A STUDY OF BACTERIA IN RELATION TO THE USE OF PAPER MILL BY-PRODUCT AS A CASING MEDIUM IN MUSHROOM CULTURE

Submitted for the degree of Doctor of Philosophy at the University of Aston in Birmingham

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SUMMARY

The gross ecology of a pulp and paper mill by-product (termed PMB) was studied in order to determine any changes, physical, chemical and biological which occurred following deposition, rendering it suitable for use as a casing medium in cultivation of Agaricus bisporus, the cultivated mushroom. PMB was most suitable after 18 months weathering. Artifical methods were explored to study the ageing process in isolation and find possible means of accelerating it.

Bacterial populations of the casing layer were studied in both PMB and peat casings and general trends were observed. Highest numbers of bacteria occurred at the onset of fruitbody formation and changes in colony types present at different stages in cropping were common to both casing media. A range of bacterial genera were isolated from PMB casing.

Quality of mushrooms from PMB casing was assessed and found to be superior to peat. Occurrence of a few diseased mushrooms necessitated pasteurisation with a steam:air mixture for one hour at 65°C to eradicate disease.

The relationship between the bacterial flora of compost and casing was examined and bacteria were found to migrate from compost into sterilised casing. Replacement of casing with fresh material at different stages could prolong cropping by removing a layer with high soluble salts and a bacterial flora inhibitory to fruiting.

Bacterial isolates from PMB casing soil were found to have positive and negative effects when interacting with pure cultures of A. bisporus, in axenic culture of A. bisporus and in normal artifical culture. Certain bacteria had stimulatory effect on fruiting and others inhibitory effects. Studies of specific properties of the bacteria indicated a link with the nutritional role of the bacteria in the casing, stimulatory cultures being able to solubilise and reduce ferri iron, solubilise phosphate and utilise ethanol.

Key words: Agaricus bisporus; Paper mill by-product; Casing layer; Bacterial ecology; Primordia stimulation.

A summary of the work submitted for the degree of Doctor of Philosophy by Patricia Ann Cresswell.

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1. INTRODUCTION

In the artificial culture of Agaricus bisporus (Lange) Sing., beds of compost when colonised with mycelium are cased or "capped" with a layer of soil or peat in order to induce the formation of the familiar mushroom fruit bodies.

The function of this casing layer has attracted much attention, but its function and the mechanisms involved in the switch from vegetative to reproductive growth are not fully known. It is generally believed that this layer of soil provides a number of conditions which are conducive to the formation of fruits. It is thought to provide (a) a reservoir of water which is used by the mycelium; (b) a suitable structure which allows gas exchange; (c) an adequate buffering capacity to maintain an optimum pH during culture and (d) a suitable medium for bacteria which are known to be associated with the developmental changes which are involved in the formation of mushroom fruitbodies.

Mushroom cultivators in this country use a sphagnum peat as the principal component of a soil for casing mushroom beds. It is neutralised by the addition of calcium carbonate as chalk or limestone before use.

In recent years there has been concern on the long term availability of peat. Estimates for reserves of peat range from 15-30 years and increasing costs of importing peat from Eire have contributed significantly to the escalation of costs in the commercial production of mushrooms.

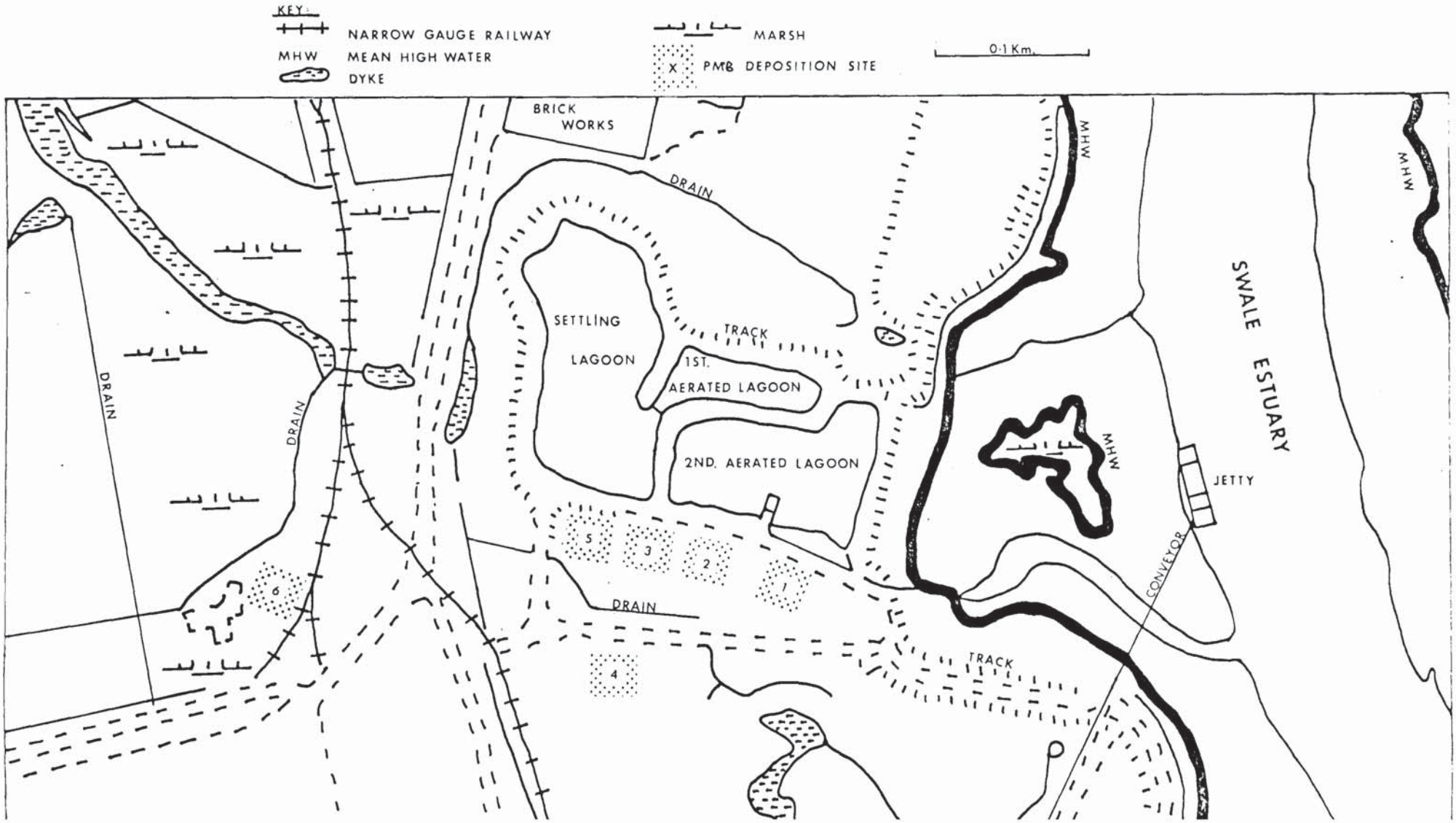
In some parts of the world where peat is not available, a loam soil is used after pasteurisation with steam. Soil however is highly variable in its performance as a casing medium.

The frequent occurrence of higher fungi growing on deposits of the solid effluents at the Bowater Paper Company (U.K.) Ltd., Kemsley, Kent, led to preliminary investigations by W.A.Hayes in 1974 and he demonstrated the possibility of using deposits which had been sufficiently weathered as a casing layer in mushroom culture. It was concluded that it offered the most satisfactory alternative to peat and probably could be processed to provide a product which was less variable than peat and conforming to given standards which could lead to improvements in the artificial techniques of mushroom culture.

The solid effluents at the Kemsley Mill (see site map) form part of the total effluent from the pulp and paper making process. When they are pumped from the mill, they are suspended in the liquid effluents which are contained in large open lagoons. The solids float to the surface and are skimmed off and deposited on nearby wasteland. After deposition the nature of the solids changes following further drainage and the influence of weather. Approximately 18 months after deposition, green plants establish.

The nature of the changes which occur after deposition have not been investigated and the objective of this study was to determine the gross ecology of the solid wastes, termed Paper Mill By-product (PMB), from the Kemsley Mill and the relevance to its possible use as an alternative casing medium in mushroom culture. In view of the known association between bacteria and the growth and developmental processes in fruitbody formation, detailed investigations were restricted to this group, with emphasis on factors likely to be relevant in the successful development of an improved casing medium.

Plan of Kemsley Mill site, indicating main areas of PMB deposition.



2. LITERATURE REVIEW

2.1.1. Effluents of the pulp and paper industry

A summary of the problems facing the pulp and paper industry due to mill effluent was detailed by Cawley and Minch (1963). They listed differences between the various production processes involved in the manufacture of paper and indicated that the waste varied with the quality of paper produced.

One of the main problems with the effluent is its high Biological Oxygen Demand (BOD). Warren and Doudoroff (1958) developed bioassays for toxicity determinations using the common guppy as a test animal. They determined the concentrations of pulp and paper mill wastes that would not harm aquatic organisms in the receiving waters.

Ayers and Patton (1971) set up a pilot plant to study biological treatment of integrated kraft pulp and paper effluents. They compared two high rate trickling filters packed with a PVC medium, an oxidation ditch with brush-type aeration and a lined earth lagoon with mechanical surface aeration. They found the most successful was the oxidation ditch, where removal of BOD above 80% was possible at test conditions and temperatures. A conventional aerated lagoon reduced BOD by 80% at relatively short retention times.

Servizi and Gordon (1973) studied aerated lagoons for reducing toxicity of the effluent from kraft mills. They concluded that biological treatment in an aerated lagoon was capable of detoxifying kraft pulp mill effluent adequately. They also found certain types of wood had residual toxicants that were resistant to biological detoxification.

Mueller and Walden (1974) also reported that aerated lagoons reduced toxicity but that there was variation in their efficiency. Adequate supplies of oxygen and nutrients, provision for good mixing and control of sludge were important factors governing the detoxification success rate of a biological system.

Leach, Mueller and Walden (1976) reviewed other types of detoxification systems. As well as aerated lagoons and activated sludge systems, they discussed the Unox process, an activated sludge system using oxygen instead of air. This removed more than 90% of BOD from bleached and unbleached kraft mill effluents in Canada at retention times of less than three hours. The Zurn-Attisholz process used two activated sludge processes in series but was inefficient.

The rotating biological disc system (RBS) consisted of closely spaced discs mounted on a drive shaft which was supported just above the waste, so that 40-45% of each disc extended into the waste. This last system was capable of greater than 80% removal of BOD from groundwood waste. The use of activated carbon in conjunction with either chemical or biological treatments was also mentioned .

2.1.2. Toxic Products

Rogers (1973) used young sockeye salmon as test organisms and found certain resin acids present that were toxic to these fish. Various heavy metals - copper and zinc did fall within toxic boundaries at certain times. They were not considered significant factors in acute toxicity of pulp mill effluent as the calcium levels were high and the toxicity of these elements decreased with the hardness of the water.

Leach, Mueller and Walden (1976) also reported on the diterpene resin acids, diterpene alcohols, and long chain fatty acids which are some of the toxic products in the effluent. The biological treatments used were fairly consistent in reducing toxicity levels to acceptable standards.

2.1.3. Possible Uses of Effluents

Wiley, Dubey and Hughes (1950) discussed the use of spent sulfite liquor (SSL) as a growth medium for a Torula type yeast. This yeast had been used as a supplement for human food and as an animal and poultry feed in commercial scale production since 1939.

Maloney and Robinson (1961) monitored the growth and respiration of a green alga, Chlorococcum macrostigmatum in SSL and found it to be capable of assimilating various sugars from the liquor.

Various by-products have been manufactured from spent sulfite liquor. Cohn (1966) noted three marketable forms: I. Raw liquor with 8-12% solids; 2. Concentrated liquor with about 50% solids; 3. Dried powder with 5% moisture. All of these could form source material for the production of valuable by-products. These included surface-active substances used as dispersants and adhesives, emulsifiers for making insecticides, waxes, tars, asphalts, adhesives for linoleum pastes, roadbinders and many other forms. Cohn also mentioned the utilisation of SSL for eventual human consumption, vanillin, vanillic acid and vanillates, preservatives for foodstuffs and for the control of moulds and fungi.

Tonseth and Berridge (1968) wrote about the possibility of converting waste sulphite liquors into animal feeds, in conjunction with other animal feed wastes, by protein precipitation.

Waste sludge from a kraft mill, when used as a crop amendment in experimental plots increased yield of grain (Yerkes 1971). At certain levels, sludge amendment additions to soils necessitated supplementary nitrogen additions for oat nutrition (Dolar, Boyle and Keeney 1972, Faulkender et al. 1970).

Sludge amended soils gave a higher yield of beans and corn (Aspitartet and Rosenfeld 1973). Sludge, with or without bark dust competitive results when used for establishing grass stands.

Guerri (1973) discussed the effectiveness of a spray application of a 10% solids sodium base concentrated waste on to land and reported this to be successful if carefully monitored.

Kosaric, Leduy and Zajic (1973) and Leduy, Kosaric and Zajic (1974) tried growing various species of Morchella on waste sulfite liquor.

Kosaric et al. (1973) used fermentation methods and worked out the optimal pH-range for growth of morel mushroom mycelium on waste sulfite liquor (WSL) Leduy et. al. (1974) studied three species of morels (Morchella spp. M. crassipes and M. esculenta) grown in WSL and compared the spectrum of the essential amino acids and found them comparable to the F.A.O. standard except for the levels of methionine and isoleucine.

2.1.4. Microbiological aspects of the pulp and paper industry.

Much of the literature in this field deals with the microorganisms involved in the infection of the groundwood pulp at the time of incorporation into the process and subsequent infections during the process of paper making. Penicillium roquefortii and Basidiomycetes can cause woodpulp deterioration (Russell 1957). Hughes (1957) detailed the different microorganisms present in the processing and effluent. These were mainly protozoa, bacteria, algae and various fungi which were not too specific in their light and water requirements. These microorganisms which passed out into the effluent, together with microbial nutrients, led to increased microbial activity and hence to a decrease in oxygen.

Use of percolating filters and activated sludge plants should have resulted in a transformation of available nutrients into microbial tissue which could then be collected in secondary settling tanks and disposed of as sludge (Hughes 1957).

Russell (1969) detailed the microbiology of pulp and paper at Bowaters Research and Development Company at Gravesend. He noted that bacterial reduction of sulphates occurred inside the salt-water stored pulpwood indicating that these organisms could be present in the area. Some of the bacteria identified from machine systems were Aerobacter, Escherichia, Alcaligenes, Pseudomonas and also the Gram positive types Bacillus subtilis and B. megaterium.

Starkey (1961) also noted the presence of sulphate reducing bacteria of importance in pulp wastes. These were anaerobic and pulp wastes discharged into streams and waste basins promoted the development of these bacteria. This resulted in the production of large quantities of hydrogen sulphide. Starkey only found three main types belonging to the genus Vibrio.

Enebo (1959) examined the bacterial decomposition of cellulose-containing sediments of dredge pulp from a Swedish fjord and showed that anaerobic decomposition proceeded very slowly in low temperatures prevailing at the bottom of the fjord. He also reported that dredge pulp appeared to have no practical influence on the BOD of the water.

2.2.1. Mushroom cultivation.

Artificial methods of mushroom culture were first developed in France in the early sixteenth century (de Tournefort 1707). Horse manure was left for a few months after it had been taken from the stables and was then stacked into beds. It was spawned with spawn made from manure and was then capped or cased with a soil. By about 1800, the French were growing mushrooms underground in quarries. Horse manure continued to be used as a base for compost and spawn was dug from meadows where horses had been trampling, the appearance of any mushrooms was purely a matter of chance.

Although mushrooms were initially grown in caves and quarries, mushroom houses were first described by Callow (1831). He described a house in which mushrooms could be grown on a flat bed on the ground and/or or shelves, one above the other on brackets attached to the walls. The house was heated by fire heat.

Atkins (1972) and Hayes and Nair (1975) described the adoption of the standard American growing house in the 1920's. The buildings of wood were of a standard size and based on the shelf bed system.

With well insulated walls they allowed controlled temperature, humidity and aeration all the year. Since the Second World War, the tray system was developed with the bed being split up into movable tray units and this is now widely adopted by the mushroom industry in America and Europe. The most recent development is the use of disposable polythene sacks as growing units. These are easily disposed of and are useful in confining and restricting the spread of disease.

A major improvement in the techniques of mushroom culture occurred with the perfection of spawn production from mushroom tissue (Duggar 1905), thus making it possible to select and guarantee a particular strain instead of using spawn dug from areas where mushrooms were known to grow.

The technique of pure culture spawn production was improved in the U.S.A. Spores from selected mushrooms were germinated under sterile conditions and were then injected into a bottle of sterilised compost and incubated at 21^{o}C. When the compost was fully colonised, the bottle was broken and the contents were used as spawn.

Lambert (1933) reported that spawn makers for the previous thirty years had tended to make multisporous cultures to produce more vigorous spawn, and reproduce both favourable and unfavourable characteristics of the parent strain.

Spawn is now produced by Sinden's method (Sinden 1932), which used grain as a base. Water and chalk were added and the grain sterilised and inoculated with a pure culture of the mycelium. When the grain was fully colonised, it could then be added to the compost, its granular nature making it easier to mix with the compost. The compost to which the spawn is added is generally based on horse and wheat straw. Synthetic composts have been developed by Sinden (1938, 1946), Stoller (1943) and Edwards (1950).

The main purpose of composting is to provide a suitable medium for the dominant growth of mushroom mycelium, increase the nitrogen content. The heat produced promotes growth of the thermophilic organisms and destroys many pests and pathogens. Turning the compost allows good aeration and mixing to get a uniform product and the alteration in the physical nature makes it easier to pack it into trays or shelves (Atkins 1972).

After packing into trays, the compost is allowed to heat again (the peak heating or pasteurisation stage). This drives off excess moisture and kills off fungal pathogens and pests.

After the compost has cooled and inoculated with spawn, it is kept at 25-28^{o}C for the spawn to colonise the compost. After about ten days, when the compost is fully colonised a 5cm deep casing layer is applied to the surface of the bed and in England, consists of peat neutralised with chalk. Loam soils are still used in America. After casing, the temperature is maintained at 25^{o}C until the mushroom mycelium has reached the surface, when it is lowered to 16-18^{o}C and the growing house is ventilated.

Approximately 12 days after casing fruit initials are formed and develop into primordia. Some of these develop into the characteristic mushroom fruits which normally are fully formed at about 21 days from casing and are harvested. Fruitbodies form in periodic flushes at about weekly intervals and beds are cropped for approximately six weeks before disposal.

2.2.2. Microbiology of substrates.

1. The compost.

Waksman and his colleagues in the 1930's studied the microbiology of composted manure and the composting process. Waksman and Allen (1932) discussed the importance of temperature on the microbiological population and the interaction between bacteria, fungi and actinomycetes. Waksman and McGrath (1931) and Waksman and Nissen (1932) also found that the mushroom, Agaricus campestris, used the lignins and proteins of the compost as its main food source, unlike most common soil fungi and bacteria.

Fergus (1964) and Stanek (1968) documented some of the fungi and actinomycetes found in composts. Stanek (1972) also reported on bacteria growing in the immediate environment of the mushroom hyphae which were not present in the rest of the compost and termed this region the "hyphasphere", analogous with the rhizosphere of higher plants.

Hayes (1969) and Laborde et al. (1969) monitored the changes in populations of bacteria, fungi and actinomycetes in composting.

Hayes (1969) studied the mesophilic and thermophilic populations of bacteria, fungi and actinomycetes through the various stages of composting, recording both qualitative and quantitative changes. He also worked on the addition of sucrose to the compost at day 7 and found numbers of bacteria increased and numbers of actinomycetes declined as compared with the reverse situation in unsupplemented compost.

Laborde, Delmas and d'Hardemare (1969) studied the microbiological balance in the compost with the intention of composting in controlled atmospheric conditions where optimal biological, chemical and physical requirements could be controlled. They reported that the work of Hayes and Randle (1968) and Hayes (1969) showed that the addition of sucrose favoured the functional groups of microorganisms responsible for the protein transformation of various forms of nitrogen at the expense of proteolytic bacteria and fungi.

Stanek (1969) studied the effect of cellulose decomposing organisms on the growth of mushrooms. He found that filtrates from cultures of typical representatives of the thermophilic cellulose decomposing microorganisms stimulated growth of the mushroom mycelium and many of the cellulose decomposing actinomycetes produced various vitamins which also stimulated the growth of the mushroom mycelium.

Stanek (1972) summarised the succession of microbial colonisation in the mushroom compost as described by Hayes (1969), Laborde et al. (1969) and Stanek (1969). Imbernon and Leplae (1972) also recorded the levels of fungi, bacteria and actinomycetes, both thermophilic and mesophilic and noted various general trends.

The importance of thermophilic bacteria in the fermentation of mushroom compost was investigated by Chanter and Spencer (1974). They used revolving drums (Randle and Hayes 1972) for compost preparation instead of the traditional stack, the procedure of Hayes and Randle (1969), being used for the preparation of the stack. They found numbers of thermophilic bacteria to be higher in drum composting whilst numbers of mesophilic fungi were lower and concluded that the shorter composting time needed in drums was due to the higher numbers of thermophilic bacteria.

Smith and Spencer (1977) examined the use of high energy carbon sources in rapidly prepared mushroom composts and concluded that the absolute quantities of soluble carbon and nitrogen which were utilisable by the compost microflora appeared to be more significant than the ratio of total carbon to total nitrogen.

2. The casing layer

Much of the early work on the casing layer was done by Edwards and Flegg in the 1950's. This included work on water-holding capacity and pore space (Edwards and Flegg 1953, Flegg 1954) and casing depth (Edwards 1954). Further work in this field was by Bels Koning (1950), Stoller (1952 a, b, c) and Flegg (1956). The effect of pH was studied by Bels Koning (1950). De Kleemaeker (1953), Flegg (1956), Allison and Kneebone (1962) and Park, Kim, Park and Kwak (1971). Edwards and Flegg (1953) also studied watering, as did Reeve, Backes and Schramer (1959), Flegg (1965 and 1974) and Edwards (1974). Other early work covered the areas of pore space and gas exchange (Lambert 1933, Bels Koning 1950, Tschierpe 1959 and Long and Jacobs 1969). It was obvious from all this work that carbon dioxide did exert some influence over fruitbody formation but that it was not the only factor involved (Park and Agnihotri 1969b).

The importance of microbial factors was mentioned by Eger (1961, 1965), Urayama (1961), O'Donoghue (1962) and Thomas, Mullins and Block (1964). Work by O'Donoghue (1962) indicated a possible importance of actinomycetes as stimulators of fruitbody formation.

Hayes, Randle and Last (1969) implicated Pseudomonas putida in fruitbody formation. Hume and Hayes (1972) confirmed this work using a two-phase petri-plate method to demonstrate the stimulatory ability of P.putida. Eger (1972) summarised the work on bacteria and sporophore initiation and also confirmed the action of P.putida in stimulating fruitbody formation. Urayama (1961) implicated a Bacillus species in stimulating sporophore formation, confirmed by Urayama (1967). Park and Agnihotri (1969a) tested a variety of bacteria and found several causing fruitbody formation. Curto and Favelli (1972) also examined the effect of yeasts, microalgae and bacteria upon fruitbody initiation and yield. They found increased numbers of fruitbodies were produced by spraying with these organisms. A supplementation of microalgae to spawned compost resulted in a yield increase of about 50%. A further study on fruitbody formation by Smith and Hayes (1972) using various autoclaved substrates to examine nutritional requirements for fruitbody formation as a means of bridging the gap between the Petri-plate technique of Hume and Hayes (1972) and the practical situation. Additions of suspensions of P.putida to their system resulted in increased numbers and weight of harvested mushrooms.

Hayes (1972) extended studies on the nutritional role of the casing layer and investigated the effect of various trace elements on primordium formation. Highest numbers of primoria were formed at iron levels of 200ppm. applied as ferrous sulphate, lower numbers being formed at lower levels of iron addition. Below 50ppm there was no primordium formation and above 200ppm there was a gradual decrease in numbers of primordia produced. Hayes (1972) suggested that P.putida and other stimulatory organisms could stimulate fruiting by releasing Fe^{2+}.

Angeli-Couvy (1972) also concluded that micro-organisms were essential for fruiting but postulated two theories for their action. Either they could transport an essential substance for fruiting to the mycelium, or they could partly or totally eliminate certain metabolic inhibitors emitted during mycelial growth. Her work involved the use of activated charcoal to eliminate (fixation by adsorption) metabolic products that could interfere with fruiting. Couvy (1976) suggested that activated charcoal might stimulate fruiting by elimination of inhibitory substances produced by the mycelium.

To this extent the work supported the ideas of Mader (1943), Schisler (1957) and Stoller (1952d) with the absorption of one or several inhibitory volatile metabolites produced by the mycelium of A.bisporus. The fruiting of A.bisporus with the addition of activated charcoal was as prolific as with microorganisms.

As both carbon dioxide and microbial activity have been implicated in fruitbody formation, work to link these two theories was done by Nair and Hayes (1974). They added one of the breakdown products of carbon dioxide (the bicarbonate ion as sodium bicarbonate), to the casing soil. Numbers of pseudomonads in the casing soil were counted at various time intervals after casing and significant increases were noted in samples to which bicarbonate additions had been made.

Maximum numbers were found at day 10 after casing. Various isolates of pseudomonads were then tested for an ability to reduce soluble and insoluble forms of iron following the work of Hayes (1972). They used the technique of Ottow and Glathe (1971), using iron in the fairly insoluble form of ferric oxide and the relatively soluble form of ferric chloride.

Summarising, they stated that the addition of the bicarbonate ion and the addition of spawned compost or inert glass fibre strands to the casing produced significant effects both on the initiation of sporophores and on the pseudomonad population, especially those associated with the solubilisation and reduction of iron. They visualised the possibility of a biological selection of microorganisms and chemical ions in a casing medium.

Hayes (1974) worked in more detail on the bacterial population of the casing layer and followed the changes in numbers of bacteria through the cropping cycle, using a variety of compost denisities. Bacterial numbers increased with compost density and appeared also to affect the growth characteristics of the crop, resulting in a shorter time to first picking and a greater weight and number of mushrooms. Comparisons between conventional trays and polythene sacks showed greater microbial activity and productivity from the polythene sacks. The sacks under study necessitated increased compost density as compared to the usual commercial situation and hence a more concentrated accumulation of volatile by-products in the casing layer as there was no means of escape as in the conventional tray system.

Nair and Hayes (1975) added compost colonised with A.bisporus mycelium to the casing with resultant earlier fruiting and increased yield. This effect was explained as an interaction between activity of pseudomonads in the casing and the aeration within that layer, this idea being supported by similar results obtained with additions of glass fibre to the casing.

A further study by Hayes, Short and Nair (1976) examined the proportions of the different types of bacteria at different times after casing and again reported on the effects of amendments to the casing of spawn run compost, woven glass fibre and stone gravel. All three amendments produced an increase in numbers of bacteria in the casing and increased production of mushrooms, the greatest effect being with the addition of spawn run compost and the least with stone gravel. They tested the effect of various metabolic by-products of the mushroom mycelium (as identified by Lockard and Kneebone 1962) on a selected bacterial isolate from the casing soil. Some were inhibitory, whilst carbon dioxide and to some extent, ethanol were stimulatory. Lockard (1967) surveyed the situation of volatiles from the mycelium and their role and indicated the line which further research could take.

In 1976, Nair, Short and Hayes again stressed the importance of the effect of the bicarbonate ion on bacteria in general and on the iron reducing pseudomonads in particular. They suggested an adaptation of the gaseous environment could be helpful in devising a mechanical means for harvesting mushrooms.

2.2.3. Pests and Pathogens

Within both compost and casing, the mycelium of Agaricus bisporus has to contend with a number of pathogens and competitors. Kneebone and Merek (1961) wrote a short general paper detailing the main problems encountered by the growing mushroom and control measures for most of them. Atkins and Atkins (1971) also wrote a general paper on mushroom pests and diseases. The majority of diseases described by Kneebone and Merek are fungal but there is mention of bacterial blotch and bacterial pit, the former caused by Pseudomonas tolaasi and the latter of unknown origin. Other diseases of bacterial origin are Drippy Gill disease, caused by P. agarici (Young 1970) and Mummy disease caused by a Pseudomonas species (Schisler, Sinden and Sigel 1968 a.).

Blotch disease was first described by Tolaas (1915) who identified the causal organism as a bacterium of the genus Pseudomonas. Paine (1919) described the occurrence of a similar disease in England. He described it as affecting the cap only and not affecting the underlying tissue below about ½mm and isolated the causal organism as a bacterium of the genus Pseudomonas. The similarity between Paine's bacterium and that of Tolaas led him to the

conclusion that they were the same and the name Pseudomonas tolaasi was proposed.

Due to the difficulties in the classification of and the fact that Pseudomonas tolaasi could only be distinguished by its pathogenicity to Agaricus bisporus, work on devising a specific test was begun. Lelliot, Billing and Hayward (1966) failed to distinguish P. tolaasi from other fluorescent pseudomonads and suggested that it was possibly a normal constituent of the mushroom bed microflora that produced a toxin against mushrooms in certain conditions. Gandy (1967) showed that pathogenicity was not lost by repeated subculturing on to synthetic media and disease symptoms could still be readily produced. Sciarid flies were possible vectors of the disease and it was certainly more prevalent in warmer, more humid weather, when evaporation was low. In 1968, Gandy devised a screening technique for P. tolaasi. Stoller (1958) advocated the use of hexachlorophene as an effective control against bacterial blotch. Nair and Fahy (1973) supported the idea of toxin production and suggested the presence of a substance of low molecular weight, which was heat stable, that also inhibited mycelial growth of the mushroom. In 1972, Nair and Fahy tried to isolate bacteria antagonistic to bacterial blotch for use in controlling the disease.

Three bacteria were isolated which appeared to reduce infection when applied to mushroom trays but were ineffective in vitro. Nair (1974) discussed the possibilities of biological control of the disease and viewed the prospect favourably. Nair and Fahy (1976) tested a range of substrates into which to inolculate the antagonistic bacteria and found that gamma irradiated peat was successful, giving a reasonable control of the disease and increased yields of 6-8% in some cases.

Bacterial blotch has also been reported on the cultivated mushroom grown in Taiwan (Hsu and Chen 1974), where again high humidity and temperature generally favoured the disease, although it did occur at low temperature and humidity. They also reported that the disease developed in stored, apparently healthy mushrooms.

Drippy gill is a disease which has only recently been described (Young 1970, O'Riordain, (1972), Lelliott, Bateson and Baker 1972). Young described both the disease symptoms and the nature of the causal organism - Pseudomonas agarici. This disease shows a pitting of the cap as well as exudation of bacterial slime from the gills, likening it in some ways to the weeping disease described by Wood (1950, 1951, 1952).

There is an overlapping of symptoms with these diseases and also with "wet mummy" as described by Tucker and Routien (1939), Wood (1950) attributed the cause of weeping disease to Bacterium cartovorum but in 1951, he described the formation of internal pits, such as were found with drippy gill. In 1952, Wood also considered the effects of Bacillus polymyxa, a soft rot organism. When applied to mushrooms it caused a rotting of the tissue and he suggested it could also cause spotting of the cap.

"Mummy disease" and "Watery Stipe" are two other diseases in dispute. Tucker and Routien (1939) described wet and dry forms of mummy and Kneebone (1959) itemised the symptoms and attempted to identify the causal organism. The possibility of mummy and watery stipe and various other disorders being due to virus was suggested by Gandy (1962). However, Schisler et al (1968, a, b, 1969) identified the causal organism as being a bacterium of the genus Pseudomonas. The cause of watery stipe (Gandy 1959, Storey and Lester 1959) is still unknown, there is a possibility it could be physiological or just another effect of one of the other diseases. Where the cause of some of the other diseases is unknown, it is suspected that they may be caused by viruses. Gandy (1962) speculated

on the causes of Die-back in mushrooms which may have come under the names of mummy disease, La France disease, Brown disease, Watery Stipe and X-disease. The range of symptoms varies greatly often with bent and/or elongated stipes, waterlogging of the tissues. Gandy (1962) reported on the presence of virus particles in infected sporophores, showing the symptoms of La France disease, Watery stipe and X-disease, although Kneebone, Lockard and Hager (1962) described X-disease as being distinct from watery stipe. It is now generally accepted that this complex of diseases is caused by viruses, Last, Hollings and Stone (1967), Dieleman (1969) and Nair (1972).

