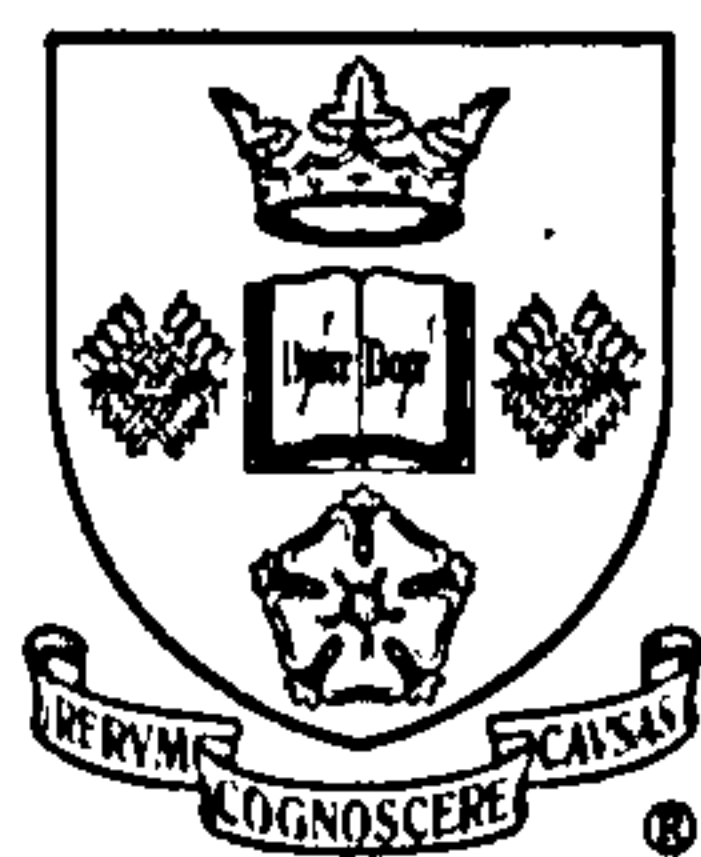


**STUDIES IN RELATION TO THE POSSIBLE
EXTRATERRESTRIAL ORIGIN OF BACTERIA**

By .

Amar Abdusalam Laswd

BSc. Omar Al Mukhtar University, Al Bayda, Libya
MSc. Al Fatah University, Tripoli, Libya



Thesis submitted in partial fulfilment for the requirements of the Degree of Doctor of
Philosophy in the Department of Molecular Biology and Biotechnology, University of
Sheffield, UK.

2008

To my beloved parents,

my wife Sabria,

my children

Muna and Abdusalam

Acknowledgments

I wish to thank my supervisor Dr, Milton Wainwright for his guidance, valuable supervision, helpful advice, encouragement and support throughout my research. I would also like to thank Prof. D. P. Hornby for making the facilities of the Department available to me. My deepest thanks go to Dr. D. J. Gilmour, for his advice during my study. Also, I wish to thank all my friends in Dr Wainwright's laboratory for their help and encouragement.

My special thanks are due to my beloved parents, and my family for their patience, support, continuous encouragement and enthusiasm throughout the period of my study. Finally, I would like to thank the Libyan Attachment at Libyan Embassy in London for their support and the Education Authority in Libya for providing me with a postgraduate scholarship.

Summary

Studies were made in relation to the theory of panspermia, in particular, the version known as cometary panspermia, which suggests that life on Earth a) originated from space (from comets) and b) continues to arrive from this source (i.e. neopanspermia).

The following conclusions were arrived at;

1) Evidence is provided to support recent findings showing that viable bacteria are present in the stratosphere at a height of 41 km (circa 25 miles). These bacteria appear in clumps of a size in excess of 10-20 microns. It is argued that it is unlikely that bacterial clumps of this size could be elevated from Earth to 41 km and that as result these clumps must be incoming from space. It is suggested that these may be unculturable and may make up the majority of unculturable bacteria that are found in the environments of Earth (this suggestion however, remains conjectural).

2) Studies were made on the Red Rain that recently fell on Kerala in India. It was confirmed that Red Rain is made up of microbial cells of morphology similar to that of algae and fungal spores. Another type of Red Rain, sampled in England, is algal in appearance, although morphologically it is completely different from the Red Rain of Kerala.

3) The chemical composition of a comet was simulated. The simulated comet was shown to be capable of supporting the growth of bacteria directly sampled from the atmosphere on Earth. Bacteria grew in the presence and absence of the PAH, naphthalene. The results suggest that Earth bacteria could grow in the nutrient conditions provided by a comet.

4) Sulphuric acid at a concentration in excess of that likely to be found in the stratosphere did not completely sterilise a culture of *Bacillus subtilis*, showing that this acid, although present in the stratosphere, is unlikely to completely inhibit bacterial survival in this environment.

5) A study was made of the microbiology of some Earth materials (i.e. amber, coals, limestone and clay). A large number of bacteria were isolated from clay (mainly species of *Bacillus*). While two bacteria were isolated from amber and one from a coal sample, no organisms were isolated from limestone. Obviously, the theory of geopanspermia, the view that bacteria are transmitted through space in rocks, requires such a demonstration that rocks on Earth do in fact harbour bacteria.

Contents

Acknowledgments	II
Summary.....	III
Contents	V
List of Figures.....	XI
List of Tables	XIV
CHAPTER ONE	1
INTRODUCTION.....	1
1.1 Introduction	2
1.2 De Maillet views, arguably the first scientific comment on panpsermia	2
1.3 The recent history of Panspermia	8
1.4 Survival of space-borne microorganisms	8
1.5 Contamination and the problem of common versus unusual microorganisms	10
1.6 The “High Cold Biosphere”	11
1.7 Problems resulting from attempts to replicate these isolations	13
1.8 The Neopanspermia paradox	14
1.9 The problem of phylogeny	15
1.10 Panspermia and the origin of life.....	16
1.11 Hoyle and Wickramasinghe’s theory of the cometary origin of life	18
1.12 Confirmation of the presence of viable but non-culturable bacteria in the stratosphere.....	20
1.13 Aims of the work reported in this Thesis	23

CHAPTER TWO	24
FURTHER EVIDENCE FOR THE PRESENCE OF MICROORGANISMS IN THE STRATOSPHERE	24
2.A Introduction to the microbiology of the stratosphere	25
2.A.1. Physical conditions in the stratosphere.....	25
2.A.1.2 Sampling the stratosphere.....	25
2.A.2 Materials and Methods	27
2.A.2.1 Stratosphere sampling.....	27
2.A.2.2 Precautions taken against contamination.....	30
2.A.3 Results and Discussion	30
2.A.3.1 Inorganic cosmic dust.....	30
2.B Use of Live /Dead and the DNA stain DAPI to confirm the biological nature of presumptive bacteria sampled from the stratosphere	39
2.B.1 Material and Methods	39
2.B.1.1 Viable staining	39
2.B.2 Results and Discussion	40
2.B.2.1 Mechanisms by which microorganisms could reach the stratosphere from Earth.....	43
2.B.2.2 Is the stratospheric microflora made up of two bacterial populations of distinct origin?	45
2.B.2.3 A mechanism by which evolution may have been speeded up by the transfer of bacteria from Earth to the stratosphere	47
2.B.3 Conclusion	48

2.B.3.1 Some suggested critiques of the above	49
2.B.3.2 The potential role of small bacteria in relation to transfer of life from the Earth to the stratosphere	50
2.C Fungi in the stratosphere.....	52
2.C.1 Introduction.....	52
2.C.2 Materials and Methods.....	54
2.C.3 Results and Conclusion.....	54
2.D Studies on the Red Rain phenomenon.....	55
2.D.1 Introduction	55
2.D.2 Materials and Methods	56
2.D.2.1 Scanning electron microscopy.....	56
2.D.2.2 Staining with DNA probes	56
2.D.3 Results and Discussion	56
Comparison of Indian and UK Red Rains.....	65
2.E Studies on the survival of <i>B. subtilis</i> in sulphuric acid in relation to the stratosphere.....	66
2.E.1 Introduction.....	66
2.E.2 Materials and Methods.....	67
2.E.2.1 The effect of sulphuric acid on freeze dried <i>B. subtilis</i>	67
2.E.2.2 Does volcanic ash protect <i>B. subtilis</i> from sulphuric acid?	67
2.E.3 Results and Discussion.....	68

CHAPTER THREE	72
STUDIES ON THE GROWTH OF BACTERIA IN A SIMULATED COMETARY NUTRIENT ENVIRONMENT.....	72
3.1 Introduction	73
3.1.1 A note on the bacterial inoculum used in these studies.....	74
3.2 Material and Methods.....	76
3.2.1 Silica gel medium (Parkinson <i>et al.</i> , 1989)	77
3.2.2 Addition of carbon source to simulate the cometary nutrient environment.....	78
3.2.3 Exposure of the silica gel medium to the atmosphere.....	78
3.2.4 Simulated comet experiments.....	78
3.2.4.1 NASA prescription for a simulated comet.	78
3.2.4.2 Silica gel experiment	79
3.2.5 Isolation of atmospheric fungi.....	80
3.3 Results and Discussion.....	81
3.3.2 Isolation of atmospheric fungi.....	82
CHAPTER FOUR.....	88
GOPANSPERMIA	88
4.1 Introduction	89
4.A Studies on the isolation of microorganisms from amber.....	90
4.A.1 Introduction	90
4.A.2 Materials and Methods	91
4.A.2.1 Nature of the amber and confirmation of its authenticity.....	91

4.A.2.2 Scoring and sterilisation of the amber	92
4.A.2.3 Incubation of the vessel and isolation of bacteria.....	92
4.A.2.4 Cracking of the amber	93
4.A.2.5 Attempted isolation of a fungus from Dominican amber	93
4.A.2.6 Experimental controls.....	94
4.A.3 Results and Discussion	94
4.A.3.1 Studies on the isolation of bacteria from Baltic amber	94
4.A.3.2 Attempts to isolate a fungus from Dominican amber.....	96
4.B Attempts to isolate bacteria from rocks and clay.....	99
4.B.1 Introduction.....	99
4.B.2 Material and Methods	99
4.B.2.1 Origin of coal, limestone and clay samples	99
4.B.2.2 Initial examination for the samples.....	100
4.B.2.3 Preparing the experiment vessels and bottles	100
4.B.2.3.a Preparing the experiment vessels.....	100
4.B.2.3.b Preparing the experiment bottles	101
4.B.2.4 Scoring and sterilization of the samples surface.....	102
4.B.2.5 Experimental design set up to control contamination.....	102
4.B.2.6 Incubation of the vessels and bottles	102
4.B.2.7 Media used in the experiments	103
4.B.3 Results and Discussion	105
CHAPTER FIVE.....	108
GENERAL DISCUSSION	108

5.1 General Discussion.....	109
5.2 Diseases from Space.....	119
5.3 Suggestion for further work.....	120
CHAPTER SIX	123
REFERENCES.....	123
APPENDIX.....	132
A. The results of 16SrDNA sequence analysis.....	133
B. Live/Dead staining.....	140

List of Figures

- Figure 1.0** A temperature profile for the atmosphere (Source, Environment Canada).12
- Figure 1.1** Original data for GC-1R57 and the predicted behavior of freeze dried bacteria.....19
- Figure 1.2** Bright (green, living) and diffuse (red, dead) fluorescent particles, having the size and morphology of bacteria, found on membranes through which stratospheric air was passed; sampled at a height of 30-39 km and (inset) 40-41 km (from Wainwright *et al.*,2003).22
- Figure 2.1** The helium-filled balloon used to sample the atmosphere over India.....26
- Figure 2.2** The cryosampler, showing cryosampler tubes (gold-topped) in carriage.....28
- Figure 2.3** Inorganic particles of cosmic dust on membranes a) Doughnut-shaped silicon particle, b) zircon crystal, c) clump of silicon-rich particles mimicking bacteria. (Height, 41 km).31
- Figure 2.4** The effect of leaving the electron beam of the scanning E/M on biological particles. Note the hole seen in Fig. 2.4b, caused by the beam, and not seen in Fig. 2.4a.34
- Figure 2.5** Particles having the morphology and chemical properties (EDAX) of bacteria, a).an amorphous, presumptive, bacterium attached to a weathered zircon particle and b) a presumptive bacterium showing signs of collapse under the pressure of the scanning E/M. (Height, 41 km).36

Figure 2.6 Presumptive fungi from the stratosphere, a) a fungal spore as seen under the light microscope, b) fungal or possible actinomycete-like hyphae from the stratosphere (22-23 km). (Kindly provided by Dr M. Harris.....	38
Figure 2.7 Live/dead staining of bacteria-like particles on membrane (41 km) Dead cells stain red and living cells stain green. The clump of green cells (mid-left of centre) is approximately 5 microns across.	41
Figure 2.8 Clumps of bacteria (circa 20 microns across) sampled from 28 km and stained with, the DNA stain, DAPI.	42
Figure 2.9 Scanning electron microscope image showing clump of bacterial cells of cross section in excess of 10 microns (from Harris <i>et al.</i> , 2001).	46
Figure 2.10 Indian Red Rain seen under the light microscope the cells are circa 5 microns across.....	57
Figure 2.11 Scanning electron micrograph showing Indian Red Rain cells (insert, shows detail of flagellum-like appendage, scale bar does not apply to insert.....	58
Figure 2.12 Sonicated Indian Red Rain cells stained with SYBER-Green 11.....	59
Figure 2.13 Sonicated Indian Red Rain cells a) unstained and b) stained with the DNA stain, DAPI.	61
Figure 2.14 Sonicated Indian Red Rain as seen under light microscope (a), and b) after staining with ethidium bromide under the fluorescence microscope.	62
Figure 2.15 Live/Dead Staining of sonicated Indian Red Rain.....	63
Figure 2.16 Exposure of Indian Red Rain to UV light for 48secs to check for autofluorescence.....	64

Figure 2.17 UK Red Rain under a light microscope (The cell is approximately 20 microns across).....	65
Figure 2.18 The effect of sulphuric acid on survival of <i>B.subtilis</i>	69
Figure 2.19 Influence of two types of volcanic ash (a, b) on the effect of sulphuric acid on <i>B. subtilis</i>	70
Figure 3.1 Hyphae of airborne fungus growing on silica gel medium and dissolving a groove in the gel.....	83
Figure 3.2 Airborne fungus growing on silica gel medium.	83
Figure 3.3 Airborne fungus growing on silica gel medium and undergoing lysis caused by similarly oligotrophically growing bacteria.	84
Figure 4.1 Typical amber samples showing fossilized insect inclusions.....	92
Figure 4.2 Fungal hyphae seen inside amber using a light microscope (X10).	96
Figure 4.3 Cracking vessel and bottle.	100
Figure 4.4 A sample of coal.	104
Figure 4.5 A sample of limestone (Flamborough Head).....	104
Figure 4.6 A sample of clay.	104
Figure 5.1 The Hoyle and Wickramasinghe cosmic life cycle.	111
Figure 5.2 Temperature profile of comet Temple (source NASA).....	117
Figure 5.3 Future space tourism- a possible future source of stratospheric samples.....	122

List of Tables

Table 3.1 The composition of the simulated comets (wetted with double distilled water).....	79
Table 3.2 Composition of silica gels containing nutrients; A, carbon free, B, containing naphthalene (0.1% w/v), C, containing glycolaldehyde dimer (0.1% w/v).	80
Table 4.1 Location of origin and carbon content of coals.....	99

CHAPTER ONE

INTRODUCTION

1.1 Introduction

The term 'panspermia' was originally used by microbiologists (of the late Victorian period) to refer to the observation that terrestrial air is full of microorganisms. Panspermia, was later used to cover the view that life on Earth originated from space; more recently however, it has been extended to describe the hypothesis that life continues to rain down to Earth from space (Wainwright, 2003). In order to avoid confusion, here the term panspermia is used in its original, astrobiological sense, i.e. relating to the origin of life, while the term neopanspermia (Wainwright, 2003) will be used to refer to the hypothesis that life continues to arrive to Earth from space. The theory of panspermia has a long history, and was probably first suggested by the Greek philosopher Anaxagoras. Lord Kelvin promoted the idea at the end of the Victorian period. It is noteworthy that Kelvin suggested that plant seeds and mosses might be transferred across the cosmos, an idea that has recently been resurrected by Tepfer and Leach (2006). In the early part of the 20th century, the Swedish physical chemist, Svante Arrhenius developed the theory, more fully. However, the first "modern" expression of panspermia seems to have been made by the French philosopher, De Maillet in the early 1700s. De Maillet's, largely forgotten work, has recently been highlighted by Wainwright (Wainwright, 2008, In Press); what follows is a revised and shortened version of this paper.

1.2 De Maillet views, arguably the first scientific comment on panspermia

The following account of the theory of panspermia suggested by the French philosopher, De Maillet is of particular interest because it was suggested at a point early in the history of science; since they have been overlooked by historians, this seems to be

an excellent opportunity to put this work on record. While it is clear that Greek philosophers, like Axegagorus, suggested the possibility that life on Earth arrived from elsewhere, it seems that De Maillet appears to have been the first to provide a full, scientific, argument in favour of panspermia.

De Maillet, a French Government official, was widely travelled and was the author of an anonymous book entitled Telliamed (De Maillet used his name backwards for the title in the hope of avoiding being recognised for his controversial views ,which might also have been seen as being antireligious). This was an imagined conversation between a fictitious oriental (Indian) and equally fictitious European, which became a highly influential early text on geology and even encompassed a primitive evolutionary theory. For many years, Telliamed circulated in manuscript form amongst French intellectuals, and was only published in 1748, ten years after De Maillet died. Even then, the edited version was much altered and an unabridged copy was only made available in 1968.

The references made by De Maillet to panspermia can be found in a marginal note to his Third Conversation in which he states:

In order to understand this natural process, imagine, Sir, that the entire extent of the air visible to our eyes: the opaque globes they perceive, and those that which they do not discover; even the portions of the inflamed globes which are not yet reached by the fires; in a word imagine that this entire space is full of seeds of everything which can live in the universe.

There is no doubt that De Malliet is here suggesting that seeds are found in the entirety of the cosmos, but what does he mean by “seeds”. When De Maillet continues,

we see that he is not referring here to plant seeds, but that he considers all life forms to have arisen from protoforms, or “seeds”. He continues:

Furthermore, these seeds are so delicate and minute, even those of animals and plants which grow to the largest sizes, that it is impossible to see them by means of our best microscopes.

The word seed then is used in its broad sense to mean a source, or beginning. It is also interesting that he speculates that such living forms might be sub-microscopic. De Maillet continues:

These seeds, spread in such a manner throughout this vast universe, are, however, more numerous around the opaque globes in thick airs and in waters than in the immense spaces, in these oceans of void by which the globes are separated, because they are not stopped there by the same arrangements which retain them around globes.

Clearly, De Maillet visualises that the seeds are concentrated around planets and held there by gravity. Now at this point we might imagine that De Maillet is merely talking about proto-life forms that merely resemble small seeds or sperm, but things become even more interesting with his next statement:

The effect produced by the spirit of life on the seeds contained in the waters is demonstrated by what may be observed in a single drop of water taken on the point of needle from any containers in which some grass has been steeped for two or three days. By means of a microscope, we see in that drop of water a fantastic number of animals, even of different species, because each kind of grass produces a different species. Some of them have a human form...some of them move swiftly and along in straight lines;

others move slowly and in circles. They may be seen to be growing because their parts increase in size appreciably from one day to another.

De Maillet is referring here to what Antonie van Leeuwenhoek and Robert Hooke observed and referred to as animalcules. Whether De Maillet is quoting these sources, or actually saw such forms himself is not obvious. What is clear however, is that he is aware that animalculae (what we would now refer to as protozoa), and also possibly bacteria, are present in water in which grass has been left. It is clear that De Maillet believes that such microscopic forms are the precursors of animals, even humans, which grow directly into the animals and plants we see all around us on Earth. These seeds he considers come initially from the air, but ultimately from space. De Maillet's ideas now begin to become clearer. What he is suggesting is that the cosmos is full of the "seeds" of all living things and these are transmitted through the universe, becoming more concentrated around rocky planets due to gravity. Once they arrive on a planet with oceans these, like a vast womb provide a beneficial climate which nurtures the seeds until they turn into small life forms observable under the microscope. These small animals and plants then grow and eventually leave the sea to colonise the land. Here, they grow to the degree of perfection we see today. De Maillet is aware however that some living forms do not survive and that:

The destruction of some of them has been, at least in the beginning, for the voracious species, the first way of preservation and development of the others.

De Maillet then states that it possible that all living things existing on Earth occur on other globes, but goes onto state that:

It is however, more reasonable to assume that all species do not exist on all globes, and that there are still many more to hope for, on Earth, as well as on each of the other globes. The globe we live on has certainly shown us only a portion of the species of animals, trees and plants whose seeds are contained in the surrounding air and sea, and we cannot doubt that the future centuries will reveal new and unknown ones.

Finally he states:

Furthermore, all the seeds do not occur around every globe. They can only be transferred from one to another by one of these revolutions mentioned previously, to which the entire system is subjected, that is, the passage of one globe from one vortex to another. If we could only see all of the species populating our planets, I am convinced that we would discover in them thousands of unknown ones.

At first sight, De Maillet's views on panspermia do not correspond with most modern versions which essentially see microorganisms as the living form which is transported around the cosmos. In the modern version, these microbes then evolve into more developed life forms, which, on Earth, reach its climax in the form of humans. De Maillet in contrast, views the cosmos as being full of planets which are populated with plants and animals, the small forms (or "seeds") of which arrived on Earth and other planets; developed at first in the oceans and then moved to the land; here they then developed directly into their mature forms, some of which fell by the wayside. Although the modern version of the Theory generally sees microorganisms as the form that is transported throughout the cosmos, it is interesting to note that some other authors have speculated on the possibility that more advanced life forms may be involved in panspermia. Lord Kelvin, for example talked of mossy stones being transported, while

Hoyle and Wickramasinghe have more recently speculated on the possibility that insects may play a role in panspermia (Hoyle and Wickramasinghe, 2000), and Tepfer and Leach (2006) have suggested that plant seeds might also be transported across the cosmos. As a result, De Maillet's views are not as far away from currently held concepts as they might initially appear.

De Maillet's views were widely discussed in certain, esoteric circles but in the end had little influence on modern thought. *Tellamed* was initially left unpublished because of De Maillet's fear of the violent reaction his ideas might evoke, especially in ecclesiastical circles; he was perhaps wise to do so, since, in addition to believing that life was not created by God, he claimed that it did not even originate here on Earth, but came from space. Such views were unlikely to make him popular amongst a philosophical and a scientific culture most of whose members firmly believed in God and his works. De Maillet in fact seems to go out of his way not to mention God, although he makes frequent reference to Bible as a way of gaining insight into the mindset of ancient peoples.

In conclusion, De Maillet was one of the first moderns to see the Earth, not as the centre of creation, but merely one part of a vast cosmic sea of life. In this he is distinguished himself from subsequent philosophers and evolutionists, including Darwin, who believed that life originated on Earth. In addition De Maillet differed from most philosopher-scientists of his age (including Buffon) when he stated that the Earth was two billion, rather than only a few thousand, years old. By being one of the first to express what might be called a post-Copernican view of biology, De Maillet's contribution deserves to be better known.

1.3 The recent history of Panspermia

The latest views on theory of panspermia have largely come about as a result of the cometary theory of panspermia, first expressed in detail by Sir Fred Hoyle and Chandra Wickramasinghe, in the 1970s (Hoyle and Wickramasinghe, 1979).

Despite the fact that belief in both panspermia and neopanspermia are becoming somewhat fashionable, many authors habitually fail to make reference to the originators of the modern view of these hypotheses, namely Hoyle and Wickramasinghe (Wainwright, 2003). Some authors instead date the resurrection of panspermia, in the widest sense, to the far more speculative, and essentially unprovable, views of Francis Crick, which relate to so-called directed panspermia (i.e. the view that life on Earth was seeded by some unknown, cosmic civilisation) (Crick, 1981). There is no doubt however that priority on the modern view of neopanspermia belongs to Hoyle and Wickramasinghe (1979, 1981, 1991, 2000). These authors also championed the view that pathogens arrived on Earth from space; an idea, termed by Wainwright, 'pathospermia' (Wainwright, 2003) that has come in for even more derision than their views relating to neopanspermia.

1.4 Survival of space-borne microorganisms

It goes without saying that any microorganisms reaching the Earth from space must have survived the extreme rigours of the space environment. Although microorganisms need not grow and reproduce in space, they obviously must survive in a form that can then reproduce once they have arrived on Earth. The ability of microorganisms to withstand environmental extremes is being increasingly widely recognised. Hoyle and Wickramasinghe (Hoyle and Wickramasinghe, 2000) pointed out that Earth

microorganisms (geomicrobes) possess all the characteristics necessary to allow them to survive in space; by reversing this argument, they then went on to suggest that the possession of such characteristics suggests that microorganisms originated in a more demanding environment, (i.e. space). This viewpoint has however, been criticised by, amongst others, Battista *et al.* (1999) who claim that microbial resistance to ionising radiation, for example, may involve DNA repair mechanisms that are evolutionary responses to other environmental factors, such as water shortage.

Although emphasis is currently being placed on the study of microorganisms, notably bacteria, that can live, and are specifically adapted to growth in, extreme environments (i.e. extremophiles), it is important to note that many common bacteria, inhabiting non-extreme environments, are able to survive extreme conditions. Such organisms have been termed extremodures (Wainwright, 2003). More recently, *Escherichia coli* has been shown to be capable of surviving exposure to high pressure (Al-Mufti *et al.*, 1984).

Exposure to radiation, notably UV, is likely to be the major factor limiting the survival of microorganisms in space. Bacteria can however, resist UV by forming clumps of individual cells (as in *Sarcina* or *Staphylococcus*), while the spores of both bacteria (e.g. *Bacillus*) and fungi are often UV-resistant. The shading provided by bacteria being surrounded by cosmic dust particles might also help protect microorganisms from UV, as might the formation of a UV-resistant layer due to the carbonisation of the outer cells of a mass of space-inhabiting bacteria (Hoyle and Wickramasinghe, 2000). Such considerations suggest that panspermic microorganisms need not be particularly unusual or especially specialised in order to survive the rigours of space. One of the frequently expressed errors in relation to the nature of panspermic

organisms is the belief that they must, by definition, be “other worldly”, or in some way connected with alien folklore or, as Wainwright (pers. comm.) has wryly commented “they must have NASA written on them”. However, if the theory of neopanspermia is correct then space organisms will be Earth organisms and possibly *vice versa*.

The greatest degree of protection from the rigours of the space environment will obviously be afforded to microorganisms which are protected inside comets, or are lying dormant within masses of interstellar dust or meteorites. It is not surprising therefore to find that bacteria-like fossils have been found in the Murcheson meteorite and in the much discussed Mars meteorite, ALH 84001. There have also been a number of claims that living bacteria (species of *Bacillus* and *Staphylococcus*) have been isolated from meteorites), although these have generally been regarded as being contaminants (Orio and Tornabene, 1965). Nevertheless, claims that ancient living bacteria exist in meteorites continue to appear (Abbot, 2001).

1.5 Contamination and the problem of common versus unusual microorganisms

Neopanspermia predicts that microorganisms are continually raining down upon our planet and that by definition most, if not all Earth microorganisms, are derived from space. Earth organisms are therefore space organisms. This view does not however, exclude the idea that a portion of the Earth’s microbial population derives from the first, panspermic, common ancestor, while new organisms have arrived at different points in the planet’s history, and continue to do so today. The hypothesis states that common Earth organisms such as species of *Pseudomonas*, *Staphylococcus* and *Bacillus* originate from space. The fact that common geomicrobes can survive under extreme conditions provides an explanation of how this might be possible. Unfortunately, a problem arises

with human perception that makes this suggestion unpalatable. Most microbiologists would assume that space-derived microorganisms would a priori be a) novel, b) primitive, and c) possess unusual, marker-physiologies. Should common, Earth organisms were to be found in space, this would doubtless induce the knee-jerk response of most microbiologists that such isolates were contaminants, or at best originated from Earth. The isolation of space-derived microorganisms would probably only be generally accepted if they proved to be novel or unusual, despite the fact that this is not required for the neopanspermia hypothesis.

Since microorganisms exist everywhere on Earth, any attempt to isolate microorganisms from space-derived samples is obviously bedevilled by problems of contamination. Any claim that geomicrobes have been isolated from space can be readily dismissed by invoking contamination, even where the most rigorous sterile technique has been employed to exclude such a possibility. Although contamination is a real problem it is often too readily used to dismiss all claims for the isolation of ancient, or space-inhabiting, microorganisms.

1.6 The “High Cold Biosphere”

The term “High Cold Biosphere” (HCB) has recently been suggested by Wainwright (Wainwright pers. comm.) to illustrate the fact that there exists in the stratosphere a biosphere in which microorganisms exist at very high altitude and under extremely cold conditions.

The stratosphere is the second layer of the Earth’s atmosphere that is found above the tropopause at an average height of around 17 km. The stratosphere is stratified, with warmer layers high up and cooler layers farther down; in this respect it is the exact

opposite of the troposphere. The stratosphere exists to a height of around 50 km. The top of the stratosphere has a temperature of about 270K (-30°C). A temperature profile for the stratosphere is shown in Fig. 1.0.

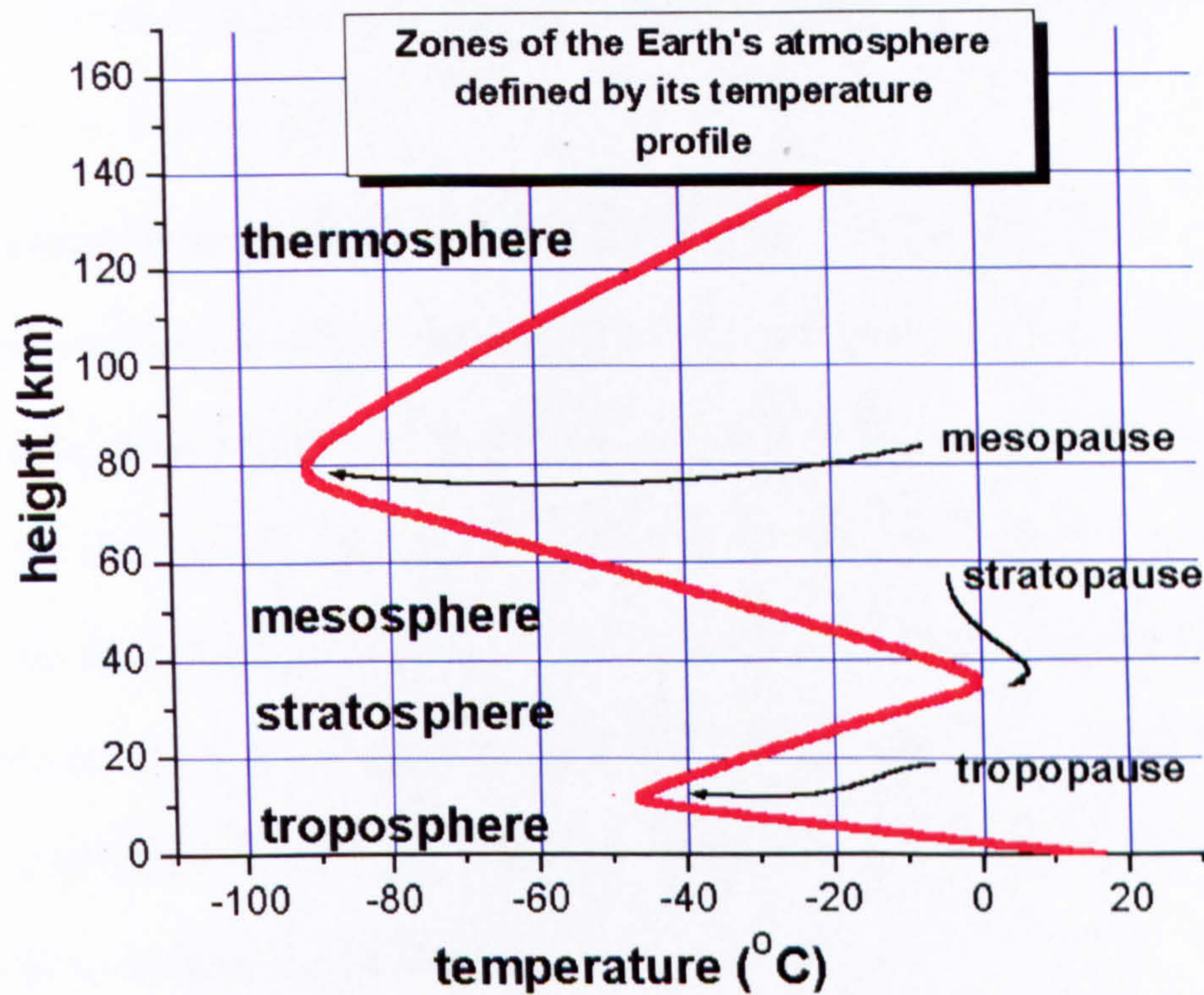


Figure 1.0 A temperature profile for the atmosphere (Source, Environment Canada).

Note that at 41 km, the height at which atmospheric air was sampled in the following studies, the temperature is around -20 to -30 degrees C. These temperatures compare with -80 to -90 degrees Celsius for the coldest Antarctic values; the Antarctic is however, much warmer than the stratosphere in summer, at around 5-15°C. It is possible that temperatures at which bacteria are exposed in particulate masses in the stratosphere will be higher than ambient temperatures, due to the fact that such particle masses act as solar collectors and thereby increase in temperature when exposed to the sun. In a

similar way, alkaline particulate masses may reduce the toxic effects of stratospheric acids and ozone, as well as the direct effects of UV light.

There is no regular turbulence in the stratosphere and air movement is largely horizontal. The ozone layer occurs at the top of the stratosphere at around 50 km. The region is also characterised by having a high concentration of sulphuric acid and high exposure to UV (Hare, 1962).

1.7 Problems resulting from attempts to replicate these isolations

If we assume that the isolates were not laboratory, or other, contaminants then they must have originated at a height of 41 km above the Earth. The obvious question that arises is - how did they get to this altitude? The two obvious conclusions are that they were carried up from Earth and remained, or were falling through, the atmosphere at 41 km from where they were isolated. Alternatively, they may have arrived from space and were sampled at 41 km before falling to Earth. Proof of the latter possibility would of course be to validate neopanspermia.

The apparently obvious conclusion is that the bacteria and the fungal isolates were carried up from Earth and floated in the stratosphere to a height of 41 km, from where they were collected. However, the only means by which bacteria can achieve a height of 41 km would appear to be via a volcanic eruption. The residence time of such particles, derived from volcanoes, would be only a matter of days. Since no volcanic eruptions occurred on Earth during the years prior to the 41 km-sampling event this would appear to rule out this possibility, although some authorities continue to believe that particles can cross the tropopause and reside at 41 km for longer periods (Gregory, 1961).

1.8 The Neopanspermia paradox

Most microbiologists would assume that the isolates, if not contaminants, must have arrived to a height of 41 km from the Earth's surface. The isolates are viewed as common Earth bacteria. The fact that the *S. pasteuri* isolate is, unlike *S. aureus* and *S. epidermidis*, capable of solubilising insoluble phosphates suggests that this isolate is not a skin contaminant, but possesses a property typical of a 'common' environment-derived, isolate. The use of the term, common in relation to microorganisms is however, questionable since microorganisms (with the exception of certain extremophiles) tend to be found ubiquitously over the Earth's surface. Even microorganisms that are regarded as being extremophiles (e.g. halophilic organisms, capable of growing in high salt concentrations) can often be readily isolated from non-saline soils. The ubiquitous distribution of microorganisms would of course support the view that such organisms are continually raining down from space, over the total surface of the Earth. Clearly the suggestion that the bacteria isolated from 41 km cannot have come from space because they are Earth organisms is in direct opposition to the neopanspermia hypothesis. If however, the Earth origin of these isolates is accepted than we are presented with a paradox since this would mean that bacteria are able to leave the Earth and infect space. Such a paradox would in fact demonstrate the correctness of the neopanspermia hypothesis and would suggest that space is contaminated by geomicrobes. Whether or not these organisms would find their way into deep space is however, debatable. In short, those who believe that organisms isolated at 41 km must be from Earth are unwittingly validating the neopanspermia hypothesis.

1.9 The problem of phylogeny

From the biologists point of view the major limitation on the view that modern microorganisms can arrive from space relates to evolution and phylogeny. Essentially, because it should have evolved, any ancient microorganism should, in terms of its nucleotide sequence, be markedly different from modern microorganisms. If an organism has a very similar nucleotide sequence to a modern organism then it must, according to current molecular biology, be a modern organism. Similarly, an organism originating from space would be expected to possess nucleotide sequences which differ from modern organisms found on Earth, simply because one would not expect the direction and rates of evolution to be the same in space as on Earth. An example of how such considerations can lead to conflict is provided by the recent findings of bacteria in ancient salt crystals. Vreeland *et al.* (2000) isolated a bacterium from a 250 million year old salt crystal. They claimed that the bacterium was not a contaminant, but was the same age as the crystal. Graur and Pupko (2001) however, claim, based on the use of molecular methods, that the bacterium is modern and, despite the fact that Vreeland *et al.* (2000) used extremely rigorous aseptic technique, must be a modern contaminant. Similar findings have been reported following the examination of Permian salt crystals by Fish *et al.* (2001). Clearly, if the bacterium is not a contaminant then there must be something wrong with our current understanding of molecular phylogeny and palaeontology. The general acceptance of the viewpoint that the nucleotide sequences of microorganisms must have changed over time (due to evolution) makes most microbiologists accept that any microorganism with nucleotide sequences close to that of modern microorganisms must, by definition, be a modern microorganism. It needs to

noted however, that the so-called “tree of life” continues to involve constant argument and modification (Maher, 2002). Invoking Occam's Razor, most biologists would state that if an Earth-like organism is found at 41 km above the Earth's surface then it is an Earth organism; anyone who believes that the tropopause acts as a barrier to such contamination must as a result, alter their own, rather than the evolutionary paradigm. Similarly, the presence of a modern organism in an ancient salt crystal must, say the biologists, indicate only thing, contamination; it should however, be noted that the whole question of molecular phylogeny is in a state of constant flux (Maher, 2002).

1.10 Panspermia and the origin of life

As was mentioned above, the term panspermia was originally used in its astrobiological connotation to refer to the view that life did not originate on Earth, but was seeded by organisms (and not just microorganisms) from space. A small number of microorganisms arriving in this way would have been capable of replicating at an incredibly rapid rate and would, in the absence of any competition from native organisms, have rapidly colonised the planet. It is noteworthy that many microorganisms have the ability to grow at very low nutrient concentrations (i.e. they are oligotrophic). Such oligotrophic growth would have been essential because nutrients to support microbial growth would have been present at very low concentrations in the uninoculated prebiotic soup. It is generally accepted that heterotrophic life predated life based on photosynthesis, a view that ties in well with the view that heterotrophs arrived exogenously.

Wainwright and Falih (1997) showed that a fungus could grow, without added nutrients, on buckminsterfullerene. Since fullerenes have been transported to Earth in

meteorites and could protect bacteria from UV, this novel carbon allotrope may have played an important role in the early origin of life on Earth. Once the conditions on Earth were suitable life took off remarkably quickly; a fact that is in agreement with the view that it arose exogenously with Earth having been continually showered with microorganisms which lay inert until the point at which conditions were suitable to support their growth.

The fact that life got going so quickly has been used recently to suggest that the origin of life is a simple and frequent event, a viewpoint that is not however, supported by any compelling evidence. Finally, it has been suggested that if living organisms did not make the journey through space to begin life on Earth then perhaps DNA or RNA may have rained down on the prebiotic Earth and given life a 'kick-start' (Gribbin, 2001).

In 1928, a certain Professor E.G. Donnes had this to say about panspermia (Wainwright, 2003): *Perhaps the chief objection to the doctrine of Panspermia is that it is a hopeless one. Not only does it close the door to thought and research, but also it introduces a permanent dualism into science and so prejudices important philosophical issues.*

Many scientists clearly continue to express this viewpoint. It is often said for example that panspermia solves nothing simply because it does not answer the question of how life arose; if life arose in space, it is argued, then it could just as easily have arisen on Earth. Of course such arguments ignore the contrast between the finite nature of Earth and the infinity of space, in terms of both time and the limitless variety of astronomical bodies on which life could have arisen.

Donne's view should be questioned when he said that panspermia closes the door to thought and experimentation. Such is the current level of theoretical and practical work being devoted to the question of neopanspermia that much new knowledge will be gained from its study, even if it does eventually prove to be a 'false doctrine' neopanspermia is a scientifically valid idea simply because it can be refuted by experimentation.

Unfortunately, microbiological studies on Earth are unlikely to provide overwhelming evidence of the validity of the neopanspermia hypothesis. This is simply because cynics can dismiss any results by invoking contamination, especially because the Hypothesis predicts that microorganisms found in space will be identical to those found on Earth. As a result, the only way to convincingly demonstrate panspermia is to conduct experiments in space where the problems of contamination can be eliminated. However, in the absence of the facilities to study microorganisms *in situ* in space, evidence can only be accumulated from Earth-based experiments. If neopanspermia is correct, then a critical mass of information will accumulate that hopefully; will be sufficient to convince even the most diehard sceptic.

1.11 Hoyle and Wickramasinghe's theory of the cometary origin of life

In the early 1980's, a spectroscopic signature of a bacterium was apparently found in interstellar dust, representing the first unequivocal demonstration that over a third of the interstellar carbon in the galaxy was tied up in the form of organic dust grains (Hoyle and Wickramasinghe, 2000). It was also claimed that such grains were indistinguishable in their spectral properties from freeze-dried bacteria as indicated, for instance, in the correspondences in Fig. 1.1.

