BIOASSAYS TO DETECT DISSIPATION AND EFFICACY OF BENOMYL ON TURF

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ABSTRACT

BIOASSAYS TO DETECT DISSIPATION AND EFFICACY
OF BENOMYL ON TURF

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Paper disc, soil-agar pellet, turfgrass-agar pellet, thatch-agar pellet, and sample agar mixture bioassays were developed and evaluated for detection of the fungicide benomyl and its major fungitoxic product methyl 2-benzimidazole carbamate (MBC) in leachate, soil, turfgrass clippings, and thatch. The bioassays could detect benomyl and MBC residues with a limit of detection of 0.2 µg/g and a limit of quantitation of 0.5 µg/g. *In vitro* soil and thatch degradation studies showed a half-life for MBC of approximately 18 days in thatch and 4 to 5 weeks in soil, depending on soil type. An adsorption equilibrium with benomyl in solution was reached within 1 h for thatch and 2 to 4 h for soils. MBC adsorbed by thatch was as much as twice that adsorbed by a Fox sandy loam which may represent a typical soil found on golf courses in Southern Ontario. When MBC was applied at 10 µg/g, up to 90% of the chemical in thatch and 68% in soil were not extractable with water. Methanol could extract up to three times the fungicide from soil or thatch as could water. Laboratory and field experiments showed that the wetting agent Aqua-Gro (AG) (polyoxyethylene ester and ether of cyclic acid and alkylated phenols) significantly reduced adsorption of benomyl by creeping bentgrass thatch. With AG, significantly less fungicide was initially adsorbed and significantly more fungicide was later desorbed from the thatch layer by 20 mm of water irrigation. Aqua-Gro increased movement, uptake, and biological availability of the fungicide and resulted in a higher residue level of fungicide in the grass clippings. Tersan 1991 (50% benomyl)

applied at 2 kg/ha with AG gave control of dollar spot disease as good as the full rate (3 kg/ha) without AG. Field studies also showed that core cultivation 1 or 7 days before fungicide application gave better and longer-lasting uptake of the fungicide by turfgrass and resulted in better control of dollar spot disease. Core cultivation one day before fungicide treatment gave the best results both in long-lasting uptake of benomyl and control of dollar spot disease.

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PREFACE

Chapters 3, 4, and 5 of this thesis were written with the intent to publish them as separate papers. As a result, there is some repetition of information in certain sections (e.g. Introduction, Materials and Methods). An attempt has been made to keep repetitiveness to a minimum by referring to other sections of the thesis, but where this would compromise the flow or completeness of a chapter, previously mentioned information has been repeated.

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CHAPTER 1. LITERATURE REVIEW

1.1 Dissipation of pesticides in soil

Dissipation of pesticides involves adsorption, transportation and degradation processes. Adsorption occurs when pesticides are adsorbed or attracted to clay or organic matter particles. It is one of the major processes affecting the interactions between pesticides and the solid phase in the soil environment (Khan, 1980). Processes of transportation result in pesticide movement from one location or medium to another. These include volatilization, leaching, runoff or erosion, and uptake by plants or soil organisms (Siegel, 1973; McEwen and Stephenson, 1979; Zbozinek, 1984; Niemczyk and Krause, 1989). Degradation processes result in structural transformations of the pesticide molecule. Photodecomposition, microbial and chemical decomposition are active degradative processes (Fleeker and Lacy, 1977; Yarden *et al*., 1990).

1.1.1 Adsorption of pesticides by soil

1.1.1.1 Soil type

Two major factors influencing pesticide adsorption in soils are the amount of clay and organic matter. Since both clay and organic matter are negatively charged and have large surface areas, they produce a sort of high cation exchange capacity (CEC). Clay and organic matter content are positively correlated with pesticide adsorption or reduction in pesticide bioavailability (Bailey and White, 1964). Many other studies also show the increase in adsorption with the increase in clay and organic matter content (e.g. Green and Obien, 1969; Brady, 1984). Higher rates of pesticide applications are often required for

pest control if soils are high in clay or organic matter. It was also reported that, among four soil properties (clay content, organic matter content, cation exchange capacity and pH), organic matter content was the best single predictor for pesticide adsorption (Aharonson and Kafkafi, 1975). A linear relationship between organic matter content and adsorption of pesticides has been found (Warren, 1970).

1.1.1.2 Soil pH

Adsorption of pesticides in soil varies with soil pH. Within a group of similar soils differing mainly in their pH, adsorption of pesticide is usually higher with a decrease in pH (McEwen and Stephenson, 1979). As the pH decreases within a certain range, the soil solution becomes more acidic, and thus pesticides become converted from negatively charged anions to uncharged molecules or to positively charged cations and thus increase their adsorption to negatively charged clay and organic particles. Adsorption of pesticides will decrease in soils with a very low pH (Bailey and White, 1964) because of numerous H^+ in soil solution that will compete for the negatively charged sites on soil colloids (Smith, 1983).

1.1.1.3 Soil moisture

Soil moisture content affects pesticide adsorption and desorption processes in soil, and both processes influence the biological activity of pesticides (McEwen and Stephenson, 1979). Generally, pesticide adsorption decreases with an increase in soil moisture content. This relationship is more evident in light or sandy soils than heavy textured or more adsorptive soils (Green and Obien, 1969; Brady, 1984).

1.1.1.4 Soil temperature

Pesticides are adsorbed by colloids through physical force or chemical bonds such as hydrogen bonds or ionic bonds. The adsorption of pesticides in soil is an exothermic process and input of energy to soil in the form of heat could break bonds and cause desorption of some pesticide molecules (Bailey and White 1964). Temperature also affects the solubility of a pesticide in a liquid medium such as soil solution. In general, adsorption decreases as solubility increases due to a rise in soil temperature (Yarden *et al*. 1989).

1.1.1.5 Chemical structure

Pesticide structure can affect its affinity for clay or organic matter and its solubility (McEwen and Stephenson, 1979). Generally most organic pesticides have low solubility in water. The acidity or alkalinity, water solubility, polarity and size of the molecule all affect adsorption-desorption by soil colloids (Bailey and White, 1964). There is a direct relationship between adsorption and solubility of pesticides within similar chemical groups. Some functional groups of a chemical affect its adsorption to soil colloids. Generally, adsorption increases with the increase of functional groups such as $CONH_2$, -OH, -NHCOR, $-NH_2$, -COOH, -OCOR, and -NHR. When the pH in soil solution is below the pK_a (ionization constant) of the pesticide (e.g. benomyl), the amino groups ($-NH_2$) may protonate and the pesticide can be adsorbed as a cation (Khan, 1980).

1.1.2 Transportation of pesticide in soil

1.1.2.1 Volatilization

Pesticides may leave the soil as a vapour. Volatility of pesticides varies greatly.

Factors influencing the volatility of a pesticide include the chemical structure which determines its vapour pressure, solubility and tendency to be adsorbed. Temperature, moisture and properties of a soil also contribute to the volatilization of a pesticide. Generally, cool, dry conditions in a soil with high clay or organic matter content result in little loss of a pesticide through volatilization (Spencer, 1987).

1.1.2.2 Leaching

There is a close relationship between leaching and adsorption of a pesticide in soil. Increased adsorption could decrease leaching (Aharonson and Kafkafi, 1975). Leaching is related to the properties of a soil. A sandy soil, for example, has larger pore sizes than a heavy textured soil or a soil with high organic matter or clay content. Leaching is also related to the solubility of a pesticide. Within certain limits, the higher the solubility of a pesticide, the quicker the pesticide may leach from the soil (McEwen and Stephenson, 1979). Leaching is also affected by movement of water through a soil. Rapid water movement may increase the potential for leaching of a pesticide, since new equilibria between the pesticide in the soil solution and that adsorbed on the soil colloid are reached during water movement (Morrod, 1982).

Somasundaram *et al*. (1991) found that soil pH significantly affects mobility of many pesticides, with increased mobility in soil with higher pH. They also concluded that both water solubility and octanol/water partition coefficient (K_{OW}) are significantly correlated with mobility of the chemicals studied.

1.1.2.3 Movement with runoff water and eroded soil

Movement of surface water on soils may carry away pesticides which desorbed

from soil colloids. In light soils, such as sandy soils, pesticide adsorption decreases as the moisture content increases (Bailey and White, 1964), and movement of a pesticide with runoff water increases as the adsorption decreases (McEwen and Stephenson, 1979). Wind and movement of surface water on soil can result in soil erosion. Pesticides adsorbed on soil particles can be carried away with soil particles by wind and runoff water over a great distance (Brady, 1984).

1.1.2.4 Uptake by plants and soil organisms

Uptake of a pesticide by plants and soil organisms accounts for a very small portion (<1%) of the dissipation of the pesticide in soil (Pimentel *et al.*, 1991). Pesticides which are less adsorbed are usually more available to plants and soil organisms and thus may be more effective. The pesticide absorbed by plants may be degraded within the plants or removed from the field with the plants. Soil organisms play a significant role in degradation of pesticides in soils (Yarden *et al.*, 1990). However the incorporation of pesticides into soil organisms is generally not significant.

1.1.3 Degradation of pesticides in soil

1.1.3.1 Microbial degradation

Microbial degradation of most pesticides has been reported to be the single most significant mode of dissipation (Brady, 1984). If pesticide molecules are biodegradable, then these molecules in soil solution at equilibrium may be readily metabolized by soil microorganisms as an energy source (Yarden *et al.*, 1990). Soil microorganisms can adapt to a biodegradable pesticide as an energy source and readily increase in numbers/activity until they have completely metabolized it. The numbers of microorganism may decrease

after available pesticide molecules have been degraded.

A logistic curve has been used to characterize the dissipation of such pesticides (McEwen and Stephenson, 1979). This curve has three phases: 1) a lag phase during which microbial or enzymatic adaptation occurs which result in increased microbial populations, 2) an log phase when the pesticide is rapidly degraded, and 3) a plateau phase resulting from low substrate concentration or accumulation of toxic metabolites. These degradation curves are usually observed only in isolated culture where the influence of other dissipation and transport precesses are limited. Under field conditions where the influence of other factors beside microbial degradation come to bear, these types of curves are usually not found (Yarden *et al*., 1989; 1990).

Any factor favouring growth of microorganisms or increasing the availability of a pesticide in soil solution may increase metabolism of the pesticide (Moorman, 1990). Organic matter may decrease the availability of a pesticide through adsorption, but it could favour the growth of soil microorganisms. Some conditions, such as optimal temperatures, adequate soil moisture and aeration, proper pH, and adequate fertility, favour growth of microorganisms and increase desorption and availability of pesticides (McEwen and Stephenson, 1979).

Among organisms responsible for the degradation of pesticides in soil, bacteria and fungi play significant roles (Aharonson *et al*., 1990). Bacteria are more important with water soluble pesticides and pesticides that are not strongly adsorbed. Fungi are more important with less water-soluble or strongly-adsorbed pesticides and are more active in the lower pH range (Yarden *et al*., 1990).

Repeated application of the same or closely related biodegradable pesticides to the same soil may result in enhanced degradation because of the selection pressure and fitness of the microorganisms (Racke, 1990). This phenomenon of enhanced degradation has in some cases resulted in economically significant pest control failures. With enhanced degradation, the lag phase is greatly shortened or there is no lag phase in the degradation curves (Yarden *et al*., 1985b).

1.1.3.2 Chemical degradation

Pesticides can be degraded chemically in soil into nontoxic compounds or in some case be activated into toxic compounds. The silicate clay fraction in soil can have a significant effect on chemical degradation of pesticides because adsorbed pesticide molecules are more easily catalyzed by soil enzymes which are located on the surface of the clay particles (Brady, 1984; Farmer and Aochi, 1987). Adsorption usually decreases the rate of microbial degradation of a pesticide because of the reduced bioavailability of the pesticide. Soil pH is another important factor influencing pesticide degradation chemically since microbial enzymes are pH dependent (McEwen and Stephenson, 1979).

1.1.3.3 Photodecomposition

The UV light absorbed by some pesticides has enough energy to break the bonds in molecules of these pesticides. Photodecomposition is important only for some pesticides in their dissipation in soil. Trifluralin, for example, is susceptible to photodecomposition when applied to the soil surface. However, irrigation or rainfall immediately after application could move the pesticide into soil layer and reduce photodecomposition (Ross and Lembi, 1985).

1.2 Benzimidazole fungicides

1.2.1 History and application

Benzimidazole fungicides include benomyl, carbendazim, thiabendazole, fuberidazole and thiophanate-methyl. These compounds lack antibacterial activity but are active towards a broad spectrum of fungi. Benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate] (Fig. 1.1) is the most effective and widely used fungicide in this group. It has a great selectivity for Ascomycetes and Deuteromycetes, and many species are highly sensitive. Most fungi of other classes, such as Basidiomycetes, or fungi in particular such as Porosporae, Annellosporae, and Oomycetes are insensitive (Bollen and Fuchs, 1970; Edgington *et al*., 1971).

Benomyl was one of the earliest systemic fungicides discovered and was first registered in 1968 (Delp and Klopping, 1968). It has been used on wide range of crops including fruit trees, field crops, vegetables, turfgrass and ornamentals. Because of its effectiveness and low mammalian toxicity (ED_{50}=10,000 mg/kg in rats), it is also used on pastures to control some fungal diseases such as *Pithomyces chartarum*, the fungus responsible for facial eczema in sheep and cattle in New Zealand (Clare, 1969). According to Douch (1973), it is unlikely that benomyl ingested from the pasture would pose toxicity or residue problems in sheep.

Commercially available forms of benomyl include Tersan 1991 and Benlate which are wettable powders containing 50% active ingredient of benomyl by weight. Tersan 1991 has been recommended for use in turfgrass to control several common diseases (Anonymous, 1990; Vargas, 1981).

Figure 1.1. Chemical or biological degradation pathway of benomyl.

1.2.2 Uptake and translocation, resistance and mode of action

1.2.2.1 Uptake and translocation in plants

Properties of a good systemic fungicide are translocation and selective toxicity towards pathogens. Uptake and translocation of benomyl in plants has been studied by many researchers (Peterson and Edgington, 1970; Erwin *et al*., 1971; Baude *et al*., 1973; Solel *et al*., 1973; Ellis and Sinclair, 1975). These studies suggested that long distance movement of fungicide in plants occurs mainly in the apoplast. The systemic movement and fungitoxic properties of benomyl in plants are due to methyl 2-benzimidazole carbamate (MBC, also known as carbendazim) which is the major degradation compound (Fig. 1.1). MBC may then be transformed into other minor metabolites (Thapliyal and Sinclair, 1971). The fungicide can be taken up through roots and leaves and is transported upward through the xylem of the plant (Erwin *et al*., 1971). Peterson and Edgington (1970; 1971) reported that benomyl, after uptake by plants, is mainly accumulated as MBC at the tips of leaves with parallel veins. Benomyl has been recommended for turfgrass as a soil drench to control diseases such dollar spot and brown patch. The fungicide must move to the root zone for root uptake and foliar translocation to provide systemic protection of the plant (Anonymous, 1990).

Solel and Edgington (1973) reported that, after foliar application of benomyl, a small proportion of MBC is translocated to roots and shoots through phloem. The concentration of MBC transported to roots and shoots is high enough to be bioassayed without any concentrating procedure. However, they indicated that application rates of benomyl are approximately 5 to 10 fold higher than normal in order to detect this type of

translocation.

Various factors affect the uptake and transportation of benomyl. Greater uptake of MBC (from benomyl application) was noticed in sand as compared to soil (Hock *et al*. 1970). The higher the clay or organic matter content of the soil, the less the uptake. Hock *et al*. (1970) also found maximum uptake and translocation at pH 3.2 with reduced uptake as the pH increased. At a pH of 9.2, uptake was completely inhibited. Water solubility in acidic pH is probably an important factor. The translocation of both benomyl and MBC from treated roots to leaves in creeping bentgrass by using the bioautograph technique has been demonstrated by Meyer *et al*. (1971). Nicholson *et al*. (1972) also found both benomyl and MBC were translocated upward to leaves of strawberry plants and also laterally to tissues of a daughter plant grown in a separate non-treated pot.

1.2.2.2 Mechanism of resistance and mode of action

Benzimidazole fungicides were a ground-breaking class of fungicides with unique and very advantageous properties such as selectivity, low application rates, activity against a range of pathogens, and with systemic activity (Delp and Klopping, 1968; Delp, 1987). However, after a few years of intensive use, field resistance to benzimidazoles was reported in many fungal pathogens. The first report was in powdery mildew of cucurbits in greenhouses after one year of application (Schroeder and Provvidenti, 1969). After two years of use, benomyl resistance in *Botrytis cinerea* was found on cyclamen in the Netherlands (Bollen and Scholten, 1971). By 1984, resistance to benzimidazoles had been reported for many pathogens on many different crops (Delp, 1987).

Studies showed that resistance to carbendazim was not due to a reduced uptake

or an increased metabolic conversion (Davidse, 1976). Benomyl and MBC have a fungistatic effect on germ tube elongation (Erwin, 1973). Studies have shown that benzimidazoles inhibit fungal mitosis (Clemons and Sisler, 1971; Hammerschlag and Sisler, 1973). Those studies, using crude extracts of *Aspergillus nidulans* and [^{14}C]-MBC, showed that binding occurred to a protein with characteristics typical for tubulin (Davidse and Flach, 1977). Further studies also supported the idea that the MBC-binding protein was fungal tubulin (Davidse, 1986; 1987). Binding affinity correlated with the sensitivity of the strain involved. Benzimidazole treatment of germinating conidia inhibited spindle formation in nuclei although the spindle pole bodies replicated, indicating that both processes are independently regulated (Kunkel, 1980). Cross resistance to most benzimidazole fungicides has been reported.

Studies of the mechanism of resistance to benzimidazole fungicides closely paralleled those on the mode of action. In *A. nidulans* three loci, *benA, benB,* and *benC,* are involved in resistance (van Tuyl, 1977). Mutations in *benA* give the highest level of resistance. Affinity changes of tubulin are associated with changes in the primary structure of β-tubulin, one of the monomers of the dimeric tubulin molecule, and it was found that the *benA* gene was the structural gene for β-tubulin. This was the first tubulin gene identified in *A. nidulans* (Sheir-Neiss *et al*., 1978). It codes for two polypeptides, β_1- and β_2-tubulin.

Binding studies with wild-type and resistant mutants of other benzimidazole-sensitive species such as *Botrytis cinerea, Fusarium oxysporum f. sp. lycopersici,* and species with natural insensitivity to benzimidazoles, such as *Alternaria brassicae* and

Pythium irregulare (Davidse and Flach, 1977; Ishii and Davidse, 1986) showed that only extracts of the sensitive types have binding activity. The affinity of the target site to benzimidazoles apparently determines antifungal activity of the fungicides.

