FINAL REPORT

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Studies on fungicides in mushroom casing in relation to disease control

M 41a

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December 2005
Commercial - In Confidence

Grower Summary

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STUDIES ON FUNGICIDES IN MUSHROOM CASING IN RELATION TO DISEASE CONTROL

Final report 2005

Project Title:	Studies on fungicides in mushroom casing in relation to disease control
Project Number:	M 41a
Project Leader:	Dr Helen Grogan, Warwick HRI
Final Report:	Dec 31st 2005
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Date Project commenced:	1st October 2002
Date Project Completed:	31st December 2005
Key words:	Prochloraz, Sporgon 50WP, *Verticillium fungicola, Agaricus bisporus*, mushroom, disease
Signed on behalf of:	**Warwick HRI**
Signature:	……
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Date:	……

Contents

Grower Summary

Headline

Microbes have been found living in fungicide spray tanks that can degrade the fungicide prochloraz. This is the active ingredient in Sporgon 50WP, which is used to control dry bubble disease.

Background and expected deliverables

The fungicide Sporgon 50WP is the only chemical available for the control of dry bubble disease of mushrooms, caused by the pathogen *Verticillium fungicola*. Recent HDC funded work (project report M 14b) showed that in Britain over 60% of isolates tested were now moderately tolerant to Sporgon 50WP, however, cropping experiments have shown that Sporgon 50WP still gives good control of these isolates (project report M 14c). Analysis of casing throughout the cropping period has shown that prochloraz concentration (active ingredient in Sporgon 50WP) in casing decreases significantly by the end of the first flush. Thus control of dry bubble with Sporgon 50WP in later flushes may be less effective. Good disease management is therefore still very important in order to keep this disease under control. The deliverables from this project are:

1. Information on the persistence of prochloraz in mushroom casing over time
2. Information on the key environmental and cultural factors affecting prochloraz persistence in mushroom casing
3. A small scale survey of the industry to indicate if prochloraz degrading microbes are common on mushroom farms

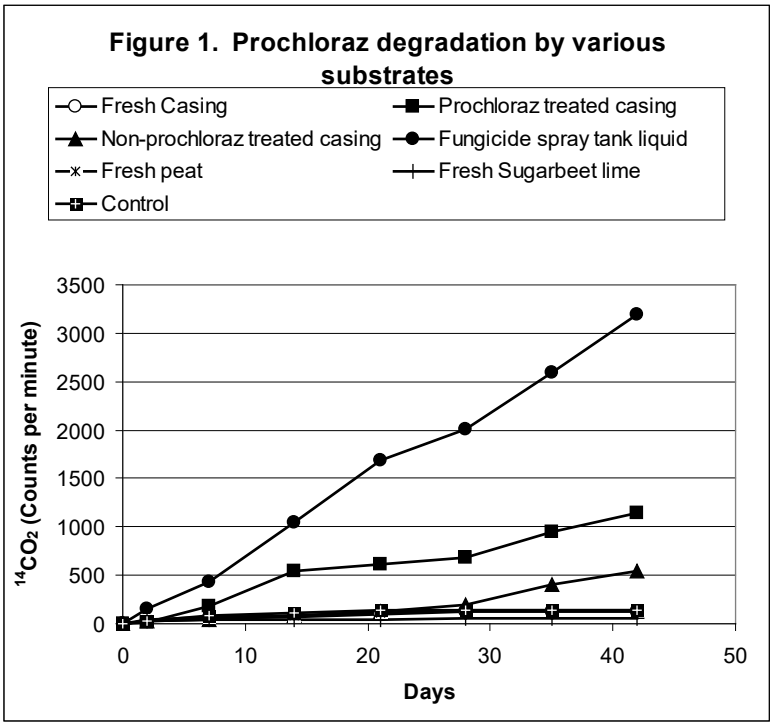
Summary of the project and main conclusions

This project looked at the factors that affect the activity and persistence of the fungicide prochloraz (Sporgon 50WP) in mushroom casing. Experiments were conducted which showed that prochloraz disappeared steadily from casing following its application when applied to a mushroom crop using standard fungicide spray equipment. However, prochloraz was more persistent in casing when applied to a small scale experimental crop using laboratory equipment. Further experiments indicated that neither *Agaricus bisporus* growth, nor casing moisture content, had any effect on prochloraz loss from casing. By the end of year two it was apparent that some unknown factor, associated with standard growing conditions, was implicated in the loss of prochloraz from casing.

The third year of this project focused on trying to identify the unknown factor responsible for prochloraz loss from casing under commercial growing conditions. Casing soil from a prochloraz-treated crop grown at Warwick HRI was shown to contain microbes which degrade prochloraz. A series of experiments was then conducted to test individual

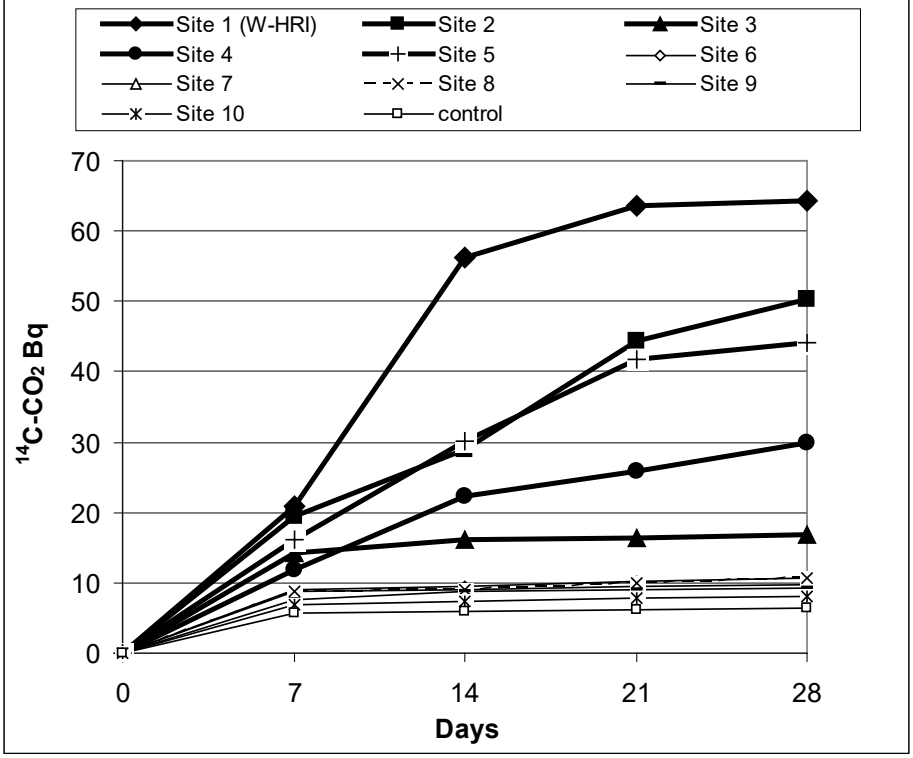
components of casing (i.e. peat, sugarbeet lime) and fungicide solution from fungicide spray tanks, to identify if any of these factors were a source of the prochloraz-degrading microbes. The results clearly indicated that prochloraz degrading microbes were present in the fungicide spray tank and not in any of the individual casing ingredients (Figure 1).

Figure 1. Prochloraz degradation by various substrates



Following on from this, 10 mushroom farms located throughout the UK were surveyed, and liquid samples were taken from fungicide spray tanks. The farms spanned a variety of growing systems and each farm managed their fungicide tanks differently. Some used them only for applying pesticides such as fungicides and nematode-based biological control agents; some used them also to apply water to crops if water pressure was variable; some used them to apply sodium hypochlorite. Two sites were organic and no longer used the tanks for pesticides, only for applying water. Samples from four of the 10 sites showed that prochloraz-degrading microbes were active in the spray tanks but their presence was not associated with any particular fungicide tank use profile (Figure 2).

Figure 2. Degradation of prochloraz by microbes in fungicide spray-tanks from 10 sites around Britain



The main **conclusions** of this work are:

- Prochloraz is relatively stable in casing under laboratory conditions but not under standard growing conditions
- *A. bisporus* does not degrade or remove prochloraz from casing
- Casing moisture content has no adverse effect on prochloraz persistence in casing
- The loss of persistence of prochloraz in mushroom casing over time is caused by microbial breakdown.
- Prochloraz-degrading microbes are not present to any significant degree in fresh casing or in individual casing ingredients.
- Prochloraz degrading microbes are present in fungicide spray tanks and have been detected at several mushroom farms around the country.

