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STUDIES ON FUNGAL BIOMASS AND ACTIVITY ASSOCIATED WITH THE DECOMPOSITION OF PLANT LITTER IN FRESHWATER

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Presented in partial fulfilment for the degree of Doctor of Philosophy to the Council for National Academic Awards, June, 1987.

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DEDICATION

To Frances for her encouragement and support

DECLARATION

I, Paul Edward Cook declare that whilst registered as a candidate for the degree of Doctor of Philosophy, at the City of London Polytechnic, I was not registered for any other award of the CNAA or of a University. The work undertaken during this period was carried out by myself, with guidance from Dr B.E.S Godfrey at the City of London Polytechnic.

Several studies were undertaken in conjunction with my research programme in partial fulfilment of the degree of Doctor of Philosophy. These included courses on fungal taxonomy and the use of botanical literature organised by the University of London Extra Mural Studies department. In addition I attended a course on non-parametric statistics at the City of London Polytechnic. I also presented several research seminars to the academic staff at the City of London Polytechnic and was involved in practical demonstrations to undergraduate students.

I attended several meetings at the British Mycological Society and meetings of the British Ecological Society and the London Freshwater Group. During my period of registration I have presented three poster communications together with an oral communication to a meeting of the Biodeterioration Society.

Paul Edward Cook

June, 1987

ACKNOWLEDGEMENTS

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Finally I would like to thank Caroline Kelly and Margaret Beyer for producing the tables and references, and Frances Booth whose painstaking work with text and figures helped see this work through to completion.

ABSTRACT

STUDIES ON FUNGAL BIOMASS AND ACTIVITY ASSOCIATED WITH THE DECOMPOSITION OF PLANT LITTER IN FRESHWATER

PAUL EDWARD COOK

A clearing and staining method using 2 stains, with one acting as a counter-stain for fungi, gave higher estimates of fungal biomass than other staining methods and it correlated well with the agar film technique. Using the method, it was possible to examine the spatial distribution of hyphae on leaves which varied between leaf types and fungal species.

Improvements were made to the agar film method by optimising extraction of hyphae from leaf litter and using a tetrazolium staining technique to detect metabolically active hyphae. An indirect method for estimating fungal biomass was developed using ergosterol as a marker. Conversion factors for ergosterol and biovolume to fungal biomass were shown to vary with fungal species, age and growth conditions, but no single parameter was found which could adequately explain this.

In field studies on flooded gravel pits biomass was 0.02-6.66% leaf dry weight depending on leaf type, biomass method and submersion time. Biomass estimated using clearing and staining was significantly correlated with ergosterol and with the agar film method; the latter gave the highest estimates. In terms of allochthonous litter inputs, fungal biomass represented 0.03-16.22 kg/ha.

Studies on a number of the gravel pit lakes demonstrated variation in communities of Ingoldian fungi both between lakes and within a single lake. Correlations were found with magnesium levels and litter inputs, but not with fungal biomass in the litter or cellulose decomposition. In one lake, decomposition of litter, fungal biomass and microbial populations were followed. No differences were found in biomass when animals were excluded although this influenced Ingoldian fungi. Higher numbers of Ingoldian fungi correlated with weight losses and biomass, and a competitive hierarchy was suggested to explain changes in fungal populations during decomposition.

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CHAPTER 1. INTRODUCTION

In many aquatic ecosystems microfungi are thought to play a major role in the degradation of allochthonous plant litter, and floristic studies have implicated the Ingoldian fungi* as being particularly important in freshwater (Triska, 1970; Bärlocher & Kendrick, 1974; Suberkropp & Klug, 1976; Padgett, 1976; Bärlocher & Kendrick, 1981; Suberkropp & Klug, 1981; Chamier & Dixon, 1982a; Chamier, Dixon & Archer, 1984). Studies on enzyme capabilities of Ingoldian fungi have shown that they possess a range of enzymes capable of maceration and disruption of leaf tissues (Suberkropp & Klug, 1980; Chamier & Dixon, 1982b; Suberkropp, Arsuffi & Anderson, 1983), and their growth and enzyme activity is thought to condition allochthonous leaf litter, increasing its palatability to aquatic invertebrates (Cummins, 1974; Bärlocher & Kendrick, 1975a; Anderson & Sedell, 1979; Bärlocher, 1982; Butler & Suberkropp, 1986).

Despite several detailed studies on the changes in microbial communities during leaf degradation in aquatic ecosystems (Morrison et al., 1977; Bärlocher & Kendrick, 1974; Padgett, 1976; Suberkropp & Klug, 1976; Chamier & Dixon, 1982a; Chamier, Dixon & Archer, 1984), only a few authors have attempted to measure microbial biomass (Suberkropp & Klug, 1976; Morrison et al., 1977; Morrison & White, 1980; Federle & Vestal, 1980 & 1982), or fungal biomass (Bärlocher & Kendrick, 1974; Lee, Howarth & Howes, 1980; Newell & Fallon, 1983; Newell, Fallon & Miller, 1986), and consequently there is a lack of information on levels of fungal biomass, particularly that associated with decomposition of allochthonous litter and especially in lakes. However, Iversen (1973), Davis & Winterbourn (1977), Bärlocher & Kendrick (1974)

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* The term Ingoldian fungi is used in preference to aquatic hyphomycetes since many of these have been found in terrestrial habitats, and not all are hyphomycetes.

and Barbe (1982) have esimated fungal biomass on allochthonous litter in rivers. Their results seem to indicate that fungal biomass may be less than 2% of leaf dry weight for a range of leaf types in rivers, although estimates of ATP suggest that total microbial biomass is 2-12% (Bärlocher & Kendrick, 1981). It seems from this that fungal biomass estimates may have been too low and therefore more information on the size of fungal biomass associated with allochthonous litter in freshwater is required, particularly as no critical studies have been made using a range of different methods.

Traditionally the estimation of fungal biomass has proved to be more difficult than that for bacteria. The filamentous nature of fungi, and their ramifying and penetrating ability, create particular problems in quantifying mycelium. Viable plate counts have been used by a number of authors, but these are known to favour the detection of reproductive propagules (Warcup, 1955; Parkinson, Gray & Williams, 1971; Wainwright, 1982).

Jones & Mollinson (1948) developed the agar film technique for the determination of fungal and bacterial biomass in soil. Since then the technique has been modified by Thomas, Nicholas & Parkinson (1965) and its application extended to estimating fungal biomass in leaf litter. Many different staining techniques have been tried (Swift, 1973a; Frankland, 1974, 1975b; Frankland, Lindley & Swift, 1978; Leonard & Anderson, 1981; Ineson & Anderson, 1982). Phenolic analine blue has proved to be the best stain for light microscopy of agar films as it appears to stain the vast majority of mycelium in preparations (Parkinson, Gray & Williams, 1971). Fluorescent stains have been used successfully to detect fungal mycelium in agar films of soil homogenates (Anderson & Slinger, 1975 a & b; Jenkinson, Powlson & Wedderburn, 1976; Roser, 1980; Ingham & Klein, 1984), and leaf litter (Leonard & Anderson, 1981; Ineson & Anderson, 1982), but few appear to give better results

than phenolic analine blue (Jenkinson, Powlson & Wedderburn, 1976).

The distinction between living and dead mycelium in agar films has presented many problems, in particular many hyphae are destroyed and cell contents disrupted during the maceration of leaf material prior to making the agar films. Jones & Mollinson (1948) claimed that phenolic analine blue only stained living hyphae, but this is now reputed to be unreliable (Parkinson, Gray & Williams, 1971; and Jenkins, Powlson & Wedderburn, 1976). Frankland (1974) observed that phase contrast microscopy revealed more mycelium ('ghost hyphae'), and she suggested that a proportion of the hyphae in agar films were not stained by phenolic analine blue and hence not visible by bright field illumination. In a further study, she estimated the proportion of hyphae with visible cell contents and used this as a measure of the living biomass (Frankland 1975a). Flanagan & van Cleve (1977) used aceto-orcein as a stain to reveal fungal nuclei in agar films and, in further work (Flanagan, 1981), found that it was correlated significantly with Frankland's method using cell contents.

Many fluorescent stains appear to stain both living and dead mycelium (Jenkinson, Powlson & Wedderburn, 1976; Roser, 1980). Anderson & Slinger (1975 a & b) used a mixture of fluorescent brightener and europium chelate to distinguish between living and dead mycelium; the fluorescent brightener staining all hyphae and the europium chelate apparently staining living cells. Few studies have critically examined the degree of differentiation between living and dead biomass by these staining methods, although Ineson & Anderson (1982) found that europium chelate stained biomass, in leaf litter, correlated poorly with a respiratory method.

Despite the improvements made to the agar film technique, it is still believed to underestimate fungal biomass, particularly in leaf litter where extraction by maceration appears to be less efficient and more

damaging than in soil (Frankland, Lindley & Swift, 1978; Leonard & Anderson, 1981; Newell & Hicks, 1982). Although poor extraction efficiencies are thought to explain lower estimates of biomass compared with other methods (Frankland, Lindley & Swift, 1978; Hicks & Newell, 1982; Newell & Fallon, 1983), few authors have considered correcting for these losses.

An alternative method to agar films was used by Hanssen, Thingstad & Goksoyr (1974); they homogenised soil and collected it on membrane filters. This approach has been adopted by a number of workers with examination being made with either light (Sundman & Sivelä,1978) or epifluorescence microscopy (Paul & Johnson, 1977; Soderstrom, 1979 a & b; Baath & Soderstrom, 1980; Frankland *et al*., 1981; Newell & Hicks, 1982). The major advantage of the membrane filter method is that a greater quantity of macerated material can be collected for enumeration and time is saved in preparation of samples, compared with agar films. Soderstrom (1977, 1979 a & b) used a membrane filter method to estimate lengths of living mycelium in leaf litter. By staining mycelium with fluorescein diacetate, collecting it on membrane filters and examining it by epifluorescence, he was able to observe lengths of fluorescing mycelium with non-specific esterase activity which has been shown to be indicative of living hyphae (Ingham & Klein, 1982 & 1984). Despite the attractiveness of this method for estimating living fungal biomass, Domsch *et al*. (1979) found it gave very low estimates of living biomass in soil when compared with other methods, presumably due to the destruction of living hyphae during preparation.

The clearing and staining of whole leaf discs or segments has been used by a number of workers (Minderman & Daniels, 1967; Bärlocher & Kendrick, 1974; Witkamp, 1974; Visser & Parkinson, 1975; Newell & Hicks, 1982) and a wide range of stains and clearing methods have been tried. This approach appears to result in lower estimates of fungal biomass than with the agar film method and it

does not enable distinction between living and dead hyphae. The advantages are that fungal reproductive structures (eg., perithecia, pycnidia) can be observed *in situ* and included in biovolume estimates (Newell & Hicks, 1982) and the spatial arrangement of mycelium on the leaf remains relatively undisturbed, hence this can be investigated and quantified.

Herrera & Ferrer (1978) mention a modification to a clearing and staining method for roots (Philips & Hayman, 1970) where the dye from stained mycelium is extracted and measured colorimetrically. However, this method does not seem to have been tried extensively, or applied to stained fungal structures in leaf material.

Apart from the problems of extracting and visualizing fungal hyphae in agar films or on membrane filters, there are also problems associated with quantification; these can include accurate estimation of hyphal diameters (Baath & Soderstrom, 1979b), the choice of magnification used to examine preparations (Baath & Soderstrom, 1980) and the use of appropriate biovolume to biomass conversion factors (Newell & Statzell-Tallman, 1982).

Problems with the use of direct microscopical methods for estimating fungal biomass has encouraged many biologists to look for indirect ways of detecting and measuring fungi (White *et al.*, 1980; White, 1983; Whipps *et al.*, 1982 & 1985). This search has been enhanced in some fields (eg., marine sediments) where the intimate association between microorganisms and detritus is so great that direct methods almost become impossible to use, other than to detect qualitative differences (White *et al.*, 1980; White, 1983; Parkes & Taylor, 1985). The complexity of many microbial assemblages in nature is often considerable and a number of authors (Swift, 1973a; White *et al.*, 1980; White, 1983) consider that the only realistic approach to studying biomass and activity of microorganisms is with the use of integrated approaches employing detailed chemical analyses coupled with isotopic

techniques.

Despite the great attractiveness of chemical methods as markers of specific microbial groups, they have been relatively ineffective in providing estimates of fungal biomass, except in pure cultures or controlled laboratory systems. This is partially due to the problems involved in identifying suitable chemical markers and determining how specific they are for particular groups or species. Knowledge of the chemistry of many groups is still so imperfect that chemical markers need to be used with caution, particularly in field studies.

Assays for fungal cell wall components (Ride & Drysdale, 1972; Swift, 1973a; Whipps et al., 1982 & 1985; Hicks & Newell, 1983) can give estimates of fungal biomass in laboratory cultures and are particularly useful in host pathogen studies. However, unlike the muramic acid assay for bacteria (Fazio, Mayberry & White, 1979) fungal cell wall and storage components cannot be used as reliable fungal biomass estimates in the field because of their widespread occurrence in other biota (White, 1983; Smith, 1980).

Adenosine nucleotides, in particular ATP, have been employed as measures of microbial biomass in a variety of terrestrial and aquatic ecosystems (Ausmus, 1973; Suberkropp & Klug, 1976; Davis & White, 1980; Karl, 1980), but their ubiquity in living organisms appears to limit their usefulness to studies of pure cultures, or total microbial biomass measures, like the chloroform fumigation method (Jenkinson & Powlson, 1976) and microcalorimetry (Sparling, 1981 a & b). The possibility of measuring prokaryotic biomass after selective lysis of eucaryotic cells and destruction of liberated ATP has been achieved in analysis of urine samples (Chappelle, Picciolo & Deming, 1978) but the potential of this method as a selective biomass method seems limited due to the wide variety of eukaryotic cell types encountered in nature.

Sterols have only recently been explored as possible

markers for fungal biomass. Ergosterol, and related C_{28} sterols, have been found in many fungal species and only to a limited extent in other organisms (Altman & Dittmer, 1972). Seitz *et al*. (1977 & 1979) developed an HPLC assay for measuring ergosterol in cereal grains; they found that the assay was more rapid and gave a greater sensitivity in estimating fungal biomass than fungal cell wall glucosamine. More recently White *et al*. (1980), in a detailed study of fungal chemical markers, concluded that sterols possibly offered the greatest potential for resolving problems of fungal biomass and community structure. Lee, Howarth & Howes (1980) and Newell, Fallon & Miller (1986) have used ergosterol as a fungal biomass marker in their studies on *Spartina alterniflora* Loisel. Despite the attractiveness of using specific sterols as markers of fungal biomass, little is known about the variation in quantities present in fungal tissues under different environmental conditions. If variation is large, as it can be in the case of glucosamine, then the use of sterols as absolute measures of biomass may be limited.

Several other chemical components of fungi seem to have potential as markers for fungal biomass in some circumstances; these include the ratio of lipid phosphate to lipid phosphonate, the ratio of polyenoic fatty acids to total fatty acids and a number of lipid carbohydrates (White *et al*, 1980; Whipps *et al*., 1982 & 1985). A major problem is that some of these components will be present in plant detritus and the fungal component could only be distinguished by incubation with suitable isotopes, followed by extraction and detection using mass spectrometry in the selective ion mode (White *et al*., 1980).

Assay for enzymes such as laccases (Wood, 1979) have also shown promise as markers for fungal biomass but the majority are either too specific (laccase) or too widespread to be of general application, but they may be of use for studying groups of species in mixed populations.

In recent years there has been interest in the use of

immunological techniques for detecting and quantifying fungal mycelium on and in plants, particularly by immunofluorescence (Warnock, 1971 & 1973; Ibbotson & Pugh, 1975; Fitzell, Evans & Fahy, 1980; Frankland et al, 1981; Chard, Gray & Frankland, 1985), but also by enzyme linked immunosorbant assay, ELISA (Johnson et al., 1982; Aldwell et al., 1983; Newell, Fallon & Miller, 1986). In particular, ELISA may have considerable potential for detecting and measuring fungal biomass.

An alternative to the direct microscopic or indirect chemical and immunological approaches to the estimation of fungal biomass has been taken by Anderson & Domsch (1973, 1974, 1975). Their method relies on measuring CO_2 respiration from substrates (soil, plant litter) to which several selective inhibitors for prokaryotes and eukaryotes are added together with a readily utilisable carbon source. The response of the microorganisms with and without groups of inhibitors is thought to differentiate the proportion of total respiration accounted for by prokaryotes and eukaryotes. By relating this to further work comparing respiration of pure cultures of known biomass, it was possible to estimate the biomass of respective groups in soils (Anderson & Domsch, 1978 a & b). The major constraints of the method concern 1) the lack of distinction between different eukaryotes (fungi, protozoa, myxomycetes, nematodes, algae, etc.); 2) the variable effects of inhibitors depending on growth states of microorganisms (Yetka & Wiebe, 1974); and 3) possible alteration of respiratory responses by release from competition (Salonius, 1981).

Clearly, there are many possible methods for estimating fungal biomass, most of them having limitations and problems. Because of this, it is likely that more than one method should be used in ecological studies in the field to avoid misinterpretations.

The present study is in two parts; the first is concerned with laboratory study of the methods and the second with field applications of the biomass methods.

The objectives of the study were:

1) To examine several approaches to estimation of fungal biomass in leaf litter, to assess their limitations and, where possible, to modify them to enable more accurate estimations of biomass.

2) To characterize the spatial distribution of biomass on leaf litter.

3) To calculate conversion factors for estimating biomass for fungi associated with allochthonous litter in freshwater. To assess the variability of conversion factors both between species and between different growth conditions.

4) To examine fungal biomass on allochthonous litter in lakes as these have been neglected in previous studies.

5) To obtain estimates of spatial and temporal variation in biomass in relation to differences in fungal communities and decomposition in a number of gravel pit lakes.

6) To study fungal biomass and activity associated with the allochthonous litter in one of these lakes in detail, and to assess the importance of abiotic and biotic variables.

CHAPTER 2. A MODEL SYSTEM FOR STUDYING FUNGAL BIOMASS ON SUBMERGED PLANT MATERIAL

2.1 Introduction

The study of microbial populations and their role in decomposition processes often requires the use of laboratory based systems which can simulate some field conditions. For studies on submerged plant material the best types of systems are those of the continuous culture type or artificial circulating river channels. Unfortunately these systems are expensive to build and run and require considerable space, thereby limiting replication.

Batch systems have become popular with microbial ecologists because they are economical and permit better replication of experiments. Among their disadvantages are possible nutrient depletion and a build up of waste metabolites and reproductive propagules. These problems may often be related to utilisation of the medium and the relative quantity of plant material; they can be overcome to some extent by using dilute mineral media and relatively small quantities of plant material. A batch type system was devised for studying the biomass and activity of fungi on submerged leaf litter.

2.2 The experimental system

Webster & Towfik (1972) and Webster (1975) studied the effects of aeration and turbulence on sporulation of 'Ingoldian' fungi in flasks. The number of spores produced varied depending on the rate of aeration and the particular species involved. Investigation revealed that turbulence in the flasks induced by the rate of aeration was more important for sporulation than the degree of oxygenation of the medium. They showed that aeration using a hypodermic needle gave a consistently

higher spore production than when using a sintered glass bulb.

Using a hypodermic needle (size 21 G) for aeration an experimental system was designed using 1 litre round-bottomed flasks (Figure 2.1). Flasks were autoclaved without medium or leaf material for 15 minutes at 121°C. After cooling, 600 ml of previously sterilized medium was added to each flask followed by the appropriate amount of sterilized leaf material. Figure 2.2 shows a typical system. Air from the pump was passed through an air filter (Millipore Corp.) before entering the flasks. The aeration rate (100 or 600 cm^3 min^{-1} depending on the experiment) for each flask was checked regularly using an air flow meter (capacity 60 to 1000 cm^3 min^{-1}) and minor adjustments made using small pinch clips. Sterility checks were made from time to time by aerating flasks with either malt extract broth or nutrient broth for periods of up to 14 days. No contamination of the flasks was encountered.

During early experiments there were some problems with hyphal films forming on the walls of inoculated flasks particularly at the air liquid interface. In later work the flask walls were siliconised using a solution of dimethyldichlorosilane (c. 2%) in 1 : 1 : 1 trichloroethane (Ugalde & Pitt, 1983) to discourage the adhesion of fungi to flask walls.

Up to 30 flasks were run for a single experiment. They were arranged randomly to reduce the effect of any variation in temperature, light or aeration. If flasks were sampled for spores or leaf material during the running of an experiment they were returned to a different position on the frame. Most experiments were conducted at a temperature of 17 ± 2°C.

2.3 Liquid medium

For most studies it was decided to use a dilute mineral medium consisting of 40 mg $Ca(NO_3)_2.4H_2O$,

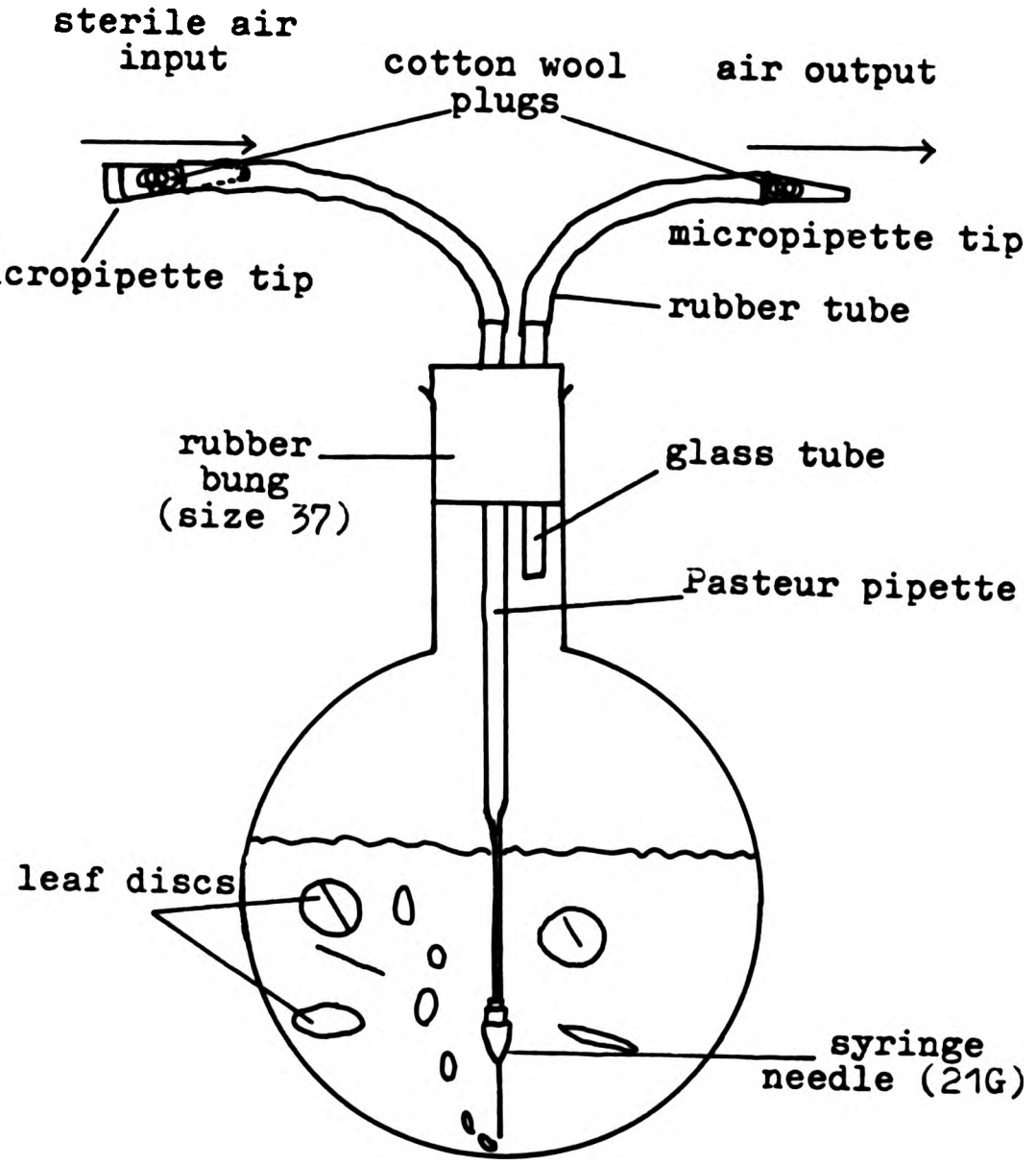
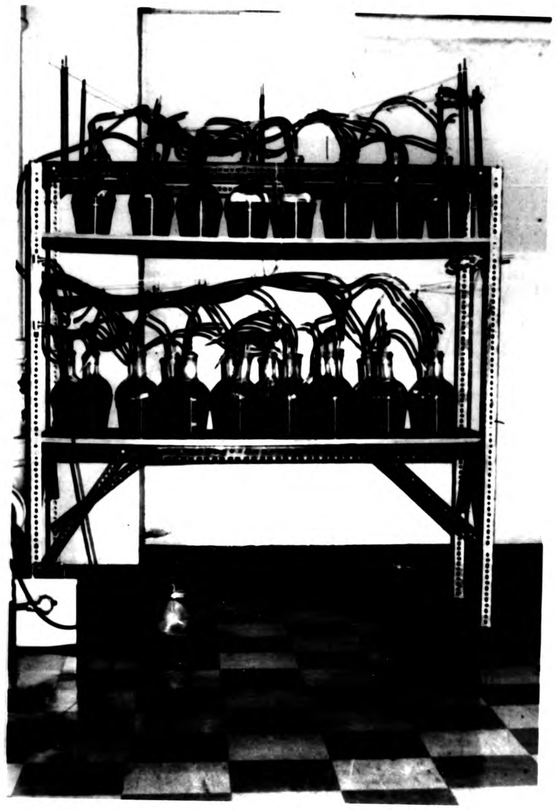


Figure 2.1. Diagram of the experimental flask (capacity 1 litre) showing the method of aeration.

Figure 2.2. Photographs showing a) the batch flask system mounted on a twin level frame and b) a close up of the flasks.

a)



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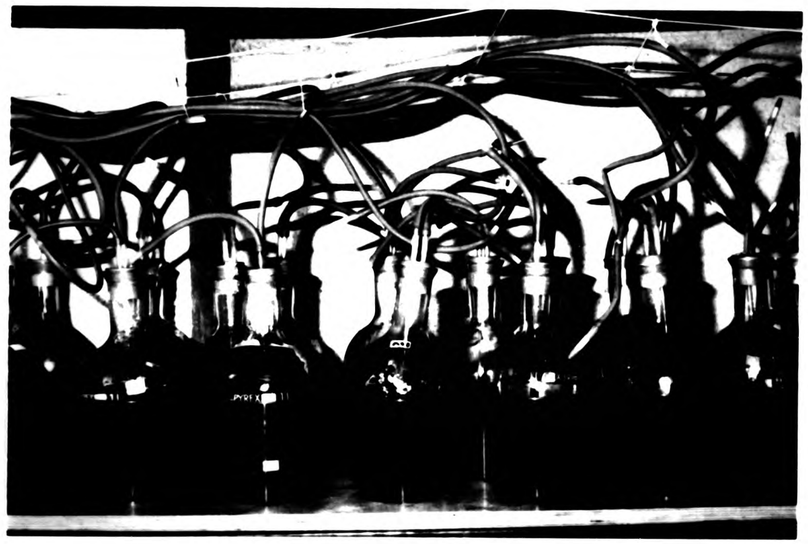


Figure 2.2. Photographs showing a) the batch flask system mounted on a twin level frame and b) a close up of the flasks.

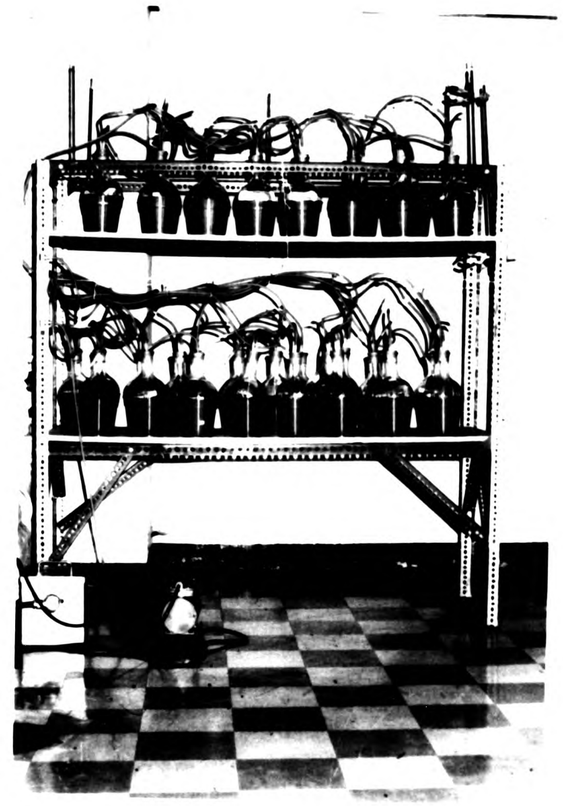
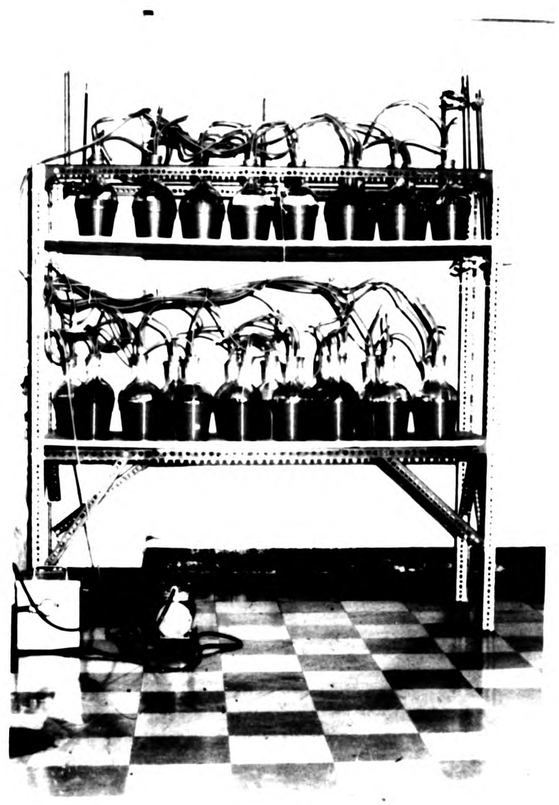


Figure 2.2. Photographs showing a) the batch flask system mounted on a twin level frame and b) a close up of the flasks.



15 mg $MgSO_4.7H_2O$, 1.5 mg KH_2PO_4, 6 mg KCl and 1 litre of distilled water. Residual materials in the leaf were thought to provide adequate trace elements. The medium was autoclaved in bulk at 121°C for 30 minutes before use. In the presence of sterile leaf material, with and without fungal inocula, the pH and conductivity of the medium remained fairly stable over a 3 week period. For some experiments freshly collected lake water (Gravel Pit No 4 at Yateley, Hampshire - see Chapter 8) was used instead of the mineral medium. The lake water was filtered (Whatman GF/C) before autoclaving in bulk at 121°C for 30 minutes.

2.4 Leaf material

Leaf material for experiments was collected during the autumn months of 1981, 1982 and 1983 from various sites near to lakes and rivers in S.E. England. Freshly fallen leaf material was collected from around the base of a few trees although some leaves were collected by suspending nets on the lower branches of selected trees. Four main species were collected, alder (*Alnus glutinosa* (L.) Gaertner), willow (*Salix fragilis* L.), oak (*Quercus robur* L.) and ash (*Fraxinus excelsior* L.). On return to the laboratory the leaves were spread out on sheets of newspaper and allowed to air dry before storing in plastic sacks at room temperature (20-25°C).

Prior to sterilization, the leaf material was soaked in distilled water for 3 days at 6°C to remove soluble components (Kaushik & Hynes, 1971). After soaking the leaves were rinsed in distilled water and air dried. For some experiments discs of 1 cm diameter were cut using a cork borer. Leaf discs, in groups of 20, were placed in separate polythene bags and sealed. Whole leaves were weighed into amounts of 1, 3 and 5 g and also sealed in plastic bags. All bags of leaf material were sterilized by gamma irradiation using a ^{60}Co source with a dose of approximately 3-4 M rad which was equivalent to about 3 days exposure to the source.

Sterility was confirmed by plating out irradiated samples on malt extract and nutrient agars. After sterilization the leaf material was stored in the dark at room temperature, for at least 4 weeks before use, to allow enzyme activity to decrease (Howard & Frankland, 1974).

2.5 Fungal inoculum

Strains of fungi were isolated from benthic litter obtained from lakes and rivers in England. Malt extract agar containing antibiotics (50 mg penicillin G and 50 mg streptomycin sulphate) was used to isolate fungi either from single spores removed by direct observation or by plating of particles of leaf material.

In some experiments flasks were inoculated with plugs of agar bearing actively growing mycelium whilst, in others, spore suspensions were used as an inoculum. Spore suspensions were produced by aerating 150 ml of sterile distilled water in conical flasks (250 ml) with plugs of active mycelium grown on malt extract and potato dextrose agars for 3-7 days at room temperature (20-25°C). A surfactant (0.01% Tween 80 v/v) was sometimes added.

Spore suspension counts were made by filtering (0.45 µm membrane filter, Millipore Corp.) 1-2 ml of each suspension, allowing the filter to dry (60°C, 15 minutes), staining with phenolic analine blue (Jones & Mollison, 1948), clearing in a few drops of lactophenol and counting the spores in 20 fields of view at x400 magnification.

The batch system described in this chapter was used to produce fungal biomass grown on submerged plant litter under a range of conditions and to provide colonised leaf material for the evaluation of quantitative techniques.

CHAPTER 3. DEVELOPMENT OF A STAINING METHOD FOR FUNGI ON BENTHIC LITTER: ITS APPLICATION TO THE MEASUREMENT OF FUNGAL BIOMASS AND DETERMINATION OF SPATIAL DISTRIBUTION OF HYPHAE ON LEAF LITTER

3.1 Introduction

A number of clearing and staining methods have been developed to investigate the presence and distribution of fungal structures in plant tissues. Most methods have been developed for living plant material infected by pathogenic fungi and only a few, including those of Hering & Nicholson (1964), Minderman & Daniëls (1967), Witkamp (1974), and Newell & Hicks (1982), have been used for leaf litter. Dead leaves contain a complex mixture of pigmented high molecular weight organic components which makes litter difficult to clear and stain. Most studies have been qualitative and there have been few attempts to obtain quantitative estimates of fungal hyphal length or biomass on leaf litter (Minderman & Daniëls, 1967; Bärlocher & Kendrick, 1974; Witkamp, 1974).

After clearing and staining of whole leaves or leaf fragments, most fungal structures are thought to remain *in situ* enabling their spatial distribution to be studied. The objective of this part of the study was to develop an effective staining method which would provide reliable estimates of biomass on leaf litter and, in addition, give some idea of fungal spatial distribution.

3.2 Materials and methods

3.2.1 Development of a whole leaf[1] clearing and staining method for fungi in leaf litter

3.2.1.1 Leaf bleaching

A range of bleaching agents were evaluated for their ability to decolourise leaf litter. These are listed in Appendix 1, Table 1 together with concentrations tried. Freshly shed leaves of alder (Alnus glutinosa (L.) Gaertner), willow (Salix fragilis L. and Salix cinerea L.), oak (Quercus robur L.) and beech (Fagus sylvatica L.) were tested together with samples of these leaf types retrieved from benthic litter of several streams and lakes. Some experiments were conducted with whole leaves but most used 1 cm diameter discs of leaf tissue. For most treatments whole leaves or discs were exposed to bleaching agents for different lengths of time at either 20 - 25 °C or 70 °C. Although a number of the bleaching treatments decolourised leaf material, only the method of Hering & Nicholson (1964) gave good results when subsequently cleared and stained. This method was adopted for all subsequent work.

After bleaching, leaves were washed in several changes of distilled water and stored in 70% alcohol. Before clearing and staining, the leaf material was dehydrated as described by Hering & Nicholson (1964) with the final step (100%) being performed under vacuum (75 cm Hg) for 15 to 30 minutes to remove traces of air from leaf tissues.

3.2.1.2 Leaf clearing

The bleaching treatment removes darkly pigmented

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1 The term 'whole leaf' is used to imply leaf material which has not been disrupted by physical maceration, and can include discs as well as whole leaves.

material but the tissues need rendering transparent. Lactophenol has been used widely for clearing plant material although methyl salicylate, clove oil and chloral hydrate have also been tried. These clearing agents were evaluated for their rapidity in rendering leaf material transparent and their suitability for use with a range of stains. Comparisons suggested that methyl salicylate and clove oil gave the best results with bleached leaf material. Of the two, methyl salicylate gave the best results during staining and was therefore adopted for use in all subsequent work.

3.2.1.3 Leaf staining

To find suitable stains for fungal structures in bleached and cleared leaf litter, a range of stains were evaluated for their ability to stain fungal structures differentially. Stains were made up at concentrations of 0.1% (w/v) in either absolute alcohol, methyl salicylate or mixtures of both. The stains tried are listed in Appendix 1, Table 2. Ideally a double stain was needed, one component staining the fungal hyphae and the other staining the plant tissue a contrasting colour. A number of stain combinations were tried and these are listed in Appendix 1, Table 3.

Staining leaf material with safranin O and fungal structures with lissamine green V gave the best differentiation between leaf tissues and fungal hyphae and was the method adopted for subsequent work. Table 3.1 presents a protocol of the stages involved in bleaching, clearing and staining of leaf litter prior to examination.

3.2.1.4 Losses of mycelium during preparation

Whole leaf methods have been criticised because they are thought to disrupt mycelium and therefore give underestimates of biomass. Many bleaching agents may cause partial destruction of plant material, particularly

TABLE 3.1 Protocol for preparing leaf material for examination

1) Bleach leaf material in a mixture of sodium chlorite, acetic acid and water (0.5ml 10% acetic acid in 40ml sodium chlorite solution (7.5mg ml^{-1}) for 3-4 hours at 60°C).
2) Rinse in distilled water for 2 minutes.
3) Dehydrate leaf material using vacuum to remove gases, 10 minutes each in 80, 90 and 95% alcohol and 20 minutes in 100% alcohol (vacuum 72 cm Hg).
4) Stain in safranin O_{*1} for 20-120 seconds.
5) Rinse in methyl $salicylate_{*2}$ for 5-10 seconds.
6) Counterstain in lissamine green V_{*3} for 20-60 seconds.
7) Rinse in methyl salicylate for 5-10 seconds to remove excess stain.
8) Mount in methyl salicylate, DPX mountant or Canada Balsam.

*1 0.03g safranin O (CI.50240) in 5ml absolute methanol made up to 30ml with methyl salicylate.
*2 Methyl salicylate can be replaced by clove oil during clearing and staining.
*3 0.03g lissamine green V (CI.44025) in 5ml methanol made up to 30ml with methyl salicylate.

at high temperatures (70 °C or more). An attempt was made to quantify any losses of mycelium during the preparation steps prior to examination by filtering (0.45 µm porosity membrane filter, Millipore Corp.) all solutions and reagents involved in the various stages.

Ten leaf discs (12 mm diameter) of oak were bleached in 85 ml of filtered sodium chlorite/acetic acid/water bleachant (see Appendix 1, Table 1) in a pre-rinsed covered beaker at 70 °C for 10 hours. The bleached discs were rinsed in 15 ml of filtered distilled water which was added to the residual bleaching solution. The leaf discs were dehydrated in filtered alcohol, cleared in methyl salicylate, stained with safranin O in methyl salicylate and counterstained with lissamine green V in methyl salicylate, as described in Table 3.1. Cleared and stained leaf discs were mounted in methyl salicylate and ten random field of view (x 40 objective) were examined for each disc. Length of visible hyphae was measured using a gridded graticule and the intersection method (Olson, 1950), see Section 3.2.3. Hyphal lengths were expressed as m g^{-1} leaf dry weight (80 °C) using 10 untreated discs for weight estimation.

Both the combined bleaching and washing solution and the dehydrating solutions were filtered to trap residual hyphae. These were then air dried under petri dish lids and stained with 3 drops of phenolic analine blue (Jones & Mollison, 1948). The covered slides were heated in an oven at 80 °C for 10 minutes followed by clearing and mounting the filters in lactophenol. Fifty fields of view were examined with x 40 objective and lengths of stained hyphae measured as above. Results were expressed as m hyphae g^{-1} leaf dry weight (80 °C).

3.2.1.5 Sectioning and staining

For accurate estimates of fungal biomass on or in leaf tissue it is necessary for the clearing and staining methods to reveal internal as well as surface hyphae. Samples of unbleached and bleached leaf material were

cleared in CNP 30 (commercial clearing agent) and, together with bleached, cleared and stained material (method in Table 3.1), were embedded in paraffin wax and sectioned (10 µm). Attempts were made to stain fungal hyphae in the unstained leaf material (unbleached and bleached) using standard methods for fungi in plant material (Vaughan, 1914; Stoughton, 1930; Dring, 1971) but these were unsuccessful. Only bleaching, clearing and staining the leaf material prior to sectioning gave satisfactory results. Attempts to bleach, clear and stain sections of untreated leaf material were also unsuccessful.

3.2.2 Other methods

Two other methods were considered for comparison with the clearing and staining technique.

3.2.2.1 Resin peels

Leaf impression methods have been used widely for studying microorganisms on surfaces (Baker, 1981). Acrylic resin peels of plant surfaces have been shown to be superior to other methods in removing the surface microflora (Birkby & Preece, 1981). The acrylic resin Primal AC 33 (Rohm & Haas Ltd, Croydon) was used in this work to make visual comparisons with leaf material which had been cleared and stained. Preparations were made as described by Birkby & Preece (1981) and results were assessed subjectively by visual comparison.

3.2.2.2 Fluorescence microscopy

Qualitative comparisons of fluorescence microscopy were made with the clearing and staining method. Leaf material was stained with various fluorescent dyes (Appendix 1, Table 4) and examined under incident ultraviolet or blue light on a Zeiss Universal Fluorescence microscope. Results were graded ona subjective scale

according to the degree of differentiation and intensity of fluorescence.

3.2.3 Evaluation of methods for quantifying fungal hyphae rendered visible by clearing and staining

The growth form of mycelial fungi has presented considerable problems in estimating their biomass using microscopical methods. Projection of fields of view onto paper enables hyphae to be drawn and measured, but the process is very time consuming. Line intercept methods (Olson, 1950; Newman, 1966; Tennant, 1975; Giovannetti & Mosse, 1980) enable direct estimation of hyphal lengths by counting the number of intercepts with lines on a eyepiece graticule. These methods have been used to measure hyphal lengths on stained membrane filters and in agar films, but their applicability to cleared and stained leaf material has not been evaluated. A comparison was made of a number of intercept methods, to see if any could be used to estimate hyphal lengths on cleared and stained leaf material.

To obtain suitably colonised leaf material a flask was set up containing 20 sterile alder leaf discs (1 cm diameter) and 600 ml of sterile mineral medium (see Chapter 2). The flask was inoculated with a spore suspension of Tetracladium marchalianum[1] (isolate 1A) to give a final concentration of 50 ± 3 spores ml^{-1}. The flask was aerated (600 cm^3 min^{-1}) during an incubation period of 7 days at 17 °C. After incubation, five leaf discs were removed, bleached, cleared and stained (see Table 3.1). Ten fields of view per leaf disc were examined (x 400) and maps of fungal hyphae in each field were made using a projection microscope. An area (13 x 13 cm) on each map was used for the quantitative study. The lengths of hyphae in these areas were measured using a map measurer; 20 maps were subjectively chosen to cover

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1 Nomenclature for 'Ingoldian' hyphomycetes follows Webster & Descals (1981).

a range of densities of fungal hyphae (130 - 2040 mm per area). This was necessary since some of the intercept methods may have responded differently at low or high densities of hyphae. Different grid patterns (random, systematic and stratified (Smartt & Grainger, 1974)), systematically arranged squares and randomly orientated lines were tried. Each was drawn on tracing paper in a square (13 x 13 cm) superimposed on the fungal hyphae maps and hyphal length was estimated using intercept methods. The results were then compared with those obtained using the map measurer. Time taken to estimate hyphal length using the intercept method was also assessed.

Table 3.2 shows that most of the intercept methods gave reasonable estimates of hyphal length. Correlations suggested that systematic and random grids gave the most consistent results over a range of hyphal densities. Intercepts with random lines gave the quickest estimates but the results were generally more variable with different densities of hyphae. Therefore in most subsequent work a systematic grid was used for estimating hyphal lengths. Suitable graticules were made using a photoreduction technique. Drawings of each graticule (13 x 13 cm) were made on tracing paper with line widths of 0.5 and 0.35 mm. Each drawing was photographically reduced to a suitable size (1 x 1 cm) using a graphics art camera (Eskoff 5506). Each grid was developed on CPF mechanical acetate film (Agfa Gevaert) and, after drying, cut to fit the microscope eyepiece.

3.2.4 Variation in the distribution of fungal biomass on leaf litter

Having established a clearing and staining method for leaf material, attempts were made to assess the variation of hyphal length on single leaves and on discs cut from a large number of leaves. These two 'scales' not only provide information on variation in hyphal lengths, but also give some indication of fungal spatial pattern on leaves.

TABLE 3.2 Comparison between different methods for estimating hyphal lengths in microscope fields of view. Each method was used to estimate the hyphal lengths on drawings of mycelium in 20 microscope fields of view projected onto paper. Estimates of hyphal length made using each method were compared to actual measured lengths made using a map measurer (Method 1).

SAMPLING METHOD	MEAN HYPHAL LENGTH(mm)	S.D.	%ERROR*	CORRELATION (r_s)	MEAN TIME (SEC)
1.Measured	950.5	693.9	0*	1.0000	311
2.Systematic grid	988.3	733.9	+3.9	0.9868	128
3.Random grid	955.3	699.7	+0.5	0.9774	143
4.Stratified random grid	931.0	684.0	-2.1	0.9560	149
5.Stratified systematic unaligned grid	905.5	650.6	-4.7	0.9744	139
6.Systematic squares	1001.5	661.8	+5.4	0.9496	67.2

SAMPLING METHOD		MEAN HYPHAL LENGTH(mm)	S.D.	%ERROR*	CORRELATION (r_s)	MEAN TIME
7.Random lines (10cm)						
No.Lines	5	910.5	732.5	-4.2	0.9225	20.6
	10	923.2	625.4	-2.9	0.9008	39.2
	15	960.2	783.2	+1.0	0.9583	63.9
	20	948.8	715.2	-0.2	0.9714	96.3
8.Random lines (5cm)						
No.Lines	5	799.8	578.9	-15.9	0.7422	12.6
	10	946.9	810.4	-0.4	0.9451	25.9
	15	977.6	707.2	+2.9	0.9579	41.9
	20	883.4	617.6	-7.1	0.9451	58.3

* Expressed as $\frac{\text{Estimated length - Measured length}}{\text{Measured length}} \times 100$

3.2.4.1 Variation on single leaves

An experiment was set up to investigate whether material cleared and stained using the method in Table 3.1 could be used to detect differences in hyphal density on leaves. Sterile alder leaves were allowed to become colonised by one or several species of fungi under laboratory conditions. Two flasks were set up, each containing 3 g of sterile alder leaves and 600 ml of sterile mineral medium (see Chapter 2). To one flask a spore suspension of Anguillospora longissima (isolate 7A) was added to give a final concentration of 50 ± 4 spores ml^{-1}. The other flask recieved spores of Anguillospora longissima (isolate 7A), Tetracladium marchalianum (isolate 1A), Clavariopsis aquatica (isolate ST1) and Articulospora tetracladia (isolate VW1) giving a final concentration of 50 ± 8 spores ml^{-1} for each species. The flasks were continuously aerated (100 cm^3 min^{-1}) and incubated at 18 ^oC for 3 weeks. After incubation the leaf material was removed, rinsed in distilled water and a single leaf chosen at random from each flask. The leaves were bleached, cleared and stained.

The number of intercepts of hyphae with a systematic grid in the eyepiece were counted at x 400 magnification. A continuous belt from the edge of the leaf (at its broadest point) toward the midrib was sampled by moving the microscope stage to give a sequence (transect) of contiguous eyepiece grids (quadrat); 128 grids were sampled for each leaf. For the leaf colonised by Anguillospora longissima alone, a transect was made on both the upper- and underside of the leaf. The leaf from the flask with four fungal species had one transect on the upperside and two on the underside of the leaf. The number of intersections with the gridded graticules was used for spectral analysis (for details see Appendix 2).

3.2.4.2 Variation based on samples of a number of leaves

The methods used to examine fine scale spatial differences are not easily applied to samples consisting of many leaves or leaf discs. In such cases methods based on the variance and mean of a number of samples could be used, and a number of methods have been proposed (Taylor, Woiwod & Perry, 1978). Taylor's power law model (Taylor, 1961) describes spatial variance (V_i) as proportional to a fractional power of mean population density (μ_i) where

$$V_i = a\,\mu_i^B$$

and for a given set of samples, can be fitted in the form of a linear regression model

$$\log S^2 = \log a + b \log \bar{x}$$

where a and b are constants (see Taylor, Woiwod & Perry, 1978). Using the slope (b) of the regressions, both spatial and temporal differences can be examined. The model has been shown to be satisfactory for describing spatial and temporal behaviour of a wide range of organisms (Taylor & Woiwod, 1982). The means and variances of fungal hyphal lengths on leaf discs cut from a number of leaves were used to describe spatial behaviour and data variability in both laboratory and field studies.

The laboratory study consisted of a single fungal species Tetracladium marchalianum (isolate 1A) colonising alder leaf discs over 21 days. Twenty-five flasks were set up, each with 10 sterile 1 cm diameter alder leaf discs in 600 ml of mineral medium (see Chapter 2). Each flask was inoculated with a spore suspension of T. marchalianum (isolate 1A) to give a final concentration of 53 ± 2 spores ml^{-1} and the flasks incubated at 18 ^{o}C for up to 3 weeks under constant aeration (600 cm^3 min^{-1}). At 3, 7, 11, 16 and 21 days

five flasks were removed and the contents sampled. Five discs were oven dried (80 ^{o}C) and weighed. The remaining 5 discs were bleached, cleared and stained and the hyphal lengths determined in 10 random fields on each leaf using a gridded graticule. This data was adjusted to m g^{-1} leaf dry weight and linear regressions were performed on $\log_{10}$ of the mean and variance of hyphal length for the leaf discs at each sample time.

To assess variability under field conditions samples of oak, willow and alder were retrieved from benthic litter in lakes and ponds and either 10 or 20 1 cm diameter leaf discs cut. Five leaf discs were bleached, cleared and stained and hyphal lengths estimated in 10 random fields of view, as above. Other discs from each sample were oven dried (80 ^{o}C) and weighed, enabling hyphal length to be expressed as m g^{-1} leaf dry weight. Linear regressions were performed on $\log_{10}$ mean and variance for this data.

3.2.5 Comparison of whole leaf clearing and staining technique with other methods for estimating hyphal lengths

3.2.5.1 Other whole leaf clearing and staining methods

The clearing and staining method described in Table 3.1 was compared quantitatively with the methods of Bärlocher & Kendrick (1974) and Ineson, Leonard & Anderson (1982). For each comparison, leaf discs (1 cm diameter) were cut from single leaves of material retrieved from benthic litter. For each method five discs were cleared and stained and the hyphal lengths determined, and five more discs were dried and weighed to allow conversion of hyphal length values to m g^{-1} leaf dry weight (80 ^{o}C).

3.2.5.2 Agar film method

Leaf discs (1 or 2 cm diameter) were cut from material (mostly willow) retrieved from several collections of benthic litter. Leaf material which had been colonised by fungi in laboratory flasks was also used. For each sample 10 leaf discs were bleached, cleared and stained, and a further 10 discs used to prepare agar films (see Section 4.2). In both cases hyphal lengths were estimated using the intersect method. Drying of further discs (at 80 ^{o}C) from each sample enabled results to be expressed as m g^{-1} leaf dry weight.

3.3 Results

These studies showed that two stains, one acting as a counterstain, gave better differentiation between leaf tissues and fungal structures than single stains (Appendix 1, Table 3). Bleached, dehydrated and cleared leaf material stained for 20 - 120 seconds with safranin O in methyl salicylate followed by counter-staining with lissamine green V in methyl salicylate for 20 - 60 seconds produced the best results. Figure 3.1 a-f shows photographs of cleared and stained leaf material colonised by fungi. In most cases the fungal hyphae and spores (stained green) can be differentiated from the leaf tissues (stained pink). Suitable staining times using the lissamine counterstain varied with leaf type and its condition prior to bleaching. Longer staining times with lissamine green (more than 30 seconds) were often necessary to enhance the contrast between fungal hyphae and leaf tissues, but this often led to some leaf tissues being stained green, e.g. stomata (Figure 3.1 a, c and d).

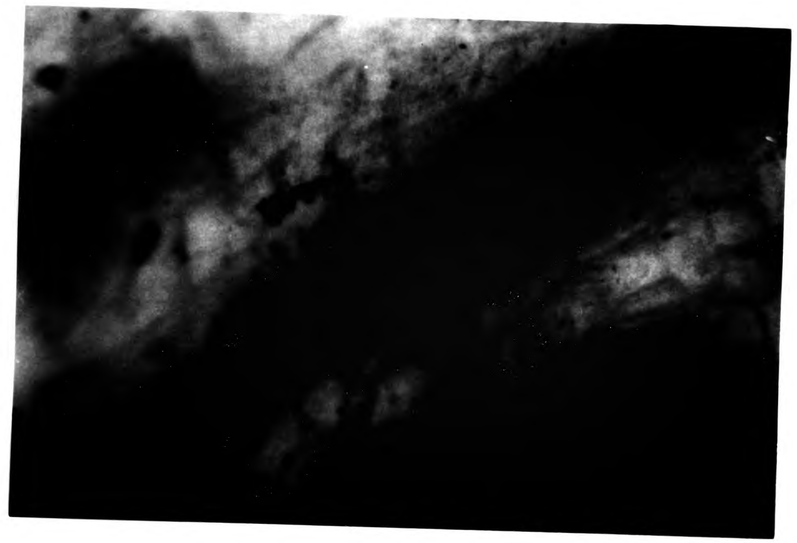
Both living and dead hyphae were found to stain equally well in comparisons of irradiated and non-irradiated samples. A test using *Bremia lactucae* Regel on *Sonchus oleraceus* L. showed that fungi without chitin wall components could also be stained. Dark pigmented

Figure 3.1 Examples of cleared leaf material stained with safranin O and lissamine green V.

a)

100 µm

b)



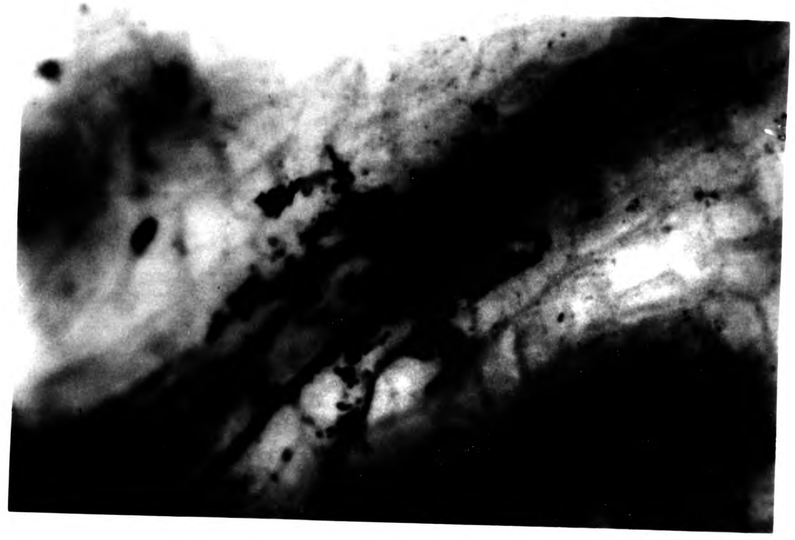
50 µm

Figure 3.1 Examples of cleared leaf material stained with safranin O and lissamine green V.

a)

100 µm

b)



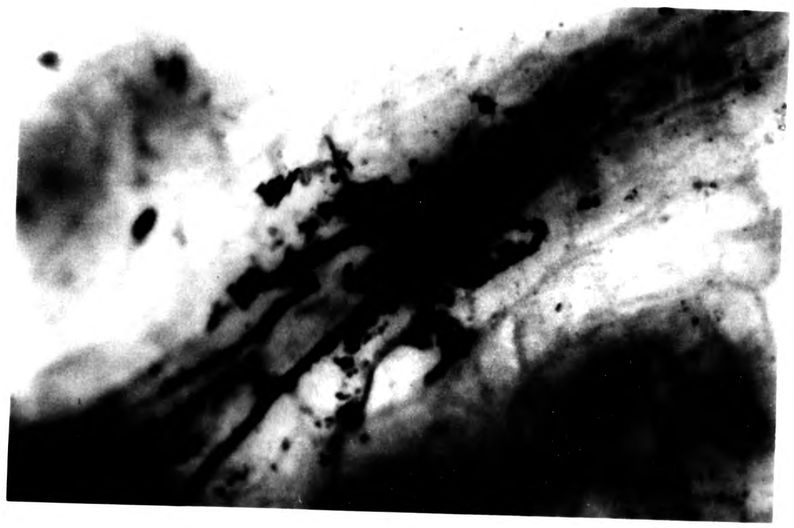
50 µm

Figure 3.1 Examples of cleared leaf material stained with safranin O and lissamine green V.

a)

100 µm

b)



50 µm

d)

c)

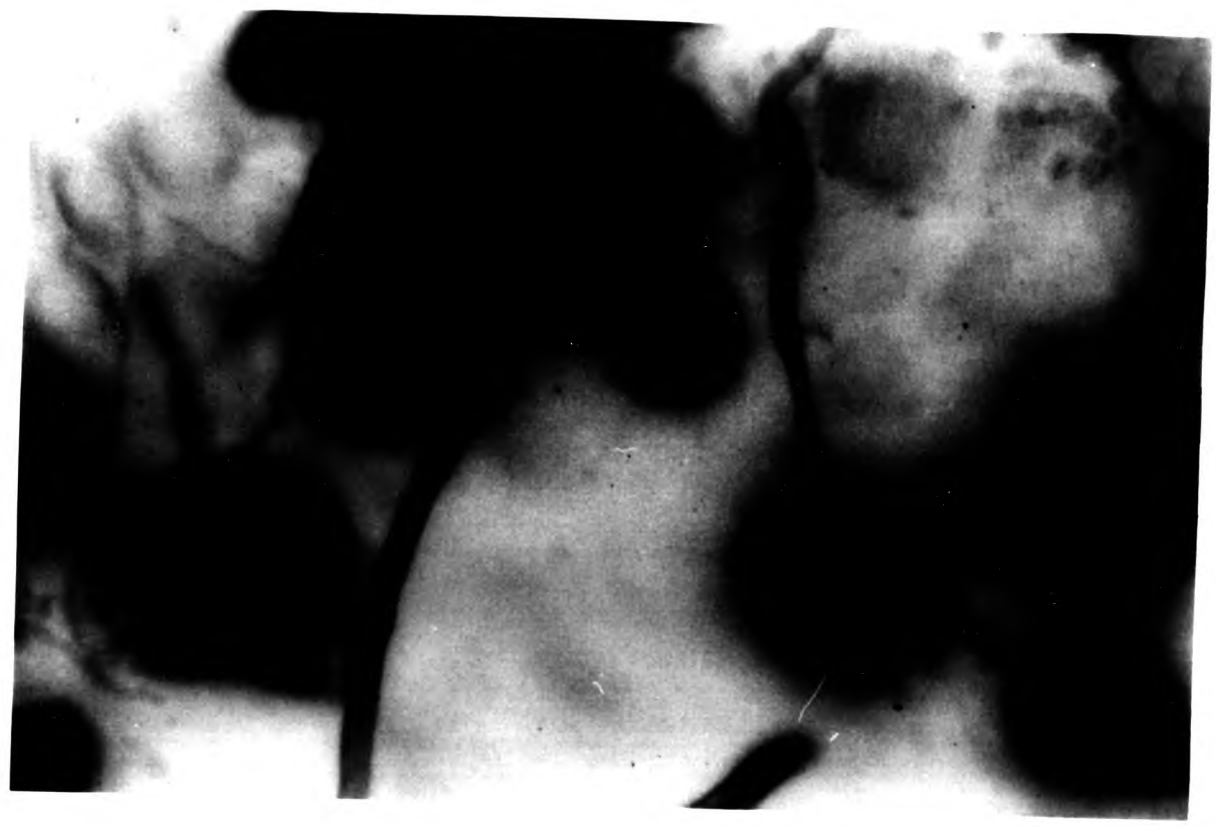
d)

c)

c)

50 µm

d)



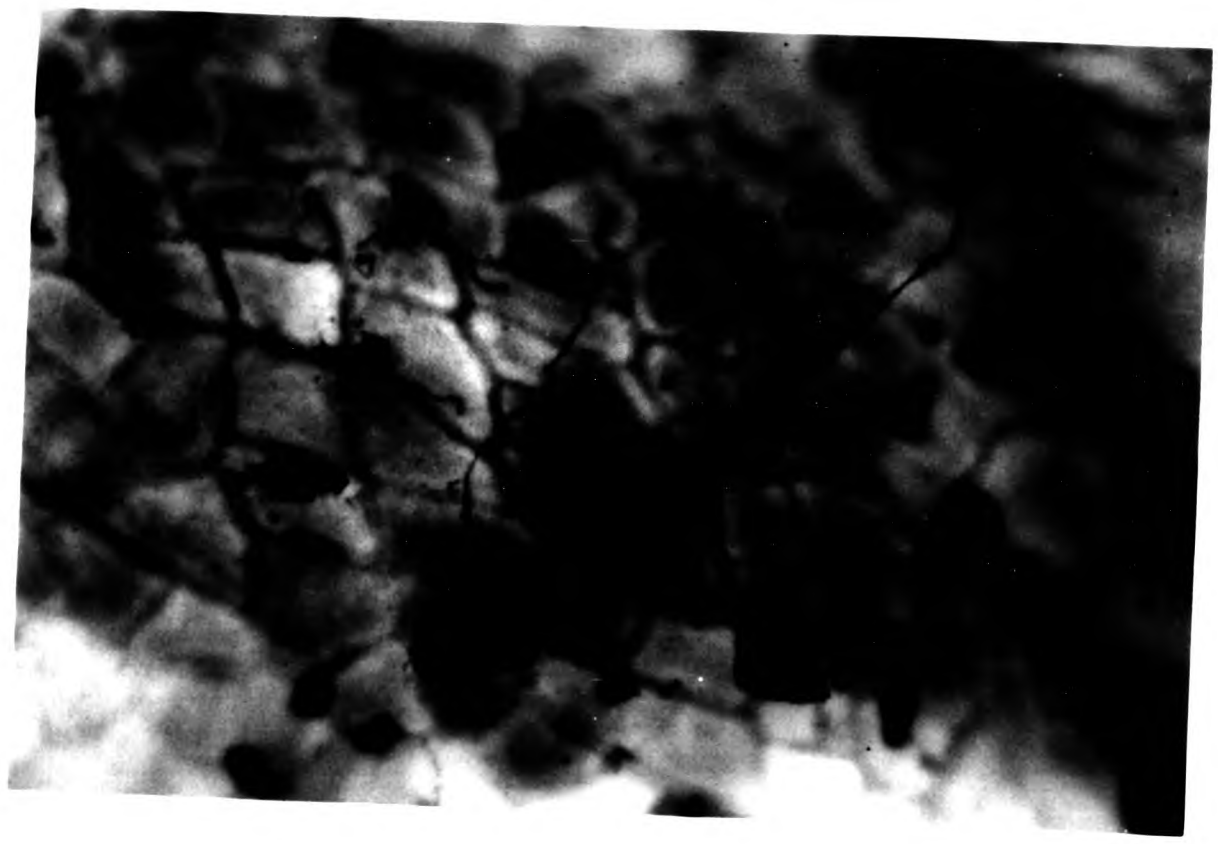
10 µm

e)

50 µm

f)

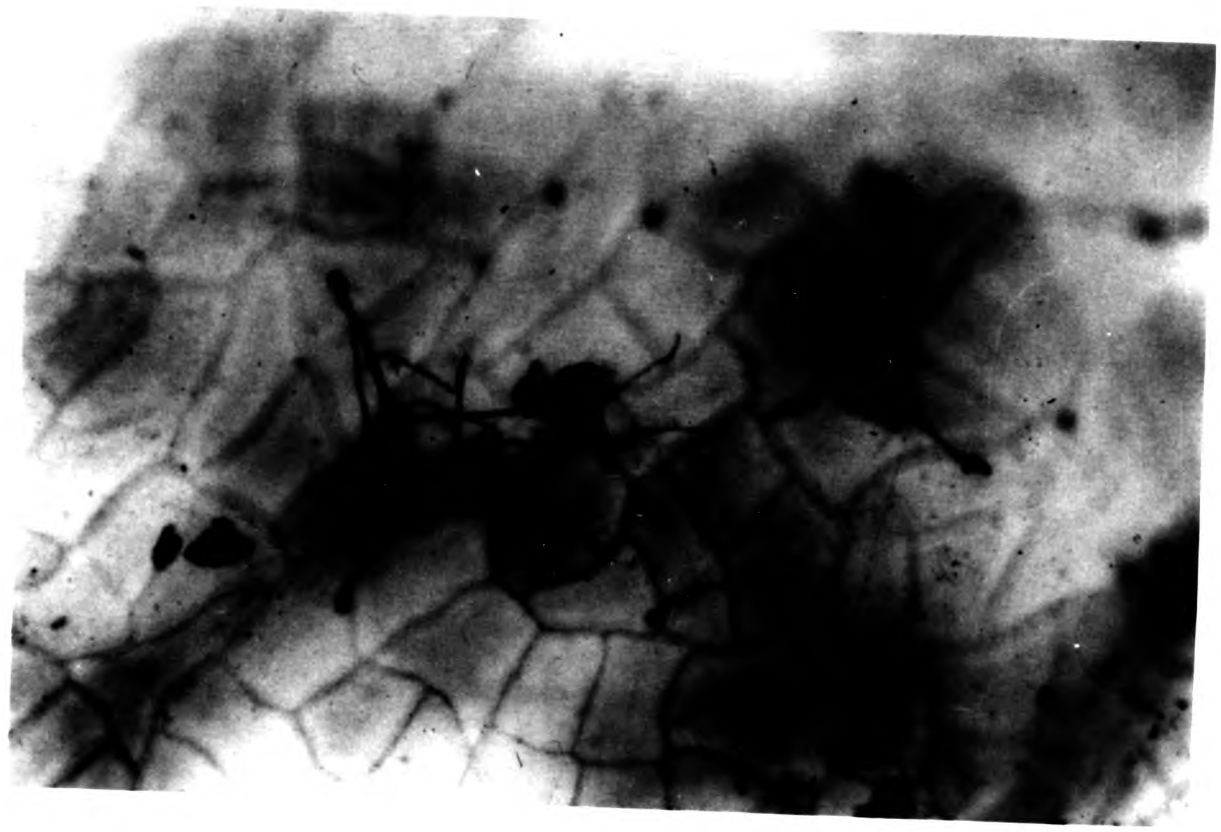
50 µm



f)

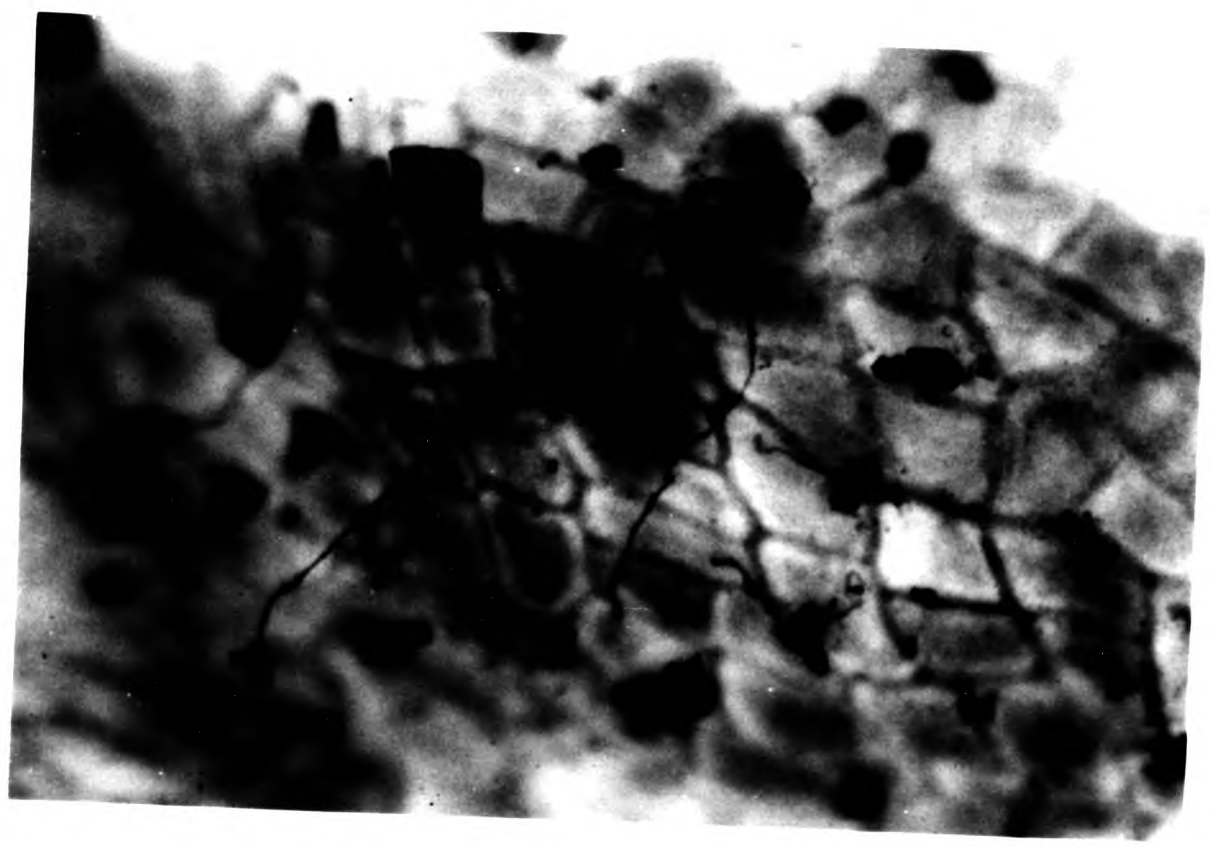
e)

e)



50 µm

f)



50 µm

hyphae and spores were not successfully bleached or stained but were clearly visible on cleared and stained leaf material.

Sections of cleared and stained leaf material revealed that both stains penetrated the internal leaf tissues. The presence of stained internal fungal hyphae mostly occurred just below the leaf surface and no extensive areas of colonisation were observed. Ascocarps and pycnidia in leaf tissues stained poorly with the lissamine stain suggesting that some fungal structures within the leaf may have stained less well than on the leaf surface. However, stained surface hyphae could often be followed into lamina and vascular tissues by adjusting the fine focusing on the microscope, and this enabled both surface and subsurface hyphae to be seen.

Damage to the leaf tissues and disruption of fungal structures during preparation appeared to be minimal. Of the hyphal length measured on oak leaf discs (1128 $\pm$ 12.6 m g^{-1} leaf oven dry weight (80°C)) only 0.58% (6.4 $\pm$ 2.0 m g^{-1} leaf oven dry weight (80°C)) could be detected in filtrates of the bleaching and dehydrating solutions. Very few fungal spores were detected in the filtrates and none were of Ingoldian fungi. In contrast, spores were often seen on stained leaf material particularly those of Ingoldian fungi (Figure 3.1 e, f). Measurements of hyphal diameters of Tetracladium marchalianum, on cleared and stained alder leaves, were very similar to those obtained with leaf material mounted in water. This suggests that the clearing and staining treatments do not change hyphal diameters.

Qualitative comparisons were made between the clearing and staining method and leaf material examined by fluorescence microscopy and acrylic resin peels. The fluorescence microscopy did not reveal any more fungal structures than the clearing and staining method and problems of quenching and masking often made this approach less convenient to use. The acrylic resin peels

suggested that the clearing and staining method gave an adequate representation of most of the leaf surface fungal structures. Bacteria and yeasts which were visible on the resin replica could not be seen on cleared and stained leaves. They may have been lost during preparation or were not stained by the lissamine green.

Figure 3.2 shows contrasting patterns of hyphal growth forms on alder leaves. In a-c the fields of view show mostly long straight hyphae with few hyphal ends, whereas in d-f the hyphae are much shorter, less straight and with more hyphal endings per field of view. Differences such as those illustrated in Figure 3.2 were often seen on leaf material and may have been related to the growth forms of different fungal species on the leaf surface (a-c) or into leaf tissues (d-f). Densities of fungal hyphae did not differ significantly ($P > 0.05$) between upper and lower surfaces of leaves. This is in contrast to the situation on aerial plant surfaces. Growth forms of fungal hyphae did not differ noticably between upper and lower surfaces of the leaf litter.

The analysis of hyphal distributions on leaf material was undertaken using spectral analysis (Appendix 2). Test runs made using data simulating known patterns helped the interpretation of experimental data. Results for the simulated data are given in Appendix 2. Figure 3.3 a-e shows the results of spectral analysis of the fungal data. In each case the raw data a-e (iii) represents counts of the number of intersections with a gridded graticule. Figure 3.3 a and b represents the transects across alder leaves colonised by Anguillospora longissima. In both cases the raw data in (iii) shows considerable fluctuations along the length of each transect. Visual inspection suggests that some pattern may be present but it is not clearly discernable. Both cumulative periodograms (a(i) and b(i)) indicate a significant decrease in spectral density with increasing frequency, as the cumulative frequencies in a(i) and b(i)

Figure 3.2 Variation in the growth form of fungal hyphae on alder leaves. (Those in a-c show hyphae growing straight with little branching, whereas those in d-f are shorter, more twisted and have more frequent branching)

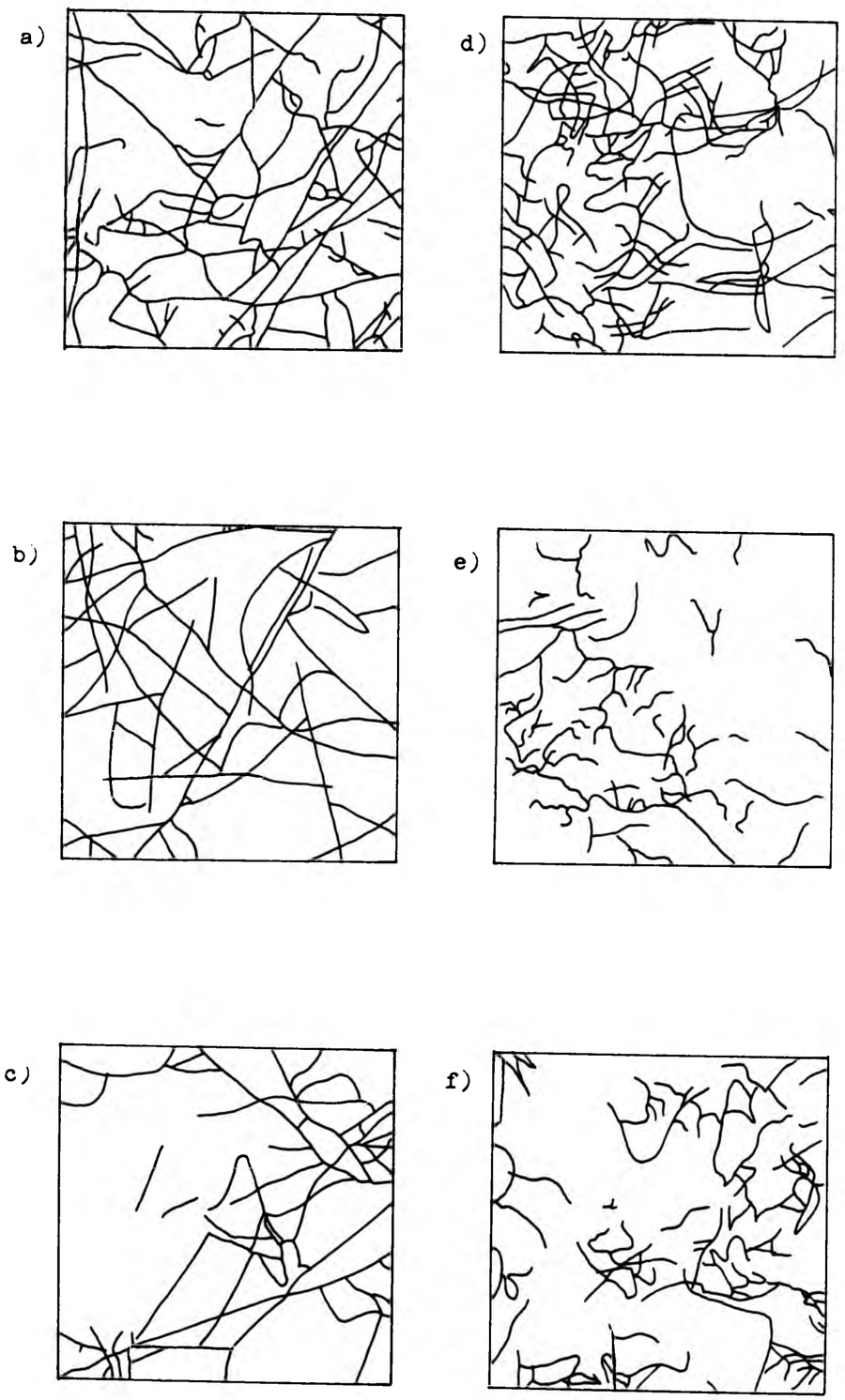


Figure 3.3. Spectral analysis and transect data of fungal distribution on leaves.

a) Anguillospora longissima on underside of an alder leaf after 3 weeks submersion.

b) Anguillospora longissima on upperside of an alder leaf after 3 weeks submersion.

c)Anguillospora longissima, Tetracladium marchalianum, Clavariopsis aquatica, Articulospora tetracladia on upperside of an alder leaf after 3 weeks submersion.

d) Anguillospora longissima, Tetracladium marchalianum, Clavariopsis aquatica, Articulospora tetracladia on underside of an alder leaf after 3 weeks submersion.

e) Anguillospora longissima, Tetracladium marchalianum, Clavariopsis aquatica, Articulospora tetracladia on underside of an alder leaf after 3 weeks submersion.

Three graphs are shown:

i) the cumulative periodogram with 95% confidence bands running diagonally.

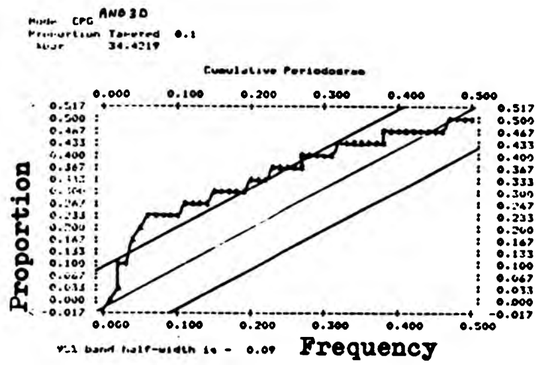
ii) smoothed spectral estimate with 95% confidence limits.

iii) a plot of the transect data from quadrat 1 to 128

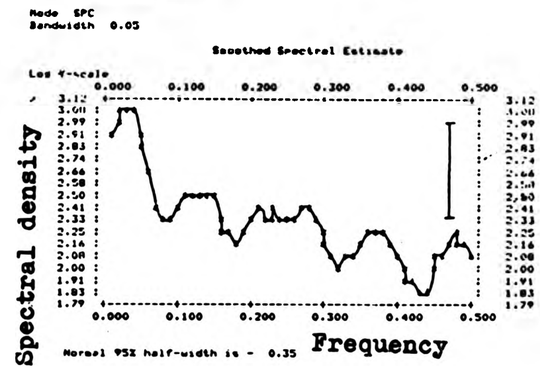
Further explanation of the Figures is given in Appendix 2.

3.3 a)

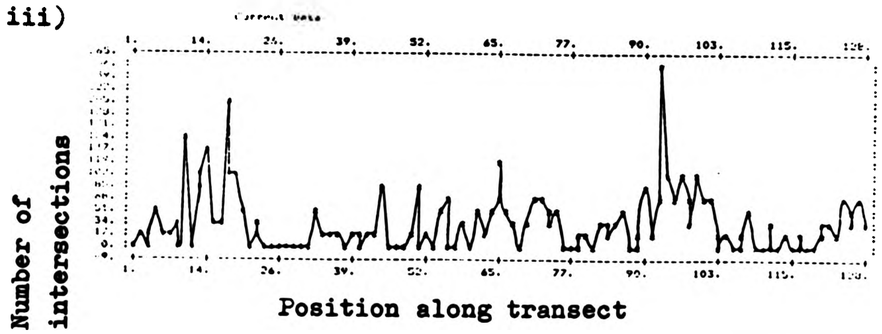
i)



ii)

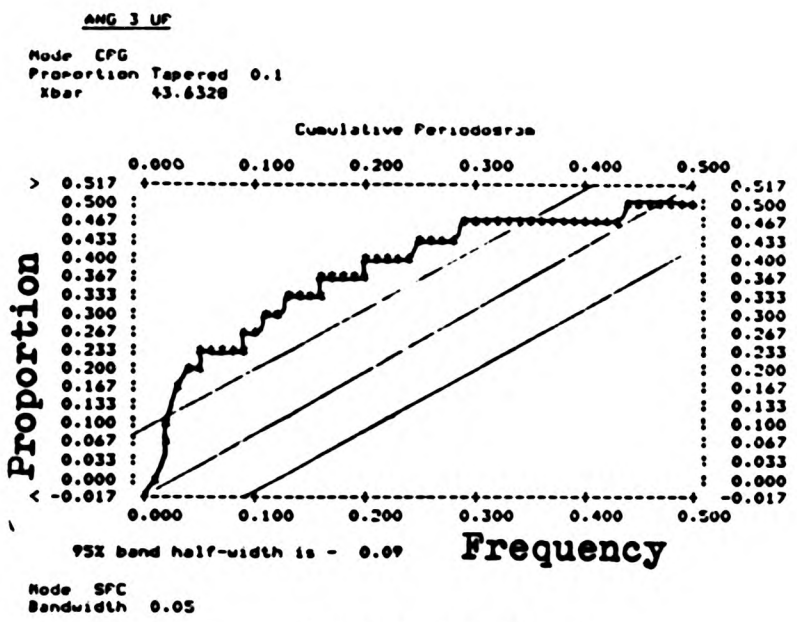


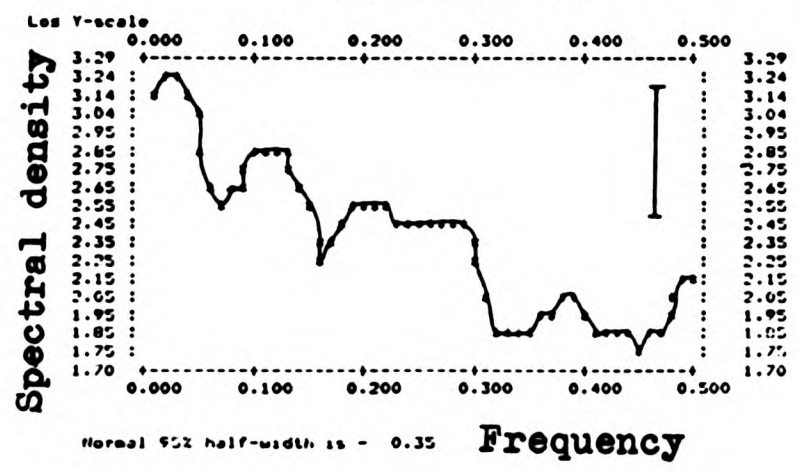
iii)



3.3 b)

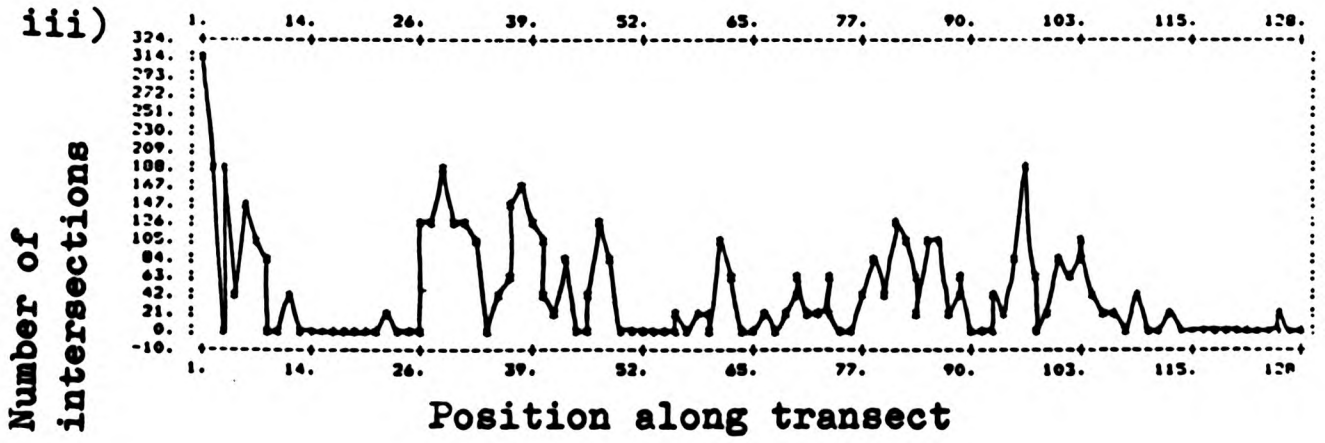
i)


ANG 3 UP
Mode CPG
Proportion Tapered 0.1
Xbar 43.6328
Cumulative Periodogram
0.000 0.100 0.200 0.300 0.400 0.500
> 0.517
0.500
0.467
0.433
0.400
0.367
0.333
0.300
0.267
0.233
0.200
0.167
0.133
0.100
0.067
0.033
0.000
< -0.017
Proportion
95% band half-width is - 0.09
Frequency


Mode SPC
Bandwidth 0.05
Smoothed Spectral Estimate
Log Y-scale
0.000 0.100 0.200 0.300 0.400 0.500
3.29
3.24
3.14
3.04
2.95
2.85
2.75
2.65
2.55
2.45
2.35
2.25
2.15
2.05
1.95
1.85
1.75
1.70
Spectral density
Normal 95% half-width is - 0.35
Frequency

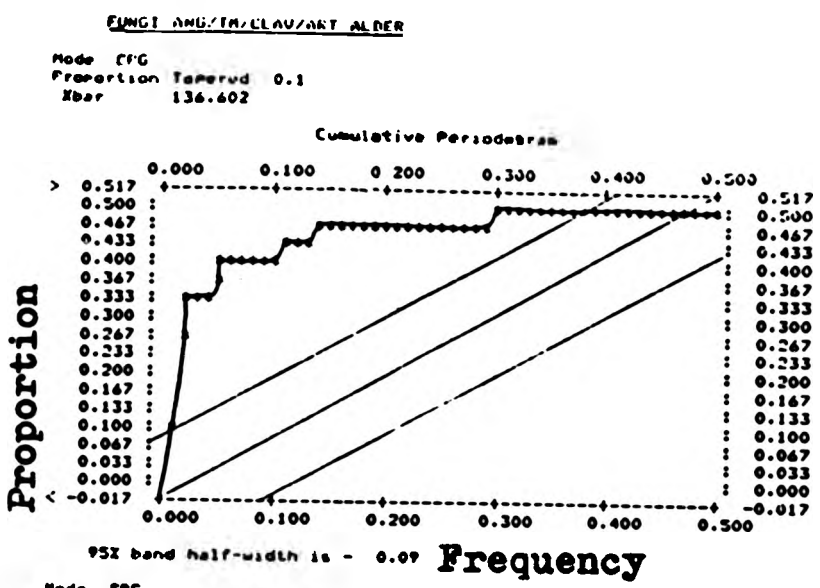
ii)

iii)

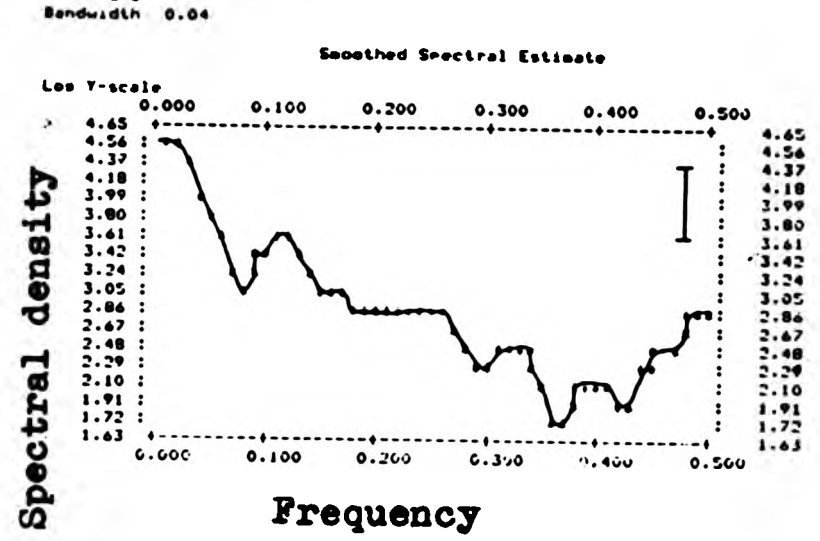

1. 14. 26. 39. 52. 65. 77. 90. 103. 115. 128.
324.
314.
293.
272.
251.
230.
209.
188.
167.
147.
126.
105.
84.
63.
42.
21.
0.
-10.
Number of intersections
Position along transect

3.3 c)

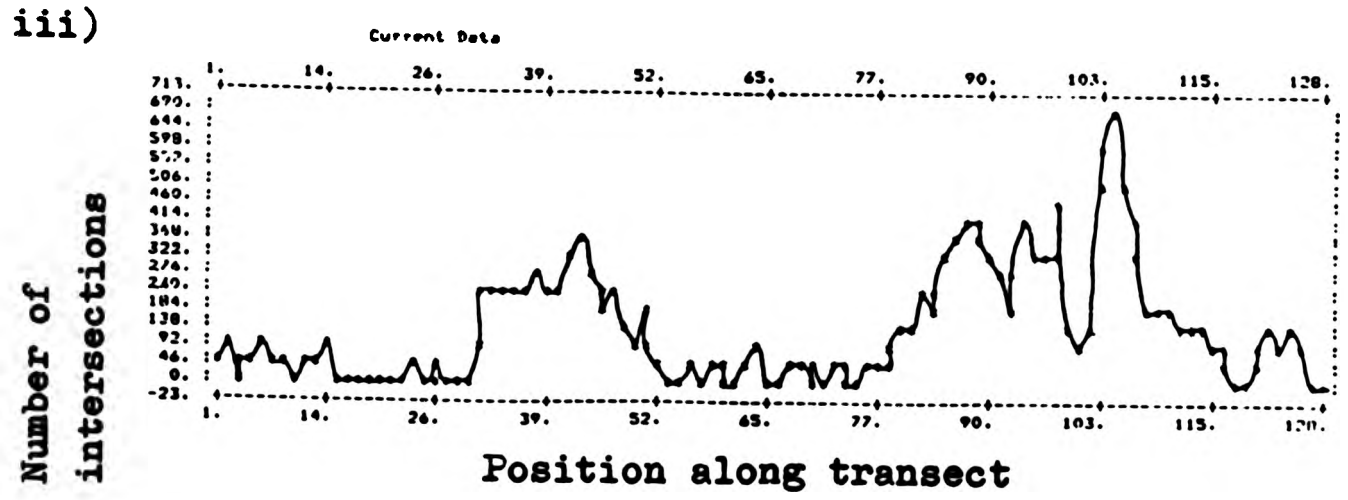
i)


FUNGI ANG/TM/CLAU/ART ALDER
Mode CPG
Proportion Tapered 0.1
Xbar 136.602
Cumulative Periodogram
Proportion
Frequency
95% band half-width is - 0.09

ii)

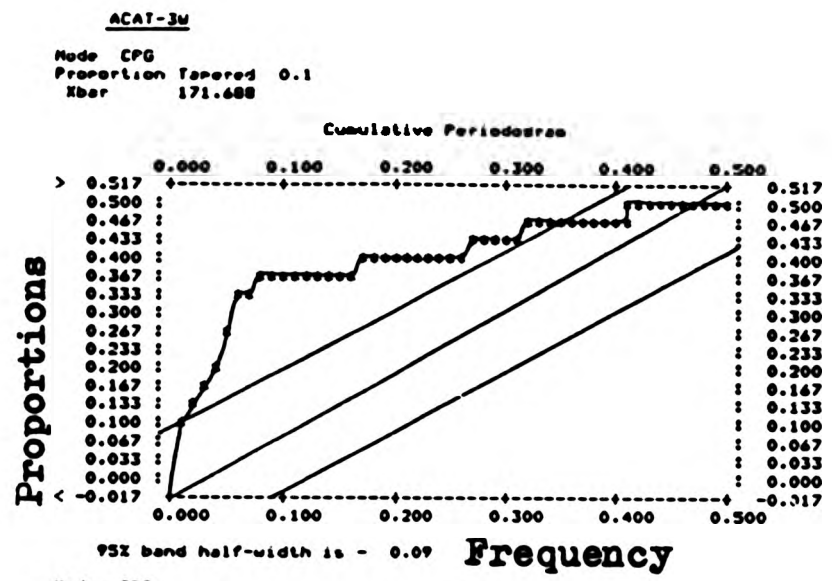

Mode SPC
Bandwidth 0.04
Smoothed Spectral Estimate
Log Y-scale
Spectral density
Frequency

iii)


Current Data
Number of intersections
Position along transect

3.3 d)

i)


ACAT-3W
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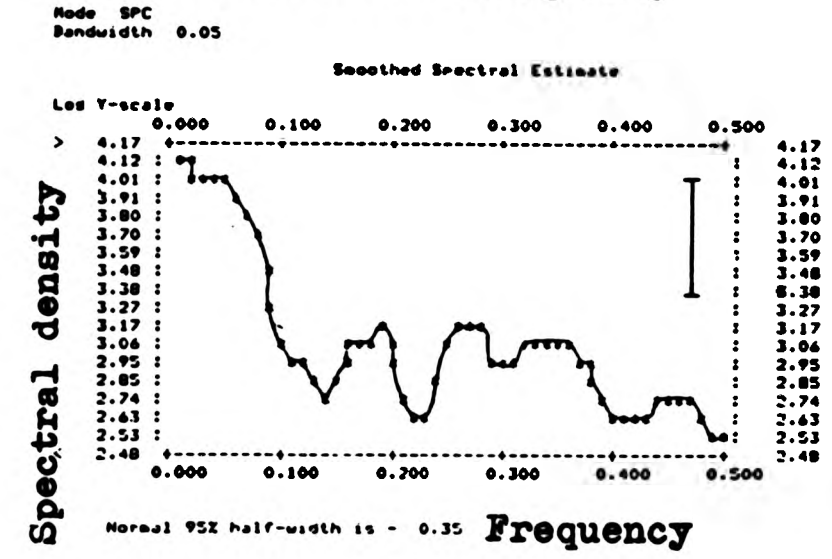
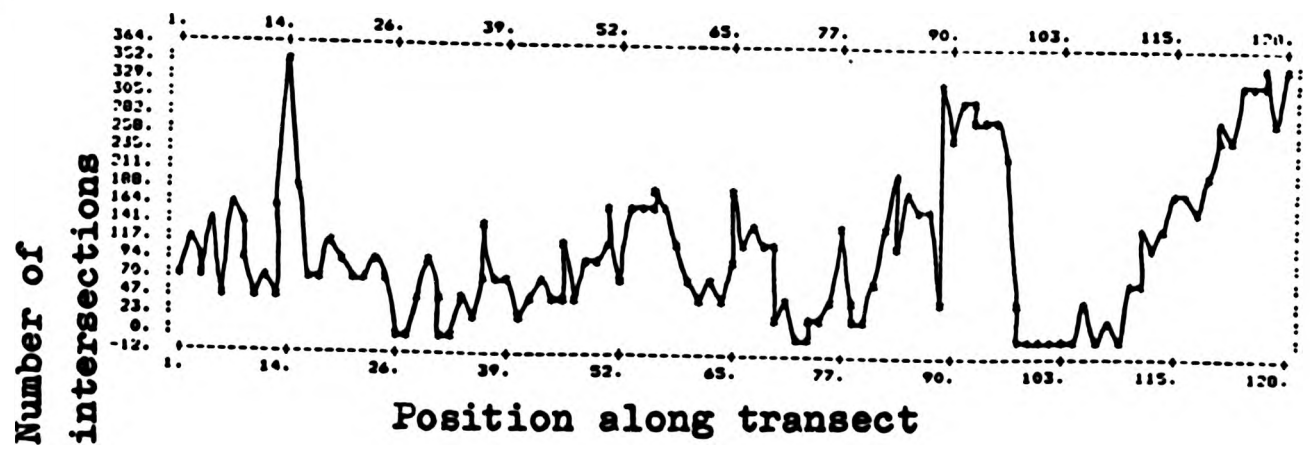
Proportion Tapered 0.1
Xbar 171.688
Cumulative Periodogram
Proportions
Frequency
95% band half-width is - 0.09

ii)

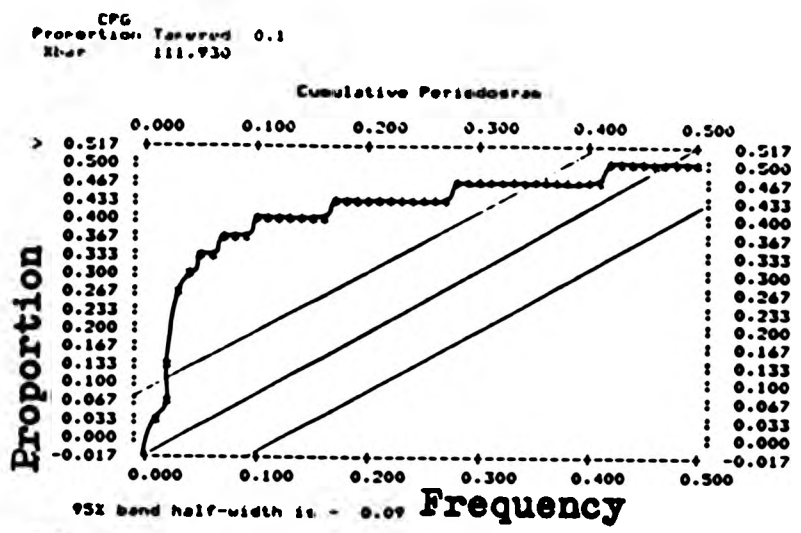

Mode SPC
Bandwidth 0.05
Smoothed Spectral Estimate
Log Y-scale
Spectral density
Frequency
Normal 95% half-width is - 0.35

iii)

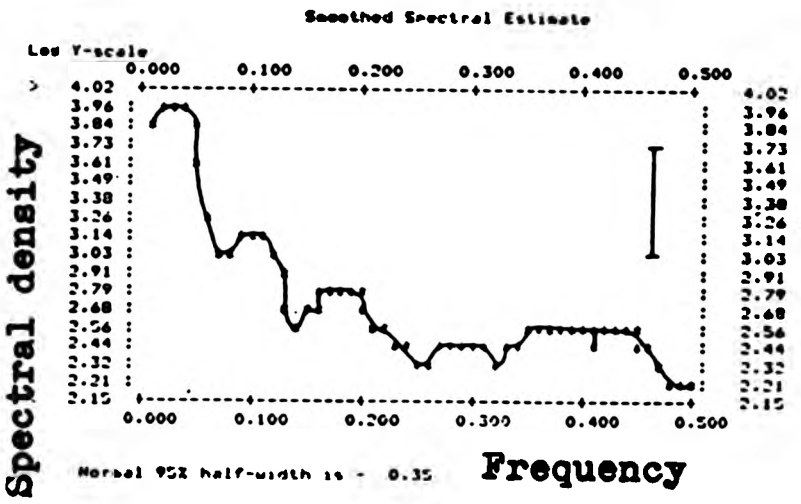

Number of intersections
Position along transect

3.3 e)

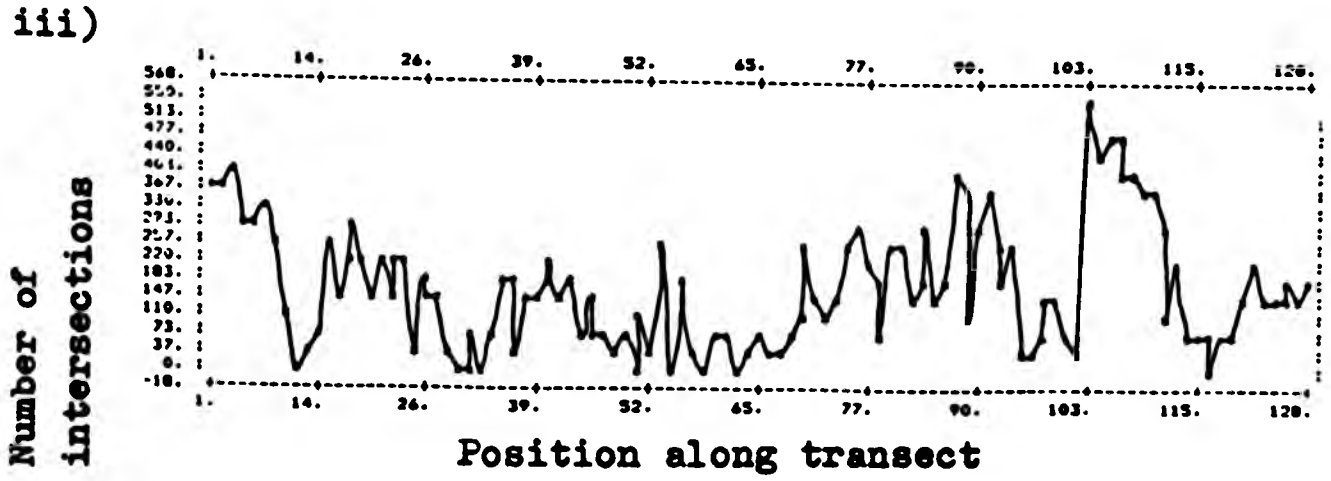
i)


CPG
Proportion Tapered 0.1
Rbar 111.930
Cumulative Periodogram
0.000 0.100 0.200 0.300 0.400 0.500
0.517 0.500 0.467 0.433 0.400 0.367 0.333 0.300 0.267 0.233 0.200 0.167 0.133 0.100 0.067 0.033 0.000 -0.017
Proportion
Frequency
95% band half-width is = 0.09
Mode SPC
Bandwidth 0.05

ii)


Smoothed Spectral Estimate
Log Y-scale
0.000 0.100 0.200 0.300 0.400 0.500
4.02 3.96 3.84 3.73 3.61 3.49 3.38 3.26 3.14 3.03 2.91 2.79 2.68 2.56 2.44 2.32 2.21 2.15
Spectral density
Frequency
Normal 95% half-width is = 0.35

iii)


1. 14. 26. 39. 52. 65. 77. 90. 103. 115. 128.
568. 513. 477. 440. 403. 367. 330. 293. 257. 220. 183. 147. 110. 73. 37. 0. -18.
Number of intersections
Position along transect

exceed the 95% confidence band. Examination of the smoothed spectral estimates, a(ii) and b(ii), indicates that no significant scales of pattern are present in either set of data, since there are no clear separations of peak heights by more than the 95% confidence limits.

