INTERACTIONS BETWEEN CYANOBACTERIA AND OTHER MICRO-ORGANISMS

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

by

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TEXT CUT OFF IN THE ORIGINAL

TO MY BELOVED PARENTS

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SUMMARY

In this study interactions between cyanobacteria and other micro-organisms, some isolated from a salt marsh, others laboratory cultures, have been considered. Results show that actinomycetes, fungi and bacteria produced 'antimicrobial agents' which were able to bring about lysis of green algal species and cyanobacteria. The interaction results between cyanobacteria and actinomycete isolates were different, depending on the media type used to grow the two groups. The vegetative cells were the most sensitive to attack by the active microbial types but heterocysts and akinetes were apparently unaffected. The resistance of these latter types of cells was not due to the presence of cellulose within the walls or to the thickness of the mucilaginous sheath.

The active compounds were present in cell-free filtrates prepared from the cultures showing that the antimicrobial agents were extracellular products. Some of these compounds whilst inhibitory to certain cyanobacteria stimulated growth, photosynthesis and nitrogenase activity of other species.

The active filtrates were extractable in various organic solvents except that from A52 (Streptomyces roseodiostaticus) which was only extractable in chloroform. The active extracts could be chromatographed on thin-layer silica gel plates. The active compound was usually present in a single spot on the chromatogram and the activity could be visualised using bioautographic techniques. These active spots could not be eluted

from the thin-layer plates since they bound tightly to the silica particles.

The activity of the different filtrates varied in their stability to heat and cold pretreatments, ie. some were autoclavable and others cold labile.

Evidence has been provided to show that it is not necessarily only antibiotics and exoenzymes released by micro-organisms which inhibit the growth of cyanobacteria and green algae, but other chemically quite different compounds. The compound released by the actinomycete A52 showed inhibitory activity against most of the cyanobacteria and green algae tested but did not affect Gram negative or Gram positive bacteria or yeasts.

The use of these active compounds to control of cyanobacteria blooms is discussed.

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CHAPTER ONE

GENERAL INTRODUCTION

Soils and water basins are inhabited by highly complex microbial populations which include bacteria, actinomycetes, fungi, cyanobacteria, algae and protozoa. Whilst many of these organisms can be transferred and grown under laboratory conditions as pure or as mixed cultures, other species that are found in nature will not easily grow in laboratory culture (Meers, 1973). This suggests that some growth determining factors present in the natural environment are difficult to simulate in the laboratory. One such factor might be the close relationship that exists between one microbe and another in the natural habitat.

Various types of association and antagonism between different microbial populations have been defined. Recent summaries of population interaction studies classify an interaction on the basis of its effect, beneficial or detrimental, on the populations. Beneficial generally means stimulatory to growth, whereas detrimental generally means inhibitory to growth (Brock, 1966; Bungay, 1968; Alexander, 1971). Nevertheless the delineations between interactions are many and confusing. Perhaps the most widely used scheme for classifying interspecific interactions is that proposed by Odum (1959). This scheme was developed as a model for interactions between two species, whereas, in natural habitats, especially in microbial habitats, several species in close juxtaposition probably interact both simultaneously and sequentially. Populations often interact in more than one way, even in laboratory situations, for instance, two populations may compete

for a common substrate and one of them may produce a factor that stimulates the growth of the other. The schemes used to define different types of microbial interactions (Odum, 1959; Meers, 1973) are as follows:-

1) Competition: a condition in which there is a suppression of one organism as the two species struggle for a limited quantity of nutrients; O_2, space or other requirements.

2) Commensalism: in which only one species derives benefit while the other is unaffected.

3) Mutualism: is similar to commensalism but in this case the growth of each species is promoted by the presence of the other, such as:

(a) Symbiosis: the two species relying upon each other and both benefiting from the relationship.

(b) Protoco-operation: an association of mutual benefit to the two species but without the co-operation being obligatory for their survival.

(c) Synergism: is the third type of mutualism in which the formation of specific products is greater in mixed than in pure populations.

4) Amensalism: in which one species is suppressed, often as a result of toxin production, by the second which remains unaffected.

5) Parasitism: occurs when one organism feeds or reproduces at the expense of the tissues or body fluids of another.

6) Predation: occurs when one organism totally engulfs and digests another organism which thereby loses the ability to reproduce itself.

Therefore, it seems probable that most organisms at times influence, or are influenced by, the behaviour of the others. Examples of microbial interactions have been known for some time, for example, the close association between cyanobacteria* and fungi in lichens and the infection of some blue-greens by Chytrids and other fungi (Canter, 1953). In the latter case, the majority of cyanobacterial hosts mentioned are species of Anabaena and in some cases infection is restricted to a particular type of cell. For example, Rhizosiphon akinetum infecting the akinetes of Anabaena affinis (Canter, 1954) whilst the heterocysts of Aphanizomenon flos-aquae are the site of infection with Chytridium cornutum (Canter, 1963).

The composition of the microflora of any habitat is governed by the biological equilibrium created by the association and interactions of all individuals found in the population. Environmental changes temporarily upset the equilibrium, but it is re-established, possibly in a modified form, as the population shifts to become acclimatised to the new circumstances.

Many micro-organisms are in close proximity to one another, and may interact in a unique way such that their behaviour is in marked contrast to the behaviour of similar pure cultures grown in the laboratory (Alexander, 1961). Therefore, it is generally accepted that soil micro-organisms live in a very complicated and competitive ecosystem and it is possible that many interactions occur between the different species which in turn affect their growth and survival (Park, 1960).

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*NB. The term 'blue-greens' and 'cyanobacteria' are used synonymously throughout this thesis.

The term "antagonism" and "antibiosis" have been used to designate the effect of one microbe upon another, resulting in reduction of growth and activities. The effects may be expressed by exhibiting temporary or permanent modifications in physiological characteristics and morphology, and in a reduction in virulence. When two organisms are grown on the same substrate, one may overwhelm and even bring about the death of the other. These antagonistic activities embrace phenomena other than mere competition for nutrients (Waksman, 1967) and may be attributed to the release of toxic compounds by the microbes into the medium. The toxic products likely to cause amensalism can be divided into two groups:

i) Inorganic substances which are usually of low potency,

ii) Organic compounds which are often toxic at a low concentration. For example, bacteria commonly excrete organic acids during growth (Meers, 1973) and this leads to a lowering of the soil pH which either inhibits or promotes the growth of other species.

The study of the interactions between soil micro-organisms in general and between cyanobacteria and other micro-organisms in particular, have been given much attention (Safferman and Morris, 1962; 1963 ; Stewart and Daft, 1971; 1976; 1977). The release of extracellular products by microbes, e.g. actinomycetes, fungi and bacteria is generally considered to be the most common cause of lysis in cyanobacteria (Granhall and Berg, 1972; Redhead and Wright, 1978; 1980). Previous workers have reported that antibiotics produced by these microbes proved the most active substances against cyanobacteria and

green algae (Foter, Palmer and Maloney, 1953; Hunter and McVeigh, 1961; Kumar, 1964; 1968; Vance, 1966; Srivastava et al., 1969; Tarar and Kelkar, 1978).

Microbes antagonistic to one another can be simultaneously isolated from almost any soil sample taken. However, it is very difficult and unusual to be aware of their antagonistic properties unless the isolates have been carefully tested under laboratory conditions. Such laboratory experiments must ensure that:

i) All the test micro-organisms have been isolated from the same soil or habitat, and are not erroneous contaminants appearing due to faulty isolation procedures.

ii) The microbial isolates to be tested are axenic.

In their natural habitat micro-organisms will be competing for nutrients, space, oxygen and other essentials. The interaction between micro-organisms in the laboratory may not be the same as that which might occur in nature as it is difficult to simulate natural conditions in the laboratory (Park, 1960; Gray and Williams, 1971).

Several studies have been carried out on the characteristics, distribution and possible contribution of cyanobacteria to the productivity of salt marshes in the United States (Webber, 1967; Zaneveld, 1966; 1972; Ralph, 1977); Canada (Bird and McLachlan, 1977); the Netherlands (Den hartog, 1967; 1968; 1971; 1973; Nienhuis, 1970; 1973; De Jonge, 1976); the Mediterranean coast of France (Van Den Hock, 1960) and in the United Kingdom (Carter, 1932; 1933a; 1933b; Chapman, 1939; 1940; 1960a; 1960b; Stewart and Pugh, 1963; Chapman and Chapman, 1973).

Detailed information on the cyanobacteria of the Dee Estuary salt marsh can be found in various reports on the Liverpool Bay area, in general, and the Dee river in particular (Recommendation of the Department of the Environment Working Party on Sludge Disposal in Liverpool Bay, 1972; Burrows and Russell, 1975). More recently Mahdi (1980) studied the distribution and nitrogen fixation by cyanobacteria in the Parkgate area of the Dee salt marsh and found that some 72 species could be isolated from this habitat.

Cyanobacterial blooms have been studied in detail during the last decade in relation to the physical and chemical parameters such as light, temperature and nutrients, which may affect their growth and primary production (see Stewart and Daft, 1977). Since the presence of cyanobacterial blooms cause problems in drinking water supplies (Ridley, 1970); amenity waters (Lund, 1972); in water used for industrial processes (Edward, 1972) in fishing waters where they cause de-oxygenation and swimming pools (Fitzgerald, 1959) research to find ways of controlling their growth has been encouraged.

Actinomycetes are a large heterogeneous group of micro-organisms, composing several families and numerous species (Waksman, 1967). These micro-organisms were known to early microbiologists (Harz, 1878; Golbig, 1888), but they were largely overlooked because of their slow growth on agar plates. This led to the tacit assumption that these micro-organisms were of little importance in soil transformation. However, these organisms received more attention in subsequent years, in particular by Krainsky, (1914); Waksman, (1916); Jensen, (1930) and Krassilnikov, (1938). More recently, due to interest in the chemotherapeutic

use of the antibiotics produced by the actinomycetes, this group has been brought under close scrutiny.

Actinomycetes are numerous and widely distributed, not only in soil, but also in a variety of other habitats including composts, rivers, muds and lake bottoms (Waksman, 1967). They are present in surface soil and also in lower horizons to considerable depths. They are second only to the bacteria in abundance and the viable count of the two is, frequently, almost equal (Alexander, 1978). In alkaline areas (especially when dry) the relative abundance of actinomycetes is high and they account for 95% of the organisms in such soil samples investigated by Johnstone (1947). Although numerous genera inhabit the soil environment only a few actinomycete genera dominate agar plates inoculated with dilution series of soil suspensions. Almost invariably Streptomyces are numerically dominant accounting for up to 70% to 90% of the actinomycete colonies on most agar media (Alexander, 1978).

The genus Streptomyces was coined by Waksman and Henrici (1943) to separate certain aerobic saprophytic aerial mycelium-producing actinomycetes from the rest of the genera of the order Actinomycetales. Streptomyces play an important role in soil. They take part in the decomposition of organic matter, in transformation processes and in the formation of humic substances. Many exhibit an antibiotic activity to a small extent even in their natural habitat. Most of the Streptomyces are acid-sensitive and like a neutral or alkaline environment (pH above 6.8). In alkaline soils, up to 60% to 70% of the total microflora

consists of Streptomyces Szabo et al. 1959). Numerous papers have been published on the number of actinomycetes, particularly Streptomyces, in soil. Most of these studies are related to the antibiotic activity of the soil microflora and the soil habitat respectively e.g. Grassland (Flaig and Kutzen, 1960), volcanic soils and even roads (Graveri et al. 1960).

Various actinomycetes have been reported to cause lysis of cyanobacteria. Safferman and Morris (1963) found 24.6% of the 403 actinomycetes that they isolated from soil, effective against cyanobacteria and 7.2% effective against both green algae and cyanobacteria. Subsequently, Rubenchik et al. (1965) found that out of 838 cultures they tested, 28 strains of Streptomyces inhibited the growth of the bloom-forming cyanobacteria Anabaena hassallii. Bershova et al. (1968) also reported various actinomycetes that were effective against cyanobacteria. These workers suggested that the actinomycetes produced antibiotics which were responsible for inhibiting cyanobacterial growth and studies with pure antibiotics have confirmed the susceptibility of cyanobacteria to such compounds (Foter et al. 1953; Edward et al. 1961; Kumar, 1964; Srivastava, 1970). For example, low concentrations of mytomycin c and neomycin sulphate inhibited the growth of the blue-green Anacystis nidulans also mytomycin inhibited division in a significant proportion of the cells of Anacystis nidulans, causing filament formation in what is a unicellular species (Kumar, 1964). Rodriguez-Lopez et al. (1970) reported the inhibitory effect of rifamycin on the growth of blue-greens and this was attributed to the direct effect of the antibiotic on the R.N.A. synthesis. Streptomycin was also found to inhibit the growth of cyanobacteria (Kumar, 1964).

Incidentally, it also bleached the cells of eukaryotic Euglena species (Embringer, 1966). Palmer and Maloney (1955) showed that cycloheximide inhibits growth of Chlorophyceae, Xanthophyceae and Bacillariophyceae, but not Cyanophyceae. However, it is active against many fungi, especially yeast, but is inactive against bacteria. Daft et al. (1973); Gunnison (1975); and Redhead and Wright (1978) have found that some actinomycete strains cause lysis of eukaryotic algae as well as cyanobacteria. However in general the cyanobacteria were found to be more sensitive to antibiotics than were the green algae and diatoms (Hunter and McVeigh, 1961). The inhibitory action of actinomycetes on both cyanobacteria and green algae can be attributed to these active substances being released into the medium since cell-free filtrates of actinomycetes are also active (Safferman and Morris, 1962; 1963; Redhead and Wright, 1978).

Bacteria have previously been reported to be able to produce anticyanobacterial agents described as antibiotics or antibiotic-like materials. These materials were found capable of destroying and lysing a range of cyanobacteria species (Granhall and Berg, 1972; Reim et al. 1974; Burnham et al. 1976; 1979), eg. Plectonema boryanum was lysed by filterable substances from a Bacillus culture.

Lytic bacteria occur abundantly in many habitats and are widely distributed throughout the world. This was first reported by Söhngen (1927) and recently by several other workers including Shilo (1970); Stewart and Brown (1972) and Gromov et al. (1972).

Cellvibrio species were isolated from the River Fyris in Sweden by Granhall and Berg (1972) and shown to be toxic towards laboratory

species of cyanobacteria. Gromov et al., (1972) noticed that Flexibacter flexilis an orange-pigmented bacterium with a low G + C ratio (ie. 35.9%) lysed species of Anabaena phormidium and Nostoc and claimed contact with the host was necessary for lysis to occur.

Whilst fruiting myxobacteria have also been shown able to lyse cyanobacteria (Wu, Hamdy and Howe, 1968; Stewart and Brown, 1970), the non-fruiting myxobacteria with high G + C ratios (ie 65% to 70%) have received the most attention (Stewart and Brown, 1969; 1971; Shilo, 1971; Daft and Stewart, 1971; Daft et al., 1975).

Some field data on the possible lysing of blue-greens by bacteria has been obtained. Studies on Lake Mendota by Fallon and Brock (1979) showed that there were periods of rapid decline in biomass of other micro-organisms in the lake during a bloom of blue-greens. The dominant cyanobacterial bloom then declined rapidly as was shown by a 50% reduction in the chlorophyll a concentration over a two to three weeks period. This destruction of the bloom was attributed to blue-green lysing micro-organisms and photo-oxidation.

Bacteria capable of lysing cyanobacteria have been shown to be very common in a number of British lakes and that the number of these bacteria increased during the period of cyanobacterial growth (Daft et al., 1975).

Two hundred isolates of bacterial cultures were isolated from the water of the Kiev reservoir by Bershova et al.,(1968) and they found that 18% of these inhibited growth of Microcystis aeruginosa, Anabaena hassallii, and Microcystis pulverea isolated from the same environment.

In addition to the antagonistic bacteria these workers also found bacteria which stimulated cyanobacterial growth and this stimulatory effect was produced not only by the cells, but by their cell-free filtrates.

The bacterial mode of action has not been studied in any detail, but the process of lysing has generally been considered to be due to the extracellular products produced. These extracellular substances have been suggested to be polypeptide antibiotics but further investigation is needed into their chemistry (Reim and Cannon, 1974; Granhall and Berg, 1972).

Thus heterotrophic bacteria and cyanobacteria probably co-exist in most ecosystems, although if the equilibrium changes markedly, as a result of change in environmental conditions, situations may arise where bacteria may play an important role in the lysis of cyanobacterial blooms.

Fungi that lyse cyanobacteria have been isolated and studied, although little is known about the effects of fungal pathogens on cyanobacteria (Saffermann and Morris, 1962; 1963; Padan et al., 1967; Stewart and Brown, 1969; Rubenchik et al., 1965; Shilo, 1970; Daft et al., 1970; Daft and Stewart, 1970; 1976; 1977). Fungal parasites of cyanobacteria have been isolated and described from lakes in England and Switzerland, Europe and the United States (Canter and Lund, 1951; Paterson, 1960; Sparrow, 1968; Canter, 1972). In all these investigations it was found that fungi causing lysis of cyanobacteria were all members of the Chytridiales with the exception of Blastocladiella anabaena which is a member of the Blastocladiales.

Detailed information on the life cycles and physiological characteristics of fungal pathogens are difficult to obtain because of the problems of getting these organisms into cultures (Canter and Willoughby, 1964). The ecological distribution of these fungal pathogens, in general, parallels the abundance of the cyanobacteria on which they occur and lyse (Canter, 1972).

Fungi have been shown capable of lysing cyanobacterial cells (Safferman and Morris, 1962; Redhead and Wright, 1978). For example, Acremonium sp. was shown to lyse Anabaena flos-aquae and several other filamentous and unicellular cyanobacteria (Parker and Bold, 1961). Lysis is mostly attributed to the fungal extracellular products and Redhead and Wright (1980) have suggested that cephalosporin antibiotics may be important. These workers extracted and partially purified β-lactam and cephalosporin c from liquid cultures of Emericellopsis salmosynnemata and Acremonium kiliense and the ability of these antibiotics to lyse cyanobacteria was confirmed.

The antimicrobial activities of a number of algae and cyanobacteria on other microbial types has also been investigated. Pratt (1942) extracted substances from Chlorella vulgaris which inhibited the growth of bacteria. This finding was confirmed by Jorgensen (1956, 1962); Allen and Dawson (1960); Sieburth (1960); Gorham (1964); Levina (1964); Mutusiak, Chrost and Krzywicka (1971). The antibacterial substance produced by this alga was found active against Gram positive and Gram negative bacteria, and could be obtained from the cell-free filtrates.

Cyanobacteria were also found able to produce antibacterial activity eg. Anabaena flos-aquae and Trichodesmium erythraeum (EHR.) against a range of Gram positive and Gram negative bacteria (Ramamurthy and Krishnamurthy, 1967; Chrost, 1974).

Anabaena and Microcystis aeruginosa inhibited the growth of both Chlorella and Anabaena (Lam and Silvester, 1979). Inhibition was probably due to the production of inhibitory extracellular products by the Microcystis aeruginosa. On the other hand, the inhibitory effect of Anabaena on Chlorella was probably due to the nutrient competition with Anabaena competing more effectively for the available phosphate in the medium.

Cyanobacteria were also found able to inhibit the growth of diatoms isolated from the same environment (Keating, 1978) and the toxin produced by Anabaena flos-aquae was reported to be toxic to crustaceans and fish (Gorham et al., 1964).

Cyanobacteria were also reported to stimulate the growth of certain bacterial strains by providing not only carbohydrates and other energy materials, but also an unidentified biologically active substance (Drewes, 1928).

Introduction to the Present Work

The inhibitory (and occasionally stimulatory) effects of various microbes on cyanobacteria have been considered in several previous studies but few studies have investigated interactions using the different types of organisms isolated from the same habitat at the same time which is the approach to be attempted in this study.

The choice of the Dee salt marsh was based on the knowledge that salt marshes have a rich microflora and that considerable information on the cyanobacterial populations of this particular area was available (see Mahdi, 1980 and Howsley, 1980).

It was hoped that it might be possible to assess whether any interactions (found in laboratory studies) between the cyanobacteria and the other micro-organisms were important in influencing the ecology and distribution of the former. As well as the microbial ecological aspects it is intended that the work should consider the mode of action of substances found to be active on the growth and physiology of cyanobacteria, and to extract and characterise such substances as far as possible. This will be done with a view to the possible commercial production of the substances and their potential use in controlling natural blooms of cyanobacterial and possibly eukaryotic algae as well.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Description of the sites

The River Dee estuary is an embayment between North Wales and the Wirral peninsula opening into the Liverpool Bay, North-west England, (National Grid Reference SJ 278779), Fig. 1.

The Parkgate area of the marsh, on the Cheshire side of the Dee Estuary, was the site of the present study.

2.2 Sampling

Twenty mud samples (approximately 30-50g) were collected randomly from the surface layer of sediment (ie the top 4-6cm) from gullies (Plate 1), pools (Plate 2), bare soil areas and under vegetation (Plate 3) within an approximately 700m^2 area at Parkgate (Fig. 2). The samples were transported back to the laboratory in sterile plastic bags and analysed immediately on return. These mud samples included a range of soil types from light coloured aerobic sandy muds to anaerobic black muds and sediments with a high organic content. The types of location from which each mud sample was taken are given in Table 1.

2.3 Isolation of micro-organisms

Preparation of mud samples for isolation

Each mud sample was thoroughly mixed in its plastic bag with a sterilized glass rod and then a 1g sample was removed and shaken for 3 min. (by hand) in 10cm^3 of sterile quarter strength Ringers solution (Oxoid) contained in a Universal bottle.

Plate 1

A typical gulley in the Dee salt marsh which was one of the mud sampling sites in this study.

Plate 2

A view of a pool with aerobic and anaerobic mud which was selected for mud sampling in this study.

Plate 1

Plate 2

Plate 3

A sampling site showing a zone of bare aerobic mud and also a region of vegetation from rich mud samples were taken. The area (A) shows a surface mat of cyanobacteria.

Plate 3

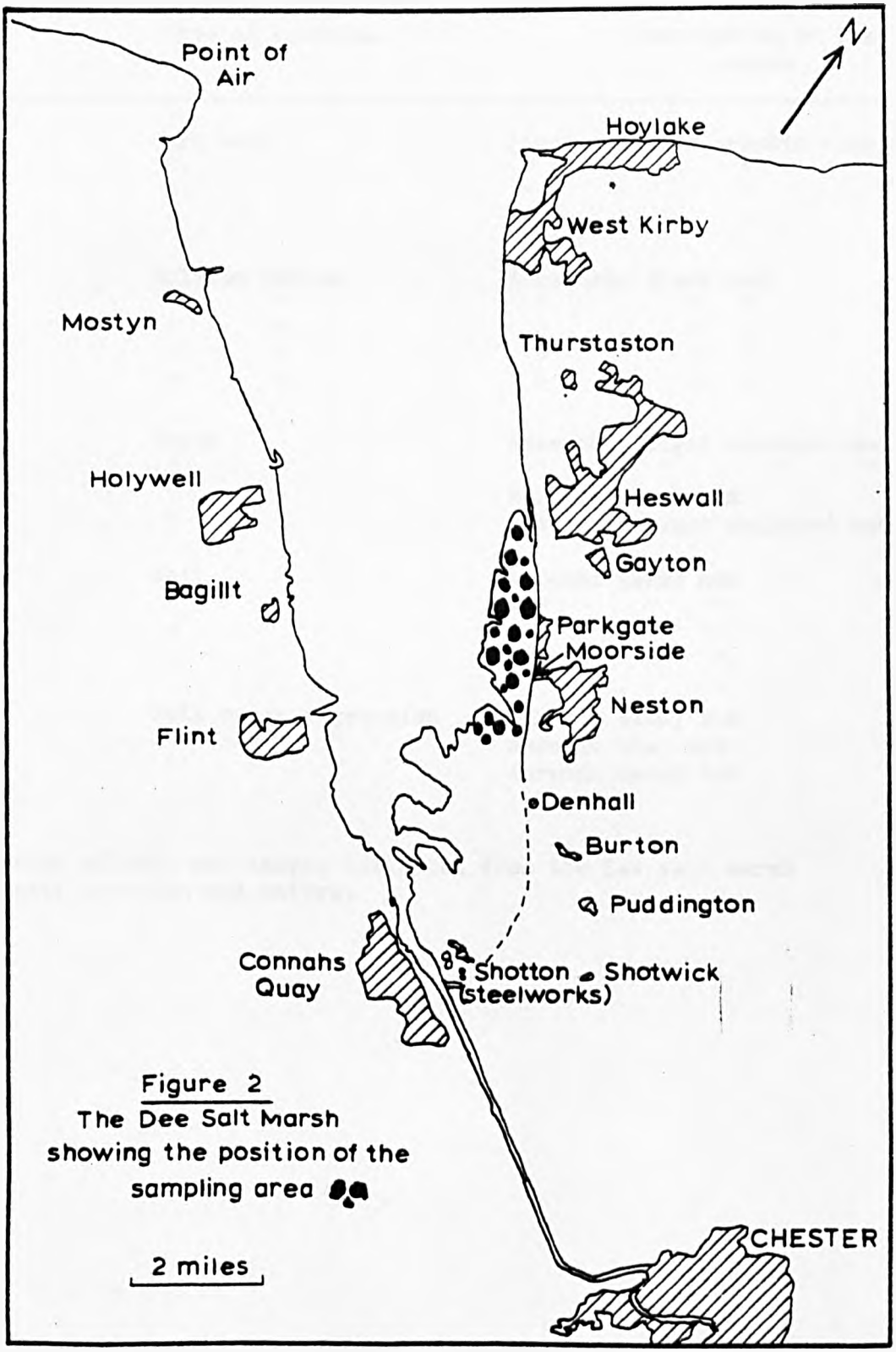


Figure 2
The Dee Salt Marsh showing the position of the sampling area

Table 1. Location and types of mud samples

Number of mud sample	Type of location	Description of the sample
1	Bare soil	Light coloured aerobic clay mud
2	" "	" " " " "
3	" "	" " " " "
4	" "	" " " " "
5	Gullies bottom	Anaerobic black mud
6	" "	" " "
7	" "	" " "
8	" "	" " "
9	" "	" " "
10	Pools	Anaerobic light coloured mud
11	"	" " " "
12	"	Aerobic black mud
13	"	Anaerobic light coloured mud
14	Soil	Aerobic sandy mud
15	"	" " "
16	"	" " "
17	"	" " "
18	Soil under vegetation	Aerobic sandy mud
19	" " "	Aerobic clay mud
20	" " "	Aerobic sandy mud

The description of each mud sample collected from the Dee salt marsh including their location and nature.

A 10-fold serial dilution series was prepared from the initial suspension by pipetting a $1cm^3$ sample into $9cm^3$ aliquots of quarter strength sterile Ringer's solution in a Universal bottle. Each dilution bottle was shaken by hand for 2 min. before a sample was removed to make the next dilution. The dilution series range was from 10^{-1} to 10^{-5} with the initial prepared mud suspension representing the 10^{-1} dilution.

Isolation and choice of media

$0.1cm^3$ portions from each of the dilution bottles were spread with a sterile glass spreader across the surface of agar plates of the appropriate medium depending on which group of microbes was to be isolated (see table 2). The plates were predried at 26°C for 24h before inoculation. Triplicate plates were prepared for each dilution in the series for each major group of micro-organisms to be isolated and incubated aerobically at 26 $\pm$ 1°C for 5 days (bacteria and fungi) and 2 weeks (cyanobacteria and actinomycetes) in a growth room under continuous illumination of approximately 65 $\mu E\ m^{-2}s^{-1}$ provided by cool white tubes. These conditions were the standard ones used in all these experiments unless otherwise stated.

Table 2. Media used for microbial isolations

Micro-organism	Medium	pH
Cyanobacteria	Allen's (1968) with and without combined nitrogen	7.8
Bacteria	Nutrient broth (oxoid)	7.4
Fungi	Potato-dextrose agar (Difco)	5.6
Actinomycetes	Casein starch	7.0

1 to 2% (w/v) Oxoid No. 3 agar (Oxoid) was used to solidify the media.

The cyanobacteria were also isolated into liquid media by placing 0.1cm^3 of the 10^{-1} dilution into 50cm^3 portions of Allens medium (with or without $NaNO_3$, 1.5 g l^{-1}) contained in 100cm^3 Erlenmeyer flasks.

Nystatin (0.02g l^{-1}) was added to the cyanobacterial medium and 0.05g l^{-1} actidione to actinomycete medium to suppress the growth of competitive micro-organisms during the isolation and purification.

2.4 Purification

Colonies of fungi, bacteria, actinomycetes and cyanobacteria appearing on the surface of the agar plates were removed using either a sterile loop or needle and streaked on to new plates of the appropriate medium. Each colony isolated and re-streaked in this way was given a reference number to facilitate comparison with the colonies obtained from either different dilution series or different mud samples. In all these purification procedures numerous transfers were necessary before axenic cultures were obtained.

Purification of the cyanobacteria proved the most difficult. The transfer of either single cells or filaments into new medium to obtain axenic cultures was aided by either growing the parent cultures in unidirectional light so that mobile filaments spread out on the plate or by adding an extra agar layer on top of the growing plate surface so that individual filaments would grow through.

2.5 Identification

Cyanobacteria :

Cyanobacterial isolates were identified to the generic level by using the method of Rippka et al. (1979), (see Appendix 2). Identification of these isolates down to the species level was performed by using the classical phycological texts of Geitler (1932); Desikachary (1959) and Bourelly (1970).

Actinomycetes :

The actinomycete isolates were identified by S. T. Williams and his group in this department. This identification was based on the isolates physiological characteristics on a numerical basis. Identification was effected using a computer probability matrix based upon X characters and MATIDEN identification programmes (see Appendix 3; Williams et al.; 1983).

Fungi :

Fungi were identified to the generic level according to their morphological characteristics (colony shape and colour etc.) visible with the light microscope. Both malt extract and potato-dextrose agar media were used in the identification stage to help in the determination of key morphological characteristics such as conidia and conidiophores (Barnett and Hunter, 1972).

Bacteria :

Some of the bacterial isolates were sent to the Torry Research Station (P.O.Box No.31, 135 Abbey Road, Aberdeen) for species identification (see Appendix 4). The choice of isolates sent for identification was based on the effectiveness of their antagonistic action towards cyanobacteria species. No systematic identification of all the bacterial isolates was attempted.

2.6 Additional culture media and growth conditions

In certain experiments additional media were used to those described for the isolation and purification of the micro-organisms. These are as follows:

i) ASM medium (Gorham et al. 1964) with and without combined nitrogen for cyanobacteria.

ii) CHU No. 10 medium (Chu, 1942) for laboratory isolates of green algae.

iii) Glycerol-asparagine agar 5 (ISP 5) and a modified recipe in which the asparagine was replaced with casitone Co. 2% (w/v) (Difco) for actinomycete growth.

iv) Malt extract agar (Oxoid) 3% (w/v) for growing fungal isolates.

v) A modified Allen's medium was sometimes used for bacterial cultivations, in which the sodium nitrate was replaced with the same molar concentration of casitone (Difco)

In all studies using liquid media $50cm^3$ aliquots were placed in $100cm^3$ Erlenmeyer flask stoppered with non-absorbent cottonwool plugs. These were continuously shaken on orbital shakers unless otherwise stated. When these additional media were to be used in a solidified form 1 to 2% (w/v) agar No. 3 (Oxoid) was added prior to autoclaving ($121^{\circ}C/15lb/inch^2$) for 15 min.

2.7 Description of microbial lawn preparation

The appropriate agar medium for each microbial group was autoclaved and then allowed to cool

to approximately 45°C with continuous swirling to keep the agar mixed. Aliquots of $5cm^3$ of homogeneous microbial suspension were added to the appropriate medium, and mixed rapidly by shaking for a few seconds before pouring into sterilised plates. These plates were kept at normal laboratory temperature (20°C) for approximately 2h. in order to allow the agar to solidify and then incubated under the appropriate conditions.

2.8 Biomass estimation for growth measurements

The cyanobacterial cell protein was determined spectrophotometrically by the method of Lowry et al. (1951), using bovine serum albumin in distilled water to obtain a standard calibration curve. Absorption was measured at 750nm in 10mm path length cuvettes using a Unicam SP600 spectrophotometer.

Cell counts of cyanobacteria were also made using an improved neubaver haemocytometer, with a ruled area of $1.0mm^2$ and a depth of 0.1mm. All counts were done in triplicate and the samples shaken or syringed when necessary to obtain a homogeneous suspension of cells or filaments prior to counting.

Chlorophyll was determined by the methanol extraction technique described by Golterman, Clymo and Ohnstad (1978). Eighty five per cent methanol used in the extraction was stored over 1% (w/v) solid magnesium carbonate.

A film of magnesium carbonate was deposited on a 2.5cm diameter GFC filter (Whatman) placed on a Swinnex millipore filter unit by applying $1cm^3$ of 0.1% $MgCO_3$ aqueous suspension and applying suction. A homogeneous suspension of cyanobacteria ($5cm^3$) was then filtered down on to the GFC pad with minimum suction. The filter plus cyanobacteria were then extracted in $10cm^3$ of methanol (contained in a graduated centrifuge tube) by heating in a water bath to near boiling in dim light for 2 min. The extract was then centrifuged, made up to the original volume of $10cm^3$ and the absorbance of the extract measured at 663nm and 750nm in 1cm path length glass cuvettes using a Unicam SP 600 spectrophotometer. Chlorophyll was calculated according to the following equation:

$$y = \frac{2.43 \times 10^6 \times \mu' \times Ve}{Kc\ Vs}$$

y = concentration of chlorophyll (mgl^{-1})

μ' = absorbance of extract corrected for absorbance at 750nm and for 1cm path length

Ve = Volume of extract (cm^3)

Kc = absorption coefficient of chlorophyll : 91

Vs = Volume of sample (cm^3)

2.9 Acetylene reduction technique

Nitrogenase activity was measured by the acetylene reduction technique (Dilworth, 1966; Scholhorn and Burris, 1966) as modified by Stewart *et al.* (1971).

Aliquots ($1cm^3$) of a homogeneous cyanobacterial suspension (obtained by gently pumping the culture in and out of a sterile $10cm^3$ syringe) were placed in either $7cm^3$ Bijou bottles or $30cm^3$ McCartney bottles fitted with rubber serum stoppers. Acetylene was injected into the gas phase of the bottles to give a final concentration of 10% (v/v).

Samples were incubated under standard growth conditions (described previously) for either 30 or 60 min. and then $1cm^3$ gas samples were removed and analysed for acetylene and ethylene by gas liquid chromatography. The gas chromatograph (Perkin Elmer F11) was fitted with either a 2.0m, Porapak R column (80 to 100 mesh) or a 0.5m long Porapak T (80 to 100 mesh) column and a dual hydrogen flame ionisation detector. The oven temperature was 60°C. The ethylene peaks obtained were quantified by reference to calibration graphs obtained by using pure ethylene gas standards (BDH Laboratory gas service).

2.10 $^{14}CO_2$-fixation measurements

$0.1cm^3$ of $NaH^{14}CO_3$ solution (Amersham) of specific activity 58.2 mCim mol^{-1} was added to 0.1365g carrier $NaH^{12}CO_3$ in $25cm^3$ of distilled water. Aliquots of $0.5cm^3$ from the resulting solution were introduced into each McCartney bottle which contained $0.5cm^3$ of a homogeneous cyanobacterial suspension and the volume made up to $6.5cm^3$ with Allen's medium. The final radioactivity in each sample was 0.4 $\mu Cicm^{-3}$. Triplicate samples of each treatment (depending on the experiment) were incubated in the light (65 $\mu E\ m^{-2}\ S^{-1}$) and three samples in the dark with continuous shaking for 30 or 60 min, at 26°C. The incubation was terminated by filtering the samples

through 2.5cm GFC pads. The filter pads were then washed with 20cm^3 0.1M hydrochloric acid, followed by 20 to 25cm^3 of cold distilled water and dried at 20°C for 8 to 10h. They were then stuck to aluminium planchets with exactly 0.1cm^3 of 0.1M sucrose to ensure even quenching and counted on a Nuclear Chicago automatic gas flow counter (model 1042).

2.11 Interaction tests between different microbial isolates

The effects of actinomycetes, fungi and bacterial isolates on the growth of either cyanobacteria or green algae were determined by three methods.

1. Block Application

Lawns of each micro-organism were prepared on agar plates, as described previously, and cores (50mm diameter) were aseptically cut out and removed using a cork borer. These cores or blocks were placed on freshly prepared or developed cyanobacterial or green algal lawns. Blocks of sterile agar medium were placed on the test lawns as controls. The media used to prepare lawns of the various types of micro-organisms were as follows: ISP5 medium and nutrient broth for actinomycetes; Nutrient broth for bacteria; Potato-dextrose agar for fungi; Solidified ASM and Allen's media for cyanobacteria and solidified CHU No. 10 for the green algae. The plates were inverted and incubated in the growth room for two to three days.

2. Filtrate application to lawns

Aliquots (30cm^3) of cell-free filtrates of the microbes growing in liquid culture were prepared by filtration through Swinnex units containing millipore membranes (pore size 0.45μ) for actinomycetes

and bacteria and GF/C filters for fungi. 0.3cm^3 aliquots of each filtrate were placed in sterilized stainless steel wells on the cyanobacterial or green algal lawns. Wells containing the appropriate sterile liquid media were used as controls. The plates were incubated in the growth room for 3 to 5 days. Loops of the filtrates were streaked on to the appropriate agar plates and incubated to check that they were genuinely cell-free. If growth occurred on these plates the interaction tests for that filtrate were ignored and repeated.

3. Filtrate addition to liquid cultures

Samples (5cm^3) of the cell-free filtrate of the test micro-organisms prepared as before were added to 50cm^3 cyanobacterial liquid cultures contained in 100cm^3 Erlenmeyer flasks and incubated under standard conditions with continuous shaking. Growth and nitrogenase activity and in some cases photosynthetic activity were estimated over 5 days by removing samples daily from the cultures. Some modifications to the growth media were required in these tests and more specific details are discussed in Chapter 4.

2.12 Light microscopy

Semi-permanent preparations of blue-green cells or filaments were made by mounting drops of the cultures in a mixture of glycerine jelly and crystal violet and allowing them to dry for 8 to 12h. with a coverslip in place. Observations were made using a Zeiss No. 61140 light microscope with camera attachment and photographs were taken using Ilford Pan F for black and white and Kodak EKTA chrome 50, Kodak Colour II or Vericolor II films.

2.13 Electron microscopy and sample preparation

Fixation :

Samples of cyanobacteria or green algae in agar were fixed for 3 to 5h in 2% (v/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde (Electron microscopic grade, TAAB Laboratories, Reading, England). Following primary fixation, the material was washed in two changes of 0.1M sodium cacodylate buffer (pH 7.2) according to the method of Karnovsky (1965). The samples were then post fixed for 2 to 5h in 1% (w/v) Osmium tetroxide (Agar Aids, Stansted, England) made up in 0.2M cacodylate buffer and then washed free of fixative in 0.2M Cacodylate buffer for 30 min. The samples were then dehydrated through a series of ethanol solutions (30% to 50% to 70% to 90% to 100% to 100%) for 15 min. each.

Embedding :

Samples were infiltrated with Spurr's resin (Spurr, 1969). A quantity of resin equal to the amount of 100% ethanol retained at the last change of dehydration was added and the solution mixed for 30 min, after which another equal volume of resin was added and the procedure of mixing repeated as summarised below:-

i) 50:50 mixture of Spurr resin/Absolute ethanol for 30 min.

ii) 75:25 mixture of Spurr resin/Absolute ethanol for 30 min.

iii)100% Spurr resin for 30 min.

iv) 100% Spurr resin (fresh change) over night.

The samples were then placed into flat embedding moulds and cured at 70°C for 16h.

Section preparations :

Sections (60 to 90nm) were cut using a glass knife on an OM-U3 Reichert ultra-microtome, and collected on 300 mesh copper grids. Sections were stained for 20 min. in uranyl acetate 0.25g (v/w) and then washed in 50% (v/v) ethanol followed by a further wash in distilled water. Sections were post-stained in lead citrate (Renolds, 1963) for 6 min. and washed in 0.02M sodium hydroxide followed by a further wash in distilled water. The sections were examined using a transmission electron microscope (Corinth 500) at 60 KV, 125 µA. Photomicrographs were taken on 70mm, black and white film (Agfa Gevaert Scientia).

2.14 Extraction and thin layer chromatography of microbial filtrates

Extraction

Samples (1 to 5L) of the test organisms were grown in either $2000cm^3$ or $8000cm^3$ conical flasks in the growth room under the usual standard conditions. After 5 to 7 days, the cultures were filtered through 0.45µ membrane filters or in the GF/C (Whatman) filters in the case of fungal cultures as described previously. The cellular material retained on the filter pads was oven dried at $30^{\circ}C$ and the biomass weight determined.

Aliquots ($800cm^3$ to $4200cm^3$) of filtrate were extracted in chloroform (polarity 4.8); ethyl acetate (polarity 6.02) or dichloromethane (polarity 8.93) as follows:
A mixture (1:1) of the microbial culture and organic solvent were placed in a $1000cm^3$ capacity separating funnel and shaken thoroughly for several minutes to facilitate extraction. After clear

separation of the organic and aqueous phases these layers were individually collected by running them off into clean flasks. The resulting extracts were evaporated to dryness using a Rotary film evaporator (Rotauor R110 No. 880502 Buchi, Switzerland) at a temperature of 40°C, and the weight of the residue determined.

The residue was re-eluted with either sterilized distilled water or 10% (v/v) ethanol and then applied to cyanobacterial lawns in stainless steel wells to assay for lytic or stimulatory activity. Samples ($0.3cm^3$) of the aqueous phase remaining after the extraction in solvent were also applied to the lawns of cyanobacteria for the same reason. The appropriate controls were included, eg.10% ethanol for extract residues redissolved in the solvent.

Thin Layer Chromatography

Portions of solvent extracted filtrates (500 to $1000cm^3$) of the micro-organisms were evaporated to dryness by rotary film, evaporation then redissolved in 5 or $10cm^3$ of the extracting organic solvent. The samples were spotted on to 0.2mm thick silica gel thin-layer chromatographic plates (MERCK). The chromatograms were run in each of the following solvents: cyclohexane (polarity 2.01); trichloro-ethylene (polarity 2.33); Diethyl ether (polarity 4.22); propan-1-ol (polarity 20.23) and methanol (polarity 32.7).

The separated spots were visualised under ultra violet light and the biological activity of these separated spots tested against the cyanobacteria using a bioautographic technique (Wagman and

Weinsten, 1973). This involved placing pieces of the chromatograms silica gel side down on freshly prepared cyanobacterial lawns which were then incubated for up to 5 days under standard conditions.

CHAPTER THREE

ISOLATION, PURIFICATION AND IDENTIFICATION OF MICRO-ORGANISMS FOUND IN SALT MARSH MUD SAMPLES

3.1 Introduction

Since the main purpose of this investigation was to study the possible interactions between cyanobacteria and other microbial groups in the Dee salt marsh, it was first necessary to isolate, purify and at least partially identify, a whole range of microbes from various salt marsh mud samples. The methods used for the isolation, purification and identification of each microbe are described in Chapter 2.

3.1.1 Cyanobacterial taxonomy

Certain taxonomic considerations warrant further elaboration particularly in relation to cyanobacteria since considerable confusion in identification has arisen in recent years.