Jung *et al*. (1987) reported that cloning and sequencing of five mutant alleles of *benA* and β-tubulin gene of *A. nidulans*, confirmed that altered sensitivity to benzimidazoles or altered stability of microtubules is due to structural changes in this gene. Mutations in the structural gene for β-tubulin are also thought to be involved in benzimidazole resistance in *Saccharomyces cerevisiae* (Thomas *et al*., 1985), *S. pombe* (Hiraoka *et al*., 1984), *Neurospora crassa* (Orbach *et al*., 1986), and *Physarum polycephalum* (Burland *et al*., 1984). However, the phenotypic effects of altered β-tubulin either on microtubule stability or on binding of benzimidazoles to tubulin are unknown. Benzimidazole resistance in one *N. crassa* strain is caused by a T-to-A base pair change at the second base of codon 167, which results in an amino acid change from phenylalanine to tyrosine (Orbach *et al*., 1986). In a strain of *Saccharomyces cerevisiae*, a G-to-A transversion at the second base of codon 241 determines resistance by changing arginine to histidine (Thomas *et al*., 1985).

A more recent study show that all benomyl-resistant field strains of *Venturia inaequalis*, *V. pirina*, *Monilinia fructicola*, *Sclerotinia homoeocarpa* and six species of *Penicillium* were found to contain a single pair mutation in their β-tubulin gene that resulted in an amino acid substitution in β-tubulin (Koenraadt *et al*., 1992).

These data suggest the β-tubulin gene mutates at specific sites. The exchange of certain amino acids within tubulin was the cause for the decreased binding affinity of a

inhibitor (MBC) to the target site. Amino acids at codon 167 for *N. crassa* and at codon 241 for *S. cerevisiae* undoubtedly are involved in binding.

Morris (1986) found that *A. nidulans* has a second β-tubulin gene (*tubC*) that codes for β_3-tubulin. Three forms of α-tubulin genes have also been found. The functions of these tubulins are not very clear although they may be associated with sensitivity to N-phenylcarbamates. Negative cross resistance between benzimidazoles and N-phenylcarbamates has been observed (Elad *et al*., 1988).

1.2.3 Dissipation of benomyl in soil and turf

1.2.3.1 Adsorption of benomyl

Adsorption of benomyl to soil is one of the major processes influencing its accessibility to target organisms and their potential to reach non-target organisms. It has been shown that uptake of benzimidazole fungicides by plants from the soil is not very efficient especially in clay soils (Fuchs *et al*., 1970). Control of vascular wilt diseases requires extremely high dosages and is not practical for deep-rooted crops (Erwin, 1973). Baude *et al*. (1974) demonstrated that residues of benzimidazole fungicides are tightly adsorbed by soil colloids and thatch. The rate of uptake of benomyl by plants is increased when they are grown on soils with low clay and organic matter content, and with higher pH.

Turfgrass, unlike other cultivated crops, usually develops a layer of thatch which is a tightly intermingled layer of dead and living roots, rhizomes, stolons and organic debris between the zone of green vegetation and the soil surface (Beard, 1973). The turfgrass thatch-soil system can adsorb significant amounts of applied pesticide and

reduce the amount reaching the roots in thatch or soil, and therefore reduced uptake of the fungicide by roots (Branham and Wehner, 1985; Krause and Niemczyk, 1989; Harrison *et al*., 1989; Cooper, 1990).

1.2.3.2 Movement of benomyl

Studies on mobility showed that benomyl and MBC are highly immobile in soil and most of the fungicide residues in soil are in the upper soil increment (Baude *et al*., 1974; Austin and Briggs, 1976). Niemczyk and Krause (1989) reported that over 90 percent of the insecticide residues studied remain in the thatch layer 57 days after treatment.

Run-off and mobility studies by Rhodes and Long (1974) showed that benomyl and MBC are immobile in soil and thatch, and do not leach or move significantly from the site of application. Over 95 percent of the applied ^{14}C-benomyl was recovered from the top 3.8 cm of soil.

1.2.3.3 Degradation of benomyl

In aqueous and nonaqueous media, benomyl is rapidly converted to methyl 2-benzimidazole carbamate (MBC) (Clemons and Sisler, 1969; Siegel and Zabbia, 1972). This side-chain hydrolysis occurs very rapidly and does not require microbial activity (Peterson and Edgington, 1969). In plants, MBC is a fungitoxic compound recovered after foliar spray or soil treatment with benomyl (Peterson and Edgington, 1970; Sims *et al*., 1969). Baude *et al*. (1973) reported that benomyl is converted to MBC on plant surfaces under summer outdoor temperature and rainfall conditions. Other metabolic studies in plants with radiolabelled compounds have shown that MBC is further decomposed via 2-

aminobenzimidazole (2-AB) into benzimidazole (Fig. 1.1) (Siegel, 1973; Ben-Aziz and Aharonson, 1974; Rouchaud *et al*., 1977). In soil, microbial degradation is an important factor affecting the persistence of benomyl (Schoen and Winterlin, 1987; Yarden *et al*., 1990). The degradation process involves the formation of 2-AB as an intermediate and the liberation of $^{14}CO_2$ from [2-^{14}C] MBC, which indicates that ring cleavage has occurred (Fig. 1.1) (Fuchs and Bollen, 1975; Helweg, 1977).

Microbial degradation of benomyl plays a very important role in dissipation of benomyl residues. Helweg (1977) concluded that MBC is a poor carbon source and suggested biodegradation of MBC is a co-metabolic process. Fungi contribute to MBC dissipation in soil, but bacteria have a greater role in enhanced biodegradation of MBC in soil (Yarden *et al*., 1987; 1990).

Photolysis is not a major pathway of benomyl degradation. When MBC in aqueous solution was exposed to sunlight and UV-light for 40 h, less than 10% was lost. When MBC was applied to leaves of corn plants and exposed to sunlight for 18 h, no photolytic products were detected in extracts of the plants (Fleeker and Lacy, 1977).

Benomyl is usually applied on turfgrass as a foliar spray followed by immediate watering of the fungicide into the root zone (Turgeon, 1985; Anonymous, 1990). The half-life of total benzimidazole residues is about 3-6 months in turf and 6-12 months in bare soil (Baude *et al*., 1974). Other reports indicated that foliar application of benomyl, at recommended rates, provides an adequate foliar residual deposit for several weeks, if rain does not wash off the fungicide (Smalley *et al*., 1973; Upham and Delp, 1973). Even with artificial rainfall (5-8 L/m^2 per week), the residue of benomyl on lettuce leaves may

be present several weeks after application (Dejonckheere *et al*., 1976). Turfgrass is usually watered and mowed frequently especially on golf course putting greens (Beard, 1982); these cultural practices may affect the dissipation of the pesticide.

Degradation studies of benomyl in thatch have not been reported, although an accelerated rate of pesticide degradation is found in thatch layer (Branham and Wehner, 1985; Potter *et al*., 1989). An increased rate of degradation of the fungicide Diazinon in thatch was observed by Branham and Wehner (1985).

Benomyl is chemically transformed into the stable MBC under both natural and artificial conditions. The transformation in water at pH 3 and 20°C was reported to be complete within 24 h (Austin *et al*. 1976). In organic solvents the rate of transformation varies. Benomyl is decomposed to MBC very quickly at low concentrations in methanol and ethyl acetate. At 10 μg/mL, for example, the half-life is less than 1 h (Chiba and Doornbos, 1974; Colmon and Sayag, 1976).

Chiba and Cherniak (1978) reported that the decomposition of benomyl to MBC in different organic solvents is reversible. Their results showed that, in organic solvents such as ethanol, methanol and ethyl acetate, benomyl does not decompose completely to MBC, and due to the reversible nature of the degradation reaction, some percentage of benomyl always remains intact. More recent studies on decomposition of benomyl to MBC showed that in mixed solutions of water with buffer, acetonitrile, or methanol, the degradation of benomyl slowed down with the increase in water concentration (Singh *et al*., 1990). Quantitative conversion of benomyl to MBC was not observed in acetonitrile and 12% of benomyl remained intact at equilibrium due to the reversible nature of the

reaction. However, their results also showed that, the degradation of benomyl to MBC in methanol was found to be quantitative, thus making this solvent more appropriate for quantitative conversion of benomyl to MBC.

In alkaline aqueous media, benomyl is partly converted to STB (1,2,3,4-tetrahydro-3-butyl-2,4-dioxo-s-triazino [a] benzimidazole) and BBU (1-(benzimidazol-2-yl)-3-butylurea) (White *et al*. 1973). In most chemical analytical procedures, conversion of benomyl to either stable MBC or STB and BBU can be achieved artificially.

1.2.4 Dollar spot disease and its control with fungicides

Dollar spot is a common and persistent disease of fine turfgrasses, especially creeping bentgrass (*Agrostis palustris* Huds.) and annual bluegrass (*Poa annua* L.). It is caused by *Sclerotinia homeocarpa* F.T. Bennett, which survives winter as mycelia in infected plants and stromata on foliage and in thatch. Under warm (>20°C) and humid weather, mycelia from infected tissue or from stromata colonize grass through cut leaf tips and natural openings. Dissemination of this fungus is restricted to movements of infected leaf debris by equipment, people, animals, water, or wind. Prolonged high humidity in the turfgrass canopy is required for fungal growth (Vargas, 1981; Smiley *et al*., 1992).

Incidence of dollar spot disease can be reduced by maintaining high nitrogen fertility during the period of dollar spot activity (Goodman and Burpee, 1991). Many fungicides are available for dollar spot disease control. Tersan 1991 (50% a.i. benomyl at 3 kg/ha) or a mixture of Tersan 1991 with Daconil 2787 (75% a.i. chlorothalonil) has been recommended for the control of this disease (Anonymous, 1990). Applications should be started at the first sign of disease and repeated in approximately 2-week

intervals (Anonymous, 1990; Smiley *et al*., 1992).

1.3 Methodology for benomyl residue analysis

Several researchers have reviewed the residue analysis of benzimidazole fungicides (Baker and Hoodless, 1973; Slade, 1975; Watkins, 1976; and Gorbach, 1980).

1.3.1 Chemical analysis of benomyl residues

1.3.1.1 Extraction of benomyl residues

Different solvents have been used for extraction of benomyl and its residues depending on the substrate, metabolites and the conversion products. Mixtures of organic solvents have also been recommended for extraction of benomyl residues (Sims *et al*. 1969). Because benomyl is transformed totally or partially into MBC, or STB and BBU during extraction, a mixture of acetone and 1 M ammonium chloride (1 + 1, v/v) is recommended by Austin and Briggs (1976) for the extraction of benomyl residues from soil. This method extracted 71.3% of the radioactive MBC applied 1 week prior to the analysis. Fernandes and Cole (1974) studied the efficiencies of some organic solvents and methods in the extraction of MBC from tobacco: among ethanol, methanol, acetone and chloroform, they found that methanol extracted the most.

1.3.1.2 Clean-up of benomyl residues

In chemical analysis, a clean-up is usually necessary before determination. Benomyl and MBC have weakly basic properties due to the =N-atom, which can be exploited for partition in the clean-up procedure (Austin and Briggs, 1976). The residue dissolved in ethyl acetate can be extracted easily with dilute hydrochloric acid and then re-extracted into ethyl acetate after adjusting the acid aqueous phase back above pH 8

(Aharonson and Ben-Azia, 1973; Austin and Briggs, 1976). Column or thin layer chromatography has been used in some cases as a clean-up procedure to eliminate interfering substances (White and Kilgore, 1972; Douch, 1973).

1.3.1.3 Determination of benomyl residues

Several methods have been reported for the detection and determination of benomyl residues. The thin-layer plate method was used by Baker and Hoodless (1973). MBC characteristically has strong absorption in the range of 260-300 nm of light, which allows for detection by spectrophotometry (White and Kilgore, 1972; Cox *et al*., 1974; Monico-Pifarre and Xirau-Vayreda, 1990). Cox *et al*. (1974) recommended measuring the absorption at the wavelengths 282 and 275 nm from a base line at 310 nm. The peak ratio at 282/275 should be constant at 1.20. Variation greater than -0.02 indicates interference from other UV-absorbing substances.

The use of fluorescence spectrophotometry for determination of benomyl residues has been reported by Pease and Holt (1971) and Aharonson and Ben-Aziz (1973). High performance liquid chromatography was also used in determination of benomyl residues (Austin *et al*., 1976; Maeda and Tsuji, 1976) as well as gas liquid chromatographic methods (Rouchaud *et al*., 1977).

1.3.2 Bioassays for the determination of benomyl residues

Thin layer plate bioassays were used by a number of authors (Ben-Arie, 1975; Ellis and Sinclair, 1975; Cox and Pinegar, 1976). In these bioassays, the extracts can be spotted onto the plates without clean-up. After thin-layer chromatography, the plates are coated with an appropriate nutrient solution and sprayed with a suspension of a test

fungus. The inhibition zones develop after an appropriate incubation period and are often well-correlated with the fungicide concentrations.

Soil plugs or soil pellet bioassays have been used by a number of authors (Munnecke, 1958; Hine *et al*., 1969; Yarden *et al*., 1985a). In these methods, the soil is made into plugs or agar pellets and placed in the centre of a Petri dish with medium. After appropriate incubation, the fungicide residues diffuse from the plugs or pellets into the medium which has been mixed or sprayed with a suspension of a susceptible test fungus. Inhibition of fungal growth has a good correlation with the fungicide concentration in soil samples. The lowest detectable concentrations of MBC was reported by Yarden *et al*. (1985a) was 0.25 µg/g.

Paper disc bioassays have been used by Thornberry (1950), Erwin *et al*. (1971), Edgington *et al*. (1973) and Black and Neely (1976). In these methods, paper discs are placed on the medium which has been mixed or sprayed with a test fungus. Solutions or supernatants of extracts from plants or soil are added to the paper discs. A good correlation can be found between inhibition of fungal growth and fungicide concentration in the samples after appropriate incubation. When using chloroform extraction of elm leaves, a concentration of benomyl of 0.1 µg/mL was detected by Black and Neely (1976).

Other bioassay methods for benomyl residue determination have also been reported. These include leaf disc bioassay (Edgington *et al*., 1973), other plant tissue bioassay (Hine *et al*., 1971; Hock and Schreiber, 1971) and wells containing fungicide solution on media (Cox *et al*., 1974).

1.4 Effects of surfactants and coring on efficacy of benomyl applied to turfgrass

1.4.1 Surfactants

Surfactants are surface active agents which act by modifying the surface tension between water and solids or other liquids. The molecules of surfactants possess hydrophilic and lipophilic parts that attract water and nonaqueous materials, respectively. Each part of the molecule orients itself to the phase that attracts it, thereby bonding surfaces that would otherwise tend to repel each other (Foy, 1989)

The primary functions of a surfactant added to a pesticide solution are to promote wetting, coverage and uptake of the pesticide by leaves. However the hydrophilic-lipophilic properties of these additives suggest that they may have more than this one function. Gillard (1987) proposed that a surfactant or adjuvant can be used to achieve better control of the target organism than is possible with the standard formulation by altering the physical characteristics, extending the period of activity, overcoming resistance developed by a target species to a pesticide, and enabling lower doses of pesticides to be used.

Rawlins and Booth (1968) showed that the surfactant Tween-20 increases the effectiveness of benomyl for the control of Verticillium wilt of cotton. Biehn and Dimond (1971) demonstrated effective field control of Verticillium wilt of potato by soil drench with Benlate plus the surfactant Tween-20.

Thatch and sand grains on golf greens are difficult to wet because the high surface tension repels water and causes localized dry spots (Miller and Wilkinson, 1977; Taylor and Blake, 1982). Wetting agents can increase infiltration rates into hydrophobic thatch and soil (Pelishek *et al.*, 1962). On sand golf greens, Aqua-Gro, a commonly used

surfactant, was found to reduce the severity of the dry spots by increasing water infiltration rate (Wilkinson and Miller, 1978). No information is available on the effect of wetting agents on efficacy of benomyl applied to turfgrass.

1.4.2 Coring

Coring is a cultivation practice involving the use of a hollow tine or spoon to remove soil cores which then leaves a hole or cavity in the sod. It has been reported to increase root growth, increase water infiltration, correct compaction, and reduce thatch (Beard, 1973).

Byren *et al*. (1965) and Canaway *et al*. (1986) found that hollow tine coring (2.5 cm diam and 15 cm depth) increased the infiltration of water into a compacted putting green. Surface compaction, hydrophobic thatch, and surface layering can severely restrict infiltration. If the coring hole traverses these restrictions, substantial increases in infiltration capacity are possible in direct response to coring. Carrow (1988) reported that hollow tine coring was the most effective of five cultivation methods for reducing penetrometer resistance. The increased infiltration capacity of cored turf is due primarily to the increased surface area resulting from coring. According to Turgeon (1985), the increase in surface area can be more than twofold when the combined areas of the walls of the holes are added to that of the turf surface.

Coring can result in an improvement of growing conditions in the immediate vicinity of the holes as long as moisture is not limiting (Turgeon, 1985). Barber and Carrow (1985) showed improved oxygen diffusion rates from hollow tine coring. After coring root growth in the 20 to 60 mm zone increased by 20 to 35% and water extraction

from this zone also increased (Wiecko *et al*., 1993).

No information is available on the effect of coring on the efficacy of benomyl applied to turfgrass. However, the improved conditions through coring may increase the movement in thatch/soil and uptake by turfgrass of benomyl. Furthermore, the effectiveness of wetting agents may be greatest for water infiltration when used after coring (Wilkinson and Miller, 1978; Shurtleff, 1981).

1.5 Research objectives

1.5.1 Turfgrass and pesticide use

Significant changes are taking place in turf industry. In 1981, there were 154,000 ha of turf, which was ranked in second place among six major cash crops in Ontario: grain corn, turf, tobacco, potato, apple and grape (Sears and Gimpelj, 1983). Because turfgrass is often grown in very stressful environments such as sports turf and golf greens, and because it is not practical to rotate turf with other crops, pests often become one of the major problems in turf management. Golf course greens, tees, fairways are the most intensively managed turf areas (Kane and Wilkinson, 1992). Disease control on these intensively managed turf areas is achieved primarily through application of fungicides. Kane and Wilkinson (1992) also reported that golf greens receive the greatest fungicide input per unit of surface area of any U.S. crop, and the dollar value of the turf fungicide market is greater than any other crop. In 1988, approximately 1.5 million kilograms and 2.5 million liters of pesticides were used on turf (Ayers and Gilmore, 1991). In Canada, it is estimated that just over 2 million kilograms of active ingredient of fungicides were used on all crops in 1990, and most of the fungicide was applied to fruits, vegetables, and

turf (Anonymous, 1992).

In Canada, the total area of golf courses was estimated to be 44,100 hectares in 1990 (Anderson *et al*., 1992). The expenditure for fungicide on golf courses was approximately 3.3 million dollars compared to 0.6 and 0.4 million dollars for herbicides and insecticides, respectively. The number of applications on greens was on average 6 times for fungicides and 1.5 and 0.4 times for insecticides and herbicides, respectively.

Appropriate use of pesticides should not only reduce the cost but also reduce the risk of environmental contamination. This becomes even more important on turfgrass, since most turfgrass is grown in urban areas and used for ornamental or recreational proposes.

Benomyl is one of the major fungicides used on turf. Its degradation, adsorption and uptake by plants in soil have been studied previously. However, little work has been done on turf which differs greatly from other crops due to its thatch development and cultural practices such as frequent moving.

