

**Factors affecting the introduction and distribution of fungi in the
Vestfold Hills, Antarctica**

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**“Antarctica is the last great wilderness, long
may it remain so.”**



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ABSTRACT

The impact of human disturbance on fungal flora around Davis Station and the Vestfold Hills in Antarctica (68° 35'S 77° 58'E) was examined by monitoring air spora and soil fungi over a 12 month period in 2001.

A number of fixed sample points were established around Davis Station from which soil was collected on a monthly basis as conditions allowed. Additionally, two transects were set up. The first transect ran 3km through the station from north to south extending from 50m beyond the limit of the station buildings, to the south of Heidemann Bay. The second transect extended 18km west to east along the length of the Ellis Fjord and Lake Druzhby as far as Trajer Ridge, 5km from the polar plateau. Each transect comprised 5 sites which were sampled on a fortnightly basis throughout 2001 using rotorod airborne particle samplers and soil dilution plates. Numbers of airborne particles were determined by counting under a light microscope. Fungi were isolated from soil using full strength potato dextrose and Czapek Dox agars as well as hair bait. Fungal isolates were identified on the basis of their general morphology and also by genetic analysis of ribosomal Internal Transcribed Spacer regions.

Fungal populations were sparse at all locations with the exception of the living quarters where numbers were consistently high. Furthermore, although seasonal variations were observed at many of the sites, no such variation was recorded at the station which would indicate a continuing source of inocula or less harsh conditions.

Increased fungal numbers near to living quarters was also observed at other stations (Law Base, Australian, Progress II, Russian and Zhong Shan, Chinese) but not beyond their confines. Also, there was little species overlap between fungi found close to station living quarters and those found farther afield, which would indicate that whilst there was a clear human impact, the fungi associated with these areas do not appear to be becoming established elsewhere.

Study of the optimum growth temperatures of fungi isolated from both soil and air showed that all isolates were psychrotrophs (i.e. were able to tolerate temperatures below 5°C) and 39% of them were psychrophiles (i.e. their optimum growth temperature was 15°C or below and they were able to grow at 0°C). All genera were capable of surviving periods of at least 28 days of desiccation.

CHAPTER 1: GENERAL INTRODUCTION

1.1. BACKGROUND

Fungi are present on every continent on Earth and many mechanisms exist for the dispersal of propagules involving both biotic and abiotic transport. Antarctica is isolated from other continents by the Southern Ocean and has a climate which prevents it from colonisation by terrestrial vertebrates. The ecosystem is therefore dominated by microbial life which is supported by a limited flora and fauna.

Since the early part of the twentieth century however, mankind has had an increasing impact upon Antarctica and several permanent settlements have become established on the continent. It was anticipated that human activities would inevitably result in the introduction of a range of exotic fungal species but that the majority of these would be unable to survive in the Antarctic habitat. This would result in a difference between those species which were present within areas of human habitation and those found beyond the confines of the station.

1.2. THE ANTARCTIC HABITAT

Antarctica is the coldest and driest continent on Earth. It is covered by an ice cap which is up to 1km thick with only 2% of the continent ice free and it is in these

areas that most of the terrestrial and lake life is concentrated. Effectively, Antarctica is a desert continent. It is completely surrounded by the Southern Ocean, which presents a physical barrier to many of the mechanisms of microbial introduction such as animal vectors. The number of migrating animal species is low and the number of potential habitats for microbial colonisation is limited. In order to be able to survive to reproduce, fungal propagules arriving in Antarctica, either in the air or carried by man or animals, must be capable of rapid adaptation or be able to tolerate cold and desiccation (Gray 1982; Zucconi *et al.*, 1996; Kochkina *et al.*, 2000).

Mean air temperatures are generally low, rarely rising above 10°C during the summer and falling to -30°C in maritime areas and -60°C in central areas during winter. Biologically available water is therefore either limited to the summer melt phase or is in lakes. Much of the ground is bare rock or covered by snow and soils, where present, vary between sites. Those of the Antarctic Peninsula may contain high proportions of organic matter due to the presence of vascular plants and therefore also support diverse microbial and invertebrate communities. Conversely, those found in the area of the Vestfold Hills, Princess Elizabeth Land, are generally poor, predominantly comprising weathered rock with poor water-holding capacity (Campbell, 2003) and generally low in N & P (Leishman & Wild, 2001). Carbon sources for microbial growth, whilst often plentiful around areas inhabited by animals, man or plants are severely limited in the poor soils. Nutrients may however, be transported by the wind (Simonson, 1995)

from areas such as penguin rookeries and presumably also from human habitations in the same manner.

1.3. HUMAN HABITATION

Bases and camps have been established on mainland Antarctica since the beginning of the 20th century. Although awareness of the ecological consequences of such habitation was not considered, the limited size of the stations meant that the associated impact was also low. Following on from the Second World War however, the number of permanent stations in Antarctica increased with scant regard for the impact upon the environment. Domestic refuse was typically dumped at sea or landfill and sewage discharged without treatment. The Antarctic treaty, established in June 1961 amidst fears of disputes over sovereignty leading to militarisation, designated Antarctica to be used for peaceful scientific purposes only. This has not only limited the number of potential outposts but, by increasing the level of scientific research on the continent, also increased awareness into the effects of habitation.

It is often not possible to study a natural system without imposing some impact upon it. There is a need therefore to identify and assess impacts and introduce measures to minimise them. Domestic practices vary between the stations of different nations, with some being better than others at adhering strictly to the protocols of the Antarctic Treaty. The following issues, common to all Antarctic habitation, however are perhaps of greatest importance.

- Treatment of domestic refuse
- Treatment of sewage
- Working practices

At Davis Station, situated in the Vestfold Hills, eastern Antarctica (Figure 1.1), domestic refuse is sorted as far as possible into various categories: glass of varying colours, steel and aluminium cans and recyclable plastics of varying type and returned to Australia for disposal. Food scraps together with other materials are incinerated and the ash retained also for removal to Australia. Larger items of refuse such as scrap metals and building materials are similarly stored for removal.

All domestic effluent passes through a maceration and biological treatment process before discharge to the sea (Smith, 2000). Sludge residue is extracted annually and spread onto an area of sea ice where solar radiation causes some degree of ultraviolet sterilisation before it enters the seawater. Although it has been shown that micro-organisms originating from human effluent can be distributed by the wind, the effects of desiccation, low temperature and high incidence of UV radiation have been shown to reduce coliform viability by more than 99% (Hughes, 2003).

Working practices dictate that recalcitrant materials such as plastics, especially expanded polystyrene are not allowed to escape into the environment (e.g. wind-

blown from unprotected skips) and similarly that care is taken with fuel transfers although spills are inevitable (Aislabie *et al.*, 2001). Poultry products are not taken into the field and all domestic and human waste is bagged and returned from field huts (Committee for Environmental Protection, 2001). Australia has been particularly conscientious at adhering to strict protocols for waste management and probably ranks among the leaders of these practices in Antarctica.

1.4. GEOGRAPHIC AREA OF STUDY

The current study was conducted at Davis Station, located in the Vestfold Hills, Princess Elizabeth Land in the Australian sector of Antarctica (Figure 1.1). Davis Station (68° 35'S 77° 58'E) was established in 1957 and is normally occupied by 60-80 personnel during the summer months and 20-25 during the winter. The Vestfold Hills is one of the larger coastal ice-free areas to be found in Antarctica, occupying an area of around 400km². The area carries numerous lakes of various sizes and depths, which range from freshwater to hypersaline (Ferris & Burton, 1988, cited by Perriss & Laybourn-Parry, 1997). The Ellis Fjord constitutes the larger part of a virtually uninterrupted natural channel, which runs from the sea and continues to Lake Druzhby and finally to the Antarctic plateau via a feature known as Trajer Ridge (Figure 1.2).

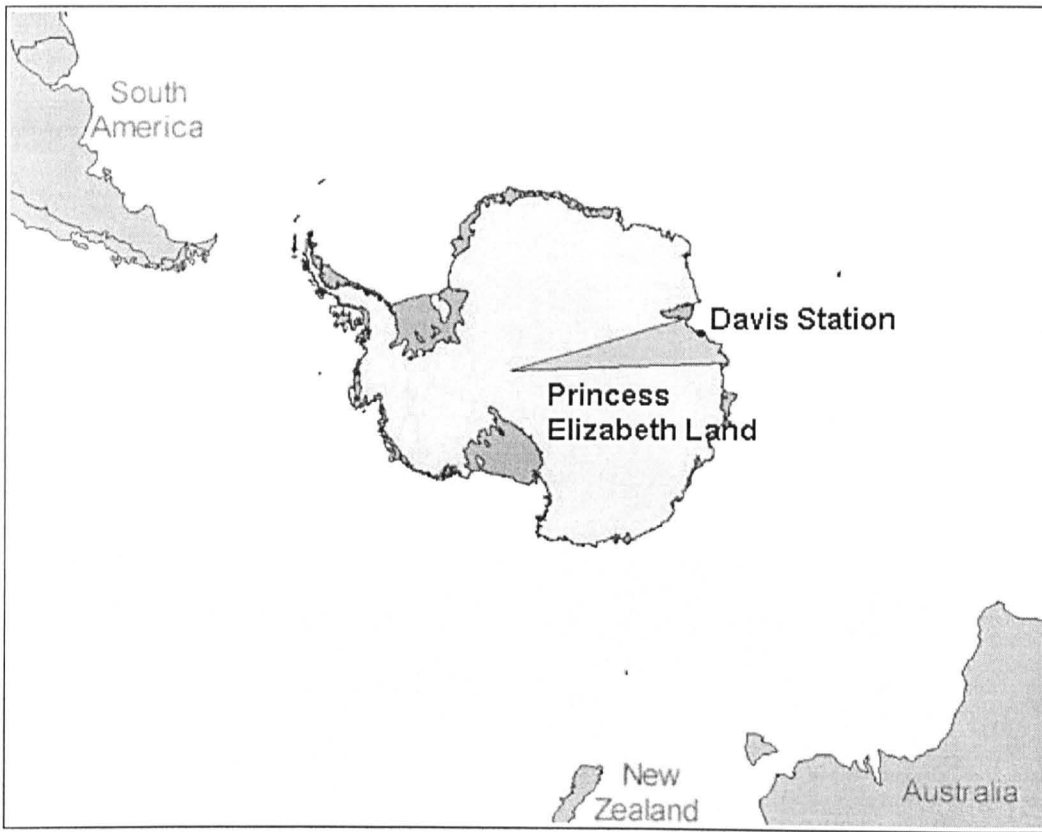


Figure 1.1. Location of Davis Station in Princess Elizabeth Land, eastern Antarctica. Map courtesy of the Australian Antarctic Division.

The underlying geology is predominantly pre-Cambrian gneiss intersected by swarms of dolerite dykes (Pickard, 1982). The area is also scattered with numerous “erratics” which have been transported by moving ice sheets (Hirvas *et al.*, 1993). Large quantities of sand and dust are present from weathered rocks, which frequently accumulate in drifts (Fitzsimons, 1995).

Temperatures range from around 5°C during the summer to -30°C during the winter, although microhabitats exist where temperatures may be increased due to the action of solar radiation on exposed rock or the presence of biotic factors such as moss beds (Walton, 1982). Winds are typically katabatic, blowing from the Antarctic Plateau towards the coast. At Davis Station, these are typically from the north east, although the Ellis Fjord channels wind along its length in a westerly direction (Targett PS, *pers com.*).

1.5. BIODIVERSITY AND BIODISTRIBUTION IN THE VESTFOLD HILLS

1.5.1. Vegetation in the Vestfold Hills

Despite the harsh environmental conditions of the Vestfold Hills, several species of moss, lichen and algae are present in sparse populations. Lichens are the most common form of vegetation with 22 species having been reported (Leishmann & Wilde, 2001); *Buellia frigida* is the most widespread within the study area, and *Xanthoria elegans* also frequently occurs. Although four moss genera have been reported in the Vestfold Hills (Pickard 1982), only one species, *Bryum algens*, was encountered within the study area, occurring at two study locations, Flat Top Peninsula and Watts Lake Hut (Appendix 2). A semi-terrestrial cyanobacterium occurs over the summer period in coastal areas (subject to melt water) associated with penguin rookeries where guano covers the rocks. At least one form of endolithic alga was observed within the study area (Plate 1.1).



Plate 1.1. Endolithic alga observed below the surface of a freshly frost-shattered shard of rock found to the north of the Ellis Fjord, Vestfold Hills, Antarctica

Mosses and lichens are generally absent from the western (coastal) areas of the Vestfold Hills (Seppelt, 1984), possibly reflecting high salt concentrations due to spray drift (McCune 1991) and also low concentrations of N and P. Vegetation is limited by available water and the concentration of soil nutrients, in particular N and P. Leishman & Wild (2001) observed that moss distribution was closely associated with available water. These authors also observed a positive relationship between lichen diversity and abundance and soil nutrient concentration associated with nesting birds. Whilst total nitrogen was found to reduce rapidly with increasing distance from birds' nests, total phosphorus declined more slowly. Nevertheless, lichens are found covering rocks well away from nesting areas. Two mechanisms of nutrient transport are probable;

permeation through melting snow and wind-blown material from nutrient rich areas.

1.5.2. Animal life in the Vestfold Hills

There are no terrestrial vertebrates which are present in the Vestfold Hills throughout the year. Animals are limited to migrating marine species which are present during the summer period only. These comprise a number of seals and birds. The Weddel seal (*Leptonychotes weddelli*) is the most widespread of the seals, many hundreds of individuals being present throughout the year. Southern elephant seals (*Mirounga leonina*) come ashore during the summer period to moult and the less common leopard seal (*Hydrurga leptonyx*) is also present during the summer where it preys upon Adélie penguins. Several species of bird are found in the Vestfold Hills although none during the winter period. Several rookeries of Adélie penguins (*Pygoscelis adeliae*) comprising many thousands of birds are found along the coast and often associated with these are Antarctic skuas (*Catharacta maccormicki*). Snow petrels (*Pagodroma nivea*) and Wilson's storm petrels (*Oceanicus oceanites*) nest among the rocks at a number of inland sites. Giant Petrels (*Macronectes giganteus*) are infrequent but occasionally found where carrion is present. No terrestrial invertebrates were observed although nematodes have been reported in the Vestfold Hills area (Rounsevell, 1981).

1.5.3. Antarctic Fungi

Despite the harsh conditions found in Antarctica, a number of habitats exist which fungi have been able to exploit. The availability of free water is the most limiting factor, with water being immobilised as ice for much of the year and fungi must therefore be able to withstand low water potentials (McRae & Seppelt, 1999) or be capable of surviving in a quiescent state (Addy *et al.*, 1997; Kochkina *et al.*, 2001).

Complex soil ecosystems are uncommon in Antarctica due to the few areas of ground which are not permanently covered by ice and also the low concentration of organic matter. Only two species of higher plants occur in Antarctica (*Deschampsia antarctica* and *Colobanthus quitensis*) and these are both restricted to the Peninsula. Moss turves are, however, found in a number of ice-free locations and in the case of mosses and higher plants, a microhabitat exists which supports fungi (as saprophytes) and also invertebrate populations which include rotifers and nematodes. In situations of low available organic nutrients, active predation of nematodes, protozoa and rotifers may occur (Gray 1985).

The remaining ice-free areas are limited to bare rock, loose frost-shattered gravels and sands, and lakes which become ice-free for much of the summer. Whilst a number of yeasts (mainly basidiomycetes) and zygomycetes have been isolated from freshwater habitats (Ellis-Evans, 1985) these are limited to less oligotrophic environments. Carbon sources elsewhere are limited to mammal

and bird dung and keratin which is produced during summer moults of seals and penguins. Del Frate & Caretta (1990) isolated four fungi (*Chrysosporium* sp., *Cryptococcus* sp. and *Thelobolus* sp.) including a white yeast from petrel and penguin dung and four species isolated from penguin and skua feathers.

1.5.4. Mechanisms of fungal propagule dispersal

Filamentous fungi isolated from Antarctic sources belong to the ascomycota, zygomycota or deuteromycota. The basidiomycota are, so far, represented only as yeasts. Filamentous fungi generally release spores directly into the air for example by means of explosive discharge (e.g. from asci) or by dehiscence of sporangia or by release of spores from conidiophores which are raised into the air current. Fungal propagules may also become dispersed either as desiccated hyphal fragments or attached to substrates such as moss fragments or soil particles when these are transported by wind or water (Marshall, 1998).

In a similar manner, lichens disperse by means of soredia, which are released into the air from disk-like structures on the thallus. As with free-living fungi, they are also distributed by fragmentation and dispersal by wind, water and fragmented substrata.

Fungi are also distributed by the activities of animals either through faeces (Davies *et al.*, 1993) or by attachment to feet, fur or birds' feathers (Sarangi & Ghosh, 1991). Scavengers in particular are important vectors of fungi since their

feeding habits involve close contact with substrates likely to contain large numbers of fungal propagules (Gary Miller, *pers. com.*). In the Vestfold Hills, the principal scavengers are giant petrels and skuas, the latter being examined as part of the current study. Man is also an important vector of fungal propagules due to the variety of materials that he transports to Antarctica. Although precautions are taken to avoid the introduction of foreign species into the continent such as the cleaning of equipment and footwear, it is impossible to prevent accidental transport of micro-organisms.

1.5.5. Effect of Antarctic climate on fungi

The Antarctic climate presents a number of physiological challenges which require considerable adaptation by the micro-organisms which colonise Antarctic habitats. Extreme low temperature is perhaps the most obvious characteristic of the continent. Enzymic reactions become slower at low temperature and the possibility of enzyme denaturation increases. It has been demonstrated that extremes of temperature and low water potential can impact negatively upon mycelium development (Donnelly & Boddy, 1997). Fresh water freezes at 0°C, bringing about a state of physiological drought as water becomes biologically unavailable. Ice forming within living cells has the potential to cause physical damage as well as increasing solute concentrations, possibly resulting in precipitation if solubility products are exceeded.

Many fungi survive freezing conditions by means of dormant stages such as spores or sclerotia, however, many are capable of surviving low temperature in a vegetative state. Although the physiological mechanisms for resistance to freezing are poorly understood (Tibbett *et al.*, 2002), fungi which are able to grow at low temperature, reduce their potential for freezing by increasing production of unsaturated lipids, extracellular glycerol (Weinstein *et al.*, 2000) and intracellular trehalose and mannitol (Tibbett *et al.*, 2002). The presence of trehalose and mannitol is attributed to the possible protection of membranes rather than cytoplasm at low temperature (Tibbett *et al.*, 2002).

Changes in solute concentrations which can be brought about by water freezing have the potential to alter osmotic gradients which may in turn, result in pH changes as buffer concentrations become altered (Smith, 1993). Production of polyhydric alcohols (polyols), in particular glycerol, has been demonstrated as a physiological response by fungi (Luard, 1982) to increased salt concentration since they are osmotically neutral and do not interact with enzyme pathways (Brown, 1978; Adler *et al.*, 1982). Increased ion uptake from saline media, in particular Na⁺ has also been suggested (Luard, 1982).

There exists within the Vestfold Hills, a phenomenon known as the Salt Line (Gore *et al.*, 1996). This is the product of a combination of marine aerosols, saturation of sediments during marine transgression and deposition of salt-saturated sediments by glacial activity. The soil organisms which exist to the

west of this NE-SW line must therefore be capable of withstanding a high osmotic gradient even when water becomes biologically available.

1.6. PREVIOUS WORK

Investigations of microbial life on the Antarctic continent have been conducted since the beginning of the twentieth century. Ekelöf (1908, cited by Corte & Daglio, 1964) described a number of filamentous fungi which he isolated from soil. Fungal isolations were also recorded by Tsiklinsky (1908), Pirie (1912) and McLean (1919, all cited by Corte & Daglio, 1964) although progress was often slow due to samples often being stored for many months before examination, thus affecting the fungal population (Corte & Daglio, 1964).

In recent years, more detailed studies have been carried out at specific locations (e.g. Meyer *et al.*, 1967; Cameron *et al.*, 1972; Pugh & Allsopp, 1982) which have provided valuable species lists. With *in situ* studies becoming more practical with increasing technology, specific habitats such as soil (Tubaki & Asano, 1965; Del Frate & Caretta, 1990), water, ice (Ellis-Evans, 1985; Ma *et al.*, 2000) and mosses (McRae & Seppelt, 1999; Tosi *et al.*, 2002) have also been studied in greater depth.

Although certain aspects of the impact of human activity such as effects on seabird populations (Micol & Jouventin, 2001) and damage caused by geological surveys (Kiernan & McConnell, 2001) have been investigated, studies into the

impact upon mycoflora are few (Kerry, 1990b; Azmi & Seppelt, 1998). Similarly, although the microbiology of Antarctic air has been studied on several occasions (Corte & Daglio, 1964; Sun *et al.*, 1978; Wynn-Williams, 1991), little work exists concerning the potential for particles to be transported long distances to Antarctica *via* the wind (Marshall, 1997).

1.7. AIMS AND OBJECTIVES OF THIS STUDY

Fungi in Antarctic environments have been examined from a number of perspectives, but studies concerning methods of introduction and human impact upon fungal populations are limited. Whilst it is accepted that this investigation was a pilot study, it was anticipated that it would identify areas which could be studied further in subsequent investigations. The investigation was divided into three broad areas of study:

- Fungal diversity and distribution
- Transport mechanisms
- Physiological adaptations

1.7.1. Fungal diversity and distribution

The first objective of this study was to examine the fungal diversity found at Davis Station and the Vestfold Hills and, by comparing fungal species, to assess the impact of the Station on more distant habitats. Two methods of examination

were used, direct sampling from the air using rotorod particle samplers (Perkins, 1957 cited by Davies, 1971) and soil dilution plates (Postgate, 1969; Shannon *et al.*, 2002). After an investigation into the use of media and the effects of antibiotics, potato dextrose agar and Czapek-Dox agar were used with a combination of streptomycin and penicillin. Although this technique is limited to isolating viable, culturable forms which are suited to those media, it is nevertheless widely used (e.g. Shannon *et al.*, 2002).

In addition to “classical” taxonomic methods based on morphological characteristics, further identification of the isolates obtained from culture plates and rotorod strips was made by genetic analysis of ribosomal DNA, Internal Transcribed Spacer (ITS) region (O’Donnell 1992; Dyer & Murtagh, 2001).

1.7.2. Transport mechanisms

A further objective was to assess the factors which affected the transport of fungal propagules such as wind, birds and human activity.

1.7.2.1. Aerial transport

Rotorods were used as a simple, cost-effective means to sample air currents from many areas over the period of a year.

1.7.2.2. Animal transport

Swabs were taken from the feet of skuas since they are opportunistic feeders which are known to cover great distances and which frequent the area in and around Davis Station.

1.7.2.3. Human introduction

Both air and soil samples were collected during the period of annual station resupply which is not only the time of greatest human activity, but the time when many materials are brought ashore having been transported from Australia and elsewhere.

1.7.3. Physiological adaptations

The effects of temperature and desiccation on fungal isolates were studied in order to determine whether isolates were capable of tolerating Antarctic conditions and were, therefore, likely to be endemic to Antarctica or else recent introductions. This required the examination of the growth rates of each isolate over a range of temperatures so that an optimum growth temperature could be determined. Additionally, isolates were desiccated to simulate the effects of physiological drought to determine which were adapted to survive under these conditions. Data are presented in Chapter 6.

This is the first comprehensive study of direct human impacts on a fungal population in Antarctica.

CHAPTER 2: THE EFFECTS OF ANTIBIOTICS ON ISOLATION OF SOIL MICRO-ORGANISMS

2.1. INTRODUCTION

Soil dilution plates are widely used in soil microbiology (e.g. Barron, 1971; Martin, 1950) as a means of enumerating microorganisms. Bacteria however, outnumber fungal propagules and reproduce at a greater rate, resulting in competition with fungi for resources and rapid over colonisation of plates. An effective means of inhibiting bacterial growth is therefore required in order to enumerate soil fungi using this method. Several solutions to this have been reported and widely used, the most popular method being acidification of the culture medium with sulphuric, citric or lactic acid (Martin, 1950) or the use of antibiotics including dyes such as Rose Bengal (Smith & Dawson, 1944). Resistances and tolerances of soil organisms to such agents, however, vary greatly and so it is not possible to produce a single solution to the problem.

Three substances, Rose Bengal, streptomycin sulphate and penicillin, were examined on the basis of their broad spectrum of effectiveness and general availability.

2.1.1. Rose Bengal

Rose Bengal (Figure 2.1) has been used as an alternative to acidification as a means to reduce bacterial growth since low pH can be inhibitory to fungi (Martin, 1950). It has also been shown to reduce the rate of fungal growth preventing faster growing varieties from over-colonising the plate and masking the growth of slower forms (Martin, 1950) although this itself may complicate fungal studies. Rose Bengal is however, an extremely efficient producer of singlet oxygen ($^1\text{O}_2$) and therefore exhibits a non-specific toxic effect when incubated in the light (Chilvers *et al.*, 1999). Since it can affect fungal growth (Smith & Dawson, 1944), Rose Bengal was examined further to establish its suitability as an antibiotic.

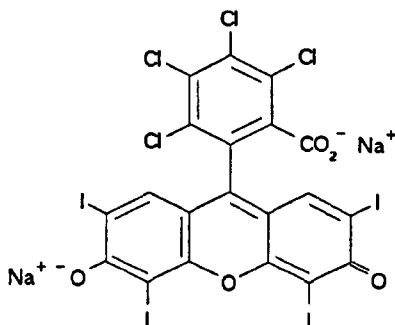


Figure 2.1. General structure of Rose Bengal (di-sodium 4,5,6,7-tetrachloro-2,3,5,7-tetraiodofluorescein) after Banks *et al.*, 1984.

2.1.2. Streptomycin

Streptomycin is commonly used in soil fungal investigations (*e.g.* Martin, 1950). It is an aminoglycoside antibiotic (Figure 2.2) first obtained from the fungus *Streptomyces griseus* and is noted for being active against both Gram-negative and Gram-positive bacteria (Schatz *et al.*, 1944). Streptomycin binds irreversibly to the 30S ribosomal subunit thus preventing correct positioning of tRNA and resulting in altered proteins (Lancini *et al.*, 1995).

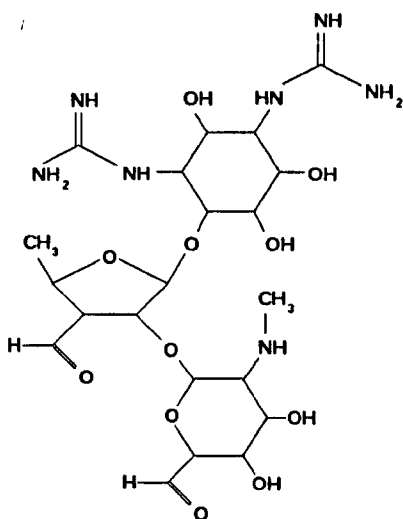


Figure 2.2. General structure of streptomycin (after Lancini *et al.*, 1995)

2.1.3. Penicillin

Penicillin (Figure 2.3) is a beta-lactam antibiotic which operates by inhibiting bacterial cell wall synthesis (Strominger & Tipper, 1965). It is highly selective against Gram-positive bacteria, but does not appear to be effective against resting cells (Schatz *et al.*, 1944).

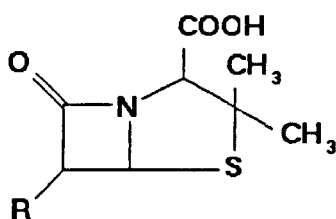


Figure 2.3. General structure of penicillin (after Grahame-Smith & Aronson, 2002)

The aim of the investigation was to determine the relative effectiveness of these antibiotic agents in order to provide a reliable and consistent means of isolating fungi from Antarctic samples. The bacteriostatic efficiency of Rose Bengal and also a range of combinations of streptomycin and penicillin was examined by conducting two separate trials. The first study consisted of a factorial experiment incorporating Rose Bengal and light interactions as factors. The second study investigated the impact of different ratios of streptomycin and penicillin when in combination.

2.2. MATERIALS & METHODS

2.2.1. Rose Bengal

Three identical sets of Petri dishes were prepared corresponding to three light treatments:

1. Incubation in the dark
2. Incubation in the light
3. Incubation in the light on media pre exposed to light

Potato dextrose agar (PDA, Oxoid) was made up to full strength according to manufacturer's instructions, under darkened conditions. Rose Bengal (Sigma) was added to the warm agar by filter sterilisation (0.2µm) to give final concentrations of 10^{-3} , 10^{-4} and 10^{-5} mol L⁻¹. Prior to inoculation, sets 1 and 2 were stored under darkened conditions whilst set 3 was placed on a bench top for 48 hours and therefore exposed to natural and artificial illumination. It was the purpose of pre-exposing the media to light to determine whether an initial reaction took place, after which the toxicity of the dye was lessened by a reduction in the amount of ¹O₂ produced

Three replicate soil samples were taken from the top 10cm of soil in grassland and woodland habitats at Sutton Bonington, Leicestershire and standard ten-fold serial dilutions prepared using sterile water. A 0.1mL inoculum of each dilution was placed directly onto the agar under darkened conditions and spread over the surface using a sterile glass rod.

Incubation was at ambient room temperature (around 20C°) for 5 days. Set 1 was maintained in the dark whilst sets 2 and 3 were placed on a bench top and therefore exposed to natural and normal laboratory (fluorescent) lighting.

At the end of the incubation period, the numbers of colonies of bacteria and fungi were recorded. Data were log₁₀ transformed to normalise the data and satisfy the requirements of ANOVA. A three-way analysis of variance (Rose Bengal concentration x light treatment x soil type) was performed using Genstat™ (v5 Release 4.1 1998)¹ to determine significance of results.

Fungal diversity was determined using Simpson's index (Krebs, 1985, Appendix 1) based on colony morphology. Ten random bacterial colonies were selected (using a numbered grid and random number generator) from each treatment combination (light, Rose Bengal and soil dilution) and sub-cultured onto nutrient agar and incubated at ambient room temperature (around 22°C) for 48 hours before Gram staining.

2.2.1.1. Gram Stain Procedure

Fresh bacterial cultures were prepared by subculturing onto nutrient agar (Oxoid) prepared according to manufacturer's instructions. Smears were made onto microscope slides using sterile water heat fixed by passing through the blue flame of a Bunsen burner.

¹ Lawes Agricultural Trust, IACR Rothamstead

Fixed samples were then stained using crystal violet for 1 minute prepared according to Hucker's method (Howard *et al.*, 1994), rinsed in deionised water and placed into Lugol's iodine for 30 seconds. Samples were rinsed and cleared in 95% ethanol for 1 minute, rinsed in deionised water and counterstained with carbolfuchsin for 30 seconds. Samples were rinsed in deionised water, blotted dry and examined under oil immersion at x1000 magnification.

2.2.2. Streptomycin and Penicillin

A preliminary sighting experiment within this study suggested that a final concentration of 65 mg L⁻¹ antibiotic is sufficient to inhibit bacterial colonisation in soil fungal studies. Based on this, a range of 14 mixtures of penicillin and streptomycin was prepared with ratios of 65:0 mg L⁻¹ penicillin (Sigma) to streptomycin (Sigma), decreasing penicillin and increasing streptomycin by 5mg L⁻¹ each time until the ratio became 0:65 mg L⁻¹ (Table 2.1).

Table 2.1. Concentrations of streptomycin and penicillin varied inversely to one another throughout the treatments. Each treatment however, contained a final concentration of 65mg L⁻¹ of antibiotic.

Treatment	Streptomycin (mg L ⁻¹)	Penicillin (mg L ⁻¹)	Units of Streptomycin (L ⁻¹)	Units of Penicillin (L ⁻¹)
1	0	65	0	103740
2	5	60	3825	95760
3	10	55	7650	87780
4	15	50	11475	79800
5	20	45	15300	71820
6	25	40	19125	63840
7	30	35	22950	55860
8	35	30	26775	47880
9	40	25	30600	39900
10	45	20	34425	31920
11	50	15	38250	23940
12	55	10	42075	15960
13	60	5	45900	7980
14	65	0	49725	0

Two agar types were used throughout this experiment to increase the range of bacteria and fungi isolated. Full-strength PDA and Czapek-Dox agar (CZA) were made up according to manufacturer’s instructions and stock solutions of each antibiotic were added to the warm agar (ca. 50°C) by filter-sterilisation (0.2µm). Fresh soil was collected from the top 10cm of soil from a grassland

habitat at Sutton Bonington, Leicestershire and, based on the results from the previous experiment, dilutions of 10^{-3} g L⁻¹ and 10^{-4} g L⁻¹ prepared using sterile water. An 0.05 ml inoculum was pipetted directly onto the agar surface and spread using a sterile glass rod. Incubation was at ambient room temperature for 6 days, after which time the numbers of bacterial and fungal colonies were counted. Fungal diversity, based on visual colony characteristics, was estimated using Simpson's index (Appendix 1). Data were log transformed and a two-way analysis of variance was performed using Genstat™ (v5 Release 4.1 1998).

2.3. RESULTS

2.3.1. Rose Bengal

No significant differences in numbers of bacterial or fungal colony forming units (CFUs) were observed between the woodland and grassland soils or between the light and dark incubations in the absence of Rose Bengal. Significant reduction ($P<0.001$) in numbers of both fungi and bacteria were, however, observed with increasing concentration of Rose Bengal irrespective of soil type and light treatment (Figure 2.4).

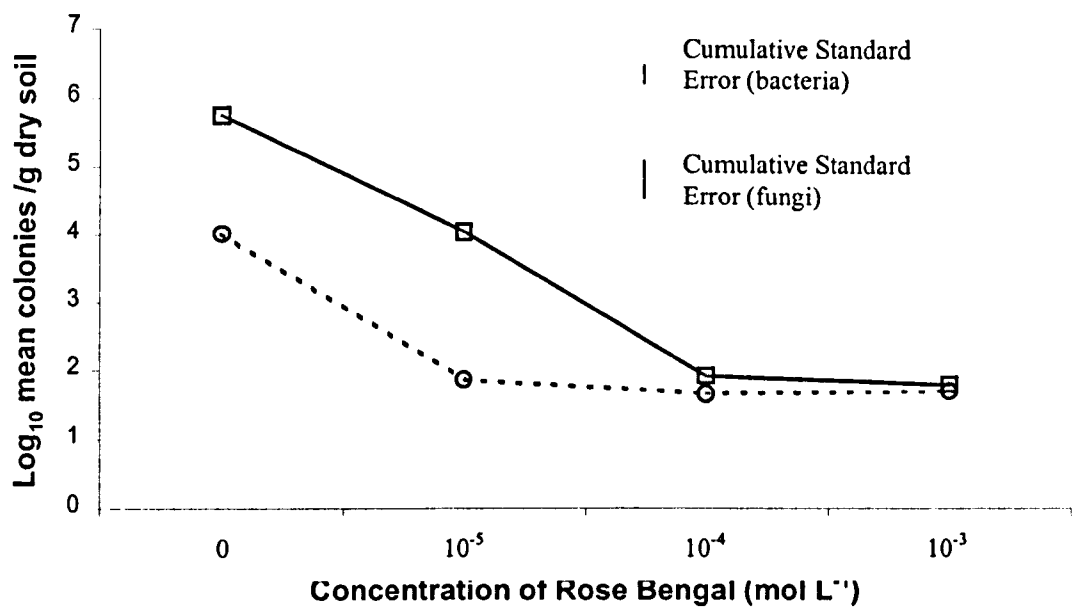


Figure 2.4. Colony numbers (\square bacteria, \circ fungi) from both soil types were reduced by the presence of Rose Bengal in both light and dark incubations. Rose Bengal was significant as a single factor, $P < 0.001$.

Rose Bengal reduced the number of fungal colony forming units in both soil types in all three light treatments. Increasing concentration of Rose Bengal from 10^{-5} to 10^{-3} mol L⁻¹ did not further reduce fungal growth.

The combined effect of increasing concentration of Rose Bengal and exposure to light also caused a significant reduction ($P<0.001$) of both bacteria and fungi (Figures 2.5 & 2.6).

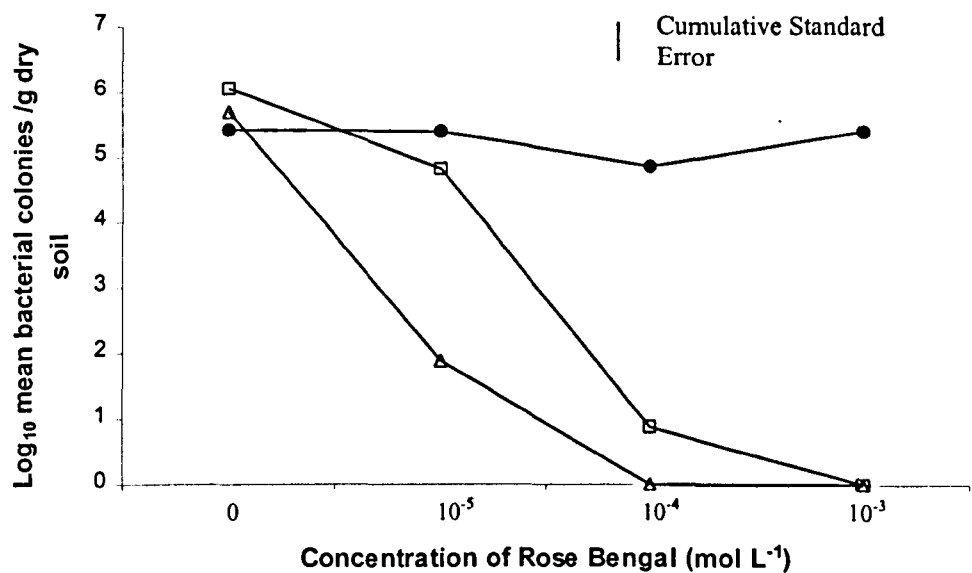


Figure 2.5. The effect of the interaction between light and Rose Bengal on bacterial colonies. ● Cultures incubated in the dark; □ cultures incubated in the light; Δ Cultures incubated in the light on growth media pre exposed to light. Light exposure x Rose Bengal interaction, $P<0.001$.

Bacterial numbers were reduced significantly ($P<0.001$) in the presence of Rose Bengal when incubated in the light whilst those incubated in the dark remained unaltered. The difference in bacterial numbers between the two light treatments was not significant.

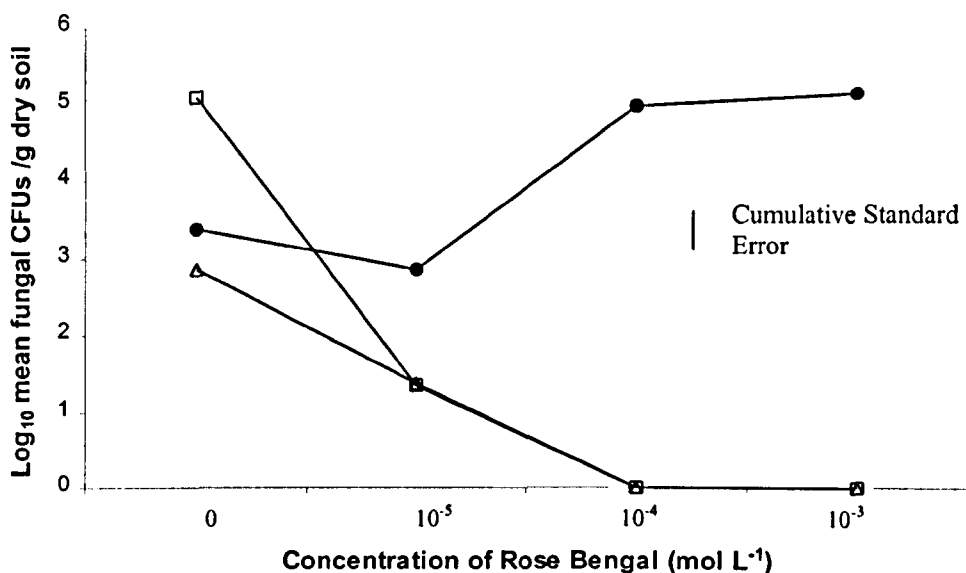


Figure 2.6. The effect of the interaction between light and Rose Bengal on fungal CFUs. ● Cultures incubated in the dark; □ cultures incubated in the light; Δ Cultures incubated in the light on growth media pre-exposed to light. Light exposure x Rose Bengal interaction, $P < 0.001$

There was a significant interaction between light treatment and Rose Bengal concentration ($P < 0.001$). More fungal colonies were recorded on plates incubated in the dark containing 10^{-4} and 10^{-3} mol L⁻¹ Rose Bengal than observed when Rose Bengal was absent from the media (Figure 2.6). There was however, no difference in the number of fungal colonies between media containing Rose Bengal which had, and had not been pre-exposed to the light.

2.3.2. Streptomycin & Penicillin

Bacterial populations were reduced significantly ($P < 0.001$) both on PDA and CZA as the concentration of streptomycin increased, despite the corresponding reduction in the concentration of penicillin. After this initial decline however, bacterial numbers levelled off after treatment 7 (30mg L⁻¹ streptomycin: 35mgL⁻¹ penicillin, Figure 2.7).

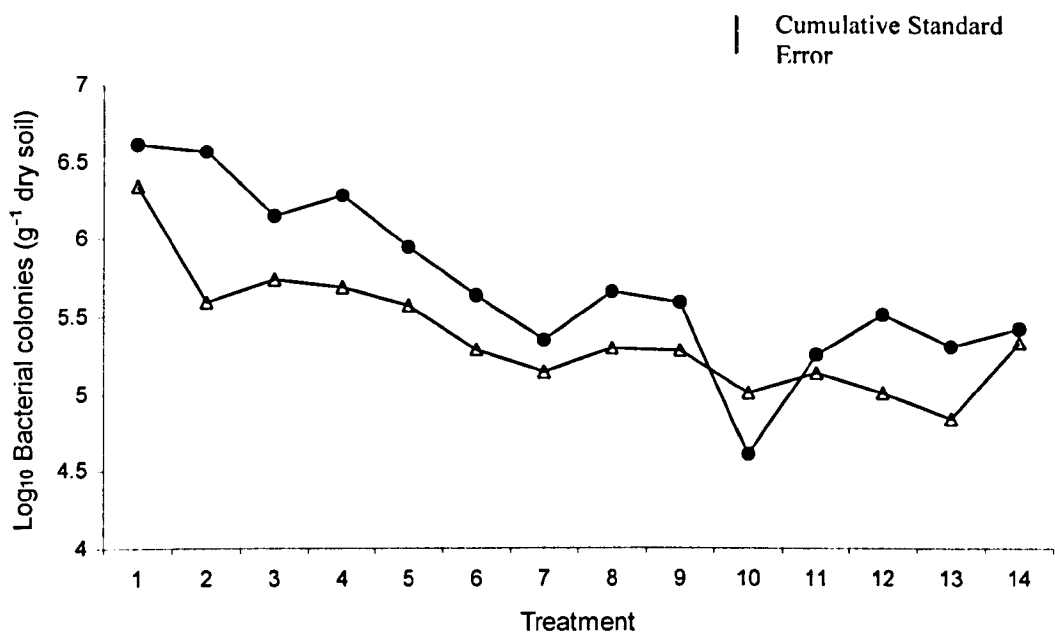


Figure 2.7. Bacterial populations with different proportions of Streptomycin and Penicillin declined until treatment seven. Δ Colonies grown on Czapek-Dox Agar; \bullet colonies grown on Potato Dextrose Agar. (NOTE: Streptomycin concentration increases with treatment number, penicillin concentration decreases).

The numbers of fungal CFUs were significantly greater ($P < 0.001$) on CZA than PDA irrespective of antibiotic treatments, a trend also noted by Fletcher *et al.*, (1985). The lowest fungal diversity recorded was with high penicillin concentration in the absence of streptomycin ($P = 0.04$).

Greatest numbers of fungal CFUs were recorded using streptomycin and penicillin at concentrations of 50mg L^{-1} and 15mg L^{-1} respectively on CZA ($2.95 \times 10^6 \text{ g}^{-1}$ dry soil) and 40mg L^{-1} streptomycin and 25mg L^{-1} penicillin on PDA ($2.53 \times 10^6 \text{ g}^{-1}$ dry soil). The cultures obtained on CZA were much slower growing than those observed on PDA (around four days) although the range of species observed was similar.