Future work

This project has identified a potential weakness in the fungicide-application system used on mushroom farms, namely the presence of prochloraz degrading microbes living inside fungicide spray tanks. Future work will identify if they have a significant impact on the efficacy of prochloraz against dry bubble disease, and if so, can they be removed from tanks by either a washing or disinfection routine.

Financial benefits

It is important to maximise the disease-controlling effects of costly fungicides by identifying the important factors that influence efficacy. The results to date will not have any financial benefits until (A) it is determined if prochloraz-degrading microbes can be eradicated from spray tanks and (B) if the elimination of prochloraz degrading microbes from spray tanks results in better control of *Verticillium* by prochloraz.

Action points for growers

At this point in time there are no recommendations for change of practice as a result of this project. A future project will examine whether or not the presence of prochloraz-degrading microbes in spray tanks reduces the efficacy of the fungicide against *Verticillium* and whether there is a need to recommend a modified tank cleaning regime. Despite the presence of prochloraz degrading microbes in spray tanks, prochloraz continues to give significant control against *Verticillium* so it is still a very valuable product for the control of this disease. Until such time as additional work can be carried out growers are reminded of the important action points to be aware of in the fight against Dry Bubble disease. Of primary importance is the fact that contamination of the casing with *Verticillium* spores **during casing preparation and/or application** is likely to be the most important route for *Verticillium* infections on a farm so that a high standard of general hygiene along with minimum dust generation will best aid disease control. The following points highlight the best practice for the control of dry bubble:

- Do not dry-sweep any areas or raise dust, **especially** when casing is being prepared and crops are being cased
- Ensure filters for fresh air at airing are in good condition
- Ensure diseasing-teams identify and treat dry bubble pieces quickly, BEFORE any watering is done, by covering any diseased areas with salt.
- Do not dry-sweep cropping rooms, especially when there is disease in a crop
- Terminate badly infected third flushes early to minimise the build up of background disease levels on the site
- Keep fly numbers down
- Ensure prochloraz (Sporgon 50WP) is applied correctly and evenly at the rates specified on the label
- Do not apply fungicide to very dry casing (i.e. between flushes) if there is a risk of run-off, and therefore under dosing; pre-water very dry casing before fungicide treatment

SCIENCE SECTION

1 Introduction

Little is known about what happens to fungicide active ingredients once they have been applied to mushroom casing. Results from HDC funded research (Grogan & Jukes 2003) suggests that prochloraz (Sporgon 50WP) concentrations in casing decrease with time during the cropping cycle so that fungicide levels are much reduced when threats from diseases are at their highest. The mushroom industry has very few approved fungicides for use and some of these are compromised as a result of fungicide resistance among pathogen populations

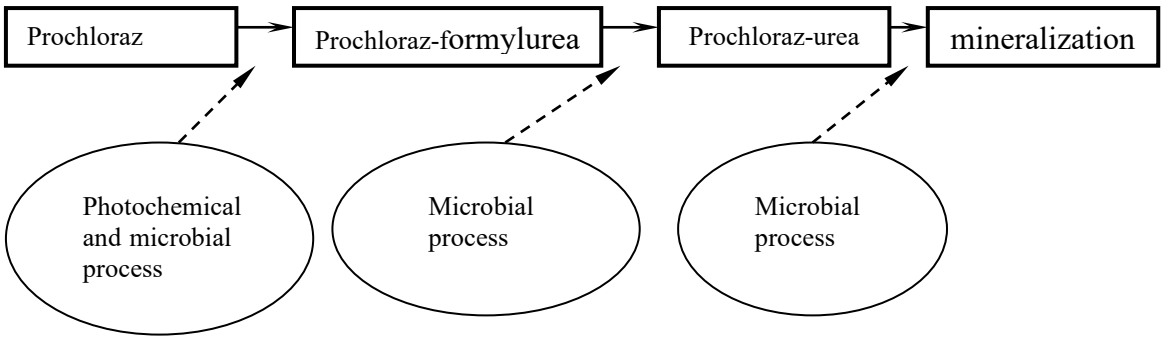
The use of Sporgon 50WP (prochloraz) in the British mushroom industry in 2003 was 1,775 kg active substance (Stoddart *et al*. 2003). At today's prices this is equivalent to around £120K, which represents approx. 0.14% of the £85m value of British mushrooms in 2004. Despite this spend, there is still considerable loss of production due to disease which, for dry bubble disease (*Verticillium*), has been estimated at £2-3million. If fungicide efficacy can be enhanced then this loss due to disease could be reduced. The aim of this project is to understand the reasons behind the loss of fungicide activity in mushroom casing.

1.1 Prochloraz characteristics in the soil

Prochloraz [N-propyl-N-{2-(2,4,6-trichlorophenoxy)-ethyl}imidazole-1-carboxamide] belongs to the group of imidazole fungicides that inhibit ergosterol biosynthesis. It is widely used to control eyespot disease and powdery mildew on cereals and it is also effective against a broad spectrum of fungal diseases on fruits as well as vegetables (Kapteyn et al. 1992; Tomlin 2000). A minor use of the compound is for disease control in mushroom growing systems. Prochloraz exhibits a half-life in soil under field conditions of between 5 and 37 days whereas in the laboratory, soil half-lives range between 92 and 171 days (Hollrigl-Rosta et al., 1999). In soil conditions therefore there is likely to be microbial degradation. The main metabolic pathway for prochloraz breakdown starts with prochloraz-formylurea, which is then hydrolysed to prochloraz-urea. Both substances have been isolated in mammalian and soil degradation studies.

1.2 Degradation of prochloraz

Degradation studies by Hollrigl-Rosta et al. (1999) demonstrated that both biotic and abiotic degradation occur concurrently for prochloraz. The overall fate of this chemical in soil is determined by a combination of photochemical and microbial processes. The importance of the microbial activity of soil in prochloraz degradation was also stressed by Bock *et al.* (1996) as they reported the formation of prochloraz metabolites (prochloraz-formylurea) after incubating a medium containing the compound and a strain of *Aureobacterium* spp. Finally, the later steps of prochloraz degradation, from the hydrolysis of prochloraz-formylurea to prochloraz-urea through to its mineralization depend only on microbial metabolism. The breakdown pathway of prochloraz is shown below:



1.3 Aim of this study

The fate of pesticides in agricultural soils has been studied extensively in last decades. However, there is limited knowledge about the fate of prochloraz in mineral soils and few researchers have looked at prochloraz-soil interactions (Hollrigl-Rosta *et al.* 1999, Roy *et al.* 2000). Furthermore, in mushroom crops where prochloraz is applied on a peat-based casing little is known about sorption, biodegradation, availability for disease control and effectiveness of this fungicide against *V. fungicola*. The overall aim of this study is:

- To understand the factors affecting the persistence of prochloraz in mushroom casing

2 Summary of results from previous interim reports

2.1 Small scale laboratory experiments

Under laboratory conditions prochloraz disappeared from mushroom casing soil slightly faster at 25°C than at 15°C, but the levels in mushroom casing and a sandy loam were still relatively high, even after 50 days (Figure 1 & 2). Prochloraz was thus fairly persistent in both peat-based casing and in the mineral soil (sandy loam) and there was no evidence of biological or chemical degradation. This was in contrast to the observations of Grogan & Jukes (2003) where prochloraz concentrations in mushroom casing dropped significantly over the life of the crop.

