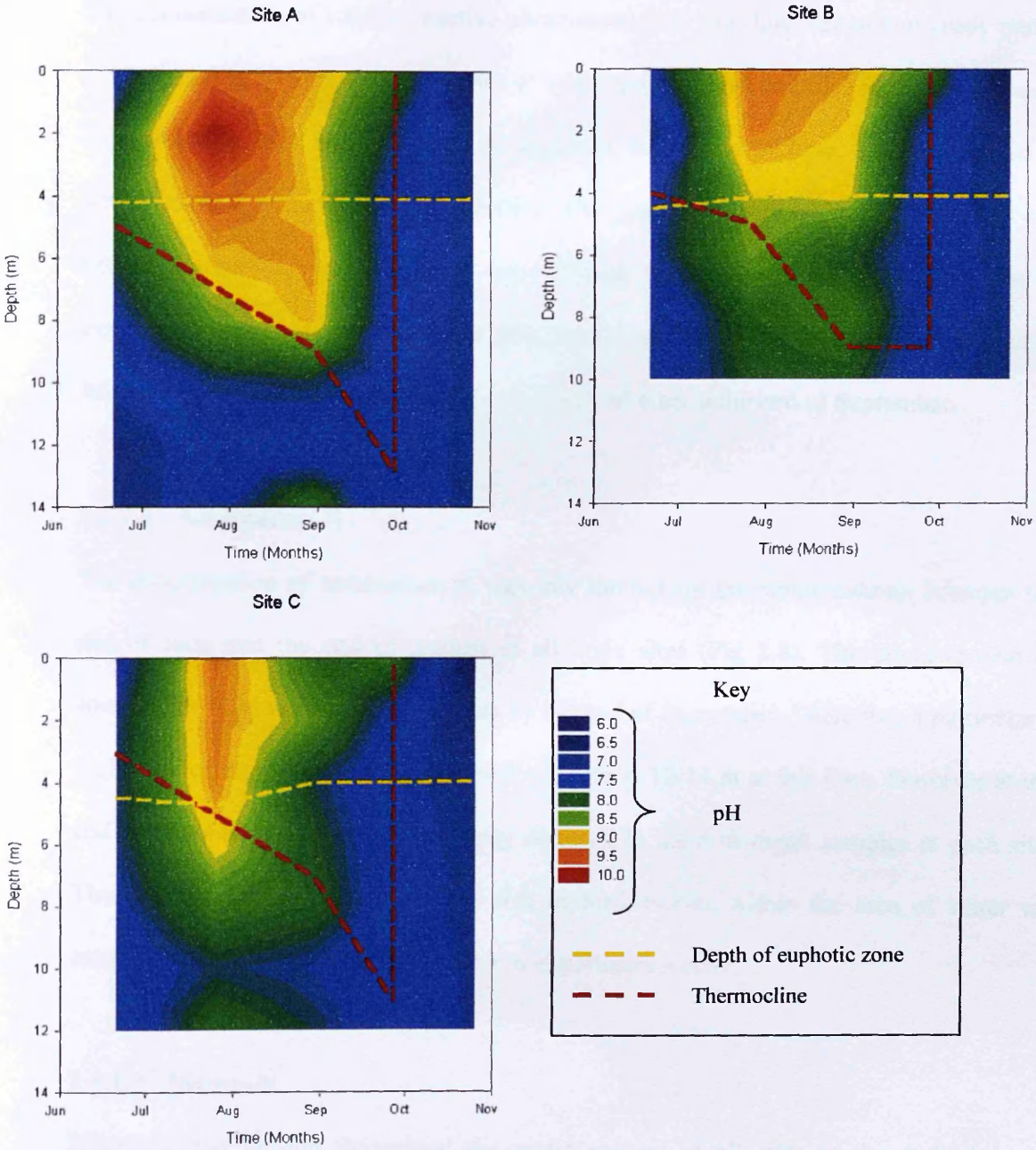


Figure 2.6 Variation in pH of the water in Esthwaite Water at the three sampling sites over the sampling period

2.4.1.5 Soluble reactive phosphate

The concentration of soluble reactive phosphorus was very low throughout study period at all sites (Fig 2.7). However, patches of water that had high soluble reactive phosphorus concentrations were identified at Site A (14 m, at the end of June, 12m, at the end of August, and 0 m, in September/October). The peak may have been due to fish or bird excreta within the area of water we sampled, they are not thought to be due to experimental error. Soluble reactive phosphorus was detectable at Site C at the end of July at 6m, and throughout the water column of all sites at the end of September.

2.4.1.6 Ammonium-N

The concentration of ammonium-N was low throughout the water column between the end of June and the end of August at all three sites (Fig 2.8). The concentration of ammonium-N had increased at all sites by the end of September. There was a particularly high peak of ammonium-N (169 μg N l^{-1}) at Site A 12-14 m at this time. However at the end of October ammonium-N was only detected in the 6 m depth samples at each site. These peaks may have been due to fish or bird excreta within the area of water we sampled, they are not thought to be due to experimental error.

2.4.1.7 Nitrate-N

Nitrate-N was present throughout the water column at all sites at the end of June, although at Site A, it was low (below 50 μg l^{-1}) at 2 and 10 m (Fig 2.9). By the end of July until the end of September the nitrate-N concentration was below the limit of detection at all sites. After the end of the period of thermal stratification, nitrate-N was detectable through the entire water column.

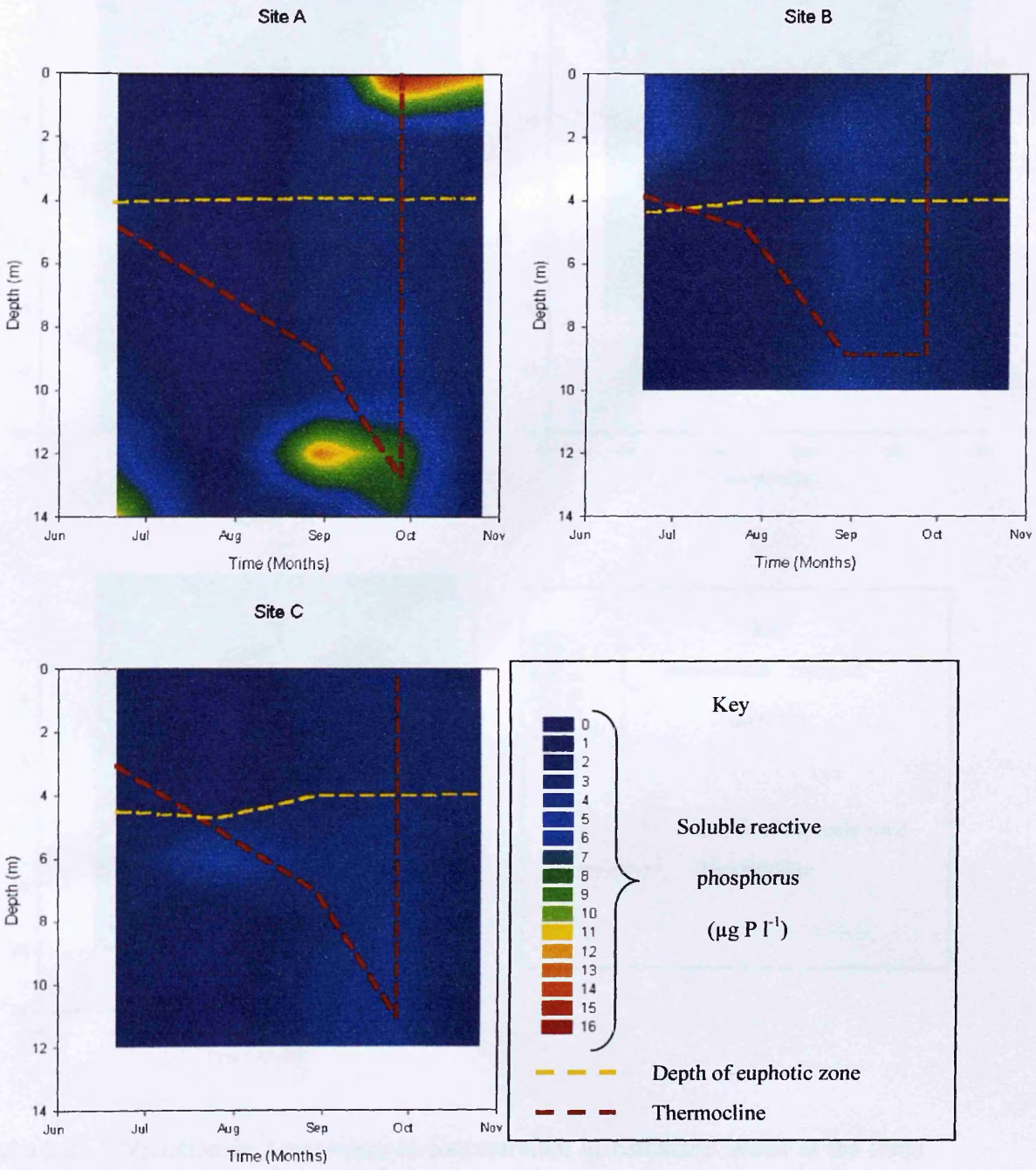


Figure 2.7 Variation in soluble reactive phosphorus concentration in Esthwaite Water at the three sampling sites over the sampling period

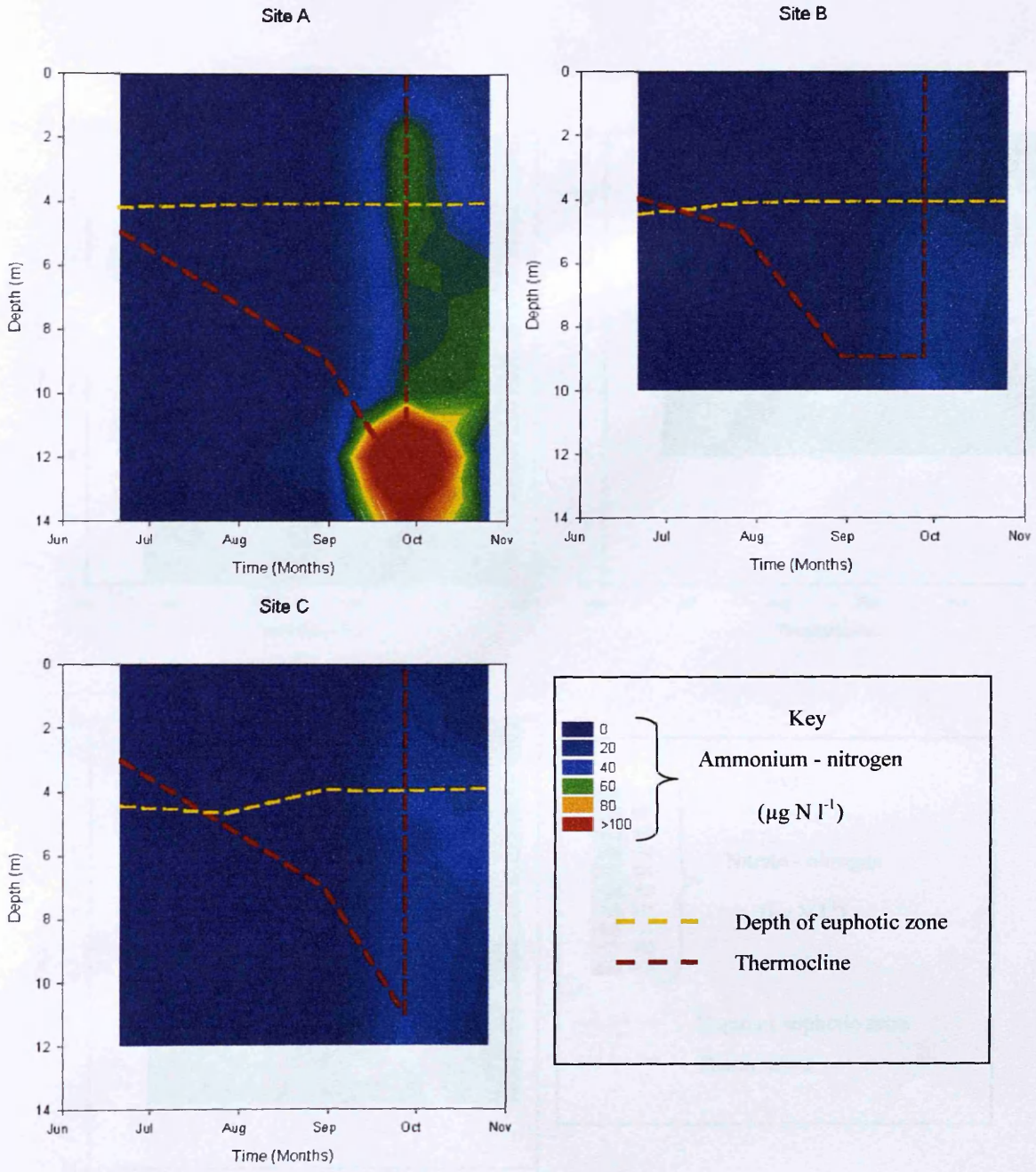


Figure 2.8 Variation in Ammonium-N concentration in Esthwaite Water at the three sampling sites over the sampling period

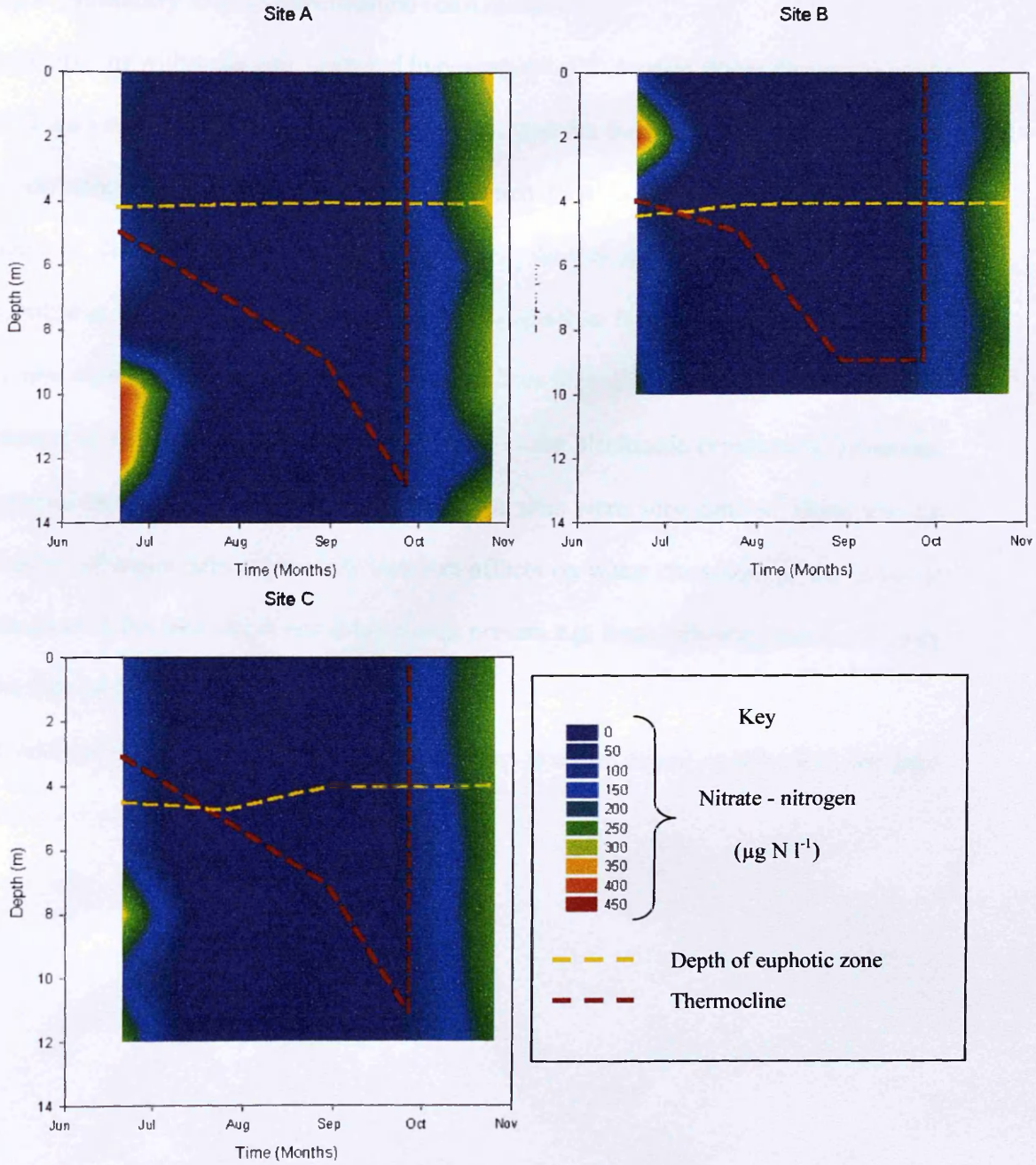


Figure 2.9 Variation of nitrate-N concentration in Esthwaite Water at the three sampling sites over the sampling period

2.4.1.8 Summary of physico-chemical conditions

The conditions within the epi- meta and hypolimnion of Esthwaite Water during the study period are summarised in Table 2.3. It is evident that the thermally stratified layers have very different physico-chemical conditions, which provide a variety of niches for the planktonic community. There were also small differences in the physico-chemical conditions at the three sites (e.g. depth of the thermocline (Fig 2.3), euphotic depth (Fig 2.4), and occasional peaks in nutrient concentrations (Figs 2.7 to 2.9), which would have presented different opportunities and challenges to the planktonic community. However, in general the abiotic characteristics of the three sites were very similar. There was no indication of major influences with localised affects on water chemistry or the physical parameters of the lake which could have been present e.g. from inflowing rivers or inputs to the fish farm.

Any additional nutrient inputs from these sources must be mixed rapidly with the lake water or assimilated by the biotic community.

Table 2.3 Summary of the physico-chemical conditions of the stratified layers

	Epilimnion	Metalimnion	Hypolimnion	After autumn overturn
Light	Available for photosynthesis	Low - but photosynthesis still possible.	Low- limiting to photosynthesis.	Light attenuation remained as during stratified period but all water circulated through light region.
Temperature	Warm (16-20°C)	Moderate (13-16°C).	Cool (9-14 °C).	Cool (11-15 °C)
Oxygenation	Oxygenated (>10mg l^{-1})	Oxygenated (4-10 mg l^{-1}).	Low - (< 4 mg l^{-1}).	Oxygenated (8-20 mg l^{-1})
pH	High (8-9.5)	High pH (8-9)	Moderate pH (7-8)	Moderate pH (7-8, occasionally 6.5)
Soluble reactive phosphorus	Low- usually < 2µg l^{-1}	Low- usually < 2 µg l^{-1}	Low- usually < 2 µg l^{-1}	Low- < 2 µg l^{-1}, except for infrequent locations.
Ammonium-N	Low (BLD during June-July)	Low (BLD).	Low (BLD).	High - particularly at Site A where concentrations reached 169 µg N l^{-1} in deep water.
Nitrate-N	Low – BLD - July and August	Low BLD - July and August.	Low – BLD - July and August).	High - (100-300 µg l^{-1})

N. B BLD = Below limit of detection. Source: Summary of information from Figs 2.3 to 2.9

2.4.2 Planktonic community structure

2.4.2.1 Colonial and filamentous cyanobacteria

This group clearly dominated the phytoplankton in terms of cell numbers during the summer algal maximum abundance (comparison of Fig 2.10 and Figs 2.11-2.14 and 2.19). They were the most abundant autotrophs in Esthwaite Water during the sampling period, reaching a peak at the end of July at Sites B (4 m, cell densities to 1.25×10^5 cells ml^{-1}, and C (6 m, cell densities to 2.5×10^5 cells ml^{-1}). At the end of August the high abundance of colonial and filamentous cyanobacteria continued at Sites B and C. At Site A at the end of August there were two smaller peaks of filamentous and colonial cyanobacteria within the epilimnion, one above, and one below the thermocline. Colonial and filamentous cyanobacteria were abundant in upper hypolimnetic samples from the end of July and end of August samples at all sites. The numbers of cyanobacterial cells present in the water column had decreased by September and October.

2.4.2.2 Autotrophic flagellates

The abundance of autotrophic flagellates was low at all sites at the end of June (Fig 2.11). However, by the end of July they were detected in the upper water at Site B (530 cells ml^{-1}), and C (69 cells ml^{-1}), and there was a peak at just over 300 cells ml^{-1} below the thermocline at Site C. At the end of August the abundance of autotrophic flagellates had increased and ranged from 1.4×10^3 to $> 4.5 \times 10^3$ cells ml^{-1} at Site A, 870 to 220 cells ml^{-1} at Site B, and 310 to 2200 ml^{-1} at Site C. By the end of September the abundance of

autotrophic flagellates had reduced to less than 1×10^3 cells ml^{-1} at Sites B and C, but remained at around 1.5×10^3 cells ml^{-1} between 6 and 10 m at Site A.

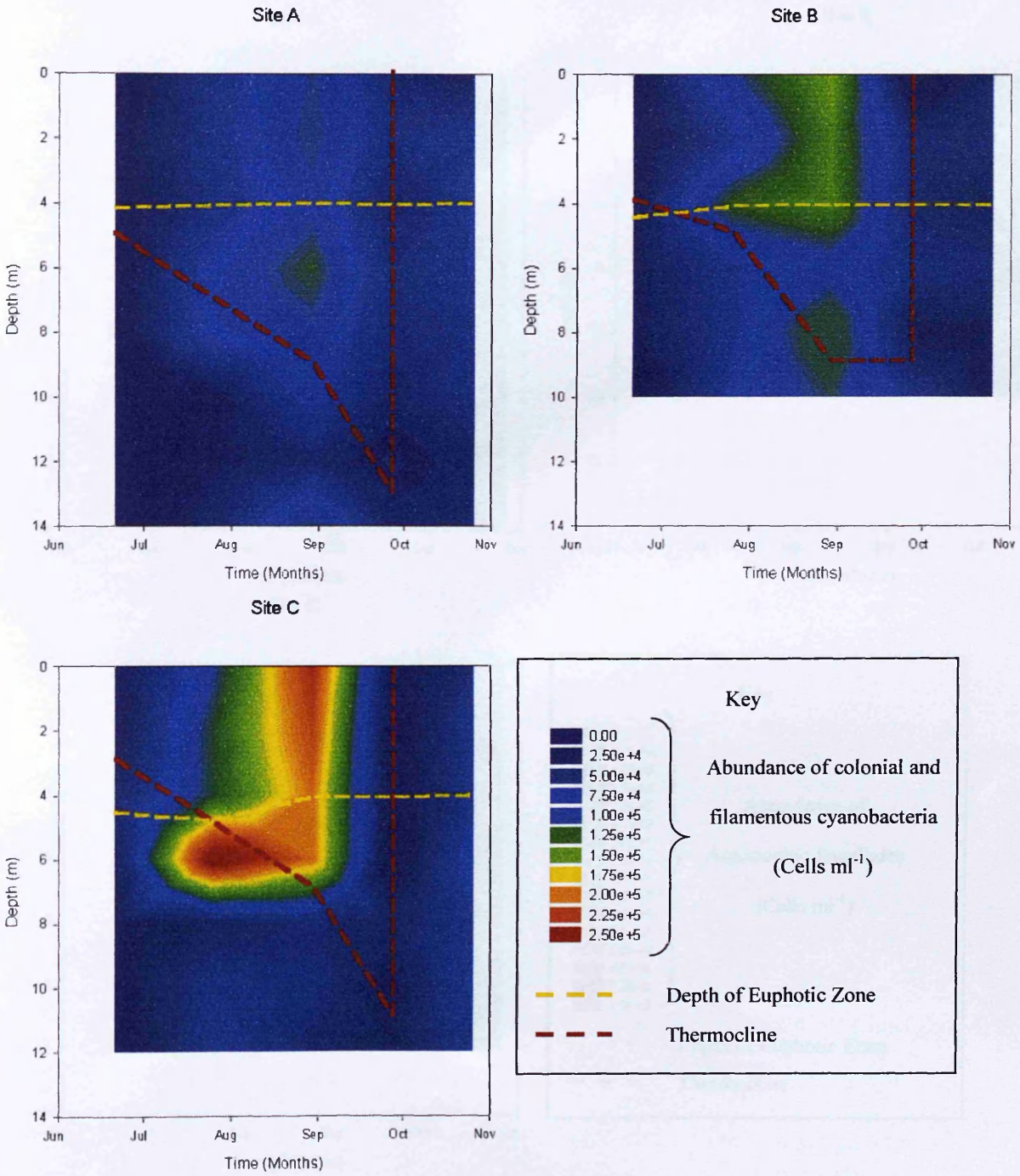


Figure 2.10 Variation in the abundance of filamentous and colonial cyanobacteria in Esthwaite Water at the three sampling sites over the sampling period

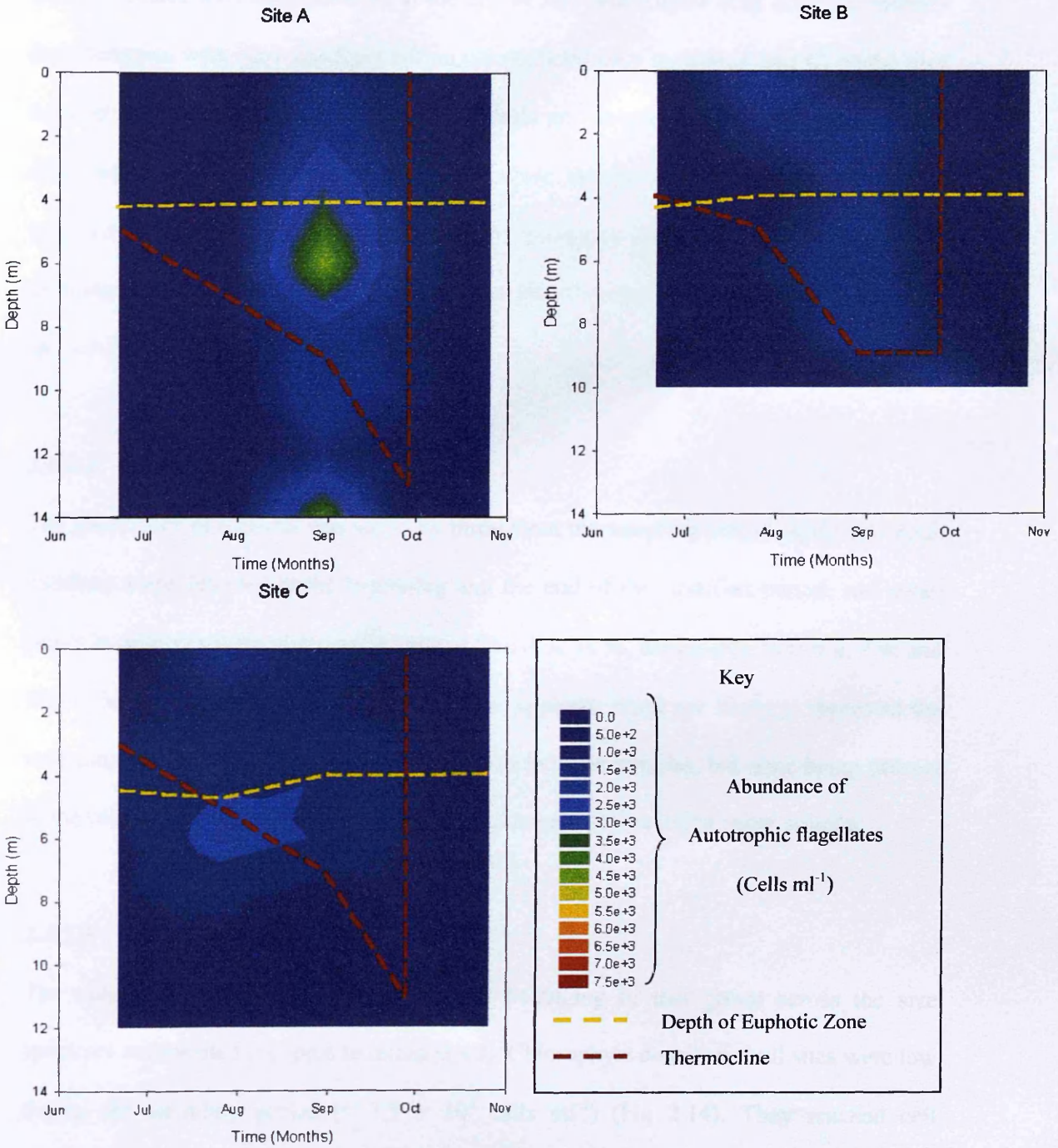


Figure 2.11 Variation in the abundance of autotrophic flagellates in Esthwaite Water at the three sampling sites over the sampling period

2.4.2.3 Dinoflagellates

Dinoflagellates were only detected at the end of July and August (Fig 2.12) Particularly high densities were recorded from within the euphotic zone at Sites A and C, where they reached 2.7 × 10^2 cells ml^{-1}, and 2.5 × 10^2 cells ml^{-1} respectively. Dinoflagellates were observed at Site B at the end of July and August, but the maximum concentration was only 1.6 × 10^2 cells ml^{-1}. During this study, *Ceratium hirudinella* was the dominant dinoflagellate enumerated, with the only other dinoflagellate observed being *Peridinium sp.*, which never exceeded 12 cells ml^{-1}.

2.4.2.4 Diatoms

The abundance of diatoms was very low throughout the sampling period. Although small numbers were detected at the beginning and the end of the stratified period, and small peaks in numbers were observed in June at Site A at 10 m, September, Site B at 8 m and Site C in October at 0-2 m. (Fig 2.13). These apparent peaks are likely to represent the recording of a very small number of individuals in some samples, but none being present in the other samples due to the very low overall concentration in the water column.

2.4.2.5 Chlorophytes

The chlorophytes included those organisms belonging to that group across the size spectrum enumerated (i.e. pico to micro sized). Chlorophyte densities at all sites were low during the sampling period (< 7.5 × 10^3 cells ml^{-1}) (Fig 2.14). They reached cell concentrations of 4.5 × 10^3 cells ml^{-1} at Site C in August, but for most of the summer phytoplankton maximum they were below limit of detection. After the end of thermal stratification an increased level of abundance of chlorophytes was recorded (Site A to 2.5

$\times 10^3$ cells ml^{-1}, at Site B to 5×10^3 cells ml^{-1} and at Site C to 7.5×10^3 cells ml^{-1}) but it was noted that the distribution of cells throughout the water column appeared patchy. Again these apparent peaks are likely to represent the recording of a very small number of individuals in some samples, but none being present in the other samples due to the very low overall concentration in the water column.

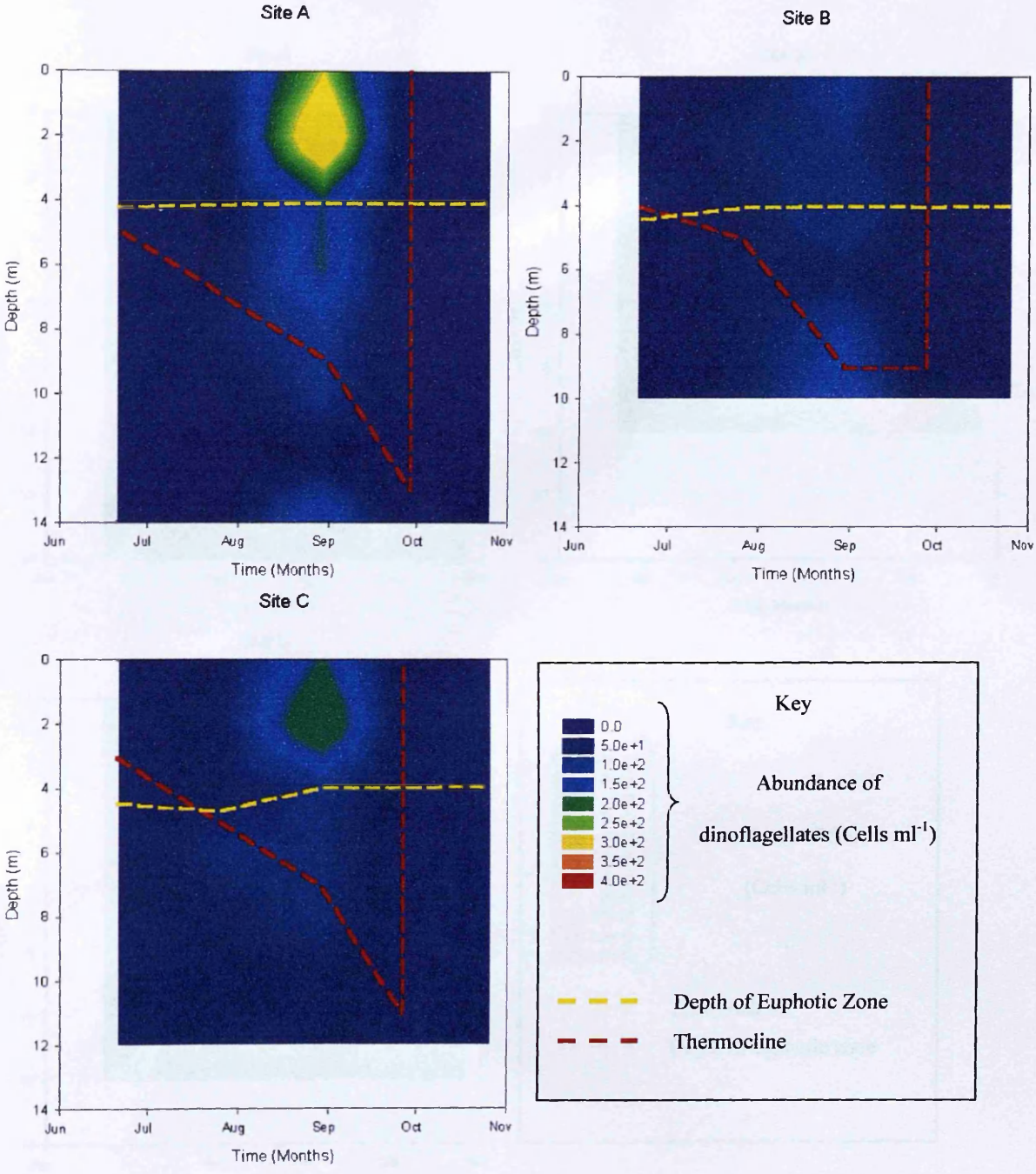


Figure 2.12 Variation in the abundance of dinoflagellates in Esthwaite Water at the three sampling sites over the sampling period

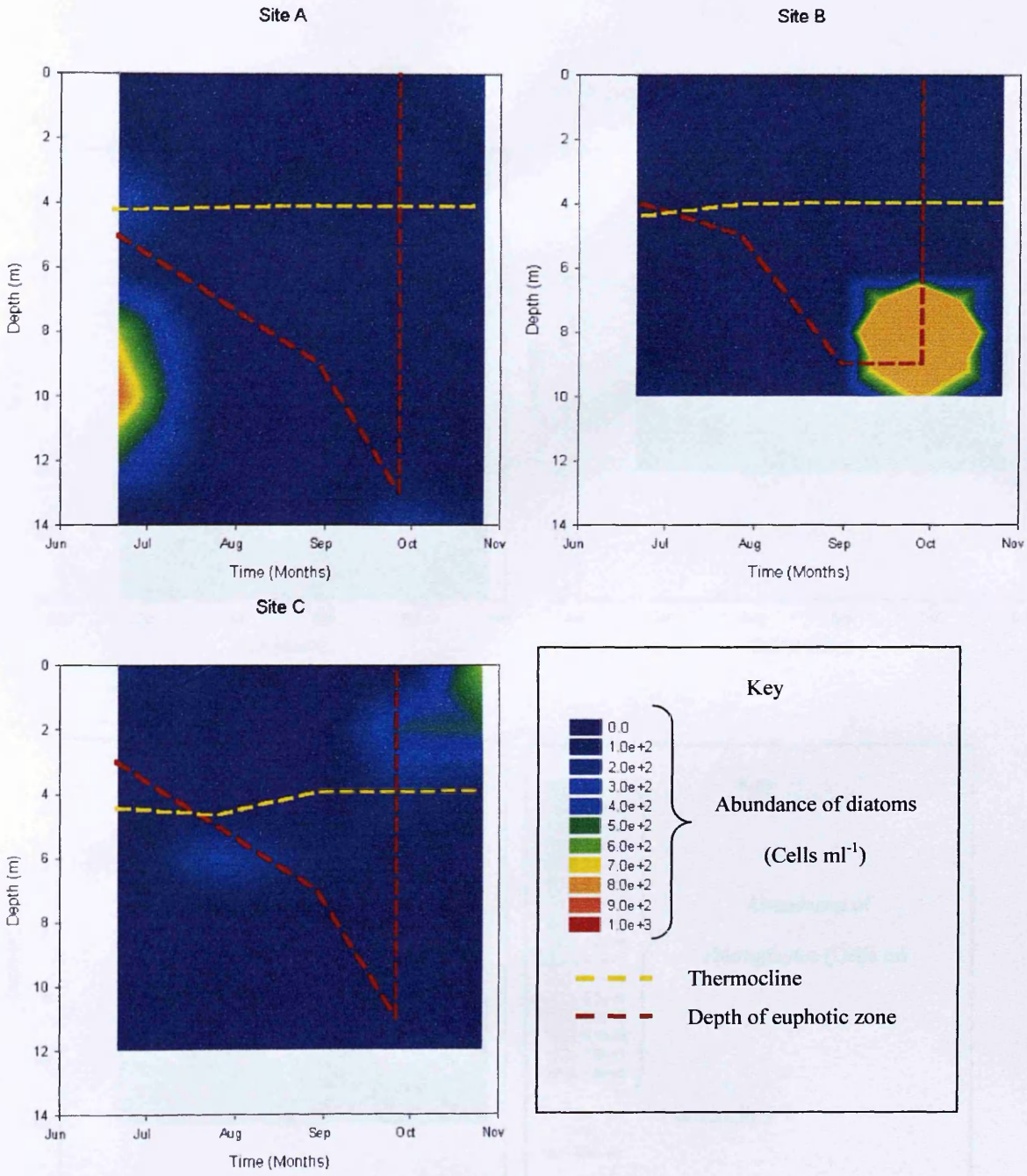


Figure 2.13 Variation in the abundance of diatoms in Esthwaite Water at the three sampling sites over the sampling period

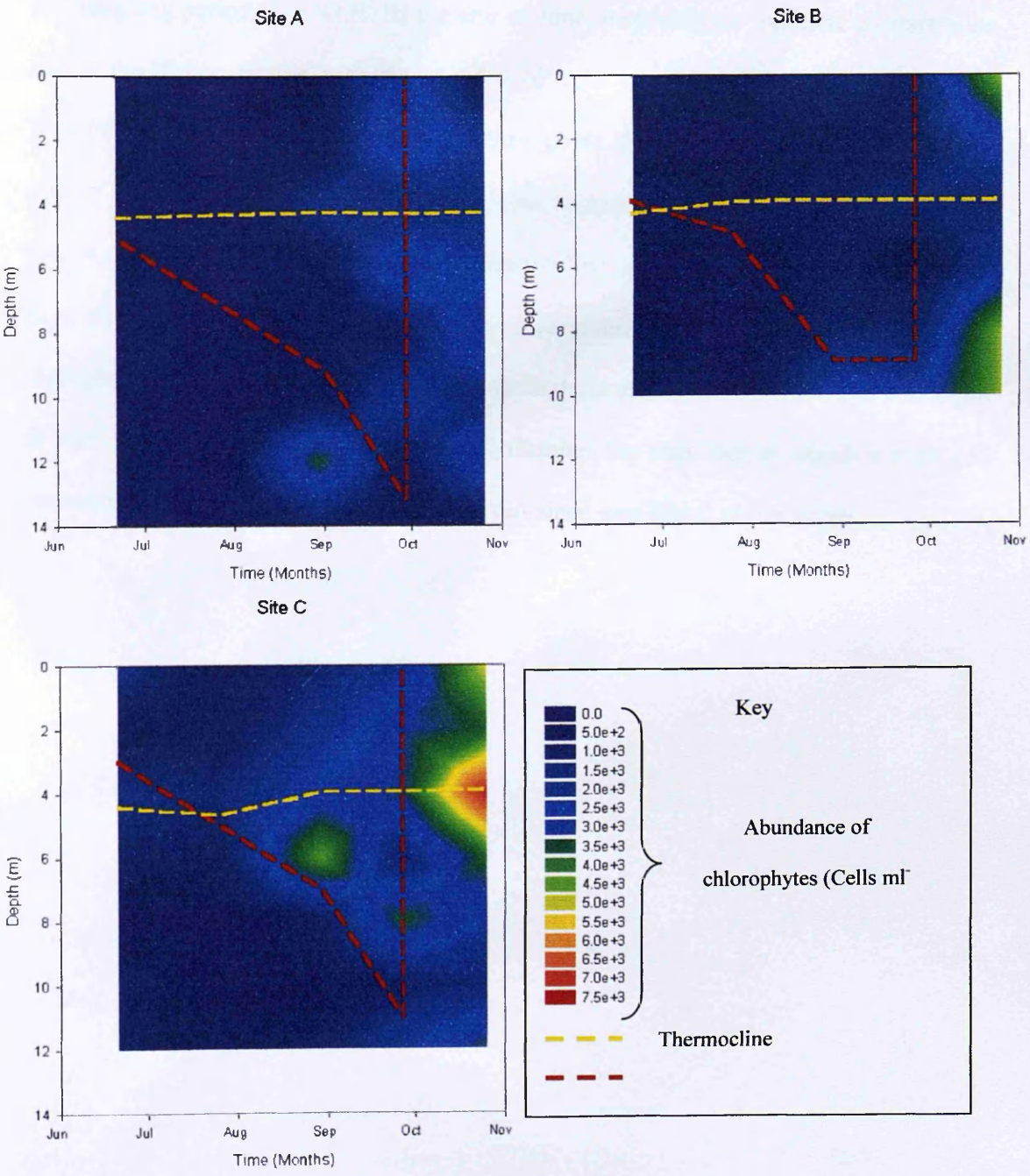


Figure 2.14 Variation in the abundance of chlorophytes in Esthwaite Water at the three sampling sites over the sampling period

2.4.2.6 Heterotrophic bacteria

The abundance of heterotrophic bacteria was > 2.5×10^6 cells ml^{-1} at all sites for most of the sampling period (Fig 2.15). At the end of June, heterotrophic bacterial concentration was at the highest level observed ($3.5\text{-}4.5 \times 10^6$ cells ml^{-1}) above the thermocline at Site A, within the hypolimnion at Site B and throughout the water column at Site C. At the end of July high cell numbers of heterotrophic bacteria were restricted to the surface of Site A, 0-2 m at Site B and beneath the thermocline at Site C. At the end of August a high abundance of heterotrophic bacteria was observed above the thermocline at Site A, throughout the water column at Site B and throughout the water column below 2 m depth at Site C. By the end of September and October the only site at which a high cell concentration of heterotrophic bacteria was remained was Site C at 4 m depth.

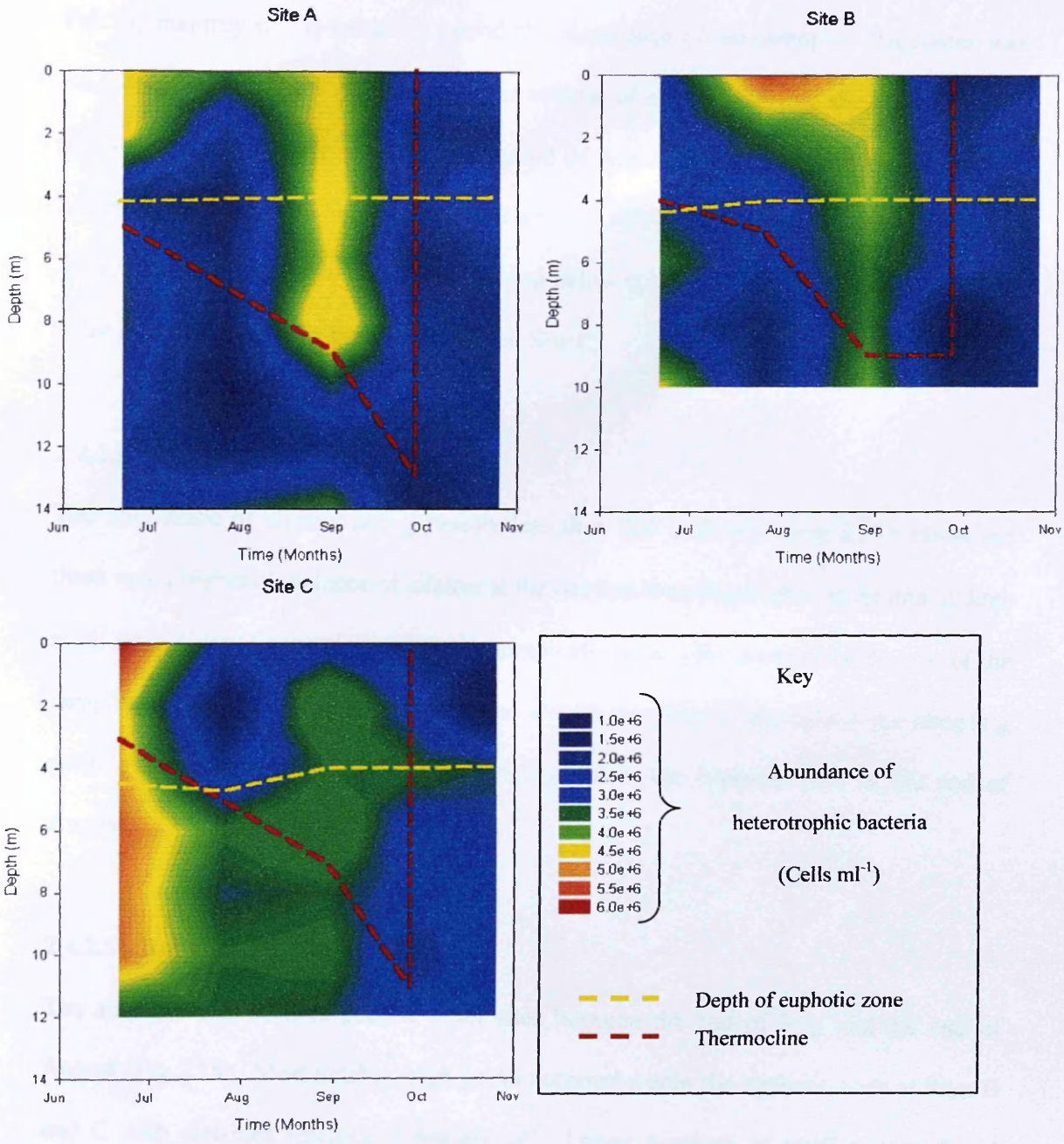


Figure 2.15 Variation in the abundance of heterotrophic bacteria at the three sampling sites over the sampling period

2.4.2.7 Heterotrophic flagellates

For the majority of the sampling period the abundance of heterotrophic flagellates was $<1.5 \times 10^2$ cells ml^{-1} throughout the water column of all the sampling sites (Fig 2.16). A main peak in abundance occurred at the end of August at all sites. Cell concentrations reached 3×10^3 cells ml^{-1} at Sites A and B and 5×10^3 cells ml^{-1} at Site C near the surface. There were peaks of lower cell concentration below the euphotic zone and thermocline at Sites A and B and below the thermocline at Site C.