2.4. DISCUSSION

Awareness of the phototoxic action of Rose Bengal with reference to its use in quantitative studies is increasing (Ottow, 1972; Banks *et al.*, 1984; Dahl, 1988; Chilvers *et al.*, 1999). Banks *et al.*, (1984) found strong evidence linking this phototoxicity with the production of $^1\text{O}_2$ since the effects were increased by treatment with deuterium oxide, a substance known to prolong the lifetime of $^1\text{O}_2$ from 2 μs to 20 μs . Similarly, treatment with $^1\text{O}_2$ quenchers L-histidine and the carotenoid crocetin, conferred some degree of protection both on bacteria and fungi. The latter is in keeping with the increased proportion of pigmented microorganisms surviving in the presence of Rose Bengal as reported by Banks *et al.*, (1984) and observed during the present study (subjective observation) due to the production of natural carotenoids. Banks *et al.*, (1984) also showed a linear relationship between increased light intensity and a decrease in dissolved oxygen concentration as a simple measure of $^1\text{O}_2$ production.

$^1\text{O}_2$ is extremely reactive and causes damage to membrane lipids as well as enzymes and other proteins and nucleic acids. Dahl *et al.*, (1988) showed that photoinactivation was up to 200 times faster in Gram positive bacteria than in Gram negative, which was attributed to the time taken for the Rose Bengal molecule to penetrate the lipopolysaccharide membrane of Gram negative bacteria. They also showed that a reduction in pH lowered the inactivation time in Gram negative bacteria which was in keeping with protonated compounds penetrating such membranes faster than anionic ones. It followed that there was a significant difference in the proportion of Gram negative to Gram positive

bacteria after treatment with Rose Bengal. In the current study however, no significant differences were observed in the proportions of Gram positive to Gram negative bacteria.

The current investigation into the effectiveness and associated phototoxicity of Rose Bengal showed that at the range of concentrations tested, it did not significantly reduce bacterial numbers unless exposed to the light. Under illuminated conditions, however, the toxic effects also extended to the inhibition of fungal growth. A similar response was noted by Chilvers *et al.*, (1988) who concluded that suppression of both bacterial and fungal colonies by Rose Bengal was dependant upon exposure to light. Furthermore, the toxic effect was increased with increasing concentration of Rose Bengal as noted by Banks *et al.*, (1984).

Chilvers *et al.* (1998) working under similar conditions of illumination found that bacterial inactivation took place after the first 60-75 minutes of illumination with no additional effects being noted after 24 hours, indicating a short-lived reaction to the light. The current data did not show this, however. Although the media were pre-exposed to light, no subsequent reduction in toxicity was observed.

Fungal colonies were reduced in the presence of Rose Bengal in the light, becoming completely absent at 10^{-4} mol L⁻¹. However, under darkened conditions, fungal colony numbers were significantly higher ($P < 0.001$) when Rose Bengal was present.

Martin (1950) investigated the use of Rose Bengal in the presence of streptomycin and reported that the latter had virtually no effect on the growth of fungi, whilst being extremely effective against bacteria. Schatz *et al.*, (1944) noted that streptomycin was active against both Gram positive and Gram negative bacteria whereas penicillin was predominantly active against Gram positive forms. Moreover, although penicillin was effective against growing cells, it had little effect against resting cells and often promoted growth of filamentous bacterial forms (Strominger & Tipper, 1965).

In the current study, streptomycin was more effective than penicillin in reducing bacteria on spread plates, although the concentration required for maximum reduction was different for each medium tested. Furthermore, high concentrations of each antibiotic were shown to impact upon fungal diversity, although little variation was observed when antibiotics were used in combination.

In practical terms, both media appear to be well suited to the culture of soil fungi having a high degree of similarity in results with each other. PDA however, produced countable colonies in a shorter time than CZA. Also, little variation in the numbers of fungal CFUs was observed between changing proportions of streptomycin and penicillin used when in combination.

Treatment number nine (40mg L⁻¹ streptomycin and 25mg L⁻¹ penicillin) effectively reduced bacterial numbers and also provided consistent numbers of

fungal colonies between replicates whilst maintaining a high level of fungal diversity.

In terms of practical use when enumerating fungi from Antarctic samples, it would appear that Rose Bengal is not suited to this type of investigation due to its sensitivity to light and the potential toxicity to fungi. Streptomycin was therefore used in combination with penicillin at 40mg L⁻¹ and 25mg L⁻¹ respectively with both PDA and CZA.

CHAPTER 3: SAMPLING BIOLOGICAL PARTICLES FROM ANTARCTIC AIR

3.1. INTRODUCTION

3.1.1 The Antarctic habitat

The continent of Antarctica is completely isolated geographically from other land masses, having become separated from Gondwanaland around 130 million years ago (Boger & Wilson, 2003). Unlike the Arctic, which comprises the northern regions of a number of land masses with large terrestrial connections, Antarctica is completely surrounded by the Southern Ocean. The biota of isolated islands or continents is limited to organisms which are capable of migration, as is the case of many Antarctic animals, or organisms capable of being carried as propagules, either by animals, the sea or by the wind (Marshall, 1996). Since the establishment of research stations in Antarctica, there is now additional potential for new species introduction through human activity. Thus, there is a pressing need to assess human impact as well as species introduction through natural means.

3.1.2 Airborne particle sampling

Collection and recording airborne particles has been undertaken for many years, usually in the context of public health (*e.g.* Le Tertre *et al.*, 2002; Li & Hou,

2002). A variety of devices has been developed for sampling particles from air (Davies, 1971) based on both active and passive techniques. Active samplers, which comprise the majority of air samplers, usually rely on air being actively drawn into a device and then trapped onto a collecting surface for observation.

The slit sampler (Bourdillon *et al.*, 1941) draws air through a narrow slit onto a rotating Petri dish containing growth medium allowing not only the total number of viable particles per unit volume to be determined but also individual capture events may be correlated with real time. Similarly, the Hirst trap (Hirst, 1952) draws air onto a continuously moving (2mm hr^{-1}) microscope slide coated with a sticky medium in order to provide both total counts and record capture events against time. The Andersen sampler (Andersen, 1958) incorporates a series of six sieves which successively remove particles of decreasing size and deposits them onto solid growth media in Petri dishes located below each sieve. Although this system provides some degree of particle size grading not offered by other samplers, limitations in design efficiency have been reported by Gregory (1961) and May (1964) (cited in Davies, 1971). The rotorod sampler (Perkins, 1957, cited by Davies, 1971) differs in that no air pump is used. The collecting surfaces are exposed directly to the air which is drawn through by a vortex induced by rotation.

Passive samplers do not rely on any mechanical means of sampling air. “Gravity slides” are glass slides coated in adhesive material such as silicone grease or petroleum jelly and placed in sheltered sites, but exposed to the air. Although of

some qualitative use, the technique is rarely, if ever, applied to serious investigations. “Settle plates” include Petri dishes containing solid media being exposed to the air and which also depend upon particles settling under gravity.

Airborne particles have been studied in Antarctica on numerous occasions. Corte & Daglio (1964) reported obtaining 214 cultures from air samples and Wynn-Williams (1991) reported a number of aerobiological sampling projects as part of the BIOTAS (Biological Investigation of Terrestrial Antarctic Systems) programme.

The aim of the current study was to determine the nature, abundance and distribution of airborne biological particles in the Vestfold Hills over the period of a year. Fungal data were compared with observations made from isolates of Antarctic fungi obtained from soil and a range of natural and introduced substrates.

3.2. MATERIALS & METHODS

Two sampling techniques were employed in order to sample airborne particles. Active sampling by rotorod air samplers and passive collectors, made from upturned “Frisbees”. Meteorological conditions were recorded for comparison against results obtained with both sampler types.

3.2.1. Design and operation of rotorod air sampler

The rotorod sampler¹ has been frequently used in aerobiology since the late 1950s (Perkins, 1957, cited by Davies, 1971) due partly to the relatively low cost per unit and the high rate of sampling (113L min^{-1} at 3000rpm). The principle of operation of the rotorod is a pair of spinning rods coated with an adhesive material on the leading edges, which capture airborne particles. The spinning rods create a vortex through which air is drawn (Figure 3.1).

The rotorods used for this study were constructed “in house” using components purchased from Farnell Electronic Components Ltd., Leeds, following a design which had previously been used successfully on Signy Island (Marshall, 1997) (Figure 3.2). Power was supplied by portable rechargeable 12v batteries (Marathon[™] from GNB Industrial Battery Co., Leicestershire).

¹ Rotorod is a registered trademark of Sampling Technologies Ltd., California.

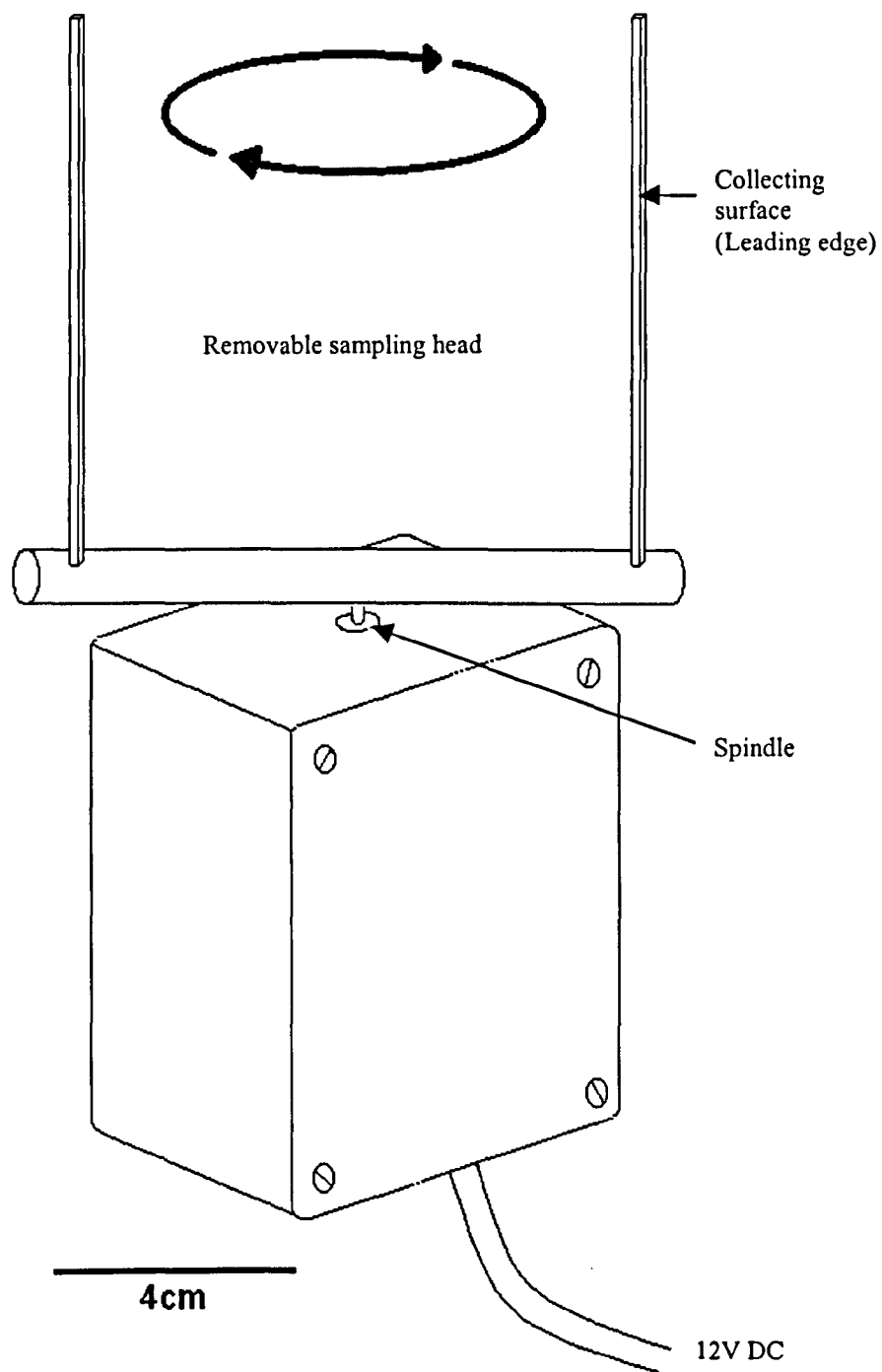


Figure 3.1. Basic view of rotorod air sampler with removable sampling head.

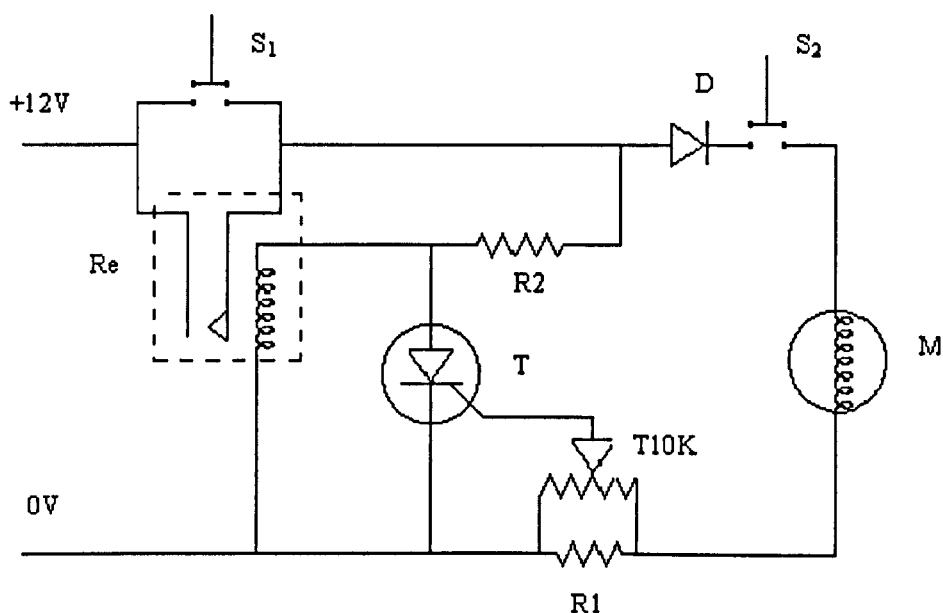


Figure 3.2. Circuit diagram for rotorod air sampler (after Marshall, 1997); Re, relay; S_x, switch; D, diode; M, motor; T, thyristor; T10k, trimmer; R_x, resistor.

The design incorporated a safety cut-out which operated in case of rime ice forming around the sampler, preventing the sampler from spinning and therefore preventing the motor from burning out. Although the speed of the motor was factory set, its performance was confirmed using a strobe timing gun prior to use.

A direct relationship exists between speed of sampler rotation and the size of particle intercepted (Operating Instructions for the Rotorod Air Sampler, 1989). Since the target particles in the current study were fungal spores with diameters in the region of 10-15 μ m, two sampler types were initially constructed, one operating at 3000 rpm, the other at 2200 rpm. Three samplers of each type were run in parallel as a pilot trial on two consecutive days in a grassland habitat in Leicestershire and the results examined directly under the microscope. The first

200 biological particles encountered by each sampler were measured and the approximate volume of each particle was calculated and abundance plotted (Figure 3.3).

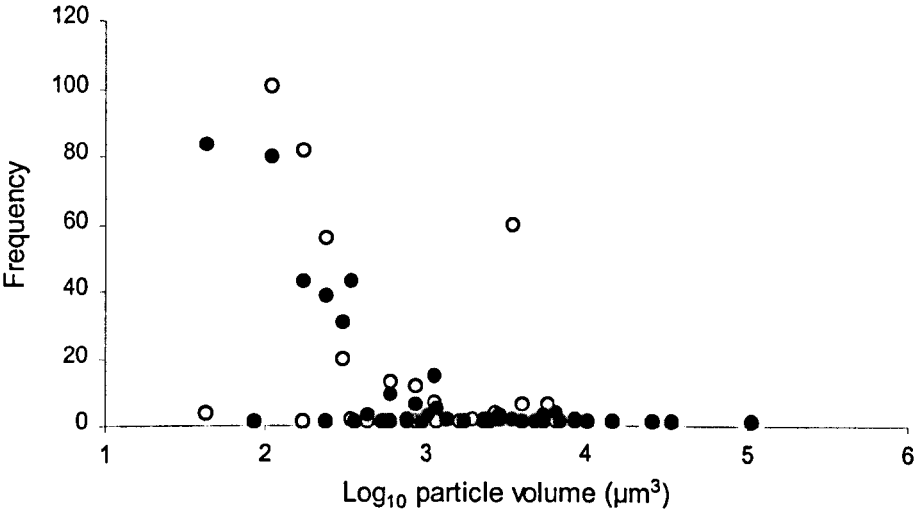


Figure 3.3. Frequencies of particles of varying sizes intercepted by six rotorods, three operating at 2,200 rpm (○) and three at 3,000rpm (●) in parallel on two consecutive days in June 2000 in a grassland habitat at Sutton Bonington, Leicestershire.

The performance of the samplers running at different speeds was similar although a greater number of smaller particles was intercepted at higher speed which was consistent with the operating profile of the sampler (operating instructions for the Rotorod air sampler, 1989) and the findings of Marshall (1997).

The volume of air being drawn through the rotorod was calculated using the General Rotorod Formula²:

$$V = t\pi ads$$

Where V = Volume (cm³)

t = time (mins)

a = total area of sampling surfaces (cm²)

d = diameter of swing (cm)

s = speed of rotation (rpm)

Since all variables except time remain constant, πads could be expressed as a constant k , which was divided by 10^6 to convert the volume to m³ (in this case $k=0.113$) so the expression was simplified to:

$$V = tk$$

Static electric fields occur naturally between airborne biological particles and contribute to their “space separation” (Benninghof, 1983), moreover, these charges have also been shown to vary with atmospheric conditions (Benninghof, 1980). The rotation of the rods through air of varying conditions generates a static charge due to the effect of charge separation which, in cold, dry conditions, could be high (Hesse *et al.*, 1992). As a measure to counteract this effect, a reed connection was included in the design which made permanent contact with the rotating spindle and was connected to earth *via* the support post (Plate 3.1).

² Operating instructions for the Rotorod sampler, Sampling Technologies Inc. 1989.

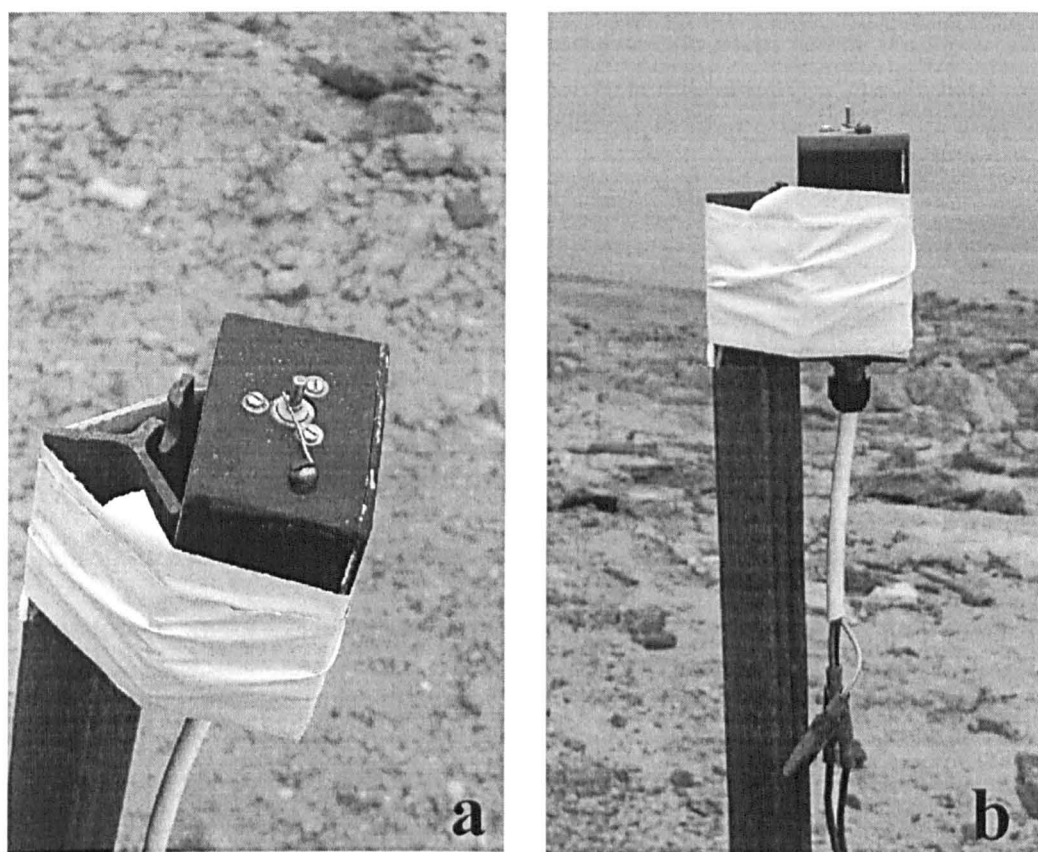


Plate 3.1. (a) Earthing reed in contact with the spindle and (b) connected to the support post to prevent static build up in the rotating rods.

The removable sampling head comprised two brass rods, 1.5mm square in cross section giving a flat leading edge, mounted 8cm apart on a nylon bar. The density of the nylon allowed the assembly to be firmly attached to the spindle of the motor simply by being pushed on.

The surfaces of the collecting arms were cleaned with acetone prior to use and strong clear adhesive tape applied to the leading edges which was trimmed to exact fit using a razor. The taped surfaces of the rods were cleaned with alcohol in order to remove dust and grease and sterilise the surface. Adhesive (Vaseline,

2 parts $\frac{1}{v}$; silicone oil, 2 parts $\frac{1}{v}$; paraffin wax, 1 part $\frac{1}{v}$; dissolved in toluene, 10 parts $\frac{1}{v}$) was evenly applied to the tape using a fine paint brush (Kapyła, 1989).

3.2.1.1. Use of rotorod air samplers in Antarctica

Two transects were established (Figure 3.4). The first transect ran 3km through the station from north to south extending from 50m beyond the limit of the station buildings, to the south of Heidemann Bay. The second transect extended 18km west to east along the length of the Ellis Fjord and Lake Druzhby as far as Trajer Ridge, 5km from the polar plateau. Each transect comprised 5 sites which were also used for the soil sampling programme (Chapter 4) and are described in Tables 3.1 & 3.2.

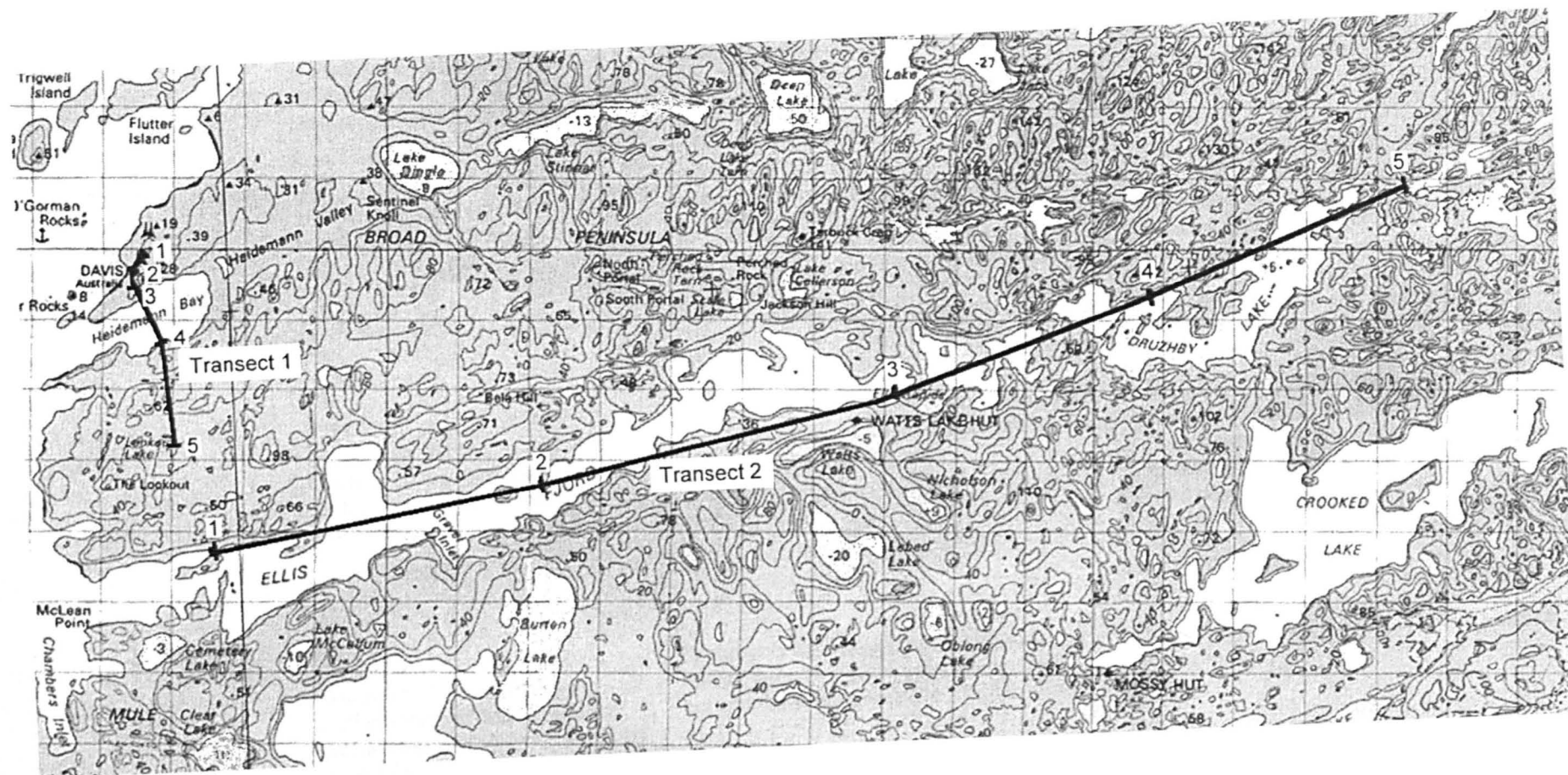


Figure 3.4. Transects 1 and 2 running through Davis Station and the Ellis Fjord respectively. Grid squares represent 1km. Map courtesy of the Australian Antarctic Division.

Table 3.1. Descriptions of five sites in and around Davis Station selected for rotorod and soil fungal study

Site	Location	Description & Underlying Geology	Human impact
1. Dingle Road	S68° 34' 54.3" E77° 58' 11.4" A slightly elevated piece of ground 100m north east from the station, set back several meters from a roadway.	Small to medium sized rocks of various sources scattered around wind-blown sand and dust.	The roadway is infrequently used by vehicles, however it is often used by walkers during the summer months when departing the station on field trips.
2. Living Quarters	S68° 34' 58.8" E77° 57' 59.4" 10m west of the main entrance to the living quarters & mess area.	A mixture of sand and small stones resulting from successive thaws of snow having been cleared from the roadway by bulldozer.	The most highly impacted area of the station with all personnel passing through this entrance at some point during the day. A parking place for vehicles which inevitably disturb the ground and standing area for field equipment prior to expeditions. Adjacent to the kitchen door where rubbish is collected on a daily basis.
3. Davis Beach	S68° 35' 59.1" E77° 58' 5.0" 50m due south of the main living quarters, on the southern reaches of the beach around 30m from the shoreline.	Predominantly sand from weathered gneiss and dolerite with organic material from marine matter and nearby elephant seal wallow.	Although the site is set back from the main part of the beach, the area is used for storage of half- height sea containers, some of which contain materials for return to Australia. The beach is also popular for recreational walking during the summer and vehicular access to the sea ice during winter.
4. Heidemann Bay	S68° 35' 10.1" E77° 58' 34.0" 1km south of the station on the northern shore of Heidemann Bay, set back from the ice/waters edge by around 3m.	Exclusively dolerite, being situated on a dyke around 8m wide. Some sand accumulated between large dolerite rocks. No lichens present.	Rarely visited by walkers or vehicles due to its location.
5. The Lookout	S68° 35' 55.9" E77° 58' 12.3" A knoll raised about 5m from the surrounding basin. Around 1km north east of a promontory known as "The Lookout".	Almost entirely gneiss with small rocks and gravel being the result of frost shattering. Lichens present mostly in cracks in rocks.	Although the basin is occasionally used by walkers when accessing The Lookout itself or the mouth of the Ellis Fjord, the knoll is rarely visited.

Table 3.2. Descriptions of five sites along the Ellis Fjord, Lake Druzhby and Trajer Ridge selected for rotorod and soil fungi study.

Site	Location	Description & Underlying Geology	Human impact
1. Ellis Narrows	<i>S68° 36' 49.2" E77° 59' 27.0"</i> A low bridge of land forming a spit extending part of the way across the seaward entrance to the Ellis Fjord	Slightly raised by 1 or 2 m with marine beaches to the east and west. Although lying on a dolerite dyke, the site is covered with small to medium sized scattered rocks of predominantly gneiss but including many erratics. Also large amounts of wind-blown sand.	The site is one of the few land bridges over which vehicles are allowed to cross. The crossing is the only vehicular access to the Ellis Fjord and the lakes beyond as far as Trajer Ridge and the polar plateau.
2. Flat Top Peninsula	<i>S68° 36' 23.8" E78° 06' 18.0"</i> The southern tip of a peninsula extending into the Ellis Fjord around 300m east of Flat Top Island.	The edge of a dolerite dyke, the site comprises large rocks with wind blown sand accumulating between them. A scattered moss bed exists around 5m to the west. Occasional lichen cover.	The site itself would receive little direct impact from walkers or vehicles, however it overlooks the Ellis Fjord which is regularly used by vehicles.
3. Watts Lake Hut	<i>S68° 35' 55.9" E78° 13' 18.5"</i> An area of low-lying beach some 40-50m north west of the field hut which lies between the Ellis Fjord and Watts Lake.	Scattered rocks of gneiss with occasional erratics. Sand and gravel from frost shattered rock with pockets of salt from evaporated marine spray. A large area of moss cover exists some 100m to the east. Although lichens are absent from the beach itself, they are common on the surrounding rocks.	The nearby hut is regularly used by walkers and drivers of vehicles. Since the approach of vehicles is over the sea ice and walkers from the north east via the land bridge, the beach itself receives little direct impact
4. Lake Druzhby	<i>S68° 35' 10.8" E78° 19' 28.2"</i> A rocky beach on the northern shore of Lake Druzhby	Large to medium sized rocks, predominantly gneiss with occasional erratics. The site is located at the waters edge during the summer and is icebound during the winter. Algal mats in various stages of desiccation are present between the rocks. Lichens are absent from the areas prone to submerging, however cover is good on the nearby rocks.	Although Lake Druzhby is frequently used by vehicles as a means of access between Trajer Ridge/polar plateau and the Ellis Fjord, the contorted nature of the lake means that the site itself is rarely approached.
5. Trajer Ridge	<i>S68° 34' 26.9" E78° 24' 28.3"</i> A raised outcrop of rock surrounded by ice at the western tip of a feature known as Trajer Ridge	Although situated on the shoulder of a slope, the rocks are raised some 2-3m above the surrounding ice. Lichen cover is widespread on the rocks themselves and surrounding areas.	The western tip of Trajer Ridge is narrow and frequently used by vehicles accessing the field hut farther to the east or the polar plateau beyond. The raised nature of the rocks however means that human impact is minimal.

Although rotorod samplers have been shown to operate effectively as single units (Eversmeyer & Kramer, 1987) two rotorods were set up at each site, fixed to rigid metal posts one meter above the ground. A rigid box, was used to transport the prepared sample heads to and from the field, alcohol-sterilised before each use and fitted with jubilee clips preventing rods from touching one another during transit. A sample head remained in the box as a control to record particles which may have entered the box when opened during field work. Sampling took place in Antarctica at fortnightly intervals throughout the entire year as conditions allowed, with additional samples being taken throughout the period of annual resupply (December 2001). At each sampling occasion, rotorods were deployed for a period of around 24 hours (largely dictated by travel time to each sample site) with exact elapsed time being recorded for each sample.

After exposure, the tape strips were removed from the leading edges of the collecting arms using a sterile razor blade. One strip from each of the two samplers at each location was placed onto a microscope slide and sealed with PVA mounting medium (Kapyla, 1989). Examination was by transmitted light at 200x magnification. Each biological particle type was drawn, measured and assigned a reference number. The occurrence of each particle was recorded by type, sample site and date.

A third strip was placed into Czapek Dox broth for incubation at room temperature (ca. 22°C). Fungal growth, where present, was observed directly on each strip and colonies counted (Plate 3.2). The last strip was placed into a sterile Eppendorf tube for storage and further examination if required.

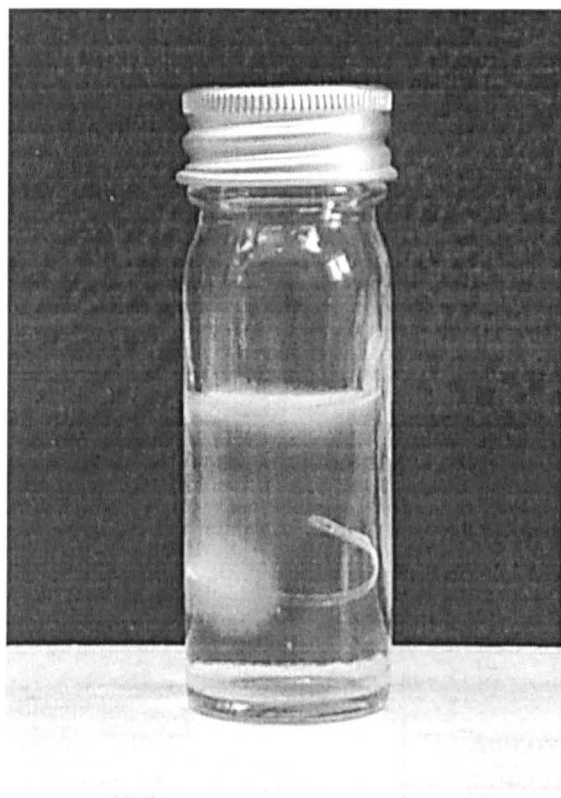


Plate 3.2. Fungal mycelium observed directly on a rotorod collector strip placed into Czapek Dox broth and incubated at 22°C for 14 days.

3.2.2. Design and operation of “Frisbee” settled particle collector

Settled particle collectors are passive samplers which rely on particles falling out of the air onto a collecting surface. The “Frisbee” is a popular toy which flies by floating on a cushion of air trapped underneath it. By inverting it, an area of still air is presented allowing airborne particles to fall out from the moving wind (Figure 3.5).

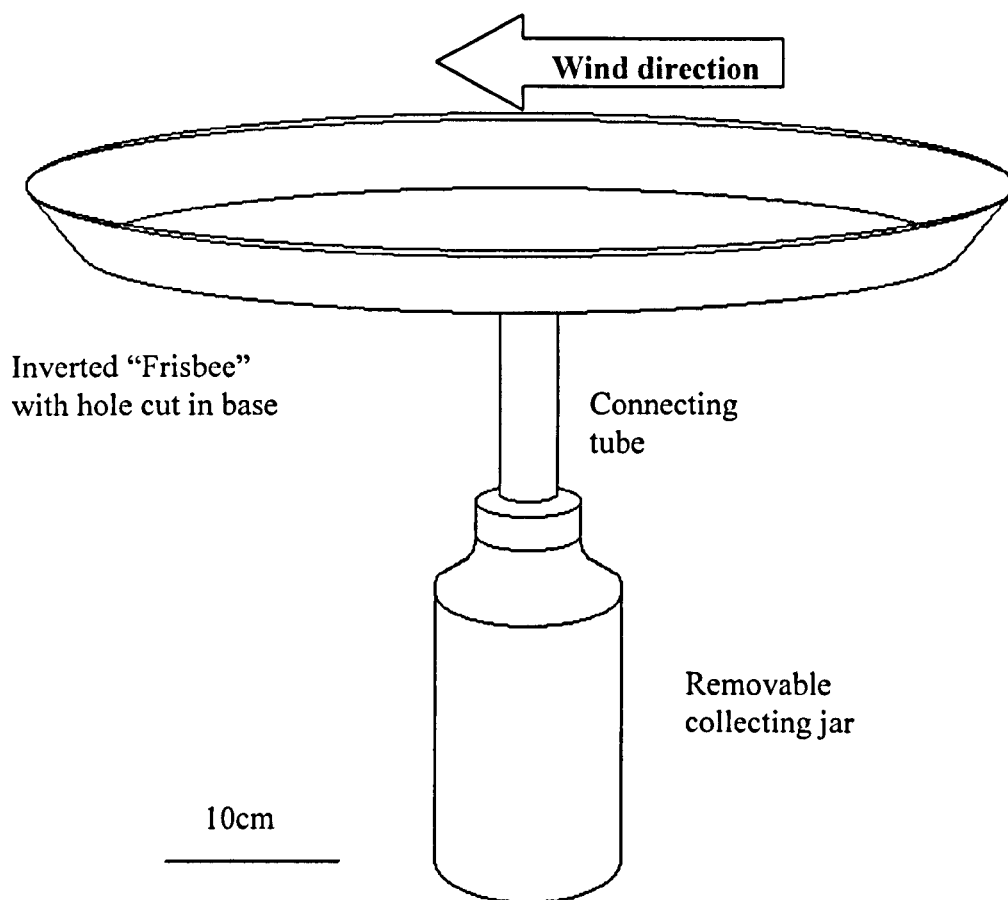


Figure 3.5. Design of “Frisbee” settled particle sampler

Prior to use, the collecting surface of the Frisbee was cleaned with alcohol and a sterile collecting jar attached. The Frisbees were mounted on metal poles one metre above the ground and left exposed for a 24 hour period (Plate 3.3). At the end of this period, the collecting surface was washed with ca. 100ml sterile water to flush settled particles into the collecting jar. The jar was then removed and returned to the laboratory.



Plate 3.3. A pair of “Frisbee” settled particle samplers in place on Davis Beach.

The water from the jars was filtered under vacuum through sterile filter paper ($10\mu\text{m}$) and then placed into Czapek Dox broth for incubation at ambient room temperature (ca. 22°C) for 14 days.

3.2.3. Treatment of results

Particle densities obtained from rotorod counts were expressed as number of particles per cubic metre of air. Linear regression analysis was carried out as required using Genstat 5 (v4.1) and cluster analysis was performed using “R”

v1.6.2³ to determine relationships between sites based on particle types following \log_{10} transformation of the data.

³ Available from <http://www.r-project.org>

3.3. RESULTS

More than eighty distinct organic particle types were found and recorded over the sampling period comprising fungal spores & lichen soredia, dinoflagellate cysts, moss fragments and feather fragments. Although the purpose of the investigation was to examine the distribution of fungal propagules, the other particles encountered are also important in understanding seasonality and the nature of airborne distribution mechanisms. A catalogue of fungal particles observed is shown in Appendix 3.

Meteorological conditions (recorded by the Australian Meteorological Bureau, Davis Station) were generally mild during 2001 with low snowfall and infrequent blizzards. The average wind speed was low, (Figure 3.6) being just over 21km hr⁻¹ and temperatures (Figure 3.7) did not fall below -30°C. Relative Humidity remained between 61-81% (Figure 3.8).

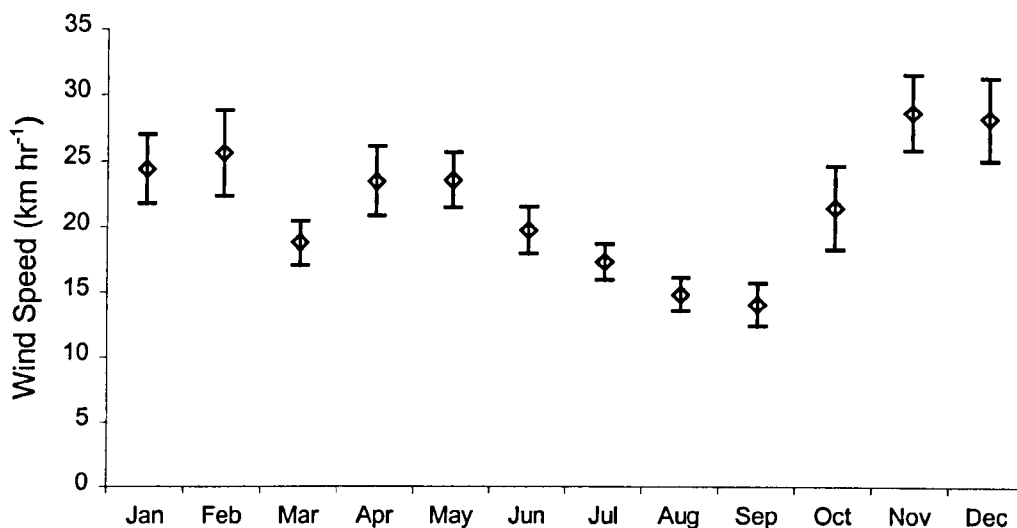


Figure 3.6. Mean wind speeds based on four-hourly recording intervals made at Davis Station during 2001.

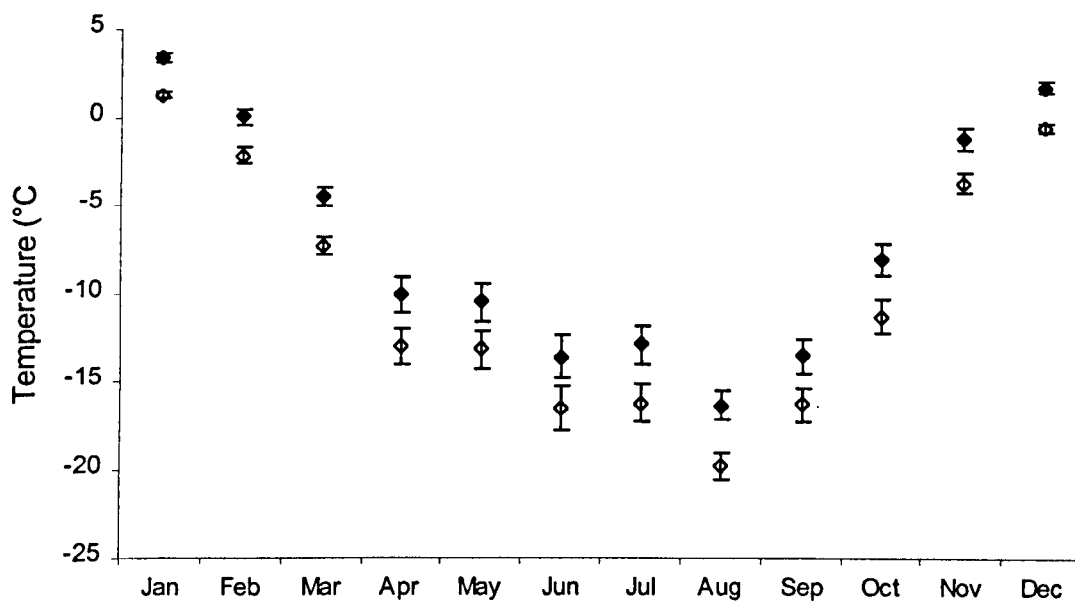


Figure 3.7. Monthly temperatures based on four-hourly recording intervals made at Davis Station during 2001. \diamond Mean temperature; \blacklozenge Mean maximum temperature.

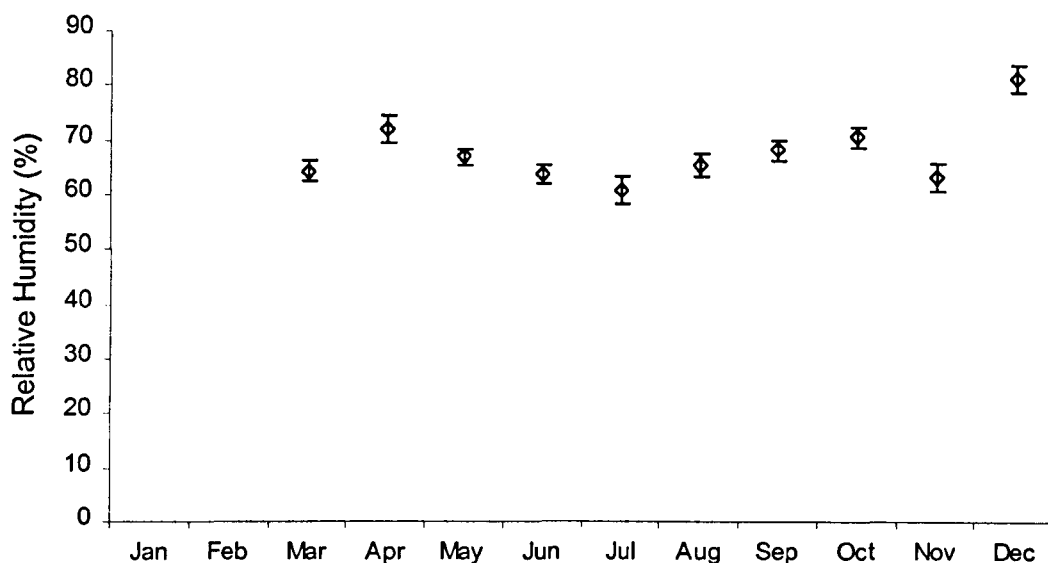


Figure 3.8. Mean relative humidity based on four-hourly recording intervals made at Davis Station during 2001. Relative humidity was not recorded during January & February.