Often the disease and pest problems encountered on farms are caused by inadequate peak heating of compost and poor cleaning of houses after cropping. This problem extended to the various pests in mushroom cultivation. Eelworms have been regarded as a pest for many years, some being fungal feeders and directly attacking the mycelium, such as Ditylenchus myceliophagus, or the more commonly found Rhabditis species. Rhabditis lambdiensis was identified as a possible vector of bacterial disease by Steiner (1933).

The presence of pests and disease organisms has drawn more attention to the pasteurisation or fumigation of a casing soil. When soil was used as a casing medium, the need for sterilisation was obvious but when peat/chalk was introduced on a large scale, sterilisation or pasteurisation was less widely used. Lambert and Ayers (1957) detailed the thermal death times for various mushroom pests and found that most were destroyed by moist heat at 130°F for sixteen hours, 140°F for six hours or 150°F for four hours. Dry heat was needed for a longer period for the same success. Steam treatment of the casing medium to produce temperatures above 65°C for 15-20 minutes was reported to be satisfactory for an adequate kill by Cairns and Thomas (1950). Most mushroom flies, mites and nematodes could not survive moist heat of 130°F for a few hours (Lambert and Ayers 1957). The main flies that are a problem are the sciarids (Lycoriella species), phorids (Megaselia species) and cecids (Heteropeza species and Mycophila species) (Atkins 1972), these latter being a particular problem as the larvae reproduce by paedogenesis so that the rate of reproduction is rapid. It is the larvae of all the flies that are the destructive stages, as they burrow into the casing and eat the mycelium and can also attack developing fruitbodies. Heteropeza pygmaea was reported as a vector of pathogenic bacterium (Hussey, Read and Hesling 1969), which fitted the description of P. agarici (Young 1970).

Hayes (1971) reported on the benefits of using methyl bromide as a method of fumigation as it struck a balance between the destruction of pathogens and pests and the preservation of beneficial organisms such as P. putida and other Group IV pseudomonads implicated in fruitbody formation (Hayes et al. 1969). One of the main difficulties being that the beneficial organisms and pathogenic bacteria were closely related pseudomonads.

The use of aerated steam for treatment of casing soils was advocated by Aldrich, Wuest and McCurdy (1974) who used a treatment of 140^{o}F for thirty minutes. The problem encountered in the treatment of soils in this way were detailed by Baker and Fuller (1976), especially the difficulty in getting an even distribution of steam uniformly through a container.

2.3. Relevance of the literature to this study.

The literature on paper mill effluents is concerned mainly with the toxicity and disposal of liquid effluents into natural waterways. Comparatively few studies have been done on the utilisation and disposal of the solid effluents after deposition. Toxicity factors are not known to be relevant to solid wastes and since fungi and plants eventually colonise deposits after weathering, their utilisation in agriculture and horticulture is worthy of exploration.

It is apparent from the literature, that the processes which govern the formation of fruitbodies are affected by a wide range of factors. However, the involvement of bacteria in the fruiting process and also in the occurrence of diseases of the mushroom crop suggest that their activity in the casing layer is particularly relevant in the possible use of paper mill wastes in commercial mushroom culture.

3. MATERIALS AND METHODS

3.1. Sampling Methods.

3.1.1. PMB from Kemsley Mill.

Samples of PMB deposited for $1\frac{1}{2}$, 3, 6, 12, 18 and 36 months were removed using a soil auger with 5cm diameter sample tubes. These sample tubes were sterilised before use by autoclaving at 121°C for 15 minutes. A sample tube was driven into the sludge to remove material from the upper 15cm and a second sample tube was used for a sample from 30-45cm depth. No samples were taken below this level. A 9 litre sample of sludge was collected as it was being deposited after removal from the settling lagoon. The samples were stored at 2°C until analysed.

In addition, larger samples sufficient to fill a 5 litre sealable plastic box were collected at each site. These were dug with a spade and were then stored at ambient temperature until analysed for nematodes and other fauna.

3.1.2. Casing samples.

A sterilised No.4 cork borer was used to take a sample through the casing soil in a growing box which was transferred to a sterilised universal bottle. Three samples were taken for each growing box or replicate unless stated otherwise.

3.1.3. Compost samples

Compost in a box or flask was carefully tipped out onto a sterilised surface and samples were removed at the required depths using sterilised forceps before transference to a sterilised universal bottle.

3.1.4. Estimates of bacterial populations and composition.

A 1g portion of the sample was transferred to 100ml of sterile phosphate buffered saline (PBS. see appendix I) solution. This was shaken for five minutes before serial tenfold dilutions were aseptically prepared in 9ml aliquots of sterile nutrient agar (Oxoid). For each dilution, eight x 0.1ml. aliquots were plated using the Colworth Droplette (Sharpe et al. 1971) and incubated for 24 hours at 18°C before counting. (See plates 3.1 and 3.2.)

For composition studies, the excess agar from the serial dilutions was poured into a Petri-plate and incubated at 18°C for forty eight hours before an estimate of colony types was made. The bacterial colonies were broadly classified on a colour basis using a dilution plate with no more than 200 colonies. Type A were cream colonies, type B - yellow, type C - orange, type D - white, type E - red, type F - pink and any different colonies were classed as other types. The percentage of each colony type on the plate was recorded. (See plate 3.3).

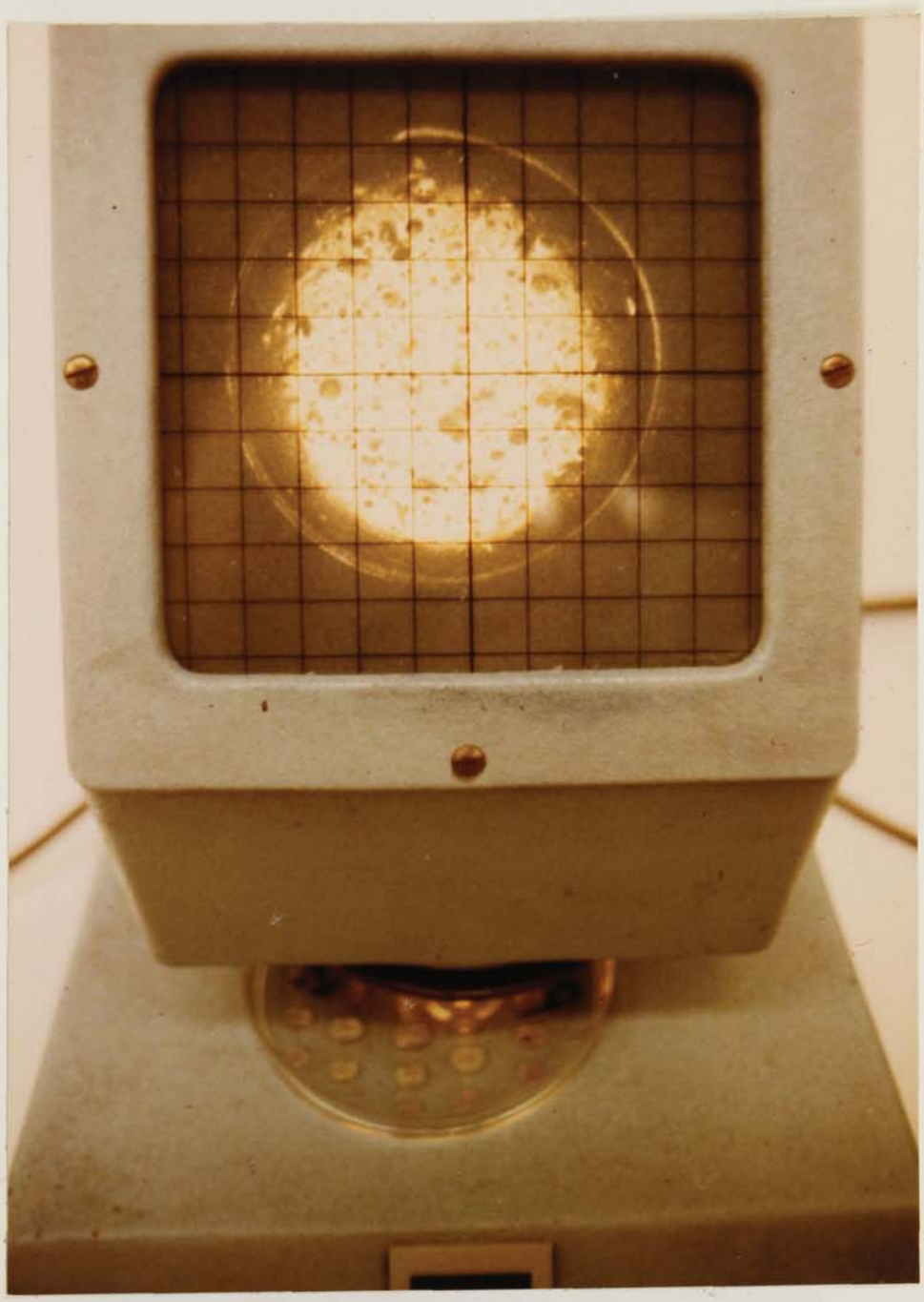


Plate 3.1. Colworth droplette with plate of agar droplets illuminated for counting.

Plate 3.2. Agar droplets feady for counting.

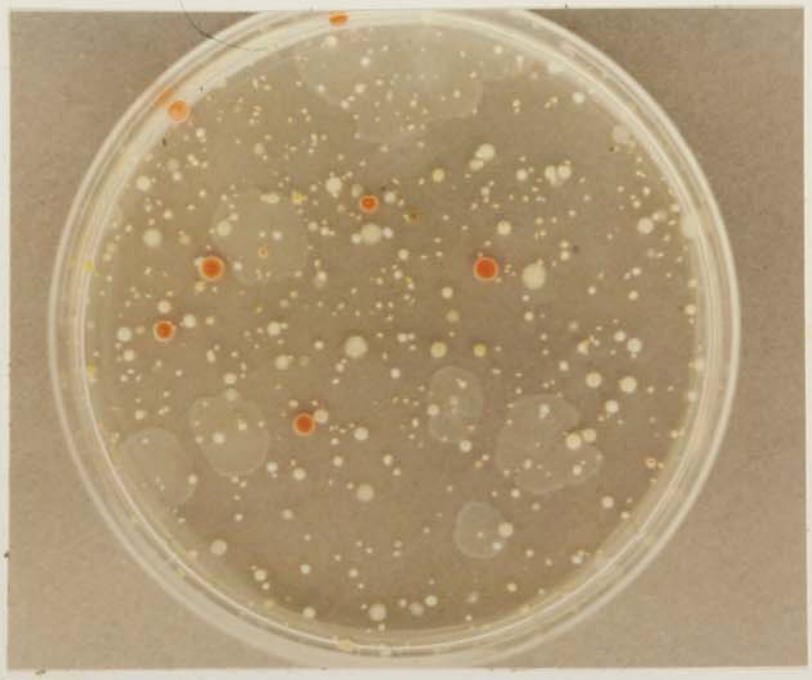


Plate 3.3. Pour plate for composition studies showing colony diversity.

For the estimation of pseudomonad populations, replicate serial dilutions were prepared in sterile PBS. 1ml of each was then pipetted into a Petri-plate and 15ml of sterile molten pseudomonad specific agar (King's B agar with the addition of antibiotics according to Sands and Rovira 1971) - see appendix I . These plates were incubated at 18°C for forty eight hours and colonies counted.

3.1.5. Subculturing and identification.

Cultures that differed in morphology, either in colour or colony form were subcultured on nutrient agar (Oxoid) and were stored at 2°C. All cultures were examined under oil immersion for shape and were tested for the Gram reaction (Lillie 1928).

Tests according to the first and second stage classification of Cowan and Steel (1970) were then applied to the cultures.

First stage tests.

(1) Catalase test Topley and Wilson 1929)

A few drops of 3% H_2O_2 were placed on a colony of the test culture. A positive reaction was indicated by bubbles of oxygen being given off.

(2) Oxidase test (Kovacs 1956)

A few drops of oxidase reagent (1% tetramethyl-p-phenyldiamine aq. solution) were placed on a colony of the test culture. A positive reaction was indicated by the development of blue colouration.

(3) Growth in air.

All cultures were isolated from aerobic dilutions.

(4) Motility (Craigie 1931)

Inoculation of the test culture was made into a central tube of sloppy (1% nutrient Oxoid) agar, after incubation at 18°C for 24 hours samples were taken from the outer tube and plated

on nutrient agar. Further samples were taken at daily intervals for one week. Samples of motile organisms grew on the plates indicating the organism had migrated from the central tube through the agar.

(5) Production of acid from glucose and oxidation and fermentation of glucose (O-F test) (Hugh and Leifson 1953).

Tubes of Hugh and Leifson's media were prepared - appendix 1 - and a sterile solution of glucose was added to give a final concentration of 1%. A Durham tube for collection of gas was placed in one tube and a second tube was covered with a layer of sterile liquid paraffin to prevent air reaching the medium. Two such tubes were prepared for each replicate. Both tubes were inoculated with a loopful of the test bacterium and were then incubated at 18°C for one week, tubes being examined daily. Change in the colour of the indicator from purple to yellow indicated the production of acid. Any gas produced could be readily detected in the Durham tube. Oxidisers showed acid production in the open tube only, fermenters showed acid production in the paraffin covered tube and usually also in the open tube.

Second stage tests.

(1) Growth at 42°C, 37°C and 5°C.

Cultures in nutrient broth (Oxoid) were placed in incubators at the specified temperatures for one week and the growth was noted.

(2) Liquefaction of gelatin (Frazier 1926)

Tubes of gelatin agar (Oxoid) were prepared and inoculated with a loopful of the test organism. The tubes were inoculated at 18°C for two weeks. The extent of liquefaction compared with the control was noted.

(3) Growth in MacConkey broth (MacConkey 1908).

Tubes of MacConkey broth (Oxoid) were prepared and a Durham tube placed in each tube. A positive effect was indicated by a colour change from purple to yellow and turbidity of the medium as compared with uninoculated control.

(4) Reduction of nitrate to nitrate with gas production (ZoBell 1932).

Nitrate broth - appendix I was prepared and a Durham tube was placed in each tube which was then inoculated with a loopful of a test organism and incubated at 18°C for one week. Gas production could be detected in the Durham tube. 1 ml of reagent A (0.8% sulphanilic acid in 5N-acetic acid) and 1 ml reagent B (0.5% naphthylamine in 5N-acetic acid) were added to each 5ml media. A red colour indicated the presence of nitrite showing that reduction of the original medium had occurred. In absence of colour 20mg powdered zinc was added, development of red colour indicated the presence of nitrate showing no reduction had occurred, absence of colour indicated nitrite was not present and the nitrate in the original medium had been reduced to nitrogen gas.

(5) Reduction of nitrite to nitrogen gas (ZoBell 1932)

Nitrite broth - appendix 1 was prepared and inoculated with a loopful of a test organism and incubated at 18°C for one week. Reagents A and B were added as in (4) with the presence of a red colour indicating the presence of nitrite and hence no reduction, absence of colour denoted a positive reaction.

(6) Utilisation of citrate (Koser 1923)

Tubes of Koser's citrate - appendix 1 - were inoculated with a loopful of the testorganism and incubated for one week at 18°C with a daily examination for turbidity. Turbidity indicated utilisation of citrate and positive cultures were confirmed by subculturing on to Koser's citrate.

(7) Utilisation of citrate (Christensen 1949)

Slopes of Christensen's media - appendix 1 - were inoculated by stabbing the butt and drawing the wire over the surface of the slope. Tubes were incubated for one week and examined daily for colour change. A magenta colour indicated utilisation of the citrate, yellow colour, no utilisation.

(8) Utilisation of starch (Skerman 1959)

Petri-plates of starch agar - Appendix 1 - were poured and then inoculated with a streak of the test culture. After one week's incubation at 18°C a few drops of iodine solution were poured on the plate. A blue colour indicated the presence of starch and a negative reaction.

(9) Utilisation of various carbohydrate sources (Hugh and Leifson 1953).

Tubes of Hugh and Leifson's media - Appendix 1 - were prepared. A sterile solution of the appropriate carbohydrate was added to give a final concentration of 1% carbohydrates used were sucrose, lactose, mannitol, dulcitol and arabinose. Durham tubes were placed in the tubes for detection of gas and a positive reaction was shown by a colour change from purple to yellow.

(10) Methyl red/Voges Proskauer reaction (Barritt 1936).

Tubes of glucose-phosphate medium - Appendix 1 - were prepared and inoculated with a loopful of a test culture. Tubes were then incubated at 30°C for 5 days.

(a) Methyl red test

2 drops of methyl red solution were added and tubes shaken and examined; they were kept for the Voges-Proskauer test. A positive reaction was shown by a red colour due to acid production from glucose. A yellow colour indicated a negative reaction caused by condensation of the pyruvic acid to form acetylmethyl-carbinol and a rise in pH.

(b) Voges-Proskauer test

After completion of the methyl red test, 0.6ml -naphthol solution (5% -naphthol in absolute ethanol) was added and 0.2ml 40% KOH aq. solution was added, tubes were shaken and then sloped and examined after 15 minutes and 1 hour. A strong red colour indicated a positive reaction caused by conversion of acid to acetylmethyl-carbinol which was then oxised to diacetyl by the addition of alkali.

Further specific tests were also done.

(1) Iron solubilisation and reduction. (Ottow and Glathe 1970).

Glucose-asparagine broth was prepared - appendix i - and iron was added in one of two forms, either in the relatively insoluble form of ferric oxide or as the relatively soluble ferric chloride. The broth was dispensed into tubes which were then sterilised.

Tubes were then inoculated with a loopful of a test organism and incubated at 18°C for 5 days. Each tube then received 1 ml of a 0.2% 2,2'-dipyridin solution in 10% acetic acid. A red colour against a pale pink control in the broth was regarded as a positive test for soluble ferrous iron.

(2) Phosphate solubilisation.

A basal medium was prepared (Collins and Lyne 1970) - appendix 1 - the components were dissolved and the medium dispensed in 50ml volumes in acid washed 100ml conical flasks. 0.05g calcium dihydrogen orthophosphate was added to each flask which was then plugged with cotton wool and sterilised at 121°C for 15 minutes. The inoculum was prepared by mixing a loopful of the test organism with 10ml sterile deionised water in a universal bottle using a Fisons Whirlimixer for 10 seconds. A loopful of this was then added to each flask.

One flask of each test organism was prepared and 13 uninoculated control flasks were also prepared, all of which were incubated at 25°C for 12 days. This was centrifuged at 5000 rpm for 10 minutes to remove the bacterial growth and residual solid.

The supernatant was collected into an acid washed polythene bottle. The pH of the remaining ten control flasks was adjusted with IM HCl and IM NaOH to give a pH range between 5.9 and 2.0, these were then treated as the test flasks. The pH of the remaining 25ml in each flask was then determined. The extracts were stored at $4^{o}C$ until phosphate determination. The total phosphate (as phosphorus) in the extracts was then determined by Auto Analysis using the molybdenum blue procedure (Allen et al. 1974), with an ammonium molybdenate-sulphuric acid reagent and stannous chloride as the reducing agent. The resultant molybdenum blue colour complex was determined spectrophotomically.

A graph was prepared using the control tubes showing the effect of pH alteration on the phosphate concentration in solution. The results of the test cultures were then compared and scored as follows:-

(a) phosphate concentration in solution greater than in unamended control tubes (with no pH alteration) and greater than expected by change in pH of the medium.

(b) phosphate concentration in solution greater than in unamended control tubes but less than would be expected due to change in pH of the medium.

(c) phosphate concentration in solution the same as in unamended control tubes.

(3) Ethanol utilisation.

Skerman's basal salts medium (Skerman 1959) was dispensed in 10ml volumes into acid washed universal bottles and sterilised at 121°C for 20 minutes. 0.1ml filter sterilised ethanol was added. A second series without ethanol was also prepared. The inoculum was prepared as for phosphate determination. Three tubes of each culture were prepared in the thanol utilisation medium and a fourth tube was also set up with the ethanol free medium as a control. The tubes were then incubated at 25°C for 7 days and then scored for growth by turbidity of the medium as compared to the control.

(4) Utilisation of bicarbonate as a sole carbon source.

Skerman's basal salts medium as prepared (Skerman 1959) with a solution of calcium bicarbonate added to give a final concentration of 1%, the solution was warmed slightly to dissolve the calcium bicarbonate.

The medium was dispensed into 10ml volume into acid washed universal bottles. A second series was prepared with no bicarbonate addition as a control. The bottles were sterilised at 121°C for 20 minutes. Tubes were inoculated as for phosphate determination and were incubated at 25°C for 7 days when they were scored for growth by turbidity of the medium as compared to the control. Tubes were further incubated for another 7 days and 0.1ml of 0.1M HCl was added to each tube to dissolve the calcium bicarbonate/carbonate precipitate to facilitate observation of growth. The tubes were then scored again for growth compared to the control tubes.

Determination of thermal death points of isolates.

Nutrient broth (Oxoid) was prepared, dispensed into 10ml volumes in Universal bottles and sterilised at 121°C for 15 minutes. Each tube was then inoculated with a stab from a bacterial colony. Three tubes were prepared for bacterial isolate. Theses cultures were then incubated at 18°C for twenty four hours. A loopful of a culture was streaked onto an agar plate to establish all cultures were viable. Cultures were then incubated at the required temperature in a water bath for one hour. A loopful of the culture was removed and steaked onto an agar plate. All plates were incubated for twenty four hours at 18°C to determine whether the cultures had been killed by the heat.

This procedure was done at 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, 80°C, 85°C and 90°C and thermal deaths were defined as one hours exposure to certain temperature.

3.1.6. Inoculation of selected isolates into casing soils.

In order to study the action of a selected bacterium in a casing soil, sterilised casing was inoculated with a pure culture of a bacterium.

A known weight of PMB was placed in a 1 litre flask and was stoppered with non-absorbent cotton wool. The flask was then sterilised by autoclaving at 121°C for thirty minutes on three consecutive days. (This was to ensure that any spores could have survived the first autoclaving and then germinated would be killed by a subsequent treatment). The flask was then tested for sterility before inoculation with 25ml of a twenty four hour broth culture of the test isolate. The flask was then incubated at 21°C for seven days to allow colonisation by the bacterium before application as a casing material.

3.1.7. Nematode extraction.

100g of PMB was placed in a double layer of muslin in a filter funnel to which a piece of rubber tubing with a clip was attached (Baermann funnel - after Peters 1955, see figure 3.1.) The funnel was filled with water to the level of the bottom of the sample. The nematodes were able to pass through the muslin and fall to the bottom of the funnel. The water was drawn off after twenty four hours by releasing the clip and the nematodes were counted directly in a Petri-plate using a binocular microscope. More water was put in the funnel and drawn off at twenty four hour intervals to ensure the extraction of all free living nematodes.

3.1.8. Extraction of other fauna.

An adaptation of the Tullgren funnel (after Peters 1955, see figure 3.2) was used for extraction of insect larvae and adults (Tipulidae and Chironomidae) amd various annelids. A 250g sample was placed in a layer of muslin on top of the autosegregators. A 40 watt. light was placed directly over the sample to act as a heat source to encourage the downward movement of the fauna. They were collected in 70% alcohol.

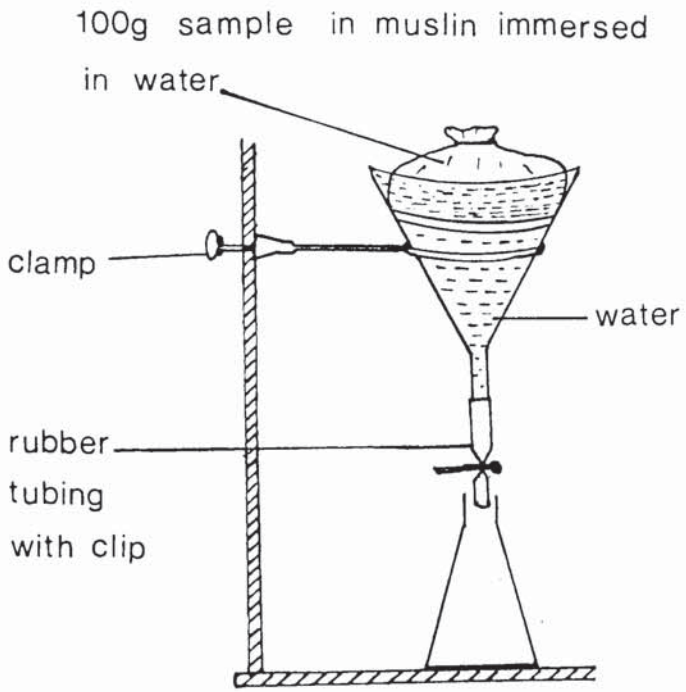


Figure 3.1 Baermann funnel for extraction of nematodes

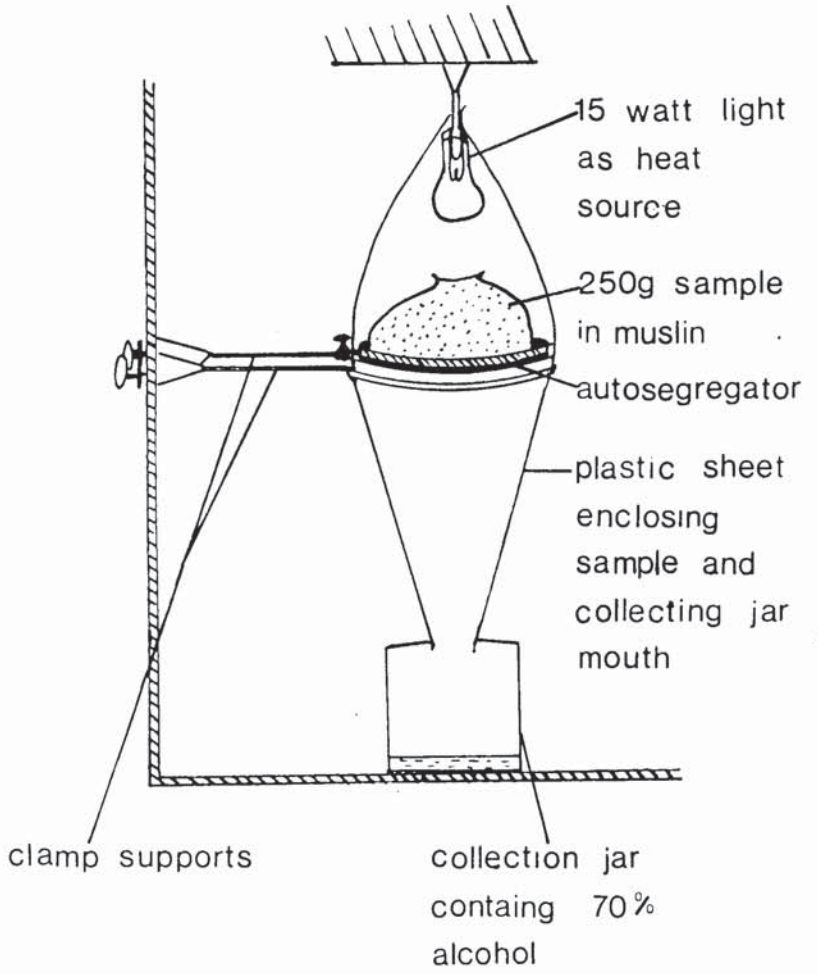


Figure 3.2 Adaptation of Tullgren funnel for extraction of fauna

3.2. Simulated ageing techniques.

PMB from Kemsley Mill was collected immediately after removal from the settling lagoon in 50 litre containers. These were taken to the laboratory where they were tipped into specially prepared funnels (See figure 3.3.) These funnels were 70 cm in diameter, holding 70cm depth of material. The base diameter where the drainage pipe was connected was 10cm. Funnel 1 contained 125kg sludge on a drainage filter 3cm thickness prepared from equal parts of bark and clinker from the Kemsley site. Funnel 2 contained 115kg sludge on an inert plastic support, drainage occurring through holes made with a number 4 cork borer. Funnel 1 contained slightly more sludge as the liquid drained so rapidly when the funnel was being filled that a greater volume could be added. These funnels were kept on the laboratory roof and the drained liquors were collected in separate graduated containers.

3.3. Mushroom culture.

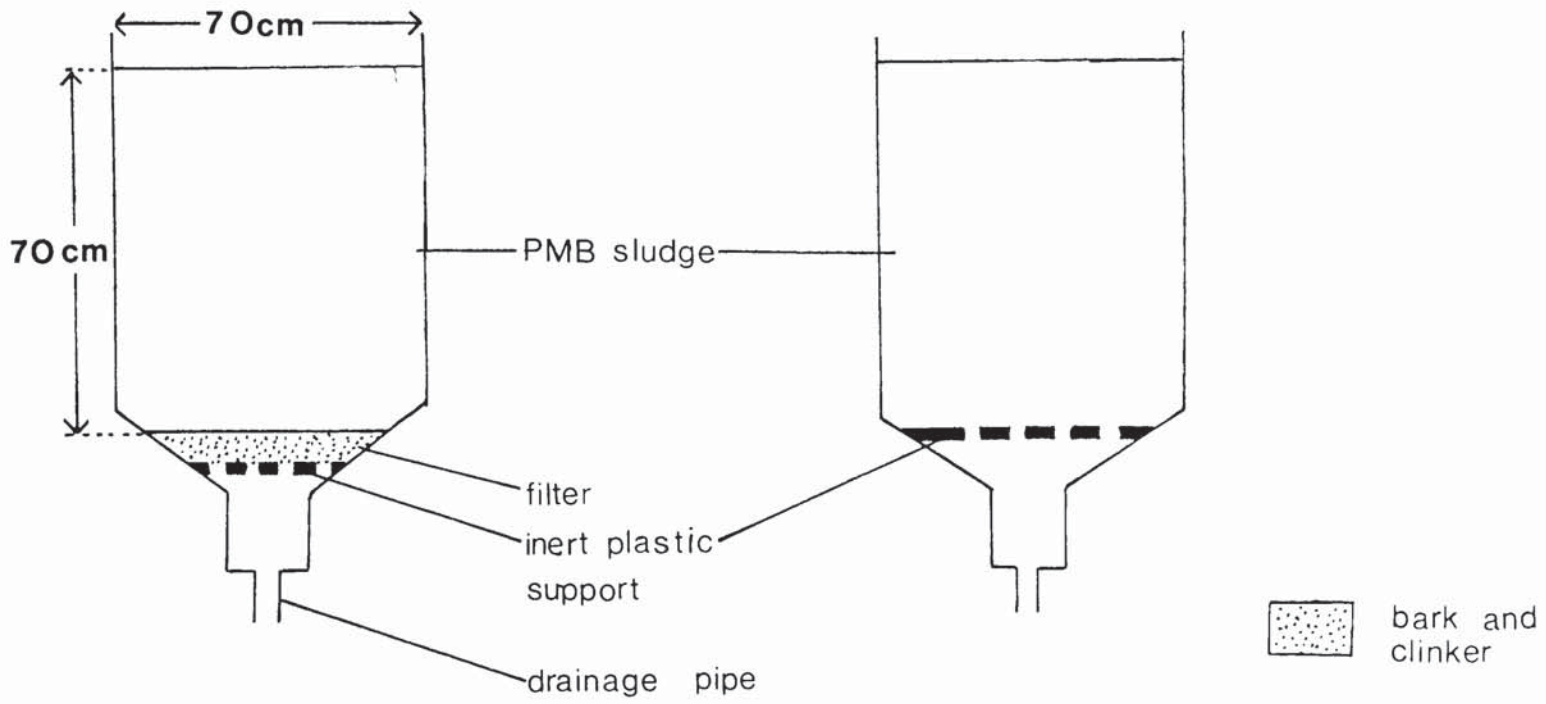
3.3.1. General techniques.

All growing experiments were done in growth cabinets where temperature, humidity and aeration could be controlled, except for the flask experiments.

The cabinets could hold eighteen large (18 x 18cm^2) boxes or thirty-two small (13 x 13cm^2). (See plate 3.4)

Figure 3.3

Isolation funnels for simulated ageing study.



