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Fungicide levels in golf putting greens and relations with arbuscular mycorrhizal fungi

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Summary

1. *Poa annua* (annual meadow grass) is the most problematic weed within sports turf and is so abundant that herbicides can not be used against it, because almost total loss of the sward would occur. It has been found that arbuscular mycorrhizal fungi have the potential to be used as biological control agents of *P. annua* in sports turf, as they reduce its growth, while increasing that of the desirable perennial grasses. However, AM fungal levels in amenity turf are very low and the reasons for their lack of occurrence need to be understood. As sports turf has a tradition of high pesticide usage, this study sought to test the hypothesis that levels of toxic elements (derived from historical fungicide applications) and/or organic fungicides in golf putting green soils are related to the low mycorrhizal abundance observed.

2. All 18 putting greens on four different golf courses were sampled. Levels of arsenic, cadmium, copper and lead were recorded, as these elements formed part of fungicidal compounds applied some years ago. Of the organic fungicides in current use, chlorothalonil, fenarimol and iprodione were found to comprise over 70 % of all compounds used and levels of these three chemicals in soils were also measured. Levels of AM colonization of *P. annua* were also measured in every green. Colonization of desirable grasses could not be obtained, because these were too rare in all the swards examined.

3. There was virtually no evidence that the abundance of AM fungi, as measured by arbuscular colonization of roots, was affected by the presence of any of the chemicals. Levels of all elements were below the ambient levels for UK soils.

4. A manipulative experiment, involving the application of chlorothalonil, fenarimol and iprodione to a putting green, was conducted over a six month period. No effects of any of the three fungicides could be found on AM colonization.

5. Synthesis and applications

It is concluded that the low levels of AM fungi in putting greens are unlikely to be due to excessive pesticide application. Levels of historically-applied compounds are very low and modern fungicides do not reduce existing AM colonization when applied to turf. Therefore, if AM fungi are applied to sports turf for the control of *Poa*, their application will not be compromised by current or past fungicide use.

Key-words: non-target effects, pesticide, *Poa annua*, sports turf, turf grass.

Introduction

One of the keys to successful sports turf management in the UK and other temperate areas of the world is control of the weed grass *Poa annua* L. (annual meadow grass or annual bluegrass) (Adams & Gibbs 1994). *P. annua* infests virtually every golf green in the UK and in many cases it can represent the majority of grass cover in the sward (Mann in press). Although selective herbicides have been tried against it (Johnson 1982), none are available in the UK and even if they were, their use would be problematic, since application would result in almost total loss of the sward, something that no golf club could allow. *P. annua* is a problem in fine turf because it is susceptible to drought, so large amounts of irrigation water must be applied to maintain the sward. Furthermore, it is also susceptible to several devastating diseases, notably microdochium patch (causative organism *Microdochium nivale* (Fr.) Samuels & I.C. Hallett) and anthracnose (basal rot) (causative organism *Colletotrichum graminicola* (Ces.) Wilson). In order to maintain fine turf on golf putting greens, fungicide application is obligatory (Perris & Evans 1996).

Currently, 16 fungicides containing 7 different active ingredients, are approved for use on turf grass in the UK (Whitehead 2000). In a recent survey, it was found that golf putting greens receive five times the amount of active ingredient of all pesticides per unit area per annum than is applied to a cereal crop (Garthwaite 1996). Therefore, such a level of pesticide application, combined with a high frequency of irrigation, means that there is the potential for leaching of chemicals and contamination of groundwater (Odanaka *et al*. 1994). Despite this theoretical risk, a recent review has found little evidence for groundwater contamination (Cohen *et al*. 1999), because chemicals may persist in the soil profile or be degraded to less harmful substances by microbial action.

Even if putting greens are not sources of groundwater contamination, there are still several reasons why approaches to turf management need to become less chemically-based and more ecologically-based. Firstly, reliance on any one chemical cannot be sustained, as problems of resistance of the target organism may occur. Indeed, fungicide resistance in *M. nivale*, the most prevalent and damaging pathogen of turf, has been reported in the USA (Vargas 1994). Secondly, many chemicals can have non-target effects on beneficial organisms, for example, fungicides applied to turf can reduce bacterial populations in the soil (Yang *et al.* 2000). A third reason is that the current EU Pesticides Review Programme may mean that a number of compounds will be lost to UK growers in the future, including products used by turf managers (Wood 2001). Over the years, pesticide strategies on turf grass have changed from the use of

inorganic compounds, such as mercuric chloride, cadmium chloride, Malachite/Bordeaux mixture (containing copper sulphate), Paris Green (containing copper acetoarsenite) and lead arsenate (Greenfield 1962) to the array of modern, less persistent organic pesticides. However, the number of currently-approved compounds is likely to diminish in the future, and thus it is critical to seek biological approaches to turf management now, so that these are in place if some currently approved products are lost. If a biological approach to *P. annua* control can be found, the need for chemical pesticide use on turf would be diminished greatly. Furthermore, it is also important to determine if such an approach is compatible with pesticide residues, derived from past or present usage.

