SOME INVESTIGATIONS INTO THE SUSCEPTIBILITY AND RESISTANCE TO INDUSTRIAL BIOCIDES OF FUNGI IMPORTANT IN TEXTILE AND POLYURETHANE BIODETERIORATION

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SUMMARY

A description is given of cotton textiles and polyurethanes as materials, and the problems associated with the biodeterioration of these materials and its prevention are discussed from both biological and economic aspects.

The necessity for biocidal protection of these materials is emphasised, and a family of new organic industrial biocides is described, with particular reference to the protection of cotton cellulose under conditions of elevated temperature, and the new synthetic polyurethane materials, especially in situations where these two types of material may be in close association.

An ecological approach has been made to the investigation into the efficacy of the new biocides. Isolation of thermophilic fungi associated with the colonisation of cotton cellulose was carried out using meadowland topsoil as a source of microbial infection. This work was extended to the colonisation of biocidally protected cellulosic substrates using the new industrial biocides, and tensile strength measurement was employed to assess the degree of biodeterioration.

Some physical and chemical properties of the new biocides which might affect their biological activity were also investigated.

Similar ecological work was carried out using polyester based polyurethane film before and after hydrolysis, and the

biological susceptibility exhibited was investigated further using respirometric methods. These studies clearly demonstrated this polyurethane material to be utilised directly by colonising fungi.

The effect of the new family of biocides in preventing such attack of polyurethanes was investigated using a similar respirometric technique, and it was demonstrated that these biocides were successful in reducing and preventing such fungal attack.

Rapid assay techniques were also sought in order to correlate biological activity and/or hydrolysis with the physical or chemical properties of the polyurethane.

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CHAPTER ONE

INTRODUCTION

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1(a) The Problems Outlined

It is well established that many materials in wide usage in the world today are susceptible to biological colonisation and the resultant damage which may occur is of considerable importance to Man's environment and economy.

Cotton textiles compose just one group of materials which are particularly susceptible to fungal attack and they have been used by man for many centuries during which time the prevention of biodeterioration has been of obvious importance.

In more recent times, progress has been made into the production and use of synthetic materials. One such group of materials is that of the polyurethanes, which, by virtue of their chemical properties may be produced in a vast number of different forms to be used for numerous purposes. As such, the polyurethanes offer great potential as new materials and it is important that these new materials should either be resistant to fungal attack, or they must be protected against biodeterioration in some way. It has already been shown that certain polyurethane formulations are susceptible to biodeterioration by fungi, (Evans and Levisohn 1968, Jones and Le Campion-Alsumard 1970 and Darby and Kaplan 1968), and it may be desirable to incorporate fungicides into these formulations to protect them from such occurrences.

The problems associated with the biodeterioration of materials and its control have, of necessity, interested a number of organisations. Catomance Ltd., who have sponsored the present

investigation, in particular have been involved in the prevention and control of biodeterioration of materials by chemical means over a number of years.

One major interest of this company has been the fungicidal protection of cellulosic materials, while with the rapid development and increasing use of modern synthetic materials, such as the polyurethanes, there is an obvious need to investigate the problems associated with any biological susceptibility in these materials.

These problems are of immediate importance to Catomance Ltd. The gradual alleviation of many primary problems, such as starvation, drought and lack of shelter, in the developing tropical countries is leading to resources becoming available to develop these countries even further. As a result, problems of biodeterioration of materials are commanding greater attention. The biodeterioration of cellulosic materials in general, and cotton textiles in particular, are of interest to Catomance Ltd., especially in situations of elevated temperatures such as occur in tropical countries and also in temperate countries where insolation can occur and, in these situations, possible prevention of such deterioration by means of a new range of organic biocides recently developed by this company will clearly be important, not only in terms of financial benefit to this company but also in further development of tropical countries.

In the case of polyurethanes, their use as modern materials, particularly in association with the more traditional cellulosic

materials, will be of interest to Catomance since, unlike many modern plastics, some polyurethane systems, especially those which are ester linked have been shown to be susceptible to fungal colonisation (Darby and Kaplan 1968). With the rapidly increasing use and development in new formulations of these polyurethane materials, again an immediate problem is posed concerning the prevention of biodeterioration.

The immediacy of these problems also serves to illustrate the role of the Interdisciplinary Higher Degrees scheme (I.H.D.) currently operating at Aston University. This type of higher degree scheme, of which this thesis is a part, is a relatively new concept, and one of the major aims of the scheme is to solve problems and carry out research of direct and immediate importance to industry, especially in situations where the industry concerned may not have the facilities or available expertise to devote to the problems involved.

There are a great number of products currently available with which materials may be treated to protect them against biodeterioration and, in addition, there is a continual search for new products which may be cheaper to produce and/or which may offer improved fungicidal activity.

It must be remembered that there is the possibility that strains of fungi may evolve which are, or become, resistant to the action of existing fungicides, and alternative protective measures will become necessary in these situations.

Other facts of importance to the fungicide manufacturing industries are those of availability of raw materials from which the fungicides are made, and possible legislation concerning chemicals which may become socially unacceptable. This latter point has already been seen to occur in the case of mercurial and arsenic compounds, and this may extend to compounds now experiencing wide usage as fungicides.

Considering these points, it is important that the fungicide manufacturers investigate the potential of new products, and in particular, find groups or families of related chemicals which may be 'tailor made' to protect specific materials under particular sets of circumstances.

Catomance are continually engaged in this search, and this has resulted in the recent development of a new range of organic fungicides, around which this research programme is centred. This new range or family of fungicides must be evaluated under two situations currently of interest to Catomance. These are, the protection of the well established material of cotton textile under conditions of elevated temperature, and protection of the new synthetic polyurethane materials.

The following investigation has therefore been carried out with particular reference to the new family of organic biocides developed by Catomance Ltd.

The project originally put forward by Catomance Ltd., was an investigation of the biodeterioration of cotton cellulose, in the form of textile, at elevated temperatures, with particular

reference to its control by means of the new family of organic biocides, together with a survey of the distribution of fungi susceptible and resistant to these industrial biocidal products. During the initial stages of the work, however, the immediate interest of the sponsoring company swung towards the biodeterioration of polyester based polyurethane systems, and, as a result, the direction of work carried out and described in this thesis was changed towards that end. This serves to illustrate again the role of the I.H.D. scheme, the major function of which is to solve the problems and carry out research of immediate interest and importance to industrial organisations.

During the course of such investigations, industrial emphases may change, and in this situation the I.H.D. scheme allows for flexibility concerning the direction of research, thus offering the greatest immediate benefit to be obtained by the industry concerned.

1(b) Cotton Textile and Polyurethanes as Materials

(i) Cotton - an old, established material

The use of cotton, a plant originally growing wild in tropical countries, is thought to have its origins in India where pieces of fabric dating back to 3,000 B.C. were discovered, and which showed extensive biodeterioration (Gulati and Turner 1920).

In Great Britain, it was not until around 1785 that cotton cloth was made in any great quantity, and this late development was due to the prominence of the wool industry in this country

at that time. The industrial revolution, however, saw the cotton industry come to the fore, and it reached a peak in 1913 with £130 million of exports. Since that time there has been a decline in the industry, and in 1965 exports were only £29 million (H.M.S.O. publication 1971). This decline was attributable to the cotton industries of other countries being increased, accompanied by a change in clothing material requirements to the use of synthetic materials.

Despite the decline in the cotton industry in the U.K., world production has increased from 10,611 million pounds weight in 1913 (Robson 1957) to 25,000 million pounds weight in 1968 (Hueck van der Plas 1971).

The price of cotton textile has also increased dramatically and as a result, any material lost or damaged due to the activities of micro-organisms will clearly lead to substantial increases in financial loss.

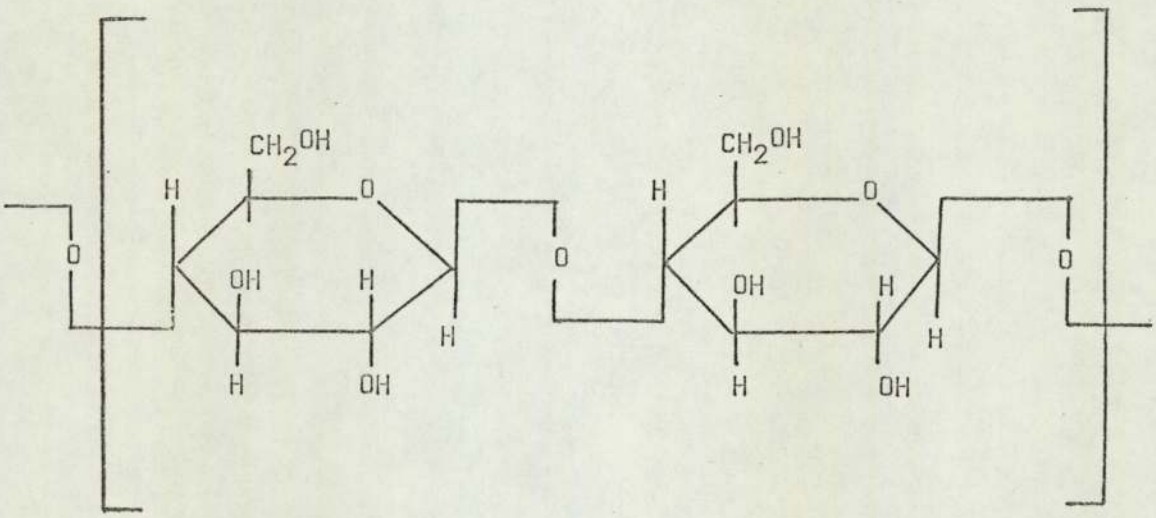
Cotton textile is manufactured from the boll of the cotton plant which is a shrub 4-6 ft in height, and indigenous to sandy soil of sub-tropical countries, and the principal producers are Southern U.S.A., India, Egypt, Brazil, West Indies, U.S.S.R. and China.

Cotton is a cellulosic material consisting basically of the seed fibre or seed hair which grows from the epidermis of the cotton seeds. The plant is a species in the genus Gossypium in the order Malvaceae, and there may be as many as 20,000 fibres

per seed with eight or more seeds per boll. These seed hairs elongate substantially upon ripening, forming long hairs ranging in size from 0.2 inches to 2.5 inches in length.

Mature cotton has a composition of which cellulose forms 94-96%, with proteinaceous and pectic constituents forming about 3%. and ash, wax and sugars making up the remainder.

Cellulose is the cell wall material, and is the principal structural polysaccharide of plants, being built up of D-glucopyranose residues forming linear polymers of high molecular weight. Upon complete hydrolysis, cellulose yields D-glucose units only, with three free hydroxyl (OH) groups per glucose residue. Partial hydrolysis yields the disaccharide cellobiose illustrated below:-



Cellobiose unit of cellulose

In the natural polymer of cellulose, the cross linkages are hydroxyl bonds or hydrogen bonds between the $-CH_2OH$ group of one chain and the OH group on the third carbon atom of its neighbour.

These bonds are strong and as there are so many bonds along the chain, the molecules are so strongly bound that cellulose itself is infusible. For this reason, cellulose is not readily soluble without previously breaking these bonds by means of powerful chemical reagents.

In natural fibres, the chain molecules are orientated into parallel bundles during the growth process. This is an important difference to the synthetic materials where orientation has to be imposed by stretching in a hot or swollen state, followed by fixation by cooling or removal of the swelling agent.

The cellulosic and fibrous nature of cotton, offers a material which is able to absorb large quantities of water together with any soluble materials and these may act as nutrient sources for micro-organisms. The fibrous nature also allows ease of penetration by micro-organisms, which may actively utilise the cellulose of the cotton as a major carbon source for growth in conjunction with the other nutrient materials present in solution.

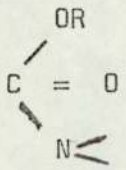
Consequently it can readily be appreciated that cotton textiles present a material which may be highly susceptible to microbial colonisation and biodeterioration, if preventative measures are not taken.

1(b) (ii) Polyurethanes - a new synthetic material

The term 'polyurethanes' refers to a class of industrially important and very useful polymers, where the repeating unit contains the urethane linkage. These materials have found

considerable commercial application only since the last war, and are now experiencing a rapid increase in development and usage.

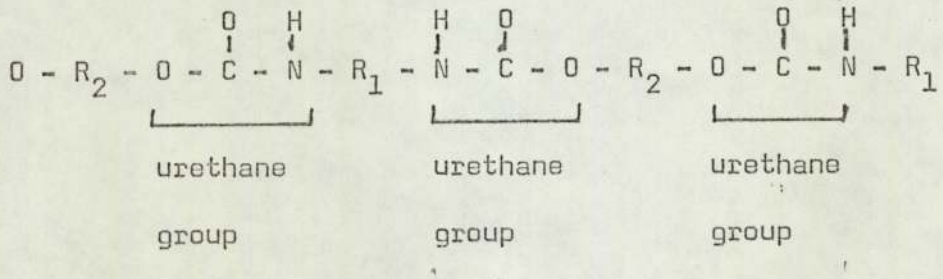
The urethane group may be considered as being derived from carbamic acid, which, due to its instability, is only found in the form of esters which are the urethanes, having the characteristic grouping of



From this structural configuration, at least three types of urethanes are possible, depending on whether groups on the nitrogen atom are represented by two, one or no hydrogen atoms.

The polymeric material may be derived from this urethane linkage by using a polyhydroxy material, such as glycol, to form an ester link, and in this way a point of possible growth is achieved. Similarly, polyfunctional nitrogen groups will offer further growth points at the amide linkage.

The simplest polyurethane is linear with the hydroxy compound and nitrogen compound having a functionality of two:-



By incorporating materials with a functionality greater than two, polymers are formed with various degrees of cross-linking, resulting in synthetic materials which vary in physical properties from soft elastomers to rigid and brittle plastics. It is thus theoretically possible to obtain a spectrum of rigidity and Saunders and Frisch (1962) note that the polyurethanes are almost unique in that cross-linking, chain flexibility and intermolecular forces may be varied widely and almost independently.

The easiest and commercially most useful method of producing the urethane linkage, is the reaction of the isocyanate radical (N=C=O) with an alcohol radical (OH), and a useful working definition of polyurethanes has been put forward by Dombrow (1965) as:-

'Polymers produced by addition reactions between polyisocyanates (difunctional or higher) and hydroxyl rich compounds (at least two hydroxyl groups per molecule) such as glycols, polyethers and polyesters'.

Polyurethane materials first came to the attention of the western allied countries after the Second World War when a review article (Kline 1945) described the progress made in the plastics field by the Germans during the war, and mentioned a new group of plastics called polyurethanes in particular.

Further development of these new materials was pursued in the U.S.A. after the war, but the major developments were made by the Germans under Dr Otto Bayer. The history of the

development of the polyurethane industry is extensively reviewed by Dombrow (1965) who estimated the civilian market for flexible and semi-flexible urethanes to be 144 million pounds weight in 1963, and predicted 269 million pounds weight in 1968. (See Appendix 1 for details).

The uses to which these new polyurethane materials may be put are numerous and diverse, including fuel tank linings, insulation of electric cables, coated fabrics, water container linings, furniture, bedding, textiles, building materials, shoes, car upholstery and packaging. It may thus be appreciated that polyurethanes offer a large and complex group of materials, and any biodeterioration of these materials will not only be aesthetically undesirable but also may result in substantial financial loss.

Examples of where losses or damage of polyurethanes may occur due to the activities of micro-organisms are likely to be found in situations of warmth and humidity which favour the growth of the organisms. Two specific examples may be found in shoes where, in addition to body heat and possible insolation, there is also a build up of perspiration which offers nutrients possibly capable of sustaining microbial growth. Upholstery in cars may become warm if left in the sun, but equally may be exposed to wet conditions after rainfall, and in the temperate climatic conditions which occur in the U.K. these two situations may in fact coincide frequently and may result in suitable conditions for microbial colonisation of the upholstery.

These two examples are of particular relevance to the present investigation since invariably the synthetic polyurethanes will be in close association with the more traditional cellulosic materials such as cotton or the fibreboard used in shoes, and it is this type of situation which is of particular interest to the sponsoring company of Catomance Ltd.

The reasons why cotton textiles and polyurethanes are associated in this thesis may therefore be summarised as follows:-

1. Polyurethanes and cotton textiles are very closely associated in some products as described above.
2. The two materials offer two different test situations for use in the evaluation of the new family of organic biocides recently developed by Catomance Ltd.
3. Polyurethanes unlike many other modern plastic materials, can be susceptible to direct degradation by fungi and hence these inherently biodegradable formulations will require to be protected by fungicides, and it is considered by Catomance Ltd. that the new family of biocides may be particularly effective in the protection of polyurethanes from microbial attack.

1(c) Biodeterioration of Materials - the concept defined and classified

Biodeterioration may be defined as 'any undesirable change in the properties of a material caused by the vital activities of organisms' (Hueck 1965).

From this definition biodeterioration may be briefly classified into three major divisions.

A material may undergo mechanical damage due to the activities of organisms where the material itself is not actually utilised as a nutrient source, but which forms a barrier between the organism and a nutrient supply, such as the gnawing action of rodents and insects on non-nutritive materials.

Secondly, the function of materials may be impaired by the physical presence of organisms, such as short circuiting of electrical instruments due to fungal colonisation of insulation materials. This phenomenon is known as soiling or fouling.

Finally, a material may be subjected to chemical attack in two different ways. The organism involved may actively utilise the material as a source of nutrients, or alternatively the organism may excrete metabolic products which are injurious to the material.

1(d) The Biodeterioration of Cotton Textiles and Thermophily

When considering the biodeterioration of cotton textiles by fungi, examples occur in two of the classes described in the previous section of this chapter. There are no examples of cotton textile undergoing damage of a mechanical nature due to fungal activity, but soiling of textiles by fungi is quite a a common occurrence. This may simply be aesthetically displeasing, such as pigmentation in and around small colonies of fungi which are not sufficient to cause a loss in actual strength of the

material, but which are nevertheless unsightly, or cause problems in further processing such as dyeing, if occurring at intermediate stages in the manufacture of the material.

The most important class of fungal deterioration associated with cotton textiles is however that of the chemical type. Here fungi secrete cellulolytic enzymes which break down the natural polymer of cellulose into simple sugars which can be assimilated by the fungal hyphae for utilisation as a carbon source for the growth of the organism (Siu 1951).

The subject of this extracellular hydrolytic breakdown of cellulose by the cellulase enzymes produced by fungi has been widely studied. It was suggested (Reese et al 1950, 1952) that the cellulase enzyme systems of fungi must be in two or more parts. These two parts would act together, first breaking down the cellulose to make it possible for the second part of the enzyme system to attack. The two parts of this enzyme system were terms C_1 and C_x and it was suggested also that each part had to act with the other and each individual part could not accomplish the breakdown by itself.

Selby (1968) isolated the two components C_1 and C_x from fungi and demonstrated that the C_1 component acted in a synergistic manner with either its own C_x or that of a different species.

Hill (1965) also describes the progress in the study of the mechanisms involved in the biodeterioration of cotton fibres, and a detailed study of the mechanisms of action of the cellulase

from Myrothecium verrucaria showed there were indeed two or more components responsible for the breakdown of cellulose, and that there were only a few sites on the cotton fibre where enzymes are able to penetrate. The attack thus proceeds by solubilisation of the cotton cellulose in the immediate vicinity of these sites.

This fact that biodeterioration of cotton cellulose is centred around localised sites is particularly important when considering the biocidal protection of cotton textiles. If the particular sites on the molecule where attack first begins can be adequately blocked by substitution with bulky molecules, then biodeterioration may be prevented.

From the literature it can be seen that the problems associated with the mesophilic biodeterioration of cotton textiles have exercised many workers over a number of years. There is however a distinct lack of literature regarding the biodeterioration of cotton textiles at thermophilic temperatures.

The effect of temperature on the growth of fungi has been the subject of numerous investigations, and fungi have been shown to grow under a wide temperature range from near freezing to 50^{o}C and above. It is also generally recognised that the optimum temperature for many fungi lies between 15^{o}C and 30^{o}C (Cochrane 1958). The fact that temperature is important to the growth of micro-organisms cannot be debated, and the relative lack of investigations at elevated temperatures appears surprising.

The characteristic of 'thermophily', which means literally a love of heat, is encountered in many different groups of organisms, such as animals which inhabit hot springs. No higher plants however are considered to be thermophilic, while the algae possess members such as the blue green algae, some of which do inhabit hot springs. Thermophilic bacteria (Allen 1953) and Actinomycetes (Hensen 1957) are also well documented and studied.

Apart from the algae, bacteria and actinomycetes displaying the phenomenon of thermophily, there are members of the fungi which also grow under thermophilic temperatures, with maxima for growth occurring at around $50^{o}C$ - $60^{o}C$.

The first thermophilic fungus, Mucor pusillus was described over 80 years ago by Lindt in 1886, but despite this, the group of thermophilic fungi has had very little recognition in the literature. Even text-books of mycology make only passing reference to this distinct group of organisms (Hawker 1950, Lilly and Barnett 1951 and Cochrane 1958).

In fact Hawker discusses the upper limits of fungal growth to be 40^{o} - $45^{o}C$, but at least five truly thermophilic species capable of growth above $50^{o}C$ were described before her book of 1950.

The reasons for an apparent lack of information concerning thermophilic fungi may lie in the fact that relatively few workers had until recently devoted a great deal of time to

this particular group. Eggins and Coursey (1961) (1964) carried out investigations into the biodeterioration of Nigerian Palm Oil which included studies of those thermophilic fungi associated with the problem.

Cooney and Emerson (1964) recognised a need for a comprehensive comparison and evaluation of all the known thermophilic fungi. They described thirteen species in detail, four of which had previously been undescribed, and three varieties of existing species which had not previously been encountered. From their studies of thermophilic fungi these workers formulated a working definition as follows:-

'A thermophilic fungus may be defined as one which has a maximum temperature for growth at or above 50°C and a minimum temperature for growth at or above 20°C.'

Some fungi, such as Aspergillus fumigatus, with maxima near 50°C but minima below 20°C were not considered by Cooney and Emerson to be truly thermophilic and were classed as thermotolerant species.

Examples of thermophilic fungi occur in the Phycomycetes, Ascomycetes, Fungi Imperfecti and there are also a few species of Coprinus in the Basidiomycetes which are able to grow at thermophilic temperatures, but which are also able to grow below 20°C and would therefore be classed as thermotolerant species according to the definition of Cooney and Emerson.

The majority of thermophilic fungi have been isolated from various types of composting plant material such as straw, hay, leaf mould and peat, and from animal faeces such as that from cows, pigs, horses, sheep, rabbits and from birds nests.

Cooney and Emerson also state that soil provides a poor source of thermophiles, a statement which may now be open to question. They do however stress the need for intensive investigation and isolation work from soils, particularly those soils of the tropics where continuous and/or intense insolation occurs to produce micro-environments favourable for the growth of thermophilic fungi.

More recently, the effect of the sun in providing favourable conditions for these fungi in cool climates has been carried out by Apinis (1963) (1965), Allsopp (1968) and Eggins and Malik (1969).

It has been demonstrated that the growth of thermophilic fungi in insolated soils is more common than at first thought, and that the distribution of thermophilic fungi is quite widespread (Eggins et al(1972)). This is in contradiction to the statement of Cooney and Emerson referred to above. The investigations also showed that although the thermophilic fungi are widespread in soil, they are not active in cooler conditions, such as shaded areas, only becoming active under insolated conditions.

It is noted in the detailed account of Cooney and Emerson (1964) that many of the known thermophilic fungi have been

isolated from rotting plant material and animal faeces. Since there is an obvious association with the degradation of material which has a high proportion of cellulose, the precise cellulolytic activity of these fungi is of importance.

Those thermophilic species which have been shown to actively break down cellulosic materials are Humicola insolens, Sporotrichum thermophile, Aspergillus fumigatus, Chaetomium thermophile, and to a more limited extent Talaromyces dupontii.

Considering the previous paragraphs it can be seen that under certain conditions, cotton textiles may well be exposed to conditions of elevated temperatures, under which the cotton cellulose may be attacked by those species of thermophilic fungi which are also cellulolytic.

Such thermophilic conditions could be encountered during the storage of textiles under warm moist conditions of the tropics, or during the transportation of textiles across the tropics as cargo in containers which may well be subject to insolation. Even in countries of colder climatic conditions thermophilic temperatures may be encountered during storage if such storage is in warehouses with glass panels in the roof, or under clear polythene sheeting. In these cases the air temperature may not approach 50^{o}C but micro-environments may well exist which do in fact reach such conditions.

It is for these reasons that there is interest in the prevention of the biodeterioration of cotton textiles due to the action of thermophilic fungi, and although the very academic

definition of Cooney and Emerson states that some species are only thermotolerant, for the purposes of this investigation, any fungi capable of growth at elevated temperatures are obviously of interest from the practical point of view.

1(e) Biodeterioration of Polyurethanes

Since the Second World War there has been an ever increasing growth in the literature concerning the microbiological deterioration of synthetic materials.

Much of the literature is contradictory and the reasons put forward to explain this situation are the lack of distinction between the susceptibility of the polymer itself to direct attack, and the susceptibility of other processing ingredients, (such as fillers, plasticisers and lubricants), and also impurities. There are also difficulties in performing reproducible experiments, and there may be some confusion between the actual effect of micro-organisms and other environmental factors such as moisture and heat.

Although these problems have been encountered by many workers, several general trends appeared from the early literature which may be summarised as follows.

(a) Those polymers which occur naturally, e.g. rubber and cellulose may be degraded by soil micro-organisms under suitable conditions.

(b) Synthetic polymers produced are not on the whole subject to biodeterioration, since in general the polymer could not

act as a carbon source - a point which needs qualification in the light of more recent studies.

(c) Many commercial polymeric compounds are susceptible to fungal attack under the correct environmental conditions for the particular fungi concerned and this is due to utilisation of a carbon source which may be either the compounds added during processing or impurities remaining on the surface of the material.

(d) It appears that only organic compounds are vulnerable to microbial colonisation, although this too is open to debate following recent investigations by Tribe (1972) where fungal growth was obtained on inorganic materials.

Recognition of microbial deterioration has been summarised by Hueck (1965). Simple observation that micro-organisms are present on a particular synthetic material is certainly no proof that these are responsible for any deterioration in the material. In order to show conclusively that the micro-organisms are directly responsible for the deterioration of the material, they must be isolated into pure culture, and the suspicious phenomena must be reproduced under controlled conditions using the pure cultures isolated. This is paralleled by Koch's postulates(1882) which relate to pathogenic organisms.

In the past, biodeterioration of materials has not been appreciated by many, if not most, engineering and chemical works but today the subject commands almost routine attention, at least by those educated in the hard fact that in certain cases biodeterioration can lead to serious financial loss.

Polyurethanes are a new class of synthetic materials finding increasingly wide usage in modern society. These particular plastic materials differ from many others in that they do not require plasticisers, which is an advantage when considering the biological susceptibility of some of these compounds.

Since polyurethanes were first produced there has been increasing interest in their biological susceptibility and resistance. Kaplan and his colleagues (1968) made a detailed review of the numerous studies carried out concerning the biodegradability of these new polymeric materials, and discussed the fallacy of attempting to make meaningfull evaluation of systems where the ingredients are unknown. They also stress the need for the incorporation of fungicides into the formulations of polyurethane systems.

The Wyandotte Chemical Corp. (1962) took an interest in the microbial degradation of polyurethanes of the polyether type, which were used as sealing compounds for pipes. Their biological tests, which consisted of broth media and soil burial for three to four weeks, indicated that the polyether type of polyurethanes were quite resistant to microbial deterioration.

Testroet (1963, 1966) investigated polyester and polyether rubbers and found the major factors responsible for failure were (i) water induced hydrolysis and (ii) breakdown of the actual polymer by fungal activity. It was also shown that the ether type of urethanes were more resistant to fungal attack than were the ester types. A number of biocides were also evaluated and pentachlorophenol proved the most effective,

but unfortunately this chemical was also detrimental to the formulation.

The work of Testroet was repeated by Kaplan and associates (1968) who washed and sterilised the samples before inoculation and incubation at 30°C for three weeks. All samples supported growth with the ether type of polyurethane being significantly less susceptible to fungal attack than the ester type. Also, the polycarbodiimide which was incorporated to inhibit hydrolysis was shown not to have any effect on fungal growth. Pentachlorophenol slowed the rate of fungal colonisation but did not completely prevent it. They were able to conclude that fungi were directly responsible for the biodegradation of the polyurethanes, which confirmed with added weight the findings of Testroet.

Three degrading phenomena may occur during burial in the soil. These are hydrolysis, oxidation and microbial deterioration.

Ossefort and Testroet (1966) showed that a polycarbodiimide can stablise polyesters against hydrolytic effects without itself undergoing biological breakdown, and they also noted that polyurethanes display a good resistance to oxidation.

Studies carried out using polyurethane elastomers and soil burial techniques (Kaplan et al 1968), taking precautions against hydrolytic and oxidative factors, still showed changes in the polyurethanes. By elimination of oxidation and hydrolysis reactions it was assumed that microbiological activity was responsible.

Kaplan also pointed out that resistance to microbial colonisation may also be a function of processing techniques. This was based on work carried out on urethane coated fabric where the coating procedure was changed with a corresponding change in biological activity. Their studies also pointed to the fact that cross-linking plays a major role in offering resistance to microbial damage of the polyurethanes.

The first steps towards a greater understanding of the biological susceptibility of chemically identified polyurethane systems was made by Darby and Kaplan (1968) who investigated one hundred laboratory synthesised polyurethanes with reference to fungal susceptibility, and demonstrated that the polymer itself provided a direct factor towards the resistance or susceptibility of a formulation.

Other recent studies involving the biodeterioration of polyurethane systems include attack of these materials by marine fungi (Jones and Le Campion-Alsumard 1970). These workers found that certain marine Ascomycetes and Fungi Imperfecti caused degradation of polyurethane covered panels submerged for up to four years in the sea near Nice. The polyurethane panels were also observed to be susceptible to a bacterium which produced a salmon pink colouration on the polyurethane.

Evans and Levisohn (1968) carried out studies on polyurethane printing rollers which were observed to become soft and deformed due to attack by fungi. Stemphylium sp. in particular was isolated for further investigation, including

evaluation of a number of fungicides which is described more fully later in this chapter.

The polyurethanes can be seen to offer a special problem compared with other synthetic polymeric materials such as polythene, nylon and polypropylene, where the polymer itself does not offer a nutritive supply, but other additives and finishes, e.g. plasticisers, do offer a potential supply of nutrients able to sustain fungal growth (Eggins et al 1971).

Polyurethanes also pose a great problem with regard to their biological activity due to the vast range of formulations available. It is unlikely that commercial polyurethanes can be guaranteed resistant to biodeterioration even if the full chemical nature of the material is known, and in such situations, if the prevention of microbial colonisation is of any importance to the application to which the material is put, and control of conditions is not possible, then incorporation of a biocide into the formulation is essential.

The choice of biocide must be made with care since compatability with the formulation may be a problem. Each polyurethane system should ideally be evaluated individually to ensure that the biocide used is active and compatible under the specific conditions of use. It is in situations such as this where Catomance are hoping to 'tailor make' biocides from a family of products which may be effective in the prevention of biodeterioration.

Evans and Levisohn (1968) tested several fungicides in conjunction with twenty different polyester-based polyurethanes and found that although all showed various degrees of improvement over the unprotected material, none of the treatments gave full protection, although 8-hydroxyquinolene appeared to offer the most adequate protection.

The findings of these workers concerning 8-hydroxyquinolene may however be misleading since this reacts with ethyl acetate, one of the more frequently used solvents in the production of polyurethanes, by a cross-linking mechanism to produce a complex with a vivid apple green colouration (Barr 1973). This of course is undesirable from many respects, unless the colouration is of no significance to the use of the end product. Unfortunately in many situations this is not the case and the colourless properties usually associated with polyurethanes are normally desirable, even if the material is to be coloured eventually.

The work of Evans and Levisohn is however of particular interest to this thesis since the polyurethanes of an ester type which are also used in this investigation are cheap and easily produced commercially. The economics of the industry must thus be carefully considered. It is obvious that a cheap product will be used preferentially, provided it fulfils all the requirements, chemically, physically and biologically, of the manufacturer. It may thus become essential to use an effective general biocide in ester type polyurethanes since although much cheaper to produce, these materials are also more susceptible to biological attack than the ether type of polyurethane.

1(f) The Economics of Biodeterioration of Cotton Textiles and Polyurethanes

It is well known that micro-organisms play an important role in nature during the recycling of naturally occurring materials. As such these organisms may be thought of as having a beneficial effect on Man's environment, for if this did not occur plant debris would simply continue to pile up. Examples of such effects are to be found in the carbon and nitrogen cycles of nature.

Cases where the effects of the activities of micro-organisms are beneficial to Man's environment and economy (biodegradation) must also be distinguished from those injurious effects which micro-organisms may have (biodeterioration).

The same organisms may be responsible for both the above phenomena. For example those cellulolytic micro-organisms responsible for the recyling of carbon from the cellulosic materials of plant debris, may also be the organisms responsible for the 'mould' growing on the cellulose pages of books stored in damp conditions, or the mould growing on damp cotton textile, wallpaper and paintwork, which many unenlightened people regard as simply dirty marks.

In the case of polyurethanes which are susceptible to degradation by fungi these may offer convenient plastic materials with a built in life span, at the end of which the polymer is broken down by the activities of micro-organism. Examples where such a system would possibly be important are to be found

in packaging where the litter problem and waste disposal currently costs a great deal, and these costs may be reduced as a result of such natural degradation of polyurethane containers.

Although biodeterioration spans vast areas of biology, not all materials are necessarily susceptible to biological attack, although recent investigations by Tribe (1972) demonstrates fungal growth on inorganic media, even after taking considerable precautions to remove any organic matter which could be utilised by the fungi for growth. Even in a humid tropical environment, which many biologists consider to be the almost ideal microbiological environment, biologically inert materials become contaminated with foreign matter on their surfaces so quickly that the so called biologically inert surfaces of certain inorganic materials and plastics can be questioned (Rasmussen, Hutton and Garner 1968).

Accepting that many, if not most, materials may be vulnerable to biodeterioration, given the right conditions, it can be appreciated how important is the initial recognition of any microbial attack, especially when considering the economic aspects of biodeterioration (Eggins 1967).

It is a fact that, although the problems of biodeterioration have been appreciated by some workers for a long time, it is only in relatively recent years that the full significance of microbial colonisation of materials has been realised by many non-biologists, and indeed by some biologists.

The situation is rapidly improving with many more people becoming aware of the fact that the 'dirty mark' on a material may in fact be the first visual indication that biodeterioration is occurring, and something more is required than simply wiping away the mark.

A particular attack may appear to be due to the activities of micro-organisms, but the problem may have been physically or chemically initiated, with the micro-organisms moving in at a later point along the chain of events occurring on the material. Alternatively a material may show damage with no obvious signs of biological activity occurring, although the damage may be directly attributable to the action of micro-organisms at an earlier stage. Such an example may be found in the leather industry, where the 'wet blue' stage of processing is particularly susceptible to fungal attack, but which may not be obvious to the biologically untrained eye. The activities of these fungi however can seriously affect further processing stages resulting in irregularities in dyeing, which can prove extremely costly.

In order to evaluate whether or not an organism is directly, indirectly or not at all responsible for the deterioration of a material, Koch's postulates may be modified and applied to the cases in question. (Koch 1882), (Eggins 1967).

Having established that the damage to a material is due to the activities of micro-organisms, the cost of this damage may be counted.

The subject of costing the effects due to biodeterioration is difficult in itself as there is no standardised procedure.

The major areas where the effects of biodeterioration costs money are:

(i) the cost of replacing a material which has undergone microbial attack,

(ii) the cost of preventing such attacks occurring initially,

(iii) the cost associated with the remedial treatments to materials which have undergone biological attack.

The cost of replacing a material may, however, be prohibitively expensive unless the material is very inexpensive in itself. Also the cost of remedial treatments can be great and the task can prove very difficult. Consequently, remedial treatments are usually limited to exceptional cases such as the restoration of highly valuable ancient cultural treasures where the expense may be justified. It is therefore the cost of the prevention of any microbial deterioration which commands the most attention and which usually proves the least expensive of the possible alternatives.

There is a notable lack of figures in the literature relating to the costs involved due to the biodeterioration of materials in general, possibly due to the fact that the economic importance of this phenomenon has only relatively recently been appreciated by many people.

Hueck van der Plas (1971) reviews the costs of some materials which are of economic importance, and which are susceptible to biological attack (Table 1a). This shows that the total value of these raw materials is 89.5×10^9 American dollars. These figures relate only to the raw materials themselves and in fact the value is increased after processing and manufacturing procedures and this will obviously increase any financial loss due to biodeterioration (Table 1b).

From these figures it can be appreciated that any loss or damage due to biodeterioration can cause substantial financial loss and measures must be taken to avoid this.

Considering the two materials relevant to this investigation, cotton textiles and polyurethanes, again there is a lack of information regarding financial losses involved. This is probably due to the difficulty in collating the necessary information from the diverse range of end points that a particular material may reach, coupled with the fact that manufacturers do not usually wish to make it known that any of their products fail in any way. This all makes any accurate costing of biodeterioration an extremely difficult task.

The cost of prevention of biological attack of materials may be partly assessed from the figures regarding the value of fungicides produced in the U.K. (H.M.S.O. 1971). In 1970 a total of £3.3 million worth of fungicides was produced and this represents a substantial expenditure in the fungicidal protection of materials in general.

Despite the lack of economic statistics regarding textiles and polyurethanes, the values concerned with these materials and their protection are considerable, and problems associated with the biodeterioration of these materials necessarily command the attention of those involved in the relevant industries.

TABLE 1a

1968 World Production Figures of some Industrially Important Materials Susceptible to Biodeterioration (Hueck van der Plas 1971)

Material	World Production in Millions of Tonnes	Estimate of Value of 10^9 U.S. Dollars
Cotton fibres	11.3	6.0
Wool	2.8	2.8
Jute fibres	2.7	0.9
Flax fibres	0.6	0.4
Synthetic fibres	7.3	9.1
Paper Pulp	23.3	3.0
Miscellaneous 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	$(1.2) \times 10^9$ cubic metres	20.0

TABLE 1b.

The Value Added to Raw Materials by Manufacturing in the U.S.A. in 1967 (Hueck van der Plas 1971)

Material or Industry	Added Value x 10^9 U.S. Dollars due to Manufacture
Textile products	18.3
Timber products	5.0
Paper products	9.6
Printing and publishing	14.3
Plastics and Synthetic Materials	3.8
Paints	1.3
Petroleum products	20.3
Rubber products	6.8
Leather products	2.6
Electrical industries	24.8
Instrumentation industries	6.3
Value added to raw materials in industries	113.1
Value added in construction and handling	42.0
Total Value added to raw material	155.1 x 10^9 $

1(g) An Appraisal of Methods of Prevention of Biodeterioration

Methods of prevention of biodeterioration of materials in general may be formulated in the light of thoughts on why materials appear not to be susceptible to biological attack (Alexander 1965, Butler & Eggins 1965).

The most frequently used methods of protection of textiles against biodeterioration are the passive treatments of providing a physical barrier, chemical modification of the cellulose molecule and the blocking of the sites of cellulase activity, and the active treatments which have a direct effect on agents of biodeterioration, i.e. treatment with toxic chemicals.

These toxic chemicals are quite diverse and a list has been published (Hueck van der Plas 1966) of many commercially available products, together with the biologically active ingredients contained by these products, and the materials to which they may be applied.

Turning to the protection of polyurethanes against biological attack, the same principles may be applied as those detailed for cotton textiles. The most commonly used methods of protection for synthetic materials are however the incorporation of biocides or the use of ingredients which have biocidal or biostatic properties.

It has been noted from the literature that deterioration due to the activity of fungi is of more importance and more common occurrence than that due to bacterial action. Consequently more

attention has been given to the fungal attack of polymeric compounds. In many cases however a chemical used as a fungicide may also inhibit or totally prevent the growth of bacteria.

An important fact that must be remembered is that there is no universal fungicide, and it is essential that fungicides for use under specific situations are chosen with care and selection in order to maintain their effectiveness.

The fungicidal compounds in general usage may be divided into groups according to their chemical structure, and Heap (1965) reviews these groups as applied to the fungicidal protection of rubber and plastics in general at that time. The major groups are the copper salts, of which copper 8-quinolinolate is in frequent use for the protection of rubber and plastics, organo mercuric compounds which have become socially unacceptable due to their toxicity and persistency, alkyl and chloro phenol derivatives, quarternary ammonium compounds and sulphur containing compounds.

1(h) Aims and Objectives of Investigation

From the preceding sections of this chapter it may be appreciated that the problems associated with the biodeterioration of both cotton textiles and polyurethanes are not entirely simple. In today's modern society, the economics of the phenomenon of biodeterioration are becoming much more widely recognised as a cause of financial loss, against which preventative measures can and must be taken in order to minimise, if not totally prevent, such occurrences.

It has been stated that there is no universal fungicide which is also socially acceptable, and there is a continuing search by industry to improve upon previous formulations and to produce new, less expensive compounds which offer equal if not improved protection. Also the fungicides currently experiencing wide usage may become ineffective due to resistant strains evolving, or alternatively these products may become unavailable due to world shortages of raw materials from which they are made, resulting in prohibitively high costs.

The investigations described in this thesis have been carried out for three major reasons:-

(i) A new family of organic biocides, recently developed by Catomance Ltd., may be evaluated employing two different situations of cotton cellulose at thermophilic temperatures, and polyurethane of an ester linked type which is a relatively new synthetic material.

(ii) Polyurethanes unlike many modern plastic materials can be biodegraded, and as such, these inherently biodegradable materials require protection by incorporation of a biocide into the formulation. It is hoped that the new family of biocides produced by Catomance Ltd., will be particularly effective when incorporated into such synthetic materials in order to tailor make a material of known susceptibility or resistance, i.e. a biodegradable plastic of known life span, after which the material may be biodegraded.

(iii) The relatively new situation of cotton cellulose and polyurethanes occurring in close association in some products needs to be investigated in the light of previous studies into the biodeterioration of both these materials, with particular reference to the new biocides produced by Catomance Ltd. This aim however proved to be beyond the scope of this thesis in the time permitted but should be encouraged as a project for further study.

1(i) Method of Investigation

An essentially ecological approach has been made to this investigation. Isolation of thermophilic fungal species associated with the colonisation of cotton cellulose was initially carried out using soil as a convenient source of micro-organisms. This work was extended to fungicidally protected cotton textile using the recently developed family of biocides produced by Catomance Ltd., and tensile strength measurement was used as the method of assessing the extent of any biodeterioration. During this ecological work a number of interesting points arose and these were investigated further.