2.4.2.8 Ciliates

The abundance of ciliates was generally less than 200 cells ml^{-1} (Fig 2.17). However, there was a higher abundance of ciliates at the deepest sampling depths at the end of June at all sites. Ciliates were then detected sporadically at all sites throughout the rest of the sampling period. However they were more abundant at Site C throughout the sampling period, and Site A within the epilimnion, but below the euphotic zone at the end of August.

2.4.2.9 Rotifers

The abundance of rotifers peaked at all sites between the end of July and the end of August (Fig 2.18). Most notably, high peaks occurred within the euphotic zone at Sites B and C with densities reaching 8 rotifers ml^{-1}. Lower numbers of rotifers were found between 4 and 10 m at these sites, but there was also a very high peak at 12 m depth (close to the lake bottom) at Site C at the end of July of 12 rotifers ml^{-1}. The rotifer concentration never exceeded 6 organisms ml^{-1} at Site A.

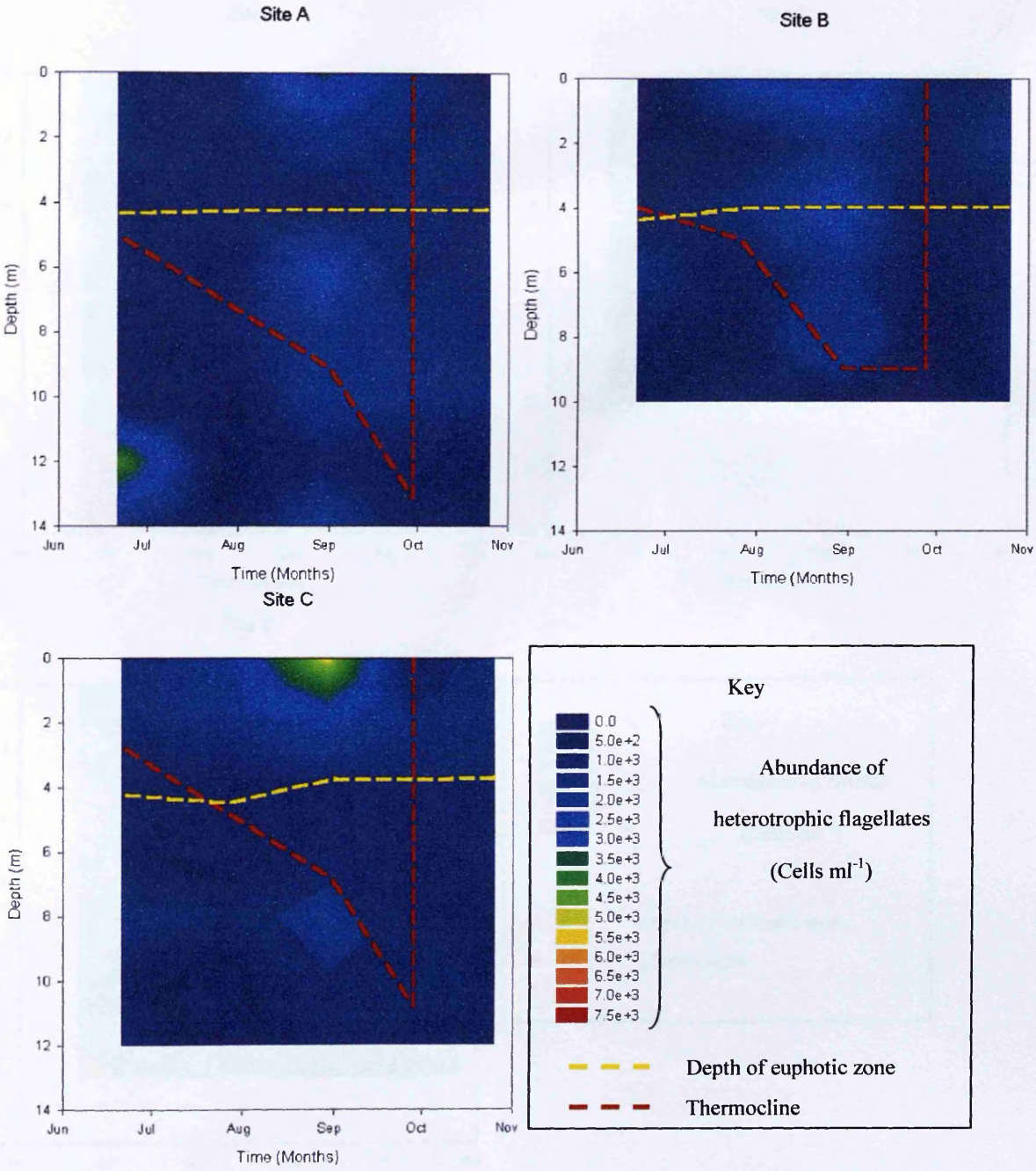


Figure 2.16 Variation in the abundance of heterotrophic flagellates in Esthwaite Water at the three sampling sites over the sampling period

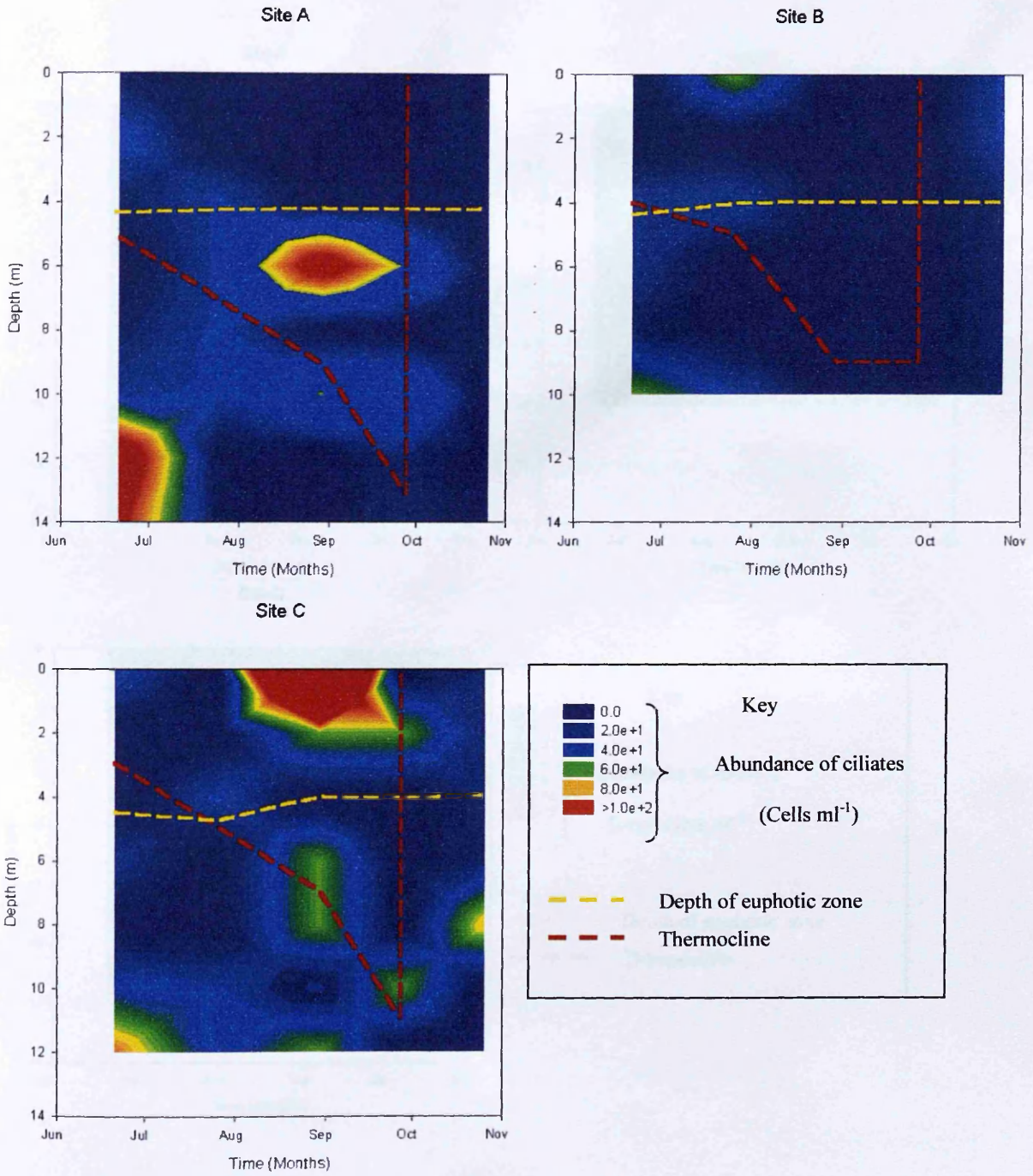


Figure 2.17 Variation in the abundance of ciliates in Esthwaite Water at the three sampling sites over the sampling period

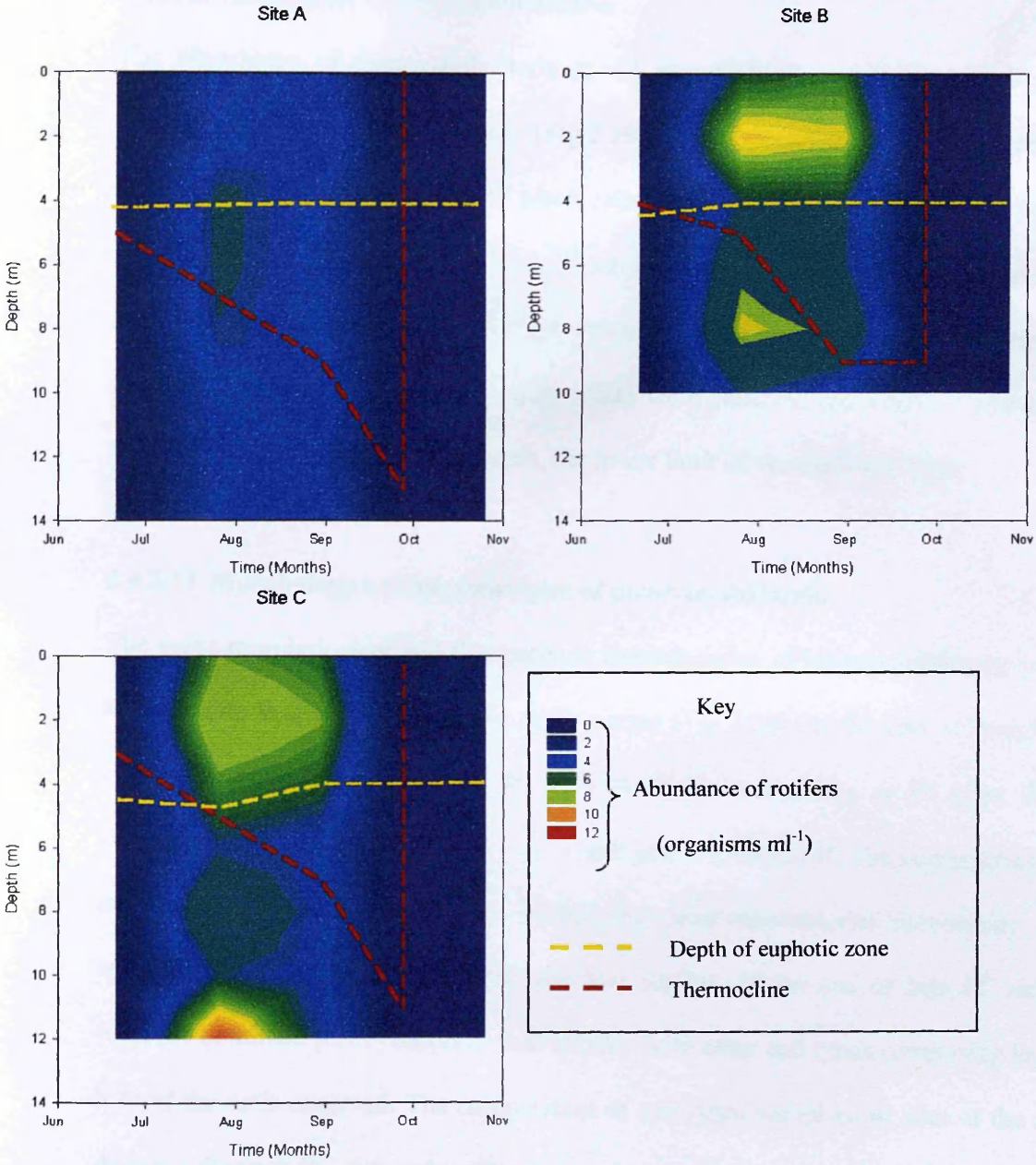


Figure 2.18 Variation in the abundance of rotifers in Esthwaite Water at the three sampling sites over the sampling period

2.4.2.10 Abundance of picocyanobacteria

The abundance of picocyanobacteria at all sampling sites in Esthwaite Water was generally less than 5×10^3 cells ml^{-1} (Fig 2.19). A peak occurred above the thermocline at Sites A and B at the end of July when cells reached 1.5- 2×10^4 cells $ml^{-1}$and more substantially below the thermocline at Site C (5.5×10^4 cells ml^{-1}). This peak diminished at Sites A and B by the end of August, however in the epilimnion at Site C the highest picocyanobacterial concentrations of the study were observed (between 5.4 and 8.5×10^4 cells ml^{-1}). The peak was at 4 m depth, the lower limit of the euphotic zone.

2.4.2.11 Morphology and pigment type of picocyanobacteria

The gross morphological and fluorescence characteristics of picocyanobacteria observed in Esthwaite Water varied over the study period (Fig 2.20). At the end of June PE rich cells were prevalent, accounting for >50 % of the community at all sites. PC rich composed 20 % at Site C, 41 % at Site A and just 5 % at Site B. The composition of the morphological and pigment types within the picocyanobacterial community of the epilimnion and hypolimnion of each site was similar. At the end of July PC rich rods were the dominant picocyanobacteria at all sites with other cell types composing less than 5 % of the cells observed. The composition of cell types varied at all sites at the end of August, although PC rich rods still composed more than 99 % of the community in the epilimnion of Site C. The picocyanobacterial community of the epilimnion of Site B was remarkable at the end of August as PC rich cells only contributed 12 % of the community. The morphological and pigment characteristics of the picocyanobacterial community at the end of September of the epilimnion of Sites A and B was composed of 30 and 37 %

PC rich cells respectively, however the hypolimnion of these sites was dominated by PC rich cells (>80 %). PE rich cells dominated both the epilimnion and hypolimnion of Site C at the end of September. The picocyanobacterial community was composed of 97 % PC rich cells at Site C, 72 % of PC rich cells at Site A and 52 % PC rich cells at Site B in October.

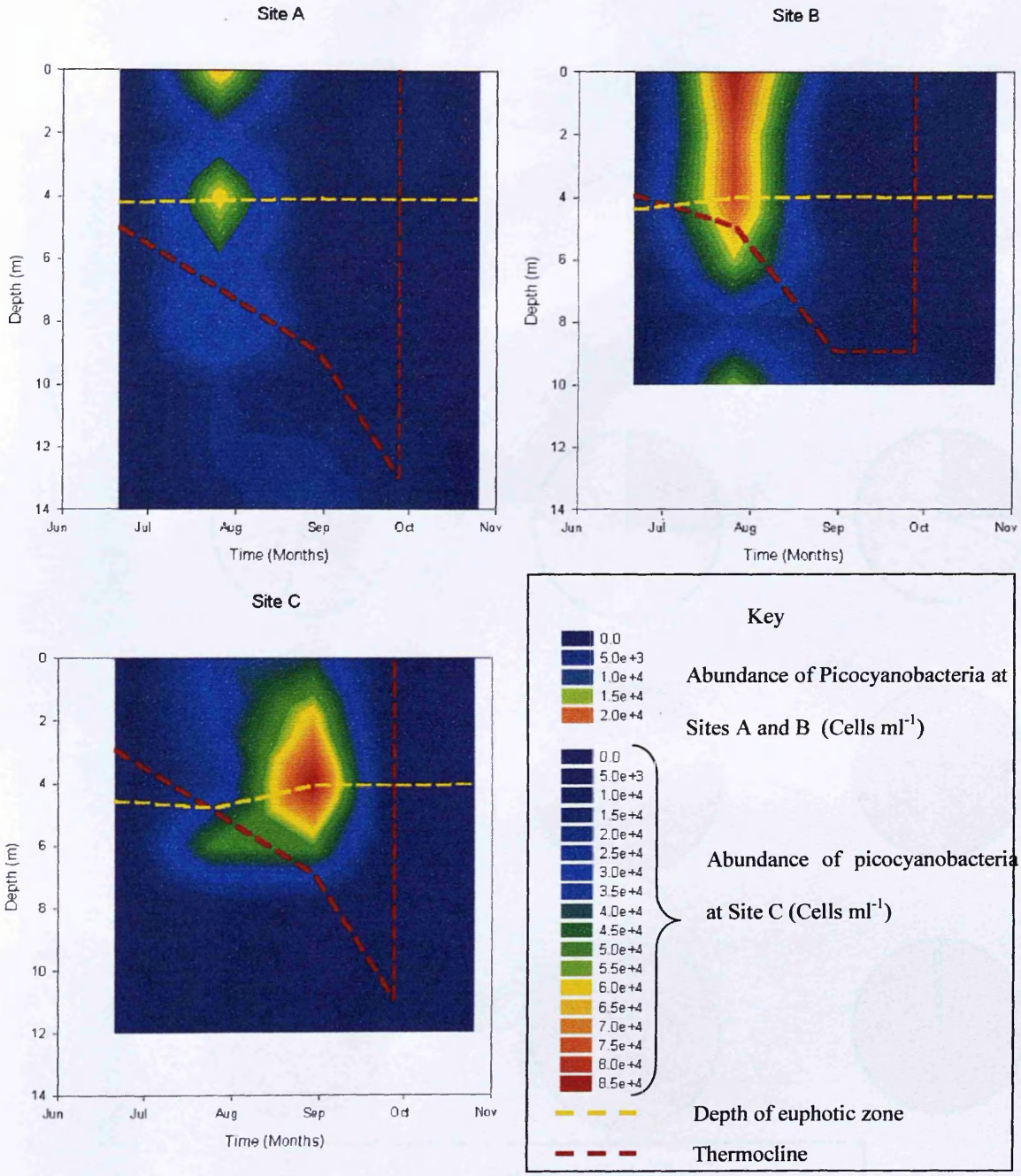


Figure 2.19 Variation in the abundance of picocyanobacteria in Esthwaite Water at the three sampling sites over the sampling period

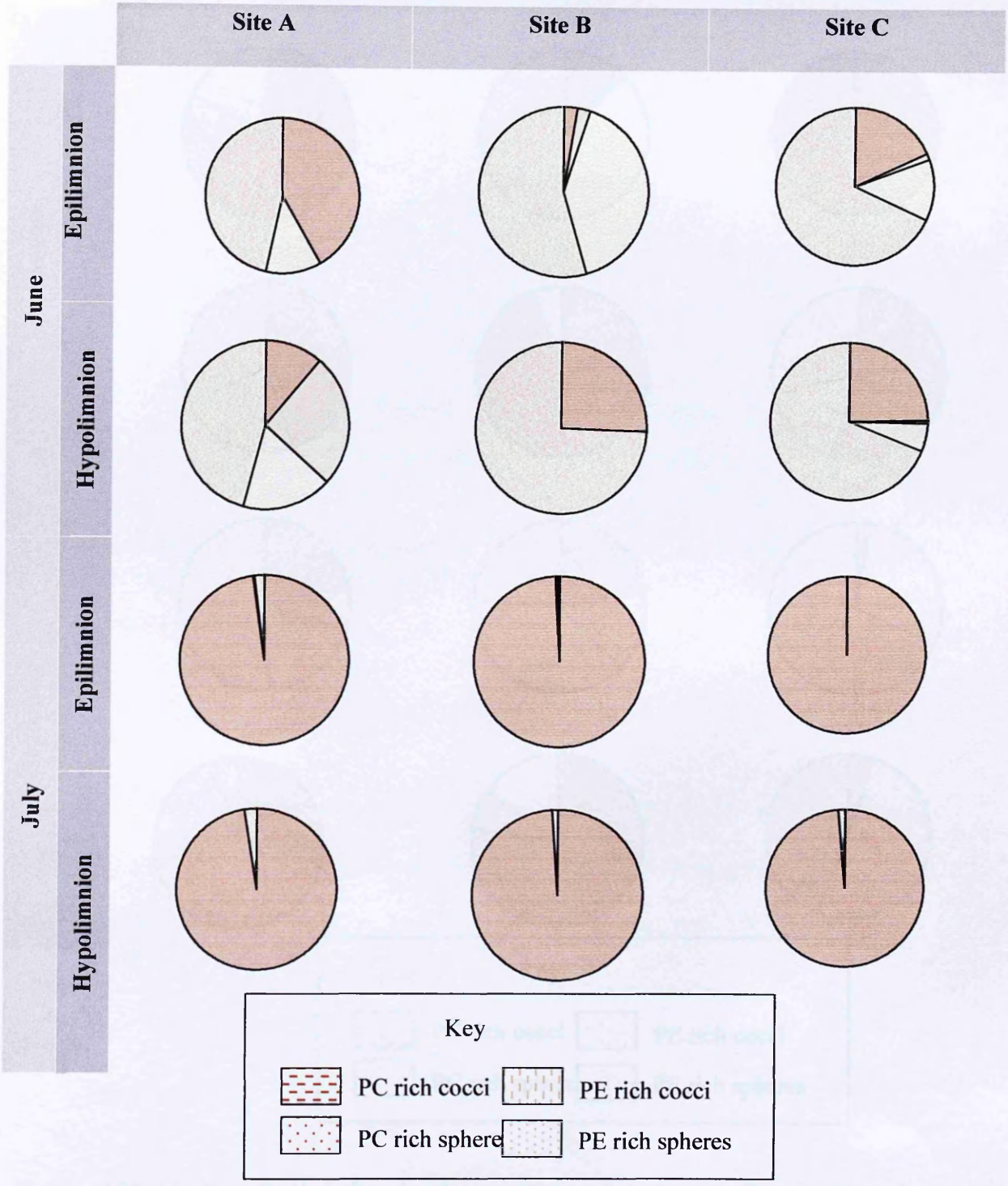


Figure 2.20 Variation in the gross morphology and fluorescence of picocyanobacteria at the three sampling sites over the sampling period

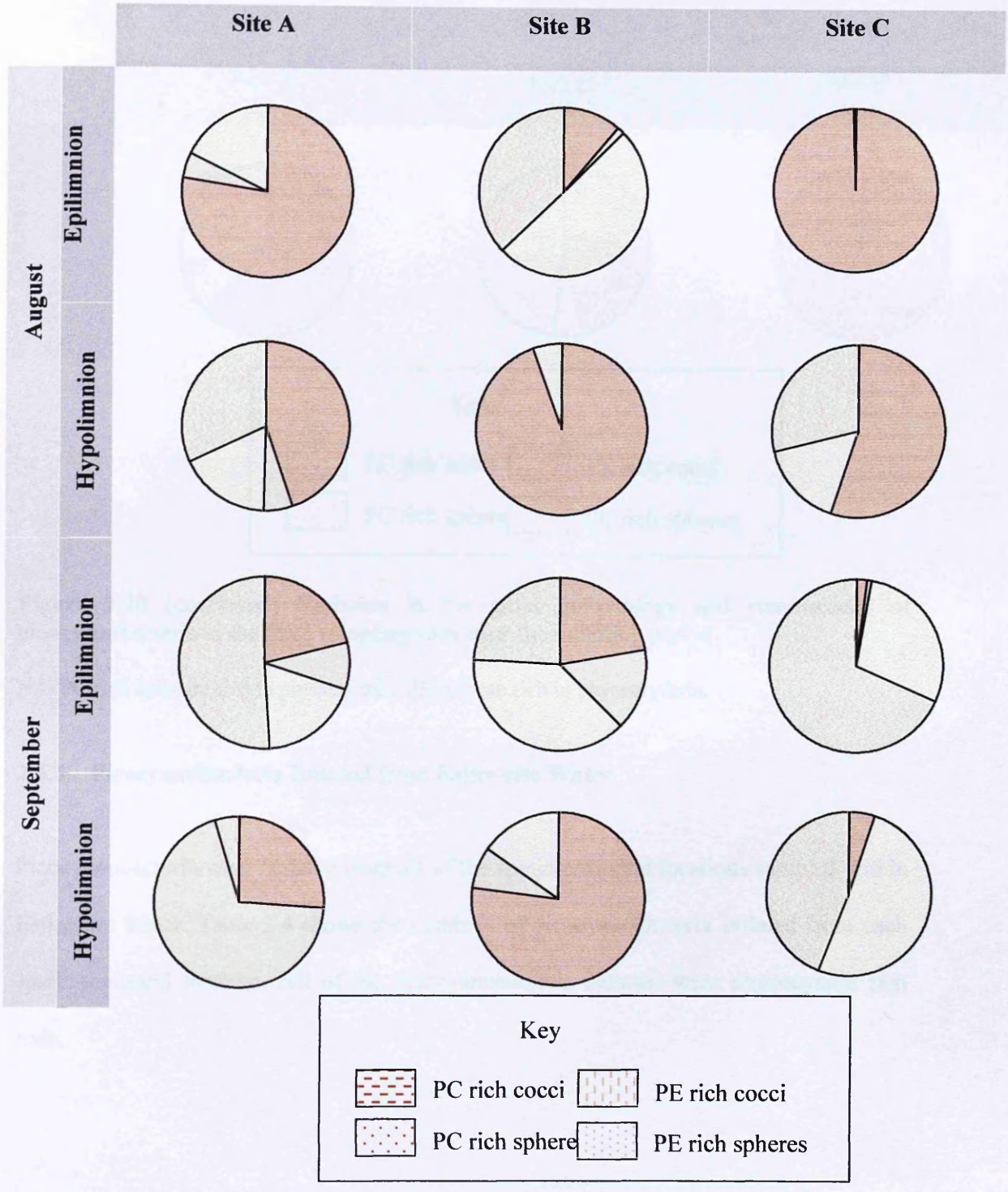


Figure 2.20 (continued) Variation in the gross morphology and fluorescence of picocyanobacteria at the three sampling sites over the sampling period

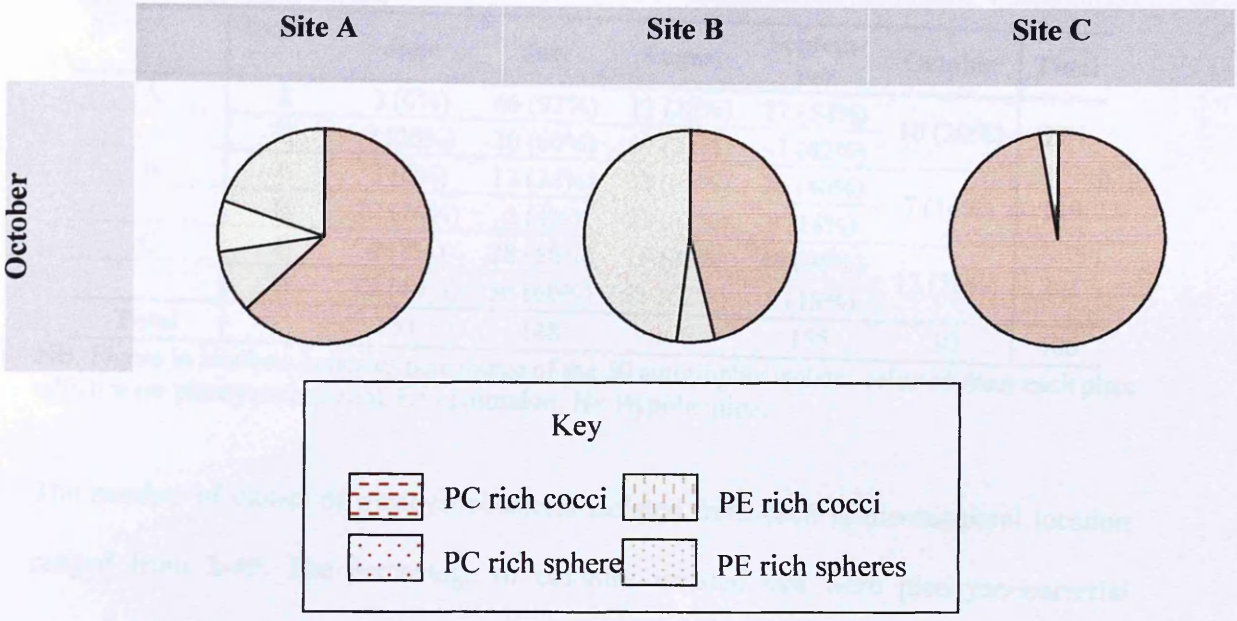


Figure 2.20 (continued) Variation in the gross morphology and fluorescence of picocyanobacteria at the three sampling sites over the sampling period

N.B PC rich cells are rich in phycocyanin, PE rich are rich in phycoerythrin.

2.4.3 Picocyanobacteria isolated from Esthwaite Water

Picocyanobacteria were isolated from all of the spatio-temporal locations sampled within Esthwaite Water. Table 2.4 shows the numbers of picocyanobacteria isolated from each spatio-temporal location. All of the picocyanobacteria isolated were phycocyanin rich rods.

Table 2.4 Number of clones of picocyanobacteria isolated from the three sites over the sampling period

Site	Depth	June	July	August	Septem-ber	October	Total
A	E	3 (6%)	46 (92%)	11 (22%)	27 (54%)	10 (20%)	224
	H	7 (20%)	30 (60%)	11 (22%)	41 (82%)		
B	E	3 (6%)	12 (24%)	33 (66%)	20 (40%)	7 (14%)	119
	H	12 (24%)	2 (4%)	21 (42%)	9 (18%)		
C	E	4 (8%)	28 (56%)	15 (30%)	49 (98%)	13 (26%)	201
	H	22 (44%)	30 (60%)	31 (62%)	9 (18%)		
Total		51	148	122	155	30	506

NB. Figure in brackets indicates percentage of the 50 autotrophic isolates selected from each plate which were picocyanobacterial. E= epilimnion, H= Hypolimnion.

The number of clones of picocyanobacteria isolated from each spatio-temporal location ranged from 2-49. The percentage of colonies isolated that were picocyanobacterial ranged from 4 % to 98 %. The non-picocyanobacterial isolates were mostly *Chlorella* type pico to nano-sized chlorophytes. There were some colonial autotrophic phytoplankton isolates which had come through the pre-filtration stage probably as single cells. These included diatoms such as *Asterionella* and other chlorophytic nanoplankton.

2.4.4 Summary of planktonic community structure

The presence of all groups of planktonic organism monitored during this study is shown at Site A in Fig 2.21, at Site B in Fig 2.22 and at site C in Fig 2.23. It is evident from these diagrams that there is a temporal succession within the community, different communities within the epilimnion and hypolimnion, and some variation between sites.

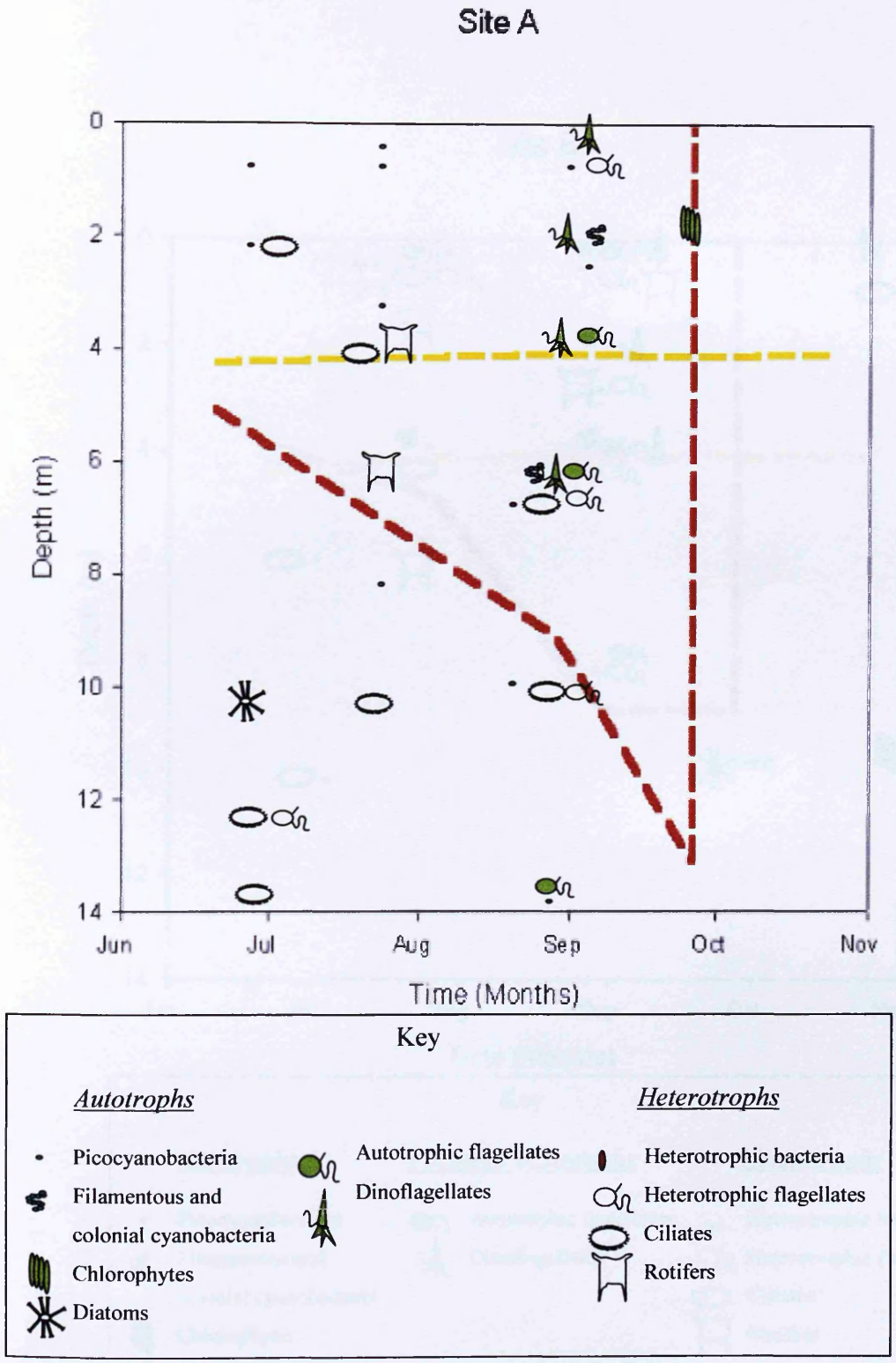


Figure 2.21 Summary of the planktonic community at Site A in Esthwaite Water during the study period

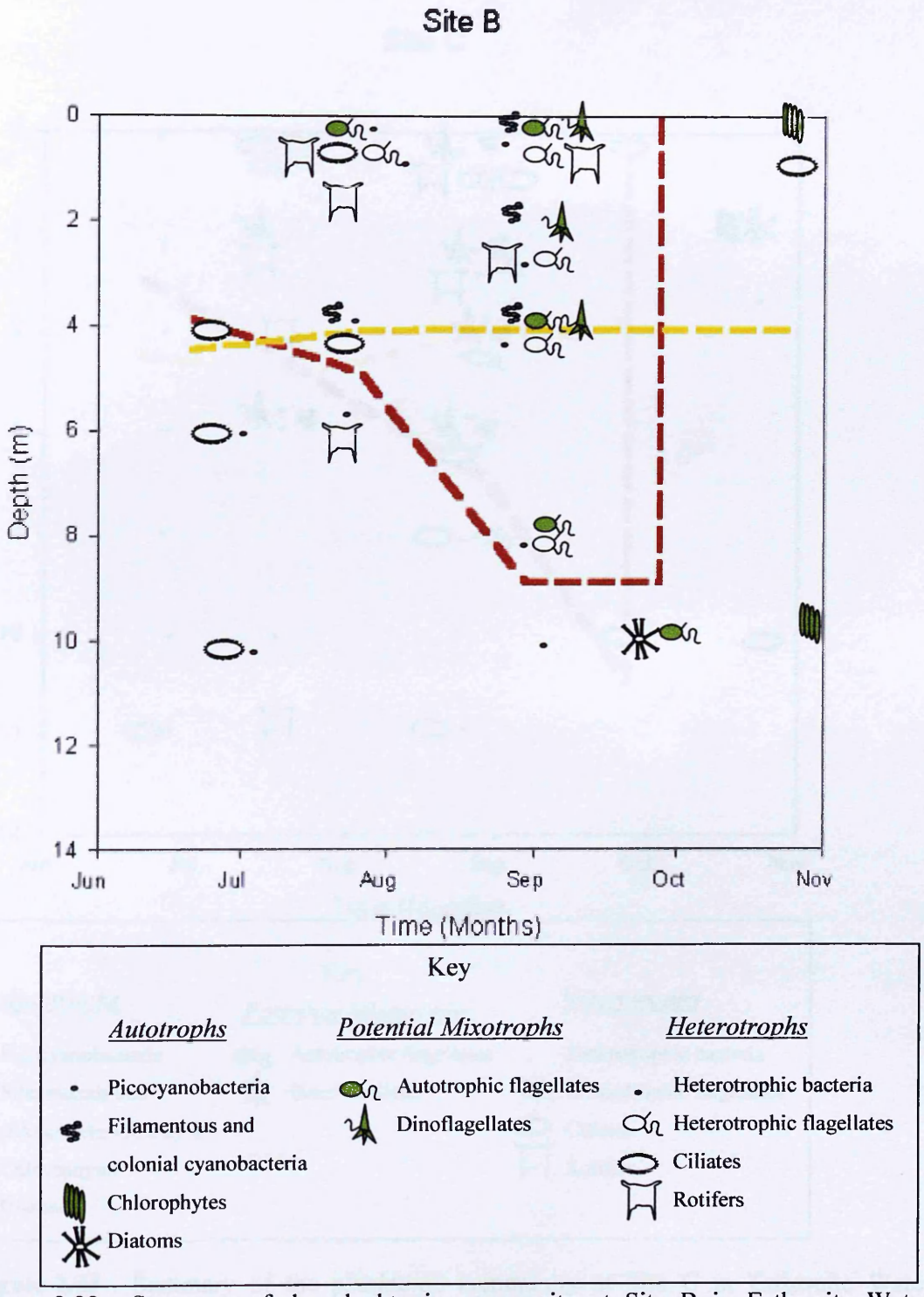


Figure 2.22 Summary of the planktonic community at Site B in Esthwaite Water during the study period

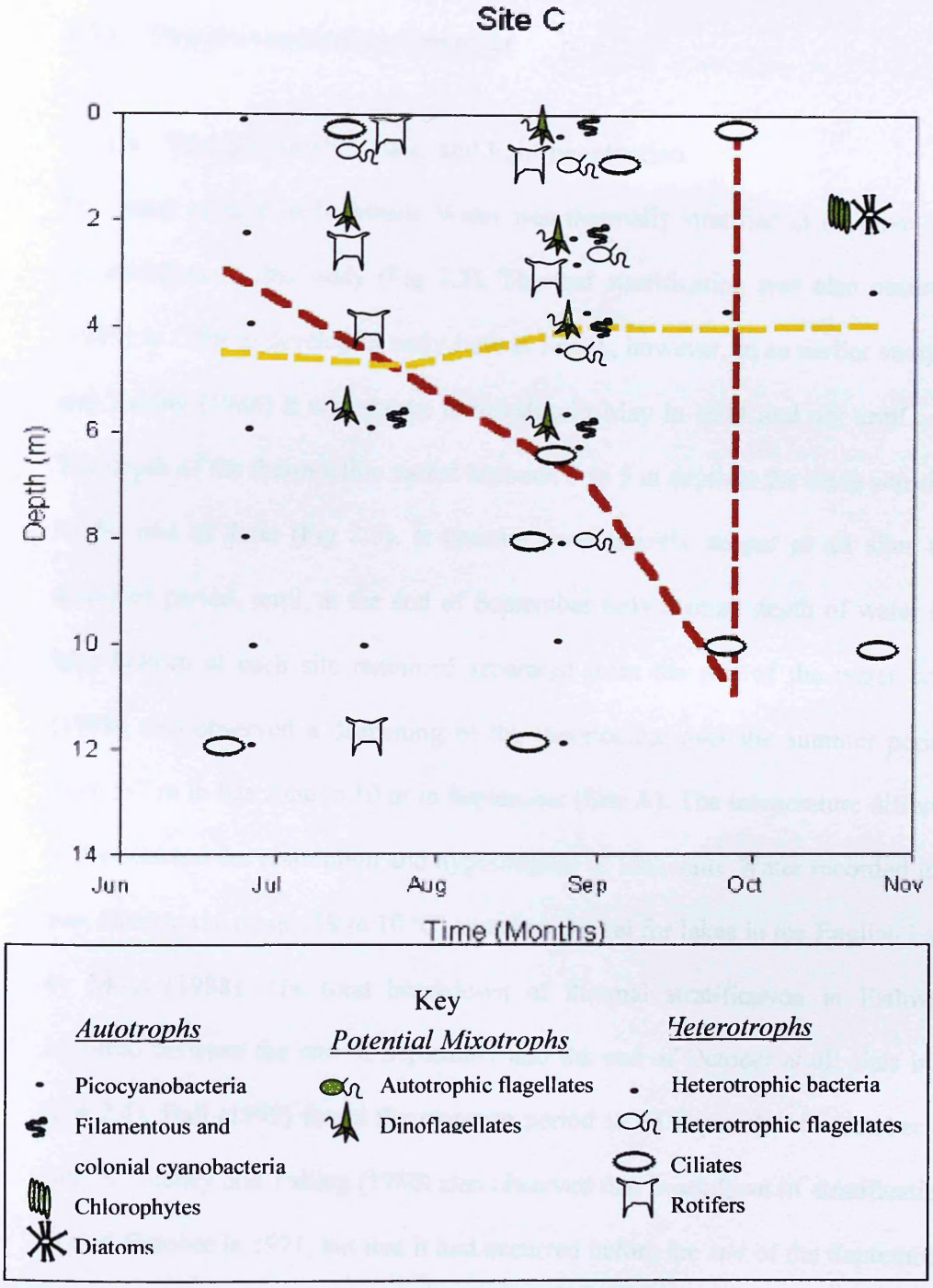


Figure 2.23 Summary of the planktonic community at Site C in Esthwaite Water during the study period

2.5 Discussion

2.5.1 Physico-chemical environment

2.5.1.1 Thermal stratification and light penetration

The water column of Esthwaite Water was thermally stratified at all three sites prior to the initiation of this study (Fig 2.3). Thermal stratification was also observed by Ball (1999) in 1996 to develop in early June at Site A, however, in an earlier study by Heaney and Talling (1986) it was shown to develop in May in 1971 and not until July in 1972. The depth of the thermocline varied between 3 to 5 m depth at the three sites in this study at the end of June (Fig 2.3). It became progressively deeper at all sites through the stratified period, until, at the end of September only a small depth of water close to the lake bottom at each site remained separated from the rest of the water column. Ball (1999) also observed a deepening of the thermocline over the summer period in 1996 from 5-7 m in late June to 10 m in September (Site A). The temperature difference (20 to 9 °C) between the epilimnion and hypolimnion of Esthwaite Water recorded in this study was close to the range (18 to 10 °C) stated as typical for lakes in the English Lake District by Moss (1988). The total breakdown of thermal stratification in Esthwaite Water occurred between the end of September and the end of October at all sites in this study (Fig 2.4). Ball (1999) found the overturn period to be September to October in 1996 at Site A. Heaney and Talling (1986) also observed that breakdown of stratification occured in mid-October in 1971, but that it had occurred before the end of the September in 1972. It is thus apparent that thermal stratification during the summer is a characteristic of Esthwaite Water, but the timing of the development and overturn is dependent on the

prevailing weather conditions. Furthermore, localised differences in the vertical structure may result from the physical morphology of the lake basin (Fryer 1991).

