SOME ASPECTS OF THE COLONISATION AND DECAY OF FUNGICIDALLY-PROTECTED COTTON TEXTILES BY SOIL FUNGI

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A thesis submitted to the

UNIVERSITY OF ASTON IN BIRMINGHAM

for the Degree of DOCTOR OF PHILOSOPHY

APRIL 1973

SUMMARY

Problems associated with the biodeterioration of cotton, including testing and economic aspects are considered. The studies focus upon the use of pentachlorophenyl-laurate (PCPL) fungicides, which are among those most widely used for textile protection.

Aspects of some major types of techniques used in the study of fungal ecology in relation to textile testing are discussed and experimental data obtained by their use is presented. A modified soil burial technique is demonstrated.

Further ecological studies centre upon the isolation of fungi from PCPL-protected cellulosic materials; agar, perfusion and soil burial techniques being employed.

Studies of the fungal colonisation and associated strength losses of cotton textile are described.

An account of the development and use of a multiple perfusion technique for the rapid initial screening of fungicides is given, together with details of its use in the study of the decay of artificially-weathered fungicidally-protected textiles.

The development of a technique combining perfusion and thin-layer chromatography for the study of fungicides is described, together with its use in an investigation of the effects of soil organisms on PCPL and the effects of heat on the efficacy of this fungicide.

The results of investigations into aspects of fungal growth and physiology in relation to test work using PCPL are given, special mention being made of effects of pH and strain differences on Trichoderma viride. Results of respirometric investigations into the effects of low levels of PCPL-based fungicides on soil organisms are set out and discussed.

Details are given of the development and employment of a simple strength-testing technique for use with perfused textile samples.

The colonisation and decay of cotton textiles by soil fungi, using different soils and varying pH is described, as is a technique designed for these studies.

To my wife, Christine and

my son, David Neville

ACKNOWLEDGEMENTS

Of the many people who have helped me in my work, I wish especially to thank the following:-

Dr. H.O.W. Eggins, for his advice, encouragement and friendship during this project.

The Directors of Catomance Limited, for their financial support and continuing interest; and individuals within that company, namely Mr. J. David, Mr. A.R.M. Barr and Mr. A.O. Lloyd, who have assisted and encouraged me in many ways.

The Staff of the Biodeterioration Centre, Department of Biological Sciences, The University of Aston in Birmingham, for their help and friendship over the years.

Dr. H.J. Hueck and his staff at Centraal Laboratorium T.N.O., Delft, the Netherlands, for their assistance and hospitality during my stay with them in 1969.

Miss Ann Lee, for typing this thesis and tolerating me and my spelling for so long.

D. Allsopp
University of Aston in Birmingham
April 1973

C O N T E N T S

Chapter 1

INTRODUCTION

INTRODUCTION

1.1. Introduction - The nature of the problem

It is well known that cotton fibres and materials manufactured from cotton, such as textiles, are susceptible to attack by fungi, with large consequential economic loss, (Marsh and Bollenbacker, 1949; Betrabet et. al. 1968; Ranganathan and Agarwal, 1969). Propagules of fungi may be present in the air, (Gregory, 1945; Hirst, 1953) or in more active states in the soil, (Waksman, 1916; Thornton, 1956; Burges, 1958; Hawker et. al., 1960; Garret, 1963). The active and varied nature of soil fungi has led to the widespread use of soil as the main environment in which to investigate the activities of fungi capable of decaying cotton and other cellulosic materials. In practice, soil burial is widely used as a severe test of the resistance of protected cotton textiles to fungal attack; the results of such tests are thought to correlate well with the life of the textile in service. (Waksman and Skinner, 1926; Bertolet, 1944; Skinner and Mellem, 1944; Dean et. al., 1960; Hueck and van der Toorn, 1965). In the prevention of biodeterioration of cotton and other materials by chemical means, a wide range of fungicides may be used, (Hueck-van der Plas, 1966) and in the testing of such protected materials, a wide range of factors may be investigated, (Hueck-van der Plas, 1965a).

The following work was therefore performed in order to study further some of those aspects of the colonisation and decay of cotton materials, by soil fungi, which are of

interest in the fields of materials protection by chemical agencies and of the testing of such protected materials.

1.2. Cotton, a textile raw material

Cotton, together with flax, hemp, jute and other fibres, is a cellulosic material of vegetable origin. It is a seed fibre from the seed pods of a species of plants contained within the genus Gossypium, perennial plants now mostly grown as annuals in cultivation. Each cotton fibre is a seed-hair, growing from the seed epidermis, each seed producing up to 20,000 fibres, the boll containing around 150,000 fibres or more. During its growth and ripening each fibre elongates, becoming a thin-walled cellulose tube with a length equal to 2,000 times its diameter.

1.2.1. The cotton fibre

At the end of its elongation period the cotton fibre develops a more complex internal structure. Layers of cellulose are added to the internal walls, a separate layer each day, each layer comprising two sub-layers, one compact and one porous. These layers are composed of spirally arranged fibrils. As the ripe boll splits, mutual pressures between fibres is released, the fibres dry, the cell walls collapse and the fibres flatten, twisting also due to the spiral arrangement of fibrils. From this it can be seen, that a finished cotton textile presents a large surface area for the absorption of water, nutrients and penetration by deteriogenic fungi, due to its fine fibrous structure, inherent from the nature of the raw material.

1.2.2. The cotton crop

With improvements in the cleaning of mechanically-harvested cotton, most of the world crop is now gathered by machine. Today, the cultivation of cotton is a major industry in sixty countires, and varieties of plants have been developed for a range of climatic conditions. Cotton plants generally require a growing period of six months, with high moisture and sunshine, followed by a dry sunny maturing period. These conditions are found generally between latitudes of 40^{o} north and south of the equator. Over 10 million tons of cotton are grown every year, the main countries being the U.S.A., U.S.S.R., China, India, Egypt, Mexico, Brazil, Pakistan, Turkey, Peru and Argentina. The U.S.A. produces almost one-third of the total crop.

On a weight basis, raw cotton accounts for 70% of all main plant fibres produced in the world, and 60% of all main natural fibres including wool. On a cost basis, the raw cotton accounts for 80% of the total value of all main plant fibres produced in the world, and 56% of all main natural fibres including wool. (Hueck-van der Plas, 1971).

1.2.3. Cotton in economic history

Some important stages in the development of the world cotton trade are shown in figure 1, the important stages in the development of the cotton manufacturing industry in Britain have been compiled and listed in table 1. (Cook, 1968).

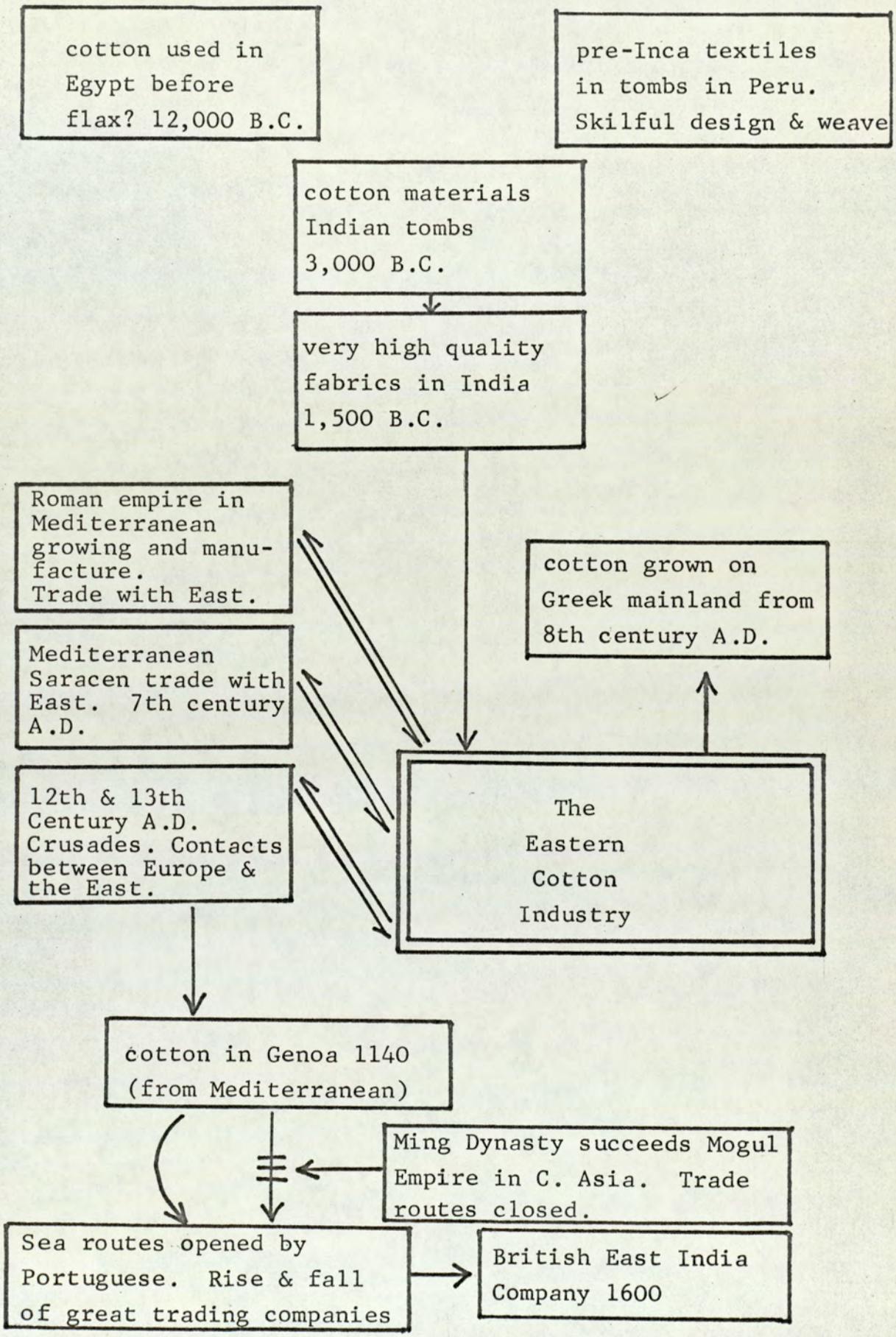


Figure 1

Some important developments in the world cotton trade.

Event	Date
Protestant artisans from Continent bring textile skills to Britain. Britain becomes an established exporter of cotton fabrics	 End of 17th Century
Abolition of Manchester act of 1700 prohibiting sale of cotton in England	1736
"Industrial Revolution". Most raw cotton from West Indies (+ India, Levant and Brazil)	Latter half of 18th Century
Cotton gin invented, removes seed from fibres. America enters market as a producer.	1793
America exports 18 million pounds of cotton to Britain, rising to 62 million pounds	1800 1811
Lancashire as cotton manufacturing centre of the world	1811-1914
Lancashire exports 7000 million yards of cotton fabric. World production of raw material 10,611 million lb.	1913

Table 1. Some major events in the cotton manufacturing industry in Britain, up to the peak of exports in 1913.

1.2.4. Historical note on the decay of textiles

The degradable nature of cellulosic textiles generally is clearly indicated by the few samples of such materials that exist from historical times, any surviving specimens usually being from tombs (see figure 1). A particularly clear reference to the fungal decay of textiles, with instructions for action are given in Leviticus (Leviticus 13: 47-49, New English Bible).

> "Where there is a stain of mould, whether in a garment of wool or linen, or in the warp or weft of linen or wool, or in a skin or anything made of skin, if the stain is greenish or reddish in the garment or skin, or in the warp or weft, or in anything made of skin; it is a stain of mould which must be shown to the priest."

The text then describes the methods for ascertaining the viability of the infection, and advocates incineration of materials colonised by viable deteriogens.

It is apparent from the notes in table 1 that the cotton manufacturing industry was one of the main developments during the so-called 'industrial revolution' in Britain. The manufacture of cotton goods in Britain for export reached its peak around 1913, when the value of cotton exports was in the region of £130 x 10^6, compared with more recent times (1965) when the value of exports was around £29 x 10^6 (H.M.S.O. 1971a). This decline in exports is due to changing fashions in clothing, the development of the cotton industry in other countries (notably in Asia) and the advent of synthetic fibres.

On a world-wide basis, however, the production of raw cotton has increased, 10,611 million lb. of cotton being produced in 1913 (Robson,1957), compared with approximately 25,000 million lb. in 1968 (Hueck-van der Plas, 1971.). Using British figures of inflation and unit price increases for cotton goods as a guideline, during the period 1913-1965, the unit price of cotton goods has increased tenfold, and the value of money has decreased to one fifth. Thus the unit value of cotton has roughly doubled in real terms over this period; any eventual losses due to biodeterioration also being increased by this amount. On a global scale, the eventual losses could also be multiplied by a factor of 2.5 if the increase in world production over this period were taken into account.

1.3. Biodeterioration of cotton

Hueck, (1965), has defined biodeterioration as "any undesirable change in the properties of a material caused by the vital activities of organisms".

Several types of biodeterioration phenomena may be distinguished in relation to cotton textiles:-

(a) Fouling or soiling

In this instance it is the presence of the organism, its body or excreta which give rise to the objection, rather than any actual decay of the material. In many cases it is in fact aesthetic deterioration of the material. An example of this type of deterioration in the field of cotton textiles, (where this phenomenon is usually associated with other decay phenomena) is the occurrence of dark fungal colonies on the surface of the material, together with stains due to the release of pigments (Anon, 1948). It is by this phenomenon that fungal attack is usually first recognised, (Ranganathan and Agarwal, 1969).

(b) Mechanical processes

Physical damage to materials by organisms not using them as a nutrient source falls within this category. There are no examples of this type of damage to textiles by fungi, but an example of physical damage caused by other types of organisms is that of damage to sacking caused by the gnawing activities of rats and mice. (Drummond, 1971).

(c) Chemical assimilatory deterioration

Here the material is used as a nutrient source by the deteriogenic organisms. The main factor in the decay of textiles, that of loss of strength due to the influences of cellulolytic enzymes of fungi, serves as an example of this extremely important type of biodeterioration. (Siu, 1951., Anon, 1949.).

It should be noted that more than one biodeterioration phenomenon (type of attack) often occurs when an organism attacks a material; it is quite possible for several or all types to occur together to greater or lesser degrees.

The categories of textiles and allied products (such as rope and cordage) which are most liable to decay are those which by nature of their structure and use tend to collect moisture and ancillary nutrients in the form of dirt during use. Examples are tentage, outdoor canvas covers, agricultural twines, canvas military equipment and cotton clothing (especially in service use in the tropics) and any form of cellulosic textiles held in poor storage conditions, (Siu, 1951., Ranganathan and Agarwal, 1969). Clothing which is in frequent use in temperate climates tends not to be prone to biodeterioration, as conditions under which fungi would have to grow change often in the washing, drying and wearing cycle.

1.4. The Economics of biodeterioration

1.4.1. Recognition of biodeterioration and costing criteria

Before any costs can be attributed to any form of biodeterioration, it is first necessary for biodeterioration to be recognised as such, and the effects delimited from the effects of chemical and physical agencies, i.e. corrosion and wear.

Some examples may be clear; the replacement of building timbers due to dry-rot attack on the originals can clearly be identified and costed as a case of biodeterioration. Spillage of grain from sacks weakened by gnawing rats may however be merely listed as 'spillage' or 'wastage'. The situation becomes even worse where microorganisms are involved in depth, as in the 'going bad' or 'going off' of materials considered to be traditionally and inevitably degradable, as shown by the term 'inherent vice', a legalistic term used in cases of deterioration of stored foodstuffs.

Assuming that these problems of the recognition of biodeterioration are able to be solved, criteria for assesment of the cost of attack must still be established. One, or a combination of the following points may be chosen. There is no standardised procedure in practice.

(a) The cost of prevention of such attacks

This may include rot-proofing, drying, cooling, sealing or sterilising the material.

(b) The cost of replacement

This is most applicable with low-cost materials.

(c) The cost of remedial treatment

Remedial treatment is almost always difficult and expensive in biodeterioration cases. The best examples of true remedial treatment, as opposed to partial replacement or prevention of further attack are to be found in restored and preserved art objects and museum specimens, i.e. costly and unique items.

The lack of cost statistics from fields such as wood preservation highlights the problem of encouraging those who are able to recognise biodeterioration to take an interest in the economic aspects. It is even more difficult where the problem is not often even recognised as biodeterioration, for example the spotting of paint films by dark-sporing fungi. There are more problems to be considered, one is the natural reluctance of manufacturers to admit to any biological problem, especially in the food and cosmetics trade, and the disbelief that anything so small as microorganisms can cause such damage (Eggins, 1967).

In the tropics, where biodeterioration is most severe it is often disregarded, as many developing countries have more immediate and pressing problems to solve. The unpredictability of losses has a nuisance value which is again difficult to cost, i.e. it is difficult to cost consumer confidence.

One way of estimating the cost of biodeterioration is to consider the value of materials which are susceptible to decay. Possibly the most quoted figures to date were given by Hueck-van der Plas in 1965(b). These figures have since been up-dated by this author and are shown in Table 2, Hueck-van der Plas (1971). Table 3 shows figures by this same author on the increases in value of materials due to manufacturing, an important factor in the increasing costs of biodeterioration as mentioned earlier.

1.4.2. World estimates

From Tables 2 and 3 it can be seen that the production of raw materials has an approximate value of 100×10^9 U.S. dollars, and that the value due to manufacture increases greatly. Figures for increases due to manufacture are not available on a world scale, but as a rough measure this has been taken as twice that of the U.S.A. in 1967 for the world for 1968. The figure thus arrived at is approximately 300×10^9 U.S. dollars. The total annual production of biodegradable materials on a world scale is therefore 400×10^9 U.S. dollars.

It has often been assumed that 1% of these materials are lost due to biodeterioration. Working on this basis, the annual world loss is in the region of 4×10^9 U.S. dollars.

It should be noted that these estimates do not include losses of stored foodstuffs, the value of which must be very large. The fouling of ship hulls, a clearly identifiable

Material	World production x 10^6 metric tonnes	Estimate of value of 10^9 U.S. dollars
Cotton fibre	11.3	6.0
Wool (greasy basis)	2.8	2.8
Jute fibre	2.7	0.9
Flax fibre	0.6	0.4
Synthetic fibre	7.3	9.1
Paper pulp	23.3	3.0
Other fibres	1.6	0.3
Skins and hides	1.6	0.5
Mineral oil	1924.0	33.5
Plastics and resins	21.8	8.7
Synthetic rubber	3.8	2.7
Natural rubber	2.7	1.6
Industrial roundwood	1.2*	20.0
Total value of raw materials		89.5×10^9 U.S. dollars

* x $10^9 m^3$.

Table 2. World production of some raw materials susceptible to biodeterioration; for industrial use, 1968. After Hueck-van der Plas, (1971).

Material or Industry	Added value x 10^9 U.S. dollars due to manufacture
Textile products	18.3
Timber and wood products (not furniture or buildings)	5.0
Paper products	9.6
Printing and publishing	14.3
Plastics and synthetics	3.8
Paints and allied products	1.3
Petroleum refining	20.3
Rubber and plastics (not as above)	6.8
Leather products	2.6
Electrical equipment	24.8
Instruments	6.3
Value added in industries	113.1
Value added in construction and further handling	42.0
Total value added:-	155.1 x 10^9 U.S. dollars

Table 3. Value added by manufacturing industries applying raw materials from table 2, in the U.S.A. in 1967. After Hueck-van der Plas, (1971).

case of biodeterioration, is not listed in the tables, but has been estimated by the T.N.O. organisation in Holland to be in the region of 10 x 10^6 U.S. dollars per year on a world scale.

1.4.3. Textiles

As any material is manufactured into smaller and smaller units, so any attempt at tracing its history, ultimate fate and therefore making an accurate costing of any biodeterioration becomes more difficult, especially in the field of consumer goods. Some general pointers can be abstracted, however, from the foregoing figures.

Table 5. Value of annual world crop of natural fibres

World Production 1968 x 10^6 metric tonnes		Value in U.S. dollars x 10^9
Cotton Fibre	11.3	6.0
Jute Fibre	2.7	0.9
Flax Fibre	0.6	0.4
Wool	2.8	2.8
Total	17.4	Total 10.1

(Added value (World) on basis of
2 x U.S. figure for 1967 :- 36.6 x 10^9 U.S. dollars)

1.4.4. Pesticides

Taking costs of prevention and eradication of biological attack on materials as an indication as to the cost of biodeterioration, the value of pesticides produced can be considered. The following figures (Table 6) are for Britain in 1970, and it should be noted that the figures given represent values of products only, and do not include the cost of application.

Table 6. Value of pesticides and allied products produced in Britain in 1970 (Values in thousands of pounds).

Type of compound	Production in quarters of year			
	1st	2nd	3rd	4th
Fungicides	1309.2	282.5	820.3	897.1
Herbicides	7967.9	7893.6	4022.5	6503.1
Pesticides*	1821.6	2315.5	1883.7	1375.7
Others	240.1	281.5	189.1	226.0
Totals	11338.8	11472.7	6915.6	9001.9

Grand Total 38729.0 i.e. over 38 million pounds

(H.M.S.O. 1971b)

* Insecticides, rodenticides, excluding sheep and cattle dips and dressings.

1.4.5. Conclusions

It can be seen from the foregoing examples that there is no clear method at the present time for the costing of biodeterioration, or indeed for the recognition of the problem, which is possibly the major factor. These general

problems in the field of biodeterioration as a whole apply equally well to the textile industry. Even allowing for some re-use of cotton textiles (for example in the paper trade), the clear fate of this material is either disposal by burning or biological decay. Textiles which yield to wear before decay are then decayed after discarding, but may well have their useful life shortened by biodeterioration, especially tentage and other outdoor fabrics. It is aspects of this decay of cotton textiles, and their biological testing, which have been studied in this thesis.

1.5. Cotton - Mode of fungal attack

The basis of the cotton fibre (and all plant fibres) is cellulose, which has the emperical formula $(C_6H_{10}O_5)_n$. It is a polymer formed by the condensation of glucose molecules as shown below:-

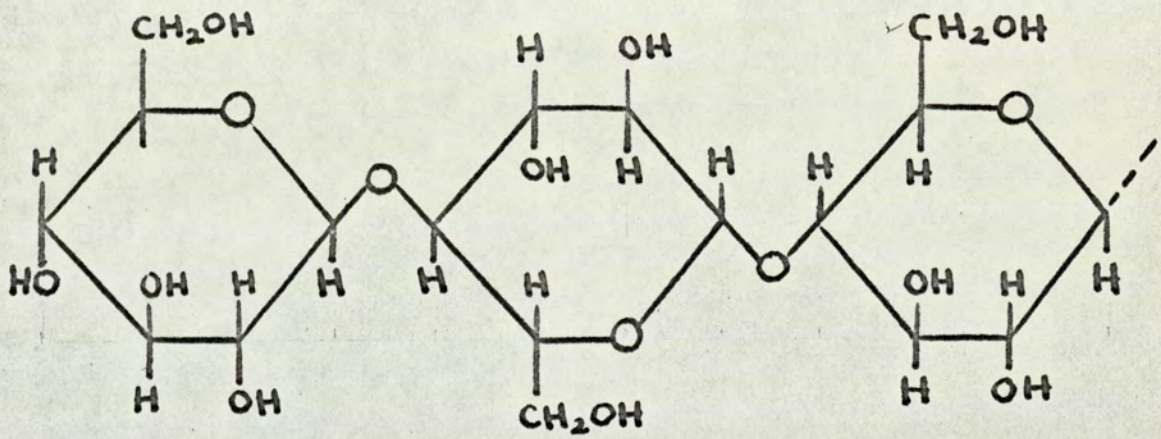


Figure 2. The cellulose molecule

The molecular weight of cellulose varies widely, depending on its source. Cellulose from cotton has been quoted as having molecular weights between 200,000 and 400,000; having between 2,000 and 3,000 glucose residues per molecule. Recent estimates put the figure for glucose residues as high as 10,000 per molecule, (Cook, 1968).

The breakdown of cellulose by fungi utilising it as a carbon (energy) source is brought about by enzymes, (Siu, 1951). The first stage in the conversion of insoluble

cellulose into soluble sugars which are able to be metabolised by the fungal cell, is brought about by extracellular enzymes. The extracellular nature of cellulases from fungi has been shown by the time-lapse photography technique of Hill (1965).

Selby (1968) has reiterated the difficulties in preparing cell-free extracts of cellulases able to cause significant decay of the highly ordered forms of cellulose such as cotton fibres. He cites Reese et. al. (1950, 1952) who suggested that the cellulolytic enzyme system must comprise at least two components. They also suggested that in order to be able to attack highly ordered forms of cellulose, a fungus must posess an enzyme component C_1 which rendered the substrate open to attack by another component C_x, which alone could perform little or no breakdown of the cellulose. Selby discusses the work which was later performed, centred around this hypothesis, and points out that some work which seems to contradict the hypothesis was carried out on much less ordered forms of cellulose than cotton fibres. Selby has isolated C_1 components from Trichoderma viride and a strain of Penicillium funiculosum (both species occurring frequently in isolation work in this thesis) and has shown the abilities of these components to act synergistically with their own and other C_x components.

Although not suggesting one single clear function of the C_1 cellulase component, Selby discusses earlier theories and puts forward one possible explanation based on the "hydrogen bondase" theory of Siu (1951), which is reinforced by new theories on the molecular structure of cotton

(Jefferies et. al., 1968). This possible mechanism is summarised in figure 3. The significant point concerning this work is that enzymic attack on a fibre is high localised, the supra-molecular structure of the highly ordered cellulose of cotton limiting the accessibility of the enzyme to it. Other work showing the localised nature of cellulose attack has been reported by Hill (1965). Attack also appears to be localised at a microscopic level, attack appearing to begin at areas of pores and crevices in the fibres themselves (van Bochove, 1967), which is of significance when considering the protection of cotton textiles by active agents such as pentachlorophenyl laurate.

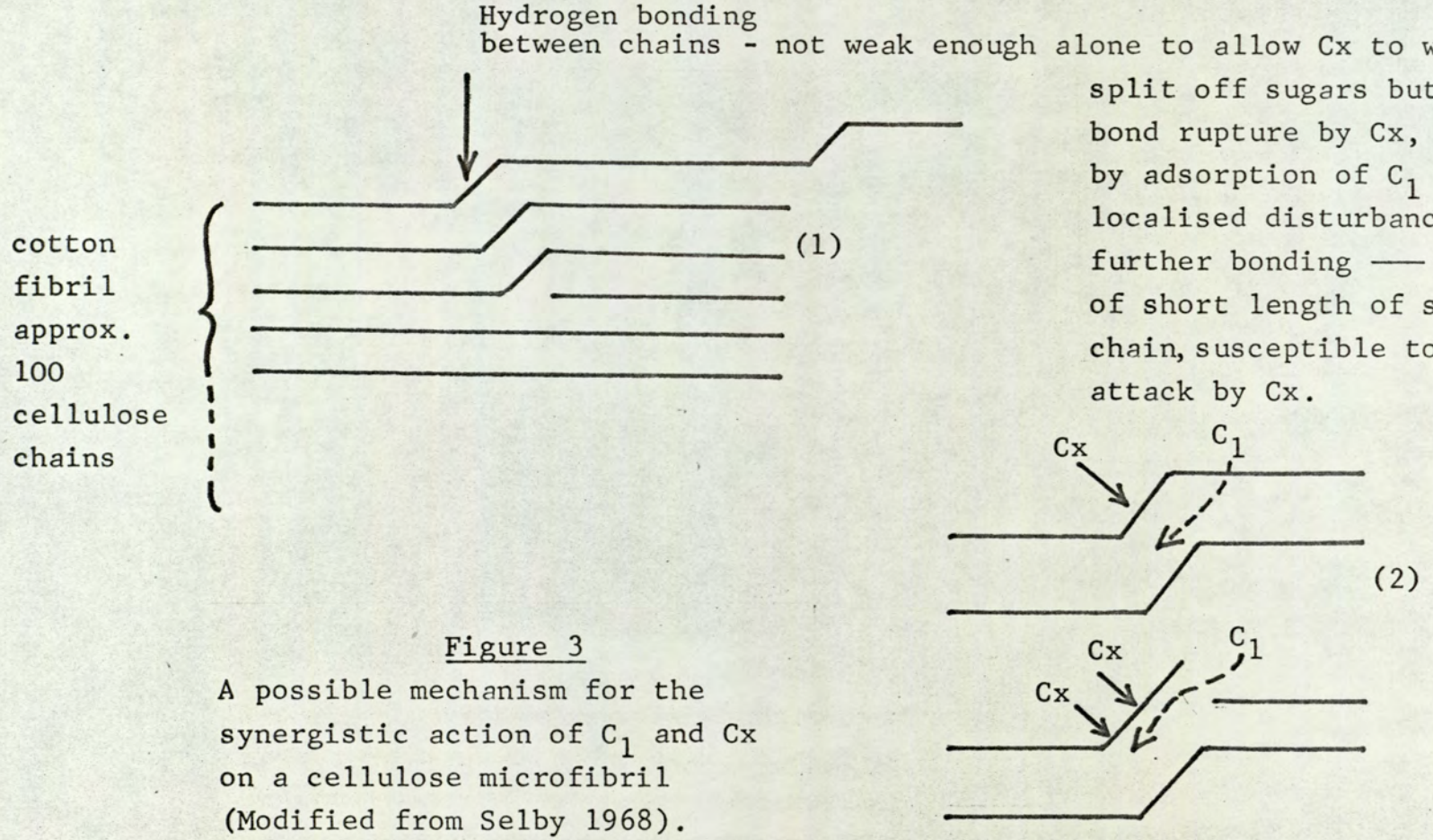


Figure 3

A possible mechanism for the synergistic action of C_1 and Cx on a cellulose microfibril (Modified from Selby 1968).

1.6. Treatments used in the protection of cotton

Protective treatments may operate in several ways. They may poison or directly inhibit the organism; they may change the nature of the cellulose so that it cannot be utilised; they may provide a physical barrier against attack; or they may block the sites of possible cellulase activity. There is no ideal method, and the one chosen in practice must be chosen with regard to the actual finished product and the uses to which it may be put. Selby (1966) has listed the characteristics of the hypothetical ideal rot-proofing treatment. These characteristics are given in expanded form below:-

The agent must:-

(i) Be toxic to a wide range of organisms (in fact to all deteriogens which it could contact) or must block their action.

(ii) Not be toxic to humans or animals by mouth or by absorption through the skin. It should be safe to use in application processes.

(iii) Not introduce any undesirable colours in the textile.

(iv) Be compatible with any other treatment given to the textile.

(v) Not affect the handle of the textile

(vi) Not weaken the textile by reducing either its tear strength or its tensile strength.

(vii) Not make the textile more sensitive to weakening by light (actinic degradation).

(viii) Be resistant to leaching (toxic agents however must have some solubility if they are to be effective).

(ix) Have a competitive cost, as low as possible.

Protective treatments may be classed as either active or passive.

(a) Passive treatments

These treatments are such as to have no toxic action on organisms, but provide a physical barrier to them, or render the material unavailable for their nutrition.

These treatments are designed to protect the cellulose from structural attack; they do not prevent surface growth by organisms not actually utilising the cellulose (van Bochove, 1967). Examples of passive treatments are given below:-

(i) Provision of a physical barrier against attack

A synthetic polymer may be coated around the fibres (Bhandari et. al., 1968). It is very difficult for total coating to be achieved, and the handle of the fabric is usually damaged, the textile becoming stiff.

(ii) Chemical modification of cellulose

Methods such as acetylation, reaction with glyoxal, cyanoethylation and carboxymethylation have been employed (Selby, 1966). The resistance to attack conferred depends upon the degree of substitution, and a minimum of 1.0 has been suggested (Levinson and Reese, 1950). This figure is open to some discussion, but it is accepted that considerable changes in the cellulose molecule must take place for this treatment to be effective, and that these changes can lead

to undesirable changes in the physical properties. This method is also rather complex and costly.

(iii) Blocking sites of cellulase activity

This is an attractive method owing to the low degree of substitution required, with correspondingly fewer alterations to the physical properties of the textile. Compounds used in tests have included phenyl isocyanate with pyridine (Hill, 1965; Selby, 1966).

(b) Active treatments

Such treatments are designed to have a direct effect on the growth and development of organisms. This inhibitory effect minimises not only structural damage but also surface growth.

Compounds which have a toxic action on fungi fall within this category. The compounds used are diverse and many, as shown by the survey of Hueck-van der Plas (1966), who lists over seventy commercially-used active ingredients used in the treatment of textiles against biodeterioration, most of which are fungicides. A similar survey was made by Wessel and Bejuki in 1959. Selby (1966) lists the main types and draws attention to their main properties. The main types of fungicidal and/or bactericidal compounds are listed below:-

Phenols, e.g.:- pentachlorophenol, O-phenyl phenol, trichlorophenol, salicylanilide, pentachlorophenyl laurate.

Phenylmercuric salts, e.g.:- acetate, naphthenate, lactate.

Pyridylmercuric salts, e.g.:- acetate, stearate, chloride.

Copper salts, e.g.:- napthenate, 8-hydroxyquin-olinate, formate, ricinoleate.

Zinc salts, e.g.:- napthenate, chloride, sulphate.

Organotin compounds, e.g.:- tributyltin oxide.

Inorganic salts, e.g.:- iron-chromium, cuprammonium hydroxide (Willesden process)

The phenolic compounds are very widely used but in conditions of strong sunlight, if used alone, show some tendency to accelerate the tendering of cotton. Organic mercury compounds are now being used less frequently owing to their high toxicity and persistency. Copper salts, although effective, impart a strong colour (usually bright green) to the textiles, and are thus unsuitable for many applications. Many organotins are easily leached, and some other types of compound, e.g.:- salicylanilide, are too volatile to impart lasting protection.

Pentachlorophenol esters, including pentachlorophenyl laurate are among the most widely used fungicides for textiles (Adema et. al., 1967). Such esters have an advantage over pentachlorophenol, due to a greater resistance to leaching (Hueck and La Brijn, 1960) and their low mammalian toxicity (see appendix). These factors have led to the further study of pentachlorophenyl laurate in this present work

1.7. The biological testing of textiles

Work on biological testing may be carried out for several reasons (Jones, 1968).

(i) To check that a material conforms with a user's specification.

(ii) To examine materials that have failed during storage. or use due to possible biological causes.

(iii) To investigate problems posed by the foregoing situations with an aim to improving the textile, its preservative treatment or the test method, in order to simulate more closely the usage conditions.

It is a basic requirement of any test that it should give some indication of the behaviour of the material when exposed to biological stress under service conditions (Lloyd, 1968). The first problem in testing arises in attempts to relate those biological stresses which occur in practice to those which are able to be applied under laboratory conditions. These stresses have been listed by Hueck-van der Plas, (1965a), who lists factors concerning the general and micro-environment, microbial populations and inocula, and nutrition. It is obviously desirable when tests are designed that the different factors influencing biological stress should be regarded, but in order to be useful with regard to the time available, stresses are often increased. The concept of severity is often difficult to define (Lloyd, 1968), especially where biocides are present.

The obvious ideal in biological testing is the simulation of natural conditions of high biological stress. Such a simulation is a complex system where many factors must be recognised and controlled. Investigation and development of such systems is not within the scope of the routine tester (Jones, 1968), but consideration of the problems involved in routine test work provides a basis for further studies. The basic problems are listed by Jones (1968) and are as follows:-

(i) Absence of assured reproducibility in comparative and successive tests.

(ii) Inadequacy of defined methods of assay.

(iii) Inadequacy of suitable controls.

These problems are quoted in relation to petri-dish and soil burial tests, and are discussed in greater detail in later chapters, together with accounts of the development of test techniques. This present work thus concerns the interaction of test materials and fungi in test techniques, i.e. aspects of the ecology of biological testing.

1.8. Aims and objects of investigation

The central aim of this investigation has been to study some of those aspects of the colonisation and decay of cellulosic materials under test conditions, by soil fungi, which are of interest in the field of materials protection by chemical agencies. Emphasis was placed on soil fungi as soil is generally the most severe and widely used test environment.

Using cellulosic materials, various fungal isolation techniques have been employed. A variety of techniques have been used, not only to isolate a wide range of fungal species for further study but also to show the selective or demonstrative nature of the techniques and the differences between them. Fungal isolations are in themselves a form of test as they show the ability of a material to be colonised and indicate suitable test organisms.

Using materials protectants (biocides or preservatives) these experiments were continued, and also the effects of other carbon sources on the system studied. Pentachlorophenyl-laurate based fungicides were the main biocides used as these are used widely in practice for the protection of cellulosic materials.

Further aims have been to develop and demonstrate techniques for the study of biodeterioration of cotton textiles and techniques for the study of the effects and detoxification of biocides, bearing in mind the difficulties often encountered in present methods of testing.

The aims of the physiological studies have been to investigate some of those aspects of the environment which have a marked effect on the activities of fungi. The topic of the effect of pH on the fungicide studied is of some significance, and is thus expanded in a later chapter. The work on respiration is of significance when treatment levels using this fungicide are considered.

The frequent requirement for tests to be quantitative led to the development of a simple laboratory strength testing machine for textile strips.

As soils in practice and in test work are subject to great variability, a range of soils was also investigated.

The work was the first study in an industrially-sponsored series, therefore one other underlying aim was to outline problems for further study.

Chapter 2

ECOLOGICAL ASPECTS OF CELLULOSE TEXTILE TESTS

2.1. Introduction

2.1.1. Agar techniques

2.1.2. Soil burial techniques

2.1.3. Perfusion techniques

2.2. Isolation work using agar techniques

2.3. Isolation work using a soil burial technique and a demonstration of the effects of substrate screening

2.4. Initial isolations of soil fungi using a perfusion technique

ECOLOGICAL ASPECTS OF CELLULOSE TEXTILE TESTS

2.1. Introduction

In studies of the colonisation and decay of materials, the fungi present in or on the substrate may be categorised either as true deteriogens or as associated or contaminant forms. True deteriogens are those fungi which pioneer the actual breakdown of the main substance of the substrate, and are able to utilise it directly as a carbon source. Associated fungi are those which are either present as passive contaminants in a dormant form, or living on compounds other than the main substance of the substrate (e.g. a starch dressing on a cotton textile), or are those which appear later in the succession, living on carbon sources which are breakdown products of the main substance of the substrate provided by the true deteriogens.

Care must be taken not to confuse this situation with other valid concepts which apply to the fungal colonisation of substrates (usually plant remains) by fungi as described below.

The nature of the substrate is usually responsible for the selection of colonisers, and so, for example, if the substrate is rich in carbohydrate, the first fungi to colonise may be the fast-growing sugar fungi. These first colonisers are termed primary colonisers. As the carbohydrate is depleted, these primary colonisers may give way to slower growing cellulolytic fungi, living on the cellulose components of the material. Although the cellulose component

may be the largest and main structural component present, these fungi are termed secondary colonisers. (Garrett, 1951). It is thus necessary to distinguish between deteriogens and non-deteriogens; and primary and secondary colonisers.

Thus, to obtain the most meaningful information concerning the colonisation of substrates by fungi a correlation is necessary between relative time of colonisation and functional aspects of fungi. This correlation is only achieved by detailed ecological studies.

Visual examination of a colonised substrate may yield little information; the fungi may not produce discrete colonies, spores or fruiting bodies, and may simply be too diffuse or small to be seen. It is also not possible to distinguish by visual means between deteriogens, non-deteriogens or passive contaminants. It is quite usual also for a group or mixture of different fungi to be involved in the colonisation and degradation of a substrate.

A technique is thus required to clarify this situation. The simplest and most widely used method for initial examination of colonised substrates is that of the agar plate. By this means fungi may be isolated from the original substrate, reproductive states induced, and identified. Using a range of nutrients within the medium, individual aspects of an organism may be investigated, and pure cultures of organisms thus isolated and investigated are available for use in the laboratory testing of materials for susceptibility to deterioration.

It is not possible, however, by simple isolation work, to distinguish between deteriogenic and non-deteriogenic colonisers. To make this distinction, demonstrative techniques must be employed, which show the ability of the organism to utilise a main component of the material as a nutrient source.

2.1.1. Agar techniques

The use of agar gels of varied composition is a universal practice in microbiological studies. A very wide range of nutrient media is available commercially, and agar may also be modified to meet specific individual needs. There is, however, no universal medium which is equally suitable for all organisms. The nutritional requirements of fungi vary so greatly that the production of a truly universal medium is impossible. There are media, however, which are widely used in fungal studies, such as potato-dextrose agar and Czapek-Dox agar, which are often regarded almost as universal media. Although very useful such media bear little relation to 'natural'materials for by processes of refining and compounding, their constituents are in highly modified forms.

Where the best possible correlation between a 'natural' material and an agar gel is required, as in studies in biodeterioration, a medium is required which is modified as little as possible. An example of such a medium is Eggins and Pugh cellulose agar (Eggins and Pugh, 1962). This medium contains inorganic nutrient salts, plus ball-milled cellulose, which is a much less modified form of cellulose

than say carboxy-methyl cellulose. By ball-milling cellulose powder into a fine form a suspension is formed which does not settle out when plates of such a medium are poured. Thus the substrate (the carbon source) is available throughout the medium. This not only allows growth but is able to demonstrate true growth on cellulose, for as the fine suspension of cellulose is dissolved away by fungal cellulases, a clear area around the colony is formed. Thus, this medium has a relatively unmodified carbon source freely available, and is also a demonstrative technique. Mere growth on an agar medium does not indicate ability of the organism to utilise the main carbon source within it; fungi are often able to grow on small quantities of other compounds present, in the medium even as unknown impurities within the supposedly non-nutrient agar gel component itself. Thus, within the field of biodeterioration studies there is a need to use demonstrative nutrient media.

Techniques employing agar have been widely used in the study of soil fungi, (Waksman, 1916; Chesters, 1940, 1948; Warcup, 1950; Chesters and Thornton, 1956). Techniques using cellulose agar as a demonstrative medium for cellulolytic activity have been used as quantitative methods (Eggins and Pugh, 1962; Rautela and Cowling, 1966).

The testing of textiles using agar techniques may be performed in several ways. Agar may be simply used as an isolation medium in the study of already colonised textiles; if the medium contains finely-divided cotton cellulose (Eggins and Pugh, 1962) cellulolytic activity may be demon-

strated and cellulolytic fungi may be selectively isolated. Agar may also be used, with or without the addition of nutrients, as a support medium on which textile samples are placed prior to inoculation with soil or pure or mixed cultures of test fungi. Studies of such test methods have been published by La Brijn and Kauffman (1971).

There are many variables inherent in agar techniques which should be taken into consideration (Hueck-van der Plas, 1965a; Jones, 1968). The possible problems in the use of agar techniques may be briefly summarised as follows:-

(i) Agar itself

The composition of the basic gel may vary depending on source (Marshall et. al., 1949) and setting problems may be encountered when it is used at low pH.

(ii) Incorporation of nutrients

When non water-soluble nutrients are used, care must be taken to ensure a fine even dispersion which does not settle out before the agar sets. (Eggins and Pugh, 1962)

(iii) In corporation of textile fungicides

Dispersion problems may also be encountered here, especially in the case of those fungicides which are normally applied to textiles from organic solvents.

(iv) Drying of the medium

This is often a limiting factor in experiments where fungicides are employed, and it is desired to maintain the culture for a long period. It is possible also that a water gradient may be set up within the agar layer as desiccation proceeds.

(v) Oxygen supply

As dissolved oxygen within the medium is used up by the fungal colony, oxygen gradients may be set up, which may further complicate observed growth patterns. This factor is of particular relevance to fungi which grow submerged in the agar.

(vi) Build-up of wastes

As an agar plate or tube is a closed system, waste metabolites from a fungal colony will accumulate in time. These may retard the growth of the colony itself, and may have an even greater effect on any other species present. This phenomenon may be readily observed, either as a clear zone of inhibition surrounding a colony, or as a coloured pigment released into the medium. Changes in pH may also occur.

(vii) Subsequent work

Any chemical analysis of the medium at the end of an experiment usually involves melting and filtering the medium. This processing may lead to further changes in the medium.

(viii) Selectivity

No isolation or growth medium can be said to be non-selective, especially such an artificial medium such as agar.

(ix) Test organisms

Test cultures of fungi, maintained for long periods for use in pure and mixed culture work may change in their characteristics. These variations, especially genetic ones have been discussed by Jones (1971).

From the above points it can be seen that several factors should be borne in mind when employing agar media for isolation and experimental work. A comparison of the advantages and disadvantages of agar techniques with other techniques is summarised in table 7.

In research into the biodeterioration of cellulosic materials, there are three main disadvantages with agar techniques. These are:-

(i) Difficulties in carrying out fine accurate work.

(ii) Difficulties in maintaining experiments for long periods.

(iii) The artificial nature of the medium, being greatly different from the actual material being studied.

The last two factors are to a great extent overcome by the techniques described below in the next section.

2.1.2. Soil burial techniques

In this type of test a usually unmodified material is brought to a mixed and unknown inoculum, a situation which constrasts with most agar methods where a pure culture of a known organism is brought to a somewhat modified material incorporated in a growth medium.

Soil burial techniques may be used to isolate deteriogenic fungi from soil and to investigate the effect of growth of soil microorganisms on materials. The advantages here are that the conditions are nearer to natural conditions than found in agar techniques, and that regarding the testing of materials, the biological stress on the material can be very severe (Bunker, 1943).

(a) Soil burial as an isolation technique

Using soil with a rich and varied mycoflora, soil burial can be a useful and convenient technique for the isolation of fungi onto test materials, as shown later in this chapter. There are, however, certain disadvantages which are discussed below.

(b) Soil burial as a test technique

The main attractions of soil burial testing and the reasons for its widespread use are that the test is not only severe and also approaches more closely natural or field conditions than agar techniques, but also that it can subsequently be made quantitative, for as solid test materials are employed, these can then be subjected to tensile and similar quantitative tests after burial. It is however widely recognised that this form of testing can be extremely

variable (Turner, 1972; Lloyd, 1968). This variability is included for discussion in the section below.

(c) Disadvantages of the soil burial technique for isolation and testing work

(i) Isolation work

In this discussion, textiles will be considered as the test substrate, but the comments apply also to paper and wood testing.

During isolation work, previously sterilised textile strips are buried in a prepared bed of soil in a covered container, and incubated. Strips are removed at intervals, cut into sections, and plated-out onto agar media for subsequent examination and identification of the colonising fungi.

The disadvantage here is that not only fungi which have actively colonised the textile appear on the agar plates, but also fungi carried passively in the adhering soil as dormant forms or resting spores. Ensuing species lists can thus contain many species which are not in fact concerned with the actual colonisation and decay of the test textile. Eggins and Lloyd (1968) suggested that screening the substrate under test from direct contact with soil by a glass fibre screen would remove the disadvantages caused by adhering soil in the soil burial technique without appreciably lessening colonisation by active cellulolytic fungi. It was also hoped that on plating out of such screened textiles, the count of passive organisms might be

lessened. An account of a practical investigation into this technique is given later in this chapter.

(ii) Testing work

A biological test which subsequently provides quantitative results, as does soil burial testing followed by tensile strength measurements, is usually attractive in an industrial application. This fact, coupled with the others previously mentioned has ensured the continuing employment of soil burial testing despite the disadvantages discussed below. Attempts have been made, with varying degrees of success, to standardise the soil burial test, but only in the aspect of comparisons of data concerning untreated and biocidally treated textiles, rather than of standardisation of practical aspects and agreement of absolute values obtained in different laboratories (Hueck and van der Toorn, 1965). It should be noted, that in all strength testing work using biocidally treated materials, that variations may also occur due to non-biological factors. Errors in the impregnation of textiles with biocides may occur, and also errors may result from the inaccurate calibration of strength testing machines. A method has been suggested by Kaplan to alleviate the latter problem, which has been described by Zinkernagel and Schmid (1968).

Non-standard soils

Unless it is clearly stated that experimental results are confined to a particular soil, which is well

described, and that indeed the flora of the soil itself is also a part of the study, problems can arise in this area. Even if the soils are prepared to a prescribed standard formula, great differences can occur with time and between different laboratories. The common concept of soil as a simple reservoir of organisms can lead to difficulties.

Unnatural soils

In order to increase the severity of this test, soils are often enriched with extra organic nutrients, such as manure or grass clippings, and to this extent the soils are somewhat artificial. A less important factor is that soils are sifted, and most of the larger animals removed. This would only be of any consequence if the results of any experiment were to be related to usage conditions where textiles were actually buried in soil, and not merely exposed to contaminating soil particles and organisms. Totally synthetic soils have been made up and used in these tests, but have proved to be even more variable in practice (Lloyd, 1970).

Packing density

Variations in the density of soils packed around the samples can lead to great variation in strength losses of the textile strips (Turner, 1972). Up to a certain point, an increase in density results in greater decay. An over-compacted bed may lead to the establishment of anaerobic conditions. Packing density is probably the factor which varies most as a result of the test being performed by different operators.

Moisture content

There are difficulties in maintaining an even and constant moisture content in a mass of soil. This is particularly apparent in small-scale soil burial work, using small amounts of soil. Turner (1972) in his statistical work on variations in this test concludes that moisture content, together with packing density are the greatest sources of variation in this type of test.

Consideration of the previous techniques has led to the development of tests which are more versatile, adaptable, and overcome many of the major disadvantages in the tests previously described, especially for use as research and investigating methods. One of the newer techniques employed in this study is described below.

2.1.3. Perfusion techniques

Although perfusion techniques are artificial in nature, they do approach natural conditions by bringing together several factors of the environment in a controlled way. The ability to be controlled gives such techniques certain advantages over soil burial tests, particularly in research or detailed screening situations.

The basic technique was devised by Eggins, Malik and Sharp (1968). It consists of a test substrate, smaller than generally used in soil burial tests, through which a low-volume flow of nutrients is maintained. Liquid nutrients

pass in water solution from a reservoir by capillary action along a glass-fibre wick. They flow through the substrate (if being used as part of the wick itself, as in the case of paper or textiles) or below and in contact with the substrate (as in the case of plastics or wood). Unused nutrients and leached waste products flow out of the colonised test substrate and accumulate in a tail wick which is exposed to the atmosphere, the rest of the system being totally enclosed. Flow of nutrients is maintained by the constant drying of the tail wick in the atmosphere. The inoculum placed on the substrate may be a pure culture or an agar plug cut from a petri dish culture, or soil organisms growing from a soil inoculum. The entire apparatus is constructed of materials which can be heat sterilised by autoclaving. The major application of this system is as a research tool or as an adaptable testing technique.

The advantages are:-

(i) Fresh nutrients are constantly supplied.

(ii) The substrate does not dry out

(iii) Toxic metabolites do not build up.

(iv) The material under study can be used as a growth substrate in its normal state.

(v) The experiment can be maintained for long periods.

(vi) The conditions (nutrients, pH, temperature, light) can be varied at will during the experiment.

(vii) With pure culture inocula, growth can be observed under sterile conditions at any time.

(viii) The equipment lends itself to modification.

The main disadvantage with this type of technique is the time required in initial preparation, which limits its usefulness as a general routine screening method. Two adaptions of this system are described in further chapters.

Technique	Advantages	Disadvantages
AGAR	1. Simple 2. Cheap 3. Adaptable	1. Prone to desiccation 2. Nutrients may be limiting over long periods. Cannot be changed 3. Wastes may build up 4. Very artificial medium
SOIL BURIAL	1. Severe 2. More 'natural than agar 3. Test material used in normal state	1. May be very variable
PERFUSION	1. Constant supply of fresh nutreints 2. Wastes are removed 3. Can be maintained for long periods 4. Conditions can be varied 5. Test material used in normal state	1. Relatively complex and time consuming to set up.

Table 7. Summary comparison of three major methods for fungal isolation and growth.

2.2. Isolation work using an agar technique

The aim of this work was simply to obtain cultures of fungi for future studies and to make an initial survey of the organisms of the test soil, using agar isolation media.

The soil used in all experiments was a meadow soil from Clent (North Worcestershire, England). This soil has been studied by other workers (Malik and Eggins, 1969, 1970) and is known to have a varied mycoflora. Fungal species were isolated from the soil using Warcup's technique on Eggins and Pugh agars at 25°C. The species isolated are listed in table 8.

Special Isolated at 25°C.	Isolation Medium	
	Cellulose Agar	Glucose Agar
Fusarium solani	X	
Humicola grisea	X	
Gliocladium roseum	X	
Mucor sp.		X
Ostracoderma stage of Peziza ostracoderma	X	
Papulaspora bulbicola	X	
Paecilomyces elegans	X	
Penicillium funiculosum	X	X
Penicillium sp.		
Trichoderma viride	X	X
Zygorynchus moelleri	X	X
Rhizopus sp.		X

Table 8. Fungal species isolated at 25°C using Warcup's technique.

Table 8 represents the results of initial agar isolation work. The experiment was mainly qualitative in nature but several points concerning frequency of isolation may be mentioned. The most common species by far was Trichoderma viride, on both cellulose and glucose agars. Other common species on cellulose agar were Humicola grisea, Fusarium solani and Penicillium sp. On glucose agar, Rhizopus sp, Zygorynchus moelleri and Penicillium funiculosum were most common. It is interestint to note that the Ostracoderma stage of Peziza ostracoderma which was isolated in this particular experiment was never isolated again during any work with this soil. It appears to be a rare organism in this soil and has only been isolated from this soil once previously (Malik, 1970).

This experiment was carried out at an initial media pH of 6.4, this being the normal pH of the medium. It has been shown by other authors (Malik and Eggins, 1970) that additions to the species list would be expected if a range of pH were used. Aspects of the effects of changes in pH are covered in further chapters.

2.3. Isolation work using a soil burial technique, and a demonstration of the effects of substrate screening

Cotton test cloth supplied by the Textile Institute, T.N.O., The Netherlands, was cut into strips 15 cm long and 2.5 cm wide (excluding ravelled margin). The strips were fastened on the outer surface of glass boiling tubes with autoclavable non-toxic glass fibre adhesive tape. Half the total number of tubes had glass fibre screening tape (non-adhesive) fastened over the textile strips (See figure 2). The completed tubes were autoclaved at 15 p.s.i. for twenty minutes before burial in the soil. Clent soil, as mentioned previously, was used in this experiment. The soil pH at the start of the experiment was 7.2 and the water content was 22% of the total weight. The soil was sifted before use and was conditioned at 25°C for one week before starting, the soil being turned each day. The temperature was maintained at 25°C throughout the experiment, the soil container being a covered polyethylene tank in a thermostatically-controlled water bath.

Subsequent isolations of fungal species from the textile test strips were made onto glucose agar and cellulose agar.

The tubes were placed in the soil, care being taken to pack soil around the tubes with as even a pressure as possible. At intervals tubes were removed from the soil, (See Plate 1) the textile strips detached, cut into sections with scissors sterilised by alcohol flaming between each cut, and plated out onto cellulose and glucose agar. Other tubes

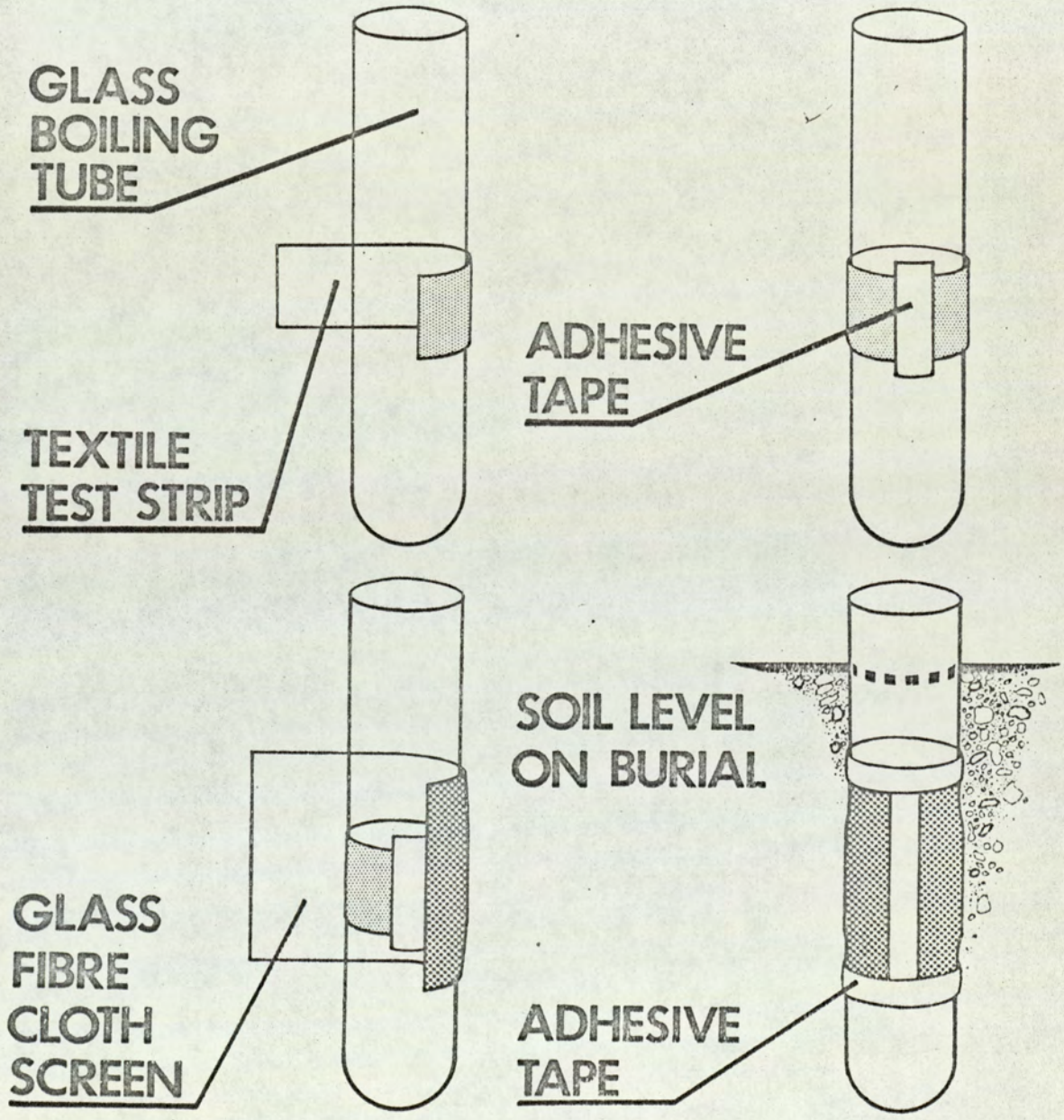


Figure 2

Stages in the construction of a screened-substrate isolation tube for soil burial.

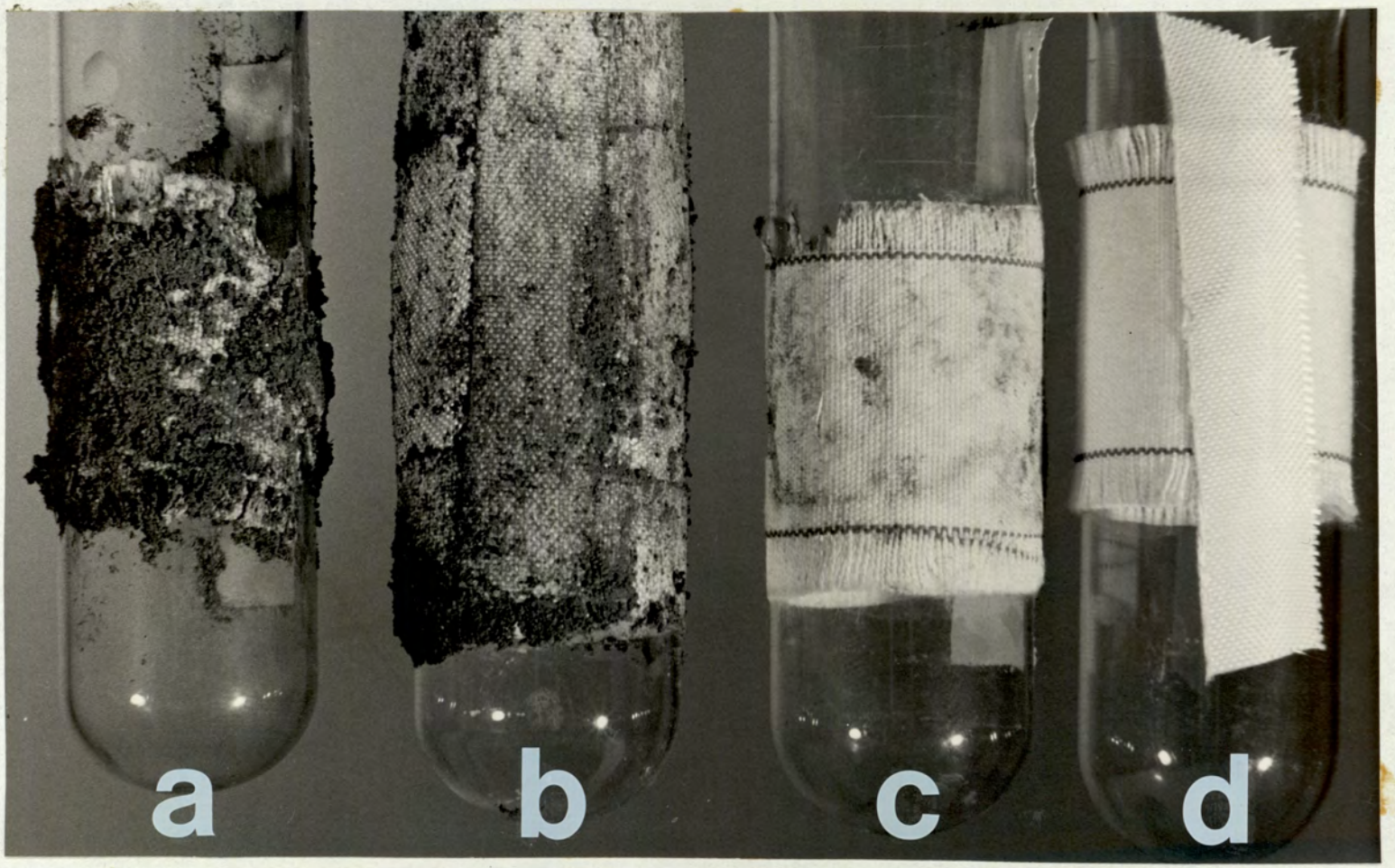


Plate 1. From left to right:-
(a) Unscreened tube on removal from soil, showing soil adhering to decayed textile strip.
(b) Screened tube on removal from soil, showing soil adhering to glass-fibre screen.
(c) Screened tube after removal of glass-fibre screen. The staining is due to fungal growth and there is no adhering soil.
(d) Unscreened tube prior to burial, showing clean textile for purposes of comparison. Note adhesive tape holding textile test strip to tube.

were also removed, the textile strips being cleaned with 70% ethanol, dried at room temperature and stored for tensile testing at the end of the experiment. After first conditioning in a temperature and humidity controlled room for three days the strips were broken on a Hounsfield tensiometer and the results plotted as breaking strength in pounds (as a percentage of initial controls) against time in days (See graph 1).

The species of fungi isolated onto agar were listed (See tables 9 and 10).

Conclusions

(a) Strength losses

(i) The textile strips lost strength after burial in the soil.

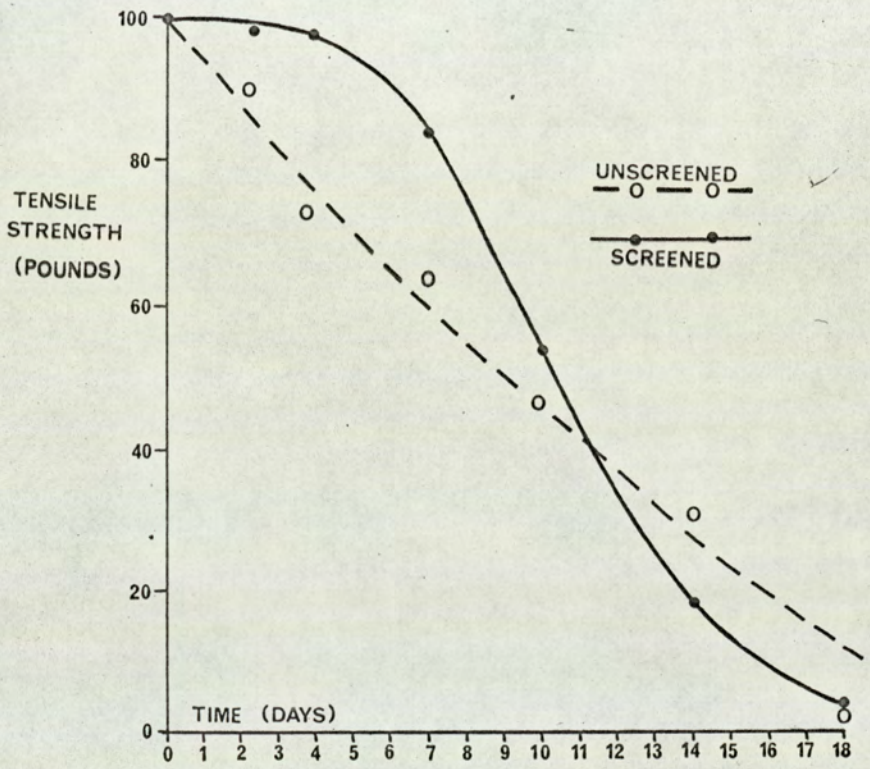
(ii) There was an initial lag in the decay of screened textile strips.