Similar ecological isolation work was carried out with polyurethane film of an ester type before and after hydrolysis, using the complementary techniques of screened substrate soil burial and perfusion colonisation. The biological susceptibility of the polyurethane film exhibited in this isolation work was investigated further using respirometric methods in situations before and after hydrolysis and in the presence of fungicides.

In association with the fungal attack of polyurethanes, rapid assay techniques were sought in order to attempt to correlate biological activity and physical or chemical properties of the polyurethane.

CHAPTER TWO

ECOLOGICAL STUDIES INTO THE BIODETERIORATION OF COTTON TEXTILES AT THERMOPHILIC TEMPERATURES

2(a) Introduction

2(b) Isolation Work using Agar Plate techniques

2(c) Isolation Work using a Screened Substrate Soil Burial Technique

2(d) Isolation Work using a Perfusion Colonisation Technique

2(e) Discussion

2(a) Introduction

Investigations into the efficacy of fungicidal compounds in the protection of materials necessitate ecological studies into any biological attack which may occur on the unprotected material under the specific conditions of interest.

This chapter describes studies into the fungal colonisation of cotton textiles at the thermophilic temperature of 50°C. In chapter one it was discussed that Catomance Ltd., are interested in the evaluation of their new family of biocides under conditions of elevated temperature. Such conditions may occur in tropical countries, and indeed countries with a predominantly temperate climate, due to the phenomenon of insolation. The biocides concerned in this thesis have already been investigated with reference to the protection of cotton textiles at mesophilic temperatures (Allsopp 1973) and it is the aim here to assess if the activity demonstrated at mesophilic temperatures will also apply under conditions of elevated temperature. For this reason the temperature of 50°C was selected for the purposes of this investigation based on the considerations of Eggins and Coursey (1964) and Cooney and Emerson (1964). It is however realised that this is just one specific temperature, and indeed in reality there is likely to be a range of conditions falling into the thermophilic category. Limitations in incubation facilities however necessitated a study at one particular temperature, and 50°C $\pm$ 1° was selected as a convenient level. Under natural conditions, micro-environments may occur where the temperature does indeed reach this level. In addition there may well be micro-environments which reach much higher temperatures

as well as lower levels, leading to a whole range of elevated temperatures where the ambient temperature may be well below such levels.

Fungal ecology with regard to colonisation of materials poses many problems in itself. For example, a fungus which is observed to be growing on a material may simply be existing passively on the material, not causing any deterioration; or alternatively it may be an active deteriogen, utilising the material directly as a nutrient supply.

For this reason, techniques to be used in the study of fungal ecology must be selected with care, and the results obtained should be analysed with equal care if spurious results and conclusions are to be avoided.

The technique which is most frequently employed in microbiological studies is that of growth studies on agar plates. This technique is particularly simple, in which fungi may be isolated from a material undergoing biological attack, and cultured in the laboratory into a pure state, from which identification can be made. Also physiological aspects of fungal ecology may be studied using this technique, by incorporating different nutrients into the agar, and by adjustment of the pH of the medium.

Agar media have been used by many workers while studying the ecology of soil microfungi (Waksman 1916, Chesters 1940, Warcup 1950, Chesters and Thornton 1956) and the agar plate

technique may be modified to be useful in the study of the biodeterioration of cotton textile.

The usual agar media employed in the study of fungi contain simple carbon sources, e.g. potato dextrose and malt extract agars. When studying the biodeterioration of cotton textile, however, the carbon source available to any potential deteriogen is that of cellulose, a much more complex form of carbon. Consequently the use of agar media containing simple sugars as the major carbon source will bear little relation to the situation that would occur in natural habitats, and their use in this particular investigation would selectively isolate the so called 'sugar fungi' rather than those cellulolytic species of importance to the current investigation.

For the agar plate technique to be of most value in the present investigations, an agar medium resembling the material substrate as closely as possible is required. This may be obtained by incorporating into the agar medium inorganic nutrient salts, together with finely divided ball milled cellulose as the major carbon source, (Eggins and Pugh 1962). The ball milled cellulose allows this more complex carbon source to be uniformly dispersed throughout the agar, and as such offers a selective medium for the isolation of cellulolytic fungi.

There are, however, disadvantages in employing agar techniques in studying the fungal colonisation of materials, and these are summarised by Allsopp (1973) and the writer (1973).

The major difficulties are those associated with precision work, maintenance of long term experiments, and the very artificial nature of the medium compared with the material under investigations.

Some of these problems may be overcome by using the material itself in an unmodified form for direct colonisation by possible deteriogenic species.

A technique which accomplishes this, is that of soil burial, where the soil acts as a mixed inoculum of micro-organisms, any one of which, may, under suitable conditions be a potential deteriogen of cotton textile which is buried in it.

The soil burial technique may readily be employed to investigate the biodeterioration of cotton textile by soil micro-fungi, and it offers a situation much nearer the natural conditions, unobtainable when using the agar plate technique.

Soil has been used by many workers as a concentrated source of micro-organisms of a diverse nature, and the use of the soil burial techniques offers a severe accelerated biological screening test for materials under natural conditions.

The soil burial technique also has certain disadvantages, however, when considering ecological aspects and testing procedures. Straightforward burial of the cotton textile in a container of soil, followed by removal after a predetermined period and plating out onto agar plates to enable identification to be carried out,

as is the usual practice in such tests, results in the isolation not only of the actively cellulolytic fungi, but also those fungi which are merely passively present on the soil particles and which do not in fact cause any biodeterioration of the cotton textile.

An attempt to overcome this problem was suggested by Eggins and Lloyd (1968) who screened cellulosic substrates from this gross contamination by soil particles with glass fibre woven ribbon which is biologically inert. This prevents the adhering soil particles causing contamination of the substrate, while allowing the fungal species directly responsible for the cellulolytic breakdown of the cotton textile to penetrate through the weave of the glass fibre fabric and colonise the cellulosic substrate. In this way, when the material is removed from the soil, and plated out onto agar plates, the fungi not responsible for the biodeterioration will interfere to a much lesser extent with the isolation of the true deteriogens.

Another slight disadvantage is that there is no constant supply of nutrients to the material. The only nutrients present would be those remaining if the textile had been soaked prior to burial, and those occurring in the soil. Usually any nutrients will rapidly diffuse from the substrate into the surrounding soil. There is also no removal of waste microbial metabolic products excreted by the fungal colonisers, any of which may inhibit the growth and development of other species which could otherwise attack the material. This is important, since leaching is a process which can commonly occur in the natural habitat, and this will influence biological colonisation.

An improvement on this technique is the addition of a constant nutrient supply to the substrate, by means of a wick from an ancilliary nutrient reservoir to the material, and then away from the site of colonisation.

In this way a model system of what actually occurs in natural habitats is approached.

The modification above led to the development of perfusion colonisation techniques (Eggins, Malik and Sharp 1968) which approach even closer the conditions encountered in natural habitats. This system, offers maintained, standard, predetermined micro-environments and consists basically of a slow controlled perfusion of supplementary nutrients along a woven glass fibre material by capillarity (Plate 2a). The nutrients pass from a reservoir to the material substrate and then away from the site of attack, by means of a tail wick which is open to the atmosphere. Evaporation from this tail wick produces the 'pull' required to maintain the slow capillary flow. In this way there is a continuous supply of fresh ancilliary nutrients to the substrate, and stale nutrients and undesirable waste metabolic products are removed from the site of biodeterioration. Cellulosic materials may be incorporated into the perfusion chain, whilst other non-porous materials must be laid on top of the glass fibre wick (as described for polyurethanes in chapter 5).

This technique provides a useful research tool as it is possible to maintain constant micro-environments which are unobtainable when using the usual microbiological cultural methods.

Plate 2(a)

Photograph of Perfusion system used in the investigation

The agar plate for example not only becomes depleted of nutrients as the fungi grow, but metabolites build up and are not removed, while there is also an aeration gradient through the layer of agar. These problems are not however encountered using the perfusion technique, which is versatile in that it may be employed as an accelerated test for the susceptibility of materials to biological attack, and for nutritional and other physiological experiments which require maintenance under controlled conditions for long periods of time.

One disadvantage of this technique however is the length of time involved in setting the apparatus up initially from the point of view of a routine technique.

The three techniques outlined and discussed in the preceding paragraphs of this chapter, have been used to make ecological studies into the biodeterioration of cotton textiles at thermophilic temperatures.

As a convenient source of a diverse range of microfungi, a soil was used during these investigations which was known to have had no artificial fertiliser treatment for a long period of time. The particular soil used was a meadowland top soil which had previously been the subject of microbiological examinations, (Malik and Eggins 1969, 1970, and Allsopp 1973). This soil was obtained from the top 6 - 8 inches from a field at Clent in Worcestershire, England.

A large quantity of soil was collected and sieved to produce a more uniform particle size. The sieved soil was then maintained

at 20° - 25°C, and covered with polythene sheet to prevent drying out. A quantity of this soil which was to be used during experimentation at the elevated temperature of 50°C was maintained at this particular temperature in the same way. In both cases the soil was turned over at regular intervals.

2(b) Isolation Studies using Agar Plate Techniques

This experiment was carried out in order to give a qualitative picture of those thermophilic fungal species which may be important in the biodeterioration of cotton textile and which occur in the soil to be used in further experimentation.

The two agar plate techniques employed in this initial isolation work, were those of Waksman (1916) and Warcup (1950). The Waksman technique would offer information concerning the rapidly growing fungi, while the Warcup technique was expected to offer additional information concerning the slower growing species which would probably include cellulolytic fungal species important to the present investigation.

The agar medium used in both these techniques was that formulated by Eggins and Pugh (1962) for the isolation of soil microfungi, and contains inorganic nutrients, and either finely divided cellulose or glucose and starch as the major carbon sources. The initial pH of this medium is 6.5.

In the Waksman technique a few particles of sieved soil were sprinkled onto the surface of the sterile agar plates, and the plates were incubated at 50°C. Observations were then

made at regular intervals. In this way, those fungi having mycelial fragments in the soil particles can be selectively isolated preferentially to those fungi which are present in the soil as spores only, or mycelia with a much slower rate of growth, the existence of which may be masked.

The Warcup technique, on the other hand, allows the slower growing species to grow more competitively with the more rapidly growing fungi. This is achieved by placing the soil particles in a petri dish prior to pouring the cooled, sterile, liquid agar on top, ensuring the soil particles are completely immersed in the liquid agar, prior to gelling.

An oxygen gradient is thus set up through the layer of agar which allows the slower growing species to compete more easily, overcoming the problem of the fast growing species overgrowing those with slower growth rates.

In both techniques, incubation was carried out at 50^{o}C for periods of twelve days. Twenty cellulose agar plates and twenty glucose/starch agar plates were inoculated with soil particles, and the percentage frequency of occurrence of the thermophilic fungal species was determined by recording their appearances on the agar plates, at intervals during incubation.

The results obtained using these two agar plate techniques to make initial isolations are detailed in tables 2(i) and 2(ii) where the figures represent the percentage frequency of occurrence. The identification of the fungi isolated and detailed in these tables was carried out using the diagnostic key to the thermophilic fungi of Cooney and Emerson (1964).

TABLE 2(i)

Fungi isolated using the Waksman Agar Plate Technique with incubation at 50°C

(figures refer to percentage frequency of occurrence)

Fungal Species Isolated at 50°C	Cellulose Agar			Glucose Starch Agar		
	4 days	7 days	12 days	4 days	7 days	12 days
Mucor pusillus (Lindt)	20	30	30	50	80	80
Penicillium dupontii (Griffon & Maublanc)	-	35	40	20	70	75
Thermoascus aurantiacus (Miehe)	20	20	20	10	30	30
Chaetomium thermophile (La Touche)	-	5	20	-	-	-
Aspergillus fumigatus (Miehe)	80	100	100	40	100	100
Humicola grisea (Traaen)	-	-	-	-	-	-
Humicola lanuginosa (Griffon & Maublanc)	-	-	5	-	20	20
Sporotrichum thermophile (Apinis)	-	5	25	-	-	10
Actinomycetes spp.	-	-	-	-	-	-

TABLE 2(ii)

Fungi isolated using the Warcup Agar Plate Technique with incubation at 50°C

(figures refer to percentage frequency of occurrence)

Fungal Species Isolated at 50°C	Cellulose Agar			Glucose Starch Agar		
	4 days	7 days	12 days	4 days	7 days	12 days
Mucor pusillus	-	5	10	20	40	40
Penicillium dupontii	10	10	20	20	50	70
Thermoascus aurantiacus	20	40	40	20	20	30
Chaetomium thermophile	20	20	60	-	-	-
Aspergillus fumigatus	40	100	100	100	100	100
Humicola grisea	10	20	20	-	20	50
Humicola lanuginosa	-	5	20	10	30	30
Sporotrichum thermophile	5	10	30	5	30	30
Actinomycetes spp.	5	40	80	20	20	20

From table 2(i) and 2(ii) it can be seen that the expected difference in colonisation patterns between the two techniques was obtained. The more rapidly growing fungi being isolated more frequently using the Waksman technique, such as T. aurantiacus, M. pusillus, A. fumigatus and P. dupontii, while the fungi with slower growth rates, such as Ch. thermophile, H. grisea, H. lanuginosa and Actinomycetes spp. were isolated with greater frequency using the Warcup technique. The growth of those fungi isolated using the Waksman technique was such that it proved difficult to examine the plates for other species, some of which may have occurred but their presence had been masked by the more rapidly growing species. This is demonstrated by the fact that when using the Warcup technique a greater variety of fungal species was isolated from the same soil. In this technique the rapidly growing fungi had been limited due to the oxygen gradient through the agar, thus enabling the slower growing species to compete more easily.

The most frequently isolated species was A. fumigatus, a fungus declared as being only thermotolerant by Cooney and Emerson (1964), rather than a true thermophile. However, for the purposes of this investigation, any fungus capable of growth at elevated temperatures and of a cellulolytic nature is of obvious commercial importance when considering the biodeterioration of cotton textiles. The frequency of isolation of this particular species is to be noted when considering further experimentation, and it was isolated on both cellulose and glucose/starch agar media. Species isolated frequently on cellulose, and showing varying degrees of cellulolytic activity by the clearing occurring

in the cellulose around the colonies, were Ch. thermophile, S. thermophile, T. aurantiacus, H. grisea and Actonomycetes spp.; plus A. fumigatus itself which displayed extensive cellulolytic activity.

It is also noted from these results that certain species not normally recognised as cellulolytic were isolated on cellulose agar. These species were probably growing on the simple sugars which are produced on the breakdown of cellulose by the cellulolytic species, and also by the action of autoclaving the agar media initially which may denature some of the cellulose. There will also be a certain quantity of nutrient material of a simple nature present in the soil particles which were used as an inoculum.

The fact that T. aurantiacus produced very slight clearing in the cellulose agar immediately surrounding the colonies was of particular interest since this species has normally been thought to be non-cellulolytic. It has however been shown to produce resin-decomposing enzymes (Cooney and Emerson 1964).

This fungus was isolated into pure culture in order to investigate this activity further but in further experiments using cellulose agar media the apparent activity was not observed. The conclusion to be drawn, is that there must have been interaction between this and a cellulolytic species when isolation was carried out using the agar plate techniques.

2(c) Isolation Studies Using a Screened Substrate Soil Burial Technique

Following initial isolation work using the agar plate techniques, which incorporate finely divided cellulose into an agar medium, isolation using the cotton textile itself as an unmodified substrate for microbial colonisation at 50°C was carried out.

The technique employed in this experiment was that described by Eggins and Lloyd (1968), the principles of which have been outlined in the introduction to this chapter, and it was performed in order to demonstrate if those organisms isolated under the rather artificial conditions of the agar plate techniques were of equal importance in the colonisation of cotton textile under conditions more nearly approaching those of natural habitats.

The cellulosic substrate used in this, and in subsequent experiments in this investigation, was supplied by Catomance Ltd. This substrate was a cotton textile in the form of standardised fabric for test purposes, produced by the Textile Institute, T.N.O. in Holland. This was known to have had no fungicidal protective treatment.

The experiment was followed as described by Eggins and Lloyd, with the modification that the cotton textile referred to above was used as the cellulosic substrate in the place of the chromatography paper used by the authors of this technique.

The samples of cotton textile used in this experiment measured approximately 7 cm in length and 3 cm in width.

The textile samples were placed against the external surface of boiling tubes, and covered with glass fibre woven ribbon which was held in place by means of glass fibre adhesive tape. Both of these materials are biologically inert and are able to withstand sterilisation by autoclaving.

Care was taken to ensure that the substrate was totally screened by the glass fibre ribbon, and the tubes were then sterilised, by autoclaving at 15 lbs per square inch for 15 minutes, prior to burial in soil. Following autoclaving, the tubes were allowed to cool to room temperature before burial was carried out.

Soil burial was performed in troughs containing previously sieved soil which had been maintained at 50°C, and which contained approximately 25% water by weight. The screened substrate isolation tubes were buried to a depth of 2 - 3 inches in this soil and the troughs were covered with polythene sheet to prevent loss of moisture by evaporation and incubated at 50°C in an incubator in which a high relative humidity was maintained throughout the incubation period.

The incubation period was fourteen days, with sacrifices being made after four, seven, ten and fourteen days. At each sacrifice, five tubes were removed from the soil, and the textile samples were carefully removed from the tubes, following the

usual aseptic techniques to prevent contamination of the cotton textile by soil particles adhering to the glass fibre materials.

Each of the five textile samples was then cut into four approximately equal parts, and these were plated out onto both cellulose and glucose/starch agar (two portions of each sample on each type of agar). Again aseptic technique was strictly adhered to during this operation to avoid spurious results due to contamination. The agar plates were then incubated at 50°C and observations were made after a further four days, in which identification and isolation of those fungi colonising the cotton textile were made.

The species isolated using this technique are listed in table 2(iii) overleaf.

From table 2(iii) it can be seen that A. fumigatus again was the most frequently identified species when the cotton textile was plated out onto both cellulose and glucose/starch agars. Also some of the non-cellulytic species were again isolated on cellulose agar, utilising the breakdown products of the cellulose attacked by those cellulolytic species. Another point to be seen from the results of this experiment is that the non-cellulolytic fungi were isolated more frequently towards the end of the incubation period, suggesting further that they are in fact secondary colonisers, moving in to utilise the simple sugars resulting from the cellulase activity of the primary colonising species.

TABLE 2(iii)

Fungi Isolated by the Screened Substrate Soil Burial Technique using Cotton Textile at 50°C

(figures refer to the number of agar plates on which the fungi were identified, Ten plates of each agar were used)

Fungal Species Isolated at 50°C	Cellulose Agar				Glucose Starch Agar			
	4 days	7 days	10 days	14 days	4 days	7 days	10 days	14 days
Mucor pusillus	2	3	1	-	6	7	7	8
Penicillium dupontii	-	2	3	2	-	4	3	6
Thermoascus aurantiacus	-	-	2	4	3	4	5	4
Chaetomium thermophile	-	2	6	5	-	-	2	-
Aspergillus fumigatus	6	10	10	10	10	10	10	10
Humicola lanuginosa	-	1	1	2	-	2	3	5
Sporotrichum thermophile	-	2	3	2	-	-	-	-
Actinomycetes spp.	1	6	7	10	-	2	-	1

As would be expected, the thermophilic cellulolytic species were not isolated with such frequency on glucose/starch agar as on cellulose agar and there is a lag before colonisation of the textile occurs due to the biologically inert screen which must be penetrated prior to colonisation.

Another point of interest is that the range of thermophilic fungi isolated in this experiment is less than in the previous techniques, with the truly cellulolytic species occurring to a greater extent.

The results from these soil burial isolation studies underline the importance of thermophilic fungi in the possible biodeterioration of cotton textile at elevated temperatures, their occurrence being quite widespread in the soil used in this investigation. If these cellulolytic species occur in the soil their spora will undoubtedly occur in the atmosphere and as such may well fall onto any cotton textile article, especially under industrial usage, and under suitable conditions may colonise and cause deterioration of the textile.

2(d) Isolation Studies using a Perfusion Colonisation Technique

The isolation work carried out using cotton textile as the cellulosic substrate for fungal colonisation, was extended to a model system offering more easily controlled standardised environments which would more closely resemble the events encountered in nature.

The technique used in this experiment was that of perfusion colonisation described by Eggins, Malik and Sharp (1968), the

principles of which have been discussed in the introduction to this chapter.

The object of this experiment was to evaluate those thermophilic fungi occurring in soil which may be important in the biodeterioration of cotton textiles at elevated temperatures, under controlled conditions and circumstances approaching those which occur in nature.

The technique was carried out essentially in the same manner as detailed by the above workers. The cellulose substrate was again cotton textile test cloth previously referred to (page 53). The dimensions of the textile samples used in this experiment were 7 cm in length, and ten warp threads in width, with a ravel on both sides of the sample. It was found that this size of sample was convenient for use in this technique as they could be easily incorporated into the perfusion chain, and were not too long to be contained flat in the base of the petri dishes used in the technique. These sample specifications were maintained in subsequent studies as they could be easily and accurately duplicated, thus allowing quantitative evaluation of the biodeterioration of unprotected and fungicidally protected cotton textile at 50^{o}C, as described in Chapter 3 in conjunction with which, this experiment was carried out.

The cotton test cloth used in this experiment was untreated with any fungicide, as in the previous technique, and the nutrient reservoir contained the medium described by Eggins and Pugh (1962), for the isolation of soil microfungi, with the omission of cellulose, glucose and agar.

In order to duplicate the results, sets of perfusion kits were prepared as shown in plate 2(a) earlier in this chapter. Upon completion, the perfusion kits were autoclaved at 15 lbs/sq. in. for 15 mins. to sterilise the substrate and nutrients in the reservoir.

Inoculation was by a small quantity of soil, which was screened from direct contact with the cotton textile by means of a piece of glass fibre woven ribbon laid on top of the cellulosic substrate. The soil was then placed on this biologically inert screen.

The kits were then incubated at 50°C under conditions of high humidity, and sacrifices were made at intervals over a three week period. At each sacrifice, five samples were each cut up, taking the usual aseptic precautions, into four approximately equal sections. Two of these sections were plated onto cellulose agar, and two onto glucose/starch agar, making a total of ten plates of each agar medium at each sacrifice. These plates were then incubated for a further two to four days at 50°C prior to exmination and identification of the fungi colonising the textile.

The results of this perfusion isolation experiment are detailed in table 2(iv).

From these results, again A. fumigatus occurs with most frequency, as occurred in the previous technique used in this chapter. The range of fungal isolates was also somewhat

TABLE 2(iv)

Fungi Isolated from Cotton Textile at 50°C using a soil inoculum and a perfusion colonisation technique

(figures refer to the number of agar plates on which the species were identified. There was a total of ten plates of each agar medium).

Fungal Species Isolated at 50°C	Cellulose Agar				Glucose Starch Agar			
	4 days	7 days	14 days	21 days	4 days	7 days	14 days	21 days
Mucor pusillus	-	1	-	2	1	3	4	2
Penicillium dupontii	1	-	2	2	-	1	3	4
Thermoascus aurantiacus	2	1	-	1	1	2	3	1
Chaetomium thermophile	-	3	4	6	-	-	1	2
Aspergillus fumigatus	4	10	10	10	6	10	10	10
Humicola lanuginosa	-	-	1	3	2	3	4	3
Actinomycetes spp.	-	4	5	8	-	1	-	-

reduced when using the perfusion colonisation technique, only A. fumigatus, Ch. thermophile and Actinomycetes spp. of the cellulolytic fungi occurring with any frequency when plated out onto cellulose agar. Again some non-cellulolytic species appear on both glucose/starch and cellulose agar.

2(e) Discussion

In this chapter, the occurrence and activity of thermophilic fungi in soil has been qualitatively evaluated, with particular reference to the cellulolytic species which may be important in the biodeterioration of cotton textiles.

The complementary techniques employed in this initial isolation work have offered an overall picture of fungal colonisation of a cellulose substrate.

The results obtained from isolation work using the agar plate techniques are in approximate agreement with the work performed by Eggins and Malik (1969) on similar soil. These workers however, using soil enrichment methods, isolated thirteen thermophilic species, while in the initial study to the present investigation, only nine thermophilic fungi were isolated from unamended meadowland topsoil. Of these nine, five displayed fairly extensive cellulolytic activity, and as such must be of importance when considering biodeterioration of cotton cellulose at elevated temperatures, if the cotton is not protected in some way.

The most frequently isolated fungal species was A. fumigatus which is not only able to grow at 50°C but also at temperatures much lower, and although classed as thermotolerant, may be particularly important in textile deterioration, especially under conditions of fluctuating temperatures which are more likely to predominate in the natural environment.

The Actinomycete species isolated also displayed quite marked cellulolytic activity, and it was noted with particular interest that this activity was quite extensive from very small mycelial masses. There was however a tendency for these species to be overgrown by the other fungi.

During the isolation work performed in this chapter, species not normally recognised as being cellulolytic were continually isolated as secondary colonisers, utilising the breakdown products of the cellulose brought about by the primary cellulolytic colonisers, This phenomenon may in fact, stimulate further cellulase activity on the part of the primary colonisers. The secondary colonisers will remove from the site of attack the simple carbon sources which they are able to utilise and this may enhance activity by the cellulolytic species with the result of more rapid deterioration of the cellulosic material of cotton textile.

From the results obtained in this chapter, it can be concluded that the thermophilic fungi display a wide distribution in this soil and under suitable conditions will be important in the biodeterioration of cotton textile at elevated temperatures.

CHAPTER THREE

ECOLOGICAL STUDIES USING BIOCIDALLY PROTECTED COTTON TEXTILE AT THERMOPHILIC TEMPERATURES

3(a) Introduction

3(b) Isolation Work using an Agar technique

3(c) Isolation Work using a Screened Substrate Soil Burial Technique

3(d) Isolation work using a Perfusion Technique

3(e) Evaluation of the degree of Biodeterioration of cotton textile at Thermophilic Temperatures

3(f) Discussion and Conclusions

3(a) Introduction

In the work of the previous chapter, an initial qualitative evaluation was made of those thermophilic fungi occurring in soil which may be important in the biodeterioration of cotton textiles. This work was extended to investigate the fungal colonisation of fungicidally protected cotton textile, and biodeterioration was quantified using the perfusion technique described in chapter two, together with tensile strength measurement.

The fungicides studied in this investigation were produced by Catomance Ltd., and included three recently developed products not yet currently commercially available, and one established product (Mystox LPL).

These four fungicides were:-

1. Mystox LPL - (PCPL) an established fungicide commercially available, being composed of a range of fatty acid esters of pentachlorophenol of which the lauryl ester predominates.

2. The ammonium sulphate compound of orthophenyl phenoxy isopropanol (Code name P_1 during this investigation).

3. Orthophenyl phenoxy isopropanol (Code name P_2 during this investigation).

4. A mixture of pentachloro phenoxy isopropanol and trichloro phenoxy isopropanol in the ratio of 70:30 (Code name P_3 in this investigation).

Chemical agents may in fact be fungicidal and/or fungistatic, and the distinction between these may often be a matter of degree of concentration and/or duration of exposure. Fungistasis is that action which is the less severe, and may be reversed, while an irreversible action constitutes the fungicidal effect. The chemical agents can be applied as eradicants or as protectants, the latter being applied to a material before any inoculum reaches the site of potential attack, often only functioning upon the germination of fungal spores. The eradicant on the other hand actually kills fungal growth already occurring on or in the material.

The lethal action of chemicals is dependent on the concentration of the compound, or a specific ion, and the time of exposure. Various fungal species exhibit great variations in ability to resist the action of certain fungicides. For example, many fungi are killed by exposure to only a few parts per million of some chemicals such as cupric ions, while a few species have been reported to grow in saturated solutions of copper sulphate. (Starkey and Waksman 1943).

The point to be made here is that there is no useful universal fungicide.

The choice of a particular fungicide depends on numerous factors, the major ones being the type of organisms to be controlled, and the nature of the material to be protected. Another important factor is the solubility of the fungicide. The most efficient preservation of cellulosic materials, such as wood and cotton textile for example, is achieved by

fungicides of low solubility, in order to extend protection over as long a period as possible, while surface sterilisation by chemical means is usually carried out by the use of highly soluble compounds. The relative sensitivity of man and fungi towards the fungicide must also be taken into account, and this limits many potentially aggressive fungicides, since they are also harmful to man or his environment.

In the past, the most widely used inorganic fungicides have contained compounds of copper, arsenic, mercury and tin, many of which are now socially unacceptable due to their substantivity and toxicity to man and natural environments. At the present time, and in the future, many fungicides of an organic nature will be increasingly used, as these offer a nearly infinite number of possible combinations.

In controlling fungal activity, there is always a possibility that organisms become tolerant of a particular compound. This means that a greater concentration of fungicide may be required to offer adequate protection, which will be much more expensive; or alternatively new compounds must be used to which the fungi have not had a chance to build up resistance. This is why it is desirable to have new fungicides in reserve, and why there is a continual search by industry to find suitable formulations which possess fungicidal activity.

The compounds used in this particular investigation are the result of such chemical research by Catomance Ltd., who are interested in the production of organic fungicides and bactericides.

The four fungicides of this present study in fact offer a family of products which may be chemically modified to offer protection under specific conditions for particular materials. In this way fungicides may possibly, in the future, be tailor made for specific purposes. This chemical modification of organic compounds is almost unlimited, which is an advantage when considering that organic fungicides tend to exhibit greater specificity than those of an inorganic nature.

In this chapter the fungal colonisation of fungicidally protected cellulose is investigated using an agar plate, screened substrate soil burial, and perfusion techniques, and the extent of any biodeterioration is quantified using tensile strength measurement.

3(b) Isolation Work using an Agar Plate Technique

The technique used in this experiment was a modification of the Warcup plate technique, with ball milled cellulose and fungicide solutions incorporated into the agar medium.

Eggins and Pugh cellulose agar was prepared in twelve flasks, each containing 200 mls of media. This was sterilised by autoclaving for 15 mins at 15 lbs per square inch, and the sterile agar was allowed to cool prior to the addition of the fungicides, which were added to give final concentrations of 1.0%, 2.0% and 4.0% by volume. The flasks were then shaken vigorously to ensure the fungicides were dispersed throughout the agar which was then poured on to soil particles in petri dishes. After setting, the agar plates were incubated at 50°C. There was a

total of twenty cellulose agar plates per fungicide concentration.

The incubation period was over fourteen days, after which time the agar plates were removed for observation, and identification of any fungi colonising the agar.

The results are detailed in tables 3(i) (ii) (iii) and (iv) and the figures in these tables represent the number of agar plates (out of a total of 20) on which the species were identified.

From the results of this experiment, it can be seen that a number of thermophilic species of fungi appear capable of colonisation of a cellulosic substrate which is protected by the four fungicides under study.

Aspergillus fumigatus again appears as the most frequently isolated species with each of the fungicides. The occurrence of Actinomycete species may also be of significance in any biodeterioration of cotton cellulose at elevated temperatures. Under conditions of quite high concentrations of fungicides there is a distinct reduction in the range and numbers of fungi identified, which is accompanied by an increase in the occurrence of Actinomycete species and bacterial colonies. This may be due to a decrease in competition, by removal of those fungi which would normally overgrow the much slower growing Actinomycetes, which appear to be able to tolerate the more adverse conditions and continue growth.

TABLE 3(i)

Fungi Isolated on Cellulose Agar Incorporating PCPL at 50°C

Fungi Isolated	Concentration of fungicide		
	1%	2%	4%
A. fumigatus	18	12	10
Ch. thermophile	5	2	-
H. grisea	3	2	1
H. lanuginosa	5	3	-
Actinomycetes spp.	8	14	18
P. dupontii	4	-	-
S. thermophile	1	2	-
T. aurantiacus	-	1	-
Bacterial colonies	12	13	17

TABLE 3(ii)

Fungi Isolated on Cellulose Agar Incorporating P_1 fungicide, at 50°C

Fungi Isolated	Concentration of fungicide		
	1%	2%	4%
A. fumigatus	20	18	20
M. pusillus	8	2	-
Actinomycetes spp.	6	17	20
Ch. thermophile	9	-	-
Bacterial colonies	4	12	18

(Figures in the tables represent numbers of agar plates, in a total of 20, on which the species were identified).

TABLE 3(iii)

Fungi Isolated on Cellulose Agar Incorporating P_2 fungicide at 50°C

Fungi Isolated	Concentration of fungicide		
	1%	2%	4%
A. fumigatus	20	17	19
S. thermophile	6	10	2
Actinomycetes spp.	2	12	15
H. grisea	3	-	2
P. dupontii	3	2	-
Ch. thermophile	8	4	-
M. pusillus	4	1	-
H. lanuginosa	1	-	-
Bacterial colones	2	13	18

TABLE 3(iv)

Fungi Isolated on Cellulose Agar Incorporating P_3 fungicide at 50°C

Fungi Isolated	Concentration of fungicide		
	1%	2%	4%
A. fumigatus	18	15	20
H. grisea	8	5	-
H. lanuginosa	3	1	-
Actinomycetes spp.	3	14	16
T. aurantiacus	3	2	-
Ch. thermophile	9	3	-
M. pusillus	2	-	2
Bacterial colonies	2	12	17

(Figures in the tables represent numbers of agar plates in a total of 20, on which the species were identified).

There was also an expected reduction in the number of fungal species identified, compared with those isolated from unprotected cellulose as described in chapter 2.

During the course of this experiment it was noted that in certain cases, the fungicides had coagulated out of the agar phase and into droplets which had fallen to the base of the petri dish prior to the completion of the gelling of the medium. This phenomenon may have affected this isolation work, in that fungi may have been isolated on plates where the fungicide concentration throughout the agar had been effectively reduced to below the levels detailed in the tables of results due to this droplet formation.

The possibility of spurious results using this technique may be demonstrated further, when considering the solubility of the fungicides. In fact only P_1 is water soluble, and as such, would be expected to be dispersed evenly throughout the agar. This is further demonstrated by the fact that there is a notable decrease in the number of different fungal species isolated when P_1 was incorporated into the agar, compared with the other fungicides.

Consequently the results obtained using this particular technique must be viewed with caution. However the general trends of increased actinomycete occurrence with the onset of more adverse conditions, together with the apparent tolerance or resistance of A. fumigatus, to all of the four fungicides, is worthy of particular note. This is important when one considers that these species display quite extensive cellulolytic activity.

3(c) Isolation Work using a Screened Substrate Soil Burial Technique

The isolation work using the agar plate technique described in 3(b) had indicated that the results may be somewhat spurious in the number of resistant species identified, and in order to gain more meaningful results, this isolation work was extended by using cotton textile test cloth which had been fungicidally protected with the same four fungicides by Catomance Ltd.

The technique employed in this experiment was essentially the same as that described in chapter 2(c), with the exception that the cotton textile cellulosic substrate had been impregnated with the four fungicides in the following manner:-

1. 1%, 2% and 4% loadings of Mystox LPL, applied from white spirit (PCPL).

2. 1%, 2% and 4% loadings of the ammonium sulphate compound of orthophenyl phenoxy isopropanol, applied from water (P_1).

3. 1%, 2% and 4% loadings of orthophenyl phenoxy isopropanol, applied from 'Imsol A'/water solution in the ratio of 2:1 (P_2).

4. 1%, 2% and 4% loadings of pentachloro phenoxy isopropanol/ trichloro phenoxy isopropanol, 70:30 mixture, applied from 'Imsol A'/water solution in the ratio of 2:1 (P_3).

The cotton textile samples were removed from the soil after an incubation period of fourteen days, and were cut up to be plated out on to cellulose agar in order to carry out fungal

identification and isolation. Again, five samples of each fungicidally protected textile were cut up into four approximately equal parts prior to plating out, giving a total of 20 cellulose agar plates per fungicidal concentration.

The results are shown in tables 3(v) (vi) (vii) (viii).

From these results it can be seen that the same trend occurs as was indicated in the agar plate technique when the fungicides were incorporated into the agar. A. fumigatus, Ch. thermophile and Actinomycete spp. appear in the case of all the fungicides to be the most frequently isolated species, and these are known to exhibit strong cellulolytic activity. There is also a distinct decrease in the number of different species isolated compared with those isolated using the agar plate technique. This reinforces the suspicions that the results obtained from the agar plate technique were not a true reflection of the activity of the fungicides, due to the droplet formation by the less soluble products already referred to in that section (3a).

A definite pattern is however shown to be developing in that the same three thermophilic species appear to be resistant to, or at least tolerant of, the four fungicides under investigation. Other species were isolated during the experiment but only occasionally, with no regular pattern, and at the higher concentrations of fungicides the Actinomycete population appeared to be able to tolerate the more adverse conditions to a greater extent than A. fumigatus. This may be possibly due to a reduction in competition from other species in addition to any tolerance or resistance to the fungicides.

TABLE 3(v)

Fungi Isolated at 50°C using the Screened Substrate Soil Burial Technique and PCPL protected Cotton Textile

Fungi Isolated	Concentration of fungicide		
	1%	2%	4%
A. fumigatus	17	18	16
Ch. thermophile	8	5	7
Actinomycete spp.	3	10	12
H. lenuginosa	2	-	-
P. dupontii	4	-	-
S. thermophile	1	-	-
Bacterial colonies	-	11	14

TABLE 3(vi)

Fungi Isolated at 50°C using the Screened Substrate Soil Burial Technique and P_1 protected Cotton Textile

Fungi Isolated	Concentration of fungicide		
	1%	2%	4%
A. fumigatus	18	20	17
Ch. thermophile	6	-	-
Actinomycete spp.	7	12	12
M. pusillus	4	-	-
Bacterial colonies	4	10	9

(Figures in the tables represent numbers of agar plates in a total of 20, on which the species were identified).

TABLE 3(vii)

Fungi Isolated at 50° using the Screened Substrate Soil Burial Technique and P_2 protected Cotton Textile

Fungi Isolated	Concentration of fungicide		
	1%	2%	4%
A. fumigatus	16	20	18
Ch. thermophile	8	2	3
H. grisea	3	1	-
Actinomycete spp.	5	11	14
S. thermophile	2	-	-
H. lanuginosa	2	-	-
Bacterial colonies	4	10	13

TABLE 3(viii)

Fungi Isolated at 50°C using Screened Substrate Soil Burial Technique and P_3 protected Cotton Textile

Fungi Isolated	Concentration of fungicide		
	1%	2%	4%
A. fumigatus	12	16	17
Actinomycete spp.	6	10	18
Ch. thermophile	8	2	4
M. pusillus	2	-	-
Bacterial colonies	-	4	12

(The figures in the above tables refer to the number of cellulose agar plates on which the species were identified out of a total of 20 for each fungicide concentration).

3(d) Isolation Studies Using the Perfusion Technique

This work was carried out in conjunction with the quantitative assessment of the biodeterioration of cotton textiles at 50°C detailed in the following section (3e), and employed the perfusion technique already described and discussed in chapter two.

The same four fungicides, at the loadings of 1%, 2% and 4%, based on the dry weight of textile to be protected had previously been applied to cotton textile test cloth by Catomance Ltd. This textile was cut into samples measuring 7 cm in length and ten warp threads in width, with a ravel on both sides of the sample to prevent fraying. These samples were incorporated into the perfusion assemblies, as the cellulosic substrate, and the nutrient salt solution contained in the reservoir was the basic media of Eggins and Pugh (1962) with the omission of any major carbon source and agar, and the reduction by half of yeast extract and L-asparagine, both of which contain carbon and may interfere with the true colonisation patterns of fungicidally protected cotton cellulose.

A total of 60 samples were incorporated into the perfusion assemblies for each fungicide concentration (in addition to a similar number of untreated cotton textile samples as controls). These samples were divided into four kits each of 15 samples. Sacrifices were made after four, seven, ten and fourteen days. Of each fifteen samples to be sacrificed, ten were used for tensile strength measurement, as described in the following section (3e), and five were removed, cut into four equal parts and plated out onto cellulose and glucose/starch agar, making a total of ten plates per agar media per fungicide concentration.

Sterilisation prior to the commencement of the experiment was carried out at 15 lbs per square inch for 20 minutes, after which time the kits were allowed to cool to room temperature. Inoculation was carried out, upon cooling, using a small quantity of soil, placed on a piece of fibre glass ribbon which acted as a screen against gross contamination of the textile sample from the soil particles, while simultaneously allowing actively growing fungi to penetrate and colonise the cotton cellulose. Incubation was at 50^{o}C under conditions of high relative humidity.

By carrying out this work in conjunction with the quantitative evaluation of the biodeterioration of cotton textile, those species directly associated with such biodeterioration could be isolated and identified.

After incubation intervals of four, seven, ten and fourteen days, one kit was removed for each fungicidal treatment, and observing the usual aseptic techniques, five of the samples were each cut into four equal pieces and plated out onto previously sterilised cellulose and glucose/starch agar. These were then incubated for a further four days to allow sporulation to occur prior to observation and identification.

The results are detailed in tables 3(ix), (x), (xi) (xii) and the figures in the tables refer to the number of plates on which the species were identified. Glucose/starch agar medium was used to selectively isolate the non-cellulolytic species which would probably be secondary colonisers, utilising the cellulose breakdown products produced by the activity of the primary cellulolytic species. The symbols C ≡ cellulose agar and G/S ≡ glucose/starch agar.