Figure 3.3 c-e represents analysis of data from alder leaves colonised by 4 fungal species. In each case the raw data, c-e(iii), suggests the presence of pattern but in all cases, except c(iii), this is not clearly discernable. The analyses of the 3 transects show significantly decreasing spectral densities with increasing frequency (c-e(ii)), see the cumulative periodograms, c-e(i). All 3 transects show significant peaks at low frequencies on the smoothed spectral estimates (difference in height exceeded 95% confidence limits). In c(ii) the peak at frequency 0.025 corresponds to about 20 quadrats (each quadrat corresponds to c. 0.5 x 0.5 mm), in d(ii) the frequency of the major peak (0.03) is about 16 quadrats and in e(ii) the major peak (0.05) is about 10 quadrats. There may be an additional scale of pattern at a frequency of between 0.1 and 0.125 corresponding to about 4 or 5 quadrats. The larger scale of pattern (10 - 20 quadrats) on the 3 transects (c-e) corresponds to clumps about 5 - 10 mm in diameter on the leaf.

The results of the plots of $\log_{10}$ mean against $\log_{10}$ variance are shown in Figures 3.4 & 3.5. In each case the least squares regressions were highly significant ($P < 0.001$). All plots showed a significant positive slope between $\log_{10}$ mean and $\log_{10}$ variance for each data set. All regression lines were above the line for a random distribution. The results shown in Figure 3.4 represents the data from the laboratory experiment (Section 3.2.4.2) with Tetracladium marchalianum grown on alder leaf discs for 3, 7, 11, 16 and 21 days. All slopes were greater than 1 (range 1.19 - 1.56) indicating that throughout the experimental period fungal hyphae showed aggregated distribution on the alder leaf discs.

Figure 3.4. Plots of the relationship between $\log_{10}$ mean and variance of hyphal densities of Tetracladium marchalianum grown on alder leaf discs for different periods of time. All units are m g^{-1} leaf oven dry weight (80°C). The data in each case was fitted by least squares regression. The regression equations obtained were:

a) 3 days, $\log_{10}s^2 = 1.71 + 1.41 \log_{10}\bar{x}$

b) 7 days, $\log_{10}s^2 = 2.15 + 1.23 \log_{10}\bar{x}$

c) 11 days, $\log_{10}s^2 = 2.70 + 1.19 \log_{10}\bar{x}$

d) 16 days, $\log_{10}s^2 = 1.73 + 1.42 \log_{10}\bar{x}$

e) 21 days, $\log_{10}s^2 = 1.46 + 1.56 \log_{10}\bar{x}$

f) all data combined, $\log_{10}s^2 = 1.69 + 1.46 \log_{10}\bar{x}$

The broken line on each graph represents the relationship for a random distribution of hyphae with $s^2 = \bar{x}$

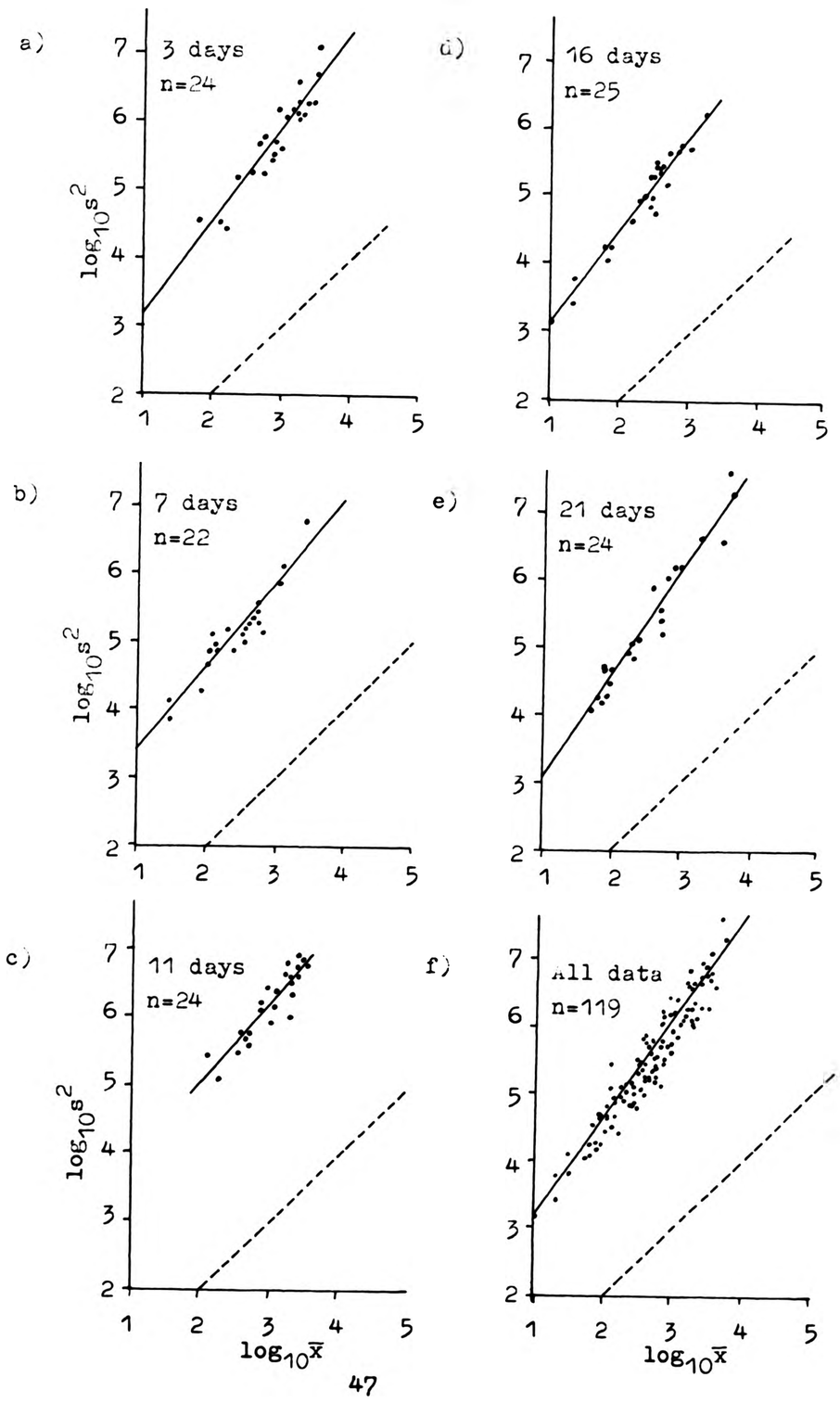


Figure 3.5. Plots of the relationship between $\log_{10}$ mean and variance of hyphal densities (m g^{-1} leaf oven dry weight (80 °C) on leaves of a) willow, b) oak, and c) alder retrieved from benthic litter in lakes and ponds. Plot d) shows all data points in a), b) and c) together with the data from the laboratory experiment (see Figure 3.4). The fitted regression lines for each set of data are:

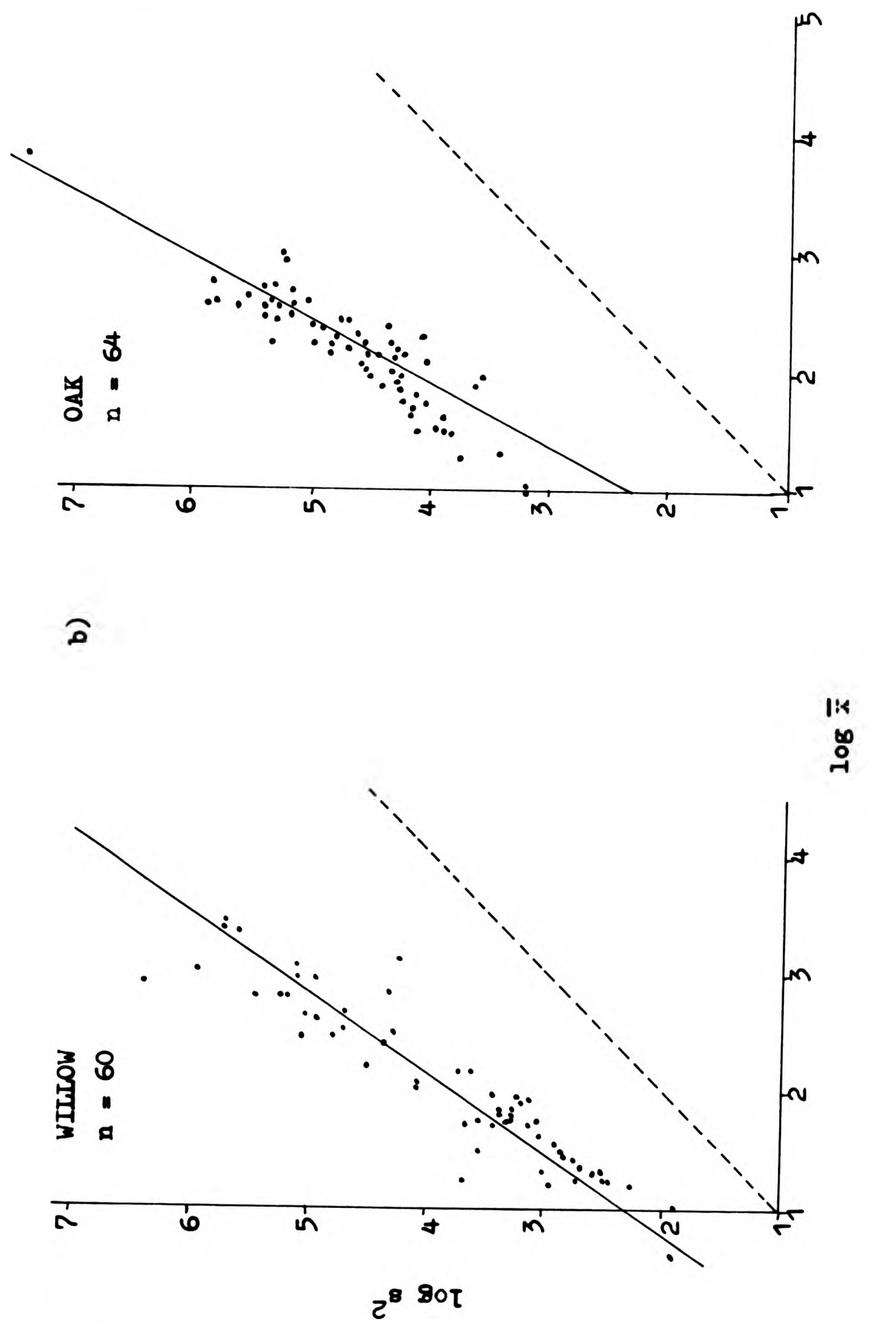
a) willow $\log s^2 = 0.83 + 1.47 \log \bar{x}$

b) oak $\log s^2 = 0.39 + 1.89 \log \bar{x}$

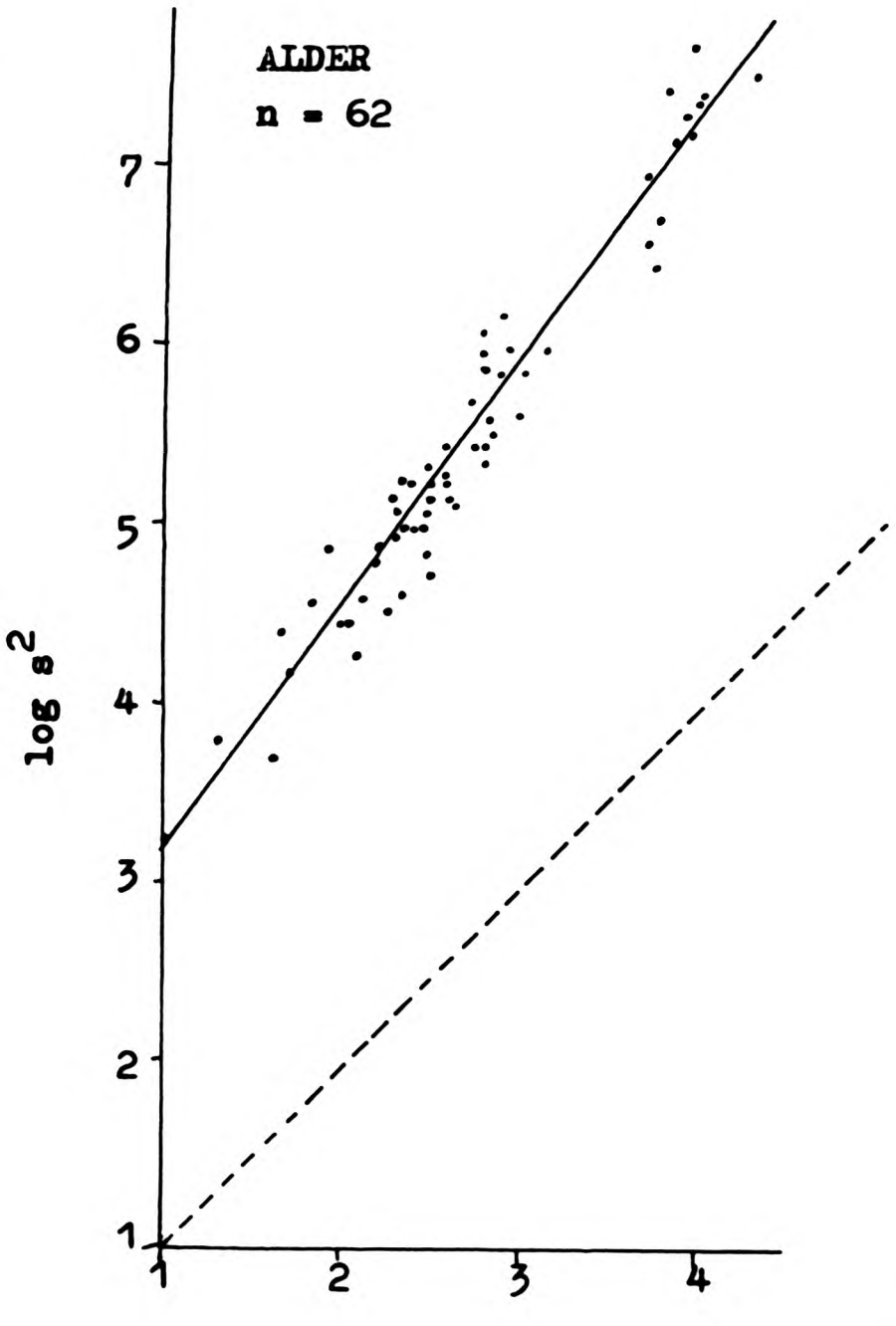
c) alder $\log s^2 = 1.78 + 1.36 \log \bar{x}$

d) total data $\log s^2 = 1.03 + 1.62 \log \bar{x}$

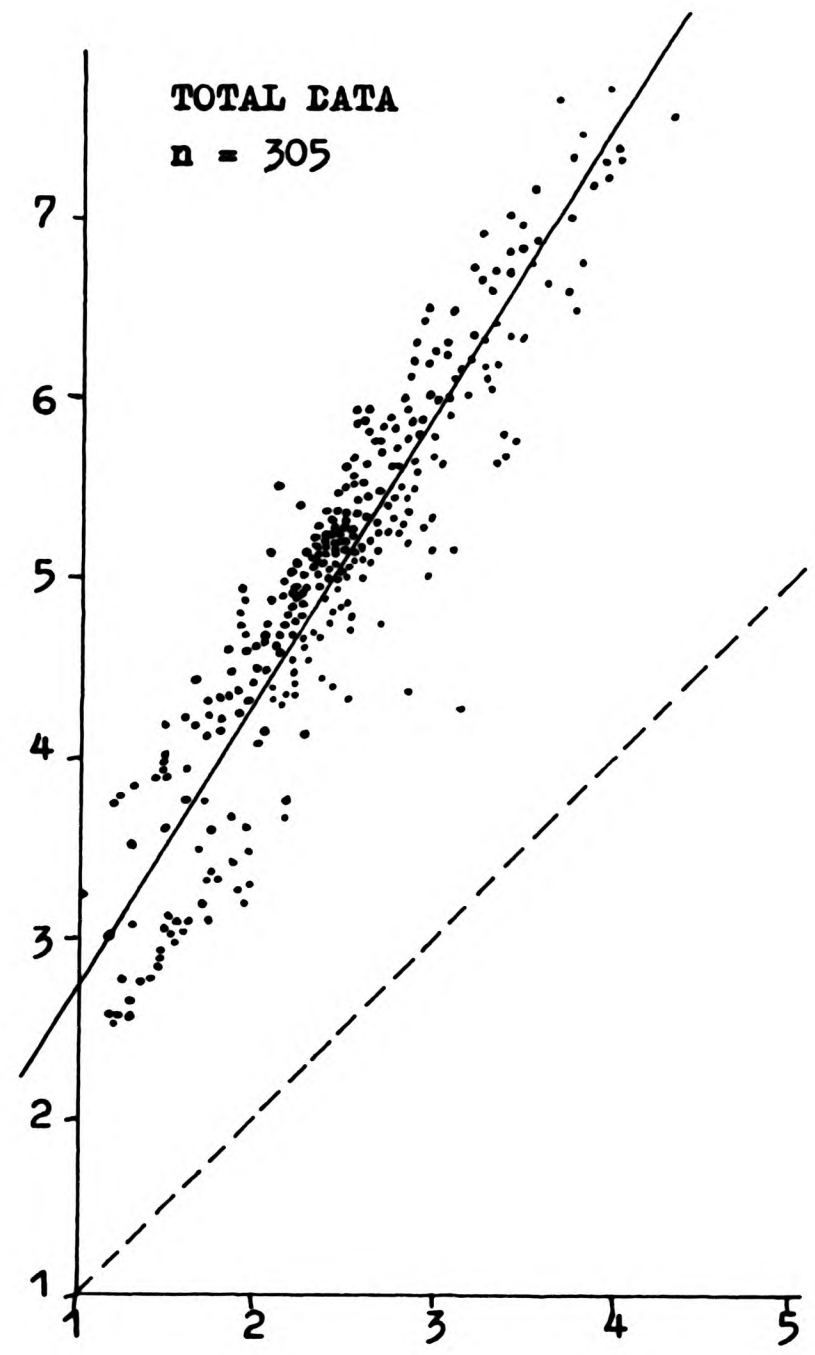
(The broken line represents the relationship for a random distibution of hyphae with $s^2 = \bar{x}$)



c)



d)



However, there were no significant temporal differences in the degree of aggregation as measured by the slope of the regression over the 21 day period.

Figure 3.5 shows plots of $\log_{10}$ mean against $\log_{10}$ variance for hyphal length data obtained mainly from field collections of leaf material. For each leaf type there was strong evidence of an aggregated distribution of fungal hyphae on the leaves. There was a wider scatter of data points with willow and oak than with alder. The slope obtained with oak (1.89 $\pm$ 0.16) was much steeper than with willow (1.47 $\pm$ 0.08) or alder (1.36 $\pm$ 0.04) suggesting that hyphae may be more aggregated on oak leaves. The plot (Figure 3.5 d) of all data points (field and laboratory data) demonstrates the wide scatter of data points, particularly at low means and variances. All data points showed higher $\log_{10}$ variance than $\log_{10}$ mean. It can be concluded that aggregation was characteristic of fungal hyphae on all leaf samples examined and it increased non-linearly with increasing hyphal density.

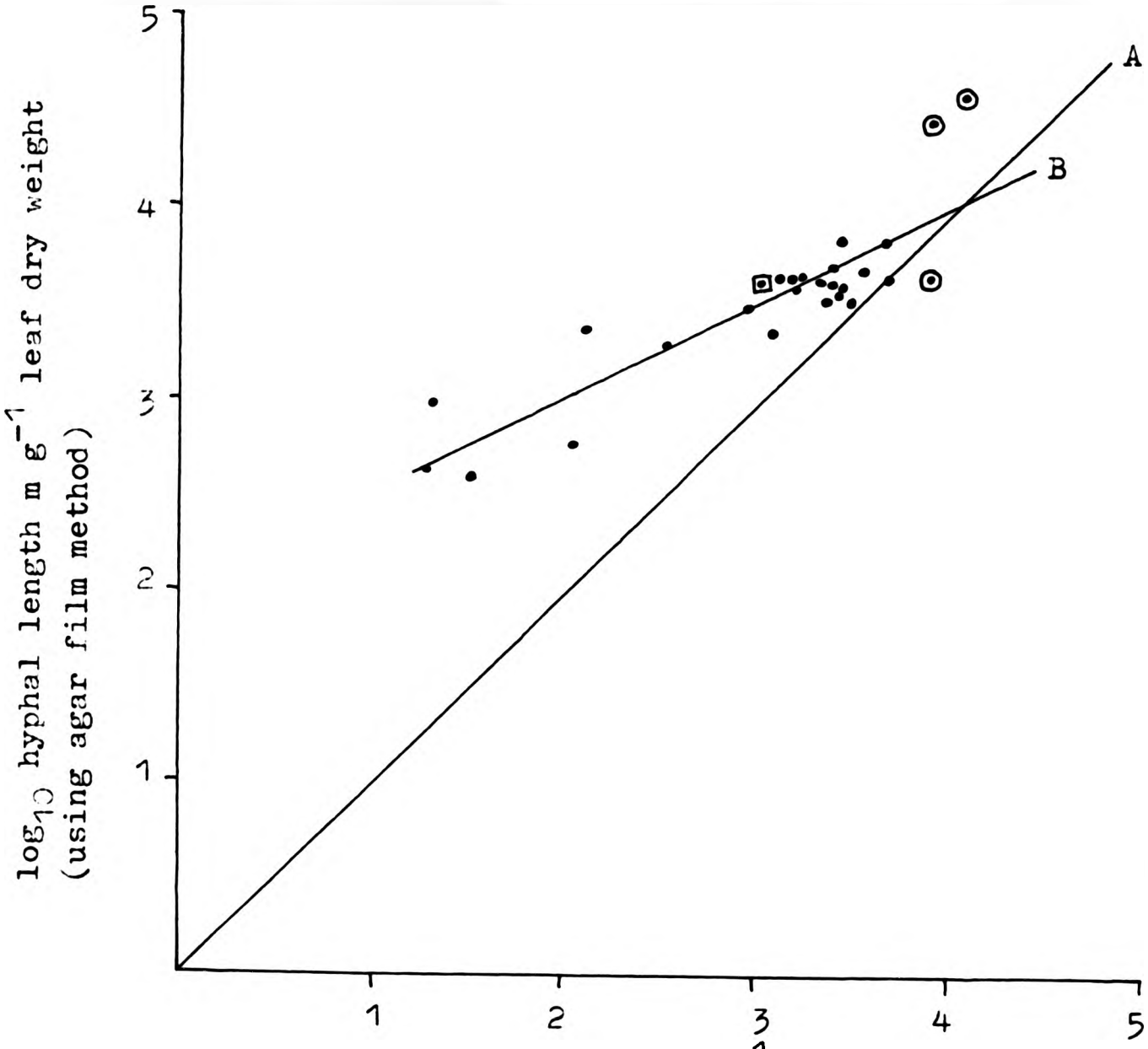
Although previous results demonstrated that the method shown in Table 3.1 could be used to detect differences in hyphal length on leaf litter, it was necessary to compare this method to others available. The results of comparisons with other clearing and staining methods are shown in Tables 3.3 & 3.4. In all cases hyphal lengths were higher with the safranin O, lissamine green V method, and this appeared to be independant of the leaf type used. Figure 3.6 shows a comparison of the clearing and staining method with the agar film technique. The data is plotted on a logarithmic scale because of the wide range in hyphal length values. High and low estimates using the clearing and staining method corresponded with high and low values estimated by the agar film technique. The slope of the regression (0.51 $\pm$ 0.06) suggested that the methods were responding differently to changes in hyphal

TABLE 3.3 Comparison of hyphal lengths (m g^{-1} leaf dry weight 80°C) on 3 leaf types using two staining methods. Values shown are the means and standard errors derived from 10 random fields on 5 leaf discs.

STAINING METHOD	LEAF TYPE		
	ALDER	WILLOW	SYCAMORE
1. Safranin O (Barlocher and Kendrick, 1974)	10.6 ±2.9	2.4 ±1.1	13.0 ±8.4
2. Safranin O Lissamine green V (Present study)	653.3 ±60.0	90.4 ±3.3	591.1 ±92.8

TABLE 3.4 Comparison of hyphal lengths (m g^{-1} dry weight 80°C) on Willow leaves using two staining methods. Values shown are the means and standard errors derived from 10 random fields on 5 leaf discs.

STAINING METHOD	LEAF TYPE
	WILLOW
1. Phenolic Analine Blue (Ineson, Leonard and Anderson, 1982)	203.6 ±42.5
2. Safranin O, Lissamine Green V (Present study)	2835.9 ±727.6



Key: ⊙ alder

⊡ oak

• willow

A Expected relationship for direct agreement between the two techniques.

B Least squares fitted regression line.
Equation is:
$\log_{10} y = 2.02 + 0.51 \log_{10} x$

R^2 = 75.4% adjusted for D.F.

Figure 3.6. Relationship between fungal hyphal length as assessed by the agar film and the clearing and staining method.

length. On samples with low hyphal lengths (<100 m g^{-1}) determined by clearing and staining, the agar film technique gave estimates over 10 times higher. On samples with higher hyphal lengths (1000 - 10 000 m g^{-1}) estimated by clearing and staining, the agar film method gave estimates only slightly higher (1 - 3 times). Because most of the leaf samples examined were willow, the relationship between the two methods may differ with other leaf types.

3.4 Discussion

Bleaching, clearing and staining of leaves has been used by a number of authors interested in estimating fungal biomass in leaf litter (Minderman & Daniëls, 1967; Bärlocher & Kendrick, 1974; Newell & Hicks, 1982). These methods have often been applied without due consideration of potential problems and limitations. Bleaching treatments have been criticised as potentially damaging to the leaf and its microflora (Baker, 1981) but there have been no attempts to determine potential losses of hyphae and spores during preparation. This work has demonstrated that a slow bleaching treatment resulted in negligable loss of hyphae and spores from leaf discs. Some hyphae and spores may have been totally destroyed by the treatment but the presence of spores attached to conidiophores on stained material suggested that any disruption and damage was minimal.

Examination of cleared and stained leaf material has often presented problems (Newell & Hicks, 1982). In many cases fungal structures may not be visible because there is insufficient contrast with the leaf tissue. The staining method proposed here has the advantage of using dyes of contrasting colours, allowing fungal structures to be clearly differentiated. The lissamine green V dye appeared to stain fungal structures in preference to leaf tissues. Lillie (1969) reported that lissamine green V does not stain cellulose in industrial

applications and low affinity for cellulose could be a reason for poor leaf staining ability of lissamine.

Fungal hyphae were sparse in stained sections of leaf material, occurring mostly just below the leaf surface. Pycnidia were observed but poorly stained. Padgett (1976) sectioned and stained leaf litter at various stages of its decomposition in a tropical river. The few hyphae observed within the leaf tissue occurred only during the latter stages of decomposition. Frankland (pers. comm.) has observed that woodland leaf litter frequently shows surface colonisation by fungal hyphae and that internal colonisation only occurs in the latter stages of leaf breakdown. It may be, therefore, that in this study the material examined in section was not sufficiently advanced in decomposition to show extensive areas of internal colonisation.

Quantitative comparisons demonstrated that the use of lissamine green V as a counterstain gave considerably higher estimates of hyphal length on leaf material than other methods of clearing and staining (Tables 3.3 & 3.4). Direct comparison of the method with the agar film technique (Jones & Mollison, 1948) revealed that clearing and staining underestimated hyphal lengths at low densities on the leaf but gave similar values to the agar film method at higher densities. Since the agar film method will include any internal hyphae and pycnidia, as well as surface hyphae, the similar values obtained using the two methods at high hyphal densities are encouraging. The differences between the two methods at lower hyphal densities possibly arises from the spatial distribution of hyphae, giving underestimates of hyphal length using clearing and staining. The problem could be overcome by increasing the number of fields of view examined. Alternatively, the agar film method may possibly overestimate hyphal length at low densities, due to confusion of leaf wall fragments and fibres with fungal hyphae. Comparisons using other leaf types are needed

before any firm relationship can be established between the two methods. The results of this work indicate that the clearing and staining approach is feasible as a method for estimating fungal hyphal length in leaf litter.

Observations and quantitative studies on the spatial distribution of hyphae on leaves suggests that fungi show clumped distributions; the scale of clumping can be estimated using spectral analysis. Clumps oı hyphae, several mm in diameter, were only detected on alder leaves taken from an experimental flask inoculated with four species. When a single species inoculum was used no significant clumping was found, although there was a departure from randomness. Bärlocher (1979) suggested that the way in which fungal hyphae are distributed on the leaf may be important in the acceptability of microbially conditioned leaves to invertebrates. Evidence for a general aggregated distribution of hyphae over a range of leaf types and conditions was provided by plots of $\log_{10}$ mean and variance. Both field and laboratory data demonstrated that hyphae are aggregated on leaf litter and that it is more pronounced on oak than on other leaf types. The range of values obtained for the slopes of the regression lines fall mostly within the range observed by Taylor, Woiwod & Perry (1978) for 156 species of animals and plants (1.45 $\pm$ 0.39 SD). Aggregation of hyphae on leaves is likely to represent a whole complex of factor including growth form, leaf quality, inter- and intraspecific interactions and the influence of grazing invertebrates. The field data was noticeably more variable than data from the laboratory experiments, probably due to complex interactions of abiotic and biotic factors.

It can be concluded that the staining method developed in this work can be applied to studies of fungal biomass in leaf litter. Its major advantage over all other methods is that fungal structures can be examined <u>in situ</u> allowing hyphal lengths and pycnidial biovolume to be estimated and spatial distribution of fungal structures to be studied.

CHAPTER 4. PREPARATION OF LEAF MATERIAL FOR AGAR FILMS AND THE APPLICATION OF STAINING METHODS TO DETECT ACTIVE FUNGAL HYPHAE.

4.1 Introduction

The agar film method (Jones & Mollison, 1948; Thomas, Nicholas & Parkinson, 1965) has been widely used as a technique for estimating biomass of microorganisms in soil and several authors (Swift, 1973b; Visser & Parkinson, 1975; Frankland, Lindley & Swift, 1978) have used it to estimate fungal biomass in leaf litter. The studies of Frankland, Lindley & Swift (1978) suggest that the method underestimates fungal biomass in litter due to inefficient maceration of leaf material and difficulty in staining and observing all fungal hyphae. Assessing which of the visible hyphae in agar films are living or dead has been a major problem. Estimating the lengths of hyphae with cell contents (Frankland 1974 & 1975a) those stained with aceto-orcein (Flanagan, 1981) and the use of various fluorochromes (Anderson & Slinger, 1975 a & b; Roser, 1980) have all been suggested, but it is not clear if any of these staining methods can adequately distinguish between living and dead, or active and inactive hyphae. The approach taken in this work has been to optimise the homogenisation of leaf material for release of fungal hyphae and to develop a staining method which would allow active hyphae to be distinguished from those which are inactive or dead.

4.2 Materials and methods

Many authors have used blenders or MSE top drive homogenisers for extracting fungal hyphae from leaf litter. Since these appear to be slow in releasing fungal hyphae (Swift, 1973b) the more powerful VIRTIS homogenisers were used (VIRTIS 45, VIRTIS Instruments, USA) in this work.

4.2.1 Homogenisation of fungal mycelium

Homogenisation of pure cultures of fungal mycelium was investigated to determine the times of optimal release of hyphae into suspension. Petri dishes containing malt extract agar and leaf extract agar (see Appendix 3) were overlaid with sterile strips of cellophane (5 x 1.5 cm, grade PF 320, D.J. Parry & Co Ltd, London) which had previously been boiled in two changes of distilled water. Each strip was centrally inoculated with a small piece of mycelium from an actively growing culture of *Trichoderma viride* Pers. (isolate Lam 23) or *Fusarium* sp. (isolate Folk 1). The plates were incubated for 14 days at 12 ^{o}C in sealed plastic bags. After incubation mycelium was scraped from each cellophane strip and 50 mg added to 10 ml of distilled water in a VIRTIS flask (capacity 100 ml). The homogeniser was run at setting 5 (approx. speed 26 000 rev min^{-1}) for periods of 30 seconds for 2 minutes and then continuously for a further 3 minutes. At each interval the homogeniser was stopped and the liquid allowed to settle for 10 seconds. Two 100 µl samples of homogenate were withdrawn and a single agar film prepared for each, as described in the protocol (Table 4.1). Agar films were stained with phenolic analine blue (PAB) and examined at x 400 with phase contrast illumination. Hyphal lengths were determined by the intersection method (Olson, 1950) and the results expressed as m ml^{-1} of homogenate.

4.2.2 Homogenisation of leaf material

Experiments were conducted on oak and alder leaves retrieved from benthic litter and on fungal colonised leaf material from laboratory flasks.

TABLE 4.1 Protocol of the stages involved in the preparation of agar films.

1) A small volume of homogenate (0.1-0.5ml) was added to a small test-tube and incubated at 60°C in a water bath. An equal volume of sterile 3% (w/v) molten agar (Ion agar, Oxoid) was added.

2) For each tube the homogenate and agar were mixed by vortexing on a whirlimixer for 2 seconds. Tubes were replaced in the water bath prior to mixing agar films.

3) Using pre-warmed pasteur pipettes a small drop of agar homogenate mixture was placed onto pre-warmed haemocytometers (depth 0.1mm, Neubauer Improved). The cover glass was pressed down and the agar film allowed to set.

4) Haemocytometers were immersed in distilled water in plastic weighing boats and the agar films floated off and teased onto microscope slides.

5) Slides were part covered with inverted petri dishes and the agar films allowed to air dry overnight at room temperature.

4.2.2.1 Benthic litter

Discs (6 mm diameter) were cut from oak and alder leaves. Twenty-five discs were homogenised in 10 ml of distilled water in a VIRTIS flask (capacity 100 ml). The homogeniser was run at setting 5 for periods of 30 seconds for 2.5 minutes. At each interval the homogenate was allowed to settle for 10 seconds and three 100 µl samples of homogenate withdrawn. Single agar films were prepared for each sample as described in the protocol (Table 4.1). Hyphal lengths were determined by the intersection method on PAB stained films examined at x 400 with phase contrast illumination. Further leaf discs from each sample were dried (80 ^{o}C) to constant weight and hyphal lengths expressed as m g^{-1} leaf dry weight.

4.2.2.2 Laboratory flasks

A flask containing 600 ml of mineral medium (see Section 2.3) and 5 g of sterile alder leaves was inoculated with spore suspensions of *Aguillospora longissima* (isolate 7A), *Articulospora tetracladia* (isolate VW1), *Clavariopsis aquatica* (isolate ST1) and *Tetracladium marchalianum* (isolate 1A) to give a final concentration of 50 $\pm$ 3 spores ml^{-1} for each species. The flask was incubated for 3 weeks at 18 ^{o}C under constant aeration (100 cm^{3} min^{-1}). After incubation, leaf material was rinsed in distilled water and treated as in Section 4.2.2.1.

4.2.3 Influence of leaf type on homogenisation efficiency

4.2.3.1 Leaf physical characteristics

Leaf tensile strength, lamina strength and lamina thickness were measured on freshly leached leaf material of alder, willow, oak and beech to aid interpretation

of homogenisation results.

Rectangles (4 x 1 cm) were cut from leaves of each species, avoiding the midrib. Tensile strengths (g) were measured on an Instron tensiometer using a 1 kg maximum load.

The weight required to penetrate the lamina tissue of leaf material was measured using a penetrometer (Appendix 4). Each test involved clamping a small piece of leaf material between two perspex plates and measuring the weight (g) required for a 2 mm diameter glass rod to penetrate through the leaf.

Leaf thickness was measured using a micrometer gauge. The midrib area of each leaf was not included in the measurements.

4.2.3.2 Correcting for inefficiency of homogenisation

Because leaf material is never completely homogenised a proportion of fungal hyphae is likely to be retained in litter fragments not incorporated into agar films. Correction factors were determined so that estimates of hyphal lengths in agar films could be adjusted to account for hyphae present in residual litter fragments. This was determined by calculating the proportion of leaf dry weight retained after passing homogenates through nylon mesh of gauge 250 µm x 250 µm (Henry Simon Ltd). It was assumed that leaf particles or fungal hyphae of less than 250 µm could be incorporated into agar films.

Ten replicates of 25 leaf discs (6 mm diameter) of alder and willow were homogenised in 10 ml of distilled water for 1 minute at setting 5 in a VIRTIS flask (capacity 100 ml). The homogenates were filtered through 250 µm mesh and the retained material washed several times and squeezed to ensure that free material passed through. The leaf material retained by the mesh was scraped off and oven dried to constant weight (80 ^{o}C). Residual material trapped in the mesh was extracted by resuspension and refiltering, followed by scraping off and oven drying.

Further leaf discs from the same pool of leaves were oven dried to constant weight (80 ^{o}C) and the proportion of leaf material retained on the nylon mesh calculated with reference to the dry weight of leaf material homogenised.

4.2.3.3 Homogeniser speed

The speed of the VIRTIS instrument was measured under different conditions using a reflective optoswitch connected to an oscilloscope (Appendix 4). The influence of leaf material on homogeniser speed was examined by homogenising 25 leaf discs (6 mm diameter) of alder, willow and oak in 10 ml of distilled water for 2.5 minutes. Readings of homogeniser speed were recorded every 30 seconds. Each run was repeated 3 times with fresh leaf discs.

4.2.4 Staining living hyphae in agar films

Tetrazolium salts have been widely used in the study of metabolically active microorganisms in soil (Smith & Pugh, 1979; Macdonald, 1980; Trevors, Mayfield & Inniss, 1982) and water (Jones & Simon, 1979; Tabor & Neihof, 1982). Macdonald (1980) demonstrated that several tetrazolium salts could be used in conjunction with the agar film technique to distinguish between active and inactive bacterial cells in soil. The potential of tetrazolium salts for distinguishing active and inactive fungal hyphae in agar films has not been investigated previously. In this work an assessment was made of their possible use for fungal biomass studies.

4.2.4.1 Screening of tetrazolium salts

Tetrazolium salts were evaluated using mycelium from an actively growing culture of *Articulospora tetracladia* (isolate VW 1). Mycelium was stripped from a 14 day old culture (grown at 20 ^{o}C) on cellophane overlying a plate

of potato dextrose agar. Mycelium (500 mg) was homogenised for 1 minute at setting 5 in a VIRTIS flask (capacity 100 ml) with 20 ml of sterile distilled water. Aliquots (0.5 ml) of homogenate were dispersed into sterile bijou bottles and 0.5 ml of triphenyl tetrazolium chloride (TTC), iodonitrotetrazolium (INT), thiazolyl blue (MTT) or tetrazolium blue (TB) (each 0.2 mg ml^{-1} in sterile distilled water) added. Replicate vials were prepared for each tetrazolium salt and controls consisted of homogenate with sterile distilled water added without the tetrazolium salt. The homogenates were incubated at room temperature for up to 17 hours in the dark. After 1.5 hours and 17 hours 50 µl were withdrawn from each bottle, mounted on slides and examined at x 100 and x 400 magnification with bright field illumination. The presence, colour and intensity of staining was noted subjectively. Further samples from each homogenate were used to prepare agar films for comparison with direct mounts.

Visual comparison of the four tetrazolium salts showed that INT and MTT were the most effective in demonstrating metabolically active hyphae, and 17 hours incubation appeared to give better results than 1.5 hours. On the basis of these results further studies were conducted with INT and MTT.

4.2.4.2 Influence of exogenous substrates on tetrazolium staining

Macdonald (1980) demonstrated that when substrates were used in combination with tetrazolium salts higher percentages of staining were obtained than with tetrazolium salts alone. These results were attributable to low levels of endogenous substrates present in some living microbial cells being insufficient for visible tetrazolium reduction. Tetrazolium staining of mycelial homogenates was tested in the presence and absence of the substrates sodium succinate, glutamic

acid, glucose, NADH and NADPH (concentrations 0.1 - 2 mg ml^{-1} in 0.1 M Tris-HCl buffer pH 7.6, containing INT or MTT (0.2 mg ml^{-1})). Only NADH and NADPH showed any noticeable improvement in tetrazolium staining compared with controls after examination of stained mycelia at x 400 magnification. The best results were obtained when NADH and NADPH were used in combination. A concentration of 0.5 mg ml^{-1} of each substrate was considered adequate for demonstrating tetrazolium reduction in living hyphae. Concentrations greater than 0.5 mg ml^{-1} did not give any noticeable improvement in the intensity of staining, formazan crystal size, or proportion of stained hyphae, therefore 0.5 mg ml^{-1} of each substrate was adopted for subsequent use.

4.2.4.3 Influence of incubation time on tetrazolium reduction.

Homogenates of Articulospora tetracladia mycelium were prepared as in Section 4.2.4.1. Aliquots (0.4 ml) were dispensed into sterile bijou bottles containing 0.1 ml of NADH and NADPH (both 4 mg ml^{-1} in 0.1 M Tris-HCl buffer pH 7.6). Aliquots (0.1 ml) of tetrazolium salts (INT or MTT, or combination of INT and MTT, 50 : 50) were added to give a final concentration of 0.2 mg ml^{-1}. The bottles were incubated at 20 ^{o}C for 20, 40, 80, 180, and 420 minutes in the dark. After incubation further tetrazolium reduction was stopped by the addition of 0.2 ml of 40% v/v formalin. The contents of each bottle were vortexed on a whirlimixer and 100 µl withdrawn and agar films prepared. Two films were prepared for each bottle and three bottles examined at each time interval for each of the three staining treatments.

Agar films were mounted in distilled water and 5 fields of view examined at x 400 magnification with bright field illumination. Lengths of hyphae showing tetrazolium staining were measured using the intersection method. The films were then stained in PAB for 30

minutes and re-examined at x 400 magnification with phase contrast illumination. Five fields of view were examined and the lengths of all visible fungal hyphae (stained and unstained) estimated using the intersection method. For each tetrazolium salt and at each time interval the percentage of active hyphae was estimated.

4.2.4.4 Staining of active hyphae in leaf material

Comparison of the tetrazolium staining of fungal hyphae on leaf material was carried out as in Sections 4.2.4.1 and 4.2.4.2. Experiments were performed at 12 ^{o} C to reduce bacterial activity. For each experiment leaf discs (6 mm diameter) were cut from alder leaves retrieved from benthic litter. Ten leaf discs were used for each comparison and all incubations were for 5 - 7 hours.

A comparison was made between incubation of leaf discs with tetrazolium salts before and after homogenisation. Ten leaf discs were homogenised for 1 minute (VIRTIS, setting 5) in 10 ml of 0.1 M Tris-HCl pH 7.6 containing 0.5 mg ml^{-1} NADH and NADPH and 0.2 mg ml^{-1} INT + MTT (50 : 50), and incubated at 12 ^{o}C for 5 hours. Ten leaf discs, not homogenised, were incubated in the above solution under the same conditions. After incubation 1 ml of formalin 40% v/v was added to each sample. The discs were removed, rinsed and homogenised in 10 ml of distilled water, as before. For each homogenate three agar films were prepared as described in Table 4.1. Films were stained in PAB, examined at x 400 magnification and the lengths of active and total hyphae estimated using the intersection method.

4.3 Results

The results of homogenising fungal cultures are shown in Figure 4.1. The optimal release of fungal hyphae was very rapid taking 1 - 1.5 minutes. Homogenisation beyond 1.5 minutes led to a reduction in hyphal

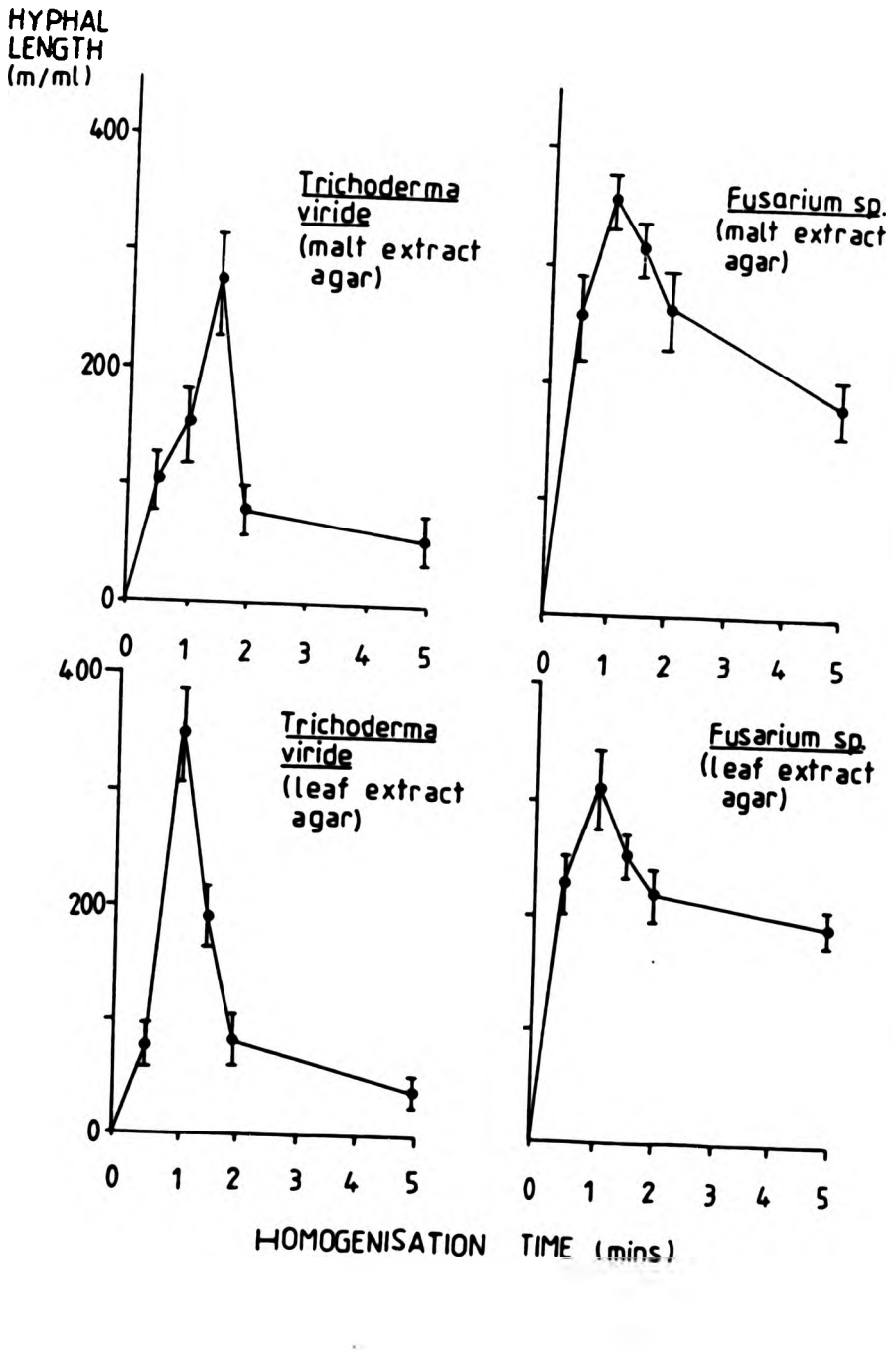


Figure 4.1. Graphs showing the influence of homogenisation time on hyphal lengths of two fungal species grown on two nutrient media. (The results are mean values with standard errors).

lengths in suspension due to destruction of hyphae. Each species appeared to show a characteristic pattern of hyphal release with homogenisation time, and this was independant of the growth medium.

Figure 4.2 shows the results of the homogenisation of fungal colonised leaf material. Optimal release of fungal hyphae from benthic litter and laboratory colonised leaf material occurred between 1 and 1.5 minutes. Homogenisation for longer than 1.5 minutes led to a marked drop in hyphal release from leaf litter. Although optimal release of hyphae was rapid, it was noticeable that leaf material was not completely broken down. Table 4.2 shows the percentage of alder and willow leaf dry weight retained by 250 µm nylon mesh after homogenisation of leaf discs for 1 minute. In both cases over 50% of the leaf material was retained by the mesh, indicating that less than half of the homogenate contributed to the agar films. For these particular leaf samples, hyphal lengths determined in agar films would need to be multiplied to account for all of the leaf material homogenised. The conversion factors would be 2.1 and 2.6 for alder and willow respectively.

The results of tensile strength and penetrometer measurements are shown in Table 4.3. They suggest that the lamina tissues of oak and beech leaves are much tougher than those of alder and willow. Alder leaves appeared to stretch during tensile strength measurements, the lamina taking much longer to break completely. Willow leaves were slightly thicker than the other species. The influence of different leaf types on homogenisation speed is shown in Figure 4.3. The presence of leaf material had relatively little influence on speed, causing a reduction of 1-6% compared with water. Leaf types appeared to break up differently; homogenisation speed increased for each species with time, but this was greater for willow and oak compared with alder, suggesting that the homogenates produced were physically different for each species.

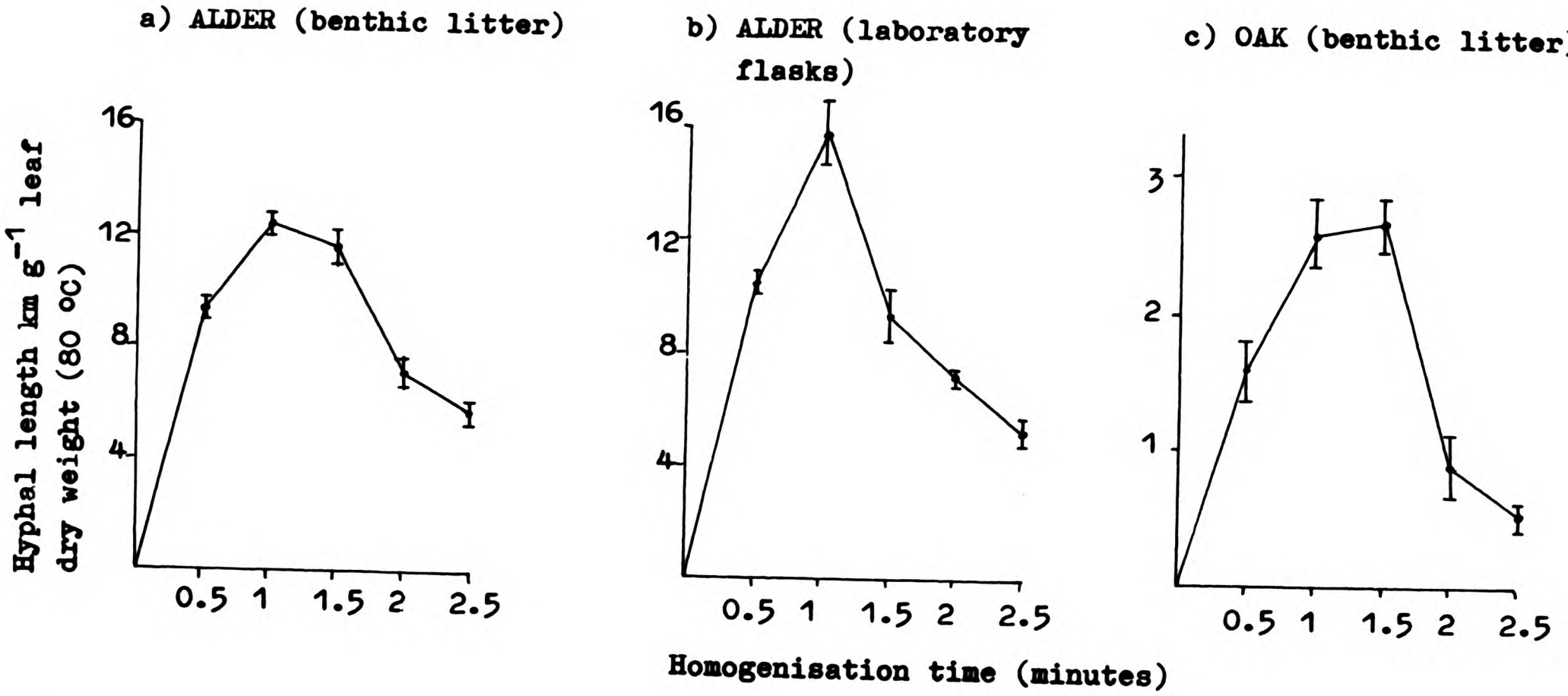


Figure 4.2. Graphs showing the influence of the duration of homogenisation (VIRTIS) on the release of fungal hyphae from alder and oak leaves. The values shown are means with standard errors.

TABLE 4.2 Percentage of leaf homogenates retained by 250µm nylon mesh. Each value for recovered leaf material represents a mean value with standard errors from 10 replicate samples. The conversion factor indicates the amount by which hyphal length estimates need to be multiplied to account for material which was not completely homogenised (i.e.>250µm).

	ALDER	WILLOW
Leaf material recovered from mesh	49.9 ± 3.6	60.6 ± 3.8
Residual material (recovered after resuspension)	2.3 ± 0.5	1.3 ± 0.2
Conversion factor	2.1	2.6

TABLE 4.3 Physical characteristics of the leaves of 4 tree species. Values are means with standard errors.

LEAF TYPE	TENSILE STRENGTH (g)	TIME REQUIRED TO BREAK (secs)	PENETROMETER (g)*1	LEAF THICKNESS (μm)*2
Alder	166.7±21.7 (n=7)	22.3±1.3	211.5±15.1	233.3±13.9
Willow	149.3±22.6 (n=4)	10.3±1.6	261.5±23.1	270.9±3.5
Oak	253.8±12.5 (n=4)	13.1±1.3	294.8±19.3	242.0±9.4
Beech	267.0±15.0 (n=2)	10.8±0.8	ND	ND

*1 n=10

*2 n=20

ND=Not determined

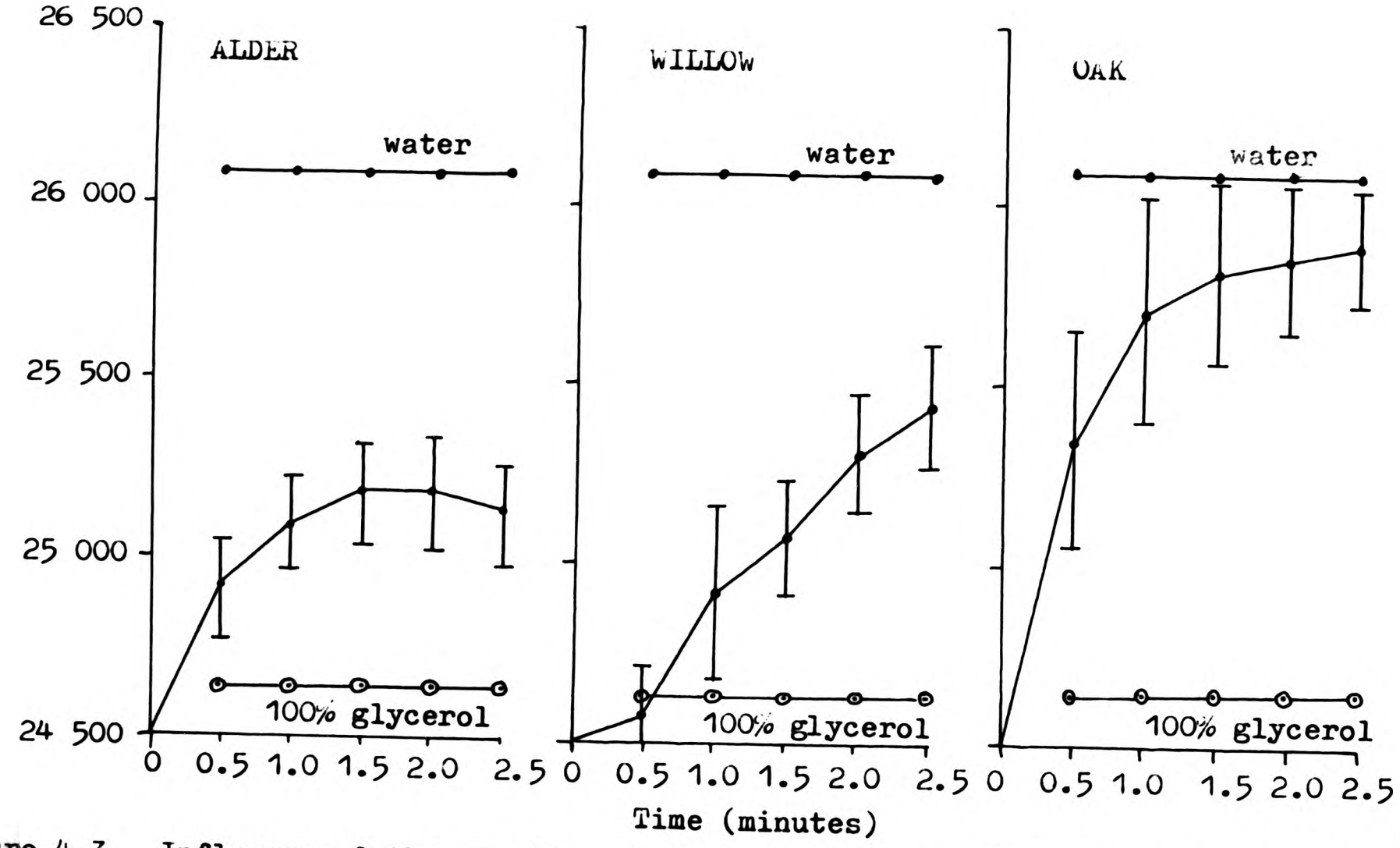


Figure 4.3. Influence of the duration of homogenisation on the speed (rev min^{-1}) with different leaf types. Values shown are means with standard deviations for 3 runs. The speed of the homogeniser in the presence of water or glycerol is shown for comparison.

The comparison with different tetrazolium salts for staining fungal hyphae demonstrated that INT and MTT gave the best results, in terms of staining intensity and colour. The incorporation of mycelium into agar films did not appear to affect the colour, contrast or persistence of tetrazolium stained hyphae. Of the substrates tested only NADH and NADPH appeared to influence the intensity and speed of tetrazolium reduction by active hyphae. Adequate results could be obtained by using NADH alone, but it was preferable to include both nucleotides in staining solutions because cells catabolising primarily by a single pathway (TCA cycle or pentose phosphate pathway) might be excluded using only one nucleotide (Macdonald, 1980). Tetrazolium staining in the presence of different concentrations of the two nucleotides suggested that incubation of samples in solutions containing 0.5 mg ml^{-1} of each nucleotide gave the best compromise between substrate concentration and staining intensity.

The influence of incubation time on tetrazolium reduction is shown in Table 4.4. For each tetrazolium salt the percentage of active hyphae initially increased with incubation time. After 3 hours the percentage of active hyphae with INT and MTT used together was higher than with INT or MTT alone. With mixed tetrazolium salts most of the crystals formed were the mauve or lilac colour of MTT formazan and fewer red crystals of INT formazan were observed. The incubation of samples with substrates and tetrazolium salts for 3 hours at 20 ^{o}C appeared to be adequate for estimating the percentage of active fungal hyphae in mycelial homogenates.

Experiments conducted using leaf material suggested that the types and concentrations of tetrazolium salts, and substrates used for optimal results with mycelial homogenates, were adequate for determining active hyphae in leaf material. Leaf material incubated with substrates (NADH and NADPH) and tetrazolium salts (INT, MTT) for 5 hours at 12 ^{o}C prior to homogenisation gave

TABLE 4.4 Dependance of the percentage of active hyphae in agar films on the incubation time of mycelial homogenates with INT, MTT or INT + MTT. The values shown are means (µm of hyphae per field of view and %hyphal length active) with standard errors.

INCUBATION TIME (mins)	INT		MTT		INT + MTT	
	HYPHAL LENGTH (µm)	%HYPHAL LENGTH ACTIVE	HYPHAL LENGTH (µm)	%HYPHAL LENGTH ACTIVE	HYPHAL LENGTH (µm)	%HYPHAL LENGTH ACTIVE
20	806.6	22.5	885.5	20.3	827.0	38.3
	±20.4	±2.5	±47.3	±6.9	±22.2	±4.5
40	814.0	48.5	918.8	45.6	828.8	60.95
	±92.5	±2.5	±27.9	±7.8	±66.6	±8.5
80	863.9	62.9	919.5	59.0	806.6	58.3
	±81.4	±5.6	±51.8	±2.5	±0	±0.45
180	836.3	63.7	925.0	62.5	849.2	69.8
	±85.1	±2.2	±10.8	±5.9	±34.9	±6.02
420	875.7	61.5	878.8	62.2	897.3	75.7
	±76.9	±4.4	±27.8	±1.9	±40.7	±0.76

INT=Iodonitrotetrazolium

MTT=Thiazolyl Blue

estimates of active hyphae (34%± 3) which were higher than those incubated after homogenisation (20%± 3). A temperature of 12 °C and incubation time of 5-7 hours for leaf material was considered a compromise between reducing the growth of bacteria, which might deplete the substrates, and the time required to demonstrate tetrazolium reduction in fungal hyphae. Figure 4.4 shows an agar film containing a tetrazolium stained hypha from homogenised leaf material. The filling of hyphae by tetrazolium formazan crystals which was observed in some mycelial homogenates, was rarely seen with hyphae from leaf material.

4.4 Discussion

Use of the agar film method for fungal biomass estimation in leaf litter presents many problems, the most significant being the treatment required to extract fungal hyphae. The results of Swift (1973b), Leonard & Anderson (1981), Newell & Hicks (1982) and the present study suggests that optimal recovery of fungal hyphae from litter depends upon the type of homogeniser, its speed and the physical properties of the leaf material being studied. Table 4.5 presents a comparison of homogenisation conditions used in the present study with those of other authors. Results of homogenising fungal cultures and leaf material with the VIRTIS demonstrates that optimal release of hyphae can be achieved in 1.5 minutes at high speed (24 000-26 000 rev min^{-1}). These results compare favourably with those of Newell & Hicks (1982) who obtained optimal release of hyphae from Spartina alterniflora Loisel in 5 minutes at 15 000 rev min^{-1}. The lower speeds of homogenisation used by most authors (Table 4.5) probably accounts for the longer times needed for optimal release of fungal hyphae (see Appendix 4 Figure 3, for example). The observed decline in hyphal lengths after optimal release (1-1.5 minutes in the present study) suggests that hyphae are

Figure 4.4. Tetrazolium and phenolic analine blue stained fungal hypha in an agar film prepared from alder leaf litter. (Note the wide spacing of tetrazolium formazan cystals along the hypha).

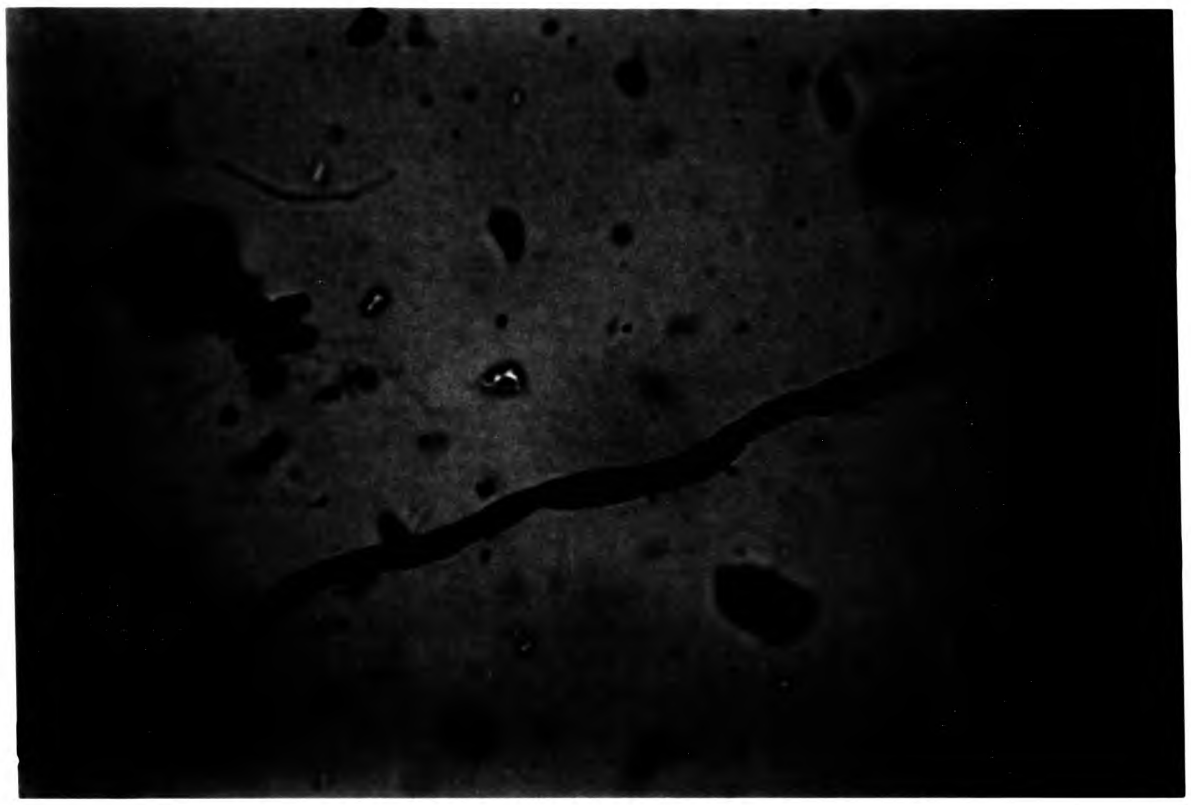


Figure 4.4. Tetrazolium and phenolic analine blue stained fungal hypha in an agar film prepared from alder leaf litter. (Note the wide spacing of tetrazolium formazan cystals along the hypha).

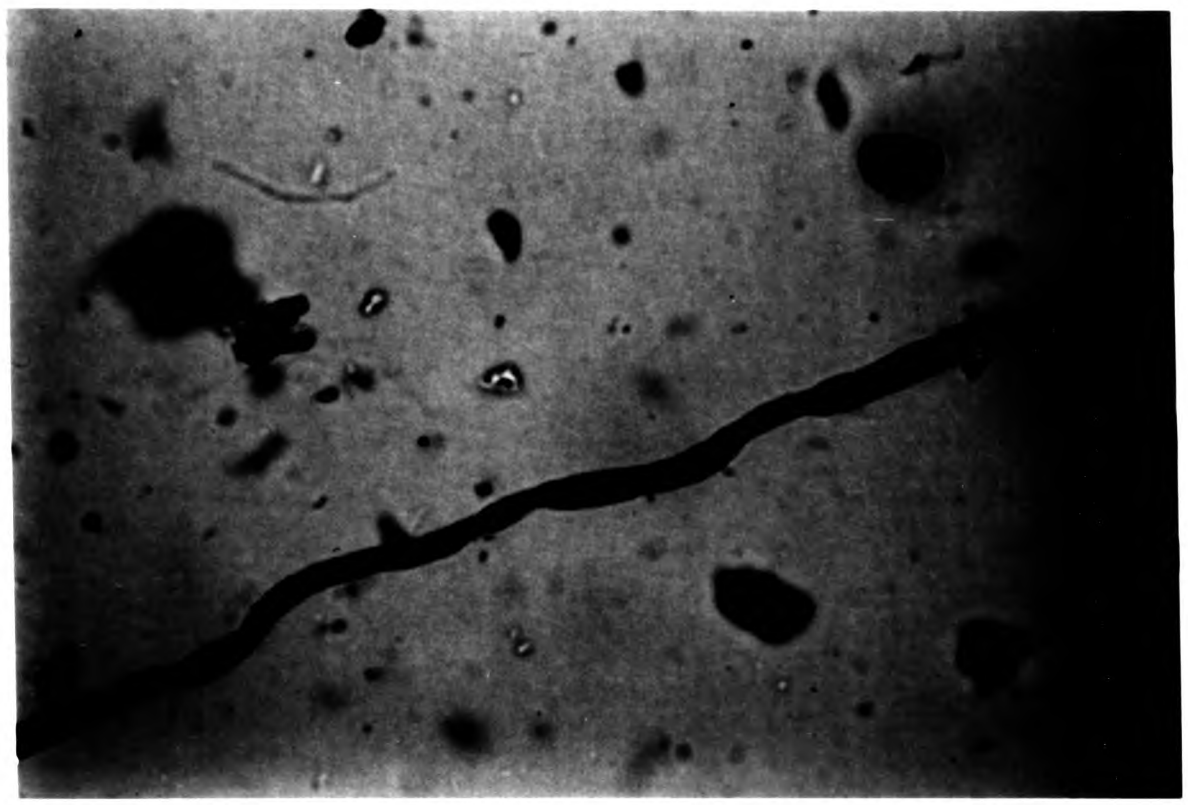


Figure 4.4. Tetrazolium and phenolic analine blue stained fungal hypha in an agar film prepared from alder leaf litter. (Note the wide spacing of tetrazolium formazan cystals along the hypha).

TABLE 4.5 The influence of homogeniser and speed on the optimum time required to release fungal hyphae from leaf litter and other organic materials.

MATERIAL	HOMOGENISER	SPEED+ (rev min^{-1})	OPTIMUM TIME (mins)	REFERENCE
Leaf litter	MSE Topdrive	12,000	6	1
Leaf litter	Blender	10,000	20	2
Leaf litter	Blender (Waring)	10,000	3	3
Leaf litter	MSE Topdrive	14,000	15	4
Lake mud*2	MSE Topdrive	14,000	10-20	5
Soil	MSE Ato-mix	6,000	2,10*1	6
Leaf	VIRTIS 45	15,000	5	7
Leaf	ROTOR STATOR	15,000	5	7
Leaf litter	MSE Topdrive	16,500	17	8*3
Leaf litter	VIRTIS 45	25,000	1	8
Leaf Litter	VIRTIS 45	25,000	1.5	8
Fungi	VIRTIS 45	25,000	1-1.5	8

REFERENCES:

1) Leonard and Anderson, 1981; 2) Swift,1973a; 3) Visser and Parkinson, 1975; 4) Frankland, Lindley and Swift, 1978; 5) Johnston, 1972; 6) Soderstrom, 1979a; 7) Newell and Hicks, 1982; 8) Present studies.

*1 Live and total hyphae respectively

*2 Actinomycete mycelium

*3 See Appendix 4 Figure 3

+ Approximate speeds, quoted by authors.

being destroyed by any further homogenisation. A similar trend was observed with pure cultures of fungal mycelium where the degree of destruction of hyphae appeared to be species specific and independant of the growth medium. These results suggest that there may be species specific differences in the toughness of mycelium and response to homogenisation.

Due to physical differences between leaf types (Table 4.3) and their influence on homogenisation speed (Figure 4.3), the proportion of total hyphae in a leaf released at optimum homogenisation time is likely to depend on the leaf type being used. Leaves of alder appeared to be homogenised more effectively than those of willow and oak. Unless corrections for homogenisation inefficiency are applied, observed quantitative differences in hyphal length estimates may reflect extraction problems rather than the lengths of fungal hyphae present.

It has been shown in the present study, and by Newell & Hicks (1982), that 50% or more of leaf material is incompletely homogenised at the time of optimal release of hyphae. It is unlikely that prolonged homogenisation of this material would release further hyphae since the fragments appeared to consist mostly of lignified tissues. Corrections of hyphal length estimates due to inefficiency of homogenisation are possible by using 250 µm as the practical upper limit of particle size for incorporation into agar films. Whether corrections can be justified can only be determined by measuring the proportions of fungal material passing through and retained by the mesh. This could be achieved by an assay for fungal chitin (Swift 1973a). Frankland, Lindley & Swift (1978) found that mycelial biomass in leaf litter estimated by the agar film method was 2-3 times lower than estimates made using a chitin assay. Since they did not examine inefficiency of homogenisation for agar films, the two techniques may have given similar results if a suitable correction

factor had been applied.

The need to measure hyphal lengths in many litter samples makes it impracticable to determine optimal release for each sample. The work presented here suggests that, at high speed, hyphae are released rapidly from leaf material of different types. Short homogenisation times (1-2 minutes) have the advantage of allowing many samples to be processed rapidly, thus providing time for estimation of the homogenisation inefficiency for each sample.

The results of staining with tetrazolium salts demonstrated that active hyphae can be detected and measured in agar films. Tetrazolium salts have been used for quantifying active bacteria in soil (Macdonald, 1980) and water (Tabor & Neihof, 1982) but there appears to have been no quantitative applications with fungi. The ability of various tetrazolium salts to stain living hyphae appears to vary considerably. INT and MTT gave the best results, particularly when used together. The reason for higher percentages of active hyphae when both tetrazolium salts were used is not clear, but it may relate to a chemical change in mixtures of the two salts or differences in enzyme specificity.

Addition of suitable substrates was important for demonstrating 'active' hyphae. Macdonald (1980) found that the presence of NADH and NADPH gave more rapid staining of bacteria and a higher proportion of active cells than with tetrazolium salts alone. Similar conclusions were drawn from the results in this work with fungi. Presumably, low metabolic activity produces tetrazolium formazan crystals which are not visible, probably because they are very small and diffuse. In contrast, many respiring bacteria appear to stain intensely with tetrazolium salts even without addition of suitable substrates (Zimmermann, Iturriaga & Becker-Birck, 1978).

Results suggest that a higher proportion of active hyphae are obtained by pre-incubation of leaf discs prior

to homogenisation than with incubation afterwards. Physical disruption of hyphae during homogenisation would not only destroy some hyphae before incubation but would expose others to osmotic changes, pH shifts and enzyme inhibition from components in the leaf material. Higher activity with pre-incubation could be explained by the water-insoluble tetrazolium crystals being less susceptible than fungal protoplasm to losses from hyphae during homogenisation. One disadvantage of incubating leaf material with tetrazolium salts prior to homogenisation is that a proportion of internal 'active' hyphae and other structures may not be stained.

Of the methods available for estimating living hyphae in agar films, tetrazolium reduction seems the most promising. Measurements of lengths of hyphae with cell contents (Frankland, 1974 & 1975a) varies with the illumination system being used, and also does not indicate the level of metabolic activity. Hyphae with large vacuolated regions with cytoplasm concentrated near to the cell wall may be overlooked using this approach. Methods based on measuring fluorescein diacetate (FDA) stained hyphae on membrane filters (Söderström, 1977 & 1979b) and in agar films (Ingham & Klein, 1984) have been proposed, but lengths of FDA stained mycelium appear to be extremely sensitive to homogenisation at low speeds, 6000 rev min^{-1} (Söderström, 1979a) and biomass estimates are low compared with other methods (Domsch *et al.*, 1979; Kjoller & Struwe, 1982). Staining of cytoplasm and DNA using aceto-orcein (Flanagan & Van Cleve, 1977) or DNA by ethidium bromide (Roser *et al.*, 1982) and europium chelate (Anderson & Slinger, 1975a & b) have been used, but since there is evidence that dead fungal cells may retain DNA, even after lysis (Scaff *et al.*, 1969; Roser, 1980) the distinction between living and dead cells becomes difficult. Ineson & Anderson (1982) examined microbial biomass in leaf litter using the agar film method and an indirect method based on glucose induced respiratory response (Anderson & Domsch, 1978a). They

suggested that the lack of a clear relationship between the two methods may have been due to europium chelate staining dead fungal cells.

Few current methods allow adequate distinction between living and dead fungal hyphae in agar films therefore, the use of tetrazolium salts seems promising. Its advantages are that most formazan crystals are semi-permanent, readily visible by light microscopy and the crystal size may indicate metabolic activity in individual hyphae.

CHAPTER 5. DEVELOPMENT OF AN INDIRECT METHOD FOR ESTIMATING FUNGAL BIOMASS IN LEAF LITTER AND FUNGAL CULTURES

5.1 Introduction

The problems of preparation and observation associated with direct microscopic methods has encouraged the search for chemical markers for detecting and quantifying fungi in plant materials. Assays for glucosamine (Swift, 1973a; Smith, 1980; Hicks & Newell, 1983) and other wall components (Whipps *et al*., 1982 & 1985) can give reasonable estimates of fungal growth under laboratory conditions, but their widespread use under field conditions is limited because of the occurrence of these markers in other biota (Smith, 1980; White *et al*., 1980).

Ergosterol ($C_{28}H_{44}O$, provitamin D_2) is potentially one of the most promising biochemical markers for fungal biomass in environmental samples (White *et al*., 1980). It is known to occur in most fungal groups, but is generally thought to be absent from the Uredinales, Perenosporales, Saprolegniales and Leptomitales (Weete, 1974; 1980), although recent studies have reported it to be present in some taxa from these groups (Warner *et al*., 1982, 1983). It is found predominantly in fungi, but it also occurs in a few species of green algae in the Chlorococales and in some protozoa, although it is not generally widespread in biota other than fungi (Altman & Dittmer, 1972).

Several assays have been developed for detecting and measuring ergosterol as a fungal marker in plant materials. Seitz *et al*. (1977 & 1979), Gordon & Webster (1984) and Matcham *et al*. (1985) used ergosterol as a marker of fungal biomass on cereal grains. When grown on grain,or in liquid culture, ergosterol correlated well with fungal mass and it enabled earlier detection of fungal growth than glucosamine. Ergosterol has been

used as a fungal marker in leaf litter of Spartina alterniflora Loisel (Lee et al., 1980), but the extraction and assay procedures used were lengthy. This study reports the development of a rapid method for the detection and quantification of ergosterol in allochthonous leaf litter in freshwater. In addition, a number of fungi isolated from the litter were screened for the presence of ergosterol.

5.2 Materials and methods

Sterols were extracted from pure cultures of fungi grown on agar and in liquid culture, and from samples of benthic litter retrieved from small lakes. Where possible, all extractions were conducted under low light intensity to minimise the possibility of structural changes in ergosterol. The ergosterol in the extracts was examined using thin layer chromatography (TLC), UV spectroscopy and high performance liquid chromatography (HPLC).

All solvents were of ANALAR grade or better. Solvents for HPLC were of HPLC grade (Rathburn Chemicals Ltd). For all work mixtures of solvents were on a v/v basis. Sterol standards were obtained from Sigma Chemicals Ltd and stored in a vacuum desiccator at 4 ^{o}C in the dark.

5.2.1 Extraction of sterols from fungal cultures

Ten fungal isolates were grown on a range of solid and liquid media (see Section 6.2 for isolates and media used). For each extraction, between 0.3 and 1 g fresh weight of mycelium was homogenised in 20 ml of methanol for 2 minutes in a VIRTIS homogeniser (VIRTIS 45, setting 5). Previous work (Figure 4.1) suggested that 2 minutes homogenisation would break up most mycelium. Each homogenate was allowed to stand for 5 minutes at room temperature (20-25 ^{o}C) then filtered (Whatman GF/C). An additional 10 ml of methanol was used to rinse the homog-

enisation flask and blades. The extract was added to a round-bottomed flask (Quickfit) together with 12.5 ml of ethanol and 5 g KOH. Each mixture was saponified by refluxing for 30 minutes and allowed to cool. After cooling, 12.5 ml of glass distilled water was added and the non-saponifiable material was extracted with 2 x 25 ml and 1 x 12.5 ml of petroleum ether (b.p. 40-60 °C). The petroleum ether extracts were combined and evaporated to dryness in a rotary evaporator (50-60 °C) under vacuum.

5.2.2 Extraction of sterols from leaf litter

To remove surface water, samples of leaf litter were shaken in muslin for 30 seconds and then blotted. Between 2 and 15 g (fresh weight) was homogenised (VIRTIS 45, setting 5) in 20 ml of methanol for 2 minutes. The homogenate was allowed to stand for 5 minutes at room temperature and then filtered, saponified, and the non-saponifiable material extracted with petroleum ether as for fungal cultures. The dry weight of leaf material extracted was calculated by oven drying (80 °C) subsamples to constant weight.

5.2.3 TLC of extracts for visualisation of sterols

The extracts were dissolved in 1 ml of benzene + acetonitrile (98:2) and a 25 µl glass syringe was used to spot or streak known volumes of extracts or standards onto pre-coated and activated (1 hr, 100 °C) TLC plates (7 x 15 cm and 20 x 20 cm, Silica G, Chem Lab.). Plates were run in benzene + acetone (90:10) for about 90 minutes, allowed to air dry and then developed with various reagents including bismuth trichloride (33% w/v in 96% v/v ethanol) with heating (100 °C, 15 minutes), chloramine-T (2% w/v in concentrated H_2SO_4) with heating (100 °C, 15 minutes), anisaldehyde (5 drops in glacial acetic acid + concentrated acetic acid (98:2)) with heating (100 °C, 20 minutes), (Lisboa, 1969; Bajaj & Ahujah, 1979), or the

Hadler histochemical reaction (Valle & Oliveira-Filho, 1975; Valle, Oliveira-Filho & Camera, 1975; and see also Appendix 5, Tables 1-2). After development spots or bands were compared with authentic sterol standards (both in visible light and under UV (265 and 356 nm)).

5.2.4 UV spectroscopy of extracts

For UV spectroscopy, the extracts were dissolved in 1 ml of benzene + acetonitrile (98:2) or dichloromethane, and 100-200 µl were loaded onto activated (100 °C, 1 hour) TLC plates (Silica G) using a 25 µl glass syringe. Plates were run in benzene + acetone(90:10) for about 90 minutes and allowed to air dry. The sterol band, located between R_f 0.37 and 0.54 (determined by running plates with sterol standards and developing with spray reagents, see Section 5.2.3), was cut out and the silica scraped into a microfiltration unit (Millipore Corp.), powdered with a glass rod and the sterols eluted with ethanol and filtered (Whatman GF/C) under vacuum. The volume of the filtered ethanol was adjusted to between 3 and 10 ml and the UV spectrum recorded with a UV spectrophotometer (Perkin Elmer 552) linked to a chart recorder.

Because of its conjugated pair of double bonds ergosterol has a characteristic spectrum between 240 and 300 nm. This contrasts with many plant sterols (eg. β-sitosterol) which absorbs very little UV light at a wavelength greater than 240 nm. The UV absorption spectrum for ergosterol in ethanol is shown in Figure 5.1). The peak height, at 282 nm, was measured for test samples and a calibration curve prepared with a range of standards (0.5-10 µg ml^{-1} ergosterol).

5.2.5 High performance liquid chromatography (HPLC)

Prior to HPLC, methods for the further purification of samples were investigated including column chromatography and TLC. With column chromatography,

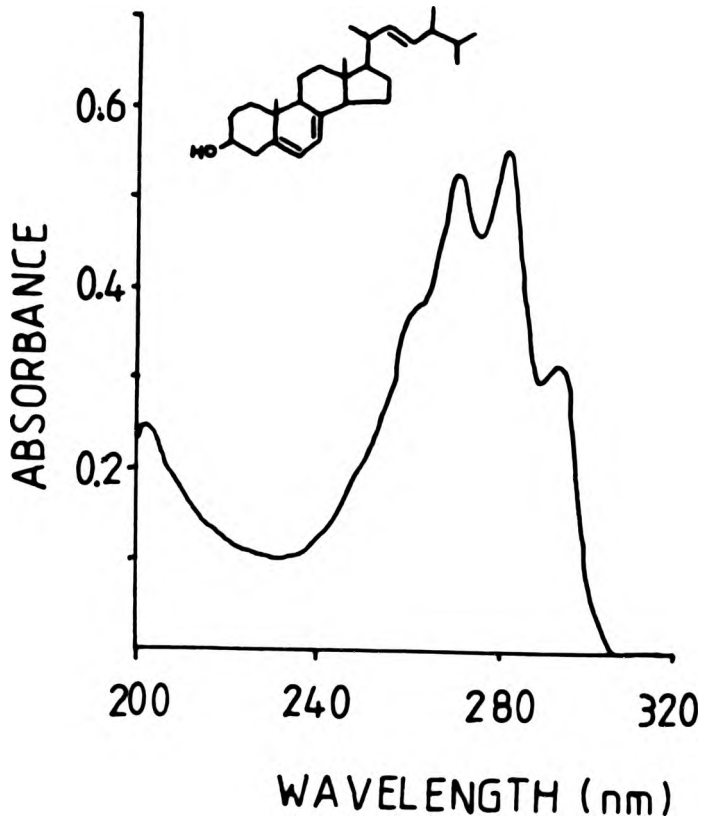


Figure 5.1. UV absorption spectrum for ergosterol in ethanol.

column separation using various supports (silica, alumina) and solvent systems were tried, but these failed to give adequate purification.

5.2.5.1 TLC of plant extracts

Sietz et al. (1977) and Matcham, Jordan & Wood (1985) used TLC for purification of ergosterol in cereal extracts prior to UV spectroscopy. These methods were investigated for the purification of leaf extracts prior to using HPLC. Extracts were dissolved in 2 ml of dichloromethane, and 80-120 µl were loaded, as a band, onto a TLC plate (Silica G, preactivated at 100 °C for 1 hour) with a glass syringe. Plates were run in a benzene + acetonitrile (90:10), dichloromethane + isopropanol (90:10), cyclohexane + ethyl acetate (75:25) and petroleum ether (b.p. 40-60 °C) + diethyl ether + glacial acetic acid (90:10:1; 70:30:1; 50:50:1). Results using various sprays (Section 5.2.3) demonstrated that, with leaf extracts, only one solvent system (petroleum ether + diethyl ether + acetic acid) gave adequate separation of sterols from other components and this was adopted for further study. When plates were run in this solvent system and examined under UV light (356 nm) an intense fluorescent band was observed with all plant extracts (over 200 samples). Comparison of mobilities with different solvent mixtures suggested that the band could be used as a relative marker for the position of ergosterol. Petroleum ether + diethyl ether + acetic acid (70:30:1) gave the best compromise between mobility, and separation of sterols and fluorescent band (Table 5.1). Attempts at using a spray (0.1% w/v dichlorofluoroscein in 96% ethanol) to visualise the sterol band prior to elution (Matcham, Jordan & Wood, 1985) gave variable results and was abandoned. After each run the silica containing the sterol band (R_f 0.314-0.438) was scraped into a micro-filtration unit (Millipore Corp.), powdered with a glass rod and the sterol eluted with ethanol and filtered

TABLE 5.1 Relative mobility (R_f) of ergosterol and a fluorescent marker band in mobile phases of differing proportions. All values are means of 10 replicates with standard deviations.

MOBILE PHASE*	RELATIVE MOBILITY R_f		
	ERGOSTEROL (A)	MARKER BAND(365nm) (B)	DIFFERENCE (B-A)
90:10:1	0.145±0.006	0.222±0.002	0.077
70:30:1	0.376±0.009	0.515±0.006	0.139
50:50:1	0.564±0.008	0.703±0.008	0.139

* Ratios of petroleum ether (b.p. 40-60°C):diethyl ether:glacial acetic acid are given.

(Whatman GF/C) under vacuum (75 cm Hg). The filtered volume was adjusted with ethanol to between 1.5 and 3 ml before HPLC.

5.2.5.2 HPLC assay

HPLC was performed on fungal extracts dissolved in ethanol (4-5 ml) and leaf extracts purified as in Section 5.2.5.1. The HPLC consisted of a Waters Associates System with a model UGK universal injector, 6000A solvent delivery system, model 660 solvent programmer and a model 441 absorbance detector. The columns (Waters Associates) used were a steel μ Bondapak C_{18} (30 cm x 0.39 I.D.) or a Radial Pak μ Bondapak C_{18} cartridge (10 cm x 0.8 I.D.). Most runs were performed using the Radial Pak cartridge in a radial compression separation unit (Z. Module, Waters Associates). Small guard columns (Z. Module RCSS Guard-Pak, C_{18} inserts (Waters Associates)) were used to improve sample purification and prolong column life. They were replaced after every 100 injections.

All solvents were degassed for 30 mins (vacuum, 75 cm Hg) and membrane filtered (0.45 μm, Millipore Corp.) before use. HPLC grade water was prepared by passing double glass distilled water through a trace organic removal cartridge (Norganic, Waters Associates) and also degassed and filtered before use. To obtain stable running conditions, the mobile phase was pumped through the HPLC for at least 1 hour before running any standards or samples.

Standards or samples (10-25 μl) were injected into the HPLC and the absorbance at 280 nm monitored on a chart recorder or integrator (Delsi Instruments). Areas were calculated for each peak produced using the integrator. Initial studies with a range of solvent systems suggested that methanol or methanol + water was suitable for separating ergosterol in fungal and leaf extracts. The retention time of ergosterol could be

reduced by increasing the solvent flow rate (Figure 5.2a) or reducing the amount of water present (Figure 5.2b). Methanol used alone appeared to separate ergosterol adequately for quantification of most fungal and plant extracts. In a few cases, addition of water to methanol was necessary to achieve adequate separation of ergosterol.

5.2.5.3 Peak identification

The presence of ergosterol in fungal and leaf extracts was identified by comparing retention times with sterol standards and spiking injected samples with known amounts of ergosterol. With some fungal extracts the peaks obtained with HPLC were sufficiently well separated from other components to allow them to be collected and concentrated. Fractions were collected from several runs of the same sample, checked for purity by re-injecting, and evaporated to dryness under a stream of nitrogen. The dried samples were used for mass spectrometry on a Jeol JMX300, mass spectrometer with an electron voltage of 70 eV. The ion masses were plotted against ion intensities, normalised relative to the base peak, and compared to runs made using authentic standards.

