## *Investigations into the niche specialisation of the coprophilous mycoflora, with reference to herbivore faeces*

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## APPENDIX.

The experiments carried out in this study were initial investigations into a subject that has hitherto been overlooked by professional mycologists. The results confirmed that some items in a 'classical succession' of coprophilous fungi could appear on samples of macerated grass from grazed fields on incubation in the laboratory.

This is a stimulating observation and there is great scope for more experimental work to be undertaken. The purpose of this appendix therefore, is to outline further work that would have been attempted had time permitted. Some of the experimental work is short-term, that is, for periods of several weeks, while other projects are long-term, for periods of a year.

## (I). Longer Term Investigations Of Fungi Appearing On Incubated Samples.

In the main study, samples of dung and grass were collected from fields and incubated in the laboratory at only one time of the year. It would be interesting to know whether there is any variation in the numbers and types of fungi (as determined by identified fruiting bodies) appearing on samples gathered from the same pasture and incubated at different times over the period of a year.

### (I.I)

Collection of samples could be carried out once every month for twelve months, beginning in early spring, and ending the next year in late winter.

Samples would have to be gathered with regard to some randomised method, involving the collection of pats and grass from different areas of the pasture. The pasture could be divided into separate areas, dependent upon the total area of the pasture. If the pasture was small enough, a sample of the deposited dung could be taken from each division, along with some grass for maceration, instead of relying on subsampling. This should tend to give a more accurate representation of the actual situation. All samples would be incubated in the laboratory in the usual way.



It might be expected that if herbivore dung was collected and incubated at different times of the year, then the fungal flora appearing would vary with the time of year when the samples were taken. However, it should

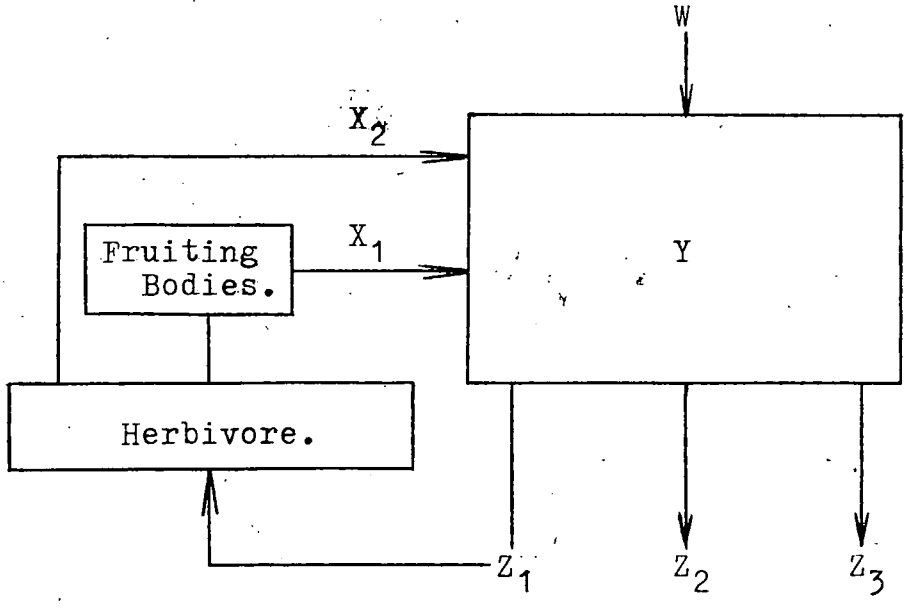
be realised that the dung is being incubated under conditions judged to be optimum for the growth and sporulation of fungi. Thus, any dung gathered from the same field, should contain roughly the same species of coprophilic fungi represented by their spores. So the types of fungi appearing on the incubated samples should be similar. In connection with this let us refer to a simple model of the dung/gut/field system.

(I.II)

The model of the dung/gut/field system is shown very simply in figure 4. It is represented as an input to and an output from a 'pool' (Y) of coprophilous fungal spores. The pool Y represents the coprophilous fungal spores attached to the growing vegetation in the grazed field. There are three main ways by which viable spores can be lost from Y; (i) loss due to grazing, $Z_1$; (ii) loss of viability due to ageing, $Z_3$; (iii) elution due to environmental factors, such as wind and rain, $Z_2$. $Z_1$ differs from $Z_2$ and $Z_3$, in that the spores may be lost for only a relative short period from the pool Y when they are held within the gut of grazing herbivores that have ingested vegetation to which spores were attached. The time spent in passage through the herbivore gut may be in the order of only a few hours. However, after the return of spore-containing faeces to the pasture there may then be a delay associated with the resettlement of spores on the surface of pasture vegetation.

Only a proportion of the spores of coprophilic fungi ingested by the grazing herbivore may be expected to be stimulated to germinate and produce fruiting bodies on the faeces returned to the pasture. The faeces may also

FIG.4. The Dung/Gut/Field System As It Affects Coprophilous Fungi.




W= Input of viable coprophilous spores* to Y from another pool.

$X_1$=Input of viable coprophilous spores to Y from fruiting bodies.

$X_2$=Input to Y of dormant viable coprophilous spores on or in deposited dung.

Y = Pool of viable coprophilous spores on pasture vegetation.

$Z_1$=Viable coprophilous spores ingested by grazing herbivores.

$Z_2$=Loss of viable coprophilous spores from Y due to enviromental factors.

$Z_3$=Loss of viable coprophilous spores from Y due to ageing.

(*coprophilous spores refers to the spores of coprophilous fungi.)

be expected to contain a proportion of ungerminated spores which may require some stimulus to which they have not yet been exposed, or not exposed to at the correct time, in order to germinate. After germination, fruiting bodies should then be produced from these spores in the usual way. Some of the dormant spores contained on or within the faeces may be redistributed over the vegetation of the pasture after the breakup of the faecal mass through the action of environmental factors such as repeated wetting and drying. These spores may then be reingested by grazing herbivores. Subsequent repetition of the previous process may then induce germination in these spores.