Cyanobacteria are prokaryotic organisms with a higher-plant type, oxygenic, photosynthesis. Although cyanobacteria are generally regarded as a single taxonomic group, they, nevertheless, show considerable diversity of form and function (Stanier, Kunisawa, Mandel and Cohen Bazire, 1971; Fogg, Stewart, Fay and Walsby, 1973; Lazaroff, 1973). Members of the group range from simple unicells to complex multi-seriate branched trichomes with varying degrees of evolutionary development (Schopf, 1970; Schopf and Blacic, 1971). The great diversity in the characteristics of the different members of this assemblage of organisms has resulted in the present flux of opinion in the taxonomic treatment of the group (e.g. Bourelly,1970; Desikachary, 1973; Drout, 1978; Rippka, Deruelles, Waterbury, Herdman

and Stanier, 1979). The present state of cyanobacterial taxonomy is bewildering and has been subjected to a lot of criticism (Drouet, 1968; 1973; 1978; Bourelly, 1970; Komarek, 1970; 1973; Stanier et al. 1971; Rippka et al. 1979). Because of their higher-plant, oxygenic, photosynthesis, cyanobacteria were placed together with green algae, as members of the Chlorospermae (Harvey, 1846), then were separated as 'Myxophyceae' by Stitzenberger (1860) simply by emphasising the formation of slime, although not all cyanobacterial species are capable of slime production. In 1874, Sachs suggested the name 'cyanophyceae' as a family of the Eukaryota, thus shifting the emphasis on to their blue colour present due to the production of cyanophycin, but this name fails to accommodate the apochlorotic and red-pigmented members. Smith (1938) suggested the name 'cyanophyta' giving the group the status of division, this was elaborated by Pascher (1914) in accordance with the Rules of the Botanical Code (Staflen, Bonner, McVaugh, Mcikle, Rollins, Ross, Schopf, Schulze, Vilmorin and Voss, 1972). On the other hand, as early as 1853, Cohn indicated that these organisms were more related to the bacteria and placed them in the kingdom prokaryota, division Schizophyta. By the time the major differences in cellular organisation between prokaryotes and eukaryotes were clearly elucidated, Stanier and Van Niel (1962), Echlin and Morris (1965) and Lang (1968) rekindled the claim that the name 'cyanophyta' was far from adequate. Stanier and his group are firm proponents of classifying these organisms along the Rules of the Bacteriological Code (Lapage, Sneath, Lessel, Skerman, Sceliger and Clark, 1975) as an order, class or division in the super-kingdom prokaryota (Stanier et al. 1971; Stanier and

Cohen-Bazire, 1977; Stanier, Sistrom, Hansen, Whitton, Castenholz, Pfennig, Gorlenko, Kondratieua, Eimhjellen, Whittenbury, Gherna and Truper, 1978; Rippka et al., 1979).

At present, about one thousand species belonging to about one hundred and fifty genera are recognized by the classical taxonomists (Bornet and Flahault, 1886-1888; Geitler, 1932; Desikachary, 1959) on the basis of morphological characters such as trichome dimensions; presence or absence of a sheath; presence, absence or location of heterocysts; and the presence and mode of branching. The taxonomy of the members of this group of organisms, on the basis of structural properties determined on the field material, could be highly variable when re-examined in growing cultures in the laboratory. This led Drouet and Daily (1956) and Drouet(1962; 1963; 1964; 1968; 1973; 1978) to revise the classification of members of the cyanobacteria denouncing many of the classical species and genera as ecophenes of other species or genera which show a considerable degree of plasticity in the field. Their efforts did not escape criticism on the grounds that they did not extend the knowledge of the group (Stanier et al., 1971) and that they relied too heavily upon herbarium specimens (Padmaja and Desikachary, 1967; Bourelly, 1970). Drouet's revision of cyanobacterial taxonomy was so drastic that too many strains have been lumped together as ecophenes of a single strain (see Stanier et al., 1971; Stam, 1979). Various other approaches have been suggested, such as the use of numerical taxonomy (Whitton, 1969), DNA base composition (Stanier et al., 1971; Herdman, Janvier, Waterbury, Rippka, Stanier and Mandel, 1979), fatty acid composition (Kenyon, Rippka and Stanier, 1972) and genome size (Herdman, Janvier, Rippka and Stanier, 1979).

The scheme presented by Rippka et al., 1979) in which cyanobacteria are divided into five major sections embodying 22 genera, relies on the use of pure cultures as valid type material. In this system, stable physiological characters are used in conjunction with structural patterns of development in the generic differentiation of the strains. As these characters are stable and are readily determinable, this scheme could prove a very useful and reliable one in the taxonomic treatment of cyanobacteria.

3.1.2 Actinomycetes

Pretreatment of mud samples in various ways has been designed to increase the chance of isolating the desired actinomycetes. Examples of treatments are heating up to 55°C for 6 minutes; 40°C for 2 to 16 hours or 100°C for 1 hour, depending on the nature of materials used to isolate actinomycetes (Williams and Wellington, 1982). Heat pretreatment of various materials has been frequently used to decrease the numbers of bacteria on actinomycete isolation plates. Another pretreatment which can be employed is filtration through membrane filter (pore size 0.45µm) whichsroutinely used to concentrate cells from water (Al-Diwany et al., 1978). However, when dealing with sea water containing very low concentrations of actinomycete propagules Okami and Okazaki (1972) found it necessary to centrifuge samples prior to filtration.

Selectivity of an isolation medium can be influenced by its nutrient composition, its pH and the addition of selective inhibitors. Incorporation of antibiotics into isolation medium is a widespread and effective means of increasing selectivity. The recognition of colonies

upon agar media is relatively simple providing the incubation period is sufficiently long.

The taxonomy of actinomycetes is also currently in a state of flux. The term actinomycete has no taxonomic validity since these organisms are classified as bacteria in a strict sense, all being Schizomycetes of the order actinomycetales, but not all genera of the actinomycetales are considered to be actinomycetes in common parlance. Recently Hunter, Eveleigh and Casella (1981) classified a huge number of actinomycete isolates, isolated from the salt marsh of Cheesequake in North East New Jersey. The taxonomic status was based on their morphology, microscopic form, and also whole cell analysis when necessary by using four taxonomic media for their growth. A five character taxonomic code consisting of aerial spore colour groups, spore chain morphology, reverse pigmentation, soluble pigments and melanin production from L-tyrosine, ie. chromogenicity, was used to group the streptomycete isolates. Substrate utilization and salt tolerance were also used as an aid in classifying isolates. More recently Alderson (1982) described the major actinomycetes cluster groups using numerical Phenetic and chemical criteria.

3.1.3 Fungi

Investigations on the fungal flora of salt marshes have been carried out by several workers (Bayliss Elliot, 1930; Saito, 1952 and Pugh, 1962). The soil dilution plate methods of Waksman (1927) and Garrett (1951) subsequently modified by Warcup (1960) have been the main isolation methods used by previous workers. By repeating the method of subculturing it is possible to obtain axenic cultures.

Fungal isolates are placed in one of three taxonomic classes, Fungi Imperfecti, Phycomycetes or Ascomycetes. The most frequently encountered group developing upon agar media are strains belonging to Fungi Imperfecti, moulds which produce spores only asexually, e.g. Aspergillus, Penicillium, Trichoderma and Fusarium. In species of this class, the mycelium is septate. and the conidial type of asexual spore is borne on specialised structures known as conidiophores. Differing from the imperfect fungi, Phycomycetes and Ascomycetes produce spores by both sexual and asexual means. The filaments of the former are usually non-septate and unicellular, and the sexual spores are borne characteristically in sporangia, e.g.Mucor, Zygorhyncus. The filaments of the Ascomycetes, on the other hand, are septate and these fungi form a definite number of ascospores in a sac-like body or ascus. Therefore, these characters of the fungal flora as well as colony shape and colour were used to identify the genera and species.

3.1.4. Bacteria

Previous workers have described isolation methods for different bacterial groups, but the plating method as described by Kelner (1948) and Warcup (1955) was considered the most reliable method.

Grouping of bacteria according to their biochemical properties was chosen as the most convenient method of identification (Gordon, 1968; Gibbs and Shapton, 1968).

3.2 Materials and methods

Twenty mud samples from different small areas of the Dee salt marsh were selected and a portion of one gram from each homogenized mud sample was diluted with $\frac{1}{4}$ strength Ringer solution (see Chapter 2). Aliquots of $0.1cm^3$ of each dilution series were transferred to the appropriate media prepared for the growth of each microbe, and incubated in the growth room under standard growth conditions.

Actinomycetes and cyanobacteria were found difficult to isolate directly from the original growing plates unless a certain amount of proper antibiotic was added to their culture media (see Chapter 2).

Single colonies of each individual micro-organism were picked off and transferred to fresh plates and the subculturing procedure repeated until pure cultures were obtained. These pure micro-organisms were identified where possible.

3.3 Results

3.3.1 Cyanobacteria

A systematic list of 20 cyanobacterial strains that have been isolated from the salt marsh and which have been kept as a part of the Liverpool University, Department of Botany Culture Collection (LBCC), are shown in Table 3. The characters used to differentiate between the various strains are given in Appendix 2. Seven species from the 27 isolated strains of cyanobacteria were found to be

Table 3. Cyanobacterial strains isolated from the Dee salt marsh environment and subsequently purified

Section[1]	Genus[2]	Strain number[3]	Classical identification[4]
I	Synechocystis	LBCC 19	Synechocystis diplococcus (Pringsh.) Bourelly
	Synechococcus	LBCC 54	Synechococcus elongatus Nag.
III	LPP group B	LBCC 11	Symploca thermalis (Kutz) Gom.
	LPP group B	LBCC 68	Symploca elegans Kutz
	LPP group B	LBCC 74	Symploca thermalis (Kutz) Gom.
	LPP group B	LBCC 41	Lyngbya halophila Hansg.
	Oscillatoria	LBCC 58	Oscillatoria lacustris (Kleb.)
IV	Anabaena	LBCC 14	Anabaena microspora Klb.
	Anabaena	LBCC 17	Anabaena minutissima Lemm.
	Anabaena	LBCC 27	Anabaena oscillaroides Bory.
	Anabaena	LBCC 28	Anabaena flos-aquae (Lyngb.) Breb.
	Nostoc	LBCC 8	Nostoc Linckia (Roth.) Born et Flah
	Nostoc	LBCC 29	Nostoc verrucosum Vauch.
	Nostoc	LBCC 34	Nostoc muscorum Ag.
	Nostoc	LBCC 37	Nostoc sphaeroides Kutz.
	Nostoc	LBCC 45	Nostoc piscinale Kutz
	Nostoc	LBCC 48	Nostoc punctiforme (Kutz) Hariot
	Nostoc	LBCC 52	Nostoc entophytum Born et Flah
	Cylindospermum	LBCC 62	Cylindospermum majus Kutz
	Nodularia	LBCC 40	Nodularia harveyana var Sphaerocarpa (Born et Flah.) Elenk

1 and 2 after Rippka et al. (1979), 3 L.B.C.C. Liverpool University Culture Collection

4 after Desikachary (1959) and Gietler (1932).

difficult to obtain in pure culture (table 4), these were Oscillatoria subuliformis, Oscillatoria anguina, Phormidium subincrustratum, Lyngbya majuscula, Anabaena doliolum, Anabaena gelatinicola and Nostoc passerinianum. They belonged to section III and IV of the Rippka et al., (1979) classification.

Of the 20 purified strains, 13 species were heterocystous and belonged to the genera Anabaena, Nostoc, Nodularia and Cylindospermum. Of the five filamentous, non-heterocystous strains one belonged to the genus Oscillatoria and the other four to LPP group B. The two unicellular isolates were identified as belonging to the genera Synechocystis and Synechococcus. All these isolates were given code numbers and stored in an illuminated cold room at 8°C on agar slopes or in liquid cultures.

3.3.2 Actinomycetes

Eighteen actinomycete strains were isolated from mud samples (table 5), of these, sixteen were identified as belonging to the species cluster Streptomyces albidoflavus (courtesy of Dr. S. T. Williams). The two remaining isolates have not as yet been satisfactorily identified. The identification of isolates into a species-cluster is explained in appendix 3. Whilst, from a numerical taxonomic point of view, it is clear that these isolates are clearly related, nevertheless there are distinct differences in their characteristics and thus they are obviously different strains. The actinomycete isolates were individually coded according to the mud sample number from which they originated.

Table 4. Strains of cyanobacteria isolated from Dee salt marsh mud samples but which could not be obtained in axenic culture

Section	Genus	Classical Identification
III	Oscillatoria	Oscillatoria anguina Bory
	Oscillatoria	Oscillatoria subuliformis Kuez
	Lpp group B	Lyngbya majuscula (Dilw.) Harvey
	Lpp group B	Phormidium subincrustratum Fritsch and Rich
IV	Anabaena	Anabaena doliolum Kleb.
IV	Anabaena	Anabaena gelatinicola lemm.
	Nostoc	Nostoc passerinianum Kutz.

Table 5. Actinomycete strains isolated from the various Dee salt marsh mud samples

Mud sample numbers	Actinomycete isolates LBCC Code No.	Species - Cluster
1	1A	Not identified
	1B	*Streptomyces albidoflavus*
	1C	*Streptomyces albidoflavus*
4	4	Contaminated
5	5A	*Streptomyces albidoflavus*
	5B	*Streptomyces albidoflavus*
6	6A	*Streptomyces albidoflavus*
	6B	*Streptomyces albidoflavus*
	6C	Not identified
9	9	Not isolated
10	10A	*Streptomyces albidoflavus*
	10B	*Streptomyces albidoflavus*
11	11A	*Streptomyces albidoflavus*
	11B	*Streptomyces albidoflavus*
	11C	*Streptomyces albidoflavus*
12		Not isolated
13	13A	*Streptomyces albidoflavus*
	13B	*Streptomyces albidoflavus*
15		Not isolated
18	18	*Streptomyces albidoflavus*
19	19	*Streptomyces albidoflavus*
20	20	*Streptomyces albidoflavus*

These identifications were performed by colleagues in Dr. S.T.Williams' actinomycete laboratory, Department of Botany, Liverpool University.

Actinomycetes were not isolated from mud samples 2, 3, 7, 8, 14, 16 and 17; isolates from mud samples 4, 9, 12 and 15 were observed on the original isolation plates but proved impossible to either isolate or purify further.

3.3.3 Fungi

It was found that bacteria and actinomycetes were usually more numerous than the fungi, and suppressed the fungal colonies growing in conventional media during the isolation procedures. Therefore, acidified solid media (pH 4.0) was used to overcome this problem.

Thirty seven fungal isolates were obtained from the salt marsh mud samples (table 6). Of these 13 were identified as belonging to the genus Trichoderma, the most dominant genus to be found among the isolates. Seven isolates belonged to the genus Penicillium, six to the genus Fusarium, five to the genus Aspergillus and two to the genus Biopolaris. Individual isolates belonging to the genera Cladosporium, Zygorhyncus, Mucor and Drechslera were also identified.

Each isolate has been given a reference number representing the mud sample number from which this was isolated, eg. isolate numbers 8A, 8B and 8C all came from mud sample No. 8 and were isolated as separate colonies. All the isolates have been kept in the Liverpool University, Department of Botany Culture Collection (L.B.C.C.)

No fungal isolates were obtained from mud samples Nos. 1 to 7 and from Nos. 19 and 20.

Isolates numbered 10B, 12C and 14B initially isolated were lost during the purification procedure. These losses could have been due

Table 6. The generic identify of fungal isolates from the various mud samples

Fungal isolates LBCC No.	Genus
8A	Trichoderma
8B	Cladosporium
8C	Biopolaris
9	Zygorhyncus
10A	Biopolaris
10B	-
10C	Trichoderma
10D	Trichoderma
10E	Penicillium
11A	Trichoderma
11B	Trichoderma
11C	Aspergillus
11D	Trichoderma
12A	Fusarium
12B	Mucor
12C	-
12D	Aspergillus
12E	Trichoderma
12F	Aspergillus
13A	Penicillium
13B	Penicillium
13C	Fusarium
13D	Trichoderma
13E	Fusarium
13F	Fusarium
14A	Drechslera
14B	-
14C	Fusarium
15A	Aspergillus
15B	Trichoderma
15C	Trichoderma
16A	Trichoderma
16B	Penicillium
16C	Trichoderma
17A	Fusarium
17B	Penicillium
17C	Penicillium
18A	Trichoderma
18B	Aspergillus
18C	Penicillium

to either the use of unsuitable media or unfavourable environmental conditions such as changes in pH and temperature affecting fungal sporulation and growth.

Isolates identified as being of the same genus might be the same or different strains. However, from examining their inhibitory and stimulatory behaviour on the cyanobacteria (see Chapter 4) it would seem that most of the isolates were different.

3.3.4 Bacteria

Twenty nine bacterial isolates were obtained and purified from the various mud samples. These isolates were coded according to the mud sample from which they were isolated, as with the other groups of micro-organisms (table 7). Of the eight bacterial isolates characterised at the Torry research station (table 8), only two, ie. 1A and 15C were given the same specific name, Bacillus pumilus (see appendix 4). These two isolates however behaved differently to one another in the interaction tests with the cyanobacterial species. This would suggest that they are not identical strains even if they are of the same species (see Chapter 4).

No bacterial isolates were obtained from mud samples 7, 10, 11, 13, 18, 19 and 20.

3.3.5 Distribution of different types of microbial isolates in the individual mud samples

The different types of microbial isolates obtained from each individual salt marsh mud sample are given in table 9. This information is essential for future experiments on the interactionsbetween the different micro-organisms and the significance of these findings will be discussed later.

Table 7. Bacterial isolates from the salt marsh mud samples.

Salt marsh mud sample number	LBCC Number of bacterial isolates obtained from each salt marsh mud sample
1	1A, 1B, 1C
2	2A, 2B
3	3
4	4
5	5A, 5B
6	6
7	-
8	8
9	9A, 9B
10	-
11	-
12	12A, 12B
13	-
14	14A, 14B
15	15A, 15B, 15C
16	16A, 16B, 16C, 16D, 16E 16F, 16G, 16H
17	17
18	-
19	-
20	-

Table 8. Identification of some of the salt marsh bacterial isolates

LBCC No. 1	Name 2
1A	*Bacillus pumilus*
2A	*Micrococcus* sp., possibly *Micrococcus luteus*
9A	*Pseudomonas* sp. or *Altermonas* sp.
15B	*Bacillus subtilis*
15C	*Bacillus pumilus*
16A	*Bacillus cereus*
16B	*Bacillus licheniformis*
16G	*Arthrobacter* sp.

1 the number represents the mud sample from which the isolate was originally isolated

2 identification carried out by Torry Research Station (Scotland)

Table 9. The different microbial types isolated from each individual salt marsh mud sample

Mud samples no.	LEGC No. and species of cyanobacteria	Bacterial isolates reference number	Actinomycete isolates reference number	Fungal isolates reference number
1	-	1A, 1B, 1C, 1D	1A, 1B, 1C	-
2	-	2A, 2B	-	-
3	-	3	-	-
4	Synechococcus elongatus(54)	4	-	-
5	Nostoc linckia (8) Anabaena gelatinicola	5A, 5B	5A, 5B	-
6	Nostoc piscinale (45)	6	6A, 6B, 6C	-
7	-	-	-	-
8	Nostoc muscorum (34) Symploca thermalis (74)	8	-	8A, 8B, 8C
9	Nostoc muscorum (34) Anabaena microspora (14) Lyngbya majuscula	9A, 9B	-	9
10	Nostoc muscorum (34) Phormidium subincrustratum	-	10A, 10B	10A, 10B, 10C, 10D, 10E
11	Oscillatoria lacustris (58) Oscillatoria anguina	-	11A, 11B, 11C	11A, 11B, 11C, 11D
12	Nostoc entophytum (52) Nostoc linckia (8) Anabaena minutissima (17) Anabaena doliolum Symploca elegans (68) Synechocystis diplococcus (19) Oscillatoria lacustris (58) Oscillatoria subuliformis	12A, 12B	-	12A, 12B, 12C, 12D, 12E, 12
13	Synechococcus elongatus (54) Nostoc muscorum (34) Nostoc passerinianum	-	13A, 13B	13A, 13B, 13C, 13D, 13E,
14	Nostoc piscinale (45) Nostoc verrucosum (29) Nostoc punctiforme (48) Symploca thermalis (74) Nodularia harveyana v.B (40)	14A, 14B	-	14A, 14B
15	Nostoc muscorum (34) Nostoc sphaeroides (36) Anabaena flos-aquae (28)	15A, 15B, 15C	-	15A, 15B, 15C
16	Nostoc punctiforme (48) Lyngbya halophila (41)	16A, 16B, 16C, 16D, 16E, 16F, 16G, 16H	-	16A, 16B, 16C
17	Nostoc muscorum (34	17	-	17A, 17B, 17C
18	Cylindrospermum majus (62) Synechococcus elongatus (54)	-	18	18A, 18B, 18C
19	Nostoc muscorum (34) Anabaena oscillaroides (27) Symploca thermalis (74)	-	19	-
20	Anabaena minutissima (17) Nostoc punctiforme (48)	-	20	-

N.B. The reference number of each microbial isolate represents the number of the mud sample from which they were originally isolated.

3.4 Discussion

It was apparent in this work that not all of the soil Micro-organisms usually present in salt marsh samples were isolated on the conventional plating media used. The microbial colonies observed by previous workers (Stevenson, 1958; Jensen, 1962) which did appear were considered to provide a representative picture of the soil microflora (a point which will be discussed more fully later). Several investigators (Conn, 1918; Waksman, 1927; Thornton and Gray, 1934; Jones and Mollison, 1948; Russell, 1950; Skinner, Jones and Mollison, 1952; Seifer, 1958), utilizing microscopic techniques for observing and counting the soil microflora, observed a discrepancy between total plate counts and values obtained by direct counts by various microscopic techniques. In general, their results indicated that only 1 - 10 percent of the soil microflora grew on the best of plating media. The reasons given to explain this were, insufficient nutrients in the plating media which reduced the colony numbers, or that at any one time, a large part of the soil microflora was dead but still stainable and thus countable with the microscope (Russell, 1950; Skinner *et al.*, 1952; Topping, 1958). Additionally, low counts on the plating media used in isolation could be due to:-

1) Autotrophic populations being present in the soil, in addition to those heterotrophic forms with which we are familiar. These autotrophs may oxidise or reduce inorganic compounds of nitrogen, sulphur, iron, phosphorus, manganese or copper without which they will not grow and which may not be present in the medium chosen.

ii) Cellulose, lignin and humic materials may support a large, heterotrophic but highly oxygen sensitive, anaerobic population which to isolate would require techniques of anaerobic isolation and cultivation similar to those described for the rumen microflora (Hungate, 1950; Bryant, 1959; Hungate, 1960; Kistner, 1960; Kistner and Gouws, 1964).

iii) Bacteria which are parasitic on other bacteria, eg. Bdellovibrio species (Stolp and Starr, 1963) and therefore require the presence of an acceptable host for their demonstration.

The isolation of micro-organisms from soil by the dilution plate method suffers from several disadvantages. First of all there is the general disadvantage, common to all such culture methods, that no single culture medium is suitable for the development of more than a small minority amongst species of soil fungi, bacteria and actinomycetes. The fungi are, in fact, frequently not found on soil dilution plates, because the culture medium may be nutritionally inadequate. The second disadvantage of the soil dilution plate technique is also common to the isolation of bacteria and actinomycetes, but it operates more significantly on the isolation of fungi. This disadvantage arises directly out of the procedure for diluting the original suspension of soil. Even before a sample of soil suspension has been pipetted to make a dilution series, most of the heavier parts of the soil may have already sunk to the bottom of the shaking bottle; this part will include the larger mineral particles, together with those soil crumbs that have not been disaggregated by the shaking procedure, as well as the heavier organic fragments. The actual samples that are finally

plated out in the petridishes are therefore suspensions of the finer soil particles, together with bacterial and actinomycetes cells, fungal spores and some hyphal fragments. Thus some microbial species aggregated with the larger particles may not be transferred to dilution series and will not be represented on the isolation plates.

Less cyanobacteria species were obtained during this work than were found by Mahdi (1980) in his study on cyanobacteria of the Dee salt marsh. This could be due to the limited amount of sampling used compared to Mahdi who collected samples from a large area and made isolations at regular intervals throughout the year. Chapman and Chapman (1973) reported that certain cyanobacterial strains are transitory during the year, which could be a reason for the absence of some particular cyanobacteria species in this study.

Cyanobacteria species were not isolated from four mud samples (ie. 1, 2, 3 and 7). This is unlikely to be due to their absence in these samples since microscopic observation showed there to be blue-greens present. It is more likely that those species present were not readily isolated in the standard media or by the isolation procedures adopted during this investigation. Another reason for the lack of blue-greens in these four mud samples could be the antagonistic effect of other microbial types on cyanobacterial growth, a point which will be discussed later. It should also be emphasised that although certain blue-greens were observed under the microscope they could not be isolated and cultured, eg. Oscillatoria anguina and Spirulina subsala belonging to section III of Rippka et al. (1979). Such species clearly have nutritional and growth requirements not provided by our methods.

Since Mahdi (1980) had also tried unsuccessfully to grow these particular species it was decided not to attempt it in this study as it was deemed more important to concentrate on other areas of the investigation. Thus we were not so interested in isolating the maximum numbers of cyanobacteria which could be obtained from mud samples, but more interested in obtaining isolates of the different microbial types from the same sampling area since this is more important in relation to the possible interactions between these different micro-organisms. Nevertheless it would seem that the chosen isolation method did yield numerous cyanobacterial species, which, when compared with the findings of Mahdi (1980), seemed representative of the cyanobacterial population.

Although it was considered necessary to purify all these cyanobacterial isolates in order to carry out the physiological and biochemical studies, it proved very difficult to obtain axenic cultures, although success was achieved with certain species by using antibiotic treatments. This purification problem was not a novel one, Echlin et al. (1965) reported that the difficulties might be due to the close interrelationships between cyanobacteria and the contaminant micro-organisms, making their physical separation difficult. Therefore, corresponding cyanobacterial strains previously isolated and purified by Mahdi (1980) were used instead of the bacterised strains isolated from cores in this study. These isolates were mostly filamentous non-heterocystous species of cyanobacteria.

It was difficult to choose a single isolation procedure to obtain all the actinomycetes likely to be living in the salt marsh mud samples.

Various methods have been suggested by previous workers to isolate these micro-organisms from soil, such as the soil plating method (Warcup, 1950); the soil drying method (Warcup, 1960); dilution plate methods (Parkinson, Gray and Williams, 1971) and others. Only one procedure and two different media (see Chapter 2) were used to isolate the actinomycete strains from the salt marsh mud samples in this study because of the limits of time and resources. Therefore, the absence of some actinomycete strains was anticipated and accepted as being inevitable.

A high density of bacterial colonies was found growing on the actinomycete isolation plates despite the use of antibacterial compounds in the media and this interference between microbial types could also have influenced the range of actinomycetes isolated. It is not usually possible to distinguish between different species of the same genus on the isolation plates. Therefore selection of large numbers of colonies can sometimes result in much duplication and wasted effort in screening programmes. Therefore, initially in the isolation procedure all the actinomycete colonies were picked out and subcultured assuming that all these colonies were different species. However, observations of the colour, colony shape and the results of frequent interaction tests suggested that many of these colonies were of identical strains and so the number of strains was reduced.

The colonies of some genera developing upon the agar surface had a firm consistency and adheredtenaciously to the solidified substratum. This caused the loss of two to three different strains since some of them were often difficult to transfer (even when

transferred with agar medium) and subsequently failed to grow on either agar plates or liquid medium. Other isolates which appeared on the isolation plates were powdery and often became pigmented when the aerial spores were produced.

Had time permitted a range of more specialised media would have been tried. As most of the actinomycetes are neutrophiles, the pH of isolation media was usually between 6.7 and 7.5. However, it would be necessary to reduce the medium pH to 4.5 to 5.0 to isolate acidophilic actinomycetes (Williams et al., 1971; Khan and Williams, 1975).

The majority of actinomycetes isolated from the salt marsh in this work were streptomycetes. Hunter, Eveleigh and Casella (1981) have suggested that the salt marsh streptomycetes population is unique and also showed that it had an ability to adjust to seasonal fluctuations in salinity. The Dee salt marsh isolates were identified by numerical taxonomic techniques and were shown to belong to the Streptomyces albidoflavus cluster. Nevertheless the data from interaction tests obtained in subsequent chapters would support the idea that many of the isolates were distinctly different strains, albeit part of the same major cluster. Alderson (1982) found that Streptomyces albidoflavus was a major cluster containing some 72 strains identified by numerical and chemical classification methods. This cluster was found to consistently contain three distinct but heterogenous subclusters with each subcluster comprising different strains of streptomyces.

The absence of fungal isolates from some Dee salt marsh mud samples in this investigation was again unlikely to be due to the total absence of fungi from these samples but a function of the choice of isolation procedure and media. Previous workers (Brierley, 1932;

James, 1935) have also noted that fungi known to fruit on soil did not occur on soil dilution plates, the technique employed in this study. As a result of this, workers are still unable to give an adequate picture of the fungal flora of a soil, because most of their knowledge of the fungi present in soil has been derived from studies using the soil dilution plate method. Although the soil fungi are not abundant in salt marshes, (Elliot, 1930; Saito, 1952), the fungal genera Penicillium, Trichoderma, Fusarium, Mucor and Aspergillus were found in decreasing order of abundance in the salt marsh soil environments of the River Dovey and the Pacific Coast (Saito, 1952). Compared with the aerobic surface muds, anaerobic muds were poorer in fungal species and numbers. This fact could be an important factor in explaining the absence of fungi in numerous mud samples in this study since several of the samples were anaerobic. The results of the fungal isolation study obtained from the Dee river mud samples were more or less similar to that of previous workers. It is difficult, however, to be sure that the genera isolated here were necessarily the dominant organisms in the natural environment. They could have simply been selected for preferentially by the choice of methods. The dominant fungi obtained all belonged to the fungi Imperfecti which consistently contributesthe greatest number of genera and species to the fungal community in general. The other fungi were not well represented amongst the Dee salt marsh fungal isolates.

The problems of isolation discussed for actinomycetes and fungi were also applicable to the isolation of bacterial colonies from soil dilution plates, despite the fact that this method is considered the

most satisfactory one for the counting and isolating of soil bacteria, because these are unicellular micro-organisms. Strictly anaerobic bacteria would not be found amongst the isolates since the isolation procedures were performed under aerobic conditions. This could explain the absence of bacterial isolates from mud samples 7, 10, 11 and 13 which were black anoxic sediments taken from the bottoms of pools and gullies. However, no isolates were obtained from the aerobic mud samples 18, 19 and 20 and in this case isolation under aerobic conditions cannot be the sole reason for the absence of bacterial isolates. In these cases other factors must be responsible.

The isolates were found to be mostly Bacillus, Micrococcus, Pseudomonas and Arthrobacter species which have been shown to grow abundantly in soil by the previous workers (Cann, 1928; Jensen, 1933; Topping, 1937; Taylor, 1938; Gibson, 1939; Lochhead, 1940; Clark, 1940). Less common species, eg. Rizobium, Azotobacter, Nitrobacter and Achromobacter were not present in the mud samples. An interesting fact arising from the identification procedure was the large number of Bacillus species amongst the salt marsh isolates. This might be attributed to the isolation method favouring the Bacillus species.

CHAPTER FOUR

ASSAYS OF DIFFERENT TYPES OF MICRO-ORGANISM INTERACTIONS WITH CYANOBACTERIA AND GREEN ALGAE SPECIES

4.1 The effects of actinomycetes on cyanobacteria and green algae

4.1.1 Introduction

The majority of actinomycetes are aerobic, saprophytic, mesophiles whose natural habitat is soil (Porter, 1971). They produce metabolites including antibiotics, extracellular enzymes, pigments and growth promoting factors (Rainbow and Rose, 1963; Lilly and Stillwell, 1965). Antibiotic production by actinomycetes is of ecological significance and has been commercially exploited by the pharmaceutical industry.

Actinomycetes are known to produce substances in the laboratory which can modify the growth of competing organisms, but it has not been fully established whether these substances are produced under natural conditions or in high enough concentrations to inhibit the growth of micro-organisms (Krassilnikov, 1959; Gottlieb, 1976). Stevenson (1956) however, claims to have shown that antibiotics produced by actinomycetes in the soil are antagonistic towards competing micro-organisms present in the same habitat. Further studies on the physiological and chemical nature of actinomycete metabolites which exhibit algicidal properties are needed (Safferman and Morris, 1962).

The aim of these experiments was to establish the effect of actinomycete metabolites on the growth of pure cultures of green algae and cyanobacteria. The latter were isolated from the same habitat as the actinomycete isolates.

4.1.2 Materials and methods

All the laboratory actinomycete strains and those recently isolated from the River Dee salt marsh were assayed against all the

laboratory and Dee salt marsh cyanobacterial isolates using the agar block and cell-free filtrate application techniques. Certain actinomycete filtrates were also individually added to specific cyanobacterial liquid cultures to assess the effect of the actinomycetes on growth rates, photosynthetic $^{14}CO_2$ fixation and nitrogenase activity. The morphological lysis effects were also investigated using light and electron microscopy. All these methods have been described in Chapter 2. Seven laboratory species of cyanobacteria originally obtained from the University of Indiana culture collection (now UTEX) used in this study were reclassified according to Rippka et al. (1979), (Table 10). These strains were given reference numbers and included in the Liverpool culture collection (L.B.C.C.).

Five green algal isolates from the University of Texas culture collection (UTEX) and the Cambridge culture collection (C.C.C.) were included in this study for comparison with the prokaryotes (Table 11).

Laboratory actinomycetes were chosen from the International Streptomyces Project (ISP) and Centraalbureau voor Schimmel cultures, Baarn, Netherlands, (CBS), (Table 12) in order to determine the effect of actinomycete isolates from the same or different habitats on cyanobacteria species.

4.1.3 Results

4.1.3.1 Assays of actinomycete isolate interactions with cyanobacteria and green algae species

Cleared zones on the cyanobacterial lawns around various actinomycete discs were detected after two or three days' incubation (Plate 4). The interaction tests were performed using different combinations of growth media for both blue-greens and actinomycetes. The effects of various actinomycete species on the growth of different

Table 10. Laboratory cyanobacteria species used in this study identified according to Rippka et al (1979).

Laboratory cyanobacterial species	1 Section	2 Genus	3 Strain new reference no.	Original culture collection and code number
Anabaena flos-aquae A37	IV	Anabaena	L.B.C.C. 85	I.C.C. 144 Tischer A37
Anabaena cylindrica		Anabaena	L.B.C.C. 86	I.C.C. 1447 MSC 251
Nostoc muscorum		Nostoc	L.B.C.C. 87	C.C.C. 456 Allison 1453/12
Plectonema boryanum	III	LPP group B	L.B.C.C. 89	I.C.C. 1542 Ailen 6306
Oscillatoria sp.		LPP group B	L.B.C.C. 91	I.C.C. 1543 Ailen 6408
Lyngbya sp.		LPP group B	L.B.C.C. 90	I.C.C. 1546 Ailen 6412
Anacystis nidulans	I	Synechococcus	L.B.C.C. 88	I.C.C. 1550 Ailen 6301

These strains were given a new reference number and have been included to Liverpool Culture Collection (L.B.C.C.)

1 and 2 see Rippka et al. (1979)

3 L.B.C.C. Liverpool University, Botany Department culture collection.

I.C.C. Univ. of Indiana culture collection (now transffered to Univ. of Texas).

C.C.C. Cambridge culture collection.

Table 11. The green algae species included with the test cyanobacteria species in this study.

Green algae species	Culture collection and species reference number
Chlamydomonas moewusii	UTEX 97
Scenedesmus obliquus	UTEX 1450
Ulothrix fimbriata	UTEX LB 638
Coelastrum microsporum	UTEX 280
Chlorella pyrenoidosa	UTEX 26

These strains were obtained from the University of Texas culture collection U.S.A.

Table 12. Laboratory species of actinomycetes used in this study.

Actinomycete species	Code No.	Culture collection
Streptomyces griseus	A50	CBS
Streptomyces roseodiostaticus	A52	CBS
Streptomyces violascens	5183	ISP
Streptomyces bellus	5185	ISP
Streptomyces goshikiensis	5190	ISP
Streptomyces globisporus	5199	ISP
Streptomyces lavendulocolor	5216	ISP

CBS : Centra alburean voor Schimmel cultures, Baarn, Netherlands.

ISP : International Streptomyces Project..

Plate 4

The effect of agar discs containing colonies of specific actinomycete isolates (A) on the growth of a cyanobacterial lawn of Anabaena microspora 14 (L). The clear area (I) shows where lysis of the cyanobacterial lawn has occurred in a zone around an applied actinomycete agar disc. Pure ISP5 agar block medium was placed in the centre of the plate as a control (C).

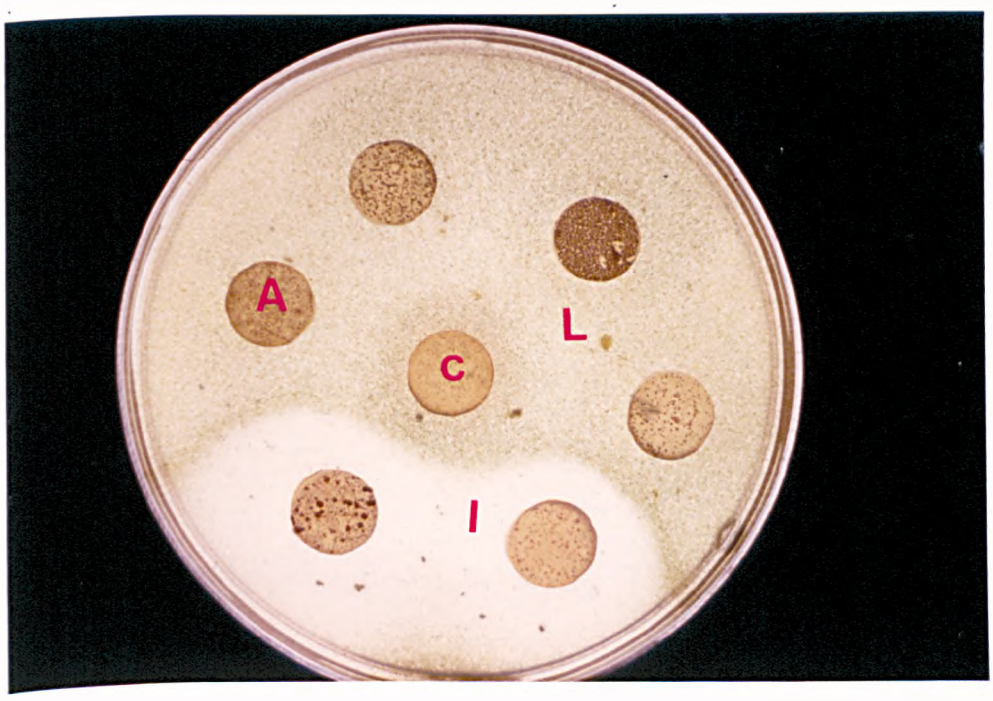


Plate 4

species of blue-greens are given in Tables 13 and 14. These data show that specific actinomycetes inhibit the growth of certain cyanobacterial species but do not affect others, eg. actinomycete isolate 1A inhibited the growth of Lyngbya halophila 41 but not Symploca elegans 68 or Plectonema boryanum 89. Actinomycete strain 190 did not show any antagonistic activity towards any of the tested cyanobacterial isolates. All the other actinomycete isolates inhibited the growth of one or more cyanobacteria but the precise species of blue-greens that were affected varied depending on the actinomycete being used.

A few of the actinomycete strains actually stimulated the growth of certain blue-green species but inhibited others, eg. actinomycete isolate 6B inhibited Anabaena minutissima 17 but stimulated the growth of Anabaena flos-aquae 28.

Similar results with these interaction tests were obtained regardless of whether mature or immature blue-green lawns were used. The application of aliquots of cell-free filtrate from the actinomycete cultures to cyanobacterial lawns in sterile stainless steel wells also gave results identical to the ones obtained from agar discs.

Different interaction results between the cyanobacteria and the actinomycetes were obtained depending on the media type used to grow the two groups. This is exemplified in Table 15 (extracted from Tables 13 and 14) and shows that the sensitivity of Anabaena microspora 14 to four different actinomycete strains varies depending on the medium type on which it is grown (ASM or Allen's medium) and also the growth medium chosen to grow the actinomycete strains (ISP5 or nutrient broth). Nostoc muscorum 87 was resistant to attack by actinomycetes when grown on ASM regardless of which medium the actinomycetes were grown on.

Table 13 The effects of agar block applications containing either laboratory or Dee salt marsh colonies of actinomycetes on lawns of all the Dee salt marsh isolates and laboratory species of cyanobacteria.