(iii) There was a greater rate of decay in the screened strips than in the unscreened strips after colonisation had begun.

(iv) The ultimate losses in both the screened and unscreened strips were approximately the same.

(b) Colonisation by soil fungi

(i) Both screened and unscreened strips were colonised by soil fungi.



Graph 1

Loss in strength of screened and unscreened textile strips in soil burial test.

SPECIES	SCREENED									UNSCREENED								
	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
Trichoderma viride			X	X	X		X			X	X	X		X	X	X		
Penicillium funiculosum						X	X	X	X					X	X		X	
Humicola grisea						X								X	X			X
Fusarium solani				X		X							X					
Rhizopus sp.														X				
Chaetomium globosum																	X	
Gliocladium roseum																		X
Papulaspora sp.														X				
Ostracoderma stage of Peziza ostracoderma						X	X											

3 weeks 3 weeks

Table 9

Fungal species isolated from textile plated out onto cellulose agar: nine sacrifices were made during a three week period.

SPECIES	SCREENED									UNSCREENED								
	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
Trichoderma viride			X	X	X		X		X	X	X	X	X	X	X	X		X
Penicillium funiculosum						X	X		X			X	X	X	X	X	X	X
Humicola grisea						X												X
Rhizopus sp.	X				X					X		X	X			X	X	
Aspergillus fumigatus	X	X	X						X	X		X		X				
Papulaspora sp.																	X	
Zygorynchus moelleri															X			
	3 weeks									3 weeks								

Table 10

Fungal species isolated from textile plated out onto glucose agar: nine sacrifices were made during a three week period.

(ii) Fungi colonising the screened strips were predominantly cellulolytic species, shown by species isolated from the strips clearing cellulose agar.

(iii) More fungi were isolated from unscreened than from screened textile strips, and the spectrum was similar to that obtained from previous isolations using Warcup's technique.

Discussion

As the soil had a rich mycoflora, great losses in strength of the textile strips were to be expected. The lag in initial decay of the screened strips is most likely due to the time required for the actively growing fungi in the soil to pass through the glass-fibre screen and colonise the textile strip. As the screen selects out only active fungi the rate of decay of screened strips is great once colonisation has taken place. This rapid rate of decay eventually reduces the strength of the screened strips to the same level as the unscreened strips. The unscreened strips are colonised not only by cellulolytic fungi, but also to a greater extent than screened strips, by sugar fungi, living perhaps on breakdown products of the initial cellulolytic colonisers or other nutrients present in the soil, or perhaps in soil as dormant forms. It may be hypothesised that screened strips decay at a faster rate than unscreened strips after colonisation as mainly cellulolytic fungi are selected out as colonisers, and there is less local competition for other nutrients.

Even the unscreened strips provide a selective environment, as shown by the greater number of species isolated from the same soil by the previous simple Warcup method. This seems to underline the fact that no isolation technique can be said to be truly non-selective.

This experiment confirms the theoretical behaviour expected for fungi colonising screened cellulose substrates, and serves to emphasise the useful nature of screened substrate techniques in the testing of materials and investigation and selective isolation of true deteriogens.

2.4. Initial isolations of soil fungi using a perfusion technique.

The previous initial work led on to studies using perfusion techniques, the results being set out in Chapter 3, together with details of the methods employed. For purposes of comparison with the previous initial isolation experiments, set out below are the results of a perfusion isolation experiment using untreated cotton textile as employed in the previous soil burials. (Table 11). The nutrient perfusate used was a solution of mineral salts plus l-asparagine and yeast extract (Eggins and Pugh, 1962) and the experiment was carried out at 25°C. Banks of perfusion units were set up in petri-dish carriers after the method of Malik and Eggins (1969), and after autoclaving were inoculated with Clent soil. The textile substrates were screened from direct soil contact by glass fibre screens. Textile strips were sacrificed at seven day intervals for five weeks and plated out on cellulose agar, the plates then being incubated at 25°C.

As can be seen from table 11, the results varied from those obtained from the soil burial isolations. The main difference was the predominance of Arthrobotrys sp. This may have been associated with the fact that nematode worms were present, many being observed on the agar plates. It may be that perfusion conditions are more favourable for the growth of these animals than on textiles in soil burial tests. The relationships between nematode worms and fungi

in the colonisation of cellulosic materials is not clear, but is worthy of further study (Stolzy and Van Gundy, 1968) and is the subject of present work (Jones, 1972).

Species	Isolations at 7 day intervals				
Sacrifice	1	2	3	4	5
Aspergillus sp.				X	
Arthrobotrys sp.		X	X		X
Chaetomium globosum		X			
Fusarium solani	X	X	X		
Humicola grisea			X		
Paecilomyces sp.				X	
Trichoderma viride		X			

Table 11. Fungal species isolated from perfused cotton textile (soil inoculum) onto cellulose agar.

Another technique was subsequently developed and used for isolation work. This technique incorporated the characteristics of a screened substrate immersion tube and the perfusion system. It is described in Chapter 8.

In this chapter, discussion of techniques has been given, together with examples of results of isolation experiments using them, employing soil organisms and cellulosic substrates. From this work it can be seen that various methods may be used for the isolation of soil fungi, and that the varying aspects of these methods can influence the results obtained.

As a result of this basic work, further aspects of textile ecology were studied as set out in subsequent chapters.

Chapter 3

ECOLOGICAL STUDIES ON FUNGICIDALLY-PROTECTED TEXTILES

3.1. Introduction

3.2. Isolation work

3.2.1. Isolation work using agar

3.2.2. Isolation work using perfusion techniques

3.3. The testing of textiles

3.3.1. Soil burial tests

3.3.2. Colonisation and strength testing of fungicidally-treated textile with the incorporation of a perfusion technique.

Chapter 3

ECOLOGICAL STUDIES ON FUNGICIDALLY-PROTECTED TEXTILES

3.1. Introduction

Following the previous fungal isolation work more detailed experiments were devised to investigate the fungal colonisation of cellulosic textiles.

In the first experimental section (3.2) isolation work was carried out using agar and perfusion techniques, using a PCPL fungicide on the test substrates. In the second experimental section (3.3) the colonisation and decay of PCPL protected textiles was further investigated using soil burial and perfusion techniques. Within this latter section the effects of alternative carbon sources on the decay of textiles was studied as one of the main aspects of the experiments.

The effects of alternative carbon sources is of interest when considering the fungal attack on finished cotton materials, as the carbon sources studies were compounds which may be used as textile finishing agents (Hall, 1952; Peters, 1967).

Pentachlorophenyl esters have been the subject of earlier studies, (Adema et. al., 1967) commercial preparations of this fungicide were screened against pure cultures of fungi and bacteria, but no ecological studies involving

isolations of organisms from soil were carried out. Some mention of the ecology of soil fungi in relation to phenolic preservatives in general may be found in literature (Lloyd, 1968), but detailed studies, particularly concerning pentachlorophenyl esters, are lacking.

3.2. Isolation work

3.2.1. Agar techniques

In a first attempt to investigate the colonisation of cellulosic materials protected by fungicides, by soil fungi, the following technique was devised, in order to establish that it was possible to isolate fungi onto PCPL-treated materials. Whatman filter paper was chosen for this experiment merely as a convenient form of cellulose.

The filter paper was cut into semicircles and immersed in a 1% solution of "Mystox E.L.C." for one hour. This concentration of fungicide emulsion was chosen in an attempt to impart only partial protection to the paper and thus allow some fungal colonisation to occur (Catomance Ltd., "Mystox" data sheet T/7). At the end of the immersion period, the papers were removed, excess emulsion shaken off and dried in sterile covered dishes. Pour plates, using both soil particles and soil solution in sterile water were prepared, using Eggins and Pugh cellulose agar (Eggins and Pugh, 1962). When the agar was at the point of solidification, the filter-paper semicircles were placed on the surface of the agar, thus ensuring firm adhesion between the paper and the agar. Replicate sets of plates were prepared and incubated at 25°C, 35°C and 50°C. The temperatures of 35°C and 50°C were included in order to investigate thermophilous and thermophilic soil fungi, which may be of importance in certain situations. By using this technique it was hoped to show:-

(i) Fungal growth on PCPL-treated material.

(ii) Cellulolytic fungi, demonstrated by clearing of the cellulose agar.

(iii) Inhibition caused by the fungicide, indicated by lack of fungal growth on the paper and in the surrounding medium.

(iv) Toleration of the fungicide, shown by growth of fungi on the treated paper.

(v) Possible preference for fibrous cellulose shown by growth on paper rather than on agar.

The results of these experiments at each temperature are given and discussed in turn below:-

25°C Results of observations are given in table 12. From these results several conclusions may be drawn:-

(i) More fungal growth resulted from a soil solution inoculum than from a soil particle inoculum.

This may be due to the initial dilution of fungal propagules inherent in soil solutions which results in less competition between closely packed fungal colonies. Nematode worms may also be less frequent, and thus more fungi survive to be observed later.

(ii) Growth was much stronger and more common on the agar than on the treated paper.

This was only to be expected, as the agar did not contain any fungicide.

TEMP:- 25°C.	PLATE NUMBER	BACTERIA		FUNGAL HYPHAE		FUNGAL SURFACE GROWTH		FUNGAL SPORULATION		SPECIES NOTED AND COMMENTS
		A	P	A	P	A	P	A	P	
PARTICULATE SOIL INOCULUM	1	-	-	+	-	-	-	-	-	(Nematodes)
	2	-	-	+	-	-	-	-	-	(Nematodes)
	3	-	-	+	-	+	-	-	-	-
	4	-	-	+	-	-	-	-	-	(Nematodes)
	5	-	+	-	-	-	-	-	-	(Nematodes)
SOIL SOLUTION INOCULUM	6	-	+	+	-	+	-	-	-	T. viride (Nematodes)
	7	-	-	+	+	+	+	+	+	T. viride
	8	-	-	+	-	+	-	+	-	T. viride (Nematodes)
	9	+	+	+	+	+	+	+	-	T. viride

Key:- Column A = agar portion of plate, Column P = treated paper portion of plate

+ = presence of growth, - = absence of growth

Table 12. Results of agar pour plate/treated paper isolations at 25°C.

(iii) Complete normal development of fungi was inhibited.

Taking the results as a whole the ratio on agar of observed hyphae, to observed growth of surface fungal colonies, to observed fungal sporulation was 8:5:3 after 7 days of incubation. This stunting of growth and the production of retarded aberrant growth forms was a phenomenon encountered throughout the investigations at this temperature. It is most probably due to a combination of the effects of the fungicide and damage caused by nematode worms.

(iv) Trichoderma viride was the dominant fungal species found at 25°C.

This was the only identifiable species of fungus to appear on the plates. This is most likely due to its vigorous growth, (Garrett, 1963) its inhibition of other species, (Weindling and Emerson, 1936), and its apparent resistance to nematode attack once established.

35°C The results of this experiment are given in table 13.

Two main differences may be seen when these results are compared with those obtained at 25°C:-

(i) On agar, growth is normal, all plates showing some fungal sporulation.

This may be due to the fact that although nematodes were present they were possible inhibited by the higher temperature of incubation.

TEMP:- 35°C.	PLATE NUMBER	BACTERIA		FUNGAL HYPHAE		FUNGAL SURFACE GROWTH		FUNGAL SPORULATION		SPECIES NOTED AND COMMENTS
		A	P	A	P	A	P	A	P	
PARTICULATE SOIL INOCULUM	1	+	-	+	-	+	-	+	-	Graphium sp.
	2	+	+	+	-	+	-	+	-	Graphium sp. (Nematodes)
	3	+	+	+	-	+	-	+	-	Graphium sp. (nematodes)
	4	-	-	+	-	+	-	+	-	Graphium sp.
	5	-	-	+	-	+	-	+	-	Graphium sp. A. fumigatus
SOIL SOLUTION INOCULUM	6	+	+	+	+	+	+	+	+	Graphium sp. A. fumigatus
	7	+	+	+	+	+	+	+	+	Graphium sp. A. fumigatus
	8	+	+	+	-	+	-	+	-	A. fumigatus (Nematodes)
	9	+	+	+	+	+	+	+	+	A. fumigatus (Nematodes)
	10	+	+	+	+	+	+	+	+	A. fumigatus Z. moelleri (Nematodes)

Key:- Column A = agar portion of plate, Column P = treated portion of plate

+ = presence of growth, - = absence of growth

Table 13. Results of agar pour plate/treated paper isolations at 35°C

(ii) The dominant fungal species were Graphium sp. and Aspergillus fumigatus. This corresponds with the optimum temperature ranges of these fungi. Previous unpublished work has shown that Graphium sp. has an optimum range of 25°C - 35°C with a maximum of 40°C, and Aspergillus fumigatus is a thermotolerant species with a wide temperature range, (Cooney and Emerson, 1964).

50°C The results of this experiment are shown in table 14. The results were the same on each plate, irrespective of the type of inoculum.

It will be noted from the results that Nematode worms were absent, and the effect of this is clearly shown. Fungal growth was not retarded in any way, and the fast growing thermophilic species Humicola grisea var. thermoidea and Chaetomium thermophile were isolated, together with the thermotolerant Aspergillus fumigatus.

Concerning the experiment as a whole, the main point of interest is that fungal species were observed growing over the surface of the fungicide treated paper.

These species were:-

25°C:- Trichoderma viride

35°C:- Aspergillus fumigatus
Graphium sp.
Zygorynchus moelleri

50°C:- Aspergillus fumigatus
Humicola grisea var. thermoidea
Chaetomium thermophile

TEMP:- 50°C.	PLATE NUMBER	BACTERIA		FUNGAL HYPHAE		FUNGAL SURFACE GROWTH		FUNGAL SPORULATION		SPECIES NOTED
		A	P	A	P	A	P	A	P	
NINE PLATES MADE, 4 PARTICULATE SOIL INOCULUM, 5 SOIL SOLUTION INOCULUM	1 - 9	+	+	+	+	+	+	+	+	A. fumigatus Chaetomium thermophile Humicola grisea

Key:- Column A = agar portion of plate, Column P = treated paper protion of plate
+ - presence of growth, - = absence of growth

Table 14. Results of agar pour plate/treated paper isolations at 50°C.

All these species with the exception of Zygorynchus moelleri demonstrated cellulolytic ability in respect to the clearing of the cellulose agar. It must be noted that their growth over paper can not with certainty be regarded as proof of ability to actually break down the fibrous cellulose, as it became apparent during the course of the experiment that agar had become interspersed between the paper fibres, especially on its under surfaces. It is apparent, however, that in situations of heavy inoculation such as this, fungi are present which are able to grow in intimate contact with a fungicidally treated material.

The fungicide did have effects however as can be seen by the fungal preference for the agar portion of the plate, and the way in which the fungi colonised the paper. Fungi first appeared on the agar portion of the plate, spreading later towards the treated paper and eventually touching it. Later still hyphae were observed growing up from the paper surface. The paper/agar boundary was particularly condusive to the growth of T. viride. The bacteria which were usually in evidence formed white, cream and yellow colonies, but never spread quickly or dominated the plates.

The vigorous growth of fungi at 50°C may be due to several reasons. The main reason is probably the absence of nematodes, a direct result of the high incubation temperature. Another factor may be the possible inactivation of the fungicide due to heat. This phenomenon is investigated further in chapters 4 and 5.

In the previous chapter, fungal growth on cellulosic materials was demonstrated; in the previous experiment the ability of fungi to grow in the presence of PCPL-based fungicide has also been established.

These experiments provided some useful initial information regarding the colonisation of fungicidally treated cellulose materials in test work and highlighted difficulties when working with aberrant fungal forms. From this work, the following experiments were evolved.

3.2.2. Perfusion Isolation Experiments

First Series

In order to investigate a different test situation which has advantages over agar techniques as described in Chapter 2, perfusion techniques were employed to isolate fungi from a soil inoculum onto screened cotton textile. The textile was used in an untreated state (control) and impregnated with 1% and 2% by weight of pentachlorophenyl laurate. Similar experiments were carried out using similarly treated and untreated chromatography paper for purposes of comparison. The cotton textile employed was T.N.O. test cloth (see appendix) supplied by Catomance Limited. The paper used was "Whatman" chromatography paper (see appendix). Both were treated with pentachlorophenyl laurate applied from white spirit, by Catomance Limited, to levels of 1% and 2% pickup by weight.

Strips of paper and textile 0.5cm wide were cut, and incorporated into perfusion kits after the manner of Malik and Eggins (1969). After sterilisation by autoclaving at 15 p.s.i. for 20 minutes and then cooling, each unit of the perfusion kit was inoculated with a small amount of soil, gently pressed onto the glass fibre screen covering the textile or paper substrate. Kits were then incubated at 25°C.

At intervals, substrates were sacrificed from each perfusion kit and plated-out onto Eggins and Pugh cellulose agar. These plates were examined after 7 days incubation at 25°C and the fungal species growing were identified and recorded. The results are shown in tables 15, 16 and 17.

From these results it can be seen that:-

(i) Fungal species from the soil colonised both paper and textile strips, treated and untreated.

(ii) More fungal species were isolated from paper than from cotton textile.

(iii) On both untreated textile and untreated paper, Fusarium sp. was the dominant species isolated.

(iv) On paper treated with 1% pentachlorophenyl laurate, Trichoderma viride was the dominant species isolated.

(v) Occurence of fungal species was generally erratic.

(vi) Bacteria became more frequent with increasing fungicide concentration.

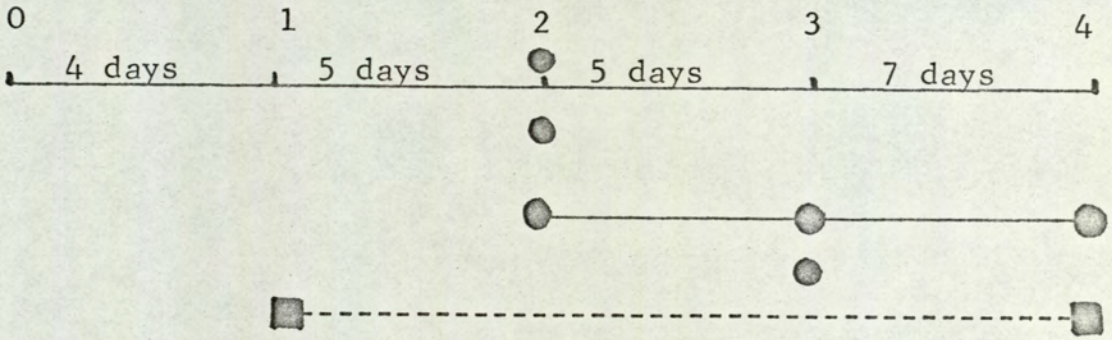
Table 15

Perfusion Isolation Experiments. 1st. Series. Isolations from perfused strips made onto E & P cellulose agar. 25°C.

UNTREATED COTTON TEXTILE (T.R.)

SPECIES

SACRIFICES



UNTREATED PAPER (P.R.)

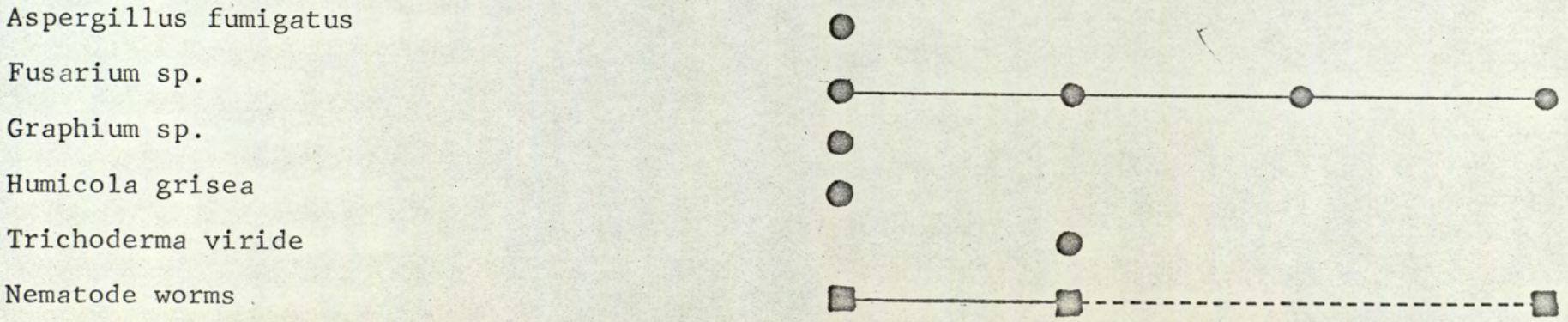
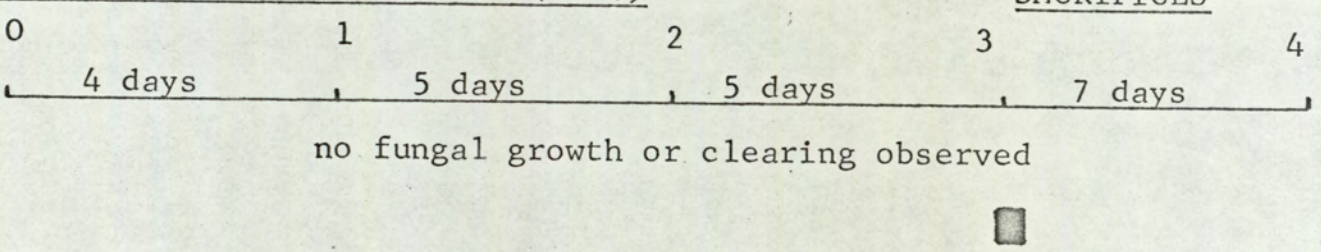


Table 16

Perfusion Isolation Experiment. 1st. Series. Isolations from perfused strips made onto E & P cellulose agar. 25°C.

TEXTILE TREATED WITH 1% PENTACHLOROPHENOL LAURATE (T.I.)



PAPER TREATED WITH 1% PENTACHLOROPHENOL LAURATE (P.I.)

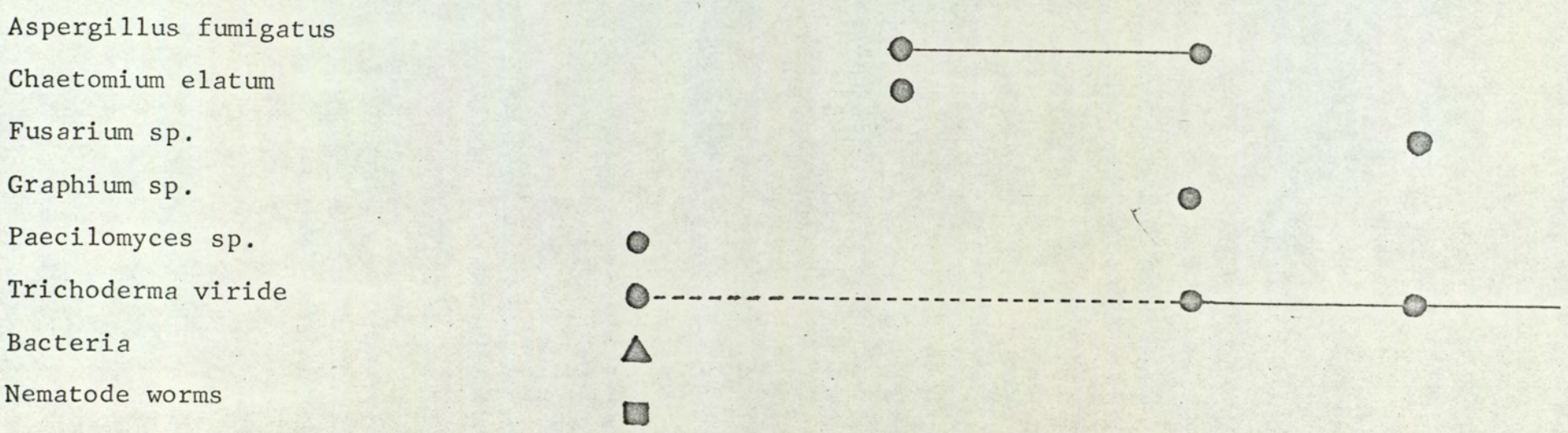
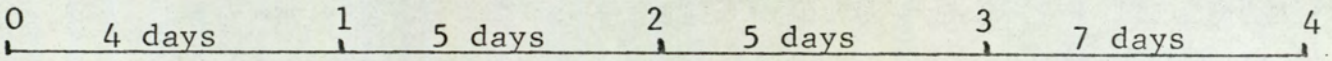


Table 17

Perfusion Isolation Experiment. 1st. Series. Isolations from perfused strips, made onto E & P cellulose agar. 25°C

TEXTILE TREATED WITH 2% PENTACHLOROPHENOL LAURATE (T.2).



Aspergillus fumigatus

Graphium sp.

Trichoderma viride

Bacteria

PAPER TREATED WITH 2% PENTACHLOROPHENOL LAURATE (D.2.)

Aspergillus fumigatus

Aspergillus sp.

Chaetomium globosum

Fusarium sp.

Trichoderma viride

Bacteria

Nematodes

(vii) In all instances, except that of 2% pentachlorophenyl laurate treated textile, nematode worms were observed to be present.

In this experiment, the test substrates were screened from direct soil contact, therefore any fungal species isolated will tend to be an active form in the soil as it is necessary for the fungus to pass through the mesh of the screen in order to colonise the test substrate. Thus, here, we have a demonstration of soil fungi which are able to actively colonise not only untreated cellulosic materials, but also those treated with fungicide. It would appear from this experiment that paper is a more readily colonised substrate than cotton textile. This may be due to the paper cellulose being less well ordered than that of the textile due to a more intensive degree of processing during its manufacture. This difference highlights the variable properties of cellulosic materials, either at a molecular and/or macroscopic level. Variations in cellulose at a molecular level are known to occur (see Chapter 1); variations due to the physical configuration of the sample may also occur (Lloyd, 1968). The common, fast growing and cellulolytic nature of Fusarium sp. may account for its dominance in untreated paper and textile samples. Trichoderma viride appears to take over this dominant role in the presence of the fungicide, a similar situation to that observed in the previous experiment using agar and paper plates. Both Fusarium sp. and Trichoderma viride have been demonstrated growing in the presence of phenolic inhibitors by Lloyd (1968). Variations in fungal form as seen in the

previous experiment were found here also in the presence of the fungicide, together with an erratic isolation pattern. As in most instances nematode worms were present a dual fungicide - nematode stress was placed on the fungi; a situation also observed in the previous experiments using agar/paper plates. It would appear that a 2% level of pentachlorophenyl laurate has no marked effect on the development of these organisms.

Series 2

In order to supplement the data provided by the first perfusion isolation experiments a second series was performed in a similar manner. As the first series of experiments showed that fungi could be isolated onto pentachlorophenyl laurate treated cellulosic materials, this aspect of fungal tolerance to the fungicide was carried further by using an additional plating-out medium; Eggins and Pugh cellulose agar containing 1% emulsified pentachlorophenyl-laurate. To supplement the data obtained at various temperatures using the agar plate/paper techniques, replicate perfusion kits were made up and incubation carried out at 25°C, 35°C and 50°C.

The results of these experiments are given in tables 18 - 23.

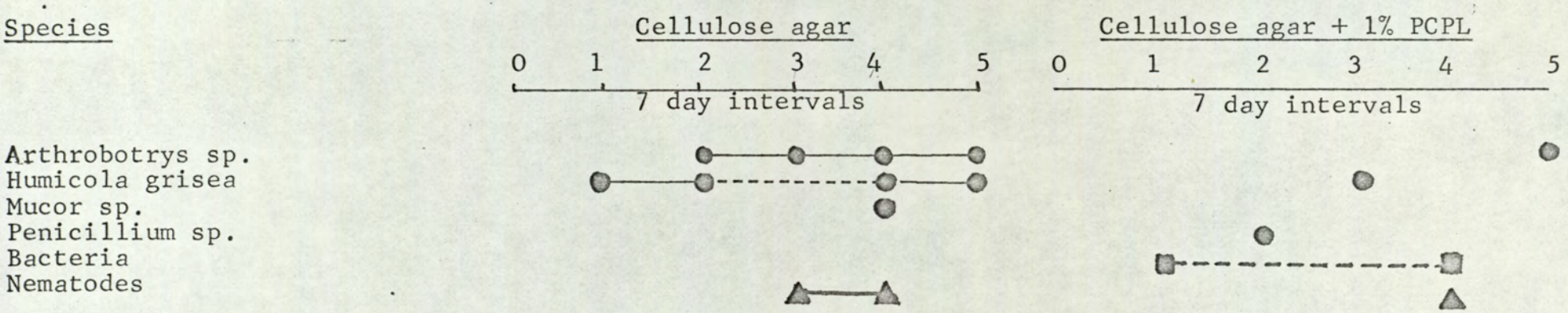
Several conclusions may be drawn from these results:-

(i) As in the first series of perfusion isolation experiments, strips of paper and textile, both treated and untreated with pentachlorophenyl laurate, were

Table 18

Perfusion Isolation Experiment. 2nd Series. Isolations made from perfused strips made onto E & P cellulose agars, and E & P cellulose agar with 1% PCPL added. 25°C.

UNTREATED COTTON TEXTILE (T.R.)



UNTREATED PAPER (P.R.)

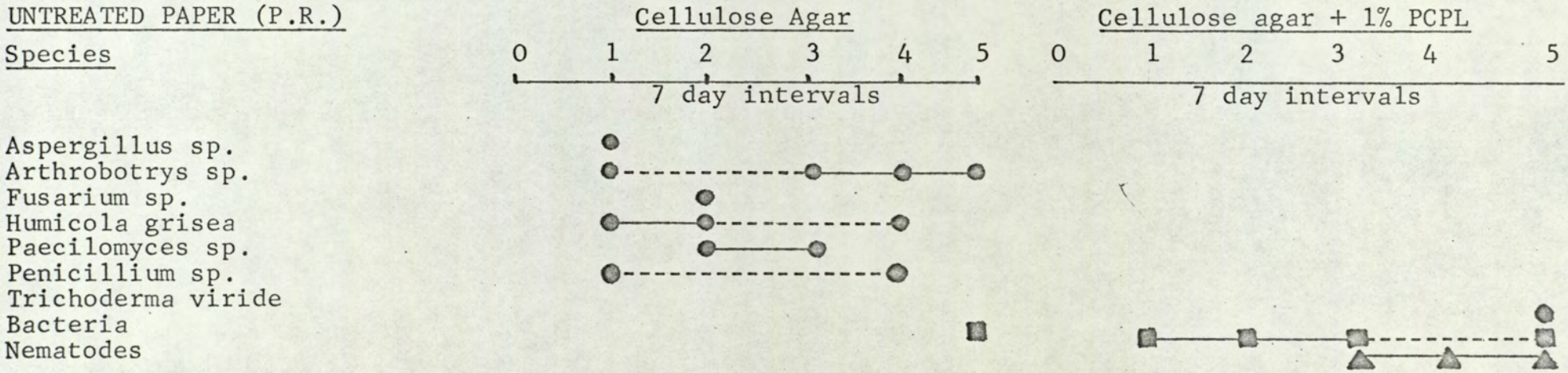
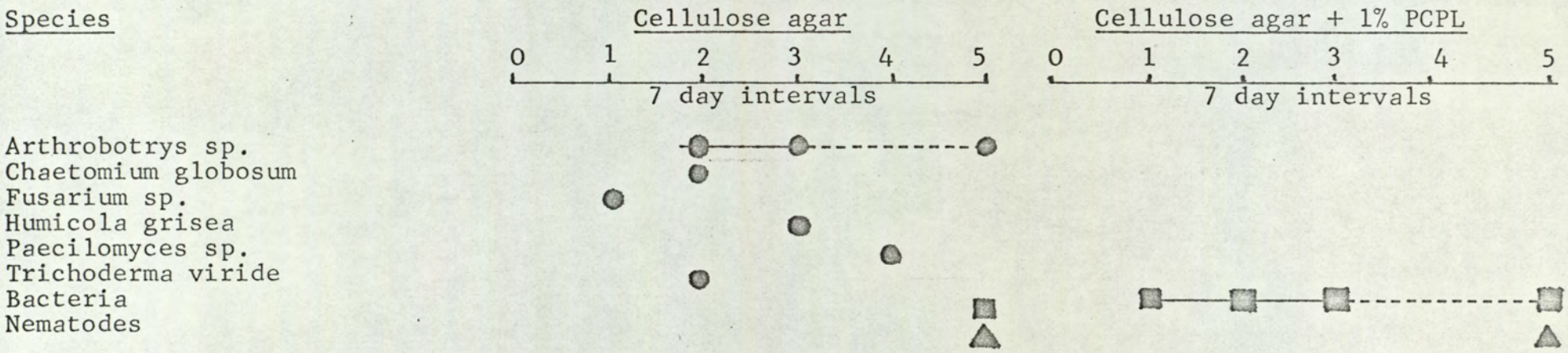


Table 19

Perfusion Isolation Experiment. 2nd Series. Isolations made from perfused strips made onto E & P agar, and E & P cellulose agar with 1% PCPL added. 25°C.

COTTON TEXTILE WITH 1% PCPL (T.I.)



PAPER WITH 1% PCPL (P.1.)

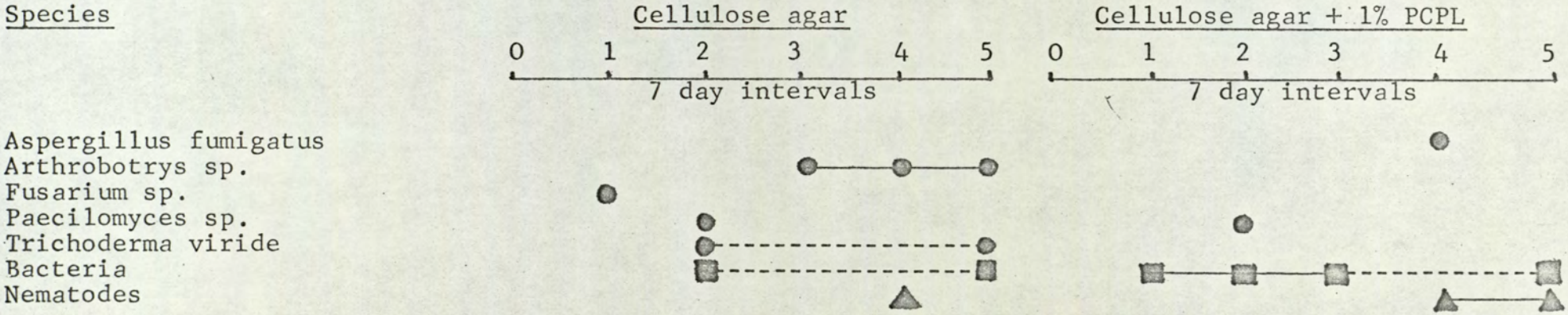
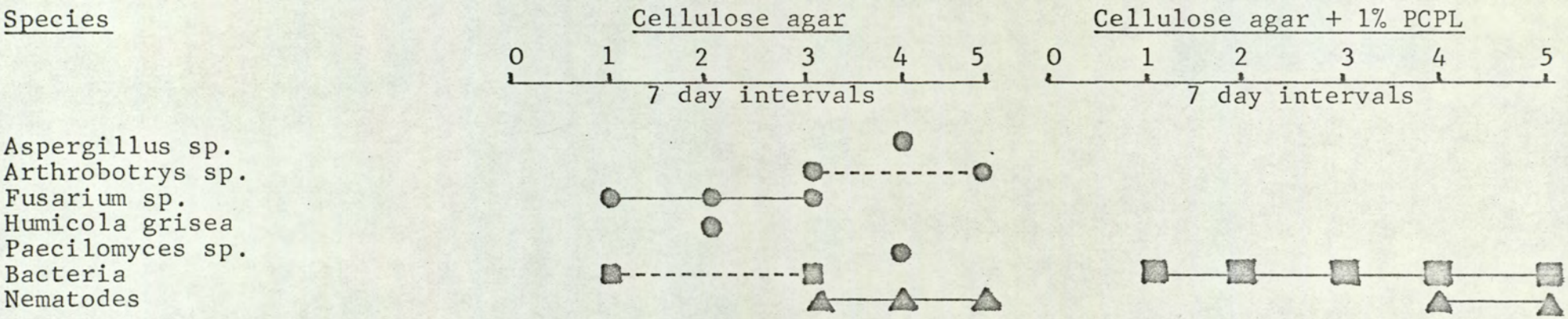


Table 20

Perfusion Isolation Experiment. 2nd Series. Isolations made from perfused strips, made into E & P cellulose agar, and E & P cellulose agar with 1% PCPL added. 25°C.

COTTON TEXTILE WITH 2% LPCP (T.2)



PAPER WITH 2% PCPL (P.2.)

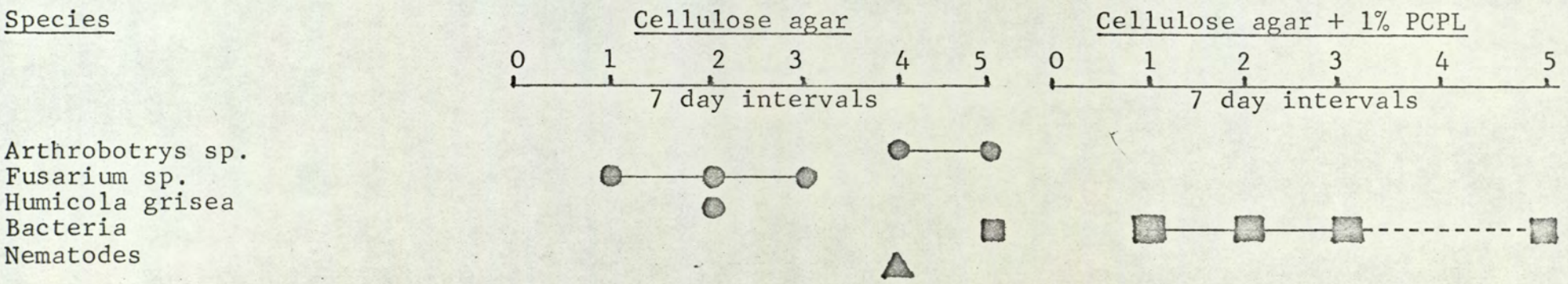
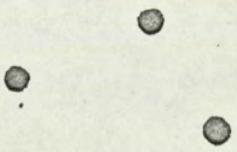
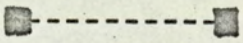
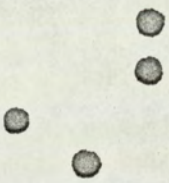
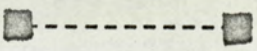
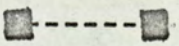
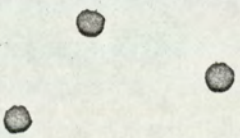
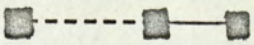
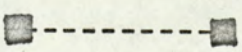
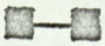
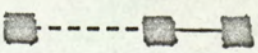


Table 21

Perfusion Isolation Experiment. 2nd Series. Isolations made from perfused strips, made onto E & P cellulose agar and E & P cellulose agar + 1% PCPL. 35°C.

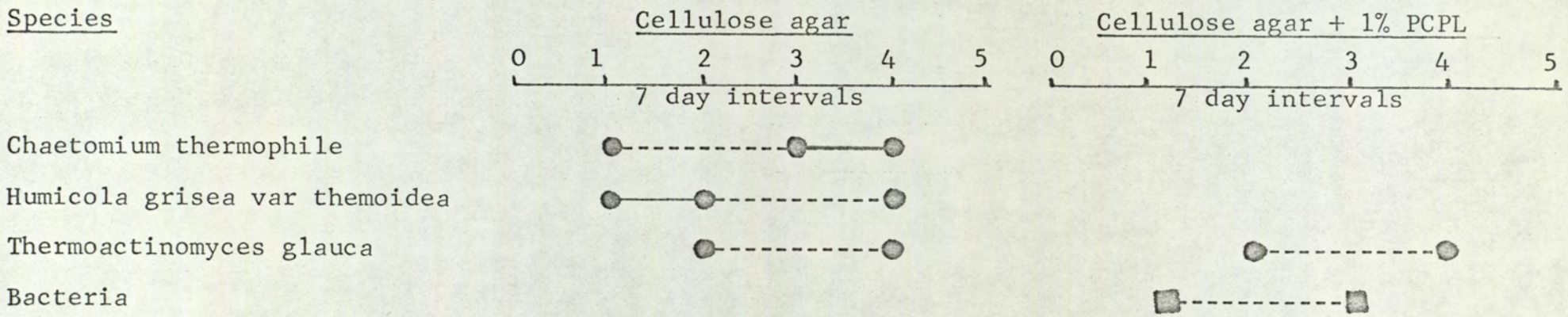
	Cellulose agar 0 1 2 3 4 7 day intervals	Cellulose agar + 1% PCPL 0 1 2 3 4 7 day intervals
TR Species Graphium sp. Mucor sp. Trichoderma viride Bacteria		
PR Species Aspergillus sp. Graphium sp. Mucor sp. Streptomyces sp. Bacteria		
T1 Species Bacteria		
P1 Species Aspergillus fumigatus Humicola grisea Mucor sp. Bacteria		
T2 Species Bacteria		
P2 Species Bacteria		

TR = UNTREATED COTTON TEXTILE PR = UNTREATED PAPER
T1 = COTTON TEXTILE + 1% PCPL P1 = PAPER + 1% PCPL
T2 = COTTON TEXTILE + 2% PCPL P2 = PAPER + 2% PCPL

Table 22

Perfusion Isolation Experiment. 2nd Series. Isolations made from perfused strips, made onto E & P cellulose agar, and E & P cellulose agar with 1% PCPL added. 50°C.

UNTREATED COTTON TEXTILE (T.R.)



UNTREATED PAPER (P.2.)

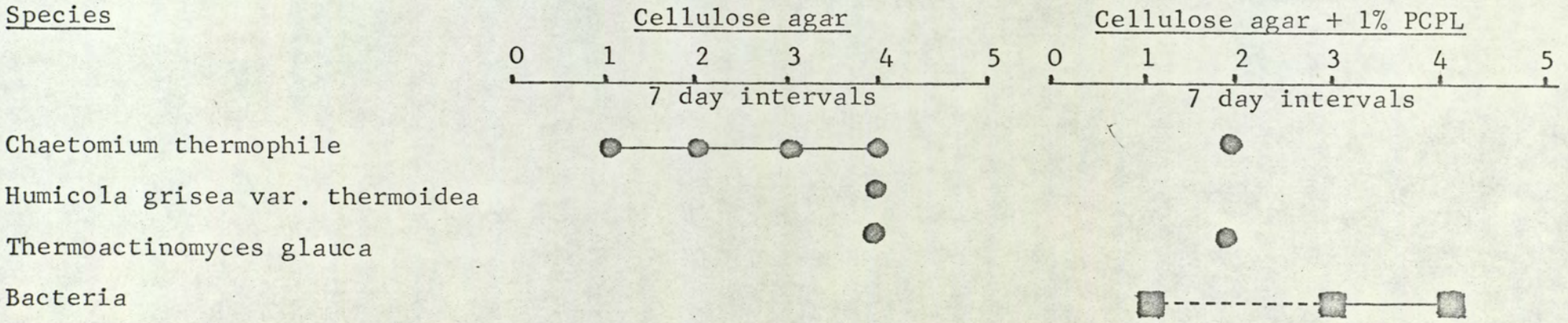
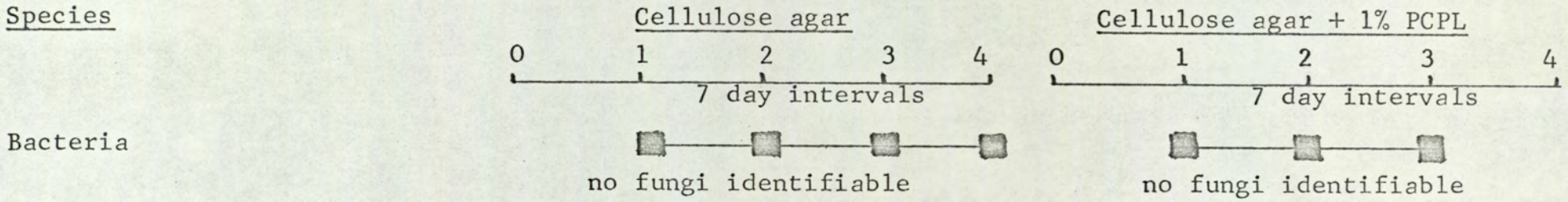


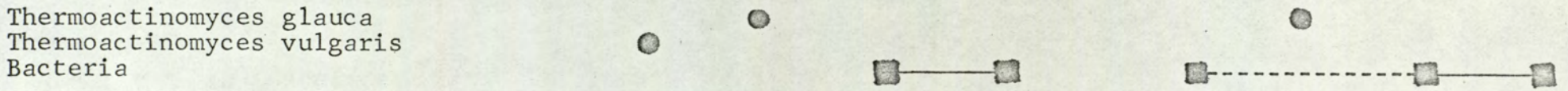
Table 23

Perfusion Isolation Experiment. 2nd Series. Isolations made from perfused strips, made onto E & P cellulose agar, and E & P cellulose agar with 1% PCPL added. 50°C

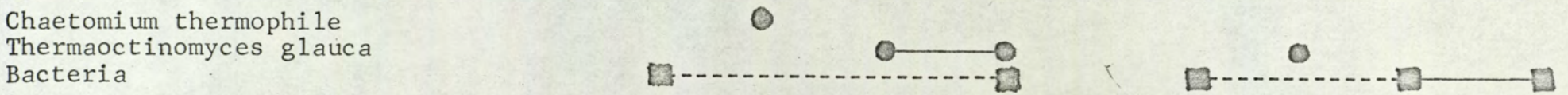
COTTON TEXTILE + 1% PCPL (T.I.)



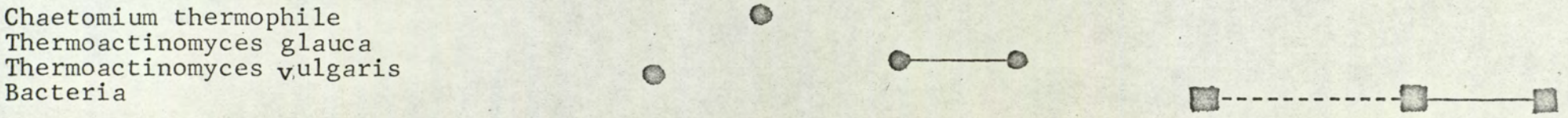
PAPER + 1% PCPL(P.1.)



COTTON TEXTILE + 2% PCPL (T.2.)



PAPER + 2% PCPL (P.2.)



colonised by soil fungi. (The species isolated are listed together in tables 24 and 25).

(ii) In this series of experiments, the dominant fungal species isolated at 25°C from untreated paper and textile were Arthrobotrys sp. and Humicola grisea. Arthrobotrys sp. remains dominant at a 1% level of PCPL in the paper and textile, to be joined by Fusarium sp. at the 2% level of PCPL in the test substrates.

(iii) From the results of experiments carried out at 35°C, no clear predominance of any fungal species was apparent.

(iv) At 50°C, Chaetomium thermophile was the predominant organism. In the presence of PCPL this fungus was joined by Thermoactinomyces glauca, an actinomycete which was also observed growing on PCPL-containing agar.

(v) Bacteria were present at all temperatures, and nematode worms were observed at 25°C and 35°C.

It is apparent from these perfusion experiments (1st and 2nd series) that different fungal species may play dominant roles even under identical experimental conditions of soil substrate, nutrient and incubation. This highlights the complex ecology of the soil and the difficulties which are encountered if attempts are made to equate soil to a simple homogenous resevoir of test organisms which can be predictably isolated (Griffin, 1972). The differences in

Key to Tables 24 and 25

A = Isolated from paper onto cellulose agar

B = Isolated from textile onto cellulose agar

C = Isolated from 1% PCPL paper onto cellulose agar

D = Isolated from 1% PCPL textile onto cellulose agar

E = Isolated from 2% PCPL paper onto cellulose agar

F = Isolated from 2% PCPL textile onto cellulose agar

G = Isolated from paper onto 1% PCPL cellulose agar

H = Isolated from textile onto 1% PCPL cellulose agar

J = Isolated from 1% PCPL paper onto 1% PCPL cellulose agar

K = Isolated from 1% PCPL textile onto 1% PCPL cellulose agar

L = Isolated from 2% PCPL paper onto 1% PCPL cellulose agar

M = Isolated from 2% PCPL textile onto 1% PCPL cellulose agar

Table 24

Species list of fungi isolated in the Perfusion Isolation Experiments (Series 1 and 2) 25°C.

Species	A	B	C	D	E	F	G	H	J	K	L	M
Aspergillus fumigatus	●	●	●		●	●			●			
Chaetomium globosum		●		●	●							
Fusarium sp.	●	●	●	●	●	●						
Graphium sp.	●	●	●			●						
Trichoderma viride	●		●	●	●	●	●					
Chaetomium elatum			●									
Paecilomyces sp.	●		●	●		●			●			
Aspergillus sp.	●				●	●						
Arthrobotrys sp.	●	●	●	●	●	●		●				
Humicola grisea	●	●		●	●	●		●				
Penicillium sp.	●							●				
Mucor sp.		●										
	no fungicide present		fungicide in test substrate				fungicide in both test substrate and agar plating medium					
Bacteria	●		●	●	●	●	●	●	●	●	●	●
Nematodes	●	●	●	●	●	●	●	●	●	●		●

Table 25 - 35°C. and 50°C.

Species list of fungi isolated in Perfusion Isolation Experiment (Series 1 and 2) 35°C.

Species	A	B	C	D	E	F	G	H	J	K	L	M
Graphium sp.	●	●										
Mucor sp.	●	●	●									
Trichoderma viride		●										
Aspergillus sp.	●											
Streptomyces sp.	●											
Aspergillus fumigatus			●									
Humicola grisea			●									
Bacteria				●	●	●	●	●	●	●	●	●

Species	A	B	C	D	E	F	G	H	J	K	L	M
							50°C					
Chaetomium thermophile	●	●			●		●					
Humicola grisea var thermoidea	●	●										
Thermoactinomyces glauca	●	●	●		●		●	●	●			●
Thermoactinomyces vulgaris			●		●							
Bacteria			●	●			●	●	●	●	●	●

no fungicide present

fungicide in test substrate

fungicide in both test substrate and agar plating medium

the environment which allow one particular species to become dominant over another may be very small. If the prime object of a biological test of a material is to apply stress; it may be argued that it is of little consequence as to which vigorous cellulolytic organism applies the stress. It is highly unlikely however that any two or more organisms will behave in exactly the same way, and thus there is value in ecological work at a species and genus level when variability in biological testing is being investigated. These experiments have shown a range of fungi which are able to colonise PCPL treated cellulose, and also that their ability to colonise varies, depending on the concentration of the fungicide present, and also on the nature of the substrate, variations being seen between cellulose agar, (as a secondary colonisation substrate in the case of these perfusion experiments) paper, and cotton textile.

The majority of fungal species were isolated at 25^{o}C; this was to be expected as mesophilic fungi are in the greatest majority in soil. The very erratic results obtained at 35^{o}C is to some extent explained by the fact that this temperature is around the borderline between true mesophilic and thermophilic ranges, and is possibly a temperature seldom maintained in nature in temperate climates, but rather passed through by a system in a self-heating state. Although there are comparatively few speices of thermophilic fungi (Cooney and Emerson, 1964) several were easily isolated at 50^{o}C, owing to the lack of competition at this temperature from other fungi (and organisms such as nematode worms), their vigorous mode of growth and their

frequent cellulolytic and widespread nature (Eggins and Malik, 1969). Throughout these experiments bacteria, being a normal part of the soil microflora, were probably always present with the fungi, but were only recorded where they were in sufficient numbers for colonies to be observed. It should be noted that as the intent of the experiment was to select out fungi, a bacteriostat, (rose bengal) was added to the agar media used (see appendix).

The lack of record of fungal species at various times during the experiments does not indicate a total absence of fungal material. Very weak growths of stunted vegetative hyphae were sometimes observed but were not recorded if they failed to develop after further incubation. The presence of such stunted growth is indicative of adverse growth conditions due to the fungicide and the presence of nematodes, as discussed earlier.

3.3. The testing of textiles

In the previous experiments fungi were isolated from soil onto cotton textile. Many of these fungi subsequently showed their cellulolytic nature by their ability to clear cellulose agar. The Clent soil used is therefore a suitable environment in which to study the breakdown of cotton textile, and was thus continued to be used in the following experiments.

3.3.1. Soil burial testing, using additional carbon sources

This experiment was designed not only to obtain basic information concerning the actual decay of cotton textiles in the soil used previously for isolation work, but also to study the effects of combinations of pentachlorophenyl laurate and alternative carbon sources upon the decay of cotton textile. The alternative carbon sources chosen were mainly starch and modified cellulose compounds, and are of interest in this context as they and similar compounds are used as commercial finishing agents for textiles.

These tests were carried out as preliminary tests on the decay of textiles to precede perfusion studies.

T.N.O. cotton test textile with the following treatments was employed in this experiment:-

A = Cotton test textile with a 5% loading of "Viscosol 220" (oxidised starch) and 1% of pentachlorophenyl laurate containing 0.3% of free pentachlorophenol.

B = Cotton test textile padded through white spirit and containing a loading of 5% "Viscosol 220",

C = Cotton test textile with a loading of 5% P.V.A. (polyvinyl acetate - "Maviol N90/98) and 1% pentachlorophenyl laurate containing 0.3% free pentachlorophenol.

D = Cotton test textile with a loading of 1% pentachlorophenyl laurate containing 0.3% of free pentachlorophenol, applied from white spirit.

E = Untreated cotton test textile padded through water only.

G = Cotton test textile with a loading of 5% maize starch.

H = Cotton test textile with a loading of 5% "Cellofas B grade B.50" (Sodium carboxymethyl cellulose).

K = Cotton test textile with a loading of 5% P.V.A. (Moviol N90/98).

These textile samples were cut into strips 6 inches long and 1 inch wide, with a 0.2 inch ravel along each long side. This ravelled edge prevents further fraying of the strip on handling and thus prevents strength loss errors due to detached warp threads. The strips were autoclaved at 15 p.s.i. for 20 minutes before burial in the soil.

The soil used in this experiment was the Clent meadow soil, contained in two polythene tanks in thermostatically controlled water baths. The soil was sifted before placing in the tanks, and turned daily for one week prior to the start of the experiment. Specimens of textile were subsequently divided between the two tanks for reasons of space, as set out below.

Test specimen groups A, C, D and H (those containing the fungicide) in tank 1, soil water content at the start of the experiment 18.5% of the dry weight, soil capable of holding 25.3% of its dry weight as water.

Test specimen groups B, E, G and K in tank 2, soil water content at the start of the experiment 21.2% of the dry weight, soil capable of holding 25.3% of its dry weight as water.

Within each tank, groups of specimens were buried in separate groups, as far apart as possible, in order to minimise any possible interactions. The samples were buried in the following manner. The soil was first turned and shaken down to the same level in each tank, each tank containing the same volume of soil. A thin wooden rule was pushed vertically into the soil to the bottom of the tank and then withdrawn. The test strip was then folded evenly over the end of the rule and pushed into the loosened soil area. The gap between the folds of the strip was then filled by trickling in loose soil (See figure 3).

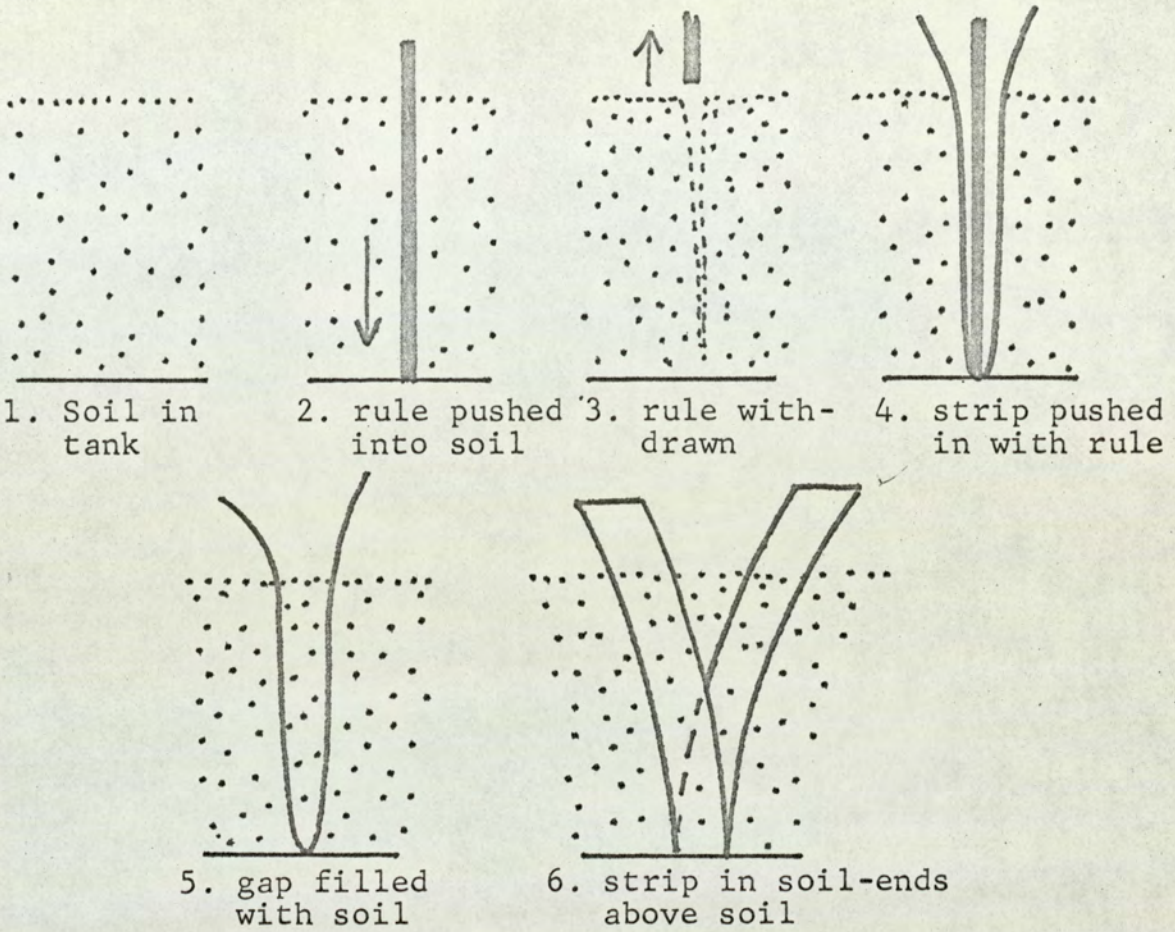


Figure 3. Method of inserting textile strips in soil in soil burial tests.

Incubation of the soil now containing the test strips was continued at 25°C. Strips were gently removed from the soil at intervals, washed in water to remove adhering soil, washed in 70% ethanol to inhibit further fungal growth, dried in air at room temperature and stored for subsequent tensile strength testing on a Hounsfield Tensometer. The results are given in graph 2. Each reading is the mean breaking strength of two similar sample strips, expressed as a percentage of the mean breaking strength of four

control strips. Separate controls were kept for each type of strip and the controls were subjected to the same autoclaving, washing and drying procedures as the test strips. All the strips were brought to a constant humidity by storing in a humidity and temperature controlled room for 72 hours before breaking.

From the results, shown in graph 2, it can be seen that:-

(i) All strips were degraded, as shown by the loss in tensile strength.

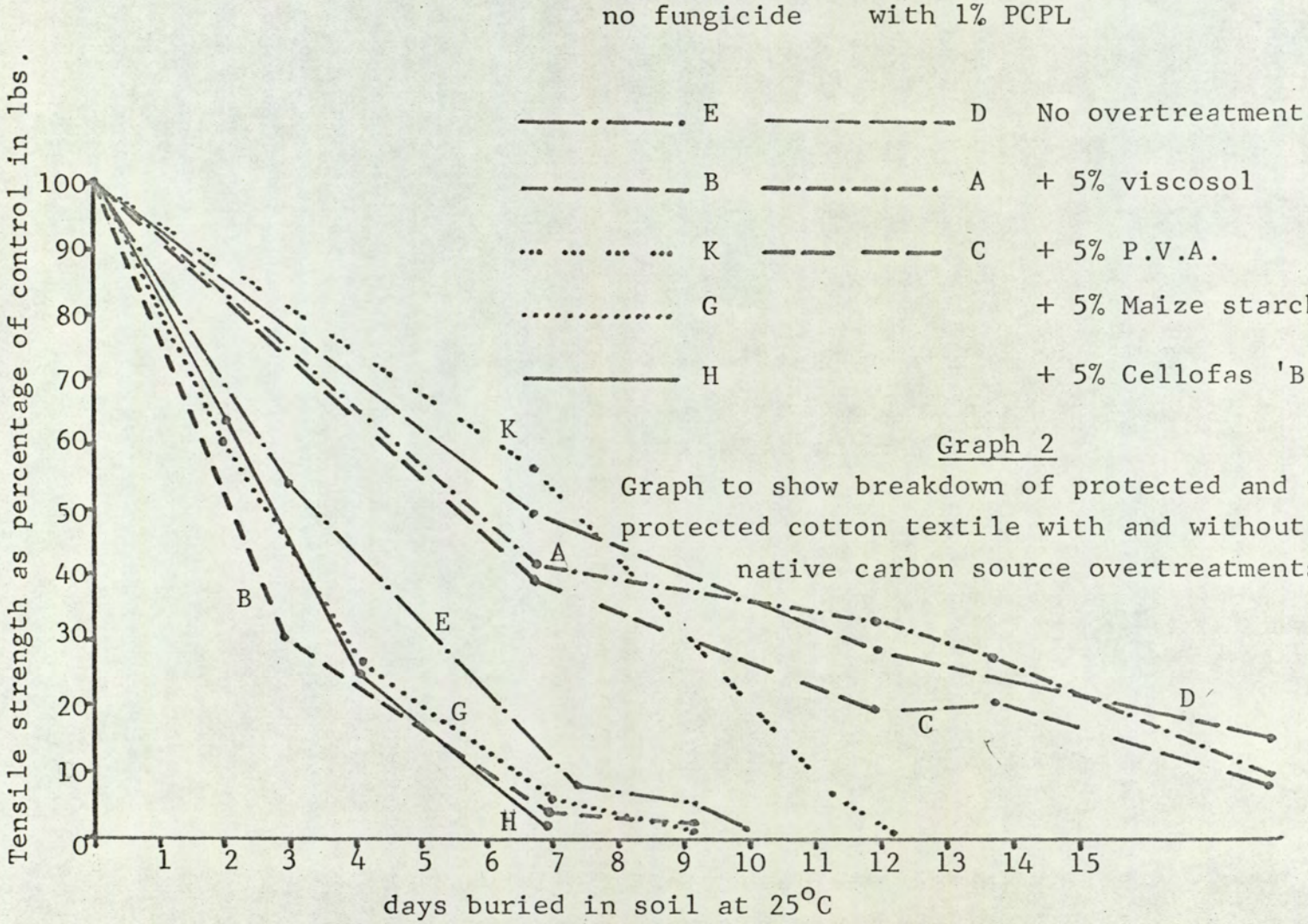
(ii) All strips containing no fungicide had lost all their strength after 12 days burial.

(iii) All strips which contained the fungicide (1% pentachlorophenyl laurate containing 0.3% free pentachlorophenol) still had approximately 10% residual strength after 18 days burial.

(iv) In the group of strips not containing the fungicide, the presence of the other carbon sources enhanced decay (except strip K - P.V.A. overtreatment).

(v) In the group of strips containing the fungicide, the presence of other carbon sources also enhanced decay

This experiment gave indications as to the most suitable intervals between sampling using this soil for such tests. With severe tests such as this, samples of cotton textile not treated with a fungicide should be taken at 2 or 3 day intervals, as decay progresses rapidly. If a fungicide is present, the interval may be extended to 3 to 4 days.