(I.III). Factors Affecting The Size Of The Pool Y.

The grazing rate of herbivores in the pasture will be one of the factors affecting the size of Y. Increasing the rate of grazing increases the rate of ingestion of spores, thus in a given time more spores will be removed from the pool. If the return of spores to the pool is at a lower rate than that of the removal of spores then it follows that Y will be reduced in size.

It must also be remembered that livestock may not be the only grazers present in the pasture. Other animals, such as rabbits and voles, may be removing significant quantities of pasture vegetation with attached fungal spores.

In order to enable interpretation of the model it is necessary to restrict the livestock to grazing solely on vegetation growing in the pasture. Introduction of outside feed such as hay would introduce errors into the analysis.

So if it can be assumed that (i), the livestock grazes at a fairly constant rate, this rate being dependent on the number of grazing animals present, and the rate at which each animal feeds; (ii) the livestock feeds only on vegetation from the field, and that; (iii) other grazing animals are excluded from the field, then it is enviromental factors which will affect the numbers of viable coprophilous fungal spores in the pool Y.

Enviromental factors will affect the number and types of fungal fruiting bodies produced on the deposited dung, which will in turn affect the input ($X_1$) of fungal spores. They may also affect the viability of spores attached to the herbage ($Z_3$). Enviromental factors may be responsible for removing spores from the surface of vegetation. Rain may wash spores down into the soil where they are unlikely to be ingested by grazing herbivores on the surface. Spores on the surface of the soil might be able to germinate if dung was subsequently deposited on them. Spores might then be produced from fruiting bodies which could then enter the pool Y. Spores can be carried out of the pool Y in air currents or through the movements of surface water.

If the species of fungi appearing at different times throughout the year did show variation when dung was incubated in the laboratory, then this might be due to changing proportions of different spores in the pool Y, with respect to time.

These differences would be directly indicated by the types of fungi appearing on the incubated samples of macerated grass, assuming that most of the fungi grow

and sporulate on this substrate and do not require passage through the herbivore gut to break dormancy. The change in different spore proportions could arise through environmental factors reducing or halting the rate of succession of fungi on dung deposited in the field, or by favouring the growth and sporulation of one or several types of fungi contributing spores to the pool Y. The proportion of spores of less abundant fungal species would still be decreased via $Z_2$ and $Z_3$, but might not be replenished at an equivalent rate if their growth and sporulation on substrata was affected detrimentally by environmental factors. A greater proportion of the spores of a certain fungus in the pool will increase the probability of spores of that species germinating and producing fruiting bodies on deposited dung. The various factors bearing upon the germination of spores are however, very complex.

(I.IV)

In addition to incubating samples of dung and macerated grass in the laboratory, samples of both types could also be collected as before, and left for observation outside the laboratory. The purpose of this would be to see the effect of presumably sub-optimalconditions on the results. Duplicate samples for study would be collected on a randomised basis from the grazed field previously studied. Preferably these experiments would be performed so as to run concurrently with the laboratory studies.

To enable comparison with the previous results, samples of macerated grass and of dung would have to be

treated in a similar fashion to those studied indoors, this would exclude the effects of a number of potential variables, such as the nature of the containers. If the samples were placed on the other side of the window used in the previous experiments then they might be expected to have a similar aspect. Variations in temperature would then be the factor differing most from previous experimentation. However, this is still a long way from conditions in the pasture.

A closer approximation would be afforded by putting samples of dung and macerated grass in similar containers as before but this time leaving them exposed to the environment by not replacing the tops on the containers. These samples would not be kept watered by the experimenter. If the original samples were taken from a pasture located nearby the site of experimentation then the conditions prevailing might be expected to be close to those in the field.

The results from these experiments would possibly demonstrate some of the points raised in the previous section. The samples of dung left exposed would more closely mimic dung pats deposited in the field as they would be subjected to similar environmental conditions. However, attack from organisms such as mycophagous invertebrates would probably be reduced in experimental samples.

Mycophagous invertebrates feed usually on fungal hyphae, thus reducing the surface area used for assimilation of nutrients. Invertebrate ravages may thus affect the rates of fungal succession on substrates.

## (II). Investigating The Fungi On Samples Of Grass And Dung From Fields Where There Are Mixed Grazing Practices.

The experiments in the main study were carried out using dung and grass samples collected from pastures where only one type of livestock was grazing. While travelling to collect samples it was noticed that many farmers had more than one type of livestock animal on the same pasture. It was possible for sheep, cows, horses, and other grazing animals to be found grazing the same pasture.

### (II.I)

It would be interesting to culture samples of macerated grass taken from these mixed-grazed fields and investigate which fungi appeared on them. Grass macerate samples would be collected on a randomised basis from the pasture, and the various dung samples from the different livestock would also be collected and incubated in the usual way for purposes of comparison. This experimentation could also be carried out on a long term basis by collecting samples once every month for a period of twelve months.

Diversity of fungal types appearing on the incubated samples of macerated grass from a mixed grazed pasture might be expected to be higher in terms of species richness than similar samples taken from a pasture grazed by a single species of livestock. This might be due to different types of dung supporting different, often characteristic, fungal species. Some fungi are apparently found to be 'restricted', appearing often on

only a certain type of dung from a single animal species. The mycoflora subsequently developed on the macerated grass samples might contain many of the specialised fungal species found otherwise only on their different characteristic dung types.

This might not be so however because of increased competition due to the greater number of fungi present. Phenomena such as hyphal interference may be more prevalent, possibly leading to reduced species richness.

In earlier discussion it was remarked that the number of species of fungi occuring on the separate macerated grass samples and on the dung samples ranged between only seven and nine. An experiment of the nature proposed, where there is presumed to be a greater number of potential fungal species present in the pool, might show that samples are capable of supporting a higher species richness than that encountered previously.