Cyanobacteria species grown on Allen's (c) media				Actinomycetes*																								
LBBC NO	Section	Genus	Classical Identification	1A	1B	1C	5A	5B	6A	6B	6C	10A	10B	11A	11B	11C	13A	13B	18	19	20	A50	A52	183	199	216	185	190
19	I	Synechocystis	Synechocystis diplococcus (Pringsh)	–	+	–	–	–	–	+	–	–	+	–	–	–	+	–	+	–	–	–	–	+	–	+	+	–	–
				+	+	+	–	–	+	+	–	+	–	–	+	–	+	+	+	+	–	–	+	+	+	+	–	–
54		Synechococcus	Synechococcus elongatus Näg	–	–	–	–	–	–	+	–	–	+	–	–	–	–	+	–	+	–	–	+	–	+	+	–	–
				–	–	–	–	–	–	–	–	+	–	–	+	–	–	+	–	–	+	–	+	–	+	+	–	–
88		Synechococcus	Anacystis nidulans	–	–	–	–	+	–	+	–	–	–	–	+	–	+	+	–	–	+	+	+	–	+	+	–	–
				–	–	–	–	–	–	+	–	+	–	–	+	–	–	–	–	–	+	–	+	–	+	+	–	–
74	III	LPPGpB	Symploca thermalis (KÜTZ) Gom.	–	–	–	–	–	–	+	–	+	+	–	+	+	+	+	–	–	+	–	+	–	+	+	–	–
				–	–	–	–	–	–	–	–	+	+	–	+	–	–	+	–	–	+	–	+	–	+	+	–	–
41		LPPGpB	Lyngbya halophila Hansg.	+	–	+	+	–	–	+	–	+	+	–	–	–	–	–	–	+	–	+	+	–	+	+	–	–
				+	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–	+	–	+	+	+	+	+	–	–
68		LPPGpB	Symploca elegans KÜTZ.	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	+	+	–	–	–	–	–
				–	–	+	–	–	–	–	–	+	–	–	–	+	–	–	–	+	–	+	+	–	–	–	–	–
89		LPPGpB	Plectonema boryanum	–	+	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	+	–	+	–	+	–	–	–
				–	+	+	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–	+	+	–	+	–	–	–
90		LPPGpB	Lyngbya spp.	–	–	–	–	–	–	+	–	–	+	–	–	+	–	–	–	–	–	–	+	–	+	+	–	–
				+	+	+	–	–	–	+	+	–	–	–	+	–	–	–	+	+	–	+	+	+	+	+	–	–
91		LPPGpB	Oscillatoria spp.	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–
				–	–	+	–	–	–	–	–	+	–	–	+	–	+	–	–	+	–	–	–	–	+	–	–	–
14	IV	Anabaena	Anabaena microspora Kleb.	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–
				–	+	+	–	+	–	–	–	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	–	–
17		Anabaena	Anabaena minutissima Lemm.	–	–	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–	+	–	+	+	–	–
				+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	+	+	–	–
27		Anabaena	Anabaena oscillaroides Bory.	–	+	–	–	–	–	–	–	+	–	–	+	+	–	–	–	–	–	–	+	–	+	+	–	–
				+	+	+	–	–	–	–	–	+	–	–	+	–	+	+	+	+	–	+	+	–	+	–	–	–
28		Anabaena	Anabaena Flos-aquae (Lyngb) Breb.	+	–	–	+	–	–	S	–	–	–	–	–	–	–	–	–	+	–	–	+	–	+	–	–	–
				–	+	–	–	–	–	S	–	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
85		Anabaena	Anabaena flos-aquae A37	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–
				–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
86		Anabaena	Anabaena cylindrica	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–
				–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
48		Nostoc	Nostoc punctiforme (KÜTZ) Hariot	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–
				–	+	–	–	–	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	+	+	–	+	–	–
52		Nostoc	Nostoc entophytum Born. et Flah	–	–	+	+	–	+	+	–	+	–	–	+	–	–	–	–	+	–	–	+	–	+	+	–	–
				+	–	+	–	–	+	+	–	+	–	+	+	+	+	–	+	+	–	+	+	–	+	–	–	–
8		Nostoc	Nostoc linckia (Roth) Born et	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–	+	–	–
				–	+	+	–	–	–	–	–	+	–	+	–	–	–	–	–	+	–	–	+	+	–	–	–	–
34		Nostoc	Nostoc muscorum Ag.	–	–	–	–	+	–	–	–	+	+	–	–	–	–	–	–	–	–	+	+	–	–	+	–	–
				–	+	+	–	–	–	–	–	+	–	–	+	+	–	–	–	+	–	–	+	–	–	–	–	–
37		Nostoc	Nostoc sphaeroides KÜTZ	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	+	–	–
				–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–
45		Nostoc	Nostoc piscinale KÜTZ	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	–
				–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
29		Nostoc	Nostoc verrucosum Vauch	–	–	–	–	–	+	+	–	–	–	+	–	–	+	–	–	–	–	–	+	–	–	–	–	–
				+	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	+	+	+	–	+	+	–	–
97		Nostoc	Nostoc muscorum	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
				–	–	+	–	–	+	+	–	–	–	+	–	+	+	–	–	+	–	–	+	–	+	–	–	–
62		Cylindospermum	Cylindospermum majus KÜTZ.	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	–	–
				–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–	–	–
40		Nodularia	Nodularia harveyana var. sphaerocarpa	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	+	–	–	+	–	–
				–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–

Blue greens growth were either inhibited [+] stimulated [s] or apparently unaffected [–] by the various strains of actinomycete

* These results are given for actinomycetes growing on ISP 5 and nutrient agar

These results were obtained from at least triplicate assays

Table 14   The effects of agar block applications containing either laboratory or Dee salt marsh colonies of actinomycetes on lawns of all the Dee salt marsh isolates and laboratory species of cyanobacteria

Cyanobacteria species grown on ASM medium				Actinomycetes*																								
LBBC No	Section	Genus	Classical Identification	1A	1B	1C	5A	5B	6A	6B	6C	10A	10B	11A	11B	11C	13A	13B	18	19	20	A40	[illegible]	16A	[illegible]	[illegible]	[illegible]	9O
19	I	Synechocystis	Synechocystis aipiococcus (Pringsh)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−	−
				−	−	−	−	−	−	−	−	−	−	−	−	−	−	−				+	+	+	+	+		
54		Synechococcus	Synechococcus elongatus Nag	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+		
				−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−		
88		Synechococcus	Anacystis nidulans	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	+	+	+	+		
				−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−		
74	III	LPPGpB	Symploca thermalis (KUTZ) Gom	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
				−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	+	−	−	−
41		LPPGpB	Lyngbya halophila Hansg	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
				−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
68		LPPGpB	Symploca elegans KUTZ	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
				−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
89		LPPGpB	Plectonema boryanum	−	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	+	−	+	−	−	−
				+	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	+	−	+	+	−	+	−	−	−
90		LPPGpB	Lyngbya spp	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	+	−	+	+	−	−
				+	+	+	−	+	−	−	−	−	−	+	+	−	−	−	−	+	−	−	+	+	+	−	−	−
91		LPPGpB	Oscillatoria spp	−	−	+	−	−	−	+	−	−	+	−	+	−	−	+	−	+	+	+	+	−	+	+	−	−
				−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	+	+	−	−	−
14	IV	Anabaena	Anabaena microspora Kleb	−	+	−	−	+	−	+	−	−	−	−	−	−	+	−	−	−	−	+	+	−	+	+	−	−
				−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−
17		Anabaena	Anabaena minutissima Lemm	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−	−
				−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+	+	−	−
27		Anabaena	Anabaena oscillaroides Bory	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
				−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
28		Anabaena	Anabaena flos-aquae (Lyngb) Breb	+	+	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
				−	−	−	−	−	−	S	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
85		Anabaena	Anabaena flos-aquae (A 37)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+	+	−	−
				+	−	+	−	−	−	−	−	−	−	+	+	−	−	−	−	+	−	−	+	+	+	+	−	−
86		Anabaena	Anabaena cylindrica	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
				−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
48		Nostoc	Nostoc punctiforme (KUTZ) Hariot	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
				−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−
52		Nostoc	Nostoc entophytum Born et Flah	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
				−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
8		Nostoc	Nostoc linckia (Roth) Born et	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
				−	+	+	−	+	−	−	−	−	+	−	+	−	+	+		+	+	+	+	−	−	+	−	−
34		Nostoc	Nostoc muscorum Ag	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−
				−	−	−	−	−	+	+	−	−	−	+	+	+	+	−	−	−	−	+	+	−	−	−	−	−
[illegible]		Nostoc	Nostoc sphaeroides KUTZ	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
				−	+	+	−	+	−	−	−	−	−	−	−	−	+	+	−	+	+	−	+	+	+	+	−	−
[illegible]		Nostoc	Nostoc piscinale KUTZ	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−	−	+	−	−
				−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−
29		Nostoc	Nostoc verrucosum Vauch	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−
				−	−	−	−	−	−	+	−	−	−	−	−	−	+	−	+	−	−	+	+	−	+	+	−	−
87		Nostoc	Nostoc muscorum	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
				−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
[illegible]		Cylindrospermum	Cylindrospermum [illegible] KUTZ	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
				−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
[illegible]		Nodularia	Nodularia harveyana var sphaerocarpa	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
				−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Blue greens growth were either inhibited [+] stimulated [s] or apparently unaffected [−] by the various actinomycete strains

* These results are given for actinomycetes growing on ISP5 and nutrient agar

These results were obtained from at least triplicate assays

Table 15. Variation in sensitivity of cyanobacteria to actinomycetes when different media are used for their growth

Cyanobacteria species	Actinomycete isolates																								Media combination	
	1A	1B	1C	5A	5B	6A	6B	6C	10A	10B	11A	11B	11C	13A	13B	18	19	20	A50	A52	183	199	216	185	190	Actino.	Cyanobacteria
Anabaena microspora	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	ISP5	Allen's medium
	-	+	+	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	-	-	N.B.	
	-	+	-	-	+	-	+	-	-	-	-	-	-	+	-	-	-	-	+	+	-	+	+	-	-	ISP5	Allen's medium
	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	N.B.	
Nostoc muscorum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	ISP5	Allen's medium
	-	-	+	-	-	+	+	-	-	-	+	-	+	+	-	-	+	-	-	+	-	+	-	-	-	N.B.	
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ISP5	ASM medium
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N.B.	

+, inhibition in growth; -, no effect

However, on Allen's media this Nostoc strain was inhibited by several actinomycetes but the effective actinomycete strain varied depending on which medium they were grown. Certain actinomycetes were more effective against blue-greens when the former were grown on nutrient broth rather than on ISP5. Nevertheless, some actinomycete strains which appeared not to inhibit cyanobacteria on nutrient broth showed remarkable inhibition on ISP5 (eg. actinomycete strain 216 against Anabaena microspora 14 and Nostoc muscorum 34) (Table 15).

The relative lysing efficiencies of actinomycetes against the blue-green species as a whole are shown in Table 16 and Figure 3. These data emphasise the effects that growth media have on the interaction results, eg. actinomycete isolate 6B grown on ISP5 lysed 12% of the cyanobacteria grown on ASM medium but 44% on Allen's medium. The laboratory strains Streptomyces A52, 199 and 216 were the most active strains against the cyanobacteria, whilst 1B, 1C, 10A, 11B and 19 were amongst the most effective of the isolates recently isolated from the salt marsh.

The overall sensitivity of individual cyanobacteria to the laboratory and salt marsh actinomycete strains (Table 17 and Figure 4) also changed with medium type.

Generally the sensitivity of cyanobacteria grown on Allen's medium was higher than when they were grown on ASM. Streptomyces A52 was the most active strain against cyanobacteria grown in either culture medium. With the actnomycetes growing on ISP5 and blue-greens on Allen's media Anabaena microspora 14 was the most sensitive species followed by Synechocystis diplococcus 19, Nostoc entophytum 52,

Table 16. Inhibitory index of each actinomycete isolate against cyanobacteria species, when both microbial types were grown on different medium

Actinomycete isolates	ISP5/Allen's medium	N.B/Allen's medium	ISP5/ASM medium	N.B/ASM medium
1A	12	28	12	12
1B	20	48	16	28
1C	16	56	8	28
5A	24	0	0	0
5B	4	4	4	16
6A	8	12	4	4
6B	44	20	12	12
6C	0	4	8	0
10A	16	52	4	8
10B	28	20	4	8
11A	4	16	0	16
11B	8	44	4	20
11C	20	20	8	4
13A	12	24	4	20
13B	16	24	8	8
18	4	20	4	8
19	16	56	8	28
20	8	16	4	8
A50	28	36	28	24
A52	88	96	64	60
183	0	28	16	20
199	72	56	56	52
216	76	40	68	28
185	0	0	0	0
190	0	0	0	0

$$\text{Inhibitory index} = \frac{\text{number of inhibited cyanobacteria}}{\text{total number of cyanobacteria tested}} \times 100$$

The total number of tested cyanobacteria species = 25.

The actinomycetes were grown on either ISP5 or NB medium and the cyanobacteria on either Allen's medium or ASM.

Figure 3

Variation in the inhibitory activity of actinomycetes grown on nutrient broth agar () and ISP5 medium () towards River Dee salt marsh and laboratory species of cyanobacteria grown on

(a) Allen's (C) medium
(b) ASM medium

Figure 3

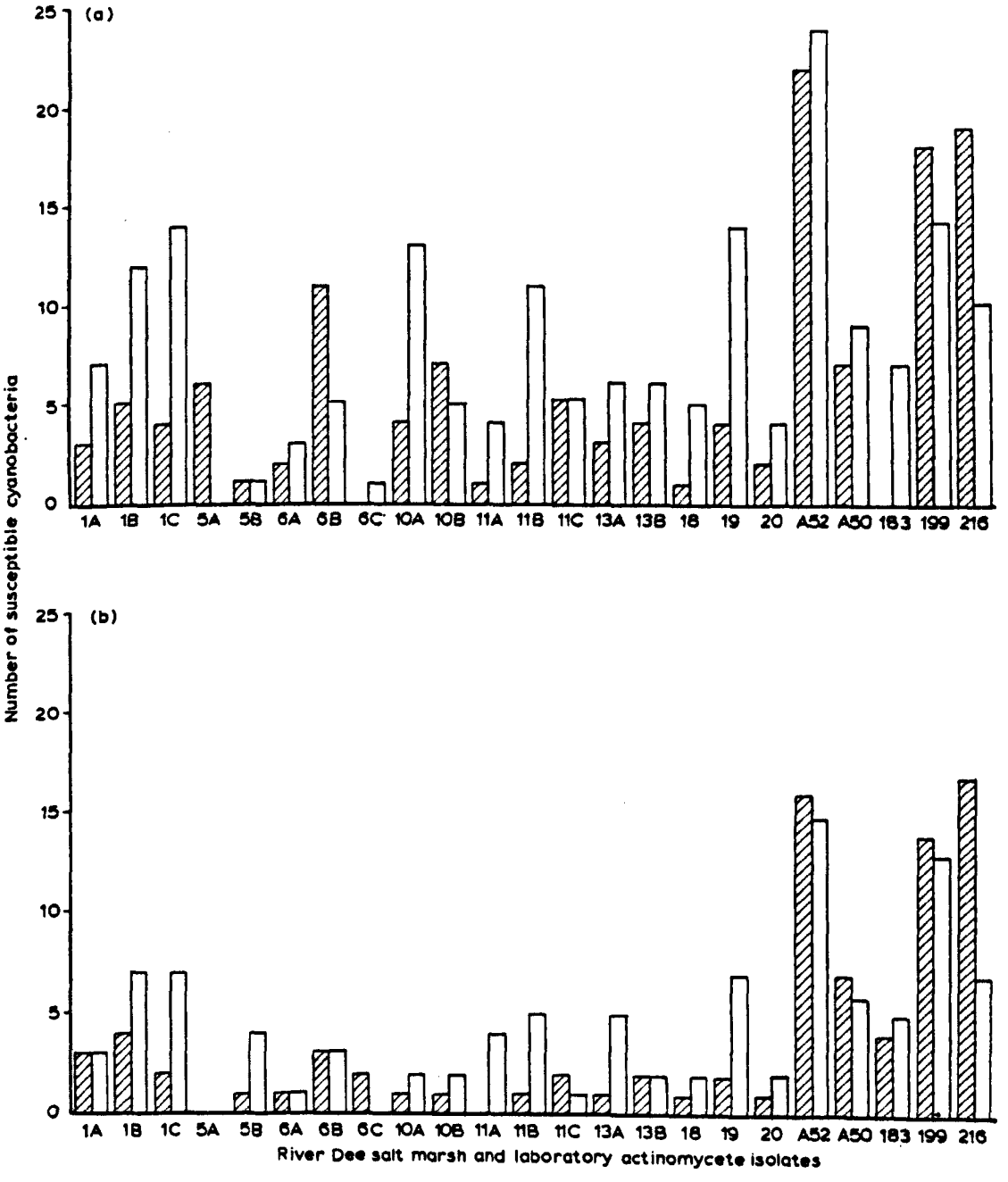

(a)
(b)
25
20
15
10
5
0
Number of susceptible cyanobacteria
1A 1B 1C 5A 5B 6A 6B 6C 10A 10B 11A 11B 11C 13A 13B 18 19 20 A52 A50 183 199 216
River Dee salt marsh and laboratory actinomycete isolates

Table 17. Sensitivity index of cyanobacteria species grown on Allen's medium and ASM medium towards laboratory species and Dee salt marsh isolates of actinomycetes grown on ISP5 and nutrient broth medium

LBCC No.	Cyanobacteria species	Sensitivity index: (%) ISP5/Allen's medium		N.B./Allen's medium		ISP5/ASM medium		N.B./ASM medium	
		Natural Act. isolates	Lab. Act. species	Natural Act. isolates	Lab. Act. species	Natural Act. isolates	Lab. Act. species	Natural Act. isolates	Lab. Act. species
19	Synechocystis diplococcus	27.7	42.8	61.1	57.1	0	28.5	0	71.4
54	Synechococcus elongatus	22.2	42.8	22.2	42.8	0	57.1	0	28.5
88	Anacystis nidulans	33.3	57.1	16.6	57.1	11.1	57.1	5.5	14.2
74	Symploca thermalis	44.4	42.8	27.7	42.8	0	14.2	11.1	28.5
41	Lyngbya halophila	38.8	57.1	22.2	71.4	0	14.2	0	14.2
68	Symploca elegans	11.1	28.5	22.2	28.5	0	0	0	0
89	Plectonema boryanum	22.2	42.8	22.2	42.8	22.2	42.8	27.7	42.8
90	Lyngbya spp.	16.6	42.8	44.4	57.1	11.1	57.1	38.8	42.8
91	Oscillatoria spp.	5.5	28.5	27.7	28.5	38.8	57.1	11.1	42.8
14	Anabaena microspora	5.5	42.8	72.2	42.8	22.2	57.1	11.1	14.2
17	Anabaena minutissima	11.1	42.8	22.2	42.8	0	42.8	5.5	57.1
27	Anabaena oscillaroides	22.2	42.8	50	42.8	0	14.2	5.5	0
28	Anabaena flos-aquae	16.6	28.5	11.1	14.2	22.2	14.2	0	0
85	Anabaena flos-aquae	5.5	42.8	0	14.2	0	57.1	27.7	57.1
86	Anabaena cylindrica	5.5	42.8	0	14.2	0	14.2	11.1	0
48	Nostoc punctiforme	5.5	42.8	22.2	42.8	0	28.5	0	42.8
52	Nostoc entophytum	38.8	42.8	61.1	42.8	0	0	0	0
8	Nostoc linckia	5.5	42.8	27.7	28.5	0	14.2	55.5	57.1
34	Nostoc muscorum	16.6	42.8	33.3	14.2	5.5	71.4	33.3	28.5
37	Nostoc sphaeroides	0	42.8	5.5	42.8	5.5	42.8	38.8	57.1
45	Nostoc piscinale	0	42.8	5.5	14.2	5.5	28.5	11.1	0
29	Nostoc verrucosum	22.2	14.2	22.2	57.1	5.5	57.1	16.6	57.1
87	Nostoc muscorum	0	14.2	38.8	28.5	0	0	0	0
62	Cylindospermum majus	0	28.5	0	28.5	0	14.2	0	0
40	Nodularia harveyana	11.1	28.5	11.1	0	0	14.2	0	0

$$\text{Sensitivity index} = \frac{\text{Number of actinomycete inhibitors}}{\text{Total no.of tested actinomycetes}} \times 100$$

Total number of laboratory actinomycetes species = 7

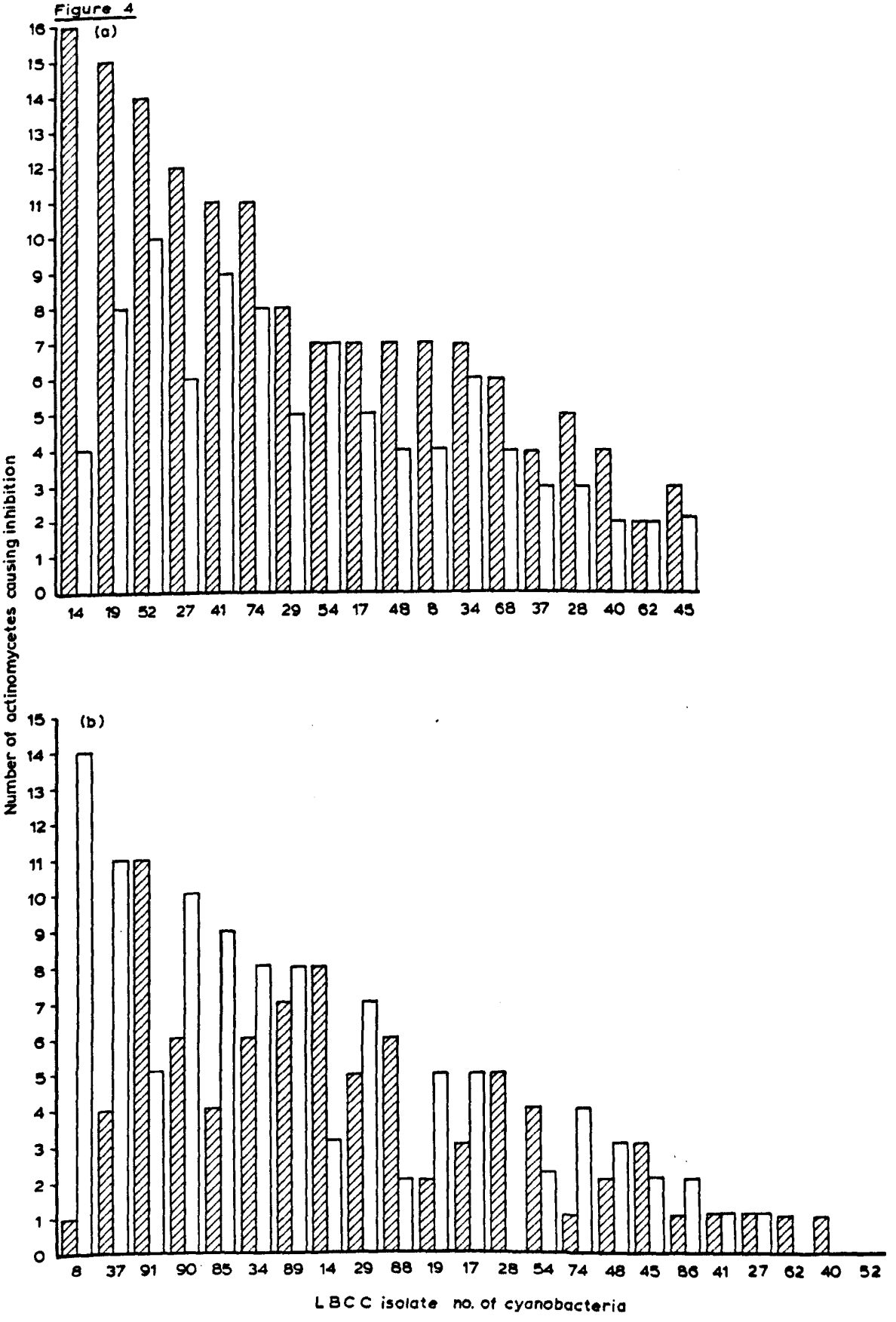
" " " *Dee salt marsh actinomycete isolates = 18*

Figure 4

Variation in the susceptibility of individual cyanobacterial isolates towards inhibition by actinomycetes growing on nutrient broth agar (□) or ISP5 medium (▨) when the cyanobacteria were grown on

(a) Allen's (C), and
(b) ASM medium

Figure 4


(a)
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
14 19 52 27 41 74 29 54 17 48 8 34 68 37 28 40 62 45
(b)
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
8 37 91 90 85 34 89 14 29 88 19 17 28 54 74 48 45 86 41 27 62 40 52
Number of actinomycetes causing inhibition
LBCC isolate no. of cyanobacteria

Anabaena oscillaroides 27 and Lyngbya halophila 41. When nutrient broth medium was used for actinomycetes cultivation, then the most sensitive blue-green was Nostoc entophytum 52, followed by Lyngbya halophila 41, Synechocystis diplococcus 19, Symploca thermalis 74 and Synechococcus elongatus 54. With cyanobacteria growing on ASM medium the most sensitive species to attack by actinomycete stains grown on ISP5 was Oscillatoria spp. 91, followed by Anabaena microspora 14, Plectonema boryanum 89, Anacystis nidulans 38, Lyngbya spp 90 and Nostoc muscorum 34. When nutrient broth medium was used for actinomycetes cultivation, the most sensitive cyanobacteria was Nostoc linckia 8 followed by Nostoc sphaeroides 37, Lyngbya spp 90, Anabaena flos-aquae 28, Nostoc muscorum 34 and Plectonema boryanum 89.

In contrast, the most resistant cyanobacterial strains to actinomycete attack, when the former were grown on Allen's media, and the latter grown on ISP5, was Nostoc muscorum 87 followed by Nostoc piscinale 45 and Oscillatoria spp. 91. When the actinomycete strains were grown on nutrient broth the most resistant strains were Anabaena flos-aquae 85 and Anabaena cylindrica 86 followed by Nostoc piscinale 45, Cylindospermum majus 62 and Nodularia harveyana 40. With the cyanobacteria growing on ASM and the actinomycete strains on ISP5 the most resistant strains were Nostoc entophytum 52, Symploca elegans 68, Nostoc muscorum 87, Nodularia harveyana 40, Cylindospermum majus 62 and Anabaena flos-aquae 28.

Accordingly, the least effective actinomycetes towards cyanobacteria species when the former were grown in ISP5 media and the latter were grown on Allen's media were 6C, 183, 185 and 190, followed by 5B, 11A and 18. The sequence of the least effective actinomycetes when grown on nutrient broth agar and Allen's medium for cyanobacterial cultivations were 5A, 185 and 190, followed by 6C and 5B. With blue-greens growing

in ASM medium the sequence of least effective actinomycetes were 5A, 11A. 185 and 190, followed by 5B, 6A, 10A, 10B and 11B when they were grown on ISP5. However, when they were grown on nutrient broth, the sequence was 5A, 6C, 185, 190,6A and 11C.

The same series of tests were repeated as before but this time the actinomycete colonies on the agar blocks were placed upside down so that they were in direct contact with the cyanobacterial lawns. Cell-free filtrates prepared from liquid actinomycete cultures were also applied to the cyanobacterial lawns in stainless steel cylinders. The results in both these subsequent test series were identical to those obtained with the agar block tests.

The same assay methods used for blue-greens were used to test the susceptibility of a few green algae to inhibition by actinomycetes. The results (Table 18) show that Chlorella pyrenoidosa was the most sensitive species, followed by Coelastrum microsporum and Chlamydomonas moewusii. The most lethal actinomycete strain found again was A52 (Streptomyces roseodiostaticus) which lysed all five green algal species used in the study.

Similar results were obtained between actinomycetes and cyanobacteria and actinomycetes and green algae when either mature or immature test lawns were used. Similar results were also found when cell-free filtrates of actinomycetes were used.

Apparent stimulation of cyanobacterial growth by actinomycetes on cores or by the addition of actinomycete culture filtrates was observed. However, stimulation was also seen around the control cores or cylinders of pure actinomycete medium (Plate 5). Thus, stimulation of cyanobacterial growth was considered to be due to the actinomycetes media rather than by metabolites released by the actinomycetes. Therefore, this

Table 18. The effects of agar block applications containing colonies of either laboratory species or Dee salt marsh isolates of actinomycetes on lawns of laboratory species of green algae.

Laboratory green algae grown on modified CHU. No. 10	Actinomycete strains grown on ISP5 medium																								
	1A	1B	1C	5A	5B	6A	6B	6C	10A	10B	11A	11B	11C	13A	13B	18	19	20	A50	A52	183	199	216	185	190
Chlamydomonas moewusii	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Scenedesmus obliquus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Ulochrix fimbriata	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Coelastrum microsporum	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	+	-	+	+	-	-
Chlorella pyrenoidosa	-	+	+	+	+	-	-	+	-	-	+	-	+	-	-	-	+	-	+	+	+	-	-	-	-

Green algae growth were either inhibited, +, or apparently unaffected, - , by the various strains of actinomycete.

Plate 5

The stimulating effect (S) of placing actinomycete blocks (A) on a cyanobacterial lawn (L). The pure actinomycete agar medium was placed in the centre of the plate as control (C), this also caused stimulation of the lawn in its area.

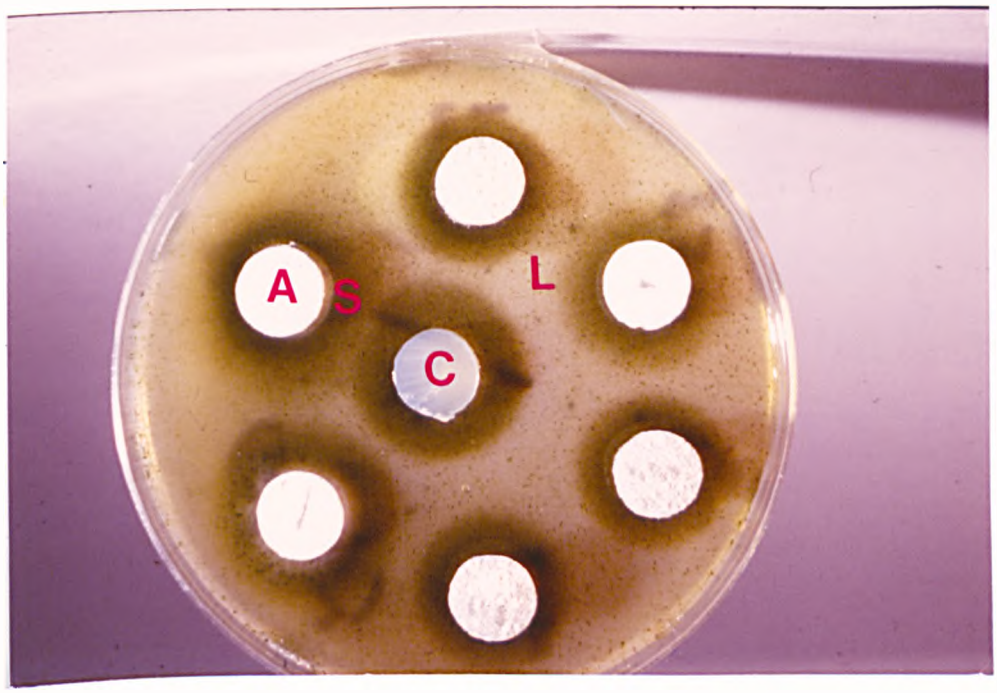


Plate 5

stimulatory effect was examined more closely as described in the next section.

The possibility that some of the resistant strains of cyanobacteria might inhibit or stimulate actinomycete growth was also investigated. In this case, cores of cyanobacterial lawns were placed on mature and immature actinomycete lawns. Aliquots of cell-free filtrates from liquid cyanobacterial cultures in stainless steel wells placed on actinomycete lawns were also used. Solid agar cores and wells of pure liquid cyanobacterial medium were used, as appropriate, for controls. In none of the experiments could cyanobacteria be shown to cause any deleterious effects on the actinomycetes. However, it should also be stated that no stimulation of actinomycetes was caused by any of the cyanobacteria used in the agar block or filtrate assays.

4.1.3.2 Studies of the morphological effects on cyanobacteria species by lytic actinomycete strains.

Samples from inhibition zones in the cyanobacterial lawns which had been caused by the activity of the actinomycete applications were removed at different times during lawn growth and observed by light microscopy. It was found that the vegetative cells of the cyanobacteria had lysed in the inhibition zones, but the heterocysts and akinetes appeared free but intact (Plate 6).

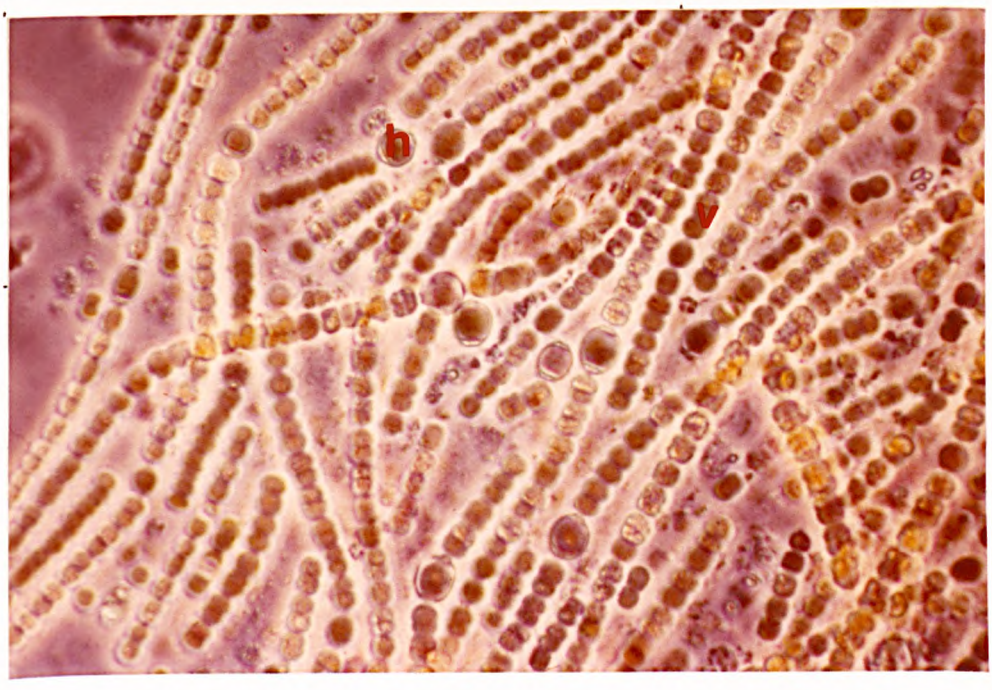
In liquid cultures the addition of actinomycete filtrates also caused lysis of vegetative cells but again heterocysts and akinetes remained intact. However, the lysis process took longer in liquid culture than on solid agar lawns and the stages of lysis could be more easily followed in samples from the liquid cultures. The lytic actinomycete filtrates first caused an increase in the size of the

Plate 6

(a) Typical unaffected filaments of Anabaena microscpora (14) x 2000, showing intact heterocysts (h) and vegetative cells (V).

(b) Lysed cyanobacterial filaments of Anaebaena microscpora (14) x 2000, after treatment with actinomycete A52 filtrate. The vegetative cells (V) were lysed but heterocysts (h) remained intact.

a



b

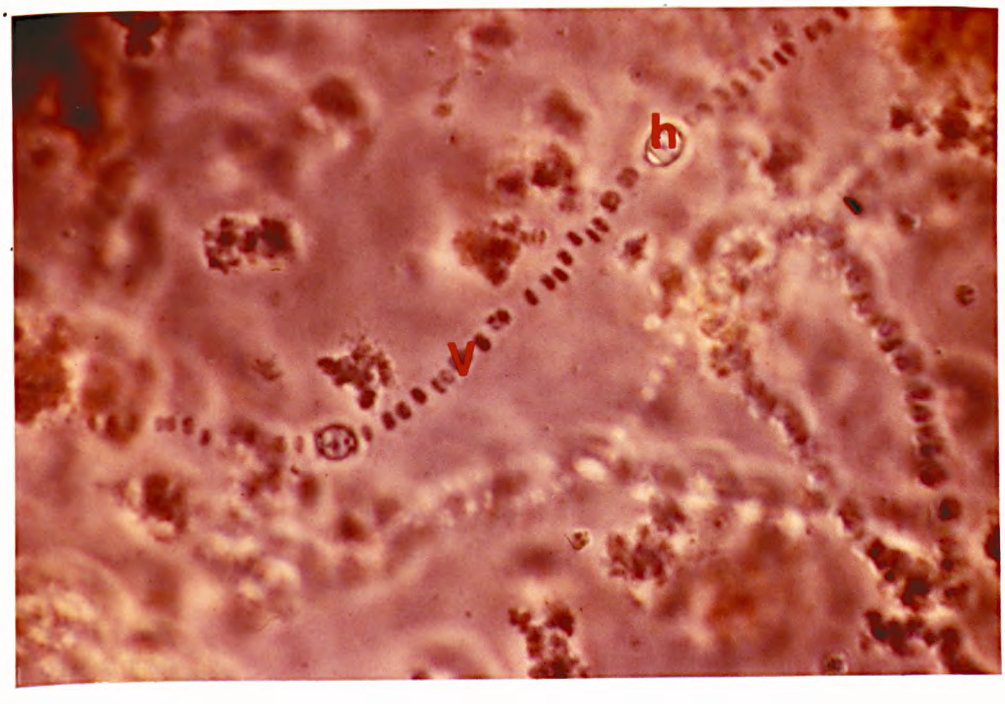


Plate 6

vegetative cells, followed by a loss of their typical shape before lysing completely. As the vegetative cells lysed, individual isolated heterocysts could be observed but they appeared to have no contents. These observations were made in many cyanobacterial isolates and typical results are those obtained with Anabaena microspora 14 (Plate 6). The results obtained with Nostoc entophytum 52, were similar but additionally the mucilaginous sheath surrounding the filaments appeared to increase in thickness after the cyanobacterial culture had been treated with actinomycete filtrate (Plate 7). This observed thickening of the sheath in no way protected the vegetative cells of the filaments from lysis by the actinomycete filtrates, in fact, Nostoc entophytum 52 was one of the most sensitive cyanobacteria to actinomycete attack (see Table 17).

4.1.3.3 Electron microscopy studies on the morphology of cyanobacteria and green algae species treated with actinomycete filtrates.

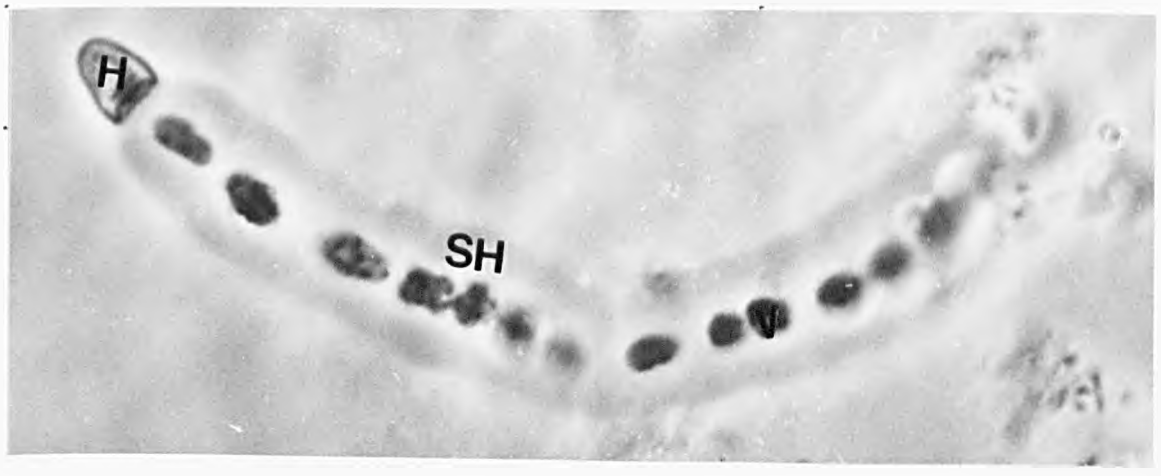
The sequence of lysing observed with the light microscope was also investigated with the electron microscope. A range of cyanobacteria sensitive to actinomycete filtrates was examined. Typical heterocysts and vegetative cells for untreated Anabaena oscillaroides culture are shown in Plate 8. The vegetative cells are surrounded by a four layered cell wall ie. L1, L2, L3 and L4, which is typical of many cyanobacterial species (Plate 9A). After the treatment of this cyanobacterial culture with a lytic actinomycete A52 filtrate the inner cell wall layers disappeared. The L2 layer disappeared first, followed by L3 and L1, although the disappearance sequence of the latter two layers was not determined (Plates 9B and 9C). The vegetative cells remained surrounded only by the L4 wall layer and the plasmamembrane. However, with time, the remaining L4 layer disappeared (or lysed) leaving the

Plate 7

(a) Lysed vegetative cells (V) of Nostoc entophytum (52) x 4400, and unaffected heterocysts (H) after treatment with actinomycete A52 filtrate. The surrounding thickened mucilaginous sheath was also clearly visible (SH).

(b) Untreated filament of Nostoc entophytum (52) x 4400

a



b

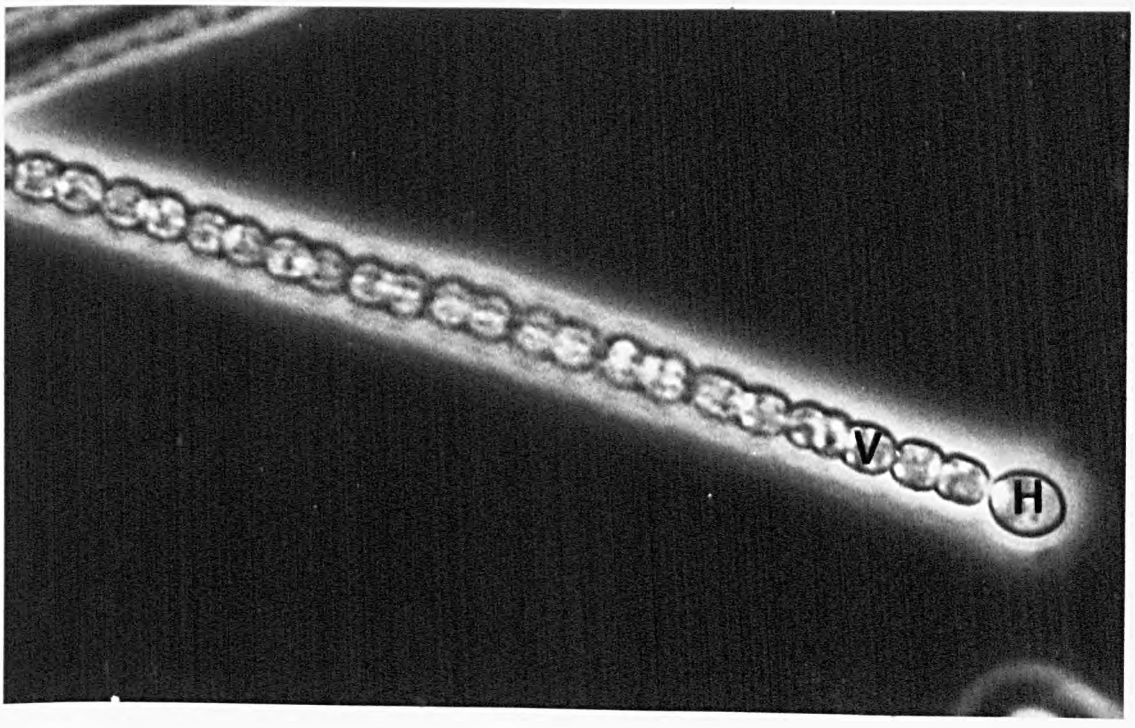


Plate 7

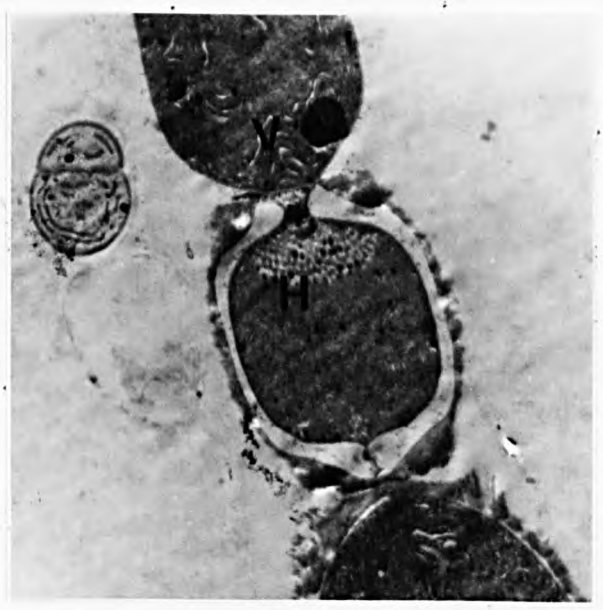
Plate 8

(a) Heterocyst (H) and an untreated vegetative cell (V) of cyanobacterium Anabaena oscillaroides (27) x 7500

(b) Typical heterocyst of untreated culture of Anabaena oscillaroides (27) x 40000

(c) Healthy filament of untreated Anabaena oscillaroides (27) x 9500

a



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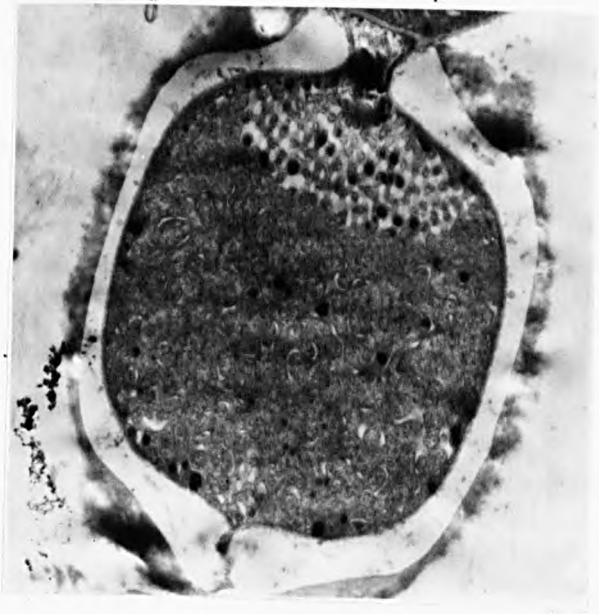


c

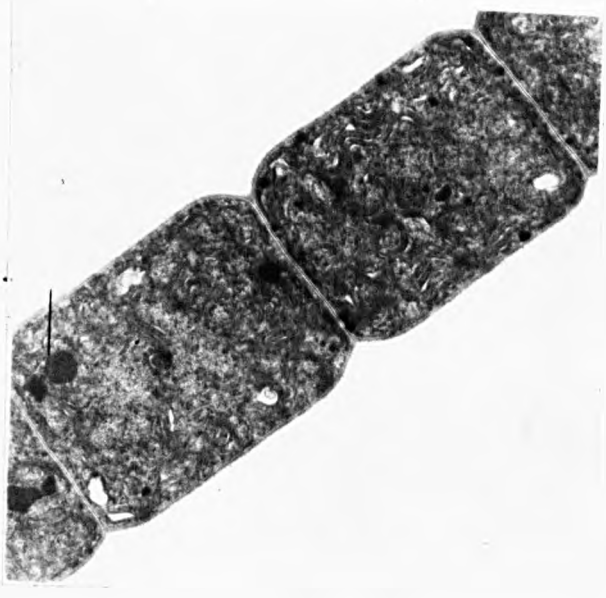


Plate 8

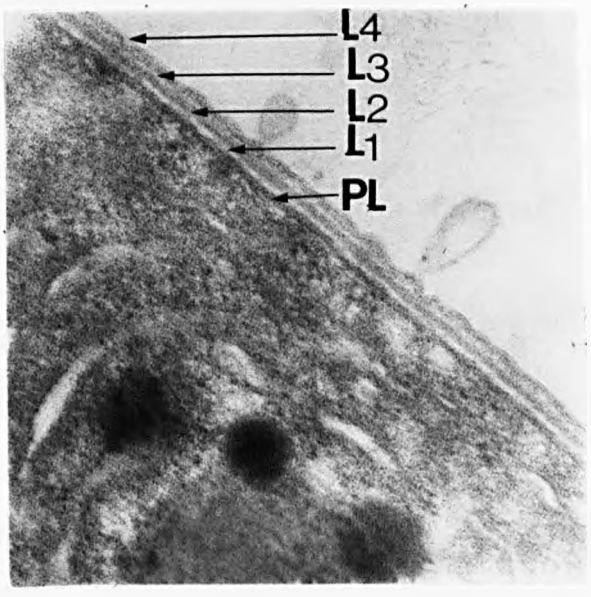
Plate 9

(a) Typical cell wall layers of the cyanobacterium Anabaena oscillaroides (27) x 110,000. All the cell wall layers (L1-4) and the plasma membrane (Pl) are visible.