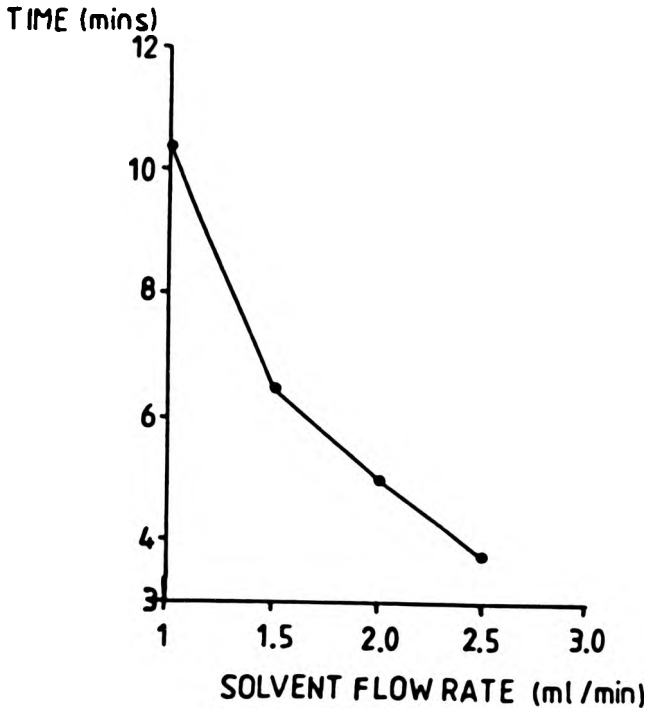
5.2.6 Recovery of ergosterol

The recovery of ergosterol from fungal cultures and leaf material was tested by adding known amounts of ergosterol (10 µg) to leaf material and fungal cultures of known ergosterol content and extracting, purifying and quantifying by HPLC as described in previous sections. This gives an indication of losses during extraction and purification.

5.3 Results

TLC was useful for detecting the presence of

a)



b)

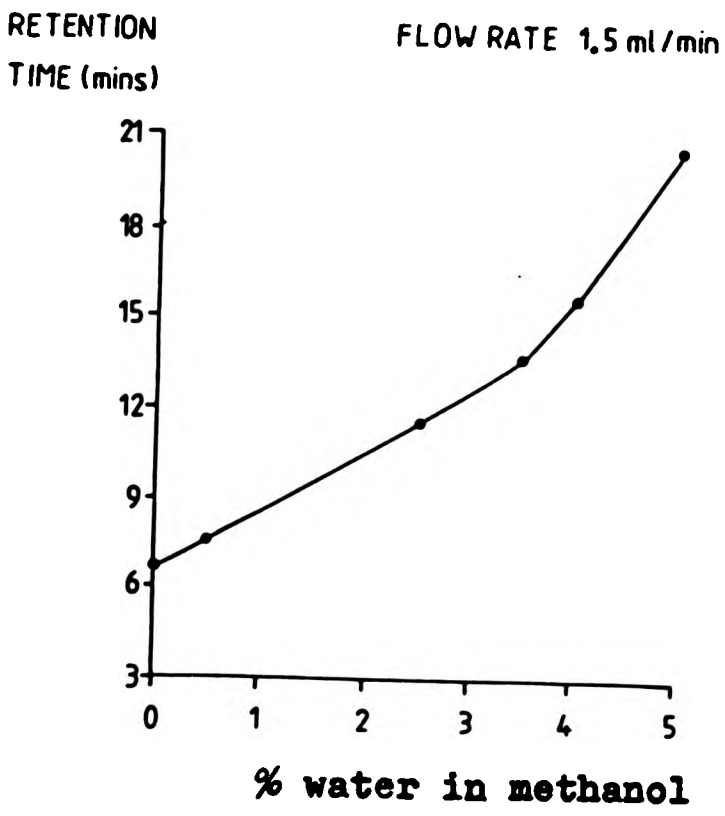


Figure 5.2. The influence of a)solvent flow rate and b) solvent composition on the retention of ergosterol using the Radial Pak C_{18} column

ergosterol in fungal cultures, but high levels of other sterols (eg. β-sitosterol) in leaf extracts masked the presence of ergosterol in most cases. Using the Hadler histochemical method (see Appendix 5) ergosterol could be identified in some plant extracts.

UV spectroscopy was adequate for assaying ergosterol in fungal cultures and concentrations of standards down to 0.5 μg ml^{-1} could be detected (Figure 5.3). Attempts to measure ergosterol in leaf extracts by UV spectroscopy were unsuccessful because of strongly absorbing components masking the ergosterol spectrum in the 200-280 nm region. Electronically derived second derivative spectra have been used to measure ergosterol in the presence of interfering compounds (Matcham, Jordan & Wood, 1985) but this approach is not entirely satisfactory for quantification and results need to be interpreted with caution.

Seitz *et al.*,(1977) used column chromatography for sample purification prior to HPLC. This method was very slow (increasing assay time by 1 hour) and, with fungal extracts, was not found to offer any advantages over direct injection of extracts in ethanol. This was not possible with the leaf extracts because it resulted in column overloading and rapid loss of column efficiency. TLC was effective in purification of sterols from plant extracts. It allowed purification despite the limited amount of material (80-150 μl) which could be run for each sample. The percentage recovery of ergosterol from TLC plates, determined by HPLC, was 97.7 ± 3.8 (S.D., n = 3).

Figure 5.4a-b shows chromatograms of HPLC separations of a fungal (a) and a plant (b) extract with methanol as the mobile phase and a flow rate of 1.5 ml min^{-1}. Under these conditions the mean retention time of ergosterol was 6.48 min ± 0.04 (S.D., n = 10). Addition of water to the mobile phase increased the retention time of ergosterol (Figure 5.2b), but in most cases addition of water was not necessary for adequate separation. This conclusion is in agreement with Colin, Gulochon & Siouffi (1979) who examined a number of solvent mixtures for the

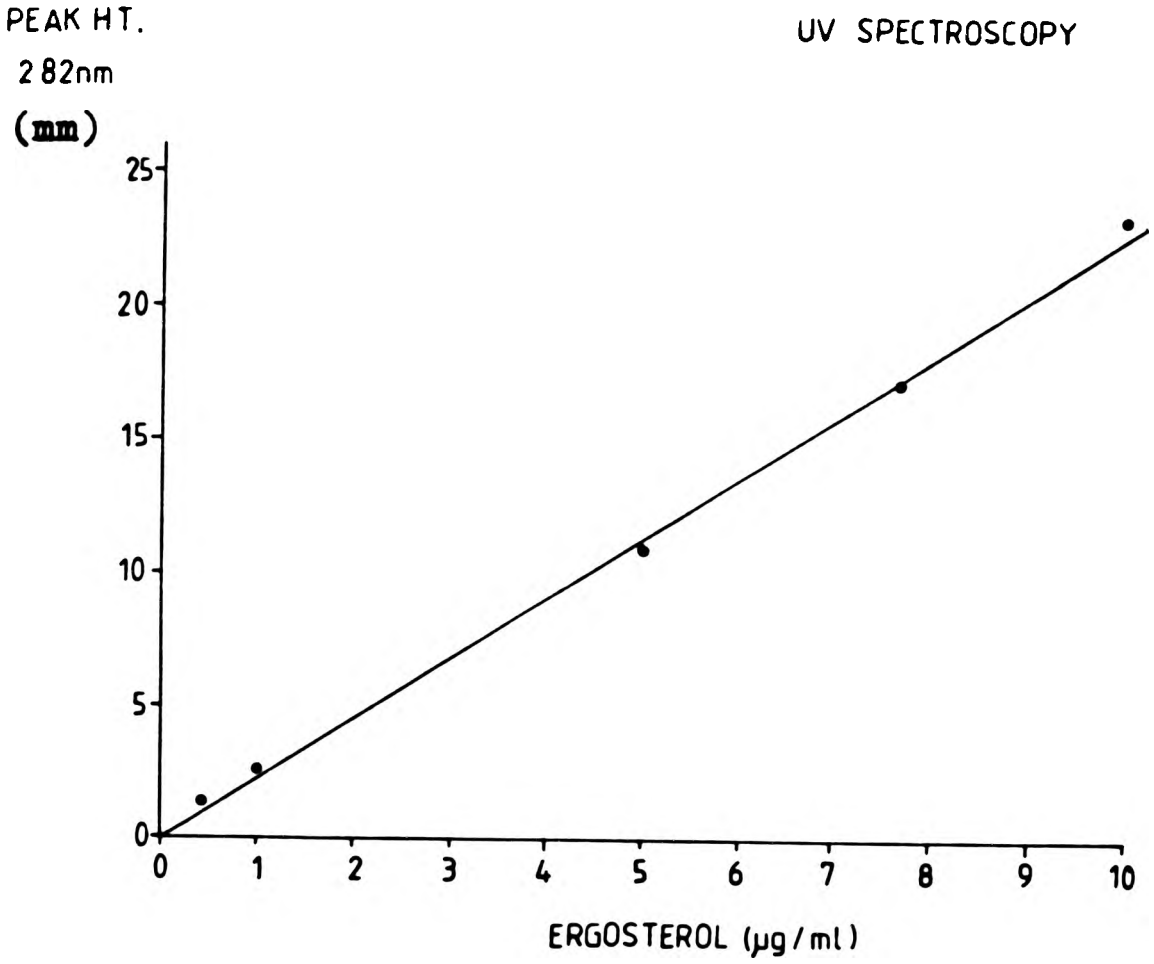


Figure 5.3. Calibration curve for ergosterol standards using UV spectroscopy at maximum sensitivity (0.005 Absorbance units full scale).

Figure 5.4. HPLC chromatograms of sterols on Radial Pak μ Bondapak C_{18} column. Solvent methanol with a flow rate of 1.5 ml min^{-1} and detection by absorbance at 280 nm.

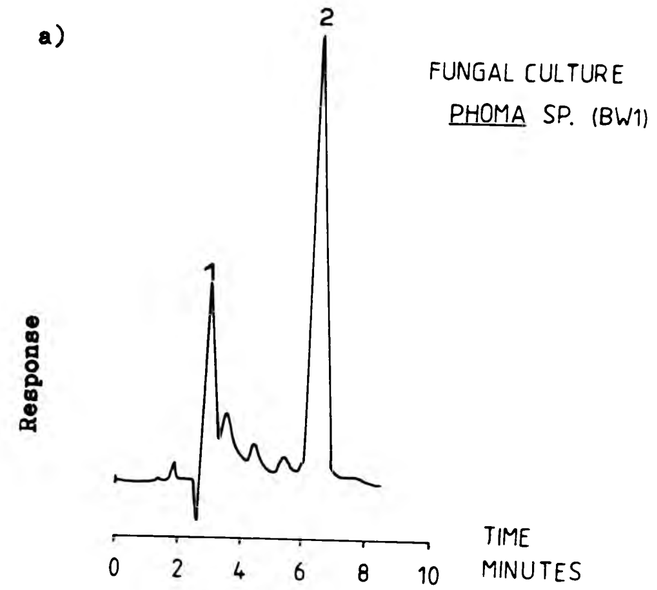
a) Extract from Phoma sp. (BW1)
(0.1 Absorbance units full scale)

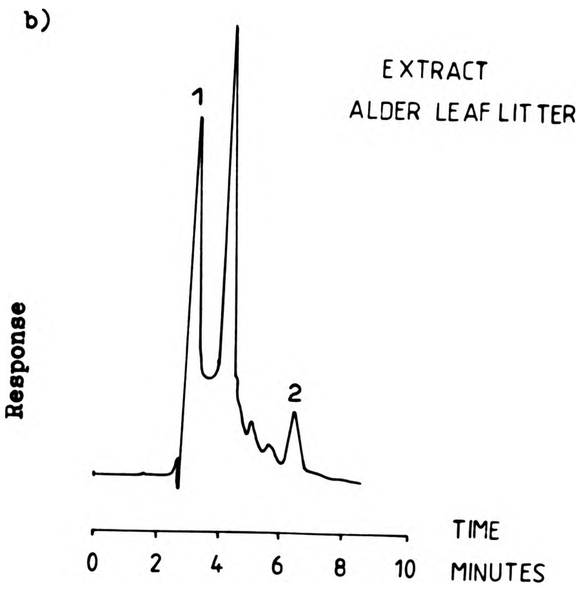
b) Extract from alder leaf litter
(0.005 Absorbance units full scale)

KEY:

Peak 1 = Solvent peak

Peak 2 = Peak with same retention time as ergosterol





separation of sterols by HPLC. For use with fungal and leaf extracts, the efficiency of the columns was adequate. The efficiency of the Radial Pak cartridge, measured as number of theoretical plates N ($N = 16$ (volume eluted before peak / eluted volume of peak width at base)^2), was 1294 with methanol as the mobile phase, and 3250 with methanol + water (95:5).

Comparison of retention times of ergosterol with standards of other sterols demonstrated that 7 dehydrocholesterol (provitamin D_3) eluted at almost the same retention time as ergosterol (6.49 min $\pm$ 0.09 (S.D., n=3). Mixtures of the two sterols could not be distinguished adequately even with addition of water to the mobile phase. Ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3) also absorb at 280 nm, but they are eluted faster 5.45 and 5.60 minutes, respectively. Other sterols tested all showed weak absorbance at 280 nm. To detect lanosterol, cholesterol and β-sitosterol quantities in excess of 1 mg needed to be injected. Their retention times were 4.15, 4.70, 5.12 minutes, respectively. In both fungal and leaf extracts peaks occurred at 3.95, 4.40, 5.05, 5.60 and 7.75 minutes, but it was not possible to assign them to particular standards. The retention time of ergosterol was checked periodically by running samples together with a known standard.

HPLC was found to be more sensitive than spraying TLC plates, or UV spectroscopy, for detecting ergosterol. As little as 1 ng of ergosterol standard could be detected, and the relationship between ergosterol concentration and peak area was linear in the range 1-100 ng (Figure 5.5).

Table 5.2 shows the percentage recovery of ergosterol from leaf material extracted with a known standard. The percentage recovery was similar for the three leaf types, and these compare favourably with recovery of ergosterol added to fungal mycelium before extraction (81.3% $\pm$ 5.2 (S.D., n = 3)).

Table 5.3 summarises the analyses carried out on

separation of sterols by HPLC. For use with fungal and leaf extracts, the efficiency of the columns was adequate. The efficiency of the Radial Pak cartridge, measured as number of theoretical plates N (N = 16 (volume eluted before peak / eluted volume of peak width at base)^2), was 1294 with methanol as the mobile phase, and 3250 with methanol + water (95:5).

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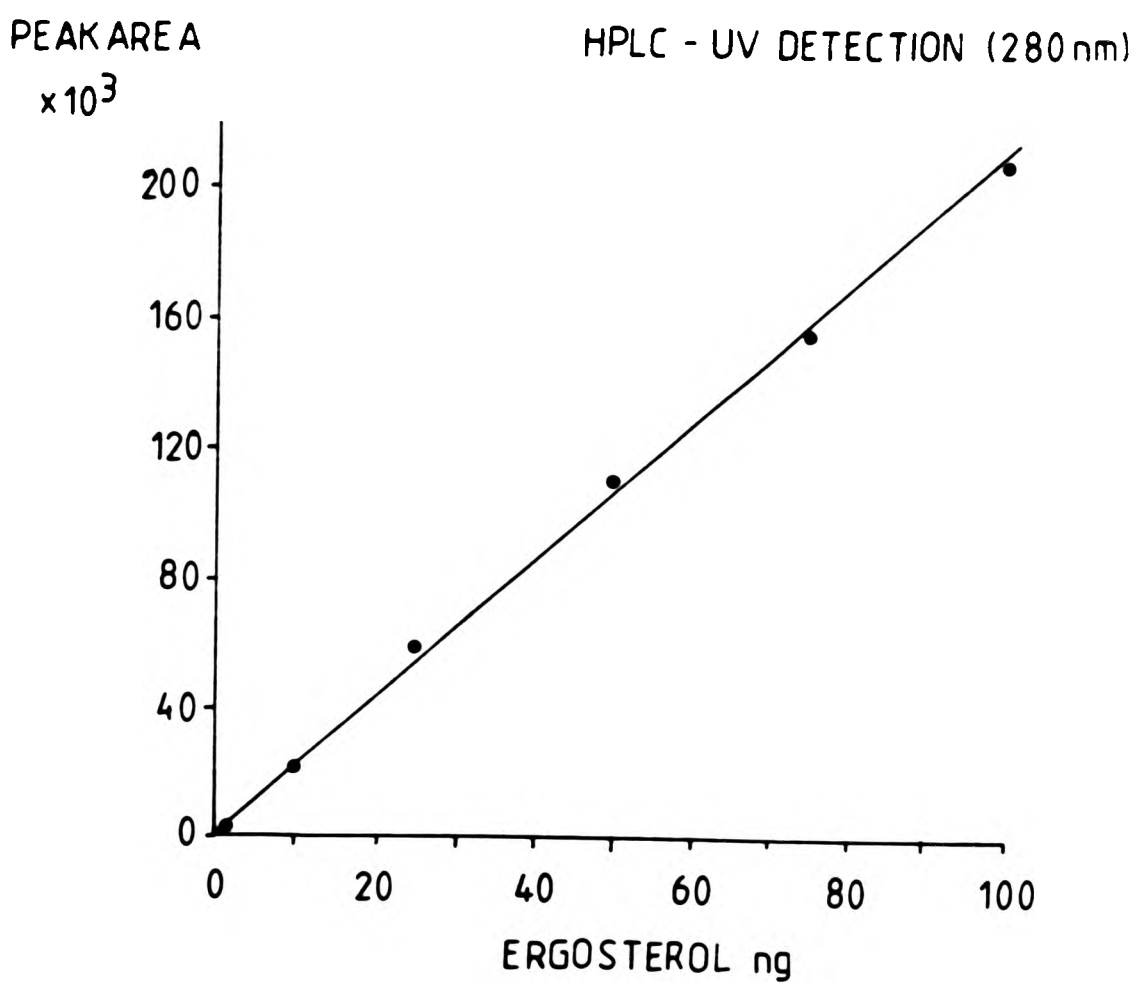


Figure 5.5. Calibration curve for ergosterol standards using HPLC at maximum sensitivity (0.005 Absorbance units full scale).

TABLE 5.2 Recovery of ergosterol added to different types of leaf litter. Ergosterol values are in µg g^{-1} leaf oven dry weight (80°C) with one standard deviation.

	LEAF TYPE		
	WILLOW (n=2)	ALDER (n=2)	MIXED SPECIES (n=3)
Ergosterol in leaf	2.85±0.89	2.59±0.54	3.86±1.18
Ergosterol in leaf + internal standard (10µg)	10.98±1.81	10.18±1.27	11.84±1.49
% Recovery *1	79.4	79.7	75.4

*1 $\%\text{Recovery} = \frac{\text{Ergosterol in leaf + internal standard}}{\text{Ergosterol in leaf + Ergosterol in leaf + internal standard}} \times 100$

TABLE 5.3 Detection of ergosterol in fungal strains using different methods.

FUNGAL SPECIES	TLC	UV Spectroscopy	HPLC Retention time	HPLC Mass Spec.
Tetracladium marchalianum (isolate 1A)	+	+	+	ND
Tetracladium marchalianum (isolate 3A)	+	+	+	ND
Tetrachaetum elegans (isolate AB27)	+	+	+	ND
Anquillospora longissima (isolate 7A)	+	ND	+	ND
Clavariopsis aquatica (isolate ST1)	+	ND	+	+
Articulospora tetracladia (isolate VW1)	+	+	+	+
Heliscus lugdunensis (isolate KA1)	ND	ND	+	ND
Tricladium splendens (isolate Yat4)	ND	ND	+	ND
Phoma sp. (isolate BW1)	ND	ND	+	ND
Fusarium sp. (isolate Folk 1)	ND	ND	+	ND

ND= Not determined

various fungal isolates for the presence of ergosterol. Ergosterol was identified in all strains when tested by HPLC, and in those when TLC and UV spectroscopy was used. With sprayed TLC plates and UV spectroscopy, only ergosterol was detected but, with HPLC, several additional peaks were seen. TLC may not detect all sterols present in extracts since the sensitivity is quite low (0.5-1 µg).

Two of the fungal extracts were used to prepare mass spectra of the HPLC sample peaks corresponding to the retention time of ergosterol standards. The mass spectra are shown in Figure 5.6a-b. Mass fragments (m/e) are shown on the horizontal scale and intensity relative to the base peak (major peak) on the vertical scale. The spectra for each isolate are similar, although the intensity of mass fragments (m/e) differs. Both isolates show m/e 396 corresponding to the molecular ion of ergosterol (molecular weight) and the major ion fragments at m/e 378,363, 337, 271 and 253. The pattern of fragmentation suggests the presence of ergosterol. The minor peaks above m/e 396 are probably due to contamination of the samples by other organic components.

5.4 Discussion

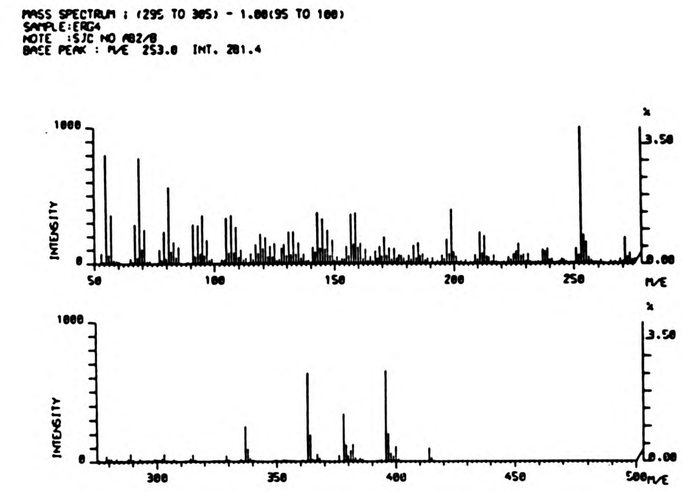
Ergosterol is a potentially useful chemical marker for fungi in environmental samples (White *et al*. 1980; Newell & Hicks, 1982; Matcham, Jordan & Wood, 1984; Newell & Fallon, 1983). Although other fungal components have been proposed as markers (Smith, 1980; White *et al*. 1980; Whipps *et al*., 1982 & 1985) they are not as specific for fungi and the assays are time consuming.

The results obtained in this study have demonstrated that, using HPLC, ergosterol could be assayed in fungal cultures and leaf litter in 2-3 hours. The assay has a high degree of sensitivity with ergosterol detectable at levels as low as 1 ng. These results are comparable to the sensitivities obtainable by gas chromatography, and the detection limits compare well with those obtained by

Figure 5.6. Mass spectra of fractions collected from HPLC peaks with the same retention time as ergosterol. Each spectrum shows the relative intensity (vertical scale 0-1000) of ion fragments m/e (horizontal scale 50-500).

a) Extract of Clavariopsis aquatica
b) Extract of Articulospora tetracladia

a)



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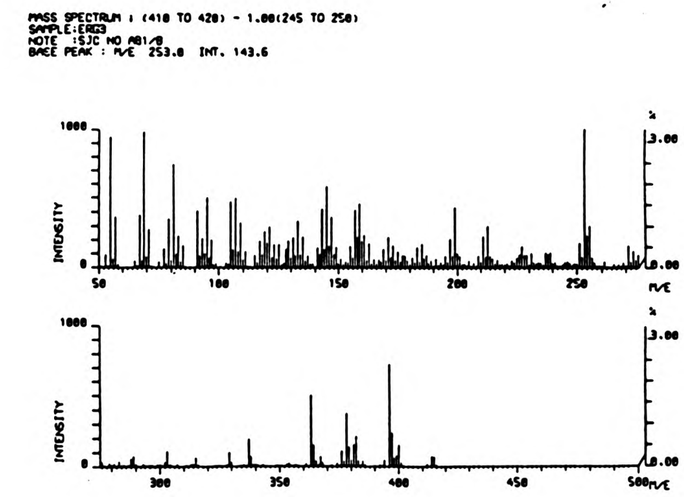


other workers using HPLC (Sietz *et al*., 1977; Colin, Gulochon & Siouffi, 1979).

The recovery of ergosterol from leaf material was consistant for several leaf types and for fungal cultures. The percentage recovery (78.2) was lower than that obtained for vitamin D metabolites in animal feeds (87.5%) by Ray, Dwyer, & Reagor (1977), possibly because these authors did not use saponification or TLC prior to HPLC. The weak absorbance of most sterols at 280 nm reduced problems of interference with ergosterol in fungal extracts and TLC purified litter samples. Based on retention times ergosterol could not be resolved from 7-dehydrocholesterol. This is unlikely to present major problems since 7-dehydrocholesterol has been reported mainly from mammalian tissue (Altman & Dittmer 1972) and as it is a C_{27} sterol it is readily separable from ergosterol, a C_{28} sterol, by mass spectrometry.

UV spectroscopy was less sensitive than HPLC for determination of ergosterol, with the limit of detection being 0.2-0.5 $\mu g\ ml^{-1}$. The method was suitable for fungal extracts but, as found by Matcham, Jordan & Wood (1985) there were problems of interference when used with plant extracts. However, Seitz *et al*. (1977) used it successfully with some cereal extracts, so the method may have potential for certain types of plant material. One problem with using UV spectroscopy is that some sterols may show spectra similar to ergosterol (eg. 7-dehydrocholesterol, 22,23-dihydroergosterol and 24(28) dehydroergosterol. The latter was shown by Woods (1971) to be present in yeasts, and to differ from ergosterol only in the 220-240 nm region of its UV spectrum. Therefore, care is needed when examining spectra particularly if sterols are extracted from mixed fungal populations.

A number of fungi isolated from allochthonous leaf litter in freshwater were found to have ergosterol present as their major sterol component. Several other

peaks were obtained in HPLC runs of fungal extracts, and these may have been ergosterol related sterols. Because of a lack of authentic standards, identification of these components would require the use of gas chromatography coupled with mass spectrometry.

The detection of ergosterol in a range of fungi isolated from benthic litter should enable it to be used as a marker for estimating fungal biomass in this habitat, providing that the relationship between fungal mass and ergosterol does not vary significantly under different growth conditions. Since chemical markers often show considerable variability under different growth conditions (Sharma, Fisher & Webster, 1977; Riemann & Wium-Anderson, 1981; Fairbanks *et al.*, 1984; Hicks & Newell, 1984), this aspect will need to be evaluated before ergosterol can be used to provide realistic estimates of fungal biomass in environmental samples.

CHAPTER 6. STUDIES ON CONVERSION FACTORS FOR ESTIMATING FUNGAL BIOMASS

6.1 Introduction

In recent years there have been significant advances in the development of new or improved methods for estimating microbial biomass. Despite this, relatively little attention has been directed towards studies of the conversion factors relating the measured unit (eg. biovolume, glucosamine, ATP, ergosterol, muramic acid) to biomass. A general lack of information about suitable biomass conversion factors, and their variability under field and laboratory conditions has, to some extent, masked the significance of biomass estimates made in natural ecosystems. More information is needed on the conditions (nutrients, temperature, species, age, grazing, interference) which may influence these conversion factors and on ways of predicting appropriate conversion factors for use in samples retrieved from the field.

Factors for converting fungal biovolume to biomass have been studied by Van Veen & Paul (1979), Newell & Statzell-Tallman (1982) and Hicks & Newell (1984) and for bacterial biovolume conversions by Bakken & Olsen (1983), Bratbak & Dundas (1984) and Bratbak (1985). Van Veen & Paul (1979), studying four fungi isolated from soil, found that the biovolume to biomass conversion factor ranged from 0.11 to 0.41 g cm^{-3}, with a mean of 0.33 g cm^{-3}. Newell & Statzell-Tallman (1982), working with fungi isolated from leaves of Spartina alterniflora Loisel, found mean conversion factors for biovolume to biomass ranging from 0.2 to 0.9 g cm^{-3} with considerable variation occurring both between species and between different ages of mycelium.

Several authors have demonstrated that biochemical markers for fungal biomass may show considerable variability. Sharma, Fisher & Webster (1977) and Hicks

& Newell (1984) showed the variation in glucosamine per unit dry weight of mycelium to vary with fungal species, age and growth conditions, the ranges being 2.3-53.5 and 8.5-92.8 $\mu g\ g^{-1}$ in the two studies respectively. Variation in ergosterol content has been less well studied than glucosamine, although Nout *et al.* (1986) reported that in *Rhizopus oligosporus* (NRRL 5905) grown in tryptone soya broth the ergosterol content of mycelium varied from 2-24 $mg\ g^{-1}$, and was influenced by medium composition, aeration and mycelial age.

The present study examined biomass conversion factors for biovolume and ergosterol content of mycelium for a number of fungi isolated from allochthonous litter. The aim was to establish a) the variation between species, b) the influence of growth conditions, c) the influence of mycelial age and d) the relationship between conversion factors and the elemental composition of mycelium. An examination of these factors was necessary before conversion factors could be applied in a study of fungal biomass under field conditions.

6.2 Materials and methods

Fungal isolates were obtained from benthic litter by particle plating or from single spores of fungi growing on leaf material incubated in sterile distilled water in petri dishes. Cultures were maintained on potato dextrose agar at 12 °C in the dark prior to use. The following species were studied: *Tetracladium marchalianum* (1A & 5A), *Tetrachaetum elegans* (27A), *Tricladium splendens* (Yat 4), *Clavariopsis aquatica* (ST1), *Articulospora tetracladia* (VW1), *Heliscus lugdunensis* (KA1), *Anguillospora longissima* (Dun 2), *Trichoderma viride* Pers. (Lam 23), *Aureobasidium pullulans* (de Bary) Arnaud (AB23 & Yat 3), *Phoma* sp. (BW1), *Fusarium* sp. (Folk 1). For details of isolates see Appendix 3, Table 1. To examine conversion factors, fungi were grown on agar media, in liquid media and on submerged leaf litter.

6.2.1 Agar media

Agar media were used to examine the influence of different nutrient conditions on conversion factors for fungal biovolume to biomass and ergosterol content. Five media were used - malt extract agar, potato dextrose agar, corn meal agar, alder leaf extract agar and willow leaf extract agar. Details of the preparation of these media are given in Appendix 3. After the media had set, each plate was overlaid with a sheet of sterile cellophane Grade PF320, (see Chapter 4) which had been boiled and washed twice prior to autoclaving. For each isolate, three plates were inoculated with several 2 mm diameter plugs of mycelium taken from the edge of actively growing cultures on PDA. Plates were sealed in plastic bags and incubated in the dark at 12 °C for 14 days. In experiments with the *Phoma* sp. isolate, plates were incubated in the dark at 12 °C for periods between 7 and 140 days.

6.2.2 Liquid media

Erlenmeyer flasks (500 ml) containing 400 ml of malt extract broth were autoclaved at 120 °C for 10 minutes. For each isolate investigated, flasks were inoculated with 2 agar plugs (4 mm diameter) taken from actively growing cultures on potato dextrose agar. Flasks were incubated as static cultures at 12 °C in the dark for periods between 7 and 140 days.

6.2.3 Submerged leaf material

Experimental flasks containing 5 g (dry weight) of sterile whole leaves of alder, willow, ash or oak together with 600 ml of sterile mineral medium were set up as described in Chapter 2. Triplicate flasks were inoculated with 3 plugs (5 mm diameter) of mycelium taken from actively growing cultures on potato dextrose agar. Flasks were incubated at 18 °C under constant aeration

($100\ cm^{-3}min^{-1}$) for periods up to 21 days.

6.2.4 Harvesting and weighing of mycelium

Mycelium was harvested from agar plates by lifting or scraping it from the cellophane covering the agar. Degradation of the cellophane was minimal on the media used. Excess moisture was removed by blotting the mycelium on filter paper. Liquid cultures were filtered (Whatman GF/C) with vacuum (75 cm Hg) and culture medium washed off with several rinses of distilled water. Mycelium was left under suction for one minute after washing to remove excess water trapped between fungal hyphae.

Mycelium from leaf cultures was harvested by shaking the flasks vigorously for 20 seconds and pouring the contents into white enamel trays. Loose tufts of mycelium were collected with fine forceps and further samples were teased or scraped from leaf surfaces and collected with a pasteur pipette. Mycelium was transferred to small nylon bags (250 µm mesh, Henry Simon Ltd) washed 3 times with distilled water for 20 seconds, and large particles of leaf debris removed with forceps. After washing, mycelium was blotted on filter paper to remove excess water. Samples of mycelium from agar and liquid cultures and submerged leaf material were stored on aluminium foil discs in petri dishes containing moist filter papers (Whatman GF/C) to reduce dehydration prior to weighing.

6.2.5 Estimation of fungal biovolume to biomass conversion factors

Conversion factors for fungal biovolume to biomass were determined using a modification of the methods of Van Veen & Paul (1979) and Newell & Statzell-Tallman (1982). The method is presented as a protocol in Table 6.1. All mycelial samples were homogenised for 1 minute (VIRTIS-45, setting 5). This has previously been shown

TABLE 6.1 Protocol for determining conversion factors for fungal biovolume to biomass.

1) Fungal mycelium weighed*. Samples preserved in 5-10ml filtered (0.22µm membrane filter, Millipore Corp) 4% v/v formalin.

2) Further samples of mycelium weighed* and oven dried (80°C) to constant weight to estimate moisture content.

3) Formalin preserved sample homogenised in 20ml 4% (v/v) formalin (0.22µm filtered) for 1 minute (VIRTIS-45, setting 5).

4) Homogenate transferred to a beaker and 2 aliquots (10ml) of distilled water (0.22µm filtered) used to rinse flask and blades. Washings were added to homogenate in beaker.

5) Between 0.1 and 1ml of homogenate added to 15ml of distilled water (0.22µm membrane filtered, Millipore Corp) and filtered (0.45µm membrane filter) under suction (vacuum 75cm Hg).

6) Filter air dried and stained with phenolic analine blue (Jones and Mollison, 1948) for 20 minutes at 40°C keeping filter protected from dust.

7) Filter cleared in lactophenol for 30 minutes.

8) Filter examined under x400 or x1000 with bright field and phase contrast illumination.

9) Estimate hyphal length on 20 microscope fields using the intersection method (Olson, 1950).

10) Measure hyphal diameter on 20 randomly selected hyphae at x1000.

11) Calculate fungal biovolume on original sample and relate it to the biomass to calculate the conversion factor.

*(Cahn Electrobalance, nearest µg).

to result in the optimal release of fungal mycelium into suspension (Figure 4.1). Newell & Statzell-Tallman (1982) observed that with some mycelial homogenates, hyphal lengths were difficult to estimate using the intersection method because of large clumps of tangled mycelium. These were observed in the present work particularly with Ingoldian hyphomycetes grown on the nutrient rich media - malt extract agar and potato dextrose agar. Attempts to re-homogenise samples or sonicate homogenates (18 μm amplitude for 3, 5 or 13 minutes on ice; Soniprep MSE) were unsuccessful, therefore a policy of examining 2 extra microscope fields for every one with tangled mycelium was adopted.

6.2.6 The ergosterol content of mycelium

The ergosterol levels in mycelium of selected fungal species were determined by HPLC, using the method described in Chapter 5. Cultures grown on agar and in liquid culture were examined. Mycelium from liquid culture was filtered (Whatman GF/C) with vacuum (75 cm Hg) before extracting ergosterol. Results were converted to mg ergosterol g^{-1} mycelial dry weight (80 ^{o}C).

6.2.7 ATP content of mycelium

ATP content was examined in mycelium grown on nutrient rich and leaf extract media for comparison with conversion factors for biovolume and ergosterol. The ATP content of six fungal isolates grown on malt extract and leaf extract agar media was determined using the luciferin luciferase enzyme method (Karl & La Rock, 1975). ATP was extracted from mycelium by boiling in 8 ml of 20 mM McIlvaine buffer pH7.7 (Bulleid, 1978) for 1 minute. The tubes were cooled on ice, pH adjusted to 7.8 with 1 M NaOH, and volumes adjusted to 10 ml with McIlvaine buffer. Samples together with ATP standards (which had been treated in the same way) were then frozen at -20 ^{o}C

until required for analysis.

The assay was conducted using commercial firefly extract (FLE-50, Sigma) which was reconstituted in 7.5 ml of 0.1 M sodium arsenate pH7.4, 5 ml of 0.5 M magnesium sulphate and 12.5 ml of distilled water. The extract was mixed thoroughly, allowed to stand for 24 hours at 4 ^{o}C and then filtered (whatman GF/C) and stored at 4^{o}C until used (within 24 hours).

ATP was determined using a scintillation counter (Beckman L.S. 7500) with a preset count of 10 seconds, a narrow window setting (280-320) and coincidence out for photoluminescence measurement. ATP determination was performed by dispensing 1 ml of sample or standard solution into a glass scintillation vial followed by 0.5 ml of enzyme extract. Exactly 1 minute after addition of enzyme extract, the photoluminescence was counted on the scintillation counter for 10 seconds. After adjusting for background, a calibration curve was prepared by plotting counts per minute against ATP concentration (see Appendix 5, Figure 1 for an example of a standard curve). Corrections for ATP losses and sample quenching were calculated according to Jones (1979). ATP was expressed as mg ATP g^{-1} mycelial dry weight (80 ^{o}C).

6.2.8 Carbon and nitrogen analysis

Oven dried (60 ^{o}C) mycelium of six fungal isolates grown on malt extract and leaf extract agar were weighed (Cahn, Electrobalance) and the percentage composition of carbon and nitrogen determined using an elemental analyser (CHN analyser, Carlo Erba). Three replicate determinations were made for each sample of mycelium.

6.2.9 Elemental composition of mycelium using X-ray microprobe analysis

The elemental composition of mycelium was examined using X-ray microprobe analysis to see if particular

elements or changes in the relative proportions of different elements could be used to predict conversion factors. Three replicate portions of oven dried mycelium (60 ^{o}C) of fungal cultures grown on agar media or submerged leaf material were mounted on double-sided Sellotape coated aluminium stubs. The stubs were carbon coated to enhance the surface conductivity of the samples and samples were analysed in a scanning electron microscope (Super Mini S.E.M., International Scientific Instruments) equipped with an energy-dispersive X-ray analyser (Lewell Electronics Ltd) and Princeton Gamma Tech system 4 computer. The samples were scanned at an acceleration voltage of 15 Kv and at a magnification of x 700 or x 1000. The beam current used for X-ray analysis was between 5 and 6 nA with a specimen tilt of 25^{o} and a working distance between sample and detector of 23 mm. Using the X-ray microprobe, areas of visible mycelium on the SEM were sampled for 100 seconds to obtain a spectrum of their elemental composition. From the resulting X-ray spectra counts were obtained for the elements Ca, Mg, Na, K, P, Si, Fe, Cl and S. The instrument does not permit the detection of elements lighter than fluorine. To allow comparison of elemental composition of different fungi grown under different growth conditions, it was necessary to normalise the X-ray counts because of differences in specimen thickness. Therefore counts for each element were expressed as a proportion of the total counts for the nine elements counted on a given sample. Controls consisted of X-ray spectra for carbon coated Sellotape on aluminium stubs.

6.3 Results

6.3.1 Conversion factors for mycelial biovolume to biomass

Tables 6.2 & 6.3 show the biovolume to biomass

TABLE 6.2 Biovolume to biomass conversion factors, hyphal diameters and mycelial moisture contents of 12* fungal isolates grown on 5 nutrient media for 14 days at 12°C. Each value represents the mean value for the 12 fungal isolates together with standard errors.

PARAMETER	MALT EXTRACT AGAR	POTATO DEXTROSE AGAR	CORN MEAL AGAR	ALDER LEAF AGAR	WILLOW LEAF AGAR
Biovolume to biomass conversion factor(g cm^{-3})	0.34 ±0.03	0.33 ±0.04	0.27 ±0.04	0.25 ±0.05 (n=10)	0.25 ±0.04 (n=10)
Hyphal diameter (µm)	2.89 ±0.20	3.06 ±0.22	3.24 ±0.22	3.75 ±0.27 (n=10)	3.52 ±0.24 (n=10)
Moisture content of mycelium (% fresh weight)	86.40 ±1.38	82.82 ±1.30	86.89 ±1.33	83.33 ±1.58 (n=11)	80.53 ±1.60 (n=11)

* (Except where number of isolates is given in parenthesis)

TABLE 6.3 Hyphal diameter, biovolume to biomass conversion factor and moisture content of 12 fungal isolates from allochthonous litter. The values shown are means for 5 different growth media (Table 6.2) together with standard errors.

FUNGAL ISOLATE	HYPHAL DIAMETER (µm)	BIOVOLUME BIOMASS CONVERSION FACTOR ($g\ cm^{-3}$)	MOISTURE CONTENT (%FRESH WEIGHT)
Tetrachaetum elegans	2.31	0.34	84.36
	±0.09	±0.08	±1.77
Articulospora tetracladia	2.34*	0.32*	84.20*
	±0.08	±0.08	±4.31
Anguillospora longissima	2.46	0.17	82.68
	±0.11	±0.02	±1.72
Tetracladium marchalianum(1A)	2.58	0.21	87.30
	±0.13	±0.02	±0.94
Tetracladium marchalianum(5A)	2.39*	0.26*	85.84+
	±0.13	±0.02	±2.49
Tricladium splendens	3.30	0.35	83.12
	±0.03	±0.08	±1.49
Heliscus lugdunensis	3.73	0.25	84.00
	±0.03	±0.05	±2.72
Aureobasidium pullulans(AB#3)	3.24	0.32	79.54
	±0.11	±0.03	±3.37
Aureobasidium pullulans(Yat3)	3.33	0.40	88.57
	±0.18	±0.08	±0.86

FUNGAL ISOLATE	HYPHAL DIAMETER (µm)	BIOVOLUME BIOMASS CONVERSION FACTOR ($g\ cm^{-3}$)	MOISTURE CONTENT (%FRESH WEIGHT)
Phoma sp	3.95 ±0.23	0.39 ±0.06	82.87 ±2.45
Fusarium sp	4.13 ±0.10	0.21 ±0.03	88.13 ±1.12
Trichoderma viride	4.24 ±0.28	0.28 ±0.07	78.21 ±2.05

* Mean and standard error based on 3 growth media.

conversion factors, hyphal diameters and percentage moisture content of fungal mycelium grown on 5 agar media. Conversion factors ranged from 0.09 to 0.60 g cm^{-3} with a mean and standard error of 0.29 ± 0.02 (n = 56). They were slightly lower for Ingoldian hyphomycetes (0.27 ± 0.02; n = 31) than for the other fungal species (0.32 ± 0.03; n = 25). Overall there was no significant difference in biovolume conversion factors between fungal species ($P > 0.05, < 0.1$) or between growth media ($P > 0.05, < 0.1$). Conversion factors were generally higher on the nutrient rich growth media (malt extract and potato dextrose agar) than on leaf extract media (Table 6.2).

Hyphal diameters varied significantly both between fungal species ($P < 0.001$) and between growth media ($P < 0.001$). Mean hyphal diameters were lowest for Tetrachaetum elegans grown on malt extract agar (2.06 µm ± 0.07) and highest for Trichoderma viride grown on alder leaf extract agar (4.93 µm ± 0.38). For all fungi, hyphal diameters were lower on the nutrient rich media than on the leaf extract media. Hyphal diameters for the Ingoldian hyphomycetes were lower (2.78 ± 0.10, n = 31) than for the other fungi (3.78 µm ± 0.12, n = 25).

Percentage moisture content of mycelium ranged from 73.1 ± 0 in Trichoderma viride grown on willow leaf extract agar to 91.7 ± 0.3 in Tetracladium marchalianum grown on corn-meal agar. There was significant variation in moisture content, both between fungal species ($P < 0.05$) and between different growth media ($P < 0.01$). The highest moisture contents of mycelium were on malt extract and corn meal agar. Percentage moisture content of Ingoldian hyphomycetes was slightly higher (84.5 ± 0.8) than for other fungi (83.5 ± 1.2). Correlations between the biovolume conversion factor for fungi grown on agar with hyphal diameter and with mycelial moisture content were not significant ($r = -0.034$, $P > 0.05$; $r = -0.187$, $P > 0.05$, respectively). Correlation between the mean biovolume conversion factor

and mean hyphal diameter of the fungi on each media was significant ($r = -0.919$, $P < 0.05$).

Table 6.4 shows the levels of ATP and percentage carbon and nitrogen in mycelium of six fungal isolates grown on malt extract and leaf extract agars. Both ATP and nitrogen were higher in mycelium grown on the nutrient rich malt extract agar, but carbon did not differ with respect to growth medium. Correlations between biovolume conversion factors and ATP content ($r = 0.336$), percentage nitrogen in mycelium ($r = -0.509$), percentage carbon in mycelium ($r = 0.136$) and carbon to nitrogen ratio of mycelium ($r = -0.100$) were not significant ($P > 0.05$).

Table 6.5 shows biovolume to biomass conversion values and hyphal diameters of mycelium at different ages grown on solid or in liquid media. The conversion factors after 7 days growth was significantly lower ($P < 0.05$) for mycelium grown on agar than in liquid medium but not after 14 days ($P > 0.05$). There were no significant differences ($P > 0.05$) in conversion factors between 7 and 14 day old mycelium grown either in liquid or on agar, but the conversion factor for mycelium at 140 days in liquid culture was significantly lower ($P < 0.01$) than that at 14 days. On both agar and in liquid culture, hyphal diameter decreased significantly ($P < 0.01$) between 7 and 14 days but not between 14 and 140 days in liquid cultures (fungi on agar plates were not examined at 140 days). On agar or in liquid medium hyphal diameters at 7 and 14 days were not significantly different ($P > 0.05$).

Biovolume to biomass conversion factors for fungi grown on submerged leaf litter are shown in Table 6.6. Significant differences ($P < 0.05$) were found between conversion factors for Anguillospora longissima, Articulospora tetracladia and Phoma sp. on different leaf types. In most cases conversion factors increased with mycelial age although this was only significant for Articulospora tetracladia ($P < 0.001$). Considering all

TABLE 6.4 Levels of ATP (mg g^{-1} dry weight), carbon and nitrogen (% dry weight) in mycelium of six fungal isolates grown on malt extract and leaf extract agars for 14 days at 12°C. Values shown are means and standard errors. The fungal isolates are listed in Appendix 5, Table 3 together with their ATP concentrations.

PARAMETER	MALT EXTRACT AGAR	ALDER LEAF EXTRACT AGAR	WILLOW LEAF EXTRACT AGAR
ATP (mg g^{-1})	1.88±0.12	0.51±0.09	0.51±0.08
% Carbon	42.10±1.20	42.50±1.30	42.90±0.97
% Nitrogen	5.20±0.50	2.00±0.10	1.90±0.20
Ratio C:ATP	224	833	841
Ratio N:ATP	28	39	37

TABLE 6.5 Comparison of the biovolume to biomass conversion factors (g cm^{-3}) for Phoma sp(BW1) mycelium at different ages, grown on malt extract agar and in malt extract broth. The values are the means of 4 replicate determinations together with standard errors. The figures shown in parentheses are the mean hyphal diameters (µm) with standard errors (n=10).

GROWTH MEDIUM	CULTURE AGE (days)		
	7	14	140
Malt extract agar	0.25±0.03	0.43±0.09	ND
	(5.02±0.15)	(3.81±0.09)	
Malt extract broth	0.55±0.06	0.43±0.02	0.27±0.05
	(5.47±0.11)	(3.96±0.16)	(4.25±0.18)

ND = Not determined

TABLE 6.6 Biovolume to biomass conversion factors (g cm^{-3}) for fungal isolates grown on submerged leaf litter for 7 or 14 days. Values are the means of 3 replicates with standard errors.

FUNGAL ISOLATE	AGE days	ALDER	WILLOW	ASH	OAK
Anguillospora	7	0.41±0.11	0.27±0.05	0.19±0.05	0.23±0.07
longissima	14	0.34±0.04	0.46±0.06	0.24±0.06	0.29±0.01
Tetracladium	7	0.20±0.05	0.12±0.05	ND	ND
marchalianum	14	ND	0.19±0.04	ND	ND
Articulospora	7	0.12±0.02	0.23±0.02	ND	ND
tetracladia	14	0.48±0.06	0.62±0.06	ND	ND
Phoma sp	7	0.82±0.05	0.50±0.05	ND	ND
	14	0.88±0.04	0.62±0.06	ND	ND

ND = Not determined

leaf types irrespective of age or species, the mean biovolume to biomass conversion factor was 0.38 ± 0.03 (n = 57). The conversion factor for all fungi grown on alder was slightly higher (0.46 ± 0.06, n = 21) than for fungi grown on willow (0.38 ± 0.04, n = 24). Conversion factors for leaf litter are considerably higher than those determined for fungi on alder (0.25 ± 0.05, n = 10) and willow (0.25 ± 0.04, n = 10) leaf extract agars (Table 6.2).

Table 6.7 shows hyphal diameters of fungi grown on the four leaf types. Mean hyphal diameter ranged from 2.05 µm ± 0.33 for Anguillospora longissima grown on alder to 5.50 µm ± 0.45 for Phoma sp. also on alder. Overall mean hyphal diameter was 3.29 µm ± 0.08 (n = 200). On alder hyphal diameter was slightly higher (3.48 µm ± 0.15, n = 80) than on willow (3.28 µm ± 0.11, n = 80) although there was no significant difference ($P > 0.05$) between hyphal diameters on any of the leaf types, irrespective of mycelial age. This contrasts with the hyphal diameters of fungi grown on agar media. Individual fungi did show changes in hyphal diameter with mycelial age. With Anguillospora longissima, Tetracladium marchalianum and Phoma sp. there was a decrease in hyphal diameter with mycelial age, although this was only significant for Anguillospora longissima ($P < 0.05$) grown on alder. In contrast Articulospora tetracladia showed a significant increase ($P < 0.05$) in hyphal diameter with mycelial age but only when grown on alder.

Correlation between mean hyphal diameter and mean biovolume to biomass conversion factor of fungi grown on leaf material ($r = 0.73$, $n = 19$) was highly significant ($P < 0.001$) which is in contrast to that obtained with cultures grown on agar media ($r = -0.034$, $n = 56$, $P > 0.05$). Combining data from 14 day old leaf and agar grown cultures of Anguillospora longissima and Phoma sp. there was a significant, although lower, correlation ($r = 0.66$, $n = 14$, $P < 0.01$) between hyphal diameter and biovolume to biomass conversion factor.

TABLE 6.7 Hyphal diameters (µm) for fungal isolates grown on submerged leaf litter for 7, 14 or 21 days. Values shown are the means of 10 measurements together with standard errors.

FUNGAL ISOLATE	AGE days	ALDER	WILLOW	ASH	OAK
Anquillospora longissima	7	3.07±0.20	2.76±0.23	2.78±0.12	3.40±0.25
	14	2.73±0.13	2.53±0.17	3.08±0.13	2.50±0.21
	21	2.05±0.33	ND	ND	ND
Tetracladium marchalianum	7	3.01±0.19	2.89±0.11	ND	ND
	14	2.98±0.15	2.95±0.18	ND	ND
	21	2.43±0.62	ND	ND	ND
Articulospora tetracladia	7	2.39±0.14	2.90±0.21	ND	ND
	14	3.34±0.13	3.51±0.17	ND	ND
Phoma sp	7	5.50±0.45	5.13±0.27	ND	ND
	14	4.19±0.30	3.56±0.31	ND	ND

ND = Not determined

6.3.2 Conversion factors for ergosterol

Table 6.8 shows the ergosterol levels in 14 day old mycelium of fungi grown on five agar media. Analysis of variance using the data for Tetracladium marchalianum, Anguillospora longissima and Fusarium sp. demonstrated that there were significant differences in ergosterol levels in mycelium both between fungal species ($P < 0.001$) and between different growth media ($P < 0.05$). The interaction between fungal species and growth medium was also significant ($P < 0.05$). The ergosterol levels ranged from 0.83 to 7.48 mg g^{-1} with a mean value of 3.55 ± 0.28 ($n = 39$). Levels of ergosterol were generally lower in mycelium grown on leaf extract media than in mycelium grown on the nutrient rich media (malt extract agar and potato dextrose agar). Levels of ergosterol in the mycelium of Anguillospora longissima were consistently higher than those in other species grown on the same media. On alder and willow leaf extract agar, levels of ergosterol in Anguillospora longissima mycelium were as high or higher than in other species even when they were grown on nutrient rich media.

Correlations between the ergosterol levels in mycelium grown on different agar media and the levels of ATP ($r = 0.57$, $n = 8$), percentage carbon content ($r = 0.14$, $n = 11$), percentage nitrogen content ($r = 0.39$, $n = 8$), carbon to nitrogen ratio of mycelium ($r = -0.34$, $n = 8$), hyphal diameter ($r = -0.42$, $n = 18$) and biovolume to biomass conversion factor ($r = -0.42$, $n = 18$) were all non-significant ($P > 0.05$).

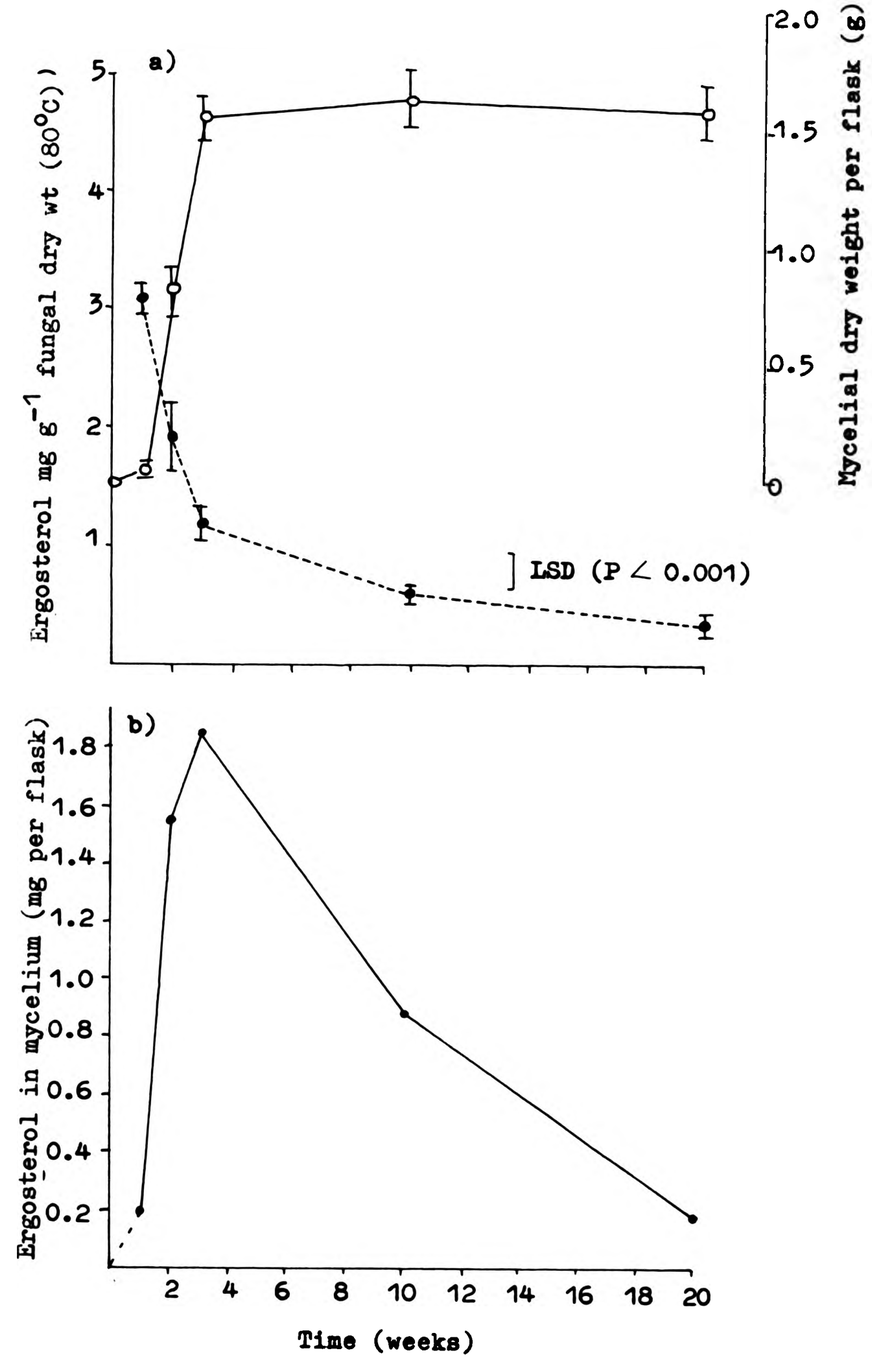
Figure 6.1a shows the change in mycelial biomass and ergosterol level in cultures of Phoma sp. grown for 140 days at 12 ^{o}C in static liquid cultures. During the incubation period there was a highly significant decrease ($P < 0.001$) in the level of ergosterol in mycelium with levels dropping significantly during the first 10 weeks. After 20 weeks the ergosterol level in mycelium was approximately 10 times lower than in the first week of incubation,

TABLE 6.8 Ergosterol levels (mg g^{-1} dry weight) in mycelium of fungal isolates grown on 5 agar media for 14 days at 12°C. Each value is the mean of at least 2 replicate determinations. Average coefficient of variation 16%.

FUNGAL ISOLATE	MALT EXTRACT AGAR	POTATO DEXTROSE AGAR	CORNMEAL AGAR	ALDER LEAF AGAR	WILLOW LEAF AGAR
Tetracladium marchalianum	2.36	2.22	3.53	2.87	1.44
Anquillospora longissima	7.41	6.14	5.90	5.43	4.83
Fusarium sp	5.08	2.99	2.09	1.30	1.04
Phoma sp	3.14	ND	ND	ND	ND
Tetrachaetum elegans	3.72	ND	ND	ND	ND
Clavariopsis aquatica	3.57	ND	3.25	ND	ND
Articulospora tetracladia	ND	ND	3.10	ND	ND

ND = Not determined

Figure 6.1. Changes in ergosterol in Phoma sp. grown in malt extract agar broth for 20 weeks at 12 °C; a) shows changes in fungal biomass o——o, and changes in ergosterol content per unit dry weight of mycelium ●------●
b) shows mycelial ergosterol content per flask. Values shown are means of 3 sets of replicate flasks, standard errors are shown in a).



whereas mycelial biomass had not changed since the third week of incubation. During the first three weeks of incubation, ergosterol per flask (Figure 6.1b) increased together with mycelial biomass (Figure 6.1a) and it was only after mycelium had reached the stationary phase of growth that the level of ergosterol per flask began to fall.

Table 6.9 compares the ergosterol levels in mycelium of Phoma sp. grown in liquid medium and on agar for 14 or 140 days. Under both growth conditions there was a significant ($P < 0.001$) decrease in ergosterol with increasing incubation time. Mycelium grown for 14 days on agar had significantly ($P < 0.05$) higher levels of ergosterol than in 14 day liquid cultures, whereas differences in ergosterol levels between growth conditions in 140 day cultures was not significant ($P > 0.05$).

Table 6.10 shows ergosterol levels in 140 day cultures of 6 fungal species grown in static cultures at 12 ^{o}C. In all cases the levels of ergosterol were low (less than 0.5 mg g^{-1} dry weight) compared to 14 day old cultures of several of these species on agar media (Table 6.8).

6.3.3 Elemental content of mycelium grown under different conditions

Figure 6.2a-b shows SEM photomicrographs of oven dried mycelium of Phoma sp. grown on a) submerged alder leaves and b) submerged willow leaves. The mycelium of both samples appeared to be uniform with little contamination by particulate leaf material. This suggested that X-ray microprobe analysis of samples would be representative of the fungal material obtained from these sources. Figure 6.3 shows examples of X-ray spectra obtained from microprobe analysis of mycelial samples of Anguillospora longissima grown on a) alder leaves and b) willow leaves for 14 days in liquid culture, photograph c) is included to aid identification of different elemental

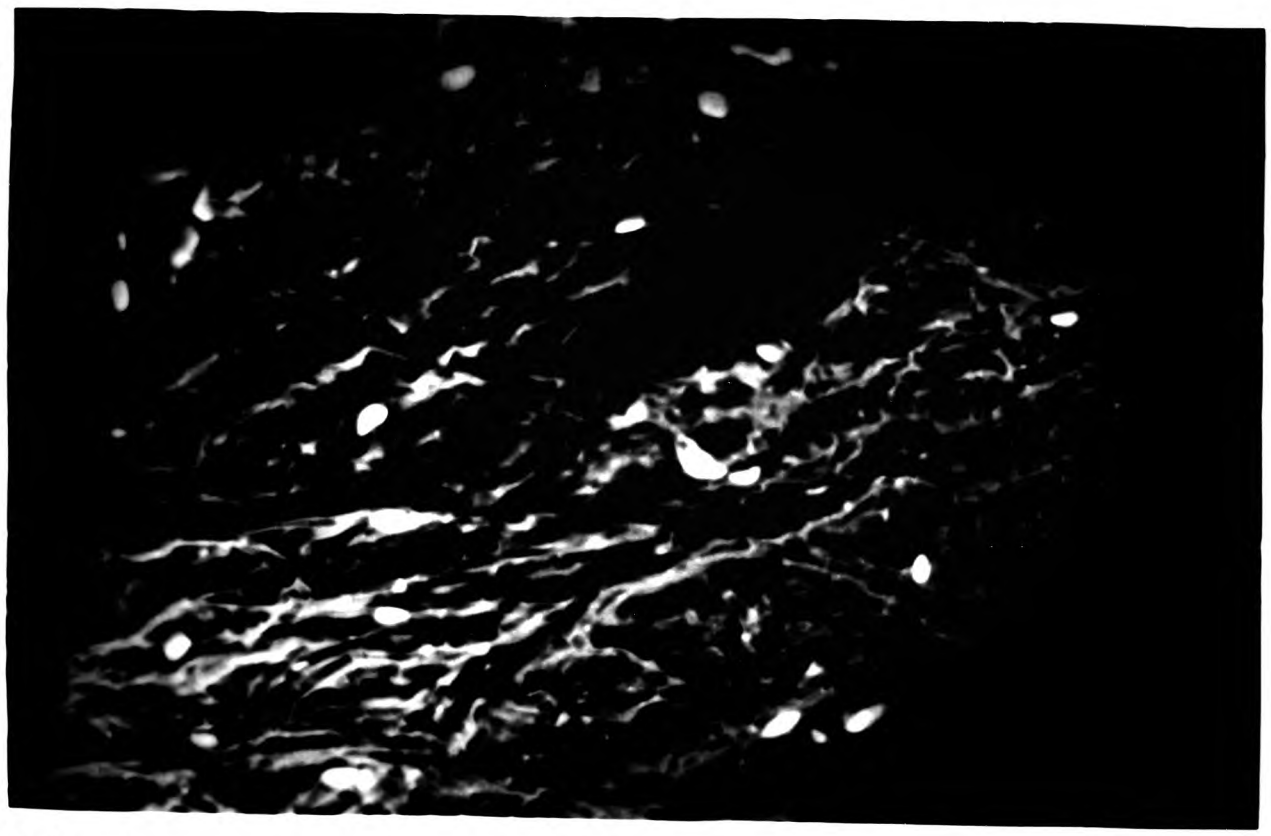
TABLE 6.9 Comparison between the ergosterol levels (mg g^{-1} dry weight) in mycelium of Phoma sp grown on malt extract agar or in malt extract broth for 14 days or 140 days. The values shown are the means of 3 replicate determinations with standard errors.

GROWTH MEDIUM	CULTURE AGE (days)	
	14	140
Malt extract agar	3.14±0.20	0.18±0.06
Malt extract broth	1.91±0.26	0.32±0.10

TABLE 6.10 Ergosterol levels (mg g^{-1}) in mycelium of 6 fungal species after 140 days in malt extract broth at 12°C. Values are the means of 3 replicate flasks together with standard errors.

FUNGAL SPECIES	ERGOSTEROL (mg g^{-1} dry weight)
Tetracladium marchalianum	0.49±0.03
Anguillospora longissima	0.21±0.06
Tricladium splendens	0.31±0.10
Articulospora tetracladia	0.38±0.08
Clavariopsis aquatica	0.36±0.11
Phoma sp	0.33±0.10

a)



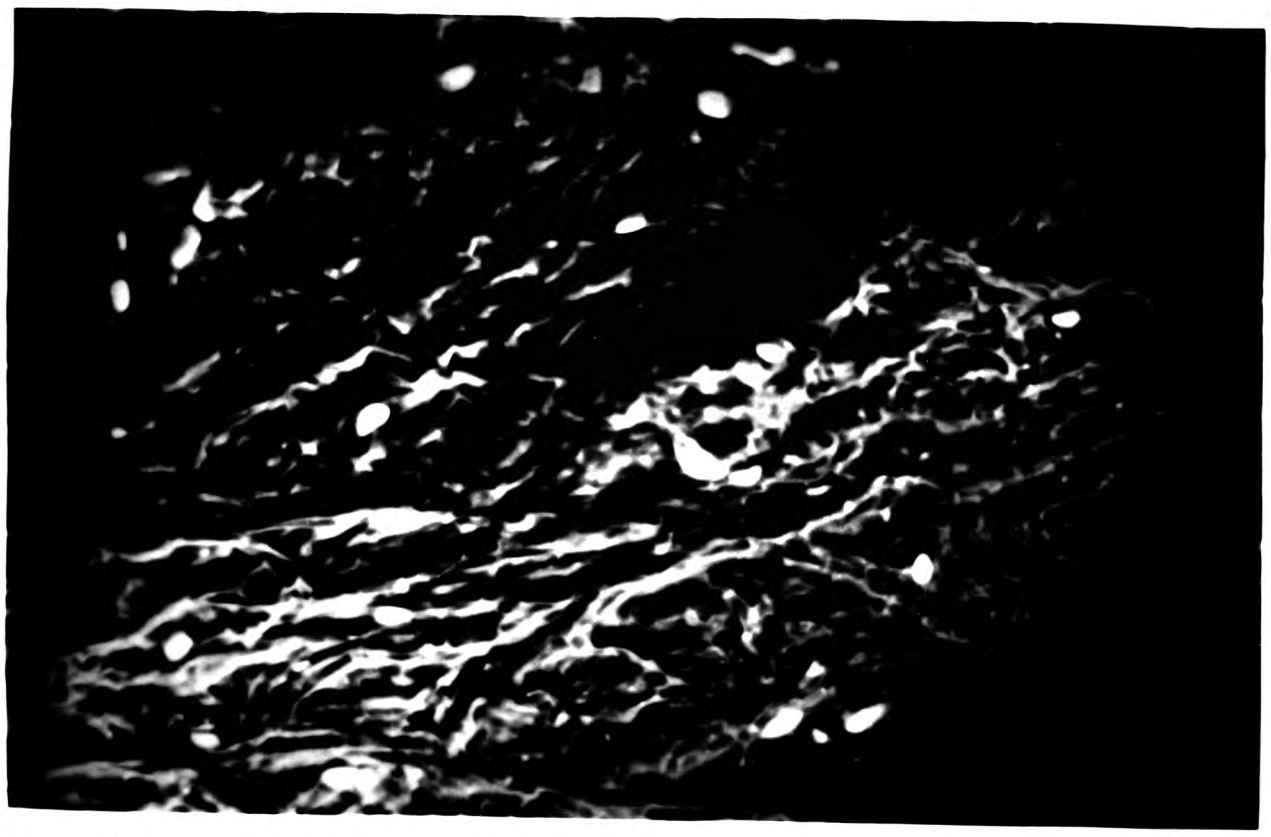
10 um

b)

10 um

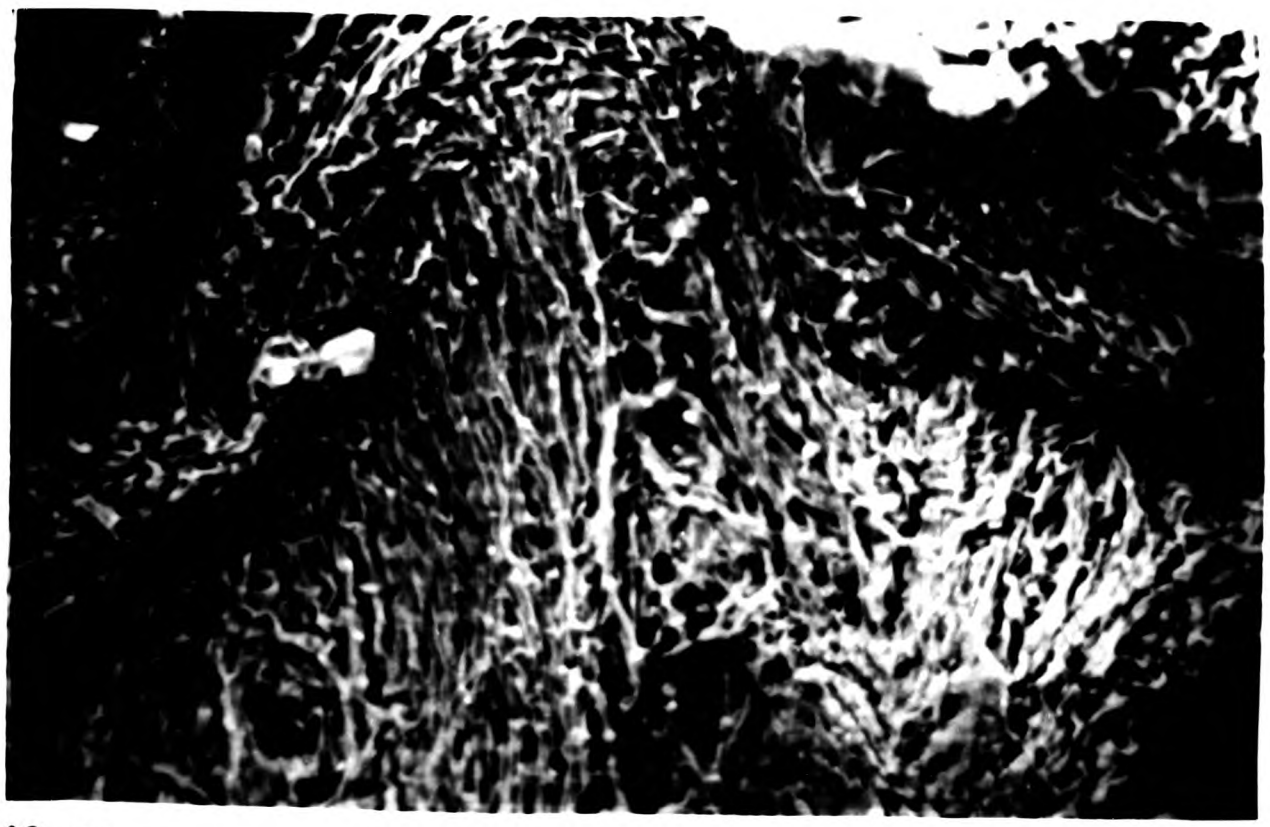
Figure 6.2. SEM photographs of carbon coated oven dried mycelium of Phoma sp. grown for 14 days on a) alder leaves and b) willow leaves.

a)



10 um

b)



10 um

Figure 6.2. SEM photographs of carbon coated oven dried mycelium of Phoma sp. grown for 14 days on a) alder leaves and b) willow leaves.

a) b) c)

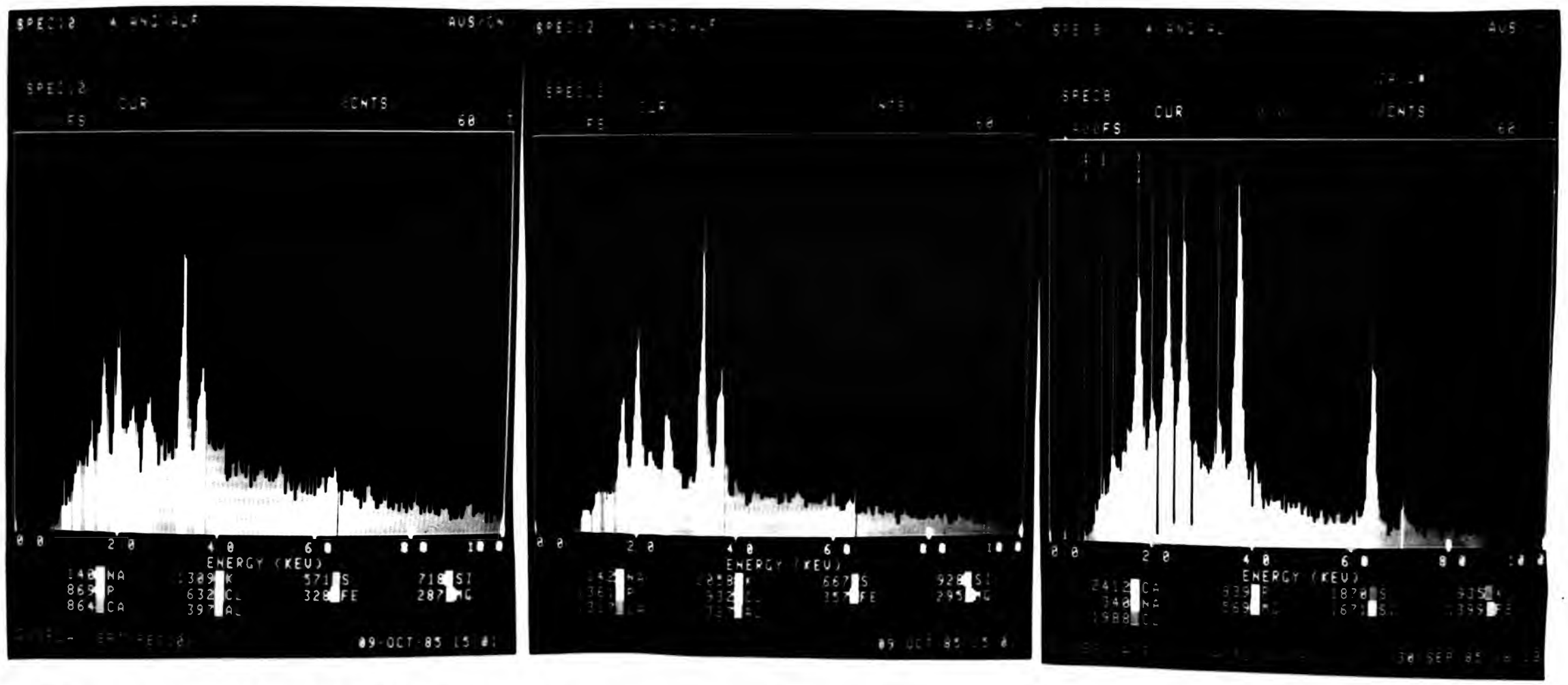
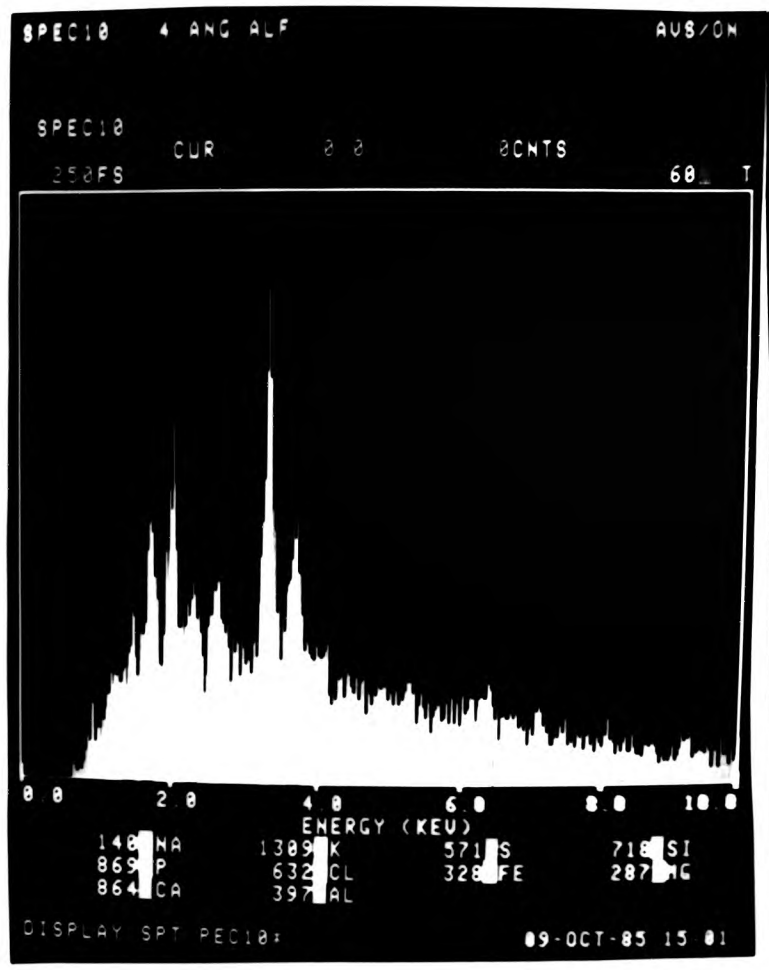
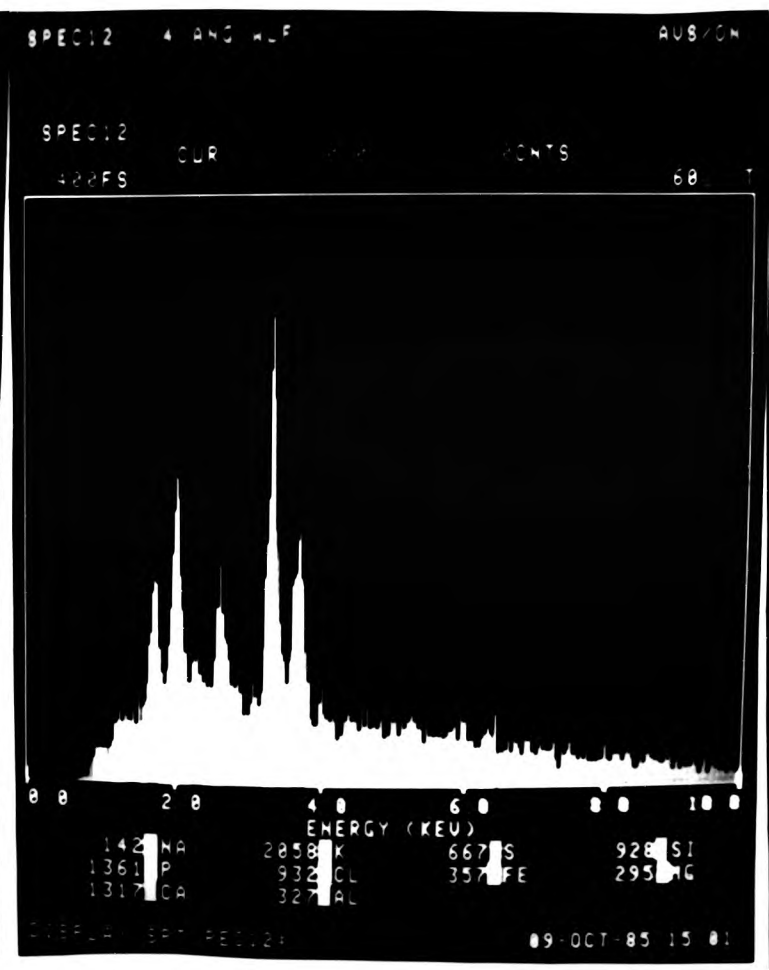


Figure 6.3. X-ray spectra showing the elemental composition of <u>Anguillospora longissima</u> grown on a) alder and b) willow leaves for 14 days in submerged cultures; c) shows an X-ray spectrum with relative positions of different elements shown as lines).

a)



b)



c)

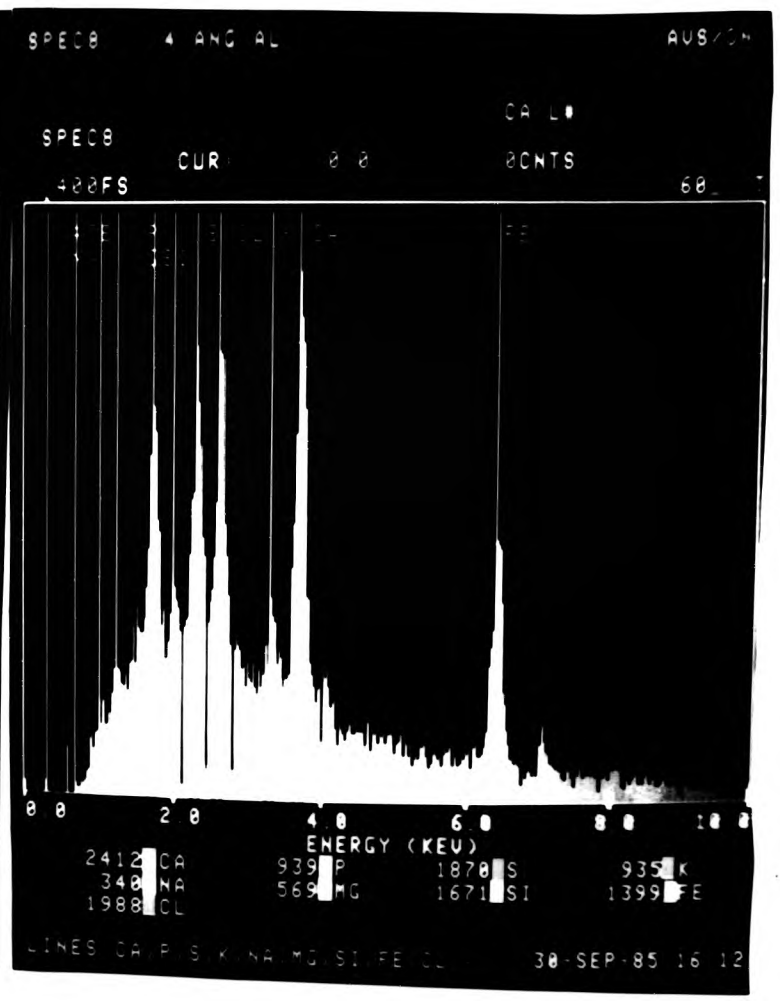


Figure 6.3. X-ray spectra showing the elemental composition of Anguillospora longissima grown on a) alder and b) willow leaves for 14 days in submerged cultures; c) shows an X-ray spectrum with relative positions of different elements shown as lines).

peaks. The spectra are essentially similar for the two growth conditions with the elements potassium, phosphorus and calcium dominating the spectra. By expressing the X-ray count for each element as a proportion of the total X-ray count for the 9 elements studied, any changes in the relative elemental composition of mycelium could be detected and attempts made to relate changes to conversion factors for biomass.

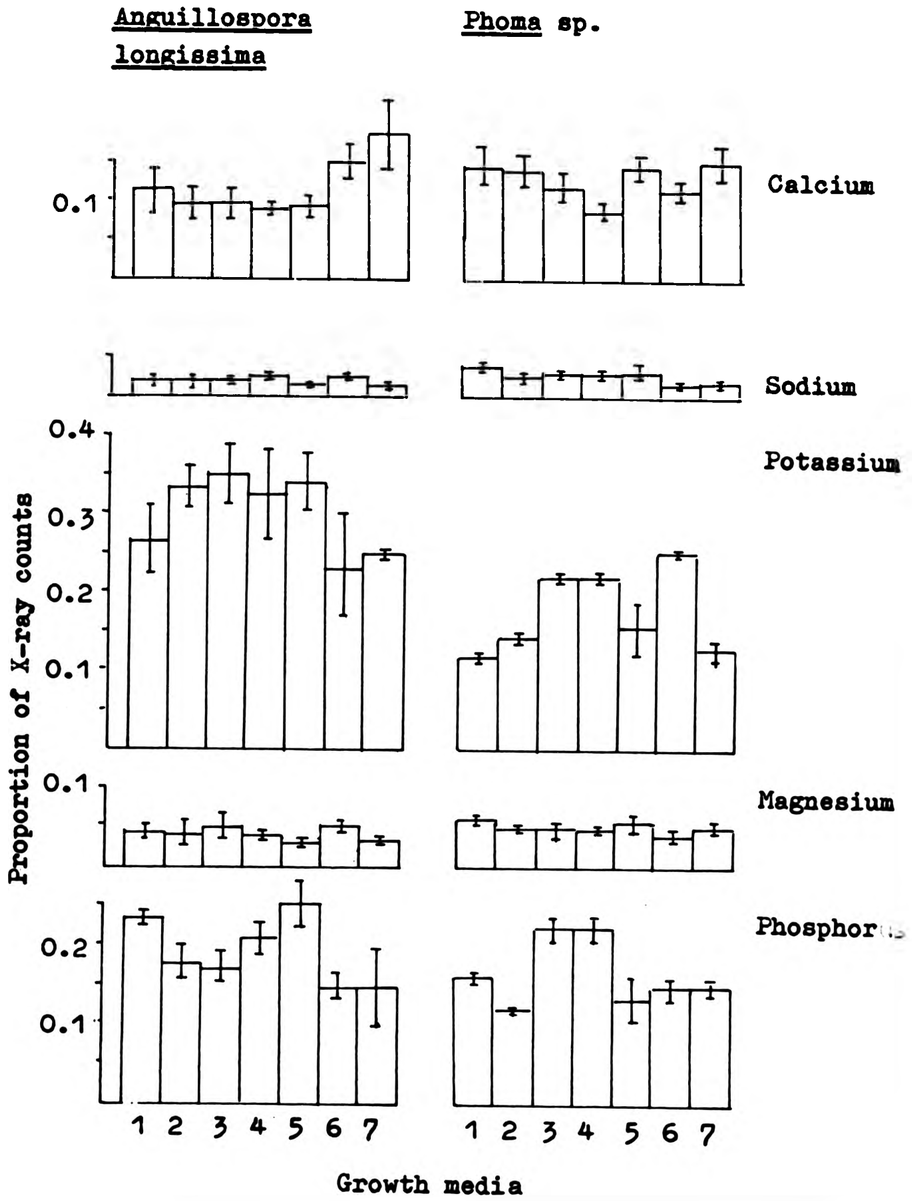
Figure 6.4 shows the results of microprobe analysis of mycelium of Anguillospora longissima and Phoma sp. grown on 5 agar media and two types of leaf litter for 14 days. There were marked differences in the relative proportions of different elements both between different growth conditions and between fungal species. In the sequence of growth conditions (left to right for each element on Figure 6.4) both fungal species showed a slight decrease in the proportion of sulphur. Anguillospora longissima showed a decrease in potassium and increases in calcium, iron and chlorine. Phoma sp. showed decreases in sodium and chlorine. The two species appeared to differ markedly in the relative proportion of different elements in their mycelium. The proportion of potassium was markedly higher in Anguillospora longissima whereas Phoma sp. had higher levels of chlorine, sulphur, magnesium, sodium, silicon and calcium. Correlations between the proportions of different elements in each fungus showed significant negative correlations between the elements calcium and potassium (Phoma sp. $r = 0.82$, $P < 0.05$; Anguillospora longissima $r = -0.81$, $P < 0.05$)

Comparisons were also performed between biovolume to biomass conversion factors, hyphal diameters, mycelial moisture contents and the relative proportion of different elements in mycelium under different growth conditions. For Anguillospora longissima there were significant correlations between the biovolume conversion factor and calcium ($r = 0.98$, $P < 0.001$) and potassium ($r = -0.80$, $P < 0.05$). With Phoma sp. there were significant correl-

Figure 6.4. Relative proportions of selected elements in the mycelium of Anguillospora longissima and Phoma sp. grown on five agar media and two types of leaf litter for 14 days. The proportions shown for each element are the means and standard deviations for 3 samples of mycelium.

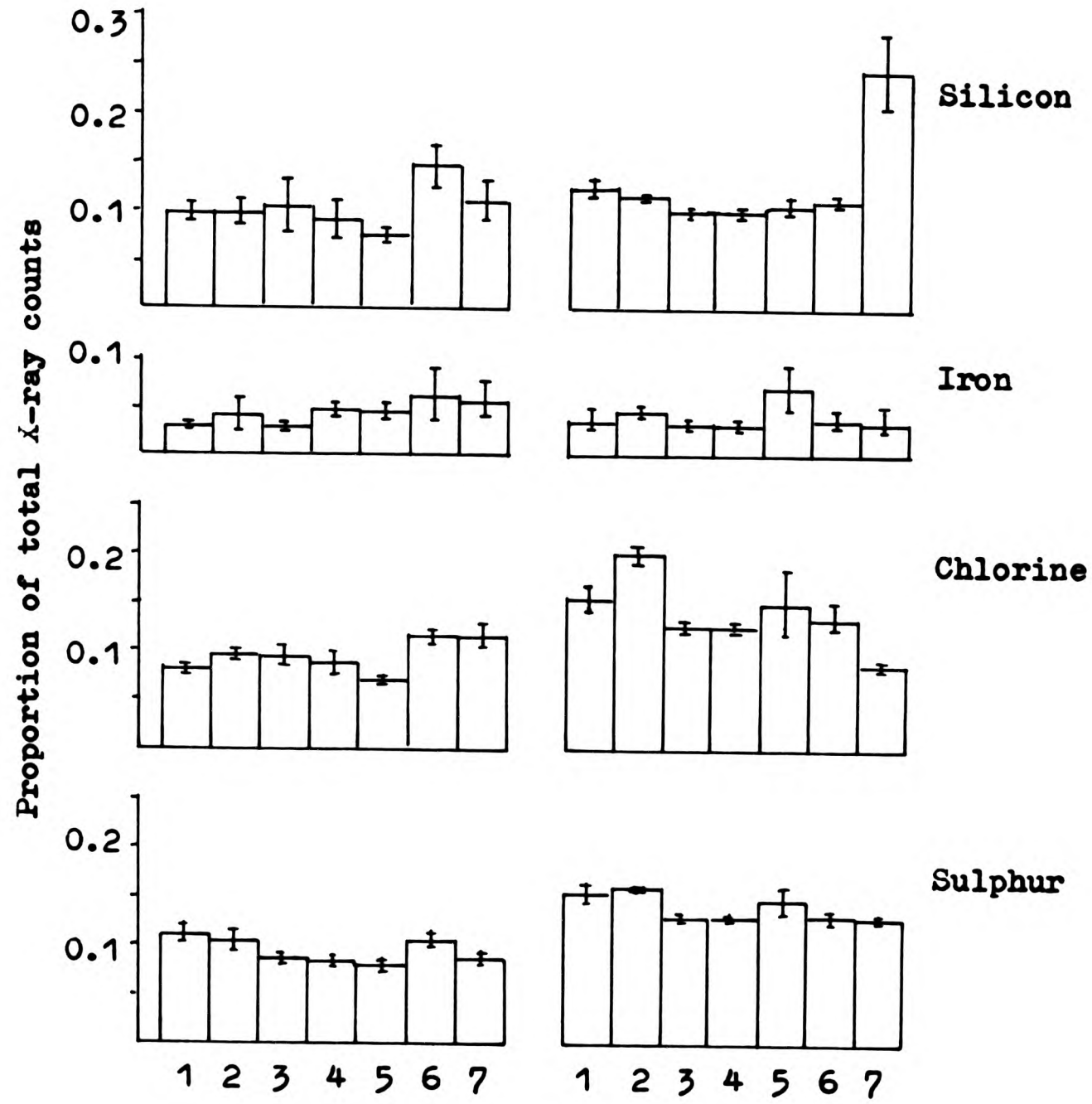
KEY:

1 Malt extract agar
2 Potato dextrose agar
3 Corn meal agar
4 Alder leaf agar
5 Willow leaf agar
6 Alder leaves
7 Willow leaves



Anguillospora longissima

Phoma sp.



a) <u>Anguillospora longissima</u>

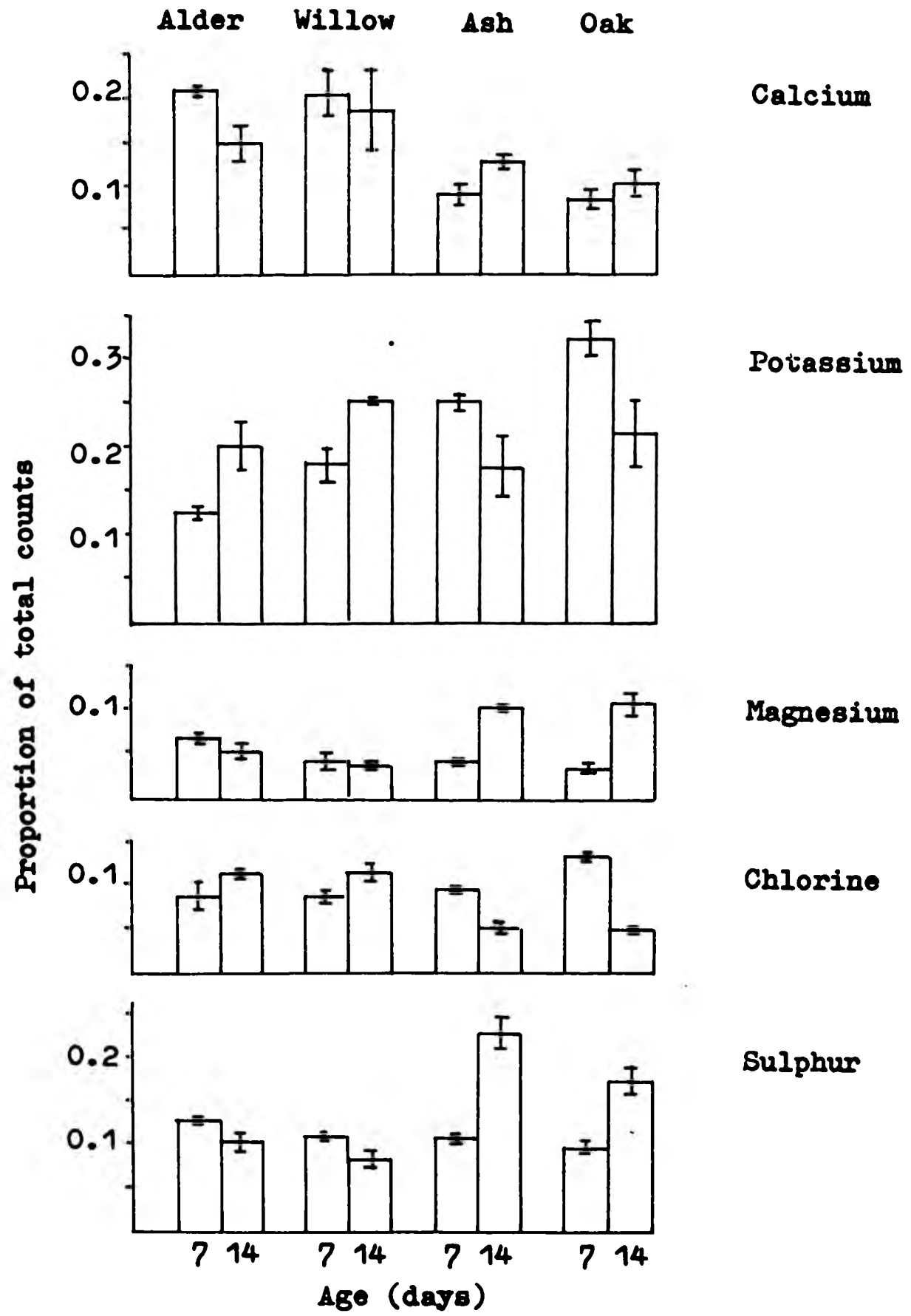
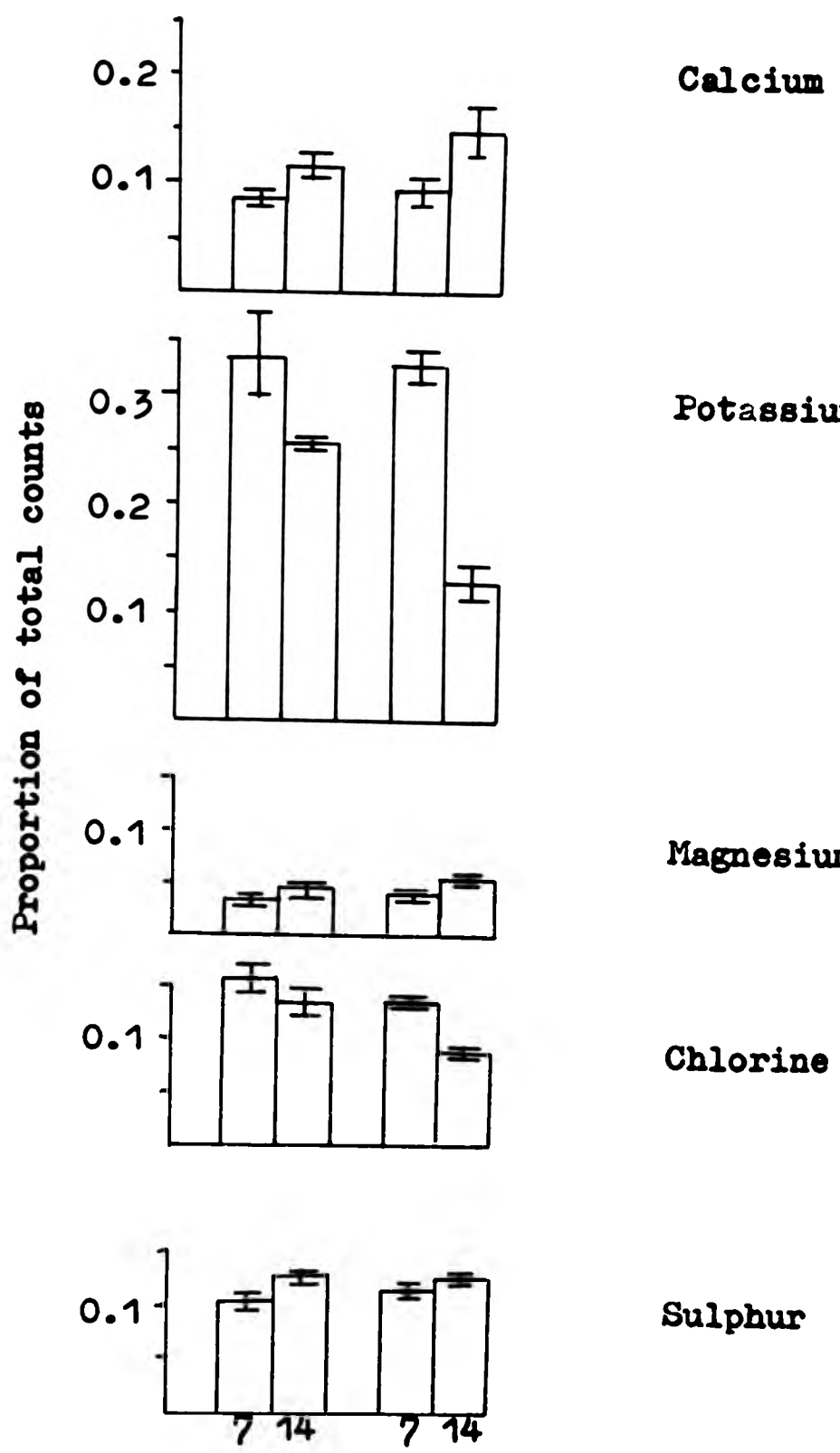


Figure 6.5. Relative proportion of selected elements in the mycelium of a) <u>Anguillospora longissima</u> and b) <u>Phoma</u> sp. (overleaf) grown on different types of leaf litter for 7 or 14 days. The proportions shown are the means and standard deviations for 3 samples of mycelium.

b) <u>Phoma</u> sp.