## (III). Investigating The Fungi Occuring On Grass Samples Where The Livestock Have Been Removed.

Pastures may be allowed to grow ungrazed after periods of exposure to grazing. Possible changes, if any, in the mycoflora of incubated samples of macerated grass taken at various intervals after the removal of grazers from the pasture can be investigated.

It would be important with this type of experiment to collect as many samples as possible and to collect these samples from all over the pasture. This would give a better indication of the numbers of different types of viable spores present in the pool. The collection of grass samples once every month for twelve months might

prove to be a convenient method for analysis.

The results from this experiment would indicate whether the pool size and composition had changed, with respect to time after the removal of grazers, compared to a similar dung/gut/field system where the grazers remain present.

(IV). The Incubation Of Dung On Samples Of Freshly Grown Grass.

Uncontaminated turves of grass could be produced by sowing a fungicide pre-treated dressed grass seed mixture onto sterilised seedling compost in clean seed trays. These trays would be covered by polythene sheets to exclude the air spora, and kept in the laboratory, watering as necessary.

After germination, when the grass has reached the tillering stage, a single pat of freshly collected herbivore dung would then be placed in the centre of some of the trays. The pats of dung should all be of similar size, presuming that similar sized pieces collected from the same situation will yield similar numbers of fruiting bodies after germination. This presumption may be inaccurate. Other trays act as controls and have no dung added to them. All trays should have loosely fitting transparent covers placed over them, these should allow a certain amount of air flow in and out of the trays. All trays would then be incubated and watered at intervals as necessary. Fungal fruiting bodies subsequently appearing on the pats would be identified by direct observation, or, if the fungi could not be immediately identified, by culturing onto dung agar. Incubation would be carried out until the fungal

succession had terminated. Then the remains of the pats would be removed, and the stems and blades of grass from each tray would be cut using scissors and collected separately. Each grass sample would then be individually macerated, thoroughly cleaning the macerator between samples. Maceration would be carried out until the grass resembled fresh horse dung in texture. These samples of macerated grass would then be incubated in trays as before. Two types of grass samples would be incubated; macerated grass from seed trays where dung was present, and macerated grass from those trays where dung was absent. Species of fungi present, if any, would be identified from their fruiting bodies. The incubated macerates from the control trays would indicate whether any fungi developed on the macerates could have arisen from sources other than the dung. These fungi could then be taken into account when considering results from the 'dung-grass' macerates.

This study would be useful because one would know that any fungi present on the macerates of grass grown with dung present are highly likely to have come only from the dung. Thus, comparisons can be made with macerate samples taken from the pasture where spores may have come from other sources besides that of the dung. Also, it is highly likely that all fungi that fruited on the dung would be represented on the incubated samples of macerated grass taken from the trays of grass in which dung was present. This is because the fungal spores are being projected onto a small area of grass, free from air currents and rain. However, in the pasture from which the samples were taken, the spores produced by fungi fruiting on dung, (representative of all the species

fruiting on the dung), are less likely to all be found to have been present subsequently on macerates of grass taken from the pasture. Thus if a difference in mycoflora composition between macerated grass samples, where, in the one, the grass was closely confined with dung, and in the other, the grass was taken from the field situation, was indicated from the results, this would imply that the field had not been sampled adequately enough to give a correct re presentation of all the species present on the dung in that field.

Similarity coefficients could be calculated for the mycoflora composition on the samples of dung and macerated grass. The method used to calculate similarity coefficients has been outlined previously in the results section of the main study. The coefficients calculated here will have more meaning than the coefficients previously calculated for different situations in the main study. These earlier calculations directly compared the mycofloras of dung and macerated grass from grazed fields. Coefficients from the proposed study using 'virgin' grass in confinement with dung should be more relevant because it is more likely that the spore inoculum from the dung should be present on the grass that is then macerated. This assumes that all the fruiting bodies produced on the dung produce viable spores, and manage to project their spores onto the grass.

## (V). The Effect Of Temperature On Numbers And Types Of Fungal Fruiting Bodies Produced On Incubated Samples Of Macerated Grass And Dung, And The Degree Of Decomposition Of Samples.