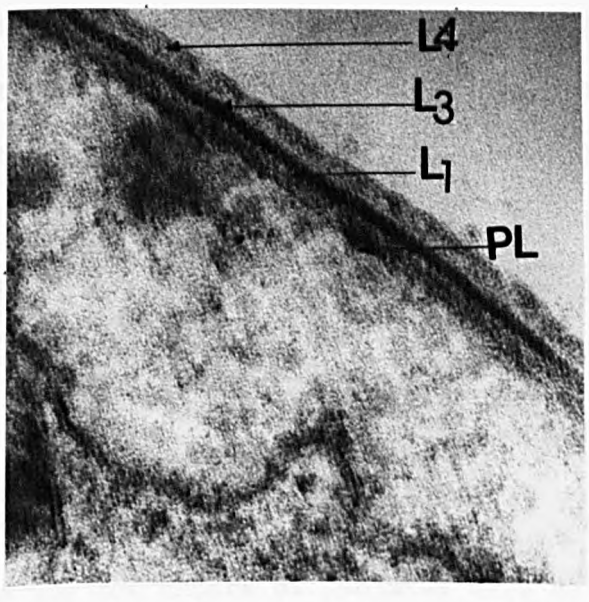
(b) First stages of lysis when the cyanobacterium Anabaena oscillaroides (27) x 110,000 was treated with the antagonistic actinomycete A52 filtrate. The L_2 layer of the cell wall is no longer visible.

(c) Later stages of lysis of Anabaena oscillaroides (27) x 160,000 by actinomycete A52 filtrate. Neither the L_2 nor L_3 layers are visible and only L_1, L_4 and the plasma membrane can be discerned.

a



b



c

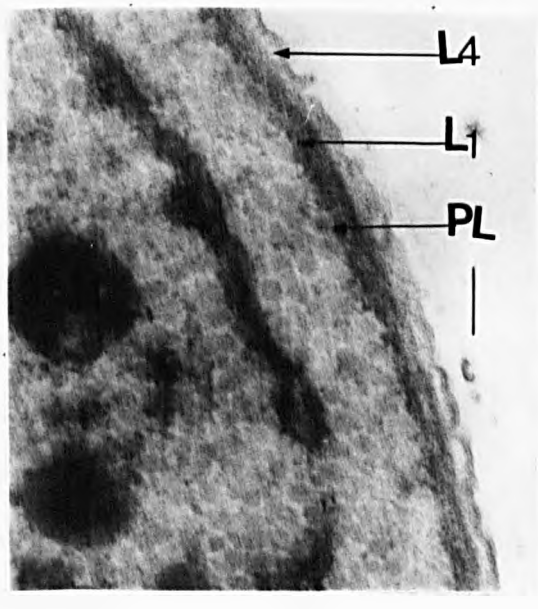


Plate 9

vegetative cells as protoplasts surrounded only by the plasmalemma (Plate 10). Ultimately, even the plasmamembrane disappeared.

The effect of lytic actinomycetes on green algae belonging to the order Chrococcales, was also investigated. These algae have a distinct two layered cell wall outside the plasmalemma (Bold and Wynne, 1978) (Plate 11), the inner wall layer being more rigid than the outer one.

The observations on the lysis of Coleastrum microsporum 280 are presented here and are typical of those obtained for all three green algae studied. After the treatment of Coleastrum microsporum with actinomycete A52 filtrate (or any other lytic actinomycete filtrate), the outer cell wall layer appeared to come away from the inner layer and membrane-bound protoplast. In later stages of lysis the inner wall, membrane and cell contents disappeared leaving just the outer cell wall layer visible (Plate 12).

4.1.3.4 The effects of cell-free filtrates of actinomycetes on the growth and physiology of cyanobacteria.

Four actinomycete isolates were selected for further study. The reasons for the choice of isolates were as follows:

(a) The laboratory actinomycete A52 caused lysis of most cyanobacterial test strains, ie. both laboratory and salt marsh isolates.

(b) Actinomycete 11B was one of the most lytic of the salt marsh isolates.

(c) Actinomycete salt marsh isolate 6B lysed certain test cyanobacteria but actually stimulated the growth of others.

(d) The least effective natural actinomycete isolate number 18 was also included as a sort of control for reference in case subsequent treatments altered the lytic properties of actinomycetes.

Plate 10

Partially lysed vegetative cells (V) of Anabaena oscillaroides (27) x 12000 treated with A52 filtrate. Only the plasmalemma (Pl) is visible surrounding the protoplast, and the cell wall layers have disappeared. A heterocyst (H) still surrounded by its wall can be observed.

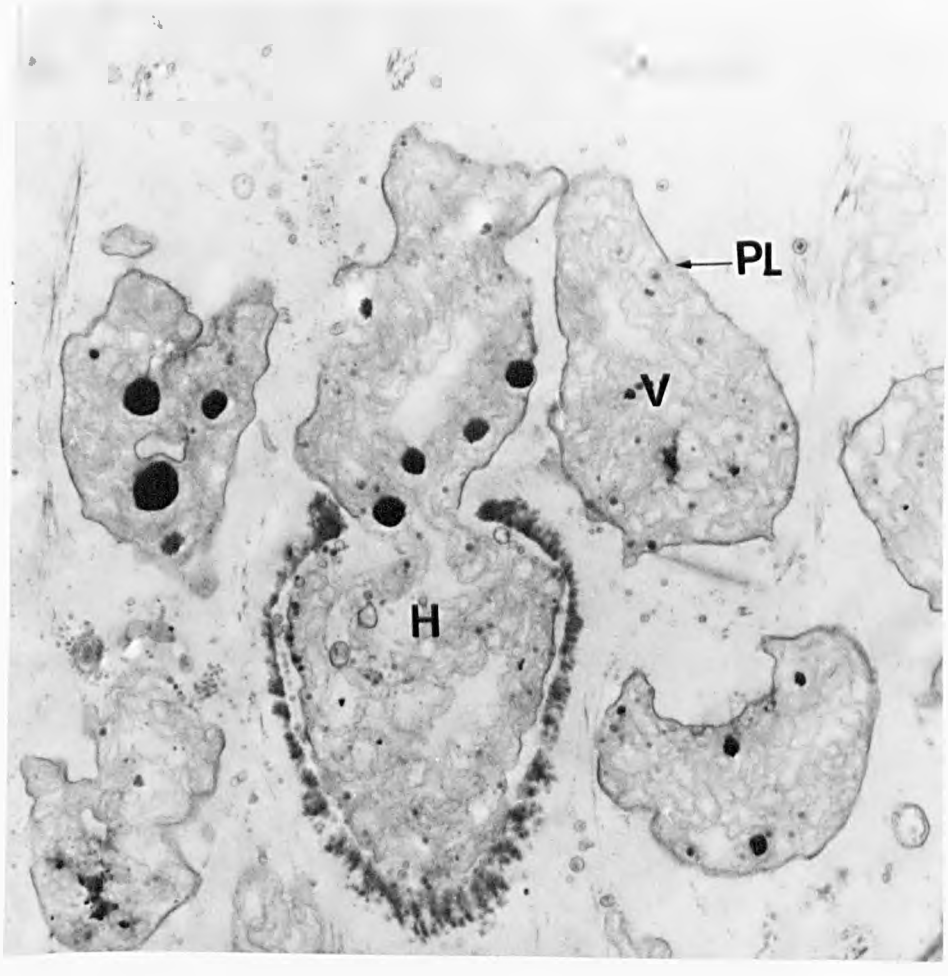


Plate 10

Plate 11

(a) Untreated vegetative cells of Coleastrum microsporum (280) x 7600 showing their typical structure.

(b) Untreated C. microsporum (280) x 42,000 cells showing details of the outer cell wall layer (OL.), the inner wall layer (IL.).

Plate 12

(a) C. microsporum (280) x 6700 treated with actinomycete A52 cell-free filtrate, & showing three stages of lysed vegetative cells (V_1, V_2 and V_3 The outer cell wall is also visible (OL).

(b) Part of the outer cell wall layer (OL.) x 30,000 which remained intact after disintegration of the vegetative cell and contents (P).

a

b

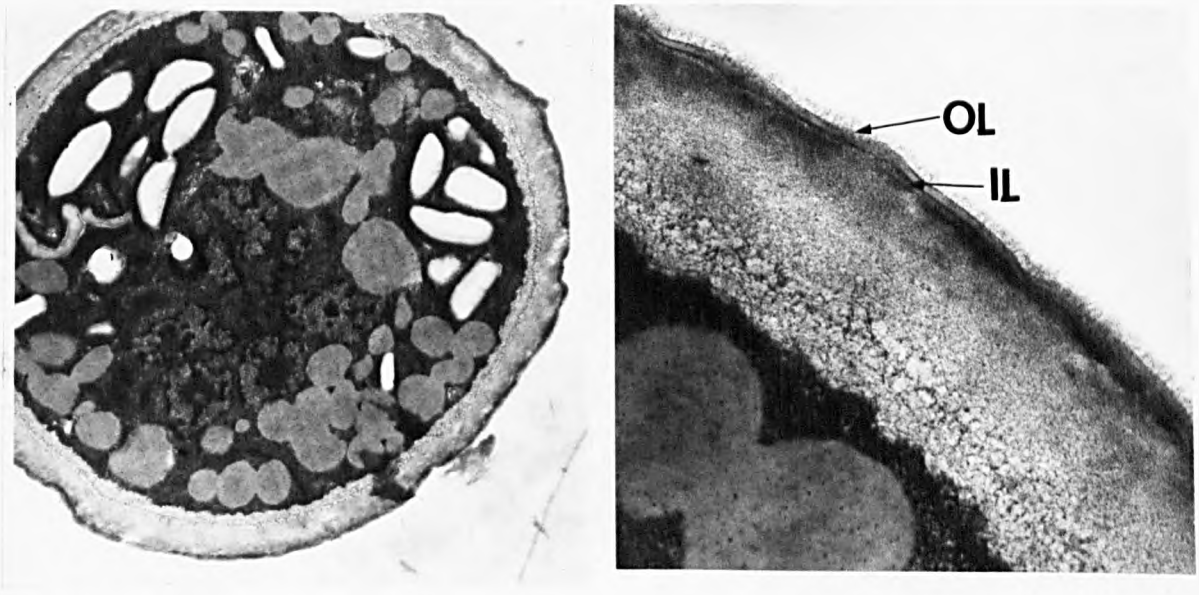


Plate 11

a

b

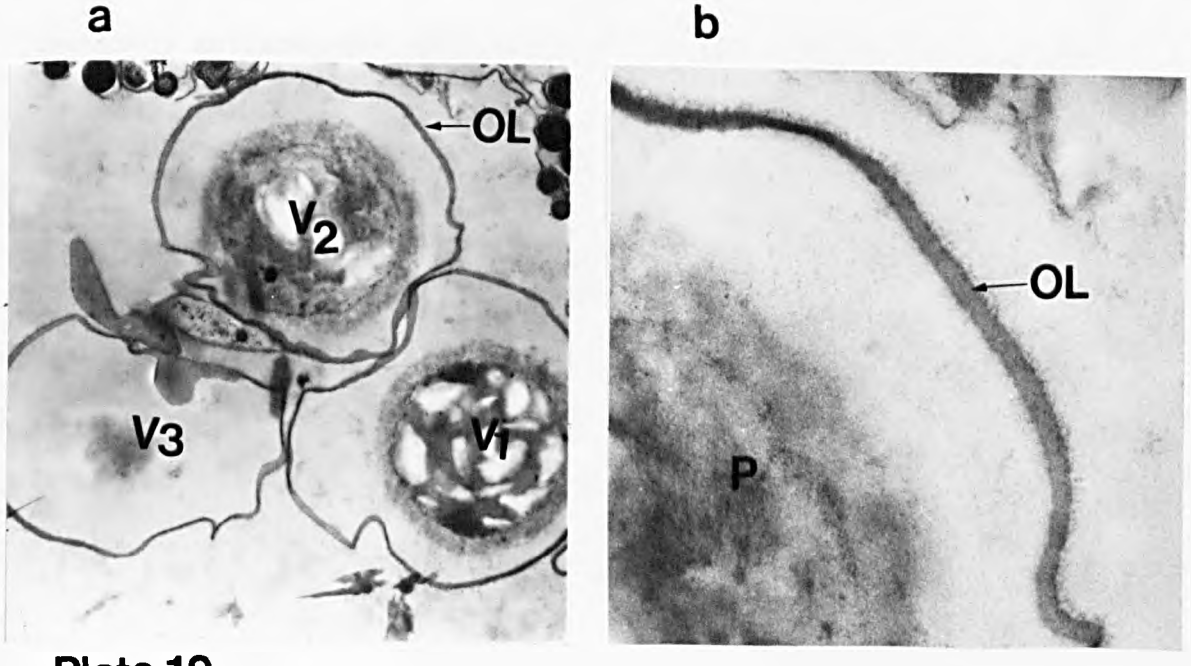


Plate 12

The choice of cyanobacteria species for test lawns was based on their showing unambiguous interaction results with the chosen actinomycetes in the original screening programme. The actual interaction combinations used in this section are presented in Table 19. These combinations of organisms were used in experiments to estimate the effect the actinomycetes have on growth, $^{14}CO_2$-fixation and nitrogen fixation of blue-greens.

Table 19. The combinations of actinomycete and cyanobacterial isolates used to investigate the effects of actinomycete filtrates on aspects of cyanobacterial metabolism and growth.

LBCC Code No.	Cyanobacterial species	Actinomycete strains
41	*Lyngbya halophila*	A52 *
14	*Anabaena microspora*	A52 *
27	*Anabaena oscillaroides*	11B
88*	*Anacystis nidulans*	11B
34	*Nostoc muscorum*	18
28	*Anabaena flos-aquae*	6B
48	*Nostoc punctiforme*	6B

* Laboratory actinomycete or cyanobacterium species.

The initial use of ISP5 media for actinomycetes cultivation, caused several problems when investigating the effect of active actinomycete filtrates on the metabolic activities of blue-greens. Small quantities of pure ISP5 medium alone (equivalent to the amount transferred to the blue-green cultures with the actinomycete filtrate) promoted the growth of the cyanobacterial cultures and it also inhibited nitrogenase activity. Subsequent experiments showed that it was asparagine in the medium that was causing both effects by acting as an additional

combined nitrogen source (Fig. 5).

The nutrient broth, which had also been used initially for actinomycete cultivation and in the interaction tests, also contained sufficiently high concentrations of combined nitrogen to similarly inhibit nitrogenase activity of the cyanobacteria. Therefore, a modified ISP5 medium was used with the asparagine being replaced by the same molar concentration of casitone, which had no effect on cyanobacterial growth and activity compared with the control medium, and did not obviously alter the growth of the actinomycete strains themselves.

The different concentrations of actinomycete filtrates (diluted with modified ISP5 medium) were added to liquid cultures of the cyanobacteria growing in Allen's medium. Growth of Anabaena microspora 14 was inhibited sequentially by increasing concentrations of the filtrate from actinomycete A52 (Fig. 6a). Nitrogenase activity (measured over 5 days) was also inhibited sequentially by the addition of increasing concentrations of the actinomycete filtrate (Fig. 6b), as was photosynthesis (Fig. 6c). The inhibited cultures turned pale yellow and lysis of the vegetative cells occurred with time. Lysis-causing actinomycete filtrate A52 showed similar effects on non-heterocystous cyanobacteria species, eg. Lyngbya halophila 41 (Fig. 7). The growth rate of this blue-green was again sequentially decreased by increasing concentrations of the A52 filtrate.

The effects of the actinomycete 11B recently isolated from the salt marsh was tested against both heterocystous and non-heterocystous cyanobacteria species, so that a comparison between the effects of a

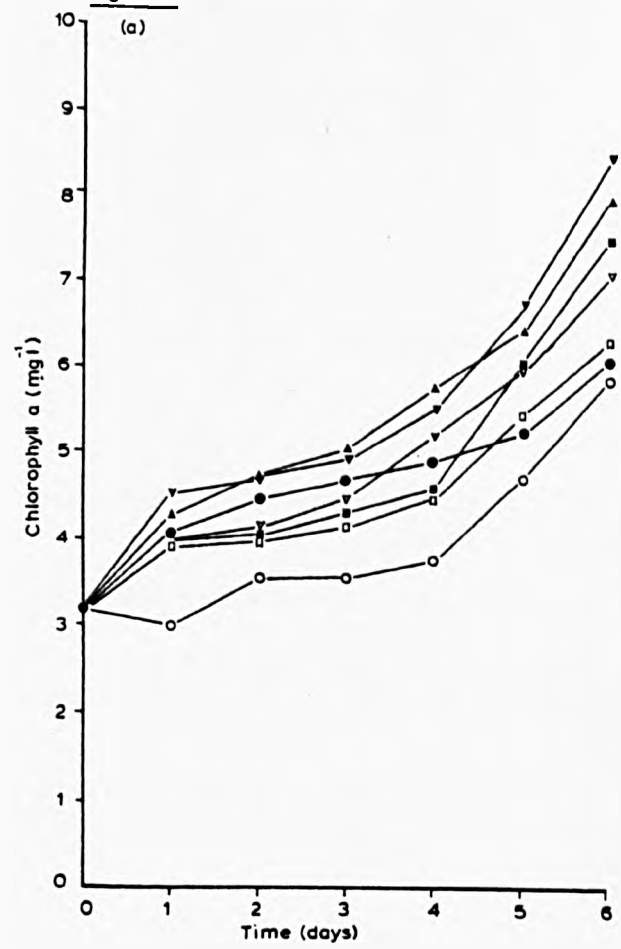
Figure 5

(a) The effect on the growth of 50cm^3 liquid cultures of Anabaena flos-aquae 85 of the addition of 5cm^3 aliquots of ISP5 medium containing o (●); 10 (o); 50 (□); 100 (▽); 250 (■); 500 (▲) and 1000 (▼); μM concentrations of asparagine.

(b) The effect of the above concentrations of asparagine in ISP5 medium on nitrogenase activity by Anabaena flos-aquae 85.

The maximum standard deviations are in (a) 0.0231 and (b) 1.022.

Figure 5


(a)
Chlorophyll a (mgl^{-1})
Time (days)

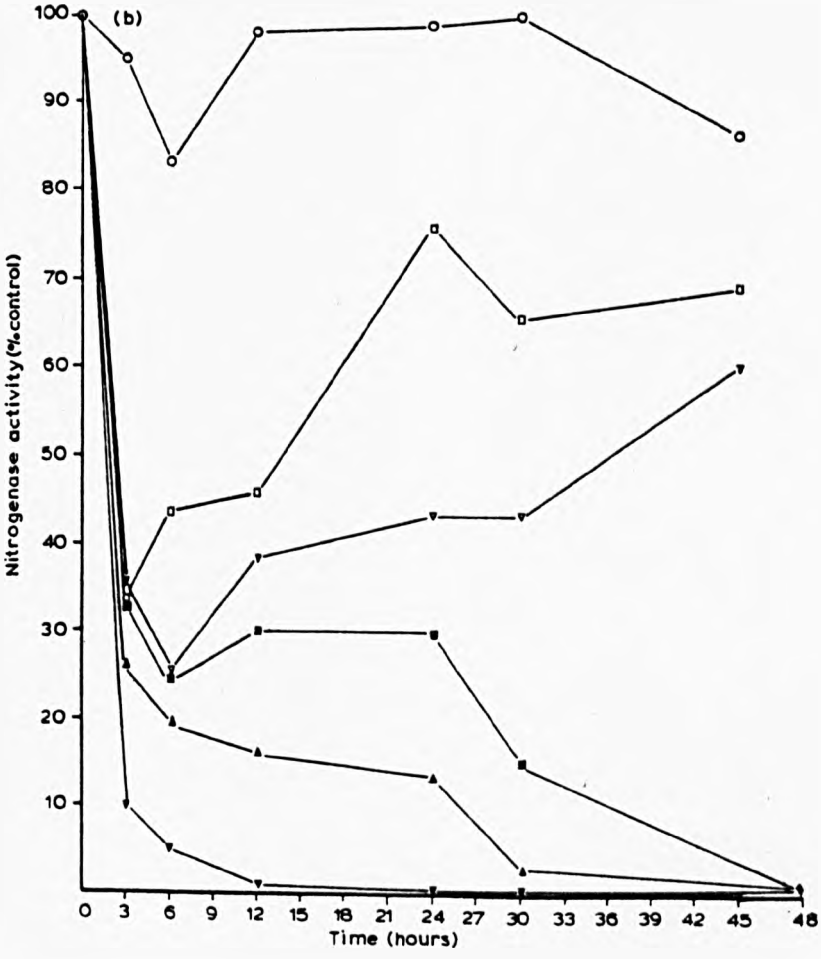

(b)
Nitrogenase activity (%control)
Time (hours)

Figure 6

(a) The effect of actinomycete A52 filtrate on the growth rate of Anabaena microspora (14) using filtrate concentrations of 10% (○), 50% (□), 100% (▲), and the control with addition of similar amount of actinomycete sterile ISP5 modified medium (●).

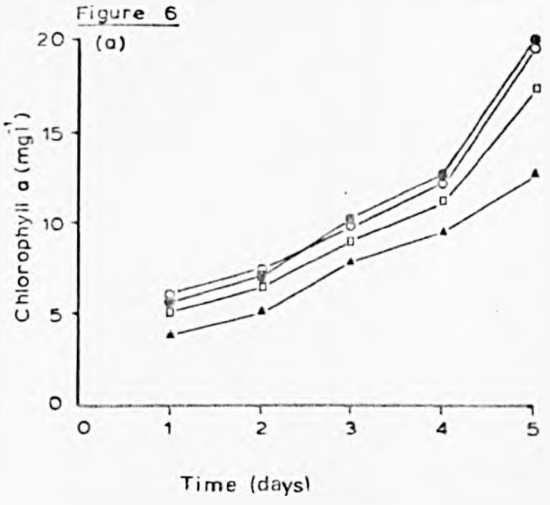
(b) The effect of actinomycete A52 filtrate on the nitrogenase activity of Anabaena microspora (14) using similar concentrations as coded in (a).

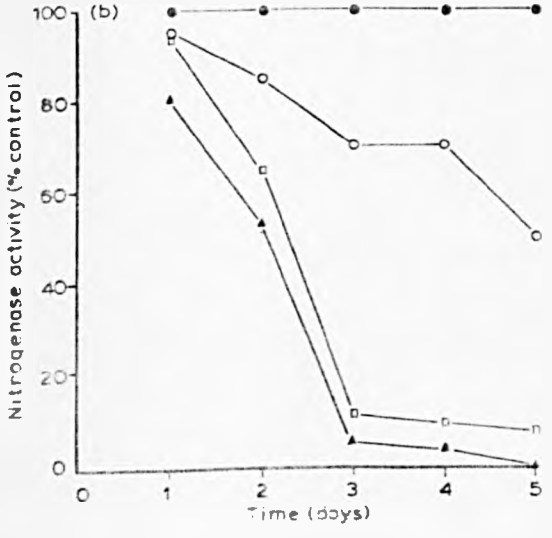
(c) The effect of actinomycete A52 filtrate on the $^{14}CO_2$ fixation of Anabaena microspora (14) using similar concentrations as coded in (a) and (b).

The maximum standard deviations are in

(a) 0.077 (b) 1.88 (c) 2.8

Figure 6


(a)
Chlorophyll a (mgl⁻¹)
Time (days)


(b)
Nitrogenase activity (% control)
Time (days)

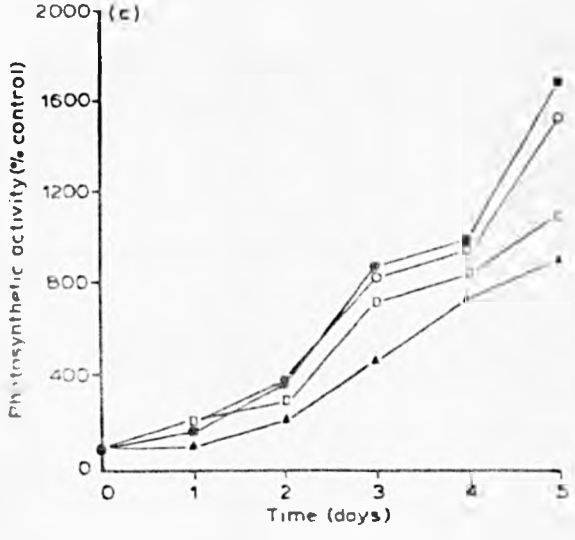
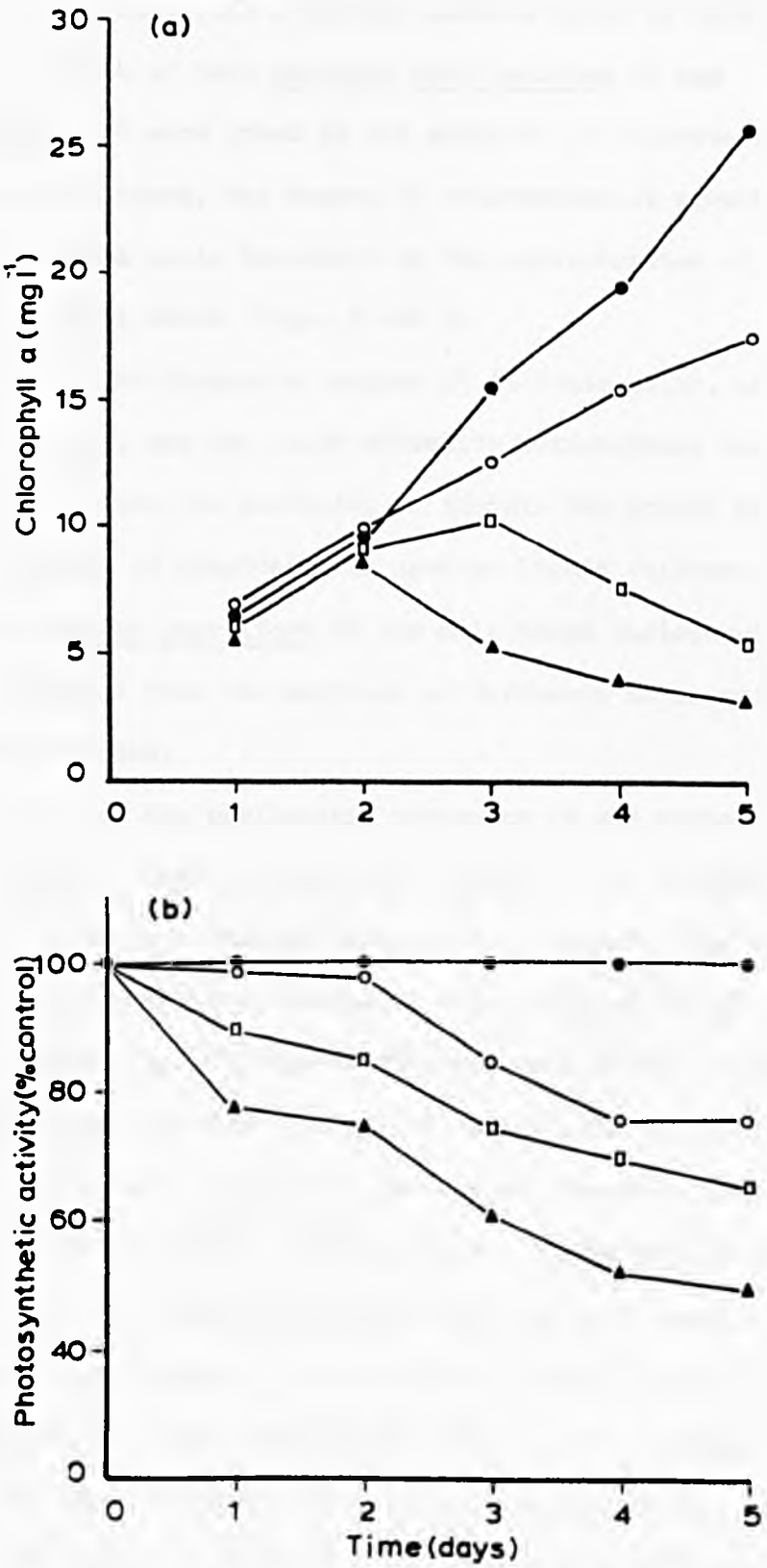

(c)
Photosynthetic activity (% control)
Time (days)

Figure 7

The effect of $5cm^3$ additions of actinomycete A52 filtrate at concentrations of 10% (o); 50% (□) and 100% (▲) of the original filtrate on the growth (Fig. a) and $^{14}CO_2$ fixation (Fig. b) of $50cm^3$ liquid cultures of Lyngbya halophila 41. A $5cm^3$ addition of sterile modified ISP5 medium was used as a control (●).

The maximum standard deviations are in (a) 0.042 and in (b) 1.210.

Figure 7


(a)
Chlorophyll a (mg l⁻¹)
30
25
20
15
10
5
0
0
1
2
3
4
5
(b)
Photosynthetic activity(%control)
100
80
60
40
0
Time(days)

laboratory culture (A52) with a natural isolate could be made.

Vegetative cells of both Anabaena oscillaroides 27 and Anacystis nidulans 88 were lysed by the addition of filtrate from actinomycete 11B cultures, the degree of retardation of growth of the cyanobacteria was again dependent on the concentration of actinomycete filtrate added (Figs. 8 and 9).

Dee salt marsh actinomycete isolate 18 filtrate which, in the initial interactions, was the least effective actinomycete isolate was used here to ensure its inability to inhibit the growth and nitrogenase activity of cyanobacteria species liquid cultures. Figure 10 shows Nostoc punctiform 34 Dee salt marsh isolate with no inhibition derived from the addition of different actinomycete 18 filtrate concentrations.

In the course of the preliminary screening it was noticed that certain actinomycete isolates which were inhibitory to a number of cyanobacteria seemed to stimulate the growth of others. The techniques used did not allow clear confirmation of this point as the actinomycete growth media itself often stimulated cyanobacterial growth (see Plate 5).

The possibility of a dual effect, ie. stimulation of some species but inhibition of others, has been investigated more carefully by adding cell-free filtrates from actinomycete cultures to liquid cultures of cyanobacteria. Actinomycete 6B (a natural Dee salt marsh isolate) was tested in this way against a range of salt marsh isolates of cyanobacteria. Typical data obtained for the growth, and nitrogenase activity of cyanobacteria in the presence of different concentrations of actinomycete filtrates are shown in Figs. 11 and 12. Filtrate from

Figure 8

The effect of $5cm^3$ additions of actinomycete 18 filtrate at concentrations of 10% (o); 50% (□) and 100% (▲) of the original filtrate on the growth (a) and nitrogenase activity (b) of $50cm^3$ liquid culture of Anabaena oscillaroides 27. A $5cm^3$ addition of sterile modified ISP5 medium was used as a control (●), and $5cm^3$ of sterile Allen's medium as another control (■).

The maximum standard deviations are in (a) 0.0323 and in (b) 1.121.

Figure 8

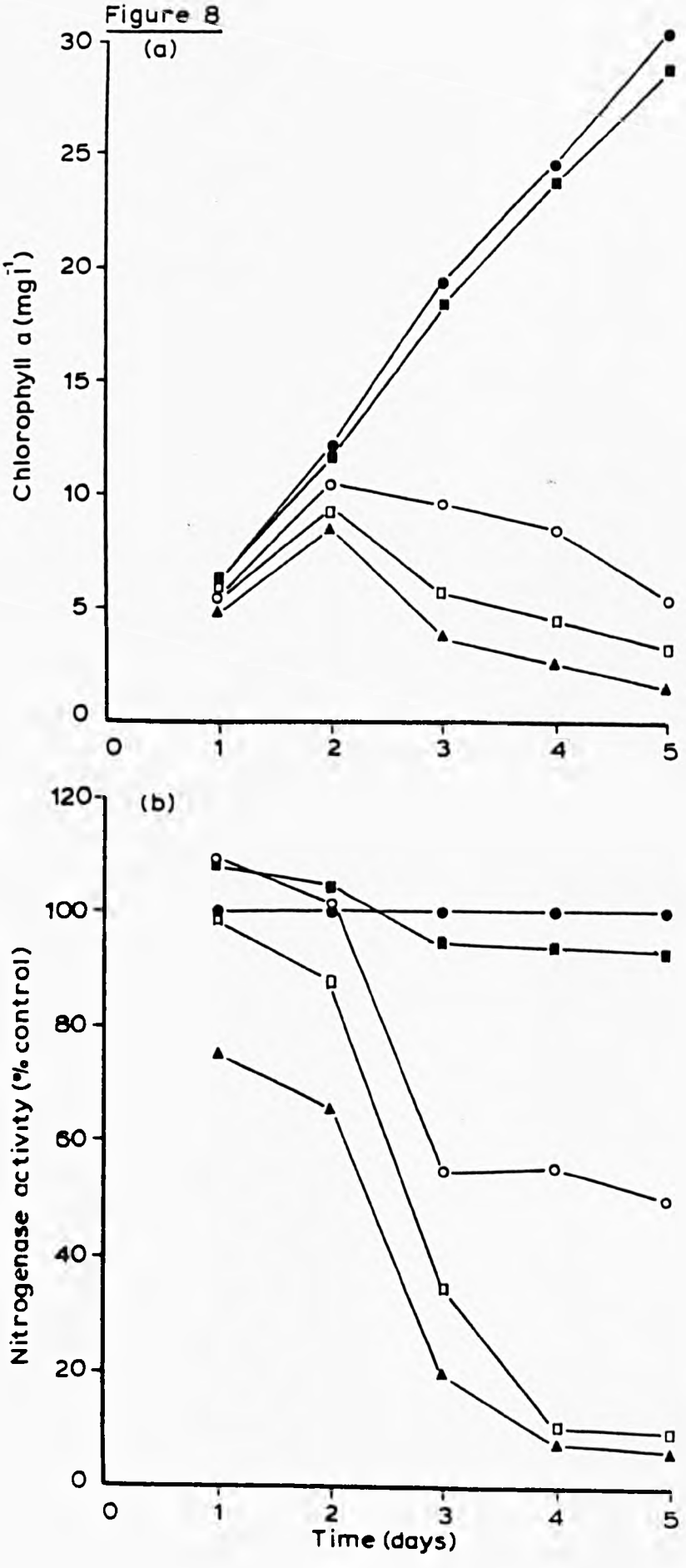

(a)
Chlorophyll a (mg l⁻¹)
30
25
20
15
10
5
0
0
1
2
3
4
5
(b)
Nitrogenase activity (% control)
120
100
80
60
40
20
0
0
1
2
3
4
5
Time (days)

Figure 9

The effect of actinomycete filtrate 11B on the growth rate of the non-heterocystous cyanobacterium Anacystis nidulans 88. The filtrate concentrations of 10% (o); 50% (□) and 100% (▲) of the original filtrate were used. A 5cm^3 addition of sterile modified ISP5 medium was used as control (●), and 5cm^3 of sterile Allen's medium was also used as another control (■).

The maximum standard deviation is 0.0251.

Figure 9

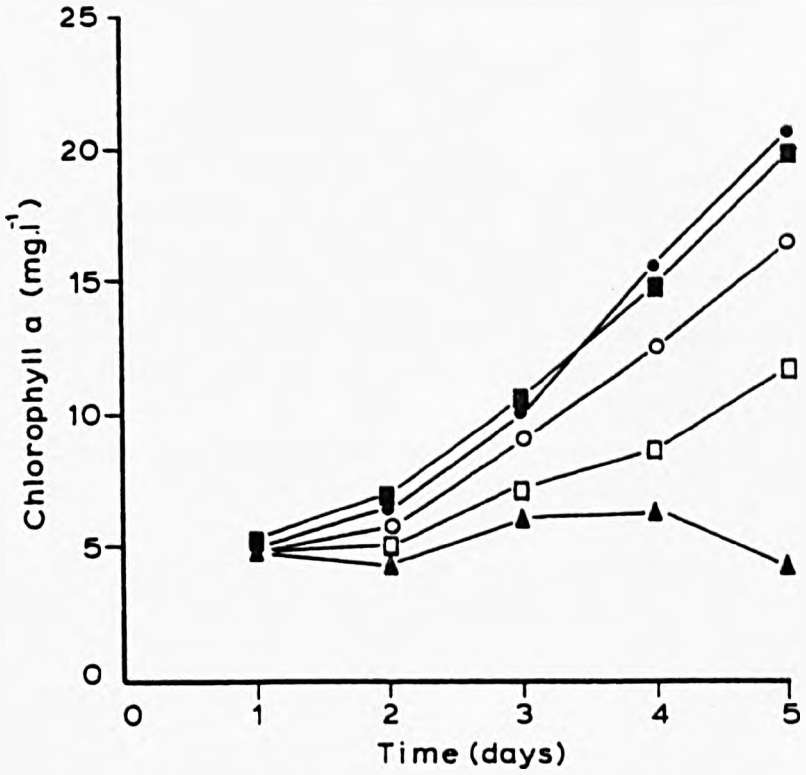


Figure 10

The effect of $5cm^3$ addition of recently isolated actinomycete 18 filtrate at concentrations of 10% (o); 50% (□) and 100% (▲) of the original filtrate on the growth (a) and $^{14}CO_2$ fixation (b) of $50cm^3$ liquid culture of Nostoc punctiforme 34. A $5cm^3$ addition of sterile modified ISP5 medium was used as a control (●).
NB. The 50% and 10% results were deleted for clarity in the figure.

The maximum standard deviations are in (a) 0.0131 and in (b) 1.802.

Figure 10

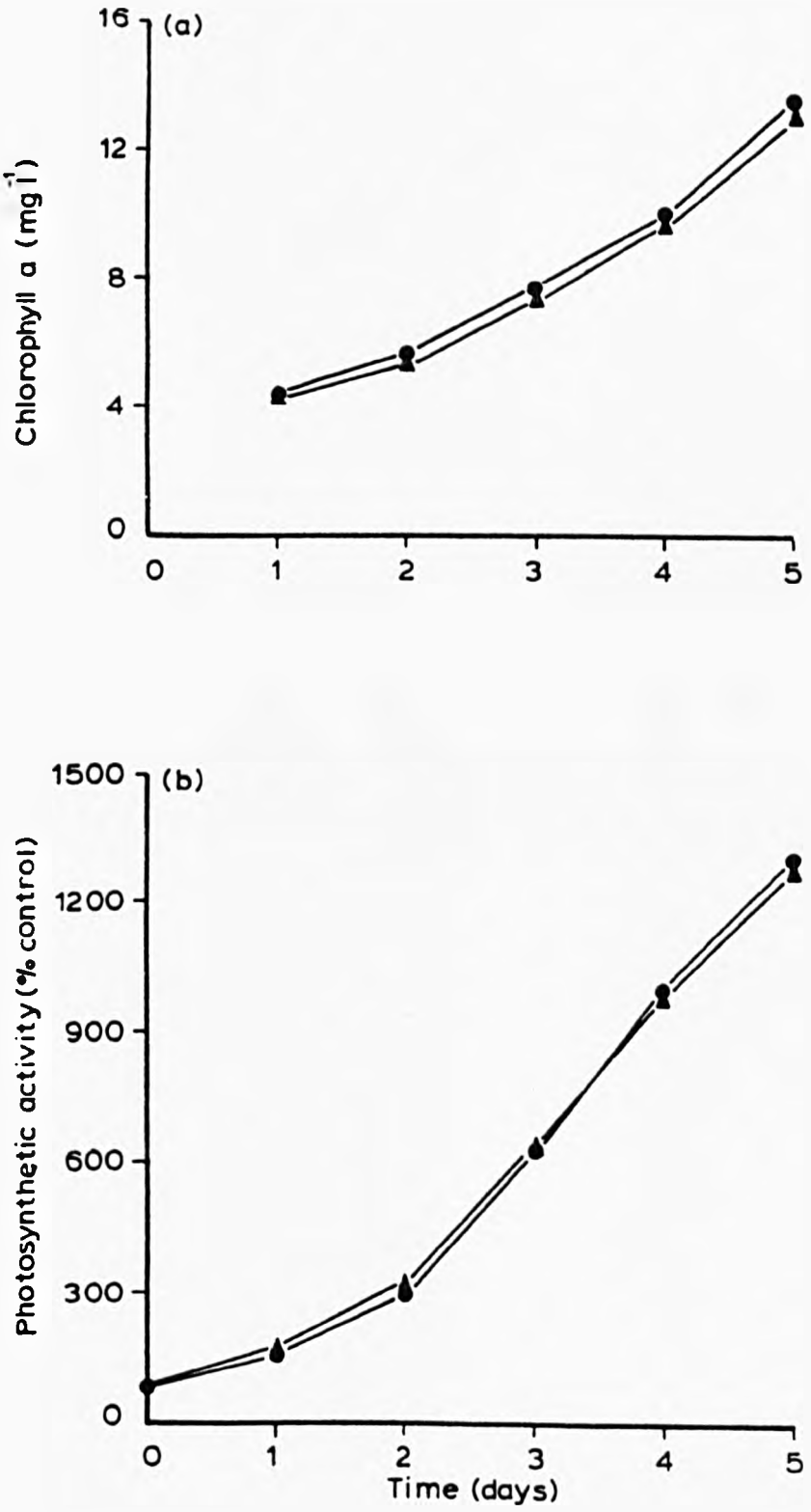

(a)
Chlorophyll a (mg l⁻¹)
16
12
8
4
0
0
1
2
3
4
5
(b)
Photosynthetic activity (% control)
1500
1200
900
600
300
0
0
1
2
3
4
5
Time (days)

Figure 11

The inhibitory effect of actinomycete 6B filtrate on the blue-green species Nostoc punctiforme 48 growth (a) and nitrogenase activity (b) using concentrations of 10% (o); 50% (□) and 100% (▲), of filtrate. A 5cm^3 aliquot of either Allen's medium (●) or modified ISP5 medium (■) was added to act as controls.