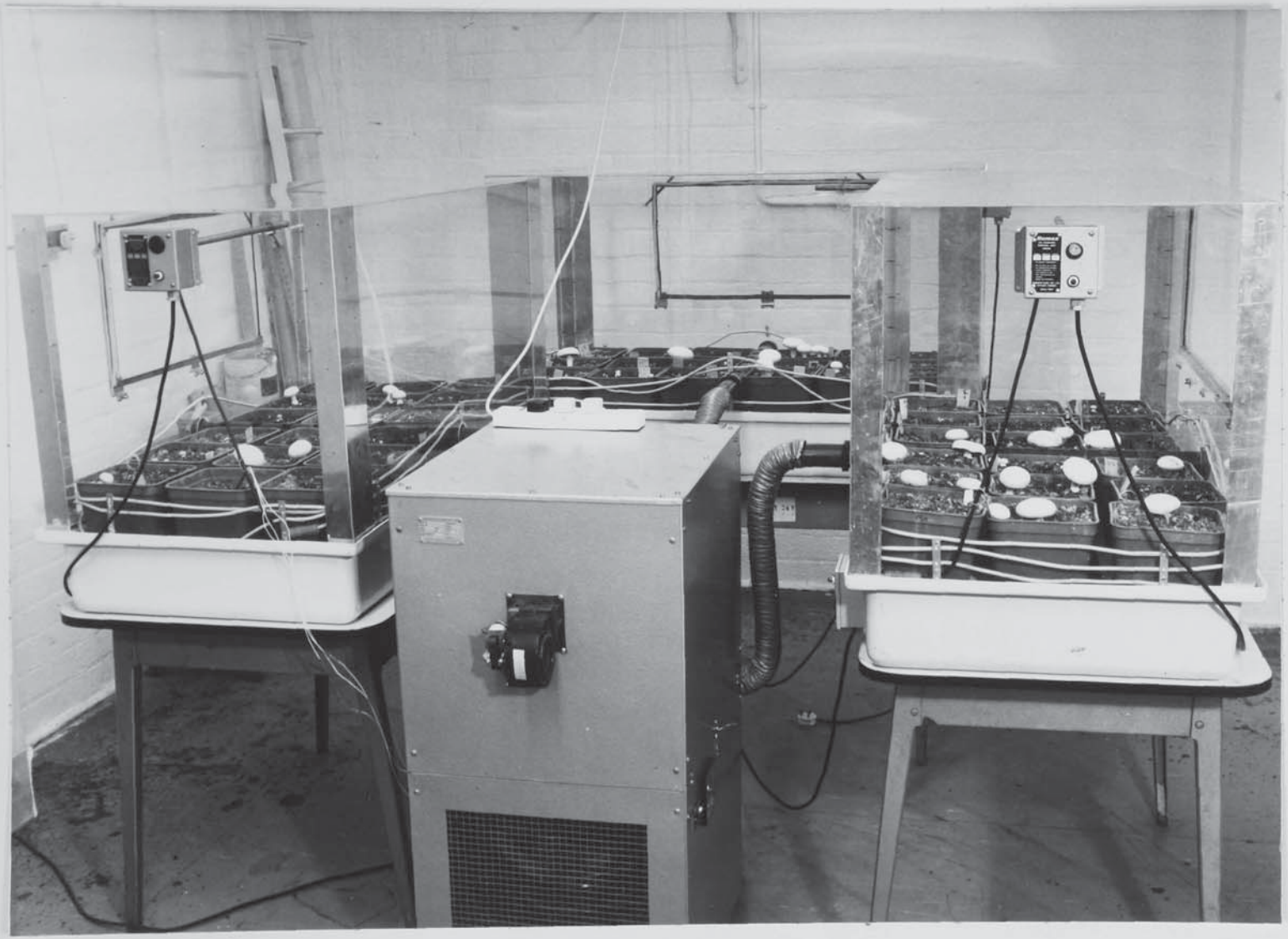


Plate 3.4. Growth cabinets for controlled humidity, aeration and temperature.

Compost was obtained commercially either after peak-heating or after spawning. Peak-heated compost was spawned in the laboratory with Darlington 649 strain (unless stated otherwise), at the rate of 1% by weight of fresh weight of compost and was incubated at 25°C until the compost was completely colonised. The compost was then packed into boxes which were placed in the growing cabinet. The temperature was kept at 25°C to allow the mycelium to regenerate.

The boxes were then cased with PMB to an even depth (2.5 cm). This was sieved to give 10mm-dust particle size, pasteurised at 65°C for sixty minutes, allowed to cool and chalk was added (Morden R) to give a pH of 7.0-7.5 (7:1 PMB:chalk by weight). Peat and chalk casing was prepared from a medium grade sphagnum peat wetted to give about 70% moisture and chalk added to obtain a pH of 7.0-7.5.

The boxes were placed in the cabinet set at 25°C. After 8-10 days, when the mycelium had grown through to the surface, the temperature was lowered by passing cool air into the cabinets. Pinheads could usually be seen 14-15 days after casing and the first mushrooms were generally harvested at the cup stage. At this stage the mushrooms were generally about 5 cm in diameter and the veil just broken.

They were brushed clean of any casing material, the minimum of base was removed to leave a clean stipe and the mushrooms were then weighed to the nearest 0.1g.

3.2.2. Special techniques.

(a) Open box culture.

The boxes used were 13 x 13cm^2, containing 500g compost with 200g casing or 18 x 18 cm^2 containing 1 kg compost with 300g casing. After filling, the compost was tamped down in the box to give an even density of compost with a level surface so that an even depth of casing could more easily be applied.

(b) Sterile flask culture.

200g compost was packed into 1 litre, flat bottomed, boiling flasks. The compost was tamped down flat and the flask was stoppered with non absorbent cotton wool in which were two L-shaped glass tubes. The exposed ends of the tubes were stoppered with cotton wool and sealed with autoclave tape. The flasks were then autoclaved at 121^{o}C for thirty minutes on three consecutive days. Random samples of compost were aseptically removed and plated out on nutrient and malt extract agars (see appendix I) to test for sterility.

The compost was then spawned with sterile grain spawn prepared from a pure culture of A. bisporus (strain Darlington 649). The rate of spawn inoculation was increased to 4g per 100g compost because growth was more difficult to establish than in open box culture. The flasks were then incubated at 25°C for about 18 days until the mycelium had grown through the compost. The flasks were then cased with inoculated casing prepared as in section 3.1.6. 150g. casing were added per flask. After about 6 days at 25°C the mycelium had grown into the casing and the flasks were aseptically connected in series to a sterile air source (see plate 3.5). The system was checked daily to ensure each flask received adequate air. The appearance of the flasks was noted daily and primordia formation was recorded.

3.4. Quality assessment.

3.4.1. Estimate of whiteness.

The whiteness of the cap at harvesting was measured by an EEL reflectometer head connected to an EEL Unigalvo (MacCanna and Gormley 1969). The instrument had a measuring aperture of 8mm diameter with a light spot diameter of 6mm. The Unigalvo was calibrated from 0:100 with a standard magnesium carbonate block.

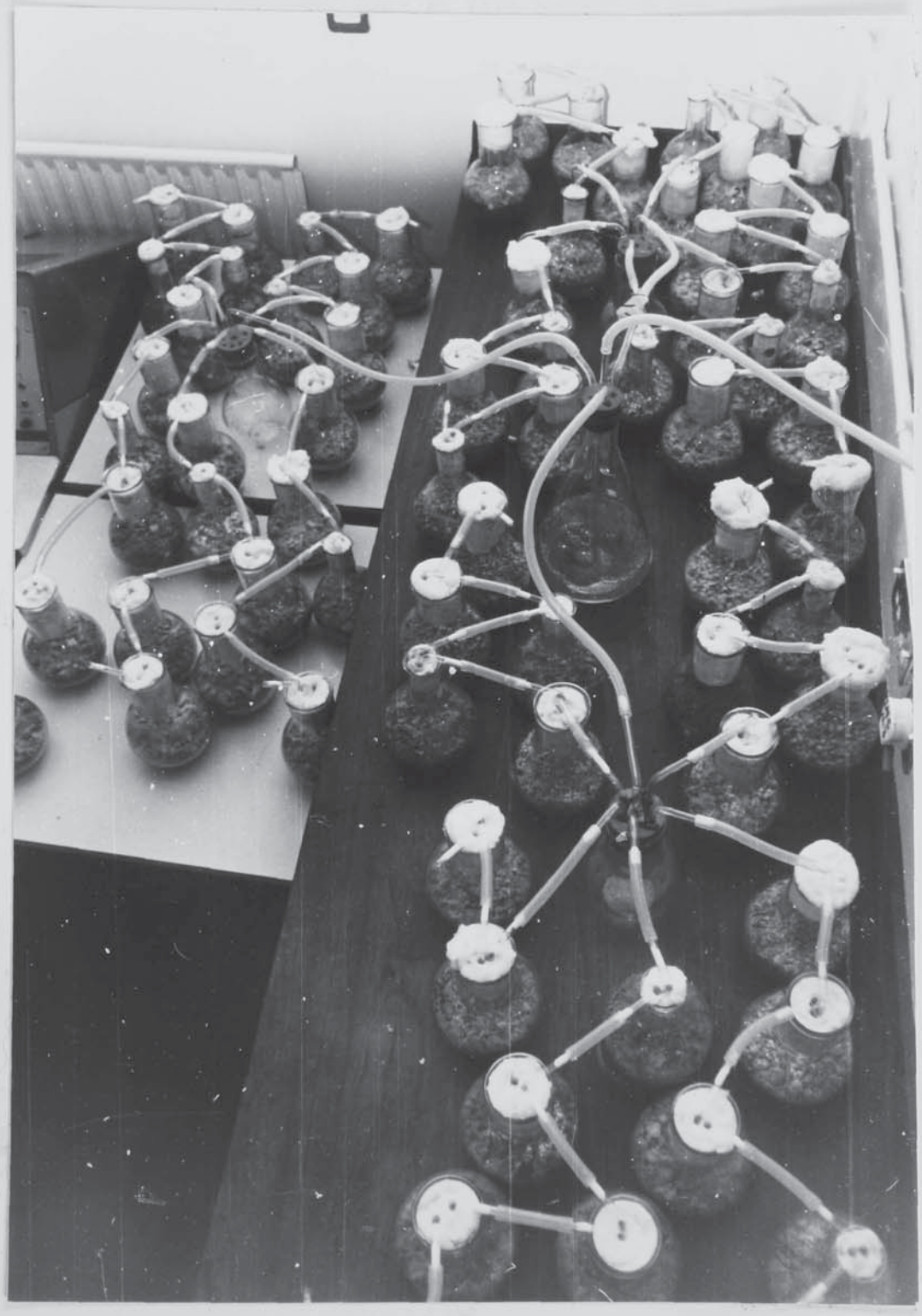


Plate 3.5. Sterile flask culture system.

Measurements were made at five points on the mushroom cap, immediately on harvesting, four around the side and one in the centre, providing the mushroom had a sufficiently flat cap surface.

The adherence of soil on harvesting and occurrence of blotch were measured on an arbitrary scale. The percentage of cap surface covered by soil at harvesting was estimated (100%, 75%, 50%, 25%, 10%, 5%, 1% or 0%) and the number of mushrooms from each treatment within these groups was recorded. Mushrooms were also categorised as having no blotch, slight blotch (1% cap affected) or bad blotch (25% cap affected).

After weighing, individual mushrooms were placed on weighed foil boats in an oven at 65^{o}C for 5 days until no further decrease in weight could be detected.

3.4.2. Screening of pathogens.

(a) Tissue blocks.

This was based on a method described by Gandy (1968), where the bacteria are screened for their effect on mushroom tissue. It was a useful method for isolating organisms capable of producing blotch symptoms when sprayed onto growing mushrooms. A block of mushroom

tissue 1 cm cube was cut from the centre of a mushroom cap using sterilised instruments and aseptic techniques. The block was carefully placed on a sterile microscope slide in a Petri-plate. A moistened filter paper in the Petri-plate prevented the block from drying out. Three blocks could be placed on a slide. A few drops of twenty four hour broth culture of a test isolate was put on the block. The Petri-plates were incubated at 21^{o}C for twenty four hours. Isolates capable of causing bacterial blotch produced a browning of the tissue. Control blocks of tissue, inoculated with sterile water or sterile nutrient broth, remained white.

(b) Inoculation into mushroom tissues.

Reinfection with organisms causing Drippy Gill was obtained by mechanically injecting a suspension of the organism into a developing mushroom with an intact veil (Young 1970). A hypodermic syringe with a fine sterile needle was used for the injection.

This technique was used with various points of inoculation to see if the bacteria would pass through the mushroom tissue. Mushrooms were injected either through the cap or stalk. Some mushrooms were injected through the mycelium and casing soil at the base of the stalk.

After two days, the mushrooms were sectioned through the point of the injection and examined. The extent of tissue invasion by the test organism was recorded.

3.5. Pasteurisation of PMB.

PMB sieved to 10mm particle size was placed in the metal bucket of the apparatus shown in figure 3.4.

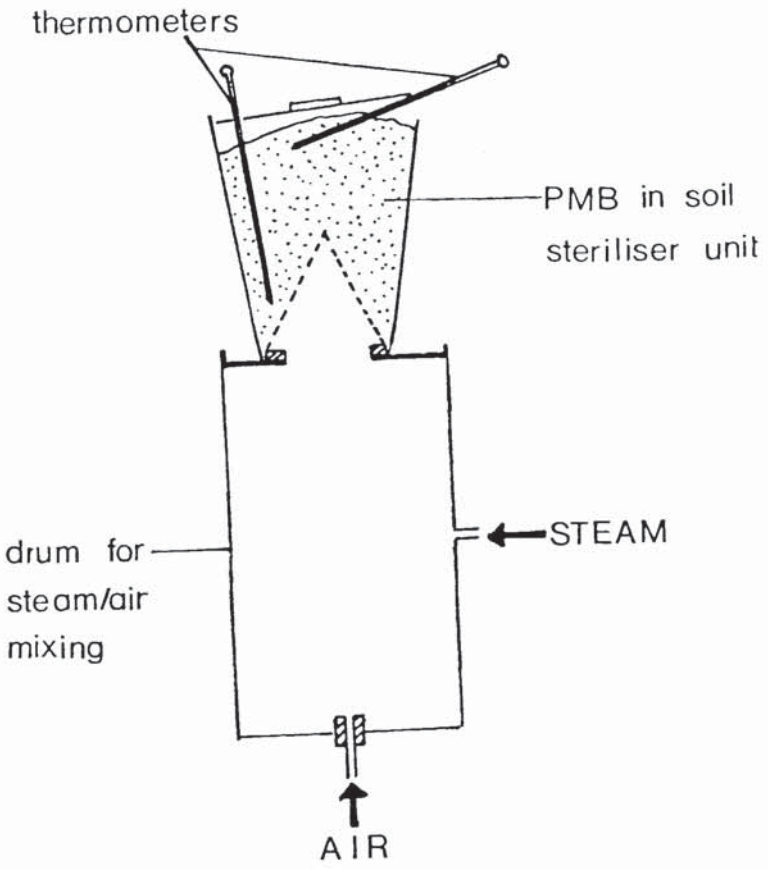


Figure 3.4 T.S. Soil steriliser unit as used for pasteurisation of PMB

The bucket with a perforated domed base fitted over a drum into which steam and air were passed. The steam was provided by a portable autoclave and the air came from

a direct line to the laboratory. A rubber gasket around the upper open end of the drum ensured a good fit when the bucket was in place. Thermometers were placed at six points to check that the temperature distribution was even. Steam was passed into the drum and forced upwards by the air flow. A temperature of 65°C was attained within ten minutes and was regulated by the manipulation of the steam air flow. The material could then be pasteurised for the desired time and temperature.

3.6. Petri plate techniques.

These techniques were used to study the interaction between A. bisporus mycelium and selected isolates in pure culture.

(a) Single phase method.

This technique was modelled on the method of Hume and Hayes (1972). Discs of A. bisporus were taken from a fourteen day pure culture using a number 4 cork borer and plated out on 2% malt extract agar (Boots Pure Drug Co.). The fourteen day cultures had been grown at 25°C and the discs were taken midway between the central inoculum and the growing edge of the colony. The inoculated plates were incubated at 25°C for fourteen days.

Isolates were cultured in a carbon free medium (Skerman 1959) supplemented with 1% ethanol or glucose (see Appendix I). The cultures were grown for fourteen days and the cells were then separated by aseptic centrifugation and washing and resuspended in sterile distilled water to give a standard suspension containing 2 x 10 cells per ml. 1ml. of this suspension was added to 19ml of sterile, molten 1% water agar and poured into a petri-plate. After the agar had set, discs were removed from the plate with a number 4 cork borer and four discs were placed at the growing edge of the A. bisporus colony. The plates were incubated for a further 21 days at 25°C. Aseptic techniques and sterile media were used throughout these procedures. The plates were examined daily and effects on vegetative growth recorded.

3.6.b. Two phase method. (Modelled on the technique of Hume and Hayes 1972).

2% malt extract (Boots Pure Drug Co.) agar was poured into one half of a divided Petri-plate. These plates were inoculated with a disc of A. bisporus taken from the edge of a fourteen day culture using a number 4 cork borer, and incubated at 25°C until the mycelial growth had extended as far as the central partition (about 12-14 days). A second medium was then poured into the adjacent half of the plate.

This second medium was based on a water agar supplemented with mineral salts (see appendix I). The mineral salts medium was filter sterilised and added to molten water agar in the proportions 7:1. This medium was gently mixed and poured into the second half of the Petri-plates. The mineral salts medium was used in different concentrations: 100% strength, 10% strength and 1% strength, in an attempt to stimulate different salt concentrations in the casing layer during stages of growth.

The plates were incubated at 25°C until the A. bisporus had grown over about half the second medium. Pure cultures of bacteria were added in the form of water agar discs (Section 3.6.a.). Two sets of control plates were prepared for each different mineral salts concentration, one set with no water agar discs and another set with water agar discs with no bacteria. Effects of individual isolates on vegetative growth were recorded.

3.7. Analysis of results.

Some of the results for this work involved qualitative rather than quantitive analysis.

Growth experiments in growth cabinets were designed so that there was a minimum of three replicates per treatment, but where appropriate, additional replicates up to a maximum of eight were used. The replicates were arranged randomly within a cabinet.

Other tests such as the screening of bacteria were replicated three times.

Three samples of casing were taken per replicate growing box, these were then mixed to provide a single sample from which dilutions were prepared. Eight replicates were prepared from this dilution for the estimation of numbers, using the Colworth Droplette technique.

Where appropriate, standard deviations for a series of results were calculated and appropriate statistical tests were applied according to Bishop (1971).

4. EXPERIMENTAL AND RESULTS

4.1.Gross changes in ecology following deposition.

4.1.1. Introduction.

Following the preliminary trials of W.A.Hayes, using PMB as a casing medium, it was thought that changes in the microbiological and/or chemical characteristics were associated with its suitability as a casing medium. Therefore the first investigations were designed to study PMB at different time intervals after deposition in order to follow any changes occurring during natural weathering. This section of the work covered changes in the bacteriology and fauna. Changes in the chemical components and fungi and higher plants were part of a parallel study by S.G.Yeo.

At deposition, the sludge is in semi-solid form with only about 8% solids and flows relatively freely (see plate 4.1). After about six months, a crust forms on the surface which leads to fissures about 45cm deep. The fissures deepen as the drying out continues and the material contracts (see plate 4.2). The first Basidiomycete fungi usually appear within these fissures (see plate 4.3), and various species are seen throughout the year up to about 14 months after which colonisation by higher plants such as Solanum nigrum and finally small shrubs and trees, such as Salix spp. (see plate 4.4).

Plate 4.1. PMB at time of deposition.from settling lagoon.

Plate 4.2. PMB six months after deposition showing fissures developing as the material contracts.

Plate 4.3. Early colonising Basidiomycetes on PMB.

Plate 4.4. PMB three years after deposition showing colonisation by shrubs and trees.

4.1.2. On site deposits at Kemsley Mill.

Estimates of numbers of bacteria and the different types of bacteria were made in material aged 0, 1½, 3, 6, 9, 12, 18 and 36 months using the techniques described in section 3.2.1. Analyses for nematodes and invertebrates were also done.

An increase in numbers of bacteria from the time of deposition up to 9 months was noted in the top core samples (see figure 4.1.1). After an initial decline, the numbers in the bottom core increased sharply at 9 and 12 months. From this time numbers in the top and bottom cores followed similar changes. Both top and bottom cores reached a minimum population at 12 months and then increased as the material aged.

The initial decline in numbers in the lower core was associated with the rapid leaching that occurred immediately after deposition, resulting in an unfavourable environment in the lower levels. The top layer drained first and so presented a more favourable environment resulting an early increase in numbers, reaching a maximum by six months.

Nematodes were not present in the raw sludge but were first seen in the lower core samples (see figure 4.1.2).

Figure 4.1.1. Estimates of the aerobic bacterial populations in PMB at different time intervals after deposition.

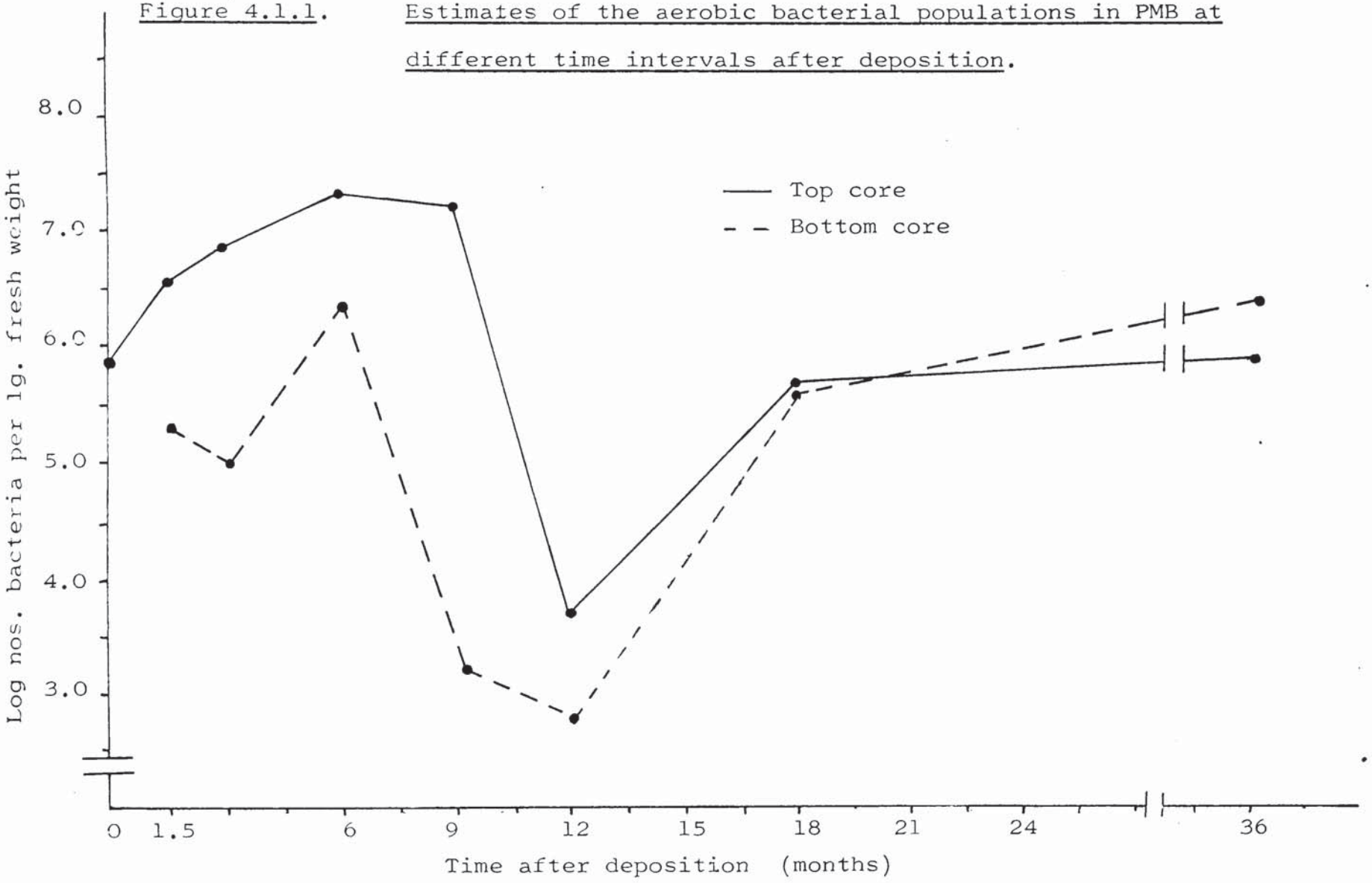
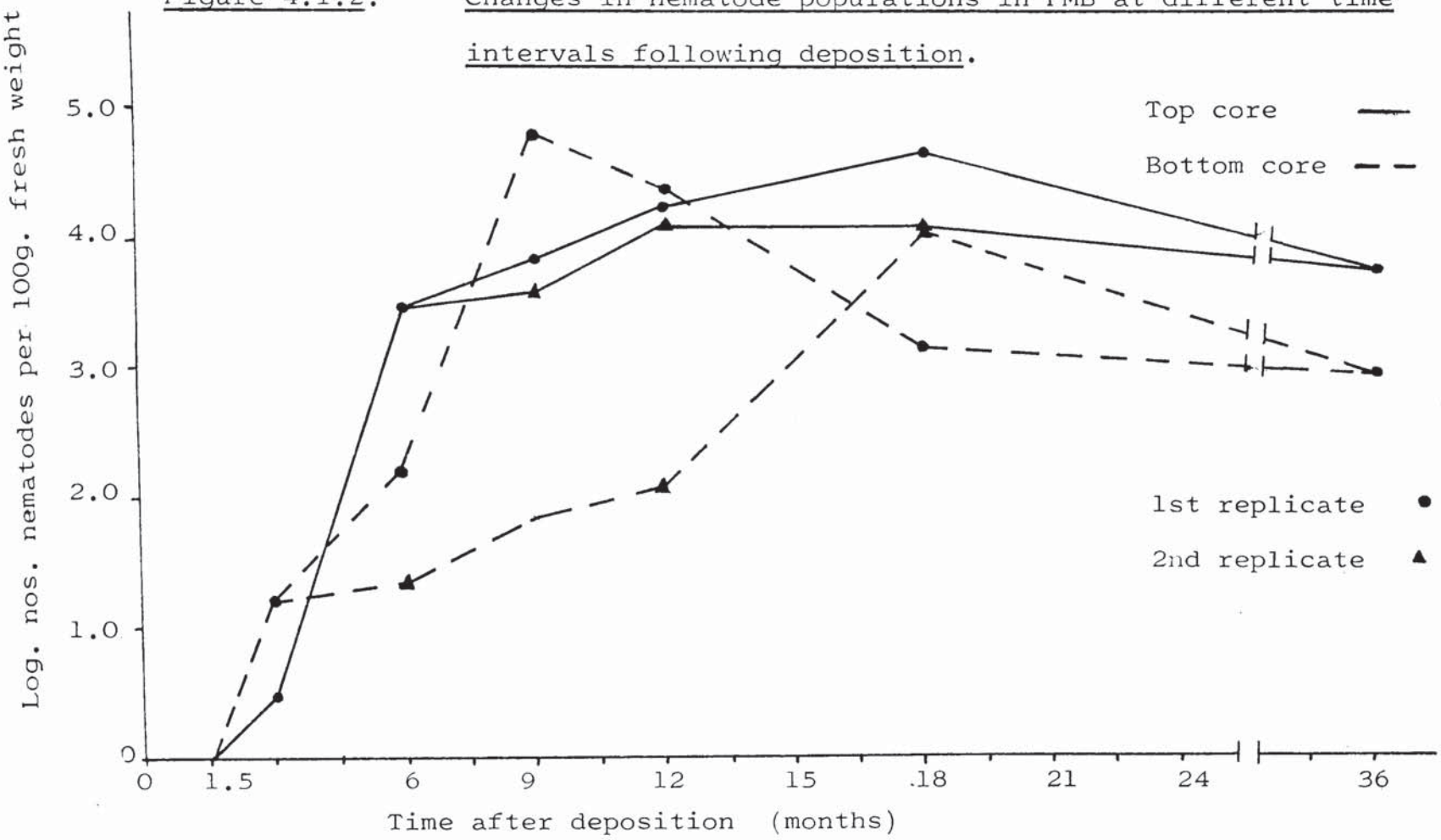


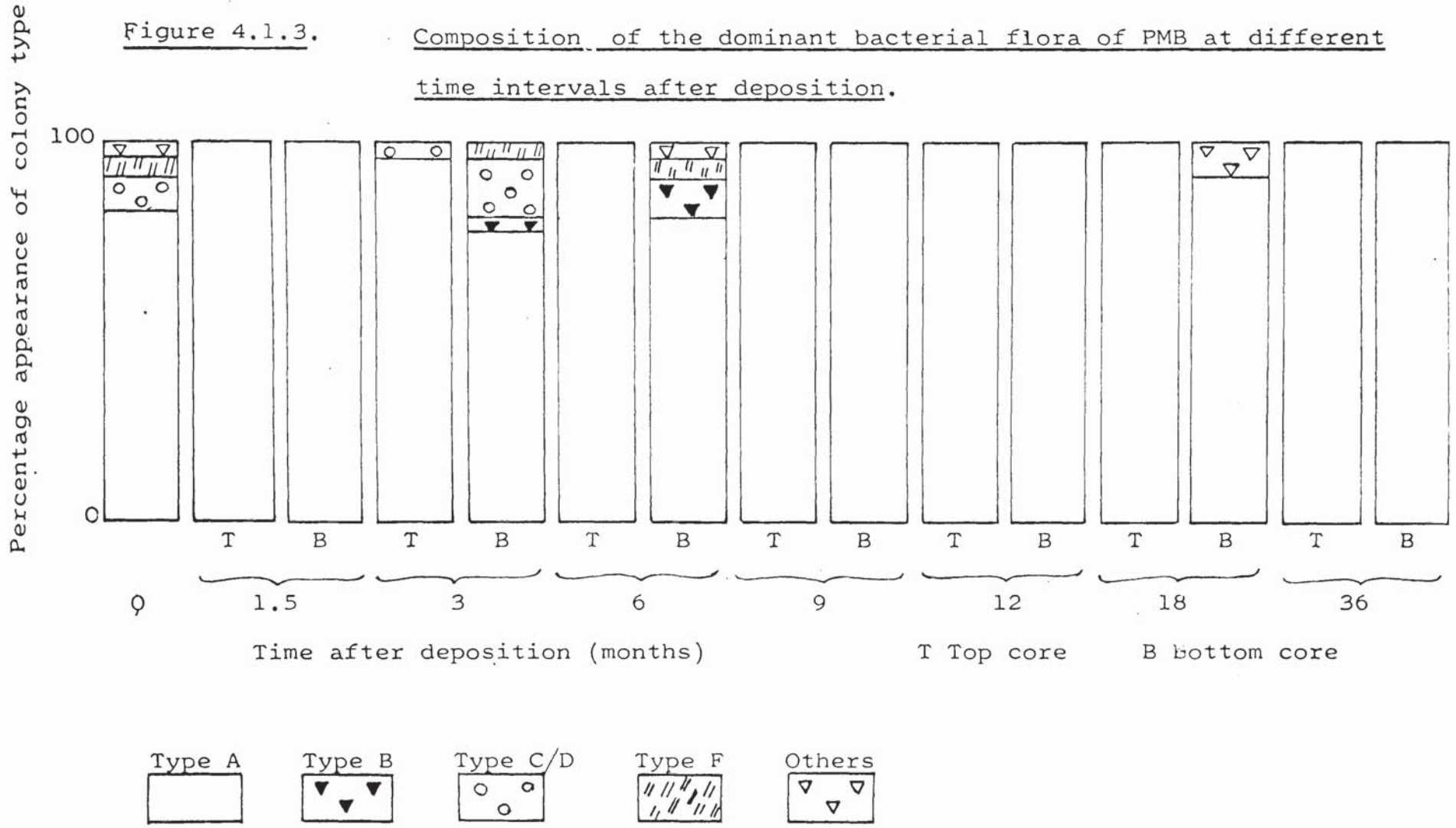
Figure 4.1.2. Changes in nematode populations in PMB at different time intervals following deposition.