Although snowfall was formally recorded (by the Australian Bureau of Meteorology, Davis Station), blowing snow was of more significance than snow cover at the sampling sites due to its adverse effects on the collecting efficiency of the rotorod samplers and this varied greatly during the sampling period.

3.3.1. Rotorod air samplers

The rotorod samplers proved the most efficient means of collecting airborne particles, although they occasionally suffered effects from adverse weather conditions. High winds during dry, summer conditions for example, resulted in heavy deposits of fine inorganic particles on the collecting surfaces making direct observation unreliable. Falling and blowing snow also adversely affected results, by blocking the collecting surfaces of the rods.

By far the most abundant biological particles (Figure 3.9) recorded were dinoflagellate cysts (Section 3.3.1.2) which were recorded in greatest numbers at the Watts Lake Hut site. So numerous were these particles that a comparison of all sites using the R program closely followed the pattern obtained for just those particles (Figure 3.19). Comparing the sites using cluster analysis with the exclusion of these particles however, showed the Living Quarters to be highly divergent from the other sites (Figure 3.10).

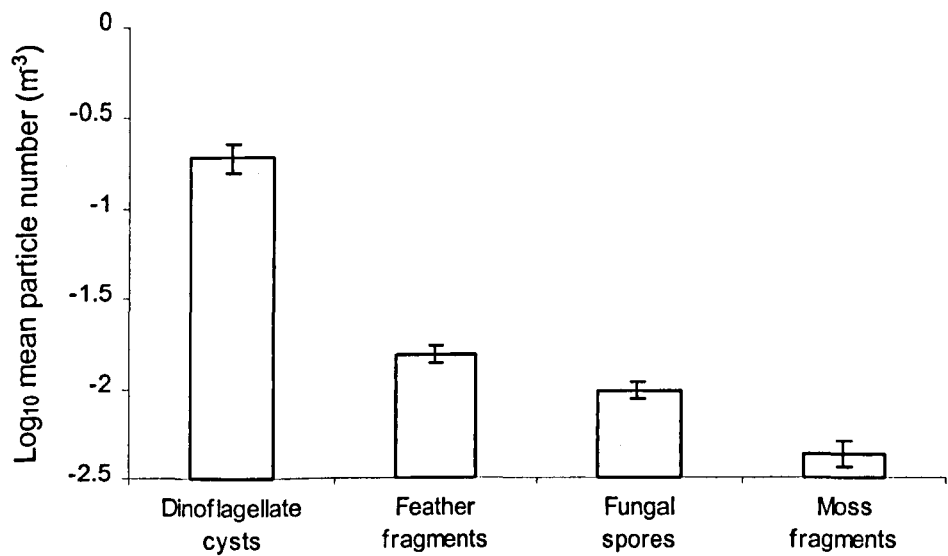


Figure 3.9. Mean abundance of four predominant particle types recorded using rotorod particle samplers at ten sites around Davis Station and the Vestfold Hills, Antarctica, throughout 2001.

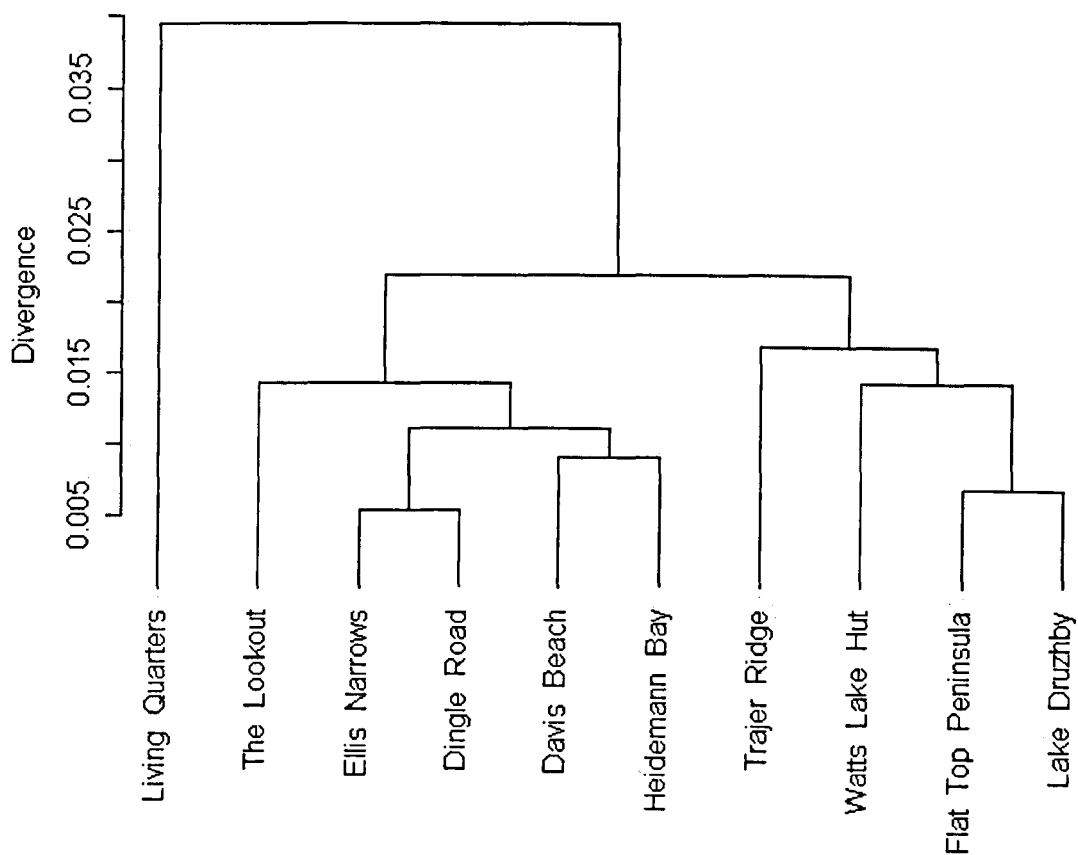


Figure 3.10. Comparison of sample sites based on the abundance and type of all particles except dinoflagellate cysts collected by rotorod samplers at ten locations around Davis Station and the Vestfold Hills during 2001.

3.3.1.1. Fungal spores & lichen soredia

Although far less common than other biological particles, twelve particle types were deemed to be of fungal origin and three more to be lichen soredia, based on their morphological characteristics. Although fungi were not observed directly in the field, several species of lichen were observed within the study area, the most common being *Buellia frigida*, *Xanthoria elegans* and *Xanthoria mawsonii*. Although standard errors are great due to the sporadic nature of the occurrence of these twelve spore types, a seasonal trend was nonetheless observed (Figure 3.11).

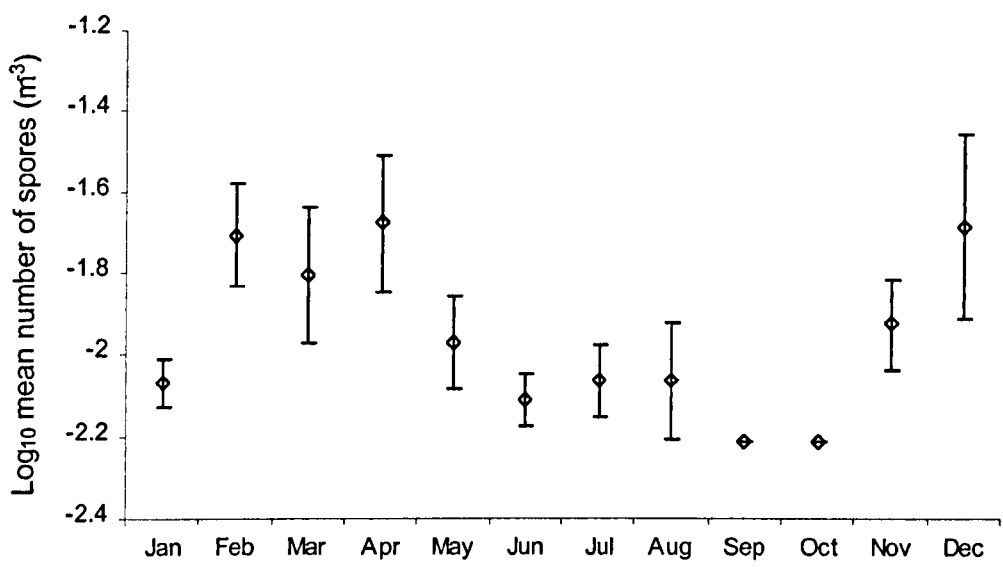


Figure 3.11. Temporal changes in the mean numbers of fungal and lichen spores (\pm standard error) recorded at ten sites around Davis Station and the Vestfold Hills during 2001.

A comparison of airborne fungal spore and lichen soredia concentrations with meteorological data is shown in Figure 3.12. Linear regression was performed using Genstat 5 and a significant relationship ($P=0.007$, $R^2=0.04$) was found between the concentration of airborne spores and mean air temperature despite a high degree of scatter. No significant relationship was found between airborne spore concentrations and other meteorological conditions.

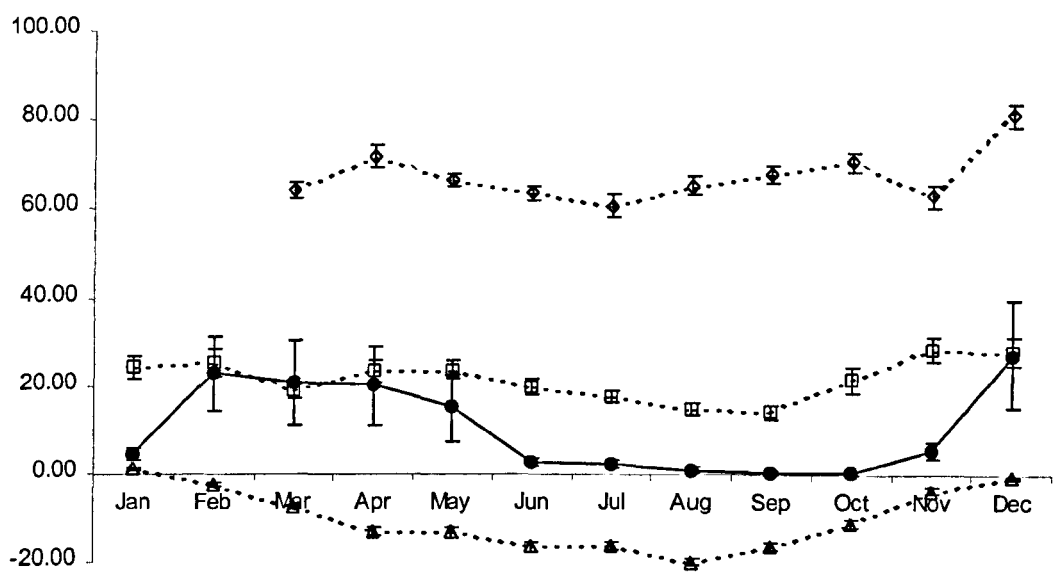


Figure 3.12. Changes in the numbers of fungal spores and lichen soredia collected at ten sites at Davis Station and the Vestfold Hills, Antarctica, with meteorological conditions recorded by the Australian Bureau of Meteorology at Davis Station during 2001. \diamond Relative Humidity (%); \square Wind speed (km hr⁻¹); Δ Temperature (°C); \bullet Mean concentration of fungal spores (m⁻³x1000). NOTE: Relative humidity was not recorded during January & February.

Trends were observed along the transects along the Ellis Fjord and across Davis Station. There was a general increase in fungal spores eastwards along the Ellis Fjord which fell sharply at Trajer Ridge at the eastern extremity (Figure 3.13).

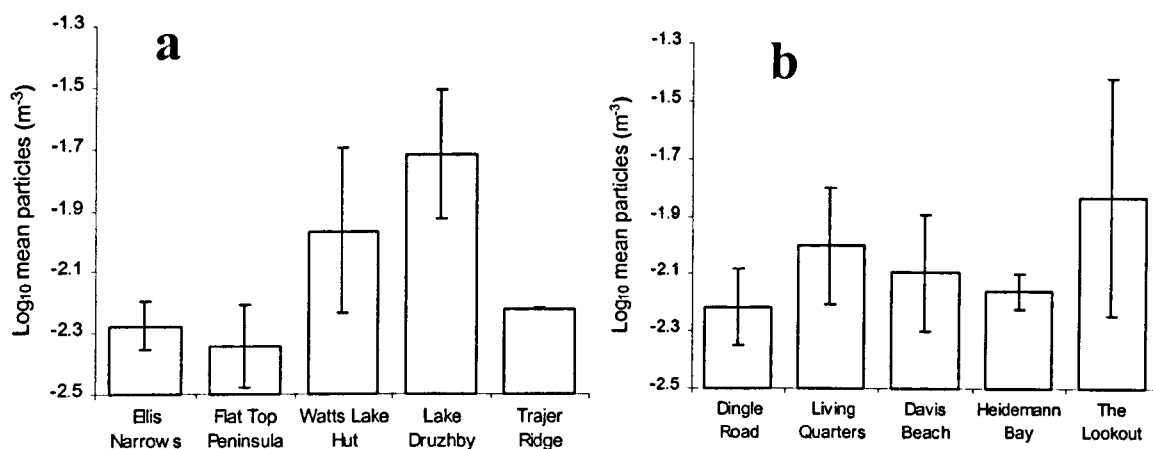


Figure 3.13. Spatial distribution of fungal spores and lichen soredia collected by rotorod sampler at ten sites within the Vestfold Hills during 2001. a) Transect running through the Ellis Fjord east to west. b) Transect running through Davis Station north to south.

Throughout the sites in the Ellis Fjord, the fungal spore most commonly encountered was a two chambered spore identified as *Phaeosphaeria* sp. which was also found in direct association with mosses (Plate 3.4). The abundance of these spores also reflects the distribution of moss fragments recorded by rotorods in the Ellis Fjord (Section 3.3.1.3). Mosses and lichens were most abundant growing around Lake Druzby and the eastern parts of the Ellis Fjord, and corresponded with the increased levels of fungal spores recorded in these areas (Figure 3.13). Also found associated with moss was an unidentified four-chambered spore which was recorded in air samples from Flat Top Peninsula and Lake Druzby (Plate 3.5).

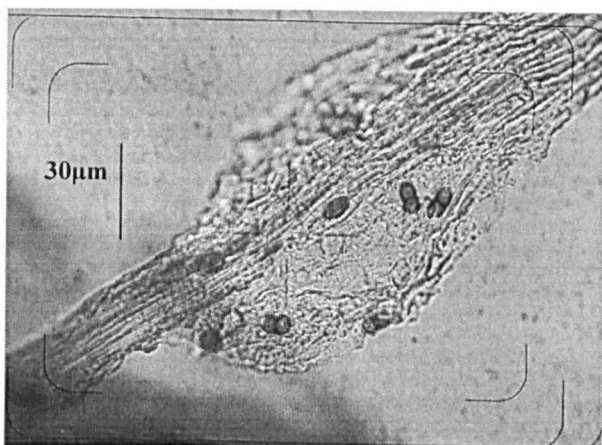
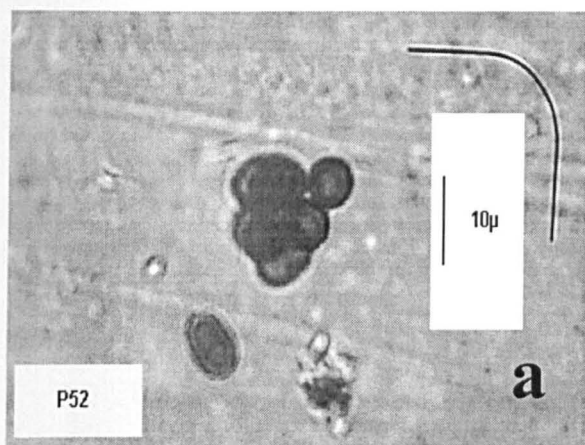


Plate 3.4. (a) Two chambered spores intercepted by rotorod sampler at the Ellis Fjord. (b) Spores observed directly in association with moss lamina.

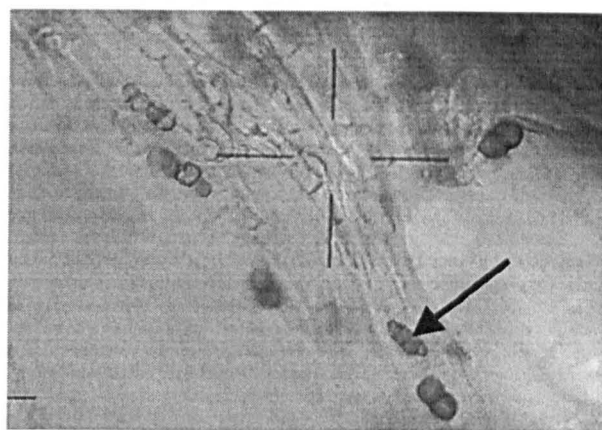
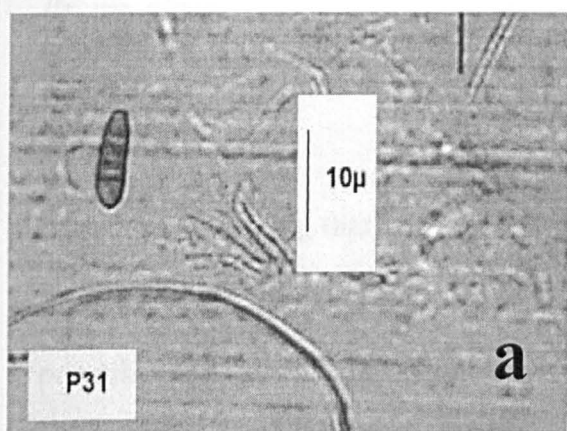


Plate 3.5. (a) Four chambered spores intercepted by rotorod sampler at the Ellis Fjord. (b) Spores observed directly in association with moss lamina.

Linear regression analysis (Genstat⁴ v5) showed a weakly significant relationship ($P=0.059$) between mean daily temperature and the abundance of the two and four-chambered spores and a significant relationship ($P=0.004$) with wind speed.

⁴ Lawes Agricultural Trust 1998, IACR Rothamstead

A mycelium was observed growing in association with moss (Plate 3.6). Whilst no sporangial structures were observed, the presence of what appeared to be progametangia suggested a zygomycete.

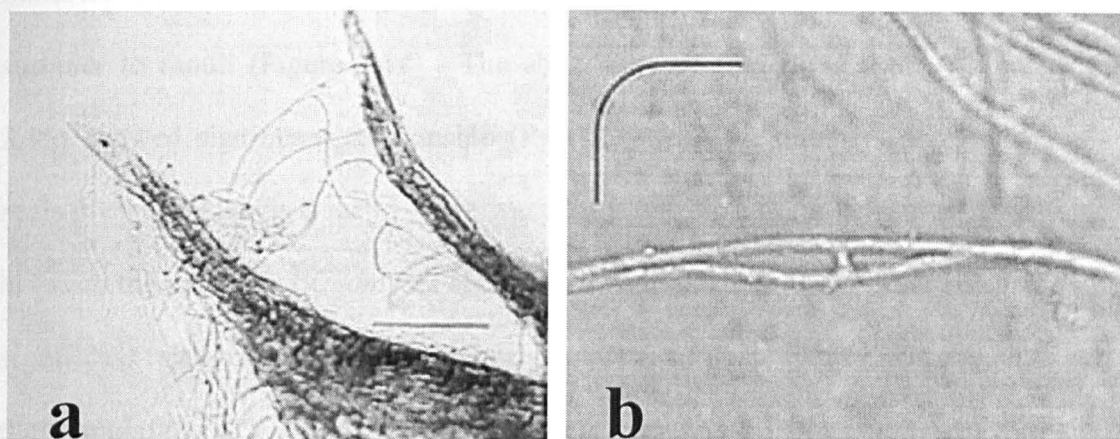


Plate 3.6. (a) Fungal hyphae (possibly zygomycete) surrounding the apex of *Bryum algens*. (b) Fused gametangia between adjacent hyphae of the same mycelium.

It was not surprising that few spores were recorded at Trajer Ridge, as it lies at the base of a ridge of snow extending from the polar plateau from where the prevailing katabatic winds originate.

A greater number of spores was recorded at the Living Quarters and Beach sites than from the Dingle Road site at the north of the station (Figure 3.13). The site at Heidemann Bay is removed from both the station and a local source of substrate and, correspondingly, the number of spores was reduced. The apparent increase in spores at The Lookout was due to an isolated increase in the number of spores of *Geomyces* sp. recorded on the 7th November, rather than an observed trend and consequently, the error bars are greatest at that site. *Geomyces* sp.

(Plate 3.7) was the most common fungal spore found at the five sites at Davis Station and likely to be associated with sources of keratin. On the beach adjacent to the station, there is a large potential source of keratin, as every year, large numbers of southern elephant seals (*Mirounga leonina*) congregate in the summer to moult (Figure 3.14). The abundance of *Geomyces* spores (Figure 3.15) showed significant relationship ($P=0.041$) with the numbers of elephant seals present on Davis Beach. There was also a significant relationship ($P=0.02$) between the number of *Geomyces* spores and windspeed. However there was no significant relationship between *Geomyces* spores and temperature. This suggests that populations of *Geomyces* were not only actively growing in association with the large increase in keratinaceous material accumulating in the seal wallows, but also able to utilise free water present due to the elevated temperature of the combined body heat of the animals. Spores would probably then be released into the air and distributed by the wind.

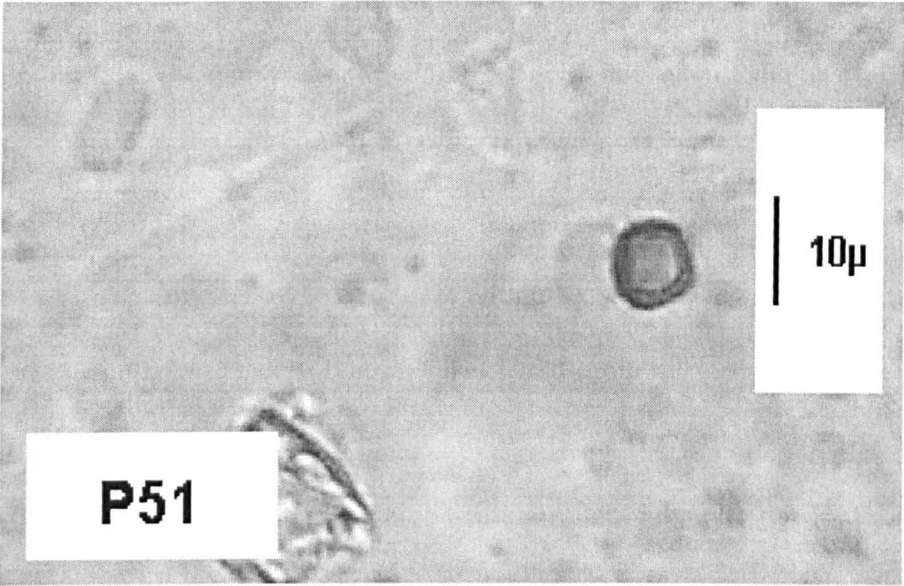


Plate 3.7. Thick-walled *Geomyces* spore intercepted by rotorod air sampler during 2001 and commonly encountered around Davis Station.

An examination of the similarity of each sample site based on the abundance and type of fungal or lichen particles was performed using the R program (Ihaka & Gentleman, 1996; Crawley, 2002). The dendrogram (Figure 3.16) showed the site at Watts Lake Hut to differ the most from the other sites, which fell broadly into two groups. The separation of the Watts Lake Hut site being due to the increased number of two-chambered spores recorded there during December which, in turn, was most likely to have been related to the nearby moss bed with which these spores were associated (Plate 3.4).

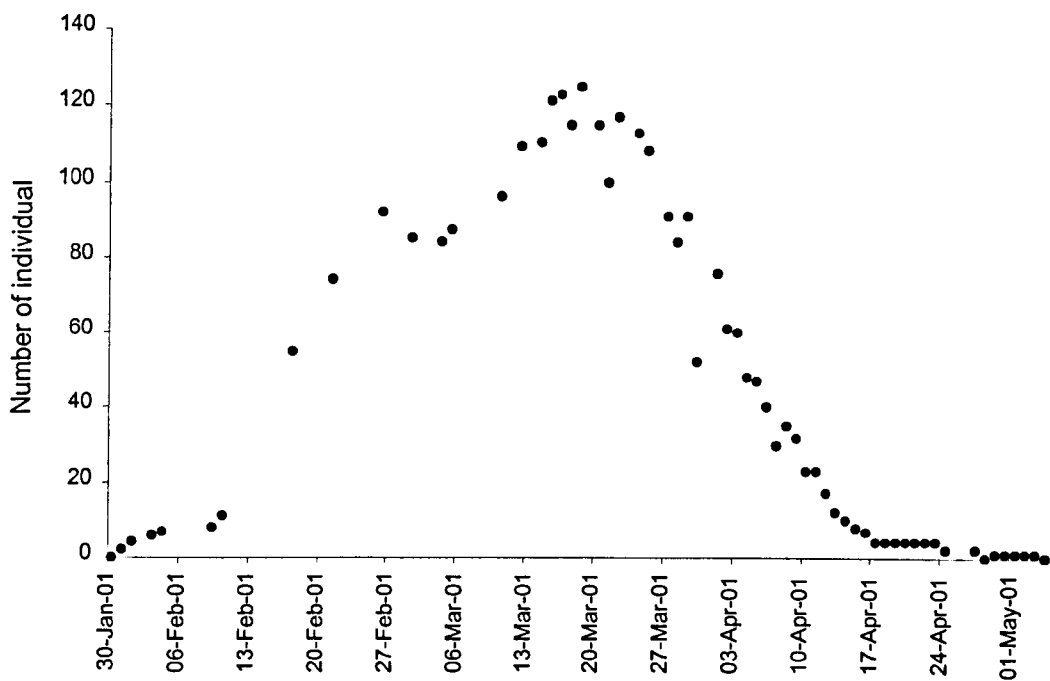


Figure 3.14. Numbers of southern elephant seals (*Mirounga leonina*) on Davis Beach during the early part of 2001.

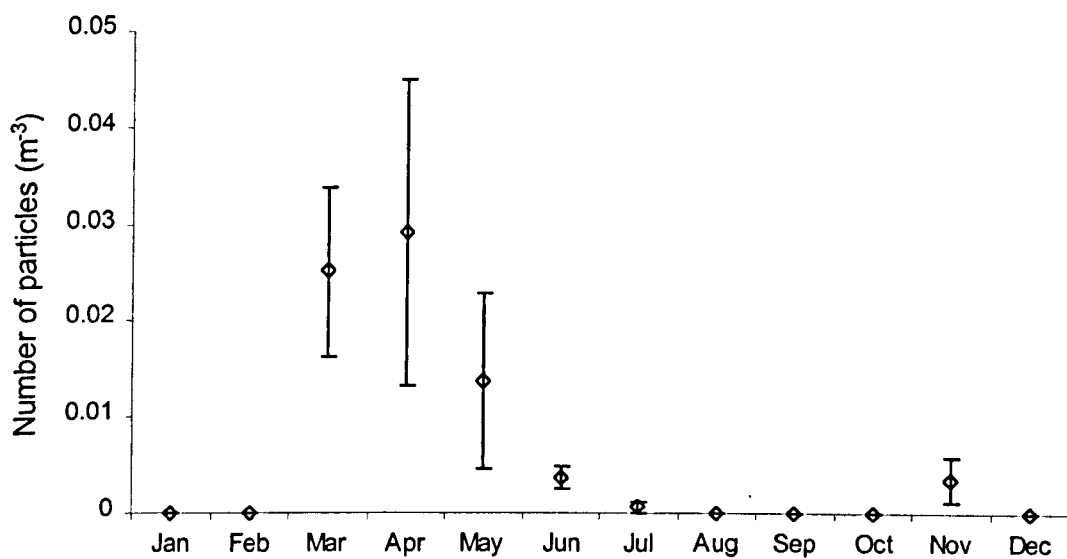


Figure 3.15. Abundance of spores of *Geomyces* sp. recorded at five sites around Davis Station during 2001.

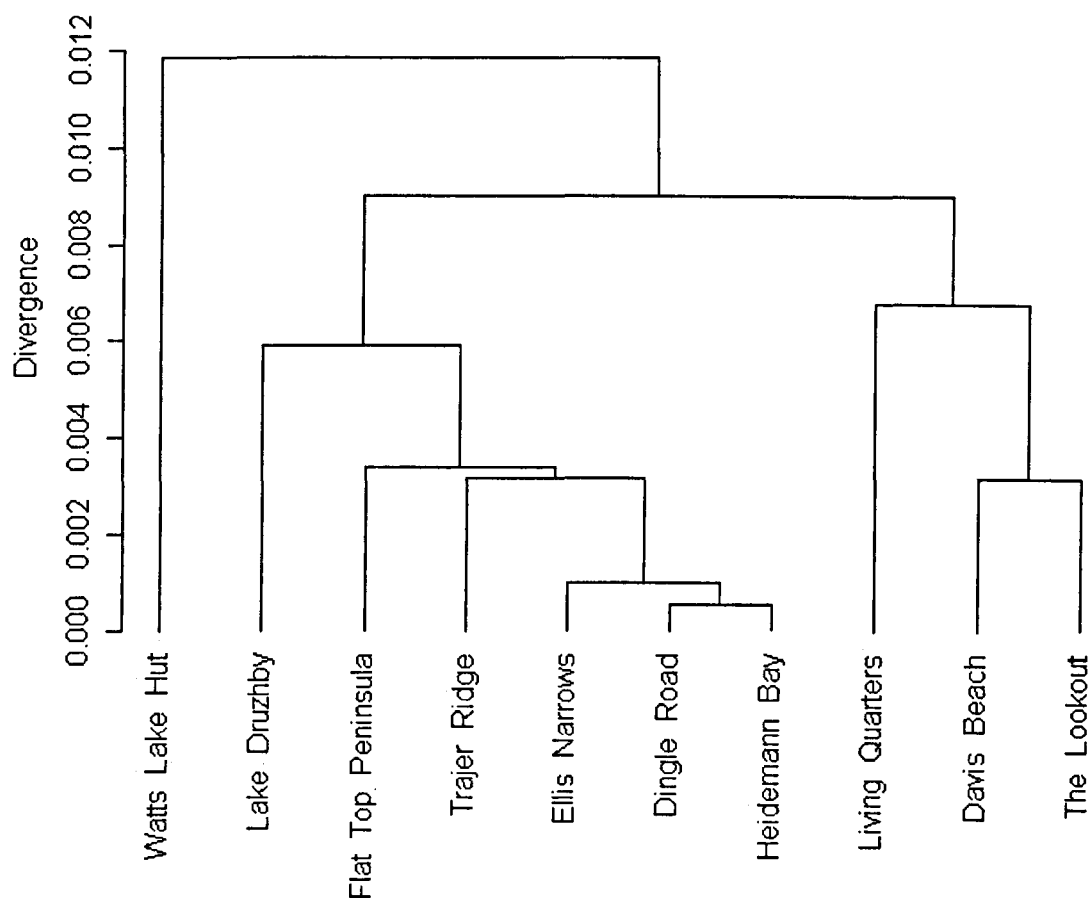


Figure 3.16. Similarity of sample sites based on the abundance and type of twelve particles of fungal or lichen origin collected by rotorod samplers at ten locations around Davis Station and the Vestfold Hills during 2001. Data were subjected to cluster analysis using the 'R' statistics package.

3.3.1.2. *Dinoflagellate* cysts

Dinoflagellate cysts (Plate 3.8) were the most prevalent particle encountered during the entire period of study and were observed at every sample location at some point during the study. Cysts were recorded throughout the year although there was a clear seasonal pattern (Figure 3.17). Although the number of cysts recorded was greatest at the eastern end of the Ellis Fjord (Figure 3.18 and 3.19), no significant relationship between particle density and distance along the transect was determined by either regression analysis or analysis of variance (Genstat 5). Linear Regression analysis did show a significant correlation ($P=0.018$) between temperature and the abundance of cysts recorded in the air samples.

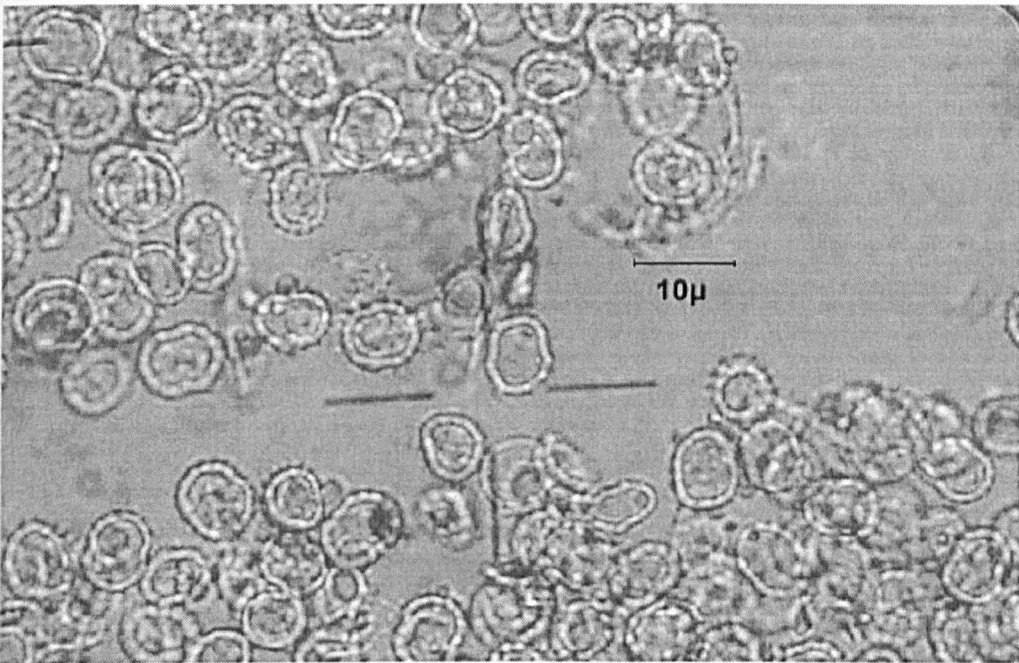


Plate 3.8. Dinoflagellate cysts collected on a rotorod strip and observed under transmitted light. Cysts were hyaline with thick walls and spines, typically ovoid with parallel sides or a median constriction.

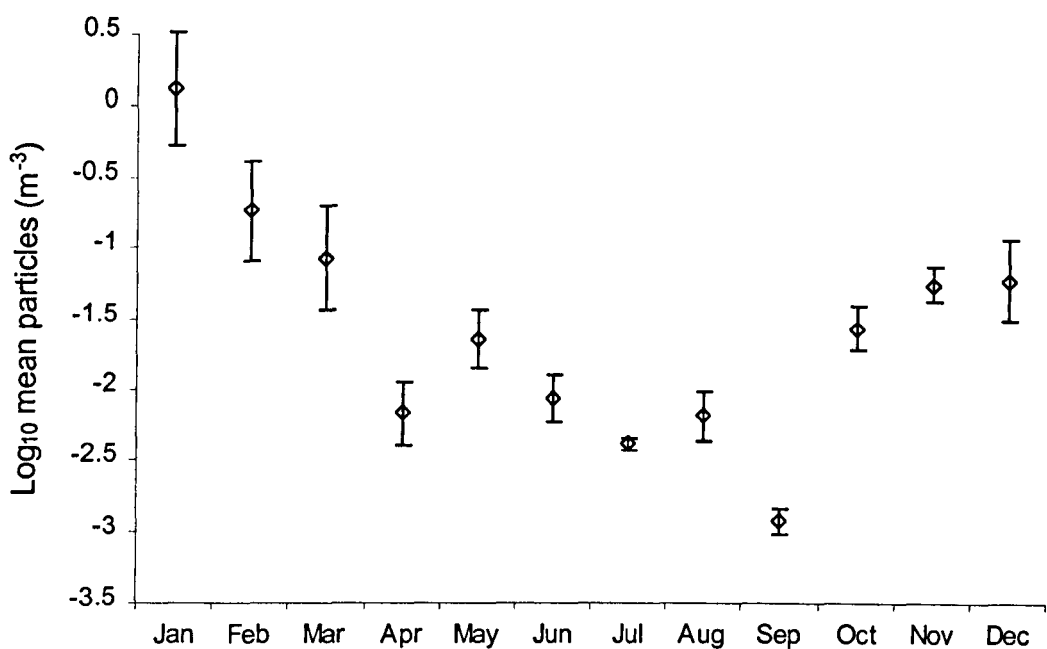


Figure 3.17. Abundance of dinoflagellate cysts recorded from 10 sites in the area of Davis Station during 2001 using rotorod air samplers.

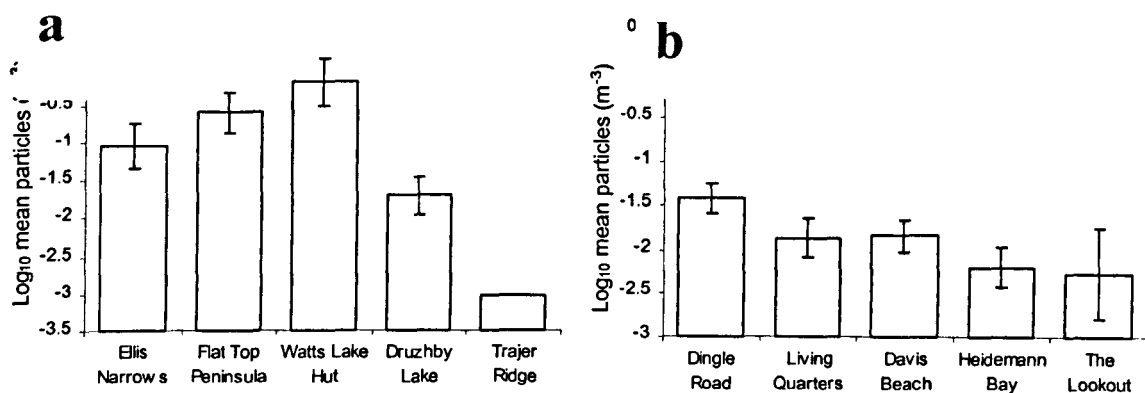


Figure 3.18. Spatial distribution of dinoflagellate cysts recorded by rotorod sampler at ten sites within the Vestfold Hills during 2001. a) Transect running through the Ellis Fjord east to west. b) Transect running through Davis Station north to south.

Although attempts were made to culture these cysts (using Bold’s Basal Medium and sterile brine) to enable species identification, this was unsuccessful. Dinoflagellates occur in many saline lakes in the Vestfold Hills (Laybourn-Parry *et al.*, 2002; Bell & Laybourn-Parry, 1999). They are also common in the Ellis Fjord (Grey *et al.*, 1997) and other fjords and marine basins (Laybourn-Parry, personal communication), as well as in the nearby marine environment (Grey *et al.*, 1997).

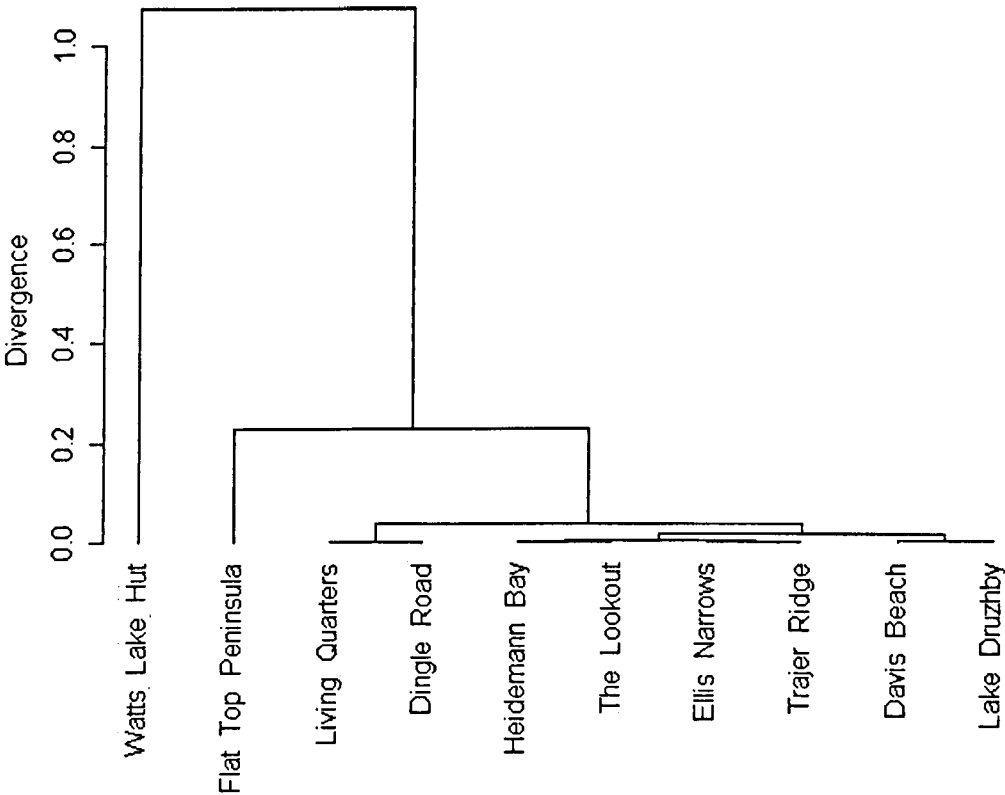


Figure 3.19. Similarity of sample sites based on the abundance of dinoflagellate cysts collected by rotorod samplers at ten locations around Davis Station and the Vestfold Hills during 2001.

Since high numbers were frequently recorded at the site near Watts Lake Hut, a transect was set up originating at the waters edge and running 100m directly inland and to the south. Although cysts were recorded, no pattern emerged from this study.

3.3.1.3. Moss fragments

Six species of moss and one liverwort have been identified as endemic to the Vestfold Hills (Seppelt 1984). In the current study, although several areas of moss growth were observed, all samples examined were found to be a single species, *Bryum algens* although it is accepted that wide variation within this genus makes accurate identification difficult. Fruiting in this species has only been observed on the Antarctic Peninsula (Filson & Willis, 1975) reproduction in the study area is limited to vegetative propagation through fragmentation (Plate 3.9).

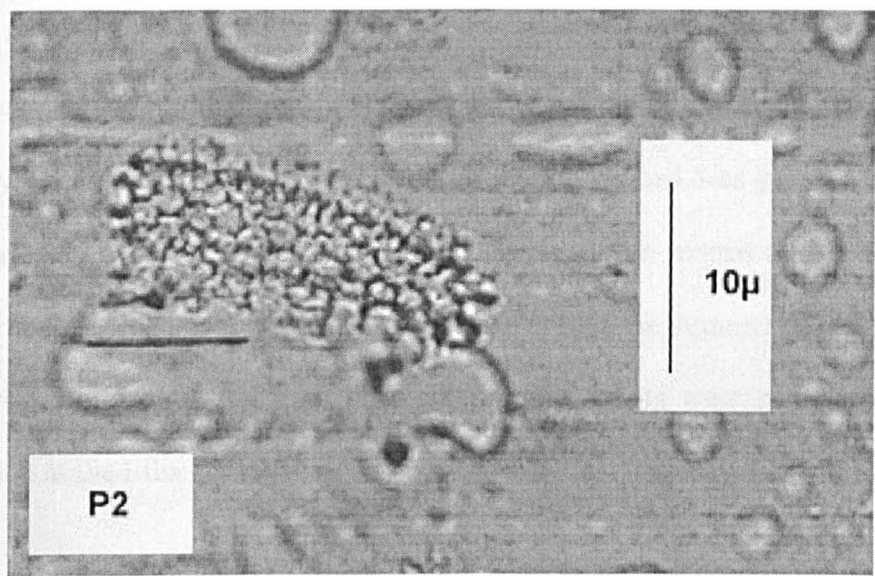


Plate 3.9. Vegetative fragment of moss tissue intercepted by rotorod air sampler in the Vestfold Hills during 2001.

The numbers of mosses encountered in the field increased easterly away from the station, with large and obvious patches occurring in the areas around Watts Lake Hut and Crooked Lake (adjacent to Druzby Lake). This was reflected in the physical distribution of fragments encountered throughout the year (Figure 3.20).