Graph 2

Graph to show breakdown of protected and unprotected cotton textile with and without alternative carbon source overtreatments.

The effect of the fungicide used here is clearly shown to be effective in inhibiting decay, prolonging the life of the textile almost twice as long as that of the untreated textile.

In work with cellulose agars (Bravery, 1968) it has been suggested that in the presence of more readily available carbon sources that cellulase production by fungi may be inhibited. The presence of alternative carbon sources in this test however appeared to have no such effect, the compounds used here being very different to those which have been implicated before (L-asparagine and yeast extract) and do not appear to inhibit decay. It can be postulated that especially in the more severe soil burial test, that the compounds used in this experiment may stimulate fungal growth in the soil, due to their potentially more easily assimilatable nature than cellulose, and because they are water soluble and can therefore easily spread into the soil in the immediate area of the strips thus forming a localised enrichment culture. Owing to their nature however, it is unlikely that they would cause rapid growth of sugar fungi to the exclusion of cellulolytic species.

Strip K (No fungicide, 5% P.V.A.) did not conform to the general pattern of increased decay. From the pattern of decay it exhibited it would appear that this was due to an initial lag in the onset of decay, a phenomenon met from time to time in such tests, and often attributed to conditions other than those of the substrate itself, such as a delay in adequate moisture reaching the textile or a fault

in soil packing at the start of the experiment (Turner, 1971). Another possible explanation in this particular case, although not applicable in the case where the fungicide was also present in the textile, is that P.V.A. is a much less 'natural' material than the other materials used for overtreatment of the textile and is less prone to decay.

3.3.2. Colonisation and strength testing of fungicidally-treated textile, with the incorporation of a perfusion technique

These experiments were designed to incorporate the severity of soil burial testing with ease of fungal isolations under more standardised conditions. Decay as shown by strength losses was able to be investigated and related to the fungal colonisation of textiles. The strength of the decayed strips was measured using simple equipment developed for this purpose, which is described in Chapter 7.

Some of the textile samples employed here were the same as used in the previous soil burial experiment for purposes of comparison. This experiment also serves as a demonstration of perfusion techniques as screening methods for new potential fungicides, three experimental compounds being investigated.

T.N.O. cotton test textile with the following treatments were used in this experiment:-

(1) Untreated textile (as E in previous experiment)

(2) Textile with a 1% loading of "Mystox LPL" (technical grade pentachlorophenyl laurate complying with BS.4024) (Catomance Ltd. "Mystox" date sheet T/1) applied from white spirit (essentially the same as sample D in the previous experiment).

(3) Textile with a 1% loading of pentachlorophenyl laurate containing 0.3% free pentachlorophenol with an overtreatment of 5% Cellofas B".

(4) Textile with a loading of 1% ammonium sulphate compound of ortho-phenyl phenoxy isopropanol applied from water (a new potential fungicide and referred to here as P.1.

(5) Textile with a loading of 1% ortho-phenyl phenoxy isopropanol, applied from "Imsol A"/water solution 2:1 (a new potential fungicide and referred to here as P.2.).

(6) Textile with a loading of 1% pentachlorophenoxy isopropanol/trichlorophenoxy isopropanol 70:30 mixture, applied from "Imsol A"/water solution 2:1 (a new potential fungicide and referred to here as P.3.).

The textile samples were cut into strips. Each strip comprised exactly ten warp threads of the textile. Strips were 7cm long and had a 3mm ravel on each side. This size of strip was chosen, as its breaking strength before the experiment fell in the upper range of the strength testing machine, the strips breaking at around 20Kg loading, the machine having a range of 0 - 25.0Kg.

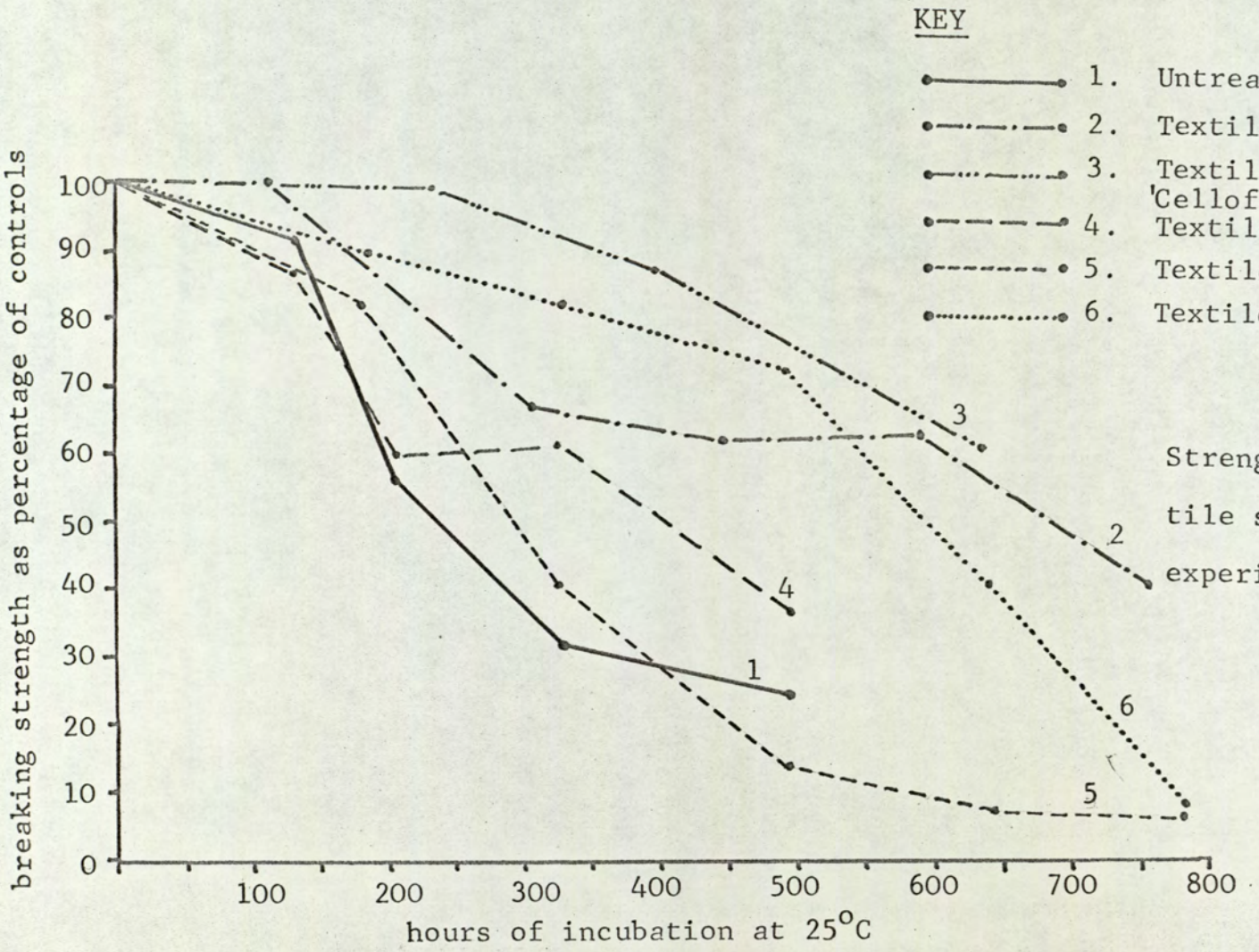
The strips were incorporated into perfusion kits as previously described, one strip to each of the 15 petri dishes in each kit. The strips were separated from direct contact with the soil inoculum by a glass-fibre screen. The liquid nutrient perfusate employed was Eggins and Pugh nutrient salts solution. After the complete kits were autoclaved at 15 p.s.i. for 20 minutes, each petri dish was inoculated by placing a small amount of Clent soil on the glass-fibre screen over the textile strip. The kits were then incubated at 25°C. The strips were sacrificed at intervals on each occassion ten strips being used for strength testing and five for plating-out on cellulose and glucose/starch agar. The strips used for strength testing were treated as follows:-

(i) After gentle removal from the perfusion kits the strips were washed in 70% ethanol to remove any adhering fungal material.

(ii) The strips were then washed in water containing a small amount (approximately 0.1%) of domestic detergent solution. This was to ensure that the strips were completely wet before strength testing, as this was the most convenient way of standardising water content.

(iii) Strips were immediately broken on the strength testing machine

The results obtained by strength testing are shown in graph 3. It can be seen from this graph that all strips lost strength during the experiment but to varying degrees. The individual treatments are discussed below.



Graph 3

Strength losses of textile strips in perfusion experiment.

Discussion - Strength losses of textile strips

1. Untreated textile

The progress of decay was according to the pattern expected. There was an initial lag, as usually found where screened substrates are employed (See chapter 2). Decay then proceeded rapidly, the rate slowing down before the experiment ended. Compared with the preceding soil burial experiment, decay took approximately twice the time to reach the same levels, but the overall pattern of decay was however very similar.

2. Textile + 1% "Mystox LPL"

Here again the decay pattern proceeded as expected, however the time taken for the textile to loose 50% of its initial strength was 3 times that taken by the untreated textile strips, as opposed to twice as long in the soil burial test experiments using the two similar textiles. This may indicate a useful feature of perfusion testing in that differences between treated and untreated materials, or between treatments, may be highlighted and therefore more clearly seen.

3. Textile + 1% PCPL + 5% "Cellofas B"

Decay was slow here, especially in the initial stages. The time taken to loose 40% of its initial strength (maximum loss at end of experiment) was almost the same as the textile with 1% "Mystox LPL" alone. (The PCPL and "Mystox LPL" used here had the same composition, the only difference being that the PCPL was a Catomance Limited laboratory preparation

of "Mystox LPL" which is produced in Catomance Limited production plant). Thus, there the presence of "Cellofas B" had little effect on decay. In the previous soil burial experiment, the difference between untreated textile and that treated with 5% "Cellofas B" alone was also small.

4, 5 and 6. Textile strips with 1% of potential fungicides P.1, P.2 and P.3.

All the strips decayed more rapidly than those treated with 1% "Mystox LPL". A comparison may be made:-

Fungicide	Time to 50% strength loss (Hours)	Time to 70% strength loss (Hours)
"Mystox LPL"	690	290
P.1	400	175
P.2	290	225
P.3	600	500

Decay patterns did vary however, as can be seen from graph 3 and last column of the above figures. The "P series" fungicides appear to be most effective in the early stages of decay, especially P3, which is much more effective than "Mystox LPL" in the early stages, and may on subsequent testing compare favourably in the longer term also.

By providing sufficient replicate test strips in this experiment it was also possible to carry out fungal isolation work in parallel with the strength testing. The results obtained from the isolation work in this experiment are shown in figures 3 - 14 inclusive, together with individual strength loss graphs for purposes of comparison.

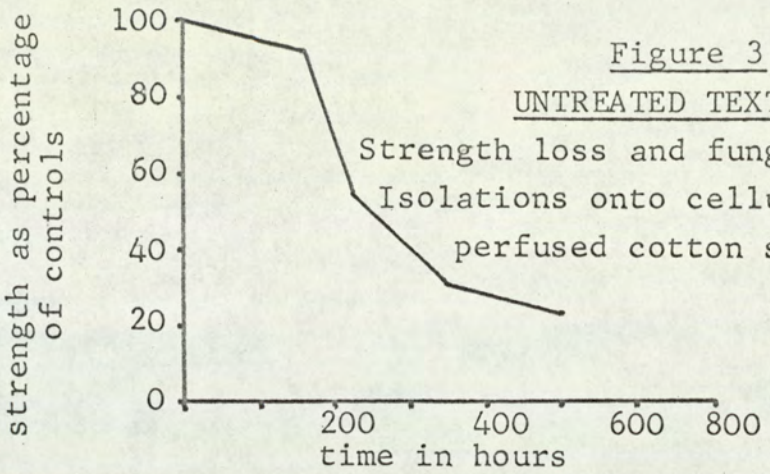
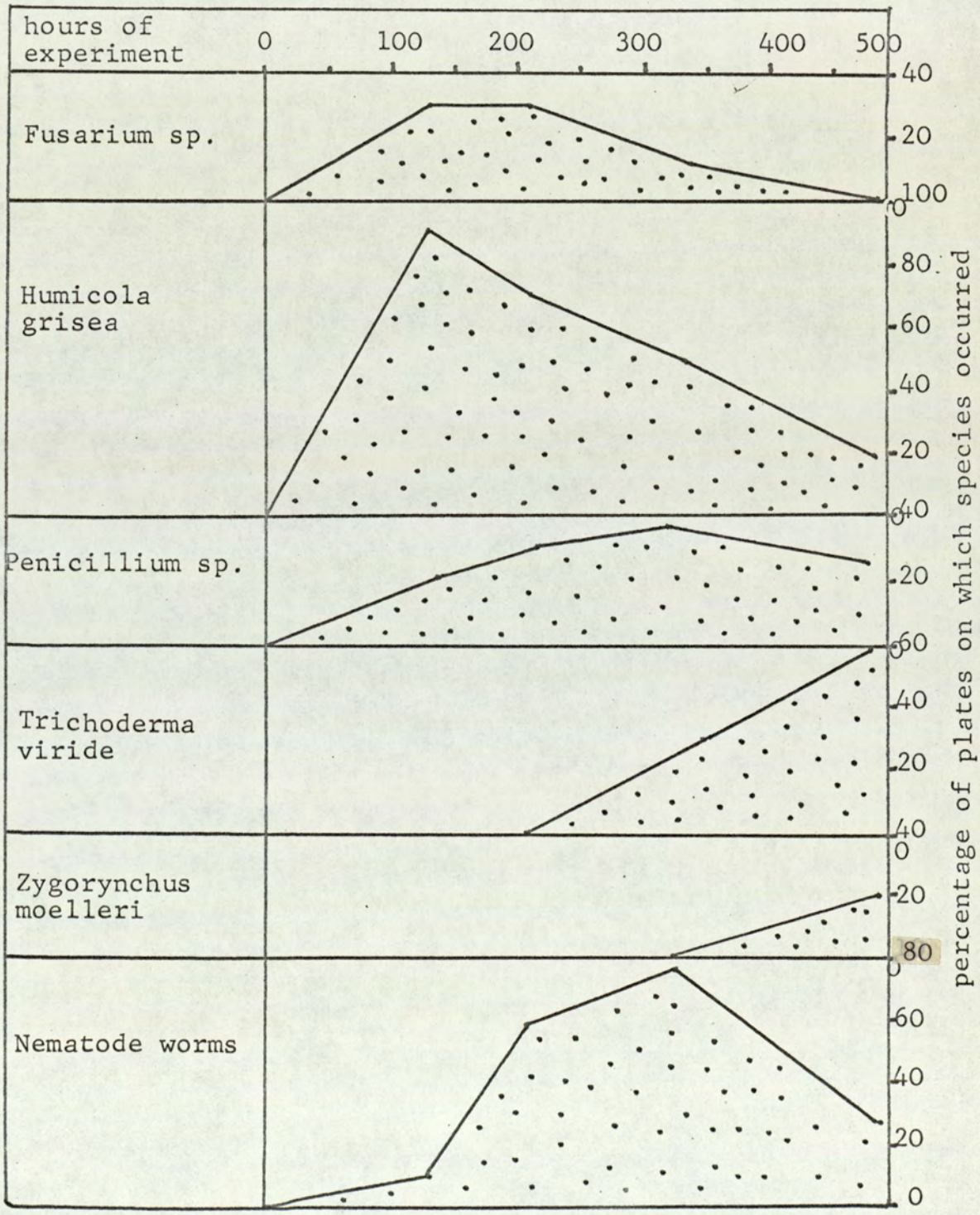


Figure 3

UNTREATED TEXTILE

Strength loss and fungal colonisation. Isolations onto cellulose agar from perfused cotton strips



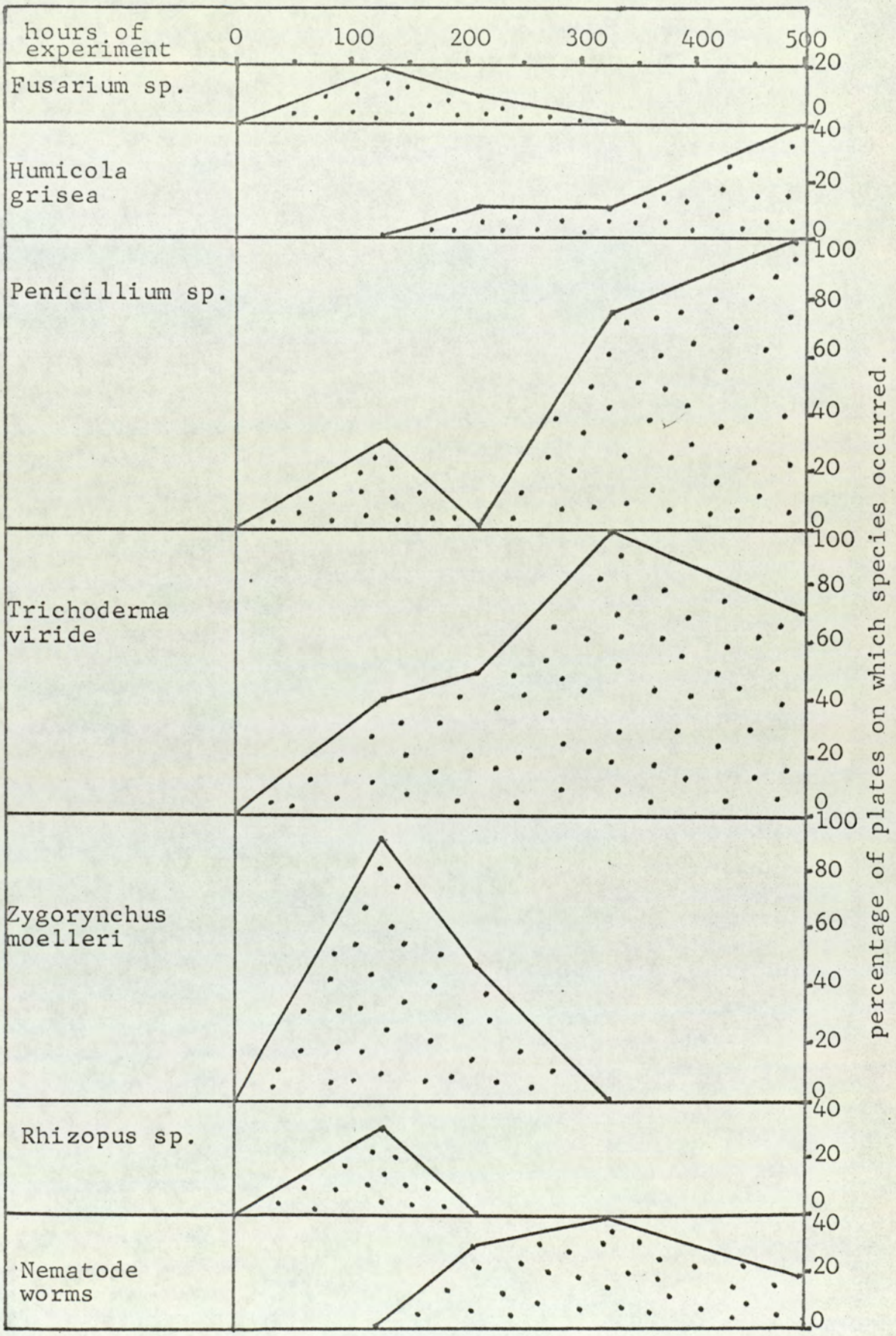


Figure 4

UNTREATED TEXTILE. Fungal colonisation. Isolations made onto glucose-starch agar from perfused cotton strips. See graph 3 for strength losses.

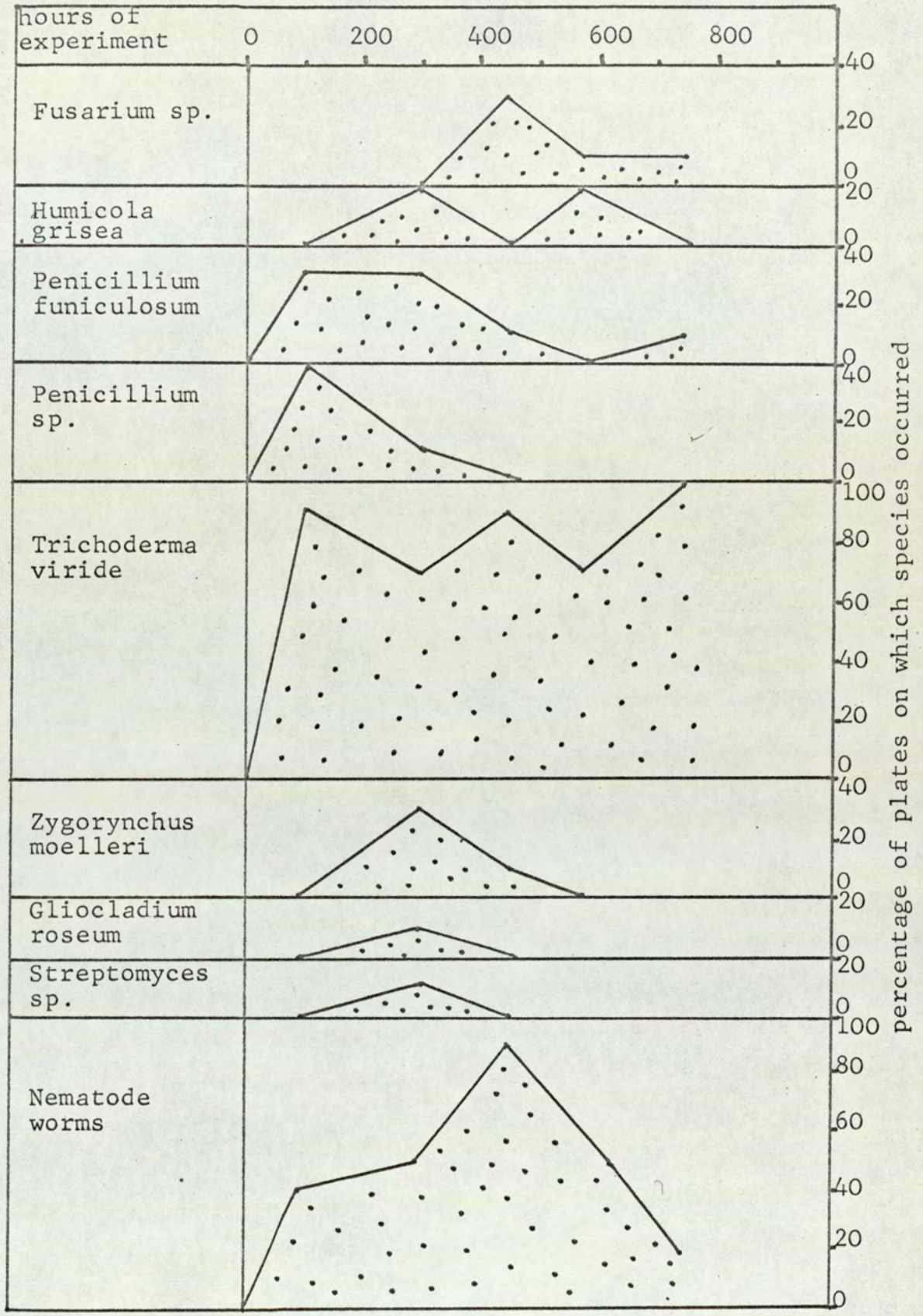


Figure 5

COTTON TEXTILE + 1% 'MYSTOX LPL'. Fungal colonisation. Isolations made onto cellulose agar from perfused textile strips. See Graph 3 for strength losses.

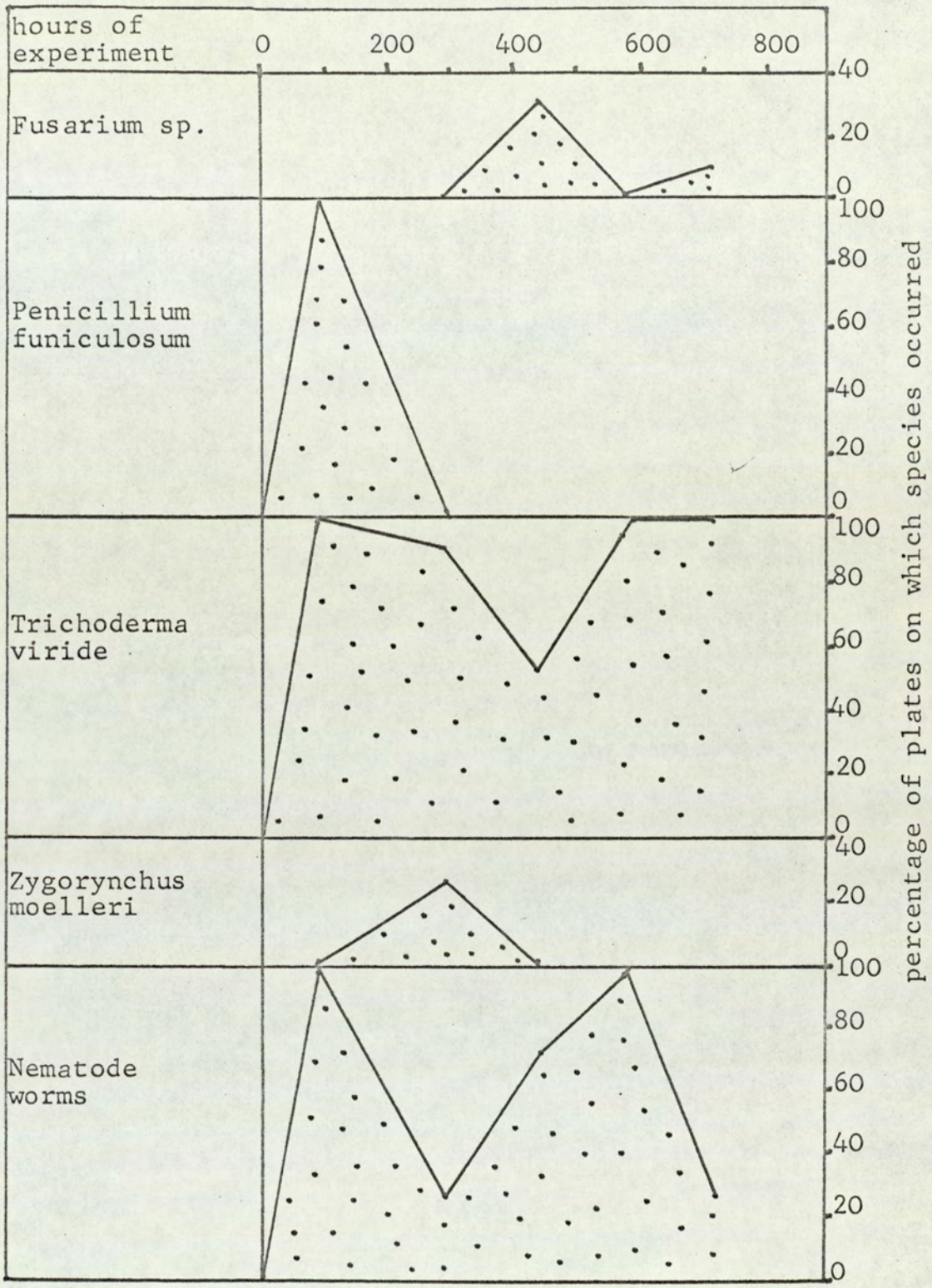


Figure 6

COTTON TEXTILE + 1% 'MYSTOX LPL'. Fungal colonisation. Isolations made onto glucose-starch agar from perfused textile strips. See graph 3 for strength losses.

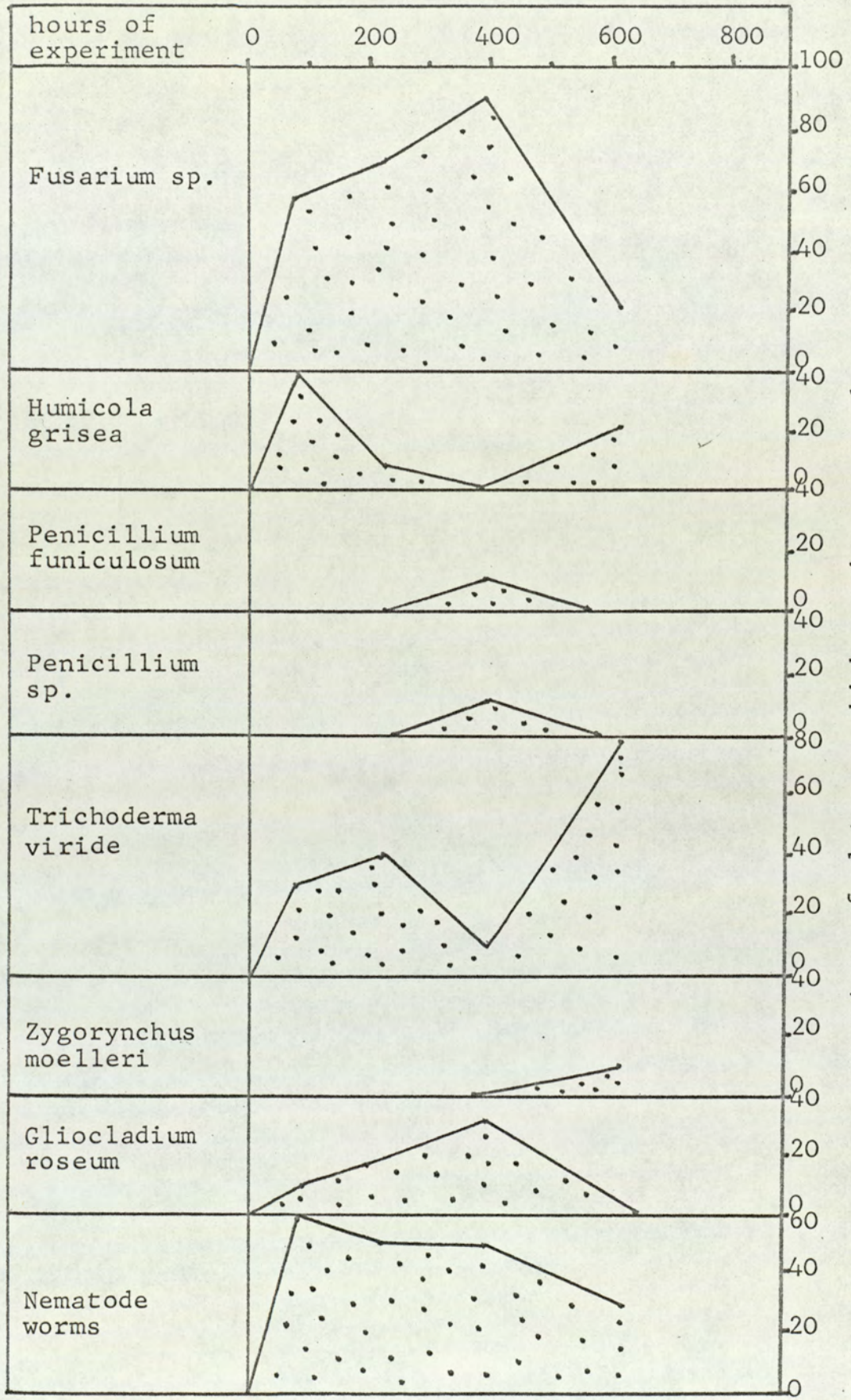


Figure 7

COTTON TEXTILE + 1% PCPL + 5% 'CELLOFAS B'. Fungal colonisation. Isolations made onto cellulose agar from perfused textile strips. See graph 3 for strength losses.

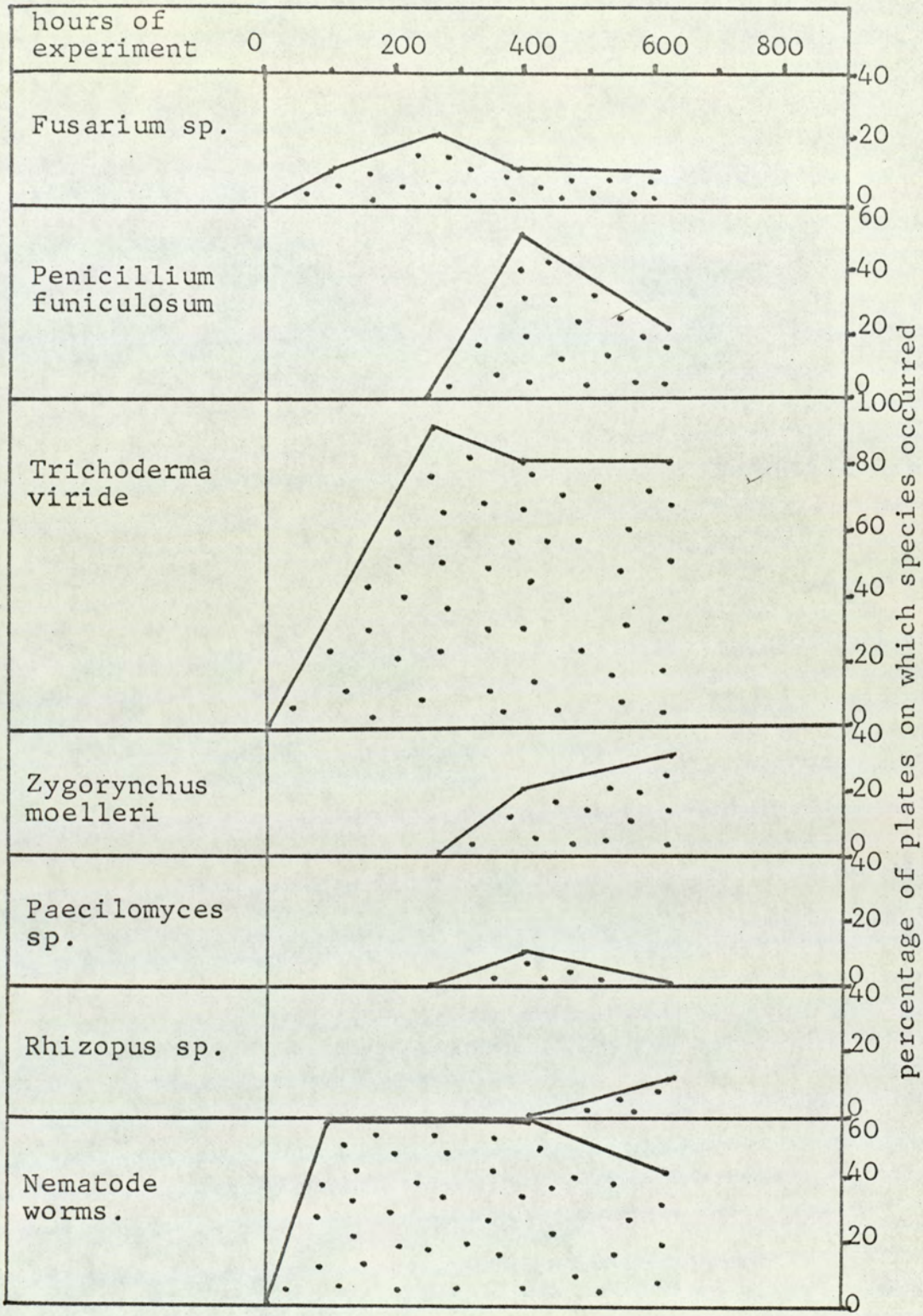


Figure 8

COTTON TEXTILE + 1% PCPL + 5% 'CELLOFAS B'. Fungal colonisation. Isolations made onto glucose-starch agar from perfused textile strips. See graph 3 for strength losses.

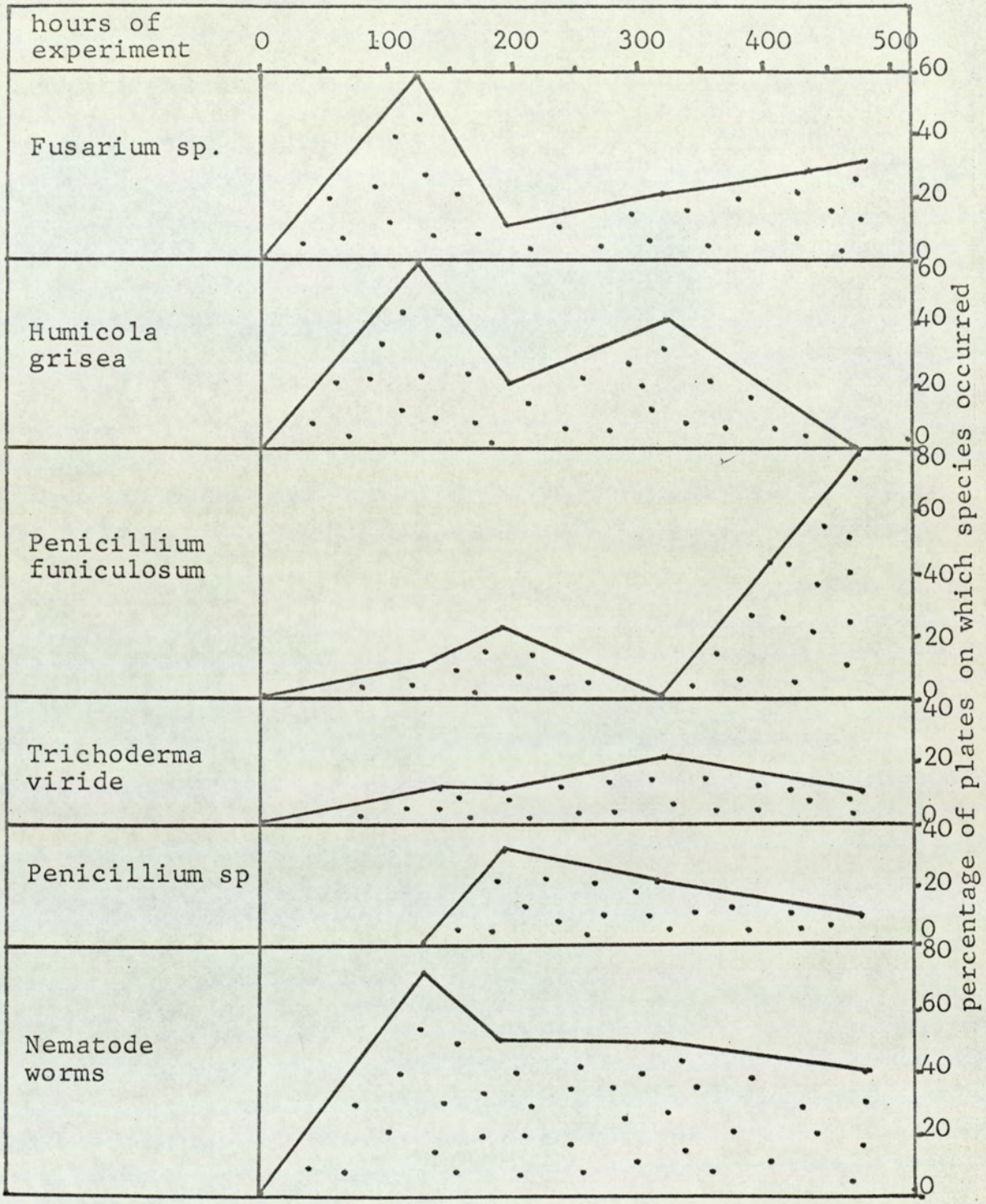


Figure 9

COTTON TEXTILE + 1% "P1" FUNGICIDE. Fungal colonisation. Isolations made onto cellulose agar from perfused textile strips. See graph 3 for strength losses.

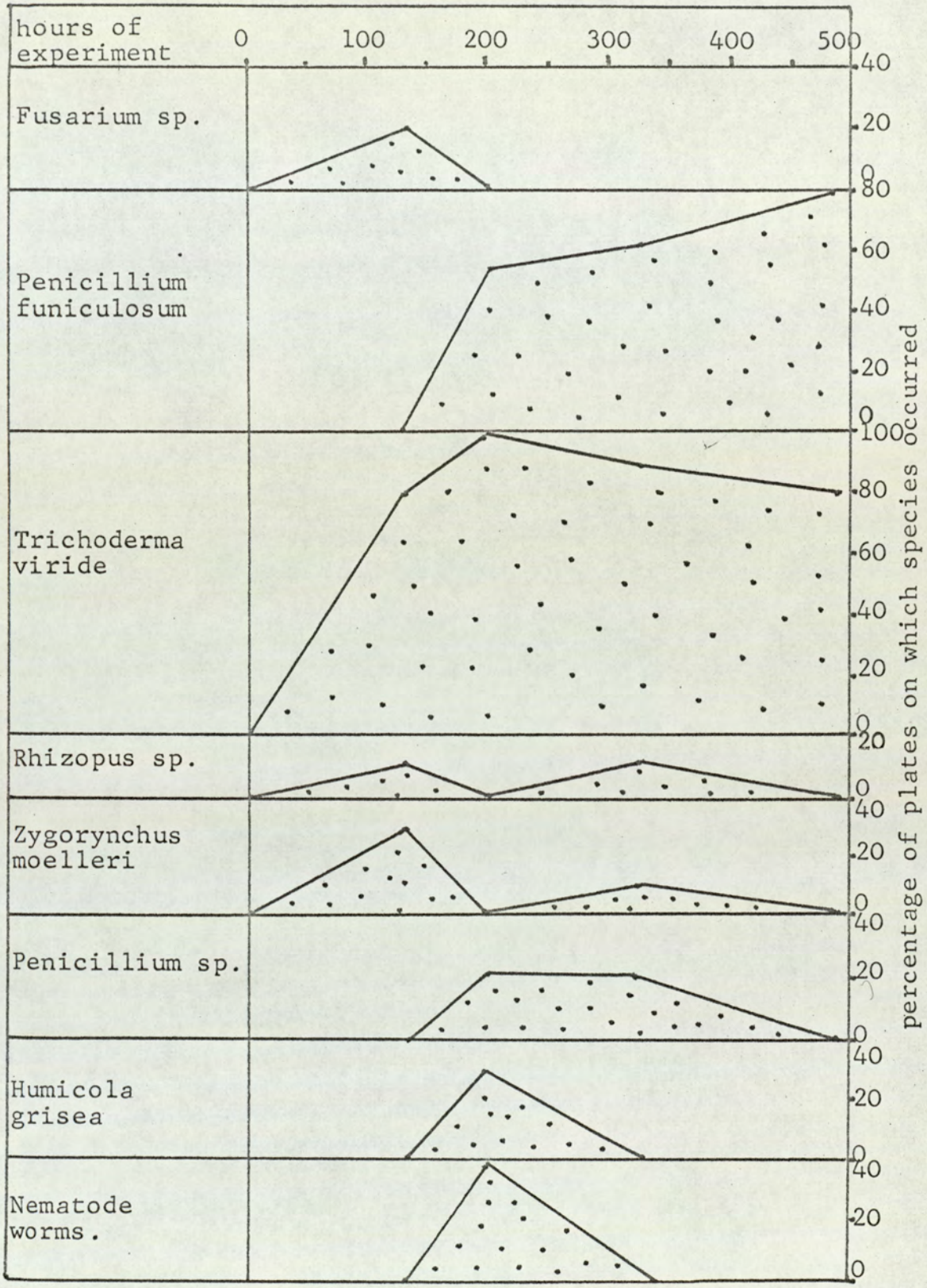


Figure 10

COTTON TEXTILE + 1% "P1" FUNGICIDE. Fungal colonisation. Isolations made onto cellulose agar from perfused textile strips. See graph 3 for strength losses.

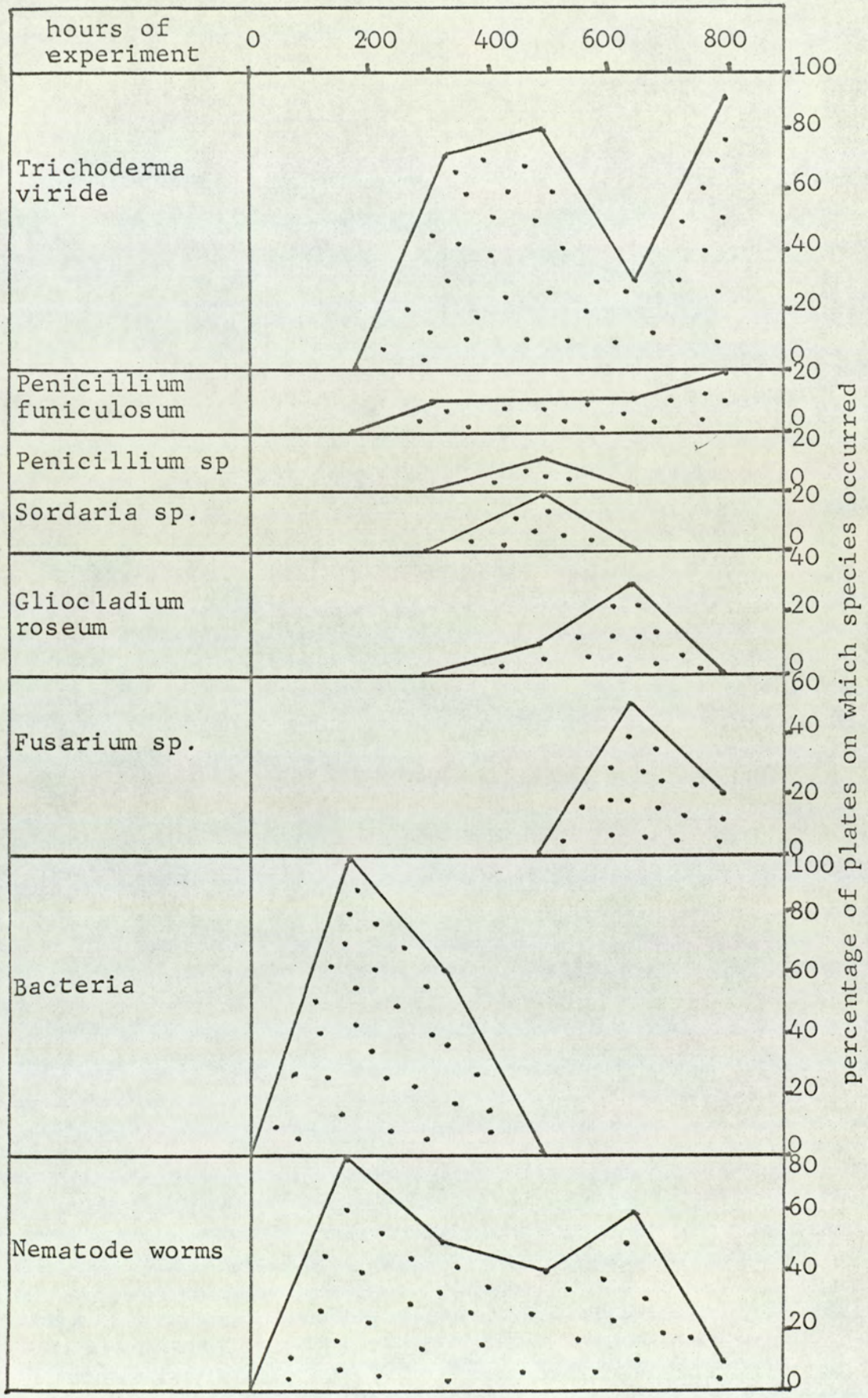


Figure 11

COTTON TEXTILE + 1% "P2" FUNGICIDE. Fungal colonisation. Isolations made onto cellulose agar from perfused textile strips. See graph 3 for strength losses.

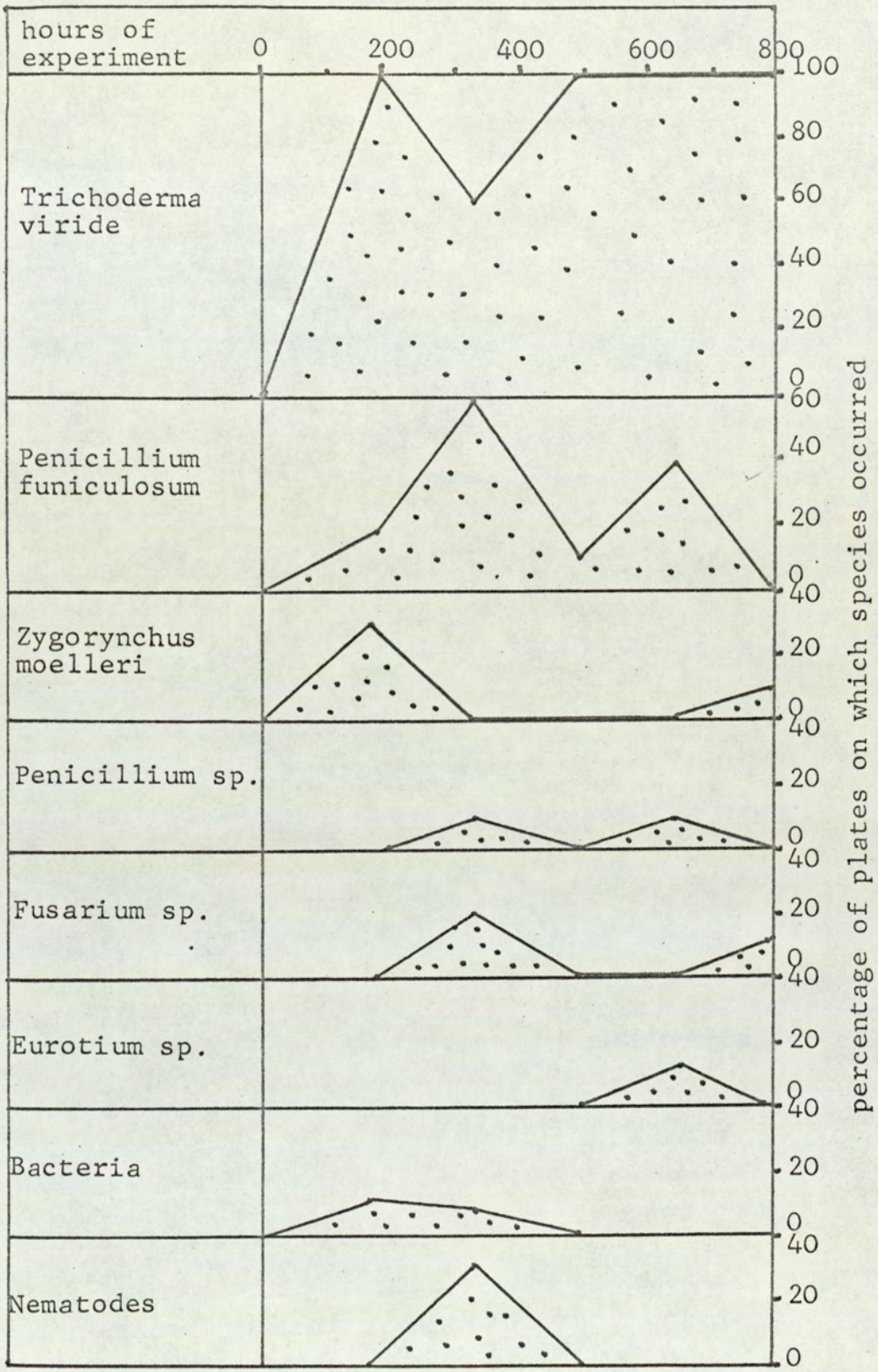


Figure 12

COTTON TEXTILE + 1% "P2" FUNGICIDE. Fungal colonisation. Isolations made onto glucose-starch agar from perfused textile strips. See graph 3 for strength losses.

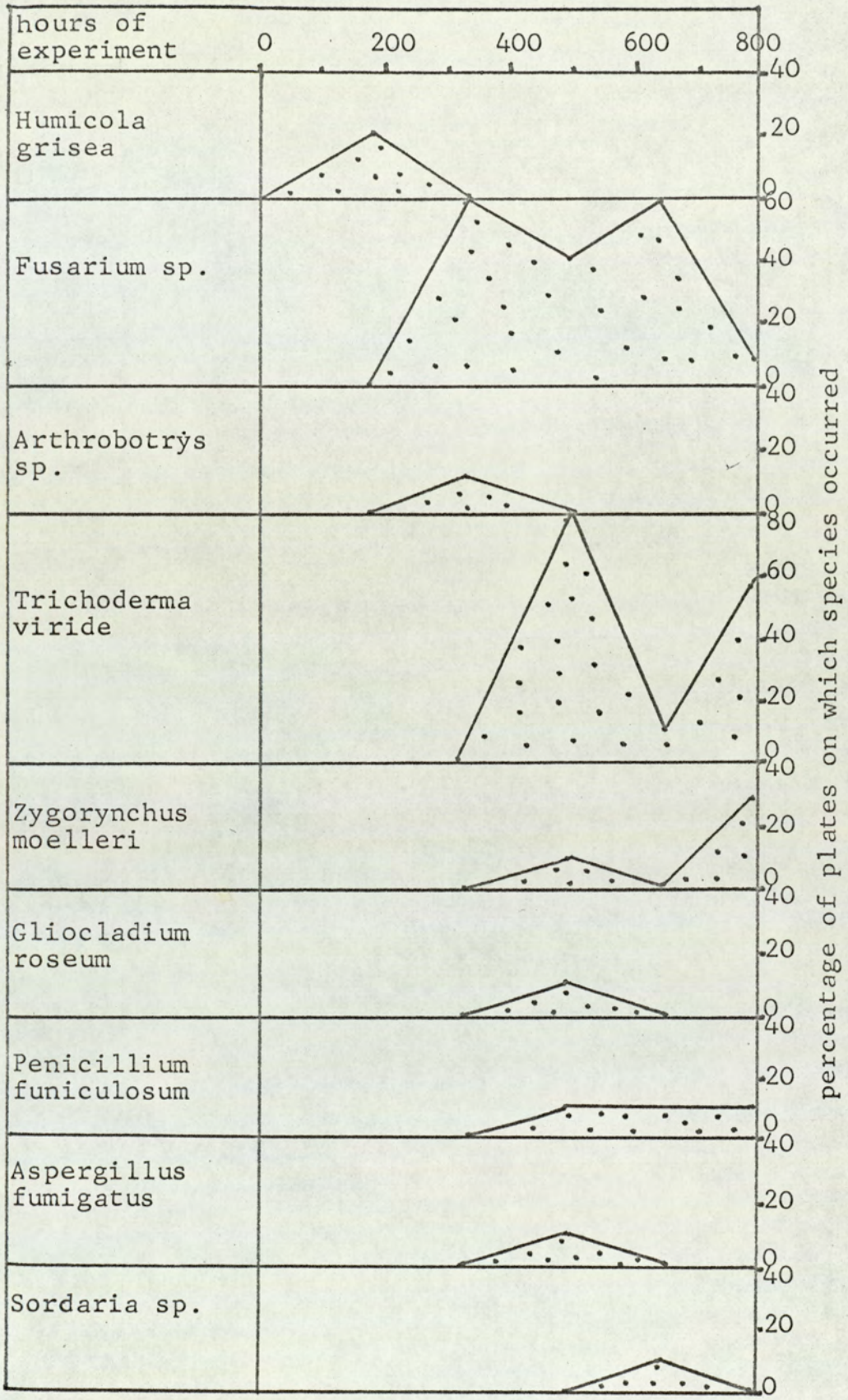


Figure 13

(Continued in figure 13A over)

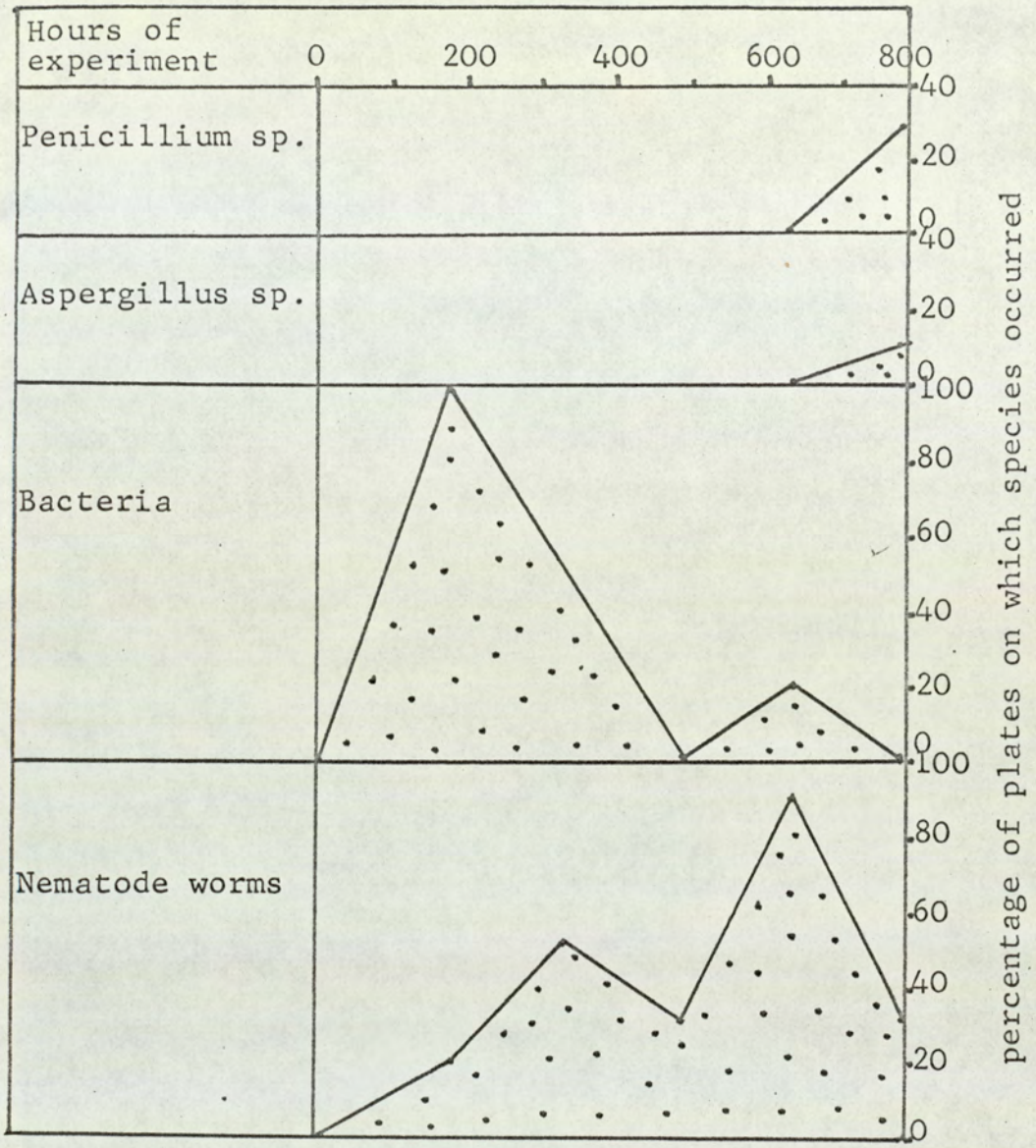


Figure 13A

Figure 13 and Figure 13A

COTTON TEXTILE + 1% "P3" FUNGICIDE. Fungal colonisation. Isolations made onto cellulose agar from perfused textile strips. See graph 3 for strength losses.

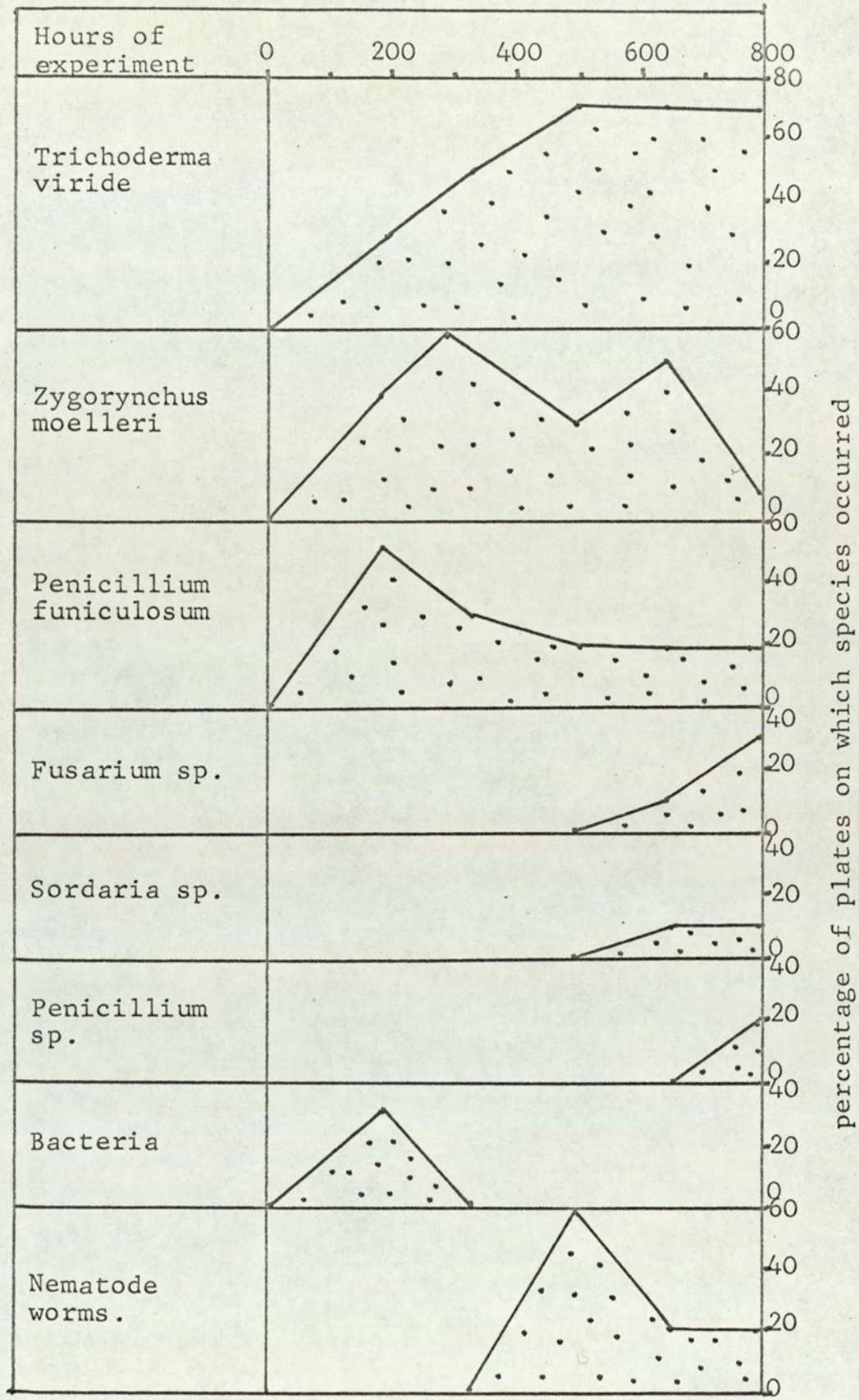


Figure 14

COTTON TEXTILE + 1% "P3" FUNGICIDE. Fungal colonisation. Isolations made onto glucose-starch agar from perfused textile strips. See graph 3 for strength losses.

Several basic conclusions may be drawn from these results.

(i) Fungi were isolated from each type of textile

(ii) Among the fungi isolated, cellulolytic species were isolated from each type of textile, as demonstrated by growth on and clearing of cellulose agar.

(iii) The dominant fungal species were similar throughout on the variously treated textile.

The individual treatments are discussed below:-

1. Untreated textile

On cellulose agar, the dominant species isolated was Humicola grisea, as in the previous perfusion isolation experiments. This species only declined on the occurrence of Trichoderma viride. A similar pattern, but to a lesser extent was followed by Fusarium sp. Only Trichoderma viride continued to flourish as the occurrence of nematode worms came to its maximum.

On glucose/starch agar the same species were isolated from textile, with the addition of Rhizopus sp, a sugar fungus to be expected on this medium. Trichoderma viride and Penicillium funiculosum were the most dominant fungi here. Humicola grisea was present in smaller quantities, but here appeared to join the dominant forms as one of the more resistant species to nematode damage. Fusarium sp. again appeared to decline in the presence of Trichoderma viride.

2. Textile + 1% "Mystox LPL"

On both cellulose and glucose/starch agars, Trichoderma viride was dominant throughout. Occurrence of other species was similar to that from untreated textile, but of a lesser magnitude.

3. Textile + 1% PCPL + 5% "Cellofas B"

On cellulose agar, Fusarium sp. was dominant, followed by Trichoderma viride. The peak in occurrence of Fusarium sp. coincided with the lowest value for the occurrence of Trichoderma viride. The peak in occurrence of Gliocladium roseum also occurred at this point. On glucose/starch agar Trichoderma viride was dominant, followed by Penicillium funiculosum. The occurrence of nematode was high and followed a similar pattern to that on cellulose agar.

4. Textile + 1% fungicide "P.1"

On cellulose agar, Fusarium sp. and Humicola grisea were predominant at first, the latter species later declining to be replaced by an increase in Penicillium funiculosum. On glucose/starch agar, Trichoderma viride was again dominant. Penicillium funiculosum being the next most abundant species.