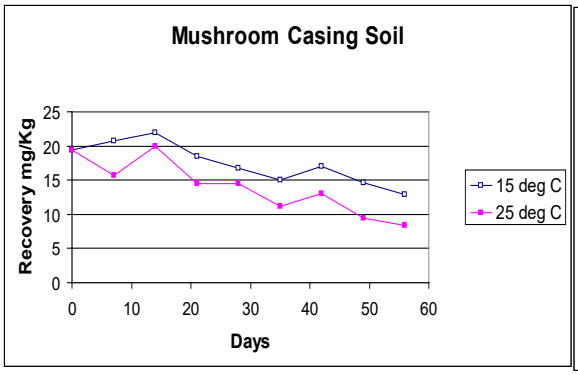


Figure 1. Prochloraz recoveries from mushroom casing at 15°C and 25°C

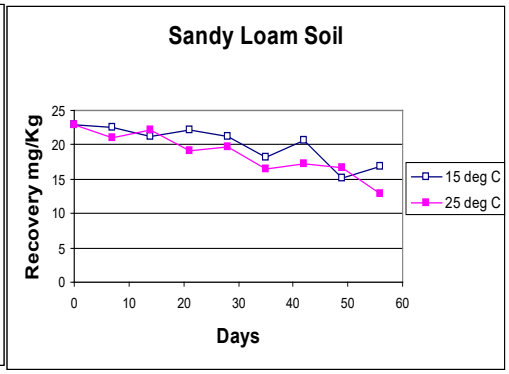


Figure 2. Prochloraz recoveries from sandy loam at 15°C and 25°C

Wetting and drying cycles are standard practice in a mushroom growing unit and moisture content influences the physico-chemical properties of casing, and consequently its relationship with pesticides (prochloraz). In laboratory experiments, we investigated whether or not the constant wetting and drying cycles could be responsible for the rapid disappearance of prochloraz from casing reported by Grogan & Jukes (2003). We found no evidence of rapid disappearance of prochloraz or any effect of wetting and drying cycles on the rate of loss. Prochloraz losses from leaching and run-off have not been investigated in this study, but Grogan & Jukes (2003) showed that prochloraz does not move through the casing layer towards the compost.

Since wetting and drying cycles did not have any impact on prochloraz recoveries and did not cause any appreciable prochloraz loss, *A. bisporus* was the next factor to be investigated for possible effects on degradation. However, in small laboratory-scale cropping experiments, *A. bisporus* had no effect on the levels of prochloraz recovered from casing (Figure 3). The activity of mushroom mycelium in the casing did not therefore exhibit any ability to remove or break down prochloraz.

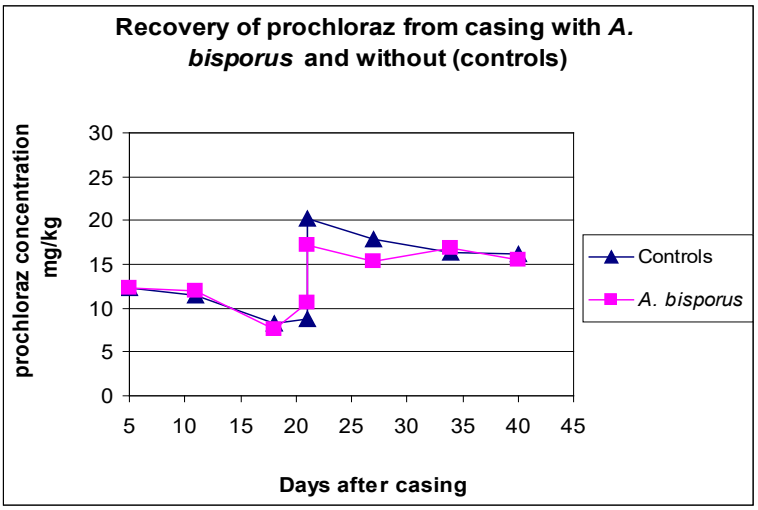


Figure 3. The recovery of prochloraz from mushroom casing in a small scale laboratory-based cropping experiment.

2.2 Prochloraz behaviour in a large scale cropping experiment

The small-scale experiments suggested that prochloraz behaviour was quite stable in mushroom casing and that it was largely unaffected by either the wetting/drying regime of the crop management practices or by the presence of *Agaricus*. This was in contrast to published results for large-scale experiments in a mushroom unit (Grogan & Jukes 2003). It was decided to repeat the work of Grogan & Jukes (2003) in order to establish if the earlier work could be reproduced. A large scale experiment was set up in HRI's Mushroom Unit using all the standard cultural techniques and equipment that are used in most commercial units in UK. The results confirmed the earlier findings that the amount of prochloraz in mushroom casing decreased rapidly with time during the life of the crop, when grown using standard commercial conditions and equipment (Figure 4)

The results firmly suggested that although prochloraz was stable in mushroom casing under laboratory conditions, it disappeared rapidly from casing in standard growing conditions. *Agaricus bisporus* had no effect on fungicide concentrations in casing, but the rapid decline in fungicide concentration was strongly suggestive of microbial activity. One of the big differences between the laboratory based experiments and the larger scale studies was the use

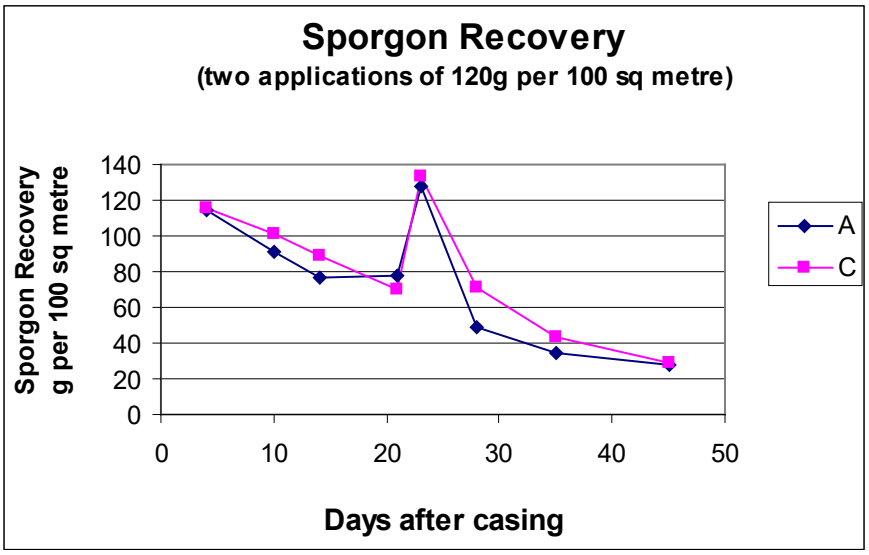


Figure 4. Recovery of Sporgon (prochloraz a.i.) from casing during the course of a mushroom crop. Sporgon had been applied at 120g/100m^2 on Day 4 and again on Day 21. Treatments included standard trays with *Agaricus* (A) and Control trays with no *Agaricus* (C).

of the HRI fungicide spray tank and casing preparation equipment. These could potentially contribute microbes that would be absent from laboratory glassware used in the laboratory scale studies. A series of experiments were therefore planned to address the questions:

1. Can we confirm that the loss of prochloraz from mushroom casing is due to microbial activity?
2. Can we identify what these microbes are?
3. Can we determine where such microbes may occur on a mushroom unit?

The results of these experiments are presented in the following sections.

2.3 Conclusions

The overall conclusions from the earlier work that has already been reported on in detail can be summarised as follows:

- Prochloraz is relatively stable in mushroom casing under laboratory conditions
- *Agaricus bisporus* does not degrade or remove prochloraz from casing
- Casing moisture content does not have any significant effect on loss of prochloraz from mushroom casing
- Some factor(s) associated with standard mushroom cultivation and /or standard equipment appear(s) to facilitate the loss of prochloraz during the life of a crop.

3 Microbial degradation studies.

3.1 Introduction

The environmental fate of pesticides in the soil is viewed with great concern today mostly due to the problems resulting from the use of persistent and mobile molecules affecting surface and ground water quality. Along with the increasing concern about chemical contamination of various ecosystems, much emphasis has been put on designing suitable methods to characterise the different processes affecting the fate of pesticides in soil (Cornejo *et al.* 2000). Ideally the chemical compounds used in crop protection should persist long enough to control target organisms and then degrade into inert products. Leaching and run-off losses, however, lead to inadequate control of target organisms as well as pollution of surface and ground waters (Vink, 1997).

Degradation research studied by Hollrigl-Rosta *et al.* (1999) reported that both biotic and abiotic degradation occur concurrently for prochloraz. The overall fate of this chemical in soil is determined by a combination of photochemical and microbial degradation processes. The importance of the microbial activity of the soil in prochloraz degradation was also stressed by Bock *et al.* (1996) as they reported the formation of prochloraz metabolites in a medium containing prochloraz and a strain of *Aureobacterium* sp. It is therefore highly likely that prochloraz degrading microbes will be selected for in places where prochloraz is used, such as on mushroom farms. Mushroom casing and compost are microbially rich substrates and could harbour organisms that are capable of utilising prochloraz as a carbon source. Fletcher *et al* (1980) reported a case where carbendazim degrading organisms were detected at high levels on one farm while an HDC report (M14c) also reported loss of carbendazim in mushroom casing over time, most likely as a result of microbial degradation (Grogan & Jukes, 2003).