TABLE 3(ix)

Ecological Studies using PCPL Protected Cotton Textile and the Perfusion Technique at 50°C

(C ≡ Cellulose Agar, G/S ≡ Glucose/Starch Agar. Figures refer to number of agar plates on which the species were observed out of a total of ten in each case)

Fungal Species Isolated	1% Fungicide loading								2% Fungicide loading								4% Fungicide loading							
	4 days		7 days		10 days		14 days		4 days		7 days		10 days		14 days		4 days		7 days		10 days		14 days	
	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S
A. fumigatus	4	6	10	10	10	10	10	10	6	8	10	10	10	10	10	10	8	6	9	10	10	10	8	9
Actinomycete spp.	4	3	8	6	8	1	7	4	4	4	10	10	9	5	6	-	5	3	8	6	10	8	8	6
P. dupontii	-	4	-	6	2	3	-	1	-	-	2	3	-	-	-	-	-	2	-	-	-	-	-	-
Ch. thermophile	3	-	6	3	8	8	3	1	4	-	2	-	3	-	-	-	-	-	-	-	-	-	-	-
H. lanuginosa	2	2	-	2	1	6	-	-	-	-	2	3	-	-	-	-	-	-	-	-	-	-	-	-
H. grisea	4	-	2	-	-	-	-	-	1	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-
S. thermophile	-	-	2	-	3	-	2	-	3	1	-	-	2	-	-	-	-	-	-	-	-	-	-	-
T. aurantiacus	-	4	-	3	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	2	-	-
Bacterial colonies	4	2	2	6	4	2	3	2	5	-	5	2	7	4	9	6	6	4	7	2	10	5	9	3

TABLE 3(x)

Ecological Studies using P_1 Protected Cotton Textile and the Perfusion Technique at 50°C

(C ≡ Cellulose Agar, G/S ≡ Glucose/Starch Agar. Figures refer to number of agar plates on which the species were observed out of a total of ten in each case)

Fungal Species Isolated	1% Fungicide loading								2% Fungicide loading								4% Fungicide loading							
	4 days		7 days		10 days		14 days		4 days		7 days		10 days		14 days		4 days		7 days		10 days		14 days	
	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S
A. fumigatus	4	4	10	10	10	10	10	10	4	5	8	8	10	10	10	10	2	3	7	6	8	8	9	8
Ch. thermophile	2	-	3	1	1	2	4	2	-	-	4	1	5	-	-	-	-	-	2	1	3	-	2	-
Actinomycete spp.	-	-	1	2	6	4	6	3	2	-	6	3	8	1	8	5	3	5	7	4	8	8	7	8
M. pusillus	-	2	2	6	-	-	-	3	-	1	2	5	-	-	-	-	-	-	-	-	-	-	-	-
H. lanuginosa	-	3	2	5	3	4	2	6	-	-	4	5	3	1	-	3	-	-	-	-	-	-	-	-
S. thermophile	2	1	3	-	4	-	1	2	2	-	4	1	3	-	-	-	-	-	2	-	1	-	-	-
T. aurantiacus	-	4	2	6	-	3	-	5	-	-	-	3	-	-	-	-	-	-	-	-	-	-	-	-
Bacterial colonies	-	-	3	4	2	3	3	1	-	2	2	1	3	2	6	4	2	4	6	7	8	5	8	6

TABLE 3(xi)

Ecological Studies using P_2 Protected Cotton Textile and the Perfusion Technique at 50°C

(C ≡ Cellulose Agar, G/S ≡ Glucose/Starch Agar. Figures refer to number of agar plates on which the species were observed out of a total of ten in each case).

Fungal Species Isolated	1% Fungicide loading								2% Fungicide loading								4% Fungicide loading							
	4 days		7 days		10 days		14 days		4 days		7 days		10 days		14 days		4 days		7 days		10 days		14 days	
	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S
A. fumigatus	3	6	9	10	10	10	10	8	4	2	4	3	4	6	5	6	5	4	6	6	6	5	7	3
Actinomycete spp.	-	-	5	4	4	3	9	5	-	-	2	4	5	8	9	6	-	2	4	3	8	4	8	6
Ch. thermophile	-	-	3	-	6	1	8	-	-	-	2	-	1	-	2	-	-	-	4	-	2	2	-	-
S. thermophile	2	-	5	2	6	1	2	2	-	-	8	4	1	2	-	1	-	-	1	-	4	1	2	1
H. grisea	-	-	-	2	1	-	2	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	2	-
H. lanuginosa	-	2	1	4	4	5	2	2	1	3	4	5	-	2	-	-	-	-	-	-	-	-	-	-
M. pusillus	-	3	-	1	-	2	2	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T. aurantiacus	-	-	-	2	-	-	-	2	-	-	1	-	-	-	1	-	-	-	-	-	-	-	-	-
Bacterial colonies	-	-	-	-	-	-	-	-	2	2	-	3	5	6	4	5	3	8	2	4	2	7	2	-

TABLE 3(xii)

Ecological Studies using P_3 Protected Cotton Textile and the Perfusion Technique at 50°C

(C ≡ Cellulose Agar, G/S ≡ Glucose/Starch Agar. Figures refer to number of agar plates on which the species were observed out of a total of ten in each case).

Fungal Species Isolated	1% Fungicide loading								2% Fungicide loading								4% Fungicide loading							
	4 days		7 days		10 days		14 days		4 days		7 days		10 days		14 days		4 days		7 days		10 days		14 days	
	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S	C	G/S
A. fumigatus	6	5	8	7	10	9	10	10	5	4	4	2	3	6	6	5	7	4	9	5	7	8	10	9
Actinomycete spp.	-	-	2	1	5	2	6	4	-	-	2	4	5	2	6	2	-	-	3	1	2	-	3	-
Ch. thermophile	-	-	3	-	4	-	4	2	-	-	2	-	3	2	-	-	-	-	-	-	4	-	-	-
S. thermophile	3	-	2	1	4	1	3	-	-	-	2	-	-	-	-	-	-	-	-	-	1	1	2	2
H. grisea	-	-	-	-	2	-	-	-	-	-	-	-	2	-	2	-	-	-	-	-	-	-	-	-
H. lanuginosa	-	1	-	3	2	2	-	3	-	-	1	1	-	2	2	3	-	-	-	-	-	-	-	-
M. pusillus	-	3	1	4	-	2	-	-	-	2	-	-	-	-	-	-	-	1	1	2	-	-	-	-
T. aurantiacus	-	2	1	4	-	-	-	1	-	-	1	-	2	1	-	-	-	-	-	-	-	-	-	-
Bacterial colonies	-	-	2	-	1	2	2	-	-	-	2	3	4	1	4	4	2	4	-	7	5	4	3	1

The results obtained from these ecological studies, show the continuation of the pattern of fungal colonisation that was indicated when employing the agar plate and screened substrate soil burial techniques. A. fumigatus together with the Actinomycete spp. and bacterial species appear to possess greater tolerance of all four fungicides than the other species identified. Chaetomium thermophile, Sporotrichum thermophile and Humicola grisea were also isolated from fungicidally protected textile, and this is important since all these species display marked cellulolytic activity. These species were however only isolated occassionally and somewhat erratically compared to the frequency of identification of A. fumigatus and Actinomycete spp. This may be due in part to the difference in natural frequency of distribution of these species in the soil, as indicated during the isolation work described in chapter two on unprotected cotton textile, where A. fumigatus, a profusely sporulating organism, was demonstrated to occur far more frequently than any other single species.

The mesophilic actinomycete population in soil is known to be quite ubiquitous in number of species, and their frequency of distribution (Hawker et al. 1967), and normally the growth of these species is significantly slower than that of other fungi. Thus there is a tendency for these other species to overgrow the actinomycetes under the artificial conditions of laboratory techniques. This phenomenon is clearly illustrated to be applicable at the elevated temperature of 50^{o}C also, and the experimentation in this chapter indicates that at the onset of adverse conditions the actinomycetes are able to compete more favourably with other species, and even tolerate or resist quite high concentrations of fungicide.

Another point of interest arising from these studies, is the increasing frequency with which bacterial colonies were identified with corresponding increasing concentrations of fungicides. This has also been observed at the mesophilic temperature of 25°C by Allsopp (1973) who used PCPL protected chromatography paper and cotton textile. This phenomenon is of particular interest since a bacteristat, rose bengal, was incorporated into the agar medium to reduce bacterial growth interfering with mycological studies (Ottow 1972).

3(e) Evaluation of Biodeterioration of Fungicidally Protected Cotton Textile at Thermophilic Temperatures

The experimental procedure of this section was carried out in conjunction with the isolation work of the previous section, which demonstrated the occurrence of thermophilic fungi on fungicidally protected cotton textile, using the perfusion technique.

Having established that there was fungal colonisation of cotton textile, which had previously been fungicidally protected, at the elevated temperature of 50°C, it was necessary to evaluate the degree, if any, of the biodeterioration of this textile. The colonisation may have been purely passive in nature with the fungi simply growing on the textile but not utilising any part of it as a nutrient source. Alternatively the fungi may have actively utilised the cellulose of the cotton fabric as a major carbon source for metabolism and in so doing will have deteriorated the cotton physically, as opposed to the aesthetic deterioration which could be displayed in the former case.

In order to assess the extent of biodeterioration of the fungicidally protected cotton textile, the perfusion technique was used as described in the preceding section. Sacrifices were made after four, seven, ten and fourteen days, corresponding to the same time intervals at which fungal isolations were carried out. At these intervals, ten standard samples were removed from the perfusion kits for each concentration of each fungicide, and were conditioned for 24 hours in a constant temperature and humidity room. The strips were then measured for any loss in tensile strength using a Hounsfield tensiometer in the same constant temperature and humidity room. The object of the conditioning procedure was to bring each of the sample strips to the same condition prior to testing to give some degree of standardisation to the measurements, as they had probably all experienced slightly different humidity conditions in the micro-environments of the different perfusion assemblies.

The average results of these tensile strength measurements are shown in table 3(xiii) where the figures represent the tensile strength at the breaking point in pounds weight, each value corresponding to the average of ten readings.

The average tensile strength detailed in table 3(xiii) are represented in graphical form in figs 3(a) and 3(b) which illustrate more clearly the pattern of biodeterioration.

It is clear that some rather erratic average results were obtained for some of the fungicides, with some values being followed after a longer incubation period by higher values, rather than the expected lower figures. This is particularly

TABLE 3(xiii)

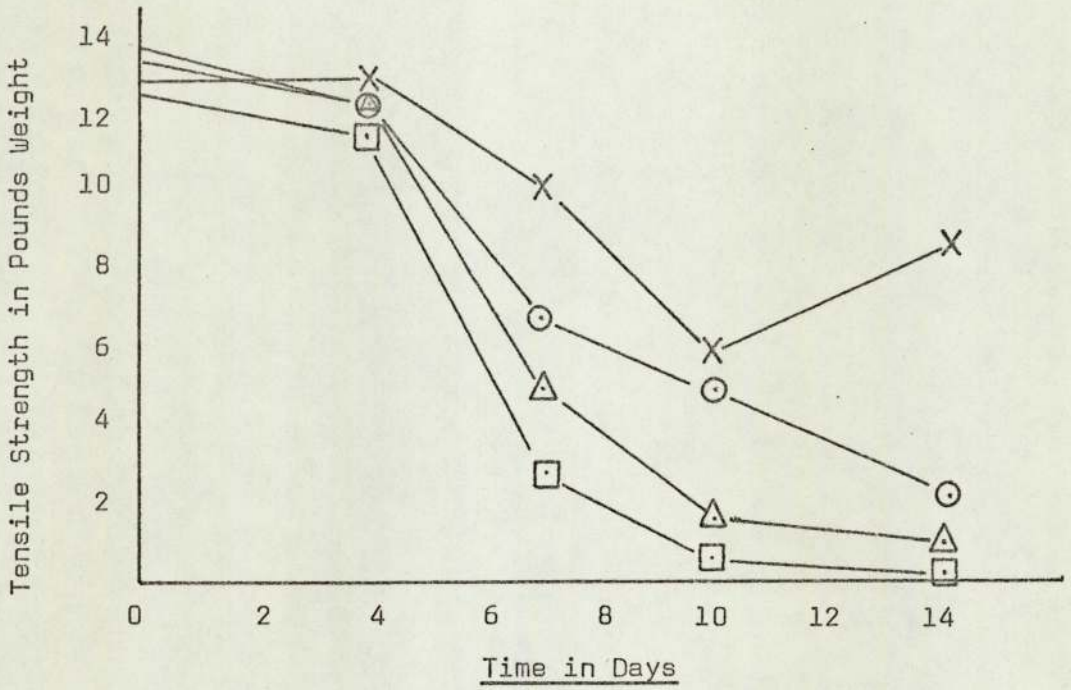
Average Tensile Strength Measurements of Fungicidally Protected Cotton Textile (in pounds weight)

Days Incubation / Fungicides	Period of Incubation at 50°C				
	0 days	4 days	7 days	10 days	14 days
Untreated Controls	12.63	11.43	2.62	0.54	-
1% PCPL	13.94	12.3	4.87	1.43	0.84
2% PCPL	13.63	12.3	6.7	4.9	2.14
4% PCPL	13.10	12.9	10.1	5.9	8.8
1% P_1	12.91	12.4	7.2	4.8	0.8
2% P_1	12.22	12.6	11.2	8.4	2.6
4% P_1	12.61	12.0	11.6	10.0	7.9
1% P_2	13.0	13.7	11.37	7.4	2.16
2% P_2	11.73	11.9	10.6	10.7	9.7
4% P_2	13.3	13.2	11.7	10.7	9.0
1% P_3	13.0	11.6	9.43	9.1	4.6
2% P_3	13.4	13.2	11.9	9.3	6.9
4% P_3	13.1	12.65	12.9	11.4	6.4

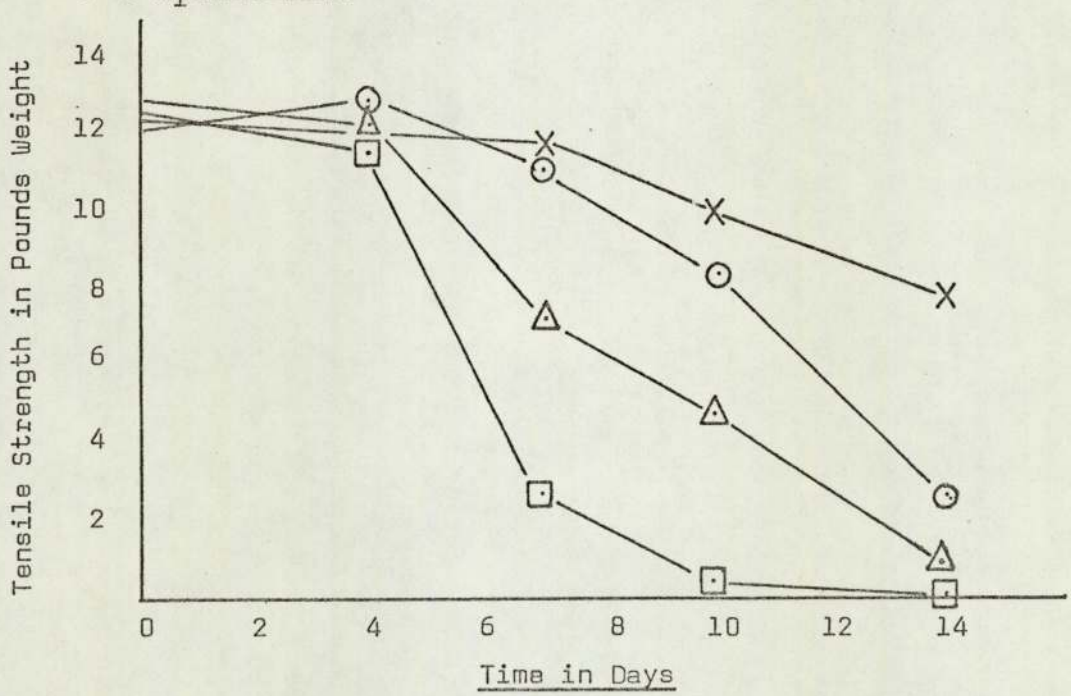
FIG. 3(a)

Average Tensile Strength Measurements in the Assessment of Biodeterioration of Fungicidally Protected Cotton Textile at 50°C

(i) PCPL



(ii) P_1 Fungicide



KEY:- —□—□— ≡ Unprotected Controls

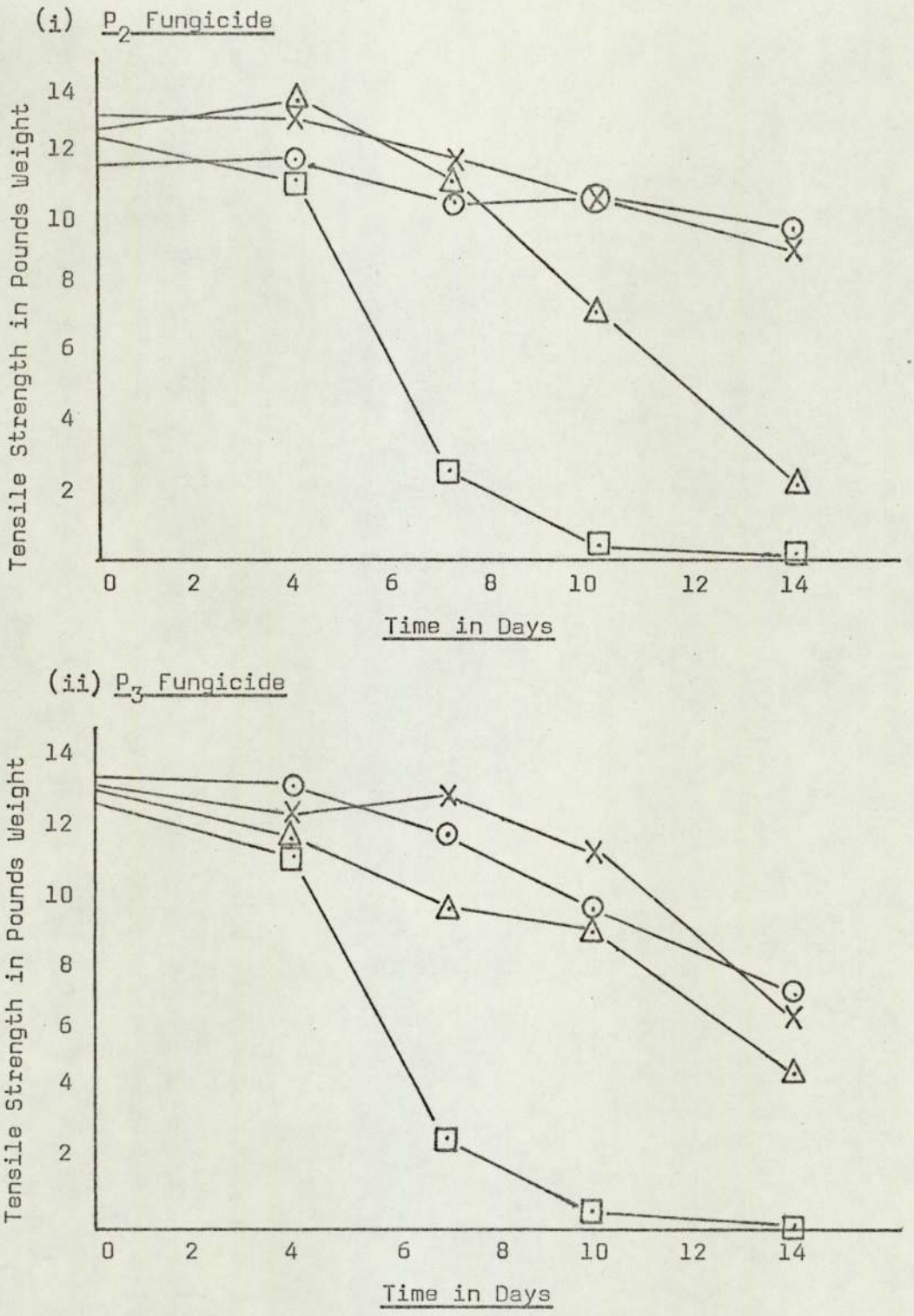
—△—△— ≡ 1% Loadings of Fungicide

—⊙—⊙— ≡ 2% Loadings of Fungicide

—X—X— ≡ 4% Loadings of Fungicide

FIG 3(b)

Average Tensile Strength Measurements in the Assessment of Biodeterioration of Fungicidally Protected Cotton Textile at 50°C



noticeable in the case of the 4% loading of PCPL at 10 and 14 day incubation periods, where the discrepancy is approximately 3 lbs.

This situation may not in fact be as extraordinary as at first appears. There is a steady trend in loss of tensile strength with time until between 10-14 days incubation where an apparent rise in strength is observed. Since the method of assessment is of a destructive type, different samples have to be sacrificed at the appropriate intervals. There may be particular species present in the soil which are not adequately controlled by this 4% level of PCPL, but distribution of fungi in the soil will probably not be uniform resulting in different species or numbers of species occurring in the different perfusion systems. As a result these differences in apparent protection may occur. To overcome this problem ten samples are tested, but this in itself may lead to problems in the final average figures.

In fact some of the ten values for any particular fungicide were high, while others were much lower and this leads to the inconsistent average figures shown on the graphs. The fact that there appear to be differences within a group of ten individual tensile strength measurements may also require explanation. Considering the experimental procedure, there may be some aspects which may lead to this variation. The individual strips were contained in separate perfusion assemblies which were made up into the kits for incubation. There is then a possibility that the rate of perfusion could differ between assemblies, and this may be reflected in the fungal colonisation, a particularly slow or intermittent rate or the stopping of the rate altogether leading to reduced colonisation and thus a higher tensile strength after

a given period. Soil was used as a mixed culture inoculum, and it is well recognised that there is no 'standard soil'. In any particular soil there is a diverse range and number of fungi, any one of which may occur in one part of that soil, but not another, and thus one species which is more cellulolytically active than another may occur on one sample but not on another. This will result in possible erratic quantitative results. There is also the possibility that the impregnation of fungicides throughout the cotton textile was not 100% efficient, resulting in areas where the fungicidal concentration was well below the true value. This may result in more rapid deterioration in these areas.

It should be noted well, therefore, that in biological testing there will be variables over which there is no adequate control and although informative and possibly significant general trends may be recognised, the limitations of quantifying biological tests must be fully recognised and stated in the interpretation of results.

Despite the difficulties outlined above, it is possible to assess the results obtained in general:-

(a) PCPL Protected textile

There is an expected pattern of increasing protection offered with increasing concentrations of fungicide. It is, however, only at the 4% loading that protection appears to be really adequate after fourteen days incubation. Certainly the lower concentrations of fungicide offers little protection after this period and the initial lag phase does not appear to be extended to any great extent.

(b) P_1 Protected textile

Again there is the expected increasing fungicidal protection with increasing fungicide concentration, but at levels of 1% and 2% there does not appear any really adequate protection offered after 14 days incubation. There is however an initial extension of the lag phase up to seven days before the biological activity appears to accelerate.

(c) P_2 Protected textile

Markedly improved fungicidal protection is offered at the 2% loading, which gives similar protection to that offered by 4% loading after 14 days incubation, but the 4% loading appears to offer better initial protection. The 1% loading, however, after a slightly extended initial lag phase, where loss in tensile strength is minimal, is seen to undergo rapid deterioration in a linear manner after approximately five days.

(d) P_3 Protected textile

An overall greater degree of fungicidal protection appears to be offered by all three concentrations. The initial lag phase is extended to approximately seven days before biological activity increases and there is then a period of greater biodeterioration which results, after fourteen days, in a definitely greater degree of protection with all concentrations offering similar protection.

Having discussed each of the fungicides, there are some interesting points of a general nature common to all.

1. There was the expected improved fungicidal protection with corresponding increases in fungicidal concentration and this was maintained throughout the fourteen day incubation period.

2. Loss in tensile strength at the elevated thermophilic temperatures was rapid, compared to the loss in tensile strength of fungicidally protected cotton textile with 1% loadings of the same fungicides at the mesophilic temperature of 25°C (Allsopp 1973).

Working with cotton textile of a similar type which had been impregnated with 1% levels of the same four fungicides and following incubation at 25°C the results obtained by Allsopp may be summarised as follows:

Fungicide	Time to 50% Strength Loss (in hours)	Time to 30% Strength Loss (in hours)
1% PCPL	690	290
1% P_1	400	175
1% P_2	290	225
1% P_3	600	500

These figures may be compared with those obtained from the present experimentation at 50°C, although the incubation period only extended to 330 hours (14 days):-

Fungicide	Time to 50% Strength Loss in hours			Time to 30% Strength Loss in hours		
	1%	2%	4%	1%	2%	4%
PCPL	146	168	226	132	144	-
P_1	192	264	-	144	240	330
P_2	264	-	-	216	330	330
P_3	288	-	-	284	288	312

It is clear from these comparisons that, in general, the biodeterioration at 50°C of fungicidally protected cotton textile is markedly more rapid than at the mesophilic temperature of 25°C. This raises the interesting questions of whether or not there is any difference in the cellulase enzyme systems of the thermophilic fungi compared to those mesophilic species, or alternatively whether there is a reduction in the efficacy of the fungicides at high temperatures due to physical/chemical factors. This phenomenon was also observed by Allsopp (1973) using PCPL protected cotton textile which had been subjected to heat treatments up to 400 hours at 70°C. This particular fungicide, however, operates on the principle of release of small quantities of pentachlorophenol and this is not the case with fungicides P_1, P_2 and P_3. However these observations may explain in part the more rapid deterioration of PCPL protected textile at 50°C and it may be necessary to increase the concentrations of fungicides accordingly in order to obtain adequate protection.

3(f) Discussion and Conclusions

The experimental work described in this chapter has shown that thermophilic species of fungi are capable of colonising fungicidally protected cellulosic substrates, and at the elevated temperature of 50°C this colonisation can result in rapid breakdown of the cellulose fibrous structure of cotton, with a resultant loss in tensile strength.

The thermotolerant species Aspergillus fumigatus together with Actinomycete spp. and bacteria were observed to be the most commonly occurring organisms, in the particular soil used in this investigation, associated with this thermophilic biodeterioration, especially at the higher fungicide concentrations. Other cellulolytic fungal species were also isolated with less regularity on unprotected and protected cotton textile, at the lower concentrations.

The fact that the thermophilic species of fungi isolated displayed extensive cellulolytic activity must be of importance when considering the storage of cotton textile under tropical conditions, and/or under conditions where insolation is likely to occur. This is particularly important when considering that the actinomycetes were observed quite frequently, and these species are generally able to tolerate much dryer conditions than other fungi. Thus even if a piece of textile may appear dry to the touch, there may be micro-environments occurring in the fibrous structure containing sufficient moisture, e.g. by condensation phenomena, to enable the growth of actinomycetes to occur. Alternatively the textiles may become damp due to

water seepage from inadequate storage buildings, and of course the storage of used material e.g. tents which are damp when placed in storage, may result in quite disastrous losses.

The rapidity of the breakdown of the cotton textile at 50°C by thermophilic fungi, and possibly bacteria, leads to the possibility of harnessing the phenomenon to the beneficial effect of industry, particularly Catomance Ltd., where there may be a possibility of detoxifying harmful industrial effluent containing obnoxious fungicides in suspension or solution. This may be achieved by means of a heated percolation system at 50°C inoculated with the thermophilic organisms known to be tolerant of or resistant to dilute concentrations of the fungicides in question. It was hoped to carry out experimentation along these lines, but the time factor forced these plans to be abandoned, but such studies may be worthy of future consideration since the effluent problem of industry is continually increasing and studies along these lines should be encouraged.

CHAPTER FOUR

INVESTIGATIONS INTO SOME PROPERTIES OF THE NEW RANGE OF BIOCIDES WHICH MAY AFFECT THE BIOLOGICAL ACTIVITY OF THESE PRODUCTS

4(a) Introduction

4(b) Investigations conerning the Solubility of a Potential Fungicide for Protection of Cotton Textile

4(c) Thin Layer Chromatographic Studies into Leaching of Fungicides from Cotton Textile at 50°C

4(d) Growth Studies with Isopropyl Alcohol at 50°C

4(e) Discussion and Conclusion

4(a) Introduction

The experimentation described in the previous chapter, has raised interesting questions concerning the fungicidal protection of cotton cellulose at elevated temperatures by the new family of fungicides produced by Catomance Ltd.

It has been demonstrated that the loss in tensile strength due to the activities of thermophilic fungi may be quite rapid, particularly in the case of unprotected textile and adequate protection appears only to be offered by relatively high concentrations of the fungicides, compared with the protection offered by the same fungicides at lower, mesophilic temperatures (Allsopp 1973).

Fungicide P_1, the ammonium salt of orthophenylphenoxy isopropanol, is the only one of the new fungicides under present investigation that is water soluble. This fact may have a bearing on the efficacy of the fungicide in the protection of cotton textile at 50°C, particularly when employing the perfusion technique to assess this efficacy. The perfusion technique maintains a continuous stream of water soluble nutrients through the textile, and in this way the fungicide may be removed from the textile by being dissolved in the water of the perfusion stream, thus rendering the textile more susceptible to fungal colonisation. It is this possibility, together with the potential fungicidal action of this P_1 fungicide, that is investigated further in this chapter.

Fungicides P_2 and P_3 are known to be insoluble in water, but the quantitative data obtained in the previous chapter indicated that little adequate protection is offered by concentrations which have been shown to be effective at the mesophilic temperature of 25°C. One possible explanation for this may be the reduction in effective protection due to the higher temperatures involved, as is the case with PCPL which operates by liberation of small quantities of pentachlorophenol from the ester. Fungicides P_2 and P_3 may have reduced efficacy in a similar manner at elevated temperatures. This possibility however has been discounted after consultation with the manufacturers who state that these products are stable to heat (Barr 1973). There is also a possibility that although recognised as insoluble in water, these fungicides may be 'flushed' rather than dissolved from the system at elevated temperatures by the action of the perfusion stream, to be deposited in the tail wick by evaporation. This possibility is investigated in the course of this chapter by extraction of these tail wicks followed by thin layer chromatographic studies of the extracts.

There is already evidence that PCPL acts partly by virtue of the hydrolytic breakdown of the fatty acid portion of the molecule by the action of soil micro-fungi thus release of small quantities of pentachlorophenol is brought about and this is the toxic component of the fungicide. From the ecological studies it appears that certain thermophilic fungi have a resistance to, or tolerance of, the new family of fungicides as a whole. The possibility therefore arises of detoxification of the fungicides by these fungi actually utilising the molecule

in part or in whole during metabolism, thus effectively detoxifying the fungicide and rendering the cotton cellulose susceptible to attack. The isopropyl alcohol radical is common to all this new family of fungicides, and the possibility of its utilisation during metabolism or as a major carbon source for nutrition by the thermophilic fungi is investigated in this chapter by growth studies with Aspergillus fumigatus which was the fungal species most frequently isolated during the ecological studies with biocidally protected textile.

4(b) Investigations concerning the Solubility of a Fungicide for Protection of Cotton Textile

The fact that P_1 fungicide is water soluble may be important in the interpretation of the results obtained in the previous chapter. The very nature of the perfusion system will tend to remove water soluble compounds from the textile under test and with subsequent loss of any water soluble fungicides, clearly the textile will be susceptible to fungal attack.

In order to ascertain whether or not this fungicide had been leached from the textile strips under test in the perfusion apparatus, and at the same time to investigate further the potential of this product as a fungicide, the perfusion experiment was repeated, and included in this repeat run was the modification of incorporating a 1% concentration of the fungicide actually within the nutrient reservoir. This ensured that the textile would be continually subjected to a constant concentration of the fungicide.

The experimental procedure was essentially the same as described in chapters 2(d) and 3(d and e), in that groups of fifteen standard cotton textile strips were incorporated into perfusion assemblies and made up into kits. A total of four kits for each treatment each containing 15 samples were set up. The nutrient supply was as previously described, with the exception that in four kits a 1% concentration of fungicide P_1 was incorporated into the nutrient reservoirs. The kits were sterilised by autoclaving for 20 mins at 15 lbs per square inch, inoculated with soil when cool, and incubated at 50°C for periods of 4, 7, 10 and 14 days, at which times textile strips were removed for tensile strength measurement in order to assess the degree of any biodeterioration which may have occurred.

The method of measuring the tensile strength of the cotton textile strips, differed from previous measurements. In the experimentation described in this chapter, all tensile strength measurements were carried out using simple laboratory equipment which had been developed at the Biodeterioration Information Centre by Allsopp specifically for this purpose (Allsopp 1973).

In addition to the kits containing 1% P_1 in the nutrient reservoir, controls of unprotected cotton textile, and P_1 (1%) protected textile without the addition of a 1% solution to the nutrient reservoir were set up.

In the case of the unprotected cotton textile controls an additional measurement of tensile strength was carried out after 2 days in view of the previously observed rapid deterioration

of this material at 50°C when inoculated with soil particles.

The average results obtained from these measurements are shown in table 4(i) below and fig 4(a). (Results given in Kg at point of break).

TABLE 4(i)

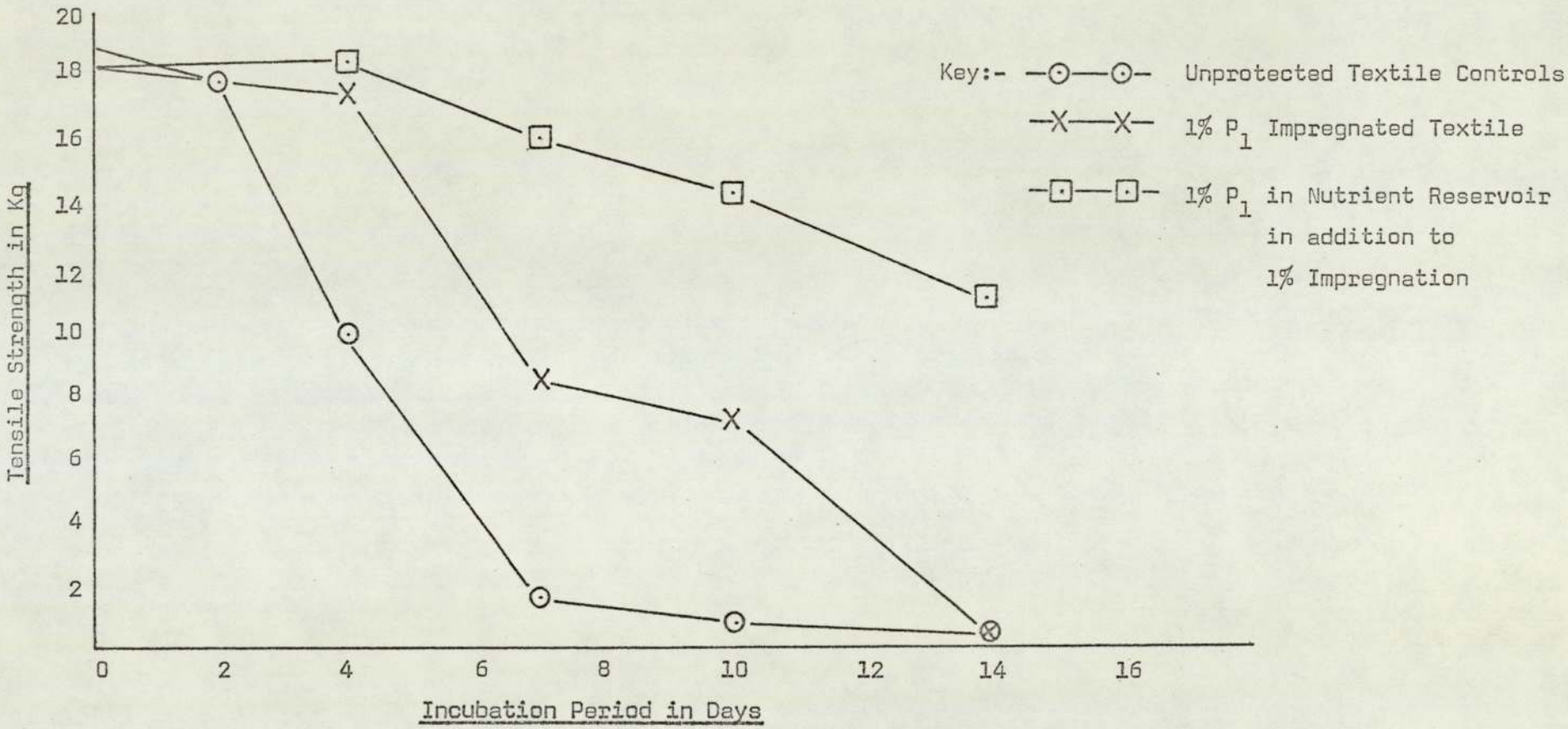
Average Tensile Strength Measurements for P_1 Protected Cotton Textile Incubated at 50°C

Fungicidal Treatment	Period of Incubation				
	0 Days	4 Days	7 Days	10 Days	14 Days
Unprotected Controls	18.8	9.8	1.4	0.7	-
P_1 Protected Textile	18.2	17.3	8.3	7.1	-
P_1 Protected Textile + 1% P_1 in Nutrient Reservoir	18.2	18.2	15.7	14.1	10.7

Average figure for tensile strength of unprotected cotton textile control strips after 2 days incubation at 50°C was 17.7 Kg. Averages calculated from a total of ten samples in each case.

The results above show that protection is offered by P_1 fungicide when impregnated onto the textile for an initial period,

Fig. 4(a) Graphical Representation of Tensile Strength Measurement of P_1 Protected and Unprotected Cotton Textile



but this protection is not maintained, and after a period of 14 days all the test samples broke on touch. This is in general agreement with the observations outlined in the previous chapter.

When 1% P_1 was incorporated into the nutrient reservoir, substantially improved protection was offered, and although there was evidence of a small degree of biodeterioration after a period of 14 days, at that point 50% loss in strength had not been attained and 30% loss in strength only occurred after ten days incubation.

There is evidence from these results to indicate that P_1 fungicide does in fact have potential fungicidal properties, under conditions where leaching is not an important factor. This experiment has also indicated that there is not complete protection even with a constant supply of the fungicide, and this may be important from an environmental outlook, in that the fungicide will have a limited protective affect, after which the protected material may undergo biological attack. Such a situation may be important for example in the problems of waste disposal.

One point which requires explanation is that the concentrations of fungicide applied to the cotton textiles are based on the dry weight of the cotton. In this experiment however the 1% solution of P_1 in the nutrient reservoir was based on the volume contained in that reservoir, and as such may have given a slightly distorted result, but meaningful general trends could be observed.

4(c) Thin Layer Chromatographic Studies into Possible Leaching of the New Fungicides from Cotton Textile

Having examined some possible explanations for the apparently poor protection offered by low concentrations of P_1 fungicide and PCPL in the protection of cotton textile against thermophilic fungal attack, attention was focussed on possible reasons for the lack of adequate protection offered by low concentrations of P_2 and P_3 fungicides which are not water soluble.

Leaching effects may partly explain the performance of P_1 fungicide, but the insoluble P_2 and P_3 fungicides should not be dissolved out of the textile strips under test by the perfusing stream of nutrients, unless possibly affected by the initial sterilisation procedure, or by the constant elevated temperature of 50°C and conditions of high relative humidity to which they had been subjected during the incubation period. As previously stated, however, the manufacturers declare these products to be heat stable, which leaves only the possibility that these fungicides may be 'flushed' out of the textile by the perfusion stream rather than actually dissolved in the aqueous phase.

In order to study this further, the perfusion experiments were repeated for P_2 and P_3 protected cotton textile, but on this occasion, the tail wicks were retained for extraction, followed by thin layer chromatographic studies, to determine if in fact these fungicides or any breakdown products of the fungicide had been removed from the textile by the perfusion stream, not necessarily in a soluble form but possibly by a flushing action or capillarity.

The perfusion kits were made up in the same manner as previously described, from kits each of 15 samples being prepared for each fungicide.

One aspect of the experiment was however modified and this was the use of a basic nutrient salt solution, with the omission of yeast extract and L-asparagine:-

KH_2PO_4	1.0g
KCl	0.5g
$MgSO_4 \cdot 7H_2O$	0.2g
$CaCl_2$	0.1g
$(NH_4)_2SO_4$	0.5g
H_2O	to one litre

In this way there was no carbon source to possibly interfere with either any fungal attack of the cotton cellulose or the chromatographic studies carried out on the tail wick extracts.

The kits thus prepared were sterilised by autoclaving at 15 lbs per square inch for 20 minutes, and on cooling were inoculated with soil particles, on top of a biologically inert fibre glass screening material which protected the cotton textile strips from gross contamination. Incubation was carried out at 50°C for periods of four, seven, ten and fourteen days, and at each time, the tail wicks were retained, and tensile strength measurements were made on ten of the cotton strips for each fungicide.

The results are shown in tables 4(ii) and 4(iii) where the figures represent the tensile strength at the point of break in Kg.

TABLE 4(ii)

1% P_2 Protected Textile - Tensile Strength Measurements

Period of Incubation				
0 Days	4 Days	7 Days	10 Days	14 Days
17.3	10.0	6.5	2.4	-
18.0	5.0	8.5	1.8	-
16.5	9.2	6.5	2.9	-
16.9	14.0	5.0	4.3	-
17.4	9.4	4.8	6.2	-
18.3	8.4	6.0	2.1	-
17.1	6.4	2.8	1.4	-
17.2	8.1	2.0	-	-
16.9	6.5	-	-	-
16.6	9.0	-	-	-
Av. 17.22	8.4	4.1	2.1	-

Figures in Kg at point of break

TABLE 4(iii)

1% P_3 Protected Textile - Tensile Strength Measurements

Period of Incubation				
0 Days	4 Days	7 Days	10 Days	14 Days
19.0	12.5	3.0	7.5	-
17.4	6.4	8.0	4.6	-
17.9	10.5	10.0	5.0	-
19.0	12.5	6.2	3.5	-
19.1	15.5	4.1	2.3	-
18.4	7.5	10.0	1.8	-
18.0	17.5	-	-	-
18.5	12.5	-	-	-
17.8	11.2	-	-	-
19.1	6.8	-	-	-
Av. 18.4	11.3	4.93	2.47	-

Figures in Kg at point of break

From these tensile strength results, it is clear that the absence of yeast extract and L-asparagine from the nutrient supply did not affect the rapidity of the fungal deterioration of the cotton cellulose by the thermophilic fungi, and the general trend was similar to that observed during the ecological studies described in chapter three. The results also justified further experimentation into extracts obtained from the tail wicks of the perfusion kits.

Extraction of Tail Wicks

This was carried out using 9 ml chloroform, 9 ml methanol and 4 ml 2NHCl per tail wick. Ten wicks per fungicide at each time interval were extracted in this way. The wicks were shaken vigorously in the solvent system, after which the solvents were decanted into a separating funnel and allowed to stand in order for the layers to separate. The lower layer was run off into a clean, dry, flask and a further 3 ml of chloroform was added to the upper layer, shaken and, after separation, run off into the clean flask. This was repeated three times in all to ensure that if any fungicide was present, it would be contained in the chloroform phase. In order to remove the 'dirt' which had accumulated in the chloroform layer, a further 2 ml of Methanol/HCl solution was added to the combined chloroform fractions, and the dirt was carried into this layer, which was then removed. The chloroform extracts were then combined and evaporated to dryness by passing a stream of nitrogen gently onto the surface of the liquid. The resultant extract was then re-dissolved in a very small quantity of chloroform/methanol mixture (1:1) prior to spotting onto the thin layer chromatography plates.

Thin Layer Chromatography

Thin layer plates were prepared by mixing 30g Kieselgel HF 254 Stahl Merk, with 60 ml of a 1% Boric acid solution, and spreading of this mixture was carried out using an automatic coater. The plates were allowed to dry in the air at room temperature prior to activiation at 110°C for 30 minutes in a hot box, after which the plates were allowed to cool to room temperature before use.

This experiment was intended only as a qualitative exercise, simply to determine whether or not any fungicide (or part of the fungicide) had been removed due to the action of the perfusion stream. For this reason no accurate quantitative spotting of the extracts onto the plates was carried out. The extracts obtained from the tail wicks were spotted onto the thin layer plates by means of a pasteur pipette, finely drawn out to deliver very small droplets, and dried using an air blower.

The developing solvent system used in the chromatography tank was a 4:1 mixture of chloroform and n-heptane.

Controls were prepared from the pure fungicides P_2 and P_3 by dissolving small portions of these products in chloroform and spotting small quantities of this solution onto the chromatograms as described above.