The euphotic zone of the water column observed at each of the sites in Esthwaite Water was also fairly typical for the lake. Ball (1999) recorded a mean euphotic depth of 4.6 m between January and December 1996. In this study, at the end of June the euphotic depth was above the thermocline at Site A, so cells would be circulated through the upper water column where light is available for photosynthesis and the darker regions below (Fig 2.4). At Sites B and C at the end of June, cells in the epilimnion would continuously be exposed to photosynthetically active radiation during the day. At the end of July the cells in the epilimnion at Sites A and B would be circulated through light and dark water, whereas at Site C again the cells would continuously be exposed to photosynthetically active radiation during the day. From the end of August onwards the depth of euphotic zone was less than the depth of the epilimnion at all sites therefore cells unable to regulate vertical position would spend some time out of the euphotic zone. Stratification of water layers shallower than the thermocline may occur during particularly warm anti-cyclonic summer days. Heaney and Talling (1986) found that high temperatures only reached by shallow water and absorption of radiant energy by the denser phytoplankton accentuate the thermal and density gradients and limit appreciable photosynthesis to a superficial (e.g. 0-2.5 m) layer.

2.5.1.2 Dissolved oxygen concentration and pH

The separation of the hypolimnion from the epilimnion by thermal stratification is evident in the dissolved oxygen concentrations at each of the three sites (Fig 2.5). Oxygenation of the epilimnion is a combined result of mixing of water with atmospheric oxygen at the

surface and the net production of oxygen from oxgenic photosynthesis. Ball (1999) found high concentrations of dissolved oxygen in the epilimnion of Esthwaite Water at Site A, but with a maximum concentration of just above 12 mg l^{-1} compared with over 20 mg l^{-1} in this study (Fig 2.3). Removal of oxygen by respiration of organisms in the hypolimnion outweighed any production by photosynthesis. Very low concentrations of oxygen were observed in the hypolimnion of all sites within the water column during July to September. Anoxic conditions, probably exisited close to or within the surface of the sediments at the lake bottom. Anoxic conditions can enable populations of anaerobic bacteria to thrive, but when they occur throughout the water column they can also be responsible for fish kills. By October, in both this study (Fig 2.3) and the study of Ball (1999) wind events had physically disrupted the stratification in Esthwaite Water and the water column became fully mixed giving a uniform oxygen concentration.

The pH of lake water is affected by processes such as photosynthesis and the assimilation of nitrate-N and phosphate, which reduce the concentration of hydrogen (H) ions and therefore cause an increase in pH (Stumm 2004). Photosynthesis was probably a causal factor of the high pH in the epilimnion of all sites during July and August (Fig 2.6). Aerobic respiration of organic matter on the other hand results in a decrease in free H ions [H^+] and hence pH falls (Stumm *et al.* 1996). In the oxygen depleted hypolimnion reductions of nitrate, manganese, iron and sulphate are likely to be responsible for increases in pH which can be seen around the sediment water interface in anoxic water of the deeper Sites A and C during July and August (Fig 2.6).

There were small differences in the pH of the study sites; these are insignificant though as pH can change rapidly as environmental conditions affect photosynthesis and other

processes. Maberley (1996) recorded pH changes of over 1 pH unit on a diel time scale in Esthwaite Water during the summer. He also noted that during stratification, episodes of high pH, typically of 1-2 weeks duration, were interspersed with periods of lower pH. He correlated these patterns with the weather, for example periods of low wind speed, low rainfall and high insolation corresponded to periods of high pH. The periods of high pH recorded during this study were found to correspond to high algal biomass concentrationss. It is interesting to note though, that the highest abundance of phytoplankton occurred at Site C during July and August did not cause a pH elevation to the extent of that at which occurred at Sites A and B. Different organisms have different pH optima so prolonged periods at a particular pH can influence species diversity at a particular location.

2.5.1.3 Nutrient availability

The concentration of nutrients (soluble reactive phosphorus, ammonium-N and nitrate-N) observed during summer in Esthwaite water were low (often below the limit of detection) from June to August 2000 at all three sites (see Table 2.3, and Figs 2.7-2.9). The techniques used to assay nutrients in this study, measured the concentration available for uptake. Other studies from Esthwaite Water (Heaney *et al.* 1986; Ball 1999) and other eutrophic lakes (Moss 1988; Suttle *et al.* 1988; Downing *et al.* 1992; Talling 1993; Kuuppo-Leinikki *et al.* 1994; Fogg 1995; Carrick *et al.* 1997) have shown that nutrients are usually difficult to detect during the spring and summer. This is due to rapid uptake by phytoplankton and heterotrophic bacteria and rapid recycling (Moss 1988; Downing *et al.* 1992; Kuuppo-Leinikki *et al.* 1994; Fogg 1995; Carrick *et al.* 1997). At the end of the stratified period high concentrations of ammonium-N and nitrate-N were detected along

with the occasional peak of phosphorus. These can be explained by a release of nutrients from the breakdown of algal cells from the summer bloom, mixing of top sediment layers with the water column, and an increase in allochthonous material washed in from the catchment with increased surface run-off typical of autumn (Moss 1988).

2.5.2 Planktonic community

2.5.2.1 Autotrophs

In Esthwaite Water, other work has shown that the planktonic community experiences a spring phytoplankton peak in abundance, which is dominated by diatoms; predominantly *Asterionella formosa* (Heaney 1986; Ball 1999). This is succeeded by a clear-water phase as suggested by the PEG model summarised in Chapter 1.5 (Sommer *et al.* 1986). The sampling of Esthwaite Water for this study probably commenced towards the end of the clear-water phase. Heaney *et al.* (1986) noted that populations of *Planktothrix* sp. flagellates and small chlorophytes could occur in deep epilimnetic maxima during this period and these phytoplankters were observed at the end of June in this study (species data not shown). Initially during this study the abundance of phytoplankton cells within the water column was low (Fig 2.10 to 2.12 and 2.19); the presence of a small population of diatoms (mainly *Fragilaria* sp.) at Site A, at the end of June was also noted by Ball (1999) at the same site in 1996 which was quite distinct from the spring maxima. Filamentous and colonial cyanobacteria dominated the autotrophic community in Esthwaite Water from the end of June until the end of October in 2000 (Fig 2.10), both in terms of abundance, but also biomass. This domination has previously been observed in Esthwaite Water (Heaney *et al.* 1986, Ball 1999), and is characteristic of meso-eutrophic

lakes and can also occur in oligotrophic lakes. There have been several theories proposed to explain cyanobacterial dominance including those related to nutrients, light, pH, CO_2, buoyancy, elevated water temperature and zooplankton grazing (see Chapter 1.6). In Esthwaite Water during the sampling period, the epilimnion of all three sites was higher than pH 9.0 between the end of July and the end of August (Fig 2.6); this coincides with the summer phytoplankton maxima and the domination of cyanobacteria (Fig 2.12). High photosynthetic activity also results in a low concentration of CO_2 in the water column; this is another condition to which cyanobacteria show high tolerance compared to other autotrophs (Shaprio 1997). A high abundance of cyanobacterial cells causes an increase in light attenuation. Some cyanobacteria regulate their position in the water column to ensure constant light availability; this often results in surface the scums of cyanobacteria that have been noted in Esthwaite Water (Reynolds *et al.* 1987). Many other species of cyanobacteria are adapted to utilising low light intensity so the prevalence of some species increases overall cyanobacterial dominance (Whitton *et al.* 2000).

The summer phytoplankton maxima in Esthwaite Water also included a significant population of dinoflagellates (Fig 2.12). The abundance of these organisms observed represents a large proportion of the biomass of the plankton community as a whole due to their large size. This was dominated by *Ceratium* sp., but a small number of *Peridinium* sp. were also observed (less than 12 cells per ml). *Ceratium* sp. is a large phytoplankter with a biovolume of approximately 43 740 μm^3 $cell^{-1}$ compared to *Anabaena circinalis* oat99 μm^3 $cell^{-1}$ (Reynolds, 1993). Although abundance of dinoflagellates was considerably lower than that of the cyanobacteria, they would still have contributed a significant portion to autotrophic carbon fixation in Esthwaite Water. The contribution of dinoflagellates to the phytoplankton maxima was greatest at Site A at the end of August,

where the cyanobacterial population was lower than at the other two sites (Fig 2.10). This may have been a result of horizontal patchiness of phytoplankton communities and the effect of wind, for example, on their populations. *Ceratium* sp. may alternatively have competed more effectively at Site A due to the shallower mixed surface layer (Fig 2.12). An inverse correlation between the abundance of *Ceratium* sp. and mixed depth has been noted by Heaney *et al.* (1988) as it is disadvantaged when unable to maintain position within the water column and suffers entrainment to water with lower temperatures and light. *Ceratium* sp. has higher light requirements for the onset and saturation of growth than other phytoplankton including some cyanobacteria (Heaney *et al.* 1985) and a high temperature threshold (Heaney *et al.* 1988). Turbulence may also inhibit cell division, however this has not been demonstrated in *Ceratium* as yet, but has been documented for *Peridinium* (Pollingher *et al.* 1981). Heaney *et al.* (1988) suggested that the *Ceratium* population of Esthwaite Water was probably limited by phosphorus as considerable depletions in cellular phosphorus were observed during the summer phytoplankton maxima. Heaney *et al.*(1986) also demonstrated that nitrogen can be limiting to *Ceratium* in the epilimnion of Esthwaite Water, and that it can move into the hypolimnion to obtain ammonium to sustain up to 25-50% of its growth demand. Migration of *Ceratium* is dependent on energy consuming swimming, and is regulated by a phototactic response. So although through swimming it has access to nutrients not available to other phytoplankton, this type of vertical migration is more restricted and maybe more expensive than the passive migration of gas vacuolated cyanobacteria (Hyenstrand *et al.* 1998). The relative costs of the two mechanisms, however, depends on the growth rate and size of the organism which probably makes flagella motility more favourable for *Ceratium* (Walsby 1994). There is great variability between *Ceratium* populations from

year to year in Esthwaite Water dependent on encystment the previous year, over-wintering conditions, water temperatures (particularly in spring), mixing and fungal parasitism and viral lysis (Heaney *et al.* 1988). If cyanobacteria have become established before *Ceratium* populations develop, *Ceratium* cannot compete well as the cyanobacteria will have caused the depletion of available nutrients (Hyenstrand *et al.* 1998). The higher numbers of dinoflagellates at Site A, and lower numbers of colonial and filamentous cyanobacteria compared to Sites B and C may be a result of a slower end to the clear-water phase at Site A, and subsequent slower release of nutrients. Incidentally, Site C is situated in closest proximity to a commercial fish farm, which would provide a source of nutrients through inputs of fish food and excreta and thus may explain the higher numbers of cyanobacteria and some other autotrophs at Site C in comparison with the other sites. The nutrient analysis from the samples collected did not demonstrate a higher availability of nutrients at site C, however due to rapid uptake and recycling by the planktonic organisms this is to be expected (Moss 1988). The shallower mixed depth at Site A may also have enabled the dinoflagellates to compete with the cyanobacteria preventing them from dominating as they did at the other sites. The dominance of the summer phytoplankton maxima by colonial and filamentous cyanobacteria and dinoflagellates is typical of maturing successions of algae which are able to exploit segregated resources of light and nutrients and conserve rather than propagate their biomass (Reynolds 1993). Communities of these phytoplankters are resistant to invaders, and able to accommodate some environmental variability. They have large cells, with low surface area to volume ratios; they are motile and have relatively high nutrient storage capacities (Reynolds 1988b).

At the end of the stratified period the populations of dinoflagellates and cyanobacteria diminished rapidly (Figs 2.10 and 2.12), no longer able to maintain a favourable position in the water column and grow as quickly in the decreasing water temperatures. Diatoms and chlorophytes were detected at the end of September and October sampling dates (Figs 2.13 and 2.14). These phytoplankton are able to grow in the lower temperatures and become circulated throughout the water column by the isothermal mixing (Padisak 2004).

2.5.2.2 Heterotrophic bacteria

The high abundance of heterotrophic bacteria at in the epilimnion of Site A, hypolimnion of Site B and throughout the water column of Site C at the end of June (Fig 2.15) was probably associated with the decline of the spring algal bloom (2.10-2.14), and the high availability of particulate organic matter from senescent diatom and chlorophyte cells. The bacterial decomposition of this material would have resulted in the production of ammonium and soluble reactive phosphorus, which fuelled the summer phytoplankton maxima. This process would however have been mostly restricted to the hypolimnion after stratification and therefore only available to phytoplankton in the hypolimnion, phytoplankton able to migrate to the hypolimnion or after mixing at the interface between stratified layers (Padisak 2004).

A high abundance of heterotrophic bacteria was recorded during this study at the same spatio-temporal locations as a high abundance of autotrophic organisms. A link between phytoplankton and heterotrophic bacteria has been made by others (Porter *et al.* 1980; Bird *et al.* 1986; Scavia *et al.* 1986; Stockner *et al.* 1986) and linked to the release of Dissolved Organic Matter (DOM) by phytoplankton which is subsequently taken up by the bacteria (Stockner *et al.* 1988). Protozoa and other heterotrophic organisms are also

known to release nutrients during grazing (messy eating) and excretion; in the marine environment at least, re-mineralisation of nutrients is thus considered to be more the result of predation and excretion than direct bacterial decomposition in the pelagic zone (Goldman *et al.* 1985).

Bacteria have the ability to sequester dissolved nitrogen and phosphorus keeping major limiting nutrients in the epilimnion and minimising losses to the hypolimnion. They can out-compete phytoplankton for phosphorus at low concentrations (Currie *et al.* 1984). Heterotrophic bacteria are involved mediating in a variety of reactions within the water column (Stumm 2004). Differences in function and activity cannot be inferred from epifluorescence counts (Poret *et al.* 1965).

2.5.2.3 Heterotrophic flagellates

The abundance of heterotrophic flagellates in freshwaters tends to build up slowly with peaks in the spring and/or summer (Laybourn-Parry 1994). Laybourn-Parry *et al.* (1990) recorded a peak of heterotrophic flagellates in Esthwaite Water of 1.4×10^3 cells ml^{-1} in July-August. During this study a peak of heterotrophic flagellates at 4.27×10^3 cells ml^{-1} was observed at 12m depth at the end of June at Site A, and a summer peak occurred at the end of August at all sites, with cell concentrations reaching 3.28×10^3 ml^{-1} and 3.1×10^3 ml^{-1} at Sites A and B and 5.14×10^3 ml^{-1} at Site C (Fig 2.16). Ball (1999) observed a higher peak of 7.01×10^3 cells ml^{-1} in Esthwaite Water in 1996. Considerable fluctuations have been observed in heterotrophic flagellate populations during some studies (Nagata 1988; Weisse 1991; Laybourn-Parry *et al.* 1993) including the study of Laybourn-Parry *et al.* 1990 in Esthwaite Water, but others have noted a single summer maximum (Pick *et al.* 1987; Carrick *et al.* 1991). It has been suggested by Laybourn-Parry (1994) that the long

sampling intervals of some studies has led some authors to miss complex fluctuations within this group of protozoa. It is possible that with a sampling interval of four weeks that this study has done the same. The peak abundance of heterotrophic flagellates recorded at the end of August (Fig 2.16) may not reflect the full extent of the peak, and other peaks may have occurred between sampling dates. Weisse (1991) demonstrated that seasonal population dynamics of heterotrophic nanoflagellates can show a recurrent pattern with identifiable seasonal phases triggered mainly simultaneous bottom up and top-down control of changing relative significance. In this study high abundance of heterotrophic flagellates often corresponded with high abundances of picocyanobacteria and heterotrophic bacteria. The abundance of heterotrophic flagellates in Esthwaite Water was higher than the abundance of ciliates. Heterotrophic flagellates normally out number ciliates in the pelagic zone of lakes where they are the major consumers of picoplankton (Stockner *et al.* 1988; Bloem *et al.* 1989; Nagata *et al.* 1996; Pernthaler *et al.* 1996).

2.5.2.4 Ciliates

The abundance of ciliates observed in Esthwaite Water during this study (Fig 2.17), often exceeded the usual range for ciliates in temperate lakes of 0.5-11 $\times$ 10^3 cells l^{-1} (references within (Laybourn-Parry *et al.* 1993), and the maximum recorded for Esthwaite Water of 9.2 $\times$ 10^3 cells l^{-1} which occurred in spring 1988 (Laybourn-Parry *et al.* 1990), this may have been linked to the higher heterotrophic flagellate numbers also recorded in this study (Fig 2.16). Abundance of ciliates throughout the summer phytoplankton maxima appeared to coincide with high picocyanobacterial and heterotrophic bacterial abundances, which also coincided with high heterotrophic

flagellate abundances (Figs 2.22 to 2.24). The ciliate community is composed of a variety of taxa with different food requirements (Weisse *et al.* 1991). Many studies have shown picoplankton to form the main constituents of the diet of ciliates, particularly those <30 μm in size (Sherr *et al.* 1991; Simek *et al.* 1995; Hadas *et al.* 1998; Simek *et al.* 2000). Laybourn-Parry (1989) found no significant link between ciliates and chlorophyll *a* concentration, but a correlation between ciliate numbers and flagellate density was apparent in Esthwaite Water.

In both Esthwaite Water and Windermere North and South basins, peak ciliate abundance has been recorded in spring to early summer (May to early June) during the peak abundance of nano-phytoplankton and low grazing pressures from macro-zooplankton (Laybourn-Parry *et al.* 1990; 1993; 1994). This would suggest that the peak ciliate abundance had past previous to the commencement of sampling during this study. There was however a relatively high abundance of ciliates observed at depth at all sites on the first sampling date (Fig 2.17). This may have been part of a decline in ciliates in the water column from a peak initiated by decomposition of diatoms and associated increase in heterotrophic bacteria and/or heterotrophic flagellates at those depths. For most of the stratified period, maximum ciliate densities were observed above the thermocline (Fig 2.18), which is consistent with the findings of Laybourn-Parry (1990).

2.5.2.5 Rotifers

Peak abundance of rotifers corresponded with high abundance of autotrophs in the upper water column which act as a vital food source (Fig 2.18). Rotifers are classified as consumers of particulate matter; potential food resources include algal and bacterial picoplankton, but also phytoplankton (ranging in size from flagellates to filamentous

cyanobacteria) ciliates, nauplii and other rotifers. Rotifers exert grazing pressure on ciliates and heterotrophic flagellates (Jurgens *et al.* 2000). They can efficiently graze on autotrophic flagellates (Bogden *et al.* 1982; Weisse 1990) and picoplankton (Simek *et al.* 1995). Grazing of filter feeders does not continuously regulate overall biomass of phytoplankton, but by discontinuously acting on smaller algae can influence the specific composition of the phytoplankton and rates at which maximum population maxima develop or collapse (Reynolds 1986, Blelham). Rotifers also play an important role in the recycling of nutrients, through phosphorus excretion (Bogden *et al.* 1982). They may play an important role in phytoplankton production rates and community structure through this process as well as through exertion of grazing pressure.

2.5.2.6 Mixotrophs

Many autotrophic flagellates also have the ability to consume food heterotrophically, in this study the abundance of autotrophic flagellates was highest in the epilimnion, where both light and picoplankton were most abundant (Fig 2.11). Fahenstiel (1991) observed autotrophic flagellates having ingested picocyanobacteria, but their contribution to grazing never exceeded 8% of total protozoan grazing. Dinoflagellates are also known to have the ability to feed heterotrophically (Jacobson 1999). No quantitative work has been done on the importance of heterotrophy to *Ceratium*, the main dinoflagellate observed in Esthwaite Water, but food vacuoles of *Ceratium hirudinella* have been found to contain bacteria, cyanobacteria, diatoms and ciliates (Dodge *et al.* 1970). This species is common in Esthwaite Water (Heaney *et al.* 1988). *Ceratium furca* has been reported to ingest particles using fluorescent beads. Observations of food vacuole contents demonstrated the presence of the ciliate *Strombilidium* spp. and tintinnids within this dinoflagellate

(Smalley *et al.* 1999). Experiments for an undergraduate dissertation at Lancaster University have indicated that increasing the concentration of picocyanobacterial cells in Esthwaite lake water can enrich for dinoflagellates even in the dark (Davies, 2000). It is evident that mixotrophy occurs in freshwater systems, but further research is required to understand its importance in a wide range of lake systems and to the functioning of aquatic microbial ecosystems.

2.5.3 Picocyanobacteria in Esthwaite Water

2.5.3.1 Abundance

The maximum abundance of picocyanobacteria in Esthwaite Water at each site in this study is compared to the abundance of picoplankton in Lakes in the English Lake District by (Hawley *et al.* 1991) and (Ball 1999) in Table 2.5. Site A sampled in this study, was also sampled by (Hawley *et al.* 1991) and (Ball 1999). However, the abundance of picoplankton reported by Hawley *et al.* (1991) was an order of magnitude less than those of Ball (1999) and the present study. This may have been due to annual variations in abundance at the site, or differences in sampling technique. Hawley *et al.* (1991) sampled 0.5 m depth using a Ruttner bottle, whereas Ball (1999) took a 0-6 m integrated sample, and the present study sampled water from 2 m depth intervals. The end of July, 0 m sample, contained the maximum abundance of picocyanobacteria at Site A in the present study, (1.71×10^4 cells ml^{-1}), however the 2 m sample did have a lower picocyanobacterial abundance of 7.89×10^3 cells ml^{-1} which was closer to the maximum abundance of Hawley *et al.* (1991). The maximum average cell density of picocyanobacteria between 0-6 m (average of samples taken at 0, 2, 4 and 6 m depth) at

Site A in this study also occurred on the end of July sampling date ($1.3x\ 10^4$ cells ml^{-1}), this was further from the data of Ball (1999) than the maximum abundance at one depth location. The pre-filtering stage of the enumeration procedure also varied between the studies, Hawley *et al.* (1991) used a 3.0 μm filter to ensure cells at the large end of the 'pico' size range passed through. Cells with diameters > 2.0 μm were omitted from counts. Ball (1999) pre-filtered samples through a 2.0 μm filter. These two studies, therefore enumerated a smaller size range of cells than enumerated in the present study (any cells which passed through 3.0 μm in size see Chapter 2.2). This maybe reflected by the higher abundance of picocyanobacterial cells enumerated in this study compared with Hawley *et al.* (1991) but it was commented by Hawley *et al.* that cells with a diameter >2.0 μm were infrequently observed during counts. Due to the method used, it would be expected that Ball (1999) enumerated cells with the smallest size range, as cells approaching 2.0 μm in diameter may have become trapped in the filter. However, the highest abundances of picoplankton were recorded at Site A were from his study, so the variation between studies is probably a reflection of annual variation and contribution by eukaryotic cells. The data of Ball (1999) and Hawley *et al*, (1991) were of the whole pico-fraction rather than just the picocyanobacteria. The abundance of picocyanobacteria observed during this study was significantly different between the three sites and the epilimnion and hypolimnion of each site on all sampling occasions (95 % confidence interval of the standard error of the mean) with the exception of the hypolimnion at Site B, and epilimnion of Site C in June which were not significantly different from each other. The picocyanobacterial abundance recorded during the peak at Site C in particular was substantially higher than observed at other spatio-temporal locations (Fig 2.19) and

indicates that abundance can vary significantly within Esthwaite Water spatially and temporally.

Table 2.5 Abundance of picocyanobacteria in lakes in the English Lake District

Lake	Trophic status	Study	Sampling date	Maximum cell concentration (cells ml^{-1})*
Ennerdale Water	Oligotrophic	Hawley *et al.* (1991)	1988	1.3×10^6
Wastwater	Oligotrophic	Hawley *et al.* (1991)	1988	8.2×10^5
Coniston Water	Mesotrophic	Hawley *et al.* (1991)	1988	4.5×10^5
Derwent Water	Mesotrophic	Hawley *et al.* (1991)	1988	2.65×10^5
Ullswater	Oligo-mesotrophic	Hawley *et al.* (1991)	1988	2.46×10^5
Thirlmere	Oligotrophic	Hawley *et al.* (1991)	1988	1.91×10^5
Windermere South Basin	Mesotrophic	Ball (1999)	1996	1.59×10^5
Winderemere North Basin	Mesotrophic	Ball (1999)	1996	1.12×10^5
Bassenthwaite	Meso-eutrophic	Hawley *et al.* (1991)	1988	7.92×10^4
Esthwaite Water (Site C)	Meso-eutrophic	Present Study	2000	$6.16 \times 10^4 (\pm 4.5 \times 10^3)$
Wastwater	Oligotrophic	Ball (1999)	1996	5.53×10^4
Esthwaite Water (Site A)	Meso-eutrophic	Ball (1999)	1996	3.32×10^4
Malham Tarn	Meso-eutrophic	Hawley *et al.* (1991)	1988	3.26×10^4
Esthwaite Water (Site B)	Meso-eutrophic	Present study	2000	$2.11 \times 10^4 (\pm 3.0 \times 10^2)$
Casop Pond	Eutrophic	Hawley *et al.* (1991)	1988	1.65×10^4
Esthwaite Water (Site A)	Meso-eutrophic	Present study	2000	$1.3 \times 10^4 (\pm 9.8 \times 10^2)$
Esthwaite Water (Site A)	Meso-eutrophic	Hawley *et al.* (1991)	1988	3.42×10^3

N. B. Figures in brackets show range recorded in this study.

During this study, peak concentrationss of picocyanobacteria mostly occurred within the epilimnion of Esthwaite Water, but were not restricted to the euphotic depth (Fig 2.19). Picocyanobacteria cannot regulate their position in the water column, so were mixed within the epilimnion through the euphotic zone and the darker water below. The two separate peaks at 0 m and 4 m at Site A at the end of July indicate that there was stratification of the water column between these peaks and hence they may have been circulated separately. At Site B at the end of July the picocyanobacteria were distributed evenly throughout the epilimnion indicating that this layer was well mixed. At Site C at the end of September, there may have been a shallow stratification of the water column that was not mixed with the rest of the epilimnion this is indicated by the lower abundance of picocyanobacteria at the surface. The surface layer would also have been constantly exposed to high light intensity during the day, which is unfavourable to many types of phytoplankton (Moss 1988).

The peak abundance of picocyanobacteria at Site C appears to have been initiated at the end of July beneath the thermocline (Fig 2.19), this pattern of population growth also occurred for filamentous and colonial cyanobacteria and autotrophic flagellates and dinoflagellates. The euphotic depth at this site was similar to the depth of the thermocline, so light penetration may have been sufficient just below the thermocline for photosynthesis to occur and nutrients may have been available from the recycling of particulate and dissolved organic matter by heterotrophic bacteria which were also prevalent at the site.

The peak abundance of picocyanobacteria at Sites B and C in August also coincided with a high abundance of other primary producers, particularly filamentous and colonial cyanobacteria and autotrophic flagellates (Figs 2.22 to 2.24). This implies that abiotic conditions were favourable for all of these organisms at these sites.

The peak abundance of picocyanobacteria at Site A, in July (Figs 2.20 to 2.22), did not coincide with high abundance of other autotrophic organisms, suggesting that environmental conditions were limiting the growth of larger phytoplankton, but were sufficient for picocyanobacteria to thrive. Freshwater picocyanobacteria are rarely limited by the availability of soluble reactive phosphorus (Wehr 1989), so as larger phytoplankton such as dinoflagellates and filamentous and colonial cyanobacteria have strategies for obtaining nitrogen (discussed above and in Chapter 1) the concentration of soluble reactive phosphorus at this site was possibly insufficient for growth of larger phytoplankton. Wehr (1991) demonstrated that the flux of phosphorus was mediated by picoplankton under low nutrient conditions, but was a function of phytoplankton biomass under conditions of phosphorus surplus.

It is interesting to note that the abundance of picocyanobacteria in Esthwaite Water during this study at Sites A and B (Fig 2.19), and Site A studied by Ball (1999) and Hawley *et al.* (1991) were similar to lakes within the eutrophic to meso-eutrophic range of lakes sampled within these studies (Table 2.5). The abundance of picocyanobacteria at Site C in this study however was higher and more typical of lakes in the oligotrophic to meso-eutrophic range (Table 2.5). Other studies have found a high abundance of picocyanobacteria in eutrophic waters (reviewed in Chapter 2.1), and it is more likely from the abundance of larger autotrophic organisms (Figs 2.10 to 2.14) that higher concentrations of nutrients were present at Site C, and to a lesser extent Site B compared with Site A. Wehr (1989; 1990; 1991) has also repeatedly demonstrated that trophic status is not a good indicator of the importance or abundance of picoplankton, but that smaller cells are relatively more abundant when phosphorus is limiting.

In terms of nutrient recycling, picocyanobacteria are able to compete for and assimilate nutrients at low concentrations. They have fast growth rates (Stockner *et al.*

1986; Fogg 1995; Raven 1998), and a negligible sinking rate (Beinfang *et al.* 1983), so nutrients within picocyanobacteria are not lost to the hypolimnion, or sediment (Stockner *et al.* 1988). The role of picocyanobacteria as a sink or link in nutrient recycling is still debated, but hinges on the flow of energy from picocyanobacteria through the food web (Fig. 2.24).

It has been found that picocyanobacteria are selected against or not digested by protozoa and other organisms (Caron *et al.* 1991; Jurgens *et al.* 2000). Thus picocyanobacteria may be a sink for nutrients until cell senescence or viral lysis occurs. Alternatively there is evidence, which suggests picocyanobacteria are grazed rapidly, so nutrients are recycled along the microbial food chain. Pernthaler *et al.* (1996) even suggested that the importance of picocyanobacteria to heterotrophic nanoflagellates as a food reserve was almost twice their contribution to the picoplankton due to rapid turnover of cells. The complexity of the microbial food chain determines the efficiency of the transport of nutrients to higher trophic levels.

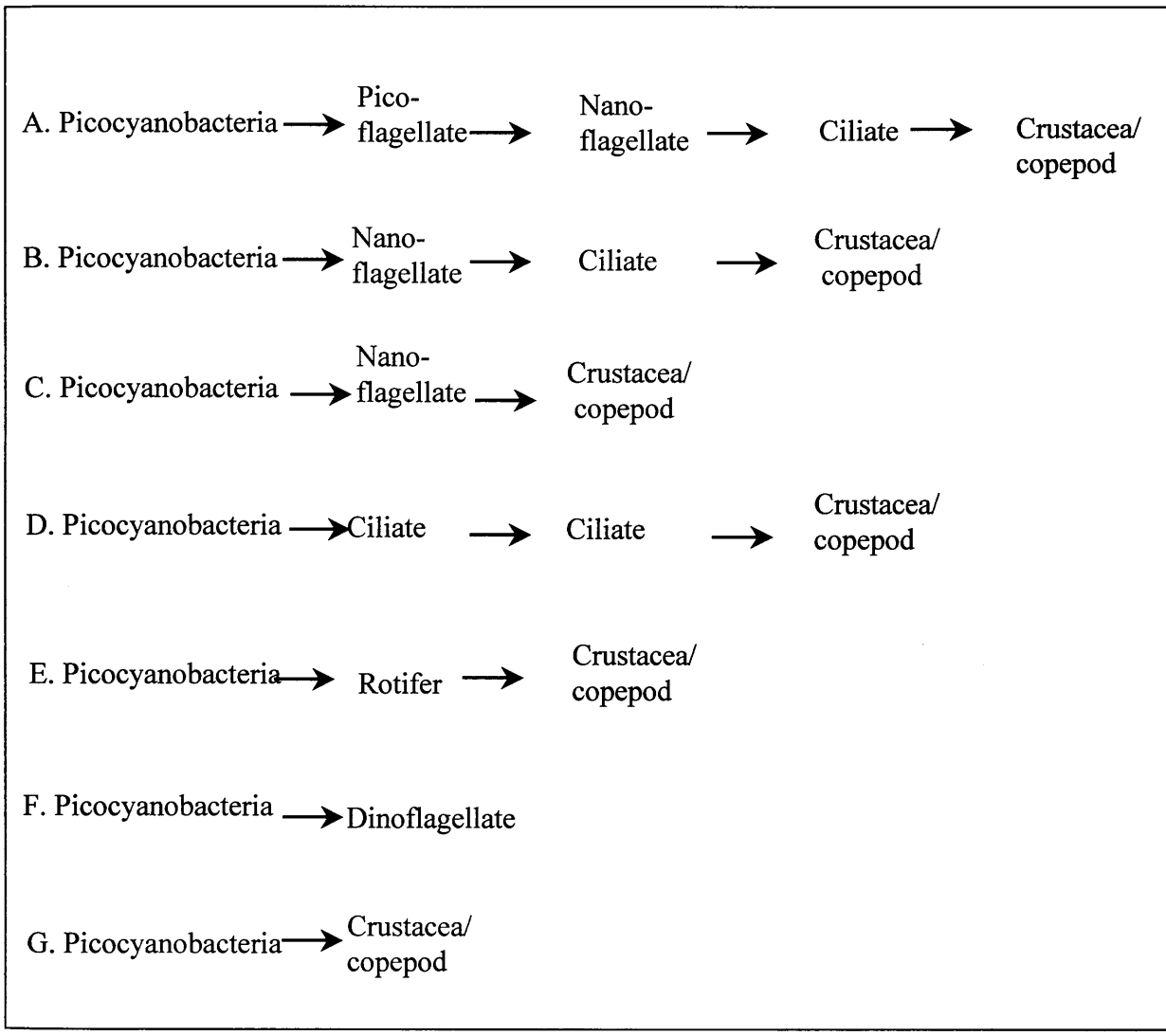


Figure 2.24 Flow of energy between picocyanobacteria and higher trophic levels N. B Sources: (Weisse 1991; Weisse *et al.* 1991; Fahnenstiel *et al.* 1992; Nagata *et al.* 1996; Simek *et al.* 2000) N.B. Crustacea , copepods and dinoflagellates are predated by fish.

The more links involved within a food chain the more energy is lost through respiration and excretion along the chain and so less reaches the higher trophic levels (Moss 1988). Nagata (1996) and Weisse (1991) for instance, both discuss how chain C in Fig 2.24 is more efficient than chain B. This increases the significance of grazing of picocyanobacteria by larger organisms like *Daphnia* even though they constitute only a small amount of the grazing losses from picocyanobacteria (Fahnenstiel *et al.*

1992). On the other hand grazing by dinoflagellates such as *Ceratium* would act as a sink for energy from picocyanobacteria as there large size provides a refuge from grazing in freshwater systems (Fahnenstiel *et al.* 1992).

Viruses and fungi were not enumerated during this study, however, they are expected to exhert some control on picocyanobacterial abundance and diversity. Dorigo *et al.* (2004) demonstrated using DGGE that 35 distinct cyanomyovirus *g20* genotypes could be elucidated from 47 sequences analysed from Lake Bourget, France. These appeared to be picocyanobacterial cyanophages.

2.5.3.2 Gross morphological characteristics and pigment types

The characteristics of the picocyanobacterial community in Esthwaite Water varied considerably over the study period. At the end of June, the community was composed of both rods and spheres, primarily rich in phycoerythrin. At the end of July and during the peak maximum picocyanobacterial abundance (i.e. including the epilimnion of Site C in September) the community was dominated by phycocyanin rich rods. As the picocyanobacterial abundance declined in August, September and October a more diverse community of picocyanobacteria, with regards to morphological and pigment types, was observed. The characteristics of the picocyanobacterial community also differed between sites and water layers. Ball (1999) found the majority (always >75%) of picoplankton cells to be eukaryotic in Esthwaite Water. Within the picocyanobacteria he observed a peak of PE-rich cells in May, followed by a peak of PC-rich cells in July 1996. Hawley and Whitton (1991) present only the percentage of cells containing PE at the maximum and minimum cell abundances to represent the type of picoplankton present in lakes. They demonstrated that the peak picoplankton abundance in Esthwaite Water, in 1988, was in May and was composed of 59% PE containing cells.

2.5.3.3 Isolation of picocyanobacteria

The number of picocyanobacteria isolated from the 50 colonies selected from each plate was not uniform through out the sampling period. However, the variation does not coincide with the spatio-temporal abundance of picocyanobacteria. This is because 50 'green' colonies were selected from a plate from each spatio-temporal sampling occasion so the variation in numbers isolated was related to the ratio of culturable picocyanobacteria to other culturable algae (e.g. chlorophytes and diatoms). As some picocyanobacterial colonies were isolated on each spatio-temporal sampling occasion the communities at each location have been represented in some way during the study. All of the picocyanobacteria isolated were PC rich, rod shaped picocyanobacteria. Most workers would classify these as *Synechococcus* spp.. This morphological form (phenotype) was the most abundant observed in Esthwaite Water during the study period and comprised most of the picocyanobacterial community at all sites during July. However, both PE and PC rich forms were present in Esthwaite Water during the study period, forming the most significant portion of the community at different times (Fig 2.20). Spherical forms of PC rich picocyanobacteria were also observed, but not isolated. This suggests that the culturing conditions used within this study were not equally appropriate for all types of picocyanobacteria and may have selected for PC rich *Synechococcus*-type. Although BG11 is suitable for the growth of a wide range of cyanobacteria and is used to maintain over 300 isolates at the Pasteur Institute (Rippka 1988) and the CCAP, (Tompkins *et al.* 1995), some constituents may make it unfavourable for some cyanobacteria e.g. high concentrations of nitrate, presence of citrate, absence of nickel, presence of copper. Other studies have also had a poor culturing success for PE rich picocyanobacteria even when the habitat sampled was dominated by PE rich forms. Ernst *et al.* (1995)

used BG11 with a reduced nitrate content and exposed the media to green light to enrich for PE rich forms, but still had a very low plating efficiency and cultivation success from Lake Constance where abundance of picocyanobacteria rich in PE is significantly higher than those rich in PC. Ernst *et al.* (1995) were able to isolate three clones of PE rich picocyanobacteria after co-cultivation of picoplankton with ciliates or exposure to high or low light intensities. This suggests that isolation of picocyanobacteria is not just affected by the media used, but as suggested by Rippka (1988), also requires other novel and innovative culture conditions to be applied. The isolation and culturing of picocyanobacteria from other habitats has also proven difficult. Douglas and Carr (1988) found that *Synechococcus* spp. from marine habitats are sensitive to high light, do not grow well on solid media, and can be inhibited by Tris and impurities in agar, glassware and water used in media preparation.

It is disappointing not have had the opportunity to compare the frequency and distribution of HIP 1 sequences from both PE and PC rich picocyanobacteria within this study. Ernst *et al.* in 2003, demonstrated that PE rich and PC rich strains can be very closely related using ITS-1 sequencing. They suggested that the ability to synthesize PE, and thus acclimatize to different light qualities, could be lost or gained by loss or gain of genes. They suggested this could occur through point mutations, extended deletions or recombination with intact genetic information provided from a closely related organism. Thus although not isolated within this study, PE rich picocyanobacteria may be closely related to the PC rich picocyanobacteria isolated.

2.6 Conclusions

This study has demonstrated that the abiotic characteristics and planktonic community of Esthwaite Water during the summer of 2000, were typical for the lake itself, and typical of meso-eutrophic lakes. The general community structure followed that suggested by the PEG model summarised in Chapter 1 (Sommer *et al.* 1986). There was some variation in characteristics between the study sites, which lead to the development of different autotrophic plankton communities. This spatial variation was particularly true of picocyanobacterial abundance, which showed a considerably larger peak at Site C than the other sites. Picocyanobacterial abundance varies between lakes, between years in the same lake and within one lake. The controlling factors appear to be complex as picocyanobacteria occur under a range of physio-chemical conditions. During this study potential predators (protozoa, rotifers and potential mixotrophs) were often present at the same spatio-temporal locations as peaks of picocyanobacterial abundance in Esthwaite Water, possibly indicating that predator-prey relationships existed between them. However, as the highest abundance of picocyanobacteria correlated with larger summer maxima of phytoplankton (Site C), this suggests that nutrients were limiting to picocyanobacteria in Esthwaite Water. Site C is situated in closest proximity to a fish farm, which would provide a source of nutrients through inputs of fish food and excreta.

The composition of the picocyanobacterial community may be a significant factor in determining the competitive ability of the group. As mentioned in the introduction, different picocyanobacteria have different growth requirements in terms of nutrients and light etc, but they may also have different susceptibilities to grazing. Morphological features such as size and hydrophobicity of prey cells affect the rate at which they are ingested by protozoa (Monger *et al.* 1999), whilst factors such as the

structure of their cell wall may determine whether they are digested by protozoan predators (Caron *et al.* 1991; Boenigk *et al.* 2001). Genetic diversity may influence the susceptibility of picocyanobacteria to cyanophage. In this study, an assessment of the morphological characteristics and pigment types within the picocyanobacteria in Esthwaite Water has shown that the population varies considerably between May and October and spatially within the lake. Although only one morphological/pigment type was successfully isolated, this study has generated over 500 picocyanobacterial isolates from Esthwaite Water for the application of HIP 1 PCR (Chapter 4).

Chapter 3 HIP 1 PCR - method optimisation and evaluation

3.1 Introduction

In Chapter 2 the community of picocyanobacteria in Esthwaite Water was distinguished into four morphological forms; phycocyanin-rich rods and spheres, and phycoerythrin-rich rods and spheres. The field survey indicated that there was spatio-temporal variation in the abundance and morphological forms of picocyanobacteria in Esthwaite Water over the summer of 2000. The data indicated that variation in picocyanobacterial community structure did not appear to follow that of other autotrophs or the absence of predators. This poses the question of whether the morphological homogeneity (only four groups easily distinguished) is masking diversity within the picocyanobacterial community. This question has been asked by others, particularly in relation to the apparent ubiquity of picocyanobacteria in almost every environment (Stockner *et al.* 1986).

Isolation of picocyanobacteria from the field study resulted in the isolation 506 clones of phycocyanin-rich rod shaped picocyanobacteria. This group dominated the picocyanobacteria in Esthwaite Water in July, August and October 2000, and composed a significant proportion of the community at other times. It is not known whether this group is homogeneous (as it appears to be morphologically), or genetically and functionally diverse. An understanding of the genetic diversity of the group may provide valuable information about the community structure. The purpose of this chapter is to optimize develop the HIP 1 PCR cyanobacterial typing technique to facilitate application to field studies.

Smith *et al.,* (1998) used PCR to analyse the frequency and distribution of the HIP 1 sequence within some isolates of cyanobacteria from the Culture Collection of Algae and Protozoa (CCAP). This chapter continues the development of the HIP 1 PCR genotyping technique by:

- evaluating different methods of DNA template preparation for HIP 1 PCR
- assessing the reproducibility and specificity of HIP 1 PCR
- increasing our knowledge of the power of discrimination between isolates of cyanobacteria
- developing a methodology to compare HIP 1 PCR products and determine whether isolates have the same or different frequency and distribution of HIP 1 sequences.

For HIP 1 PCR, genomic cyanobacterial DNA is utilised as the template for amplification. This is a crucial and time-consuming part of the HIP 1 PCR technique. In order to apply this technique to a large number of field isolates a time and cost-effective method of template DNA preparation must be selected. Smith *et al.* (1998) used a phenol-chloroform DNA extraction method to obtain reproducible PCR products. However, a number of other methods of DNA extraction have been described in the literature (Bancroft *et al.* 1989; Pickup *et al.* 1995; Rudi *et al.* 1998). To extract DNA, the cell wall and membranes must be perforated by either chemical or physical means. The DNA must then be separated from other cell components that may impair PCR or cause the degradation of the DNA. The phenol-chloroform method uses a combination of physical means of opening the cell wall, with the use of glass beads, and chemical means of removing proteins and lipids and precipitating the DNA. This method is effective at obtaining DNA for PCR, which can be stored for a

number of months or years. The length of the time required by the procedure, however, restricts the number of extractions that can be performed at once. In this chapter a number of other methods of template DNA preparation are compared with the phenol-chloroform method.

The specificity and reproducibility of the HIP 1 PCR typing technique are also assessed in terms of the constituents within the reaction, the annealing temperature and the presence of non-target DNA.

The reproducibility is a measure of robustness of the technique; it pertains to the likelihood of the same results being achieved if the same isolate is typed on a number of occasions. There are a number of factors which cause variation between genetic typing results, these include factors which alter the amplification of specific PCR products, amplification of non-specific PCR products, and differences between separation and visualisation of products. A study of the reproducibility of PCR based techniques is advised prior to application to studies of diversity (Meunier *et al.* 1993). The effect of altering the concentration of a number of the components of PCR was tested by Smith *et al.* (1998). To ensure reproducibility between workers those components that affect the specificity of the PCR have been repeated and/or expanded within this study.