Figure 11

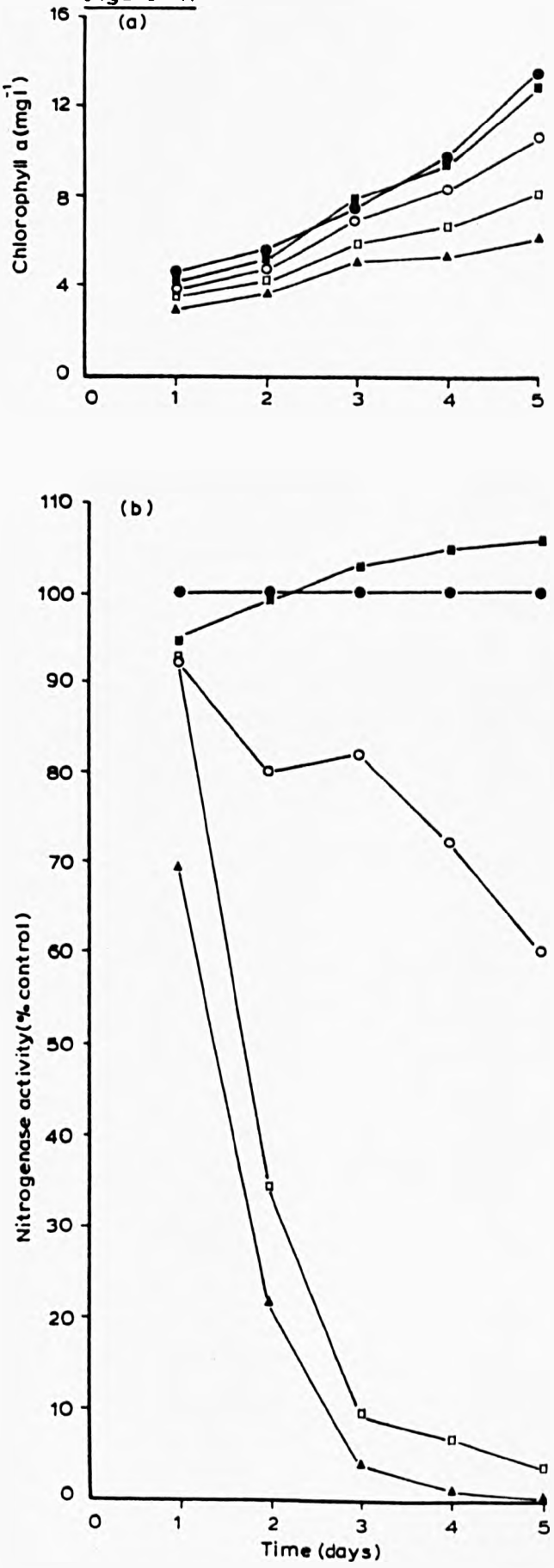
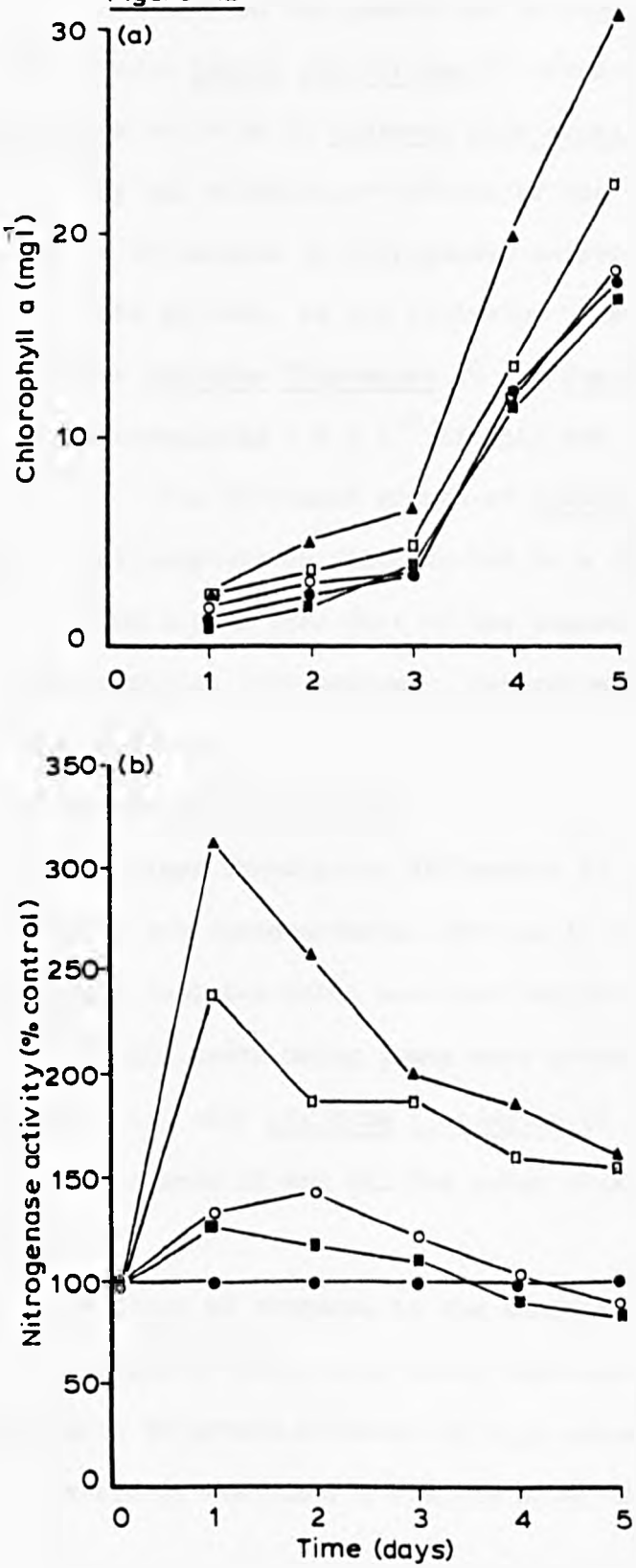

(a)
Chlorophyll a (mg l⁻¹)
16
12
8
4
0
0
1
2
3
4
5
(b)
Nitrogenase activity (% control)
110
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
Time (days)

Figure 12

The stimulatory effect of actinomycete 6B filtrate on the growth rate (a) and nitrogenase activity (b) on *Anabaena flos-aquae* 28 using filtrate concentrations of 10% (o); 50% (□) and 100% (▲). A 5cm^3 aliquot of either Allen's medium (●) or modified ISP5 medium (■) was added to act as controls.

The maximum standard deviations are in (a) 0.0132 and (b) 1.035.

Figure 12


(a)
Chlorophyll a (mgl⁻¹)
30
20
10
0
0
1
2
3
4
5
(b)
Nitrogenase activity (% control)
350
300
250
200
150
100
50
0
0
1
2
3
4
5
Time (days)

actinomycete 6B which inhibited the growth and nitrogenase activity of cyanobacteria Nostoc punctiforme 48 actually stimulated growth and nitrogenase activity in Anabaena flos-aquae 28.

These inhibitory and stimulatory effects of the filtrate were not solely the result of changes in nitrogenase activity, which in turn altered the growth pattern, as the filtrates caused the same effects on growth when Anabaena flos-aquae 28 and Nostoc punctiforme 48 were grown on medium containing 1.5 g l^{-1} nitrate and exhibiting no nitrogenase activity. The increased growth of Anabaena flos-aquae 28 in the presence of actinomycete 6B filtrate led to a dry weight yield (after 5 days) 15.6% higher than that of the controls inoculated initially with just modified ISP5 medium. Heterocysts frequently increased from approximately 4.5% of the total cell count in the controls to 6.9% in 6B treated cultures.

Because of this clear unambiguous difference in response of actinomycete 6B to the two cyanobacterial strains in liquid culture all the cyanobacterial isolates which were not inhibited by 6B in the preliminary screening tests using lawns were retested against 6B in liquid culture. Still only Anabaena flos-aquae 28 showed a stimulatory response towards 6B and all the other blue-greens behaved as before.

There was a gradient of response to the addition of actinomycete filtrates to the blue-green cultures in these experiments such that most of the inhibition of growth occurred at high concentration of filtrate and this response was clearly visible after three days of

treatment. It has been suggested by Bershova et al., (1968) that bacterial filtrates which were inhibitory to other microbes at high concentrations caused stimulation when diluted but the details of the methods and the amount of dilution of the filtrates were not given. Actinomycete isolates which caused inhibition or had no effect on the blue-greens in our experiments were further tested using a dilution series of their filtrates (down to 0.1%) and applying these dilutions to cyanobacterial lawns. The results obtained gave no evidence of stimulation when the filtrates were diluted up to a thousand times.

4.2 The effects of fungi on cyanobacteria

4.2.1 Introduction

Certain fungal pathogens of cyanobacteria have been studied in detail and shown to be quite host specific. For instance, Rhizophydium ubiquetum only lysed Anabaena solitaria (Canter 1968; 1972). The fungi which cause lysis of blue-greens are predominantly (although not extensively), members of the Chytridiales. One notable exception is Blastocladiella anabaena, which is a member of the Blastocladiales and has been shown to lyse Anabaena solitaria and Aphanizomenon sp. (Canter, 1972).

Many habitats have been investigated for sources of pathogenic fungi active against blue-greens including the English and Swiss lakes (Canter and Lund, 1951). The lysing of cyanobacteria by fungi is considered to be due to the release of antibiotics. However, the concentrations of the antibiotics active against the blue-greens varies with the particular blue-green species tested (Srivastava, 1969; 1970; Kumar, 1963; Srivastava and Nizam, 1969; Reddy, 1975; and Redhead and Wright, 1980).

Redhead and Wright (1978) have reported that members of the genera Acremonium, Emericellopsis and Verticillium also lyse cyanobacteria, and suggest that diffusible extracellular products are the lytic agents. They also observed that with many lytic compounds the heterocysts and akinetes seem more resistant to attack than the vegetative cells.

4.2.2 Materials and methods

The natural salt marsh fungal isolates were tested against both the laboratory and the Dee salt marsh cyanobacteria species. The same techniques were employed here as in the last section. Therefore, both agar blocks containing fungi and cell-free filtrates from liquid fungal cultures were applied to the cyanobacterial lawns. Cell-free filtrates of the fungi were also added to liquid cultures of cyanobacteria and the effects on growth and nitrogenase activity determined (see Chapter 2).

Although potato dextrose agar media was used to cultivate the fungi in the initial screening tests, malt extract broth was used to grow the fungi when cell-free filtrates were to be used since potato dextrose showed some evidence of stimulating the growth of the blue-greens. As before, Allen's medium was used to grow the cyanobacteria on agar plates or in liquid culture.

4.2.3 Results

4.2.3.1 Interaction tests between Dee salt marsh fungal isolates and cyanobacteria species

On certain cyanobacterial lawns clear inhibition zones appeared around some of the fungal blocks or wells containing cell-free filtrates within three days (Plate 13). The results of the basic interaction tests

Plate 13

The effect of agar discs containing fungal hyphae of specific fungal isolates (F) on the growth of a cyanobacterial lawn of Synechococcus elongatus 54 (L). The clear inhibition zone (I) shows where lysis of the cyanobacterial lawn has occurred. Pure potato dextrose agar block medium was placed in the centre of the plate as a control (C).

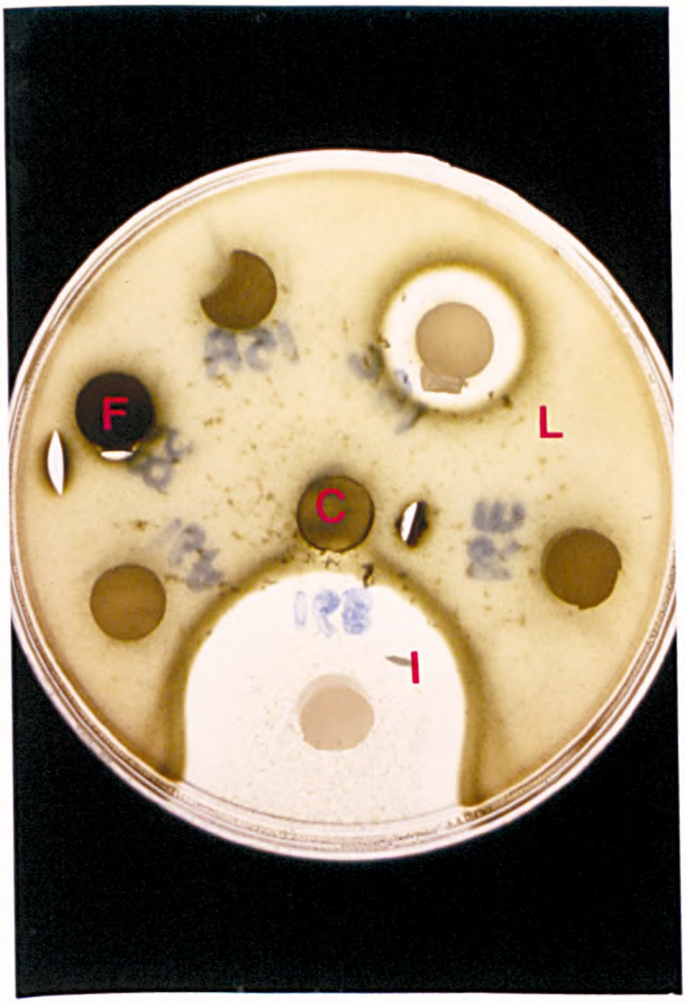


Plate 13

between fungi and cyanobacteria are given in Table 20. These data show that, like actinomycetes, the fungal isolates show a pattern of lytic activity against cyanobacteria. Not all fungal isolates showed lytic activity, e.g. fungal isolate 8B showed no lytic activity against cyanobacteria species. In contrast to 8B isolates 13A and 13B showed lytic activity against a wide range of different cyanobacterial strains. However, all the laboratory and natural cyanobacterial isolates were susceptible to at least two fungal isolates.

The overall index of relative sensitivity of individual cyanobacterial species to lysis by the fungal isolates are given in Table 21. These data show that Nostoc entophytum 52 was the most sensitive species being lysed by nearly 48% of the test fungi. The next most sensitive species were Nostoc muscorum 34 and Ancystis nidulans 88. In contrast the least sensitive cyanobacteria species were Synechococcus elongatus 54, Anabaena oscillaroides 27 and Nostoc sphaeroides 37.

An inhibitory index showing the effectiveness of each fungus against the test cyanobacteria as a whole is given in Table 22. These data show that Penicillium 13A and Penicillium 13B possessed the most effective lysing agents followed by Trichoderma 11B, Aspergillus 15A and Trichoderma 15C. However, seven fungal isolates, ie. Zygorhyneus 9, Biopolaris 10A, Cladosporium 8B, Trichoderma 12E, Fusarium 13C, 13F and 14C did not show any lytic activity against any of the tested cyanobacteria.

Table 20 The effects of agar block applications containing colonies of Dee salt marsh isolates of fungi on lawns of all the Dee salt marsh and laboratory species of cyanobacteria

Cyanobacteria species grown on Allen's (c) media				Fungi grown on PDA medium																																				
BCC N^O	Section	Genus	Classical identification	8A	8B	8C	9	10A	10C	10D	10E	11A	11B	11C	11D	12A	12B	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
19		Synechocystis	Synechocystis diplococcus (Pringsh.)	-	-	-	-	-	-	+	-	-	+	-	+	-	+	-	-	-	-	-	-	+	-	-	-	-		+	+					-			+	
54	I	Synechococcus	Synechococcus elongatus	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	-	-	-	-	-	-	-	-			+	+		-			+				
88		Synechococcus	Anacystis nidulans	-	-	-	-	-	+	+	-		+		+	-	+		-		+	+	-	-		-	-	-	+	+	+		-	-	-	+				-
74		LPPGpB	Simploca thermalis (KUTZ) GOM	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-		+	+	-	+	-	-			+	-	+		-		-	-	-	+	+	+
41		LPPGpB	Lyngbya halophila Hansg	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-		+	-	-	-	-	+	+	-		-			-		+	+	+
68	III	LPPGpB	Symploca elegans KUTZ	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	+	-	S	+	-	-	-	-	+	+	+	-	+	-		-	-	-	-	-
89		LPPGpB	Plectonema boryanum	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	+	+	-			-	+	+			+
90		LPPGpB	Lyngbya spp	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	+	-	+	-	-		-	+	+	+
91		LPPGpB	Oscillatoria spp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	+	+	-	-	-	-	+	S	S	-	-
14		Anaboena	Anaboena microspora Kleb	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+
17		Anaboena	Anaboena minutissima Lemm	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	S	-	-	-	+
27		Anaboena	Anaboena oscillarioides Bory	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-
28		Anaboena	Anaboena flos-aquae (Lyngb.) Breb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
85		Anaboena	Anaboena flos-aquae A37	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-		+		
86		Anaboena	Anaboena cylindrica	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-	+	-	+	+	-			
48		Nostoc	Nostoc punctiforme (KUTZ) Hariot	-	-	-	-	-	-	-	-	-	+	+	-	-	+		-	-	-	+	-	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-		-
52		Nostoc	Nostoc entophytum Born et Flah	-		+			+	-	+	-	-	+	-	-	-	+	-		+	-		-	-	-	+	-	+	-	-	-	+	+	+	+	+			-
8	IV	Nostoc	Nostoc linckia (Roth.) Born et			-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	
34		Nostoc	Nostoc muscorum Ag	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-		
37		Nostoc	Nostoc sphaeroides KUTZ	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+				
45		Nostoc	Nostoc piscinale KUTZ	-	-	-	-	-	-	-	-	+	+	-	+	-		-	-	-	+	+	-	-	-	-	-	-	+	+	+	+						-		
29		Nostoc	Nostoc verrucosum Vauch	-		-	-	-	+	+	-	-	+	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	+	-	+	-	-	-	-	-				
87		Nostoc	Nostoc muscorum	-	-	-	-	-	+	+	-	-	+	-				+	-	+	+	+	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	+	+	+
62		Cylindospermum	Cylindospermum majus	-	-	-	-	-	-	-	-	-	+	-	-		-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
40		Nodularia	Nodularia harveyana var. sphaerocarpa		-	-	-	-	-	-	-	-	+		+	-		-	-	-	+	+		+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Blue green growth was either inhibited (+) stimulated (s) or apparently unaffected (−) by various fungal isolates
These results were obtained from at least triplicate assays

Table 21. The sensitivity index of cyanobacteria species grown on Allen's medium towards Dee salt marsh fungal isolates grown on P.D.A. medium.

L.B.C.C. Code numbers	Cyanobacteria species	No.of different fungal isolates causing lysis	Sensitivity index %
19	Synechocystis diplococcus	9	24.3
54	Synechococcus elongatus	3	8.1
88	Anacystis nidulans	13	35.1
74	Symploca thermalis	9	24.3
41	Lyngbya halophila	11	29.7
68	Symploca elegans	8	21.6
89	Plectonema boryanum	6	16.2
90	Lyngbya sp.	11	29.7
91	Oscillatoria sp.	5	13.5
14	Anabaena microspora	6	16.2
17	Anabaena minutissima	5	13.5
27	Anabaena oscillaroides	3	8.1
85	Anabaena flos-aquae	2	5.4
28	Anabaena flos-aquae	3	8.1
86	Anabaena cylindrica	9	24.3
48	Nostoc punctiforme	11	29.7
52	Nostoc entophytum	18	48.6
8	Nostoc linckia	5	13.5
34	Nostoc muscorum	4	10.8
37	Nostoc sphaeroides	3	8.1
45	Nostoc piscinale	12	32.4
29	Nostoc verrucosum	9	24.3
87	Nostoc muscorum	14	37.8
62	Cylindospermum majus	4	10.8
40	Nodularia harveyana v.p.	5	13.5

$$\text{Sensitivity index} = \frac{\text{Number of inhibitory isolate of fungi}}{\text{Total number of tested fungi}} \times 100$$

NB. Total number of test fungi = 37

Table 22. The inhibitory index of fungal isolates against laboratory species and Dee salt marsh isolates of cyanobacteria.

Fungal isolates L.B.C.C. No.	Fungal genera	Inhibitory index against cyanobacteria
13A	Penicillium	76
13B	Penicillium	72
11B	Trichoderma	56
15A	Aspergillus	56
15C	Trichoderma	52
17B	Penicillium	40
18C	Penicillium	40
10C	Trichoderma	36
11D	Trichoderma	36
15B	Trichoderma	36
13D	Trichoderma	32
18B	Aspergillus	28
16B	Penicillium	28
18A	Trichoderma	24
10D	Trichoderma	20
17A	Fusarium	16
11C	Aspergillus	16
12B	Mucor	16
12D	Aspergillus	12
12F	Aspergillus	12
17C	Penicillium	8
8A	Trichoderma	8
16A	Trichoderma	8
8C	Biopolaris	4
10E	Penicillium	4
11A	Trichoderma	4
12A	Fusarium	4
13E	Fusarium	4
14A	Drechslera	4
16C	Trichoderma	4
9	Zygorhyncus	0
10A	Biopolaris	0
12E	Trichoderma	0
13C	Fusarium	0
13F	Fusarium	0
14C	Fusarium	0
8B	Cladosporium	0

The cyanobacteria species were grown on Allen's medium and fungi were grown on P.D.A. medium

$$\text{Inhibitory index} = \frac{\text{Number of the inhibited cyanobacteria}}{\text{Total number of cyanobacteria species tested}} \times 100$$

N.B. Total number of tested cyanobacteria species = 25

When fungi cell-free filtrates were applied the results were identical to those obtained with agar cores. As with the actinomycetes, no direct contact between fungal hyphae and cyanobacterial cells was required for lysis to occur. Clearly the lytic agent is released from the fungal hyphae into the medium.

Observations with the light microscope showed that, as with lytic actinomycete filtrates, the fungi lysed vegetative cells leaving the akinetes and heterocysts isolated but apparently intact. Plate 14 shows examples of typical results obtained when various blue-greens were treated with certain fungi.

Swelling of the sheath surrounding filaments of Nostoc entophytum 52, first noted when it was treated with actinomycete filtrates, occurred when fungal filtrates were added to the liquid blue-green culture (see Plate 7).

4.2.3.2 The effects of cell-free filtrates of fungi on the growth and nitrogenase activity of cyanobacteria species.

The antagonistic effects of fungal filtrates on the growth and nitrogenase activity of certain cyanobacterial species was investigated. Two fungal isolates Aspergillus 15A, Trichoderma 18A and four species of cyanobacteria, Anabaena flos-aquae 85, Nostoc punctiforme 48, Synechocystis diplococcus 19 and Anabaena oscillaroides 27 were used. The choice of isolates was based on the inhibitory or stimulatory effects shown by these fungi on particular cyanobacteria species during the preliminary screening programme.

A series of dilutions of fungal filtrates was prepared using malt extract liquid medium as a dilutant. The experimental procedure used to test the effect of these filtrates was basically the same as that used with actinomycete filtrates.

Plate 14

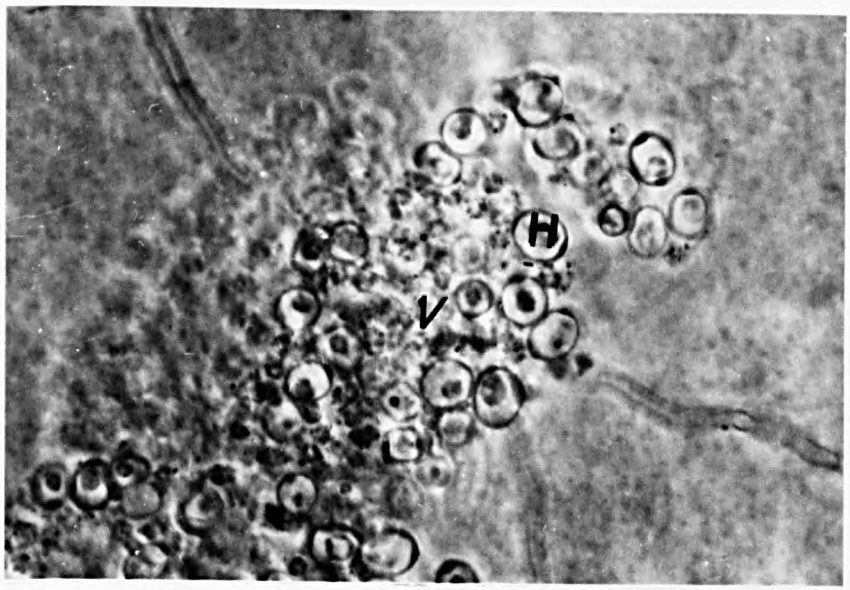
The morphological effects of the salt marsh fungal isolates on different blue-greens isolated from the same habitat.

(a) Vegetative cells (V) of Nostoc verrucosum (29) x 1700 lysed by Penicillium 13A. The heterocysts (H) remained unaffected.

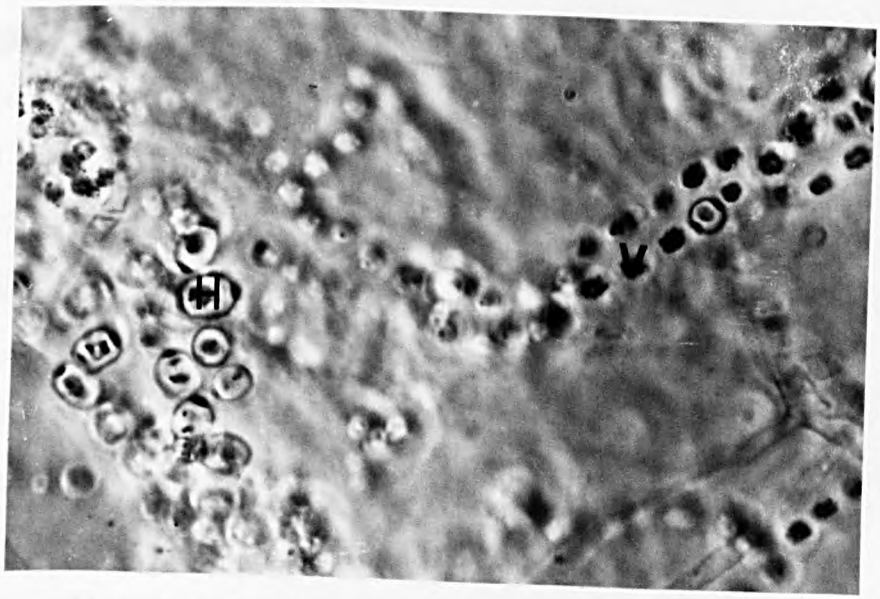
(b) Vegetative cells (V) of Anabaena microspora (14) x 1700 lysed by the addition of Fusarium 17A. The heterocysts (H) remained intact.

(c) Vegetative cells (V) of Nostoc punctiforme (48) x 1700 lysed by the addition of Aspergillus 15A. The heterocysts (H) and the akinetes (K) remained unaffected.

a



b



c

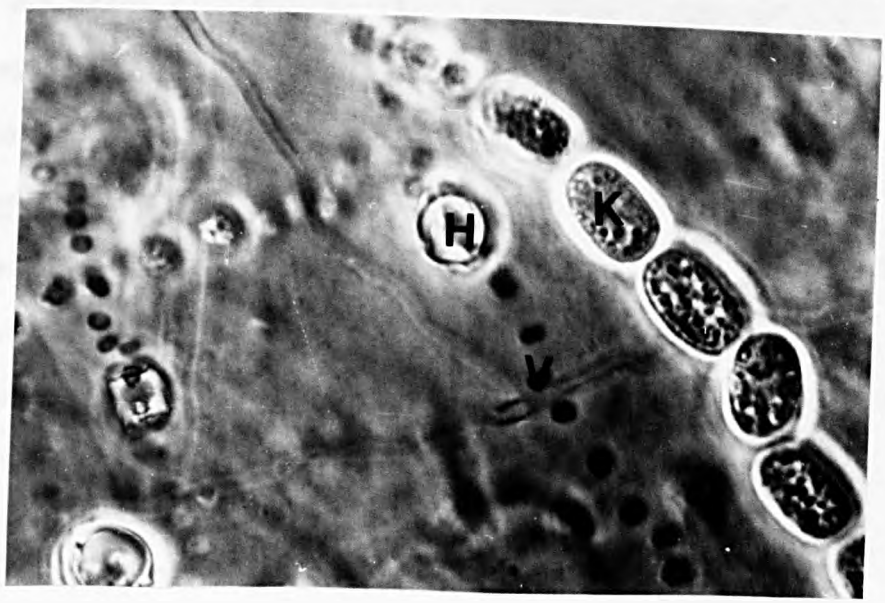


Plate 14

Typical effects of lytic fungal filtrates on the growth and nitrogenase activity of heterocystous cyanobacteria in liquid culture are shown by Aspergillus 15A on both Anabaena flos-aquae and Nostoc punctiforme (Fig. 13). The degree of inhibition of cyanobacterial growth and nitrogenase activity was directly proportional to the concentration of the fungal filtrate added. A 50% strength filtrate of Aspergillus 15A completely suppressed the growth of cyanobacteria. The vegetative cells were lysed completely within five days in Nostoc punctiforme and after a slightly longer time in Anabaena flos-aquae. Nostoc punctiforme was observed to be more susceptible to attack by Aspergillus 15A filtrate than Anabaena flos-aquae, and the latter showed resistance to 10% concentration of the filtrate Growth and nitrogenase activity in Nostoc punctiforme was increasingly inhibited by increasing filtrate.

The addition of Trichoderma 18A filtrate to liquid cultures of Symploca thermalis 74 and Nostoc muscorum 87 caused a concentration dependent inhibition of growth and nitrogenase activity (Fig. 14), as was found with Aspergillus 15A. When Trichoderma 18A filtrate (in malt extract) was added to liquid cultures of Anabaena oscillaroides 27 it caused stimulation of growth and nitrogenase activity which could not be attributed to the medium (Fig. 15). This stimulation was specific to Anabaena oscillaroides 27 and was not observed with any other blue-greens used in the general interaction tests. Increases in chlorophylla were supported by increases in dry weight but as with previous findings the stimulation of growth was less when expressed on a

Figure 13

The effect of 10% (○), 50% (□) and 100% (▲) concentrations of *Aspergillus* 15A filtrate on the growth (a) and nitrogenase activity (b) of *Anabaena flos-aquae* 85 and the growth (c) and nitrogenase activity (d) of *Nostoc punctiforme*.
(■) and (●) are presenting the control.

The maximum standard deviations are in (a) 0.0180, (b) 0.005, (c) 0.0123 and (d) 0.0045.

Figure 13

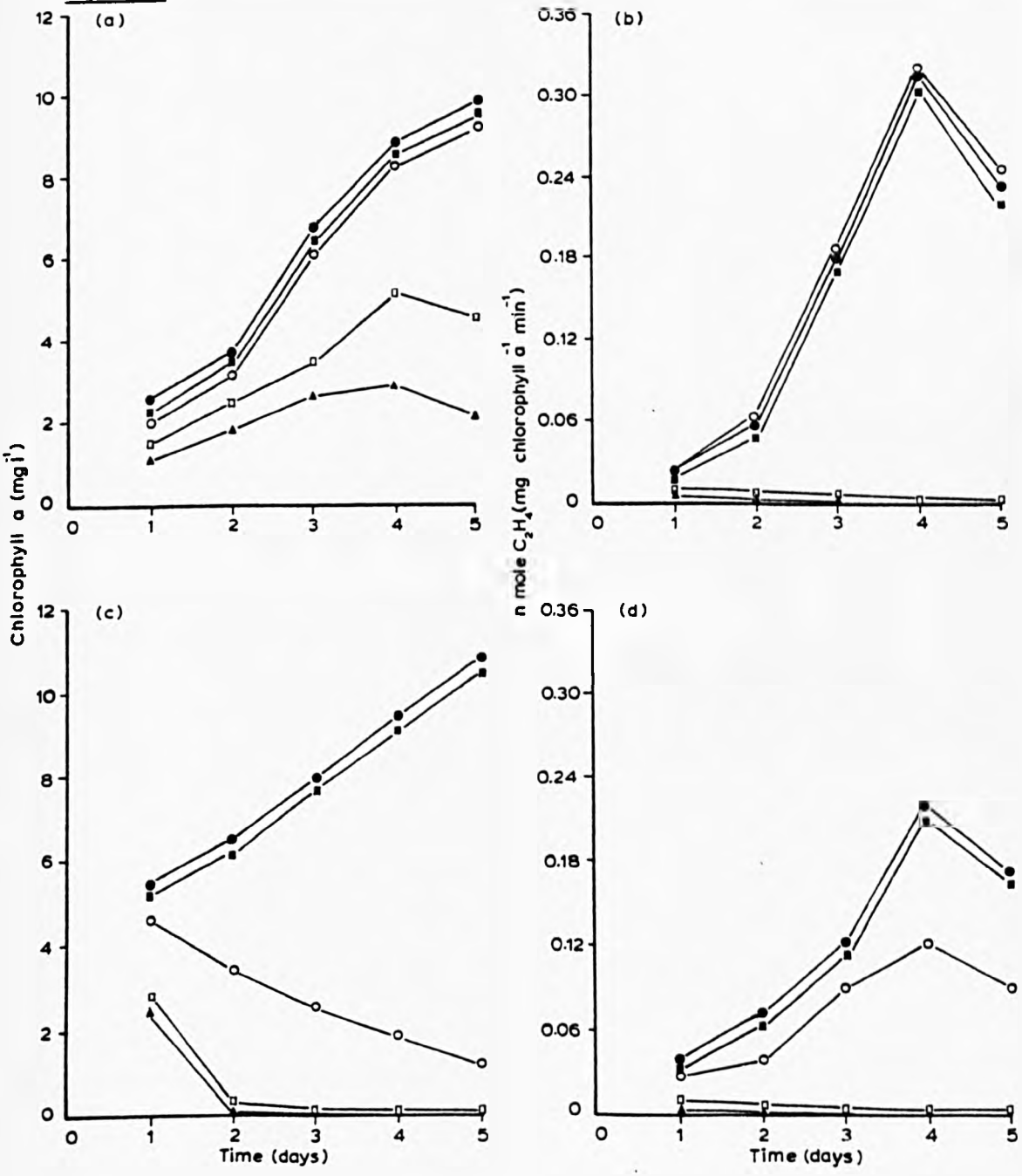

(a)
(b)
(c)
(d)
Chlorophyll a (mgl^{-1})
n mole C_2H_4(mg chlorophyll a^{-1} min^{-1})
Time (days)
Time (days)

Figure 14

The effect of Trichoderma 18A filtrate on the growth rate of Symploca thermalis 74 (A) and both growth rate and nitrogenase activity of Nostoc muscorum 87 (B and C). The filtrate concentrations used were 10% (o), 50% (□), 100% (▲), and 0% the control (●).

The maximum standard deviations are in (a) 0.0123 (b) 0.0118 and (c) 0.0052

Figure 14

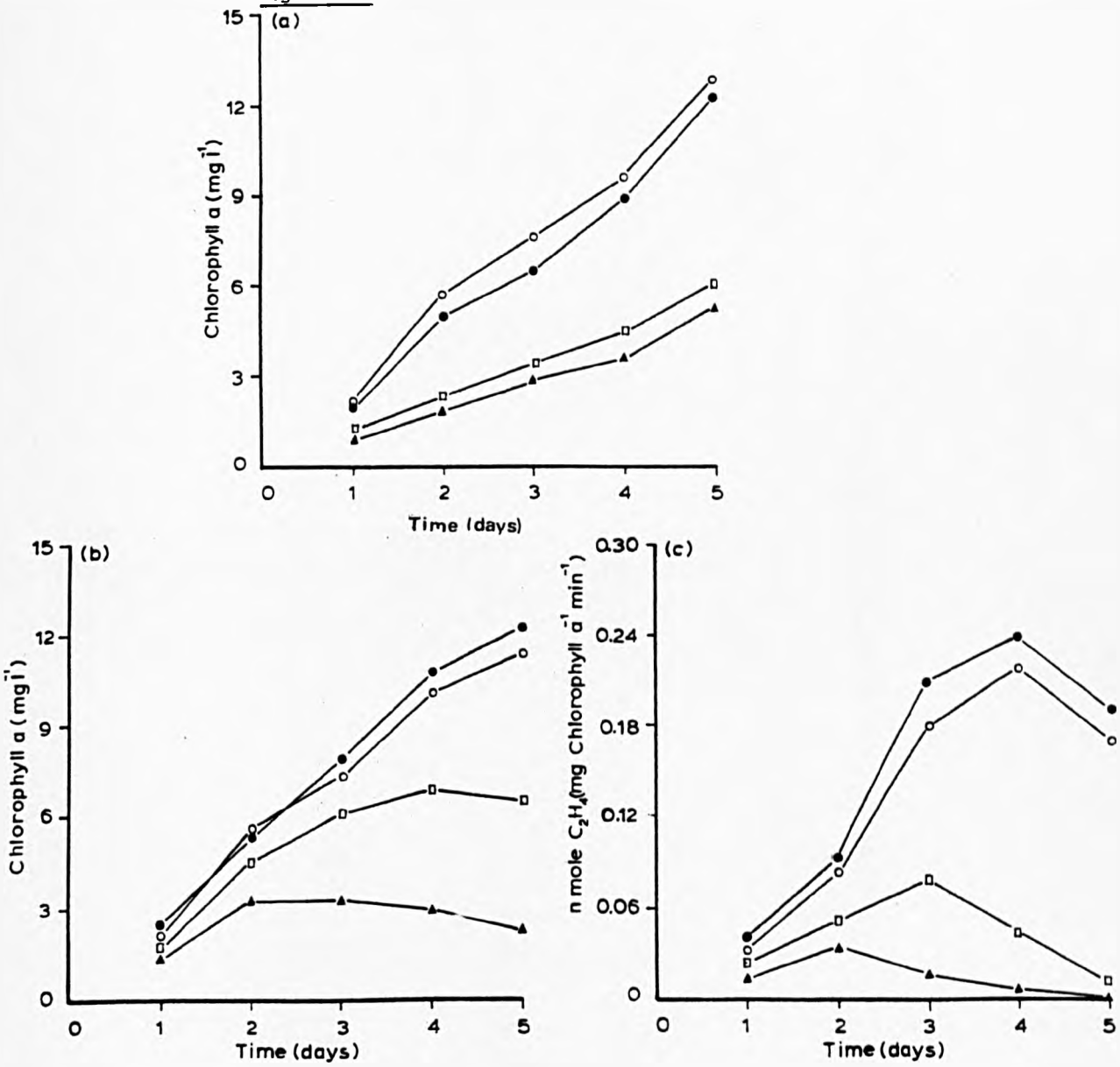
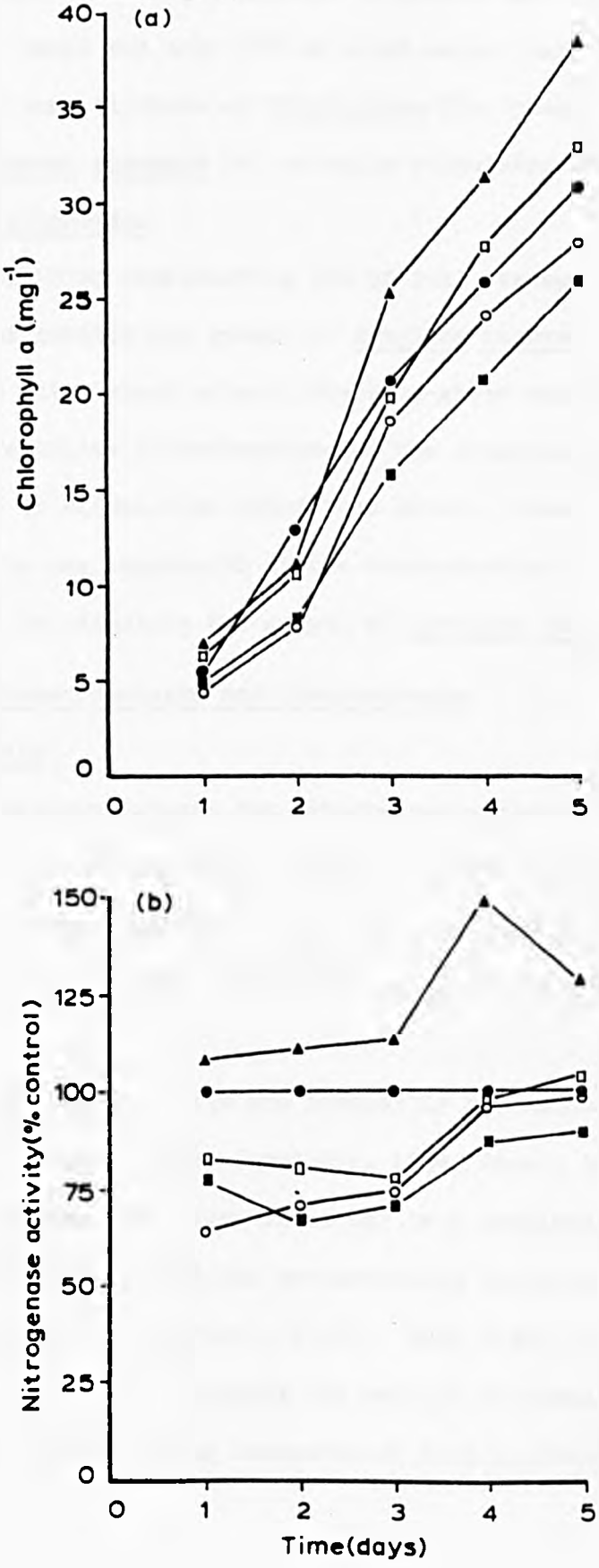

(a)
Chlorophyll a (mg l⁻¹)
Time (days)
(b)
Chlorophyll a (mg l⁻¹)
Time (days)
(c)
n mole C₂H₄/mg Chlorophyll a⁻¹ min⁻¹
Time (days)

Figure 15

The stimulatory effect of Trichoderma 18A filtrate on the growth (a) and nitrogenase activity (b) of Anabaena oscillaroides. The fungal filtrate was added to the liquid cultures at concentrations of 10% (○), 50% (□) and 100% (▲). Either malt extract broth (■) or Allen's medium (●) was added to the control flasks.

Figure 15


(a)
Chlorophyll a (mg l⁻¹)
40
35
30
25
20
15
10
5
0
0
1
2
3
4
5
(b)
Nitrogenase activity(% control)
150
125
100
75
50
25
0
0
1
2
3
4
5
Time(days)

dry weight basis. Maximum stimulation in growth was 48% on a chlorophyll a basis but only 30% on a dry weight basis. Thus aliquots of the same filtrate of Trichoderma 18A lysed Symploca thermalis and Nostoc muscorum but actually stimulated the growth of Anabaena oscillaroides.

Since a dilution representing 10% of full strength Trichoderma 18A filtrate did not inhibit the growth of Symploca thermalis 74 and in fact showed a slight stimulatory effect, the blue-green was retested against a series of more dilute concentrations of the original Trichoderma 18A filtrate to see if stimulation would then occur. However, a series of dilutions down to one thousandth of the concentration of the original filtrate failed to stimulate the growth of Symploca thermalis.