Alder
Willow
Calcium
0.2
0.1
Potassium
0.3
0.2
0.1
Proportion of total counts
Magnesium
0.1
Chlorine
0.1
Sulphur
0.1
7
14
7
14
Age (days)

ations between the biovolume conversion factors and sodium ($r = -0.80$, $P < 0.05$) and between hyphal diameter and calcium ($r = -0.81$, $P < 0.05$), magnesium ($r = -0.79$, $P < 0.05$) and potassium ($r = 0.80$, $P < 0.05$).

Figure 6.5a-b shows the relative proportions of elements in mycelium of Anguillospora longissima and Phoma sp. grown for 7 or 14 days on different types of leaf litter. For each fungal species there were marked differences in the proportions of elements present in the mycelium. With Anguillospora longissima there was a decrease in the proportions of calcium, magnesium and sulphur between 7 and 14 days when grown on both alder and willow, in contrast to the proportions of potassium and chlorine which increased. On ash and oak this trend was completely reversed (Figure 6.5a). With Phoma sp. grown on alder and willow there was an increase in the relative proportions of calcium, magnesium and sulphur and a decrease in potassium and chlorine between 7 and 14 days. These results suggest that changes in the relative proportions of different elements with mycelial age appear to be dependant on both the fungal species and the leaf type on which it is growing. The shift in the proportions of elements in 7 and 14 day old mycelium of Phoma sp. on alder and willow is similar to that in the mycelium of Anguillospora longissima on ash and oak, but is completely opposite to the shift on alder and willow for Anguillospora longissima.

6.4 Discussion

Many workers using direct methods to estimate fungal biomass have used conversion factors based on determinations of moisture content and specific gravity. Published figures for specific gravities of fungal mycelia range from 1.0-1.5 with a mean value of 1.2 (Saitô, 1955; Parkinson, Gray & Williams, 1971; Warnock, 1971; Faegri, Torsvik & Goksoyr, 1977). Specific gravities of fungal mycelia are difficult to estimate and consequently many

authors have assumed previous values together with approximate estimates of mycelial dry weight (Frankland, Lindley & Swift, 1978; Bååth & Söderström, 1979a). The conversion factors obtained in the present work ranged from 0.08 to 0.89 g cm^{-3}, with a mean and standard error of 0.29 ± 0.02 (n = 56) for fungi grown on agar, and 0.38 ± 0.03 (n = 57) for fungi grown on leaf litter. Use of these values would result in biomass estimates between 29 and 69% higher than in previously published estimates using 0.22 (1.1 specific gravity; dry weight 20% of wet weight) as a conversion factor.

Few studies have examined conversion factors with different growth conditions and mycelial ages. Newell & Statzell-Tallman (1982) and Hicks & Newell (1984) found wide variation in biovolume to biomass conversion factors, both between fungal strains and between growth conditions. A Fusarium sp. and Phaeosphaeria typharum (Desm.) Holm both showed an increase in conversion factors with mycelial age, in contrast to Buergenerula spartinae Kolm et Gessner which showed a decrease. In the present study there was also considerable variation between fungal species and between fungi grown under different growth conditions. As in the previously mentioned studies, no clear pattern emerged between conversion factors and growth conditions, mycelial ages, moisture contents or hyphal diameters.

Analysis of the elemental composition of mycelium grown under different conditions did reveal some differences between fungi in the relative proportions of elements. Significant correlations were found with biovolume conversion factors, but there was no evidence to suggest that a particular element was suitable for predicting conversion factors in mixed fungal populations.

Many factors may be responsible for variation in the dry weight of a unit of mycelium and it is unlikely that a single variable (hyphal diameter, levels of a particular element) could be used to predict conversion factors particularly in environmental samples. Given uniformity

in cell wall thickness, it would be logical that thinner hyphae should be more dense because there would be a greater proportion of dry wall material relative to biovolume. Hyphal diameters were narrower and biovolume conversion factors higher on nutrient rich agar media than on leaf extract agars (Table 6.2), but on submerged leaves this trend was reversed. In both the present study, and that of Newell & Statzell-Tallman (1982), there were problems in preparing mycelium for biovolume estimation. Mycelium grown on agar is tightly packed, particularly on nutrient rich media, and this may influence the efficiency of homogenisation. With submerged leaf material, mycelium may be contaminated with microparticulate organic debris which would interfere with the weight of mycelium per unit volume.

Many parameters are likely to affect biovolume conversion factors and more studies are needed, in particular, variation in hyphal wall thickness under different growth conditions and the proportion of mycelium occupied by protoplasm.

Conversion factors for ergosterol levels to mycelial biomass also demonstrated appreciable variation. Significant differences were found in ergosterol levels, both between different fungal species and between fungi grown under different nutrient conditions. These results are in agreement with the limited number of studies where authors have investigated differences in ergosterol in fungi grown under different conditions (Woods, 1971; Seitz et al., 1979; Nout et al., 1986)

Measurement of ergosterol in mycelium of Phoma sp. during different stages of growth in static liquid culture suggested that there was a decline in ergosterol once growth had reached the stationary phase. Previous workers (Seitz et al., 1979; Matcham, Jordan & Wood, 1985; Nout, et al., 1986) have examined the relationship between ergosterol levels and biomass of fungi. Generally there was good agreement between ergosterol levels and biomass although studies were usually conducted during the active

growth phase of the mycelium. In the present study ergosterol levels in flasks increased with biomass during the active growth phase, but decreased dramatically once the stationary phase had been reached (Figure 6.1a-b). Low levels of ergosterol (less than 0.5 mg g^{-1}) were also found in a range of other fungi after 140 days in liquid culture and a fall in the level of ergosterol was observed with cultures of Phoma sp. grown on agar. These results suggest that ergosterol is associated with the living component of mycelium, since 140 day cultures would probably contain mostly dead or senescent mycelium, in contrast to the young actively growing mycelium present in the early stages of growth. Sterols are mostly found in the cytoplasmic membrane of eukaryotes where they interact with other lipids and proteins (Poralla & Kannenberg, 1987) and ergosterol is known to be associated with membranes in fungi (Weete, 1973; Griffin, 1982). It is not known how long the cytoplasmic membrane will persist after hyphae become inactive or die, but the data of Miller, Young & Trenholm (1973) examining ergosterol and viable plate counts in cereal grain, suggested that ergosterol levels remain constant even after a loss of viability. If ergosterol is measuring predominantly living and recently dead fungal mass then this may explain the results of Lee, Howarth & Howes (1980) who found that ergosterol gave lower estimates of biomass than a direct counting method. However, the differences between the two methods could have been due to the use of inappropriate conversion factors, particularly as Newell, Fallon & Miller (1986) found ergosterol to give higher estimates than a direct counting method.

The ergosterol levels measured in mycelium in the present study ranged from 0.04-7.6 mg g^{-1} with a mean and standard error of 2.19 $\pm$ 0.27 (n =62). Table 6.11 gives examples from the literature of ergosterol levels in a range of fungi grown under different conditions. Clearly there is appreciable variation in ergosterol content both between different fungi and between growth conditions for

TABLE 6.11 Comparison between the levels of ergosterol reported for a range of different fungi. Values represent the range of ergosterol levels (mg g^{-1}) found in each study. Single values represent only one determination.

SPECIES	ERGOSTEROL (mg g^{-1})	REFERENCE
Alternaria alternata	3.8-4.4	1
Aspergillus flavus	2.3-3.3	1
Aspergillus amstelodami	4.9-5.9	1
Fusarium solani	1.7-2.4	2
Drechslera spp.	0.4-1.0	3
Claviceps purpurea(sclerotia)	0.5-0.7	4
Saccharomyces cerevisiae	9.8-13.3	5
Agaricus bisporus	2.0-2.7	6
Volvariella volvacea	2.5-6.3	7
Lentinus edodes	0.6-2.7	7
Auricularia auricula	0.7	7
Rhizopus oligosporus	2.0-24.0	8
Rhizopus arrhizus	1.1	9
Monodictys pelagica	3.9	10
Spaerulina oraemaris	5.0	10
Leptosphaeria oraemaris	3.8	10
Dendryphiella salina	3.8	10
Various fungi	0.04-7.6	Present study

1) Seitz et al, 1979; 2) Kok, Norris and Chu, 1970; 3) Gordon and Webster, 1985; 4) Seitz et al, 1983 (cited by Nout et al. 1986); 5) Woods, 1971; 6) Matcham, Jordan and Wood, 1985; 7) Huang, Yung and Chang, 1985; 8) Nout et al, 1986; 9) Weete, Lawler and Laseter, 1973; 10) Miller et al, 1984.

the same fungus and this variability cannot be disregarded if fungal biomass is to be accurately estimated in field samples. Use of the range of values encountered in the present study would give ergosterol to biomass conversions of between 132 and 25 000.

Attempts to use glucosamine to estimate fungal biomass has resulted in similar problems of choosing appropriate conversion factors (Swift, 1973a; Whipps & Lewis, 1980; whipps et al., 1982 & 1985; Hicks & Newell, 1984). Hicks & Newell (1984) demonstrated that by using the range of conversion factors for glucosamine from the literature, fungal biomass in Spartina could account for between 2.6 and 256% of leaf dry weight. These are extreme values but there is obviously a need for better ways of predicting conversion factors for chemical markers, particularly under field conditions.

Mycelium growing in organic substrates in laboratory microcosms or under field conditions is likely to represent a range of different age classes for each particular fungal species, but it is not usually possible to make a visual distinction between ages. In situations where mycelium is grazed by invertebrates it may be maintained in a young state by continual cropping, or alternatively younger mycelium may be selectively grazed leaving an accumulation of older mycelium. These and other factors (eg. microbe-microbe interactions) are difficult to take into account when trying to determine realistic conversion factors because of the difficulty of measuring any effects under pure culture conditions. An additional problem is that conversion factors for surface mycelium may be different from mycelium within the leaf (Swift, 1973b) yet, most field studies will want to include both surface and subsurface mycelium and other structures (eg. pycnidia) in estimates of biomass.

Studies on fungal biomass should attempt to use a sliding scale of values for conversion factors depending on the fungal species present, nutrient status and mycelial age. In many field situations the most abundant

and 'active' fungal species can be isolated and identified, and studies in laboratory systems can provide some information on the influence of nutrient conditions on conversion factors under different growth conditions. The most important parameter to measure in field and microcosm experiments would seem to be mycelial age since this appears to have a major influence on conversion factors, particularly for chemical components (Sharma, Fisher & Webster, 1977; Whipps *et al.*, 1982; Newell & Fallon, 1983; Nicks & Newell, 1984; Whipps *et al.*, 1985).

Particular elements, ratios or organic components which can be measured in individual hyphae on the leaf surface may be related to mycelial age. Microprobe analysis was shown in this work to detect differences in the elemental composition of mycelium grown on submerged leaf litter of different types, and a more detailed study might reveal particular trends with mycelial age. Ways of obtaining organic and inorganic spectra of individual bacterial cells have recently been developed (Seydel *et al.*, 1982; Lindner & Seydel, 1983 & 1984; Heldal, Norland & Tumyr, 1985), and it may be possible to apply these methods to obtain fingerprints of fungi on the leaf surface. Analysis of such data could indicate, not only the types of fungi present, but also the age and nutritional status of fungal hyphae. Accurate conversion factors are essential for estimation of fungal biomass in environmental samples, and future studies should endeavour to find suitable ones which are representative of the fungi and the conditions under which they are growing.

CHAPTER 7. THE CONTRIBUTION OF ALLOCHTHONOUS PLANT LITTER TO THE ORGANIC MATERIAL IN A FLOODED GRAVEL PIT

7.1 Introduction

A number of studies have shown that leaves and other allochthonous organic matter are the predominant source of energy for unpolluted woodland streams (Kaushik & Hynes, 1968 & 1971; Cummins *et al*., 1973; Cummins, 1974; Fisher & Likens, 1973). Estimates suggest that allochthonous organic matter may represent 40-99% of the energy entering streams in wooded regions, of which 40-50% consists of autumn shed leaves (Nelson & Scott, 1962; Cummins *et al*., 1966; Fisher & Likens, 1973).

In small lakes the allochthonous litter inputs may also be significant, particularly if phytoplankton productivity is low (Hodkinson, 1975a; Gasith & Hasler, 1976; Hanlon, 1981). This contrasts with the situation in large eutrophic lakes where allochthonous inputs are thought to be relatively insignificant in relation to phytoplankton production (Szczepanski, 1965; Pieczynska, 1972).

Many flooded gravel pits are small in size (less than 4 ha, Figure 8.1) and have a large proportion of their banks occupied by trees and shrubs, in pits over 25 years old. Although these lakes show some eutrophic features they are frequently poor in soluble nutrients, PO_4-P, NO_3-N + NO_2-N (Appendix 6, Tables 1-3) and consequently phytoplankton productivity may be low. In such situations, allochthonous inputs may become an important source of organic and inorganic material, contributing significantly to the energy entering these lakes.

In terrestrial ecosystems fungal biomass has been estimated in leaf litter at a number of sites where the annual inputs of plant litter are known (Frankland, 1975b; Flanagan, 1981; Kjoller & Struwe, 1982) but

similar comparisons have not been made in freshwater ecosystems. The present study was undertaken to estimate the allochthonous litter input into a small flooded gravel pit, as a preliminary to estimating the significance of fungal biomass and activity associated with decomposition.

7.2 Materials and methods

7.2.1 Site description

Yateley 4 is a small flooded gravel pit (1.5 ha, depth 1.3 ± 0.2 m) situated in the complex of flooded gravel pits (Yateley-West) on the Hampshire side of the River Blackwater (altitude 55 m O.D., Grid Ref. SU 823 614). (For details of the site and location of the gravel pit complex see Section 8.2 and Figure 8.2). Figure 7.1 shows an outline map of Yateley 4 together with depth contours and profiles at different points around the lake margin. In places, the banks shelve steeply and allochthonous litter fallen or blown into the lake tends to accumulate at the bottom of these slopes about 2-4 m from the bank. This contrasts with many natural or semi-natural lakes where litter often accumulates around the shallow lake margin.

About 79% of the margin of Yateley 4 has trees and shrubs (Figure 7.2) and in many places these overhang the lake as in Figure 8.6. There is a wide diversity of trees and shrubs present including Salix fragilis L., Salix cinerea L., Salix viminalis L., Salix spp., Alnus glutinosa (L.) Gaertner, Quercus sp., Betula sp., Fraxinus excelsior L., Populus sp. and Spiraea salicifolia L. Around the lake margin, emergent vegetation includes Typha latifolia L., Typha angustifolia L., Iris pseudocornis L., Juncus effusus L., Carex pseudocyperus L. Carex vesicaria L., Calamagrostis epigejos (L.) Roth and Equisetum fluviatile L. Two clumps of Nymphaea alba L. occur along the south eastern edge of the lake. During

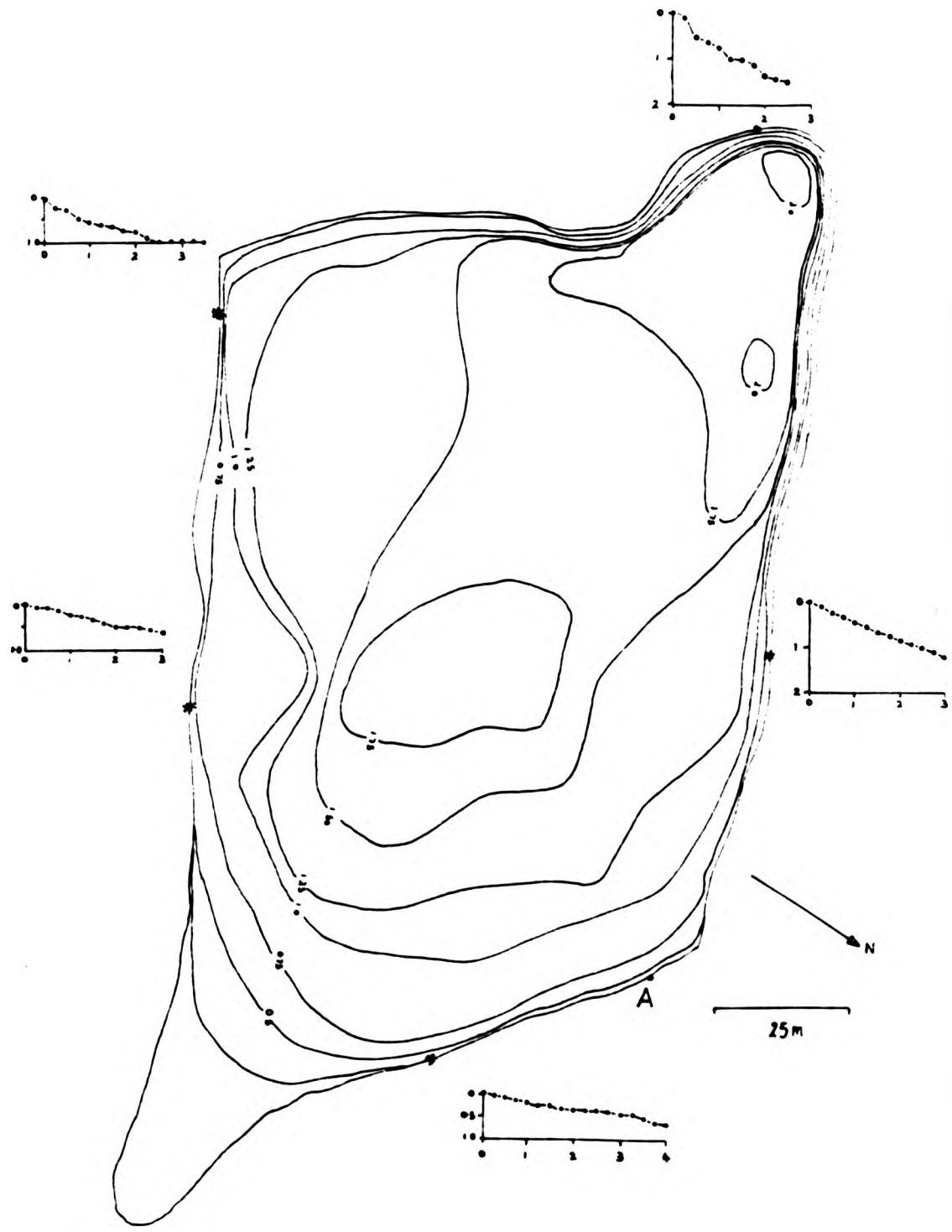


Figure 7.1. Map showing outline of Yateley 4 with contours of water depth (m). Depth profiles are provided for selected points around the lake (*) (y axis is depth in m and x axis is distance from lake edge in m. Contours are based on data provided by Dr M.J. Burgis.

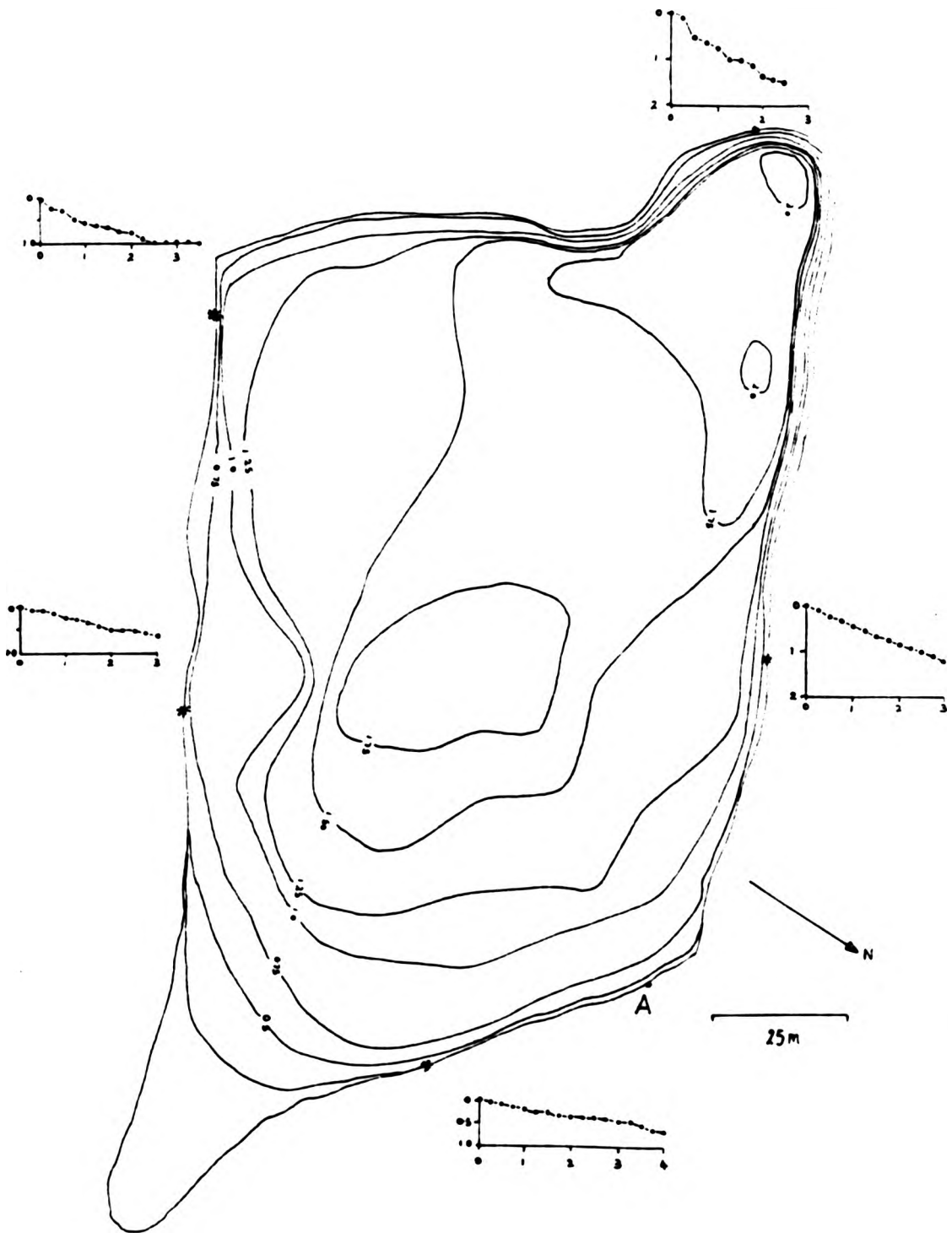


Figure 7.1. Map showing outline of Yateley 4 with contours of water depth (m). Depth profiles are provided for selected points around the lake (*) (y axis is depth in m and x axis is distance from lake edge in m. Contours are based on data provided by Dr M.J. Burgis.

Figure 7.2. View of Yateley 4 showing the dense cover of trees around the lake margin (looking from S.W. to N.E. on Figure 7.1).

Figure 7.3. Photograph showing circular litter traps on Yateley 4 during the autumn of 1983.

Figure 7.2. View of Yateley 4 showing the dense cover of trees around the lake margin (looking from S.W. to N.E. on Figure 7.1).

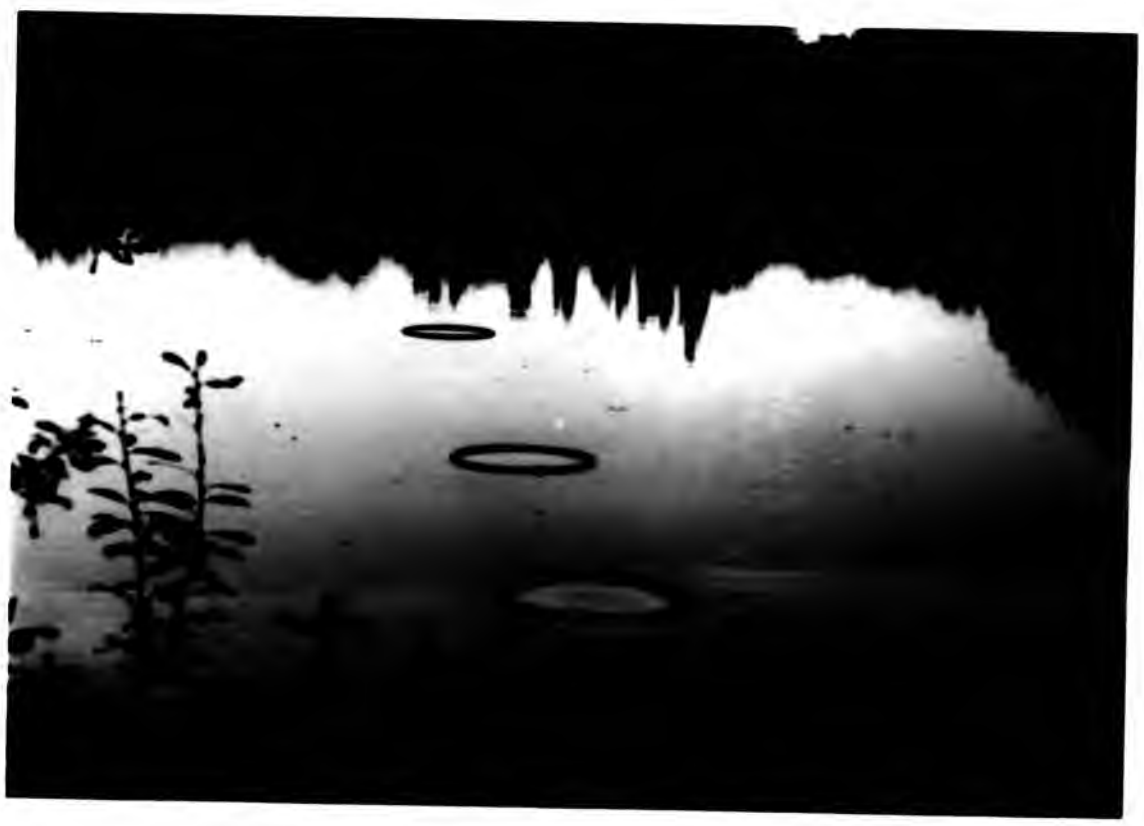


Figure 7.3. Photograph showing circular litter traps on Yateley 4 during the autumn of 1983.

Figure 7.2. View of Yateley 4 showing the dense cover of trees around the lake margin (looking from S.W. to N.E. on Figure 7.1).

Figure 7.3. Photograph showing circular litter traps on Yateley 4 during the autumn of 1983.

the summer months the lake often contains large amounts of submerged macrophytes including Ceratophyllum demersum L., Elodea canadensis Michx., Myriophyllum spicatum L. and, in the shallow areas, Elatine hexandra (Lapierre) DC. Blanket weed (Cladophora spp.) is often abundant during the summer months.

7.2.2 Estimation of allochthonous plant litter entering the lake

Allochthonous plant litter entering Yateley 4 was estimated using litter traps during the autumn of 1983. The litter traps consisted of polyester mesh nets (2.5 mm pore size) attached to floating hollow plastic rings (1 m diameter). Each ring was allowed to float on the lake surface and was held in position by nylon twine connected to weights on the lake bottom. Litter traps were placed in lines 1, 3, 5, 9, 13 and 21 m from the bank at 3 sites around the lake margin. Figure 7.3 shows 3 of the litter traps placed in position. The sites selected for study were chosen for lack of accessibility from the bank to prevent interference with the experiment. Random placement of nets was not possible, but sites were chosen to be as representative of the lake margin, where trees and shrubs were present, as far as possible.

The traps were emptied weekly between October 20 and December 7. Observations had revealed that there was negligible leaf fall before October 20. The collected litter was sorted into different leaf types, twigs, flowers, seeds and fruits and oven dried at 80 ^{o}C to constant weight.

7.2.3 Seasonal changes in chlorophyll a, soluble phosphate and soluble nitrate + nitrite

Seasonal changes in chlorophyll a, soluble phosphate (PO_4-P) and soluble nitrate + nitrite (NO_3-N + NO_2-N)

were monitored during 1983 and 1984 using the methods described in Section 8.2.2.

7.2.4 Phytoplankton productivity

Estimates of phytoplankton productivity were made during the spring and early summer of 1984. On each occasion photosynthetic rate was estimated during a six hour period using the light and dark bottle method (Vollenweider, 1971). Six depth integrated samples of water were collected from random points in the lake using a perspex column (2 x 0.06 m) mixed in a bucket and 100 ml stoppered bottles were filled and suspended at depths of 0.5, 0.75 and 1.5 m below the lake surface. Changes in oxygen levels in the bottles after 6 hours were estimated using the Winkler titration method (Mackereth, Heron & Talling, 1978) and net productivity per day estimated from changes in the oxygen levels in the bottles and the number of daylight hours.

7.2.5 Macrophyte productivity

During late August and early September 1984 much of Yateley 4 had extensive growth of submerged macrophytes (greater than 80% surface area). On September 7 the area of the lake occupied by macrophytes was estimated subjectively and 3 representative areas close to the margin were chosen for biomass sampling. The macrophytes in 3 areas (10.5 x 1.5 m) were harvested using a large chain and garden rakes to drag the vegetation onto the bank. Each area was sampled three times to ensure that all the vegetation was removed. The macrophytes were washed to remove sediments, sorted into species and oven dried at 80 ^{o}C to constant weight. Emergent and floating macrophytes were not sampled.

7.3 Results

Figure 7.4 shows the total quantity of airborne

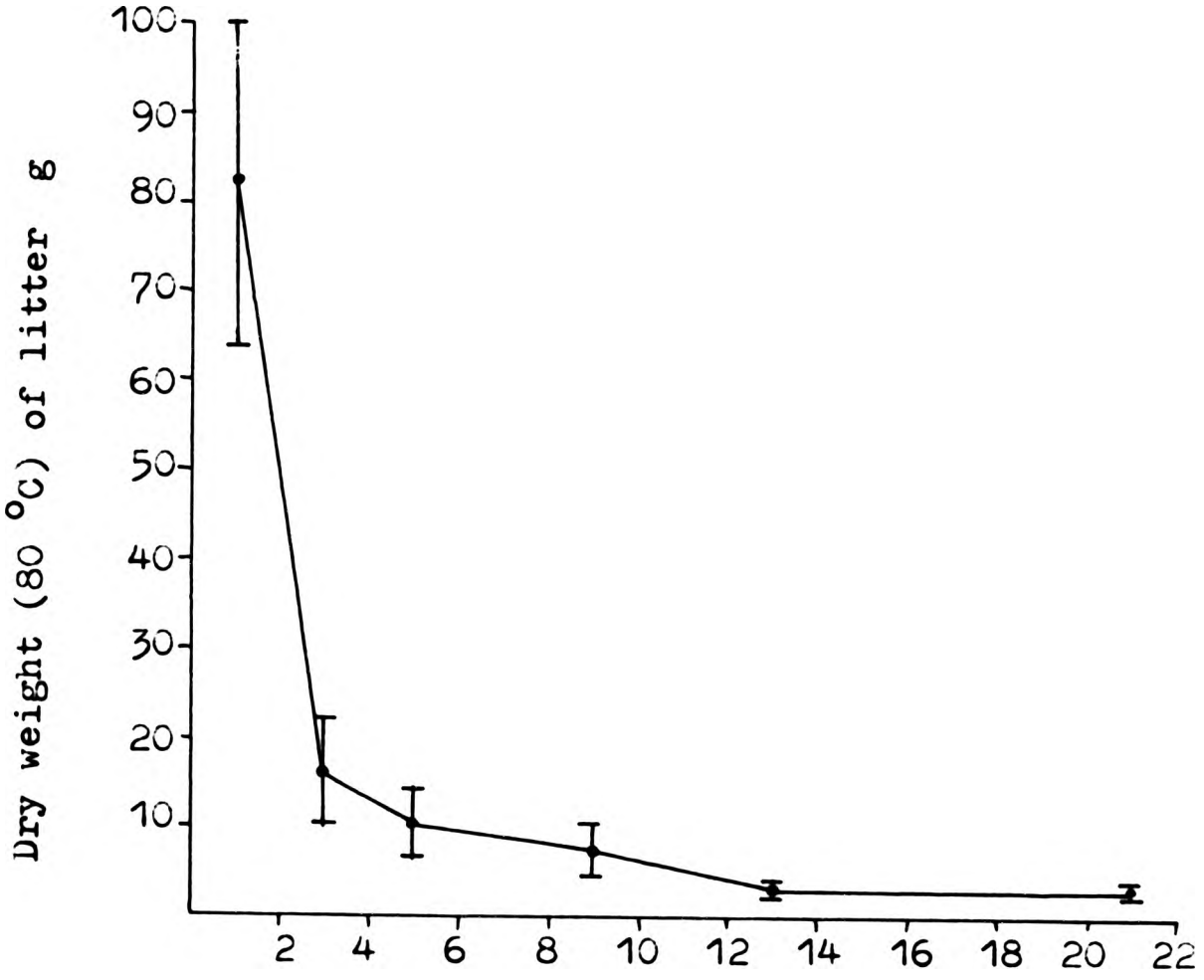


Figure 7.4. Graph showing the quantitiy of airborne plant litter caught by litter traps (1 m diameter) at different distances from the shore. (Each point represents the mean dry weight of litter caught in three traps during the study period together with the standard error).

plant litter caught by litter traps at different distances from the lake margin during the study period. It was assumed that litter collected in each trap was derived from 1 m of bank. Most of the litter caught, fell within the first 3 m and only a small percentage was recovered at 13 and 21 m from the margin. The total weight of litter entering the lake per metre of shoreline with trees and shrubs was calculated from the area under the curve by calculus. The estimated total dry weight of litter input was 348.8 g m^{-1} of wooded shoreline, which represents a total of 254.8 kg dry weight of litter entering the lake, or 17.0 g m^{-2} lake surface.

Figure 7.5 shows the pattern of litter fall based on the weekly collections. Maximum litter fall occurred during the first week in November. Strong winds during the last week in November resulted in a higher litter fall than the previous week and a larger proportion of twigs. The leaves of alder appeared to fall earlier than the other species and twigs and branches became increasingly important components of total litter fall by late November - early December.

Table 7.1 shows the composition of the total litter fall collected over the study period. Leaf input consisted predominantly of willow (Salix spp., 64.6%), alder (11%) and Spiraea (6%) with most other leaf types forming only a small percentage (less than 4%) of the total litter input. Twigs and branches formed a significant input (14%), whereas flowers, fruits and seeds were only of minor importance (1.5%).

Seasonal changes in chlorophyll a, PO_4-P and NO_3-N + NO_2-N in Yateley 4 are shown in Appendix 6, Figure 1. The levels of chlorophyll a were generally low (less than 10 µg l^{-1}) for much of the year, with the highest levels occurring during the spring and summer months. Levels of PO_4-P and NO_3-N + NO_2-N were low throughout the year (less than 14 µg l^{-1} and less than 140 µg l^{-1}, respectively). Net phytoplankton productivity measured during April and May was 0.393 and 0.131 g C m^{-2} day^{-1}, respect-

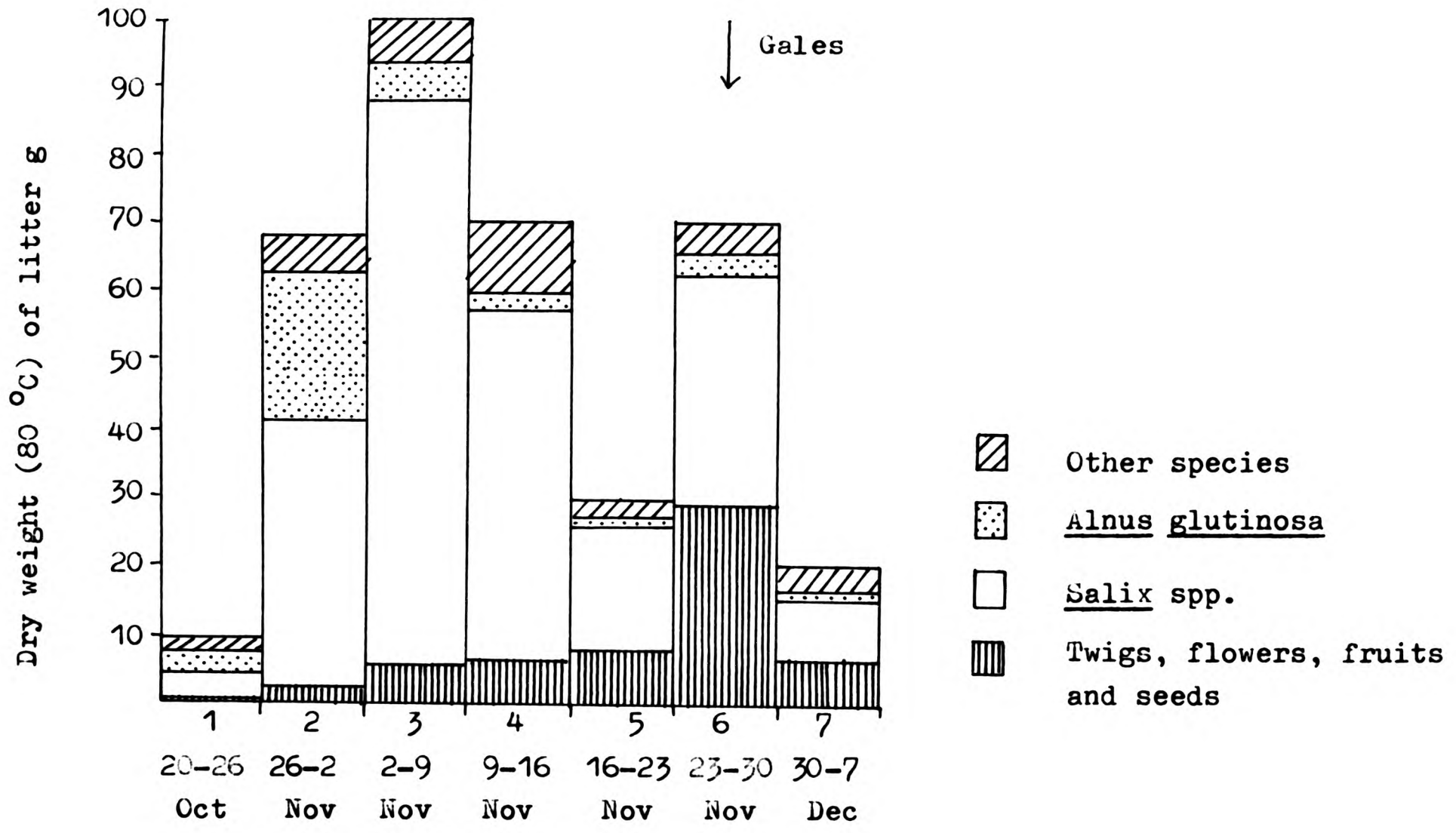


Figure 7.5. Dry weight of plant litter recovered from all traps during the autumn leaf fall into Yateley 4 in 1983.

TABLE 7.1 Percentage composition of the major types of plant litter recovered from traps during the autumn leaf fall into Yateley 4.

LITTER TYPE	%COMPOSITION
Salix spp	56.54
Twigs and branches	13.94
Alnus glutinosa	11.16
Salix fragilis	7.10
Spiraea salicifolia	6.30
Quercus spp	1.60
Flowers, fruits and seeds	1.45
Betula spp	0.90
Viburnum opulus	0.35
Populus sp	0.30
Fraxinus excelsior	0.13
Crataegus monogyna	0.05
Other species	0.06

ively. Taking the mean of these values and assuming that there was net productivity for only 6 months of the year gave an estimate of net productivity of 47.8 ± 23.9 g C m^{-2} yr^{-1}. This possibly overestimates phytoplankton productivity since, for much of the year, chlorophyll *a* and PO_4-P levels were very low, less than 10 and less than 5 µg l^{-1}, respectively, in Yateley 4 (Appendix 6, Figure 1). The three transects for macrophyte biomass yielded 261, 152.4 and 33.0 g dry weight m^{-2}. Assuming carbon to be 45% of oven dry weight (Westlake, 1963) then the estimated productivity, based on biomass, was 66.9 ± 22.6 g C m^{-2} yr^{-1} consisting of 95% *Ceratophyllum demersum*, 4% *Elodea canadensis* and 1% *Myriophyllum spicatum*.

7.4 Discussion

The quantity of airborne plant litter entering Yateley 4 during the autumn of 1983 was estimated to be 254.8 kg or 17.0 g dry weight m^{-2}. Although the autumn 'pulse' of litter represents the major input of allochthonous litter, twigs, branches, flowers, fruits, seeds, bud scales and some leaves may be shed throughout the year. Estimates of the proportion of total allochthonous input shed in the autumn vary between 70 and 80% (Mason, 1970; Fisher & Likens, 1973; Gasith & Hasler, 1976) which would give a value of approximately 340 kg or 22.6 g m^{-2} of litter entering Yateley 4 each year, using 75% as the amount of litter entering the lake during the autumn. Assuming that approximately 45% of the allochthonous litter is organic carbon (Westlake, 1963) then annual contribution of airborne plant litter to the carbon budget of the lake would be 153 kg C or 10.2 g C m^{-2} yr^{-1}. Herbs, grasses and leaf litter on the bank would also contribute some carbon to the lake by being blown or washed in, so the figures obtained from airborne litter will underestimate the true input of allochthonous litter.

The results obtained in the present study suggest that the majority of allochthonous litter falls within

the first 3 metres from the shore, and that the amount of litter fall follows an exponential decline with increasing distance outward from the bank. Similar results were found by Gasith & Hasler (1976) and Hanlon (1981) although, in these studies, litter fall did not decline as sharply with increasing distance from the margin. Yateley 4 is surrounded by other wooded lakes and the influence of wind on the dispersal of leaves may have been reduced, causing most leaves to fall near to the lake margin.

The input of plant litter into lakes varies considerably. It depends on the surface area, the degree of shoreline development (Gasith & Hasler, 1976), the proportion of shoreline with trees and shrubs, and the degree of exposure of the site. Table 7.2 shows a comparison between the input of plant litter and phytoplankton productivity in lakes of different sizes. In larger lakes (greater than 100 ha) the carbon input from airborne litter is clearly less important than phytoplankton productivity, but in smaller water bodies such as Yateley 4 and Beaver Pond (Hodkinson, 1975a) it becomes more significant. Gasith & Hasler (1976) have demonstrated that (assuming similar levels of phytoplankton productivity and percentage of shoreline with trees and shrubs) the relative importance of allochthonous plant litter compared to phytoplankton production increases as lakes become smaller, due to a decrease in surface area relative to lake perimeter. Many of the flooded gravel pits in south east England are small water bodies of less than 4 ha (Figure 8.1) and they are often low in nutrients (Appendix 6, Table 2). Phytoplankton productivity may be low in many of these lakes and, as in the case of Yateley 4, may be exceeded by macrophyte productivity. In such waters the input of carbon from allochthonous litter may make a significant contribution (greater than 8% of phytoplankton + macrophyte net primary production) to potential energy entering these lakes.

Allochthonous plant litter may be a valuable source

TABLE 7.2 Comparison of airborne litter fall and phytoplankton productivity for lakes of different sizes.

LAKE	AREA (ha)	A*1	B*2	C*3	D*4
1) Beaver Pond (Hodkinson,1975a)	0.07	100	27	7.55	28
2) Yateley 4 (Present study)	1.50	79	48	10.20	21
3) Llyn Frogach (Hanlon,1981)	7.20	23	15	0.63	0.4
4) Findley (Rau,1976)	11.40	70	6	1.53	25.6
5) Mirror (Jordan & Likens,1975)	15.00	90	78	4.30	5.5
6) Winga (Gasith and Hasler,1975)	131.00	75	400	0.92	0.2
7) Mikolajki (Szczepanski,1965)	459.00	52	330	0.36	0.1

*1 A = Percentage of shoreline with trees

*2 B = Phytoplankton Primary Production (g C m^{-2} yr^{-1})

*3 C = Airborne litter fall (g C m^{-2} yr^{-1})

*4 D = Litter fall as a percentage of Primary Production

of nitrogen, phosphorus, potassium, calcium and other elements in small lakes, and particularly in gravel pits where there is often little potential for groundwater inputs because of very narrow strips (3-5 m) of land between the lakes. The addition of allochthonous litter to lakes and rivers can be regarded as a type of ecological disturbance (Pugh, 1980) since a residue of allochthonous litter bearing microorganisms is suddenly mixed with a large mass of new resources for potential colonisation. The response of fungal populations to this disturbance may be quite marked and is well illustrated by the observed increases in numbers of spores of Ingoldian hyphomycetes which coincides with the autumn pulse of leaf litter into rivers and streams (Iqbal & Webster, 1973 & 1977; Chamier & Dixon, 1982a).

In the following chapters the fungal communities associated with allochthonous leaf litter and cellulosic substrates in flooded gravel pits will be examined together with studies on fungal biomass and activity during the time course of decomposition in these lakes. The estimate of allochthonous litter input to Yateley 4 should allow estimates of fungal biomass and activity associated with the decomposition of these resources to be seen in a wider context.

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CHAPTER 8. VARIATION IN FUNGAL BIOMASS, COMMUNITY STRUCTURE AND CELLULOSE DEGRADATION IN A SERIES OF SMALL FLOODED GRAVEL PITS.

8.1 Introduction

In studies on freshwater ecosystems it has been established that Ingoldian fungi form part of the characteristic mycoflora of decaying allochthonous plant litter and their important rôle in the decomposition of these resources is now recognised (Barlocher & Kendrick, 1974, 1976 &1981; Suberkropp & Klug, 1976; Chamier & Dixon, 1982a). Rivers and streams have been widely surveyed for these fungi (Webster & Descals, 1981) but few authors have attempted to measure fungal biomass or cellulose degradation together with a description of the fungal communities.

Evidence suggests that both water chemistry and allochthonous litter input may have a significant influence on fungal communities (Roberts, 1963; Iqbal & Webster, 1973; Barlocher & Rosset, 1981; Wood-Eggenschwiler & Barlocher, 1983; Shearer & Webster, 1985a, b) and that water chemistry influences the degradation of cellulosic substrates (Egglishaw, 1968 & 1972; Hofsten & Edberg, 1972; Fleischer & Kerstin, 1974). Less is known about the processes in lakes than in rivers, particularly in relation to fungal communities.

The present work examined the communities of Ingoldian fungi on allochthonous plant litter in a series of small flooded gravel pits in S.E. England. The close proximity of these lakes, on similar geological formations, provided an excellent opportunity to examine the Ingoldian fungi, fungal biomass and cellulose degradation as a preliminary to a more detailed study in one of these lakes.

8.1.1 The gravel pit lakes

Prior to 1973 there was a dramatic increase in the demand for sand and gravel in the United Kingdom. Demand increased from 3 million tonnes in 1920 to 129 million tonnes in 1973. Archer (1972) calculated that, since 1895, 2300 million tonnes of sand and gravel had been extracted in the United Kingdom and that production had doubled since 1957. Titmus (1979) estimated that 1600 ha of land were excavated annually, of which more than 1000 ha remained as wetlands. The continued extraction of aggregates and the low return of land to agriculture has meant that flooded gravel pits are fast becoming one of our largest water resources. Most extraction of aggregates has been concentrated in regions with extensive valley gravels, particularly from deposits in the Thames and Trent valleys and, to a lesser extent, from the floodplains of the Ouse and its tributaries.

Flooded gravel pits are usually relatively small in area (Figure 8.1) and they occur in small complexes in river valleys as a result of the sequence of land aquisition, extractive processes and restoration regimes. Many pits are less than 30 years old, shallow ($\angle$ 4 m deep), with a steeply shelving littoral zone (see Figure 7.1). The substratum consists of variable proportions of silt, organic matter, gravel and clay depending on extraction and restoration methods. Because most pits are shallow, thermal stratification is rare and there is rapid warming and cooling of the waters at different times of the year. Water levels may fluctuate in many pits because of a dependence on rainfall and groundwater, there being little surface inflow.

Banoub (1978, 1981) has demonstrated that gravel pit water chemistry is influenced by the surrounding groundwater. Most gravel pits are generally classified on a nutrient basis as meso-eutrophic (Banoub, 1978 & 1981; Northcott & O'Grady, 1984) although the range of variation

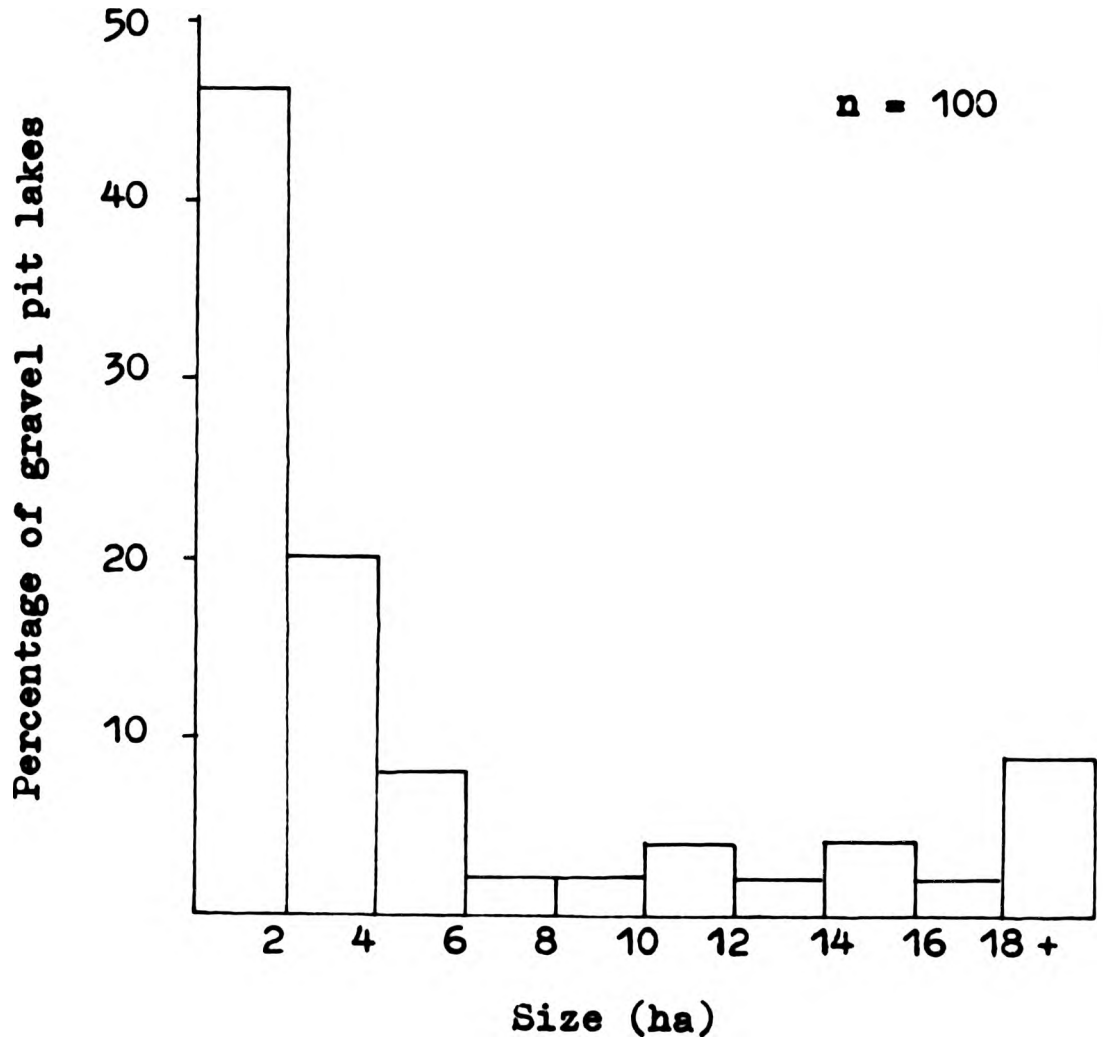

Figure 8.1. Frequency distribution of 100 gravel pit lakes in terms of size. The data are for lakes in S.E. England and were obtained from Gee (1978), Powell & South (1978) and the Leisure Sport Rule and Guide Book (1984-85 season)

has not been defined.

Studies on the ecology of flooded gravel pits have mostly been concerned with animals (Barber, 1976; Gee, 1978; Powell & South, 1978; Cook, 1979; Titmus, 1979; Northcott, 1981) and phytoplankton (Karim, 1960; Anderson, 1981) and only one study has examined fungal populations in these lakes (Sultana, 1976; Godfrey & Mehdi (née Sultana), 1982).

8.2 Materials and methods

8.2.1 Study sites

The gravel pit lakes studied are located in the Blackwater valley in Hampshire, Berkshire and Surrey, about 50 km west of London. Two small complexes of lakes were investigated; a group of 14 lakes at Yateley (Grid Ref. SU 8261) and six lakes at Frimley (Grid Ref. SU 8757) about 6 km south-east of Yateley. The lake complexes at Yateley and Frimley are shown in Figure 8.2a & b, respectively.

Figure 8.3 shows the study area at Yateley (it is an enlargement of the area enclosed within the broken line in Figure 8.2a). The lakes studied are numbered from 1-14, lakes 1-9 being referred to as Yateley West and lakes 10-14 Yateley East. Details of the Frimley lakes are shown in Figure 8.4. At this site, 6 lakes were studied and the numbering system, 18A, 18B, 19-21, 21A, was adopted from Northcott (1981) and Burgis (1983).

Most of the lakes are small (< 5 ha) and vary in age from 10 to 30 years (Appendix 6, Table 1). Many follow old field boundaries and are separated by narrow strips of land (Figure 8.5). Many of the pits have extensive plantings of trees and shrubs around the shorelines and, in many places, these overhang the littoral zone by 1-2 m (Figure 8.6). The most frequent trees and shrubs are Alnus glutinosa (L.) Gaertner, Salix fragilis L. and Salix spp. A more extensive list of the vegetation

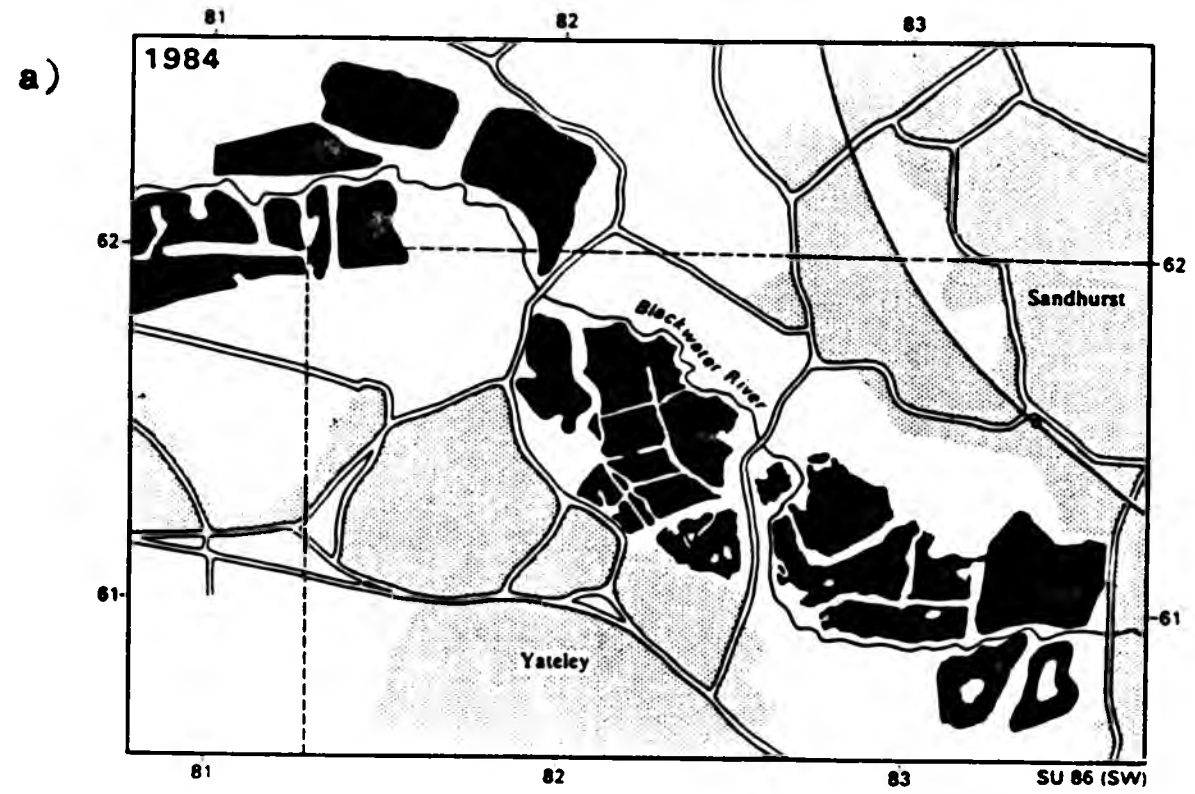

Figure 8.2. Maps showing the complex of lakes at a) Yateley and b) Frimley in 1984 and 1983, respectively. The lakes enclosed by the broken lines on each map were those used in the present study. The scale on each map is indicated by the 1 km National Grid reference numbers around the border of each map, 100 km^2 reference is SU

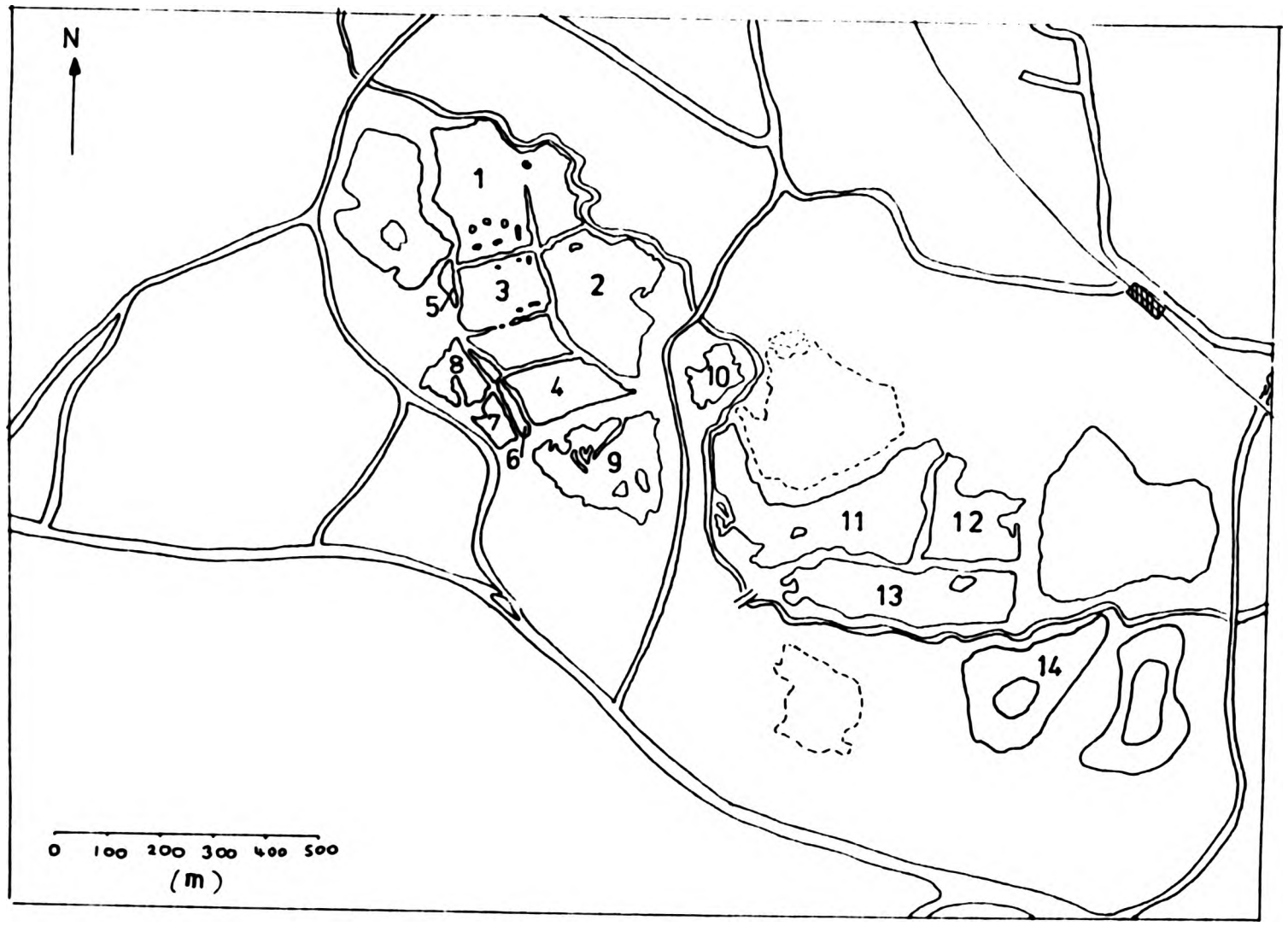

Figure 8.3. Detailed map of part of the Yateley complex of gravel pit lakes. The lakes studied in the present survey are numbered from 1 to 14. The map is an enlargement of the portion of Figure 8.2 enclosed by broken lines.

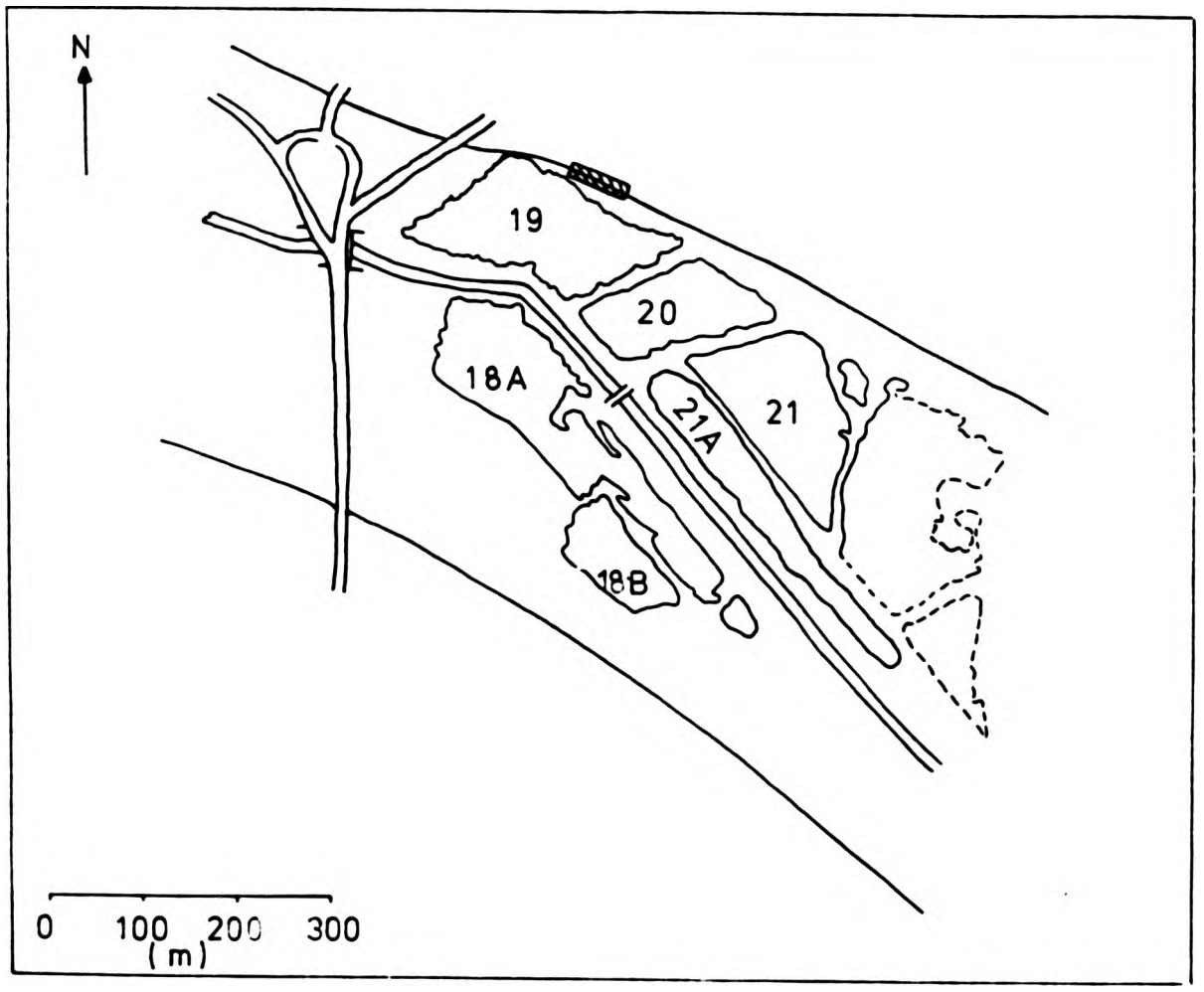

Figure 8.4. Detailed map of part of the Frimley complex of lakes showing the reference numbers, relative positions and sites of the 6 lakes studied. The map is an enlargement of the portion of Figure 8.2 b) enclosed by broken lines.

Figure 8.5. Aerial photograph of the Yateley lake complex in 1979. The photograph was taken looking S.W., with Yateley 2 in the foreground.

Figure 8.6. Photograph showing overhanging vegetation around the edge of a typical gravel pit (Yateley 3).

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Figure 8.6. Photograph showing overhanging vegetation around the edge of a typical gravel pit (Yateley 3).

for one of the lakes, Yateley 4, is given in Section 7.2.1.

8.2.2 Physical and chemical analyses

8.2.2.1 Age, area and shoreline development

The age and area of each gravel pit lake was obtained either from large scale Ordnance Survey maps or from data in Gee (1978), Northcott (1981) or Burgis (1983). The degree of shoreline development was estimated according to the method in Gasith & Hasler (1976) using information on lake area and perimeter. Lake perimeter was estimated by enlarging Ordnance Survey maps and using a map measurer to estimate shoreline length.

8.2.2.2 Water chemistry

The water chemistry of each lake was investigated during October, February, May and August. All lakes were sampled on at least 3 occasions. Water samples were collected in polyethylene bottles (Azlon, 250-1000 ml) which had been acid washed (5% v/v HCl) and rinsed in distilled water. At each lake a water sample was collected from 2 or 3 accessible places around the lake edge, 0.5-1 m from the bank at a depth of about 10 cm. Each sample was transported to the laboratory on ice before commencing analyses.

Conductivity and pH were measured using standard instrumentation. The sample alkalinity was determined by titration with 0.01 N HCl to a pH of 4.5 (using a pH electrode) and the alkalinity calculated according to Mackereth, Heron & Talling (1978). Cations and anions were determined after filtering the water samples (Whatman GF/C). Calcium and magnesium were determined by atomic absorption spectrophotometry and sodium and potassium by flame emission photometry using appropriate standards and conditions in each case. Soluble reactive phosphorus was low in all of the lakes studied (< 50 $\mu g\ l^{-1}$), therefore the extractive method of Mackereth,

Heron & Talling (1978) was used. Soluble nitrate and nitrite were determined together using a modification of the rapid brucine method (Ranney & Bartlett, 1972). To 1 ml of sample or standards (0.05-2.5 mg l^{-1} as KNO_3-N) 100 µl of brucine in methanol (4% w/v) was added followed by 1 ml of concentrated H_2SO_4 (Aristar Grade, BDH chemicals). After incubation for 1 hour at room temp. absorbance was measured and compared to the standard curve (linear up to 1 mg l^{-1} NO_3-N). Chlorine was determined by a modification of the Mohr titration (Williams, 1979).

8.2.2.3 Chlorophyll a

The chlorophyll *a* in each water sample was determined by filtering 100-200 ml of sample (Whatman GF/C, 3 cm diameter) with vacuum suction (75 cm Hg), extracting in 20 ml of methanol (boiling for a few seconds) and measuring the chlorophyll *a* quantitatively by absorbance at 660 nm. Corrections and calculations were made according to Jones (1979).

8.2.3 Fungi on allochthonous litter in different lakes

8.2.3.1 Allochthonous litter input

Estimates of annual allochthonous litter input into each lake were made by comparing the length of wooded shoreline around each lake to that of Yateley 4 for which allochthonous litter input was known (Chapter 7). An estimate of the proportion of wooded shoreline around each lake was obtained from Burgis (1983), aerial photographs, or by measuring the length of wooded shoreline. The input of allochthonous litter into Yateley 4 per metre of wooded shoreline was then used to estimate inputs into each lake and results were expressed in terms of g C m^{-2} yr^{-1}, as in Chapter 7.

8.2.3.2 Collection of allochthonous litter

Samples of benthic litter were removed from each lake during October, February, May and August. On each occasion between 20 and 50 g (wet weight) of benthic litter was collected from 4 or 5 representative sites in the littoral zone around each lake, 0.5-1.5 m from the bank. Each group of samples from a lake were then pooled and placed in plastic bags and kept on ice during transfer to the laboratory. On return, each sample was emptied into a large enamel tray and silt, mud, twigs and invertebrates carefully removed; the sample was rinsed in distilled water and then divided into two portions, one being deep frozen (-70 ^{o}C) for ergosterol analysis, the other being used to detect sporulating fungi.

8.2.3.3 Fungi sporulating on allochthonous litter

Leaf material obtained from each lake was added to six petri dishes (9 cm diameter) containing 20 ml of sterile distilled water. The plates were incubated for 14 days at 6, 12 or 20 ^{o}C depending on the temperature of the lake at time of collection. Each plate was examined every 2-3 days using a microscope (x 10 objective) and any Ingoldian fungi recorded. Attempts were made to filter (8 µm membrane filter, 25 mm diameter, Millipore Corp.) 100-500 ml volumes of lake water to detect Ingoldian fungi (Iqbal & Webster, 1973) but the densities of spores were too low ($\angle$ 10 l^{-1}) to make the method practicable for use with lake water samples.

8.2.3.4 Ergosterol in allochthonous litter

Samples of litter collected in February, May and August were used for ergosterol extraction and purification according to the method given in Sections 5.2.2 & 5.2.5.1 for leaf litter. Ergosterol was quantified using the

HrLC method as described in Section 5.2.5.2. Care was taken to ensure that no macro-invertebrates were included with the litter being extracted. Dry weight of litter samples was estimated by drying subsamples at 80 ^{o}C until constant weight.

8.2.4 Fungi on allochthonous litter in a single lake

8.2.4.1 Sample collection

Variation in species composition and fungal biomass in allochthonous litter in a single lake (Yateley 4) was investigated on June 29, 1984. Four sites were selected for study using random number tables to select positions around the lake perimeter. At each site a steel framed quadrat (1 x 1 m) divided in 4 sections (0.25 x 0.25 m) was positioned on the lake bottom 1.5 m from the bank. Prior to placement of the quadrat, the frame was covered in aluminium foil and strings with polystyrene floats attached to the edges and centre of the frame. The aluminium foil, in conjunction with polystyrene floats, enabled the frame to be positioned accurately and be seen clearly.

The allochthonous litter in each of the four sections of the quadrat was sampled once using an Ekman grab (Aparatabau, GmbH, W. Germany). This was lowered into the centre of each section and sampled an area 0.15 x 0.15 m. The contents of each section were emptied into plastic bags and kept on ice during transfer to the laboratory, where samples were treated as in Section 8.2.3.2.

8.2.4.2 Fungi sporulating on allochthonous litter

Samples of allochthonous litter (10-15 g wet weight) were placed in experimental flasks (see Chapter 2) containing 600 ml of sterile distilled water and

aerated (100 cm^{-3} min^{-1}) for 3 days at 18 °C. After incubation large particles of leaf material were allowed to settle and two 50 ml samples of water removed from each flask and separately filtered (8 µm membrane filter, 25 mm diameter, Millipore Corp.) with vacuum suction (75 cm Hg) and the number of spores quantified as in Section 2.5. Residual leaf material, together with the water in each flask, was filtered (Whatman GF/C) and the retained material dried to constant weight at 80 °C.

8.2.4.3 Fungal biomass

Ergosterol in allochthonous litter was estimated as in Section 8.2.3.4. The whole leaf clearing and staining method was also used, and the method was that described in Chapter 3. Hyphal diameters were estimated on 30 randomly selected hyphae from a pooled sample of leaf discs.

8.2.5 Degradation of cellulose in different lakes

8.2.5.1 Changes in weight, area and tensile strength

Degradation of cellulose substrates was monitored in eight lakes (Yateley 2, 4, 8, 11, 12; Frimley 18A, 18B, 19) which were representative of the range of physical and chemical variation present in the lakes studied in this work. For each lake, ten litter bags were made (22 x 11 cm; mesh size 1 x 1 cm NETLON) containing one filter paper (Whatman No 1, 9 cm diameter) and a 15 cm length of natural jute garden twine (Type C3 Soft Natural, Anderson & Firmin Ltd., London) which was stapled to the mesh of the bag. The 10 bags were grouped into 5 pairs and each attached at one end to a 2 metre length of nylon monofilament line and placed in the lakes at 5 randomly selected sites around the perimeter. Each pair of bags was positioned on the lake bottom, approximately 1.5 m from the bank, and secured with the nylon line to small

pegs in the bank. The bags were placed in the lakes on August 2 and retrieved after 35 days, each pair of bags being placed in a separate plastic bag, prior to being transported to the laboratory on ice.

The filter papers were immersed in distilled water and invertebrates, gravel, silt and mud gently rinsed off with the aid of an air jet, all filters being cleaned under the same conditions for the same length of time. Clean unused filter papers presoaked for 2 hours in sterile lake water were treated in the same way as the samples. After cleaning, the percentage of filter paper area lost during the 85 days was estimated using graph paper, by comparing the number of mm^2 occupied by control and degraded filter papers. The filters were then oven dried to constant weight at 80 ^{o}C.

The twine samples were rinsed in distilled water and a 10 cm portion cut for tensile strength determination. Each sample was tested using a Hounsfield Tensiometer (Tensiometer Ltd, Croydon) with a full scale beam of 70 Newtons and the force required to break the twine recorded. Control samples consisted of twine incubated for 2 hours in sterile distilled water prior to tensile strength testing. The results were expressed as percentage loss in tensile strength relative to the controls.

8.2.5.2 Fungal colonisation of cellulose substrates

Fungal colonisation of cellulose filter papers in 2 of the lakes (Yateley 4 & 8) were investigated using techniques of direct observation, hemp seed baiting and plating of material on nutrient media. Mesh bags containing filter papers were placed in each lake for 35 days at 5 random locations, as described in Sections 8.2.5.1. On retrieval, 50 discs (6 mm diameter) were cut from the filter papers from each lake using a flamed cork borer, and the discs rinsed in 3 changes of sterile distilled water. Fifteen of the discs were

incubated in 20 ml of sterile distilled water in a petri dish for 7 days at 18 °C, and a further 15 discs incubated under the same conditions but with 10 sterile hemp seeds. The remaining discs were homogenised in 20 ml of sterile distilled water (VIRTIS 45, setting 5, 1 minute) followed by serial dilution of the homogenate in sterile distilled water and spreading of aliquots of appropriate dilutions onto potato dextrose agar containing antibiotics, lake water agar and cellulose agar (Park, 1973; see Appendix 3). Plates were incubated in sealed plastic bags at 18 °C and examined weekly for the appearance of fungal colonies.

Short segments of twine (2 cm) were cut from material incubated in the lakes (Section 8.2.5.1), rinsed in 3 changes of sterile distilled water and incubated in 20 ml of sterile distilled water in petri dishes for 7 days at 18 °C. Material was examined periodically (x 100) for the appearance of sporulating fungi.

8.2.6 Data analysis

The floristic data for Ingoldian fungi was analysed by reciprocal averaging ordination (Hill, 1973b). Ordinations were performed on presence-absence data for the different lakes and on quantitative data (% spore frequency) for the different sites within Yateley 4. Ordination methods enable floristic or environmental data sets to be reduced in complexity to a few axes, or components, representing the majority of information present in the data (Gauch, 1982). They often reveal trends not apparent by usual inspection of data and they can enable the generation of hypotheses about factors influencing the distribution of species (Greig-Smith, 1983). An outline of the method of reciprocal averaging is given in Appendix 7. The analysis was performed using the program in Orloci (1978) modified for the Polytechnic VAX 750/780 system. A listing is provided in Appendix 7.

Data for percentage loss of filter area and weight, and percentage loss in tensile strength of twine, were transformed (arcsine) prior to analysis of variance. Data for hyphal biomass, ergosterol, species number and spore production were also transformed (log n + 1) prior to analysis of variance.

8.3 Results

8.3.1 Physical and chemical features of the lakes

The physical and chemical features of the gravel pit lakes are summarised by the data presented in Appendix 6, Tables 1-3. Despite the close geographical location of the lakes, there were marked differences in water chemistry. The pH range was 7.3-8.4, alkalinity ranged from 1.09 to 2.19 m equiv l^{-1}, and calcium was 18.2-48.1 mg l^{-1}. This corresponds to 'medium to hard' lakes in the classification of Dussart (1976). Levels of soluble reactive phosphorus were variable, 1.8-20 µg l^{-1}, and low for lakes in S.E. England. Chlorophyll *a* varied considerably, both between sample dates and between lakes (range was 5.3-63.2 µg l^{-1}), although Burgis (1983) did not find any significant difference between summer levels of chlorophyll *a* in eight of the lakes.

Relative levels of cations differed between the gravel pits and, when expressed in terms of a ratio of divalent to monovalent cations, there was a significant negative correlation with gravel pit age (Appendix 6, Figure 2). This suggests that there may be a fall in the levels of calcium and magnesium, and a rise in the levels of sodium and potassium, with increasing age. No other environmental factors showed any significant correlation with gravel pit age.

Shoreline development expresses the irregularity of the shoreline in terms of lake area, and is an important factor for allochthonous litter input, if trees and shrubs are present (Section 7.3). There was a significant

correlation between shoreline development and allochthonous litter inputs ($r = 0.82$, $P < 0.001$) suggesting that lakes with more irregular shorelines had higher litter inputs. Mean levels of soluble reactive phosphorus (PO_4-P) were also significantly correlated with allochthonous litter inputs ($r = 0.86$, $P < 0.001$) and shoreline development ($r = 0.72$, $P < 0.001$), but not with mean levels of chlorophyll _a_ in each lake ($r = -0.16$, $P > 0.05$). Allochthonous litter may be an important source of soluble phosphorous in the gravel pit lakes, particularly if there is only a small input from surrounding groundwater or release from the sediments is low. However, in Yateley 4, soluble phosphate was generally low throughout the year (Appendix 6, Figure 1) and there was no detectable increase in PO_4-P coinciding with the autumn leaf fall.

8.3.2 Fungi on allochthonous litter in the different lakes

Ingoldian fungi observed sporulating on allochthonous litter in the 20 gravel pit lakes are listed in Table 8.1. Only one species, _Alatospora acuminata_, was observed sporulating on litter in every lake and this species appeared to be the most abundant in terms of spore production, although in Frimley 18A filiform species, particularly _Anguillospora_ spp., were the most abundant. Several species occurred in most of the lakes, although 50% of the species were found in 4 lakes or less. The distribution of Ingoldian fungi on the leaves often appeared to be patchy with partly decomposed leaves of alder and willow often producing no sporulating hyphomycetes. This appears to contrast with the situation in many running waters where most allochthonous leaves seem to be colonised by Ingoldian fungi.

Examination of Table 8.1 does not reveal any obvious pattern in the distribution of Ingoldian fungi between the lakes, but some differences were revealed by the reciprocal averaging ordinations (Figure 8.7-8.10). Figure 8.7 shows a scatter diagram of the first two axes

TABLE 8.1 Ingoldian fungi sporulating on allochthonous litter from 20 gravel pit lakes. Nomnclature follows Webster and Descals, 1981.

SPECIES	YATELEY(WEST)								
	1	2	3	4	5	6	7	8	9
Alatospora acuminata(Aa)	+	+	+	+	+	+	+	+	+
Clavatospora longibrachiata(Cl)	+	+	+	+	+	+	+	+	+
Clavatospora stellata(Cs)	+	+	+	+	+	+	+	+	
Anguillospora longissima(Al)	+	+	+	+	+	+	+	+	+
Clavariopsis aquatica(Ca)	+	+	+	+		+	+		
Tricladium splendens(Ts)	+	+	+	+	+		+	+	
Tetracladium marchalianum(Tm)	+	+	+	+	+	+			
Lemonniera aquatica(La)	+	+		+			+		
Flagellospora curvula(Fc)			+	+	+		+		
Varicosporium elodae(Ve)			+	+	+		+		+
Articulospora tetracladia(At)			+	+	+		+	+	+
Tetrachaetum elegans(Te)				+	+		+		
Margaritispora aquatica(Ma)					+		+		
Fusarium spp(Fs)					+				+
Anguillospora pseudolongissima(Ap)					+				
Triscelophorus monosporus(Tm)									
Anguillospora crassa(Ac)				+					+
Lemonniera terrestris(Lt)				+					

SPECIES	YATELEY(WEST)								
	1	2	3	4	5	6	7	8	9
Tetracladium setigerum(Ts)				+					
Lunulospora curvula(Lc)		+		+					
Dimorphospora foliicola(Df)				+					
Unknown sp.1(U1)									
Unknown sp.2(U2)									
Unknown sp.3(U3)									
Unknown sp.4(U4)									
Candida sp.(Caq)				+					
Number of species(TOTAL 26)	8	12	13	19	6	8	10	7	6

SPECIES	YATELEY(EAST)				
	10	11	12	13	14
Alatospora acuminata(Aa)	+	+	+	+	+
Clavatospora longibrachiata(Cl)	+	+	+	+	+
Clavatospora stellata(Cs)	+	+	+	+	+
Anguillospora longissima(Al)	+	+	+	+	+
Clavariopsis aquatica(Ca)	+	+	+	+	
Tricladium splendens(Ts)	+	+	+	+	
Tetracladium marchalianum(Tm)		+	+	+	+
Lemonniera aquatica(La)		+	+	+	
Flagellospora curvula(Fc)			+	+	
Varicosporium elodae(Ve)	+	+			
Articulospora tetracladia(At)			+	+	
Tetrachaetum elegans(Te)		+		+	

SPECIES	YATELEY(EAST)				
	10	11	12	13	14
Margaritispora aquatica(Ma)			+	+	
Fusarium spp(Fs)	+				
Anguillospora pseudolongissima(Ap)		+			
Triscelophorus monosporus(Tm)	+			+	
Anguillospora crassa(Ac)					
Lemonniera terrestris(Lt)					
Tetracladium setigerum(Ts)					+
Lunulospora curvula(Lc)					
Dimorphospora foliicola(Df)					
Unknown sp.1(U1)					
Unknown sp.2(U2)					
Unknown sp.3(U3)					
Unknown sp.4(U4)					
Candida sp.(Caq)					
Number of species(TOTAL 26)	9	11	11	13	6

SPECIES	FRIMLEY						
	18A	18B	19	20	21	21A	A*1
Alatospora acuminata(Aa)	+	+	+	+	+	+	20
Clavatospora longibrachiata(Cl)	+	+		+	+	+	19
Clavatospora stellata(Cs)	+	+	+	+		+	18
Anguillospora longissima(Al)	+	+		+	+		18
Clavariopsis aquatica(Ca)	+	+	+	+	+	+	16
Tricladium splendens(Ts)	+	+		+	+	+	16
Tetracladium marchalianum(Tm)		+		+	+		13
Lemonniera aquatica(La)	+	+	+	+	+		12

SPECIES	FRIMLEY						
	18A	18B	19	20	21	21A	A*1
Flagellospora curvula(Fc)	+	+	+	+	+	+	12
Varicosporium elodae(Ve)	+	+			+	+	11
Articulospora tetracladia(At)				+			9
Tetrachaetum elegans(Te)	+			+		+	8
Margaritispora aquatica(Ma)	+					+	6
Fusarium spp(Fs)			+				4
Anguillospora pseudolongissima(Ap)		+			+		4
Triscelophorus monosporus(Tm)				+			3
Anguillospora crassa(Ac)	+						3
Lemonniera terrestris(Lt)	+						2
Tetracladium setigerum(Ts)							2
Lunulospora curvula(Lc)							2
Dimorphospora foliicola(Df)				+			2
Unknown sp.1(U1)			+			+	2
Unknown sp.2(U2)	+						1
Unknown sp.3(U3)	+						1
Unknown sp.4(U4)				+			1
Candida sp.(Caq)							1
Number of species(TOTAL 26)	15	11	7	14	10	10	

*1 A - Nos of occurrences for a particular species in the 20 lakes.

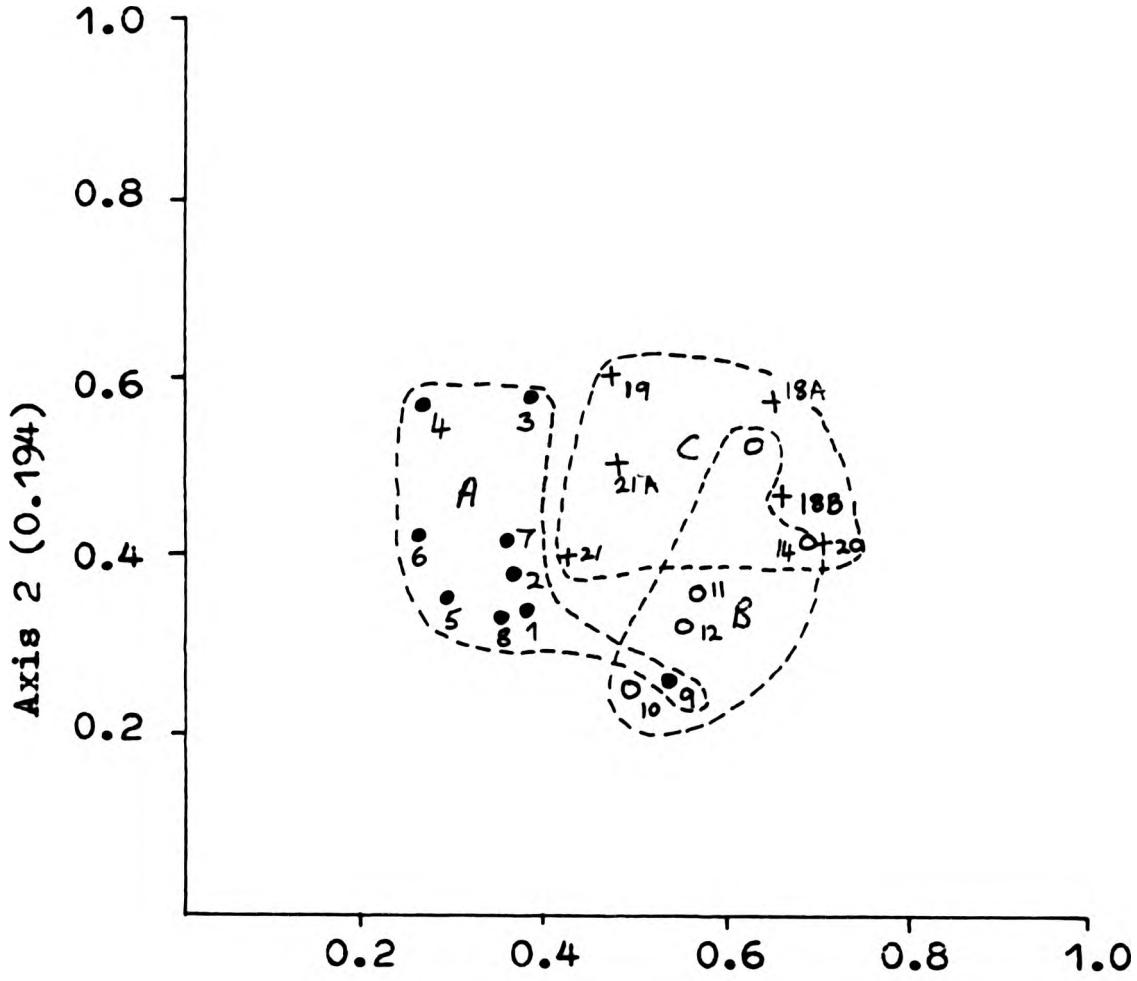


Figure 8.7. Reciprocal averaging ordination of the 20 gravel pit lakes studied based on the presence or absence of Ingoldian fungi. Plot of axis one against axis two. Eigenvalues are shown in parentheses.

KEY:

Yateley West, Group A	•
Yateley East, Group B	o
Frimley, Group C	+

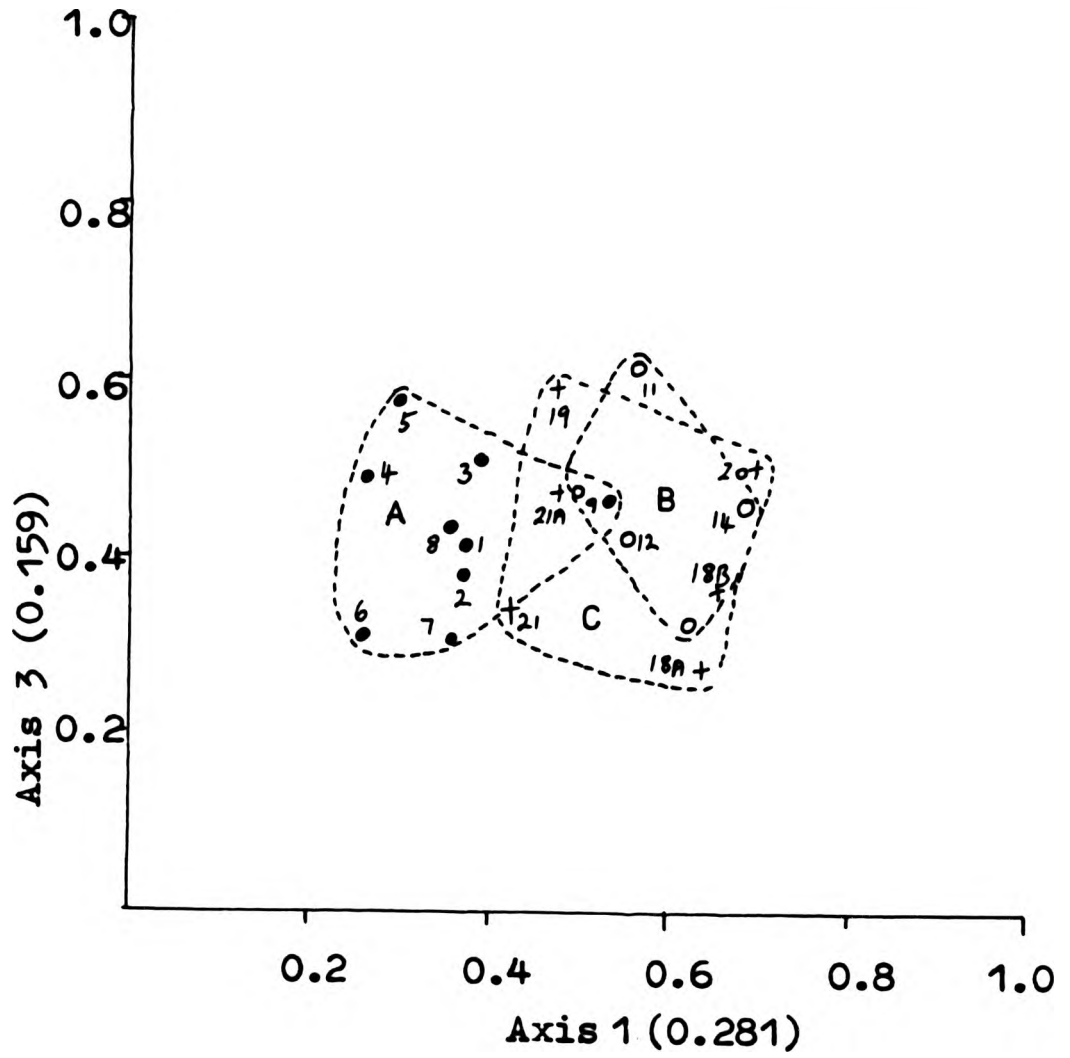


Figure 8.8. Reciprocal averaging ordination of the 20 gravel pit lakes studied, based on the presence or absence of Ingoldian fungi. Plot of axis 1 against axis 3. Eigenvalues for each axis are shown in parentheses.