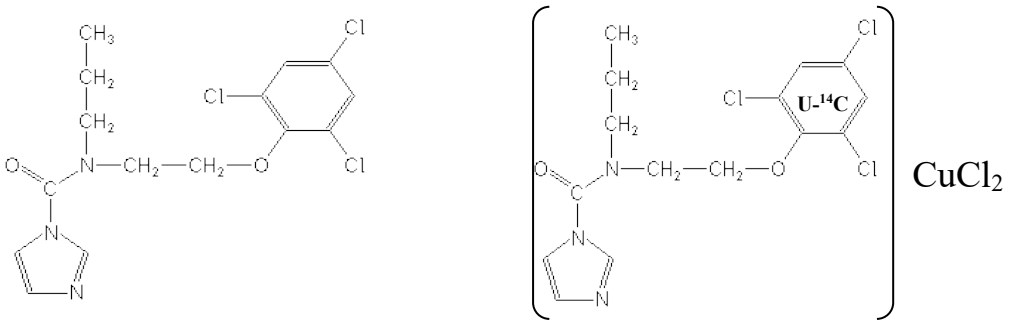
In view of the fact that prochloraz is steadily lost from mushroom casing following its application, in a manner suggestive of microbial degradation, it was decided to try and prove conclusively that this was the case and to try and identify where such micro-organisms might be. This section deals with several experiments looking at microbial degradation of prochloraz with respect to mushroom casing and mushroom growing conditions on British farms.

3.2 Evaluation of the prochloraz degrading potential of a mushroom casing sample

3.2.1 Materials and Methods

Chemicals

Prochloraz [N-propyl-N-{2-(2, 4, 6-trichlorophenoxy)-ethyl} imidazole-1-carbox-amide] analytical grade (98% purity, Greyhound Chromatography & Allied Chemicals UK) was used in all liquid media. Ring-labelled prochloraz [Tetrakis (N-(propyl-N-[2-(2, 4, 6-trichloro [U-C^{14}] phenoxy) ethyl] imidazole-1-carboxamide) (99.3% radio-purity, Scynexis Inc., USA) copper salt] was also used in respirometric studies.



Prochloraz ^{14}C-ring labelled Prochloraz

Mineral media

A liquid Mineral Salts Medium (MSM) was prepared using three stock solutions (Table 1); 100ml of stock 1 was added to 780 ml of distilled de-ionised water and the solution was sterilised by autoclaving; when cool, 100 ml of stock 2 and 20 ml of stock 3 were added under sterile conditions (Bending *et al* 2003).

Preliminary assessment of prochloraz-degrading microbial activity in mushroom casing

Microbial degradation of prochloraz was studied by adding a small quantity of mushroom casing soil (taken from a prochloraz-treated crop on Day 27 after casing) to a mineral medium containing prochloraz as the sole source of carbon. The prochloraz medium was prepared as follows: Analytical grade prochloraz was added to 2 ml of ethanol to give a concentration of 150 mg ml^{-1}. A 200µl aliquot of this stock was added to a sterile Duran bottle, which was then left open in a laminar air flow bench until the ethanol had completely evaporated. One litre of MSM was then added to give a final prochloraz concentration of 30 mg L^{-1}. The bottle

Table 1. Preparation of stock solutions.

	De-ionised water	Mineral Salt	weight
Stock 1	500ml	KH_2PO_4	11.35g
		$Na_2HPO_4 \cdot 12H_2O$	29.85g
		NaCl	5.00g
Stock 1. Dissolve mineral salts in water; sterilise by autoclaving for 20 minutes at 121°C			
Stock 2	500ml	$MgSO_4 \cdot 7H_2O$	2.50g
		$CaCl_2 \cdot 2H_2O$	0.05g
		$MnSO_4 \cdot 4H_2O$	0.10g
Stock 1. Dissolve mineral salts in water; sterilise by autoclaving for 20 minutes at 121°C			
Stock 3	1000ml	$FeSO_4 \cdot 7H_2O$	0.25g
Stock 3. Dissolve mineral salt in water, sterilise by passing through 0.2µ millipore filter (Nalgene). Store at 5 °C.			

was shaken at 100 revolutions per minute (rpm) for 1h on a Wrist-Action® Shaker (Burrell Scientific) to ensure the prochloraz was completely dissolved in the MSM. The concentration of the medium was checked by HPLC. ^{14}C labelled prochloraz was then added to the prochloraz medium to facilitate the measurement of ^{14}C-labelled CO_2; this would give an indication of the level of prochloraz mineralisation taking place as a result of microbial degradation. A ^{14}C ring-labelled prochloraz stock solution in methanol was made to give a radioactivity concentration of 4.7MBq/ml. A 10.6 µl aliquot of the stock solution was pipetted into a sterile Duran bottle and left open in a laminar flow-bench until the methanol had evaporated. Then 500 ml of prochloraz-amended MSM prepared earlier was then added to the bottle and shaken, as before, for 1h. The final media had a concentration of 30 mg L^{-1} prochloraz and a radioactivity of 100Bq ml^{-1}. The addition of ring-labelled prochloraz did not change the concentration of prochloraz in the solution due to the ultra-low volume of ^{14}C-prochloraz added.

Respirometer units

For monitoring the mineralization of radio-labelled prochloraz a simple respirometric method was used (Reid *et al* 2001). Each respirometer consisted of a 500 ml or 250 ml screw-cap Duran bottle in which a hole was drilled in the centre of the plastic lid and fitted with a stainless steel threaded rod to which a crocodile clip was attached (Figure 1). The rod was held in place with washers, nuts and a wing on top of the lid to produce an airtight seal. A ^{14}C-CO_2 trap, consisting of a plastic scintillation vial containing 1ml NaOH 1M, was attached to the crocodile clip. Prochloraz-amended liquid medium (100ml) containing ring-labelled prochloraz was placed in the respirometer unit. Microbial inoculum was then added to the units with treatments consisting of the following:-

Figure 1. Respirometric flask system: Duran bottle, screw cap, scintillation vial, crocodile clip, threaded rod, wing nut, Teflon liner (Reid *et al*. 2001)

1. 1 g of casing soil taken from a prochloraz-treated crop on Day 28 after casing
2. 1 g of casing soil from the same source that was γ-irradiated (sterilised)
3. no inoculum

Three replicates were prepared for each treatment. The lids were screwed tightly into place and the bottles were placed in an orbital shaking incubator set at 70 rpm and 25 ºC.

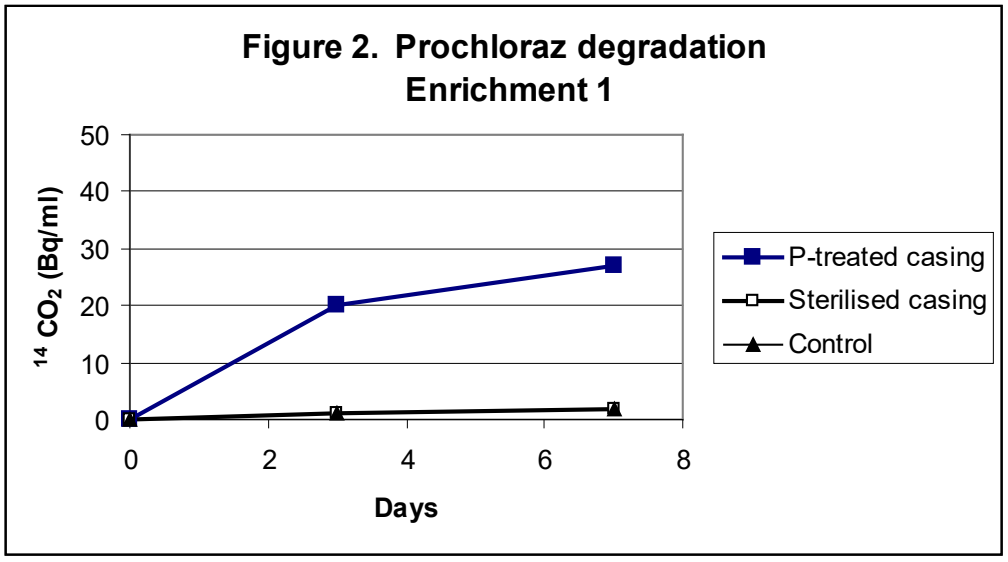
At selected time intervals of between 1 and 7 days, the lids of the respirometer units were unscrewed in a class II safety cabinet and the ^{14}C-CO_2 traps were quickly removed and replaced with new ones. The scintillation vials were wiped externally with ethanol to avoid contamination and the quantity of trapped ^{14}C-CO_2 was measured using a scintillation counter. A 1 ml aliquot of the prochloraz-amended culture medium were also removed and tested for prochloraz by high performance liquid chromatography (HPLC). The sample was placed in an Eppendorf tube and centrifuged at 1000 rpm for 20 min to remove solid materials from the culture medium. A 0.75 ml aliquot of the supernatant was mixed with 0.75ml of the HPLC mobile phase {[acetonitrile:water: orthophosphoric acid] [85: 15: 0.25] v/v} before injecting into the HPLC (Kontron series 300 with a Pinnacle II C-8 column (5µm, 150x4.6mm, Restek) at a flow rate of 1 ml/min. Prochloraz was quantified by UV absorbance at 220nm wavelength. The software used for processing the results was KromaSystem 2000.