Benomyl is not stable in water and may be quickly but partially converted to MBC. The coexistence of the two compounds makes it difficult to analyze one or both compounds using chemical methods due to different chemical and physical properties. However, in trial experiments, we found that benomyl and MBC were equally toxic to fungus *Penicillium expansum*. Therefore it is possible to detect both benomyl and MBC with *P. expansum* in a bioassay.

The effects of surfactants on the coverage and uptake of pesticides by plant leaves have been well studied (Anderson and Girling, 1983; Shurtleff, 1981). However, little

work has been done on the effects of surfactants or core cultivation on pesticide movement in soil and uptake by roots. Information on effective uptake of benomyl by turfgrass roots is lacking. Frequent applications of benomyl are required to give effective control of some diseases such as dollar spot (Vargas, 1981), which could be changed if benomyl uptake could be improved.

1.5.2 Objectives

The objectives of this work are: 1) to evaluate and develop bioassay techniques for the determination of biologically available and total benomyl/MBC residues in turfgrass/thatch/soil; 2) to examine dissipation and translocation of benomyl/MBC in turf thatch/soil under lab and field conditions; 3) to examine the effects of a wetting agent, Aqua-Gro, and coring on the movement of benomyl/MBC in thatch/soil, and on uptake and disease control.

CHAPTER 2. DEVELOPMENT AND EVALUATION OF BIOASSAYS FOR THE DETECTION OF BENOMYL RESIDUES

2.1 Abstract

Paper disc, soil-agar pellet, turfgrass-agar pellet, thatch-agar pellet, and sample-agar mixture bioassays were modified or developed and evaluated to detect the fungicide benomyl and its fungitoxic residue methyl 2-benzimidazole carbamate in water, soil, turfgrass clippings, and thatch. The bioassays could detect biologically available benomyl and/or MBC residues with a limit of detection of 0.2 μg/g and a limit of quantitation of 0.5 μg/g. Over 100 samples can be tested by a single person within 2 days.

2.2 Introduction

Chemical analysis of pesticides in soil and plant tissue involves many extraction steps, which may be laborious, and costly, and only a few samples can be processed in a short period (Gorbach, 1980). Chemical analysis usually cannot quantify the amount of fungicide which is biologically available to target and non-target organisms. Bioassays, on the other hand, are simple, less costly, and the biologically available fungicide in samples can be detected using target or non-target organisms sensitive to the fungicide (Bennett and de Beer, 1984).

Bioassay techniques have been previously developed to detect the residues of pesticides in plant and soil (Munnecke, 1958; Peterson and Edgington, 1969; Erwin *et al*., 1971; Edgington *et al*., 1973; Yarden *et al*., 1985a). The detectable concentration of the fungicide benomyl was reported to be as low as 0.1 μg/mL when using a paper disc assay

(Black and Neely, 1976) or 0.25 μg/mL when using a soil-agar pellet assay (Yarden *et al*., 1985a). Many previously reported bioassays were conducted using leaf disc diffusion plate techniques to detect the pesticide residues in plant leaves (e.g. Erwin *et al*., 1971; Edgington *et al*., 1973); Such techniques are, however, suitable only for plants with large leaves.

Bioassays are based on the diffusion of fungicide from a paper disc or sample agar-pellet to media inoculated with spores of the test fungus. The fungicide can be determined quantitatively from the resulting inhibition zone of growth of the fungus. The inhibition zone from a known quantity of fungicide permits calculation of a standard dosage-response curve from which unknown concentrations of the fungicide can be estimated (Thornberry, 1950; Edgington *et al*., 1973; Yarden *et al*., 1985a). The process of fungicide diffusion from paper disc or sample agar-pellet to media could be similar to that in soil where the fungicide diffuses from soil to nearby roots.

Detection of benomyl through chemical analysis is complicated because it decomposes rapidly in solution, plants and soil, or during the analysis, to methyl 2-benzimidazole carbamate (MBC) (Clemons and Sisler, 1969; Peterson and Edgington, 1970; Singh *et al*., 1990).

The objective of the present work was to develop and evaluate bioassays for the detection of benomyl and its fungitoxic residue MBC in soil, turfgrass, thatch and leachate and to evaluate the technique by comparison with UV-spectrophotometry.

2.3 Materials and methods

2.3.1 Materials

2.3.1.1 Chemicals

Technical grade Methyl 2-benzimidazole carbamate (MBC), and the commercial product Tersan®1991 (50% active ingredient benomyl) were provided by Du Pont Canada, Inc. Mississauga, Ontario. Pesticide grade methanol was purchased from Fisher Scientific, as were analytical grade HCl, NaOH, and NH_4OH.

2.3.1.2 Fungi

Sclerotinia homoeocarpa F.T. Bennett and *Penicillium expansum* Link were used as test organisms because of their sensitivity to MBC and benomyl (Black and Neely, 1976). *Sclerotinia homoeocarpa*, the causal agent of dollar spot disease on turfgrass represents non-sporulating fungi and *P. expansum* represents fast growing and sporulating fungi. Six isolates of *S. homoeocarpa* were selected from our culture collection. These isolates were isolated from dollar spot disease on turfgrass at different locations. The isolate of *P. expansum* was obtained from Dr. G. Barron, Department of Environmental Biology, University of Guelph, Guelph, Canada.

2.3.1.3 Turfgrass, thatch and soil

Turfgrass, thatch and soil came from a 13-year-old sand green seeded with `Penncross' creeping bentgrass at the University of Guelph, Cambridge Research Station, in Ontario. The soil was Fox sandy loam (76.9% sand; 17.0% silt; 6.1% clay; 1.7% organic matter; pH 5.2; CEC 12.5 c/kg). The site was maintained at 5 mm cutting height, mowed daily and irrigated as needed to minimize stress.

2.3.1.4 Other materials

Cellulose paper discs (12.7 mm in diameter) for bioassay were purchased from Fisher Scientific (Unionville, Ontario, Canada). Tissue culture plates (24-well) with a flat bottom (16 mm in diameter) were purchased from Corning Glass Works (Corning, New York) and used to make turfgrass- and thatch-agar pellets. Plastic rings (12.7 mm in diameter and 10 mm in height) cut from a plastic tube were used to make soil pellets. Potato dextrose agar (PDA) was from Difco. PU8800 UV/Visible Spectrophotometer (Philips Scientific & Analytical Equipment, Pye Unicam Ltd, Cambridge, England) was used for chemical analysis of the fungicide.

Organic solvents may interact with pesticides in bioassays (Stratton, 1985). Methanol was chosen as a solvent in this study because it did not show interaction with MBC. Methanol is also less toxic to test organisms (Bowman *et al*., 1981) and can extract significantly more benomyl or MBC from plant tissue than acetone or chloroform (Fernandes and Cole, 1974).

2.3.2 Methods

2.3.2.1 Paper disc bioassay

This bioassay was a modification of the method used by Edgington *et al*. (1973). Standard concentrations of MBC were prepared in methanol (0, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 20, 50, and 100 μg/mL) and in distilled water (0, 0.25, 0.5, 1, 2, 4, 6, 8, and 10 μg/mL). A 7-day-old culture of *P. expansum* grown on PDA was flooded with a small quantity of autoclaved 0.1% (v/v) Tween 20 in distilled water and rubbed gently with a blunt transfer needle to liberate spores. The number of spores was counted with a haemocytometer and

adjusted to 2 x 10^6 conidia/mL.

Potato dextrose agar was autoclaved at 121°C for 15 min, cooled, and then 1 mL of the *P. expansum* spore suspension mixed with 99 mL PDA at 45°C. The seeded PDA (10 mL) was transferred into a Petri dish and the Petri dish swirled in a circular motion for 5 seconds to thoroughly distribute the medium. After the PDA solidified, it was sliced into parallel strips (Fig. 2.1). Two strips of 15.5 mm width were retained in the dish and the rest of the PDA removed, giving a 7 mm gap between the strips. A paper disc (12.7 mm diam) was placed in the middle of each strip, and 70 µl of the standard concentration of MBC in water or methanol added to the disc. There were eight replicates at each concentration.

Plates were incubated in the dark at 4°C for 24 h. This step allowed the fungicide to diffuse from the paper disc into the medium while fungal growth was temporarily inhibited at the low temperature. The plates were subsequently incubated in the dark for 24 h at 23°C. The inner diameter of fungal growth was measured and used to calculate the diameter of zone of inhibition (diameter of fungal growth less diameter of paper disc). Standard curves were established by plotting the mean net diameter, square of net diameter, and area of the zone of inhibition against the log concentration of MBC using the procedure of "best of fit" in Sigma Plot software (Anonymous, 1992).

2.3.2.2 Sample-agar pellet bioassay

2.3.2.2.1 Soil-agar pellet bioassay

This bioassay was a modification of Munnecke's (1958) method using soil pellets. The following standard concentrations of MBC in soils were prepared by mixing 1000

Figure 2.1. Top: Paper disc bioassay on PDA strips [numbers are concentrations of MBC in methanol (μg/mL)]. Bottom: Paper disc bioassay (left), agar-pellet bioassay (middle), and soil-agar pellet bioassay (right) with full plate PDA.

g air-dried and sieved (2 mm) soils with standard solutions of MBC in methanol. The final concentrations of MBC in soil were 0, 0.25, 0.5, 1, 2, 4, 6, 8, 10 μg/g. The samples were air-dried in the fume hood overnight to allow methanol to evaporate, and then kept at -20°C.

Eight samples of air-dried soil (0.5 g each) with different MBC concentrations were used to make soil-agar pellets. Preparation of the PDA plates was the same as stated previously. Plastic rings (12.7 mm in diameter and 10 mm in height) were placed onto the centre of the PDA plates or in the middle of PDA strips in Petri dishes with gentle pressure, to ensure firm contact of the ring to the medium (Fig. 2.2). Soil pellets were made by placing soil samples into the rings followed by adding 0.3 mL of molten water agar (containing 1.5% agar and 30 μg/mL streptomycin sulphate) dropwise into the soil in the rings. Agar pellets without soil but amended with an amount of MBC equal to that in the soil pellets were also tested. This was to test biological availability of MBC by aqueous diffusion.

The water agar used for making agar pellets was prepared by adding 1 mL of MBC methanol solution to 100 mL of hot water agar (90°C). After the water agar cooled down to 45°C, 1 mL streptomycin sulphate stock solution (3000 μg/mL) was added. The water agar for soil-agar pellets was made in the same way except that fungicide was added to soil instead of water agar (Fig. 2.1 and 2.2).

The rings were removed after water agar in the pellets had solidified, and the plates were incubated following the same procedure as in the previous experiment. The net diameter of fungal inhibition was measured and regressed on the concentration of

Figure 2.2. Top: A 24-well culture plate for making turfgrass- or thatch-agar pellets (left) and a Petri dish with a plastic ring for making soil-agar pellets (right). Bottom: Turfgrass- or thatch-agar pellet bioassay and soil-agar pellet bioassay.

MBC in the samples. The standard curves were established using the procedure of "best of fit" in Sigma Plot software (Anonymous, 1992).

Soil with known MBC concentrations was subjected to degradation *in vitro* and tested by soil-agar pellet bioassays and UV-spectrophotometry (Section 3.3.2.1). The results from the two methods were compared.

2.3.2.2.2 Turfgrass-agar pellet bioassay

Samples of freshly mowed turfgrass clippings (100 g) from creeping bentgrass were sprayed uniformly with MBC-methanol solutions (10 mL, which contains 0.01% surfactant Aqua-Gro) with different concentrations of MBC using a small pressure spray pack (100 mL). The concentrations ranged from 0.25 to 10 μg/g. The clippings were well mixed and left in a fume hood overnight to allow methanol to evaporate, and then stored in a freezer at -20°C.

Eight samples of clippings (0.2 g fresh weight) with different MBC concentrations were each placed into a well of a 24-well culture plate followed by mixing with 0.8 mL of water agar using a steel needle (Fig. 2.2). After the agar solidified, pellets were transferred onto the middle of PDA strips or into the centre of a Petri dish (10 cm diam) containing 10 mL of PDA which had been mixed with 1% (v/v) conidial suspension (2 x 10^6 conidia/mL) of *P. expansum*. Agar pellets without turfgrass but amended with an amount of MBC equal to that in turfgrass pellets were also tested. The plates were incubated following the same procedure as previously stated. The diameter of fungal growth was measured and standard curves were established.

Turfgrass clippings with different concentrations of MBC were tested by both UV-

spectrophotometry after extraction with methanol (Fernandes and Cole, 1974), and the turfgrass-agar pellet bioassay. The results from the two methods were compared.

2.3.2.2.3 Thatch-agar pellet bioassay

Thatch collected from creeping bentgrass was air dried and cut into small pieces (< 3 mm). Standard concentrations of MBC in thatch were prepared as for turfgrass clippings. The thatch was then kept at -20°C.

Eight samples of thatch (0.2 g dry weight) with different MBC concentrations were placed into wells of 24-well culture plates (Fig. 2.2). To each well, 1 mL water agar was added and mixed with thatch in the well using a steel needle. Agar pellets without thatch but amended with an amount of MBC equal to that in thatch pellets were also tested. After the water agar had solidified, pellets were transferred onto the middle of PDA strips or the centre of Petri dishes containing 10 mL PDA which had been amended with 2 x 10^4 *P. expansum* conidia/mL. Plates were incubated as in the previous experiment. The net diameter of fungal inhibition was measured and standard curves were established as in the previous experiment.

2.3.2.3 Sensitivity of *S. homoeocarpa* to MBC

The following standard concentrations of MBC were made in methanol: 0, 1.0, 2.5, 5.0, 10 and 15 µg/mL. One milliliter of each fungicide solution was mixed with 100 mL of PDA (90°C). The final concentrations of MBC in PDA were 0, 0.01, 0.025, 0.05, 0.025, 0.10, 0.125 and 0.15 µg/mL. After the PDA cooled (45°C), it was amended with streptomycin sulphate to make a concentration of 30 µg/mL. Ten milliliters of the amended PDA was added to each plate (10 mm diam) and the plate swirled thoroughly

to distribute the agar. After the PDA in the plates solidified, mycelial plugs (4 mm in diameter) of *S. homoeocarpa* were placed onto the centre of plates. There were 8 replicates at each concentration. Plates were incubated at 23°C for 4 days. The diameter of fungal mycelial growth (DFMG) was measured, and the percent of inhibition of fungal growth (DFMG of control less DFMG of treated all divided by DFMG of control) and the concentration of MBC were used to establish a standard curve.

2.3.2.4 Statistical analysis

Regression analyses between diameter of zone of inhibition, or the square of diameter of zone of inhibition, or area of zone of inhibition and concentration of MBC were performed in both linear and log scales using the "best of fit" procedure in Sigma Plot software (Anonymous, 1992). The best fit nonlinear regression line between concentration of MBC and fungal growth inhibition for sample-agar mixture bioassay was established (Gomez and Gomez, 1984). The EC_{50} values were estimated from the regression equation. All tests for significance were performed at P=0.05.

2.4 Results and discussion

In agreement with Cox *et al*. (1974), preliminary studies showed that benomyl and MBC were equally toxic on a molar basis to *Penicillium expansum*, though this might be due to the rapid conversion of benomyl to MBC in the assay samples or media (Section A.1.4.8). Throughout this thesis, MBC was used in place of benomyl in most cases. The concentration of benomyl calculated as 1.52 times the concentration of MBC, (1.52 is the ratio of molecular weights of the two compounds).

2.4.1 Paper disc bioassay

In published papers on fungicide bioassays, various fungal growth parameters have been used to establish standard curves with fungicide concentrations. These parameters include diameter of zone of growth inhibition (DZI) (Hine *et al*., 1969; Edgington *et al*., 1973; Cox *et al*., 1974; Black and Neely, 1976), square of diameter for zone of inhibition (SDZI) (Yarden *et al*., 1985a), and area of zone of inhibition (AZI) (Erwin *et al*., 1971; Moldzynska and Rejman, 1977). In this study, regression analyses were conducted on these parameters and the best fit regression lines were used as standard curves.

Curvilinear relationships were found between concentrations of MBC and DZI (Fig. 2.3A), or SDZI (Fig. 2.3C) or AZI (Fig. 2.3E). If the concentration of MBC was transformed to log scale, linear relationships were found between log concentration of MBC and DZI, or SDZI, or AZI (Fig. 2.3B, 2.3D and 2.3F). When the concentration of MBC was less than 10 ppm, a near-linear relationship and steepest slope were observed (Fig. 2.3A). This suggested that the sensitivity of the bioassay was the highest at concentrations less than 10 ppm. All three regression lines had highly significant r^2 values, which indicated a close relationship between fungicide concentration and each of the three parameters used. However, when SDZI and AZI were used instead of DZI, most points were further away from the regression lines especially at low concentrations such as 0.5 ppm or very high concentrations such as 100 ppm (Fig. 2.3D and 2.3F). Therefore, the best fit line between DZI and log concentration of MBC was used as a standard curve. This line gave the highest coefficient of determination (r^2) among the

Figure 2.3. Relationships between concentration of MBC and diameter of zone of inhibition (DZI) (A, B), or square for diameter of zone of inhibition (SDZI) (C, D), or area of zone of inhibition (AZI) (E, F). Concentrations of MBC in methanol ranged from 0.5 to 100 μg/mL and tested with paper disc bioassay.

regression lines (Fig. 2.3). The linear relationship between DZI of fungal growth and the logarithm of the concentration of benomyl or MBC has also been reported by others (Edgington *et al*., 1973; Fernandes and Cole, 1974).

When the MBC concentration in methanol was greater than 30 µg/mL, DZI was increased only slightly with further increase in MBC concentration, which indicated that sensitivity of the bioassay was reduced when MBC concentrations were greater than 30 µg/mL. However, most soil treated with benomyl in the field will contain MBC at less than 10 µg/g (given application rates less than 6 kg a.i/ha, soil bulk density of 0.95 g/cm^3, and soil depth of infiltration 2 cm). Also, for most residual analyses, attention is usually directed to the lower range of residual concentrations (Cox *et al*., 1974; Yarden *et al*., 1985a). Therefore a range of 0.5 to 10 µg/mL of MBC was used to establish standard curves for the estimation of fungicide residues in soil solution (Fig. 2.4). Compared to the concentration range of 0.5 to 100 µg/mL (Fig. 2.3), the smaller range of 0.5 to 10 µg/mL resulted in more realistic regressions (Fig. 2.4).

MBC when dissolved in methanol gave larger zones of inhibition than when dissolved in H_2O at the same concentrations using paper disc bioassay on PDA plates (Fig. 2.5). This was likely due to the dispersion or high solubility of the fungicide in methanol (Colmon and Sayag, 1976). The results also indicated that when the amount of MBC was larger than 8 µg/mL (solubility of MBC in water at pH = 7) and less than 10 µg/mL in water, the diameter of zone of inhibition did not increase significantly in most cases (Fig. 2.5).

Analysis of the regression lines of MBC in methanol and that of MBC in water

Figure 2.4. Relationships between concentration of MBC and diameter of zone of inhibition (DZI) (A, B), or square for diameter of zone of inhibition (SDZI) (C, D), or area of zone of inhibition (AZI) (E, F). Concentrations of MBC in methanol ranged from 0.5 to 10 μg/mL and tested with paper disc bioassay.