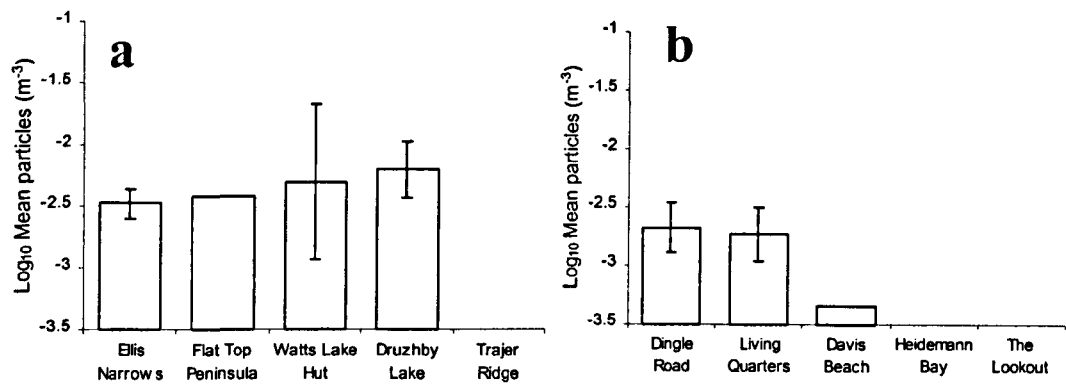


Figure 3.20. Spatial distribution of moss fragments intercepted by rotorods at ten sites at Davis Station and the Vestfold Hills during 2001. a) Transect running through the Ellis Fjord east to west. b) Transect running through Davis Station north to south.

Since mosses are typically found in sheltered areas, the major moss patches were permanently covered by snow during the Antarctic winter because drifting snow readily accumulated in these areas. A diffuse moss bed was present between large rocks at the Flat Top Peninsula site, less than ten metres to the west, the plants themselves however were only visible during the summer period. Since the prevailing wind was from the east, particle counts were similar to those recorded at the Ellis Narrows.

Moss fragments were also recorded in July (Dingle Road, Davis Station) and August (Ellis Narrows) but only as isolated fragments. The weather conditions

on both days were fine with low relative humidity (35.9% & 51.6% respectively).

3.3.1.4. Feather fragments

Feather fragments (Plate 3.10) were commonly found in rotorod samples throughout the year (Figure 3.21). With the exception of occasional wandering emperor penguins, no birds over-winter in the Vestfold Hills. During the summer months however, several large rookeries of adelic penguins are present on nearby islands and large numbers of adult birds periodically come ashore at Davis. By late summer, the chicks have lost their juvenile down and the adult birds are moulting; many do this on shore rather than on the islands. By the end of March, the last of the adelines have left the rookeries (Johnstone *et al.*, 1973) and returned to the open sea to overwinter. Feathers are slow to break down and so often accumulate in large deposits where the wind traps them. These deposits are usually buried by snow or at least immobilised by ice and so account for small numbers of particles found throughout the winter.

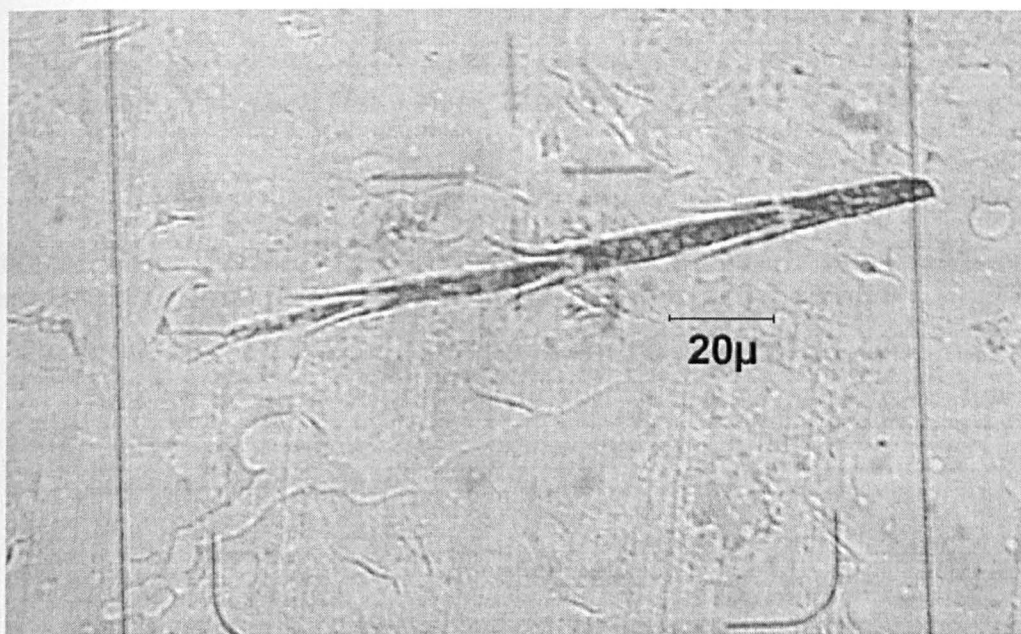


Plate 3.10. Fragment of pigmented feather (probably adelic penguin) collected by rotorod sampler at Davis Station.

Although visiting avian species included southern skuas (*Catheracta maccormicki*), giant petrels (*Macronectes giganteus*), storm petrels (*Oceanites oceanicus*) and snow petrels (*Pagodroma nivea*), it is likely that most of the feather fragments had originated from the penguin rookeries. The distribution of feather fragments both temporal and spatial, reflects the occurrence of the birds in the vicinity of the Vestfold Hills.

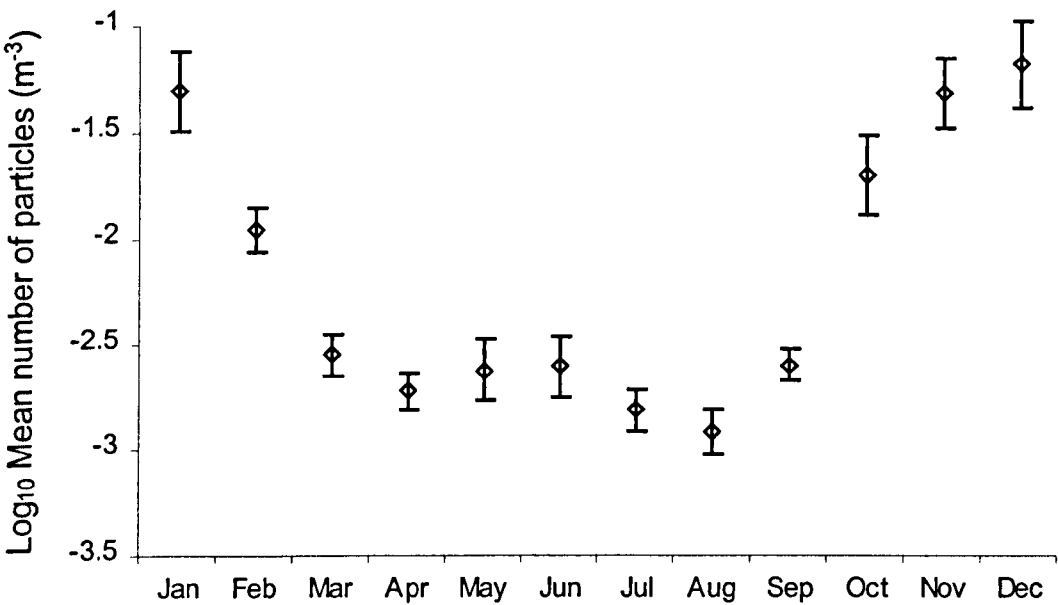


Figure 3.21. Temporal distribution of feather fragments intercepted by rotorod particle samples at ten sites at Davis Station and the Vestfold Hills.

3.3.1.5. Effects of annual resupply

Davis Station undergoes a major resupply at the beginning of each working year depending upon shipping times and is the period of greatest activity at the station and the largest number of personnel coming ashore. The resupply for 2002 took

place between December 1st and 4th 2001 during intermittent blizzard conditions. Rotorod samples were collected from the sites within Davis Station on November 28th and December 1st, 2nd and 3rd, blizzard conditions preventing sampling on the 4th. Although there was a significant increase ($P=0.01$) in the total numbers of biological particles recorded over this period (ten-fold), no corresponding increase in particles deemed to be of fungal origin was observed.

3.3.2. “Frisbee” settled particle collector

In practice, the dry, dusty environment coupled with strong winds made this sampler impractical for use at the majority of sample sites for much of the year. Although several samples were collected, the majority either contained too much wind-blown material or else were continually being blown over or blasted clean by strong winds. No reliable results were therefore obtained from this type of sampler.

3.4. DISCUSSION

Davies (1971) cited a number of studies testing the efficiency of the rotorod air sampler. He noted that rotorods had been found to be less efficient than slit samplers at low wind speeds, although they became more efficient than Cascade impactors at higher wind speeds. Since the mean airspeed recorded in the Vestfold Hills was 21.7 km hr^{-1} , it was appropriate that rotorods were used in favour of larger and more complex samplers. Davies (1971) also cited literature concerning the use of "Settle plates". Although standard Petri dishes were used rather than 25cm "Frisbees", he noted that settle plates were more suited to lower airspeeds, if they were not to become biased toward heavier particles. Indeed, in the current study, the use of settle plates proved inappropriate for the conditions, either becoming unstable on the rocky substrate in high winds, or else collecting large volumes of heavy wind-blown inorganic material.

Atmospheric conditions affect the microflora of the air. Sreeramulu & Ramalingam, (1969) showed a direct relationship between the aerial concentration of spores of a number of leaf-infecting fungi and changes in relative humidity and temperature. Such pronounced changes reflect the ability of fungi to undergo several sporulations in a short period of time and therefore increase the numbers of spores liberated to the air by several orders of magnitude. The Antarctic climate, although variable, does not show rapid variations in either relative humidity or temperature, limiting the degree to which sporulation can take place, so increases in spore numbers are correspondingly smaller than in warmer climates. Nevertheless, a relationship was observed

between the mean number of spores intercepted and temperature. Not only is elevated temperature required for the processes necessary to produce spores, but water is also frequently a limiting factor, being immobilised as ice during the winter and therefore biologically unavailable. Despite this, microhabitats exist where temperatures are raised above ambient, for example, mosses are strongly pigmented and many of the rocks supporting lichen growth are black dolerite. Adsorption of sunlight results in a local warming of these materials allowing water to exist in liquid form (Robinson, 2001). The presence of fauna such as elephant seals also provides a microclimate where temperatures are raised above ambient and liquid water may exist. A relationship was demonstrated between wind speed and the number of spores intercepted. It seems likely that this was the result of spores having been redistributed from settled material after having been released.

There were distinct differences in the types of fungal spore found in the Vestfold Hills and near Davis Station using rotorods. Several species of fungi do seem to have associations with mosses in Antarctica (Tosi *et al.*, 2002). Two species were seen associated with mosses in the Vestfold Hills during this study. One of these, *Phaerosphaeria*, was found associated with mosses sampled at the eastern end of the Ellis Fjord. Spores from this species were recorded at all the rotorod sites along the Ellis Fjord transect, including Lake Druzhby and Trajer Ridge, but not at the Ellis Narrows close to the seaward end of the fjord, where mosses are absent. Mosses are also absent close to Davis Station and *Phaerosphaeria* was not recorded by rotorods at Davis. Thus the evidence indicates that this species

is dispersed locally by the wind and that the process is influenced by meteorological conditions.

Close to the station, *Geomyces* was common. This species produces keratinase enzymes and has previously been shown to be associated with keratinaceous substrates in Antarctic soils (Marshall, 1998). Its abundance near the station appears to be associated with an elephant seal wallow, and possibly also material from several penguin rookeries on the islands close to Davis. Again, spores were probably being dispersed locally by the wind from several actively reproducing populations during summer. The deposition of seal skin and feathers close to Davis Station is cumulative, so the seasonal increase in *Geomyces* spores in summer is likely to be related to the availability of free water. The presence of the spores appeared to be linked with the presence of the elephant seals which huddle together in the wallows in order to conserve heat, providing a microhabitat of keratin substrate, elevated temperature and available water. During winter, all water is locked up in ice and is not biologically available. At this time, the fungi survive in a quiescent state, indeed the majority of fungi isolated during this study were capable of surviving desiccation (Chapter 6).

The total number of fungal spores recorded from around Davis Station, although greater than from sites immediately surrounding it (Dingle Road and Heidemann Bay), was similar to that recorded at the eastern part of the Ellis Fjord and Lake Druzhby. Whilst the nature of the particles was different between the two locations, the similarity in spore counts between the sites contrasts with the data obtained from soil-borne fungi (Chapter 4).

The high abundance of dinoflagellate cysts is particularly interesting. They are a group that readily forms cysts. In Ace Lake, one of the saline meromictic lakes in the Vestfold Hills, they use the cysts as a mechanism for overwintering (Bell & Laybourn-Parry, 1999). Although dinoflagellates are a common element in the plankton of the Ellis Fjord, the inshore marine environment and saline lakes ranging in salinity from around 4‰ to around 65‰ (Grey *et al.*, 1997; Perriss & Laybourn-Parry 1997; Bell & Laybourn-Parry 1999; Laybourn-Parry *et al.*, 2002), they do not occur in the more hypersaline lakes. For most of the year, the sea and the less saline lakes and fjords are ice covered. It is only the most saline lakes do not form ice covers and since these do not support dinoflagellate populations, they cannot contribute cysts into the air. It is possible that less saline lakes which support dinoflagellates (and lose their ice for 3-4 weeks in most years) may contribute spores to the air when the waters are subject to wind induced turbulence that produces spray. It is however, more likely that the main source of the cysts is from the sea, which is partially ice-free while the lakes are completely so, for two or more months in summer. Moreover, the sea is likely to generate more wind blown spray. Certainly dinoflagellate cysts were more abundant in rotorod samples during the summer months. They were present in smaller numbers during winter and may have simply been redistributed from ground settled material.

The large numbers of dinoflagellate cysts is particularly interesting from the aspect of lake colonisation. The lakes of the Vestfold Hills were formed by isostatic uplift after the last major glaciation some 10,000 years ago (Adamson &

Pickard, 1986). Pockets of seawater trapped in closed basins gave rise to the saline lakes that have undergone a complex evolution since their isolation from the sea. Whilst they would have contained marine derived communities that have undergone significant simplification through time (Laybourn-Parry *et al.*, 2002) it is likely that they have been repeatedly recolonised by airborne cysts, since dinoflagellates are a conspicuous feature of their communities.

CHAPTER 4: ISOLATION AND ENUMERATION OF FUNGI FROM ANTARCTIC SOIL

4.1. INTRODUCTION

Antarctica is the coldest, driest continent on planet Earth. Effectively, much of it is a polar desert. For most of the year, there is no free water. Only in summer is there thaw and ice melt that produces liquid water to support terrestrial life. Consequently, terrestrial organisms in Antarctica have a short window of opportunity for growth, while for the rest of the year they survive in a quiescent state (e.g. Arora, 1991; Jennings, 1993; Dix & Webster, 1995). In the terrestrial environment, which in ice free areas is bare rock and a sparse soil, daily temperature ranges may be high (Walton, 1982).

Fungal propagules have reached Antarctica in the air (Marshall, 1996) and successfully colonised an extreme environment, however, around stations, man has also introduced fungal propagules (Azmi & Seppelt, 1998), some of which may have become established. The current study was conducted to ascertain the endemic fungal communities in the Vestfold Hills, as well as human impacts in terms of 'exotic' fungal colonisation around stations.

Antarctic soils support a number of fungal communities (Vincent, 1988) and isolation of fungi from soils has been achieved by many researchers (e.g. Fletcher *et al.*, 1985; Azmi & Seppelt, 1998). The Antarctic environment also

supports a number of fungal communities on a range of other natural substrates (Tubaki & Asano, 1965; Del Frate & Caretta, 1990). In the current study, fungi were isolated from soil taken from each of the rotorod sites as part of the airborne particle sampling study (Chapter 3) as well as from 20 sites around Davis Station. A number of miscellaneous sources including several natural materials, building materials and food were also examined on an ad-hoc basis.

The aim of the investigation was to determine what fungi were present in soils and other materials at Davis Station and the Vestfold Hills and record changes in populations and distribution throughout the year.

4.2. MATERIALS & METHODS

Two transects were established as part of the airborne particle sampling experiment (Chapter 3). Transect 1 initially comprised three sites extending from the north east of the station to the beach. The transect was extended 2 km to the south with two new sites in May 2001 as additional samplers were installed for airborne particle monitoring (Table 4.1).

Table 4.1. Locations of transect points running through Davis Station (Transect 1)

Transect Site	Name	Description
1	Dingle Road	80-100m north east of the station, site set back from an access road infrequently used by vehicles and pedestrians.
2	Living Quarters	Around 10m from the main entrance to the living quarters, adjacent to a roadway in constant use by people and vehicles.
3	Davis Beach	20-25m from the shoreline in a sheltered area. Infrequently visited by pedestrians but around 10m from an area occupied by southern elephant seals during the summer period.
4	Heidemann Bay	1km due south of the station, on the coast infrequently visited by pedestrians
5	The Lookout	A raised knoll about 500m east of a hill known as “The Lookout”, 3km south of the station. The area is occasionally visited by walkers although it is unlikely that the site itself would be approached.

Transect 2, initially of three sites, extended the length of the Ellis Fjord from The Narrows at the western (seaward) end to the eastern end (near Watts Lake Hut). The transect was extended to the east by two additional sites, in January and June 2001 respectively (Table 4.2). Transects 1 and 2 were approximately 1.5km apart (Figure 3.4).

Table 4.2. Locations of transect points running through Ellis Fjord (Transect 2)

Transect Site	Name	Description
1	Ellis Narrows	The western (seaward) end of Ellis Fjord on a piece of land exposed to the catabatic winds which blow along the fjord from the plateau. The area is frequently passed by vehicles, however walkers normally follow different routes.
2	Flat Top Peninsula	The tip of a narrow peninsula extending into the Ellis Fjord near Flat Top Island. The area is rarely approached by vehicles or walkers.
3	Watts Lake Hut	A rocky shoreline on the Ellis Fjord some 30m from Watts Lake field hut. Although the hut is frequently occupied, the shore is not on a direct walking route.
4	Lake Druzhby	The northern shore of Lake Druzhby (a freshwater lake between the polar plateau and the Ellis Fjord). Although the lake is frequently used as an access way, the site itself was not on a direct route.
5	Trajer Ridge	A rocky promontary in a narrow valley at the western tip of a snowy ridge extending to the polar plateau. The route was frequently used as an access, the raised nature of the site itself however reduced the likelihood of human interference.

An additional set of twenty sites was established around Davis station (Figure 4.1). These included areas which were either likely to receive a great deal of activity throughout the year (such as the living quarters), or only during the summer periods (such as the summer ablutions block) or very little activity (such as the wharf).

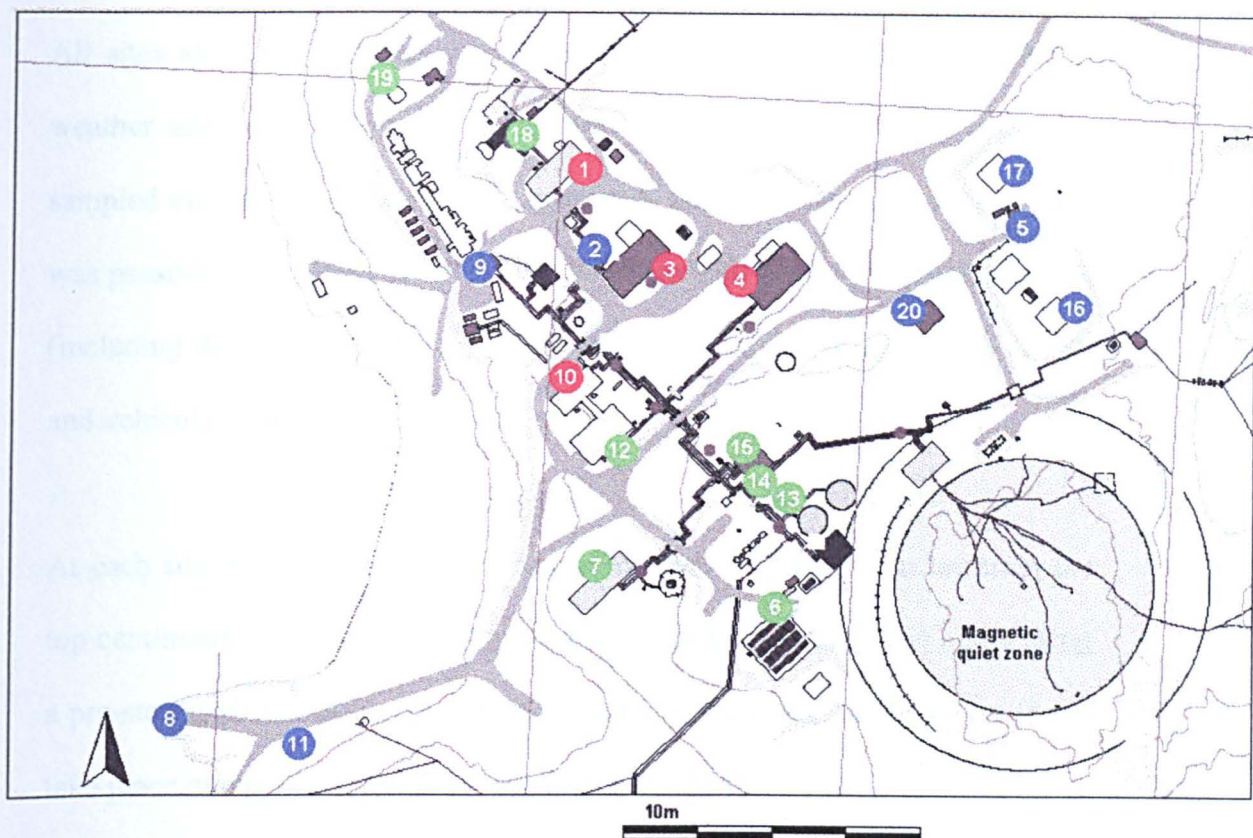


Figure 4.1. Twenty locations set up around Davis Station for monthly soil sampling. Each site has been categorised according to the estimated human impact during the course of the winter period. Red – high impact, Green - medium impact and blue – low impact. Map courtesy of the Australian Antarctic Division.

High Impact – Visited regularly by the majority of personnel

- 1 Science building - south entrance
- 3 Main store - south entrance
- 4 Workshops - steps to loading platform
- 10 Living Quarters - main entrance

Medium Impact – Visited regularly by a few personnel

- 6 Fuel farm
- 7 Operations building - main entrance
- 12 Sleeping/Medical quarters - south west entrance
- 13 Hydroponics - base of steps
- 14 Services building - south entrance
- 15 Services building - north entrance
- 18 Meteorology - main entrance
- 19 Field store - north entrance

Low Impact – Rarely visited by personnel

- 2 Main store - north entrance (under ducting)
- 5 Helipad - steps to office
- 8 Wharf edge
- 9 Summer ablutions – base of steps
- 11 Wharf (drum store for return to mainland)
- 16 Helipad - southern pad
- 17 Auxiliary helipad
- 20 Emergency vehicle store - main entrance

All sites along the transects were sampled fortnightly throughout the year as weather and access permitted. The twenty sites around Davis Station were sampled on a monthly basis, when snow was absent and access to the ground was possible. Additional samples were taken from all sites around Davis Station (including those forming part of the transect) at times of resupply when human and vehicular activity were greatest.

At each site, a composite sample of approximately 25g was collected from the top centimetre of soil from an area ca. 20cm² into a sterile universal bottle using a pre-sterilised stainless steel spatula. In cases where the examination could not take place immediately, samples were frozen at -20°C.

Two techniques were used during the investigation, spread plates (Martin, 1950; Barron, 1971) and hair bait (Orr, 1969) the latter to isolate keratinophylic forms. Potato dextrose agar (PDA, Oxoid) and Czapek-Dox agar (CZA, Oxoid) were used for the spread plates amended with a mixture of penicillin and streptomycin at 25mg L⁻¹ and 40mg L⁻¹ respectively (Chapter 2).

4.2.1. Surface spread agar plate technique

PDA and CZA were made up to full strength according to manufacturer's instructions (after preliminary investigations showed no significant differences in the number of CFUs observed between full strength media and 10⁻¹ dilution). A stock solution of antibiotics was made up using 500mg penicillin (7.98 x10⁵ units) and 800mg streptomycin (6.12 x10⁵ units) in 100ml sterile water. A 5ml

aliquot was filter sterilised (0.2 μ m) and added to 1 litre of warm agar to achieve a final concentration of 25mg L⁻¹ penicillin and 40mg L⁻¹ streptomycin.

A dilution of 0.01g L⁻¹ soil was found to provided the most countable numbers of fungal colonies during preliminary investigations, so suspensions were made up using 0.25g soil in 24.75mL sterile water and shaken vigorously by hand for around 15 seconds. Aliquots of 0.05ml were pipetted onto the surface of both agar types in triplicate and evenly spread over the surface of the agar using a sterile glass rod. Incubation was at ambient room temperature (ca 22°C) for five to ten days. Colonies were counted periodically and the numbers of each type recorded based on colony morphology. Diversity was determined using Simpson's Diversity Index (Krebs, 1985). Each morphologically distinct type was isolated into axenic culture, transferred to an agar slope and submerged under paraffin oil allowing preservation for subsequent genetic investigation (Onions, 1971).

4.2.2. Hair bait technique

Human hair was used throughout the investigation since horse hair (Orr, 1969) was unavailable. Unaltered (i.e. not dyed or bleached) dark hair cuttings (around 2-3cm) were thoroughly washed in detergent and rinsed in deionised water before being autoclaved and oven dried at 60°C in foil packages. The hair was then evenly scattered onto sterile filter paper in a Petri dish. Around 0.5g soil from each site was sprinkled onto the hair and moistened with approximately 2ml sterile water containing 25mg L⁻¹ penicillin and 40mg L⁻¹ streptomycin.

The plates were incubated at ambient room temperature (ca. 22°C) for 21 days and re-moistened with sterile water (containing antibiotics) as necessary. Since this is not a quantitative method, fungal growth was observed by eye or with the aid of a hand lens and recorded using a subjective scale of 0 (absent) to 3 (greatest cover). Fungi obtained in this way were isolated into pure culture using Sabouraud's agar (Del Frate & Caretta 1990) and transferred to agar slopes and stored under paraffin oil (Onions, 1971).

Fungi are important decomposers and play a role in the breakdown of vegetable material, animal bodies and excreta. They are likely to be present in imported materials either having been introduced themselves or opportunistically utilising the imported material. It is possible therefore that these substrates are providing inocula for the fungi found elsewhere in the Vestfold Hills. In order to examine this hypothesis, several miscellaneous samples were collected from a variety of natural and human influenced locations and examined for the presence of fungi.

Samples were placed either directly onto agar or into moist chambers i.e. Petri dishes containing sterile filter paper moistened with sterile water containing 25mg L⁻¹ penicillin and 40mg L⁻¹ streptomycin (Del Frate & Caretta, 1990). Incubation was at ambient room temperature (ca. 22°C) for up to 21 days and re-moistened as required. Examination was under low-power (x10) stereo microscope and fungi, where present, recorded using an arbitrary scale between 0 and 3.

Samples examined by moist chamber:

- Feathers & faeces from adelic penguins (*Pygoscelis adeliae*)

Samples were collected from Gardner Island penguin rookery during the early part of the winter after the adult birds had moulted and returned to the ocean.

- Skin and hair from moulting southern elephant seals (*Mirounga leonina*)

Elephant seals habitually come ashore at Davis beach during the summer in order to moult. Shed skin is plentiful and was collected directly from the beach.

- Marine macro algae

Several species of macro alga are deposited along Davis beach including red, brown and green genera. Red thalloid algae are the most common forms and frequently build up in deposits along the strand line during the summer. Sections of thalli approximately 2-3 cm were collected.

- Moss (*Bryum algens*)

Although mosses are slow-growing and infrequent in the Vestfold Hills several individual shoots were excised from cushions growing near Watts Lake Hut.

Samples examined by direct plating onto agar:

- Swabs from feet of south polar skua (*Catharacta maccormicki*)

Skuas are scavengers and cover a wide range and are therefore potential vectors for a variety of organisms. The foot swabs were collected during the summer in

collaboration with Gary Miller, University of New Mexico; ASAC project 1336, South polar skuas as vectors of disease.

- Spoiled fruit and vegetables

Several types of fruit and vegetables are refrigerated at Davis station including apples, pears, grapefruit, potatoes and onions. Inevitably, a proportion of these succumb to fungal infection and so are destroyed by incineration. Samples were collected on an ad-hoc basis as infected fruit and vegetables were found.

- Decaying vegetable matter from station hydroponics facility

The station hydroponics facility is maintained around 26°C and is very humid. A variety of produce is grown including cucumbers, lettuce and tomatoes. As with stored produce, fungal infection is inevitable. Samples of infected tissues were collected from tomato and lettuce plants on an ad-hoc basis as infections occurred.

- Dust collected from ventilation system of living quarters

Domestic dust consists of a high proportion of human skin cells and therefore usually contains a range of microorganisms associated with this. During 2001, repainting and recabling operations took place, during which time a great deal of disturbance occurred within the Living Quarters. Dust was sampled from the cavity walls and also from the ventilation system using a sterile spatula and sterile vials.

- Saline lake sediment (Ace Lake & Organic Lake)

Lakes provide a large surface area for settled particles which accumulate in sediments (Ellis-Evans, 1985). Although saline lakes are non-ideal candidates for preservation of fungal spores due to their osmotic potential, an opportunity arose to collect sediment samples in collaboration with Marco Coolen, University of Utrecht; ASAC project 1166, Interaction between carbon and sulphur cycles in Antarctic stratified lakes and fjords biology.

Soils were also collected from neighbouring stations in the Larseman Hills to the south of Davis, Law Base (Australian), Progress II (Russian) and Zhong-Shan (Chinese). Since visits to these stations were infrequent and only during the summer, soils were collected on a one-off basis from areas likely to have high human activity and examined in the same way as soils from Davis Station.

4.2.3 Data Analysis

Colonies were counted by eye and mean values expressed as colony forming units per gram dry soil. Data were \log_{10} transformed to normalise them in order to satisfy the requirements of ANOVA. A two way ANOVA (site x growth medium) was performed on data collected throughout the year to test the significance between sites. Data for each month were pooled and used as replicates in order to reduce the large numbers of zero values which would otherwise invalidate the analysis. Fungal diversity was determined by counting

the numbers of each species (based on colony morphology) isolated and using Simpson's diversity index¹ (SI) to obtain a value between 0 and 1.

¹ Appendix 1

4.3. RESULTS

Although not all samples produced culturable fungi, isolates were obtained from all soil sampling sites at various times throughout the year.

4.3.1. Davis Station Transect

The numbers of colony forming units isolated from the sites along the transect at Davis Station are shown in Figure 4.2. The numbers of fungal CFUs isolated was highly variable both in terms of sample site but also time of year. Of the five sites comprising the transect, the three sites within Davis Station (Dingle Road, Living Quarters & the Beach), particularly the latter two, which were subject to the greatest human activity.

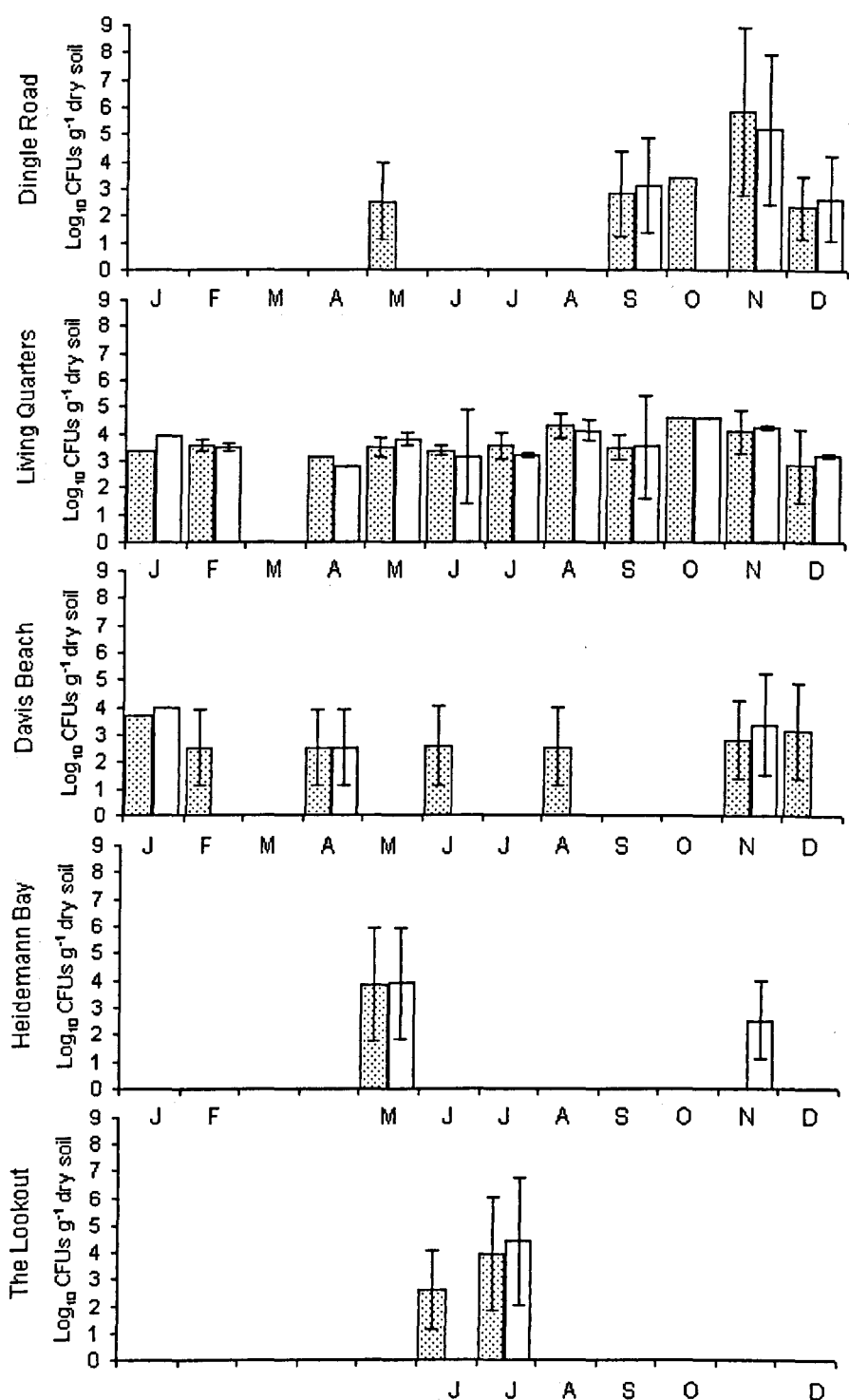


Figure 4.2. Mean fungal colony forming units (\pm standard error) isolated from soils collected in 2001 from a transect passing through Davis Station. Shading represents colonies isolated on PDA, clear bars represent colonies isolated on CZA. Months omitted from the x-axis represent periods when no samples were collected.

The site at Dingle Road was predominantly upwind of the station and infrequently used by people or vehicles. No fungi were isolated from this location during the latter part of the Antarctic summer, the single colony isolated in May almost certainly being artefactual. Furthermore, no fungi were recorded from this site during the winter period, despite the ground lacking snow cover and therefore exposed to particle settlement. Frequency of fungal isolations did, however, increase with the onset of summer. The increase in colonies isolated during November was due in part to a pink yeast (identified as CBS 8923 by a 99% match with a record contained within the BLAST database, Chapter 5).

The greatest number of fungal CFUs isolated over the year were from the site immediately opposite the main entrance to the living quarters which, with the exception of May, yielded fungal isolates on every sampling occasion throughout the year. Fungal diversity was also greatest at this site (Figure 4.3) especially when compared with the sites at Dingle Road, where diversity was only recorded during September and November ($SI=0.22$) and at Davis Beach during January ($SI=0.05$).

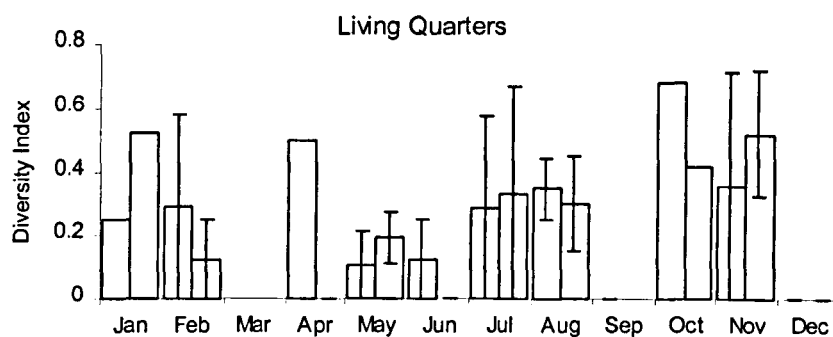


Figure 4.3. Changes in fungal diversity measured using Simpson's Diversity Index (\pm standard error) over a twelve-month period recorded outside the Living Quarters at Davis Station. Shading represents colonies isolated using PDA, clear bars represent colonies isolated using CZA.

The sampling sites at Heidemann Bay and The Lookout were established in May (Winter) and consequently no data were recorded for the end of the summer period. Fungi were far less frequently isolated from these sites compared with those from within Davis Station during the same period of time, although fungi were isolated from both sites during the winter period with only a single colony being isolated during the summer from Heidemann Bay.

Hair baited samples yielded fungi from all three sites within Davis Station, showing no decrease throughout the winter period (Table 4.3). Although the beach was frequented by moulting southern elephant seals (*Mirounga leonina*) during March and April, no corresponding increase in keratinophiles was observed using hair bait.

Table 4.3. The abundance of fungi isolated from the transect running through Davis Station during 2001 using the hair bait technique. Since colonisation was not in the form of discrete colonies, a scale between 0 and 3 was adopted.

	Dingle Road	Living Quarters	Davis Beach	Heidemann Bay	The Lookout
Jan	0	0	2	-	-
Feb	1	1.5	2	-	-
Mar	0	2	1.5	-	-
Apr	0	1	1	-	-
May	0	2	0	0.5	0
Jun	3	3	3	0.5	0
Jul	1.5	2	1	0	0
Aug	2.5	2.5	0	0	0
Sep	1.5	1.5	1.5	0	0
Oct	1.5	1	1	-	0
Nov	3	1.5	1.5	2	0
Dec	2.3	1	1.5	0	0

4.3.2. Ellis Fjord Transect

All sites on the Ellis Fjord transect yielded fungi although less frequently than those at Davis Station (Figure 4.4).

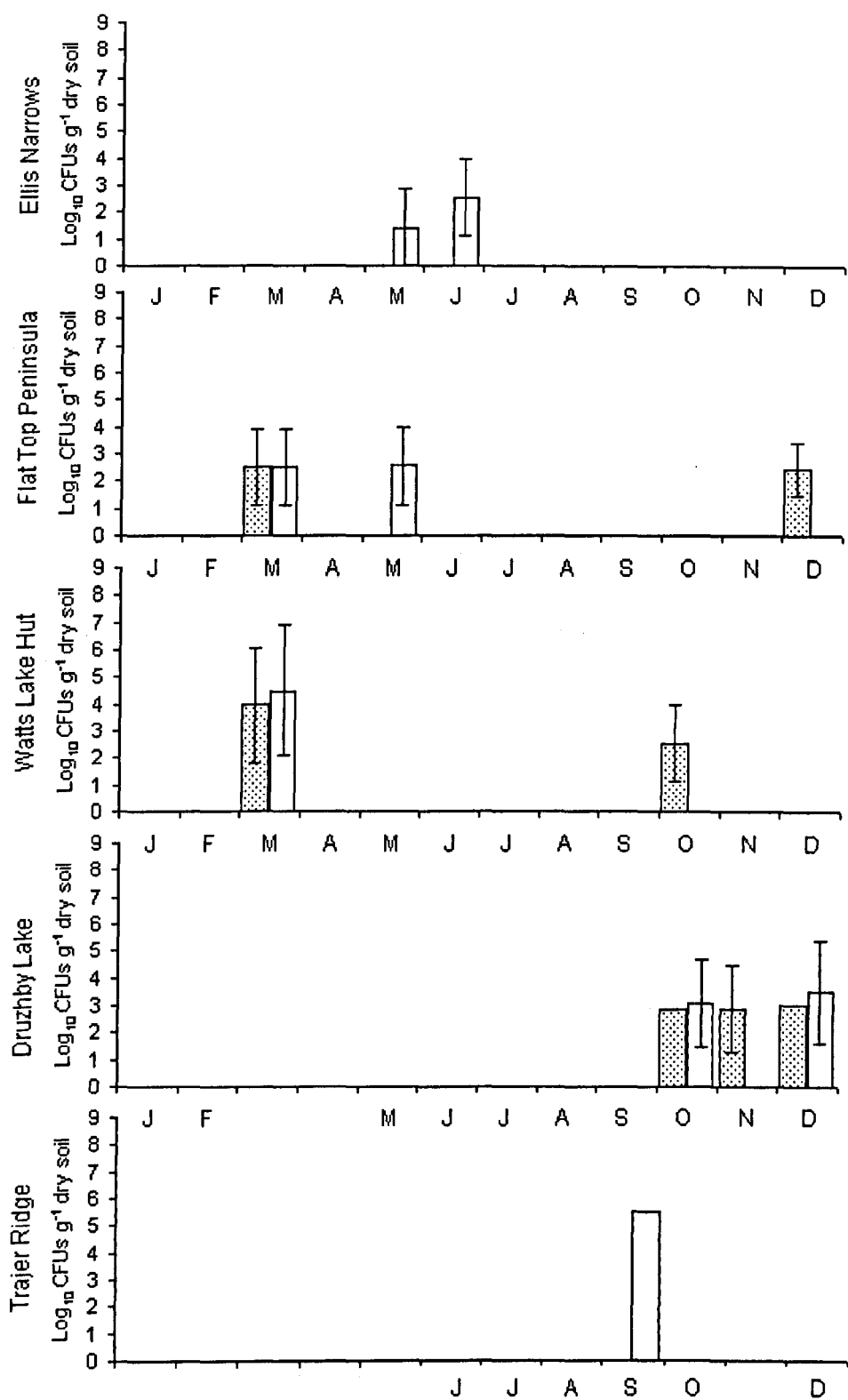


Figure 4.4. Mean colony forming units isolated from soils collected in 2001 from a transect passing through the Ellis Fjord. Shading represents colonies isolated on PDA, clear bars represent colonies isolated on CZA. Months omitted from the x-axis represent periods when no samples were collected.

The site at the Ellis Narrows is 1.5 km south of The Lookout (the southernmost site of the Davis Station transect). Fungi were only isolated from this site during the winter period. Fungi were also isolated during the winter at Flat Top Peninsula, however, colonies were also isolated during the mid and late summer periods, in keeping with those isolated from the Watts Lake Hut site.

Fungi were recorded at the Lake Druzhby site consistently during the early and mid summer period although no samples were collected during March and April due to problems gaining access to the site at that time of year. The site at Trajer Ridge was set up in June (mid winter) and so no data were available for the end of the summer period, however *Penicillium* sp. was observed on one occasion in September.

Since single colonies were isolated from the Ellis Narrows and Flat Top Peninsula, there was no species diversity. Although several colonies were isolated from Watts Lake Hut they were of a single species and so no diversity could be calculated. Three colonies were isolated from the Druzhby Lake site during December however, comprising two species, giving a diversity index of 0.25 using Simpson's index.

A significantly greater number of fungal CFUs ($P < 0.001$) was isolated from the site outside the Living Quarters over the study period on both PDA and CZA. Davis Beach was the next most prolific site over the 12 month period (Figure 4.5).

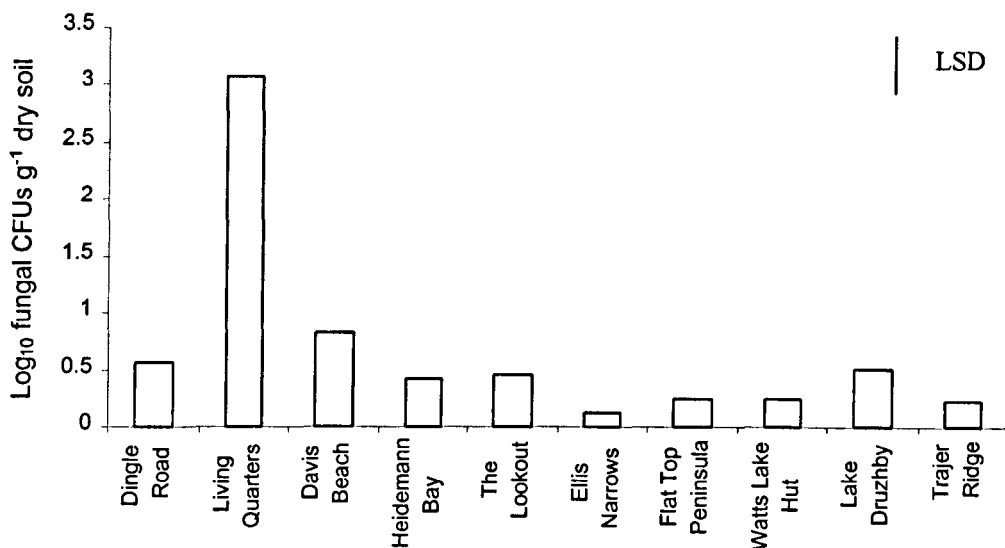


Figure 4.5. The mean numbers of fungal CFUs recorded at all transect points throughout the year of 2001. Site was a significant single factor ($P<0.001$) in ANOVA.