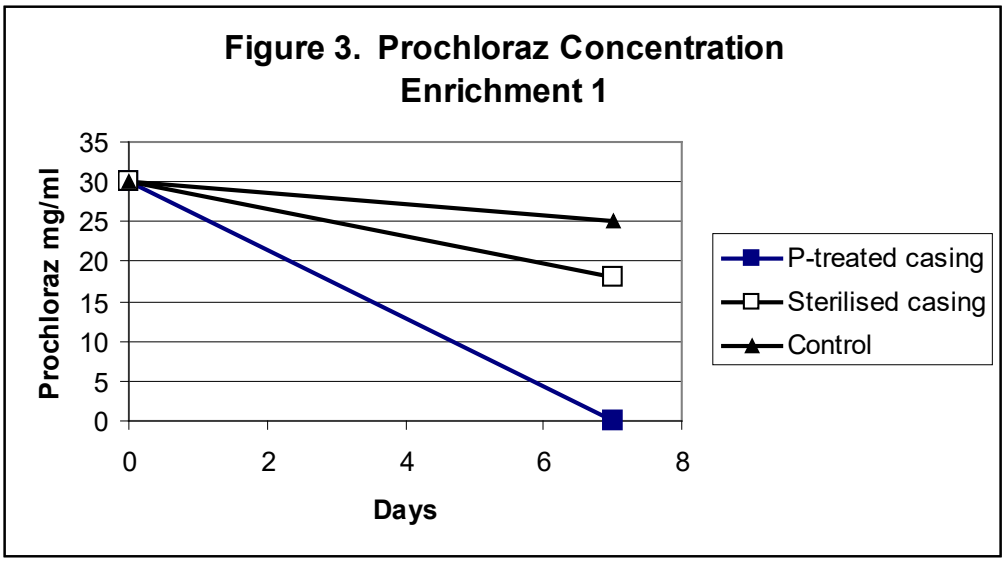
When > 90% of the prochloraz had been degraded, a second enrichment culture was set up by transferring 1ml from the first enrichment culture into a new respirometer unit containing 99

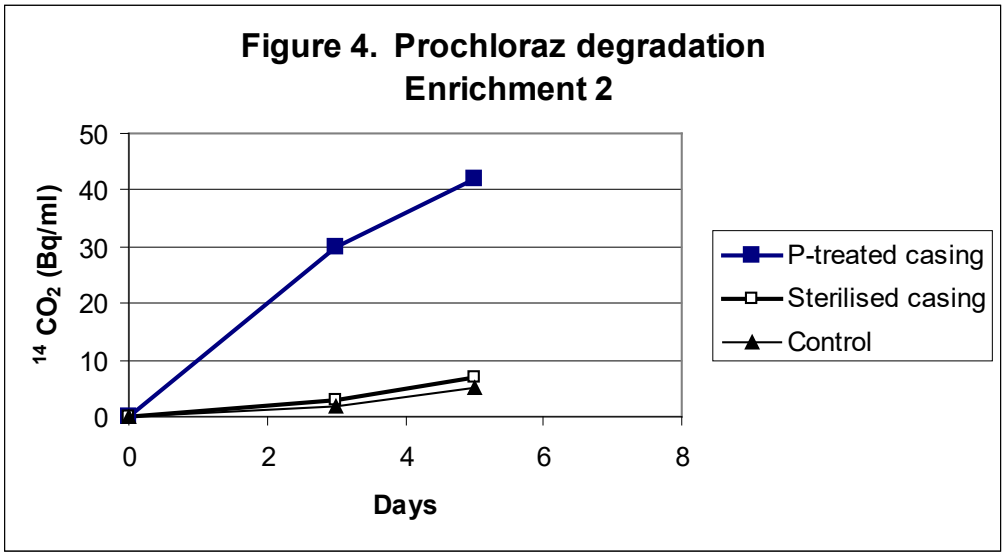
ml of fresh prochloraz-amended MSM (30 ml L^{-1} prochloraz and 100Bq ml^{-1} ^{14}C-prochloraz). Again, when >90% of the prochloraz had been degraded a third enrichment culture was set up in a similar fashion.

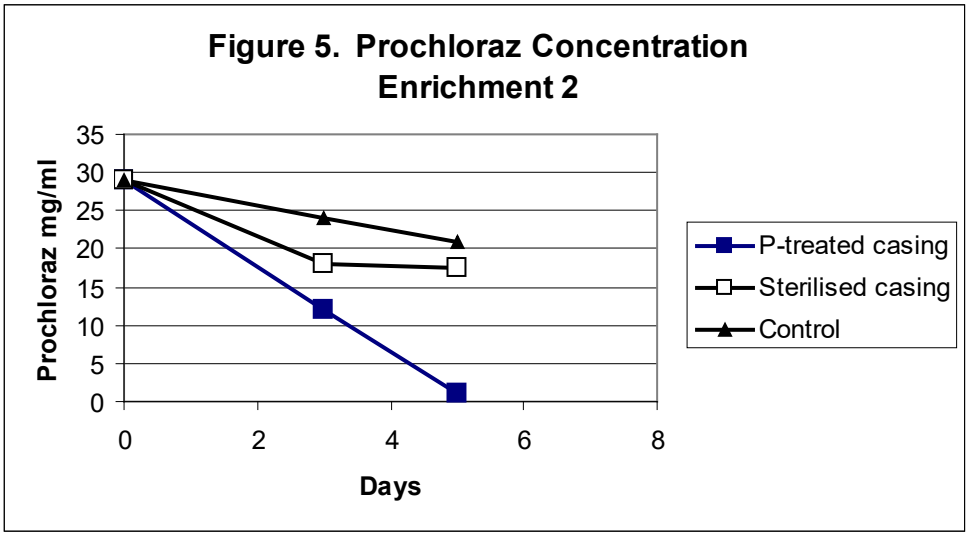
3.2.2 Results & Discussion

Prochloraz degradation, as measured by $^{14}CO_2$ production from ^{14}C-labelled prochloraz in the respirometer units, was detected in the presence of a prochloraz-treated casing sample (P-treated casing), but not when the sample had been sterilised (thereby killing all microbes), or in the controls (Figure 2). This indicates that the prochloraz-treated casing contained living organisms capable of utilizing the ^{14}C-labelled prochloraz as a carbon source. Analysis of Enrichment 1 growing medium by HPLC also indicated that no prochloraz remained by Day 7 in the medium containing the prochloraz-treated casing (Figure 3). Some loss of prochloraz occurred in the sterilised casing sample and the control, which may have been due to adsorption of prochloraz by the casing and/or physical breakdown of the prochloraz. Similar results occurred in Enrichment 2 (Figures 4-5) and Enrichment 3, however additional sampling times during these enrichments indicated that all the prochloraz was removed by Day 5, or 4, respectively. The speed of loss suggests that the sample interval for the enrichment studies should have been daily in order to determine how rapidly the prochloraz was being degraded.









3.3 Isolation and characterisation of prochloraz degrading organisms

The microbes present in the third prochloraz-amended enrichment cultures described in the previous section were isolated and characterised by several methods.