The plates were developed in chromatography tanks until the developing solvent front had advanced to within one inch of the top of the plates, after which the plates were removed and

air dried before observations were carried out.

Examination of the plates was carried out under ultra violet light which showed up any organic compounds as a series of spots. Upon examination under this light, any spots or larger more diffuse areas occurring on the plates, together with the initial starting spot, and the final position of the developing solvent front were carefully marked using a very fine dissecting needle. This enabled measurement of R_F values to be made more conveniently than could otherwise be carried out while the plates were under the U.V. light.

The results of this experiment were as follows:-

P_2 biocide (pure control) was observed to separate into three distinct spots on the chromatogram, with two additional more diffuse areas, when the plates were developed.

The R_F values were obtained using the formula:

$$R_F = \frac{\text{migration distance of substance}}{\text{distance of developing front from start}}$$

The R_f values obtained for the three major components of P_2 fungicide for five duplicates were:-

	R_f Values				
	1	2	3	4	5
1st Spot from Starting Point	0.22	0.32	0.31	0.20	0.25
2nd Spot from Starting Point	0.38	0.46	0.45	0.37	0.37
3rd Spot from Starting Point	0.64	0.69	0.65	0.63	0.62

P_3 fungicide (pure control) was observed to migrate into only one zone and the R_F values of this spot for four duplicates were:-

R_F Values

1	2	3	4
0.95	0.96	0.85	0.8

Having obtained R_F values for the pure fungicide, the extracts from the tail wicks were run on the chromatograms. Upon developing and observation under the U.V. light, however, the original spots applied to the thin layer plates had not migrated from the original position, with no spots or diffuse areas further up the plate. This was found to be the case for both P_2 and P_3 fungicide, and indicated that these insoluble fungicides are not removed from the cotton textile by the perfusion stream.

There was however a possibility that the low concentrations of fungicide involved may have been too low to show up on the chromatogram. To investigate this possibility ten strips of P_2 and P_3 protected textile (1% loadings) were extracted in the same manner as previously described, and the extracts were run on the chromatogram. The results of this set of controls was in general accordance with the pure fungicide controls. In this case however the spots were not so well defined, being much reduced in intensity and very diffuse. This led to difficulty in making precise measurements from which is determined the R_F values. The areas did however visually correspond to those previously obtained. This exercise illustrates the difficulties involved

in attempting to identify such small concentrations of fungicide, but considering the evidence that no spots or even diffuse areas were observed on examination of the chromatograms of the tail wick extracts, it may reasonably be concluded that the fungicides do in fact remain in association with the cotton textile strips, and are not flushed from the system.

4(d) Growth Studies with Isopropyl alcohol - a constituent group common to all the new family of fungicides under investigation

Following the results obtained in the preliminary studies using the new fungicides produced by Catomance Ltd., an explanation for apparent inadequate protection offered to cotton textile at 50°C was sought. It was decided to study the properties of isopropyl alcohol as a carbon source for fungal nutrition, since this was a chemical grouping common to all the new fungicides of this investigation.

From the literature it appears that the lower monohydric aliphatic alcohols - methanol, ethanol, etc., are not generally utilised by microfungi, although Aspergillus niger has been shown to make limited growth with both methanol (Baba 1941) and ethanol (Tamiya 1932). The most extensive studies of the alcohols is that of Stahl and Pessen (1953) who observed that Aspergillus versicolor grows poorly with ethanol and not at all with other primary alcohols from C_1 through to C_{11}. Alcohols with 12 to 18 carbon atoms support some growth of A. versicolor and the optimum chain length appeared to be 13 to 15.

There is some evidence that the biocide, pentachlorophenyl laurate (PCPL) is actively attacked by soil micro-organisms, this attack being directed towards the breakdown of the lauric acid side chain of the molecule. (Allsopp 1973).

With this phenomenon in mind coupled with the fact that some cellulolytic thermophilic fungi have been shown in this investigation to be tolerant of or resistant to the new biocides, the possibility of detoxification of these fungicides arises. Since the results of ecological studies in this thesis have shown inadequate fungicidal protection to be offered by lower levels of the new compounds in general, some thermophilic species may be able to detoxify the new range of fungicides by attacking that part of the molecule common to each of the new products - namely the isopropanol group.

Aspergillus fumigatus was used to investigate this possibility. This organism is a thermotolerant species known to display aggressively cellulytic activity, and has been shown in this thesis to be most frequently associated with the biodeterioration of the fungicidally protected cotton textile at elevated thermophilic temperatures. A liquid culture technique was employed and the growth of A. fumigatus was measured by mycelial dry weight production at intervals over a 14 day incubation period, which was carried out at $50^{\circ}C$.

25 ml portions of Eggins and Pugh nutrient salts were pipetted into 100 ml conical flasks. These were plugged with cotton wool and sterilised by autoclaving for 15 mins at 15 lbs per square inch. After sterilisation, the culture flasks were allowed to

cool, and isopropanol was added, taking the usual aseptic precautions to avoid contamination, at levels of 0.1%, 0.2%, 0.4%, 0.8%, 1.0% and 2.0% by volume. The above concentrations were prepared in duplicate. Measurement of mycelial growth was made after 4, 7, 10 and 14 days incubation. The flasks were then inoculated with 0.5 ml of a standard spore suspension obtained from a pure culture of A. fumigatus isolated from fungicidally protected cotton textile. The culture flasks were then incubated at 50°C under conditions of high relative humidity.

After each incubation period, the contents of the culture flasks were filtered through sinter glass funnels of porosity 10, which had previously been dried for 2 hours at 110°C and weighed accurately. After filtration of the mycelial mass, the funnels were again dried for two hours at 110°C, and re-weighed to determine the dry weight of mycelium produced. Control flasks were set up which contained the basis nutrient salts, but isopropanol was omitted.

The results of this growth study are shown in table 4(iv), where the figures represent dry weight of mycelium in mg..

The dry weights obtained are quite low, and at such levels a possibility of inaccuracies in weighing arises. This may account for the discrepancies within the same concentration level, where less growth appears to have occurred after a longer period. This may also be due in part to variation between culture flasks, as each determination was carried out on different flasks, i.e. estimation at each period was

destructive in nature, sacrifices being made of different flasks at each incubation period.

TABLE 4(iv)

Growth Studies with A. fumigatus using Isopropanol as a Carbon Source for Nutrition (figures in mg dry weight)

Isopropanol Concentration	Period of Incubation 4 Days Incubation	7 Days Incubation	10 Days Incubation	14 Days Incubation
-	6	3	4	6
0.1%	9	10	7.5	10
0.2%	10	10	9	9
0.4%	10	7	8	7
0.8%	8	7	9	7
1.0%	8	4	9	11
2.0%	8	3	10	7

From these results it is shown that growth occurred in the control flasks containing no isopropanol. This growth must have been made by the utilisation of the yeast extract and L-asparagine as carbon sources, as these were the only componants of the nutrient salts which contained carbon, and although not constituting a major carbon source, could support limited growth. Also from the mycelial dry weight results, it can be seen that in the case of the flasks containing the isopropanol as a carbon source for nutrition, slightly greater growth had occurred, although the increase was very small compared with the controls,

and may be regarded only as limited growth. It is interesting to note however, that the isopropanol did not appear to prohibit any growth, even at the 2.0% concentration. At the same time, as only limited growth occurred, it cannot be shown that this fungus will actively utilise the isopropyl radical as a major carbon source for nutrition. There is however an indication that, in the presence of other major carbon sources and subsidiary nutrients, the isopropyl radical may well have a part to play in the metabolism of the fungi associated with the thermophilic biodeterioration of fungicidally protected cotton textile. Fungal growth from spores may only be limited in the presence of isopropanol alone, but already established mycelial growth may possibly possess enzyme systems capable of utilising or breaking down this radical, and thus possibly detoxifying the fungicide.

Further experimentation along these lines, together with more intensive biochemical investigations may possibly offer evidence for the above hypotheses but this line of research did not fall in the scope of the present investigation and was abandoned for aspects of greater, direct concern to the sponsoring company.

4(e) Discussion and Conclusions

In this chapter, initial studies have been made to look more closely into the results obtained in the earlier ecological work described in previous chapters.

The solubility of P_1 fungicide has been shown to explain, in part, the poor protection offered by this product in the

thermophilic biodeterioration of cotton textile. This information has resulted in Catomance Ltd., developing further products, based on P_1 fungicide, but incorporating other less soluble salts into the molecule. These products have, however, not been studied during the present investigation. The experiment with P_1 fungicide in the nutrient reservoir also demonstrated the effective fungicidal properties of this fungicide, and it is hoped that the less soluble modifications of P_1 fungicide will prove effective under conditions where a leaching effect may occur, while P_1 fungicide may have a useful role to play in situations where leaching is not a possibility.

The chromatographic studies on P_2 and P_3 fungicides demonstrated that these products are not 'flushed' or 'leached' from the cotton textile (under the particular conditions of the perfusion technique). Although earlier ecological work (chapter 3) indicated that low concentrations of 1% of these products do not offer really adequate protection at 50°C, improved protection was offered by greater concentrations of 4%, but this concentration was also subject to a limited degree of biodeterioration. This phenomenon may however be advantageous from an environmental outlook. Under the present social state of Man it is considered by many that built in biodeterioration of materials is advantageous, if not essential. The fact that fungicide formulations do eventually undergo biodegradation thus becomes highly desirable, and this ensures that at the end of the useful life of a particular fungicidally protected material, there is an ultimate recycling of the material by the action of micro-organisms. If the fungicides had total substantivity, there would be a

build up of such fungicides in the environment and also of protected materials which had no further useful life, resulting in a gross distortion of natural cycles in addition to the problems associated with the disposal of such materials.

Thermophilic growth studies using isopropanol as a carbon source were only superficial but did show that this compound did not prohibit growth. Indeed it has been indicated that it may be utilised to a very limited extent by A. fumigatus, and the possibility arises of the reaction of an already established mycelial colony to break down the isopropanol radical.

More detailed biochemical investigations into the fungicidal properties of these new fungicides may lead to a greater understanding into the differences in the rate of biodeterioration, of cotton textile protected by these new products, at mesophilic and thermophilic temperatures. Unfortunately this type of study is beyond the confines of this investigation which is primarily of an ecological nature, but should be encouraged in the future.

CHAPTER FIVE

ECOLOGICAL STUDIES WITH POLYURETHANE FILM

5(a) Introduction

5(b) Ecological Studies using Polyurethane Film as Prepared

(i) Isolation work using a screened substrate soil burial colonisation technique

(ii) Isolation work using a perfusion colonisation technique

5(c) Ecological Studies using Polyurethane Film following Accelerated Hydrolysis

(i) Isolation work using a screened substrate soil burial colonisation technique

(ii) Isolation work using a perfusion colonisation technique

5(d) Discussion

5(a) Introduction

In chapter one, the rapid development of the polyurethane industry was outlined, together with the economic importance of these materials and their great versatility. It is this versatility of application which has, in recent years, become more and more important to those industries concerned with the utilisation of this material in articles experiencing everyday domestic and industrial usage. Catomance Ltd., have recently become involved with uses of polyurethanes as surface coating films applied to materials which may be susceptible to biodeterioration. An example of such a situation is the surface coating of cellulosic materials, such as cotton textile, with a film of polyurethane.

Catomance Ltd., have been involved with the biocidal protection of materials in general for a number of years, and now a new situation arises where more and more frequently the cellulosic materials are found in close association with the relatively new synthetic polyurethane materials. The fact that cotton cellulose is highly susceptible to fungal attack if not adequately protected is well known (Siu 1951, Hill 1965) as is the susceptibility of some polyurethane systems (Darby & Kaplan 1968), although it has generally been thought in the past that these new synthetic materials are in fact resistant to microbial damage (Heap 1965).

As a result of this new situation, Catomance Ltd., are concerned not only with the fungicidal protection of cellulosic materials, but also with ensuring that the polyurethanes used in association with these cellulosic materials are not themselves

susceptible to fungal attack. In situations where a particular polyurethane material is susceptible to biodeterioration, it may be essential that some form of protection is offered. It is for this reason that Catomance Ltd., have been engaged in the development of new fungicides which may be incorporated into the polyurethane system to offer adequate fungicidal protection, while at the same time not adversely affecting the desirable chemical and physical properties of the material. Conversely there is also interest in the preparation of polyurethanes which are known to be susceptible to attack by micro-organisms and by means of protection by biodegradable biocides, systems may result offering modern plastic materials of known resistance with a built in useful life span, after which they may undergo biodegradation and possible recycling.

One aspect associated with the chemistry of the ester linked polyurethanes as used in these investigations, is the relative ease with which hydrolysis of the synthetic material is brought about, resulting in a change of the properties for which they are used, e.g. elasticity or rigidity. This hydrolysis may be brought about by simple contact with water or under humid conditions over a period of time, or by more accelerated catalysed chemical reactions. This ease of hydrolysis may be important when considering the biodeterioration of polyurethane systems, and in this chapter ecological investigation is made of fungal colonisation of polyurethane film following periods of accelerated hydrolysis.

The deterioration of polyurethane films which may occur in the natural environment may follow the hypotheses listed overleaf,

based on consideration of the general concepts of biodeterioration (Hueck 1965).

1. The polyurethanes may be directly attacked by micro-organisms resulting in a loss in physical and/or chemical properties.

2. Polyurethanes may be colonised by micro-organisms without any significant loss of physical and/or chemical properties, but the presence of which are aesthetically undesirable. Here the microbial growth may possibly be sustained by detritus and trace nutrients occurring on the surface of the material, some of which may be release agents used in the manufacturing processes.

3. Hydrolysis of polyurethanes may occur due to physical and/or chemical factors (Testroet 1963, Ossefort & Testroet 1966 and Hole 1969). This may allow fungi to utilise the products of such hydrolysis in their metabolism.

4. The hydrolysis may actually be directly enhanced by the activities of micro-organisms either during direct utilisation of the material or by the action of metabolic products such as organic acids.

In this chapter a qualitative ecological study is made of the mesophilic fungal colonisation of an ester linked polyurethane film, prepared and supplied by Catomance Ltd., for use in these investigations. This study is concerned with fungal attack of polyurethane as prepared, and following various degrees of hydrolysis, offering a preliminary qualitative picture of any microbial colonisation of this synthetic material.

5(b) Ecological Studies Using Polyurethane Film as Prepared

In this study, the screened substrate soil burial colonisation and perfusion colonisation techniques were employed as previously described and used in the investigations concerned with fungicidally protected cotton textile in chapters two and three.

The ester linked polyurethane film used was prepared by Catomance Ltd., and was a two component polyurethane which was cast onto clean polythene sheeting. In this way a very thin film 0.1 mm in thickness was obtained and which offered ease of release. A thin film was used in order to simulate the type of film which may be expected to occur as a surface coating of cellulosics such as cotton textile. The polythene sheeting was used since this offered much greater ease of release of the polyurethane when fully cured than was obtained using other material surfaces, e.g. glass and poly-tetra-fluoro-ethylene (PTFE) onto which the film was cast.

The screened substrate soil burial technique was employed as described and discussed in chapter two using similar meadowland soil which had been maintained at 25^{o}C since its collection. Screened soil burial tubes were prepared, with one inch squares of polyurethane, sufficient to give twenty samples at five incubation periods on three agar media. The incubation periods at which sacrifices were made and fungal colonisation recorded were 4, 7, 10, 14 and 21 days. The three agar media onto which the samples were placed at these intervals were glucose/starch, cellulose and basic nutrient salts agar. This enabled a study to be made into the effect of simple sugars and cellulosic carbon

sources, in addition to the carbon compounds of the polyurethane, on the fungal colonisation of the synthetic material.

The perfusion colonisation technique was also used in this ecological study, and was again carried out in the form of kit assemblies as described in chapters 2 and 3, with the exception that, since the synthetic polyurethane material is not porous, it had to be placed on top of the perfusion wick and was not incorporated as an integral part of the perfusion chain itself in the way that the cellulosic material of cotton textile was in previous work. Inoculation was made using sieved meadowland soil maintained at 25°C. Sufficient perfusion kits were prepared to offer 20 samples at 4, 7, 10, 14 and 21 days intervals, at which times the samples were removed from the perfusion assemblies, taking the usual aseptic precautions, and plated out onto basic nutrient salts agar containing no major carbon source. Lack of sufficient adequate incubation facilities prevented duplication of this part of the experiment to include the effects of cellulose and simple sugar carbon sources on any fungal colonisation.

The results of this ecological survey demonstrated that the most frequently occurring fungi associated with the colonisation of this polyurethane film by microfungi contained in the meadowland topsoil were:-

Fusarium sp.

Gliocladium sp.

Penicillium funiculosum

Trichoderma viride

Aspergillus fumigatus

Penicillium lilacinum

Humicola grisea

Mucor sp.

Actinomycete spp.

Aspergillus terreus

Other fungal species were identified during these studies, but their isolation was extremely infrequent. These fungi included:-

Sporotrichum sp.

Cunninghamella sp.

Aspergillus versicolor

Eurotium sp.

Aspergillus niger.

Chaetomium globosum

Zygorhynchus sp.

Cephalosporium sp.

Arthrobotrys sp.

Paecilomyces sp.

The different agar isolation media appeared to have little influence on the colonisation patterns although a lower total number of species was isolated when a basic nutrient salts agar with no major carbon source, and the perfusion technique were

employed, compared with the use of a full alternative carbon source, as would be expected. It was interesting to note that the actinomycete population were isolated frequently only when using the perfusion technique and the basic nutrient salts agar. This may be due to their occurrence being masked or inhibited when employing agar media with alternative carbon sources other than the polyurethane itself, where the other fungi tended to exhibit extensive and profuse growth, overgrowing the actinomycetes which had a much slower rate of growth.

5(c) Ecological Studies Using Polyurethane Film after Accelerated Hydrolysis

Screened substrate and perfusion colonisation techniques were again employed in this experiment, which was carried out simultaneously with that of section 5(b) of this chapter.

Identical polyurethane film was used and accelerated hydrolysis was achieved by autoclaving the samples at 15 lbs/sq. in. for periods of 30 mins, 1 hour, 2 hours and 3 hours. (Hole 1969 and SATRA Test Method CM 28). In this way, an ecological study was carried out into the fungal colonisation at various stages of hydrolysis. It was found that after a 3 hour period of accelerated hydrolysis the thin film had substantially altered in properties, being a tacky mass and although remaining in the original shape of the sample when wet, changed to a rigid state when dry with minute crazing occurring over the whole surface.

The experimental procedure was identical to that described in the previous section of this chapter.

Screened Substrate Soil Burial

After only a short period of accelerated hydrolysis (30 mins), similar patterns of fungal colonisation were observed as those in section 5(b) of this chapter, with the same frequently occurring species of Fusarium sp., Gliocladium sp., Penicillium lilacinum, Trichoderma viride occurring. Other fungi were isolated, but only infrequently compared with the predominating species. The Actinomycete spp. did however occur with much greater frequency than previously observed (5b) especially after a longer period of incubation when these slower growing species were able to overcome to some degree the extensive growth of other species. This was particularly noticeable when basic nutrient salts agar was employed with no major carbon source available other than that of the polyurethane film. In this case competition was markedly reduced and the Actinomycete spp. were isolated with a frequency of 90%. Cephalosporium sp. was also observed with regularity which was not the case previously (5b).

This situation remained similar after the one hour period of accelerated hydrolysis with the Actinomycete spp. being the most frequently isolated organisms after an incubation period of about 10 - 14 days. Penicillium funiculosum which was only observed relatively infrequently after the $\frac{1}{2}$ hour accelerated hydrolysis treatment was observed to occur much more frequently after the 1 hour treatment, and this was also the case with Humicola grisea.

After a two hour accelerated hydrolysis period followed by screened soil burial procedures it was noticeable that Mucor sp.

which had only been observed erratically previously, now occurred with regularity and was in fact one of the most frequently occurring fungi. Again the same major species predominated although Gliocladium sp. was not observed in the early periods of incubation only being identified after a 10 day incubation period. This may be due to unavailability of particular nutrients after the hydrolysis treatment or to competition for available nutrients between species. Species of Aspergillus were also observed with more regularity although these fungi did not constitute any of the predominant species.

Following the 3 hour hydrolysis treatment again Mucor sp. was observed with regular frequency during the incubation period together with Fusarium sp., P. funiculosum, P. lilacinum, Actinomycete spp., H. grisea, Gliocladium sp. and Aspergillus sp. In addition, A. terreus was observed to occur with increasing frequency while T. viride was only identified occassionally.

The general trends thus observed in these studies were that the predominant fungal species remained the same as those described in the previous section to this chapter after the various accelerated hydrolysis treatments, but in addition there was a gradual increase in the number of other fungi observed with increasing degrees of hydrolysis. This may be explained by the fact that hydrolysis is gradual and even after the 3 hour treatment the reaction is probably not 100%. As such there will remain a certain amount of unhydrolysed substrate suitable for colonisation by those species observed with greatest frequency on the unhydrolysed polyurethane film. At the same time however the hydrolysis

reaction may change the chemical nature of the polyurethane film offering a different substrate for utilisation by fungi originally unable to do so, resulting in an increase in the number of species encountered.

Perfusion Colonisation of Polyurethane following Accelerated Hydrolysis

A similar pattern as that described above was obtained using this complementary technique with the initial predominant species remaining as such throughout. This was accompanied by a gradual increase in the number of species isolated and an increase in the frequency with which these species were encountered. The major species isolated were Fusarium sp., P. lilacinum, Gliocladium sp., Humicola grisea and A. fumigatus. The species displaying a gradual increase in frequency were Aspergillus spp., Actinomycete spp., Paecilomyces sp., T. viride, Mucor sp. and A. terreus.

5(d) Discussion

In this chapter, screened substrate soil burial of the polyurethane film has shown this material to be quite susceptible to fungal colonisation. The range and frequency of occurrence of those colonising soil micro-fungi were also shown to be similar when plated onto glucose/starch, cellulose and nutrient salts agars after incubation periods in the soil. This indicates that those fungi colonising the polyurethane film obtained adequate carbon supplies from that film and were relatively unaffected by the presence of alternative carbon sources, with regard to range of species and their frequency of occurrence. The extent of the growth however was seen to be markedly increased in the case of

the glucose/starch plates as would be expected, while the extent of growth on cellulose and nutrient salts agar was similar. The most frequently isolated fungi were Fusarium sp., Gliocladium sp., Penicillium lilacinum, Penicillium funiculosum, and Trichoderma viride. Perfusion of the same polyurethane film demonstrated similar fungal colonisation patterns, with Actinomycete spp., Cephalosporium sp. and Aspergillus spp., being isolated in addition to those above throughout the incubation periods.

Screened substrate soil burial of the polyurethane film after periods of accelerated hydrolysis demonstrated fungal colonisation by the same predominating species as were isolated on the unhydrolysed material. There was also an increasing frequency of occurrence of Humicola grisea and P. funiculosum and Mucor sp. with increasing degrees of hydrolysis, while T. viride was observed with less frequency as the degree of hydrolysis increased. When the perfusion colonisation technique was employed, using hydrolysed polyurethane film, again a similar range of predominant fungi was isolated, although the total range of different fungi was reduced.

The results of this ecological study have demonstrated that both the unhydrolysed and hydrolysed ester based polyurethane film were susceptible to fungal colonisation by the soil micro-fungi occurring in the meadowland topsoil used in the experiments described in this thesis. A similar range of predominant fungi were isolated after the various hydrolysis treatments. Some species isolated only occasionally on the unhydrolysed material

either did not occur on the hydrolysed polyurethane or occurred to an even more limited extent. This may possibly be due to the fact that these species may have occurred as contaminants on the film prior to investigation. There was also a succession of species which increased in frequency of occurrence with increasing degrees of hydrolysis.

Microscopical observation of the samples of polyurethane following fungal colonisation did not reveal any great depth of penetration of the fungal hyphae into the polyurethane film but was mainly limited to surface etching. The polyurethane film was very thin(0.1 mm)and this made such observations as to the degree of penetration of fungal hyphae difficult. These findings were however similar to those observed by Evans & Levisohn (1968) who recorded that Aspergillus niger, Fusarium sp. and Cephalosporium sp. produced only a limited amount of penetration when inoculated onto thin slabs of ester based polyurethane.

The results of this ecological study have illustrated the extensive range of soil micro-fungi which are capable of colonising both unhydrolysed and hydrolysed polyurethane of an ester linked type. The work of Evans and Levisohn (1968) resulted in the isolation of Aspergillus spp. and Penicillium spp. (P. citrinum in particular) in addition to Phoma sp., Fusarium sp., Cephalosporium sp. and Stemphylium sp. from polyester polyurethane printing rollers. The fungi identified in this thesis associated with the colonisation of the ester based polyurethane film include some of the same fungi with the exception of Phoma sp., Stemphylium sp. and P. citrinum, as recorded by Evans and Levisohn.

It is interesting to note that these workers observed Stemphylium sp. to be the most aggressive biodeteriogen of polyurethane, making tunnels deep into the material within two days of incubation and even quicker following hydrolysis by autoclaving. This organism was not identified during the studies described in this thesis, but since it was previously demonstrated to be such an aggressive deteriogen of polyurethane a culture of S. lanuginosum was obtained from the Commonwealth Mycological Institute for further studies involving the polyurethanes and their biocidal protection.

The investigations of Darby and Kaplan (1968) into the fungal susceptibility of 100 polyurethane systems involved pure culture test organisms of Aspergillus niger, A. flavus, A. versicolor, P. funiculosum, Pullularia pullulans, Trichoderma sp. and Chaetomium globosum. The results did not differentiate between these organisms after incubation but simply referred to the extent of gross fungal growth, with the exception of Ch. globosum which was observed to produce perithecia when grown on 3-methyl-2,4-pentanediol and tolylene 2,4-diisocyanate (T.D.I.), a polyether polyurethane. Thus the precise nature of the fungal colonisation is not available for comparison of colonising species to be made.

The fact that fungal colonisation of the polyurethane film has been shown to be extensive, outlines the necessity for some form of fungicidal protective treatment of this material. This becomes particularly important if the synthetic material is to be used in close association with

cellulosic materials such as cotton textile, which although probably protected itself, may not be adequately protected if there should be a heavy inoculum of fungi due to fungal colonisation of the polyurethane component of the total system.

Little information as to the possible mechanisms of fungal colonisation has been obtained from these ecological studies, but the experiments have shown the extensiveness and ease with which this material is colonised under suitable microbial conditions. In order to investigate this colonisation further, the predominant species were isolated into pure culture for use in further experimentation, which is described in subsequent chapters.

CHAPTER SIX

RESPIROMETRIC INVESTIGATIONS INTO THE BIOLOGICAL ACTIVITY ASSOCIATED WITH POLYURETHANE FILM

6(a) Introduction

6(b) Manometric Technique

6(c) Experiments using Unhydrolysed Polyurethane Film as Prepared

6(d) Experiments using Polyurethane Film Following Accelerated Hydrolysis

6(e) Discussion and Conclusion

6(a) Introduction

In the previous chapter, the susceptibility of polyurethane film to fungal attack was demonstrated, both on unhydrolysed and hydrolysed material, with a difference in colonisation pattern on the two materials being observed. This colonisation may occur due to direct utilisation of the polyurethane polymer by the fungi during nutrition. Alternatively, colonisation may be due to utilisation of contaminating compounds and detritus present on the surface of the polyurethane, and it may be these compounds which are utilised by the fungi in order to sustain growth, while not directly utilising the synthetic material itself. During this indirect colonisation, the secretion of metabolic products may cause or catalyse hydrolysis of the polyurethane film itself, the products of which may then be utilised either by the same fungi or other, secondary colonising species. The possibility also exists that the polyurethane undergoes chemical change due to physical and/or chemical factors prior to colonisation by fungi. Another factor which may influence fungal colonisation is the possible incomplete curing of the polymer, a situation which was in fact carefully guarded against during the preparation of the polyurethane film. The possibility of fungal growth on contaminating detritus on the surface of the polyurethane film was also guarded against by thoroughly washing the material with distilled water prior to use in the experiments.

The fungi occurring most frequently in the ecological studies described in chater five were isolated into pure culture for use in the experiments described in this and the following chapters. It was considered that respirometric studies may offer further

information concerning the mechanism and type of fungal colonisation on both unhydrolysed and hydrolysed polyurethane film. The fungi used were Fusarium sp., Gliocladium sp., Penicillium lilacinum, Trichoderma viride and Aspergillus sp.. Also included were Stemphylium lanuginosum and Chaetomium globosum, both species having been associated with the fungal colonisation of polyurethane articles (Evans and Levisohn 1968 and Darby and Kaplan 1968.) Aspergillus niger was also included in these studies as a species isolated from the meadowland topsoil but not occurring with any frequency during the ecological isolation experiments. This species was used as a possible example of non-utilisation of this particular polyurethane film. The micro-organisms occurring generally in the soil, referred to in previous chapters, were also used as a mixed inoculum in this experimentation.

The aim of the experiments in this chapter was to demonstrate the direct utilisation of the polyurethane film by the mixed microbial inoculum occurring in the soil, and those fungi isolated in previous work as listed above, and to study the effects of hydrolysis upon fungal colonisation.

In order to carry out such an investigation, an accurate and sensitive measure of biological activity was required. Manometric technique was considered to offer such a measure, using oxygen uptake during respiration as a measurement of microbial activity. This form of assessment has been widely used (Kaplan 1964) and the Warburg respirometer has been found a suitable piece of equipment for such studies, while modifications on the same principle have been used for similar studies concerning

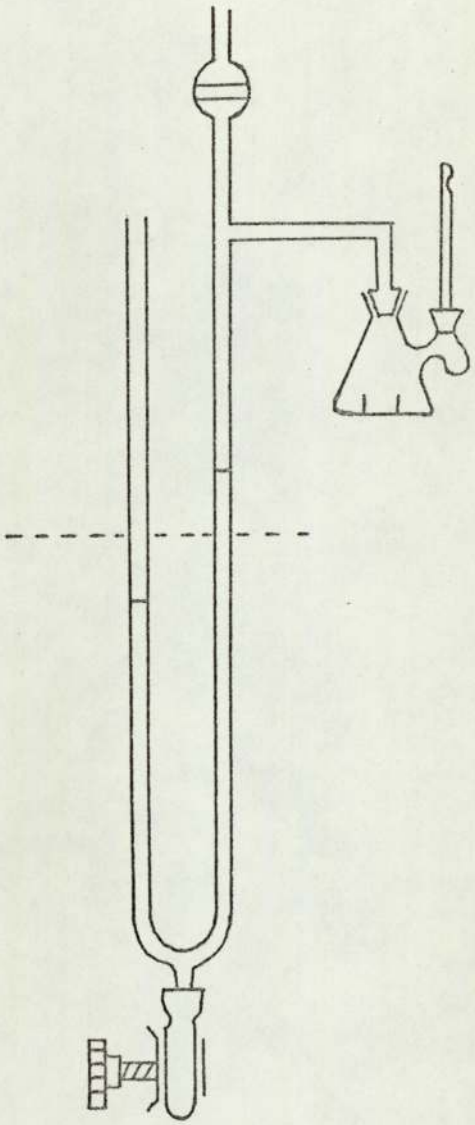
the biological activity associated with other polymeric materials (Burgess and Darby 1964, 1965).

6(b) Manometric Technique

A Braun, circular, Warburg apparatus was used in these respirometric experiments, and the principles of the technique are briefly described here.

The apparatus consists basically of a capillary U-shaped tube, at the base of which is a reservoir of Brodies solution (Appendix II) Fig. 6(i). The left hand limb of this U-tube is open to the atmosphere, while the right hand limb can be closed using a 3-way tap. There is also facility for the attachment of a flask at the top of the right hand limb, by means of a ground glass joint at the end of a short side arm. A scale marked in mm is situated behind the glass U-tube to enable measurement of a rise or fall of the manometer fluid in the open (left hand) limb. The zero point of this scale is halfway up the U-tube, and before any reading is taken of the fluid level in the left hand limb, the fluid in the right hand limb is always returned to the zero position by adjusting the pressure on the fluid reservoir. In this way a constant volume of gas (V_g) is maintained in the manometer and flask. The value of V_g for any manometer and flask is given by $(V_o - V_f)$, where V_o is the volume of the flask, manometer side arm, and that portion of the right hand manometer limb between the upper tap and the zero point, while V_f is the volume occupied by the liquid in the flask. The gas producing or gas consuming reaction takes place in the liquid of the manometer flask, and this liquid is in equilibrium

Fig. 6(i) Diagram of Warburg Respirometer



with the gas phase enclosed so that utilisation of dissolved gas is reflected as a decrease in gas pressure and vice versa.

When gas is taken up during the reaction in the flask, there is an accompanying rise in the level of the manometer fluid in the right hand limb of the manometer, and a fall in the level of fluid in the left hand limb. The meniscus in the right hand limb is then adjusted to the zero point so that the actual decreases in pressure at constant volume may be obtained, by allowing some manometer fluid to return to the reservoir. This reading may be quantitatively related to the gas absorbed in the manometer flask and to obtain an almost continuous record of gas uptake, such readings are taken at regular and frequent intervals during the course of reaction.

The manometer flask has a closable tap-stopper situated on a side arm which enables reactants to be tipped into the main flask compartment, but which was not used in this way during these experiments, remaining closed throughout each experimental run. In the main flask compartment is a centre well into which is placed a gas absorbent, here KOH was used to absorb CO_2. The flask contains a small volume of reaction mixture and constant shaking, by an oscillation facility of the respirometer apparatus, allows a larger liquid-gas interface to be maintained. This aids rapid diffusion and equilibrium of free undissolved gases.

As the readings are made on the manometer, similar readings are also taken with a thermobarometer (a 'blank' manometer) the flask of which contains only water and no microbial inoculum.

In this way compensation may be made for any small fluctuations in atmospheric pressure and/or the temperature of the water bath during the experiment.

Any pressure change in the manometers are therefore equivalent to distances moved by the manometer fluid in the left hand limb either up for positive changes or down for negative responses. Any movement of the meniscus of the thermobarometer is subtracted from those recorded on the other manometers; the resulting distances in mm then being translated into micro litres of dry gas at S.T.P. This is achieved by multiplication by the manometer constant. This constant, K, must be calculated for each individual manometer and re-calculated when any change in the system occurs, e.g. the nature of the gas under investigation, temperature, or volume of liquid in the flask. To avoid continuous re-calculation of these constants in the experiments described in this thesis, all the factors were maintained constant, and the same flasks were always used with the same manometers throughout the experiments.

The manometer constant (K) is obtained using the equation:-

$$K_{o_2} = \frac{V_g \, {}^{273}/_{T} + V_f \circ \alpha_{o_2}}{P_o}$$

where

V_g = Gas volume in μl.

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

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

P_o = Normal atmospheric pressure, expressed in mm manometer fluid rather than mm Hg (Brodies solution gives P_o = 10,000 mm).

V_f = Volume of fluid in manometer flask in μl.

Since the solubility of the exchanged gas is dependent on temperature, this is taken into account by using its absorption coefficient (α) relevant to the temperature at which the experiment is carried out.

The oxygen uptake in the manometer flask is given by the equation:-

$$V = h \times K$$

where

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

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

K = Flask Constant

In all the respirometric experiments carried out, the Warburg apparatus (model V166) was set at a shaking rate of 70 cycles/minute, and the water bath was controlled at 30°C by a sensitive thermostat. This temperature was selected because it was the lowest temperature that could be constantly maintained by the equipment in the laboratory since insolation factors raised the ambient room temperature to almost this level on some occasions.

The micro-organisms used in this study were those cultures isolated in previous ecological work (Chapter 5) and the mixed multitude of organism in the meadowland topsoil.

6(c) Experiments using unhydrolysed polyurethane film

The polyurethane film was thoroughly cleaned by washing under the tap followed by distilled water, and allowed to dry in a clean

desiccator before being cut into half inch squares for use in these experiments. Four such square samples were placed in the manometer flasks (including the thermobarometer) to give a total of one square inch per flask.

When soil was used as an inoculum, it was made up as a thick suspension of 25g sieved soil in 100ml liquid, which was either distilled water or E. and P. basic nutrient salts solution without any major carbon source. Four mls of this suspension were added to each flask using a pipette that had the tip broken to avoid blockage of the fine orifice by the soil particles. The centre well of the manometer flask contained 0.5 ml 20% KOH to absorb CO_2, and this was aided by the addition of a standard piece of filter paper, 2 cm x 4 cm, rolled up and placed in the centre well, effectively increasing the surface area for CO_2 absorption. The thermobarometer contained 4 mls of sterile distilled water in the main compartment, 0.5 ml 20% KOH in the centre well and four half inch square samples of polyurethane only. There was no microbial inoculum in the thermobarometer. Before setting the flasks and manometers up in the above manner all the flasks were thoroughly cleaned using chromic acid followed by extensive washing under the tap and three rinses in distilled water. After this cleaning treatment the flasks were autoclaved and thoroughly dried. In this way any possibility of cross contamination by the micro-organisms used was eliminated as was the possibility of growth occurring on contaminating organic matter on the glass itself.

When pure cultures of fungi, previously observed to be associated with the colonisation of polyurethane film, were used

as an inoculum, a spore suspension was prepared using 14 day old cultures. This suspension was again prepared in both sterile distilled water and E. and P. basic nutrient salts solution. Again, 4 mls of these spore suspensions were pipetted into the main compartment of the manometer flasks, while 0.5 ml 20% KOH was pipetted into the centre well, using similar standard pieces of filter paper to increase the surface area for CO_2 absorption.

Before each experimental run, when all the flasks had been assembled with their contents on the manometers, they were allowed to equilibrate for a period of one hour, when all taps were open to the atmosphere. After this period all taps were closed and the experiment was run over a period of two days in the case of unhydrolysed polyurethane film and three days when using hydrolysed film, readings being taken at regular intervals depending on the trends in microbial activity taking place in the flasks. Overnight the taps were again opened to the atmosphere to avoid the possibility of the manometer fluid being carried over into the flask in the event of a high rate of oxygen uptake. The following morning the taps were closed again and a new set of readings taken throughout that day.

Results

The data obtained from these experiments is shown in graphical form, Figs 6(ii) - (x), where average oxygen uptake in μl at S.T.P. is recorded over the duration of each experimental run. The major points to be observed from these results are as follows:-

The unhydrolysed polyurethane is utilised during growth of the mixed inoculum of micro-organisms occurring in the meadowland

topsoil. This activity was seen to occur with or without the presence of ancillary essential nutrients in the form of E. and P. basic nutrient salts solution. When sterile distilled water was used there were probably sufficient nutrients present in the soil suspension itself to promote the microbial growth recorded. The growth pattern was similar at the beginning of the experiment for both the situation where polyurethane film was accompanied by ancillary nutrients and where there was no polyurethane. At this stage, the micro-organisms probably preferentially utilise the most readily available nutrient, and after a period of about 12 - 14 hours it was observed that the microbial growth sustained on the basic nutrient salt solution alone gradually reduced as the nutrient supply became depleted. The microbial activity associated with the polyurethane film however increased in a linear manner as would be expected in terms of available substrate and enzyme reaction if the material is utilised. A similar phenomenon was also demonstrated when distilled water alone was used.