KEY:

Yateley West, Group A	•
Yateley East, Group B	o
Frimley, Group C	+

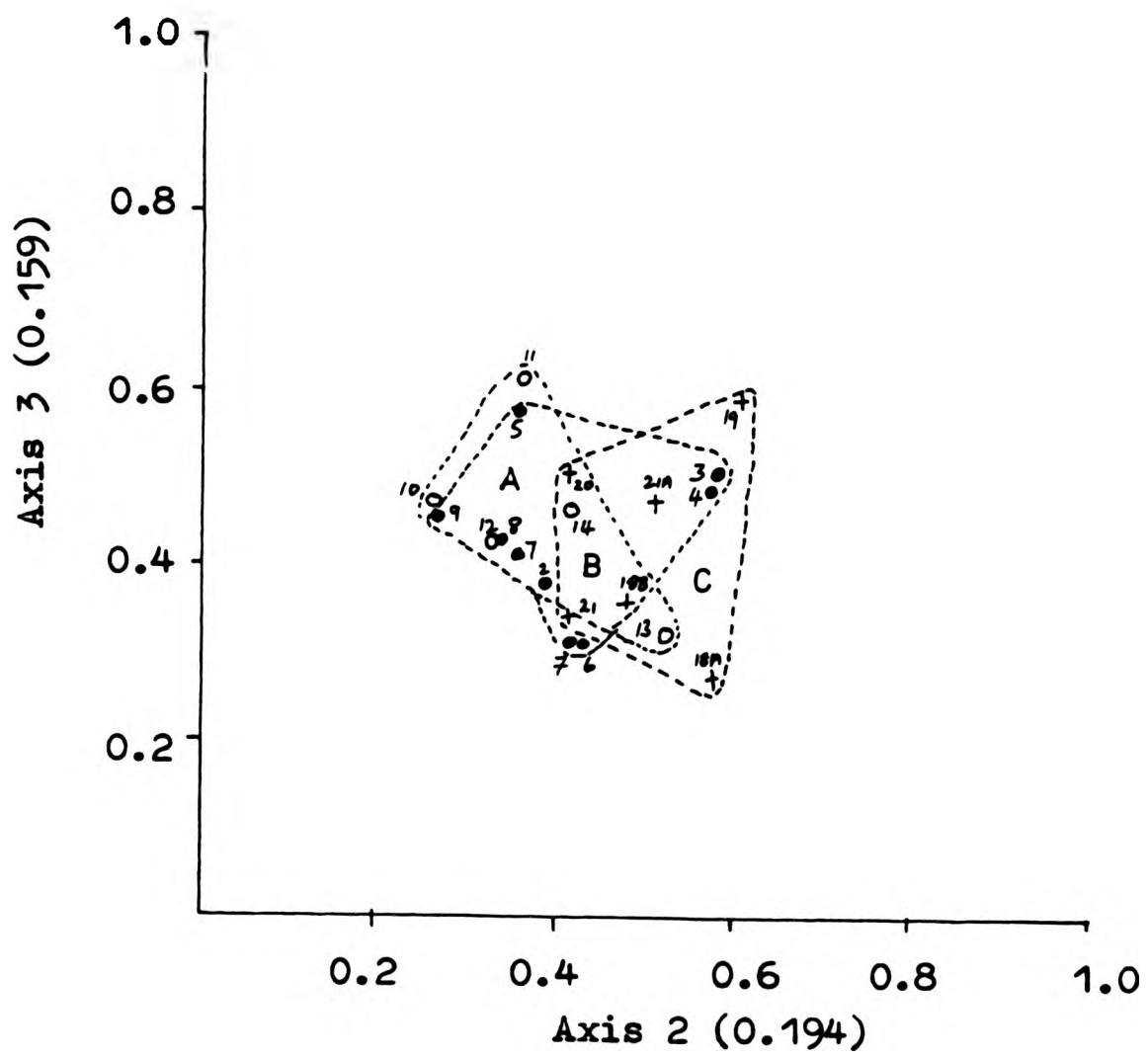


Figure 8.9. Reciprocal averaging ordination of the 20 gravel pit lakes studied, based on the presence or absence of Ingoldian fungi. Plot of axis 2 against axis 3. Eigenvalues are shown in parentheses.

KEY:

Yateley West, Group A	•
Yateley East, Group B	o
Frimley, Group C	+

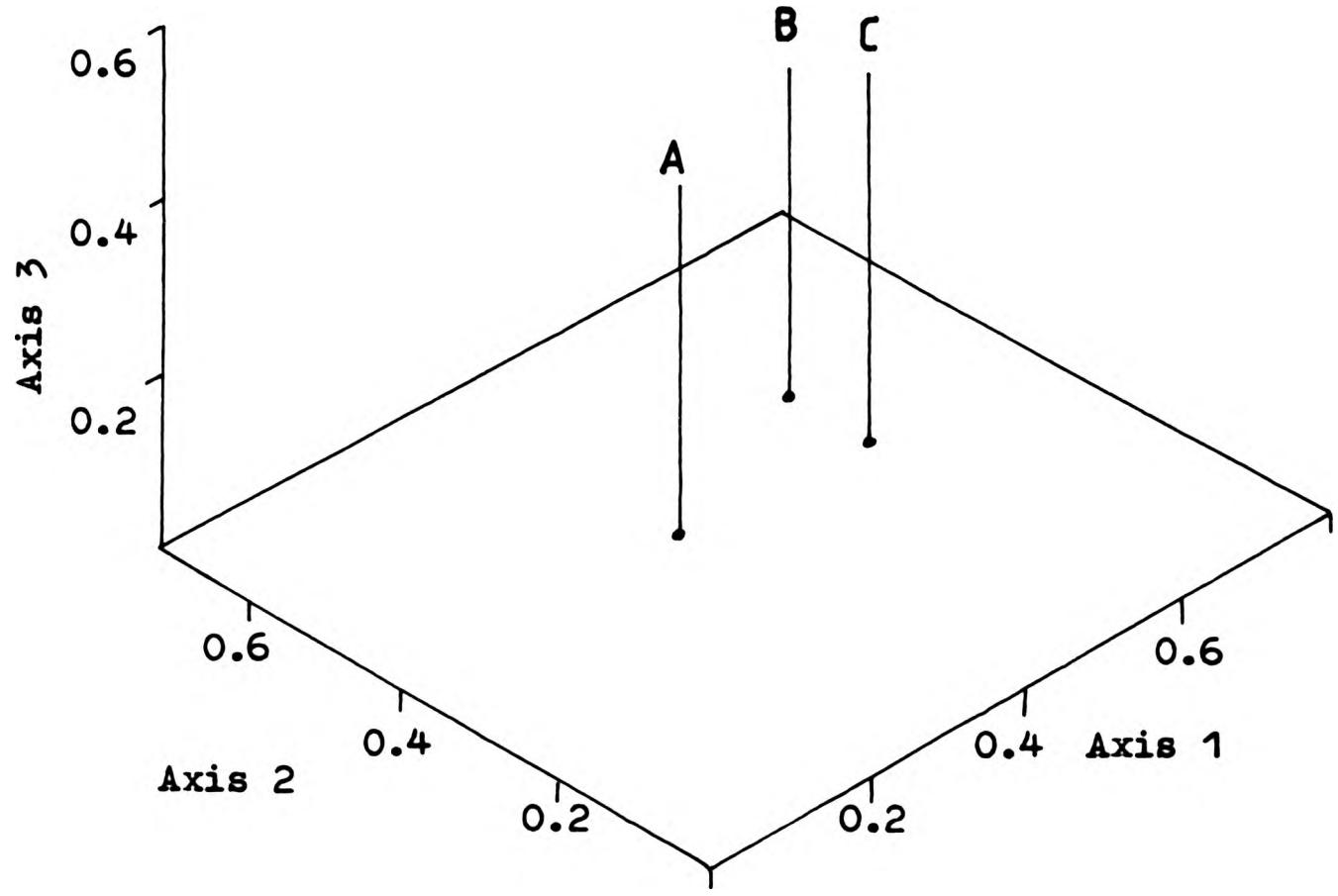


Figure 8.10. Reciprocal Averaging ordination plot (axis 1 by axis 2 by axis 3) of the three groups of lakes (Yateley West = A, Yateley East = B and Frimley = C) using centroids for axis scores for each group (ie., mean axis score within a lake group).

of the reciprocal averaging ordination. All of the lakes are clustered around the central region of each axis indicating that most of the lakes had a similar floristic composition. Examination of positions of different groups of lakes suggests that there is some separation in the floristic composition according to geographical location. The lakes in group A (Yateley 1-9) appear to form a fairly homogenous group, except for lake 9 which has more in common with group B (Yateley East). Although groups A and B are much closer geographically than either is to group C (Frimley), there is considerable overlap between groups B and C, suggesting that factors other than geographical location are responsible for differences between the lakes. Certain lakes in each group were clustered close together on the ordination (eg. 1 and 2, 11 and 12, and 9 and 10) reflecting their close geographical proximity, some being separated by only a few metres.

Further plots of ordination axis scores are shown in Figures 8.8-8.10. Axis 1 plotted against axis 3 (Figure 8.8) again reveals a separation of group A from group B and C, although in this plot there is more overlap between the groups. Axis 2 plotted against axis 3 (Figure 8.9) shows even less variation between the lakes, all 3 groups overlapping considerably. This suggests that most of the floristic variation between the lakes is summarised in the first 2 axes. Figure 8.10 summarises the ordination by plotting the mean axis score of each group of lakes for the first 3 ordination axes. This plot of the 3 groups clearly demonstrates that group B and C (Yateley East and Frimley) have more in common, in terms of their Ingoldian fungal flora than either has to group A (Yateley West).

The scatter plot of the first two axes of the reciprocal averaging species ordination are shown in Figure 8.11. In contrast to the ordination of lakes in Figure 8.7, the species plot is more scattered and there is a poor correpondence between the 2 plots (compare Figure

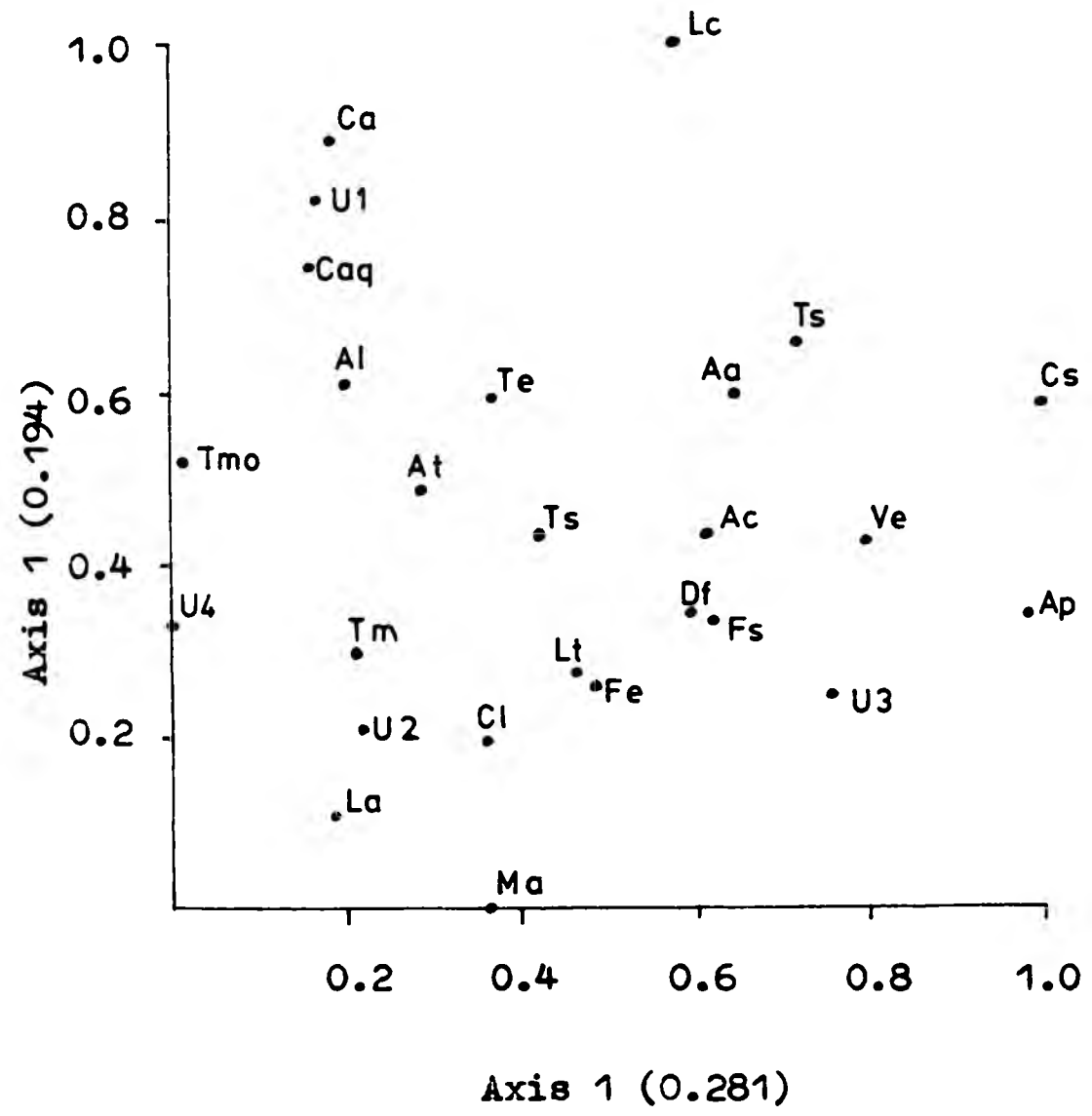


Figure 8.11. Reciprocal averaging ordination of the Ingoldian fungi based on their presence or absence in the twenty gravel pit lakes. Plot of axis one against axis two. Species initials refer to those given in Table 8.1. Eigenvalues are shown in parentheses.

8.7 and 8.11). In reciprocal averaging, the site and species ordinations are usually similar, particularly if there is a marked difference in species composition between sites. In the species ordination (Figure 8.11) only 9 of the 26 species occur in the same region of the plot of axes 1 and 2 as the lakes in Figure 8.7, suggesting that differences in species composition between the lakes are only minor and that particular species do not characterise particular lakes.

Because the 3 groups of lakes appear to differ slightly in their composition of Ingoldian fungi the relative levels of chemical, physical and biological parameters in each group of lakes were examined to see if there were any differences. Table 8.2 summarises the mean levels of these variables in the 3 groups of lakes. The Yateley West lakes (group A) differ from the others by having higher allochthonous litter inputs, fewer species of Ingoldian fungi, higher alkalinity and soluble reactive phosphorus and lower levels of calcium, magnesium and potassium. In contrast the Frimley lakes (group C) are generally smaller in size and have lower litter inputs, alkalinity and soluble reactive phosphorus but higher levels of chlorophyll a, conductivity, numbers of Ingoldian fungi, calcium, magnesium, sodium, potassium, chlorine and soluble nitrate. Clearly there are marked physical, chemical and biological differences between the different groups of lakes.

The axis scores of reciprocal averaging ordinations often reflect underlying environmental gradients (Cook, 1981; Greig-Smith, 1983) and correlations were made between the ordination axis scores of the lakes (for axes 1 & 2) and 22 environmental variables relating to the physical, chemical and biological features of the lakes. The results of the most significant correlations are listed in Table 8.3.

In reciprocal averaging, axis 1 usually reflects the 'strongest gradient' of any underlying environmental factor influencing species distribution, and successive

TABLE 8.2 Comparison of the mean values for selected physical, chemical and biotic parameters of the three groups of gravel pit lakes. Values are the means with standard errors for each group of lakes.

PARAMETER	YATELEY WEST (n=9)	YATELEY EAST (n=5)	FRIMLEY (n=6)
Lake area (ha)	1.94±0.56	2.63±0.68	0.95±0.13
Lake age (yrs)	23.11±1.73	17.80±3.60	21.83±2.02
Litter input ($g\ C\ m^{-2}\ yr^{-1}$)	13.77±3.78	5.89±3.45	4.40±2.34
No.of sp. of Ingoldian fungi	9.89±1.41	10.00±1.18	11.17±1.20
Chlorophyll *a* ($\mu g\ l^{-1}$)	19.30±4.80	19.65±1.87	32.90±8.03
Conductivity (μmhos, 25°C)	367.70±30.10	377.80±21.20	455.00±25.20
pH	7.92±0.11	8.00±0.02	7.80±0.05
Alkalinity ($m\ equiv\ l^{-1}$)	1.70±0.11	1.64±0.16	1.66±0.09
Calcium ($mg\ l^{-1}$)	30.18±2.26	33.42±4.14	35.28±2.39
Magnesium ($mg\ l^{-1}$)	3.77±0.16	6.48±0.45	7.28±0.20
Sodium ($mg\ l^{-1}$)	26.36±3.06	18.62±2.05	27.37±2.67
Potassium ($mg\ l^{-1}$)	5.36±0.37	5.74±0.70	7.42±0.29

PARAMETER	YATELEY WEST (n=9)	YATELEY EAST (n=5)	FRIMLEY (n=6)
Chlorine (mg l^{-1})	21.67±1.86	24.84±1.92	22.42±1.45
Soluble ortho-phosphate (PO_4-P; µg l^{-1})	8.63±1.56	5.62±1.06	3.45±0.66
Soluble nitrite + nitrate, NO_2-N + NO_3-N; µg l^{-1}	285.90±146.40	261.20±142.40	494.00±145.21

TABLE 8.3 Variables significantly correlated with axis score on the reciprocal averaging ordination of lakes. Figures in parentheses are the percentages of variance in axis scores accounted for by each variable.

VARIABLE	CORRELATION COEFFICIENT(r)	PROBABILITY
AXIS 1		
Magnesium	+0.74 (55)	P<0.001
Allochthonous litter input	-0.57 (33)	P<0.01
Soluble phosphate	-0.56 (31)	P<0.05
Divalent to monovalent cation ratio	+0.50 (25)	P<0.05
AXIS 2		
No. of fungal species	+0.54 (29)	P<0.05
Soluble phosphate	-0.48 (23)	P<0.05
Potassium	+0.45 (20)	P<0.05

axes usually reflect less strongly marked gradients. The analysis revealed that there was a highly significant correlation ($r = 0.74$, $P < 0.001$) between ordination axis 1 score for each lake and mean levels of magnesium in the lake water, the correlation accounting for more than 55% of the variation. (The relationship between axis 1 score and magnesium levels in the lakes is shown diagrammatically in Figure 8.12).

Inputs of allochthonous litter were also correlated with lake position on axis 1 ($r = -0.57$, $P < 0.01$) with higher inputs into Yateley West than Yateley East or Frimley (Table 8.2). Soluble reactive phosphorus ($r = -0.56$, $P < 0.05$) and divalent, monovalent cation ratio ($r = 0.50$, $P < 0.05$) were also correlated with axis 1 score, although they accounted for only 31 and 25% of the variation, respectively.

Number of fungal species, soluble reactive phosphorus and potassium were all significantly correlated ($P < 0.05$) with lake positions on axis 2, although no variable accounted for more than 30% of the variation.

8.3.3 Fungal biomass on allochthonous litter in the different lakes

Table 8.4 shows the mean levels of ergosterol in the 3 groups of lakes during February, May and August. In the Yateley West group of lakes ergosterol levels declined from 14.57 ± 5.19 $\mu g\ g^{-1}$ in February to 11.77 ± 2.31 in May and 6.02 ± 2.09 in August. In contrast, the February levels in Yateley East were much lower (0.96 ± 0.26 $\mu g\ g^{-1}$) but rose to 10.50 ± 3.14 in May before falling to 7.83 ± 3.24 in August. There was a similar trend for the Frimley lakes, with a low level of ergosterol in February, a peak level in May, followed by a fall in August. Overall, ergosterol levels were higher in the Yateley West lakes (10.79 ± 2.06) than in Yateley East (6.43 ± 1.82) or Frimley (6.08 ± 1.22) although these differences were not statistically significant ($P > 0.05$).

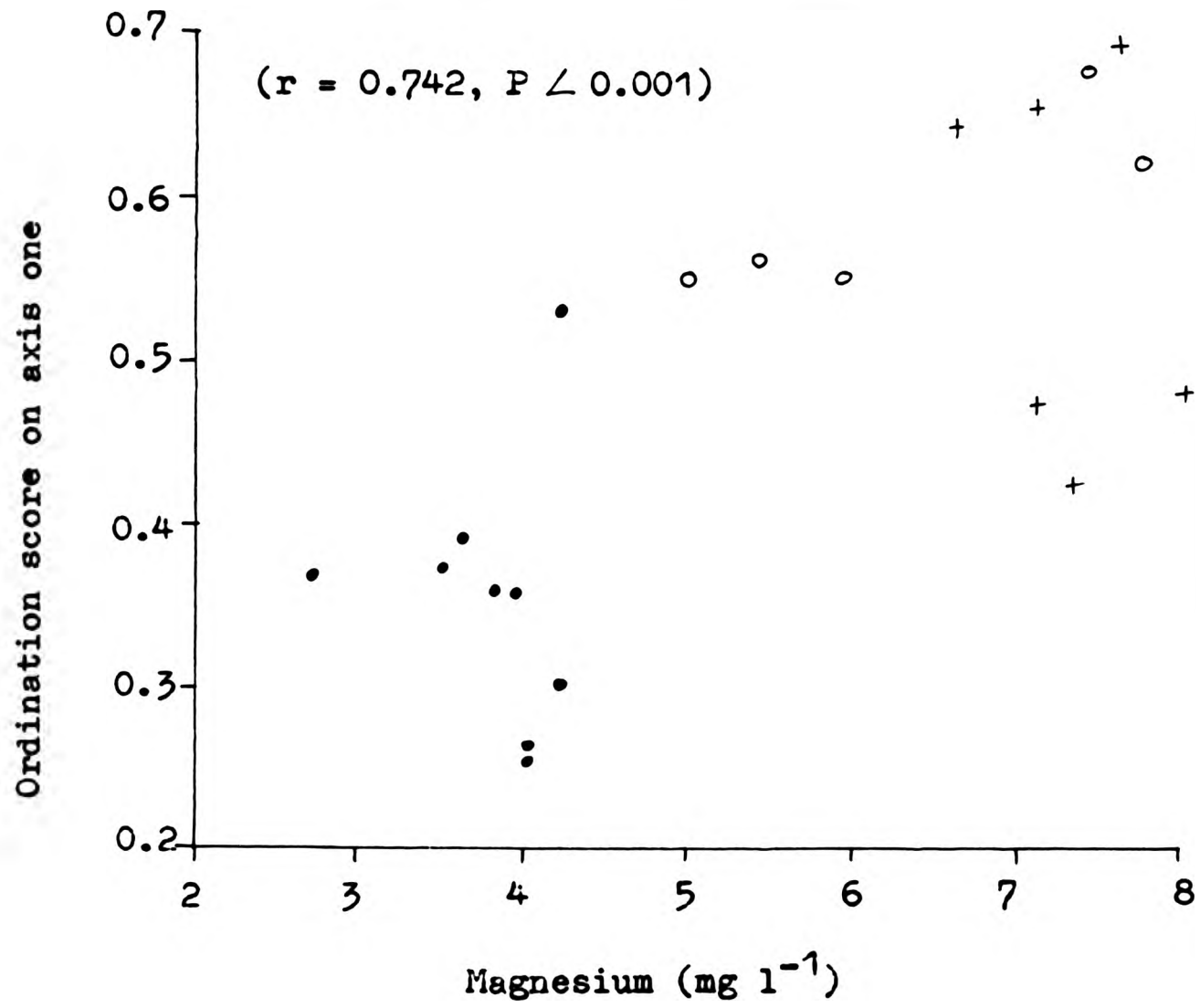


Figure 8.12. Comparison between the magnesium levels in the water and the score on axis one of the reciprocal averaging ordination of Ingoldian fungi in the gravel pit lakes.

KEY: Yateley West •
Yateley East o
Frimley +

TABLE 8.4 Ergosterol levels ($\mu g\ g^{-1}$ leaf oven dry weight, 80°C) in three groups of lakes during February, May and August. Each value is the mean for each group of lakes together with the standard error.

LAKE GROUP	FEBRUARY	MAY	AUGUST	OVERALL
Yateley (West) (n=9)	14.57±5.19	11.77±2.31	6.02±2.09	10.79±2.06
Yateley(East) (n=4)*	0.96±0.26	10.50±3.14	7.83±3.24	6.43±1.82
Frimley (n=6)	2.15±0.74	8.46±1.75	7.64±2.61	6.08±1.22

*(n=4 Yateley East 14 not sampled for ergosterol)

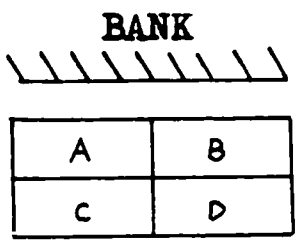
For all lakes the measured ergosterol levels ranged between 0.47 µg g^{-1} and 44.41 µg g^{-1} with a mean and standard error of 7.98 ± 1.14 (n = 57). The lowest mean level was recorded in Frimley 21A and the highest in Yateley West 3. Mean ergosterol levels in the lakes were only weakly correlated with lake ordination axis 1 score ($r = -0.44$, $0.05 < P < 0.10$) suggesting that the communities of Ingoldian fungi observed sporulating on allochthonous litter in different lakes were poor indicators of the levels of ergosterol in the litter.

8.3.4 Fungi on allochthonous litter in a single lake

Table 8.5 shows the percentage frequency of Ingoldian fungi sporulating on allochthonous litter retrieved from 4 sites in Yateley 4 in June 1984. Only Anguillospora longissima, Clavariopsis aquatica, Lunulospora curvula and Dimorphospora foliicola were found at all four sites in the lake and no species were encountered which had not previously been detected in litter samples from the lake (Table 8.1).

The results of reciprocal averaging ordinations based on the frequencies of spores are presented in Figures 8.13a-d. The scatter diagram of sample scores on axis 1 and 2 is shown in Figure 8.13a, and reveals a good separation of the 4 sites, the positions of the 4 samples at each site being linked together. Sites 1, 2 and 4 are distinctly separated from each other and there is a reasonable grouping of samples within each site, particularly for site 2. Site 3 exhibits some overlap with site 1, but the low numbers of species and spores in site 3 samples has resulted in a wide scatter of this group of samples on the plot, with samples A and C from site 3 showing little similarity to each other, or to samples B and D. Scatter diagrams using other axis combinations (Figure 8.13b-c) showed fewer differences between the sites than axis 1 and axis 2, although on all three scatter diagrams site 2 was distinct from sites 1

TABLE 8.5 Frequencies (percentages) of Ingoldian fungi sporulating on allochthonous litter retrieved from 4 sites in Yateley 4 in June 1984. The letters A-D refer to a group of contiguous quadrats at each site. Nomenclature follows Webster and Descals, 1981.



SPECIES	SITE 1			
	A	B	C	D
1 Anguillospora longissima(Al)	16.2	42.2	34.1	76.2
2 Clavariopsis aquatica(Ca)	32.3	39.1	29.5	6.3
3 Alatospora acuminata(Aa)	5.1	4.3	1.2	-
4 Lunulospora curvula(Lc)	-	-	32.6	-
5 Tetrachaetum elegans(Te)	2.8	-	-	-
6 Flagellospora curvula(Fc)	-	-	-	-
7 Lemonniera aquatica(La)	1.1	-	0.6	-
8 Anguillospora sp(Asp)	-	-	-	-
9 Articulospora tetracladia(At)	-	-	-	-
10 Clavatospora stellata(Cs)	-	-	0.2	1.3
11 Clavatospora longibrachiata(Cl)	-	-	-	-
12 Tricladium splendens(Ts)	-	-	-	-
13 Tetracladium marchalianum(Tm)	-	-	-	-
14 Fusarium sp(Fs)	-	-	-	-
15 Dimorphospora foliicola(Df)	42.4	-	0.6	10.1
16 Enteromorpha sp(Es)	-	14.3	0.8	6.3
17 Unknown species (U1, U2, U3)				

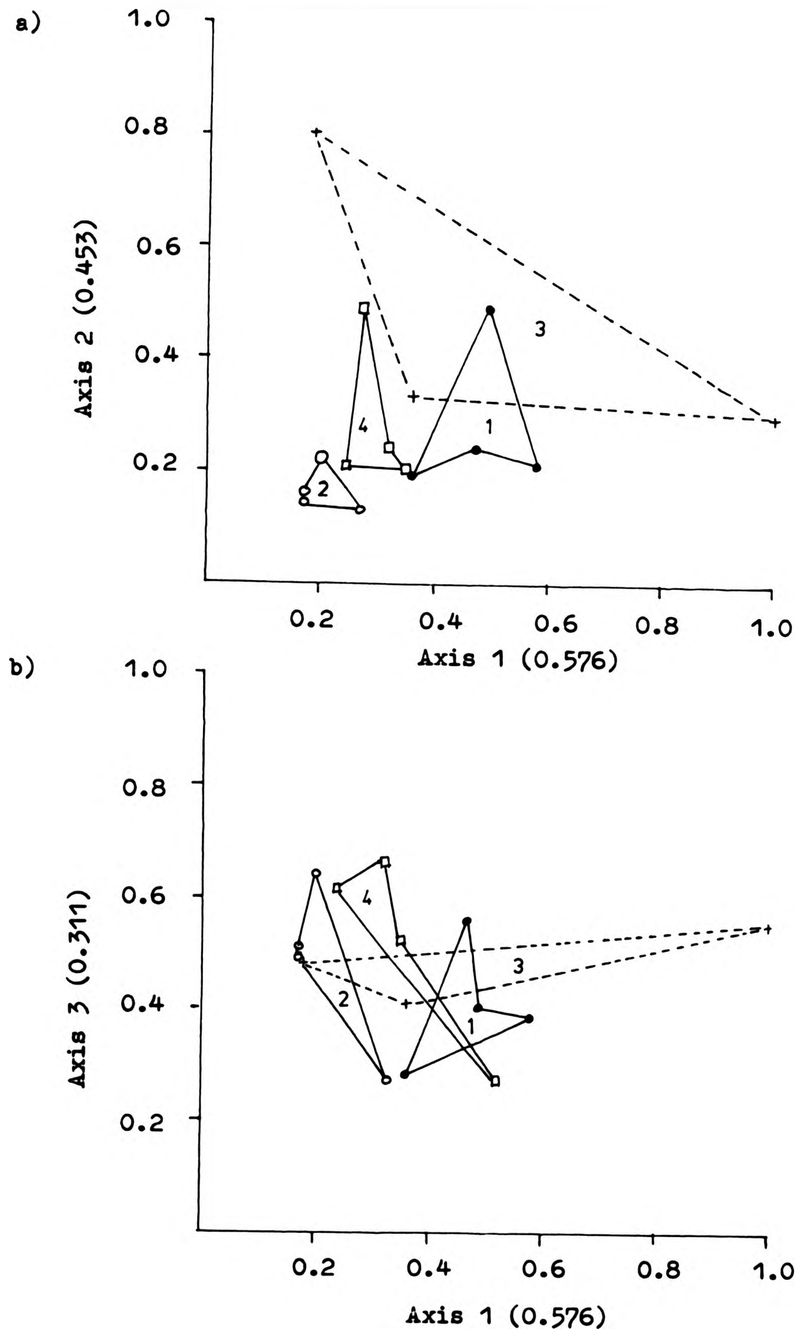
SPECIES	SITE 2			
	A	B	C	D
1 Anquillospora longissima(Al)	44.7	10.0	1.8	7.1
2 Clavariopsis aquatica(Ca)	2.8	-	5.4	-
3 Alatospora acuminata(Aa)	-	-	5.4	-
4 Lunulospora curvula(Lc)	-	-	0.3	-
5 Tetrachaetum elegans(Te)	0.7	-	-	-
6 Flagellospora curvula(Fc)	-	-	-	-
7 Lemonniera aquatica(La)	-	-	-	-
8 Anquillospora sp(Asp)	-	-	-	-
9 Articulospora tetracladia(At)	-	-	-	-
10 Clavatospora stellata(Cs)	-	-	-	-
11 Clavatospora longibrachiata(Cl)	-	-	1.0	-
12 Tricladium splendens(Ts)	-	-	-	-
13 Tetracladium marchalianum(Tm)	-	-	0.3	-
14 Fusarium sp(Fs)	-	-	-	-
15 Dimorphospora foliicola(Df)	14.9	64.6	85.4	58.3
16 Enteromorpha sp(Es)	36.9	24.6	-	34.5
17 Unknown species (Ul, U2, U3)		$(0.8)_1(0.3)_3$		

SPECIES	SITE 3			
	A	B	C	D
1 Anquillospora longissima(Al)	37.5	-	-	-
2 Clavariopsis aquatica(Ca)	12.5	100	-	100
3 Alatospora acuminata(Aa)	-	-	-	-
4 Lunulospora curvula(Lc)	12.5	-	75.2	-
5 Tetrachaetum elegans(Te)	-	-	-	-
6 Flagellospora curvula(Fc)	12.5	-	-	-
7 Lemonniera aquatica(La)	-	-	-	-
8 Anquillospora sp(Asp)	-	-	-	-
9 Articulospora tetracladia(At)	-	-	-	-
10 Clavatospora stellata(Cs)	-	-	-	-
11 Clavatospora longibrachiata(Cl)	-	-	-	-
12 Tricladium splendens(Ts)	-	-	-	-
13 Tetracladium marchalianum(Tm)	-	-	-	-
14 Fusarium sp(Fs)	-	-	-	-
15 Dimorphospora foliicola(Df)	25	-	24.8	-
16 Enteromorpha sp(Es)	-	-	-	-
17 Unknown species (U1, U2, U3)				

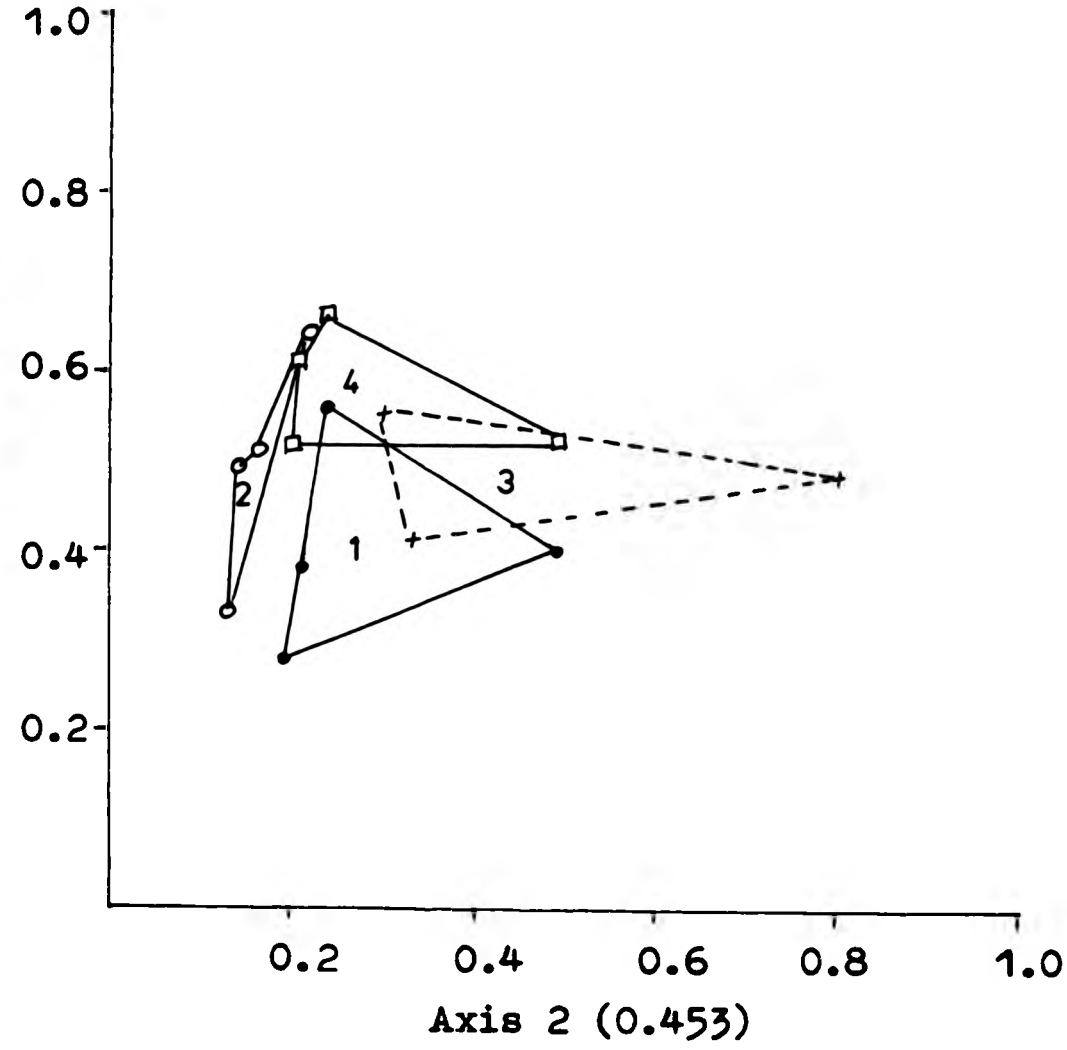
SPECIES	SITE 4			
	A	B	C	D
1 Anquillospora longissima(Al)	24.5	12.8	10.7	12.7
2 Clavariopsis aquatica(Ca)	13.4	10.2	8.6	4.7
3 Alatospora acuminata(Aa)	8.2	14.0	0.7	4.0
4 Lunulospora curvula(Lc)	0.3	4.1	35.3	-
5 Tetrachaetum elegans(Te)	2.4	15.0	4.1	10.0
6 Flagellospora curvula(Fc)	3.4	1.2	0.4	0.7
7 Lemonniera aquatica(La)	-	1.5	2.2	4.7
8 Anquillospora sp(Asp)	12.4	2.3	-	4.7
9 Articulospora tetracladia(At)	0.5	14.0	0.7	-
10 Clavatospora stellata(Cs)	-	-	-	-
11 Clavatospora longibrachiata(Cl)	-	1.2	-	-
12 Tricladium splendens(Ts)	4.5	-	-	-
13 Tetracladium marchalianum(Tm)	-	-	-	-
14 Fusarium sp(Fs)	-	0.3	0.4	1.3
15 Dimorphospora foliicola(Df)	25.8	20.4	34.6	56.7
16 Enteromorpha sp(Es)	4.7	2.9	1.9	0.7
17 Unknown species (U1, U2, U3)			$(0.4)_2$	

Figure 8.13. Reciprocal averaging ordinations of litter samples from sites 1-4 in Yateley 4 based on the frequencies of Ingoldian fungal spores. The eigenvalue for each axis are shown in parentheses on each axis.

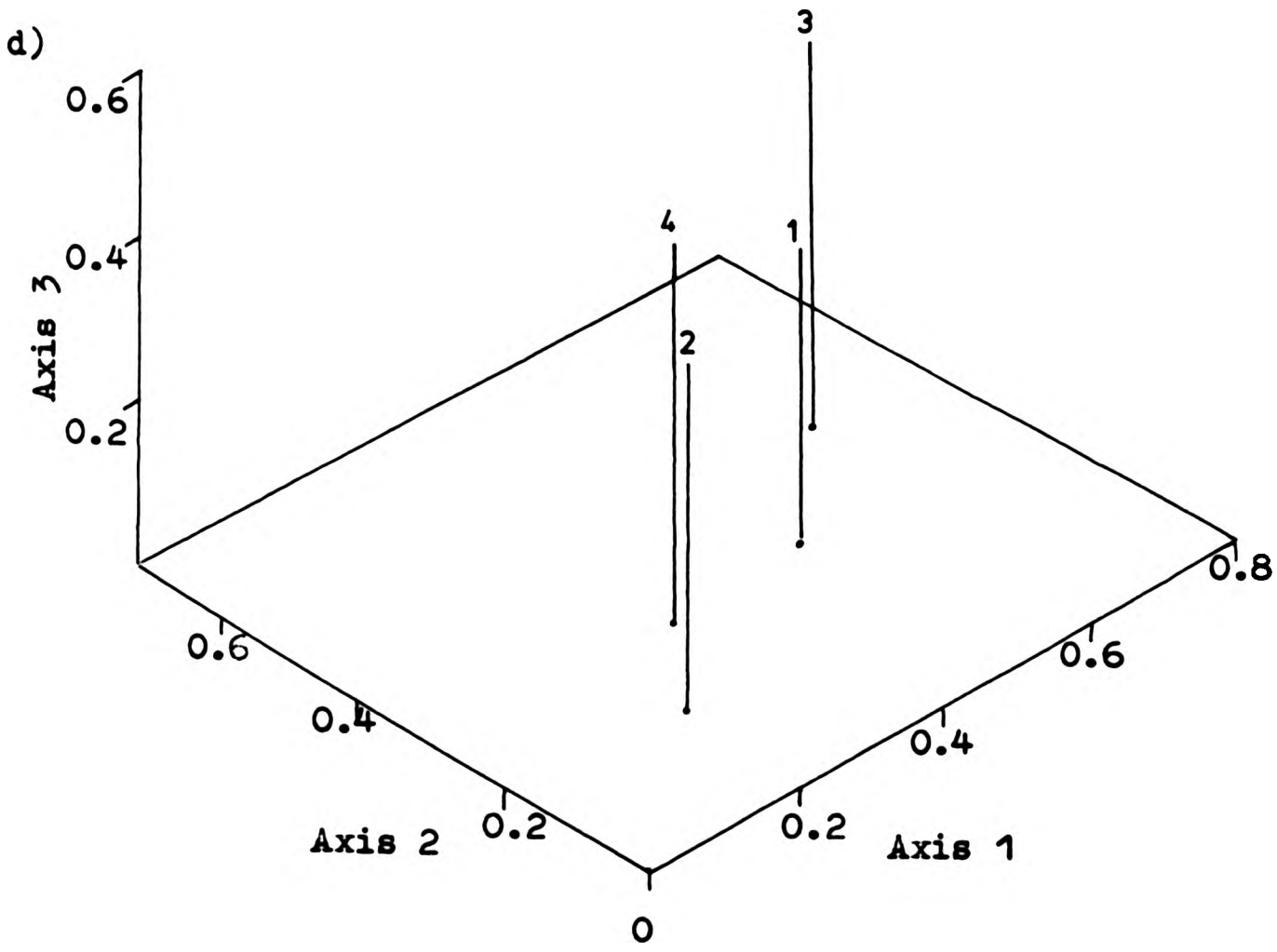
a) Axis 1 against axis 2
b) Axis 1 against axis 3
c) Axis 2 against axis 3
d) Ordination plot (axis 1 by axis 2 by axis 3) of the 4 site centroids (mean position for 4 subplots at each site)



c)


1.0
0.8
0.6
0.4
0.2
Axis 3 (0.311)
1
2
3
4
0.2
0.4
0.6
0.8
1.0
Axis 2 (0.453)

d)


3
4
1
2
0.6
0.4
0.2
Axis 3
0.8
0.6
0.4
0.2
Axis 2
0.2
0.4
0.6
0.8
Axis 1
0

and 4. Figure 8.13d summarises the relative positions of the 4 sites based on the mean axis position of each group of 4 samples on the first 3 axes of the ordination.

Figure 8.14 shows a scatter diagram of the reciprocal averaging species ordination for axes 1 and 2. There is a reasonable correspondance between the scatter plots of the species and sample ordinations (compare Figure 8.13a to 8.14) with most species scattered in the region of the ordination plot occupied by samples from sites 1, 2 and 4. The relative positions of the outlying species Clavariopsis aquatica (Ca) and Lunulospora curvula (Lc) reflects the wide scatter of samples from site 3 in which these species formed significant components of the flora.

Comparison of the species ordination for Yateley 4 (Figure 8.14) with that based on the presence or absence of Ingoldian fungi in the different lakes (Figure 8.11) reveals that in both ordinations Lunulospora curvula Clavatospora stellata and, to a lesser extent, Clavariopsis aquatica occur towards the periphery of the scatter diagrams. Similarities in the relative proximity of species on the 2 ordination plots include Tetracladium marchalianum and Clavatospora longibrachiata, Tricladium splendens and Alatospora acuminata, and Articulospora tetracladia and Tetrachaetum elegans.

8.3.5 Fungal biomass associated with allochthonous litter in a single lake

Table 8.6 summarises the data for hyphal biomass, ergosterol, spore output and species number for fungal populations at the four sites in Yateley 4. Hyphal biomass ranged from 0.04 to 1.28 mg g^{-1} with a mean value of 0.40 ± 0.09. Site 3 had the highest hyphal biomass overall (0.79 ± 0.27) but differences between sites were not significant ($P > 0.05$). Ergosterol ranged from 1.22 to 10.37 µg g^{-1} with a mean level of 5.46 ± 0.77. Site 1 had the highest mean level of ergosterol (7.42 ± 0.47 µg g^{-1}) and site 3 the lowest (3.20 ± 1.70

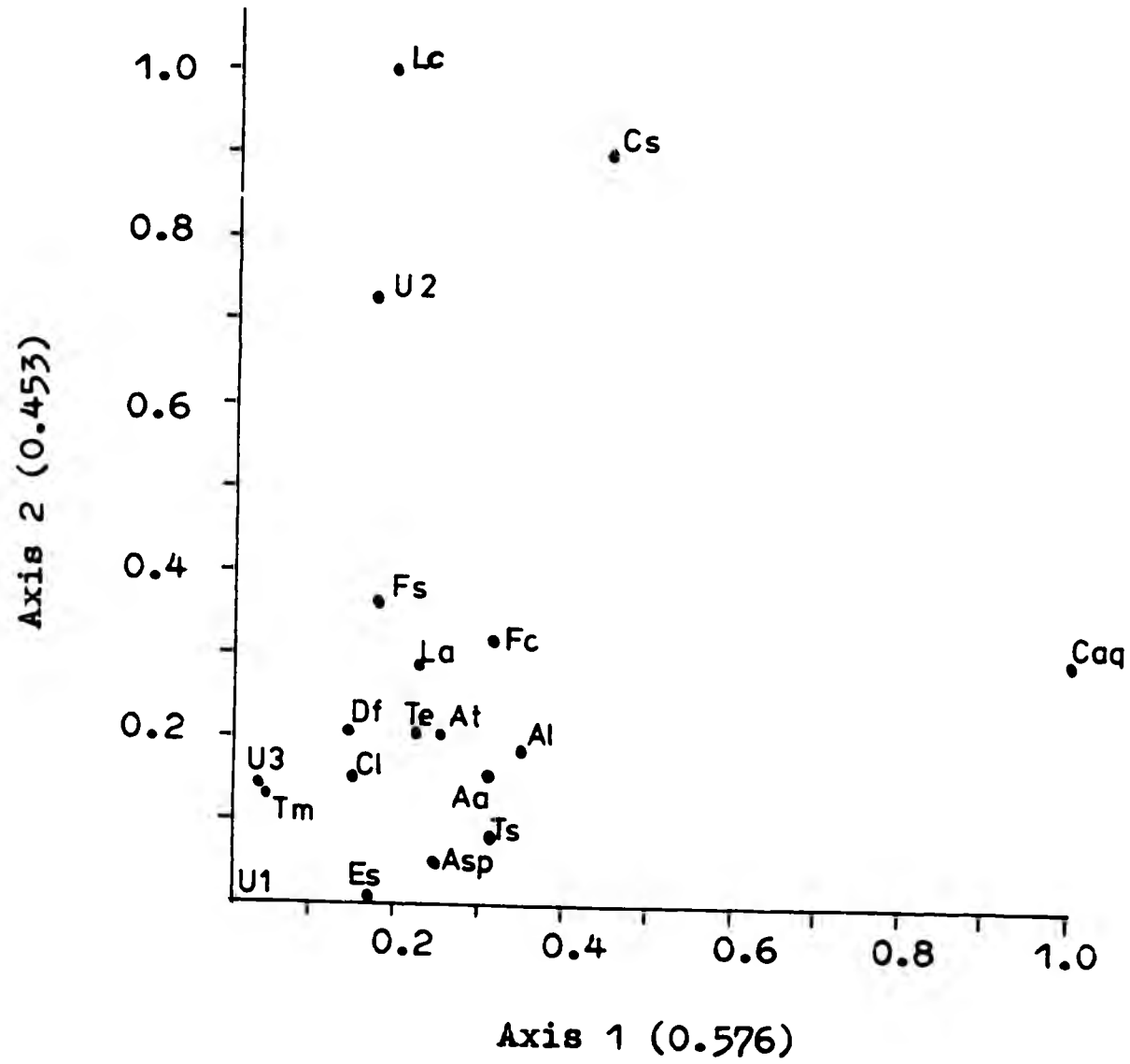


Figure 8.14. Reciprocal averaging ordination of Ingoldian fungi based on their frequency of sporulation at 16 sites in Yateley 4. The plot is of the first two axes of the ordination. Species initials refer to those given in Table 8.1. Eigenvalues are shown in parentheses.
The ordination is based on litter samples taken on June 29 1984.

TABLE 8.6 Hyphal biomass, ergosterol, number of species of Ingoldian fungi and number of spores on allochthonous litter at 4 sites in Yateley 4 in June. Values shown are means and standard errors.

SITE	HYPHAL*1 BIOMASS ($mg\ g^{-1}$)	ERGOSTEROL ($\mu g\ g^{-1}$)	NO. SPECIES	NO. FUNGAL SPORES (mg^{-1})
1	0.24±0.06	7.42±0.47	5.75±0.85	44.50±18.32
2	0.25±0.07	4.20±0.70	5.00±1.08	50.13±16.93
3	0.79±0.27	3.21±1.62	2.25±0.95	3.20±1.70
4	0.33±0.11	7.01±1.95	11.00±0.71	40.08±10.79

*1 Assumption: biovolume to biomass conversion factor 0.29 $mg\ cm^{-3}$.

$\mu g\ g^{-1}$) but there was no significant difference between any of the sites ($0.1 > P > 0.05$).

Hyphal biomass was not significantly correlated with ergosterol ($r = -0.13$), or with number of spores ($r = -0.39$). Ergosterol was significantly correlated with the number of fungal species ($r = 0.59$, $P < 0.05$) and the percentage frequency of Anguillospora longissima spores ($r = 0.62$, $P < 0.05$).

The number of fungal species differed between sites, the highest numbers of species being at site 4, and the lowest at site 3. The numbers of fungal spores obtained from litter samples, by aeration, ranged from 0.2×10^3 g^{-1} with significantly lower numbers ($P < 0.001$) of spores being detected in samples from site 3.

The ordination axis 1 scores for samples at the different sites showed a highly significant correlation with the percentage frequency of Clavariopsis aquatica spores ($r = 0.99$, $P < 0.001$) and a significant negative correlation with frequency of Dimorphospora foliicola spores ($r = -0.67$, $P < 0.01$). Scores on axis 3 showed a strong negative correlation with the frequency of Anguillospora longissima spores ($r = -0.83$, $P < 0.001$). Spore production by Anguillospora longissima was significantly correlated with spore production by Clavariopsis aquatica ($r = 0.74$, $P < 0.01$).

8.3.6 Cellulose decomposition in the lakes

Table 8.7 shows the results of the cellulose decomposition studies measuring loss in area and weight of cellulose filter papers and tensile strengh loss of twine. Loss of tensile strength varied significantly between lakes ($P < 0.01$) and between sites in the lakes ($P < 0.05$). Yateley West 4 had the lowest tensile strength loss ($60.7\% \pm 7.5$) and Frimley 18A the highest ($100\% \pm 0$). Loss of filter paper weight and area also varied significantly between lakes ($P < 0.001$) and sites ($P < 0.001$) and, in addition, there was a significant

TABLE 8.7 Percentage loss in tensile strength of twine, filter paper weight and area after submersion in 8 gravel pit lakes for 35 days. Values shown are means with standard errors.

LAKE NO.	% LOSS IN TENSILE STRENGTH	% LOSS IN FILTER WEIGHT*2	% LOSS IN FILTER AREA
2	83.4±7.1	44.7±18.5	48.4±19.1
4	60.7±7.5	44.6±5.0	13.5±11.20
8	94.5±5.5	46.1±6.6	31.8±8.1
11	73.5±4.1	23.4±7.7	10.5±2.5
12	98.4±1.0	65.4±11.7	59.8±17.2
18A*1	100±0	100±0	100±0
18B	61.1±13.2	42.7±14.4	20.7±19.8
19	78.7±5.0	46.5±13.4	24.3±18.9

*1 Tensile strength below detection limit of instrument therefore given at 100%.

*2 Weight loss adjusted for area loss.

interaction ($P < 0.001$) between lake and site, ie. the weight loss and decomposition at particular sites also depended on the particular lake. Mean weight loss in the different lakes varied between 42.7% ± 14.4 in Frimley 18B to 100% in Frimley 18A, although 5 of the 8 lakes had losses between 42 and 47%. Mean percentage weight loss of filters in the different lakes was significantly correlated with percentage loss in tensile strength of twine ($r = 0.84$, $P < 0.01$). There was no significant correlation between either weight loss or tensile strength loss in the different lakes with any of the physical, chemical or biological variables described previously, although there was a correlation between the tensile strength loss and the lake scores on ordination axis 3 ($r = -0.71$, $P < 0.05$).

Microbiological analysis of the cellulosic materials incubated in the lakes revealed colonisation by a range of different fungi. Direct examination of twine incubated in water revealed the presence of Ingoldian fungi, Fusarium sp., Saprolegnia sp. and Pythium spp. (Table 8.8). Sporulating fungi were observed on all twine samples except those from Frimley 18B. There was no obvious pattern of species in relation to the tensile strength losses of the samples, with Frimley 18A (100% tensile strength loss) having similar species to Yateley East 11 (73.5% tensile strength loss). The presence or absence of sporulating Ingoldian fungi did not appear to influence the tensile strength, with Yateley West 4 and Yateley East 12 both lacking Ingoldian fungi, yet having very different tensile strength losses (60.7% and 98.4%, respectively).

Examination of cellulose filter papers for evidence of colonisation by microfungi was confined to studies of samples from Yateley West 4 and 8. Both these lakes had different percent losses in tensile strength, filter paper area and filter paper weight (Table 8.7). The results of applying different detection methods for fungi on filter papers are shown in Table 8.9. Each method revealed a

TABLE 8.8 Fungi observed by direct observation on twine samples after 35 days submersion in 8 gravel pit lakes and 1 week in sterile distilled water.

FUNGAL SPECIES	YATELEY WEST LAKES			YATELEY EAST LAKES		FRIMLEY LAKES		
	2	4	8	11	12	18A	18B	19
Anquillospora longissima	+		+	+		+		+
Lunulospora curvula	+							
Fusarium sp	+			+		+		
Dimorphospora foliicola			+					
Unknown spp			+					+
Pythium spp		+	+	+	+	+		
Saprolegnia spp				+				

TABLE 8.9 Comparison of the fungal flora associated with filter papers after 35 days submersion in 2 gravel pit lakes (Yateley 4 and 8). The fungal flora was examined by direct observation, baiting with hemp seeds and plating of homogenates on agar media.

FUNGAL TAXA	DETECTION METHODS									
	BAITING		DIRECT OBSERVATION		CELLULOSE AGAR		POTATO DEXTROSE AGAR*		LAKE WATER AGAR	
	4	8	4	8	4	8	4	8	4	8
Penicillium							+	+		+
Acremonium						+		+		
Trichoderma						+	+	+		
Cladosporium						+	+	+	+	
Acremonium							+	+	+	
Epicoccum							+			
Fusarium				+	+	+				
Paecilomyces							+			
Alternaria							+		+	
Nigrospora								+		
Geotrichum							+			
Sphaeropsidales						+				
Tetracladium marchalianum			+							

	DETECTION METHODS									
FUNGAL TAXA	BAITING		DIRECT OBSERVATION		CELLULOSE AGAR		POTATO DEXTROSE AGAR*		LAKE WATER AGAR	
Pythium	+	+	+	+						
Achyla		+								
Saprolegnia	+			+						
Sterile mycelium			+	+		+	+		+	+
Mucorales							+	+		+

* Containing antibiotics, see Appendix 3.

different spectrum of microfungi, the greatest contrast being between the baiting and direct observation methods compared with plating of homogenates onto potato dextrose agar. Pythium, Saprolegnia, Achlya and Tetracladium marchalianum were only detected using baiting or direct observation, whereas Fusarium was detected by direct observation and plating on cellulose agar. Plating of homogenates revealed a wide range of fungi, particularly hyphomycetes, with most being detected on potato dextrose agar. Plate counts of fungi per cm^2 of filter paper based on dilutions spread onto 20 replicate plates of potato dextrose agar for each lake were $9.19 \times 10^4 \pm 2.28 \times 10^4$ for Yateley 4, and $6.35 \times 10^5 \pm 1.53 \times 10^5$ for Yateley 8. Yeasts represented 88.1% of the counts for Yateley 4 and 53.5% for Yateley 8.

8.4 Discussion

Ingoldian fungi form part of the characteristic mycoflora developing on allochthonous litter in running waters and, although they have been reported from lakes and ponds (Willen,1958; Suzuki & Nimura, 1960, 1961 a-b; Willoughby & Collins, 1966; Nilsson, 1964; Khan, 1981; Webster & Descals, 1981), few authors have examined their distribution and abundance on benthic litter in these habitats. Results of the present study indicate that these fungi are abundant on allochthonous litter in a number of lakes of quite recent origin. Examination of water samples from the lakes revealed the presence of of only a few Ingoldian fungal spores per litre, confirming observations made by Willoughby & Collins (1966) and Kahn (1981) also working on lakes. The abundance of spores of Ingoldian fungi in running waters has led to an emphasis of their importance in rivers and streams yet, despite low counts in lake water, they may have an equally important rôle in these ecosystems as incubation of leaf material from the gravel pits demonstrated that many leaves collected produced spores

of these fungi and aeration of leaf material collected in Yateley 4 in June produced up to 9.7×10^4 spores per gram of leaf material.

Communities of Ingoldian fungi on allochthonous litter in gravel pit lakes were similar to those found in rivers and streams in temperate climates (Chamier, 1980; Webster & Descals, 1981), although the number of species recorded was lower than for many rivers. Within the group of gravel pit lakes studied, reciprocal averaging ordination demonstrated that geographically separated groups of lakes could be differentiated by their communities of Ingoldian fungi present on allochthonous litter. It was further demonstrated that a significant proportion of the variation in the floristic elements between the different lakes could be accounted for by magnesium levels in the water. Previous studies have shown that streams of different chemical composition may have different Ingoldian fungi communities (Barlocher & Rosset, 1981; Wood-Eggenschwiler & Barlocher, 1983). However, the streams studied by these authors varied considerably in their water chemistry and hence differences in Ingoldian fungal communities were more marked than in the present study. Wood-Eggenschwiler & Barlocher (1983) demonstrated that levels of magnesium contributed towards explaining the species richness of Ingoldian fungi in streams, although other chemical features (conductivty, alkalinity, calcium and pH) were also important.

Allochthonous litter input into the different lakes also seemed to be related to the Ingoldian fungal flora of the lakes (from ordination and correlation analyses). Allochthonous litter inputs may influence fungal communities by 1) providing new subtrates for exploitation 2) providing a diversity of resource types (leaves of different species, twigs, fruits, seeds, bud scales) or even different resource sizes, eg., the composition of fungal communities have been shown to be significantly influenced by the size of the resource in both freshwater (Sanders & Anderson, 1979; Barlocher & Schweizer, 1983)

and terrestrial ecosystems (Zak & Parkinson, 1984; Wildman, 1987), or 3) enrichment of the water with inorganic (eg. PO_4-P) or organic (eg. amino acids, Bengtsson, 1982) components.

The diversity of allochthonous litter input has been the subject of a previous study (Barlocher & Rosset, 1981) but no clear relationship was established between hyphomycete communities and the diversity of litter inputs. The leaves of one deciduous tree species may be as suitable as any other for colonisation by Ingoldian fungi, although some evidence for substrate preference has been provided by Gönzczöl (1975), Bengtsson (1983), Rossi et al. (1983) and Butler & Subberkrop (1986).

Reciprocal averaging of the Ingoldian fungi on litter samples from a single lake demonstrated that species composition of samples from a single site were more similar to each other than to samples from other sites. Similar evidence for differences in the spatial distribution of Ingoldian fungal communities has been provided by Shearer & Webster (1985a) in studies on the River Teign. These authors found that there were different numbers of conidia in the water and different numbers of species on the leaf material at 3 different sites in the river. They attributed this to differences in water chemistry and riparian vegetation. In the present study there were no marked differences in the water chemistry or in trees and shrubs between sites, and approximately 80% of the litter sampled at each site consisted of a single leaf type (Salix sp.). However, observations made whilst estimating the contribution of allochthonous litter to the lake (Chapter 7) suggested that many leaves landing on the lake surface are carried by prevailing winds to particular areas within the lake before sinking. If different parts of the lake receive a disproportionate mass of leaf litter, then the disturbance brought about by the autumn leaf fall (Pugh, 1980) may be more localised in its impact on the resident microflora, thus possibly accounting for different fungal communities

between different sites in a lake.

Fungal biomass measured as ergosterol varied considerably, both between the lakes and within Yateley 4, yet no significant differences could be detected. Using a conversion factor of 2.19 mg of ergosterol per g fungal dry mass (Section 6.3.2) the biomass in different lakes ranged from 0.22-20.30 mg g^{-1} with an overall mean and standard error of 3.65 $\pm$ 0.52 mg g^{-1}. In Yateley 4 the range was 0.56-4.74 mg g^{-1} with a mean level of 2.50 $\pm$ 0.35. These estimates are similar to those reported by Lee, Howarth & Howes (1980) who obtained values ranging from 0.5-26 mg g^{-1} for fungal biomass in Spartina alterniflora Loisel using ergosterol as a marker. The temporal trends in ergosterol levels in the present study were also similar to those observed by Lee, Howarth & Howes (1980) and Newell, Fallon & Miller (1986) in their studies on Spartina.

With the study in Yateley 4, the biomass based on measuring fungal biovolume was much lower than that using ergosterol as a marker. Clearing and staining usually reveals pycnidia and internal hyphae, but these may have been absent or poorly stained on this particular leaf material. Yeasts and fungal spores present on the leaf surface were not included in the biovolume estimates, although they are known to contain ergosterol (Woods, 1971; and Weete & Laseter, 1974, respectively). A second possibility for the difference between estimates is the use of inappropriate conversion factors. It has previously been shown (Chapter 6) that conversion factors can vary considerably depending on fungal species, growth conditions and mycelial age. The conversion factors for biovolume to biomass can vary by a factor of 10, but for ergosterol to biomass it may be as high as 200 (Section 6.3). Differences in the fungal community, population age structure and resource state between sites are all factors which may influence conversion factors and mask any clear relationship between different techniques.

Studies on cellulose degradation in the lakes

demonstrated differences both between lakes and between sites within lakes, and there was good agreement between filter paper weight loss and decrease in tensile strength of twine ($r = 0.85$, $P < 0.01$). Egglishaw (1968, 1972) has demonstrated that water chemistry has a significant influence on the rate of degradation of cellulose in rivers. Egglishaw (1972) observed that additions of magnesium chloride to river water increased the breakdown of cellulose more than additions of calcium chloride or sodium phosphate, either singly or combined. Clearly, more information is needed on the influence of divalent cations on the distribution of fungi in freshwater, particularly as Chamier & Dixon (1983) and Rosset & Barlocher (1985) have demonstrated that calcium may influence growth and pectolytic enzyme production by Ingoldian fungi *in vitro*. Leaf pack size could also be important, eg., Reice (1974) and Benfield, Paul & Webster (1979) have shown that size of leaf packs may have a significant effect on rate of leaf breakdown in streams, and similar effects may occur in lakes. However, in the present study neither water chemistry nor the allochthonous litter input was significantly correlated with weight loss of filter papers or reduction in tensile strength of twine, although leaf pack size was not studied.

Ingoldian fungal community structure and fungal biomass were also not significantly correlated with loss in filter paper weight and twine tensile strength, in this study. Direct observation of filter paper and twine samples revealed the presence of Ingoldian fungi, although there did not appear to be any pattern of species occurrence with the observed weight or tensile strength losses. Egglishaw (1972) found that polycentric chytrids (Cladochytriaceae) were the dominant microorganisms colonising cotton in streams; these fungi were not observed in the present study. Other fungi recorded by direct observation in the present study included *Pythium* spp. and Park (1975, 1976) has demonstrated that a cellulolytic *Pythium* could be isolated from filter papers

exposed in a river. However, most fungi isolated by plating of filter paper homogenates were members of the soil and litter flora and Park (1974) found no evidence that 'soil fungi' and 'leaf fungi' actively colonised or broke down cellulose filter papers placed in a river, and he attributed their occurrence as being due to passive accumulation of propagules. In the present study quantitative differences in viable counts from homogenised filters from two lakes was large (x 7) and this suggests that 'soil' and 'leaf' fungi may have been active or that that the propagule numbers in the water or sediment of the 2 lakes were very different.

Barlocher & Kendrick (1974), Sultana (1976), Rossi et al. (1983) and Godfrey (1983) have demonstrated that some 'leaf' and 'soil' fungi may have the potential for cellulose degradation, and Godfrey & Mehdi (1983) and Godfrey (1983) have suggested that 'leaf' fungi may become active as cellulose decomposers in shallow lakes during summer months. The shallow nature of many gravel pit lakes means that thermal stratification is rare and Northcott (1981) has recorded temperatures up to 24 °C in Yateley 4 in summer months. Since leaf degradation is often slower in lakes than in rivers (Petersen & Cummins, 1974; Hodkinson, 1975b; Gasith & Lawacz, 1976; Hanlon, 1982) and spores and colonisation by Ingoldian fungi appears to be more sparse, then leaf fungi may have an active rôle in the decomposition of allochthonous litter in these lakes. It remains to be seen how these, and other fungi, fit into the sequence of fungal activity associated with decomposition.

CHAPTER 9. AN EXPERIMENTAL STUDY OF FUNGAL BIOMASS AND ACTIVITY AND THE DECOMPOSITION OF ALLOCHTHONOUS LEAF LITTER IN A SMALL FLOODED GRAVEL PIT

9.1 Introduction

Despite the lack of quantitative data on fungal biomass in freshwater ecosystems, several authors have considered that fungi play an important rôle in the 'conditioning' of allochthonous plant litter in that they render it more acceptable and palatable to aquatic invertebrates (Bärlocher & Kendrick, 1973a & b, 1975a; Cummins & Klug, 1979; Anderson & Sedell, 1979; Bärlocher, 1985). Additionally, exposure to increases in invertebtate grazing have been shown to influence the composition and biomass of microbial communities markedly in estuarine environments (White *et al*., 1979; Morrison & White, 1980; Smith *et al*., 1982) and a similar influence may occur in freshwater ecosystems.

The purpose of the present study was to examine leaf degradation and fungal biomass in more detail, particularly in relation to the influence of invertebrate grazing. By following changes in fungal biomass and activity on litter during leaf degradation, it was hoped that the importance of grazing could be assessed. Litter was enclosed in bags of different mesh size to control the numbers of colonising invertebrates. The study also provided the opportunity to compare the biomass methods developed in this work under field conditions and to examine the relationship of these with other variables associated with the decomposition of plant litter in freshwater.

9.2 Materials and methods

9.2.1 Site description

Experiments were carried out on Yateley 4 (a small flooded gravel pit descibed in detail in Chapter 7), as other aspects of its freshwater ecology had been studied and the annual allochthonous litter contribution to the lake was already known.

9.2.2 Experimental design

Freshly fallen or easily detached leaves of alder (*Alnus glutinosa* (L.) Gaertner), willow (*Salix fragilis* L.), willow 2 (*Salix cinerea* L.) and ash (*Fraxinus excelsior* L.) were collected between late October and mid November from trees around the margin of Yateley 4. On return to the laboratory, the leaves were air dried at room temperature and stored in black plastic sacks until required. Sub-samples were oven dried (80 ^{o}C) to calculate an air dry to oven dry conversion factor for use in weighing samples.

Four mesh sizes were used in the experiment :

Extra large (EL)	10 x 10 mm
Large (L)	2.5 x 2.5 mm
Medium (M)	0.98 x 0.98 mm
Fine (F)	0.26 x 0.26 mm

and these are shown in Figure 9.1. Plasic netting (Netlon Ltd, Blackburn) was used for the extra large mesh and polyester netting (Fryma Fabrics Ltd, Nottingham) for the other sizes. Using the mesh materials, litter bags (20 x 10 cm) were made and filled with whole air-dried leaves to give 5 g oven dry weight of leaf material in each bag. The bags were sealed with polyester thread and bias binding (100% polyester).

Five rectangular aluminium frames (1 x 0.75 x 0.75 m) were covered in 20 x 20 mm platic mesh (Netlon) and sealed

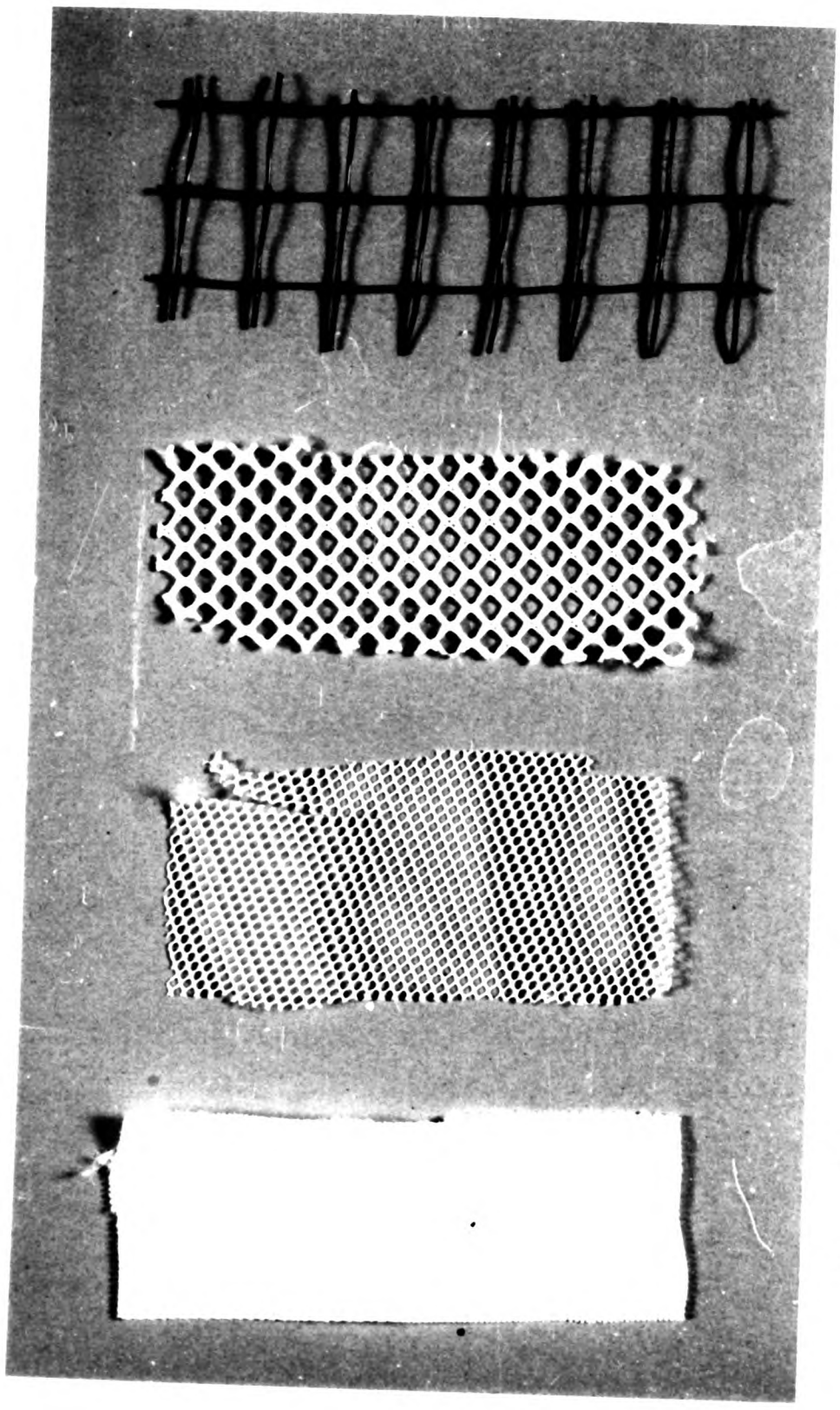
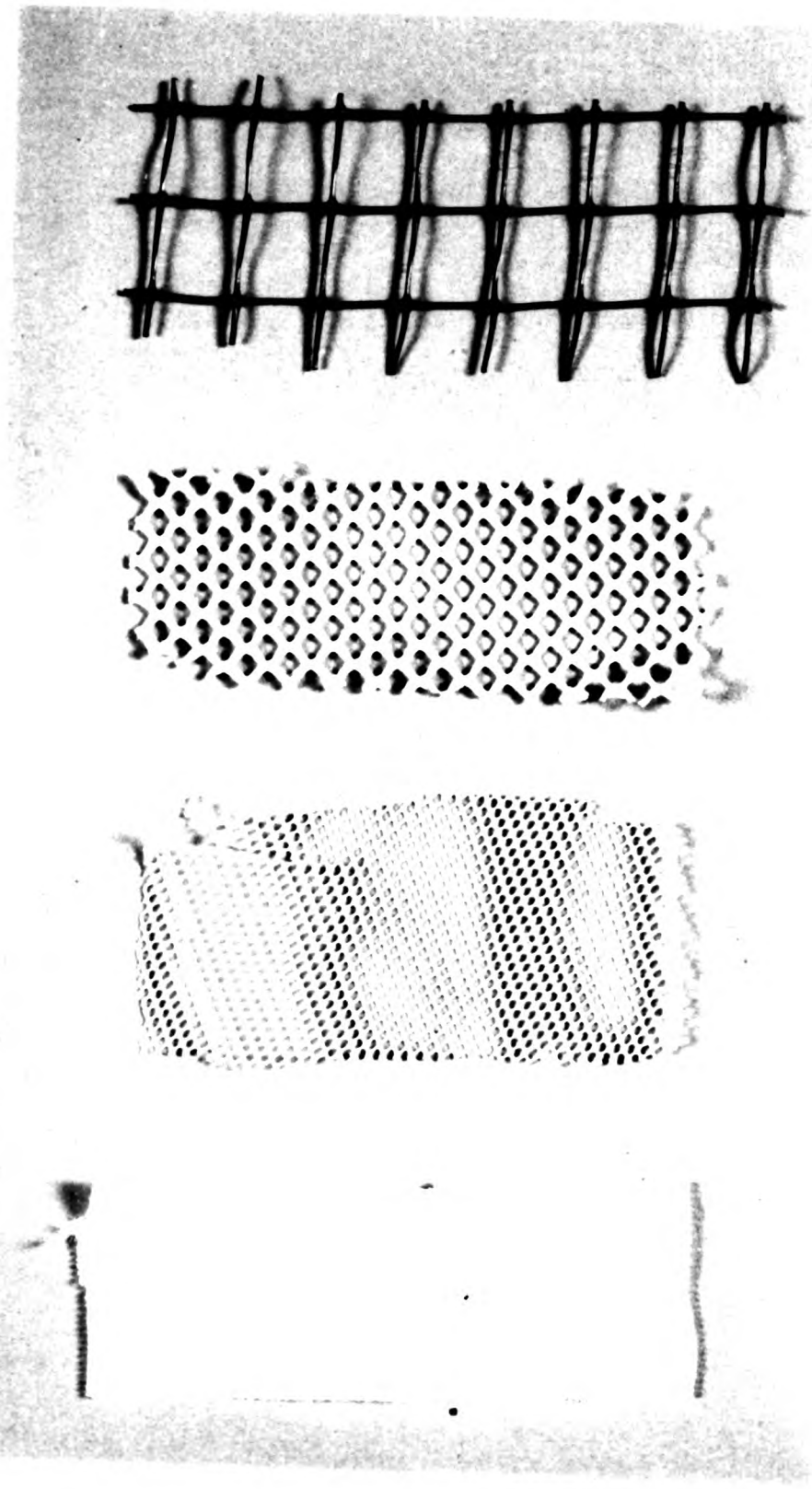


Figure 9.1. Photograph showing the 4 mesh sizes used in the study.



Extra large
(10 x 10 mm)

Large
(2.5 x 2.5 mm)

Medium
(0.98 x
0.98 mm)

Fine
(0.26 x
0.26 mm)

Figure 9.1. Photograph showing the 4 mesh sizes used in the study.

to form a cage. Four cylindrical clay drainage pipes (25 x 8 cm) were attached to the bottom corner of each cage so that each cylinder ran parallel to the longest side of the cage. The purpose of these pipes was to stabilise the position of the cage on the lake bottom and to reduce the possibility of litter bags being immersed in the mud. Each cage was filled through a hole in the top (30 x 20 cm), before being sealed with netting, with 162 litter bags comprising 76 bags of alder (19 x 4 mesh sizes), 76 bags of willow (19 x 4 mesh sizes), 5 bags of willow 2 (one mesh size, EL) and 5 bags of ash (one mesh size, EL).

Prior to the start of the experiment, the cages were left overnight at 16 ^{o}C and beside the lake for 1-2 hours to allow some absorption of moisture and reduce fragmentation. Losses due to fragmentation during transit and handling were estimated as 0-0.6% for alder, 0-2.4% for willow and 0.8% for willow 2 and ash.

The cages were put into the water of Yateley 4 on December 7, at 5 randomly determined sites around the lake perimeter (30, 559, 163, 270 and 313 metres from point A on Figure 7.1, in an anticlockwise direction; they were designated as sites 1-5, respectively). Each cage was positioned 2-3 m from the bank with the longest side parallel to it. Water depths at each site were 1.9, 1.1, 1.5, 2.1 and 1.1 m, respectively.

At 2 week intervals up to 24 weeks, and then at 28 and 40 weeks, 1 bag of each type of each mesh type of alder and willow was removed from each cage for microbiological analysis. For leaf pack weight loss, 1 bag of each type of litter and mesh size was removed after 2, 8, 18, 28 and 40 weeks submersion in the lake. When sampling, the bags were removed randomly from each cage using a 0.75 m helping hand device (Helping Hand, Tonbridge) inserted through the top of each cage. Bags were transferred to individual unused plastic freezer bags and held on ice during transit to the laboratory (1½-2 hours). In some cases the cages had to be lifted

off the lake bottom to facilitate removal of litter bags. This was done vertically from a boat and care was taken in the removal and replacement of the cage.

9.2.3 Environmental factors

On each visit to the lake water samples were taken from beside each cage using an open-closing bottle. Temperature, alkalinity, pH, dissolved oxygen, conductivity, sodium, potassium, calcium, magnesium, chlorine, soluble orthophosphate, soluble nitrate + nitrite and chlorophyll *a* were monitored at each site using standard methods described in Section 8.2. Maximum-minimum thermometers were attached to the cages at sites 1 and 4 to detect any temperature fluctuations between sample dates.

Fluctuations in the mean levels (5 sites combined) of environmental variables during the study period are shown in Figure 9.2 and mean values for each site in Table 9.1. Although there was considerable fluctuation in environmental variables between sample dates, the mean levels and variability (coefficient of variation) of each site were similar (Table 9.1).

9.2.4 Handling of samples

On return to the laboratory, each litter bag was opened and sorted in an enamel tray in a laminar flow-hood. Leaf packs were separated after flooding in sterile lake water. Aquatic invertebrates were removed and preserved in 70% ethanol after counting. Leaf packs for weight loss determination were then placed on aluminium foil trays and dried to constant weight at 80 ^{o}C.

For microbial analysis, leaf discs (6 mm diameter) were cut from each leaf pack using flamed cork borers and transferred to sterile petri dishes containing 20 ml of sterile lake water. For each sample, 10 leaf discs were oven dried (80 ^{o}C) to constant weight and weighed

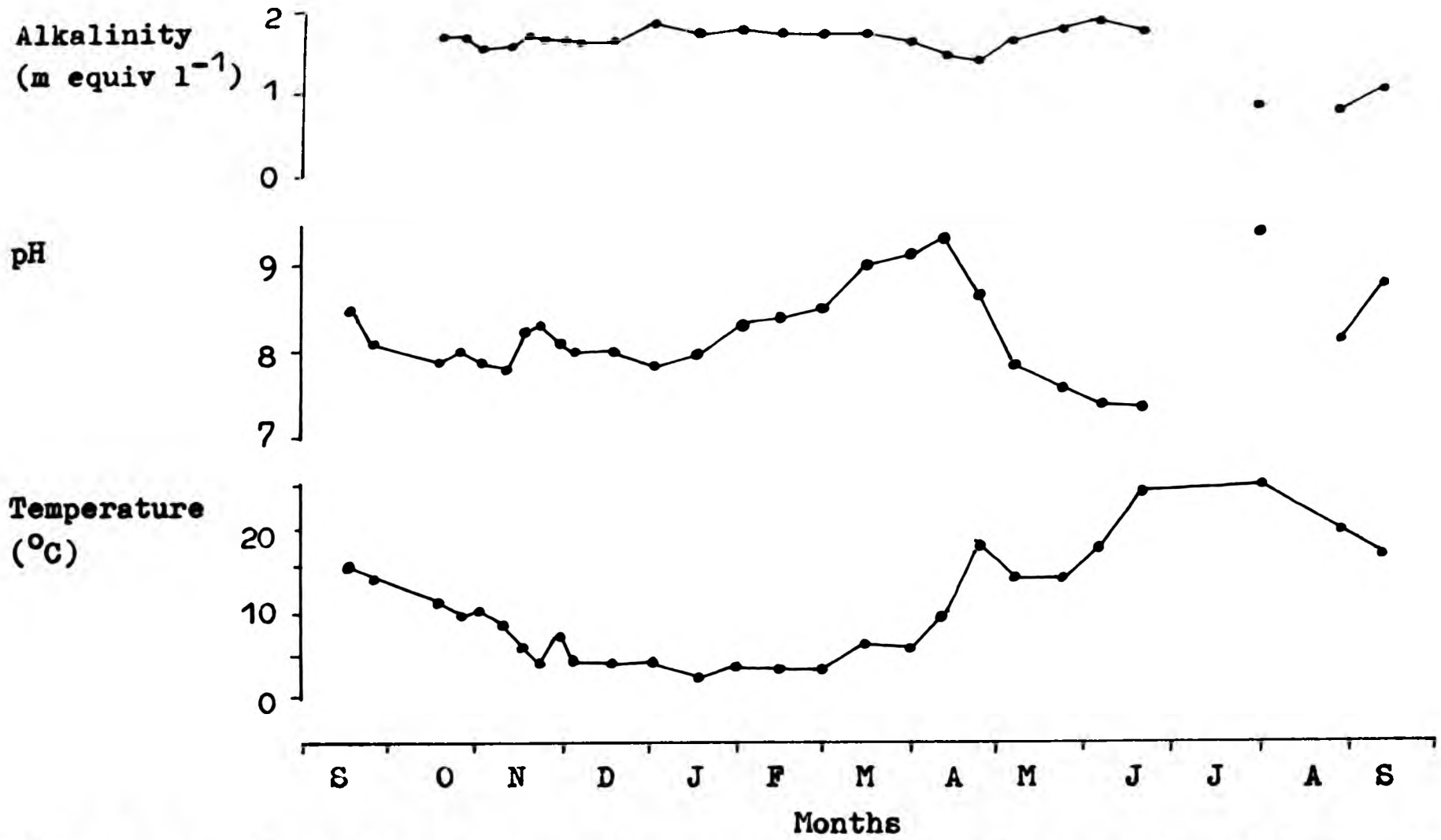
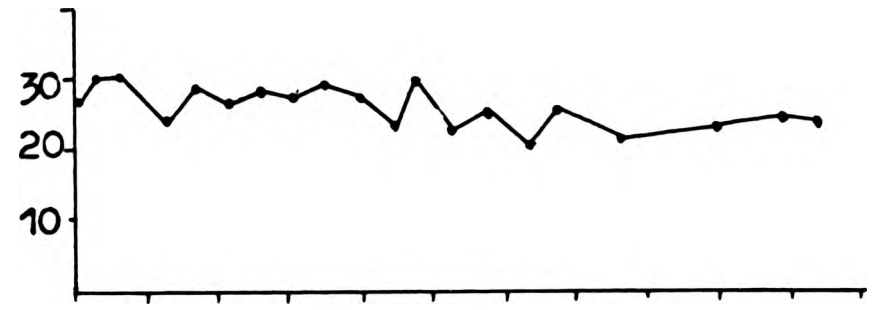
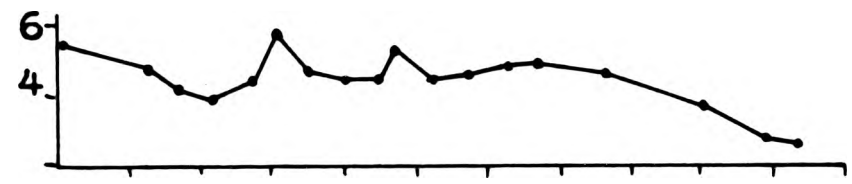


Figure 9.2. Fluctuations in 9 environmental variables (more overleaf) during the study period at Yateley 4. Each point represents the mean for 5 sites.

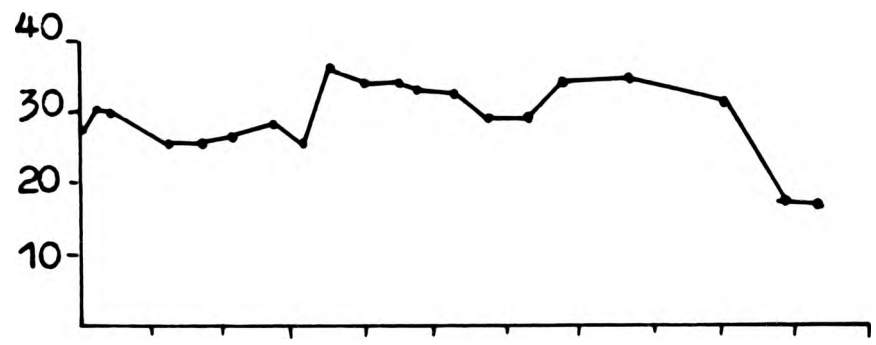
Sodium
(mg l^{-1})



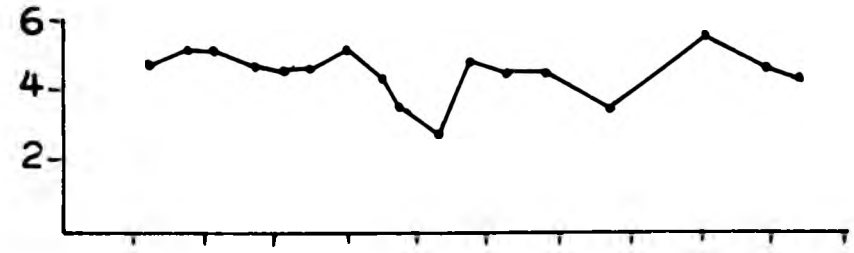
Potassium
(mg l^{-1})



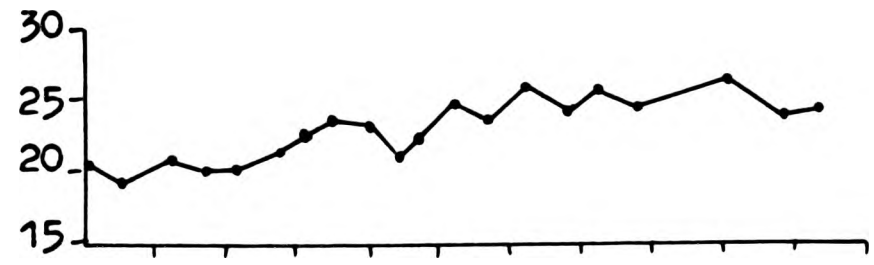
Calcium
(mg l^{-1})



Magnesium
(mg l^{-1})



Chlorine
(mg l^{-1})



Conductivity
(umhos 25 °C)

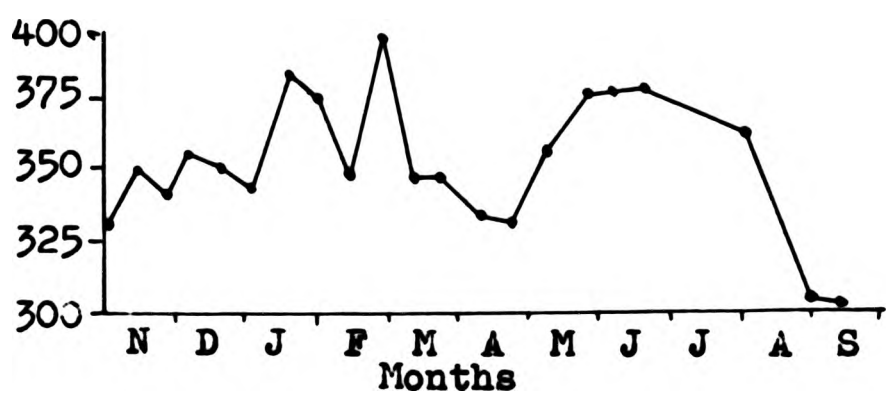


TABLE 9.1 Variation in environmental parameters in Yateley 4 during the study period (December - September). The values shown for each variable at the 5 sites are means with standard deviations (n=15). Coefficients of variation (expressed as a percentage) are shown in parenthesis.

VARIABLE	SITE				
	1	2	3	4	5
pH	8.29	8.37	8.21	8.21	8.27
	±0.59	±0.58	±0.57	±0.65	±0.67
	(7.2)	(6.9)	(7.0)	(7.9)	(8.1)
Alkalinity	1.62	1.63	1.61	1.61	1.57
(m equiv l^{-1})	±0.29	±0.33	±0.21	±0.26	±0.29
	(18.2)	(20.1)	(13.3)	(15.9)	(15.7)
Conductivity	335.1	357.4	359.7	359.4	356.3
µmhos(25°C)	±30.2	±31.7	±25.9	±27.5	±29.2
	(8.5)	(8.9)	(7.2)	(7.6)	(8.2)
Oxygen	104.4	105.2	102.8	103.7	103.2
%saturation	±14.3	±19.8	±13.6	±16.2	±20.8
	(14.1)	(18.9)	(13.1)	(15.6)	(20.1)
Calcium	30.9±6.1	29.3±5.9	29.7±5.1	30.2±4.2	29.6±4.8
mg l^{-1}	(19.6)	(20.2)	(17.0)	(13.9)	(16.3)
Magnesium	4.7±0.5	4.7±0.6	4.6±0.6	4.6±0.7	4.6±0.6
mg l^{-1}	(10)	(13.3)	(12.1)	(14.1)	(12.2)
Sodium	27.1±3.6	27.5±5.4	25.9±1.9	25.6±2.2	27.4±2.8
mg l^{-1}	(13.2)	(19.8)	(7.3)	(8.6)	(10.3)
Potassium	4.5±0.6	5.2±1.6	4.7±1.0	4.4±0.7	4.6±0.6
mg l^{-1}	(13.2)	(31.4)	(21.8)	(16.5)	(12.9)

VARIABLE	SITE				
	1	2	3	4	5
Soluble Phosphorus $\mu g\ l^{-1}$	3.9±4.9 (127)	2.7±2.9 (111)	2.6±3.2 (124)	2.7±3.1 (113)	4.0±5.8 (143)
Soluble Nitrate + Nitrite $\mu g\ l^{-1}$	52.4 ±51.6 (98.4)	53.7 ±35.7 (66.6)	53.8 ±32.6 (60.6)	49.4 ±43.2 (87.5)	51.3 ±43.5 (84.9)
Chlorphyll a $\mu g\ l^{-1}$	12.1±13.6 (113)	17.1±24.6 (144)	15.1±16.6 (110)	16.6±11.9 (72)	17.6±17.1 (96.9)

to the nearest µg using an electrobalance (Cahn Instruments, USA).

9.2.5 Microbial biomass and activity

9.2.5.1 ATP

ATP was measured to provide an indication of total microbial biomass. ATP was extracted from leaf material using the cold H_3PO_4, EDTA method of Karl & Craven (1980). This method was used because nucleotide extracts from aquatic environments low in inorganic phosphate are prone to degradation from alkaline phosphatase when traditional extraction methods (eg. H_2SO_4, EDTA) are used (Karl & Craven, 1980).

For each extraction, 10 leaf discs (6 mm diameter) were added to 5 ml of 1M H_3PO_4 and incubated at 4 °C for 1 hour. After incubation, 1 ml of 48 mM EDTA was added to chelate organic and inorganic ions which interfere with the luciferin luciferase reaction. The extracts were decanted into a 20 ml measuring cylinder and the pH adjusted to 7.8 with 4 M NaOH. The volume of each extract was adjusted to 15 ml with 20 mM Tris-HCl pH 7.8 and the extracts frozen at -20 °C until assayed. ATP standards were made up in the extracting solution, neutralised to pH 7.8 and frozen with each batch of samples. Internal standards were used with some leaf extracts to estimate extraction efficiency.

Samples were assayed for ATP using the luciferin luciferase enzyme method described in Section 6.2.5. Each sample was assayed with and without a known ATP standard and corrections for ATP losses and sample quenching calculated according to Jones (1979). Mean efficiency for all treatments of alder and willow was 54.2% (range 10.1-81.7) and 49.7% (range 15.1-83.4), respectively. Mean extraction efficiency from leaves in which known amounts of ATP were added before extraction was 41.2% for alder and 74.2% for willow.

ATP concentrations were corrected and expressed per g dry weight of leaf material.

9.2.5.2 Fluorescein diacetate (FDA) hydrolysis

Spectrophotometric assays for FDA hydrolysis have been used to estimate microbial activity in soil (Schnurer & Rosswall, 1982) and on leaf surfaces (Swisher & Carrol, 1980), and staining by FDA forms the basis of methods for assaying numbers of active bacteria (Lundgren, 1981) and lengths of metabolically active hyphae (Soderstrom, 1977; Ingham & Klein, 1982, 1984). A spectrophotometric assay was used in the present work as an indication of microbial activity on leaf litter although it is realised that the method is likely to underestimate the metabolic activity of heterotrophic gram negative bacteria (Chrzanowski et al., 1984).

Ten leaf discs (6 mm diameter) of each litter sample were added to boiling tubes containing 10 ml of 60 mM sodium phosphate buffer, pH7.7 and 8 $\mu g\ ml^{-1}$ of FDA. Tubes were shaken for 60 minutes at 180 rev min^{-1} at room temperature (23-25 ^{o}C) and then transferred immediately to a bed of ice where the contents of each tube were allowed to settle for 5 minutes. Aliquots (2 ml) of solution were withdrawn from each tube and mixed with 1 ml of acetone, vortexed for 10 seconds and allowed to stand before reading the absorbance at 492 nm against a blank sample run with no leaf discs. Results were expressed as absorbance units (492 nm) per 10 leaf discs.

9.2.6 Fungal biomass

9.2.6.1 Ergosterol

Ergosterol was extracted from leaf material of alder and willow after 8, 18 and 28 weeks submersion in Yateley 4. The method for extraction and quantification

of ergosterol is described in Section 5.2.

9.2.6.2 Whole leaf clearing and staining

The clearing and staining method developed in Chapter 3 (Table 3.1) was used to estimate fungal biomass in leaf litter of alder and willow after 8, 18 and 28 weeks submersion. Five random microscope fields (x 400) were examined on 10 leaf discs (6 mm diameter) for each sample. Hyphal lengths were estimated using the intersection method with a gridded graticule (10 x 10 units). Pycnidial biovolume was estimated by measuring the mean diameter and assuming the biovolume approximated to the volume of a sphere ($4/3\pi r^3$). Hyphal diameters measured in agar films of each sample (see Section 9.2.6.3) were used to convert hyphal lengths to biovolume. Biovolumes were then converted to biomass, assuming a biovolume to biomass conversion factor of 0.29 g cm^{-3} (Section 6.3).

9.2.6.3 Agar film

Sample bags of litter were taken from all sites after 8, 18 and 28 weeks submersion and from site 1 at each sample date between 2 and 28 weeks. For each sample, 25 discs (6 mm diameter) were incubated in the dark for 4 hours at 20 °C in 2 ml of 0.1 M Tris-HCl, pH 7.6, containing 1 mg each of NADH and NADPH and 0.4 mg each of the tetrazolium salts INT and MTT (see Section 4.2 for details). These conditions and concentrations were considered optimal for demonstrating metabolically active hyphae (see Chapter 4). After incubation, 3 drops of formalin (40% v/v) were added to fix the samples.

Leaf discs were homogenised for 1 minute in 10 ml of filtered distilled water (0.45 µm membrane filter, Millipore Corp.) at setting 5 in a VIRTIS 45 homogeniser Section 4.2.1). The homogenate together with distilled water used to rinse the flask were filtered through

nylon mesh (250 µm, Henry Simon Ltd) and the volume measured. Five agar films were produced for each sample as described in Table 4.1 and these were stained and mounted in phenolic analine blue (Jones & Mollison, 1948) before examination.

Total hyphae were observed at x 400 magnification with phase contrast illumination and those stained by tetrazolium salts by bright field. Hyphal lengths were estimated in 2 fields of view on each film by the intercept method, using a gridded eyepiece graticule (Olson, 1950) and expressed as m g^{-1} leaf dry weight. Five hyphal diameters were measured for each leaf sample and the biovolume calculated and converted to biomass, assuming a biovolume to biomass conversion factor of 0.29 g cm^{-3} (Section 6.3.1).

9.2.6.4 Agar film corrected

Agar film estimates of hyphal length were corrected for inefficiency of homogenisation by estimating the proportion of leaf material in each sample which was retained by 250 µm mesh (Section 4.2.3.2). Retained material was dried (80 °C) to constant weight and expressed as a proportion of the estimated dry weight of leaf material homogenised. Biomass estimates were then corrected to account for all leaf material in a sample.