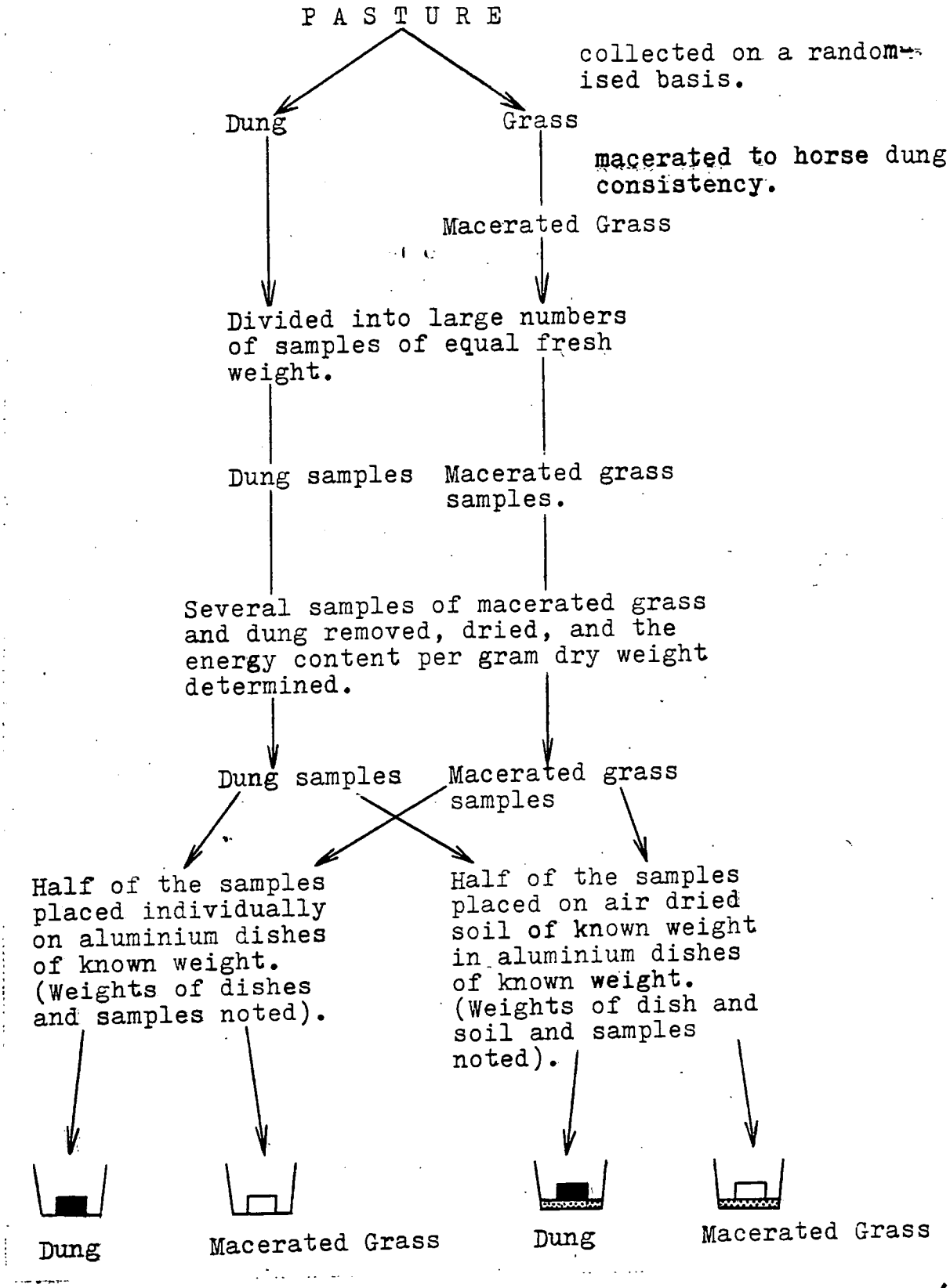
In the main study, samples of macerated grass and of dung were incubated at the temperature of the laboratory, a temperature prone to fluctuation. Light levels in the laboratory also varied, primarily on a diurnal rhythm. However, because the samples of macerated grass and of dung were both exposed to the same conditions, a comparison could be made between them concerning the mycoflora developed on them. This initial experimentation took place at what would be called 'room temperature' though this has no fixed value. Thus it would be interesting to know the effect of a range of known temperatures on the results. One suitable method might be as shown in the flow diagram on page numbers 14 and 15.

In this method samples are also incubated on soil. This should give some indication as to whether the soil micro-organisms have any effect on the species of fungi appearing on the samples and on the amount of decomposition occuring in those samples.

The procedures shown in the flow diagram would be carried out until the succession of fungal fruting bodies on the various samples had been completed. This might take a different length of time at each temperature, and using the results the following points could be considered;-

(i) Are there differences between the mycofloras of macerated grass samples when they are both on aluminium dishes, at the same temperature? This comparison could be

FIG.5. A Flow Diagram Illustrating The Method Used in Section V.




Equal numbers of all four types of dishes would then be incubated at one of a series of temperatures in an incubator in a 12/12 light regime; they would be kept adequately watered. Possible suitable temperatures

might be 0°C, 10°C, 20°C and 37°C. (37°C is near the optimal temperature for many coprophilous fungi to grow at). The samples would be observed periodically and any fungi present on them identified and noted. At intervals, one of each type of sample at each different temperature would be removed, dried, and its energy content (in $Jg^{-1}$ dry weight) determined using the bomb calorimeter. Using the value for initial energy content per gram dry weight, the loss in energy per gram initial dry weight could be determined at each stage for each type of sample at the different temperatures.

made for each of the temperatures used.

(ii) Are there differences between the mycofloras of macerated grass samples and dung samples when they are both on the surface of soil in aluminium dishes at the same temperature? This comparison could be made for each of the temperatures used.

(iii) As above, but between grass on aluminium weighing dishes and grass on the surface of soil.

(iv) As above, but between dung on aluminium weighing dishes and dung on the surface of soil.

(v) Are there differences between the mycofloras of macerated grass samples on aluminium weighing dishes at each of the different temperatures used?

(vi) As above, but between mycoflora compositions of macerated grass samples on the surface of soil at the different temperatures used.

(vii) As above, but between mycoflora compositions of dung samples on aluminium weighing dishes at the different temperatures used.

(viii) As above, but between mycoflora compositions of dung samples on the surface of soil at the different temperatures used.

Thus all combinations of differences within a treatment and between treatments could be determined.

(ix) Does temperature affect the length of time before fruiting bodies are observed?

(x) Does temperature affect the duration of the succession of fungal fruiting bodies?

(xi) Is the total amount of energy lost (mainly as

respiration) equal for each of the different samples at the same temperature?

(xii) Does the incubation of a sample on damp soil affect the mycoflora composition?

(xiii) Does incubation of a sample on damp soil affect the energy loss?

Some previous work has been carried out similar to this type of experiment. Wicklow & Moore (1974) incubated fresh rabbit faeces at different temperatures to determine the amount of energy lost as respiration from the faeces. They found that rabbit faeces lost a far greater percentage of weight during the first month of incubation at low temperatures than did decomposing plant materials incubated at the same temperatures for longer periods. It would be interesting to know whether this is also true for decomposing macerated grass when compared with the faeces of herbivores feeding on the grass. It would be expected that macerated grass should be much more readily decomposed than intact plant material. Maceration entails tissue damage which encourages decomposition by micro-organisms. Wicklow & Moore showed that faeces incubated at 24°C and at 37.5°C had average daily decomposition rates of 0.61% and 0.87% respectively during the first thirty days of incubation. (The figures refer to the percentage of total calories in the sample that had been liberated in respiration.) They also found that faeces incubated on the surface of damp soil lost more weight than those incubated on aluminium weighing dishes over the same period of time. It would be interesting to know if this was also true for

samples of macerated grass incubated on soil compared with samples incubated on bare aluminium dishes at the same temperature. Samples incubated on the surface of the soil might be losing more energy than those not incubated on the soil because,

(a) Some soil micro-organisms could be invading and so contributing to the decomposition of the sample;

(b) waste metabolites that would otherwise cause staling arising from fungi growing on the sample might be diffusing from the sample and into the soil, reducing possible inhibition of fungal growth;

and, or,

(c) energy rich substances, such as soluble sugars, may be lost from the samples into the soil by diffusion and/or leaching.