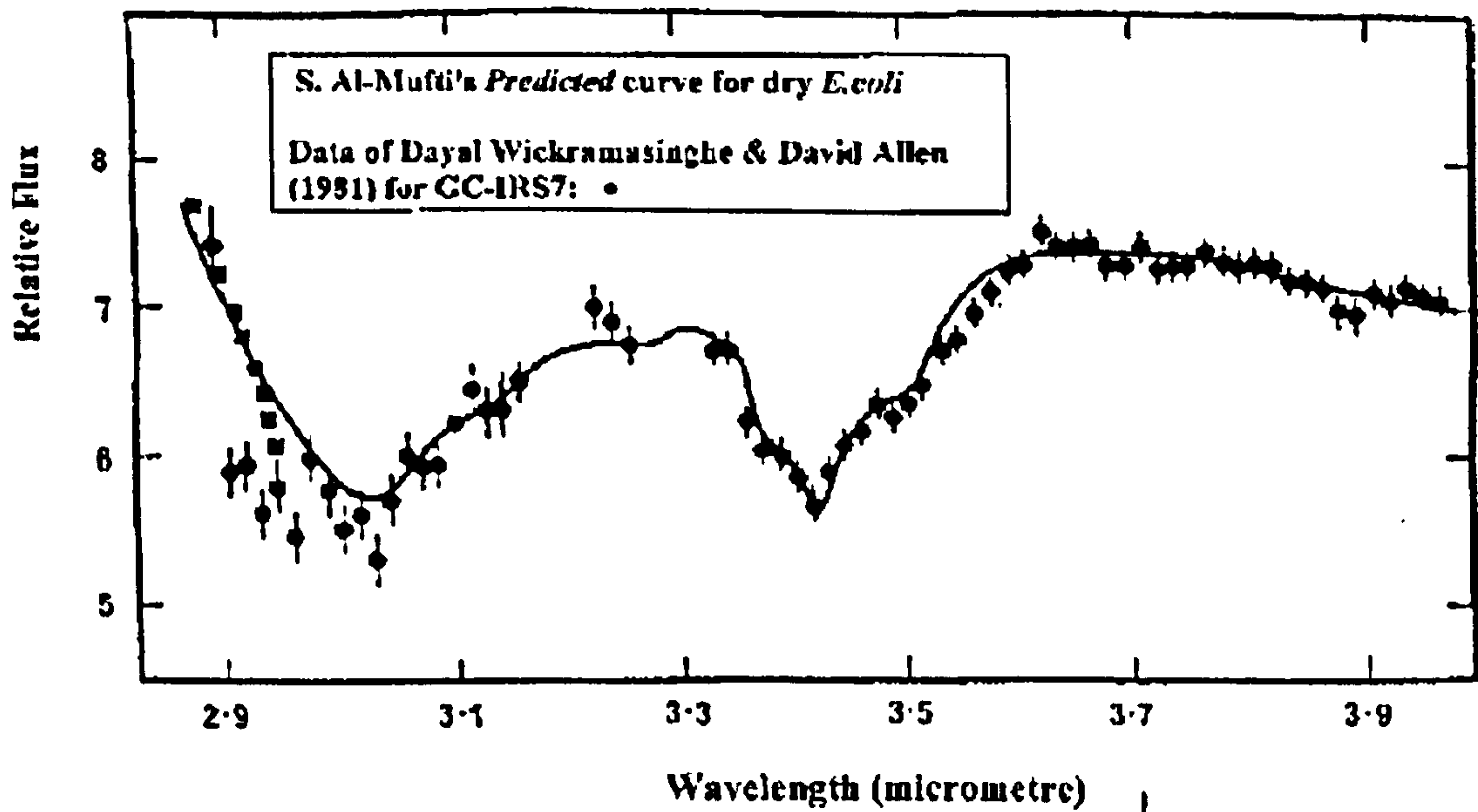


Figure 1.1 Original data for GC-1R57 and the predicted behavior of freeze dried bacteria.

Figure 1.1 shows the GC-1R57 spectrum for a laboratory bacterial culture agrees with the relative flux at these wavelengths seen in space.

Comets, it was claimed, mop up interstellar dust at the time of their formation. Any viable fraction of interstellar bacteria incorporated in such comets replicate on a huge scale, during a very short time scale within cometary cores, cores that are melted transiently due to the energy released by radioactive heat sources (Hoyle *et al.* 1982). Such biological particles, in a freeze-dried state, are then returned to space in comet tails, either to enter the inner regions of a planetary system, or to return into the interstellar medium from which new stars originate. Comets forming at every star-forming site, incorporating such material, would ensure propagation of cosmic biology because, the small but finite surviving fraction, would be re-cycled and greatly amplified within the comet. Apparently, only one in ten of the bacteria picked up from the

interstellar medium, need to retain viability for every one of about 100 billion comets in order to contain enough bacteria for such regeneration to occur. This would provide a positive feed-back of biological material back into space. As we have seen, panspermia theory predicts that comets carry microorganisms that are being transported through the atmosphere onto the Earth even at the present day.

1.12 Confirmation of the presence of viable but non-culturable bacteria in the stratosphere

The presence of viable bacteria in air sampled from the stratosphere at a height of 41 km has been previously reported (Harris *et al.*, 2001). Subsequently, two bacteria (*Bacillus simplex* and *Staphylococcus pasteurii*) and a fungus (*Engyodontium album*) were independently cultured from the samples (Wainwright *et al.*, 2004). These reports show that living organisms occur at a height of 41 km, i.e. well above the tropopause (17 km). Electron microscope studies have also provided tentative evidence for the presence of bacteria at a height of 25 km (Bigg, 1984); however Bigg made no attempt to stain or grow these supposed bacteria.

Microorganisms present at 41 km above the Earth could have originated from Earth or space (or possibly a combination of both); the view that such microorganisms originated from space is obviously controversial. Studies on bacteria in the upper atmosphere are also relevant to the transport of animal, plant and human pathogens around the globe. Here we describe experiments aimed at confirming the presence of viable bacteria in the stratosphere. The work reported by Harris *et al.* (2001) involved the use of the fluorescent membrane potential sensitive dye, carbocyanine. Using this stain, small clumps of coccoid cells of the dimension of bacteria (1-2 μm) were seen on membranes

through which stratospheric air had been filtered. From these observations, Harris *et al.* (2002) concluded that the observed fluorescing particles were viable bacteria; they did not, however, comment on their culturability.

The aim of the work described here was to attempt to provide independent confirmation of the presence of such viable bacteria in samples obtained from the stratosphere.

The Live/Dead probe used here utilizes mixtures of a green fluorescent nucleic acid stain and the red fluorescent nucleic acid stain, propidium iodide. When used alone, the green stain generally labels all bacteria, including those with intact or damaged cell membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes causing a reduction in the green stain when both dyes are present; as a result, the probe stains live cells fluorescent-green, while dead bacteria stain red.

Figure 1. 2. shows that both green fluorescent and red staining bodies were present on the membrane filters through which stratospheric air, at both heights, had been filtered. By careful focusing of the microscope these were seen to consist of individual cocci, or clumps of a small number of coccoid shaped cells, having both the dimensions (circa 1 μm) and appearance of bacteria. No similar, fluorescent green or red staining bodies were seen on newly opened membranes, or control membranes exposed to the same handling protocols. As a result, we conclude that the green fluorescent cells observed on the space-derived samples are viable bacteria, and that by using an approach to viable staining, differing from that used by Harris *et al.*, (2001), workers in this laboratory have confirmed the presence of viable bacteria in samples derived from the stratosphere.

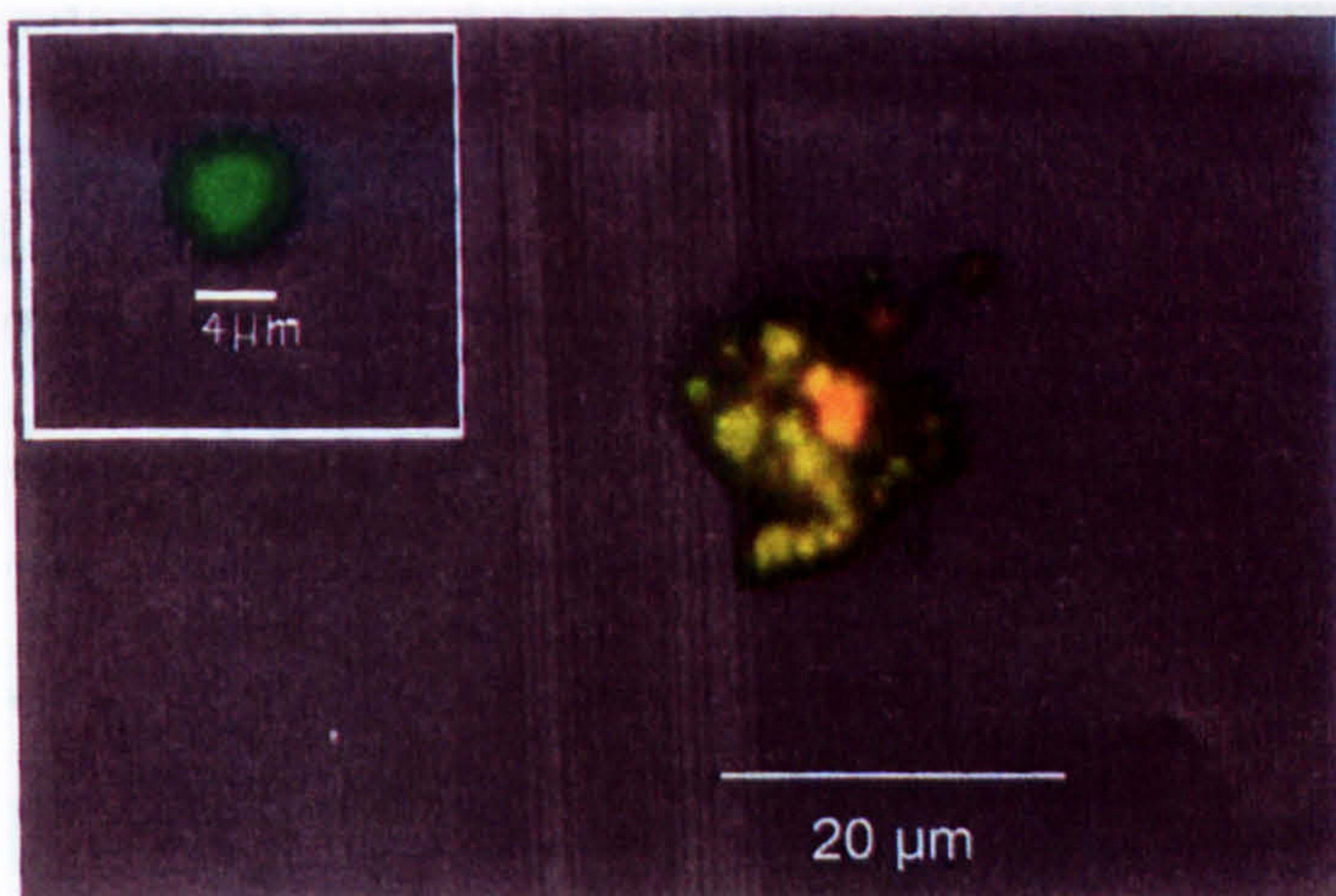


Figure 1.2 Bright (green, living) and diffuse (red, dead) fluorescent particles, having the size and morphology of bacteria, found on membranes through which stratospheric air was passed; sampled at a height of 30-39 km and (inset) 40-41 km (from Wainwright *et al.*, 2003).

Neither bacterial colonies nor non-colony forming bacteria were isolated from the membranes using the media and protocols employed. Within the limits of our studies, the fluorescent-staining coccoid cells, seen on the membranes, can be regarded as being viable but non-cultureable (VBNC) bacteria. Of course, culturability depends on the type of media and conditions employed; as a result, the bacteria observed might have grown had different media and growth conditions been employed. Two bacteria (*Bacillus simplex* and *Staphylococcus pasteurii*) and a single fungus (*Engyodontium album*) have been isolated (by employing some of the media used here) from some of these stratosphere samples (Wainwright *et al.*, 2003). However, the fact that viable microorganisms were only cultured on only one occasion, from a single membrane, suggests that most of the stratospheric bacteria present on the membranes are VBNC cells. Wainwright *et al.*, (2004), using scanning E/M, also concluded that nanobacteria can be isolated from the stratosphere.

In conclusion, the above results confirm that bacteria are present in the stratosphere. Clearly, further probes need to be sent to the heights sampled here before the issue of whether such bacteria originate from Earth or space can be concluded; to this end, we are currently attempting to conduct carbon isotope fractionation studies on the membranes to determine if the bacteria seen on them are of terrestrial origin.

1.13 Aims of the work reported in this Thesis

The aim of the work reported in this Thesis was to investigate a variety of factors relevant to the possibility that bacteria have an extraterrestrial origin. To achieve this aim the following questions were investigated:

- 1) Can further evidence be provided for the existence of microbes in the stratosphere?
- 2) Does the so-called Red Rain event of Kerala in India provide evidence for neopanspermia?
- 3) Can bacteria, from the atmosphere of Earth, grow in the nutrient conditions expected to be found in comets?
- 4) Is there any evidence that Earth's materials (i.e. amber, coals, limestone and clay) contain bacteria which may be ejected and transported throughout the cosmos via the process of geopanspermia?

CHAPTER TWO

FURTHER EVIDENCE FOR THE PRESENCE OF MICROORGANISMS IN THE STRATOSPHERE

2.A Introduction to the microbiology of the stratosphere

If the theory of neopanspermia is correct, and microorganisms are continually raining down to Earth from space it follows that we should be able to find microorganisms above the Earth's surface at various heights into the stratosphere. Since we know that Earth-derived microorganisms occur in the lower atmosphere, it follows that if we wish to demonstrate that microorganisms are incoming to Earth from space then we will have to go to higher elevations. It should of course be possible then to detect microorganisms using space rockets; Burch, (1967) and Imshenetsky *et al.*, (1978) claimed to have done this. However, for practical sampling reasons the search of the upper atmospheric regions is limited to the stratosphere. Very recent studies (Griffin, 2007) have confirmed that Eubacteria, including species of Staphylococci and *Micrococcus* can be isolated from the stratosphere at 20 km, findings which go against the often expressed view that staphylococci are incapable of surviving in extreme environments.

2.A.1. Physical conditions in the stratosphere

The stratosphere is defined as the region of the atmosphere between 17 and 50 km. (Note the lower height, that is the height of the tropopause, varies with seasons and is thus variously quoted; in this Thesis, at an average height of 17 km is assumed.

2.A.1.2 Sampling the stratosphere

The work reported here was restricted to sampling the stratosphere at a maximum height of 41 km (circa 25 miles). This sampling was achieved using a helium-filled balloon which was launched from India (Fig. 2.1). Forty one kilometres is close to the maximum height that such balloons can reach. It could be argued that rockets, because they can go higher, might be used in preference to balloons.



Figure 2.1 The helium-filled balloon used to sample the atmosphere over India.

However, the advantage of a balloon-launched sampler lies in the fact that sampling is achieved at relatively low speeds and less damage to microorganisms is therefore likely to occur during the sampling process. In contrast, the high speed of rockets is likely to ensure that any biological entities are smashed against any rocket-borne sampling devices. However, as has already been stated, Imshenetsky *et al.*, (1978) claimed to have successfully used such rockets to isolate viable, culturable bacteria from the stratosphere. The use of balloons to sample the stratosphere, is neither cheap, nor a routinely available approach and the use of this sampling method here depended upon opportunity. In this case, the Indian scientific research community had planned to use this approach for stratospheric sampling and were willing to supply samples for our use. It is worth noting that, although NASA has the technology to undertake such searches for life in the stratosphere, no research programme in this respect is ongoing, or

apparently planned. As a result, we remain in the surprising position of not knowing to what height the Earth's biosphere extends.

Previous studies in this laboratory had shown the following:

- 1) That bacteria can be seen, using scanning electron microscopes, and DNA-specific staining on membranes on which samples from the stratosphere had been collected.
- 2) That two bacteria, *Staphylococcus pasteuri* and *Bacillus simplex*, and single fungus, *Engyodontium album*, were isolated from such membranes. This fungus is known to be widely distributed on Earth, but is not a common laboratory contaminant (de Hoog, 1978). Further studies, by Wainwright, in collaboration with Indian workers, confirmed the presence of culturable bacteria in similar samples, obtained at a height of 41 km (Shivaji *et al.*, 2006.). From these and other published findings, the fact that bacteria, and possibly fungi, exist in the stratosphere can be regarded as having been established.

At the end of the above-mentioned research, a number of unused membranes, containing stratosphere-related samples were available for use. No attempt was made to further isolate microorganisms from these membranes.

The aim of the work described here was to use scanning E/M and DNA-specific stains to further investigate the presence of microorganisms on the membranes, and thereby add to our knowledge of the microbiology of the stratosphere.

2.A.2 Materials and Methods

2.A.2.1 Stratosphere sampling

Air samples were collected over Hyderabad, India on 20 January 2001 at various heights up to 41 km. The collection involved the deployment of a balloon-borne cryosampler (Fig. 2.2).



Figure 2.2 The cryosampler, showing cryosampler tubes (gold-topped) in carriage.

The cryosampler consisted of a 16-probe manifold, each probe being made of high-quality stainless steel capable of withstanding pressures in the range 10^{-6} mb (ultravacuum) to 200 b. The probes and all their components were thoroughly sterilized. They were flushed with acetone and heat sterilized to temperatures of 180°C for several hours. The entrance to each probe was fitted with a metallic, motor driven (Nupro) valve that could be opened and shut on ground telecommand. The payload trailed at a shallow angle of elevation behind the balloon gondola, being tethered by a sterilized 100-m-long rope. As a further precaution against the possibility of collecting any traces of out-gassed material from the balloon surface, a sterilized intake tube 2-m-long formed an

integral part of the cryosampler ensemble. The intake to each probe was covered during the balloons ascent through the atmosphere.

Throughout the flight the probes remained immersed in liquid Ne, in order to create a cryopump effect, allowing ambient air to be admitted when the valves were opened. Air was collected into a sequence of probes during ascent; the highest altitude reached being 41 km. The cryosampler manifold, once the probes were filled, was parachuted back to ground. The probes were stored at -80°C until the laboratory work began.

Laboratory analysis relating to only two probes is discussed here:

1. Probe A: Collection between 30 and 39 km altitude, a total quantity amounting to 38.4 L of air at normal temperature and pressure (NTP).
2. Probe B: Collection between 40 and 41 km altitude, a total quantity amounting to 18.5 L of air at NTP.

Two procedures were used to extract aerosols aseptically from the probes.

1. Procedure 1: The air from the exit valve of each probe was passed in a sterile system in a microflow cabinet sequentially through a $0.45\ \mu\text{m}$ and a $0.22\ \mu\text{m}$ micropore cellulose nitrate filter (filter diameter 47 mm).
2. Procedure 2: following the completion of Procedure 1, the probes were injected with sterile phosphate buffer solution, agitated for several hours in a shaker to dislodge particles adhered to the walls, and the liquid syringed out and passed sequentially through three filters (i) $0.7\ \mu\text{m}$ glass microfibre filter, (ii) $0.45\ \mu\text{m}$ cellulose acetate filter, and (iii) $0.2\ \mu\text{m}$ cellulose acetate filter.

Most of the aerosols are expected to have been collected in Procedure 1.

2.A.2.2 Precautions taken against contamination

Standard microbial techniques, employing a laminar air cabinet, were used throughout. All plates were used within 8 h of pouring and were checked for contamination, both by light microscope examination and by culturing. Control membranes were also analysed after being subjected to identical transfer procedures used for stratosphere-derived samples. In addition, the isolation experiments were repeated in a separate laboratory, by a technician who was not informed of any expected outcomes.

2.A.3 Results and Discussion

2.A.3.1 Inorganic cosmic dust

(Some of the data discussed here were obtained from photographs obtained by Dr Melanie Harris, from the same membrane sample batch as those examined here in Sheffield. These photos were presented to Dr Wainwright by Dr Melanie Harris, formally of the University of Cardiff. They were produced as a collaborative effort conducted by Prof. David Hughes and Prof. Chandra Wickramasinghe of the same University. They have not been previously assessed, or published and are used here with the permission of Professor Wickramasinghe).

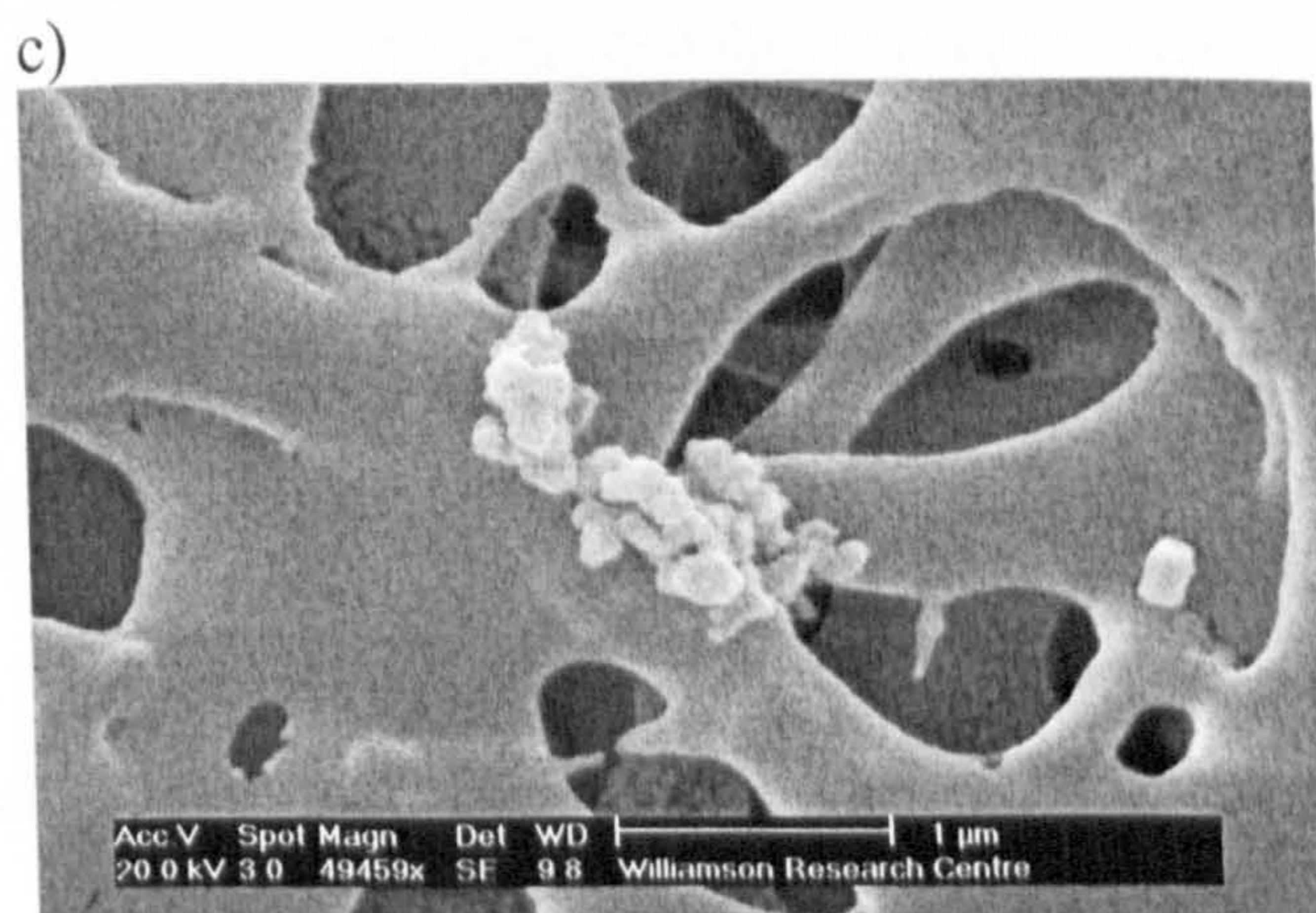
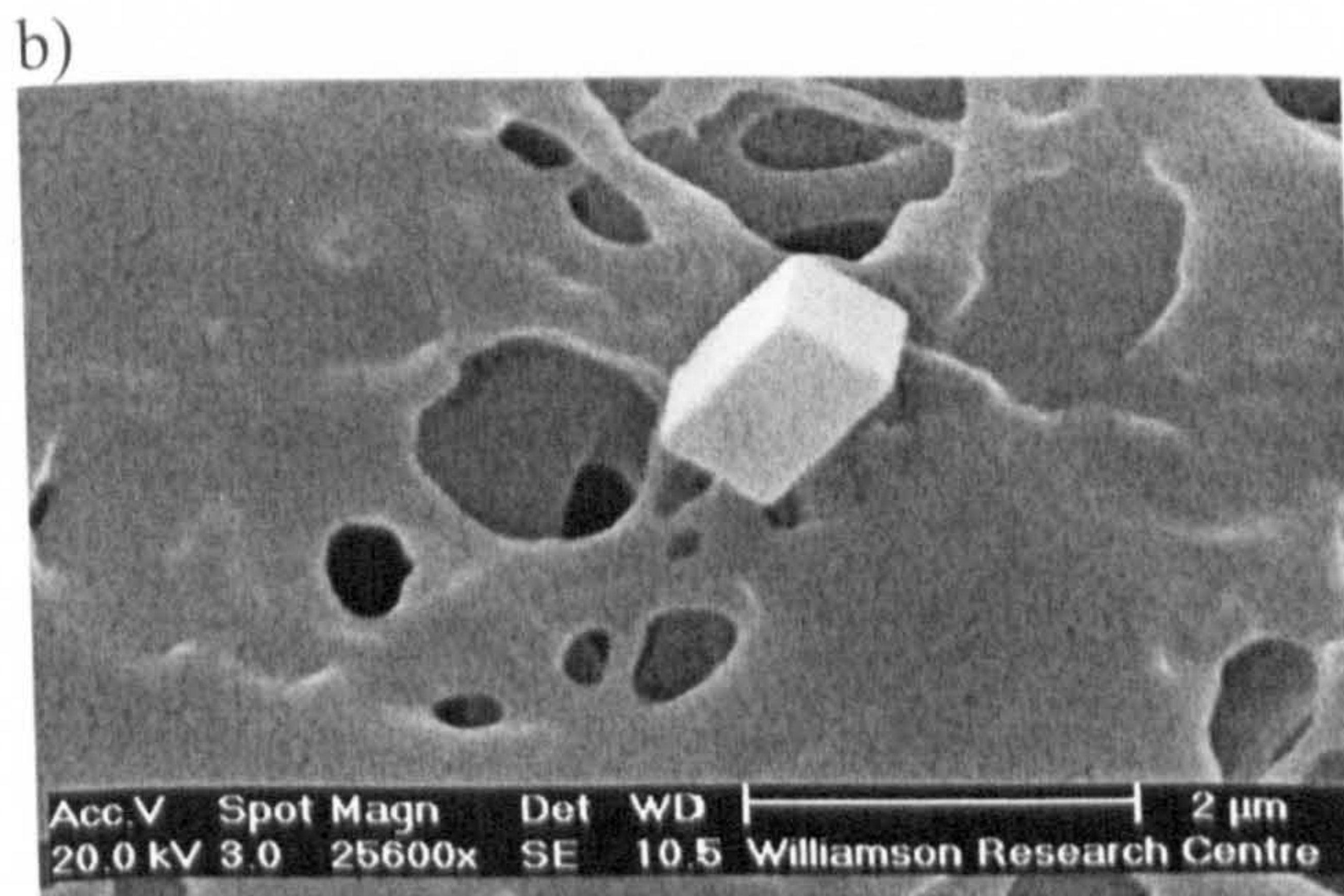


Figure 2.3 Inorganic particles of cosmic dust on membranes a) Doughnut-shaped silicon particle, b) zircon crystal, c) clump of silicon-rich particles mimicking bacteria. (Height, 41 km).

It is important to emphasise that the majority of particles seen under scanning E/M on membranes of material collected on stratospheric membranes (hereafter referred to as membrane material) is composed of inorganic material. The sheer amount of this material along with its chemical composition and non-biological nature shows this material to be cosmic dust. Figure 2.3a for example, shows an individual doughnut-shaped silicon-rich particle, while a remarkable oblong-shaped particle which, under EDAX analysis, was seen to be rich in zircon is shown in Fig. 2.3b. It is extremely easy, based on superficial morphology alone, to conclude that some of these inorganic particles are bacteria. Figure 2.3c, for example shows a clump of inorganic dust that is superficially bacteria-like. Clumps of similar material have however, been shown (by Nano-Sims analysis) to be inorganic in nature (Wainwright *et al.*, 2004). Extreme caution must therefore be applied, and some analysis must be undertaken, before any particle found on the stratospheric membranes is referred to as a bacterium. In these studies, we applied some, or all, of the following criteria before concluding that any observed membrane particle is bacterial in nature:

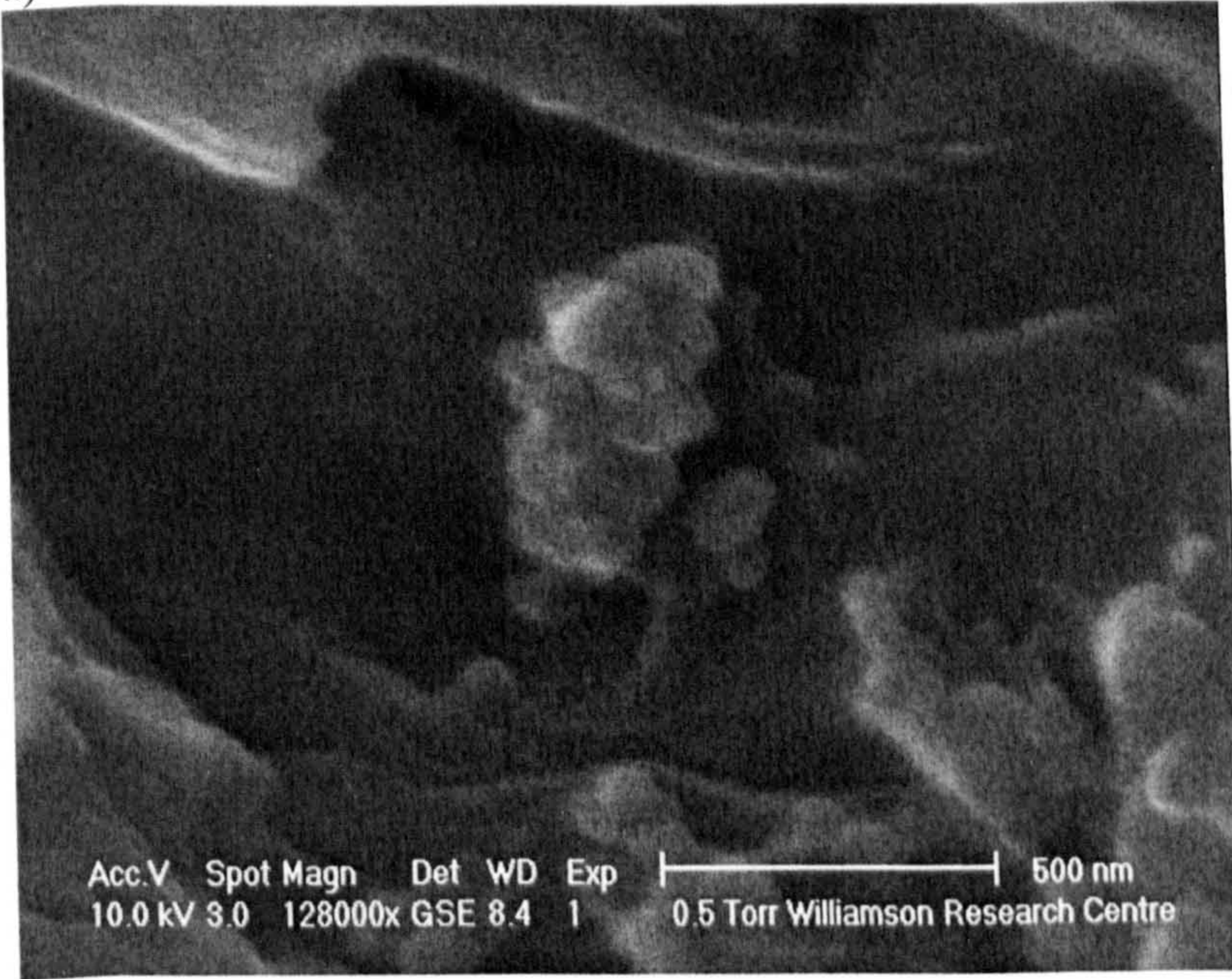
- 1) The particles must look morphologically like, and be of the size typical of bacteria. It should be remembered however, that sub-micron bacteria may be present; as a result, no size limit for presumptive bacterial cells should be applied *a priori*.
- 2) Morphological characteristics typical of bacteria should be found to be present. Examples include fimbriae, flagella or bacterial slime.
- 3) Analytical tests, such as EDAX (associated with scanning E/M) and (less routinely), Nano-Sims analysis should be applied to make sure that the suspected biological particles does not consist solely of inorganic elements, such as Ca, Fe, and Si. Biological

particles would be expected to contain carbon and only small amounts of inorganic ions. An exception may exist where bacteria are coated with inorganic materials such as calcium or silicon.

4) Biological particles may be distinguished from mineral particles by the, relatively extended, application of the electron beam from the scanning E/M. Cosmic, or other dust, will not withstand such treatment, while biological materials, including bacteria, will be holed, or denatured, by the extended exposure to an electron beam. This effect is shown in Fig. 2.4. Here, particles obtained from rain water (collected in Sheffield) were exposed to relatively long periods of exposure to the electron beam of the SEM.

Fig. 2.4a shows a clump of particles before beam exposure, while Fig. 2.4b shows a hole formed in the particle clump following application of the beam. From the result of this treatment, it is concluded that the clump of particles under examination, is biological in nature, (i.e. it includes a bacterium).

a)



b)

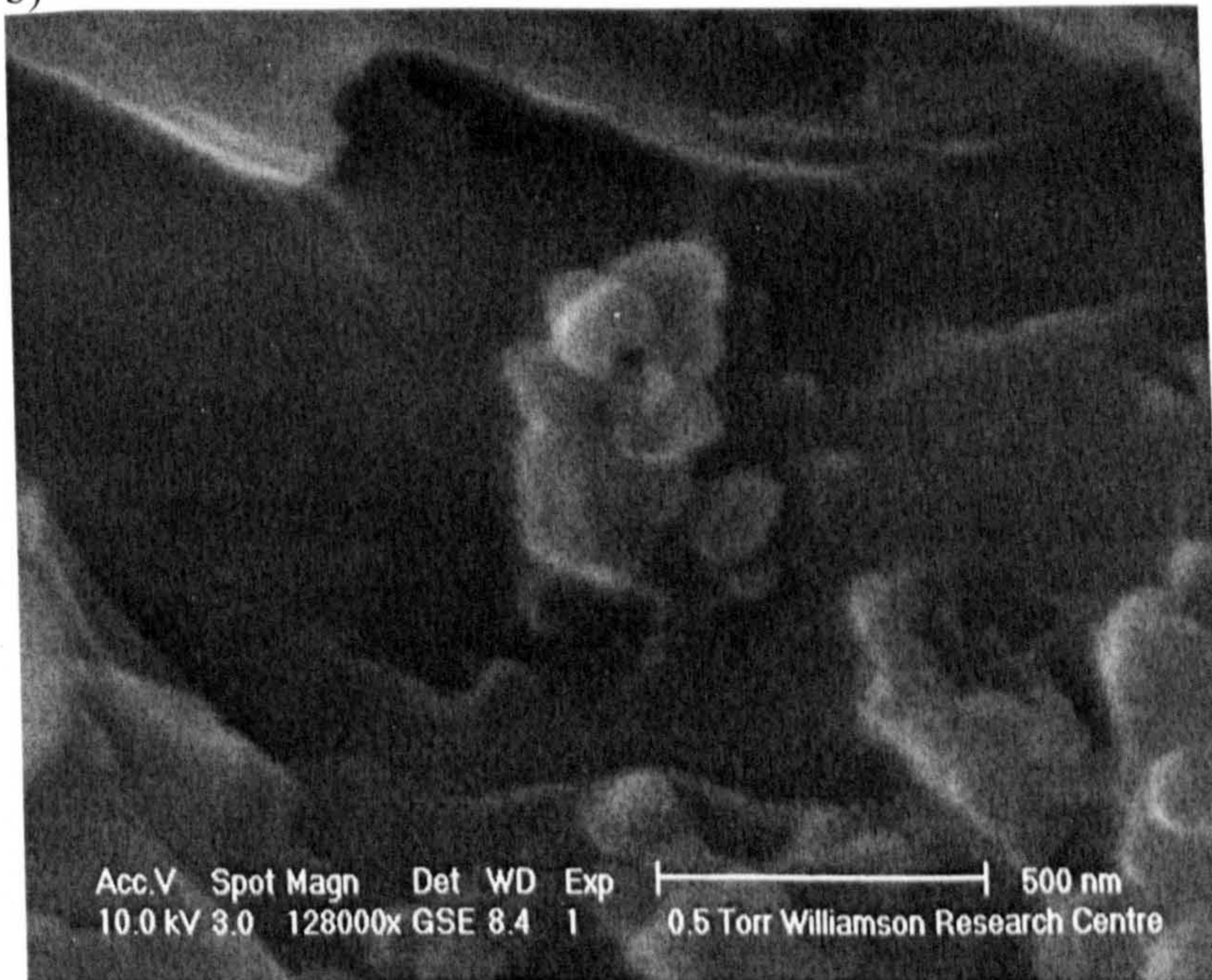
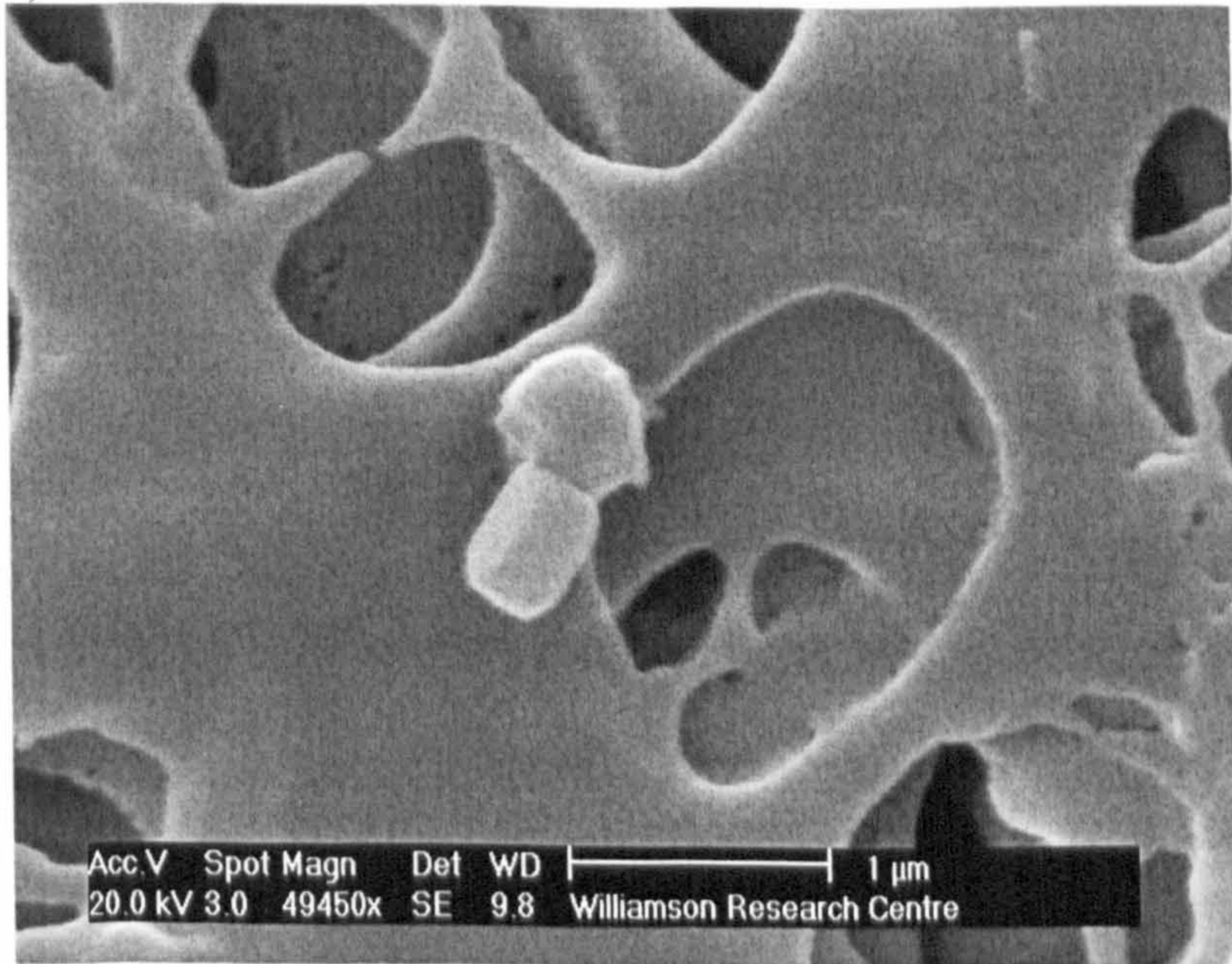


Figure 2.4 The effect of leaving the electron beam of the scanning E/M on biological particles. Note the hole seen in Fig. 2.4b, caused by the beam, and not seen in Fig. 2.4a.