Magnesium chloride concentration affects the stringency of primer-template interaction within the PCR. The annealing temperature affects the specificity of primer annealing. Higher temperatures during the annealing stage of the PCR result in the amplification of fewer non-specific PCR products (Erlich 1989). Extension of HIP 1 primers, with three randomly selected bases at the 5' end produces primers that have a higher temperature optimum so the annealing temperature can be increased. These primers are known as ONHIP primers. This introduces a 64 fold redundancy effectively reducing the primer concentration by 64 fold. Increasing primer

concentration also increases non-specific binding so there is a balance between finding the correct temperature and concentration required.

Understanding the specificity of HIP 1 PCR to cyanobacteria, and the potential for amplification of HIP 1 PCR products from non-cyanobacterial origin is important in evaluating the reproducibility of the technique. Producing axenic cultures is technically difficult and time consuming (Rippka 1988), so most cultures are contaminated with heterotrophic bacteria. Other algae can also be isolated with, and coexist in cyanobacterial cultures, particularly when studying the freshwater environment.

The discrimination is the power of resolution of the technique. It has been demonstrated that HIP 1 PCR resolves between isolates of the same species of cyanobacteria (Smith *et al.* 1998), this work was expanded during this study by increasing the number of isolates from the CCAP analysed within this chapter and through application to isolates from a field study in Chapter 4.

How much variation between PCR products should be regarded as constituting a different genetic type? How much variation is a result of differences in amplification and separation of PCR products from the same organism? Reproducibility has the potential to affect the discrimination of cyanobacteria as variation between results from a single HIP 1 type of cyanobacteria could result in artificial separation of that type. The complexity, time required, sample capacity and cost of this technique will also be evaluated in determining the suitability for application to field studies.

Although the laboratory application of this technique is reasonably suitable for application to a large number of isolates (simple, quick and low cost), to date most studies which have used repetitive sequences to type microorganisms have analysed only a small number of isolates (heterotrophic bacteria: (Versalovic *et al.* 1991; Brujin 1992), cyanobacteria: (Rouhiainen *et al.* 1995)). This is because a number of PCR

products of different sizes are often yielded from each isolate. Comparison of PCR products from a large number of isolates, which have been separated by electrophoresis on different gels is difficult manually. Studies using HIP 1 PCR to distinguish between cyanobacteria have also so far compared a small number of isolates (Robinson *et al.* 1995; Smith *et al.* 1998), and considered them 'the same' or 'different' after visual comparison of the PCR products. To enable the HIP 1 PCR typing technique to be applied to ecological studies, or to the maintenance culture collections, the analysis of large numbers of isolates is required. Comparison of PCR products generated over time and separated on different gels is essential. Sophisticated database software is now available to standardize and analyse complex gel patterns. This enables the analysis of large numbers of isolates, separated on different gels, obtained over extended time intervals and even in different laboratories (van Belkum *et al.* 1998). Molecular markers run in several positions on each gel are used for standardisation, so PCR products can be compared accurately to those on the same, and different gels, which may have been separated to greater or lesser degrees by electrophoresis. Mathematical analysis of gel patterns produces quantitative similarities between isolates, and enables the construction of dendrograms so isolates can be clustered. Differences and similarities between complex PCR products are therefore easier to detect and thus the isolation of new strains or changes within isolates in culture can be monitored more readily. Application of software to analyse HIP 1 PCR products is an important development of the technique required to apply the technique to large numbers of field isolates. The analysis of HIP 1 PCR products, however, must be appropriate to the information yielded about the genome by these PCR products. HIP 1 PCR amplifies regions of DNA between HIP 1 sequences, so the profiles represent the frequency and distribution of HIP 1 sequences in the template DNA. Changes to the PCR products amplified can result from changes to the HIP 1

sequences themselves or the DNA (coding or non-coding) between the sequences. Therefore isolates which generate very similar HIP 1 PCR product profiles are likely to be very closely related (possibly derived from the same clone or strain?). However, one would need high certainty that PCR products were the same (e.g. perform Southern blotting and hybridization) and possibly test the genetic similarity of the isolates in a different way, to be confident they represented the same clone or strain. Isolates which produce the results from typing techniques whether based on genetic or biochemical characteristics, are often referred to as the representative of the same 'type'. For instance Streulens *et al.(1996)* defined a 'type' as characterised by a specific pattern/set of marker scores displayed by a strain on application of a particular typing technique. As in this study, there is only sufficient time to apply one typing technique to the isolates analysed, it may be more appropriate to assign them to a 'type', so further analysis could increase the certainty of their identity as a particular strain or species later.

On the other hand those isolates, which generate very different HIP 1 PCR product profiles, can be identified as different from each other, or distinct (this is the power of discrimination of the technique). This poses two questions, the first is bearing in mind the fact there will always be some variation in PCR profiles from isolates even where derived from the same clone due to differences in PCR constituents, thermal cycling conditions and gel electrophoresis (i.e. reproducibility issues), what level of similarity is considered significant enough for the isolates to be considered to be derived from the same clone, or represent the same strain? This chapter sets out to determine this by analysing variation in HIP 1 PCR profiles from the same PCR reaction separated on different gels. Others have considered this question during genotyping studies (van Belkum *et al.* 1998; Soll 2000; Rementaria *et al.* 2001). Generally the approach applied is to compare PCR product profile using similarity coefficients through

sophisticated computer programs and determining a percentage similarity which represents a reliable threshold above which it is thought PCR products are sufficiently similar to have been generated from isolates derived from the same clone. In this chapter different similarity coefficients are tested for matching the isolates. The algorithms used for presenting the results of the comparisons as dendrograms are also compared. A threshold is then selected above which isolates will be considered belonging to the same HIP 1 "type". The second question which arises from a comparison of isolates and determination of whether they are 'the same' or distinct is can the HIP 1 PCR profiles be used to indicate how similar or different isolates determined as distinct are from each other? This question is often asked of genetic information. Genetic sequence data from ribosomal DNA is commonly used to determine phylogenetic relationships between organisms. Sequence data from other locations of the genome, particularly coding regions has also been used for this purpose. This is because where changes to nucleotide sequences happen infrequently a relationship can be drawn up between those which diverged recently (few changes expected) and those which diverged further back in time (expected to be more different). Data from PCR products amplified from primers based on repetitive sequences can not be used in this way, this is because the mechanisms which influence the frequency and distribution of HIP 1 PCR sequences may be varied. These include those that affect the propagation of HIP 1 itself within the genome (Robinson *et al.* 2000) and those which affect the stretches of DNA between the sequences. Thus HIP 1 PCR cannot be used to generate relationships between isolates that are assessed as different.

3.2 Aims

The aim of the work described in this chapter was develop the HIP 1 PCR genotyping technique so it can be applied to isolates from the field. This was accomplished by:

- comparing a number of methods of DNA extraction for cost, time, and quality to enable selection of a suitable method for application to the field study.
- assessing the specificity and reproducibility of HIP 1 PCR.
- furthering the understanding of the discrimination of HIP 1 PCR of cyanobacteria.
- developing a method of comparing the PCR products of isolates of cyanobacteria for the field study.

3.3 Standard Methods

3.3.1 Cyanobacteria and culture conditions

Cyanobacterial cultures were obtained from the CCAP at the Centre for Ecology and Hydrology (CEH) in Windermere (now based at the Scottish Association for Marine Science, Oban), UK. They were routinely maintained in BG11 media (Stanier *et al.* 1971) in 50 ml flasks at 20°C ± 2 °C with a 12:8 hour light:dark cycle. Light was provided using 65 Watt fluorescent tubes (General Electric). The cultures were agitated on an orbital shaker at 300 rpm. For the preparation of template DNA for HIP 1 PCR, stationary phase cultures were generally used, however, experiments demonstrated that reproducible PCR products were produced from cultures of different growth phase (results not shown).

3.3.2 Standard PCR method

Each standard 50 µl reaction contained 2.5 units of recombinant Taq DNA polymerase (Gibco-BRL), one-tenth volume of 10x PCR buffer with 2 mM MgCl, 40 nM of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP and dTTP) (Amersham), 6 nM of an extended HIP 1 primer, and template DNA. Thermal cycling was carried out using an Ericomp Delta Cycler II thermal cycler or GeneAmp Perkin Elmer cycler according to the following temperature profile: initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 0.5 min, annealing at 30°C or 2°C below the melting-point (t_m) of the primer (unless stated otherwise), extension at 72°C for 1 minute, then a final extension period of 72°C for 10 minutes.

Negative controls consisting of the PCR standard mixture without template DNA were included in every reaction. Reactions without primer were carried out periodically.

3.3.3 Visualisation of PCR products

PCR products were separated using gel electrophoresis with 50% Nuseive 3:1 low melting temperature agarose (FMC Bioproducts) and 50% agarose (NBL Gene Sciences) at a final combined concentration of 3% w/v. DNA was stained with ethidium bromide (contained in the TBE running buffer (Sambrook *et al.* 1989)). A 123 bp ladder molecular marker was separated in three lanes on each gel as recommended by Schmidt-Gerner (1998). Visualisation of the PCR products was performed using a Pharmacia gel documentation system. Variation of product separation between gels was analysed using the BioNumerics (Applied Maths, Belgium) gel analysis software Version 2.5 (2000).

3.4 Experimental methods

3.4.1 Preparation of template DNA for HIP 1 PCR

Five methods of preparing DNA for HIP 1 PCR for suitability for use in field studies were assessed. These included the phenol-chloroform method, a method based on a kit using magnetic beads, lysozyme and direct PCR from cells.

3.4.1.1 Phenol-chloroform method

DNA was initially routinely isolated using a phenol-chloroform extraction method (Smith *et al.* 1998), where 10 ml of culture was pelleted by centrifugation (microfuge; 3500 rpm, 10 minutes), washed twice in TE buffer (pH 7.2) and re-suspended in 750 µl of TLES buffer. The re-suspended cells were added to a screw-capped Eppendorf

with 500 µl of glass beads and shaken (Griffin flask shaker; 300 rpm, 7 minutes). Cell debris and glass beads were concentrated by centrifugation (microfuge; 13200 rpm, 8 minutes). The supernatant was removed and added to an equal volume of phenol-chloroform-isomyl alcohol (25:24:1 by volume) and mechanically mixed for 1 minute to remove protein and lipid contamination. After centrifugation (microfuge; 13200 rpm for 15 minutes) the aqueous supernatant was removed to a fresh tube before the phenol-chloroform extraction was repeated. The aqueous supernatant was added to 50 µl of 5 M potassium acetate and placed on ice for 10 minutes until a turbid precipitate of polysaccharide formed which was removed by centrifugation (microfuge; 13200 rpm, 15 minutes) and the supernatant retained. Two volumes of absolute ethanol were added and the sample placed at -20 ^{o} C for at least 20 minutes to precipitate the DNA. The sample was washed with 70 % ethanol then dried in an airflow cabinet at room temperature. The DNA was re-suspended in 20 µl of TE buffer containing DNAase free RNAase A (25 µg ml^{-1}). Preparations were stored at -20 ^{o} C and further diluted prior to PCR.

3.4.1.2 Dynabeads DNA DIRECT^{TM} system I

Approximately 2 × 10^{8} cells were pelleted by centrifugation and the supernatant removed. 1 unit of Dynabeads (200 µl) was added and the tube was pre-incubated in a water bath at 65^{o} C for 15 minutes. The tube was kept at room temperature for 5 minutes whilst the DNA/Dynabead complex formed. The magnet (Dynal MPC) was used to pellet the complex and the supernatant was removed. The pellet was washed twice with 200 µl of "washing buffer" before re-suspension in 20-40 µl of "re-suspension" buffer. DNA was eluted from the beads by an incubation of 5 minutes at 65^{o} C. The beads were concentrated at the base of the tube using the magnet and the supernatant containing the extracted DNA was removed to a clean tube and stored at

-20°C. The DNA was diluted up to a factor of 1:50 before it was used as a template for the PCR.

3.4.1.3 Lysozyme extraction

Lysozyme digestion of gram negative cell walls has been used to prepare template DNA for PCR (Bancroft *et al.* 1989). Cells were incubated with different concentrations of lysozyme (2 – 50 mg ml^{-1}) in sphaeroplast buffer (17 % sucrose, 0.05 M Tris (pH 8.0), 0.005 M NaH_2PO_4, 0.005M K_2HPO_4, 0.01 M $MgCl_2$) over night at 37 ° C. The cells were then pelleted, re-suspended in a small amount of TE (pH 7.2), and used as template for a 50 µl PCR reaction.

3.4.1.4 Direct PCR

Untreated cells were used directly for PCR after concentration by centrifugation and washing in TE buffer (pH 8.0) (Rudi *et al.* 1998).

3.4.2 HIP 1 PCR sequences in cyanobacteria from the CCAP.

DNA was extracted from a number of isolates of colonial and fialmentous cyanobacteria and picocyanobacteria from the CCAP collection (Table 3.1) using the phenol-chloroform extraction method (Chapter 3.4.1.1). The DNA was used as template for the PCR using the standard method (Chapter 3.3.2) using the HIP 1 primer HIPCA, and visualised using gel electrophoresis (Chapter 3.3.3). The PCR products were analysed using BioNumerics software with the Pearson correlation coefficient and UPGMA algorithm. The filamentous and colonial cyanobacteria were matched separately from the picocyaobacteria.

Table 3.1 Cyanobacteria from the CCAP analysed by HIP 1 PCR

CCAP Accession Number	Species	Form of cyanobacteria	Contamination with other organisms
1403/B	*Anabaena flos-aquae*	Filamentous	Bacteria
1403/E	*Anabaena flos-aquae*		Bacteria
1403/F	*Anabaena flos-aquae*		Bacteria
1403/G	*Anabaena flos-aquae*		Bacteria
1443/1	*Limnothrix redekei*	Filamentous	Bacteria
1443/3	*Limnothrix redekei*		Bacteria
1443/2	*Limnothrix redekei*		Bacteria
1443/4	*Limnothrix redekei*		Bacteria
1450/1	*Microcystsis aeruginosa*	Colonial	Bacteria
1450/3	*Microcystsis aeruginosa*		Bacteria
1450/4	*Microcystsis aeruginosa*		Bacteria
1450/6	*Microcystsis aeruginosa*		Bacteria
1450/8	*Microcystsis aeruginosa*		Bacteria
1450/11	*Microcystsis aeruginosa*		Bacteria
1450/12	*Microcystsis aeruginosa*		Bacteria
1450/15	*Microcystsis aeruginosa*		Bacteria
1453/15	*Nostoc elipsosporum*	Filamentous	Bacteria
1453/16	*Nostoc elipsosporum*		Bacteria
1453/17	*Nostoc elipsosporum*		Bacteria
1453/18	*Nostoc elipsosporum*		Bacteria
1453/119	*Nostoc elipsosporum*		Bacteria
1459/11A	*Oscillatoria argardhii*	Filamentous	Bacteria
1459/14	*Oscillatoria argardhii*		Bacteria
1459/15	*Oscillatoria argardhii*		Bacteria
1459/16	*Oscillatoria argardhii*		Bacteria
1459/17	*Oscillatoria argardhii*		Bacteria
1459/21	*Oscillatoria argardhii*		Bacteria
1459/22	*Oscillatoria argardhii*		Bacteria
1459/23	*Oscillatoria argardhii*		Bacteria
1459/24	*Oscillatoria argardhii*		Bacteria
1459/36	*Oscillatoria argardhii*		Bacteria
1459/37	*Oscillatoria argardhii*		Bacteria
1480/1	*Synechocystis minima*	Picocyanobacteria	Bacteria
1480/14	*Synechocystis sp.*	Picocyanobacteria	Bacteria
1499/3	*Synechococcus capitatus*	Picocyanobacteria	Bacteria
1479/12	*Synechococcus sp.*	Picocyanobacteria	Bacteria
1479/13	*Synechococcus sp.*		Bacteria
1479/14	*Synechococcus sp.*		Bacteria
1479/11	*Synechococcus sp.*		Bacteria
1479/9	*Synechococcus sp.*		Bacteria
1479/5	*Synechococcus sp.*		Bacteria
1450/7	*Synechococcus sp.*		Bacteria
1405/1	*Synechococcus leopoliensis*	Picocyanobacteria	Bacteria

N.B *Oscillatoria* since renamed *Planktothrix* (Suda *et al.* 2002)

3.4.3 Specificity and reproducibility of HIP 1 PCR

3.4.3.1 Magnesium chloride concentration

The standard PCR was carried out with the following series of final concentrations of the $MgCl_2$ (supplied with *Taq* DNA polymerase (Gibco BRL)): 1.0, 1.25, 1.5, 1.75, 2.0, 2.5*, 3.0 and 4.0 mM. (N.B.* Indicates concentration used in standard reaction mixture).

3.4.3.2 Effect of annealing temperature

The PCR was carried out with a gradient of annealing temperatures using an Eppendorf Master Cycler Gradient thermal cycler. One mixture of the standard PCR was aliquoted into wells across a 96-well plate and inserted into the machine. Wells across the plate were subjected to different annealing temperatures. These temperatures represented 2° C intervals from (above and below) the Tm (melting point) of the primer.

3.4.3.3 HIP 1 sequences in other micro-organisms

DNA was extracted from a number of environmental isolates of heterotrophic bacteria (isolated by Elaine Summerfield, MPhil student, Lancaster University 1999-2000) and freshwater Eukaryotes from the CCAP collection (Table 3.2) using the phenol-chloroform extraction method (Chapter 3.4.1.1). The DNA was used as template for the PCR using the standard method (Chapter 3.3.2) using extended HIP 1 primers OnHIPGC and OnHIPCA and visualised using gel electrophoresis (Chapter 3.3.3).

Table 3.2 Eukaryotic microorganisms from the CCAP analysed by HIP 1 PCR

CCAP Accession Number	Species	Class /Order	Contamination with other organisms
933/27	*Ochromonas tuberculata*	Chrysophyceae	Bacteria
257/7	*Oocystis* sp.	Chlorophyceae	Bacteria
917/1	*Dinobryan divergens*	Chrysophyceae	Bacteria
24/9	*Eudorina* sp	Chlorophyceae	Bacteria
995/3	*Rhodomonas lacustris*	Rhodophyceae	Bacteria
1110/4	*Ceratium hirudinella fo. Furcoides*	Pyrrhophyceae	Bacteria
1624/15	*Euplotes daidaleos*	Ciliate	Bacteria
979/46	*Cryptomonas* sp.	Cryptophyceae	Bacteria
1005/13	*Asterionella formosa*	Bacillariophyceae	Bacteria
211/12	*Chlorella vulgaris*	Chlorophyceae	Axenic
11/32B	*Chlamydomonas reinhardtii*	Chlorophyceae	Axenic
1224/5Z	*Euglena gracilis*	Euglenophyceae	Axenic

3.4.3.4 DNA polymerase concentration

The standard PCR was performed on two cyanobacterial templates (CCAP 1405/1, and 1479/10) with the following series of final concentrations of the *Taq* DNA polymerase (Gibco BRL) 1.0, 1.5, 2.0, 2.5*, 3.0 and 3.5 units. (N.B.* Indicates concentration used in standard reaction mixture).

3.4.3.5 Primer concentration

To analyse the effect of primer concentration on the PCR product the standard PCR was carried out with the following series of final concentrations of the extended primer ONHIPGC: 100, 200, 300, 400, 500, 600*, 700, 800 nM. (N.B.* Indicates concentration used in standard reaction mixture).

3.4.3.6 Variability between PCR products run on different gels

This was assessed during the development of a method for grouping HIP 1 PCR products. For this, PCR products were separated on a number of gels (Chapter 3.4.4)

then matched using the BioNumerics software. Variability resulting from gel electrophoresis is discussed in Chapter 3.6.4.4.

3.4.4 Analysis of PCR products

In order to evaluate the PCR product clustering techniques and variability between PCR products separated on different gels, PCR products of known identity were analysed. A PCR was carried out using the standard mixture and conditions outlined in Chapter 3.3.2 on templates of five isolates of cyanobacteria from the CCAP (Table 3.3). To assist with labelling of tubes I assigned a single digit reference number to the strains. This is shown for the strains used in this work in Table 3.3, and on the dendrograms.

Table 3.3 Cyanobacteria isolates used to develop PCR product analysis techniques.

CCAP Accession Number	Species	My reference number
1459/21	*Oscillatoria arghardhii*	7
1459/17	*Oscillatoria arghardhii*	8
1459/14	*Oscillatoria arghardhii*	10
1479/14	*Synechococcus* sp.	4
1405/1	*Synechococcus leopoliensis*	5

N.B *Oscillatoria* has since been renamed *Planktothrix* (Suda *et al.* 2002)

Two 50 µl reactions for each isolate were pooled into one tube to create sufficient uniform product to load six gels. All of the gels contained three lanes of 123 bp ladder (lanes 1,7 and 14). The first gel was the reference gel; it contained one lane of each of the isolates. The remaining five gels were loaded with a random distribution of the PCR products from the five isolates.

To determine the most appropriate method of comparing similarities and clustering HIP1 PCR products from cyanobacteria using the BioNumerics software, and to examine the reproducibility of the technique (or variability between gels), a series of cluster analyses were carried out on these gels. The effects of matching using

different similarity coefficients, optimisation of the clustering, altering the length of profile matched, and the use of different algorithms to create the dendrograms were examined. Parameters tested are summarised in Table 3.4.

3.4.4.1 Gel processing

Gel images in Tag Image File Format (TIFF) with inverted colours (so PCR products appeared dark on a light background) were loaded into the BioNumerics software. Lanes were assigned to the gels to follow the route of PCR products from each well. Each band within the molecular size markers which had been separated in three lanes (the two outside, and a central lane) as recommended by Gerner-Smidt *et al.,* (1998) were assigned to the appropriate size in molecular weight. Normalization was performed by the software so PCR products which had been separated on different gels could be compared through calibration with the molecular size markers. For comparison with band-based similarity coefficients the software was first allowed to automatically assign bands to peaks on the gels. Visual observation of the detection of bands was used to make amendments where aberrations in the gels had been detected as bands.

3.4.5 Effect of similarity coefficient

Similarity coefficients either compare the characteristics of PCR product profiles between lanes (profile-based coefficients) or the presence or absence of bands (band-based) that have migrated the same distance within each lane. Unlike band-based coefficients, the profile-based similarity coefficients take into account the width and intensity of each band as well as location within the lane relative to the molecular standards. Pearson and Cosine profile-based similarity coefficients were tested.

Table 3.4 Similarity coefficients, length of profile matched and optimization and tolerance settings used for comparisons.

Similarity coefficient	Type (band or profile based)	Length of profile matched	Optimization setting	Tolerance setting
Pearson	Profile	Whole	Off	Off
Pearson	Profile	Whole	On	Off
Pearson	Profile	29.9- 100%	Off	Off
Pearson	Profile	29.9- 100%	On	Off
Cosine	Profile	Whole	On	On
Cosine	Profile	29.9- 100%	On	On
Dice	Band	Whole	Off	On
Dice	Band	Whole	On	On
Jaccard	Band	29.9- 100%	On	On
Jaccard	Band	Whole	On	On

Dice and Jaccard band-based coefficients were tested. The BioNumerics software has elements that allow the optimization of comparisons. This feature sets the shift you will allow between the full length of any two patterns, within which the program will look for matching. This allows for PCR products within specific lanes to have moved at a slightly different rate compared to the molecular markers see Fig 3.1 for an example. Tolerance (also shown in Fig 3.1) is the maximal shift in percentage shift in pattern length allowed between individual bands to consider them 'the same'. Optimization and tolerance settings can be manually set by the user or the program can generate the optimum settings. For this test matches were performed without these settings, then the software was allowed to select the appropriate optimum and tolerance settings (Table 3.4)

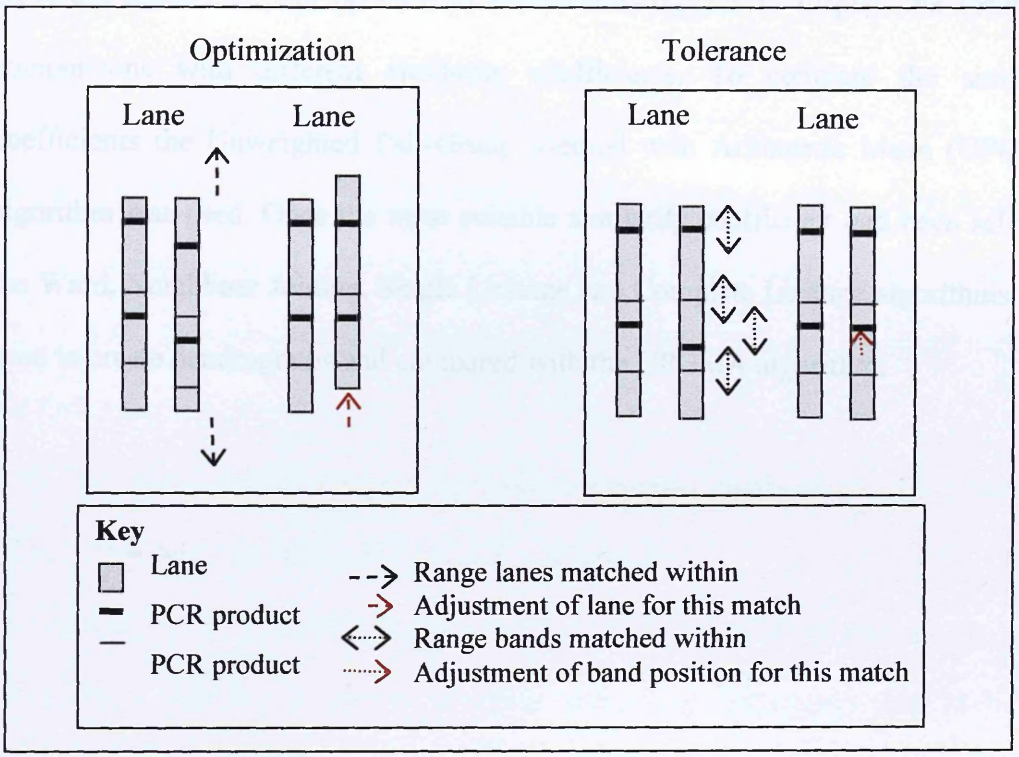


Figure 3.1 Diagram to show how optimization and tolerance features within in BioNumerics software affect the comparisons.
N. B Tolerence is not an option for matches with most profile based correlation coefficients.

The length of the lane within which PCR products migrated can also be restricted so that only PCR products of specific sizes are matched. The whole lane length is split into 100, with 0 being closest to the loading wells and 100 being the far extreme of the lane. The user can specify what proportion of the lane should be matched; the full lane is equal to 100% of the profile. The user can specify that a shorter length, or a number of shorter lengths of the lane, in which the PCR products have been separated be matched. For this test the whole profile was matched, then the larger molecular weight zone where PCR products amplified from heterotrophic bacteria were generally separated to were screened out by restricting the match to 29.9% of the lane length (Table 3.4).

3.4.6 Affect of dendrogram algorithm

Different algorithms can be used to create dendrograms to display the results of comparisons with different similarity coefficients. To compare the similarity coefficients the Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) algorithm was used. Once the most suitable similarity coefficient had been selected, the Ward, Neighbour Joining, Single Linkage and Complete Linkage algorithms were used to create dendrograms and compared with the UPGMA algorithm.

3.5 Results

3.5.1 Preparation of template DNA for HIP 1 PCR

The results of a comparison of the methods of DNA extraction tested in this study are summarised in Table 3.5. Only the methods based on a phenol-chloroform extraction and Dynabeads system I DNA extraction produced template that yielded reproducible PCR results. PCR results were achievable with the direct PCR and lysozyme methods, but they were very inconsistent.

Both the phenol-chloroform and Dynabeads system I methods produced template DNA which could be stored at -20 °C for later analysis.

The capacity of DNA extractions per run (i.e. number of isolations that can be carried out at once with the equipment available within our laboratory) was 24 for the Dynabead system I method, compared to 10 for the phenol-chloroform method. The length of the time to complete an extraction run was also considerably shorter for the Dynabead method; the total time for each extraction was only 1.6 minutes, compared to 50 minutes per extraction for the phenol-chloroform method.

The cost, simplicity and hazard rating of the Dynabead method were all lower than the phenol-chloroform method. Magnets were required, but once purchased were totally re-usable and maintenance-free. The phenol-chloroform method on the other hand required an orbital shaker, which would require routine electrical safety checking, and more expensive consumables (screw-capped tubes and glass beads) were required.

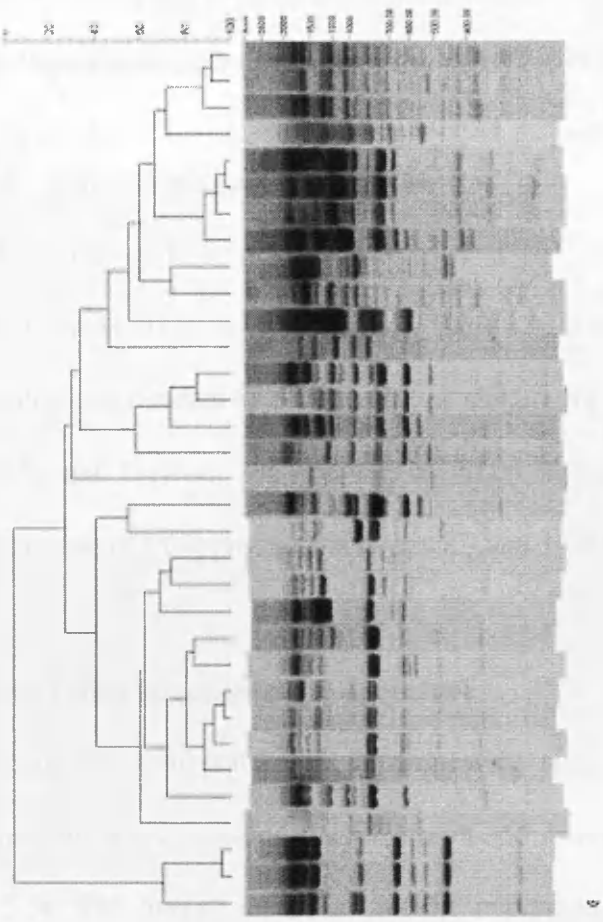
Table 3.5 *Comparison of DNA extraction methods.*

DNA extraction method	Reproducible PCR product	DNA for storage	Extractions per run	Time		Special equipment required*	Cost per extraction	Complexity of procedure**	COSHH assessment
				Per run	Per extraction				
Phenol-chloroform	Yes	Yes	10	5 h	30 mins	Orbital shaker	£0.75	High	High risk
Dynabeads System I	Yes	Yes	24	40 mins	1.6 mins	6 Dynal MPC magnets Water bath	£0.31	Low	Low risk
Direct PCR	No- occasional results but inconsistent	No	High	N/A	N/A	N/A	N/A	N/A	N/A
Lysozyme 2mg/ml to 50mg/ml	No- occasional results but inconsistent	No	High	15 – 30 mins	Overnight	None	£0.12- £0.99	Low	Low risk

N.B * Other than available in most laboratories. ** Techniques were rated against each other.

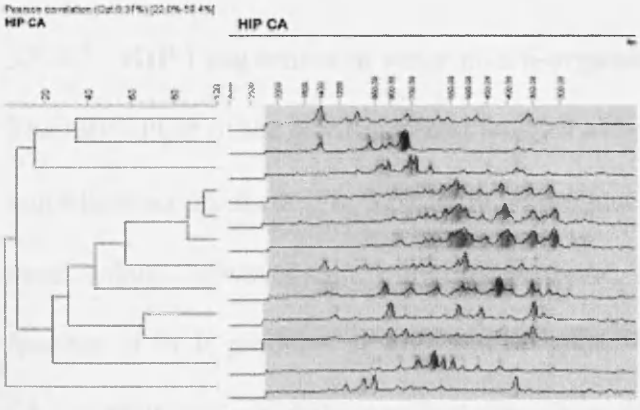
3.5.2 HIP 1 Sequences in cyanobacteria from the CCAP collection

The isolates of cyanobacteria from the CCAP analysed generated complex banding patterns of HIP 1 PCR products (Fig 3.2). The PCR products generated from isolates of the same species of filamentous and colonial cyanobacteria were matched together to varying degrees on the dendrogram. Isolates of *Oscillatoria (Planktothrix) agardhii* generated particularly homeogenous PCR products, with the isolates all being grouped together on the dendrogram. Three isolates of *Limnothrix redekei* also produced homeogenous PCR products and were grouped. Isolates of *Microcystis aeruginosa*, *Anabaena flos aquae* and *Nostoc elipsosporum* however were separated on the dendrogram. The PCR products generated from the picocyanobacteria were distinct from each other (Fig 3.3).



CCAP No.	Species	Isolation origin
1443/1	Limnothrix redekei	Germany
1443/3	Limnothrix redekei	Germany
1443/2	Limnothrix redekei	Germany
1450/1	Microcystis aeruginosa	USA
1453/16	Nostoc ellipsosporum	Unknown
1453/18	Nostoc ellipsosporum	USA
1453/17	Nostoc ellipsosporum	Unknown
1453/15	Nostoc ellipsosporum	USA
1403/13D	Anabaena flos-aquae	England
1443/4	Limnothrix redekei	Germany
1453/19	Nostoc ellipsosporum	Unknown USA?
1450/3	Microcystis aeruginosa	England
1450/9	Microcystis aeruginosa	Canada
1450/12	Microcystis aeruginosa	Unknown
1450/4	Microcystis aeruginosa	Canada
1450/6	Microcystis aeruginosa	Canada
1450/11	Microcystis aeruginosa	Japan
1450/10	Microcystis aeruginosa	England
1459/24	Oscillatoria argardhii	Sweden
1459/37	Oscillatoria argardhii	Norway
1459/22	Oscillatoria argardhii	Norway
1459/15	Oscillatoria argardhii	N. Ireland
1459/14	Oscillatoria argardhii	England
1459/21	Oscillatoria argardhii	England
1459/17	Oscillatoria argardhii	England
1459/16	Oscillatoria argardhii	England
1459/23	Oscillatoria argardhii	England
1459/11A	Oscillatoria argardhii	England
1459/36	Oscillatoria argardhii	Norway
1450/15	Microcystis aeruginosa	England
1403/13F	Anabaena flos-aquae	England
1403/13E	Anabaena flos-aquae	Wales
1403/13G	Anabaena flos-aquae	England

Figure 3.2 HIP 1 PCR products from isolates of colonial and filamentous cyanobacteria from the CCAP.
N.B *Oscillatoria* has since been renamed *Planktothrix* (Suda *et al.* 2002)



CCAP No.	Species	Isolation origin
1479/11	*Synechococcus* sp.	Unknown
1479/9	*Synechococcus* sp.	England
1479/14	*Synechococcus* sp.	USSR
1479/5	*Synechococcus* sp.	Unknown
1480/14	*Synechocystis* sp.	Unknown
1430/1b	*Synechococcus* sp.	Unknown
1450/7	*Synechococcus* sp.	Unknown
1405/1	*Synechococcus leopoliensis*	USA
1480/1	*Synechococcus* sp.	Unknown
1499/3	*Synechococcus capitatus*	Unknown
1479/12	*Synechococcus* sp.	USSR
1479/13	*Synechococcus* sp.	England

Figure 3.3 HIP 1 PCR products from isolates of picocyanobacteria from the CCAP.
N.B The shape of the profiles matched are indicated on this gel.

3.5.3 Specificity and reproducibility of HIP 1 PCR

3.5.3.1 Effect of magnesium chloride

Amplification of PCR products occurred at all magnesium chloride concentrations tested (1 mM to 4 mM) (Fig 3.4). Low (<1.5 mM) and high (4 mM) $MgCl_2$ concentrations resulted in the reduced production of PCR products, particularly those > 738 bp and between 246 and 492 bp. There appeared to be little difference in the amplification of PCR products between 1.5 and 3 mM magnesium chloride.

3.5.3.2 Effect of annealing temperature

Increasing the temperature of the annealing stage of the PCR has the effect of reducing the amplification of some bands and increasing the amplification of others (Fig 3.5). The overall effect is that the maximum number and intensity of bands amplified occurs at the middle range of temperatures tested which were around the predicted melting point ™ of the primer tested.

3.5.3.3 HIP 1 sequences in other micro-organisms.

The HIP1 PCR of the environmental isolates of heterotrophic bacteria produced some amplification products (Fig 3.6). HIPCA produced two significant large products for most isolates, however some only produced one. Two (lanes 5 and 7) also produced a number of PCR products at low concentration. HIPGC produced one, two or three intense PCR products from most isolates of heterotrophic bacteria, but six isolates also produced a number of PCR products at low concentration with this primer. The approxiamate size of the largest and most frequently amplified of these PCR products could not be estimated as the molecular weight mark had not separated sufficiently to

compare band sizes. Some to the isolates however also produced a PCR product of approxiamately 700 bp.

Very few PCR products resulted from the PCR of Eukaryotic microbial DNA with primer HIPCA (Fig 3.7). Although templates of *Oocystis* sp., *Dinobryon divergens, Rhodomonas lacustris* and *Ceratium hirudinella fo. Furcoides* produced a high molecular weight smear and *Cryptomo*nas sp. produced two significant PCR products. Primer HIPGC produced a small number of PCR products of low concentration with *Ochromonas tuberculata, Oocystis* sp., *Dinobryan divergens,* and *Cryptomo*nas sp. Significant PCR banding patterns were produced by template DNA of *Ceratium hirudinella fo. Furcoides* and *Euplotes daidaleos* with the HIPGC primer.

3.5.3.4 DNA polymerase concentrations

Concentrations of *Taq* DNA polymerase less than 2 units per reaction for template *Synechoccocus leopoliensis* (CCAP 1405/1) and 2.5 units per reaction for template *Synechoccocus* sp. (CCAP 1479/10) resulted in a reduction in the number of amplification products resulting from the PCR (Fig 3.8). These concentrations also resulted in a decrease in concentration of other amplification products for the respective templates. The products that underwent no/less amplification ranged in size across the size range of all of the PCR products.

There was no visible difference between the products amplified using *Taq* DNA polymerase concentrations of > 2.5 units per reaction.

3.5.3.5 Primer concentration

Primer concentrations less than and greater than 400nM resulted in a reduction in the number of bands amplified (Fig 3.9). Although it is not possible to see from the gel,

the higher the concentration of primer used, the higher the concentration of a small molecular weight product (primer-dimer) was produced. This is a result of the primer binding and amplifying itself during the PCR reaction rather than the template DNA.

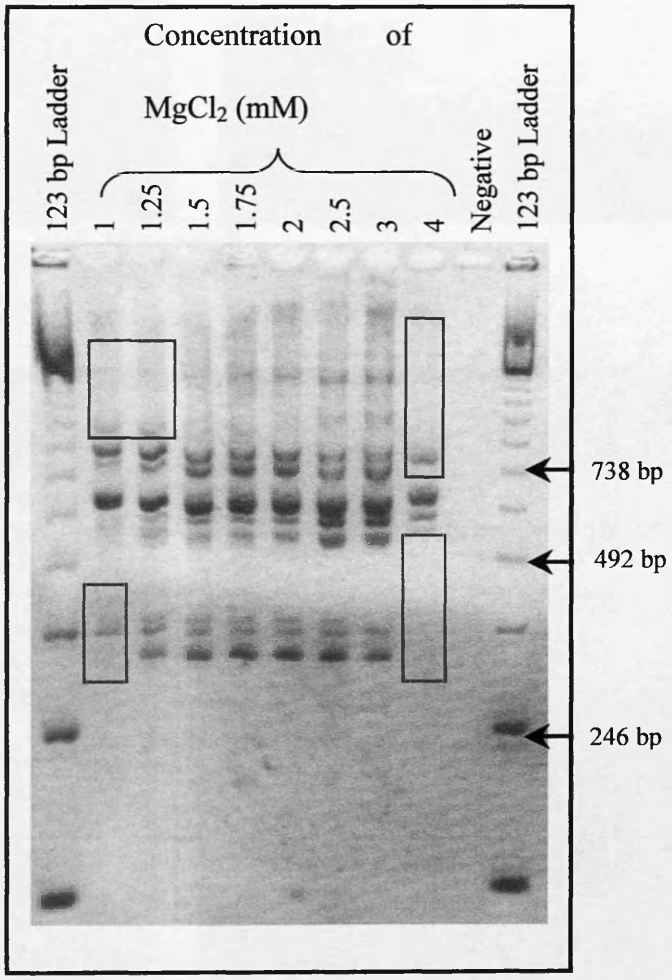


Figure 3.4 Concentration of $MgCl_2$
Synechoccocus leopoliensis (CCAP 1405/1) N. B. Primer used: HIPCA. Red boxes highlight areas of PCR product profile where amplification of PCR products differs from mid-range concentrations of $MgCl_2$.

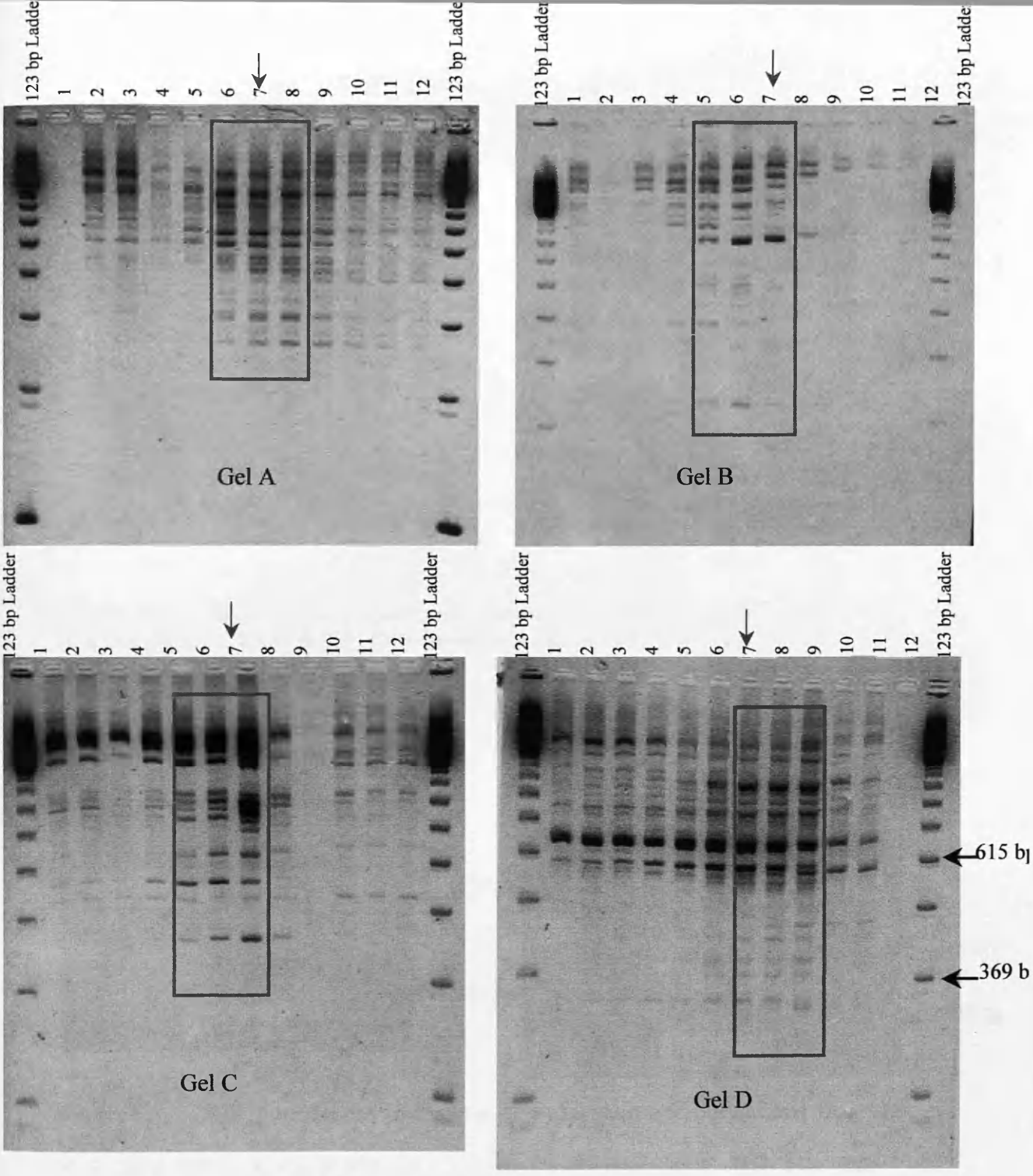


Figure 3.5 Affect of temperature on PCR products

N. B Gel A, template: *Synechocystis* sp. (CCAP 1480/4); primer ONHIP CA, Gel B, template: *Synechocystis* sp. (CCAP 1480/4); primer ONHIP GC, Gel C, template: *Synechoccocus leopoliensis* (CCAP 1405/1); primer ONHIP CA, Gel D, template: *Synechoccocus leopoliensis* (CCAP 1405/1); primer ONHIP GC. Red arrow indicates melting point (Tm) of primer, the reaction in each lane to left was at a further 2°C lower, each lane to right was at a further 2°C higher. Blue box indicates lanes with reproducible PCR products.