Numbers then increased steadily for 9 months after deposition when they stabilised. They were identified as Rhabditis species which are bacterial feeders. The increase in numbers of nematodes coincided with the decline in numbers of bacteria and could account for this. Numbers of nematodes subsequently remained at a relatively constant level. The initial inoculation of nematodes could have been from beneath and this was supported by the results of the simulated ageing experiment.

Any changes in colony type of less than 1% were not recorded. The raw sludge had a varied composition with organge, yellow and white colonies being present as well as the dominant cream type A. The greatest changes figure 4.1.3. occurred during the first six months after deposition when the more intense drainage occurred. There was a change from a varied flora to a dominance of the cream type followed by a return to different varied composition. This change at about 3-6 months coincided with the colonisation by Ascomycete and Basidiomycete fungi as indicated by the work of Yeo (Pers. Comm.). The further change in composition at 18 months was also associated with the gradual colonisation by grasses and other higher plants.

Figure 4.1.3. Composition of the dominant bacterial flora of PMB at different time intervals after deposition.



The initial decline in the conductivity was inversely related to the increase in bacterial population. By 18 months after deposition, when the bacterial population had stabilised, the electrical conductivity had also stabilised (Yeo, Pers. Comm.).

Protozoa were noted in all samples, mainly Paramoecium species, and rotifers (Philodina species) were seen in samples of 18 months and older. This genus of rotifer is a vortex feeder, feeding on minute particles of detritus.

A number of mites and Chironomid larvae were found in samples of 9 months. These are all fungal feeders. Chronomidae and Tipulidae adults were found in samples of 12 months and older.

4.1.3. Isolation funnels.

The study of different aged deposits of PMB (section 4.1.2) provided interesting information about the changes occurring in the natural weathering at the Kemsley site. This indicated that the changes in the bacterial flora were associated with:-

(a) colonisation by other organisms

(b) the nature of the sub-soil

(c) the leaching of salts from the sludge

This investigation was designed to study the changes in isolated PMB exposed to weathering, removing the effect of the sub-soil and reducing the effects of other colonising organisms.

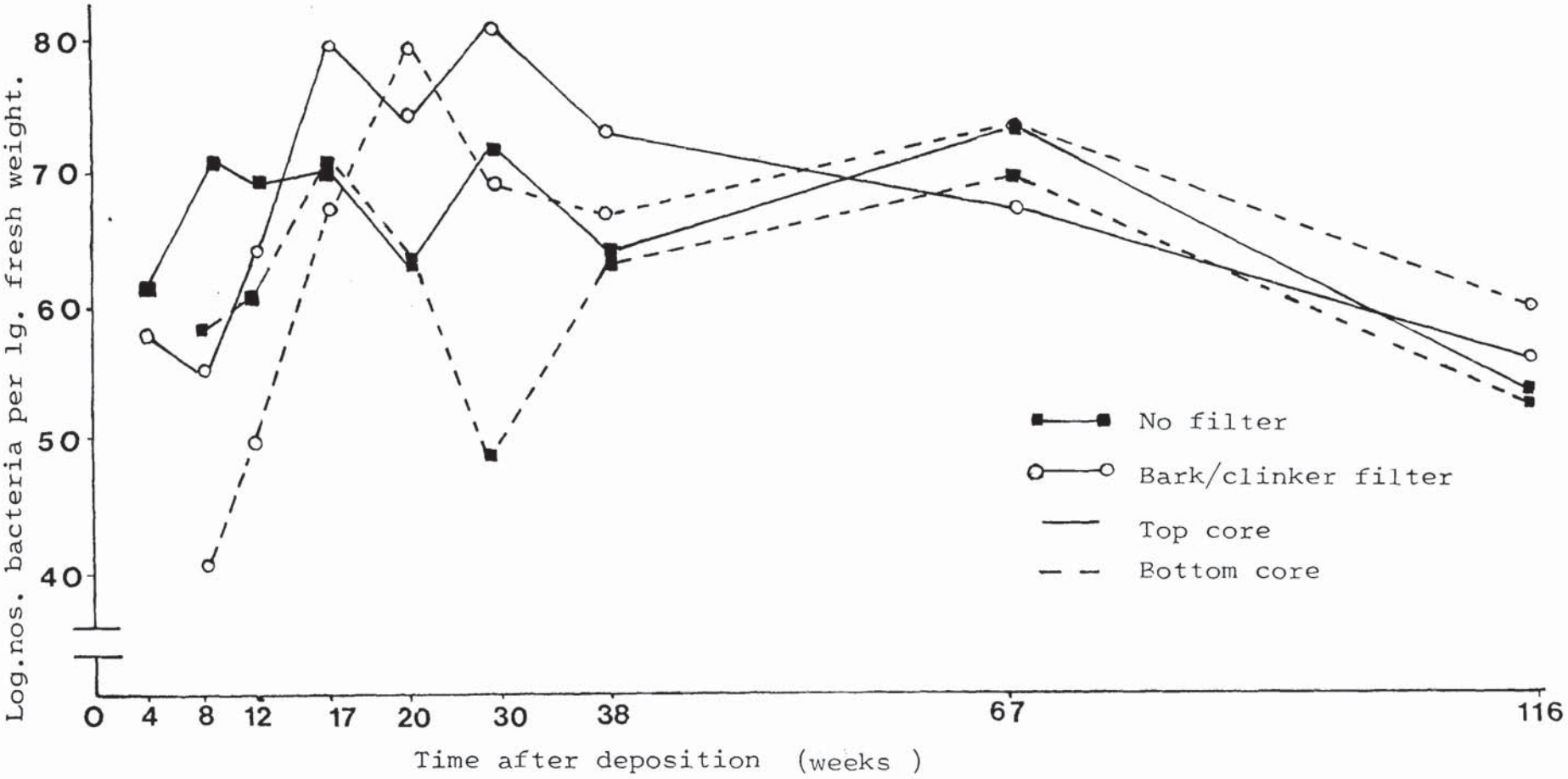
The establishment of the bacteria and the effect of leaching of soluble salts could be studied in detail.

Sample cores were initially taken every 4-6 weeks from the upper and lower horizons and bacterial populations were estimated and nematodes counted. (figure 4.1.4)

70-85% of the liquid effluent was collected in the first 5-6 days (appendix II) with more being collected after each period of heavy rainfall (appendix III). The volume of material in the funnels decreased rapidly and shrank away from the sides of the containers. The total volume decrease was about 1/4. A hard crust formed on the surface of the material.

After 4 weeks, the bacterial population in funnel 1 was higher than any of the samples taken from Kemsley Mill.

Figure 4.1.4. Numbers of bacteria in PMB during simulated ageing experiment.



Funnel 2 had a population between that of the $1\frac{1}{2}$ and 3 month samples from Kemsley Mill. After 17 weeks, this exceeded the highest population from the Kemsley Mill samples. The bacterial populations, both with and without filter, showed similar trends after initial differences. The better drainage from the funnel with the filter and so a greater leaching effect of salts then resulted in the higher bacterial population in the funnel with a filter.

The top core generally had the higher bacterial population except for the 20 week samples where the top and bottom cores had similar levels of bacteria. The high numbers of bacteria were associated with the absence of nematodes and other bacterial feeders. The absence of nematodes in both funnels suggested that colonisation could have been from the sub-soil.

Coprinus impexi appeared on both funnels after 15 weeks and Peziza species appeared after 7 months. After $7\frac{1}{2}$ months, Stropharia semiglobata was seen on funnel 1 and after 9 months a yellow mould grew on funnel 2. Coprinus impexi has been identified as one of the first colonisers of the material at Kemsley Mill (Yeo, Pers. Comm.).

There was an absence of higher plants colonising the material, but this material was less susceptible to colonisation due to its isolated position. The absence of many colonising Basidiomycetes to break down the material would also render it less suitable to higher plants.

4.2. PMB as a casing medium

Following the preliminary work of Hayes(Pers.Comm.) further investigations were necessary to assess the performance of PMB as a casing medium in mushroom culture.

It is known that a casing medium must possess a number of different properties in order to sustain fruitbody formation. These relate to physical, chemical and biological factors. In view of microbiological activity shown in section 4.1., emphasis was given to the bacterial changes which occurred subsequent to casing mushroom beds. Bacteria, in particular Pseudomonads, are known to be associated with productivity and fruitbody formation in peat based casing soils (Hayes 1974).

The structure of a casing medium is also known to influence the formation of mushroom fruits and Nair and Hayes (1974) linked different structures to bacterial activity.

PMB may be processed to a given structure, this factor was taken into account in the design of an experiment to elucidate the bacterial changes occurring in PMB when used as a casing medium.

Changes in the total bacterial population and *Pseudomonas* population were followed in both peat and PMB casings when applied to spawned compost (Section 3.3.2) Different structural properties were conferred on PMB before neutralising with chalk, by sieving to different particle sizes. These were:-

(1) 20mm - dust

(2) 10mm - dust

(3) 5mm - dust

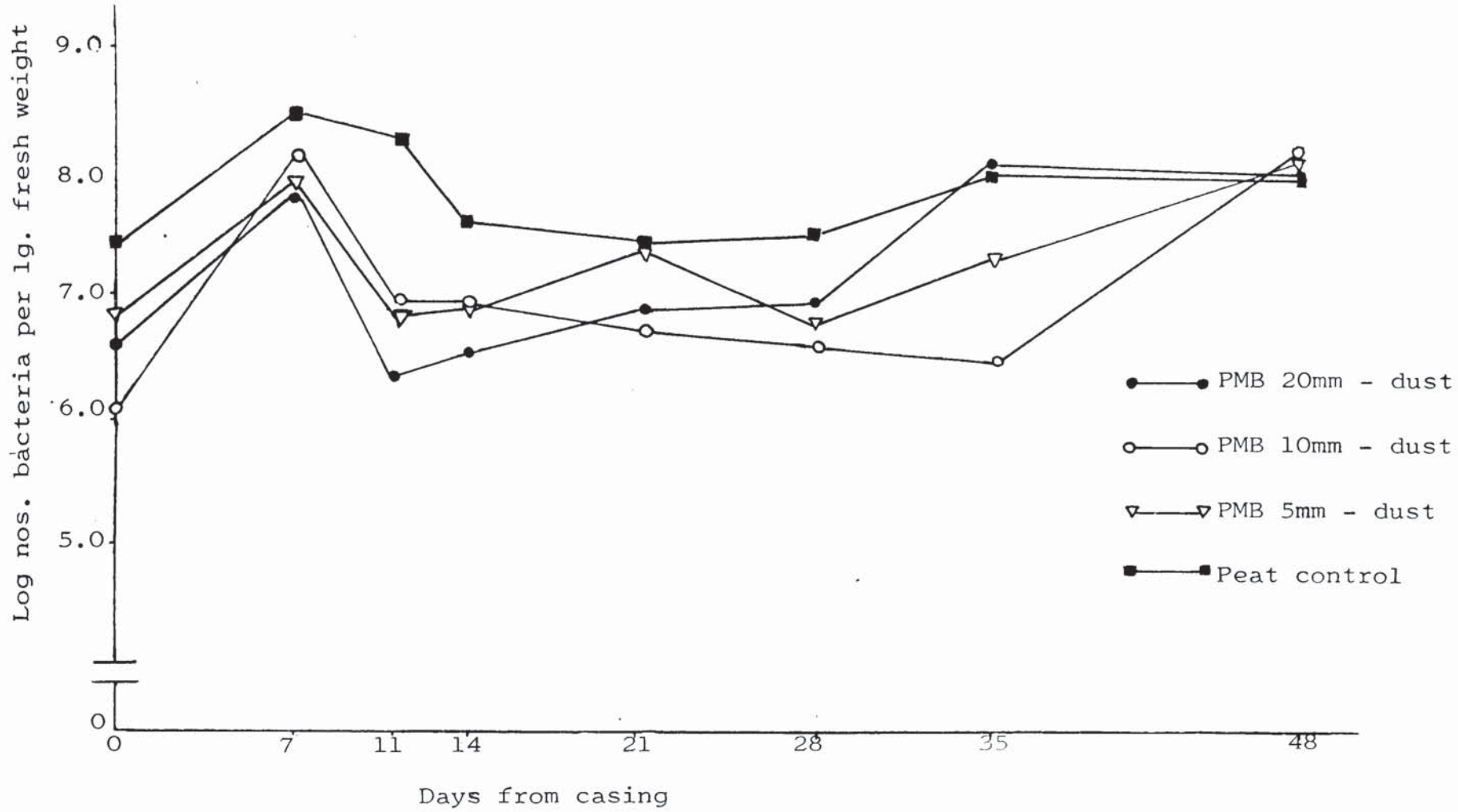
Peat neutralised with chalk was used as a control.

4.2.1. Changes in bacterial populations.

Samples were taken through the casing soil on days 0, 7, 11, 14, 21, 35 and 48 after casing (Section 3.1.2).

Numbers of bacteria increased to a maximum at day 7-10 after casing (figure 4.2.1) Peat casing had a higher initial population than all PMB casings, and it maintained this through to an increase at day 11-14. The population then declined to a level above all PMB casings until day 28 when there was an increase in numbers by day 35 coinciding with the third break which stabilised up to day 48. Numbers in PMB casing also increased from day 0-7 but then declined by day 11. Highest numbers were obtained from the 10mm-dust treatment, but differences between casing treatments were relatively small.

Figure 4.2.1. Changes in bacterial populations in PMB casing of different particle sizes.

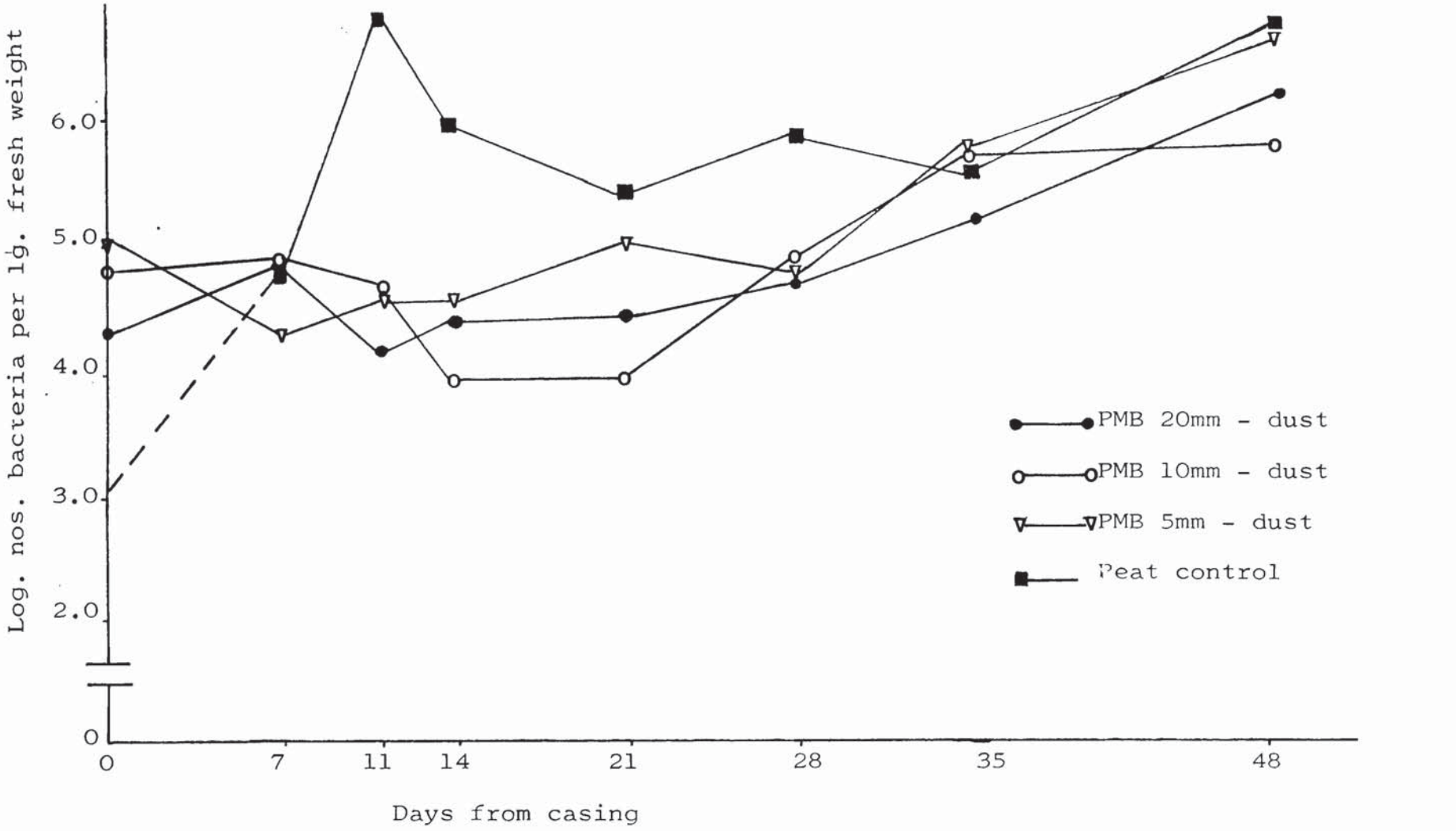


There was then a slight increase at day 21 when the first break was picked in treatments of 20mm and 5mm particle sizes. Numbers of bacteria in the treatment with 10mm particle size followed a similar pattern to peat casing until day 28. In PMB 20mm, the population increased to a higher level than peat by day 35 and in PMB 5mm, the population increased at a similar rate to peat casing. In PMB 10mm the population declined slightly but the increase after day 35 and by day 48 was the highest. All treatments had similar numbers of bacteria at day 48 and the higher numbers present coincided with the decline in yield particularly in peat casing and PMB 10mm particle size.

4.2.2. Chances in the Pseudomonad population.

Numbers of pseudomonads in peat increased rapidly from the day of casing, when the population was below 1×10^4, to a level greater than 1×10^6 by day 11 (figure 4.2.2). Numbers then declined gradually by day 21, with another slight increase at day 28 then a rise and fall in numbers, until another marked increase from day 35-68. Numbers of pseudomonads in PMB casing were originally higher than peat, but after day 7 they remained at levels well below peat casing and there was no increase in numbers at day 11-14.

Figure 4.2.2. Changes in Pseudomonad populations in PMB casing of different particle sizes.



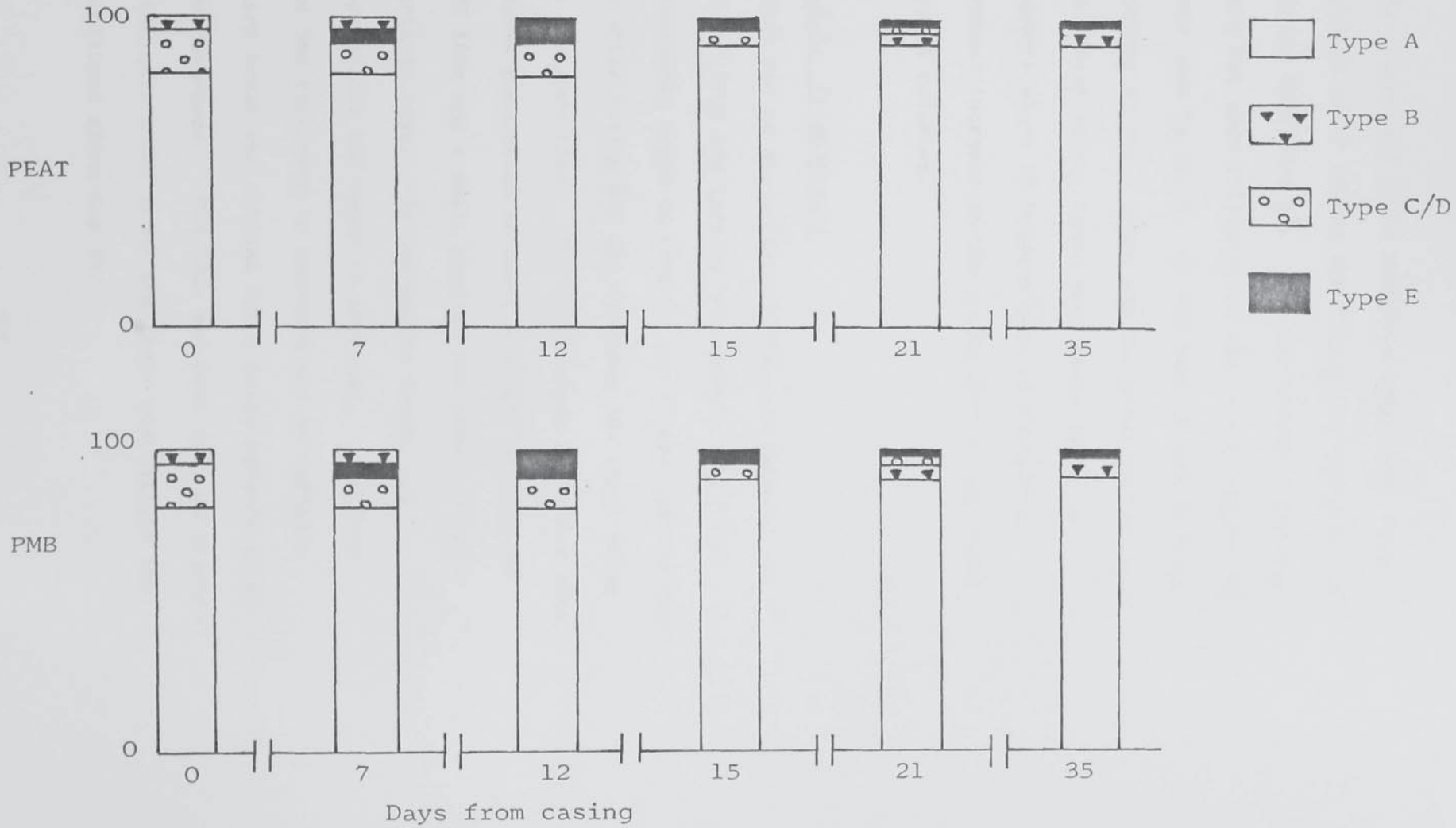
Numbers increased and declined slightly up to day 28 when there was an increase in numbers similar to that in peat and in PMB of 20mm and 5mm particle sizes. In PMB 10mm, population increased from day 28-35 and then stayed at similar levels to day 48.

The initial peak in pseudomonad numbers in peat casing occurred at day 11 rather than at day 7 as with the total numbers. Pseudomonad bacteria in PMB casing did not increase sharply at day 7-14 as did the total numbers and similar relationships between total and pseudomonad populations were evident as peat was higher at the same times after casing.

4.2.3. Composition of the bacterial flora.

Based on colony form, there was no discernable difference in the composition of the bacterial flora in peat or PMB casings (figure 4.2.3). There were changes in the bacteria found immediately after casing, during cropping and towards the end of cropping. Cream, type A colonies occurred in high proportions throughout the growing cycle. Yellow, type B colonies were seen immediately after casing and then again at the end of cropping. As less than 1% appearance was not recorded, the yellow colonies would have been present in very low numbers at other times.

Figure 4.2.3. Changes in the occurrence of colony types from the casing layer.

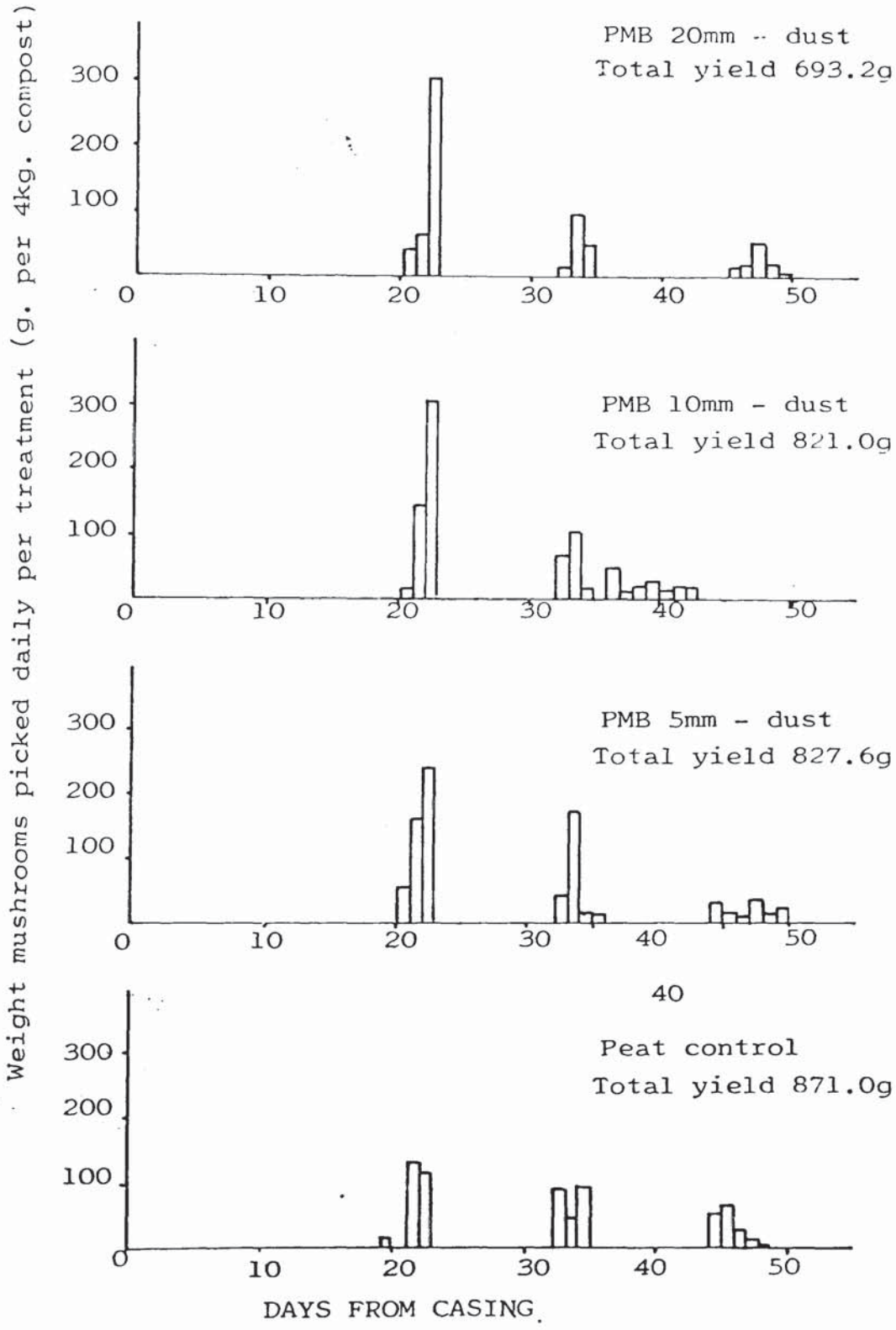


The orange and white colonies (type C/D) were present after casing and during the mid-cropping period until day 35. The red colonies (type E) were not seen initially in the casing sample but were seen by day 7. At the time of the peak in numbers at day 7 there was the greatest variation in colony types, there were fewer types in numbers above 1% between days 12-15 with a gradual increase in the proportion of the cream type A colonies.

4.2.4. Productivity.

There was no significant difference between the yields from the various treatments. All PMB treatments began to crop on day 21 and peat on day 20 after casing but all finished the first break at the same time. The first break coincided with lowest population in peat, a slight decline in PMB 10mm and a small peak in PMB 20mm and 5mm particle size. At the second break, all populations had begun to increase. PMB 10mm and 5mm continued to increase and no definite third break was noticed but a daily harvest of a few mushrooms. PMB 20mm and peat casings produced a definite third break and their populations had stabilised since day 36.

Figure 4.2.4. Effect of particle size on the daily yield from PMB casing.



4.3. Quality and pathological assessments.

The quality of mushrooms produced is an important aspect of a casing. Ideas of quality vary greatly but the main factors are the whiteness and cleanness of the cap, texture of the mushroom and freedom from disease (Pearson and Hayes 1976). The whiteness of the cap is influenced by the adherence of soil, mechanical damage and the presence of bacterial disease and animal vectors. Soil tends to adhere more readily to damp cap surfaces and harbours bacteria which readily infect the cap surface with diseases such as bacterial blotch and animal vectors such as mites and nematodes. As the mushroom grows through the casing, it may be damaged mechanically by the casing or damaged on picking or post-harvest handling.
This results in bruising and subsequent discolourition due to action by the enzyme O-diphenol oxidase converting tyrosinase to the pigment melanin (Goodenough 1976). Cap damage prior to harvesting can also allow easy penetration by bacteria and animal vectors.

The aim of this study was to compare the dry weights of mushrooms grown on PMB and peat casings and to measure the whiteness of the cap in terms of colour and soil coverage at picking. Presence of diseases and animal vectors was recorded. Humidities and temperatures higher than standard were used to encourage the development of bacterial blotch.

4.3.1. Quality assessment.

Studies were made as in section 3.4.1. on the moisture content, colour and soil coverage of mushrooms grown on peat an PMB casings. The incidence of blotching was recorded as it detracted from the whiteness and many other diseases and animal vectors were noted.

Total yields and numbers and weight of mushrooms harvested (table 4.3.1 and figure 4.3.1) showed a higher yield from PMB casing than from peat casing and the varied growing conditions did not affect this difference. PMB yielded fewer, heavier mushrooms than peat casing and they had a significantly higher dry matter content (table 4.3.2). These mushrooms had a firmer texture and were less prone to bruising during harvesting and handling, they also were significantly whiter (table 4.3.3) and had less

Table 4.3.1.

Effect of varied temperature and humidity upon productivity from peat and PMB casings.

Days from casing	Daily weight (g) harvested			
	Control conditions		Varied conditions	
	PMB	Peat	PMB	Peat
16	62.8	-	-	-
17	105.6	28.8	-	-
18	360.7	153.7	89.3	28.8
19	963.9	556.9	92.8	75.0
20	183.9	378.3	171.7	-
21	11.2	187.5	158.1	68.1
22	81.2	18.7	463.4	756.9
23	-	-	15.1	489.0
24	-	-	1.8	24.1
25	-	-	-	16.3
26	77.8	27.6	40.0	80.1
27	113.0	-	85.6	-
28	228.0	183.2	141.3	-
29	34.2	132.9	21.0	-
30	29.5	164.5	37.9	54.7
31	30.8	280.4	58.0	93.0
32	81.8	15.3	96.2	114.0
33	-	-	-	54.2
34	-	-	-	57.9
35	-	-	102.2	82.8
36	-	-	-	32.4
37	-	-	46.6	-
38	27.5	-	171.2	-

to be continued.

Table 4.3.1. contd.

	Control conditions		Varied conditions	
Days from casing	PMB	Peat	PMB	Peat
39	22.0	-	32.4	-
40	-	-	-	-
41	-	-	-	-
42	129.1	64.0	88.9	119.7
43	14.8	69.6	36.2	66.8
44	30.4	111.5	36.2	47.2
45	17.3	41.0	26.9	32.1
46	-	34.4	22.6	-
47	-	-	-	-
48	-	-	-	-
49	38.6	4.2	90.6	114.5
Total yield	2117.4g	2452.5g	2089.8g	2432.6g
Mean per kg compost	235.6g ± 19.81	272.5g ± 27.46	232.2 ± 20.22	270.29g ± 21.28

Significant difference ($0.05 > P > 0.02$) found between peat and PMB casings in control conditions.

Significant difference ($0.1 > P > 0.05$) found between peat and PMB in varied conditions.

No significant differences occurred with one treatment in different conditions.

Figure 4.3.1.

Effect of varied temperature and humidity on the productivity of PMB casing.