Figure 2.5. Sensitivity of paper disc bioassay for the detection of MBC concentrations in methanol using PDA in strips and full plate PDA, and in water using full plate PDA bioassay.

indicated that there were no significant differences between slopes although intercepts were significantly different. This suggested that there was no interaction between MBC and methanol using the paper disc bioassay. Therefore, both regression lines can be used to estimate the concentration of MBC in methanol and water respectively.

2.4.2 Sample-agar pellet bioassay with full plate PDA

2.4.2.1 Soil-agar pellet bioassay

Standard curves for agar pellet and soil-agar pellet bioassays are shown in Fig. 2.6. Linear relationships were found between the log concentration of MBC in agar pellets or soil-agar pellets and DZI. The slopes in agar-pellet and soil-agar pellet assays were not significantly different from each other, which indicated that the amount of fungicide adsorbed by soil was proportional to the concentration of MBC in soil within the range of fungicide concentrations studied. Since the two regression lines were parallel, the percentage of MBC adsorbed to soil at different MBC concentrations was similar. Using the equations of the two regression lines ($Y_1 = 31.8 + 26.0 \text{Log} X_1$ and $Y_2 = 10.1 + 25.1 \text{Log} X_2$), the calculated percentages of adsorbed fungicide in Fox Sandy loam were listed in Table 2.1 (see detailed calculating procedure in APPENDIX 3).

2.4.2.2 Turfgrass-agar pellet bioassay

Standard curves for the turfgrass-agar pellet bioassay are shown in Fig. 2.7. There was a linear relationship between diameter of zone of inhibition and log concentration of MBC for both agar-pellet and turfgrass-agar pellet bioassays. The slopes for the regression lines were not significantly different, which indicated that the proportion of fungicide adsorbed or bound to turfgrass, or that which could not diffuse

Figure 2.6. Relationships between diameter of zone of inhibition and concentration of MBC in soil tested by agar pellet bioassay and soil-agar pellet bioassay using full plate PDA. Results from Fox sandy loam are presented.

Figure 2.7. Relationships between diameter of zone of inhibition and concentration of MBC in turfgrass clippings tested by agar pellet bioassay and turfgrass-agar pellet bioassay using full plate PDA.

Table 2.1. Applied MBC and proportion `adsorbed' by soil, turfgrass clippings, and thatch *in vitro* estimated by sample-agar pellet bioassay.

Turfgrass		Thatch		Soil	
MBC (μg/g)	Adsorbed (%)	MBC (μg/g)	Adsorbed (%)	MBC (μg/g)	Adsorbed (%)
0.4	84.9†	0.5	46.2	0.5	19.9
0.8	85.3	1.0	48.0	1.0	44.3
1.6	85.6	2.0	49.7	2.0	61.7
2.4	85.8	4.0	51.3	4.0	73.6
3.2	85.9	6.0	52.3	6.0	78.8
4.0	86.1	8.0	52.9	8.0	81.8
8.0	86.4	10.0	53.5	10.0	83.9
16.0	86.7	20.0	55.0	20.0	88.9
		40.0	56.5	40.0	92.4
		50.0	56.9	50.0	93.2

† See Appendix 3 for detailed calculation procedure.

out of the pellets, was related to the concentration of MBC in grass blades. Plant-bound pesticide residues are not biologically available (Khan *et al*., 1984; Khan and Dupont, 1987). Using the two regression equations, the bound fungicide was estimated as 46.2 to 56.9% of that originally applied (Table 2.1). The variation was caused by the slight difference in slopes which were not significantly different for the two regression lines.

For turfgrass clippings that were spiked with a known amount of MBC, the extracts did not differ significantly in MBC concentration when tested either by the turfgrass-pellet bioassay or the UV-spectrophotometry method (Table 2.2). Therefore the turfgrass-pellet bioassay can be considered reliable.

2.4.2.3 Thatch-agar pellet bioassay

Standard curves for thatch-agar pellet and agar-pellet bioassays were established

Table 2.2. MBC in turfgrass clippings estimated by turfgrass-agar pellet bioassay and UV-spectrophotometry.

Method	Concentration of MBC (µg/g)			
	Sample 1	Sample 2	Sample 3	Sample 4
Turfgrass-agar pellet	0.8a[†]	2.3a	4.8a	8.2a
UV-spectrophotometry	0.9a	2.1a	4.5a	7.9a

[†] Means with the same letter in a column are not significantly different from each other at P=0.05. Each number is the mean of 8 replicates for turfgrass-agar pellet bioassay and 4 replicates for UV-spectrophotometry.

as shown in Fig. 2.8. A linear relationship was found between DZI and the log concentration of MBC in thatch. Slopes of the regression lines were significantly different from each other, which suggested that the proportion of fungicide adsorbed by thatch was not constant with increased fungicide levels. This indicated that the thatch adsorbed a large portion of the fungicide and that this adsorbed portion was not mobile in aqueous phase. Niemczyk *et al*. (1988) reported that over 90% of applied insecticide is adsorbed by the thatch layer under turfgrass. The current study showed the adsorbed MBC ranged from 19.9% to 93.2% depending on concentration of the fungicide in the thatch (Table 2.1).

2.4.3. Sample-agar pellet bioassay with PDA in strips

The sensitivity of bioassays was increased when strips were used instead of the full plate. This was demonstrated by the significant difference (P=0.05) between the intercepts of the regression lines from strip bioassays (Fig. 2.9) compared to full plate bioassay (Fig. 2.6, 2.7 and 2.8). This was due to the higher fungicide concentration available when the fungicide diffused in two direction in strips instead of 360° in full

Figure 2.8. Relationships between diameter of zone of inhibition and concentration of MBC in thatch tested by agar pellet bioassay and thatch-agar pellet bioassay using full plate PDA.

Figure 2.9. Comparison of soil-agar pellet, turfgrass-agar pellet, and thatch-agar pellet bioassays using PDA in strips.

plate PDA. The limits of quantitation of MBC with PDA in strips ranged from 0.1 to 0.5 ppm depending on bioassay methods.

2.4.4 Sensitivity of *S. homoeocarpa* to MBC

In most toxicity experiments for animals, the concentrations of the chemicals under study are transformed to a log scale before regressing on mortality to give a linear relationship (McEwen and Stephenson, 1979). There was no linear relationship between log concentrations of MBC and growth inhibition of *S. homoeocarpa* in this study. Here, a standard curve was established by plotting the percent inhibition of fungal growth (relative to a fungicide-free control) against the concentration of MBC in PDA, which gave an exponential relationship (Fig. 2.10). We also plotted the probit scale for inhibition against concentration of MBC or log concentration of MBC, but we found that the curve with exponential relationship gave the best fit line and a highly significant R^2 value.

The effective concentration that reduces the growth of a fungus by 50% (EC_{50}) is a commonly used indicator of the sensitivity of a fungus to a fungicide (Edgington *et al*., 1971). The EC_{50} value of MBC calculated using the regression equation (Fig. 2.10) was 0.09 μg/mL MBC for the six isolates of *S. homoeocarpa* tested.

2.5 Conclusions

The bioassays developed were fast, less costly and could be used directly to detect the biologically-available benomyl and MBC in samples. The results obtained by bioassays were found to be the same as those obtained by spectrophotometry analysis. The entire process did not require sterile conditions because the plates were inoculated

Figure 2.10. Relationship between concentration of MBC in medium and percent inhibition to the mycelial growth of *S. homoeocarpa* in linear scale (A) and probit scale (B). Dotted lines indicate 95% confidence interval.

with high concentrations of a fast growing fungus. Over 100 samples could be processed

by one person in two days.

The paper-disc bioassay can be used to determine the fungicide level in aqueous solutions such as sprays, leachates, or extracts. Sample-agar pellet bioassays can be used to detect fungicide residues directly from soil, thatch or turfgrass without extraction. The process of fungicide diffusion from the pellets to the PDA is perhaps similar to the process *in situ* of diffusion from soil or thatch to the roots of turfgrass.

The sensitivity of paper-disc or sample agar-pellet bioassay was improved when using PDA strips instead of full plate PDA. The strip bioassay was suitable for the detection of lower levels of fungicide residues (<10 ppm). At higher fungicide levels, the DZI became too large and the inhibition zone sometimes reached the edge of the Petri dish, causing variation in results. This was overcome by diluting the fungicide concentration in test samples.

The major disadvantage of these bioassays was that they were not benomyl- or MBC-specific. Other fungicides or toxic substances may affect the results. Therefore, they are not recommended for MBC detection when mixtures are used unless the bioassay fungus is insensitive to the other fungicides. These bioassays can be used as tools for the studies on benomyl/MBC dissipation, adsorption, movement in soil, and uptake by plants.

Other chemical or physical factors can affect the result of a bioassay. Appendix 1 presents these factors in more detail.

CHAPTER 3. ADSORPTION AND PERSISTENCE OF BENOMYL/MBC IN SOIL AND THATCH

3.1 Abstract

Dissipation, adsorption and biological availability of the fungicide benomyl in soil and thatch were studied using bioassay techniques. *In vitro* soil and thatch degradation studies showed a half life for MBC of approximately two and a half weeks in thatch and 4 weeks in soil depending on the soil type. Less than 10% of the applied fungicide was degraded chemically. The amount of MBC adsorbed by soil and thatch varied, depending on soil type and fungicide concentration. An equilibrium with fungicide in solution was reached within 1 h for thatch and 2 to 4 h for soils. MBC adsorbed by thatch was as much as twice that adsorbed by Fox sandy loam which represents a soil often found on golf courses. When MBC was applied at 10 µg/g, as much as 90% of MBC in thatch and 68% in soil were not extractable with water. Methanol extracted up to three times the fungicide from soil or thatch compared to water.

3.2 Introduction

Turfgrass, unlike other cultivated crops, usually develops a layer of thatch which is a tightly intermingled layer of dead and living roots, rhizomes, stolons and organic debris that develops between the zone of green vegetation and the soil surface (Beard, 1973). The turfgrass thatch-soil system can adsorb significant amounts of applied pesticide, and reduce the amount of pesticide reaching the roots in the thatch layer or soil underneath (Branham and Wehner, 1985; Krause and Niemczyk, 1989; Harrison *et al.*,

1989; Cooper, 1990). Furthermore, an accelerated rate of pesticide degradation has been found in thatch (Branham and Wehner, 1985; Potter *et al*., 1989). Niemczyk and Krause (1989) reported that over 90 percent of insecticide residues remained in the thatch layer at 91 days after treatment.

Adsorption of benomyl and its fungitoxic residue MBC in soil is one of the major processes influencing accessibility to target organisms and the potential to reach non-target organisms. Fuchs *et al*. (1970) found that uptake of benzimidazole fungicides by plants from the soil is not very efficient especially in clay soils. The rate of uptake of benomyl by plants was increased when plants were grown on soils with low clay and/or organic matter content, or with a higher pH. Studies on mobility showed that benomyl/MBC were highly immobile in soil and that most of the fungicide residues were in the upper 2 cm of the soil (Baude *et al*., 1974; Austin and Briggs, 1976).

Microbial degradation plays a very important role in the dissipation of fungicide in soil or thatch. Fungicide not degraded by microorganisms could be that which is tightly adsorbed or bound to thatch. This tightly bound portion usually cannot be extracted with water or even methanol (Khan and Dupont, 1987; Gorbach, 1980), and is generally considered biologically unavailable (Khan, and Dupont, 1987; Khan *et al*., 1984).

Detection of benomyl by chemical analysis is complicated by its decomposition to MBC, which can occur in solution, plants, soil, or during analysis (Clemons and Sisler, 1969; Peterson and Edgington, 1970; Singh *et al*., 1990). The complete conversion of benomyl to MBC takes a period of time measured in hours (Chiba, 1977) to days (Baude *et al*., 1973). Chiba (1977) reported that the conversion of benomyl to MBC reached

equilibrium in a given solvent. This suggested that both benomyl and MBC could exist in soil or soil solution after application of benomyl.

The biologically available fungicide residue in soil refers to that which is readily taken up by plants or soil-inhabiting organisms (Khan and Dupont, 1987). Fungicide extracted with water, which, by definition, does not include any that has been tightly adsorbed by the soil particles, should be readily available to plants and soil organisms (Bennett and de Beer, 1984).

MBC is also fungitoxic (Siegel and Zabbia, 1972; Cox *et al*., 1974; Austin and Briggs, 1976). Cox *et al*. (1974) found that, on a molar basis, benomyl and MBC were similarly toxic to *Penicillium citrinum* in bioassays, which suggested that both benomyl and MBC can be determined with a bioassay at the same time (Section A.1.4.8).

Certain fungicide bioassays are based on the diffusion of fungicide from the sample through agar medium inoculated with spores of the test fungus. A standard dosage-response curve is established by plotting inhibition against concentration of the fungicide (Thornberry, 1950; Munnecke, 1958; Edgington *et al*., 1973; Yarden *et al*., 1985a). The fungicide concentration in unknown samples is then determined from the inhibition of fungal growth using the standard curve.

The objectives of the present work were to use bioassay techniques (developed in Chapter 2) for the detection of adsorption, persistence and amount of biologically available MBC residues in soil and thatch, and to evaluate the techniques by comparing the results to those obtained using UV-spectrophotometry.

3.3 Materials and methods

3.3.1 Materials

3.3.1.1 Chemicals

Technical grade methyl 2-benzimidazole carbamate (MBC) and the commercial product Tersan 1991 (50% active ingredient benomyl) were provided by Du Pont Canada. Pesticide grade methanol, acetone and ethyl acetate were purchased from Fisher Scientific, as were analytical grade HCl, NaOH, and NH_4OH. Unless otherwise noted, MBC in the text refers to both originally amended MBC and MBC from benomyl.

3.3.1.2 Bioassay fungus

Penicillium expansum, the bioassay organism, was obtained from Dr. G. Barron, Department of Environmental Biology, University of Guelph. *Penicillium expansum* was used in this study because benomyl and MBC were equally toxic to this fungus on molar basis (Section A.1.4.7). It is very sensitive to benomyl (Moldzynska and Rejman, 1977) and has a rapid and high sporulating capacity.

3.3.1.3 Other materials

Soils used in this study were a Guelph loam (24.1% sand; 53.0% silt; 23.0% clay; 5.7% organic matter; pH 7.2; CEC 19.9 c/kg) and a Fox sandy loam (76.9% sand; 17.0% silt; 6.1% clay; 1.7% organic matter; pH 5.2; CEC 12.5 c/kg). These soils have not been treated with benomyl over the past 10 years. These are two typical soils in Ontario. They were air-dried and passed through a 2 mm sieve. Thatch was collected from a creeping bentgrass green at the Cambridge Research Station, University of Guelph.

A PU8800 UV/Visible Spectrophotometer (Philips Scientific & Analytical Equipment, Pye Unicam Ltd, Cambridge, England) was used in chemical analysis.

Bioassay paper discs (12.7 mm in diameter), Whatman No. 1 filter papers (90 mm in diameter) from Fisher Scientific were used in the bioassay.

3.3.2 Methods

3.3.2.1 Dissipation of benomyl/MBC in soil and thatch

Two kilograms of air-dried sterilized or non-sterilized soil and thatch were separately amended with NaOH or HCl solution to adjust the pH to 7.0. This was determined by further dilution and testing in water of a subsample. The soil and thatch were then mixed with a benomyl-methanol solution to obtain a concentration of 15.2 μg/g which is equivalent to 10.0 μg/g of MBC. The samples were placed in a fume hood overnight to allow methanol evaporation; 400 g were placed in a biometer flask (Bartha and Pramer, 1965) and adjusted to a moisture content of approximately 80% field capacity with sterilized water. The top opening of the flask was sealed with parafilm. In the side arm, 30 mL of sterilized water was added to maintain a constant moisture level in the soil and thatch. The flasks were incubated in the dark at 23°C. Four replications were run for each type of soil or thatch.

At weekly intervals for 7 weeks, 60 g samples of soil and thatch were removed from each respective flask and kept at -20°C until testing. The soil or thatch was then air-dried and four sub-samples (0.5 g for soil and 0.2 g for thatch) tested for MBC concentration with the soil- or thatch-agar pellet bioassay (Section 2.3.2.2) and three sub-samples (12 g each) with spectrophotometry. Extraction was done with acetone and ammonium chloride (1:1, v/v). The clean-up was done with ethyl acetate, and the pH adjusted with concentrated HCl or 6.5 N NaOH. For spectrophotometry, MBC was

measured in 0.1 N HCl at 282 nm. The detailed steps for extraction, clean-up and detection using spectrophotometry are modified from Austin and Briggs (1976) and are listed in Fig. 3.1.

Benomyl in sand (Gradation index 4.2, mid-particle diameter 0.3, and pH 6.4), Guelph loam, and sand mixed with Guelph loam were also examined for degradation rates. Sand and Guelph loam were mixed in ratios of 3:1, 1:1 and 1:3 (w/w). Aliquots of a benomyl-methanol solution were then added so that the mixture contained benomyl at 15.2 µg/g (equal to MBC at 10 µg/g). The mixture was air-dried in a fume hood overnight. The samples of 400 g of sand, soil or the mixtures were added to biometer flasks. Water was added to each flask to bring the soil to 80% of field capacity. Constant moisture levels were maintained in the flasks by adding 30 mL of water in the side arm. The flasks were incubated in the dark at 23°C. Approximately 5 g of sand was taken out at weekly interval for 7 weeks and kept at -20°C. The soil was air dried and tested for MBC by the soil-agar pellet bioassay (Section 2.3.2.2).

3.3.2.2 Adsorption of benomyl/MBC by soil or thatch

3.3.2.2.1 Test for equilibria of MBC in water

Four samples of 25 g air-dried soil or 15 g air-dried thatch were placed into 250 mL flasks. A water suspension of 150 mL or 90 mL with 6.1 µg/mL benomyl, equivalent to 4.0 µg /mL MBC, was added into flasks with soil or thatch. The flasks were shaken for 10 minutes and the pH adjusted to 7.0 using HCl or NaOH. The flasks

Figure 3.1. Extraction steps for MBC from soil or thatch for spectrophotometry. Modified from Austin and Briggs (1976).

were then stoppered and shaken at 23°C. At 1, 2, and 4 h, 2 mL of water from the suspensions were taken out and analyzed using the paper disc bioassay (Section 2.3.2.1).

At 6 h, 22 mL of soil- or thatch-water suspension were taken out and tested by the paper disc bioassay and spectrophotometry after clean up. The procedures for spectrophotometry were modified from those of Austin and Briggs (1976) with differing amounts of samples and solvents.

3.3.2.2.2 Effect of MBC concentration on its adsorption by thatch and soil

The same procedure as the test for equilibria was followed, except that multiple benomyl concentrations were tested. Benomyl was suspended in water to achieve a concentration series of 3.0, 6.1, 9.1, and 12.1 µg/mL which is equivalent to 2.0, 4.0, 6.0 and 8.0 µg/mL MBC respectively. Soil (25 g) and thatch (15 g) were added to 250 mL flasks containing 150 and 90 mL of fungicide suspension, respectively. The pH was adjusted to 7.0. Flasks were then stoppered and shaken for 6 h at 23°C. The filtered suspension was tested using paper disc bioassay. Soil and thatch were filtered out, air-dried and tested using soil- or thatch-agar pellet bioassay (Section 2.3.2.2).