5. Textile + 1% fungicide "P.2"

On cellulose agar, Trichoderma viride was the dominant form. The point of its lowest occurrence coincides with the peaks of Fusarium sp. and Gliocladium roseum. Bacteria

appeared to delay colonisation here at first. Trichoderma viride was also the dominant species on glucose/starch agar.

6. Textile + 1% fungicide "P.3"

From the isolations made onto cellulose agar, it appears that colonisation of the textile was delayed by bacterial growth in the first instance, the only fungus present being Humicola grisea. Fusarium sp. later became dominant, later declining to Trichoderma viride. There later appeared to be some undecided competition between these two species. On glucose/starch agar, Trichoderma viride, Penicillium funiculosum and Zygorynchus moelleri were common at first, Penicillium funiculosum declined somewhat at a later stage; Zygorynchus moelleri varied in frequency of occurrence, declining eventually, Trichoderma viride remaining as the dominant species.

Colonisation patterns

Within the environment of this experiment, a group of predominant fungi became apparent. This would appear to be due to the fact that they were all vigorous forms, and that they are mainly cellulolytic fungi in regard to cellulose agar, and now also appear to have an affinity for cotton cellulose. This group comprises Trichoderma viride, Fusarium sp., Humicola grisea and Penicillium funiculosum. By the nature of the experiment, it would also appear that these fungi are among the potential deteriogens most resistant to the fungicides employed. There is a tendency for Trichoderma viride to be antagonistic towards Humicola grisea and Fusarium sp. especially if established first.

A strong existing growth of Fusarium sp. may reverse this effect, but this is much less common.

Another group of fungi may be seen, the group of common 'secondary' fungi. These are Zygorynchus moelleri, Gliocladium roseum, Arthrobotrys sp. and Rhizopus sp. Zygorynchus moelleri and Rhizopus sp. usually fulfill here the expected role of secondary sugar fungi, and are rarely primary colonisers. The occurrence of Arthrobotrys sp. may be linked with the presence of nematode worms, as members of this genus are known to prey on nematode worms (Talbot, 1971), these worms being frequently found in this soil. Gliocladium roseum was present on the two occasions when Fusarium sp. was antagonistic to Trichoderma viride, and may play some part in this phenomenon.

Resistance to nematode attack is best shown by Trichoderma viride and Penicillium funiculosum. Although Trichoderma viride has a well known antagonism to other fungi by the production of antibiotic substances (the work since 1932 being summarised by Garrett, 1963) it is not known whether there is any inhibition of nematodes by this means even if indirectly by removing food bacteria. It may be simply due to its rapid growth and tolerance to inhibitors. Penicillium funiculosum frequently releases a deep red pigment into the media used here, but again a possible explanation is its vigorous growth.

Comparison of the results of decay of the textiles, together with the colonisation results shows a correlation between loss in strength and predominance of cellulolytic

fungi and underlines the importance of this group of organisms in the breakdown of fungicidally treated and untreated cotton textiles.

General discussion

The experiments in this chapter have demonstrated several points concerning the colonisation and decay of PCPL-treated cellulosic materials. Throughout, fungi have been isolated which are not only cellulolytic but are also resistant to the fungicide. These fungi were also shown to be resistant to varying degrees, as demonstrated by less frequent occurence of species on the highest concentrations of fungicides used on materials or in situations where the fungicide was also used in subsequent agar growth media. By the use of substrate screening, further differentiation of fungal isolates was possible, true colonisers being able to be isolated and identified.

The experiments also served to show the complex nature of ecological studies using soil, shown by variations in colonisation patterns even using the same technique. Increases in such variation are also shown when a toxic component is added to the system, resulting in erratic or aberrant growth by fungi. Within these experiments it also proved possible to demonstrate that alternative carbon sources generally had little effect on the decay of cotton cellulose and also that the efficacy of the PCPL fungicide declined at high incubation temperatures.

By designing experiments to give parallel results both for textile decay and fungal colonisation, it also proved possible to correlate these two features and further confirm the importance of several fungal species as cotton textile deteriogens.

Chapter 4

THE DEVELOPMENT AND USE OF A MULTIPLE PERFUSION TECHNIQUE FOR THE RAPID INITIAL SCREENING OF TEXTILE FUNGICIDE TREATMENTS

4.1. Introduction

4.2. Assembly of perfusion kits, and the use of the technique on a comparison of various fungicide treatments.

4.3. Use of the technique in the study of decay of artificially-weathered fungicidally-treated textiles

THE DEVELOPMENT AND USE OF A MULTIPLE PERFUSION TECHNIQUE FOR THE RAPID INITIAL SCREENING OF TEXTILE FUNGICIDE TREATMENTS

4.1. Introduction

An important problem in the biological testing of materials is the provision of a constant environment under which the attack microorganisms on materials can quickly and easily be observed and compared. This technique was developed to provide a means whereby three material specimens can be subjected to biological stress under identical conditions, and a rapid indication of the organism's ability to maintain surface growth, liberate pigment, sporulate, or perform other biological activity, can be obtained and compared with controls. Due to the nature of this technique, it may be employed over long periods, if necessary. This is of particular significance in the testing of fungicidally protected textiles.

Fungi grown on damp textile samples in petri-dishes tend to grow in an erratic manner, possibly due to the complex physical nature of woven textiles (as compared to agar) and local variations in moisture content. Thus, measurement of colony diameter as an indication of growth may be difficult. The steady flow of liquid nutrients through a perfused strip, however, tends to even out the colony form as it responds to the steady and constant flow, and a neat regular front of hyphae usually progresses along the test strip, thus facilitating measurement

4.2. Assembly of perfusion kits, and the use of the technique in a comparison of various fungicide treatments.

The perfusion kits followed the general principles of Eggins, Malik and Sharp (1968). The configuration of the kits is shown in figure 15 and plates 2 and 3. The important feature of this technique is the divided or multiple perfusion bed contained within the petri-dish, which carries nutrients to separate material samples under test. These perfusion beds were cut from glass-fibre tape, and edged with a strip of silicone rubber adhesive, applied from a tube via an applicator nozzle and allowed to set to form a ridge on the underside edge of the glass-fibre bed. This edging serves to prevent fraying to facilitate handling, washing and subsequent re-use of the bed, and also to raise the bed from the surface of the lower valve of the petri-dish, thus allowing more aeration and avoiding cross-flow of metabolites or other substances liberated and carried in the nutrient flow from textile strips under test. The portion of the tape carrying nutrient solution from the reservoir to the petri-dish was cased in 'layflat' tubing to prevent contamination from the atmosphere.

Strips of textile were cut and fastened to the perfusion bed by small spots of silicone rubber adhesive at the corners. After assembly of the kits, the reservoirs were filled two-thirds full of Eggins and Pugh nutrient salts solution. Glass microscope slides were laid across the textile strips and perfusion beds to prevent buckling during sterilisation, and the entire closed assemblies were autoclaved at 15 p.s.i. for 15 minutes.

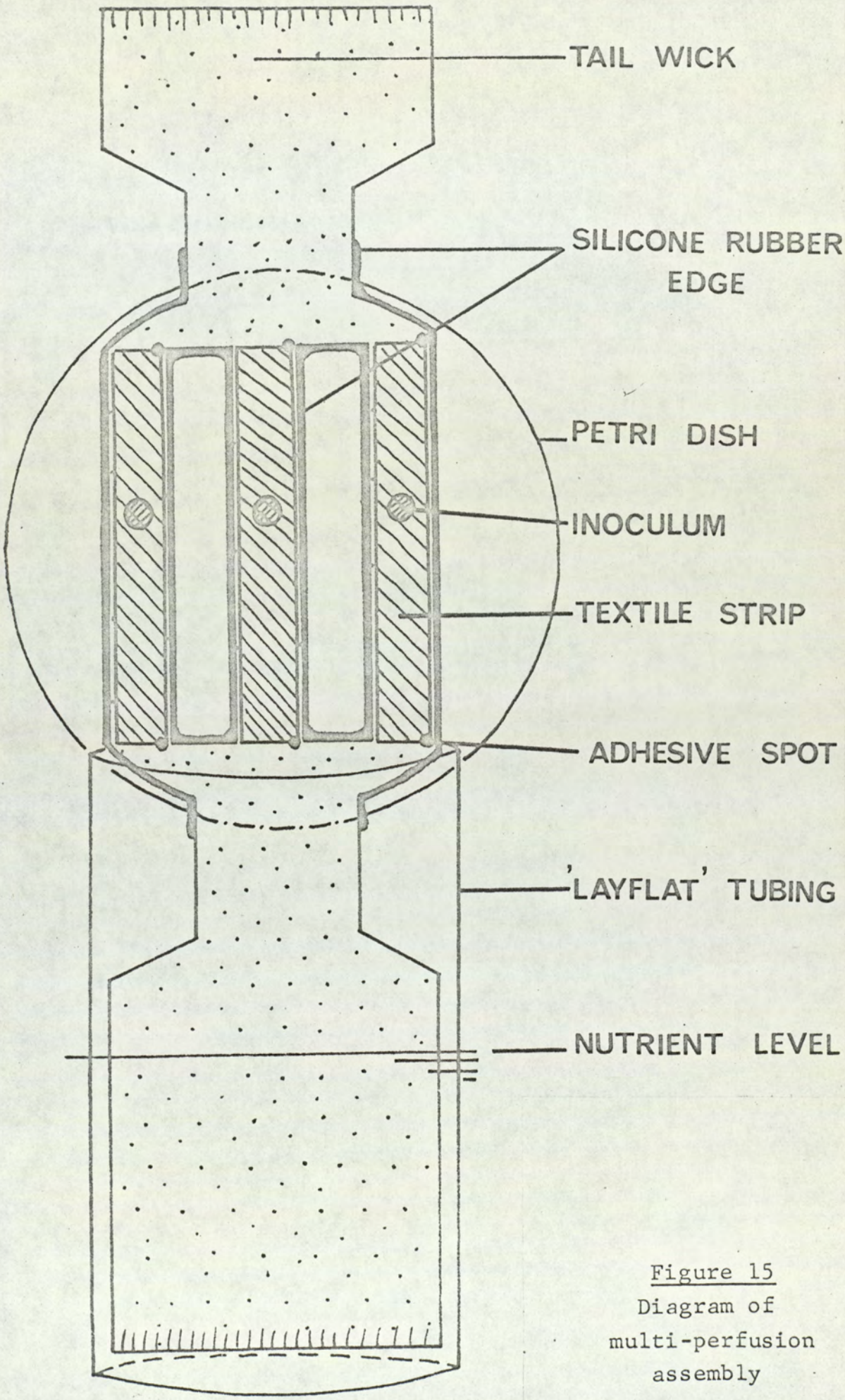


Figure 15
Diagram of multi-perfusion assembly

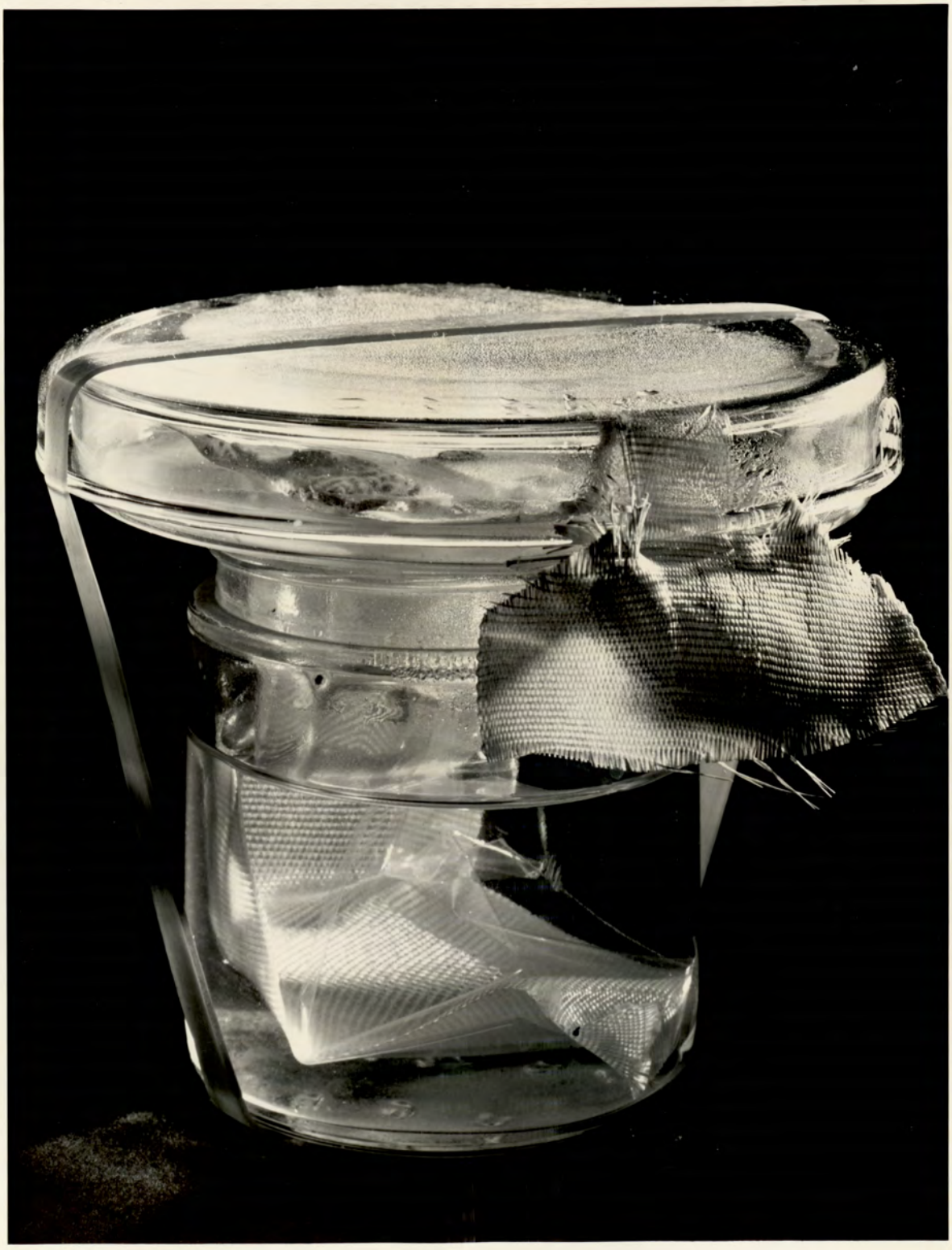


Plate 2

A completed perfusion kit in use. (Multi-perfusion assembly). Note lower liquid nutrient reservoir and free wick.

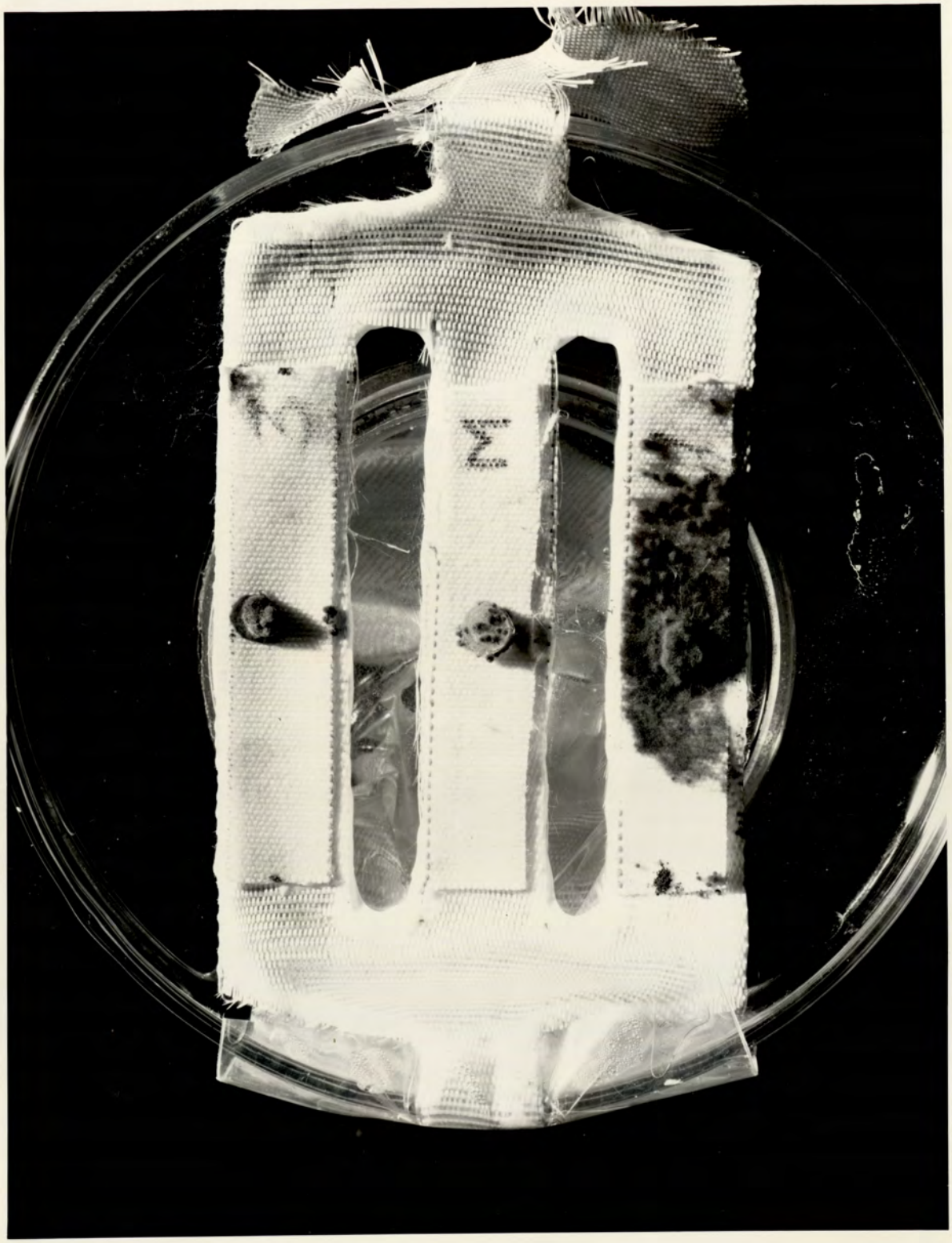


Plate 3

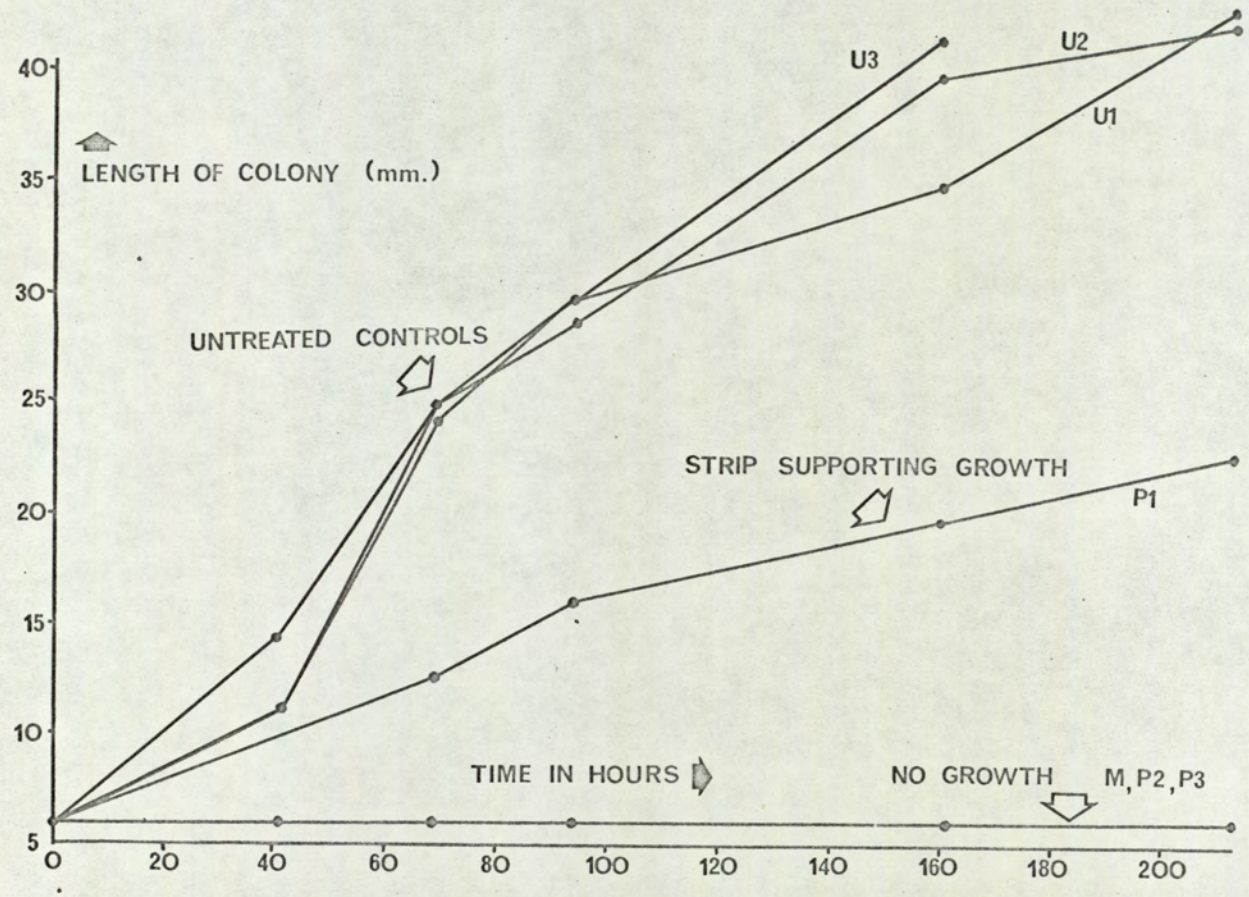
Chaetomium globosum growing on treated textiles on multi-perfusion bed. Growth inhibited by fungicides (left and centre) heavy growth on untreated control (right).

After cooling, each strip in each kit was inoculated with the test organism, in this case Chaetomium globosum, a fungus which shows compact vegetative growth and clearly recognisable sporulating tissue. Inocula were cut from the edge of a single colony growing on cellulose agar, using a sterile 6mm diameter stainless steel punch. These discs of agar were placed mycelium downwards on the textile strips, in the positions shown in figure 15. This position was not central, as previous experience had shown that the colony tends to grow in the direction of fresh nutrients, and so allowance was made for this factor. The perfusion kits were incubated at 25°C and examined at intervals. Growth of the colony was measured by observation under a low-power binocular microscope. This was usually possible without removing the upper valve of the petri-dish. The maximum distance which hyphae had grown at any point along the length of the strip was noted. As the textile strips had a fine and constant weave, measurement was greatly facilitated by using the threads as a built-in measure. In this experiment, three different fungicide treatments were compared with untreated textile and a 'standard' fungicide whose activity is well known. In each individual kit was one strip of untreated textile, one treated with the 'standard' fungicide and one strip treated with a fungicide of little known activity. Three kits for each of the fungicides of little known activity were made up. The measurements of colony length were plotted against time, each point on the graph being the average of 3 readings.

From the results plotted on graph 4, it can be seen that the untreated control strips U1, U2 and U3 supported fungal growth to similar degrees, only one fungicide-treated strip P1 (1.0% ammonium sulphate compound of ortho-phenyl phenoxy isopropanol applied from water) supported fungal growth. The rest, P2 (1.0% ortho-phenyl phenoxy isopropanol applied from Imsol A/water in ratio 2:1), P3 (1.0% pentachlorophenoxy isopropanol/trichlorophenoxy isopropanol 70:30 mixture applied from Imsol A/water in ratio 2:1) and the 'standard fungicide M(1.0% "Mystox" LPL) completely inhibited any fungal growth on the textile strips. The rate of growth of Chaetomium globosum on strip P1 was less than half that on the untreated control strips. The experiment was concluded when the colonies on the control strips had almost covered the total length of the strips, after nine days of incubation.

The technique employed

As previously mentioned, fungal growth on textile buried in soil has the disadvantages of being erratic or non-observable. When a textile strip is perfused however, fungal growth is sufficiently even to be easily observed and measured. Although colony size is no indication of the degree of breakdown of the material under test, this technique provides a simple quick method whereby a material can be subjected to biological stress, and where the materials ability to support surface growth, or induce liberation of pigment from colonising organisms can be rapidly assessed; these latter points being of great interest in biodeterioration studies (see chapter 1).



Graph 4

Graph of colony size (distance grown along strip) plotted against time.

U1, U2, U3 = Untreated textile control strips.

M, P1, P2, P3 = Average textile strips treated with various fungicides.

This technique also provides a convenient method for the culture of organisms on the test material in its normal form (i.e. not powdered or shredded), and also a means whereby samples of deteriorated materials, produced under identical conditions, can be obtained at intervals for any further examination desired.

Perfusion techniques are particularly applicable to fungicide treatment studies, as the constant supply of fresh nutrients and the constant removal of metabolites enables testing to continue under standardised conditions for much longer than in agar plate methods. It is possible that this technique may also lend itself to use with other groups of organisms, such as algae or perhaps lichens.

The fungicides employed

Over a short period of time such as that employed in this test (9 days) it was to be expected that 1.0% applications of most fungicides would inhibit growth of the test organism. This proved to be the result in all cases except one. The treated textile which supported growth (P1) was treated with a fungicide which is readily soluble in water. The results obtained here correlate well with previous work (see graph 3, chapter 2,). Future tests may show increased effectiveness of this compound in a static system, but in this instance, one of the factors which must be recognised in explaining its lack of effectiveness must be its likely susceptibility to leaching; such a physical stress being present during the initial autoclaving and the subsequent action of the perfusion flow.

4.3. Use of the technique in the study of the decay of artificially-weathered fungicidally-treated textiles

Cotton textiles are susceptible to actinic degradation due to sunlight, this 'tendering' process being enhanced by some fungicides, including phenolic compounds such as pentachlorophenyl laurate (Selby, 1966). Whilst weathering due to physical agencies is in progress, cotton develops resistance to microbial degradation. (Abrams, 1951; Bayley and Wetherburn, 1946, 1947; Yelland, 1951). The exact mechanisms for the increased microbial resistance of weathered cotton have not been determined, but Kaplan et. al. (1970) have shown that photochemical activity during weathering exposure tests transforms the cellulose into an altered substrate that prevents access of enzymes to susceptible sites of the cellulose molecule. It is of interest to note that the cellulases used in this latter work were obtained from a strain of Trichoderma viride.

The following experiments were devised to investigate some further points arising from considerations of the above phenomena; those of the efficacy of weathered rot-proofing treatments on weathered cotton.

The materials used in the test were as follows:-

(i) T.N.O. cotton test textile, containing 1.5% of pentachlorophenyl laurate (applied as "Mystox LPL"). This was the control textile and was labelled "C".

(ii) Treated textile "C" after heating at 70°C at 60% R.H. for 400 hours, labelled "70".

(iii) Treated textile "C" after exposure to Xenon arc light for 400 hours, labelled "X".

These samples were prepared and supplied by the Biology Department, Centraal Laboratorium T.N.O., Delft, The Netherlands.

Three different fast growing fungi were chosen as the test organisms. The first two had been used in testing before, the third was chosen from isolation work in progress at the same time. The species were:-

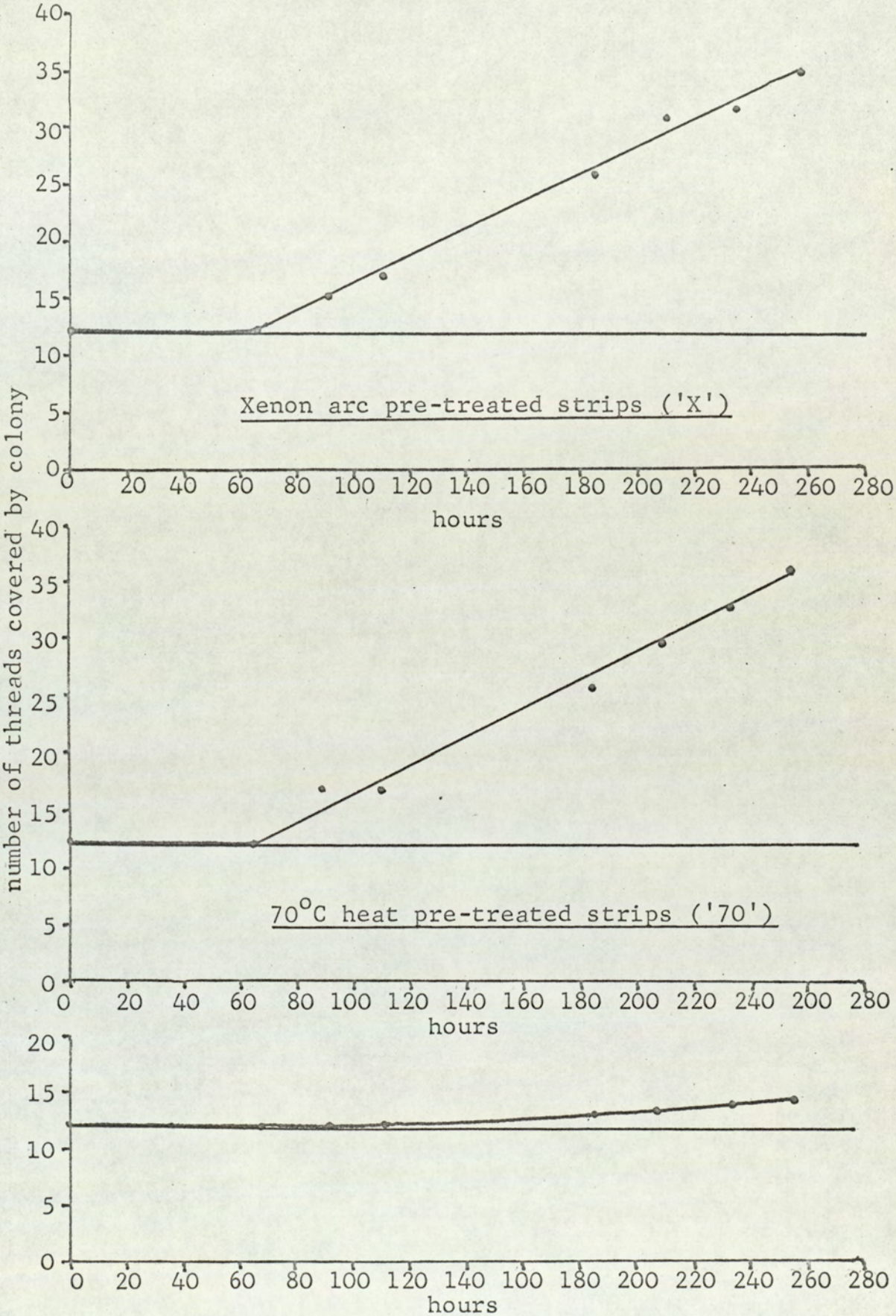
Chaetomium globosum (G)
Trichoderma viride (H)
Geotrichum candidum (R)

Several multi-perfusion kits, as previously described, were set up for each species, each kit having one strip each of textile treatments "C", "70" and "X". The kits were incubated at 25°C and readings of the colony spread were taken at intervals, as were readings relating to the onset and spread of sporulation within the colony where applicable. These results are shown in graphs 5 - 8.

From these results, several conclusions may be drawn.

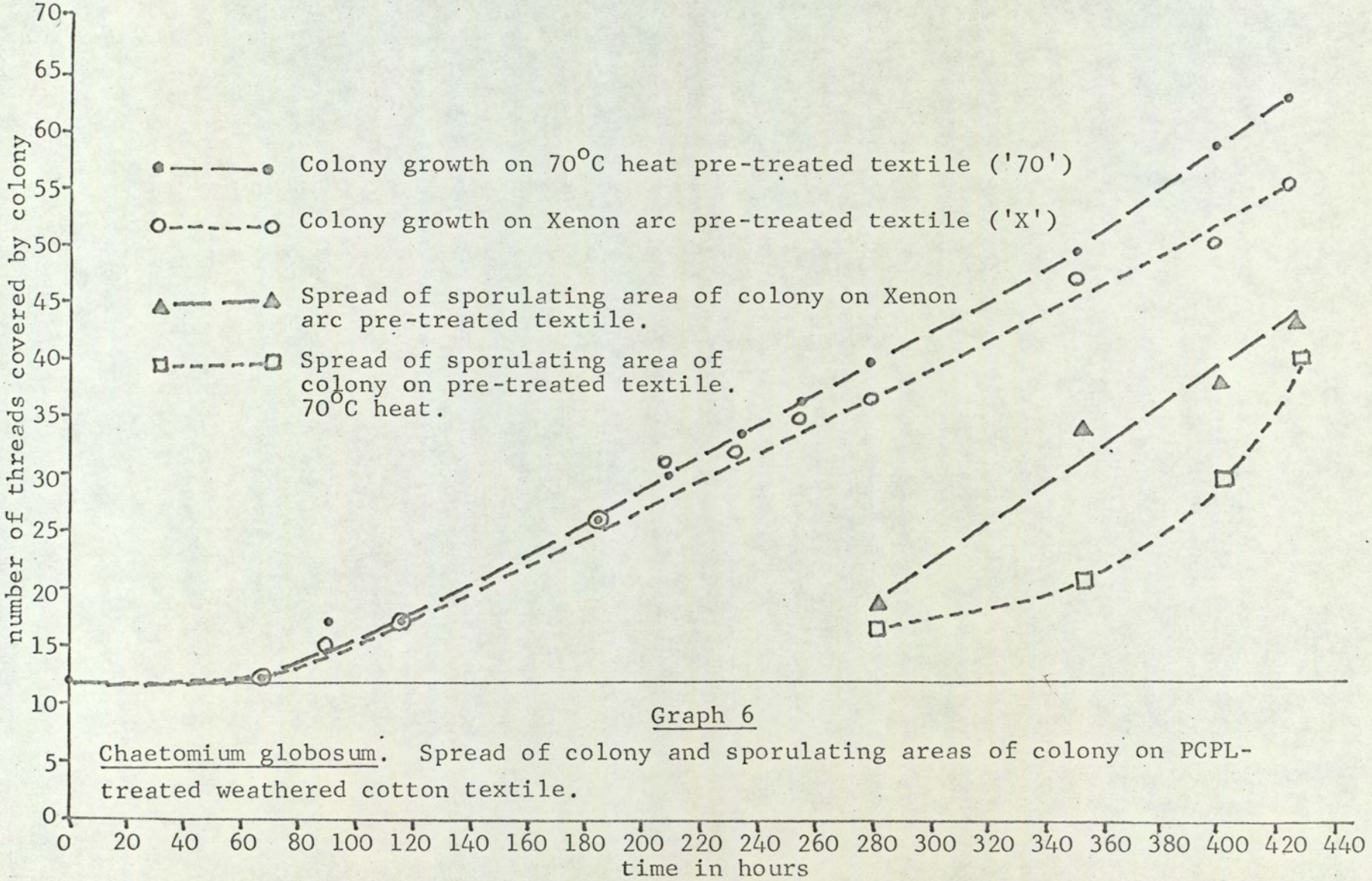
(a) Vegetative fungal growth

(i) All species grew on textile strips that had been subjected to light or heat.



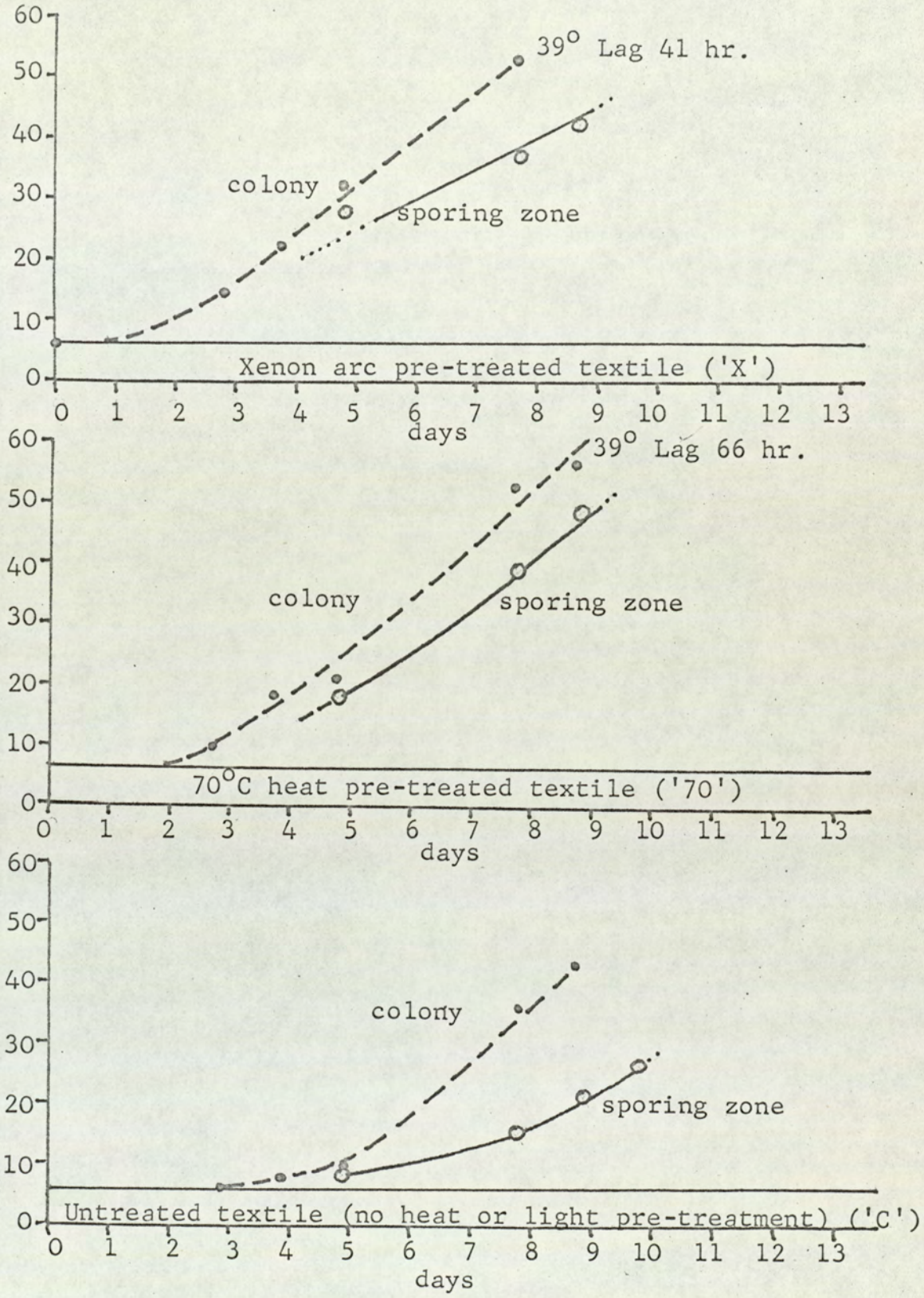
Graph 5

Chaetomium globosum. Spread of colony on weathered PCPL treated cotton textile.



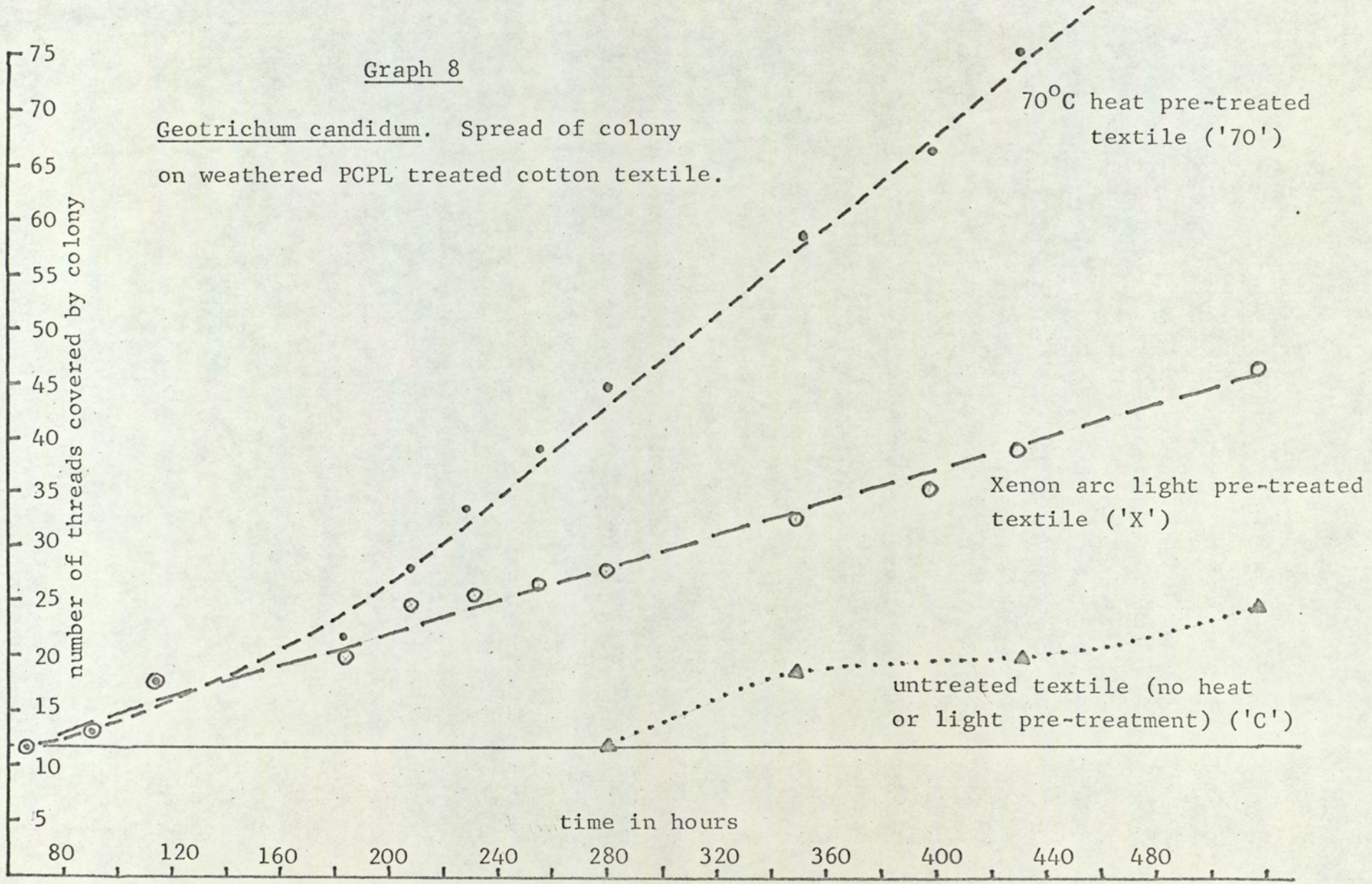
Graph 6

Chaetomium globosum. Spread of colony and sporulating areas of colony on PCPL-treated weathered cotton textile.



Graph 7

Trichoderma viride. Growth and sporulation of colonies on PCPL treated weathered cotton textile.


Graph 8
Geotrichum candidum. Spread of colony on weathered PCPL treated cotton textile.
70°C heat pre-treated textile ('70')
Xenon arc light pre-treated textile ('X')
untreated textile (no heat or light pre-treatment) ('C')
number of threads covered by colony
time in hours
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
80
120
160
200
240
280
320
360
400
440
480

(ii) Only Trichoderma viride grew well on the control strips.

(iii) There was little difference between rates of growth on heat and light treated textile.

(iv) There were considerable differences between growth rates of the different fungal species.

(b) Sporulation

The two species which formed dark, easily observable reproductive structures were studied, namely Chaetomium globosum and Trichoderma viride.

(i) Trichoderma viride produced spores on all textile samples.

(ii) Although the vegetative growth rate of Trichoderma viride was greatest on the unweathered control, sporulation was at its lowest level on the control strip.

(iii) Chaetomium globosum did not produce reproductive structures on the unweathered control strips.

(iv) Sporulation of Chaetomium globosum on heat treated textile was more erratic than on light-treated textile, the rate of production of sporulating areas being slower at first, increasing later.

From the above results and conclusions it is apparent that the effects of heat and light upon textiles treated with pentachlorophenyl laurate fungicides are generally detrimental to the inhibitory qualities of those compounds. The lag phase preceding the onset of colonisation is shortened,

and the rate of growth greater. It appears also that the ability of a fungus to sporulate is more easily inhibited by this fungicide than is its potential for vegetative growth. This has also been noticed earlier in other experiments. This marked inhibition of sporulation isperhaps expected as the processes involved are obviously more complex and more sensitive to external factors than those involved in vegetative growth.

Tensile strength measurements.

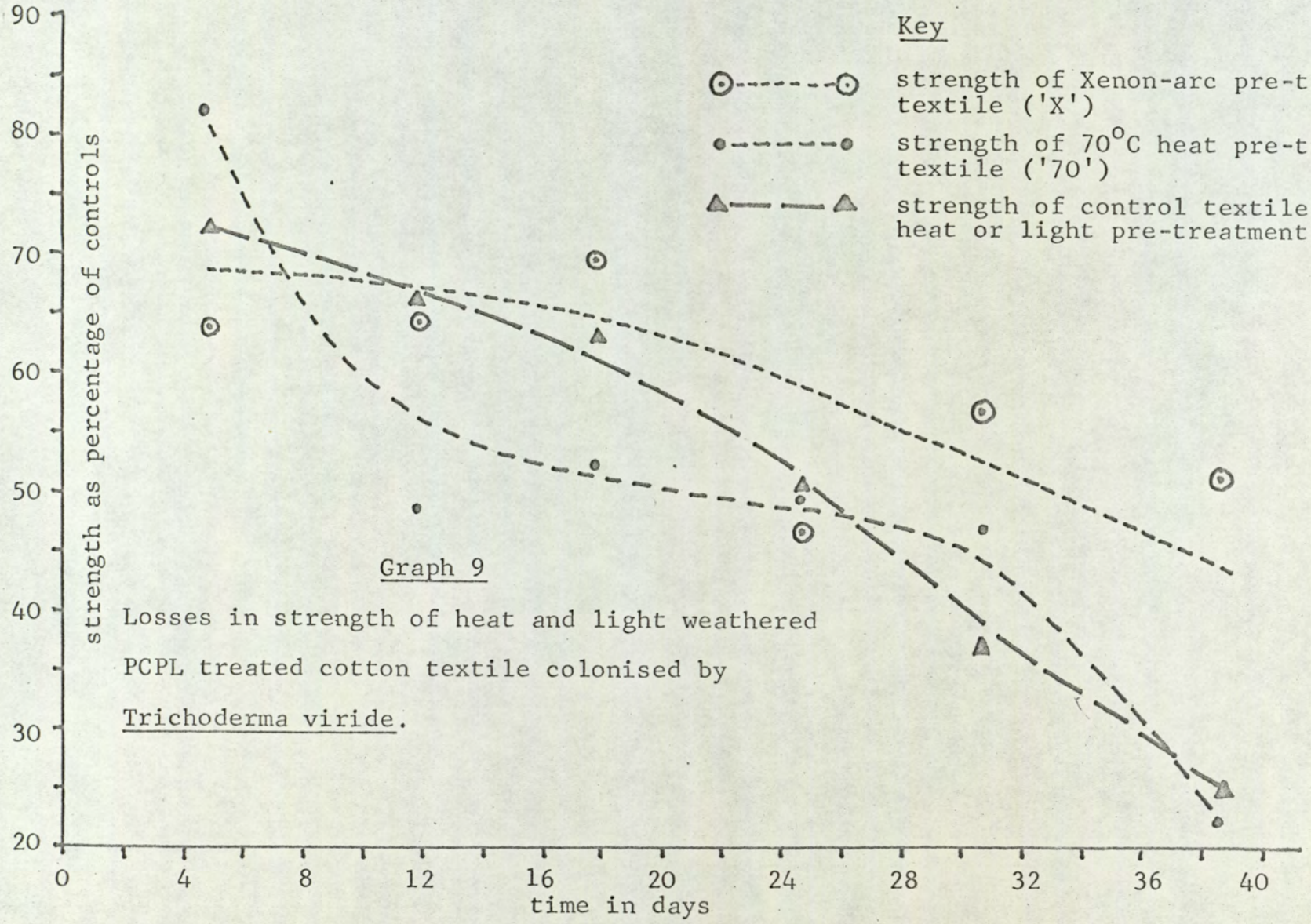
A replica of the previous experiment was run at the same time, in order to obtain samples for tensile strength measurements. Textile strips were removed at intervals, washed in 70% ethanol to arrest further growth, stored over saturated NaCl agar at 4°C to standardise the water content, and broken at the end of the experiment. The results, which varied greatly, are shown in graphs 9 - 11. Each reading is the average of three strips, as a residual percentage of strength as compared with undecayed controls. The actual average values of these controls were as follows.

Control strips "C" = 19.55 Kg.
Heat treated strips "70" = 18.2 Kg.
Light treated strips "X" = 12.45 Kg.

The initial actinic degradation of the light-treated textile is very clear from these figures.

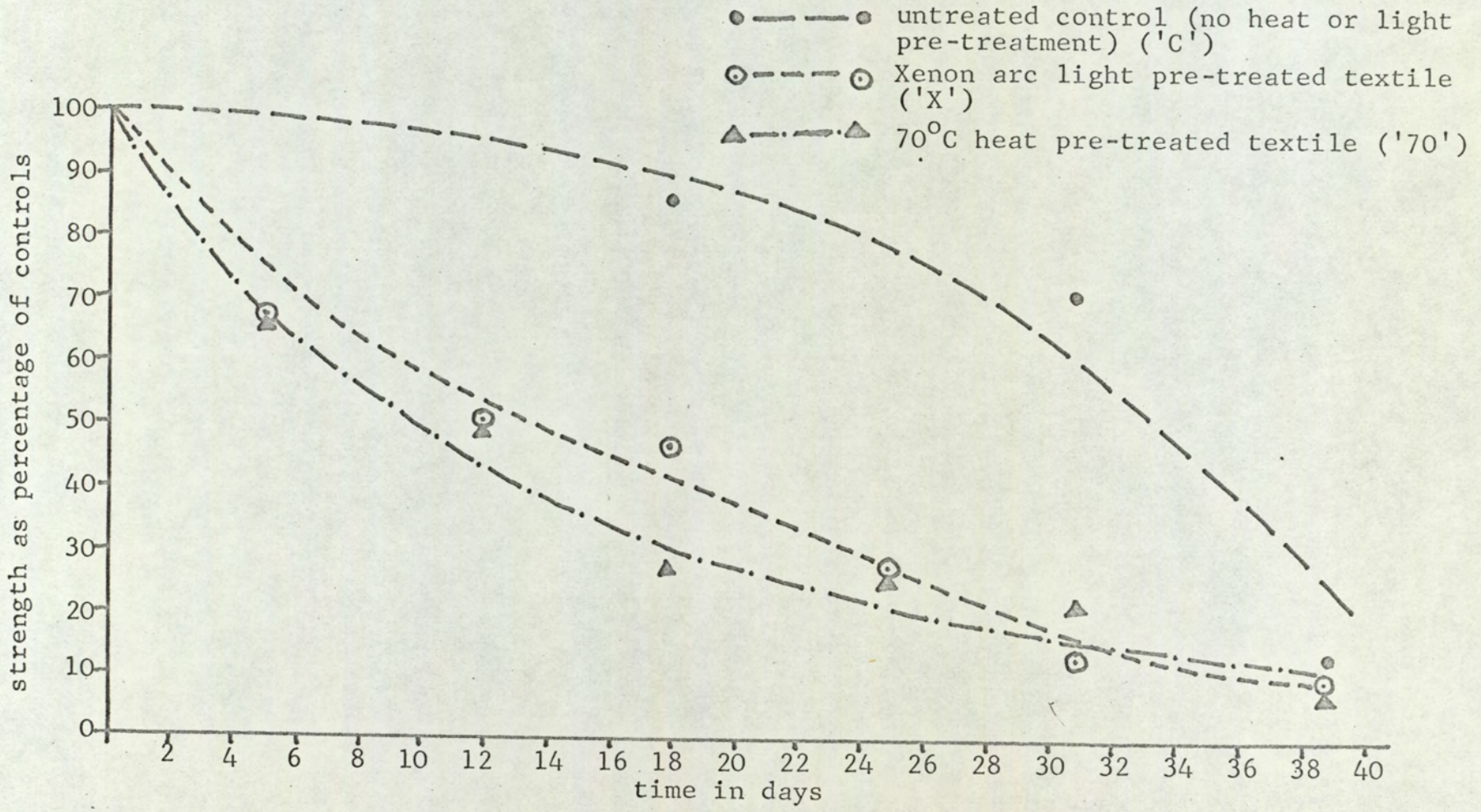
Chaetomium globosum

Heat and light treated strips decayed at similar rates, there being no appreciable lag period preceding the onset of



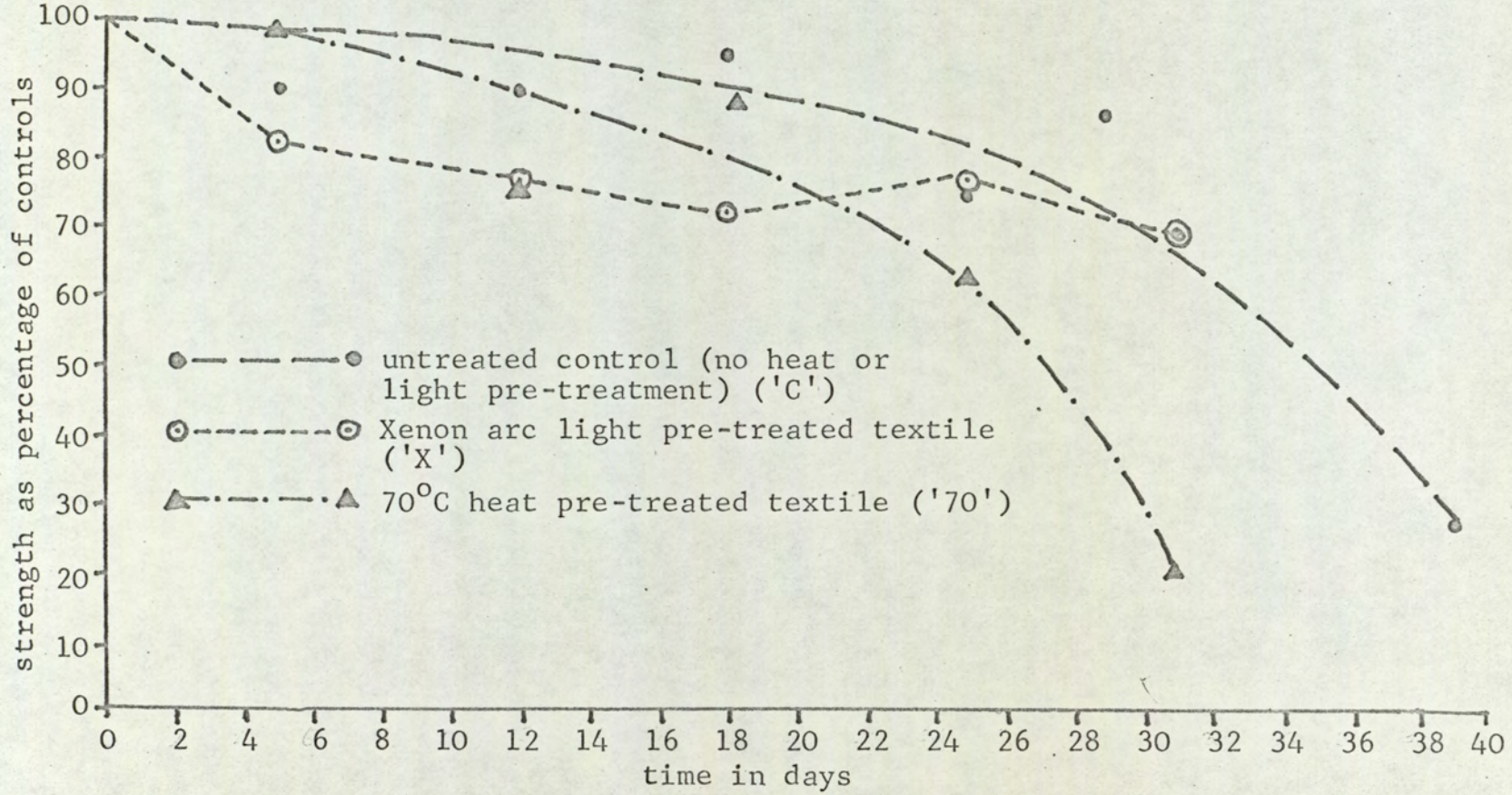
Graph 9

Losses in strength of heat and light weathered PCPL treated cotton textile colonised by *Trichoderma viride*.



Graph 10

Losses in strength of heat and light weathered PCPL treated textile colonised by Chaetomium globosum.



Graph 11

Losses of strength in heat and light weathered PCPL-treated textile colonised by Geotrichum candidum.

decay. The control strip maintained its strength for a long lag period, and then decayed rapidly. All strips had a residual strength of only 10% after 39 days of test. The general decay pattern here compares well with the surface growth patterns seen in the same periods during the previous experiment.

Trichoderma viride

The results obtained were erratic compared to those from Chaetomium globosum. The light treated textile proved to be the most resistant to decay over the longer period of the test, but little significance can be attached to this owing to the erratic nature of the decay observed.

Geotrichum candidum

The results were similar to those obtained for Trichoderma viride. The greatest similarity is that the light-treated textile again showed the best resistance to decay over the total period of the 39 days of test.

One hypothesis to explain any difference in the decay of light treated textiles and heat treated textiles is as follows:- Both light and heat may affect the surface of a cotton textile, but light is less able to penetrate in depth. Therefore, over a short term test (such as the first part of this experiment, 9 days surface growth) the effects of the two treatments may appear similar (as in fact they were). Over a longer term, however, the effects of the all pervasive heat treatment becomes apparent, and light treated textiles

prove less susceptible to decay. This may be due to changes in the cellulose only, as mentioned in the opening of this chapter, but it is possible that the fungicide may become modified or perhaps more evenly distributed in some way also. Arguing this way, it would appear that the effects of heat and light in the long term must be considered to have differing effects, heat enhancing later decay, light treatment retarding it. Even if it were shown conclusively that light is able to enhance the effect of this fungicide in some way to act synergistically with the weathered cotton in increasing resistance to decay, it would have little or no practical value, as any increased resistance would be far outweighed by the phenol-compound-accelerated actinic degradation brought about in the first instance.

Both the experiments in this chapter serve to show the useful nature of this multiple perfusion technique. As identical conditions can be guaranteed within each kit, a quick and easily assessed comparison between different fungicide treatments may be made. The ability of one small compact piece of apparatus to give a visual comparison between treatments is of advantage in demonstrations to non-technical staff in industry and also in teaching. In the research field, average results from several perfusion kits provide useful data on rates of surface growth of fungi, the technique also providing a means of culturing organsims in relative bulk in a pure form on test materials, and being sufficiently flexible to allow its use with organisms other than fungi, and materials other than textiles.

Chapter 5

THE INTERACTION OF A TEXTILE FUNGICIDE WITH SOIL FUNGI, AND THE INFLUENCE OF INITIAL HEAT TREATMENT ON ITS ACTIVITY

5.1. Introduction

5.2. Study of PCPL as a material protectant using a perfusion technique.

5.2.1. Introduction

5.2.2. An investigation into the effects of soil organisms on pentachlorophenyl-laurate applied to cotton textile.

5.2.3. The effects of heat on the efficacy of pentachlorophenyl-laurate.

THE INTERACTION OF A TEXTILE FUNGICIDE WITH SOIL FUNGI, AND THE INFLUENCE OF INITIAL HEAT TREATMENT ON ITS ACTIVITY

5.1. Introduction

In this chapter, some of the difficulties encountered in fungicide studies are discussed and the development and use of methods of biological investigation of the fungicide component of protected materials described. The underlying theme is that of a fungicide which is degradable. It will be seen that this degradability is a necessary part of the toxic action of PCPL-based fungicides, but the implications of this are somewhat deeper. As in the case of the insecticide DDT breaking down into more toxic compounds such as DDE, this PCPL fungicide is shown to release pentachlorophenol (PCP) which is more toxic. However, unlike DDE the ultimate fate of PCP would appear to be one of further degradation and inactivation in the soil, and also probably tolerance by soil organisms. (Cserjesi, 1967, 1972; Chu and Kirsch, 1972; Kirsch and Etzel, 1972; Lloyd, 1968). Its final distribution in the environment is also much less widespread than many other biocides, due to its use as a material protectant rather than a generally spread insecticide or herbicide.

A biodegradable fungicide is not a contradiction in terms; the real meaning is that the life of a fungicide should ideally be geared to the expected service life of the material it protects. The use of a fungicide is of course a factor

to be considered when predicting service life, but it will not usually increase resistance of the material to physical or chemical agencies, and so these latter factors can be used as yardsticks.

Part of a code of practice for fungicide (and other biocide) use therefore should be as follows:-

(i) Fungicides in present use should be investigated as to their degradation characteristics; those leaving persistent and proven harmful residues being phased out of use.

(ii) New fungicides should have built in characteristics of degradability.

(iii) All fungicides should be matched to the finite (even if long) service life of the material they are to protect.

As previously mentioned, other factors must also be taken into account when choosing the hypothetical ideal fungicide (Selby, 1966). The aspects of persistence (as defined by those who may be affected by this property) and degradability after use should ideally now be included in these factors to be considered, in order to avoid harmful side effects to other living systems at a later time.

5.2. Study of PCPL as a material protectant, using a perfusion technique.

5.2.1. Introduction

Much of the experimental work carried out using biocides has been concerned with determinations of efficacy in conjunction with the materials for which the biocide is ultimately intended. This is an essential aspect of biocide study, but if such studies prove satisfactory from a usage point of view, more fundamental information may never be sought. As more concern is now being shown as to the mode of action and possible detoxification of biocides, both from protectant and efficiency and also environmental pollution aspects, it is becoming more necessary to look deeper into the action of biocides.

There are many techniques available to the analyst for the investigation of chemical compounds. These techniques are made much more difficult however when investigating living systems by the fact that many extranous compounds are often present, over and above those being investigated. If we consider a fungicide supporting fungal growth on an agar plate, several problems arise. There is the problem of the many compounds that may be present, those formed by the colony itself, those of the agar and nutrient medium, and those of the fungicide. The separation of these compounds is difficult as extraction and isolation is usually preceded by re-melting the agar, leading to an intermixing or even an actual change in the compounds. There are also problems associated with physical changes owing to the nature of an agar gel itself.

The following technique, which is a combined perfusion and chromatographic method, was developed to investigate the breakdown of pentachlorophenol laurate by soil fungi, and serves to demonstrate a technique for biocide study and also establish a basic point in the theory of the action of this fungicide.

5.2.2. An investigation into the effects of soil organisms on pentachlorophenyl laurate applied to cotton textiles.

Pentachlorophenyl esters are the main ingredient in many well-known material fungicides. These fungicides have a wide application, especially in connection with the preservation of textiles and paper. Aspects of the toxic action of such fungicides thus merits attention.

The toxic action of pentachlorophenyl laurate has long been assumed to be due to the release of free pentachlorophenol, the ester being used in practice since it is much less susceptible to leaching than pentachlorophenol itself. It has been suspected that the release of pentachlorophenol from pentachlorophenyl laurate is due to hydrolysis caused by microbial activity (Adema, Meijer and Hueck, 1967), and the work described below supports this hypothesis. Difficulties in demonstrating this in the past have included those of collection of the small amounts of pentachlorophenol released, in a pure state, from a living system containing pentachlorophenyl laurate of sufficiently low concentration to allow growth of microorganisms, and also of differentiating any pentachlorophenol resulting

from hydrolysis from "contaminant" pentachlorophenol, which is often present in pentachlorophenyl laurate preparations.