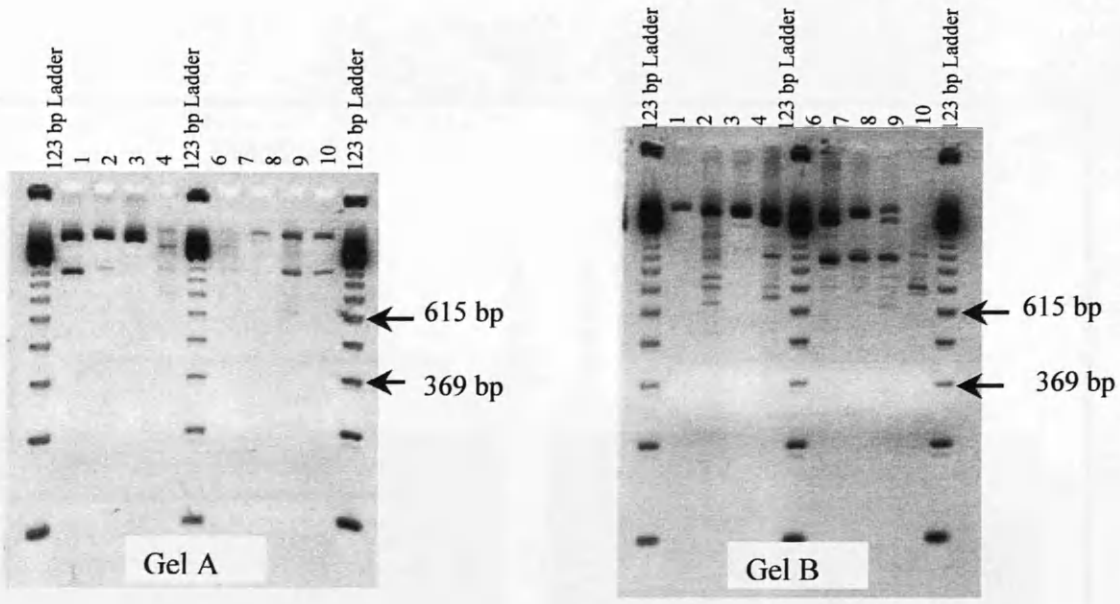


Figure 3.6 Environmental isolates of heterotrophic bacteria
N. B Gel A HIP1 CA, Gel B HIP1 GC. Annealing temperature 30 °C.

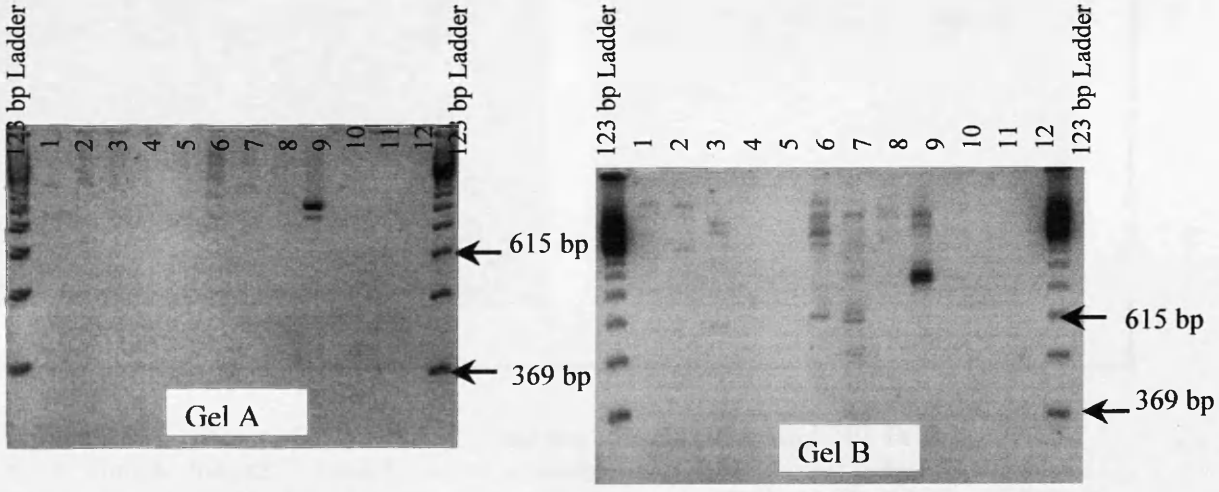


Figure 3.7 HIP 1 sequences in isolates of Eukaryotic microorganisms from the CCAP.
N. B Gel A HIP1 CA, Gel B HIP1 GC. Annealing temperature 30 °C. Both gels: Lane 1: *Ochromonas tuberculata* (CCAP 933/27), 2: *Oocystis* sp. (CCAP 257/7), 3: *Dinobryon divergens* (CCAP 917/1), 4: *Eudorina* sp. (CCAP 24/9), 5: *Rhodomonas lacustris* (CCAP 995/3), 6: *Ceratium hirudinella fo. Furcoides* (CCAP 1110/4), 7: *Euplotes daidaleos* (CCAP 1624/15), 8: *Cryptomonas* sp. (CCAP 979/46), 9: *Asterionella formosa* (CCAP 1005/14), 10: *Chlorella vulgaris* (CCAP 211/12), 11: *Chlamydomonas reinhardtii* (CCAP 11/32B), 12: *Euglena gracilis* (CCAP 1224/5Z).

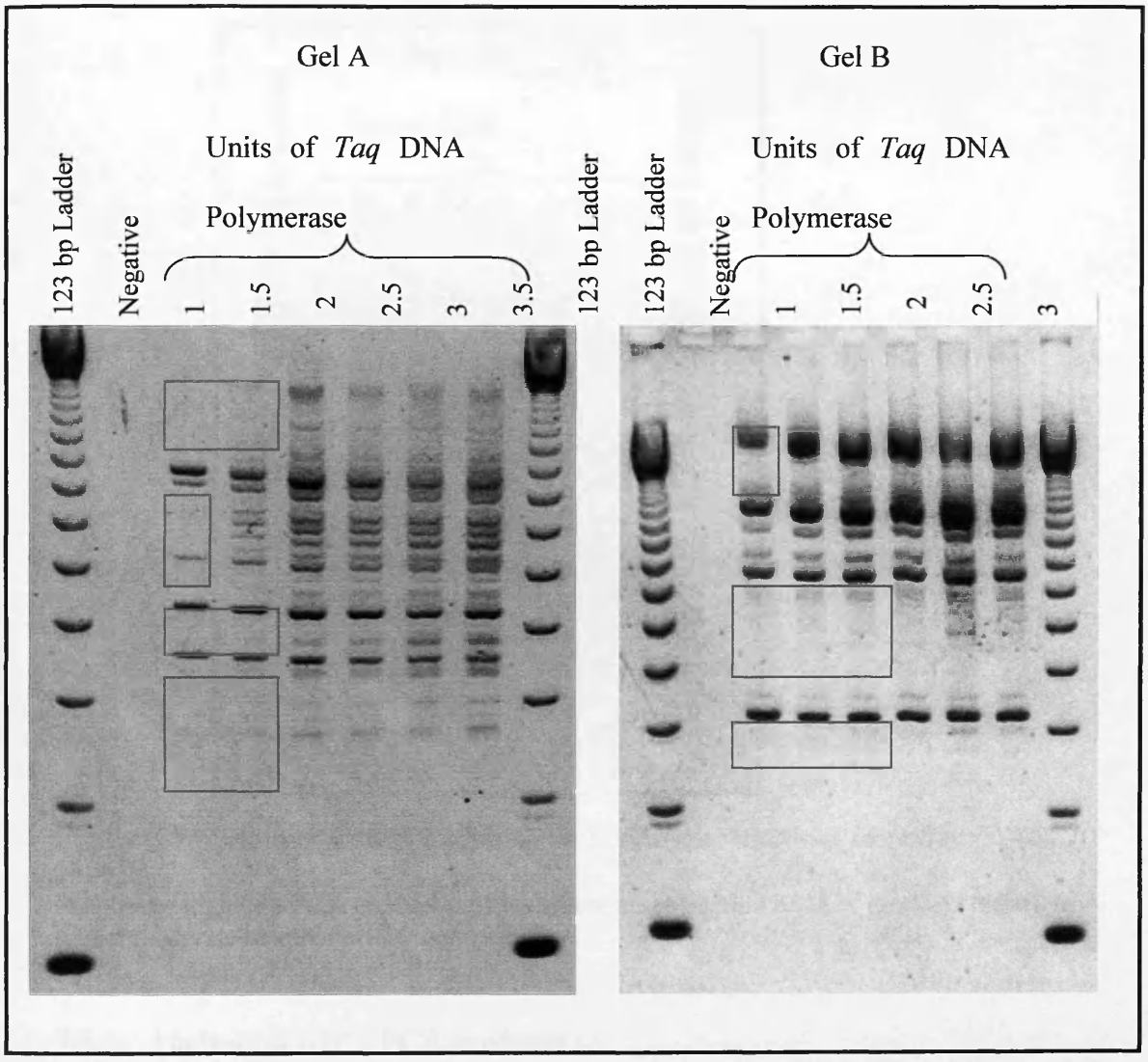


Figure 3.8 Effect of *Taq* DNA polymerase concentration on HIP1 PCR
N. B Gel A template: *Synechococcus leopoliensis* (CCAP 1405/1), Gel B template: *Synechococcus* sp. (CCAP 1479/10). Primer for both gels: HIP1 CA. Red boxes highlight areas of PCR product profile where amplification of PCR products differs from higher range of *Taq* DNA polymerase concentration.

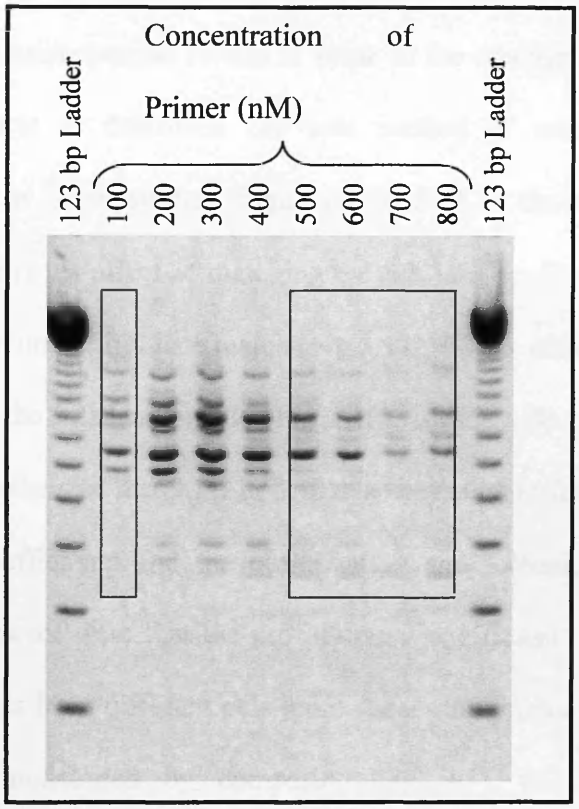


Figure 3.9 Primer Concentration ONHIPGC *Synechoccocus leopoliensis* (CCAP 1405/1)
Red boxes highlight PCR product profiles where amplification of PCR products differs from higher moderate range of primer concentration.

3.5.4 Analysis of HIP 1 PCR products

3.5.4.1 Gel processing

The procedures to prepare the gels for comparison in the software were relatively straightforward. However automatic band assignment did require significant user intervention to ensure aberrations were not detected as bands as this would have made the PCR products appear less similar. The manual amendment of band detection brought user decision making into the comparison, which may have been subject to inconsistency and error.

3.5.4.2 Affect of similarity coefficient

Table 3.6 summarises the results of some of the comparisons of the repeated lanes of PCR products to determine the best method of matching HIP 1 products for application to larger studies. Figures 3.10 to 3.13 show a sub-set of the results to demonstrate i) the affect of matching the full lane profiles (Fig 3.10), ii) the affect of optimization on the full lane match (Fig 3.11), iii) the affect of matching a shorter lane length than the full lane profile (Fig 3.12), iv) the affect of a band-based coefficient (full lane, optimized match) (Fig 3.13). It was evident from the comparisons with the different coefficients and the optimization and tolerance options provided by the BioNumerics software that the profile-based coefficients tested matched the lanes of PCR products from different gels more successfully than the band-based coefficients. This is demonstrated by comparing Fig 3.11 (the whole lane profile-based comparison) where all of the lanes of each isolate were clustered together on the dendrogram, with Fig 3.13, (the whole lane band-based comparison) where a lane of isolate 4 was not clustered correctly and the similarity values between the lanes are significantly lower. Table 3.6 shows that the Pearson, optimized, full lane match was the only comparison where all of the clusters were intact. Fig 3.11 shows that the 80% cut-off could be used if isolates were more similar to each other, but no lanes were matched as identical. Although it did not match lanes of all isolates into clusters, the use of the Jaccard coefficient did show that lanes with PCR products from some of the isolates to be identical to other lanes of the same isolate which did not happen with the profile-based coefficients (Fig 3.13).

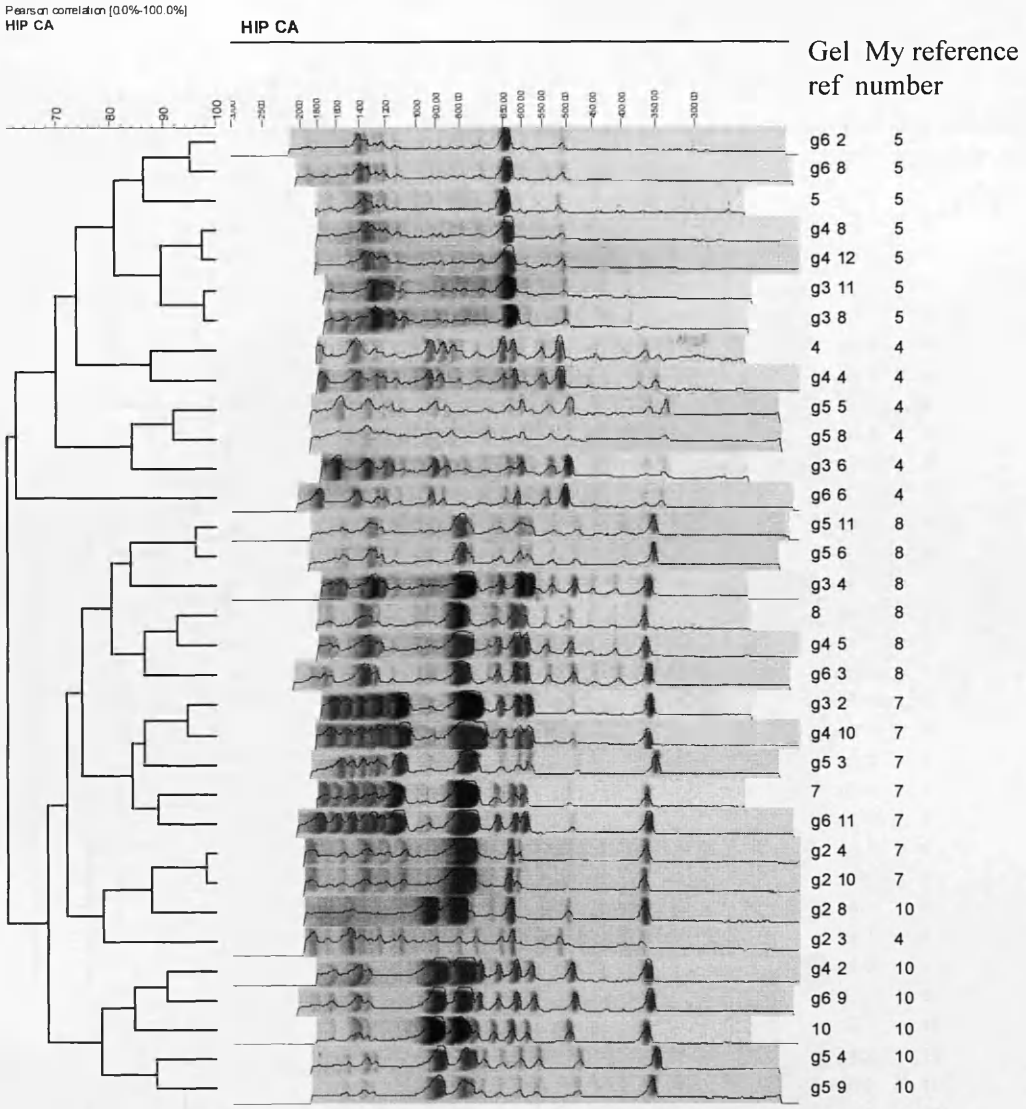


Figure 3.10 Affect of Pearson correlation coefficient on matching repeated PCR products from five isolates

N. B. Full profile matched, no optimization performed. My reference number relates to CCAP accession code (see Table 3.3).

Gel reference refers to the number gel (gx) and lane (following digit) the PCR products were separated on. The control gel (equivalent to gel 1, where all isolates were separated) is simply numbered by my isolate reference number.

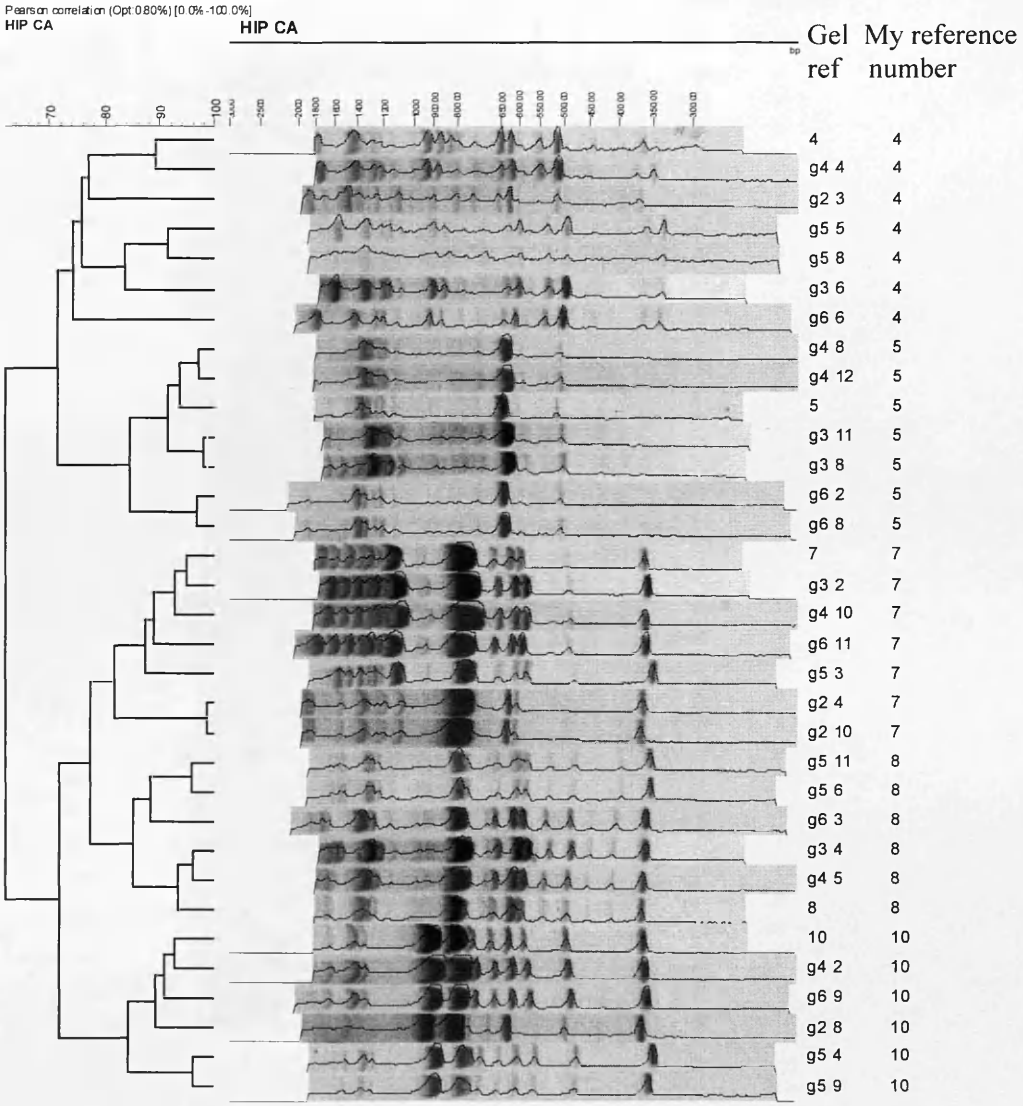


Figure 3.11 Affect of Pearson correlation coefficient on matching repeated PCR products from five isolates

N. B. Full profile matched, optimization performed. My reference number relates to CCAP accession code (see Table 3.3).

Gel reference refers to the number gel (gx) and lane (following digit) the PCR products were separated on. The control gel (equivalent to gel 1, where all isolates were separated) is simply numbered by my isolate reference number.

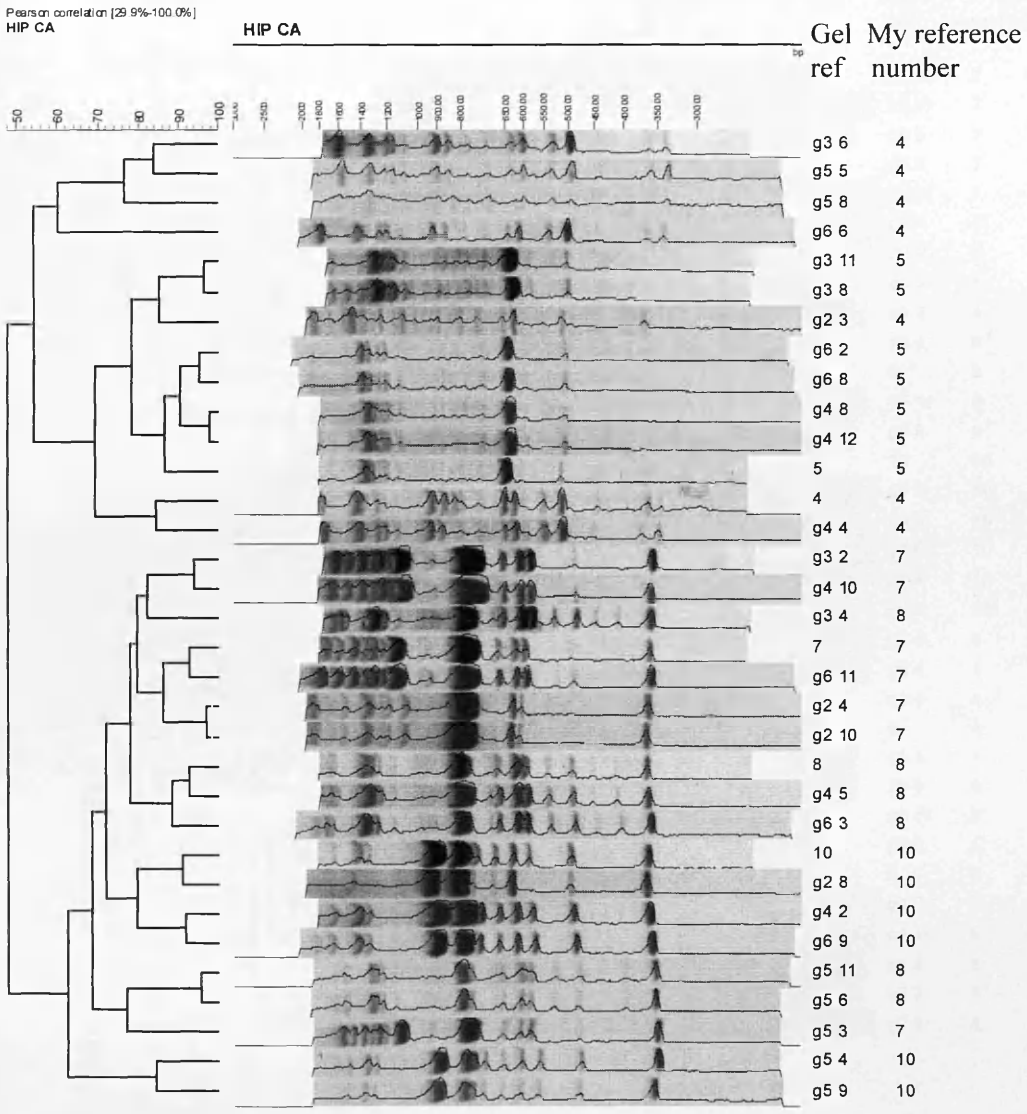


Figure 3.12 Affect of Pearson correlation coefficient on matching repeated PCR products from five isolates

N. B. shortened profile matched, no optimization performed. My reference number relates to CCAP accession code (see Table 3.3).

Gel reference refers to the number gel (gx) and lane (following digit) the PCR products were separated on. The control gel (equivalent to gel 1, where all isolates were separated) is simply numbered by my isolate reference number.

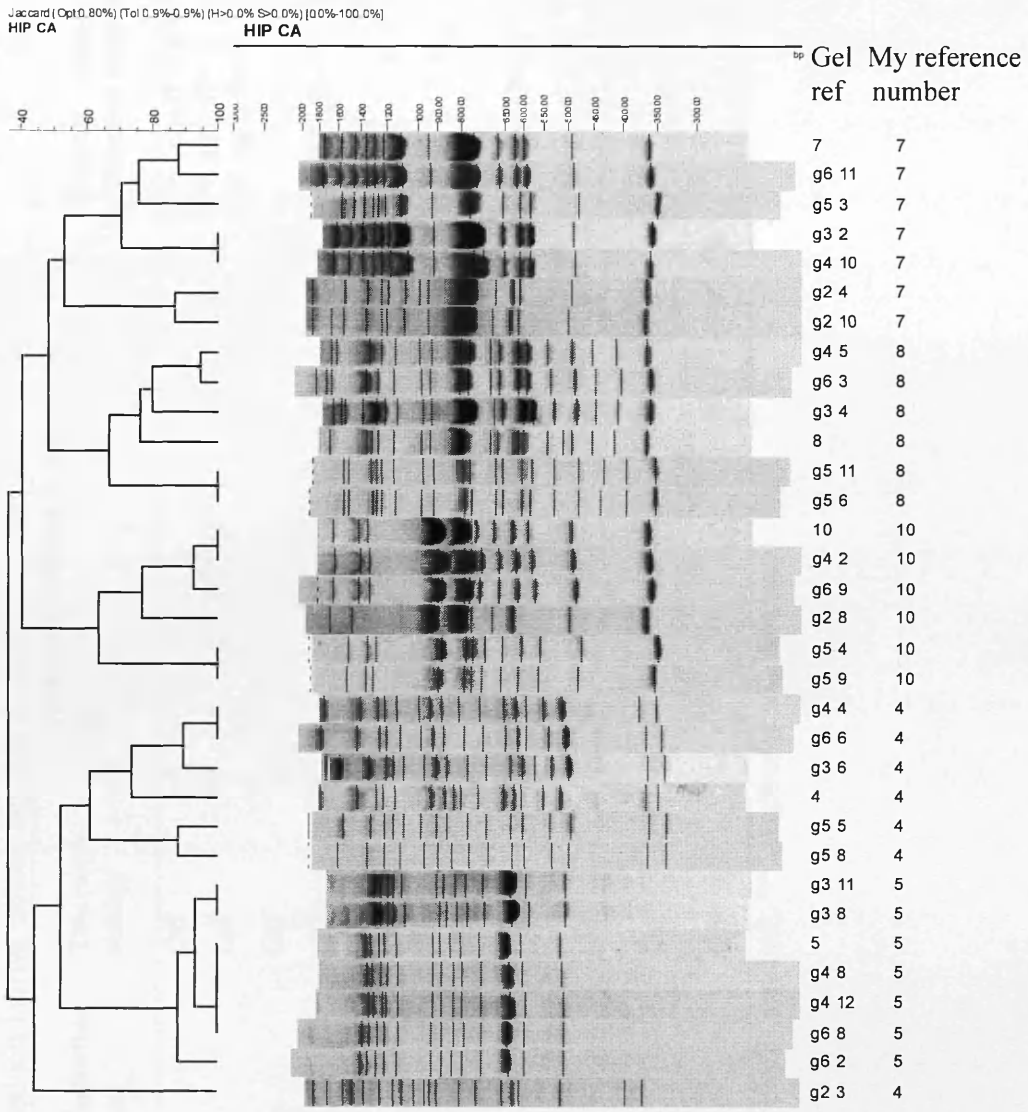


Figure 3.13 Affect of Jaccard coefficient on matching repeated PCR products from five isolates

N. B. Full profile matched, optimization and tolerance performed. My reference number relates to CCAP accession code (see Table 3.3).

Gel reference refers to the number gel (gx) and lane (following digit) the PCR products were separated on. The control gel (equivalent to gel 1, where all isolates were separated) is simply numbered by my isolate reference number.

Table 3.6 Summary of results of analysis of PCR products known isolates separated on different gels.

Similarity coefficient	Figure	Type *	Length**	Optimization setting	Tolerance setting	Resolution of isolates to discrete clusters					Potential isolate similarity delineation threshold
						4	5	7	8	10	
Pearson	3.10	Profile	Whole	Off	Off		✓		✓		80% cut-off = 5, 8
Pearson	3.11	Profile	Whole	On	Off	✓	✓	✓	✓	✓	80% cut-off = 5, 7, 8, 10 72% cut-off = 5, 7, 8, 10, 4
Pearson	3.12	Profile	29.9- 100%	Off	Off						
Pearson	Data not shown	Profile	29.9- 100%	On	Off		✓			✓	80% cut-off = 5, 10
Cosine	Data not shown	Profile	Whole	On	On	✓	✓				
Cosine	Data not shown	Profile	29.9- 100%	On	On						
Dice	Data not shown	Band	Whole	Off	On		✓		✓		60% cut-off = 8
Dice	Data not shown	Band	Whole	On	On				✓	✓	70% cut-off = 5 60% cut-off = 5, 8
Jaccard	Data not shown	Band	29.9- 100%	On	On			✓	✓	✓	70% cut-off = 5 60% cut-off = 5, 8
Jaccard	3.13	Band	Whole	On	On			✓	✓	✓	70% cut-off = 5 60% cut-off = 5, 8, 10

N.B * Type of coefficient (bands matched or densitometric profile matched), ** Length of profile matched.

3.5.4.3 Affect of dendrogram algorithm

The effect of algorithm type on resolution of isolates with cut-off thresholds is summarised in Table 3.7. The lanes of isolates separated on different gels were compared by Pearson correlation coefficient and then clustered using a number of dendrogram algorithms. The Ward algorithm produced a dendrogram which resolved the PCR products to the appropriate isolates (Fig. 3.14). A cut-off threshold at 70% similarity could be used to delineate the isolates 8 (CCAP 1459/17 *O.* (now *P.*) *arghardhii*), 10 (1459/14 *O.* (now *P.*) *arghardhii*) and 5 (CCAP 1405/1 *S. leopoliensis*), but not isolates 4 (CCAP 1479/14 *Synechococcus* sp.) or 7 (CCAP 1459/21 *O.* (now *P.*) *arghardhii*).

The Neighbour Joining algorithm produced a dendrogram which did not enable the resolution of PCR products to the appropriate isolates (Fig 3.15.). Two lanes of isolate 7 were deemed more clustered more closely to isolate 10 than 7 (both *O.* (now *P.*) *arghardhii*). No isolates would have been delineated correctly by a 70% similarity cut off.

The single linkage algorithm produced a dendrogram that did not resolve the isolates (Fig 3.16), lanes of isolate 4 (*Synechococcus* sp.) and 7 (CCAP 1459/21 *O.* (now *P.*) *arghardhii*) were clustered away from the rest of those isolates. Consequently a cut-off at 70% similarity would not have delineated any of the isolates.

The complete linkage algorithm produced a dendrogram that resolved all the PCR products into clusters accurately (Fig 3.17), with the exception of placing a lane of isolate 4 (*Synechococcus* sp.) with the lanes of isolate 7(CCAP 1459/21 *O.* (now *P.*) *arghardhii*). Isolates 5 (*S. leopoliensis*), 8 and 10 (both *O.* (now *P.*) *arghardhii*) could be delineated by a 70% similarity cut off.

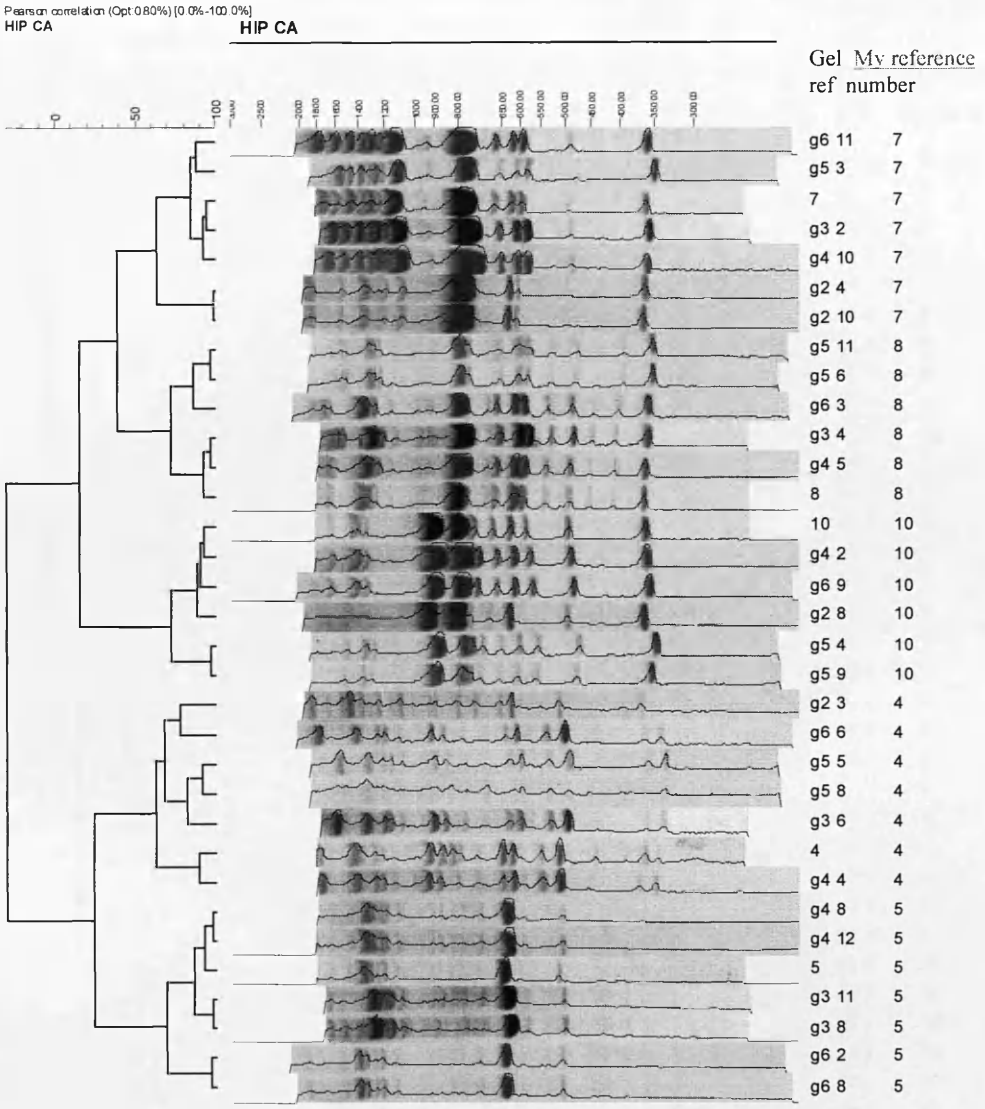


Figure 3.14 Affect of Ward dendrogram algorithm on clustering of isolates.
N.B similarity values generated from isolates matched as Fig 3.11. My reference number relates to CCAP accession code (see Table 3.3).
Gel reference refers to the number gel (gx) and lane (following digit) the PCR products were separated on. The control gel (equivalent to gel 1, where all isolates were separated) is simply numbered by my isolate reference number.

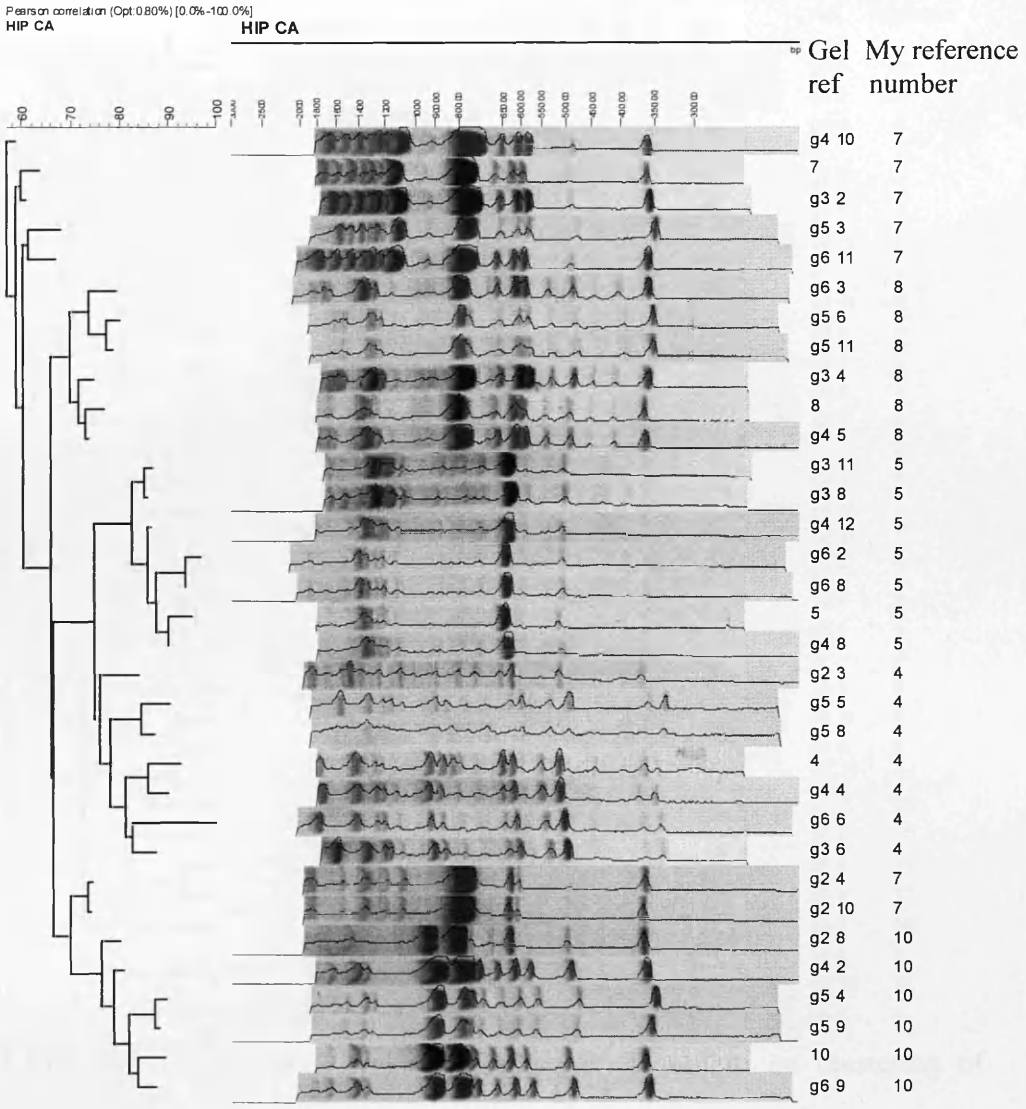


Figure 3.15 Affect of Neighbour Joining dendrogram algorithm on clustering of isolates.

N.B similarity values generated from isolates matched as Fig 3.11. My reference number relates to CCAP accession code (see Table 3.3).

Gel reference refers to the number gel (gx) and lane (following digit) the PCR products were separated on. The control gel (equivalent to gel 1, where all isolates were separated) is simply numbered by my isolate reference number.

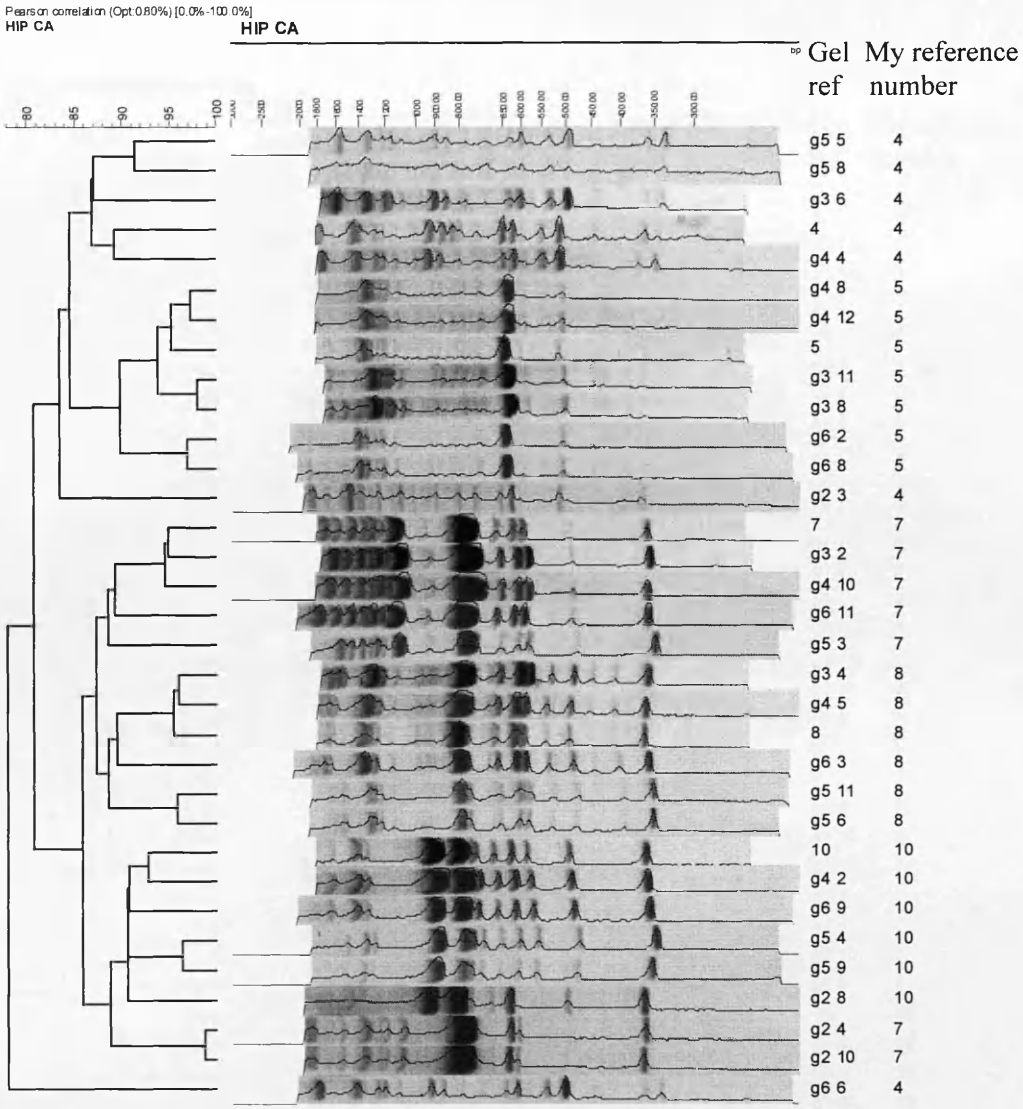


Figure 3.16 Affect of Single Linkage dendrogram algorithm on clustering of isolates.

N.B similarity values generated from isolates matched as Fig 3.11. My reference number relates to CCAP accession code (see Table 3.3).

Gel reference refers to the number gel (gx) and lane (following digit) the PCR products were separated on. The control gel (equivalent to gel 1, where all isolates were separated) is simply numbered by my isolate reference number.