Fungi were isolated using the hair bait technique predominantly during the summer periods (Table 4.4). A notable exception to this being Lake Druzhby where keratinophiles were isolated between May and August. Keratinophiles were also isolated from Flat Top Peninsula and Watts Lake Hut during August.

Table 4.4. The abundance of fungi isolated from the transect running through the Ellis Fjord during 2001 using the hair bait technique. Since colonisation was not in the form of discrete colonies, an arbitrary scale between 0 and 3 was adopted.

	Ellis Narrows	Flat Top Peninsula	Watts Lake Hut	Druzhby Lake	Trajer Ridge
Jan	0	2	0.5	0.5	-
Feb	0.5	0	0	0	-
Mar	0	0.5	0	-	-
Apr	0	0	0	-	-
May	0	0	0	0.5	-
Jun	0	0	0	0.5	0
Jul	0	0	0	2	0
Aug	0	2	0.5	1	0
Sep	0	0	0	0	0
Oct	0.5	0.5	1	0	0
Nov	0	0.5	0	0	-
Dec	0	0	0	0	0

4.3.3. Fixed sample sites within Davis Station

Fungi were isolated from all twenty fixed points at various stages throughout the year (Figure 4.6 to 4.9), however, several areas were identified as yielding higher numbers of CFUs and higher diversity. The greatest number of colonies consistently obtained was from the site outside the main entrance to the living quarters (Site 10), although this did not correspond with the highest diversity. Sites with high numbers of CFUs and high diversity were the main entrance to the science building (Site 1), the south entrance to the main stores building (Site 3), the entrance to the workshops (Site 4), the summer ablutions building (Site 9) and the south entrance to the services building (Site 14). Although only used during the summer months, the ablutions building provided both high numbers of CFUs and high diversity when sampled during the winter.

Amongst the lowest numbers of CFUs (and the lowest diversity) were the extreme edges of the station, such as the two sites at the wharf, the helipads and the entrances to the Operations and Meteorological buildings.

Annual station resupplies took place in October 2000 and December 2001. During these periods, some changes in number of CFUs and diversity were noted, however these were not pronounced. An increase in fungal CFUs was recorded at the cargo store located at the wharf (Site 11), where a number of sea containers were removed prior to return to Australia. An increase in fungal CFUs was also recorded at the main store where supplies are unloaded from sea containers. This increase was however, accompanied by a fall in diversity, indicating an increase of a small number of species.

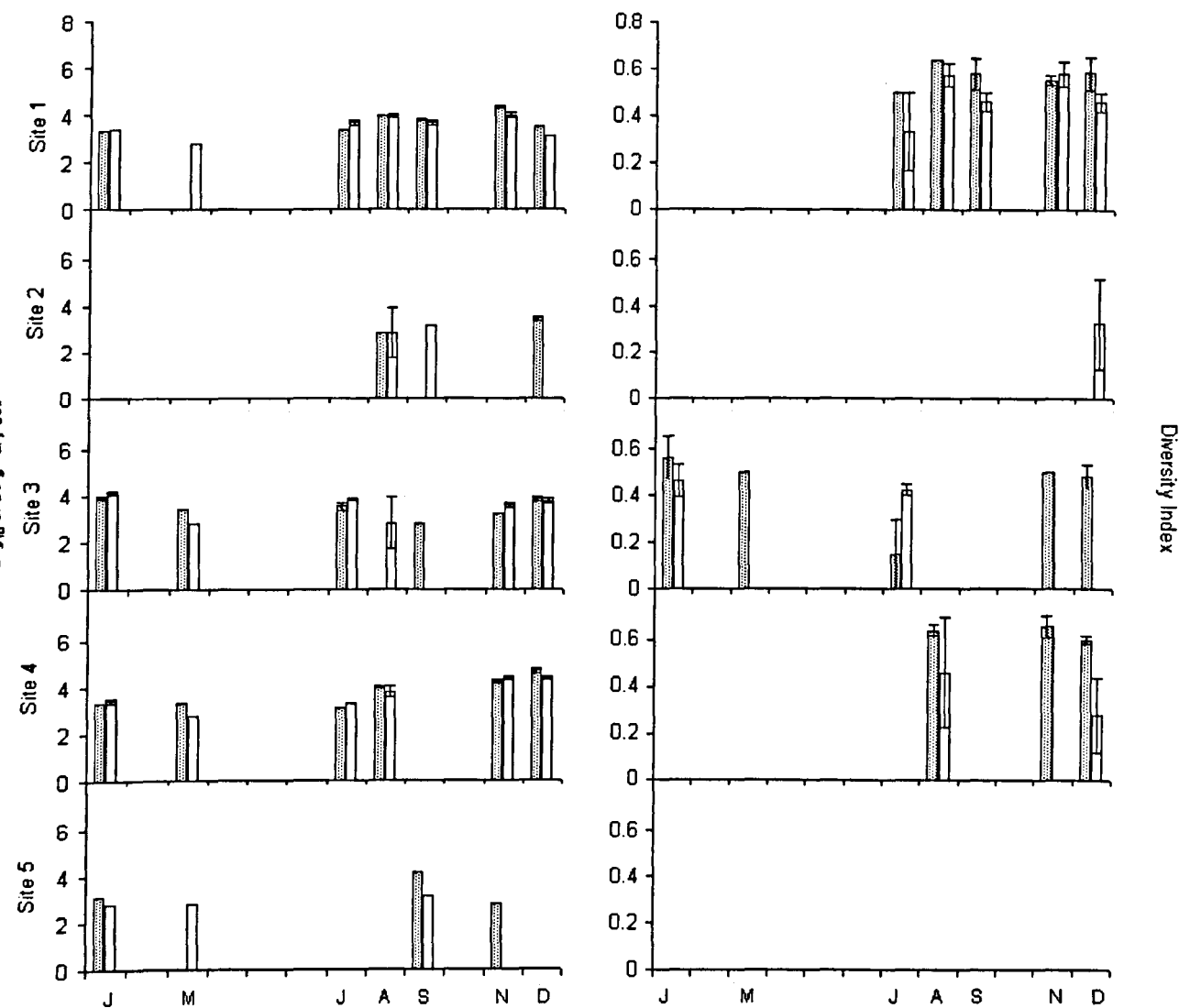


Figure 4.6. Fungal CFUs and diversities (\pm standard error) for soils sampled from five sites at Davis Station recorded during 2001 on seven months as conditions allowed. Shading represents colonies isolated using PDA, clear bars represent colonies isolated using CZA.

- Site 1. Science building - south entrance**
- Site 2. Main store - north entrance (under ducting)**
- Site 3. Main store - south entrance**
- Site 4. Workshops - steps to loading platform**
- Site 5. Helipad - steps to office**

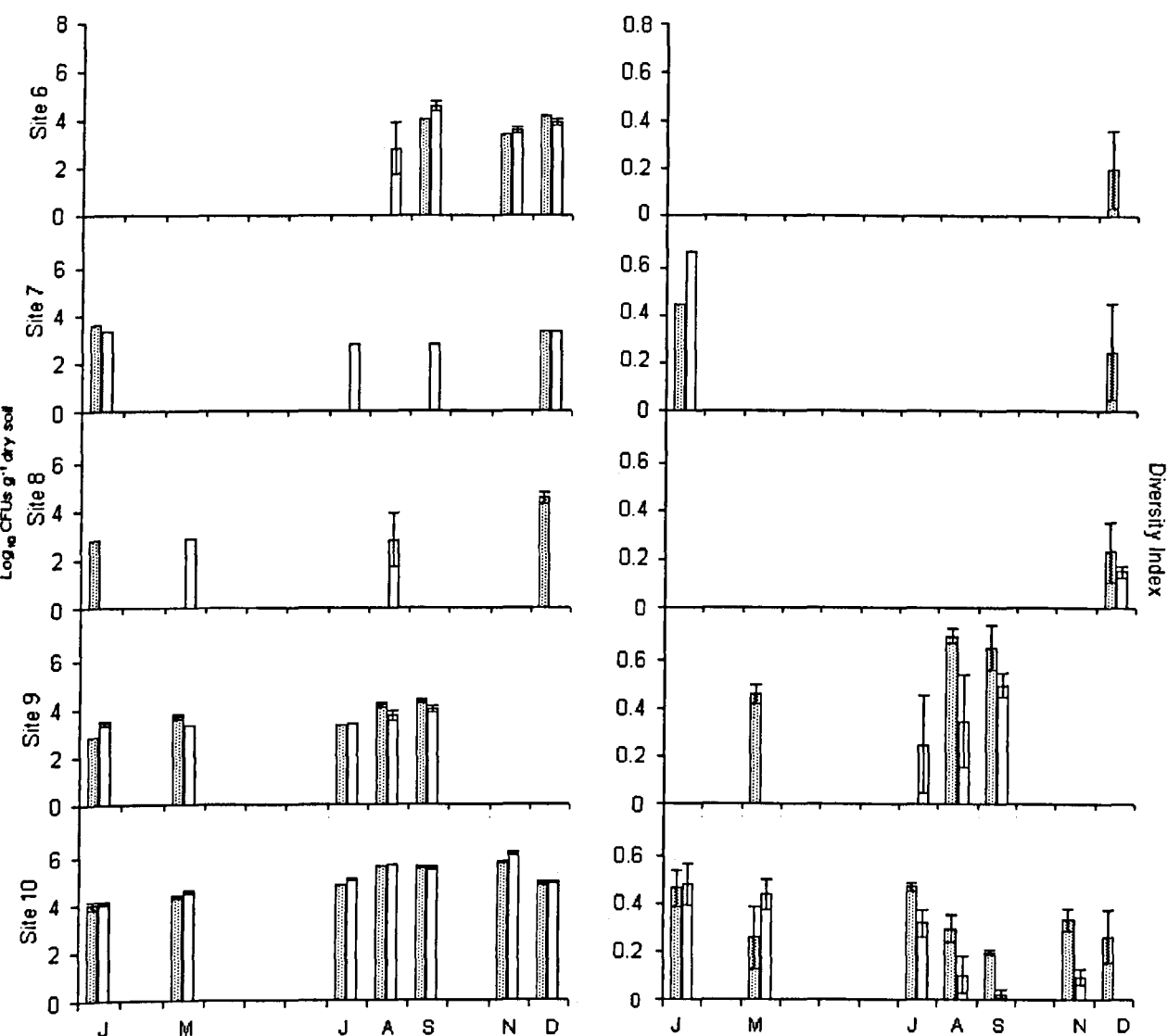


Figure 4.7. Fungal CFUs and diversities (\pm standard error) for soils sampled from five sites at Davis Station recorded during 2001 on seven months as conditions allowed. Shading represents colonies isolated using PDA, clear bars represent colonies isolated using CZA.

Site 6. Fuel farm
 Site 7. Operations building - main entrance
 Site 8. Wharf edge
 Site 9. Summer ablutions - base of steps
 Site 10. Living Quarters - main entrance

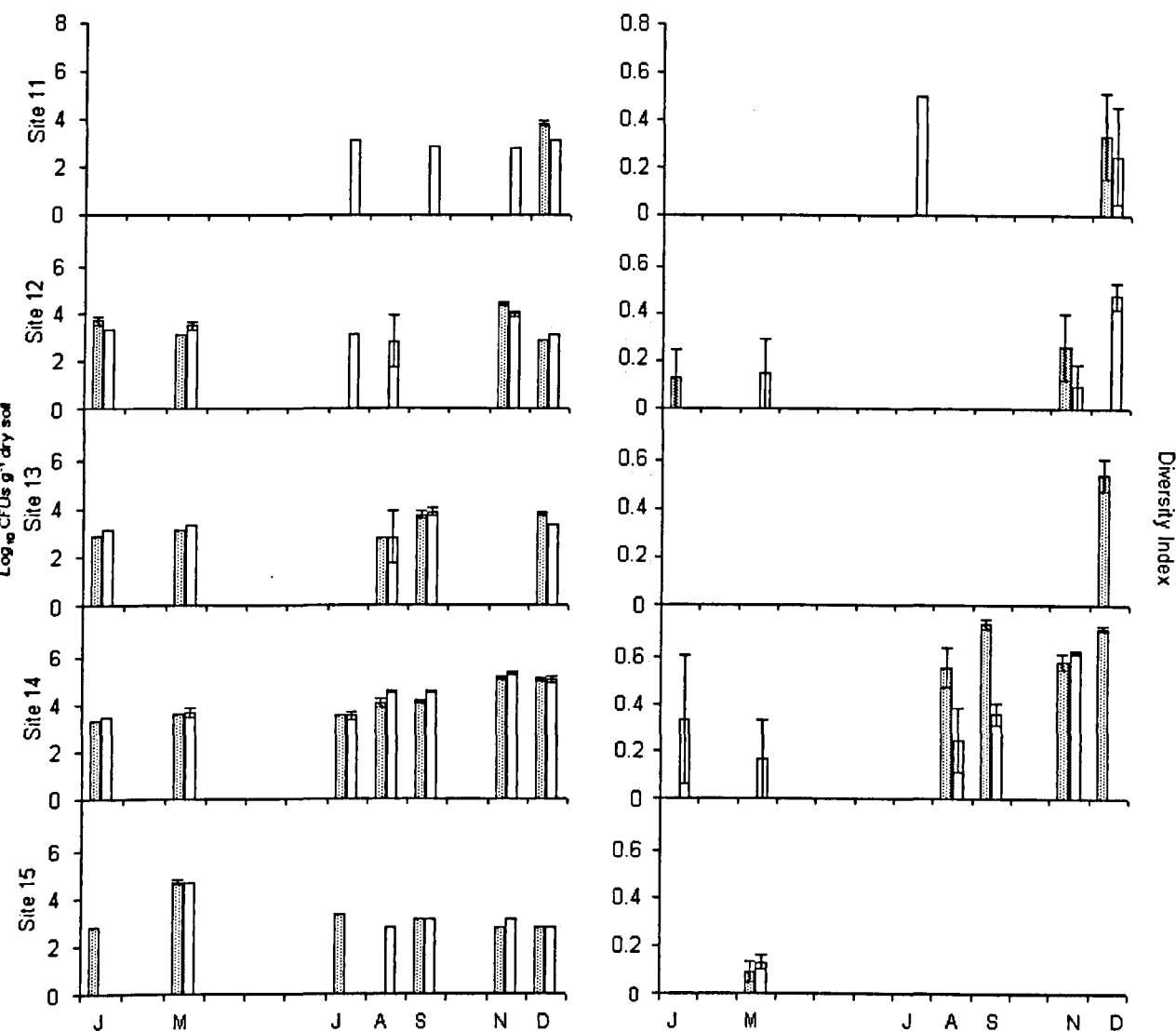


Figure 4.8. Fungal CFUs and diversities (\pm standard error) for soils sampled from five sites at Davis Station recorded during 2001 on seven months as conditions allowed. Shading represents colonies isolated using PDA, clear bars represent colonies isolated using CZA.

Site 11. Wharf (drum store for return to mainland)

Site 12. Sleeping/Medical quarters - south west entrance

Site 13. Hydroponics - base of steps

Site 14. Services building - south entrance

Site 15. Services building - north entrance

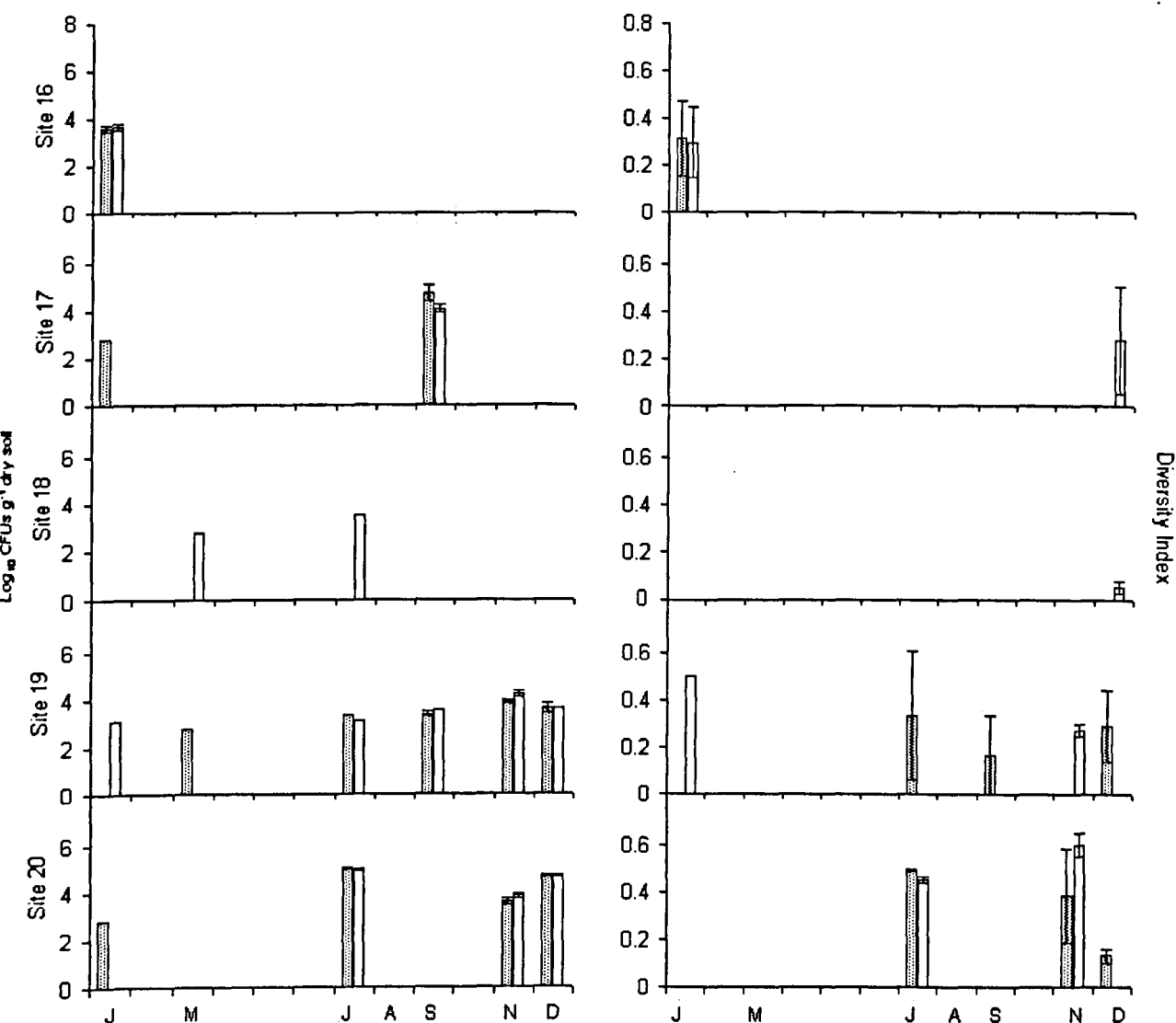


Figure 4.9. Fungal CFUs and diversities (\pm standard error) for soils sampled from five sites at Davis Station recorded during 2001 on seven months as conditions allowed. Shading represents colonies isolated using PDA, clear bars represent colonies isolated using CZA.

Site 16. Helipad - southern pad
 Site 17. Auxiliary helipad
 Site 18. Meteorology - main entrance
 Site 19. Field store - north entrance
 Site 20. Emergency vehicle store - main entrance

The mean number of colony forming units obtained throughout the entire sampling period together with mean diversities were determined and sorted into ascending order for comparison against sites within Davis Station and the expected human impact (Figure 4.10).

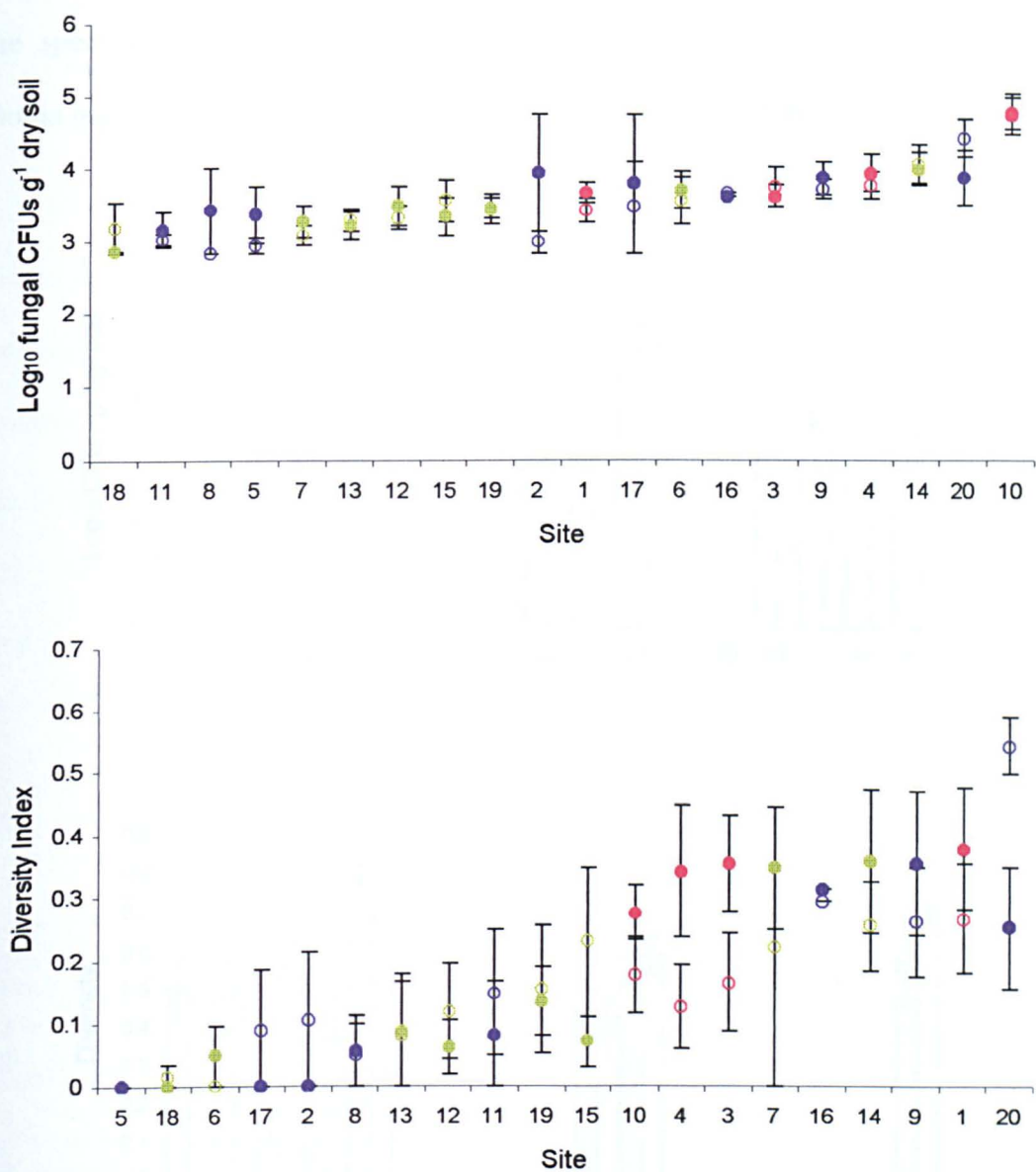


Figure 4.10. Mean fungal CFUs and diversities from twenty locations around Davis Station recorded over a sixteen-month period. Solid circles represent colonies grown on PDA whilst open circles represent colonies grown on CZA. In each graph, sample sites have been sorted into ascending order of CFUs and diversity respectively. Colours used on the graphs correspond to the areas of high, medium and low human impact as described in Figure 4.1.

4.3.4. Miscellaneous Samples

Fungi were isolated from opportunistic samples taken at three stations in the Larsemann Hills, Law Base (Australian Antarctic Division), Progress II (USSR) & Zhong Shan (Chinese National Antarctic Research Expedition) (Figure 4.11). The species identified included *Pseudogymnoascus roseus* from Law Base, *Phoma macrostoma* from Progress II and *Podospora minuta* from Zhong Shan.

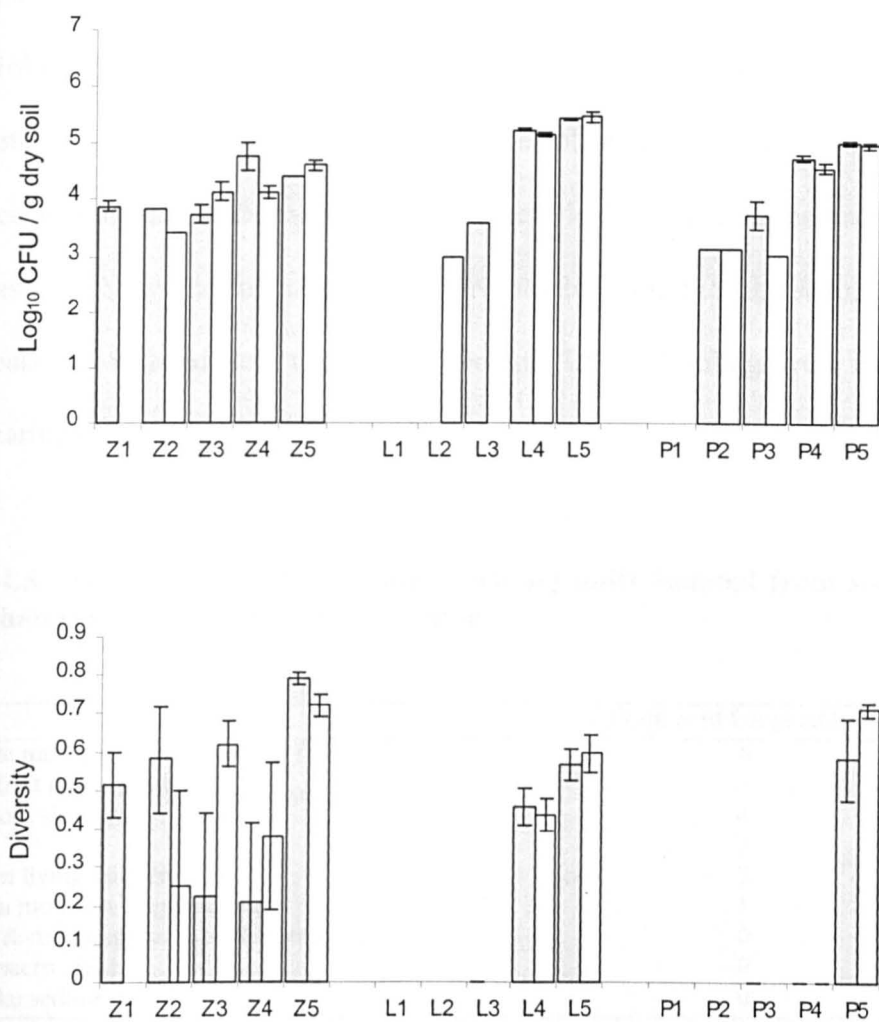


Figure 4.11. Fungal CFUs and fungal diversities soils collected at three stations in the Larsemann Hills during 2001. Shading represents colonies isolated using PDA, clear bars represent colonies isolated using CZA.

Zhong Shan

Z1	Path outside main building
Z2	Helipad
Z3	Near sleeping quarters
Z4	Outside mess
Z5	Road between buildings

Law Base

L1	Adjacent to glacier
L2	In between living quarters
L3	50m south of base
L4	Outside living quarters
L5	Entrance to main building

Progress II

P1	Beach near station
P2	Road to Law Base
P3	Outskirts of base
P4	Back of kitchen
P5	Front of kitchen

The results from the miscellaneous samples are shown in Table 4.5. No fungi were isolated from penguin feathers or droppings. One unidentified species of filamentous fungus was isolated from moulted elephant seal skin using the moist chamber technique. Swabs taken from skua feet yielded four isolates, including one yeast. Although no fungal colonies were isolated from the lake sediments, a pink yeast was isolated from the shore of Organic Lake. No fungi were isolated from marine macro-algae.

Table 4.5. Summary of fungal colony forming units isolated from sources other than soil in the area of Davis Station

Source	Number of Fungi isolated
Vegetable matter from hydroponics facility	6
Spoiled fruit and vegetables	5
Swabs from skua feet	4
Moss	2
Dust from living quarters	2
Hair from moulting elephant seals	1
Feathers & droppings from adelic penguins	0
Marine macro algae	0
Saline lake sediment	0

4.4 DISCUSSION

The presence of a varied fungal community on continental Antarctica has been observed on a number of occasions (e.g. Fletcher *et al.*, 1985; Azmi & Seppelt, 1998). Human activities inevitably involve the introduction of new materials and therefore substrates and have been shown to introduce new microbial species (Kerry, 1990b). Kerry (1990b) also reported fewer fungal genera present at Davis Station than found at other Australian stations (Casey or Mawson) which was attributed to the dry, saline nature of the soils present in the Vestfold Hills. In the current study, fungi were enumerated by isolation onto agar or hair. It must be understood however, that a large proportion of fungal propagules present in soil may be unviable, unculturable or simply not suited to the conditions provided. The results are therefore limited to fungal propagules which are viable and culturable using the methods applied in the current and other studies.

Greater numbers of fungi and higher diversities were recorded from the sites within Davis Station than from the Ellis Fjord, suggesting that, although the fungal diversity is generally low, that the presence of the station has had an impact upon the mycoflora. The presence of fungi was strongly linked with biologically influenced areas, either through human activity or animal or plant colonisation as reported by other workers (e.g. Fletcher *et al.*, 1985; Azmi & Seppelt, 1998). A large number of *Mycelia sterilia* were found as reported by Pugh & Allsopp, (1982). The most commonly encountered genus however, was *Penicillium* sp. contrary to the findings of Del Frate & Caretta, (1990), who did not encounter this genus in any of the samples taken at Victoria Land (Inexpressible Island to Cape King). Del Frate & Caretta (1990) also used the dilution

plate technique including PDA with a combination of streptomycin and penicillin and found a total of 15 species as compared with the 22 isolated during the current study.

The site at Dingle Road was established as a reference site, since human activity there was low and the site was predominantly upwind of the station. No fungi were isolated from this site during the first part of the year, however, the numbers of fungi including keratinophiles increased after the midwinter period. Although it is possible that fungi could have been introduced during the sampling process, care was taken to ensure that soil was collected from previously undisturbed ground. The sampling sites located outside the main entrance to the living quarters (both as part of the Davis transect and from the fixed sample point) provided the greatest numbers and diversities of fungi isolated from any of the sites. This was to be expected since this area received the greatest human impact, being in regular use by all persons at the station. The site was also used as a vehicle parking area and loading area for field equipment prior to any excursions. These results are similar to fungal counts obtained from the living areas at Law Base, Zhong Shan and Progress II. Thus there is a clear human impact in terms of fungal colonisation associated with stations

The presence of the keratinophile *Geomyces pannorum* was reported in the soil at Davis Station by Kerry (1990b) and in soil from three locations in Victoria Land by Del Frate & Caretta (1990). In the current study, this species was isolated on several occasions from the living quarters and also Zhong Shan Station, indicating a strong connection with biotically influenced environments. It was also isolated from skua feathers on Signy Island (Marshall, 1998), although it was not encountered in feathers or faecal samples associated with penguins and skuas during the current study, the latter being in

keeping with Fletcher *et al.*, (1995) who attributed this to the presence of acrylic acid, a fungal inhibitor.

A high fungal diversity was recorded from the site at the main entrance to the science department (fixed sample site 1), although this site did not produce the highest number of colony forming units. The science building was regularly used by personnel handling equipment brought from outside Antarctica and unloading samples being returned from the field. The main station store (fixed sample site 3) was used in a similar manner, as it was visited on a regular basis for a variety of station supplies. This site was also notable for high numbers of colony forming units and high diversity.

The presence of *Phoma* sp. has been reported from The Windmill Islands near Casey Station (Azmi & Seppelt, 1998) and from the Vestfold Hills (Kerry, 1990b), where it has been associated, although not exclusively, with mosses. In the current study, *Phoma* sp. was isolated from two localities, Davis Station (soil outside the main store) and Progress II Station (soil outside the kitchen). Both of these sites are strongly linked with human activities and far removed from moss beds, although Del Frate & Caretta, (1990) reported the unexpected presence of *Phoma* sp. in soils and also from skua feathers.

Keratinophilic fungi were isolated from the living quarters throughout the year, showing an increase during the winter period, consistent with the findings of Marshall (personal communication). At the beach site however, keratinophiles were found predominantly during the summer. Davis beach is occupied during these months by around a hundred male southern elephant seals (*Mirounga leonina*), which come ashore to moult. During this time, large pieces of shed skin and hair become scattered over the beach or collect in

sheltered areas by wind action. Although samples of skin placed in moist chambers did not appear to yield culturable fungi, it is reasonable to assume that keratinophiles would be present and sporulating during these periods.

The transect sites along the Ellis Fjord provided a strong contrast to those at Davis Station in terms of fungal yield. Fungi were only isolated as single colonies on two occasions from the Ellis Narrows and only isolated on two occasions from Watts Lake Hut. The sample collected in March from Watts Lake Hut yielded a large number of fungi, but all *Penicillium* sp., again suggesting a local and temporal event rather than the presence of an established population. No effects were observed which could be attributed to the site's proximity to the field hut.

Lake Druzhby is a freshwater lake ranked as hyper-oligotrophic, that is, having waters with low inorganic nutrients, dissolved organic carbon and low biotic concentrations (Laybourn-Parry & Bayliss, 1996; Henshaw & Laybourn-Parry, 2002). Nutrients, together with fungal and algal propagules, are however, undoubtedly washed into the lake with glacial meltwater (Ellis-Evans, 1985) and indeed, algal mats were observed during the summer period in the shallow waters around the rocky shoreline. Yeasts have also been noted in DAPI stained plankton samples at the time of ice breakout in the summer (Laybourn-Parry, personal communication). This probably accounts, at least in part, for the increase in fungal activity recorded at the lake during the summer. One species, possibly *Oidiodendron tenuissimum* (identified by 97% DNA sequence match) isolated from the lake edges was also isolated from moss patches near Watts Lake Hut, suggesting an association with mosses. Del Frate & Caretta, (1990) reported an association of *Cladosporium* sp. with moss beds and Azmi & Seppelt, (1998) reported

an exclusive association of *Athrobotys* sp. with mosses. Neither of these species however, were found during the current study. The almost total absence of culturable fungi from the site at Trajer Ridge is not surprising. Although the site was only established in June, its location, a rocky promontory 5km from the edge of the polar plateau, would only expose it to microbial introduction by the wind, birds or humans.

Keratinophiles were isolated from all sites with the exception of Trajer Ridge and The Lookout. The distribution throughout the winter period at Lake Druzhby was similar to that observed near the living quarters at Davis Station, again in keeping with Marshall (1998). The lack of significant differences between the sites in the Ellis Fjord reflects the similarity of the sites in terms of their lack of organic material in the soil and their geographic separation from the human impacted area at Davis Station.

The two periods of station resupply took place in October 2000 and December 2001. Efforts are made to minimise the chances of introduction of alien species into Antarctica, with strict rules governing biological materials being carried in belongings coupled with vacuuming of clothing and luggage and washing of footwear in a solution of bleach prior to disembarking from the ship (ANARE Operations Manual, unpublished). Despite these precautions however, vehicles, sea containers and equipment which have been loaded in Australia frequently carry soil, leaves and spider's webs (which trap a wide variety of biological particles). It was therefore expected that these periods would correspond with an influx of new inocula, coupled with the period of greatest activity around the station and that the areas around the living quarters, main store and wharf would show the greatest impact, since these were the areas of greatest activity. An increase in fungal numbers, but not diversity, was recorded from the drum

store at the wharf. These drums and half-height sea containers contained waste materials being returned to Australia for disposal. Although the contents were poor substrates for fungal growth, the soils under and around these containers had lain undisturbed for several months and had themselves acted as wind traps for blown materials. An increase in fungal CFUs was recorded at the main store, coupled with a drop in diversity, indicating either an influx of fungi or the disturbance and proliferation of fungi due to spore dispersal. Surprisingly, a decrease in fungal CFUs was recorded in the area around the living quarters.

Soils were not sampled from the fixed sample sites at the time of the second resupply due to blizzard conditions. Soil samples were taken from the transect sites throughout the three-day resupply operation but fungi were only isolated from beach and living quarters on the second day of activity and from Dingle Road on the third day. With high winds and blowing snow, little significance can be placed on these results.

Fruit and vegetables are ozone treated (ANARE operations manual, unpublished) before export to Antarctica. This sterilisation technique has been shown to be more effective against premature rot than treatment with dilute acetic acid (Scholberg & Gaunce 1995). Despite this, however, washed potatoes spoil faster than those which have been brushed, although the latter still retain a coating of agricultural soil. It follows that many micro-organisms including *Mucor* sp. and *Fusarium* sp. are introduced with the fruit and vegetables and associated soil. With the exception of *Penicillium* sp., however, none of the fungi isolated from these sources were isolated from elsewhere beyond the station.

The dust obtained from the wall cavities in the living quarters, like dust in buildings elsewhere, contains a large proportion of sloughed human skin cells. The two fungi isolated, *Penicillium commune* and an unidentified white *Mycelia sterilia* were in keeping with plates left exposed in the laboratory to examine the extent to which airborne contamination was likely to interfere with results. No keratinophiles were found using hair bait.

It has been reported that skuas (*Catheracta* sp) are carriers of fungal propagules on their feet (Azmi & Seppelt, 1998). Skuas are regarded as particularly important transporters of propagules into Antarctica since their scavenging and predacious nature ensures that they visit a number of sites likely to support fungi, often covering large distances between sites. They are also commonly associated with human dwellings. From the twelve skuas examined, only three filamentous fungi were isolated. *Cladosporium* sp. and *Penicillium* sp., both from a freshly dead specimen collected from the ice and *Eurotium* sp. from a healthy individual. Only one yeast, *Kondoa aerea* was isolated from a foot swab. This low diversity may simply reflect a limited number of organisms present at feeding sites.

It seems reasonable to assume that fungi together with natural weathering and the action of ultra violet light are the principal agents responsible for the decomposition of feathers. During the current study, no fungi were isolated from penguin feathers using moist chambers, which is contrary to the findings of Del Frate & Caretta, (1990) who isolated *Chrysosporium verrucosum* using the same method. It should be noted however, that they also isolated this species from soil and penguin dung samples and

CHAPTER 5: EXTRACTION, PURIFICATION AND AMPLIFICATION OF FUNGAL DNA FOR IDENTIFICATION BY SEQUENCING

5.1. INTRODUCTION

Following the investigations into soil fungi (Chapter 4) and airborne fungal spores (Chapter 3), more than eighty isolates were preserved and returned for study. It was anticipated that the isolates would contain fungi endemic to Antarctica (or at least those which had become established prior to man's occupation). As well as macroscopic "classical" taxonomic analysis, the isolates were subject to molecular analysis.

For many years, the classical approach to fungal taxonomy has been based upon morphological characteristics, either at the macroscopic or microscopic level. Although this approach has become greatly refined and even automated (Dorge *et al.*, 2000), it can also be unreliable due to similarities between closely related species and phenotypic variation between cultures isolated on different media. Physiological characters such as the production of secondary metabolites, including mycotoxins, are also important in fungal taxonomy (Guarro *et al.*, 1990; Christensen *et al.*, 1999; Middlehoven *et al.*, 2000) however, it is accepted that organisms found in wide ranging habitats may exhibit different physiological responses (Murtagh *et al.*, 2002) that prevent accurate classification. Studies of

conserved regions of DNA (i.e. regions which show little variation) are now becoming more widespread as levels of expertise increase and primers become more available (Shen *et al.*, 1998).

Ribosomal DNA (rDNA) has been widely studied in a variety of organisms and is frequently used as an indicator of phylogenetic relationship due to its conserved nature. Eukaryotic ribosomes comprise two subunits, the 40S and 60S which are made up of ribosomal DNA (rDNA) molecules of varying sizes which carry the genes for ribosomal RNA (rRNA) and a number of proteins. The large subunit (LSU) is composed of 3 DNA molecules, the 5S, 5.8S and 28S whereas the small subunit (SSU) contains only a single DNA molecule, the 18S. Between the 18S and 28S RNA molecules of the two subunits are non-coding regions of RNA known as Internal Transcribed Spacer (ITS, Figure 5.1). These regions are commonly used for identification at the inter- and intra-species level (Mitchell *et al.*, 1995), since they are highly conserved except where mutations occur (White *et al.*, 1990; O'Donnell, 1992).

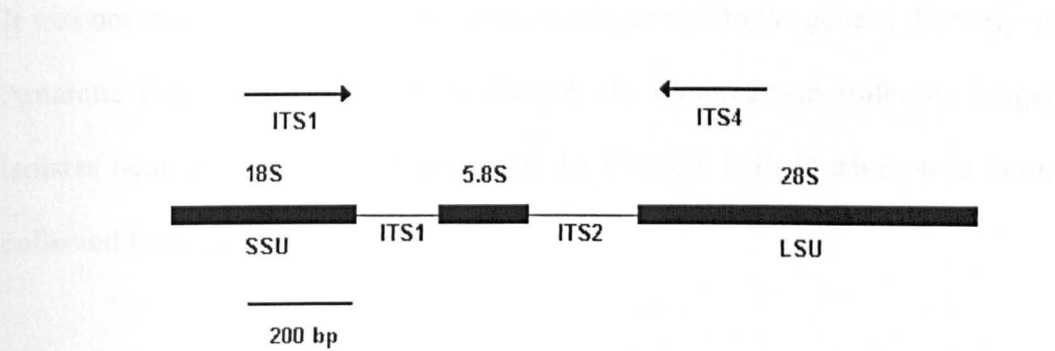


Figure 5.1. The positions of the Internal Transcribed Spacer regions (ITS1 and ITS2) between the small subunit (SSU) and large subunit (LSU) and the direction of operation of the ITS1 and ITS4 primers.

Although analysis of the ITS region is more suited to identifying closely related organisms (Valente *et al.*, 1996; Skouboe *et al.*, 1999) it was used in the current study due to the availability of the ITS primers and the established level of expertise available at the University of Tasmania. Additionally, the D1/D2 region of the large subunit has been shown to be useful in the examination of yeasts (Thomas-Hall & Watson, 2001) and was therefore used for the yeast cultures.

The purpose of the current study was two fold. Firstly to use the ITS sequences as a tool to aid the identification of the fungi cultured. Once the fungi were confidently identified to genus or species level, the findings were examined in the context of previous investigations. Secondly, by comparison against a database of archived ITS sequences, the degree to which the cultured organisms differed from previously recorded strains was determined. In this way, the presence of new or adapted species could be demonstrated.

It was not intended that this study be an investigation into the genetic diversity of Antarctic fungi, but as a means to identify the airborne and soilborne fungal isolates obtained from Davis Station and the Vestfold Hills in addition to those collected from other sources.

5.2. MATERIALS & METHODS

Fungi were obtained from locations around Davis Station, the Ellis Fjord, neighbouring Law Base (Australian) Zhong Shan (Chinese) and Progress II (Russian) stations (Appendix 2) and also a number of miscellaneous sources including swabs from skua feet (Chapter 4). Isolates were cultured using potato dextrose and Czapek-Dox agar and stored under mineral oil at 4°C (Onions, 1971) prior to returning to Australia for molecular analysis.

5.2.1. Extraction and Purification of DNA

Fresh cultures of fungi were grown from preserved specimens returned from Antarctica, on appropriate agar medium (corresponding to those used for initial isolation) and allowed to mature so that maximum biomass could be harvested. Plates were frozen overnight (-20°C) allowing some initial lysis of tissue and to make harvesting the material easier. Between 0.25g and 0.5g of fungal tissue was scraped from the surface of the agar using a sterile scalpel and placed into an Eppendorf tube to which 400µl saline EDTA extraction buffer (Appendix 4) was added.