From the results obtained using pure cultures as an inoculum, a general trend was observed in all cases. During the first 10 hours of incubation the more readily available ancillary nutrients were preferentially utilised, together with any traces of dissolved nutrients carried over during preparation of the original spore suspensions. This initial activity slowed down during the second day of incubation in the case of the controls giving a plateau appearance to the graphs, while the activity associated with the polyurethane film, was maintained at an increased rate and in a linear manner. Also observed was the very low level of activity when the fungal species were incubated in sterile distilled water only, which in all cases was practically zero. Incubation of

Fig. 6(ii) Utilisation of Unhydrolysed Polyurethane Film by Soil Micro-organisms

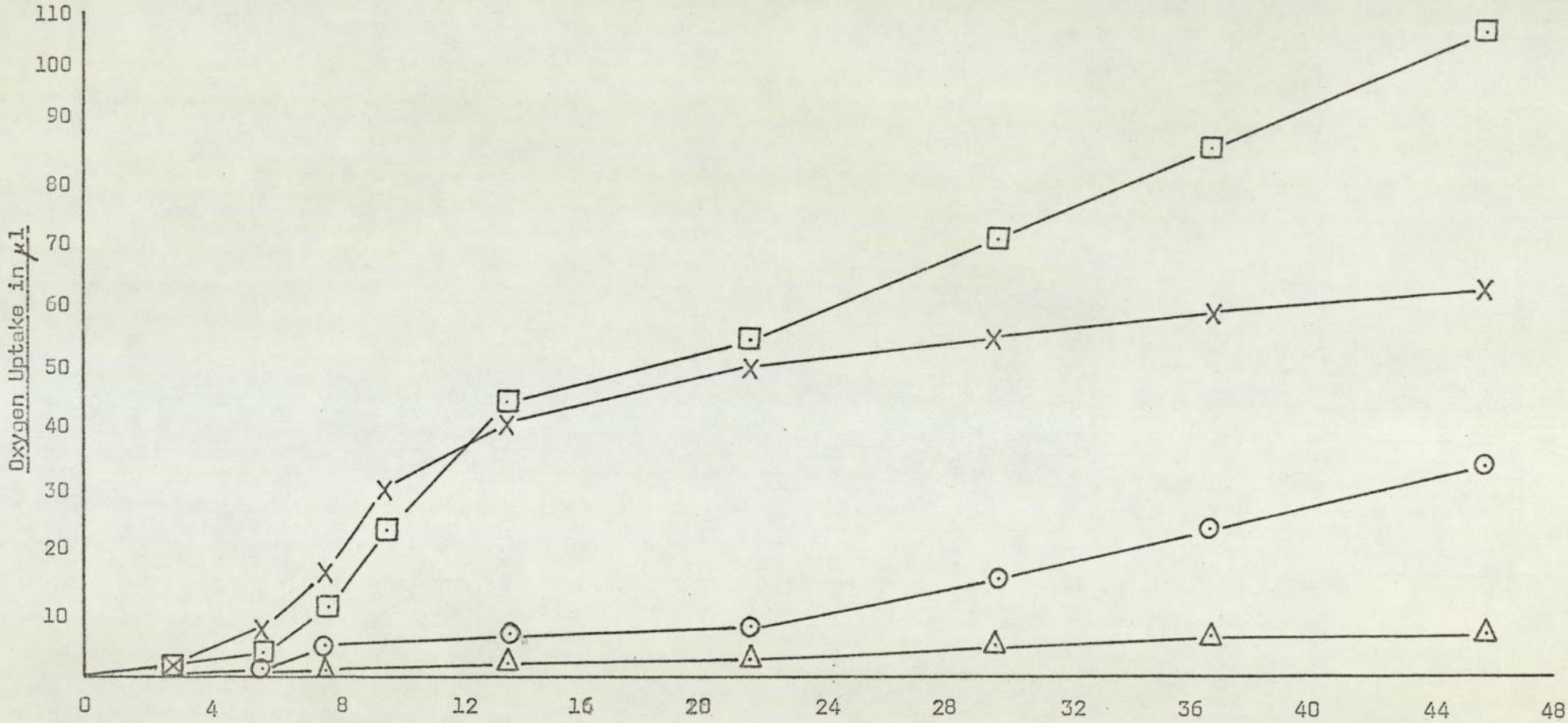
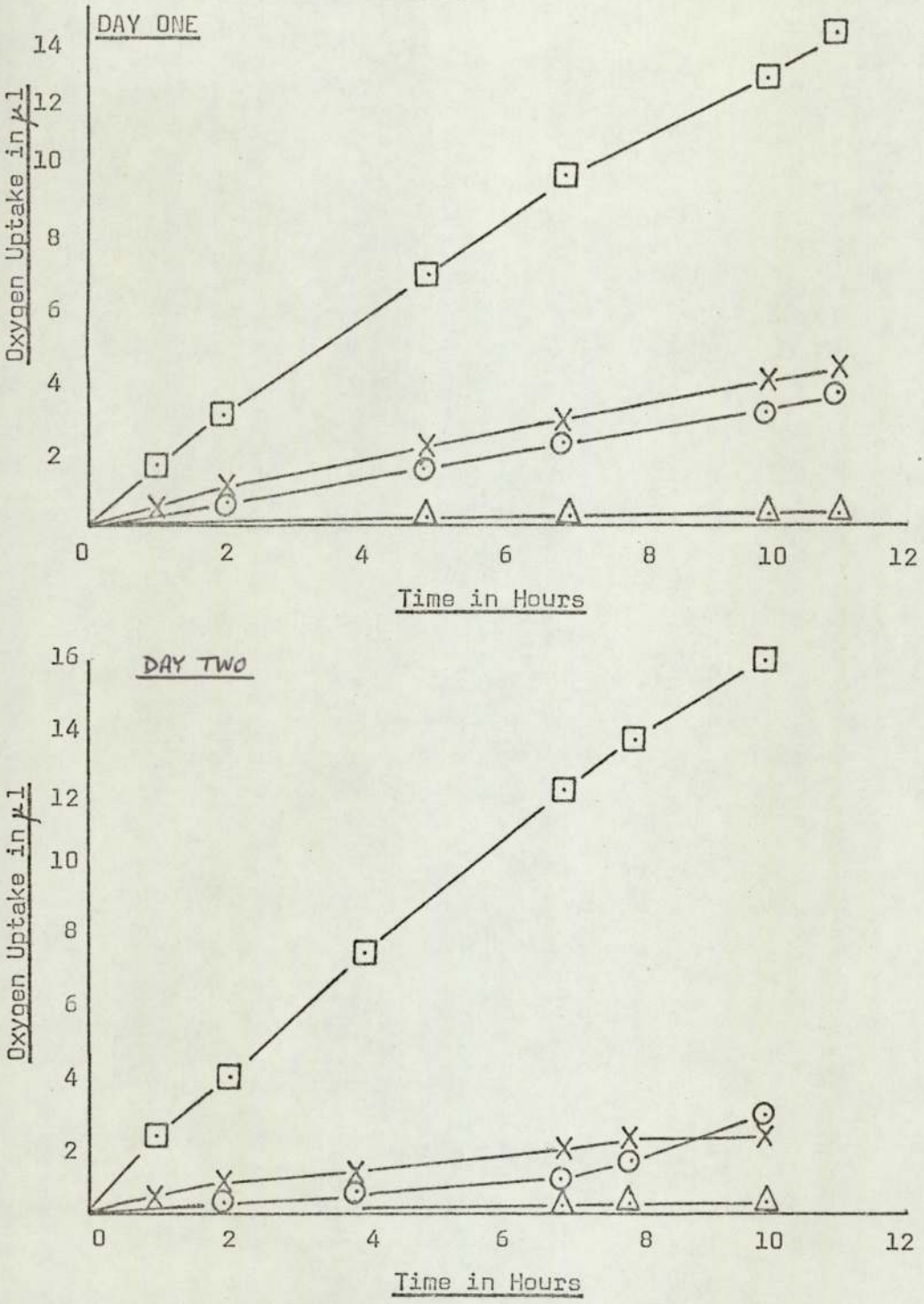


Fig. 6(iii) Utilisation of Unhydrolysed Polyurethane Film by Gliocladium sp.



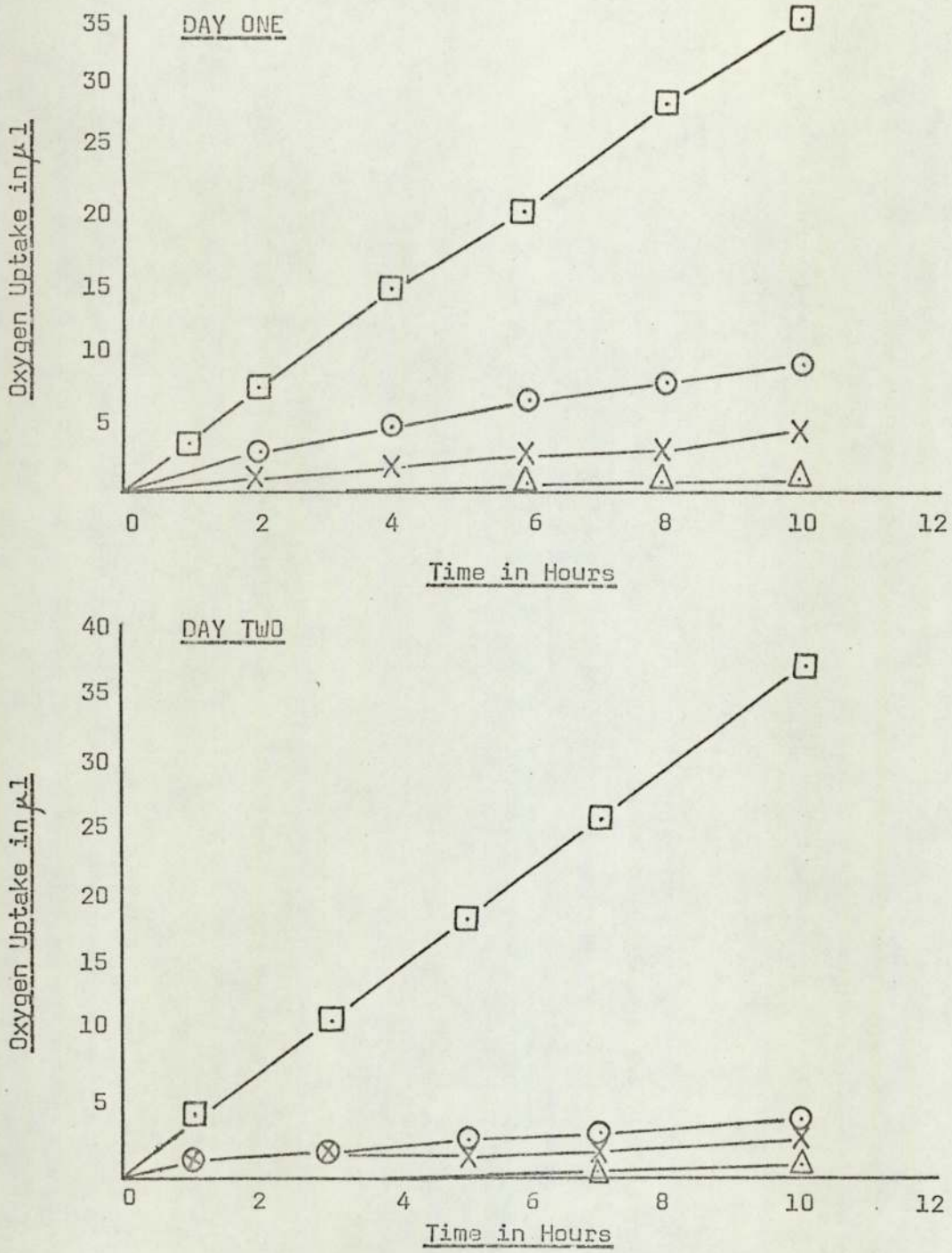
Key:- —X—X— = Gliocladium + E & P Nutrient Salts only

-⊡—⊡- = Gliocladium + E & P Salts + Polyurethane

—△—△— = Gliocladium + Distilled Water only

—⊙—⊙— = Gliocladium + Distilled Water + Polyurethane

Fig. 6(iv) Utilisation of Unhydrolysed Polyurethane Film by Penicilium Lilacinum



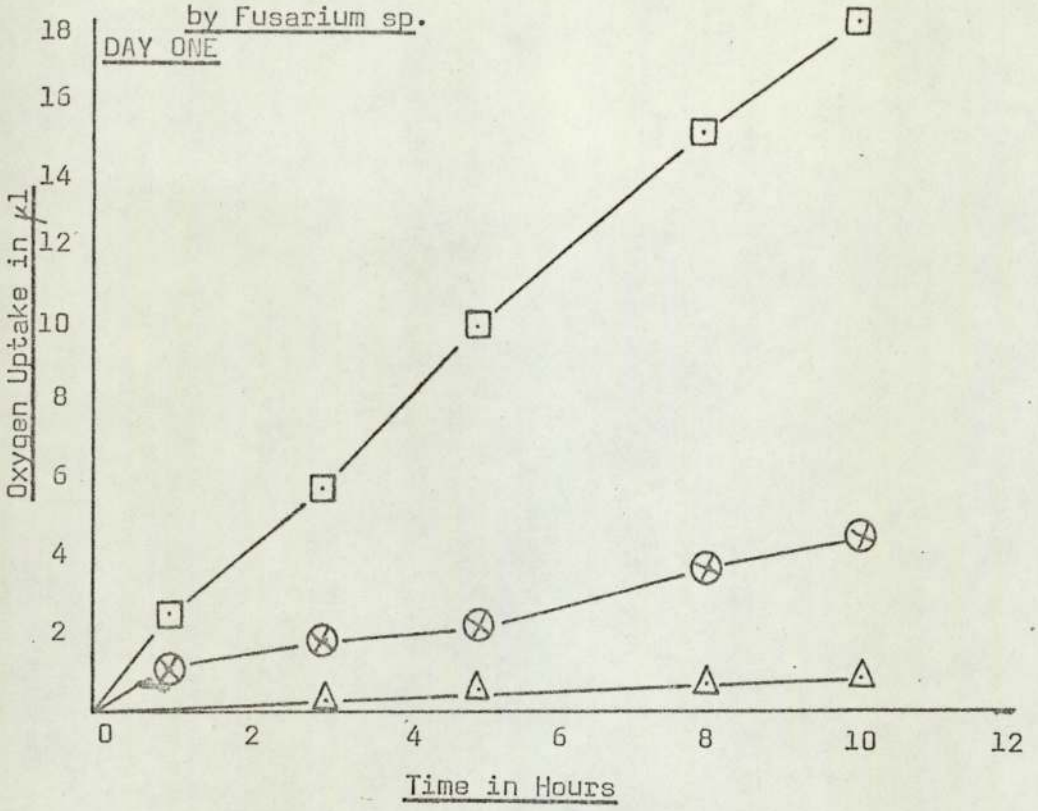
Key:- —X—X— = P. lilacinum + E & P Nutrient Salts only

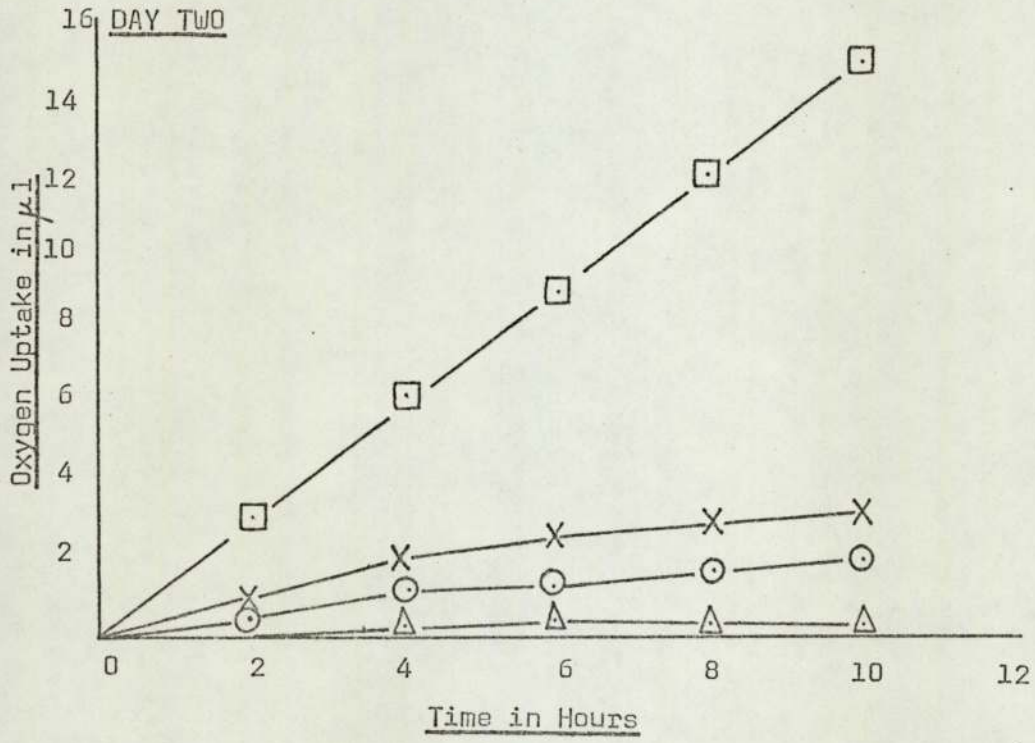
—⊡—⊡— = P. lilacinum + Polyurethane + E & P Nutrient Salts

—△—△— = P. lilacinum + Sterile Distilled Water only

—⊙—⊙— = P. lilacinum + Polyurethane + Sterile Distilled Water

Fig. 6(v) Utilisation of Unhydrolysed Polyurethane Film by Fusarium sp.





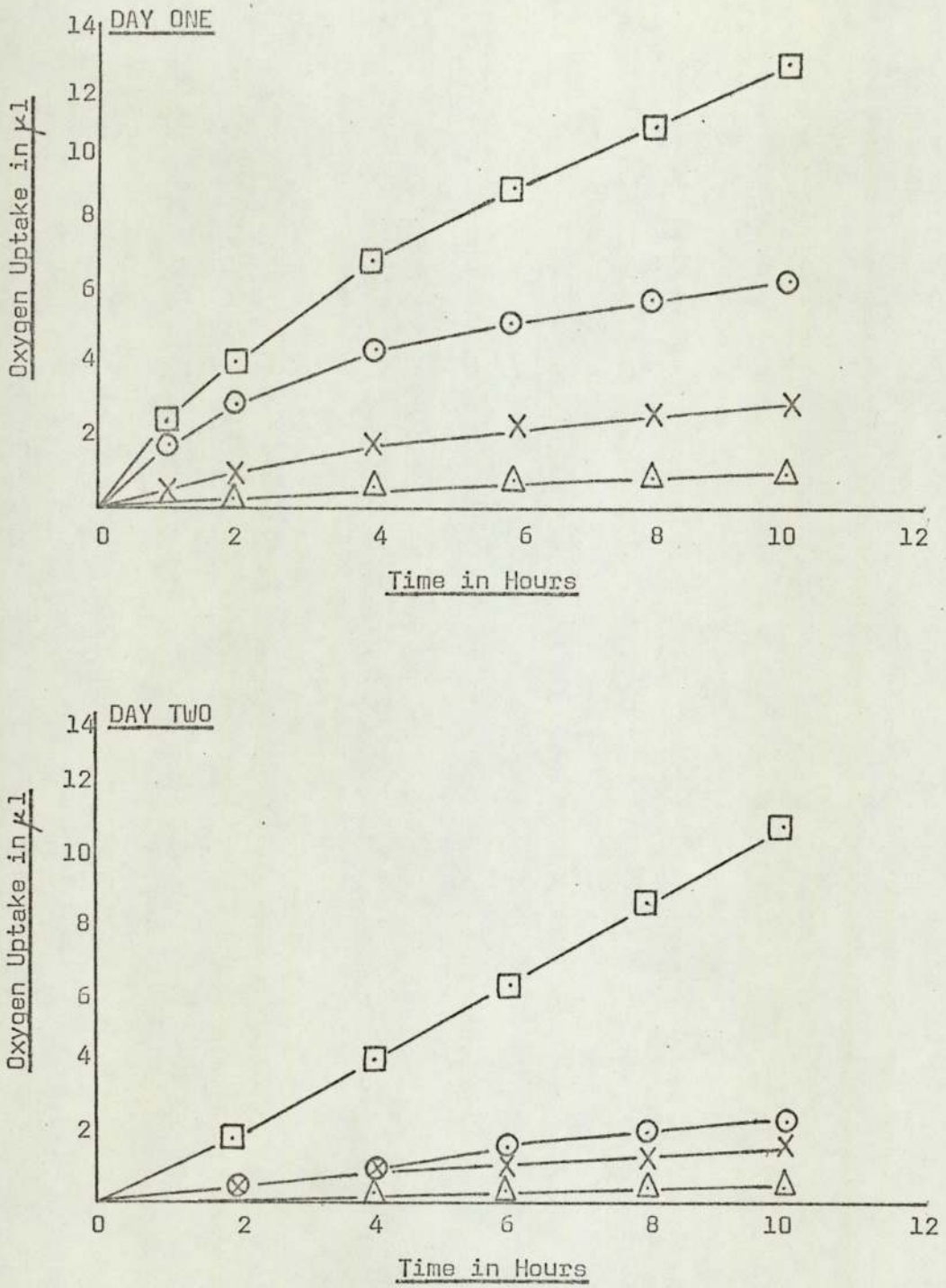
Key:- -X—X- = Fusarium sp. + E & P Nutrient Salts only

-⊡—⊡- = Fusarium sp. + E & P Nutrient Salts + Polyurethane

-△—△- = Fusarium sp. + Distilled Water only

-⊙—⊙- = Fusarium sp. + Distilled Water + Polyurethane

Fig. 6(vi) Utilisation of Unhydrolysed Polyurethane Film by Trichoderma viride



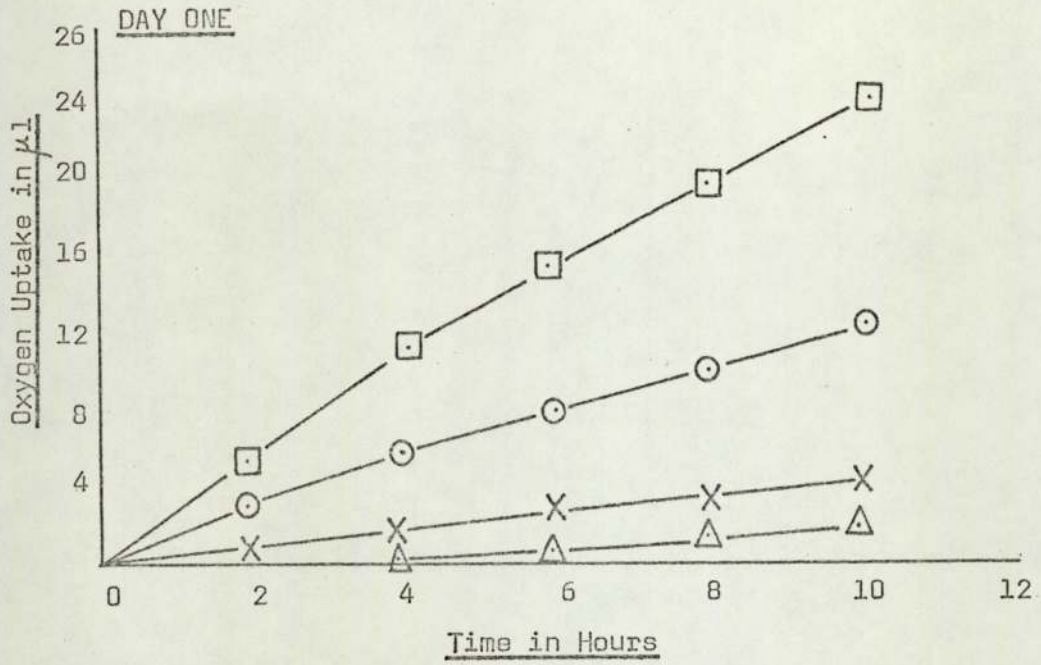
Key:- -X—X- = T. viride + E & P Nutrient Salts only

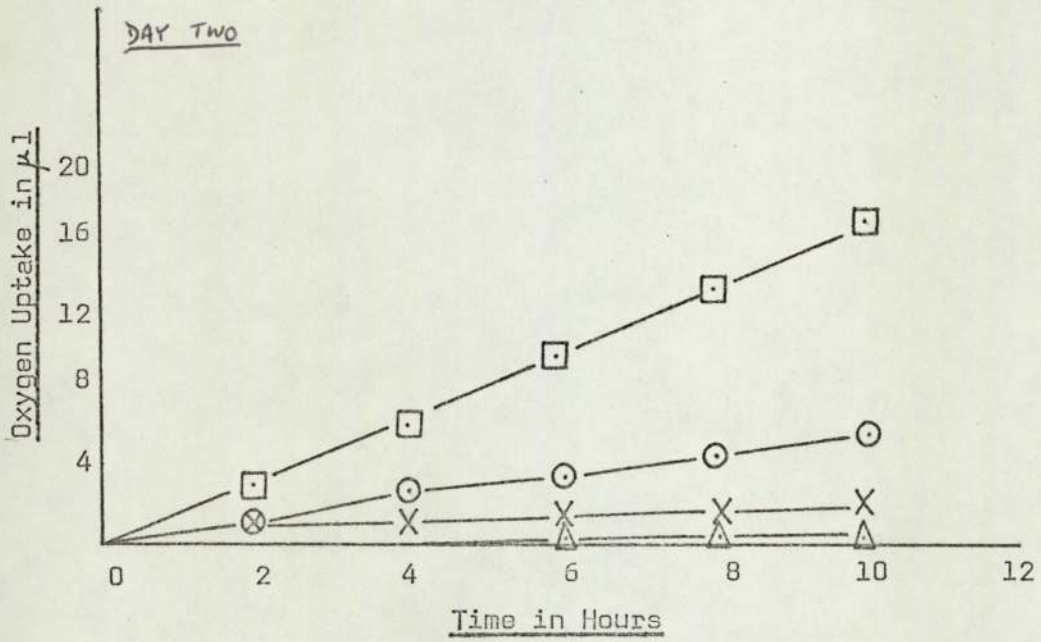
-□—□- = T. viride + E & P Nutrient Salts + Polyurethane

-△—△- = T. viride + Distilled Water only

-⊙—⊙- = T. viride + Distilled Water + Polyurethane

Fig. 6(vii) Utilisation of Unhydrolysed Polyurethane Film by Aspergillus sp.





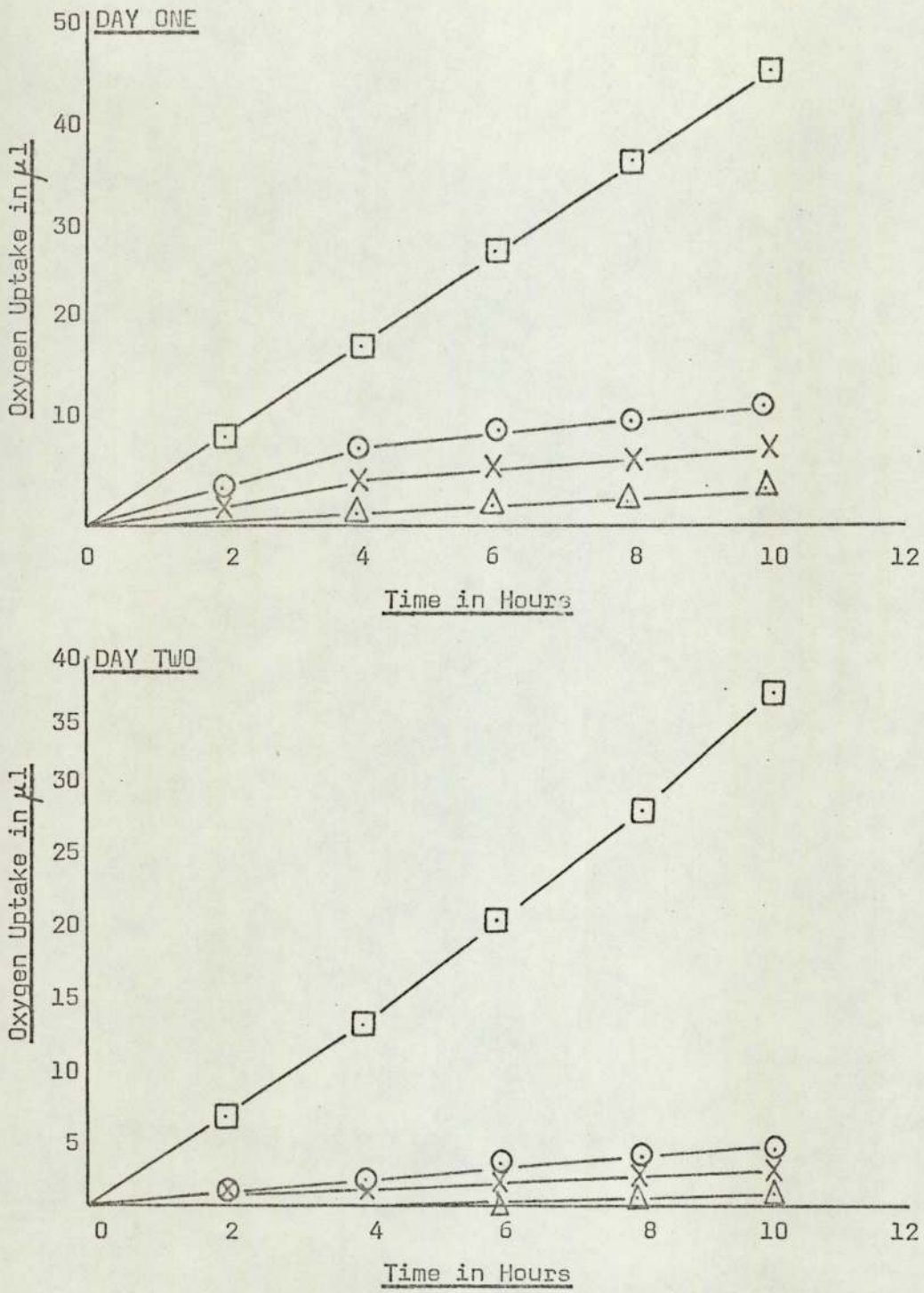
Key:- —X—X— = Aspergillus sp. + E & P Nutrient Salts only

-⊡—⊡- = Aspergillus sp. + E & P Nutrient Salts + Polyurethane

-△—△- = Aspergillus sp. + Distilled Water only

-⊙—⊙- = Aspergillus sp. + Dstilled Water + Polyurethane

Fig. 6(viii) Utilisation of Unhydrolysed Polyurethane Film by Stemphylium lanuginosum



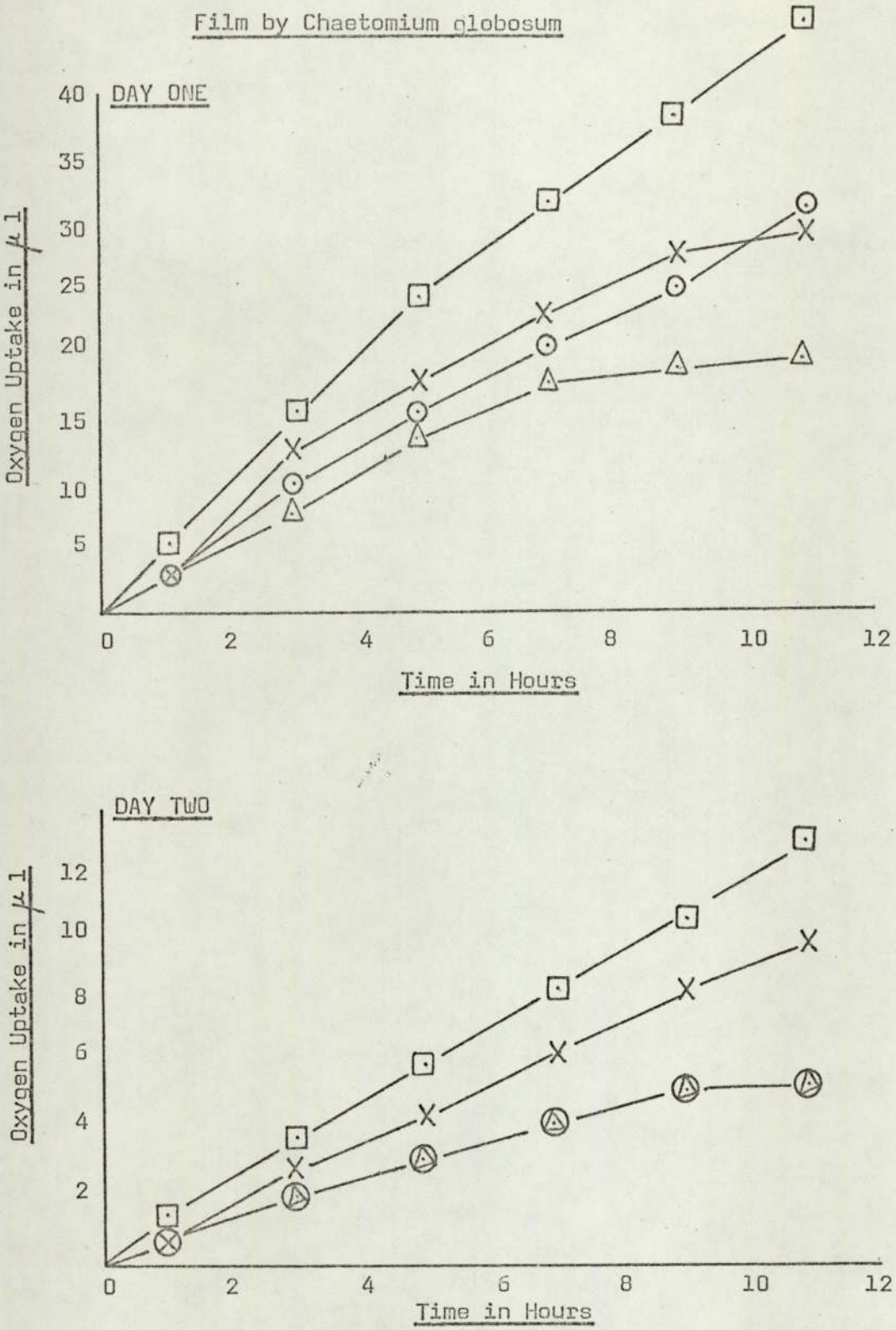
Key:- -X—X- = S. lanuginosum + E & P Nutrient Salts only

-⊡—⊡- = S. lanuginosum + E & P Nutrient Salts + Polyurethan

-△—△- = S. lanuginosum + Distilled Water only

-⊙—⊙- = S. lanuginosum + Distilled Water + Polyurethane

Fig. 6(ix) Utilisation of Unhydrolysed Polyurethane Film by Chaetomium globosum



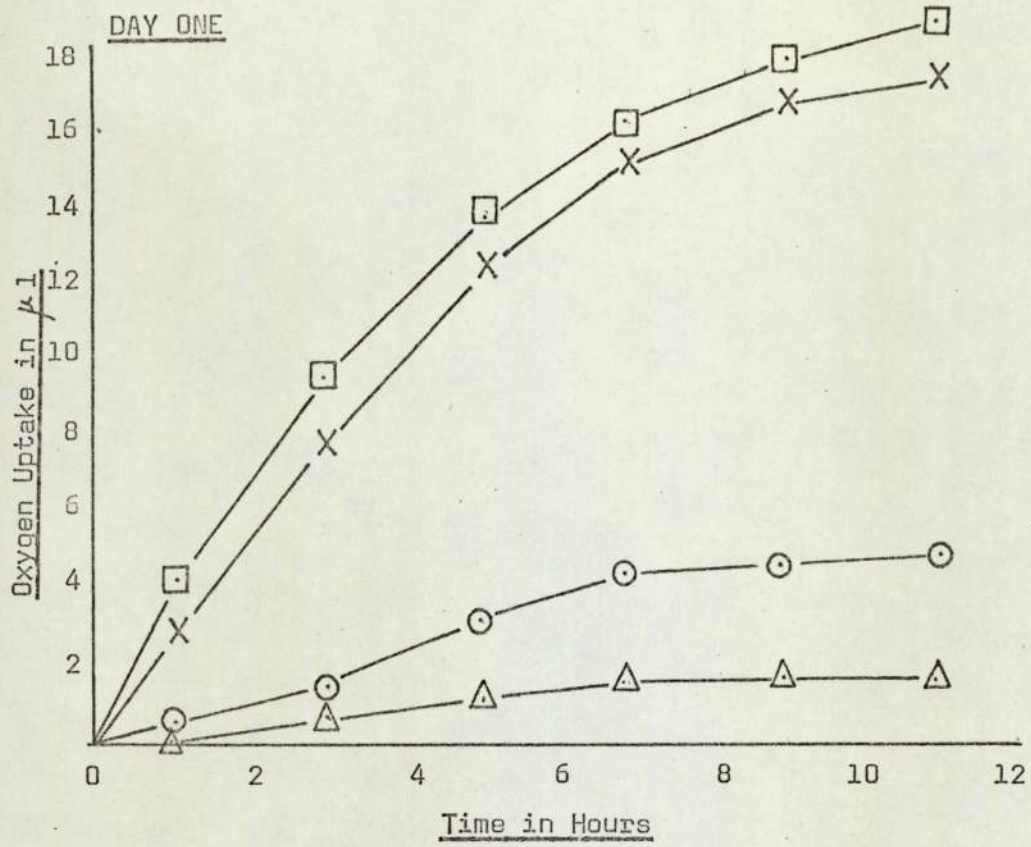
Key:- —X—X— = Ch.globosum + E & P Nutrient Salts only

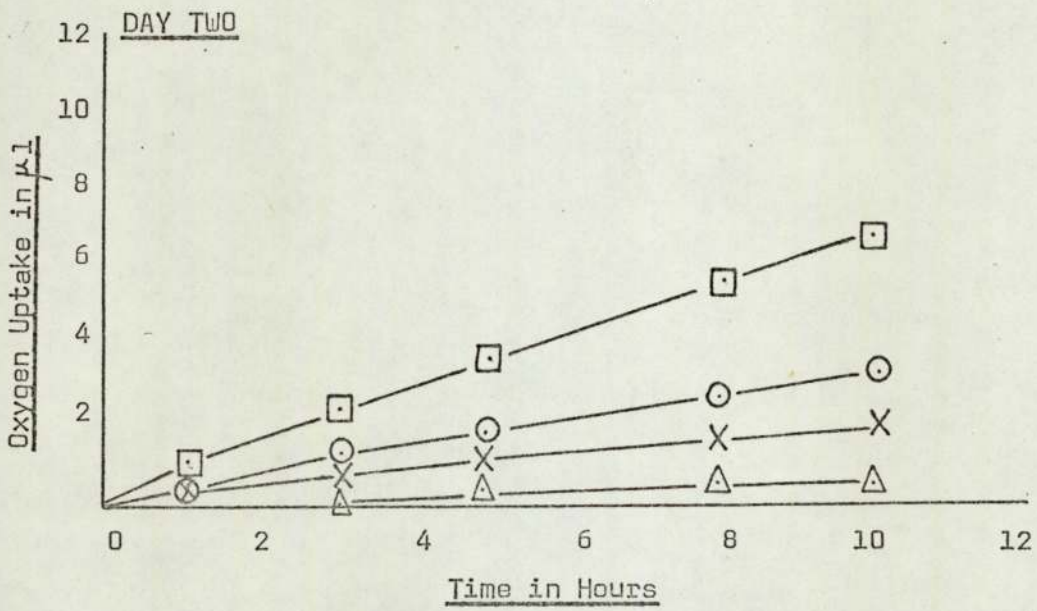
—⊡—⊡— = Ch. globosum + E & P Nutrient Salts + Polyurethane

—△—△— = Ch. globosum + Distilled Water only

—⊙—⊙— = Ch. globosum + Distilled Water + Polyurethane

Fig. 6(x) Utilisation of Unhydrolysed Polyurethane Film by Aspergillus niger





Key:- —X—X— = A. niger + E & P Nutrient Salts only

—⊡—⊡— = A. niger + E & P Nutrient Salts + Polyurethane

—△—△— = A. niger + Distilled Water only

—⊙—⊙— = A. niger + Distilled Water + Polyurethane

the polyurethane in the presence of sterile distilled water resulted in a slight increase in growth activity which was however still rather limited compared to the microbial activity observed when both the polyurethane film and the ancillary nutrient supply were present. These observations demonstrate that those fungal species isolated during ecological studies in association with the frequent colonisation of the polyurethane film appear to actively utilise this synthetic material during growth. This growth is more adequately sustained when there is a balanced nutrient supply, and is rather limited when these ancillary nutrients are absent, i.e. the polyurethane material is unable to offer all the essential nutrient requirements for fungal growth, but probably offers a major carbon source to certain fungi with a possibility that the nitrogen inherent to this synthetic polymer may also be available to some species.

Aspergillus niger was included in these studies because, although a widely occurring species in the soil, it was not associated with the fungal colonisation of the polyurethane material in the previous ecological isolation studies. It was therefore thought that this species may offer a suitable example of the respirometric responses associated with non-utilisation of this particular material. It can be seen from the appropriate graph (6(x)) that although a species prolific in spore formation, and use of a heavy inoculum, the microbial activity was in fact very low. This species grows readily in the presence of simple carbon sources such as sugar, but in this experiment it was observed that growth in the presence of basic nutrient was not great, probably due to the absence of a major simple carbon source. When the small quantities of possible nutrients, in

the form of E & P nutrient salts, were depleted after germination of the spores, the oxygen uptake was even more markedly reduced. During the second day of incubation there was however a slight increase in activity recorded in the presence of the polyurethane film. It may be concluded from this that A. niger is not able to utilise the complex carbon source of this particular synthetic polyurethane material to the same extent, if at all, as the other species investigated.

6(d) Experiments using polyurethane film following accelerated hydrolysis

The same manometric technique previously described in this chapter was used to investigate the microbial activity associated with hydrolysis of the esterlinked polyurethane film. The same fungal species as in the previous experiment were used and meadowland topsoil again provided a diverse range and number of micro-organisms in a natural, mixed inoculum.

Accelerated hydrolysis of the polyurethane film was obtained by autoclaving the material at 15 lbs/square inch (121^{o}C) for periods of 30 minutes, 1, 2, and 3 hours (Hole 1969) and SATRA Test Method CM 28). In this way the hot moist conditions would bring about varying degrees of hydrolysis. Periods of accelerated hydrolysis treatment longer than 3 hours resulted in a gross distortion of the material, with holes appearing in the film which became viscous when wet but on drying was very brittle with fine crazing over its surface. The material at this stage was difficult to handle and these irregularities in shape were considered undesirable for the purposes of comparative investigation and were omitted.

Clean polyurethane film was cut into half inch squares prior to the hydrolysis treatment, and placed on clean fibre glass ribbon, which was found to facilitate release and removal of the samples after treatment. Following the accelerated hydrolysis, the samples were transferred immediately to the dry, clean manometer flasks to give a total of one square inch of polyurethane film per flask.

The fungal and soil inocula were prepared in the same way as previously described in sterile E & P nutrient salts solution. The manometer flasks and thermobarometer were also set up in the same way, containing samples of polyurethane film after each period of accelerated hydrolysis. In addition, a control containing only the microbial inoculum and nutrient salts solution was set up.

Results

The processed data from these experiments is illustrated in the form of tables 6(a) - (i) on the following pages (graphical representation proved to be confusing since many of the points for different degrees of hydrolysis coincided or were extremely close, making clear examination difficult). From these results the following general points were noted:-

Taking the incubation period as a whole, the micro-organisms occurring in the soil appeared to utilise the polyurethane film which received the longest hydrolysis period (3 hrs) to the greatest degree. On the first day of incubation, however, the least hydrolysed polyurethane was utilised more readily, but this situation gradually changed during the course of incubation and

on the third day the microbial activity on the 2 hour hydrolysed film was nearly half as much again as the 30 min treatment, and the value of oxygen uptake after 3 hours hydrolysis was almost twice that at 30 mins. The controls of soil plus nutrients only show that throughout the incubation period, the polyurethane film is utilised to different degrees after all hydrolysis treatments. This would be expected since it has already been shown in the previous section of this chapter that the unhydrolysed material is susceptible to direct microbial utilisation and after the hydrolysis treatments there is likely to be a certain percentage of unhydrolysed material present. The important point here is that microbial activity is shown to increase with increasing degrees of hydrolysis. This infers that the meadowland soil under investigation contains microbial species which are able to utilise the unhydrolysed material, and others, or possibly the same species, which are able to utilise products of hydrolysis.