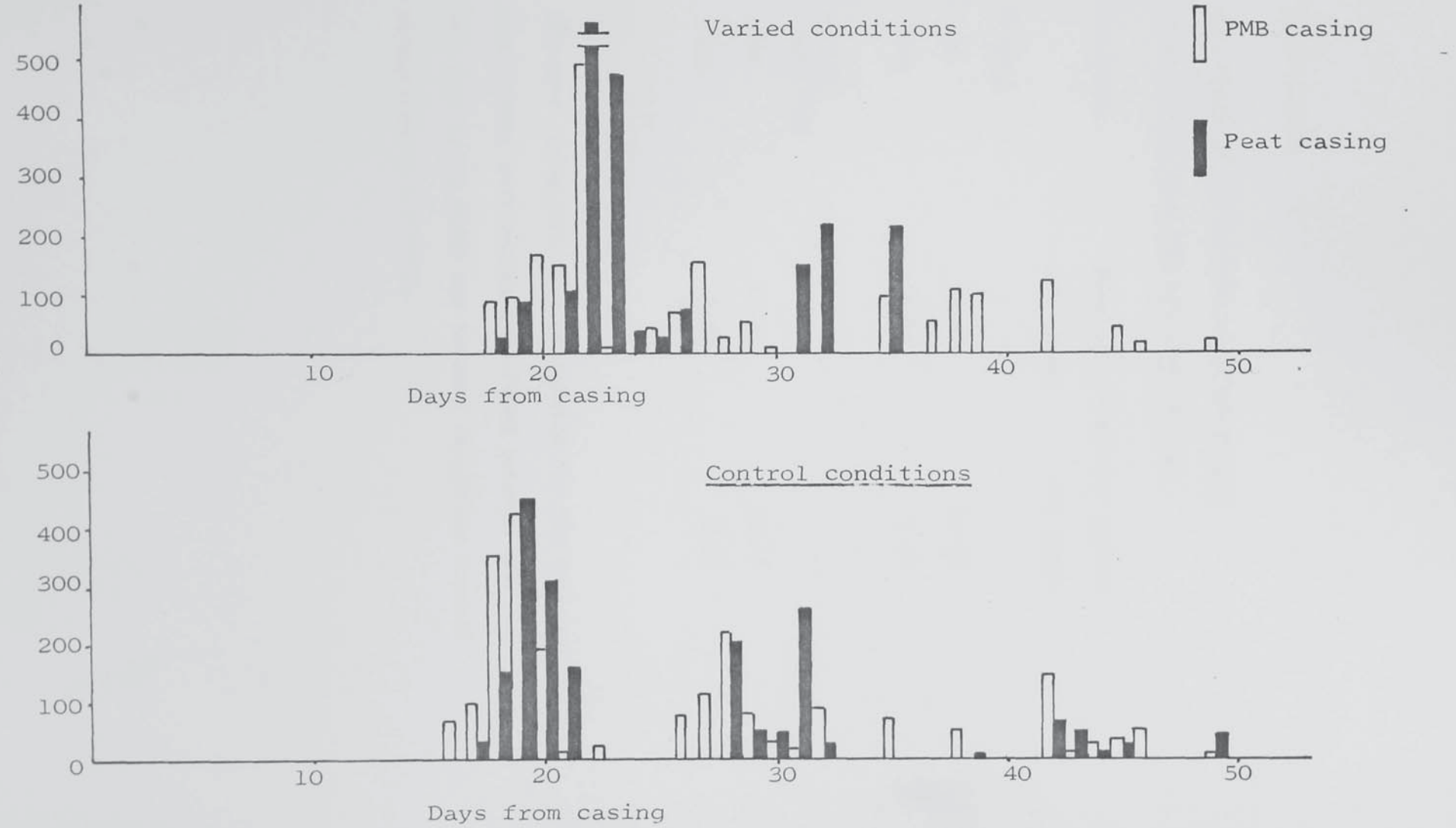


Table 4.3.2.

Dry weight as percentage of fresh weight of mushrooms from PMB and peat casings.

Treatment	Mean percentage dry matter	
	1st break	2nd break
Control		
PMB	10.43	10.76
Peat	8.30	7.17
Varied Conditions		
PMB	11.36	13.18
Peat	8.33	9.22

Standard deviations were calculated for the data and significant differences were found between PMB and peat ($p < 0.005\%$) and between varied and control conditions ($p < 0.005\%$).

Table 4.3.3.

Whiteness* of mushroom caps from PMB and peat casing soils.

Treatment		1st break (mean)	2nd break (mean)
Control Conditions	PMB	73.48 ± 1.13	86.4
	PEAT	54.10 ± 1.13	72.1
Varied Conditions	PMB	75.2 ± 0.11	79.9
	PEAT	63.8 ± 0.11	64.9

There were significant differences between PMB and Peat ($0.01 > P > 0.001$) and between the different conditions ($0.05 > P > 0.01$)

* Whiteness was measured (section 3.4) with the instrument calibrated from 0 to 100 with a standard block of magnesium carbonate for 100% reflectance (Pearson and Hayes 1976).

casing material adhering to the cap (table 4.3.4) than mushrooms from peat casing.

4.3.2. Pathological assessment.

(a) Bacterial diseases.

PMB produced more mushrooms with bacterial blotch (table 4.3.5 and plate 4.5) in conditions of high temperature and humidity than in standard culture conditions.

A tissue block method (Gandy 1968) was used successfully to screen bacteria isolated from PMB and peat casings and from infected fruitbodies for pathogenic activity on the mushroom cap. Different degrees of browning were evident indicating the presence of different strains of Pseudomonas tolaasi, casual agent of bacterial blotch (see plate 4.6).

A direct inoculation method was used to screen bacteria for effect on gill tissue. Isolates of P. agarici were confirmed using this method described by Young (1970) when symptoms of drippy gill were found two days after inoculating through the cap of a growing mushroom. (section 3.4.2.b). No reinfection was obtained when inoculations were made into the stipe tissue.

Table 4.3.4.

Extent of covering of cap by casing soil at picking

Extent of cap surface covered by casing soil	Percentage of mushrooms with casing soil on cap			
	Control conditions		Varied conditions	
	PMB	Peat	PMB	Peat
100%	0	8.2	0	2.3
75%	0	9.4	0	11.2
50%	0	11.55	15.0	14.2
25%	7.9	2.4	2.5	7.1
Less than 25%	6.29	32.42	13.2	20.0
No soil	85.81	36.03	69.3	45.2

Table 4.3.5. Incidence of blotch from PMB and peat casing soils.

Control Conditions	PMB	0.7% of mushrooms had slight blotch (1% cap affected).
	Peat	No blotch
Varied Conditions	PMB	13.1% of mushrooms had blotch on 25% of cap surface 6.8% of mushrooms had blotch on 1% of cap surface
	Peat	0.6% of mushrooms had blotch on 1% of cap surface

Plate 4.5. Mushroom infected with bacterial blotch.

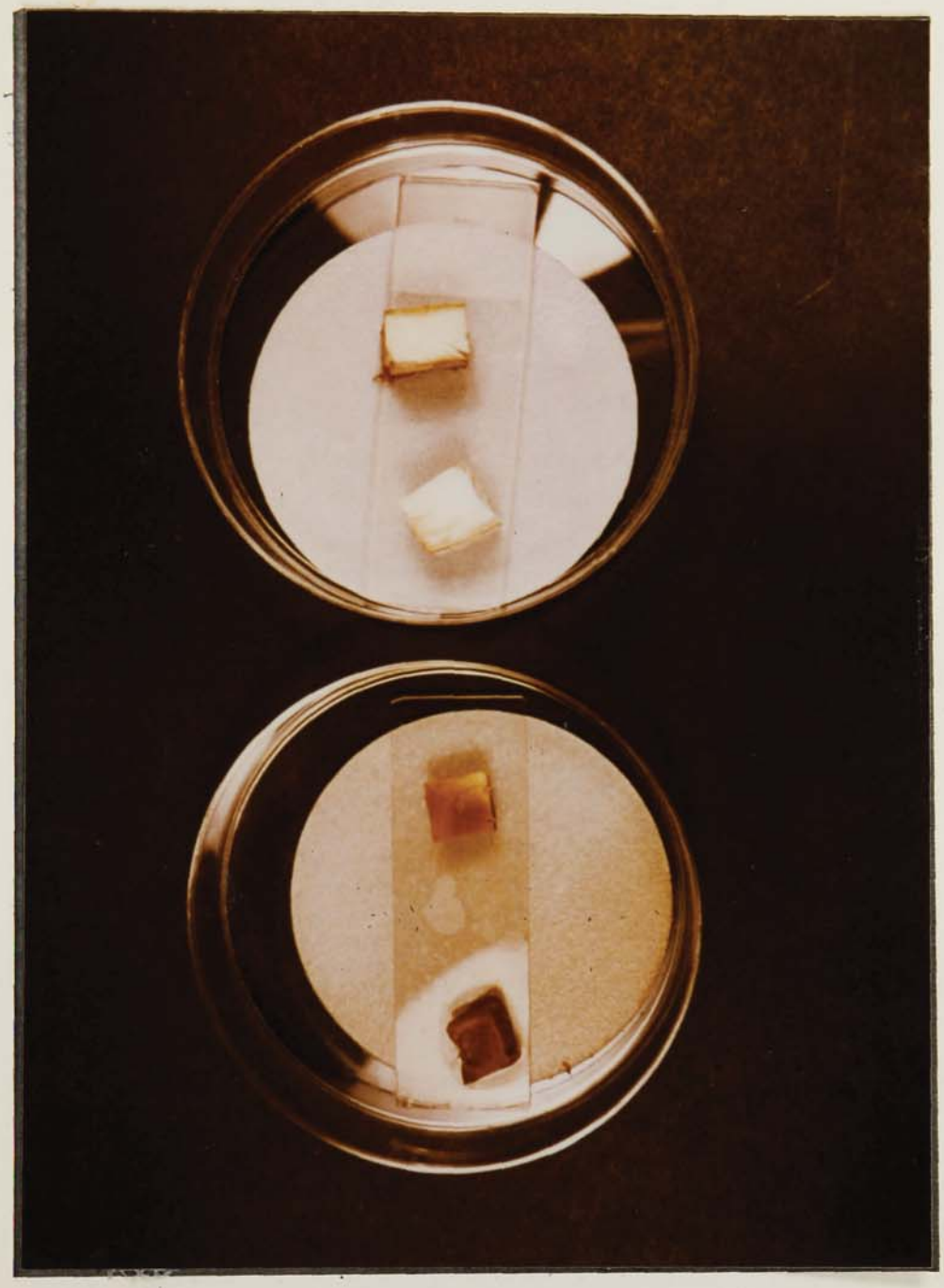


Plate 4.6. Tissue block method for screening bacteria pathogenic to mushroom cap tissue.
Block B is a control, showing no browning, other blocks show different degrees of browning from very severe (D) to weak (A).

Infection of the gills and the production of bacterial slime was found two days after injecting into the casing soil and mycelium at the base of the stipe. This suggested that the bacteria probably entered the mushroom through the stipe and passed up the stipe and then out to the gills. Mushrooms grown in the laboratory and infected with drippy gill often had a black speckling on the cap. Close examination of the specks revealed internal pits. These occasionally broke through to the surface, resulting in a similar appearance to bacterial pit (plate 4.7). In severe cases, the pitting was deeper and quite extensive and occurred radially on the cap supporting the idea that the initial infection passed up the stipe and radiated across the cap. The damage to the gills was occasionally so severe that they disintegrated completely. Cecid larvae (Heteropeza pygmaea) have been noted in association with this disease, both in Aston laboratory and by Hussey et al. 1969). When mushrooms were injected with bacteria that had caused browning in the tissue block method and were then sectioned through the point of infection, there was no effect on the gills but there was a browning of the internal tissues around the line of the inoculation. Isolates that had produced no reaction in the tissue block did not produce any reaction in this test. (plate 4.8).

Plate 4.7. Mushroom infected with drippy gill.

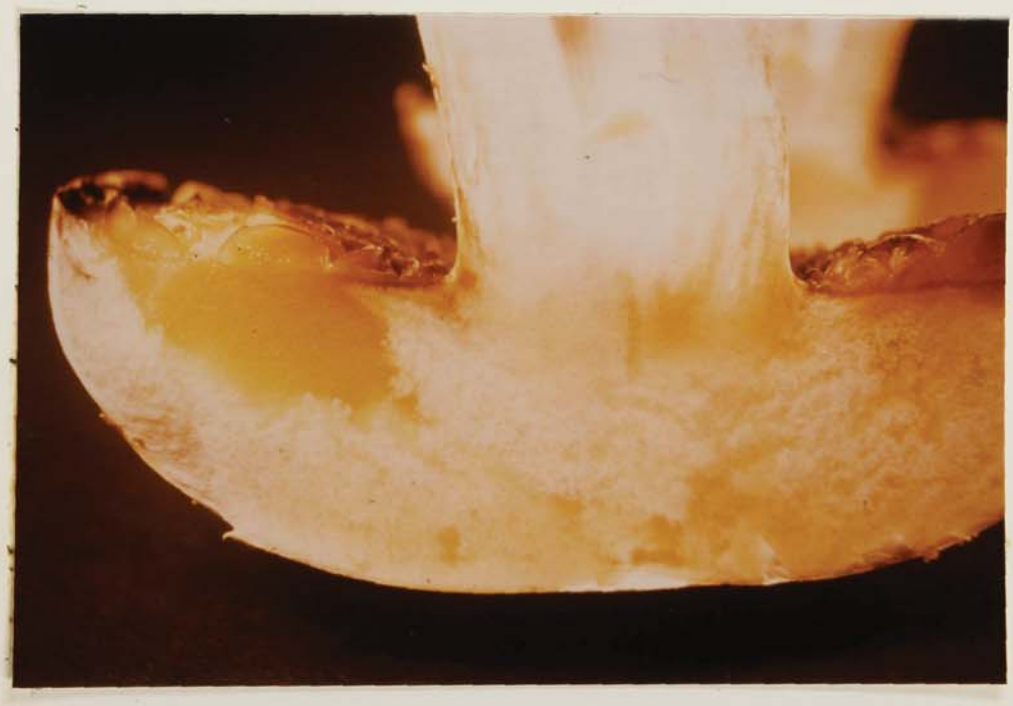


Plate 4.8 Mushroom inoculated with Pseudomonas agarici and sectioned to show the extent of tissue infection.

Drippy gill symptoms were seen on mushrooms grown on PMB and peat casings.

(b) Fungal diseases.

Experiments with PMB revealed a new fungal disease whose causal organism was identified as a Gliocladium species belonging to the Gliocladium deliquescens series (Yeo and Hayes 1978). This disease was seen on PMB and peat casings.

(c) Animal vectors.

Nematodes and mites were not seen during this experiment but the mites that had been isolated from PMB from Kemsley were Rhabditis species. These are saprophytic nematodes, feeding on bacteria and whilst they may act as disease vectors, they are not primary pathogens.

4.3.3. Conclusions

This study showed that PMB casing produced better quality mushrooms than peat casing. The mushrooms were whiter, with less adhering casing and had a firmer texture. This meant that they were less susceptible to mechanical damage. Bacterial blotch was more common on PMB casing than on peat and the occurrence of other diseases such as drippy gill and Gliocladium was noted.

PMB casing did not support large numbers of mites and pathogenic nematodes and there were no problems with other diseases such as Verticillum, Mycogone and bacterial pit. The presence of saprophytic nematodes which are disease vectors indicated some treatment of the casing to suppress these as well as the bacterial pathogens without a harmful effect on the beneficial bacteria.

4.4. Pasteurisation of PMB

From the experiment to assess the quality of mushrooms from PMB casing (section 4.3.2), it appears that this material supports various pathogenic organisms. Nematodes and insect larvae are also present in the material, and whilst they are not pathogenic, may be vectors for other pathogens. It was hoped that a suitable regime could be found that would suppress the harmful bacteria but not at the expense of the beneficial bacteria. As bacteria are important in the casing layer for fruitbody initiation, it is important that the stimulatory bacteria are not killed by any pasteurisation regime.

4.4.1. Effect of heat on bacterial isolates.

The thermal death points of the bacterial isolates were determined before choosing a pasteurisation regime in order to establish that the non-pathogenic bacteria would not be destroyed by the temperatures necessary to kill the pathogenic bacteria. (Table 4.4.1)

Table 4.4.1.

Thermal deaths* of bacterial isolates

Culture	Thermal death
(a) Pathogenic isolates	
Pseudomonas tolaasi	55°C
Pseudomonas agarici	55°C
(b) Non pathogenic isolates	
27 Pseudomonas	55°C
28 Pseudomonas	55°C
74 Pseudomonas	55°C
62 Alcaligenes	55°C
81 Alcaligenes	60°C
64 Bacillus	85°C
51 Serratia	55°C
7 Flavobacterium	65°C
10 Enterobacteriaceae	65°C

* All thermal deaths resulted from treatment at the named temperature for one hour

The determination used the method in section 3.1.5. Pathogenic bacteria were killed by treatment at 55°C for one hour, several non-pathogenic bacteria were also destroyed at this temperature with others being destroyed at 65°C and 85°C.

Using these results, the following experiment was used to assess the effect of a range of pasteurisation treatments on the quality and quantity of the bacterial flora.

The following regimes were chosen.

50°C for thirty minutes

65°C for thirty minutes

65°C for sixty minutes

80°C for thirty minutes

80°C for sixty minutes

Untreated material was used as a control and the PMB was pasteurised to the chosen regimes using the methods outlined in section 3.5.

4.4.2 (1) Bacterial population studies.

Using the methods in section 3.1.4. estimates of bacterial populations were made immediately after pasteurisation treatments (Figure 4.4.1). Treatment at 50°C had no significant effect of numbers of bacteria. The treatment at 65°C did cause a significant reduction in numbers but there was little difference between the treatment for thirty or sixty minutes. Treatment at 80°C for thirty minutes caused great reduction in numbers of bacteria and treatment at 80°C for sixty minutes reduced numbers to less than one hundredth of the untreated material.

4.4.2 (2) Composition of the bacterial flora.

The composition of the bacterial flora was examined using the methods described in section 3.1.4. The treatment at 50°C had no effect on the composition of the flora (Figure 4.4.2). Treatment at 65°C resulted in a decline in the numbers of colony type A, increase in proportion of type B/C and the appearance of a type F. This colony was present in the untreated material but in very low proportions (included in "others"). Treatment at 80°C for thirty minutes resulted in the elimination of all but two colony types. Treatment at 80°C for sixty minutes eliminated all but type A (cream colonies).

Figure 4.4.1. Effect of steam/air pasteurisation on numbers of bacteria in PMB.

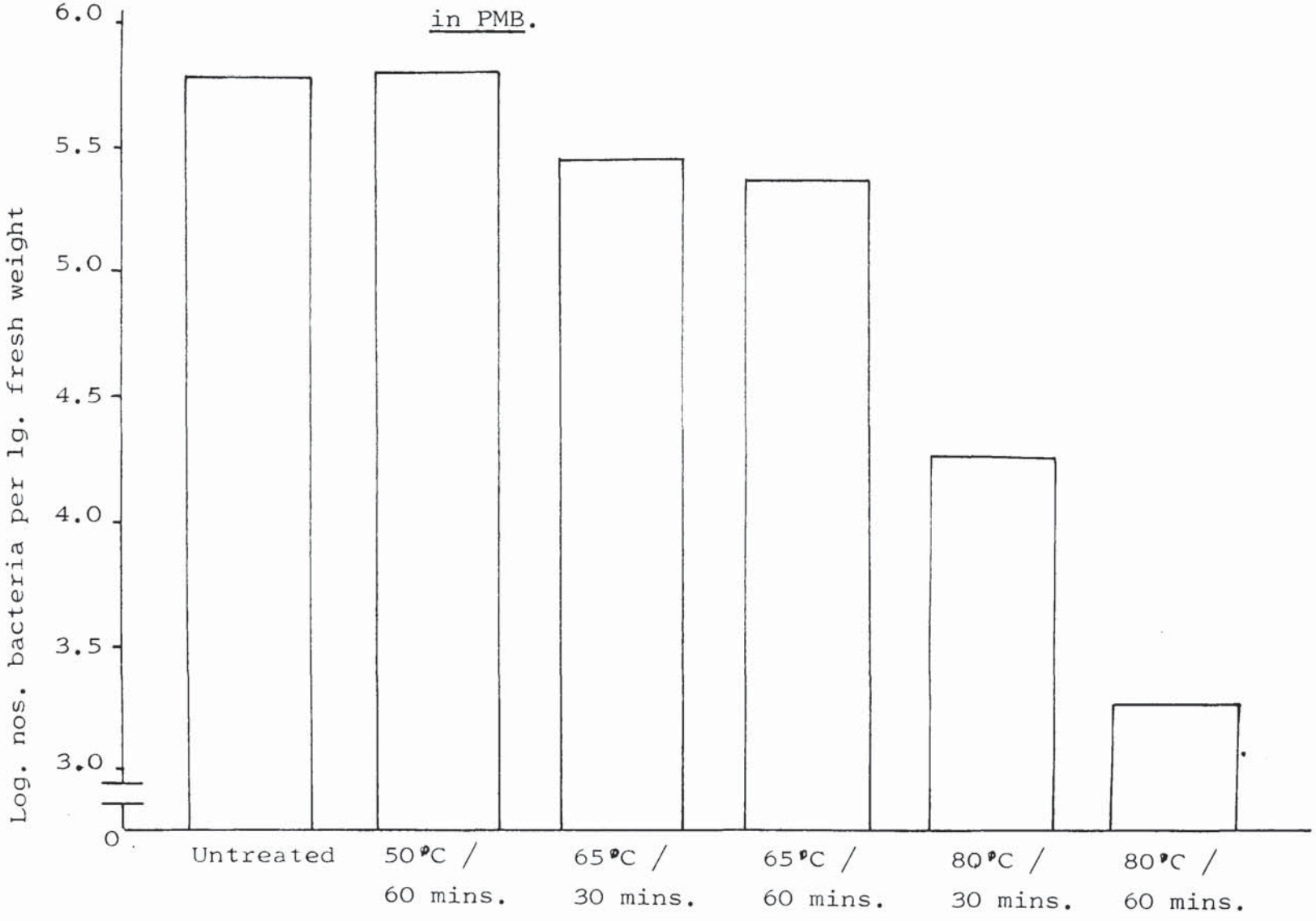
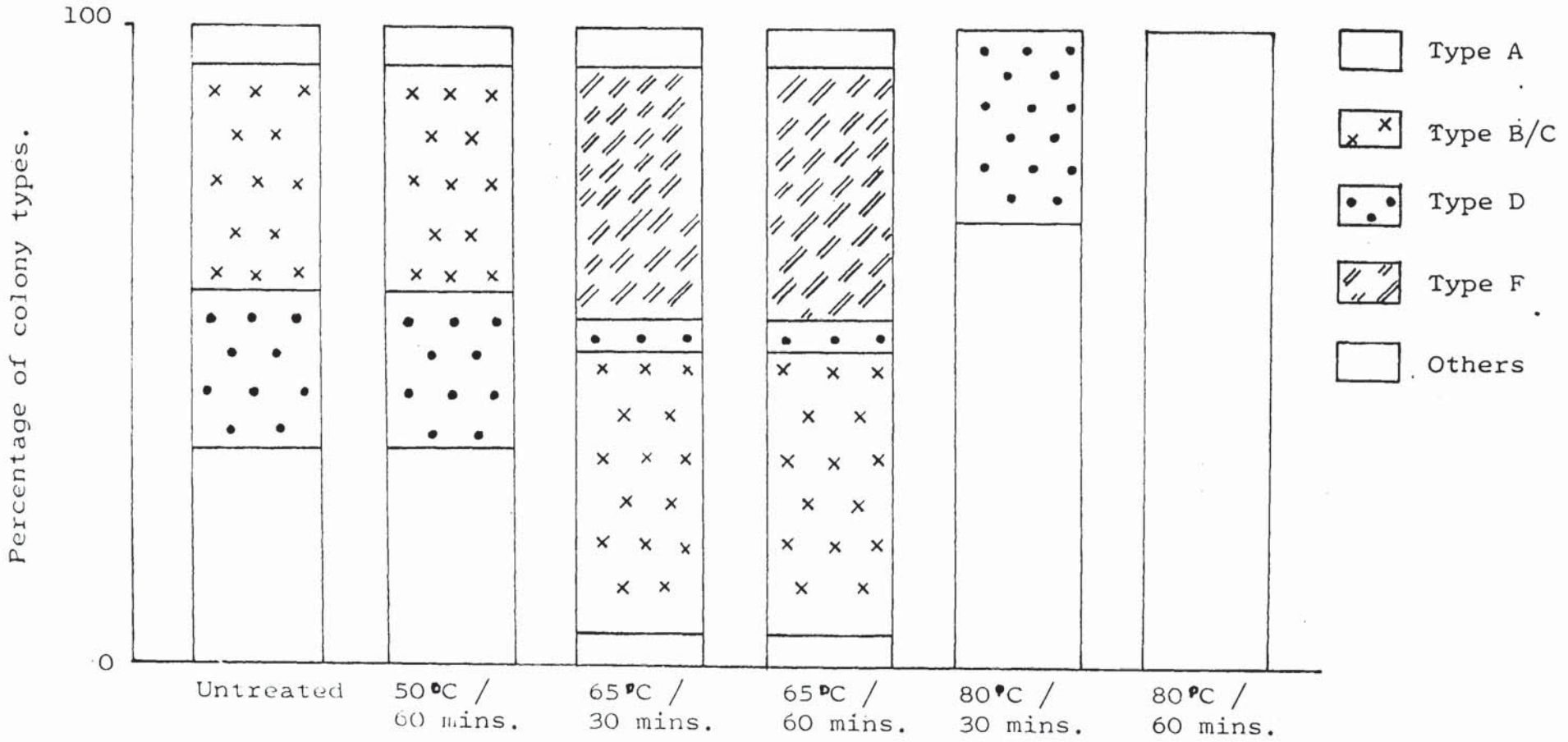


Figure 4.4.2. Effect of steam/air pasteurisation on colony types.



4.4.3. Pasteurised PMB in mushroom culture.

The aim of this experiment was to see the effect of recolonisation after pasteurisation, when the material was applied to a mushroom bed as a casing layer. Monitoring of the population build-up could possibly give an indication of the first colonising organisms.

PMB was pasteurised as described in section 3.5.according to the following regimes:-

$65^{o}C$ for sixty minutes

$85^{o}C$ for sixty minutes

Untreated material was used as a control. The pasteurising casing was used to case trays 56 x $56cm^{2}$ each containing 8kg compost. (see section 3.3.2 (a)).

4.4.3.1. Population studies.

Samples of 10g were removed from different areas of the tray and transferred to sterile universal bottles. From these larger samples, 1g samples were taken and serial dilutions were prepared as in section 3.1.4. for estimates of population. The samples were taken immediately after pasteurisation and then on days 3, 8 and 12 after application to the mushroom bed.

The results (figure 4.4.3) show that steam:air pasteurisation resulted in higher bacterial populations in the casing. Although the treatment had 80°C resulted in lower initial numbers of bacteria, the population soon rose to a similar level to the 65°C treatment. Both pasteurised and untreated material showed the same basic trends in numbers of bacteria.

4.4.3.2. Composition of the bacterial flora.

Although the initial colony types varied from one treatment to another (figure 4.4.4) by day 8 the colony types were the same on all treatments. This indicated that a casing soil appears to have a certain composition regardless of the initial bacterial flora.

4.4.3.3. Productivity.

The yield data showed that the pasteurisation improved the yield and also resulted in heavier mushrooms (table 4.4.1). Gliocladium disease was seen on the untreated material by second flush, but no trace was seen on any of the other treatments. There were no outbreaks of any other diseases so the chosen regimes were effective in this respect.

Figure 4.4.3. Estimates of bacterial populations from raw and heat treated PMB casing soils.

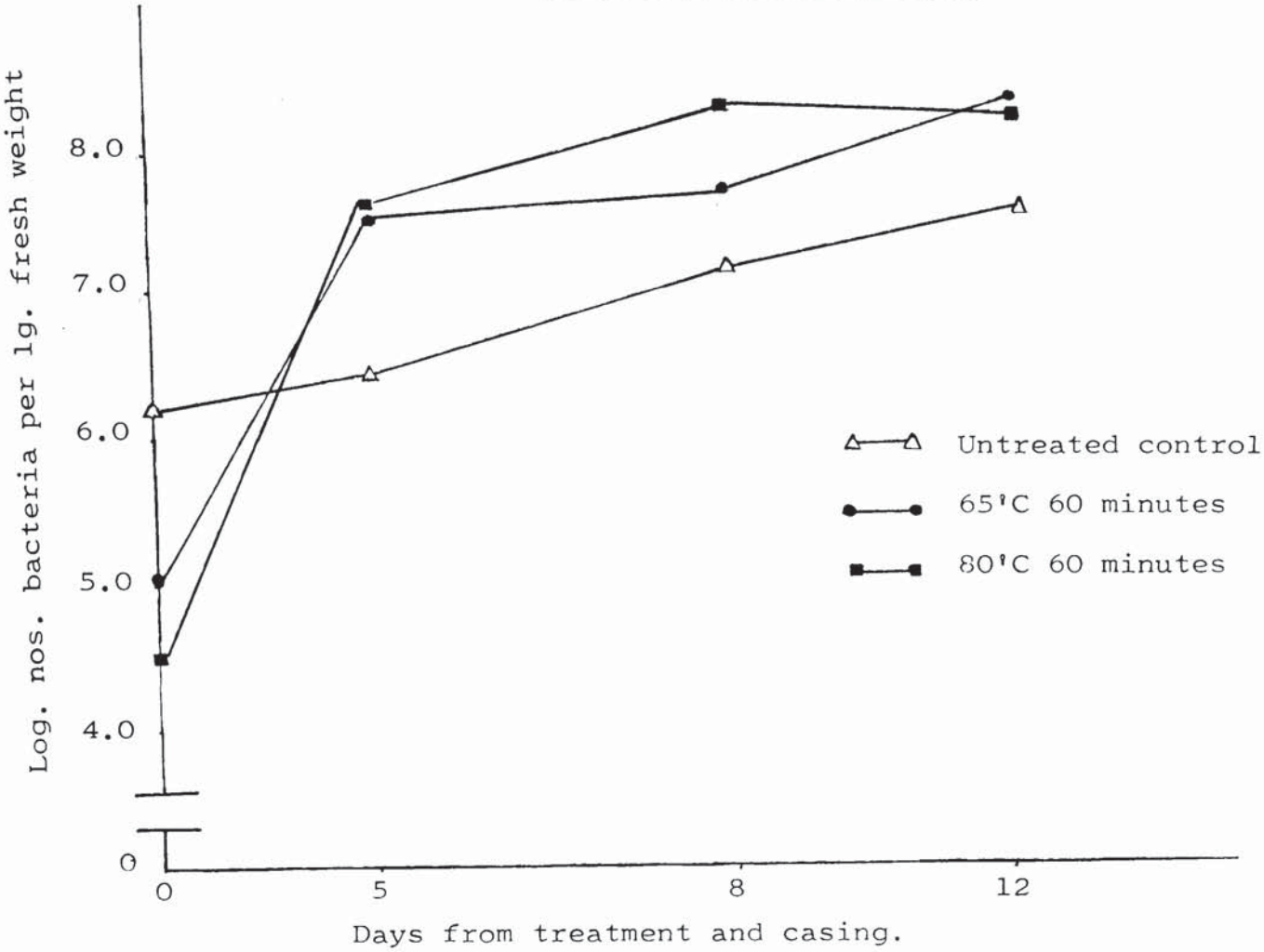


Figure 4.4.4. Frequency of occurrence of different colony types during recolonisation of heat treated PMB casing soil.