3.3.1 Materials and Methods

Isolation of bacteria from third enrichment culture

A 500µl aliquot was sampled from each of the three replicates of the third enrichment culture and added to 4.5ml of Ringer's solution (NaCl 0.9 g, KCl 0.42 g, CaCl 0.25 g, in 100 ml sterile/deionised water) in one of six wells on a microtitre plate. After mixing with a pipette 500 µl of this solution was transferred to a new well, containing 4.5 ml of Ringer's solution, and mixed. Further dilutions were made in the same way creating six serial dilutions d_1, d_2, d_3, d_4, d_5 and d_6. For each of the dilutions (d_1 to d_6), for each of the three replicates, 200µl were spread onto agar Petri dishes (1% w/v R_2A agar, Sigma) and incubated at 20ºC for 7 days. At the end of the incubation time the Petri dishes were scanned under a low-power microscope. The total number of bacterial colony forming units (c.f.u) was counted and colony types were assigned to 5 distinct phenotypic groups according to a number of properties such as colour, shape and size.

Determining the prochloraz-degrading ability of bacteria

Forty individual colonies, representing 8, 7, 11, 4 & 10 replicates of each of Phenotypes 1, 2, 3, 4 & 5, respectively, were picked off with a sterile loop into individual wells on a microtitre plate containing 5ml of prochloraz-amended MSM (with 30mg/L prochloraz). The numbers varied depending on the relative abundance of each type. In addition, a consortium of all bacterial types was prepared by washing cells from plates. The consortium was subject to five tenfold serial dilutions and each dilution was inoculated into 4.5 ml prochloraz-amended MSM in microtitre wells. Nine uninoculated wells with 5ml of prochloraz-amended MSM only, served as controls. The plates were incubated in an orbital shaker at 25ºC and 70 rpm. Prochloraz concentration was measured in all wells after eleven and 28 days of incubation.

Molecular Profiling

PCR-16S rRNA-denaturing gradient gel electrophoresis (DGGE) was conducted in order to determine whether organisms proliferating following treatment with prochloraz could be

identified by molecular profiling. DNA was extracted from 100-500 µl-samples from each of the three replicates obtained for the first three enrichments (One replicate from a fourth enrichment that was subsequently prepared, was also included), using a Cambio Ultraclean DNA extraction kit. Partial eubacterial 16S rRNA gene fragments were amplified using primers described by Muyzer *et al.* (1993) at positions 341f and 534r (*Escherichia coli* numbering), using a Hybaid Omnigene thermocycler (Ashford, UK). DGGE gels were set up according to Muyzer *et al*. (1993) using an Ingeny PhorU System (Amsterdam) with 8% acrylamide, and a denaturant gradient of 20 - 70% (100% denaturant was equivalent to 7 M urea with 40% vol/vol formamide). The gels were run at 70 V and 60ºC for 18 hours. The gels were stained with ethidium bromide (0.5 mg l^{-1}) and visualised under UV light on an Imago Imaging system (B and L systems, the Netherlands).

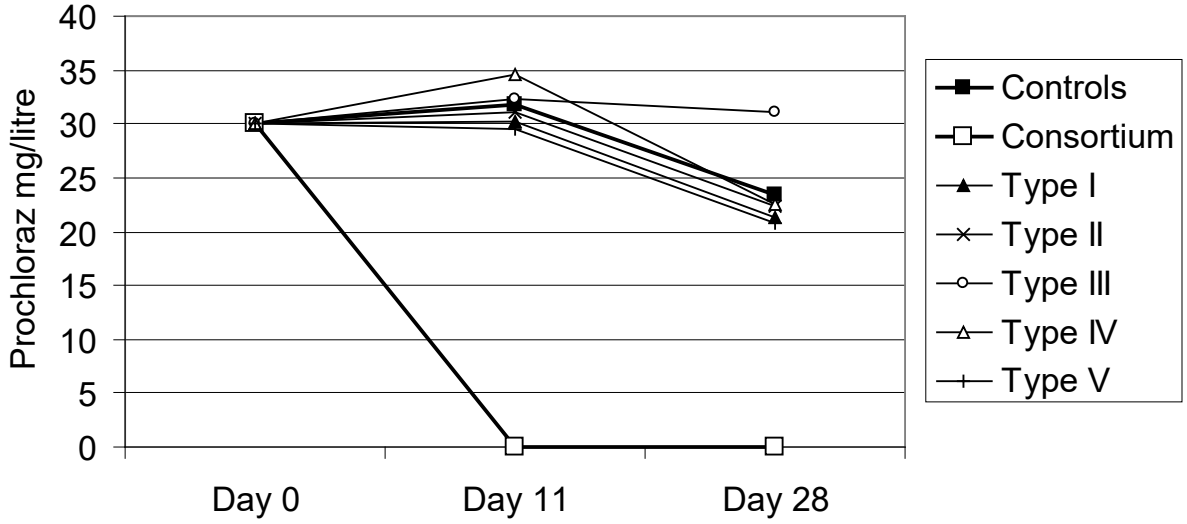
The PCR products were purified using a QIAquick PCR purification kit (Qiagen Ltd, Dorking, UK), and then cloned using a TOPO TA Cloning kit (Invitrogen, Paisley, UK). Sequencing reactions were performed according to manufacturer's instructions, on a Hybaid PCR multiblock system (Hybaid, Middlesex, UK), using a PRISM BigDye Terminator Cycle Sequence reaction kit (Applied Biosystems, Warrington, UK). Products were analysed on an Applied Biosystems 377 DNA sequencer.

3.3.2 Results and Discussion

Only the consortium of bacteria was capable of degrading prochloraz (Figure 8) and this was complete by the first sampling time of 11 days. No single microbe type had a significant effect on degradation when present in isolation with prochloraz. It is not unusual for pesticide degradation to be carried out as a result of several microbial degradation steps, requiring several different organisms. Some bacteria may be able to start the process of degradation while others may only be capable of degrading specific elements of the compound. Some microbes may also require other nutrients or growth factors to enable them to attack prochloraz and these can often be by-products from other microbes. If the necessary microbes for degradation are present in the casing as a heterogeneous population, then the process of degradation will progress. The data in Figure 8 suggests that a complex of organisms is required to degrade prochloraz. It may be that only some of the consortium

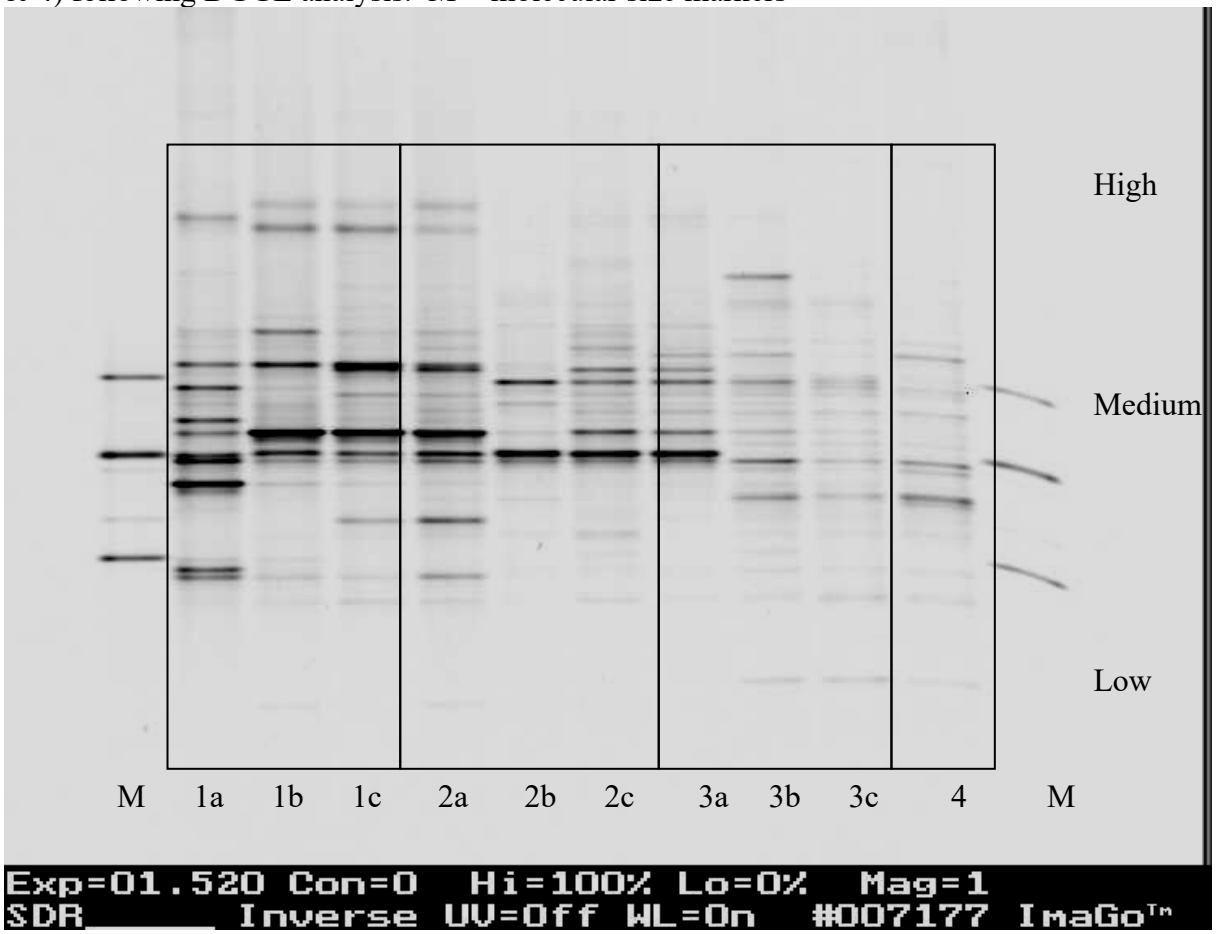
organisms were necessary and further work would be required to identify which types are specifically needed by looking at the degrading ability of all possible combinations of the five types of microbes.