4.3 Interactions between bacteria and cyanobacteria

4.3.1 Introduction

The interactions between the cyanobacteria species and bacteria have frequently received special attention (Voroshilova and Dianova, 1934; Razumov, 1948; Novozhilova, 1957; Gasilina, 1963; Bershova et al., 1968; Daft and Stewart, 1971, 1973; Daft et al., 1975; Christensen and Cook, 1978).

Cultures of cyanobacteria are frequently contaminated with other micro-organisms (Gromov, 1954; Perminova, 1964; Paerl, 1976) and some workers have suggested that there may be a symbiotic relationship between the cyanobacteria and the contaminating bacteria (Jones, 1930; Mikhailova, 1961, 1963; and Paerl, 1976). Bunt (1961) noted that the bacterium Caulobacter increased the rate of nitrogen fixation by Nostoc species, although being incapable of fixing nitrogen itself.

Micro-algae and cyanobacteria have also been shown to stimulate the growth of heterotrophic bacteria (Shtina and Yung, 1963).

Some bacteria have an antagonistic effect on cyanobacteria species causing inhibition of growth and cell lysis, examples of these come from many different habitats (Parker and Bold, 1961; Echlin, 1965; Lockshine and Burris, 1965; Burnham et al., 1968; Wu et al., 1968; Fitzgerald, 1969; Stewart and Brown, 1970; Shilo, 1970, 1971; Gromov et al., 1972; Granhall and Berg, 1972; Daft and Stewart, 1971, 1973; Burnham et al., 1980, 1981; Daft et al., 1975: Yamamoto, 1977; Burnham and Locker, 1976).

4.3.2 Materials and methods

In this section all the bacterial species isolated from the Dee salt marsh were tested against the same cyanobacteria previously tested against actinomycetes and fungi using the same basic techniques as were described previously in materials and methods (Chapter 2).

4.3.3 Results

The results of interaction tests when agar cores of the bacterial isolates were placed on lawns of cyanobacteria are presented in Table 23. As in the previous interaction tests with the other microbial groups, inhibition of cyanobacterial growth was detected by a cleared zone around the bacterial core sample. Compared with the results from the actinomycetes and fungi, the cyanobacteria seemed generally more resistant to lysis by the various bacterial isolates. Individual bacterial isolates varied in the number and types of species of blue-greens they lysed. Eight bacterial isolates causing lysis were identified (see Chapter 3), of these Bacillus species proved the most

Table 23 The effects of agar block applications containing Dee salt marsh colonies of bacteria on lawns of all the Dee salt marsh and laboratory cultures of cyanobacteria

Cyanobacteria species grown on Allen's (c) media				bacteria grown on nutrient broth agar media																												
LBBC NO	Section	Genus	Classical Identification	1A	1B	1C	1D	2A	2B	3	4	5A	5B	6	8	9A	9B	12A	14B	14C	15A	15B	15C	16A	16B	16C	16D	16E	16F	16G	16H	17
19		Synechocystis	Synechocystis diplococcus (Pringsh)	-	-	-	-	-	+	+	-	-	-	-	+	-	-	+	-	-	+	-	+	+	+	+	-	-	-	-	-	-
54	I	Synechococcus	Synechococcus elongatus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-
88		Synechococcus	Anacystis nidulans	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-
74		LPPGpB	Symploca thermalis (KÜTZ) GOM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
41		LPPGpB	Lyngbya halophila Hansg	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
68		LPPGpB	Symploca elegans KÜTZ	-	-	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	-	-	-	-	-
89	III	LPPGpB	Plectonema boryanum	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-	-	-	-	-	-
90		LPPGpB	Lyngbya spp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
91		LPPGpB	Oscillatoria spp.	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
14		Anabaena	Anabaena microspora Kleb.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17		Anabaena	Anabaena minutissima Lemm.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27		Anabaena	Anabaena oscillaroides Bory.	-	+	-	-	+	+	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	+	-	+	+	-	-	+	+	-
28		Anabaena	Anabaena flos-aquae (Lyngb) Breb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
85		Anabaena	Anabaena flos-aquae A37	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
86		Anabaena	Anabaena cylindrica	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	-	-	-	-	-
48		Nostoc	Nostoc punctiforme (KÜTZ) Hariot.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-	-
52	IV	Nostoc	Nostoc entophytum Born et Flah.	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
8		Nostoc	Nostoc linckia (Roth) Born et.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
34		Nostoc	Nostoc muscorum Ag.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
37		Nostoc	Nostoc sphaeroides KÜTZ.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
45		Nostoc	Nostoc piscinale KÜTZ.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+	-	-	-	-
29		Nostoc	Nostoc verrucosum Vauch.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
87		Nostoc	Nostoc muscorum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
62		Cylindospermum	Cylindospermum majus	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
40		Nodularia	Nodularia harveyana var. sphaerocarpa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Some blue greens were inhibited [+] or apperently resistant to bacterial attack [−]
These results were obtained from at least triplicate assays.

efficient lysers and Bacillus pumilus 15C the most effective isolate of the genus. Not all the isolates were identified and several of the unidentified isolates were active against blue-greens eg. 1C, 8, 12A, 16C (Table 24). The sensitivity of the cyanobacterial species to the bacteria are presented as sensitivity indices in Table 25. This shows that Anabaena oscillaroides 27 was the most susceptible blue-green followed in decreasing order of sensitivity by Synechocystis diplococcus 19, Anabaena cylindrica 86, Symploca elegans 68, Plectonema boryanum 89 and Nostoc punctiforme 48. Conversely several cyanobacteria species were totally resistant to attack by the bacteria, ie. those species with a sensitivity of 0.

The results obtained using bacterial cell-free filtrates on cyanobacterial lawns were the same as those for the bacterial cores. This confirms the observations found in all the tests presented here, that direct contact between the bacteria and cyanobacteria is not necessary for lysis to occur.

Vegetative cells of the cyanobacteria again proved the most sensitive cell-type to lysis by the bacteria with heterocysts and akinetes remaining largely unaffected. Plate 15 shows that the vegetative cells of Anabaena cylindrica were lysed completely by Bacillus pumilus 1A whilst heterocysts remained apparently intact.

There was some evidence of stimulation of blue-green growth in the interaction tests but since the control cores of pure bacterial medium caused considerable stimulation the results were not included in Table 23. This point will be considered again later.

Table 24. The inhibitory index of Dee salt marsh bacterial isolates against cyanobacteria species.

Dee salt marsh bacterial isolates grown on nutrient agar medium	Bacterial isolates L.B.C.C. number	Inhibitory index (%) against cyanobacteria species
Bacillus pumilus	1A	12
-	1B	4
-	1C	12
-	1D	0
Micrococcus luteus	2A	4
-	2B	8
-	3	16
-	4	0
-	5A	0
-	5B	4
-	6	0
-	8	12
Pseudomonas sp.	9A	0
-	9B	0
-	12A	12
-	14B	0
-	14C	0
-	15A	12
Bacillus subtilis	15B	8
Bacillus pumilus	15C	36
Bacillus cereus	16A	12
Bacillus licheniformis	16B	24
-	16C	28
-	16D	12
-	16E	8
-	16F	0
Arthrobacter sp.	16G	8
-	16H	4
-	17	0

The cyanobacteria species were grown on Allen's medium.

$$\text{Inhibitory index} = \frac{\text{Number of the cyanobacteria lysed}}{\text{Total number of cyanobacteria tested}} \times 100$$

NB. The total number of cyanobacterial isolates tested = 25

Table 25. The sensitivity index of cyanobacteria species towards the Dee salt marsh bacterial isolates grown on nutrient agar medium.

L.B.C.C. No.	Cyanobacteria species grown on Allen's medium	No. of bacterial isolates causing lysis	Sensitivity index (%)
19	Synechocystis diplococcus	9	31
54	Synechococcus elongatus	2	6.89
88	Anacystis nidulans	2	6.89
74	Symploca thermalis	0	0
41	Lyngbya halophila	2	6.89
68	Symploca elegans	6	20.6
89	Plectonema boryanum	6	20.6
90	Lyngbya sp.	0	0
91	Oscillatoria sp.	2	6.89
14	Anabaena microspora	0	0
17	Anabaena minutissima	1	3.44
27	Anabaena oscillaroides	11	37.9
85	Anabaena flos-aquae	0	0
28	Anabaena flos-aquae	0	0
86	Anabaena cylindrica	6	20.6
48	Nostoc puntiform	3	10.34
52	Nostoc entophytum	2	6.89
8	Nostoc linkia	0	0
34	Nostoc muscorum	1	3.44
37	Nostoc sphaeroides	0	0
45	Nostoc piscinale	3	10.34
29	Nostoc verrucosum	1	3.44
87	Nostoc muscorum	0	0
62	Cylindospermum majus	2	6.89
40	Nodularia harveyana v.p.	0	0

$$\text{Sensitivity index} = \frac{\text{Number of inhibitory isolates of bacteria}}{\text{Total number of tested bacteria}} \times 100$$

NB. Total number of bacterial isolates tested = 29

Plate 15

Lysed vegetative cells (V) and intact heterocysts (H) caused when *Bacillus pumilus* 1A was added to a culture of *Anabaena cylindrica* x 2000. Both micro-organisms were isolated from the Dee salt marsh but not necessarily from the same microhabitat.

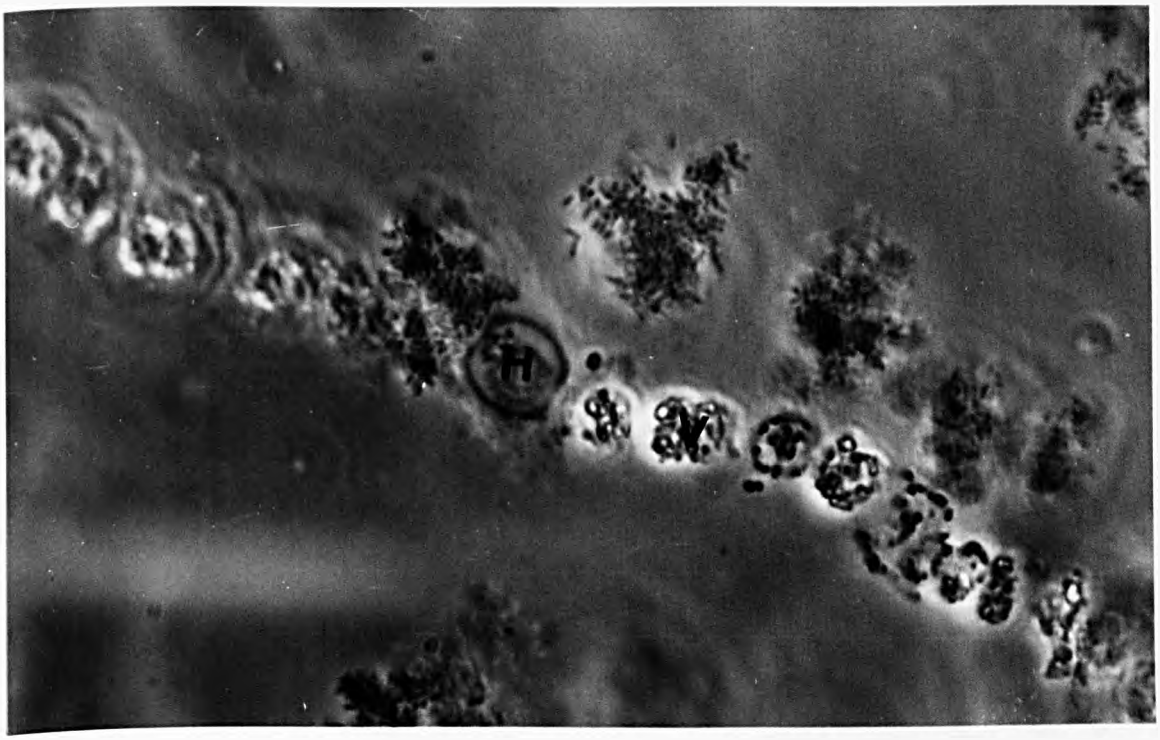


Plate 15

It was observed during these tests, that some cyanobacteria which were initially inhibited by bacteria and which had lysed in the inhibition zone were able to re-invade this zone from outside and even grow on the original bacterial cores. One such example was where Anabaena cylindrica re-invaded the inhibition zone produced by treatment with Bacillus pumilus 15C.

Some of the species of bacteria which were shown to be biologically active against cyanobacterial species in these preliminary screening experiments were studied further. Filtrates from four bacterial liquid cultures were tested against liquid cultures of four different cyanobacterial species (Table 26) and the effect on growth investigated.

Table 26. The combinations of bacteria and cyanobacterial isolates used to investigate the effects of bacterial filtrates on growth of cyanobacteria

Bacteria species code numbers	Bacteria species	Cyanobacteria species
16A	Bacillus cereus	Synechocystis diplococcus
16B	Bacillus licheniformis	Anabaena cylindrica
15C	Bacillus pumilus	Lyngbya halophila
2B	unidentified	Anabaena oscillaroides

The media initially used for bacterial growth was changed because of the stimulatory effect it seemed to have on cyanobacterial growth. It was substituted with Allen's media containing 0.2% casitone as both carbon and nitrogen source. This new modified bacterial media had no effect on bacterial growth and did not alter the observed preliminary interaction results showing cyanobacterial lysis. It did however eliminate any evidence of stimulation of the blue-greens by

bacteria. It must be concluded therefore that any stimulation of the blue-greens by bacteria in the preliminary tests was probably due to the medium itself.

Aliquots of bacterial cell-free filtrates were prepared and added to liquid cyanobacterial cultures in Allen's medium. A reduction in cyanobacterial growth was observed which was roughly proportional to the degree of dilution of the bacterial filtrates and representative results of these experiments are presented in Figure 16. The observed reductions in growth were due to the amount of lysis of the vegetative cells.

The bacterial filtrates which did not attack cyanobacterial species were serially diluted down to 0.1% of the original filtrate concentration and added to cyanobacterial cultures. No stimulation was shown by any of these bacterial filtrates or any of the dilutions.

4.4 Discussion

Actinomycetes, fungi and bacteria isolated from the salt marsh habitat were found able to inhibit the species of cyanobacteria obtained from the same or different habitats. This inhibition was due to the lysis of the vegetative cells by substances produced by these other microbial types and released into the medium. The use of cell-free filtrates of the micro-organisms in these interaction studies provides evidence that no direct cell to cell contact is required between any of the inhibiting microbes and the sensitive

Figure 16

The effects of filtrates of bacterial cultures (■) on the growth of cyanobacteria. The control cultures (●) received sterile bacterial medium:

a) Bacillus cereus 16A filtrate was added to Synechocystis diplococcus 19.

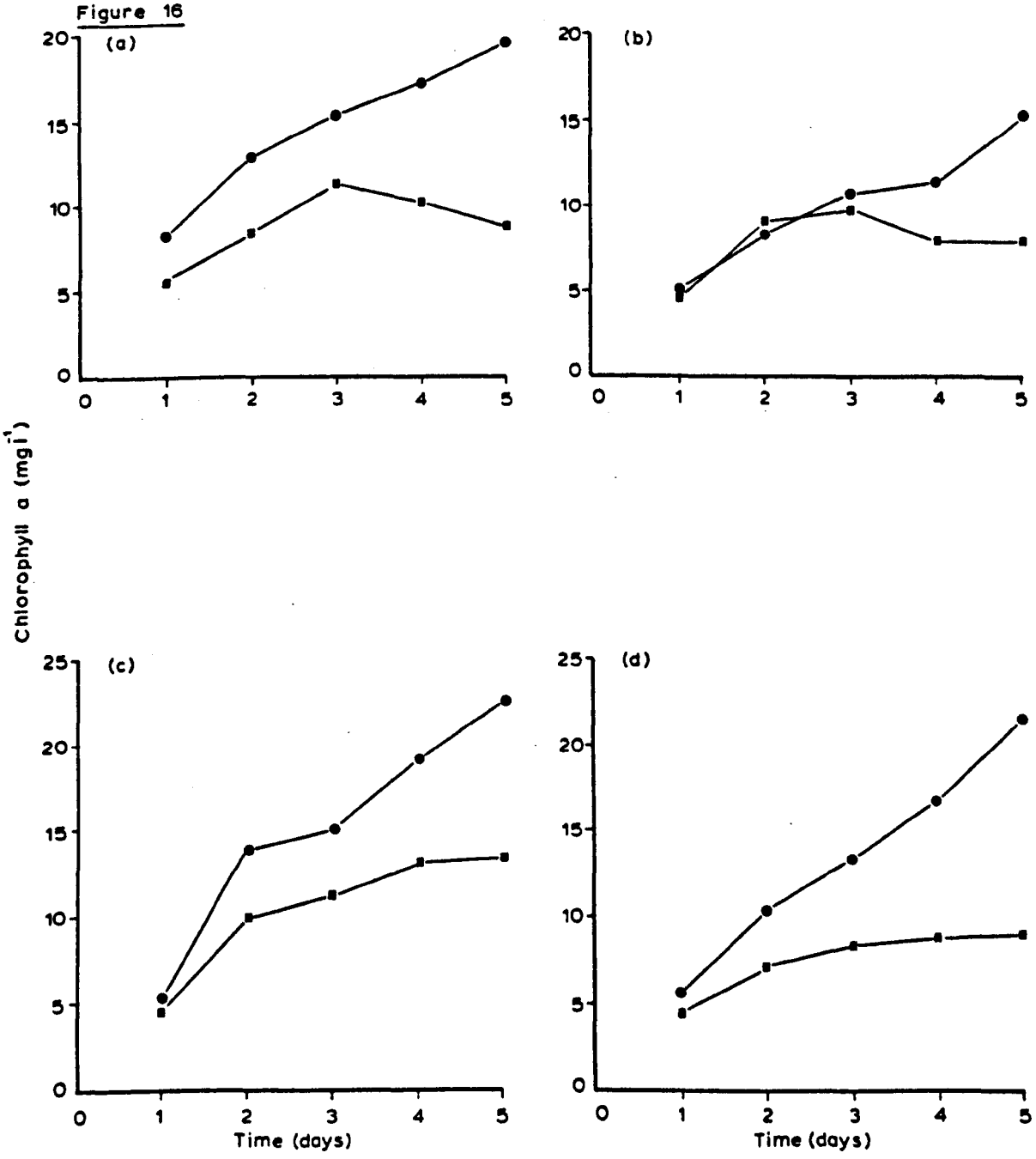
b) Bacillus licheniformis filtrate was added to Anabaena cylindrica 86.

c) Bacillus pumilus 15C filtrate was added to Lyngbya halophile 41.

d) Bacterial isolate 2B filtrate was added to Anabaena oscillaroides 27.

The maximum standard deviations are in (a) 0.023, (b) 0.051, (c) 0.042 and (d) 0.022.

Figure 16



cyanobacterial strains. These findings contrast with those of Daft and Stewart (1971); Fackrell and Robinson (1972 and 1973) who reported that physical contact with the inhibiting bacterial strains was essential for lysis of sensitive cyanobacteria species to occur. However, similar results to those presented here were obtained by Bershova et al. (1968), Stewart and Brown (1969), Granhall and Berg (1972), Safferman and Morris (1963) and Burnham, Stetak and Locker, (1976) in interaction tests between bacteria or actinomycetes and cyanobacteria. Redhead and Wright (1978) obtained similar results between filtrates from fungal isolates and blue-greens.

Microbial filtrates have been reported to contain various substances which might be the cause of the inhibitory or stimulatory responses shown by cyanobacterial species. These substances can be classical antibiotics such as streptomycin and penicillin (Kumar, 1964; Srivastava, 1970) and exo-enzymes (Strominger and Ghuysen, 1976; Shilo, 1971).

The number of cyanobacteria species lysed by a specific actinomycete isolate depended on the medium on which the two microbial groups were grown. In general, the actinomycetes showed greater lytic properties on nutrient broth than on ISP5 medium. This is probably due to the composition of the nutrient medium which has an essential effect on the amount of enzymes or antibiotic produced by the actinomycetes (Waksman, 1962; Vlahov, 1976). Nutrient broth medium is nutritionally richer than inorganic ISP5 medium and this allowed better actinomycete growth. Therefore, higher quantities of diffusible extracellular products might be expected to be produced into the medium.

The amount of the enzyme oxidase increased when actinomycetes were grown on soya meal medium and synthetic medium with potassium nitrate added (Vlahov, 1976). This indicates the importance of some nutrients on the production of certain microbial metabolites. The production of an antibiotic is sometimes only evident when the micro-organism is grown in special media under certain culture conditions. Streptomyces albidoflavous is known to produce antibiotics of the Streptothricin type (Norris & Richmond, 1981).

Since all the salt marsh actinomycete isolates belonged to the Streptomyces albidoflavus cluster it is possible that Streptothricin may be responsible for the lytic activity of certain of the strains. However, we have no proof that it is important in all the lytic reactions observed and clearly it is not produced by all strains belonging to the S. albidoflavus cluster since some of the salt marsh strains were ineffective. Furthermore, others showed variation in their lytic activity towards different cyanobacterial strains. The production of lytic substance, and thus possible streptothricin, is clearly affected by the choice of media since different results were obtained with nutrient borth and ISP5 media. There is not simply a general increase in activity when the actinomycetes are grown on nutrient agar rather than ISP5. For example, actinomycete 5A which lyses Anabaena microspora 14 when the former is grown on ISP5 failed to do so when grown on nutrient agar. In contrast, actinomycete 5B which failed to lyse Anabaena microspora 14 on ISP5 medium did so when grown on nutrient agar.

Altering the cyanobacterial growth media also aftered the pattern of interactions between the actinomycetes and the cyanobacteria. Therefore, the effects of using different media are not simply to

increase the production of inhibitory compounds by the actinomycetes but specific precursors or nutritional requirements may be needed which differ depending on the active compounds involved. The metabolic and nutritional state of the cyanobacterium itself could also alter its susceptibility and this may depend on the medium in which it is growing or the nutritional effects of the actinomycete medium in the vicinity of the interaction zone. Inorganic compounds present in the cyanobacterial media could either activate or alter the activity of the inhibitory substances produced by actinomycetes. Furthermore, these compounds may regulate the activity of the actinomycetes in several ways; they may be toxic; they may serve as sources of inorganic material for growth and they may act as chelating agents.

Extracellular substances released by the cyanobacteria could do the same (see later in this section).

Only a single type of medium was used to grow the cyanobacteria, fungi and bacteria cultures during the preliminary screening programme and the effect of using different media on the pattern of interaction results was not considered. Alteration in the interaction results might be expected if different media were used, as was found with the actinomycete tests.

Light and electron microscope studies confirmed that it was the vegetative cells of cyanobacteria which were lysed regardless of which group of micro-organisms the lytic agent came from. It was found that the cell wall layers of the vegetative cells disappeared sequentially, leaving the protoplast surrounded by the plasmalemma which also finally disintegrated. Similar findings were noted by Daft and Stewart (1973). It has been suggested by these workers that it is the differences in cell wall composition and structure of vegetative cells

compared with heterocysts and akinetes that accounts for the relative differences in susceptibility to lysis shown by the various cell types.

On the basis of histochemical tests. several groups of workers have referred to the presence of cellulose in the cell walls of heterocysts and its absence in vegetative cells (Fritsch, 1945; Daft and Stewart, 1971: Granhall, 1972 and Granhall and Berg, 1972). Granhall (1972) produced some evidence that bacteria which were capable of producing cellulase enzymes were able to decompose the heterocysts and akinetes as well as vegetative cells of cyanobacteria. Polyglucosides, but not cellulose or chitin have been reported as present in the envelopes of akinetes but are not confirmed as present in vegetative cell walls (Miller and Lang, 1968). However, no comprehensive chemical analysis of the walls of the various cell types have been made (see Haselkorn, 1978). Daft and Stewart (1971) and Granhall and Berg (1972) have suggested that the resistance of heterocysts to attack by certain bacterial strains might be due to the presence of the cellulose in the wall which the microbes are unable to degrade. The green algae have two distinct wall layers distinguishable with a light microscope, an inner, rather firm layer and an outer, capsular layer which may be stratified. These layers, formerly thought to consist entirely of polysaccharides, have been shown to contain from 10-69% protein (Gotelli and Cleland, 1968) and also hydroxyproline glucosides (Miller *et al.* 1972). Roberts (1974) has discussed the cell wall of algae with special references to their glucoprotein composition.

Earlier rather crude chemical tests (sulphuric acid and iodine) indicated that the inner cell wall was composed of cellulose (polymerized glucose) in the green algae. More recent investigations (Kreger, 1962; O'Colla, 1962; Parker, 1964, 1969, 1970; Preston, 1968; Hanic and Craigie, 1969; Miller *et al.* 1972; Siegel and Siegel, 1973; Catt *et al.* 1976), on a relatively few number of green algae, indicate that while cellulose is present in the walls of some algae (e.g. Chaetomorpha), it is absent from others

(e.g. Chlamydomonas, Acetabularia and Bryopsis). Instead, some of them have walls that are largely composed of polymers of xylose or mannose which occur as fibrils embedded in a non-fibrillar matrix, the latter being composed of hemicellulose. In this study the active actinomycete filtrates lysed the inner cell wall layer of green algae which is composed of cellulose but left the outer layer intact. The resistance of heterocysts and akinetes to the antimicrobial agents is therefore unlikely to be directly related to the presence of cellulose.

Yamamoto and Suzuki (1977) have reported the lysis of heterocysts by Pseudomonas strains and Granhall and Berg (1972) reported cellulase activity by Cellvibrio strains. It is therefore unlikely that the presence of cellulose gives general protection against lysis by micro-organisms. If cellulose is not the key substance preventing lysis then it will be necessary to look again at the chemical nature of the walls of the vegetative cells of tolerant cyanobacteria species and also of heterocysts and akinetes.

Granhall and Hofsten (1969) suggested that most lytic activity is attributable to the release of lytic enzymes by the micro-organisms. They argue that one reason for resistance of the blue-greens to attack is the absence of appropriate adsorption sites on the blue-green cell wall which are vital if the lytic enzymes are to attach to the wall and degrade a wall constituent.

It was thought that perhaps the mucilaginous sheath might be a factor in preventing lysis in certain insensitive cyanobacterial species. If this is the case then there must be different chemical components in the sheaths of different blue-green species which afford protection to lysis. Protection is unlikely to be directly related to sheath thickness as the addition of a lytic filtrate from certain actinomycete or fungal isolates to cyanobacterial specie caused swelling of the mucilaginous sheath but still brought about vegetative cell lysis. In fact, one of the most sensitive species of blue-green Nostoc entophytum 52 appeared to produce the thickest sheath in response to the addition of lytic filtrates. Whether this increase in thickness of the sheath

was caused by the inhibitory filtrates stimulating mucilage or sheath production or whether existing material simply swelled due to some chemical reactions with the filtrate was not confirmed.

The differences in the chemical structure of either the sheath, cell wall or cell membrane in different cyanobacterial species could account for the differences in sensitivity of certain blue-greens to specific microbial isolates. Since the sensitivity changes with culture conditions, this would have suggested that the composition of these boundary structures changes and this is related to the condition of cultures. A whole range of extracellular products is known to be produced by blue-greens, eg. polypeptides, peptides, polysaccharides, amides, organic acid, amino acids (Whitton, 1963; 1964; Hellebust, 1974; Walsby, 1965) and extracellular enzymes. B-lactamase which is capable of degrading penicillin and thus permitting growth in the presence of added antibiotic is known to be produced by different strains of cyanobacteria (Krushner and Brenil, 1977). Therefore, the differential sensitivity of blue-greens could be due to their ability to produce extracellular compounds capable of either degrading the different lytic substances or adsorbing them and thus inactivating them. These extracellular substances from cyanobacteria do not directly control the production of anticyanobacterial compounds by the other micro-organisms since the differential sensitivities shown by the various cyanobacteria were obtained when cell-free filtrates from the other microbes were used. Thus, any modifying effects on the activity of lytic compounds added to the cyanobacteria cultures must be on substances already produced by the other micro-organisms, ie. already present in the lytic filtrate.

Inhibitory filtrates seemed unable to cause stimulation when diluted and thus the differences in response between different blue-greens species is not simply due to responses to different concentrations of the same active substance. It was observed that when blue-greens were treated with lytic actinomycetes, fungi and bacteria the inhibition zone on the lawns was sometimes

surrounded by another zone of increased cyanobacterial growth. This increase in growth is unlikely to be due to lower filtrate concentrations at the perimeter of the inhibition zone since diluting the filtrate caused no stimulation in liquid cultures. This apparent increased growth zone could have been due in some cases to mobility of the cyanobacterial filaments brought about by a chemotactic response but not in all cases since several of the cyanobacteria showing increased growth outside the invasion zone were non-motile species.

The differential zones on the plates could be due to stimulatory substances diffusing further out into the lawn than the inhibitory ones. However, these filaments of cyanobacteria were observed, after several days of interaction, to re-invade the inhibition zone. This might be due to the lytic compounds which diffused out from the microbial block becoming de-activated or adsorbed after lysing some of the blue-green cells on the lawn. The lytic zone would then be an area high in nutrients since they would not have been used up before the original filaments of the lawn had died.

The extracellular substances produced by a few isolates of actinomycetes and fungi which lysed certain blue-greens caused stimulation of others when applied to liquid and solid cultures. This observation cannot be explained by the different blue-greens being sensitive to different concentrations of the same inhibitory compound with the compound becoming more stimulatory at low concentrations because a series of dilutions down to a concentration of 0.1% of the original filtrate concentration failed in all cases to change

an inhibitory effect into a stimulatory one or even a neutral effect into a stimulatory one. Thus either a single substance can act quite differently on different blue-green strains or several substances are present in the filtrate, one or some of which are active against certain blue-greens. Examples of substances which were found to stimulate certain species of cyanobacteria are 7-azatryptophan, rafimpicin (0.025 μg ml^{-1}) (Mitchison and Wilcox, 1973; Wolk and Quine, 1975; Bothe and Eisbrenner, 1977; Stacey et al., 1979). These may act either externally or internally after assimilation by the blue-green cells. Increase in nitrogenase activity in cyanobacterial cultures coincided with an increase in the concentrations of these substances over a limited range.

Whilst the overall number of blue-greens tested was relatively small compared with the total number of species known, and different numbers of isolates for the various groups were tested, cyanobacteria from group IV were generally the most sensitive. However, having said this, the group IV species Cylindospermum majus 62 and Nodularia harveyana 40 were the least sensitive of all the blue-greens tested. Although all the green algae tested were susceptible to actinomycete A52, apart from this, they seemed less sensitive to the other actinomycetes than the cyanobacteria. This is in line with the findings of Hunter and McVeigh (1961) who also showed that Eukaryotic algae were less sensitive to antibiotics produced by actinomycetes.

Actinomycete isolates produced the most effective cyanobacterial-lysing agents. However, evidence was obtained in this study that

strains of blue-greens sensitive to actinomycetes were not necessarily the strains which were sensitive to either fungal or bacterial lysins.

CHAPTER FIVE

CHARACTERIZATION OF SUBSTANCES INHIBITORY OR STIMULATORY TO CYANOBACTERIA AND GREEN ALGAE

5.1 Introduction

The results obtained from the preliminary screening tests (see Chapter 4), showed that the actinomycetes, bacteria and fungal isolates produced substances in their growth medium which lysed blue-greens. A few isolates produced substances which stimulated the growth of certain cyanobacterial species (although the same substances also inhibited other cyanobacterial strains). The inhibitory substances which have variously been called antibiotics, toxins, lysins or bacteriolysins, vary considerably in their physical, chemical and biological properties. Certain of these compounds are destroyed by heating and exposure to light; others are stable to both heat and ultra-violet light. Some are water soluble, others insoluble in water but soluble in various organic solvents (Waksman, 1967).

In this chapter some basic information on the physiological stability of these inhibitory and stimulatory substances will be sought and an attempt made to elucidate some of the physico-chemical characteristics exhibited by these compounds.

5.2 Materials and methods

5.2.1 Actinomycetes

Four actinomycetes strains which lysed the greatest number of species of green algae and cyanobacteria were used in this part of the investigation. They were in decreasing order of effectiveness A52, 216, 11B and 10A (see Chapter 4). Also included was the isolate 6B which showed both lytic and stimulatory activity depending on the

cyanobacterial isolates used. Twentyfive cm^3 aliquots of cell-free filtrate of the actinomycetes prepared as before (see Chapter 2) were either heated to 60°C or 100°C for five minutes in a water bath, or autoclaved for fifteen minutes at 121°C/15Lb/$inch^2$. Other aliquots of each of the filtrates of the actinomycete isolates were also maintained at 24°C, 15°C, 8°C, 4°C, 0°C and -10°C for two days. Then 0.3cm^3 amounts of the various filtrates subjected to the different pretreatments were applied in stainless steel cylinders to freshly seeded cyanobacterial lawns.

The cyanobacterial strains used to make lawns were Symploca thermalis (74); Anacystis nidulans (88); Lyngbya halophila (41); Nostoc entophytum (52); Anabaena oscillaroides (27) and Anabaena microspora (14). In certain cases the actinomycetes were also tested against lawns of the gram negative bacteria, Pseudomonas fluorescens P 2 and Escherichia coli Eq; the gram positive bacteria Bacillus subtillis B2 and Staphylococcus aureus S9 and the yeast Saccharomyces sp. All/1, all obtained from the culture collection of the Department of Botany, University of Liverpool.

The extractibility of the active agents from washed cells or cell-free filtrates of the actinomycetes was tested at pH 4, 7 and 9 using three different organic solvents (chloroform, ethyl acetate and dichloromethan). A total of 1 litre of the filtrate was extracted by shaking equal volumes (200cm^3) of it with solvent for three to five minutes and allowing the aqueous and organic phases to separate unless otherwise stated. The organic phase was run off and the aqueous phase re-extracted twice more with the appropriate solvent and the separate

organic fractions obtained for each pH combined into a single extract. The extracts of each individual organic solvent were taken to dryness under vacuum using a rotary film evaporator. The residues were redissolved in a small volume (5 - 10cm^3) of the solvent initially used in the extraction step and the pH re-adjusted to pH 7 if necessary by the addition of 4M sodium hydroxide or hydrochloric acid.

The antimicrobial activity of each individual solvent extract was tested by applying it to sterile 1cm diameter filter paper disc, drying it and repeating the procedure a total of five times and placing the filter paper disc on the surface of freshly prepared lawns of cyanobacteria or other test micro-organisms. The aqueous phase was also tested for lytic activity, after re-adjusting the pH to 7, by applying it to lawns in sterile stainless steel wells. Sterile medium was also extracted using the same organic solvent and applied to the cyanobacterial lawns as controls.

The absorption spectrum for actinomycete A52 filtrate residues in chloroform was obtained using an Sp-1800 Perken Elmer Spectrophotometer.

Alumina and silica gel thin layer plates were used for chromotographic separation of the extracts from the various isolates using five different organic running solvents of different polarity (see Chapter 2). The anticyanobacterial activity of the separated spots on the dried chromatograms were tested, by the bioautographic technique of Wagman and Weinstein (1973). The chromatograms were placed in sterile petridishes overlaid with cyanobacterial suspension in agar and incubated under the standard growth conditions.

5.2.2 Fungi

Three fungal genera which showed differing abilities to lyse cyanobacterial species were used in this part of the investigation, they were Trichoderma 18A; Penicillium 13B and Fusarium 13E. Trichoderma 18A was also used in stimulation experiments with certain species of cyanobacteria. The test cyanobacterial species used in this investigation were Anabaena oscillaroides (27); Nodularia harveyana (40) and Symploca thermalis (74).

The methods used in the heat and cold stability experiments and the extraction and separation of the active compounds were the same as those described for actinomycetes.

5.2.3 Bacteria

The filtrates and extracts from the bacterial isolates Bacillus pumilus 15C; Bacillus licheniformis 16B and Bacillus cereus 16A were chosen for further studies. The test cyanobacterial strains used were Synechosystis diplococcus (19); Plectonema boryanum (89) and Anabaena oscillaroides (27). Again the methods used were identical to those described for actinomycetes and fungi.

5.3 Results

5.3.1 Actinomycetes

The A52 (Streptomyces roseodiostaticus) strain was tested against the gram negative and gram positive bacteria and yeast using both agar block and cell-free filtrate applications (Table 27). The results showed no lytic activity was apparent against any of the test organisms. Glaxo Group Research Limited confirmed that this streptomycete strain showed no classical antibiotic behaviour, and the C.B.S. culture collection had reported that no antibiotic was produced by this strain.

Unlike the results for A52, simple tests using the actinomycete isolates 11B, 10A and 216 showed varying abilities to lyse the test bacteria and yeast (Table 27). These results showed that Streptomyces lavindulocolor 216 was able to lyse all the test microbes, but that the 10A and 11B (Streptomyces albidoflav us) only lysed the gram positive bacteria and the yeast.

Table 27. The lysis of different gram negative and gram positive bacteria and a yeast by various actinomycete strains.

Actinomycete strain	Pseudomonas fluorescems (-)	Escherichia coli (-)	Bacillus subtilis (+)	Staphylococcus aureus (+)	Saccharomyces sp.
* 216	+	+	+	+	+
11B	-	-	+	+	+
10A	-	-	+	+	+
* A52	-	-	-	-	-

\+ = inhibition in growth of tested microbes

\- = no inhibition in growth of tested microbes

* = laboratory actinomycete strain

The lytic properties of heat treated actinomycete filtrates were assessed using four sensitive strains of cyanobacteria (Table 28). The results showed that all the cyanobacteria tested with A52 filtrate were lysed except when Nostoc entophytum (52) and Anabaena oscillaroides (27) were incubated with filtrate pretreated at 100°C or autoclaved. Strain 216 (Streptomyces lavindulocolor) still showed a wide lytic activity after pretreatments at high temperatures and autoclaving. Filtrates of salt marsh isolates 11B and 10A (Streptomyces albidoflav us) remained active after high temperature pretreatment and autoclaving except in their action on Nostoc entophytum (52).

Table 28 The effect of temperature pretreatments on the lytic activity of cell-free filtrates of certain actinomycetes

Cyanobacterial strains	LBCC No	Actinomycete isolate numbers and pretreatment temperatures															
		24°C (Control)				60°C				100°C				Autoclaving 121°C/15 lb/inch²			
		A52	216	11B	10A	A52	216	11B	10A	A52	216	11B	10A	A52	216	11B	10A
Symploca thermalis	74	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Anacystis nidulans	88	+	+	+	−	+	+	+	−	+	+	+	−	+	(+)	+	−
Lyngbya halophila	41	+	+	−	+	+	+	−	+	+	+	−	+	+	+	−	+
Nostoc entophytum	52	+	+	+	+	(+)	+	(+)	(+)	−	+	−	−	−	+	−	−
Anabaena oscillaroides	27	+	+	+	+	(+)	+	+	+	−	+	+	+	−	+	+	+

+ = good lysis (+) = weak lysis − = no effect

The same type of experiment was performed on the filtrates pretreated at temperatures below the normal growth conditions. The results (Table 29) indicate that cooling the A52 filtrate to 15°C did not cause any reduction in its lytic activity, however, cooling to 8°C and below drastically reduced or completely removed its lytic capacity. The filtrates from actinomycetes 216, 11B and 10A all remained active after cooling pretreatments.

5.3.1.1 Extraction

The results of extracting actinomycete A52 filtrates with organic solvents and testing them for lytic activity against two cyanobacterial species are presented in Table 30. These show that only chloroform extracted the lytic substances from the A52 filtrate. After extraction with ethyl acetate and dichloromethane, lytic activity was retained in the aqueous phase when the extracts were treated against the susceptible cyanobacterial species. When the aqueous phases were re-extracted with chloroform, the anticyanobacterial activity partitioned into the chloroform layer. No lytic activity was observed in the controls in which just sterile actinomycete medium was extracted with the various organic solvents and the different fractions applied to the lawns of cyanobacteria.

The weight of the residue remaining after rotary film evaporation to dryness of the various solvent extractions are presented in Table 31. These data show that the weight of the residue from the chloroform extractions were higher than the residue weights remaining from extraction in either ethyl acetate or dichloromethane. When half the volume of the aqueous phases remaining after initial extraction in either ethyl acetate

Table 29 The effect of cooling and freezing temperature pretreatments on the lytic activity of cell-free filtrates of certain actinomycetes

Cyanobacterial strains	LBCC No	Actinomycete isolate numbers and pretreatment temperatures																					
		24°C(Control)				15°C				8°C				4°C				0°C				-10°C			
		A52	216	11B	10A	A52	216	11B	10A	A52	216	11B	10A	A52	216	11B	10A	A52	216	11B	10A	A52	216	11B	10A
Simploca thermalis	74	+	+	+	+	+	+	+	+	(+)	+	(+)	+	−	+	+	+	−	+	+	+	−	+	+	+
Anacystis nidulans	88	+	+	+	−	+	+	+	−	(+)	+	+	−	−	+	+	−	−	+	+	−	−	+	+	−
Lyngbya halophila	41	+	+	−	+	+	+	−	+	(+)	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+
Nostoc entophytum	52	+	+	+	+	+	+	+	+	(+)	+	+	+	−	+	+	+	−	+	+	+	−	+	+	+
Anabaena oscillaroides	27	+	+	+	+	+	+	+	+	(+)	+	+	+	−	+	+	+	−	+	+	+	−	+	+	+

+ = good lysis (+) = weak lysis − = no effect

Table 30 Extraction of lytic compounds of actinomycete A52 cultures with different organic solvents

Sample extracted	Extracting solvent	Partitioned fractions	Test Cyanobacteria species and LBCC No	
			Anacystis nidulans (88)	Anabaena microspora(14)
A52 filtrate	None	None	+	+
ISP5 medium	Chloroform	Aqueous phase	—	—
		Organic phase	—	—
A52 filtrate	Ethyl acetate	Aqueous phase	+	+
		Organic phase	—	—
A52 filtrate	Dichloromethane	Aqueous phase	+	+
		Organic phase	—	—
A52 filtrate	Chloroform	Aqueous phase	—	—
		Organic phase	+	+
Aqueous phase of ethyl acetate from A52 filtrate	Chloroform	Aqueous phase	—	—
		Organic phase	+	+
Aqueous phase of dichloromethane from A52 filtrate	Chloroform	Aqueous phase	—	—
		Organic phase	+	+

Organic fractions were added to filter paper discs and dried prior to applications to cyanobacteria lawns. Aqueous phases were applied to the lawns in sterile stainless steel wells.

+ = inhibition of growth — = no effect

or dichloromethane were bulked and extracted with chloroform, the weight of the residue on taking to dryness was close to the weight of the residue obtained when the A52 filtrate was extracted directly with chloroform. Thus it would seem that chloroform extracts different compounds than either of the other two solvents.

The residues were easily dissolved in water except that obtained with chloroform when some of the residue remained undissolved even after boiling. The lytic activity of the water soluble materials were assayed against the same cyanobacteria but the results were all negative. This might indicate that the lytic agent of this actinomycete strain is a water-insoluble substance or substances only soluble in chloroform. Therefore, the residues from chloroform extractions were dissolved in a series of ethanol concentrations from pure ethanol down to 10% aqueous ethanol. When the lytic activity of these ethanol solutions were tested against the cyanobacteria all caused inhibiton of growth. Controls of equivalent concentrations of just ethanol were also applied to the lawns and whilst the high concentrations inhibited cyanobacterial growth, inhibition was not caused by the 10% ethanol concentration. This confirms that the lytic factors extracted by chloroform and not soluble in water will dissolve in ethanol. A chloroform extract gave a single absorption peak between 290nm and 360nm with a maxima at 335nm (Fig. 17).

Table 31. Dry weights of residues of A52 (Streptomyces roseodiostaticus) extracted with different organic solvents.