9.2.7 Viable counts

Viable counts were obtained for mycelial fungi, yeasts, total aerobic bacteria and pectinolytic bacteria on alder and willow litter samples from site 1 for all sample dates up to 28 weeks submersion. For each sample, 10 leaf discs (6 mm diameter) were homogenised (VIRTIS 45, setting 5) for 1 minute in 10 ml of sterile lake water and the homogenate was serially diluted (10 fold) to 10^{-8} in sterile lake water. At appropriate dilutions, 0.1 ml was spread onto duplicate plates of nutrient agar

(Oxoid) for total bacteria, pectin agar (Hankin et al., 1971; Appendix 3) for pectinolytic bacteria and potato dextrose agar containing antibiotics (Appendix 3) for mycelial fungi and yeasts. Plates for bacterial counts were incubated at 37 °C for 24–48 hours and those for fungi at 12 °C for 1–2 weeks. Results were expressed as number of colony forming units per g leaf dry weight. Sporulating fungal colonies were identified to genera and yeasts catagorised on the basis of colony colour.

After 8, 22 and 28 weeks submergence, 25 leaf discs (6 mm diameter) of each alder and willow sample were plated on potato dextrose agar containing antibiotics. Plates were incubated for 1–2 weeks at 12 °C and the percent frequency calculated for the presence of different fungal species on the leaf discs.

9.2.8 Fungal spore production

9.2.8.1 Ingoldian fungi

Spore production by Ingoldian fungi on allochthonous leaf litter in aquatic ecosystems is likely to reflect a whole series of variables influencing the biomass and activity of mycelium. These may include temperature, water and substrate quality, turbulence, fungal species composition, other microorganisms and invertebrate grazing. Because of these factors, spore numbers are unlikely to reflect the amount of fungal biomass in a substrate but may be indicative of a change in metabolic activity of at least part of the mycelium.

Methods of quantifying spore production from allochthonous leaf litter have included membrane filtration (Bärlocher & Schweizer, 1983), sedimentation tubes (Chamier & Dixon, 1982a), Sedgewick rafter chamber (Willoughby, 1978), haemocytometer (Kahn, 1981) and direct counts on leaf material (Shearer & Lane, 1983). Attempts to use these methods with samples from litter bags were unsuccessful because of the low numbers of

spores, therefore an alternative method was used.

For each sample, 100 leaf discs (6 mm diameter) were transferred to 20 ml of sterile lake water and incubated for 5 days at 6 °C for samples taken after 2-16 weeks submersion, 12 °C after 18-24 weeks submersion or 23-25 °C after 28 and 40 weeks submersion. These temperatures were chosen to correspond to those of the lake water at each sample week (Figure 9.2). The contents of each petri dish were mixed for 5 minutes each day (60 rev min^{-1}, orbital shaker) to disperse any 'clumps' of leaf discs or spores. After 5 days each plate was examined at x 100 magnification for 6 minutes and any spores of Ingoldian fungi counted. In most cases the majority of the plate could be scanned in this time. Preliminary trials with leaf discs gave no more than 10% error between replicate samples in numbers of spores counted per unit time, and at least 90% of the species present were observed during 6 minutes observation. Where no species or spores were observed in a six minute period, a further 4 minutes search was undertaken. Any species observed during this time were given a spore count of 1 regardless of the number of spores seen. The presence of other fungi in the plates was also noted during observation. Spore counts were expressed as relative counts per 100 leaf discs per 6 minute observation.

9.2.8.2 <u>'Aquatic' phycomycetes</u>

A semi-quantitative baiting method was used to obtain an indication of the presence of these fungi on leaf material. After counting spores for Ingoldian fungi, 10 sterile hemp seeds were added to each plate and incubation continued for 7 days under the same conditions as previously. The number of hemp seeds out of 10 which were colonised by these fungi were recorded for each sample.

9.2.9 Fungal biomass and activity in the lake sediment

Cores of sediment were taken in early June for comparison of sediment fungal biomass and activity with that associated with leaf material from litter bags.

Four cores of sediment (6 cm diameter) were extracted using a Gilson mud sampler at 2, 4, 6 and 8 m from the bank at three randomly selected sites around the lake margin. Cores were transferred to perspex cylinders (6 cm diameter) and transported to the laboratory on ice. The sediment in each core was extracted down to the underlying clay and the 4 samples from each site combined and sieved through 1 mm and 0.5 mm mesh (Endecotts Test Sieves Ltd). Stones and twigs were removed from the 1 mm mesh and the retained material on each mesh rinsed in sterile lake water.

The sediment material in 3 fractions ($>$ 1 mm, $<$1 mm and $>$0.5 mm, $<$ 0.5 mm) were re-suspended or adjusted to 500 ml in sterile lake water. Aliquots (20 ml) of the 9 suspensions (3 fractions x 3 sites) were filtered (GF/C, Whatman), dried (80 ^{o}C) and the weight of retained material calculated.

Three aliquots (2 ml) of each suspension were used to estimate FDA hydrolysis. Each aliquot was mixed with 8 ml of 75 mM sodium phosphate buffer, pH 7.6, containig 10 μg ml^{-1} FDA. The assay was performed as in Section 9.2.5.2 except that samples were filtered prior to reading absorbances. For agar films, 10 ml of each sample was centrifuged (2000 x g) and the pellet used for tetrazolium staining, homogenisation and agar film preparation as in Section 9.2.6.3. Fungi present in each sediment fraction were identified after diluting each suspension to 10^{-1}-10^{-2} and spread plating 0.2 ml on potato dextrose agar containing antibiotics, and lake water agar (Appendix 3). Incubation of aliquots of each suspension in sterile petri dishes at 18^{o}C for 7 days was used for detecting fungi by microscopic examination.

9.2.10 Data analysis

9.2.10.1 Leaf pack weight loss

The model most commonly used to express weight loss from litter bags, in both terrestrial and aquatic systems is:

$$W_t = W_0 \tfrac{1}{2} e^{-kt}$$

where W_t is the weight at time t, W_0 is the initial weight at time t = 0 and k is the decay coefficient for a specific litter type. The decay coefficients for the leaf material in bags of different mesh sizes were calculated by fitting a linear regression to $\ln(W_t/W_0)$ and t.

9.2.10.2 Fungal biomass and other factors

Data were analysed by analysis of variance for 2 factors (time and mesh size) for each leaf type. Data which was not normally distributed was transformed using suitable transformations ($\sqrt{n+1}$, $\log(n+1)$ or $-1/(n+1)$) before analysis. Relationships between biotic and abiotic variables were examined by correlations.

9.3 Results

9.3.1 Leaf breakdown

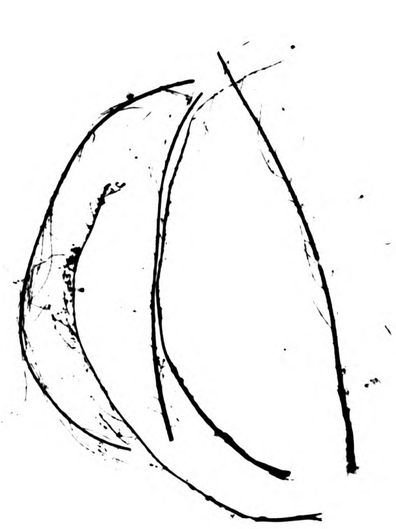
Figure 9.3 shows the appearance of litter bags after 40 weeks submersion in Yateley 4. Bags of all mesh sizes still contained leaf material and the bags had remained intact throughout the study period. Examination of the fine mesh x 100 revealed that the holes had not enlarged or become blocked during the study period. Figure 9.4 a-h shows photographs of leaves of willow (a-d) and alder (e-h) after 40 weeks submersion in litter bags. In all

Figure 9.3. Appearance of the litter bags after 40 weeks submersion in Yateley 4.

Figure 9.3. Appearance of the litter bags after 40 weeks submersion in Yateley 4.

Figure 9.3. Appearance of the litter bags after 40 weeks submersion in Yateley 4.

Figure 9.4. Appearance of leaf material after 40 weeks submersion in Yateley 4. Photographs a) to d) are willow and e) to h) are alder.



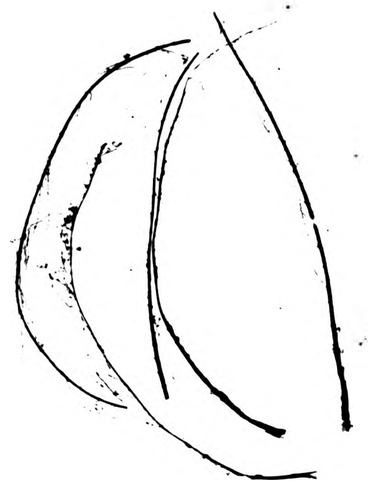
a) Extra large mesh (Site 4)

b) Large mesh (Site 5)

c) Extra large mesh (Site 1)

d) Fine mesh (Site 1)

Figure 9.4. Appearance of leaf material after 40 weeks submersion in Yateley 4. Photographs a) to d) are willow and e) to h) are alder.



a) Extra large mesh (Site 4)

b) Large mesh (Site 5)

c) Extra large mesh (Site 1)

d) Fine mesh (Site 1)

e) Extra large mesh (Site 3)

f) Medium mesh (Site 1)

g) Fine mesh (Site 3)

h) Extra large mesh (Site 5)

e) Extra large mesh (Site 3)

f) Medium mesh (Site 1)

g) Fine mesh (Site 3)

h) Extra large mesh (Site 5)

cases, leaves showed signs of degradation but this was different between mesh sizes and between different sites. In some extra large mesh bags (eg., a and e) mostly vascular tissue remained and the original leaf shape was not apparent. In contrast, in the fine mesh bags (d and g), and to some extent in some extra large bags (c and h), the leaf shape could be seen clearly despite degradation of lamina tissue.

Figure 9.5 shows the changes in leaf pack weight for leaf material in bags of different mesh size during the 40 weeks of submersion. In the first 2 weeks, 14-20% of alder, 20-25% of willow, 21% of willow 2 and 19% of ash dry weight had been lost, probably due to removal of soluble components. After this initial fall, leaf pack dry weight declined steadily with submersion time for all leaf types and mesh sizes. After 40 weeks all leaf packs, other than those in fine mesh bags, had lost 75% or more of their original dry weight.

Table 9.2 summarises the decay parameters for each of the weight loss curves shown in Figure 9.5. Ash in extra large mesh bags showed the highest rate of decomposition ($k = 0.00744$) and alder in fine mesh bags the lowest ($k = 0.00315$). With both alder and willow the decomposition rate constants (k) were lower for litter in fine mesh bags, but the difference was only significant in the case of alder. The 50% and 95% life of leaf material in each bag (Table 9.2) clearly demonstrates the influence of mesh size on weight loss. The 95% life of alder in fine bags was 2.1 times that in extra large mesh bags, in contrast to willow where the relative difference was 1.5 times. On the basis of the decomposition model and the predicted 95% life for leaf material in litter bags, there would be a net annual accumulation of allochthonous litter in Yateley 4.

Figure 9.6 shows the mean weight of alder and willow leaf discs cut from leaf material submerged in Yateley 4 over a 28 week period. The equations for the fitted linear regressions are presented in Table 9.3. For all mesh

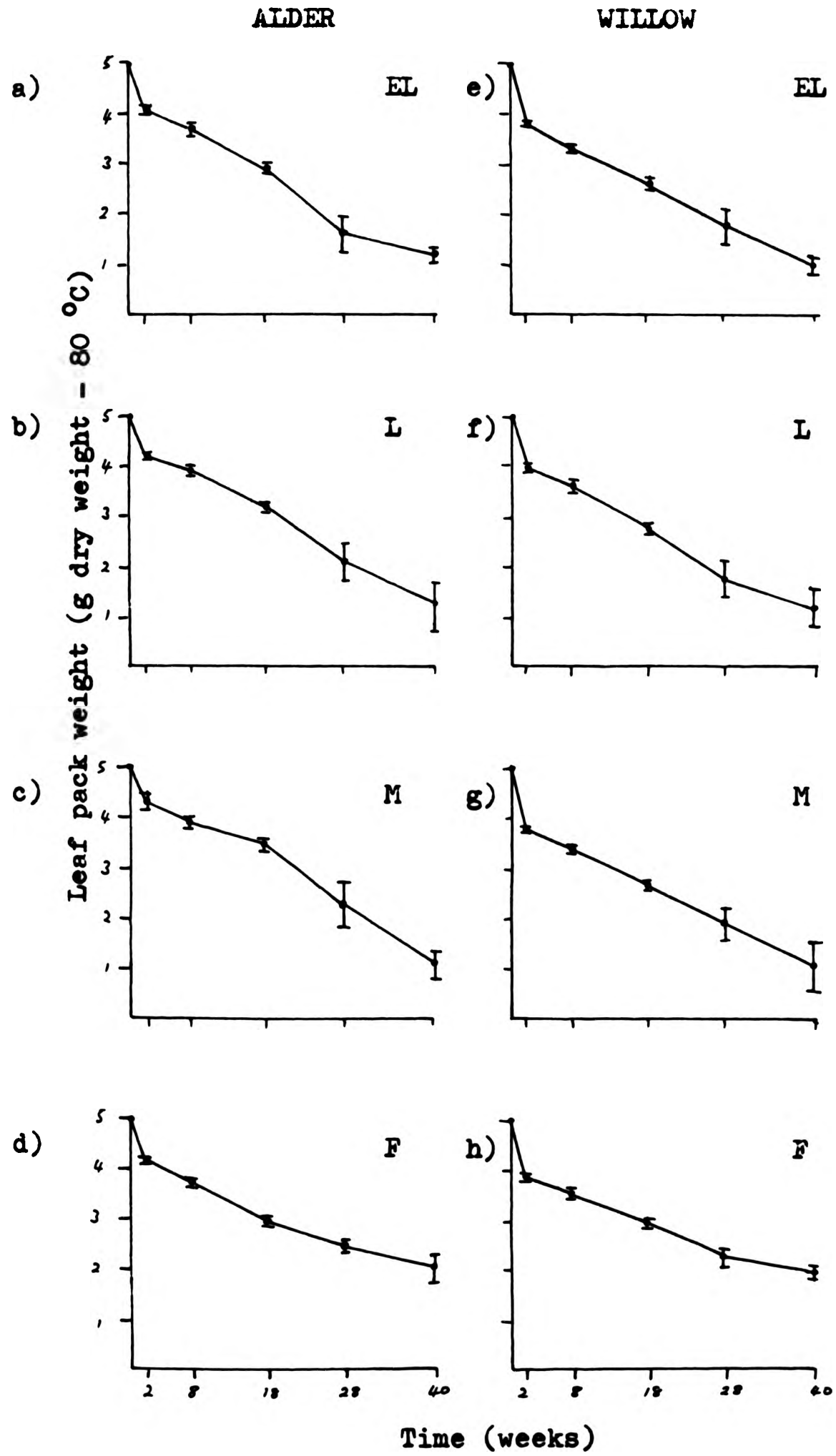


Figure 9.5

P.T.O.

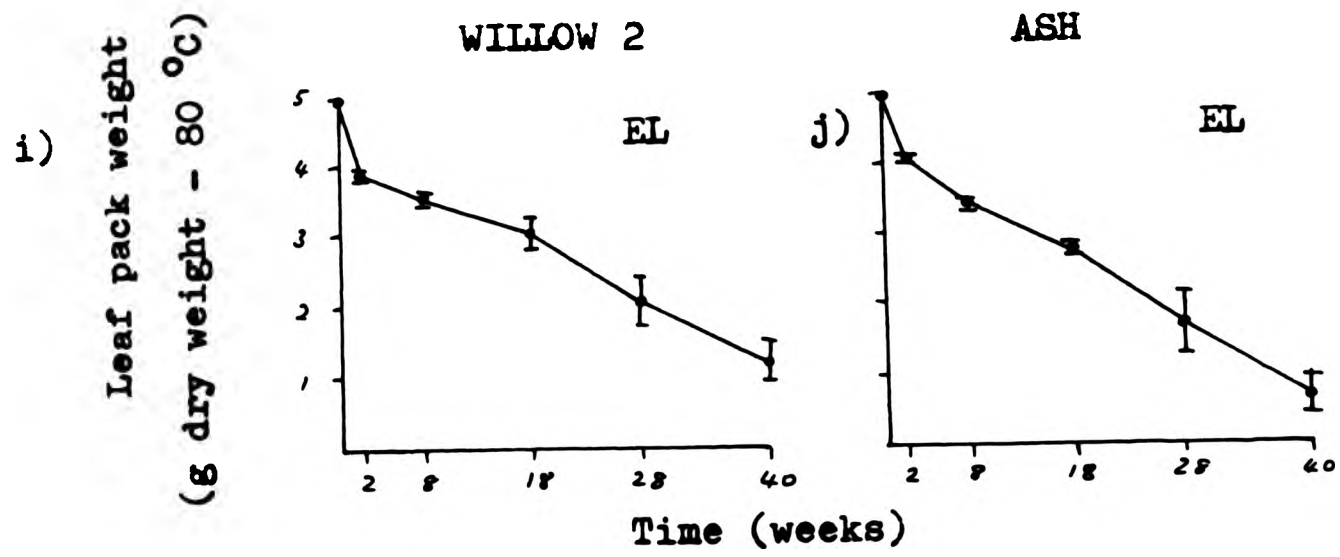


Figure 9.5. Graphs showing leaf pack weights of alder (a-d), willow (e-h), willow 2 (i) and ash (j) over a period of 10 months submersion in Yateley 4 in bags of different mesh size (EL extra large, L large, M medium, F fine). Values are means for 5 sites with standard errors.

TABLE 9.2 Decay parameters for leaf packs in extra large (EL), large(L), medium(M) and fine(F) mesh bags over a 10 month period of submersion in Yateley 4. (95%*1 life values are shown in parenthesis in the 50% life column).

LITTER	MESH	REGRESSION EQUATION*3	r^2	F*4*5	k*2	50%*1 LIFE
Alder	EL	y=5.07exp(0.00665x)	0.709	68.2	0.00665 ±0.00165	104.2 (451.1)
Alder	L	y=5.17exp(0.00555x)	0.598	41.7	0.00555 ±0.00176	124.9 (540.5)
Alder	M	y=5.30exp(0.00578x)	0.727	74.5	0.00578 ±0.00132	119.9 (519.0)
Alder	F	y=4.57exp(0.00315x)	0.834	140.6	0.00315 ±0.00054	220.0 (952.0)
Willow	EL	y=5.10exp(0.00674x)	0.658	54.0	0.00674 ±0.00188	102.8 (445.1)
Willow	L	y=5.02exp(0.00585x)	0.582	39.1	0.00585 ±0.00192	118.5 (512.8)
Willow	M	y=4.94exp(0.00611x)	0.601	42.3	0.00611 ±0.00193	113.4 (491.0)
Willow	F	y=4.72exp(0.00453x)	0.489	26.8	0.00453 ±0.00179	153.0 (662.3)
Ash	EL	y=5.21exp(0.00744x)	0.716	70.7	0.00744 ±0.00181	93.1 (403.2)
Willow2	EL	y=5.20exp(0.00485x)	0.702	66.0	0.00485 ±0.00122	142.9 (618.6)

*1 Time in days 0.5=0.693/k 0.95=3/k

*2 k = decomposition rate constant with 95% confidence limits

*3 y = weight loss, x = time in days

*4 F = test result for the significance of the slope of the regression

*5 All figures significant at $p<0.01$

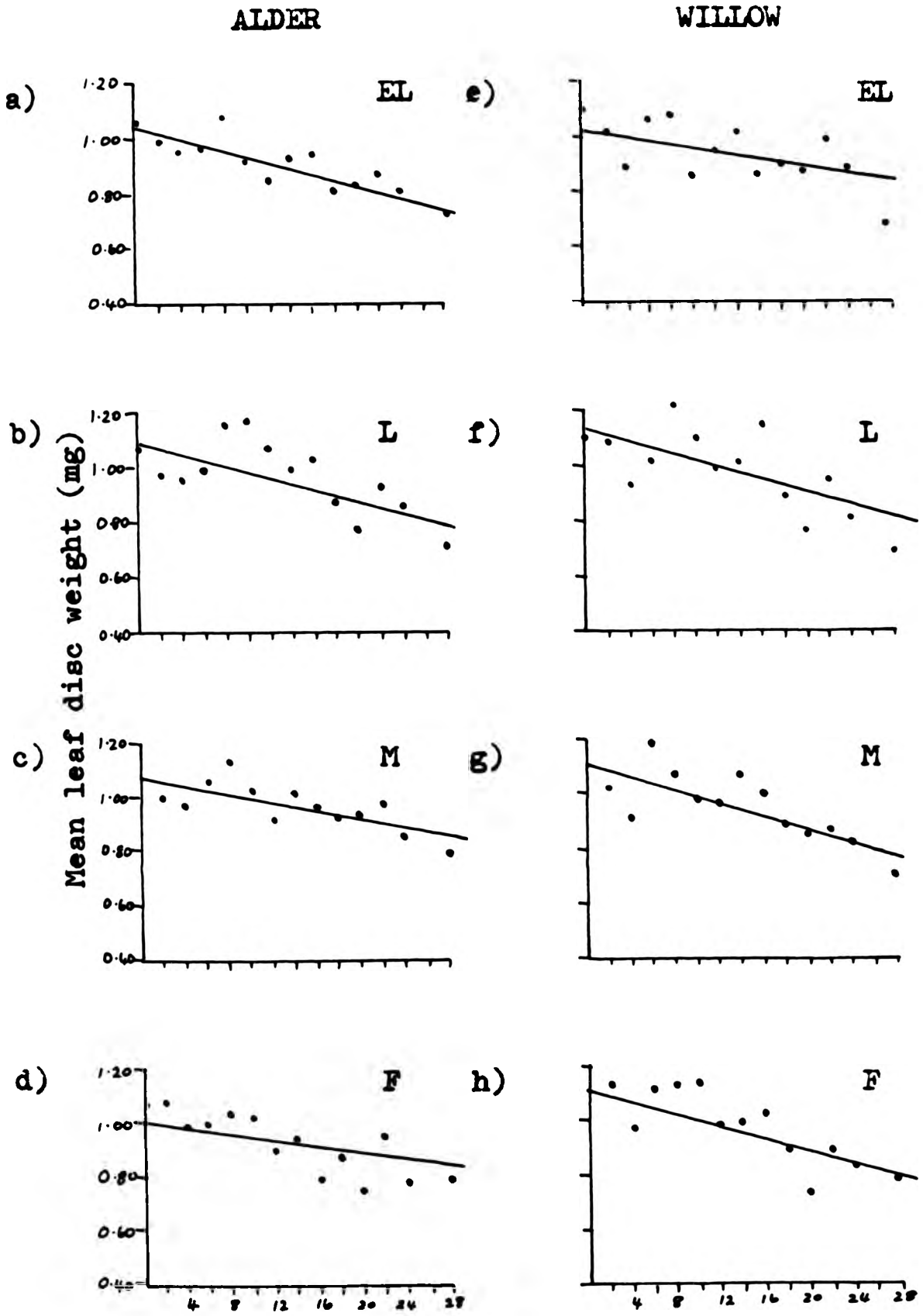


Figure 9.6. Graph showing mean leaf disc weight of alder (a-d) and willow (e-h) against time of submersion in bags of different mesh size (EL extra large, L large, M medium, F fine). The fitted regression lines are shown for each set of data.

TABLE 9.3 Decay rates of alder and willow leaf discs (6mm diameter) over a 7 month period in bags of different mesh size. Linear regressions were fitted using the least squares method. Data plotted in Figure 9.6.

LITTER SPECIES	MESH	REGRESSION EQUATION	r^2	F*1	P	SLOPE WITH 95% CONF. LIMITS
Alder	EL	y=1.04-0.0097x	0.698	25.4	p<0.001	-0.0097 ±0.0042
Alder	L	y=1.10-0.0108x	0.440	8.6	p<0.05	-0.0108 ±0.0081
Alder	M	y=1.07-0.0076x	0.527	12.3	p<0.01	-0.0076 ±0.0048
Alder	F	y=1.07-0.0047x	0.669	22.3	p<0.001	-0.0047 ±0.0029
Willow	EL	y=1.04-0.0080x	0.371	6.5	p<0.05	-0.0080 ±0.0069
Willow	L	y=1.16-0.0131x	0.469	9.7	p<0.01	-0.0131 ±0.0092
Willow	M	y=1.12-0.0120x	0.575	14.9	p<0.01	-0.0120 ±0.0068
Willow	F	y=1.17-0.0141x	0.689	24.4	p<0.001	-0.0141 ±0.0063

*1 F=test result for the significance of the slope of regression

sizes of alder and willow there was a significant decrease in mean leaf disc weight with time of submersion. Examination of the 95% confidence limits for the slope of the regressions (Table 9.3) does not indicate any significant difference between mesh sizes of alder or willow but does suggest a difference between alder and willow in fine mesh bags. There was no significant correlation between the slope of the regression (Table 9.3) for leaf disc weight loss and the decomposition constant (k) for whole leaf packs (Table 9.2), suggesting that the two parameters were measuring different aspects of leaf degradation.

9.3.2 Microbial biomass and activity

Figure 9.7 shows the levels of ATP associated with alder and willow leaf litter in bags of different mesh size. With all mesh sizes, ATP was initially low (15-45 $\mu g\ g^{-1}$) and increased gradually during the period of submersion to reach a peak level of between 180-380 $\mu g\ g^{-1}$ after 20-22 weeks, followed by a decline. Conversion of ATP to microbial biomass (ATP to carbon 1:250, carbon 36.7% of dry weight; (Anderson & Domsch, 1980)) would give biomass estimates of between 122.6 and 258.9 $mg\ g^{-1}$ leaf dry weight at peak levels (20-22 weeks) or 12.3-25.9% of leaf dry weight.

Figure 9.8 shows the ATP levels associated with willow and alder leaves at the 5 sites in Yateley 4. In all, ATP levels were initially low and rose to a peak after 20-22 weeks. This is a similar trend to that in Figure 9.7. Comparison of the ATP levels at different sites suggests there is more variation between sites than between leaf types at a particular site. Sites 2 and 3 show similar trends on both alder and willow but these are different to those shown by the leaf types at site 1 and 4. Site 5 appears to differ by showing a marked difference in ATP levels on the 2 leaf types.

Figure 9.9 shows the fluorescein diacetate hydrolysis

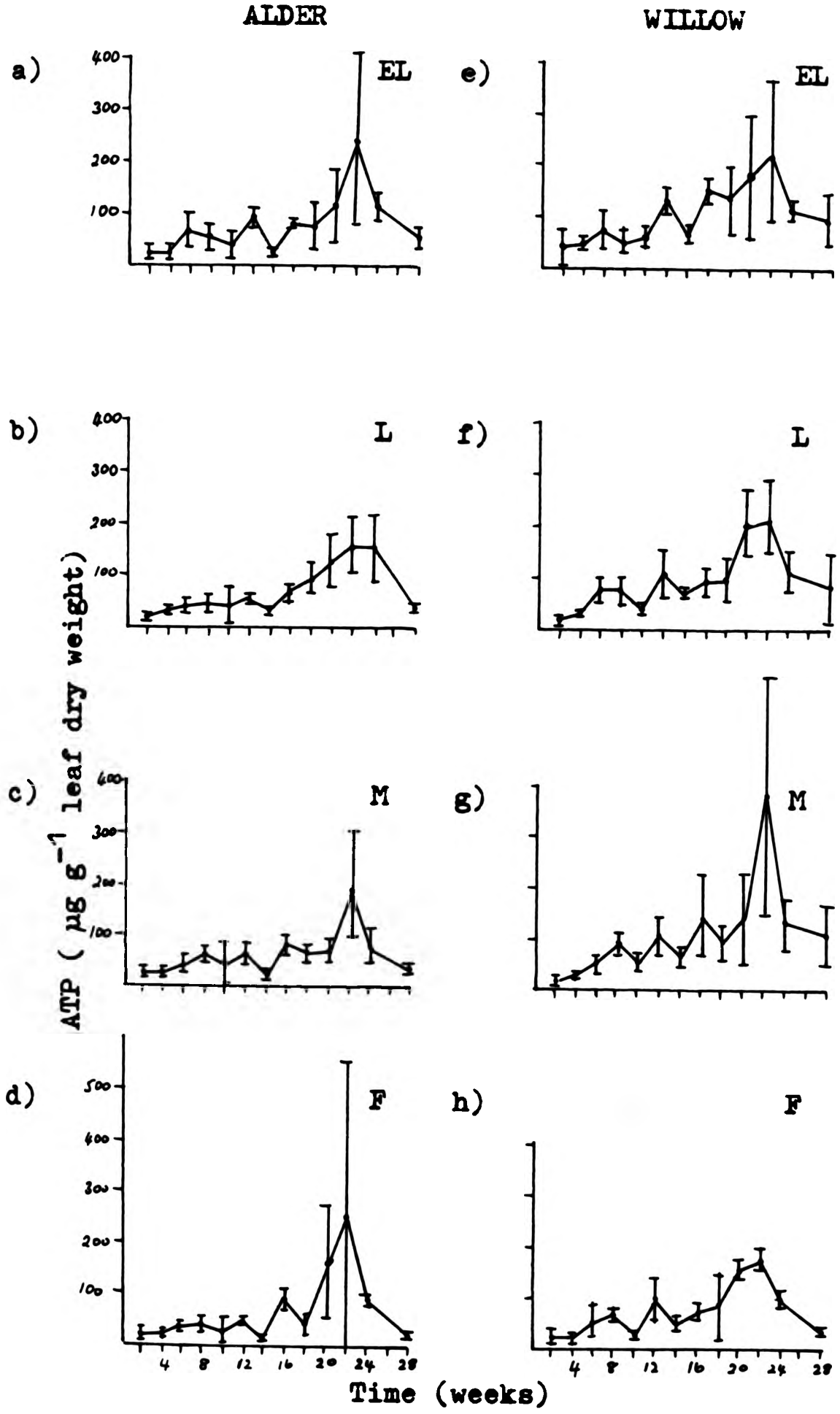


Figure 9.7. ATP on alder and willow leaf litter over a period of 7 months submersion in Yateley in bags of different mesh size (EL extra large, L large, M medium F fine) Values are means (n = 5) with standard errors.

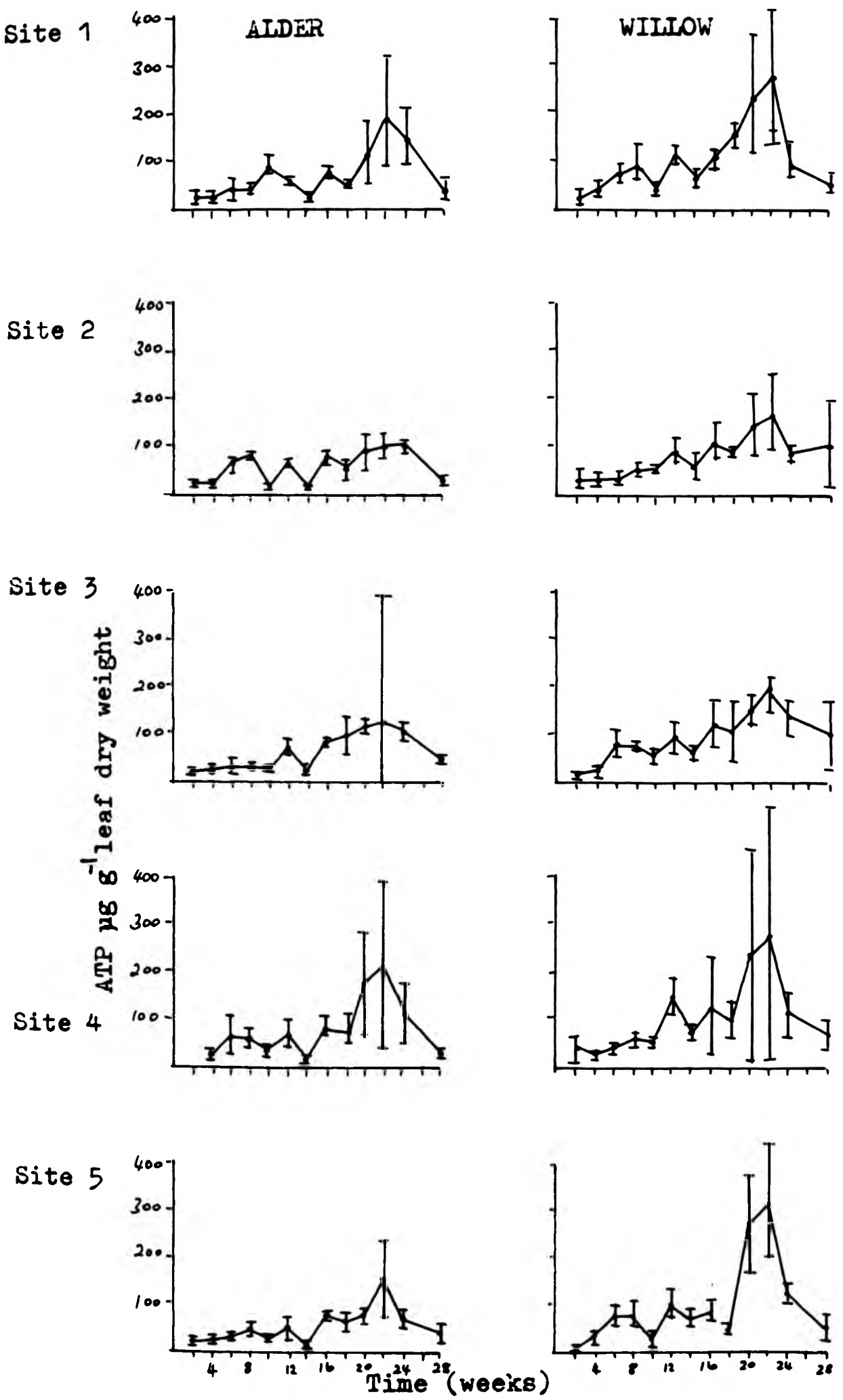


Figure 9.8. ATP ($\mu g\ g^{-1}$) on alder and willow leaf litter over a period of 7 months submersion at different sites in Yateley 4. For each site mean values are shown with S.E. (n=4). 246

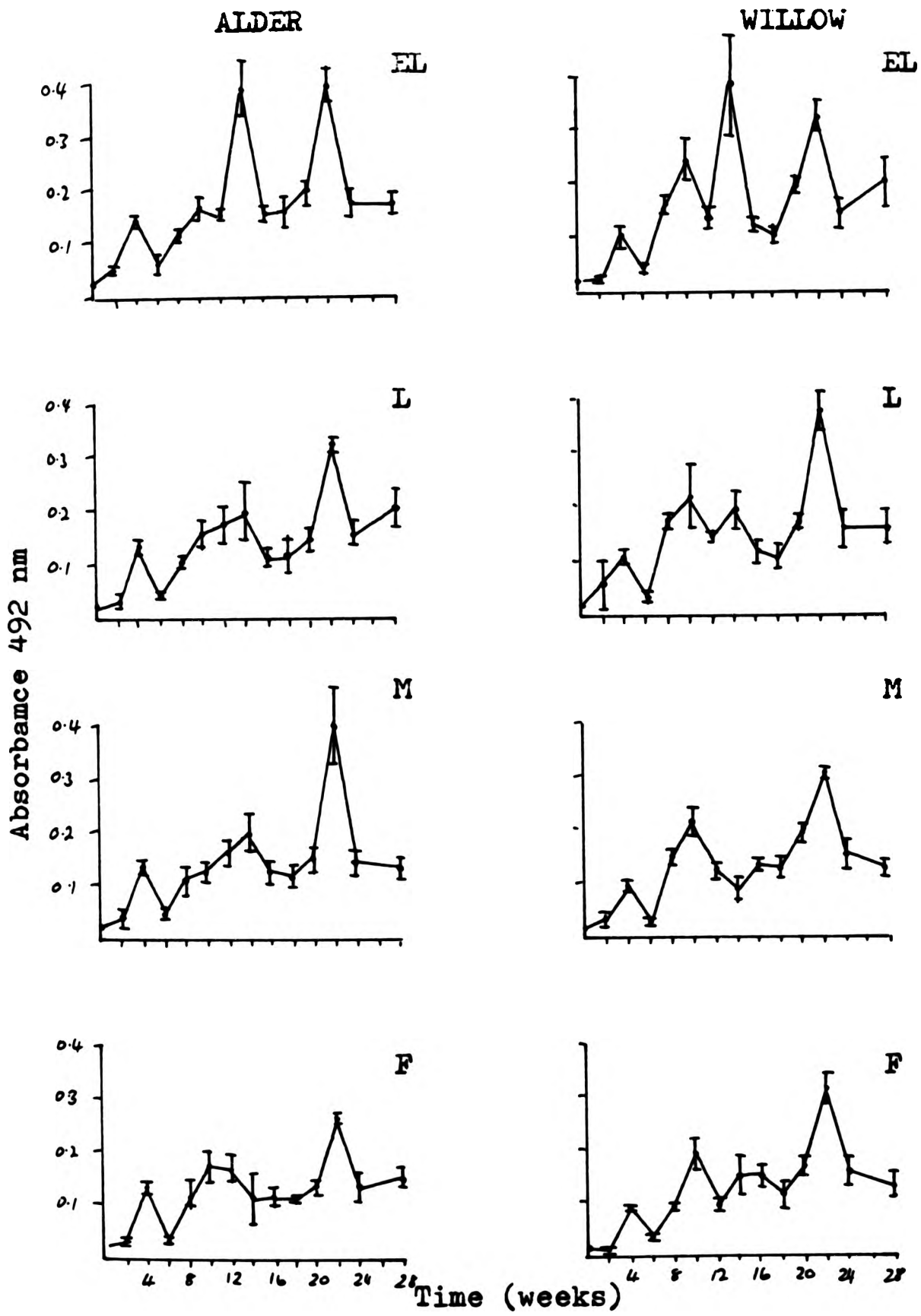


Figure 9.9. FDA hydrolysis associated with alder and willow leaf litter over a period of 7 months submersion in Yateley 4. Values shown for each mesh type are mean absorbance at 492 nm (arbitrary units) per 10 leaf discs with standard errors

associated with alder and willow leaf litter during 28 weeks of submersion. In all cases there was an increase in microbial activity with submersion time with a peak level of activity on most leaf samples being reached after 22 weeks. With extra large mesh bags, and to a lesser extent with other mesh sizes, there was another major peak of FDA activity after 12-14 weeks. This is in contrast to the trend shown by ATP, where there was only one major peak at 20-22 weeks. Figure 9.10 shows the FDA activity at different sites in Yateley 4. It increased at all sites with major peaks at 10-14 and 22 weeks. There appeared to be less variation between sites and more between leaf types, suggesting that FDA activity was not closely related to ATP microbial biomass at each site in the lake.

9.3.3 Fungal biomass

The levels of fungal biomass determined by the agar film method are shown in Figure 9.11. Plots are shown for total biomass and active biomass (tetrazolium stained) for alder and willow leaf litter in bags of different mesh size. In most cases, total biomass increased between 8 and 18 weeks, and then declined at 28 weeks. At 18 weeks the mean levels of biomass were similar on both leaf types and for all mesh sizes (between 2-2.5% of leaf dry weight). Active biomass also increased with submersion time, and peak levels were reached at 8, 18 or 28 weeks depending on leaf type and mesh size. Active biomass was between 0.3 and 0.65% of leaf dry weight. Including time 0, percentage of total biomass which was active varied between 5 and 38%, with the highest percentage activity being at 18 and 28 weeks. Mean hyphal diameters ranged between 3.06 µm $\pm$ 0.27 for hyphae on alder in extra large mesh bags to 3.85 $\pm$ 0.39 for hyphae on willow in large mesh bags. There was no significant difference between hyphal diameter on alder or willow or between hyphae on litter in bags of different mesh

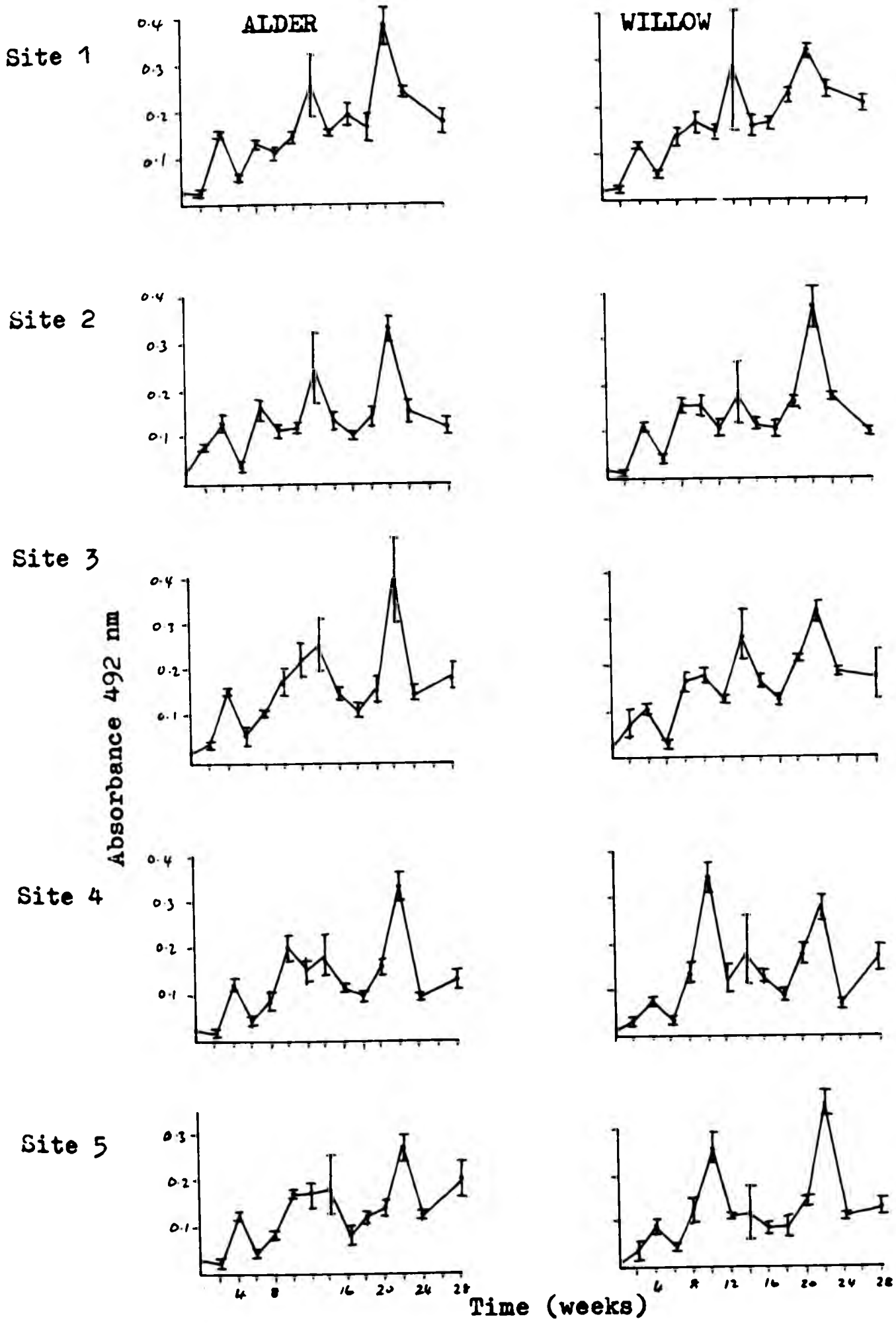


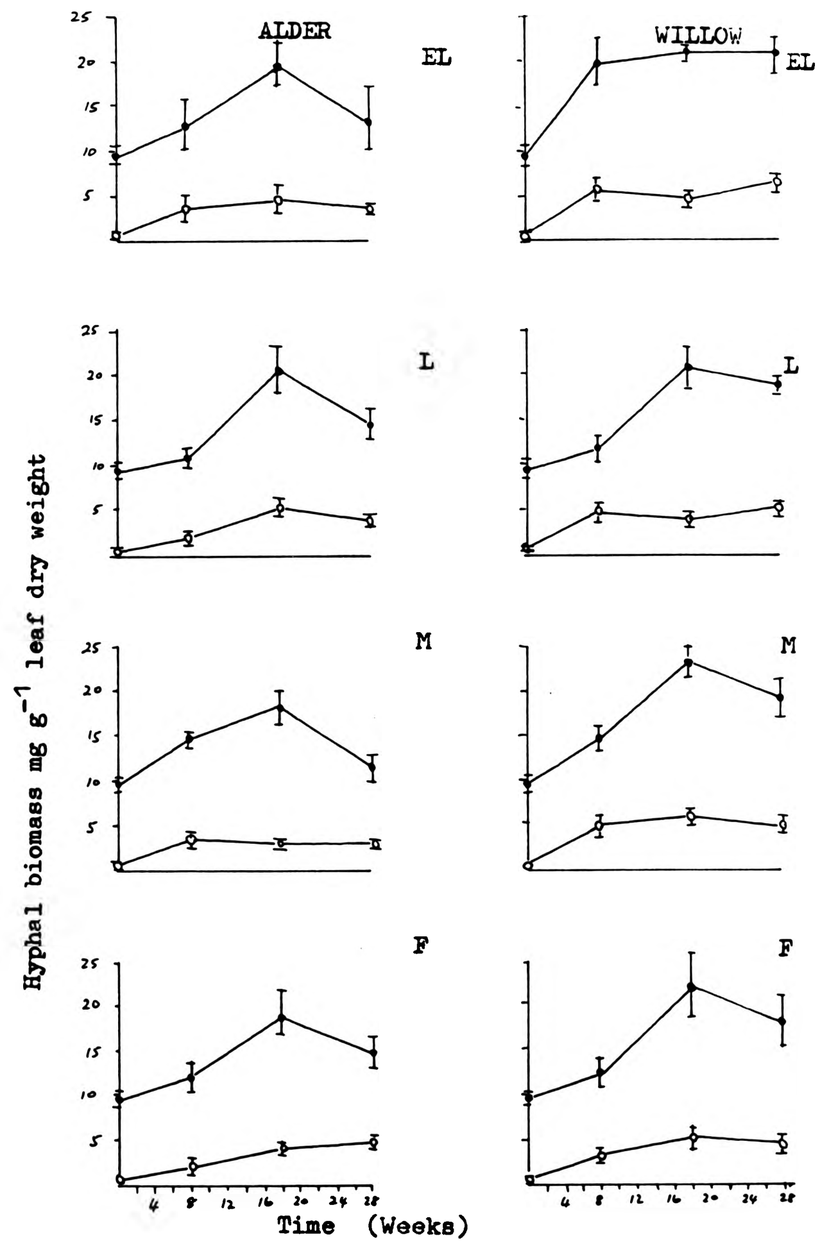
Figure 9.10. FDA hydrolysis on samples of alder and willow leaf litter over a period of 7 months submersion at different sites in Yateley 4. Values are arbitrary units of absorbance at 492 nm/ 10 leaf discs with S.E. n = 4

Figure 9.11. Agar film fungal biomass on alder and willow leaf litter over a period of 7 months submersion in Yateley 4. The values shown for each mesh type (mg g^{-1} dry weight *1) are means together with standard errors (n = 5).

Agar film total

Agar film active

*1 assumption: Biovolume to biomass conversion factor = 0.29 g cm^{-3}



sizes.

Figure 9.12 shows the dependence of the leaf weight retained by 250 µm mesh on the time of submersion in Yateley 4. For both leaf species and all mesh sizes there was a significant decrease in the percentage of leaf material retained at different times. After 28 weeks only 16.5% ± 1.2 of alder and 24.4% ± 1.3 of willow homogenates were retained by 250 µm mesh. This contrasts with 50% ± 2.8 for alder and 60.3% ± 4.0 for willow after 2 weeks submersion. This indicates that the correction factor for inefficiency of homogenisation changed with the degree of degradation of leaf material. Softening of leaf tissues by microbial enzymes may be responsible for the reduction in leaf material retained with increasing incubation time. The slopes of the fitted regression lines were steeper for willow than for alder, irrespective of the mesh size, indicating that the degree of leaf 'softening' was species specific.

Values for agar film biomass were corrected for inefficiency of homogenisation using conversion factors derived from the fitted regressions for each leaf species and mesh size and the results are shown in Figure 9.13. In most cases the corrected agar film total biomass increased between 0 and 18 weeks and then declined to a level at or below that at time 0. At peak levels, corrected agar film total biomass was between 2.5 and 3.7% of leaf dry weight. Corrected active biomass reached a peak level at 8 or 18 weeks, depending on the leaf type and mesh size. There was a decline in corrected active biomass after the peak but, in all cases, levels remained above that for time 0, thus contrasting with corrected total biomass. The maximum corrected agar film active biomass was between 0.5 and 1.1% of leaf dry weight.

Figure 9.14 shows the ergosterol levels associated with alder and willow litter in bags of different mesh size. In all cases, mean levels of ergosterol increased during the study period with peak levels between 5.5 and 18 µg g^{-1} being reached after 18 or 28 weeks

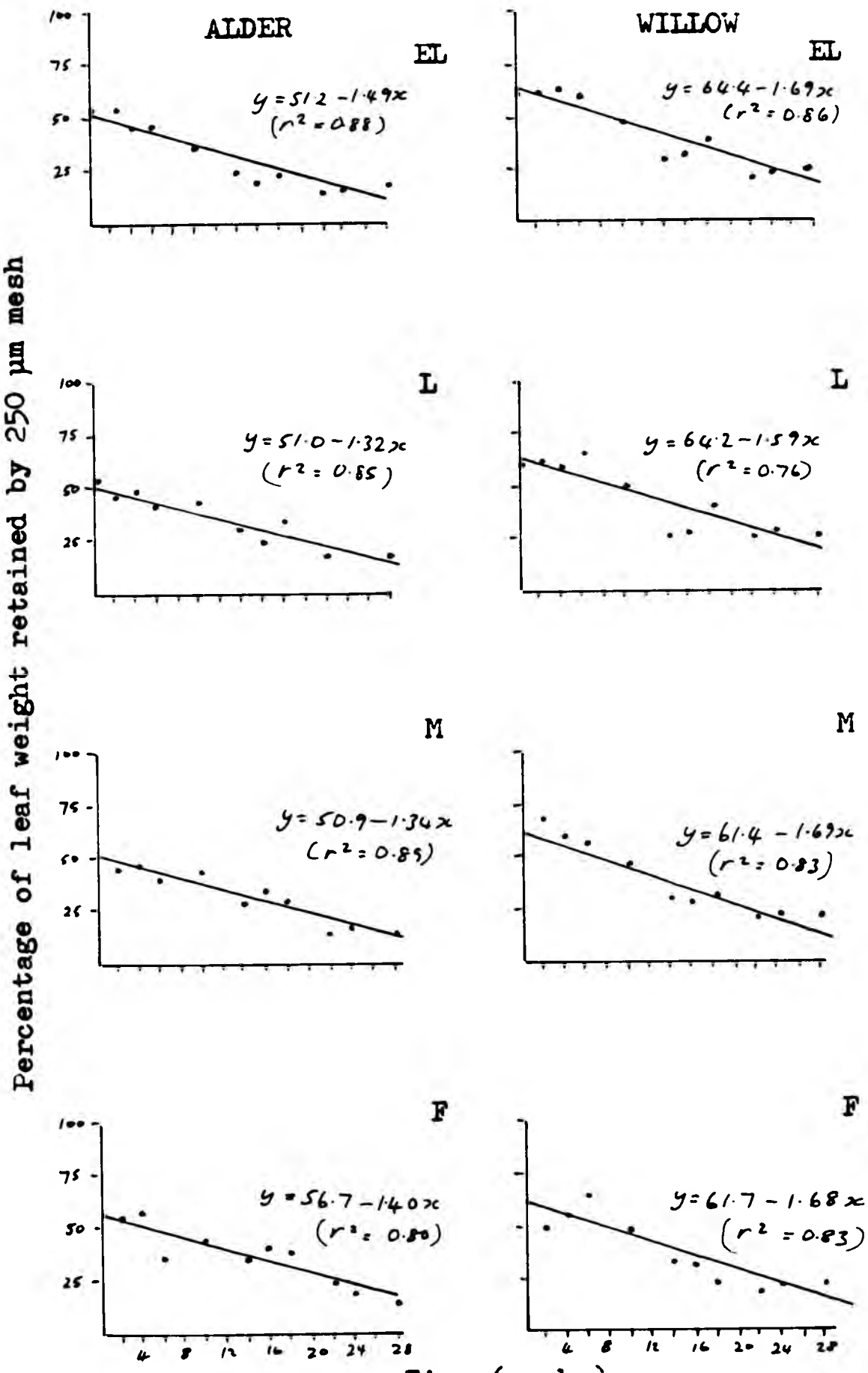


Figure 9.12. Dependence of the percentage of leaf weight retained by 250 µm mesh after homogenisation on the time of submersion of leaf litter in Yateley 4. For each mesh type the data (means of 5 replicates) are plotted against period of submersion. Fitted linear regression lines and equations are included

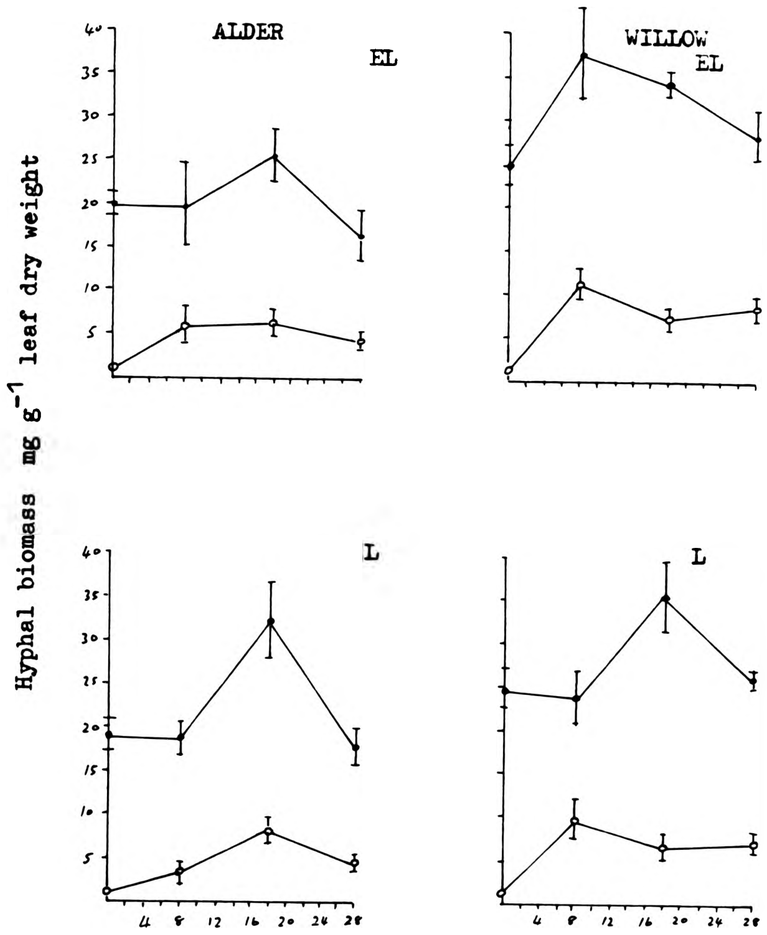
Figure 9.13. Corrected *1 agar film fungal biomass on alder and willow leaf litter over a period of 7 months submersion in Yateley 4. The values shown for each mesh type (mg g^{-1} dry weight *2) are means together with standard errors (n=5).

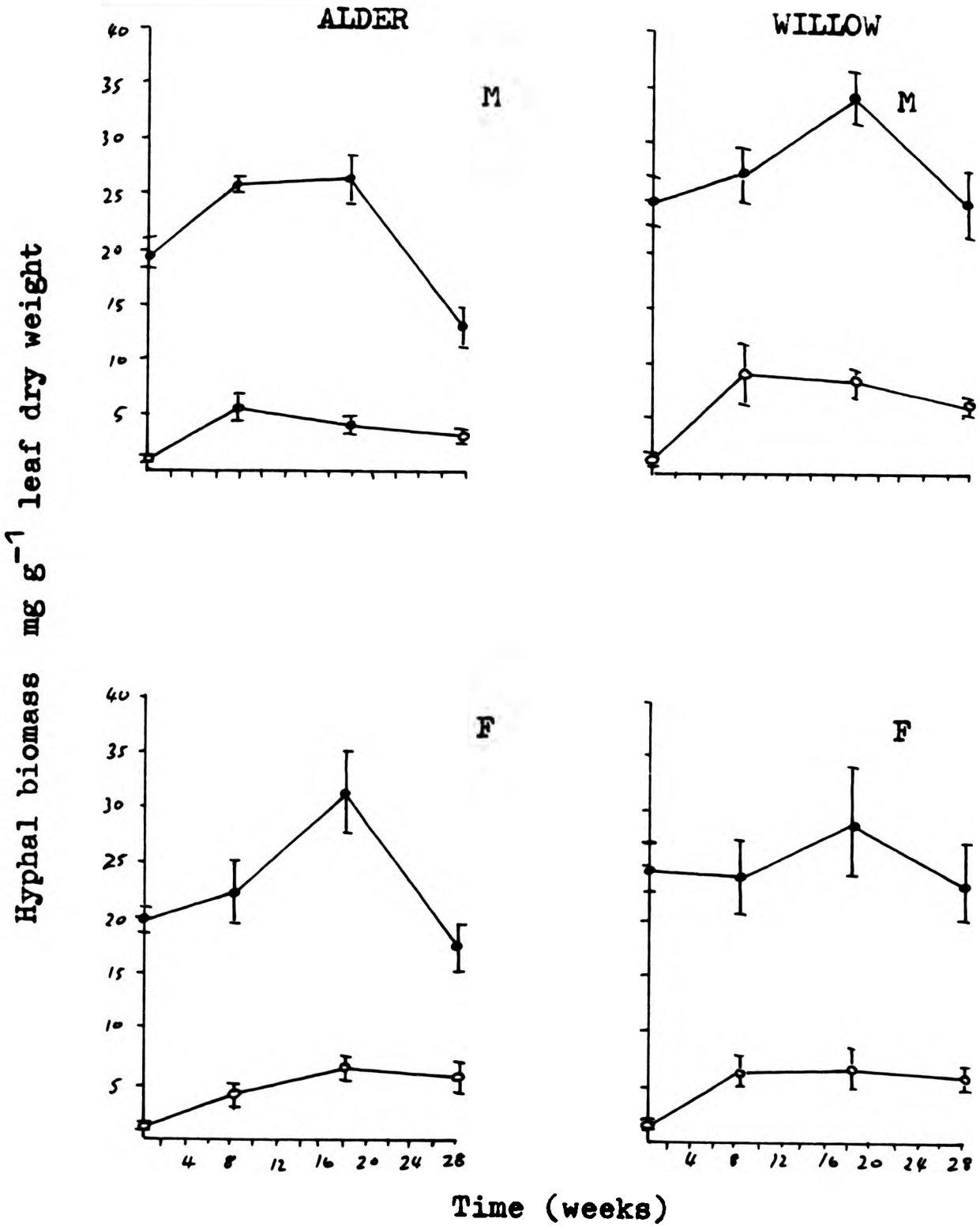
•——• Agar film total biomass

o——o Agar film active biomass

*1 assumption biovolume to biomass conversion factor = 0.29 g cm^{-3}

*2 corrected for inefficiency of homogenisation




ALDER
WILLOW
M
M
F
F
40
35
30
25
20
15
10
5
4
8
12
16
20
24
28
Hyphal biomass mg g^{-1} leaf dry weight
Time (weeks)

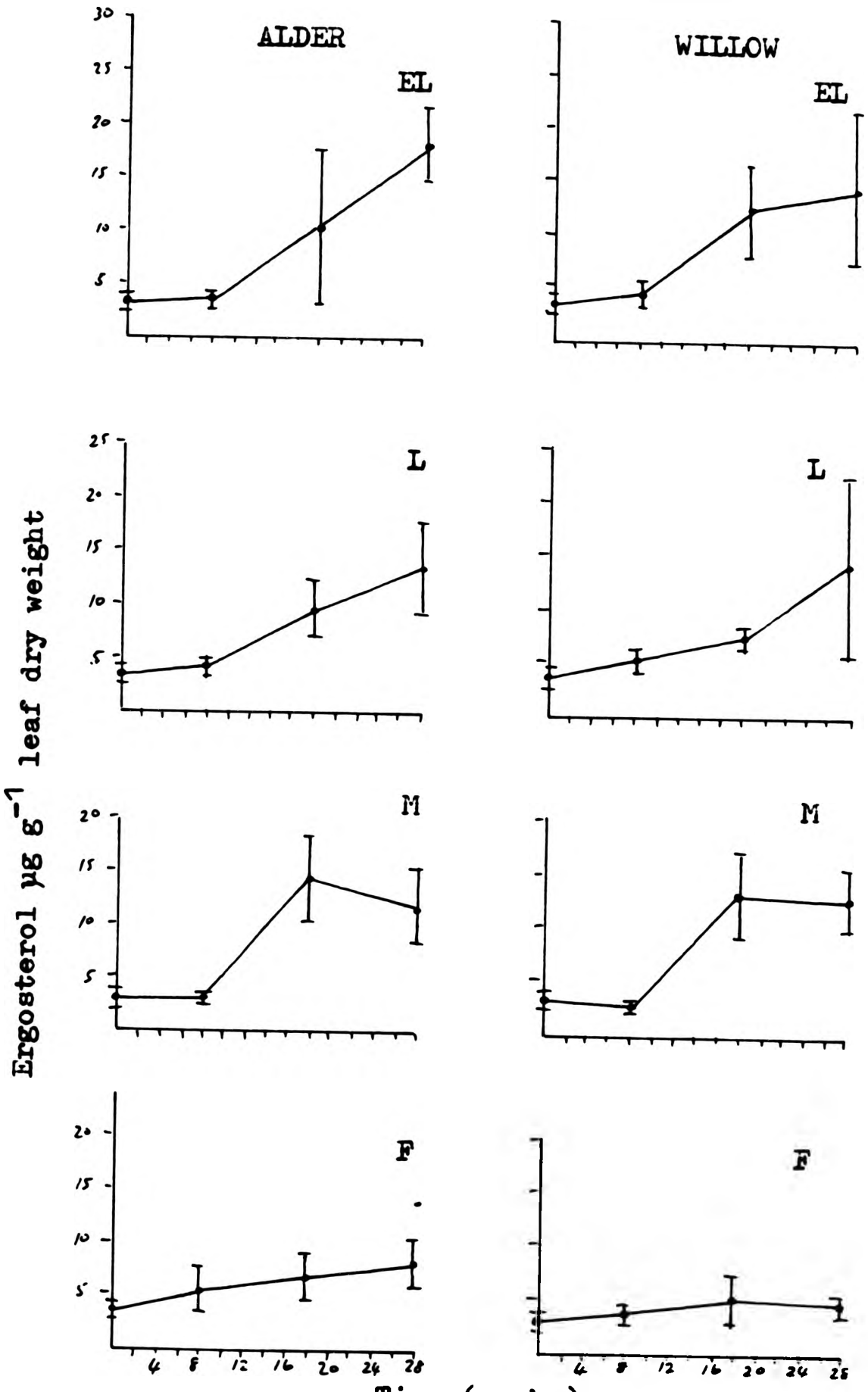


Figure 9.14. Ergosterol associated with alder and willow leaf litter over a period of 7 months submersion in Yateley 4. The values shown for each mesh type are means with standard errors (n=5)

depending on leaf species and mesh size. Levels of ergosterol were lower in fine mesh bags than in bags of other mesh sizes after 18 and 28 weeks. Using a factor of 2.19 mg g^{-1} to convert ergosterol to fungal mass (see Section 6.3.2), these values would give a fungal biomass of between 2.5 and 8.4 mg g^{-1} leaf dry weight, representing between 0.25 and 0.84% of leaf dry weight.

Fungal biomass estimated using the whole leaf method is shown in Figure 9.15. Each value was derived from the biovolume of both hyphae and pycnidia using a conversion factor of 0.29 mg cm^{-3} (Section 6.3.1). As with ergosterol, fungal biomass increased during submersion with peak levels of 12-16 mg g^{-1} (1.2-1.6% leaf dry weight) being reached after 28 weeks.

Pycnidial biomass formed a significant proportion of the biomass, on average 31.6% and 33.5% of total biomass on alder and willow, respectively.

Table 9.4 summarises the results of analysis of variance for different biomass measurements on alder and willow leaf litter. Most estimates increased significantly with submergence time of litter (age) but there were few significant effects of mesh size. Agar film active biomass showed no significant increase with submersion time or effect of mesh size. ATP showed a significant difference between mesh sizes for both alder and willow with litter in fine mesh bags having lower levels of ATP than in bags of larger mesh size. Whole leaf biomass on alder and FDA hydrolysis on willow both showed significant differences between mesh sizes, with litter in fine mesh bags having lower biomass and FDA hydrolysis than other mesh sizes.

Differences between biomass estimates also showed some effects. The ratio of ATP to ergosterol on willow decreased significantly with submersion time, suggesting that fungi were forming a larger proportion of the biomass. The difference between total biomass by the agar film method and that by the whole leaf method showed a

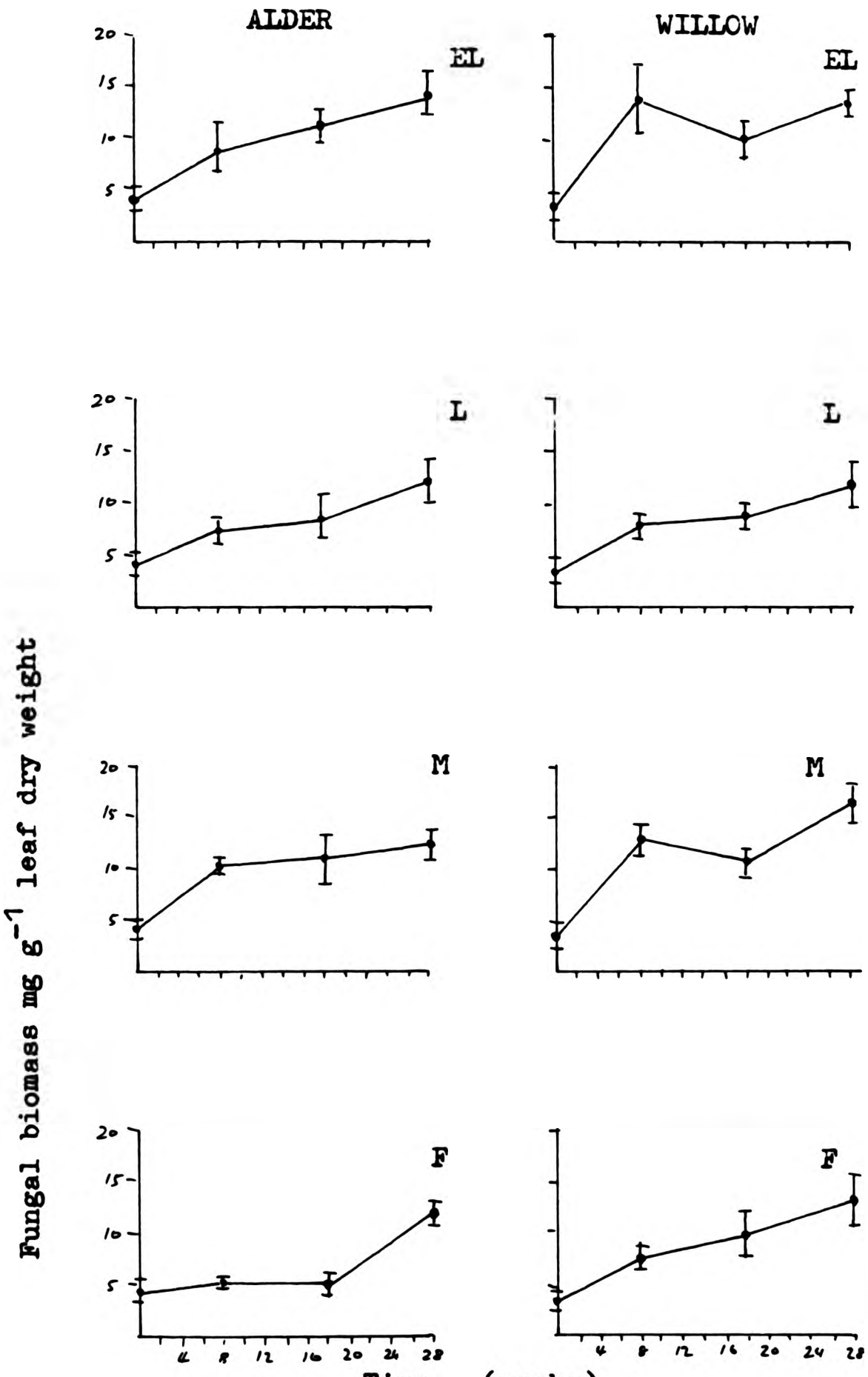


Figure 9.15. Whole leaf clearing and staining fungal biomass*1 on alder and willow leaf litter over a 7 month period of submersion in Yateley 4. The values shown for each mesh type (mg g^{-1} dry weight*2) are means with standard errors (n=5).

*1 includes pycnidial biomass *2 assumption biovolume to biomass conversion factor = 0.29 g cm^{-3}

TABLE 9.4 Results of analysis of variance for biomass and activity measurements in terms of period of submersion (age) and mesh size. Analyses based on submersion at times 8, 18 and 28 weeks.

VARIABLE	ALDER (n=60)			WILLOW (n=60)		
	AGE	MESH	AGExMESH	AGE	MESH	AGExMESH
ATP	***	**	NS	*	*	NS
Ergosterol	***	NS	NS	***	NS	NS
Agar film total	***	NS	NS	***	NS	NS
Agar film active	NS	NS	NS	NS	NS	NS
Whole leaf total	***	*	NS	*	NS	NS
Whole leaf pycnidia	***	NS	NS	***	NS	NS
FDA hydrolysis	**	NS	NS	*	*	NS
ATP/Ergosterol	NS	NS	NS	***	NS	NS
Ergosterol/Agar film active	*	NS	NS	NS	NS	NS
ATP-Agar film active	*	NS	NS	**	*	NS
Agar film total-whole leaf total	***	NS	NS	***	NS	NS
Whole leaf total-pycnidia	NS	NS	NS	NS	NS	NS

Key: *** $p<0.001$

** $p<0.01$

* $p<0.05$

NS Not significant; $p>0.05$

significant change with submersion time, there being a larger difference at 18 weeks than at 8 or 28 weeks.

Correlations between different biomass methods, irrespective of leaf type, mesh size or period of submersion, are shown in Table 9.5. There were significant positive correlations between agar film total and ATP, ergosterol and whole leaf total biomass, ergosterol and whole leaf pycnidia, agar film total biomass and whole leaf total biomass, agar film total biomass and agar film active biomass, agar film active biomass and FDA hydrolysis, and whole leaf total biomass and pycnidia. Other significant positive correlations were found with differences or proportions of variables. Whole leaf total biomass minus whole leaf pycnidia was significantly correlated with with agar film total biomass minus agar film active biomass, and with FDA hydrolysis. The proportion of agar film total biomass which was active was significantly correlated with FDA hydrolysis. Most significant correlations were fairly low (< 0.4), suggesting that there was a significant amount of variation between variables.

9.3.4 Viable counts and fungal biomass at one site

A study made at one site (Site 1) in Yateley 4 examined viable counts of bacteria and fungi together with fungal biomass. Viable counts on agar media were determined for mycelial fungi, yeasts, total aerobic bacteria and pectinolytic bacteria. Fungal biomass was estimated using the agar film method with tetrazolium staining for active hyphae.

Figure 9.16 shows the numbers of viable bacteria on alder and willow leaf material in bags of different mesh size over a period of 28 weeks submersion at site 1. Total aerobic bacteria increased markedly during the first half of the study period, reaching a peak of 10^{12} g^{-1} leaf dry weight after 14 weeks. This was followed by a marked decline in numbers and by 18 weeks

TABLE 9.5 Correlation coefficients (r) between different measurements of fungal biomass and activity.

VARIABLE+	1	2	3	4	5
1 ATP					
2 Ergosterol	0.000				
3 Agar film total	0.218*2	0.096			
4 Agar film active	0.062	0.064	0.658*3		
5 FDA hydrolysis	-0.024	0.105	0.145	0.222*2	
6 Whole leaf total	0.077	0.189*1	0.208*2	0.231*2	0.188*1
7 Whole leaf pycnidia	0.065	0.273*2	0.075	0.167	0.084
8 Agar film total-agar film active	0.241*2	0.089	0.937*3	0.354*3	0.078
9 Agar film active-agar film total	-0.104	0.034	-0.042	0.644*3	0.338*2
10 Whole leaf total-pycnidia	0.051	0.016	0.236*2	0.181	0.196*1
11 ATP/ ergosterol	0.446*3	-0.510*3	0.070	-0.013	-0.025

VARIABLE+	6	7	8	9	10
1 ATP					
2 Ergosterol					
3 Agar film total					
4 Agar film active					
5 FDA hydrolysis					
6 Whole leaf total					
7 Whole leaf pycnidia	0.742*3				
8 Agar film total-agar film active	0.152	0.016			
9 Agar film active-agar film total	0.112	0.170	-0.351*3		
10 Whole leaf total-pycnidia	0.762*3	0.131	0.209*1	0.001	
11 ATP/ ergosterol	0.025	-0.078	0.093	-0.109	0.042

KEY: *1 $p<0.05$; *2 $p<0.01$; *3 $p<0.001$; + n= 120

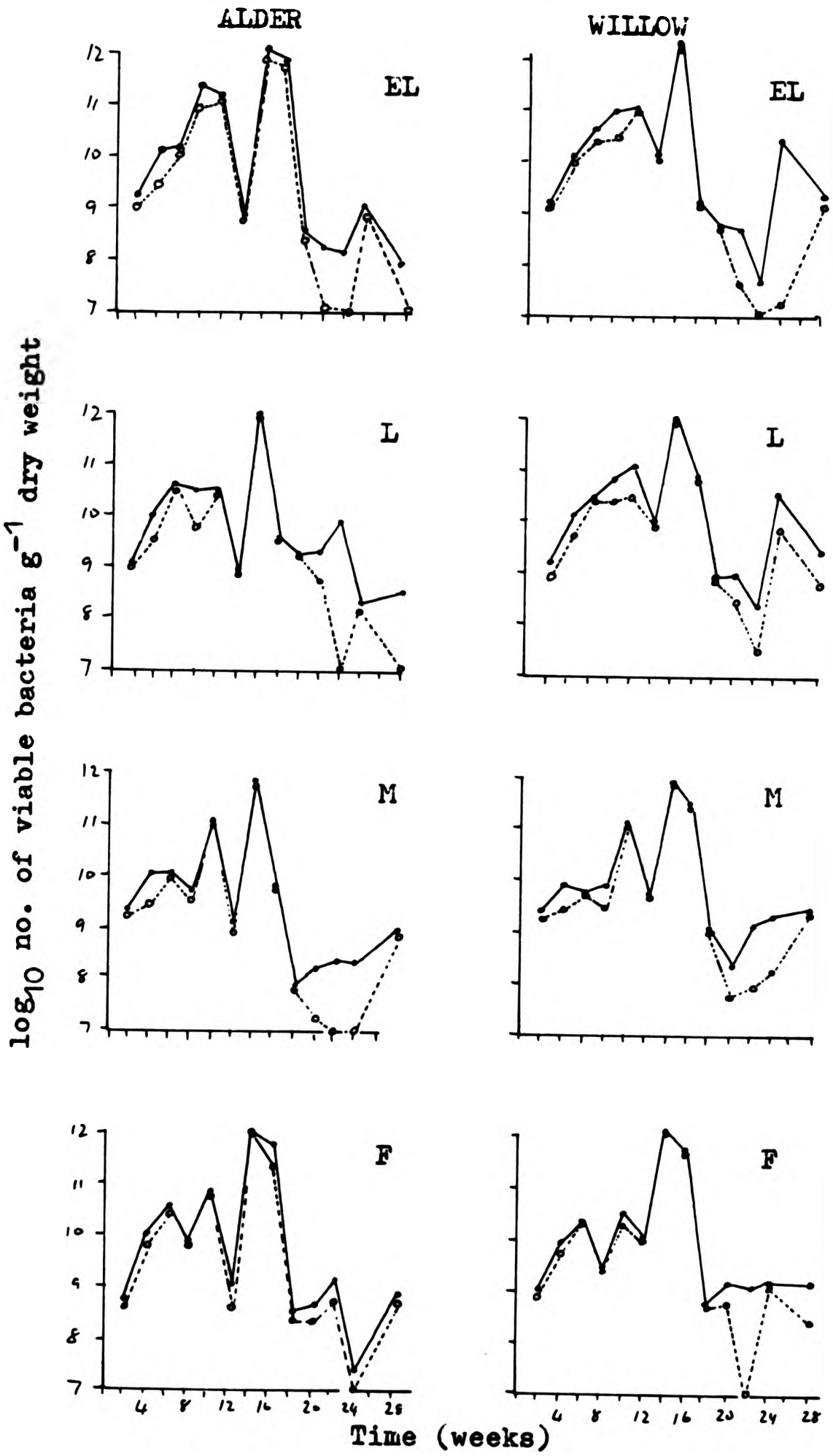


Figure 9.16. Number of viable bacterial colony forming units on alder and willow leaf litter over a period of 7 months submersion at site 1 in Yateley 4.

—●— Total number of aerobic bacteria

o·····o Numbers of aerobic pectinolytic bacteria

bacterial numbers were at or below the levels of samples in weeks 2 and 4. This trend was apparent for all mesh sizes on both leaf types. Pectinolytic bacteria formed a significant component of the total aerobic bacteria particularly in weeks 10-18. In the early stages of colonisation (weeks 2-10) and particularly after peak numbers had fallen (weeks 18-28) they formed a smaller proportion of the total aerobic bacteria.

Figure 9.17 shows the number of fungal colony forming units associated with alder and willow litter over the study period. In most cases numbers of mycelial colonies increased sharply during the first 12-14 weeks of the study and then remained fairly constant until week 28. In the case of alder litter in fine mesh bags there was a suggestion of a decline in numbers after 16 weeks. Numbers of viable colonies were slightly higher on willow than on alder leaves, but there was no clear evidence of a difference in viable counts in bags of different mesh size. Numbers of viable yeasts fluctuated more than numbers of mycelial fungi. In most cases they showed peak numbers of 10^7-10^8 g^{-1} dry weight after 10-16 weeks of submersion although in the case of alder litter in large mesh bags peak numbers were recorded after 2 weeks. On all samples there was a decline in yeast numbers after 18 weeks, to levels between 2 and 100 times lower than in the second week of submersion.

Figure 9.18 shows agar film total and active biomass for alder and willow litter at site 1 in bags of different mesh size. Fungal biomass increased on all leaf material irrespective of leaf species or mesh size and, in most cases, reached a peak after 20-22 weeks. In the case of willow in large and medium mesh bags there was an extra peak after 14 weeks. Agar film biomass on litter in fine mesh bags of alder and willow were similar but not for these species in large and medium mesh bags. In extra large bags of alder and willow litter the peak level of biomass was less well defined than with litter from bags of other mesh sizes. Total biomass

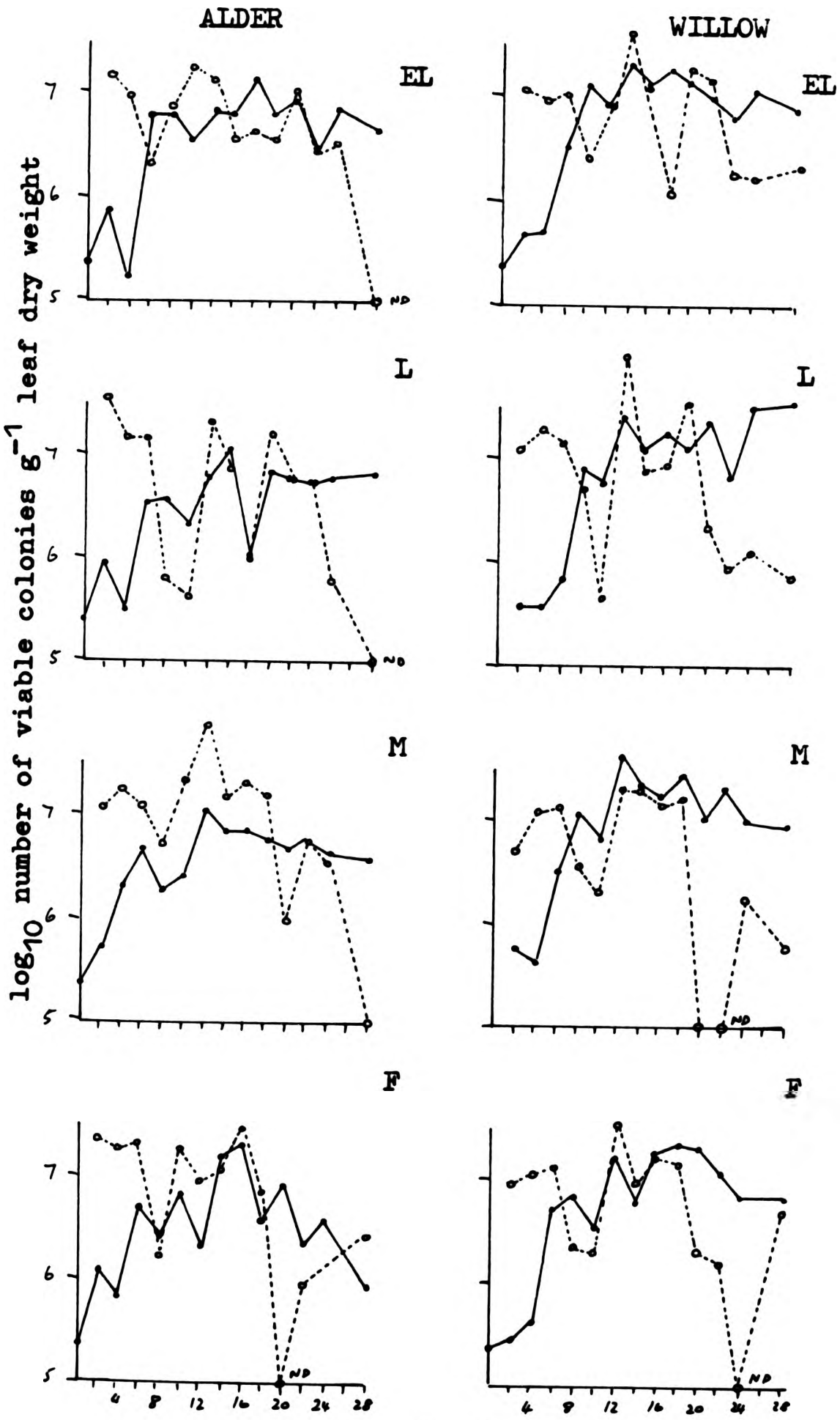


Figure 9.17. Numbers of viable fungal colony forming units on alder and willow leaf litter over a period of 7 months submersion at site 1 in Yateley 4.

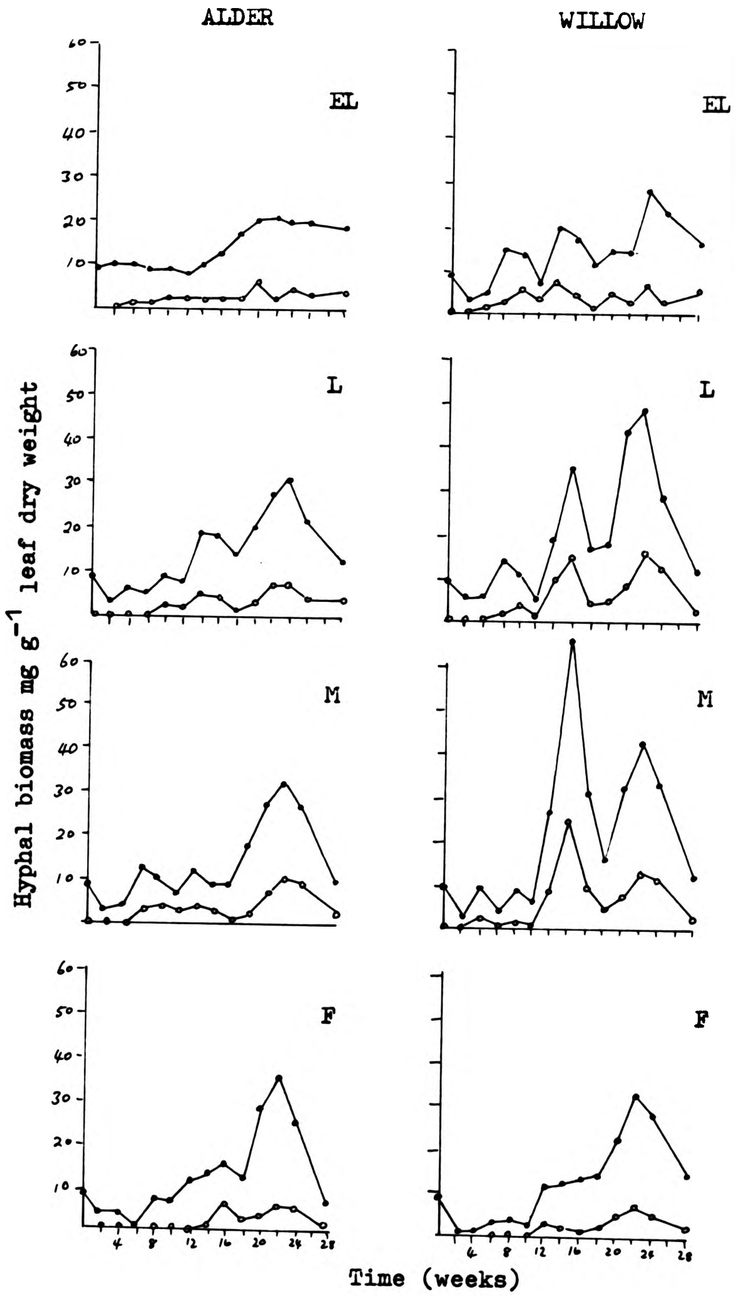
Mycelial fungi •——•, Yeasts o·····o, ND = not detected

Figure 9.18. Agar film fungal biomass on alder and willow leaf litter over a 7 month period of submersion at site 1 in Yateley 4. The values shown for each mesh type are means (mg g^{-1} dry weight*1)

Agar film total biomass

Agar film active biomass

*1 assumption biovolume to biomass conversion factor 0.29 cm^{-3}



ranged from 1.2-35.4 mg g^{-1} on alder and from 1.5-66.3 mg g^{-1} leaf dry weight on willow with means of 14.5 mg g^{-1} $\pm$ 1.2 and 17.6 mg g^{-1} $\pm$ 1.9, respectively. Active biomass showed a similar trend to total biomass but levels were lower, ranging from 0.2-10.5 mg g^{-1} on alder and from 0.2-24.8 mg g^{-1} on willow with means of 3.4 mg g^{-1} $\pm$ 0.4 and 4.9 mg g^{-1} $\pm$ 0.7, respectively. These values indicate that during the 28 weeks submersion, total fungal biomass was between 0.12-6.6% and active biomass 0.02-2.48% of leaf dry weight. Mean hyphal diameters were 3.23 µm $\pm$ 0.31 for alder and 3.85 µm $\pm$ 0.39 for willow.

Figure 9.19 shows the agar film biomass corrected for inefficiencies of homogenisation. Each graph shows the same trend as the uncorrected biomass estimates (Figure 9.18) although many of the peaks are more pronounced. There is no evidence of an increase in fungal biomass in either of the extra large mesh bags using the corrected figures. Corrected total biomass estimates ranged from 3.0-45.6 mg g^{-1} on alder and 3.0-95.5 mg g^{-1} on willow, with mean values of 20.1 mg g^{-1} $\pm$ 1.4 and 27.1 mg g^{-1} $\pm$ 2.5, respectively. The corresponding active biomass ranged from 0.3-13.9 mg g^{-1} on alder and 0.4-35.8 mg g^{-1} on willow, with mean values of 4.7 $\pm$ 0.5 and 7.5 $\pm$ 0.9, respectively. These values indicated that the corrected total biomass was 0.3-9.6% and active fungal biomass 0.03-3.58% of leaf dry weight during the 28 weeks of submersion.

Table 9.6 shows correlations between viable counts for fungi and bacteria, fungal biomass, ATP and FDA hydrolysis at Site 1 over the 28 weeks period. Data from all mesh sizes of alder and willow are combined for the analysis. Both agar film total biomass and agar film active biomass show significantly positive correlations with fungal viable counts, FDA hydrolysis and ATP. The agar film total biomass and FDA hydrolysis both showed a significant negative correlation with the numbers of viable yeasts, but showed significant positive

Figure 9.19. Corrected*1 agar film fungal biomass on alder and willow leaf litter over a period of 7 months submersion in Yateley 4. The values shown for each mesh type are means (mg g^{-1} dry weight*2).

Agar film total biomass

Agar film active biomass

*1 Corrected for inefficiency of homogenisation

*2 Assumption: biovolume to biomass conversion factor = 0.29 g cm^{-3}

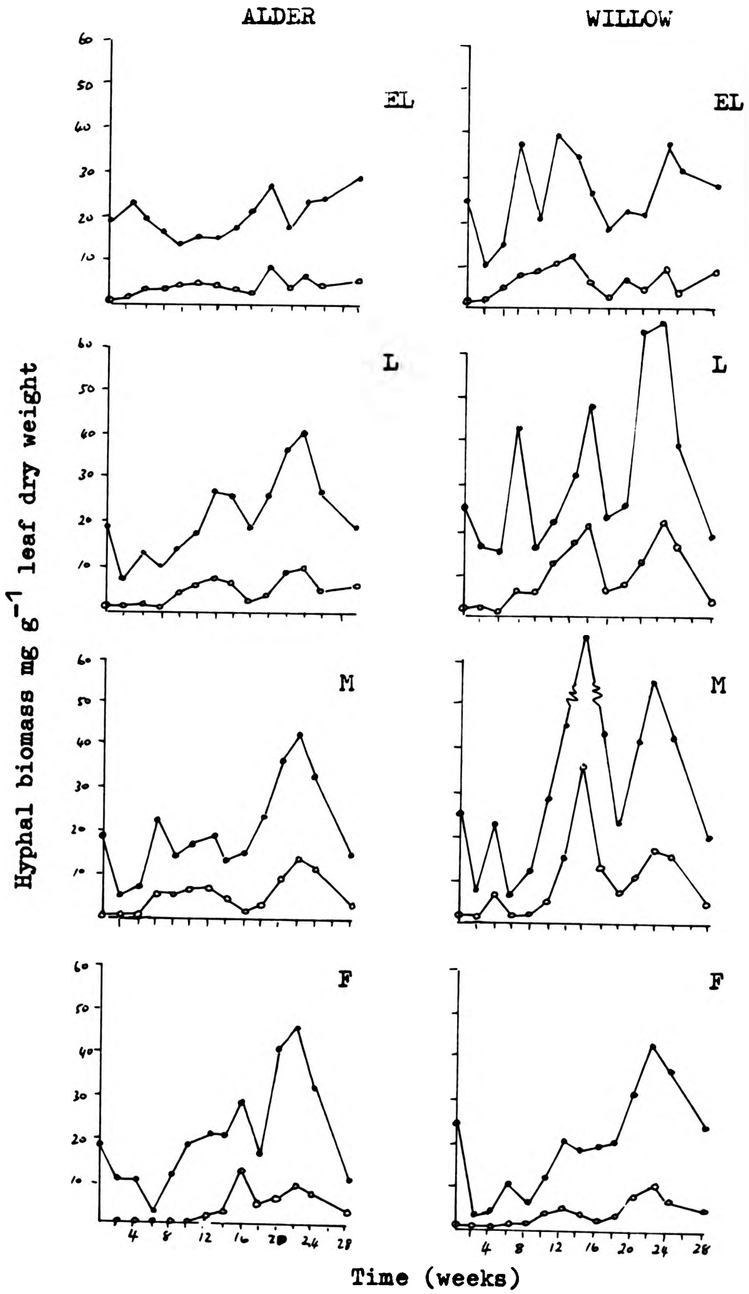


TABLE 9.6 Correlation coefficients (r) between different measurements of biomass and activity on leaf litter at Site 1 in Yateley 4.

VARIABLE[+]	1	2	3	4	5
1 No. Fungal cfu´s					
2 Log No. Fungal cfu´s	0.812[*3]				
3 No. Yeasts	0.263[*2]	0.133			
4 Log_{10} No. Yeasts	0.105	0.234[*1]	0.697[*3]		
5 No. Bacteria	0.163	0.229[*1]	0.003	0.136	
6 Log_{10} No Bacteria	0.169	0.157	0.100	0.248[*1]	0.699[*3]
7 FDA hydrolysis	0.208[*1]	0.332[*2]	-0.229[*1]	-0.280[*2]	0.366[*3]
8 ATP	0.420[*3]	0.450[*3]	0.088	-0.306[*2]	-0.178
9 Agar film total[*+]	0.490[*3]	0.503[*3]	-0.075	-0.249[*1]	0.111
10 Agar film active[*+]	0.526[*3]	0.495[*3]	-0.096	-0.113	0.156

VARIABLE[+]	6	7	8	9
1 No. Fungal cfu´s				
2 Log No Fungal cfu´s				
3 No. Yeasts				
4 Log_{10} No. Yeasts				
5 No. Bacteria				
6 Log_{10} No Bacteria				
7 FDA hydrolysis	-0.062			
8 ATP	-0.300[*2]	0.430[*3]		
9 Agar film total[*+]	-0.071	0.379[*3]	0.553[*3]	
10 Agar film active[*+]	0.041	0.281[*2]	0.430[*3]	0.885[*3]

KEY: *1 $p<0.05$; *2 $p<0.01$; *3 $p<0.001$; + n=104; *+ not corrected for inefficient homogenisation.

correlations with bacteria. ATP showed significant positive correlations with viable counts of fungi, but significant negative correlations with numbers of viable bacteria and yeasts. Numbers of pectinolytic bacteria (not shown) were significantly correlated with total numbers of aerobic bacteria ($r = 0.983$, $P < 0.001$) and FDA hydrolysis ($r = 0.321$, $P < 0.001$). Agar film total biomass showed a highly significant correlation with agar film active biomass.

9.3.5 Colonisation of litter bags by invertebrates

Figure 9.20 shows the changes in numbers of invertebrate animals in bags of alder and willow leaf litter during 28 weeks submersion at the 5 sites in Yateley 4. In extra large and large mesh bags of alder and willow there was a rapid increase in the numbers of invertebrates during the first 8-10 weeks with maximum numbers (51.6 ± 32.5 - 71.4 ± 89.8) occurring between 14 and 18 weeks. With medium mesh bags colonisation was much slower with maximum numbers on alder and willow being reached after 28 weeks (58 ± 30.5 and 58.4 ± 35.5, respectively). Few animals were found in fine mesh bags (maximum 6) and these were either Oligochaetes or Chironomid larvae. All litter bags showed a decline in the numbers of animals between 28 and 40 weeks. Both *Gammarus pulex* L. and *Asellus aquaticus* L. formed a significant proportion (up to 50%) of the animals in each bag. Peak numbers of these invertebrates occurred after 20-24 weeks. The most abundant invertebrate overall was the triclad *Dugesia tigrina* (Girard).

9.3.6 Colonisation of litter by Ingoldian fungi

Colonisation of litter by Ingoldian fungi was monitored at all sites in Yateley 4 over a 40 week period. Figure 9.21a shows the influence of submersion time on the number of litter bags colonised.