Some indication of the likelihood of (a) could possibly be obtained by placing sterilised pieces of dung on the surface of soil in dishes as before. If fungal growth subsequently occured on the dung, then, assuming that the dung was sterile and that air spora effects were minimal, it would be shown that it was at least possible for fungi to pass from the soil to the sample.

To test hypothesis (b) the soil from an aluminium dish on top of which had previously been incubated a sample bearing fungi, could be sterilised by heat. Treating them separately after cooling, enough distilled water would be added to each soil sample until it was of a sludge like consistency. The mixtures would be stirred and left for a length of time to allow any substances present to enter solution. Then each would be filtered through muslin and the clear liquid - extract collected. Each type of liquid would then be incorporated into a

potato-dextrose agar mixture and a dung agar mixture, and these used to produce plates in petri-dishes, as described in the main study. Petri-dishes containing dung agar and potato-dextrose agar without any extract included would also be set up. Cultures of fungi known to have been present on samples could then be streaked onto each of these different types of agar plates, and these plates then incubated at temperatures used previously. Subsequent growth, if any, of fungi on these plates would then be examined. If there was seen to be evidence of inhibition of growth of fungi on the agar mixtures containing the extract from the soil upon which samples had been incubated, when compared with the fungal growth on the agar mixtures containing extracts from soil not previously incubated with samples, then this would indicate that an inhibitory substance had passed from the samples of dung or macerated grass into the soil. A comparison of the growths of fungi on agar plates without added soil extracts, and on agar plates where extract from soil incubated without an added sample above it would indicate whether this effect was produced by adding any extract of the soil. If there was little apparent difference between them then it would imply that inhibitory compounds were not present in the soil.

A problem with this experiment might be that the inhibitory substances might not be water-soluble. An organic solvent, such as alcohol, might be necessary to elute them. Also, if an inhibitory substance were present within the soil used, there is the possibility that it might be destroyed or its growth inhibitory properties reduced when the soil was sterilised. However, this sterilisation has to be carried out, otherwise the micro-

organisims present in the soil might be cultured on the agar plates and so obscure the results. Sterilisation by irradiation might be preferable to sterilisation by heat. It may also be possible that by incorporating the soil extracts with agar, any inhibitory substances present in them might be diluted to a concentration ineffective in fungal inhibition.

## (VI). The Effect Of pH On Fungi Growing On Samples Of Macerated Grass.

It might be expected that coprophilic fungi would grow and reproduce most successfully in the pH range encountered on the dung on which they are most often found. However, it is most likely that the pH of macerated grass and dung differ - dung having passed through the alimentary canal of a grazer. Inside the herbivore the food is affected by gut secretions and processes and, when finally deposited as dung, it usually has a pH of above 6.5 (Hudson, 1972). It is an established fact that micro-organisms have favoured pH ranges, outside of which they may not be able to grow. Thus it would be interesting to know what effect pH difference has on the composition of the mycoflora developed on incubated macerated grass samples at various pH values compared with that developed on incubated fresh dung.

One suitable method of testing this might be as follows. Samples of grass and dung would be collected on a randomised basis from the same grazed pasture. The pH of the samples of fresh dung would then be found using a conventional pH-meter. The grass would be macerated as before to the consistency of fresh horse dung and the pH of the substance

found. If there was found to be a difference in pH between the dung and the macerated grass, the pH of the macerated grass would then be made equivalent to that of the dung by the addition of necessary buffers, perhaps using $PO_4^{3-}$ buffer. The samples of dung and macerated grass plus buffer would be incubated as in the main study, a control sample of unaltered macerated grass also being incubated. The samples would be kept moist and the fungal successions on each of the three samples would be carefully studied and compared. After the completion of each succession it would be interesting to know whether there was any difference in the composition of the mycoflora appearing on the buffered macerated grass and that appearing on the unbuffered macerated grass, and whether or not the fungal succession on the buffered macerated grass was closer to that occuring on the dung. If this was found to be true it would imply that pH is an important factor when considering the growth of fungi on macerated grass samples and on dung. Additional experiments could be carried out by incubating buffered macerated grass over a wide range of pH values, not just that of fresh dung. This would be expected to show exactly what effect different pH values have on fungal succession.

## (VII). The Effect Of The Length Of Period Of Maceration On The Fungal Succession On Macerated Grass.

A factor possibly affecting the succession of fungi, and the types of fungi found to appear on incubated macerated grass samples might be the texture of those samples. The consistency of the samples is dependent on the extent of maceration of the grass used to make them,

the samples of grass used in the main study were macerated to the consistency of fresh horse dung. This took about fifteen minutes with the macerator used. Prior to maceration the culms and blades of grass were cut to a suitable size for the machine, using scissors. It would be interesting to investigate whether the degree of maceration does have an effect on fungal succession.

One suitable way of studying this may be as follows. Samples of grass would be obtained on a randomised basis from a grazed pasture. These would first be cut to a manageable size using scissors and then macerated in the macerator for different periods of time. Suitable periods might be; one minute, two minutes, five minutes, ten minutes, and fifteen minutes. The separate macerated grass samples would then be incubated in the laboratory as in the main study. Fungi appearing on the different samples would be identified and the time before the fruiting bodies appeared would be noted.