Source of residue	Weight of residue ($g\ l^{-1}$)
Cellular material remaining after filtration of 1 l of culture	0.432 ($\pm$ 0.012)
Chloroform extract	0.2 ($\pm$ 0.02)
Ethyl acetate extract	0.14 ($\pm$ 0.004)
Dichloromethane extract	0.116 ($\pm$ 0.005)
1:1 combined aqueous phases from ethyl acetate and dichloromethane re-extracted in chloroform	0.19 ($\pm$ 0.011)

The figures in brackets are the standard errors obtained from at least 4 replicates.

5.3.1.2 Chromatography and purification

When concentrated chloroform extracts from A52 filtrates were chromatographed no separation of the original spot occurred on thin layer alumina plates with any of the running solvents. However, on silica gel plates, the original spot separated into a maximum of 5 spots depending on the running solvent used (Table 32). The best separation was achieved with cyclohexane, whilst other solvents brought about separation of the original sample, resolution was poor.

The lytic activity of the separated spots on the cyclohexane developed chromatograms, was tested against the three susceptible cyanobacterial species by applying strips of the thin layer chromatograms

Figure 17

Absorption spectrum for the active compound (in chloroform) extracted from streptomycete A52 filtrate.

Figure 17

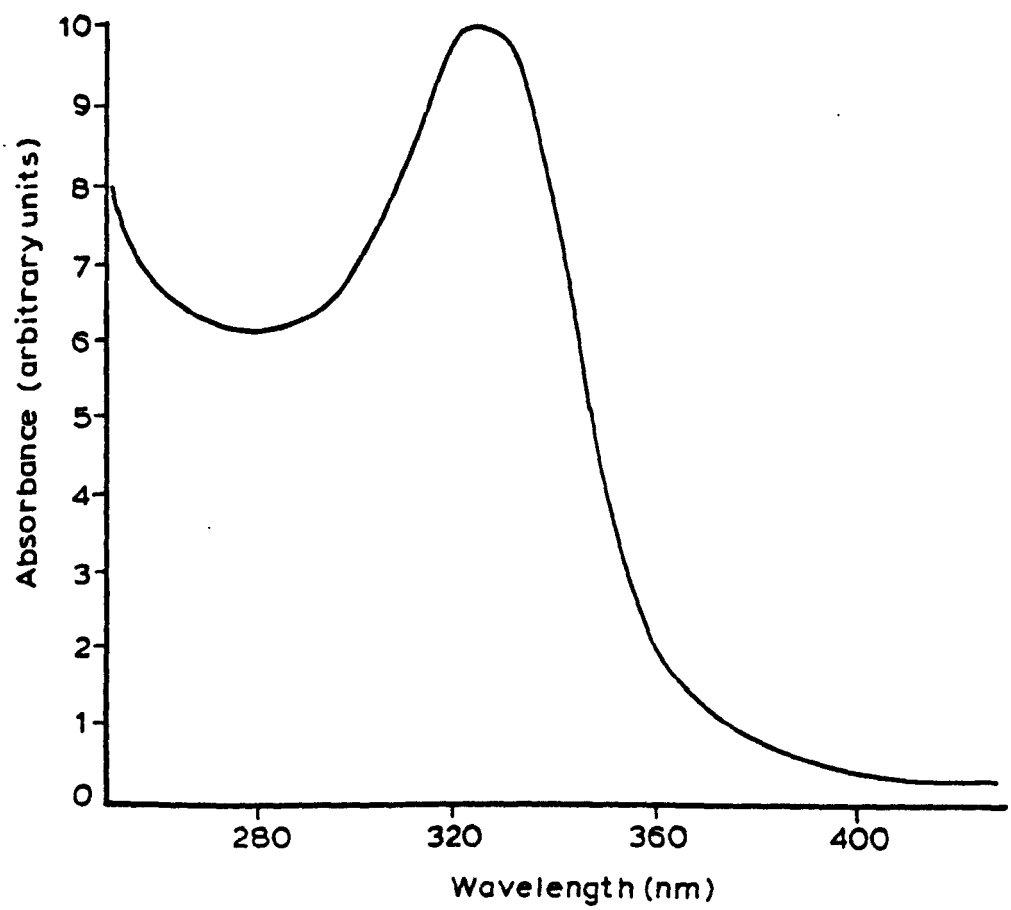

Absorbance (arbitrary units)
10
9
8
7
6
5
4
3
2
1
0
280
320
360
400
Wavelength (nm)

Table 32 R_f values for separated spots of chloroform extracts of A52 (Streptomyces roseodiostaticus) run on silica gel plates using different running solvents

Sample extracted	Extracting solvent	Running solvent	R_f values of spots	Test Cyanobacteria species and LBCC No	
				Symploca thermalis (74)	Lyngbya halophila (41)
A52 cell-free filtrates	Chloroform	Cyclohexane	0.710	+	+
			0.420	−	−
			0.100	−	−
		Trichloroethylene	0.916	−	−
			0.750	+	+
			0.500	−	−
			0.083	−	−
		Propan-1-ol	0.916	−	−
			0.875	+	+
			0.830	−	−
			0.750	−	−
			0.500	−	−
		Diethyl ether	0.950	−	−
			0.833	−	−
			0.789	+	+
		Methanol	0.000	+	+

+ = Lysis − = no effect

to lawns of blue-greens after first allowing the running solvent to evaporate off. Maximal lytic activity (ie. a clear zone on the lawn) was associated with a single spot on the chromatogram with an R_f value of 0.71. This activity was visible within five days (Plate 16).

Inhibition zones on the lawns were also seen on chromatograms developed in the other running solvents at spots corresponding to approximate R_f values of 0.75, 0.789 and 0.875 respectively for trichloroethylene, diethyl ether and propan-1-ol although both the resolution of the spots and the inhibition zones was poor. An inhibition zone was detected around the origin on the methanol run chromatogram on which no separation had occurred.

Several chromatograms were prepared as before using cyclohexane as the running solvent, so that a number of active spots could be obtained. These spots were cut from the silica plates, extracted in a range of different organic solvents (Table 33) at 24°C and 40°C for 10 to 15 minutes with continuous shaking to aid elution of the anticyanobacterial agents. These eluents were evaporated to dryness under vacuum and redissolved in fresh solvent and tested for lytic activity against the same cyanobacteria mentioned previously. The eluted strips of the silica gel plates were also tested by placing them on to freshly seeded cyanobacterial lawns. Since none of the eluents caused lysis it would seem that the solvents used failed to elute the anticyanobacterial substances adsorbed on to the silica particles. In contrast the chromatogram strips containing the active spots still caused lysis of the test lawns.

Plate 16

The results of the autobiographic test showing lysis of the cyanobacterial lawn around the active spot on the chromatogram run in cyclohexane.

O = origin, Sf = solvent front,
I = inhibition zone, L = cyanobacterial lawn.

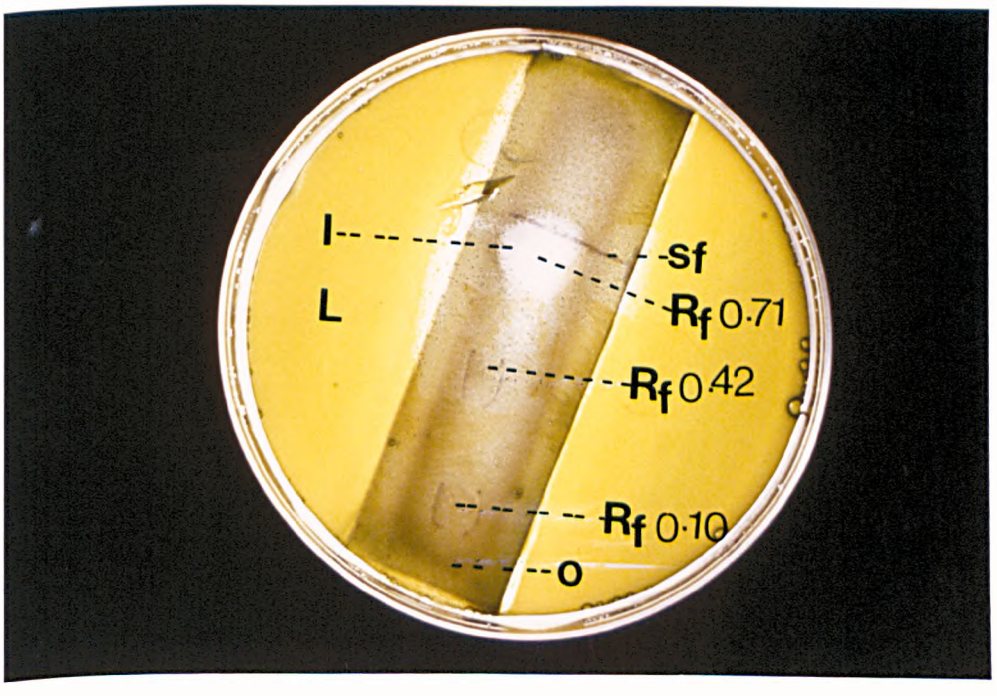


Plate 16

Table 33. Organic solvents used to elute the anticyanobacterial compounds from the active spot on the developed chromatograms

Eluting solvents	Polarities
Carbon tetrachloride	2.24
Benzene	2.30
Diethyl ether	4.22
Chloroform	4.80
Ethyl acetate	6.00
Benzyl alcohol	13.10
n-Butanol	17.50
Acetone	20.70
Methanol	32.70

The isolate 6B (*Streptomyces albidoflavus*) which exhibited both antagonistic and growth promoting properties (depending on the test cyanobacterial species used) was also investigated. The same organic solvents were used to extract the active substances from 6B as were used with A52. Both chloroform and ethyl acetate were capable of extracting the active compounds into the organic phases but with dichloromethane the activity stayed in the aqueous phase (Table 34).

The chloroform and ethyl acetate extracts were evaporated to dryness and the residues redissolved in water and loaded on to sterile 1cm diameter filter paper discs. When these discs were placed on freshly seeded cyanobacterial lawns they caused either inhibition or stimulation of growth in a similar way to the previous results obtained with the agar block or cell-free filtrate applications of this actinomycete strain. This contrasts with the findings for A52 where the active compounds only extracted in chloroform and could not be re-dissolved in water.

Table 34 Extraction of lytic and growth promoting compounds of actinomycete 6B cultures with different organic solvents

Sample extracted	Extracting solvent	Partitioned fractions	Test Cyanobacteria species and LBCC numbers			
			Lyngbya halophila (41)	Synechocystis diplococcus(19)	Anabaena flosaquae (28)	Nostoc entophytum(52)
6B filtrate	None	None	+	+	S	+
ISP5 medium	Chloroform	Aqueous phase	—	—	—	—
		Organic phase	—	—	—	—
6B filtrate	Chloroform	Aqueous phase	—	—	—	—
		Organic phase	+	+	S	+
6B filtrate	Ethyl acetate	Aqueous phase	—	—	—	—
		Organic phase	+	+	S	+
6B filtrate	Dichloromethane	Aqueous phase	+	+	S	+
		Organic phase	—	—	—	—

Organic fractions were added to added to filter discs and dried prior to applications to cyanobacterial lawns. 0.3 cm^3 aliquots of the aqueous phases were applied to the lawns in sterile stainless steel wells.
+ = inhibition to growth — = no effect and S = stimulation to growth of cyanobacteria species

The dry weight of the materials extracted by each solvent system are given in Table 35. These data show that the amounts of material extracted by chloroform and ethyl acetate and which contain the active compound was more than that extracted by dichloromethane (and which was inactive).

The weighed residues were then re-eluted with the same solvent in which they had been extracted and applied to thin layer silica gel plates. The same set of running solvents was used here as was used with A52.

Separation of both chloroform and ethyl acetate extracts was obtained with propan-1-ol, diethyl ether, cyclohexane and trichloro ethylene but not methanol. The best separation was obtained with propan-1-ol. The bioautographic technique was again used to visualise the active spots. Both the inhibitory and stimulatory responses were obtained with the same spot on the chromatogram, although the R_f value of the active spot differed slightly depending on whether the original extract was made in chloroform (R_f 0.545) or ethyl acetate (R_f 0.509). Again, it should be emphasised that the type of response depended on the species of cyanobacteria used to make the lawns (Table 36).

Similar procedures were followed to investigate the extractability, solubility and chromatographic separation of the anticyanobacterial agents produced by actinomycete isolates 10A and 11B. The results showed that again only ethyl acetate and chloroform were able to extract the active compounds. These substances were also soluble in water, and propan-1-ol produced the best separation on the chromatograms. Five different spots

Table 35. Dry weights of various residues of 6B (Streptomyces albidoflavous) extracted with different organic solvents.

Source of residue	Weight of residue ($g\ l^{-1}$)
Cellular material remaining after filtrationg of 1 l of culture	0.368 ($\pm$ 0.012)
Chloroform extract	0.015 ($\pm$ 0.001)
Ethyl acetate extract	0.012 ($\pm$ 0.0013)
Dichloromethane extract	0.009 ($\pm$ 0.0005)

The figures in brackets are the standard errors obtained from at least 4 replicates.

were visualised under the ultra violet light and only one of the spots was active against cyanobacteria. For 11B it had an R_f of 0.755 when initially extracted in chloroform and an R_f of 0.722 in ethyl acetate. The R_f values for 10A were 0.66 and 0.755 for extracts in chloroform and ethyl acetate respectively (Table 37).

All attempts to re-elute the inhibitory compounds adsorbed on to the silica particles of the chromatograms failed.

5.3.2 Fungi

All the fungal filtrates lost their activity on autoclaving (Table 38), but, with the exception of filtrates of Fusarium 13E, retained their activities after heating to 100°C for five minutes. With cooling and freezing pretreatments the filtrate of Penicillium 13B lost activity at 0°C and -10°C. However, filtrates of Trichoderma 18A and Fusarium 13E lost their lytic activities at 4°C and below (Table 39).

Table 36 The results of chromatographic separation and bioautographic tests on extracts from isolate 6B

Extracting solvent	Running solvent	R_f values	Test Cyanobacteria species and LBCC No	
			Anabaena flos-aque (28)	Lyngbya halophila (41)
Chloroform	Propan-1-ol	0.872	—	—
		0.545	S	+
		0.181	—	—
Ethyl acetate	Propan-1-ol	0.874	—	—
		0 509	S	+
		0.254	—	—
Chloroform	Methanol	0.000	S	+

+ = inhibition — = no effect and S = stimulation for growth of cyanobacteria species.

Table 37 Bioautographic tests of active spots separated on developed chromatograms from cell-free filtrates of different actinomycetes

Sample extracted	Extracting solvent	Running solvent	R_f values of spots	Test Cyanobacteria species and LBCC No		
				Symploca thermalis(74)	Nostoc entophytum(52)	Anabaena oscillaroides(27)
Isolate 11B filtrates	Chloroform	Propan-1-ol	0.944	−	−	−
			0.755	+	+	+
			0.666	−	−	−
	Ethyl acetate	Propan-1-ol	0.888	−	−	−
			0.722	+	+	+
			0.444	−	−	−
Isolate 10A filtrates	Chloroform	Propan-1-ol	0.666	+	+	+
	Ethyl acetate	Propan-1-ol	0.755	+	+	+

+ = inhibition — = no effect on Cyanobacterial growth lawns

Table 38 The effect of heat pretreatments on the lytic activity of cell-free filtrates of certain fungi on cyanobacteria species

Test Cyanobacteria species	LBCC No	Fungal isolate LBCC numbers and pretreatment temperatures											
		13B Penicillium				18A Trichoderma				13E Fusarium			
		24°C Control	60 °C	100 °C	Autoclaving 121°C/15lb/inch²	24°C Control	60 °C	100 °C	Autoclaving 121°C/15lb/inch²	24°C Control	60 °C	100 °C	Autoclaving 121°C/15lb/inch²
Anabaena oscillaroides	27	+	+	+	—	S	S	(S)	—	—	—	—	—
Nodularia harveyana	40	+	+	+	—	—	—	—	—	+	(+)	—	—
Symploca thermalis	74	+	+	+	—	+	+	+	—	—	—	—	—

+ = Lysis — = no effect S = stimulation (+) = weak lysis and

(S) = weak stimulation of growth of cyanobacterial lawns

Table 39 The effect of cooling and freezing pretreatments on the lytic activity of different fungal cell-free filtrates on cyanobacteria species

Test Cyanobacteria species	LBCC No	Fungal isolate LBCC numbers and pretreatment temperatures																	
		13B Penicillium						18A Trichoderma						13E Fusarium					
		24°C Control	15°C	8°C	4°C	0°C	-10°C	24°C Control	15°C	8°C	4°C	0°C	-10°C	24°C Control	15°C	8°C	4°C	0°C	-10°C
Anabaena oscillaroides	27	+	+	+	+	—	—	S	S	(S)	—	—	—	—	—	—	—	—	—
Nodularia harveyana	40	+	+	+	+	—	—	—	—	—	—	—	—	+	+	(+)	—	—	—
Symploca thermalis	74	+	+	+	+	—	—	+	+	(+)	—	—	—	—	—	—	—	—	—

+ = Lysis — = no effect S = stimulation (+) = weak lysis and (S) = weak stimulation for growth of cyanbacterial lawns

5.3.2.1 Extraction

The substances present in the filtrates from fungal cultures which caused either stimulation or inhibition of cyanobacteria were extractable with chloroform and ethyl acetate but not with dichloromethane. In the latter solvent the activity remained in the aqueous phase (Table 40). A control of pure fungal medium was also extracted with these solvents and the extracts tested against cyanobacteria to prove that no lytic or stimulating activity was due to either compounds in the culture medium or the extracting solvents.

The dry weights of the residues extracted from the various fungal filtrates by different organic solvents are presented in Table 41. The dry weights of extracted material obtained from the same fungal filtrate with either ethyl acetate or chloroform were similar but in comparison the weight of the material extracted with dichloromethane was very small.

The active components extracted by the solvents and thus present in the residues were all water soluble (and gave results similar to those in Table 40), although not all of the residue in the ethyl acetate extract re-dissolved in water. The remaining non-water soluble substances were tested against the susceptible cyanobacterial strains by re-dissolving them in the extracting solvent and applying them to filter discs as before. No inhibitory or stimulatory activity could be observed.

5.3.2.2 Chromatography and purification

The same extraction procedures were used as before but larger quantities of fungal filtrates were extracted. This was to try and

Table 40 Extraction of lytic and growth promoting compounds of different fungal culture filtrates with different organic solvents

Sample extracted	Extracting solvent	Partitioned fractions	Test Cyanobacteria species and LBCC No			
			Anacystis nidulans (88)	Symploca thermalis (74)	Anabaena oscillaroides (27)	Nodularia harveyana (40)
Malt extract medium	None	None	—	—	—	—
Malt extract medium	Chloroform	Aqueous phase	—	—	—	—
		Organic phase	—	—	—	—
	Ethyl acetate	Aqueous phase	—	—	—	—
		Organic phase	—	—	—	—
	Dichloromethane	Aqueous phase	—	—	—	—
		Organic phase	—	—	—	—
Penicillium 13B filtrates	None	None	+	+	+	+
Penicillium 13B filtrates	Chloroform	Aqueous phase	—	—	—	—
		Organic phase	+	+	+	+
	Ethyl acetate	Aqueous phase	—	—	—	—
		Organic phase	+	+	+	+
	Dichloromethane	Aqueous phase	+	+	+	+
		Organic phase	—	—	—	—
Fusarium 13E filtrates	None	None	—	—	—	+
Fusarium 13E filtrates	Chloroform	Aqueous phase	—	—	—	—
		Organic phase	—	—	—	+
	Ethyl acetate	Aqueous phase	—	—	—	—
		Organic phase	—	—	—	+
	Dichloromethane	Aqueous phase	—	—	—	+
		Organic phase	—	—	—	—
Trichoderma 18A filtrates	None	None	—	+	S	—
Trichoderma 18A filtrates	Chloroform	Aqueous phase	—	—	—	—
		Organic phase	—	+	S	—
	Ethyl acetate	Aqueous phase	—	—	—	—
		Organic phase	—	+	S	—
	Dichloromethane	Aqueous phase	—	+	S	—
		Organic phase	—	—	—	—

Organic fractions were added to filter paper discs and dried prior to application to cyanobacterial lawns. 0.3 cm^3 aliquots of the aqueous phases were applied to the lawns in sterile stainless steel wells.
+ = inhibition — = no effect and S = stimulation to growth of cyanobacteria species.

Table 41 The dry weight of residues extracted from fungal cultures by organic solvents

Fungal isolates	Dry weight gl^{-1} of cellular materials filtered from cultures	Dry weight of residue (gl^{-1}) extracted from the filtrates by:-		
		Chloroform	Ethyl acetate	Dichloromethane
Penicillium 13B	8.35	0.136	0.134	0.020
Fusarium 13E	7.21	0.203	0.210	0.083
Trichoderma18A	9.53	0.251	0.223	0.076

obtain larger amounts of the toxic or stimulatory substances and thus make the chromatographic separation and purification easier. The same chromatographic solvents were used as in previous separations of the other microbial filtrates. Propan-1-ol gave the best separation of the Trichoderma 18A extracts regardless of whether they had been originally extracted in chloroform or ethyl acetate. No separation was obtained with the other running solvents and the biological activity remained at the origin in these cases. With propan-1-ol three separate spots could be detected under the ultra-violet light (Table 42). Activity was again visualised bio-autographically using Symploca thermalis to test for inhibition and Anabaena oscillaroides for stimulation. Both stimulatory and inhibitory activity was attributable to the spot with an R_f of 0.725.

Solvent extracts of Fusarium 13E and Penicillium 13B were also chromatographed and separated into various spots. A single active spot but with different R_f values depending on the running solvent was observed (Table 43).

As in previous studies it was not possible to elute the active substances from the thin layer plates since the active compounds remained bound to the silica gel particles.

5.3.3 Bacteria

The results presented in Table 44 show that Bacillus cereus 16A and Bacillus licheniformis 16B lost their antagonistic activities after pretreatment at 100°C and autoclaving. However they retained their activities at 60°C. Filtrates from Bacillus pumilus 15C were however heat stable, remaining active even after autoclaving. The cold stability

Table 42 The results of chromatographic separation and bioautographic test on extract from Trichoderm 18A

Sample extracted	Extracting solvent	Running solvent	R_f values of spots	Test Cyanobacteria species and LBCC No	
				Symploca thermalis (74)	Anabaena oscillaroides (27)
Trichoderma 18A filtrate	Chloroform	Propan-1-ol	0.891	—	—
			0.725	+	S
			0.450	—	—
	Ethyl acetate	Propan-1-ol	0.892	—	—
			0.725	+	S
			0.423	—	—

+ = Lysis — = no effect S = stimulation

Table 43 The results of chromatographic separation and bioautographic test on extracts from two fungal isolates

Sample extracted	Extracting solvent	Running solvent	R_f values of spots	Test Cyanobacteria species and LBCC No	
				Symploca thermalis (74)	Nodularia harveyana (40)
Penicillium 13B filtrates	Chloroform	Propan-1-ol	0.916	+	+
	Ethyl acetate	Propan-1-ol	0.809	+	+
			0.666	−	−
	Chloroform	Diethyl ether	0.906	+	+
			0.613	−	−
			0.533	−	−
	Ethyl acetate	Diethyl ether	0.895	+	+
			0.723	−	−
			0.509	−	−
Fusarium 13E filtrates	Chloroform	Propan-1-ol	0.000	−	+
	Ethyl acetate	Propan-1-ol	0.000	−	+
	Chloroform	Diethyl ether	0.642	−	+
			0.557	−	−
			0.285	−	−
			0.214	−	−
			0.142	−	−
	Ethyl acetate	Diethyl ether	0.926	−	−
			0.838	−	−
			0.720	−	+

+ = inhibition — = no effect on growth of Cyanobacterial lawns

Table 44 The effect of heat pretreatments on the lytic activity of cell-free filtrates of certain bacterial isolates

Test Cyanobacteria species	LBCC No	Bacterial isolates and the filtrate pretreatment temperatures											
		Bacillus pumilus (15C)				Bacillus cereus (16A)				Bacillus lichenformis (16B)			
		24°C Control	60 °C	100 °C	Autoclaving @ 121°C/15 lb/inch²	24°C Control	60 °C	100 °C	Autoclaving @ 121°C/15 lb/inch²	24°C Control	60 °C	100 °C	Autoclaving @ 121°C/15 lb/inch²
Synechocystis diplococcus	19	+	+	+	+	+	+	−	−	+	+	−	−
Plectonema boryanum	89	+	+	+	+	−	−	−	−	+	+	−	−
Anabaena oscillaroides	27	−	−	−	−	+	+	−	−	−	−	−	−

+ = Lysis − = no effect

of these filtrates were also variable (Table 45), Bacillus cereus 16A and Bacillus licheniformis 16B lost their lytic activities below 4°C but Bacillus pumilus 15C remained active even after the freezing pretreatment. The active agents present in Bacillus pumilus 15C filtrates therefore appeared more resistant to the different pretreatments than the other bacterial filtrates tested.

5.3.3.1 Extraction

The extractability of the anti-cyanobacterial agents from the bacterial filtrates by organic solvents (Table 46) was found to be similar to that of the fungi. The active substances present in the bacterial filtrates were extractable with chloroform and ethyl acetate but not dichloromethane. As with the fungi the active substances remained in the aqueous phase after treatment with dichloromethane.

The dry weights of the residues obtained after extraction of the bacterial filtrates with different organic solvents are presented in Table 47 and show that the dry weights of the materials obtained by extracting equal volumes of the same filtrate with either chloroform or ethyl acetate were very similar. In contrast the residues after extraction with dichloromethane were very small. However, the residues extracted by all the organic solvents were water soluble.

5.3.3.2 Chromatography and purification

Solvent extracts of filtrates of Bacillus pumilus (15C) and Bacillus cereus (16A) were subjected to chromatographic separation on thin layer silica gel plates using the same solvents as for the actinomycetes and fungi.

Table 45 The effect of cooling and freezing pretreatments on the lytic activity of bacterial filtrates

Test Cyanobacteria species	LBCC No	Bacterial isolates and their filtrates pretreatment temperatures																	
		Bacillus pumillus (15C)						Bacillus cereus (16A)						Bacillus licheniformis (16B)					
		24°C Control	15°C	8°C	4°C	0°C	-10°C	24°C Control	15°C	8°C	4°C	0°C	-10°C	24°C Control	15°C	8°C	4°C	0°C	-10°C
Synechocystis diplococcus	19	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	−	−	−
Plectonema boryanum	89	+	+	+	+	+	+	−	−	−	−	−	−	+	+	+	−	−	−
Anabaena oscillaroides	27	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−

− = Lysis − = no effect

Table 46 Extraction of lytic compounds of different bacterial culture filtrates with different organic solvents

Sample extracted	Extracting solvent	Partitioned fractions	Test Cyanobacteria species and LBCC No		
			Synechocystis diplococcus (19)	Plectonema boryanum (89)	Anabaena oscillaroides (27)
Modified Allen's medium	Chloroform	Aqueous phase	−	−	−
		Organic phase	−	−	−
	Ethyl acetate	Aqueous phase	−	−	−
		Organic phase	−	−	−
	Dichloromethane	Aqueous phase	−	−	−
		Organic phase	−	−	−
Bacillus pumilus 15C filtrate	None	None	+	+	−
Bacillus pumilus 15C filtrate	Chloroform	Aqueous phase	−	−	−
		Organic phase	+	+	−
	Ethyl acetate	Aqueous phase	−	−	−
		Organic phase	+	+	−
	Dichloromethane	Aqueous phase	+	+	−
		Organic phase	−	−	−
Bacillus cereus 16A filtrate	None	None	+	−	+
Bacillus cereus 16A filtrate	Chloroform	Aqueous phase	−	−	−
		Organic phase	+	−	+
	Ethyl acetate	Aqueous phase	−	−	−
		Organic phase	+	−	+
	Dichloromethane	Aqueous phase	+	−	+
		Organic phase	−	−	−
Bacillus lichenformis 16B filtrate	None	None	+	+	−
Bacillus lichenformis 16B filtrate	Chloroform	Aqueous phase	−	−	−
		Organic phase	+	+	−
	Ethyl acetate	Aqueous phase	−	−	−
		Organic phase	+	+	−
	Dichloromethane	Aqueous phase	+	+	−
		Organic phase	−	−	−

Organic fractions were added to filter paper discs and dried prior to applications to cyanobacterial lawns. 0.3cm^3 aliquots of the aqueous phases were applied to the lawns in sterile stainless steel wells.
+ = inhibition − = no effect

Table 47 The dry weights of residues extracted from bacterial cultures by organic solvents

Bacterial isolate	LBCC No	Dry weight(g) of cellular material filtered from 1 litre cultures	Dry weight of residues(g) extracted from 1 litre of the filtrates by:-		
			Chloroform	Ethyl acetate	Dichloromethane
Bacillus pumilus	15 C	0.332	0.095	0.103	0.039
Bacillus cereus	16A	0.371	0.107	0.102	0.029
Bacillus lichenformis	16B	0.353	0.121	0.109	0.031

Propan-1-ol gave the clearest separation of the extracts of both bacteria regardless of which solvent was used to make the original extracts (Table 48). No separation was obtained with methanol, cyclohexane or trichloroethylene as the running solvents. Diethyl ether separated the extract into 5 spots two more than was obtained with propan-1-ol but the clarity of separation was inferior with some overlapping of the spots which made interpretation of the bioautographic analyses difficult. The inhibitory activity after separation in propan-1-ol was attributable to single spots, the spot R_f 0.733 for Bacillus pumilus and R_f 0.7 for Bacillus cereus.

Again the re-elution of the substances from the active spots was unsuccessful despite using different organic solvents as mentioned before (see section 5.3.1).

5.4 Discussion

Antibiotic screening tests performed at the C.B.S. culture collection had previously shown that the strain A52 (Streptomyces roseodiostaticus) did not appear to produce any classical antibiotics. This finding seemed to be confirmed by the results of some simple tests carried out in this study which showed that A52 did not cause the lysis of the test bacteria and yeast in either agar block or cell-free filtrate applications. Furthermore, work by Glaxo Group Research Limited confirmed that no "conventional antibiotic" behaviour could be attributed to A52 using their screening procedures. Therefore, the antialgal and anticyanobacterial substances produced by A52 may be chemically quite different from the classical antibiotics

Table 48 The chromatographic separation of bacterial filtrates and bioautographic testing of the separated spots

Sample extracted	Extracting solvent	Running solvent	R_f values of spots	Test Cyanobacteria species and LBCC No	
				Synechocystis diplococcus(19)	Anabaena oscillaroides(27)
Bacillus pumilus (15C) filtrates	Chloroform or Ethyl acetate	Propan-1-ol	0.733	+	—
			0.520	—	—
			0.332	—	—
		Diethyl ether	0.742	+	—
			0.550	—	—
			0.352	—	—
			0.226	—	—
			0.120	—	—
Bacillus cereus (16A) filtrates	Chloroform or Ethyl acetate	Propan-1-ol	0.700	+	+
			0.500	—	—
			0.300	—	—
		Diethyl ether	0.720	+	+
			0.533	—	—
			0.383	—	—
			0.216	—	—
			0.102	—	—

+ = growth inhibition — = no effect

produced by actinomycetes. In contrast to A52 the other actinomycete strains used in these experiments viz 216 (Streptomyces lavindulocolor), 10A and 11B (Streptomyces albidoflavus) have all been reported to produce classical antibiotics. Streptomyces lavindulocolor was reported to produce streptothricins and antifungal and antibacterial activity was exhibited by Streptomyces albidoflavus (see Bergey's manual of determinative bacteriology 8th edition, 1975). More recently Norris and Richmond (1981) reported that Streptomyces albidoflavus like Streptomyces lavindulocolor were also able to produce streptothricins. Cell-free filtrates of these actinomycete strains were able to inhibit the growth of different test bacteria, yeast, cyanobacteria and green algae. Therefore, these results might indicate that a range of different types of lytic substances are produced by these isolates, some of which may or may not be identifiable as established antibiotics.

The antimicrobial activity present in cell-free filtrates of A52 was found to be thermostable against certain cyanobacteria. It was reduced or lost, however, when boiled or autoclaved and sited against Nostoc entophytum (52) and Anabaena oscillaroides (27). The various effects of lysis might be due to the degree of sensitivity of these cyanobacteria species to the lytic substances which would appear to lose some potency under the more extreme heating pretreatments.

The total loss of lytic activity in A52 filtrate after the cooling pretreatments at and below $4^{\circ}C$ is unusual, however, this may be attributable to changes in the chemical configuration of the compounds.

It was interesting to note that the active substance in cell-free filtrates of A52 (unlike all the other isolates) could only be extracted with chloroform and gave a single clear absorption peak with a maxima of 335nm. This would suggest that only a single organic substance is involved in any antimicrobial activity. In contrast to A52 the filtrates from streptomycete isolates 216, 10A and 11B (known to produce antibiotics) remained active under both heat and cold pretreatment conditions although their effectiveness against the Nostoc species was again reduced. These cold and heat pretreated filtrates retained levels of activity against the bacteria and the yeast, similar to the original filtrates. Since streptothricins (produced by these actinomycetes) are stable to such pretreatments and are active against these blue-greens it is likely that the antimicrobial activity shown by these isolates is due to the action of these "classical" antibiotics, particularly since the bacteria and yeast were also inhibited like the cyanobacteria and green algae.

Although so-called classical antibiotics are known to be produced by different actinomycete strains and to cause the lysis of various cyanobacterial species (Palmer and Maloney, 1955; Foter, Palmer and Maloney, 1953; Hunter and McVeigh, 1961; Srivastava and Nizam, 1969; Srivastava, 1970, this study suggests that other compounds not recognised as typical antibiotics are produced and cause lysis of cyanobacteria species. The nature of these compounds, however, has not been established.

Enzymes are known to be produced by different microbes including actinomycetes, but they are unlikely to be the active substance in these isolates, since enzymes do not survive autoclaving.

The inhibitory action of isolate 6B (Streptomyces albidoflav us) on the growth and metabolic activities of several different cyanobacterial species is in stark contrast to the stimulatory action this isolate has on Anabaena flos-aquae (28). This reaction highlights the specificity of such actinomycete isolates which clearly do not have a blanket effect on all cyanobacteria. Why in repeated experiments Anabaena flos-aquae (28) should show this different behaviour is difficult to explain. Waksman (1959) pointed out that some microbial strains are capable of producing both antimicrobial and growth promoting agents and it is possible that Anabaena flos-aquae (28) can produce a compound capable of denaturing the lytic compound whilst at the same time benefiting from the release of a stimulatory compound. Unfortunately it was not possible in the time available to investigate whether Anabaena flos-aquae (28) did produce an anti-lytic compound. Simple tests could have been performed to see if filtrate from Anabaena flos-aquae (28) cultures could, when added to sensitive isolates of blue-greens, prevent their lysis by 6B filtrate. It should be noted, however, that the stimulatory and inhibitory activity could be related to the same active spots after chromatographic separation of the extracts and this would suggest, although not conclusively, that inhibition and stimulation could be attributed to the same compound.

Fungi are known to produce a great variety of antibiotics. Among the most active fungal genera are Penicillium, Aspergillus and Trichoderma. The antibiotics produced by the genera were found to be able to lyse certain species of cyanobacteria and green algae (Srivastava, 1970; Kumar, 1964). Recently, Redhead and Wright (1980) isolated

cephalosporin C (which behaved similarly to B-lactamase) from Emericellopsis salmosynnemata and Acremonium kiliense which proved capable of lysing Anabaena flos-aquae. In contrast Fusarium species are known to produce the enzyme inhibitor fusaric (fusarinic) acid (Umezawa, 1972). This compound could be the anticyanobacterial agent active in this study since protein inhibitors are known to have a broad spectrum of activity.

Although Trichoderma (18A) appeared to inhibit the growth of certain cyanobacterial species, it stimulated the growth of Anabaena oscillaroides (27). It is possible that Trichoderma produces both stimulatory and inhibitory agents and in certain cases the cyanobacterium is not sensitive to the inhibitor but stimulated by another compound. However, as with chromatographic separation of extracts of the actinomycete filtrates, stimulatory and inhibitory activity seem to be attributable to the same active spot.

All the fungal isolates under investigation lost their activities after autoclaving pretreatments. However, cell-free filtrates from Penicillium and Trichoderma survived 100°C for five minutes (unlike Fusarium) but showed reduced potency. In the case of cooling down to 4°C and below only the Penicillium filtrate survived these pretreatments, and remained active, confirming the different nature of the anticyanobacterial agents produced by the different fungal isolates. They could however all be extracted in the same organic solvents and could (with the exception of Fusarium) be best separated with the same chromatography solvent, namely propan-1-ol.

The active bacterial strains produced soluble extracellular lysing compounds. The 15C (Bacillus pumilus) filtrate was diffusible through agar, heat stable and cold labile. In contrast filtrates of 16A (Bacillus)

licheniformis) lost their active properties against the cyanobacteria after the pretreatment at 100°C and autoclaving. Again this indicates that different anticyanobacterial agents were present in the filtrates of the different bacterial isolates. Since autoclaving did not destroy the activity of all the bacterial extracts it is possible that some of the active agents were enzymes or antibiotics. It has been reported that Cellvibrio, Flexibacter, Myxobacter xanthus and Myxobacter 44 produce soluble extracellular compounds which lyse cyanobacteria, while other bacteria such as CP1 and FP1 required mechanical contact with the host for lysis to occur (Shilo, 1970; Daft and Stewart, 1971, 1973).

The extracellular substances produced by bacteria include a variety of extracellular enzymes, some or all of which may be important in the lytic process. The rapid dissolution of the host cell wall layers indicates that a lysozyme type of enzyme could be important in some cases and the organisms show proteolytic activity as evidenced by their capacity to liquify gelatine. They can also break down peptone, casitone and soluble starch (Daft et al. 1975). A contact mechanism may be particularly advantageous to these bacteria because it results in a concentration of the enzymes at the point where they are required most. Certain bacteria depend, however, on truly extracellular enzymes for lysis and the most detailed study of this type of organism has been carried out by Stewart and Brown (1971) on Myxobacter 44. This bacterium produces an extracellular lysozyme-like enzyme and cell-free filtrates caused lysis of cyanobacterial species.

In addition to its lysozyme-like activity Myxobacter 44 shows proteolytic, alginase, cellulase and chitinase activities (Stewart and Brown, 1971). Granhall and Berg (1972) reported that antibiotic-like substances formed by Cellvibrio cause lysis of the vegetative cells of blue-greens. Furthermore, cyanobacteria have been previously reported to be lysed by gramicidin s. an antibiotic probably produced by Bacillus brevis (Reim et al. 1974). Although it was not possible to characterise the active substances from the bacterial isolates in this study, it was shown that the active substances could be extracted with organic solvents from cell-free filtrates and are thus extracellular compounds. No contact between the bacterium and the cyanobacterium was necessary for lysis to occur confirming the suggestion of Chapter 4.

CHAPTER SIX

DETERMINATION OF THE FORM AND ARRANGEMENT OF MICRO-ORGANISMS IN SOIL SALT MARSH MICROHABITATS

6.1 Introduction

In the original isolation studies the various microbial types were isolated from a particular mud core after it had been thoroughly mixed. No account was taken of any possible spatial distribution of the various microbial types within the core which might coincide with different types of microhabitats. Furthermore, the interaction studies showed that lytic microbes could be isolated from the same cores as cyanobacteria which were apparently sensitive to them.

Therefore, in the following experiments, cores taken from the salt marsh were vertically sectioned into 0.2cm deep pieces and isolation of the microbial groups made on each individual piece. In this way it was hoped to gain some information on the distribution of individual microbial groups with depth in the cores and also on the relative distribution between the groups.

6.2 Materials and methods

Simple mud cores were produced from $10cm^3$ capacity disposable plastic syringes by cutting off the needle attachment end and sharpening the cut edge of the barrel. Prior to use, three corers were surface sterilised in methanol (80% v/v) for 24h. They were then allowed to dry by evaporation in a laminar flow sterile hood and wrapped in sterile aluminium foil before transportation to the field site.

Three mud cores were taken in close proximity to one another (Plates 17 and 18) and transported back to the laboratory in the syringes with the open ends sealed with sterile caps (Plate 19). The surface 1cm zone of each core was then sectioned into five separate 0.2cm thick sections (Plate 21) by placing the corer into a simple microtome (Plate 20) extruding all but the top 1cm of the core (ie, the zone nearest to the plunger) and cutting this off with a sterile razor blade. The 0.2cm sections from the surface 1cm zone were then collected by sequentially cutting off each 0.2cm of the core as it extruded from the end of the syringe. These sections were coded 1, 2, 3, 4 and 5 starting from the surface.

The individual mud sections were immediately serially diluted down to 10^{-5} with quarter strength Ringers solution and $0.3cm^3$ from each dilution (in triplicate) was spread onto the plates of the various media depending on which microbial group was to be isolated. The colonies appearing on the plates were counted, ignoring colonies of other microbes on the actinomycete plates and so on. The various organisms were then isolated and purified using the same procedures as employed previously (Chapter 2).

The differences in the counts obtained for each microbial group in different sections of the same core were investigated for statistical significance. The parametric ANOV test (Zar, 1974) was performed. The above test assumes certain distribution of the micro-organisms, and is sensitive to departures from this assumption, and to homogeneity of the variance. Therefore, the non-parametric Kruskal-Wallis test was also performed to avoid violating these assumptions (Siegal, 1956). Both of these tests were performed using

Plate 17

Sterilized $10cm^3$ syringes were used to collect mud samples from the Dee salt marsh environment.

Plate 17

Plate 18

A modified $10cm^3$ capacity syringe used to take the mud cores from the salt marsh.

Plate 19

A mud sample taken with a sterilized $10cm^2$ capacity syringe. The open end was resealed with a sterile cap.