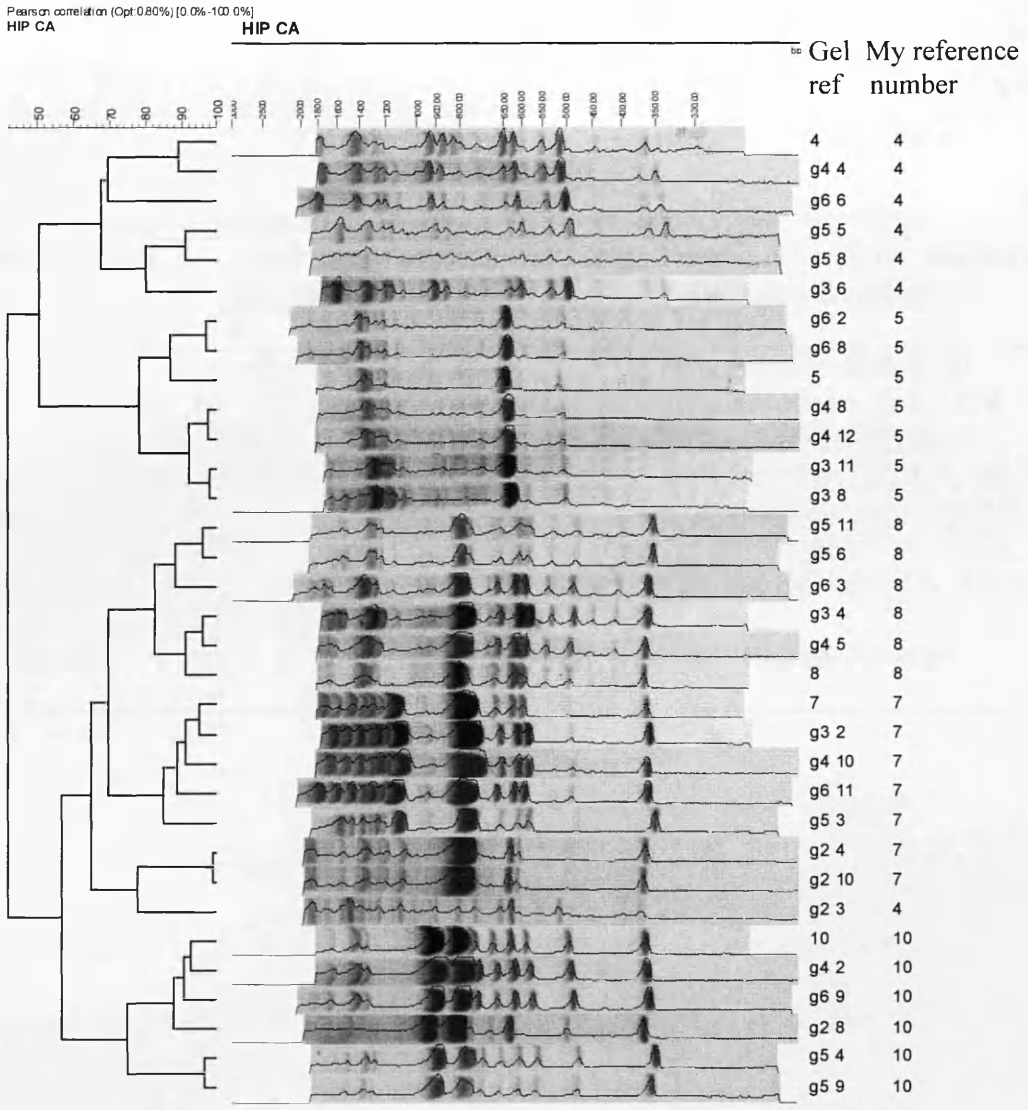


Figure 3.17 Affect of Single Linkage dendrogram algorithm on clustering of isolates.

N.B similarity values generated from isolates matched as Fig 3.11. My reference number relates to CCAP accession code (see Table 3.3).

Gel reference refers to the number gel (gx) and lane (following digit) the PCR products were separated on. The control gel (equivalent to gel 1, where all isolates were separated) is simply numbered by my isolate reference number.

Table 3.7 Comparison of clustering depicted by different dendrogram algorithms

Algorithm	Figure	Resolution of isolates into discrete clusters					Potential isolate similarity delineation threshold
		4	5	7	8	10	
UPGMA	3.11	✓	✓	✓	✓	✓	80% cut-off = 5, 7, 8, 10 72% cut-off = 5, 7, 8, 10, 4
Ward	3.14	✓	✓	✓	✓	✓	70% cut-off = 5, 8, 10 60% cut-off = 5, 7, 8, 4, 10,
Neighbour Joining	3.15	✓	✓		✓		Not possible
Single Linkage	3.16		✓		✓	✓	85% cut-off = 5
Complete Linkage	3.17		✓		✓	✓	70% cut-off = 5, 8, 10

3.6 Discussion

3.6.1 Preparation of template DNA for HIP 1 PCR

As a technique suitable for the extraction of DNA for genotyping of field isolates it is important that the method used consistently produces DNA from which reproducible PCR products can be generated. It is also useful to be able to store the DNA for future analysis. From the techniques compared for this study, these criteria rule out the direct PCR and lysozyme extraction methods (see Table 3.5). This means the cyanobacteria to be analysed by HIP 1 PCR must be in culture. To analyse the diversity of picocyanobacteria by HIP 1 PCR the cells need to be in culture to ensure there is sufficient clonal template for amplification. However for studies of larger cyanobacteria PCR can yield reproducible PCR products from single filaments. This method was used for HIP 1 PCR by Zheng *et al.* (2002). Along with reproducibility the cost and speed of the DNA extraction procedure extractions are also important criteria to consider when selecting a technique to apply to a large number of samples in a restricted time period. For these reasons, the Dynabead method of DNA extraction was chosen over the phenol-chloroform method, which had previously been used for HIP 1 genotyping studies in this laboratory.

Labour costs were not taken into account during this study as it was not an important factor in selecting the technique for application to further experiments for this study. However, the direct PCR was the least labour consuming, followed by lysozyme extraction. The phenol-chloroform method was the most labour intensive as required preparation pre-extraction (e.g. filling tubes with glass beads to even weights) as well

as intensive work during the procedure. The Dynabeads system 1 came with pre-prepared buffers and thus was relatively low labour in comparison the with the phenol-chloroform method. The labour costs for the Dynabeads system could be reduced by the use of plate magnets rather than single eppendorf magnets, however this would increase the initial start up costs and thus was not possible for this study. Moreover, a dramatic reduction in labour costs could be achieved by the automation of pipetting, but again at a higher cost.

3.6.2 HIP 1 sequences in cyanobacteria from the CCAP

HIP 1 PCR products generated from the cyanobacteria from the CCAP (Fig 3.2 and 3.3) demonstrated that this technique has the potential to discriminate both a broad range of cyanobacteria and within isolates of the same species. The isolates of the species of filamentous and colonial cyanobacteria were grouped together more closely when isolated from smaller geographical areas. The isolates of *Oscillatoria (*now *Planktothrix) argardhii* were clustered together, most of these isolates were from England, and all were from Europe. Isolates of species that originated from across the world were scattered through the dendrogram. This demonstrates the point that HIP 1 PCR can not be used to study phylogenetic relationships. The separation of isolates of *M. aeruginosa* through the dendrogram does not imply that some isolates are more similar to other genera. It implies that the variation in HIP 1 sequence distribution is wide within the isolates of *M. aeruginosa* analysed. The PCR products generated from isolates of *M. aeruginosa*, although maybe of more similar molecular weight and quantity to some generated by isolates of different genera may also be derived from different regions of the genome and thus represent distinctly different PCR products.

The isolates of picocyanobacteria generated very distinct PCR product profiles compared with the larger cyanobacteria, indicating that HIP 1 PCR diversity within this group of isolates is high. Although two isolates were from England and two from the USSR, the geographical isolation origin of the other isolates is unknown. Studies using sequencing have also indicated that organisms assigned to *Synechococcus* are very diverse (Iteman *et al.* 2000), probably even polyphyletic. These results do indicate that the discriminatory power of HIP 1 PCR has the potential to be suitable for differentiating picocyanobacteria as well as larger cyanobacteria during field studies.

3.6.3 Specificity and reproducibility of HIP 1 PCR

The variations in magnesium chloride, Taq DNA polymerase and primer concentration and annealing termperature all resulted in variation in the PCR products generated from the template DNA (Figs 3.4, 3.5, 3.6 and 3.7). This was expected from the analysis performed by Smith *et al.* (1998) and is common to PCR in general (Saiki 1989). These variations may have resulted from the effect of magnesium chloride concentration on Taq DNA polymerase, the availability of catalyst for the reaction, ratio of primer to target binding sites and temperature impacts on the specificuty of primer binding/denaturation of DNA secondary structure. The variations observed however appear minimal within the margins of error around the usual concentrations/temperatures used for HIP 1 PCR.

3.6.3.1 HIP1 sequences in other microorganisms

A small number of HIP 1 PCR products were obtained from heterotrophic bacteria in this study (Fig 3.6). PCR products were also obtained from environmental isolates of

heterotrophic bacteria by Smith *et al.* (1998), but not from *E. coli* by Robinson *et al.* (1995). Some of the cultures of eukaryotic algae tested in this study also produced products with HIP 1 extended primers (Fig 3.7). The results do not conclusively indicate that these products were derived from the eukaryotic templates as they were only produced from non-axenic cultures. Cyanobacterial isolates from the CCAP and field study were clonal but not axenic so any influence on PCR products can only have been derived from heterotrophic bacteria within the cultures.

The small number of bands produced by heterotrophic bacteria confirms the findings of a search of database entries by Robinson *et al.* (1995) that showed that over-representation of HIP 1 was unique to cyanobacteria among bacterial entries. Most of the PCR products produced from non-cyanobacterial source were of high molecular weight (>841 bp), though these do overlap with the size range of bands produced by cyanobacteria. A few bands of smaller molecular weight were also amplified. The amplification of PCR products from contaminating sources may affect the reproducibility and typeability of HIP 1 PCR as mentioned by Smith *et al.* (1998), but it may be possible to reduce the impact of the majority of these bands by restricting matching of PCR products to those smaller than 841 bp. This would result in a reduced amount of information from the cyanobacterial template being compared, but fewer non-cyanobacterial bands would be included. The effect of amplification of non-cyanobacterial bands could be investigated further by quantifying the template DNA of heterotrophic bacteria required within a cyanobacterial DNA reaction to produce detectable PCR products. It may be that competition between more stringent binding sites on cyanobacterial template would outcompete non-specific binding sites on heterotrophic bacterial DNA.

3.6.4 Analysis of PCR products

3.6.4.1 Effect of coefficient

It was clear from many of the matches that isolates 4 and 5 were distinctly different from isolates 7, 8 and 10 (Figs 3.11-3.17). All of the comparisons plus algorithms tested except the Dice (band-based) coefficient, and the Single Linkage algorithm, separated these two isolates from the others with the first branch. Isolates 7, 8 and 10 were all isolates of *Oscillatoria (*now *Planktothrix) arghardhii* (7= CCAP1459/21, 8= CCAP 1459/17, 10= 1459/14), which generated a number of bands of similar size and intensity, but varied by generation of a small number of PCR products. Isolates 4 and 5 were *Synechococcus* sp. (CCAP 1479/14) and *Synechococcus leopoliensis* (CCAP 1405/1). They had a small number of PCR products of similar size, but were otherwise quite distinct. The ability for the similarity coefficient to split isolates 4 and 5 from the others did not appear to be linked to the type of information used by the coefficient (Table 3.5). Both Pearson (densitometric curve based) and Jaccard (band based) coefficients separated these two sets of isolates with first order branches.

Optimisation and tolerance values generated by the software applied to matches increased the ability of all of the similarity coefficients to group the lanes into isolate single clusters. It appeared to reduce the effect of lane similarity resulting from separation on the same gel, but increased the similarity values generated between PCR product of the same isolate separated on different gels. These features thus assist in reducing differences between PCR products generated merely because they have been separated on different gels.

Reducing the length of the lane compared reduced the similarity found between the same PCR products separated on different gels. Thus although this may mean larger PCR products which could have been generated by PCR from contaminating

heterotrophic DNA could be screen out, it may reduce the ability of the software to match isolates of the same HIP 1 type.

3.6.4.2 Which is the most appropriate coefficient?

Pearson and Jaccard coefficients were able to group the lanes into isolate clusters with the highest accuracy (Table 3.5). Although Jaccard only miss-placed one lane, it was only possible to delineate three of the clusters at a 60% cut-off threshold, compared with four at 80% and all at 72% with the Pearson correlation coefficient. The difference between the two band-based coefficients Jaccard and Dice, is that the former weights matching bands higher than mismatches, whereas Jaccard gives equal weights to matches and mismatches. The over-weighting of matches verses mismatches of the Dice coefficient may have produced higher similarity values for lanes of different isolates which have bands of similar molecular weight, but are essentially different (thus this may explain why it did not separate isolates 4 and 5 from the rest). As PCR products amplified by HIP 1 PCR are from different regions of the genome, HIP 1 PCR products of the same size are not necessarily amplified from the same region. It is therefore inappropriate to weight apparent matches more highly than mismatches.

The bands assigned for the band-based comparisons during this study were first automatically assigned by the software, and then visually amended by visual inspection; this would require a considerable amount of time for a large number of samples. It is also subject to user decision-making, which may not be consistent, particularly if more than one user is involved. The allocation of bands to minor/low intensity PCR products can be particularly difficult and cause variation in matches.

Densitometric curve based similarity coefficients are a measure of the simple linear correlation between the overall density profile of two different lanes (Seward *et al.* 1997) so both the molecular weight and the intensity of PCR products are compared. PCR products from HIP 1 PCR do vary considerably in intensity (Smith *et al.* 1998) so this type of coefficient does provide more information to the matches than band based coefficients. In carrying out the comparisons in this study, it was also found that disabling the background subtraction feature for the analysis of the densitometric curves increased the accuracy of the matching.

3.6.4.3 Dendrogram algorithms

The comparison of dendrogram algorithms used to cluster the similarity coefficients generated by the Pearson coefficient which was optimised by the software, demonstrated that only the UPGMA and Ward algorithms clustered the lanes into isolates accurately (Figs 3.11, 3.14-7, Table 3.6). It was then possible to delineate four of the isolates using an 80% cut-off threshold with the UPGMA and all five isolates by 72%. A 60% threshold was required to delineate all of the clusters using the Ward algorithm. The UPGMA has frequently been used to generate dendrograms for genotyping methods (Soll 2000). It is proposed that due to the results of these comparisons that it is the most suitable for clustering HIP 1 PCR products. The approach of separating isolates into groups based on clusters which exceed a similarity threshold has been applied by others (Rementaria *et al.* 2001). This method of delineating isolates of picocyanobacteria into groups was applied to isolates from the field study in Chapter 4. Visual comparison of the diversity within and between the groups was then used to assign isolates to HIP 1 'types' based.

3.6.4.4 Factors affecting the likelihood of PCR products being matched

This work has demonstrated that the Pearson correlation coefficient and UPGMA dendrogram are the most effective at comparing and clustering the repeated lanes of each isolated tested. However, it was evident from the dendrograms in Figs 3.10 to 3.14 that some of the repeated lanes of some isolates were matched more frequently by the similarity coefficients and dendrogram algorithms tested than those of other isolates. In order to understand the variability between PCR products that should be accepted from unknown isolates so one can define clusters as a 'groups' or HIP 1 'types' one needs to consider the factors which affected the matching between the repeated lanes of the test isolates. Variation between the repeated lanes of each individual isolate can be a consequence of variation incurred during separation between lanes on the same and different gels. Table 3.8 compares for each test isolate, the number of lanes separated in total, with the mean number of lanes per gel (lanes separated/gels these lanes spread between). It can be seen that on average 1.2 lanes per gel were separated for each isolate, however 1.8 lanes were separated per gel for isolate 5. As it was split between less gels this probably contributed to the high similarity value generated between lanes of this isolate. Only six lanes of isolate 8 and 10 were separated (both *O.* (now *P.*) *arghardhii*). This may contribute to the high similarity values generated between repeated lanes of these isolates. Isolates 5 (*S. leopoliensis*), 8 and 10 (both *O.* (now *P.*) *arghardhii*) were also the most frequently clustered accurately on the dendrograms. I had expected the isolates, which generated the most PCR products to have been matched most effectively. However, this was not the case, with this parameter not tallying with the isolates which were the most frequently matched or given the highest similarity values. This maybe because the

match is based on more than presence or absence of bands and that all the isolates produced relatively different and complicated HIP 1 PCR products.

Table 3.8 Comparison of PCR products of isolates 4,5,7,8 and 10.

Isolate number *	Ranking based on no. of PCR products generated** (1 = least PCR products)	Ranking based on no. of lanes per gel*** (1= most lanes per gel)	Ranking based on similarity between lanes**** (1= most similar)	Ranking based on most frequently clustered
4 (7)	5 (18)	2 (1.2)	3 (72 %)	4
5 (7)	2 (13)	1 (1.8)	1 (88%)	2
7 (7)	3 (15)	2 (1.2)	2 (82%)	4
8 (6)	4 (16)	2 (1.2)	1 (88%)	1
10(6)	1 (12)	2 (1.2)	1 (88%)	3

* Figure in brackets is number of lanes separated, ** Figure shown in brackets is number of peaks in PCR product *** Figure shown in bracket is number of lanes/number of gels on which they were separated, ****Figure shown in brackets is mean % similarity of all lanes of the isolate according to match shown on Fig 3.11. See Table 3.3 for CCAP accession number and species names of the isolates.

Thus, the higher the number of gels, isolates with similar PCR products are separated on, the higher the variability between the PCR product and lower similarity values should be expected. This experiment did not take into account the variability that can result from different PCR mixtures and conditions within different thermal cycling runs. Seward *et al.* (1997) suggested that to combat variability between PCR products caused by gel electrophoresis that a DNA size marker should be loaded into each lane. As the HIP 1 PCR products are so complex this is not appropriate for this study as it may interfere with the analysis of the products themselves.

The PCR products of the isolates with the highest similarity values were most frequently clustered on all of the dendrograms. This gives confidence that using the threshold cut-off technique is a useful approach for grouping the isolates. However, some lenience will need to be given in terms of what similarity values to be accepted to cluster isolates into groups or types as variability will come from the PCR reaction,

the gel electrophoresis and the genetic makeup of the isolate. It is proposed therefore using the following method:

- Process gels (assign lanes, calibrate using MW size markers)
- Allow software to generate optimization value (% lane length)
- Compare full size range of PCR products using Pearson correlation ceofficent
- Cluster using UPGMA algorithm
- Use 70% similarity as a threshold to define 'groups' of >5 isolates
- Assign all other isolates to 'no group'
- Use visual observation and reasoning to check coherency of 'groups'/'no group' delineation and make manual adjustment.
- Assign manually adjusted 'groups' as HIP 1 types.

3.7 Conclusions

This chapter describesthe development of procedures to allow HIP 1 typing to be applied to a field study. A method for template DNA preparation and the analysis of PCR products have been selected. These results have also increased our understanding of the level of discrimination, specificity and reproducibility of HIP1 PCR. It appears that it can elucidate different isolates or strains of the same species of cyanobacteria, whilst the results should be susceptible to minimal interference by contaminants. The use of good laboratory practice should ensure that the results are representative of the targets isolated and reproducible. After this work therefore HIP 1 PCR continues to show the potential to be a useful technique for discriminating between cyanobacteria within field studies.

Chapter 4 HIP 1 diversity of picocyanobacteria in Esthwaite Water

4.1 Introduction and context of the Chapter

This chapter combines the outcomes of Chapters 2 and 3; the HIP 1 PCR genotyping technique (optimized as described in Chapter 3) is applied to picocyanobacteria isolated from Esthwaite Water (during the field study described in Chapter 2).

4.2 Aims of the Chapter

The aims were to:

- analyse the HIP 1 diversity of picocyanobacteria isolated from Esthwaite Water
- to continue the development and evaluation of HIP 1 PCR as a cyanobacterial genotyping technique by application to a field study.

4.3 Methods

4.3.1 Picocyanobacteria

The picocyanobacteria isolated from Esthwaite Water i(Chapter 2) were maintained in culture according to the method outlined in Chapter 3.3.1.

4.3.2 HIP 1 typing

The methods for DNA extraction, HIP 1 PCR, separation and visualisation of PCR products and gel processing have been described in Chapter 3.3.2, 3.3.3 and 3.4.4.9. The isolates were analysed by PCR using the extended HIP 1 primer HIP CA.

4.3.3 Analysis of results

4.3.3.1 Initial assignment of groups

As proposed from the developmental work described in Chapter 3, the HIP 1 PCR products generated from the isolates, were compared using the Pearson correlation similarity coefficient and clustered using the UPGMA algorithm. Isolates were initially grouped into clusters of greater than five isolates with a similarity of greater than 70%.

4.3.3.2 Assignment of HIP 1 types

The groups assigned using the 70% similarity cut-off threshold were scrutinised by the user. Amendments were made based on diversity within the groups, similarity to other

groups, and similarity to isolates not assigned to a group, and the group separation statistics.

4.3.3.3 Nomenclature

As outlined above in this study the HIP 1 PCR products of the isolates of picocyanobacteria cultured from the field samples from Esthwaite Water 2000 were compared. Initial groups were established based on predetermined criteria using the similarity of the PCR products of each isolate. These groups of isolates were labelled A to R from the top to bottom of the dendrogram (Fig 4.2. Those isolates not meeting the criteria were referred to as not belonging to a 'group'. After visual scrutiny and some manual reassignment of the groups, the groups were referred to as HIP 1 types. The HIP 1 types were labelled 1 to 21 from the top to bottom of the dendrogram (Fig 4.7). There were isolates that remained not associated within a HIP 1 type, these were referred to as not assigned to a type.

4.4 Results

4.4.1 HIP 1 PCR products

The HIP 1 PCR typing technique produced amplification products from 501 of the 506 isolates from the field study (Chapter 2). The number of PCR products amplified and detected per isolate ranged from 1 to 19 (see Figure 4.1). Seventy-eight percent of the isolates produced between 6 and 15 PCR products, with 19% producing less than 5 PCR products and 3% producing more than 16 PCR products.

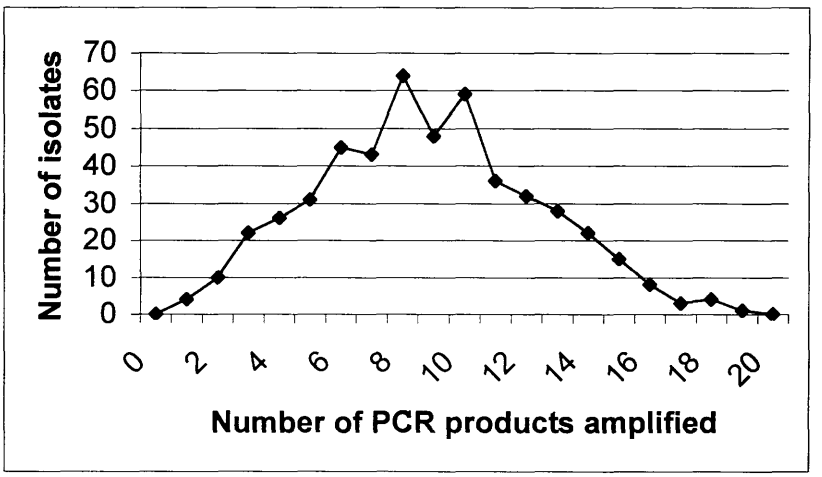


Figure 4.1 Number of HIP 1 PCR products amplified per isolate.

4.4.2 Summary of overall diversity and initial assignment to groups

The comparison of the isolates with Pearson correlation similarity coefficient showed all of the isolates to be greater than 4.61 % similar to each other (Fig 4.2). Seventy percent of the isolates were greater than 43.71% similar. Using the 70% similarity cut-off threshold (as method proposed from Chapter 3) groups were defined as clusters of more than 5 isolates with greater than 70% similarity of HIP 1 PCR products. A total of 351 (70%) of isolates were classed as within a group whilst 150 isolates were not

assigned to a group. The groups were dispersed throughout the dendrogram and ranged in size from 6 to 75 isolates (Fig 4.2). The diversity of HIP 1 PCR products generated by isolates within the groups varied.

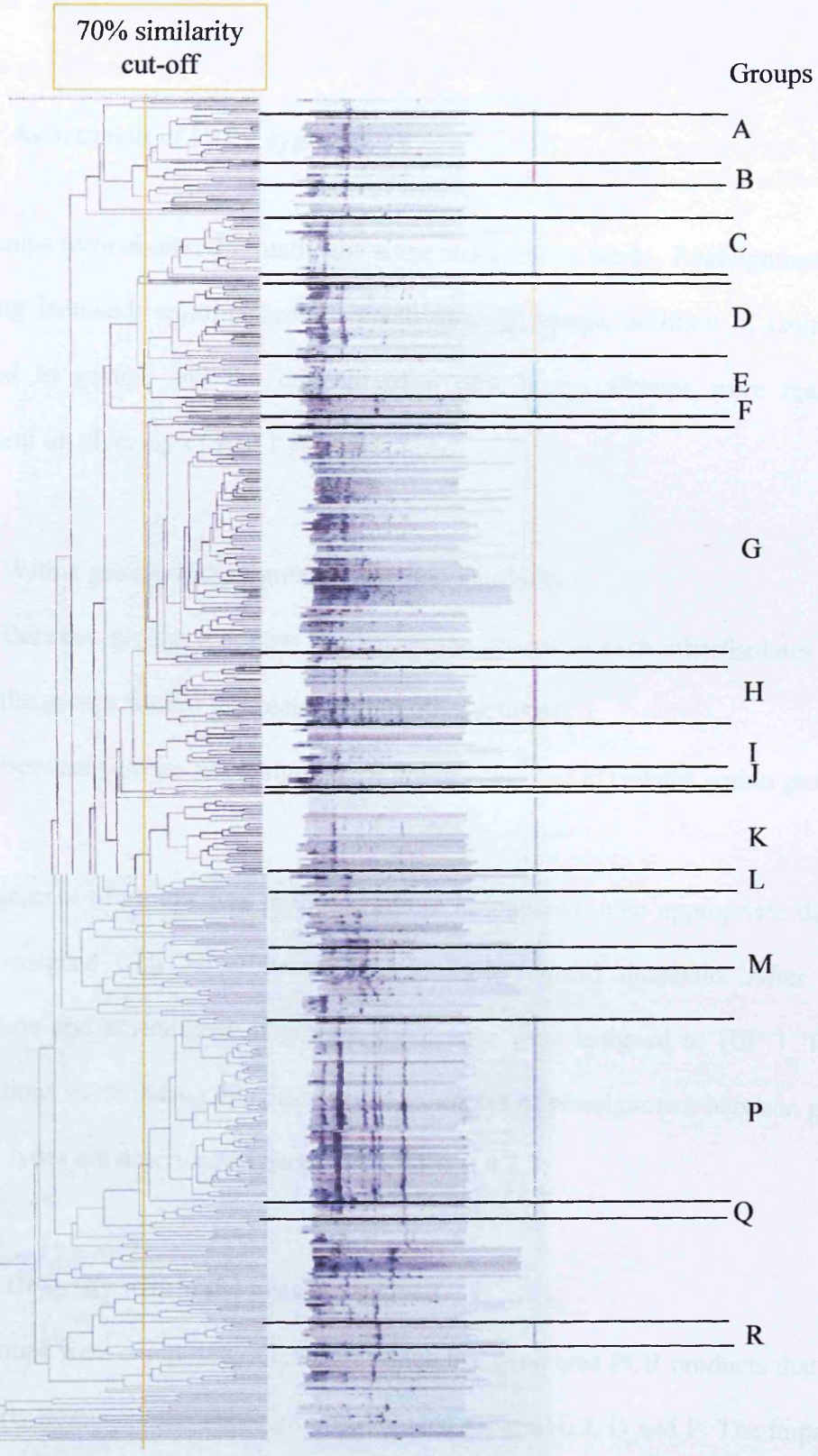


Figure 4.2 Clustering of HIP 1PCR products from isolates of picocyanobacteria.
N. B. Groups as initially defined by clusters of > 5 isolates of > 70% similarity.
Isolates between the groups were not assigned to a group.

4.4.3 Assignment of HIP 1 types

The groups were observed visually and some amendments made. Reassignment of the grouping included; splitting groups, amalgamating groups, addition of isolates not assigned to groups and the creation of a new group. Groups were reassigned dependent on diversity of HIP 1 PCR products:

- Within groups with of greater than 70% similarity
- Between groups (i.e. how similar are the groups to each other/isolates within the groups similar to isolates within other groups)
- Between isolates not assigned to a group compared to isolates within groups

Re-assignment of groups was proposed where it appeared more appropriate than the groups assigned with the arbitrary 70% similarity cut-off threshold. After visual observation and amendment of groups, the groups were assigned as HIP 1 'types'. Observations made during this process and examples of reassignment between groups to HIP 1 types are described in Section 4.4.3.1 to 4.4.3.3.

4.4.3.1 Diversity within the groups assigned

Some groups were composed of isolates, which had generated PCR products that were particularly homogenous. These groups included groups H, J, Q and P. The impact of reassignment was generally to leave these groups as initially assigned, or combine them with a small number of additional isolates not previously assigned to a group if

they were visually very similar. Fig 4.3 shows group P and the two additional isolates that were combined with this group to form HIP 1 type 18.

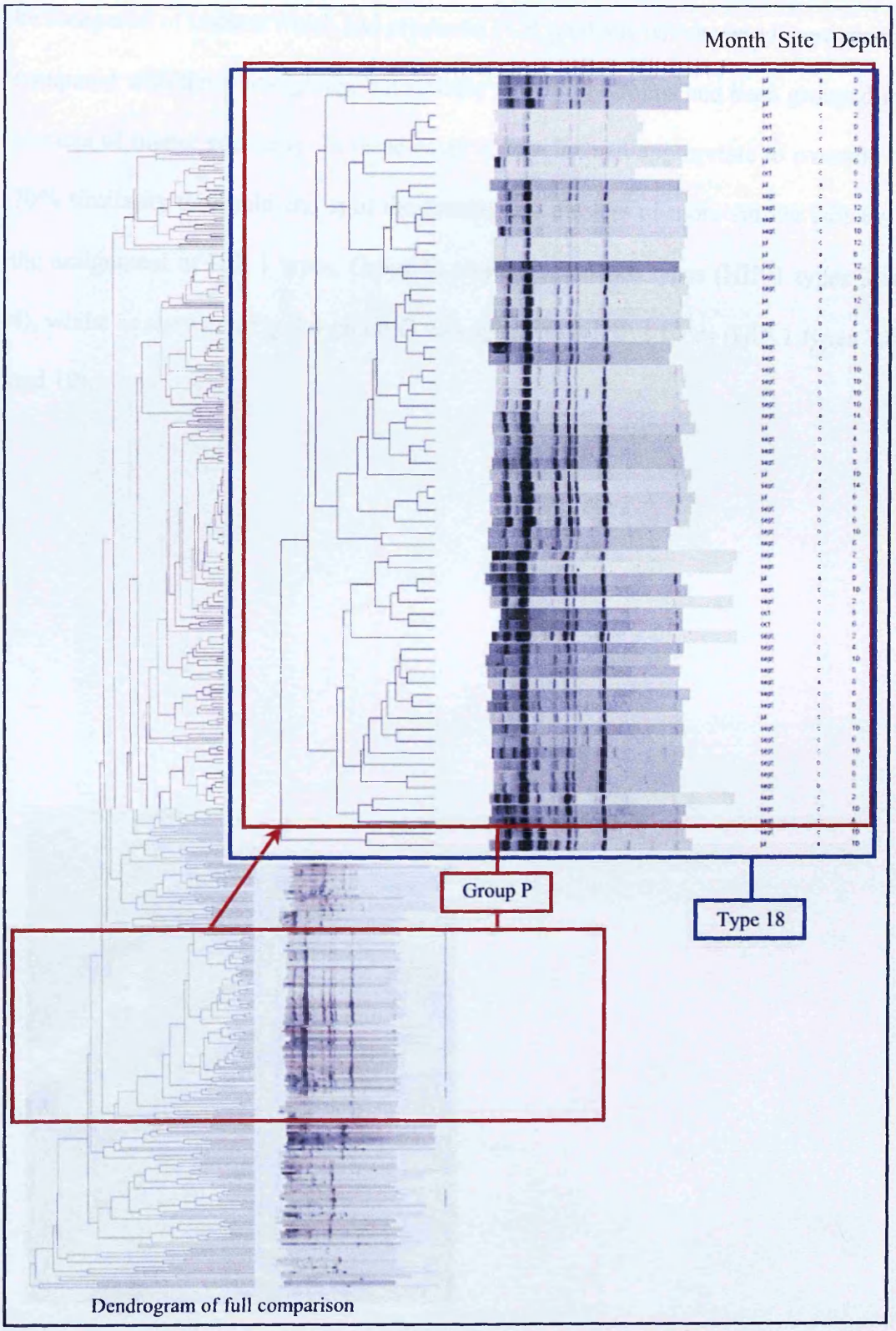


Figure 4.3 Reassignment of group P to HIP 1 type 18 (addition of two isolates).

Some groups although assigned with the 70% similarity cut-off threshold appeared to be composed of isolates which had produced PCR products which were heterogeneous compared with the whole group, but isolates within the groups had been grouped into clusters of higher similarity. In these cases it was deemed appropriate to override the 70% similarity threshold and split the groups into clusters of more similar isolates in the assignment of HIP 1 types. Group D was split into two types (HIP 1 types 3 and 4), whilst as shown in Fig 4.4 group G was split into 4 HIP 1 types (HIP 1 types 7,8,9 and 10).

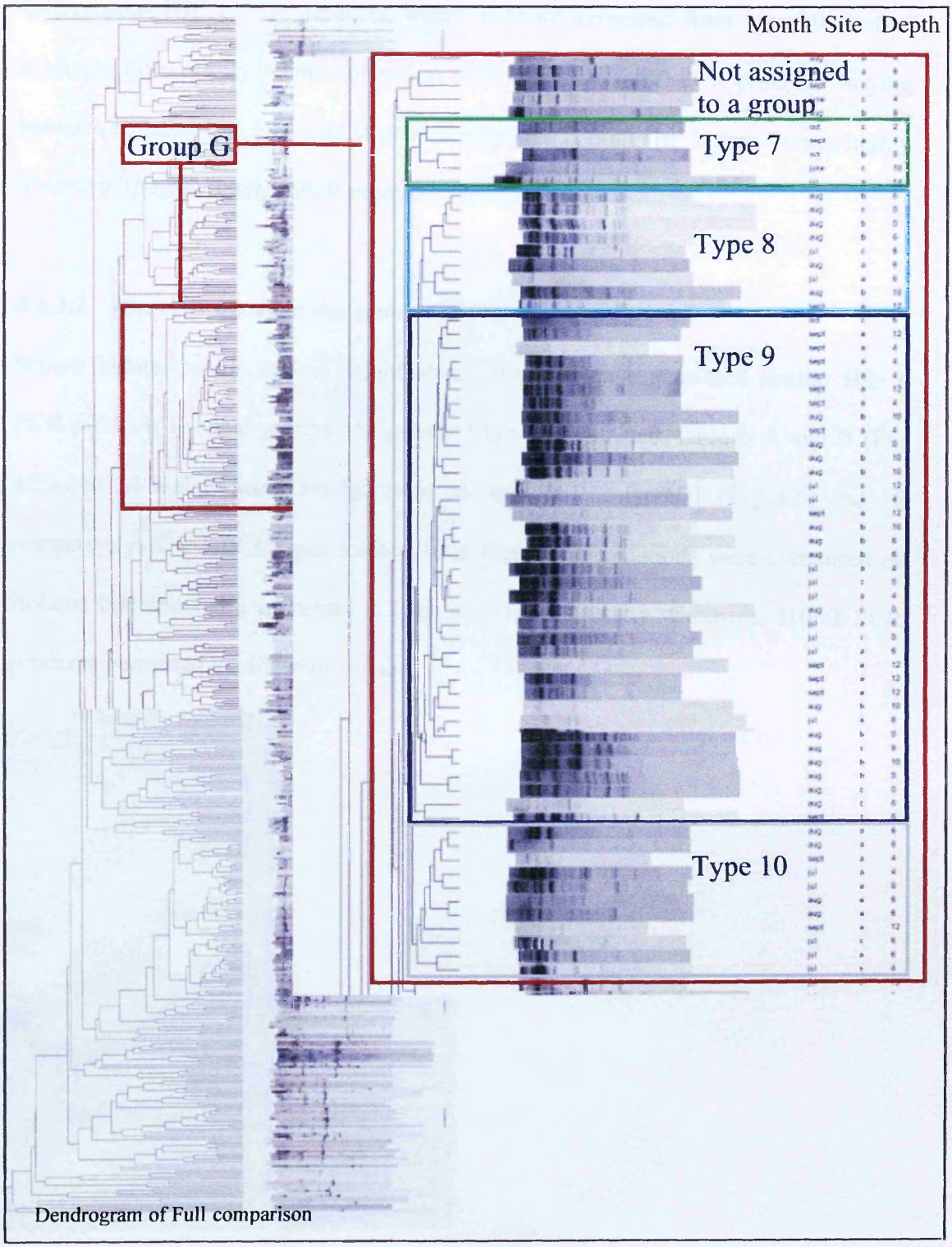


Figure 4.4 Reassignment of group G to four HIP 1 types.

The diversity within the HIP 1 types generated from these split groups varied. For instance HIP 1 types 8 and 9 were composed of isolates which had generated quite

homogenous HIP 1 PCR products, where as those generated from type 10 showed moderate diversity with some intense as well as minor (faint) PCR products varying between isolates. The HIP 1 PCR products generated from HIP 1 type 7 were highly diverse with many intense PCR products varying between isolates.

4.4.3.2 Diversity between the groups

Where isolates within groups neighbouring appeared have generated similar HIP 1 PCR products to other groups, the groups were amalgamated. Groups A and B (Fig 4.5) and M and N were amalgamated to form HIP 1 types 1 (Fig 4.5) and 16 respectively. The HIP 1 types formed from these amalgamations were composed of isolates that showed a moderate to high degree of diversity within the HIP 1 PCR products generated by different isolates.

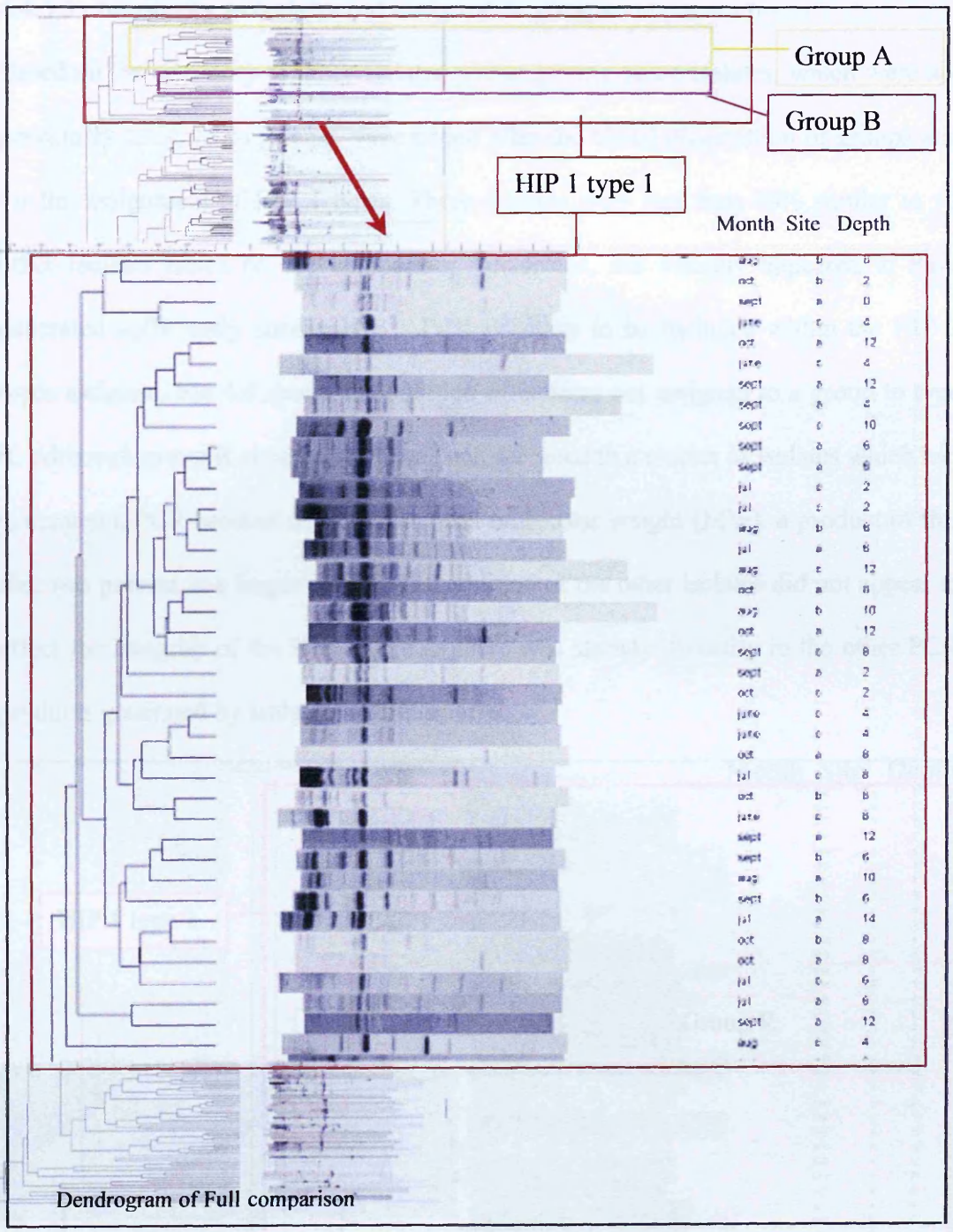


Figure 4.5 Amalgamation of groups A and B to HIP 1 type 1.

4.4.3.3 Similarity to isolates not assigned to groups

Based on the similarity to other isolates within groups, some isolates, which were not previously assigned to groups, were added after the visual observation of groups and for the assignment of HIP 1 types. These isolates were less than 70% similar to <5 other isolates based on the comparison performed, but visually appeared to have generated sufficiently similar HIP 1 PCR products to be included within the HIP 1 types assigned. Fig 4.6 shows the addition of isolates not assigned to a group to type R. Although group R appeared to have been assigned to a cluster of isolates which had generated a PCR product of similar approx molecular weight (MW), a product of this size was present in a larger cluster. The addition of the other isolates did not appear to affect the integrity of the HIP 1 type as there was already diversity in the other PCR products generated by isolates within group R.

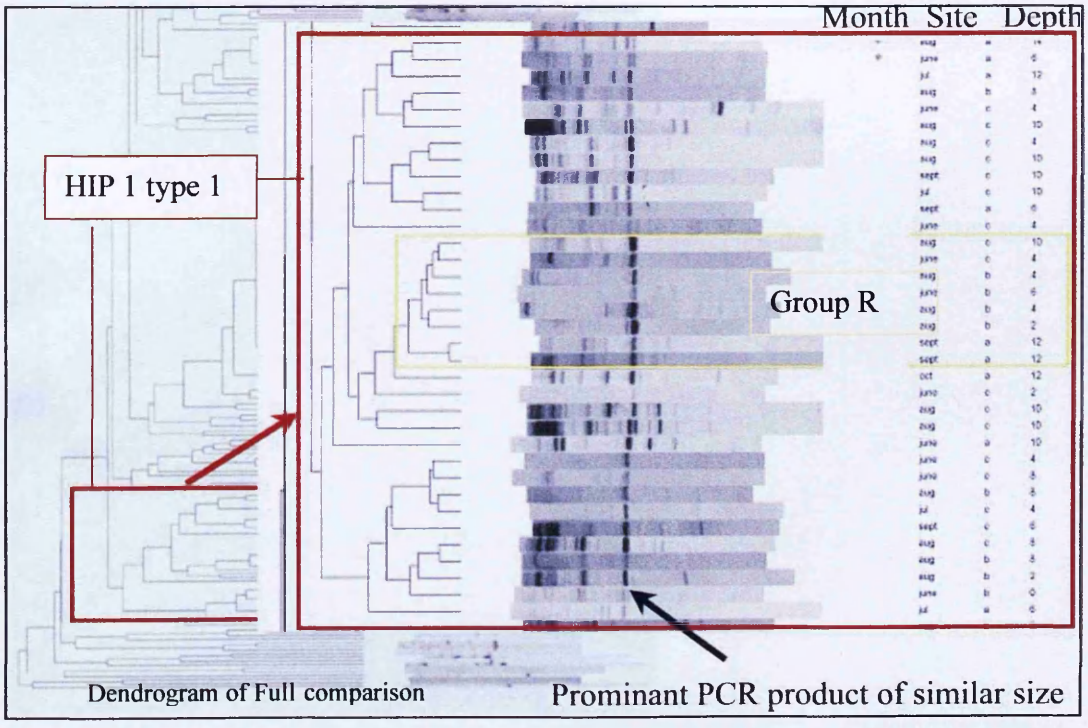


Figure 4.6 Group R initially composed of 8 isolates was increased in size by the addition of 27 others originally not assigned to a group.
N.B Yellow box highlights group R assigned with >70% similarity, Red box highlights isolates selected to represent HIP 1 type R after visual observation.

Table 4.1 summarises the changes made to all the groups in the assignment to HIP 1 types. Figure 4.7 shows the final dendrogram of twenty-one HIP 1 types. This assignment of HIP 1 types resulted in the assignment of ten types, which could be considered composed of isolates that were fairly homogenous in terms of HIP 1 products generated, six of moderate homogeneity of HIP 1 products and five HIP 1 types composed of isolates that generated PCR products, which could loosely be described as similar. 18% of isolates (ninety isolates) generated PCR products which were not similar enough to those generated by >4 other isolates to be assigned into a HIP 1 type.