The main difficulties were overcome using a perfusion system (Eggins, Malik and Sharp, 1968), modified to the form described below. (See figure 16). The test substrate, in this case a cotton textile treated with pure pentachlorophenyl laurate enclosed in a petri-dish, is perfused with a nutrient salts solution flowing from a reservoir via a woven glass-fibre wick. Another wick, the tail wick, is attached to the other end of the strip of fabric, and carries the flow of exhausted nutrients, metabolic wastes, and breakdown products out of the system. The constant drying of this tail wick in the air maintains the flow of nutrients through the system. The nutrient salts solution used in this experiment was a modified Eggins and Pugh nutrient salts solution as suggested by Bravery (1968). This medium was originally suggested as a means of improving cellulase production but was used here as it does not contain yeast extract or L-asparagine which were feared might complicate the subsequent solvent extraction processes. The medium as used was:-

KH_2PO_4	1.0 g
K Cl	0.5 g
$MgSO_4 . 7H_2O$	0.2 g
$Ca\ Cl_2$	0.1 g
$(NH_4)_2SO_4$	0.55 g
Thiamine hydrochloride	0.001 g
Distilled water to	1 l

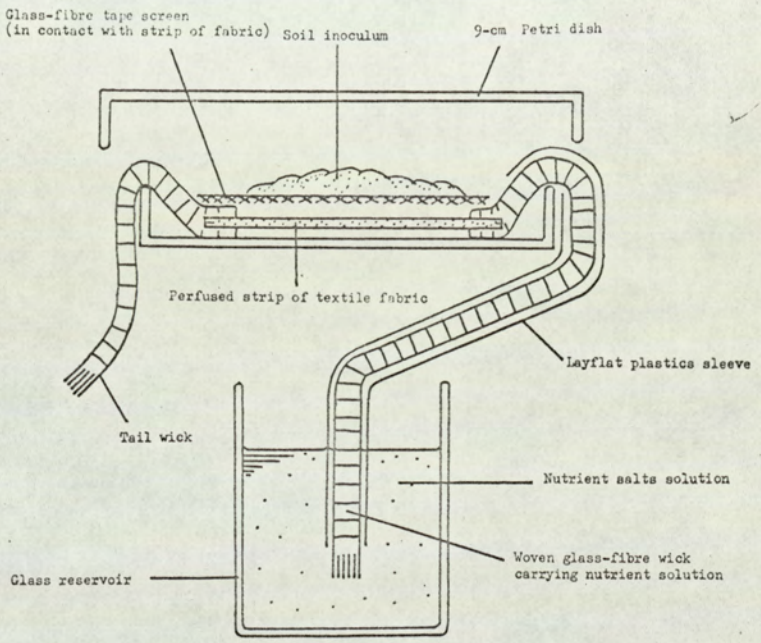


Figure 16

Diagram (exploded) of perfusion assembly. In practice, the closed dish rests on the reservoir rim, and is held in place by a rubber band. The materials used permit sterilization by autoclaving to be performed on the complete assembly before inoculation.

A source of colonising soil organisms was provided by inoculation of the perfused textile substrate with soil. Direct contact between soil and the textile was avoided by employing a glass-fibre screen (Eggins and Lloyd, 1968).

The perfusion assemblies were incubated at 25°C. Textile samples were collected at intervals and were observed to be colonised by soil fungi; fungi isolated from the meadow soil used included species of Chaetomium, Humicola, Trichoderma, Fusarium, Aspergillus, and Penicillium.

More species and more decay (as measured by tensile-strength testing) were noted on the control textile (un-treated) than on pentachlorophenyl-laurate protected textile.

Tail wicks were removed at intervals, dried in air, and extracted with organic solvents. These extracts were examined by means of a thin layer chromatography technique. The development and use of the extraction and chromatographic technique is described below.

Extraction technique

The problem posed was as follows, any pentachlorophenol or pentachlorophenyl laurate present in the tail wick needed to be extracted free from other impurities.

The following method was tried first of all:-

70ml methanol

30ml distilled water

100ml chloroform

Upon standing, the solution separates into two layers, methanol/water on top and chloroform below. A pentachlorophenol solution was used to test this solvent mixture. From previous experience it was expected that the pentachlorophenol would be contained in the chloroform layer, and other compounds in the methanol/water layer. It was found by chromatography of both layers that the pentachlorophenol was taken up by the methanol/water layer, and so this first method would be likely to prove unsatisfactory in use.

A second solvent system was devised. This comprised:-

70ml methanol

30ml 2N HCl

100ml chloroform

Upon standing, the mixture separates into two layers, methanol/HCl on top, and chloroform below. (This mixture was tested as it had proved satisfactory when used to extract similar biocide residues from the larvae of Tribolium confusum at Centraal Laboratorium T.N.O.).

It was hoped that in this case, pentachlorophenol and pentachlorophenyl laurate residues would have much less affinity for acidified methanol and would be taken up in the chloroform layer, remaining residues of compounds from the growth medium being taken up by the acidified methanol.

The method used for extraction (using pure pentachlorophenol here to test the system was as follows:-

To the material for extraction add 2ml of methanol/HCl and 2ml of chloroform. Shake well on a mechanical agitator. Allow the tube to stand and the layers to separate. Remove the lower layer and transfer to a fresh tube. To the residual upper layer in the first tube add 2ml of chloroform. Mix, allow to separate, remove the lower layer and once more and add to the first lower layer in the second tube. Perform this procedure once more (i.e. three times in all). This repeating of the procedure is in fact a washing of the methanol/HCl layer with chloroform to remove any remaining PCP or PCPL left after the initial extraction. Now, 2ml of Methanol/HCl is added to the combined chloroform fractions (lower layers), that is a reverse washing is carried out to remove any 'dirt' carried over into the chloroform.

The upper layer (Methanol/HCl last added) is removed after separation and combined with the other Methanol/HCl fractions in the other tube.

Both tubes were then evaporated to dryness by the use of a surface stream of nitrogen.

For spotting out on plates, a small measured quantity of a Chloroform/Methanol 1:1 mixture was used to dissolve the extract and one quarter of this was used, allowing for three more chromatograms to be made from each sample if required.

The method was tested for clear separation of PCP and also PCPL into a single solvent layer. This proved satisfactory. The method was also checked using pure PCPL to ensure no detectable dissociation of PCPL occurred due to the acidified methanol. No dissociation was detected. Tests were also run to establish correct concentrations of comparison markers required, to test the purity of the PCPL on the initial textile samples, and also to ensure PCP was not lost during the evaporation process.

PLATES

Plate 4 (LI 2529)

Purity check on 1% PCPL treated textile showing no PCP, comparison markers in correct range, and at least 400 µgm of LPCP in the standard textile sample used for extraction.

Plate 5 (LI 2530)

As above, but for 2% PCPL treated textile, showing no PCP and at least 800 µgm of PCPL in the sample.

Plate 6 (LI 2534)

A technique check using PCP, showing its absence in the methanol/HCl layer.

Plate 7 (LI 2539)

A technique check using PCPL, showing its presence in the chloroform layer.

Plate 8 (LI 2532)

Showing PCP from the water /methanol layer of the first technique tested. The technique was not employed as many of the impurities on the tail wick would probably

Reference Markers 10 50 100 µgm PCP | 1/10 1P | Reference Markers 10 50 100 µgm PCPL

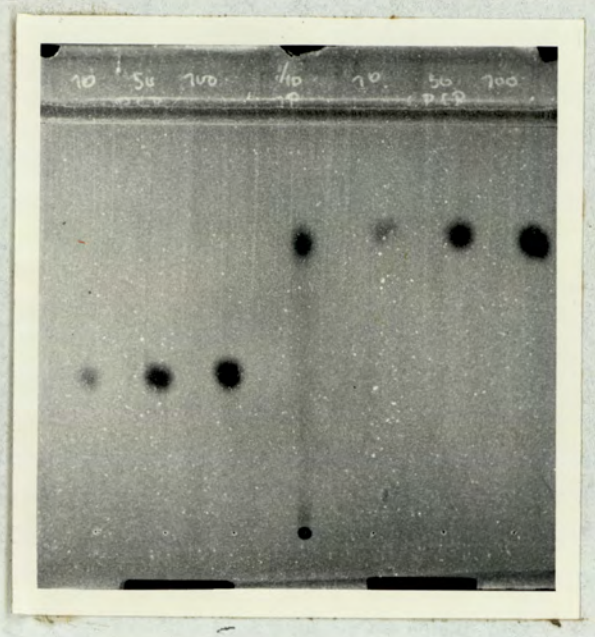


Plate 4

(L1.2529)

Purity check on 1% PCPL-treated textile showing no PCP, comparison markers in correct range and at least 400 µg of PCPL in the standard textile used for extraction.

Reference Markers 10 50 100 µgm PCP | 1/10 2P | Reference Markers 10 50 100 µgm PCPL

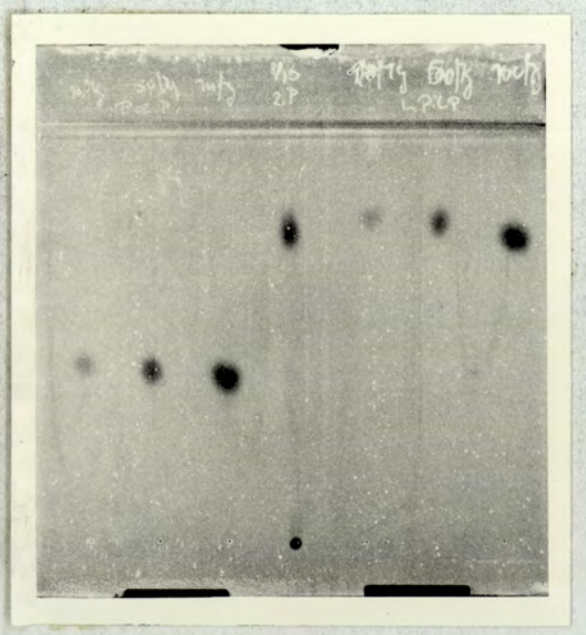


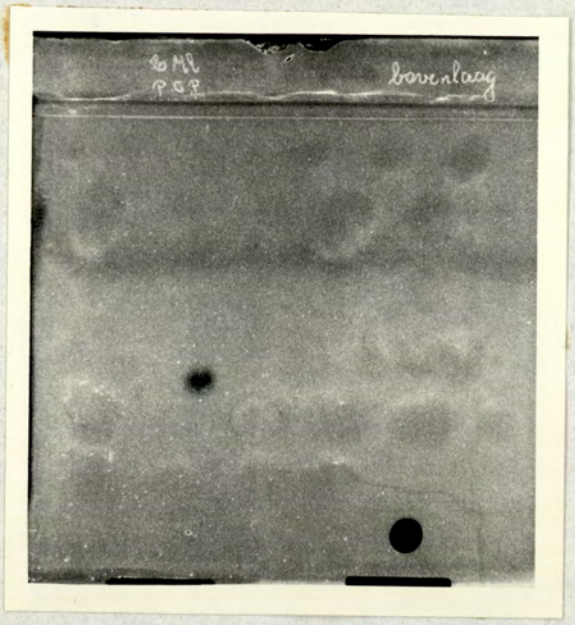
Plate 5

(L1.2530)

As Plate 4, but for 2% PCPL-treated textile, showing no PCP and at least 800 µg of PCPL in the sample

Plate 6

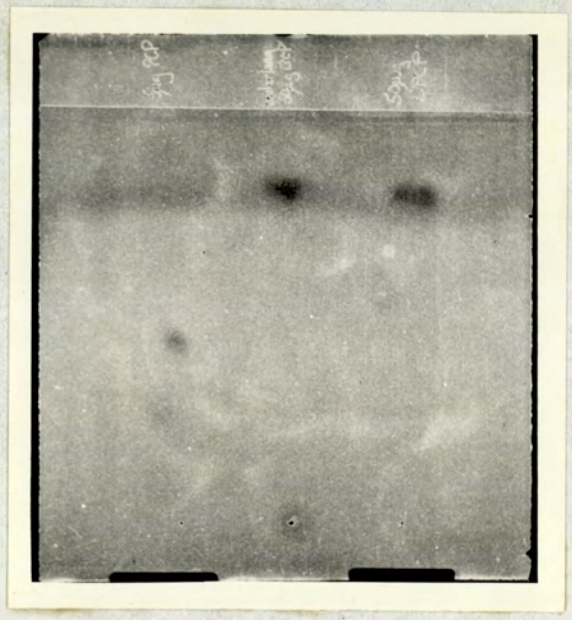
(L1.2534)



A technique check using PCP, showing its absence in the methanol/HCl layer.

Plate 7

(L1.2539)



A technique check using PCPL, showing its presence in the chloroform layer.

Top Bottom 20 µg PCP

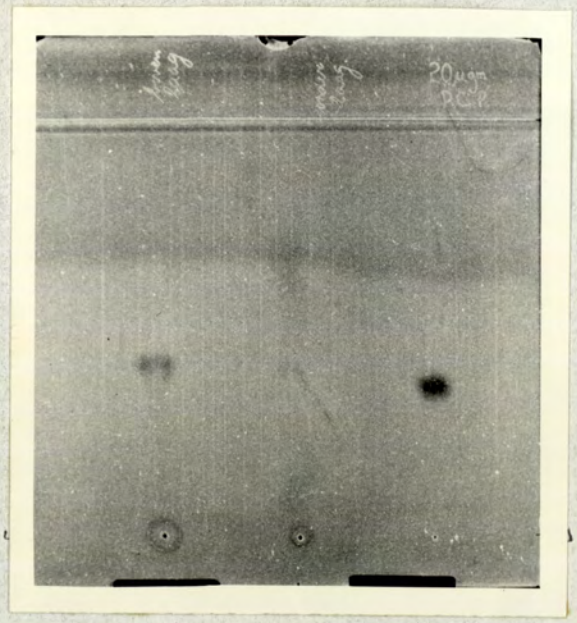


Plate 8

(L1.2532)

Showing PCP from the water/methanol layer of the first technique tested. The technique was not employed as many of the impurities on the tail wick would probably have been taken up in the water/methanol layer, together with the PCP (and PCPL) in preference to the chloroform layer.

have been taken up in the water/methanol layer together with the PCP (and PCPL) in preference to the chloroform layer.

The method described was adopted for use, the chloroform layer being used for chromatography. The amounts of solvents used in the process on each wick were:-

Chloroform	9ml
Methanol	9ml
2N HCl	4ml

Chromatography

The layer medium was made up by dissolving 1gm of boric acid crystals in 99ml of distilled water and mixing 60ml of this solution with 30gm of Kieselgel HF 254 Stahl (Merk). After spreading, this was activated by heating at 110°C for 30 minutes. The solvent used in the chromatography tank was chloroform: n-heptane 4:1.

The spots on the chromatograms were visible in ultra-violet light. Photographs were made of chromatograms. Plates were illuminated by two Hanau ultra-violet lamps, 35 watts, 220 volts, giving a light of 254 mμ wavelength. The lights were positioned on either side of the plate to be photographed at an angle of 45° mid line to mid line, 12 inches away.

The camera used was a Linkof Technica with a F 3.5, 105mm Tessar lens and Super Rollex Rollfilm back, giving a

film format of 56 x 72mm. Film used was Agfa IFF Isopan film speed 25 A.S.A. The exposure time was 5 minutes at f 5.6 using a 2X yellow filter and an ultra violet filter.

Investigations using the perfusion and chromatography technique.

This technique was used to investigate two main areas, the suspected hydrolysis of PCPL and the effect of heat on this biocide when applied to textiles.

Evidence of the hydrolysis of Pentachlorophenyl laurate by soil microorganisms.

Perfusion kits were set up as previously described, and the tail wicks removed and extracted at intervals. By using this semi-quantitative method, two facts were first established.

(1) The initial textile samples did not contain any detectable pentachlorophenol.

(2) The initial textile samples contained either approximately 400 µg of pentachlorophenyl laurate (1% treatment) or approximately 800 µg of pentachlorophenyl laurate (2% treatment).

Including the initial control, five sacrifices of tail wicks were made over 25 days, and the results of hydrolysis i.e. amounts of free pentachlorophenol were estimated chromatographically.

Photographs of the T.L.C. plates are shown in plates 9 - 13.

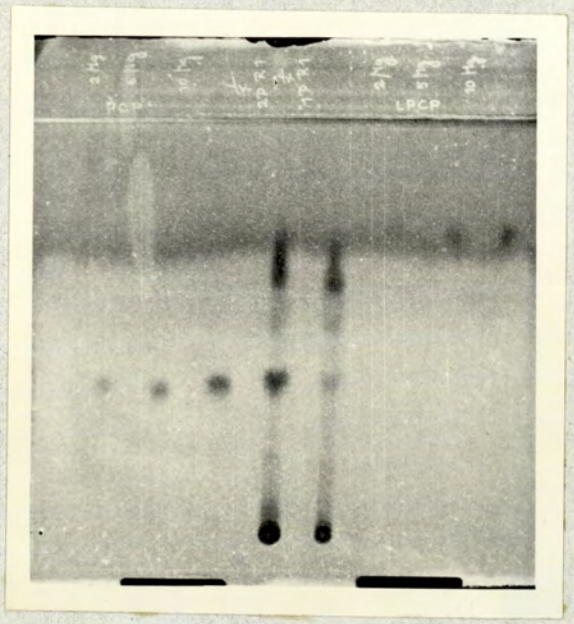
1/4 1/4

2, 5, 10 µg PCP 2P R.I. 1P R.I. 2, 5, 10 µg PCPL

Plate 9

(L1.2540)

1st sacrifice of PCPL-treated textile



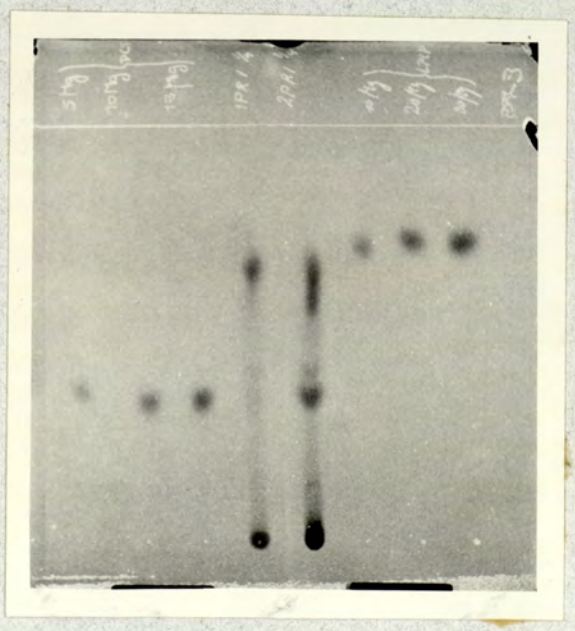
1/4 1/4

5, 10, 15 µg PCP 1P R.I. 2P R.I. 10, 20, 30 µg PCPL

Plate 10

(L1.2543)

As above, different reference markers.

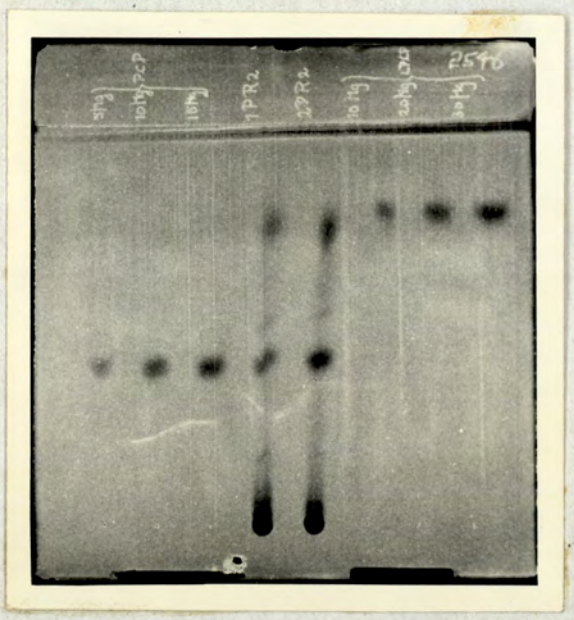


1/4 1/4

5,10,15 µg PCP 1P R.2 2P R.2 10,20,30 µg PCPL

Plate 11

(L1.2546)



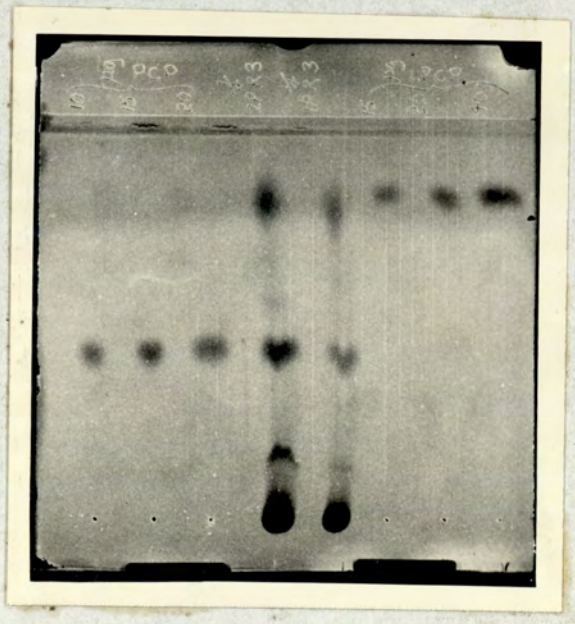
2nd sacrifice of PCPL-treated textile

1/4 1/4

10,15,20 µg PCP 2P R.3 1P R.3 15,25,40 µg PCPL

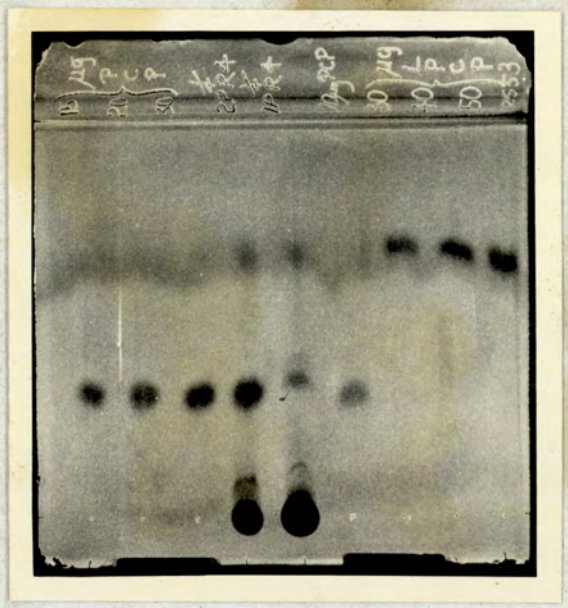
Plate 12

(L1.2549)



3rd sacrifice of PCPL-treated textile.

15,20,30 µg PCP	1/4 2P R.4	1/4 1P R.4	10 ug PCP	30,40,50 µg PCPL



4th sacrifice of PCPL-treated textile

Amounts of PCP liberated were estimated by comparison of the size and intensity of the spots obtained with standards on the same plate. This was carried out using the actual plates as well as the photographs. Photographs alone should not be used as variations in printing can lessen the accuracy of the method.

The results are shown in graph 12. It can be clearly seen from the graphs that:-

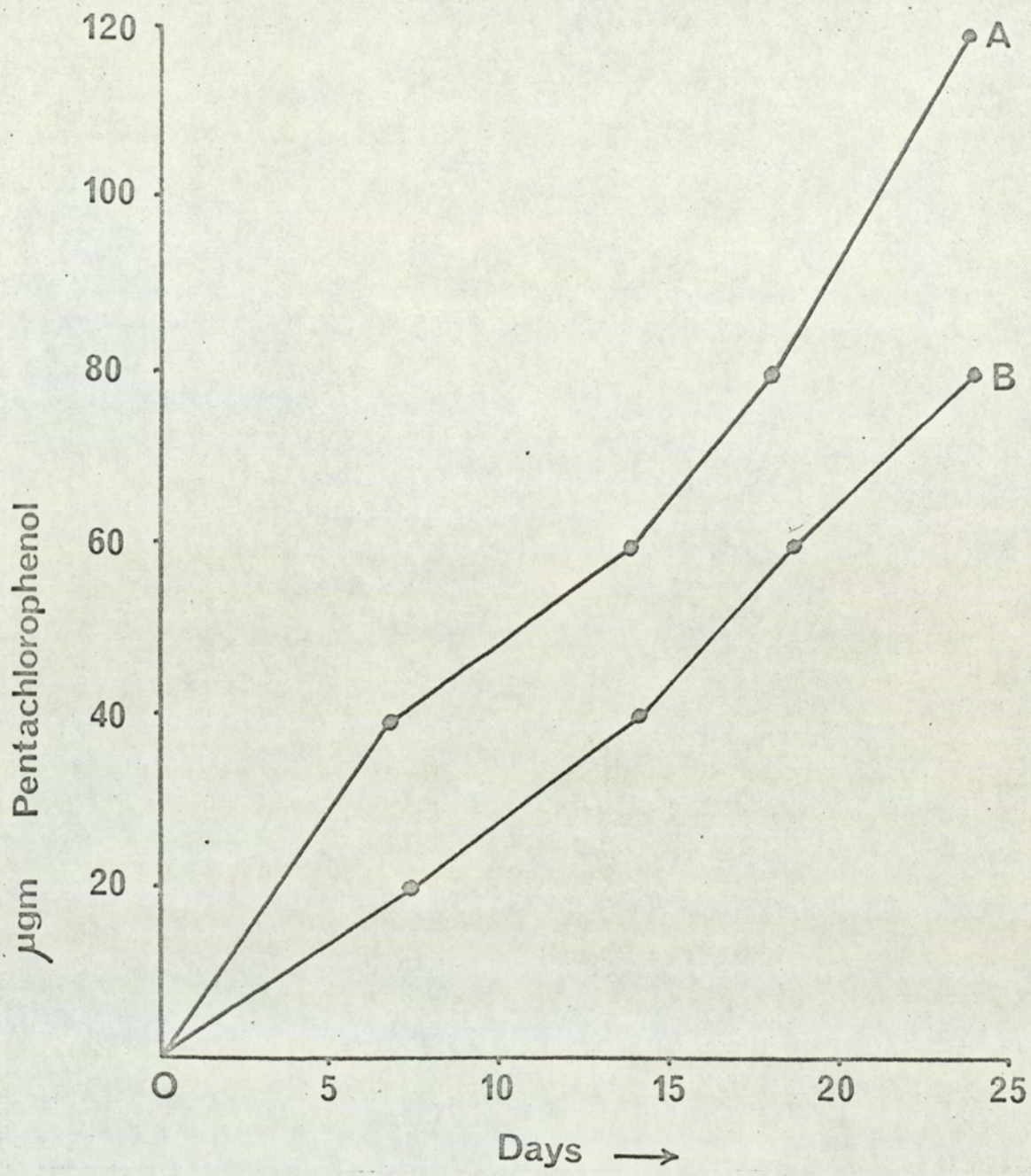
(1) Free PCP is liberated from the PCPL treated textiles.

(2) The total amount of PCP liberated increased with time.

(3) More PCP was liberated from textile treated with 2% PCPL than from textile treated with 1% PCPL.

These results are taken to be an indication of hydrolysis of pentachlorophenyl laurate by soil micro-organisms. It can be postulated that the observed hydrolysis of pentachlorophenyl laurate was due to a purely chemical effect caused by the soil itself. Circumstantial evidence suggests that this mechanism is unlikely and that the observed hydrolysis was, in fact, due to biological activity in the soil especially as fungal growths were observed on the test textile used here.

The hydrolysis of pentachlorophenyl laurate is only the first stage in the degradation of this compound, and it is fitting that further mention is made of work that has been carried out on pentachlorophenol. Compared with other chlorinated phenols, pentachlorophenol is relatively



Graph 12

Graph of results obtained by using a semi-quantitative chromatography technique and showing approximate amounts of pentachlorophenol liberated from textile samples treated with pentachlorophenyl laurate

Graph A : 2% pentachlorophenyl laurate, approximately 800 µg in sample originally.

Graph B : 1% pentachlorophenyl laurate, approximately 400 µg in sample originally.

resistant to degradation (Alexander and Aleem, 1961) and could therefore be considered a potential pollutant. Cserjesi (1972) reviews the work carried out on the detoxification of chlorinated phenols and states that the main process is probably one of biological methylation (Engel et. al. 1966; Curtis et. al, 1972; Suzuki and Nose, 1971; Cserjesi and Johnson, 1972). Other processes mentioned are biological conjugation of derivatives, a 'natural' detoxification mechanism as shown by Williams (1966) and possibly by the phenol oxidases produced by lignin-decay fungi (Lyr, 1962, 1963).

5.2.3. The effect of heat on the efficacy of pentachlorophenyl laurate.

In this experiment, textile samples containing originally 1% or 2% of pentachlorophenyl laurate and having been subjected to artificial weathering at 25°C or 70°C at R.H. 60% were perfused and colonised by soil fungi. The PCP released from these strips was examined chromatographically, and compared with the amounts released in the previous experiment, where the textile was not pre-weathered. The results are shown in graphs 13, 14, and 15.

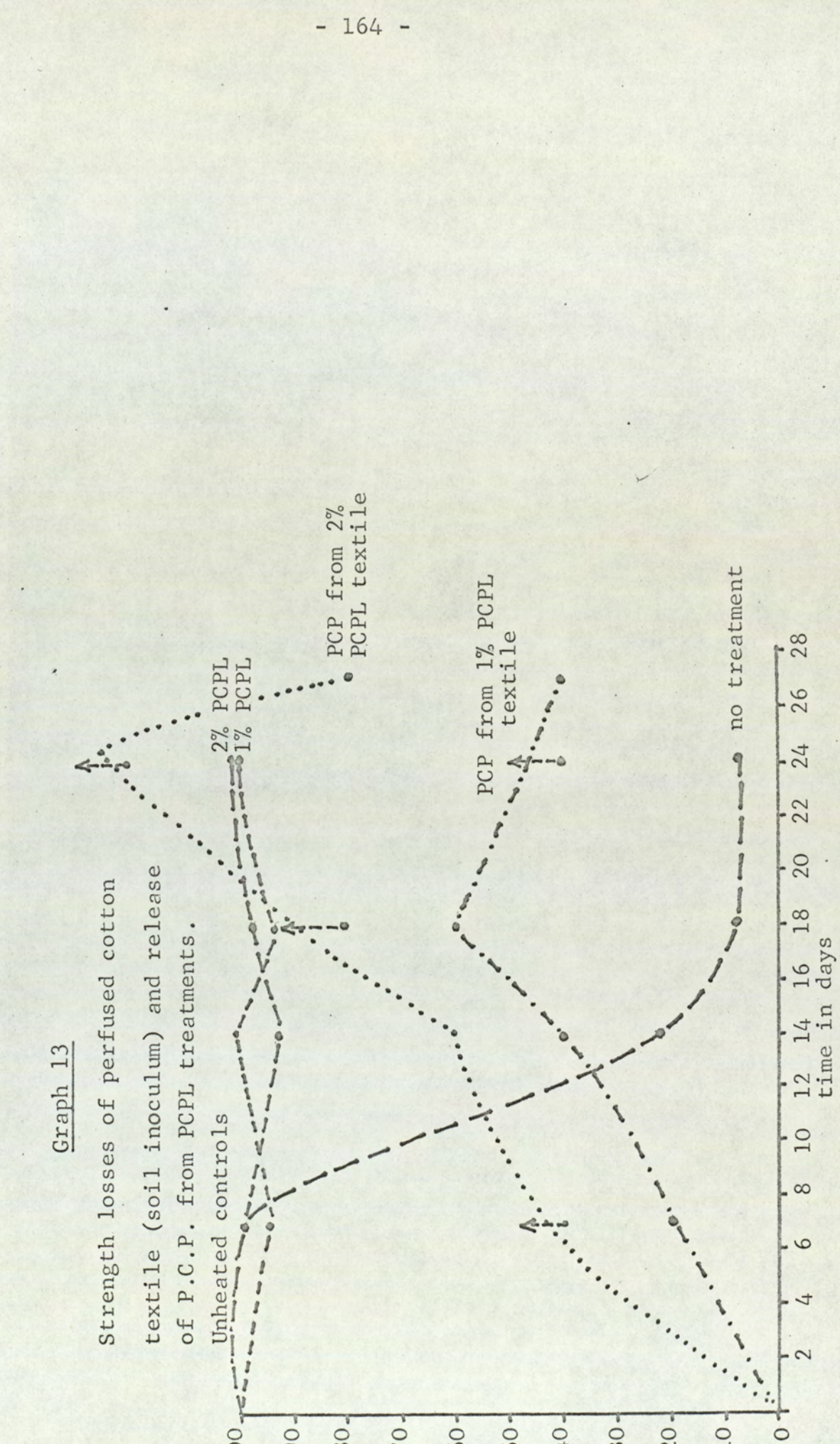
From the graphs it can be seen that:-

(1) Decay as measured by loss of tensile strength increased as PCPL strips had more heat applied in pretreatment.

(2) Control strips (no PCPL fungicide) decayed in similar manners to each other.

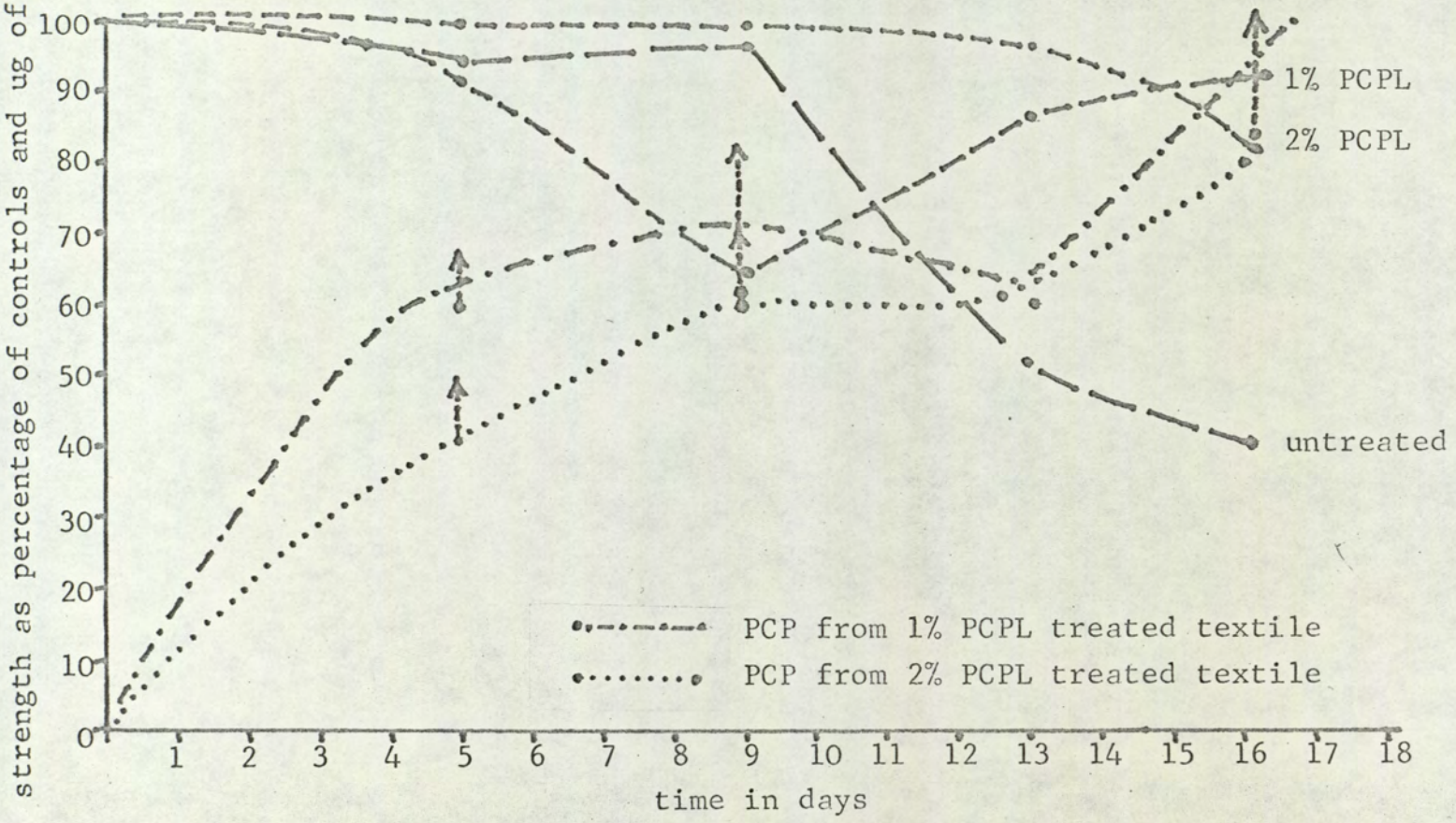
Graph 13

Strength losses of perfused cotton textile (soil inoculum) and release of P.C.P. from PCPL treatments.



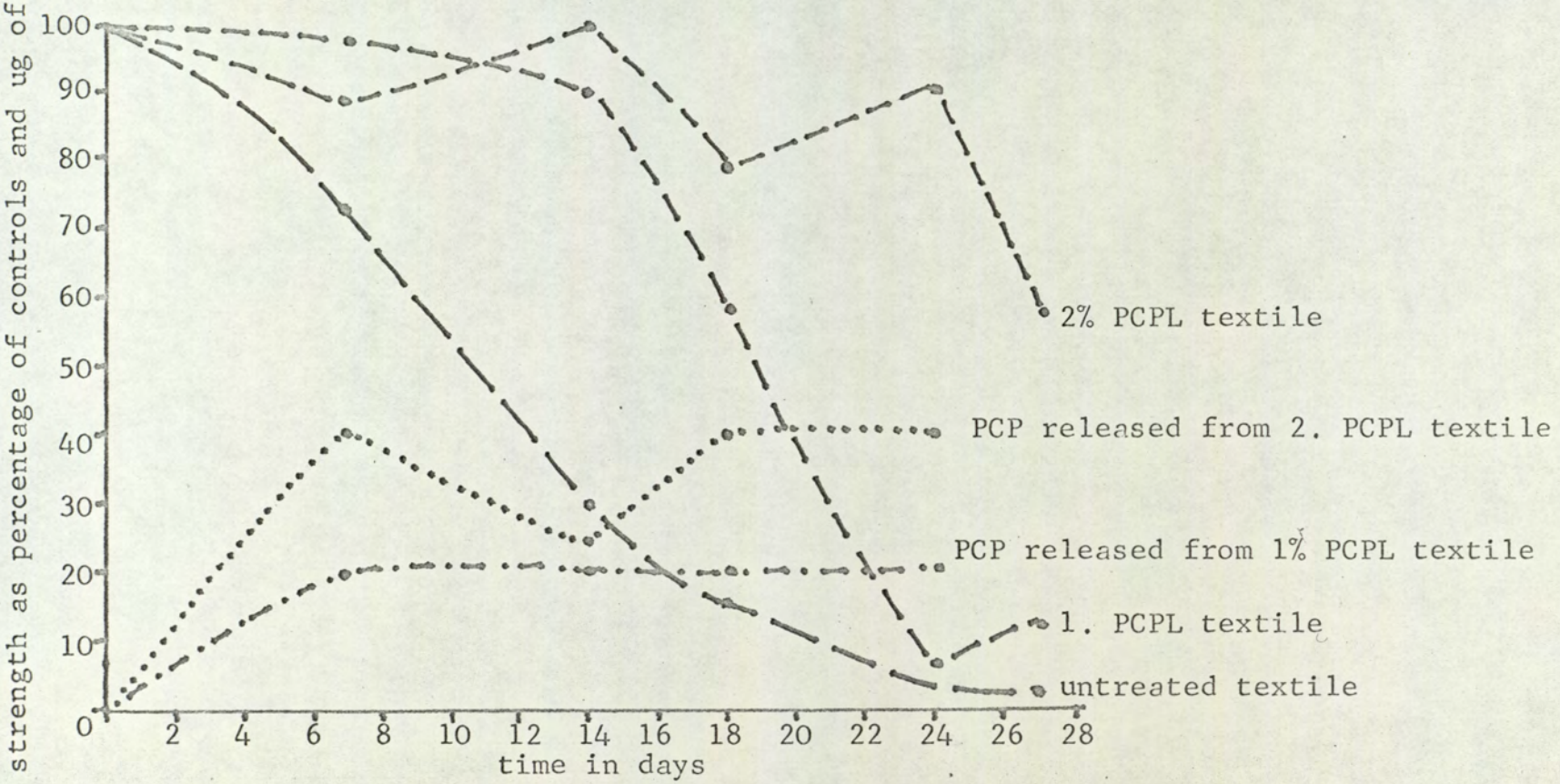
Graph 14

Strength losses of perfused cotton textile (soil inoculum) and release of PCP from PCPL treatments. 25°C heat treatment of strips before test.



Graph 15

Strength losses of perfused cotton textile (soil inoculum) and release of PCP from PCPL treatments. 70°C heat treatments of strips before test.



(3) With strips that had been subjected to most heat (70°C for 400 hours) the lag phase of decay was much reduced. This phenomenon has been noticed in earlier work. (Chapter 4).

(4) Unheated (i.e. room temperature handled textile) and treated at 25°C textile were similar in performance; the 70°C textile was the most different.

(5) PCP released from the 70°C cloth increased very little with time.

The effects of heat and weathering on PCPL protected textiles must be regarded as being of a complex nature, and experimental results in this area are rarely clear cut. The results of this experiment, however, serve to underline some basic assumptions that are being made for PCPL biocides. Firstly, that heat is detrimental to the biocidal activity of the biocide. This can be seen by increased decay and also by the fact that less free PCP is released from heated and weathered cloth, assuming that the toxic action of PCPL is by release of free PCP as shown by the previous experiment. The fate of PCPL on heating and weathering is not wholly clear. It is reasonable to suppose that any PCP present or formed is subject to evaporation and also that other fractions of the fungicide formed upon weathering may be lost to the atmosphere. The volatile nature of PCP is clearly demonstrated by the fact that untreated textiles stored in the vicinity of textiles treated with PCP will pick up detectable amounts of PCP over periods of a few weeks. It has also been suggested (Hueck-personal communication) that the PCPL fungicide or components of the fungicide may become firmly

bound onto the fibres as a result of heat. This may be so, especially deeper in the textile but if so, the end result appears to be the same, that less PCP is easily hydrolysed and available for protection. Any suggestion that heating treated textiles for long periods to increase their resistance to decay would probably be countered by the fact that extreme physical influences would increase the physico-chemical processes also responsible for strength losses of textiles. This point leads on here to the second point, that of the lag phase at the start of decay. The apparent slower rate of decay of heated PCPL treated textiles is due to the fact that little lag phase is observed, although the final decay result is the same. This may be due to a combination of two factors, firstly, the partial inactivation or removal of the fungicide and secondly the chemical decay of the fibres due to oxidation by weathering (see untreated heated textile) easing the onset of biological attack which would in this case of less protection able to be initiated by the first colonisers, little build-up of fungal species being necessary before decay manifests itself. (See also here Chapter 4, effects of heat and light). These points underline the fact that in the study of decay processes, the whole of the decay process should be observed, especially the important initial periods, where little change may be apparent, and that observations on rates of decay should not just be made considering the active or mid phase of the decay process.

From experiments in this chapter, it would appear that the concept of PCPL acting as a reservoir of toxic PCP is essentially correct. This has significance in several ways-;

the toxic component is not susceptible to leaching-out in its entirity from the start of protection; if the hydrolysis of PCPL is indeed biological as suggested, the PCP thus released is where required, that is, in intimate contact with the fungus; and also from an application and operator view, the use of a fungicide of low initial toxicity to humans is of advantage (see appendix on toxicity of PCPL and PCP).

Although PCPL-based fungicides have been used widely for many years, it would appear that they have attractive features in their patterns of use and action which would be most desirable in new protective compounds.

Chapter 6

ASPECTS OF THE GROWTH AND PHYSIOLOGY OF FUNGI IN RELATION TO TEST WORK USING PENTACHLOROPHENYL-LAURATE BASED FUNGICIDES.

6.1. General introduction

6.2. Comparison of growth of fungal isolates using agar growth media.

6.3. Effect of pH on the growth of Trichoderma viride on PCPL-containing agars.

6.4. Comparison of the decay abilities of two strains of Trichoderma viride, using a pure culture soil burial technique.

6.5. The effects of low levels of PCP and PCPL on the respiration of soil organisms.

ASPECTS OF THE GROWTH AND PHYSIOLOGY OF FUNGI IN RELATION TO TEST WORK USING PENTACHLOROPHENYL-LAURATE BASED FUNGICIDES.

6.1. General Introduction.

When investigations are made into the effects of biocides on living systems, it is often desirable to express any effects in such a way as to give an easy and valid comparison between a 'biocidal' and a 'non-biocidal' situation. It is usually necessary to express this difference in a quantitative way, and thus one of the readily measurable aspects of the living system, such as rate of increase in colony size, or rate of oxygen uptake, can provide a useful indicator of the effects of a biocide.

In the following experiments, several methods of investigation yielding quantitative data were employed. Comparison of the growth rates of previously-isolated fungi is obtained quickly, but also in such a form that can be quickly checked, for instance in the screening of test species for strain differences. It is this quick reproducable nature of agar methods, as mentioned earlier, which is one of their main advantages. Using similar agar techniques, the effects of various pH on the growth of Trichoderma viride was also investigated. This pH influence on growth was chosen as it is of great importance to fungal physiology (see Chapter 8). Two strains of T. viride were also tested quantitatively for strain differences in decay ability, using a modified pure culture soil burial test

and tensile testing, in order to highlight one more variable in testing work.

The final quantitative method employed here was in the study of the oxygen uptake of soil organisms in the presence of low levels of PCP and PCPL, in order to obtain data regarding the thresholds of inhibition of these fungicides.

6.2. Comparison of growth of fungal isolates using agar growth media.

Following previous experiments, comparisons have been made concerning fungal species in relation to their abilities to be isolated from soil into PCPL containing media. It was therefore decided to make a controlled comparison between individual species, in order to obtain more data concerning them, and also to choose test organisms for other studies.

Eight vigorous species of fungi from Clent soil were chosen as follows:-

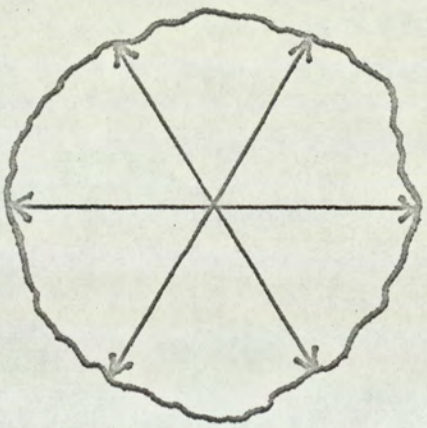
(i)	Penicillium funiculosum	'A'
(ii)	Penicillium sp.	'B'
(iii)	Fusarium solani	'F'
(iv)	Chaetomium globosum	'G'
(v)	Trichoderma viride	'H'
(vi)	Zygorynchus moelleri	'N'
(vii)	Geotrichum candidum	'R'
(viii)	Alternaria sp.	'V'

These fungi were grown on malt agar (Difco), from which young colony-edge inocula 6mm in diameter were cut using a sterile cork-borer. The inocula were placed mycelium-downwards on plates of the following media:-

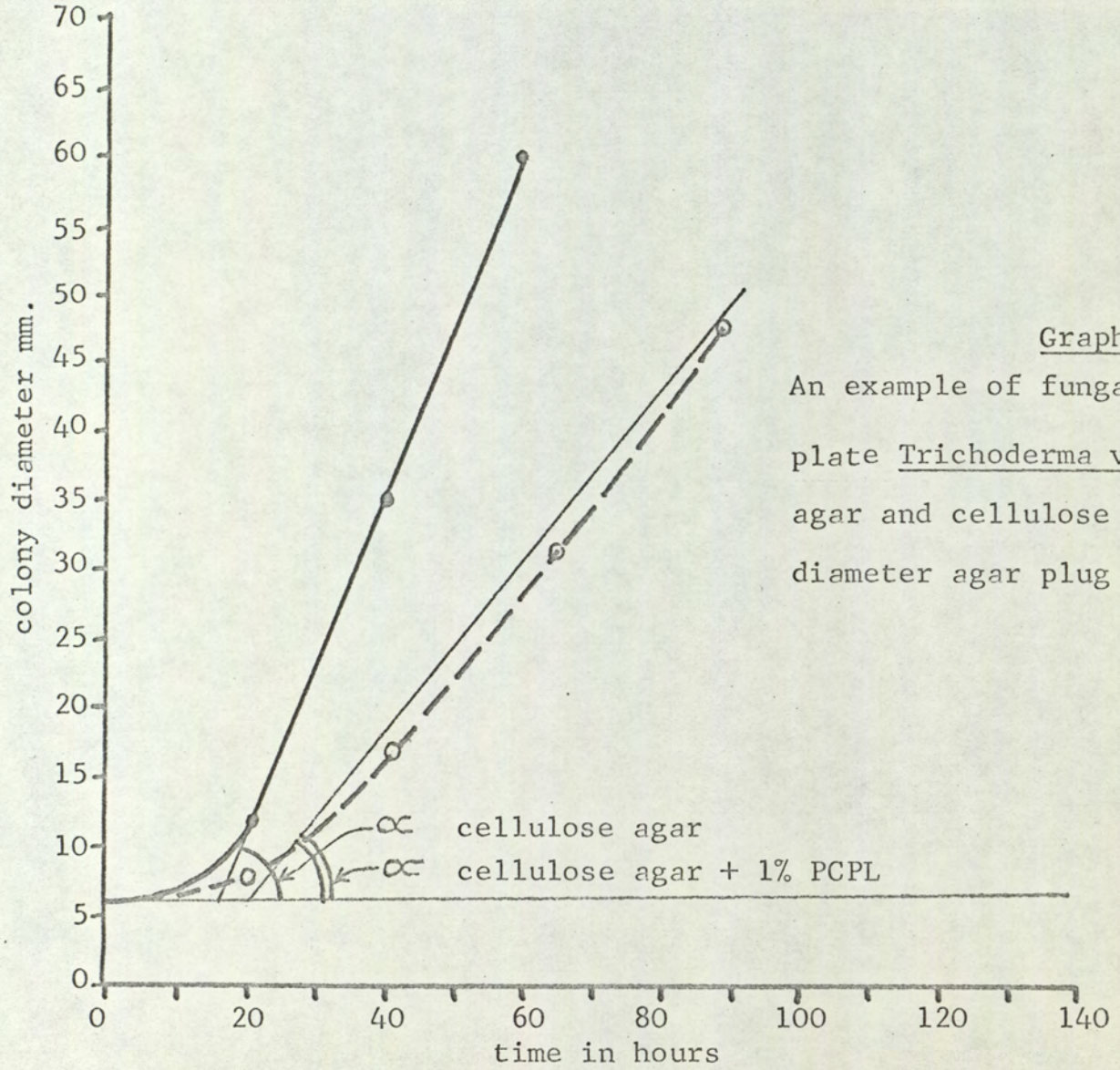
1. Malt agar (Difco)
2. Malt agar (Difco) + 1% PCPL as a 'Mystox') preparation

3. Eggins and Pugh cellulose agar.
4. Eggins and Pugh cellulose agar + 1% PCPL as a 'Mystox' preparation.

All plates were then incubated at 25°C. Measurements of colony diameters were taken at daily intervals. For each species on each medium, the measurement noted on each occasion was the average of colonies on each of three similar plates, the reading from each plate being the result of three measurements taken as shown below.

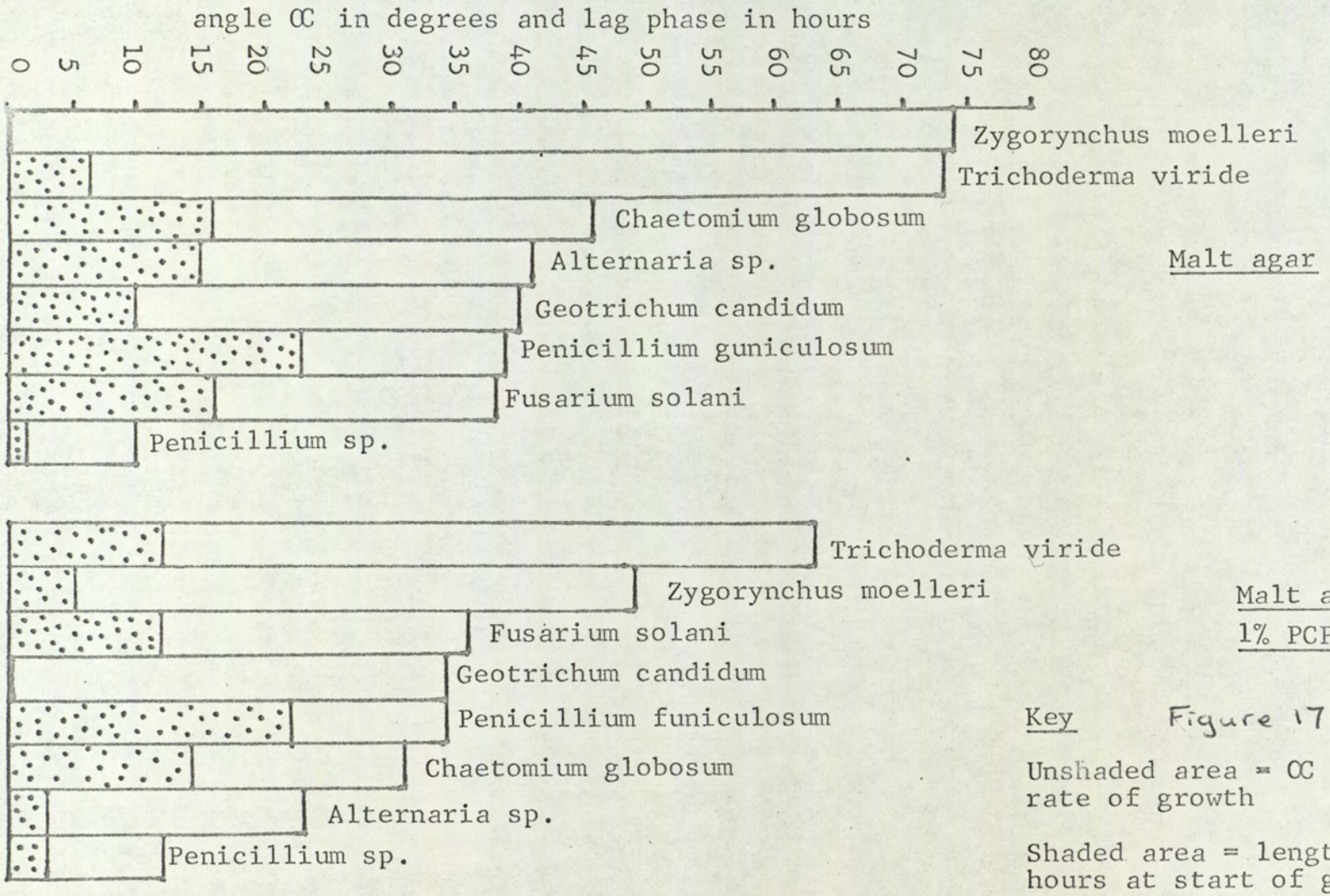


The results were plotted in graph form, and in order to aid comparison of rates of growth, it was decided to compare the angles subtended by the best-straight-line through the exponential or logarithmic growth phase (or the nearest approximation to this phase present) with the x-axis of the graph (angle α). Another factor concerning growth, that of the lag period prior to the start of the exponential phase was also noted. The best-straight lines were calculated using an Olivetti Programma desk calculator, drawn in the graphs, and the angle measured. An example is given in graph 15a. The results are summarised in figure 17 (malt agars) and figure 18 (cellulose agars)



Graph 15 (a)

An example of fungal growth on an agar plate Trichoderma viride on cellulose agar and cellulose agar + 1% PCPL (6mm diameter agar plug inoculum).

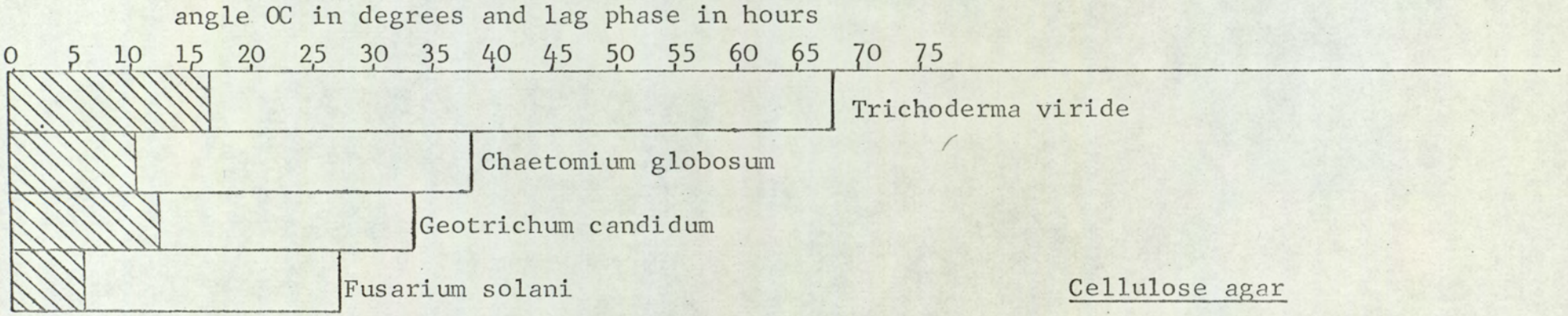


Key

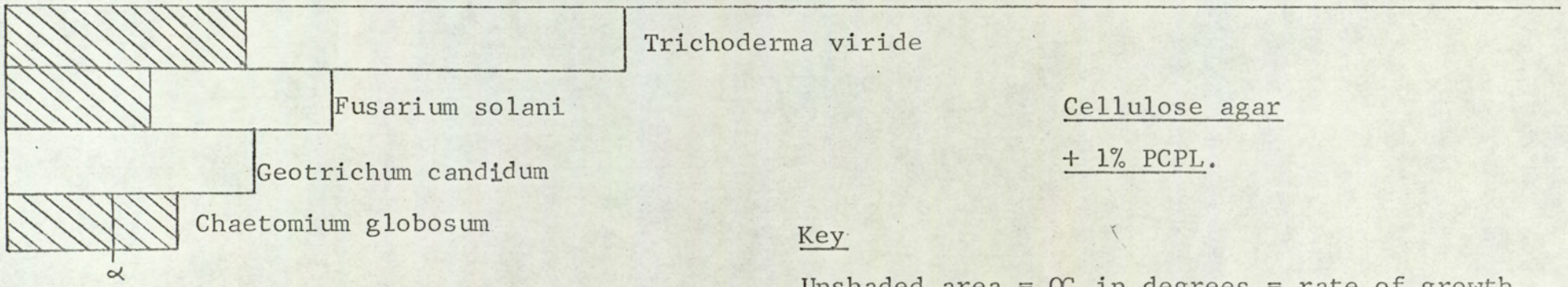
Unshaded area = ∝ in degrees = rate of growth

Shaded area = length of lag phase in hours at start of growth of colony.

Figure 17



These species all grew on and cleared cellulose agar.



Key

Unshaded area = OC in degrees = rate of growth

Shaded area = length of lage phase in hours at start of growth of colony.

Figure 18

From these results, several conclusions may be drawn:-

(i) Fewer fungal species grew on cellulose agar than on malt agar.

(ii) Growth on malt agar was more rapid than on cellulose agar.

(iii) Species were inhibited to different extents upon the addition of PCPL to the media.

As malt agar is easily assimilated by most fungi it was to be expected that growth upon it would be more rapid than on cellulose agar, and also that more species would be able to grow on malt agar. On malt agar, two species, Zygorynchus moelleri and Trichoderma viride, were by far the fastest growing species. Z. moelleri is a sugar fungus and such growth is to be expected; T. viride is able to utilise a wide range of nutrients. Upon addition of PCPL the vigorous growth and fungicide resistance of T. viride give it an advantage compared with Z. moelleri. The majority of fungi tested had similar growth rates on malt agar, this intermediate group comprising Chaetomium globosum, Alternaria sp., Geotrichum candidum, Penicillium funiculosum and Fusarium solani. Upon the addition of PCPL to the medium, the differences in growth rates became somewhat more marked, F. solani being least affected, and Alternaria sp. and C. globosum

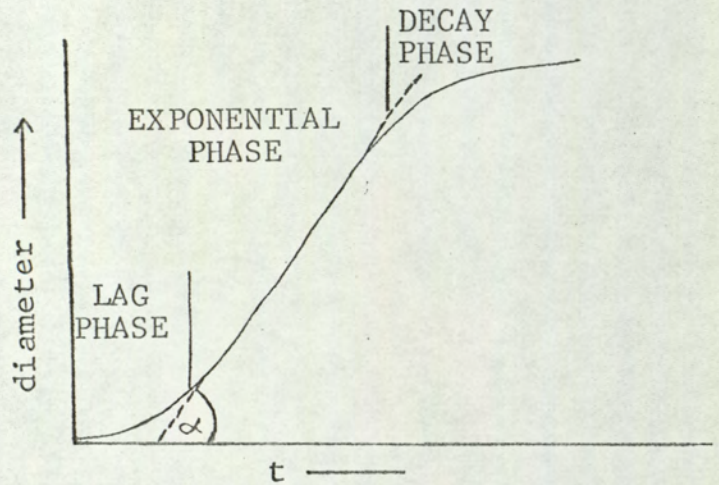
being most inhibited. The remaining species tested on malt was a species of Penicillium, which was the slowest growing species. The addition of PCPL to the medium increased the growth rate of this species. This anomaly is most probably due to the low accuracy of this method of investigation when very slow growing fungi with a poorly defined exponential growth phase are studied. Such cases are discussed in the following section on this method.

It will be seen from figures 17 and 18 that the lag periods of fungal species before strong growth commenced varied greatly, some fungi having no appreciable lag phase at all. These variations are significant when considering isolation work. A species which has a short lag phase, especially if it is a vigorous species, will have a marked competitive advantage on a mixed culture plate or similar system where different species are present.

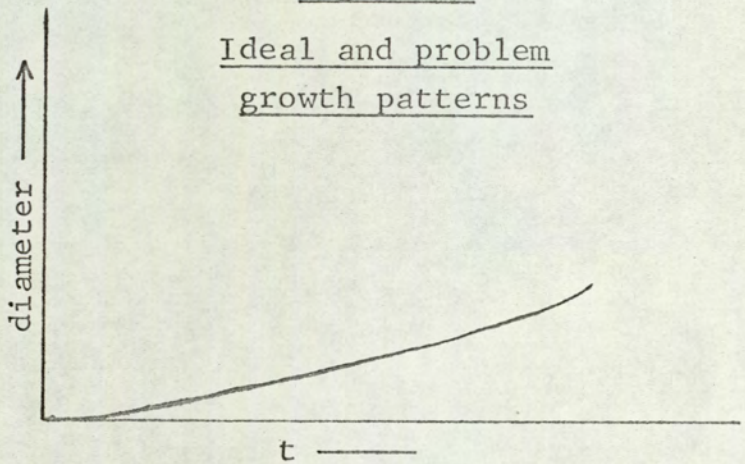
On cellulose agar, comparisons are simplified due to the fewer species which were able to grow. Those which did grow demonstrated their cellulolytic activity by clearing this medium. T. viride was the dominant species on both cellulose agars. Upon addition of PCPL F. solani again showed most tolerance to the fungicide. These results reinforce similar observations of PCPL tolerance by these species made in earlier chapters. The three fungi with the neatest growth habits, namely T. viride, G. candidum and C. globosum, which grew on cellulose

Figure 19

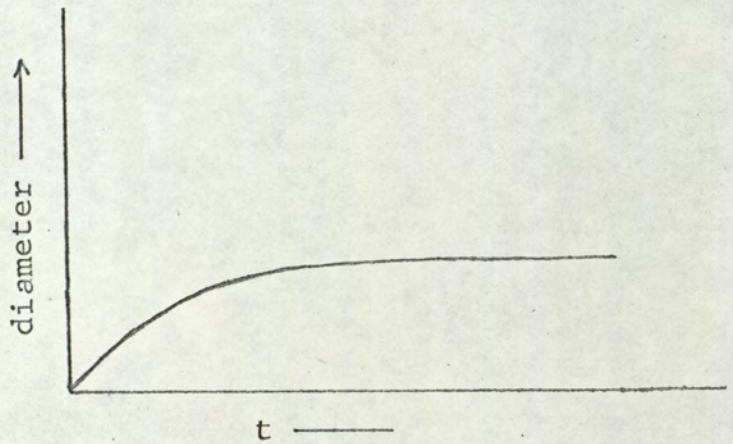
Ideal and problem growth patterns



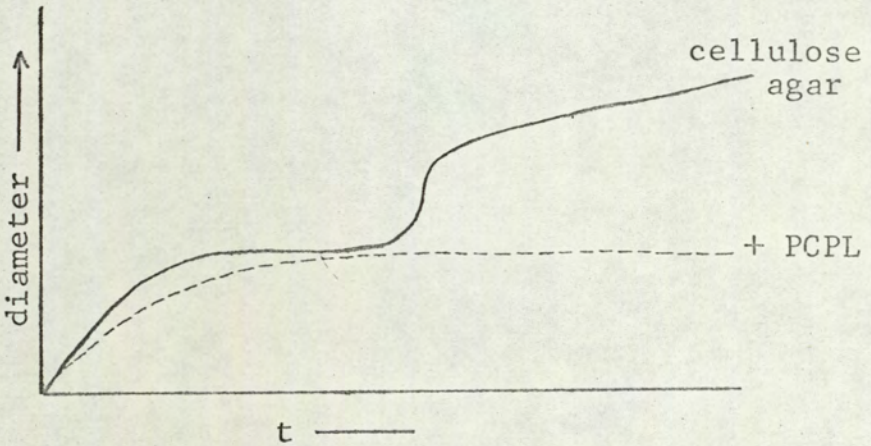
(a) ideal growth pattern



(b) slow growth - no distinct phases



(c) convex slope



(d) erratic growth. (Penicillium sp.)

agar + 1% PCPL were chosen as test organisms for use in the multiple perfusion technique (See Chapter 4).