The zircon particle shown in Fig. 2.3b did not undergo similar degradation in the electron beam showing this is inorganic and not a bacterium. Presumptive bacteria are also shown in Fig. 2.5. In Fig. 2.5a, another example of a zircon particle is shown which, unlike that seen previously (Fig. 2.3b), appears to have undergone erosion of its edges. Associated with this clearly crystalline zircon particle is an amorphous particle which did not EDAX as a mineral and is considered to be a presumptive bacterium. The suggestion that the particle adhering to the zircon crystal is a bacterium is further strengthened by the presence of what appears to be bacterial slime at the point of contact with the zircon crystal. From these findings, it is concluded that the particles attached to the zircon crystal is a bacterium of diameter of around 0.5 micron. The bacterium and zircon crystal must have become attached at some point. Did this attachment occur in space, on Earth or during the sampling procedure? It is worth noting that, because of the mass of the zircon crystal, the zircon-bacterium mass will fall out of the stratosphere extremely rapidly. It is unlikely that particle mass, of this size, could exit the stratosphere. The crystal bacterial mass therefore appears to have originated in space or formed during the sampling period. Another presumptive bacterium is shown in Fig. 2.5b, again of a size approximating to 0.5 micron.

a)



b)

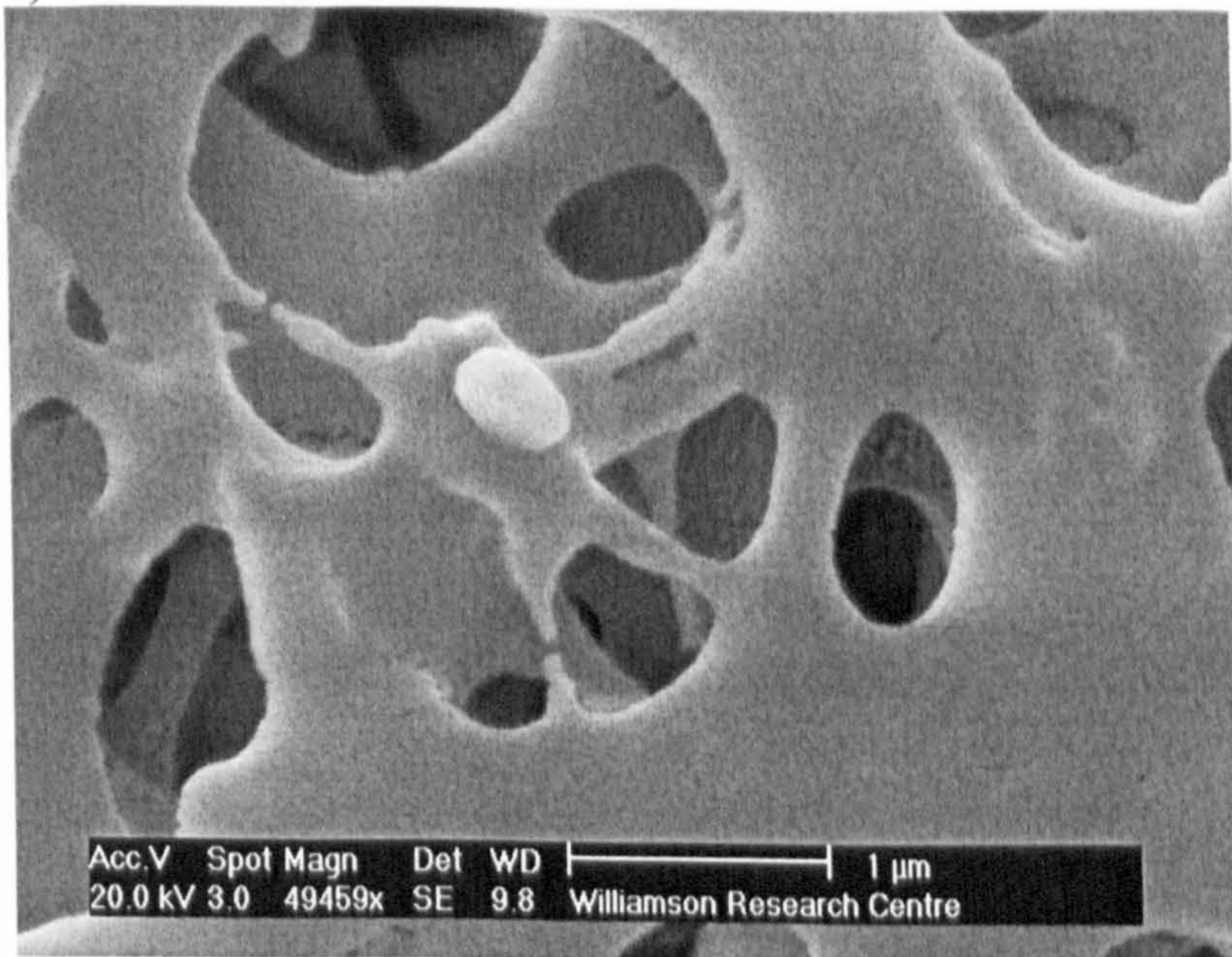
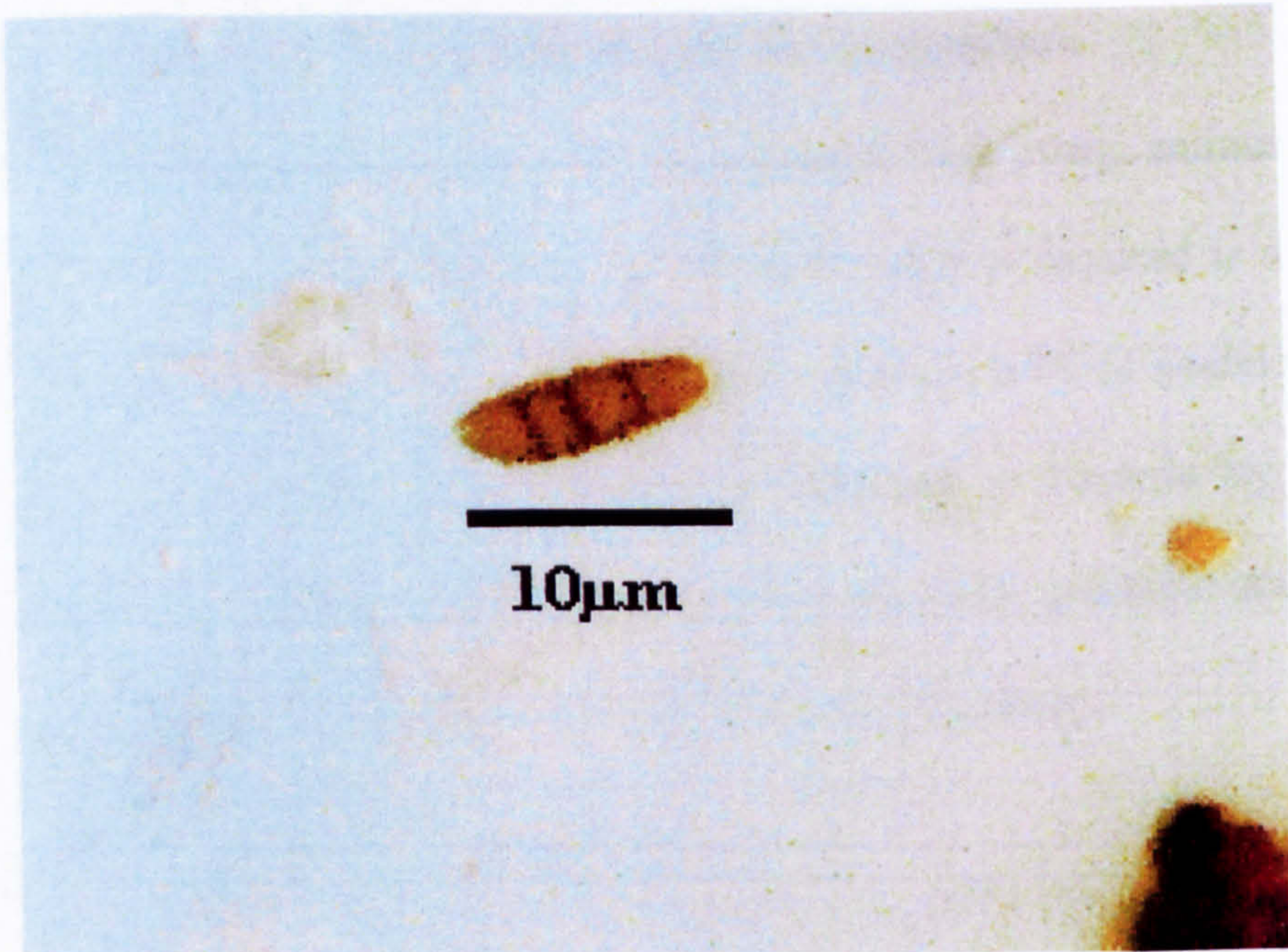


Figure 2.5 Particles having the morphology and chemical properties (EDAX) of bacteria, a).an amorphous, presumptive, bacterium attached to a weathered zircon particle and b) a presumptive bacterium showing signs of collapse under the pressure of the scanning E/M. (Height, 41 km).

Morphological evidence for the presence of fungi in the stratosphere is provided in photographs shown in Fig. 2.6. A fungal spore, (observed using the light microscope) similar to a member of the genus *Helminthosporium* or possibly *Alternaria* is shown in Fig. 2.6a; this spore was found on membranes obtained from the 22-23 km sampling height. What appears to be mass of fungal hyphae is shown in Fig. 2.6b, sampled low down in the stratosphere, at a height of 22-23 km. Although this tangled hyphal mass is assumed to be fungal, the hyphae are thinner than might be expected of a fungus (at least when grown under nutrient-rich conditions); this suggest the possibility that it may be an actinomycete. On the other hand, the hyphal mass may represent a fungus growing under low nutrient conditions; which tend to induce the formation of fine hyphae (Wainwright *et al.*, 1991).

a)



b)

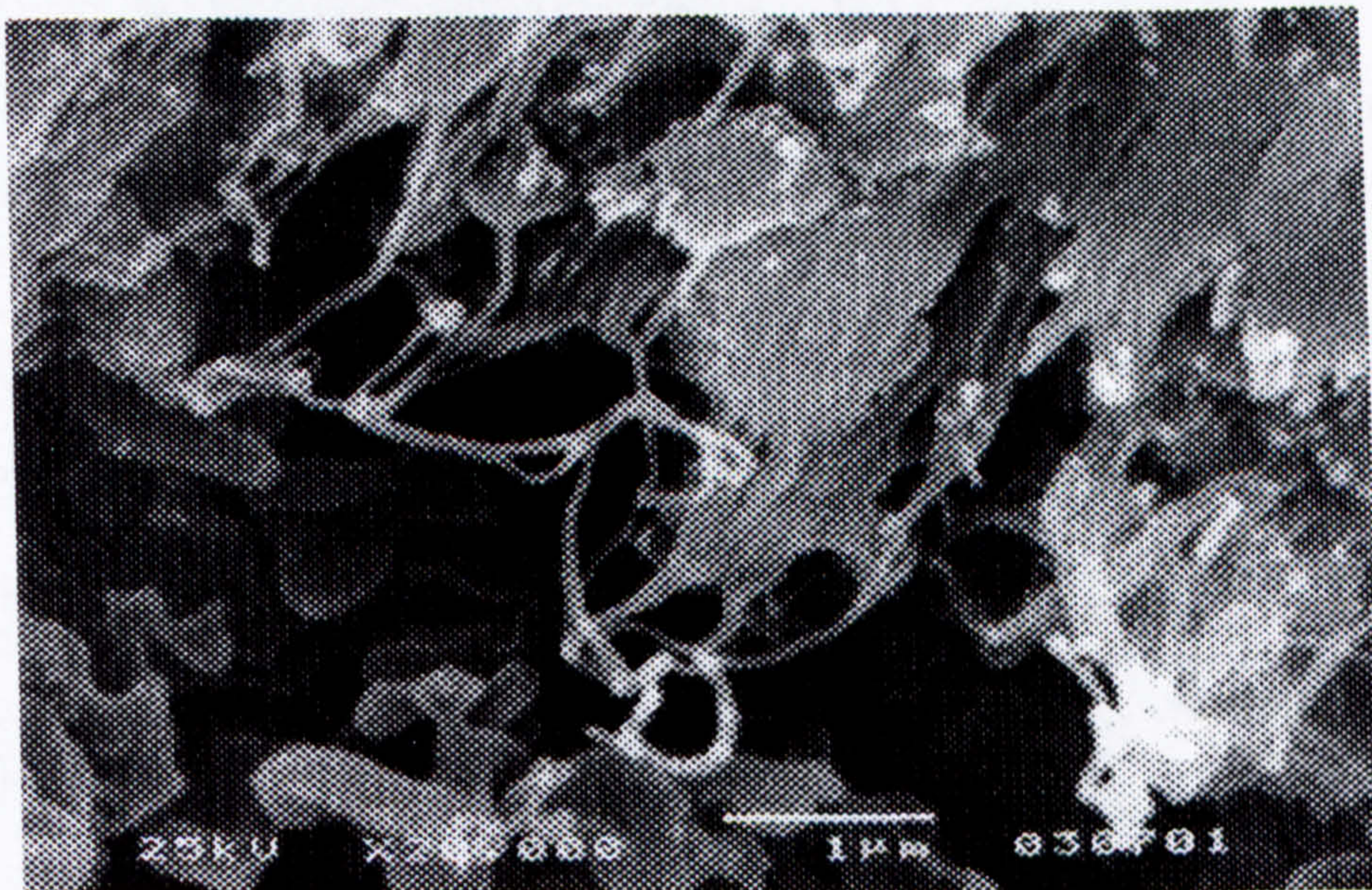


Figure 2.6 Presumptive fungi from the stratosphere, a) a fungal spore as seen under the light microscope, b) fungal or possible actinomycete-like hyphae from the stratosphere (22-23 km). (Kindly provided by Dr M. Harris).

2.B Use of Live /Dead and the DNA stain DAPI to confirm the biological nature of presumptive bacteria sampled from the stratosphere

The presumptive evidence for the presence of living entities on the stratospheric membranes can be discussed *ad infinitum*, what is required is a test that confirms the presence of a biological marker, such as protein, ATP or nucleic acids. The aim of the following work was to confirm the presence of bacteria by staining some of the membranes with Live/Dead Stain, a commercially available stain which stains living bacteria fluorescent green and dead bacteria red orange.

2.B.1 Material and Methods

2.B.1.1 Viable staining

The air samples collected from the stratosphere were passed through 0.2 and 0.45 μm cellulose acetate membrane filters. Some of these samples were used by Harris *et al.* (2001) to detect the presence of viable bacteria; studies on some of the remaining samples are discussed here.

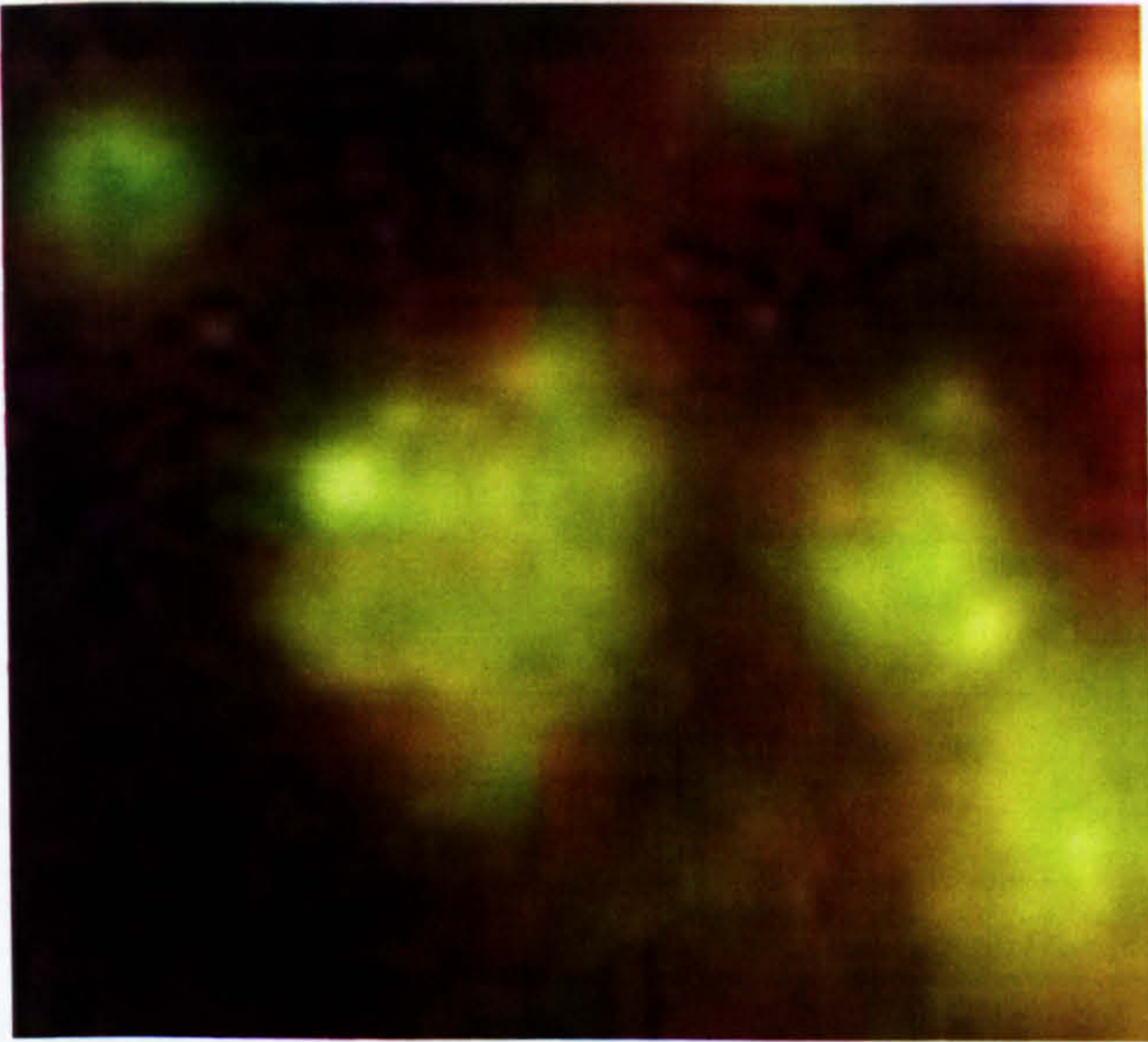
Sterile distilled water (1 ml) was added to samples of portions of membrane (of varying sizes) contained in sterile plastic Universal bottles. Membranes through which air passed at both heights were used. The bottles were shaken vigorously for 15 minutes and the presence of viable microorganisms was determined by using a Live/Dead BacLight Bacterial Viability Kit (Molecular Probes Inc., Netherlands), as described in the maker's instructions. Two control membranes were included: (a) a sample of membrane that had been exposed to the same handling procedures as above, but which had not been exposed to the atmospheric air sample and (b) portions of a new, unused membrane, Samples of the stained solutions were then examined under oil immersion

(100x) using a fluorescent microscope (Olympus BX41, fitted with a U-RFL-T lamp. using a wide blue filter). An equal amount of time was devoted to examining both test and control samples, photographs were taken using a digital camera (Olympus C4040 200M, calibration carried out using Olympus DP-soft imaging software).

DNA Staining was achieved using DAPI. Samples from the filters were transferred onto premium microscope slides that had been washed, cleaned and polished. A stock solution containing 0.1 mg^{-1} DAPI stain was prepared, applied and left to develop for 15 minutes, prior to being examined under the fluorescent microscope.

2.B.2 Results and Discussion

Figure 2.7 shows that when Live/Dead stain is applied to the stratospheric membrane some particles stain red, while others stain bright green and orange. This figure provides evidence for the presence of a) living cells, having active membranes, and b) dead cells having non-functional membranes; since the particles are of the correct size and morphology typical of bacteria this picture provides further evidence that bacteria were sampled from the stratosphere at a height of 41 km. It is important to note that a large proportion of the particles on this membrane stained red and were therefore dead bacteria. This is not surprising considering the extreme environment provided by the stratosphere, a subject that will be discussed at length below.

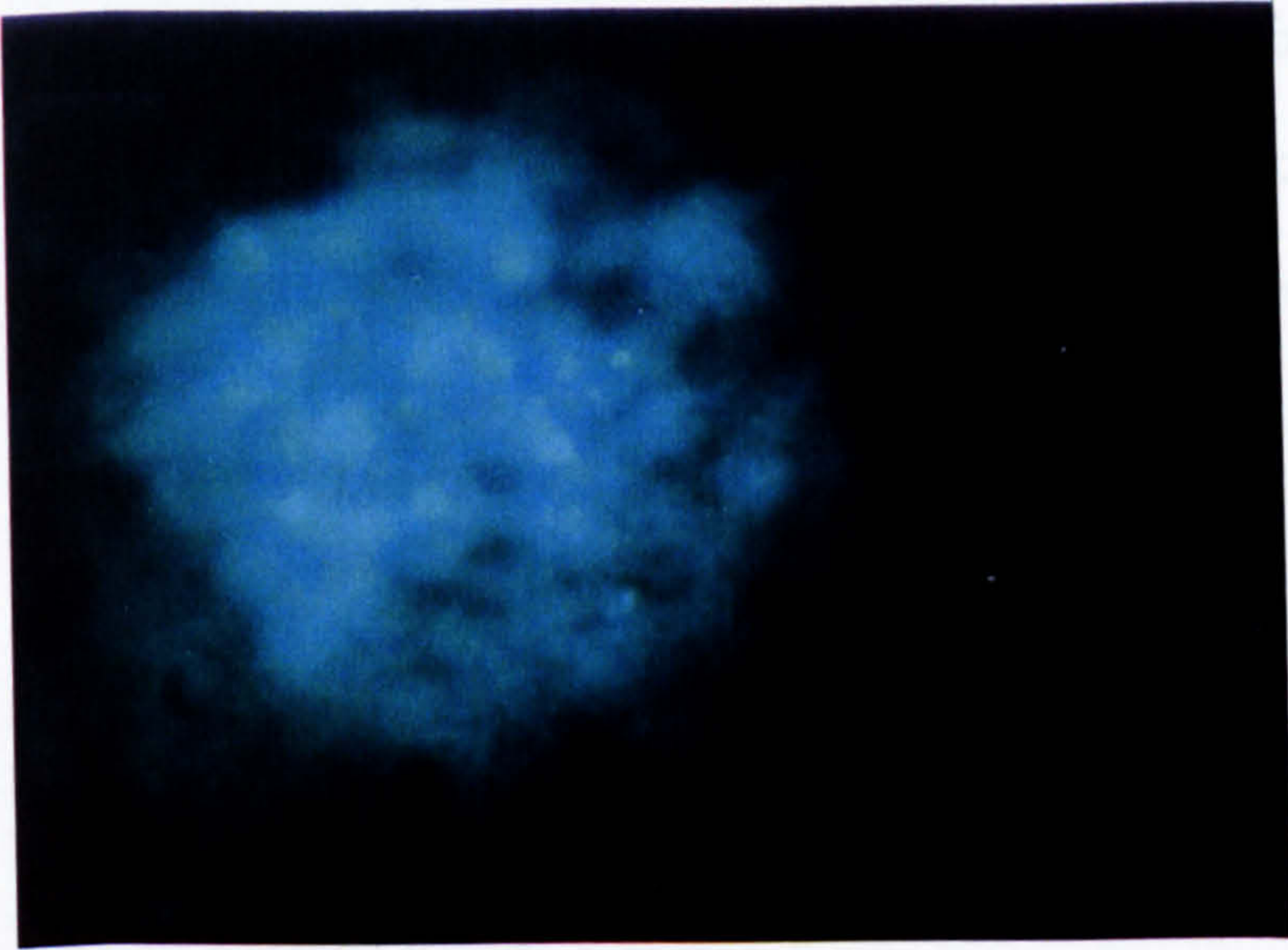


UNIVERSITY
OF SHEFFIELD
LIBRARY

Figure 2.7 Live/dead staining of bacteria-like particles on membrane (41 km) Dead cells stain red and living cells stain green. The clump of green cells (mid-left of centre) is approximately 5 microns across.

When the membranes on which stratospheric samples had been deposited were stained with DAPI, fluorescent blue clumps of cells were seen, indicating that these clumps contain DNA. Of particular importance (as will be discussed later) is the fact that these clumps of bacterial cells exceed a diameter of 20 microns (Fig. 2.8).

a)



b)

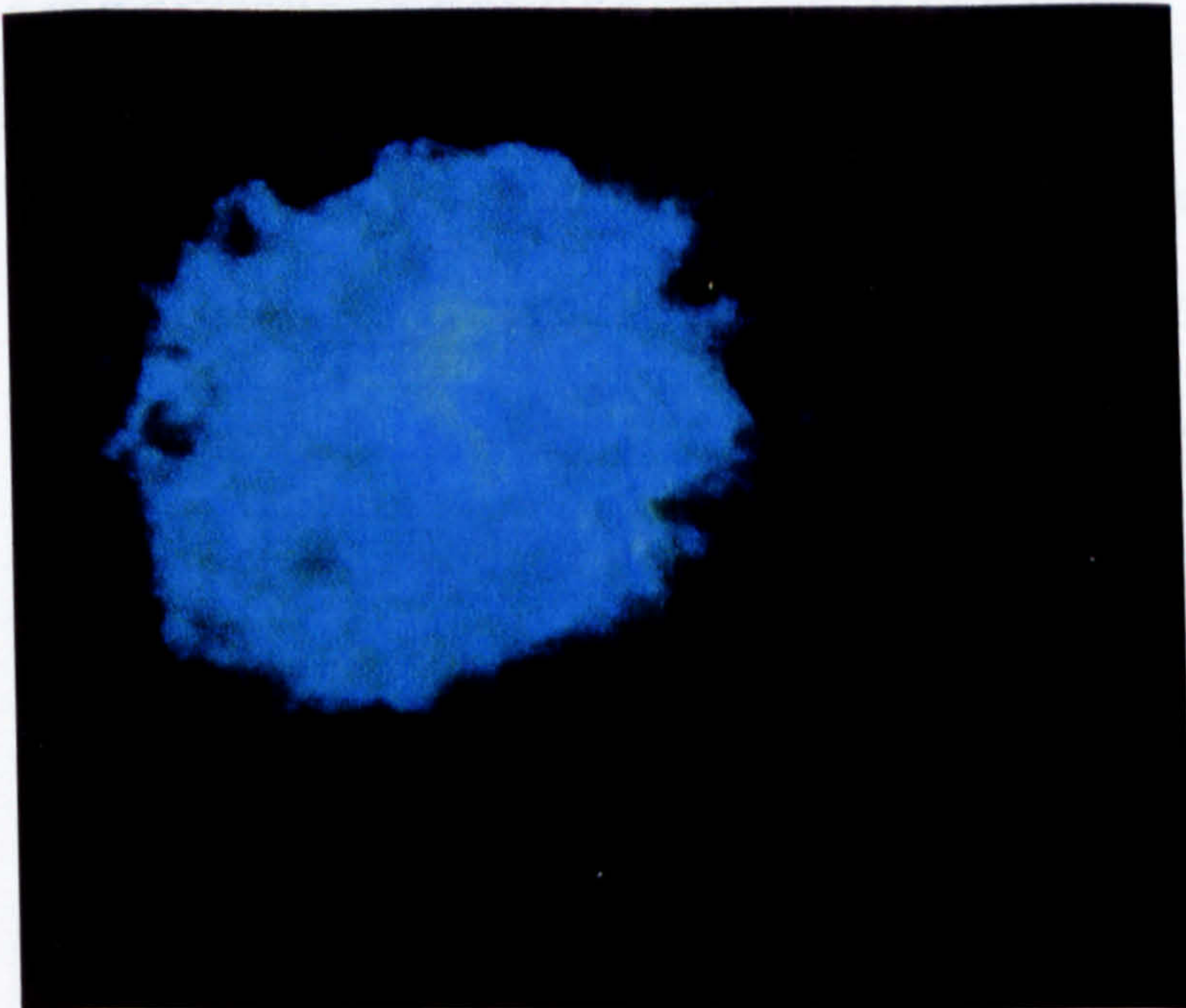


Figure 2.8 Clumps of bacteria (circa 20 microns across) sampled from 28 km and stained with, the DNA stain, DAPI.

When the above findings were presented at internal Departmental seminars there was comment that Live/ Dead and DNA stain should not be used in the way they were applied above, and can only be confidently applied to stain particles known to be bacteria. As will be seen below, we have used DNA stains throughout our studies (they were also used in similar studies mentioned above by Harris *et al.* (2001) at Cardiff University) and we can see no logical reason why they cannot be used to stain DNA in unknown, or presumptive bacteria. As a result, we are confident that the use of DNA stains confirms the previously described scanning E/M work and we conclude that bacteria can be found in the stratosphere at a height of 41 km.

2.B.2.1 Mechanisms by which microorganisms could reach the stratosphere from Earth

The possibility that the above findings result from contamination is obviously reduced by the fact that independent studies have demonstrated the presence of a stratospheric microflora. Since, however, a negative cannot be proven, it is impossible to rule out sampling or laboratory-based contamination. However, if we accept that microorganisms are indeed present in the stratosphere we are left to answer the following question: how did they get there? The obvious answer-that they were carried up from the Earth – unfortunately, presents a number of problems. Firstly, the tropopause (at an average height of 17 km) is generally thought to act as a barrier to the upward movement of particles (although not necessarily gases or volatiles). As a result, particles must be ejected with force before they can pass through this barrier. Volcanic eruptions provide an obvious means by which particles could be ejected through the tropopause into the stratosphere. However, such eruptions occur infrequently and, since any particles

reaching the stratosphere are subject to sedimentation under gravity, such eruptions cannot provide a constant supply of stratospheric microorganisms.

Although there seems to be no obvious means by which microorganisms could regularly reach 41 km; a number of possibilities can be suggested. For example, it is possible that particles, including microorganisms, are transported to the stratosphere in the updraft resulting from so-called blue lightning strikes. These are conical blue jets that travel upwards from cloud tops and travel at a hundred thousand metres per second to a height of 70 km, charging the atmosphere as they go (Pasko *et al.*, 2002); whether microorganisms could survive such charging and the high rate of upward movement is of course open to question.

Microorganisms might also be carried above the tropopause when thunderstorms and forest fires occur together. Apparently, forest fires strengthen thunderstorms, which then pump immense plumes of smoke and soot (as well as presumably microorganisms) into the stratosphere (Fromm *et al.*, 2004).

Finally, so-called gravito-photophoresis (GP) might provide yet another mechanism by which microorganisms could reach the stratosphere (Rohatshek, 1996). As a result of GP, soot can be carried to a height of 10-20 km, and beyond. Calculated vertical velocities exceed settling velocities by a factor of 30, and it takes about 30 years to transport soot from 10-20 km, and a further 20 years to transport it from 20-80 km. The effect of GP is strongly altitude dependent with the lifting force most effective at an altitude of between 10 and 85 km. It is therefore possible that small microorganisms could be carried by this mechanism in soot particles. Such transfer would be particularly advantageous, since a coating of soot would protect the organisms from the damaging

effects of ultraviolet radiation. However, a major problem with this suggestion is that GP only works on soot particles of diameter around 1 μm ; submicrometre-sized bacteria might however, reach the stratosphere by this mechanism, either independently or when travelling on soot particles. This caveat immediately excludes the possibility that fungal spores, which are relatively large, are carried up from the Earth to the stratosphere in this way. It is possible, however, that microscopic fragments of fungal hyphae are carried from Earth to this region and then, infrequently, cultured from stratospheric air samples. It is particularly noteworthy that bacteria and fungi growing under low nutrient (i.e. oligotrophic) conditions are usually much smaller than equivalent cells grown in nutrient rich environments (Wainwright *et al.*, 1991); oligotrophically growing microorganisms are therefore likely to be the requisite size to enable them to be transported through the stratosphere.

2.B.2.2 Is the stratospheric microflora made up of two bacterial populations of distinct origin?

The view that all of the microorganisms found in the stratosphere originate from Earth is questionable for the following reasons. Firstly, many of the clumps seen by scanning electron microscopy are relatively large. Harris *et al.* (2001), for example, reported clumps (exceeding 10-20 μm across) of viable, but unculturable, bacteria. It is difficult to see how any mechanism could carry such large masses into the stratosphere (Fig. 2.8, 2.9).

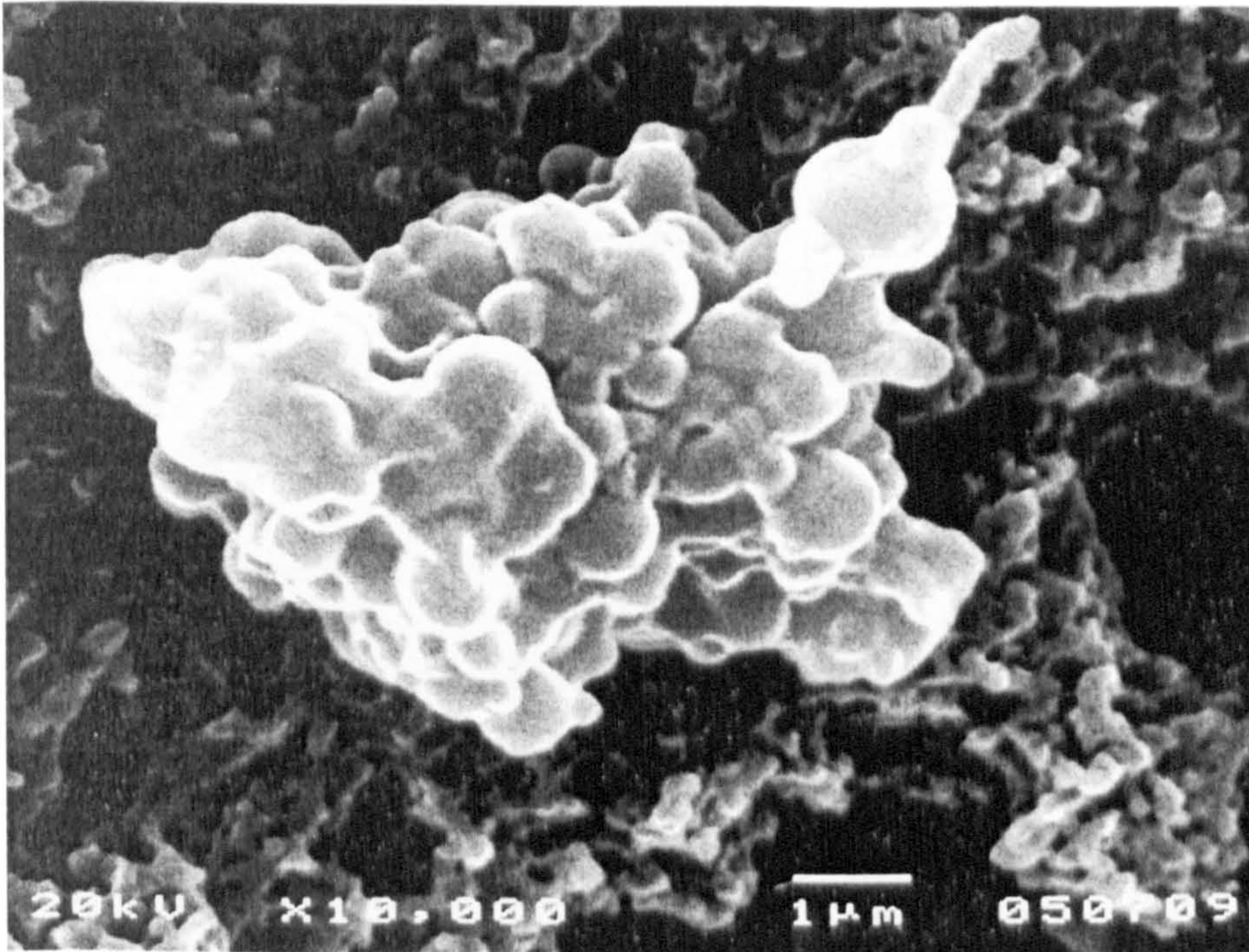


Figure 2.9 Scanning electron microscope image showing clump of bacterial cells of cross section in excess of 10 microns (from Harris *et al.*, 2001).

It is also unlikely that a single bacterium, carried up to 41 km, for example, would replicate to form such a clump under the extreme conditions prevailing at this altitude. Such clump formation might possibly have occurred as the samples were being processed in the laboratory, as they were being exposed from freezing to room temperature and prepared for scanning electron microscopy. However, since no nutrients were added at this stage, such replication and growth to form clumps would have had to have occurred under oligotrophic conditions; presumably such actively dividing bacteria would have been readily cultured, which was not the case (alternatively, bacteria might have physically clumped together).

If such relatively large clumps of bacteria do in fact occur in the stratosphere then we are left with the possibility that they originate from space. Provisional evidence in support of this view was recently provided by Narlikar *et al.* (2003). Based on the

determination of numbers of presumed bacteria at two heights in the stratosphere, they concluded that the viable, but non-culturable, bacteria that occur as clumps must be incoming to Earth, rather than exiting.

2.B.2.3 A mechanism by which evolution may have been speeded up by the transfer of bacteria from Earth to the stratosphere

It is possible that the transfer of bacteria (and other microorganisms) from Earth to the stratosphere may have impacted (and continue to impact) on bacterial evolution (Wainwright, 2007). The continued development of life on Earth has depended upon the presence of an atmosphere that shields the biota from the lethal effects of ultraviolet radiation. Such protection will necessarily have reduced the exposure of bacteria to the potentially mutagenic ultraviolet and other radiation. This problem can obviously be overcome if a mechanism exists whereby bacteria can be transferred from the relatively benign environment of Earth to the highly mutagenic conditions found in the stratosphere. The above described mechanisms provide a means by which such transfer of bacteria between Earth and the stratosphere can occur. Those bacteria that are mutated, but not killed, by exposure to stratospheric ultraviolet radiation (a mixture of UV-A, B and C), will be returned to Earth as the result of sedimentation. The novel, multiple or increased number of mutations they carry with them will increase the mutation pool that is available for natural selection to act upon and, as a result, the rate of bacterial evolution will have been greater than if such evolution was solely dependent on mutations occurring below the stratosphere. It is likely that such mutations will occur most often in the lower regions of the stratosphere where numbers of bacteria are highest, and where residence times allow for rapid mutation, but not sterilization. Of

course, such a view is dependent on a large number of bacteria being exchanged between the Earth and the stratosphere. However, even if we consider volcanic transfer alone, the number of bacteria transferred is likely to be extremely large over the aeons that bacteria have existed on the Earth (Wainwright, 2007).

2.B.3 Conclusion

Recent findings relating to the microbiology of the stratosphere suggest that there exist at least two separate populations of stratospheric microorganisms. One population consists of common Earth bacteria and fungi that are carried on a relatively regular basis (by phenomena such as blue lightning, fire-associated storms and GP) to heights above 17 km; these organisms can be cultured, albeit rarely, from stratospheric air samples. The fact that such bacteria are, essentially, genetically identical to the same species derived from Earth suggests to us that they are from Earth, as it would seem highly unlikely that space-derived bacteria would have evolved at exactly the same rates as their Earth-derived counterparts. Occam's Razor can thereby be invoked: Earth-like bacteria come from Earth (Wainwright *et al.*, 2003).

The second component of the stratospheric bacterial population consists of bacteria that exist as relatively large clumps; this population is viable, but non-culturable. It is assumed here that such clumps (around 10-20 microns across) are too large to have originated from Earth and their distribution in the atmosphere indicates that they have a non-terrestrial origin. Since the majority of bacteria found on Earth have yet to be cultured, the possibility exists that an unknown fraction of these bacteria may have originated from space. The possibility still remains however, that all components of the stratospheric microflora are incoming to Earth. I recognize of course that these views are

highly controversial and will need to be verified by further studies of the microbiology of the stratosphere.

2.B.3.1 Some suggested critiques of the above

The above argument is based on the view that the samples did not grow during sample processing. The samples were collected by a cryosampler which means that they were obtained under conditions of low temperature and high pressure. It would seem highly unlikely that microorganisms could have grown under these conditions. Since the tubes were kept frozen prior to sampling it also remains unlikely that the 10-20 micron-plus bacterial clumps could have arisen during this period. This leaves the possibility that growth occurred during the sampling period, when the tubes were air-evacuated or were washed out. These processes took place at room temperature over a period of around 4h. It again appears unlikely that the bacterial clumps could have formed during this period, or while the membranes were stored at freezing prior to examination under the SEM. This is especially the case, since no nutrients were added at any stage during this processing, so the bacteria would need to have grown oligotrophically on the membranes. While all of these possibilities may have occurred, the balance of probabilities is that the ten microns clumps were sampled at 41 km and were not artefacts produced during laboratory processing.

Finally, it is possible that the 10-20 microns plus clumps came from high flying aircraft, or from space debris associated with spent rockets and satellites etc. However, most commercial aeroplanes fly at 30,000 ft (approx. 10 km), well below the height of 41 km sampling point. It is likely that the outer surface of any high flying, military aircraft would undergo surface sterilization during heating as they approach the speed of sound.

Similarly rockets are likely to undergo sufficient surface heating during passage through the atmosphere to sterilise their surfaces. However, the possibility that the 10 micron-plus particle masses arose from the above sources cannot be ruled out. Again it is a question of probability. What is the probability of a relatively small amount of space junk or high flying aircraft contributing particles to the balloon sampler? Although not impossible, this would appear unlikely. This is especially the case since Narlikar *et al.* (2003) calculated, based on the number of DNA-stainable particles on stratospheric filters, that the daily mass input of bacteria onto Earth would be around a third of tonne. If this calculation is correct, then it is clearly unlikely that all such bacteria could have originated from space debris, rockets and aeroplanes.

It should be noted that considerable effort went into avoiding contamination of the collection apparatus by microorganisms from the balloon; such efforts were based on calculations which showed that the sampling device would never pass through the airspace under the balloon in the time taken for microbes to fall and contaminate the sampling airspace.

2.B.3.2 The potential role of small bacteria in relation to transfer of life from the Earth to the stratosphere

Most commonly studied bacteria, when grown in the laboratory are of dimension around 1µm. This fact has tended to cloud the perception of microbiologists to view this dimension as the “normal” size of bacteria. However, it is becoming increasingly clear that sub-micron bacteria exist in the environment. The importance of this fact to astrobiology lies in the fact that the smaller the bacterium, the more likely it is to be elevated from Earth to the stratosphere. Thus, while it seems difficult to explain how a

one micron-sized particle could be transferred from Earth to 41 km (in the absence of volcanoes and impact events), it becomes increasingly more likely that a sub-micron particle could be elevated to this height, with the probability of this occurring increasing with decreasing bacterium size. Similarly, it is likely that a sub-micron bacterium would have a longer residence time in the stratosphere, compared to a micron-plus sized bacterium.

Small forms of bacteria have been reported from all terrestrial environments. A particularly interesting study was reported by Bakken and Olsen (1987), who found that the majority of bacteria, seen by direct microscopic counts, were small forms (less than 1 μm); they concluded that the majority of these small bacteria are not small forms of ordinary bacteria induced by starvation which then resort to "normal" sized bacteria on nutrient-rich media. While soil plate counts tend to count medium-large bacterial cells the small forms are almost totally excluded from this isolation approach. It is well known that starvation reduces the size of both bacteria and fungi and that microorganisms growing oligotrophically are also much smaller than similar, copiotrophically, growing cells. We thus appear to have present in the environment, stable small bacteria and bacteria whose size depends upon the nutrient conditions under which they are growing. Many natural environments are oligotrophic; it is expected that in these, small (nutrient dependent) forms of bacteria will predominate. The work of Bakken and Olsen (1987) and others clearly shows that there exists a population of stable, small bacteria (ultramicrobacteria) in the oceans. Whatever their origin, such small forms are likely to be more readily transferred to the stratosphere than are one micron-plus sized bacteria; it is therefore important to determine whether such small

forms are common in the Earth environment. Recent work in this laboratory has confirmed findings reported from elsewhere, showing that sea water is a particularly good source of so-called “filterable bacteria”, that is bacteria that can pass through 0.45 and 0.22 μm membrane filters. There have also been a limited number of reports of marine bacteria or bacterial forms being capable of passing through 0.1 μm filters (Lillis and Bissonette, 2001).

Fischer and Velimorov (2000) concluded that the rod morphotypes have more potential to produce viable cells with minimal volumes than spherical morphotypes. It would therefore be expected that filterable bacteria would be predominantly rod-shaped, and this appears to be borne out by the literature. As a result, we might also expect bacteria in the stratosphere to be predominantly rod-shaped, and again there are indications that species of *Bacillus* predominate, although not exclusively, the stratospheric bacterial flora (Shivaji *et al.*, 2006).

As a generalisation then, it is more likely that a small, sub-micron bacteria would have a greater chance of being elevated from Earth to the stratosphere, than a “normal”, micron-plus, sized bacterium; this assumption is independent of the mechanism involved in such a transfer. As a result, it would be of obvious interest to determine if sub-micron bacteria can be regularly isolated from environments on Earth, notably sea water.

2.C Fungi in the stratosphere

2.C.1 Introduction

If the enigma of how bacteria get to heights of 41 km in the stratosphere presents a problem, then the question of how fungi achieve such heights is even more difficult to

explain. This is simply because fungi are bigger, both in terms of their vegetative biomass (i.e. the hyphae) and their reproductive forms, namely spores. It has already been noted that Imshenetsky *et al.* (1978) isolated a range of fungi from heights up to, and exceeding, 60 km, while the presence of these organisms in the stratosphere at heights of 20 and 41 km has more recently been confirmed. If we assume that fungal hyphae and spores have an average diameter of 4 μm , a size which would have difficulty crossing into the stratosphere, then we are presented with the problem of finding smaller forms of these organisms that might be able to cross the tropopause by the various mechanisms described above in relation to the transport of bacteria into the stratosphere. We are therefore left needing to find small forms of hyphae and or spores than can be lifted to heights above 17 km; such small forms, as is the case with bacteria, would need to be sub-micron in diameter.