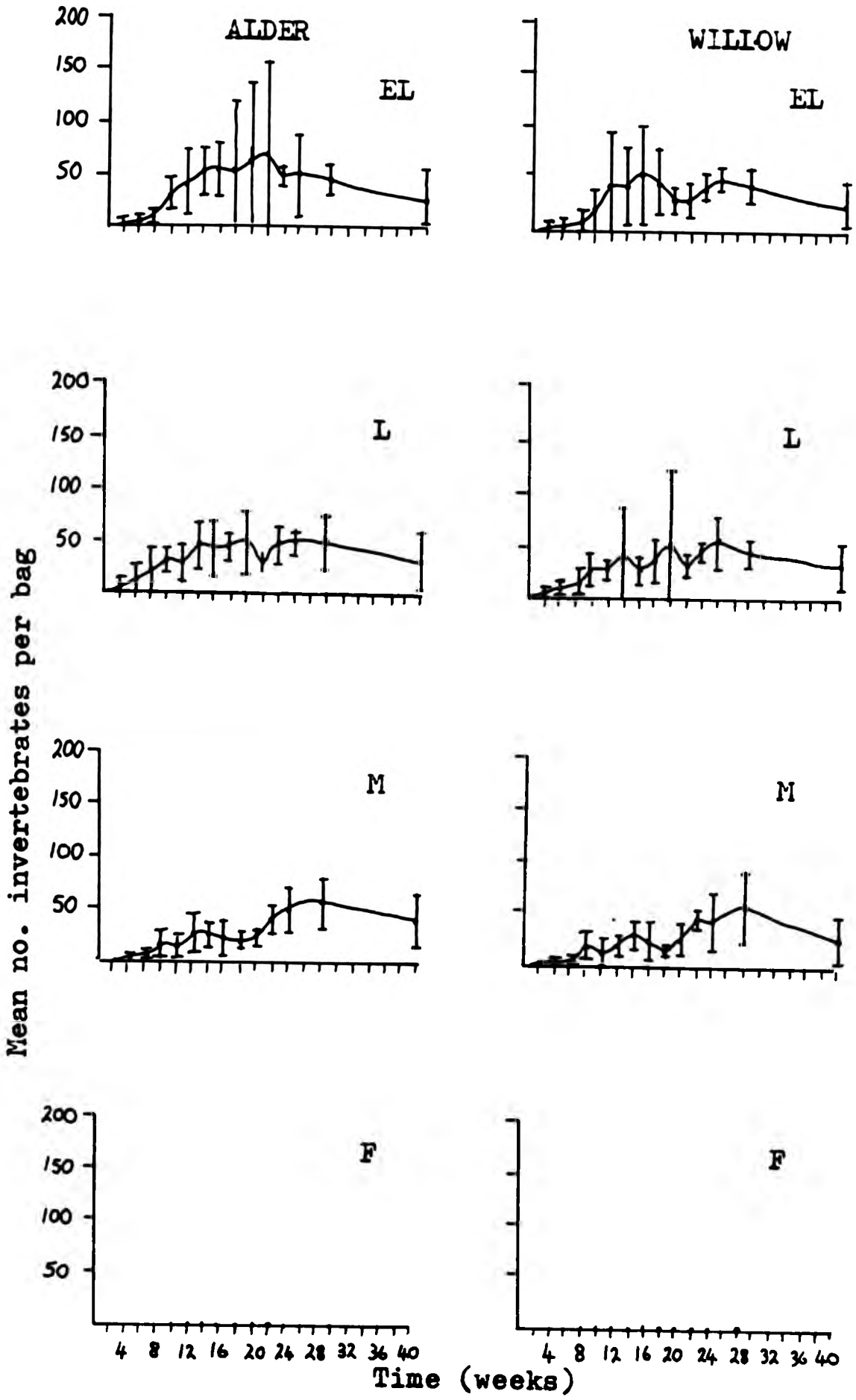
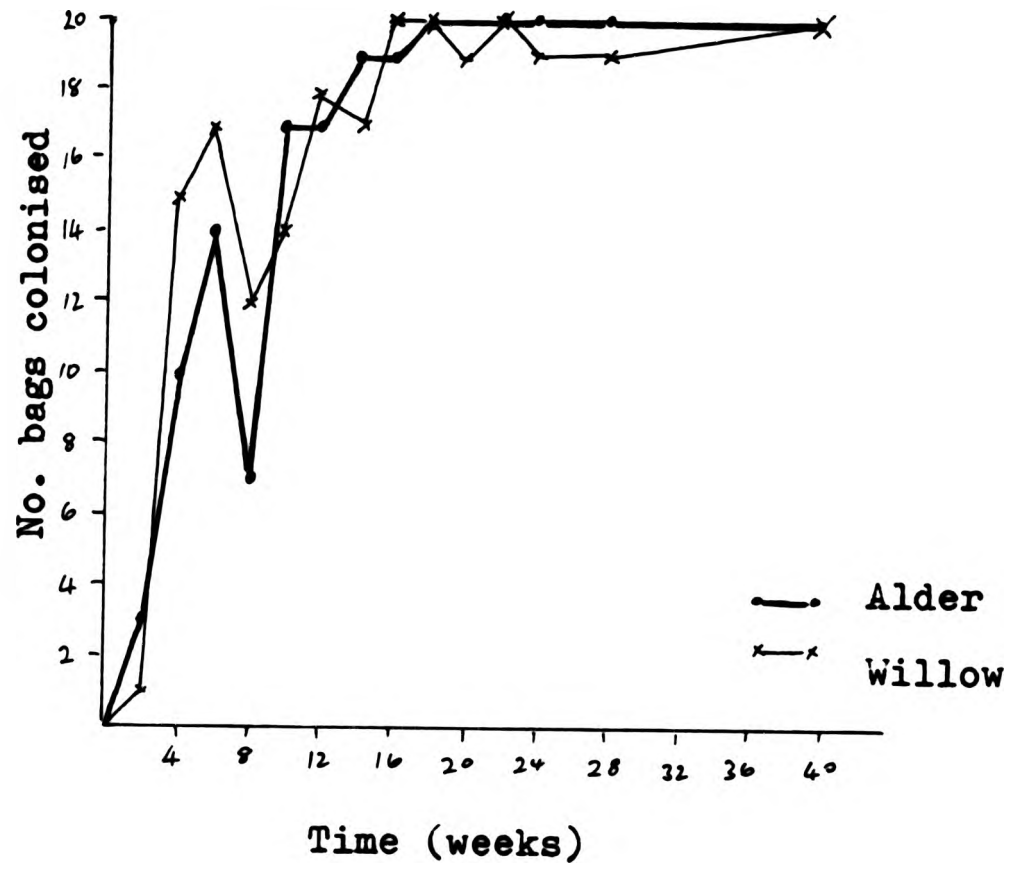


Figure 9.20. Numbers of invertebrates colonising alder and willow litter bags over a period of 10 months submersion in Yateley 4. Values shown for each mesh type are the mean number of invertebrates per bag together with standard deviations.

a)



b)

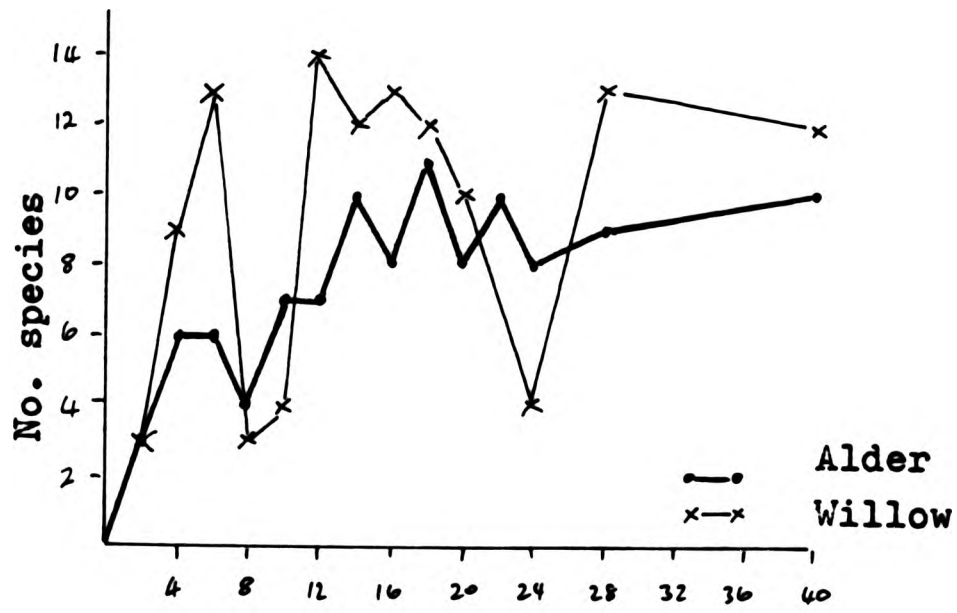


Figure 9.21. Influence of submersion time on a) the number of litter bags colonised and b) the number of species of Ingoldian fungi on alder and willow litter.

Colonisation was very rapid with 50% of alder and 75% of willow litter bags being colonised in the first 4 weeks of submersion. By 16 weeks almost all the litter bags had been colonised. Figure 9.21b shows the number of species on the two leaf types over the study period. Although both leaf types showed a steady increase in numbers of species the trend for willow fluctuated much more than for alder. On alder, species number increased for the first 14-16 weeks and then oscillated around a mean number of species until week 40. Willow generally had more species than alder but the numbers were sometimes lower (eg., week 24).

Figure 9.22 shows a) the number of new species per sample date and b) the species turnover rate for Ingoldian fungi on alder and willow leaf litter over the 40 week period. It can be seen in a) that there is a decline in the addition of new colonising species with increasing submersion time, the trend being similar for both alder and willow. Most species colonised in the first 12 weeks followed by a period of 12 weeks when there were few new species. Percentage species turnover rate (Figure 9.22b) was highest for alder (67%) between weeks 6 and 8 and highest for willow (66%) between weeks 8 and 10. Between weeks 12 and 14 there was a marked fall in species turnover, to below 30% for both species, but it increased after week 20 to reach a peak of 40% for alder between weeks 24 and 28, and 54% for willow between weeks 22 and 24.

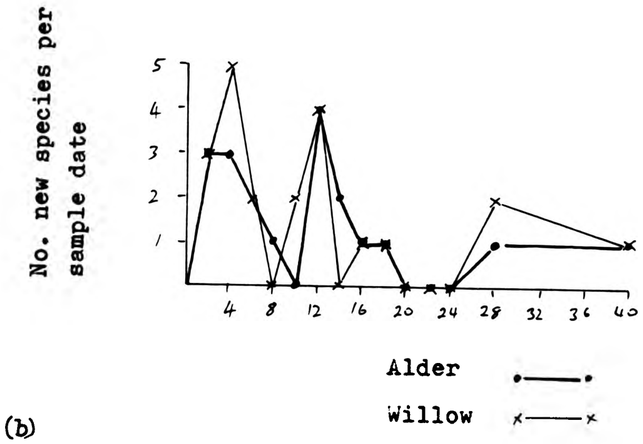
Figure 9.23 shows the influence of mesh size on the number of Ingoldian fungi colonising alder and willow litter after different times of submersion. There was an increase in the number of species on litter of alder and willow for all mesh sizes, but species number fluctuated more on willow than on alder. In the extra large and large mesh bags there was a higher maximum number of species (12 and 11, respectively) on willow than on alder (7 and 8, respectively), but there were no differences in maximum number of species between leaf

Figure 9.22. Number of new species (a) and species turnover rate, % (b) for Ingoldian fungi colonising alder and willow leaf litter over a period of 10 months submersion in Yateley 4.
Number of new species represents difference in species number between sampling dates, previous colonists are included.
Species turnover (QS) was estimated using the modified Sorensen's formula (Glowacinski & Jarvinen, 1975)

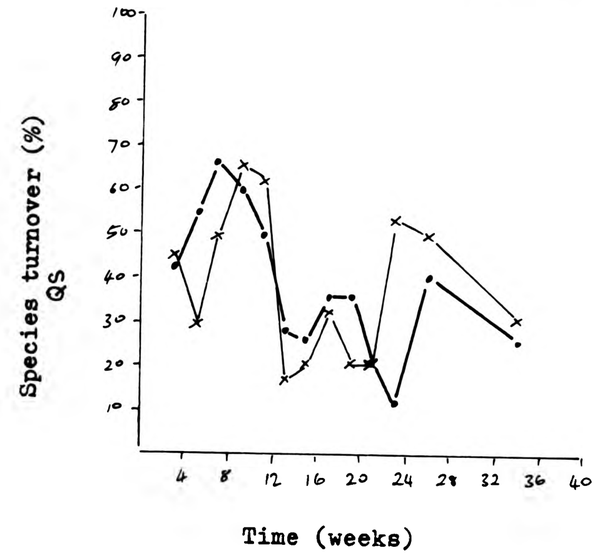
$$QS = 100\ (a + b) / a + b + 2c$$

where a and b are the numbers of species which are present in either assemblage (ie., at time a or b) but not in both, and c is the number of species common to both assemblages (ie., present at both time a and b).

(a)



(b)



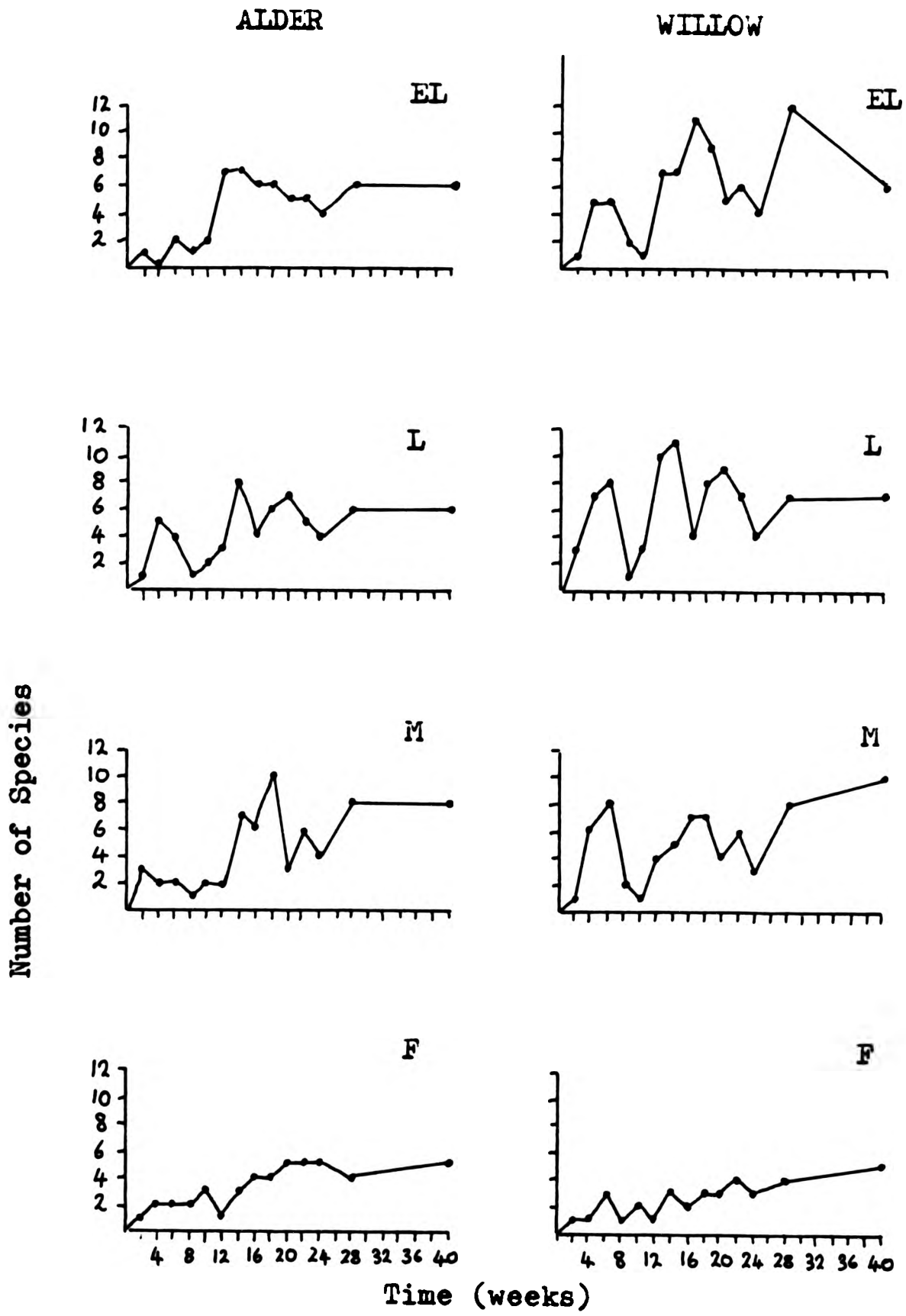


Figure 9.23. Influence of mesh size on the number of Ingoldian fungi colonising alder and willow leaf litter over a period of 10 months submersion in Yateley 4. The numbers of species represents the total recorded for a particular mesh type.

types with medium or fine mesh bags. With species number at 8, 18 and 28 weeks there was a significant effect of submersion time for both alder and willow ($P < 0.001$). There was also a significant difference in numbers of species between mesh sizes, both for alder ($P < 0.05$) and for willow ($P < 0.01$).

Figure 9.24 shows the influence of mesh size on the cumulative number of species of Ingoldian fungi recorded on a) alder and b) willow over a 40 week period of submersion. In the case of alder, the cumulative number of species on litter in all mesh sizes increased until week 28 when it reached a maximum of 18 species in medium mesh bags. Ingoldian fungi in fine mesh bags show a similar cumulative species curve as the other mesh sizes, reaching a maximum of 15 species after 28 weeks. In the case of willow the cumulative species species curves were still increasing after 40 weeks submersion in the lake with 22 species reached in large and medium mesh bags. Fine mesh bags had a considerably lower number of species than the other mesh sizes with only 11 species recorded after 40 weeks. The difference in these cumulative number of species in fine mesh bags relative to other mesh sizes suggests that the presence of animals in the litter bags had a significant influence on colonisation of willow leaves by Ingoldian fungi.

Table 9.7 shows the species of fungi associated with alder leaf litter during 40 weeks submersion in Yateley 4. *Alatospora acuminata* and *Anguillospora longissima* were found during most of the study, occurring on litter in a large proportion of the bags. *Tetracladium marchalianum*, *Clavatospora stellata*, *Clavatospora longibrachiata* and *Varicosporium elodae* became most frequent after 12-14 weeks and *Clavariopsis aquatica*, *Flagellospora curvula* and *Lemonniera aquatica* after 28 weeks. *Fusarium* spp. were found in the first 20 weeks of submersion and aquatic phycomycetes in the first 8 weeks. *Helicodendron* sp. was observed after 40 weeks submersion. Table 9.8 shows the fungal species associated with willow leaves.

Figure 9.24. Influence of mesh size on the cumulative numbers of species of Ingoldian fungi detected on a) alder and b) willow leaf litter over a period of 10 months submersion in Yateley 4.

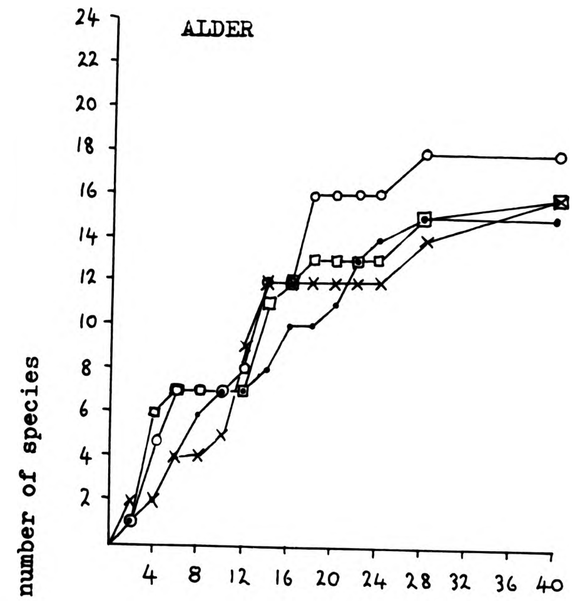
Medium

Fine

Large

Extra large

a)



b)

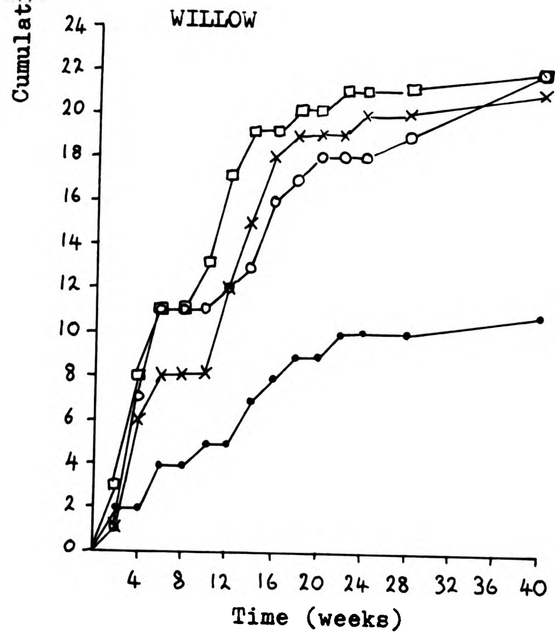


TABLE 9.7 Fungi associated with Alder leaf litter over a period of 10 months submersion in Yateley 4. Species were recorded by direct observation after 5 days incubation in sterile lake water in the laboratory. The numbers at each sample date refer to the number of bags of litter (maximum 20,5x4 mesh sizes) on which fungi were observed sporulating.

	TIME WEEKS													
NO. SPECIES	2	4	6	8	10	12	14	16	18	20	22	24	28	40
1 Alatospora acuminata		2	12	6	15	16	18	18	20	19	18	15	14	14
2 Anquillospora longissima		1	2	1		5	9	11	15	15	18	13	19	13
3 Tetracladium marchalianum						2	2	4	2	4	1	2		
4 Clavariopsis aquatica													7	5
5 Flagellospora curvula			1										2	7
6 Lemonniera aquatica		1	1				1	1	3				2	13
7 Articulospora tetracladia	1	4					2			1			1	1
8 Dimorphospora folliicola				1					1					2
9 Clavatospora stellata		1			1	9	7	4	11	2	7	3	5	
10 Clavatospora longibrachiata						2	5	4	6	3	3	2	2	

NO. SPECIES	TIME WEEKS 2	4	6	8	10	12	14	16	18	20	22	24	28	40
11 Varicosporium elodae							3		3	3	2	1	3	1
12 Tetrachaetum elegans								3		3	1	1		
13 Tricladium splendens							1		1					1
14 Margaritospora aquatica						1					1	1		
15 Tetracladium setigerum						2			3					
16 Tricladium spl							1							
17 Tricladium sp2									1					
18 Filiform spl											1			
19 Unknown sp6					1									
20 Unknown sp7					1									
21 Fusarium spp	2	3			2		3	2			1			
22 Helicodendron sp														4
23 Achlya spp			1											
24 Pythium spp			1	1										
25 Saprolegnia spp 1														
26 Candida aquatica						2								

TABLE 9.8 Fungi associated with willow leaf litter over a period of 10 months submersion in Yateley 4. Species were recorded by direct observation after 5 days incubation in sterile lake water in the laboratory. The numbers at each sample date refer to the number of bags of litter (maximum 20,5x4 mesh sizes) on which fungi were observed sporulating.

	TIME (WEEKS)													
NO. SPECIES	2	4	6	8	10	12	14	16	18	20	22	24	28	40
1 Alatospora acuminata	1	7	16	12	13	18	16	20	18	16	9	8	14	9
2 Anquillospora longissima		7	4	1		9	8	13	15	10	16	8	14	11
3 Tetracladium marchalianum		4	5			4		2	1	1	1		3	
4 Clavariopsis aquatica													7	4
5 Flagellospora curvula		1	2			1			1	1	1		1	5
6 Lemonniera aquatica		1					1	1	3	1			1	13
7 Articulospora tetracladia	1	1	1					1						3
8 Dimorphospora folliicola			2			1								1
9 Clavatospora stellata		2				1	3	1	4	1	6	8	4	1
10 Clavatospora longibrachiata						2	3		3	5	3		1	2

	TIME WEEKS													
NO. SPECIES	2	4	6	8	10	12	14	16	18	20	22	24	28	40
11 *Varicosporium elodae*					1	1	3	1	4		1		3	
12 *Tetrachaetum elegans*						1	2	1	3	2		3	3	
13 *Tricladium splendens*						2	3	6	3	1	1		7	
14 *Margaritospora aquatica*						1		2		1	1		2	1
15 *Tetracladium setigerum*					1	2	1	1						
16 *Filiform* spl			1					1						
17 *Filiform* sp2		4												
18 Unknown sp3			1											
19 Unknown sp4 (El)							1							
20 Unknown sp5												1		
21 *Helicodendron* sp														2
22 *Arthrobotrys* sp			4			1								
23 *Acaulopage* sp			1											
24 *Alternaria* sp									1					
25 *Penicillium* sp														1
26 *Fusarium* spp	1	4	1	1		2	3	2	1		8			
27 *Pythium* spp			8				1							
28 *Saprolegnia* spp			1											
29 *Candida aquatica*	1				1									

Again both Alatospora acuminata and Anguillospora longissima were present throughout most of the study on willow litter in a large proportion of bags. Several species occurred sporadically during the study, but Clavatospora stellata, Clavatospora longibrachiata, Varicosporium elodae, Tetrachaetum elegans, Tricladium splendens and Magaritispora aquatica became noticeably more frequent after 12 weeks submersion. Clavariopsis aquatica was only found after 28 weeks and Helicodendron sp. only after 40 weeks. Fusarium spp. occurred on litter for the first 28 weeks and aquatic phycomycetes were found until week 14. Both Alternaria sp. and Penicillium sp. were observed sporulating on the edge of leaf discs of willow. For alder and willow there were clearly distinct changes in the species composition of the fungal communities at different stages of leaf decomposition.

Figure 9.25 i) shows the total spore production by Ingoldian fungi on alder and willow litter and ii) and iii) shows spore production by the two most abundant species. Total spore production increased with submersion time, with the highest number of spores being recorded in extra large and large mesh bags of alder. Spore production from litter in fine mesh bags was generally lower than that from litter in other mesh sizes. Analysis of variance for spore production after 8, 18 and 28 weeks indicated that, for both alder and willow, there was a significant effect of submersion time (age) ($P < 0.001$) and a significant effect of mesh size ($P < 0.001$ and $P < 0.05$ for alder and willow, respectively). However, the shape of the spore production curves appeared to differ more between leaf types than between mesh sizes, suggesting that leaf type was an important factor influencing spore production.

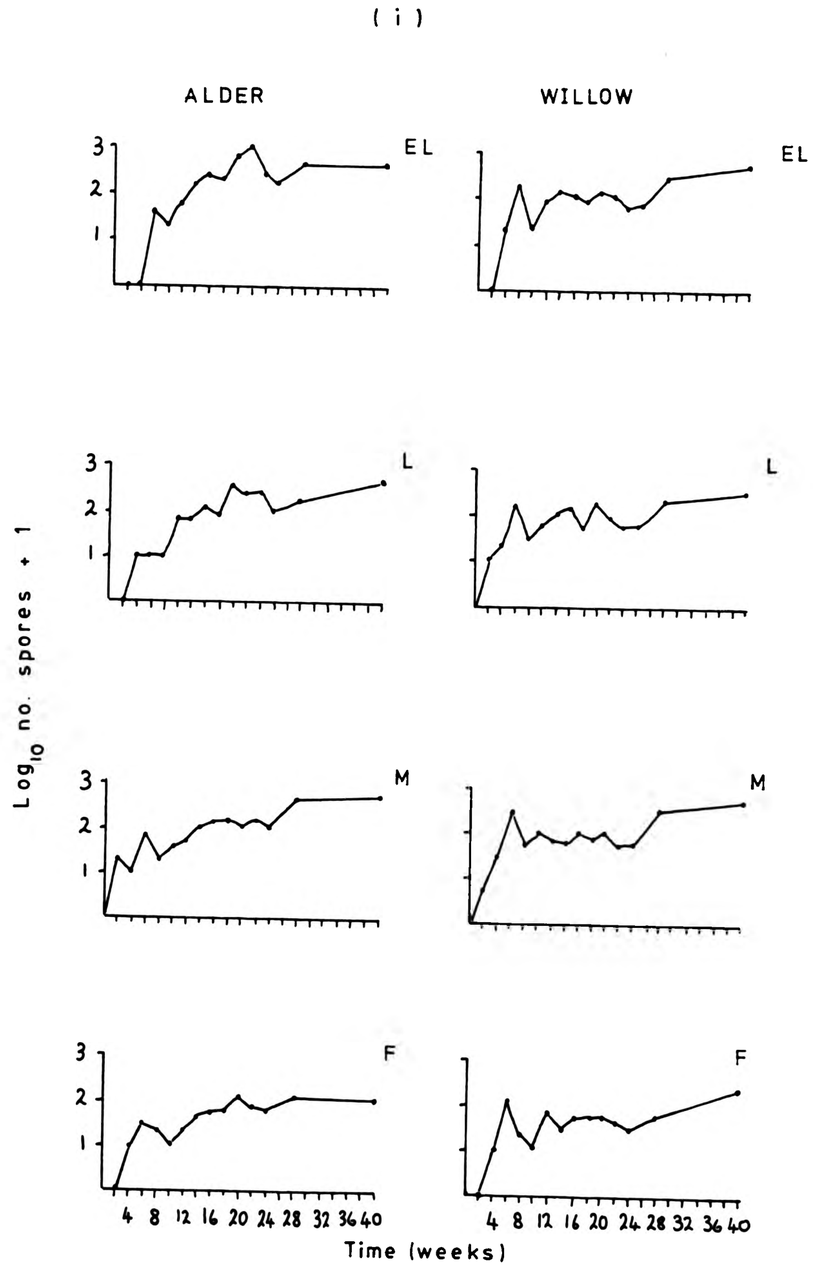
Figure 9.25 ii) shows the spore production curves for Alatospora acuminata. On alder, maximum spore production occurred after 18-28 weeks and on willow after 6 weeks. Spore production by this species

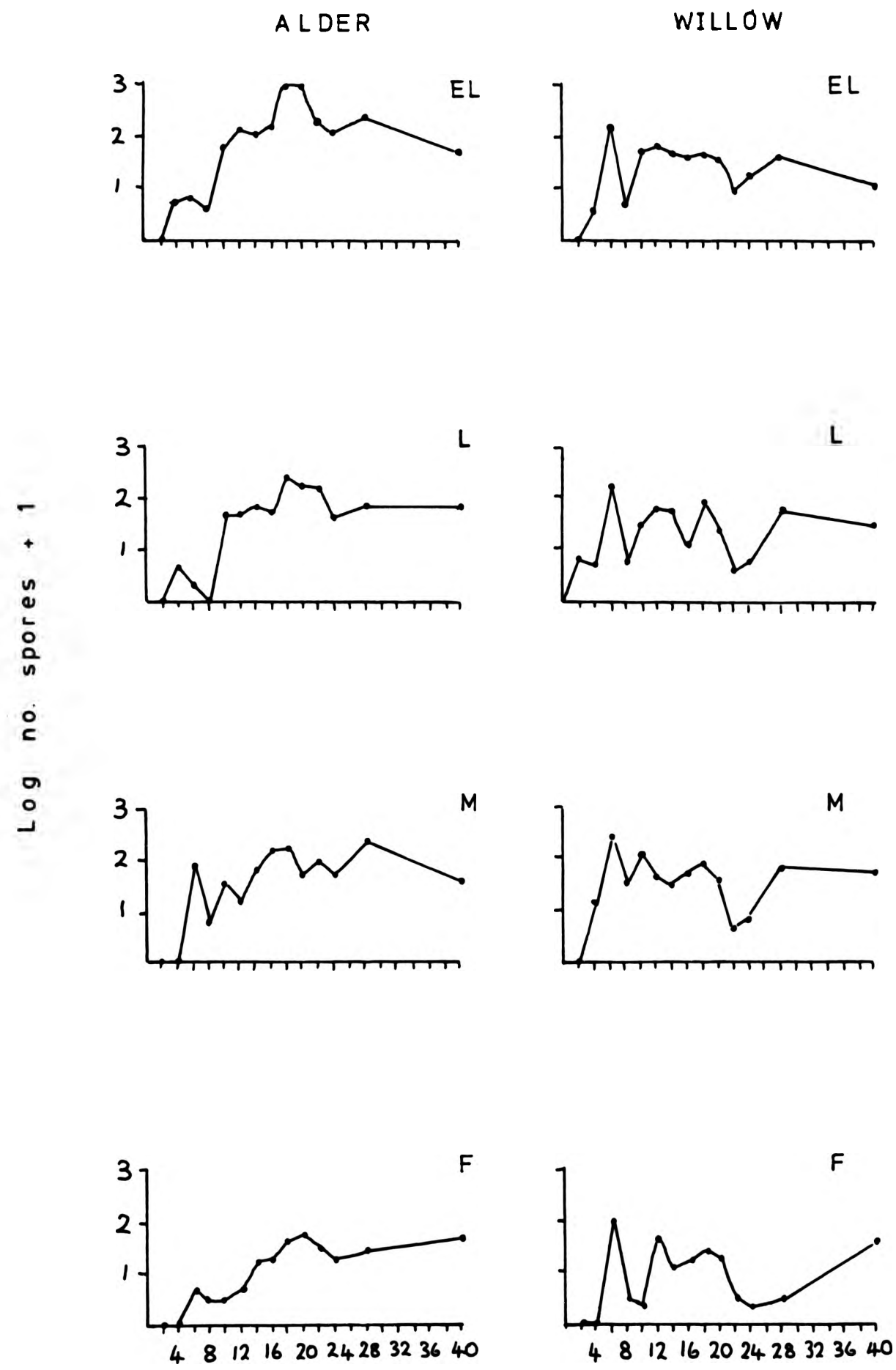
Figure 9.25. Spore production by Ingoldian fungi on alder and willow leaf litter retrieved from Yateley 4 over a 10 month period. Plots are of $\log_{10}$ number of spores + 1 against time, for each mesh type for

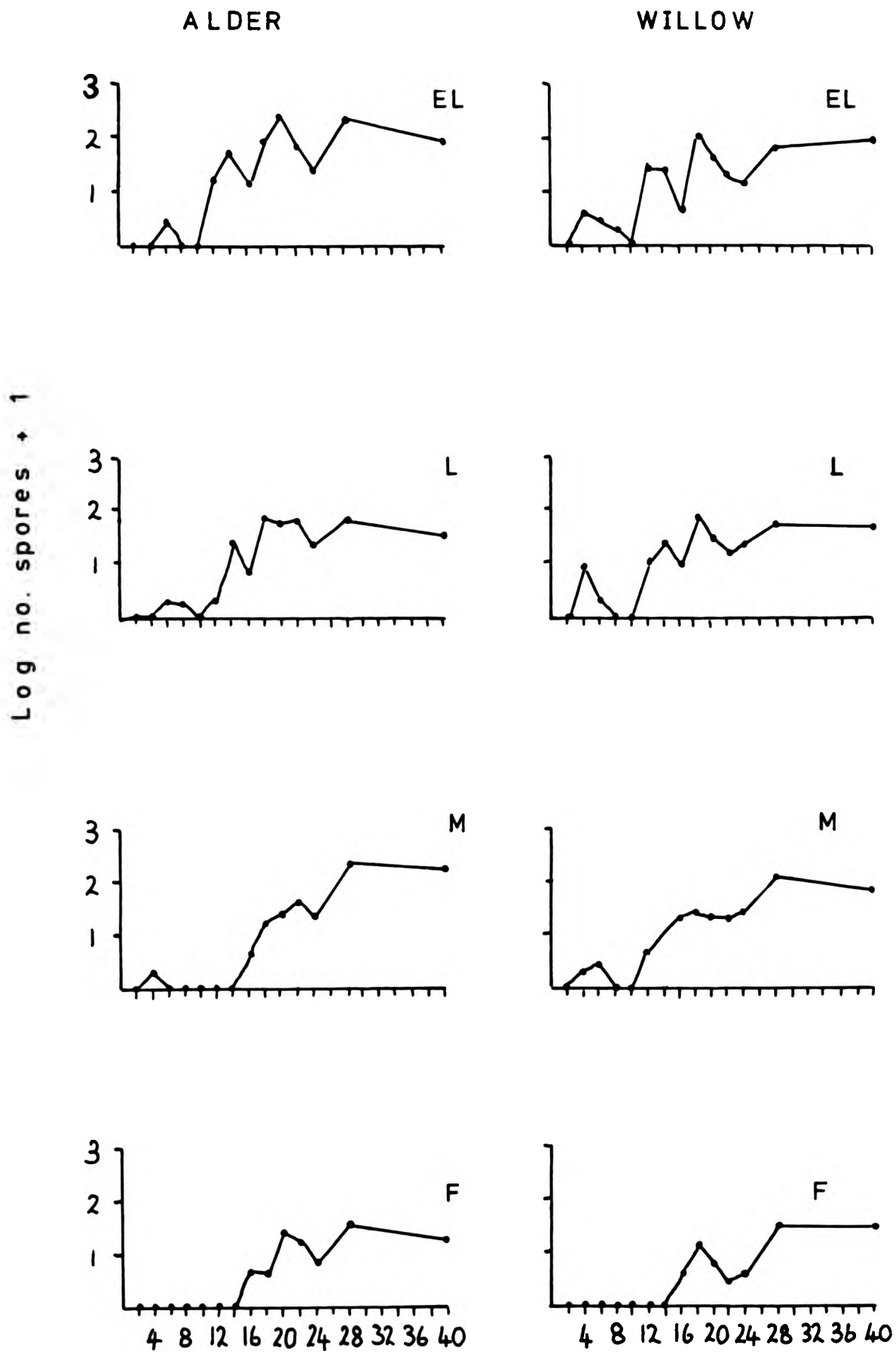
i) total Ingoldian fungi

ii) Alatospora acuminata

iii) Anguillospora longissima




ALDER
WILLOW
EL
L
M
F
3
2
1
Log no. spores + 1
4 8 12 16 20 24 28 32 36 40
Time (weeks)

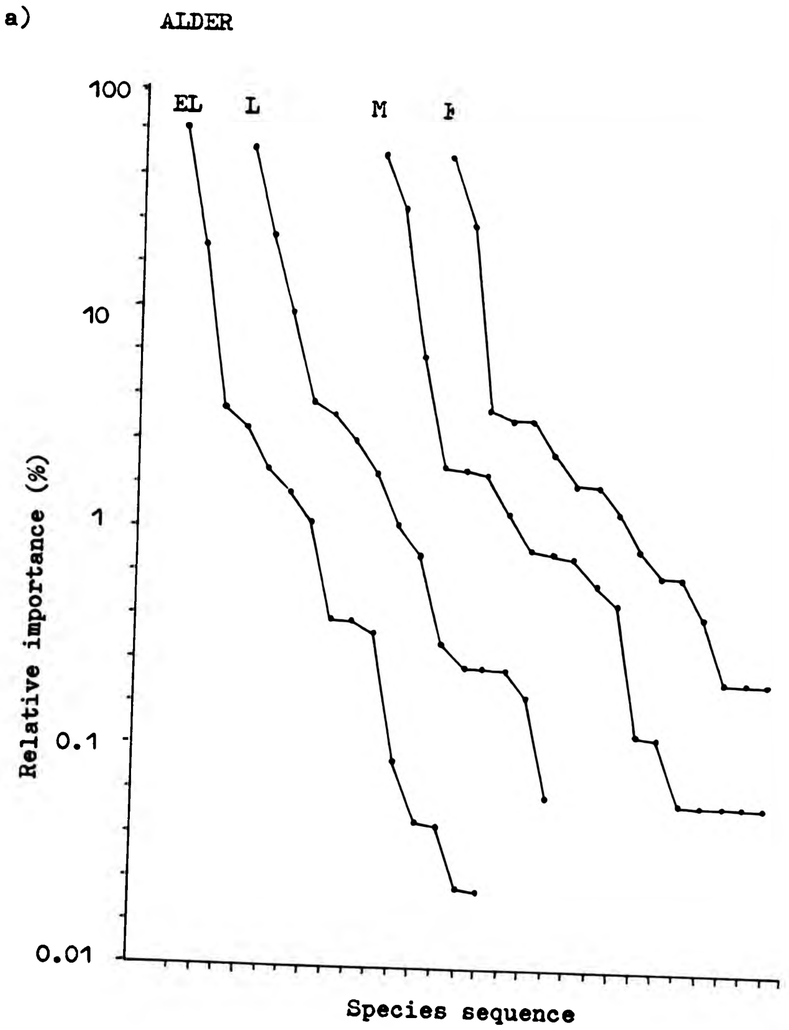

ALDER
WILLOW
EL
L
M
F
Log no. spores + 1
3
2
1
4 8 12 16 20 24 28 32 36 40
Time (weeks)

fluctuated more on willow than on alder and this appeared to be irrespective of mesh size. Spore production was lower on willow than on alder and lower on litter in fine mesh bags than on litter in other mesh sizes. Figure 9.25 iii) shows the pattern of spore production for Anguillospora longissima. Spore production by this species was sporadic for the first 10-14 weeks but increased sharply to reach a peak after 20-28 weeks. As in the case of Alatospora acuminata, spore production by Anguillospora longissima was lower on litter in fine mesh bags, but difference in spore production between alder and willow was less marked. At 8, 18 and 28 weeks spore production was significantly correlated ($r = 0.618$, $P < 0.001$) with the number of Ingoldian fungal species and spore production by Alatospora acuminata was significantly correlated with spore production by Anguillospora longissima ($r = 0.383$, $P < 0.001$). With the study at Site 1 over 28 weeks, total spore production was again correlated with the number of Ingoldian fungal species ($r = 0.551$, $P < 0.001$) and spore production by Alatospora acuminata was correlated with that of Anguillospora longissima ($r = 0.511$, $P < 0.001$).

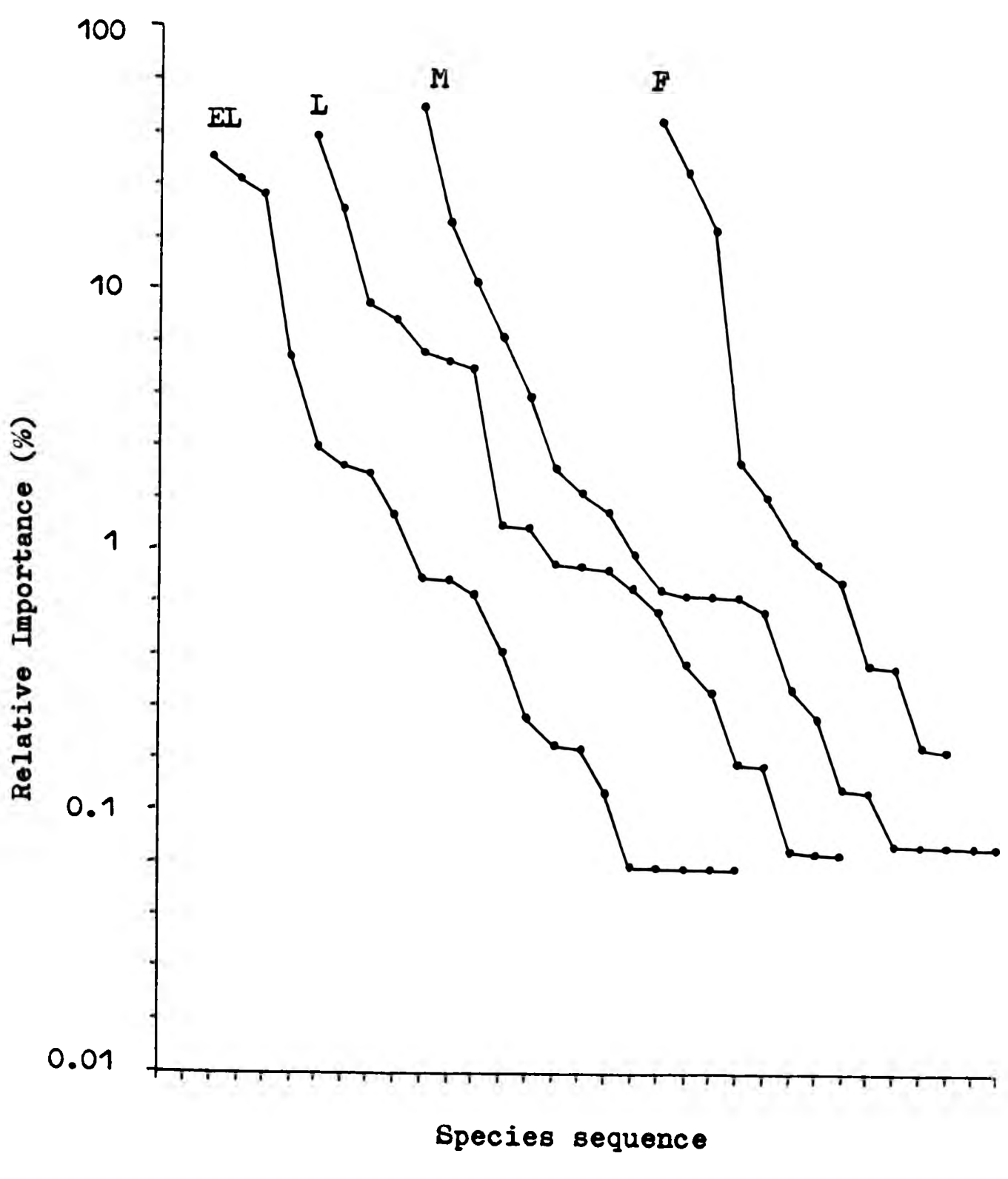
Figure 9.26 shows the importance values curves based on spore production by Ingoldian fungi on a) alder and b) willow leaf litter in bags of different mesh size. The shapes of these curves indicates the degree of homogeneity or evenness between species based on their reproductive output. In all cases, but particularly with willow, the curves are intermediate between those of the geometric and log normal distributions for species abundances (Whittaker, 1975; Christensen, 1981). With alder, communities on litter in bags of different mesh size are all dominated by 1 or 2 species of high frequency, hence the sharp initial drop in importance values with increasing species sequence. In the case of willow there are more species with high importance values and the curves for species sequences on litter in extra large, large and medium mesh bags are less steep than the

Figure 9.26. Importance value curves for Ingoldian fungi on alder and willow leaf litter over a period of 10 months submersion in litter bags in Yateley 4. The curves for different mesh sizes are shown for alder in a) and for willow in b). Each species is represented by a point located by that species' relative importance (the percentage that a particular species represents of the total spore production of all species on litter in all bags of a particular mesh size) on the vertical axis , and its position in the sequence of species from highest to lowest importance values, on the horizonatal axis. Note that the starting position for each species sequence is arbitrary and relates only to the particular importance value curve.

a)



b) WILLOW



corresponding ones for alder. This suggests that there are differences in the communities developing on the two leaf types and that these differences are largely independant of mesh size. In the case of willow in fine mesh bags the trend is less clear because of the much lower number of species present.

9.3.7 Aquatic phycomycetes

Figure 9.27 shows the number of hemp seeds colonised by aquatic phycomycetes after exposure to alder and willow leaf litter submersed for different periods of time. In both cases there appeared to be a marked increase in the inoculum being produced from colonised leaf material. The trend was similar on the two leaf types with a peak after 10 weeks and a steady rise in colonisation after week 12. Analysis of variance demonstrated that there was a significant increase in the number of colonised hemp seeds between 8, 18 and 28 weeks, for both alder and willow litter. Microscopic examination of colonised hemp seeds revealed that most of the colonising genera were Achlya and Pythium with Saprolegnia and Dictyuchus being more frequent in the first 10 weeks of study. There was no significant difference in the number of hemp seeds colonised from litter in bags of different mesh sizes of either alder or willow.

9.3.8 Changes in fungal populations isolated from homogenates of leaf material

Fungal genera were identified from colonies appearing on agar plates of litter homogenates from Site 1 over a 28 period of submersion. Fluctuations in numbers of viable colonies have already been described in Section 9.3.4 and illustrated graphically in Figure 9.17. Table 9.9 shows the presence of different fungal genera on alder and willow leaf litter after different submersion times. The number of fungal

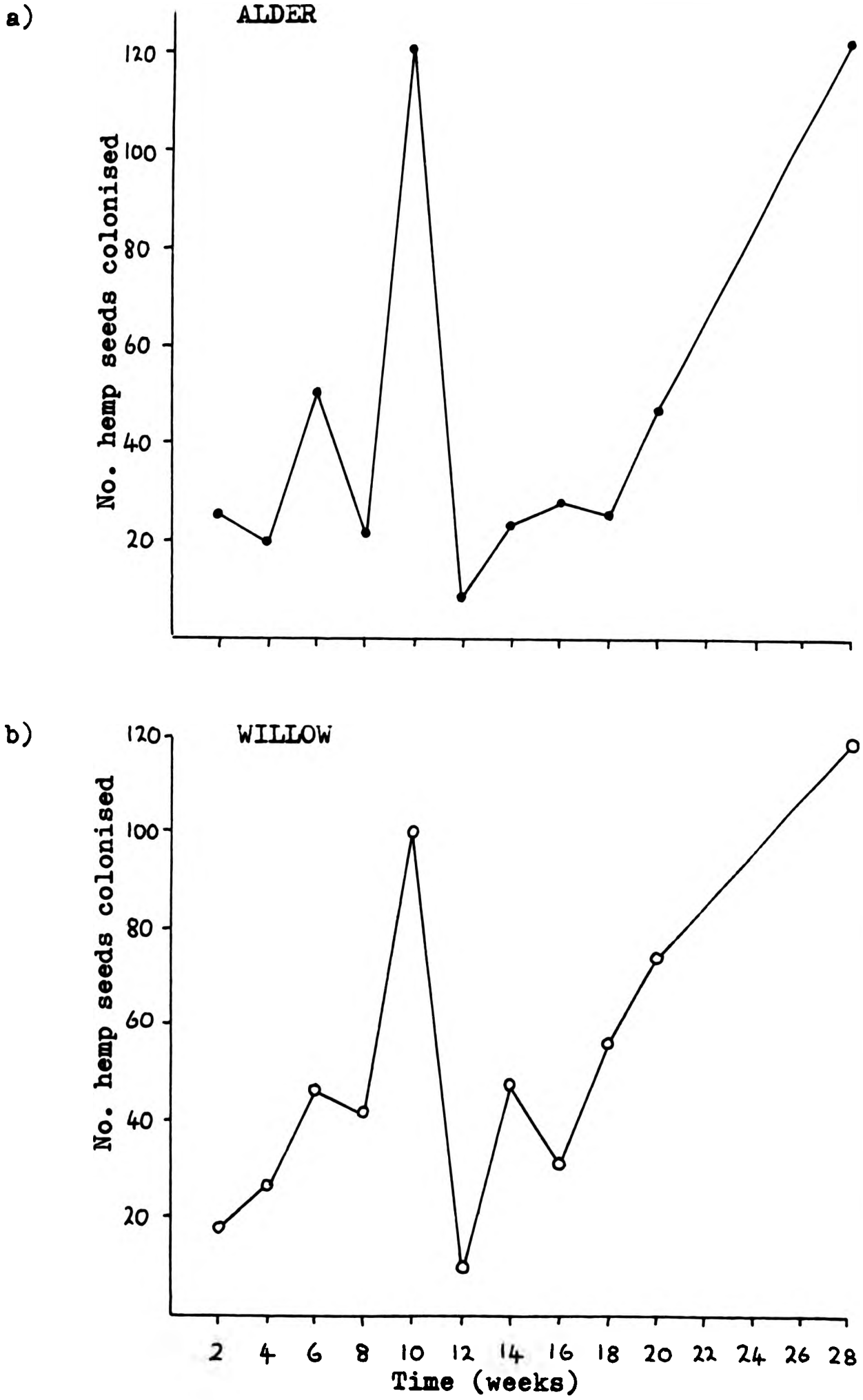


Figure 9.27. Number of hemp seeds colonised by aquatic hyphomycetes (maximum 200) on exposure to leaf material of a) alder and b) willow after different times of submersion in Yateley 4. Data for all mesh sizes was pooled.

TABLE 9.9 Fungal genera detected by viable plate counts of alder and willow leaf litter homogenates. Samples were taken over a period of 7 months at one site (site 1) in Yateley 4. The data from different mesh sizes were pooled together.

FUNGAL GROUPS	TIME (weeks)												
	2	4	6	8	10	12	14	16	18	20	22	24	28
Aureobasidium	*+	*+	*+	*+	*+	*+	*+	*+	*+	*+	*+	*+	
Cladosporium	*+	*+	*+	*+	*+	*+	*+	*+	*+	*+	*+	*+	+
Alternaria	*+	*+	*+	*+	*+	*+	*+	*+	+			+	+
Epicoccum	+	+	*+	+	*+	*+	+	*	+		+		+
Fusarium	+	+	*+	+	*	+	*+	+			+		+
Penicillium			+	*+	*+	+	+	*+	*	*+	*+	*+	
Phoma	+	+	*	*+	*+	+	+	+	*+				
Mucor					*	+	*	*	*+				
Acremonium					*	*			*		*+		+
Stemphylium				*+			+		*				*+
Botrytis		+				+		*+					
Pythium	*			*				+					
Verticillium							+		*+				
Gliocladium							*			*			
Aspergillus										+	*	+	
Trichoderma								*					*
Arthrobotrys	*	*											
Fusidium								+					
Sclerotopsis													+
Paecilomyces												+	
Mortierella											+		

FUNGAL GROUPS	TIME (weeks)												
	2	4	6	8	10	12	14	16	18	20	22	24	28
Lemonniera													+
Anguillospora										*	*		+
Tricladium										+	*	*+	
Clavariopsis								*		+			+
Varicosporium												+	
Tetracladium								+	+	+			
Unknown spl					*+			*+	+				
Sterile mycelium	+	*		+	*+		*+	*+	*+	*+	*+	*+	*+
Yeasts Black	*+	+	*				+						
Pink	*+	*+	*+	*+	*+	*+	*+	*+	*+	*+	*	+	
Yellow	*+	*+	*+	*+	*+	*+	*+	*+	*+	*+	*+	*	+
White	*+	*+	*+	*+	*+	*+	*+	*+	*+	*+	*+	*+	+

* Present on alder

+ Present on willow

genera identified increased with increasing submersion time, although by weeks 24 and 28 there were a large number of sterile colonies. Most genera were found on both alder and willow although Acremonium, Trichoderma and Gliocladium were more frequent on alder, and Phoma, Fusarium and Ingoldian fungi were more frequent on willow. There was some evidence of a change in the species composition on the litter with increasing submersion time. Alternaria, Fusarium and Epicoccum and black and pink yeast colonies appeared to decline, whereas other genera, Phoma, Penicillium, Acremonium, Stemphylium, Mucor and unknown sp.1 were recorded more often after several weeks of submersion. Ingoldian fungi were only found after 16 weeks but were present in all subsequent samples.

Figure 9.28 shows the changes in the numbers of viable colonies of Aureobasidium, Cladosporium and Alternaria increasing submersion time of alder and willow litter in bags of different mesh size. All 3 genera showed an initial increase (x 10) in numbers on both leaf types in all mesh sizes. They showed a general decline in numbers after 16-18 weeks, but in most cases numbers were higher than at time 0. There was no obvious difference in the abundance of these genera on litter in bags of different mesh size although, after submersion, Alternaria appeared to be more frequent on willow than on alder.

Table 9.10 shows the frequency of Aureobasidium, Cladosporium, Epicoccum and Mucor on plated discs of leaf material of alder and willow from bags of different mesh size. After 8 weeks submersion all species showed an increase in frequency but with continued submersion for 22 weeks all species, except Cladosporium, showed a decline. After 28 weeks all genera, except Cladosporium, were less frequent than before submersion. These trends appeared to be independent of the leaf type or mesh size.

Figure 9.28. Numbers of viable fungal colony forming units of selected fungi on alder and willow leaf litter over a period of 7 months submersion at site 1 in Yateley 4, in bags of different mesh size for

a) Aureobasidium pullulans

b) Cladosporium spp.

c) Alternaria alternata

For each species, viable colony forming units are shown on a log scale between 10^5 and 10^7.

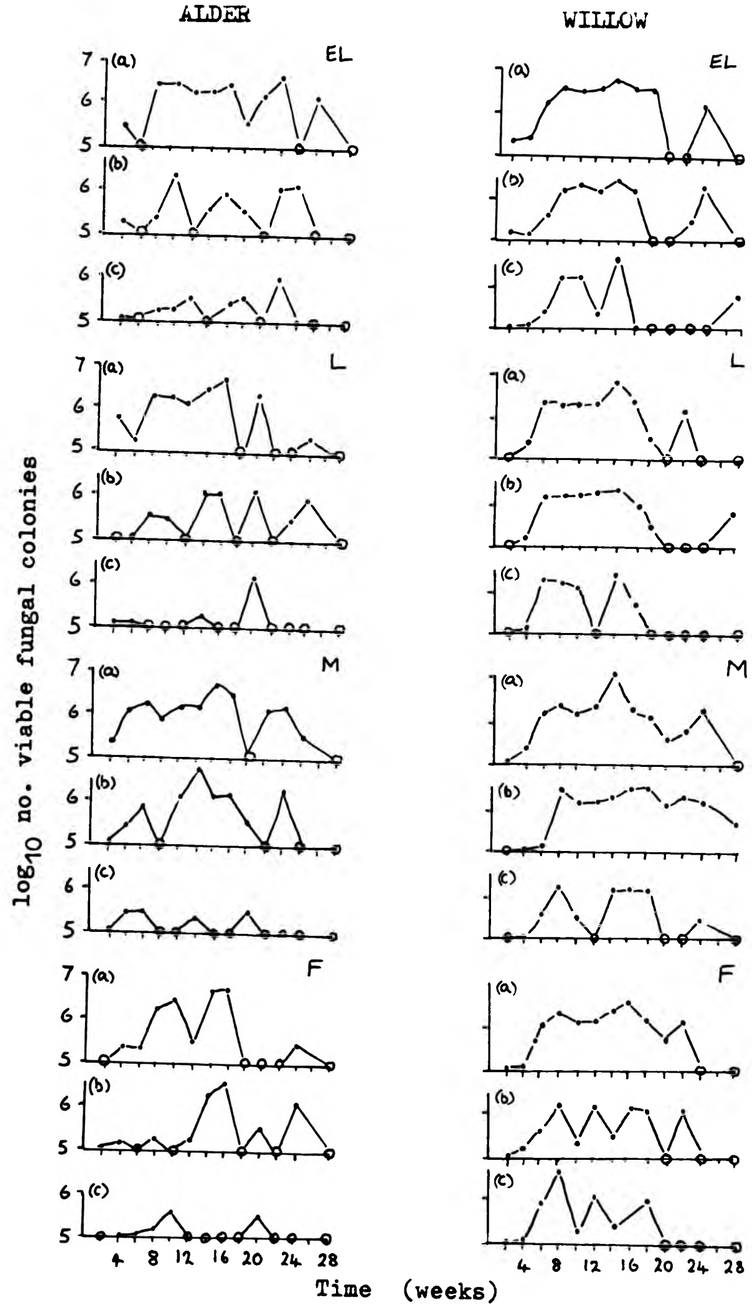


TABLE 9.10 Frequency (%) of 4 fungal taxa on 25* leaf discs (6mm diameter) of alder and willow plated on potato dextrose agar containing antibiotics. Frequencies are shown for different periods of submersion in Yateley 4 (site 1) and for different mesh sizes.

		ALDER				WILLOW			
TIME	WEEKS	Ap	Csp	Ep	Muc	Ap	Csp	Ep	Muc
a)	0	95	71	12	9	98	94	72	11
	8	100	96	52	40	100	90	96	40
	22	12	100	40	8	40	100	24	4
	28	0	52	0	0	0	52	28	16
b)	0	95	71	12	9	98	94	72	11
	8	100	100	40	60	100	96	88	48
	22	44	100	16	40	44	80	24	48
	28	0	68	4	12	0	64	4	20
c)	0	95	71	12	9	98	94	72	11
	8	100	100	70	64	100	100	60	36
	22	24	100	20	16	28	100	16	4
	28	0	80	4	8	0	80	12	0
d)	0	95	71	12	9	98	94	72	11
	8	100	88	55	30	100	100	92	48
	22	24	100	12	16	32	100	16	8
	28	0	72	12	4	0	92	16	4

KEY: Ap = Aureobasidium pullulans; Csp = Cladosporium spp.; Ep = Epicoccum purpurascens; Muc = Mucor spp.

a) = Extra large mesh; b) = Large mesh; c) = Medium mesh; d) = Fine mesh

* Time 0, n=100 discs

9.3.9 Correlations between fungal biomass and biotic and abiotic factors

Table 9.11 shows the most significant correlations of abiotic and biotic variables with measures of fungal biomass on alder and willow litter at 8, 18 and 28 weeks after submersion. Most measures of biomass were significantly correlated with aspects of the water chemistry, although few of the variables accounted for more than 20% of the variation in fungal biomass. Both ergosterol and whole leaf total biomass were significantly correlated with calcium concentration in the water and with water temperature and temperature change. The agar film total biomass showed negative correlations with both alkalinity and magnesium levels and ergosterol was negatively correlated with soluble nitrate and soluble nitrate + phosphate. Agar film active biomass (not shown) was not significantly correlated with any of the abiotic or biotic variables. Both ergosterol and whole leaf total biomass were significantly correlated with the number of species of Ingoldian fungi, but the correlation for the whole leaf total biomass was only significant for alder. Spore production by Anguillospora longissima on alder and willow (not shown) was also significantly correlated with ergosterol ($r = 0.210$, $P < 0.01$). There were no significant correlations between biomass and numbers of animals colonising the bags.

Correlations between abiotic and biotic factors and ATP and FDA hydrolysis are not shown in Table 9.11. ATP showed significant negative correlations, on alder and willow, with alkalinity ($r = -0.306$, $P < 0.001$), conductivity ($r = -0.233$, $P < 0.01$), soluble phosphate + nitrate ($r = -0.214$, $P < 0.01$) and calcium ($r = -0.209$, $P < 0.01$) and significant positive correlations with pH ($r = 0.258$, $P < 0.01$) and number of Ingoldian fungi ($r = 0.204$, $P < 0.01$). FDA hydrolysis on alder and willow showed a significant negative correlation with pH ($r = -0.326$, $P < 0.001$) and significant positive

TABLE 9.11 Correlations (r) between measures of fungal biomass and abiotic and biotic factors associated with the weight loss of alder and willow leaf packs, at 8, 18 and 28 weeks after submersion in Yateley 4. Each analysis includes all mesh sizes.

VARIABLE	ALDER[+A]	WILLOW[+A]	ALDER + WILLOW[+B]
a) Ergosterol			
Water temperature	0.459[*3]	0.219[NS]	0.339[*3]
Temperature change[+C]	0.447[*3]	0.212[NS]	0.330[*3]
PO_4-P+NO_3-N	-0.422[*3]	-0.318[*1]	-0.370[*3]
NO_3-N	-0.419[*3]	-0.317[*1]	-0.368[*3]
Calcium	0.381[*2]	0.158[NS]	0.291[*2]
No. species of Ingoldian fungi	0.396[*2]	0.271[*1]	0.329[*3]
b) Agar film total			
Alkalinity	-0.534[*3]	-0.371[*2]	-0.436[*3]
Magnesium	-0.436[*3]	-0.219[NS]	-0.316[*3]
c) Whole leaf total			
Water temperature	0.463[*3]	0.286[*1]	0.365[*3]
Temperature change[+C]	0.471[*3]	0.288[*1]	0.370[*3]
Conductivity	0.403[*2]	0.326[*1]	0.357[*3]
pH	-0.327[*1]	-0.322[*1]	-0.319[*3]
Calcium	0.457[*3]	0.162[NS]	0.292[*2]
No. species of Ingoldian fungi	0.479[*3]	0.045[NS]	0.241[*2]

*1 $p<0.05$; *2 $p<0.01$; *3 $p<0.001$; NS Not significant; +A n=60; +B n=120; +C Change in water temperature over previous month.

correlations with conductivity ($r = 0.317$, $P < 0.001$), calcium + magnesium ($r = 0.288$, $P < 0.01$), calcium ($r = 0.278$, $P < 0.01$), temperature ($r = 0.274$, $P < 0.01$) and number of species of Ingoldian fungi ($r = 0.220$, $P < 0.01$).

With the study over 28 weeks at Site 1 there were significant correlations between viable fungal counts and number of species of Ingoldian fungi ($r = 0.418$, $P < 0.001$), FDA analysis and numbers of Gammarus and Asellus ($r = 0.4$, $P < 0.001$) and total animals ($r = 0.395$, $P < 0.001$), ATP and number of Gammarus and Asellus ($r = 0.433$, $P < 0.001$) and total animals ($r = 0.340$, $P < 0.001$), and between agar film total biomass and numbers of Gammarus and Asellus ($r = 0.261$, $P < 0.01$) and total animals ($r = 0.219$, $P < 0.05$). Numbers of viable yeasts showed a significant negative correlation with numbers of animals ($r = -0.374$, $P < 0.001$). Numbers of animals were significantly correlated with the number of Ingoldian fungal species ($r = 0.502$, $P < 0.001$) and with total spore production ($r = 0.358$, $P < 0.001$). Correlations with abiotic factors were not calculated for Site 1.

9.3.10 Relationship between weight loss of leaf packs and abiotic and biotic variables

Table 9.12 shows the most significant correlations between abiotic factors and weight loss of alder and willow leaf packs after 8, 18 and 28 weeks submersion in Yateley 4. Correlation coefficients with abiotic variables were similar for both alder and willow. Temperature and temperature change accounted for most of the variation in leaf pack weight loss, with the most significant chemical variables being calcium and calcium + magnesium. Levels of soluble phosphate + nitrate were negativiely correlated with leaf pack weight loss.

Table 9.13 shows the relationship between leaf pack weight loss and biotic factors. Biomass estimates were only significantly correlated with alder leaf pack

TABLE 9.12 Correlations (r) between leaf pack weight loss and physical and chemical factors of leaf pack environment. Data from all mesh sizes were included in the analysis.

VARIABLE	ALDER[+A]	WILLOW[+A]	ALDER + WILLOW[+B]
Temperature	0.828[*3]	0.826[*3]	0.811[*3]
Temperature change[+C]	0.768[*3]	0.767[*3]	0.753[*3]
Calcium	0.769[*3]	0.616[*3]	0.666[*3]
PO_4-P+NO_3-N	-0.640[*3]	-0.654[*3]	-0.634[*3]
Calcium + Magnesium	0.732[*3]	0.586[*3]	0.633[*3]
NO_3-N	-0.637[*3]	-0.652[*3]	-0.632[*3]
Conductivity	0.652[*3]	0.602[*3]	0.616[*3]
pH	-0.502[*3]	-0.443[*3]	-0.465[*3]

*3 $p<0.001$; +A n=60; +B n=120; +C Change in water temperature over previous month

TABLE 9.13 Correlations (r) between leaf pack weight loss and a) fungal biomass, b) No. Ingoldian fungal species and spore production and c) numbers of invertebrate animals. The data from all mesh sizes were used in the analysis.

VARIABLE	ALDER^{+1}	WILLOW^{+1}	ALDER + WILLOW^{+2}
a)			
1 Ergosterol	0.489^{*3}	0.219^{NS}	0.344^{*3}
2 FDA hydrolysis	0.443^{*3}	0.153^{NS}	0.305^{*3}
3 Whole leaf total	0.349^{*3}	0.196^{NS}	0.299^{*2}
4 Pycnidial biomass	0.372^{*2}	0.207^{NS}	0.298^{*2}
b)			
1 No. species Ingoldian fungi	0.377^{*2}	0.645^{*3}	0.498^{*3}
2 No. conidia	0.234^{NS}	0.643^{*3}	0.284^{*2}
3 No conidia *Anquillospora* spp.	0.367^{*2}	0.472^{*3}	0.370^{*3}
4 No. conidia *Alatospora acuminata*	0.133^{NS}	0.336^{*2}	0.085^{NS}
5 No. Phycomycete colonized hemp seeds	0.468^{*3}	0.275^{*1}	0.389^{*3}
c)			
Total animals	0.317^{*1}	0.319^{*1}	0.297^{*2}
No. *Asellus* sp + *Gammarus* sp	0.427^{*3}	0.381^{*2}	0.378^{*3}

*1 P<0.05; *2 P<0.01; *3 P<0.001; NS not significant;
+1 n=60 +2 n=120

weight loss, with ergosterol showing the highest correlation. The number of species of Ingoldian fungi showed significant correlations with weight loss as did spore production by Anguillospora longissima and number of phycomycete colonised hemp seeds. Total spore production and spore production by Alatospora acuminata were only significant for willow leaf packs. Both total animals and numbers of Gammarus and Asellus were significantly correlated with weight loss. Most of the biotic factors accounted for considerably less of the variation in leaf pack weight loss (maximum 42%) than temperature (maximum 69%) or water chemistry (maximum 59%).

9.3.11 Fungal biomass and activity in the lake sediment

Figure 9.29 shows a sediment core from Yateley 4 with the litter and detritus layer above black mud and gravel and overlying clay. Cores taken from 3 sites in Yateley 4 gave a mean sediment depth (above the clay) of 7.4 cm $\pm$ 1.08 (n = 12).

Table 9.14 shows the sediment composition, fungal biomass and FDA hydrolysis associated with different size fractions of sediment. About 97.7% of the sediment consisted of fine silt of less than 0.5 mm particle size; leaf litter, twigs and other detritus formed less than 3% of the sediment by weight. Leaf litter consisted mostly of small fragments of lamina or petiole and no retted leaf fragments were found. FDA hydrolysis, although low, was mostly associated with the larger particle sizes of sediment (greater than 0.5 mm), being significantly lower in the less than 0.5 mm fraction. The agar film total biomass and agar film active biomass was lower in the less than 0.5 mm fraction as was mean hyphal diameter. However, there was no significant differerence between biomass or hyphal diameter in any of the three size fractions of sediment.

Table 9.15 shows the frequency of fungal genera associated with the different size fractions of sediment. Fewer genera were isolated from the fractions less than

Figure 9.29. Sediment core from Yateley 4 showing relative proportions of a) litter and organic detritus b) black mud and gravel and c) clay. The core was taken approximately 3 metres from the bank.

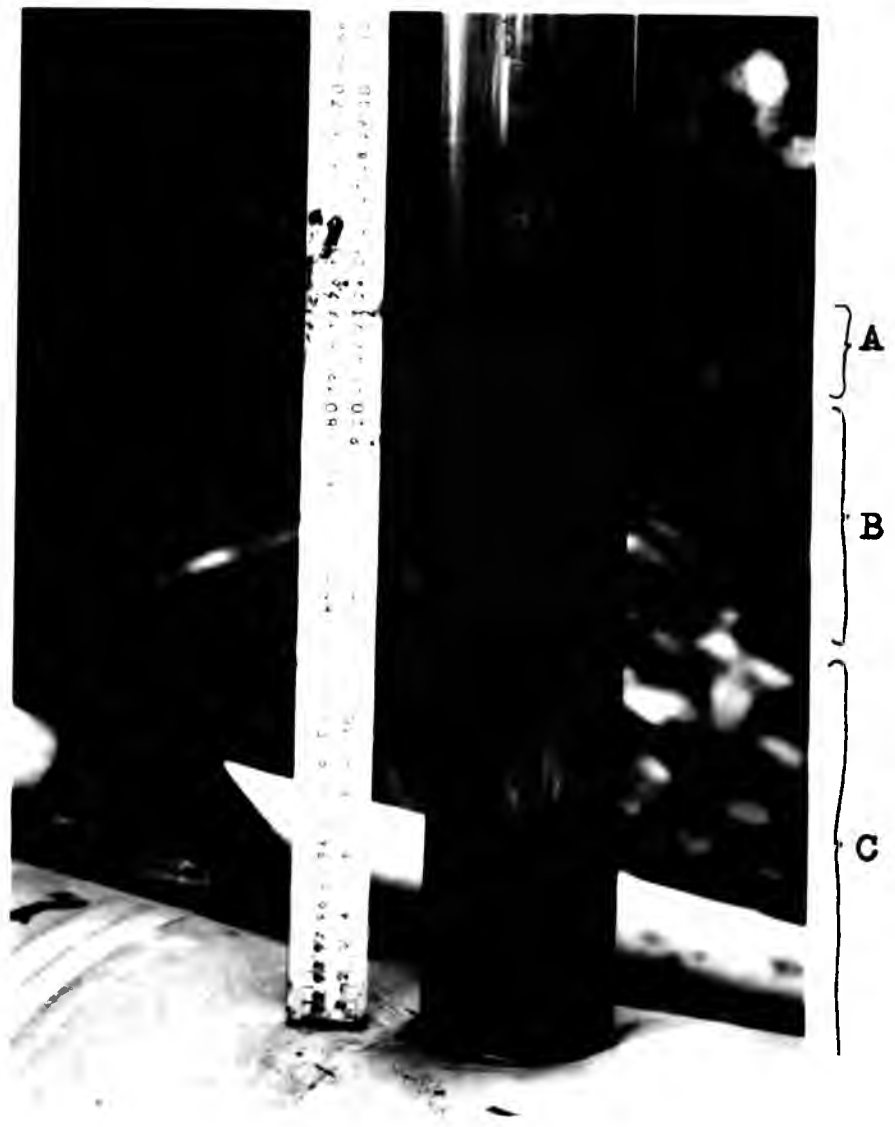


Figure 9.29. Sediment core from Yateley 4 showing relative proportions of a) litter and organic detritus b) black mud and gravel and c) clay. The core was taken approximately 3 metres from the bank.

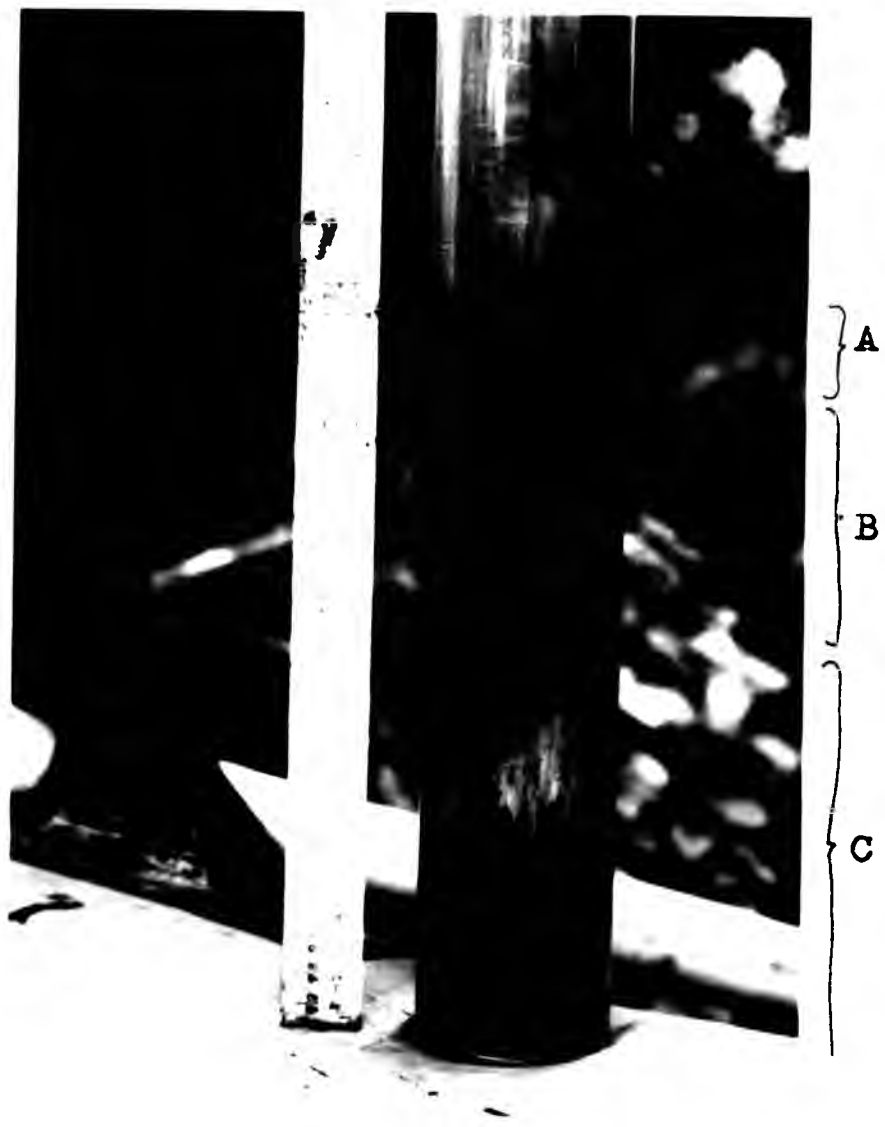


Figure 9.29. Sediment core from Yateley 4 showing relative proportions of a) litter and organic detritus b) black mud and gravel and c) clay. The core was taken approximately 3 metres from the bank.

TABLE 9.14 Fungal biomass and activity associated with different size fractions of sediment in Yateley 4.

VARIABLE	SIZE FRACTION		
	>1mm	<1>0.5mm	<0.5mm
Sediment composition(%) by weight	1.38±0.39	0.88±0.32	97.73±0.76
FDA activity absorbance units 492nm g^{-1} sediment	0.02±0.002	0.03±0.005	0.01±0.001
agar film total*1 mg g^{-1}	27.6±7.7	25.6±8.5	19.2±3.8
Agar film active*1 mg g^{-1}	4.9±1.4	4.5±1.5	3.3±0.7
Hyphal diameter µm (n=15)	3.45±0.42	3.48±0.47	3.19±0.47

*1 Assumption: biovolume to biomass conversion factor 0.29g cm^{-3}. Values not corrected for inefficient homogenization.

TABLE 9.15 Frequency of fungi (%) in different size fractions of lake sediment in Yateley 4.

FUNGAL GROUP	SIZE FRACTION		
	>1mm	<1>0.5mm	<0.5mm
Cladosporium	0.15	0	0
Phoma	0.22	0	3.90
Fusarium	0.31	0	0
Aureobasidium	3.40	4.33	7.80
Penicillium	0.24	0.85	8.90
Acremonium	5.16	6.50	3.03
Trichoderma	0	0.11	0
Unknown sp.1	0.85	0	0
sp.2	0.07	0	3.03
sp.3	0.07	0	0
sp.4	0	0.20	5.90
sp.5	0	0.48	0
Yeasts pink	18.73	1.86	5.00
yellow	33.38	25.60	29.80
grey/white	37.50	59.70	32.60
No of mycelial colonies $x10^3$ g^{-1} dry weight	12.7±4.5	15.0±1.7	98±38.9

1 mm and less than 0.5 mm , and there seemed to be some variation in % frequency in different size fractions of the sediment. Penicillium and Aureobasidium became more frequent with decreasing particle size, whereas Cladosporium, Fusarium and several unidentified isolates were only found with particles greater than 1 mm. The frequency of pink yeasts decreased with decreasing particle size. Numbers of mycelial colonies were 6-8 times higher in the 0.5 mm fraction than in either of the larger particle sizes. Direct examination of incubated samples of each fraction failed to reveal any spores of Ingoldian fungi although many of the leaf fragments were only partly decomposed and appeared suitable for colonisation.

9.4 Discussion

9.4.1 Weight losses

Several authors have noted that decomposition rates are slower in lakes than in rivers (Petersen & Cummins, 1974; Hodkinson, 1975b; Gasith & Lawacz, 1976; Hanlon, 1982) and comparison of the data in the present work with that in the literature (Petersen & Cummins, 1974) is in agreement with this. In rivers, allochthonous litter might be expected to degrade faster since water currents can lead to a high degree of fragmentation, abrasion and exposure of new surfaces for colonisation. Degradation rates for Salix in Yateley 4 were higher than in many other lakes (Hodkinson, 1975b; Gasith & Lawacz, 1976; Hanlon, 1982). This cannot be due to wave action or exposure as Yateley 4 is sheltered from strong winds by dense surrounding vegetation, hence microbial and invertebrate processing must be important factors.

The results of degradation of leaf packs and leaf discs demonstrated that there were differences in weight loss between species. There were also some differences in the degradation of leaf material in packs in bags of

different mesh size, ie., weight losses were greater in packs from extra large, large and medium mesh bags and differences between replicate samples became larger with increasing submersion time. These differences appear to be due principally to leaf skeletonisation by invertebrates (Figure 9.4 a & e). Animals may scrape and abrade surfaces (Winterbourn, 1978) as well as shredding leaf tissues, but leaf discs from litter in fine mesh bags did not degrade significantly slower than in bags of larger mesh size (in contrast to weight loss of leaf packs) suggesting that microorganisms, rather than animals, are primarily responsible for observed weight losses in the discs. Other authors (Mathews & Kowalczewski, 1969; Mason & Bryant, 1975; Buth & de Wolf, 1985) have attributed the bulk of leaf degradation in their studies to microbial activity as they found weight losses in packs from fine mesh bags did not differ from those allowing access of invertebrates.

In Yateley 4 leaf decomposition appears to be due primarily to the action of microorganisms, but invertebrates may accelerate weight losses in leaf packs.

9.4.2 Microbial and fungal biomass

Total microbial biomass as ATP did not appear to differ markedly between leaf types, but did between fine meshed bags and other mesh sizes on alder and willow, and between different sites in Yateley 4. This was in contrast to FDA hydrolysis which showed more differences between leaf types and only slight differences between sites and between willow litter in different mesh sizes. Although FDA has been used to estimate microbial biomass in soil (Schnürer & Rosswall, 1982) and on leaves (Swisher & Carroll, 1980), it probably provides a better indication of the metabolic state of microorganisms (Ingham & Klein, 1982 & 1984) and this may account for the lack of a clear relationship between the two methods in the main study at 8, 18 and 28 weeks, although the

methods were significantly correlated in the study at site 1.

Access of animals to leaf litter has been shown to result in both increases and decreases in microbial biomass and metabolic activity depending on the grazing pressure (Hanlon & Anderson, 1979; Morrison & White, 1980; Ineson, Leonard & Anderson, 1982; Smith et al., 1982), although change in activity does not always occur in the presence of animals (Mason & Bryant, 1975). In the present study, both ATP and FDA hydrolysis were higher on litter in bags which allowed access of invertebrates, although the differences were not large. Apart from response to direct grazing on microorganisms, an increase in microbial biomass and metabolic activity could occur because of damage to the leaf. Scraping and abrading leaf surfaces may facilitate the entry of microorganisms to internal tissues (Bärlocher, 1980) and fine particulate material produced during this process is likely to show high microbial activity, particularly by bacteria (Hargrave, 1972; Olah, 1972; Mason & Bryant, 1975).

Fungal biomass did not show any clear differences between litter in different mesh sizes, although it increased significantly with submersion time. With high numbers of invertebrate animals, particularly 'shredders'(Gammarus and Asellus), colonising litter bags between weeks 12 and 28 (Figure 9.20) it might have been expected that fungal biomass would decline in all litter bags other than in fine ones, but this did not seem to be the case (Figures 9.11-9.15). There is a suggestion that at site 1 fungal biomass was being grazed in the extra large bags (Figures 9.18-9.19) but litter in the other mesh sizes allowing access of invertebrates generally showed a similar trend to litter in fine mesh bags. If fungal biomass was not being depleted by invertebrates then it was either a) not being consumed, b) not palatable, c) unavailable, or d) consumption of leaf material was independent of its fungal colonisation

or e) biomass consumption was met by increased fungal productivity.

Many studies have shown that fungal conditioning of autumn shed leaves significantly affects their palatability to invertebrates (Cummins et al., 1973; Cummins, 1974; Bärlocher & Kendrick, 1975 a & b, 1976, 1981; Bärlocher, 1985) although certain types of leaf are known to be more palatable to aquatic invertebrates than others (Bärlocher, 1985). Beech leaves and conifer needles generally require extensive conditioning before they are acceptable, whilst some leaves such as alder have a high protein content (Bengtsson, 1982) and are very palatable, even without prior conditioning (Bärlocher, 1985), so consumption of leaves is not necessarily dependant on prior microbial colonisation.

Particular invertebrates may show preferences for particular fungal species (Suberkropp, Arsuffi & Anderson, 1983; Arsuffi & Suberkropp, 1984), as different fungi may vary in their palatability, both between species (Suberkropp, Arsuffi & Anderson, 1983) and between periods of conditioning (Suberkropp & Arsuffi, 1984). Most palatability studies have been conducted with pure cultures of fungi on leaf material heavily colonised by mycelium of single species and less is known about the palatability of mixtures at lower densities. It may be that mixtures of Ingoldian and other fungi differ appreciably in their palatability to invertebrates and this may be particularly marked if one or several species of fungi produce toxic metabolites. There is apparently little evidence of this in Ingoldian fungi (Bärlocher & Kendrick, 1981).

Few authors have examined the importance of other organisms in the diet of invertebrates, although Findlay, Meyer & Smith (1986) mention that algae may make an important contribution to the diet of the freshwater isopod (Lirceus sp.), and Asellus is known to feed on diatoms and other algae (Moore, 1975).

The distribution of fungal biomass could be important to grazing invertebrates, since this may influence the detection of the fungi by grazing animals. Pycnida formed a significant proportion of the whole leaf total biomass on both alder and willow, and this fraction of the biomass may have been largely unavailable to grazing animals because it was embedded in the leaf tissues. In Chapter 3, it was shown that fungi exhibit differences in the aggregation of hyphae on leaf surfaces. If surface hyphae are sparse or in dense clumps, then it may not be so easily perceived by grazing animals, particularly if the leaf itself is palatable, as in the case of alder. Linear regressions of log variance on log mean (Section 3.2.4.2) for whole leaf estimates of hyphal lengths on leaf discs of alder gave slopes of 1.45 and 1.42 for litter in extra large and fine mesh bags, respectively. This indicated that there was little difference in the spatial arrangement of mycelium exposed and unexposed to invertebrate animals and suggests that the animals were not specifically grazing the mycelium on the leaf material, since a change in spatial arrangements in response to grazing might be expected.

There was no evidence to suggest that fungal productivity increased in response to grazing on mycelium since agar film active biomass did not increase significantly with time or between different mesh sizes.

Table 9.16 summarises the overall estimates of fungal and microbial biomass associated with alder and willow litter at 8, 18 and 28 weeks after submersion. Figures for the ranges include the data from site 1. For most methods the mean levels of biomass were similar for the 2 leaf types, although the ranges suggest that they were more variable on willow than on alder. ATP indicated that total biomass was on average 3.7 and 5.8% of leaf dry weight for alder and willow respectively, with values ranging from 1.0-24.9% for alder and 0.9-34.5 for willow.

TABLE 9.16 Fungal and microbial biomass associated with alder and willow leaf litter in Yateley 4. Estimates are based on samples from litter bags (all mesh sizes) over a 5 month period (February-June) from 5 sites in Yateley 4. Each value represents mg g-1 dry weight (unless otherwise indicated) with standard errors (and ranges*5).

METHOD	ALDER (n=60)	WILLOW (n=60)
Agar film total*1	15.2±0.7(1.2-35.4)	18.3±0.7(1.5-66.3)
Agar film total*1*2 (corrected)	22.4±1.2(3.0-45.6)	28.5±1.1(3.0-95.5)
Agar film active*1	3.6±0.3(0.2-10.5)	4.8±0.3(0.2-24.8)
Agar film active*1*2 (corrected)	5.3±0.4(0.3-13.9)	7.7±0.5(0.4-35.8)
Ergosterol ($\mu g\ g^{-1}$)	8.6±1.0(0.6-30.6)	7.7±1.0(1.1-43.0)
Ergosterol biomass*3	3.9±0.5(0.3-14.0)	3.5±0.5(0.5-19.6)
Whole leaf total*1	9.8±0.6(1.1-19.6)	11.5±0.6(3.5-23.7)
Whole leaf pycnidia*1	3.1±0.3(0-11.2)	3.9±0.4(0-16.1)
ATP($\mu g\ g^{-1}$)	54.6±3.5(14.0-366.1)	85.8±5.9(13.4-506.2)

METHOD	ALDER (n=60)	WILLOW (n=60)
Total biomass (ATP)*4	37.2±2.4(9.5-249.3)	58.4±4.0(9.1-344.7)
FDA hydrolysis absorbance units (492nm)	0.136±0.007 (0.020-0.490)	0.139±0.007 (0.020-0.670)

*1 Assumption: biovolume to biomass conversion factor 0.29 g cm^{-3}

*2 Corrected for inefficiency of homogenization (proportion of homogenate retained by 250µm mesh)

*3 Assumption: Ergosterol content of mycelium 2.19 mg g^{-1} (Section 6.3)

*4 Assumption: ratio of ATP to cell carbon 1:250 and carbon 36.7% of cell dry weight (Anderson and Domsch, 1980)

*5 Ranges include data from Site 1 study.

A number of authors have measured ATP in their studies on the decomposition of allochthonous litter in water. Conversion of their estimates to microbial biomass (ATP to carbon 1:250, Lee et al., 1971; carbon 36.7% of microbial cell dry weight, Anderson & Domsch, 1980) would give estimates of microbial biomass as percentage of leaf dry weight of 6.4–12.9% for Carya glabra, 1.7–3.6% for Quercus alba (Suberkropp & Klug, 1976), 0.6–6.2% for Carex (Federle & Vestal, 1982) 1.8–3.4 for Robinia pseudo-acacia, 1.4–2.7% for Betula lenta (Meyer & Johnson, 1983) and 0.2–0.9% for Pinus elliotii and 0.1–0.6% for Quercus virginiana (Morrison et al., 1977). Clearly there are some differences in biomass levels on different types of leaf and Suberkropp , Godshalk & Klug (1976) attributed the differences between ATP on Carya and Quercus, in their study, to differences in leaf chemistry. It is known that the tannin content of leaves can influence growth of fungi markedly (Harrison, 1971).

In the present study, the estimates of fungal biomass were all lower than for ATP biomass, but the methods showed a similarity in biomass levels between leaf types (Table 9.16). Irrespective of leaf type, fungal biomass represents about 0.4–2.8% of leaf dry weight based on mean values and 0.13–9.55% based on the ranges. These values are all generally lower than the corresponding ones based on ATP but encompass the range of values reported for fungal biomass on allochthonous litter in freshwater (Bärlocher & Kendrick, 1981). There is a marked difference between the fungal biomass estimates based on ergosterol and agar film active biomass and those based on ATP. This suggests that they either underestimated the fungal biomass, or that the fungal biomass is low in relation to that of bacteria, algae, protozoa, nematodes etc., or that the ATP biomass is overestimated. Other organisms may have formed a significant component of the microbial biomass, and the problems of ATP measurement and interpretation are well known (Karl, 1980; Riemann & Wium-Anderson, 1981; Fairbanks,

et al., 1984). Each of the methods used for fungal biomass is known to have limitations (see previous chapters), with probably the most significant being the choice of conversion factors (Chapter 6). The choice of conversion factors is particularly critical in estimates of biomass based on ergosterol. For direct methods, a conversion factor of 0.29 g cm^{-3} was used, this being based on fungi grown on agar media (Section 6.3.1). A higher conversion factor was obtained for fungi grown on submerged litter (0.38 g cm^{-3}, Section 6.3.1) and use of this in the present study would increase estimates by 1.31 times. Use of the range of conversion factors found for ergosterol in this study (Section 6.3.2) would give biomass estimates of between 0.007 and 107.5% of leaf dry weight; this emphasises the problems associated with extrapolating relative measures to biomass. In this study, ergosterol gave comparable estimates of biomass to agar film active biomass, and this would be expected if ergosterol was a measure of living mycelium and the conversion factors for each method were appropriate.

Corrections for agar film biomass seemed justifiable on the grounds that homogenisation of leaf material was inefficient. Results of the present study suggest that conversions for inefficiency of homogenisation are dependant on both the leaf species and the stage of decomposition. Use of the corrections appears to inflate the estimates during the early stages of decomposition, prbably when most leaf material contains very little internal mycelium. This may explain why corrected agar film biomass estimates, after 28 weeks, were sometimes lower than for time 0.

Although the estimates of biomass differed in magnitude, there were some significant correlations between different methods suggesting that biomass, and in particular fungal biomass, was being detected. The correlations between ATP and agar film total and active biomass and between ATP and fungal viable counts suggests that fungi were forming an increasingly important

component of the total biomass associated with the leaf material during decomposition and that that the methods used were detecting these changes.

Levels of fungal biomass in the leaf packs were similar to those in the sediment fraction where particle size was > 0.5 mm, but biomass associated with smaller particle sized fractions was not, suggesting that the biomass was associated with the fractions containing plant material. However, most of the sediment (c. 98%) contained small particle sized fractions.

9.4.3 Microbial colonisation of leaf material

There have only been a few studies regarding microbial successions in lakes (Mason & Bryant, 1975; Federle & Vestal, 1980 & 1982; Federle, McKinley & Vestal, 1982) and the present studies have demonstrated that there are distinct changes in microbial populations associated with the decomposition of allochthonous litter.

Studies at site 1 revealed that there was a distinct pattern of colonisation by bacteria on alder and willow and that the pattern was independant of mesh size for each litter type. The bacterial numbers were usually higher than 10^9 g^{-1} leaf dry weight during the first 16 weeks of study, with pectinolytic bacteria forming a large proportion (> 80%) of the total bacterial count during the weeks 12-16; there was a dramatic fall in total numbers of bacteria after week 16.

The high bacterial numbers, particularly of those able to utilise pectin, coupled with peaks in FDA hydrolysis at site 1 (Figure 9.9), suggest that bacteria may have contributed to weight loss of leaf material during the first 12-16 weeks of leaf submersion. The reason for the decline is not clear, but it may reflect the exhaustion of utilisable substrates, interactions with fungi, or change in environmental factors. Pairing on agar media of 8 morphologically distinct bacterial colonies isolated during the study with a range of leaf and

Ingoldian fungi failed to demonstrate any interactions, so antagonism is not suggested as a reason for decline in numbers.

Bacterial numbers associated with allochthonous litter in streams ranged from 10^7-10^9 g^{-1} leaf dry weight (estimated from data provided in Kaushik & Hynes, 1971; Suberkropp & Klug, 1976; Chamier & Dixon, 1982a). In most cases bacterial numbers have been reported to increase steadily following litter submersion in the streams, with peak numbers usually being preceded by high numbers of fungi, this being different to the present study. The maximum number of bacteria found by Chamier & Dixon (1982a) was 10^9 g^{-1} leaf dry weight, with 50% being pectinolytic bacteria.

In this study the numbers of viable fungal colonies increased up to week 8-14 depending on the species and then appeared to oscillate around 10^7 g^{-1} leaf dry weight for most of the study period. Numbers were significantly correlated with ATP, FDA hydrolysis and agar film total and active biomass, suggesting that fungi became an increasingly important component of the biomass with increasing submersion time. Witkamp (1974) also observed a correlation between fungal viable counts in litter and an estimate of hyphal length based on a clearing and staining method.

Although the Ingoldian fungi were found on leaf material after 2 weeks submersion time (Tables 9.7 & 9.8) spores did not appear in significant numbers until week 12. This is possibly because the levels of inoculum were low and the colonisation of new resources in lakes is much slower than in streams. The observed changes in numbers of species on leaf packs with submersion time clearly indicates that there is an initial period of rapid colonisation, followed by a plateau with an oscillating number of species. The form of the colonisation curves were similar to those observed in a number of other studies (Dickinson & Pugh, 1965; Bärlocher, 1980; Zak & Parkinson, 1984; Wildman, 1987) and appeared

to be consistent with the general form predicted by the theory of island biogeography (MacArthur & Wilson, 1967). The levelling of the colonisation curves (Figures 9.21 a & b, 9.23) may indicate the attainment of an equilibrium between immigration and losses of species from leaf packs, but could also be related to a saturation of the leaf habitat by the majority of species in the species pool, ie., all those present in the lake. The latter case does not seem to be the case, since the cumulative species curves demonstrated that most potential colonists in the lake (Table 8.1) were found on the leaf packs at some time, but the mean number of species present on alder or willow, or on litter of these species in all mesh sizes, at any one time was always much lower (Figure 9.21 & 9.23). The number of new species per sample week and species turnover both demonstrated that there was considerable immigration and losses of species on the litter during the submersion period. These results suggest that the number of colonisable niches were rapidly filled and establishment of new species was dependant on the losses of already established ones.

The cumulative number of species on alder was similar for litter from bags of all mesh sizes but, with willow, fine mesh bags had considerably fewer species than litter in the other bags. This suggests that, in the case of willow, the presence of animals is particularly important for colonisation by Ingoldian fungi, possibly because they facilitate access to the leaf tissues by damaging the epidermis (Bärlocher, 1980). It was demonstrated in Chapter 4 (Table 4.3) that willow leaves are thicker than alder and appear to have a tougher lamina. The greater dependence on animals for fungal colonisation on willow may help to explain the greater fluctuations in species on willow than on alder (Figure 9.21 & 9.23).