The ease and accuracy of this method depends upon a 'normal' growth pattern being made by the organism under investigation. A regular uniform colony must be formed, as near a perfect circle as possible for measurements to be meaningful. The plotted graph of colony size against time must show a clear exponential phase (figure 19(a)) through which a line may be drawn and measured. Problems may arise where the colony is very slow growing, where phases of growth are not clear (figure 19(b)). Where very slow growth occurs the effects of fungicides may not be marked as occurred with a very slow growing Aspergillus species investigated at the time of the experiment. It is possible that a convex slope may result; this may perhaps be interpreted as a lack of a lag phase, followed by a short exponential phase and a long decay phase, this is open to argument, however, and comparisons with other species growing 'normally' are difficult (figure 19(c)). Due to factors not identifiable, growth may be erratic and results cannot be compared. An example of this was produced by a Penicillium species whose performance was not compared with others for this reason (figure 19(d)). It may often be the case that exceptions to rules are of interest, and so any technique employed should allow

for their inclusion. This technique, however, is a useful means of comparing the growth of fast growing species and appears to have worthwhile validity when used for this purpose.

6.3. Effect of pH on the growth of Trichoderma viride on PCPL-containing agars.

The pH of a medium is known to be one of the critical factors which affect the growth of fungi. This factor has been further considered in different ways during the course of this investigation, but in this particular instance, the growth of one particular fungus, Trichoderma viride has been studied. Trichoderma viride was chosen as it is a particularly common fungus isolated from soil and also because it is a very vigorous species and shows a marked tolerance to pentachlorophenyl laurate biocides; it is thus an ideal test organism.

The experiment was prompted by indications of pH change of media supporting growth of Trichoderma viride. The particular medium used initially was Eggins and Pugh agar. This medium contains ammonium sulphate at a concentration of 0.5g/l. It is known that as this compound is utilised, free sulphate ions are liberated which may lower the pH of the medium. This would tend to occur in media supporting a well established growth of fungus, but the change due to this factor alone would appear to have little effect under normal circumstances, as fungal growth proceeds well on this medium. It appears therefore that this fungus is able to induce a low pH in an agar medium, possibly due to the excretion of acidic metabolites. Whatever the cause, it was observed that Trichoderma viride can induce

and prefers low pHs. This was initially observed in the following way. During isolation experiments using 1% 'Mystox ELC' in agar media, it was noticed that media containing a bacteriostat, Rose bengal, which is usually bright pink, had become discoloured to a pale brown colour. Plates on which this had occurred were supporting strong growths of Trichoderma viride. Upon checking the pH of the discoloured media, it was found that the pH had dropped to just below 3 (Starting pH around 7.0). It was therefore decided to choose Trichoderma viride as the test organism to investigate the effect of pH on the effect of 'Mystox ELC' in agar media.

Trichoderma viride was grown on agar plates at 25°C. The media used were malt extract agar, cellulose agar and Eggins and Pugh nutrient salts agar (Eggins and Pugh 1964). These media were also used containing 1% 'Mystox ELC'. These were buffered and adjusted in pH from 4.5 to 8.0. The diameters of the resulting colonies were measured at intervals and plotted in graph form against time. The pH of the media was checked at intervals and plotted on the same graphs as growth.

Malt-extract agar, Eggins and Pugh cellulose agar and Eggins and Pugh nutrient salts agar (not excluding yeast extract and L-asparagine) were made up. The cellulose used here was 'Avicel-RC' in its commercial form. This was used by way of an experiment and if ball milling is not carried out before use, care must be taken to agitate the medium as it sets to avoid settling-out of the cellulose. McIlvane's citrate-phosphate buffer was used in the media. The amounts of buffer used, however, had to be sufficiently low so as not to change the osmotic potential of the medium unduly, leading to a situation where the buffering effect was not optimal. Eggins and Pugh media however, are to a great extent self-buffering and all media kept in general within a range of 1.5pH units from the initial pH in practice.

1% 'Mystox ELC' was added as the following solution:- 10 parts 'Mystox ELC', 2 parts 'Tween-80', 8 parts distilled water. This produces a less viscous emulsion which facilitates measuring and handling. The concentration of 'Tween-80' used was determined so as to not exceed 0.05% in the final medium, as this is its generally accepted maximum non-inhibitory concentration in agar media. The pH of the medium was measured before and after autoclaving using a temperature-compensated pH meter. It is important to measure pH immediately after autoclaving, for as far as can be seen from the results, there is usually a drop in pH of around 1 full pH unit as a result of autoclaving.

Inocula were cut from colony edges of Trichoderma viride growing on cellulose agar. The isolate used was obtained from 'Mystox ELC' treated chromatography paper which had been in contact with refuse-dump waste pour plates made with 1% 'Mystox ELC' cellulose agar. The plates were incubated at 25°C and the colony size and pH measured at 3, 5 and 10 days. pH was ascertained by gently melting the agar from the colony area and using a temperature compensated pH meter.

The results were plotted in graph form (See graphs 16-18 and plates 14-16).

Several conclusions may be drawn from these results:-

(a) The fungus grew well on all the media not containing a fungicide (i.e. the controls).

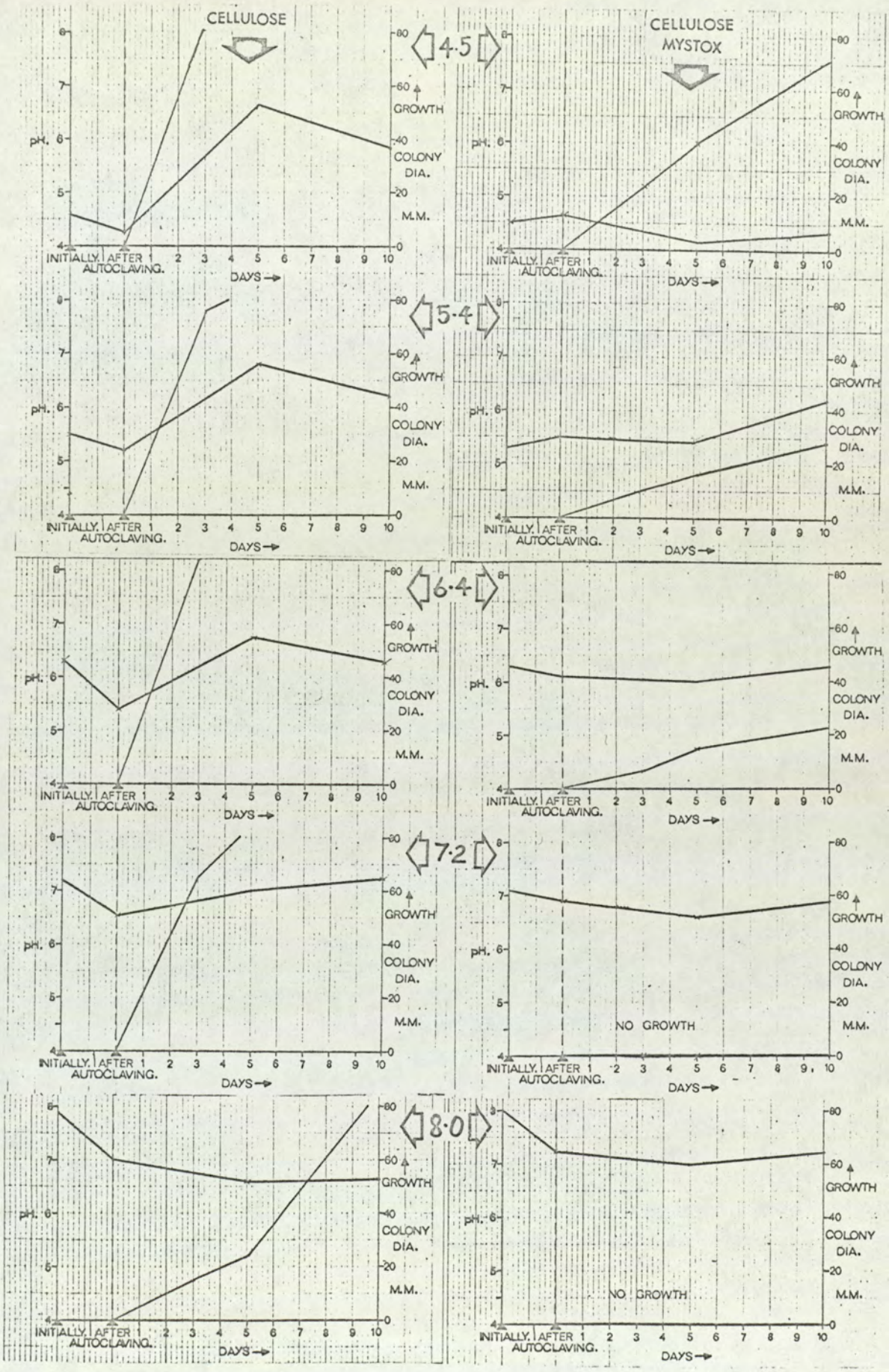
(b) Most rapid growth occurred below pH 7, i.e. under acid conditions.

(c) On media containing 1% 'Mystox ELC' maximal growth was as follows:-

Malt extract agar :- pH 4.5-6.4

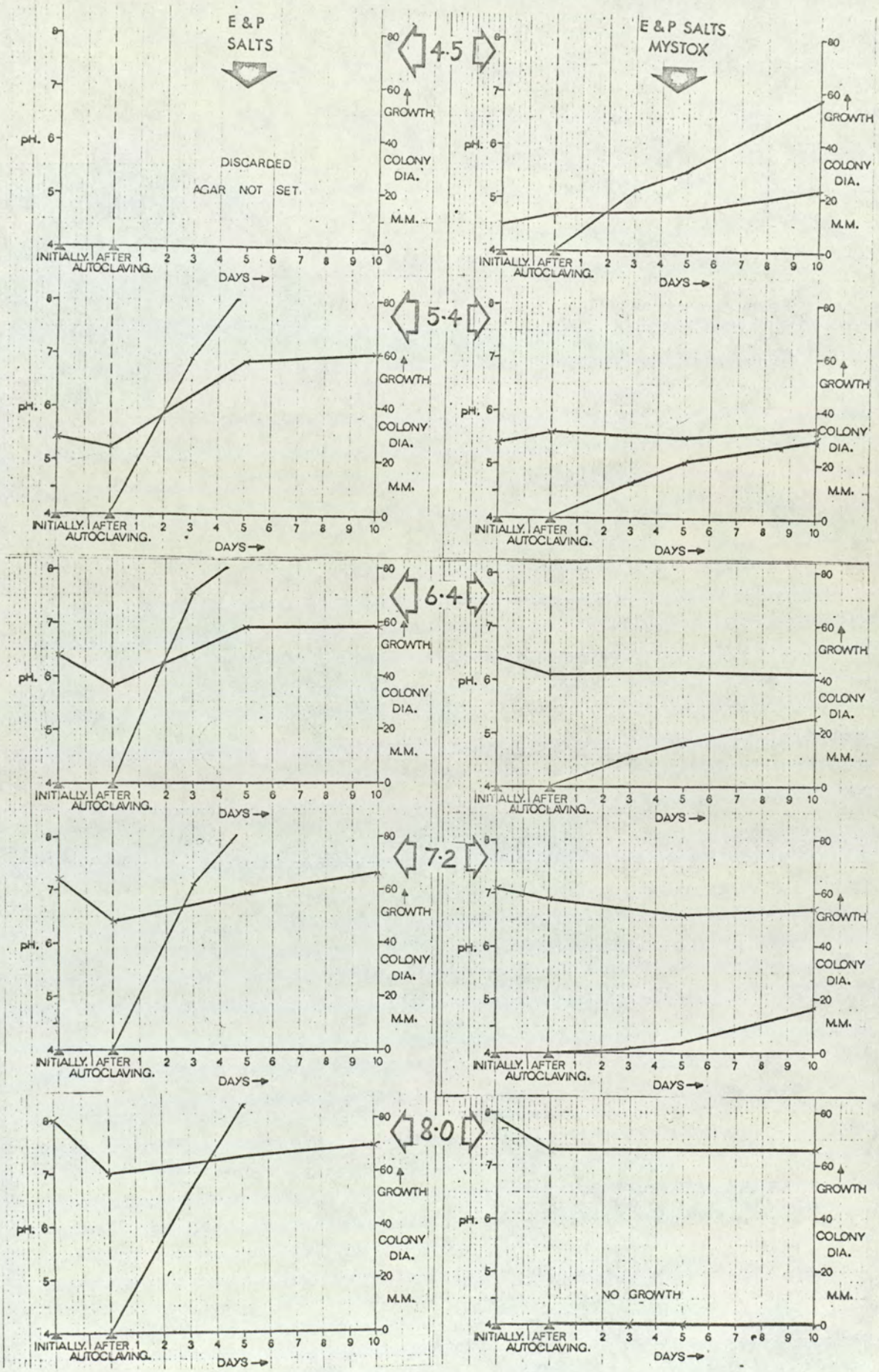
Cellulose agar :- pH 4.5

E & P Salts agar :- pH 4.5



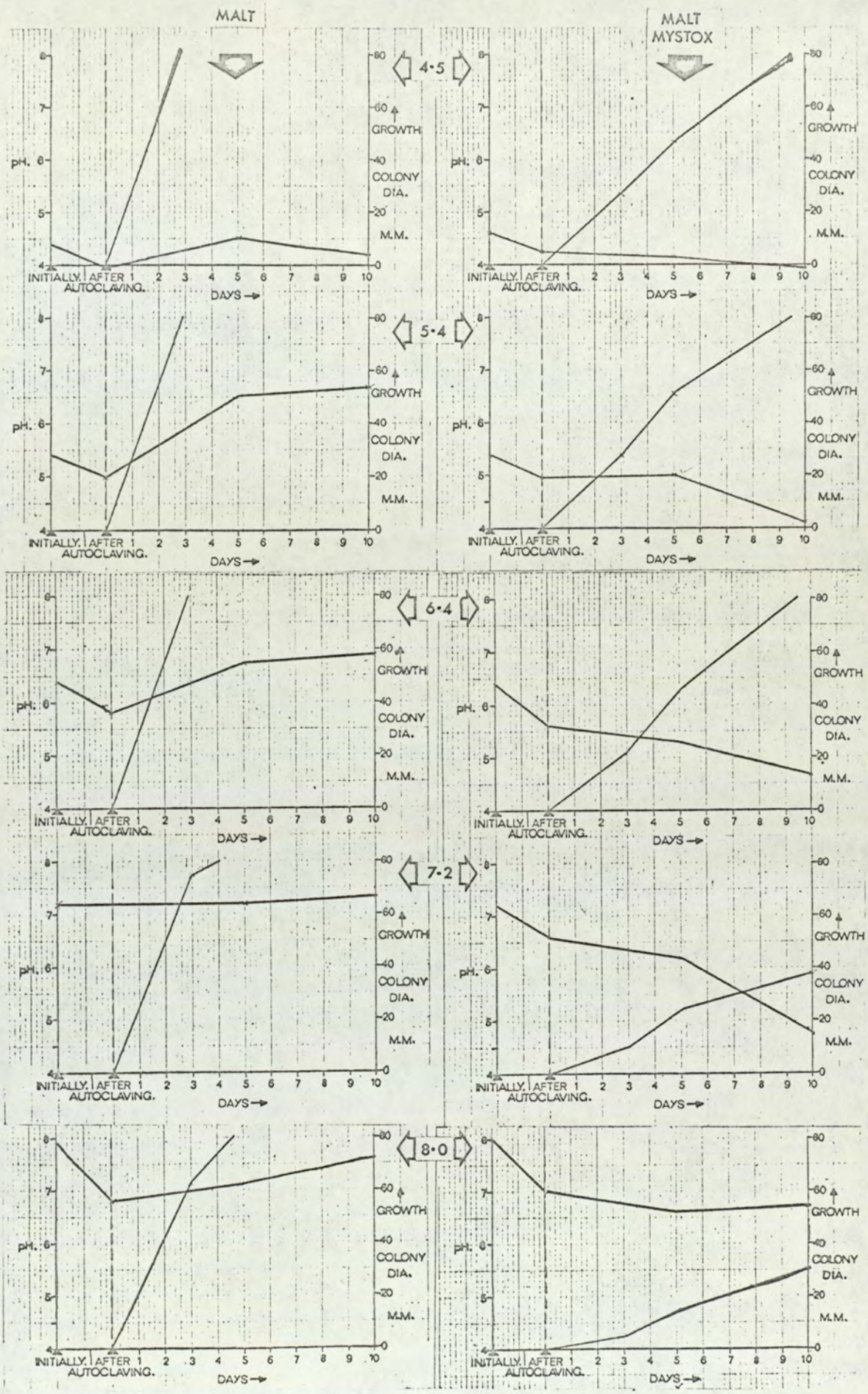
Graph 16

Growth of T. viride on cellulose agar at various pH, with and without fungicide added. pH changes in media are shown.



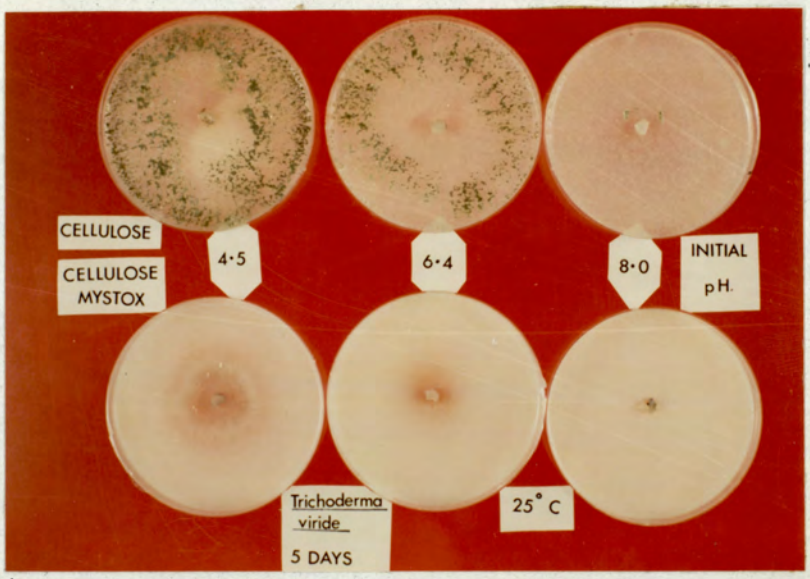
Graph 17

Growth of T. viride on E & P salts agar at various pH, with and without fungicide added. pH changes in media are shown.



Graph 18

Growth of T. viride on malt agar, at various pH, with and without fungicide added. pH changes in media are shown.



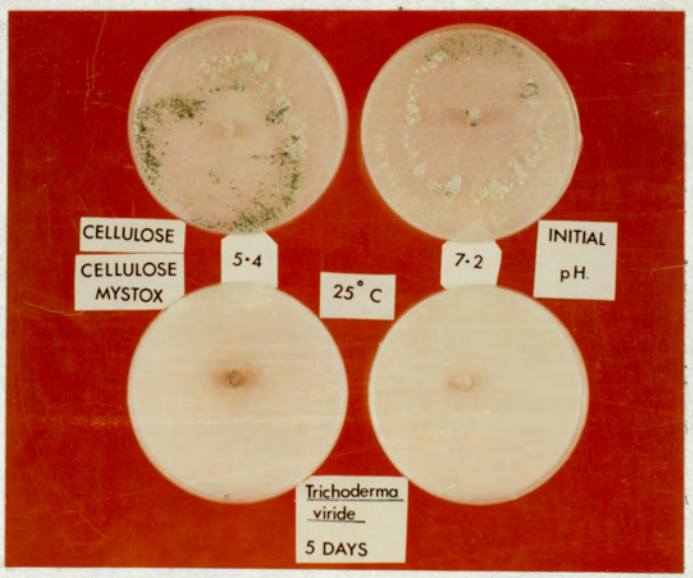
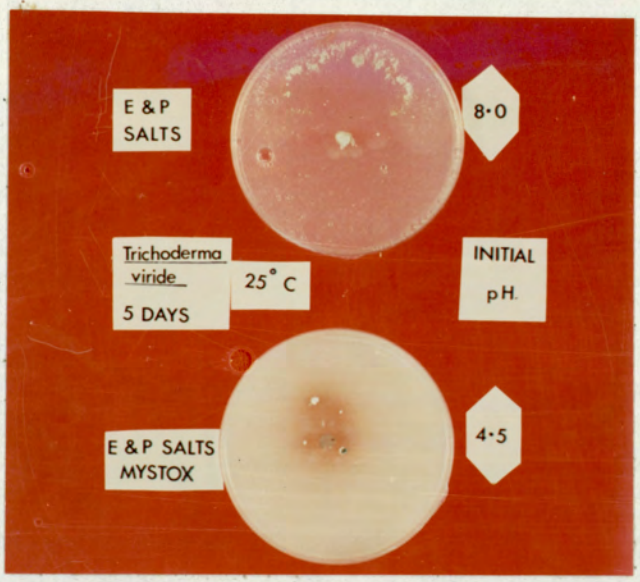


Plate 14

Growth of Trichoderma viride on cellulose agar at various pH, with and without addition of PCPL fungicide



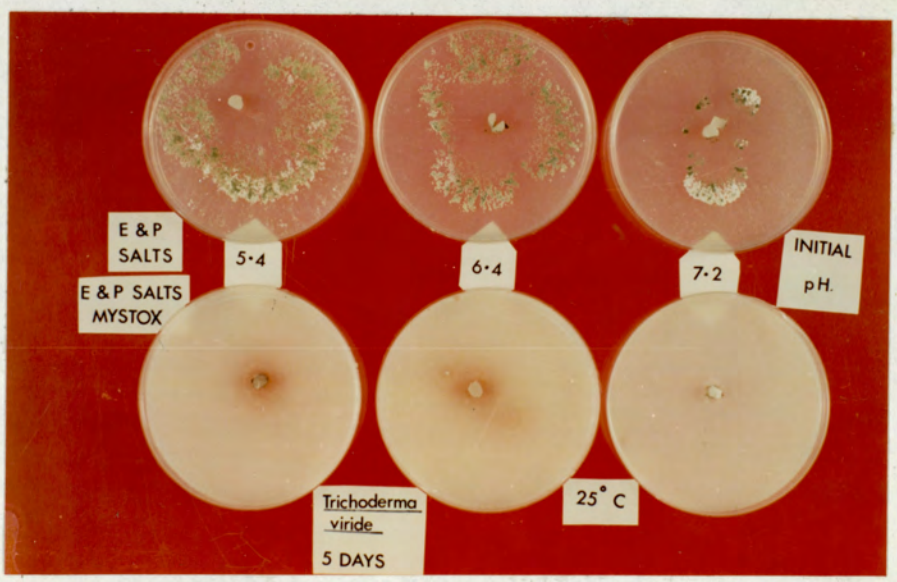


Plate 15

Growth of Trichoderma viride on Eggins & Pugh salts agar at various pH, with and without addition of PCPL fungicide

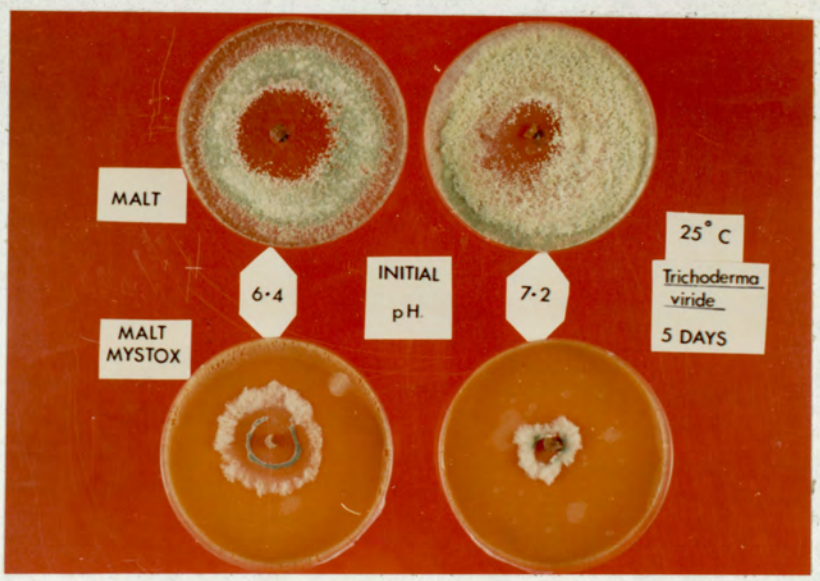


Plate 16

Growth of Trichoderma viride on malt agar at various pH, with and without addition of PCPL fungicide

Two clear points emerge from this experiment, firstly the growth of the fungus is greatest under acid conditions, and secondly that growth in the presence of the biocide is greatest under conditions even more acid.

Good growth under acid conditions in normal media is to be expected as fungi tend to prefer acid conditions for growth. The need for greater acidity in the presence of this biocide is a more complex situation. The factors which may be involved are as follows.

(1) The combination of two unfavourable conditions, high pH and the biocide together, leading to reduction in growth.

(2) The release of toxic pentachlorophenol from the biocide may be more easily effected at higher pH (See Chapter 5 on mode of action of pentachlorophenyl laurate biocides). It is known that this hydrolysis can occur chemically under highly alkaline conditions, and so this experiment serves to demonstrate that the efficacy of this biocide is lessened as conditions become more acidic, where T. viride is the test organism. This effect is also seen in Chapter 8, where cotton textile decayed most is from the two soil samples with the lowest pH (5.2 and 6.0). T. viride was abundant in both soils.

These results raise several points for discussion. The first, which may in fact cloud the following points, is the presence of T. viride. This appears to be a resistant organism to chlorinated phenols and has been reported as degrading PCP.

Degradation of PCP by T. viride was reported by Duncan and Deveral (1964) using analysis of residual PCP in wood blocks after fungal growth. Levtritz (1965) questioned these results, suggesting that losses were due to leaching and water displacement. However, Unligil (1968) obtained results with T. viride similar to those of Duncan and Deveral in a more controlled experiment. Cserjesi (1967) also showed depletion of PCP by several Trichoderma species.

Bearing these factors concerning T. viride in mind the second point is that the study here is of pentochlorophenyl laurate and not pentachlorophenol.

Cserjesi and Johnson (1972) quote from unpublished work that they have also observed an increase in the efficiency of pentachlorophenol as pH falls. They compared growth in liquid culture at pH 5 and 6 and showed a ten-fold difference. They were, however, using two different forms of pentachlorophenol, the phenol at pH 5 and sodium pentachlorophenol at pH 6, which makes direct comparison difficult.

At pH lower than 5, chlorinated phenols are mainly non-dissociated in aqueous solutions, and as the pH rises, they become more dissociated and less toxic, as shown by Wessels and Adema (1968) using pentachlorophenol, and Wolf and Schaffer (1968) using trichlorphenol. Using PCPL, however, inhibition of growth was greatest at alkaline pH.

The argument is one of proportion. PCPL was least effective at pH 4.5, but nevertheless inhibited growth in each case by at least a factor of 2. Also, notwithstanding the fact that the PCP moiety released from PCPL may be different from forms used by other workers in studies on PCP, the PCPL here acts as a reservoir of toxic PCP, perhaps releasing more in conditions condusive to hydrolysis, chemical and biological, at higher pH and in the presence of resistant fungi. Due perhaps to this action it may be that PCPL based (esterified) biocides have a wider range of activity than PCP based ones, extending the range of chlorinated phenol biocides in the more alkaline range. It may also be a factor that for the PCP to be freed, hydrolysis must first occur, and as this may often be biological, the newly released PCP, in intimate contact with the organisms involved, may be more effective due to its chemical form and proximity to organisms than the PCP in biocide formulations.

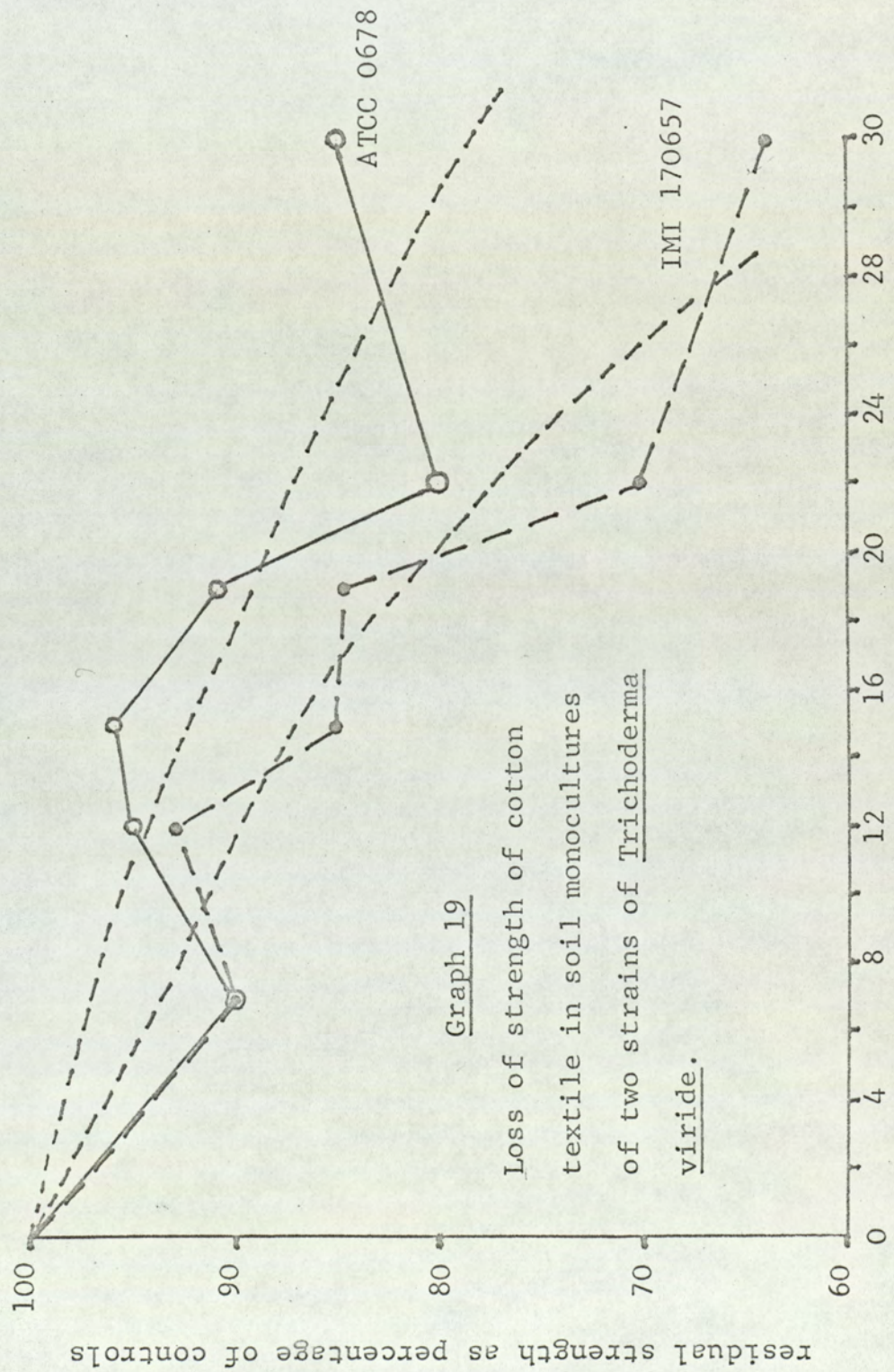
6.4. Comparison of the decay abilities of two strains of Trichoderma viride, using a pure culture soil burial technique.

Differences in strains of the same fungal species may be a source of variation in testing work (Jones, 1968). The frequent occurrence, vigour and fungicide resistance of the Trichoderma viride isolated from Clent soil and used in previous experiments led to a comparison of its activity with another known strain. The Clent strain was identified by the Commonwealth Mycological Institute, Kew, England, and has been allocated the reference number I.M.I. 170657. The strain used for comparison was the test strain used at Centraal Laboratorium T.N.O., Delft, The Netherlands, which is ATCC 0678, TNO culture number E3070.

Both cultures were plated-out and grown up on Difco malt extract agar, incubated at 25°C. Covered beakers were filled with a well drained soil and autoclaved at 20 p.s.i. for 1 hour. The sterility of the soil was checked by plating out samples. The beakers of sterile soil were divided into two groups, and into each beaker was mixed the fungus covered agar from one plate, together with 100 ml of sterile water. The beakers were incubated

at 30°C in a high-humidity room, the soil being mixed each day for one week prior to insertion of cotton textile test strips. Thus, by using a large inoculum on a nutrient base in sterile soil, monocultures of both fungal strains were obtained. This technique was chosen as it is less artificial than agar techniques, giving the fungi more chance to exhibit their full decay potential. During the course of the experiment isolations were made onto cellulose agar using decayed textile from the beakers. No other fungi were isolated than the T. viride strains.

The cotton test textile used in this experiment was T.N.O. cotton textile treated with 1% PCPL as a 'Mystox' preparation, containing 0.13% free PCP. The textile was cut into strips 1.5 cm wide with 0.5 cm ravelled edges, and 8 cm long. These strips were then fixed singly to the outside of glass boiling tubes with adhesive glass-fibre tape, and after autoclaving at 15 p.s.i. for 20 minutes, were buried in the beakers of soil, 11 tubes in each beaker. Strips were removed at intervals, washed in 70% ethanol to arrest further growth of the colonising fungi, dried at room temperature and after conditioning for 24 hours in a humidity and temperature controlled room, were broken on a tensiometer. The results are shown in graph 19. Each point is the average of three results as a percentage of the average control breaking strength.



Graph 19

Loss of strength of cotton textile in soil monocultures of two strains of *Trichoderma viride*.

It can be seen from graph 19 that the decay abilities of these two strains varied. The actual graphs as plotted are rather uneven; this is probably due to the small numbers of replicates (3) used for each reading, however, one curve is consistently lower. Even though it would be difficult to state with certainty that the two strains always differ, it is clear that under the conditions of this experiment there was a marked difference. In growth on agar there was also a difference, I.M.I. 170657 showing greater pigmentation and more abundant sporulation than ATCC 0678. Whether these differences are truly inherent in the strains or whether they are brought about by methods of handling, culturing and storage, would be a subject for further investigation. The central point is that variation occurred in two strains of the same fungal species. This serves to underline the problem of strain differences in test work; a problem which may often be overlooked due to shortage of time.

6.5. The effects of low levels of pentachlorophenyl-laurate on the respiration of soil organisms.

When pentachlorophenyl-laurate biocides are used as textile protectants, they are normally applied at concentrations of 1-2% by weight. This concentration is designed to give a useful length of protection at economic cost. Under some circumstances the cost of the biocide may be critical, and it is thus important to know the minimum useful concentration necessary. Each individual case is of course different, but this study goes some way in showing at which biocide concentrations inhibition of organisms is initiated. This factor is also of importance when considering application of a biocide; if distribution of the biocide is not even upon the textile, then there may be areas (on a micro-scale) which are liable to attack, even though the textile as a whole has taken up a 'safe' concentration.

Before any of these practical points could be investigated further, however, it is necessary to investigate the effects of low levels of biocides. To do this it was found that a more sensitive test than agar plates could provide was required. It was therefore decided to measure respiration (uptake of oxygen) as an indication of microbial activity in the presence of the biocides under investigation.

Soil was used as the 'living' part of the system under investigation as it contains the whole range of organisms to which textiles may be exposed. It also proved more convenient to use than spore suspensions which are in fact less typical as an inoculum even if possibly more severe under certain circumstances (i.e. a dense concentration of spores of a vigorous organism.

Measurement of carbon dioxide evolution or oxygen uptake is a well known method of assessing microbial activity. Early techniques used chemical absorption and titration in the assessment of carbon dioxide evolution from soils (Huekelekian and Waksman, 1925; Dubos, 1928). Later workers found the Warburg respirometer a convenient tool (Bunt and Rovira, 1955); its use being extended by modification for longer-term studies, over weeks rather than hours (Chase and Grey, 1957), other types of manometers being used for similar studies (Burgess and Darby, 1964, 1965). Although useful research tools, manometric methods are somewhat complex, and current thinking on respirometry is reverting once more to simple methods of chemical absorption of evolved carbon dioxide and titration (Kaplan, 1964; Kibble, 1966; Stewart and Walsh, 1971).

A particular use of respirometric techniques is in estimating microbial activity, where the organisms are intermixed with their substrate; they are thus ideal tools in soil studies.

The Warburg constant volume manometer

The apparatus is a capillary U-tube with a reservoir of manometer fluid (in this case Brodie's solution) at its base. The left hand limb of the U-tube is left open to the atmosphere, while the right hand limb may be closed by a 3-way stop tap. Via a side arm from the top on the right hand limb a flask is attached to the manometer. A scale is fixed behind the U-tube to enable a rise or fall of the fluid in the open limb to be measured. The zero point on the scale is about half-way up the U-tube and the fluid in the right-hand limb is always returned to this zero point by adjusting pressure on the flexible reservoir before any reading is taken of the level of fluid in the left hand limb. This maintains a constant volume of gas (Vg) in the manometer and flask. The value of Vg for any manometer and its flask is given by (Vo-Vf) where Vo is the volume enclosed by the flask plus manometer side arm plus that portion of the right-hand manometer limb between the stop tap and the zero calibration point, while V_l is the volume of the liquid contents of the flask. The gas-producing or gas-consuming reaction proceeds in the liquid contents of the manometer flask. This liquid is in equilibrium with the enclosed gas phase so that utilisation of dissolved gas is reflected as a decrease in gas pressure, and vice versa. If gas is taken up during the reaction in the flask, the level of the manometer fluid rises in the right hand limb of the manometer U-tube, causing a fall in

the level of fluid in the left-hand limb. To read the actual decrease in pressure at constant volume, the meniscus in the right-hand limb must be returned to the zero point. This is done by allowing some manometer fluid to return to the reservoir. The fall in the level of the fluid in the left-hand limb can be quantitatively related to the amount of gas absorbed in the flask. As such readings are taken at frequent intervals during the course of the reaction an almost continuous record of the course of the gas uptake (in this case oxygen) is obtained.

The manometer flask has a closeable tap-stopper, a side arm for reactants (not used here) and a centre well to hold gas absorbents, in this case KOH to remove CO_2. The flask contains a comparitively small volume of reaction mixture and is constantly shaken by the oscillation mechanism of the respirometer. Thus, a large liquid-gas surface is maintained to promote rapid diffusion and equilibration of free and dissolved gases at the set temperature.

Concurrent readings to those taken from manometers attached to reaction flasks are taken with a thermobarometer, a 'blank' manometer (i.e. water, biocide and KOH here) whose flask here contained no soil. These readings are used to compensate the experimental readings for fluctuations in atmospheric pressure or bath temperature during the course of the experiment.

The pressure changes in the manometer are thus translated into distances moved by the manometer fluid in the

left hand limb in either an upward (positive) direction or downward (negative) direction (as in this study). Movements in the similar meniscus in the thermobarometer are substracted from those recorded in the experimental manometers. The resulting distance (in mm) is then translated into micro-litres of dry gas at S.T.P. by multiplication by a constant (the manometer constant). The value of this constant, K, is made for each individual manometer and must be calculated afresh whenever any of the following factors change.

(i) The nature of the gas whose partial pressure changes in the manometer and flask;

(ii) The temperature of the experiment;

(iii) The volume of the liquid in the flask (Vf).

In order to avoid fresh calculation at each stage of the study, these are kept constant.

Calculation of the manometer constant.

The value of the manometer constant (K) relating to a specific gas in a particular manometer at a single temperature is obtained by substitution in the following equation:-

$$K = \frac{Vg \frac{273}{T} + Vf}{Po}$$

Where:-

Vg = volume of available gas space in μl

T = experimental temperature in degrees absolute (^{o}K)

Vf = volume of fluid in manometer in flask in ul

α = absorption coefficient of the exchanged gas in the liquid contents of the manometer flask at temperature T

Po = normal atmospheric pressure (i.e. 760mm Hg), expressed in mm of manometer fluid (The fluid is made up so that Po = 10,000mm manometer fluid).

The effect of temperature on the solubility of the exchanged gas is taken into account by using the value of its absorption coefficient (α) that is relevant to the experimental temperature.

In both solubility and partial pressure the exchanged gas is wholly independent of the concurrent presence of other gases, and this independence extends to the manometer constant. Thus the manometer constant (K_{O_2}) to be used when following the utilisation of oxygen by respiring tissues in a given manometer, has the same value irrespective of whether the atmosphere in the manometer flask consists of pure oxygen, air or an oxygen plus carbon dioxide mixture.

Calculation and plotting of results.

The amount of oxygen consumed in the flask of a Warburg manometer during each interval between readings is calculated as follows:-

$V = h \times K$

Where V = volume of gas exchanged in µl at S.T.P.

h = distance in mm by which the meniscus moves in the left-hand limb of the manometer

K = the relevant flask (manometer) constant.

(As this study is of oxygen uptake, h has a negative value and thus V also has a negative value, i.e. the volume of gas in ul at S.T.P. taken up.)

Results are calculated as follows:-

(i) Note the amount by which each thermobarometer reading differs from that immediately previous to it.

(ii) Note the 'interval changes' in the readings of all the experimental manometers (as in (i)). Enter each result in column (i) for each manometer (See specimen results).

(iii) Subtract the thermobarometric interval changes from the corresponding interval changes for the remaining barometers. Enter this figure in column (ii).

(iv) Multiply the true interval changes in the manometric readings (column (ii)) by the relevant manometer constants to give the gas volume interval changes in µl at S.T.P. Enter this figure in column (iii)).

(v) Sum the gas volume interval changes (column (iii)) to give the total change in the gas volume at S.T.P. Enter this figure in column (iv). (Column (iv) for each manometer, either each reading or the final total enables a comparison to be made between manometers, and thus detection of any malfunction).

(vi) Add the values of column (iii) for each manometer at each reading. Divide this sum by the number of experimental manometers to obtain an average for each reading. Add each reading to give cumulative totals at each time interval.

(vii) Plot each cumulative total volume of gas absorbed against time.

See figure 20. Specimen results and calculations.

Use of the Warburg respirometer

The respirometer used was a Braun circular Warburg apparatus, Model V166, set at a shaking rate of 70 cycles/minute. The motor bath was held at 30°C by a heater/thermostat. 30°C was chosen as the incubation temperature as it was the lowest temperature above the ambient laboratory temperature that could be maintained by the equipment.

The same stock of sieved and watered Clent soil was used throughout this study; the soil being stored at around 30°C between experiments.

TIME mins	THERMO-BAROMETER reading	change	MANOMETER 1 K = 1.0					MANOMETER 2 K = 1.2					MANOMETER 3 K = 1.1				
			reading	(i)	(ii)	(iii)	(iv)	reading	(i)	(ii)	(iii)	(iv)	reading	(i)	(ii)	(iii)	(iv)
0	15.0	0	15.0	0	0	0	0	15.0	0	0	0	0	15.0	0	0	0	0
10	14.8	-2	14.6	-2	-0	-0	-0	14.5	-5	-3	-3.6	-3.6	15.0	0	+2	+2.2	+2.2
20	14.6	-2	14.3	-3	-1	-1	-3	14.3	-2	0	0	-3.6	14.8	-2	0	0	+2.2
30	14.8	+2	14.3	0	-2	-2	-5	14.3	0	-2	-2.4	-6	14.7	-1	-3	-3.3	-1.1
40	14.8	0	14.2	-1	-1	-1	-6	14.3	0	0	0	-6	14.7	0	0	0	-1.1
50	15.0	+2	14.2	0	-2	-2	-8	14.3	0	-2	-2.4	-8.4	14.7	0	-2	-2.2	-3.3

Figure 20 (Continued Figure 20A

Specimen results. Oxygen uptake of a 30% soil solution in water, 30°C - first six readings of a six hour run. Six experimental manometers used.

MANOMETER 4 K = 1.1					MANOMETER 5 K = 1.1					MANOMETER 6 K = 1.0					SUM (iii)s	DIVIDE BY 6 (average)	CUMULATIVE TOTAL $\mu l\ O_2$ taken up
reading	(i)	(ii)	(iii)	(iv)	reading	(i)	(ii)	(iii)	(iv)	reading	(i)	(ii)	(iii)	(iv)			
15.0	0	0	0	0	15.0	0	0	0	0	15.0	0	0	0	0	0.0	0.00	0.00
14.7	-3	-1	-1.1	-1.1	14.7	-3	-1	-1.1	-1.1	15.0	0	+2	+2	+2	-3.6	-0.60	-0.60
14.4	-3	-1	-1.1	-2.2	14.4	-3	-1	-1.1	-2.2	14.6	-4	-2	-2	0	-5.2	-0.87	-1.47
14.4	0	-2	-2.2	-4.4	14.6	+2	0	0	-2.2	14.9	+2	+1	+1	+1	-11.1	-1.85	-3.32
14.3	-1	-1	-1.1	-5.5	14.7	+1	+1	+1.1	-1.1	14.9	0	0	0	+1	-1.0	-0.17	-3.49
14.4	+1	-1	-1.1	-6.6	14.7	0	-2	-2.2	-3.3	14.9	0	-2	-2	-1	-11.9	-1.99	-5.48

Figure 20A

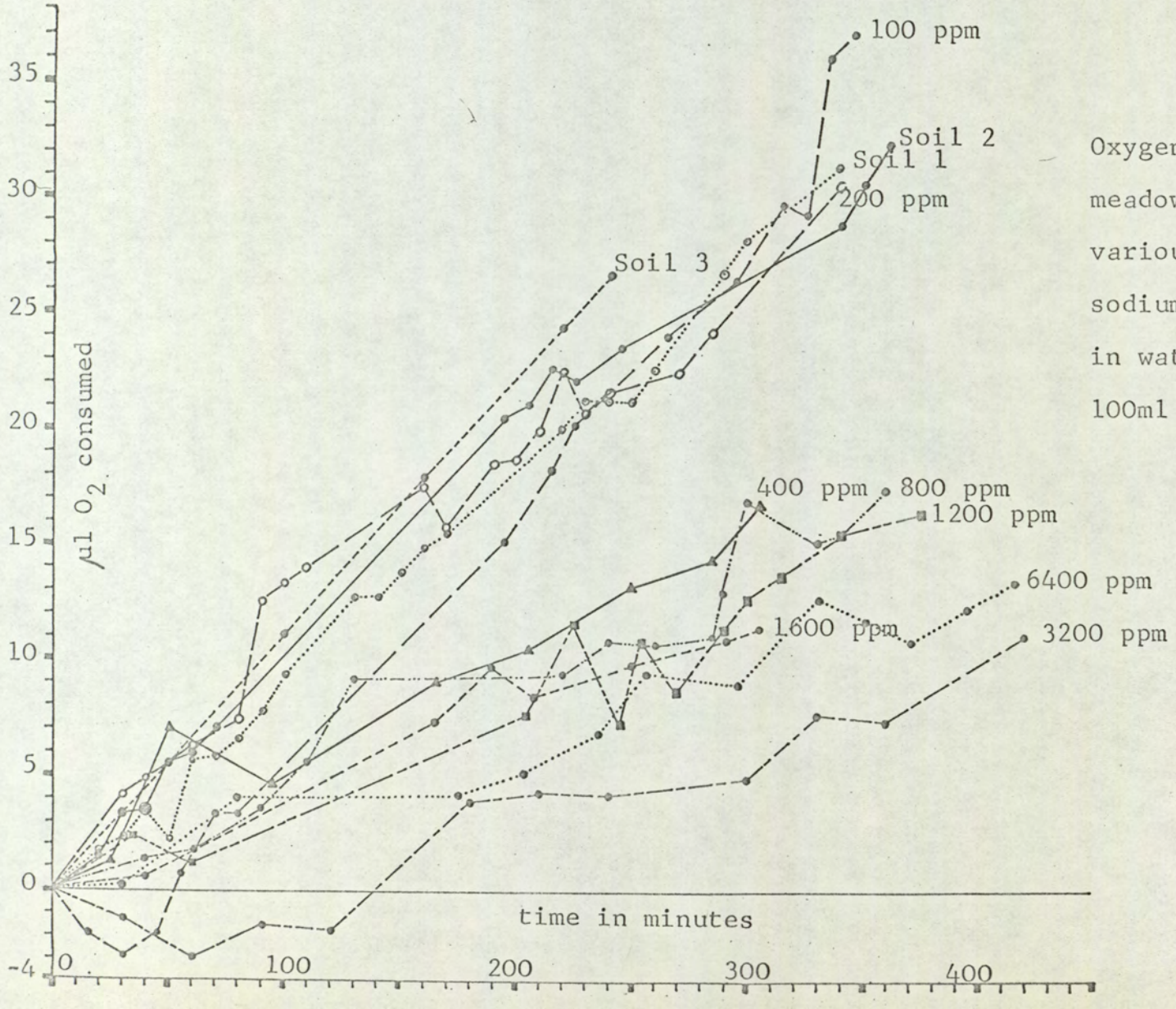
In order to have the maximum activity from the soil organisms, for ease of measurement it was necessary to make up a thick soil suspension for use in the experimental flasks. The maximum concentration of soil which could be pipetted with ease was 30g of soil in 100ml of water or biocide solution. Soil solutions were made up freshly each time immediately before each experiment.

4mls of soil solution were used in each flask, with 0.5ml of 20% KOH solution in each centre well. A standard roll of filter paper 5cm x 2cm was also placed in each centre well to increase the surface area of the KOH solution.

The thermobarometer contained either 4mls of water or 4mls of biocide solution to correspond with the experimental flasks and also 0.5mls of KOH and the paper strips in the centre well.

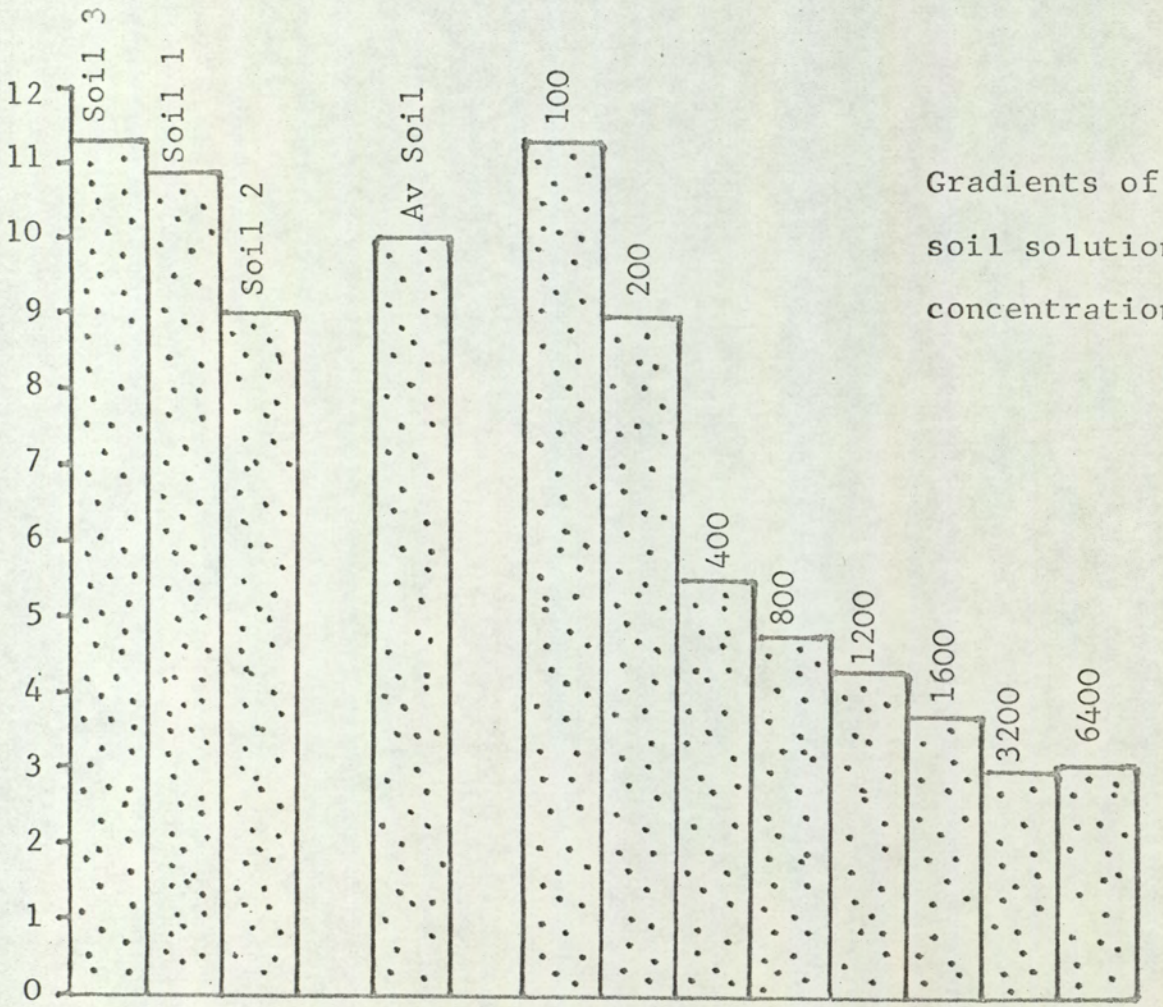
Before each experiment, the assembled manometers were allowed to equilibriate for one hour, with all taps open to the atmosphere. Each experiment was then run for a period of about six hours, readings being taken at intervals. Frequency of readings depended on the activity of the soil solution in different experiments.

The results obtained in this study are shown in graph 20.



Graph 20

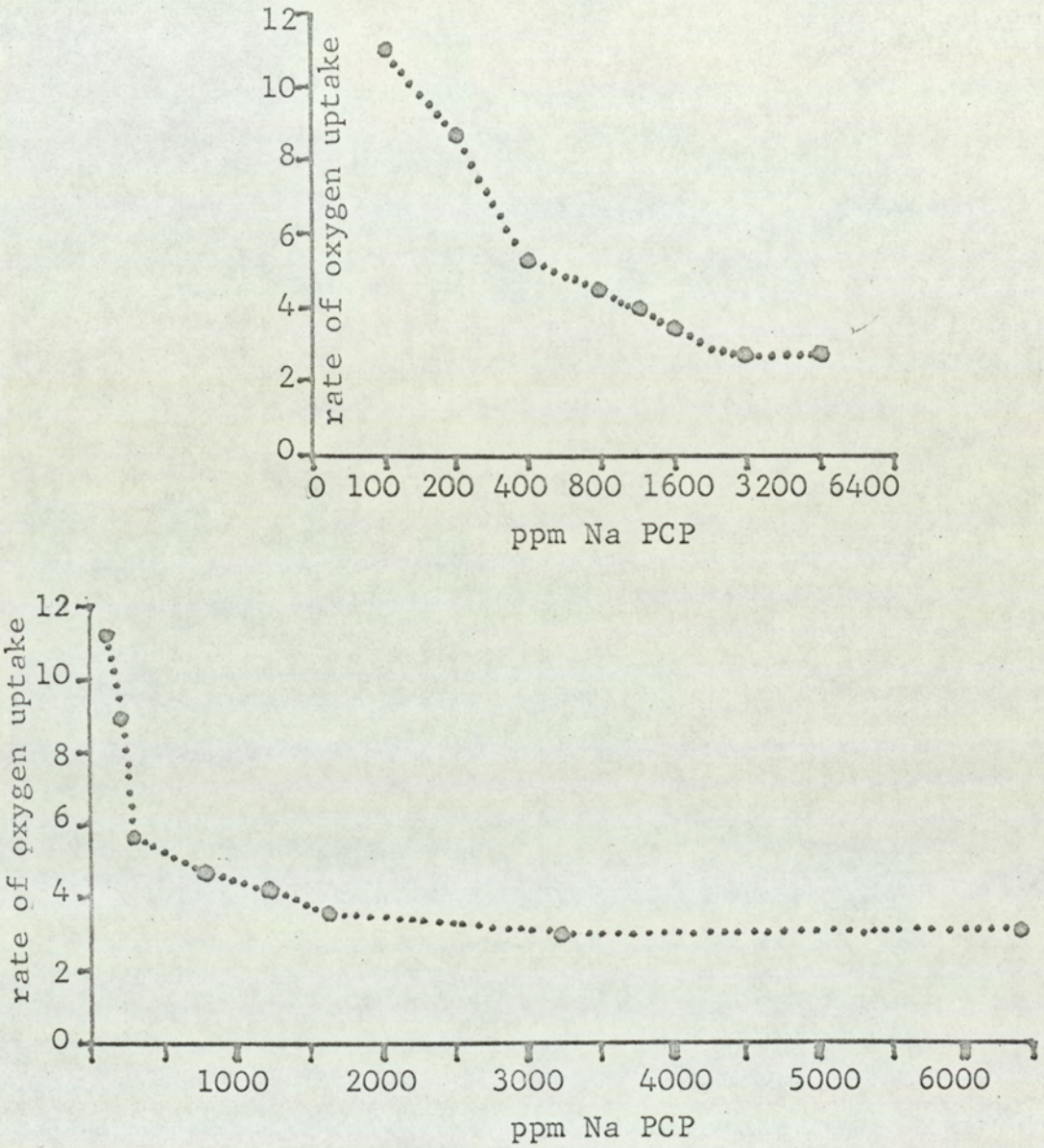
Oxygen uptake of 4ml of meadow soil solution in various concentrations of sodium pentachlorophenate in water 30g of soil in 100ml of water/fungicide 30°C.



Graph 20A

Gradients of oxygen uptake curves of soil solutions containing various concentrations of sodium pentachlorophenate.

(See graph 20)



Graph 20B

Rates of oxygen uptake of soil solutions containing various concentrations of sodium pentachlorophenate. (See graphs 20 and 20A).

From the experiments with Sodium pentachlorophenol several conclusions may be drawn:-

1. The soil solutions showed uptake of oxygen with time.

2. The rates of oxygen uptake varied with different amounts of the biocide.

3. The rates of oxygen uptake fell with increasing concentrations of the biocide.

4. Very low levels of the biocide, 100 and 200 ppm had no clear effect on oxygen uptake.

5. At concentrations of 400 ppm and higher, respiration dropped to around half its normal rate.

6. Respiration tended to be more erratic at higher levels of the biocide.

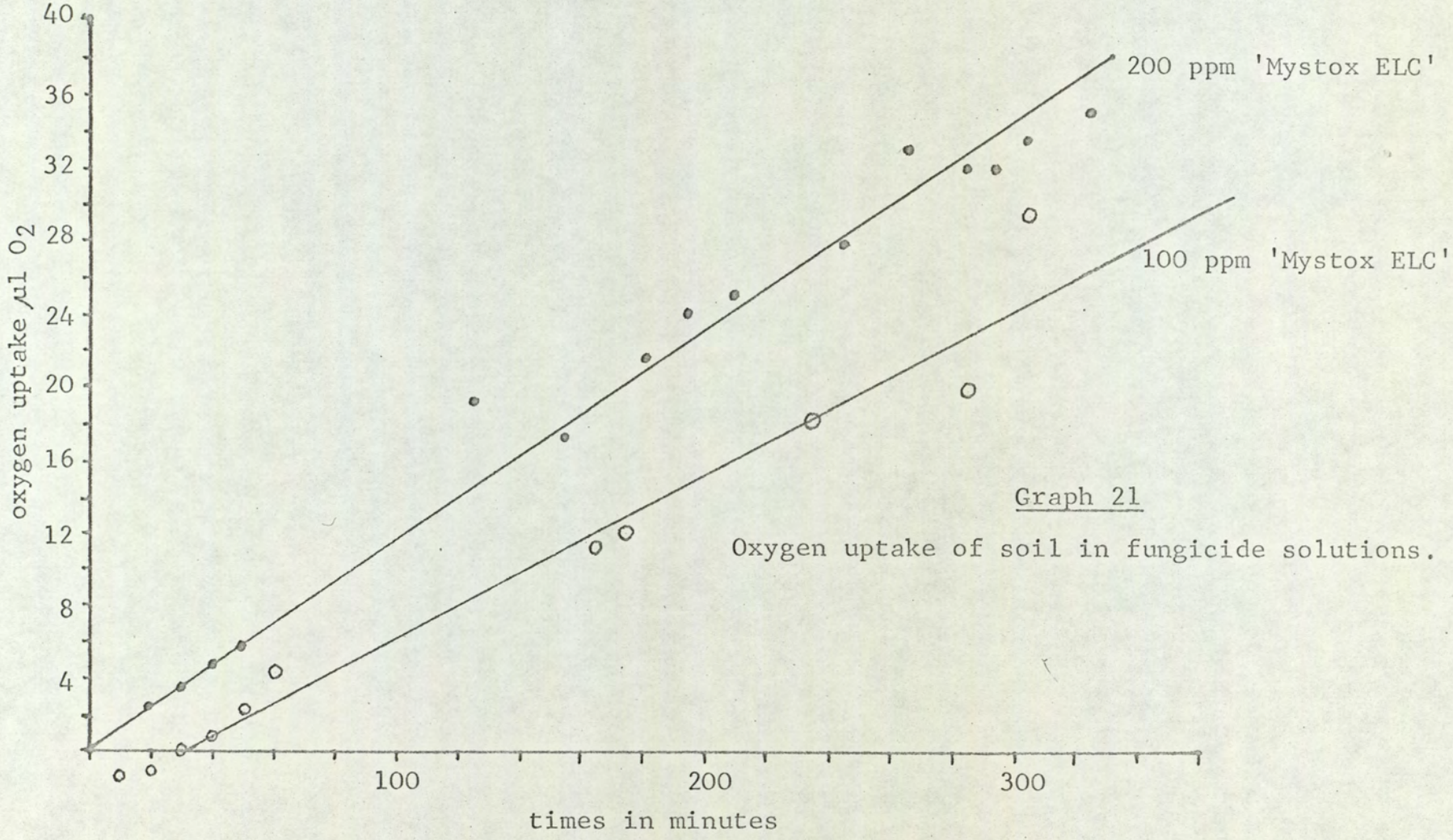
It was to be expected that soil solutions would take up oxygen with time. It was known from previous work that this soil had a rich varied flora and that the fungi and bacteria were active. As sodium pentachlorophenol is known to be toxic it was also to be expected that it would have an inhibitory effect on the microorganisms in the soil, shown by a decrease in the rate of oxygen uptake and

therefore in respiration with an increased biocide level. Chlorinated phenols generally upset the normal regular metabolic activities of fungal cells. PCP is an uncoupler of oxidative phosphorilation (Weinback, 1965; Lyr and Ziegler, 1959) and is thus toxic to all life. As PCP has to enter cells to exert this toxic action, penetration must occur, and as correlations have been found between toxicity and lipid solubility of toxic chemicals, it may be assumed that the cytoplasmic membrane is involved (Miller, 1969).

It appears from this study that very low concentrations, 100 and 200 ppm of sodium pentachlorophenol are simply insufficient to suppress respiration, although this does not mean that such concentrations would have no effect on soil if present for long periods of time. It is possible that such concentrations could affect other aspects of the metabolism or life cycles of microorganisms in the long term.