3.3.2.3 Biologically available fungicide from soil and thatch

Twenty-five grams of Guelph loam or 10 g thatch with known concentrations of MBC (4, 6, and 10 µg/g for soil, 10 µg/g for thatch), prepared as previously described (Section 3.3.2.1), were placed into 250 mL flasks. Water or methanol at 12.5, 25, or 50 mL was added into different flasks with soil; and water or methanol at 25, 50, or 100 mL was added into different flasks with thatch. The flasks were shaken for 6 h. The water or methanol suspensions were tested with the paper disc bioassay. Four replications were run for each treatment.

3.3.2.4 Statistical analysis

Chemical analysis using spectrophotometry was compared to bioassay results using *t*-tests. Slopes and intercepts of simple linear regression lines of concentrations of MBC on absorbance at 282 nm were tested. In persistence and adsorption studies, Duncan's Multiple Range Test was used to separate treatment means when a significant *F*-value was generated. All tests for significance were performed at $P=0.05$.

3.4 Results and discussion

3.4.1 Dissipation of benomyl/MBC in soil and thatch

The amount of fungicide residues in the two types of soil and one of thatch tested by spectrophotometry and soil-agar pellet bioassay were not significantly different from each other at each time interval (Fig. 3.2). Results using both methods showed a half life for MBC of four to five weeks in soil and two and half weeks in thatch.

Data from chemical analysis at different time intervals were slightly lower than those using the soil-agar pellet bioassay. The lower values from chemical analysis could be due to the incomplete decomposition of benomyl to MBC during the incubation and extraction process (Cox *et al*., 1974). In the bioassay, both benomyl and MBC were equally toxic to *P. expansum* on a molar basis, which allowed determination of the total amount of benomyl and MBC. Good agreement between the soil-agar pellet bioassay and chemical analysis for MBC using HPLC has also been reported by Yarden *et al*. (1985a).

There seemed to be a lag phase in the first few weeks of degradation of the fungicide in the two soils (Fig. 3.2). In Fox sandy loam, the lag phase lasted longer than

Figure 3.2. *In vitro* degradation of MBC in non-autoclaved thatch and two soils, determined by soil-agar pellet bioassay (open symbols) and spectrophotometry (shaded symbols). Soil and thatch were extracted with acetone and ammonium chloride (1+1), cleaned with ethyl acetate, and measured in 0.1 N HCl.

in Guelph loam or thatch. This could have been due to the higher organic matter content in Guelph loam or thatch resulting in higher microbial populations (Mancino *et al*., 1993).

Wainwright and Pugh (1974) reported that benomyl at 4 and 20 kg/ha applied to a field soil of pH 5.3 and 3.1% organic matter resulted in an increase in fungal propagules 4 weeks after treatment: the rate of degradation was faster after the population of microorganisms increased and adapted to the fungicide as an energy source.

Degradation of benomyl/MBC is also related to the clay content in soil. At its peak, the Fox sandy loam, with a lesser clay content, showed a faster rate of degradation than the Guelph loam. The strong adsorption of the fungicide by clay particles or organic matter makes it unavailable for use by soil organisms. The degradation rate slowed down after a few weeks to a level similar to that of Guelph loam. This may be explained by the reduced availability of carbon in the Fox sandy loam. Perhaps the microorganisms use other carbon sources as well as benomyl/MBC as energy sources. This phenomenon has been reported by van Faassen (1974). This interpretation was further confirmed by trials on the degradation of MBC in pure sand and sand amended with Guelph loam. In pure sand, the lag phase was much longer than that amended with Guelph loam. Soil with 3/4 sand and 1/4 Guelph loam had longer lag phase than that with 1/2 of each, 1/4 of sand and 3/4 Guelph loam, and Guelph loam alone (Fig. 3.3).

In a trial with autoclaved soil, the level of MBC tested with the soil-agar pellet bioassay did not change significantly during the time of incubation (Table 3.1). At 7 weeks after incubation, less than 10% had degraded chemically in soil or thatch, which suggested that chemical degradation did not play a significant role in the dissipation of

Figure 3.3. *In vitro* degradation of MBC in non-autoclaved sand, 3/4 sand + 1/4 Guelph loam, 1/2 sand + 1/2 Guelph loam, 1/4 sand + 3/4 Guelph loam, and pure Guelph loam, determined as MBC by soil-agar pellet bioassay.

MBC in soil, at least under our experimental conditions, and that microbial breakdown may be more important.

Table 3.1. Chemical degradation of MBC in autoclaved soil and thatch. MBC was tested by sample-agar pellet bioassay.

	Concentration of MBC (μg/g)							
	weeks of incubation							
Soil type	0	1	2	3	4	5	6	7
Fox sandy loam	10.0†	10.0	10.0	10.0	9.8	9.8	9.7	7.9
Guelph loam	10.0	10.0	10.0	9.9	9.7	9.6	9.6	4.9
Thatch	10.0	9.8	9.7	9.6	9.6	9.5	9.3	2.9

† Values are averages of four replicates.

The relatively greater degradation in Guelph loam and thatch (Table 3.1) could be related to the higher amount of organic matter which is highly correlated to pesticide adsorption (Brady, 1984; Aharonson and Kafkafi, 1975). Adsorption may increase the rate of chemical degradation of some pesticides because adsorbed pesticide molecules can be more easily catalyzed by soil-borne enzymes on the surface of clay or organic particles (Farmer and Aochi, 1987).

3.4.2 Adsorption of benomyl by soil and thatch

An adsorption-desorption equilibrium was established within approximately 1 hour for thatch, 2 h for Guelph loam and 4 h for Fox sandy loam (Fig. 3.4). Adsorption increased rapidly at the beginning and slowed down as it approached equilibrium. Very little fungicide was adsorbed by the thatch after it reached equilibrium. Rapid adsorption was also observed during the first hour for the two soils. Surface adsorption may have contributed to the initial rapid response where the cations on the clay or organic matter

were replaced by the fungicide. The slower rate prior to equilibrium may have been caused by reduced surface area for adsorption and the breakdown of soil aggregates with shaking and diffusion into smaller stable soil aggregates (Green and Obien, 1969).

At equilibrium, the fungicide adsorbed by thatch was up to twice that adsorbed by the soils (Fig. 3.4). This may explain why higher rates of fungicides (e.g. 1.5 kg/ha a.i. benomyl) are applied to turf with an extensive thatch compared to crops grown in bare soil (e.g. 1 kg/ha a.i. benomyl) to achieve effective control of target organisms (Anonymous, 1990).

Adsorption of benomyl/MBC by two types of soils and thatch tested by the paper disc and sample-agar pellet bioassays are shown in Fig. 3.5. An almost linear relationship was found between the amount of fungicide adsorbed and the concentration of the fungicide in solution at equilibrium. Because of this linearity, the distribution coefficients (K) of MBC (adsorbed MBC / MBC in solution), which describe the adsorptive capacity of both soils and thatch (McEwen and Stephenson, 1979), were the same as the regression coefficients in our studies. The K value of thatch was significantly higher than that of Guelph loam which was significantly higher than that of Fox sandy loam (P=0.05). This was most likely due to higher clay content in Guelph loam and higher organic matter content in thatch resulting in a higher exchange capacity (Bailey and White, 1964). The pH of the soil or thatch may also have significant effects on

Figure 3.4. Equilibration time for adsorption of MBC (4.0 μg/mL in water) on thatch, Guelph loam and Fox sandy loam, determined as MBC by paper disc bioassay.

Figure 3.5. Adsorption of benomyl/MBC by thatch and two soils, determined as MBC by soil- or thatch-agar pellet bioassay (open symbols) and paper disc bioassay (shaded symbols).

pesticide adsorption (Austin and Briggs, 1976), but in this study they were adjusted to the same level in soil solutions.

Analysis using spectrophotometry following the procedure of Austin and Briggs

(1976) indicated that absorbance at 282 nm was highly correlated with the concentration of MBC extracted from thatch and two types of soils (Fig. 3.6). The intercepts for the four regression lines were not significantly different from zero while the slopes did differ (P=0.05). Therefore, the amount of unextractable fungicide varied with the substrate and was proportional to the fungicide concentration. With bioassay, adsorption of MBC by thatch or soil was also found to be closely related to the concentration of MBC before adsorption (Fig. 3.7).

The results of the adsorption trial, determined with bioassays, had trends similar to those found by Aharonson and Kafkafi (1975) with fluorescence spectrophotometry. In chemical analysis, extraction and clean-up processes are involved and these processes could have potential for loss of fungicide. Furthermore, benomyl in soil, thatch or solution may not be totally degraded into MBC (Baude *et al*., 1973; Singh *et al*., 1990) which was the actual compound measured at 282 nm with a spectrophotometer in some chemical analysis. This meant that the levels of benomyl and its residue MBC, determined by certain chemical analyses, might have been lower than the actual amounts present in the samples.

3.4.3 Biologically available fungicide

The amount of fungicide extracted with methanol or water was significantly different (Table 3.2). MBC extracted with methanol was on average twice as high as that

Figure 3.6. Relationship between UV-absorbance and concentration of MBC in acetone and ammonium chloride (control), thatch, Guelph loam, and Fox sandy loam, determined by spectrophotometry. Soil and thatch were extracted with acetone and ammonium chloride (1+1), cleaned with ethyl acetate, and measured in 0.1 N HCl.

Figure 3.7. Relationship between concentration of MBC in soil or thatch at equilibrium and concentration of MBC in water before adsorption, determined by soil-agar pellet and paper disc bioassays.

Table 3.2. Concentration of MBC in water and methanol at equilibrium in different ratios of solvents and soil (Guelph loam) or thatch, as tested by paper disc bioassay.

Type	Concentration of MBC (µg/g)	Solvent	Ratio Type:solvent (w/v)	Equilibrium MBC (µg/mL)	Extracted (%)
Soil	4	water	2:1	0.7[†]	8.8
Soil	4	water	1:1	0.6	15.0
Soil	4	water	1:2	0.5	25.0

Soil	6	water	1:1	1.3	21.7		
Soil	10	water	2:1	2.3	11.5		
Soil	10	water	1:1	2.0	20.0		
Soil	10	water	1:2	1.6	32.0		
Thatch	10	water	1:2			0.4	8.0
Thatch	10	water	1:5			0.2	10.0
Thatch	10	water	1:10			0.1	10.0
Soil	4	methanol	2:1	2.3	28.8		
Soil	4	methanol	1:1	1.2	30.0		
Soil	4	methanol	1:2	0.7	35.0		
Soil	6	methanol	1:1	2.2	36.7		
Soil	10	methanol	1:1	3.6	36.0		
Thatch	10	methanol	1:2			0.9	18.0
Thatch	10	methanol	1:5			0.4	20.0
Thatch	10	methanol	1:10			0.3	30.0

† Values are averages of four replicates.

extracted with water. In chemical analysis, organic solvents are usually used to extract pesticides. However the amount extracted with organic solvents may be misleading since some of it may have been biologically unavailable.

At different soil-water ratios, the concentration of fungicide in solution at equilibrium did not vary greatly (Table 3.2). The lower ratio of 2 parts soil to 1 part water (w/v) might best represent the field condition (Aharonson and Kafkafi, 1975). The mixture of 2 parts water to 1 part thatch gave a thatch containing a similar amount of water to that collected after 20 mm of irrigation. Under these conditions, some of the fungicide adsorbed by thatch could be desorbed into the water and taken up by plants (Section 4.4.1).

At different concentrations of MBC in soil, 8.8-32.0% was extracted with water and 18.0-36.0% was extracted with methanol. Similar results were found for thatch: 8.0-

10.0% was extracted with water and 18.0-30.0% with methanol. The difference between solvents could be due to differential solubility of benomyl/MBC in water or methanol (Fernandes and Cole, 1974). At lower concentrations of benomyl or MBC in soil, less fungicide was extracted with water, and this was probably related to the tight binding to clay or organic particles. At high concentrations, the adsorption sites would become saturated and thus the fungicide may not be as tightly bound and hence more easily extracted.

When fungicide was extracted from thatch or soil (containing 10 μg/mL MBC) at the same solvent dilutions, much less fungicide was extracted from thatch than from soil. No more than 10% of the MBC was extracted by water from thatch, and even after increasing the water ratio from 1:5 to 1:10, the percent extracted remained at 10%. This indicated that when the concentration of MBC in thatch was 10 μg/g, 90% of the fungicide was tightly adsorbed and might not be biologically available.

3.5 Conclusions

In vitro studies showed that a large portion of applied fungicide was adsorbed and degraded by microorganisms in thatch. The half life for MBC was approximately two and half weeks in thatch and 4 weeks in soil depending on soil type. Less than 10% of MBC was degraded in 7 weeks when autoclaved soil and thatch were used. The amount of MBC adsorbed by soil and thatch varied, depending on soil type and fungicide concentration. Thatch adsorbed as much as 4 times the fungicide than soil. An equilibrium with fungicide in water solution was reached within 1 h for thatch and 2 to 4 h for soils. MBC adsorbed by thatch was as much as twice that adsorbed by Fox sandy loam. Up to 90%

of MBC in thatch and 68% in soil applied at 10 μg/g were not extractable with water. Methanol extracted up to three times the fungicide from soil or thatch than did water.

The results further confirmed that paper disc and sample-agar pellet bioassays, modified from previous studies (Munnecke, 1958; Edgington *et al*., 1973), were simple, rapid and well-correlated with results using chemical analysis. These methods could be used in the studies of the adsorption and persistence of benomyl/MBC in soil. Both bioassays could determine the total amount of benomyl/MBC to a concentration of 0.5 μg/g. These methods can also be adapted to the studies of adsorption and persistence of other fungicides.

CHAPTER 4. EFFECTS OF A WETTING AGENT ON EFFICACY OF BENOMYL APPLIED TO CREEPING BENTGRASS

4.1 Abstract

Laboratory and field experiments showed that the wetting agent Aqua-Gro (AG) (polyoxyethylene ester and ether of cyclic acid and alkylated phenols) significantly (P=0.05) reduced the adsorption of the fungicide Tersan 1991 by creeping bentgrass thatch. With AG, significantly less fungicide was initially adsorbed and significantly more fungicide was desorbed from the thatch layer after 20 mm of water irrigation. Aqua-Gro applied at 5 L/ha increased movement, uptake, and biological availability of the fungicide and resulted in a higher residual level of fungicide in the grass clippings. For dollar spot disease, Tersan 1991 (50% benomyl) applied at 2 kg/ha with AG gave as good control as the full rate (3 kg/ha) without AG.

4.2 Introduction

The primary functions of a surfactant (wetting agent) added to a pesticide solution are to promote wetting, coverage and uptake of the pesticide by leaves. However, the hydrophilic-lipophilic properties of surfactants may impart additional properties. Gillard (1987) proposed that a surfactant can be used to give better control of the target organism than is possible with the standard formulation alone. This is achieved by altering the physical characteristics, extending the period of activity, and enabling lower doses of pesticides to be used. Rawlins and Booth (1968) showed that the surfactant Tween 20 increases the root uptake of benomyl for the control of Verticillium wilt of cotton.

Nowacka *et al*. (1979) demonstrated that the surfactants Euphytan® and Triton CS® permitted a three-fold reduction of spray concentration of benzimidazole fungicides without lowering the efficacy of those compounds for the control of apple scab. There are no previously published reports on the effects of a wetting agent on fungicide movement in turfgrass.

Turfgrass is a unique cropping system in that it possesses thatch. Thatch is a tightly intermingled layer of dead and living stems and roots that develops between the zone of green vegetation and the soil surface (Beard, 1973). Thatch and sand grains on greens are difficult to wet because the surface of thatch and sand grain particles are water-repellent which can lead to localized dry spots (Miller and Wilkinson, 1977; Taylor and Blake, 1982; Miyamoto, 1985). Wilkinson and Miller (1978) reported that the infiltration rate within the dry spots was 20% of that for normal turf. Pelishek *et al*. (1962) and Miller and Letey (1975) also reported that wetting agents increase infiltration rates into hydrophobic thatch and soil.

Aqua-Gro (AG) is 50% polyoxyethylene ester and 50% polyoxyethylene ether of cyclic acid and alkylated phenols with silicone anti-foam emulsion produced by Aquatrols Corp. of America, Pennsauken, NJ. It is a nonionic surfactant and has been used on turf to increase the water infiltration into poorly wettable thatch and sand (Endo *et al*., 1969; Wilkinson and Miller, 1978). Aqua-Gro has low phytotoxicity and does not reduce root growth and quality of annual bluegrass at application rates of 4.2 or 8.4 L/ha (Cooper *et al*., 1987). It has been shown to be tightly adsorbed to the top 1 cm of soil and has a long-lasting effect (Miller and Letey, 1975).

Tersan® 1991 (containing 50% benomyl) is a xylem-mobile compound (Solel *et al.*, 1973) that has been recommended for turfgrass as a soil drench to control diseases such as dollar spot and brown patch (Anonymous, 1990). The fungicide must move to the root zone for root uptake and foliar translocation to provide systemic protection of the plant. Limited foliar uptake has also been observed (Baude *et al.*, 1973). Surfactants have been shown to improve fungicide mobility (Morrod, 1982; Bateman, 1984) and increase uptake of systemic pesticides by roots in soil (Rawlins and Booth, 1968). In turfgrass, however, little work has been done on the effects of wetting agents on pesticide movement in thatch and uptake by roots.

The objective of this investigation was to examine the effects of AG on the adsorption, movement, uptake of benomyl, and the combined effects of AG and benomyl on dollar spot disease control.

4.3 Materials and methods

4.3.1 Adsorption and desorption of benomyl from thatch in laboratory experiments

Turf used in this experiment was from a 13-year-old sand green seeded with `Penncross' creeping bentgrass (*Agrostis palustris*) at the Cambridge Research Station, University of Guelph, Guelph, Ontario. The soil was Fox sandy loam. The site was mowed daily at 5 mm height (clippings removed) and irrigated as needed. Thatch thickness was not uniform and localized dry spots were found.

Thatch thickness at different locations was initially determined using a soil sampler (2.5 cm in diameter). Three cores were then taken with a putting green cup cutter (10 cm in diameter) from each location. The thatch and soil were separated, and thickness

and weight of the thatch layers were recorded. Thatch plugs were then placed in porcelain funnels (10 cm in diameter), and the wall of each funnel was coated with octadecyl chlorosilane to form a water-proof barrier.

Three rates (0, 2 and 3 kg/ha) of Tersan 1991 in water were mixed with two rates of AG (0 and 5 L/ha) and applied at 1250 L/ha. The suspension was applied to a thatch plug in the funnel with a small (100 mL) pressurized spray pack. To simulate sprinkler irrigation, a volume of water equivalent to 20 mm was applied after fungicide treatment. The volume of water was divided into 3 parts and applied at 30-minute intervals. Leachates from watering at different time intervals were mixed and tested for benomyl and MBC using the paper disc bioassay (Section 2.3.2.1; Liu and Hsiang, in press). The concentration of fungicide in the leachate was used to estimate the amount of fungicide remaining in the thatch. The thatch was then air dried to the same weight as prior to fungicide application and kept in a moist box at 20°C. After 3 days, irrigation and collection of leachate were repeated.