The most obvious means by which small forms of fungi might be generated is by either starvation, or oligotrophic growth. Here we define starvation as a period of nutrient-deficiency imposed after a period of nutrient sufficiency (Wainwright *et al.*, 1991). Oligotrophic growth in contrast is the state in which fungi are permanently growing under low nutrient conditions and are thereby adjusted to this condition; oligotrophy does not thereby constitute a stressed state for fungi, in fact it is the state in which fungi most frequently exist in the common environments of Earth, which are oligotrophic, (e.g. fresh waters, seawater and soils) and away from decomposing organic matter or the rhizosphere. Under both conditions, fungal hyphae and fungal spores have been found to decrease in size. Under oligotrophic conditions, fungi also lose their ability to produce complex spore bearing structures such as the phialide-masses found in

species of *Penicillium*, instead they resort to the less nutrient-consuming state of microcycle conidiation, in which small spores are formed on simple hyphal tips (Wainwright *et al.*, 1991)

The aim of the work discussed below was therefore to determine if by starvation or growth under oligotrophic conditions, fungi could be induced to produce sub-micron forms that could be readily elevated to the stratosphere.

2.C.2 Materials and Methods

A culture of *Penicillium* Sp. was grown without nutrient conditions for a period of 7 days. The small amount of biomass formed was divided into two. One portion was then examined under the scanning electron microscope, while the other was transferred to a Universal bottle, to be broken down. This was achieved by adding glass beads to the bottle containing mycelium and water and the bottle was shaken overnight at high speed. The resultant smashed hyphae was then transferred to a filter apparatus (0.4 μm) and allowed to pass through the filter under gravity into the bottom half of the apparatus which contained sterile Czapek Dox medium. The apparatus was then incubated at 25°C and periodically checked for the presence of fungal growth.

2.C.3 Results and Conclusion

The SEM studies of oligotrophically grown *Penicillium* show that fungal growth, in the absence of added nutrients, led to a reduction in the size of both hyphae and spores. However, the size reduction was only in the region of a fifty percent decrease, with the result that both growth forms were reduced to a size of approximately 2 μm . Growth was not observed in the medium in the bottom of the filtration apparatus; this shows that

when the oligotrophically-grown mycelium was smashed, no viable pieces of less than 0.4 micron were produced.

It appears that oligotrophic growth does not lead to a sufficiently small reduction in the size of both fungal hyphae and spores to achieve the size (less than 1 μm) which would be readily transferred from Earth to the stratosphere. Evidence for the presence of fungi in the stratosphere, therefore remains a problem, which, currently, cannot be explained. If it is difficult to explain how a 1 micron-sized bacterial cell can reach a height of for example, 41 km, then it is even more difficult to explain how fungi can do so when they occur in the Earth's environment at a size approaching 2 μm . The only obvious solution is that fungi are coming into Earth from space. This conclusion can, of course, still be argued against if it assumed that the findings of Imshenetsky (1978), Wainwright *et al.* (2003) and Griffin (2004, 2007), result from contamination, either in the collection, or processing, of samples.

2.D Studies on the Red Rain phenomenon

2.D.1 Introduction

Between July and September, 2001 an unusual Red Rain fell on the State of Kerala in India (Anon, 2001). Scientific interest in Red Rain is based largely on claims by Louis and Kumar (2006) that Red Rain is biological in nature, is relatively rich in silicon, but lacks DNA. As the result of these, and some other properties of Red Rain, these authors suggested that Red Rain may have arrived to Earth from space. The exclusive opportunity was given to us to examine the biological properties of Red Rain, when samples were forwarded to Sheffield by Dr Louis.

2.D.2 Materials and Methods

2.D.2.1 Scanning electron microscopy

The cells were air dried using a dryer directly onto an aluminium stub and examined using an environmental scanning electron microscope.

2.D.2.2 Staining with DNA probes

Red Rain cells were re-suspended in Milli-Q purified, distilled water and a 1 ml sample was sonicated on ice for 8x20 seconds at amplitude of 15 microns using a Sanyo Soniprep 150 (MSE). Ten second cooling intervals ensured that the sample was not overheated. Approximately 1 in 10 cells were lysed. The samples were the subjected to a further 6 periods of sonication at the same amplitude, each lasting 30 seconds with a 10 second cooling interval. SYBR Green II was diluted 1:10 in 50 mM Tris-HCl buffer, pH 8.0 and examined using a fluorescent light microscope.

2.D.3 Results and Discussion

Red Rain cells, as seen under the light microscope are shown in Fig. 2.10. Under these conditions the red cells seen with naked eye look greenish. They are about 5 microns in diameter and have thick cell walls.

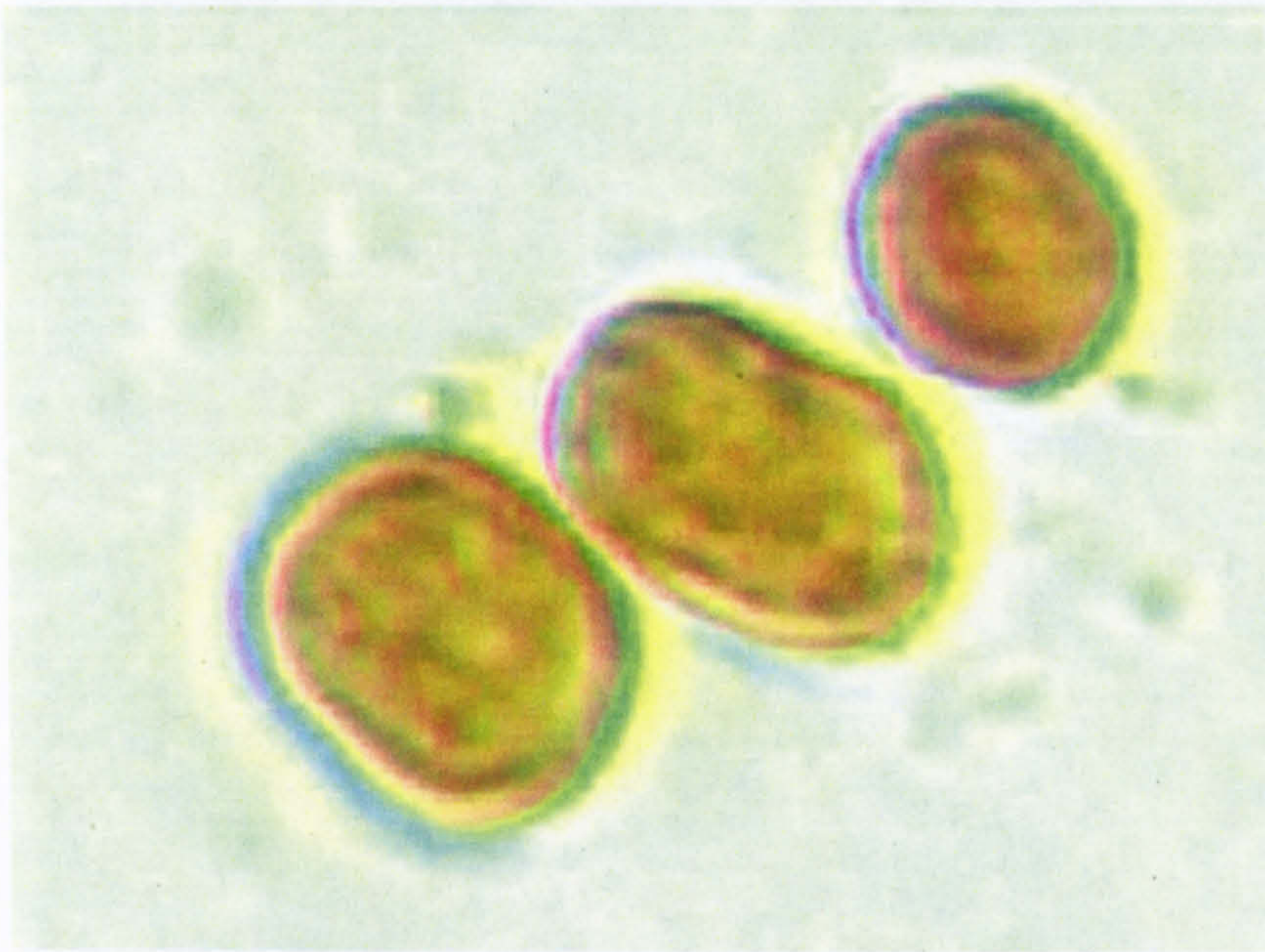


Figure 2.10 Indian Red Rain seen under the light microscope the cells are circa 5 microns across.

Since all organisms on Earth contain DNA, the claims that Red Rain lacks DNA is of obvious interest. As a result, the work reported here was mainly focused on determining if DNA could be detected in Red Rain cells using sensitive, fluorescent microscope stains. Some uninformed, media opinion has suggested that the Red Rain is dust, fat globules or red blood corpuscles. However, the studies by Louis and Kumar (2006) clearly show that the redness of the Kerala rain is due to the presence of cells. This is confirmed by the scanning electron microscope image show in Fig. 2.11; the suggestion that these cells are erythrocytes is doubtless based on the annular-like appearance induced by exposure to the high vacuum of the scanning electron microscope.

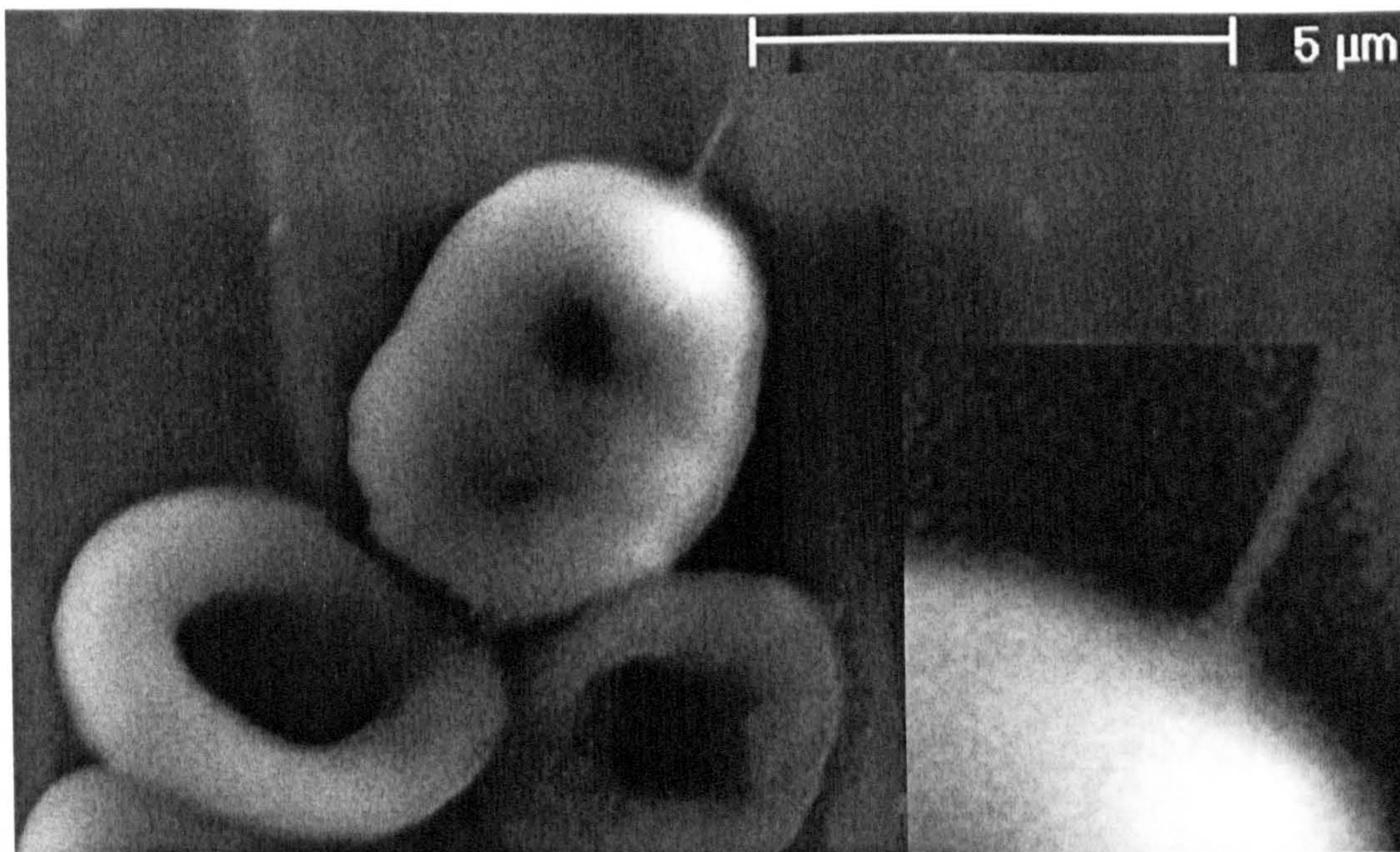


Figure 2.11 Scanning electron micrograph showing Indian Red Rain cells (insert, shows detail of flagellum-like appendage, scale bar does not apply to insert).

However, as Fig. 2.11 shows, some cells are sufficiently rigid to avoid this effect and appear as spheres, approximately 4 μm in diameter. These cells were found to be insoluble in carbon tetrachloride, and are clearly not fat globules. The cells also do not stain with Gram's iodine, suggesting that they lack starch, a typical storage product of green algae. The cells are morphologically similar to red algae (notably species of *Porphyridium*) but do not contain the stellate chloroplasts that are typical of this alga. The morphology of the cells, taken together with other biological characteristics, clearly show that Red Rain is not made up of dust particles, a fact confirmed when EDAX analysis of the cells indicated the presence of carbon, but no mineral elements, including silicon; this last result that contradicts the findings of Louis and Kumar (2006) that the Red Rain cells contain silicon. Red Rain cells generally lack any external structures, although very occasionally (Fig. 2.11), flagellum-like appendages are seen emerging

from the cell wall. Scanning electron microscope studies also show that clumps of Red Rain cells are often held together by a mucilage-like material.

Red Rain cells proved very resistant to the cellular disruptive effects of sonication, a fact that presents a problem since adequate cell-disruption is essential if the cellular components of Red Rain, including DNA, are to be sufficiently exposed to allow for them to be detected.

After exposure to a high degree of sonication, disrupted Red Rain cells were treated with a variety of fluorescent stains to determine if, as suggested by Louis and Kumar (2006), they lack DNA. As Figure 2.12 shows that the cells fluoresced when exposed to SYBR Green II fluorescent stain, a stain that detects both DNA and RNA.

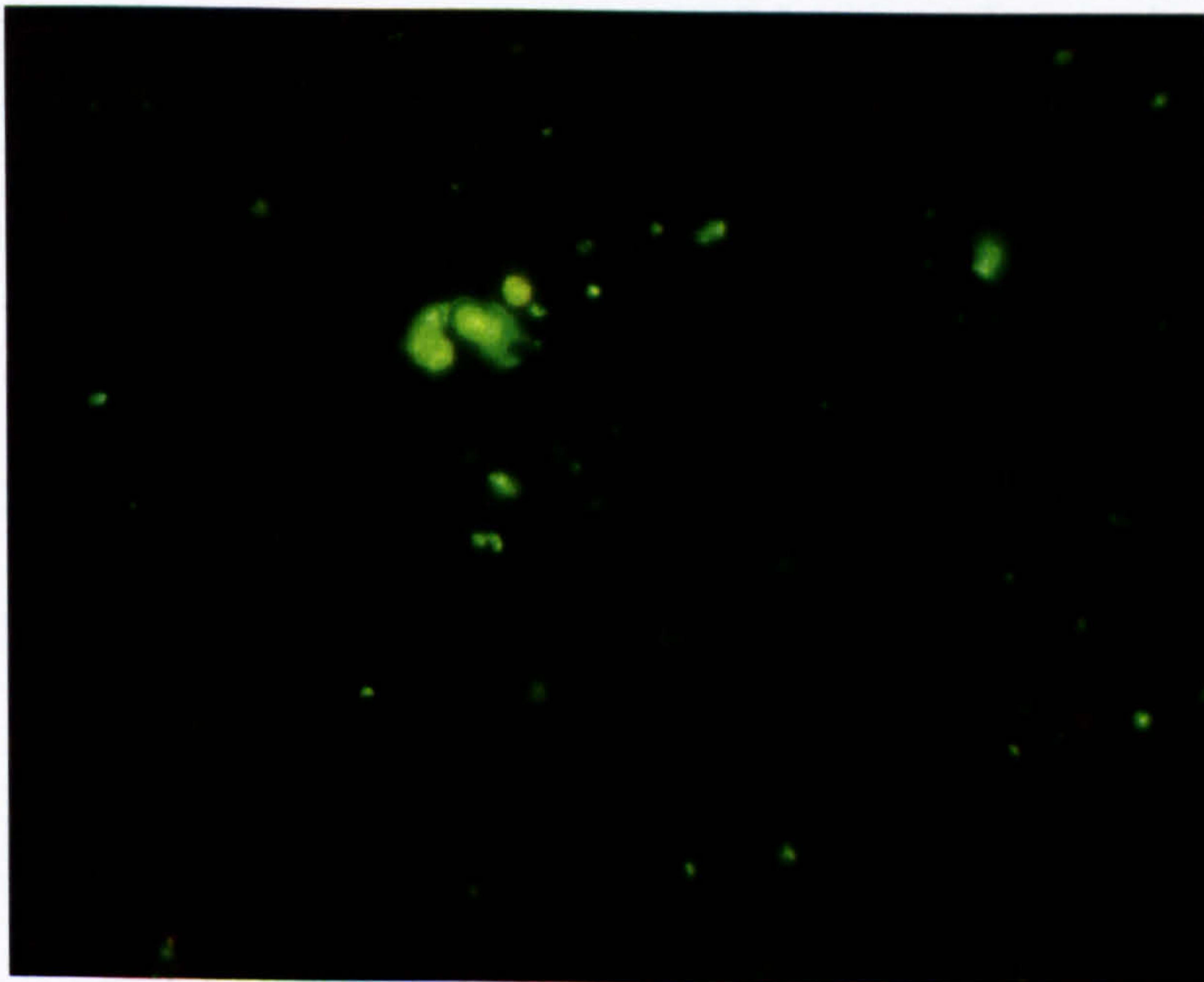


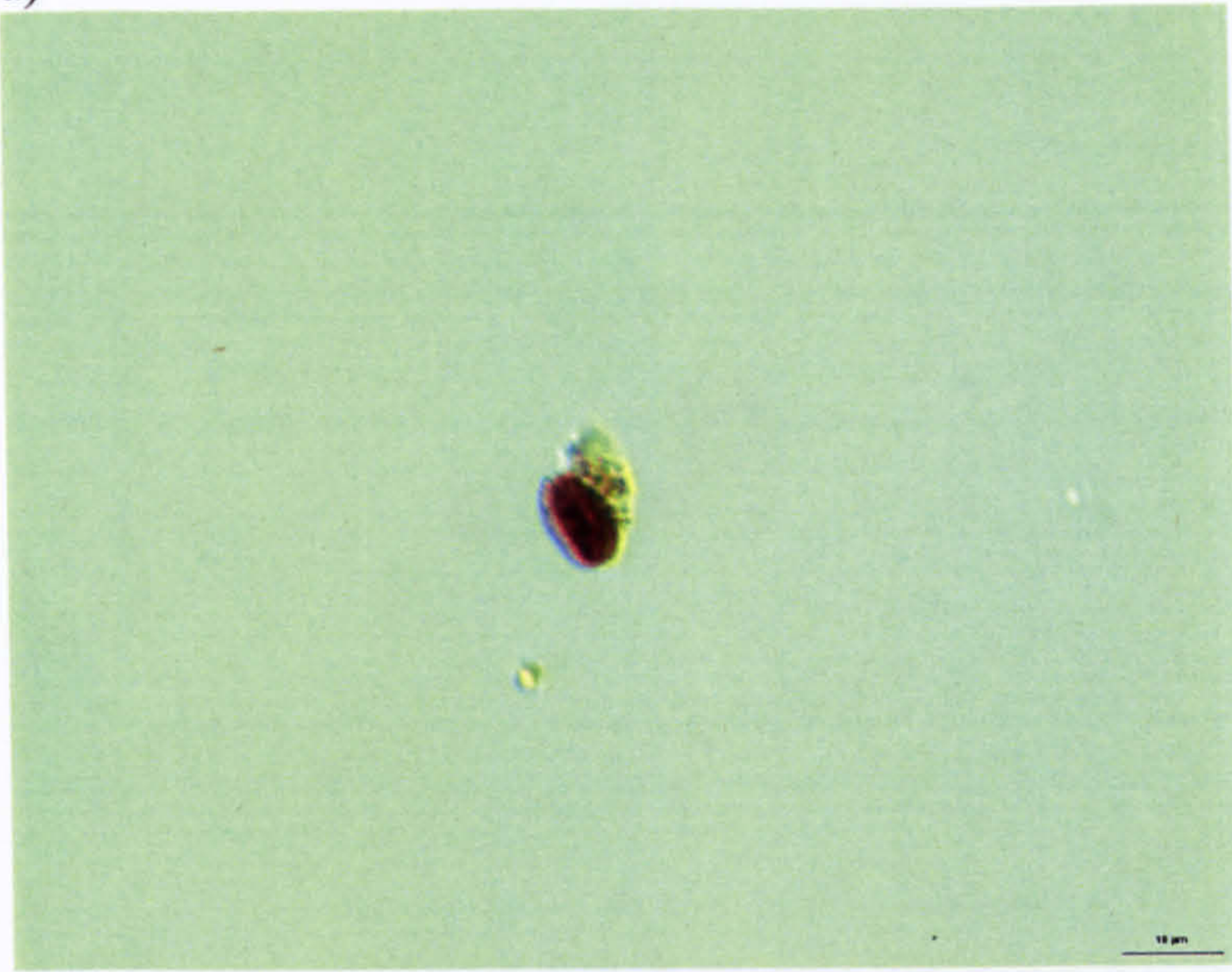
Figure 2.12 Sonicated Indian Red Rain cells stained with SYBER-Green 11.

Images taken using white light and UV, show that the fluorescence exactly corresponded to the location of the cells. Similar fluorescent staining of the Red Rain

cells was achieved using the DNA sensitive stains DAPI (Vectashield, Mounting Medium with DAPI, H-1200), (Fig. 2.13) ethidium bromide and Live/Dead Stain BacLight (Molecular Probes) (Figs. 2.14, 2.15). Not surprisingly, the sonicated cells did not stain green, to indicate the presence of intact, functioning membranes, following the application of live /dead stain. No evidence was found to suggest that these results were due to auto fluorescence by Red Rain cells. Tests for autofluorescence in unstained Indian Red Rain cells when exposed to UV light were always negative, as shown in Fig. 2.16. These results show that, contrary to the findings of Louis and Kumar (2006), Red Rain does in fact contain DNA.

The Red Rain cells look similar to small uredospores (also termed urediniospores) of rust fungi, large numbers of uredospores are released, and form extensive air-borne clouds, in various parts of the world where wheat and other, rust susceptible crops, are grown. However, the cells do not appear to contain chitin (as indicated by lack of fluorescence when stained with Calcaflor, Sigma), nor have any germ tubes, typically found when uredospores germinate, been seen emerging from the Red Rain cells.

a)

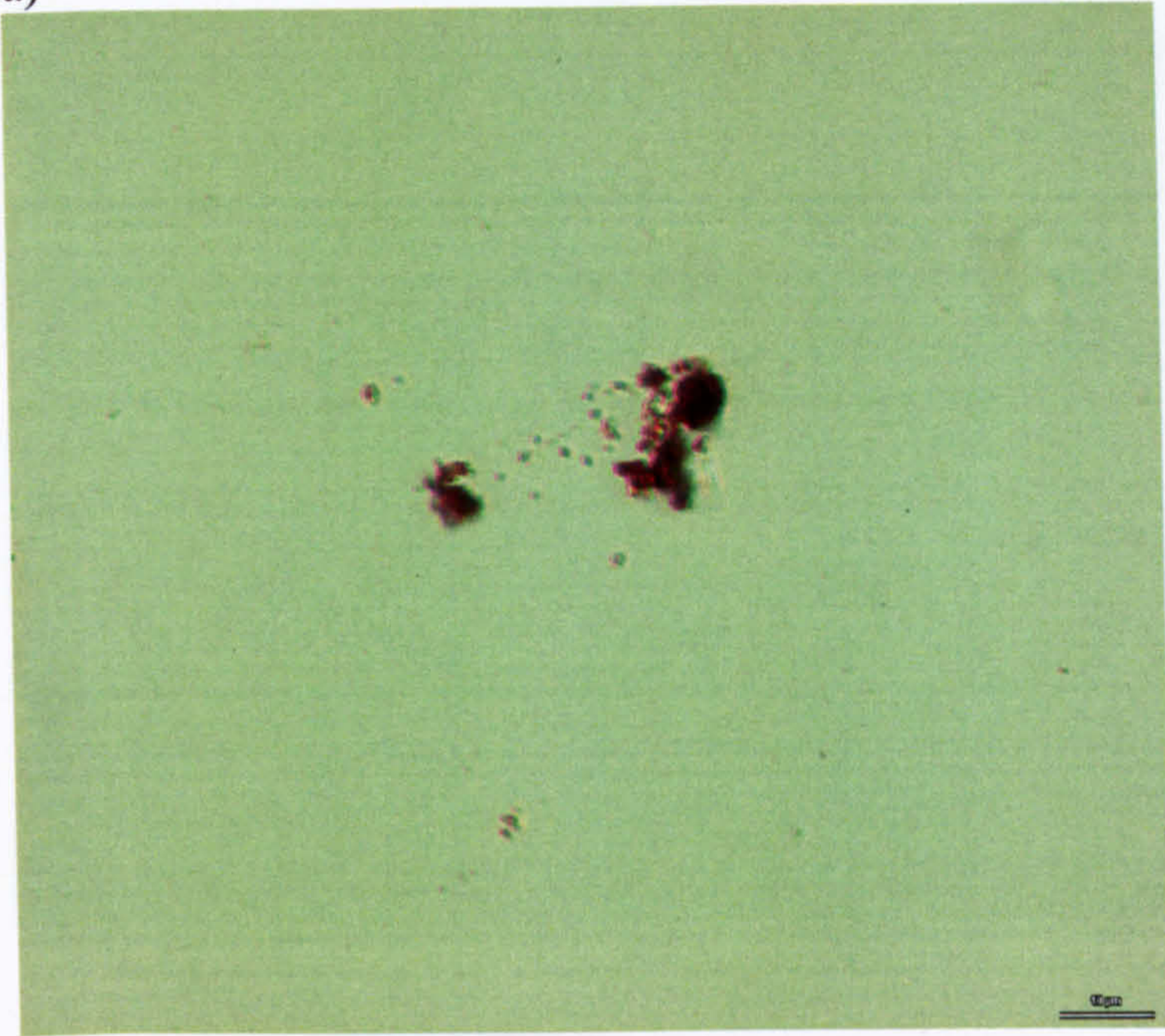


b)



Figure 2.13 Sonicated Indian Red Rain cells a) unstained and b) stained with the DNA stain, DAPI.

a)



b)



Figure 2.14 Sonicated Indian Red Rain as seen under light microscope (a), and b) after staining with ethidium bromide under the fluorescence microscope.

a)

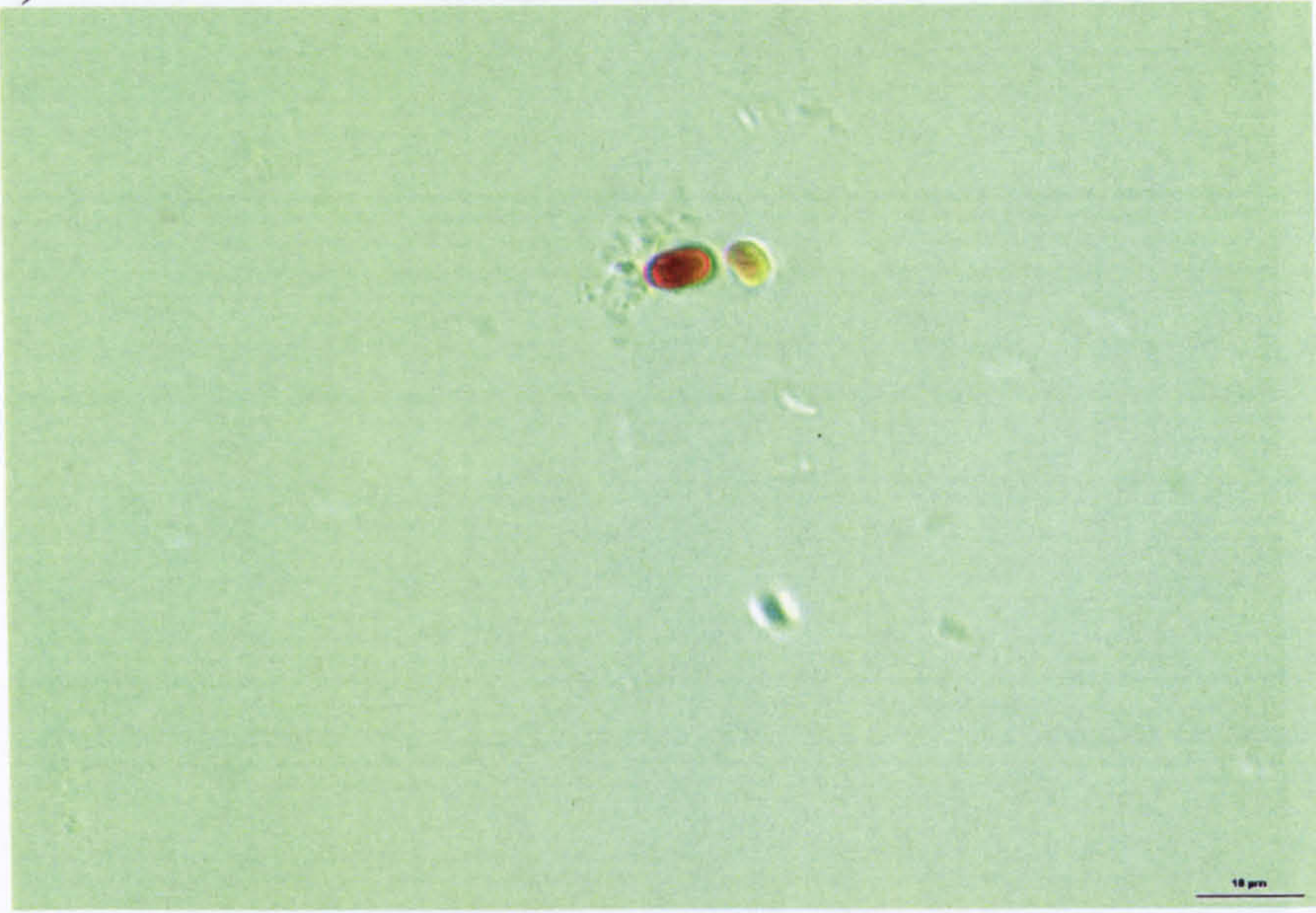


Figure 2.16 Exposure of Indian Red Rain to UV light for 48secs to check for autofluorescence.

b)



Figure 2.15 Live/Dead Staining of sonicated Indian Red Rain.

There have also been suggestions that the Red Rain of Kerala is an alga of the genus

Figure 2.15 Live/Dead Staining of sonicated Indian Red Rain.

explain how this alga could have reached the stratosphere in such large quantities.

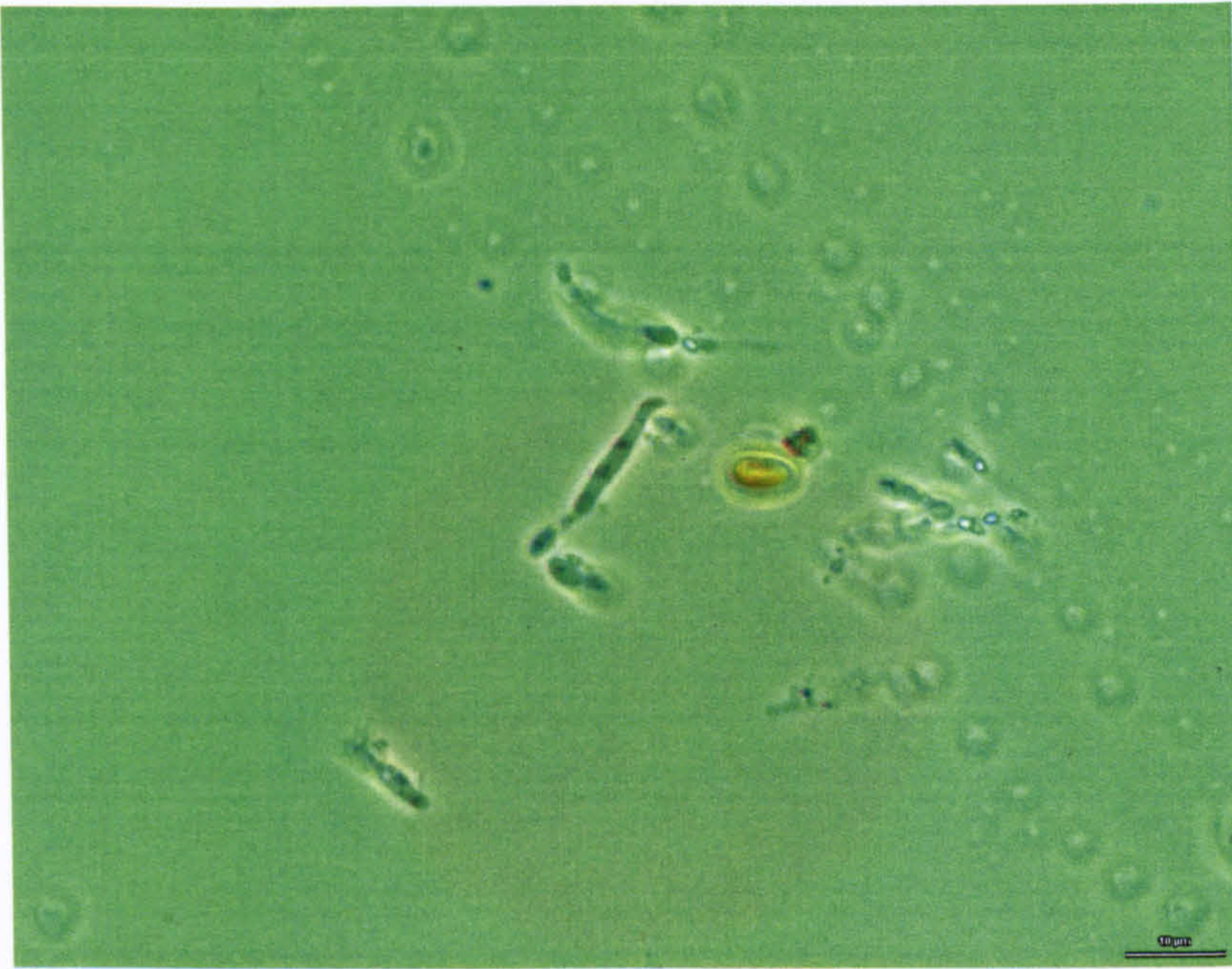


Figure 2.16 Exposure of Indian Red Rain to UV light for 48secs to check for autofluorescence.

These results tell us nothing about the origin of Red Rain. The fact that the Red Rain cells contain DNA obviously suggests the possibility that they originated from Earth. It is important however, to note that the theory of panspermia does not necessarily suggest that microorganisms arriving to Earth from space will be novel to the extent of being devoid of DNA. Louis and Kumar (2006) point out that the appearance of Red Rain in Kerala was associated with a meteorite-associated air burst; further claims by them that Red Rain exhibits unusual biological properties (including growth at 300°C) and has a panspermic origin. Should these claims be verified by further work, then the Red Rain cells are indeed unusual.

There have also been suggestions that the Red Rain of Kerala is an alga of the genus *Trentepohlia* (Sampath *et al.*, 2001); however, if this is the case, it remains difficult to explain how this alga could have reached the atmosphere in such large quantities.

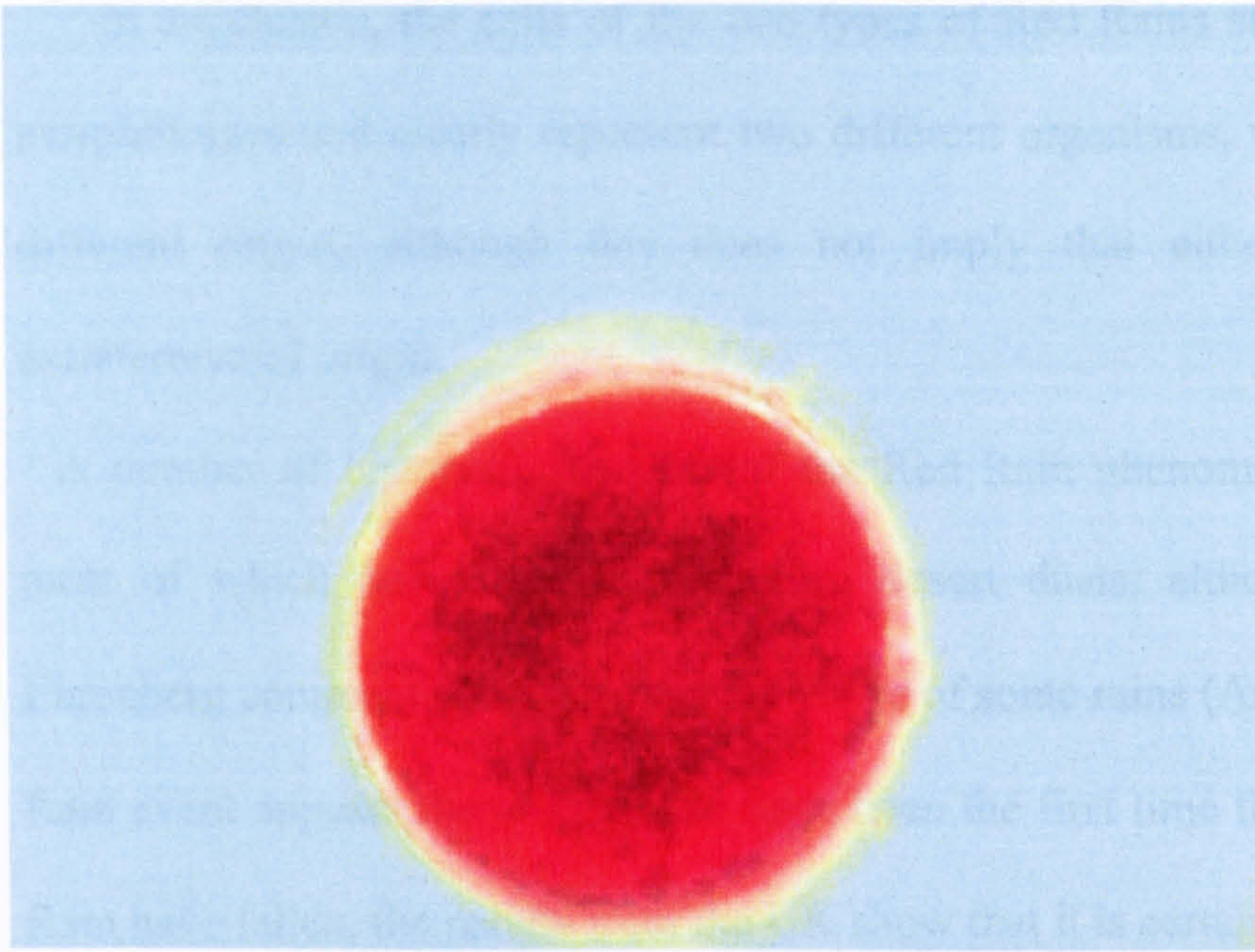


Figure 2.17 UK Red Rain under a light microscope (The cell is approximately 20 microns across).

Comparison of Indian and UK Red Rains

The two types of rain are substantially different with regard to their morphologies. Both types of rain are clearly red when viewed with the naked eye, although when viewed under the light microscope, the UK rain is clearly brighter and redder in colour than the Indian version (Fig. 2.17). Under the light microscope, the two types of rain are again seen to be substantially different (Compare Fig. 2.10 and 2.17). The Indian Red Rain is approximately 5 microns in diameter compared to the much UK rain cells, which is approximately 20 microns in diameter. The UK Red Rain is also spheroid compared to the more ovate morphology of the Indian Red Rain. Both cells appear to have contained within them, smaller, or daughter, cells. The UK Red Rain is also noticeably a richer mix of organisms, bacteria, algae and fungi, than is the Indian Red Rain, which is less heavily contaminated.

In conclusion, the cells of the two types of Red Rains studied here differ in their morphologies and clearly represent two different organisms, which presumably have a different origin, although this does not imply that either has by necessity, an extraterrestrial origin.

A number of historical accounts of the Red Rain phenomenon have been reported, most of which have been attributed to desert dusts, although in the early 1800s, Ehrenberg commented on a biological nature of some rains (Anon, 1852). The Kerala Red Rain event appears therefore not to have been the first time that large quantities of Red Rain have fallen, the results from the UK show that it is certainly not the last and that the deposition of large amounts of biological material to Earth in rain water may be a common event.

2.E Studies on the survival of *B. subtilis* in sulphuric acid in relation to the stratosphere

2.E.1 Introduction

As has already been mentioned, the stratosphere can be regarded as a very extreme environment for such growth and survival of bacteria and other microorganisms. Since the residence time of bacteria in this region is likely to be short, we need here to emphasise the factors that may influence their survival, however, residence times for particles the size of very small bacteria may reach 6 months, thereby allowing time for growth to occur should conditions be suitable. The questions that need to be asked-are: could bacteria a) survive in the stratosphere and then be capable of growing when transferred to media on Earth? and b) could they grow, while present in the stratosphere,

from very small forms to the large, 10 micron-plus clumps found on stratospheric membranes?

Amongst the major factors that might limit the survival and growth of bacteria, and other microorganisms in the stratosphere is the presence of strong acids, e.g. sulphuric, hydrochloric and nitric acids. The aim of the research reported in this section of the Thesis was therefore to determine the effects of concentrated sulphuric acid on the survival of *Bacillus subtilis*, and to determine if volcanic ash is able to protect bacteria from any potential lethal effect of sulphuric acid.

2.E.2 Materials and Methods

2.E.2.1 The effect of sulphuric acid on freeze dried *B. subtilis*.

A liquid culture for *B. subtilis* was prepared for freeze dried. Freeze dried *B. subtilis* (0.02 g) was placed in eight sterilized tubes. Ten ml of (0.0, 0.25, 0.50, 1.00, 1.25, 2.50, 5.00, and 10.00%) concentrations of sulphuric acid were added, and the tubes were then incubated for seven days at 25°C. An aliquot of 0.1 ml from each tube was next plated onto LB Agar medium, four plates of each concentration, and then incubated for two days at the above mentioned temperature.

2.E.2.2 Does volcanic ash protect *B. subtilis* from sulphuric acid?