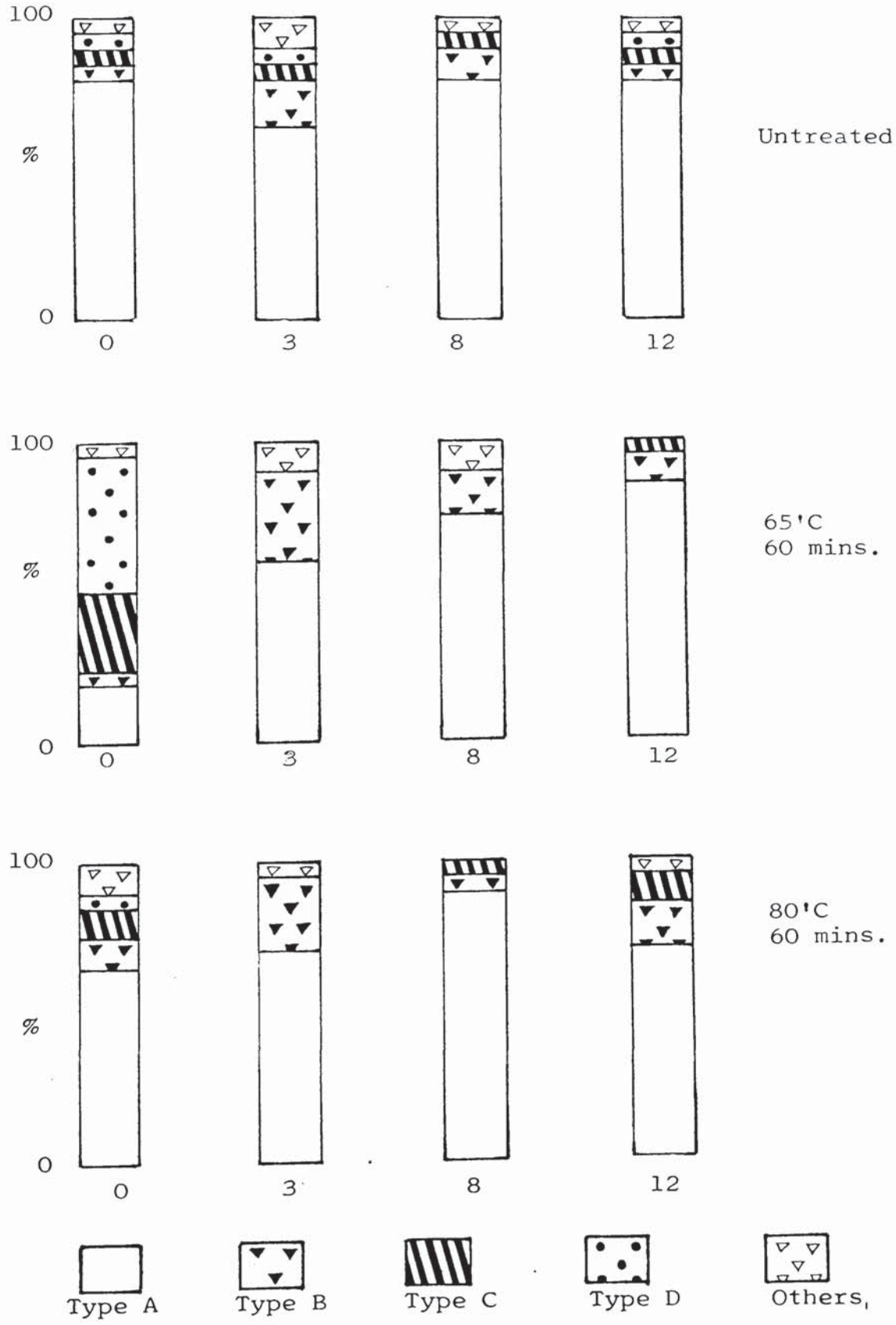


Table 4.4.2. Effect of heat treatment on yield from PMB casing.

Daily yield from raw and heat treated PMB casing soil.(8kg. fresh weight compost)

Days from casing	Untreated	65°C for 60 mins.	80°C for 60 mins.
20	276.3g.	-	-
21	249.0g.	-	-
22	112.6g.	169.0g.	74.4g.
23	18.9g.	508.4g.	128.9g.
24	-	135.7g.	647.1g.
25	-	-	-
⋮			
32	565.5g.	-	-
33	9.5g.	542.1g.	71.8g.
34	-	109.1g.	314.6g.
35	-	-	-
36	-	-	102.0g.
⋮			
42	188.9g.	214.2g.	156.7g.
Total yield	1420.7g.	1678.5g.	1504.5g.
Number of mushrooms	242	206	176
Mean weight per mushroom	5.866g.	8.148g.	8.548g.

There were no significant differences between treatments.

Various considerations had to be made when selecting a suitable regime of pasteurisation, namely yield and disease effects.

Most general observations indicated that the 65°C treatment for one hour was the most useful, giving little trouble with disease and pests and also good comparable yields.

4.5. Relationship between the bacterial flora of the compost and casing layers.

Work by Hayes *et al.* (1969) showed that no fruiting occurred in totally sterile conditions but sterile casing on non-sterile compost yielded mushroom fruitbodies. The experiments to date have indicated that numbers and different colony types of bacteria reached similar levels regardless of the casing material used. A peak in numbers of bacteria was seen at the time of primordia formation followed by a decline in populations to steady levels during cropping with a second peak in numbers coinciding with a decrease in yield. There were different colony types present in this second peak suggesting that the compost acted as a reservoir of bacteria which could then colonise the casing when conditions were suitable. At various stages of growing, there were bacterial colony types that had not been present at the time of casing. These types could have been present in proportions less than 1% or been present in the compost and moved upward into the casing. This study was designed to study the bacterial flora of the compost and casing layer and to examine any relationship between the two layers.

4.5.1. Populations of the compost and casing layers.

Five flasks were filled with spawned compost (section 3.3.2.b) which was not sterilised and were cased with sterile casing. One flask was sampled at each of five sampling times (days 0, 7, 16, 21 and 32)after casing. Numbers of bacteria and colony types were recorded.

The results (figure 4.5.1) showed that there was rapid colonisation of sterile casing by bacteria from the compost. By day 7 there was a higher bacterial population in the casing than the compost and after day 20 they remained at similar levels. The types of colonies present in the compost were also seen a few days later in the casing. Initially the proportions were similar but by day 21 there was no decline in the proportions of yellow type B in the casing as in the compost. By day 32, orange and pink colonies (C and F) were noticed in the compost and pink colonies indicated that they were present in very low numbers before day 32. (Figure 4.5.2).

4.5.2. Effect of casing renewal on bacterial populations and productivity.

Renewal of the casing would replace a layer colonised by bacteria that could be inhibitory to fruitbody formation with a fresh layer with a relatively low bacterial population and low electrical conductivity.

Figure 4.5.1. Numbers of bacteria in the compost and casing layer after casing.

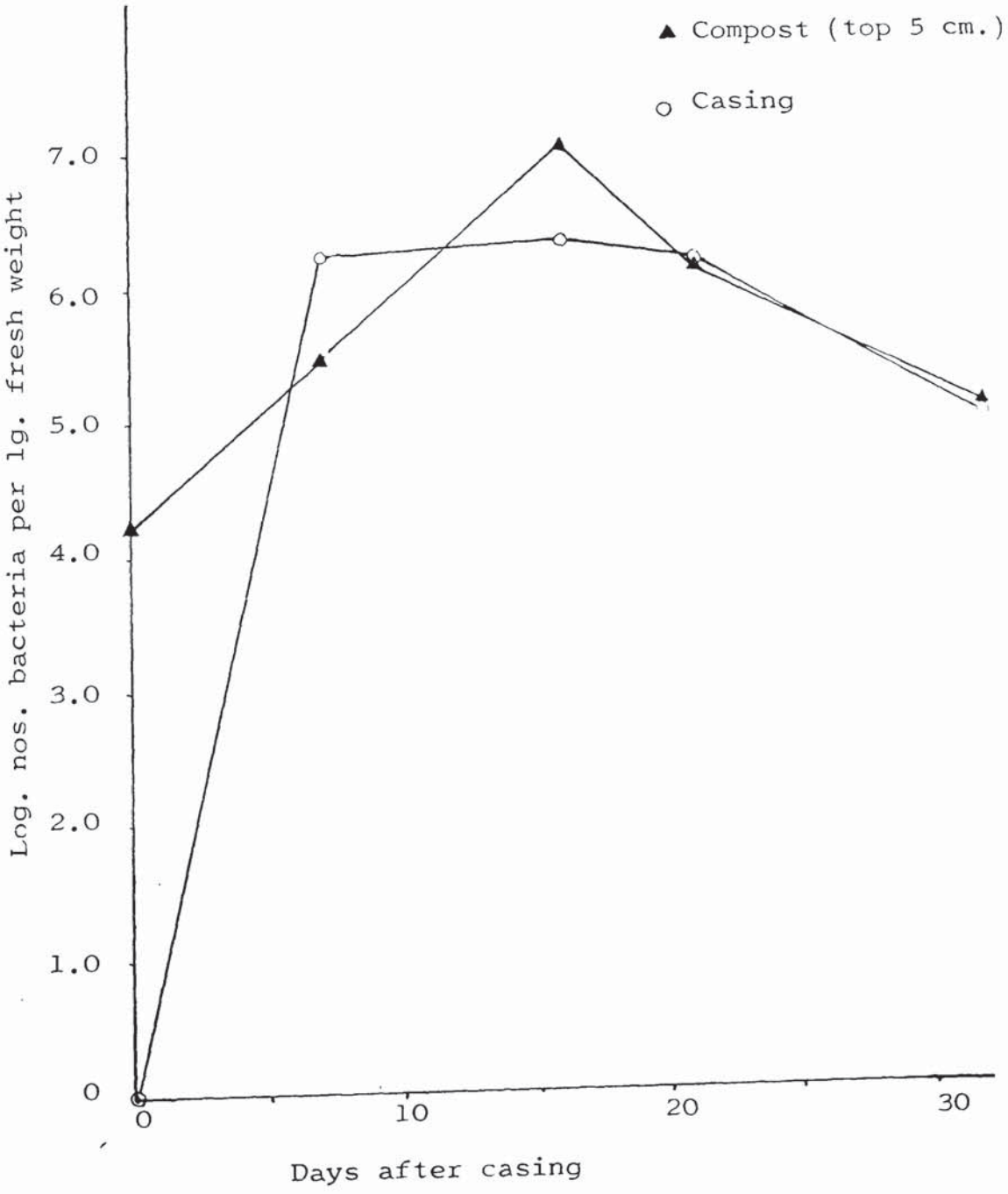
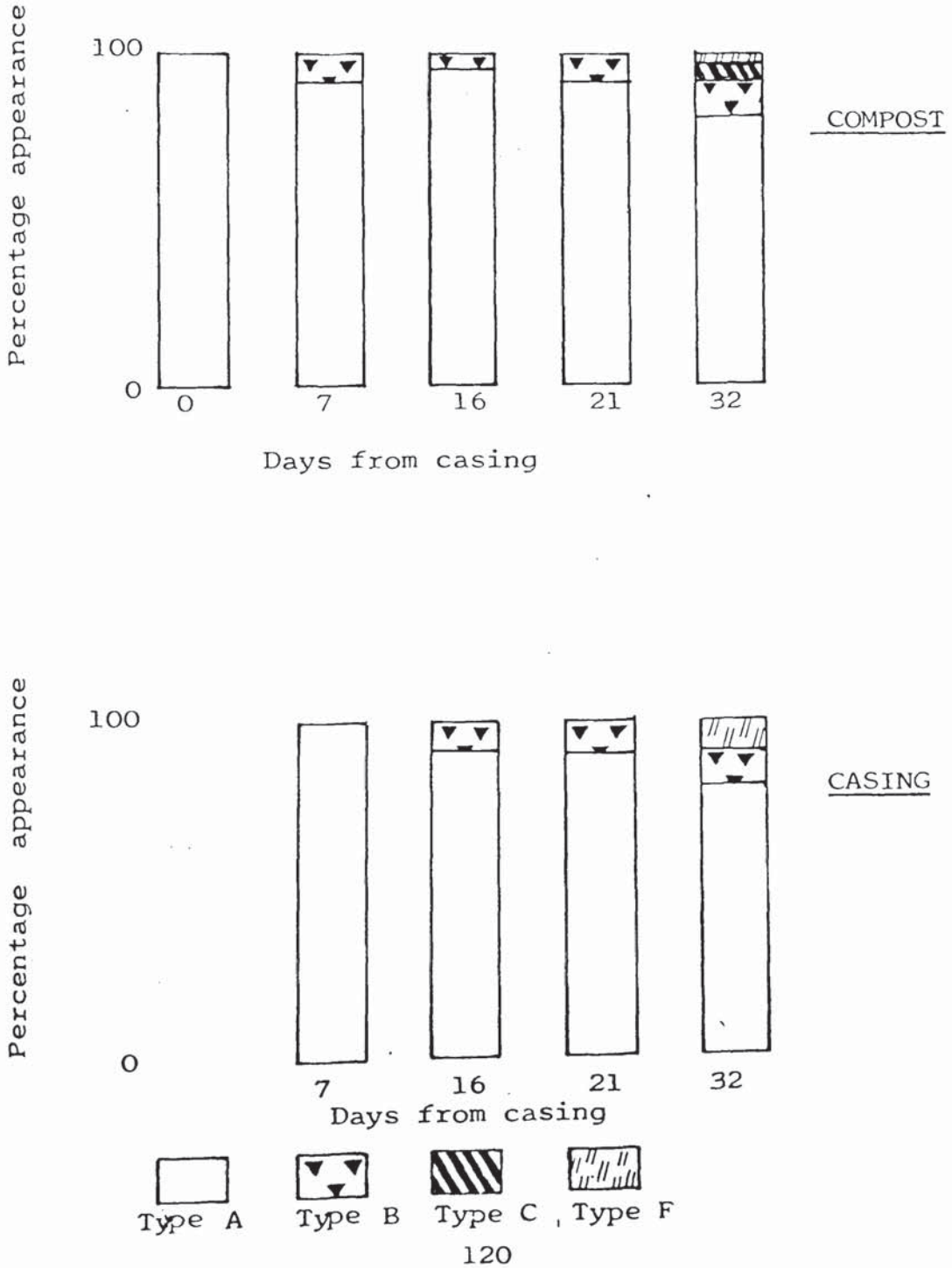


Figure 4.5.2. Composition of the bacterial flora in the compost and casing layer



This experiment was designed to study the effect of a fresh casing on the microflora at different times during cropping. The casing was removed after the first break, second break and third break and was replaced with fresh casing material. Numbers of bacteria were measured at the time of casing and then at seven day intervals after casing.

Growing techniques as in section 3.2.1. were used with 13 x 13cm^2 boxes. The boxes were cased with 200g of pasteurised PMB casing material.

Four treatments were used -

(a) control - no casing removal

(b) Removal of casing after first break

(c) Removal of casing after second break

(d) Removal of casing after third break

Eight replicate boxes were used per treatment.

Core samples were taken through the casing at the time of casing (day 0) and then on days 7, 15, 21 after each casing. Samples were taken from the control boxes at similar times as from each test box. Numbers of bacteria were estimated.

Figure 4.5.3 indicated the numbers of bacteria in the different treatments. Each treatment had a low bacterial population after the recasing but after seven days had a population higher than that of the control. The populations then declined slightly so that all treatments had similar numbers of bacteria.

After each recasing, the mycelium had to grow into the fresh casing, during which time no fruitbodies were produced. The first break of mushrooms produced on the fresh casing was higher than was produced by the control, i.e. the third break produced when recasing after the second break was higher than the third break produced by the control. The total yield from the (table 4.5.1.) treatment with casing removed after the third break was higher than the other two treatments which had reduced yield as compared with the control. Darlington 649 strain was used which produces a heavier second break and so recasing after the first break would be expected to cause greater disruption.

Casing renewal after the third break appeared to affect the growth least and the time for the mycelium to grow into the casing was shorter.

Figure 4.5.3. Populations of bacteria in PMB casing when casing was replaced during cropping

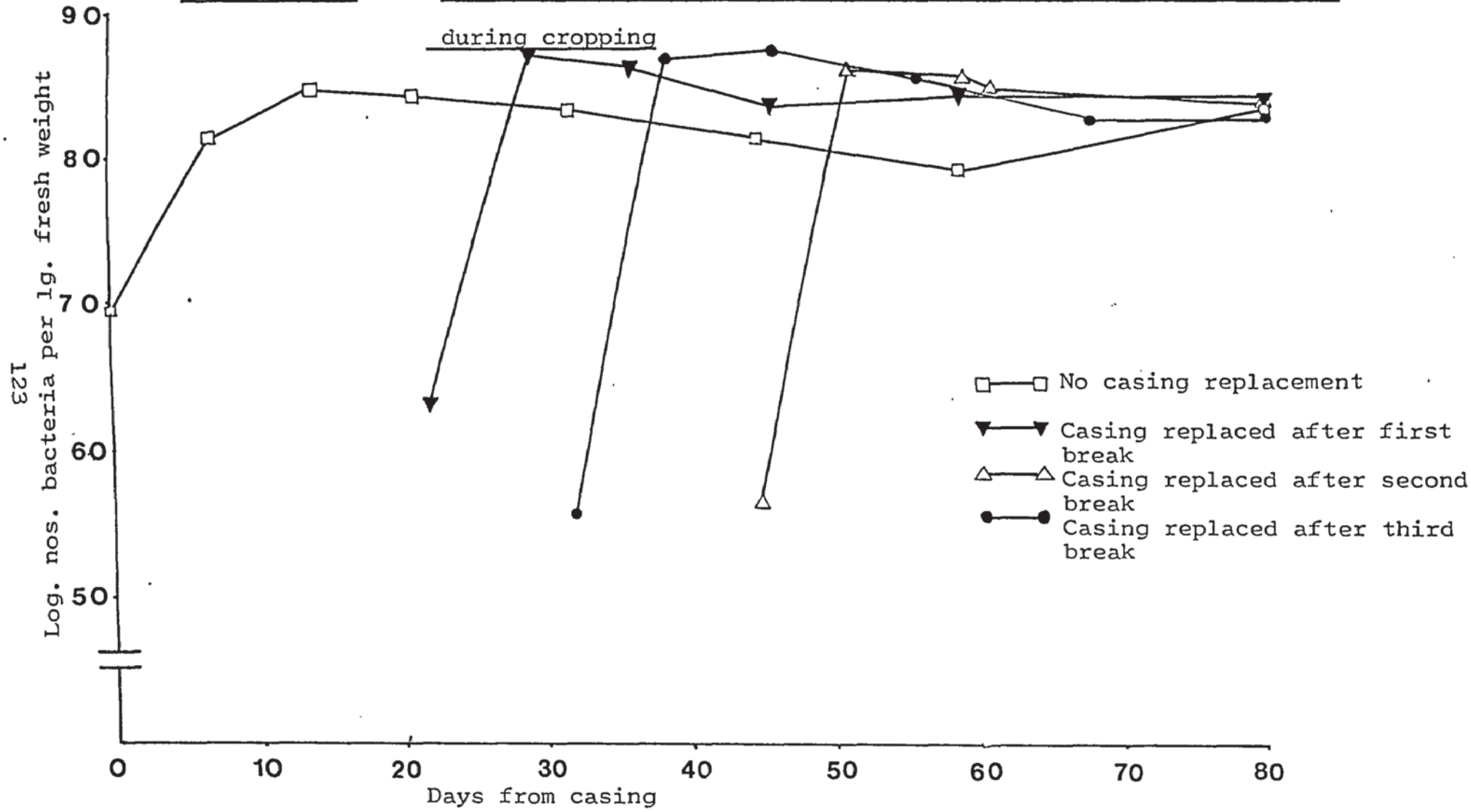


Figure 4.5.3. Populations of bacteria in PMB casing when casing was replaced during cropping

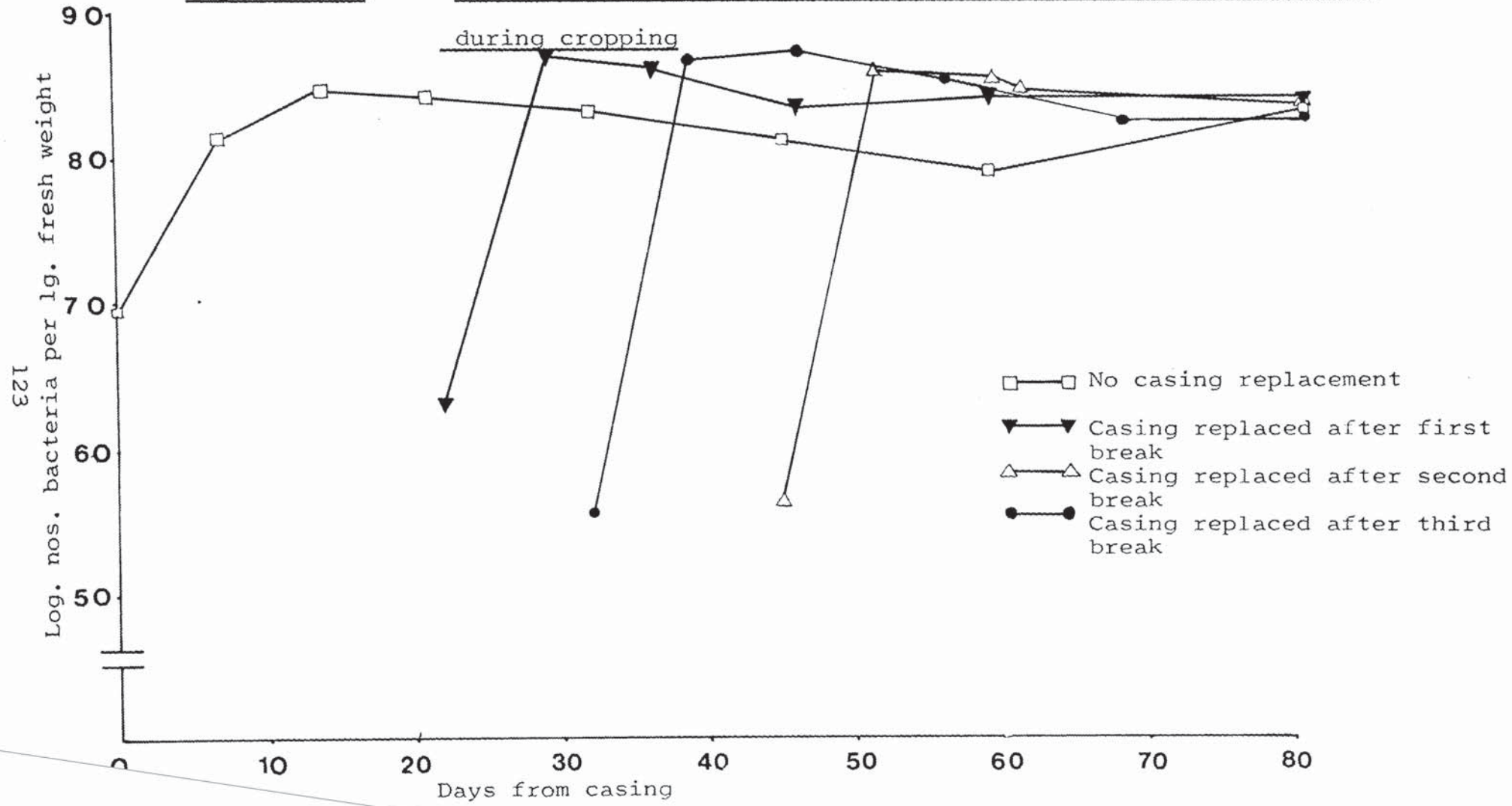


Table 4.5.1.

Effect of recasing at different times during cropping upon the productivity of PMB casing.

	Daily weight (g) picked per 8kg. compost			
Days from casing	Control	Recased after 1st break	Recased after 2nd break	Recased after 3rd break
19	21.7	64.2	19.2	48.9
20	301.9	239.0	221.1	277.3
21	88.1	149.0	171.4	102.2
⋮				
27	-	-	46.0	71.3
28	-	-	48.3	-
29	232.2	-	107.0	93.8
30	170.2	-	72.9	184.4
31	47.4	-	202.0	108.2
32	-	-	-	9.2
⋮				
35	5.6	-	-	-
⋮				
38	20.6	-	-	23.4
39	14.8	-	-	8.9
40	29.7	-	-	22.7
41	-	28.0	-	73.7
42	55.4	-	-	39.7
⋮				
45	39.0	57.3	-	30.7
46	14.8	144.4	-	-
⋮				
49	-	40.2	-	-
50	20.4	17.5	-	-
51	-	-	-	-
52	-	26.9	36.6	-
53	40.3	-	7.0	-

Table 4.5.1. contd.

Days from casing	Control	Recased after 1st break	Recased after 2nd break	Recased after 3rd break
54	30.1	9.4	31.0	-
55	15.0	52.5	52.5	-
56	18.2	86.1	40.2	-
57	23.8	47.2	-	-
58	-	-	-	-
59	-	35.1	-	-
60	17.1	-	44.2	-
61	-	-	17.0	-
62	21.1	-	22.8	-
63	30.0	17.9	43.8	60.7
:				
66	-	-	92.9	79.9
67	16.3	8.6	-	10.5
68	-	-	-	-
69	-	16.5	-	-
70	-	18.0	-	23.0
71	12.0	-	15.6	22.3
72	11.5	-	11.3	21.2
73	-	41.8	-	16.1
74	11.8	26.5	23.8	-
75	-	-	-	-
76	37.6	-	19.5	51.8
77	-	-	11.2	33.7
78	-	14.9	8.7	-
79	8.3	48.9	-	-
80	-	7.6	-	-
Total yield	1354.1	1197.5	1366.0	1413.6

Table 4.5.1. contd.

Days from casing	Control	Recased after 1st break	Recased after 2nd break	Recased after 3rd break
Mean per 1kg compost	169.26g ± 8.01g	149.70g ± 18.22g	170.63g ± 16.15g	176.29g ± 12.73g

Comparing Control with recasing after 1st break

t=1.93 $0.1 > P > 0.05$

Comparing control with recasing after 2nd break.

t=0.016 $P>0.1$ not significant

Comparing control with recasing after 3rd break.

t=0.968 $P > 0.1$ not significant

Comparing recasing after 1st break with recasing after 3rd break.

t=2.386 $0.02 > P > 0.05$ highly significant difference

Comparing recasing after 2nd break with recasing 3rd break

t=0.592 $P > 0.1$ not significant

The primordia began to form before the growth had reached the surface indicating that a shallower casing could have been added. Only eighteen days were lost by recasing after the third break as compared to twenty days lost with the other two treatments. The high numbers of bacteria after recasing were associated with the higher yield after recasing.

The electrical conductivities had increased initially and then declined at each recasing. They increased again after recasing but never reached the levels of the control (Yeo, pers. comm.).

4.6. The characteristics of casing bacteria and their association with A. bisporus.

During this study emphasis was given to gross changes in numbers of bacteria in a casing layer prepared from PMB and where appropriate comparisons were made with peat casing. Compositional changes have also been observed by comparing gross changes in the colony types detected from samples taken from a wide range of experiments reported in sections 4.1 - 4.5.

In view of the fact that PMB acts as a highly satisfactory casing medium, it is clear that the bacteria known to be required and associated with fruitbody formation in the casing layer are present and functional, in addition to those already considered in section 4.3 which were shown to be the agents in the well known diseases, bacterial blotch and drippy gill.

The precise role of these bacteria which are required for fruitbody formation are not known. Using peat casings, the work of Hayes *et al*. (1969) indicated that *Pseudomonas putida* and *Pseudomonas* Group IV organisms were the principal bacteria active in forming fruitbodies.

During the course of these studies, a representative range of all colony types were isolated into pure culture in an attempt to characterise further the nature of the bacterial populations in PMB soils and to study their action in pure culture and in association with *A. bisporus*.

4.6.1. Action of isolated bacteria in pure culture

Tests were carried out according to the first and second stage classification of Cowan and Steel (1970) (Section 3.1.5.) in order to determine the genera of a representative range of isolates. The results have been shown in tables 4.6.1 and 4.6.2. A number of the isolates tested were Pseudomonas species, with various other genera - Alcaligenes, Bacillus, Serratia and the Enterobacteriaceae group also represented.

A wide range of bacteria had been isolated from PMB from the Kemsley Mill site and from casing experiments and this identification showed that the dominant type A in population studies included Pseudomonas species, and bacteria of the Enterobacteriaceae group. Less dominant were types B and C (Flavobacterium species), Type D (Alcaligenes species), Types E and F (Serratia species) and other types including the Actinomycetes. These tests, however, gave very little indication of any effect with A. bisporus or functional aspects of these bacteria which could give information about their effect in cases soils

Table 4.6.1.

First stage classification according to Cowan and Steel (1970).

Code no.	Origin of isolate	Colony type	Shape of colony/ Grams reaction
27	PMB	A	-ve rod
28	PMB	A	-ve rod
31	PMB	A	-ve rod
32	PMB	A	-ve rod
59	Peat	A	-ve rod
60	PMB	A	-ve rod
74	PMB	A	-ve rod
94	PMB	A	-ve rod
3	PMB	A	-ve rod
4	PMB	A	-ve rod
10	PMB	A	-ve rod
46	PMB	A	-ve rod
98	PMB	A	-ve rod
5	PMB	A	-ve rod
47	PMB	A	-ve rod
92	PMB	A	-ve rod
12	PMB	D	-ve rod
16	PMB	D	-ve rod
62	PMB	D	-ve rod
81	PMB	D	-ve rod
93	PMB	D	-ve rod
51	PMB	E	-ve rod
7	PMB	B/C	-ve rod
64	PMB	A	+ve rod

Table 4.6.1. contd.

Code no.	Aerobic growth	Catalase reaction	Oxidase reaction	Acid from glucose	Oxidation/ fermentation	Motility	Suggested genus
27	+	+	+	-	O	+	Pseudomonas
28	+	+	+	-	O	+	Pseudomonas
31	+	+	+	-	O	+	Pseudomonas
32	+	+	+	-	O	+	Pseudomonas
59	+	+	+	-	O	+	Pseudomonas
60	+	+	+	-	O	+	Pseudomonas
74	+	+	+	-	O	+	Pseudomonas
94	+	+	+	-	O	+	Pseudomonas
3	+	+	-	+	F	+	Enterobacteriaceae
4	+	+	-	-	F	+	Enterobacteriaceae
10	+	+	-	+	F	-	Enterobacteriaceae
46	+	+	-	+	F	+	Enterobacteriaceae
98	+	+	-	+	F	+	Enterobacteriaceae
5	+	+	+	+	F	+	Alcaligenes
47	+	+	+	-	F	+	Alcaligenes
92	+	+	+	+	F	+	Alcaligenes
12	+	-	+	-	-	+	Alcaligenes
16	+	-	+	-	-	+	Alcaligenes
62	+	-	+	-	-	+	Alcaligenes
81	+	-	+	-	-	+	Alcaligenes
93	+	+	-	-	-	+	Alcaligenes
51	+	+	-	-	F	+	Serratia
7	+	+	-	-	O	-	Flavobacterium
64	+	+	-	-	-	+	Bacillus

Table 4.6.2.

Second stage classification according to Cowan and Steel (1970

Code no.	Gelatin liquefied	Citrate (Koser)	Citrate (Christensen)	Growth on MacConkey	NO_3-NO_2	NO_2-N_2 gas	Methyl red	Voges Proskauer	42 °C growth	38 °C growth	5 °C growth
27	-	+	-	-	-	-			+	+	+
28	+	-	-	-	+	-			-	+	+
31	+	-	-	-	-	-			+	+	+
32	+	+	+	-	+	-			+	+	+
59	-	-	-	-	+	-			+	+	+
60	+	-	-	-	+	-			+	+	+
74	+	-	-	-	-	-			-	+	+
94	+	+	-	-	+	-			+	+	+
3	-			-			-	-			
4	+			-			-	+			
10	+			+			-	+			
46	+			+			-	+			
98	+			+			+	-			
5	+			+							
47	+			+							
92	+			+							
12	+			-							
16	+			-							
62	+			-							
81	+			-							
93	+			-							
51	+			-		+	-				
7	+			-		+	-				
64	+			-							

Table 4.6.2. contd.

Code no.	Starch utilisation	Acid from carbohydrates 1) sucrose	2) lactose	3) mannitol	4) dulcitol	5) arabinose
27	-	+	+	-	-	-
28	-	+	+	-	-	-
31	+	+	+	-	-	-
32	+	+	+	-	-	-
59	+	-	-	-	-	-
60	+	-	-	-	-	-
74	-	-	-	-	-	-
94	+	-	-	+	-	-
3	-	-	-	-	-	-
4	+	-	-	-	-	-
10	-	+	+	+	-	-
46	+	+	+	+	-	+
98	-	+	+	+	-	+
5	-	-	-	-	-	-
47	+	-	-	-	-	-
92	-	-	-	-	-	-
12	-	-	-	-	-	-
16	+	-	-	-	-	-
62	+	-	-	-	-	-
81	+	-	-	-	-	-
93	-	-	-	-	-	-
51	-	+	+	+	-	-
7	-	-	-	-	-	-
64	+	-	-	+	-	-

+ positive reaction

- negative reaction

4.6.2. Interaction between selected bacterial isolates and A. bisporus.

(a) Effects on growth in Petri-plate culture.