4.3.2 Adsorption and uptake of benomyl in field experiments

On the previously mentioned creeping bentgrass green, the same treatments as in the previous experiment were applied in late July 1992 and 1993 to 1 x 2 m plots of an area with relatively uniform thatch thickness. The plots were laid out in a Randomized Complete Block design. Fungicide was applied to each of four plots using a bicycle-wheel-mounted air pressure sprayer (1 m in width, 140 kPa). Plots were watered (20 mm) immediately after treatment and every three days thereafter, depending on rainfall. Turfgrass clippings were collected at 1, 3, 7, 14 and 21 days after fungicide treatment. At

each sampling time, 10 cores (2.5 cm in diameter) were taken from each plot and the thickness of thatch from each core recorded. Clippings collected from sets of ten cores were mixed together and frozen at -20°C for 24 h, as was the thatch. Four subsamples from each of the clipping and thatch mixtures were then tested for fungicide concentration using the agar-grass and agar-thatch pellet bioassays (Section 2.3.2.2).

4.3.3 Control of dollar spot disease by benomyl

To determine the effects of benomyl as a preventive control of dollar spot disease, each turfgrass plot was inoculated one day after treatment and then weekly for three weeks. Inoculum was prepared by soaking chicken (cereals) scratch in water for 24 h, autoclaving twice in canning jars, and then inoculating with five strains of *Sclerotinia homoeocarpa* separately. After incubating for 3 weeks at 23°C, the chicken scratch was air-dried, ground, mixed and passed through a 1 mm sieve. For a uniform distribution, 20 g of soil was mixed with 2 g of inoculum and applied to each 2 m^2 plot. The development of dollar spot disease was evaluated visually at 1, 7, 14, 21, and 28 days after fungicide treatments. This experiment was done in 1992 and 1993.

4.3.4 Statistical Analysis

The average concentration of benomyl in thatch of each plot was adjusted to account for varying thatch thickness using covariance analysis (Gomez and Gomez, 1984). Thickness of thatch has a significant effect on pesticide adsorption (Niemczyk *et al*., 1988). Analysis of variance (ANOVA) was conducted on fungicide concentration in turfgrass clippings and the log number of dollar spots in each plot. Duncan's Multiple Range test was used to separate the treatment means when the ANOVA *F*-test indicated

that the treatment effect was significant. All tests for significance were performed at P=0.05.

4.4 Results and discussion

4.4.1 Effect of AG on adsorption and movement of benomyl in thatch

The thickness of the thatch layer had a significant effect on fungicide adsorption *in vitro*. A near linear relationship was found between the percentage of fungicide adsorbed and the thickness of thatch (Fig. 4.1). The percent of applied fungicide that was adsorbed increased with thatch thickness. When the thatch layer was 20 mm thick, and in the absence of AG, over 80% of the fungicide was adsorbed; this was approximately 30% more than when AG was used (Fig. 4.1). When the thatch thickness was over 16 mm, the difference in adsorption with and without AG was much greater than the difference in adsorption between the two fungicide rates (2 and 3 kg/ha) (Fig. 4.1).

Fungicide adsorbed by the thatch *in vitro* was desorbed from the thatch layer with irrigation water applied three days after initial treatment (Fig. 4.2). Up to 45% of the fungicide adsorbed in the thatch layer was washed off three days after initial application of fungicide and AG. The amount of fungicide desorbed increased with thatch thickness (Fig 4.2).

When AG was not used, significantly less fungicide (of the amount that was adsorbed) was desorbed from the thatch layer (Fig. 4.2). This could have been due to the hydrophobic nature of the thatch layer which has a low infiltration rate (Miyamoto, 1985). Fungicide and irrigation water tended to stay within the thatch layer and not penetrate into the hydrophobic sandy soil. The adsorbed fungicide could not be washed

Figure 4.1. Effect of thatch thickness on adsorption of benomyl applied with and without Aqua-Gro (AG) *in vitro*. Benomyl levels were detected using paper disc bioassay on leachate immediately after fungicide treatment.

Figure 4.2. Effect of thatch thickness on desorption of benomyl applied with and without Aqua-Gro (AG) *in vitro*. Benomyl levels were detected using paper disc bioassay on leachate form irrigation 3 days after fungicide treatment.

off with irrigation. When AG was used, the thatch layer became less hydrophobic, and more fungicide could be desorbed until the water flow stopped. The fungicide would then move down with the mass flow of the water to the soil layer (Morrod, 1982). Aqua-Gro could form micellae which could transport the benomyl through thatch (D. Moore, Aquatrols Corp. of America, personal communication).

The reason for reduced benomyl adsorption and the subsequent increased desorption by thatch when applied with AG is not certain. Valoras *et al*. (1969) reported that the equilibrium between Pachappa sandy loam soil and AG solution was either complete or nearly complete after one hour. This suggested that AG has a strong tendency to be adsorbed compared to benomyl which has a slower equilibrium time of 4 to 6 hours in Fox sandy loam soil (Section 3.4.2; Aharonson and Kafkafi, 1975). According to Miller and Letey (1975), AG was strongly and irreversibly adsorbed within the top 1 cm of a sandy loam soil. They suggested that AG could give a long wetting effect of the thatch layer and maintain a high infiltration rate in subsequent watering. Similar results have been reported by Miyamoto (1985) and Mane *et al*. (1993). These results suggest that the fungicide may not reach an equilibrium with the thatch because of the fast movement of irrigation water which acts as a carrier for the fungicide.

4.4.2 Effect of AG on uptake of benomyl by turfgrass

Aqua-Gro had a significant effect on the uptake of benomyl by turfgrass *in situ*. One day after treatment with benomyl and AG, the fungicide concentration in turfgrass clippings could be detected by bioassay (Table 4.1). However, the concentration was not detectable at day 1 if no AG was applied. This was likely due to the wetting effect of AG

on plants, since benomyl may be adsorbed or even absorbed by foliage shortly after application (Nowacka *et al*., 1979).

Table 4.1. Concentration of benomyl in turfgrass clippings (µg/g fresh clipping weight) *in vitro*.

Concentration of benomyl (µg/g)[@]					
Tersan 1991	Days after treatment				
Treatment	1	3	7	14	21
2 kg/ha	ND[†]	0.6c[‡]	2.8b	0.4c	ND
3 kg/ha	ND	1.0b	3.5a	0.9b	ND
2 kg/ha+AG[¶]	0.6a	1.2b	2.6b	1.1b	ND
3 kg/ha+AG	0.9a	1.7a	3.3a	1.8a	0.7

[@] Concentrations are expressed in benomyl, but MBC was actually detected here.
[†] ND - not detectable.
[‡] Means followed by the same letter in a column are not significantly different from each other at P=0.05. Each mean is derived from 4 replicates.
[¶] AG is Aqua-Gro applied at 5 L/ha.

If no AG was used, the fungicide in the leaf blades was not detected until 3 days after treatment. This could be due to the slow uptake by roots, stems or stolons of the grass. After this initial period, however, the rate of uptake was very fast, and at the same application rates there were no significant differences (P=0.05) between the amount of fungicide in clippings at seven days after treatment with fungicide alone or those treated with fungicide and AG (Table 4.1). Figures 4.1 and 4.2 indicate that, if AG was not used, more fungicide was adsorbed by the thatch layer and less was desorbed and leached down to the soil layer. This meant that the concentration of fungicide in thatch was higher in plots treated with fungicide alone. This is in agreement with the results of bioassay on

thatch where higher levels of benomyl were found for treatments without AG (Table 4.2). Therefore, it is likely that the fungicide taken up by grass treated with fungicide alone was mainly from the reservoir that stayed in the hydrophobic thatch layer.

Table 4.2. Concentration of benomyl in thatch (μg/g dry thatch weight) *in vitro* at different time after treatment.

Concentration of benomyl (μg/g)[@]				
Tersan 1991	Days after treatment			
Treatment	1	3	7	14
2 kg/ha	17.2b[†]	14.4b	9.5b	4.1a
3 kg/ha	25.6a	21.0a	12.7a	4.6a
2 kg/ha+AG[‡]	6.2d	4.5d	2.8c	0.8b
3 kg/ha+AG	9.9c	6.5c	3.2c	0.8b

[@] Concentrations are expressed in benomyl, but MBC was actually detected here.
[†] Means followed by the same letter in a column are not significantly different from each other at P=0.05. Each mean is derived from 4 replicates.
[‡] AG is Aqua-Gro applied at 5 L/ha.

When AG was used, significantly higher concentrations of fungicide were found in the grass 14 days after treatment (Fig. 4.1). Less adsorption, more desorption and improved movement of fungicide into soil (Bateman, 1984), as has also been shown for herbicides (Bayer, 1967), could increase the fungicide level in soil beneath the thatch where the actively growing roots are located.

Microbial activity is one of the main factors for the degradation of benomyl (Yarden *et al*., 1987). The rate of microbial degradation of benomyl and other pesticides in soil is much lower than that in thatch (Section 3.4.1; Branham *et al*., 1993), which is

likely due to the higher populations of microorganisms in thatch (Mancino *et al*., 1993). These results suggested that fungicide levels in soil last longer compared to thatch. This may contribute to the higher concentrations of fungicide in grass clippings at 14 days after treatment with fungicide plus AG because of translocation. After 14 days, the concentration of fungicide in grass decreased quickly in all treatments. At the lower fungicide application rate (2 kg/ha), the concentration could not be detected (< 0.2 ppm) by bioassay 21 days after treatment with or without AG. Only for the treatment with 3 kg/ha of Tersan 1991 plus AG, was benomyl detected at 21 days (Table 4.1).

4.4.3 Effect of AG on control of dollar spot disease by benomyl

The development of dollar spot disease was reduced by treatment with Tersan 1991 and AG (Table 4.3). Dollar spot patches were seen on day 14 after treatment with

Table 4.3. Number of dollar spot patches at different time after treatment on 2 m^2 plots.

	Dollar spot patches per plot				
	Days after treatment				
Tersan 1991 Treatment	1	7	14	21	28
2 kg/ha	0	0	2b[†]	18c	41b
3 kg/ha	0	0	0c	3d	19c
2 kg/ha+AG[‡]	0	0	0c	2d	17c
3 kg/ha+AG	0	0	0c	0d	8d
AG	0	4	13a	42b	117a
Control	0	2	16a	63a	122a

[†] Means followed by the same letter in a column are not significantly different from each other at *P*=0.05. Each mean is derived from 4 replicates.
[‡] AG is Aqua-Gro applied at 5 L/ha.

Tersan 1991 at 2 kg/ha without AG. With AG, dollar spot patches were not seen until day 21 after treatment, and the rate of development was slower compared with fungicide alone. Because more fungicide was taken up by turfgrass in the first week after treatment (Table 4.1), and because of faster microbial degradation of benomyl in thatch and mowing of turfgrass (Section 3.4.1; Branham *et al*., 1993)), there was likely not enough fungicide in thatch and soil to be taken up and to maintain a threshold of fungicide in the turfgrass two weeks after treatment with fungicide alone.

Aqua-Gro was found to extend the residual efficacy of benomyl (Table 4.3). Turfgrass treated with 3 kg/ha Tersan 1991 plus AG did not show dollar spot patches on day 21 after treatment. At 3 kg/ha Tersan 1991 without AG, dollar spot patches were not seen until day 21, and patch development was not significantly different from that at 2 kg/ha Tersan 1991 plus AG.

4.5 Conclusions

The wetting agent Aqua-Gro significantly reduced the adsorption of the fungicide Tersan 1991 by creeping bentgrass thatch. When Aqua-Gro was applied with the fungicide, significantly less fungicide was initially adsorbed and significantly more fungicide was leached through the thatch layer after 20 mm of water irrigation. Aqua-Gro increased movement, uptake, and biological availability of the fungicide and resulted in a higher residual level of fungicide in the grass clippings. The results suggest that when Tersan 1991 is applied at two-week intervals, fungicide application can be reduced by approximately 30 percent of the recommended rate if applied with AG and irrigated immediately after.

CHAPTER 5. EFFECTS OF CORE CULTIVATION ON EFFICACY OF BENOMYL APPLIED TO CREEPING BENTGRASS

5.1 Abstract

Prior to benomyl application, coring was conducted in a field trial to study the effect of coring and time of coring on the movement and uptake of benomyl and on control of dollar spot disease. Core cultivation 14 days before benomyl treatment (DBBT) did not have a significant effect on the uptake of benomyl during 14 days after fungicide treatment. Core cultivation 7 or 1 DBBT gave better uptake and long-lasting residue levels in turfgrass clippings. This also resulted in better control of dollar spot disease. Core cultivation 1 DBBT gave the best results both in long-lasting uptake of benomyl and control of dollar spot disease. Fungicide levels in turfgrass clippings, thatch and soil close to the coring holes were significantly higher in most cases than those farther away from the coring holes.

5.2 Introduction

Pesticides are often applied as soil drenches for the control of harmful organisms in soil or foliage of plants. This is usually achieved by applying a large volume of water to act as a carrier for the active ingredient. The biological efficacy of a pesticide depends critically on the distribution of the pesticide in the soil and the rooting habit of the plant (Morrod, 1982). Because infiltration is usually low on hydrophobic thatch and sand (Taylor and Blake, 1982; Miyamoto, 1985) and because thatch adsorbs large amounts of applied pesticides (Niemczyk and Filary, 1988; Niemczyk *et al*., 1988), the movement of

pesticides through thatch to soil may be limited. Niemczyk and Filary (1988) applied nine insecticides to turfgrass and found that despite 1.25 cm irrigation immediately after treatment, 96-99% of the pesticide residues remained in the thatch during the seven days following application. Other studies have been conducted to increase the efficacy of pesticides in turf by applying wetting agents (Miyamoto, 1985) or subsurface application of pesticides (Niemczyk, 1993).

Coring involves the use of a tine or spoon to displace or remove soil and thatch which then leaves a hole or cavity in the sod. The depth of penetration is usually 7 to 10 cm with the cores spaced on 5- to 15-cm centres (Beard, 1973). Core cultivation is commonly used on turfgrass sites during the growing season especially spring or fall to increase water infiltration, to stimulate root and shoot growth, to alleviate compaction and to control thatch (Turgeon, 1985).

Byren *et al*. (1965) and Canaway *et al*. (1986) found that coring increased the infiltration in compacted putting greens. They suggested that surface compaction, hydrophobic thatch, and surface layering can severely restrict infiltration. If the coring hole traverses these restrictions, substantial increases in infiltration capacity are possible in direct response to coring. Carrow (1988) reported that hollow tine coring was one of the most effective cultivation methods for reducing penetrometer resistance. The increased infiltration capacity of cored turf is due primarily to the increased surface area resulting from coring. According to Turgeon (1985), the increase in surface area can be more than doubled when the combined areas of the walls of the holes are added to that of the turf surface.

Coring can result in an improvement of growing conditions in the immediate vicinity of the holes as long as moisture is not limiting (Turgeon, 1985). Barber and Carrow (1985) showed improved oxygen diffusion rates from hollow tine coring. Root growth in the 20 to 60 mm zone increased by up to 35% and water extraction from this zone also increased (Wiecko *et al*., 1993).

There has been limited investigation into the effects of core cultivation on the movement and uptake of pesticides by turfgrass. The increased infiltration and increased root growth of turfgrass may increase the efficacy of a pesticide. Therefore, the objective of this study was to use benomyl as a model to examine the effect of core cultivation on the movement in thatch/soil and uptake of pesticide by turfgrass.

5.3 Materials and methods

5.3.1 Effect of core cultivation on uptake of benomyl by turfgrass

Turf used in this experiment was from a 13-year-old sand green seeded with `Penncross' creeping bentgrass (*Agrostis palustris*) at the Cambridge Research Station, University of Guelph, Guelph, Ontario. The soil is Fox sandy loam (76.9% sand; 17.0% silt; 6.1% clay; 1.7% organic matter; pH 5.2; CEC 12.5 c/kg). The site was mowed daily at 5 mm mowing height and irrigated as needed.

Each turf plot (1 x 2 m) was cored once with a hollow tine coring machine (Ryan Greensaire, 12.5 mm diam, 7 cm depth, and 7 cm apart) at specified times in mid to late August before fungicide application. The soil cores were removed from the plots. Fungicide Tersan 1991 (50% a.i. benomyl) at a rate of 3 kg/ha was applied in water at 1250 L/ha. The fungicide was applied with a bicycle-wheel-mounted air pressure sprayer

to plots that were either not cored, or cored 1 day, 7 days, or 14 days before benomyl treatment (DBBT). The plots were watered (20 mm) immediately after fungicide treatment. There were four replicates for each coring treatment. The experiment was done in 1992 and 1993.

One day after fungicide treatment, samples of turfgrass clippings 0-10, 11-20, and 21-30 mm away from the coring holes were collected and stored at -20°C. Samples of the top 10 mm thatch and top 10 mm soil were also collected and stored at -20°C. The thatch and soil were air-dried before testing both benomyl and methyl 2-benzimidazole carbamate (MBC), which is the principal fungitoxic degradation compound of benomyl, using sample-agar pellet bioassay (Section 2.3.2.2). Samples of turfgrass clipping mixtures from each plot were also collected at 1, 3, 7, and 14 days after fungicide treatment and frozen at -20°C before analysis using the bioassay.

5.3.2 Effect of core cultivation on control of dollar spot disease by benomyl

To determine the effects of coring on the efficacy of benomyl for control of dollar spot disease, each plot was inoculated with inoculum of *Sclerotinia homoeocarpa* one day after fungicide treatment and then weekly for three weeks after treatment. The inoculum was prepared by soaking chicken scratch in water for 24 h, autoclaving twice in canning jars, and then inoculating with five strains of *S. homeocarpa* separately. After incubating for 3 weeks at 23°C, the chicken scratch was air-dried, ground, mixed and passed through a 1 mm sieve. For a uniform distribution, 20 g of soil was mixed with 2 g of inoculum before inoculation and applied to each plot. The development of dollar spot disease was evaluated visually at 1, 7, 14, 21, and 28 days after fungicide treatment.

5.3.3 Statistical analysis

Analysis of variance (ANOVA) was conducted on fungicide concentration in turfgrass clippings, thatch and soil, and on the log number of dollar spots in each plot. Duncan's Multiple Range test was used to separate the treatment means when the ANOVA *F*-test indicated that the treatment effect was significant. All tests for significance were performed at P=0.05.