The effect of the weathered volcanic ash on the interaction between sulphuric acid and *B. subtilis* was next determined. Two samples of weathered volcanic ash were collected from Mt Tiede on Tenerife (3000 meter above sea level). Ten g samples were then transferred to a 250 ml flask; triplicates were made for each soil. Then 50 ml of distilled water was added to each flask which was then autoclaved for 15 minutes in order to sterilize the soil. *B. subtilis* (1 ml) was added to each flask and incubated at 25°C for two

days. All of the contents of the flasks content were poured in sterilized filters (0.02 μm) and left at 25°C for seven days till the soil on the filters became air dried. Then 2 g of each dried soil were put in eight sterilized tubes. Ten ml of the following concentrations (0.0, 0.25, 0.50, 1.00, 1.25, 2.50, 5.00, and 10.00%) of sulphuric acid were added to the soil in the tubes then incubated for seven days at 25°C. Aliquots (0.1 ml) from each tube was plated onto LB agar medium, three plates of each concentration, and then incubated for two days.

2.E.3 Results and Discussion

Figure 2.18 shows that sulphuric acid, at a concentration of 0.5%, markedly reduced the colony count of *B. subtilis*, although some bacteria survived; concentrations above 1.5% however, completely inhibited the growth of this bacterium. An interesting paradoxical effect was seen at the 1% concentration where numbers of bacteria increased compared to the 0.5% concentration. It is clear therefore that a substantial number of cells of *B. subtilis* survived these high concentrations of acid. Although it is difficult to determine the likely concentration of sulphuric acid in the stratosphere; it is however, unlikely ever to reach a value as high as 1%v/v, a fact indicating that bacteria are unlikely to be completely inhibited in the stratosphere by the presence of sulphuric acid. It is not clear why a proportion of the bacterial inoculum, used here, survives acid exposure. Survival of *B. subtilis* in the stratosphere in the presence of sulphuric acid is likely to depend on the number of bacteria present and the proportion of these that are resistant to the acid, such variables are impossible at the moment to determine. However, we can conclude that there is no *a priori* reason why *B. subtilis* should not survive in the presence of sulphuric acid in the stratosphere.

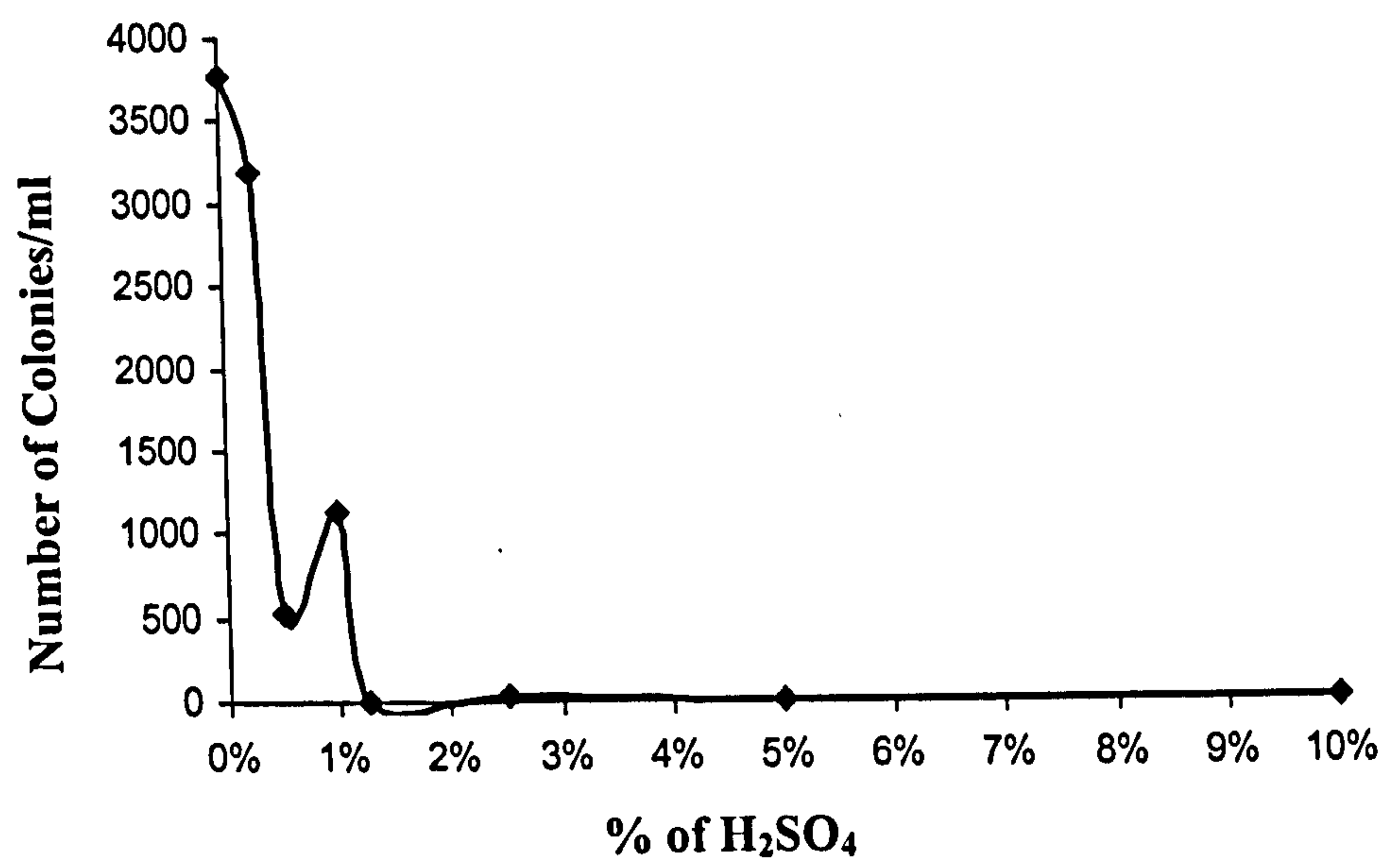
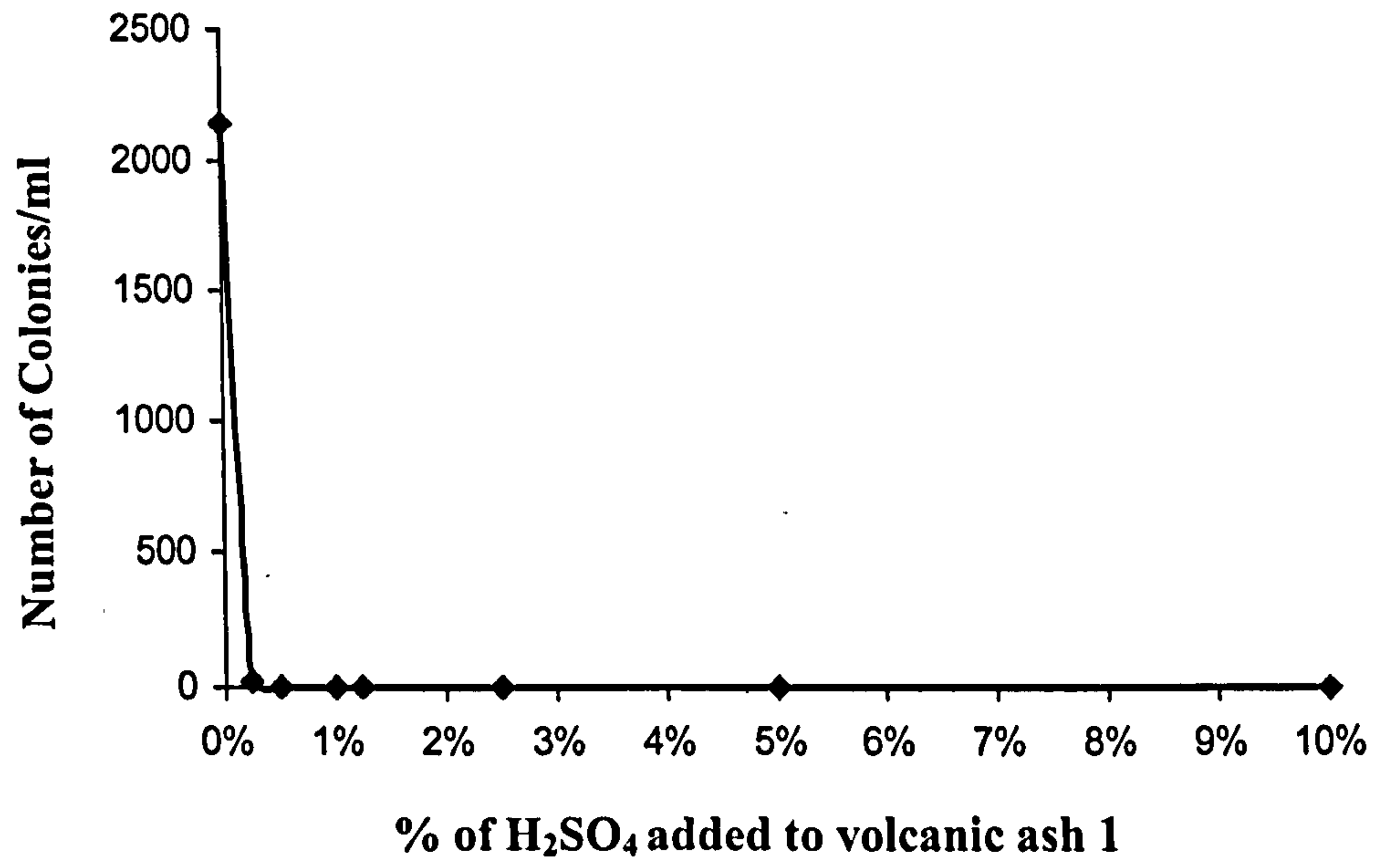


Figure 2.18 The effect of sulphuric acid on survival of *B. subtilis*.

a)



b)

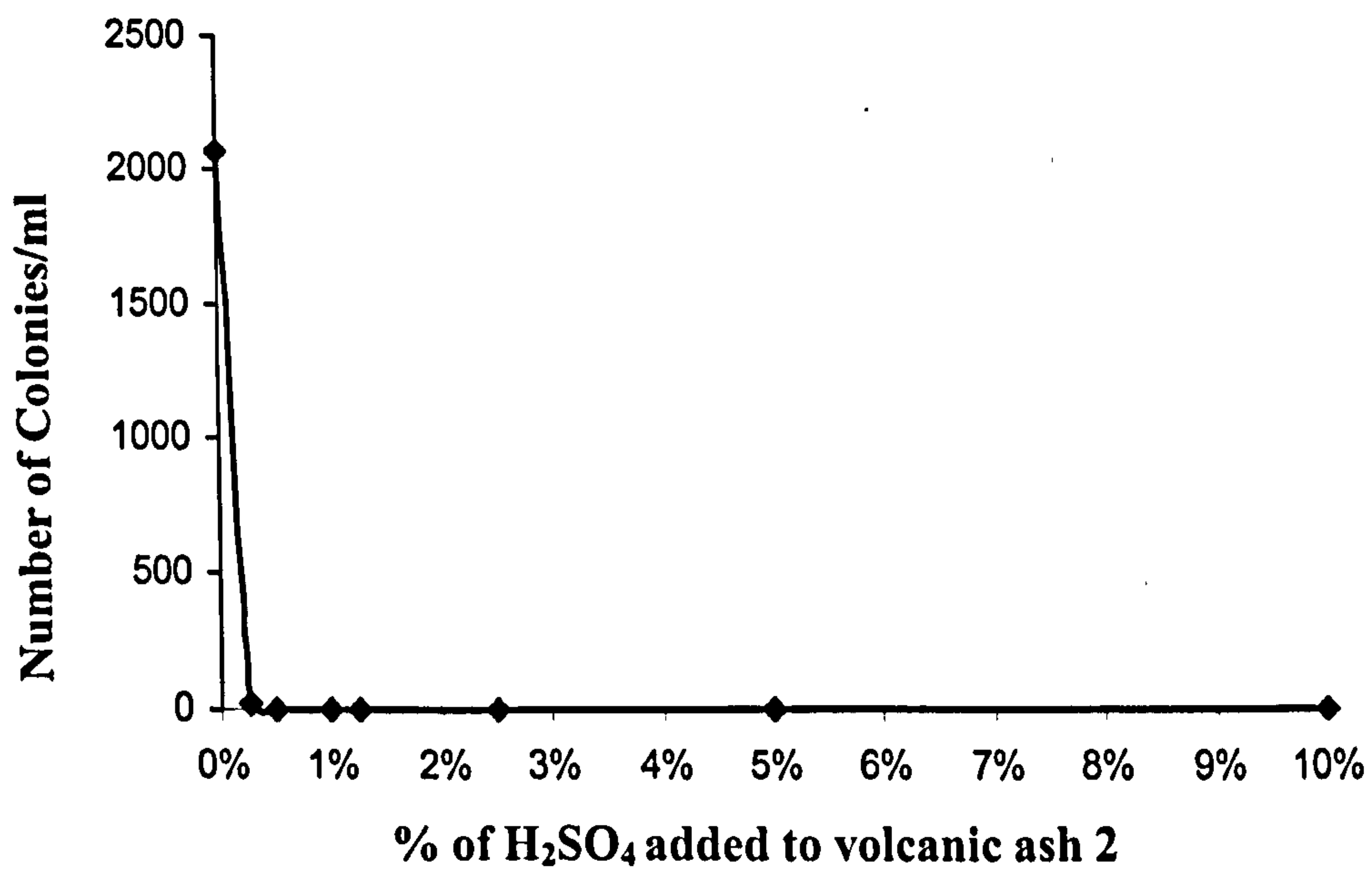


Figure 2.19 Influence of two types of volcanic ash (a, b) on the effect of sulphuric acid on *B. subtilis*.

It was expected that the addition of alkaline volcanic ash might reduce the toxic effect of sulphuric acid on *B. subtilis*. As Fig. 2.19 shows however, the opposite was the case, since in the presence of two types of volcanic ash no bacteria survived at any concentration of acid. Possibly toxic materials, such as heavy metals, were leached from the ash by the acid and this added a further toxic parameter to the system. These results show how difficult it is to accurately simulate an environment like the stratosphere, whose physical and chemical make up is often unknown, but is expected to be highly variable. Ozone, for example is a potentially important biocide which is found in the stratosphere and troposphere. In the former region, it is formed when molecular oxygen is split into free radicals by the action of the UV present in solar radiation; some of this ozone is transported to the tropopause, where it can also be formed by the photochemical oxidation of hydrocarbons. Stratospheric ozone plays an important role in protecting the biosphere of Earth from UVB and UVC (Staehelin *et al.*, 2006). Ozone is however, capable of inhibiting bacteria and fungi, although it is not clear if such inhibition would occur at the low temperatures found in the stratosphere; clearly this is an important area for future research

Of course the simple answer to the question- do bacteria survive the presence of sulphuric acid, and other potential limiting factors, such as ozone, is yes. This conclusion is based on the fact that, as we have seen, viable bacteria have been isolated from this region by a number of different workers.

CHAPTER THREE

**STUDIES ON THE GROWTH OF BACTERIA IN A
SIMULATED COMETARY NUTRIENT ENVIRONMENT**

3.1 Introduction

The Theory of cometary panspermia of Hoyle and Wickramasinghe suggests that comets act as the breeding ground and source of bacteria and other microorganisms which are then spread throughout the cosmos via the process of neopanspermia (Wickramasinghe, 2005). If this theory is correct then it must obviously be possible for bacteria to live and replicate in comets. The general understanding of comets is that they are cold and inhospitable places in which life could not exist. Recent studies on comets, including information provided by the Temple 1 mission however, show that such views are misplaced, and that comets could act as ideal “incubators” for bacterial life, as suggested by the Hoyle and Wickramasinghe hypothesis (Wickramasinghe, 2005). The aim of the work discussed in this section of the Thesis was to determine if Earth bacteria could grow under the nutrient conditions likely to prevail in comets. It should be noted that the general conditions found in comets were not simulated in these experiments. The initial aim was to determine if bacteria could grow under cometary, nutrient conditions. Obviously if they were incapable of doing so, then further studies on cometary growth of bacteria under a variety of physical conditions would be pointless, since it could be argued that bacteria simply could not be sustained by the nutrient conditions available in comets.

Studies have shown that comets contain a mix of carbonaceous components, including hydrocarbons, polyaromatic hydrocarbons, HCN, formaldehyde and carbon monoxide (Mumma and Reuter, 1989 and Fomenkova *et al.*, 1994), all of which can act as substrates for microbial growth. Halley’s comet also contains particles rich in the light elements, hydrogen, carbon and nitrogen. Although it is unclear if free oxygen exists in

comets, they certainly contain water and can reach temperatures of 40°C, factors which substantially increase their suitability as life supporting environments. Glycolaldehyde was used in the following studies, because it is a common compound found in space and comets (Hudson and Moore, 2005) and might therefore act as a carbon source for space microbes.

3.1.1 A note on the bacterial inoculum used in these studies

The type of bacterial inoculum used in these studies is extremely important since it is necessary to avoid using bacteria that have become acclimatised to the high nutrient conditions prevailing when bacteria are grown in the laboratory. Such growth leads to bacteria that are essentially laboratory conditioned and have nutrient-utilisation characteristics that are markedly different from bacteria which have grown continuously under natural environmental conditions. This is particularly the case, since many natural environments are nutrient limited and the bacteria they support grow oligotrophically, under low nutrient conditions. To avoid using such lab-conditioned bacteria, a novel approach had to be used to provide the inoculum for these experiments. Essentially the simulated cometary nutrient environment was merely exposed to the atmosphere (on windless nights) and any bacteria floating down into the Petri dishes acted as the (potentially mixed) inoculum. Any bacteria that grew (assuming some did) in the nutrient solution were then identified and regarded as being capable of living in the nutrient conditions prevailing in comets. This approach had the advantage of using bacteria that were not conditioned to laboratory growth and which had probably been growing oligotrophically in an Earth environment. Of course, the possibility exists that such bacteria originated from comets, or other panspermic origins; however, since no

method of demonstrating this possibility could be devised, it is assumed that any bacterium growing in the simulated nutrient environment originated from Earth.

Oligotrophy is the ability of heterotrophic microorganisms to scavenge carbon substrates (liquid and gases) from their environment. By using this growth strategy, oligotrophic microorganisms can live in environments lacking readily available carbon or nutrients. Since it is likely that many environments in space will contain only small amounts of nutrients oligotrophy is obviously relevant to the study of the microbial colonization of other planets.

Oligotrophy is extremely common amongst terrestrial bacteria and fungi, with most microorganisms being facultative in this respect; i.e. they can grow, producing large amounts of biomass, in the presence of copious amounts of substrates and then switch to oligotrophic growth when carbon substrates are rare (Wainwright *et al.*, 1991). Oligotrophy is usually considered in relation to carbon (i.e. oligocarbotrophy) but microorganisms can also scavenge traces of other nutrients such as nitrogen (oligonitrotrophy) and phosphorus (oligophosphotrophy); indeed it seems that all nutrients required by microorganisms can be scavenged oligotrophically; either from liquids, volatiles or gases. The ability to scavenge nitrogen oligotrophically allows microorganisms to exist in low nutrient environments even if they lack the ability to fix dinitrogen.

Oligotrophy is not synonymous with “starvation” which is a state properly defined as “a period of nutrient stress imposed on microorganisms after a period of growth under nutrient sufficient conditions”. Rather than starving, oligotrophs grow in balance with the available nutrients. In order to achieve this balance, oligotrophs produce only small

amounts of biomass and are generally much reduced in size compared to when growing in nutrient rich conditions (so-called copiotrophy) . Under oligotrophic conditions, fungi, for example, produce very fine hyphae and form complex mycelial networks (referred to as gossamers), both of which lead to an increase in surface area which facilitates efficient nutrient scavenging. Fungi also tend to avoid producing complex spore-bearing structures when growing oligotrophically, and instead reproduce by microcycle conidiation, a growth strategy which results in the formation of very small spores (Wainwright *et al.* 1991).

Oligotrophy is obviously relevant to the question of microbial growth in space, since in many such environments carbon, and other nutrients will always, or periodically, be in short supply and under these conditions heterotrophic microorganisms will need to scavenge trace amounts of whatever nutrients are available. In order do so bacteria and fungi have developed mixotrophy that is the ability to scavenge and utilise trace amounts of a wide variety of nutrients (Wainwright *et al.* 1991).

The aim of the work reported in this thesis was to determine if airborne microorganisms are able to grow oligotrophically in the absence of an added carbon substrates and also in the presence of carbon substrates that might be available in the space environment notably comets. In this case, the cometary nutrient environment was based on recent findings showing that comets are rich in silicon and water and contain a range of organic compounds, notably polyaromatic hydrocarbons.

3.2 Material and Methods

Silica gel medium was used in these studies to act as solid surface substrate. This is similar to agar, but is composed of colloidal silica and is free of added nutrients (see

below). The silica gel medium was prepared using ultra pure water and was poured into glass petri dishes, pre-cleaned to remove organic contaminants using sulphuric acid (80%v/v) and then washed again with ultrapure water.

3.2.1 Silica gel medium (Parkinson *et al.*, 1989)

To make silica gel medium, three solutions need to be prepared.

1- Salts solution

The following table shows the composition of Salts Solution which is added to 1000 ml of double purified distilled water (pdw) then autoclaved

No	Substance	Quantity (g)	Note
1	KH ₂ PO ₄	1.0	
2	KCl	0.5	
3	MgSO ₄ .7H ₂ O	0.5	
4	FeSO ₄ .7H ₂ O	0.01	Added after autoclaving

2- Orthophosphoric Acid

Orthophosphoric acid (20 ml) was added to 100 ml of pdw.

3- Potassium silicate

Potassium hydroxide (7 g) was dissolved in 100 pdw then added to 10 g of silicic acid

Medium preparation:

Salts solution (10 ml was mixed with 10 ml of potassium silicate solution in each Petri dish. After the addition of these quantities to all the Petri dishes needed in the experiments, 2 ml of orthophosphoric acid solution was added to each mixture and immediately mixed. Then the gel set in about 15 minutes.

3.2.2 Addition of carbon source to simulate the cometary nutrient environment

The surface of the gel was amended with (0.1 ml) of one of the following carbon sources (0.01% w/v), naphthalene or glycolaldehyde.

3.2.3 Exposure of the silica gel medium to the atmosphere

The lid of the glass Petri dish was removed and the surface of the gel was exposed to the outside ambient air (for 8 hours), allowing microorganisms to fall from the atmosphere onto the gel surface; these then acted as the sole microbial inoculants. After such exposure, the petri dish (sterilized) lid was replaced and the dish was incubated at 25°C. The experiment was repeated, but in this case the plates were incubated in gas jars anaerobically in an atmosphere of carbon dioxide and hydrogen. Ten dishes were incubated under each condition and ten controls were included which the nutrient medium lacked any added carbon source. After exposure, the plates were examined at intervals for surface growth using a low power microscope. Further examination was conducted using high power and environmental scanning electron microscopy. Bacteria growing on the surface of the exposed silica gel plates were transferred to Nutrient Agar (Oxoid) for culture (25°C).

3.2.4 Simulated comet experiments

3.2.4.1 NASA prescription for a simulated comet.

A simulated comet mixture (lacking added carbon and other nutrients) was prepared as given in (Table 3.1). (From NASA, How to Make Comet Soup: www.nasa.gov/mission_pages/deepimpact/multimedia/ingredients090705.html).

The simulated comet mixture was divided into three portions, each being added to one of three sterile glass Petri dishes (washed with concentrated sulphuric, to remove traces

of carbon and then ultrapure water). Double purified water (20 ml) was then added to each Petri dish; all dishes were then left uncovered, outdoors overnight (in a calm dry night). The Petri dishes were then covered with new sterilized glass covers and incubated for 24 hours at 25°C. A sample (0.1 ml) of the liquid from each dish was then taken and plate onto Nutrient Agar (Oxoid) medium and then incubated, aerobically, for 48 hours at 25°C.

Table 3.1 The composition of the simulated comets (wetted with double distilled water).

Composition	Weight (g)
Enstalite	1.0
Olivine	1.0
Dolomite	1.0
Magnesium aluminate, single crystal substrate <111>, 99.99%	0.18

3.2.4.2 Silica gel experiment

Silica gel media was prepared. Some plates were used with nutrient free silica gel. Others received non-carbon nutrients A. To gel B was added naphthalene (0.1% w/v) and gel C, glycolaldehyde dimer (Table 3.2) as potential carbon sources. Six plates of each type were exposed to air outside the laboratory overnight (on a windless night).

Table 3.2 Composition of silica gels containing nutrients; A, carbon free, B, containing naphthalene (0.1% w/v), C, containing glycolaldehyde dimer (0.1% w/v).

No	A	B	C
1	Ammonium sulphate (NH ₄) SO ₄	Ammonium sulphate (NH ₄) SO ₄	Ammonium sulphate (NH ₄) SO ₄
2	Calcium phosphate (monobasic)	Calcium phosphate (Monobasic)	Calcium phosphate (monobasic)
3	Sodium sulphate anhydrous	Sodium sulphate anhydrous	Sodium sulphate anhydrous
4	Potassium hydrogen carbonate (KHCO ₃)	Potassium hydrogen carbonate (KHCO ₃)	Potassium hydrogen carbonate (KHCO ₃)
5		Naphthalene	Glycolaldehyde dimer

Sterilized distilled water (10 ml) was added to each plate and then left for 15 minutes then 0.1 ml were taken from each plate was then plated onto solid Nutrient Agar (Oxoid) medium and incubated in aerobic and anaerobic conditions for 24 hours. Triplicates were made for each plate.

3.2.5 Isolation of atmospheric fungi

Nutrient-free silica gels were also exposed to the atmosphere and unintentionally left at room temperature (circa 18°C) and fungi were isolated (Note, we have noticed that fungi often prefer a variable room temperature/light regime for growth than constant 25°C).

3.3 Results and Discussion

3.3.1 Simulated Comet Experiments

3.3.1.1 NASA prescription for a simulated comet

Bacteria were isolated using Nutrient Agar, from the nutrient and carbon free simulated comet mixture which had been left overnight outside. The bacterium was a single species, present at a density of 1.5×10^4 colonies per ml of extract (1.5×10^3 per gram of comet mixture). The bacterium was independently identified, using 16SrDNA as *Acinetobacter* (genome species 3).

Recent studies have shown that comets contain a wide variety of carbon nutrient, for bacteria growth, including polyaromatic hydrocarbons (PAHs), like naphthalene, and carbon rich gases, like methane (Kawakita,2003). It has been suggested that the building blocks for life on Earth were, in fact, delivered by comets and the range of carbon compounds present includes sugar and amino acids. This study shows that bacteria, present in the Earth atmosphere, can grow on simulated comet mixtures which lack added carbon and nitrogen (although potassium and phosphorus are present in the rock material added). Clearly, if bacteria can grow in comet mixtures to which no carbon and nitrogen has been added, then they can grow in native comet material, which we know contains a large amount of a variety of carbon and nitrogen compounds. As a result, we can confidently predict that bacteria can grow in comets, at least with regard to their nutrient status. Note of course, that when the mixture was exposed to the air, it was exposed to normal atmospheric nutrients, which might have supported bacterial growth; however, such nutrients are present in far lower concentrations in the Earth's atmosphere

than in comets. The experiment described above, of course tells us nothing about potential bacterial growth under the physical conditions prevailing on comets.

3.3.1.2 Silica Gel experiments

Nutrient free silica gel and gels containing nutrients, but not a carbon source (A) (Table 3.2) and gels containing nutrients plus a carbon source in the form of naphthalene (B), and glycolaldehyde dimer (C) were exposed to the Earth's atmosphere as in the comet mixture experiments described above. Bacteria were only isolated from (B), that is, silica gel containing nutrients and naphthalene as the carbon source; the average number of bacteria (from 6 exposed gel plates) was 2.92×10^7 colonies per ml (aerobic) and 1.06×10^7 colonies per ml (anaerobic). The aerobic colonies comprised a single bacterium which was independently identified as *Sphingomonas aerolata* (see Appendix A), (the anaerobic bacterium was not identified, but has identical morphology to aerobic isolate.

3.3.2 Isolation of atmospheric fungi

Nutrient-free silica gels were exposed to the atmosphere and unintentionally left at room temperature (circa 18°C). After 7 days of incubation, the surface of the silica gel was examined (visually and using a microscope), for fungal and bacterial growth. Fungal colonies (restricted to less than ten per plate) were seen on all of the plates after they had been aerobically, anaerobically (in an atmosphere of CO₂ and H₂). The fungi seen (Fig. 3.1) usually consisted of very fine (circa 1 µm) sterile, hyphae which formed grooves in the silica gel; this is assumed to be due to solubilisation of the gel by the hypha. On some plates, colonies of *Penicillium* species and *Stemphyllium* sp were observed. In the case of the former, microcycle conidiation was seen and the large spore bearing structures typical of *Penicillium* grown on rich nutrient media were reduced to single phialides.

Similar structures have been reported when fungi are grown oligotrophically, that is on low-nutrient media, or under apparently nutrient-free conditions. Bacterial colonies were relatively numerous on all plates and under the low power microscope were seen to be made up of cells exhibiting a typical Chinese Character appearance; these lysed the fungi on the gels, from which they presumably gained nutrients (Fig. 3.3).

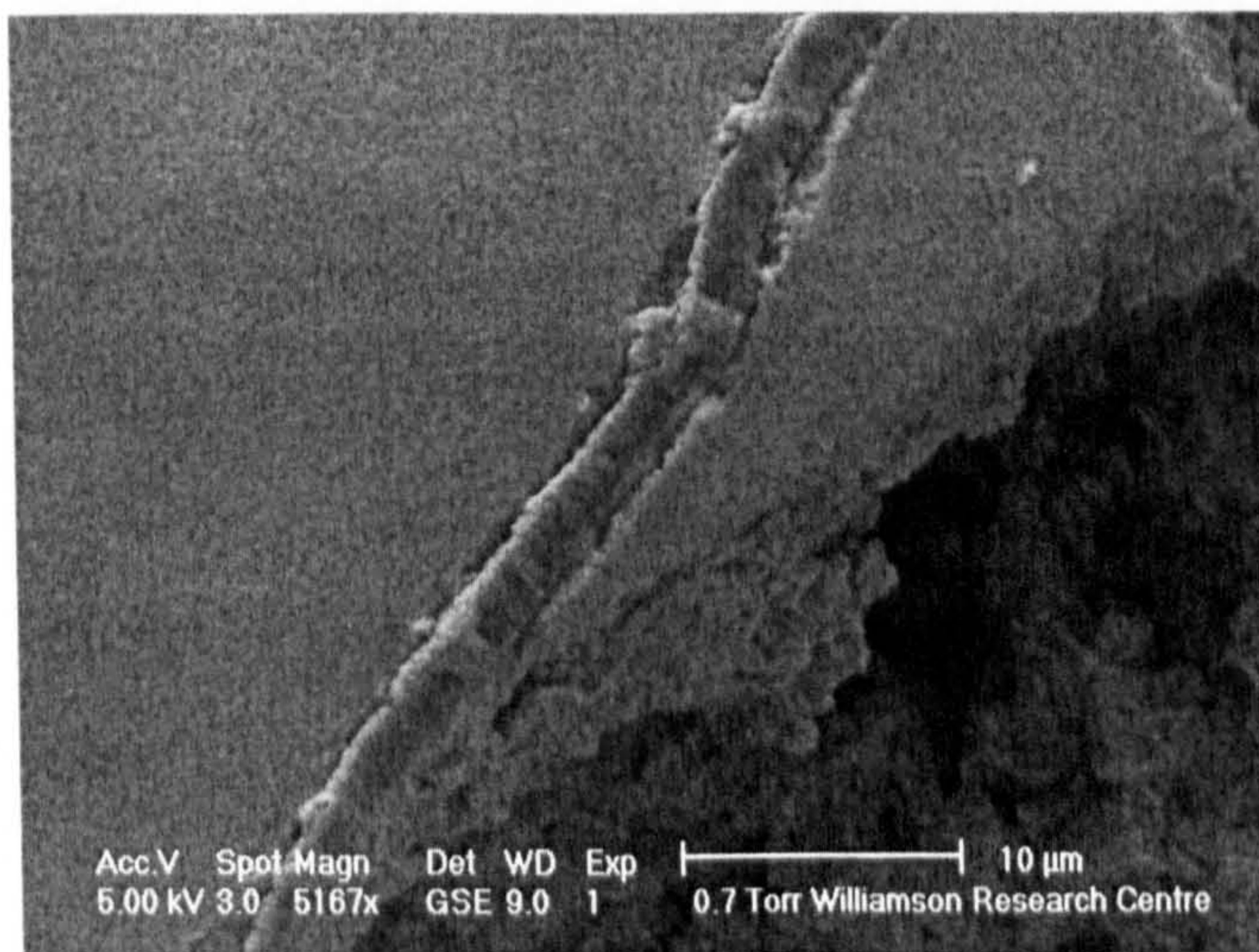


Figure 3.1 Hyphae of airborne fungus growing on silica gel medium and dissolving a groove in the gel.



Figure 3.2 Airborne fungus growing on silica gel medium.

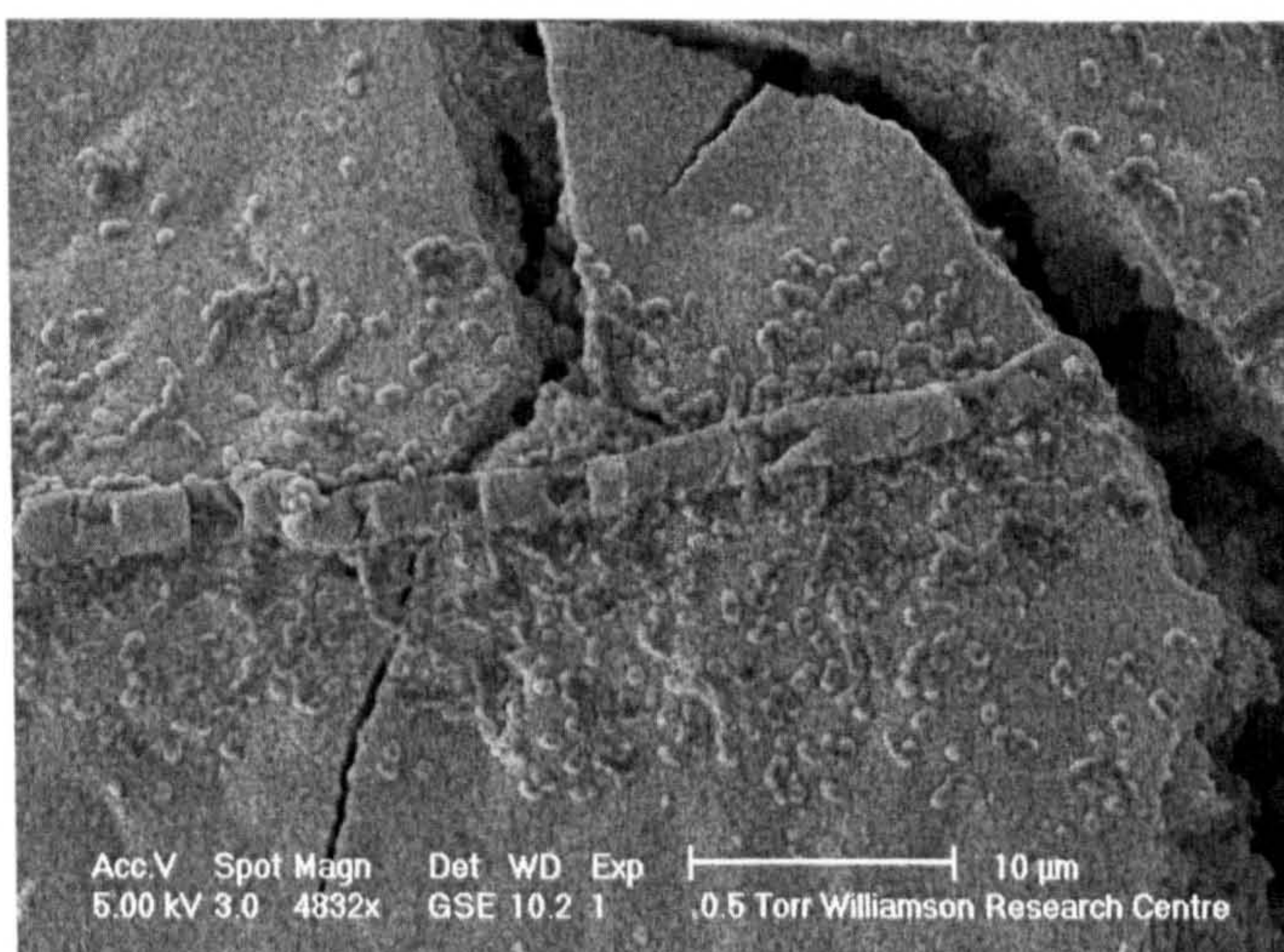


Figure 3.3 Airborne fungus growing on silica gel medium and undergoing lysis caused by similarly oligotrophically growing bacteria.

The above results show that air-borne fungi and bacteria can grow on naphthalene, but not glycolaldehyde as sole carbon source. It is important to note that no evidence was found to associate any microbial colonies with atmospheric particles which might have provided alternative carbon sources. The PAH, naphthalene has been found in comets, while the sugar, glycolaldehydes has been found to be widely distributed throughout space. The results therefore show that fungi and bacteria found in the atmosphere near the Earth's ground level can grow on carbon substrates found in comets and space generally.

The fact that bacteria and fungi colonies were occasionally seen growing on silica gel lacking any carbon source confirms the ability of microorganisms to grow in the apparent absence of added carbon substrates, i.e. as oligocarbotrophs (see below). Microorganisms growing under these conditions, scavenge carbon and other nutrients from the local atmosphere. As a result, although the presence of carbon sources led to an increase in the frequency of colonies, limited growth can occur in the absence of added

nutrients. The ability of microorganisms to scavenge nutrients in space is likely to be important as this will allow for limited growth where only trace amounts of nutrients occur.

Airborne microorganisms were used in this experiment, despite the fact that this meant that a variable inoculum of unknown composition was used. The importance of this approach was that it allowed us to avoid the use of bacteria and fungi that had been grown under nutrient-rich conditions and which had become generally habituated to laboratory growth. There is little doubt that comets clearly contain ample source of nutrients for bacteria. In fact comets would seem to provide an ideal nutrient source for microbial growth under both aerobic and anaerobic conditions. Microorganisms could also grow (oligotrophically, by scavenging) in cometary niches which contain only trace amounts of carbon and other nutrients. Although the physical conditions such as Eh, pH and temperature would obviously influence microbial colonisation of comets, it is probable that variations in such conditions would allow for a diverse range of microbial types including both extremophiles and non-extremophiles; comets for example, experience a wide range of temperatures including niches at 40°C. It may be that there exist niches where compounds such as formaldehyde and naphthalene occur at inhibitory concentrations (since both are biocidal), although it is also likely that niches will occur where suitable dilute concentrations of these occur and provide microbial carbon sources.

It is also possible that after long periods of growing under oligotrophic conditions some space organisms will develop into obligate oligotrophs, and will be poisoned by the excess quantities of nutrients present in most isolation media. As a result it would be

prudent to include oligotrophic media when attempts are being made to isolate microorganisms from space. The ability of microorganisms to grow under low nutrient conditions will also influence their ability to survive in the space environment. Bacteria for example, can survive exposure to UV for longer periods when grown under low carbon nutrient conditions than when grown copiotrophically (Joux *et al*, 1999). This fact should obviously be borne in mind when space environments are being simulated and exposure to UV, and other potentially inhibitory factors, is being investigated. The fact that very small, or thin, cells are produced when bacteria and fungi grow under oligotrophic conditionings will also influence the movement of these cells through space and the rate at which they deposit during passage through atmospheres, such as Earth.

The results of this study do not of course prove that microorganisms grow in comets. They do however, show that there is no *a priori* reason, based on nutrient conditions, and also probably physical conditions, why microorganisms, found in the Earth's atmosphere, could not grow in comets. Although only two carbon sources were used here, it is well established that microorganisms can use a wide range of PAHs and other carbon sources that are known to occur in comets. The corollary of this, is that there is again no *a priori* reason, based on nutrient supply, why bacteria and fungi present in the Earth's atmosphere could not have originated in comets and been delivered to Earth, by the mechanisms discussed at length by Hoyle and Wickramasinghe (Wickramasinghe, 2000).

Astrobiologists tend to consider that microorganisms found in space must necessarily be both novel and extremophiles, however, there seems to be no reason why many of the microorganisms commonly found on Earth could not survive in space or grow in the

cometary environment; it has, for example, recently been suggested that lichens could survive the rigours of space (de Vera *et al.*, 2004). The finding that airborne microorganisms can grow on carbon sources found in comets, and space generally, may be considered by some to be axiomatic or even trivial; it is however, a fact that needs to be established, since it is certain that, had we been able to show that microorganisms cannot grow in the nutrient conditions prevailing in comets, critics would have been quick to suggest that comets cannot act as habitats for microbial growth.

Finally, although many authorities (with the exception of Wickramasinghe and co authors) have commented that organics in comets could act as the building blocks of life, the possibility that such organics result from the presence and or metabolism of cometary microorganisms continues to be assiduously ignored.

CHAPTER FOUR

GEOPANSPERMIA

4.1 Introduction

The concept of geopanspermia (also called impact panspermia or lithopanspermia) suggests that rocks are ejected into space from planets by impact events; these rocks then travel through space and deliver microorganisms (or possibly other life forms) to other planets. The total annual flux of material falling to Earth from Mars is estimated at half a tonne, and Martian material has rained down on Earth throughout its history (Melosh, 1999). The obvious question is-do Earth rocks contaminate Mars and other areas of the cosmos? The most lightly shocked rocks of a planet will be those occurring at the surface where, conveniently, most of the biosphere exists. It is unlikely that large organisms could withstand the acceleration associated with such impact events, so the only life forms likely to be ejected from a planet will be microbial in nature. Natural orbital perturbations supply the means for ejected rocks to spread throughout the solar system. Any microorganisms in such a rock could colonise this new planet, provided that is it could survive the journey and the impact of the fall to its new destination. Small rocks, of the size of the meteorites that reach Earth from Mars, are the ones (by definition) most likely to survive such impact and even the sparse atmosphere of Mars would result in some slowing down of the rock to avoid its volatilization. Geopanspermia therefore provides an entirely feasible theory that suggests that life came (and perhaps continues to come) to Earth protected inside a meteorite.

The attraction of this form of panspermia lies in the fact that the microorganisms are enclosed within rocks and are thereby better protected from the rigours of passage through space. The fact that most planets of the solar system (and presumably planets elsewhere) undergo frequent bombardment with meteorites, asteroids and comets is well

established. Such impacts lead to large amounts of solid material being ejected from planets, like, Earth into space. The Earth (and most notably the Moon) is known to have undergone periods of intensive bombardment. It is even believed that most of the water on Earth was delivered by comets during such impact events (Roy-Poulsen, 1981). At present, there is an ongoing debate as to the possibility that life was seeded to Mars from Earth or *vice versa*.

The presence of rocks on Earth containing microorganisms is an obvious pre-requisite for the transfer of microorganisms from Earth to other parts of the solar system and beyond. Soils, of course, contain a large number and wide variety of microorganisms. At the outset of the experiments described below, we considered that it would be extremely straightforward to show that the rocks studied contain bacteria and fungi, probably in large numbers and will possess a wide, species diversity.