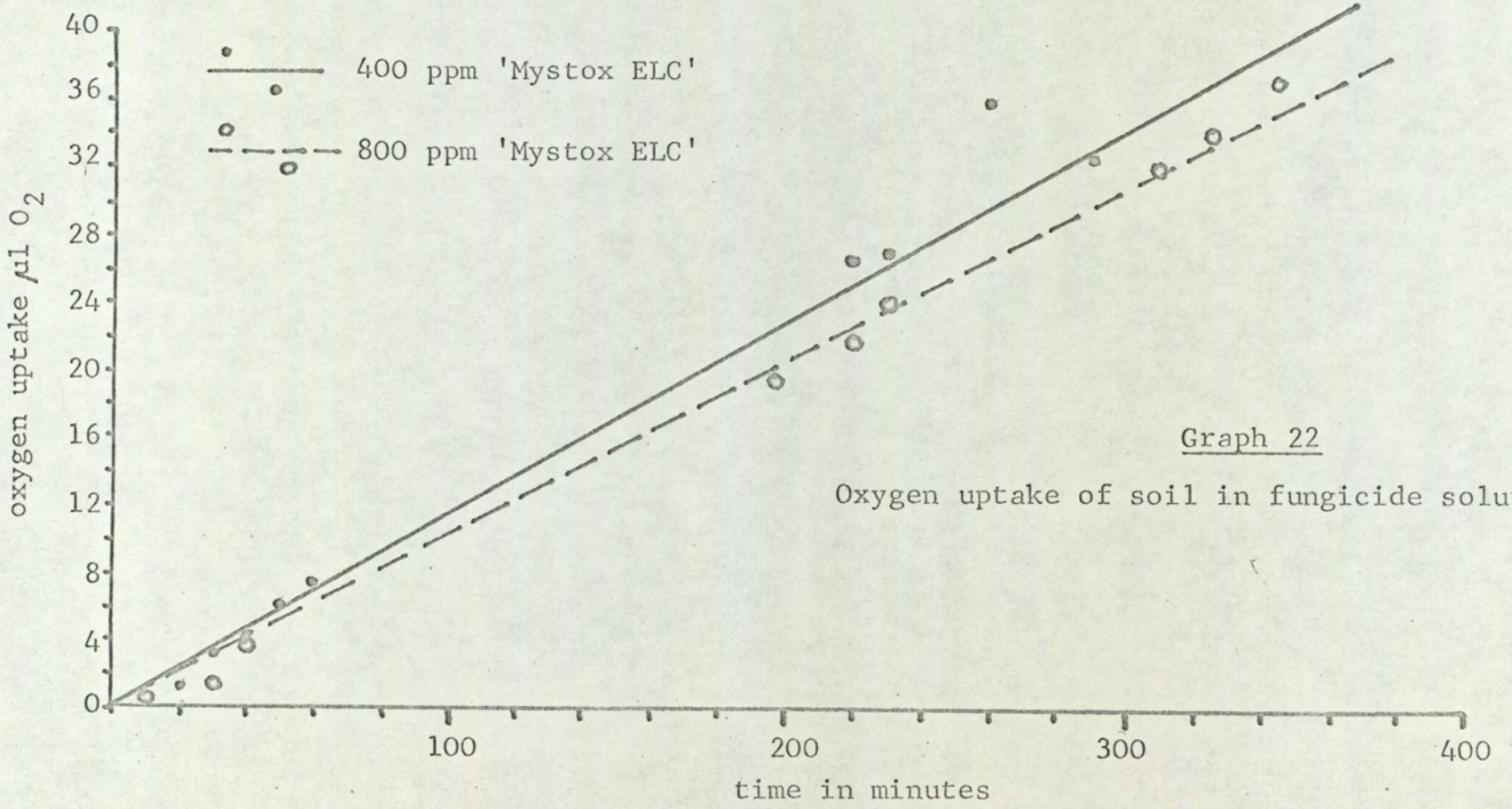
From the results it may appear that there is a clear suppression of respiration from the beginning, i.e. at 200 ppm, but as the actual figures are within the range found for untreated soil itself, they must be thought of as being more fortuitous for the experimenter than in fact significant.

At concentrations of 400 ppm and over, however, a marked effect is noted. (This can be clearly seen by the various plottings of the approximate gradients). Respir-



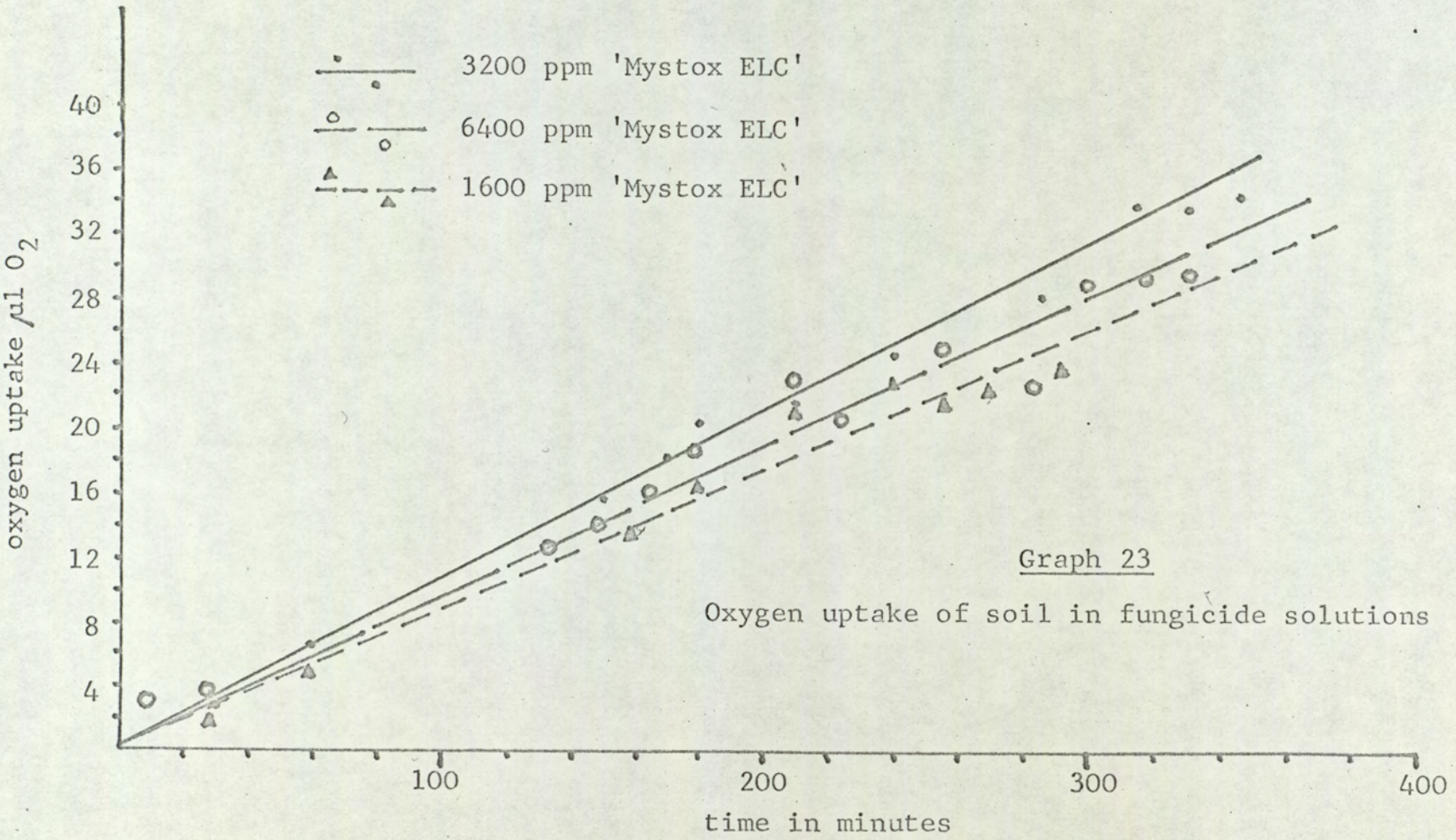
Graph 21

Oxygen uptake of soil in fungicide solutions.



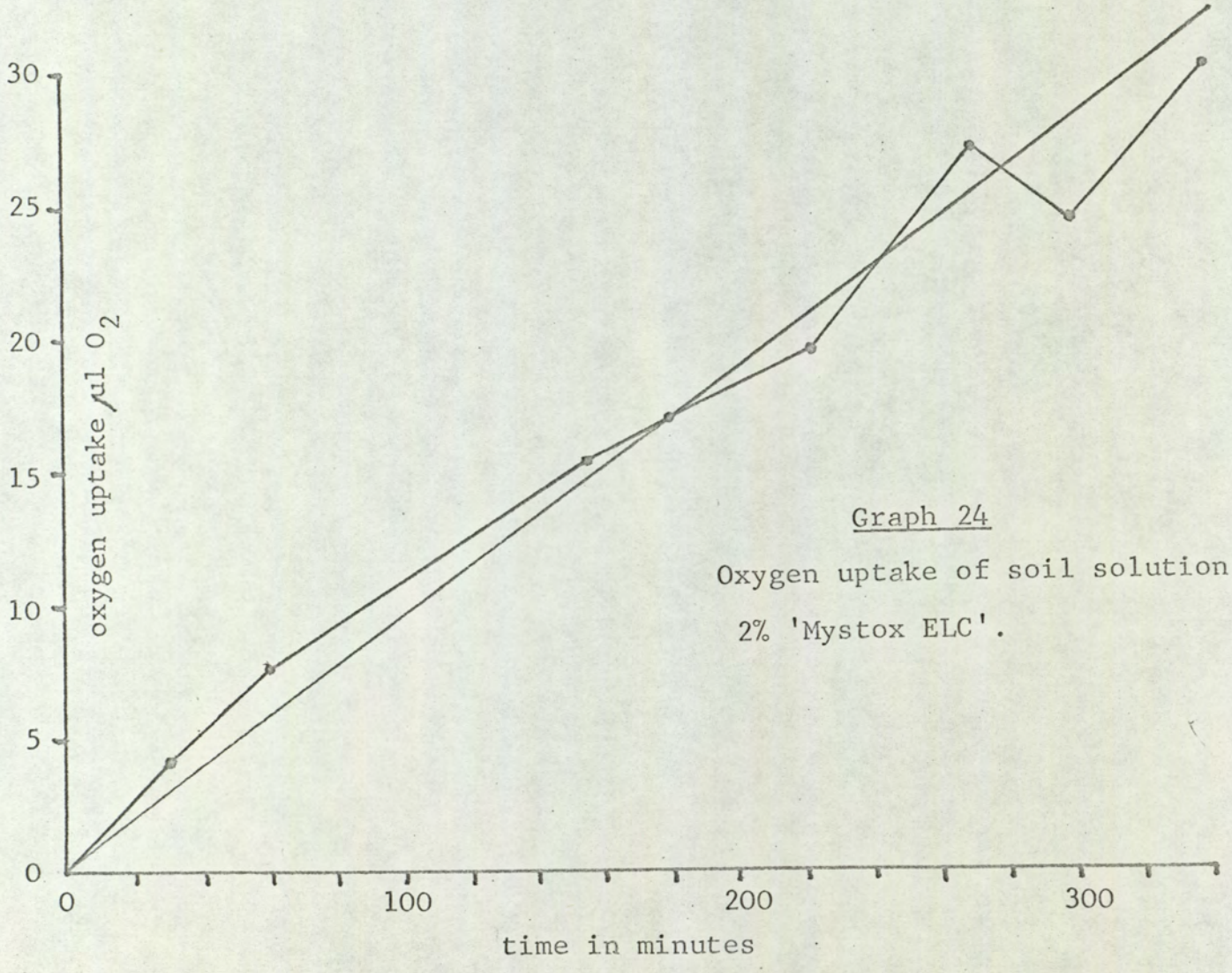
Graph 22

Oxygen uptake of soil in fungicide solutions



Graph 23

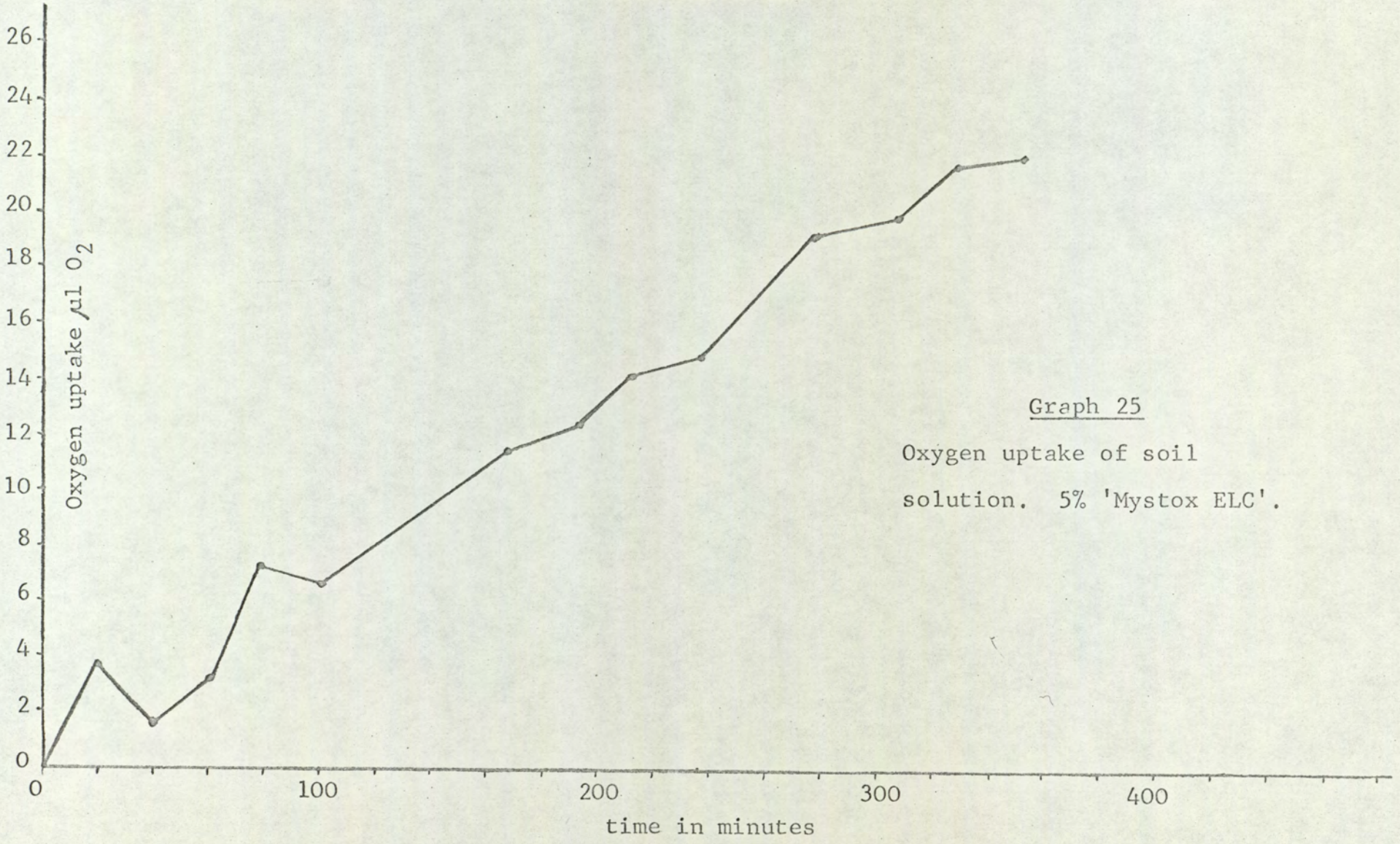
Oxygen uptake of soil in fungicide solutions



Graph 24

Oxygen uptake of soil solution

2% 'Mystox ELC'.



Graph 25

Oxygen uptake of soil solution. 5% 'Mystox ELC'.

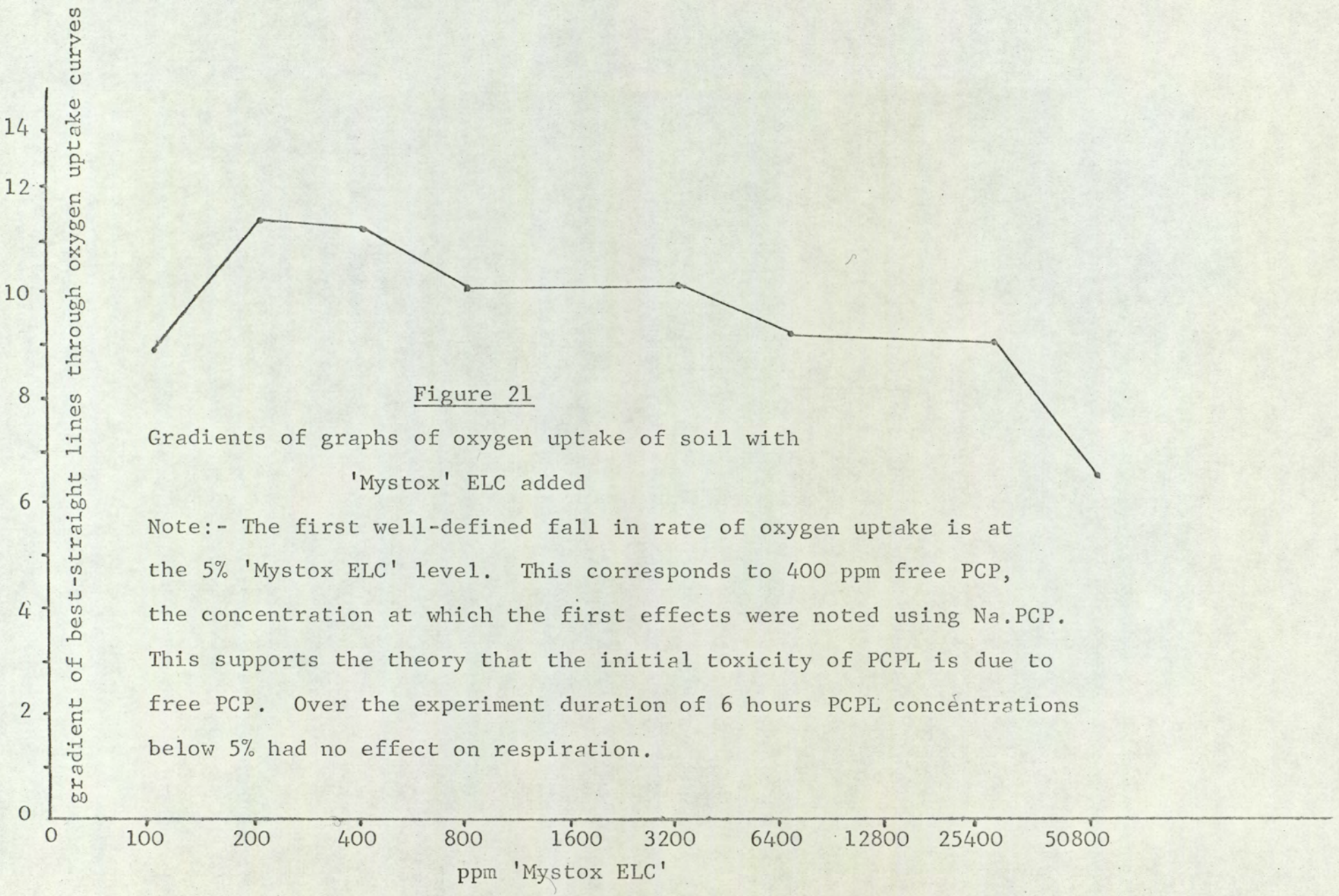


Figure 21

Gradients of graphs of oxygen uptake of soil with 'Mystox' ELC added

Note:- The first well-defined fall in rate of oxygen uptake is at the 5% 'Mystox ELC' level. This corresponds to 400 ppm free PCP, the concentration at which the first effects were noted using Na.PCP. This supports the theory that the initial toxicity of PCPL is due to free PCP. Over the experiment duration of 6 hours PCPL concentrations below 5% had no effect on respiration.

ation rate fell to around half its average normal level and it can be said that sodium pentachlorophenol takes noticable affect at around this concentration.

As the concentration increased respiration tended to become more erratic. This serves to show the unbalanced nature of a poisoned system and is to be expected. It is interesting to compare this effect with the similar one found relating to growth of a fungal colony. At inhibitory concentrations of the biocide, growth and respiration are not only slowed down but become more erratic. This appears to occur not only with mixed cultures (as in the respiration experiments) but with pure cultures also (as in the case of colony growth).

Experiments with 'Mystox ELC' (A pentachlorophenyl laurate emulsion, containing 0.8% free pentachlorophenol) (See graph 21-25 and figure 21).

1. The soil solutions showed uptake of oxygen with time.

2. The rates of oxygen uptake varied with different amounts of the biocide .

3. Between the two lowest levels of this biocide 100, 200 ppm there was no inhibition of respiration observed.

4. At higher levels of this biocide, 400, 800, 3,200, 6,400 and 25,00 there was a slight decrease in

respiration observed but the actual levels were within those of untreated soil.

5. At the highest level of biocide used, 50,000 ppm (5%) there was a marked drop in respiration.

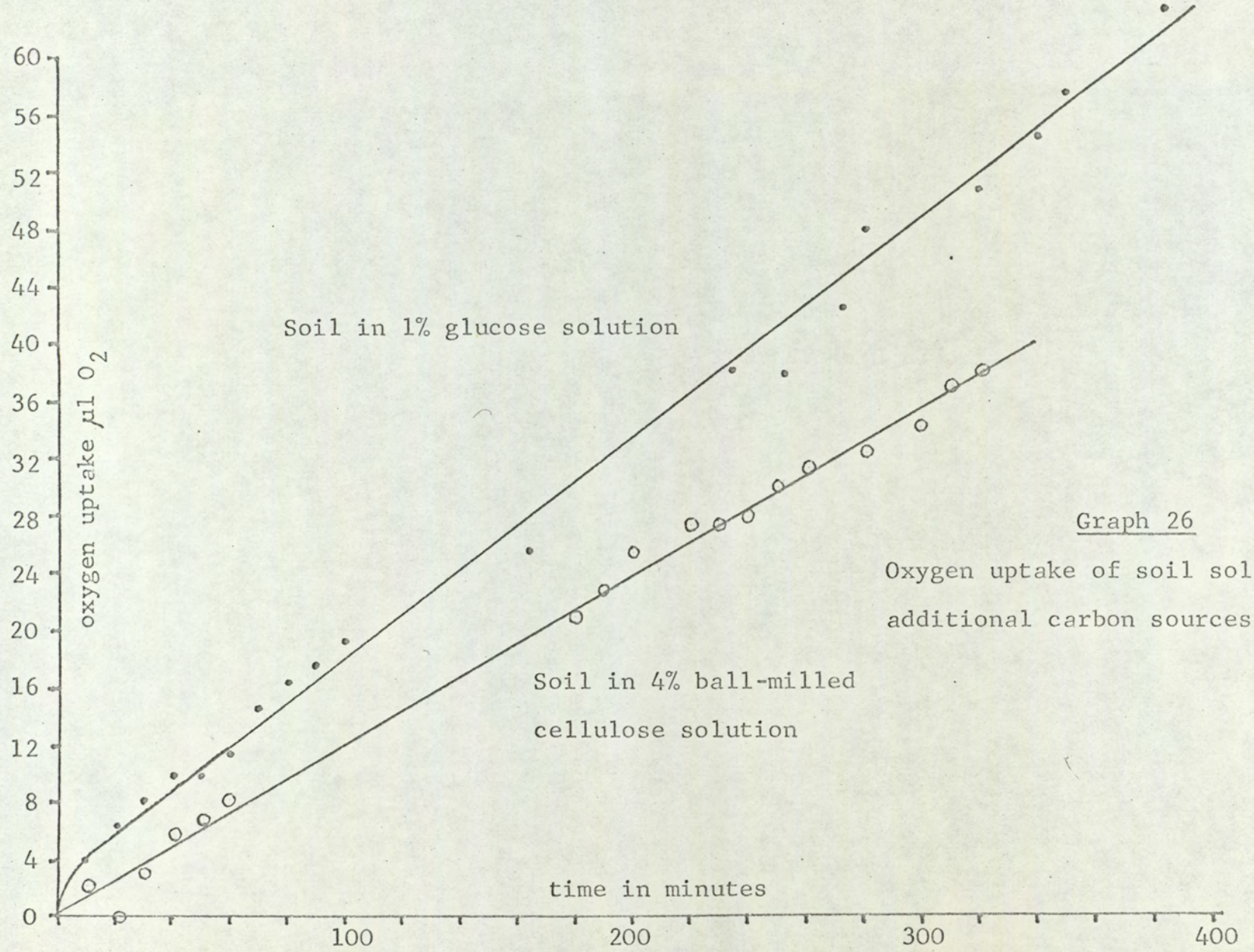
The significance of this experiment is to be seen when comparisons are made with the previous similar work using sodium pentachlorophenol, and also the work on the hydrolysis of pentachlorophenyl laurate. It would appear from previous work that the toxic action of pentachlorophenyl laurate biocides is now confirmed as being due, in the early stages of colonisation, to the residual free pentachlorophenol in the biocide. The fungicide used here contained 0.8% free pentachlorophenol. Therefore using 6,400 ppm of 'Mystox ELC', the free pentachlorophenol present was in the order of 50 ppm. From the previous work, it cannot be expected that this will have any effect on respiration. From the previous work, the first effects of pentachlorophenol can be expected at around the 400 ppm level,i.e. 51,200 ppm 'Mystox ELC'. This was in fact the case; using 5% 'Mystox ELC' (50,000 ppm) the first clear fall in respiration was noted. This confirms the assumption that free pentachlorophenol provides the initial toxicity of 'Mystox ELC' and the two experiments correspond quantitatively.

It is of interest to note finally, the work of Cserjesi (1972), who performed experiments using liquid

cultures of species of two genera of common soil fungi, Aspergillus and Penicillium. The concentration of sodium pentachlorophenate at which growth visibly decreased was between 320 and 640 ppm, which fits well with the figure of 400 ppm for sodium pentachlorophenate obtained by respirometry studies.

Effects of Glucose and Cellulose on respiration of soil.

In order to put in perspective any variations in respiration of untreated soils (which may be due in part to variations in the concentration of easily assimilated nutrients in the soil) a small control experiment was carried out using glucose and ball-milled cellulose at concentrations similar to those used in agar culture media. The great increase in respiration observed (graph 26) due to the addition of glucose (more easily assimilated than cellulose) served to show that in the soils used there was no great variation in the readily available nutrients present. The effect of cellulose was to increase respiration to a level slightly above the average rate obtained for unaltered soils, a rate only just greater than one of the basic soil rates obtained. This experiment confirms the idea that respirometry is not a technique which should be used to assess the degradability of complex nutrients or biocides when using freshly made cultures. It also suggests that any complex compounds or variations in them will have little effect on results of studies similar to the ones carried out here. This is useful in this instance,



Graph 26

Oxygen uptake of soil solutions with additional carbon sources. 30°C.

as it is not necessary to attempt to adjust inhibition caused by higher PCPL concentrations with respect to the possible increase in respiration caused by increase in the fatty acid concentration. It would appear that this factor would have little effect in such short-term experiments (under 6 hours) as those carried out here.

Chapter 7

DEVELOPMENT OF A TESTING TECHNIQUE ALLIED TO PERFUSION SYSTEMS

DEVELOPMENT OF A TESTING TECHNIQUE ALLIED TO PERFUSION SYSTEMS

Perfusion systems provide a means whereby solid substrates can be used to provide colonised samples for further study. Textiles colonised by fungi in perfusion systems can be observed in situ and the degree of colonisation noted, as shown in Chapter 4. To obtain further information such as a record of species colonising the textile from soil, the substrates need to be removed from the perfusion system and plated out onto nutrient media. Accounts of such experiments are given in Chapter 3. Quantitative measurements may also be made; such as the strength testing also listed in Chapter 3.

The rapid decay of substrates in perfusion systems is not only due to the provision of near optimal conditions for fungal growth but where strength losses are to be measured, due also to the small sizes of the test substrates (Lloyd, 1968). This small size of test strip can have disadvantages. Many tensiometers are designed to handle much larger and stronger strips and unless one of the more sophisticated tensiometers is available there may be difficulty in making strength-loss measurements.

As the perfusion systems were designed to provide a simple, cheap, but yet very effective means of studying aspects of material decay, it was decided to devise a strength testing system along the same lines to be used to test the small substrate strips after colonisation. The results of

this system in use are given in Chapter 3. The details of the design, construction, testing and use of the simple laboratory tensiometer are given below. (See figure 21).

The machine consists of an 'Avery' dial spring balance with a scale calibrated in 100g divisions from zero to 25Kg. The spring balance is attached to a heavy steel frame by means of a bolt fitted through its upper suspension bracket. The frame is bolted to a workbench. The frame baseplate is fitted with two specimen clamps, which hold the test specimen horizontally across a central cut-out gap. A specially shaped steel breaking bar is then attached to the machine, the upper end being hooked onto the balance, the actual breaking projection resting on the upper surface of the test specimen, and the lower end being attached to a weight container. If required, an initial height adjustment may be made at this point by means of the upper suspension bolt, to ensure that the breaking projection is just touching the test specimen but causing no downwards displacement.

A load is then applied to the specimen by running water into the weight container at a constant rate, until the specimen breaks. The load applied is then noted as the direct reading indicated on the balance scale.

Some features of this machine require further explanation:-

1. Specimen clamps and breaking bar

(See figures 22 and 23)

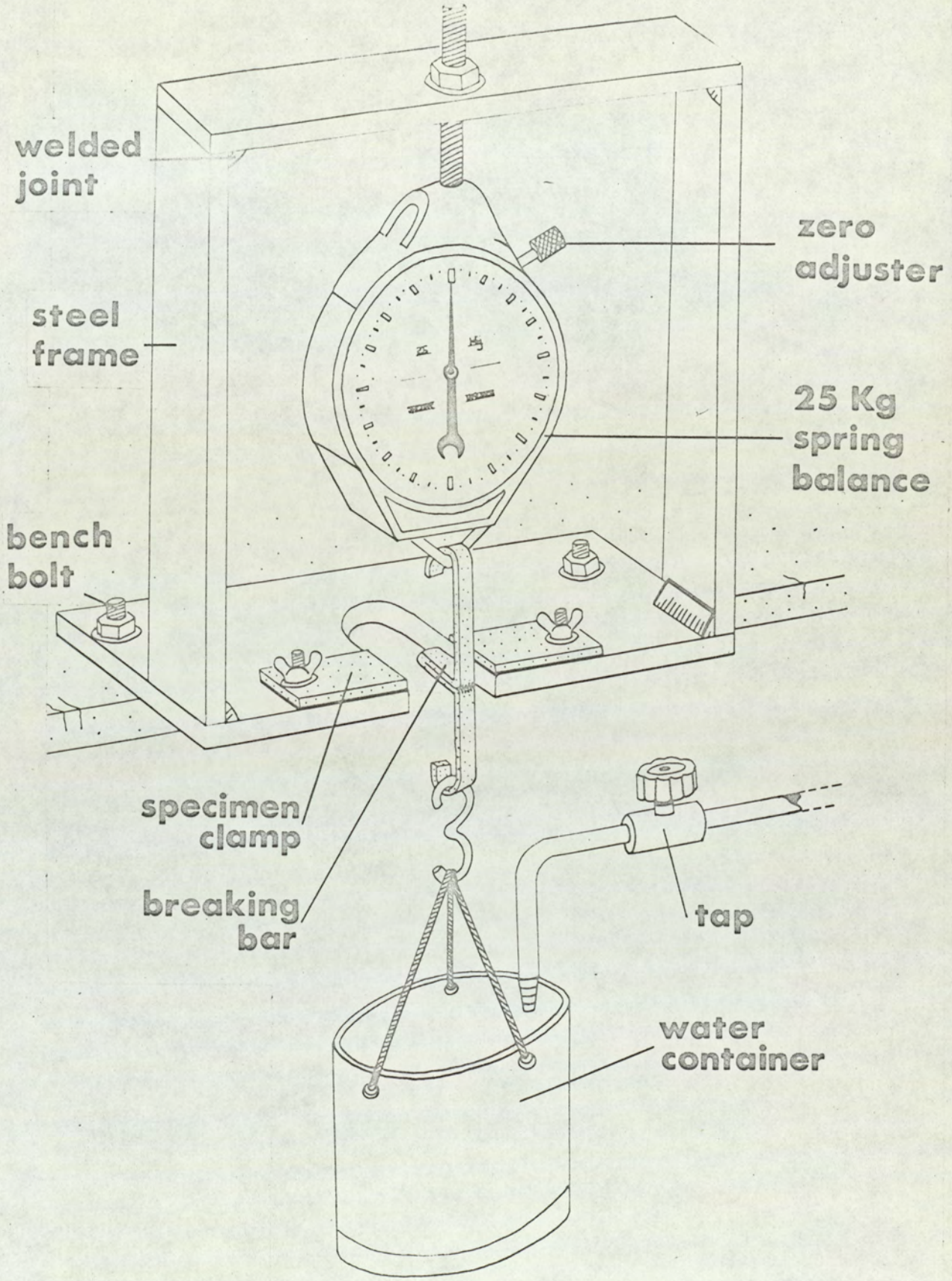


Figure 21

A simple strength testing machine for textile strips

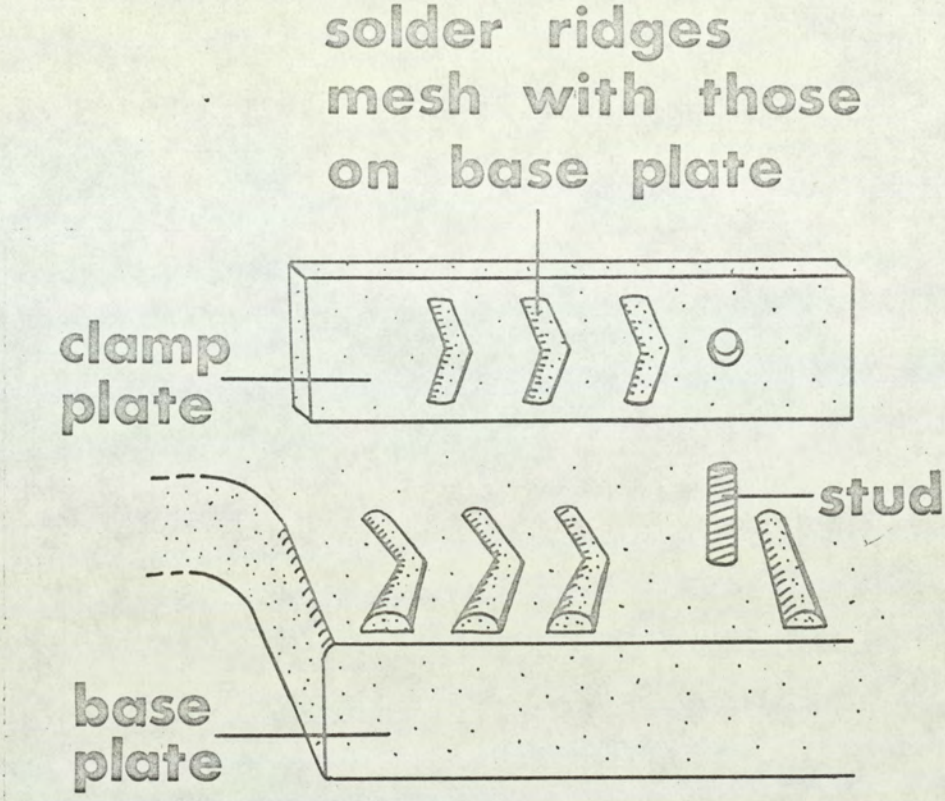


Figure 22. Diagram of gripping surfaces of clamp and base plates

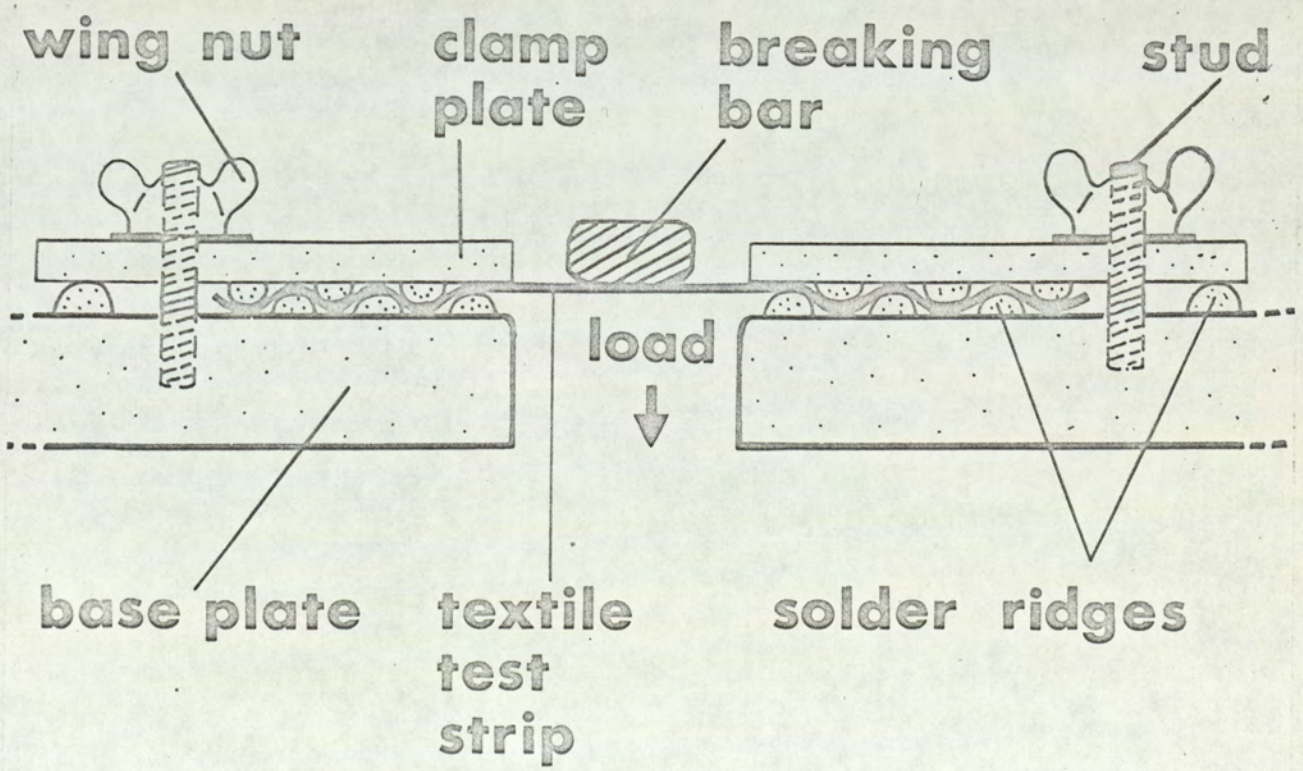


Figure 23. Section through clamps and specimen before load is applied

Due to the comparatively high loading of specimens on this machine, considerable difficulty was experienced due to slipping specimens. This was observed directly and also by sudden increases in apparent loading, indicated by increased pointer movement on the balance under a constant loading. Merely clamping wet textile specimens between the brass and steel surfaces of the clamp and base plates proved unsatisfactory, and various other methods were devised for increasing the friction between the specimen and the clamping surfaces. Abrasive cloth bonded to the clamping surfaces using epoxy resin proved unsatisfactory, as the abrasive grit on the cloth quickly wore away under wet conditions. The problem was finally solved by soldering intermeshing strips of solder onto the inner surfaces of the clamp plates. The strips of solder themselves were periodically roughened by coarse filing and can easily be renewed when necessary. This system proved most satisfactory and was not affected by the presence of moisture from the test samples.

After manufacture, it was noted that the breaking bar was rectangular in section, having sharp edges. If used in this state, a cutting effect may have been observed, and to avoid this, the breaking projection was filed to a rounder section, such that at no time during contact with the specimen as a load was applied was any sharp edge in contact with the test specimen. This rounding of edges was also carried out on the clamp edges. In practice this worked as the test specimen invariably broke at a point about midway between the clamp edge and breaking bar, on either side.

2. Loading

The problem of applying a large load (up to 25Kg) to the test strip at a constant even rate was solved by using a water system. Loading by means of a chain, paid out from a drum, was considered but proved impractical. The initial loading on the specimens was that of the steel water container (about 2Kg), the rest of the load being added at a constant rate by running water into the container. In practice, this process can be speeded up, by pre-loading the container with metal weights up to about one third of the estimated breaking strength of the samples, adding the rest of the load by running in water. This practice, however, depends on operator experience with previous experimental samples, as initial overloading may lead to premature sample breakage.

Textile strips were always broken in a wet condition as this was the easiest way to standardize conditions. This was carried out by soaking the test strips in a weak solution of detergent for several minutes before breaking.

Testing of the machine

This machine was not designed to replace more elaborate and accurate machines, but as a simple and cheap method of obtaining quantitative comparative strength measurements. The actual strength value obtained for each specimen cannot be compared to one obtained on a tensile strength testing machine, for a straight pull on the sample is not made; a somewhat different set of forces are applied. It was, however, desirable to make some comparison between this machine, and

one known to be "accurate" in the normally accepted sense. The test cloth used is usually accepted as having a maximum variation in strength of around 10% on either side of its mean breaking strength, and it was desirable to see if results obtained exceeded this greatly, as figures in excess of this must be due to the machine and its operator. To check the machine 60 ten-thread strips were broken upon it; and 60 similar strips were broken on a Hounsfield Tensometer (Serial W3710) at Catomance Limited, Welwyn Garden City.

As can be seen from the following histograms (figures 24 and 25) both sets of results contained over 94% of the results within 2 standard deviations either side of the arithmetical mean, and 10% variation on either side was contained within the 2 standard deviations.

This is taken as an indication that the strength testing machine is usefully and consistently accurate in practice when used for comparative testing.

As detailed in Chapter 3 this machine was used to study the loss in strength of cotton textile samples treated with various fungicides and nutrient materials, after attack by soil fungi. Used in this manner, for this and other studies, such machines can provide biological data of a quantative nature.

The use of strength testing apparatus is widely made in research and industry, as a means of determining the

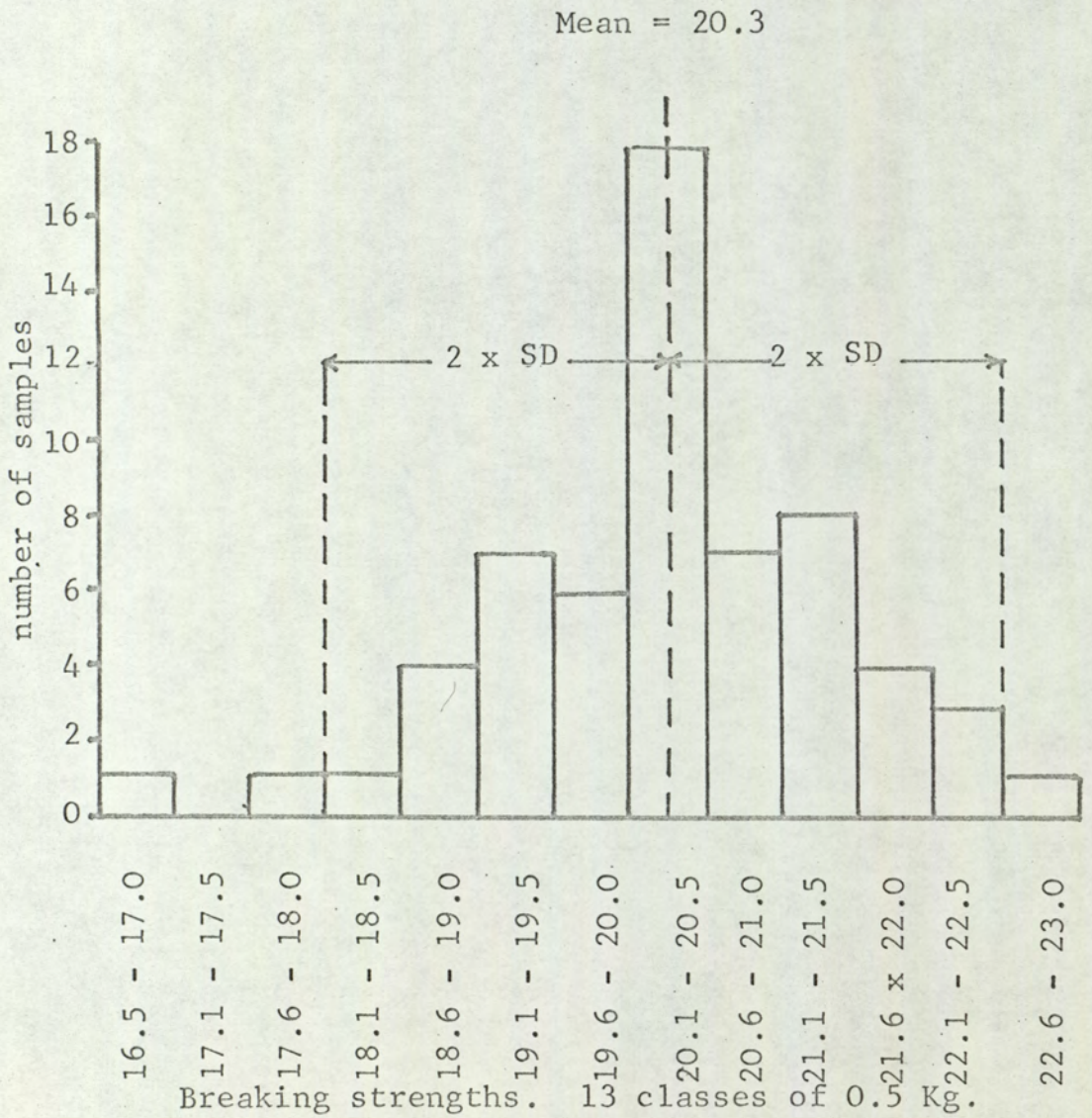


Figure 24

Breaking strengths of 60 samples of TNO test textile as measured by the simple laboratory tensiometer.

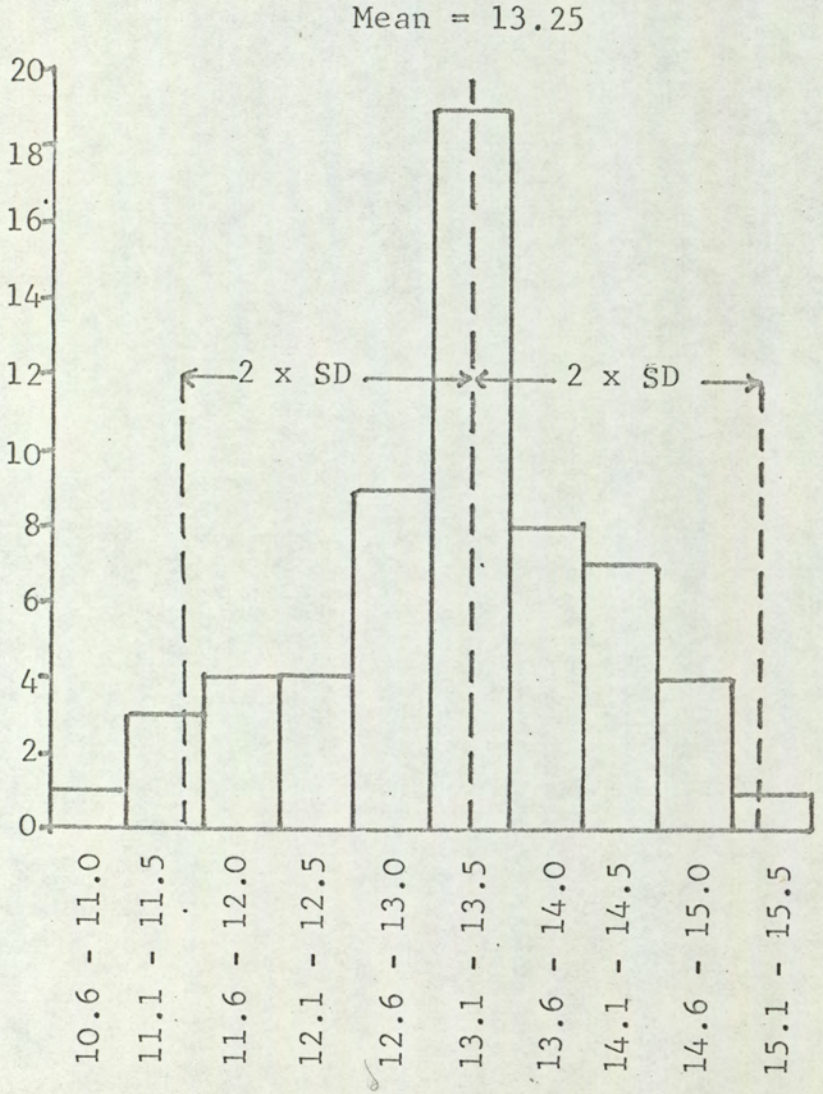


Figure 25

Tensile strengths of 60 samples of TNO test textile as measured by a 'Hounsfield Tensometer' (Serial W3710).

extent of fungal decay. Such usage is generally limited to the testing of materials after a given time of exposure to attack, and not to study the course of decay as it progresses. Even if the species responsible for decay (or perhaps merely all those present) are isolated from the final sample, progressive isolations coupled with progressive strength testing of replicate samples are rarely carried out.

Detailed study of the patterns of strength losses coupled with details of the colonisation of a material by differing fungi are obviously not practicable or necessary for the routine screening of well known material protectants but research studies could surely benefit by more work on these lines. It may well be that future biocides are not those which are more toxic or more persistent, but are those which are geared to general colonisation patterns, details of which are still little understood. It is to this end, that of the monitoring of decay, that such simple devices as this strength testing machine are made. Their usefulness is due in no small part to their general availability. It is then comparatively simple to produce results as in figure 26, which is an example of a colonisation and strength loss experiment sampled at intervals.

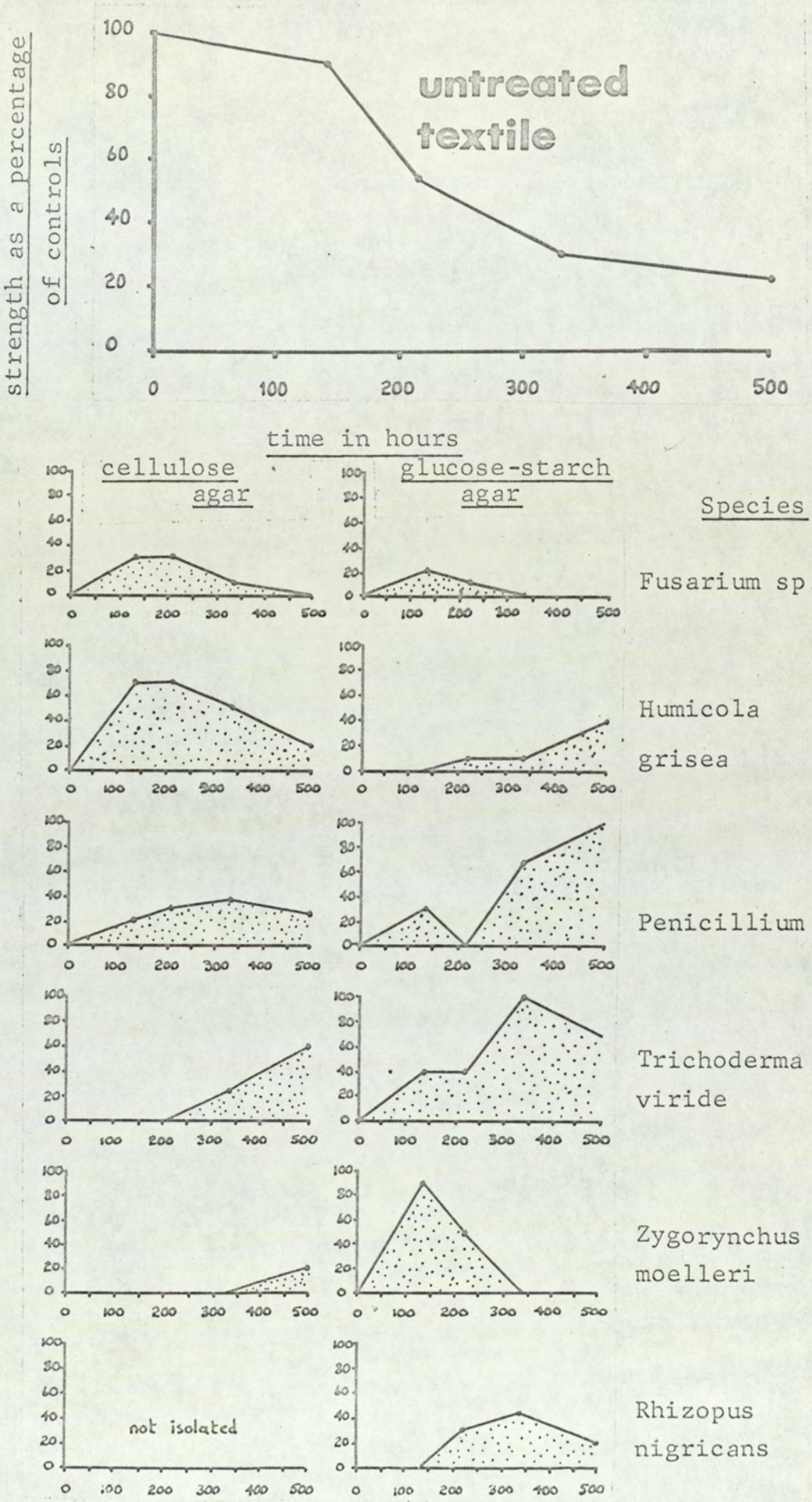


Figure 26. Example of textile decay/colonisation

Chapter 8

AN INVESTIGATION OF THE COLONISATION AND DECAY BY SOIL FUNGI OF COTTON TEXTILE USING DIFFERENT SOILS AND VARYING THE pH

8.1. Introduction

8.2. Development and use of a technique for the study of the effects of pH.

Chapter 8

AN INVESTIGATION OF THE COLONISATION AND DECAY BY SOIL FUNGI OF COTTON TEXTILE USING DIFFERENT SOILS AND VARYING THE pH

8.1. Introduction

As discussed in Chapter 2, it is well recognised that different soils may possess different degrees of aggressiveness in regard to the decay of cotton textiles. This is generally thought to be due to variations in soils in regard to their abilities to support a varied and active flora of deteriogenic fungi. The main variations in soils are usually considered to be those of nutrition and aeration (Lloyd, 1968; Stolzy and Van Gundy, 1968; Turner, 1971). In order to maximise and regulate these factors, test soils are often amended from their natural state. This amendment may take the form of addition of compost or manure to increase the available nutrients and often to improve drainage, make water holding more even and improve aeration. In the case of heavy soils, sand may be added to improve drainage and aeration. Thus, it is often the policy of a test laboratory to prepare an agressive but probably unique soil stock to suit its own particular requirements, as constant as possible regarding aeration and nutrients.

The pH of this soil may often be noted, but attempts to investigate the effects of changes in one soil, or variations between different soils are not usually made; changing and maintaining the pH of a soil in bulk is difficult to perform accurately and quickly. The variation of pH within any one

soil, or between different soils is a recognised important variable in testing work and in field conditions (Wessels and Adema, 1968). For this reason, it was decided to make further investigation into this factor.

It is of importance that such work was carried out in the context of this present study, for by demonstrating the effect of such a major environmental factor as pH, the other studies within this thesis can be more easily compared with results obtained elsewhere; the results being more meaningful when set in a more well defined environmental context.

8.2. Development and use of a technique for the study of the effects of pH.

In the following experiment, a technique was devised to isolate fungi from soils at differing pH, not by altering the soil itself, but by maintaining the test substrates in the soil at various pH levels. Thus there was no large initial shock to the mycoflora of the soil as a whole due to general pH changes, but species which were able to colonise the cotton textile at a changed pH were encouraged and selectively isolated.

The experiment was designed to show the variations in fungal species and the decay resulting from their colonisation of substrates from different soils, the normal pH of each soil being known. Also shown was the effect of changes in pH of substrates in each soil within a range of 1.5 pH units below and above the normal soil pH. Soils were kept in each case near their normal maximum water-holding capacity, but at a constant temperature of 26°C, and so far as is possible, at a standard packing density under test conditions.

Preparation of isolation tubes

The isolation tubes designed for this experiment combine several principles:-

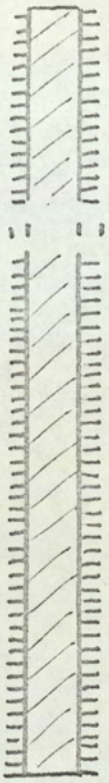
(i) Isolation - they are designed to isolate soil fungi onto a solid material.

(ii) Screened substrates - as previously described (Chapter 2) the test materials were screened from direct soil contact to isolate only active fungi.

(iii) Immersion - they are buried in soil in bulk, which has the advantage of being closer to natural conditions and also conventional soil burial tests than agar plate isolations.

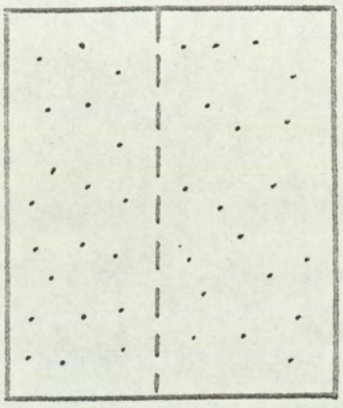
(iv) Perfusion - as described in previous chapters, constant nutrient and pH conditions were maintained in the test substrates by this technique.

The tubes were constructed as shown below

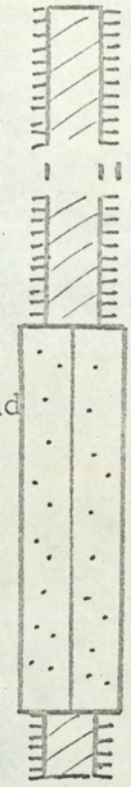


10 warp thread strip of test textile with ravel along each edge

aluminium foil

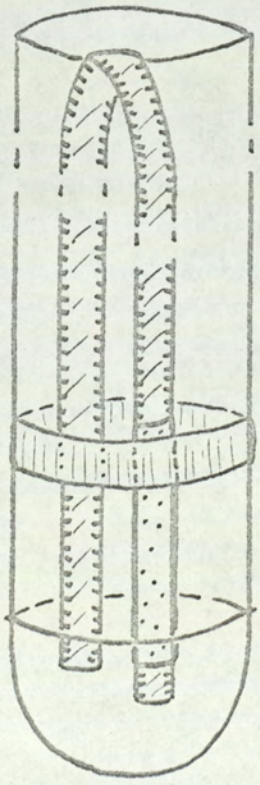


foil is folded round strip and free edges folded under.



(1) (2)

(3) Six such units are attached to a boiling tube with a strip of adhesive tape.

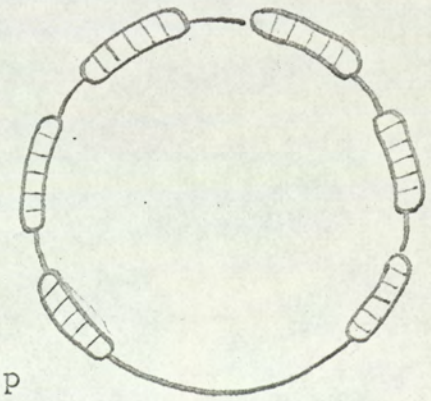


one strip shown for clarity. Free end folded inside tube to form wick.

tape (also holds screen)

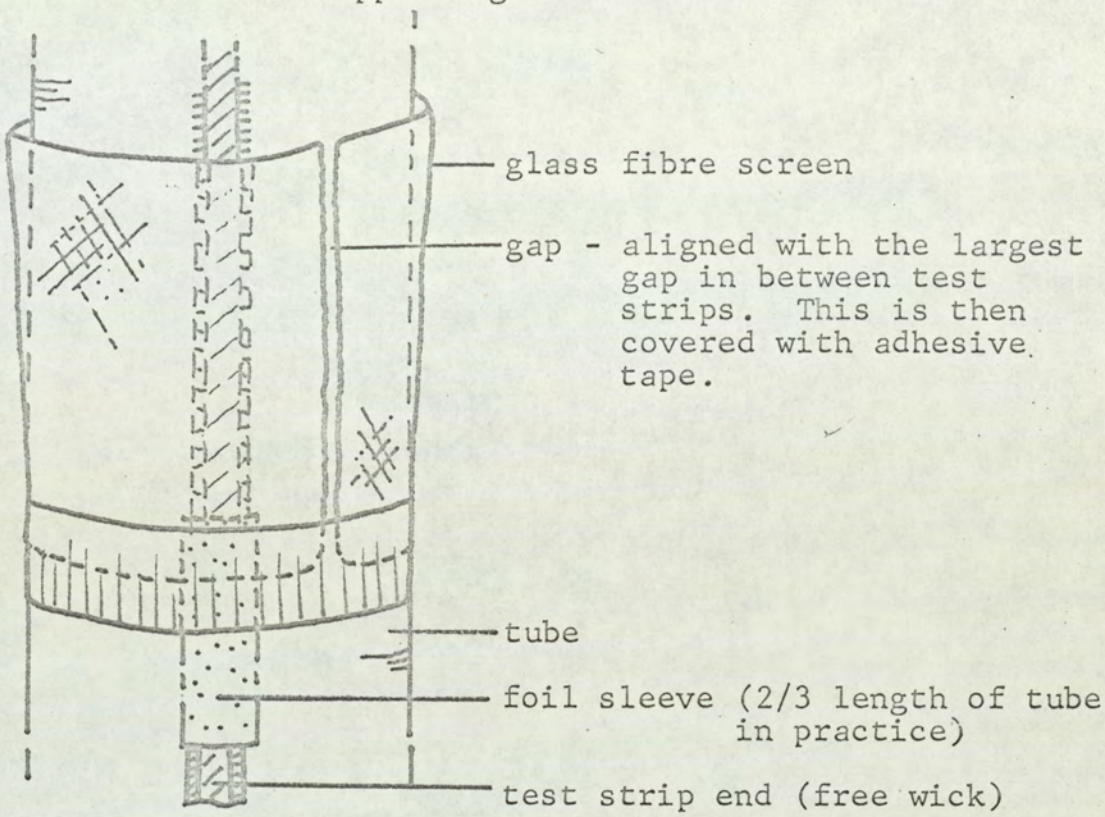
temporary rubber band to hold strip for fixing

six strips are arranged thus:-

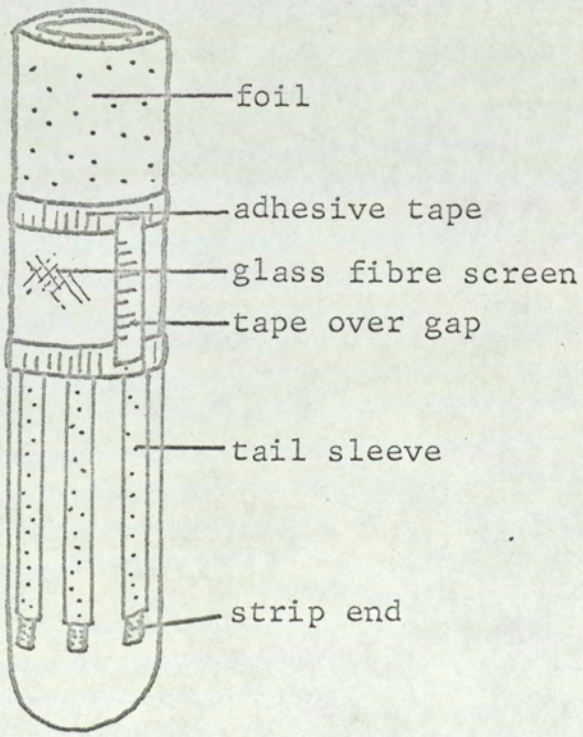


1 longer gap left.

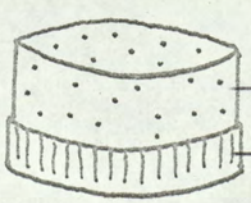
(4) As the first adhesive tape strip is fixed over the foil of the six test strips the glass fibre screen is also fixed under its upper edge.