Zirconia-Silicon beads (0.5mm diameter) were added in roughly equal volume to the fungal material and the mixture vigorously shaken on a Bead Beater™ for 20 seconds at 5000 rpm. In order to prevent the mixture from overheating (causing damage to the DNA), the process was split into two 10 second treatments

separated by 2 to 5 minutes. The mixture was separated by centrifugation at 20160G for 10 minutes and the liquid phase transferred to a fresh Eppendorf (the bead mixture was retained frozen at -80°C so that further DNA could be recovered if required at a later stage).

mainly

Excess proteins and lipids were removed from the liquid by the addition of 400µl chloroform & isoamylalcohol (24:1). The mixture was thoroughly mixed using a Vortex Mixer and then centrifuged at 20160G for 10 minutes. The aqueous phase was transferred to a fresh Eppendorf and the process repeated.

procedure

In order to remove excess salts introduced with the saline EDTA (which interfere with the sequencing process), the sample was flooded with 1ml Prep-a-Gene™ binding buffer (Appendix 4) and 10µl of a suspension of diatomaceous earth added (vortex mixed prior to use to ensure an even suspension). The mixture was thoroughly vortex mixed and allowed to stand for around 10 minutes to allow DNA to bind with the diatomaceous earth. The mixture was centrifuged (20160G for 1 minute) and the supernatant discarded. A further 750µl of binding buffer was added, the diatomaceous earth resuspended by vortex mixing and recentrifuged (20160G for 1 minute), the supernatant again being discarded.

The pellet of diatomaceous earth to which the DNA had been bound, was washed

twice using 750µl Prep-a-Gene™ wash buffer (Appendix 4), vortex mixed and centrifuged (20160G for 1 minute), the supernatant being discarded each time.

The last centrifugation was extended to 2-3 minutes and excess buffer removed with a pipette.

The DNA was returned to solution by the addition of 50µl sterile water maintained at 37°C for 15- 20 minutes. After centrifugation (20160G 2-3 minutes), 45µl of the supernatant was transferred to a fresh Eppendorf and the diatomaceous earth discarded.

The presence of DNA in the cleaned sample was visualised by gel electrophoresis (Vanden-Heuval 1998). Agarose gel (1%) was made up by heating with 25ml 1x TAE buffer (Appendix 4) and prestained with 200µl (0.5µg ml⁻¹) ethidium bromide (Sigma). The gel was poured whilst hot into horizontal electrophoresis trays (Horizon 58, Life Technologies) and allowed to solidify with two eight-pin combs inserted in order to create wells when removed. Aliquots of 3µl of each DNA sample were mixed with an equal volume of optical dye (indian ink in sucrose solution) to enable the process to be monitored under visible light. The gel was flooded with excess TAE buffer (1x) and the dyed DNA templates pipetted into each of the wells. A 90mV current was applied for approximately 30 minutes, monitored by the movement of the dye. The gel slab was removed and examined under short-wave ultraviolet light on a transilluminator and photographed with a digital camera (Plate 5.1). The remaining purified template DNA in solution was frozen at -80°C until required.

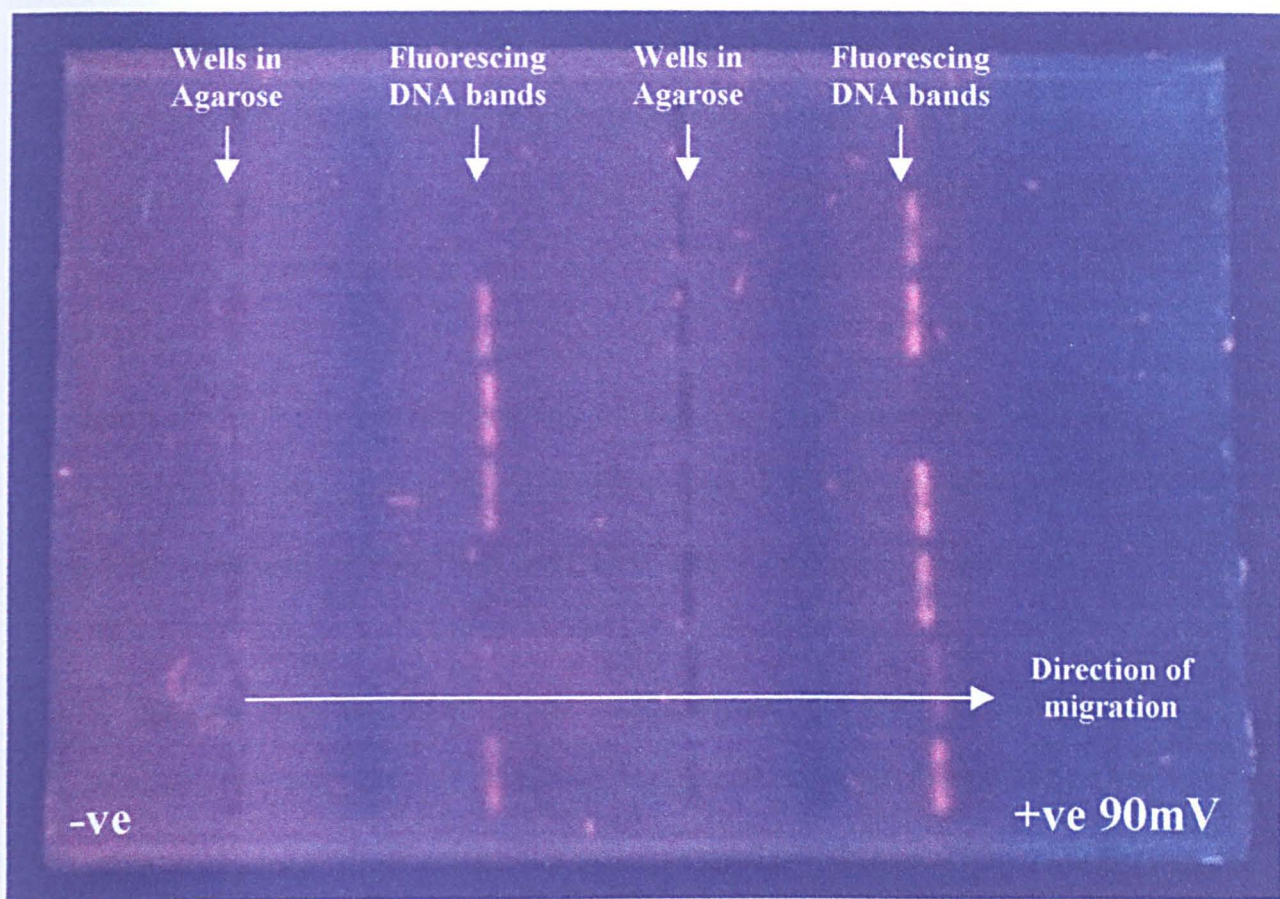


Plate 5.1. Agarose gel photographed under short-wave ultraviolet light. DNA, where present, fluoresces as bright bands having migrated from the wells into which they had been introduced.

5.2.2. Amplification of extracted DNA using the Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is a method of vastly increasing the number of copies of a target genetic sequence by the successive addition of new bases following on from specific oligonucleotide primers. DNA strands are separated by heating (denaturing) and site-specific primers become annealed to

complimentary regions during cooling. Subsequent heating allows the extension of the oligonucleotides by deoxynucleotide triphosphates (dNTPs) mediated by a thermostable DNA polymerase (Taq), theoretically doubling the target region. Repeated heating and cooling cycles therefore result in an exponential increase in the number of target sections.

Before the PCR process was initiated, the concentration of template DNA was determined by absorption using a Biorad Smartspec™ 3000 spectrophotometer so that the volume of template DNA to be used in the reaction could be calculated.

The Quiagen™ *Hot Start* PCR kit was used throughout this study, following the manufacturer's protocol. A premixed solution was prepared (Appendix 4. Quiagen™ *Hot Start* PCR mixture) which contained two primers ITS1 & ITS4 (Table 5.1) corresponding to the start of the target sequence from each direction, *Hot Start* solution (dNTPs, supplied by the manufacturer), "Q" solution (manufacturer's reagent containing magnesium, required for the function of Taq) and water.

Table 5.1. Oligonucleotide primers used for the amplification of fungal DNA. ITS1/ITS4 after White *et al.*, (1990), D1/D2 after manufacturer's label.

Primer	Sequence (5'→3')
ITS1	TCCGTAGGTGAACCTGCGG
ITS4	TCCTCCGCTTATTGATATGC
D1	CGATATCAAGCGGAGGAA
D2	GGTCCGTGTTTCAAGACG

A 22µl aliquot of the premixed solution was added to ca. 3µl each DNA template (depending on template DNA concentration) and 25µl *Hot Start* solution (containing Taq). The mixture was then subjected to a sequence of heating and cooling using a PTC 200 Gradient Cyclor (MJ Research).

PCR thermal cyclor program

95°C for 15 minutes		
94°C for 1 minute	} Repeated 24 times	Denaturation of DNA
58°C for 1 minute		Annealing of primers
72°C for 1 minute		Extension of sequence
Hold at 4°C		

Amplified PCR products were cleaned using Prep-a-Gene™ binding buffer, diatomaceous earth and wash buffer in the same manner as initial purification. The final concentration of amplified DNA was again determined colourimetrically using a Biorad Smartspec™ 3000 spectrophotometer.

5.2.3. Sequencing of amplified fungal DNA

The first stage of the sequencing process uses the Polymerase Chain Reaction. The amplified DNA templates were mixed with dNTPs which contained a proportion of bases tagged with coloured dyes. These tagged bases can be detected spectrographically and also prevent the addition of further bases, thus terminating the PCR process for that template molecule. Since many copies of the template are amplified in this way, many copies of the sequence will result, each terminated at a different point by a tagged base. Statistically, a tagged section should therefore be formed which corresponds to every increment of the base series.

A premixed solution containing primer, DTCS Quick Start Master Mix (Beckman Coulter) and water was prepared according to manufacturer's guidelines (Appendix 4). An 18µl aliquot was added to 2µl template DNA (PCR product) and the mixture subjected to a cycle of heating and cooling using a PTC 200 Gradient Cycler.

Dye-Terminated thermal cycler program

96°C for 20 seconds	} Repeated 40 times	Denaturation of DNA
50°C for 20 seconds		Annealing of primer
60°C for 4 minutes		Extension of sequence
Hold at 4°C		

A “stop Solution” (EDTA & sodium acetate in equal volumes) was made up and a 4µl aliquot added to each sample together with 1µl glycogen. The mixture was cleaned by treatment with 200µl cold 95% ethanol, vortex mixed and centrifuged at 4°C for 10 minutes. The supernatant was carefully removed by pipette and a further 200µl cold 75% ethanol added and centrifuged (4°C for 10 minutes) without prior mixing. The supernatant was again removed by pipette and discarded and the resulting pellet dried in a rotary vacuum drier for 40 minutes and resuspended in 40µl formamide Sample Loading Solution (Beckman Coulter).

The sequences were determined colorimetrically using a Beckman Coulter CEQ™ 2000XL DNA Analysis System. The output was examined and manually checked and edited as required using BioEdit™ software¹ (Hall, 1999) and the sequences analysed by BLAST (Basic Local Alignment Search Tool) via the

¹ Available at <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>

internet² in order to provide identification and confidence limits to genus or species level (Altschul *et al.*, 1997).

Bootstrapped Neighbour Joining trees (Saitou & Nei, 1987; Hall 2001) were produced using ClustalX³ v1.81 software (Chenna *et al.*, 2003; Jeanmougin *et al.*, 1998, Thompson *et al.*, 1997) and edited using NJplot (included within ClustalX). Bootstrap values greater than 80% were considered as being significant (Hall, 2001).

² <http://www.ncbi.nlm.nih.gov/BLAST/>

³ Available at <http://inn-prot.weizmann.ac.il/software/ClustalX.html>

5.3. RESULTS

DNA was successfully extracted and sequenced from a total of 81 fungal strains, six of which were yeasts. After PCR amplification, an average of more than 100µg ml⁻¹ DNA was obtained, which yielded sequences of between 500-600 bp. After duplications had been ignored, forty unique sequences were obtained. Strains were matched with existing database sequences to provide identification to the species level. Four yeast genera were identified and eighteen hyphal genera belonging to zygomycota, ascomycota and deuteromycota.

Table 5.2. Fungal genera isolated from Davis Station and the Vestfold Hills, Antarctica identified by ITS sequencing based on nucleotide match against the BLAST database. Whilst the majority of matches were > 98%, all matches were >90%.

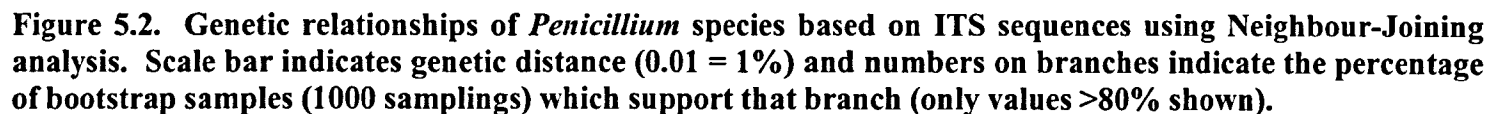
Hyphal genera		Yeast genera
<i>Antarctomyces</i>	<i>Phaeosphaeria</i>	<i>Bensingtonia</i>
<i>Aspergillus</i>	<i>Phialophora</i>	<i>Cryptococcus</i>
<i>Cladosporium</i>	<i>Phoma</i>	<i>Exophiala</i>
<i>Eurotium</i>	<i>Podospora</i>	<i>Udeniomyces</i>
<i>Fusarium</i>	<i>Pseudogymnoascus</i>	
<i>Graphium</i>	<i>Sclerotinia</i>	
<i>Mucor</i>	<i>Tetracladium</i>	
<i>Oidiiodendron</i>	<i>Ulocladium</i>	
<i>Penicillium</i>	<i>Verticillium</i>	

Although not every isolate was successfully sequenced, all of the genera identified (Table 5.2) were isolated from Davis Station with only a few exceptions. Two filamentous fungi, *Cladosporium* and *Eurotium* and the yeast *Bensingtonia* were isolated from swabs obtained from skua feet but not from elsewhere at Davis Station or the Vestfold Hills. Furthermore, *Phaeosphaeria*

was only found associated with mosses near Watts Lake Hut. The only genus to be isolated from both Davis Station and the Vestfold Hills was *Sclerotinia* sp., isolates of which were obtained from a mould found on courgette plants (*Cucurbita* sp.) being grown in the hydroponics facility and also from a rotorod strip which was exposed at the Ellis Narrows.

Although sequencing the ITS region is a useful tool for taxonomic purposes when applied to a range of genera, the principal application is to determine variations between closely related species. Since the primary purpose of the current investigation was concerned with fungal distribution, very few replicate cultures were obtained limiting the scope for intraspecific comparisons.

Although few isolates were retained from what appeared to be identical species (based on morphology), fifteen isolates of *Penicillium* from a variety of habitats were preserved, ultimately being found to represent six species (Figure 5.2).



Of the six species of *Penicillium*, seven isolates of *P. commune* were divided into two groups which differed from each other at 6 loci (1% variation).

The yeast species returned for examination were not closely related and there was no duplication of genera so that no intraspecific relationships could be shown (Figure 5.3).

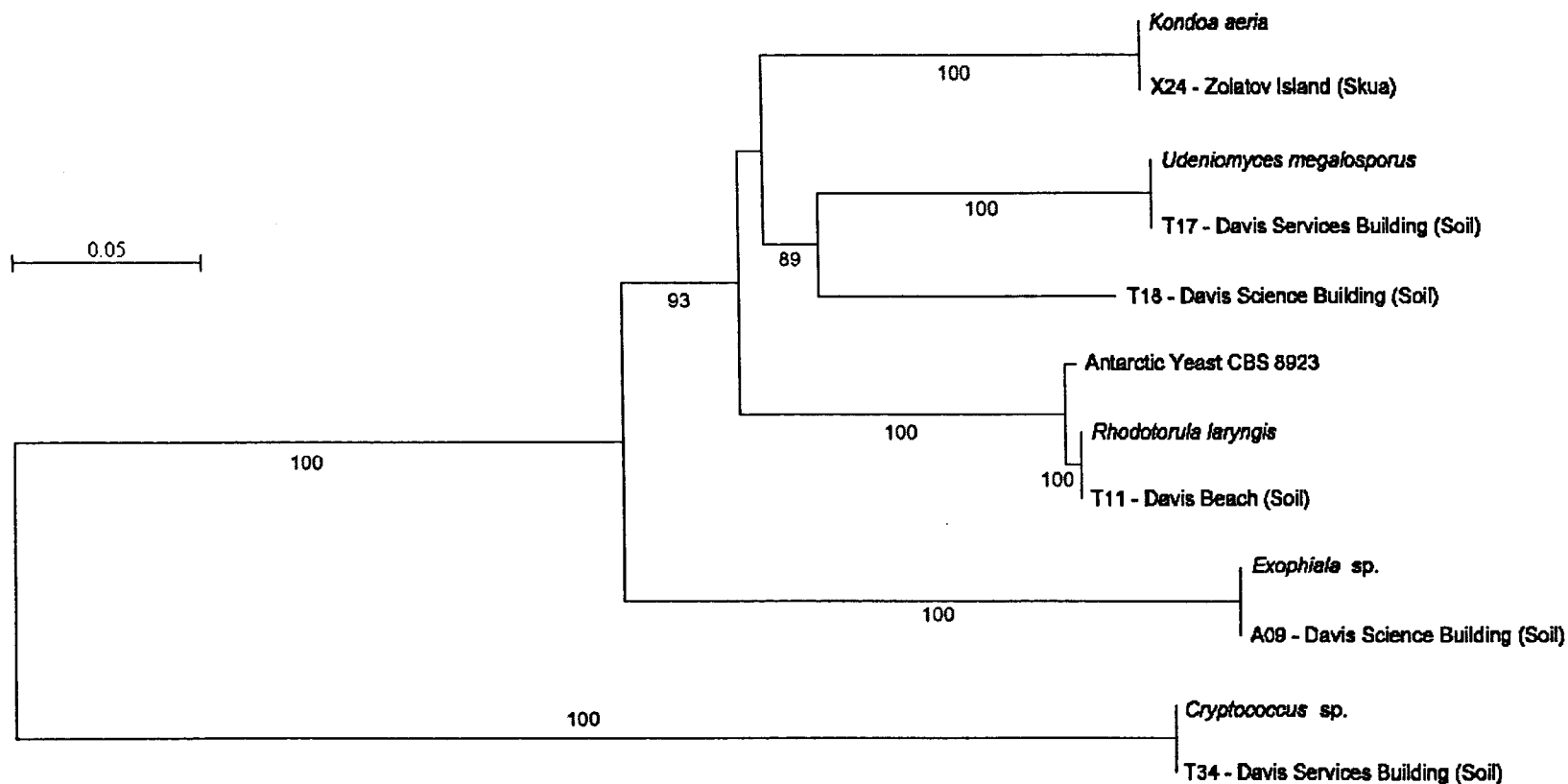


Figure 5.3. Genetic relationships of yeast species based on D1/D2 LSU sequences using Neighbour-Joining analysis. Scale bar indicates genetic distance (0.01 = 1%) and numbers on branches indicate the percentage of bootstrap samples (1000 samplings) which support that branch (only values >80% shown).

5.4. DISCUSSION

Several concepts have been proposed to define a species (Taylor *et al.*, 2000), the Morphological Species Concept and Biological Species Concept having been widely accepted for many years, although it is generally accepted that there is no single definition which can be relied on exclusively. Fungi, in particular can present problems since morphology can be nondescript in the absence of spore structures and mating types may be unknown or absent (Petersen & Hughes, 1999).

The use of genetic sequences for identification of species (Phylogenetic Species Concept) is becoming more widespread since the materials required are increasingly available and consequently, databases of species examined are growing. Although this technique has provided a great deal of insight into phylogenetic relationships and evolutionary pathways, it introduces the concept of species complexes. This is because species which have previously been accepted as morphologically and physiologically identical, contain genetic variations within geographic regions or even populations. In such cases, the term *species* becomes more subjective since genetic drift is continuous and proportional to both genetic isolation and population size (Taylor *et al.*, 2000).

The degree of species separation according to the Phylogenetic Species Concept is based on the speed of the 'genetic clock' (Burnett, 2003), i.e. the time taken for a sufficient number of mutations to occur at a given locus. This assumption

is however dependant upon a number of factors. The number of life cycles, and hence the time required for changes to occur, may vary greatly according to the organism and also the conditions under which it is living. Antarctic fungi are subject to extreme environmental conditions, in particular low temperature and limited availability of free water. Consequently, the number of life cycles over a given period is limited and likely to be fewer than that of a temperate species. It follows therefore that the scope for mutation is also limited.

The analysis of the ITS region of the isolates obtained from Davis Station and the Vestfold Hills was successful insofar as being able to provide identification to genus level in all cases and species level in most cases (Table 6.1) (based on >90% match against the BLAST database). It is however, important to note that results obtained from comparisons against any database are dependent upon the data held within it and therefore it is likely that a number of identifications may be inaccurate.

A limitation of the current study was the low number of isolates with which to provide intra-specific comparisons. Since it was the principal objective of the study to examine the distribution, inter-species diversity and seasonality of Antarctic fungi, only single examples of non-identical morphotypes were retained for further study. Thus the scope for examining the genetic variation within Antarctic populations was limited.

Not all species isolated during the study were successfully sequenced, however, all species identified by ITS sequence showed greater than 90% similarity with sequences obtained from the BLAST database. Neighbour-joining analysis did not show any strains which differed enough from those recorded in BLAST to be considered new to science. It should be noted, however that the closest match offered by BLAST may not always imply closest relationship with the study sequence (Koski & Golding, 2001). Furthermore, it must be noted that even a high (>98%) level of similarity to a previously recorded strain may be sufficiently different to be considered a new species.

Whilst species were encountered which could be considered as native, such as *Antarctomyces* (Stchigel *et al.*, 2001), and Antarctic yeast (CBS 8923), some seemed to be non-Antarctic or even phytopathogenic (e.g. *Phaeospharia* and *Oidiodendron*) in origin indicating that they had been introduced.

The majority of the genera identified corresponded to those which have been previously recorded in Antarctica such as *Aspergillus*, *Cladosporium*, *Phialophora* and *Phoma* (Sun *et al.*, 1978) indicating a high probability that these organisms are either indigenous or have become endemic. The presence of fungi associated with vegetable matter such as *Mucor* sp. and *Sclerotinia* sp. at Davis Station is perhaps not surprising since vegetables are imported, stored and in certain cases grown on site.

Those genera considered to be non-Antarctic in origin included *Phaeosphaeria* (isolated from Antarctic moss), normally associated with eye spot in maize and *Oidiodendron* (isolated from airborne spores at Davis Living Quarters) forms mycorrhizal associations within the Ericaceae. It would seem unlikely that either of these genera would occur naturally in an Antarctic habitat due to their associations with angiosperms and equally unlikely that they might have occurred as laboratory contaminants. The ITS sequences of both isolates extended to around 500bp with no 'gaps' and no other similar matches were offered by BLAST indicating a close similarity with known phylogenetic species. Whilst the possibility exists that these genera were indeed present, a more likely alternative would be that either the BLAST identification was unreliable (Koski & Golding, 2001) or that the DNA sequencing procedure was flawed.

The two groupings of *Penicillium commune* (as obtained from the BLAST database) to which six isolates were attributed, differed at six loci, each being a single base substitution. All isolates of *Penicillium* (with one exception) were obtained from sites within Davis Station indicating a strong likelihood that these are not only closely related but possibly introduced organisms. Unfortunately, an insufficient number of separate isolates precludes an investigation into the possible degree of genetic drift which may be present within the species representing this genus.

CHAPTER 6: THE EFFECTS OF TEMPERATURE AND DESICCATION ON FUNGAL GROWTH

6.1. INTRODUCTION

6.1.1. The Antarctic habitat

Antarctica is essentially a cold desert imposing many physiological stresses on the organisms which survive there (Robinson, 2001). Water is biologically unavailable for most of the year and nutrients are frequently severely limited. Damage to stratospheric ozone causes high UV radiation in spring extending into summer, which may be inhibitory to fungal growth (Hughes *et al.*, 2003). Although temperatures are generally low, there is the potential for extreme temperature fluctuations, especially in microhabitats (Walton, 1982) due to low air temperatures causing widespread freezing and the incidence of sunlight on dark rocks. No higher plants are found in the Vestfold Hills and vegetation is limited to sparse assemblages of mosses, lichens and free-living fungi, cyanobacteria and algae (Pickard, 1982). For any organism to survive under these conditions, it must have undergone a high degree of adaptation (Convey, 1998).

Fungi are important decomposers and therefore their activity affects the cycle of nutrients within an ecosystem (Kerry, 1990a). This is of particular importance in the nutrient-poor ecosystems of Antarctica, especially as natural processes are slowed at

low temperatures due to reduced enzyme activity. Factors affecting the growth of fungi therefore ultimately affect the balance of the ecosystem.

6.1.2. Adaptations associated with low temperature

Low temperatures reduce the rate of enzyme-mediated reactions, in severe cases causing protein denaturation. Ice formation may occur within tissues depending upon the freezing point of the cellular solution and the availability of nucleation sites. As ice crystals form, the concentration of solutes and osmotic potentials increase possibly resulting in solute precipitation if solubility products are exceeded. This may, in turn, result in pH changes as buffer concentrations become altered (Smith, 1993). Fungi are able to overcome many of the problems associated with low temperature by the formation of cryoprotective carbohydrates. Indeed, fungal propagules have been found to be able to undergo cryopreservation in frozen soils and glacial ice for several thousand years (Ma *et al.*, 2000; Kochina *et al.*, 2001). Weinstein *et al.*, (2000) noted several physiological changes occurring in fungi growing at low temperature, such as the production of intracellular trehalose and extracellular glycerol and also changes in unsaturated lipid concentration. Increased amounts of melanin have also been observed in fungi that occur in low temperature environments (Jumpponen & Trappe, 1998).

Fungi are broadly described as psychrophiles, mesophiles or thermophiles, depending on their thermal preferences (Zucconi *et al.*, 1996). Although it is accepted that most Antarctic fungi are mesophiles (i.e. show optimum growth above 15°C), they may also be described as psychrotrophs in that they are able to tolerate temperatures below 5°C

regardless of their optimum growth temperature. A proportion of the fungi isolated from Antarctica however, may be introduced species having been carried by animals, by meteorological events or as a result of human activity. Although some of these introduced organisms may be psychrotrophs, they are likely to be less well adapted to survive the freeze-thaw conditions of an Antarctic habitat than endemic species. Similarly they may not possess the physiological or biochemical mechanisms to withstand desiccation over the winter period (Robinson, 2001) and may therefore only survive as opportunists for the summer period or within artificial environments such as Antarctic stations.

6.1.3. Adaptations associated with desiccation

In a desert environment like Antarctica, organisms have to possess some mechanism of withstanding long phases when liquid water is unavailable. Indeed many terrestrial polar organisms, such as insects (Ramløv & Lee, 2000; Watanabe *et al.*, 2002), exploit desiccation as a survival strategy.

The ability of fungi to achieve optimum potential (i.e. utilise a nutrient source, compete with other organisms and reproduce) is dependant on a number of factors such as temperature, pH and water activity (Gock *et al.*, 2003). For fungi to be able to grow at any temperature, there must be free water. Water activity (a_w) is defined as the ratio of water vapour pressure of a material compared with that of pure water under the same conditions (Magan & Lacey, 1984). Although spores of *Eurotium*, *Aspergillus* and *Penicillium* have been shown to survive at $a_w < 0.58$, a value of 0.6 is generally accepted to be the limit of cell growth (Carlile *et al.*, 1994). Fungi may

counter the effects of low water activity by accumulating glycerol and other polyhydric alcohols (polyols) within their hyphae (Adler *et al.*, 1982). High concentrations of polyols can be tolerated within cells since they do not interact with proteins and therefore do not interfere with normal cell enzyme pathways (Brown, 1978).

The effects of temperature and desiccation on fungal growth and adaptations to low temperature have been studied before (e.g. Kerry, 1990a; Zucconi *et al.*, 1996; Magan & Lacey, 1984). The purpose of this investigation however, was two-fold. Firstly to determine the range of thermal preferences which exist within an Antarctic community and the proportion of true psychrophiles and non cold-tolerant mesophiles present. Secondly, to determine if the isolates were capable of surviving total desiccation.

Airborne and soil fungi were collected at Davis Station (68° 35'S 77° 58'E) and its environs using rotorod air samplers (Chapter 3) and soil dilution plate methods (Chapter 4). The growth rates of the fungi isolated were examined over a range of temperatures in order to determine optimum growth rates. Isolates were also totally desiccated and reinoculated onto fresh agar to determine viability.

6.2. MATERIALS & METHODS

Fungi were isolated from a number of sites around Davis Station and the Vestfold Hills during 2001 (Chapters 3 and 4). All fungal isolations were performed as described in Chapters 2 to 4.

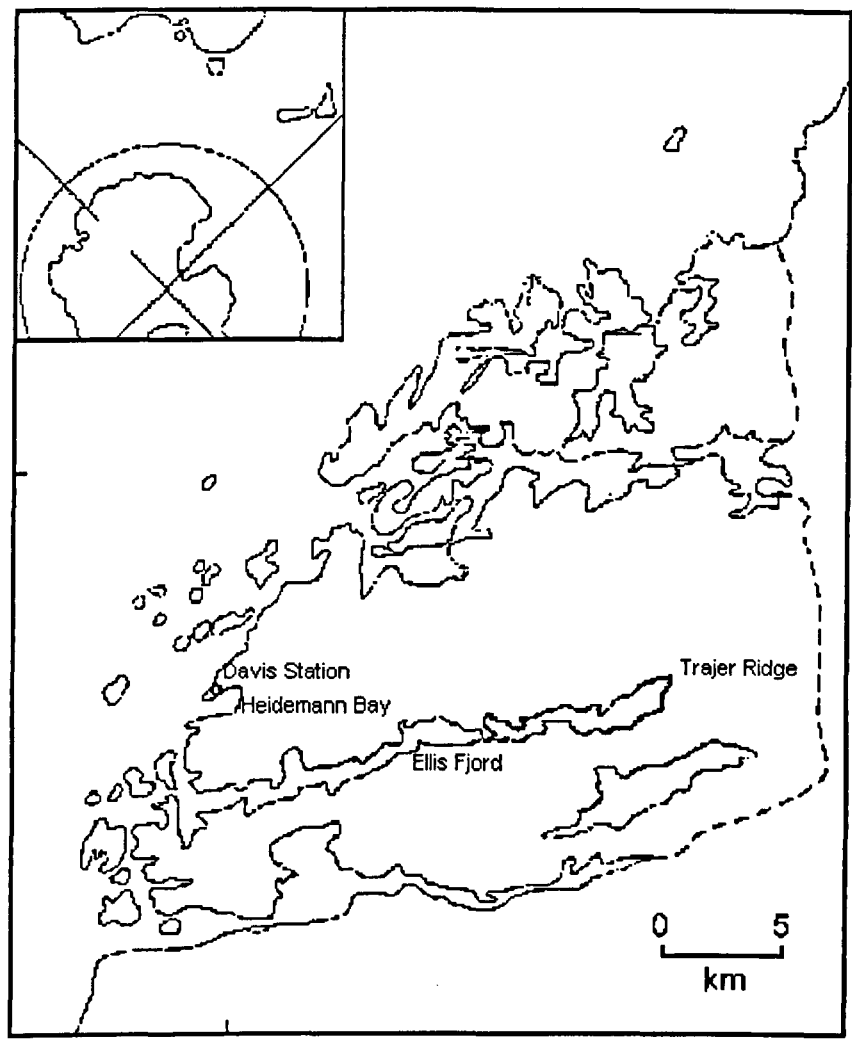


Figure 6.1. Davis Station and the Vestfold Hills. Map courtesy of the Australian Antarctic Division.

Colonies obtained were subcultured to produce pure isolates which were transferred to agar slopes and then stored refrigerated under liquid paraffin until required (Onions, 1971). Identifications of fungal cultures were made to the genus or species level by ITS sequencing (Chapter 5).

Since fungi were isolated throughout the twelve-month period of study, the growth investigations were carried out in two parts. The first part of the investigation, carried out at Davis Station, Antarctica, was conducted at 0°, 2°C, 5°C, 10°C, 15°C, 20°C, 30°C and 37°C. The second part, carried out at the University of Nottingham, was conducted at 2°C, 10°C, 15°C and 22°C.

Prior to examination, fresh cultures of each isolate were prepared from the refrigerated samples which were in turn subcultured in order to ensure that metabolic pathways were restored after the preservation period. Unfortunately, a small number of isolates were either lost in transit from Antarctica due to damaged containers or else failed to grow when inoculated onto fresh media, a number of yeasts fell into the latter category.

Sections of agar approximately 5mm² were excised from the margin of colonies and placed upside down in the centre of an agar plate and incubated for 28 days with observations made at 48 hour intervals. Hyphal growth was recorded to the nearest half millimetre against a baseline inscribed on the base of the Petri dish. Measurements were continued until the edge of the Petri dish was reached by the mycelium or else until reliable measurements were no longer possible due to the formation of daughter colonies resulting from spore release from the main colony. No

attempt was made to determine the point at which spores were formed since many isolates appeared to be sterile.

The means of three replicates were used as a measure of colony growth at each temperature. Mean growth was plotted against time and mean growth rate determined by regression analysis using Microsoft Excel[®] v97.

Following the growth experiment, the cultures were dried in a low temperature oven (37°C) and placed in desiccators for a further 28 days. After this period, viability was determined by reinoculation onto fresh agar and incubation at 20°C for 14 days, since this temperature had been shown to result in colony growth in all isolates. Sections of agar were removed which contained desiccated hyphae from the edges of the mycelium. It was inevitable however, that spores would also be transferred from those isolates which produced them.

6.3. RESULTS

A total of 57 isolates were examined (Table 6.1), of which only 3 showed optimal growth at 10°C. A further 19 showed an optimum growth temperature of 15°C and another 14 around 20-22°C. Two isolates were found to have optimal growth rates of 30°C whilst the remaining 19 could only be described as optimally growing at >22°C using the range of temperatures available at Nottingham. Lag phases were observed in a number of isolates especially at low temperature, however this period rarely lasted for more than 3-4 days.

The three isolates which had optimum growth temperatures around 10°C were identified as *Penicillium commune* (two strains) and a yeast designated as “Antarctic Yeast” CBS 8928. Although seven strains of *P. commune* in total were isolated and identified, each strain was found to have a different growth rate. Isolates T01 & R02 (Figure 6.2.a) both collected from within Davis Station showed similar growth rates with an optimum growth temperature of 10°C. A further four isolates of *P. commune*, including three obtained from within Davis Station and one obtained from moss plants in the Ellis Fjord also showed similar growth rates however their optimum growth temperatures are at 15°C (Figure 6.2.b). The final isolate of *P. commune*, D07, was obtained from a dust sample collected from a cavity wall in the living quarters at Davis Station and was found to have an optimum temperature greater than 22°C (Figure 6.2.c) indicating possible adaptation to the artificial climate of human habitation.

Table 6.1. Optimum growth rates and temperatures for 57 fungal isolates obtained from Davis Station, the Vestfold Hills and neighbouring Law Base, Progress II Station and Zhong Shan Station in the Larsemann Hills, Antarctica.

Isolate	Optimum growth temp (°C)	Optimum Growth rate (mm/day) ± standard error		Species (if identified)	Location of original isolation	Date collected	Medium	Original substrate
T18	10	0.21	±2.01 x10 ⁻³	Antarctic yeast	Davis Site 3	28-Nov-00	CZA	Soil
R02	10	0.38	±4.31 x10 ⁻³	<i>Penicillium commune</i>	Davis Living Quarters	08-Dec-00	CZA	Rotorod
T01	10	0.44	±2.38 x10 ⁻³	<i>Penicillium commune</i>	Davis Site 2	30-Mar-01	PDA	Soil
T13	15	0.28	±2.01 x10 ⁻³		Davis Living Quarters	08-Dec-01	CZA	Soil
T30	15	0.44	0		Davis Site 7	06-Jul-01	CZA	Soil
D05	15	0.52	±1.00 x10 ⁻²	<i>Penicillium commune</i>	Davis vegetable store	11-Apr-01	CZA	Pear
T14	15	0.61	±6.04 x10 ⁻³		Davis Living Quarters	08-Dec-01	CZA	Soil
T07	15	0.61	±2.01 x10 ⁻³		Davis Living Quarters	08-Dec-00	CZA	Soil
T16	15	0.66	±2.38 x10 ⁻³		Davis beach	08-Dec-00	CZA	Soil
X29	15	0.67	±6.20 x10 ⁻³	<i>Pseudogymnoascus roseus</i>	Law Base	08-Jan-01	PDA	Soil
T28	15	0.72	0	<i>Penicillium commune</i>	Davis Site 3	06-Jul-01	PDA	Soil
T12	15	0.82	±5.61 x10 ⁻³	<i>Tetracladium furcatum</i>	Davis Living Quarters	08-Dec-00	CZA	Soil
T15	15	0.87	±2.01 x10 ⁻³		Davis Living Quarters	08-Dec-01	CZA	Soil
T02	15	0.87	±2.01 x10 ⁻³		Davis Living Quarters	08-Dec-00	CZA	Soil
D06	15	0.93	±4.31 x10 ⁻³	<i>Penicillium commune</i>	Davis Living Quarters	10-Apr-01	CZA	Cavity wall dust
T03	15	0.96	±2.01 x10 ⁻³	<i>Phialophora malorum</i>	Davis Site 10	15-Jan-01	PDA	Soil
T10	15	1.01	±2.01 x10 ⁻³		Davis Living Quarters	08-Dec-00	PDA	Soil
T08	15	1.18	±3.81 x10 ⁻³	<i>Graphium rubrum</i>	Davis Site 9	15-Jan-01	PDA	Soil
T27	15	1.46	0	<i>Antarctomyces</i>	Davis Site 1	06-Jul-01	PDA	Soil
X14	15	1.69	±1.72 x10 ⁻⁸	<i>Podospora minuta f. tetraspora</i>	Zhong Shan (helipad)	26-Dec-01	PDA	Soil
X26	15	9.00	0		Davis vegetable store	26-Jan-02	PDA	Potato (soil)
LMI	15	11.0	0		Davis hydroponics	22-Dec-01	PDA	Lettuce
T17	20	0.13	0	<i>Udeniomyces megalosporus</i>	Davis Site 14	10-Oct-00	PDA	Soil
T05	20	0.90	0		Davis Site 1	08-Mar-01	CZA	Soil
T04	20	1.05	0	<i>Verticillium lecanii</i>	Davis Site 2	11-May-01	CZA	Soil
T06	20	1.55	0		Davis Site 3	09-Mar-01	CZA	Soil
A16	22	0.94	±2.16 x10 ⁻²	<i>Penicillium commune</i>	Ellis Rapids	17-Jun-01	PDA	Moss

Isolate	Optimum growth temp (°C)	Optimum growth rate (mm/day) ± standard error		Species (if identified)	Location of original isolation	Date collected	Medium	Original substrate
T23	22	1.00	±2.79 x 10 ⁻³	<i>Penicillium kojigenum</i>	Davis Site 4	15-Jan-01	CZA	Soil
A17	>22	0.40	±8.63 x 10 ⁻³	<i>Phaeosphaeria oreochloae</i>	Ellis Rapids	17-Jun-01	PDA	Moss
X06	>22	0.46	±4.31 x 10 ⁻³	<i>Eurotium niveoglaucum</i>	Skua	06-Dec-01	PDA	Swab
A8	>22	0.53	0	<i>Oidiodendron griseum</i>	Davis Living Quarters	11-Dec-00	CZA	Rotorod
R05	>22	0.58	0		Watts Lake Hut	20-Dec-00	CZA	Soil
D07	>22	0.58	±3.14 x 10 ⁻²	<i>Penicillium commune</i>	Davis Living Quarters	10-Apr-01	CZA	Cavity wall dust
A18	>22	0.81	±3.13 x 10 ⁻²	<i>Penicillium commune</i>	Davis Living Quarters	21-Jan-02	CZA	Rotorod
T19	>22	0.92	±1.42 x 10 ⁻²	<i>Penicillium glabrum</i>	Davis Site 15	15-Jan-01	PDA	Soil
D09	>22	1.10	±2.77 x 10 ⁻²	<i>Penicillium brevicompactum</i>	Davis hydroponics	30-Mar-01	PDA	Lettuce
D02	>22	1.24	±1.73 x 10 ⁻²		Davis vegetable store	26-Mar-01	PDA	Potato
T31	>22	1.25	±1.03 x 10 ⁻²	<i>Phoma</i> sp.	Davis Site 3	06-Jul-01	PDA	Soil
T26	>22	1.29	0		Davis Site 1	06-Jul-01	PDA	Soil
CT1	>22	1.32	±2.89 x 10 ⁻²	<i>Sclerotinia sclerotiorum</i>	Davis hydroponics	22-Dec-01	PDA	Courgette
X31	>22	1.32	±4.31 x 10 ⁻³	<i>Phoma</i> sp.	Davis Site 15	21-Jan-02	CZA	Soil
CPI	>22	1.33	±2.35 x 10 ⁻²	<i>Penicillium brevicompactum</i>	Davis hydroponics	22-Dec-01	PDA	Capsicum
D01	>22	1.34	±7.02 x 10 ⁻²	<i>Penicillium polonicum</i>	Davis vegetable store	26-Mar-01	PDA	Potato
R03	>22	1.41	0	<i>Graphium rubrum</i>	Davis Living Quarters	08-Dec-01	CZA	Rotorod
A6	>22	1.47	±4.31 x 10 ⁻³	<i>Uncultured fungus</i>	Davis Living Quarters	04-Apr-01	CZA	Rotorod
T29	>22	1.52	±1.31 x 10 ⁻²	<i>Pseudogymnoascus roseus</i>	Davis Site 9	06-Jul-01	CZA	Soil
X01	>22	1.78	±9.43 x 10 ⁻³	<i>Mucor racemosus</i>	Davis vegetable store	26-Jan-02	PDA	Potato (soil)
R04	>22	2.33	±1.76 x 10 ⁻²	<i>Penicillium glabrum</i>	Davis beach	10-Dec-01	CZA	Rotorod
A5	>22	2.5	±3.99 x 10 ⁻²	<i>Aspergillus ochraceus</i>	Davis Site 12	27-Dec-01	PDA	Soil
X28	>22	2.65	±1.43 x 10 ⁻²	<i>Phoma macrostoma</i>	Progress II (outside kitchen)	09-Jan-01	CZA	Soil
D08	>22	2.71	±3.84 x 10 ⁻²	<i>Penicillium digitatum</i>	Davis vegetable store	12-Mar-01	PDA	Grapefruit
A4	>22	2.83	±1.07 x 10 ⁻²	<i>Ulocladium botrytis</i>	Davis Site 6	27-Dec-01	CZA	Soil
TMO	>22	3.09	±3.59 x 10 ⁻²	<i>Fusarium oxysporum</i>	Davis hydroponics	22-Dec-01	CZA	Soil
D03	>22	3.75	±3.20 x 10 ⁻²	<i>Fusarium tumidum</i>	Davis vegetable store	26-Mar-01	PDA	Potato
D10	>22	8.11	±2.22 x 10 ⁻¹	<i>Penicillium brevicompactum</i>	Davis hydroponics	30-May-01	PDA	Lettuce
T11	30	0.22	0	Antarctic yeast	Davis Beach	03-Jan-01	PDA	Soil
T09	30	0.22	±3.81 x 10 ⁻³		Davis Living Quarters	08-Dec-01	CZA	Soil

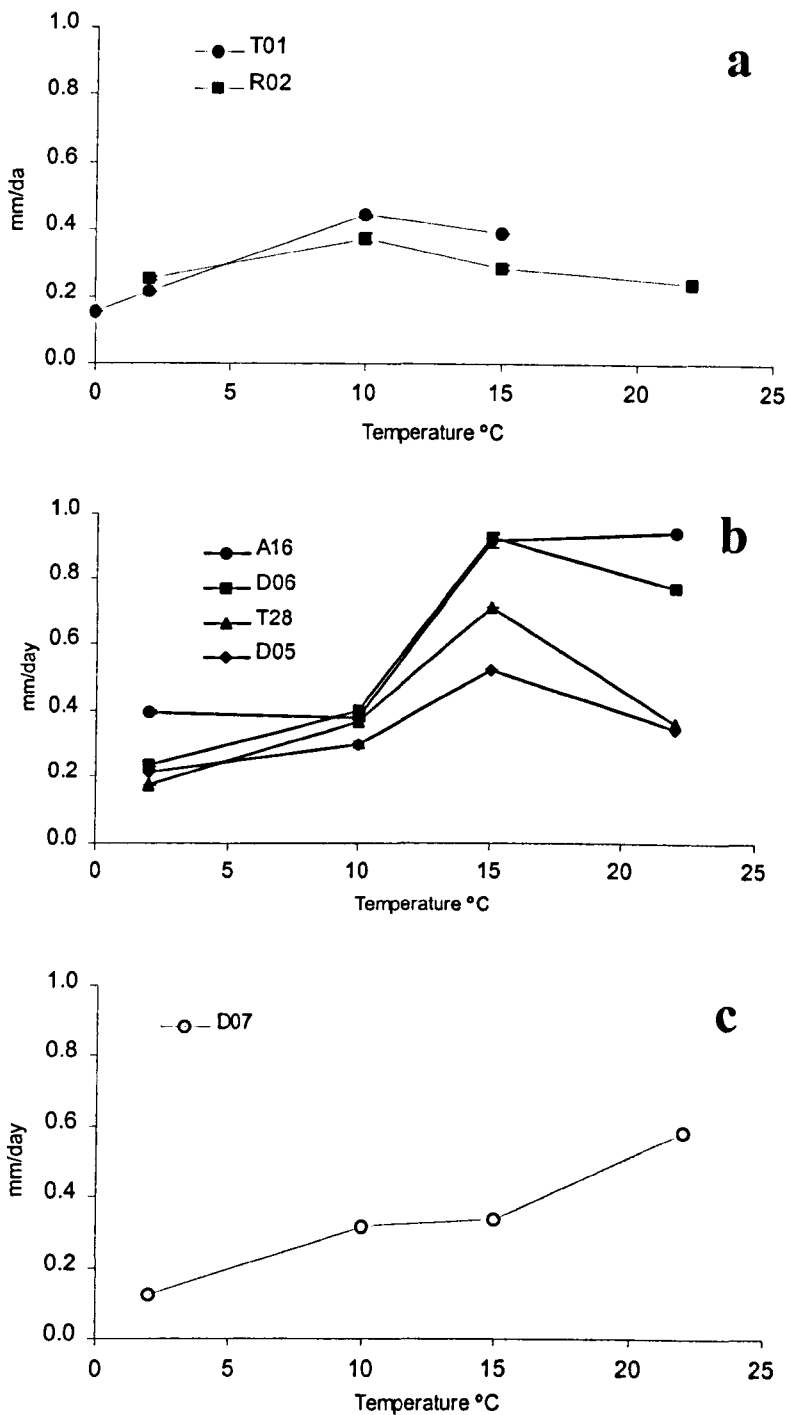


Figure 6.2. Different growth rates recorded for seven isolates of *Penicillium commune* from a range of habitats. a) T01 (soil near Main Store) and R02 (rotorod outside Living Quarters) show optimum growth at 10°C. b) A16 (moss bed near Watts Lake Hut), D06 (dust from cavity wall in Living Quarters), T28 (soil from Davis Science Building main entrance) and D05 (pear from Davis vegetable coldstore) show optimum growth at 15°C. c) D07 (dust from cavity wall in Living Quarters) shows optimum growth at 22°C. Values are shown \pm standard error. Where error bars not visible, values are too small to show.