The aim of the work reported here was, to use a novel approach, which ensures sterility, to determine if rocks commonly found on Earth, i.e. coal and limestone, as well as amber contain bacteria and fungi.

4.A Studies on the isolation of microorganisms from amber

4.A.1 Introduction

Amber has been shown to contain both fossilised and living bacteria and fungi. While it is claimed such isolates are as old as the amber itself (i.e. at least 25- 40 million years), not surprisingly, critics have suggested that they are modern contaminants.

Here, we discuss experiments aimed at isolating microorganisms from Baltic and Dominican amber using an experimental approach designed to avoid all chance of contamination. The experiments were not designed to isolate all of the bacteria present

in the amber samples used, or to determine the frequency, or variety, of microorganisms in amber. Since two bacterial isolates were isolated from ancient amber, I took this as confirmation that amber contains bacteria and did not vary the media, or conditions used, in order to obtain further isolates.

4.A.2 Materials and Methods

4.A.2.1 Nature of the amber and confirmation of its authenticity

Samples of Baltic amber, containing insect inclusions, were obtained from a variety of suppliers (Fig. 4.1). The authenticity of the individual amber samples was confirmed by the “salt water test” i.e. the ability to float in a saturated solution of sodium chloride (Ross, 1998) and by the fact that they emitted a pine resin odour when exposed to a hot wire or when scored with a serrated blade. The absence of surface cracks in the amber was confirmed using a hand lens and low power microscope.

In order to release the inclusions into media, the amber was cracked open and broken into small pieces using the vessel shown in Fig. 4.3a. This consisted of a thick glass-walled, tissue homogenising vessel, sealed with a metal cap. A plunger passed through the cap and touched the bottom of the vessel. The top of the cap was covered with part of an autoclave bag (using a corner cut from a large autoclave bag), attached and sealed closed using autoclave tape attached to the cap and the top of the plunger. The ability of the autoclave bag cover to act as an airtight seal, and thereby prevented ingress of contaminants, was checked by inverting it under water and pumping air into it via the cracking vessel cap; absence of air bubbles confirmed the air tight nature of the seal. For the isolation of bacteria, Nutrient broth (Oxoid, 10 ml) was added to the cracking vessel which was then autoclaved for 20 minutes at 120°C.



Figure 4.1 Typical amber samples showing fossilized insect inclusions.

4.A.2.2 Scoring and sterilisation of the amber

A shallow central indentation was made in the surface of the amber and shallow grooves were scored (with a serrated knife) from this indentation around the amber. The amber was immersed in domestic bleach (10% v/v) for 20 minutes and then transferred to a closed bottle containing sterile distilled water (500 ml) and washed vigorously. The amber was then removed and immersed in membrane-filtered (0.22 μm) alcohol and transferred to a flame, using flame-sterilised forceps; the residual alcohol was then ignited. The sterilised amber was finally transferred to the growth medium (10 ml) in the cracking vessel.

4.A.2.3 Incubation of the vessel and isolation of bacteria

After inserting the sterile amber, the cracking vessel was left for 4 days at 25°C. All vessels in which bacteria grew in the medium were considered to be contaminated and therefore discarded. Where no bacterial growth appeared in the medium, the vessels were opened in a laminar air-flow cabinet and a small amount of medium was poured

onto a Nutrient Agar (Oxoid) plate; this was then incubated at 25°C for a further 4 days. If no bacteria appeared on the Nutrient Agar, or in the Nutrient Broth, over this time period the amber was cracked *in situ* in the vessel. If, following this period of incubation, bacterial growth appeared in the Nutrient Broth the vessel was opened, the neck of the vessel was thoroughly flame sterilised and a small amount of broth was aseptically transferred to Nutrient Agar; this was then incubated at 25°C until growth appeared. Any bacterial isolates were then purified by streaking and were independently identified (NCIMB, Aberdeen) using 16SrDNA analysis. All transfers were done in a laminar air flow cabinet, the sterility of which was checked periodically.

4.A.2.4 Cracking of the amber

The sterilised amber was cracked *in situ* in the cracking vessel by placing the bottom of the plunger into the surface indentation and applying a sharp tap to the top of the plunger with a light-weight hammer (Fig. 4.3a). The force cracked the amber along the scored grooves to expose any fossilised insects inside (Fig. 4.1). Further crushing of the amber was then achieved by manually applying force to the top of the plunger, until the original amber was crushed to form a suspension of very small particles.

4.A.2.5 Attempted isolation of a fungus from Dominican amber

An authenticated piece of Dominican amber containing a fungus was sterilised and cracked (as described above) in Czapek Dox liquid medium (Oxoid) containing 0.2g l⁻¹ mycological peptone (Oxoid). The cracking vessel was incubated at 25°C for 10 days when the contents were transferred to a sealed, sterile bottle (1litre) containing distilled water (500 ml); the bottle was then incubated at 25°C for a further 20 days.

4.A.2.6 Experimental controls

Although the experiments provide an “internal control” against contamination, 10 amber cracking vessels containing just medium and another 10 amber cracking vessels containing medium and uncracked amber were also exposed to the same handling procedures described above.

4.A.3 Results and Discussion

4.A.3.1 Studies on the isolation of bacteria from Baltic amber

Twenty different pieces of Baltic amber, containing unidentified insect inclusions, were studied. After cracking, bacterial growth occurred in the medium in only one case; the media in the other 19 vessels remained clear. No growth occurred in any of the 20 amber cracking vessels that acted as controls. Two bacteria were isolated from the vessel in which growth occurred; these were independently identified by 16SrDNA analysis as *Bacillus amyoliquifaciens* or *B. atrophaeus* (these bacteria are too close to separate) and *B. cereus*. Since the medium was checked for sterility prior to cracking, and the tube remained sealed throughout the experiment, we conclude that these bacteria originated from within the amber. It could be argued that un-germinated spores were present on the surface of the amber prior to it being cracked. This could be the case, although plating prior to cracking showed that the medium itself was not contaminated in this way. The amber sterilisation technique was also checked by placing sterilised samples on Nutrient Agar; no bacterial growth appeared around the embedded amber. While the presence of a single contaminating spore, of each of the two bacteria, at some stage in the experiment can never be ruled out, the sterility checks used are rigorous as we can make them. The question of contamination naturally arises in studies such as these,

where apparently ordinary, modern bacteria are isolated from ancient or highly extreme environments. The advantage of the approach used here is that the system contains an internal control, in that the amber is only cracked if the medium is seen to be uncontaminated; any bacterial growth following cracking of the sample must, as a result, have come from inside the amber.

Other workers have isolated bacteria from amber (e.g. Cano *et al.*, 1997, Cano, 2003, Cano, 1995). Although no attempt was made here to demonstrate the antiquity of our isolates, others (by using genome analysis) have claimed that bacteria isolated from amber are millions of years old. Lambert *et al.* (1998) for example, isolated a species of a *Staphylococcus* (*S. succinus*) from 25-40 million year Dominican amber while Cano and Borucki (1997) reported the isolation of *Bacillus sphaericus* (a species phylogenetically close to *B. amyoliquifaceans*); Greenblatt *et al.* (2000) have also isolated a wide range of bacteria from Dominican and 120 million year old amber from Israel. Although the authors of these studies discount the possibility of contamination, their work has nevertheless been criticised on this basis. Contamination can clearly never either be ruled out or be adequately controlled for. For example, it is possible that, despite a thorough microscope examination of the amber used here, a minute crack existed in the amber in which bacterial spores could reside, protected from the amber sterilisation process.

Since the sample of amber that resulted in bacterial growth contained enclosed air bubbles, it is noteworthy that Ascaso *et al.* (2003) reported observing bacteria-like inclusions in amber air pockets. It may be that bacteria survive better in such air bubbles than they do in the insect inclusions; as a result, amber samples containing such bubbles

may be a more productive source of bacterial isolates than amber that is free of such inclusions.

It might be assumed that any microorganisms isolated from amber must be able to grow under anaerobic conditions. However, survival, rather than growth, under anoxic conditions is the essential requirement. Since both of the bacteria isolated here are facultative anaerobes, survival in the anaerobic environment assumed to be present deep in amber, should not present a problem, at least in relation to this possible limitation.

4.A.3.2 Attempts to isolate a fungus from Dominican amber

Microscope examination (x10 magnification) of the sample of Dominican amber studied clearly shows the presence of a mass of fungus mycelium (Fig. 4.2). The fungus occurs as a mass of very fine hyphae showing numerous anastomoses (i.e. connections), so as to form a web-like network. Such networks, or gossamers, are typically seen when fungi

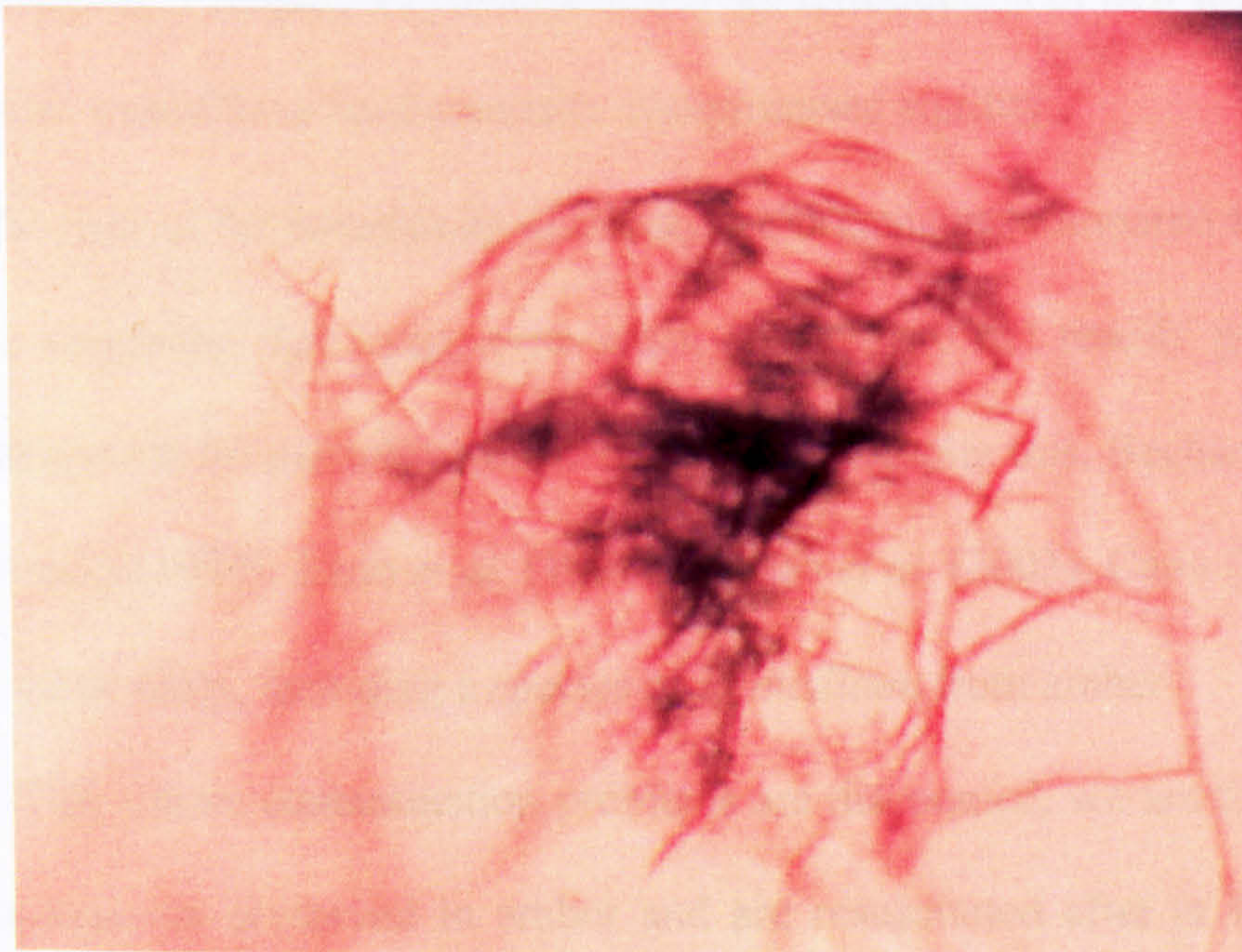


Figure 4.2 Fungal hyphae seen inside amber using a light microscope (X10).

are grown under oligotrophic (i.e. low-nutrient) conditions. No fungi (or bacteria) were isolated in the original medium after 10 days incubation, or following dilution of the original medium in sterile water and a subsequent 28 day incubation period. The dilution step was introduced in case the fungus was inhibited by the original nutrient-rich medium and preferred instead to grow as an oligotroph. Fig. 4.2 shows that fungus was clearly present in the amber prior to cracking; this was presumably dead, or did not grow under the nutrient conditions imposed. It should be emphasised that only one attempt was made here to isolate fungus from amber; this compares with the finding that bacteria were isolated on only one occasion when 20 pieces of amber were cracked; clearly a fungus might be isolated should a large number of fungus-containing pieces of amber be sacrificed. It could also be argued that the amber-fungus might have been induced to grow had a range of media been used. While this is true, only one sample of amber, containing a fungus, was available and the introduction of new medium into the cracking vessel would have fundamentally compromised sterility.

In view of the complexity of eukaryotic organisms, compared to bacteria, it is perhaps not surprising that fungi, even when present, are not readily grown from amber after entrapment for periods of millions of years. Despite this, Bechanbach (1995), Cano and Borucki (1997); Cano *et al.* (1991); Ascaso *et al.* (2003); Cano (2003), all claim to have isolated fungi, including a species of *Penicillium* from amber.

If the above contamination problems are discounted, we are then left to conclude that bacteria are preserved in amber and are resuscitated after at least 40million years of stasis. Despite the known ability of spores of *Bacillus* species to survive over long periods this possibility remains difficult to accept. However, set against this is the fact

that bacteria, notably *Bacillus* species, have now been isolated, using a range of different experimental protocols, from geographically distinct amber samples. Added to this are reports of the isolation of *Bacillus* species from ancient salt crystals (Vreeland *et al.* (2000). However, absolute proof of the existence of recoverable bacteria in 40 million year old amber will presumably have to await an experimental approach that allows for the detection of viable bacteria within the amber itself.

4.B Attempts to isolate bacteria from rocks and clay

4.B.1 Introduction

This section is devoted to discussing work which is essentially a repeat of the amber cracking experiments discussed above; here however, a larger cracking vessel was employed in order to accommodate the larger rock samples studied.

4.B.2 Material and Methods

4.B.2.1 Origin of coal, limestone and clay samples

Coal samples were obtained from six different mining locations in the UK. Table 4.1 shows the locations in the UK where they were mined and the percentage carbon contents of the coals.

Table 4.1 Location of origin and carbon content of coals.

N	Carbon Source	Carbon Content (%)
1	CORTONWOOD	87.2
2	CWM	90.3
3	CYNHEIDRE	95.2
4	NADINS	80.1
5	THORESBY	85.1
6	TILMANSTONE	92.4

The limestone samples were taken from the cliffs at Flamborough Head (North Landing), East Yorkshire. The clay sample was obtained from Filey Brig, North Yorkshire.

4.B.2.2 Initial examination for the samples

The absence of large surface cracks in the rock samples was confirmed using a hand lens and low power microscope.

4.B.2.3 Preparing the experiment vessels and bottles

4.B.2.3.a Preparing the experiment vessels

In order to release the organisms, from the coal samples, into liquid media, the samples were cracked and broken into small pieces using the rock cracking vessel shown in Fig. 4.3a.

a) Cracking vessel



b) Cracking bottle



Figure 4.3 Cracking vessel and bottle.

The vessel consisted of a thick glass-walled, tissue homogenising vessel, sealed with a metal cap. A plunger passed through the cap and touched the bottom of the vessel. The top of the cap was covered with part of an autoclave bag (using a corner cut from a large

autoclave bag), attached and sealed closed using autoclave tape attached to the cap and the top of the plunger. This autoclave bag provides the vessel with the required airtight sealing and thereby prevented ingress of contaminants; also, it provides the vessel with the ability to move the plunger up and down to break the amber and the coal. This ability of the autoclave bag cover to act as an airtight seal was checked by inverting it under water and pumping air into it via the cracking vessel cap; absence of air bubbles confirmed the air tight nature of the seal. For the isolation of microorganisms, 10 ml of a variety of liquid medium was added to the cracking vessel, which was then autoclaved for 20 minutes at 120°C.

4.B.2.3.b Preparing the experiment bottles

In order to release the organisms, from the core of the lime stone samples, into liquid media, the samples were cracked and broken into small pieces using Cracking Bottles (Fig. 4.3b). Bottle consisted of a thick glass-walled, tissue homogenising bottle, sealed with a plastic cap. A drill head passed through the cap and touched the bottom of the bottle. The top of the cap was covered with part of an autoclave bag (using a corner cut from a large autoclave bag), attached and sealed closed using autoclave tape attached to the cap and the top of the drill head. This autoclave bag provided the bottle with the required airtight sealing and thereby prevented ingress of contaminants. This ability of the autoclave bag cover to act as an airtight seal was checked by inverting it under water and pumping air into it via the Cracking Bottle cap; absence of air bubbles confirmed the air tight nature of the seal. For the isolation of microorganisms, 20 ml of a variety of liquid medium was added to the Cracking Bottle, which was then autoclaved for 20 minutes at 120°C.

4.B.2.4 Scoring and sterilization of the samples surface

A shallow central indentation was made in the surface of the sample, coal and limestone, and shallow grooves were scored (with a serrated knife) from this indentation around the sample. The sample was immersed in domestic bleach (10% v/v) for 20 minutes and then transferred to a closed bottle containing sterile distilled water (500 ml) and washed vigorously. The sample was then removed and immersed in membrane-filtered (0.22 μm) alcohol and transferred to a flame, using flame-sterilized forceps; the residual alcohol was then ignited. The sterilized sample was finally transferred to the growth medium (10 ml) in the cracking vessel or bottle.

4.B.2.5 Experimental design set up to control contamination

To minimize the possibility of contamination, an experimental design was used for the vessel and bottle experiments. This design involves eight different vessels and bottles for each experiment. Their distribution was as follows:

- 1- Two vessels and two bottles containing just liquid medium (Control 1)
- 2- Six vessels and six bottles containing liquid media and the samples (coal or limestone), three of each six left as a second control, in which the samples inside were not cracked throughout the experiment to check the effectiveness of the surface sterilisation.

4.B.2.6 Incubation of the vessels and bottles

After inserting the surface-sterile samples into the cracking vessels and bottles, these vessels and bottles were left for 4 days at 25°C and 37°C. All vessels or bottles in which bacteria grew in the medium were considered to be contaminated and therefore discarded. Where no bacterial growth appeared in the medium, the vessels or bottles

were opened in a laminar air-flow cabinet and small amounts of medium were poured onto a variety of medium plates; then were incubated at 25°C and 37°C, respectively, for a further 4 days. If no bacteria appeared on the vessels, bottles or plates over this period of time the sample was cracked *in situ* and incubated at 25°C and 37°C for 4 days then for another 7 days if there is no growth. If, following these incubation times, bacteria growth appeared in one of the cracking vessels or bottles, a small amount of the liquid media was transferred to solid medium plates in laminar airflow cabinet then incubated at 25°C and 37°C until growth appeared. Any bacterial isolates were then purified by streaking and were independently identified (NCIMB, Aberdeen) using 16SrDNA analysis.

4.B.2.7 Media used in the experiments

In these experiments a variety of media were used (For further information see Appendix 1):

1- Liquid Medium used in the cracking vessels and bottles:

a) %100 LB, b) 20% of LB, c) 10% of LB ,d) Nutrient Broth

2- Semi Liquid LB Medium for anaerobic experiments (0.005 Agar added to the LB liquid medium).

3- Solid Media: LB, 20% of LB, 10% of LB, Nutrient Agar medium.



Figure 4.4 A sample of coal.

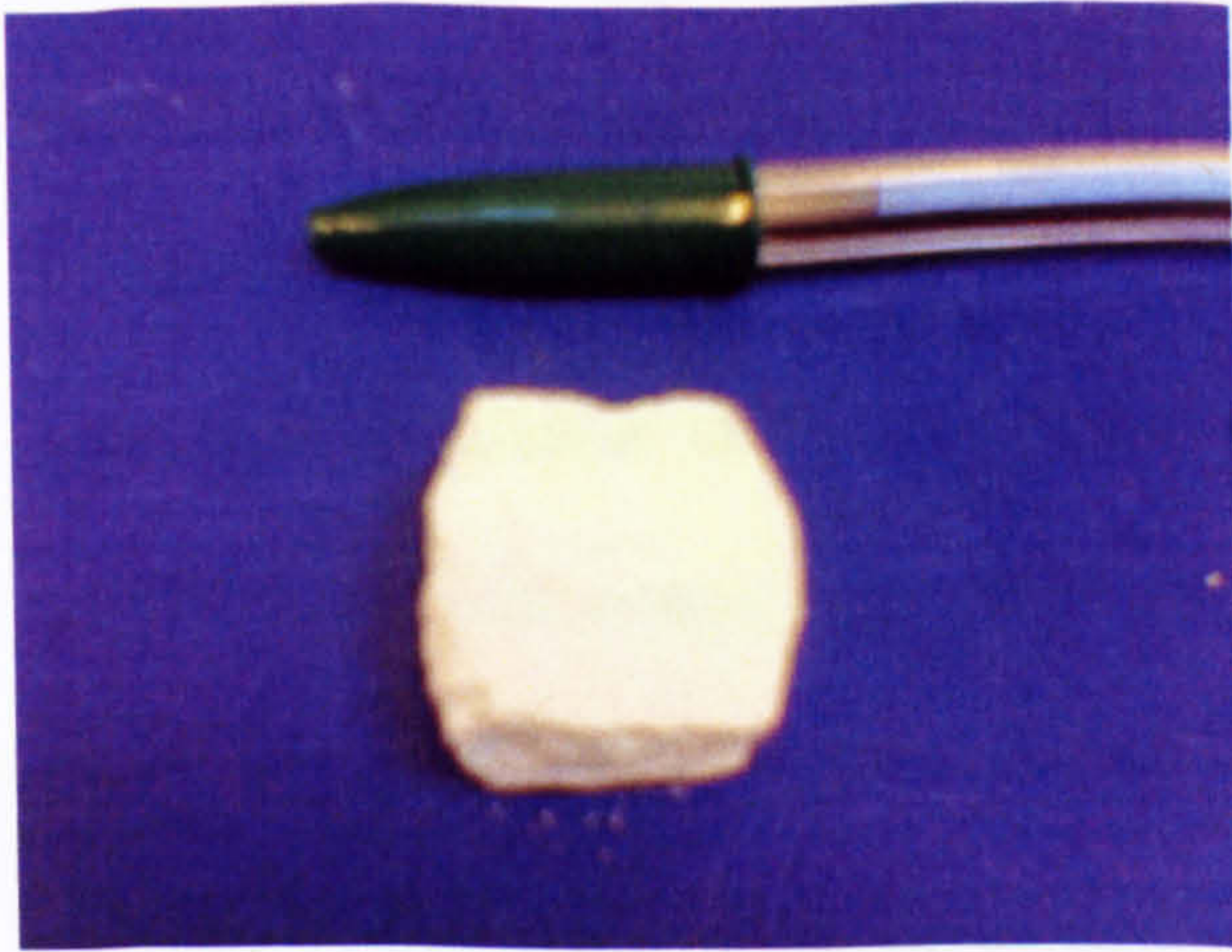


Figure 4.5 A sample of limestone (Flamborough Head).



Figure 4.6 A sample of clay.

4.B.3 Results and Discussion

The above described isolation of bacteria from amber suggests that this material could act as vehicle for the transfer of bacteria from Earth to other areas of the cosmos, via geopanspermia. It is obvious however, that the transfer of naked amber in this way would present a problem, since amber is inflammable and would not resist re-entry into a planet which possesses the reasonable amount of atmosphere necessary to slow down the infall of rocks. As a result, the only way in which amber could act as a panspermic vehicle would be when encased in other flame resistant rocks. The same proviso obviously also applies to coal.

The clay sample contained a large number of bacteria, (mainly species of *Bacillus*) equivalent the number likely to be found in soils. A single bacterium was isolated from an individual coal sample; this was independently identified using molecular methods as, *Bacillus subtilis subtilis* (see Appendix A).

Bacteria were not isolated from any of the cracking vessels in which limestone (Fig. 4.5) was cracked under sterile conditions. The fact that the medium used remained sterile in the bottles containing the limestone prior to cracking shows that the method used is rigorous and that the absence of bacteria following cracking is due to either the absence of bacteria in the samples, or to their inability to grow on the medium chosen

Our inability to isolate bacteria from limestone came as a considerable surprise, since it was, not unreasonably, expected that this rock (being porous) would contain substantial numbers of a variety of bacteria and fungi. It could be argued that the medium used selected against the organism in this rock type, or that the surface heating, applied to sterilise the rocks, effectively sterilised the whole substrate, with the effect

that no microorganisms grew from the samples. However, while the medium used here, like all media, selects only a fraction of the soil microbial population it would nevertheless have been expected to support the growth of those bacteria that might be expected to have entered limestone in ground water. Similarly, it is unlikely that the small period of heating employed would have sterilised the whole limestone piece that was added to the cracking vessel.

The conclusion which obviously results from this study is that the limestone samples used here do not contain bacteria and fungi capable of being isolated by the medium employed; i.e. most heterotrophic, aerobic bacteria found in soils. If this finding is verified then it follows that limestone could not act as vehicle for the transfer of bacteria from Earth to other planets.

A single bacterium, (*B. subtilis*) was however, isolated from a coal sample (Nadin), which agrees with previous reports from the literature. Farrell and Turner (1932) for example, found that anthracite coal contains a range of bacteria; Lipman (1931) claimed that living bacteria could be isolated from coals, as well as ancient rocks (and meteorites). He maintained that the bacteria he found in coals had been there from the carboniferous period, when the coal measures had been first laid down, an idea that was criticized by Burke and Wiley (1937).

Myers *et al.* (1966) reported that bacteria can penetrate rocks; Folk (1993) also reported that bacteria and nanobacteria can be found in carbonate sediments and rocks; while Frederickson and Onstott (1996) have reported that microorganisms inhabit water-yielding rock formations. Of course, it could be argued that, since none of these reports involved the use of cracking vessels, like the one used here, designed to ensure non-

contamination, that such reports of the presence of bacteria in rocks are the result of laboratory based contamination. This would lead to the inevitable conclusion that the results presented here are correct and that limestone does not contain bacteria.

These findings obviously have a major impact on the theory which suggests that rocks, containing microorganisms, can be blasted from the Earth's surface and act as a vehicle for panspermia. Clearly, if Earth rocks, like limestone, do not contain bacteria, then this possibility is non-existent. It is hardly believable however, that most Earth rocks are sterile; and as we have seen clay, amber and coal could act as a panspermic vehicle. Clearly, it would be desirable to study a wider range of rocks than the ones used here.

The finding of a bacterium in coals suggests that this rock could act as a vehicle for panspermia. However, since coal is somewhat fragile (some types of course are very soft, while anthracite is harder) and is readily burned (for example during re-entry into a planet possessing an atmosphere) it follows that coals would have to be encased in a coating of a more resistant rock; such encased coals might then act a suitable vehicle for geopanspermia and the bacteria contained in such coals might then be released during impact, or subsequent weathering.

CHAPTER FIVE

GENERAL DISCUSSION

5.1 General Discussion

During the period 1974-78, Hoyle and Wickramasinghe (Wickramasinghe, 2000) developed the idea that grains present in space are made up of complex organics and that their presence in 100's of billions of comets enabled prebiotic matter to develop into primitive bacterial cells. They argued that pre-existing, viable bacterial cells derived from interstellar space may have become added to comets in the primitive solar system, when it was found that the extinction properties of interstellar dust matched with what was described as being "uncanny" precision to the expected behavior of freeze-dried bacteria. Hoyle and Wickramasinghe observed that the 2175 Å ultraviolet extinction band may be better explained by an assemblage of biological aromatics than by spherical graphite grains previously thought to be involved. In particular, two obvious spectroscopic features of dust appeared to show that living material is present everywhere in the cosmos. Other astronomical evidences, e.g. the complex organic composition of cometary dust, and the extended red emission in the red rectangle also served to add weight to this conclusion; despite this evidence, the theory of cometary panspermia has yet to gain widespread acceptance.

The theory of panspermia dates back to the Greeks and possibly even earlier. It was, however, first scientifically discussed by De Malliet in the 1700s, and by Lord Kelvin and Hertzog in the late Victorian period and then, in more detail during the early 20th century by Svante Arrhenius. Arrhenius' argument that bacterial spores could be transported across the galaxy was criticized by, for example, Becquerel, who using laboratory data, argued that bacteria could not survive space conditions, most notable, exposure to UV radiation. This, and other criticisms of panspermia, has been met by

counter-arguments by Hoyle and Wickramasinghe (2000). For example, a thin coating of carbonaceous matter around a bacterial grain (which they suggested would inevitably form in space), would act as a screen against ultraviolet light. The effects of ionizing radiation (cosmic rays) might be more difficult to avoid, but still achievable. During an average residence time of 10 million years in a typical location in interstellar space, the cumulative radiation dose received by a bacterium is estimated as 10⁵ rads. Although many terrestrially adapted bacteria would not survive such a large radiation dose, some, like *Deinococcus (Micrococcus) radiodurans* can withstand a million rads under laboratory conditions, and other bacteria exist that can similarly thrive in working nuclear reactors. It is also not certain if our understanding of the radiation susceptibility of microorganisms can be directly transferred to interstellar conditions, most notably, whether radiation damage to bacteria is strictly a linear process. For example, exposure to millions of years of extremely low intensities of interstellar ionizing radiation may be far less damaging than short pulses of high intensity radiation delivered to laboratory-grown bacteria. What is clear is that bacteria, or even entire microbial groupings, encased within comets or fragments of comets, would be protected from the cosmic radiation in space, and could as a result be safely transported between well-separated stellar and planetary systems.

Cometary collisions may also have injected life onto the Earth on several occasions during the period 4.3-3.83 billion years. It is an interesting possibility that bacterial phyla that survived under such episodes of periodic evaporation were thermophiles, bacteria which are found at the base of the currently suggested phylogenetic tree. These

presumably were the life forms that survived to give rise to the evolution of later organisms.

The Hoyle and Wickramasinghe theory requires life to have been introduced to Earth by comets, rather than originating here *in situ*, some 4 billion years ago, with an ongoing input of microorganisms from this source continuing to the present. Recent discoveries of organic molecules and fragile bacteria-like structures within the Mars meteorite ALH84001 tend to support the viewpoint that microbial life could indeed have been transferred in viable form between objects within the solar system. It should however, be emphasized that there has always been considerable debate over whether such fossil-like minerals do in fact represent small bacteria (McSween, 1997).

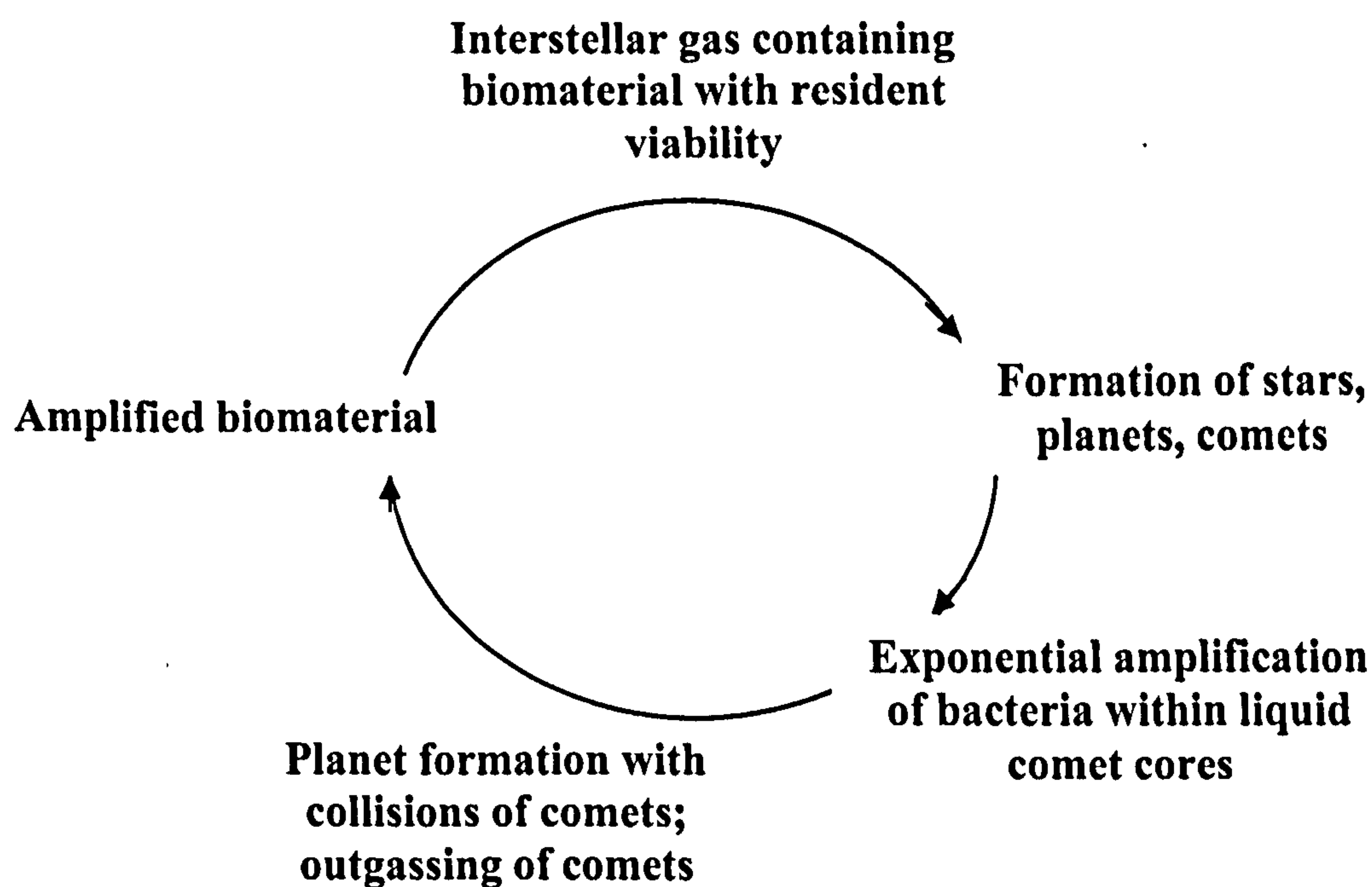


Figure 5.1 The Hoyle and Wickramasinghe cosmic life cycle.

Microbes are found in vast array of environments on Earth; in geothermal vents, the ocean floor, in radioactive dumps and in the Antarctic soil, and have been recovered from depths of 8 km beneath the Earth's crust. Laboratory studies have shown that bacteria can survive pressures at ocean depths of several kilometers or more (Horikoshi, 1998).

The long- term survivability of bacteria has also been extended to by the finding that they can be entrapped in salt crystals. Direct proof of the survival of bacteria exposed to radiation in the near-Earth environment has also been demonstrated using NASA's Long Exposure Facility. What is now required is a direct demonstration that viable microbes exist within cometary material and that they are being transferred to the Earth.

The hypothesis of the extraterrestrial origin (and continued arrival) of microorganisms from space, while still controversial, is becoming increasingly more acceptable to the scientific community. The bacterial material, cultured in the balloon experiments, and detected earlier through fluorescence microscopy, can be regarded as forming part of the cometary material known to reach the Earth. Critics of panspermia may argue that 3 μm plus radius particles get burnt through frictional heating and end up as meteors. Some fractions may do, but others would not. Survival depends on many factors such as angle of entry and mode of deposition in the very high stratosphere. Several methods of entry can be considered that would allow for intact injection into the Earth's stratosphere, possibly starting off as larger aggregates released from comets which would then disintegrate into a cascade of smaller, slow-moving clumps at heights above 270 km; here, frictional heating would be expected to be negligible. Evidence for such disintegrations has been available for many years, and more recent studies of Brownlee

particles collected using U2 aircraft have also shown the survivability of extremely fragile organic structures.

Based on the cells detected on some filters, a crude estimate can be made of the amount of such material falling in at such heights, which Narlikar *et al.*, (2003) suggest is around 3 tones per day over the entire globe. Whatever the source of the clumps might be, such an infall or fallback rate from 39 to 41 km would seem inescapable. With an average of 2.4×10^6 g of bacteria (deemed viable) collected per filter, it would indeed be somewhat surprising to find that they are all non-culturable.

The aim of the work described in this Thesis was to add data towards vindicating the theory of panspermia, i.e. the view that life originated from space. In addition, the work was focused on the theory of cometary panspermia of Hoyle and Wickramasinghe which states that, not only did life on Earth originate from space, but that life (in the form of microbes) continues to rain down on the planet from this source.

If this latter suggestion is correct then it obviously must be possible to obtain microorganisms in a viable state at heights above the Earth before they enter the atmosphere. If it could be demonstrated, beyond all doubt, that microbes could not reach such heights from the Earth (or from man-made objects in space) then it follows that such organisms must be incoming from space; if this possibility could be proven then such data would vindicate the view of Hoyle and Wickramasinghe (1981), that microbes are indeed currently incoming to Earth. The problem arising from such attempts to demonstrate the last point relate to the problems relating to the isolation of microbes using rockets, since the collision velocity between a solid rocket and a particle would be 40 km per hour; clearly sufficient to smash such fragile living particles. Such a

calculation would suggest that the results of Russian workers, who used sounding rockets, that microorganisms can be isolated in the stratosphere at heights up to 60 km are dubious; these must have either resulted from contamination, unless of course bacteria and fungi can withstand such collision velocities. It is for this reason that balloons have been used in the sampling trips referred to here, since balloons reduce the impact velocity to near zero. While this solves the impact velocity problem, it limits the height at which material can be collected to around 40-50 km. Such restrictions then impose sampling heights at which arguments can then be made about the possibility of upward contamination of the stratosphere from Earth. While it is generally accepted that particles of a size greater than 1µm could not reach even the lower levels of the stratosphere, it could nevertheless always be argued that there must be some unknown mechanism by which such transfer could occur; if samples could be obtained at altitudes of, say, 100 km, this argument would surely have no foundation. For the moment however, we are left with maximum sampling heights, using balloons of 41 km.

The results presented in Chapter 1 clearly suggest that microorganisms, both bacteria and fungi are present in the stratosphere at heights up to 41 km, and that some of these bacteria (as indicated by viable, fluorescent staining techniques) are viable. This confirms previous findings, from this laboratory and Cardiff University. Taken together with results obtained from other laboratories, it seems that there is little doubt that bacteria and fungi can be isolated from heights of, at least, 41 km.

We are then left with the obvious question: from where did the bacteria and fungi found at 41 km originate? While the knee jerk reaction would be Earth, arguments have been presented here to back up previous claims (Wainwright, 2007) that there exists a

mixed population of bacteria in the stratosphere, one being elevated upwards from Earth, by an unknown mechanism, and one descending to Earth from space. The suggestion that the latter may have originated from rockets, spy planes and space debris, seems unlikely to be true, but is again an ideal refuge for anyone who wishes to reject, a priori, the view that stratospheric microorganisms must have a non-Earth origin. The suggestion that this mixed population might contain microbes from Earth is in fact given here to pacify critics; it must be emphasised that there remains no known mechanism by which bacteria, and, especially, fungi could reach the stratosphere from Earth. Critics might argue then that we microbiologists have unwittingly provided a stimulus to atmospheric scientists to believe that such a mechanism must exist, and this provides encouragement for them to prove this to be the case. Even if they did so, it would remain extremely difficult, to the point of impossibility, to argue that a bacterial mass of around 10-20 microns in diameter could reach heights of 41 km above Earth, from the Earth (in the absence i.e. of volcanic ejection).

The results presented here tend to confirm the view therefore that microorganisms are continually raining down to Earth from space. Clearly further sampling trips are required to make such a claim beyond any doubt.

One problem relating to the input of large 10 μm bacterial masses relates to "burn-up" during entry. The normally accepted entry speed of small particle is about 40 km per second, a speed which would enable a cell of the size around 1 μm to avoid burning up in the Earth's atmosphere. In exceptional circumstances (i.e. entry on so-called favourable orbits) however, a bacterial mass of 60 μm could safely enter the Earth's atmosphere without burning up (Hoyle and Wickramasinghe, 1981); on this basis, the

10-20 μm bacterial masses observed here, on membranes sampled at 41 km, would have no problem in avoiding damaging frictional- heating.

The results on bacterial survival in the stratosphere in relation to the presence of sulphuric acid aerosols show that the presence of aerosols of this acid should not prevent bacterial survival in this region; this would follow of course if bacteria have been isolated from the stratosphere. Suggestions that UV and ionizing radiation would necessarily kill bacteria in space have been countered by Hoyle and Wickramasinghe (1981). The fact that bacteria are often seen associated with clumps of cosmic dust suggest a means by which they could be protected, while resident in the stratosphere, from the first of these damaging types of radiation.

The studies reported here (Chapter 4) showing the absence of microorganisms in limestone were somewhat surprising, as this rock might be expected to possess a rich microbial population. These studies were directed to the idea (lithopanspermia) that microbe-rich rocks could, after an impact event, be transferred from Earth to other planets (and presumably, *vice versa*). It is not immediately obvious why limestone was found to lack a microbial population. The fact that other workers have found microbes in terrestrial rocks, and bacteria were found, in this study, in clay, amber and coal, means that the findings presented here, do not *a priori* deny the possibility of lithopanspermia.

Chapter 3 of this Thesis is devoted to answering the question—can the nutrient status likely to exist in comets support microbial life. We are fortunate that new data on the composition of comets continues to arrive and this has greatly altered our view of cometary composition. It is now known that comets contain water, and hydrocarbons, in the form of polyaromatic hydrocarbons. The question of whether comets have any free

oxygen has not yet been answered however. Certainly comets are not, throughout the period of their existence, the extremely cold environments of common expectation. And can reach temperatures as high as 40°C (Fig. 5.2).