It might be expected that those samples of grass which had been macerated for the longest periods of time would be most easily decomposed by the fungi. However, what effect this would have on species richness is uncertain, though the succession might be expected to take place in a shorter period of time. Maceration may increase species richness in that it may make available a greater variety of substrates. However, the consistency (texture) of the samples will affect the rate of diffusion of gases into and out of them. This might be important because most fungi are aerobic organisms. Oxygen availability and the rate of removal of waste gases may affect the succession. Samples of grass macerated for

longer periods of time will have a smaller pore-size within them. This can result in increased retention of water making gaseous diffusion slower. Griffin (1963) grew a species of Curvularia in a series of aluminium oxide grits which had different moisture retaining characteristics determined by the pore-size between the grit particles. He found greater fungal growth where the spaces between the particles were predominantly air filled rather than water saturated. Where the spaces between the particles were smaller, water saturation was more likely. Griffin tentatively concluded that the factor affecting fungal growth in the grits was the degree of restriction of gaseous exchange. Gaseous exchange was affected by the degree of water saturation of the pores.

Dickinson & Underhay (1977) working on cow dung, which has a very small internal pore-size, put forward the hypothesis that dung moisture was the main factor affecting the spread of hyphae into the centre of dung pats. They said that oxygen rapidly became limiting towards the centre of the pat because of the high water content restricting diffusion. Water content in the dung pats also closely influenced the extent of sporulation of the fungi.

The pores present in grass macerated for longer periods of time will have smaller radii than those pores present in grass macerated for a shorter length of time. A decrease in pore radius will increase the forces between water and solid, the so-called 'matric-potential' according to the equation;

$$\tau = \frac{2\sigma}{r}$$

(where; $\sigma$ = surface tension (dynes cm ), $\tau$ = matric potential (dynes cm ), $r$ = pore radius).

(After Garret, (1972)).

If the findings of Dickinson and Underhay are applicable, the fruiting bodies of a larger number of species of fungi will be observed on samples of grass that have been macerated to give an optimum, relatively large, pore-size. There should then be seen to be a decrease in the diversity and extent of fungal growth produced on the samples the longer they have been macerated, giving smaller pore-sizes. Increased maceration will be followed by reduction in water potential of samples as water deficit develops. It is known that decreasing the water potential increases the length of time of the latent period before germination of fungal spores occurs, until, at some specific potential, germination is inhibited indefinitely. This is not clear-cut, however, because in the process of maceration, fungal spore walls may be ruptured, acting as a stimulus to germination. Whilst in operation the macerator tends to get warm, and this heat could also act as a stimulus to germination. Maceration is thus serving to stimulate spores to germinate through the action of wall rupture and warmth, while simultaneously making conditions for them to then grow and produce fruiting bodies less favourable by reducing the pore-size. However, whilst pore-size may have some effect on fungal growth, whether pore-size is reduced to an inhibitory size by maceration is uncertain. Macerated grass samples may still be very fibrous, with enough relatively large pore-sizes, even after twenty minutes of maceration.

## (VIII). Determination Of The Amount Of Energy Loss From Incubated Samples Due To Coprophilous Fungi.

It has previously been discussed that energy loss from incubated samples cannot be attributed solely to fungal respiration; a proportion of this loss may also be due to the metabolic processes of other organisms such as bacteria, invertebrates, and myxomycetes. It would be useful to obtain more accurate values for the amount of energy that the coprophilous mycoflora is respiring from the samples of dung and macerated grass. The way to do this would be to suppress or remove the other organisms present in the samples which are also causing decomposition. The only other organisms contributing to the loss of energy to any large extent would be the bacteria and invertebrates.

Samples of macerated grass and dung could be incubated in the laboratory for the determination of energy loss, as was outlined in the main discussion, in this experiment many replicate samples would have to be set up.

One set of samples would be incubated in the normal manner, a sample would be removed every four days for determination of dry weight and energy content. Another set of samples would be treated or sprayed with a suitable broad range insecticide. An acaricide could also be added to the samples, making sure that it was well distributed throughout the samples. These samples would then be incubated and their energy content per gram dry weight would be determined every four days.

A set of samples could also be treated with a broad range bacteriocide such as chloramphenicol; this could

be made up to a suitable concentration and then added to the samples so that it was well distributed throughout each sample. These samples would also be incubated and their energy content determined.

Another set of samples could be treated with a mixture of insecticide, acaricide, and bacteriocide, so that most of the bacteria and invertebrates would be eliminated. These samples would be incubated and their energy content determined as before.

The results from these experiments would indicate how much of the loss of energy from the samples, by respiration, is caused by invertebrates and bacteria. This, however, is rather an oversimplification; there are many problems associated with this assumption. The bacteriocide may not affect all of the bacteria present, and its action may not be clear cut, it might also affect the growth of some of the fungi present. It could also be that some of the mycoflora depends upon bacteria, or the products of some of the eliminated bacteria to grow. The insecticides may kill the mites and flies present but they might not affect eggs of these organisms present in the samples, these eggs are thus left free to hatch at a later date, possibly when the concentration of insecticide has decreased to a sub-lethal level. A possible drawback to these experiments might be that the substrates not used by the other suppressed organisms, could provide for enhanced respiration by fungi; so that the values for energy loss from samples with inhibitors might exceed those for samples without inhibitors.

## (IX). The Inoculation Of Pure Cultures Of Fungi Onto Samples Of Macerated Grass.

The inoculation of pure cultures of fungi onto samples of fresh and autoclaved macerated grass, as performed in the main study, provided scant positive evidence that different coprophilic genera could grow and fructify on these samples after inoculation. These results would seem to be at variance with the results obtained on incubation of fresh macerated grass from livestock grazed pastures; but, the lack of production of fungal fruiting bodies might have been because of several reasons:-

(i) The transference of the cultures of fungi could have resulted in contamination of the substrate samples, and this might have prevented establishment of the fungi.

(ii) The flamed inoculating needle could have destroyed a significant portion of the culture being transferred; thereby decreasing the amount of inoculum and making establishment less certain.