In order to study the functional aspects of bacterial isolates in association with A. bisporus in casing soils, more information was needed about the interaction between these bacteria and the fungal mycelium. This study was designed to examine this interaction in pure culture in Petri-plates. Various bacteria were chosen for this study which had all been isolated from PMB. Because of the importance of Pseudomonas species in fruitbody formation (Hayes et al 1969), various Pseudomonas isolates were selected, and also a representative selection of the other genera. The isolates selected were cultures:-

27 Pseudomonas

28 Pseudomonas

74 Pseudomonas

10 Enterobacteriaceae group

62 Enterobacteriaceae group

81 Alcaligenes

51 Serratia

7 Flavobacterium

64 Bacillus

The single phase method demonstrated the influence of bacteria upon the vegetative growth of A. bisporus, whereas the two phase method was used to illustrate the variation in growth of A. bisporus on a relatively nutrient rich medium (malt extract agar) and a nutrient poor medium (water agar) analogous to the compost and casing layer of normal artificial culture. The effect of bacteria upon these two phases of growth could also be studied in this latter method. Supplementation with mineral salts at different strengths was used to simulate the casing layer at different times during cropping.

1. Malt agar plates - the single phase method.

The single phase method (section 3.6.a) revealed an inhibition of the growth of A. bisporus by some of the bacteria. Others did not influence vegetative growth. Effects may be summarised as follows:-

(i) Weak inhibition, the fungal mycelium gradually grew over the bacterial inoculum - cultures 74 and 51.

(ii) Strong inhibition of A. bisporus mycelium with no growth within 2mm of the inoculum and no growth over the inoculum - cultures 28, 81 and 10 (see plate 4.9).

(iii) No effect on the growth of A.bisporus - shown by cultures 27, 62, 7 and 64 (see plate 4.10).

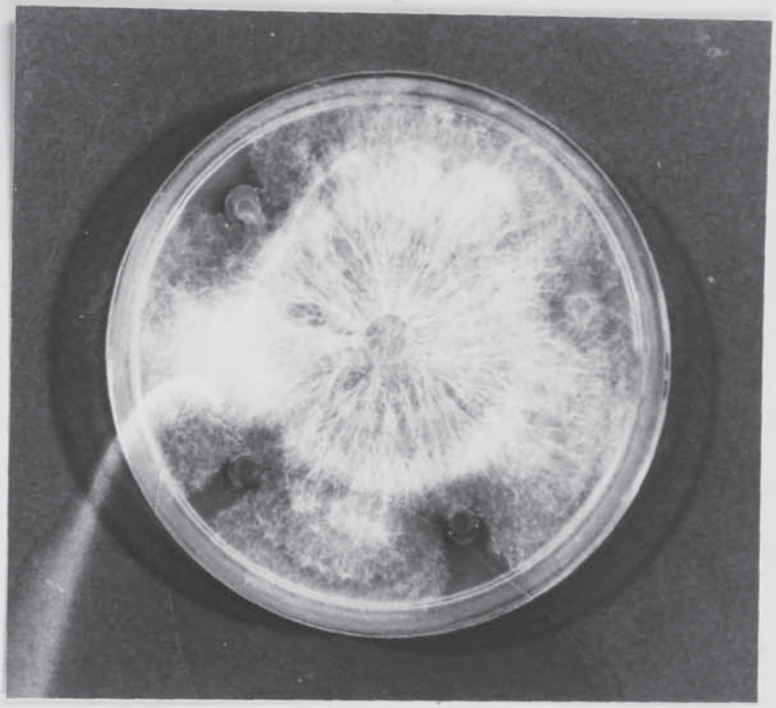


Plate 4.9. Strong inhibitory effect in single phase culture due to isolate 62 (Alcaligenes).

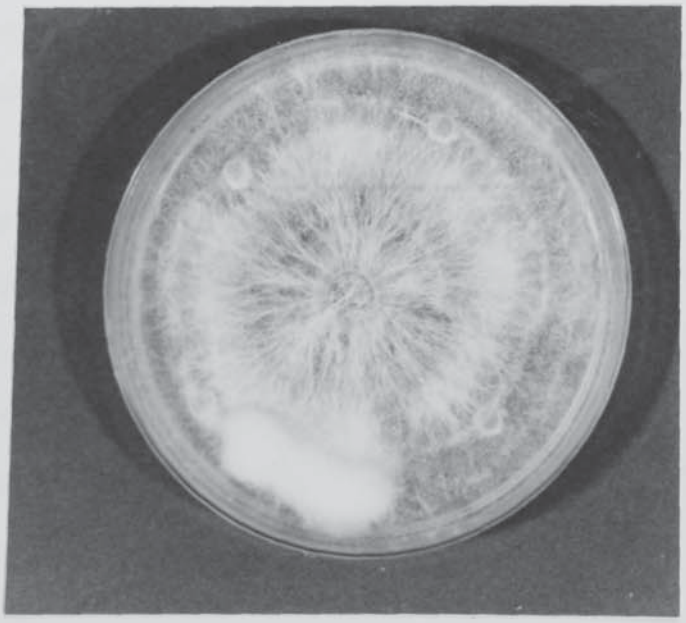


Plate 4.10. No inhibition of A.bisporus growth near bacterial inoculum.

2. Partitioned plates using malt agar and water agar

The two phase method (section 3.6.b) showed an acceleration of growth initially towards the inoculum and then the formation of thickened hyphal strands and hyphal aggregates. These effects were produced in varying degrees with all bacterial cultures. Where the medium was supplemented with mineral salts, there was an increase in fungal growth (table 4.6.3) depending on the strength of salts used. This effect was not influenced by the presence of bacteria. (See plates 4.11 to 4.14).

Using a mineral salts supplementation there was an initial increase in growth, with thickening of the hyphal strands and the formation of hyphal aggregates. Strands being defined as thickened hyphal elements and hyphal aggregates as clumps of normal hyphal elements forming distinct aggregates. The bacteria which produced an inhibitory effect on the growth of A. bisporus on the relatively nutrient rich layer in the single phase method and stimulated growth effect in the nutrient poor water agar layer in the two phase method. The presence of bacteria resulted in the thickening of hyphae and formation of strands as occurred in the media supplemented with mineral salts.

(b) Effects on pure cultures of A. bisporus in casing soil.

Following a study on the effects on pure cultures of A. bisporus in Petri-plates, an investigation of the effects in casing soil was necessary.

Table 4.6.3.

Effect of mineral salts addition to growth of A. bisporus in Petri-plate culture

Days of Growth	Mean* growth(mm/day) on water agar in two phase culture			
	1% strength	10% strength	100% strength	Control - no salt added
3	3mm	4mm	5.5mm	2mm
6	4.5mm	5.5mm	11.5mm	3.5mm
8	5.2mm	8.2mm	15mm	6 mm
16	8mm	14.5mm	22.5mm	12 mm

* Mean values from six plates per treatment

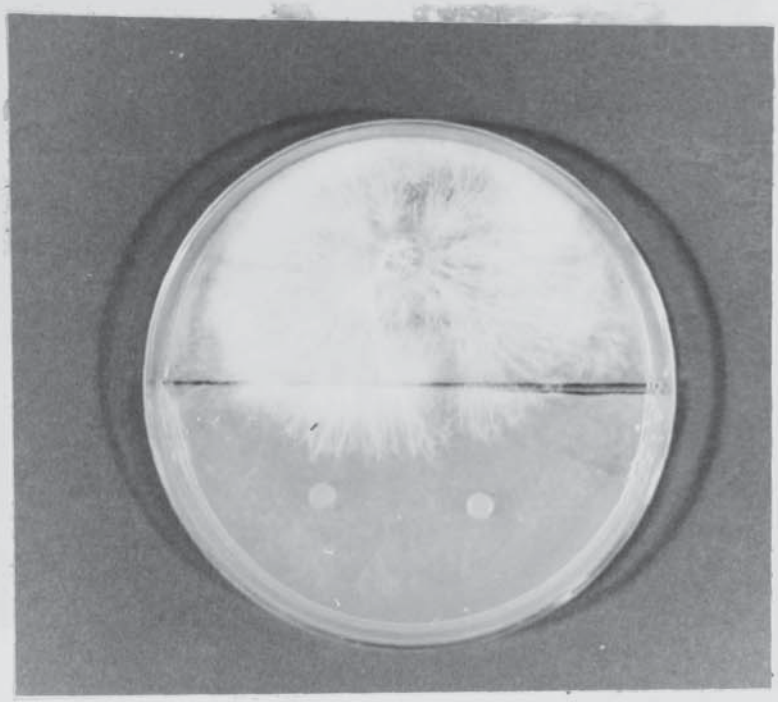


Plate 4.11. Control - A. bisporus growing in two phase culture on water agar.

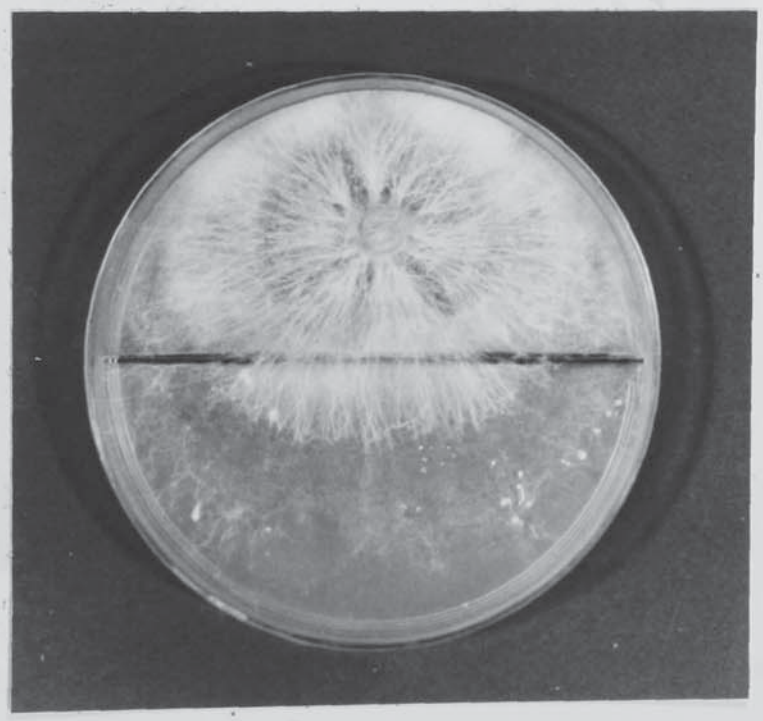


Plate 4.12. Control - A. bisporus in two phase culture growing on water agar supplemented with 100% strength mineral salts.

Plate 4.13. Culture 62 (Alcaligenes) on water agar, slight formation of strands only.

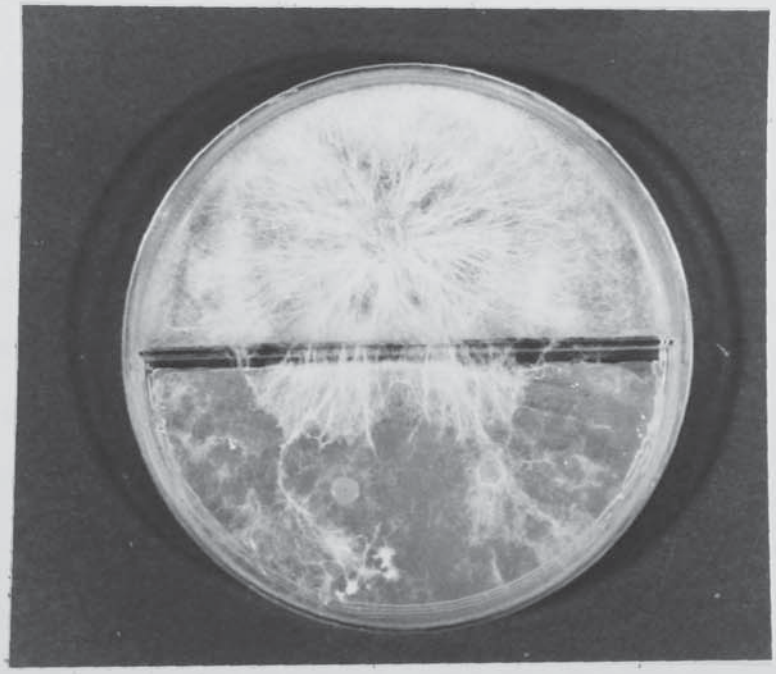


Plate 4.14. Culture 62 on water agar with 10% mineral salts, hyphal aggregates have formed at lower edges near bacterial inoculum.

Pure cultures of A. bisporus in sterilised compost were grown in flasks (section 3.3.2.b) which had been cased with sterilised casing inoculated with a selected bacterium. The flasks were examined daily for effects on growth of mycelium and formation of strands, hyphal aggregates, primordia and finally mature fruitbodies. In mushroom beds, many primordia form but not all develop to maturity, in this study the production of fruitbodies was carefully monitored as some flasks produced primordia, many of which did not develop. Other flasks produced few or no visible pins but finally yielded mature fruitbodies. The time taken to grow into the casing layer was also recorded. The results have been summarised in tables 4.6.4. and 4.6.5. (see plate 4.15). Cultures 27, 29 and 81 although producing no visible primordia yielded some fruitbodies whereas other cultures produced visible primordia which subsequently matured. Certain cultures, particularly 10, had a stimulatory effect on fruiting and strong primordia development followed by a good yield.

(c) Effects in normal artificial culture.

Techniques described in sections 3.3.1 and 3.3.2.a were used to prepare 13 x 13cm^2 boxes which were then cased with inoculated casing soil (section 3.1.6). Three boxes were prepared per isolate and six control boxes.

Table 4.6.4.

Effect of bacterial isolates on primordia formatio formation in sterile flask culture.

Culture	Nos. visible primordia at day 17 (mean 4 reps.)	(standard deviation)
Pseudomonas 27	0	(0)
Pseudomonas 28	0	(0)
Pseudomonas 74	6.25	(0.96)
Alcaligenes 62	7.25	(2.75)
Alcaligenes 81	3.75	(0.96)
Bacillus 64	9.25	(0.5)
Serratia 51	14.5	(1.73)
Flavobacterium 7	14.75	(3.20)
Enterobacteriaceae IO	29.0	(4.16)
Sterile control	0	(0)
Non-sterile control	52.25	(4.57)

Table 4.6.5.

Effect of bacterial isolates on time to harvest and yield in sterile flask culture.

Culture	Harvest time	Yield (g. fresh weight) 3 reps.total
Pseudomonas 27	-	0
Pseudomonas 28	-	0
Pseudomonas 74	-	0
Alcaligenes 62	-	0
Alcaligenes 81	33 days	19.25g.
Bacillus 64	24 days	17.7g.
Serratia 51	33 days	39.8g.
Flavobacterium 7	24 days	31.2g.
Enterobacteriaceae 10	24 days	56.45g.
Sterile control	-	0
Non-sterile control	20 days	60.08g

Plate 4.15. Sterile flask culture showing developing primordia.

Three control boxes had sterile casing and three others had normal pasteurised casing soil.

Core samples were taken through the casing on days 0, 5, 15, after casing and estimates of number and type of bacteria were made as in section 3.1.4.

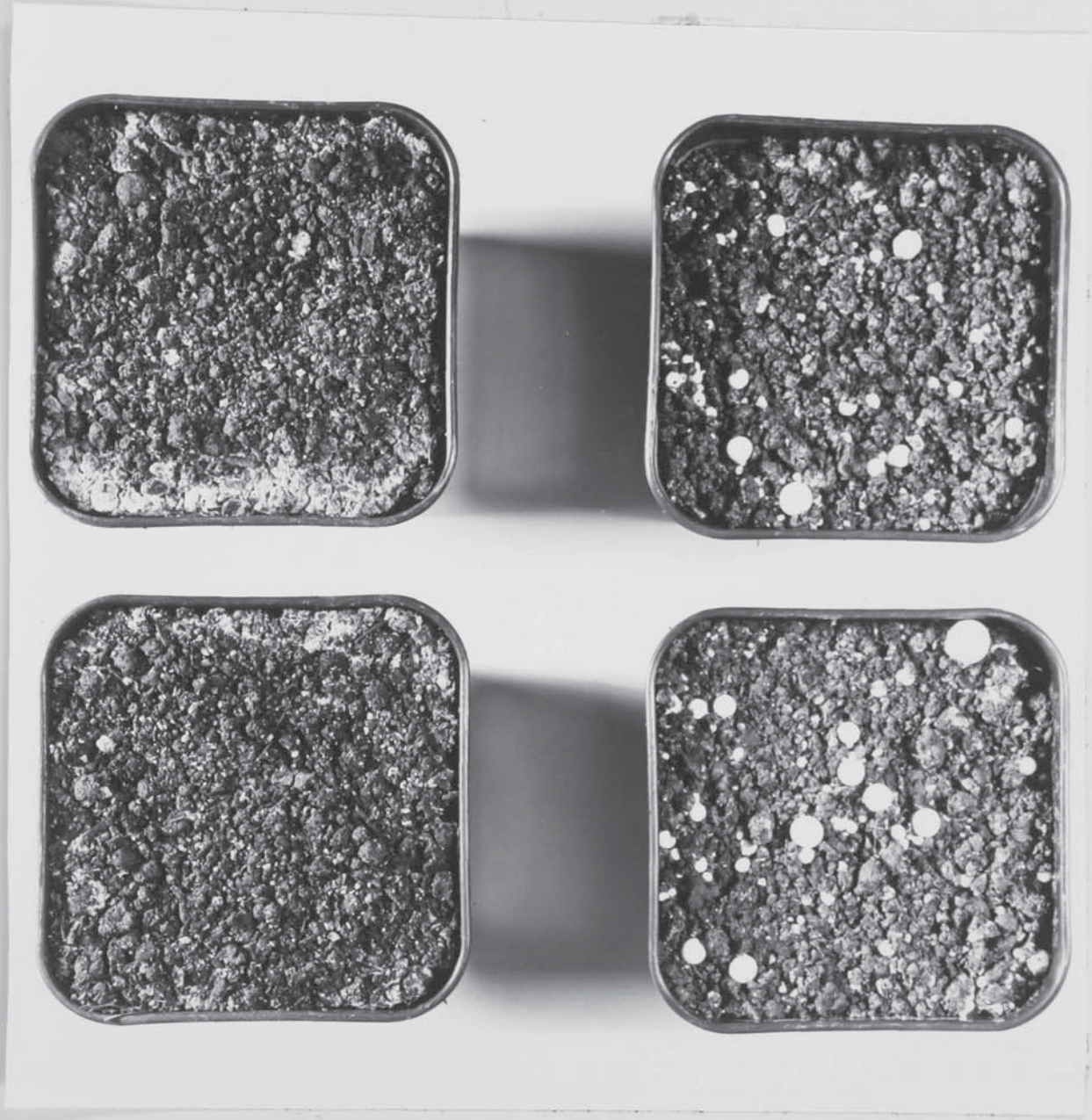
Growth of the mycelium through the casing, development primordia, final yield and time taken to produce fruitbodies per treatment were recorded. (plate 4.16)

As culture 51 - Serratia species could be easily identified due to its red pigmentation, it was chosen as an indicator.

Composition of the bacterial flora was expressed in terms of the proportion of the inoculated bacterium as compared to the rest of the flora using culture 51 to examine this effect.

Tables 4.6.6. and 4.6.7. summarised the effects on growth of primordia and yield and figure 4.6.1. illustrated the duration of dominance of an inoculated bacterium.

The results showed that groups other than Pseudomonas were also active in stimulating fruitbody formation including Alcaligenes, Flavobacterium, Serratia and Enterobacteriaceae group. The techniques used in these studies have indicated that these bacteria



A B

Plate 4.16. Open box culture with bacteria exerting a negative (A) or positive (B) effect on primordia formation.

Table 4.6.6.

Effect of bacterial isolates on primordia formation in normal artificial culture.

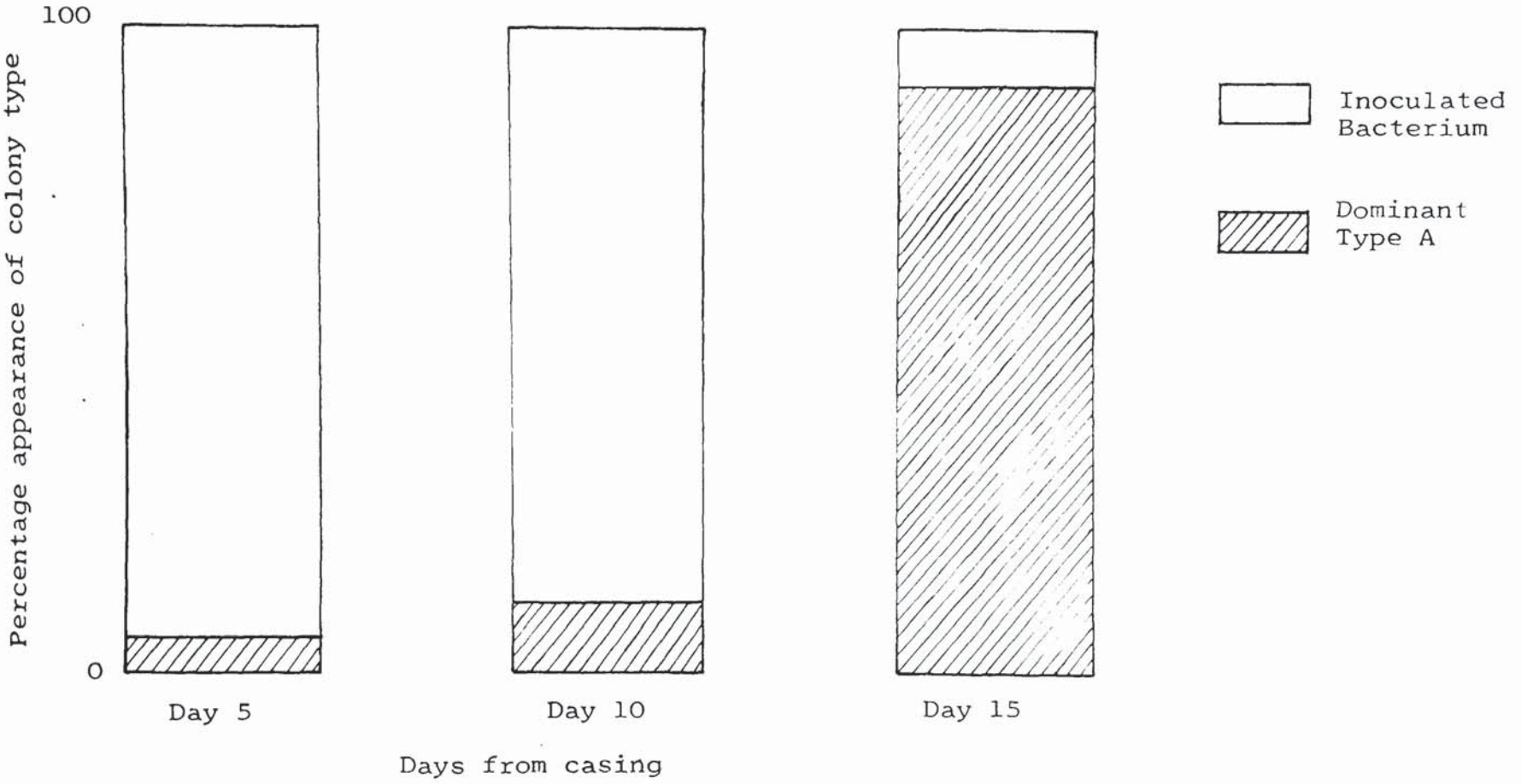
Culture	Nos. visible primordia at day 16 (mean 3 reps.)	(standard deviation)
Pseudomonas 27	0	(0)
Pseudomonas 28	0	(0)
Pseudomonas 74	2.67	(0.58)
Alcaligenes 62	6.33	(2.31)
Alcaligenes 81	14.0	(1.00)
Bacillus 64	10.33	(2.31)
Serratia 51	15.0	(1.73)
Flavobacterium 7	13.67	(0.58)
Enterobacteriaeae 10	23.33	(3.21)
Sterile control	2.67	(1.15)
Non-sterile control	28.67	(2.31)

Table 4.6.7.

Effect of bacterial isolates on time to harvest and yield in normal artificial culture.

Culture	Harvest time	Yield (g. fresh weight) mean 3 reps.	
Pseudomonas 27	24 days	22.6g	(9.43)
Pseudomonas 28	25 days	42.43g	(9.61)
Pseudomonas 74	24 days	33.63g	(7.97)
Alcaligenes 62	23 days	37.20g	(6.89)
Alcaligenes 81	33 days	19.20g	(17.06)
Bacillus 64	24 days	36.27g	(8.15)
Serratia 51	23 days	47.80g	(12.46)
Flavobacterium 7	25 days	30.13g	(6.21)
Enterobacteriaceae 10	23 days	48.33g	(3.69)
Sterile control	33 days	7.43g	(12.87)
Non-sterile control	22 days	59.3g	(11.17)

Figure 4.6.1. Establishment of dominant Type A bacteria in sterilised inoculated PMB casing soil.



are not always present in high numbers and the rapid decline in the proportion of an inoculated bacterium indicated that any effect must manifest itself before the fifteenth day after casing as by this time the dominant flora was the same as in the normal control.

Different degrees of effect were noted with the various cultures and in particular, culture 10 produced good primordia formation and yield, supporting the results of the previous section. Culture 81, however, had a markedly negative effect on yield and primordia formation.

4.6.3. Specific properties of some bacterial isolates from PMB casing soils

A wide range of genera have been identified from PMB but although some of their general properties were examined (section 4.6.1) their properties in relation to effects in casing soil were not investigated. Section 4.6.2. demonstrated a variation in effect of these bacteria on A. bisporus in vegetative and reproductive media but the mechanisms of these effects are unknown. Hayes (1972) and Nair and Hayes (1974) indicated the importance of iron in stimulating fruitbody formation and levels of phosphate have also been found to alter during cropping (Yeo, Personal communication), with phosphate levels in compost declining during cropping and no detectable levels in the casing layer. Availability of the bicarbonate ion was shown to have an effect on microbial activity of the casing layer (Nair and Hayes 1974, Perally 1978) and the presence of ethanol (Hayes et al 1969, Hayes 1976) has also been implicated in fruitbody formation. This investigation was designed to study these factors in association with the selected bacteria.

The methods outlined in section 3.1.6. were used.

(i) Iron - table 4.6.8 - eight out of nine cultures reduced soluble iron as $FeCl_3$ and three of these cultures 10, 62 and 7 also solubilised and reduced insoluble iron as Fe_2O_3. Culture 81 had no effect on iron in either form. Measurement of water soluble iron from the flasks in section 4.6.2.b revealed differences in levels in the casing as compared with the control (Mosley, Personal communication). Cultures 81 and 64 had significantly higher levels than the normal control ($P=0.05$) and the reducers of iron were significantly lower than the control.

(ii) Phosphate - table 4.6.9. - five out of nine cultures solubilised phosphate, two cultures, 74 and 7 produced no change in phosphate concentration other than would be caused by a change in pH of the medium, culture 81 solubilised phosphate but less than would be expected by change in pH (Culture 64 sample was damaged before determination).

(iii) Bicarbonate and ethanol utilisation - growth in the media was scored as in table 4.6.10. Only culture 81 was able to utilise bicarbonate as sole carbon source. Cultures 27, 62, 10, 7 and 81 utilised ethanol as their carbon source.

Table 4.6.8.

Effect of bacterial isolates on the reduction and solubilisation of ferric iron.

Culture		Reduction of soluble iron	Reduction of insoluble iron
Pseudomonas	27	+	-
Pseudomonas	28	+	-
Pseudomonas	74	+	-
Alcaligenes	62	+	+
Alcaligenes	81	-	-
Bacillus	64	+	-
Senatia	51	+	-
Flavobacterium	7	+	+
Enterobacteriaceae	10	+	+

+ reduction - no reduction

Table 4.6.9.

Effect of bacterial isolates on solubilisation of phosphate.

Culture		Solubilisation of PO_4	ppm	PO_4
Pseudomonas	27	+	55	
Pseudomonas	28	+	64	
Pseudomonas	74	-	49	
Alcaligenes	62	+	55	
Alcaligenes	81	+	60	
Bacillus	64	Sample damaged	-	
Serratia	51	+	127	
Flavobacterium	7	-	51	
Enterobacteriaceae	10	+	78	
Control			47.3 $\pm$	3.84

+ solubilisation of PO_4 - no solubilisation

Table 4.6.10

Effect of bacterial isolates on utilisation of ethanol and solubilisation of bicarbonate

Culture		Ethanol utilisation	Bicarbonate utilisation
Pseudomonas	27	+	-
Pseudomonas	28	+	-
Pseudomonas	74	-	-
Alcaligenes	62	+	-
Alcaligenes	81	+	+
Bacillus	64	-	-
Serratia	51	-	-
Flavobacterium	7	+	-
Enterobacteriaceae	10	+	-

+ solubilisation and utilisation - no solubilisation and utilisation

Table 4.6.11 (Appendix IV) summarises the various properties studied in this section and any relationship between ability to stimulate growth, primordia formation and these various specific properties. The bacteria able to solubilise and reduce iron were consistently good at producing fruitbodies in flask culture and in normal artificial culture. Four cultures (28, 74, 51 and 64) that did not utilise ethanol also did not solubilise ferric iron. There was no relationship between phosphate utilisation and primordia and fruitbody production shown by these tests. Generally similar results were obtained in both sterile flask and open box culture methods.

5. GENERAL DISCUSSION AND CONCLUSIONS

At present there are no acceptable commercial uses for the solids which are separated from the total effluent arising from pulp and paper manufacture. The solids, which consist primarily of lignin cellulose fibres and clay, account for approximately 8% by weight of the total effluent and at Bowaters (U.K.) Ltd., Paper Mill at Kemsley in Kent, approximately 100 tons of dry solids are produced per week. These are deposited on land near to the mill. This study has explored the possible use of these solids, now referred to as Paper Mill By-product (PMB), as a casing medium in mushroom culture.

The satisfactory functioning of the casing layer is known to be related to its water holding properties, physical, chemical and biological characteristics. In this study, emphasis has been given to the biological properties related to bacterial activity which appear to be essential for the formation of fruitbodies of the commercially cultivated mushroom, Agaricus bisporus.

PMB is only suitable as a casing medium some 18 months after its deposition on waste land and thus the biological factors which are associated with this change during the ageing are of relevance not only in identifying the factors, which are of importance in its

subsequent usage in mushroom culture, but may provide a basis for accelerating the ageing process. Parallel studies by Yeo have revealed important changes in the chemical properties of PMB and will be discussed where relevant to findings revealed in this work.

Relevant changes in the flora of PMB following deposition

Except for the very early stages after deposition, the numbers of bacteria followed a similar pattern irrespective of depth of sample, but as the solids became progressively drier, numbers increased to a maximum 6 months after deposition and then declined to the lowest level during the following 6 months. After this time numbers increased gradually, the increase in numbers of bacterial feeding nematodes followed the pattern of decline in bacterial populations.

The appearance of Basidiomycete fungi precedes the colonisation of the solids by green plants and since the solids are at this time (18 months after deposition) most suited for use as a casing medium, it can be seen that their suitability coincides with the natural ecological sequence of dominant life forms viz. bacteria, nematodes - fungi - green plants.

While the natural ecology of PMB at Kemsley Mill site is influenced by local factors, it was of interest to observe that when the solids were exposed in funnels at the University of Aston, fungi also developed after 7 months. However, unlike the Kemsley Mill site, nematodes were absent, suggesting that these invade from the underlying land. Although the experience in funnels was limited in scope, it clearly indicated that the principal controlling factor in the colonisation of PMB by bacteria and fungi was the decline in soluble salts as a result of leaching by rainwater. It also indicated possible approaches to the acceleration of the natural weathering process by artificial means.

The bacterial ecology of PMB when used as a casing layer in mushroom culture.

From the quasi-qualitative estimates of the bacterial flora in both the traditionally used peat based casing soil and PMB, bacterial composition did not differ markedly in the two soils. No major differences were apparent in the changes in total numbers throughout cropping when both soils were compared. As in other studies (Hayes 1974), a peak in numbers formed at the onset of fruitbody formation, but following the first harvest a marginal decline in numbers in the casing layer was followed by a peak in numbers following the third break (Figure 4.2.1). Changes in the composition of the bacterial flora were based on colony type and certain types, primarily

groups A - Pseudomonads and the Enterobacteriace and C/D - Acaligenes were evident at primordia formation but following the third break and coinciding with the decline in cropping, a different flora was evident including Flavobacterium sp. and Serratia sp.