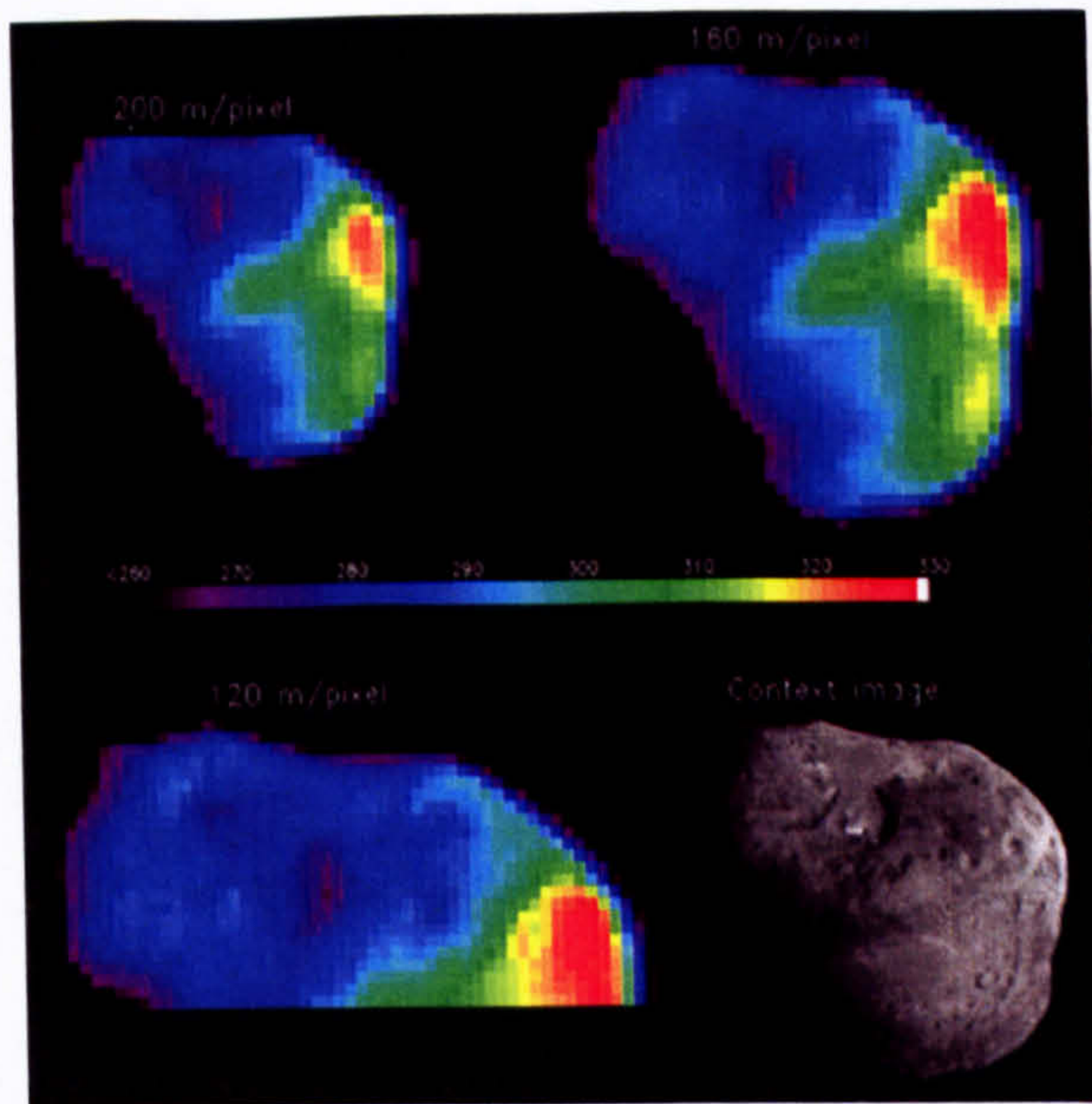


Figure 5.2 Temperature profile of comet Temple (source NASA).

There is no theoretical reason therefore why microorganisms cannot live and replicate in comets as is suggested by the theory of cometary panspermia. The results presented here show that airborne bacteria sampled on Earth can grow in simulated cometary nutrient environments. This fact could of course easily have been predicted from just looking at the composition of simulated comets, but it is a fact that has to be demonstrated nevertheless as, in its absence, critics would argue that although comets appear to provide an excellent nutrient environment this cannot be assumed without experimental proof; this proof we believe has been provided here. The simulated cometary nutrient mix used here provides a rich nutrient environment for microbial growth; in fact, considering the ability of microorganisms to grow under oligotrophic

conditions, the nutrients are in excess and it is likely that microorganisms could live and reproduce in a much less-rich cometary environment, assuming these exist.

In conclusion, the results presented here show that bacteria and fungi exist in the stratosphere at heights up to 41 km, and provide good theoretical evidence to suggest that there exists, in the stratosphere a mixed population of microorganisms, some possibly coming from Earth (although the mechanism by which this can be achieved is unknown) and some, large bacterial masses (and presumably fungi), are incoming from space. It is statistically unlikely that the microorganisms isolated from the stratosphere, as detailed in the four available published reports, all come from space debris, space remains the most likely source of these microorganisms. If, in future, this is confirmed then it would provide evidence for neopanspermia, the view, expressed by Hoyle and Wickramasinghe, that microorganisms are continually raining down to Earth from space. Clearly, more sampling trips of the stratosphere, and beyond, are required in order to convince the scientific community of the validity of these findings. The cometary theory of panspermia is further strengthened by the finding that the nutrients they contain can support bacterial growth and also, that the physical conditions found within comets are not a priori inhibitory for microbial growth. The cometary theory of panspermia has been strengthened by these findings, but there is a long way to go before the suggestion that life on Earth originated from space, and that microorganisms continue to enter Earth from this source is finally accepted by the scientific community at large.

5.2 Diseases from Space

An interesting extension of the theory of neopanspermia, not covered by the research described in this Thesis, but nevertheless worth discussion, is the idea that diseases occurring on Earth may have originated from space; a concept termed pathospermia (Wainwright, 2003). This Theory has once again been extensively worked out by Hoyle and Wickramasinghe (Hoyle and Wickramasinghe, 1979) and has received even more criticism, bordering on derision from the scientific community than has the general theory of cometary panspermia. Pathospermia suggests that some of the bacterial and viral pathogens that cause disease in Man and, possibly also, animals originated from space and come to Earth in the mass of cosmic dust that continuously arrives from this source.

The evidence for pathospermia, provided by Hoyle and Wickramasinghe can be summarised as follows:

- 1) The historical association between disease occurrence and the sighting of comets suggests that disease may arrive from space
- 2) The inexplicable speed of transfer of diseases like the Black Death from one part of the Earth's surface to another in a period when rapid transport was not available indicates the extraterrestrial, or atmospheric transfer of pathogens over long distances.
- 3) The fact that most new diseases originate in Southern China (around Hong Kong) which is known to be the "Earth's sink hole" into which material from space tends to be focused is again indicative that pathogens may enter our planet at this point.

4) The distribution of diseases, like influenza in remote populations which cannot apparently be explained on the basis of person to person contact suggest an extraterrestrial source, or atmospheric transfer.

Perhaps not surprisingly, these aspects of the Theory have been challenged, although not as rigorously as might be expected. It has often been simply taken for granted that these ideas must be wrong. The usual over-riding criticism that is made of the idea that virus infections, such as influenza, or SARS might arrive from space is that a) pathogenic viruses would not survive the rigours of such a trip and b) the fact that space viruses can cause disease in humans presupposes some connection between host and pathogen. It obviously seems unlikely that a space-derived virus would be adapted to an unknown host, i.e. Man. It is noteworthy however, that viruses are now being shown to be able to survive in various Earth-extreme environments. The well-known ability of viruses to undergo rapid mutation would also presumably account for why they might be able to rapidly adjust to a previously unknown host on a distant planet.

5.3 Suggestion for further work

Any future work on the main topic of this Thesis will obviously depend on the availability of samples; unfortunately, at present, it is unlikely that such samples will be forthcoming. The balloon flight, discussed here, has been costed at around £1million! As a result, it is unlikely that, because of these high costs involved, a large number of balloon sampling flights will be conducted in the near future. This problem is compounded by the fact that the Indian Government research agencies involved have decided that, in the future, all the sampling and microbiological analysis will be done "in house". While this decision is entirely understandable (since the Indian Government is

paying for the sampling trips) it means that workers in the UK, both here in Sheffield, as well as in Cardiff will not have access to future stratospheric samples.

As a result of these sampling limitations, future studies on panspermia, conducted here in Sheffield, will probably be limited to simulation experiments, notably in relation to the question of the survival of microorganisms in space. Much of this work has already been conducted (Horneck, 1998) and there is no pressing need for it to be replicated. Perhaps further studies could be conducted on simulated comets, especially as future comet-probes will doubtless provide us with a better understanding of the possible biological potential of the cometary environment.

While future work, from this laboratory, on the microbiology of the stratosphere looks unlikely for the above reasons, it is noteworthy that NASA has already (August, 2007) conducted sampling flights into the stratosphere at around 20 km, in order to further study the microbiology of this region; we obviously await the results with great anticipation. Such studies may confirm and, hopefully, extend the results obtained from the balloon sampling trip discussed here.

There remains one possible means by which workers, in this laboratory, and elsewhere, could in future gain stratospheric samples. This relates to so-called "space tourism". In the very near future, it will be possible for tourists to be elevated to around 40 km, from where they will be able to parachute to Earth. The French adventurer, Michel Fourier (Fig. 5.3) will soon attempt to break a number of altitude and height records (for parachutists) by jumping from around 40 km. It is possible therefore that collaboration with space tourist firms, or adventurers like Fourier, could provide stratospheric samples for further study.

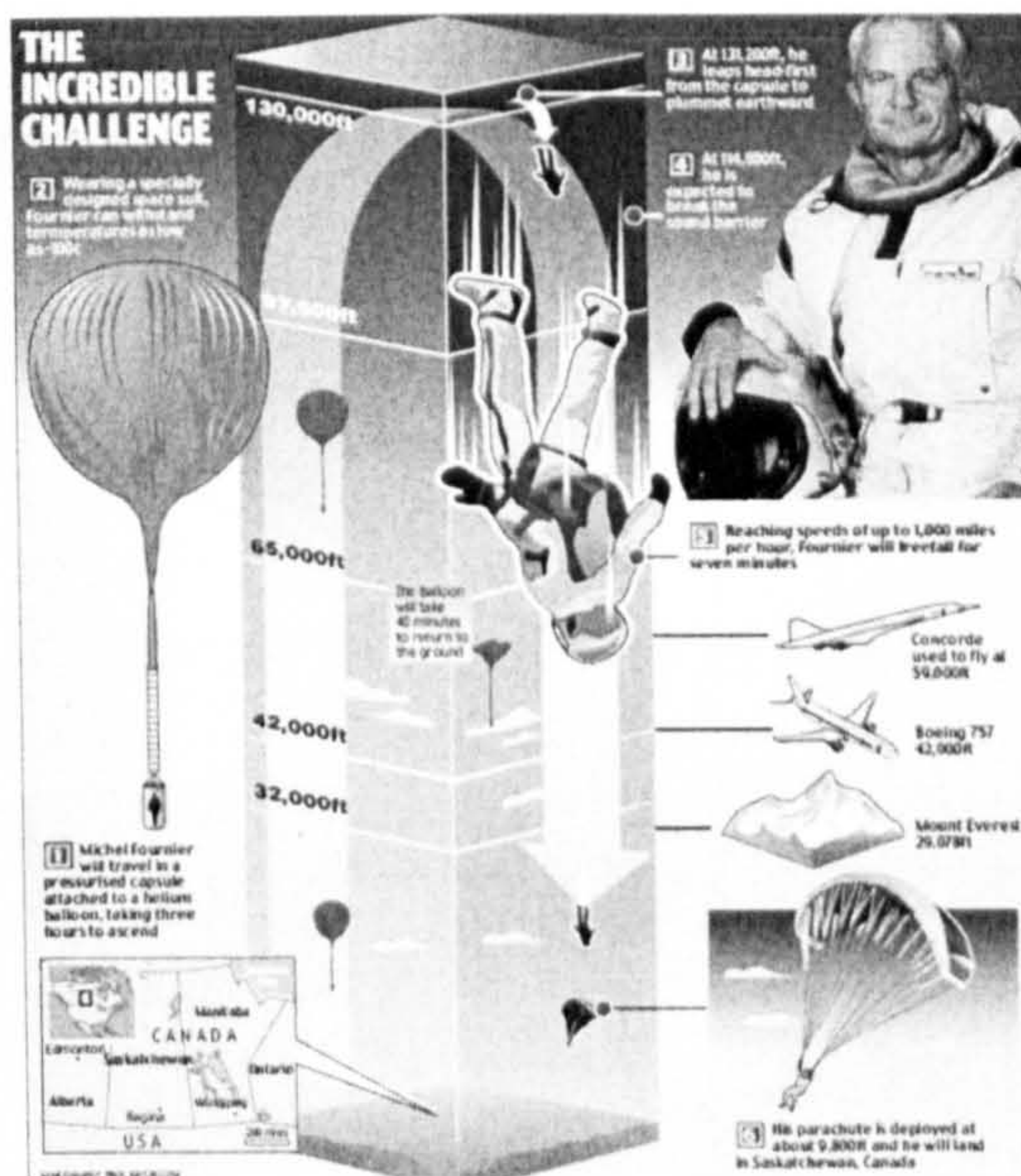


Figure 5.3 Future space tourism- a possible future source of stratospheric samples.

Fortunately there exist opportunities for doing further studies which do not require atmospheric samples. For example, it would be desirable to repeat the “rock cracking study” to confirm that coals, limestone clays, and possibly other rocks, do in fact lack culturable microbes. Similarly, further studies could be conducted on the effects of acids, ozone and UV on bacteria and fungi at levels likely to be found in the stratosphere. Another intriguing possibility is provided by what might be termed “archeapanspermia” that is the transfer of life from Earth in man made materials. A substantial portion of the Earth is covered in large amounts of concrete, building bricks and tarmac. Might these materials act as vehicle for the transfer of microorganisms from Earth in the event of an impact event?

Finally, it goes without saying that confirmation that microorganisms continually reach Earth from space will have a major impact on mankind’s view of the origin and evolution of life.

CHAPTER SIX

REFERENCES

- Abbot, A.: 2001, Resuscitated 'alien' microbes stir up Italian storm, *Nature* **411**, 229.
- Al-Mufti, S., Hoyle, F. and Wickramasinghe, C.: 1984, The survival of *E.coli* under extremely high pressures, in: C. Wickramasinghe (ed.), *Fundamental Studies and the Future of Science*, Cardiff, University of Cardiff Press, pp. 342-352.
- Anon, (2001). Red Rain leaves Indian scientist battling demons. *Nature* **412**, 671.
- Ascaso, C., Wierzchos, J.C., Corral, J., Lopez Raphael, A.J. (2003). New applications of light and electron microscope techniques for the study of microbiological inclusions in amber. *J. Paleontol.* **77**, 1182-1192.
- Bakken, L.R. and Olsen, R.A. (1987) The relationship between cell size and viability of soil bacteria. *Microbial. Ecol.* **13**, 103-114.
- Battista, J.R., Earl, A.M. and Park, M.J.: 1999, Why is *Deinococcus radiodurans* so resistant to ionizing radiation? *Trends in Microbiol.* **7**, 362-365.
- Bechanbach, A.T. (1995). Age of bacteria in amber. *Science* **270**, 2015-2016.
- Bigg, E.K. (1984). Particles in the upper atmosphere. In: *Fundamental Studies and the Future of Science*. (Wickramasinghe, C, Ed.), pp. 38-51. University College Cardiff Press, Cardiff.
- Burch, C.W. (1967) Microbes in the upper atmosphere and beyond. *Symp. Soc. Gen. Microbiol.* **17**, 345-373.
- Burke, V. and Wiley, A.J. (1937). Bacteria in coal. *J. Bacteriol.* **34**, 475-81.
- Cano, R.J., Borucki, M.K. (1997). Ancient Microorganisms, US Patent: 5 593 883.
- Cano, R.J. (2003). The microbiology of amber: a story of persistence. In: Greenblatt C. and Spiegleman, M., Eds. *Emerging Pathogens*, Oxford, University Press, Oxford.

- Cano, R.J. and Borucki, M.K. (1995). Revival and identification of bacterial spores in 25-40 million year old Dominican amber. *Science* **268**, 1060-1064.
- de Hoog, G.S. (1978) Notes on some fungicolous hyphomycetes and their relatives. *Persoonia* **10**, 33-81.
- de Vera, J.R., Horneck, G, Rettberg, P. and Ott, J. (2004). The potential of the lichen symbiosis to cope with the extreme conditions of outer space. *Adv. Space Res.* **33**, 1236-1243.
- Donnes, F.G. (1928), The mystery of life, *Review of Reviews* **76**, 349-354.
- Driks, A (1999) *Bacillus* spore coat. *Microbiol. Mol. Biol. Rev.* **63**, 1-20.
- Farrel, M.A. and Turner, H.G. (1932). Bacteria in anthracite coal. *J. Bacteriol.* **23** 155-162,
- Fish, S.A., Shepherd, T.J., McGenity, T.J. and Grant, W.D.: 2001, Recovery of 16sSrRNA fragments from ancient halite. *Nature*, **2002**, 432-436.
- Fischer, U.R. and Velimoriv, B. (2000). Comparative study of the abundance of various bacterial morphologies in a eutrophic freshwater environment determined by ADC and TEM. *J. Microb. Meth.* **39**, 213-224.
- Folk, R.L. (1993). SEM imaging of bacteria and nanobacteria in carbonate sediments and rocks. *J. Sed. Res.* **63**, 990- 999.
- Fomenkova M.N., Chang, S. and Mukhin, L.M. (1994). Carbonaceous components in the Comet Halley *Geochim. Cosmochim. Acta* **202**, 4503-4512.
- Frederickson, J.K. and Onstott, T.C. (1996). Microbes deep inside the Earth. *Scientific American* **275**, 68-73.

- Fromm, M., Bevilacqua, R., Stocks, B. and Servanckx, R. (2004). New directions - eruptive transport to the stratosphere: add fire-convection to volcanoes. *Atmos. Environ.* **38**, 163-165.
- Graur, D. and Pupko, T. (2001). The Permian bacterium that isn't, *Mol. Biol. Evol.* **18**, 1143-1146.
- Greenblatt, C.L., Davis, A., Clement, B.G., Kitts, C.L., Cox, T and Cano, R.J. (2000). Diversity of microorganisms isolated from amber. *Microbial. Ecol.* **38**, 58-68.
- Gregory, P.H. (1961). *The Microbiology of the Atmosphere*, Leonard Hill, London.
- Gribbin, J. (2001). *Space*, BBC Books, London.
- Griffin, D.W. (2004). Terrestrial microorganisms at an altitude of 20,000 m in Earth's atmosphere. *Aerobiologia* **20**, 135-140.
- Griffin, D.W. (2007). Non-sporing eubacteria isolated from an altitude of 2000m in Earth's atmosphere. *Aerobiologia* **19**, 143-157.
- Hare, F. K. (1962). The Stratosphere. *Geogr. Rev.* **52**, 525-547.
- Harris, M.J., Wickramasinghe, N.C., Lloyd, D., Narlikar, J.V., Rajaratnam, P., Turner, M.P., Al-Mufti, S., Wallis, M.K., Ramadurai, S. and Hoyle, F. (2001). The detection of living cells in stratospheric samples. *Proc. SPIE. Conference.* **4495**, 192-198.
- Horikoshi, K. (1998). Barophiles. *Curr. Op. Microbiol.* **1**, 291-295.
- Hornek, G. (1998) Exobiological experiments in Earth orbit. *Adv. Space Res.* **22**, 317-326.
- Hoyle, F. and Wickramasinghe, N.C.: 1979, *Diseases from Space*, Dent, London.

- Hoyle, F. and Wickramasinghe, N. C., (1981). In: *Comets and the Origin of Life* (ed. C. Ponnamparuma) D. Reidel., Amsterdam.
- Hoyle, F. and Wickramasinghe, C. (1991). *The Theory of Cosmic Grains*, Kluwer Dordrecht.
- Hoyle, F. and Wickramasinghe, N.C. (2000) *Astronomical Origins of Life: Steps towards Panspermia*. Kluwer, Amsterdam.
- Hoyle, F., Wickramasinghe, N.C., Al-Mufti, S., Olavesen, A.H., and Wickramasinghe, D.T., 1982. *Astrophys. Sp. Sci.*, 83, 405.
- Hudson, R.L. and Moore, M.H. and Cooke, A.N. (2005). IR characterization and radiation chemistry of glycaldehyde and ethylene glycol ices. *Adv. Space Res.* 36, 184-189.
- Imshenetsky, A.A., Lysenko, S.V. and Kazakov, G.A. (1978). Upper boundary of the biosphere. *Appl. Environ. Microbiol.* 35, 1-5.
- Joux, F., Jeffrey, W.H., Laberon, P. and Mitchell, D.L. (1999). Marine bacterial isolates display diverse responses to UV-B radiation. *Appl. Environ. Microbiol.* 65, 3820-3827.
- Kawakita, H., (2003). Saturated hydrocarbons in Comet 153P/Ikeya - Zhang: Ethane, Methane, *Astrophys.J.* 590, 573-578.
- Lambert, L.H., Cox, T., Mitchell, K, Rossello-Mora, R., Del Cueto, C., Orkland, P. and Cano, R.J. (1998). *Staphylococcus succinus* sp.nov. isolated from Dominican amber. *Intern. J. Syst. Bacteriol.* 48, 115-118.

- Lillis, T.O. and Bissonette, G.K. (2001). Detection and characterization of filterable heterotrophic bacteria from rural groundwater. *Lett. Appl. Microbiol.* **32**, 268-272.
- Lipman, C.B. (1931) Living microorganisms in ancient rocks. *J. Bacteriol.* **22**, 183-198.
- Louis, G. and Kumar, A.S. (2006). The Red Rain phenomenon of Kerala and its possible extraterrestrial origin, *Astrophys. Space Sc.* **302**, 175-187.
- Maher, B.A. (2002). Uprooting the tree of life. *Scientist* **16**, 26-27.
- McSween, H.Y. (1999). Evidence of life in martian meteorite. *GSA Today*, **7**, 1-7.
- Melosh, H.J. (1999). Swapping rocks: exchange of surface material between planets. *The Planetary Report*, Planetary Society, July, 1999.
- Mumma, M.J. and Reuter, D.C. (1989). On the identification of formaldehyde in Halley's comet. *Astrophys. J.* **344**, 940-948.
- Myers, G.E. and McCready, R.G.L. (1966). Bacteria can penetrate rocks. *Can. J. Microbiol.* **12**, 477-484.
- Nariikar, J.V., Wickramasinghe, N.C., Wainwright, M. and Rajartnam, P. (2003). Detection of microorganisms at high altitudes. *Current Sci.* **85**, 25-30.
- Narlikar, J.V., Ramadurai, S., Bhargava, P., Damle, S.V., Wickramasinghe, N.C., Lloyd, D., Hoyle, F. and Wallis, D.H. (1998) The search for living cells in stratospheric samples. *Proc. SPIE Conf.* **3441**, 301-305.
- Orio, J. and Tornabene, T.: 1965, Bacterial contamination of some carbonaceous meteorites, *Science* **150**, 1046-1048.

- Pasko, V.P., Stanley, M.A., Mathews, J.D., Inan, U.S. and Wood, T.G. (2002). Electrical discharge from thundercloud tops to the lower ionosphere. *Nature* **416**, 152-154.
- Roberts, w.: 1874, Studies on biogenesis, *Proc. Roy. Soc.* **22**, 289-291.
- Rohatschek, H. (1996). Levitation of stratospheric and mesophilic aerosols by gravitophotophoresis. I. *Aerosol Sci.* **27**, 467-475.
- Ross, A. (1998) *Amber*, Natural History Museum, London.
- Roy-Poulson, H. (1981). Meteorites and the origin of water and reduced carbon Earth. *Phys.Ser.* **23**, 1113-1117.
- Sampath, S., Abraham, T.K., Sasi Kumar, V and Mohanan, C.N. (2001). *Coloured Rain: A Report of the Phenomenon*, CESS-PR-114-2001, Centre for Earth Science Studies and Tropical Botanical Garden and Research Institute, Palode, India.
- Shivaji, S., Chaturvedi, P., Suresh, K., Reddy, G.S.N., Dutt, C.B.S. Wainwright, M., Narlikar, J.V. and Bhargava, P.M. (2006). *Bacillus areus* sp.nov etc. isolated from cryogenic tubes used for collecting air samples from high altitudes *Intern. J. System. Evolut. Microbiol.* **56**, 1465-1473.
- Staehelin, J. Harris, N.R.P, Appenzeller, C, Eberhard, J. (2006). Ozone trends: A review. *Rev. Geophys.* **39**, 231-290.
- Tepfer, D. and Leach, S. (2006). Plant seeds as model vectors for the transfer of life through space. *Astrophys. Space Sci.* **306**, 69-75.
- Vreeland, R.H., Rosenzweig, W.D. and Powers, D.W. (2000). Isolation of a 250 million year old halotolerant bacterium from a primary salt crystal, *Nature* **407**, 897-900.

- Wainwright, M, Wickramasinghe, C., Narlikar, J.V., Rajaratnam, P. (2003). Microorganisms cultured from stratospheric air samples obtained at 41 km. *FEMS Microbiol. Lett.* **218**, 161-65.
- Wainwright, M. (2003). A microbiologist looks at Panspermia. *Astrophys. Space Sci.* **285**, 563-570.
- Wainwright, M. and Al Falih, A.M.K. (1997). Fungal growth on buckminsterfullerene, *Microbiology* **143**, 2097-2098.
- Wainwright, M., Barakah, F., Al-Turk, I. and Ali, A.A. (1991). Oligotrophic microorganisms in industry, medicine and the environment. *Sci. Progress* **75**, 313-322.
- Wainwright, M., Wickramasinghe, N.C., Narlikar, J.V. and Rajaratnam, P. (2004). Are these stratospheric nanoparticles bacteria? *Microbiology* **150**, 756-758.
- Wainwright, M. (2001). In praise of Hoyle and Wickramasinghe, *Microbiology Today* **28**.
- Wainwright, M., Weber, P., Smith, J., Hutcheon, I., Klyce, B., Wickramasinghe, C., Narlikar, J. and Rajaratnam, P. (2004). Studies on bacteria-like particles in the stratosphere. *Aerobiologia* **20**, 237-240.
- Wainwright, M. (2007). How do bacteria reach the stratosphere? *Proc. SPIE Conf.* **6694 OT-1**, San Diego.
- Whisler, B.A. (1940). The efficacy of ultra-violet light sources in killing bacteria suspended in air. *Iowa State Coll. J. Sci.* **14**, 215-231.
- Wickramasinghe, C. (2001). *Cosmic Dragons: Life and Death on Our Planet.*: Souvenir Press, London.

Wickramasinghe, C. (2004). The universe: a cryogenic habitat for microbial life.

Cryobiology 48, 113-125.

Wickramasinghe, C. (2005). *A Journey with Fred Hoyle*, World Scientific, London.

Wickramasinghe, C. (2006). *The Spread of Life Throughout the Cosmos*, New York, Springer.

APPENDIX

A. The results of 16SrDNA sequence analysis

CONFIDENTIAL

22nd May 2006

Our Ref: NCSQ 22499
Your Ref: MBB024602

Client: Dr Milton Wainwright
University of Sheffield
Dept of Molecular Biology
& Biotechnology
Firth Court
Western Bank
Sheffield
S10 2TN

Isolate Codes: Sample 1

Date Received: 9.5.06
Date Analysed: 18.5.06

Methodology: 16S rDNA sequence analysis was carried out on these samples using NCIMB Ltd Internal work Instructions; WI-NC-68, 134, 138,147,149 and 191

Identification Summary:

Isolate Code	Identification	% Similarity	Match
Sample 1	<i>Sphingomonas aerolata</i>	99.3	species

Comments:

Searching the MicroSeq database did not give a genus level match and therefore the sequence obtained for the isolate was searched against the EMBL database (Appendix 1).

Responsible Scientist: Vikki Mitchell

Authorized and approved on behalf of NCIMB Ltd: 

This report shall not be reproduced except in full, without the approval of NCIMB Ltd., and applies only to the isolates listed.
Furthermore, nothing in this report shall be taken to imply any endorsement by NCIMB Ltd as to the fitness for purpose of any product to which the report applies.

NCSQ 22499.DOC

Page 1 of 3

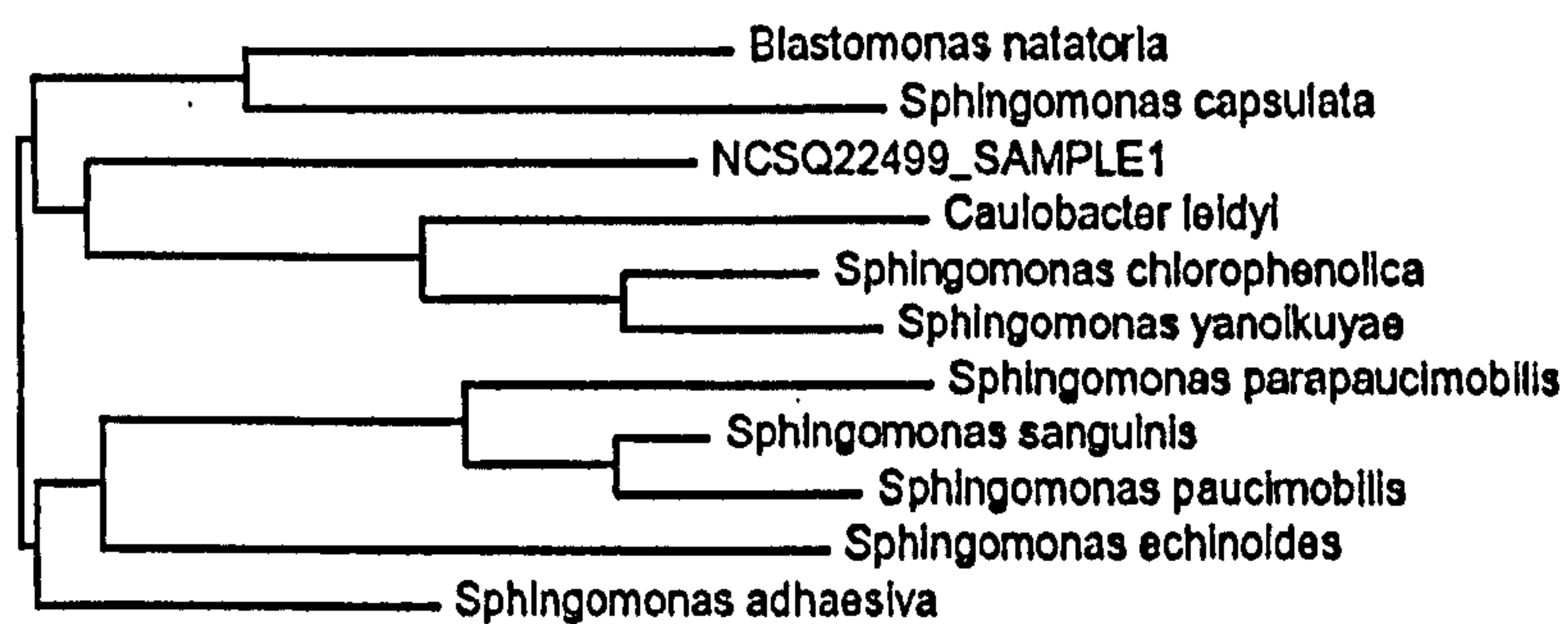
CONFIDENTIAL

Sample 1 TOP 10 HITS MicroSeq™ 500

Sequence Name	% Match	Sequence Name	% Match
<i>Sphingomonas adhaesiva</i>	96.06	<i>Sphingomonas sanguinis</i>	94.44
<i>Sphingomonas chlorophenolica</i>	95.14	<i>Caulobacter leidyi</i>	94.44
<i>Sphingomonas parapaucimobillis</i>	94.91	<i>Sphingomonas echinoides</i>	93.98
<i>Sphingomonas capsulata</i>	94.91	<i>Blastomonas natatoria</i>	93.98
<i>Sphingomonas yanoikuyae</i>	94.68	<i>Sphingomonas paucimobillis</i>	93.29

Phylogenetic Tree

Specimen : NCSQ22499_SAMPLE1
N.Join: 3.0%



CONFIDENTIAL

Appendix 1 Sequence alignment of Isolate Sample 1

EM_PRO:SPH429240 AJ429240.1 *Sphingomonas aerolata* part (1472 nt)
initn: 2083 init1: 1885 opt: 2105 Z-score: 1148.0 bits: 223.3 E(): 3.6e-56
banded Smith-Waterman score: 2105; 99.306% identity (99.306% similar) in 432 nt
overlap (1-432:19-448)

```

                10      20      30      40
SAMPLE          AATGAACGCTGGCGGCATGCCTAACACATGCAAGTCGAACGA
                ::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO AGAGTTATCATGGCTCAGAATGAACGCTGGCGGCATGCCTAACACATGCAAGTCGAACGA
                10      20      30      40      50      60

                50      60      70      80      90      100
SAMPLE AGTCCTTCGGGGCTTAGTGGCGCACGGGTGCGTAACGCGTGGGAATCTGCCCTTGGTTC
                :: ::::: ::: ::::::::::::::::::::::::::::::::::::::::::::
EM_PRO AG-CCTTC-GGGTTTAGTGGCGCACGGGTGCGTAACGCGTGGGAATCTGCCCTTGGTTC
                70      80      90      100     110

                110     120     130     140     150     160
SAMPLE GGAATAACAGTTGAAACGACTGCTAATACCGGATGATGACGTAAGTCCAAAGATTTATC
                ::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO GGAATAACAGTTGAAACGACTGCTAATACCGGATGATGACGTAAGTCCAAAGATTTATC
                120     130     140     150     160     170

                170     180     190     200     210     220
SAMPLE GCCGAGGGATGAGCCCGTAGGATTAGGTAGTTGGTGTGGTAAAGGCGCACCAAGCCGA
                ::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO GCCGAGGGATGAGCCCGTAGGATTAGGTAGTTGGTGTGGTAAAGGCGCACCAAGCCGA
                180     190     200     210     220     230

                230     240     250     260     270     280
SAMPLE CGATCCTTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGAC
                ::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CGATCCTTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGAC
                240     250     260     270     280     290

                290     300     310     320     330     340
SAMPLE TCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAAGCCTGATCCAGCAATG
                ::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO TCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAAGCCTGATCCAGCAATG
                300     310     320     330     340     350

                350     360     370     380     390     400
SAMPLE CCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTACCCGGGATGATAATGACA
                ::::::::::::::::::::::::::::::::::::::::::::::::::::
EM_PRO CCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTACCCGGGATGATAATGACA
                360     370     380     390     400     410

                410     420     430
SAMPLE GTACCGGGAGAATAAGCTCCGGCTAACTCC
                ::::::::::::::::::::::::::::
EM_PRO GTACCGGGAGAATAAGCTCCGGCTAACTCCGTCAGCAGCCGCGTAATACGGAGGGAG
                420     430     440     450     460     470
```


CONFIDENTIAL

20th December 2005

Our Ref: NCSQ 22048
Your Ref: MBB021972

Client: Dr Milton Wainwright
University of Sheffield
Dept of Molecular Biology
& Biotechnology
Firth Court
Western Bank
Sheffield
S10 2TN

Isolate Codes: 37-1

Date Received: 8.12.05

Methodology: 16S rDNA sequence analysis was carried out on these samples using NCIMB Ltd Internal work instructions; WI-NC-68, 134, 147,149 and 191

Identification Summary:

Isolate Code	Identification	% Similarity	Match
37-1	<i>Acinetobacter genomospecies 3</i>	100	species

Comments:

Responsible Scientist: Pauline Littlewood

Authorised and approved on behalf of NCIMB Ltd: *J. Baxter*

This report shall not be reproduced except in full, without the approval of NCIMB Ltd., and applies only to the isolates listed.
Furthermore, nothing in this report shall be taken to imply any endorsement by NCIMB Ltd as to the fitness for purpose of any product to which the report applies.

NCSQ 22048

Page 1 of 2

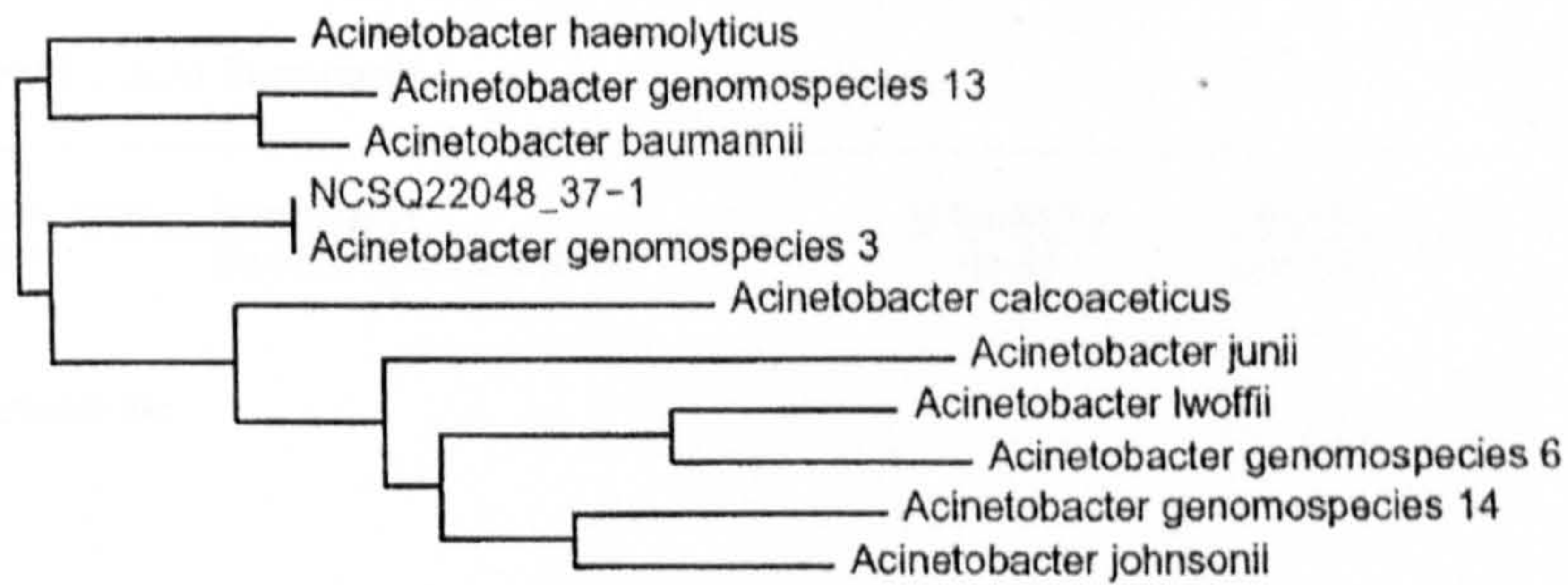
CONFIDENTIAL

NCSQ 22048 TOP 10 HITS MicroSeq™ 500

Sequence Name	% Match	Sequence Name	% Match
<i>Acinetobacter genomospecies 3</i>	100	<i>Acinetobacter johnsonii</i>	95.84
<i>Acinetobacter genomospecies 13</i>	97.92	<i>Acinetobacter genomospecies 14</i>	95.43
<i>Acinetobacter haemolyticus</i>	97.71	<i>Acinetobacter junii</i>	95.43
<i>Acinetobacter baumannii</i>	97.51	<i>Acinetobacter lwoffii</i>	94.8
<i>Acinetobacter calcoaceticus</i>	96.88	<i>Acinetobacter genomospecies 6</i>	94.7

Phylogenetic Tree

Specimen : NCSQ22048_37-1
 N.Join: 2.5%



Concise Alignment

Entry Name	
NCSQ22048_37-1	No Mismatch
<i>Acinetobacter genomospecies 3</i>	No Mismatch

CONFIDENTIAL

30th November 2006

Our Ref: NCSQ 23050
Your Ref: MBB028428

Client: Dr Milton Wainwright
University of Sheffield
Dept of Molecular Biology & Biotechnology
Firth Court
Western Bank
Sheffield
S10 2TN

Isolate Codes: MW1

Date Received: 22.11.06
Date Analysed: 28.11.06

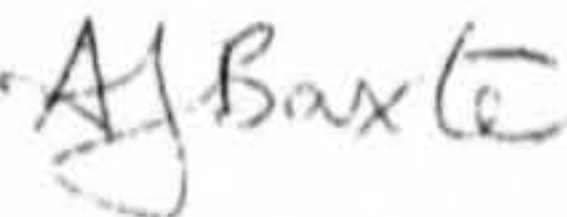
Methodology: 16S rDNA sequence analysis was carried out on these samples using NCIMB Ltd internal work instructions; WI-NC-68, 147, 149 ,191,214 and 215

Identification Summary:

Isolate Code	Identification	% Similarity	Match
MW1	<i>Bacillus subtilis subtilis</i>	99.91	species

Comments:

Responsible Scientist: Vikki Mitchell

Authorised and approved on behalf of NCIMB Ltd: 

This report shall not be reproduced except in full, without the approval of NCIMB Ltd., and applies only to the isolates listed.
Furthermore, nothing in this report shall be taken to imply any endorsement by NCIMB Ltd as to the fitness for purpose of any product to which the report applies.

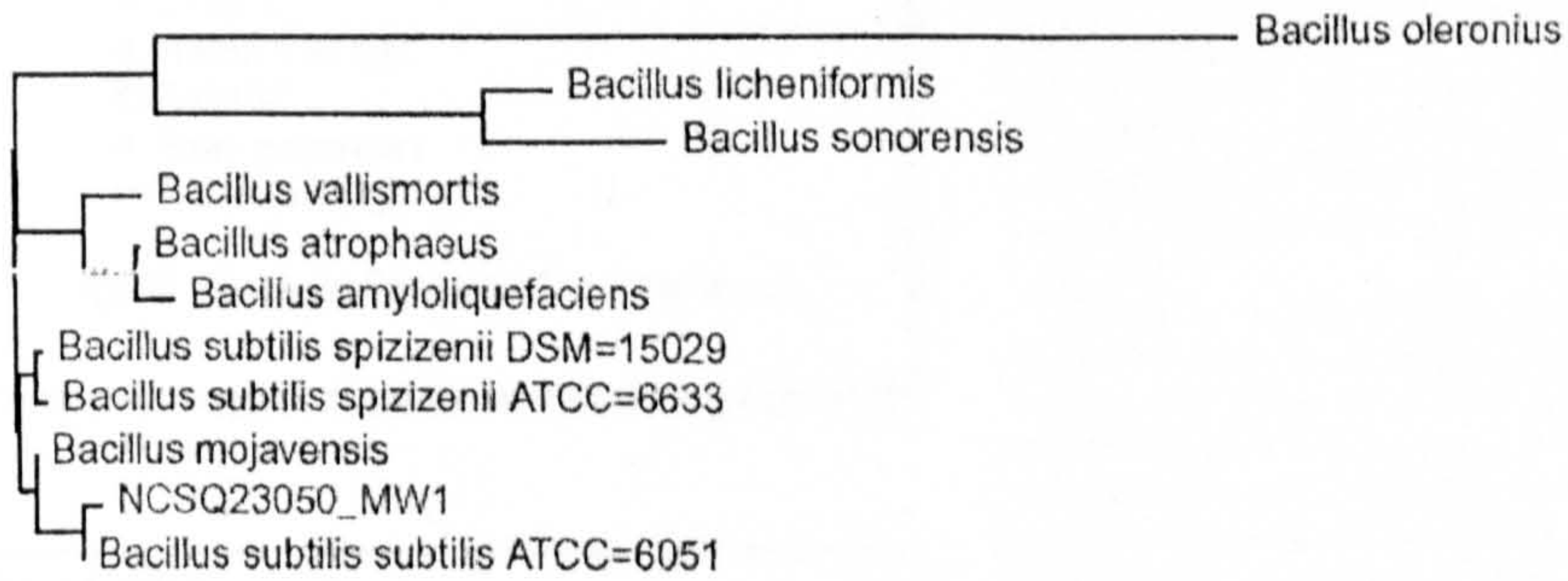
CONFIDENTIAL

MW1 TOP 10 HITS MicroSeq™ 500

Sequence Name	% Match	Sequence Name	% Match
<i>Bacillus subtilis subtilis</i>	99.91	<i>Bacillus atrophaeus</i>	99.18
<i>Bacillus mojavensis</i>	99.68	<i>Bacillus amyloliquefaciens</i>	98.98
<i>Bacillus subtilis spizizenii</i> DSM=15029	99.68	<i>Bacillus licheniformis</i>	97.07
<i>Bacillus subtilis spizizenii</i> ATCC=6633	99.59	<i>Bacillus sonorensis</i>	96.6
<i>Bacillus vallismortis</i>	99.26	<i>Bacillus oleronius</i>	94.1

Phylogenetic Tree

Specimen : NCSQ23050_MW1
N.Join: 2.5%



Concise Alignment

NCSQ23050_MW1

Entry Name	24	43	45	
NCSQ23050_MW1	Y			
<i>Bacillus subtilis subtilis</i> ATCC=6051 99.91	C	A	T	

B. Live/Dead staining



Product Information

Revised: 15-July-2004

LIVE/DEAD® BacLight™ Bacterial Viability Kits

- L7007 LIVE/DEAD® BacLight™ Bacterial Viability Kit *for microscopy*
L7012 LIVE/DEAD® BacLight™ Bacterial Viability Kit *for microscopy and quantitative assays*
L13152 LIVE/DEAD® BacLight™ Bacterial Viability Kit *10 applicator sets*

Quick Facts

Storage upon receipt:

Kits L7007 and L7012

- ≤ -20°C
- Protect from light

Kit L13152

- Room temperature
- Protect from light

Note: Do not use Component C as immersion oil.