A similar result was observed with three isolates of *P. brevicompactum* obtained from the hydroponics installation at Davis Station which showed very different growth rates (Figure 6.3) but a similar temperature response.

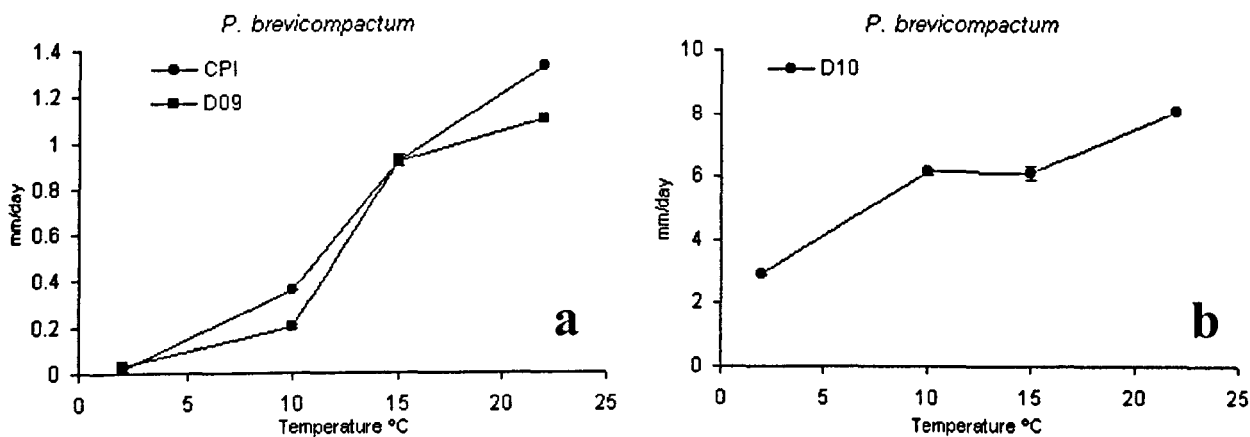


Figure 6.3. Comparison of the growth rates of three isolates of *Penicillium brevicompactum* collected from the hydroponics facility at Davis Station. a) CPI (capsicum plants) & D09 (lettuce plants). b) D10 (lettuce plants). Although the optimum growth temperatures have not been reached, the rate of growth of isolate D10 is more than an order of magnitude greater than the other isolates.

The majority of the isolates examined were found to be cold-tolerant mesophiles. *Phaeosphaeria oreochloae* was isolated from mosses growing near Watts Lake Hut (eastern end of the Ellis Fjord). Although its optimum growing temperature was greater than 22°C, it showed sustained growth at 2°C and an almost linear increase thereafter. Conversely, *Aspergillus ochraceus* showed very slow growth below 10°C followed by an exponential increase above 10°C. An optimum point was not seen in the range 2-22°C indicating an adaptation to higher temperatures (Figure 6.4).

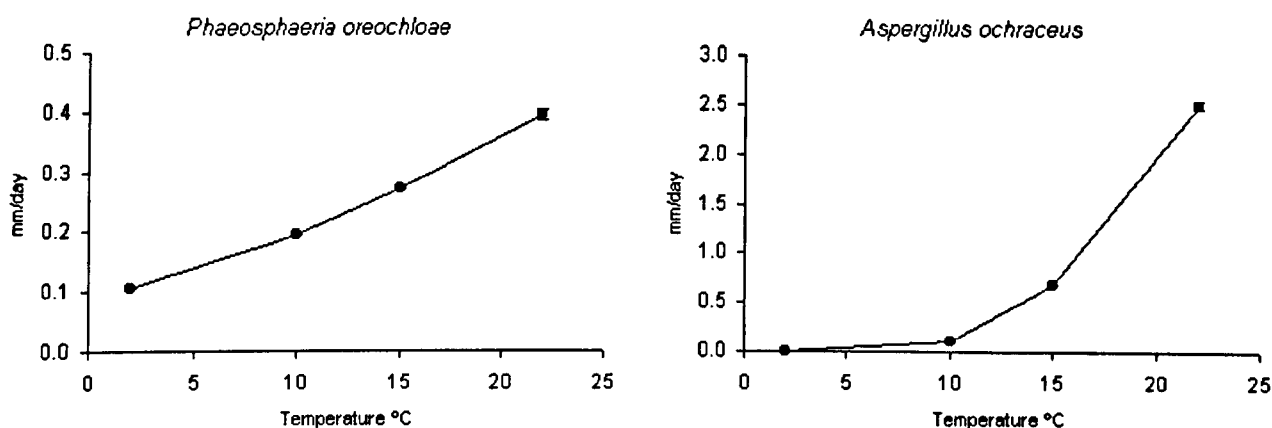


Figure 6.4. Changes in growth rate with increasing temperature in *Phaeosphaeria oreochloae* isolated from moss beds near Watts Lake Hut and *Aspergillus ochraceus* isolated from outside the Living Quarters at Davis Station.

No distinct changes in morphology were observed with respect to the temperatures tested other than the stage at which pigmentation and spores were formed, which was slower to develop at low temperatures.

Following desiccation, all of the isolates, with only a single exception remained viable and produced vigorous growth. The isolate which failed to regrow was T28, one of six isolates of *Penicillium commune* and was originally isolated from the main entrance to the Science building at Davis Station. This would suggest an experimental artefact rather than a fundamental property of the isolate.

6.4. DISCUSSION

It is accepted that the majority of Antarctic fungi are cold-tolerant mesophiles (Kerry, 1990a) and the findings from this study support this. The distinctions between thermal preferences in fungi are indistinct and opinions differ as to where boundaries should be drawn.

Morita (1975), classed organisms with an optimum growth temperature of 15°C or below and able to grow at 0°C as psychrophiles. Furthermore, any fungus capable of growth at 5°C irrespective of optimal temperatures was considered psychrotrophic. All of the 57 isolates examined in the current study were capable of growth at 5°C, although in the majority of cases the optimum growth rate was above 20°C. Nineteen isolates had an optimum temperature of 15°C and three at 10°C and were capable of growth at 0°C, therefore fitting Morita's definition of psychrophiles.

Whilst all isolates fell into the category of psychrotroph (Morita, 1975), the presence of a significant proportion of psychrophiles (33%) is not surprising. Evidence outlined in Chapters 3 & 4 indicate that there is a marked human impact as evidenced by species found around Davis Station not being recorded elsewhere in the Vestfold Hills away from human influence. However, it is likely that many more fungal propagules are introduced than is indicated by the strains isolated. Many will simply be unable to grow, even in the atypical environment of an Antarctic station.

Baross & Morita, (1978) (cited by Zucconi *et al.*, 1996) noted that psychrophiles were characteristic of stable cold environments. The microhabitats within the sampling locations in the current study however, were highly variable and subject to wide temperature variations. Although the original isolations in the current study were performed at room temperature, a factor which can influence the variety of fungi isolated (Carrero & Koske, 1992), it was anticipated that a number of fungi would be isolated which would exhibit a range of thermal preferences due to the variability of the habitats from which they were obtained.

Latter & Heal (1971) noted a thicker aerial mycelium formed at 1°C than at 25°C and suggested a measure of total biomass as an alternative to mycelial expansion when observing growth relative to temperature. Whilst this would be an interesting future study, the scope of the current study would not have extended to include the number of replicates required to perform accurate measurements over time since the determination of biomass by dry weight from fluid culture is destructive. Moreover, Widden & Parkinson (1978) noted different results when comparing solid and liquid media. Whilst neither is directly analogous to a soil environment, it is generally accepted that constant external conditions results in linear growth on solid media whereas growth kinetics in submerged culture are more variable (Smith & Berry, 1974).

Zucconi *et al.*, (1996) reported a series of strains within isolates of *Acremonium* sp. isolated from Victoria Land, Antarctica, which exhibited different thermal preferences. This phenomenon was also observed in the current study within isolates of *Penicillium*

sp. Isolates of both *P.commune* and *P. breicompactum* exhibited different temperature-growth profiles, although several of these isolates were obtained from similar habitats. Some of these isolates were however obtained from vegetable material in the hydroponics facility where the temperature is maintained at around 25°C. These inocula were probably introduced by human activities and were not isolated from any other location.

Whilst certain isolates obtained from similar locations displayed similar thermal preferences (Figure 6.5), others were better adapted to thermally unstable environments which was reflected by the broadness of their temperature growth curves (Figure 6.6). This corroborates the findings of Zucconi *et al.*, (1996).

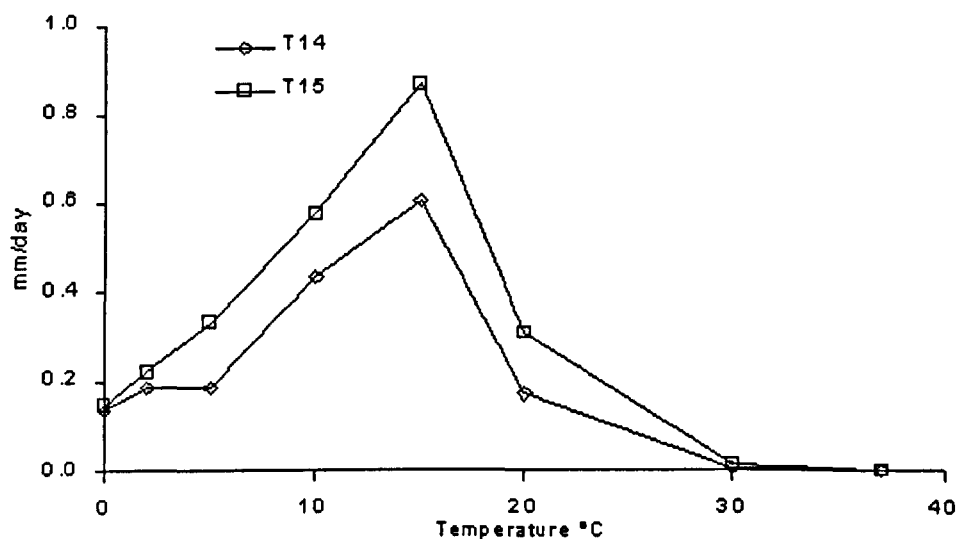


Figure 6.5. Comparison between the growth rates of two unidentified isolates obtained from soils outside Davis Living Quarters showing almost overlapping temperature response curves.

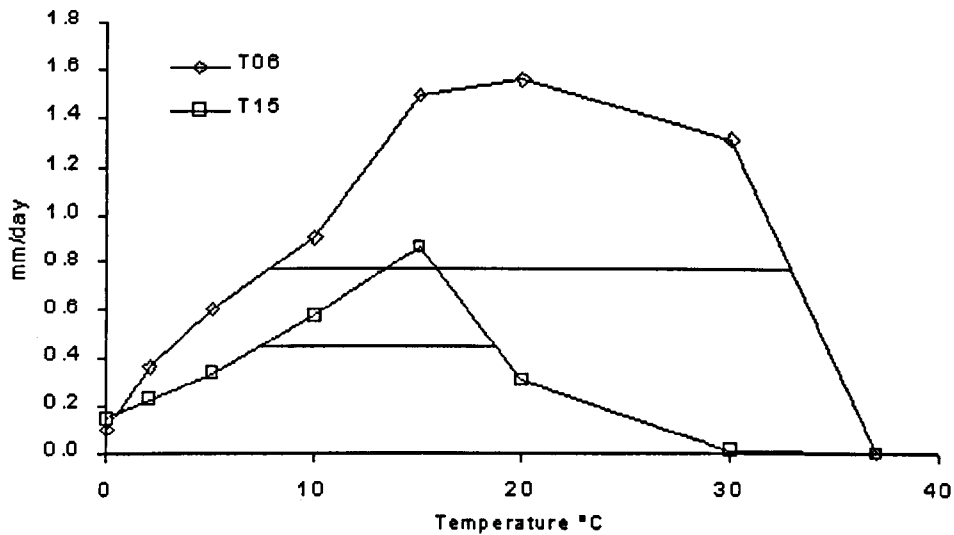


Figure 6.6. Comparison between the growth rates of two unidentified isolates obtained from soils at Davis Station. T06 (outside Davis Science Building) & T15 (outside Davis Living Quarters). The width of the curve at half the optimum rate of T06 is twice that of T15 indicating greater adaptation to thermally unstable environments.

The presence of different thermal responses within the single species *Penicillium commune* (Figure 6.2) is particularly interesting since it suggests physiological adaptation with the existence of distinct strains within a species. Latter & Heal (1971) suggested that fungi isolated from Antarctic soils (Signy Island) were predominantly cosmopolitan species which had become adapted to low temperature by natural selection.

In addition to being psychrotrophic, all the isolates tested (with one possible exception) also appear to be xerotolerant either vegetatively, in the case of sterile mycelia, or through a combination of vegetative means or xerotolerant spore

production in those forms which fruited. This is perhaps not unexpected since low temperature and low available water are the prime factors which limit biological activity in Antarctica.

The most xerotolerant genus is generally accepted to be *Aspergillus* (Dix & Webster, 1995). The genus *Penicillium* also contains a number of xerotolerant forms however, Magan & Lacey, (1984) noted that *Aspergillus* can only compete effectively with *Penicillium* at higher temperatures when water activity (a_w) is low. The Antarctic environment is a combination of predominantly low temperature and low water availability. Whilst *Penicillium* is an extremely cosmopolitan species, this observation may account for the predominance of *Penicillium* over *Aspergillus* throughout the sample sites.

The isolate of *P. commune*, which failed to regrow, whilst possibly an experimental artefact, may rely on spores rather than vegetative hyphal propagules in order to survive desiccation. It is possible that spores were not produced by the culture prior to desiccation, which would account for its failure to recover.

CHAPTER 7: GENERAL DISCUSSION

7.1 BACKGROUND

The fungal flora of Antarctica has been studied on a number of occasions (e.g. Fletcher *et al.*, 1985; Del Frate & Caretta, 1990; McRae & Seppelt, 1999) and isolates representing all fungal divisions with the exception of the oomycota and chytridiomycota, have been identified (although the basidiomycota are so far only represented as yeasts). The majority of these investigations however, have centred around particular habitats such as animal colonies, moss beds, fresh water lakes and glacial ice.

Early expeditioners to Antarctica had poor understanding and therefore scant regard for the fragility of this environment and the consequences of their activities (Draggan & Wilkniss, 1992). With increasing awareness of the ecosystems present, several studies into human impact have been made, for example seabird populations (Micol & Jouventin, 2001), the effects of geological surveys (Kiernan & McConnell, 2001) and the effects of discharge to the marine environment (Jiménez *et al.*, 1999; Smith, 2000).

To date however, studies into the impact of Antarctic stations upon fungal ecosystems have been limited. Kerry (1990b) examined the fungal populations close to and away from human interference at both Davis and Mawson Stations including various packing materials and petroleum contaminated soils. She found relatively few species in the soils and moss beds but found far greater

diversity associated with the stations, which she attributed to the effects of human activity. Aislabie *et al.*, (2001) recorded that *Philalophora* sp. was more abundant on soils contaminated by oil spills whereas *Chrysosporium* sp. was dominant elsewhere in the coastal soils of Scott Base, Ross Sea.

The current investigation examined the fungal populations found in the Vestfold Hills and compared these with those found around Davis Station over a period of 12 months.

7.2. GENERAL FINDINGS

The investigation into the factors affecting the introduction and distribution of fungi in the Vestfold Hills involved a number of different experimental procedures for the isolation, identification and examination of the fungi present. A summary of the individual experiments is given in Table 7.1.

Table 7.1. Summary of main findings from experiments described in Chapters 2-6

	Main Findings
2. The effects of antibiotics on isolation of soil micro-organisms	<ul style="list-style-type: none"> • There was significant interaction ($P < 0.001$) between Rose Bengal and light. • Rose Bengal was toxic to both bacteria and fungi when exposed to the light. • Streptomycin was more effective at reducing bacterial numbers than penicillin. • High concentrations of Penicillin reduced fungal diversity. • A final concentration of 40mgL^{-1} Streptomycin and 25mgL^{-1} Penicillin was found to be adequate for suppressing bacterial growth without impacting upon fungal growth.
3. Sampling of biological particles from Antarctic air	<ul style="list-style-type: none"> • Rotorod samplers were more suited to sampling in the Vestfold Hills than “Frisbee” samplers. • A number of biological particles were present in Antarctic air of which the most prevalent were dinoflagellate cysts, being found at all sample locations. • Seasonal changes were observed with fungal spores. • <i>Geomyces</i> was the most common spore found at Davis station. A relationship existed between numbers of southern elephant seals and numbers of spores collected. • <i>Phaerospharia</i> was found in association with moss beds at the eastern part of the Ellis Fjord.
4. Isolation and enumeration of fungi from Antarctic soil	<ul style="list-style-type: none"> • Fungal propagules were isolated from all sites within the Vestfold Hills • Imported materials were found to carry fungal propagules. • A greater number of fungal propagules were found around the living areas of Davis station as well as neighbouring stations than in the Ellis Fjord. • A relationship existed between the number of fungal propagules isolated and the degree of human impact within Davis Station. • Seasonal variation was observed with numbers of fungal and other particles recorded.
5. Extraction, purification and amplification of fungal DNA for identification by sequencing	<ul style="list-style-type: none"> • DNA was obtained from 49 filamentous and 6 yeast species representing 19 hyphal genera and 4 yeast genera. • <i>Penicillium</i> was the most prevalent genus amongst the isolates although mainly found at Davis Station. • A relationship was shown between optimum growth temperatures and genetic groupings in <i>Penicillium</i>.
6. The effects of climate and desiccation on fungal growth	<ul style="list-style-type: none"> • A total of 57 isolates were examined and a range of temperature growth curves was obtained. • All isolates were psychrotrophs, 22 were psychrophiles, the remaining 35 were mesophiles. • More than one temperature growth profile exists for species of <i>Penicillium</i> with different optimum growth temperatures found • A genetic relationship was found between strains of <i>Penicillium</i> with similar temperature growth profiles. • All isolates (with only one possible exception) were tolerant of complete desiccation, either through physiological adaptation of the tissues or the production of resistant spores.

7.2.1. Microbial introduction into Antarctica by natural methods

A number of mechanisms exist by which micro-organisms can be introduced into a continent, however Antarctica is very different from other continents. The Southern Ocean not only presents a broad physical barrier but the temperature of the seawater is low and ice surrounds the continent for much of the year. Consequently, soil quality is poor and vegetation is sparse and of low diversity.

For a micro-organism to colonise the continent, it must be able to survive being carried by a migrating animal or be transported by wind or sea. With the exception of the Antarctic peninsula, the distance from other land masses is great, so that objects drifting in the sea must remain afloat in brine for prolonged periods and be able to exploit a substrate upon arrival. To date, no evidence of maritime fungal introduction has been recorded.

Wynn-Williams (1991) and Marshall (1996) examined meteorological events as a means of fungal introduction into Antarctica. Residence time for fungal propagules in the atmosphere is largely dependant upon physical dimensions with durations as long as 5 years being reported (Imshenetsky *et al.*, 1978, cited by Wynn-Williams, 1991). Marshall (1996) reported a dramatic influx of material into Antarctica which was correlated with a mass air movement from South America.

All of the avian fauna and most of the mammalian Antarctic species are migratory providing another potential means of propagule transport. Skuas are

scavengers which feed on a variety of prey and carrion (Votier *et al.*, 2003) and have also established intimate associations with Antarctic stations due to the feeding potential. Mund & Miller, (1995) observed a direct relationship between human refuse found in regurgitated pellets and faeces and distance from McMurdo Station. Skuas occur naturally around penguin rookeries and snow petrel nesting sites where they prey upon young birds. In the current study, four fungal isolates including one yeast were recovered from swabs taken from skua feet, indicating that at least in the area around Davis Station, skuas are capable of acting as vectors for fungal propagules.

7.2.2. Microbial introduction into Antarctica by human activity

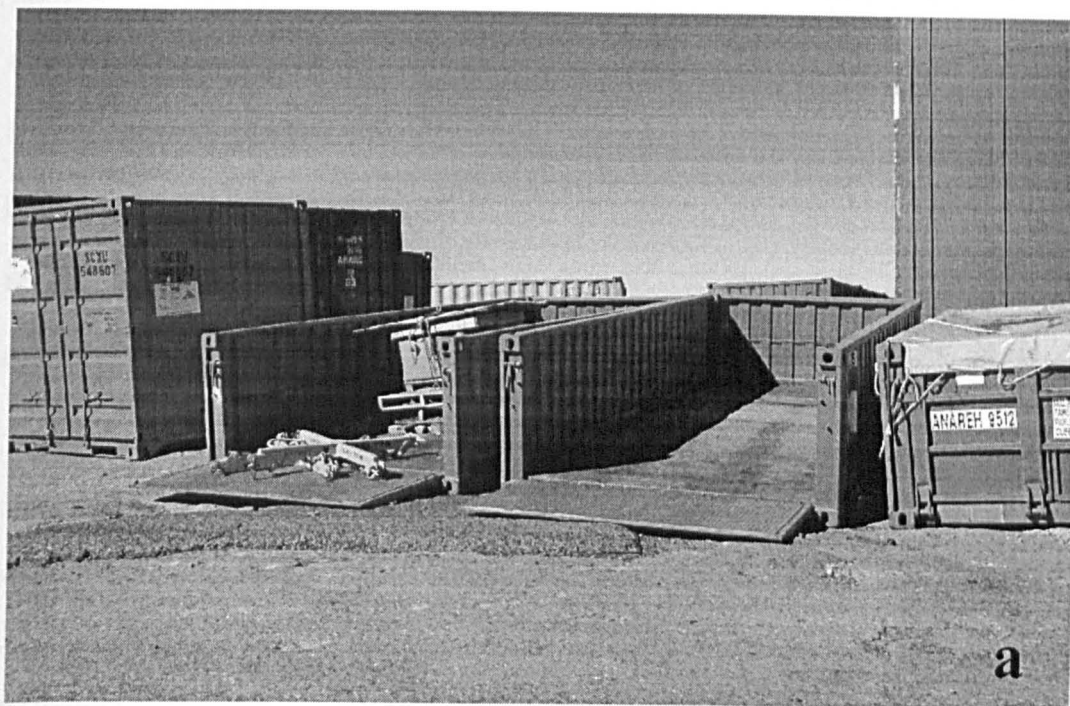
Human activity in Antarctica has increased greatly throughout the 20th century which has inevitably resulted in the introduction of micro-organisms to the environment. Dogs were also used for transport on the continent from the earliest explorations until their use was prohibited by the Antarctic Treaty (1991 Environmental Protocol, annex II).

Introduction of an inoculum may not be the only issue however. The presence of an Antarctic station also provides potential for microhabitats of elevated temperatures and where water availability may not be a limiting factor. Increased carbon sources are also inevitable due to human and domestic waste coupled with pollution incidents such as fuel oil spills (Kerry, 1990b; Aislabie *et al.*, 2001).

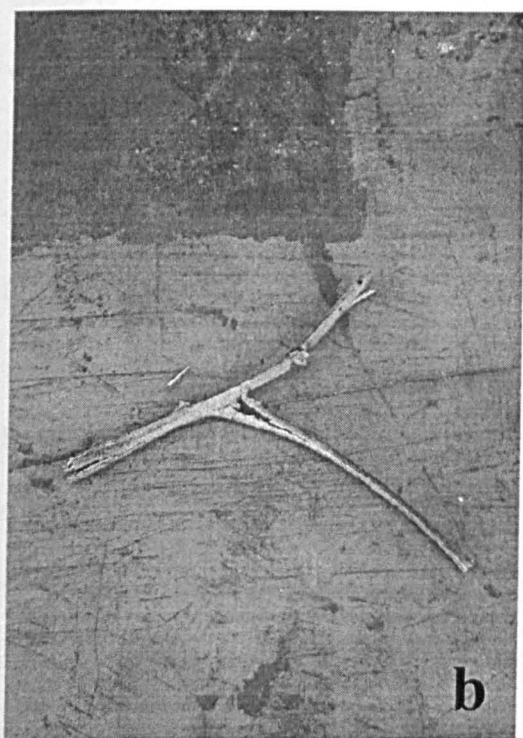
In the current study, increased numbers of fungal CFUs, airborne fungal spores and fungal diversities were found associated with Davis Station especially outside the living quarters. Similar results were found at Law Base, Progress II and Zhong Shan stations. When compared with the results obtained from the Ellis Fjord, these data represented a significant difference ($P < 0.001$).

7.2.2.1. Effect of resupply

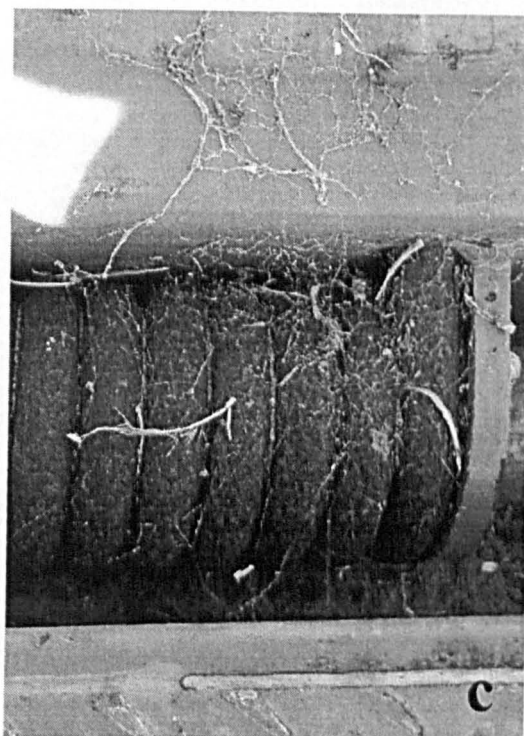
Antarctic stations typically receive one major resupply every year. During this period, there is a large influx of new materials coupled with a period of high activity around the station. The ground is constantly being disturbed by people and vehicles as new sea containers are brought ashore and discarded materials, which may have been stored for long periods, are removed. Sea containers (Plate 7.1) have often been allowed to stand exposed before shipment to Antarctica and in so doing, may have collected a variety of contaminants ranging from soil around the base, to leaves and debris accumulating within them. Spiders' webs are also common which are known to be efficient collectors of micro-organisms as well as other organic material (Handley & Roe, 1994). Although less frequent than sea containers, vehicles are occasionally delivered to Antarctic stations which may also carry foreign materials on their tyres and wheel arches.



a



b



c

Plate 7.1. (a) Half-height sea containers having been unloaded. (b) Tree debris in the base of a sea container. (c) Spiders' webs accumulating organic material around a sea container.

Two annual station resupply operations were examined during the period of study. The first resupply (25th to 29th October 2000) was studied by soil sampling only with samples being collected from a number of sites around the station before and after the event. The same approach was adopted for the second resupply (1st to 4th December 2001) but with additional rotorod samples being collected throughout the event (Chapter 3).

Although it was generally accepted that this is the busiest time around the station with the greatest number of personnel and the greatest activity, few changes were observed over the period of the first resupply. A significant increase in fungal CFUs ($P < 0.001$) was observed in soil outside the main store and also from the wharf area ($P = 0.03$) where sea containers were brought ashore and those which had been stored there were removed for shipping. No increase in fungal CFUs was observed in the other areas of high activity such as the Living Quarters or the helipads.

Extreme weather was responsible for the poor data obtained during the second resupply since high winds caused a large amount of blown dust and snow. Large quantities of wind blown dust render the collecting surfaces of the rotorod arms almost impossible to examine by eye under the microscope whilst snow has the effect of blocking them and preventing particles from being collected. No significant differences were observed with fungal CFUs from the soil sample sites used during the first resupply.

It would appear therefore, that although organic matter was being imported and human activity was high, the actual period of resupply did not greatly impact upon the mycoflora present around Davis Station.

7.3. LIMITATIONS

Whilst the techniques employed throughout the investigation have been used routinely in the past by other investigators, it is important to understand the limitations of these techniques, the difficulty of working in a remote environment and the degree to which these could impact upon a study.

7.3.1. Limitations of sampling

Sampling from remote field sites was limited by two factors; weather and ground conditions. Falling snow or high winds causing drifting snow not only made trips into the field hazardous but also blocked the collecting surfaces of the rotorods preventing reliable sampling. High winds during dry conditions also resulted in large volumes of dust accumulating on rotorod collecting surfaces making accurate counting by eye impossible. The availability of personnel and transport was also occasionally a limiting factor. Fieldwork requires a minimum of two persons for safety reasons and transport of equipment into the field required either additional personnel or mechanised transport. During the summer period, many projects are run in parallel which leads to high demands on transport and logistic resources.

The number of sample sites and consequently transects was limited by the number of available rotorod samplers and batteries, the time required to set them up and the time and consumables required to process them on return to the laboratory.

7.3.2. Limitations of culture

A major limitation of the investigation was that the isolation techniques used were limited to showing only viable and culturable fungi. A great proportion of fungi are known to be unculturable under artificial conditions (Kaeberlein *et al.*, 2002) and may require a molecular approach to identification (Gao & Moore, 1996). Many fungal spores, which may be present in any given ecosystem may not be viable and the likelihood of this is almost certainly increased in extreme environments such as that of Antarctica. Such limitations are faced in all soil fungal studies since no 'ideal' procedure exists which can provide reliable values for total numbers present. The use of soil dilution plates, reflects general community changes (Klironomos &) although a bias towards r-strategists may skew results.

The soil dilution plate study was restricted to the use of two agar media (potato dextrose & Czapek Dox) and a single soil dilution due to resource limitations. The technique did however, provide a simple and consistent method which allowed a cost and labour effective long-term investigation of the study areas. Whilst molecular techniques maybe employed for specific assays for future

studies, many of the sample sites had not been investigated before and therefore a pilot study of this type was necessary.

7.4. CONCLUSIONS

The fungal populations found in the soil and air around Antarctic stations (Davis Station, Law Base, Zhong Shan and Progress II) are significantly different from those found elsewhere in the Vestfold Hills. Since the fungi encountered in and around the stations was different from those found in the surrounding area, it is reasonable to assume that the differences in numbers is not merely the product of a different habitat, but a microflora which has been introduced through human activity and sustained either by reinoculation from stored materials or sustained by an artificial habitat. Whilst several types of fungi were identified from the Ellis Fjord, it is not possible to determine exactly when these became established and what the means of initial inoculation was.

Whilst the fungal flora was significantly higher around areas of human habitation, the current study failed to detect species which occurred both around the station and elsewhere in the Vestfold Hills. Furthermore, the absence of a decreasing zone of contamination around the station would indicate that few, if any fungal strains were able to survive, even in a quiescent state, in the surrounding area.

The possibility that exotic fungal species have become established in Antarctic habitats cannot be ruled out. Indeed the potential for the accidental introduction

of a micro-organism is always present. It is therefore important that the current precautions, especially those where wildlife is, concerned are continued.

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ASSESSMENT OF FUNGAL DIVERSITY

$$D = 1 - \sum_{i=1}^s (\rho_i)^2$$

where D = diversity

s = number species

ρ_i = proportion of the total sample belonging to the i^{th} species

LOCATIONS OF THE TRANSECT SAMPLE SITES

Two transects were set up each comprising five sites. The first transect ran through Davis Station from Dingle Road in the north, through the station and extended across Heidemann Bay to the south (Figure A1 & Plate A1).

Sample site: Dingle Road

Location: $S68^{\circ} 34' 54.3'' E77^{\circ} 58' 11.4''$

A slightly elevated piece of ground 100m north east from the station, set back several meters from a roadway.

Sample site: Living Quarters

Location: $S68^{\circ} 34' 58.8'' E77^{\circ} 57' 59.4''$

10m west of the main entrance to the Living Quarters & mess area.

Sample site: Davis Beach

Location: $S68^{\circ} 35' 59.1'' E77^{\circ} 58' 5.0''$

50m due south of the main living quarters, on the southern reaches of the beach around 30m from the shoreline.

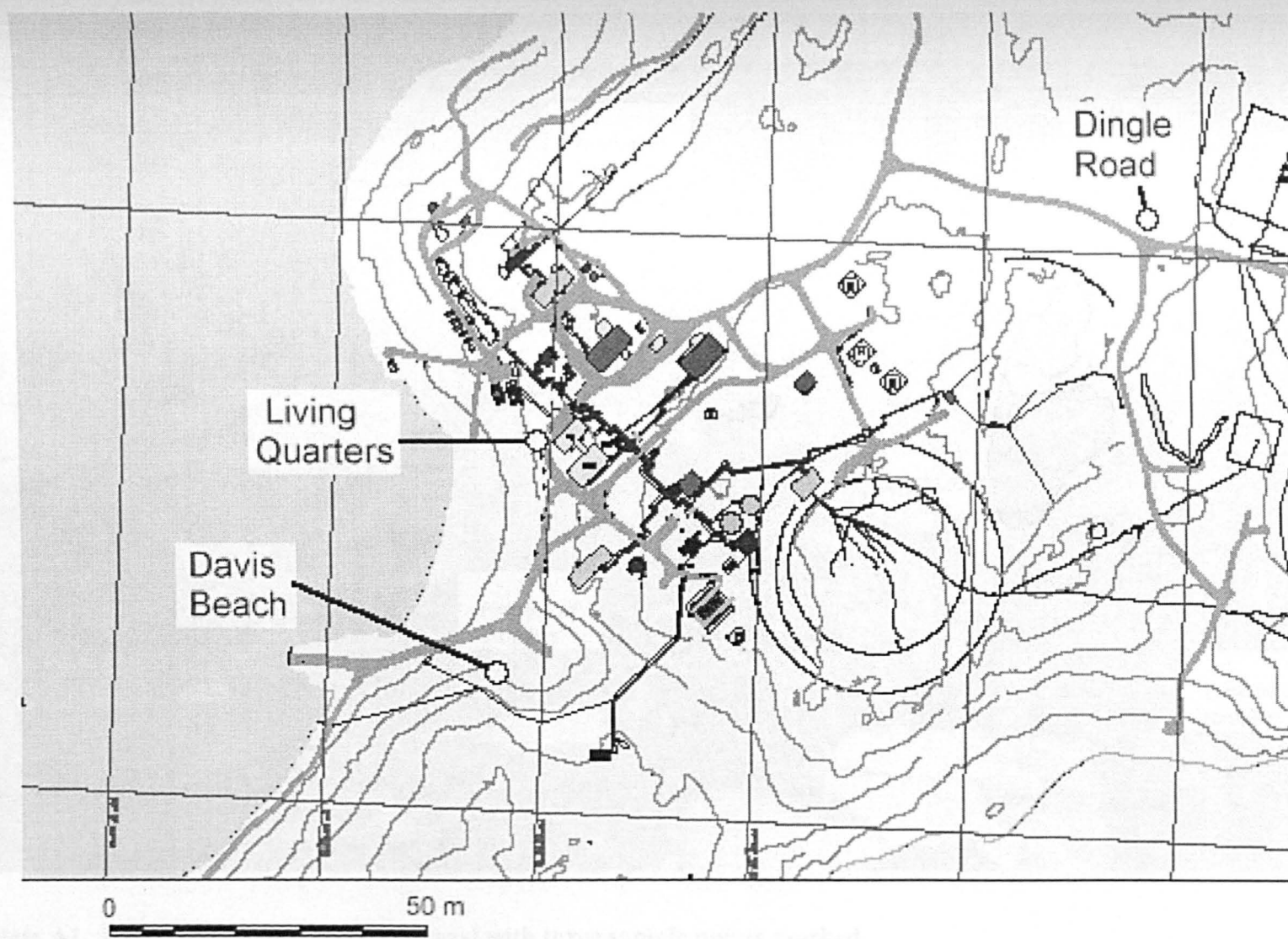


Figure A1. Davis Station showing three sites from a transect which extended south across Heidemann Bay.

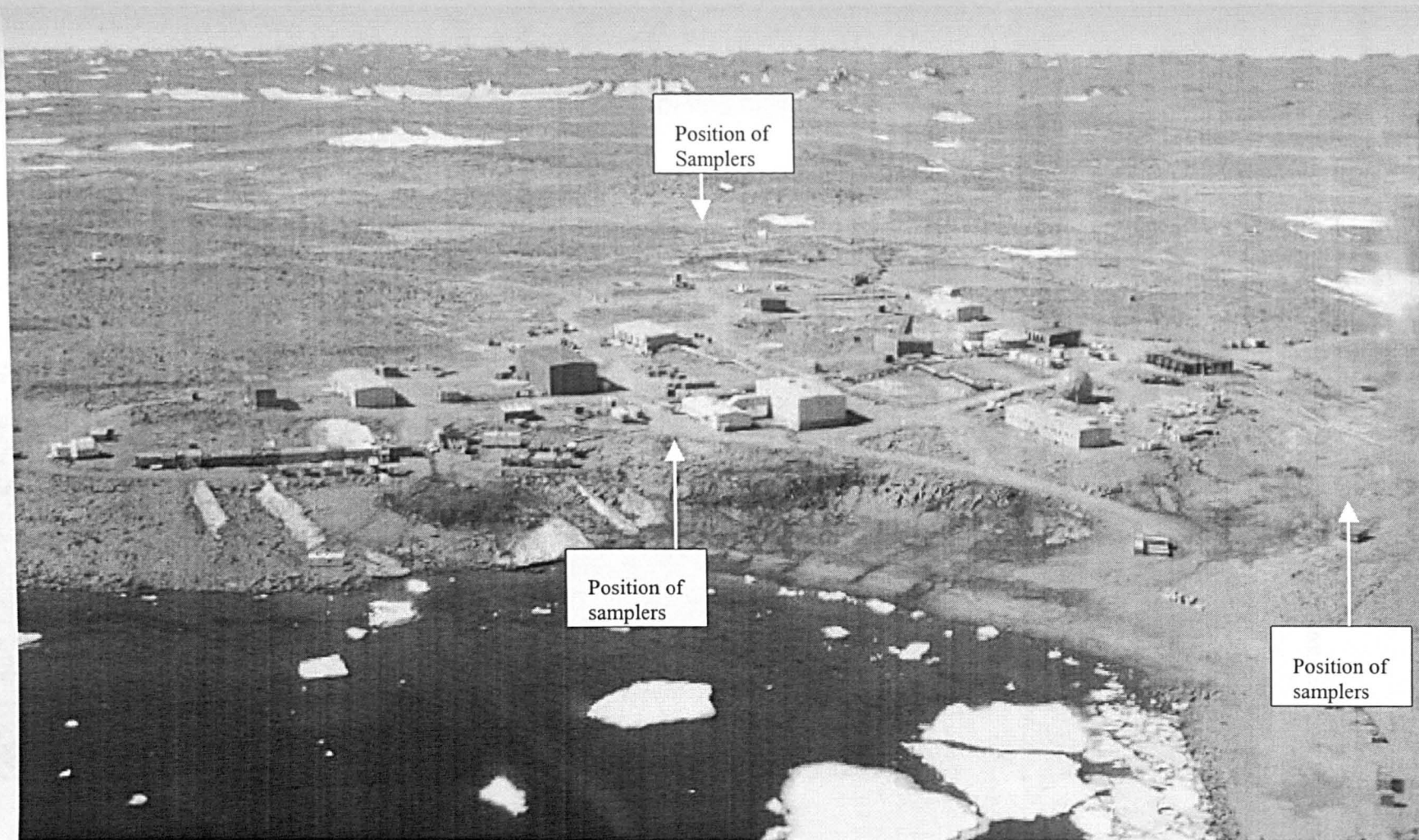
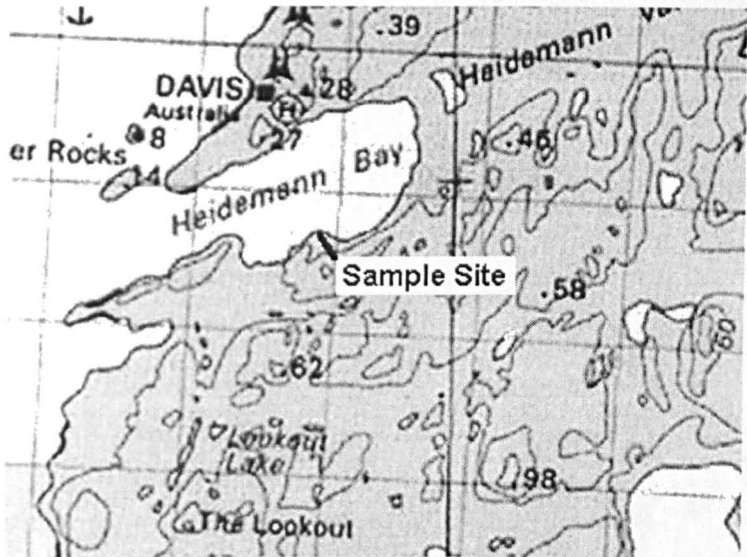


Plate A1. Davis Station, looking north east with three sample points marked.

Sample site: Heidemann Bay

Location: $S68^{\circ} 35' 10.1'' E77^{\circ} 58' 34.0''$

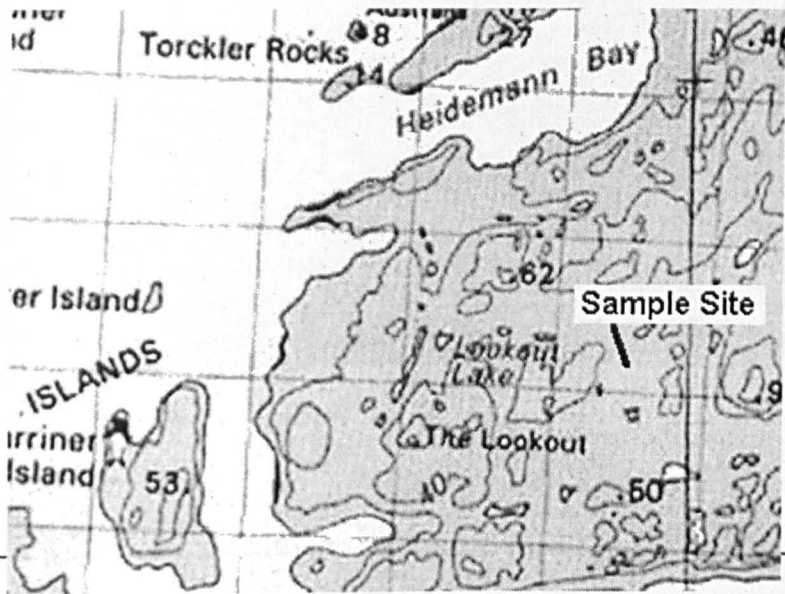
1km south of the station on the northern shore of Heidemann Bay, set back from the ice/waters edge by around 3m.