It was also demonstrated that Gliocladium sp. was able to utilise the polyurethane film after all four accelerated hydrolysis treatments. Again this would be expected as it has been shown in section 6(c) of this chapter that this species appears capable of direct utilisation of the unhydrolysed material. Since hydrolysis is probably not 100% after the treatments, there will be some unhydrolysed material remaining to act as a suitable substrate for the microbial enzyme systems. There is no clear evidence that there is increasing utilisation of the synthetic material with increasing hydrolysis. After the two hour treatment there is a two-fold increase in microbial activity, while after the three-hour treatment this activity is only marginally greater

TABLE 6(a) Utilisation of Polyurethane Film by Soil Micro-organisms, following Accelerated Hydrolysis

(Figures represent average Oxygen Uptake in μl)

Treatment \ Time in Hours	Day One				Day Two				Day Three			
	1	3	5	7	1	4	6	7	1	3	5	7
30 mins Accelerated Hydrolysis	1.9	6.4	10.8	15.1	1.1	6.8	8.9	10.9	1.1	3.18	5.4	7.3
1 hour Accelerated Hydrolysis	1.5	4.8	8.1	11.1	0.9	5.4	7.1	8.7	1.0	2.9	5.5	7.1
2 hours Accelerated Hydrolysis	1.1	4.6	7.8	10.8	1.2	6.1	7.9	9.7	1.4	4.3	7.8	10.0
3 hours Accelerated Hydrolysis	1.6	4.7	7.9	10.9	1.4	7.0	9.1	11.0	1.7	5.3	9.7	13.1
Soil + Nutrient Salts only	1.0	3.1	5.6	7.6	0.3	2.5	3.4	4.3	0.7	1.32	2.4	3.0

TABLE 6(b) Utilisation of Polyurethane Film by Gliocladium sp. following Accelerated Hydrolysis

(Figures represent average Oxygen Uptake in μl)

Treatment \ Time in Hours	Day One				Day Two				Day Three			
	2	4	6	8	2	4	6	8	2	4	6	8
30 min Accelerated Hydrolysis	2.97	6.7	9.87	12.83	2.57	5.4	8.3	10.8	3.6	8.0	10.6	14.25
1 hour Accelerated Hydrolysis	2.6	5.57	8.26	10.67	2.2	4.4	6.74	8.6	2.4	5.4	7.3	10.0
2 hours Accelerated Hydrolysis	2.8	5.8	8.8	11.5	2.8	5.7	8.9	12.2	7.1	16.0	22.0	29.1
3 hours Accelerated Hydrolysis	2.73	6.0	8.3	10.8	2.32	4.8	7.3	9.9	4.64	8.61	12.5	16.5
Gliocladium sp. + E & P Nutrient Salts only	1.2	4.9	7.0	8.3	0.5	1.0	1.6	2.2	0.4	0.8	1.0	1.5

TABLE 6(c) Utilisation of Polyurethane Film by Fusarium sp., following Accelerated Hydrolysis

(Figures represent average Oxygen Uptake in μl)

Treatment \ Time in Hours	Day One				Day Two				Day Three			
	1	3	5	7	2	4	6	7	1	3	6	7
30 mins Accelerated Hydrolysis	0.45	2.16	5.2	9.62	2.63	5.44	8.36	9.89	1.72	6.54	11.2	13.8
1 hour Accelerated Hydrolysis	0.35	1.83	4.32	8.35	3.01	5.8	8.72	10.23	1.55	5.26	11.35	13.5
2 hours Accelerated Hydrolysis	0.53	2.1	4.7	8.85	2.3	4.65	7.12	8.3	1.54	5.22	11.07	13.24
3 hours Accelerated Hydrolysis	1.62	4.23	7.21	10.3	1.66	3.25	4.86	5.72	0.93	4.43	8.2	10.2
Fusarium sp. + Nutrient Salts only	0.31	1.63	3.72	6.67	0.15	1.54	2.09	2.48	-	0.5	1.4	1.5

TABLE 6(d) Utilisation of Polyurethane Film by Trichoderma viride, following Accelerated Hydrolysis

(Figures represent average Oxygen Uptake in μl)

Treatment \ Time in Hours	Day One				Day Two				Day Three			
	1	3	5	7	2	4	6	8	1	3	5	7
30 mins Accelerated Hydrolysis	0.5	2.1	6.1	11.14	2.7	5.2	7.75	10.3	1.6	5.0	8.16	11.55
1 hour Accelerated Hydrolysis	0.2	2.0	7.1	12.2	2.1	4.0	6.4	9.1	1.6	4.7	8.0	11.3
2 hours Accelerated Hydrolysis	0.6	3.3	7.84	13.2	3.0	6.2	9.5	12.7	3.0	8.6	14.2	20.3
3 hours Accelerated Hydrolysis	0.4	2.3	7.84	14.4	4.5	9.6	14.8	20.1	4.8	14.5	23.2	33.2
Trichoderma viride + Nutrient Salts only	0.08	1.4	4.9	8.4	1.1	2.25	3.3	3.9	0.7	1.5	2.4	2.7

TABLE 6(e) Utilisation of Polyurethane Film by Aspergillus sp., following Accelerated Hydrolysis

(Figures represent average Oxygen Uptake in μl)

Time in Hours / Treatment	Day One				Day Two				Day Three			
	2	4	6	7	1	3	6	7	2	3	5	6
30 mins Accelerated Hydrolysis	5.2	13.5	24.5	28.3	1.57	5.0	10.7	12.55	5.4	7.9	11.84	17.54
1 hour Accelerated Hydrolysis	4.73	12.0	20.5	24.5	1.23	4.3	9.05	10.63	4.86	6.9	11.76	14.75
2 hours Accelerated Hydrolysis	10.7	19.3	25.2	26.8	1.8	6.03	11.16	13.46	7.92	11.47	19.2	24.6
3 hours Acclerated Hydrolysis	5.7	18.9	23.8	27.4	2.14	7.18	15.7	18.46	9.1	13.14	22.1	26.6
Aspergillus sp. + E & P Nitrient Salts only	1.4	5.0	11.63	14.5	0.31	1.16	2.82	3.3	0.93	1.3	2.25	2.8

TABLE 6(f) Utilisation of Polyurethane Film by Stemphylium lanuginosum, following Accelerated Hydrolysis

(Figures represent average Oxygen Uptake in μl)

Treatment \ Time in Hours	Day One				Day Two				Day Three			
	1	3	5	7	1	3	5	7	2	3	5	7
30 mins Accelerated Hydrolysis	0.3	2.1	4.0	7.2	2.7	7.7	12.4	17.6	5.0	7.2	12.1	17.0
1 hour Accelerated Hydrolysis	0.6	2.2	5.3	11.6	2.6	7.4	12.4	17.2	4.3	6.0	10.7	14.9
2 hours Accelerated Hydrolysis	0.65	2.1	4.1	6.7	2.7	7.9	13.1	18.0	4.8	7.2	11.8	17.0
3 hours Accelerated Hydrolysis	0.8	3.4	8.4	16.5	2.9	8.4	14.1	20.0	6.5	9.4	15.5	21.7
Stemphylium lanuginosum + Nutrient Salts only	0.7	2.5	4.8	7.7	0.5	1.6	2.6	4.0	1.16	1.6	2.4	3.1

TABLE 6(g) Utilisation of Polyurethane Film by Penicillium lilacinum following Accelerated Hydrolysis

(Figures represent average Oxygen Uptake in μl)

Treatment \ Time in Hours	Day One				Day Two				Day Three			
	1	3	5	7	1	3	5	7	1	3	5	7
30 mins Accelerated Hydrolysis	4.3	12.73	20.68	28.64	7.3	20.7	34.8	46.3	6.4	17.3	26.5	36.2
1 hour Accelerated Hydrolysis	3.6	9.0	14.6	20.7	5.2	14.3	24.0	33.0	5.0	14.0	22.0	32.0
2 hours Accelerated Hydrolysis	4.7	13.7	23.0	33.0	7.3	20.0	33.8	43.7	6.8	17.6	26.0	35.8
3 hours Accelerated Hydrolysis	3.9	11.0	18.8	26.9	8.1	22.5	38.1	50.5	7.1	18.0	26.0	33.0
P. lilacinum + Nutrient Salts only	1.24	3.3	4.8	6.2	0.5	1.5	2.7	3.5	0.5	1.1	1.2	2.2

than that recorded after 30 mins hydrolysis. This anomaly may be explained in terms of the mechanism of hydrolysis. There may be intermediate products formed during hydrolysis which can be readily utilised by the species, but which are only transient and after 3 hours accelerated hydrolysis these products may be changed again.

Fusarium sp. also appears to utilise the polyurethane film after all hydrolysis treatments. The interesting point from this part of the study is that there does not appear to be any increase in activity with increasing degrees of hydrolysis. In fact, there is a slight reduction in oxygen uptake during the third day of incubation after a 3 hour hydrolysis treatment. These observations infer that in the case of this species the unhydrolysed part of the material becomes less readily available with increasing hydrolysis, resulting in reduced microbial activity.

Trichoderma viride was shown to have increased activity with increasing degrees of hydrolysis. After the two hour hydrolysis treatment the microbial activity was almost twice that associated with the 30 minute treatment, while after 3 hours hydrolysis the oxygen uptake showed a three-fold increase over that after a 30 min treatment.

Aspergillus sp. also showed increase in oxygen uptake with increasing degrees of hydrolysis. There was in this case, however, a reduction in microbial activity after a 1 hour hydrolysis treatment. This anomaly may be due to operator error in pipetting

the spore suspension when initially setting up the experiment. Alternatively there may be transient intermediates produced during the hydrolysis which tend to either inhibit this species or at least cause the material to be less readily accessible to the enzyme systems of this species.

Stemphylium lanuginosum was shown to utilise the polyurethane film again after all four hydrolysis treatments and there was also a slight increase in microbial activity after the 3-hour hydrolysis treatment. Since this was not very marked and there appeared to be no change in activity after the two-hour treatment it appears that activity of this species is not affected by the extent of hydrolysis.

Penicillium lilacinum showed very similar rates of oxygen uptake after all four hydrolysis treatments and it was demonstrated that this species is capable of utilisation of the polyurethane film both in an unhydrolysed state and after hydrolysis has taken place. This utilisation is however not apparently increased or decreased when hydrolysis has occurred.

Chaetomium globosum was demonstrated to be able to utilise the polyurethane film following hydrolysis, and this activity increased with increasing degrees of hydrolysis. There were however discrepancies observed after the one hour treatment, where a reduction of oxygen uptake was recorded. There was also a reduction after three hours hydrolysis compared to that after two hours, but this reduced activity remained nearly twice that recorded after 30 minutes hydrolysis. These variations may

TABLE 6(h) Utilisation of Polyurethane Film by Chaetomium globosum, following Accelerated Hydrolysis

(Figures represent average Oxygen Uptake in μl)

Time in Hours / Treatment	Day One				Day Two				Day Three			
	1	3	5	7	1	3	5	6	1	3	5	7
30 mins Accelerated Hydrolysis	1.1	4.6	9.3	13.1	1.0	3.3	6.1	7.4	0.97	3.4	6.2	8.8
1 hour Accelerated Hydrolysis	1.6	4.2	6.8	9.2	1.0	3.6	6.1	7.5	0.91	2.5	4.6	6.3
2 hours Accelerated Hydrolysis	2.1	4.9	8.4	10.7	1.2	3.5	6.8	8.3	2.7	7.8	13.7	18.7
3 hours Accelerated Hydrolysis	1.73	8.1	17.5	26.3	2.6	7.5	12.35	14.56	2.4	6.1	10.5	14.0
Ch. globosum + E & P Nutrient Salts only	0.9	4.0	7.7	11.5	0.23	0.7	1.2	1.3	0.1	0.4	1.08	1.47

TABLE 6(i) Utilisation of Polyurethane Film by Aspergillus niger, following Accelerated Hydrolysis

(Figures represent average Oxygen Uptake in μl)

Time in Hours / Treatment	Day One				Day Two				Day Three			
	1	3	5	7	1	3	5	7	1	3	5	7
30 mins Accelerated Hydrolysis	2.2	7.8	12.8	17.4	1.4	4.0	6.6	9.3	1.4	5.0	8.8	12.3
1 hour Accelerated Hydrolysis	1.8	7.1	12.2	16.8	1.4	4.2	7.0	9.8	1.76	6.1	10.7	15.6
2 hours Accelerated Hydrolysis	1.9	7.7	13.2	17.5	1.7	5.2	9.1	13.0	2.7	8.8	14.8	21.7
3 hours Accelerated Hydrolysis	1.0	5.0	9.1	13.9	1.4	4.2	7.0	9.6	1.8	5.8	10.2	15.3
A. niger + Nutrient Salts only	0.15	0.85	1.8	2.9	0.5	1.5	3.0	3.5	0.1	0.7	1.4	2.6

probably be attributed to the difficulty in obtaining a uniform spore suspension when using this spceies, since the spores tend to aggregate in clusters. This may result in different levels of inoculum, and although steps were taken to avoid this possibility, by using glass beads and one drop of Tween 80 to aid disperal of the spores when preparing the suspension, nevertheless it may not have been entirely successful. Another possibility is that transient intermediate products of hydrolysis may have an effect on microbial activity.

Aspergillus niger also showed ability to utilise the polyurethane film after all four hydrolysis treatments, with the activity being comparable after all the treatments with the exception of the activity after two hours hydrolysis. In this case the level of activity was twice as great as that recorded after the 30 min treatment. This may be due to an error when initially pipetting out the spore suspension, but this does not seem to be confirmed by the oxygen uptake figures although the values for the 3 hour treatment are somewhat reduced compared to the others. This reduced activity after 3 hours is maintained through the incubation period although after 3 days the difference has been reduced, indicating that although not shown in the figures there is more increased activity with the most hydrolysed polyurethane and that the low values obtained may have been due to pipetting errors. One may conclude therefore that this species is more easily able to utilise the polyurethane film following increased hydrolytic breakdown.

Comparison of microbial activity associated with the unhydrolysed ester based polyurethane system and that of the

polyurethane following hydrolysis showed that the most frequently encountered species during the ecological studies described in chapter 5 were able to utilise the unhydrolysed polymeric material as a nutritive source. The degree of utilisation by these species was also similar following hydrolysis of the polyurethane inferring that it is the polymer itself which is undergoing biological attack rather than utilisation of any products of hydrolysis.

In the case of those fungal species which were observed rather less frequently during the ecological work, the utilisation of the unhydrolysed material has been shown to be limited. A general trend has been shown however with these species displaying increased microbial activity, following hydrolysis of the polyurethane and this increase is sustained with increasing degrees of hydrolysis. This implies that the ability of these species to utilise the unhydrolysed material is poor, but the products of hydrolysis appear to offer an adequate nutrient supply, in the presence of ancillary nutrients, to sustain fungal growth. It should be pointed out here that the ancillary nutrients did not contain any major carbon source, and it may therefore be concluded that the polyurethane served as the major carbon source for fungal metabolism.

These observations give added weight to the tentative conclusions drawn from the isolation work carried out using the unhydrolysed and the hydrolysed polyurethane film (Chapter 5).

6(e) Discussion and Conclusions

The experimental work described in this chapter has demonstrated that those fungi isolated most frequently during the ecological studies described in chapter five are able to directly utilise polyurethane film as a nutritive source. This utilisation is limited when no ancillary nutrients are present, but microbial activity is substantially increased when other essential nutrients for fungal growth are present. Those species isolated with slightly less frequency during ecological studies also showed ability to utilise this material and their infrequency may be due to competitive factors where the more frequently occurring species possibly have more active enzyme systems. Stemphylium lanuginosum and Chaetomium globosum, which were included in these respirometric studies but not isolated during the previous ecological experiments, also displayed marked ability to utilise this polyurethane material, in agreement with observations made by other workers (Evans and Levisohn 1968), (Darby and Kaplan 1968).

The studies using polyurethane film that had been subjected to accelerated hydrolysis demonstrated that those species most frequently isolated and able to readily utilise the unhydrolysed material did not show any marked increase in microbial activity with increasing degrees of hydrolysis. Other species isolated less frequently were shown however to display increasing activity with increasing degrees of hydrolysis. This may provide an indication as to the reasons why these species were isolated less frequently, since although able to utilise the unhydrolysed material when present in pure culture, they grow more prolifically when partial hydrolysis has occurred. This hydrolysis may be

brought about by the enzyme activity of the most frequently isolated fungi or by physical phenomena allowing increased colonisation by the 'secondary' colonising species.

Work carried out by Evans and Levisohn (1968) demonstrated a number of fungi were able to utilise polyester based polyurethane slabs quite freely, and these workers also found that fungal growth was quicker and more vigorous on autoclaved samples suggesting that hydrolytic deterioration of the polyurethane hastened fungal attack. These ideas were based on observation of growth on slabs of polyurethane and no accurate measurements of growth were recorded to add weight to the observations and conclusions drawn. They also observed that fungal growth was more prolific in water surrounding the polyurethane samples demonstrating that a nutrient fraction of the polyurethane was extractable with water. The results of work described here are in general agreement with the observations of Evans and Levisohn and the respirometric studies may give added weight to such observations. In addition the number of fungal species capable of attacking polyester polyurethane has been shown to be quite diverse.

The major conclusions to be drawn from the experiments so far are that this ester linked polyurethane material is highly susceptible to colonisation by the microfungi occurring in the meadowland soil. This colonisation occurs on both unhydrolysed and hydrolysed material, and the fungi responsible are known to be widely distributed species (Eggins and Malik 1969). It becomes clear therefore that there is indeed an important problem concerning the microbial susceptibility of this material, particularly in applications where this new synthetic material is in close

association with the established material of cotton textile as previously discussed in this thesis. This is particularly important when considering that some of the fungi associated with the direct colonisation of polyurethane also possess strong cellulolytic properties (Siu 1951).

CHAPTER SEVEN

INVESTIGATIONS INTO THE FUNGICIDAL PROTECTION OF POLYURETHANES, AND THE PHYSICAL ASSESSMENT OF BIOLOGICAL ACTIVITY AND HYDROLYTIC SUSCEPTIBILITY ASSOCIATED WITH THESE MATERIALS

7(a) Introduction

7(b) Respirometric studies using polyurethane film with the new fungicides

- (i) Mixed soil inocula
- (ii) Pure fungal culture inocula

7(c) Tensile Strength Measurement

7(d) Infra red spectrophotometric analysis

7(e) Dielectric constant measurement

7(f) Dynamic visco elastometer measurement

7(g) Discussion and Conclusions

7(a) INTRODUCTION

In the previous chapter, it was demonstrated that the polyurethane film under investigation was susceptible to direct fungal attack, and that this colonisation was likely to occur on the unhydrolysed and hydrolysed material. It was also found that some of the fungi involved in this colonisation of the polyurethane material were also known to exhibit cellulolytic properties, e.g., Fusarium sp., Chaetomium globosum and Trichoderma viride. In cases where the fungi were isolated with reduced frequency, the colonisation of the synthetic material was found to be enhanced after partial hydrolysis of the material.

These observations clearly indicate the important problems associated with the use of this polyurethane material, particularly when considering situations where the new synthetic material is used in conjunction with cellulosic materials such as cotton textile under conditions which may be favourable for microbial colonisation to occur. One such example is to be found in the shoe industry where there is increasing usage of new polyurethane materials such as poromerics which are basically made of polyurethane elastomers together with various textile fibres. They are multi layer structures with the urethane impregnated textile component forming a flexible and mechanically strong substrate. These are now being used as alternatives to the traditional shoemaking material of leather. Even when leather itself is used, it is frequently finished with synthetic materials such as polyester and polyether polyurethane films to improve the aesthetic appearance of the finished article and improve the resistance of the leather to scuffing and

other abrasion. In this situation cellulosic materials and synthetic polyurethanes are in close association since the former are widely used in shoe manufacture as linings, interfacings and support structures. There will also tend to be warm, moist conditions occurring in this situation, accompanied by a build up of perspiration, and under these conditions the polyurethane material may be rapidly hydrolysed (Hole 1969).

Catomance Ltd., are concerned with the biocidal protection of cellulosic materials in general, as previously discussed in chapter one, and under these new situations which are increasingly arising, the interest of this company is being extended into the problems arising from such associations with reference to susceptibility to biodeterioration. As a result of this interest, coupled with the susceptibility of the ester based polyurethanes to fungal attack, a development programme was carried out by the chemists at Catomance Ltd., to formulate fungicides for incorporation into polyurethane materials in order to offer adequate protection against fungal attack, while at the same time, not adversely affecting the more desirable properties of the polymeric material.

Such chemical research produced three possible fungicides designed particularly for the protection of polyurethane. These were:-

Code letter

A = Cumyl phenoxy isopropanol

B = Cumyl phenoxy isopropyl acetate

C = Iso propyl derivative of benzthiazole.

For convenience these code letters will be used to refer to these products in further descriptions and discussion.

This new family of fungicides is similar to those already investigated in chapter three with reference to the fungicidal protection of cotton textiles, the isoprpyl radical being the common portion of the molecule around which modification is made.

The aim of these experiments was to evaluate the efficacy of the new organic fungicides with particular reference to the protection of ester linked polyurethanes, firstly using the general mixed microbial population present in the soil, and secondly using pure cultures of those fungi already shown to be associated with the direct colonisation of this synthetic material in either unhydrolysed or hydrolysed form.

Another interesting problem associated with ester based polyurethane film was that of assessing the extent of hydrolysis and biological attack of this material by physical methods. This problem is also of interest to Catomance Ltd., from the point of view of possibly predicting the microbial susceptibility of a particular material or degree of hydrolysis so that a tailor-made product may be produced by changing the polymeric material itself and/or by the addition of suitable fungicidal chemicals, thus offering a range of materials of known susceptibility.

It has been demonstrated in this thesis that the polyester polyurethane under investigation is susceptible to direct colonisation by microfungi. Any physical technique therefore which assists assessment of such activity would be of value not only as a routine

procedure, but also as a possible research tool, monitoring biological activity in a quantitative manner.

The aim of the experiments described in this chapter in addition to those described above was to attempt to monitor the hydrolysis of the polyurethane film after accelerated hydrolytic treatment, and to correlate these observations with those obtained following biological colonisation by the microfungi known to actively utilise the polyurethane film.

7(b) Respirometric Studies using Polyurethane Film and the new Fungicides

(i) Mixed soil inocula

In this experiment the manometric technique described in chapter six was used to measure oxygen uptake in investigating the effect of the new fungicides on the general microflora of meadowland topsoil concerning their ability to utilise the ester based polyurethane film.

The soil suspension was prepared in the same manner as previously described (chapter 6), using 25g of soil in 100 ml sterile nutrient salts solution (Eggins and Pugh 1962). The fungicides were pipetted into this suspension to give final concentrations of 0.5%, 1.0%, and 2.0% based on the volume of the soil suspension. Controls were also set up containing soil in nutrient salts only, and soil, nutrient salts and polyurethane film but without fungicide. There was no major carbon source present in the nutrient salts, other than which might occur in the soil itself and be carried over in the preparation of the initial suspension.

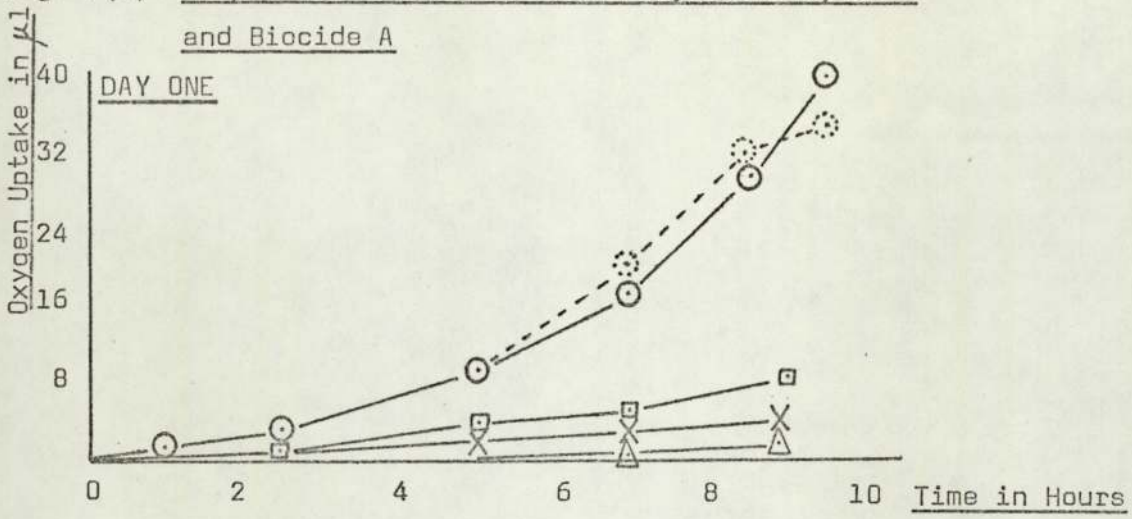
The polyurethane film samples were also prepared in the same way as described in previous experiments with a total of one square inch of the synthetic material in each flask, which had previously been cleaned with chromic acid, thoroughly washed in tap and distilled water, sterilised by autoclaving and dried in a desiccator, prior to each experimental run.

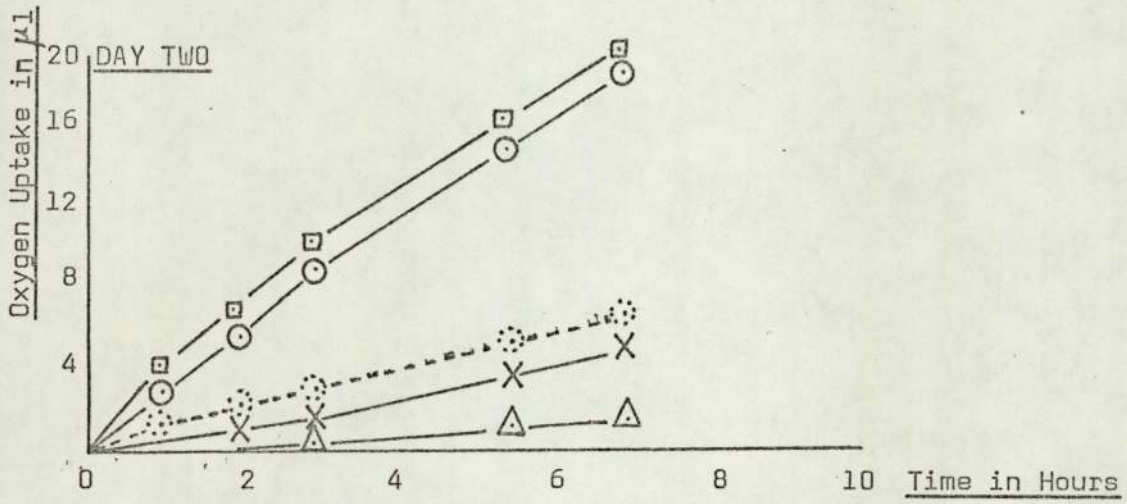
4 ml portions of the soil suspensions were pipetted carefully into the main compartment of the manometer flasks containing the polyurethane film with 0.5 ml 20% KOH and the standard size piece of filter paper being placed in the centre well of the flask. The manometers were assembled in the usual way and allowed to equilibrate, at 30^{o}C with an oscillation rate of 70 cycles/minute, for a period of one hour. After this period, all taps were closed before experimental observations were carried out. Each experimental run was over a three day incubation period, readings being taken at regular intervals throughout this period. During each night, the taps were opened to prevent the manometer fluid being drawn into the reaction flask in the event of exceptionally rapid oxygen uptake.

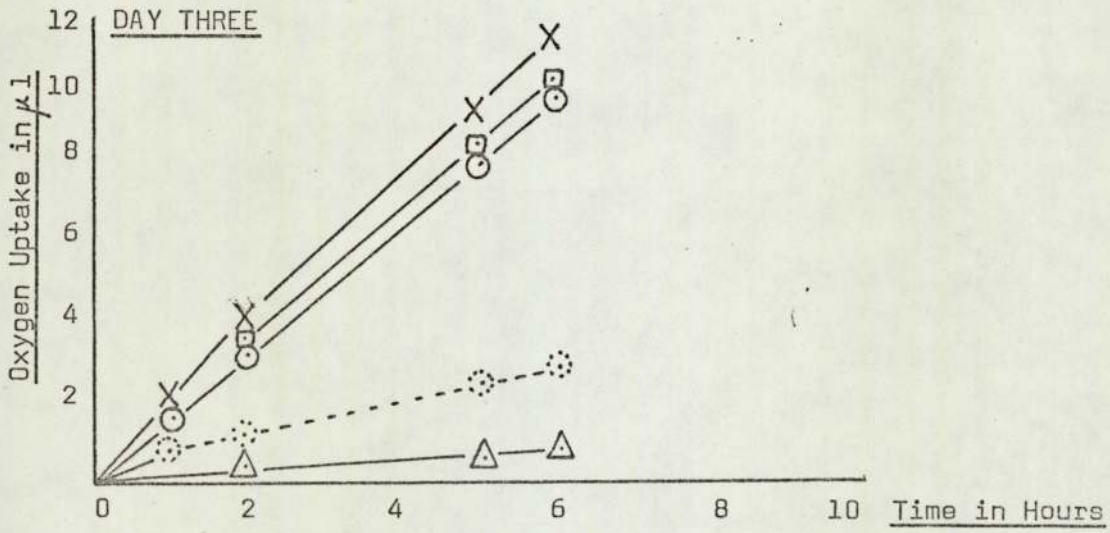
Results

The raw data was obtained and processed as described in chapter six to determine the average oxygen uptake throughout the experimental period. These average values are shown represented in graphical form (Figs. 7(i) - (iii) and from which the following general points may be observed.

Fig. 7(i) Respirometric Studies with Polyurethane, Soil and Biocide A







KEY:- —⊙—⊙— = Polyurethane + nutrient salts + soil only

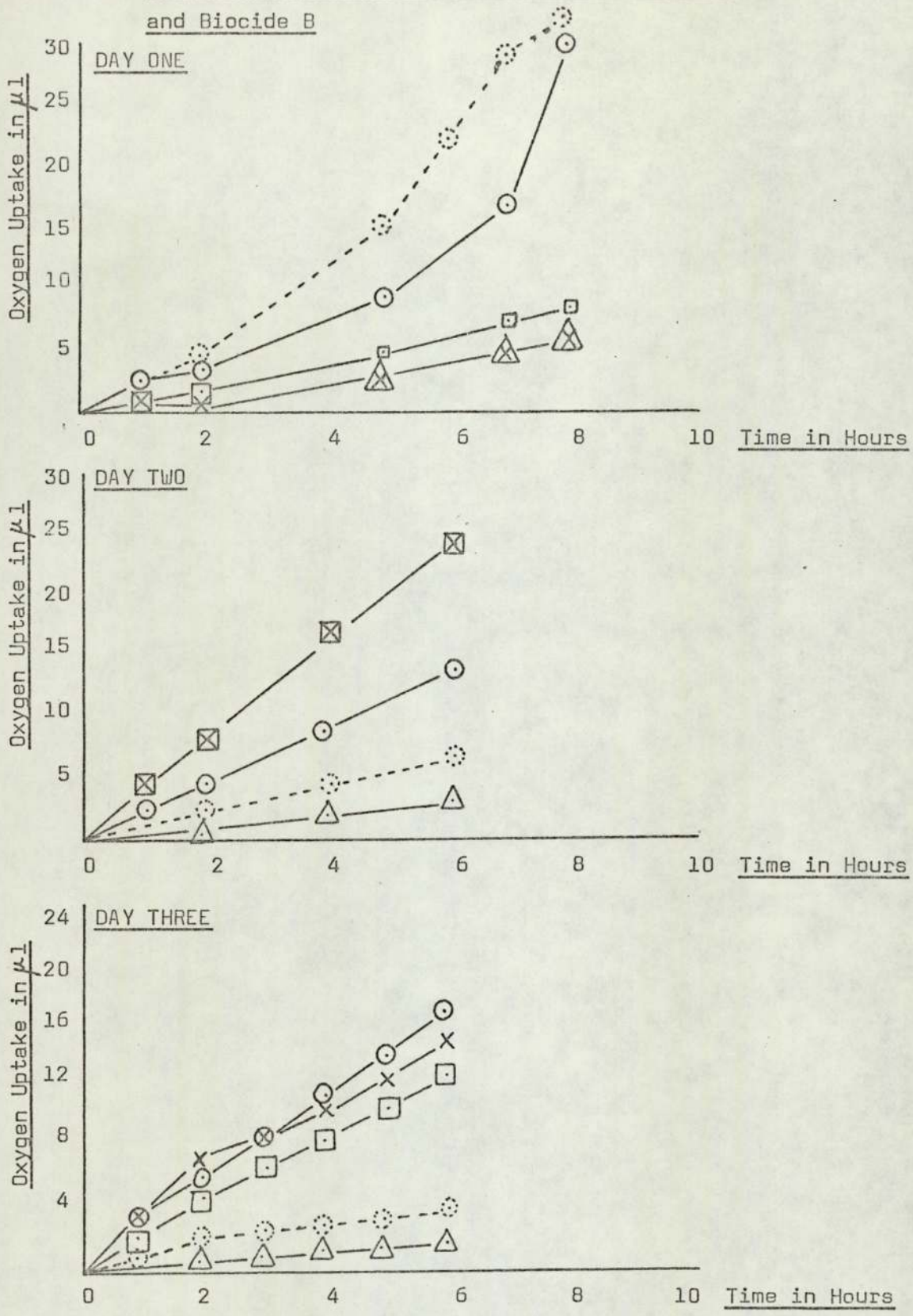
—□—□— = Polyurethane + nutrient salts + 0.5% Biocide A

—X—X— = Polyurethane + nutrient salts + 1.0% Biocide A

—△—△— = Polyurethane + nutrient salts + 2.0% Biocide A

--⊙---⊙-- = Control (Nutrient salts + soil only)

Fig. 7(ii) Respirometric Studies with Polyurethane, Soil and Biocide B



KEY:- —⊙—⊙— =Polyurethane, nutrient salts and soil only

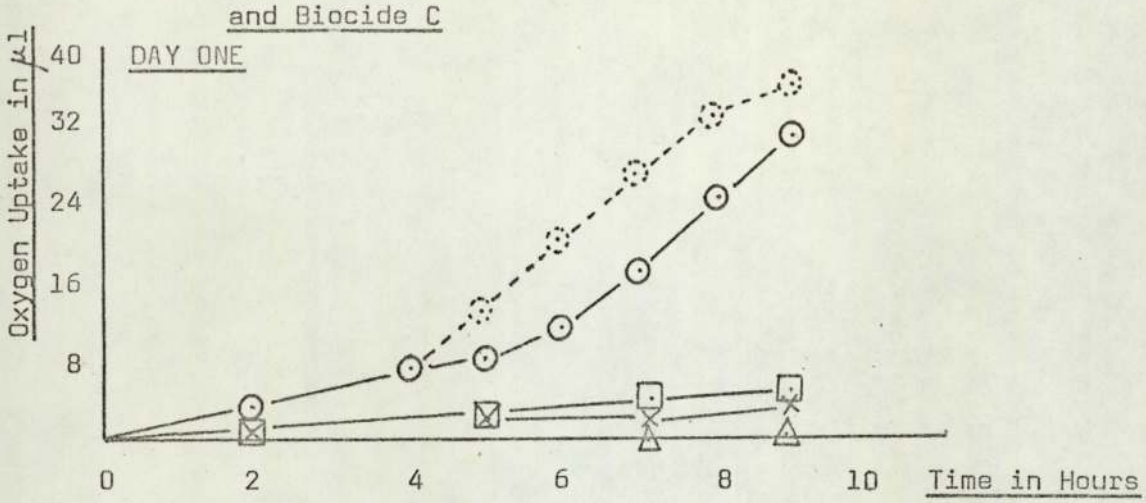
—⊡—⊡— =Polyurethane, nutrient salts + 0.5% Biocide B

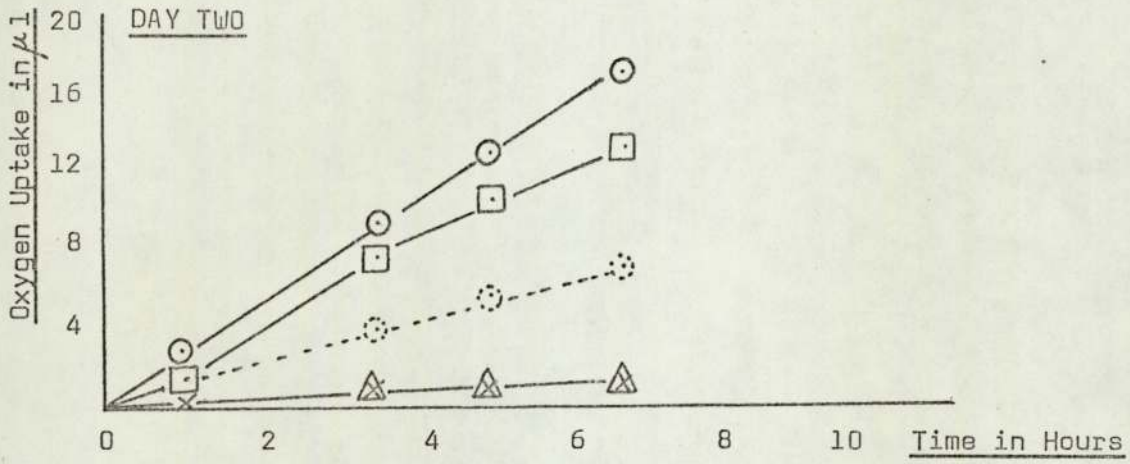
—X—X— =Polyurethane, nutrient salts + 1.0% Biocide B

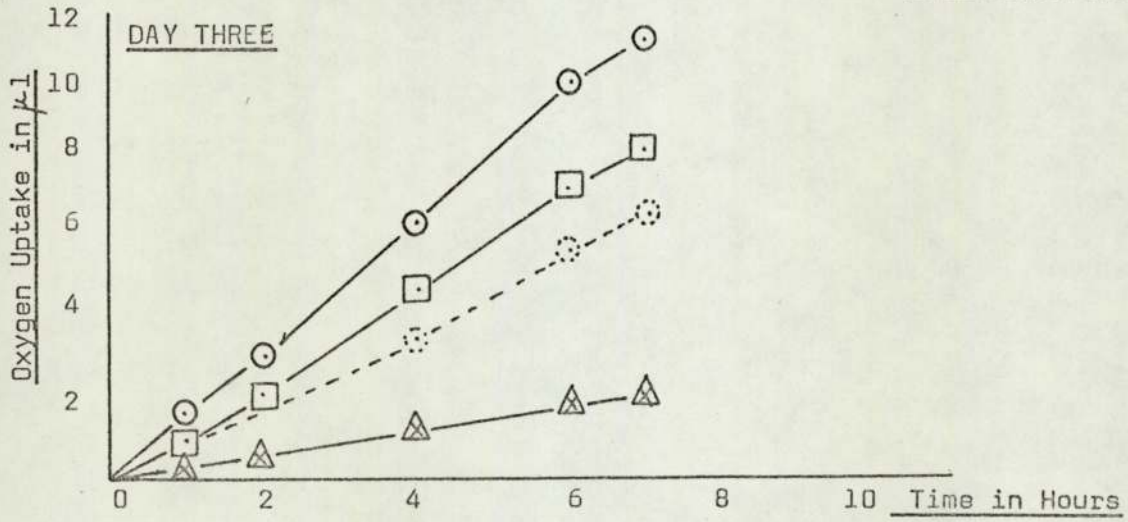
—△—△— =Polyurethane, nutrient salts + 2.0% Biocide B

---⊙---⊙-- =Nutrient salts + soil only

Fig. 7(iii) Respirometric Studies with Polyurethane, Soil and Biocide C







KEY:- —⊙—⊙— = Polyurethane, nutrient salts and soil only

—⊡—⊡— = Polyurethane, nutrient salts + 0.5% Biocide C

—X—X— = Polyurethane, nutrient salts + 1.0% Biocide C

—△—△— = Polyurethane, nutrient salts + 2.0% Biocide C

---⊙----⊙-- = Nutrient Salts + Soil only

Fungicide A Fig.7(ii):- On the first day of incubation, all three concentrations of the fungicide prevented the start of microbial growth, while the controls displayed the expected activity. During the second day, only the 1.0% and 2.0% concentrations offered adequate suppression of microbial activity, and this was reduced still further when only the 2.0% level offered protection on the third day of incubation. The control of polyurethane film, soil suspension and nutrient salts, demonstrated again the ability of the soil micro-organisms to utilise the polymeric material and this was also observed when microbial activity commenced in the presence of the lower concentration of the fungicide.

During the second and third day of incubation it was observed that the microbial activity associated with the 0.5% and 1.0% concentrations of fungicide appeared to be greater than that of the control without any fungicide present. This may be explained in terms of the delay in microbial activity caused by these fungicides resulting in the rapid (log) phase of growth, when the ancillary nutrients are used to a great extent, occurring at a time when the controls are undergoing a reduction of activity as the plateau of the growth curve is approached, thus leading to the observations made during these experiments. Alternatively the fungicide may in fact actually stimulate increased growth responses at these concentrations, after the initial suppression of activity.

Fungicide B, Fig. 7(ii):- A similar pattern of microbial activity to that described above was recorded. There was a suppression of activity during the first day of incubation, but not with the same effectiveness as fungicide A. During the second day of

incubation only the 2.0% level of fungicide prevented microbial growth, and this was maintained for the remainder of the incubation period. Again utilisation of the polyurethane film was demonstrated by reference to the controls.

Fungicide C. Fig 7(iii):- Again a similar pattern of activity was recorded. The controls displayed the usual growth curve response and utilisation of the polyurethane film. Only the 1.0% and 2.0% levels of fungicide proved effective throughout the duration of the experiment, while the 0.5% level proved effective in delaying microbial growth only during the first day of incubation.

Comparing the three fungicides, it appeared that product C offered the most adequate protection against microbial growth at levels of 1.0%, followed by fungicide A which was effective at 1.0% and 2.0% after two days incubation but only the 2.0% level proved effective over the three day period. Product B offered adequate protection only at the 2.0% level even after only one day incubation period.

One observation made during the course of this experiment was that when the higher concentrations of fungicide were pipetted into the manometer flasks, there was a small amount of adhesion of the fungicide to the glass wall of the pipette. The extent of this adhesion was minimal and the majority remained as an emulsion in the spore suspension, but this may have had an effect on the final concentrations of fungicide. Despite this difficulty however, the general trends outlined may be considered to hold.

It may be concluded from these preliminary studies that all three new fungicides offer a broad effectiveness, concentrations of 2.0% aopearing to offer most adequate protection over the short duration of these experiments. This situation may however alter after longer term incubation, but limited time prevented long term observations to be carried out. However since these experiments were carried out under microbiologically very favourable conditions using heavy inocula it was considered to be a severe accelerated test under conditions which would not normally be expected to occur continually in the natural environment, and as such may be considered adequate to gain information concerning general trends.

7(b) (ii) Pure Fungal Culture Inocula

In these experiments, the Warburg respirometer was used to investigate the effect of the new family of organic fungicides on the fungal colonisation of polyurethane film. The fungi used were those already shown to actively utilise the synthetic material, and the experimental method was carried out in the same manner as described in chapter six of this thesis.

The spore suspensions used in these experiments were prepared, as previously described (chapter six), in Eggins and Pugh basic nutrient salts solution, and the fungicides were added to give final concentrations of 0.5%, 1.0% and 2.0%, based on the volume of the suspension. Two sets of controls were set up containing i) the spore suspension and nutrient salts only and ii) polyurethane film, spore inoculum with nutrient salts but without added fungicide. There was again no major carbon sources offered in the nutrient salts solution.

The polyurethane samples were also prepared as before and placed in the main compartment of clean, dry and sterile manometer flasks prior to inoculation with 4 mls of the spore suspensions with and without added fungicides. 0.5 ml 20% KOH was again pipetted carefully into the centre well of the flask accompanied by a standard sized piece of filter paper for improved CO_2 absorption. The apparatus was assembled in the usual way, and allowed to equilibrate for one hour before the taps were closed and experimental observations commenced. The duration of each experiment was again over a three day period with the taps being opened overnight.