5.4 Results and discussion

5.4.1 Benomyl residues in the mixture of turfgrass clippings of each plot

5.4.1.1 Coring 14 days before benomyl treatment

Coring had a significant effect on the uptake of benomyl by turfgrass. Among all coring treatments, plots cored 14 DBBT gave the highest benomyl concentration in turfgrass clippings 1 and 3 days after the fungicide treatment (Fig. 5.1) and the concentration was significantly higher than that cored one DBBT and non-cored control. However, for the same treatment, the concentration of benomyl in clippings was not significantly different from non-cored control 7 and 14 days after the fungicide treatment. This could be due to the short-lived effect of coring. Soil and roots may have filled the holes and reduced the water infiltration and fungicide movement into the soil (Turgeon, 1985). Root growth is improved by coring (Murphy and Rieke, 1987), therefore the high fungicide concentration in turfgrass 1 and 3 days after the fungicide treatment was most likely due to the increased uptake by new roots produced within the coring holes. However, the amount of fungicide entering the holes was limited due to the filling of the holes and did not provide long-lasting effects. Benomyl/MBC could not be detected by

Figure 5.1. Effect of time of coring (days before benomyl treatment DBBT) on levels of benomyl in turfgrass clippings tested at different time after treatment with Tersan 1991 (3 kg/ha).

bioassay at 21 days after benomyl application for plots 14 DBBT or non-cored control.

Rapid uptake by turfgrass after benomyl application could be beneficial for disease control. However, if a pesticide is not transported into soil through the coring holes, then most of it would be adsorbed by the thatch layer (Niemczyk and Filary, 1988). Thus, the uptake of the fungicide by actively growing roots in the soil beneath the thatch would be limited.

5.4.1.2 Coring 7 or 1 day before benomyl treatment

Coring 7 DBBT gave significantly the highest level of fungicide in turfgrass clippings at 7 days after the treatment (Fig. 5.1). By 14 days after fungicide treatment, the fungicide level decreased sharply and there was no significant difference between coring treatment of 1 and 7 DBBT. On day 21 after fungicide treatment, the fungicide level was still detectable by the bioassay, but it was significantly lower than coring 1 DBBT.

The results over the whole experimental period showed that coring 7 DBBT gave the highest peak concentration, but coring 1 DBBT gave the longest lasting detectable levels of benomyl. The most recent cores would have the largest surface area and could allow greater inflow of fungicide with the subsequent watering (20 mm). The one-day-old holes were almost the entire 7 cm depth which would allow fungicide to flow directly into the soil. The degradation of fungicide in soil is much slower than in the thatch (Section 3.3.2.1; Liu and Hsiang, in press). Therefore, a greater amount of fungicide would reach the rhizosphere and the fungicide could persist longer in the turfgrass as it is translocated to the grass tips (Peterson and Edgington, 1971). Unlike other crops, turfgrass, especially creeping bentgrass, is frequently mowed and fungicide in leaf blades is easily removed

with the clippings. A high and long-lasting uptake of fungicide is essential for an effective control of foliar diseases.

5.4.2 Benomyl residues in turfgrass clippings, thatch and soil at various distances from coring holes

5.4.2.1 Benomyl residues in turfgrass clippings

One day after treatment with Tersan 1991, the clippings collected within 10 mm around cores from plots cored 14 DBBT had significantly higher levels of benomyl than those of plots cored 7 or 1 DBBT (Table 5.1). This was similar to the result when all clipping mixtures from each plot were tested 1 and 3 days after fungicide treatment (Section 5.4.1; Fig. 5.1). The higher level of benomyl is likely due to the increased root growth or root hair activity from core cultivation (Wiecko *et al*., 1993) and the fungicide reaching to the roots through the coring holes.

Benomyl level in clippings from plots of all coring treatments decreased as the distance increased away from the cores (Table 5.1). Between plots cored 14 and 7 DBBT, no significant differences were found in clippings collected from 11-20 and 21-30 mm away from the cores. Benomyl in clippings from plots cored 1 DBBT and collected at 11-20 and 21-30 mm away from the cores, was not detectable by bioassay. Because of good aeration porosity from coring, the mass of newly-grown roots near cores cultivated 14 DBBT was likely higher than that of 7 and 1 DBBT (Agnew and Christians, 1993; Wiecko *et al*., 1993). This may have resulted in significantly higher uptake of fungicide as shown 1 and 3 days after fungicide treatment (Fig. 5.1). Further away from

Table 5.1. Benomyl in turfgrass clippings (fresh), thatch (10 mm deep, air-dried) and soil (10 mm deep, air-dried) at different distances from coring holes 1 day after the benomyl treatment.

cores, no significant difference was found. This may be explained by the fact that root growth within and on the top of the holes was promoted the most from core cultivation (Murphy and Rieke, 1987).

For plots cored 1 DBBT, major root growth was not likely to have occurred within two days. Therefore, only turfgrass clippings closely surrounding the core holes (0-10 mm) had a detectable level of benomyl one day after fungicide treatment (Table 5.1). However, for this coring treatment, the fungicide level in clipping mixtures increased very quickly in the first week and maintained the highest level when tested on days 14 and 21 after fungicide application (Fig. 5.1). This again suggested that increased root growth and the diffusion of benomyl from coring holes and thatch to roots away from the holes play important roles in the uptake of the fungicide.

5.4.2.2 Benomyl residues in thatch

The level of benomyl in thatch was different from that in grass clippings (Table 5.1). For all coring treatments, fungicide levels decreased with increasing of distance away from cores. This again suggested that coring improved the fungicide movement especially around the coring holes. Within 10 mm of the cores, benomyl concentrations in thatch cored in 3 time periods were significantly different from each other. This was likely related to the initial inflow of the fungicide into coring holes via water, which acted as carrier of the fungicide (Morrod, 1982). The highest value was found from thatch cored 1 DBBT followed by 7 and 14 DBBT.

For thatch 11-20 and 21-30 mm away from the cores, the fungicide levels were not significantly different from each other among three coring treatment intervals. The

higher levels of fungicide in thatch for all coring treatments also confirmed the report that a large proportion of the applied pesticide stays in the thatch layer (Niemczyk and Filary, 1988; Niemczyk *et al*., 1988).

5.4.2.3 Benomyl residues in soil

At various distances from the coring holes, soil cored 1 DBBT had significantly higher concentration of benomyl than that cored 7 or 14 DBBT. Benomyl level, however, decreased with increasing distance from coring holes. Core cultivation increased water infiltration capacity (Byrne *et al*., 1965) and thus the mass flow of water from irrigation could take the fungicide through the holes down to the soil layer where most actively growing roots are located. This would increase uptake by plant compared with non-cored plots as shown in Fig. 5.1. Compared to soil cored 7 and 14 DBBT, relatively higher fungicide levels in soil 11-20 and 21-30 mm away from the cores cultivated 1 DBBT were likely due mainly to the diffusion of the fungicide from soil near the coring holes rather than movement through thatch layer.

5.4.3 Effect of coring on control of dollar spot disease by benomyl

For dollar spot disease control, the results were in a good agreement with the level of benomyl in turfgrass clippings. All plots treated with Tersan 1991 had significantly fewer dollar spot patches than non-treated control (Table 5.2). However, except at 14 days after treatment, disease severity on plots cored 14 DBBT was not significantly different from that of non-cored plots. Again this may have been due to the in-growth of cores cultivated 14 DBBT, which resulted in lower levels of benomyl in clippings. Plots cored 1 or 7 DBBT showed less disease, and dollar spot patches were not seen until day 21 for

both treatments. Coring 1 DBBT with fungicide gave the best disease control. This was likely due to the relatively higher level of benomyl residues in turfgrass clippings at 14 and 21 days after fungicide treatment (Fig. 5.1).

Table 5.2. Development of dollar spot patches after fungicide treatment on 2 m^2 plots.

Tersan 1991 (3 kg/ha)	Number of dollar spot patches per plot Days after fungicide treatment 1	7	14	21	28
Untreated	0	3a[†]	19a	51a	103a
Not cored	0	0b	7b	21b	48b
Cored 14 days	0	0b	2c	19b	43b
Cored 7 days	0	0b	0d	8c	18c
Cored 1 day	0	0b	0d	5c	9d

[†] Numbers with the same letter in a column are not significantly different from each other at P=0.05. Each number is the mean of four replicates.

5.5 Conclusions

Core cultivation increased the efficacy of benomyl applied to creeping bentgrass. Time of coring had significant effects on the efficacy of benomyl. Coring 1 day and 7 days before benomyl treatment gave significantly higher and longer-lasting benomyl residues in turfgrass clippings one week after fungicide treatment than non-cored control and coring 14 days before fungicide treatment. Increased infiltration and root growth from core cultivation could contribute to increased efficacy of benomyl. The number of dollar spot patches was related to fungicide levels in the turfgrass clippings. Fungicide application 1 day after core cultivation gave the best control of dollar spot. In general,

fungicide levels in turfgrass clippings, thatch and soil close to the coring holes were higher than those away from the coring holes.

CHAPTER 6. GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

6.1 Bioassays

Under many situations, turfgrass, especially that found on golf courses, needs intensive management. Turfgrass is different from other field crops because it is usually a perennial cover and cannot be rotated, and thus pesticide use rather than soil cultivation is the major tool to control pests. Turfgrass is also characterized by the development of a thatch layer (Beard, 1982), which may adsorb most of the pesticides applied, thus making them biologically unavailable (Niemczyk *et al*., 1988; Niemczyk, 1993).

Efficacy of a pesticide depends on its biological availability. Compared to chemical analysis and other techniques, bioassays may be the best and most reliable method for detection and determination of the biologically available pesticide residues. In this study, different bioassays were developed and evaluated in studies of benomyl in turfgrass clippings, thatch and soil. These bioassays included paper disc bioassay, soil-agar pellet bioassay, turfgrass-agar pellet bioassay, thatch-agar pellet bioassay, and sample-agar mixture bioassay.

Paper disc bioassays for pesticides have previously been used (Thornberry, 1950; Edgington *et al*., 1973) and are relatively easy to conduct. In this study, paper disc bioassay give very low standard errors. Compared to the other bioassays, it was best used to detect MBC in liquid solutions such as leachates. For the detection of fungicide in turfgrass, thatch or soil by conventional chemical analysis or paper disc bioassay,

extraction is necessary. However, the fungicide extracted from substrates using organic solvents, as is done in most chemical analyses, may not represent the fungicide in its *in situ* state, or the actual biological availability to target or non-target organisms. Thus other bioassays were developed to test solid substrates.

Soil-agar pellet bioassay was used to detect benomyl or MBC in soil without extraction, and has been previously used by Munnecke (1958) and Yarden *et al*. (1985). The method was modified and used here to detect the amount of benomyl or MBC adsorbed by soil. Soil-agar pellet bioassay can be used to detect fungicide residues directly from soil without extraction. The process of fungicide diffusion from the pellets to the PDA is perhaps similar to the process *in situ* of diffusion from soil or thatch to the nearby roots of turfgrass. Therefore the amount of fungicide detected by soil-agar pellet bioassay may model the exposure of roots to biologically available fungicide in soil.

Benomyl or MBC in turfgrass or thatch were detected by thatch-agar or turfgrass-agar pellet bioassays respectively. There have been no previous reports using these types of bioassays for detection of fungicides. Residues which were tightly adsorbed or bound to thatch or turfgrass tissues did not diffuse out of the pellets and thus were biologically unavailable in the sense of water soluble deposits.

Because the *Penicillium expansum*, the assay fungus, was equally susceptible to benomyl and its fungitoxic residue MBC, it could be used to detect both benomyl and MBC in samples at the same time. Although methods of chemical analysis have been developed for the simultaneous detection of benomyl and MBC (Chiba, 1977; Chiba and Singh, 1986), the complicated extraction, clean-up, detection processes and required

equipment limit their use. Bioassays, on the other hand, were easy to operate and did not need expensive equipment. Bioassays developed here can have a limit of detection of 0.2 ppm and a limit of quantitation of 0.5 ppm for MBC. The results from our bioassays were not significantly different from those of UV-spectrophotometry.

The disadvantages of the fungicide bioassay were that it is not chemical specific and may be affected by various chemical and physical factors. Media quantity and incubation temperature had the most significant effect on the results of the bioassays. Herbicide 2,4-dichlorophenoxy acetic acid (2,4-D) up to 2000 ppm or in a mixture with Tersan 1991 (benomyl) did not have significant effects on the results of paper disc bioassay. Daconil 2787 (chlorothalonil) is commonly mixed with Tersan 1991 when fungicide mixtures are applied to turfgrass (Anonymous, 1990). When the concentration of chlorothalonil in water suspension was less than 31.3 μg/mL, no significant effect on bioassay fungus was found. When chlorothalonil at over 12.5 μg/mL was mixed with benomyl, chlorothalonil enhanced the toxicity of benomyl and gave a larger zone of inhibition than benomyl alone (Section A.1.4.8 and A.1.4.9).

6.2 Degradation, adsorption and biological availability of benomyl

MBC, when applied as benomyl *in vitro*, has a half life of approximately 2 and a half weeks in thatch and 4 weeks in soil depending on soil type. Chemical degradation did not play an important role in the dissipation of benomyl in soil. Less than 10% of the original applied fungicide was chemically degraded from soil and thatch *in vitro* within 7 weeks.

Thatch and soil differed in adsorption of MBC. As much as twice the fungicide

was adsorbed by thatch compared to Fox sandy loam. Adsorption of MBC by soil depends on soil type. The distribution coefficient of MBC for thatch (in water) was significantly higher than that for Guelph loam, which was then significantly higher than that of Fox sandy loam. The equilibrium for fungicide adsorption between water and thatch or soil was reached within 1 h for thatch and 2 to 4 h for two soils. The amount of fungicide adsorbed by thatch or soil was linearly related to the fungicide level in thatch or soil. Within a certain range, the more fungicide applied, the more adsorbed. Approximately 90% of the applied fungicide (10 μg/g) could not be extracted with water from thatch compared to 70 to 90% of that in Guelph loam respectively depending on the amount applied. Methanol extracted two or three times MBC from thatch or soil respectively than did water.

6.3 Effects of Aqua-Gro and coring on efficacy of benomyl

Although most of the applied benomyl was adsorbed by the thatch layer, the efficacy of the fungicide was improved by using a wetting agent such as Aqua-Gro or through core cultivation before fungicide treatment.

The wetting agent Aqua-Gro significantly reduced the adsorption of the fungicide Tersan 1991 by creeping bentgrass thatch. With AG (at 5 L/ha), significantly less fungicide was initially adsorbed and more fungicide was leached through the thatch layer. Aqua-Gro increased movement and uptake of the fungicide and resulted in a higher residue level of fungicide in the grass clippings. For dollar spot disease, Tersan 1991 applied at 2 kg/ha with AG gave as good control as the full rate (3 kg/ha) without AG.

Because of possible increased root growth and water infiltration from core

cultivation, better uptake and longer-lasting residue levels were found in turfgrass clippings with core cultivation 7 or 1 day before fungicide treatment. This also resulted in a better control of dollar spot disease. Coring 14 days before fungicide treatment did not have a significant effect on the levels of benomyl 7 and 14 days after fungicide treatment. Core cultivation one day before fungicide treatment gave the best results both in long-lasting uptake and control of dollar spot disease.

6.4 Recommendations for future research

Bioassays developed here are not specific for benomyl detection when mixtures of benomyl and other fungicides were applied. Therefore, it may be beneficial to develop bioassays specifically for benomyl detection when a fungicide mixture is used, and bioassays for other fungicides.

Since both wetting agents and core cultivation can increase water infiltration, treatment of coring plus wetting agent may give better efficacy than each of them applied separately. Application of Aqua-gro several times before the fungicide treatment may also increase the efficacy of benomyl. Since soil drench of pesticides depends very much on the volume of water applied after pesticide treatment (Morrod, 1982), the effect of increasing water volume should be examined.

Papers on the effects of wetting agents or core cultivation on pesticide efficacy are limited; therefore, studies of these effects on other pesticides are recommended.

Turfgrass on greens is usually mowed daily with clippings removed. The clippings can contain relatively high levels of fungitoxic residues due to the accumulation of fungicide at tips of turfgrass leaves. It could be beneficial both environmentally and

economically to develop a method to return clippings containing fungicide to the turfgrass.

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APPENDIX 1. FACTORS AFFECTING THE RESULTS OF FUNGICIDE AGAR DIFFUSION BIOASSAYS

A.1.1 Abstract

Many factors can affect the results of a fungicide bioassay where fungi are used as test organisms. The factors studied have included concentration of spores of the test fungus in medium, incubation time and temperature, medium quantity in Petri dishes, wetting agent, organic solvent in media, herbicide 2,4-D, and fungicide mixture of Tersan 1991 and Daconil 2787. These factors showed effects on fungal growth inhibition to various degrees. The incubation temperature and quantity of medium were found to have the greatest effect on diameter of zone of inhibition of *Penicillium expansum*. Benomyl and its fungitoxic breakdown product MBC were tested for their toxicity to *P. expansum*, and were found equally toxic to this fungus on a molar basis.

A.1.2 Introduction

Bioassays are simple and inexpensive. They can detect biologically available fungicide using target or non-target organisms sensitive to the fungicide (Bennett and de Beer, 1984). Different bioassay techniques have been previously developed and widely used to detect residues of pesticides in plant or soil (Peterson and Edgington, 1969; Erwin *et al*., 1971; Edgington *et al*., 1973; Yarden *et al*., 1985a). These methods were developed based on the diffusion of fungicide from the disc or pellets to the agar medium inoculated with spores of the test fungus. The fungicide residue level was determined from the resulting inhibition zone of the growth of the fungus. Standard dosage-response curves

derived from known quantities of fungicide may then be used to estimate the quantity of the fungicide of unknown samples.

One of disadvantages of a bioassay method is that it is affected by many physical and chemical factors. The growth of a bioassay agent, usually a fungus, is affected by factors such as temperature, moisture and the length of incubation. Other factors include concentration of fungal spores in media, quantity of media in Petri dishes, concentration of organic solvents in media, herbicide or fungicide other than the one studied. The objective of this study was to evaluate the effect of some major physical and chemical factors on fungicide diffusion in agar and to determine the most appropriate conditions for a fungicide bioassay.

A.1.3 Materials and methods

Tersan 1991 and Technical grade methyl 2-benzimidazole carbamate (MBC) was provided by Du Pont Canada. Pesticide grade methanol, bioassay paper discs (12.7 mm in diameter), and potato dextrose agar (PDA) were purchased from Fisher Scientific.

Penicillium expansum (from Dr. G. Barron, Department of Environmental Biology, University of Guelph) was maintained on PDA and used as a test fungus for most bioassays. *Sclerotinia homoeocarpa* was used as another test fungus.

A.1.3.1 Procedures used for paper disc bioassay

The chemical and physical factors in our procedure were chosen after considering previous publications (e.g. Yarden *et al*., 1985a; Edgington *et al*., 1973).

Standard concentrations of MBC were prepared in methanol or distilled water. A spore suspension (1 mL, 2 x 10^6 conidia/mL) of *P. expansum* was mixed with sterilized

warm PDA (100 mL, 45°C) to give a medium containing 2 x 10^4 conidia/mL. Ten milliliters of this PDA was poured into a Petri dish (10 mm diam) which was then swirled to thoroughly distribute the media. The procedure did not have to be conducted under sterile conditions because of the rapid growth of *P. expansum*.