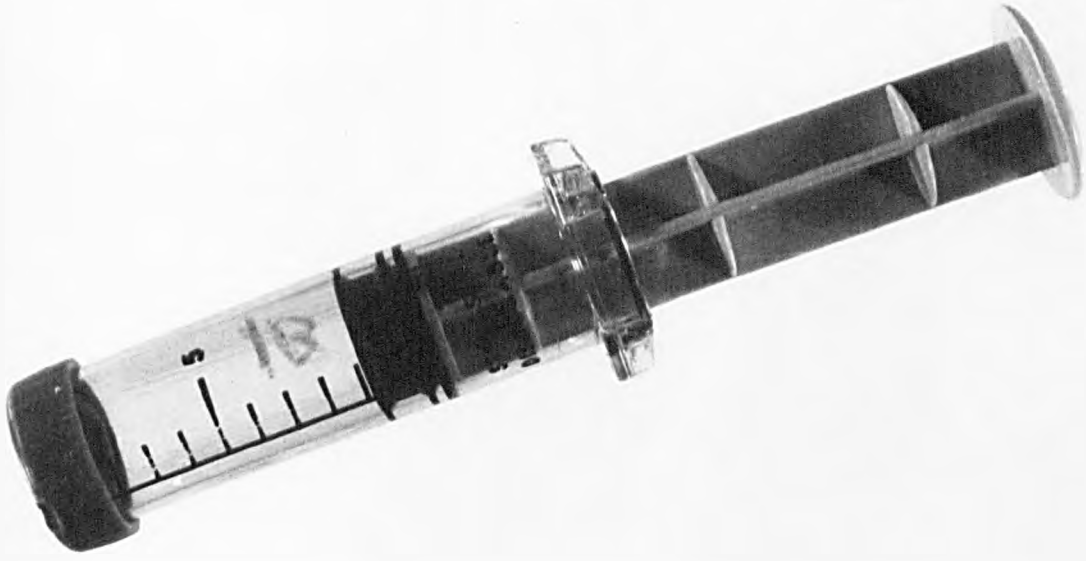


Plate 18

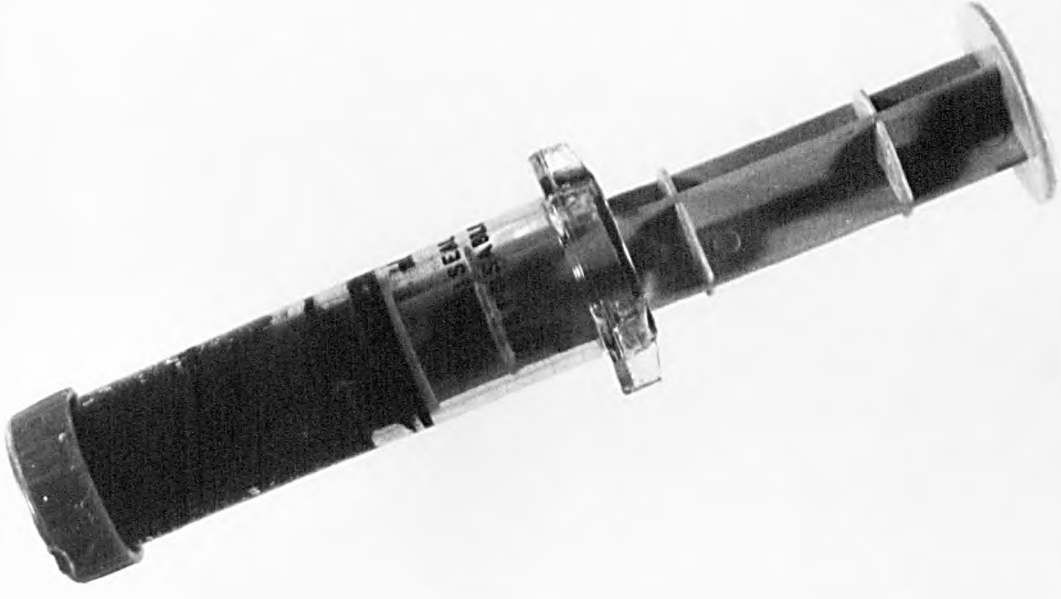


Plate 19

Plate 20

The simple microtome used to section the mud sample inside the sterilized syringe.

Plate 21

A core mud sample showing how it was to be sectioned into five subsections (S_1 to S_5) each 0.2cm thick.

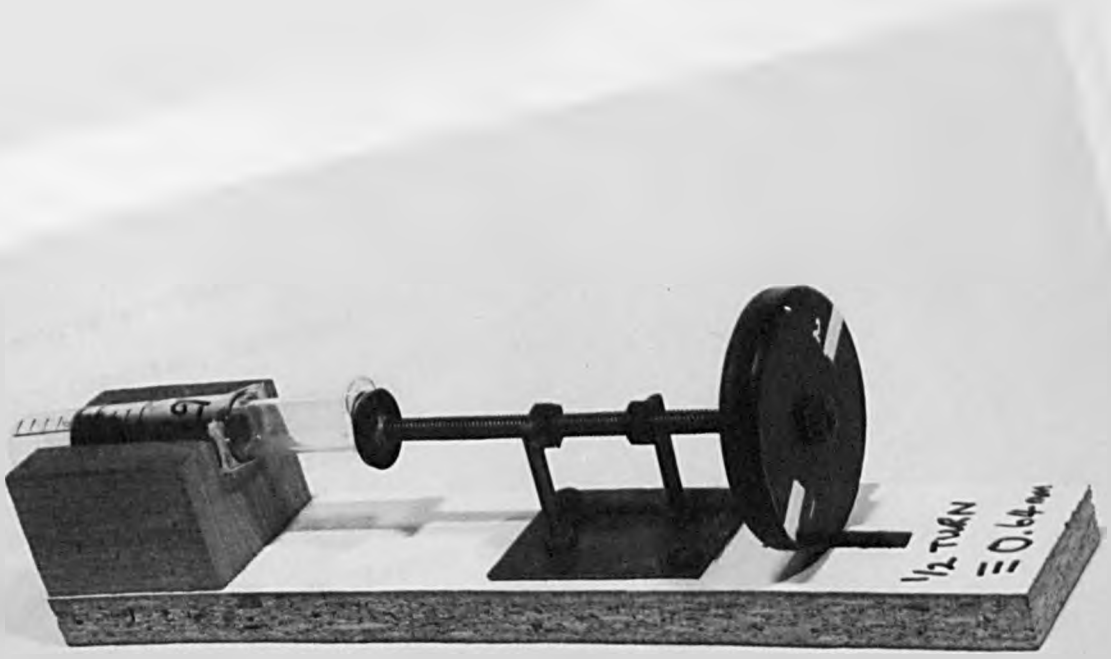


Plate 20

Plate 21

the procedures provided by the SPSS statistical package available on the ICL Computer, Liverpool University.

Interaction tests between the cyanobacterial isolates and the actinomycetes obtained from the same and different sections of the same core or different sections from different cores were made using the same procedure as in Chapter 2.

6.3 Results

Isolates of the different microbial groups were obtained from each of the five 0.2cm sections comprising a single sediment core. The number of isolates comprising each of the microflora groups were counted for each section and compared with the numbers of isolates of the same group obtained at different depths in the same core or at the same depth in different cores.

More bacterial isolates were found than any other group followed by the actinomycetes, fungi and cyanobacteria and this sequence was obtained in every section of every core. The results of Kruskal-Wallis one-way analysis of variance (table 49) showed that there was no significant difference between the numbers of a particular microbial type found at different depths within a core.

Isolates of actinomycetes and cyanobacteria were chosen at random from individual sections in each core and purified. These were each given a reference number denoting the number of core and section from which the isolate was originally obtained. Interaction tests were performed between the cyanobacteria and actinomycete isolates obtained from the same 0.2cm section, from different sections within the core and from sections from different cores. The results (table 50) show that

Table 49. Testing of differences in numbers of the different microbial groups between sections in each core. Results of Kruskol-Wallis one way analysis of variance

Microbial types	Sections	Core sample "A" Mean	S.D.	95% Conf. int.for Mean	Core sample "B" Mean	S.D.	95% Conf. int.for Mean	Core sample "C" Mean	S.D.	95% Conf. int.for Mean
Bacteria	1	588190	817509.514	-426865.6412 to 1603245.6412	50370	50904.0470	-12834.6957 to 11.3574.6952	632458	893806.4969	- 477331.3189 to1742247.3189
	2	112260	131646.7926	- 51498.4273 to 275718.4273	107880	255005.2392	-118745.6809 to 514505.6809	177050	259962.0405	- 145730.2627 to 499830.2627
	3	312980	431695.6706	-223032.2643 to 848992.2643	189560	345469.2577	-239389.7711 to 618509.7711	343192	649684.5945	- 463484.8660 to1149868.8660
	4	201012	512920.903	-345853.0727 to 927877.0727	285352	351707.8269	-151343.8520 to 722047.8520	65660	74851.8403	- 27279.3254 to 158599.3254
	5	183464	227332.0138	- 98801.3915 to 465729.3915	914152	1446526.3342	-881918.4928 to 2710222.4928	566390	1083457.5952	- 778878.4343 to1911658.4343
Fungi	1	4314	5218.6186	- 2165.6656 to 10793.6656	6844	9165.4613	- 4536.2384 to 18224.2384	3778	4487.7522	- 1794.1898 to 9350.1598
	2	4118	5372.8130	- 2553.1201 to 10789.1201	702	1294.9981	- 905.9264 to 2309.9264	4324	8771.8231	- 6567.4800 to 15215.4800
	3	322	431.1844	- 213.3775 to 857.3775	1110	1719.1568	- 1024.5804 to 3244.5804	464	862.8325	- 607.3307 to 1535.3307
	4	316	435.0632	- 224.1936 to 856.1936	1466	2163.1643	- 1219.8797 to 4151.8797	790	1278.8667	- 797.8969 to 2377.8969
	5	3026	4219.9976	2213.7340 to 8265.7340	8574	12646.6549	- 7128.6410 to 24276.6410	2256	4347.3762	- 3141.8928 to 7653.8928
Actinomycetes	1	8	17.8885	- 14.2112 to 30.2112	496	610.8028	- 262.3995 to 1254.3995	604	866.0716	- 471.3524 to 1679.3524
	2	2594	4218.1370	- 2643.4238 to 7831.4238	244	433.2205	- 293.9056 to 781.9056	6656	13110.4416	- 9622.4989 to 22934.4989
	3	1264	2651.1281	- 2027.7568 to 4555.7568	6066	13380.1076	- 10547.3280 to 22679.3280	2312	4331.0184	- 3065.5822 to 7689.5822
	4	2112	3894.9608	- 2724.1541 to 6948.1541	1930	3957.5245	- 2983.8358 to 6843.8358	1106	2182.9292	- 1604.4206 to 3816.4206
	5	3284	4532.2489	- 2343.4389 to 8911.4389	2206	4378.0338	- 3229.9586 to 7641.9586	2638	4180.6961	- 2552.9355 to 7828.9355
Cyanobacteria	1	348	470.6591	- 236.3910 to 932.3910	98	173.2628	- 117.1307 to 313.1307	4654	8671.0195	- 6112.3179 to 15420.3179
	2	304	443.9369	- 247.2116 to 855.2116	44	60.6630	- 31.3218 to 119.3218	312	437.8584	- 231.6642 to 855.6642
	3	46	87.0632	- 62.1015 to 154.1015	128	216.6102	- 140.9528 to 396.9528	54	87.0632	- 54.1015 to 162.1015
	4	8	17.8885	- 14.2112 to 30.2112	182	303.5128	- 194.8548 to 558.8548	28	43.8178	- 26.4061 to 82.4061
	5	24	43.3590	- 29.8364 to 77.8364	1848	2671.8009	- 1469.4251 to 5165.4251	10	22.3607	- 17.7640 to 37.7640

Table 50. Interactions between actinomycetes and cyanobacteria isolated from sectioned sediment cores from the Dee salt marsh

Cyanobacterial isolates		Actinomycetes reference number of isolates [1]									
Reference number [1]	Genus	A/1/1	A/2/1	A/3/1	A/4/1	A/5/1	B/1/1	B/2/1	B/3/1	B/4/1	B/5/1
A/1/1	Anabaena	-	-	-	-	-	+	-	-	-	+
A/1/2	Oscillatoria	+	+	-	-	-	+	-	-	-	+
A/1/3	Lyngbya	+	+	+	-	-	+	-	-	-	+
A/2/2	Oscillatoria	+	-	+	+	-	+	-	-	-	+
A/2/1	Nostoc	+	-	-	-	-	+	-	-	-	+
A/3/2	Lyngbya	+	-	+	-	-	+	-	-	-	+
A/3/1	Nostoc	-	-	+	-	-	-	-	-	-	+
A/4/1	Anabaena	-	-	-	-	-	-	-	-	-	+
A/4/2	Oscillatoria	+	-	-	-	-	+	-	-	-	+
A/5/2	Anabaena	+	+	-	-	-	-	-	-	-	-
A/5/1	Lyngbya	+	+	+	-	-	+	-	-	-	+
B/1/1	Oscillatoria	+	+	-	-	-	+	-	-	-	+
B/2/2	Nostoc	-	-	+	-	-	+	-	-	-	+
B/3/2	Oscillatoria	+	-	-	-	-	-	-	-	-	+
B/4/1	Lyngbya	+	-	+	-	-	+	-	-	-	+
B/5/1	Nostoc	+	+	+	-	-	+	-	-	-	+
B/5/2	Nostoc	-	-	-	-	-	+	-	-	-	-
B/5/3	Nostoc	+	-	+	-	-	-	-	-	-	-
B/5/4	Lyngbya	+	+	+	-	-	+	-	-	-	+

1. The letter in the code represents the core from which the isolate came, the first number is the 0.2cm core section from which the isolate came numbering from the surface and the second number identifies the isolate.

The interaction tests were performed using the agar block method.

interactions occurred between actinomycetes and cyanobacteria isolated from the same core section as well as between isolates from different sections or different cores.

6.4 Discussion

Although the number of isolates of the different microbial types was different between the sections of the cores, statistical analysis on isolate numbers showed there to be no apparent variation or pattern in distribution with depth for any of the groups in any of the cores at least within a 0-1cm deep zone from the surface.

The interaction tests showed that lysis of the cyanobacteria by actinomycete isolates occurred even when the test organisms were originally obtained from the same section. This might suggest that cyanobacterial distribution even within a thin section of a core cannot be linked to the sensitivity of the cyanobacteria to other microbial isolates. However, such a statement requires considerable qualification. It is possible that even with a narrower sections under natural conditions the zones of influence of the different microbes do not necessarily overlap and thus such a core section itself may contain numerous microhabitats. Furthermore, one cannot be sure that the lytic substances produced under laboratory conditions are produced or are effective under natural conditions. It is also possible that if microbial interactions do exert some control over the pattern of distribution of cyanobacteria, then those organisms isolated here may not be the effective ones and were missed in the isolation programme. Another point worth mentioning is that the

isolates obtained in this study were all isolated under aerobic conditions and yet it was clear from visual observation of the cores that black anaerobic zones were present within the top 1cm zone. Thus obligate anaerobes may have been missed which could have been exerting microbial control.

CHAPTER SEVEN

GENERAL DISCUSSION

In this study the cyanobacteria species, particularly the unicellular and filamentous non-heterocystous species, proved to be the most difficult micro-organisms to obtain free from other bacteria. This was due to the apparently close physical relationship between the contaminating bacteria and the cyanobacteria. It has been suggested that contaminating bacteria may play an important role in the general physiology of certain cyanobacteria. For example, bacteria seen attached to the polar regions of heterocysts of Aphanizomenon flos-aquae and Anabaena circinalis, (Pearl, 1976) probably assimilate substances released by the heterocysts and may in return benefit the blue-greens by scavanging oxygen in the vicinity of the heterocysts which house the extremely oxygen sensitive nitrogenase enzyme (Bershova et al. 1968; Bunt, 1961; Schwabe and Mollenhaver, 1967). In general, the role of attached bacteria is little understood since the physiology of blue-greens is usually studied in axenic culture. Kuentzel (1969) and Lang (1967) reported that CO_2 evolution resulting from bacterial mineralization of organic compounds stimulated the growth of algal hosts. Furthermore, Fogg (1952) and Fogg and Pattnaik (1966) suggested that at least part of the organic carbon excreted by cyanobacteria was mineralized by the association bacteria.

A positive decision to evaluate the sensitivity of blue-greens to the effects of various other microbes using axenic cultures of cyanobacteria was made even though it was acknowledged that, the close association of blue-green and contaminant might significantly regulate any interaction under field conditions. This decision was made because

it was considered that there was no means of being sure that the contaminant was real or a laboratory introduced one and because even if the contaminant was real, under field conditions it may not be the major or only contaminating strain.

The anticyanobacterial agents discovered in this work have not been chemically characterised or their absolute concentration determined and therefore it is difficult to show that an organism is more susceptible to one agent than to another simply on the basis of the size of the inhibitory zone produced. Certain points were noted, however, when carrying out the interaction tests:-

i) The composition of the nutrient medium must be standardised, since certain known antibiotics are affected by specific compounds contained in laboratory media. The depth of medium and the degree of hydration of the agar gel must also be standardised.

ii) The inoculum must be of constant size.

iii) The incubation time and temperature must be constant.

In general, the production and potency of antibiotic substances is known to be affected by medium composition. It should be re-emphasised that in this study the number of cyanobacterial species lysed by a specific actinomycete isolate depended on the medium in which both interactants was grown. This could be due to the effects of nutrients on the production of antibiotic or any modifying compounds produced by either of the micro-organisms. Vasilieva et al. (1979) reported the effects of components of the nutrient medium on the diffusion properties of mycoheptin and thus its effect on the growth of Candida utilis. It was found that the content of sodium and potassium

chlorides, sodium sulphate or glucose in the medium had a significant effect on the size of the inhibition zones of the test cultures. Green and Waksman (1948) studied the effects of nutrient medium on the inhibitory action of streptomycin and found that salt concentrations had the most significant effect on its potency. The salt concentration not only affected the streptomycin itself but also the medium, which in turn influenced the growth of the host organism. For example, the inhibitory action of streptomycin on the growth of E. coli was affected by the salts of succinic, formic and malic acids; E. coli and P. vulgaris were largely protected by the organic acids against the action of streptomycin, whereas Anabaena aerogenosa and Subtilis aureus were only slightly affected. Furthermore, Reddy (1977) reported on the effects of the concentration of the nitrogen source (KNO_3, $NaNO_3$ and NH_4Cl) on streptomycin and penicillin toxicity.

The data available suggests that it is difficult to predict whether species produce substances which have an inhibitory potential if the chemical nature of its environment is not known. In this study the effect of different individual components of the nutrient medium on the inhibitory and stimulatory activity of actinomycete metabolites was not attempted because of the limited amount of time available.

Many cyanobacterial and green algal species used in this study were found to be resistant to attack by certain antimicrobial agents produced. It is possible that extracellular products released by these resistant strains could inactivate the toxic compounds, e.g. B-lactamase antibiotic (Kushner and Breuil, 1977). Furthermore, the contaminant bacteria, which may be present in the mucilaginous

sheath, might play an important role in preventing the cyanobacteria from attack, by producing extracellular compounds which either kill the competing microbes or de-activate the toxic substances present in the culture medium. Whitton (1965, 1967) has found that extracellular products released by Anaebaena cylindria markedly increase the resistance of this microorganism to polymyxin B, a substance to which it is otherwise relatively sensitive. More recently, Gunnison and Alexander (1975a) suggested that the production of toxins did not account for the resistance of these cyanobacteria and the "differing susceptibilities to decomposition may be related to the relative biodegradabilities of specific components of the algal walls".

The characteristics of resistant strains of cyanobacteria have not yet been identified. Kumar (1964) isolated a streptomycin and penicillin resistant strain of Anacystis nidulans by serially subculturing the organism into increasing concentrations of the respective antibiotics. Singh et al. (1965) produced resistant strains to the same antibiotics in one subculturing into streptomycin and after several subculturings into penicillin. Both studies claim mutational origin of resistant strains. Anacystis nidulans used in this study which was different from the strain used by Kumar (1964) was sensitive to various numbers of micro-organisms. However, Gunnison and Alexander (1975a) reported that the same species was the most resistant to microbial attack by different micro-organisms.

Blue-greens are known to possess antibiotic properties. They are effective against gram-positive and gram-negative bacteria, e.g. Bacillus subtilis and Bacillus typhosus respectively. These blue-greens

are Oscillatoria princes and Lyngbya majuscula (Gupta and Srivastava, 1965). In this study, however, no inhibitory or stimulatory activity by any of the blue-greens was shown on any tested fungi, bacteria or actinomycetes by using either their cell-free filtrates or block applications methods. Jorgensen (1962) extracted substances from some green algae which were very effective against the genus Bacillus and he suggested that the compounds were chlorophyllides. Other workers (Ramamurthy, 1970; Patnaik, 1967; Perminova, 1963, 1964; McCracken et al., 1979; Pratt, 1941; Scuit, 1964; Debro and Ward, 1979; Sieburth, 1971; Chrost, 1974; Meffert and Overbeck, 1979; Bell and Lang, 1974; Ramamurthy et al. 1967; Sieburth, 1964; Keating, 1978; Harris, 1971; Lam and Silvester, 1979) have reported antibiotic-like properties attributable to cyanobacteria or green algae which cause the inhibition of growth of other micro-organisms.

An extensive review of the literature dealing with interactions between micro-organisms would be too lengthy for discussion. The work of Akehurst, 1931; Pratt and Fong, 1940; Levring, 1945; Denffer, 1948; Lefevre et al. 1952; Jakob, 1954; McVeigh and Brown, 1954; Rice, 1954; Allen, 1956; Jorgensen, 1956; Fogg, 1957; Protector, 1957a & b, include reports of the co-action of algae. Notable among the references dealing with biotic relationships between algae and other micro-organisms are the papers of Pratt and Fong, 1940; Flint and Moreland, 1946; Henricksson, 1951; Pratt et al. 1951; Watanabe et al. 1951; Harder and Opermann, 1953; Bond and Scott, 1955; Dodd, 1955; Steeman-Nielsen, 1955; De and Mandel, 1956; Emeis, 1956; Gibor, 1956; Collins, 1957; Maclachlan and Yentsch, 1959; Mclaughlan and Zahl, 1957, 1959; Hutchison and Hammen, 1958; Ahmadjian, 1959a & b; Ahmadjian and Henricksson, 1959; Pratt and Sestak, 1959;

Golueke, 1960. It does not seem relevant to this study to review all the results of these investigators because:

i) Most of the studies have dealt with organisms which were not isolated from the same identical habitat.

ii) Most of the studies employed either highly complex nutrient media or strictly inorganic mineral media, both of which are unnatural and, hence inadequate for in vitro ecological studies.

iii) Many of the studies have not attempted to ascertain the causal mechanisms for the phenomena reported.

While results obtained in this study showed that microbes were able to attack the cyanobacteria living in the same micro-habitat, some recent reports have been included here which discuss biotic relationships between microbes isolated from the same or different habitats, for example, interactions between cyanobacteria and other micro-organisms were carried out, some of which were isolated from the same habitat (Safferman and Morris, 1963; Daft et al. 1973, 1975; Gunnison and Alexander, 1975b) and the others from completely different habitats (Safferman and Morris, 1962; Wu et al. 1968; Bunt, 1961; Bershova et al. 1968; Stewart and Brown, 1970, 1971; Gromov et al. 1972; Granhall and Berg, 1972; Burnham et al. 1976; Shilo, 1970, 1971; Daft and Stewart, 1971, 1973; Yamamoto and Suzuki, 1977; Yamamoto, 1978; Redhead and Wright, 1978, 1980 and Burnham et al. 1981). These workers suggested that the antialgal agents were either antibiotics or exoenzymes produced by different microbes. No definite identifications of the antialgal agents were made by these investigators with the exception of Redhead and Wright (1980) who partially purified a cephalosporin c antibiotic from certain fungal cultures.

Although in this study positive interactions were observed from microbes isolated from the same microhabitat, it is still not possible to determine whether these antimicrobial metabolites are produced in the natural habitat.

The classical antibiotics like streptomycin and penicillin are commercially isolated from different micro-organisms. Their lytic activity has been demonstrated against different strains of cyanobacteria and green algae (Kumar, 1964, 1968; Rodrigues-lope and Munoz, 1970; Srivastava, 1970; Vedajanani and Sarma, 1977; Tarar and Kelner, 1978; Vance, 1966; Foter et al. 1953; Tchan and Gould, 1961: Hunter et al. 1961; and Srivastava and Nizam, 1969). Various respon-es were observed which included the actual lysis of the vegetative cells although heterocysts and akinetes were usually unaffected. In general, the cyanobacteria species were found to be more susceptible to attack to these antibiotics than the green algae and diatoms.

Despite numerous studies, it has proved difficult to demonstrate the presence in soil of specific inhibitory metabolites or antibiotics. It has been suggested that an accumulation of metabolites might occur in soil because of the growth of micro-organisms, but confirmatory evidence has proved hard to find (Williams, 1982). Balis and Kouyeas (1968) stated that evidence for any inhibitor in soil is circumstancial. The leaching of soil with water fails to reduce antagonistic activity and sterile soil extracts are usually, but not always, without activity (King and Coley-Smith, 1969). Lockwood (1914) states that clear evidence for fungistatic activity in soil would require the following minimum evidence :

i) demonstration of inhibitory activity in extracts made with mild reagents from a wide range of soils.

ii) demonstration of inhibitory activity by such extracts against a wide range of micro-organisms.

It is difficult to imagine that the unusual, and in most cases, complicated antibiotic molecules, which are biologically very active, do not have any role or physiological function in the producing organisms. According to Krassilnikov (1959) these substances play an important role among the competing microbes in nature. It is known, however, that in nature antibiotics are not produced in high concentrations or according to some authors (e.g. Gottlieb, 1976) they are not formed in the soil at all. Furthermore, it is difficult to agree with those who suppose that some antibiotics serve as detoxifying substances (Livitov, 1957), since the synthesis of an antibiotic requires the production of a number of specific enzymes and is thus bioenergetically inefficient (Barbas, 1980). It is more economic for the microbes to synthesise a single enzyme (e g. B-lactamase) which can destroy or inactivate the toxic substances.

In this study all the stimulatory or inhibitory substances affecting blue-greens were found to be present in the filtrates from microbial cultures and were thus extracellular products. There is a need therefore for further investigation into the possible role of such compounds in the control of algal blooms and thus the commercial production of such compounds using processes similar to those used for the manufacture of antibiotics.

The compound produced by the actinomycete A52, which was very active against cyanobacteria, showed some peculiar characteristics, e.g. it was partially or completely denatured by cooling down to 4°C and below. Thus its use in the control of cyanobacterial blooms in natural environments in temperate climates may be restricted. However, its chemical characterisation could lead to an understanding of the basis of its activity and subsequent chemical modification might eliminate the cold labile property. Alternatively, it may be possible to synthesise a similar type of compound directly. However, it should be remembered that cyanobacteria cause many nuisance blooms in warm temperate and tropical climates where cold liability would be of no significance.

Further developments

Evidence provided here suggests that some of the active compounds isolated in this study were not classical antibiotics but represented a new type of antimicrobial compound. It would be invaluable, therefore, to characterise fully some of these compounds. It would also be useful to see if the antimicrobial activity can be sustained under field trial conditions to regulate the growth of natural microbial populations. Furthermore, it is important to study the pilot scale production of a compound like that obtained from A52 to determine how it is actually produced by the microbe and the optimum conditions for its production. Such factors are important to the eventual commercial production of such a compound.

In this investigation obligate anaerobes, notably the bacteria, were not considered, therefore it is possible that these micro-organisms could produce anticyanobacterial substances since blue-greens are present in many anaerobic habitats. These active compounds produced by an obligate anaerobe may be stable under aerobic conditions once they have been synthesised. Thus, they might be useful to control cyanobacterial blooms in the environment.

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APPENDIX 1

(A) Modified Allen's (1968) medium used in the isolation and cultivation of the cyanobacterial strains reported in this work.

Salt	Concentration ($g\ l^{-1}$)
$NaNO_3$	1.500 *
Na_2CO_3	0.020
$Na_2SiO_3.9H_2O$	0.058
Citric acid	0.006
Ferric acid	0.006
E.D.T.A.	0.001
$CaCl_2$	0.027
$MgSO_4.7H_2O$	0.075
K_2HPO_4	0.039

Microelements	$\mu g\ l^{-1}$
H_3BO_3	618.0
$Na_2MoO_4.2H_2O$	504.0
$MnCl_2.4H_2O$	720.0
$ZnSO_4.7H_2O$	230.0
$CoCl_2.2H_2O$	80.0
$CuCl_2.2H_2O$	0.034

* For N-free medium this was replaced by 58.44g NaCl. Analar grade reagents were used. The total volume of the medium was made up to 1.0 l with distilled water. The pH of the medium is 7.8. 1.5% Agar No. 3 (Oxoid) was added to make solid medium.

(B) ASM medium used in the cultivation of the cyanobacterial strains during interaction tests with actinomycetes (Gorham et al. 1964).

Salt	Concentration (μ moles/125 ml)
$NaNO_3$	2000 *
$MgSO_4$	200
$MgCl_2$	200
$CaCl_2$	100
K_2HPO_4	100
Na_2HPO_4	100
$FeCl_3$	4
H_3BO_3	40
$MnCl_2$	7
$ZnCl_2$	3.2
$CoCl_2$	0.08
$CuCl_2$	0.0008
Na_2EDTA	20

* For N-free medium this was replaced by 58.44 g NaCl
Analar grade reagents were used. The total volume of the medium was made up to 1.0 l with distilled water. The pH of the medium is 7.4 - 7.8.

(C) CHU. No. 10 modified medium after Gerloff *et al* (1950) used in the cultivation of the green algae species.

Salt	Concentration ($g\ l^{-1}$)
$Ca(NO_3)_2.4H_2O$	0.040
K_2HPO_4	0.010
$MgSO_4.7H_2O$	0.025
Na_2CO_3	0.020
$Na_2SiO_3.9H_2O$	0.025
Ferric Citrate	0.003
Citric acid	0.003

The pH of the medium is 6.5 - 7.0

(D) Casein-starch medium used in the isolation and cultivation of the actinomycete isolates reported in this work.

Salt	Concentration ($g\ l^{-1}$)
Starch	10.00
Casein	0.30
KNO_3	2.00
NaCl	2.00
K_2HPO_4	2.00
$MgSO_4.7H_2O$	0.05
$CaCO_3$	0.02
$FeSO_4.7H_2O$	0.01

1.2% Agar No. 3 (Oxoid) was added to make solid medium

The pH of the medium is 7.0

(E) Glycerol-asparagine agar 5 medium (ISP5) used in the isolation and cultivation of the actinomycete strains reported in this work.

Salt	Concentration ($g\ l^{-1}$)
L-Asparagine (anhydrous)	1.0
Glycerol	10.0
K_2HPO_4	1.0

*Trace salt solution.

Salt	Concentration (g/100ml)
$FeSO_4.7H_2O$	0.1
$MnCl_2.4H_2O$	0.1
$ZnSO_4.7H_2O$	0.1

* 1 ml trace salt solution was added to the medium and has been made up to 1 litre by addition of distilled water

2% agar No. 3 (Oxoid) was added to make solid medium.

The pH of the medium is 7.0 - 7.4.

APPENDIX 2 Properties of the 18 cyanobacterial strains isolated from the Dee Estuary salt marsh and maintained in culture

(1)

Strains of Section I

Synechococcus nageli

LBCC No. (2)	Cell dimensions W.XK. (m)	Cell shape (4)	Heterotrophic growth Glucose	Heterotrophic growth Sucrose	Microaerobic nitrogenase synthesis	Tolerance sulphide (HIC) (5)
LBCC 54	2.3 - 2.9 d	sph.	-	-	+	193 - 255

Synechocystis sauvageau

LBCC no.	Cell dimension W.XL. (µm)	Cell shape	Heterotrophic growth Glucose	Sucrose	Microaerobic nitrogenase	Tolerance to sulphide (HIC)
LBCC 19	1.6 - 1.8 d	sph.	-	-	-	124 - 131

Strains of Section III

The LPP group B [8]

LBCC No.	Cell dimensions W.XL. (µm)	Cell shape	Presence of sheath	Heterotrophic growth Glucose	Heterotrophic growth Sucrose	Microaerobic synthesis of nitrogenase	Presence of gas vacuoles	Constriction between cells	Tolerance to sulphide (HIC)
LBCC 41	0.9 - 1.2 x 2.6 - 4.3	cyl.	-	-	-	-	-	-	n.d.
LBCC 68	1.1 - 1.4 x 2.3 - 4.5	cyl.	-	-	-	-	-	-	n.d.
LBCC 74	1.1 - 1.4 x 1.0 - 1.4	i	-	-	-	-	-	-	n.d.

Strains of Section IV All strains develop heterocysts in the absence of a combined nitrogen source, and all strains fix nitrogen

Anabaena Bory de St. Vincent

LBCC No.	Vegetative cell dimensions (WXL) (µm)	Cell shape	Heterocyst dimensions (µm)	Heterotrophic growth Glucose	Sucrose	Presence of gas vacuoles	Constriction between cells	Tolerance to sulphide (HIC)
LBCC 14	3.8 - 6.2 x 3.8 - 7.2	sph	5.4 - 7.9 x 5.8 - 9.4	-	-	+p	+	n.d.
LBCC 17	2.2 - 2.6 x 3.0 - 3.5	i	3.2 - 3.8 x 4.2 - 4.8	+	+	-	+	43 - 44
LBCC 27	5.8 - 6.7 x 5.4 - 5.9		6.0 - 7.0 x 7.0 - 7.7	-	-	-	+	n.d.
LBCC 28	5.3 - 8.3 x 3.2 - 3.5	cyl.	4.2 - 5.8 x 8.3 - 9.9	-	-	-	+	25 - 32
Nostoc Vaucher								
LBCC 8	3.6 - 6.2 x 3.6 - 4.7	sph.	4.3 - 6.1 x 3.6 - 7.2	-	-	-	+	n.d.
LBCC 29	3.2 - 4.7 x 2.9 - 3.6	sph.	3.8 - 6.1 x	+	+	+	+	22 - 28
LBCC 34	3.0 - 4.3 x 3.6 - 5.4	i	5.5 - 6.1 x 5.4 - 7.2	-	-	-	+	38 - 41
LBCC 37	3.8 - 4.5 x 4.3 - 4.9	i/cyl.	5.4 - 6.1 x 6.4 - 7.0	-	-	+	+	n.d.
LBCC 45	3.5 - 4.7 x 3.6 - 4.7	i	3.5 - 4.0 x 4.0 - 4.3	-	-	-	+	n,d.
LBCC 48	3.6 - 4.0 x 3.5 - 7.9	ov.	4.3 - 5.8 x 4.5 - 6.4	-	-	-	+	46 - 49
LBCC 52	2.9 - 3.6 x 3.6 - 7.2	cyl.	3.4 - 3.9 x 3.6 - 4.3	+	+	-	+	25 - 27
Nodularia Mertens								
LBCC 40	3.8 - 4.5 x 1.9 - 3.2	disc	6.1 - 7.4 x 3.8 - 4.5	-	-	-	+	n.d.
Cylindospermum Kutzing								
LBCC 62	3.4 - 4.0 x 4.7 - 6.8	cyl.	4.0 - 5.4 x 6.5 - 9.2	-	-	-	+	n.d

NOTES

1. See Rippka et al (1979)
2. See Materials and Methods
3. d = diameter
4. cell shapes : sph. = spherical; rod = rod-shaped; disc = disc-shaped; cyl. = cylindrical; i = isodiametric; ov = oval.
5. Data kindly provided by R. Howsley (pers. comm.). HIC (Half Inhibitory Concentration) values were determined for each isolate from $^{14}CO_2$ fixation values after 60 minute incubations with a range of sulphide concentrations, at pH 8.0. The linked HIC values represent the means of two separate experiments. Fur further details, see Howsley and Pearson (1979).
6. n.d. = not determined
7. p = polar gas vacuoles present
8. See Rippka et al (1979)

Appendix 3

The chemical tests performed in order to identify the actinomycete isolates

	Dee salt marsh actinomycete isolates																
Tests	1A	1B	1C	5A	5B	6A	6B	6C	10A	10B	11A	11B	11C	13	18	19	20
Adonitol	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Rhamnose	-	+	-	+	-	-	-	-	-	+	+	+	+	-	+	-	+
Raffinose	-	-	-	+	-	-	-	+	-	-	+	+	-	-	+	-	+
Inositol	+	+	+	-	-	+	-	-	-	+	-	+	+	-	-	+	+
NA Azide 0.01%	+	+	+	-	+	+	-	-	-	+	+	+	-	-	-	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NaCl 7%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phenol 0.1%	+	+	-	-	-	+	-	+	-	-	+	-	-	-	-	-	+
Growth 45°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rifampicin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Neomycin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arbutin	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Xanthine	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Allantoin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inhibit A. niger	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-
Inhibit S. murinus	+	+	+	+	-	+	+	-	+	+	-	-	+	+	+	+	-
Nitrate reduction	+	-	-	+	+	-	-	+	-	-	+	+	+	-	-	-	+
H_2S production	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+
Pectin hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lecithinase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-hydroxyproline	+	-	-	+	-	+	+	-	-	-	-	-	-	-	+	-	-
L-histidine	+	+	+	-	-	-	-	-	-	+	-	+	+	-	+	+	-
D-Z-Aminobutyric acid	+	-	+	-	-	-	+	-	-	-	-	-	-	+	+	+	-
Fragmenting mycellium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melanin production	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-
Subst. Pigt. R/O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inhibition B. subtilis	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Spore mass, GR.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spore mass, red	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spore surface, RG	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spore surface, Sm	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Spore chain, BIV	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spore chain, Sp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spore chain, RA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spore chain, Rf	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inulin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Subst. pigt. y/Br	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Spore mass, Gn	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

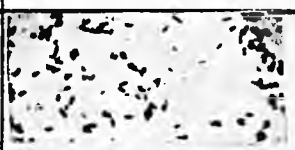
Rf = Rectus flexibilis; RA = retinaculum-apertum; Sp = Spira; BIV = Biverticillus, sm = smooth; RG = ridged;
GR = Grey; GN = Green; y/Br = yellow/Brown; R/O = red/orange

Appendix 4

IDENTIFICATION
OF THE BACTERIAL DEE SALT MARSH ISOLATES.

FIRST STAGE

Morphological descriptions are from growth on Oxoid CM3 Nutrient Agar except as stated

Isolate	1A	2A	9A	15B
°C incubation	30	30	30	30
Cell morphology × 630	 cells are 1 day at 30°C on CM_3 by Gram stain	 cells are 5 hours at 30°C on CM, plus 0.75% agar by phase contrast		 Cells are 1 day at 30°C on CM_3 by Gram stain
Gram	variable	+	-	-
Spores	+	-	-	+
Motility Flagella EM		-	+ 1 polar	
Colonial morphology	off-white smooth flat entire circular translucent 1mm 1 day	orange smooth convex entire circular translucent <½mm 1 day	off-white smooth convex entire circular translucent ½-1mm 1 day	off-white rough flat erose circular translucme 1 - 1½mm 1 day
°C, growth	45° + 50° -	37° + 41° -	5° - 45° + 50° -	55° + 60° -
Catalase	+	+	+	+
Oxidase, Kovacs	-	-	-	weak +
O-F glucose		No growth	oxidative	
First stage identification	Bacillus	Coccus	Gram negative non-fermentative	Bacillus

IDENTIFICATION
OF THE BACTERIAL DEE SALT MARSH ISOLATES

FIRST STAGE

Morphological diescriptions are from growth on Oxoid CM3 Nutrient Agar except as stated

Isolate	15C	16A	16B	16G
°C incubation	30	30	30	30
Cell morphology x 630	 cells are 1 day at 30°C on CM_3 by Gram stain		 cells are 3 days at 30°C on CM_3 by Gram stain	 (a)  (b)
Gram	variable	-	-	+
Spores	+	+	+	-
Motility Flagella EM		+	+	-
Colonial moprhology	off-white smooth flat entire circular translucent ½ & ½mm 1 day	off-white smooth low convex entire - undulate irregular almost transpatent 2-5mm 1 day	off-white smooth kow convex entire - undulate irregular almost transparent ½ - 1mm 1 day	creamy smooth low convex entire circular transparent 1mm 1 day
°C, growth	45° + 50° -	37° + 41° weak +	55° + 60° -	37° + 41° -
Catalase	+	+	+	+
Oxidase, Kovacs	-	weak +	-	-
O-F glucose				-
First stage identification	Bacillus	Bacillus	Bacillus	Coryneform

Gram-negative fermentatives - second stage and Coccus

Tests \ Isolates	2A		2A		2A
°C incubation	30				
KCN		Gas glucose	-	Acid + Gas	
Urease		ONPG	+	Adonitol	-
PPA		Arg Møller	-	Aesculin	-
H_2S TSI		Lys Møller		Arabinose	-
Gluconate		Orn Moller		Dulcitol	-
Malonate		NO_3^- to NO_2^-	-	Glucose	-
Citrate Koser		NO_3^- to N_2	-	Glycerol	-
Sensitivity		DNAase	+	Inositol	-
Penicillin G	+	Gel stab 20°	+	Lactose	-
Streptomycin	+	Gel plate	+	Maltose	-
Chloramphen.	+	Casein	No growth	Mannitol	-
Tetracycline	+	Starch	-	Raffinose	-
Novobiocin	+	Lecith egg	No growth	Rhamnose	-
Polymyxin B	+	Lipase egg	No growth	Salicin	-
O/129	+	NH_3	-	Sorbitol	-
Coagulase	-	Indole	-	Sucrose	-
Glucose CSU	-	MR	-	Trehalose	-
Glycerol-erythromycin acid	No growth	VP	-	Xylose	-
		Haemolysis-B	-	Dextrin	-
Mannitol:-		Horse blood	-	Fructose	-
Acid aerobically	-	Tween 80	-	Galactose	-
Acid anaerobically				Inulin	-
Anaerobic growth:				Sorbose	-
Evans & Kloos	No growth			Cellobiose	-
Glucose broth	-			Starch	-

References : 1. Bergey's Manual of Determinative Bacteriology, 8th edn.(1974). (R.E.Buchanan & N.E.Gibbons, eds.) Baltimore: Williams & Wilkins

2. Cowan, S.T. & Steel, K.J. (1974). Manual for the Identification of Medical Bacteria. Cambridge University Press.

Bacillus - second stage and conclusion

Tests \ Isolates	1A	15B	15C	16B	16A
Tests at °C	30	30	30	30	30
Spore shape [a]	E	E	E	E	E
Sporangium distinded distinctly	-	-	-	-	-
Spore position dominant [b]	T	T	T	T	T
Rod width, µm					/µm
Intracellular globules [c]					+
Anaerobic growth [c]	-	-	-	+	+
Growth in 5% NaCl	+	+	+	+	+
" " 7% NaCl	+	+	+	+	+
" "10% NaCl					
" " pH 5.7 broth	+	+	+	+	+
Acid from glucose [d]	+	+	+	+	+
Gas from glucose [d]	-	-	-	-	-
VP (acetoin)	+	+	+	+	+
Egg yolk agar opacity	-	-	-	-	
Casein decomposition	+	+	+	+	+
Gelatin decomposition	+	+	+	+	+
Tyrosine decomposition					
Starch hydrolysis	-	+	-	+	+
NO_3^- to NO_2^-	-	+	-	+	+
Indole	-	-	-	-	-
Citrate, Koser's	-	-	-	-	-
Arginine dihydrolase, Møller's	-	-	+	+	-
pH in VP broth	5.2	5.7	5.2	8.0	5.2

Reference : Bergey's Manual of Determinative Bacteriology, 8th edn. (1974) (R.E.Buchanan & N.E.Gibbons, eds.) Baltimore: Williams & Wilkins.

[a] E elliptical or cylindrical; S, spherical or nearly so;

[b] C central; T, terminal or subterminal; CT, central to terminal

[c] On glucose agar; [d] Peptone water sugar, Andrade's indicator

Second stage

Gram-negative non-fermentatives & Coryneforms

Tests \ Isolates	9A	16G		9A	16G	
°C incubation	30	30				
			Acid glucose	-	-	
Phycocyanin	-		Gas glucose	-	-	
Fluorescence	-		ONPG	weak +		
L-Arg CSU	+		Arg Møller	+		
Betaine CSU	+		Lys Møller	-		
Glucose CSU	+	-	Orn Møller	-		
Lactate CSU	+	-	NO_3^- to NO_2^-	+	-	
Acetate CSU	+		NO_3^- to N_2	-	-	
Sensitivity			DNA ase	-		
Penicillin G	-		Gel stab 20°	-		
Streptomycin	+		Gel plate	-	+	
Chloramphen.	+		Casein	-	+	
Tetracycline	+		Starch	-	+	
Novobiocin	-		Lecith egg	-	NG	NG = No growth
Polymyxin B	+		Lipase egg	-	NG	
O/129	-		NH_3	+	-	
Levan		-	Indole	-	-	
Growth factor requirement	-		H_2S (TSI)	-		
			Tween 80			
			Urease		-	

References : 1. Bergey's Manual of Determinative Bacteriology, 8th edn. (1974) (R.E.Buchanan & N.E.Gibbons, eds). Baltimore: Williams & Wilkins.

2. Cowan, S.T. & Steel, K.J. (1974). Manual for the Identification of Medical Bacteria. Cambridge University Press.