(iii) The process of autoclaving, the purpose of which is to destroy micro-organisms that could cause antagonism, may have affected the chemical composition of the macerated grass, so that the resulting substrate was unfavourable for the growth and sporulation of most of the coprophilic genera used in the study.

(iv) Many of the cultures used in the inoculation experiments in the main study were supplied by the Commonwealth Mycological Institute in the freeze-dried state. This method of preservation might have had a detrimental effect on the ability of the fungus to establish itself and produce fruiting bodies on macerated

grass. The results from the inoculation of cultures onto macerated grass samples should be compared with the success of establishment of these cultures on dung agar and other standard culture media. Future inoculation experiments should be carried out using cultures that do not have to be revived after preservation, or which after freeze-drying, have been allowed to grow successfully on other standard media.

The method of inoculation may determine whether the fungus can successfully establish itself on the substrates, attention was not paid to this detail when the study was carried out. In further experiments the inoculum should be introduced onto or into the sample in several ways:-

(i) If inoculum is supplied as spores; it should be well diluted with water, and then spread throughout the substratum using sterilised inoculating apparatus.

(ii) The mycelium should be well 'broken-up', and then distributed throughout the substratum in the same manner.

(iii) The spores/mycelium should be streaked onto the surface of the substratum.

(iv) The spores/mycelium should be stabbed into the substratum - the stabbing action producing micro-aerophilic conditions.

## (IX.I). Experiments Designed To Elucidate Factors Controlling The Establishment And Production Of Fruiting Bodies Of Fungi Inoculated Onto Macerated Grass.

Because so few genera of fungi produced fruiting bodies when they were inoculated onto the samples of fresh and

autoclaved macerated grass, further experiments could be carried out to elucidate the factors controlling the establishment and production of fruiting bodies of these fungi after inoculation on macerated grass.

For these experiments, fresh and autoclaved macerated grass would be produced in the manner described in the main study and inoculation onto samples carried out using both spores, and mycelium for each species, if obtainable.

The experiments would be performed using several states of inoculum, that is; (i) spores collected from fruiting bodies and left for different periods of time before inoculation; as fresh spores may be viable but in a state of enforced dormancy, the degree of which could decrease with time; (ii) fresh spores and mycelium; (iii) spores which had been abraded in some manner, this could be carried out by shaking and rubbing spores against fine sandpaper, and; (iv) spores that had been heat-shocked - experiments have shown that in Phycomyces, the percentage of germination of spores is increased by subjecting them to a temperature of 50°C for fifteen minutes. An increased percentage of spore germination in other species might also be brought about by subjecting their spores to this treatment.

### (IX.II). The Effect Of The Period Of Maceration Of Samples Of Grass On The Production Of Fruiting Bodies By Fungi Inoculated Onto Them.

As discussed in section VII of this appendix; the texture of macerated grass samples might be a factor affecting the types of fungi found to appear after incubation of these samples.

The grass used in the inoculation experiments in the main study was cut to manageable sized pieces, and macerated until it had the consistency of freshly deposited horse dung; which took about fifteen minutes. The effect that the period of maceration of samples has on whether fungal fruiting bodies would appear after inoculation could be studied.

One suitable way of studying this may be as follows. Grass could be cut to manageable size pieces and then macerated in the macerator for different periods of time. Suitable periods might be; one minute, two minutes, five minutes, ten minutes, and fifteen minutes. The samples of grass that had been macerated for different periods of time would then be divided into replicate samples, and half of these, from each maceration period, would be autoclaved. The fresh and autoclaved samples would then be inoculated using mycelium and spores of various coprophilous fungi, and inoculation would be carried out using each of the methods described. The samples would then be incubated at room temperature for several weeks, watering them at intervals as necessary, and the production (if any), and the length of time before the production of fungal fruiting bodies noted.

The results from this study would indicate whether the texture of the samples of fresh or autoclaved macerated grass was a factor determining whether fruiting bodies would be produced by fungi inoculated onto them. They would also show whether the method of inoculation (in conjunction with the period of maceration) also had an effect.

However, firm conclusions about the importance of these factors cannot be drawn from the results for the fungi inoculated onto samples of fresh macerated grass. If fruiting bodies of the inoculated fungi did not appear on these samples after incubation, then it may have been that this was caused by fungal antagonism due to the other fungi present in the sample. These fungi should not be present in the samples of autoclaved grass because they should have been destroyed during the process of autoclaving.

## (IX.III). Experiments To Determine The Effect Of Temperature On The Production Of Fruiting Bodies By Fungi Inoculated On Samples Of Autoclaved Macerated Grass.

In the main study, samples of macerated grass were inoculated with cultures of fungi and incubated at the temperature of the laboratory. As the number of fungi that produced fruiting bodies was so few, it would be interesting to know the affect of a range of known temperatures on the results. A possible method for determining this might be as follows.

Replicate samples of autoclaved macerated grass could be separately inoculated with cultures or spores of coprophilous fungi, using all the methods of inoculation described, and then incubated at different temperatures in incubators with a 12/12 light regime. Possible suitable temperatures might be; $0^{o}C$, $10^{o}C$, $20^{o}C$, $37^{o}C$, and also room temperature, (which is prone to fluctuation), to act as a comparison. The samples would be incubated for several weeks, and the production, and the period of time before the appearance of any fruiting bodies, noted. Using the results the following points could be considered;-

(i) Does the incubation temperature have an affect on whether fruiting bodies are produced on the samples?

(ii) Of the temperatures tested, which was the one at which the most number of species were found to produce fruiting bodies ?

(iii) Does the method of inoculation affect whether fungal fruiting bodies are produced on the substrates at the various temperatures?

(iv) Does the period of time before the appearance of

fruiting bodies of a species differ at each of the temperatures?