Figure 8. Prochloraz degradation by selected microbes



Molecular profiling of the organisms in the enrichment cultures indicated that as the enrichments progressed, the molecular profile of the organisms present did not change very much. Some elements appeared to disappear (high bands at the top of Enrichment 1a, 1b, 1c and 2a; Figure 9) but most of them persisted (medium and low bands) in Enrichments 3a,b,c & 4. Consequently, it was not possible to identify organisms that could have contributed to prochloraz degradation.

Figure 9. Molecular profiles of microbes in four Enrichment cultures (1a,b,c, 2a,b,c, 3a,b,c & 4) following DGGE analysis. M = molecular size markers



3.4 Localization of prochloraz degraders within mushroom casing and fungicide application processes.

The small scale laboratory experiments indicated that prochloraz degradation did not occur in mushroom casing that had no contact with the mushroom growing unit at HRI. In the small scale experiments prochloraz was applied to mushroom casing using clean laboratory glassware and pipettes so there was a possibility that the fungicide spray tank may have been a source of prochloraz degrading microbes. However there was also the possibility that the bacteria that are responsible for prochloraz degradation are present in mushroom casing ingredients at a low level, and given time and substrate, the population could increase on mushroom unit facilities. We conducted experiments to favour the growth of prochloraz degrading microbes by providing prochloraz as the sole source of carbon. Various different sample types were tested including peat, sugarbeet lime, freshly mixed casing and fungicide spray tank liquid. The experiment and results are described below.

3.4.1 Materials and Methods

Six substrates were selected for this study and are listed in Table 2 below.

Table 2.

Treatment	**Source**
Fresh casing	Unopened 50kg bag of fresh casing (TunnelTech English)
Prochloraz-treated casing	Casing taken from a prochloraz treated crop on day 32 after casing. Crop had been sprayed with Sporgon 50WP on day 3 and day 21, at a rate of 120g x $100m^{-2}$ in 180 litres. Prochloraz levels in the casing were shown to have decreased to a very low level by the time the sample was taken
Non-prochloraz-treated casing	Casing sample taken from a crop that was cased with the same batch of casing as the previous sample, but one which had **not** been treated with prochloraz
Fresh peat	Deep dug black peat used in preparation of 50kg bags of Tunnel Tech English casing mix
Sugarbeet lime	Sugarbeet lime ingredient used in preparation of 50kg bags of Tunnel Tech English casing mix.
Fungicide spray tank liquid	Aqueous liquid containing some solid sediment from the pump-housing and tank-bottom of the fungicide spraying equipment
Control	No substrate addition

A further six control treatments consisted of the six substrate samples which were sterilised by γ-radiation (as described earlier) to eliminate any microbes which might be present.

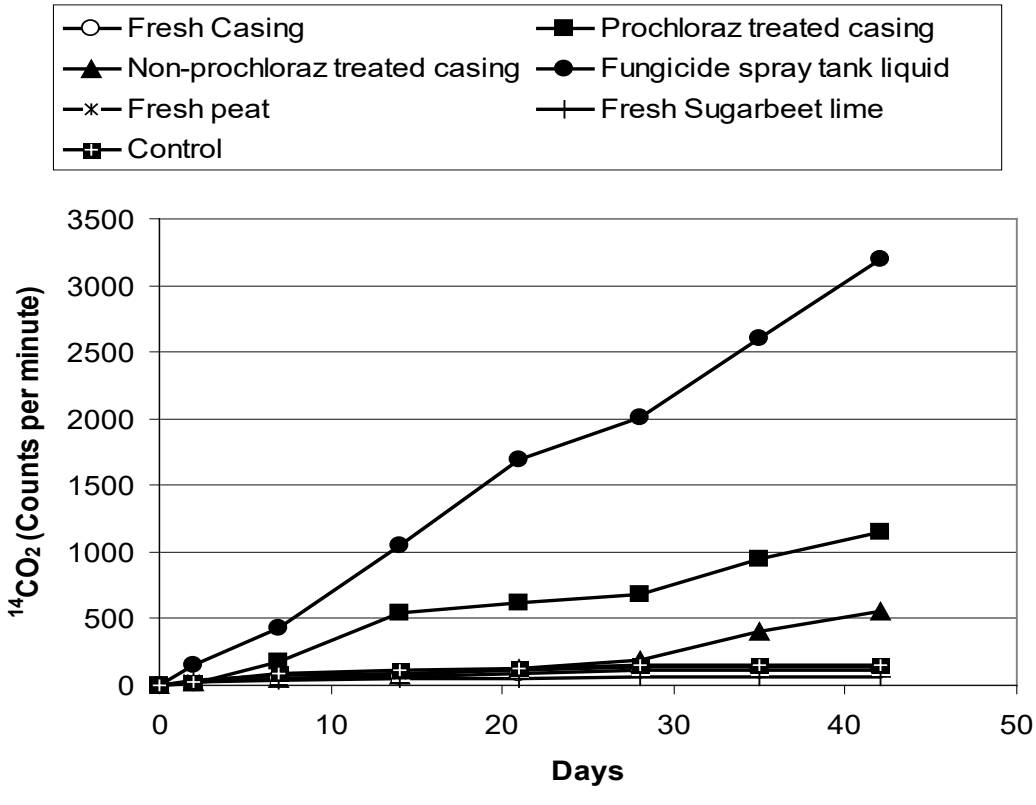
Experimental set up.

Respirometer units were prepared as described in section 3.2.1 containing 20 ml of prochloraz-amended MSM with 30 mg L^{-1} prochloraz and ^{14}C-prochloraz at 100 Bq ml^{-1}. Three replicates were prepared for each sample with 1 g of substrate (or 1ml of liquid) as microbial inoculum. The respirometer units were placed in an orbital shaking-incubator at 25°C and 50 rpm. $^{14}CO_2$ evolution was recorded as counts per minute.

3.4.2 Results and Discussion

After 28 days incubation only two samples demonstrated an ability to degrade prochloraz; they were the liquid from the fungicide spray tank and the sample of casing taken from a prochloraz treated crop (Figure 10). This data strongly indicates that there are prochloraz degrading microbes living in the fungicide spray tank and that these microbes are applied to casing along with the fungicide. Neither fresh casing, nor any of the mushroom casing

Figure 10. Prochloraz degradation by various substrates



ingredients contained microbes capable of degrading the prochloraz provided in the respirometer units. Mushroom casing from a crop treated with prochloraz, using the same spray tank, also contained the microbes necessary to degrade prochloraz but mushroom casing from a crop that was not treated with prochloraz did not have the microbes needed to degrade prochloraz, at least for the first 28 days of incubation. After this time the microbes in this casing sample also started to degrade prochloraz. There may have been a very small population of prochloraz-degrading microbes in this casing sample, which took 28 days to build up to a stage where they were numerous enough to have a significant impact. This non-prochloraz treated casing sample was taken from mushroom trays that were placed in the same room as the prochloraz-treated trays so there is a possibility that the microbes in the prochloraz-treated casing were distributed around the growing room at low levels during the course of the crop prior to the taking of the samples. Microbes are easily transmitted within humid mushroom houses where air circulation ensures uniform mixing of the air within the room and daily picking of mushrooms, and the watering operations of the crop can also transmit microbes from one tray to another.