After the PDA solidified, it was sliced with a special cutter, and two separate agar strips (15.5 mm in width) were left in each dish. A paper disc was placed in the middle of each strip followed by adding 0.07 mL of MBC standard solution in methanol. Eight plates were used for each concentration of the fungicide.

Plates were incubated in the dark at 4°C for 24 h. This step allowed the fungicide to diffuse from the paper disc into the medium while fungal growth was inhibited by the low temperature. The plates were then incubated in the dark for 24 h at 23°C. The diameter of the zone of inhibition (DZI), which is the diameter of fungal mycelial growth less the diameter of the paper disc, was measured and the average was used to estimate the inhibitory effect of the fungicide. Standard curves were established by regressing the DZI against the log concentration of the fungicide.

A.1.3.2 Effect of physical and chemical factors on the DZI

A.1.3.2.1 Effect of conidia concentration in medium on DZI

A conidial suspension of *P. expansum* was prepared in sterilized distilled water. Then a small amount this concentrated spore suspension was added to warm PDA (45°C) for a dilution of 2 x 10^6 conidia/mL which was determined with a haemocytometer. More PDA was added so that the following concentration series of conidia in PDA were prepared: 2 x 10^2, 2 x 10^3, 2 x 10^4, and 2 x 10^5 conidia/mL. Paper disc bioassay was

performed (Section 2.3.2.1). After incubation, DZI was measured and used for comparison.

A.1.3.2.2 Effect of incubation time and temperature on DZI

Following the procedure for paper disc bioassay (Section 2.3.2.1), plates were prepared. MBC-methanol solution with a concentration of 6 μg/mL MBC was transferred to paper discs. Plates were incubated at 4°C for 0, 24 or 48 h followed by incubating at 23°C for 24 or 48 h. The DZI was measured and used for comparison. Four plates were used as replicates in each combination of temperature and incubation time.

A.1.3.2.3 Effect of medium quantity in Petri dishes on DZI

Plates with 5, 10, 15 or 20 mL of PDA were prepared for paper disc bioassay (Section 2.3.2.1). MBC in methanol (0.07 mL, 6 μg/mL) was transferred to each paper disc. The plates were incubated following the bioassay procedure. The DZI was measured and was used for comparison.

A.1.3.2.4 Effect of a wetting agent (Aqua-Gro) on DZI

Standard concentrations of MBC (1, 2, 4, 6, 8 and 10 μg/mL) were prepared in water. To each MBC solution, Aqua-Gro, a wetting agent, was added to make the following concentrations: 0, 0.01, 0.1 and 1 % (v/v). Plates were prepared and incubated for paper disc bioassay (Section 2.3.2.1). The DZI was measured and used for comparison.

A.1.3.2.5 Effect of methanol on *S. homoeocarpa*

A solution of MBC (5 ppm) was made in water. One milliliter of this solution was mixed with 99 mL of warm PDA (50°C) to give 0.05 ppm MBC. Methanol was then

added to the PDA to make the following concentrations of methanol: 0 (control), 0.25, 0.5, 1, 1.5 and 2% (v/v). Ten milliliters of PDA were poured into each Petri dish. After the PDA had solidified, mycelial plugs (4 mm in diameter) of *S. homoeocarpa* were taken from the growing edge of 7-day-old cultures and transferred onto PDA in the Petri dish and the dishes incubated at 23°C in dark for 4 days. The colony diameter was measured from the edge of the mycelial plug to the edge of the mycelial growth. Eight plates were made for each concentration of methanol in medium and the mean was plotted against the concentration of methanol in PDA.

A.1.3.2.6 Effect of storage time of *P. expansum* spore suspension on DZI

Conidial suspensions of *P. expansum* (2 x 10^6 conidia/mL) were kept in dark at 4°C. At 0, 1, 2 and 4 months, aliquots of the suspension were mixed with PDA (45°C) and poured into plates. Standard concentrations of methanol were tested by paper disc bioassay (Section 2.3.2.1). Diameter of zone of inhibition was measured used for comparison.

A.1.3.3 Toxicity of MBC and benomyl to *P. expansum*

Standard concentrations of MBC and benomyl (0.25 to 10 μg/mL) were prepared in methanol and tested immediately by paper disc bioassay (Section 2.3.2.1). The concentration of benomyl was converted to an MBC equivalent by multiplying by 0.659 (0.659 is the ratio of molecular weight of MBC to molecular weight of benomyl). Diameter of zone of inhibition was measured and the mean of four replicates was used to plot against concentration of MBC. The regression lines for both MBC and benomyl were established in the same plot. Slopes and intercepts of the two lines were tested.

A.1.3.4 Effect of herbicide 2,4-D on the results of bioassay

Herbicide 2,4-D amine was mixed with water to make the following concentrations: 0, 5, 10, 50, 100, 1000 and 2000 μg/mL. The solution with each concentration of 2,4-D was divided into 3 parts. The first part contained 2,4-D only. In the second and the third parts, MBC suspension were added to make the solutions with 1 μg/mL and 8 μg/mL of MBC respectively. All three parts were tested with paper disc bioassay. Four samples from each concentration were tested. Diameter of zone of inhibition was measured and used for comparison.

A.1.3.5 Effect of Daconil 2787 on the results of bioassay

When Tersan 1991, a systemic fungicide, is mixed with a protective fungicide, Daconil 2787 is commonly used (Anonymous, 1990). According to the recommended ratio and rate of application (Anonymous, 1990), the following solutions with different concentrations of Daconil 2787 and Tersan 1991 were made in water: group 1 contained the following concentrations of Daconil 2787 only: 0.87, 1.57, 3.13, 6.26, 12.5, 18.8, 25, and 31.3 μg/mL; group 2 contained the following concentrations of Tersan 1991 only: 0.25, 0.5, 1, 2, 4, 6, 8, and 10 μg/mL; and group 3 contained both Daconil 2787 and Tersan 1991 in the same concentrations as above respectively.

The three groups of fungicide solutions were tested by paper disc bioassay. Diameter of zone of inhibition was measured and the mean of four replicates was used for plotting against concentrations.

A.1.4 Results and discussion

A.1.4.1 Effect of conidia concentration on DZI

The maximum DZI was obtained when the concentration of *P. expansum* was 2 x 10^4 conidia/mL in medium (Table A.1). At lower concentrations of conidia, there were not enough colonies to give distinct clear zone in the medium and the standard error was high. At higher concentrations of conidia, the fungus may adsorb or degrade part of the fungicide and give a smaller DZI. Therefore the spore concentration of 2 x 10^4 conidia/mL was found to be the best for bioassays.

Table A.1. Effect of concentration of conidia in medium on diameter of zone of inhibition. MBC concentration of 6.0 µg/mL in methanol was tested.

Number of Conidia per mL medium	Mean diameter of zone of inhibition (mm)
2 x 10^2	27.6(±2.8)†
2 x 10^3	28.3(±1.6)
2 x 10^4	29.4(±0.8)
2 x 10^5	27.5(±1.0)
2 x 10^6	25.2(±0.7)

† Means (±SE), n=4.

A.1.4.2 Effect of incubation time and temperature on DZI

Length of incubation at lower temperatures has a significant effect on bioassay results (Table A.2). Plates pre-incubated at 4°C for 24 or 48 h gave significantly larger DZIs than these without pre-incubation at 4°C. There was no significant difference among plates which received at least 24 h of pre-incubation at 4°C and at least 24 h of incubation

at 23 °C. Therefore incubation at 4 °C for 24 h and then at 23 °C for 24 h was found to be the best for bioassays.

A.1.4.3 Effect of medium quantity on DZI

A plate with 5 mL of medium gave a significantly higher DZI than those with 10,

Table A.2. Effect of pre-incubation time at 4 °C and incubation time at 23 °C on diameter of zone of inhibition. MBC concentration of 6 μg/mL in methanol was tested.

Pre-incubation time at 4 °C (h)	Mean diameter of zone of inhibition (mm) Incubation time at 23 °C 24 h	48 h
0	21.3(±1.1)	19.2(±1.7)[†]
24	29.4(±0.8)	28.0(±0.9)
48	32.6(±1.9)	30.4(±1.6)

[†] Means (±SE), n=4.

Table A.3. Effect of medium quantity in a Petri dish on diameter of zone of inhibition.

Volume of medium in a Petri dish (mL)	Mean diameter of zone of inhibition (mm)
5	48.2(±2.3)[†]
10	29.4(±0.8)
15	21.7(±0.9)
20	15.5(±1.2)

[†] Means (±SE), n=4.

15 or 20 mL of medium (Table A.3), it also had the highest standard error because the

medium was too thin and parts dried out during the incubation period. On the other hand, too much medium in a Petri dish would dilute the fungicide diffusing out of the paper disc and thus give a smaller DZI. Plates with 5 mL of medium could be used for detection of lower concentrations of MBC in samples. However they would have a large variation unless a proper moist environment is maintained. The DZI obtained with 10 mL of medium in a Petri dish was significantly higher than that obtained with 15 or 20 mL of medium in a Petri dish. Based on these results, 10 mL PDA was used through this study.

A.1.4.4 Effect of Aqua-Gro on DZI

Aqua-Gro (AG), increased DZI of the bioassay (Fig. A.1). At 0.01% Aqua-Gro, the DZI was not significantly different from that without AG when benomyl concentration was 6 µg/mL or less. As fungicide concentration in water (without AG) increased, the DZI also increased but at increasingly lower rates. This may be due to the limited solubility of MBC in water (which is 8 µg/mL at pH = 7). The dispersing effect of AG and possibly increased solubility of MBC in AG-water suspension may contribute to the larger DZI. Further increase of AG above 0.1% did not increase the sensitivity of the bioassay significantly, which suggested that the maximum effect of AG may have been reached at a concentration of 0.1%. These results indicate that AG, at certain concentrations, increased the movement of benomyl or MBC in PDA. In this study, AG (5 L/ha) was applied to turf and resulted in much lower concentrations of AG in thatch or soil samples. Therefore the results of soil- or thatch-agar pellet bioassay were not

Figure A.1. Effect of Aqua-Gro (AG) in water suspension of benomyl (6 μg/mL) on diameter of zone of inhibition of *P. expansum*, as determined with paper disc bioassay. The concentrations of AG are 1.0% (v/v), 0.1%, 0.01%, and 0%.

likely affected by AG. The increased movement of fungicide by AG was also observed

in thatch/soil (Section 4.4.1).

A.1.4.5 Effect of methanol on *S. homoeocarpa*

Methanol in PDA had a significant effect on the mycelial growth of S. homoeocarpa when concentration of methanol in PDA was 0.25% (v/v) (Fig. A.2). However further inhibition on mycelial growth was not significant when concentration of methanol in PDA was increased from 0.25% to 1.0%. When concentration of methanol was larger than 1.0%, the inhibition was linearly related to the concentration of methanol in PDA. The reason is not very clear. It may related to the changes of mode of action by the toxicity of methanol. In this study, methanol-MBC solution was added to hot PDA (90°C) to allow methanol to evaporate and therefore reduced the effect of methanol on the result of the bioassay.

A.1.4.6 Effect of storage time of spore suspension on DZI

The storage of spore suspensions of *P. expansum* at 4°C for up to 4 months did not have a significant effect on the DZI (Fig. A.3). These results suggested that it is possible to just make one spore suspension, and if kept at 4°C at dark, it can be used for 4 months without affecting the bioassay results. Variation in preparing and using new conidial suspensions can be reduced by using the same spore suspension over the entire bioassay period. In this study the maximum storage time for any single spore suspension was 4 months.

A.1.4.7 Toxicity of MBC and benomyl to *P. expansum*

MBC and benomyl were equally toxic to *P. expansum* on a molar basis (Fig.

Figure A.2. Effect of methanol concentration in PDA with 0.05 mL MBC on the mycelial growth of *S. homoeocarpa* when incubated at 23°C for 4 days.

Figure A.3. Effect of storage time of spore suspension at 4°C on the diameter of zone of inhibition of *P. expansum*, as determined with paper disc bioassay. Bars with the same letters are not significantly different from each other at P=0.05 by t-test.

Figure A.4. Effect of benomyl and MBC in methanol on diameter of zone of inhibition of *P. expansum*, as determined with paper disc bioassay. Concentrations of benomyl were converted to concentrations of MBC for presentation in the same graph.

A.4). This was demonstrated by testing the intercepts and slopes of the two regression

lines, which showed no significant differences. Benomyl is not stable and is easily converted to MBC by losing a side chain (Fig. 1.1). The toxicity of benomyl to *P. expansum* may be due to the fungitoxic compound MBC (Peterson and Edgington, 1971; Sims *et al*. 1969). In our studies, when benomyl (Tersan 1991) was used instead of MBC, an MBC equivalent was derived by multiplying by 0.659.

A.1.4.8 Effect of 2,4-D on the results of bioassay

Herbicide 2,4-D up to 2000 μg/mL did not give any inhibition of the growth of *P. expansum* (Table A.4). When 2,4-D solutions up to 2000 μg/mL were mixed with MBC at 1 μg/mL or 8 μg/mL, the DZIs were not significantly different from those of control (Tersan 1991 alone), which suggested that 2,4-D did not interact with MBC within the concentration limits studied.

Table A.4. Effect of 2,4-D concentration on diameter of zone of inhibition.

	Mean diameter of zone of inhibition (mm)					
	Concentration of 2,4-D Amine in water (μg/mL)					
Treatment	0	10	50	100	1000	2000
2,4-D alone	0	0	0	0	0	
	0					
With 1 μg/mL MBC	10.8a[†]	11.6a	11.1a	10.7a	11.3a	[illegible]
With 8 μg/mL MBC	32.8a	33.1a	33.3a	34.0a	33.8a	[illegible]

[†] Numbers with the same letters in a row are not significantly different from each other at P=0.05 by *t*-test. Each number is the average of four replicates.

A.1.4.9 Effect of Daconil 2787 on the results of bioassay

Daconil 2787 (a.i. chlorothalonil) did not inhibit the growth of *P. expansum* when concentrations were less than 6.26 µg/mL (Fig. A.5). Only small DZIs (1.3-3.8 mm) were produced when the concentrations ranged from 6.26 to 31.3 µg/mL (the highest tested) and the DZIs were not significantly different from each other.

DZIs produced from Tersan 1991 (a.i. benomyl) alone and from the mixture with Daconil 2787 were not significantly different from each other at lower concentrations of Daconil 2787 (equal or less than 12.5 µg/mL) (Fig. A.5). However when concentrations of Daconil 2787 were greater than 12.5 µg/mL (along with higher Tersan 1991 concentration), significant differences were found. The slopes of the two regression lines were significantly different, which indicated that there was some interaction between Daconil 2787 and Tersan 1991. In general, the addition of Daconil 2787 additively increased the DZI.

Figure A.5. Effect of benomyl, chlorothalonil, and mixture of benomyl and chlorothalonil on diameter of zone of inhibition of *P. expansum*, as determined with paper disc bioassay.

APPENDIX 2. *IN VITRO* EFFECTS OF AQUA-GRO ON THATCH MICROBIAL POPULATIONS

A.2.1 Materials and methods

Air-dried thatch (10 g) was placed in 50-mL flasks. Twenty milliliters of Aqua-Gro-water solution, with the following AG concentrations: 0, 100, 200, 500, 1000, and 5000 μL/kg were added to flasks with thatch. The flasks were stoppered with parafilm and kept in dark at 25°C. There were four replicates for each concentration of AG. Wet thatch (1 g) was taken out for fungal and bacterial counts using the dilution plate technique (Black *et al*., 1965) at days 0, 2, 7, and 14.

A.2.2 Results and discussion

When concentration of AG was less than 1.0 mL/kg (in air-dried thatch), the populations of fungi and bacteria were not significantly different from the control (without AG) (Table A.5). When AG in thatch was 5.0 mL/kg, significantly fewer fungal colonies were found 7 and 14 days after incubation. Also at this concentration of AG, significant effects on bacteria population was found only at 7 days after incubation.

Aqua-Gro has been shown to be tightly adsorbed to the top 1 cm of soil (Miller and Letey, 1975). When applied on turf, it is likely to be adsorbed by the thatch layer. In our field experiment (Chapter 4), when AG was applied at a rate of 5 L/ha, the concentration of AG in thatch was not more than 200 μL/kg (assuming the turf with a thatch layer of 15 mm in depth). Therefore the results in this *in vitro* studies suggested that AG did not have significant effects on microbial populations when applied at a rate of 5 L/ha.

Table A.5. Fungal and bacterial populations in 1 g air-dried thatch at different times after Aqua-Gro treatment.

AG conc in thatch (mL/kg)	Fungi(x10^4/g) on day 0	2	7	14	Bacteria (x 10^6/g) on day 0	2	7	14
0	45a[†]	47a	52a	46a	56a	55a	58a	62a
0.1	46a	45a	51a	47a	54a	49a	48a	56a
0.5	44a	48a	52a	47a	57a	62a	59a	63a
1.0	47a	51a	55a	45a	60a	66a	69a	63a
5.0	45a	44a	31b	31b	56a	52a	43b	58a

[†] Numbers with the same letters in a column are not significantly different from each other at P=0.05 by DMRT. Each number is the average of four replicates.

APPENDIX 3. PROCEDURE FOR THE CALCULATION OF MBC ADSORBED BY SOIL, TURFGRASS AND THATCH PRESENTED IN TABLE 2.1

In our sample-agar pellet bioassay, agar-pellets and sample- (soil-, thatch- and turfgrass-) agar pellets contained the same amount of fungicide (Section 2.3.2.2). However, the mean diameter of zone of inhibition was not the same. This is likely due to the adsorption of the fungicide by soil, thatch or turfgrass in the sample-agar pellets. The amount of fungicide adsorbed can be calculated using the regression equations for agar-pellet and sample-agar pellet bioassays (Fig. 2.6, 2.7 and 2.8). For example, when reading from the dosage-response curve (Fig. 2.6) that the soil contains 4.0 µg/g of MBC, the amount of MBC adsorbed by the soil is calculated as follows:

The regression equation for agar-pellet bioassay is:

$Y_1=31.8+26.0\text{Log}X_1$, X_1 is MBC amended to agar-pellet.

The regression equation for soil-agar pellet bioassay is:

$Y_2=10.1+25.1\text{Log}X_2$, X_2 is MBC concentration in soil.

Let $Y_1=Y_2$; then, if $X_2=4.0$ µg/g, X_1 can be calculated by combining the two equations:

$31.8+26.0\text{Log}X_1=10.1+25.1\text{Log}(4.0)$, therefore $X_1=0.558$.

$$\text{The amount adsorbed MBC by soil} = \frac{X_2 - X_1}{X_2} \times 100$$

$$= \frac{4.0 - 0.558}{4.0} \times 100$$

$$= 86.1\%$$

APPENDIX 4. CLIMATOLOGICAL DATA

Figure A.6. Air temperature, ground temperature, and dewpoint during the growing season in 1992 at the Cambridge Research Station. (Source: Guelph Turfgrass Institute 1992 Research Report, University of Guelph)

Figure A.7. Rainfall, relative humidity, and hours of leaf wetness during growing season in 1992 at the Cambridge Research Station. (Source: Guelph Turfgrass Institute 1992 Research Report, University of Guelph).