If, for example, after inoculation with spores of a certainfungus, it was found that fruiting bodies were produced on incubation of samples at 37°C, but not at the other temperatures; then this would seem to indicate that either; incubation at this temperature is required to break spore dormancy; or, it is a prerequisite for the production of fruiting bodies, or that it is needed for both of these processes. It is not easy to prove or disprove these hypotheses.

If after incubation for some period of time, fruiting bodies had not been produced, then the sample could be analysed to determine whether fungal growth was proceeding throughout the substrate. This could be done using the method devised by Dickinson & Underhay (1977), which has been discussed in the main study. For this experiment a macerated grass/agar mixture would have to be made if a macerated grass sample was to be examined. The results of this experiment would give a guide as to whether the spores had germinated, but, problems are that contamination of the sample may have occured and the mycelium that was being observed could have been that of a different fungus.

Microscopic examination could help to answer this question. The mycelium present in the samples could be isolated and stained with a suitable dye, such as lactophenol cotton blue, which would show -up the structure of the hyphae. The determination of the structure of the hyphae would provide information as to which group the fungus belonged. It would not provide information as

to which genus the fungus belonged. The mycelium of the Phycomycetes has nonseptate mycelium that is lacking in cross walls. All other groups of fungi apart from the Phycomycetes have distinct cross walls occuring at regular intervals along the hyphae. The mycelium of Basidiomycete fungi will be able to be identified by its characteristic clamp connections.

(IX.IV). Experiments Concerned With The Production Of Fruiting Bodies By Fungi Inoculated On Samples Of Autoclaved Macerated Grass With Different pH Values.

It might be expected that fungi inoculated onto samples of macerated grass might grow and produce fruiting bodies most successfully if the pH of the samples was altered to that encountered on the dung on which they are most often found.

As has been previously described, one can alter the pH of samples of macerated grass by treating them with phosphate buffer. The pH of replicate samples of autoclaved macerated grass could therefore be altered so that it was the same as that of fresh herbivore dung. Cultures of fungi could then be inoculated onto these samples using all the methods described. The samples would then be incubated in the laboratory and the production, and the time before appearance of fruiting bodies noted. The results from this experiment could indicate whether the above hypothesis was correct, and additional experiments could be carried out by incubating buffered macerated grass over a wide range of pH values, not just that of fresh dung.

## (IX.V). Experiments To Determine Fruiting Body Production By Pancreatin Treated Spores Inoculated Onto Samples Of Autoclaved Macerated Grass.

Some of the spores of coprophilous fungi inoculated onto autoclaved grass samples, may not have subsequently produced fruiting bodies, even though they were inoculated onto many replicate samples.

If these fungi had previously been observed to be able to grow on macerated grass samples then this result might suggest that the process of autoclaving the sample may be affecting it, so that it is unsuitable for the germination and growth of the fungi tested, or that; the spores needed to be exposed to some pre-treatment before they could germinate. This pre-treatment might consist of the passage of spores through the gut of a grazing herbivore.

To simulate passage through the gut to some extent, the spores of various coprophilous fungi could be pre-treated with pancreatin for five hours at 37°C. To determine whether this treatment had broken spore dormancy, these pre-treated spores could then be inoculated onto replicate samples of autoclaved macerated grass, which would be subsequently incubated in the laboratory. Untreated spores would also be inoculated onto samples and these would be incubated in the same manner to act as a comparison.

If the treated spores then produced fruiting bodies after inoculation onto the samples, while the untreated spores did not, then this would lend weight to the hypothesis that the spores need to travel through the herbivore gut before they can germinate.

(IX.VI).

Only a limited number of inoculation experiments are presented here, there are many others which could be carried out. For instance, the effects of various combinations of different factors on the production of fruiting bodies by fungi inoculated onto samples could also be studied. For example; this could consist of macerating samples of grass for different periods of time, autoclaving these samples, inoculating them with various fungi, and then incubating the samples at various temperatures. It could be that only when the samples had been macerated to a certain texture and incubated at a certain temperature, that the inoculated fungi would grow and produce fruiting bodies.

## (X). The Inoculation Of Autoclaved Substrates With Mixtures Of Fungi To Determine Possible Interactions Between Species.

The inoculation of an autoclaved substrate with a single fungal species reveals solely whether that fungus can grow and produce fruiting bodies on the material. In nature, however, it is very rare that a fungus should grow in isolation from other species that may be competing for the same nutrients. The purpose of this set of experiments therefore, is to observe the results of inoculating autoclaved samples with pairs of different fungal cultures.

Aliquot samples of autoclaved macerated grass and autoclaved dung would be produced; and two different cultures inoculated onto the same autoclaved sample of dung and of grass, using all of the methods described. Many replicate samples would be inoculated in this way. A distance of about two centimetres would be left between the two inoculation points, and various combinations of fungi inoculated onto samples.

The fungi would also be inoculated singly onto samples of autoclaved macerated grass and autoclaved dung, their performance on these samples could then be compared with that of paired inocula.

Also, two fragments of cultures of fungi could be removed from their agar culture plates and mixed together in 5ml of sterile water. This culture mixture would then be inoculated onto samples of autoclaved macerated grass and autoclaved dung, and incubated in the laboratory with the other samples.

One would then observe which species of fungi produced fruiting bodies, and the amount of fruiting bodies produced.

If none, or very few, fruiting bodies of a species were produced, then this could have been due to antagonistic effects caused by the other species. Any interaction between the fungi affecting the production of fruiting bodies, should be more apparent in the latter method of inoculation, because, using this method, the fungal mycelia are already in close contact, even before the inoculation process. The hyphae do not have to touch for antagonistic effects to be apparent. The fungi digest their food extracellularly, secreting enzymes and absorbing digestion products, so that there will be competition for nutrients. Also, a fungus may produce antibiotics that could diffuse throughout the sample. There are various possible types of interactions between associated fungi. Of course, many replicate inoculation experiments would have to be carried out, because it is possible that due to chance a fungus might not have become established and its absence might not be ascribable to effects caused by the other fungus.