Table 4.1 Summary of changes to 70% delineated groups to assign final types

Initial Group		**Changes to 70% delineated groups**				**Final Type**	
Name	No. of isolates	No change	Amalgamation with other groups	Addition/removal of isolates not in groups	Splitting of groups	New groups	Name	No. of isolates
A	16		+ B	+ 17			**1**	39
B	6		+A	+17				
C	21	✓					**2**	21
D	24			- 4	2 Types		**3**	10
							4	10
E	12	✓					**5**	12
F	5	✓					**6**	5
G	75			-4	4 Types		**7**	5
							8	9
							9	48
							10	11
H	19	✓					**11**	19
I	14	✓					**12**	14
J	6	✓					**13**	6
K	25			+ 3			**14**	28
L	14	✓					**15**	14
M	18		+N	+2			**16**	28
N	8		+M	+2				
O	9			+5			**17**	14
P	65			+2			**18**	67
Q	6	✓					**19**	6
NA	-					✓	**20**	10
R	8			+27			**21**	35
No Group	150			-60			**No Type**	90

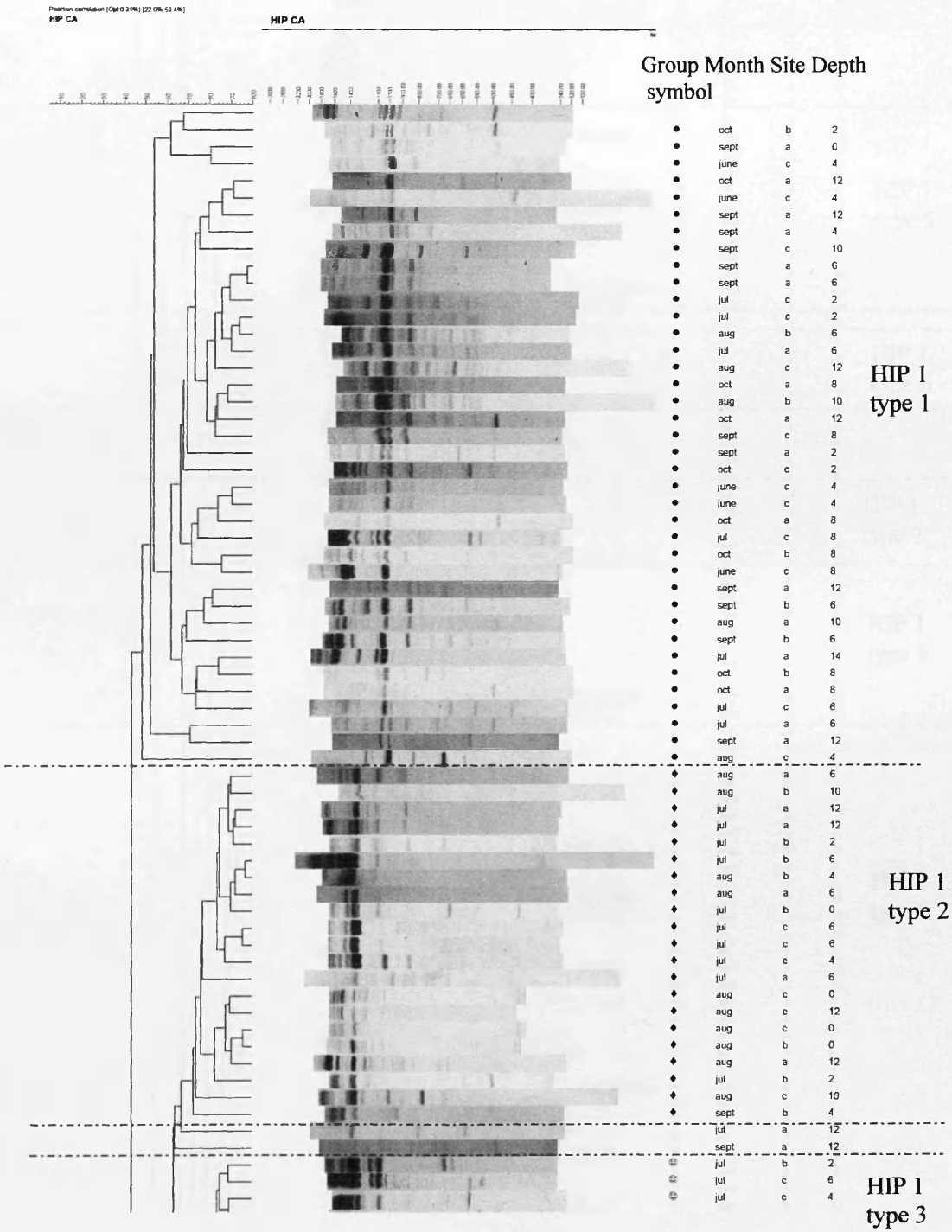


Figure 4.7 Full dendrogram of HIP 1 types

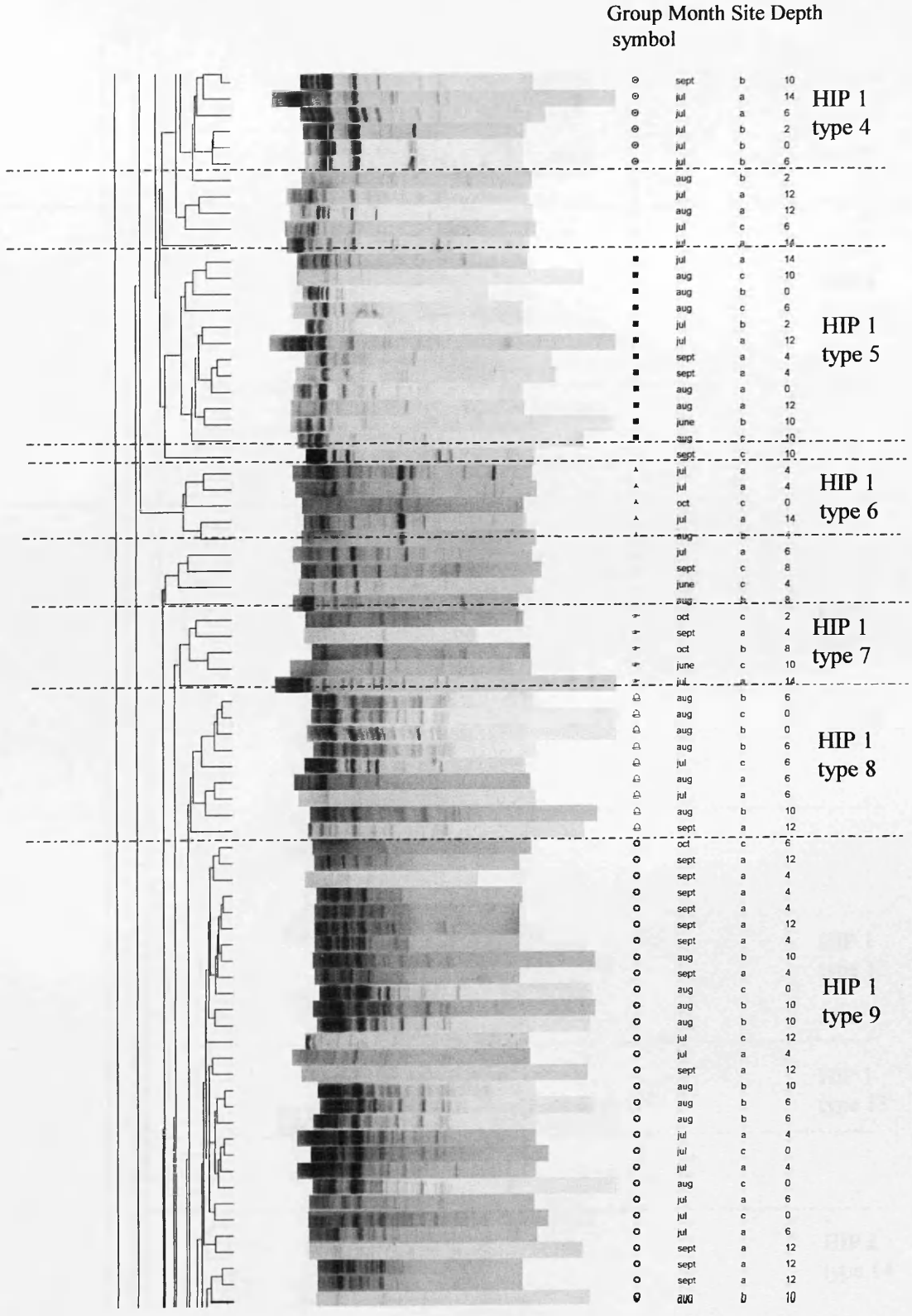


Figure 4.7 Full dendrogram of HIP 1 types (continued)

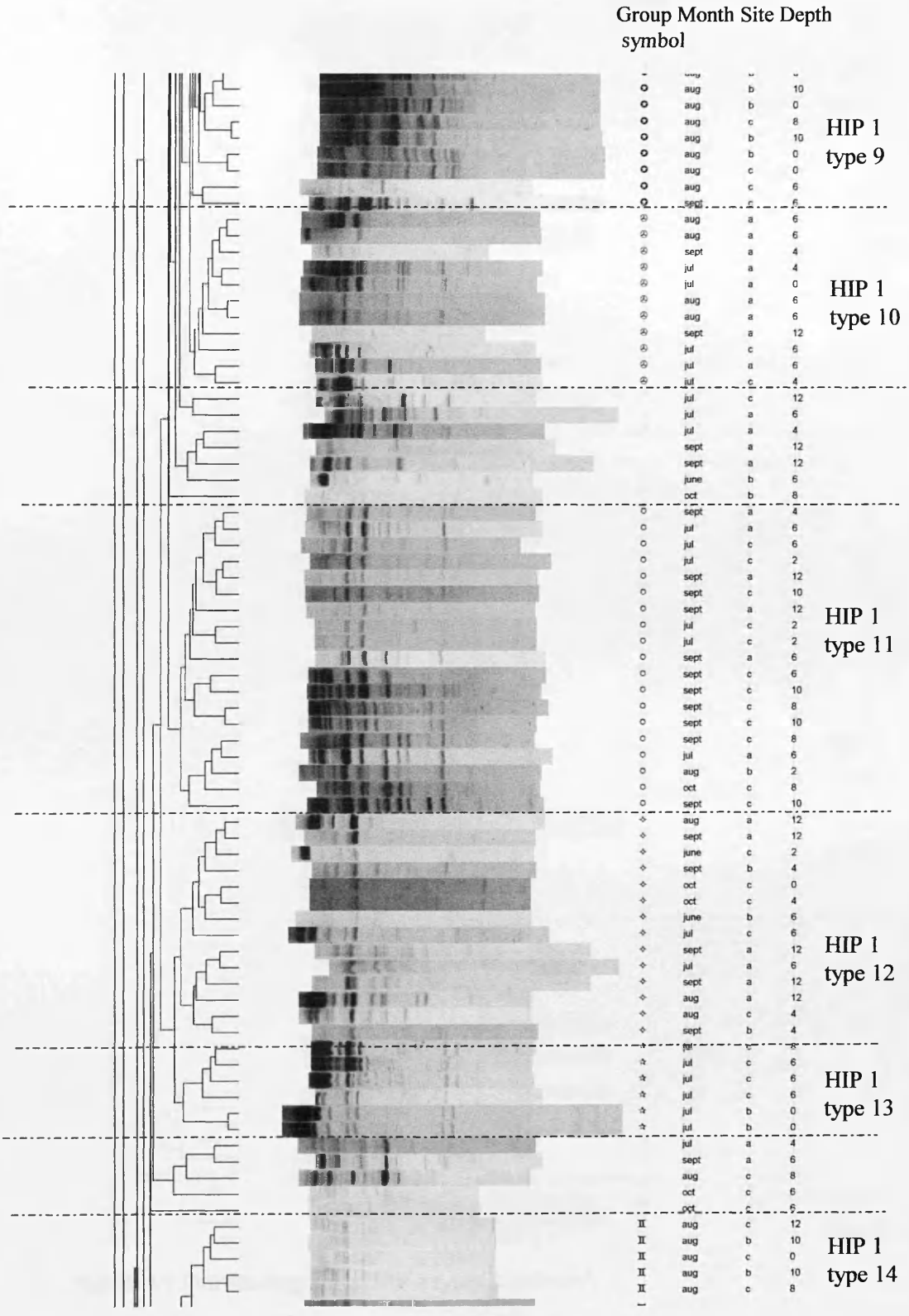


Figure 4.7 Full dendrogram of HIP 1 types (continued)

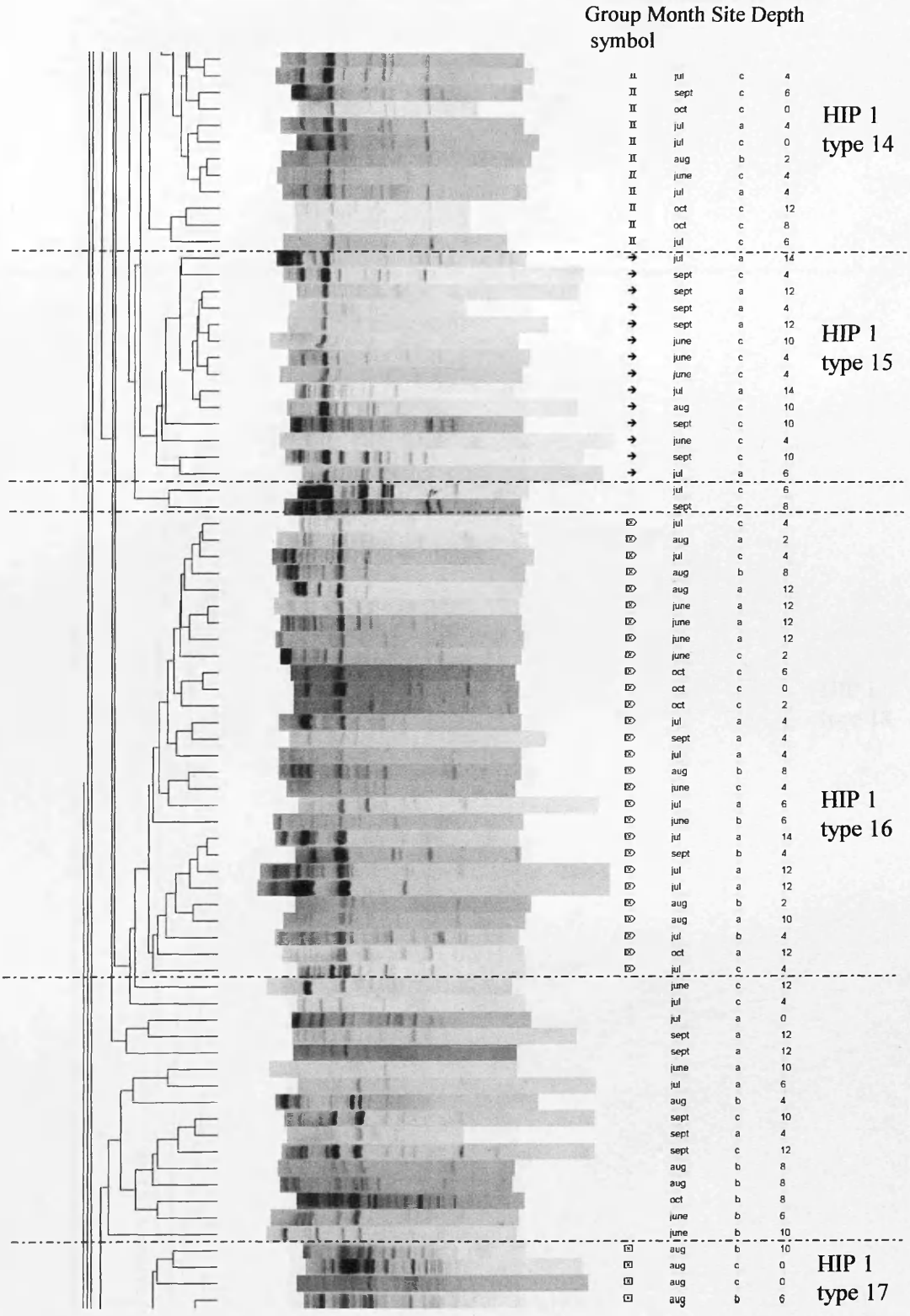


Figure 4.7 Full dendrogram of HIP 1 types (continued)

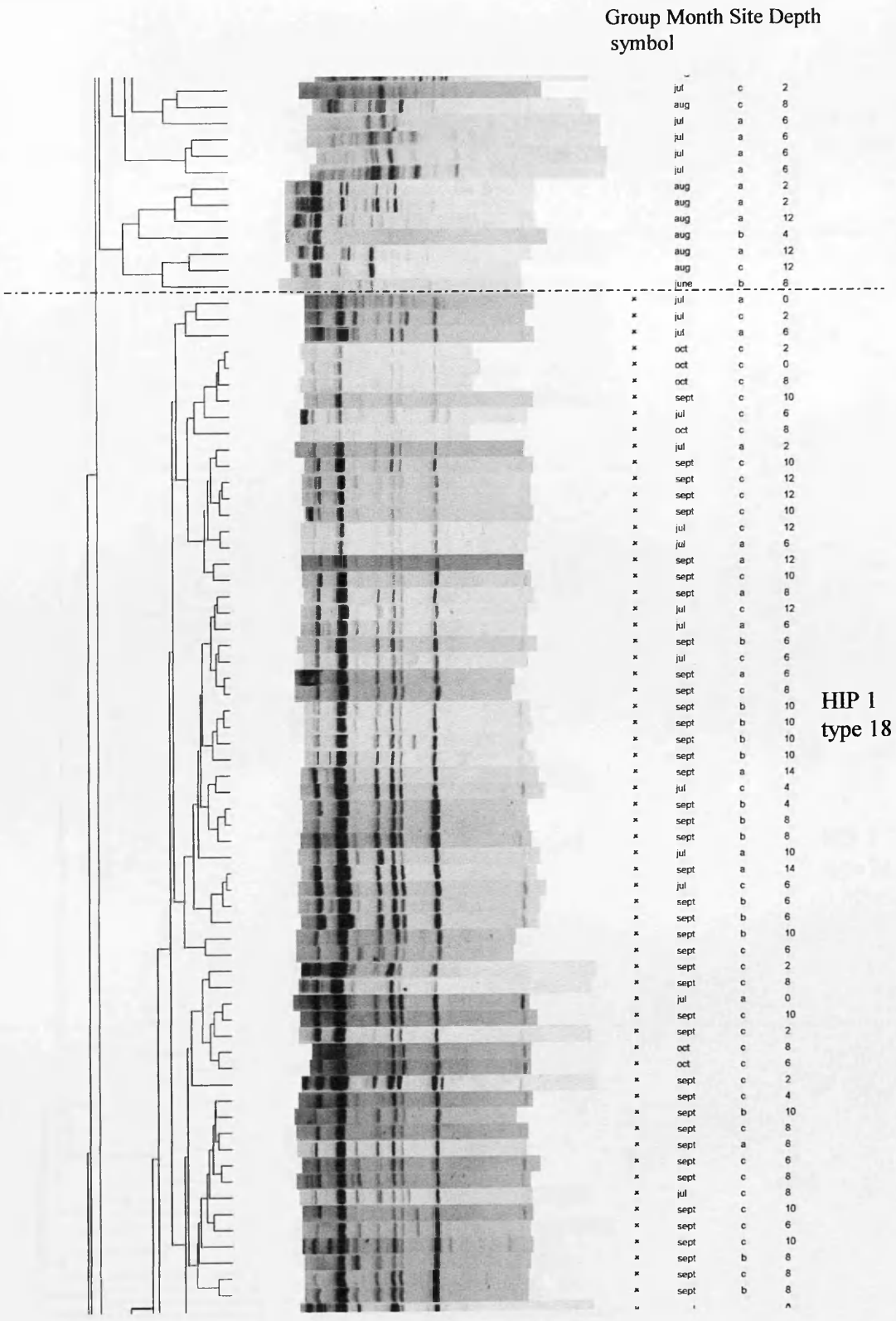


Figure 4.7 Full dendrogram of HIP 1 types (continued)

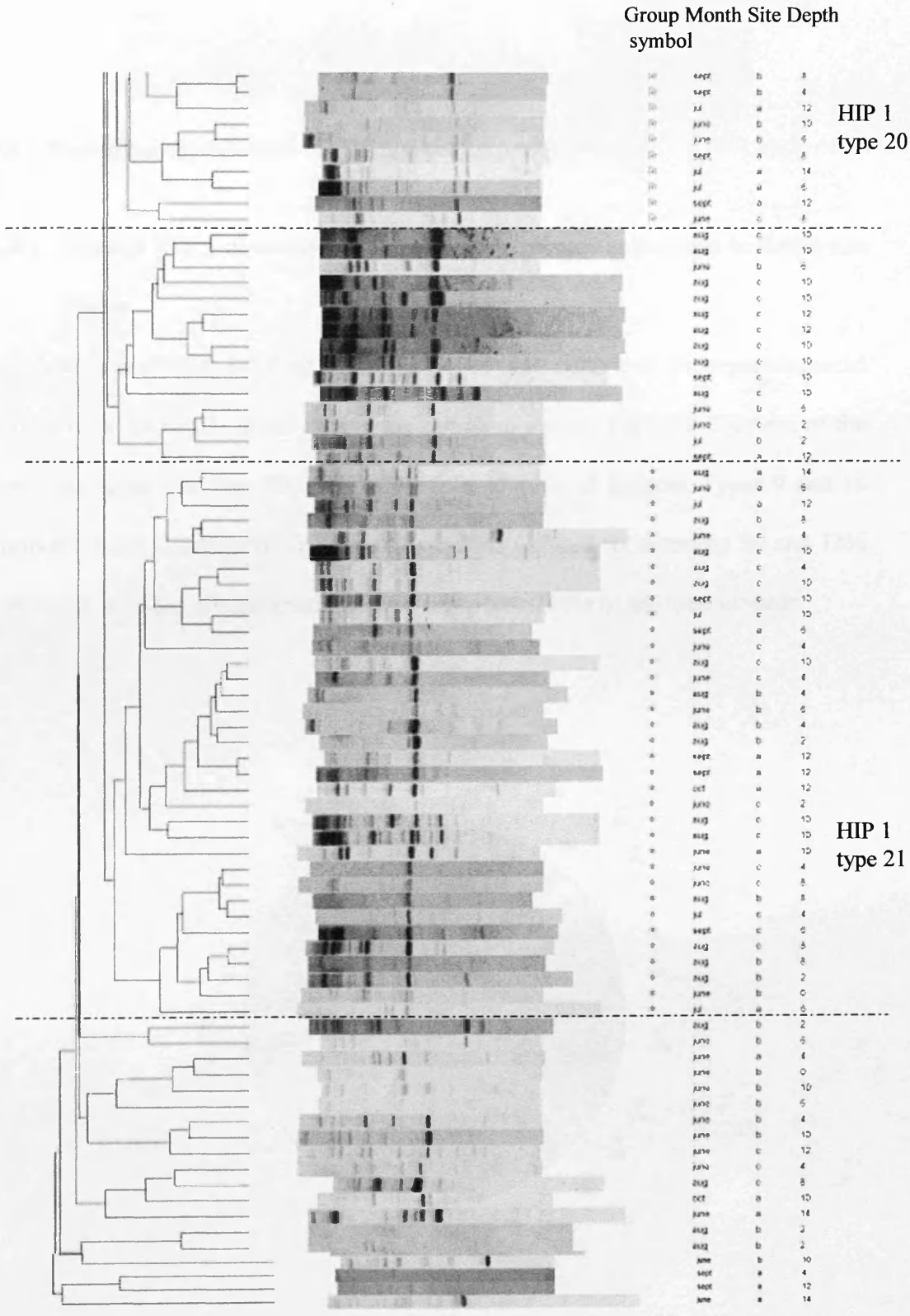


Figure 4.7 Full dendrogram of HIP 1 types

4.4.4 Ecological significance of HIP 1 types

4.4.4.1 Overall HIP 1 diversity of the culturable picocyanobacteria in Esthwaite Water

Each type contributed between 1 and 13% of the culturable picocyanobacterial community of Esthwaite Water during the sampling period (Fig 4.8). Fourteen of the types contributed less than 5% each to the total number of isolates. Types 9 and 18 contributed most significantly in terms of numbers isolated representing 10 and 13% respectively. Isolates not assigned to a type contributed 18% of the total isolates.

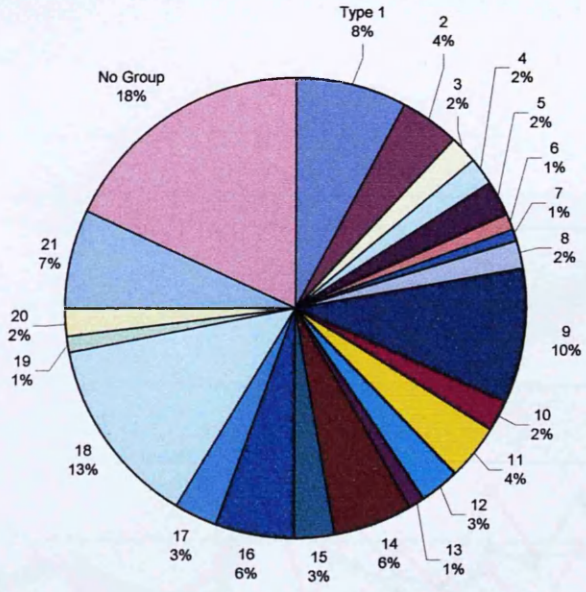


Figure 4.8 Pie chart to compare contribution of each type to total number of isolates

4.4.4.2 Does the HIP 1 diversity of spatio-temporal locations vary?

In general the contribution of each type to the picocyanoabcteria isolated from each site followed the same overall pattern (Fig 4.9). The highest peaks representing the groups isolated most frequently (Types 18, 9 and 1). The significance of Type 18 to the representatives of the community isolated at Site C, however was around 10% higher than at Sites A and B. Types 1 and 9, contributed a smaller proportion to the isolates at Site C than at A and B. Type 13 was not isolated from Site A. Types 10 and 15 were not isolated from Site B and Type 4 was not isolated from Site 5.

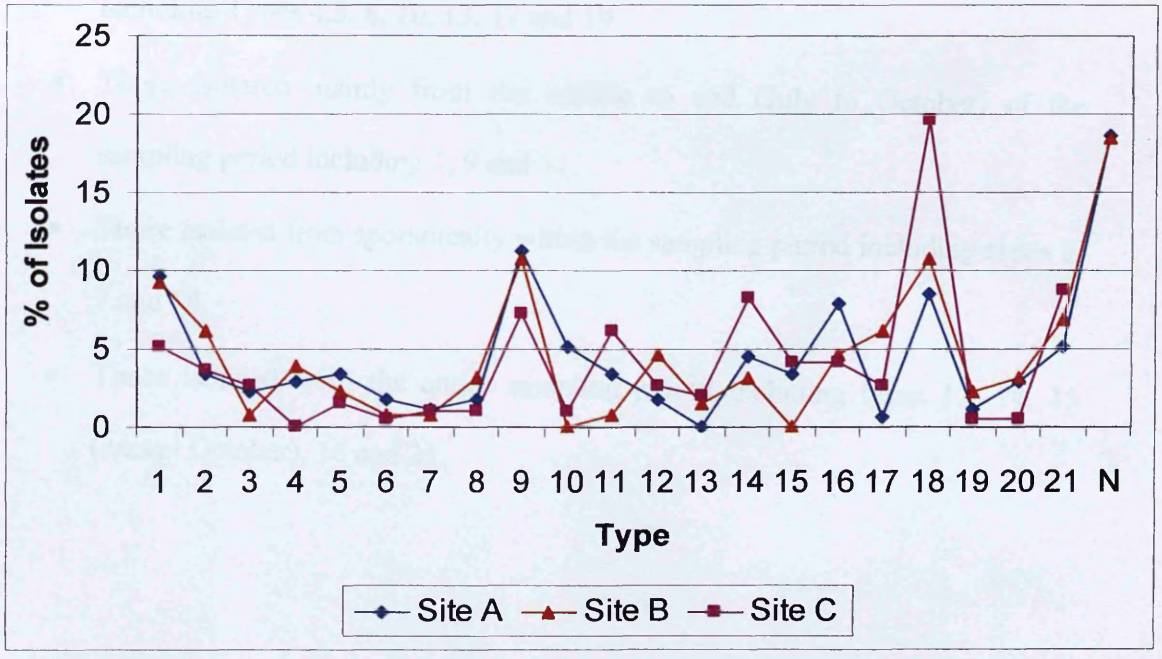


Figure 4.9 Contribution of each type to total isolates from each site
N. B N = No type, % of isolates = % isolated from each site.

The relative percentage of each type isolated at each site from the epilimnion or hypolimnion is indicated in Tables 4.2 to 4.4. In general, most types were isolated in small numbers over the sampling period.

Type 2 was isolated from all sites and both the hypolimnion and epilimnion. Types 21 and 16 were isolated from the hypolimnion of all sites. Forty percent of isolates of Type 6 were isolated from the epilimnion of Site A in June. Other types were only present at one or two sites or not at all.

On close observation of Tables 4.2 to 4.4 it appears that many of the types assigned seem to have been isolated over a discrete portion of the sampling period. These can be summarised as follows:

- Those isolated early (June to August) in the sampling period only, including Types 2, 3, and 20.
- Those isolated in the middle (July to September) of the sampling period only, including Types 4,5, 8, 10, 13, 17 and 19.
- Those isolated mainly from the middle to end (July to October) of the sampling period including 1, 9 and 11.
- Those isolated from sporadically within the sampling period including types 6, 7 and 18.
- Those isolated from the entire sampling period including types 12, 14, 15 (except October), 16 and 21.

Table 4.2 Relative isolation success of each type from the spatio-temporal sampling locations at Site A

	June						July						August						September						October					
Epilimnio	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
	7	8	9	10	11	12	7	8	9	10	11	12	7	8	9	10	11	12	7	8	9	10	11	12						
	13	14	15	16	17	18	13	14	15	16	17	18	13	14	15	16	17	18	13	14	15	16	17	18	7	8	9	10	11	12
	19	20	21	N	A		19	20	21	NT	All		19	20	21	NT	All		19	20	21	NT	All							
Hypolimni	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	13	14	15	16	17	18
	7	8	9	10	11	12	7	8	9	10	11	12	7	8	9	10	11	12	7	8	9	10	11	12						
	13	14	15	16	17	18	13	14	15	16	17	18	13	14	15	16	17	18	13	14	15	16	17	18	19	20	21	NT	All	
	19	20	21	NT	All		19	20	21	NT	All		19	20	21	NT	All		19	20	21	NT	All							

Table 4.3 Relative isolation success of each type from the spatio-temporal sampling locations at Site B

	June						July						August						September						October					
Epilimnio	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
	7	8	9	10	11	12	7	8	9	10	11	12	7	8	9	10	11	12	7	8	9	10	11	12						
	13	14	15	16	17	18	13	14	15	16	17	18	13	14	15	16	17	18	13	14	15	16	17	18	7	8	9	10	11	12
	19	20	21	N	A		19	20	21	NT	All		19	20	21	NT	All		19	20	21	NT	All							
Hypolimnion	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	13	14	15	16	17	18
	7	8	9	10	11	12	7	8	9	10	11	12	7	8	9	10	11	12	7	8	9	10	11	12						
	13	14	15	16	17	18	13	14	15	16	17	18	13	14	15	16	17	18	13	14	15	16	17	18	19	20	21	NT	All	
	19	20	21	NT	All		19	20	21	NT	All		19	20	21	NT	All		19	20	21	NT	All							

Table 4.4 Relative isolation success of each type from the spatio-temporal sampling locations at Site C

	June						July						August						September						October					
Epilimni	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
	7	8	9	10	11	12	7	8	9	10	11	12	7	8	9	10	11	12	7	8	9	10	11	12						
	13	14	15	16	17	18	13	14	15	16	17	18	13	14	15	16	17	18	13	14	15	16	17	18	7	8	9	10	11	12
	19	20	21	N	A		19	20	21	NT	All		19	20	21	NT	All		19	20	21	NT	All							
Hypolimnion	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	13	14	15	16	17	18
	7	8	9	10	11	12	7	8	9	10	11	12	7	8	9	10	11	12	7	8	9	10	11	12						
	13	14	15	16	17	18	13	14	15	16	17	18	13	14	15	16	17	18	13	14	15	16	17	18	19	20	21	NT	All	
	19	20	21	NT	All		19	20	21	NT	All		19	20	21	NT	All		19	20	21	NT	All							

Colours denote percentage of type isolated on each occasion

0 | 1-10 | 11-25 | 26-50 | 50-75 | 76-100

Numbers indicate type.

A similar range of types appeared to be represented within isolates from the epilimnion and hypolimnion during June (Fig 4.9). However the contribution of the types that were represented within both to total isolates from each depth range (epilimnion or hypolimnion) did differ. For instance type 3 represented 30% of the isolates from the epilimnion in June, but only 10% of those isolated from the hypolimnion. Some types were only isolated within one depth zone e.g. types 3 and 7 were only isolated from the epilimnion, whilst types 1, 14 and 15 were only isolated from the hypolimnion. Types 4, 7, 8, 9 and 10, and 17, 18 and 19 were not isolated in June.

Although some types e.g. HIP 1 types 2, 10 and 18 were represented equally in those isolated from the epilimnion and hypolimnion in July, there appeared to be a distinctly different type composition between the epilimnion and hypolimnion in this month (Fig 4.10). Types 3, 4 and 13, although present in both depth ranges, contributed a greater proportion to the culturable picocyanobacteria from the epilimnion than hypolimnion. The reverse was true of types 9 and 14. Type 6 was not isolated at all in July and types 5, 6, 7, 8, 9, 12, 17 and 19 – 21 were only represented by a small number of the isolates.

In August, the picocyanobacteria isolated from the epilimnion and hypolimnion contained representatives from a very similar range of types in similar proportions (Fig 4.11). However, small numbers of Types 2, 6 and 11 were only isolated from the epilimnion. Types 3, 4, 7, 13 and 18 were not isolated in August.

In September Types 1, 9, 11, 14 and 15 were isolated from both the epilimnion and hypolimnion, but contributed individually less than 16% to the total number of isolates from each site that month (Fig 4.12). Type 18 however was also isolated from

both locations but contributed over 23% to the epilimnetic culturable isolates and 42% to those isolated from the hypolimnion. Types 2, 3, 6, 13, 17, 19 and 20 were not isolated in September.

Type 1 contributed over 25% of the isolates from October (Fig 4.13). Type 18 contributed 17%, and between 5 and 15% of the isolates were represented by Types 6, 12, 14 and 16. Types 6, 9, 11 and 21 contributed less than 5% to the isolates from October. The other eleven types were not isolated from the October samples.

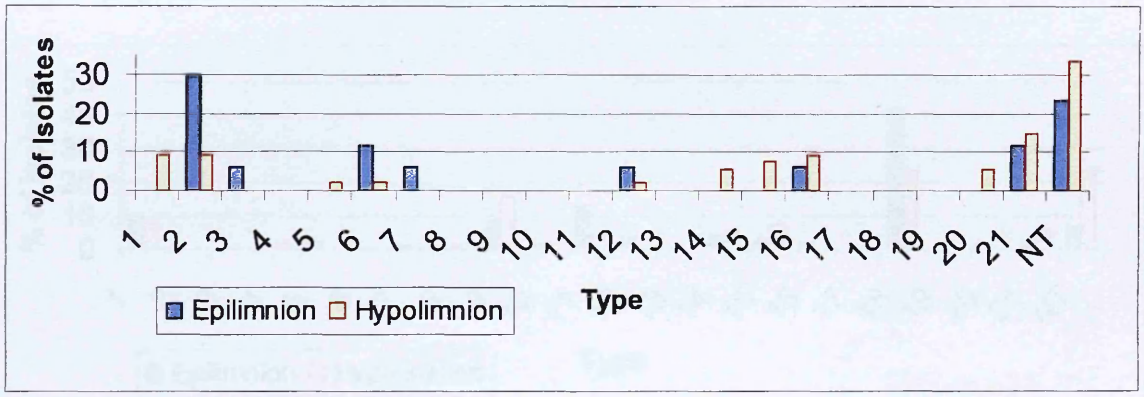


Figure 4.10 Contribution of types to picocyanobacteria isolated in June 2000 (NT = Not assigned to a type).

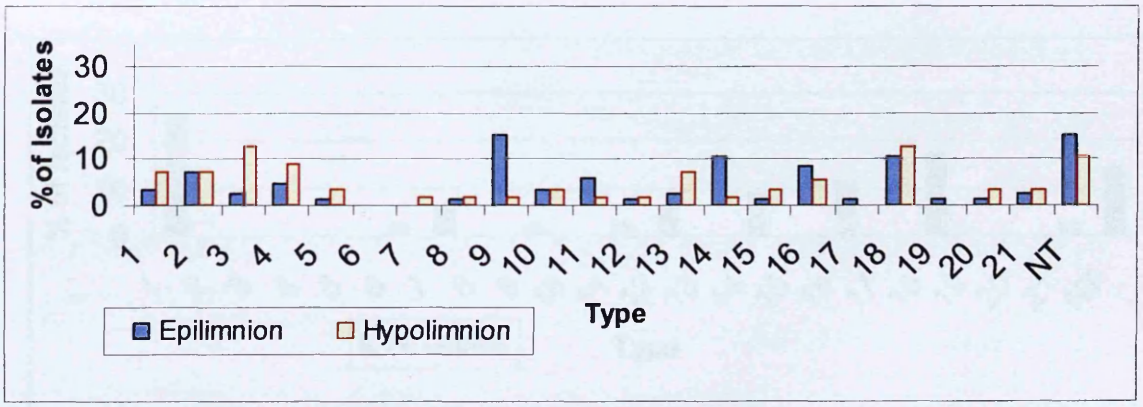


Figure 4.11 Contribution of types to picocyanobacteria isolated in July 2000 (NT = Not assigned to a type).

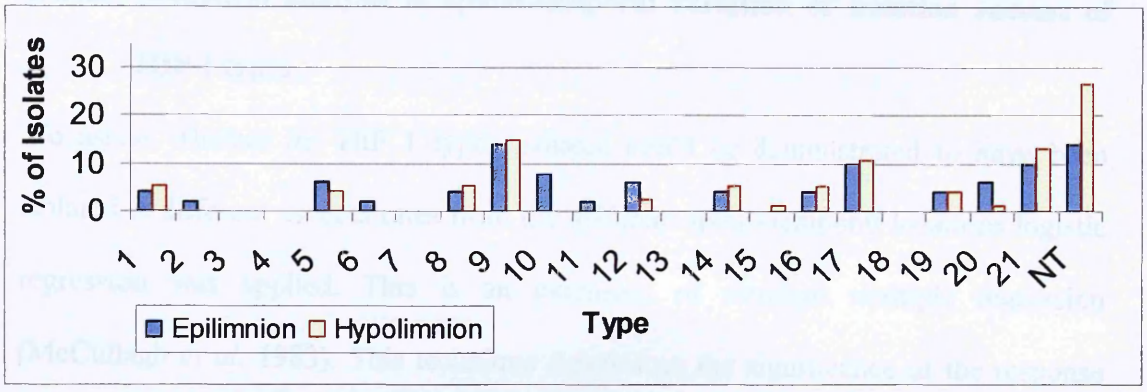


Figure 4.12 Contribution of types to picocyanobacteria isolated in August 2000 (NT = Not assigned to a type).

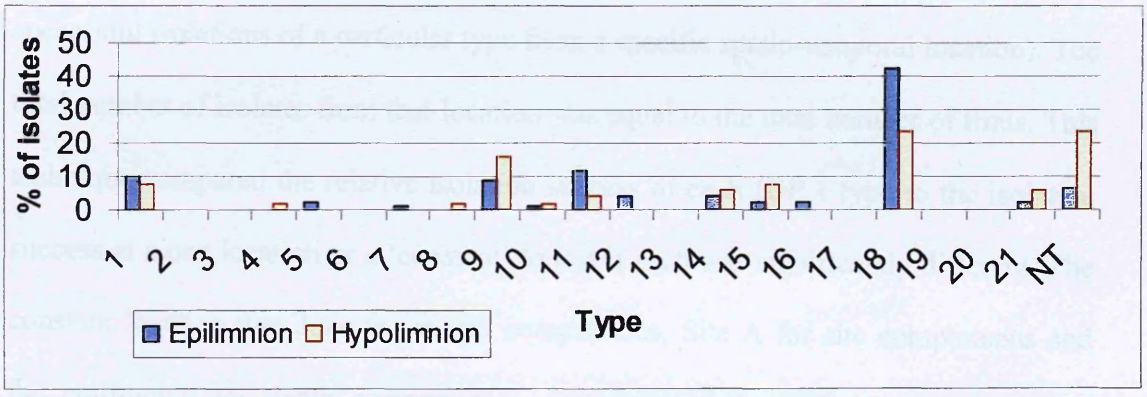


Figure 4.13 Contribution of types to picocyanobacteria isolated in September 2000 (NT = Not assigned to a type).

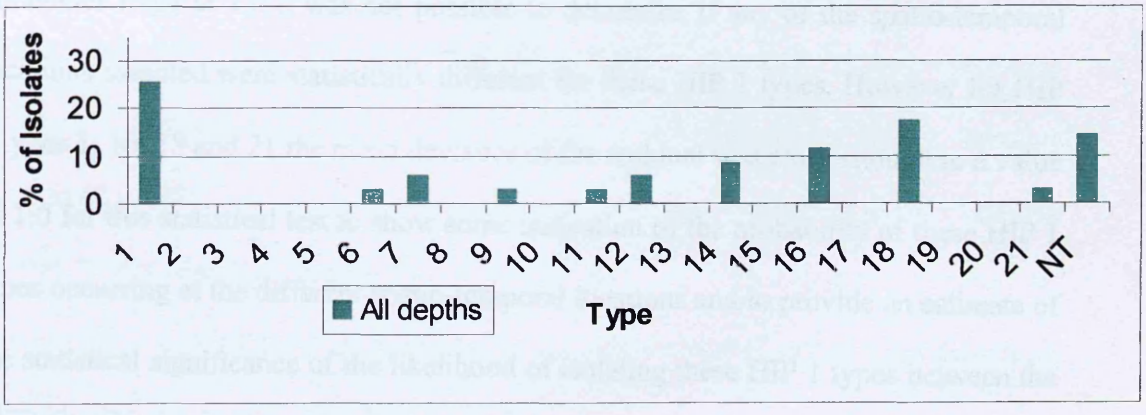


Figure 4.14 Contribution of types to picocyanobacteria isolated in October 2000 (NT = Not assigned to a type).

4.4.4.3 Statistical analysis of spatio-temporal variation of isolation success of HIP 1 types

To assess whether the HIP 1 types isolated could be demonstrated to have been isolated at different success rates from the different spatio-temporal locations logistic regression was applied. This is an extension of standard multiple regression (McCullagh *et al.* 1983). This technique determines the significance of the response which is a number of successes out of a total number of trials. The number of isolates from a single spatio-temporal location was equal to the number of successes (or successful isolations of a particular type from a specific spatio-temporal location). The total number of isolates from that location was equal to the total number of trials. This technique compared the relative isolation success of each HIP 1 type to the isolation success at a one location or a 'constant' to test if each was significantly different. The constant location was June for month comparisons, Site A for site comparisons and the epilimnion for depth comparisons. For most of the HIP 1 types, the mean deviance of the residual was outside of the acceptable range and thus with a dispersion parameter fixed at 1.0 it was not possible to determine if any of the spatio-temporal locations sampled were statistically different for these HIP 1 types. However for HIP 1 types 1, 16, 18 and 21 the mean deviance of the residual was close enough to a value of 1.0 for this statistical test to show some indication of the probability of these HIP 1 types occurring at the different spatio-temporal locations and to provide an estimate of the statistical significance of the likelihood of isolating these HIP 1 types between the locations. Table 4.5 summarises the results from these four HIP 1 types.

Table 4.5 Significance of isolation success of specific HIP 1 types using standard multiple regression

HIP 1 Type	Mean deviance of Residual	Site(s) significantly different from constant*
Type 1	1.092	None
Type 16	0.9602	July (T=-2.47) September (T=-2.76)
Type 18	1.194	Site C (T=3.86) Hypolimnion (T=3.09)
Type 21	1.046	July (T=-2.95) September (T=-2.8)

* Constant = June for month comparisons, Site A for site comparisons, and the epilimnion for depth comparisons. T value > 2 = significant.

Thus although there was sufficient data for Type 1 it was not isolated at significantly greater frequencies from any spatio-temporal locations throughout the sampling period. Isolates of HIP 1 Type 16, 18 and 21, however, were isolated in significantly higher/lower proportions of the total numbers of isolates from some spatio-temporal locations. This demonstrates that isolates assigned to these three HIP 1 types may exhibit different ecological characteristics from each other and the remaining isolates as a whole.

Secondly the effect of abiotic conditions (detectable nutrient concentrations, temperature, light, dissolved oxygen and pH) and the abundance of potential predators (groups of protozoa and rotifers) in the lake at the time of isolation on the isolation success of the HIP 1 types was tested. However the mean deviance of the residual was outside of the acceptable range for all isolates and thus with a dispersion parameter fixed at 1.0 it was not possible to determine the significance of any of these factors on isolation success on any of the HIP 1 types.