Thus, with an appropriate mixture of the SYTO 9 and propidium iodide stains, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. The excitation/emission maxima for these dyes are about 480/500 nm for SYTO 9 stain and 490/635 nm for propidium iodide. The background remains virtually nonfluorescent. Furthermore, although the dye ratios suggested for the LIVE/DEAD BacLight Bacterial Viability Kits have been found to work well with a broad spectrum of bacterial types, these kits also accommodate fine-tuning of the dye combinations so that optimal staining of bacteria can be achieved under a variety of environmental conditions.

A common criterion for bacterial viability is the ability of a bacterium to reproduce in suitable nutrient medium. Exponentially growing cultures of bacteria typically yield results with the LIVE/DEAD BacLight bacterial viability assay that correlate well with growth assays in liquid or solid media. Under certain conditions, however, bacteria having compromised membranes may be able to recover and reproduce - such bacteria may be scored as "dead" in this assay. Conversely, some bacteria with intact membranes may be unable to reproduce in nutrient medium, and yet these may be scored as "alive."⁵

The LIVE/DEAD BacLight Bacterial Viability Kits have been thoroughly tested with a variety of organisms and under several different conditions (see *Bacteria That Have Been Tested*, below). The kits are well suited for use in fluorescence microscopy or for use in quantitative analysis with a fluorometer, fluorescence microplate reader, flow cytometer⁶ or other instrumentation. In our original LIVE/DEAD BacLight Kit (L7007), the dyes are provided mixed at different proportions in two solutions. Kit L7007 is still available for customers who have already developed protocols using that formulation. Kit L7012, however, is more flexible because it provides separate solutions of the SYTO 9 and propidium iodide stains. Having separate staining components facilitates the calibration of bacterial fluorescence for quantitative procedures. For added convenience, our LIVE/DEAD BacLight kit (L13152) contains the separate dyes premeasured into pairs of polyethylene transfer pipets. Besides having the convenience of being packaged in handy applicator pipets, kit L13152 has a formulation that does not require dimethyl sulfoxide (DMSO), nor does it require refrigerated storage.

The LIVE/DEAD BacLight Bacterial Viability Kits are intended as research tools and our Technical Assistance Department welcomes any feedback on the performance of these kits with bacterial strains and environmental conditions not described in this enclosure.

Introduction

Molecular Probes' LIVE/DEAD® BacLight™ Bacterial Viability Kits provide a novel two-color fluorescence assay of bacterial viability that has proven useful for a diverse array of bacterial genera. Conventional direct-count assays of bacterial viability are based on metabolic characteristics or membrane integrity. However, methods relying on metabolic characteristics often only work for a limited subset of bacterial groups,¹ and methods for assessing bacterial membrane integrity commonly have high levels of background fluorescence.² Both types of determinations suffer from being very sensitive to growth and staining conditions.^{3,4} Because of the marked differences in morphology, cytology and physiology among the many bacterial genera, a universally applicable direct-count viability assay has been very difficult to achieve. Our LIVE/DEAD BacLight Bacterial Viability Kits now allow researchers to easily, reliably and quantitatively distinguish live and dead bacteria in minutes, even in a mixed population containing a range of bacterial types.

The LIVE/DEAD BacLight Bacterial Viability Kits utilize mixtures of our SYTO® 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide. These stains differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cells. When used alone, the SYTO 9 stain generally labels all bacteria in a population - those with intact membranes and those with damaged membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present.

MP 07007

LIVE/DEAD® BacLight™ Bacterial Viability Kits

Materials

Kit Contents for Viability Kit, L7007

- SYTO 9 dye, 1.67 mM / Propidium Iodide, 1.67 mM (Component A), 300 μ L solution in DMSO
- SYTO 9 dye, 1.67 mM / Propidium Iodide, 18.3 mM (Component B), 300 μ L solution in DMSO
- BacLight mounting oil (Component C), 10 mL, for bacteria immobilized on membranes. The refractive index at 25°C is 1.517 \pm 0.003. DO NOT USE FOR IMMERSION OIL.

Kit Contents for Viability Kit, L7012

- SYTO 9 dye, 3.34 mM (Component A), 300 μ L solution in DMSO
- Propidium Iodide, 20 mM (Component B), 300 μ L solution in DMSO
- BacLight mounting oil (Component C), 10 mL, for bacteria immobilized on membranes. The refractive index at 25°C is 1.517 \pm 0.003. DO NOT USE FOR IMMERSION OIL.

Note that a 1:1 mixture of Components A and B of kit L7012 is exactly equivalent to a 1:1 mixture of Components A and B of kit L7007.

Kit Contents for Viability Kit, L13152

- SYTO 9 dye (Component A), stabilized as a solid in 10 sealed applicator pipets
- Propidium Iodide (Component B), as a solid in 10 sealed applicator pipets
- BacLight mounting oil (Component C), 10 mL, for bacteria immobilized on membranes. The refractive index at 25°C is 1.517 \pm 0.003. DO NOT USE FOR IMMERSION OIL.

For use of the applicator pipets provided in kit L13152, snip off the sealed ends and dissolve the contents in deionized water, as described in the protocols below.

Number of Tests Possible

At the recommended reagent dilutions and volumes, kits L7007 and L7012 contain sufficient material to perform \geq 1000 individual tests in 96-well assay plates, many more tests by fluorescence microscopy or \sim 200 tests by flow cytometry. In kit L13152, each applicator pair contains sufficient dye to perform 50 individual tests in a 96-well assay plate, \sim 1000 assays by fluorescence microscopy or 10 tests by flow cytometry.

Storage and Handling

For either kit L7007 or L7012, the DMSO stock solutions should be stored frozen at \leq -20°C and protected from light. Allow reagents to warm to room temperature and centrifuge briefly before opening the vials. Before refreezing, seal all vials tightly. When stored properly, these stock solutions are stable for at least one year.

For kit L13152, store at room temperature, protected from light. The new stain formulation is solid phase and is chemically stable when stored at 37°C for more than six months, protected from light. The dissolved dye solutions are stable for up to a year, when stored frozen at \leq -20°C and protected from light.

The BacLight mounting oil may be stored at room temperature, and is stable indefinitely.

Caution: Propidium iodide and SYTO 9 stain bind to nucleic acids. Propidium iodide is a potential mutagen, and we have no

data addressing the mutagenicity or toxicity of the SYTO 9 stain. Both reagents should be used with appropriate care. The DMSO stock solutions should be handled with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues. We strongly recommend using double gloves when handling the DMSO stock solutions. As with all nucleic acid stains, solutions containing these reagents should be poured through activated charcoal before disposal. The charcoal must then be incinerated to destroy the dyes.

Experimental Protocols, General Considerations

The following protocols are provided as examples to guide researchers in the development of their own bacterial staining procedures. Researchers at Molecular Probes have used these procedures and found them to be simple and reliable for both gram-positive and gram-negative bacteria.

Culture Conditions and Preparation of Bacterial Suspensions

Note: Care must be taken to remove traces of growth medium before staining bacteria with these kit reagents. The nucleic acids and other media components can bind the SYTO 9 and propidium iodide dyes in unpredictable ways, resulting in unacceptable variations in staining. A single wash step is usually sufficient to remove significant traces of interfering media components from the bacterial suspension. Phosphate wash buffers are not recommended because they appear to decrease staining efficiency.

1.1 Grow 30 mL cultures of either *Escherichia coli* or *Staphylococcus aureus* to late log phase in nutrient broth (e.g., DIFCO catalog number 0003-01-6).

1.2 Concentrate 25 mL of the bacterial culture by centrifugation at 10,000 \times g for 10-15 minutes.

1.3 Remove the supernatant and resuspend the pellet in 2 mL of 0.85% NaCl or appropriate buffer.

1.4 Add 1 mL of this suspension to each of two 30-40 mL centrifuge tubes containing either 20 mL of 0.85% NaCl or appropriate buffer (for live bacteria) or 20 mL of 70% isopropyl alcohol (for killed bacteria).

1.5 Incubate both samples at room temperature for 1 hour, mixing every 15 minutes.

1.6 Pellet both samples by centrifugation at 10,000 \times g for 10-15 minutes.

1.7 Resuspend the pellets in 20 mL of 0.85% NaCl or appropriate buffer and centrifuge again as in step 1.6.

1.8 Resuspend both pellets in separate tubes with 10 mL of 0.85% NaCl or appropriate buffer each.

1.9 Determine the optical density at 670 nm (OD_{670}) of a 3 mL aliquot of the bacterial suspensions in glass or acrylic absorption cuvettes (1 cm pathlength).

1.10 For suggested concentrations of *E. coli* or *S. aureus* suspensions, please refer to the section appropriate for your

instrumentation: fluorescence microscope, fluorometer, fluorescence microplate reader or flow cytometer.

Bacteria That Have Been Tested

The LIVE/DEAD BacLight Bacterial Viability Kits have been tested at Molecular Probes on the following bacterial species: *Bacillus cereus*, *B. subtilis*, *Clostridium perfringens*, *Escherichia coli*, *Klebsiella pneumoniae*, *Micrococcus luteus*, *Mycobacterium phlei*, *Pseudomonas aeruginosa*, *P. syringae*, *Salmonella oranienburg*, *Serratia marcescens*, *Shigella sonnei*, *Staphylococcus aureus* and *Streptococcus pyogenes*. All of these bacterial types have shown a good correlation between the results obtained with the LIVE/DEAD BacLight Bacterial Viability Kits and those obtained with standard plate counts. These tests were performed on logarithmically growing cultures of organisms. In addition, we have received favorable reports from researchers who have used these kits with: *Agrobacterium tumefaciens*, *Edwardsiella ictaluri*, *Eurioplasma eurylytica*, *Lactobacillus* sp., *Mycoplasma hominis*, *Propionibacterium* sp., *Proteus mirabilis* and *Zymomonas* sp.

Optimization of Staining

The two dye components provided with the LIVE/DEAD BacLight Bacterial Viability Kits have been balanced so that a 1:1 mixture provides good live/dead discrimination in most applications. Occasionally, however, the proportions of the two dyes must be adjusted for optimal discrimination. For example, if green fluorescence is too prominent in the preparation, we suggest that you try either lowering the concentration of SYTO 9 stain (by using less of Component A) or by raising the concentration of propidium iodide (by using more of Component B).

To thoroughly optimize the staining, we recommend experimenting with a range of concentrations of SYTO 9 dye, each in combination with a range of propidium iodide concentrations. In the case of Kits L7007 and L7012, you may wish to try staining 1.0 mL of the bacterial suspension with 3 µL of dye pre-mixed at different Component A Component B ratios. In the case of kit L13152, separate dye solutions can be made by dissolving the contents of one Component A pipet in 2.5 mL filter-sterilized dH₂O and the contents of one Component B pipet in 2.5 mL filter-sterilized dH₂O. These separate solutions can be blended at different ratios, and then the mixtures applied 1:1 with the bacterial suspension.

Fluorescence Microscopy Protocols

Selection of Optical Filters

The fluorescence from both live and dead bacteria may be viewed simultaneously with any standard fluorescein longpass filter set. Alternatively, the live (green fluorescent) and dead (red fluorescent) cells may be viewed separately with fluorescein and Texas Red bandpass filter sets. A summary of the fluorescence microscope filter sets recommended for use with the LIVE/DEAD BacLight Bacterial Viability Kits shown in Table 1.

Staining Bacteria in Suspension with either Kit L7007 or L7012

2.1 Combine equal volumes of Component A and Component B in a microfuge tube, mix thoroughly.

2.2 Add 3 µL of the dye mixture for each mL of the bacterial suspension. When used at the recommended dilutions, the reagent mixture will contribute 0.3% DMSO to the staining solution. Higher DMSO concentrations may adversely affect staining.

2.3 Mix thoroughly and incubate at room temperature in the dark for 15 minutes.

2.4 Trap 5 µL of the stained bacterial suspension between a slide and an 18 mm square coverslip.

2.5 Observe in a fluorescence microscope equipped with any of the filter sets listed in Table 1.

Staining Bacteria in Suspension with Kit L13152

3.1 Prepare a 2X stock solution of the LIVE/DEAD BacLight staining reagent mixture by dissolving the contents of one Component A pipet (containing yellow-orange solids) and one Component B pipet (containing red solids) in a common 5 mL-volume of filter-sterilized dH₂O.

3.2 Combine a sample of the 2X stock solution with an equal volume of the bacterial suspension. The final concentration of each dye will be 6 µM SYTO 9 stain and 30 µM propidium iodide.

Table 1. Characteristics of common filters suitable for use with the LIVE/DEAD BacLight Bacterial Viability Kits.

Omega Filters*	Chroma Filters*	Notes
XF25, XF26, XF115	11001, 41012, 71010	Longpass and dual emission filters useful for simultaneous viewing of SYTO 9 and propidium iodide stains
XF22, XF23	31001, 41001	Bandpass filters for viewing SYTO 9 alone
XF32, XF43 XF102, XF108	31002, 31004 41002, 41004	Bandpass filters for viewing propidium iodide alone

* Catalog numbers for recommended bandpass filter sets for fluorescence microscopy. Omega® filters are supplied by Omega Optical Inc. (www.omegafilters.com). Chroma filters are supplied by Chroma Technology Corp. (www.chroma.com).

3.3 Mix thoroughly and incubate at room temperature in the dark for 15 minutes.

3.4 Trap 5 μ L of the stained bacterial suspension between a slide and an 18 mm square coverslip.

3.5 Observe in a fluorescence microscope equipped with any of the filter sets listed in Table 1

Fluorescence Spectroscopy Protocols

Staining Bacteria with either Kit L7007 or L7012

4.1 Adjust the *E. coli* suspensions (live and killed) to 1×10^8 bacteria/mL (~ 0.03 OD₆₀₀) or the *S. aureus* suspensions (live and killed) to 1×10^7 bacteria/mL (~ 0.15 OD₆₀₀). *S. aureus* suspensions typically should be 10-fold less concentrated than *E. coli* for fluorescence spectroscopy.

4.2 Mix five different proportions of the bacterial suspensions in 1 cm acrylic, glass or quartz fluorescence cuvettes (Table 2). The total volume of each of the five samples will be 3 mL.

4.3 Prepare a combined reagent mixture in a microfuge tube by adding 30 μ L of Component A to 30 μ L of Component B.

4.4 Add 9 μ L of the combined reagent mixture to each of the five samples (5 samples \times 9 μ L = 45 μ L total) and mix thoroughly by pipetting up and down several times.

4.5 Incubate at room temperature in the dark for 15 minutes.

Staining Bacteria with Kit L13152

5.1 Adjust the *E. coli* suspensions (live and killed) to 2×10^8 bacteria/mL (~ 0.06 OD₆₀₀) or the *S. aureus* suspensions (live and killed) to 2×10^7 bacteria/mL (~ 0.30 OD₆₀₀). *S. aureus* suspensions typically should be 10-fold less concentrated than *E. coli* for fluorescence spectroscopy.

Table 2. Volumes of live- and dead-cell suspensions to mix to achieve various proportions of live:dead cells for fluorescence spectroscopy.

Ratio of Live:Dead Cells	mL Live-Cell Suspension	mL Dead-Cell Suspension
0:100	0	3.0
10:90	0.3	2.7
50:50	1.5	1.5
90:10	2.7	0.3
100:0	3.0	0

5.2 Mix five different proportions of the bacterial suspensions in 1 cm acrylic, glass or quartz fluorescence cuvettes (Table 2). Note that when using kit L13152, only one-half of the cell suspension volume (1.5 mL) listed in Table 2 will be used.

5.3 Prepare a 2X working solution of the LIVE/DEAD BacLight staining reagent mixture by dissolving the contents of one

Component A pipet (containing yellow-orange solids) and one Component B pipet (containing red solids) in a common 5 mL-volume of filter-sterilized dH₂O.

5.4 Mix 1.5 mL of the 2X staining reagent mixture with an equal volume (1.5 mL) of each bacterial suspension. Note that, as described above, two applicator sets will be needed (5 samples \times 1.5 mL = 7.5 mL total), however, it may be possible to use smaller volumes.

5.5 Incubate at room temperature in the dark for 15 minutes.

Fluorescence Spectroscopy and Data Analysis

6.1 Measure the fluorescence emission spectrum (excitation 470 nm, emission 490–700 nm) of each cell suspension (F_{cell}) in a fluorescence spectrophotometer (Figure 1a).

6.2 Calculate the ratio of the integrated intensity of the portion of each spectrum between 510–540 nm (em1: green) to that

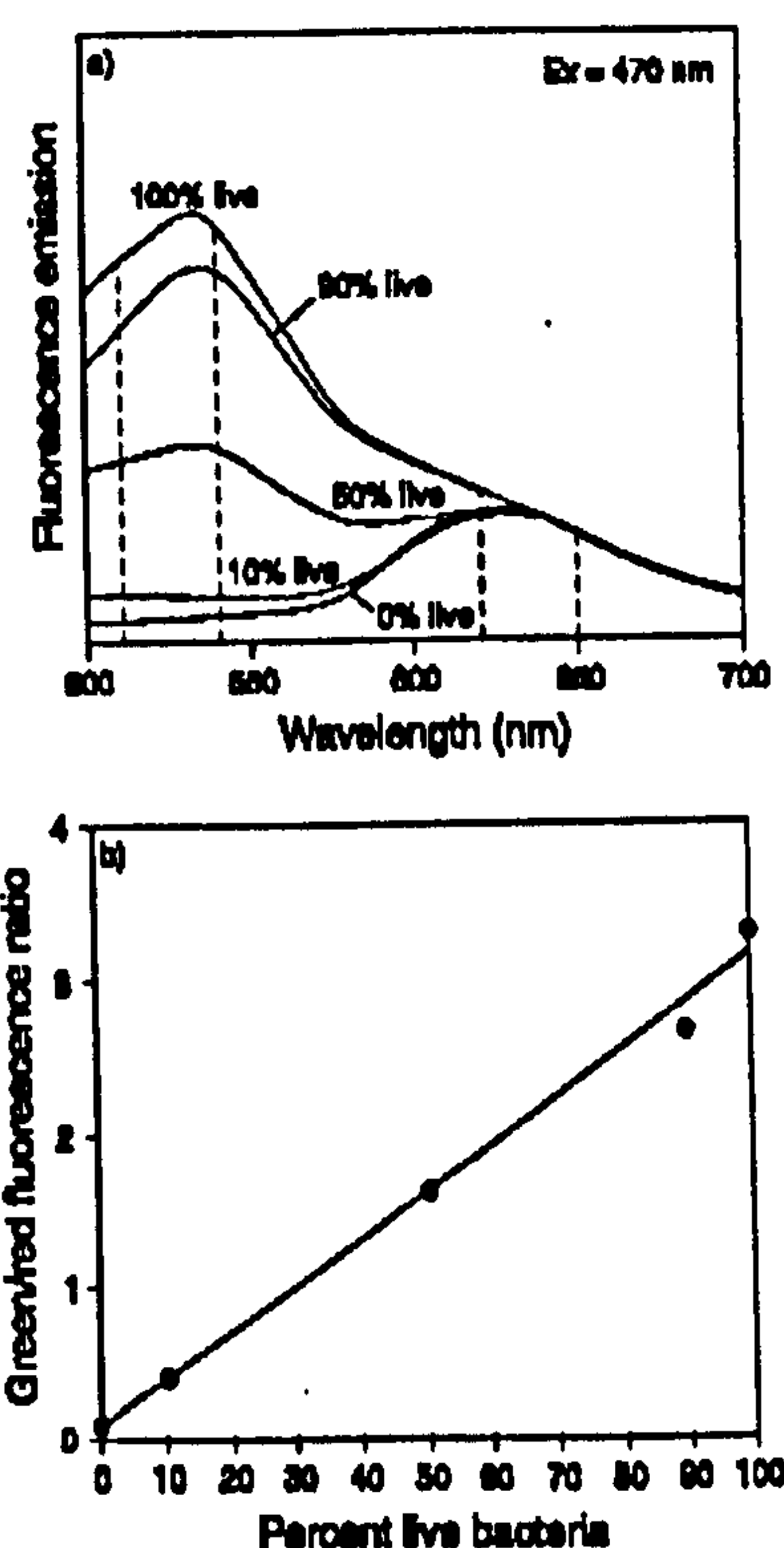


Figure 1. Analysis of relative viability of *E. coli* suspensions by fluorescence spectroscopy. a) Emission spectra of suspensions of various proportions of live and isopropyl alcohol-killed *E. coli* were obtained from samples prepared and stained as outlined in the text. Integrated fluorescence emission intensities were determined from the spectral regions indicated by dashed vertical lines. b) Integrated intensities of the green (510–540 nm) and red (620–650 nm) emission were acquired, and the green/red fluorescence ratios (Ratio_{GR}) were calculated for each proportion of live/dead *E. coli*. The line is a least-squares fit of the relationship between % live bacteria (x) and Ratio_{GR} (y).

between 620–650 (em2, red) for each bacterial suspension.

$$\text{Ratio}_{\text{GR}} = \frac{F_{\text{cell,em1}}}{F_{\text{cell,em2}}}$$

6.3 Plot the ratio of integrated green fluorescence to integrated red fluorescence (R_{GR}) versus percentage of live cells in the *E. coli* suspension (Figure 1b).

Fluorescence Microplate Readers

Conditions required for measurement of fluorescence in microplate readers are very similar to those required for fluorescence spectroscopy of bacterial cell suspensions. As in fluorescence spectroscopy experimental protocols, reagent concentrations are the same as those recommended for fluorescence microscopy, and the ratio of green to red fluorescence emission is proportional to the relative numbers of live bacteria.

Staining Bacterial Suspensions with either Kit L7007 or L7012

7.1 Adjust the *E. coli* suspensions (live and killed) to 2×10^8 bacteria/mL ($\sim 0.06 \text{ OD}_{600}$) or the *S. aureus* suspensions (live and killed) to 2×10^7 bacteria/mL ($\sim 0.30 \text{ OD}_{600}$). *S. aureus* suspensions typically should be 10-fold less concentrated than *E. coli* when using a fluorescence microplate reader.

Table 3. Volumes of live- and dead-cell suspensions to mix to achieve various proportions of live:dead cells for fluorescence microplate readers.

Ratio of Live:Dead Cells	mL Live-Cell Suspension	mL Dead-Cell Suspension
0:100	0	2.0
10:90	0.2	1.8
50:50	1.0	1.0
90:10	1.8	0.2
100:0	2.0	0

7.2 Mix five different proportions of *E. coli* or *S. aureus* (Table 3) in 16×125 mm borosilicate glass culture tubes. The total volume of each of the five samples will be 2 mL.

7.3 Mix 6 μL of Component A with 6 μL of Component B in a microfuge tube.

7.4 Prepare a 2X stain solution by adding the entire 12 μL of the above mixture to 2.0 mL of filter-sterilized dH_2O in a 16×125 mm borosilicate glass culture tube and mix well.

7.5 Pipet 100 μL of each of the bacterial cell suspension mixtures into separate wells of a 96-well flat-bottom microplate. We

recommend that you prepare samples in triplicate. The outside wells (rows A and H and columns 1 and 12) are usually kept empty to avoid spurious readings.

7.6 Using a new tip for each well, pipet 100 μL of the 2X staining solution (from step 7.4) to each well and mix thoroughly by pipetting up and down several times.

7.7 Incubate at room temperature in the dark for 15 minutes.

Staining Bacterial Suspensions with Kit L13152

8.1 Adjust the *E. coli* suspensions (live and killed) to 4×10^8 bacteria/mL ($\sim 12 \text{ OD}_{600}$) or the *S. aureus* suspensions (live and killed) to 4×10^7 bacteria/mL ($\sim 60 \text{ OD}_{600}$). *S. aureus* suspensions typically should be 10-fold less concentrated than *E. coli* when using a fluorescence microplate reader.

8.2 Mix five different proportions of *E. coli* or *S. aureus* (Table 3) in 16×125 mm borosilicate glass culture tubes.

8.3 Prepare a 2X working solution of the LIVE/DEAD BacLight staining reagent mixture by dissolving the contents of one Component A pipet (containing yellow-orange solids) and one Component B pipet (containing red solids) in a common 5 mL volume of filter-sterilized dH_2O .

8.4 Pipet 100 μL of each of the bacterial cell suspension mixtures into separate wells of a 96-well flat-bottom microplate. We recommend that you prepare samples in triplicate. The outside wells (rows A and H and columns 1 and 12) are usually kept empty to avoid spurious readings.

8.5 Using a new tip for each well, pipet 100 μL of the 2X working stain solution (from step 8.3) to each well and mix thoroughly by pipetting up and down several times.

8.6 Incubate the sample at room temperature in the dark for 15 minutes.

Fluorescence Measurement and Data Analysis

9.1 With the excitation wavelength centered at about 485 nm, measure the fluorescence intensity at a wavelength centered at about 530 nm (emission 1; green) for each well of the entire plate.

9.2 With the excitation wavelength still centered at about 485 nm, measure the fluorescence intensity at a wavelength centered about 630 nm (emission 2; red) for each well of the entire plate.

9.3 Analyze the data by dividing the fluorescence intensity of the stained bacterial suspensions (F_{em1}) at emission 1 by the fluorescence intensity at emission 2.

$$\text{Ratio}_{\text{GR}} = \frac{F_{\text{cell,em1}}}{F_{\text{cell,em2}}}$$

9.4 Plot the Ratio_{GR} versus percentage of live cells in the *E. coli* suspension (Figure 2).

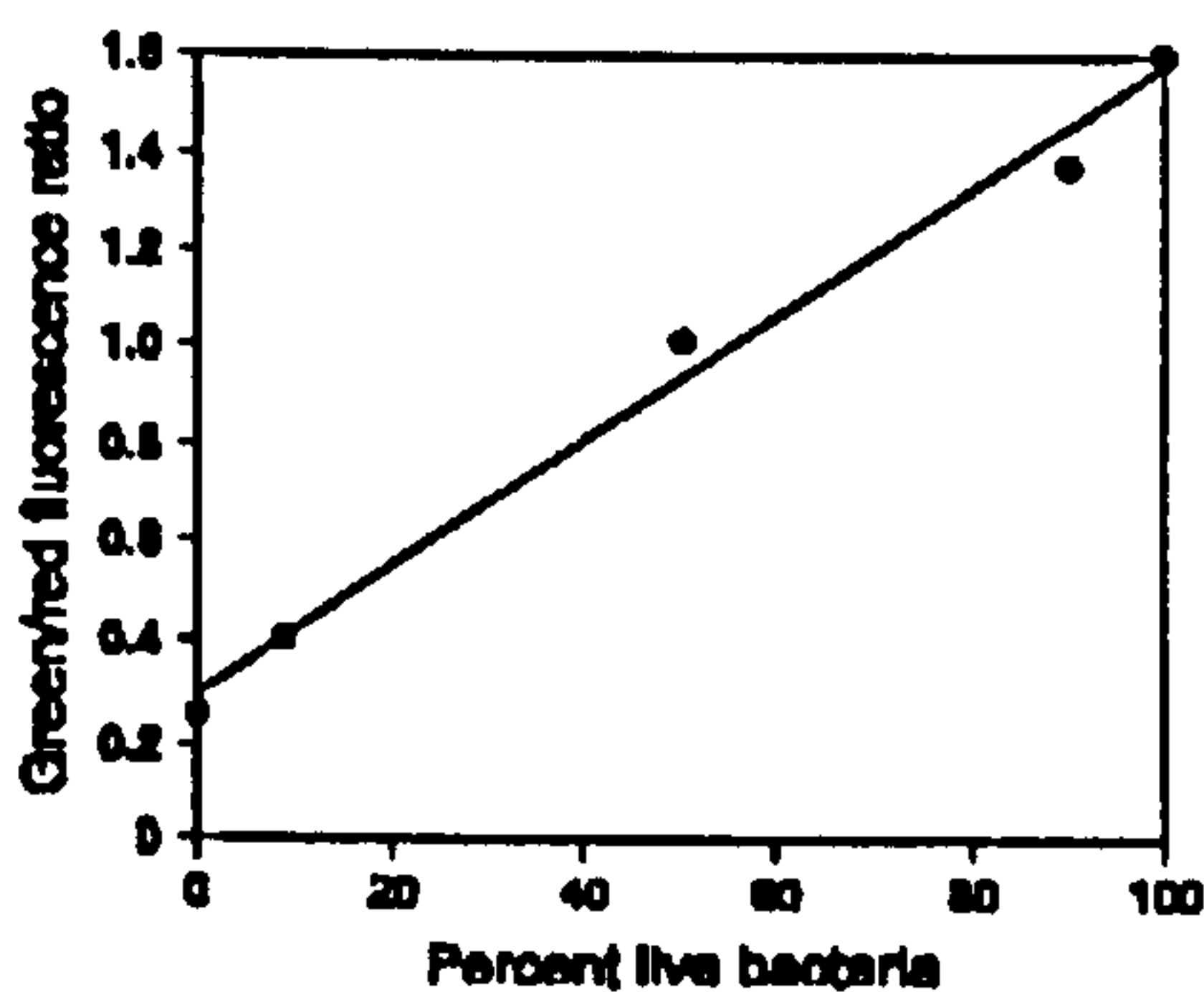


Figure 2. Analysis of relative viability of *E. coli* suspensions in a fluorescence microplate reader. Samples of *E. coli* were prepared and stained as outlined in the text. The integrated intensities of the green (530 ± 12.5 nm) and red (620 ± 20 nm) emission of suspensions excited at 485 ± 10 nm were acquired, and the green/red fluorescence ratios ($Ratio_{GR}$) were calculated for each proportion of live/dead *E. coli*. Each point represents the mean of ten measurements. The line is a least-squares fit of the relationship between % live bacteria (x) and $Ratio_{GR}$ (y).

Flow Cytometry

Instrument capabilities may vary considerably but the techniques and parameters established here should aid considerably in setting up similar analyses in the majority of flow cytometers now in use, both in research and clinical environments.

Table 4. Volumes of live- and dead-cell suspensions to mix to achieve various proportions of live:dead cells for flow cytometry.

Ratio of Live:Dead Cells	mL Live-Cell Suspension	mL Dead-Cell Suspension
0:100	0	2.0
10:90	0.2	1.8
20:80	0.4	1.6
30:70	0.6	1.4
40:60	0.8	1.2
50:50	1.0	1.0
60:40	1.2	0.8
70:30	1.4	0.6
80:20	1.6	0.4
90:10	1.8	0.2
100:0	2.0	0

Staining Bacterial Suspensions with either Kit L7007 or L7012

10.1 Adjust the *E. coli* suspensions (live and killed) to 1×10^8 bacteria/mL (~ 0.03 OD₆₀₀), then dilute them 1:100 in filter-sterilized dH₂O to reach a final density of 1×10^6 bacteria/mL.

10.2 Mix 11 different proportions of *E. coli* in 16×125 mm borosilicate glass tubes according to Table 4. The volume of each of the 11 samples will be 2 mL.

10.3 Mix 35 μ L of Component A with 35 μ L of Component B in a microfuge tube. If Kit L7012 is used, it may be desirable to prepare additional bacterial samples for staining with Component A alone and with Component B alone.

10.4 Add 6 μ L of the combined reagent mixture to each of the 11 samples (11 samples \times 6 μ L = 66 μ L total) and mix thoroughly by pipetting up and down several times.

10.5 Incubate at room temperature in the dark for 15 minutes.

Staining Bacterial Suspensions with Kit L13152

11.1 Adjust the *E. coli* suspensions (live and killed) to 1×10^8 bacteria/mL (~ 0.03 OD₆₀₀), then dilute them 1:50 in filter-sterilized dH₂O to reach a final density of 2×10^6 bacteria/mL.

11.2 Mix 11 different proportions of *E. coli* in 16×125 mm borosilicate glass tubes according to Table 4. Note that when using kit L13152, only one-half of the cell suspension volume (1.0 mL) listed in Table 3 will be used.

11.3 Prepare a 2X working solution of the LIVE/DEAD BacLight staining reagent mixture by dissolving the contents of one Component A pipet (containing yellow-orange solids) and one Component B pipet (containing red solids) in a common 5 mL-volume of filter-sterilized dH₂O. It may be desirable to prepare additional bacterial samples for staining with Component A alone (dissolved in 5 mL of filter-sterilized dH₂O) and with Component B alone (dissolved in 5 mL of filter-sterilized dH₂O).

11.4 Mix 1 mL of the 2X working solution of the LIVE/DEAD BacLight staining reagent mixture with an equal volume (1 mL) of the bacterial suspension. Note that, as described above, three applicator sets will be needed (11 samples \times 1 mL = 11 mL total); however it may be possible to use smaller volumes.

11.5 Incubate the sample at room temperature in the dark for 15 minutes.

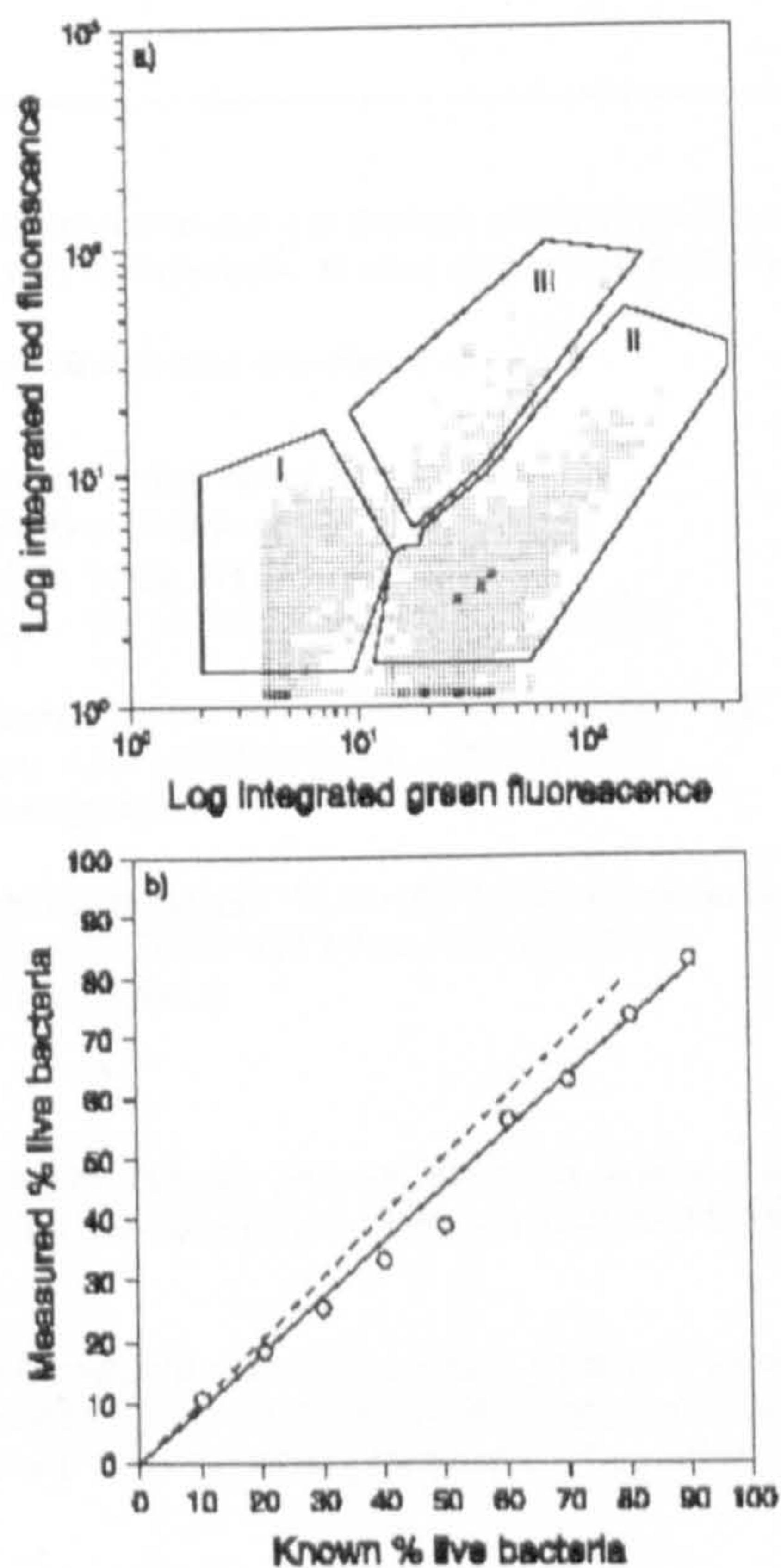
Instrument Parameters

The data shown in this example were acquired with a Coulter EPIC S V™ flow cytometer equipped with an argon-ion laser at 488 nm and 400 mW output. Data acquisition and analysis were controlled using Cytomation CICERO software and a hardware interface. The emission light path contained a 515 nm blocking filter, 590 nm dichroic filter before the Green PMT and a 610 nm absorbance filter before the Red PMT. The density of the *E. coli* bacterial suspension was 1×10^6 cells/mL and the sampling rate was ~300 particles/sec. The sheath fluid was distilled water and the flow tip was a 76 µm air tip.

Fluorescence Measurements and Data Analysis

Because both live and dead cells exhibit green fluorescence, the signal discriminator was set at 15% of the log-integrated green fluorescence (LIGFL) to eliminate debris. Populations of bacteria were discriminated as three regions of the log-integrated red fluorescence (LIRFL) versus LIGFL plot (Figure 3a), and the numbers of bacteria found within these regions were used to estimate the percentage of viable organisms in the population (Figure 3b).

Figure 3. Analysis of relative viability of *E. coli* suspensions by flow cytometry. Samples of *E. coli* were prepared, stained and analyzed as outlined in the text. a) A two-parameter comparison of the green and red components of fluorescence emission of individual bacteria from a population containing 70% "killed" organisms indicates two major regions (I & II) and one minor region (III). The majority of the bacteria are represented by region I (dead cells) and region II (live cells), which have similar red fluorescence intensity and different proportions of green fluorescence. *E. coli* organisms appearing in region III generally represent less than 5% of the population and are as yet uncharacterized in terms of viability. b) Known viability is defined as the proportion of "live" to "killed" bacteria. Measured viability is defined by the following equation: Measured % live bacteria = (# of bacteria in region II/# of bacteria in regions I+II) × 100. A least-squares fit extrapolated to the 100% "live" point suggested a 13% component of dead bacteria in the "live" population. The upper (dashed) line is corrected for a 13% dead component in the "live" cell suspension.



References

1. J Appl Bacteriol 72, 410 (1992); 2. Lett Appl Microbiol 13, 58 (1991); 3. Curr Microbiol 4, 321 (1980); 4. J Microbiol Methods 13, 87 (1991); 5. Microbiol Rev 51, 365 (1987); 6. J Med Microbiol 39, 147 (1993).

Product List Current prices may be obtained from our Web site or from our Customer Service Department.

Cat #	Product Name	Unit Size
L7007	LIVE/DEAD® BacLight™ Bacterial Viability Kit *for microscopy* *1 000 assays*	1 kit
L7012	LIVE/DEAD® BacLight™ Bacterial Viability Kit *for microscopy and quantitative assays* *1000 assays*	1 kit
L13152	LIVE/DEAD® BacLight™ Bacterial Viability Kit *10 applicator sets*	1 kit

Contact Information

Further information on Molecular Probes products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Leiden, the Netherlands. All others should contact our Technical Assistance Department in Eugene, Oregon.

Please visit our Web site — www.probes.com — for the most up-to-date information

Molecular Probes, Inc.

29851 Willow Creek Road, Eugene, OR 97402
Phone: (541) 465-8300 • Fax: (541) 335-0504

Customer Service: 6:00 am to 4:30 pm (Pacific Time)
Phone: (541) 335-0338 • Fax: (541) 335-0305 • order@probes.com

Toll-Free Ordering for USA and Canada:
Order Phone: (800) 438-2209 • Order Fax: (800) 438-0228

Technical Assistance: 8:00 am to 4:00 pm (Pacific Time)
Phone: (541) 335-0353 • Toll-Free: (800) 438-2209
Fax: (541) 335-0238 • tech@probes.com

Molecular Probes Europe BV

Poortgebouw, Rijnsburgerweg 10
2333 AA Leiden, The Netherlands
Phone: +31-71-5233378 • Fax: +31-71-5233419

Customer Service: 9:00 to 16:30 (Central European Time)
Phone: +31-71-5236850 • Fax: +31-71-5233419
eurorder@probes.nl

Technical Assistance: 9:00 to 16:30 (Central European Time)
Phone: +31-71-5233431 • Fax: +31-71-5241883
eurotech@probes.nl

Molecular Probes products are high-quality reagents and materials intended for research purposes only. These products must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Please read the Material Safety Data Sheet provided for each product; other regulatory considerations may apply.

Several Molecular Probes products and product applications are covered by U.S. and foreign patents and patents pending. Our products are not available for resale or other commercial uses without a specific agreement from Molecular Probes, Inc. We welcome inquiries about licensing the use of our dyes, trademarks or technologies. Please submit inquiries by e-mail to busdev@probes.com. All names containing the designation [®] are registered with the U.S. Patent and Trademark Office.

Copyright 2004, Molecular Probes, Inc. All rights reserved. This information is subject to change without notice.