Sample site: The Lookout

Location: $S68^{\circ} 35' 55.9'' E77^{\circ} 58' 12.3''$

A knoll raised about 5m from the surrounding basin. Around 1km north east of a promontory known as "The Lookout".



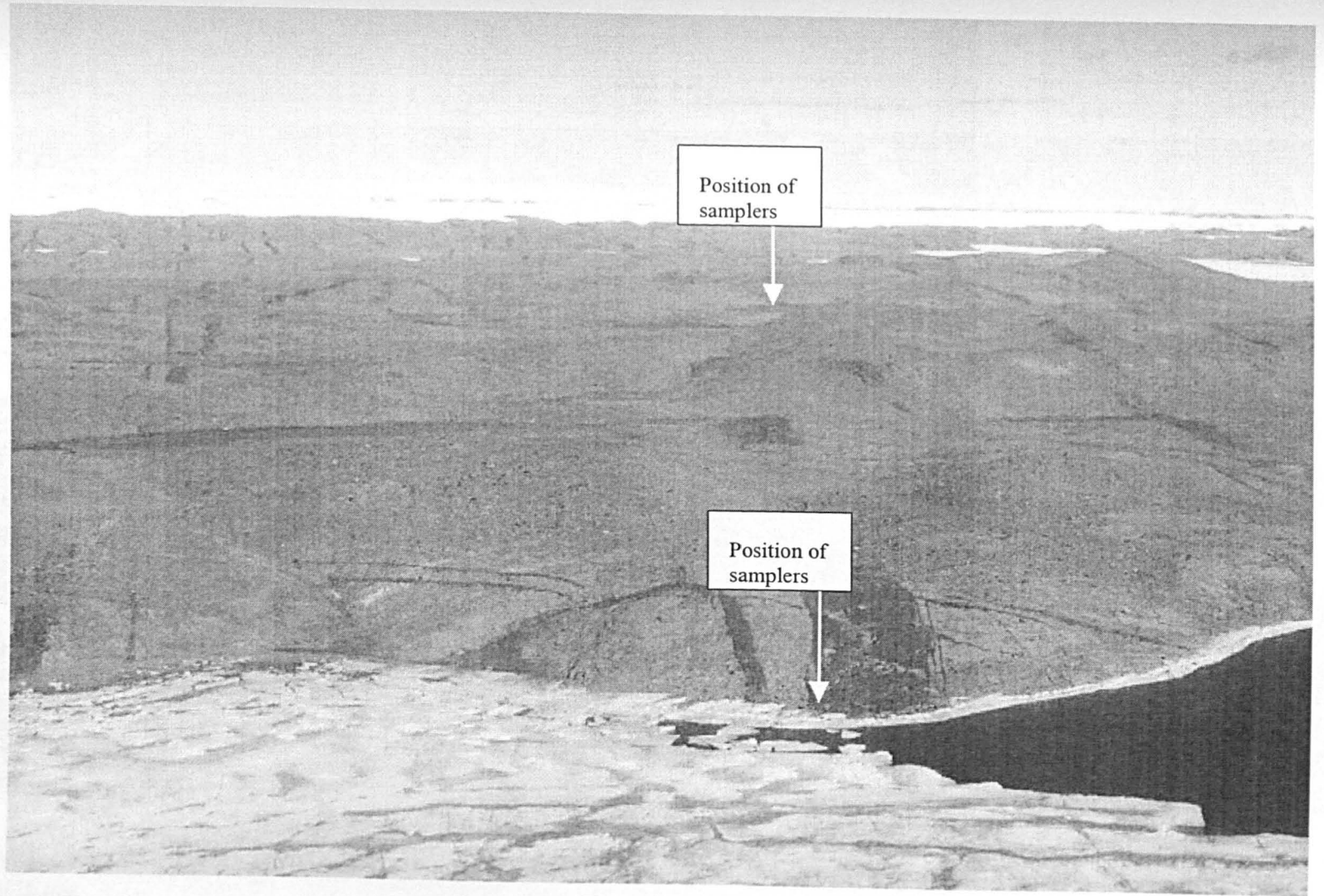


Plate A2. Heidemann Bay looking south towards the Ellis Rapids showing the locations of the sample sites at Heidemann Bay and "The Lookout"

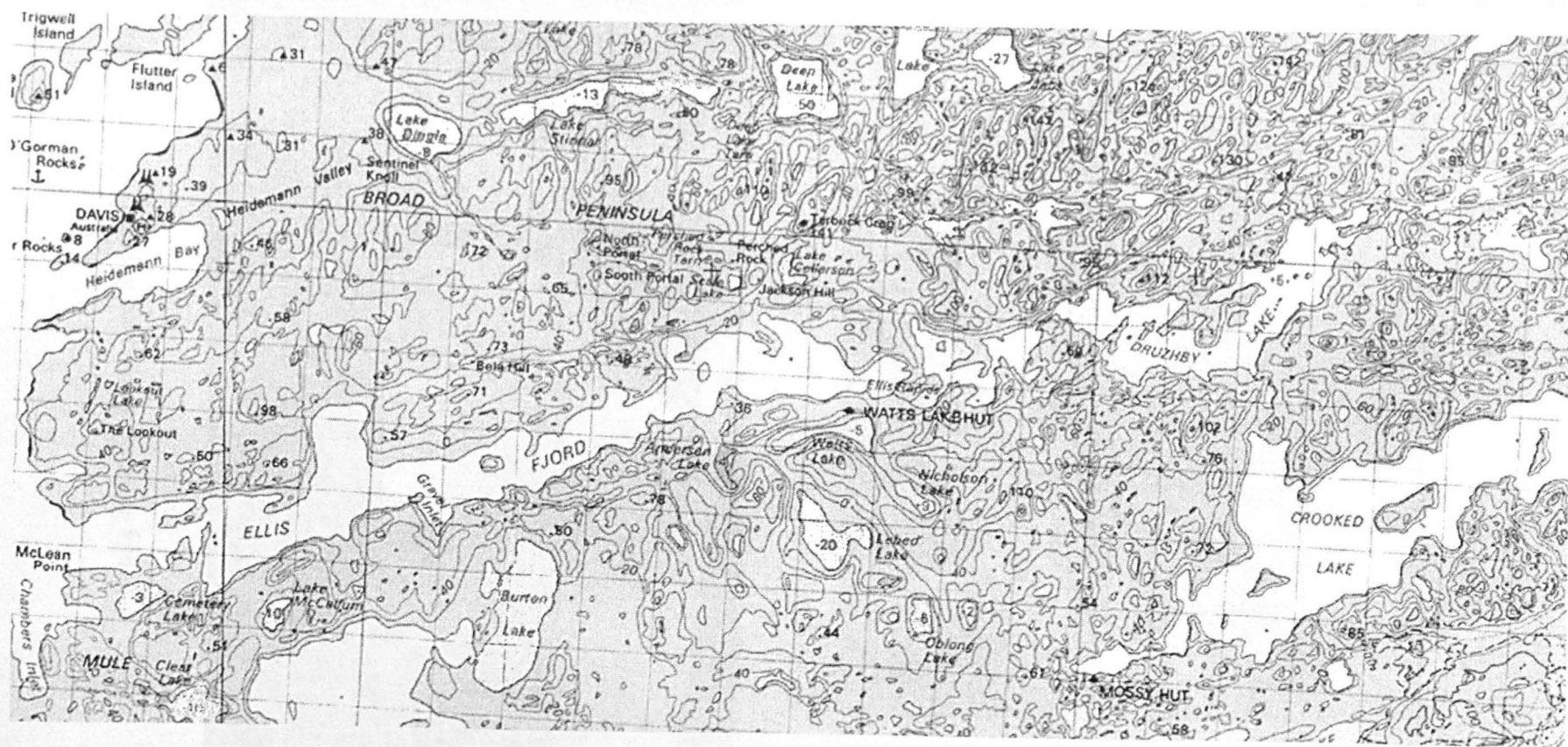


Figure A1. The position of Davis Station relative to the Ellis Fjord. Map courtesy of the Australian Antarctic Division.

Sample site: Ellis Narrows

Location: $S68^{\circ} 36' 49.2''$ $E77^{\circ} 59' 27.0''$

A low bridge of land forming a spit extending part of the way across the seaward entrance to the Ellis Fjord

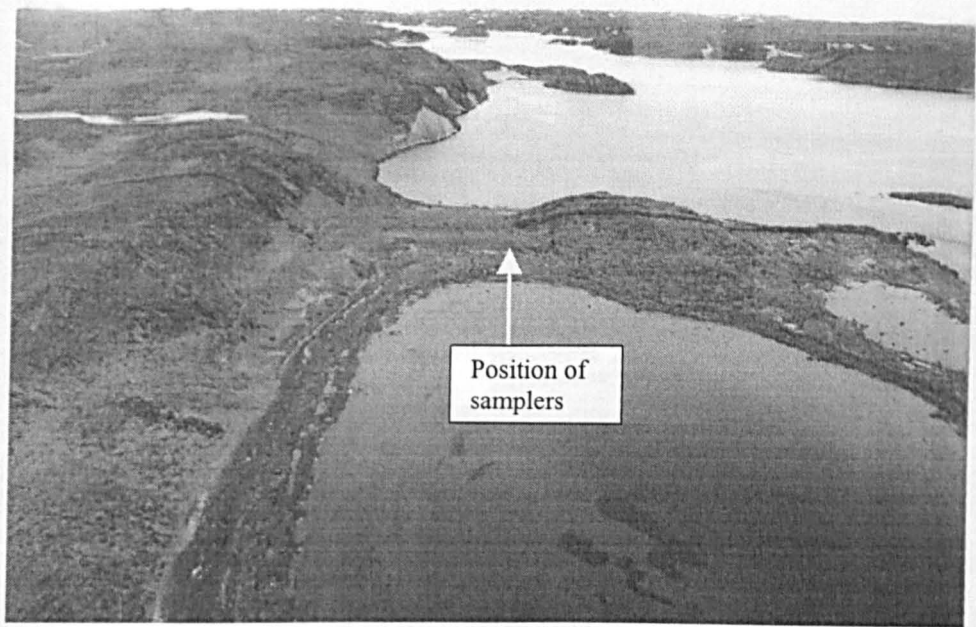
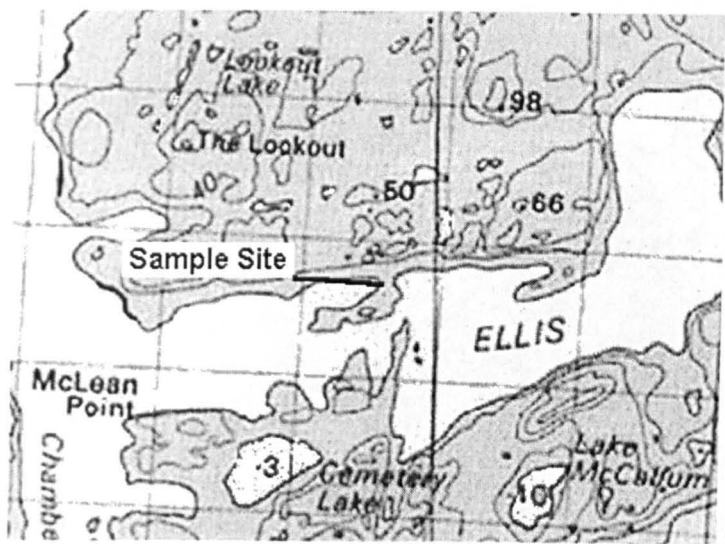


Plate A3. Ellis Narrows, looking east along the Ellis Fjord.

Sample site: Flat Top Peninsula

Location: $S68^{\circ}36'23.8''$ $E78^{\circ}06'18.0''$

The southern tip of a peninsula extending into the Ellis Fjord around 300m east of Flat Top Island.

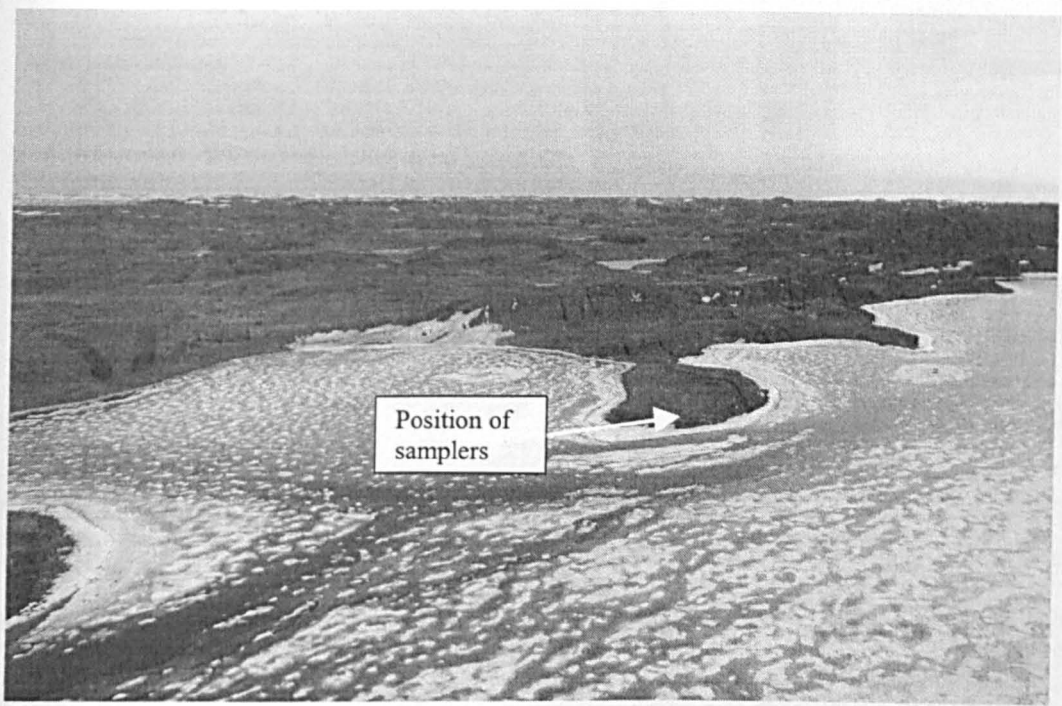
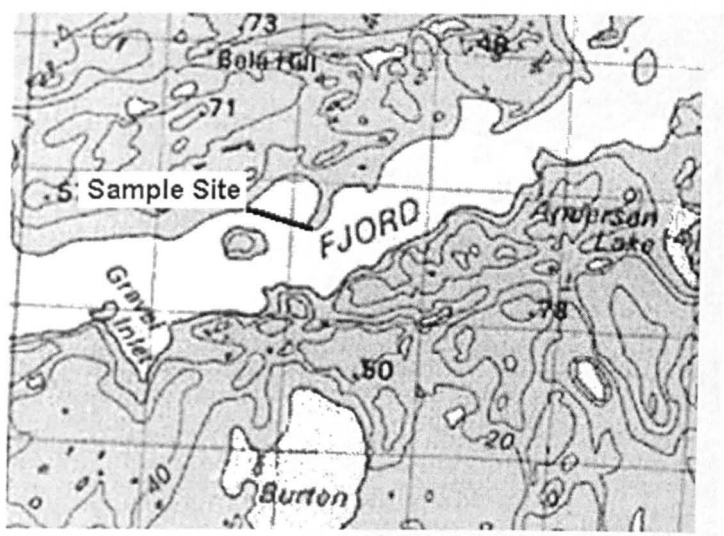


Plate A4. Flat Top Peninsula, looking north east from the Ellis Fjord. Flat Top Island may be seen at the left of the picture.

Sample site: Watts Lake Hut

Location: $S68^{\circ} 35' 55.9''$ $E78^{\circ} 13' 18.5''$

An area of low-lying beach some 40-50m north west of the field hut which lies between the Ellis Fjord and Watts Lake.

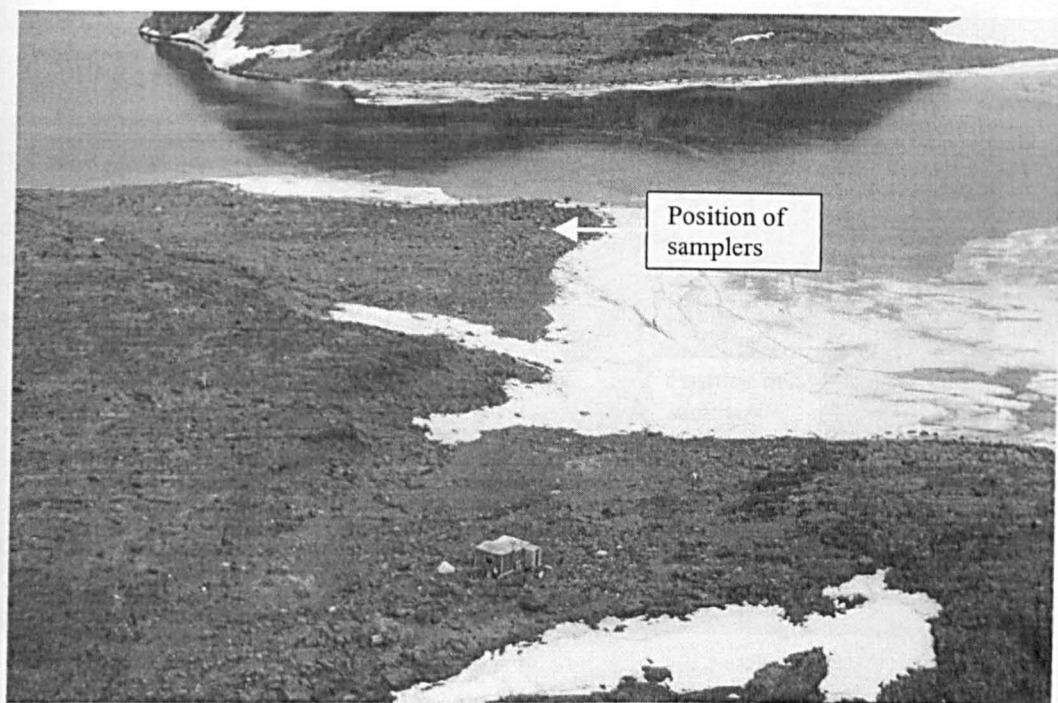
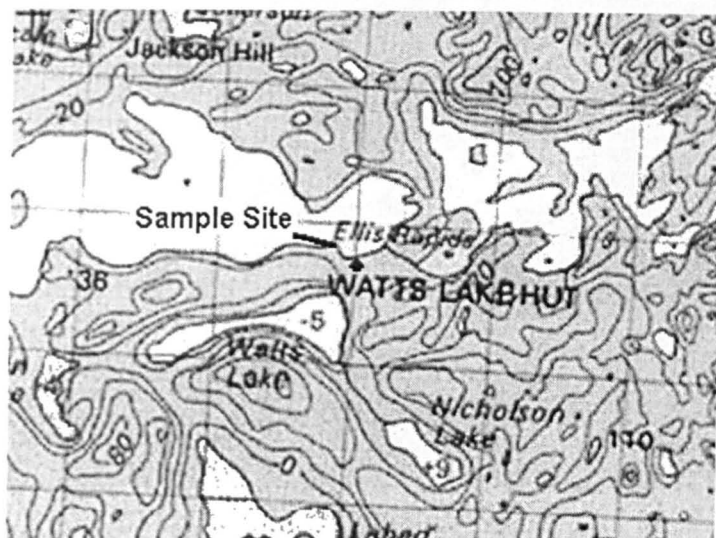


Plate A5. Eastern tip of Ellis Fjord looking north. Watts Lake Hut may be seen in the foreground of the picture, Watts Lake lies c.a. 50m to the south.

Sample site: Lake Druzhby

Location: $S68^{\circ} 35' 10.8''$ $E78^{\circ} 19' 28.2''$

A rocky beach on the northern shore of Lake Druzhby

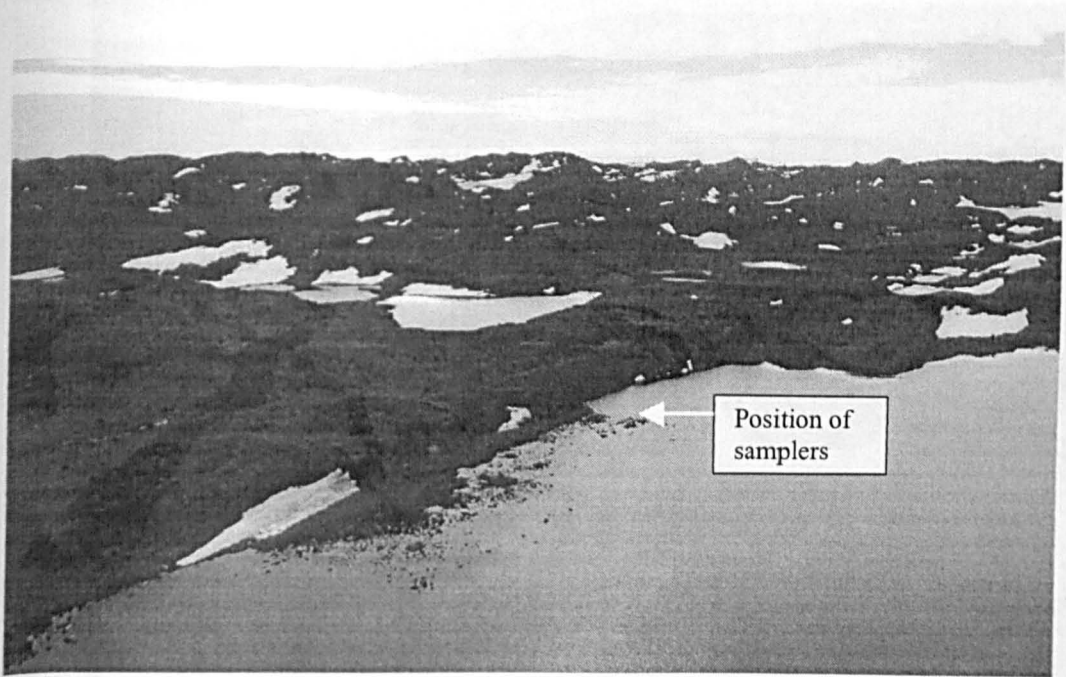
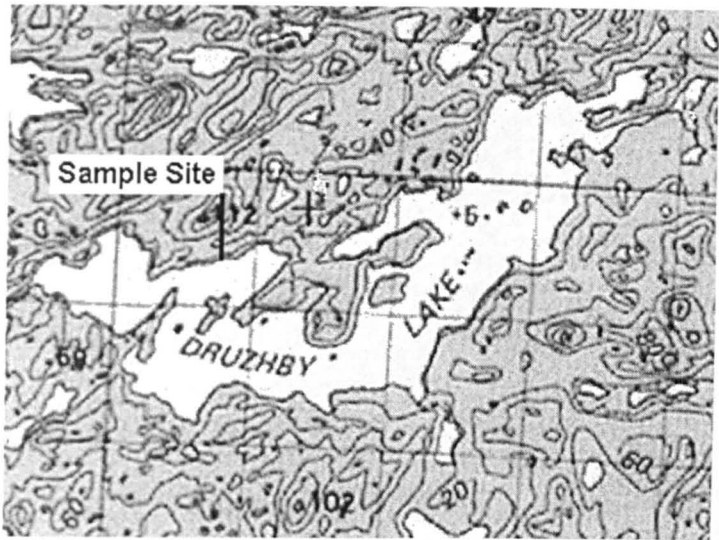


Plate A6. Northern shore of Lake Druzhby looking north east

Sample site: Trajer Ridge

Location: $S68^{\circ} 34' 26.9''$ $E78^{\circ} 24' 28.3''$

A raised outcrop of rock surrounded by ice at the western tip of Trajer Ridge

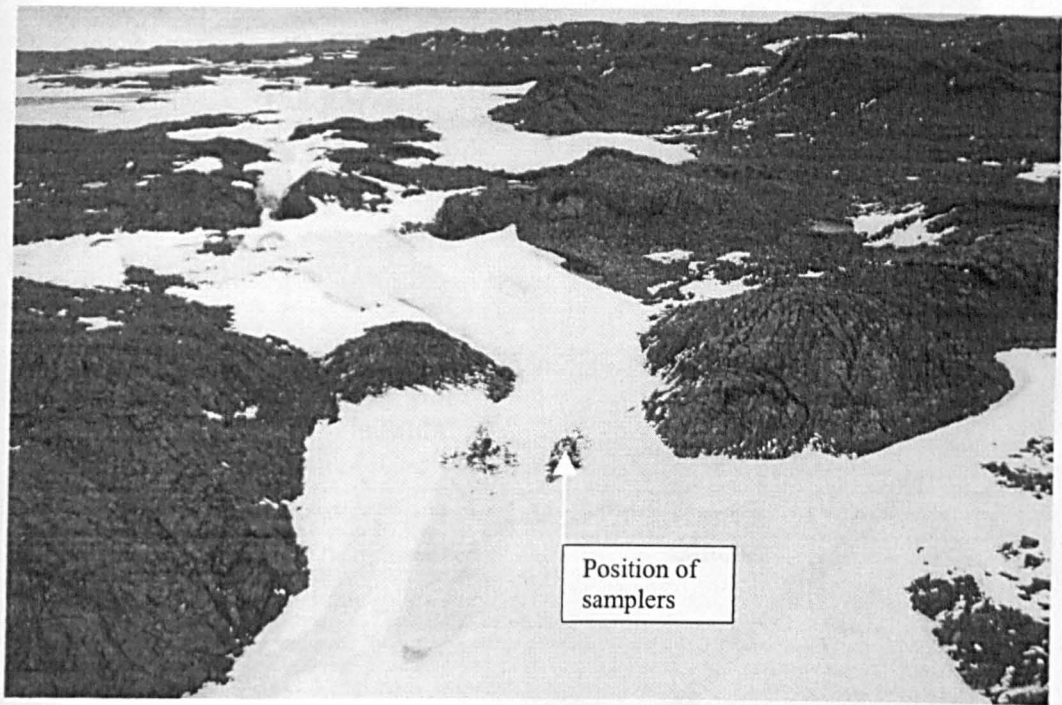


Plate A7. Western tip of Trajer Ridge, looking west over Lake Druzhby.

Sample site: Law Base, Zhong Shan & Progress II Stations

Location: $S69^{\circ} 23'$ $E76^{\circ} 23'$

Standing within 5km of each other in the Larsemann Hills to the south of Davis Station.

Law Base is about 1km inland on a raised area of ground, it comprised a collection of temporary huts. Zhong Shan and Progress II are both on coastal locations and typically accommodate 20 to 30 persons.

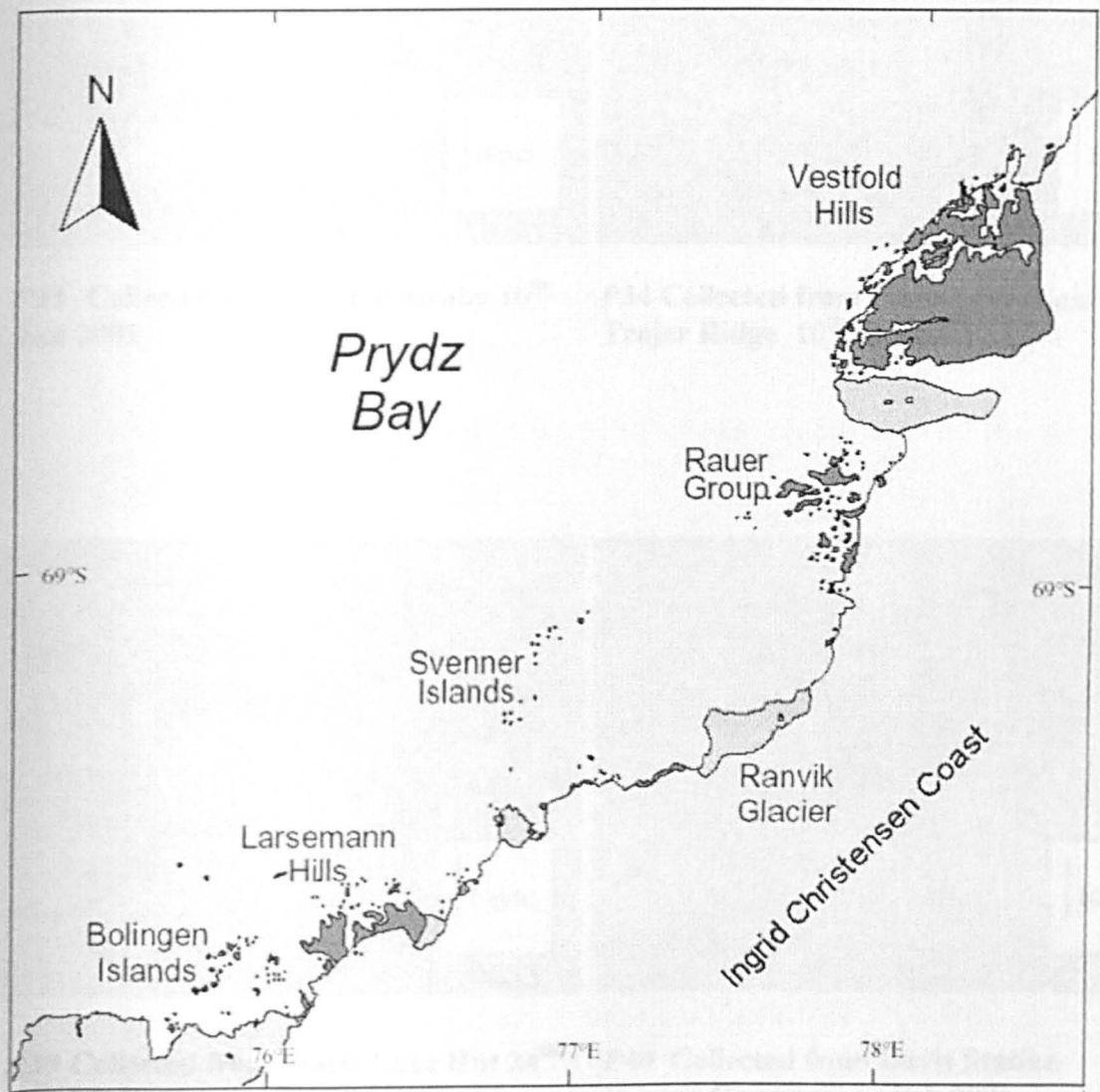
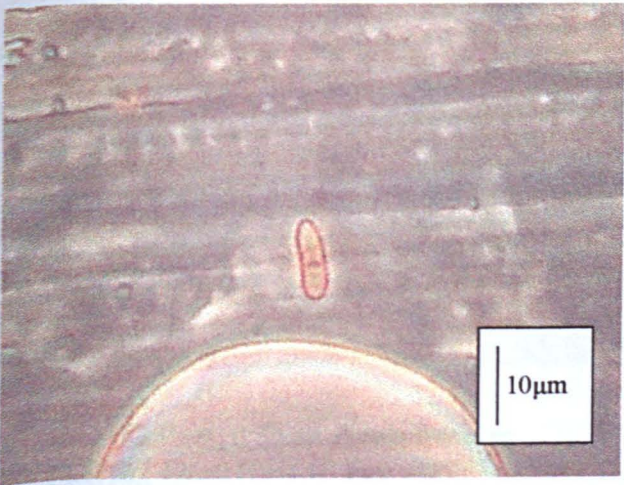


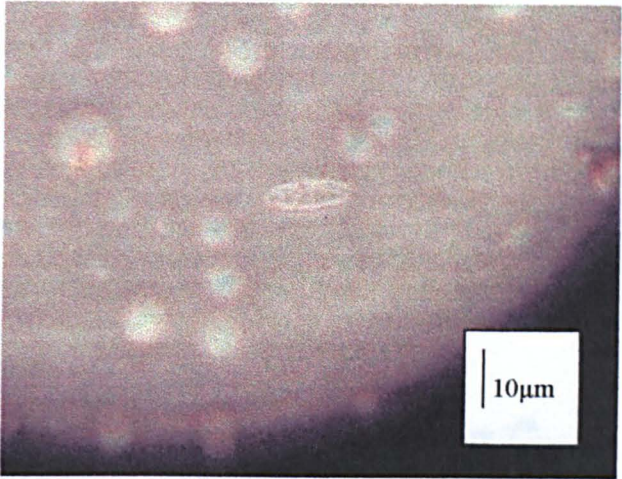
Plate A8. Location of the Larsemann Hills with respect to Davis Station and the Vestfold Hills. (Map courtesy of the Australian Antarctic Division).

APPENDIX 3

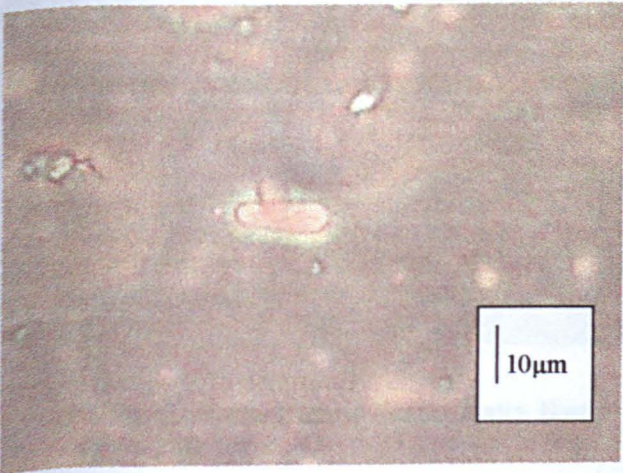
Fungal particle catalogue



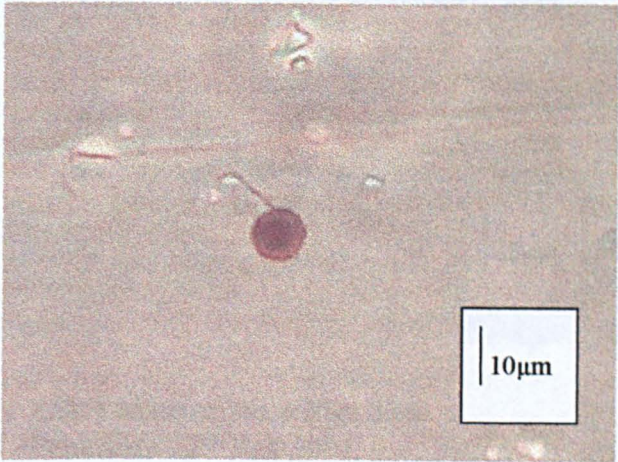
P31 Collected from Lake Druzhby 10th Jan 2001



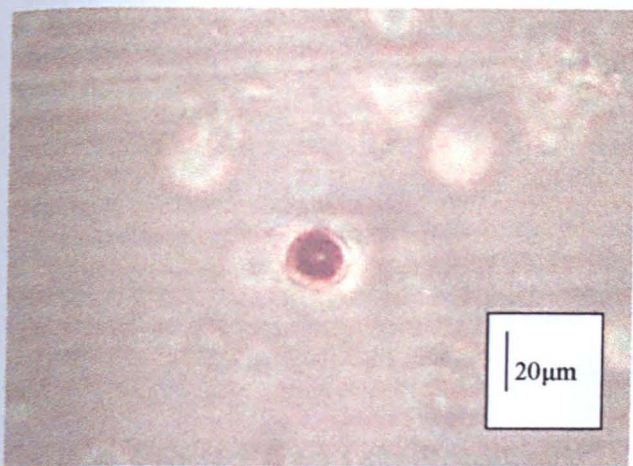
P34 Collected from Pauk Lake, Near Trajer Ridge 10th Jan 2001



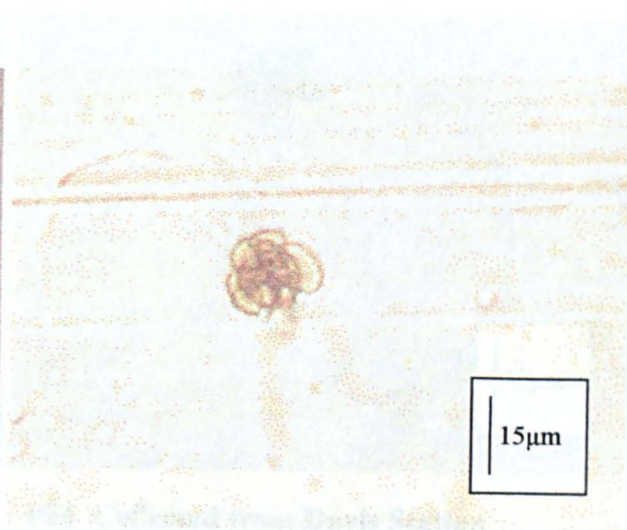
P39 Collected from Watts Lake Hut 24th Jan 2001



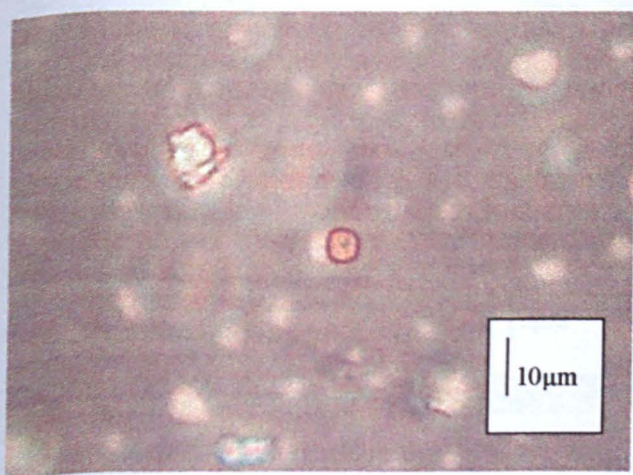
P40 Collected from Davis Station (Living Quarters) 1st Feb 2001



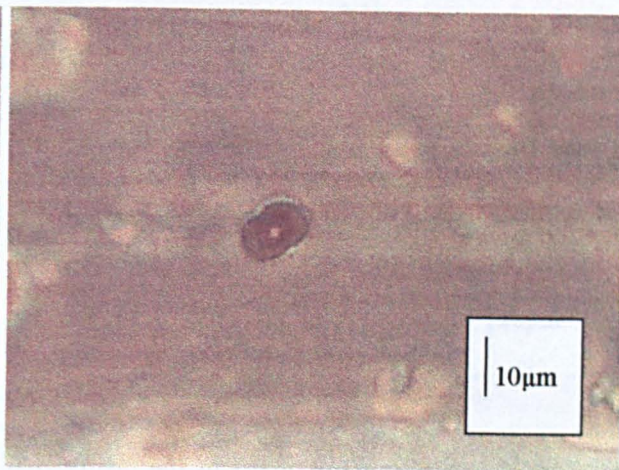
P41 Collected from Davis Station
(Living Quarters) 1st Feb 2001



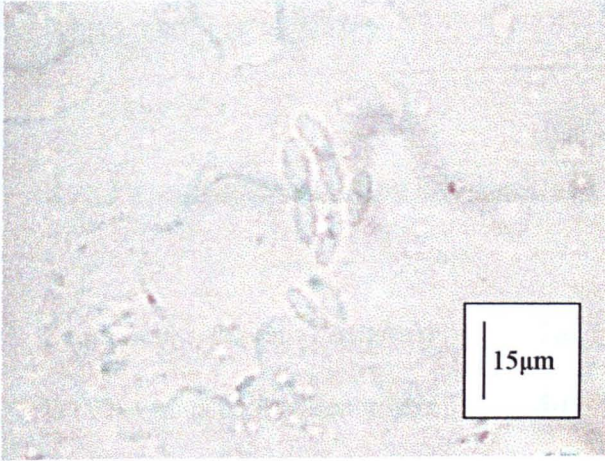
P42 Collected from Lake Druzhby 7th
Feb 2001



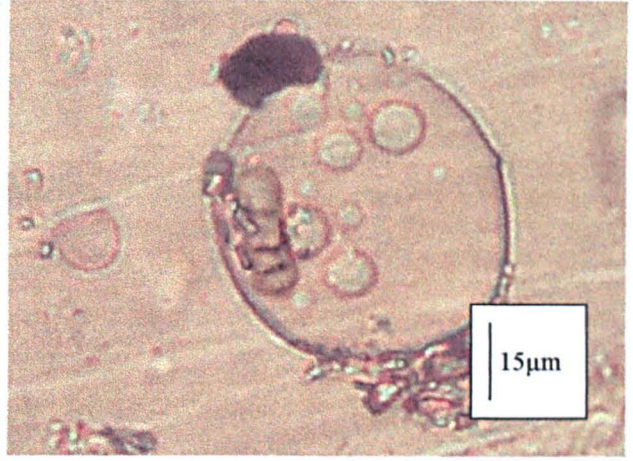
P51 Collected from Watts Lake Hut
13th Feb 2001



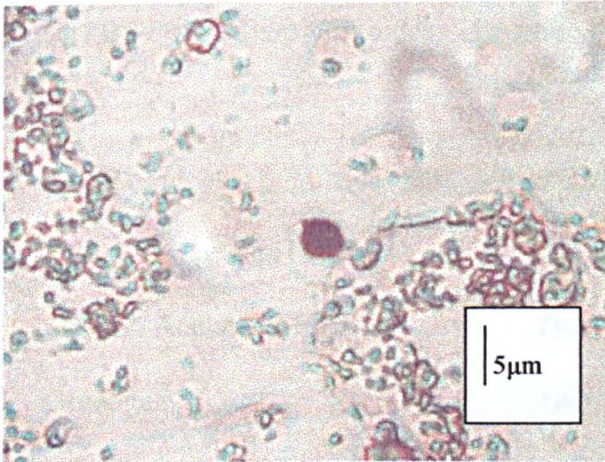
P52 Collected from Flat Top
Peninsula 22nd Feb 2001



P53 Collected from Lake Druzhby 22nd
Feb 2001



P54 Collected from Davis Station
(Dingle Road) 26th Feb 2001



P57 Collected from Davis Station
(Dingle Road) 2nd Mar 2001



P87 Collected from Flat Top Peninsula
11th December 2001

APPENDIX 4

REAGENTS USED DURING DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

Dye Terminator Sequencing kit - Beckman Coulter™ CEQ 2000

Sequencing primer (1.6µg/ml)	2µl
DTCS Quick Start Master Mix	4µl
Water	12µl

For use with 2µl DNA template. Changes in template or primer volume due to concentrations used should be compensated by adjustments to the water content.

(DTCS Quick Start Master Mix is supplied with the Quiagen™ *Hot Start* Kit)

Dye Terminator Sequencing kit - Beckman Coulter™ CEQ 2000 - Stop Solution

EDTA 100 mM pH 8	2µl
Sodium Acetate 3M pH 5.2	2µl
Glycogen	1µl

Prep-a-Gene™ Binding Buffer

Tris-HCl	3.94g	(50mM)
Na ₂ EDTA	1.86g	(10mM)
NaClO ₄	421.32g (367.20g anhydrous)	(6M)
Water	500ml	

Adjusted to pH 8 and autoclaved

Prep-a-Gene™ Wash Buffer

Tris-HCl	1.576g	(20mM)
Na ₂ EDTA	0.372g	(2mM)
NaCl	22.177g	(0.8M)
Water	500ml	

Autoclaved, then 500ml ethanol added.

Quiagen™ Hot Start PCR mixture

Forward Primer ITS4 (50pmol μl^{-1})	0.5 μl
Reverse Primer ITS1 (100pmol μl^{-1})	0.25 μl
<i>Q solution</i> *	2.5 μl
<i>Hot Start</i> *	25 μl
Water	18.75 μl

For use with 3 μl DNA template. Changes in template or primer volume due to concentrations used should be compensated by adjustments to the water content. (**Q solution* and *Hot Start* are supplied with the Quiagen™ *Hot Start* Kit)

Saline EDTA

NaCl	2.19g	(0.15M)
Na ₂ EDTA	0.93g	(0.01M)
Water	250ml	
Adjusted to pH 8		

TAE buffer (50x)

Tris base	242g
Acetic acid (glacial)	57.1ml
EDTA (0.5M) pH 8	100ml
Water	842.9ml

Tris buffer is dissolved in 700ml water and the acid and EDTA added. Made up to 1 litre with water.