Conclusions

The main conclusions from the microbial degradation studies are:

1. Prochloraz degradation in mushroom casing requires the presence of a consortium of microbes.
2. Prochloraz degrading microbes are localised in fungicide spray tanks used to apply the chemical.
3. Prochloraz degrading microbes are not present at any significant level in fresh casing or casing ingredients.

Survey of fungicide spray tanks around Britain.

Introduction

The microbial degradation studies reported in the previous chapter strongly suggested that prochloraz degrading organisms were localised in the fungicide spray tank used to apply the chemical to mushroom crops and that they were not naturally present in mushroom casing or casing ingredients. However, before embarking on work to determine how to eradicate the microbes from the tank, it was considered important to establish first of all if the phenomenon of prochloraz-degrading microbes in fungicide spray tanks was more widespread within the industry or localised to the experimental mushroom unit at Warwick HRI. To this end the milestones of the original project were changed and a survey of fungicide spray tanks within the industry was undertaken instead.

Materials and Methods

Ten mushroom farms were selected around the country, which encompassed a wide variety of growing systems, fungicide application regimes and fungicide spray tanks. Two organic farms were included who no longer used their tanks to apply fungicides but whose tanks would at some time in the past have been used for that purpose. Visits were made to all sites over a two-week period in May/June 2005. Growers were questioned about their uses of the spray tanks, how frequently they applied prochloraz, if the tank was used to water the crop, apply nematodes or other chemicals such as sodium hypochlorite. A 250-500 ml sample of tank liquid was obtained from each tank and a small scraping of the tank sides was taken as microbes would lodge on the tank surfaces and in the pump housing in between uses. Details of the fungicide spray tank uses at the different sites are given below in Table 3.

Tank samples were stored in the fridge for up to six weeks before analysis. Respirometer units were set up as described in section 3.2.1 using 20 ml of prochloraz-amended MSM containing 30 mg L^{-1} prochloraz and 100 Bq ml^{-1} ^{14}C-ring-labelled prochloraz. A 1 ml aliquot of the combined tank liquid and tank scraping samples was added to the respirometer. Three replicates were prepared for each of the farms in the survey. The evolution of $^{14}CO_2$ was measured over a period of 28 days.

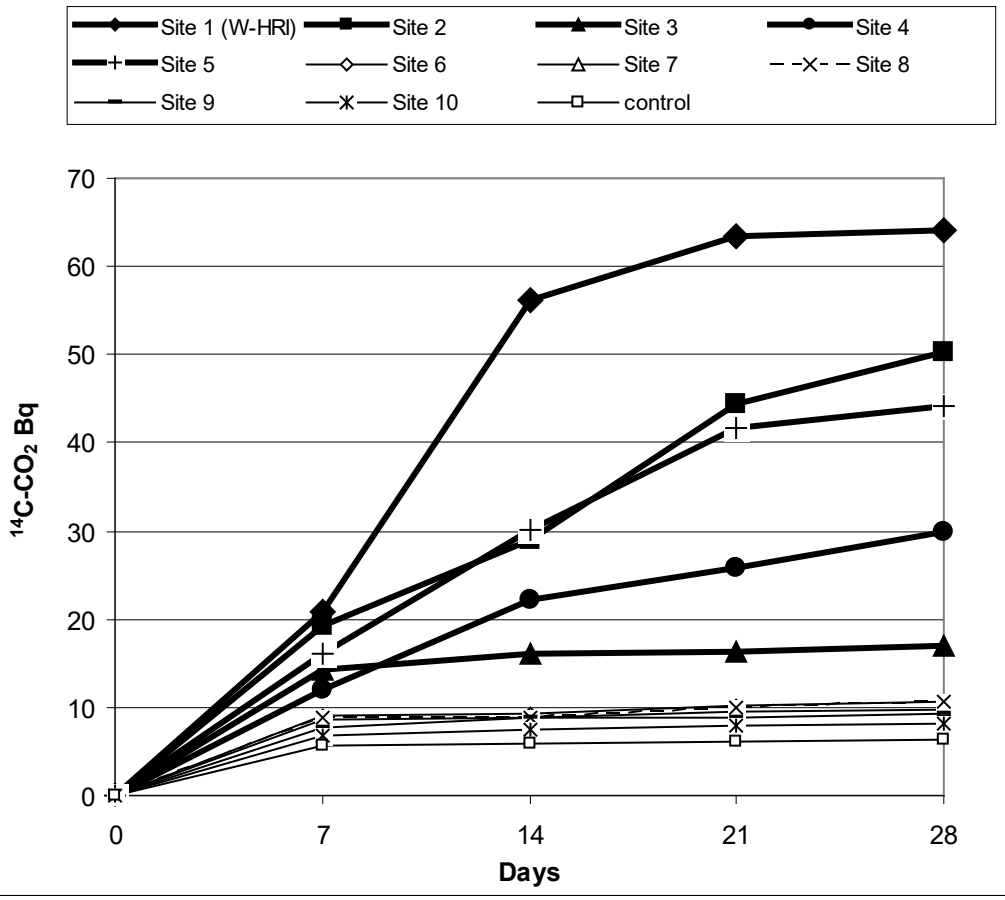
Table 3. Details of fungicide tank uses by 10 different mushroom farms in Britain

Farm No	**Frequency of Prochloraz application**	**Last application**	**Tank used to water crop**	**Tank used to apply** Na^+ClO^-	**Tank used to apply nematodes**
1 (W-HRI)	When needed	> 1 month ago	no	no	Yes
2	When needed	> 1 month ago	yes	yes	Yes
3	Organic	Organic	yes	---	Yes
4	Every crop	< 1 week	yes	yes	Yes
5	When needed	< 1 week	no	no	No
6	Every crop	< 1 week	no	no	No
7	Every crop	< 1 week	yes	no	No
8	Every crop	< 1 week	yes	yes	Yes
9	Organic	Organic	yes	---	No
10	Every crop	< 1 week	no	yes	No

Results and Discussion

Microbes capable of degrading prochloraz were detected in the liquid samples from four of the spray tanks surveyed at Site 1 (Warwick HRI), Site 2, Site 4 and Site 5 (Figure 11). The microbes present in the tank from one organic farm, Site 3, showed some ability to degrade prochloraz initially but they were unable to proceed with degradation after 7 days. There appeared to be no correlation between the presence of prochloraz degrading microbes and tank-use activities. For example Sites 2 and 4, which contained prochloraz-degrading microbes, used their tanks to apply water and sodium hypochlorite to crops, two activities that one might think would dilute and minimise any microbial activity in the tank, while Site 5 only used their tank to apply fungicides. Similarly, other sites that also applied water and or sodium hypochlorite to crops with their tanks showed no presence of prochloraz-degrading microbes (Sites 7, 8, 9, 10).

Figure 11. Degradation of prochloraz by fungicide spray-tank microbes



One explanation for the heterogeneity may be the fact that the tanks were sampled at different stages in the crop cycle depending on the time of the visit. Thus, some growers had just applied a chemical or water, or on some sites the tanks were empty. Another factor may have been whether or not growers used mains water or had their own water source, but this information had not been recorded. However the key finding is that four sites around the country out of 10 sampled showed the presence of prochloraz degrading microbes residing in the fungicide spray tanks. This result in itself means that there may be an opportunity to eradicate such microbial populations by reviewing the washing and disinfection regimes for fungicide spray tanks. Future work is planned in this area.

Conclusions

1. Prochloraz degrading microbes are present in many fungicide spray tanks around the country.
2. There is no obvious explanation as to why some tanks support prochloraz degrading microbes and some tanks do not.
3. Further work is needed to identify any factors that contribute to the presence of prochloraz degrading microbes in spray tanks and whether a review of spray tank management could eliminate such microbes and improve the efficiency of fungicides

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