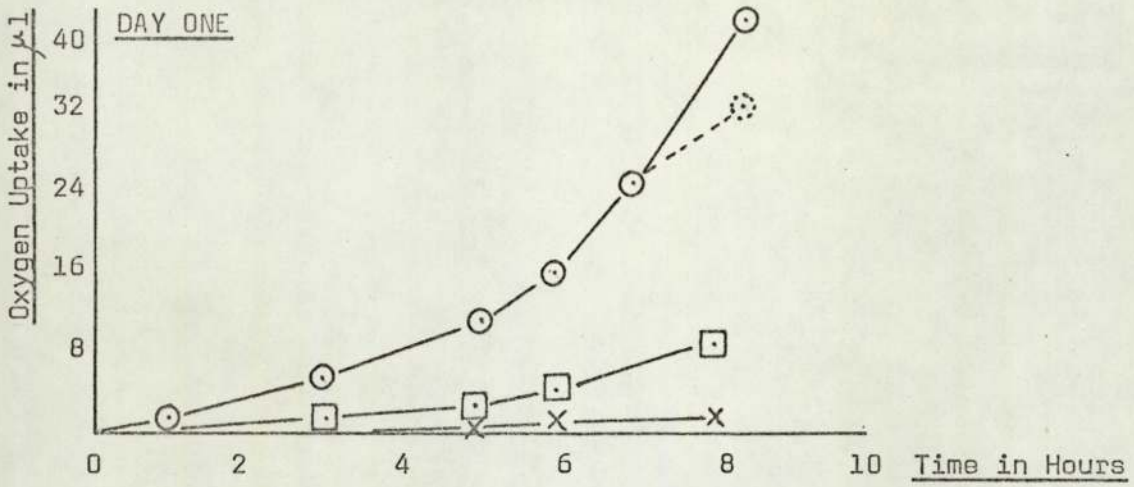
Lack of time prevented the desired comprehensive study of all the fungal species with all three fungicides over long periods and these initial evaluation studies had to be confined to those results described here.

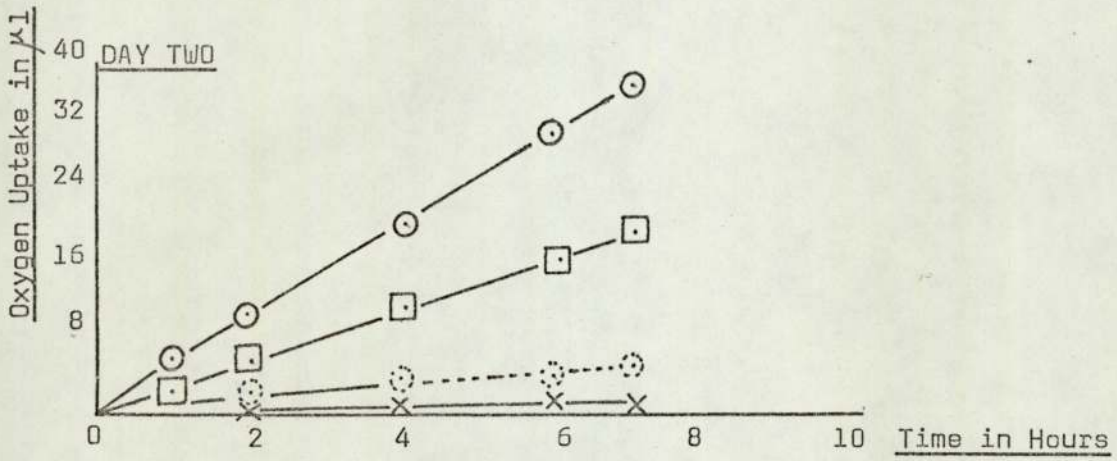
Results

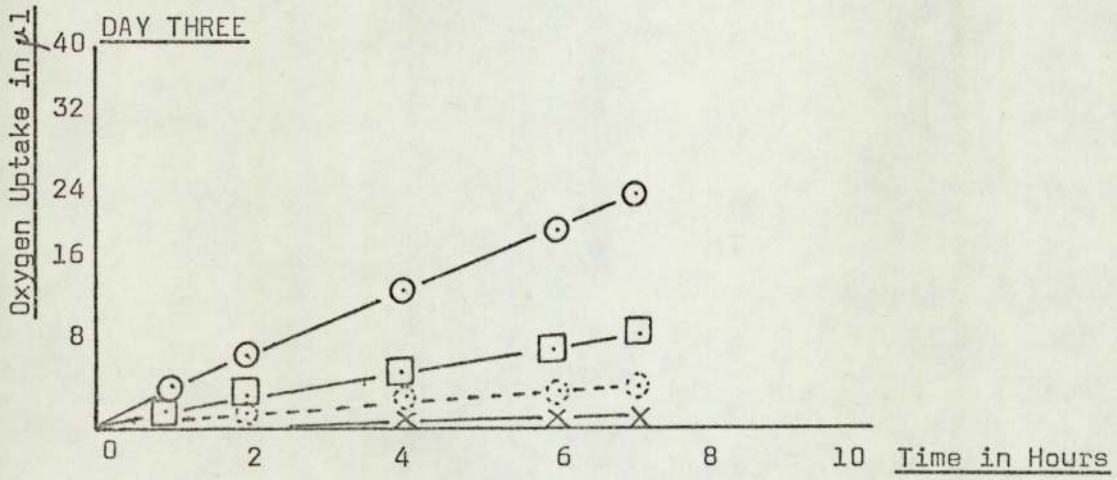
The average oxygen uptake values against time of incubation are represented in graphical form (Figs 7(iv) - (xiii)). The major points of interest arising from this experimentation are as follows:-

Gliocladium sp. was effectively inhibited by 1.0% and 2.0% concentration of fungicide A throughout the three day incubation period. The 0.5% concentration delayed the start of microbial activity but proved inadequate once microbial growth had begun. The direct utilisation of the polyurethane film was again demonstrated by this species. Fungicide B proved slightly less

Fig. 7(iv) Respirometric Studies using Polyurethane, Gliocladium sp. and Biocide A







KEY:- —⊙—⊙— = Polyurethane, nutrient salts and Gliocladium sp. only

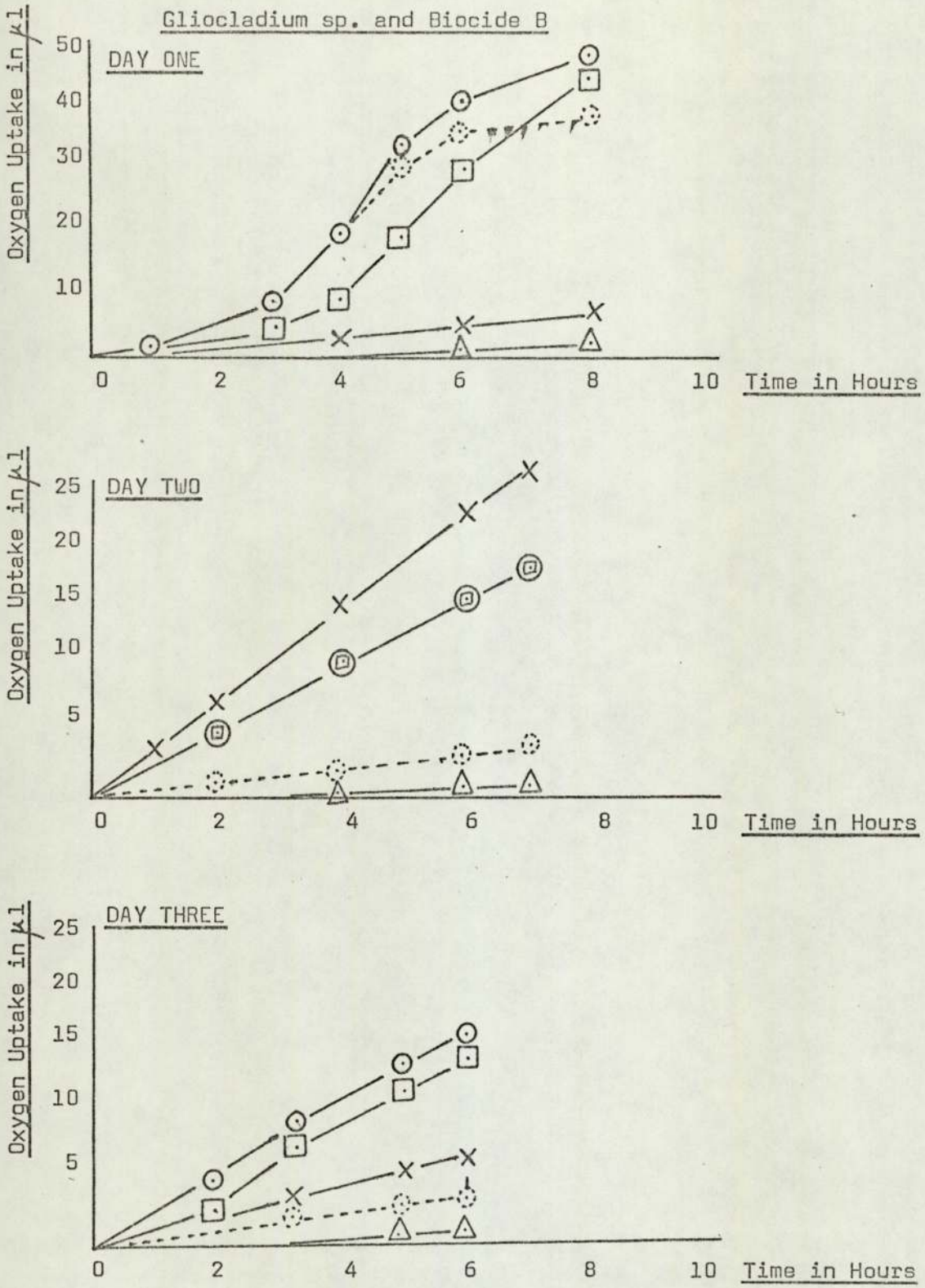
—⊡—⊡— = Polyurethane, nutrient salts + 0.5% Biocide A

—X—X— = Polyurethane, nutrient salts + 1.0% Biocide A

—△—△— = Polyurethane, nutrient salts + 2.0% Biocide A

--⊙--⊙-- = Nutrient salts + Gliocladium sp. only

Fig. 7(v) Respirometric Studies using Polyurethane, Gliocladium sp. and Biocide B



KEY:- —⊙—⊙— = Polyurethane, nutrient and Gliocladium sp. only

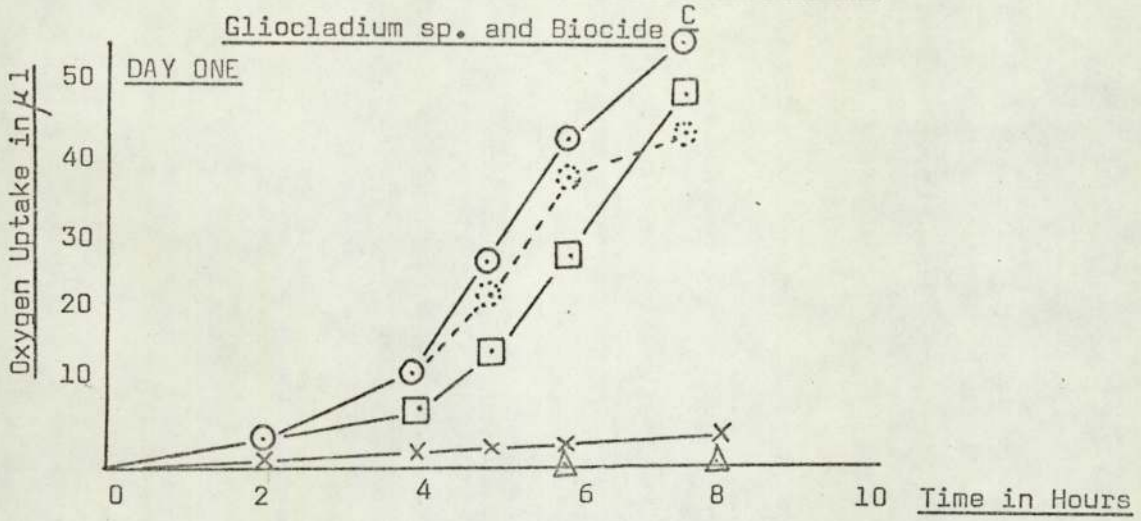
—⊡—⊡— = Polyurethane, nutrient salts and 0.5% Biocide B

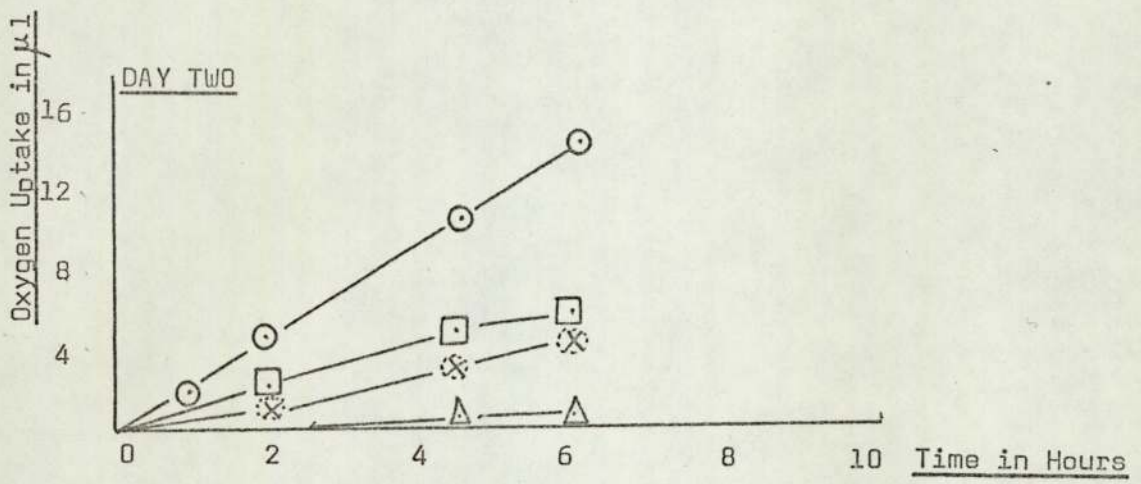
—X—X— = Polyurethane, nutrient salts and 1.0% Biocide B

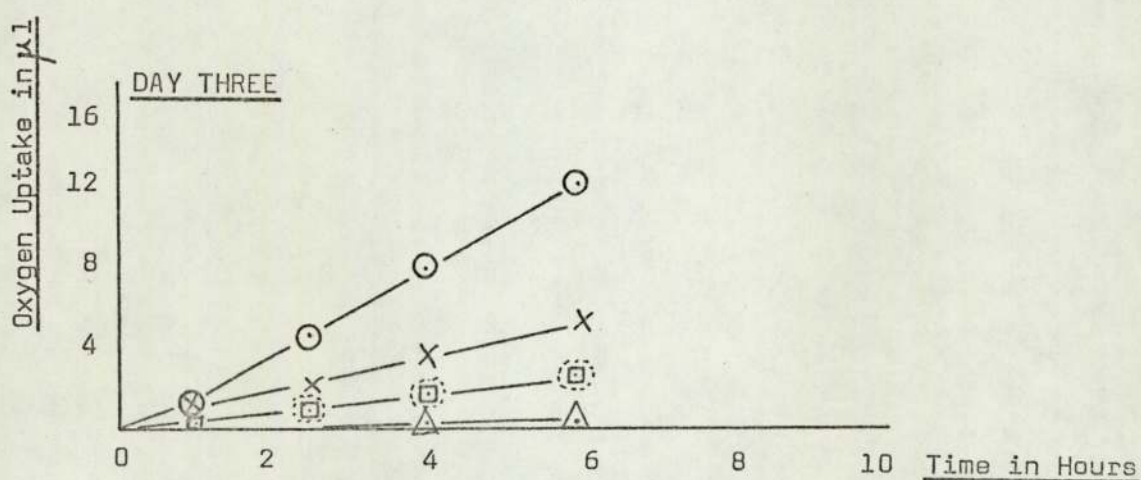
—△—△— = Polyurethane, nutrient salts and 2.0% Biocide B

---⊙---⊙--- = Nutrient salts + Gliocladium sp. only

Fig. 7(vi) Respirometric Studies using Polyurethane, Gliocladium sp. and Biocide C







KEY:- -⊙—⊙- = Polyurethane, nutrient salts and Gliocladium sp. only

-⊡—⊡- = Polyurethane, nutrient salts + 0.5% Biocide C

-X—X- = Polyurethane, nutrient salts + 1.0% Biocide C

-△—△- = Polyurethane, nutrient salts + 2.0% Biocide C

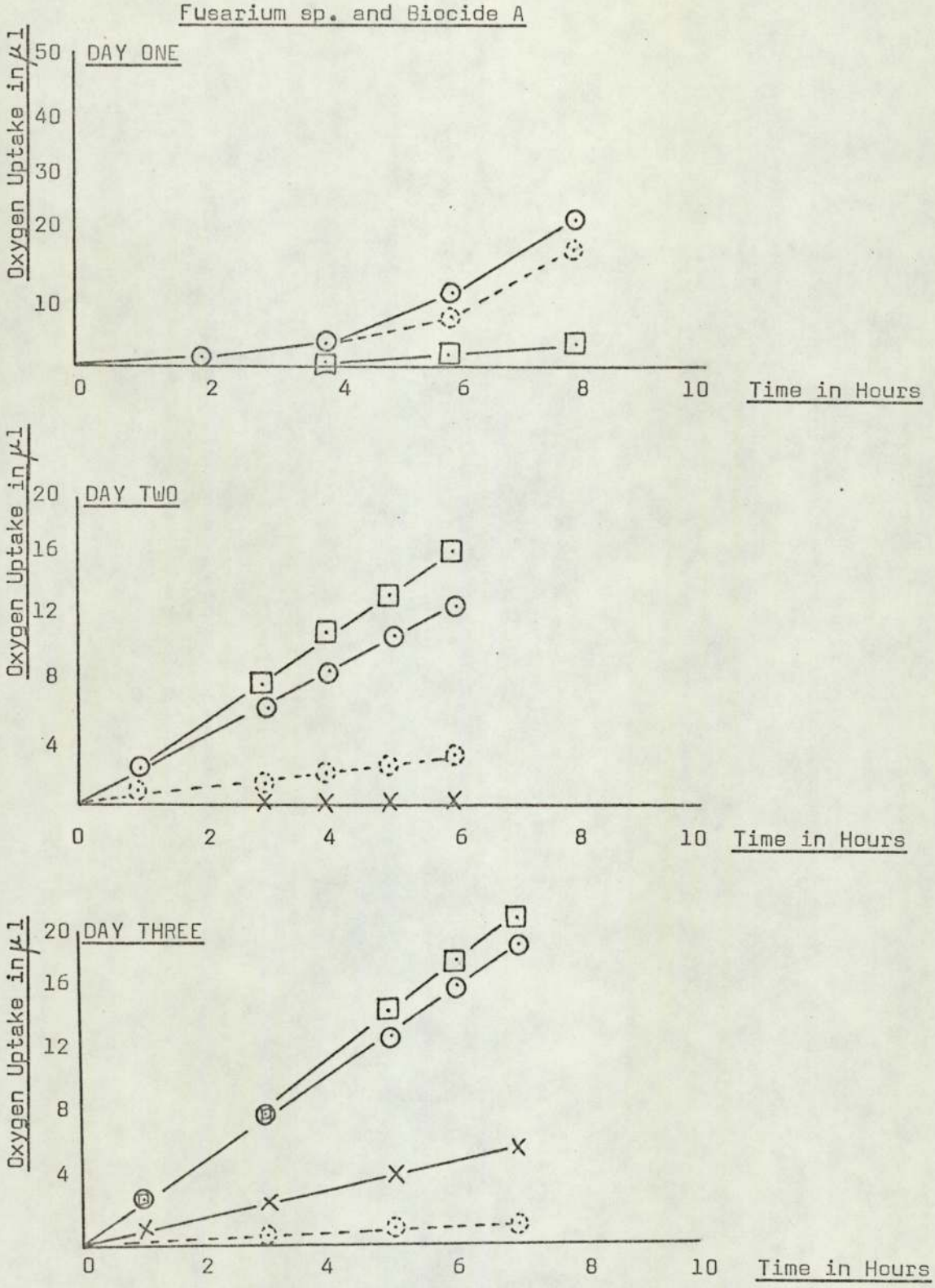
--⊙---⊙--= Nutrient Salts + Gliocladium sp. only

effective offering adequate protection only at the 2.0% concentration, although the 1.0% level did inhibit the microbial activity to a limited degree, but not entirely. The anomalies of apparent increased microbial activity with fungicide compared to the controls was also observed during the second day of incubation. This may be explained as outlined in the discussion of the results concerning the soil inoculum and fungicide A (page 177). Fungicide C produced a similar pattern of microbial activity with only the 2.0% level offering adequate protection throughout the incubation period.

Fusarium sp. was also inhibited by fungicide A, but only the 2.0% concentration offered adequate protection throughout the three day experimental period. The 1.0% level did however delay the start of microbial activity during the first and second day of incubation, but this was not maintained on day three.
A similar pattern of fungal growth was also obtained in the presence of fungicide B, with similar anomalies of increased activity occurring in the presence of 0.5% and 1.0% fungicide compared to controls, which may again be explained as described elsewhere in this chapter. Fungicide C also appeared to have a similar effect on microbial activity, but from the results appeared to be more effective at the 1.0% concentration compared with products A and B, and the 2.0% level of product C offered the most adequate degree of protection.

Owing to limited time available for these studies, the remainder of the fungi investigated here were studied only in the presence of fungicide C. This product was considered by Catomance Ltd., to have the greatest possibilities regarding the fungicidal protection of polyurethane systems in general, and

Fig. 7(vii) Respirometric Studies using Polyurethane, Fusarium sp. and Biocide A



KEY:- —⊙—⊙— = Polyurethane, nutrient salts and Fusarium sp.only

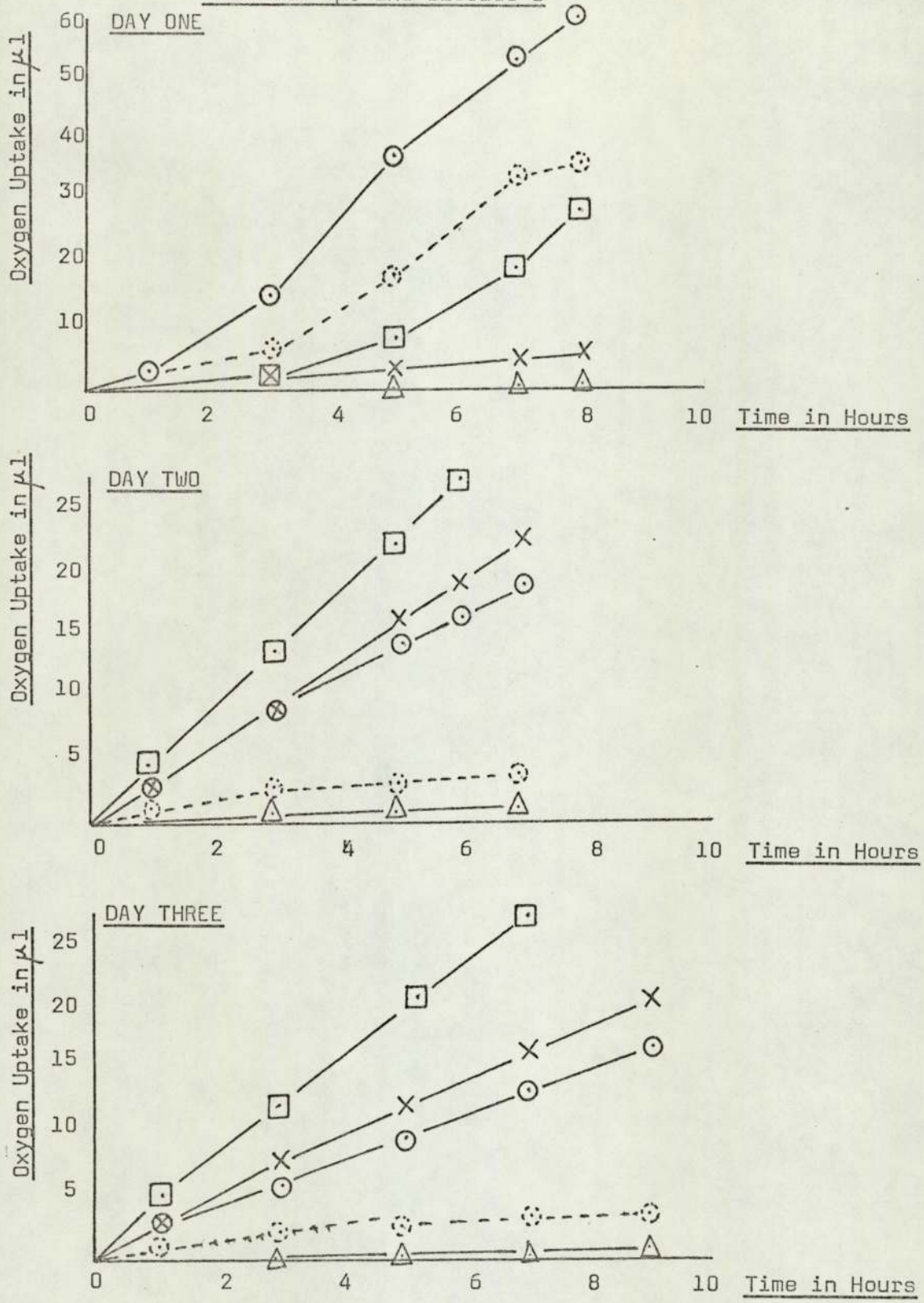
—⊡—⊡— = Polyurethane, nutrient salts + 0.5% Biocide A

—X—X— = Polyurethane, nutrient salts + 1.0% Biocide A

—△—△— = Polyurethane, nutrient salts + 2.0% Biocide A

--⊙--⊙-- = Nutrient Salts + Fusarium sp. only

Fig. 7(viii) Respirometric Studies using Polyurethane, Fusarium sp. and Biocide B



KEY:- —⊙—⊙— = Polyurethane, nutrient salts and Fusarium sp.only

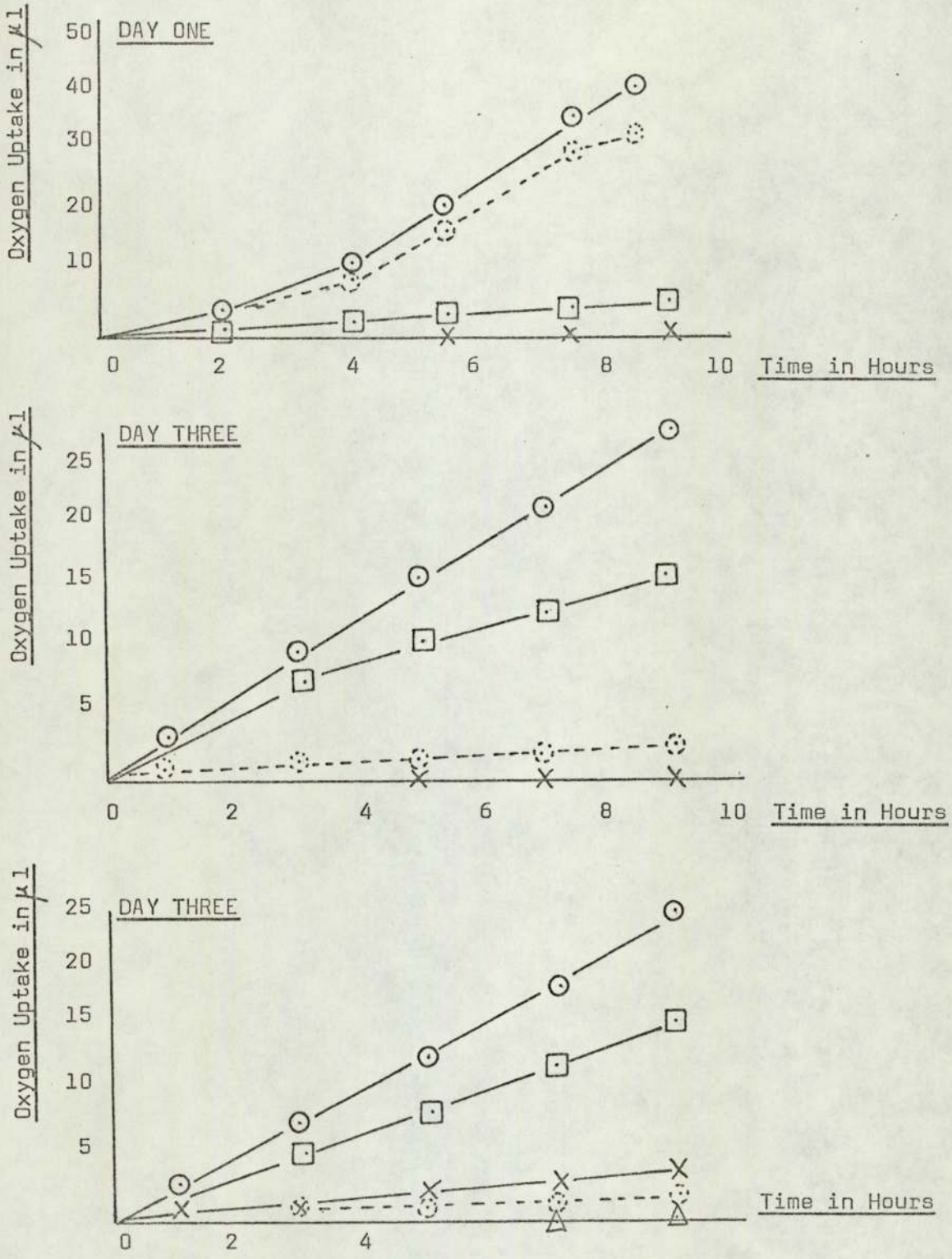
—⊡—⊡— = Polyurethane, nutrient salts + 0.5% Biocide B

—X—X— = Polyurethane, nutrient salts + 1.0% Biocide B

—△—△— = Polyurethane, nutrient salts + 2.0% Biocide B

--⊙---⊙-- = Nutrient salts + Fusarium sp. only

Fig. 7(ix) Respirometric Studies using Polyurethane, Fusarium sp. and Biocide C.



KEY:- —⊙—⊙— = Polyurethane, nutrient salts and Fusarium sp. only

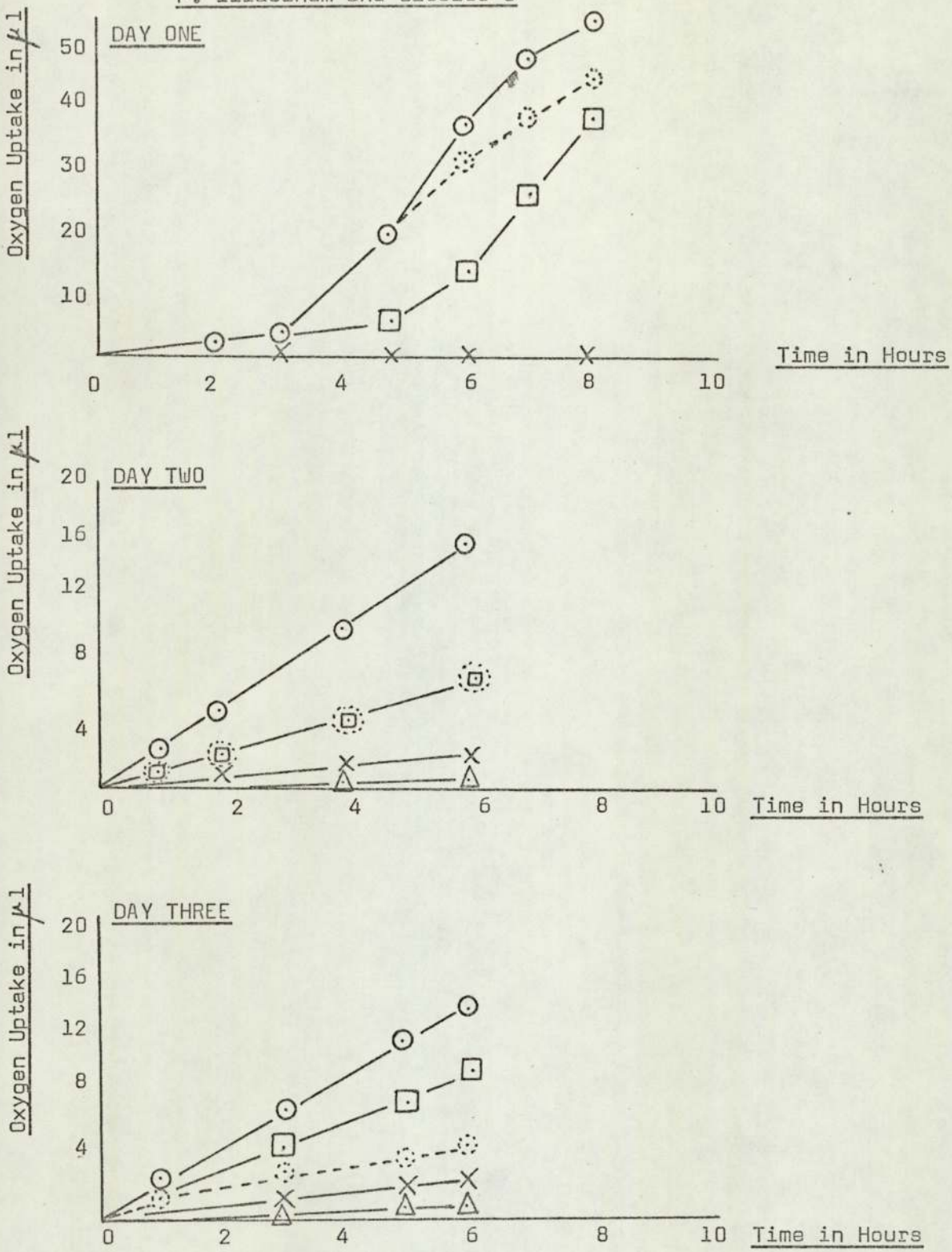
—⊡—⊡— = Polyurethane, nutrient salts + 0.5%Biocide C

—X—X— = Polyurethane, nutrient salts + 1.0% Biocide C

—△—△— = Polyurethane, nutrient salts + 2.0% Biocide C

--⊙---⊙-- = Nutrient salts + Fusarium sp. only

Fig. 7(x) Respirometric Studies using Polyurethane, P. lilacinum and Biocide C



KEY:- -⊙—⊙- = Polyurethane, nutrient salts and P. lilacinum only

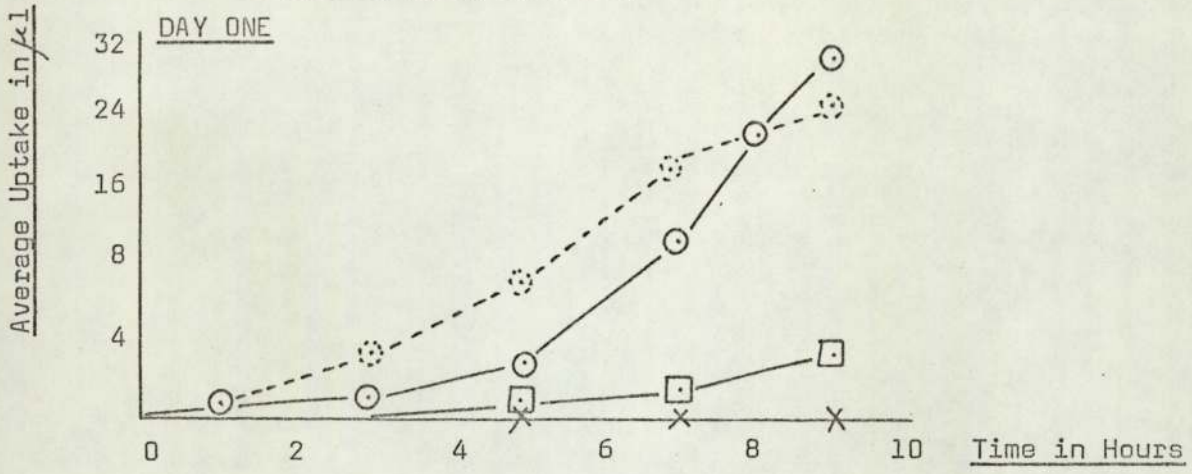
-⊡—⊡- = Polyurethane, nutrient salts + 0.5% Biocide C

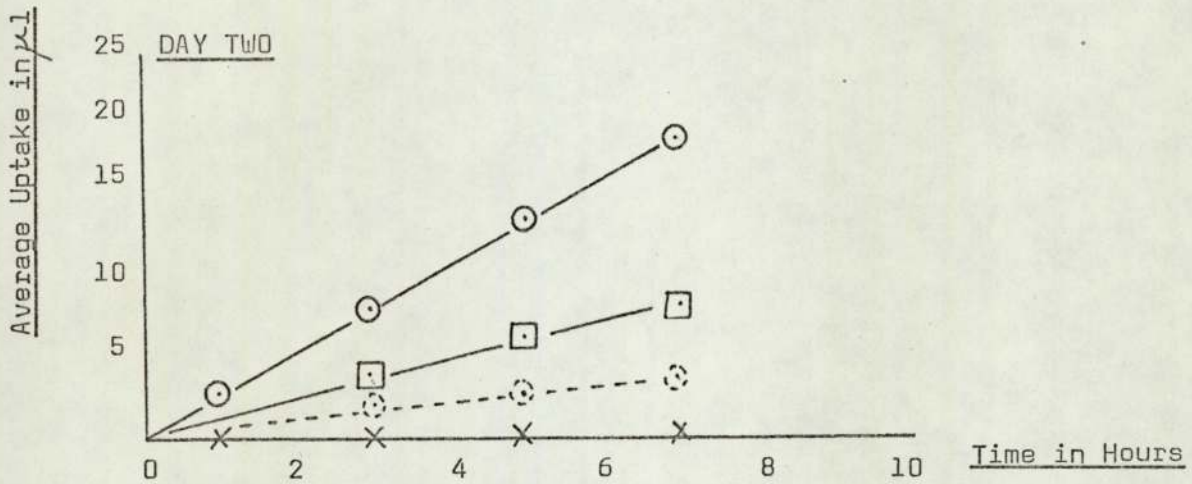
-X—X- = Polyurethane, nutrient salts + 1.0% Biocide C

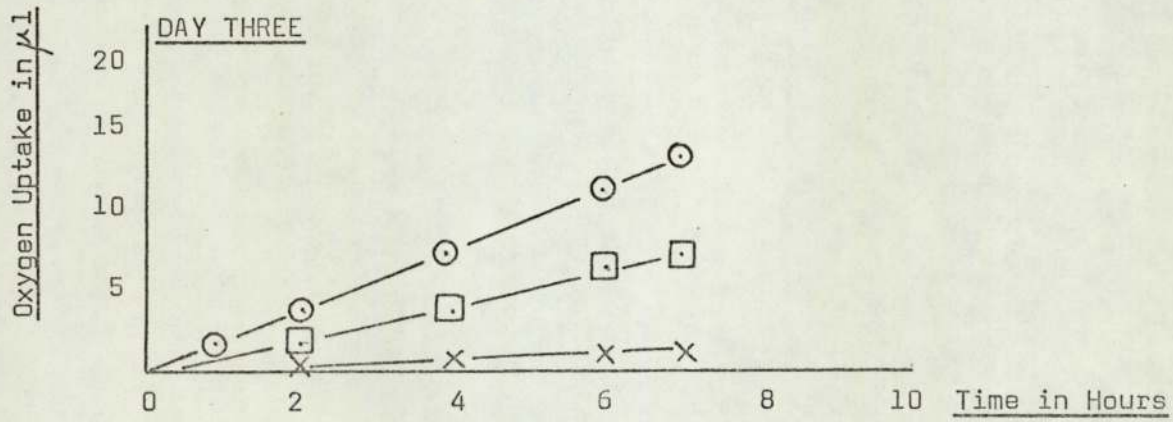
-△—△- = Polyurethane, nutrient salts + 2.0% Biocide C

--⊙---⊙-- = Nutrient salts + P. lilacinum only

Fig. 7(xi) Respirometric Studies using Polyurethane, T. viride and Biocide C







KEY:- —⊙—⊙— = Polyurethane, nutrient salts and T. viride only

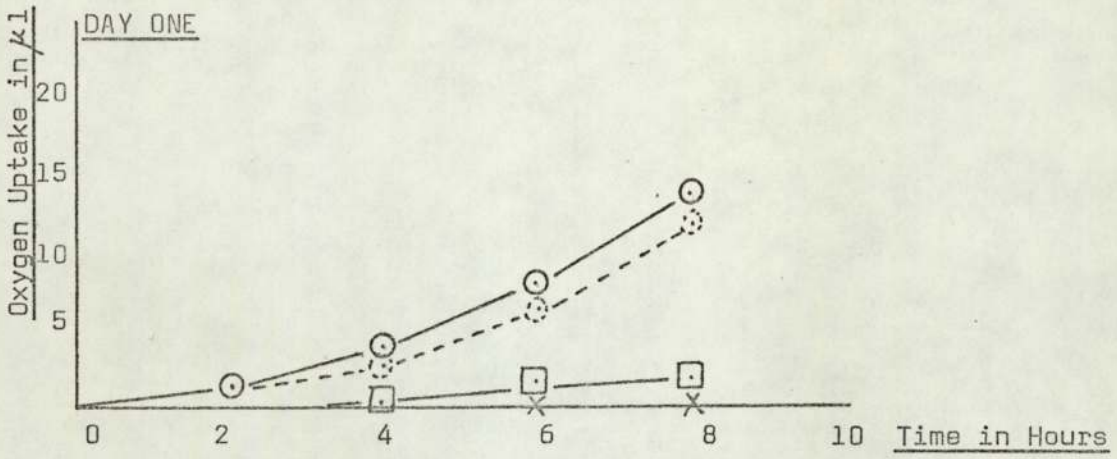
—⊡—⊡— = Polyurethane, nutrient salts + 0.5% Biocide C

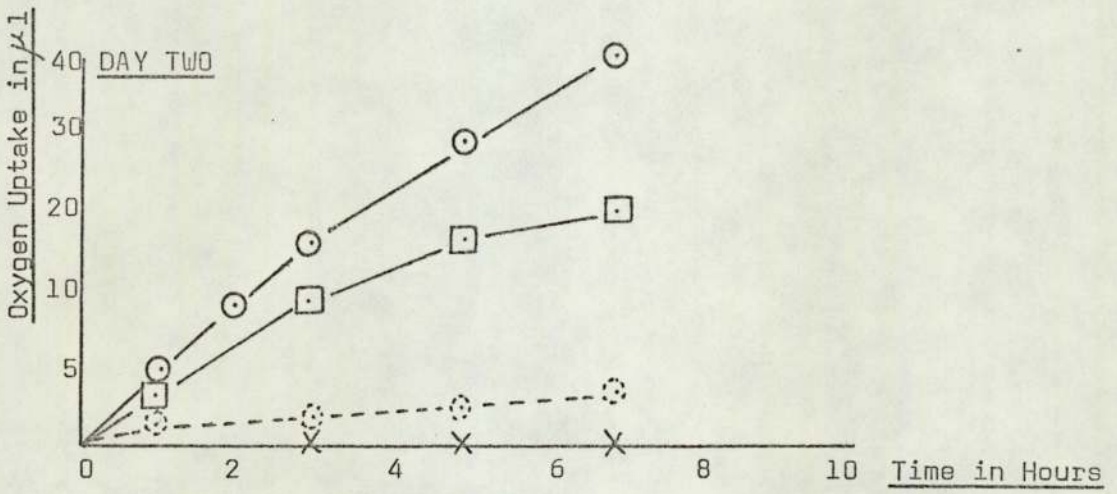
—X—X— = Polyurethane, nutrient salts + 1.0% Biocide C