Although productivity expressed in fresh weight of harvested fruitbodies were the same for the two casing soils, PMB casing soils produced fewer primordia at the induction of fruitbody formation, probably a reflection of the marginally lower numbers of bacteria and fewer fruitbodies were harvested. As a result, larger fruitbodies with higher dry matter were produced (table 4.3.2.). In addition to this quality characteristic, generally whiter fruitbodies were produced on PMB casing soil (table 4.3.3). This characteristic is of considerable relevance in the commercial production of mushrooms in the U.K. (Pearson and Hayes 1978). The appearance of occasional blotched mushrooms on raw PMB indicated the presence of the common and commercially significant condition known as bacterial blotch, caused by Pseudomonas tolaasi, as frequently found in peat. In addition, another bacterial disease known as "drippy gill", caused by Pseudomonas agarici, was observed in fruitbodies from PMB and this, together with a new fungus disease caused by Gliocladium deliquescens series

(Yeo, personal communication) indicated the requirement for a pasteurisation treatment in order to eradicate these diseases.

For this purpose, the use of a mixture of steam and air was adopted in an attempt to prevent the incidence of these organisms. Thermal death point studies of the pathogenic organisms in pure culture indicated that these were destroyed when maintained at temperatures at 55°C for one hour. Pasteurisation for one hour at 65°C was found to be effective in the control of the diseases and this treatment would seem to be the most appropriate for commercial application. Heat treatment of soils however is known to affect the biological and chemical balance of soils and may seriously influence plant growth (Baker 1971). This effect was also shown to be applicable to A. bisporus culture using PMB. Treatment at 80°C for one hour resulted in a 12% reduction in the productivity of PMB casing compared to 65°C for one hour treatment. Reduced numbers of bacteria and/or the demonstrated compositional changes could have been responsible for this effect.

The influence of the compost layer on the bacterial ecology of the casing layer.

A casing soil when sterilised does not support the development of fruitbodies (Eger 1961, Hayes et al. 1969). This effect was repeatedly confirmed in these studies. Up to the time of the present study, single isolates from a peat based soil, when reinoculated into a pure culture of A. bisporus restored the capacity of A. bisporus to fruit, indicating that some isolates are directly concerned with the critical "switch" from vegetative to reproductive growth (Hayes et al, 1969).

Peat based casing soils were shown by Hayes et al (1969) to be dominated by Pseudomonads, some species of which were responsible for fruitbody formation and concluded that these were selected for, following application of the casing soil on to a mushroom bed.

While single isolate studies are of relevance in identifying the action of different isolates, in practice, the casing soil is a mixed culture containing both beneficial and harmful organisms. The compost layer also acts as a rich source of bacteria (Stanek 1969, and Hayes 1977) even when colonised following vegetative growth. In the course of this study a significant relationship between these two layers was established. The bacteria colonising the

upper layers of compost were found to be comparable to those colonising a sterilised casing layer following its application to a nonsterilised commercial compost. Numbers reached similar levels after 12 days (figure 4.5.3) indicating a comparatively rapid colonisation, the compost layer serving as an inoculum and possibly a direct migration of bacteria is possible. It was suggested in section 4.2. that the small peak in the bacterial flora that occurred after third break was associated with the decline in cropping which is characteristic of mushroom cropping patterns. It was shown that this decline could be checked by recasing the beds at different time intervals during cropping. Significantly, when done after third break, more fruitbodies and a better yield were obtained over a 80 day cropping period compared with the control casing soil. Furthermore, it was demonstrated that after recasing the populations rose initially to levels above the control but then declined slightly and maintained similar levels despite recasing at different times. Other factors - physical condition and chemical factors (Yeo, personal communication) showed that water holding capacity was reduced and there was an increase in levels of soluble salts (Yeo, personal communication) and these together with the changes in bacterial populations proved that the casing layer, rather than the popularly held view

that decline in nutrient levels, was responsible for the decline in crop yield.

The significance of the bacterial ecology of the casing soil.

Ecological studies of bacteria in natural substrates are of necessity restricted to gross changes in groups. Little information can be gained on changes in the activity or occurrence of specific isolates at a given time or under a given set of conditions. Enrichment techniques cannot be readily applied since very little information is available on the specificity or requirements of bacteria which are associated with fruitbody formation. While in this study, the total effect of the bacterial population in PMB casing soils has been monitored and related to different effects on the development of fruitbodies in casing soils, the techniques of estimating number and composition facilitated the isolation of representative types and permitted the study of single isolates in pure culture.

In the range of bacteria isolated from PMB, a number of bacterial genera were represented. Some isolates when tested on a malt agar medium were shown to be inhibitory to the advance of A. bisporus mycelium but when placed alongside A. bisporus on a mineral salts medium in the divided Petri plate technique, no inhibitory effects could be demonstrated. However, the inclusion of salts in the

medium designed to mimic the salt in the casing layer stimulated growth but also appeared to intensify strand formation. The aggregation of hyphae to form initials and occasionally primordia was caused by the addition of bacteria.

Whilst these plate studies clearly demonstrated the relevance of the chemical interaction of bacteria with A. bisporus mycelium, more significance could be attached to their action in flask culture where axenic cultures of A. bisporus could be maintained. However the same isolates which induced formation of hyphal aggregates and primordia did not cause formation of primordia in sterile flasks, clearly indicating that for primordia formation in a casing soil of conventional cultures, other factors are operating in addition to the bacteria. A marked difference was found between the individual isolates (table 4.6.4.). Of the three Pseudomonads selected for test, only one induced primordia formation but no fruitbodies were produced. Similarly, isolate 62, a species of Alcaligenes, produced only primordia whilst another isolate of Alcaligenes (81) developed fruitbodies.

Three isolates, namely Serratia (51), Flavobacterium (7) and Enterobacteriaceae (10) were exceptionally effective

in stimulating both primordia and fruitbodies.
Two other cultures, Bacillus (64) and Alcaligenes (81) also produced fruitbodies but the yield was much lower, and the time to harvest varied with two isolates taking 33 days to produce mature fruitbodies compared with the 24 days for the other isolates. These findings suggest that some species act only in the formation of primordia whilst others are effective in inducing their development to fruitbodies. The superior isolates in flask culture were also superior in open box culture and the role of the unsterilised compost layer in influencing the composition of the casing layer was also observed in the open box system. In this non-axenic system, other bacteria may have colonised the casing layer. The compost layer has already been discussed as a reservoir for invasion.

Evidence from the easily observed isolate (51) (Figure 4.6.1) a superior isolate, strongly suggests that dominance is maintained for only about 12 days, thus suggesting that the action of this isolate, at least, is expressed in the initial phase.

Problems relating to the function of bacteria in the formation of fruitbodies.

The functions of bacteria associated with primordia and fruitbody formation has not been resolved by these studies.

However, the variability of different isolates from PMB casing soil strongly suggests that in the artificial culture system, different species exert different effects some acting negatively and others positively. A successful and high yielding culture would thus be more influenced by the positive bacteria rather than by those exerting a negative effect. Such an effect could explain the variability in crop performance encountered in commercial practices.

It was not possible to relate specific properties of an isolate to the degree of its action on primordia and fruitbody formation (table 4.6.11). Emphasis was given to those properties which have been postulated by various workers to be involved in fruitbody formation. However, the most positive isolates in these culture tests, an Enterobacteriaceae species (10), was shown to solubilise iron, reduce ferric iron, solubilise phosphate and utilise ethanol. In natural conditions of artificial culture it seems likely that the mixed population of bacteria exert a total effect as noted above.

Concluding Remarks.

This research demonstrated the possibility of utilising paper waste as an alternative to peat as a casing medium in mushroom culture. When pasteurised at 65^{o}C for one hour, pathogens and animal vectors of mushroom diseases

are eliminated. Mushroom fruitbodies harvested from PMB based casing soils are of superior quality to mushrooms harvested from peat. Large scale commercially based trials are justified.

The suitability of PMB as a casing medium is dependent on an 18 month ageing process during which changes in bacterial populations, physical and chemical properties occur.

Following its application to the compost layer in mushroom culture, the composition and population changes in bacterial numbers follow the same pattern as in peat based soils. The influence of the bacteria in the compost layer has been demonstrated in this study and the benefits of replacing the casing layer during cropping may be related to bacterial ecology, but other factors such as salt accumulation (Yeo, personal communication) may also contribute to the benefit. Such a practice deserves further study, especially since it could maximise productivity from deep beds. It has been shown that different isolates exert different effects in plate culture, sterile flask culture and in open box culture. In addition different isolates exert qualitatively different effects on primordia and fruitbody formation. No individual isolate tested produced primordia or fruitbodies equal to or better than

the normal culture and in view of the fact that no common factor could be related to their action in the formation of fruitbodies in the casing layer, it is suggested that the complementary effect of a mixed population may be more relevant to an understanding of mechanisms.

Mineral salts have been demonstrated to influence the morphology of developing colonies in plate culture. Consideration should be given to other chemical factors likely to relate to bacterial activity in the casing layer, amino acids, vitamins and other organis compounds that may be secreted into the environment of the casing layer.

APPENDICES.

APPENDIX I - Media

Phosphate buffered saline solution (PBS)

NaCl	8.0g
KH_2PO_4	0.34g
K_2HPO_4	1.21g
H_2O (deionised)	1000ml

pH adjusted to 7.3 Media sterilised at 121°C for 15 minutes after dispensing into required volumes.

2% Malt extract agar.

Malt extract (Boots Pure Drug Co.)	20g
Agar (Oxoid No.3)	15g
H_2O (deionised)	1000ml

pH adjusted to 7.5 by the addition of N/10 sodium hydroxide before autoclaving.

Water agar.

Agar (Oxoid No.3)	15g
H_2O	1000ml

Mineral salts solution for addition to water agar.

$NaNO_3$	3.2g
KCl	0.8g
$MgSO_4$	0.8g
KH_2PO_4	1.6g
$FeSO_4$	0.016g
H_2O (deionised)	200ml

pH adjusted to 7.5 by addition of N/10 sodium hydroxide.

King's B agar

Proteose peptone	20g
Glycerol	10g
$MgSO_4 7H_2O$	1.5g
K_2HPO_4	1.5g
H_2O	1000ml

pH adjusted to 7.0 with sodium hydroxide and 20g Oxoid agar no. 3 added. Medium sterilised at 121°C for 15 minutes.

Antibiotics added according to Sands and Rovira (1970)

Penicillin	75 units/ml
Albamycin (Novobiocin)	45 g/ml
Actidione (Cycloheximide)	75 g/ml

Add to 3ml 95% ethanol to dissolve and make up to 50ml with dionised water and filter sterilise.

Add 5ml to 95 ml media (Kings B agar) mix gently and pour plates as required.

Skerman's carbon free basal salts medium

Prepare the following solutions:-

1. N NaOH 1.1 Sterilise at 121°C for 20 mins.
2. 0.074M H_3PO_4 1.1 Sterilise at 121°C for 20 mins
3. Using soln. 2 above, take 200ml and dilute to 2000 ml (0.0074M), sterilise as above.
4. Using soln. 3 above, take 1000 ml and neutralise with N NaOH, sterilise as above.
5. NaCl 3.0g solvent 0.0074M H_3PO_4
6. $(NH_4)_2SO_4$ 6.6g solvent 0.074M H_3PO_4
7. $LiCl_2$ 21.0g solvent 0.0074M H_3PO_4
8. $CuSO_4 5H_2O$ 80.0mg solvent 0.0074M H_3PO_4
9. $ZaSO_4 7H_2O$ 106.0mg solvent 0.0074M H_3PO_4
10. H_3BO_4 600.0mg solvent 0.0074M H_3PO_4

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Hugh and Leifson's O-F medium (Hugh and Leifson 1953)

Peptone	2.0g
NaCl	5.0g
K_2HPO_4	0.3g
Agar (Oxoid No.3)	3.0g
H_2O (deionised)	1000ml

Bromthymol blue 0.2% ag soln. 15ml

Dissolve the solids by heating in the water, adjust pH to 7.1, filter, add the indicator and sterilise at 115°C for twenty minutes. Add aseptically a filter sterilised solution of the appropriate carbohydrate to give a final concentration of 1%.

11.	$Al_2(SO_4)_3.18H_2O$	123mg solvent - 0.0074M H_3PO_4
12.	$NiCl_2.64_2O$	110mg solvent - 0.0074M H_3PO_4
13.	$CoSO_4.74_2O$	109.0mg solvent - 0.0074M H_3PO_4
14.	$TiCl_4$	60.0mg solvent - 0.084M H_3PO_4
15.	KBr	30.0mg solvent - H_2O(deionised)
16.	Kl	30.0mg solvent - H_2O(deionised)
17.	$MnCl_2.4H_2O$	629.0mg solvent - 0.074M H_3PO_4
18.	$MgSO_4.7H_2O$	1.4g solvent - H_2O(deionised)
19.	$SnCl_2.2H_2O$	36.0mg solvent - H_2O (deionised)
20.	$FeSO_4.7H_2O$	300.0mg solvent - H_2O (deionised)

Step 1: Pipette into a 1-l standard flask the following amounts of solutions: 10ml of solutions 5 and 6 and 0.1 ml each of solutions 7 through 14

Step 2: Add approximately 600ml of solution 3 and 210 ml H_2O (deionised).

Step 3: Adjust the pH to 7.0 with N NaOH (solution 1).

Step 4: Add 0.1 ml of solutions 15 and 16

Step 5: Take 0.1ml of solution 17, add 9.9ml of solution 2, adjust pH to 7.0, autoclave for twenty minutes at 121^{o}C and filter. Add filtrate to the medium.

Step 6: Add 10ml of solution 18, and 0.1ml of 19 and 20.

Step 7: Using solution 4, make the final volume to 1l.

Step 8: Sterilise at 121°C for twenty minutes and store until required.

The medium was dispensed in 50ml volumes into 100 ml acid washed conical flasks. The flasks were stoppered with cotton wool and sterilised at 121°C for twenty minutes. Filter sterilised ethanol solution was added aseptically to give a concentration of 1%.

Citrate, Christensen's (Christensen 1949).

Sodium citrate	3.0g
Glucose	0.2g
Yeast extract	0.5g
Cysteine Hydrochloride	0.1g
Ferric ammonium citrate	0.4g
KH_2PO_4	1.0g
NaCl	5.0g
$Na_2S_2O_3$	0.08g
Agar	20.0g
H_2O (deionised)	1000ml
Phenol red, 0.2% soln.	6ml

Dissolve the solids in the water by heating, filter, adjust pH to 6.8-6.9, add the indicator and dispense into tubes. Sterilise at 115°C for 20 minutes.

Nitrate broth

KNO_3	1.0g
Nutrient broth (Oxoid)	100 ml

Dissolve the KNO_3 in the broth, distribute into tubes containing inverted Durham's tubes and sterilise at 115°C for twenty minutes.

Nitrite broth

$NaNO_2$	0.01g
Nutrient broth (Oxoid)	1000ml

Dissolve the $NaNO_2$ in the broth, distribute into tubes and sterilise at 115°C for twenty minutes.

Citrate, Koser's (modified from Koser 1923)

NaCl	5.0g
$MgSO_4.7H\ O$	0.2g
$NH_4H_2PO_4$	1.0g
K_2HPO_4	1.0g
H_2O (deionised)	1000ml

Dissolve the salts in the water. Add 2.0g citric acid to the salts solution, adjust to pH 6.8 with N NaOH. Filter through a sintered glass funnel. (All glassware used should be acid washed). The medium is then dispensed into tubes and sterilised at 115°C for twenty minutes.

Starch agar

Potato starch	10g
H_2O (deionised)	50ml
Nutrient agar (Oxoid)	1000ml

Triturate the starch with the water to a smooth cream and add to the molten nutrient agar. Mix and sterilise at 115°C for 15 minutes. Distribute into Petri plates.

Glucose-phosphate medium MR and V-P.

Peptone	5.0g
K_2HPO_4	5.0g
H_2O (deionised)	1000ml

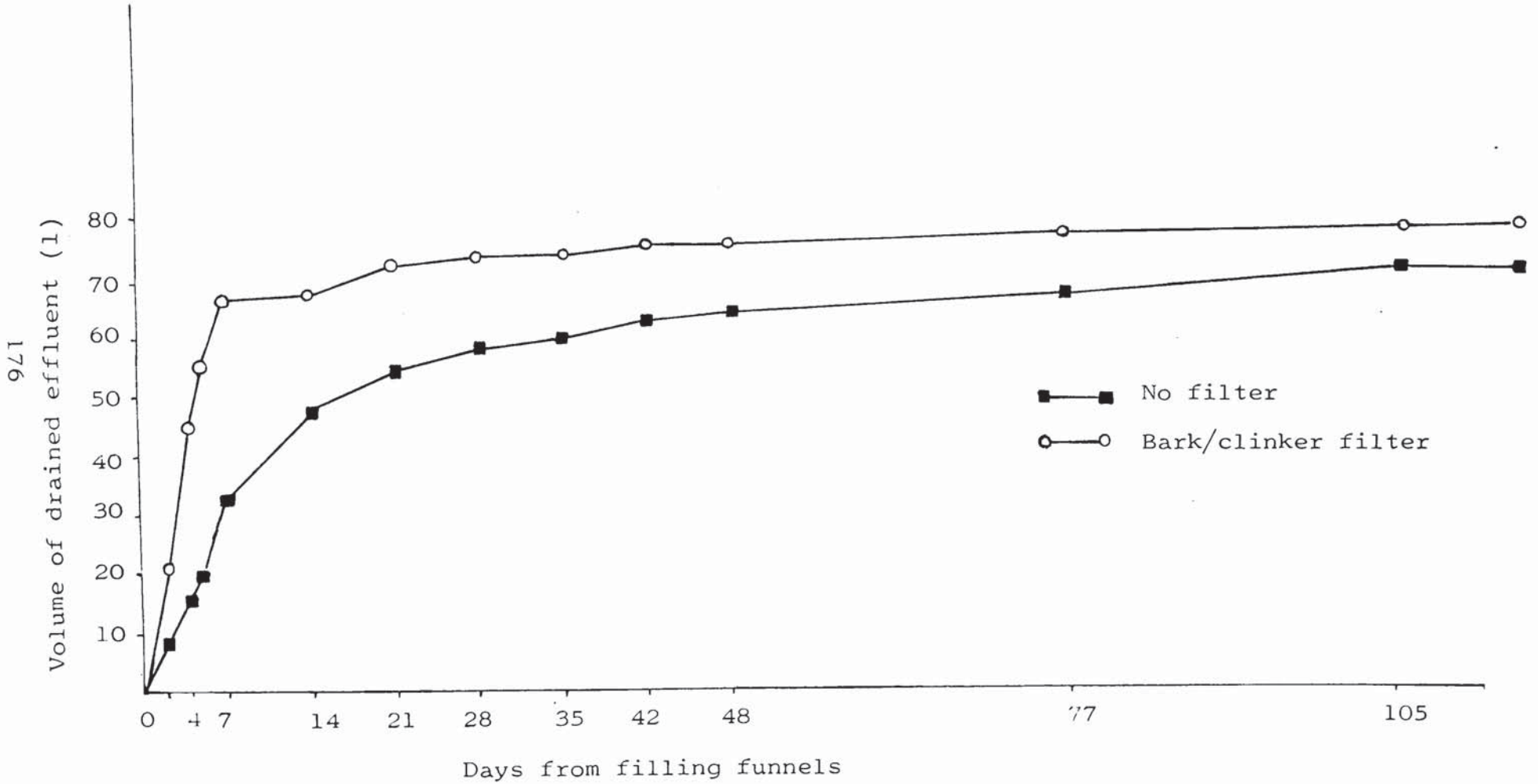
Steam until the solids are dissolved, filter and adjust to pH 7.5. Add 5.0g glucose, mix and distribute 1.5ml volumes into tubes. Sterilise at 115°C for 10 minutes. For sterilisation the tubes must be placed in a solid bottomed container to protect them from contact with the boiling water, otherwise the medium becomes straw yellow in colour.

Glucose asparagine broth (Ottow and Glathe 1971)

Glucose	20.0g
Asparagine	5.0g
K_2HPO_4	3.0g
KH_2PO_4	0.8g
$MgSO_4.7H_2O$	0.2g
H_2O (deionised)	1000ml

Ferric iron is then added as either Fe_2O_3 - 1.0g or $FeCl_3$ - 1.0g The medium is dispensed into tubes and sterilised at 115°C for twenty minutes.

Appendix II Drainage of liquid effluent from isolation funnels.



Appendix III Weekly rainfall during simulated ageing experiment

Appendix IV - Additional data.

Table 4.1.1.

Numbers of bacteria in PMB at different times after deposition.

Time after deposition	TOP CORE		BOTTOM CORE	
	1st sample	2nd sample	1st sample	2nd sample
Raw sludge	9.66×10^5			
1½ months	4.27×10^6		2.2×10^5	
3 months	8.36×10^6		1.2×10^5	
6 months	25.0×10^6	11.04×10^6	2.5×10^6	1.82×10^7
9 months	19.3×10^6	5.1×10^5	1.6×10^3	3.0×10^4
12 months	5.6×10^3	2.1×10^4	6.0×10^2	1.8×10^3
18 months	6.0×10^5	4.6×10^3	5.0×10^5	3.0×10^5
36 months	9.5×10^5		2.6×10^6	

Table 4.1.2.

Numbers of nematodes in PMB at different times after deposition.

Time after deposition	TOP CORE		BOTTOM CORE	
	1st. sample	2nd. sample	1st. sample	2nd. sample
Raw sludge	0		0	
1½ months	0		1	
3 months	3		18	
6 months	3,130		168	25
9 months	6,812	3,726	64,285	77
12 months	17,510	12,968	23,939	164
18 months	45,056	12,269	1,400	10,555
36 months	5,472		833	

Table 4.1.3.

Numbers of bacteria in PMB in isolation funnels.

Sample age (weeks)	FUNNEL 1		FUNNEL 2	
	Top core	Bottom core	Top core	Bottom core
4	12.8 $\times 10^6$		6.1 $\times 10^6$	
8	1.2 $\times 10^8$	5.6 $\times 10^7$	3.0 $\times 10^7$	1.0 $\times 10^5$
12	7.49 $\times 10^7$	5.02 $\times 10^7$	1.91 $\times 10^7$	9.87 $\times 10^5$
17	9.6 $\times 10^7$	1.04 $\times 10^8$	8.1 $\times 10^8$	4.8 $\times 10^7$
20	17.8 $\times 10^6$	18.4 $\times 10^6$	19.5 $\times 10^7$	79.8 $\times 10^7$
30	13.0 $\times 10^7$	6.5 $\times 10^5$	9.8 $\times 10^8$	8.75 $\times 10^7$
38	3.9 $\times 10^7$	2.2 $\times 10^7$	16.3 $\times 10^7$	3.83 $\times 10^7$
67	19.6 $\times 10^7$	8.3 $\times 10^7$	5.0 $\times 10^7$	16.8 $\times 10^7$
116	21.5 $\times 10^5$	18.2 $\times 10^5$	3.17 $\times 10^6$	10.18 $\times 10^6$

Table 4.2.1.

Effect of particle size on total numbers of bacteria in PMB casing. (Numbers of bacteria per 1g. fresh weight)

Days from casing	Casing treatment			
	PMB 20mm.	PMB 10mm.	PMB 5mm.	Peat/chalk control
0	4.08 $x10^6$	1.23 $x10^6$	7.44 $x10^6$	26.0 $x10^6$
7	76.5 $x10^6$	143.0 $x10^6$	82.0 $x10^6$	312.0 $x10^6$
11	2.5 $x10^6$	9.05 $x10^6$	7.0 $x10^6$	192.0 $x10^6$
14	3.25 $x10^6$	9.0 $x10^6$	7.54 $x10^6$	40.0 $x10^6$
21	10.08 $x10^6$	5.40 $x10^6$	28.0 $x10^6$	31.36 $x10^6$
28	11.96 $x10^6$	4.35 $x10^6$	6.38 $x10^6$	34.36 $x10^6$
35	148.0 $x10^6$	3.5 $x10^6$	21.9 $x10^6$	104.5 $x10^6$
48	128.0 $x10^6$	238.0 $x10^6$	154.5 $x10^6$	104.5 $x10^6$

Table 4.2.2.

Effect of particle size on numbers of Pseudomonad bacteria (numbers of bacteria per 1g. fresh weight).

Days from casing	PMB 20mm.	PMB 10mm.	PMB 5mm.	Peat/chalk control
0	2.0×10^4	7.5×10^4	10.0×10^4	$< 10^4$
7	8.3×10^4	8.0×10^4	2.0×10^4	7.0×10^4
11	1.5×10^4	6.0×10^4	4.0×10^4	8.0×10^6
14	3.0×10^4	1.0×10^4	4.0×10^4	1.0×10^6
21	3.0×10^4	1.0×10^4	12.0×10^4	29.0×10^4
28	6.0×10^4	9.5×10^4	7.5×10^4	89.0×10^4
35	19.0×10^4	47.0×10^4	65.4×10^4	41.5×10^4
48	2.34×10^6	76.0×10^4	7.0×10^6	8.0×10^6

Table 4.4.3.

Effect of steam/air pasteurisation on numbers of bacteria.

Treatment	No. bacteria per 1g fresh weight
50°C 60 mins.	6.25×10^5
65°C 30 mins.	3.003×10^5
65°C 60 mins.	2.507×10^5
80°C 30 mins.	1.87×10^5
80°C 60 mins.	1.91×10^5
Untreated	5.95×10^5

Table 4.4.4.

Effect of steam/air pasteurisation on numbers of Pseudomonad bacteria

Treatment	No. bacteria per 1g fresh weight
50°C 60 mins	1.3×10^3
65°C 30 mins	$< 10^2$
65°C 60 mins	$< 10^2$
80°C 30 mins	$< 10^2$
80°C 60 mins	$< 10^2$
Untreated	7.8×10^3

Table 4.4.5.

Effect of heat treatment on numbers of bacteria in PMB casing soil.

Treatment	Days from treatment and casing			
	0	3	8	12
65°C 60 mins	8.86×10^4	32.6×10^6	55.0×10^7	20.31×10^7
85°C 60 mins	2.25×10^4	38.4×10^4	20.5×10^7	17.78×10^7
Untreated	1.59×10^6	26.9×10^5	15.22×10^6	37.39×10^6

Table 4.5.2.

Populations of bacteria in PMB casing when recased at different time intervals after casing.

Days from casing	Recased after 1st break	Recased after 2nd break	Recased after 3rd break	Control not recased
0	21.3_5 (22) x 10^5	35.8_4 (32) x 10^4	49.15_4 (45) x 10^4	86.5_5 x 10^5
7	53.0_7 (29) x 10^7	50.86_7 (39) x 10^7	41.64_7 (52) x 10^7	13.80_7 x 10^7
14	44.71_7 (36) x 10^7	53.82_7 (46) x 10^7	36.97_7 (59) x 10^7	31.2_7 x 10^7
After 1st break 22	24.2_7 (46) x 10^7	38.17_7 (56) x 10^7	30.71_7 (71) x 10^7	27.2_7 x 10^7
After 2nd break 32	28.10_7 (59) x 10^7	22.13_7 (68) x 10^7		22.75_7 x 10^7
After 3rd break 45				12.79_7 x 10^7
After 4th break 59				86.23_4 x 10^4
After 5th break 80				22.59_7 x 10^7

Number in brackets denote number of days from original casing

Figure 4.5.4.

Effect of recasing with PMB at different times from casing upon the yield.

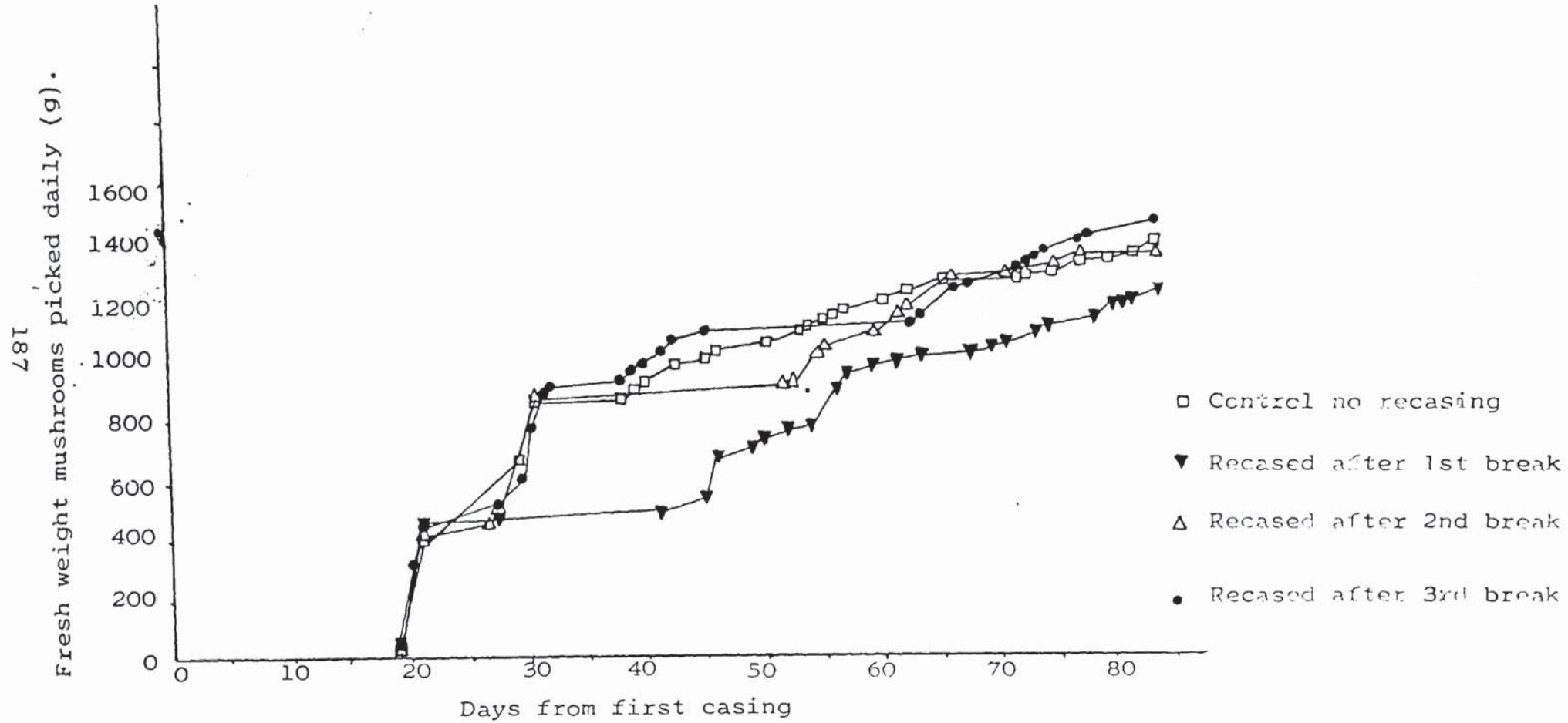


Table 4.6.11.

Some properties of bacterial isolates from PMB casing soils.

Culture code	27	28	74	62	81	64	51	7	10
Test									
Soluble Fe reduction	+	+	+	+	-	+	+	+	+
Insoluble Fe reduction	-	-	-	+	-	-	-	+	+
Phosphate solubilisation	+	+	-	+	+	0	+	-	+
Bicarbonate utilisation	-	-	-	-	+	-	-	-	-
Ethanol utilisation	+	-	-	+	+	-	-	+	+
Primordia formation in sterile flasks	-	-	+	+	+	+	+	+	+
Yield from sterile flasks	-	-	-	-	+	+	2+	2+	3+
Primordia formation in normal culture	-	-	+	+	+	+	+	+	+
Yield from normal culture	-	+	+	+	+	+	3+	2+	3+

+ ⟶ 3+ increasing yield

\- no yield/negative reaction

\+ positive reaction

0 sample damaged

Culture codes

27 Pseudomonas
28 Pseudomonas
74 Pseudomonas
62 Alcaligenes
81 Alcaligenes
64 Bacillus
51 Serratia
7 Flavobacterium
10 Enterobacteriaceae

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