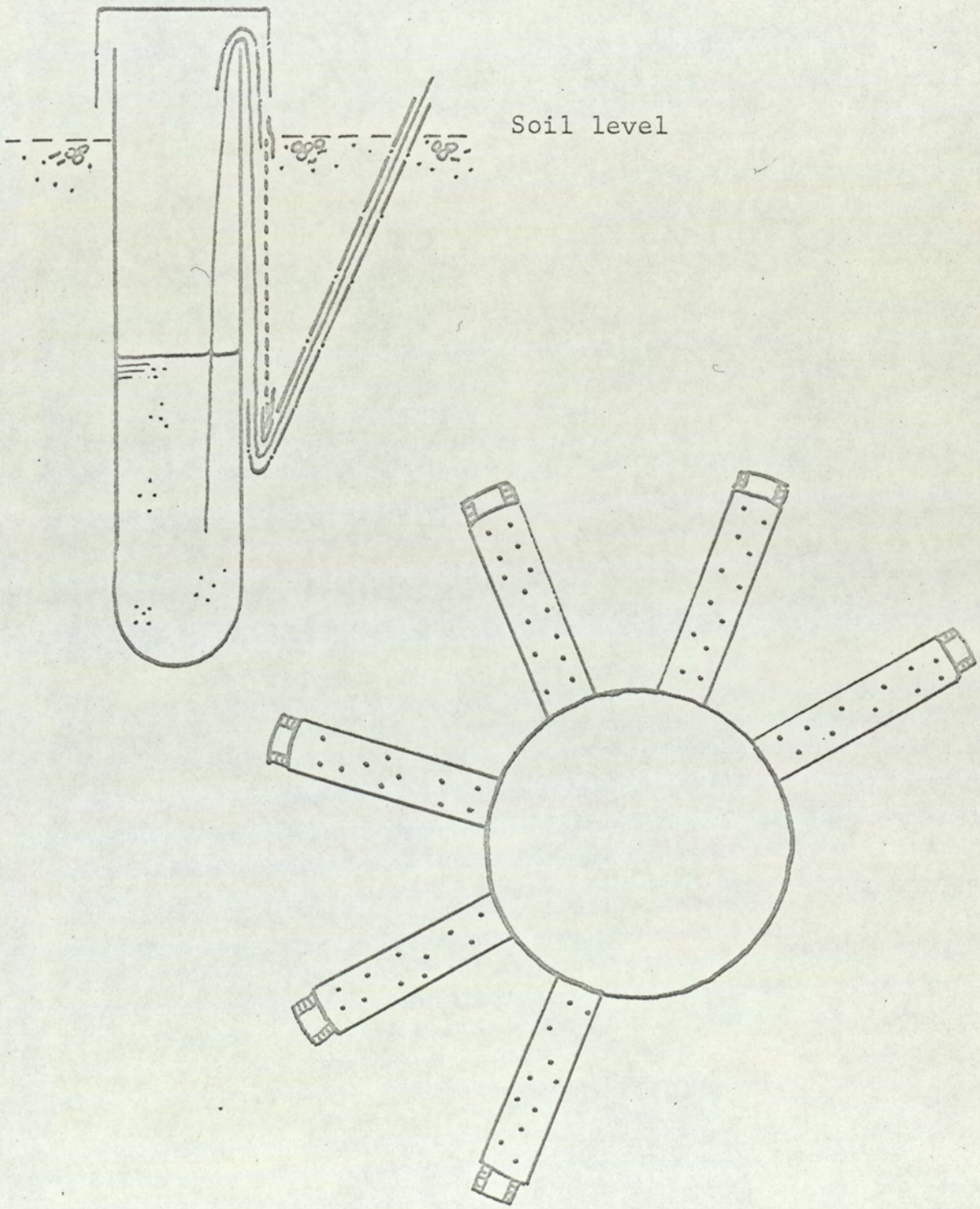
(5) Aluminium foil is then wrapped over the upper portion of the tube, overlapping the screen at its lower edge, and folded over the tube top. The join with the screen is covered with adhesive tape.



(6) A foil cap is then made by folding and edged with adhesive tape - this fits over the boiling tube mouth



(7) After filling the tube with liquid nutrients the wicks were folded up flat against the sides of the tube. The tubes are autoclaved, being held in metal test tube racks. Before burial in soil the six tail wicks are folded part of the way down again as shown below:-



Two features of this assembly should be noted.

(i) The specimen acts as a wick, no additional attached glass fibre wicks are needed, this speeds and simplifies construction greatly and helps even flow.

(ii) The use of aluminium foil as a sleeving material speeds construction, is cheap, and also when folded is stiff, thus the final bends of the wicks are maintained sufficiently to ease burial of tubes.

For burial in each soil, tubes containing untreated textile and also 1.5% pentachlorophenyl laurate treated textile were prepared.

The tubes were filled with Eggins and Pugh nutrient salts solution, adjusted to each pH required. The tubes were then autoclaved at 15 p.s.i. for 20 minutes. The wicks were folded up over the screen during this process. This facilitates filling the tubes in racks, and also shields the screen from any splashing which may occur. At the same time 20 control strips of both treated and untreated textile were autocalved in Eggins and Pugh solutions in petri dishes, at the average, and highest and lowest pH used in the experiment. These dishes were then sealed and kept at the incubation temperature for the duration of the experiment.

After cooling, the tubes were placed in 600ml beakers, the wicks folded out and soil was packed round the tubes. The soil was packed as evenly as possible, and lightly, with just sufficient pressure in each case to ensure all round

contact with the tubes. The tubes were then topped up with sterile nutrient solution of the appropriate pH, to compensate for autoclaving losses and losses due to the initial wicking-up of the solution. The tubes were then incubated for 10 days at 26°C. As a check for any change occurring, the pH of each nutrient salts solution was also noted after autoclaving. This factor was also checked before the experiment, the changes found being very slight.

Soils used in the experiment.

Five different soils were obtained, being taken from depths not exceeding six inches. All vegetation was removed and the soils passed through a 9mm mesh sieve. The soils were then loosely packed in previously used clean clay pots, and brought up to their maximum water content by watering with distilled water over a period of several days. A final watering was given and the pots allowed to drain freely for 24 hours before samples were taken for estimation of water content and pH.

Water content was estimated by drying to constant weight at 105°C and weighing. The pH was determined by adding 50 mls of distilled water to 10g of soil, shaking well at intervals over 1 hour, centrifuging and determining the pH of the resulting supernatant using a pH meter.

The soils used were as follows:-

(1) From Catomance Limited

Topsoil from waste ground close to the lauric acid plant. A clay soil.

(2) Garden soil

Topsoil from a Birmingham garden, left fallow for several years. A sandy soil.

(3) From Oldbury, Worcestershire

Topsoil from waste ground by a canal in an industrial area. A dark gritty soil.

(4) From Clent, Worcestershire

Topsoil from a meadow, as used previously. A sandy soil.

(5) Woodland soil

Topsoil from a mixed woodland/parkland in Birmingham. Composed mainly of decayed leaf litter. Trees in immediate vicinity, Holly and Mountain Ash. Other nearby trees were Oak, Sycamore and Beech.

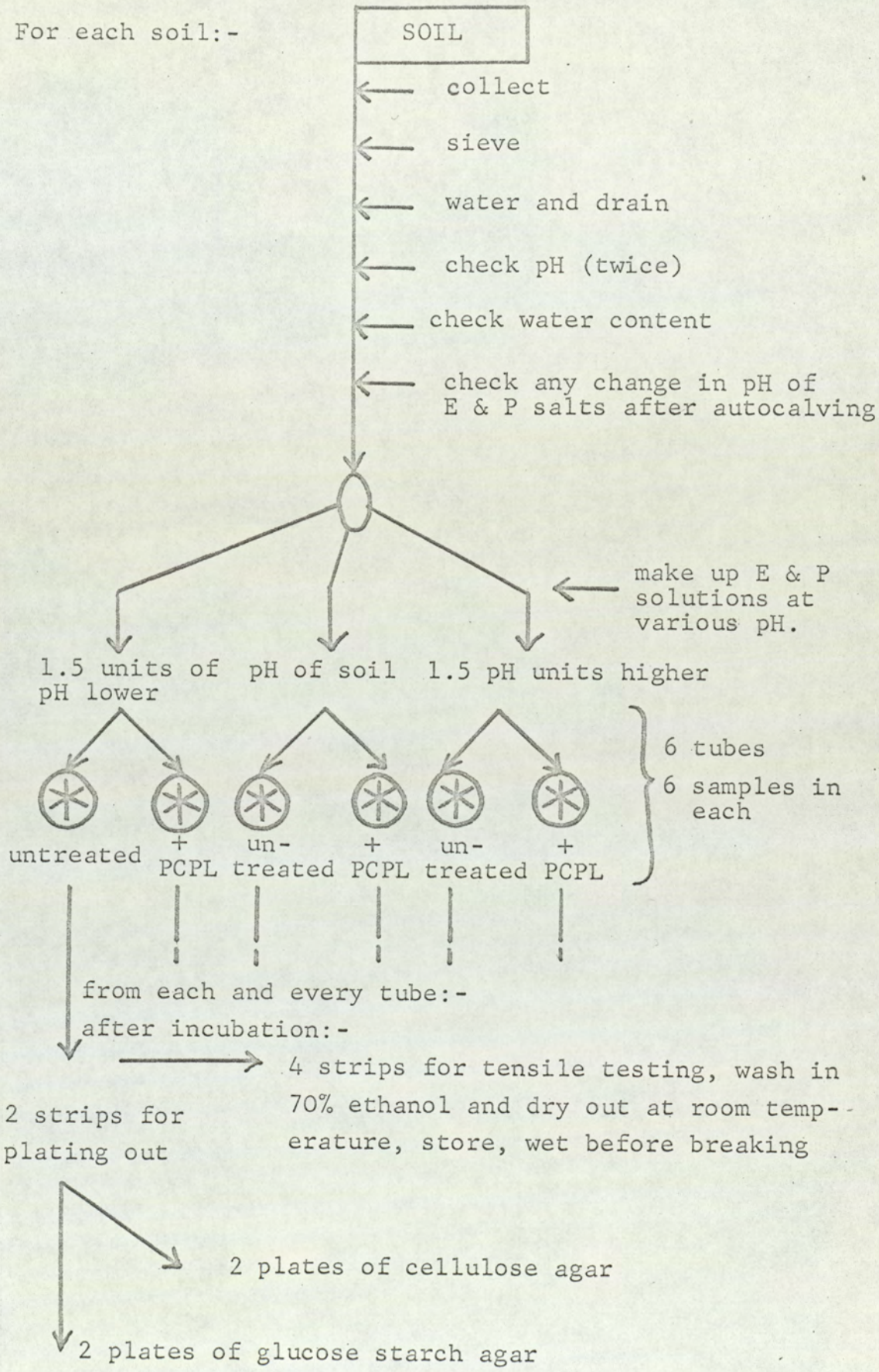
Agar for isolation plating

Two media were used.

(i) Eggins and Pugh Cellulose Agar

(ii) Eggins and Pugh Glucose/Starch Agar.

Owing to the composition of these media no extra buffering salts were added. They were adjusted to each required pH by addition of N/10 HCl or N/10 KOH. As noted in previous experiments, the pH of these agars tends to fall after autoclaving and some allowance was made for this when adjusting the original pH. The actual final pH of each agar was noted before use. The variation in pH of agar emphasises the usefulness of the perfusion principle, where variation on sterilisation is much less.


For each soil:-
SOIL
collect
sieve
water and drain
check pH (twice)
check water content
check any change in pH of E & P salts after autocalving
make up E & P solutions at various pH.
1.5 units of pH lower
pH of soil
1.5 pH units higher
6 tubes
6 samples in each
untreated
+ PCPL
un- treated
+ PCPL
un- treated
+ PCPL
from each and every tube:-
after incubation:-
4 strips for tensile testing, wash in 70% ethanol and dry out at room temperature, store, wet before breaking
2 strips for plating out
2 plates of cellulose agar
2 plates of glucose starch agar

	Maximum Water content as % of Dry Weight	Maximum Water content as % of Wet Weight
Garden	35	26.4
Clent.	37.5	27.4
Oldbury	56.3	36.1
Catomance	42	29.6
Woodland	107	53.4

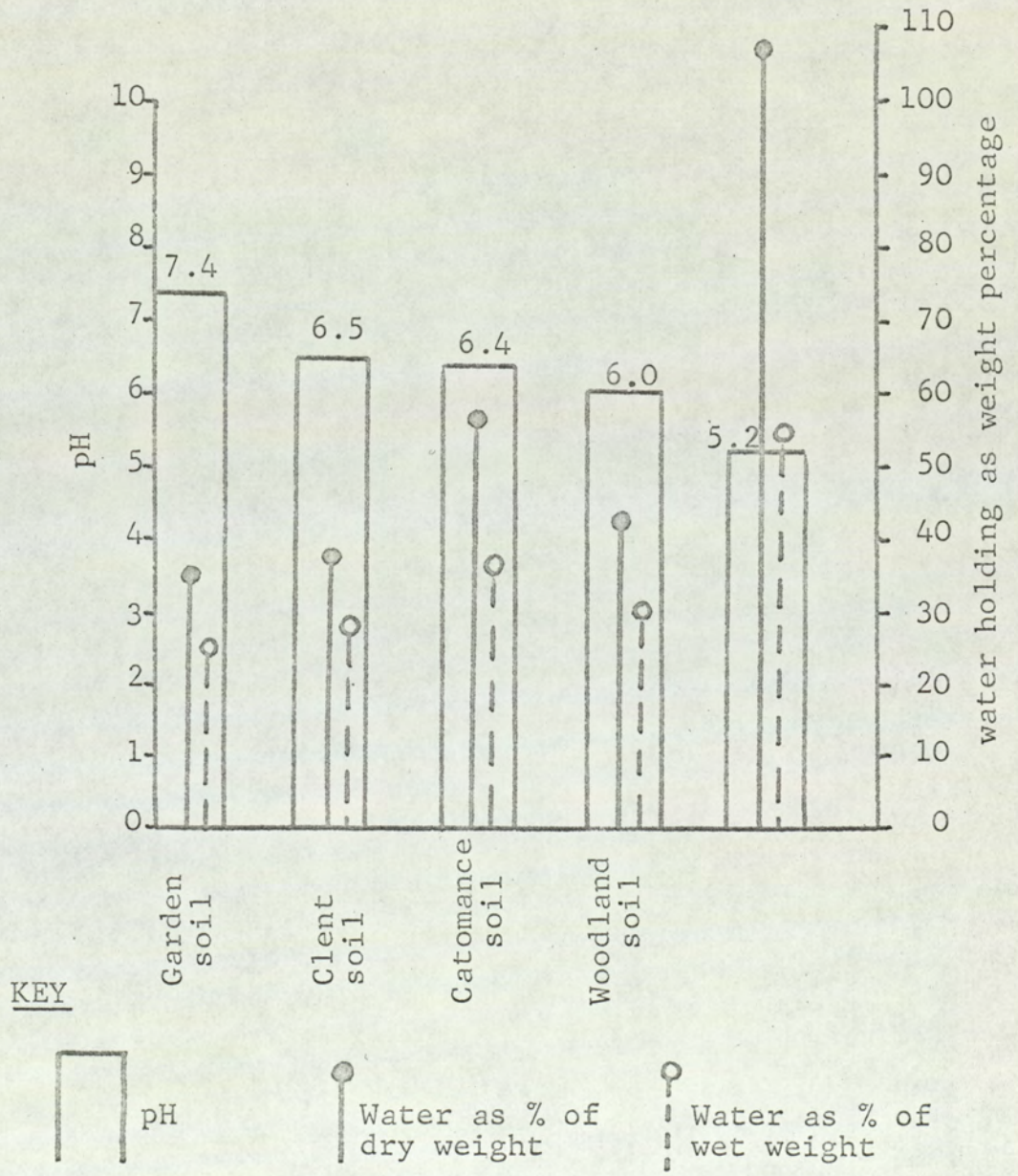


Figure 28. Normal pH and maximum water contents of various soils.

pH

		NORMAL	IDEAL	PERFUSATE	AUTOCLAVED G/S MEDIA	AUTOCLAVED CEL. MEDIA
WOODLAND	LOW	-	3.7	4.15	5.1	5.3
	NORMAL	5.2	5.2	5.44	5.5	5.6
	HIGH	-	6.7	6.75	6.55	6.85
CATOMANCE	LOW	-	4.5	4.9	5.5	5.7
	NORMAL	6.0	6.0	6.1	5.7	5.7
	HIGH	-	7.5	7.3	7.25	7.5
OLDBURY	LOW	-	4.9	5.15	5.65	5.65
	NORMAL	6.4	6.4	6.3	5.95	6.0
	HIGH	-	7.9	7.65	7.5	7.5
CLENT	LOW	-	5.0	5.3	5.6	5.8
	NORMAL	6.5	6.5	6.6	6.35	6.45
	HIGH	-	8.0	7.8	7.6	7.6
GARDEN	LOW	-	5.9	6.1	5.8	5.7
	NORMAL	7.4	7.4	7.2	7.25	7.2
	HIGH	-	8.9	9.1	7.6	8.65

Table 26

pH of soils, liquid and solid media

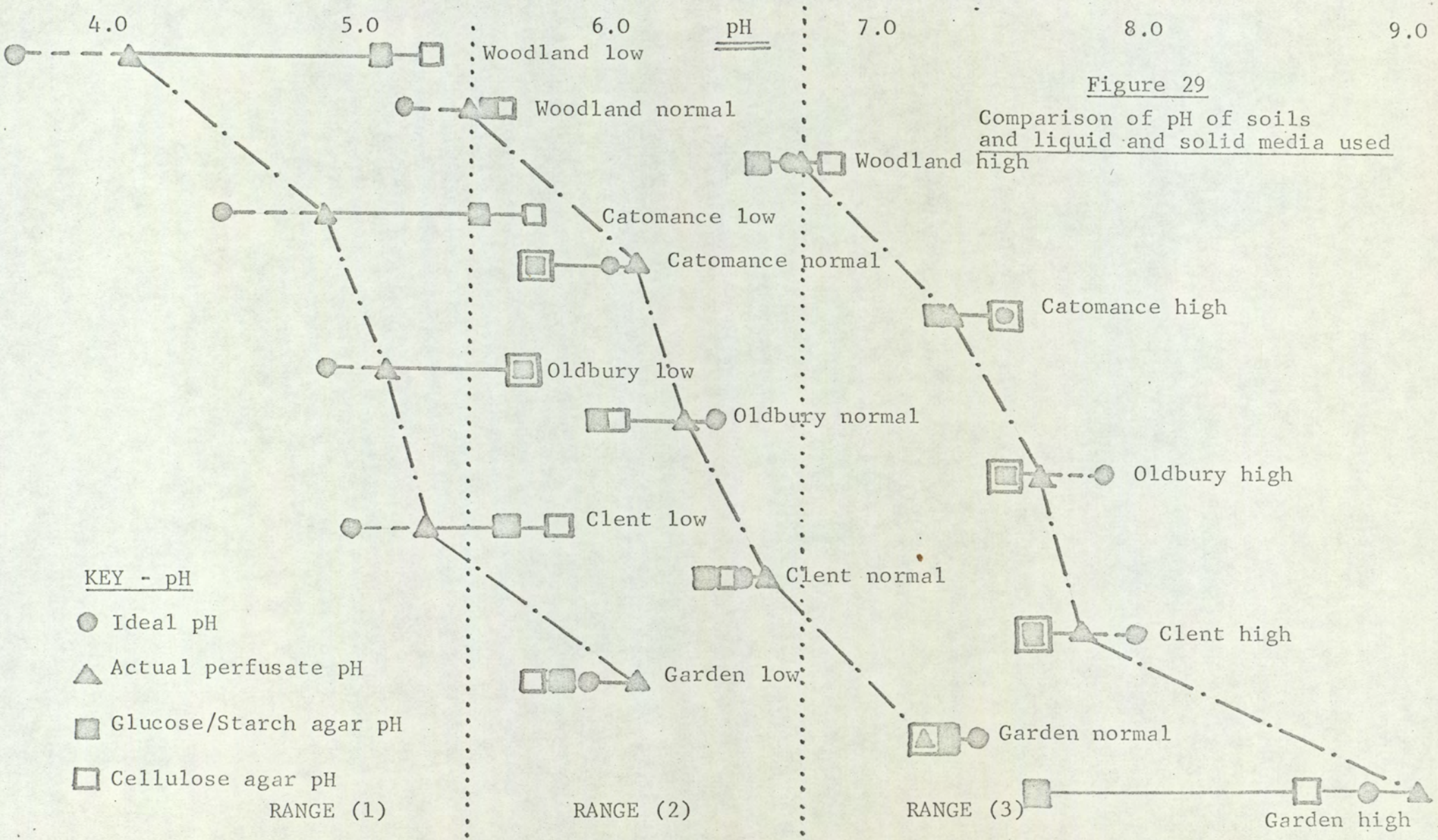


Figure 29

Comparison of pH of soils and liquid and solid media used

pH RANGES (PERFUSATE)

RANGE (1)	pH	4.15 - 5.45
WOODLAND	LOW	4.15
CATOMANCE	LOW	4.9
OLDBURY	LOW	5.15
CLENT	LOW	5.3
WOODLAND	NORMAL	5.44

RANGE (2)	pH	5.46 - 6.75
GARDEN	LOW	6.1
CATOMANCE	NORMAL	6.1
OLDBURY	NORMAL	6.3
CLENT	NORMAL	6.6
WOODLAND	HIGH	6.75

RANGE (3)	pH	6.76 - 9.1
GARDEN	NORMAL	7.2
CATOMANCE	HIGH	7.3
OLDBURY	HIGH	7.65
CLENT	HIGH	7.8
GARDEN	HIGH	9.1

Ranges as above defined by point of overlap of low/normal and normal/high ranges in figure 29.

Table 27

pH ranges of perfusate

Results of fungal isolations

After incubation for ten days in soil, the tubes were removed and the test strips removed for plating-out and for strength testing. Those to be plated out were handled with sterile forceps, cut into sections with sterile scissors and plated out, four small portions on each plate. Plates were examined after seven days, and the species occurring were noted. Frequency of occurrence and vigour of growth were indicated by the approximate scheme below.

+ + + +	very abundant
+ + +	abundant
+ +	common
+	scarce
0	absent

In the following tables, the figures are additions of the two values from the two similar plates for each treatment, e.g. + + + + on one plate and + + on the other gives a value of 6.

The figures for bacteria and nematodes merely indicate presence or absence on each plate thus a note:-

0	=	absent on both plates
1	=	present on one plate
2	=	present on both plates

The isolation results are presented in the following tables, firstly the results of untreated and treated textile at the normal pH, and then together with results at the modified pH levels.

(See tables 28 - 34)

Untreated Textile

Species	WOODLAND pH 5.2		CATOMANCE pH 6.0		OLDBURY pH 6.4		CLENT pH 6.5		GARDEN pH 7.4	
	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)
Arthrobotrys sp.	0	0	0	0	3	0	7	4	3	6
Eurotium sp.	0	0	0	0	0	2	0	0	0	0
Humicola grisea	0	0	3	0	6	0	0	0	5	4
Gliocladium roseum	0	0	1	0	1	1	0	0	0	0
Rhizopus sp.	0	6	4	6	2	6	0	0	0	0
Trichoderma viride	4	3	0	6	3	4	0	0	0	0
Penicillium funiculosum	0	1	0	0	0	1	0	2	0	0
Chaetomium globosum	0	0	1	0	0	0	0	0	0	0
Dicoccum sp.	6	6	3	0	0	0	0	0	0	0
Sordaria sp.	0	0	0	0	0	0	0	0	1	0
Phoma sp.	0	0	0	0	0	0	0	0	4	0
Zygorynchus moelleri	0	1	0	0	0	0	0	0	0	0
Cunninghamella elegans	0	0	1	0	0	0	0	0	0	0
Nematodes	1	2	1	2	2	2	2	2	2	2

For fungal species:- Column (i) = frequency/strength of colonies on cellulose agar, 0-8 scale.

Column (ii) = frequency/strength of colonies on glucose/starch agar, 0-8 scale.

Nematodes:- (and bacteria in other tables) For either medium
0 = not seen, 1 = on one plate,
2 = on both plates.

Table 28. Isolations on cellulose and glucose starch agar at normal pH.

Treated Textile 1% PCPL + 0.3% free PCP

Species	WOODLAND pH 5.2		CATOMANCE pH 6.0		OLDBURY pH 6.4		CLENT pH 6.5		GARDEN pH 7.4	
	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)	(i)	(ii)
Trichoderma viride	5	8	0	7	2	4	0	0	0	2
Penicillium funiculosum	0	0	0	0	0	2	0	1	0	0
Fusarium sp.	0	0	2	2	2	4	0	2	4	0
Rhizopus sp.	0	0	0	1	0	1	0	0	0	0
Gliocladium roseum	0	0	0	0	0	0	0	0	1	0
Cladosporium sp.	0	0	0	0	0	0	0	0	1	0
Graphium bulbicola	0	0	0	0	0	0	0	0	1	0
Nematodes	0	2	2	1	2	1	2	1	2	2
Bacteria	0	0	0	0	1	0	1	2	1	2

Key:- as table 28.

Table 29. Fungal isolations on cellulose and glucose/starch agar at normal pH.

Note:- The numerical value in each case is the result of addition of the + marks, given to each species on each plate, as an approximate indication of frequency of occurrence and vigour.

Table 30

Occurrence of species isolated from untreated and PCPL treated textile from soil at differing pH.

	WOODLAND SOIL	Species	LOW pH 4.15 Cel	LOW pH 4.15 G/s	NORMAL pH 5.44 Cel	NORMAL pH 5.44 G/s	HIGH pH 6.75 Cel	HIGH pH 6.75 G/s
Untreated Textile	TOTAL SPECIES	Diococcum sp.	7	0	6	6	7	6
	LOW pH 4	Fusarium sp.	0	0	1	0	0	0
	NORMAL pH 6	Penicillium sp.	0	1	0	1	0	0
	HIGH pH 4	Trichoderma viride	3	1	4	3	3	3
		Rhizopus sp.	0	7	0	6	0	2
		Zygorynchus moelleri	0	0	0	1	0	0
	Nematodes 0, 1 or 2 plates		1	1	1	2	2	2
	Bacteria 0, 1 or 2 plates		0	1	0	0	0	0
PCPL treated textile	TOTAL SPECIES	Penicillium sp.	2	1	0	0	0	0
	LOW pH 3	Fusarium sp.	3	0	0	0	0	0
	NORMAL pH 1	Rhizopus sp.	0	0	0	0	0	1
	HIGH pH 2	Trichoderma viride	8	8	5	8	0	8
	Nematodes 0, 1 or 2 plates		1	2	2	0	2	2

CATOMANCE SOIL	Species	LOW pH 4.9		NORMAL pH 6.1		HIGH pH 7.3		Untreated Textile
		Cel	G/s	Cel	G/s	Cel	G/s	
Total species	Chaetomium globosum	0	0	1	0	0	0	
LOW pH 3	Cunninghamella elegans	0	0	0	0	1	0	
NORMAL pH 6	Humicola grisea	5	0	3	0	4	2	
HIGH pH 3	Diococcum sp.	0	0	3	0	0	0	
	Gliocladium roseum	0	0	1	0	0	0	
	Rhizopus sp.	4	7	4	6	4	8	
	Trichoderma viride	0	3	0	6	0	0	
	Nematodes	2	1	1	2	2	1	
	Bacteria	2	0	0	0	1	0	

Table 31

Occurrence of species isolated from untreated textile from soil at differing pH

PCPL Treated Textile

CATOMANCE SOIL	Species	LOW pH 4.9		NORMAL pH 6.1		HIGH pH 7.3	
		Cel	G/s	Cel	G/s	Cel	G/s
Total species	Dicoccum sp.	1	0	0	0	0	0
LOW pH 5	Fusarium sp.	3	1	2	2	2	1
NORMAL pH 3	Rhizopus sp.	0	0	0	1	0	0
HIGH pH 3	Trichoderma viride	0	4	0	4	0	3
	Penicillium sp.	0	4	0	0	0	1
	Stysanus sp.	0	1	0	0	0	0
	Nematodes	2	1	2	1	2	1
	Bacteria	0	0	0	0	1	2

Table 31A

Occurrence of species isolated from PCPL treated textile from soil at differring pH

Table 32

Occurrence of species, isolated on untreated and PCPL treated textile from soil at differing pH.

	CLENT SOIL	Species	LOW pH 5.3 Cel	LOW pH 5.3 G/s	NORMAL pH 6.6 Cel	NORMAL pH 6.6 G/s	HIGH pH 7.8 Cel	HIGH pH 7.8 G/s	
	Total species	Arthrobotrys sp.	2	0	7	4	8	8	Untreated Textile
	LOW pH 4	Fusarium sp.	0	2	0	0	0	0	
	NORMAL pH 3	Humicola grisea	0	2	0	0	0	0	
	HIGH pH 2	Penicillium sp.	0	0	0	2	0	1	
		Trichoderma viride	7	8	0	4	0	0	
		Nematodes	0	0	2	2	2	2	
		Bacteria	0	0	0	0	2	0	
	Total species	Fusarium sp.	4	2	0	2	5	3	PCPL Treated Textile
	LOW pH 4	Gliocladium roseum	1	0	0	0	0	0	
	NORMAL pH 2	Penicillium ochraceus	0	2	0	0	0	0	
	HIGH pH 2	Penicillium sp.	4	3	0	1	0	0	
		Trichoderma viride	0	0	0	0	2	0	
		Nematodes	1	1	2	1	0	0	
		Bacteria	0	0	1	2	2	2	

GARDEN SOIL	Species	LOW pH 6.1		NORMAL pH 7.2		HIGH pH 7.1		Untreated Textile
		Cel	G/s	Cel	G/s	Cel	G/s	
Total species	Arthrobotrys sp.	0	0	3	6	1	3	
LOW pH 4	Geotrichium candidum	7	0	0	0	0	0	
NORMAL pH 4	Gliocladium roseum	0	0	0	0	2	0	
HIGH pH 4	Humicola grisea	6	2	5	4	7	0	
	Eurotium sp.	0	1	0	0	0	0	
	Phoma sp.	1	1	4	0	1	0	
	Sordaria sp.	0	0	1	0	0	0	
	Nematodes	2	2	2	2	2	2	
	Bacteria	0	1	1	2	2	0	

Table 33

Occurrence of species isolated from untreated textile from soil at differing pH

GARDEN SOIL	Species	LOW pH 6.1		NORMAL pH 7.2		HIGH pH 7.1		
		Cel	G/S	Cel	G/s	Cel	G/s	
Total Species	Gliocladium roseum	2	1	1	0	0	0	PCPL Treated Textile
LOW pH 5	Graphium bulbicola	0	0	1	0	0	0	
NORMAL pH 4	Fusarium sp.	2	4	4	0	2	2	
HIGH pH 2	Penicillium sp.	0	2	0	0	0	0	
	Humicola grisea	2	0	0	0	0	0	
	Trichoderma viride	1	2	0	0	1	2	
	Cladosporium sp.	0	0	0	1	0	0	
	Nematodes	0	2	2	2	2	2	
	Bacteria	0	1	1	2	2	2	

Table 33A

Occurrence of species isolated from PCPL treated textile from soil at differing pH

Table 34

Occurrence of species isolated from un-treated and PCPL treated textile from soil at differing pH.

	OLDBURY SOIL	Species	LOW pH 5.15		NORMAL pH 6.3		HIGH pH 7.65		
			Cel	G/s	Cel	G/s	Cel	G/s	
Untreated Textile	Total species	Arthrobotrys sp.	8	0	3	0	0	0	
	LOW pH 5	Eurotium spp.	0	2	0	2	0	0	
	NORMAL pH 7	Fusarium sp.	0	3	0	0	6	0	
	HIGH pH 3	Rhizopus sp.	1	5	2	6	0	6	
		Gliocladium roseum	0	0	1	1	0	0	
		Humicola grisea	0	0	6	0	6	3	
		Nematodes	1	0	2	2	0	2	
		Bacteria	0	2	0	0	0	2	
PCPL Treated Textile	Total species	Fusarium sp.	6	4	2	4	3	1	
	LOW pH 2	Trichoderma viride	0	4	2	4	0	0	
	NORMAL pH 4	Penicillium sp.	0	0	0	2	0	0	
	HIGH pH 1	Rhizopus sp.	0	0	0	1	0	0	
		Nematodes	0	1	2	1	1	2	
		Bacteria	0	0	1	0	1	2	

Strength Losses (See figures 30 and 31)

Control Strips	Strength in pounds	
	Untreated	PCPL Treated
Low pH 4.15	13.15 (Av. of 16)	13.13 (Av. of 18)
Medium pH 6.3	12.63 (Av. of 18)	13.04 (Av. of 19)
High pH 9.1	13.36 (Av. of 19)	13.53 (Av. of 17)

The total numbers of strips producing these averages vary as some samples in each case were lost due to a machine malfunction.

The maximum difference between these averages is around 10%, and as this is the normal variation in the initial textile it can be said that storage at different pH did not affect the textile. Also, it perhaps could have been expected that if any effect at all occurred at high or low pH, it would have been to weaken the textile, both averages however were higher, most probably a chance effect, and reinforcing the above statement that these acid and alkaline conditions had no significant effect.

As the final average control strength therefore the average chosen was that of all control samples, this being:-

13.14 lb. breaking strength.

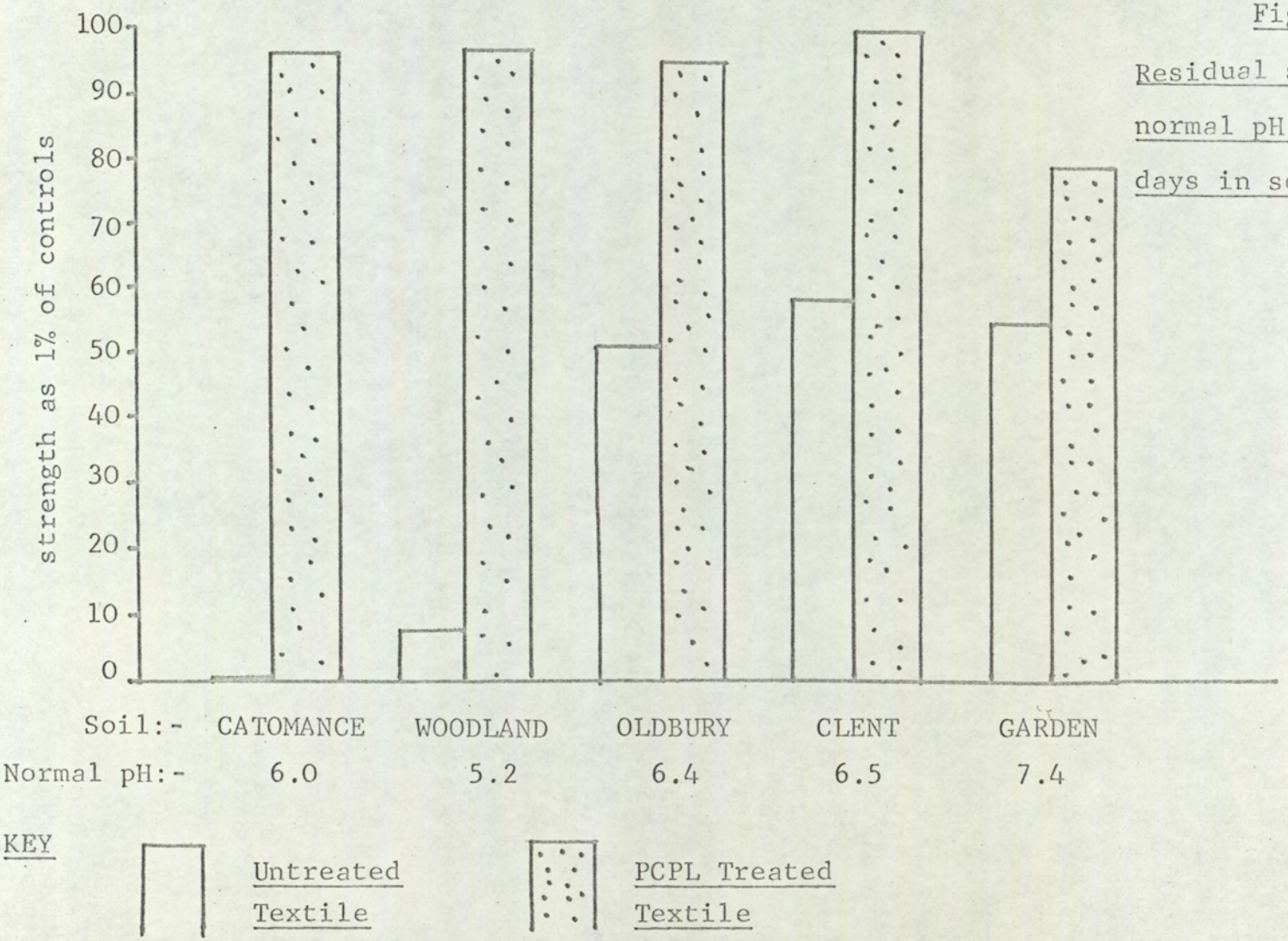


Figure 30

Residual strength at normal pH after 10 days in soil at 26°C

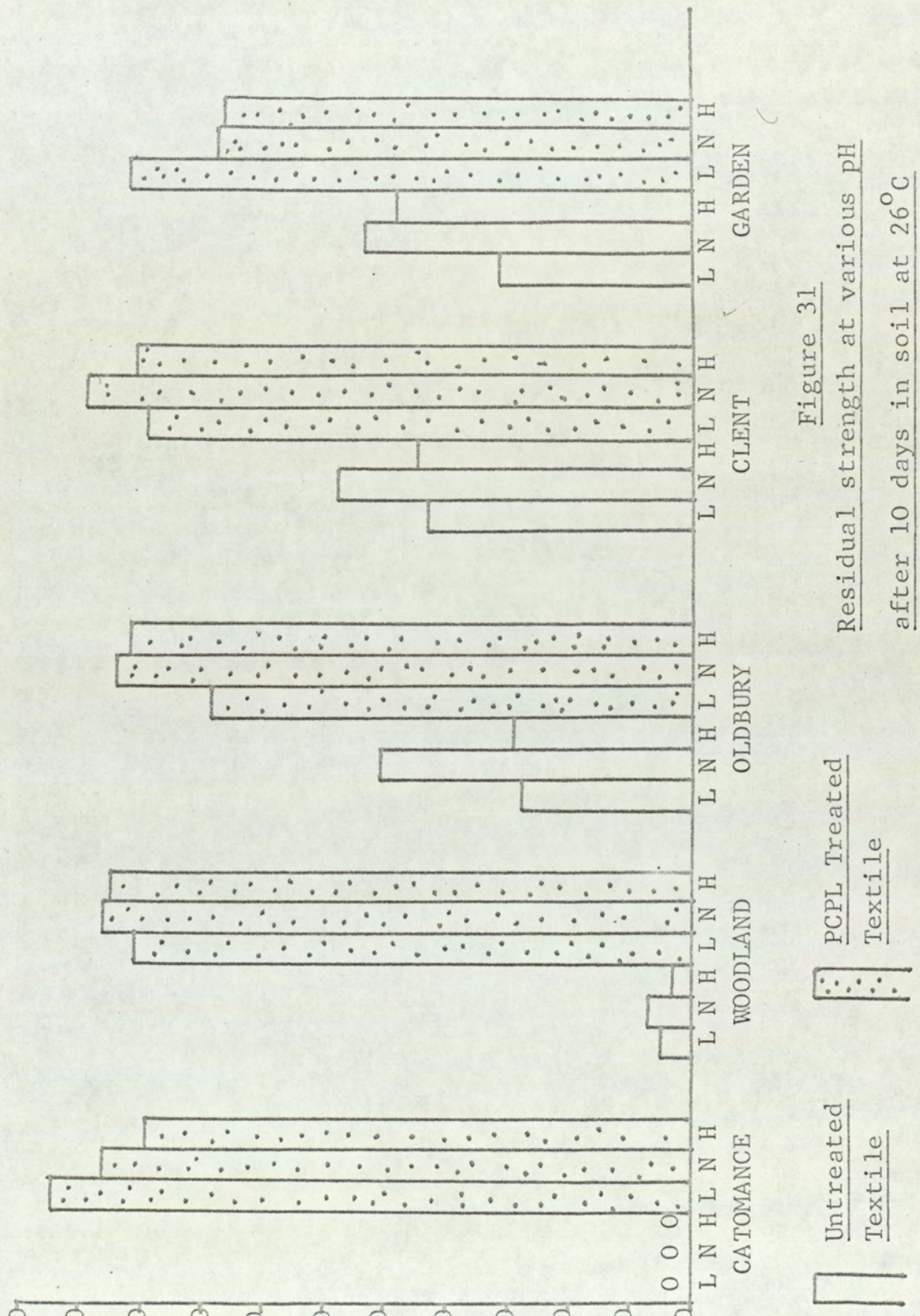


Figure 31

Residual strength at various pH after 10 days in soil at 26°C

Several conclusions may be drawn from the isolation work -

In general:-

(i) Fungal species were isolated from untreated textile samples after exposure to each soil.

(ii) Fungal species were isolated from treated (1.5% pentachlorophenyl laurate) textile samples after exposure to each soil.

(iii) More species were isolated from untreated textile than from treated textile after exposure to each soil.

For individual soils:-

(iv) Woodland Soil

From untreated textile, more species were isolated at the normal pH than at the higher and lower levels. The predominant species were Dicoccum sp. Trichoderma viride and Rhizopus sp.

From treated textile, most species were isolated at the lowest pH. Fungal species were fewer than from untreated textile. The predominant species was Trichoderma viride.

(v) Catomance soil

From untreated textile, more species were isolated at the normal pH than at higher and lower levels. The predominant species were Humicola grisea, Rhizopus sp. and Trichoderma viride.

From treated textile, most species were isolated at the lowest pH. Fungal species were fewer than from untreated textile. The predominant species were Fusarium sp. and Trichoderma viride.

(vi) Clent soil

From untreated textile, most species were isolated at the lowest pH (one more than at the normal pH). The predominant species were Arthrobotrys sp., especially where nematodes were present, and Trichoderma viride. From treated textile, most species were isolated at the lowest pH. Fungal species isolated were fewer than from untreated textile. The predominant species was Fusarium sp., the only species isolated at all three pH levels.

(vii) Garden Soil

From untreated textile, the same number of fungal species were isolated at each pH. The most predominant species was Humicola grisea.

From treated textile, most species were isolated at the lowest pH. The most predominant species was Fusarium sp.

(viii) Oldbury soil

From untreated textile, most species were isolated at the normal pH. The predominant species was Rhizopus sp., several cellulolytic fungi being also isolated but somewhat less frequently. From treated textile, most species were isolated at the normal pH. Fewer fungal species were isolated than from untreated textile. The most predominant species was Fusarium sp.

Regarding pH:-

(ix) In general, for untreated textile, most species were isolated at the normal pH.

(x) In general, for treated textile, most species were isolated at the lowest pH.

Regarding cellulolytic ability:-

(xi) The predominant species mentioned above, with the exception of Rhizopus species, were all cellulolytic, shown by their ability to grow on, and clear, cellulose agar.

Regarding Nematodes and Bacteria:-

(xii) These occur commonly. Arthrobotrys sp. and nematode worms often occur together.

Conclusions may also be drawn with relation to strength losses:-

Regarding Treatment:-

(i) Untreated textile lost strength after exposure to soil

(ii) Treated textile (except one sample) showed loss in strength after exposure to soil.

(iii) Untreated textile showed a very marked loss in strength.

(iv) Treated textile showed only small losses in strength.

Regarding soils and pH:-

(v) The soils showing the greatest agressiveness by far to untreated textile were the Catomance soil and the Woodland soil. These had the lowest pH of the soils investigated, 6.0 and 5.2 respectively.

Regarding modified pH:-

(vi) Untreated textile showed the least losses in strength at the normal pH for each soil.

(vii) In addition to loosing much less strength than untreated samples, the treated samples showed no clear pattern as regards the effect on strength losses as related to modification of pH. In three cases least loss occurred at the normal pH of the range for the soil, and in the other two at the lowest pH of the range.

It was to be expected that more fungal species and most decay occurred on untreated textile than on that treated with the PCPL fungicide. Considering the differing natures of the soils used, it was also to be expected that different species in different numbers and producing differing amounts of decay would be found.

This experiment was designed to investigate the effects of pH and fungal species of soils in the agressiveness in textile decay. From the results it would appear that there is some correlation between these factors. It is generally accepted that fungi tend to prefer acid conditions, and with regard totheir decay abilities, this does in fact appear to be the case here. The two soils which had the lowest pH were markedly more agressive than the others at higher pH. Although different in nature and water holding, these two soils had a

similar mycoflora, with similar predominances of vigorous cellulolytic species. Trichoderma viride was common in both soils, and from previous work in this thesis it can be seen that this fungus is very active at low pH. It is possible that as pH is depressed two factors concerning fungal species become more important. Firstly that fungi which prefer a lower pH become more widespread, and as a result of this become more vigorous due to lack of competition.

One may hypothesise therefore, that a lowering of pH would tend to increase fungal decay activity. When pH was lowered for each soil this proved to be the case, however, decay activity also increased as pH was raised. It is possible that a different mechanism is at work here. In a soil in its natural state, changes are usually slow and gradual, and fluctuations in the decay ability of the mycoflora will gradually even out as a mature steady state is reached. In this experiment however, a sharp change in substrate pH occurred at a single point in time, and the experiment itself continued only for a short time. There was therefore some degree of shock imposed on the mycoflora. It is probable that less vigorous species growing within the soil were less able to adapt to this change in the short time allowed, thus the more vigorous primary colonisers, cellulolytic in nature, became in a state of less competition and therefore were able to colonise the substrate more effectively than at the normal pH of the soil, being also less affected themselves by the change in pH due to their normal vigour and ability to grow over wider ranges of pH.

This effect is less marked when considering the decay of the treated textiles, but as fungal growth on these samples was small and is often erratic on protected samples, this is perhaps to be expected.

Several trends may be seen concerning individual fungal species. Trichoderma viride showed its common nature and vigour, especially at the three lower pH levels on untreated textile, extending its range when the textile was treated with PCPL. This serves to show that this species is not an anomalously agressive fungus, restricted to the Clent soil used in other experiments. Rhizopus sp. occurred together with Trichoderma viride on untreated textile, and it may be filling the niche of a secondary coloniser here. Another species which was active at lower pH on untreated textile was Dicoccum sp., but this does not appear to have the fungicide resistance of Trichoderma viride. Humicola grisea, found over the pH range on untreated textile also appears to be less tolerant to the fungicide than Trichoderma viride. Fusarium sp. appeared to fill the niche left by the less fungicide-tolerant species on treated textile, especially at the higher pH levels, this is of interest as this fungus is a common and troublesome deteriogen, not only being cellulolytic but also being responsible for much pink-staining of cotton.

A final point of interest concerning fungal species is the coincidence of Arthrobotrys sp. with nematode worms, at the three higher pH levels on untreated textile. This fungal species is known to live in association with nematodes on

which it may prey, and its occurrence here serves as a reminder of the complex ecological situation in soil, and the need for wider considerations to be taken into account in future work.

As regards the technique employed in this experiment, it proved simple in use and as it combines several principles which encourage the growth of vigorous fungi under steady and adaptable conditions, and is nearer to natural conditions than some techniques, such as agar plate techniques, it is suggested that this technique, or aspects of it, could be considered as possible modifications to the notoriously variable soil burial test.

Chapter 9

GENERAL DISCUSSION

Chapter 9

GENERAL DISCUSSION

The work set out in this thesis was performed in order to study further some of those aspects of the colonisation and decay of cotton materials, by soil fungi, which are of interest in the fields of materials protection by chemical agencies, and of the testing of such protected materials.

The work centres upon cotton, a biodegradable natural fibre, which is still the most important plant fibre produced in the world (Hueck-van der Plas, 1971). Its importance has been shown in the context of rising raw material prices and the increasing importance of prevention of biodeterioration. It is important to stress this latter point here for as raw materials are processed into more and more sophisticated products and the demand for these products increases, so the final prices rise, making losses due to biodeterioration greater. The problems concerned with the economics of biodeterioration; those of recognition of the problem and the establishment of costing criteria are still great (Eggins, 1967). The underlying reason may really be that, in those countries where biodeterioration is greatest, i.e. in the tropics, the more pressing identifiable problems of population, food production, housing and health overshadow considerations of the losses due to biodeterioration. As these problems are alleviated, more resources may be able to be utilised in the study of biodeterioration and its prevention and the knowledge applied to alleviate further

the basic problems in the countries most affected. Much more work on the economic aspects of the interaction of organisms and materials needs to be carried out and is to be encouraged.

There is an understandable tendency in biodeterioration studies for materials to be treated as though they were pure homogenous chemical compounds. Cotton can be seen as a complex natural material, (Cook, 1968) and the descriptions of its physico-chemical nature gives an insight into its biodegradable nature. In the listing of methods for the protection of cotton one becomes aware that chemical protection by means of fungicides is by far the most useful method of imparting resistance to fungal attack (Hueck-van der Plas, 1966). This being so, the need for more detailed knowledge of present and proposed fungicides is highlighted, from the point of view of efficiency in use, their ultimate fate and their safety both in application and use (Selby, 1966).

The fungi are the group of organisms most involved in the decay of cotton (Marsh and Bollenbacker, 1949; Betrabet et. al., 1968; Ranganathan and Agarwal, 1969). They are ubiquitous and are found in great variety and numbers in soils (Hawker et. al, 1960; Garret, 1963). Soil inhabiting species were the natural choice of organisms for use in this work, not only because soil is the most widely used test environment, (Dean et. al., 1960; Hueck & van der Toorn, 1965) but also because soil is the most likely contaminant of cotton textiles in use.

The concept of a material as a heterogenous environment for fungal growth leads to considerations of the complex nature of the soil, which cannot be regarded as a mere source of agents of decay. Thus ecological studies of textile decay become necessary. By outlining the problems posed by biological testing the bias of the practical work is established, the fundamental approach being basically ecological, with the object that this approach leads to a greater understanding of biodeterioration problems and problems concerned with the design, execution and interpretation of biological tests.

The basic work on fungal isolations from soil using a variety of techniques proved adequate in providing test organisms and in demonstrating the variations in results which occur when different methods are used. It is always desirable that any isolation medium used in biodeterioration studies should have as large a resemblance to the material being studied as possible. Thus, cellulose agar was used extensively in the work, as it resembles soiled cotton in many ways nutritionally and is also a demonstrative medium (Eggins and Pugh, 1962). Isolations onto cellulose agar are therefore a test in their own right, as the ability of an organism to secrete cellulolytic enzymes is demonstrated.

Considering the large number of fungal species known to occur in soil, the investigator may be tempted to continue primary isolation work longer than is necessary for the main deteriogens to be identified. The aims of this work did not include an exhaustive survey of soil organisms; emphasis

was placed on the most common and vigorous species. This proved to be valid in that later isolation work carried out incidentally to other experiments compared well with the initial isolation work and in that the main species commonly found proved to be potent deteriogens. Some main fungi falling into this category are Trichoderma viride, Humicola grisea and Fusarium sp. The variety of fungi which may be isolated from soil and the time required to carry out such work usually excludes isolation work being used as an initial test. This is an unfortunate situation, as such work may often be of much greater practical significance than that carried out using laboratory test organisms. By using set test organisms only, the ecological approach is lost, and the organisms may become regarded as standard reagents. Organisms kept for long periods are subject to changes in their properties (Jones, 1971), and for reasons of practical convenience organisms originally chosen as test organisms for one material may be used for new totally different materials. The need for constant review of test methods at a high level must be emphasised, as the degree of understanding regarding biological testing in some industrial applications leaves much to be desired at the present time.

As soil features prominently in this thesis the detailed discussions on the soil burial test were considered to be appropriate. The soil burial test is widely used but may be very variable (Lloyd, 1968; Turner, 1972). The simplicity and severe nature of this test however (Bunker, 1943) has led to its wide adoption and often to the exclusion of other tests. Its name implies some standardisation, but as

the soil used varies the test may only be the same within one laboratory at one period in time. The provision of samples suitable for strength testing and thus the provision of quantitative results is another factor in its popularity.

There is no doubt that soil burial testing is a valuable tool and will continue to be used for a long time. It is hoped therefore that the benefits of such a simple but useful modification as substrate screening (Eggins and Lloyd, 1968) to select out only active and mainly cellulolytic fungi from the soil will be apparent from these studies, especially where any subsequent isolation work is contemplated, and that its more widespread use will be encouraged.

Mycological studies over the years have led to the development of many nutrient media to enable fungi to make luxuriant but often atypical growth in the laboratory. This ease of fungal culture has probably discouraged mycologists from carrying out studies where a fungicide is incorporated in the medium, for it can be seen from this present work that the growth of fungi in the presence of sub-lethal concentrations of a fungicide leads not only to slower growth but also to erratic and distorted growth patterns and forms. This makes studies using agar difficult, and as studies using the agar plate have been central to laboratory studies in mycology for so long, other techniques have lagged behind in their development. In studies with soil it can be seen that the total soil ecology is affected by fungicides; bacteria and nematode worms becoming more apparent. Agar techniques however, have shown that some

fungi are tolerant of PCPL based fungicides to varying degrees, and this in itself is valuable information.

The limitations in agar techniques, notably here the drying of the medium and the difficulty in ensuring even distribution of a fungicide led to the adoption of perfusion techniques (Eggins, Malik and Sharp, 1968). The constant slow flow of nutrients through the material under test enables experiments to be maintained in a healthy state for long periods and for fungal growth to be studied on a substrate close to or even the same as the material of interest. It should be clearly stated however that perfusion systems in their present physical form do not lend themselves to use as routine screening methods. The principle however is valid for such uses, and once the assembly time is reduced and re-use made easier a more sophisticated screening tool will be available. The variety of perfusion systems which have been devised in one laboratory over the last few years (Eggins, Malik and Sharp, 1968; Malik and Eggins, 1969; Sharp and Eggins, 1970; Malik and Eggins, 1970a) shows the flexibility of the system and the uses to which it may be put.

In this present work, perfusion systems have been used to study fungal growth and decay of cotton textiles; to provide samples for chromatographic investigation of the breakdown of PCPL fungicides and developed as a possible system for the rapid initial screening of material samples. This latter employment of perfusion systems made use of the fact that fungal growth on perfused textiles is more compact and regular than on textiles in a static system where they

are merely dampened with liquid nutrients. This even growth is not an obvious but a useful feature of such systems. Perfusion systems have only been used in mycological work to date, but there is no reason why they could not be used to good effect in bacterial and algal studies.

One of the results of the work in the preceding chapters has been that designs have now been made for pre-assembled perfusion systems for commercial laboratory use, and it is hoped that such future developments will lead to the more widespread use of perfusion systems.

Work on forms of cellulose or forms of cotton textile is of value to those concerned with its chemical protection. A finished article in use however does not consist of cotton alone. One of the major additions may be some form of textile finishing agent or dressing (Hall, 1952). This fact prompted the work on the decay of cotton in the presence of alternative carbon sources. Although by no means exhaustive in scope, this work has given some insight into the type of results which may be expected. Further work in this area is needed even though the results may only truly apply to specific types of finished article.

In order to put any fungicide to its most efficient and economical use, data are required on its distribution within and upon a protected material. Such information is of immediate interest to those who compound and apply the fungicide, for its distribution at a microscopic and macroscopic level depends to a large extent on their knowledge.

With a fungicide such as pentachlorophenyl laurate, which is often applied as an emulsion in water, problems of variations in concentration at a microscopic level present themselves. As fungal spore germination and hyphal proliferation occur at a microscopic level, it is of value to learn the concentrations of fungicide at which inhibition begins to take place. This is of particular significance with the fungicide in question, as the work in this thesis employing perfusion and chromatographic techniques has confirmed that its initial toxicity is due to small amounts of pentachlorophenol (PCP) present in commercial preparations as a necessary impurity. The same experiments have shown the mode of action of pentachlorophenyl-laurate (PCPL) as one of a biodegradable reservoir of active PCP, and the question of the PCP released by fungal hydrolysis being perhaps more active than may have been expected has been raised. The nature of this 'nascent' PCP is of interest, and may merit further study and clarification.

The use of perfusion systems as an analytical tool is a new concept which may merit further development. Metabolites from organisms, the breakdown products of the substrate or a biocide can be detected in many growth systems, but a most useful feature of the perfusion system is that such products are concentrated in a convenient form by the normal functioning of the system, free from many contaminants.

The pH of any soil, be it either cause or effect, is a major factor in its fertility and linked with this, from the results of the foregoing studies, in the cellulose decay

abilities of fungi and the efficacy of fungicides in lessening decay. Soils have been investigated with regard to variations in pH using the new technique of the screened substrate perfusion immersion tube, and the results have shown the value of investigations of such environmental factors before using soils in test work. The differences in the agressiveness of different soils to cotton textiles have been shown to be great, thus highlighting the value of investigation of such environmental factors before a particular soil is used in test work.

In practice, biological tests, such as soil burial, are usually performed to give one end result, for example, the loss in strength of a textile sample after fourteen days of burial. In more detailed work, and in the investigation and development of tests, it is desirable to monitor the progress at intervals. The development of a simple but effective strength testing machine to be used with textile samples from perfusion systems was one attempt to enable such monitoring to be carried out easily. This machine proved most useful during the course of the present studies and it was suggested that small laboratories and colleges embarking on biodeterioration work might find such apparatus useful. This has subsequently proved to be the case, as several such organisations have expressed their intention to develop equipment along these lines.

Throughout these studies, emphasis on soil ecology has been directed to showing, and to some extent clarifying, the nature of the soil environment, as the soil is the largest

variable factor in test work, and as mentioned earlier is all too often regarded as merely a constant source of agents and decay.

Although the work in this thesis has been concerned primarily with fungi from the soil, other soil organisms, notably the nematode worms, have been mentioned. The frequency of nematodes in soils both in the field and in test situations, their known interactions with fungi and their apparent resistance to PCPL fungicides has led to a further study of these aspects elsewhere (Jones, 1972).

As a result of this work, it is suggested that several areas of study may merit future attention. One is that of testing materials in finely controlled and monitored environments; another is that of fundamental study being made of the interactions of fungi and new potential fungicides. Other more general areas for possible future work may be those which encompass the effects of temperature on the colonisation and decay of textiles; the biodegradability of PCPL fungicides and perhaps more important, fungicides generally and also the general biological mode of action of fungicides, related to soil organisms as a whole.

APPENDIX 1. MATERIALS

Non-standard materials used

Knitted glass-fibre sleeving for use as perfusion wicks:-
'Vidaflex 99' 9mm and 4mm width electronic glass-fibre sleeving.

Silicone rubber tubing for casing perfusion wicks:-
'Vidaflex SS400' silicone rubber tubing, bore 3mm, wall thickness 0.5mm, transparent.

Jones Stroud & Co. Ltd.,
Long Eaton,
Nottingham.

Adhesive glass-cloth tape for use in perfusion kits and screened substrate soil burial tubes:-
No. 365 ½ inch adhesive glass cloth tape.

General Fabrications Ltd.,
26 Orphanage Road,
Erdington,
Birmingham 24.

Glass cloth tape for use as a substrate screen and for multiple perfusion systems:-
'Duraglass' electrical 'E' grade glass cloth plain weave .003 inches x 2.0 inches wide. ECT.101.
Turner Brothers Asbestos Co. Ltd.,
Rochdale,
Lancashire

Lay-flat tubing for use as wick casing in multiple perfusion systems:-

'Melinex' film, 'rusty' variety, 100 guage 2 inch wide lay-flat tubing.

Secol Ltd.,
Industrial Estate,
Thetford,
Norfolk.

Silicone rubber adhesive for use in perfusion kits:-

'Silastoseal'
Midland Silicones Ltd.,
Barry,
Glamorgan.

Polyethylene backed paper for use in perfusion kits:-

'Whatman' 3mm polythene backed chromatography paper.

W. & R. Balston Ltd.,
Springfield Hill,
Maidstone,
Kent.

Specification of standard T.N.O. cotton test textile

Raw material	cotton, equal to or corresponding with good middling
Warp	
yarn-count	Ne 32/2 (2 x 18 Tex)

turns/inch-single yarn	23
turns/inch-twofold	19

Weft

yarn-count	Ne 20/2 (2 x 30 Tex)
turns/inch-single yarn	16
turns/inch-twofold	16

Fabric-construction

weave	plain
ends per 10 cm	340 $\pm$ 7 i.e. warp threads
picks per 10 cm	170 $\pm$ 5 i.e. weft threads
weight per m^2 in grammes	255 $\pm$ 13

Composition of Eggins and Pugh Agars

Rose bengal	15ml	(Stock solution to give 670 µg/l of agar)
Potassium dihydrogen phosphate		1.0g
Ammonium sulphate		0.5g
Potassium chloride		0.5g
L-asparagine		0.5g
Yeast extract		0.5g
Magnesium sulphate hydrate		0.2g
Calcium chloride		0.1g
Agar		20.0g

Either Cellulose 10g or 250ml 4% suspension
or:- Glucose 10g
or:- Glucose 5g + Soluble starch 5g

Distilled water to 1 litre

APPENDIX 2

Toxicological properties of pentachlorophenyl laurate as "Mystox LPL".

(Data supplied by Catomance Limited, Welwyn Garden City)

The oral and dermal toxicity of this compound was assessed in rats and compared with that of pentachlorophenol and sodium pentachlorophenate. The toxicity of "Mystox", like pentachlorophenol and sodium pentachlorophenate, varies with the vehicle used in its administration. "Mystox" is less toxic than either of the compared compounds and, although skin application of large amounts produced pathological lesions, lethal effects were not produced through the skin.

Oral Toxicity

This was assessed in groups of five female rats weighing approximately 160g. Acute poisoning causes fever which may lead to exhaustion and death. One animal given "Mystox" had a temperature of 110°F at death. Survivors seemed to make a complete recovery within 48 hours.

Female Rat L.D. 50 mg/kg

Vehicle	Mystox	Pentachlorophenol	Sodium Pentachlorophenate
Polyethylene glycol	1080	-	750
Maize oil	608	282	245

Skin Absorption

Six applications of undiluted "Mystox" to the dehaired dorsal skin of three female rats at a daily dosage of 4 g/kg were without clinical effect. Post mortem examination showed no gross abnormality but histological examination of the liver

revealed moderate damage characterised by some centrilobular necrosis, and degeneration and death of scattered liver cells. This is in keeping with changes caused by lesser doses of pentachlorophenol. No effect was caused by a single application of 2 g/kg in maize oil.

By contrast when maize oil was used as a vehicle for pentachlorophenol and sodium pentachlorophenate, the percutaneous L.D. 50 of the former was approximately 500 mg/kg and that of the latter 175 mg/kg. Both compounds were without effect at a dosage of 500 mg/kg when dissolved in polyethylene glycol.

Skin Irritation

The daily application of "Mystox" for two weeks either undiluted or as a 50% solution in either maize oil or acetone caused only mild erythema and moderate thickening of the shaven skin of the animal's ear.

PROBABLE EFFECTS IN MAN

The material has a moderate systemic toxicity when given orally and some absorption may occur through the intact skin. However, it should prove considerably less toxic than either pentachlorophenol or sodium pentachlorophenate.

Eye Instillation

A drop of the undiluted material was instilled into the eyes of three rabbits. This caused mild initial irritation in all, and in one animal some clouding of the cornea was noted. All the eyes were normal within 48 hours.

APPENDIX 3

Composition of the blended fatty acids with emulsifiers as used in "Mystox" preparations.

(Data from "Mystox ELC"; supplied by Catomance Limited, Welwyn Garden City).

22.5% Blended fatty acid emulsion.
1.0% An ethylated castor oil (Texaphore D 10)
1.0% An ethylated nonyl phenol (Ethylan 77)
0.25% An ethoxylated soya bean amine (Ethymene S 15).
Water to 100%

Analysis of Fatty Acids

C_6 0.9%
C_8 31.7%
C_{10} 23.1%
C_{12} 11.1%
C_{14} 1.3%
C_{16} 3.8%
C_{18} 0.5%
C_{18} - 2 unsaturated hydrogen bonds 23.9%
C_{18} - 4 unsaturated hydrogen bonds 2.9%
C_{18} - 6 unsaturated hydrogen bonds 0.4%
C_{20} - 0.4%

Molecular Weight 187

APPENDIX 4

Some technical details of 'Mystox' preparations

(Supplied by Catomance Ltd., Welwyn Garden City).

Mystox ELC

Composition

90% commercial Pentachlorophenyl Laurate (PCPL) with cationic-nonionic emulsifying agents.

Description

Light brown, non-corrosive, self-emulsifying oil.

Function

Fungicide, bactericide, insecticide, mothproof.

Fields of application

Substantive treatment of textiles, particularly yarns in hank or package form.

Preservation of latices and adhesives.

Combined application with Mystolene CW where waxing is also required.

Concentration

1.4-2.8% on dry weight of goods assuming 80% exhaustion. To meet the requirements of BS.2087:1963 the following concentrations would be required:

Light 1.4% Mystox ELC to deposit 1% PCPL

Normal 2.35% Mystox ELC to deposit 1.7% PCPL

Heavy 2.8% Mystox ELC to deposit 2% PCPL

Latices and Adhesives:

1.1% Mystox ELC on total weight of mix.

Mystox LSE

Composition

25% technical Pentachlorophenyl Laurate (PCPL, nonionic emulsifying agents and water.

Description

Free flowing, pale cream coloured emulsion posessing high wetting power.

Function

Fungicide, bactericide, insecticide, mothproof.

Fields of application

Mystox LSE is recommended where its high wetting power is advantageous in assisting penetration; for example on fire hose and tightly woven or intrinsically hydrophobic materials. Also for application to yarns during winding or doubling and loose materials by spray application.

Concentration

4-8% according to the degree of protection required or to meet the requirements of BS 2087:1963.

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