4.5 Discussion

4.5.1 Can HIP 1 PCR be used to type culturable picocyanobacteria from Esthwaite Water?

The application of HIP 1 PCR to picocyanobacteria from Esthwaite Water resulted in the generation of HIP 1 PCR products from 501 out of 506 isolates. This is a low failure rate for a typing technique. Dombek *et al.* (2000) used primers based on the ERIC and BOX sequences for PCR to analyse 208 isolates of *E. coli.* Fingerprints suitable for analysis were only achievable from 125 isolates. This is a 38% failure rate, compared to <2% failure to produce results in this study. The failure to produce HIP 1 PCR products from five isolates probably resulted from a failed PCR due to contamination of tubes, insufficient or damaged constituents or damaged or contaminated template DNA or a problem with the thermal cycling. This high success rate however is positive attribute of HIP 1 PCR as a typing technique for application to field studies.

The number of PCR products generated ranged from one to nineteen, and these varied in molecular weight between approximately 350 and 2000 bp. In order for the technique to be useful to discriminate between picocyanobacteria the results must have the parameters to represent a specific type and be different from those of other types. A low number of PCR products generated would make discrimination and identification of isolates to types easier by eye. It would also make HIP 1 an appropriate genotyping technique if the diversity amongst picocyanobacteria is low. However if the picocyanobacterial community is composed of genetically and ecologically diverse organisms, typing techniques require sufficiently complicated

markers to enable differentiation. As HIP 1 PCR products are generated from dispersed locations from around the genome it is possible that PCR products of the same molecular weight, and thus separated to the same distance by gel electrophoresis could have been amplified from different regions of the genome. Thus amplification of more than one PCR product per isolate is useful. Less than 8% of the isolates which generated HIP 1 PCR products produced 4 or fewer PCR products, so generally isolates generated sufficient products to compare. At the other end of the scale, typing techniques based on repetitive sequences can generated too many products to be useful for typing micro-organisms. The isolates analysed for the work of Dombek *et al.,* in 2000 generated generally between 25 to 30 PCR products, but up to 40 bands were obtained from some isolates. Twenty five percent of these bands were common to most isolates. This compares to a maximum of 20 PCR products detected from isolates from Esthwaite Water. This demonstrates that large differences exist between the discriminatory power or typeability of different techniques depending on the diversity of the target to be differentiated.

The overall similarity of the HIP 1 PCR products generated by the isolates was very low (less than 5%). This is because a few isolates produced very different HIP 1 PCR products from the rest. These isolates were not re-isolated on more than one or two occasions so they could have represented picocyanobacteria which were rare within the community, or picocyanobacteria that were not suited to the culturing conditions used for isolation, or the PCR products resulted from erroneous PCR or amplification from contaminants. Further work to investigate which of these theories is true could include repeating the PCR to ensure the PCR products are representative from that isolate (re-isolation of DNA from the culture would be advisable first). To determine the contribution of these genetic types to the community one could now develop a

probe for the isolate and utilise a technique which does not require the organisms to be in culture (e.g. DGGE or FISH type techniques).

The remaining isolates generated PCR products that were greater than 43% similar. Using the similarity threshold of 70% between PCR products compared with the Pearson correlation coefficient, 70% of isolates were clustered into groups of greater than five isolates. After reassignment to HIP 1 types, 82% were assigned to a type. This reassignment process was necessary because the 70% cut-off threshold was an arbitrary threshold proposed through matching replicates of PCR products from five isolates separated on a small number of different electrophoresis gels. The products themselves however were amplified within the same reaction. The variation between PCR products which were generated over a number of months and were separated over a larger number of gels is likely to be higher than within the test when the threshold cut-off was generated.

Examples of how groups were reassigned to HIP 1 types (split, amalgamated, added to with isolates not initially assigned to groups etc) are given in the results section. This was a subjective process comparing the similarities of PCR products generated by isolates within the groups with others within the group, other groups and other isolates not assigned to a group. In this case it was performed by one user on review of the entire set of HIP 1 PCR products. The process was difficult to perform and ensure that types could be justified on the same criteria when PCR products and heterogeneity of PCR products differed so much within the groups. Two thirds of the final types assigned could still be defined by the 70% cut-off threshold. Although the user did not affect the match (similarity values) therefore, as would have been the case if a band based coefficient was used, the assignment of types was open to judgement and two different users would most likely have assigned different clusters of isolates to types.

This process resulted in the assignment of 21 HIP 1 types, comprising 82% of isolates. Isolates within these types had generated PCR products with varying homogeneity. Limitations of this process for the analysis of HIP 1 PCR are that only those isolates which were positioned in close proximity on the dendrogram were assigned to the same group. The UPGMA algorithm simplifies the results of similarity coefficients by clustering isolates by an averaging method. The two most similar gel profiles are matched together, followed by any other gel profiles or groups of gel profiles which are similar to those two. This means that isolates may be placed in groups, then those groups placed with other groups which contain some isolates which are less similar to isolates in the first group than they are to isolates in other groups in the match in other clusters. This grouping is done one dimensionally so that the clustering can be presented on paper. However there may be several isolates or groups of isolates which have the same similarity of HIP 1 PCR products to one isolate. Alternative approaches based on multidimensional comparisons could be performed, but would require a simple method of assigning types to be developed to make it useful for application to HIP 1 PCR for typing cyanobacteria.

4.5.1.1 Genetic significance of HIP 1 types

The HIP 1 types defined within the culturable picocyanobacteria isolated from Esthwaite Water in 2000 could be grouped based on the similarity of the PCR products generated by within the type. Ten HIP 1 types generated PCR products which formed distinct, complex banding patterns. These types were composed of between six and sixty-seven isolates. The largest group in particular (HIP 1 type 18) demonstrates that HIP 1 PCR can be used to generated reproducible typing results over a long period. Variability that was detected between isolates within these types is associated with one or two bands which vary between PCR products from these

isolates. Repeating the PCR would determine if these bands really indicate diversity between the frequency and distribution of HIP 1 sequences within the genomes of these organisms or if they are artefacts. DNA purification should be performed again to ensure this is the case and the cultures could be examined for contaminant algae. Heterotrophic bacteria within the cultures could also be screened for the presence of HIP 1 sites to ensure these are not the cause of erroneous PCR products.

These HIP 1 types represent picocyanobacteria with relatively similar frequency and distribution of HIP 1 sites selected for by the primer HIP CA. In order to investigate the diversity of this group further the diversity of HIP 1 sites could be assessed with the use of other HIP 1 extended primers. This may result in the necessity to split the currently assigned types into smaller numbers and further HIP 1 types. The genetic similarity of other locations of the genome could also be assessed to determine how discriminatory HIP 1 PCR is and how homogenous these isolates are.

The other HIP 1 types can be described as either comprised of isolates with moderate or low similarity between PCR products. Those with moderate similarity show similarity in the size and intensity of some PCR products generated by the isolates. This includes six HIP 1 types which between them include 12% of the isolates from which PCR products were generated. It is likely that further isolation of picocyanobacteria would mean these types could be redefined in the future. However, it would be interesting to examine the homology between PCR products generated by the isolates to determine if they have some PCR products in common or if they have coincidently generated products of a particular molecular weight from different areas of the genome. HIP 1 types with low similarity of HIP 1 PCR products generated just one to three PCR products of similar size and intensity to other isolates within that type. Twenty-seven percent of isolates which generated PCR products were assigned to one of five HIP 1 types that can be described in this way. It is very likely that these

isolates can be reassigned to further HIP 1 types in the future. Thus although 21 HIP 1 types have been assigned at current, it is thought that there is probably some diversity within the HIP 1 types which appear homogenous, and the other types represent isolates which will probably be reassigned to a larger number of HIP 1 types with just the HIP CA primer in the future. Within just the phycocyanin-rich rod-shaped picocyanobacteria in Esthwaite Water this work has therefore indicated there is substantial diversity within the frequency and distribution of the HIP 1 sequence in the genomic DNA of these organisms.

The isolates that are matched within each comparison influences the clusters produced. For example matching a selection of the 501 isolates may mean that isolates would be assigned to different types. Thus it is important to verify the assignment of groups and to be aware that the delineation of groups or types is likely to be an evolving process. It is evident that the types assigned in this study that produced homogenous PCR banding profiles (see Table 4.6) represent isolates with very similar frequency and distribution of HIP 1 CA sequences. However, even isolates within these types produced some differentiating bands, which could be used to increase the resolution of typing of these organisms. After further confirmation of similar identity and reproducibility of HIP 1 PCR products from these homogenous types it may be possible to recognised specific isolates as 'typical of these types and match new isolates to them. In the more diverse types it is likely that with further isolations, further splitting of these types would be appropriate. Thus definition of isolates into types requires user intervention, which is subjective and could lead to different assignment of types in different studies. The genetic changes between isolates which lead to the amplification of different HIP 1 PCR products, and the issue of artificially assigning types within a genetic continuum are discussed in Chapter 5.

Table 4.6 Summary of HIP 1 characteristics of types and suggestions for future developments

HIP 1 homogeneity of isolates within types	Types	Percentage of isolates	PCR banding pattern			Further analysis suggested	Future changes likely
			Nature of pattern	Description of heterogeneity within type	Similarity to other types		
High	3, 8, 9, 11, 13, 14,17, 18 and 19, 20	43% (6-67)	Distinct complex banding pattern generated by isolates of each type.	Some heterogeneity within minor PCR products.	Varied from low to moderate.	Reproducibility of variability between isolates could be verified by repeated HIP CA PCR. Other primers could be used for further resolution.	Variability of minor PCR products could be used for further resolution. Sub-types may be assigned.
Moderate	2, 4, 5, 6, 10, 12	12% (5-21)	Similarity of size and intensity of some PCR products.	Moderate to high variability of minor PCR products.	Low similarity with other types.	Southern blotting and hybridisation could be used to test homology of PCR products generated by those with fewer/different bands to the rest of isolates within the type.	May be split into new types or sub-types assigned.
Low	1, 7, 15, 16, 21	27% (5-39)	Isolates generated 1 to 3 PCR products of similar size and intensity.	High variability of minor PCR products.	Low to moderate similarity with other types.	Southern blotting and hybridisation or sequencing could be used to test homology of key PCR product.	May be split into new types or sub-types assigned.
Very Low	No Type	18% (90)	Isolates generated PCR naming patterns that were unique or similar to <5 other isolates.	High variability of PCR products, although, products from some pairs and triplets of isolates were similar.	Low.	Southern blotting and hybridisation or sequencing could be used to test homology of PCR products within isolates not assigned to a type and types assigned.	New types could be assigned with further isolations. More isolates may also be assigned to no type.

Other studies have used HIP 1 typing to discriminate between isolates of cyanobacteria (Neilan *et al.* 1995; Smith *et al.* 1998; Zheng *et al.* 2002), and Chapter 3 describes its application to a number of cultures from the CCAP collection. However, this is the first time it has been applied to picocyanobacteria isolated from the field. The results demonstrate that the culturable PC rich rods can be discriminated into types based on HIP 1 PCR products. Although 18 % of the culturable population were isolated on <5 occasions. The studies using HIP 1 PCR to date mainly demonstrated that the cyanobacteria analysed could be typed as the same, similar or different. Smith *et al.* (1998) demonstrated that isolates of *Nostoc elipsosporum* and *Anabaena flos aquae* could be typed using HIP 1 PCR. The isolates of *N. elipsosporum* were found to be more heterogeneous than those of *A. flos aquae.* Results shown in Chapter 3 of this work also demonstrated that HIP 1 PCR can be used to differentiate between isolates of other cyanobacterial genera and species from the CCAP (Fig 3.2). Some isolates, however, do demonstrate very similar banding patterns (e.g. *A. flos aquae* and *Oscillatoria (*now *Planktothrix) agardhii*). Zheng *et al.* (2002) demonstrated that 24 different HIP TG fingerprints could be generated from 93 cyanobacterial samples derived from cycads. Orcutt *et al* (2002) used a combination of two HIP 1 PCR primers to differentiate between isolates of *Trichodesmium.* They recognised that some isolates were 'similar but distinguishable from each other' whilst other isolates displayed patterns that were distinctly different from each other. Two strains produced identical banding patterns. Neilan *et al.* (1995) also used a mixture of two HIP 1 primers to resolve diversity between isolates of *Cylindrospermopsis raciborskii.* They used a binary matrix based on the presence or absence of bands to separate the strains into two clusters. Further diversity was

observed within these clusters, but could not be statistically proven using the binary matrix technique.

Guillane-Gentil et al (2002) applied PCR using the REP sequence to 38 isolates of *Bacillus sporothermococlurans* from UHT and sterilized milk sources. They found that the PCR products could be separated into two clusters, one which could be discerned visually and by a similarity level of 80%. The second were less than 80% similar to each other with the exception of two isolates. This separation however did delineate the isolates from the two sources.

The BioNumerics software enabled the analysis of 501 isolates which would not have been possible manually. The standardisation of the gels with molecular markers enabled PCR products which were amplified and separated on different gels, and over an extended time period (3 months) to be compared. The use of the similarity coefficient and dendrogram enabled similar isolates to be displayed in close proximity and different isolates to be separated by automated procedure. The 70% cut-off threshold, and grouping restriction of >5 isolates enabled initial groups to be assigned using a predetermined set of criteria

HIP 1 was first discovered at the junctions of a gene *smtB* repressor deletion event (Gupta *et al.* 1993) however the purpose and means of propagation of the sequence are still unclear. Robinson *et al.* (2000) discussed whether HIP 1 plays a functional role in cyanobacteria (e.g. in termination, stability and regulation of transcription, the promotion of genetic rearrangements, or chromosomal integration of DNA and the organisation of the bacterial genome) or whether it is parasitic. They suggested that there is more evidence to indicate that it is a functional sequence than parasitic.

The HIP 1 PCR technique compares the diversity of the frequency and distribution of HIP 1 over the entire genome. It is therefore sensitive to DNA variations which effect the number of HIP 1 sites and the genetic material between them. Thus cyanobacteria which generate different HIP 1 PCR products may have considerably different DNA composition. The next section and Chapter 5 review the spatio-temporal origins of the isolates to see if the results may indicate a different functionality between the HIP 1 types.

4.5.2 Environmental significance of types

In general many of the types were isolated from a number of different spatio-temporal sampling locations (Tables 4.4 to 4.2). This applied to both the types isolated in large numbers and those isolated in smaller numbers. The re-isolation of types over the sampling period demonstrates that there was some stability within the HIP 1 sequence frequency and distribution within members of the picocyanobacterial community. As although 21 HIP 1 types were isolated, (and it is thought they actually represent a greater diversity than indicated by this number), several fairly homogenous types were re-isolated over a period of five months. Some trends could be discerned from the relative isolation success of each type from the different spatio-temporal locations. There appeared to be a temporal succession of successful isolation of types; with some being isolated early, some middle, some middle to end, some throughout and some sporadically through the sampling period (listed in Chapter 4.3.8). There appeared to be a spatial difference in the success of isolation from the epilimnion and hypolimnion. Most types showed a higher isolation success from the epilimnion than hypolimnion, however this may be due to the range of sample points taken in each,

particularly as the thermocline deepened through the sampling period increasing the number of sampling points in the epilimnion.

On different sampling occasions the isolation success of the different types and of number of types varied considerably between sites. For instance Type 6 was only isolated from Site A in June, but these isolates constituted 60% of all of the isolates assigned to Type 6 over the entire period. In July a much broader range of types were isolated from Site A than Sites B or C, and the same can be said of Site C in October. The isolation success may be taken to represent the times at which the types were most abundant in the lake or when they were most viable. Thus it appears from these results that the genetic types were more successful in some spatio-temporal locations. Most types appeared to demonstrate a peak or a number of peaks in isolation success, however the range of site, depth and month variables meant that although 501 isolates were analysed, only a small number of isolations of each type were successful at any one spatio-temporal location. This meant the peaks are not very pronounced, and that it was not possible to demonstrate a statistical significant relationship between isolation success and spatio-temporal location in most cases, and a relationship between isolation success and abiotic and biotic variables was not possible in for any of the HIP 1 types. Four types however had been isolated frequently enough to enable statistical analysis of isolation success verses success of isolating the picocyanobacteria as a whole. Three of these isolates were isolated with greater success on from particular spatial or temporal locations. Type 18 for instance was isolated more frequently from Site C than the other sites sampled and the hypolimnion rather than the epilimnion in comparison to the other isolations of picocyanobacteria. This suggests that this type may have contributed a significant abundance during the bloom of picocyanobacteria at Site C. Type 16 and 21 were isolated statistically less

often during July and September in comparison to the number of other isolates within those months. This suggests there may be a temporal shift between HIP 1 types. Further isolations and HIP 1 genotyping of picocyanobacteria within Esthwaite Water is required to increase the understanding of how the community diversity alters. Laboratory analysis of growth characteristics and susceptibility to predation may also elucidate more about the ecophysiological diversity of HIP 1 types and the picocyanobacterial community.

4.6 Conclusions

HIP 1 PCR has been used to compare the genetic similarity of 501 picocyanobacteria isolated from Esthwaite Water in 2000. Twenty-one types were discriminated and 90 isolates were not assigned to a type. On further isolation it is expected that this number of types would increase due to the diversity within some of the types assigned within this analysis. The internal diversity of these HIP 1 types varies from very homogenous groups to groups based on similarity of the size and intensity of 1 PCR product. Further work is required to examine the diversity within and between the groups.

The HIP 1 types assigned during this study were re-isolated from two or more sampling occasions (each was separated by four weeks). HIP 1 types appeared to show an isolation pattern of early, mid, late, through-out or sporadic isolations through the sampling period. It was statistically possible to demonstrate that three HIP 1 types were isolated at different suceess rates to the rest of the picocyanobacteria isolated at some spatial or temporal locations. This indicates that at least some types may have different functional roles e.g. growth characteristics (bottom up) or

susceptibility to predators (top down), or that the abundance of HIP 1 types oscilliates within the community for some other reason.

The HIP 1 genotyping technique did yield a high rate of typing results from picocyanobacteria within the field study. The reproducibility of the technique appears good as one type was re-isolated and very homogenous PCR products generated on 69 occasions over a number of months. The technique was able to discriminate between at least 21 HIP 1 types (probably more) and 90 isolates were identified as having generated PCR products, which were too dissimilar to others to be assigned to a HIP 1 type. Thus this technique appears to have quite a high power of discrimination using only one extended primer. The clustering methodology was useful to group such a large number of isolates, but still required user checking and decision-making and thus may be inconsistent between different workers. Once 'types' are recognised however the process should become one of comparing to known 'types' and assigning new ones if appropriate rather than starting with a blank sheet.

Chapter 5 General Discussion

5.1 Context and aims

The overall aim of this work was to investigate the diversity of the picocyanobacterial community. This comprised of studying a larger number of picocyanobacterial isolates than previous studies, from one habitat, so an overview of the dynamism of the diversity could be ascertained.

The HIP 1 PCR typing technique was selected to assist with the analysis of diversity. However, before application of this technique to a field study, it required further optimimzation and development.

5.2 Summary of the findings

Chapter 2: Field study to investigate the spatio-temporal distribution of picocyanobacteria in Esthwaite Water

A field study was performed over the summer of 2000 to set the context for the picocyanobacterial diversity and isolate picocyanobacteria for genotyping.

- The physical and chemical factors measured during the summer of 2000 demonstrated that the field study was based on a summer with typical conditions for Esthwaite Water and eutrophic lakes of its kind. There were small detectable differences between the sites for example in the thermal stratification and the mixed depth in relation to the euphotic zone recorded on the sampling dates.
- The succession of the planktonic organisms generally followed the PEG model (Sommer *et al.* 1986), however the composition and abundance of organisms

varied between the sites. In particular, the peak abundance of autotrophs at Site C was considerably larger at Site C than Sites A and B.

- Picocyanobacterial abundance also varied between sites, with the peak occurring at Sites A and B in July, and a much larger peak recorded at Site C in July and August.
- The morphological and pigment characteristics of the community also varied through the sampling period with all types being present in the community early and late summer. The community present in July and August (during the peak abundance) was dominated by picocyanobacteria characterised by PC-rich pigment type and rod-shaped morphology.
- Five hundred and six picocyanobacteria were isolated during the field study, however the pigment/morphological characteristics of all the cultures was of PC-rich cells of rod-shaped morphology.

Chapter 3: HIP 1 PCR cyanobacterial typing technique: method optimisation and evaluation

The HIP 1 PCR genotyping technique was optimised and developed for application to a field study, by selection of methods for DNA extraction and data analysis and assessment of the discrimination and reproducibility of the technique.

- Several methods of DNA extraction were compared; the Dynabeads DNA Direct System 1 was selected for use in the field study as it produced the most reliable template for PCR at the lowest processing time and cost.

- HIP 1 PCR products were generated from a range of filamentous and colonial cyanobacteria and picocyanobacteria from the CCAP collection. Discrimination was possible between strains assigned to the same genera (including *Synechococcus)* and strains of the same species.
- Although some variability was detected within PCR products when different concentrations of constituents and thermal cycling conditions were used, it was concluded that under standard experimental conditions variability should not be too significant to prevent matching of isolates of the same HIP 1 type.
- HIP 1 PCR with templates generated from heterotrophic bacterial and eukaryotic organisms generated some PCR products, however these were of large molecular weight and low in number. It was thought these were generated from non-homologous primer binding and would not be significant for in terms of reproducibility from cyanobacterial templates.
- A method was devised for assessing the similarity of PCR products amplified by HIP 1 PCR for application to the field study isolates. This method was demonstrated as sufficient to appropriately group the PCR products from four different templates of cyanobacterial DNA separated repeatedly on different electrophoresis gels.

Chapter 4: HIP 1 diversity of picocyanobacteria in Esthwaite Water

The HIP 1 PCR was applied to the picocyanobacteria isolated from the field study described in Chapter 2 using the methods developed and selected as described in Chapter 3.

- HIP 1 PCR products were generated from 501 (99%) of the 506 isolates of picocyanobacteria.

- Eighteen groups were initially delineated using the analysis techniques and criteria suggested in Chapter 3. These groups were reassigned to 21 HIP 1 types after visual assessment of coherency.
- The HIP 1 types were composed of between 5 and 67 isolates. Ten of the types assigned (comprising 43% of isolates) were composed of isolates which had generated PCR products in complex banding patterns which were very homogenous. Five of the types assigned (comprising 12% of the isolates) generated PCR products with moderate homogeneity. A further four types were assigned (to 27% of isolates) had generated a PCR product of similar size and intensity, but were otherwise heterogeneous. The remaining 18% of isolates produced PCR products that were not sufficiently similar to >4 other isolates to assign them to a type.
- Many of the types were re-isolated on several occasions and from different spatial locations within Esthwaite Water over the 5-month sampling period. The isolation of the types varied across the sampling period, with some types isolated more successfully early in the sampling period, some within the middle, some sporadically and some consistently through the sampling period. Different HIP 1 types were isolated from different depth/site locations at the same sampling occasion demonstrating spatial variation within the lake.
- The composition of HIP 1 types within the culturable picocyanobacterial community at the three sites was similar when averaged across the entire sampling period. However on each sampling occasion there was variation between the communities at each site and within the epilimnion and hypolimnion.

- It was possible to demonstrate that that three HIP 1 types were isolated at different success rates to the rest of the picocyanobacteria isolated at some spatial or temporal locations. This indicates that at least some types may have different functional roles e.g. growth characteristics (bottom up) or susceptibility to predators (top down), or that the abundance of HIP 1 types oscilliates within the community for some other reason.

5.3 Discussion

5.3.1 Picocyanobacteria

5.3.1.1 Abundance and Diversity

This study demonstrated that there maybe a general pattern of succession of HIP 1 types throughout the summer in Esthwaite Water which may be similar to the succession described for larger phytoplankton by the PEG model (Sommer *et al.* 1986) and the review of phytoplankton periodicity by Reynolds (1983). This implies that these HIP 1 types may have different ecophysiological characteristics. This study focused on a 'broad' assessment of the physical and chemical parameters and planktonic community structure based on sampling 2 m depth intervals, at three dispersed sites in Esthwaite Water, at monthly time intervals. Due to differences between the sampling locations and the relatively high diversity of picocyanobacteria isolated, the information yielded cannot be accurately used to demonstrate correlations between the abundance of types and factors measured in the field study. However, it is possible to speculate about the ecophysiological differences that may exist between the HIP 1 types based on this data and other studies on picocyanobacteria. The picocyanobacteria within specific HIP 1 types may vary in ability to survive in specific environmental conditions analogous to the Beta functional guilds described by Wilson (1999). For instance HIP 1 types may differ in their ability to exist in environments with different light intensity (as unlike large phytoplankton they are unable to compete with other picocyanobacteria for light). Variation in light intensity within the environment in which picocyanobacteria survive can occur in short timescales (e.g. diurnal cycles and cloud cover, or as a result of

vertical mixing of the water column, medium timescales (e.g. due to changes in absorption of light due to accumulation of phytoplankton biomass in the water column through the summer period) or longer timescales (due to seasonal changes or changes in trophic status which may take several years to occur). Phycocyanin rich picocyanobacteria have been observed to respond rapidly to changes in light intensity through changes in pigment composition (Tandeau de Marsac *et al.* 1993). Contradictory observations of other picocyanobacteria have suggested that isolates of *Synechococcus* may have low (Waterbury *et al.* 1986) or high light optima (Kana *et al.* 1987b). Postius *et al.* (1998) however demonstrated that two genetically different isolates of PE rich picocyanobacteria from lake Constance had differing abilities to grow under high light intensities. One was able to grow quickly, and was characterised by a rapidly reduced the PE/chlorophyll ratio while maintaining a constant zeaxanthin content, both of which may have protected it against high light intensity. The other isolate demonstrated a slow growth rate and underwent a slow reduction in PE/chlorophyll ratio; zeaxanthin content of the cells was significantly reduced. Genetic diversity and ecophysiological differences may therefore explain the contradictory results of Kana *et al.* (1987b) and Waterbury *et al.* (1986). This type of ability to withstand high light intensity may enable early HIP 1 types to survive when frequently circulated through water with high light intensity. It may also explain high PE rich picocyanobacterial contribution to the community at this time, or assist picocyanobacteria to survive if they become thermally separated into very shallow surface water with minimal shading by phytoplankton during the summer. This type of capability probably does not give competitive advantage to picocyanobacteria through most of the midsummer period below the surface layer as shading quickly reduces light intensity.

Alternatively HIP 1 types may vary in ability to compete for resources. Wilson (1999) also described Alpha guilds based on competition for resources. Organisms within the same guild are unlikely to be found together if resources are limiting. HIP 1 types of picocyanobacteria may vary in the form of nitrogen used for growth. Studies have shown that isolates of picocyanobacteria from freshwater and marine environments have the different abilities to use different sources of nitrogen. Some freshwater isolates have been observed to lack the ability to use nitrate (Probyn *et al.* 1985; Miller *et al.* 2001). Rippka *et al.* (1979) also included three freshwater isolates of *Synechococcus* which had the ability to synthesize nitrogenase in their review of properties of cyanobacterial cultures and Gilbert *et al.*(1990) demonstrated that two marine clones of *Synechococcus* had different growth rates on nitrate and ammonium. The nitrogen form available for uptake in lakes is known to shift from nitrate to ammonium between spring and into summer (Blomqvist *et al.* 1994). The available nitrogen form may also vary spatially through a lake, with urea and amino acids also providing a useful source to some cyanobacteria (Berman *et al.* 1999). Marine *Synechococcus* capable of swimming motility and positive chemotaxis towards nitrogenous compounds have been recorded (Willey *et al.* 1989), although not observed within freshwater picocyanobacteria it may exist and characterise particular genetic types.

The HIP 1 types may have different uptake abilities for either nitrogen or phosphate. Wehr (1989) demonstrated, using mesocosms, that picocyanobacteria are rarely limited by phosphate concentration. This feature may be common to the group as a function of their size and surface area to volume ratio (Fogg 1995; Raven 1998) or related to the physiological capacity of the organisms to utilise each nutrient. If the latter is the case, the apparent ability of the picocyanobacteria analysed by Wehr

(1989) to sequester phosphate may have been common to the type of picocyanobacteria to which the selected advantage had been provided by the conditions in the mesocosm, rather than the picocyanobacterial community in general. The uptake capacity of different HIP 1 types for specific nutrients may be one of the factors that influenced the specific distribution patterns observed during this study. The types, which were recorded throughout the season, may be similar to those studied by Wehr (1989) that were able to sequester phosphate even at low concentrations. The abundance of these types was greater when phosphate was detected. It is likely that due to excretion by messy-eating predators like rotifers (Bogden *et al.* 1982) that types which can assimilate phosphate at very low concentrations will never be limited by phosphate concentration during the summer in Esthwaite Water. Figure 5.1 represents how relative phosphate and nitrogen concentrations may influence community structure in favour of superior competitors for nitrogen or phosphate or coexistence.

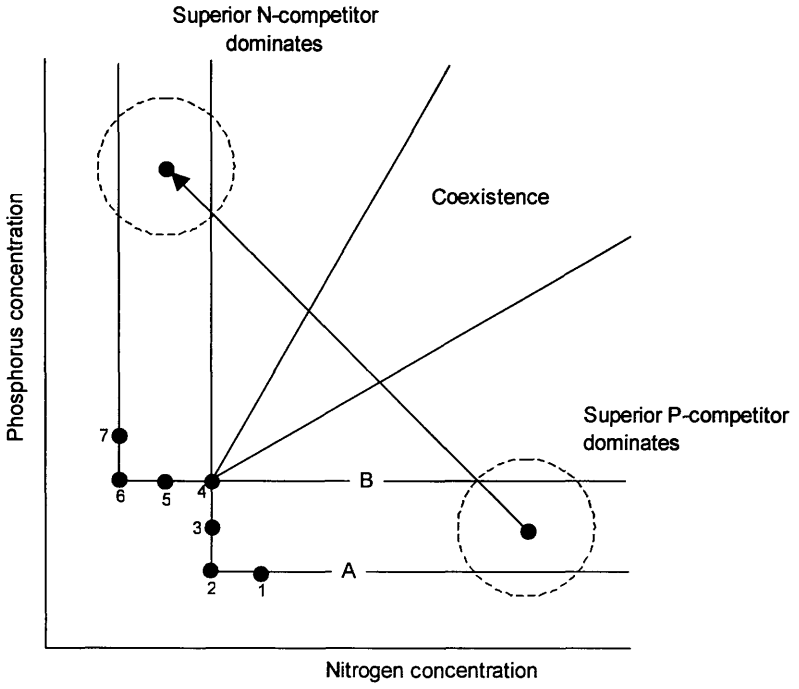


Figure 5.1 The changing nitrogen-to-phosphate supply ratio hypothesis.
N.B Axes represent nutrient concentrations. Supply clouds (dashed circles) are plotted at points representing total resource concentrations in the absence of uptake; supply rates are presumed proportional. When supply rates change from high nitrogen but low phosphate (supply cloud on bottom right) to low nitrogen but high phosphate (top left) the community should shift from dominance by superior phosphate but inferior nitrogen competitor (isocline A) to coexistence between competitors to dominance by superior nitrogen but inferior phosphate competitor (isocline B). The points 1-7 represent different times during the season. During 1-3 the superior phosphate competitor dominates, at 4 the competitors coexist, and during 5-7 the superior nitrogen competitor dominates. Reproduced from (Sterner 1989)

Top down control may also influence the community structure of HIP 1 types. Potential predators were frequently detected in the same locations as picocyanobacteria during the study. Size is one factor which has been shown to influence prey selection in many organisms (Takamura *et al.* 1983; Vanni 1987; Wehr 1991; Kuuppo-Leinikki *et al.* 1994) this factor may not differentiate between HIP 1 types (as they probably overlap in size range), but several studies have shown that freshwater heterotrophs (particularly protozoa) select their prey based on other characteristics such as surface hydrophobicity (Monger *et al.* 1999). The genetic composition of bacterial communities has also been observed to alter with grazing pressure (van Hannen *et al.* 1999). Some types may also be more edible to different

types of predators. Changes in the abundance of heterotrophic flagellates, ciliates and rotifers as well as potential mixotrophs such as dinoflagellates and autotrophic flagellates occurred over the spatio-temporal sampling locations. For instance do the rotifers and/or dinoflagellates reduce the abundance of the early types to below the limit of detection during July and August at Site A, and in the hypolimnion of sites B and C in August? More complex changes in species abundance were not recorded during this study, but may have a significant impact on the grazing pressure on picocyanobacteria and potentially specific types. Ciliates in particular vary considerably in terms of the size and diet of each species. In a similar study to this, Simek *et al.* (2001) monitored changes in epilimnetic bacterial population at 9 stations at 1.8 km equidistance intervals through a reservoir on two sampling occasions. They concluded that three major factors were apparently acting in parallel – complicated hydrology (related to river flow rate, water temperature and chemistry), downstream changes in substrate availability (allo- versus autochthonous carbon) and protistan bactivory to produce a spatial succession of genetic types.

Phage abundance and specificity was not measured in this study, however, it is known that they do contribute to cyanobacterial losses. Moreover, phage contribute to controlling cyanobacterial community diversity through genetic specificity of host range. The majority of the research on cyanophage which infect picocyanobacteria has been performed in the marine environment where infectious cyanophage concentrations can occur between 10^4 to 10^5 ml^{-1} (Suttle *et al.* 1993; Waterbury *et al.* 1993), and it is estimated that viral lysis could be responsible for 30% of cyanobacterial mortality (Proctor *et al.* 1990). Although not monitored routinely, viruses that infect freshwater picocyanobacteria are thought to be as widespread as other freshwater cyanophage (1 to 2 orders of magnitude lower than marine

cyanophage) (Suttle 2000) but at present relatively little is known about their contribution to picocyanobacterial losses or diversity.

In order to determine whether the HIP 1 types can represent different functional guilds as described by Wilson (1999), or whether succession due to changes in allogenic factors (water chemistry, temperature, light etc) or autogenic factors (physiological and life history characteristics, competition, predation, parasitism and other factors under biological control) as described by Tilman *et al.* (1982) within the PC rich picocyanobacteria really occurs requires further investigation. Laboratory experiments should be performed to demonstrate whether isolates within each type and between the types demonstrate different requirements for nutrients or uptake and assimilation abilities. Ingestion and digestion by a range of predators and susceptibility to phage may also allude to the functional role these picocyanobacteria play in Esthwaite Water. If HIP 1 types are found to have different ecophysiological characteristics, this may assist with understanding why so many surveys of picocyanobacterial abundance have produced contradictory results (see review in Chapter 2). It may also explain the apparent ubiquity of *Synechococcus* in every environment (Stockner *et al.* 1986). If HIP 1 types represent groups of picocyanobacteria with specific ecophysiological characteristics rather than genetic types arbitrarily sub-divided, it would demonstrate that beneath the apparent morphological uniformity hides genetic, physiological and functional diversity. The role of picocyanobacteria in aquatic food chains may also be more complicated than explained by simple pathways as different HIP 1 types may be involved in different food chains; and the answer to the frequently asked question; "do picocyanobacteria act as a sink or source in nutrient recycling?" may be "both" depending on the genetic type in question.

Regardless of controlling factors, the abundance of picocyanobacteria probably underestimates the role they play in nutrient turnover in lakes due to quick growth rates and grazing. The significance of individual types may also be underestimated or overestimated by disproportionate grazing on different types. The isolation methods used may also have influenced the relative proportions of types isolated (Rippka *et al.* 1979; Ernst *et al.* 1995). The reliance on isolation of cyanobacteria prior to HIP 1 typing is a limitation of the technique discussed in Chapter 5.3.2.

Further HIP 1 assisted ecological field studies could also advance our understanding of the role of picocyanobacteria in freshwater environments. The aim of this study was to survey the community of picocyanobacteria in Esthwaite Water during the summer. However Ball (1999) also demonstrated that picocyanobacteria are abundant in Esthwaite Water during spring. HIP 1 PCR could be used to compare the diversity of the picocyanobacteria in spring, with that observed in summer during this study. This study broadened the understanding of picocyanobacterial abundance within lakes previously based on sampling of one spatial location, using mixed depth epilimnetic water samples (Hawley *et al.* 1991; Ball 1999). Variation of HIP 1 types between sites on particular sampling occasions was recorded. Thus restricting the study to one site would have resulted in a lower estimate of the diversity within the lake, and the variability in abundance and types within present within the lake at any one time would have been missed. The variation in the community structure spatially within the lake may indicate that different processes are occurring in a heterogeneous patchy environment experienced by the cells within the lake. A field survey of the picocyanobacterial diversity in relation to abiotic factors and the planktonic community structure over densely sampled transects within the lake on a single sampling occasion, or more frequent (e.g. daily) sampling of a single location may

provide more information about community dynamics, relationships with other factors and scales of disturbance to add to the information gained from this study (Jones *et al.* 1980). The four weekly sampling period of this study extends greatly beyond the generation time of picocyanobacteria, therefore the community may have altered several times within the interim to different environmental conditions not detected by this work. Reynolds (1993) suggested that environmental constancy over 12-16 generations may permit a climactic condition to be achieved within planktonic communities. Picocyanobacterial generations have been recorded on sub daily (12-16 hours) (Stockner *et al.* 1986) so climactic populations would be expected to have become dominant after less than 16 days. The relatively high diversity of HIP 1 types recorded however (21 types + 90 isolates not assigned to a type) may be a consequence of the constantly changing conditions experienced by the picocyanobacteria as stable conditions would generally lead to dominance by one type (Tilman *et al.* 1997), whereas changing conditions prevents the establishment of the climactic community (as proposed by the intermediate disturbance hypothesis (Sommer *et al.* 1993)). Similar factors (hydrology, substrate availability and predation) concluded as responsible for bacterial diversity by Simek (2001) in a eutrophic reservoir may be responsible for maintaining this diversity within this lake. Gradients of these factors are probably magnified in Esthwaite Water as it is a relatively small lake with inputs from the catchment varying from each direction, river inflows and a commercial fish farm, plus uni- or bi-directional wind impacts and bathymetric variables. Work by Frost and Weins (Frost *et al.* 1988; Weins 1989) on spatial scaling in freshwater ecology could be used to assist with design of future studies. Expansion of this work to application of HIP 1 PCR to the picocyanobacteria

within a range of lakes with varying trophy would also greatly aid our understanding of the community.

5.3.2 Evaluation of HIP 1 PCR cyanobacterial typing technique

HIP 1 PCR typing has been tested through technique development and application to a field study. Table 5.1 summarises the strengths and limitations of the HIP 1 PCR typing technique found within this work. Suggestions of further work which could advance the understanding of the results of HIP 1 PCR and its potential usefulness as a cyanobacterial typing technique are also made.

Overall HIP 1 PCR has been demonstrated to be a useful tool for elucidating the diversity of picocyanobacteria during a field study within the constraints of a PhD project. The major advantages of the technique compared to others are the discrimination, cost, speed and simplicity of the technique and capacity for analysis of large numbers of isolates. The major limitation of the technique is the requirement for isolation of cyanobacteria. Within this study HIP 1 PCR directly on cyanobacterial cells did not yield reproducible results. This may have been due to the cell wall structure of the isolates or polysaccharide interference with the polymerase chain reaction. Other workers have yielded reproducible results form cyanobacterial cells for HIP 1 PCR and other molecular studies (e.g. Zheng *et al.* 2002). The significance of the diversity of community elucidated by HIP 1 PCR could be analysed by techniques which can be applied to natural mixed community samples without isolation (Ferris *et al.* 1996; Ferris *et al.* 1997; Muyzer 1999).

HIP 1 PCR is a useful technique for application to ecological field studies. In particular, as also suggested by Zheng *et al* (2000), it may provide a vital screening role to assess diversity of communities before the application of more labour and cost

intensive techniques based on both genetic and phenotypic characterisation of cyanobacteria. Further analysis of the picocyanobacteria within Esthwaite Water would provide more information on the significance of HIP 1 types. The limitations of the technique should also be recognised specifically in terms of the restriction to the culturable population and definitive assignment of types.

Table 5.1 Overall evaluation of HIP 1 PCR

Factor	Strengths	Limitations	Suggestions for further developments
Discrimination	High level of discrimination: • between isolates of a species of filamentous and colonial cyanobacteria • between isolates of picocyanobacteria from the CCAP collection • between picocyanobacteria isolated from natural samples Knowledge of the diversity of the community is not required previous to application of the technique The same primer can be used to discriminate between a wide range of cyanobacteria. Different primers used alone or in combination offer flexibility for further resolution. HIP 1 types appear to occupy different ecophysiological niches.	The number of HIP 1 sequences within the genome of cyanobacteria limits the resolution of the technique. This varies between cyanobacteria. The frequency and distribution of HIP 1 sequences does not necessarily characterise specific species or genera (resolution is generally higher). HIP 1 types should be recognised as evolving entities rather than definitive groups. Further isolation and analysis of genetic diversity and ecophysiology will provide advanced understanding of the significance of these groupings and the variability of isolates within and between the groups. HIP 1 diversity within and between types compared with sequence diversity within rDNA or other gene loci is not understood. Ecophysiological characteristics of HIP 1 types requires further study. The requirement for isolated cyanobacteria within this study limited the application of HIP 1 PCR to the culturable community. Thus a significant proportion of cyanobacteria in Esthwaite Water were not characterised.	A comparison of HIP 1 diversity with sequence homology of rDNA of other gene loci. Ecophysiological characterisation of HIP 1 types through laboratory experiments. Probes for DGGE/TGGE could be developed for HIP 1 types, then applied to determine the significance of the proportion of the community analysed by HIP 1 PCR.
Reproducibility	Although PCR is susceptible to variation through different concentration of constituents, thermal	A small number of PCR products were generated from HIP 1 PCR with non-	The impact of adding bacterial template to cyanobacterial HIP 1

Factor	Strengths	Limitations	Suggestions for further developments
	cycling conditions or contamination, this was not thought to be a significant issue for HIP 1 PCR under usual circumstances.	cyanobacterial templates. These were probably the result of non-homologous binding of primers to template DNA. They may not be significant when homologous binding sites are present, but may lead to some variability in results.	PCR in increasing concentration could be tested.
Technique procedure	Simple, based on one PCR step and gel electrophoresis.		
Cost	The cost of HIP 1 PCR was sufficiently low to enable development of the technique and application of over 500 isolates within a PhD project.	1) The cost of the software for data analysis is high. The analysis of the results was initially impeded within the study by lack of access to a software package with the appropriate capability. Studies elsewhere could also be restricted to small numbers of isolates without this access.	
Time	The simplicity of the procedure make this technique extremely fast. Turnover of results can be achieved within a day. The sample processing time was sufficiently low to enable development of the technique and application of over 500 isolates within a PhD project.	Data analysis of PCR products derived from large numbers of isolates can be time consuming.	
Data analysis	The BioNumerics software enabled the development of a standard procedure for initial matching of PCR products from large numbers of isolates. PCR products can be matched if generated over a number of months and separated on different electrophoresis gels.	Visual verification and user interference is required to assign isolates to appropriate groups. Final groups are subjective not definitive at this stage.	Further isolations, ecophysiological and genetic characterisation is required to determine the significance of these groups.

5.4 Conclusions

This study has expanded our knowledge of the diversity of picocyanobacteria by genotyping over 500 isolates from a small lake in the English Lake District isolated from a large number of spatio-temporal sampling locations over one summer.

HIP 1 PCR has demonstrated that genetic diversity exists within picocyanobacteria which are characterised morphologically by rod shaped cells and high PC pigment content. Twenty-one HIP 1 types were assigned after the analysis of 506 isolates of picocyanobacteria from Esthwaite Water in 2000. Many of the types were re-isolated over the sampling period showing there is some stability within the community, however, there was a much greater diversity of HIP 1 types than was discernable morphologically. Some of the 21 HIP 1 types assigned were particularly homogenous with respect to PCR products generated from the isolates. Other types however were more heterogeneous and with further isolation and analysis, these types are likely to be split into more types. There appeared to be a temporal shift of HIP 1 types between June and October in Esthwaite Water. Three HIP 1 types were isolated at statistically different success rates from some spatial or temporal locations. The study indicated that the physico-chemical environment, planktonic community and picocyanobacterial abundance and diversity varied spatially within the lake on each sampling occasion. However the conditions did appear to be typical for Esthwaite Water during the summer stratified period.

The HIP 1 PCR typing technique has proved useful for application to a field study and has yielded some interesting information about the diversity of picocyanobacteria in

Esthwaite Water. It is likely to provide a useful technique amongst the armoury of methods for assessing the genetic and phenotypic diversity of cyanobacteria. The advantages and limitations of HIP 1 PCR mean that for some studies it is likely to provide a useful tool to assess the diversity alone, and in others it may be combined with different techniques to provide most optimum tools to advance ecological research.

5.5 Summary of suggestions for further work

- Laboratory analysis of ecophysiological characteristics of HIP 1 types
- Further field studies using HIP 1 PCR to examine the distribution of HIP 1 types over longer time scales and lakes of different trophic status.
- Further field studies using HIP 1 PCR to PCR to analyse picocyanobacterial community dynamics with more frequent sampling occasions.
- DNA sequencing of HIP 1 types to further understanding of the diversity between isolates within and between different HIP 1 types.
- Development of probes for HIP 1 types for techniques such as DGGE/TGGE to estimate the significance of HIP 1 types and non-culturable picocyanobacteria within lake communities.

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