The importance of animals in establishment is emphasised by the fact that the numbers of Ingoldian fungi weresignificantly lower in fine mesh bags of both

leaf types and number of species and spore production were significantly correlated with numbers of animals and with numbers of Gammarus and Asellus. The pattern of development of Ingoldian fungi on alder and willow suggests that after 12-14 weeks of submersion a 'unit community' (sensu Swift, 1976, 1984) of between 6-8 species was present on both leaf types and it persisted until week 24-28. Alatospora acuminata and Anguillospora longissima were the dominant species on both leaf types, in terms of spore production, although the importance values curves suggest that there was slightly less 'dominance' by these species on willow. These results agree with observations made in rivers that the unit community of aquatic fungi consists of a stable association of a few species together with others which occur irregularly and sporadically (Suberkropp & Klug, 1976; Sanders & Anderson, 1979; Bärlocher, 1980; Chamier & Dixon, 1982a). It is suggested, from examination of the sediment, that Ingoldian fungi did not persist throughout leaf decay. Either they do not persist on the recalcitrant leaf material or they are unable to grow on small fragments of litter which eventually become incorporated into the sediment. The last possibility is most likely, particularly as Field & Webster (1983) have demonstrated that Ingoldian fungi generally appear to be intolerant of low oxygen tensions.

Leaf and soil fungi (Park, 1972; 1974) have been implicated in the decomposition of leaf litter in freshwater and several authors have demonstrated that these fungi are capable of leaf degradation in vitro (Godfrey, 1983; Rossi et al., 1983; Chamier, Dixon & Archer, 1984). These fungi were detected throughout the the present study on submerged leaf litter and on particulate litter in the sediment of the lake. Plating of leaf discs and homogenates, and direct observation, suggested that some of these fungi may have been active on the leaf material, particularly before the appearance of Ingoldian fungi on agar plates. After submersion for

several weeks, several genera of fungi appeared which were either uncommon or were not observed in allochthonous litter before submersion, and these included Fusarium, Phoma, Penicillium, Acremonium and unknown sp. 1 (Table 9.9). These species appeared to form an intermediate group between the leaf fungi and the Ingoldian fungi, and evidence from increasing pycnidial biomass and the appearance of Fusarium spores by direct observation suggest that some of these were active during leaf degradation. The correlation between colony counts and ATP, FDA hydrolysis, agar film total biomass and active biomass suggests that both the leaf and intermediate fungi may have been active during leaf degradation, particularly as these fungi formed the bulk of the colony counts on agar plates (Figure 9.17).

Several authors have postulated that the ability of Ingoldian fungi to grow and sporulate at low temperatures may explain their predominance on allochthonous litter in streams (Bärlocher & Kendrick, 1974 & 1981) and that leaf and soil fungi are thought to be more active on allochthonous litter in freshwater at higher temperatures (Bärlocher & Kendrick, 1974; Godfrey, 1983; Chamier, Dixon & Archer, 1984). However, the results obtained in the present studies, by direct observation of spores and the appearance of colonies on agar media, suggest that Ingoldian fungi become more active with increasing submersion time coincident with rising temperatures, and that there is a shift in the types of fungi present, from leaf fungi to intermediate ones and then to Ingoldian fungi. Competitive interactions may be important, and antibiosis has been postulated as an important factor in microbial succession (Park, 1967; Wicklow & Hirschfield, 1979 a & b).

Table 9.17 shows features of the groups of fungi associated with leaf (A), intermediate (B) and Ingoldian (C) stages of succession on allochthonous litter in Yateley 4. Not all the isolates were obtained from the

lake, but the species were representative of the different ecological groups. There were differences in the growth rates and hyphal diameters of the three groups, but the most striking difference was in the interactions between fungi in the different groups. Whereas antagonism was generally low within groups (A with A, B with B, and C with C), that between groups was more marked, and Ingoldian fungi clearly showed more interactions with groups A and B than with themselves. Totalling the columns for each group gives an overall measure of the aggressiveness of each group and totals for rows gives a measure of each groups sensitivity to others. If a succession proceeds on the litter from A-C then there would be an expected increase in aggressive interactions between fungi, if they all persisted, but a slight fall in susceptibiliy. Horn (1977) has introduced the concept of a competitive hierarchy to describe successional patterns where later successional plant species exert a dominating influence over early successional species, and similar processes have been postulated for fungal communities (Wicklow & Hirschfield, 1979 a & b). If interactions occur on allochthonous leaf litter in fresh-water, as observed in Table 9.17, then a competitive hierarchy may result. The recognition of the existence of strategies in autotrophic plants (Grime, 1977 & 1979) has recently led to the concept being applied to fungi and their role in communities (Pugh, 1980; Cooke & Rayner, 1984; Swift, 1984). Ingoldian fungi, although exhibiting slow growth rates and strong competitive or combative interactions (C-type), also show ruderal (R-type) strategies with their prolific sporulation and exposure to grazing disturbance. Cooke & Rayner (1984) have suggested that fungi showing these characteristics may be regarded as having secondary strategies (C-R types), and they give examples of fungi colonising burnt ground or dung. These are the same habitats where previous studies have demonstrated the existence of competitive hierarchies of fungi (Wicklow & Hirschfield,

TABLE 9.17. Characteristics of groups of fungi associated with allochthonous litter during its decomposition in freshwater. Values shown for growth rates and hyphal diameters are means with standard errors. Figures in parentheses are the number of isolates used. Details of scoring fungal interaction are given in Appendix 8.

FUNGAL GROUP	GROWTH RATE*1*3 mm day^{-1}	HYPHAL*2*3 DIAMETER µm	ANTAGONISM*4 GROUP A	B	C	SENSITIVITY OF EACH GROUP (row totals)
A) leaf fungi (n = 4)	4.49 ± 1.17 (n = 3)	3.43 ± 0.35 (n = 4)	2	20	30	52
B) intermediate fungi (n = 5)	3.49 ± 1.06 (n = 2)	3.86 ± 0.26 (n = 4)	5	21	32	58
C) Ingoldian fungi (n = 5)	1.34 ± 0.22 (n = 4)	2.24 ± 0.05 (n = 5)	4	18	17	39
AGGRESSIVENESS OF EACH GROUP (column totals)			11	59	79	

*1 n = 3

*2 n = 20

*3 Potato dextrose agar containing 0.1 M sodium phosphate buffer, pH 6.65

*4 Score of fungal interactions

1979 a & b), and therefore a link between fungal strategies and competitive interactions seems likely.

9.4.4 Relationship between abiotic and biotic factors in leaf degradation

Correlations suggested that both temperature and water chemistry were major factors associated with the weight losses of leaf packs in Yateley 4. Although the biotic factors appear important, they account for considerably less of the variation in leaf pack weight loss than temperature and water chemistry, suggesting that the environmental factors are of overriding importance, probably influencing the actions of micro-organisms and animals. The correlations between the number of Ingoldian fungal species and spore production and leaf pack weight loss suggest that the unit community of these fungi is an important factor in weight loss, and that higher numbers of species appear to result in an increased biomass (as significant correlations were found with ergosterol, whole leaf total and ATP). Bärlocher & Kendrick (1974) also found that mixtures of fungal species caused more weight loss of leaf discs than single species, and suggested that this was due to synergism. The protein content of their leaf discs was also higher with mixed cultures, suggesting that biomass was also greater. These results appear to agree with the findings in the present study and suggest that the development of communities of these, and other fungi, on allochthonous litter in gravel pit lakes is important in the degradation of allochthonous litter and the production of fungal biomass.

CHAPTER 10. DISCUSSION

The methods developed in this work have provided several improvements in the estimation of fungal biomass in allochthonous leaf litter. Many whole leaf clearing and staining methods are thought to give lower estimates of fungal biomass than other techniques but, as demonstrated in Chapter 3, improvements in staining can result in better estimates of fungal biomass. Correlations with the agar film methods in the laboratory, and with agar film and ergosterol in the field study, suggest that the method does detect differences in fungal biomass, although estimates are still lower than with the agar film method. Many methods destroy the sample during preparation (eg., agar film, ergosterol, glucosamine) and consequently, there is a loss of information about the biomass when using these procedures. Since more information is needed on the rôle of fungal biomass in natural communities, the ability to detect changes in its magnitude, yet examine it *in situ*, is important.

It was demonstrated in Chapter 9 that pycnidia formed a significant proportion of the biomass in allochthonous litter during decomposition and a similar conclusion was reached by Newell & Hicks (1982) working on *Spartina*. Use of agar films, ergosterol or glucosamine would not have detected this component specifically, since hyphae and reproductive structures would be encompassed in a single estimate. The abilty to examine hyphal growth patterns in cleared and stained leaves was shown, in Chapter 3, to provide information on the spatial distribution of mycelium on leaves; it revealed variation both between different leaf types and between different mixtures of fungi. It was suggested that the application of quantitative methods to analyse spatial differences in fungal biomass on the 'fine scale' level may help in understanding interactions between species and their response to disturbance, both in laboratory microcosms and in natural communities.

Although the major problems with the agar film method are recognised (Swift, 1973b; Baath & Soderstrom, 1979b, 1980; Newell & Hicks, 1982) few authors have examined them in detail or have suggested improvements. In the present work it was demonstrated that high speed homogenisation led to an optimal release of hyphae from both mycelium and leaf material in 1-1.5 minutes. These times are considerably shorter than those used by other authors and, advantageously, there appeared to be only slight differences in the time of optimal release of hyphae from mycelium of different fungal species or from different leaf types.

Correcting for inefficiency of homogenisation was suggested by Newell and Hicks (1982) and this aspect was examined in the present studies. Results suggest that, although a correction for inefficiency of homogenisation could be used, the correction required appears to differ both between leaf types and between different stages of decomposition. Therefore, to apply corrections to agar film estimates would require the efficiency of homogenisation to be determined for each sample. Further work is required to determine how much mycelium is retained by residual leaf material, particularly since the studies in Chapter 9 suggest that the use of correction factors may result in the overestimation of hyphal lengths in leaf material with little internal colonisation. The proportions of fungal mycelium retained by partly homogenised leaf fragments could possibly be estimated using an assay for glucosamine.

Tetrazolium staining has been used for counting metabolically active bacteria in agar films (Macdonald, 1980), but there appears to have been no previous applications of tetrazolium staining with an agar film method for fungi. The method developed in the present work demonstrated that, in field samples of allochthonous litter, between 5 and 38% of fungal hyphae were metabolically active with mean values of 24 and 27% for alder and willow, respectively. These figures are

comparable to estimates of living hyphae in woodland litter based on hyphal counts (Frankland, 1975a) but much higher than those using fluorescein diacetate to estimate active hyphae (Soderstrom, 1977, 1979a). One possible reason for higher proportions of active hyphae with tetrazolium staining is that staining is performed prior to homogenisation, and insoluble tetrazolium formazan crystals may become lodged in hyphae and render active lengths less prone to disruption. The insolubility of the formazan crystals in water may enable losses of active mycelium during preparation to be accounted for since solvents could be used to extract formazan crystals from different fractions; the absorbance could be read colorimetrically and related to losses.

Previous studies have suggested that ergosterol is a suitable marker for fungal growth (Seitz et al., 1977, 1979) and a method was developed in the present study for its detection in allochthonous leaf material. In field studies, ergosterol was significantly correlated with the whole leaf method and with pycnidial biomass and showed similar biomass estimates to the agar film active biomass. The major advantages of the method are that it is sensitive and capable of detecting very small differences in ergosterol, enabling small changes in fungal biomass to be detected.

Conversion factors may have an important influence on biomass estimates, and the study made in Chapter 6 demonstrated that there was considerable variation in these between species and between growth conditions. Norland, Heldal & Tumyr (1987) found an allometric relationship between bacterial dry matter and biovolume, with smaller bacteria having higher dry mass relative to volume. Clear evidence for a similar relationship between fungal dry mass and biovolume was not found in the present study, or in that of Newell & Statzell-Tallman (1982), and it may be that problems in obtaining accurate estimates of fungal biovolume masked a clear

relationship between these variables.

Ergosterol conversion factors were more variable than for fungal biovolume and significant variation was found between fungal species, growth on different nutrient media and between different ages of mycelium. Examination of the relationship between ergosterol and fungal biomass during growth in liquid cultures suggests that ergosterol levels decline after growth ceases, and that this is related to falls in the amount of living mycelium. Previous studies have followed the relationship between ergosterol and biomass, but only to the stationary phase of growth when most mycelium would still be living. Clearly there is a need to reconcile these findings with the observations in the field study, and with those of Lee, Howarth & Howes (1980) and Newell, Fallon & Miller (1986), that ergosterol gives lower estimates of biomass in plant litter than other biomass methods. This could involve a closer examination of the ergosterol content of mycelium and the proportion of mycelium which is living or active. By using methods such as respirometry, ATP content and FDA hydrolysis, it should be possible to establish which fraction of the total biomass ergosterol is measuring.

Use of the 3 biomass methods in the field suggested that, irrespective of leaf types, fungal biomass was 0.4-2.8% of leaf dry weight based on the mean values of different methods and 0.13-9.55% based on the ranges. Ergosterol measurements in a number of different lakes gave estimates of 0.02-2% of dry weight. Total biomass as ATP was 3.7 and 5.8% for alder and willow, respectively and ranged from 1-24.9%. These values are slightly higher than those reviewed for fungal and microbial biomass in streams by Barlocher & Kendrick (1981), but in general they indicate that the fungal biomass is not large in comparison to total microbial biomass and leaf mass.

A biomass that has a faster turnover and is removed by grazing could account for a relatively low biomass on

litter, but experimental studies with bags of different mesh size suggest that selective grazing was not important in the removal of fungal biomass from leaf litter in Yateley 4, although animals did consume leaf material. Findlay, Meyer & Smith (1986) found that fungal biomass on leaf litter under laboratory conditions was insufficient to meet the carbon needs of a freshwater isopod (Lirceus sp.), and they suggested that the fungal modification of the leaf is more significant than the magnitude of the biomass. The fungi are seen as mediators of carbon flux, rather than direct contributors to higher trophic levels. In this respect, it is interesting to note that there was a substantial amount of fungal mycelium present in the sediment of Yateley 4, possibly originating from allochthonous litter. Clearly, more information is needed to establish the interrelationships between animals and fungi, and such information might come from reciprocal transplant experiments. By exposing previously protected litter and protecting previously exposed litter, it may be possible to, not only measure the impact of invertebrates on fungal biomass, but also gain some understanding of the relationship between these organisms and decomposition processes.

Although the fungal biomass on allochthonous litter in Yateley 4 was quite low, it was 2-10 times higher than that per unit dry weight of litter in a deciduous woodland (Frankland, 1975b). However, conversion of the biomass figures from Yateley 4 in terms of litter inputs (Table 10.1), indicates that the mean fungal biomass (in kg ha^{-1}) in the litter layer is only between 6-41% of that in the deciduous woodland, emphasising the overriding influence of carbon inputs on the magnitude of biomass on litter in these ecosystems. The soil horizons below the litter in deciduous woodlands appear to contain the bulk of the fungal biomass (Frankland, 1975 b) and examination of the sediment in Yateley 4 suggests that a similar situation may be true for some lakes, because of the large mass of these components

TABLE 10.1 Estimates of fungal and microbial biomass associated with allochthonous leaf litter in Yateley 4. Estimates (kg ha^{-1} dry weight) are based on samples from 60 litter bags for each species, retrieved from the lake over a 5 month period (February - June). Values are the means with standard errors and ranges*7.

METHOD	ALDER*6 (n=60)	WILLOW*6 (n=60)
Agar film total*1	2.59±0.12	2.85±0.12
	(0.21-6.01)	(0.26-11.28)
Agar film total*1 *2	3.80±0.20	4.85±0.19
(corrected)	(0.51-7.75)	(0.51-16.22)
Agar film active*1	0.61±0.05	0.82±0.05
	(0.03-1.79)	(0.03-4.22)
Agar film active*1 *2	0.89±0.07	1.31±0.09
(corrected)	(0.05-2.40)	(0.07-6.08)
Ergosterol biomass*3	0.66±0.09	0.65±0.09
	(0.05-2.38)	(0.09-3.33)
Whole-leaf total*1 *4	1.66±0.10	1.92±0.10
	(0.19-3.33)	(0.60-4.03)
Total biomass (ATP)*5	6.32±0.41	9.12±0.68
	(1.60-42.4)	(15.83-58.39)

*1 Assumption: biovolume to biomass conversion factor 0.29g cm^{-3}

*2 Corrected for inefficiency of homogenisation (proportion of homogenate retained by 250µm mesh).

*3 Assumption: Ergosterol content of mycelium 2.19 mg g^{-1}

*4 Includes biomass of pycnidia

*5 Assumption: Ratio of ATP to cell carbon 1:250 and carbon 36.7% of cell dry weight (Anderson and Domsch,1980)

*6 Values for **Alder** and Willow are calculated on the basis of 169.87kg of allochthonous litter entering 1 hectare of Yateley4 annually (see Chapter 7).

*7 Ranges are for all data obtained in Yateley 4. i.e includes data from Site 1 study.

relative to allochthonous litter. More studies are needed to establish if the sediment is an important source or sink for fungal biomass.

It has been shown in Chapters 8 and 9 that Ingoldian fungi formed characteristic communities on allochthonous litter in gravel pit lakes, and that they appear to be actively associated with the decomposition of these resources. Although temperature and water chemistry appear to be overriding factors in the decomposition of allochthonous litter in these lakes , the Ingoldian fungi appear to make a significant contribution to weight loss and the development of fungal biomass.

A competitive hierarchy is postulated to account for observed changes in fungal species during decomposition of allochthonous litter in these lakes and it appears to have an important influence on the development and possibly the magnitude of fungal biomass. However, fungal biomass is only likely to be seen in perspective by obtaining a greater understanding of the roles of particular species in decomposition. The development of immunological methods may offer the greatest potential for a better understanding of the ecology of individual fungal species, and already ELISA has been used for identifying fungi (Aldwell et al., 1983) and estimating the biomass of individual species under field conditions (Newell, Fallon & Miller, 1986).

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APPENDIX 1

Table 1 Survey of potential agents used for bleaching leaf litter.

AGENT	METHOD	BLEACHING* EFFICIENCY	COMMENTS
A)LIQUID			
1) Sodium hydroxide (0.1M)	Discs were boiled for 10 minutes.	+	Limited bleaching, some leaf damage.
2) Sodium hypochlorite (3%,6% aq v/v)	Discs were immersed for varying periods of time.	+++	Rapid but damaged leaves.
3) Hydrogen peroxide (3%,6%,30% aq v/v)	Discs were immersed for varying periods of time.	++	Leaves blistered, many turned pale green.
4) Methanol, chloroform, lactic acid (1:1:1)	Discs were immersed in the mixture.	+	Removed some pigment but leaf not bleached.
5) Sodium chlorite/ acetic acid/ water(Hering & Nicholson, 1964)	0.5ml of 10% acetic acid was added to 40ml of an aqueous solution of sodium chlorite(7.5mg/ml).	+++	Fairly slow, less disruptive than method 2).

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5) Sodium chlorite/ acetic acid/ water(Hering & Nicholson, 1964)	0.5ml of 10% acetic acid was added to 40ml of an aqueous solution of sodium chlorite(7.5mg/ml).	+++	Fairly slow, less disruptive than method 2).

AGENT	METHOD	BLEACHING* EFFICIENCY	COMMENTS
6) Sodium hydroxide, Hydrogen peroxide (Newell & Hicks, 1982)	Discs heated in NaOH (5% aq w/v) at 80°C for 5 minutes, rinsed and bleached in 30% H_2O_2	+++	Rapid bleaching, some leaf blistering.
7) Hydrogen peroxide, ammonia water (Witkamp, 1974)	Discs bleached in mixture, 10ml of H_2O_2(30% v/v) and ammonia(20% v/v) with 30ml distilled water.	+++	Slower than 6) but similar results.
B) <u>DRY</u>			
8) Sodium isocyanuriate (Baker, 1981)	Discs were exposed to chlorine gas produced by mixing 3g of sodium isocyanuriate with 30ml of distilled water.	++	Slow, many leaves bleached to a pale green colour after 1-2 weeks.

*KEY: + poor; ++ fair; +++ good; ++++ excellent (subjective scale).

Table 2 Solubility, colour intensity and differentiation between fungi and leaf tissues of various stains made up in methyl salicylate*[1].

STAIN	C.I.*[2] NUMBER	SOLUBILITY	STAINING COLOUR INTENSITY	DIFFERENTIATION BETWEEN FUNGAL AND LEAF TISSUE
Safranin O	50240	+++	+++	+
Methyl Violet 2B	42535	+++	++++	+
Victoria Blue	44045	+++	+++	+
Erythrosin	45425	+	++++	+
Carbolan-Brilliant Green	42040	+++	++++	+
Page Blue 83	42660	++	+++	+++
Analine Blue	42755	+	+	+
Blebrick Scarlet	26905	+	+	+
Chlorazol Black E	30235	+	+	+
Trypan Blue	23850	+	+	+
Lissamine Green V	44025	++	+++	+++
Lissamine Green B	44090	++	+++	++
Lissamine Rhodamine	45100	++	+++	++
Lissamine Fast Yellow	18965	+++	+++	+

KEY - based on subjective assessment by eye:

+ poor; ++ fair; +++ good; +++ excellent

*1 All stains tested at 0.01% w/v

*2 C.I.: Colour Index Number, Society of Dyers and Colourists Colour Index, 2nd Edition. (1956-58, Supplement 1963) Bradford, England.

Table 3 Effectiveness of different staining combinations in differentiating fungi from plant tissues. All staining treatments were performed using stains made up in methyl salicylate at 0.05% w/v.

STAIN	C.I.*2 NUMBER	COLOUR FUNGUS	COLOUR PLANT TISSUE	DIFFERENTIATION*1
Safranin O Lissamine Green V	50240 44025	Green	Red	+++++
Safranin O Lissamine Green B	50240 44090	Green	Red	++++
Methyl Violet 2B Lissamine Green V	42535 44025	Green	Violet	+++
Lissamine Fast Yellow Lissamine Green V	18965 44025	Green	Pale yellow	++
Lissamine Rhodamine	45100	Dark red	Red	++

STAIN	C.I.*2 NUMBER	COLOUR FUNGUS	COLOUR PLANT TISSUE	DIFFERENTIATION*1
Methyl Violet	42535	Violet	Violet	+
Safranin O	50240	Red	Red	+

*1 Degree of differentiation between fungal structures and leaf tissues: (+++++) very good; (++++) good; (+++) fair; (++) difficult to separate smaller structures; (+) poor, hyphae not detectable. All subjectively determined by eye.

*2 C.I.Number, Society of Dyers and Colourists Colour Index, 2nd Edition (1956-58, Supplement 1963) Bradford, England.

Table 4 Ability of various fluorochromes to stain fungi on leaf discs under UV or blue light.

STAIN	UV LIGHT*$_1$	BLUE LIGHT*$_2$
Nucleic Acid Intercalators		
Acridine Orange (100 µg ml^{-1},aq)	++ Red	++++ Orange/Green
Berberine Sulphate (100µg ml^{-1},aq)	-	+ Yellow
Ethidium bromide 6.5µg ml^{-1} (0.1M phosphate buffer pH 6.8)	+ Red	++ Red
Protein Binders		
Fluorescein isothiocyanate (0.3mg ml^{-1}, O.1M phosphate buffer pH 7.2)	++ Yellow	++++ Yellow
Membrane active		
Photine H.V. *$_3$ diluted 1:10(aq)	++++ Blue	-
Primulin (2mg ml^{-1}, aq)	++++ Blue	-

STAIN	UV LIGHT*$_1$	BLUE LIGHT*$_2$
<u>Enzyme mediated</u>		
Fluorescein diacetate ($10\mu g\ ml^{-1}$, 0.06M phosphate buffer, pH 7.4)	++ Yellow	++++ Yellow
3-O-methyl fluorescein phosphate ($10mg\ ml^{-1}$, aq)	+ Yellow	++ Yellow

*$_1$ G365 excitation filter, FT365 beam splitter and LP420 barrier filter.

*$_2$ BP436/8 excitation filter, FT460 beam splitter and LP470 barrier filter.

*$_3$ Gift from Hickson and Welch Ltd (Castleford)

KEY: - negative; + weak; ++ clear; +++ bright; ++++ intense
all assessed on subjective scale by eye.

APPENDIX 2

Spatial analysis on single leaves.

When it is said that the distribution of a species or group of species shows pattern it is meant that a departure from randomness is shown. The species may be more clumped than expected or more regularly spaced than expected.

The following methods of pattern analysis were considered for assessing fungal distributions on leaves:

1) Greig-Smith's block size method using grids or transects (Greig-Smith, 1952).
2) Two-term local variance, a modified version of Greig-Smith's method (Hill,1973a) applied to transect data.
3) Mead's two within four randomisation test (Mead, 1974).
4) Spectral analysis in one dimension (Ripley, 1978) applied to transect data.
5) Spectral analysis in two dimensions (Renshaw and Ford, 1984) applied to data in grids.

The first of these methods has been used widely in plant ecology (Greig-Smith, 1983) and in microbiology to examine spatial distribution of fungi on leaf surfaces (Cox, 1976), aggregation of fungal zoospores (Porter and Shaw, 1978), the distribution of bacteria on leaf surfaces (Newman and Bowen, 1974) and the spatial pattern of Botrytis cinerea Sardinia on Vicia faba L. (Gilligan,

1982). Mead's method has been used to study the effect of herbicides on the aggregation of bacteria in soil (Polonenko, Pike and Mayfield, 1978).

Despite the use of these methods to study the spatial distribution of microbial populations, few authors have acknowledeged that the various methods may give different results on the same data set (Ripley, 1978). The major problems with the methods are the bias due to starting position (methods 1 and 3), inadequate statistical testability (methods 1 and 2), and the limited number of scales of pattern that can be examined (methods 1 and 3). For these reasons, ecologists have often employed several methods in a study, limiting the amount of data that can be analysed, and making interpretation more difficult.

A comparative study of the various methods with real and artificial data has concluded that methods based on spectral analysis (methods 4 and 5) are by far the most efficient for the analysis of data for patterns (Ripley, 1978). Spectral methods are not dependant on the starting position of a transect or grid and it is possible to test the results statistically. For the work in this thesis spectral analysis in one dimension was used (Ripley, 1978). The analysis in two dimensions (Renshaw and Ford, 1984) provides more information (e.g. the directional trend of any pattern present) than the one dimensional analysis, but the collection of data and the analysis and interpretation are very time consuming.

Spectral analysis

Spectral analysis was originally developed for describing a series of events in time but is also applicable to a spatial series. The analysis in one dimension regards the observed values in quadrats along a transect as determined by a number of functions each showing a wave-form of variation along the transect. The wavelength will differ from two to the complete length of the transect so that the observed value x_i in a quadrat is:

$$x_i = c_0 f_0(i) + c_1 f_1(i) + c_2 f_2(i)....$$

Where the f(i)'s are the values of the functions for quadrat i and the c's are constants calculated for the particular set of data. The only wave system for which the starting system is not crucial is that of sine and cosine waves which form the basis of spectral analysis. The observations $x_i.....x_m$ are expressed as:

$$x_i = c_0 + \sum_{j=1}^{m/2-1} \{c_j.\cos(2\pi ij/m) + s_j.\sin(2\pi ij/m)\} + c_{m/2}(-1)^i$$

The constants are calculated

$$c_0 = (x_1 + ...x_m)/m$$

$$c_j = \{\sum_{k=1}^{m} x_k.\cos(2\pi jk/m)\}2/m \qquad j=1.....m/2$$

$$s_j = \{\sum_{k=1}^{m} x_k . \sin(2\pi jk/m)\}2/m \qquad j=1.....m/2$$

Including c_0, as many constants are calculated as there are quadrats in each transect, providing a suitable number (>100) are present. The central tool of spectral analysis is the periodogram ($I_j=(c_j^2+s_j^2)m/8\pi$) which is proportional to the reduction in the sum of squares obtained by fitting sine and cosine waves of period m/j i.e. those with j complete cycles in the transect.

The interpretation is made in terms of the calculated value of the periodogram for different scales, and is plotted as a smoothed function of the periodogram against frequency or block size of quadrats. Confidence limits can be obtained for the smoothed spectral function and although strictly valid for normally distributed data, can provide a good approximation for spectra calculated from long transects of counts or cover scores. A plot of the cumulative periodogram gives an indication of the overall spectrum and any non-randomness in the data can be detected by confidence bands. Examined together with the smoothed spectral function the plots indicate if any pattern is present and at which scale.

Analysis of data

Analysis of transect data was performed using a spectral analysis program written in Fortran 77 (supplied by Professor B.D.Ripley, Mathematics Department,

Strathclyde University).

To aid interpretation of real data a number of test runs were made using artificial data sets simulating different types of pattern. Three types of data were examined in transects 128 units long:

a) Random data

b) Random data with a systematic component

c) Systematic presence/absence data.

The results are shown in Appendix 2, figures 1a-c. Three items are shown for each data type:

(i) <u>The cumulative periodogram with 95% confidence band.</u>

The vertical axis represents cumulative frequencies and the horizontal axis the frequency of different quadrat numbers (blocks) e.g.

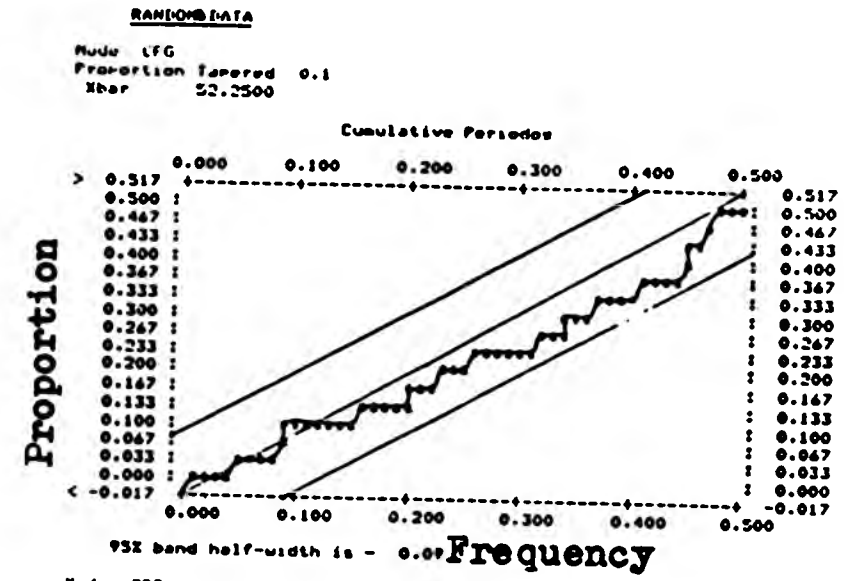
Frequency value	Number of Quadrats (Blocks)	Number of cycles in transect
0.500	1	64
0.250	2	32
0.125	4	16
0.063	8	8
0.032	16	4
0.016	32	2

(ii) <u>The smoothed spectral estimate.</u>

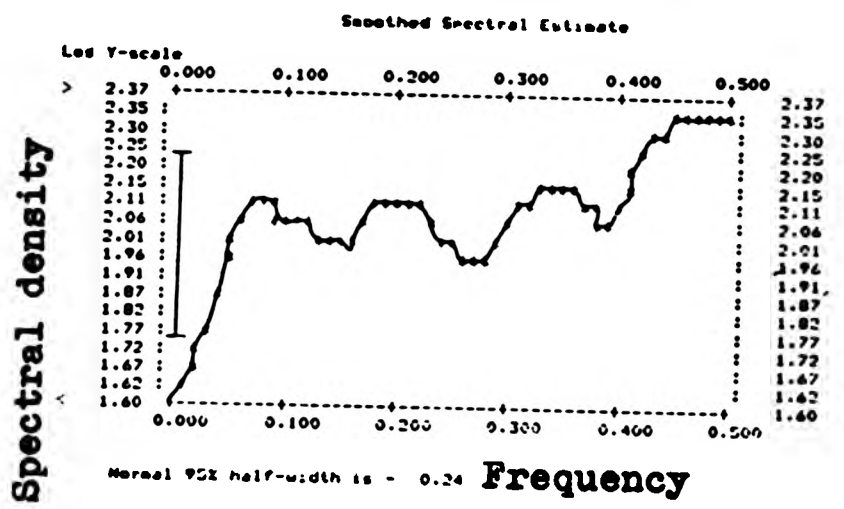
This shows the change in spectral density (vertical axis) with changes in quadrat frequency (horizontal axis). The 95% confidence limits are shown for each spectrum. Significant peaks are those which are separated by more than the 95% confidence limits.

Figure a)

i)



ii)



iii)

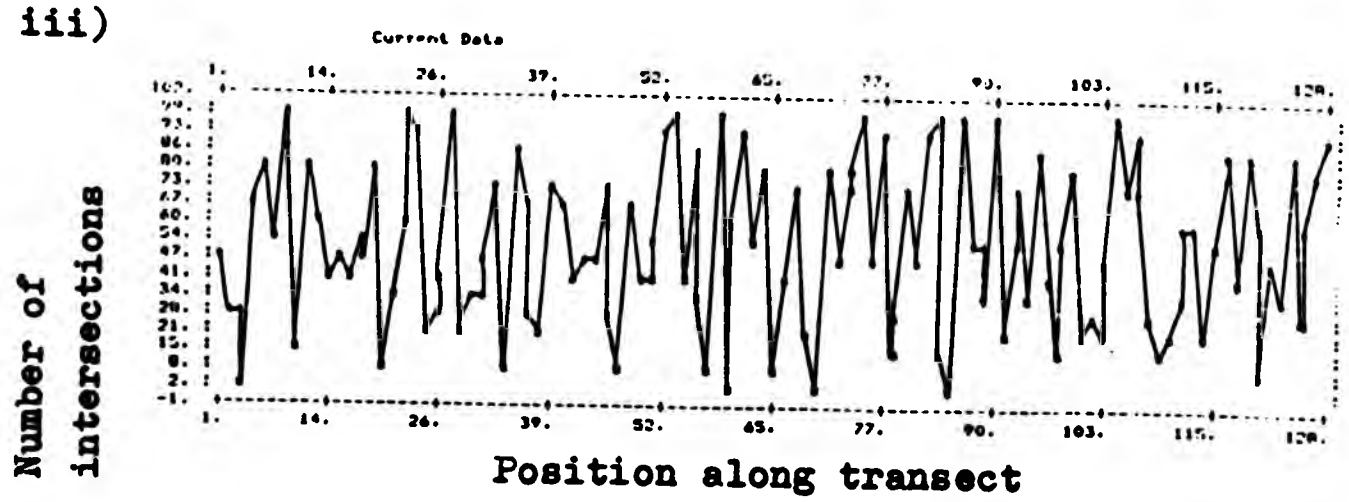
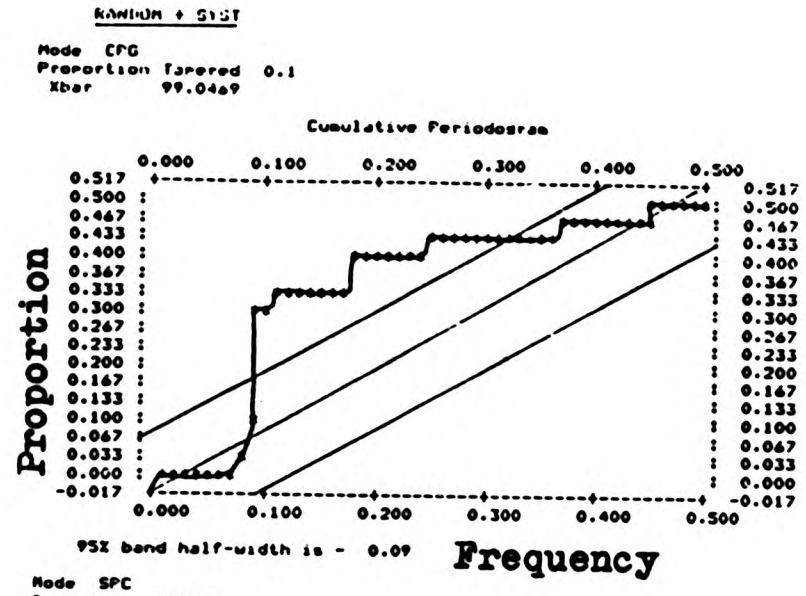


Figure b)

i)



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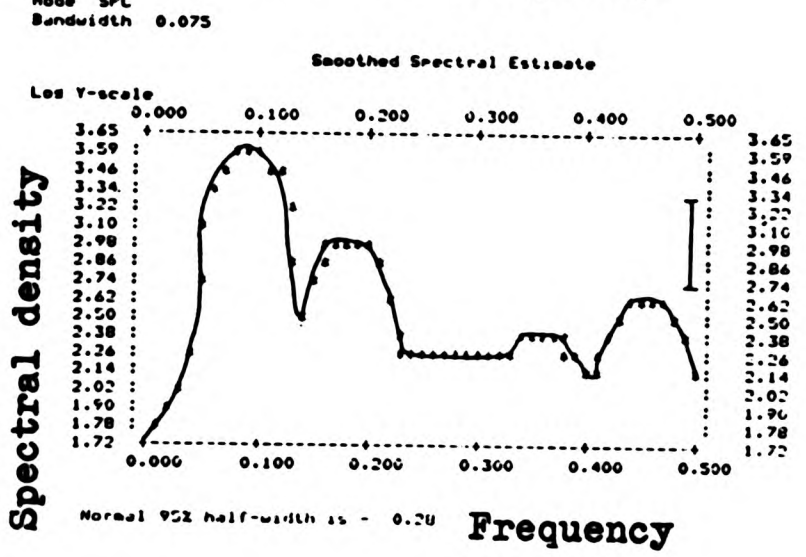

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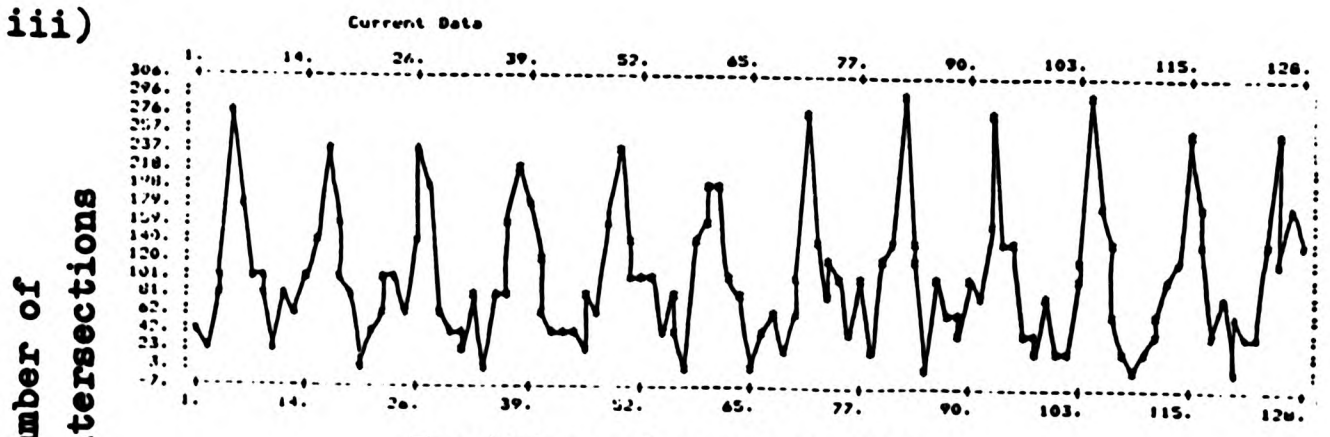
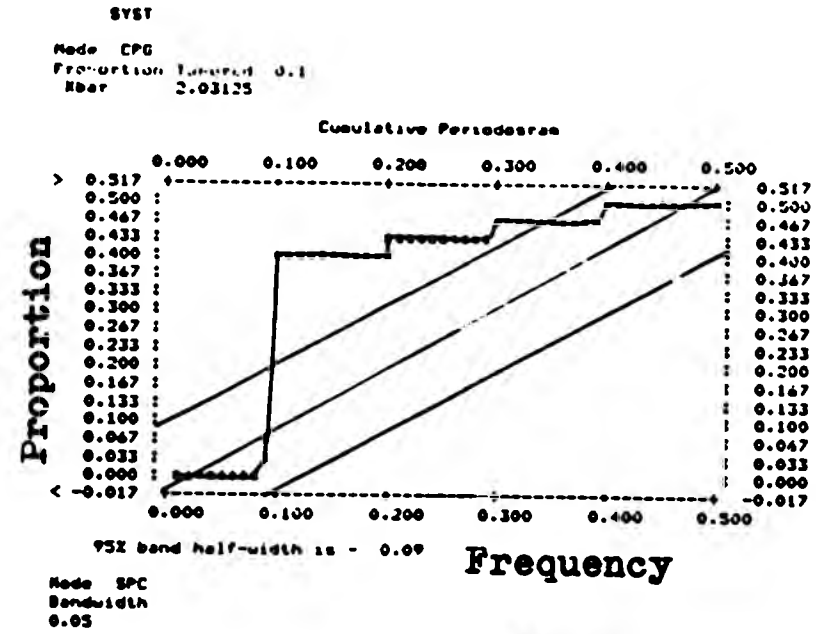


Figure c)

i)



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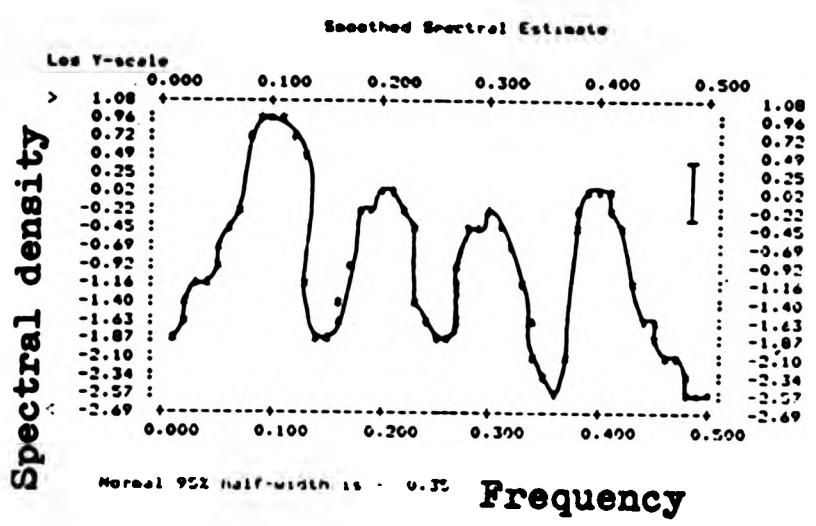
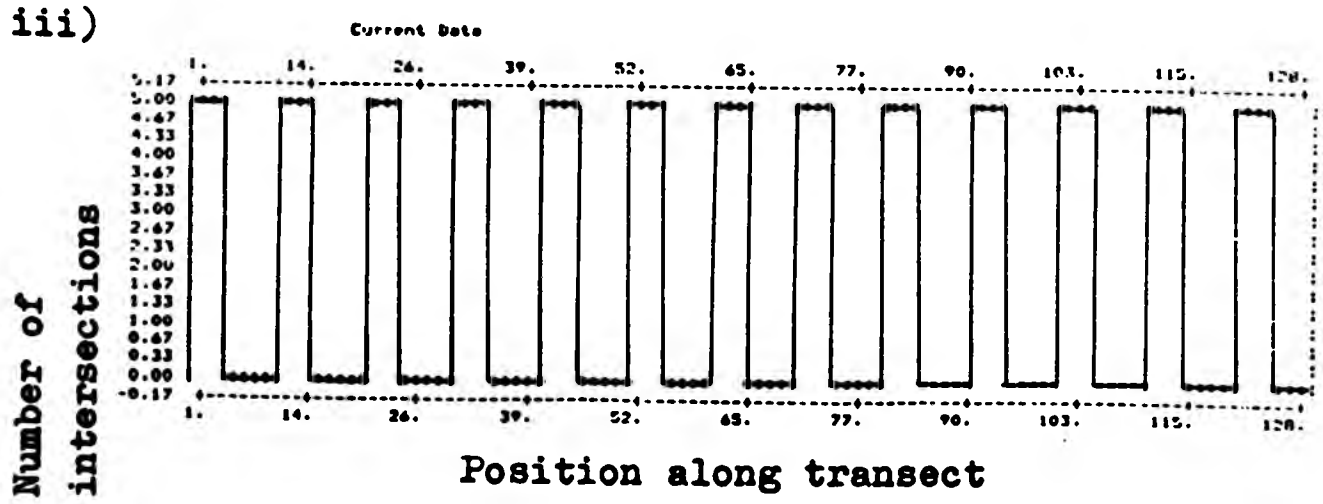


iii)



(iii) A plot of the transect data.

This shows the sequence of data from quadrat 1 to 128.

Interpretation

Figure a) Random data.

Inspection of the raw data (random values between 0 and 100) shows no obvious indication of large scale pattern. It is not possible by visual inspection to tell if small scale pattern (2-8 quadrats) is present or not. The analysis shown in (a)(i) and (ii) demonstrates that no significant pattern is present in the data. The cumulative periodogram (i) shows that the spectral estimate data does not exceed the 95% band. None of the peaks in the smoothed spectral estimate (ii) are significantly different from each other indicating that no pattern is present.

Figure b) Random data and systematic component.

To simulate pattern against a background of 'noise', a systematic component was added to the random data described in the previous example. Each systematic component consisted of 5 values 50, 100, 200, 100, 50 in sequence followed by a sequence of 6 values when the systematic component was absent (i.e. random data 0-100). This pattern was repeated for the whole length of the transect. The raw data is shown in (b)(iii). Pattern is obviously present by visual inspection, but the scale is masked by the underlying random component in the data. The analysis also

detects the presence of pattern. Both the cumulative periodogram (note deviation from 95% confidence band) (b)(i) and the smoothed spectral estimate (b)(ii) indicate a significant peak at a frequency of 0.09-0.1 indicating a pattern at a scale of about 5 quadrat units, which was the 'scale' added to the random data.

Figure c) Systematic presence/absence data.

This class of artificial pattern consists of regular blocks of presence (4 units) or absence (6 units) simulating the occurrence of fungal hyphae in 4 quadrats followed by absence from the next 6 (see (c)(iii) for raw data). The sharp jump in the cumulative periodogram (c)(i), and the strong peaks in the smoothed spectral estimate, (c)(ii), suggest the presence of a 'strong' regular scale of pattern of 4 to 5 units. The peaks at frequencies of 0.2, 0.3 and 0.4 are significantly different, by the 95% confidence limits, from the major peak at about 0.1 but not from each other. These peaks represent harmonics of the dominant peak at 0.1. This feature is common in data with very regular patterns.

These simulations with artificial patterns clearly demonstrate that spectral analysis is capable of detecting the presence and approximate scale of pattern in transect data. The method can be applied to examine the distribution of fungal hyphae on leaf litter for the presence of pattern as described in Chapter 3.

APPENDIX 3 Media used in the study

(i) Solid media

Malt extract agar, potato dextrose agar, cornmeal agar and nutrient agar were obtained from commercial suppliers (Oxoid Ltd) and made according to the instructions supplied with each product. All media were sterilized at 121°C for 15 minutes except media with malt extract which were sterilized at 115°C for 10 minutes.

a) Potato dextrose agar containing antibiotics

Potato dextrose agar (Oxoid)	39g
Penicillin G (1650 IU)	0.1g
Streptomycin sulphate	0.1g
Distilled water	1 litre

The antibiotics were filter sterilized and added to the medium just before the plates were poured.

b) Malt extract agar containing antibiotics

Malt extract agar (Oxoid)	20g
Agar (Oxoid No.3)	9g
Penicillin G (1650 IU)	0.1g
Streptomycin sulphate	0.1g
Distilled water	1 litre

The antibiotics were added as in the previous medium. Chloramphenicol was occasionally used at 0.06g l^{-1} and sterilized with the medium.

c) Leaf extract agar

Whole leaves (air dried)	10g
Agar (Oxoid No.3)	15g
Distilled water	1 litre

Leaves were soaked in 500ml of distilled water for 48 hours at 6°C. The water was discarded and the leaves rinsed in fresh distilled water. Leaves were added to a blender (ATO-MIX, MSE) together with 500ml of distilled water and homogenized for 2 minutes at top speed (c.12000 rev min^{-1}). The homogenate was allowed to stand for 5 minutes at room temperature followed by filtering and squeezing the homogenate through doubled muslin. The filtrate was adjusted to 1 litre with distilled water before adding the agar and autoclaving.

d) Lake water agar

Agar (Oxoid No.3)	15g
Lake water, filtered (GF/C, Whatman)	1 litre

e) Leaf extract lake water agar

The medium was made up as in c) except that the leaves were macerated and made up to 1 litre in filtered lake water (GF/C, Whatman).

f) Cellulose isolation agar (Park, 1973)

KH_2PO_4	1.0g
KCl	0.5g
$MgSO_4.7H_2O$	0.2g
$CaCl_2$	0.1g
Yeast extract (Oxoid)	0.5g
Agar (Oxoid No.3)	20.0g
Cellulose (ball milled)	10.0g
Distilled water	1 litre

g) Pectin isolation agar (Hankin, Zucker and Sands, 1971) (for pectinolytic bacteria)

$(NH_4)_2SO_4$	2.0g
KH_2PO_4	4.0g
Na_2HPO_4	6.0g
$MgSO_4.7H_2O$	0.2g
$FeSO_4.7H_2O$	0.001g
$CaCl_2.2H_2O$	0.001g
H_3BO_3	0.00001g
$MnSO_4.4H_2O$	0.00001g
$ZnSO_4.4H_2O$	0.00007g

$CuSO_4$	0.00005g
MoO_3	0.00001g
Pectin (250 grade, BDH)	10.0g
Agar (Oxoid No.3)	15.0g
Yeast Extract (Oxoid)	2.0g
Distilled water	1 litre

(ii) Liquid media

Malt extract broth was obtained from commercial suppliers (Oxoid) and made according to supplied instructions.

TABLE 1. List of fungal isolates used in this study

SPECIES	ISOLATE NUMBER	LEAF+	SOURCE*	LOCATION
Tetracladium marchalianum	1A	O	L	Epping, Essex
Tetracladium marchalianum	5A	O	L	Epping, Essex
Anguillospora longissima	7A	O	L	Epping, Essex
Anguillospora longissima	Dun 2	W	L	Dungeness, Kent
Clavariopsis aquatica	ST1	W	L	Stodmarsh, Kent
Articulospora tetracladia	VW1	A	L	Virginia Water, Berks
Heliscus lugdunensis	KA1	W	R	Dover, Kent
Tricladium splendens	Yat 4	A	L	Yateley, Hants
Tetrachaetum elegans	27A	A	R	Aber, Gwynedd
Fusarium sp.	Folk 1	S	R	Folkestone, Kent
Fusarium sp.	Yat F1	W	L	Yateley, Hants
Phoma sp.	BW1	A	R	Hindley, Lancs
Acremonium sp.	Yat 9	A	L	Yateley, Hants
Unknown sp. 1	Yat G17	W	L	Yateley, Hants
Trichoderma viride	Lam 23	A	R	Newbury, Berks
Cladosporium cladosporioides	Yat 37	A	L	Yateley, Hants
Epicoccum purpurascens	Yat 15	W	L	Yateley, Hants

SPECIES	ISOLATE NUMBER	LEAF+	SOURCE*	LOCATION
Alternaria alternata	Yat 23	W	L	Yateley, Hants
Aureobasidium pullulans	AB23	A	R	Aber, Gwynedd
Aureobasidium pullulans	Yat 3	A	L	Yateley, Hants

\+ Leaf type (O = oak, W = willow, A = alder, S = sycamore)

* R = river/stream, L = lake/pond

APPENDIX 4

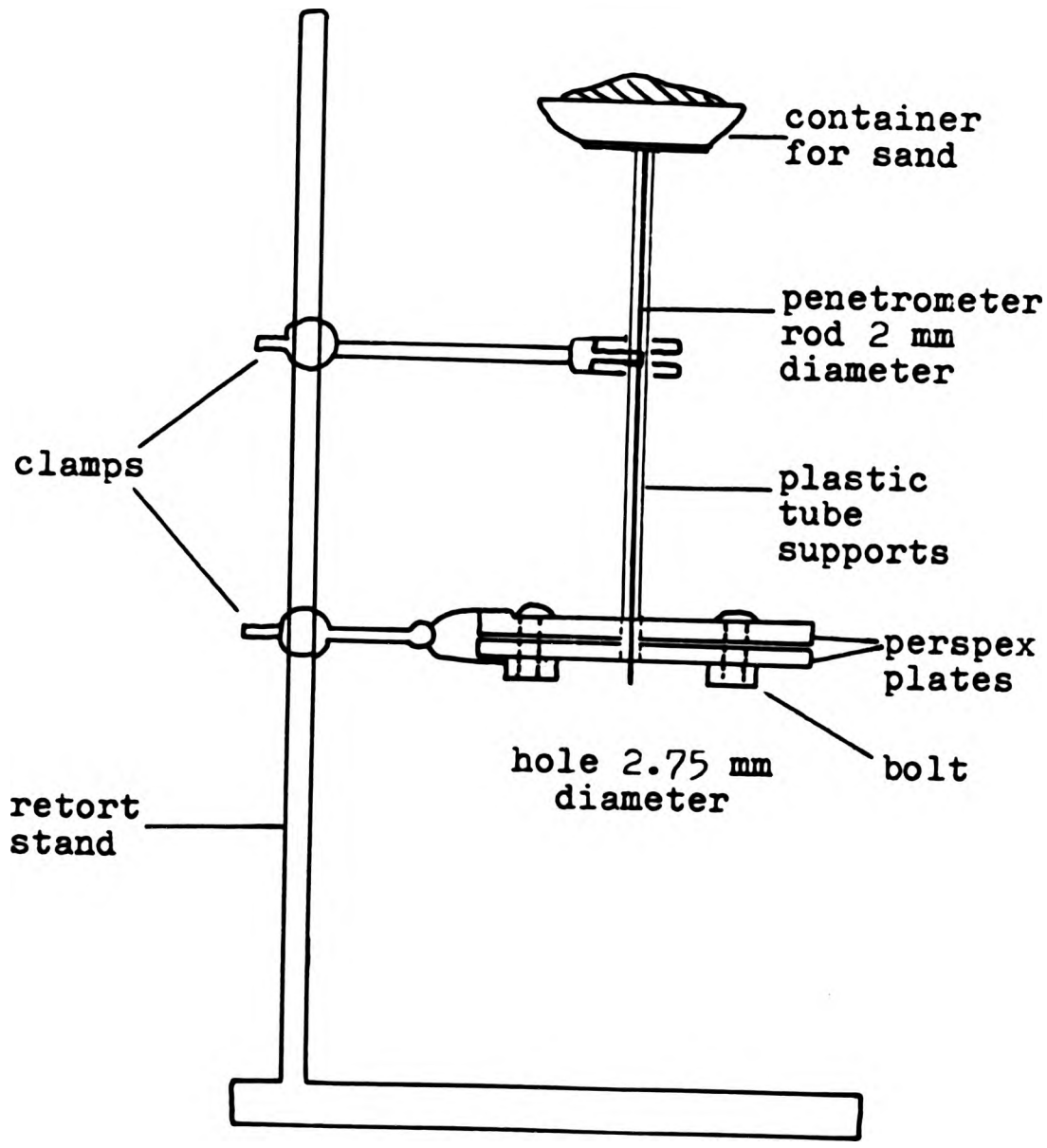


Figure 1. Penetrometer used for measuring leaf lamina toughness.

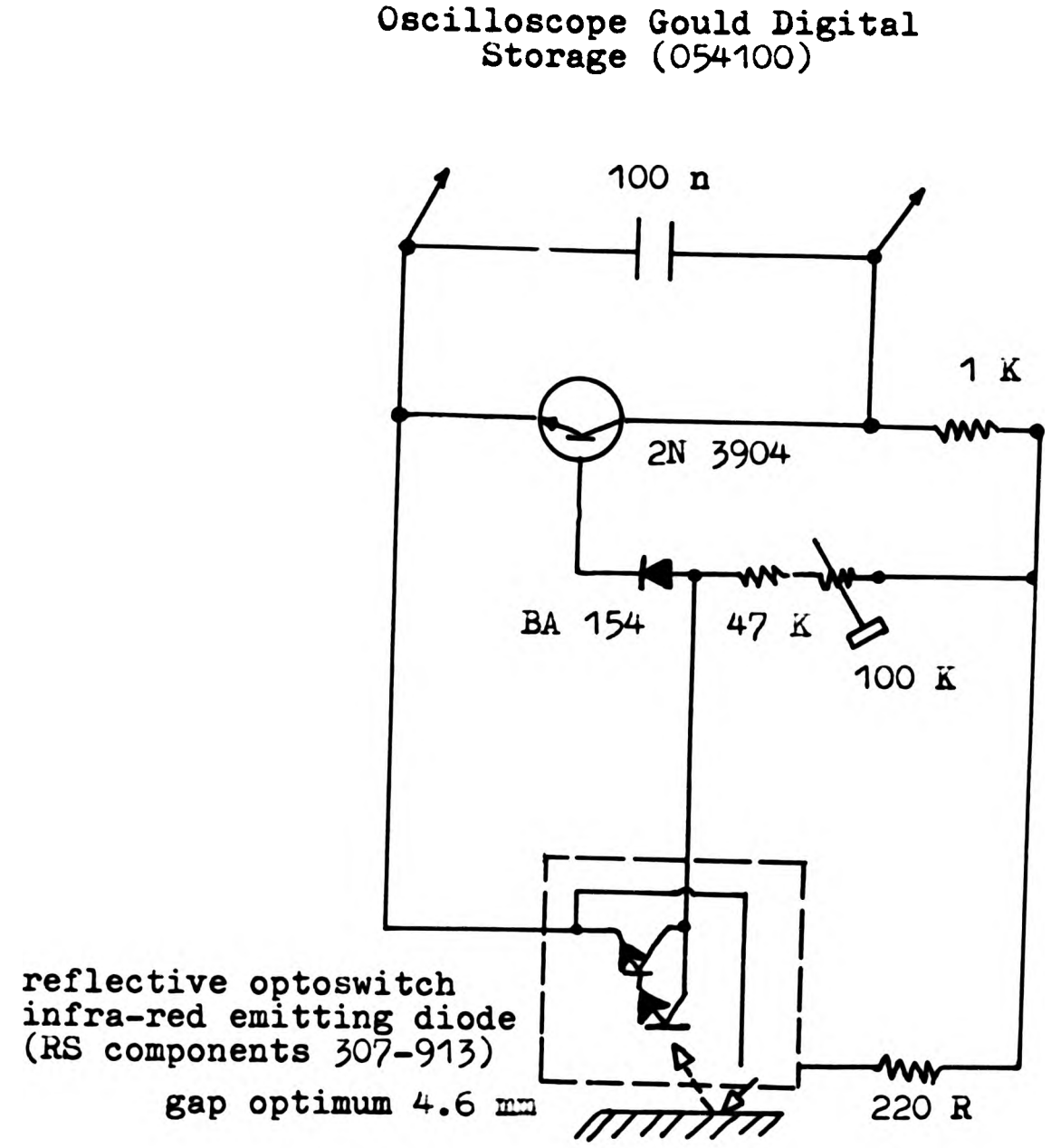


Figure 2. Circuit diagram of reflective optoswitch for determining speeds of homogenisation.

Homogenisation speed determination

To determine speed of homogenisation, an area of the homogenisation shaft was first painted black and a small white reflective line painted over it parallel to the length of the shaft. The reflective optoswitch (Figure 2) was located directly opposite the line at a distance of approximately 4.6 mm. A single revolution of the homogeniser shaft generated a waveform on the oscilloscope which could be readily measured on a time scale of 0.5-5 milliseconds. Data were converted to revolutions per minute for comparisons of leaf homogenates.

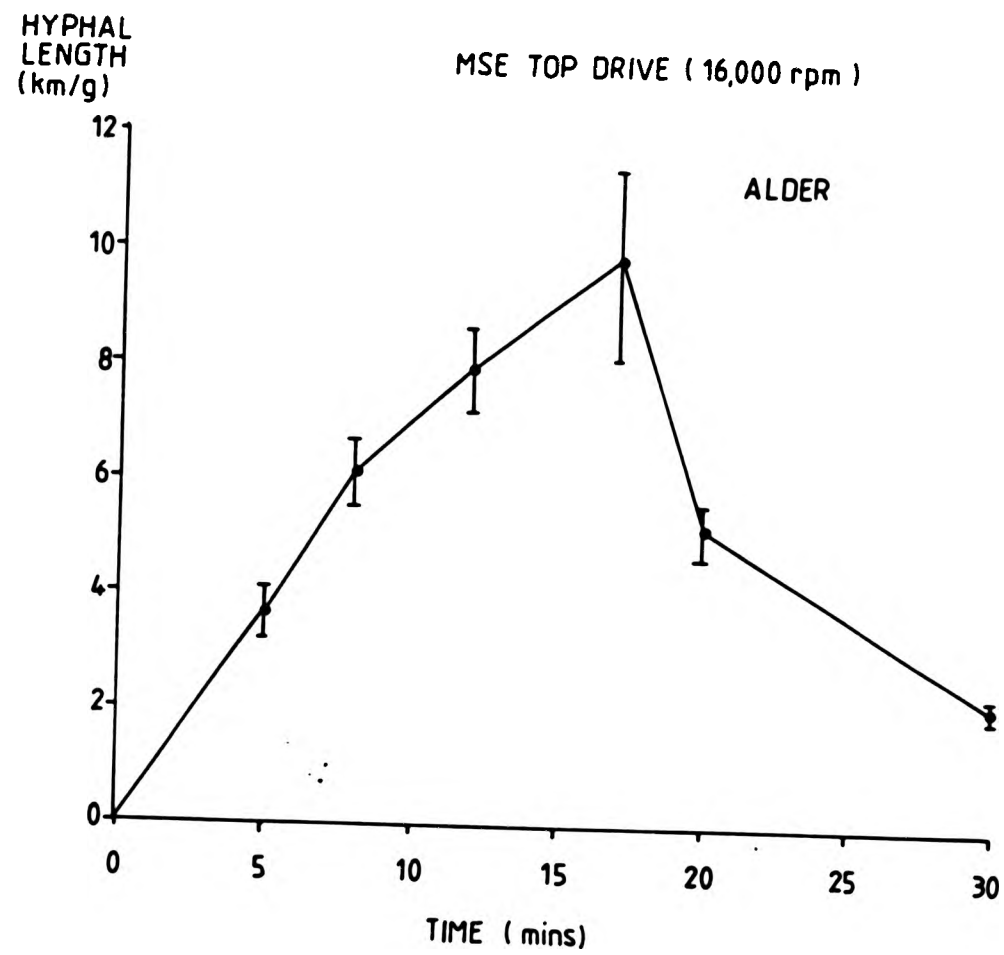


Figure 3. Influence of homogenisation time on hyphal release from alder leaf material retrieved from benthic litter. (Ten leaf discs were homogenised in 20 ml of distilled water).

APPENDIX 5

TABLE 1 Results of the Hadler histochemical reaction for 7 Sterols on Silica G thin layer plates. The numbers refer to each stage in the preparation, see Appendix 5, Table 2.

	1	2	3	4
STEROL	BROWNING REACTION	TOLUIDINE BLUE	HEAT 110°C 15 mins	UV 356nm
DESMOSTEROL	+	+	DEEP RED	BROWN
ERGOSTEROL	+	-	STRAW	YELLOW
CHOLESTEROL	+++	+	STRAW	BROWN
DIHYDROCHOLESTEROL	+	+	WHITE	-
βSITOSTEROL	+++	++	DEEP RED	BROWN
STIGMASTEROL	++	+	DEEP RED	BROWN
LANOSTEROL	+++	+	PALE BROWN	PALE-BROWN

Strength of staining judged subjectively:

- none, + moderate, ++ strong, +++ very strong

TABLE 2 Procedure for the Hadler histochemical reaction for detecting sterols on TLC plates (after Valle and Oliveira-Filho, 1975)

1) Spray with permanganate-sulphuric acid oxidative solution (0.12SN H_2SO_4 +0.038N $KMnO_4$, 1:1 pH 1.30 followed by heating for 2 mins at 110°C and record degree of browning.

2) Spraying with hydrogen sulphite solution (5% aq) and reheating to discolour plates. Spray with toluidine blue (0.5% w/v in 1N H_2SO_4; pH 0.5-0.6) and record results.

3) Heat at 110°C for 15 minutes and record the results.

4) Expose plates to ultraviolet (UV) light (356nm).

TABLE 3 ATP levels (mg g^{-1} mycelial weight (80°C)) in six fungal strains grown on malt extract and leaf extract agars. The values shown are the means and standard errors based on three replicate determinations.

SPECIES	GROWTH MEDIA		
	MALT	ALDER	WILLOW
Tetracladium marchalianum	1.92±0.21	0.39±0.01	0.42±0.04
Tetrachaetum elegans	2.36±0.18	0.65±0.04	0.38±0.03
Articulospora tetracladia	2.23±0.10	0.31±0.03	0.31±0.01
Heliscus lugdunensis	1.34±0.22	0.25±0.02	0.35±0.06
Phoma sp.	1.53±0.14	0.71±0.07	0.60±0.04
Fusarium sp.	1.70±0.09	0.85±0.09	0.79±0.11

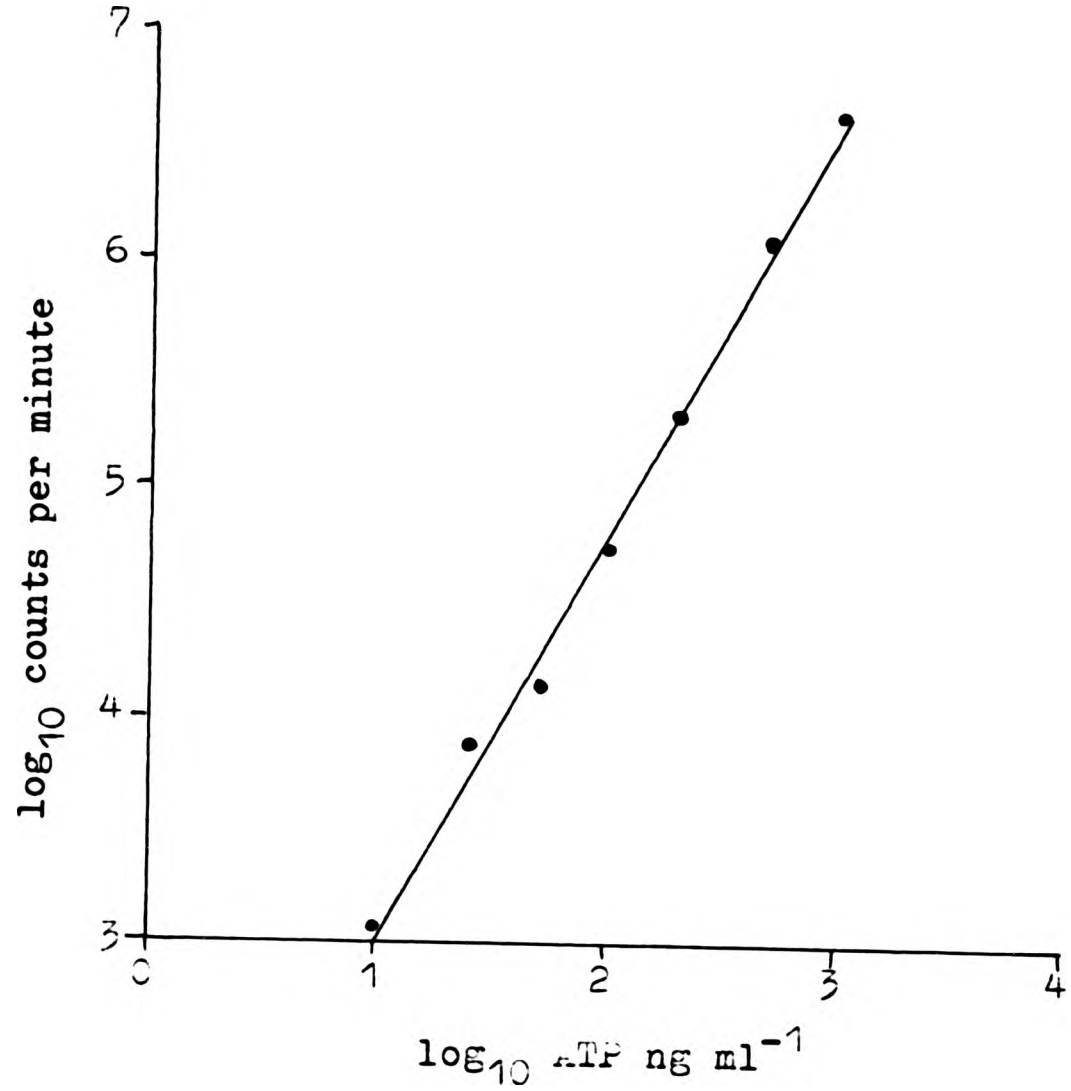


Figure 1. Standard curve for ATP determination using the luciferin luciferase assay with scintillation counting of photoluminescence.

(Samples were counted for 10 seconds one minute after addition of enzyme mixture to the standard or sample)

APPENDIX 6

TABLE 1 Physical and chemical characteristics of the Blackwater Valley gravel pits in 1984. Values for Chlorophyll a are based on at least 3 determinations.

LAKE	AGE YRS (1984)	AREA (ha)	SHORELINE* DEVELOPMENT	LITTER** INPUT g $cm^2 yr^{-1}$	CHLOROPHYLL a mg l^{-1}
1	26	4.70	1.56	10.39	14.6
2	14	3.70	1.39	3.20	5.3
3	24	3.10	1.57	6.29	14.8
4	26	1.50	1.29	10.20	6.3
5	26	0.10	6.17	38.96	28.6
6	26	0.30	6.82	24.80	12.7
7	26	0.46	1.50	13.35	23.9
8	26	0.80	1.82	11.43	52.3
9	14	2.80	1.77	4.69	15.1
10	14	0.71	4.72	10.04	23.9
11	15	4.86	2.04	3.79	21.7
12	26	2.02	1.74	7.71	12.7
13	26	3.04	1.96	6.72	21.9
14	8	2.53	1.49	1.18	22.6
18A	28	1.09	1.70	2.16	63.2
18B	28	0.51	1.35	6.47	12.8
19	21	1.00	1.35	1.41	22.6
20	19	0.70	1.08	3.47	51.1
21	18	1.00	1.44	6.62	26.8
21A	17	1.40	1.95	6.27	21.1

* Shoreline development = 0.5 x perimeter $(\pi \text{ area})^{-1/2}$

** Litter input estimated from input per m of shorline into Yateley 4 and proportion of even shoreline with trees and shrubs.

TABLE 2 Chemical characteristics of the Blackwater Valley gravel pits in 1984. Values are means of at least 3 determinations.

LAKE	CONDUCTIVITY (µmhos, 20 °C)	pH	ALKALINITY (m equiv l^{-1})	PO_4-P (µg l^{-1})	NO_3N/NO_2-N (µg l^{-1})
1	362	7.7	1.41	10.8	110.1
2	214	8.4	1.09	5.1	75.6
3	371	8.0	1.68	6.1	171.7
4	374	8.0	1.72	6.0	40.7
5	242	7.3	1.55	20.0	25.2
6	447	7.7	1.72	9.3	1320.5
7	486	7.8	2.01	5.2	687.8
8	442	8.1	1.91	6.9	103.7
9	371	8.3	2.19	8.3	38.0
10	337	7.9	1.16	9.7	65.0
11	322	8.1	1.78	4.7	34.5
12	397	8.1	1.62	5.5	741.1
13	437	7.9	2.12	3.8	437.2
14	396	8.0	1.50	4.4	28.4
18A	392	7.9	1.56	1.8	42.3
18B	364	7.8	1.27	3.0	58.7

* Shoreline development = 0.5 x perimeter $(\pi \text{ area})^{-1/2}$

** Litter input estimated from input per m of shorline into Yateley 4 and proportion of even shoreline with trees and shrubs.

TABLE 2 Chemical characteristics of the Blackwater Valley gravel pits in 1984. Values are means of at least 3 determinations.

LAKE	CONDUCTIVITY (μmhos,25°C)	pH	ALKALINITY (m equiv l^{-1})	PO_4-P ($\mu g\ l^{-1}$)	NO_3N/NO_2-N ($\mu g\ l^{-1}$)
1	362	7.7	1.41	10.8	110.1
2	214	8.4	1.09	5.1	75.6
3	371	8.0	1.68	6.1	171.7
4	374	8.0	1.72	6.0	40.7
5	242	7.3	1.55	20.0	25.2
6	447	7.7	1.72	9.3	1320.5
7	486	7.8	2.01	5.2	687.8
8	442	8.1	1.91	6.9	103.7
9	371	8.3	2.19	8.3	38.0
10	337	7.9	1.16	9.7	65.0
11	322	8.1	1.78	4.7	34.5
12	397	8.1	1.62	5.5	741.1
13	437	7.9	2.12	3.8	437.2
14	396	8.0	1.50	4.4	28.4
18A	392	7.9	1.56	1.8	42.3
18B	364	7.8	1.27	3.0	58.7

LAKE	CONDUCTIVITY (μmhos, 25°C)	pH	ALKALINITY (m equiv l^{-1})	PO_4-P (μg l^{-1})	NO_3N/NO_2-N (μg l^{-1})
19	472	7.8	1.78	2.1	623.9
	508	7.9	1.78	3.2	865.0
21	505	7.8	1.88	4.5	757.9
21A	489	7.6	1.71	6.1	616.2

TABLE 3 Chemical characteristics of the Blackwater Valley gravel pits in 1984. Values are means of at least 3 determinations.

LAKE	CALCIUM (mg l^{-1})	MAGNESIUM (mg l^{-1})	SODIUM (mg l^{-1})	POTASSIUM (mg l^{-1})	BALANCE*	CHLORINE (mg l^{-1})
1	30.5	3.5	24.6	4.7	1.16	21.8
2	20.2	2.7	11.6	4.2	1.45	15.7
3	29.5	3.6	27.3	5.0	1.03	23.4
4	31.6	4.0	28.0	4.7	1.09	24.2
5	18.2	4.2	13.1	5.6	1.20	13.2
6	38.1	4.0	36.3	5.0	1.02	24.0
7	33.5	3.8	34.8	7.5	0.88	32.5
8	33.7	3.9	36.5	6.8	0.87	21.6
9	36.3	4.2	25.0	4.7	1.36	18.6
10	26.6	6.0	15.1	3.6	1.74	29.5
11	30.5	5.4	17.6	5.6	1.55	22.7
12	25.5	5.9	17.6	5.5	1.34	21.6
13	36.4	7.7	26.6	8.0	1.28	29.5
14	48.1	7.4	16.2	6.0	2.50	20.9
18A	30.8	6.6	19.6	7.4	1.39	20.8
18B	25.9	7.1	20.0	8.1	1.17	16.1

LAKE	CALCIUM ($mg\ l^{-1}$)	MAGNESIUM ($mg\ l^{-1}$)	SODIUM ($mg\ l^{-1}$)	POTASSIUM ($mg\ l^{-1}$)	BALANCE*	CHLORINE ($mg\ l^{-1}$)
19	37.3	7.1	33.3	7.8	1.08	25.4
20	38.9	7.6	35.3	7.4	1.09	23.7
21	37.0	7.3	27.7	7.7	1.25	25.5
21A	41.8	8.0	28.3	6.1	1.45	23.0

* expressed as $\frac{\text{Calcium + Magnesium}}{\text{Sodium + Potassium}}$

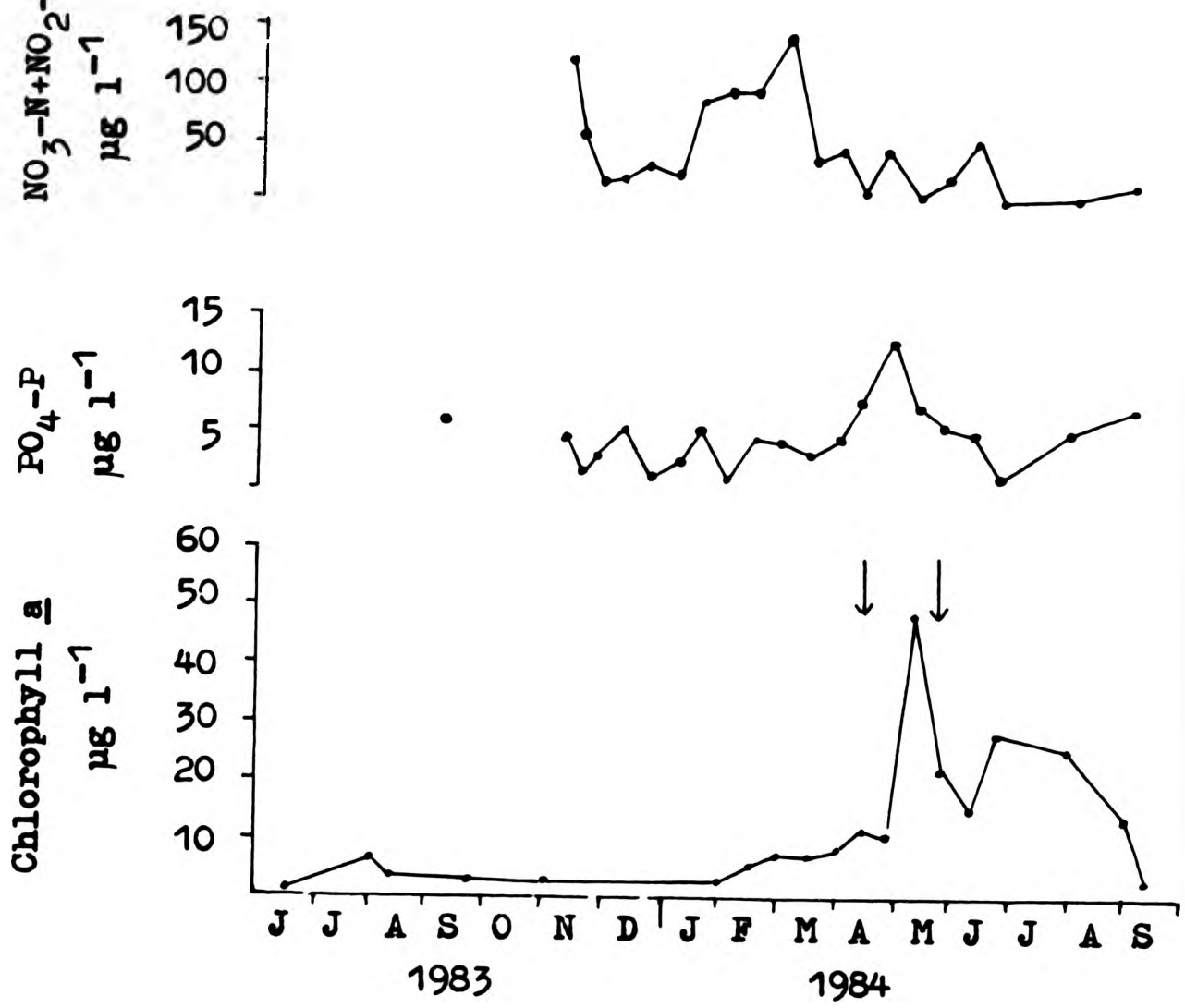


Figure 1. Seasonal changes in chlorophyll a, PO_4-P and NO_3-N + NO_2-N in Yateley 4 during 1983 and 1984. The arrows show the dates when phytoplankton productivity was estimated.

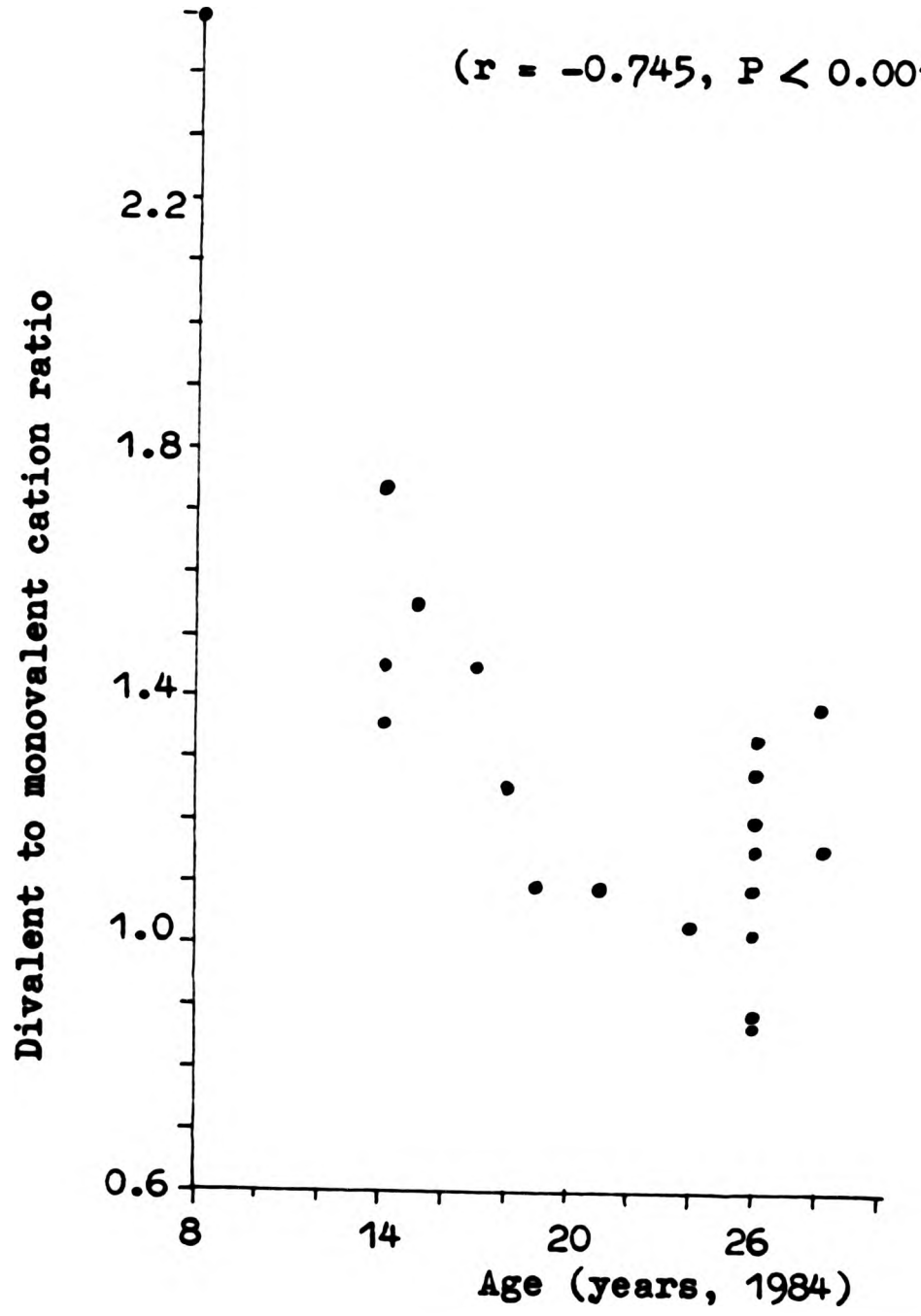


Figure 2. Relationship between age of flooded gravel pits in the Blackwater Valley and the ratio of divalent to monovalent cations.

$$\frac{Ca + Mg}{Na + K} = \text{Divalent : monovalent cation ratio}$$

APPENDIX 7. Reciprocal averaging ordination

Reciprocal averaging is an eigenvector ordination method similar to that of principal components, but differing in that ordination scores for species are averages of the sample (lakes) ordination scores and reciprocally, the sample (lake) ordination scores are averages of the species ordination scores (Hill, 1973b). Using an iterative procedure based on weighted averages, stable species and sample scores are usually achieved after 20-200 iterations, when there is no further change with further iteration. These scores represent the first axis of the ordination and are usually scaled from 0-100. The contraction in the range of species or sample scores in one iteration after convergence is reached is the eigenvalue for that axis. Successive axes can be generated by the same iterative method except that the scores are corrected for the first axis; a third axis may then be calculated whilst correcting for the first two axes and so on, for as many axes as are required. Most information is usually contained in the first few axes, and plots of the ordination axes scores as two dimensional scatter diagrams reveals the relationship between species or samples (lakes), those having similarities in distribution or species composition being clustered close together. More details of the method are given in Hill (1973b).

Program for reciprocal averaging ordination (after Orloci, 1978).

```
$ type rqt.bas
0010 PRINT "PROGRAM NAME--- RQT"
0020 REM--- THIS PROGRAM COMPUTES THE EIGENVALUE AND VECTOR
0030 REM ALGORITHM OF RECIPROCAL ORDERING WITH OPTIONS FOR
0040 REM ADJUSTMENTS TO GIVE WEIGHT TO SPECIES AND FOR
0050 REM CONVERSION TO SCORES IN RECIPROCAL AVERAGING.
0060 REM THE DATA ARE READ FROM DISK FILE RAWD WITH ELEMENTS
0070 REM ARRANGED AS R SETS OF C NUMBERS. R INDICATES THE NUMBER
0080 REM OF SPECIES AND C THE NUMBER OF QUADRATS. SPECIES SCORES
0090 REM ARE WRITTEN INTO DISK FILE SOMS AND THE QUADRAT SCORES INTO
0100 REM DISK FILE COMS.
0110 PRINT "======================================================"
0120 REM FILES RAWD, SOMS, COMS
0122 OPEN 'RAWD' FOR INPUT AS FILE #1
0124 OPEN 'SOMS' FOR OUTPUT AS FILE #2
0126 OPEN 'COMS' FOR OUTPUT AS FILE #3
0140 DIM X(21,21),R(21,21),B(21,21),T(21,21),Q(21)
0150 DIM C(21,21),S(21),O(21),D(21,21),U(21,21)
0160 MAT S=ZER
0170 MAT O=ZER

0190 REM---EXPLANATION OF ARRAY SYMBOLS:
0210 REM         X, D - R*C ARRAYS
0220 REM         T, C - C*R ARRAYS
0230 REM         R, B, U - R*R ARRAYS
0240 REM         Q, S - R-VALUED VECTORS
0250 REM         O - A C-VALUED VECTOR
0270 REM  ---READ DATA
0280 PRINT "NUMBER OF SPECIES R";
0290 INPUT R
0300 PRINT "NUMBER OF QUADRATS C";
0310 INPUT C
0320 PRINT "TYPE 1 IF PRINTING OF SCALAR PRODUCTS REQUIRED"
0330 PRINT "ELSE TYPE 0";
0340 INPUT Z2
0350 PRINT "TYPE 1 IF TRANSFORMATION TO RECIPROCAL"
0360 PRINT "AVERAGING SCORES REQUIRED ELSE TYPE 0";
0370 INPUT Z3
0380 IF Z3=1 THEN 450
0390 PRINT " IF COMMON OR RARE SPECIES ARE GIVEN HIGH WEIGHT"
0400 PRINT " TYPE 0,TYPE 1 IF NO WEIGHTING REQUIRED";
0410 INPUT Z4
0420 PRINT "IF RARE SPECIES ARE GIVEN HIGH WEIGHT"
0430 PRINT "TYPE 1 ELSE TYPE 0";
0440 INPUT Z5
0450 PRINT
0460 LET L=C
0470 LET N=R
0480 MAT B=IDN
0490 LET A=0
0500 FOR I=1 TO R
0510 FOR J=1 TO C
0520 INPUT #1,Q
0530 LET X(I,J)=Q
0540 LET D(I,J)=Q
0550 LET S(I)=S(I)+Q
0560 LET O(J)=O(J)+Q
```

```
0570 NEXT J
0580 LET A=A+S(I)
0590 NEXT I
0600 LET Q2=A
0610 FOR I=1 TO R
0620 FOR J=1 TO C
0630 LET X(I,J)=X(I,J)/SQR(S(I)*O(J))
0640 LET X(I,J)=X(I,J)-SQR(S(I)*O(J))/A
0650 NEXT J
0660 NEXT I
0670 MAT T=TRN(X)
0680 MAT R=X*T
0690 IF Z2=0 THEN 720
0700 PRINT "SCALAR PRODUCTS"
0710 MAT PRINT R;
0720 REM---EIGENVALUE AND VECTOR PROCEEDURE
0730 LET A=0.00000001
0740 LET C=0
0750 FOR I=2 TO N
0760 FOR J=1 TO I-1
0770 LET C=C+2*(R(I,J)^2)
0780 NEXT J
0785 NEXT I
0790 LET Y=SQR(C)
0800 LET O=(A/N)*Y
0810 LET T=Y
0820 LET D=0
0830 LET T=T/N
0840 FOR Q=2 TO N
0850 FOR P=1 TO Q-1
0860 IF ABS(R(P,Q))<T THEN 1170
0870 LET D=1
0880 LET V=R(P,P)
0890 LET Z=R(P,Q)
0900 LET E=R(Q,Q)
0910 LET F=.5*(V-E)
0920 IF F=0 THEN 950
0930 LET G=-(SGN(F))
0940 GO TO 960
0950 LET G=-1
0960 LET G=G*Z/(SQR(Z^2+F^2))
0970 LET H=G/(SQR(2*(1+SQR(1-G^2))))
0980 LET K=SQR(1-H^2)
0990 FOR I=1 TO N
1000 IF I=P THEN 1080
1010 IF I=Q THEN 1080
1020 LET C=R(I,P)
1030 LET F=R(I,Q)
1040 LET R(Q,I)=C*H+F*K
1050 LET R(I,Q)=R(Q,I)
1060 LET R(P,I)=C*K-F*H
1070 LET R(I,P)=R(P,I)
1080 LET C=B(I,P)
1090 LET F=B(I,Q)
1100 LET B(I,Q)=C*H+F*K
1110 LET B(I,P)=C*K-F*H
1120 NEXT I
1130 LET R(P,P)=V*K^2+E*H^2-2*Z*H*K
1140 LET R(Q,Q)=V*H^2+E*K^2+2*Z*H*K
1150 LET R(P,Q)=(V-E)*H*K+Z*(K^2-H^2)
1160 LET R(Q,P)=R(P,Q)
```

```
1170 NEXT P
1175 NEXT G
1180 IF D<>1 THEN 1210
1190 LET D=0
1200 GO TO 840
1210 IF T>0 THEN 830
1220 FOR I=1 TO N
1230 LET G(I)=I
1240 NEXT I
1250 LET J=0
1260 LET V1=0
1270 LET J=J+1
1280 FOR I=1 TO N-J
1290 IF R(I,I)>=R(I+1,I+1) THEN 1370
1300 LET V1=1
1310 LET V2=R(I,I)
1320 LET R(I,I)=R(I+1,I+1)
1330 LET R(I+1,I+1)=V2
1340 LET P=G(I)
1350 LET G(I)=G(I+1)
1360 LET G(I+1)=P
1370 NEXT I
1380 IF V1<>0 THEN 1260
1390 IF Z3=0 THEN 1420
1400 GOSUB 1730
1410 STOP
1420 FOR J=1 TO N
1430 IF R(J,J)<0 THEN 1560
1440 LET K=G(J)
1450 PRINT " EIGENVALUE";R(J,J)
1460 PRINT " SET",J,"OF SPECIES SCORES"
1470 FOR I=1 TO N
1480 IF Z5=0 THEN 1500
1490 LET B(I,K)=B(I,K)*(G2/S(I))
1500 IF Z4=0 THEN 1520
1510 LET B(I,K)=B(I,K)/SQR(S(I)/G2)
1520 PRINT B(I,K);
1530 PRINT #2,B(I,K)
1540 NEXT I
1550 PRINT
1560 NEXT J
1570 LET C=L
1580 MAT T=TRN(D)
1590 MAT C=T*B
1600 FOR J=1 TO R
1610 IF R(J,J)<0 THEN 1710
1620 PRINT " SET ",J," OF GUADRAT SCORES"
1630 LET K=G(J)
1640 FOR I=1 TO C
1650 LET G=C(I,K)/(D(I)*SQR(R(J,J)))
1660 PRINT #3,G
1670 PRINT G;
1680 NEXT I
1690 PRINT
1700 NEXT J
1710 GOSUB 2210
1720 STOP
1730 FOR J=1 TO N
1740 IF R(J,J)<0 THEN 2200
1750 LET K=G(J)
1760 FOR I=1 TO N
1770 LET B(I,K)=B(I,K)/SQR(S(I))
1780 NEXT I
```

```
1790 PRINT "   RANGE";R(J,J)
1800 PRINT "SET",J,"OF SPECIES SCORES"
1810 LET M1=10^20
1820 LET M2=-10^20
1830 FOR I=1 TO N
1840 IF M1<=B(I,K) THEN 1860
1850 LET M1=B(I,K)
1860 IF M2>=B(I,K) THEN 1880
1870 LET M2=B(I,K)
1880 NEXT I
1890 LET M3=M2-M1
1910 LET M5=1/M3
1920 FOR I=1 TO N
1930 LET U(I,K)=(B(I,K)-M1)*M5
1940 PRINT U(I,K);
1950 NEXT I
1960 PRINT
1970 GOSUB 2090
1980 PRINT "--OR--"
1990 PRINT "  SET",J,"OF SPECIES SCORES"
2000 FOR I=1 TO N
2010 LET U(I,K)=U(I,K)*R(J,J)
2020 PRINT U(I,K);
2030 PRINT #2,U(I,K)
2040 NEXT I
2050 PRINT
2060 GOSUB 2090
2070 NEXT J
2080 RETURN
2090 PRINT "  SET",J,"OF QUADRAT SCORES"
2100 FOR G=1 TO L
2110 LET G=0
2120 FOR I=1 TO N
2130 LET G=G+D(I,G)*U(I,K)
2140 NEXT I
2150 LET G=G/O(G)
2160 PRINT G;
2170 PRINT #3,G
2180 NEXT G
2190 PRINT
2200 RETURN
2210 IF Z4=0 THEN 2280
2220 PRINT "  CANONICAL CORRELATIONS"
2230 FOR J=1 TO N
2240 IF R(J,J)<0 THEN 2270
2250 PRINT "SET",J,"R(X,Y)=",SQR(R(J,J))
2260 NEXT J
2270 PRINT "GRAND TOTAL IN MATRIX D",G2
2280 END
$
```

APPENDIX 8.

Interactions between fungi

Discs of agar (6 mm diameter) bearing actively growing mycelium of test fungi were placed in pairs (3 cm apart) on potato dextrose agar containing 0.1 M sodium phosphate buffer, pH 6.65. Each fungus was paired with itself and 13 other isolates (see Table 1) to give 14 x 14 combinations. Plates were incubated at 12 °C for up to 3 weeks and the aggressiveness of each fungus scored using the method of Wicklow & Hirschfield (1979a) as given below:

INTERACTION	SCORE
Mutual intermingling of the 2 mycelia	0
Mutual inhibition on contact, space between colonies small but clearly marked	1
Mutual inhibition at a distance	2
Inhibition on contact, antagonist continues to grow unchanged, or at a reduced rate through colony of inhibited organism	3
Inhibition of one organism at a distance, the antagonist continues to grow through the resulting clear zone at an unchanged or reduced rate	4

TABLE 1. Interactions*1 within and between groups of fungi on potato dextrose agar.

FUNGAL SPECIES	A*2	B*3	C*3
a) LEAF FUNGI			
Aureobasidium pullulans (AB23)	0	0	0
Cladosporium cladosporioides (Yat 37)	0	2	3
Epicoccum purpurascens (Yat 15)	1	3	0
Alternaria alternata (Yat 23)	1	0	1
b) INTERMEDIATE FUNGI			
Phoma sp. (BW1)	4	3	0
Acremonium sp. (Yat 9)	0	0	1
Fusarium sp. 1 (Folk 1)	8	6	5
Fusarium sp. 2 (Yat F1)	8	8	12
Unknown sp. 1 (Yat G17)	0	4	0
c) INGOLDIAN FUNGI			
Tetracladium marchalianum (1A)	0	2	0
Tetrachaetum elegans (27A)	8	12	4
Anguillospora longissima (Dun 2)	4	6	2
Clavariopsis aquatica (ST1)	12	12	8
Articulospora tetracladia (VW1)	6	0	3

*1 The scores shown for a particular species are the total for interactions of that species with members of the same group and with members of the other groups. Eg., *Epicoccum* has a score of 1 with the species in its own group, A, a score of 3 with all the fungi in group B and 0 with those in C.

*2 Max. score for a species is 16 (4 x score of 4)

*3 Max. score for a species is 20 (5 x score of 4)

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END