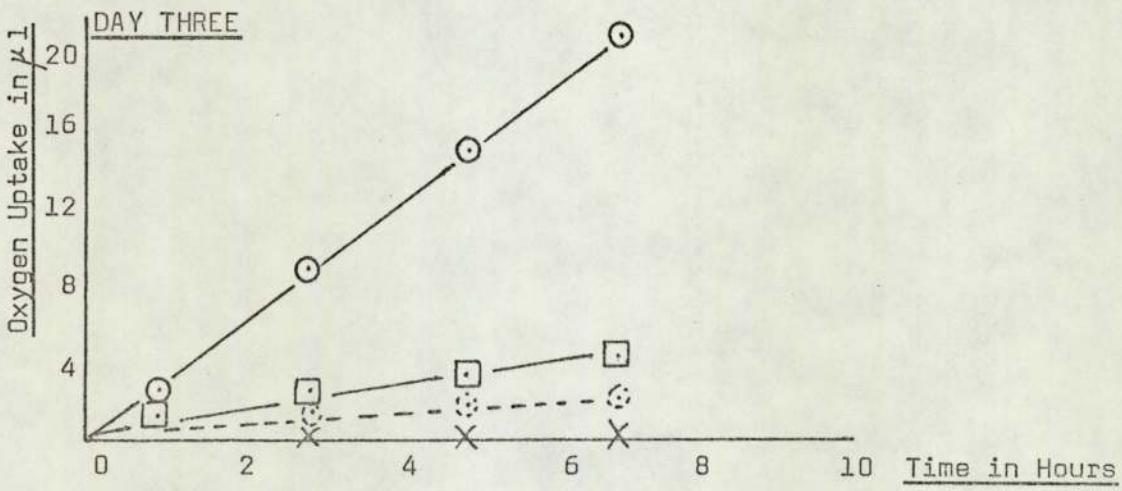
—△—△— = Polyurethane, nutrient salts + 2.0% Biocide C

--⊙--⊙-- = Nutrient salts and T. viride only

Fig. 7(xii) Respirometric Studies using Polyurethane, natural salts and S. lanuginosum







KEY:- —⊙—⊙— = Polyurethane, nutrient salts and S. lanuginosum onl

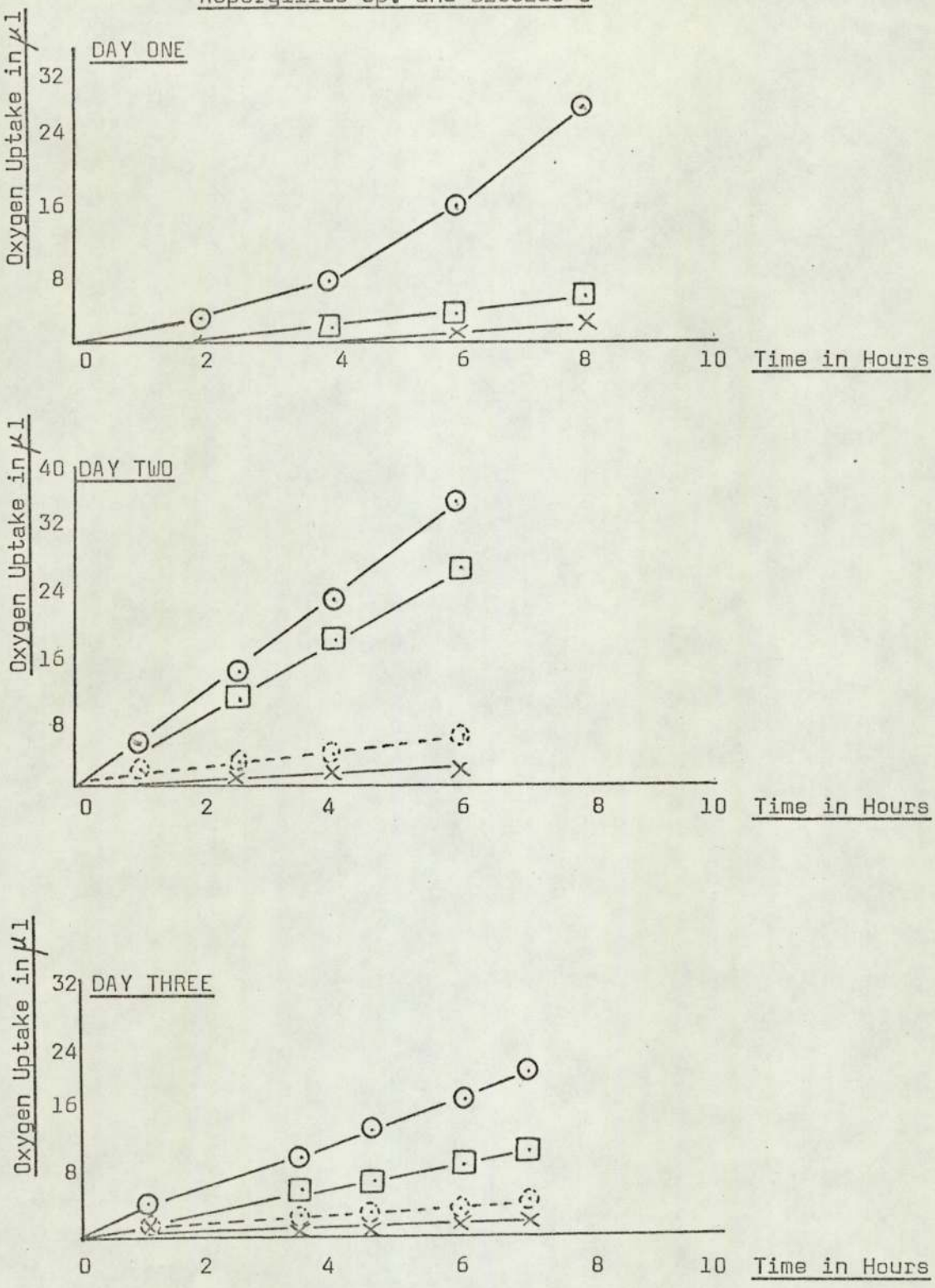
—⊡—⊡— = Polyurethane, nutrient salts + 0.5% Biocide C

—X—X— = Polyurethane, nutrient salts + 1.0% Biocide C

—△—△— = Polyurethane, nutrient salts + 2.0% Biocide C

---⊙---⊙-- = Nutrient salts + S. lanuginosum only

Fig. 7(xiii) Respirometric Studies using Polyurethane, Aspergillus sp. and Biocide C



KEY:- —⊙—⊙— = Polyurethane, nutrient salts and Aspergillus sp.

—⊡—⊡— = Polyurethane, nutrient salts + 0.5% Biocide C

—×—×— = Polyurethane, nutrient salts + 1.0% Biocide C

—△—△— = Polyurethane, nutrient salts + 2.0% Biocide C

--⊙---⊙-- = Nutrient salts + Aspergillus sp. only

which appeared to be the most effective on the basis of the results obtained using soil inocula and Fusarium sp. the fungus which occurred with most frequency during the ecological studies described earlier.

Similar general results were obtained for Penicillium lilacinum, Trichoderma viride, Stemphylium lanuginosum and Aspergillus sp.. In these cases the ability to utilise the polyurethane film was again demonstrated by the controls while the 1.0% and 2.0% concentrations of fungicide C proved adequate in protection throughout the experiment. The 0.5% levels did not however offer complete protection although inhibiting the growth to a limited extent.

7(c) Tensile Strength Measurement

The first technique to be considered as a possible way of assessing and monitoring the extent of hydrolysis of the polyurethane film was that of tensile strength measurement. This is a widely used technique for evaluating materials in general, and as such it is accepted by industry as a recognised test procedure, and may add support to biological observations of an ecological nature.

The polyurethane film as prepared was 0.1 mm in thickness, and samples were cut in the usual manner using a metal die punch which produced samples 5 mm in width and 7.0 cm in length. By using the same punch, this size of sample was maintained as standard during these studies.

The polyurethane samples were then subjected to tensile strength measurement at constant temperature ($22^{o}C$) and humidity using an Instron tensiometer, with a chart recorder displaying the relevant data in the form of a curve.

It was found that the samples were extremely elastic, and the break point was between the limits of 0 - 0.5 lb. This order of magnitude was only just within the limits of the instruments available. The film samples also proved to be so elastic that the initial length of 7.0 cm stretched to a length of approximately 60 cm again this order of increase in length was only just within the limits of the instrument. These phenomena resulted in very erratic values, with some samples not breaking within the extension limits of the instrument and others breaking before any record of load was made on the recorder chart.

Under such circumstances no meaningful results could be obtained to plot the increase in length at half the break load, which was the initial intention in connection with this particular study. This technique was therefore abandoned as a possible method of assessment during the present investigation into the hydrolysis of polyurethane film. Some of the difficulties may have been overcome by altering the dimensions of the sample, in particular the thickness of film, but this was not possible as the film was already preprepared by Catomance Ltd., and facilities were not available to cast thicker films, while at the same time it was the very thin surface coating films which were of particular interest to these investigations.

7(d) Infra-red Spectrophotometric Analysis

Having found that the tensile strength technique proved inadequate to the requirements of this investigation, it was decided to look into the modifications which may occur within the polymeric polyurethane material itself during the course of hydrolysis.

In order to make such observations, infra-red spectrophotmetric techniques were employed, analysing the spectra of the polyurethane film prior to and after various stages of hydrolysis. The important factor in this case was that the technique was non-destructive, and the same sample could be observed initially prior to treatment and again after any hydrolysis treatment had taken place. This of course was impossible in the case of the tensile strength method where the samples were extended to the break point. The importance of this factor is even more evident when considering the unreproducibility frequently encountered when working with biological systems. Using this technique, the changes occurring in each individual sample may possibly be accurately and continually monitored.

The polyurethane film was cut into circles, approximately 1 inch in diameter, and these were mounted between sodium chloride discs in the usual way for analysis using the Unicam SP 200 infra-red spectrophotometer. A chart recorder automatically plotted a graphical representation of the transmittance over the range 4000 - 650.

The polyurethane was then subjected to various degrees of accelerated hydrolysis by autoclaving at 15 lbs/sq. in. for different periods of time, after which the samples were thoroughly dried and the scanning operation repeated.

From the results obtained, although there were very minute differences in the spectra, these were not considered adequate to be able to distinguish chemical modification which may have occurred. The small differences may also be due to operating differences when carrying out the measurements since the instrument was in continual use, and it was necessary to re-adjust the instrument prior to each piece of work.

If the small differences are assumed to be chemical changes occurring in the polymeric material, although insufficient and difficult to use for assessment of the extent of hydrolysis, it may indicate that the effects of hydrolysis on the polymer are very small and subtle with the major gross polymeric structure being relatively unchanged and so masking these very small changes.

This technique was investigated further using other polyurethane films, but with similar end results and after extensive studies in this direction the inconclusive results obtained led this line of approach to the problem to be terminated.

7(e) Dielectric Constant Measurement

Following the disappointing results from the previous section, alternative physical or chemical techniques of a

non-destructive type were sought. One such technique considered to offer possibilities in this direction was that of dielectric constant measurement. It was thought that if a chemical change occurs in the polyurethane material during hydrolysis, there should be a corresponding change in the dielectric constant of the material since this parameter is specific to particular materials.

The principle of the method is that of measuring the capacitance of insulating materials, of which polyurethane is one, over a range of frequencies. By means of mathematical manipulation the dielectric constant may be calculated from the results obtained using a dielectric measuring instrument. The complex theory behind the technique and a description of the instrument are discussed by Hartshorn and Ward (1936). The Marconi dielectric test set, type TF 704C, was used for the measurements.

The polyurethane film samples were prepared as circles, 53 mm in diameter, as this size was the most suitable for use with the instrument.

The method is that of capacitance variation in a tuned circuit, with a thermionic voltmeter which detects resonance. Adjustments are made using two micrometer condensers, one plate condenser in which a sample is placed, and the other a cylinder which measures sharpness of resonance.

The sample is held between two plate electrodes, and observations are made both at the resonant frequency and at specific points off resonance, to determine the width of the resonance curve. The specimen is then removed and the observations are repeated with air as the dielectric. By comparing the two resonance curves, the properties of the dielectric (polyurethane in this case) may be determined.

The thickness of the material used with the dielectric test instrument is not important, except that it should be greater than 2 mm, since it is difficult to obtain accurate results with specimens outside this limit. Unfortunately the polyurethane film was only 0.1 mm thick, and repeated attempts to make the required measurements resulted in a very erratic and meaningless scatter of results. To overcome this problem of insufficient thickness, several samples were placed on top of each other in order to bring the thickness within the limits of the instrument. This however proved unsuccessful since, even though great care was taken to avoid any air being trapped between each sample, some air did remain and this affected the measurement.

The result of these preliminary measurements, using the dielectric test instrument, was to abandon the technique in connection with the present investigation. It may however be a useful technique when dealing with polymeric materials of greater thickness and may be worthy of further investigation along these lines.

7(f) Dynamic Visco Elastometer Measurement

Having been unsuccessful using the previous techniques described in this chapter, the direct reading dynamic visco elastometer was considered as a further possibility in offering a suitable non-destructive method of assessment of any changes occurring in the polyurethane film during hydrolysis or biological attack.

There has been a rapid development in the field of high polymer materials such as plastics, rubbers, fibres and polyurethanes, and the measurement of tan δ and complex modulus is used as one of the methods of examining the physical properties of these synthetic materials. This measurement has been made relatively simple using the Rheovibron (Model DDVII) direct reading dynamic visco elastometer by which the temperature dispersion of complex modulus of elasticity $[E^*]$ and the tan δ profile may be used to study transition phenomena and various states of polymeric materials.

The purpose of this instrument is to measure the temperature dependence of the complex modulus at a definite frequency. If tensile strain is applied on one end of the polymeric material sample in a viscoelastic state, the stress is generated at the other end of the sample and the phase angle δ is found between the strain and the stress. By using this instrument, tan δ may be read directly from a meter, and the storage modulus and loss modulus may be calculated from the amplitude of stress and strain, and the δ value.

For the purposes of the present investigation into changes taking place in polyurethane film after varying degrees of hydrolysis and biological activity, these measurements were carried out at room temperature only.

Both ends of the samples are fixed to two strain gauges (fig7(xiv), one of which is a transducer of displacement (T-7) and the other is a transducer of generated force (T-1). After absolute values of the electrical vectors transduced from force and displacement are adjusted to give a full scale deflection on the meter, vector subtraction is made by changing the connection of the output circuit of the two strain gauges and the value of tan δ may be read directly from the meter.

The sample dimensions suitable for use with this instrument should lay within the following limits:-

Length 0.5 - 6 cm, Thickness max 1 mm and Breadth max. 5 mm and the sample size used in this experiment, using polyurethane film were 3 cm x 0.5 cm x 0.1 mm.

The determination of the dynamic elastic modulus $[E^*]$ is obtained by substitution in the equation

$$[E^*] = 2.0 \times \frac{1}{A \times D} \times 10^9 \times \frac{L}{S} \text{ dynes/cm}^2$$

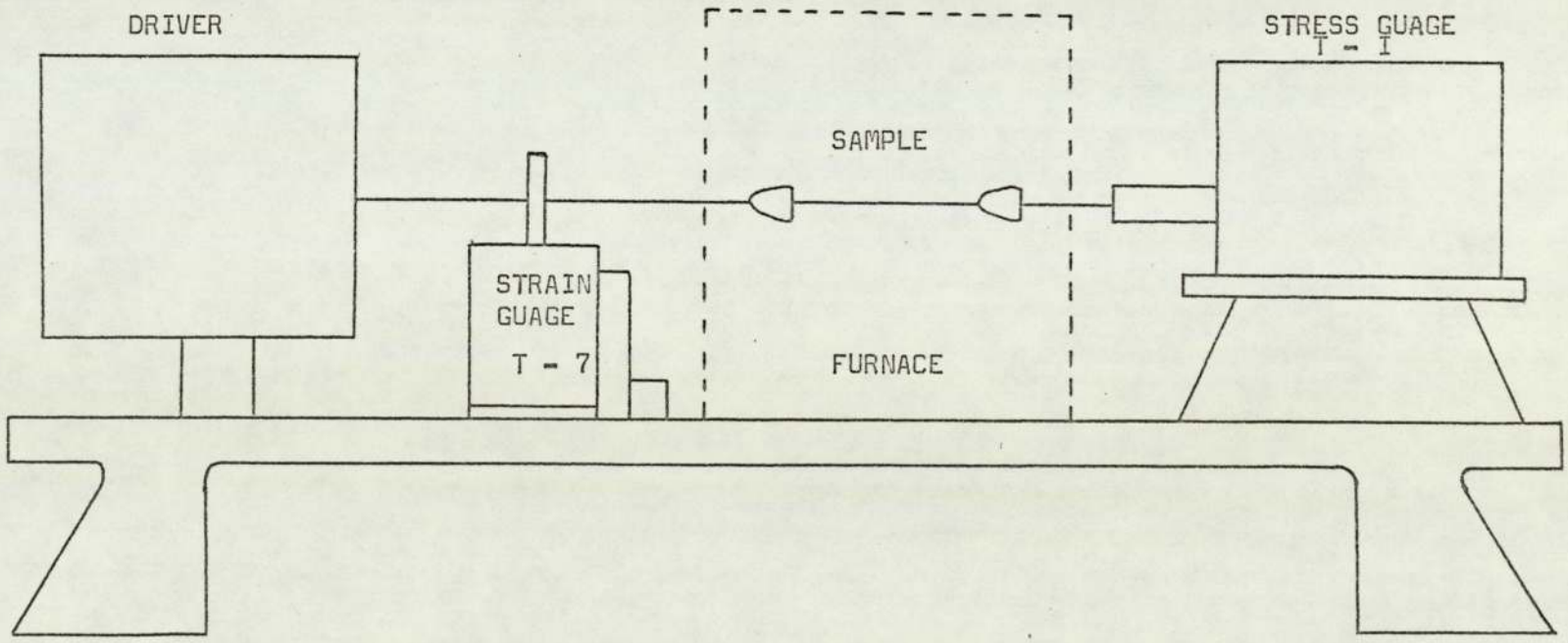
where L = Sample length between clamps, measured using a vernier scale on the instrument (cm)

S = Sample section area in cm^2

A = Value of amplitude factor when measuring tan δ

D = Value of dynamic force dial on instrument at the time of measurement of tan δ.

Fig. 7(xiv) DIAGRAM OF DYNAMIC VISCO ELASTOMER APPARATUS



The calculation of the dynamic complex modulus including error constant K is obtained by substitution in the equation

$$\left[E^*\right] = \frac{2}{A_o D - K} \times \frac{L}{S} \times 10^9 \text{ dyne/cm}^2$$

The dynamic elastic modulus E' is given by

$$E' = \left[E^*\right] \cos \delta .$$

and the loss modulus E is given by

$$E'' = \left[E^*\right] \sin \delta .$$

For convenience, a computer programme, in the form of a punched tape, was used to carry out the calculation of complex, elastic and viscous moduli.

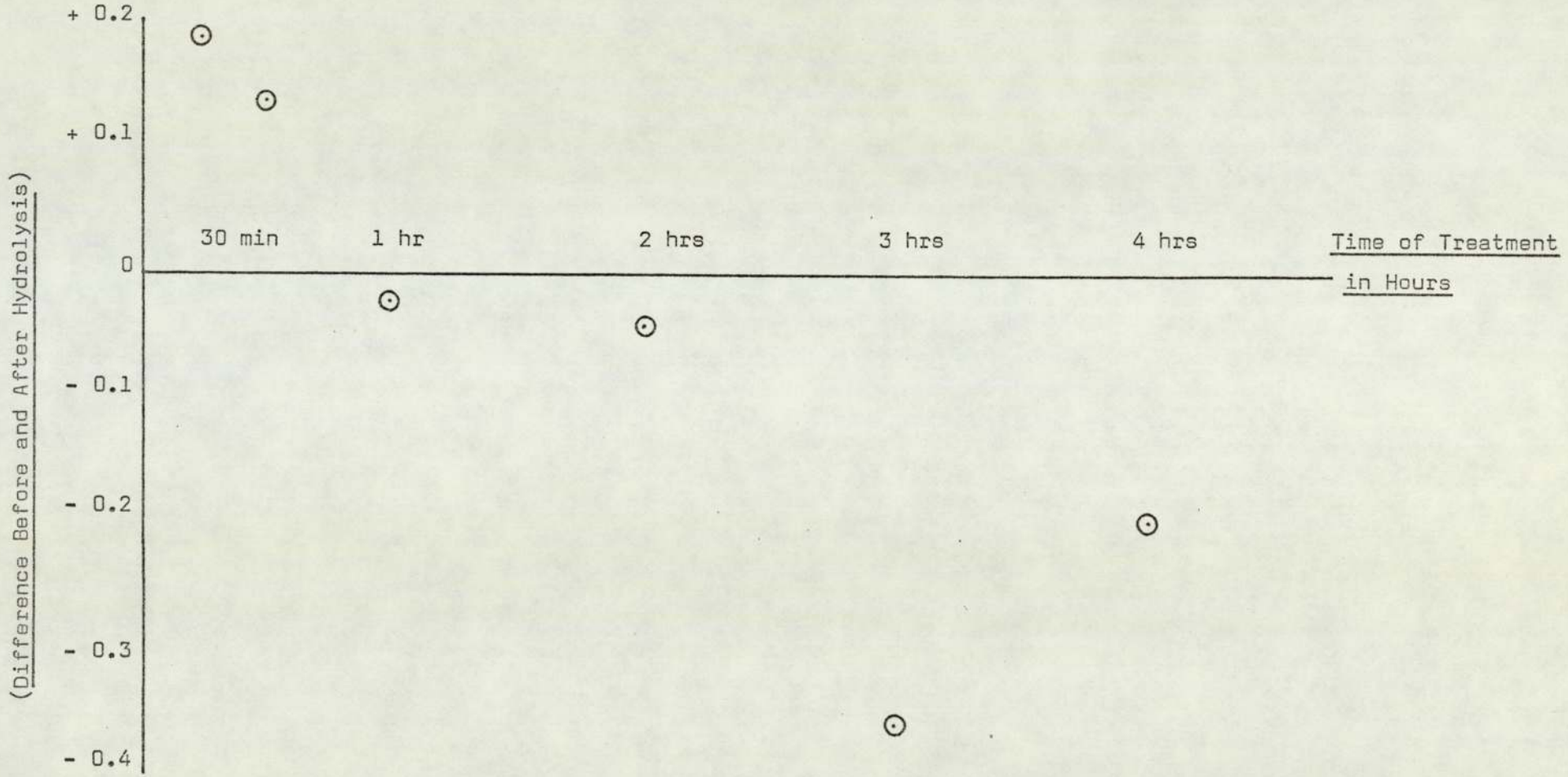
The average results obtained using the Rheovibron instrument are illustrated in table 7(a).

The results showed considerable variation in the initial value of the moduli, prior to any experimental treatment. This in itself possess problems in making meaningful interpretation of any results. However the scatter diagram (fig 7(xv) (page 202) showing the differences in viscous modulus before and after accelerated hydrolysis treatment reveals that there may be a relationship between degree of hydrolysis and the change in viscous modulus. In order to make definite conclusions, further work would be required, since the variation between the samples initially may lead to rather erroneous results. Unfortunately time prevented further experimentation along these lines, but this initial experiment may indicate that the technique could offer a suitable means of assessing changes

TABLE 7(a) Average Results Obtained Using Dynamic Viscoelastometer Before and After Accelerated Hydrolysis

Treatment	Complex Modulus (Dynes/cm^2 x 10^9)			Elastic Modulus (Dynes/cm^2 x 10^9)			Viscous Modulus (Dynes/cm^2 x 10^8)		
	Before	After	Difference	Before	After	Difference	Before	After	Difference
15 mins Accelerated Hydrolysis	0.164	0.319	+ 0.155	0.1628	0.317	+ 0.155	0.21	0.395	+ 0.185
30 mins Accelerated Hydrolysis	0.236	0.355	+ 0.119	0.213	0.353	+ 0.140	0.305	0.443	+ 0.138
1 hour Accelerated Hydrolysis	0.318	0.307	- 0.011	0.315	0.304	- 0.011	0.413	0.390	- 0.023
2 hours Accelerated Hydrolysis	0.389	0.342	- 0.047	0.386	0.339	- 0.047	0.502	0.4522	- 0.049
3 hours Accelerated Hydrolysis	0.181	0.451	+ 0.270	0.179	0.446	- 0.267	0.262	0.625	- 0.358
4 hours Accelerated Hydrolysis	0.318	0.391	+ 0.073	0.297	0.387	- 0.089	0.346	0.552	- 0.206

Fig. 7(xv) Scatter Diagram Based on Changes in Viscous Modulus after Accelerated Hydrolysis Treatment



taking place in the polyurethane film, and is probably worthy of more intensive evaluation in the future. One disadvantage is the fact that the instrument is expensive and although quite versatile, may not justify the high capital outlay. If the technique is shown to be suitable for such evaluation of polyurethane film it may be possible to modify the set up and produce a simple, cheap, laboratory apparatus on the same principles.

7(g) Discussion and Conclusions

The results obtained from the respirometric experiments described in this chapter have shown the new fungicides to be effective in inhibiting fungal growth at 0.5% and 1.0% concentrations, and preventing any microbial activity at the 2.0% concentration. Fungicide C appeared to be the most effective of these new products. Insufficient time prevented further experimentation with other fungi associated with the colonisation of the polyurethane film and the new fungicides which might have offered a more comprehensive picture of the efficacy of these fungicides. These initial investigations, however, have offered some evidence as to the general trends in fungicidal properties displayed by the new fungicides, which at the time of writing had been developed on the laboratory scale, in very small quantities only.

Another factor which must be considered when interpreting the results is that the fungicides were applied as a percentage of the spore suspension by volume, and not actually incorporated into the polyurethane film, and although these experiments show the fungicidal effectiveness of the new organic products, this

may differ when they are incorporated into the formulation of the synthetic polyurethanes.

The reasons for carrying out these experiments in such a way, was that these products had only been developed towards the end of this three year research programme, and the chemical research and development programme concerning these products was in its infancy at the sponsoring company who had not had an opportunity to incorporate these products into the synthetic material itself.

The possibility may exist that when these products are successfully incorporated into the polyurethane material, adequate protection may be obtained at reduced concentrations compared to those used in these experiments, since they will be in closer association with the material which they are protecting. There is, however, the alternative possibility that the fungicidal properties may be reduced or even lost when these products are incorporated into the polyurethane system. Should this occur, it will be the task of the chemists to develop suitable modifications either in the polyurethane system itself or, in the fungicide formulation.

It was intended to extend this research to study the situation where polyurethane and cotton textile were in close association as a synthetic film on the surface of a cotton fabric. The study of fungal colonisation patterns and interaction between fungi, materials and various combinations of fungicides were planned. Unfortunately, such study was beyond the scope of these present investigations, but may offer interesting

lines of further investigation in the future. Another item of interest for future work would be the study of the susceptibility of these polyurethane systems to thermophilic fungi which may be important when the polyurethane material is subject to conditions of insolation and/or tropical climatic conditions.

With regard to the physical methods for assessing the degree of hydrolysis and extent of biological activity, several techniques were evaluated and concerned changes which may occur in the polymeric structure of the polyurethane film. These changes may be due to chemical hydrolysis in the presence of moisture and/or changes resulting from the direct biological activity associated with this material.

The widely used method of tensile strength measurement proved unsuitable in this situation due to the polyurethane film being particularly thin and very elastic. As a result of these physical properties, the load at the break point was very low and outside the limits of the instruments available. At the same time the extension produced was so great that this also lay outside the limits of the instrument. For the purposes of this investigation therefore, this approach was abandoned and alternative techniques sought.

Infra-red spectrophotmetry was considered as a possible line of approach, looking at changes in the spectra of the polyurethane material during hydrolysis treatment, with the aim of extending this to investigating changes caused by the enzyme activity associated with colonising fungi. This technique

appeared to offer a more suitable method of monitoring any changes, especially since the method was non-destructive, allowing the same sample to be analysed throughout, while also being a straightforward procedure. After extensive experimentation with the polyurethane film, it was found that any differences in the spectra before and after accelerated hydrolysis treatments were very small, and it was questionable if these were due to chemical changes or to inconsistency in operation of the instrument. Certainly the differences were too small to make this method suitable as a routine tool for the particular purposes envisaged, and as a result investigations along these lines were terminated.

Dielectric constant determination was also a possible method considered, but here again the physical properties of the polyurethane film caused problems in that the material was too thin, falling outside the limits for accurate measurement to be carried out, and this method also had to be abandoned for the purposes of the present material under investigation.

The dynamic visco elastometer was considered as an instrument offering a non-destructive test, and which has been used to examine the properties of synthetic polymeric materials. The physical dimensions and elastic properties of the polyurethane also lay within the limits of the instrument. This technique appeared to be the most promising so far, but the limited and rather inconsistent results do not show any clear indication as to the suitability of this method for the purposes in mind. There was however sufficient evidence to indicate a potentially worthwhile method. Unfortunately the time factor prevented

further more extensive investigations along these lines, and it may be worthwhile in the future to investigate this potential further. There is, however, a disadvantage in the high cost of such an instrument for such routine operations, but if found to be a suitable method, smaller less complex apparatus may be developed along similar principles as those described earlier.in this chapter.

It must be noted however that although the tensile strength test and dielectric constant test proved inadequate during the present investigation due to the properties of the particular material being studied, these difficulties may be overcome or not encountered when studying other materials of the polyurethane type, and here these methods may offer greater potential than was indicated in this investigation.

The tests referred to in this chapter were carried out using polyurethane film before and after accelerated hydrolytic treatment. It was planned to extend these studies further to investigate changes occurring in the polyurethane film as a direct result of fungal colonisation and microbial enzyme activity. However such an investigation was found to be beyond the limits of the present research programme, but such a project should be encouraged in the future.

CHAPTER EIGHT

GENERAL DISCUSSION

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GENERAL DISCUSSION

The work described in this thesis was undertaken in order to investigate the biodeterioration of cotton textile by soil micro-organism under conditions of elevated temperature, and the biodeterioration of a relatively new synthetic material of ester based polyurethane film, with particular reference to the control of such deterioration by means of a new family of organic biocides recently developed by Catomance Ltd., who have sponsored this research programme.

The association between cotton textile and polyurethane film in this thesis is a direct result of the immediate interest of the sponsoring organisation. Polyurethanes and cotton textile are closely associated in some products, and these new situations are important to Catomance Ltd., concerning possible biological activity. Cotton and polyurethane also provide two quite different test situations for use as substrates in the evaluation of the new family of organic biocides, and finally polyurethanes are very different to many modern synthetic plastic materials in that they can be biodegraded. Hence inherently biodegradable products would require to be protected in some way, such as the incorporation of a biocide.

Evaluation of the new fungicides described in this thesis has already been studied at mesophilic temperatures with reference to protection of cotton cellulose (Allsopp 1973) showing these products to be effective in this situation and

Catomance Ltd., are interested in the performances of these products under conditions of elevated temperature, such as occurs in tropical countries, and under insolation conditions found in more temperate climates. Biodeterioration of materials in general is far greater in tropical countries and now that many of the more urgent problems of the developing countries are being alleviated (e.g. famine and housing), more time and expertise is available for studying problems of biodeterioration which if prevented may allow still further development of these countries.

The description of both cotton cellulose and polyurethane in previous chapters has illustrated that these materials are complex and cannot be thought of as homogeneous when considering any problems involving microbial colonisation, and the prevention of microbial attack on these materials may be achieved in a number of ways (Eggins 1967), the most useful and widely used method being that of chemical protection using biocides.

The experimental work described in this thesis concerns the activities of soil microfungi and a convenient source of such organisms was found in meadowland topsoil which had been the subject of previous investigation (Eggins and Malik 1969), and was known to contain a wide range and large numbers of micro-organisms. Soil was employed as a source of microfungi since it is from the soil that many of the air spora originate prior to contamination of materials and subsequent colonisation, if not from direct contact with the soil itself.

The temperature of 50°C was selected for the studies involving cotton cellulose and thermophilic fungi. This temperature

may be considered as being on the high side for normal conditions of elevated temperature encountered in tropical countries, but under situations of insolation, micro-environments may be created where the temperature rises to very high levels. For the purposes of this investigation, the term thermophilic was extended to include thermotolerant species such as A. fumigatus which under the definition of Cooney and Emerson (1964) are not classed as true thermophils, but which none the less may be important in the biodeterioration of cotton textile under conditions of elevated temperature especially since this species displays aggressive celluloltyic properties.

A basically ecological approach has been made towards this investigation concerning the problems associated with the biodeterioration of cotton cellulose and ester based polyurethane film, using complementary techniques to give a broad picture of the patterns of fungal colonisation of these materials. A number of techniques may be employed in ecological work of this nature and the advantages and disadvantages of some of these have been discussed by the author (Hollingsworth et al 1973). One of the important points when carrying out ecological work in the laboratory is to approach as closely as possible the conditions that may normally be expected to occur in the natural environment. For this reason, isolation work was carried out using the complementary techniques of agar plate studies, screened substrate soil burial (Eggins and Lloyd 1968), and screened substrate perfusion colonisation studies (Eggins, Malik and Sharp 1968). The latter technique may be considered as offering the closest situation similar to that which occurs in the natural environment.

Initial isolation work using cellulose agar (Eggins and Pugh 1962) and unprotected cotton textile, demonstrated the occurrence of thermophilic fungi in the meadowland topsoil used for these studies. This isolation work was in approximate agreement with previous studies on similar soil (Eggins and Malik 1969). When isolations were carried out using the demonstrative medium of cellulose agar, the ability of some thermophilic species to secrete cellulolytic enzymes was clearly demonstrated, by areas of clearing occurring around the colonies in the cellulose agar, and this will clearly be important in any fungal colonisation of cotton textile at elevated temperatures.

Because of the length of time involved in ecological isolation studies these are frequently ignored, laboratory cultured 'test' organisms being preferred for convenience and ease of identification. This is an unfortunate practice in some respects since organisms originally isolated from one material may subsequently be used to evaluate a totally different system, and such a situation may bear little if any resemblance to the natural environmental circumstances. It is therefore advisable to commence with at least a brief ecological isolation study in order to isolate the main organisms actively involved in the particular situation of interest.

Having demonstrated that the meadowland topsoil provided a good source of potential thermophilic fungal deteriogens with reference to cotton cellulose, the isolation work was extended using cotton textile which had been fungicidally protected with the new family of biocides associated with this investigation.

In this way species which were possibly resistant to these fungicides were isolated, and an evaluation as to the efficacy of the fungicides could be carried out by means of tensile strength measurements which offered a quantitative indication as to the extent of deterioration as a loss in strength over an incubation period at thermophilic temperatures. These studies indicated the fungicides to be effective, but concentrations higher than those found to be adequate at mesophilic temperatures (Allsopp 1973) were required. Certain species were also observed to be resistant to or tolerant of the new fungicides with the Actinomycete spp. and A. fumugatus in particular occurring under the more severe conditions. The activity of the Actinomycete spp. is particularly interesting since during these studies it has been noted that these organisms are able to tolerate much drier conditions for growth than other fungi, and that the enzyme activity associated with the Actinomycete spp. was quite extensive from relatively minute mycelial masses. This phenomena may be important in connection with the biodeterioration of cotton cellulose under adverse conditions or over long periods of storage when relatively little moisture is available.

Several interesting points arose from the investigations into the efficacy of the new fungicides and these were pursued further. One interesting fact was that one fungicide (P_1) was water soluble and this may have influenced the results obtained if it was leached from the textile which was incorporated in the perfusion chain. By incorporating this fungicide in the nutrient reservoir of the perfusion system a constant level could be maintained. This resulted in marked improvement in effectiveness and demonstrated the potential of this product

under circumstances where leaching would not be encountered and if the product was modified to give a less soluble chemical. The sponsoring firm of Catomance Ltd., acted on these results to produce further salts of this product which were in fact much less soluble.

Further experiments using thin layer chromatographic techniques and water insoluble products P_2 and P_3 demonstrated that these were not leached or flushed from the textile in the same way as may occur with P_1 which was water soluble.

Since low concentrations of the fungicides did not appear to offer adequate protection (1%), the possibility of detoxification of the biocides by thermophilic fungi arose. This was further investigated using isopropanol as the major carbon source for fungal growth. This radical was common to all the new family of biocides, but it was shown that this could not be readily utilised by the fungi associated with the thermophilous deterioration of cotton textile which had been protected by the new biocides.

Ecological isolation studies using polyester polyurethane film before and after accelerated hydrolysis indicated a wide range of fungi were capable of utilising both the unhydrolysed polymer and the products of accelerated hydrolysis. Similar species were isolated in these studies as reported by other workers in previous experiments (Evans and Levisohn 1968) but although extensive fungal colonisation of this synthetic material was observed with a succession of species occurring particularly

with increasing hydrolysis, no evidence as to the mechanism of fungal attack could be gained from such ecological work.

Respirometric methods added weight to the tentative conclusions drawn concerning the fungal colonisation of the polyurethane film, demonstrating the direct utilisation of the polymeric material by some species, and the products of hydrolysis by others, thus leading to the succession of fungi observed in the previous ecological studies.

Following the investigations into the utilisation of the polyurethane as a major nutrient supply, there was clearly a need for the protection of these inherently biodegradable modern materials. It was considered that the family of biocides produced by Catomance Ltd., would offer adequate protection in association with the polyurethane film. Respirometric techniques using the new biocides in conjunction with this polyurethane film revealed this to be the case, offering adequate protection from attack by either the general microbial population occurring in soil and pure fungal cultures obtained from a similar soil.

Although the respirometric technique offers a useful research tool it requires quite substantial attention over long periods of time when applied to problems such as those studied here, although development of automatic respirometers may relieve some of these problems.

Rapid methods of assessing the extent of biological attack and the degree of hydrolysis of polyurethane films, were sought.

Unfortunately problems were encountered with tensile strength and dielectric constant measurements due to the physical size of the film which was extremely thin (0.1 mm). The changes in the infra-red spectra were examined before and after hydrolysis but these were so small relative to the unchanged portion of the material that any differences could not be easily detected. The dynamic visco elastomer offered potential as a non-destructive test method but unfortunately the results obtained did not realise this potential and time prevented further comprehensive evaluation of this particular piece of equipment but further studies should be encouraged.

From this work information has been gained which has been of direct and immediate importance to the sponsor, in addition to opening up further areas where investigation may result in more effective fungicides and possible methods of assessing biological activity. Unfortunately time prevented many of these avenues being explored, but the general activity of thermophilic fungi associated with cotton textile has been demonstrated and shown to be important in the deterioration of cotton, even in temperate climatic conditions. In addition the susceptibility of polyester based polyurethanes has been demonstrated and this may involve a very wide range of organisms. However the initial investigation of the new biocides reveals that these certainly have potential in the protection of such new synthetic materials. It was planned to extend these studies further to the situation where cotton cellulose and polyurethane film were in association, but this proved beyond the scope of the present investigation, and should be encouraged in future work.

APPENDICES

APPENDIX I

MARKETS FOR URETHANES

(DOMBROW 1965)

(a) Flexible and Semi Flexible Polyrethanes

Market	Estimated U.S. Consumption in millions of pounds	
	1963	1968
Furniture	65	100
Bedding	20	65
Textiles	18	30
Rug and Floor underlay	2	15
Car Industry	36	54
Packaging	1	2
Miscellaneous	2	3
TOTAL	144	269

(b) Rigid Polyurethanes

Refrigeration (appliances)	7	23
Refrigeration (commercial)	2	5
Refrigeration (transportation)	8	15
Refrigeration (construction)	7	22
Construction (commercial)	2	8
Construction (residential)	-	1
Flotation	2	5
Car Industry	-	1
Packaging	1	3
Containers, cups etc.	1	2
TOTAL	30	85

(c) The breakdown with respect to uses in 1964 was:-

Flexible foams	60%
Rigid foams	13%
Elastomers	2%
Coatings	5%
Export from U.S.A.	20%

(d) A market survey carried out in the U.S.A. claims the following overall picture for total urethane market.

	1963	1968
Flexible foams	143	250
Rigid foams	43	125
Elastomers	14	39
Spandex	6	20
Binders	9	18
Coatings	10	30
Others	1	1
TOTAL	226	484

Figures given above are in millions of pounds weight.

(e) The Methylene Diisocyanate (M.D.I.) applications breakdown is as follows for:

	1963	1968 (%)
Elastomers	45	38
Spandex	28	25
Rigid forms	23	35
Others	5	5

(f) The Tolylene Diisocyanate applications breakdown is as follows:

	1963	1968 (%)
Flexible foams	60	50
Rigid foams	15	35
Surface Coatings	5	5
Others	20	5

The individual usage of M.D.I. for 1963 is not known but was estimated at 15 million pounds which was predicted to treble by 1968.

(g) Production of Polyurethanes, Polyethylene and Polyvinylchloride in the United Kingdom

(figures in thousand tons)

Polymer	1962	1963	1964	1965	1966	1967	1968	1969	1970	1971
Polyurethanes	11.5	15.8	20.8	23.3	25.1	26.8	32.8	30.9	32.8	36.3
Polyethylene	N.A.	185.6	216.1	231.4	255.6	279.5	309.1	334.8	416.0	379.7
P.V.C.	115.0	147.1	176.8	193.7	198.7	224.0	267.0	278.7	309.6	314.3

SOURCES:- 'Annual Abstracts of Statistics'

'Business Monitor P.21 Synthetic Resins and Plastic Materials'

(Polyethylene figures for 1962 are combined with those for polypropylene in the official figures, and so they have been omitted above, as they cannot readily be separated out)

APPENDIX II

FORMULA FOR BRODIES SOLUTION

This solution was used in the respirometer manometer capillary tubes as described in chapters six and seven.

NaCl	23 g
Bile salts (Sodium tauroglycocholate)	5 g
Water to	500 ml

Colour with crystal violet (or other stain)

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