It has recently been shown that arbuscular mycorrhizal (AM) fungi may have potential for use in reducing the amount of *P. annua* in putting green turf (Gange 1998; Gange, Lindsay & Ellis 1999b). These fungi generally form mutualistic associations with about 70% of vascular plants (Hodge 2000), but for most plants there exists a continuum of responses to fungal colonization, from positive (i.e. beneficial) to negative (i.e. antagonistic) (Gange & Ayres, 1999). In fine turf, it appears that AM fungi are antagonistic to the growth of *P. annua*, while being beneficial to the growth of desirable grasses such as *Agrostis* spp. (Gemma *et al*., 1997; Gange, 1998). The mechanism is thought to be one in which carbon outflows to the mycorrhiza exceed nutrient inflows in *P. annua*, thus resulting in a net reduction in plant growth (Gange *et al*., 1999b). AM fungi therefore have the potential to be an important part of an integrated control programme for *P. annua*. However, for this to be successful, management techniques must be developed to encourage and sustain high levels of these fungi in sports turf.

There is a potential conflict in the use of turf fungicides and AM fungi, because many of the commonly-applied chemicals have been shown to reduce AM abundance in ecological experiments (e.g. chlorothalonil (Venedikian *et al*. 1999) and iprodione (Gange, Brown & Farmer 1990)). Furthermore, if lack of pesticide leaching from golf greens is indicative of chemical retention within the soil profile (Cohen *et al*. 1999; Armbrust 2001), it is also important to determine if this is a cause of the relative lack of these fungi in turf soil. Certainly, levels of AM fungi in putting greens are considerably lower than those of less intensively-managed areas (Koske, Gemma & Jackson 1997a; Gange *et al*. 1999b) and this could be due to persistent elements such as cadmium, lead or arsenic derived from compounds applied many years ago, or high levels of modern organic fungicides. However, to date, no study has examined whether application of fungicides to fine turf affects the colonization of grass roots by AM fungi.

The aims of this paper are to describe recent fungicide application patterns at four golf courses in southern England and to determine which chemicals are most commonly applied. In addition, we have measured levels of arsenic, cadmium, copper and lead and three of the most commonly used organic pesticides in putting green soils and related these to abundance of AM fungi. Clearly, it is not desirable or relevant to perform manipulative experiments involving the application of historically-applied compounds such as lead arsenate or cadmium chloride. Therefore, we have taken an observational approach and sought relations between elemental levels and AM colonization in a similar manner to that of Weissenhorn, Mench & Leyval (1995). In contrast, it is relevant to perform manipulative experiments with modern fungicides and we report the results of a study in which three of the most commonly used chemicals were applied to a working golf green and AM levels measured over a six month period. This experiment was designed to test the hypothesis that fungicide application will reduce AM colonization in sports turf, given that one of the most-commonly used chemicals (iprodione) has been shown to do so in a natural plant community (Gange *et al*. 1990).

Materials and methods

COURSE SPECIFICATIONS

Four 18-hole golf courses were selected for the study. As each course wishes that fungicide application details remain confidential, they are hereafter referred to as A, B, C and D. Courses A, B and C are of similar age, being opened in 1896, 1894 and 1928. Each of these three courses has greens in which the rootzone was constructed using local soil. This gave pH ranges of 5.6 – 7.9 (course A), 6.6 – 7.4 (course B) and 4.5 – 6.8 (course C). Course D is of more recent construction (1991) and the rootzone in the greens comprises a mixture of 80% sand and 20% milled peat, known as 'United States Golf Association (USGA) specification' (Bengeyfield 1989). Bicarbonate-extractable P concentrations were 19.4 ± 1.2 μg g^{-1}, 26.3 ± 3.6 μg g^{-1}, 24.7 ± 5.4 μg g^{-1} and 17.9 ± 2.3 μg g^{-1} respectively. Greens were mown daily in summer to a height between 4 and 5 mm and all were equipped with automatic irrigation systems.

FUNGICIDE APPLICATION RECORDS

The pesticide application records of each course were inspected for the years 1993 to 2000. Applications of all fungicides (whether for fungal or earthworm control) were recorded per year and summarised to provide information on the relative amount of use of each. These data showed that chlorothalonil, iprodione and fenarimol were the most widely used

chemicals and so these three were selected for soil analyses (see below). Differences in the mean number of applications per year between courses were examined with one factor ANOVA, to determine if the extent of fungicide use varied between courses.

ELEMENTAL ANALYSIS

Two soil cores (5 cm deep x 2.5 cm in diameter) were taken from random positions on each of the 18 greens on each course. Duplicate cores were also taken at random from two greens per course, for use in assessing the sampling precision of both elemental and organic analysis. A reference (background) soil was sampled in the same way from the apron (edge) of each green which was untreated with fungicides, and these cores were used to repair greens.

Each core of soil was split into two halves. One half was stored for organic fungicide analysis (see below), while the other sub-sample required for analysis of copper, cadmium, lead and arsenic was placed in an oven at 50°C for 24 hours, before being crushed and passed through a 500 μm mesh sieve. One gram (± 0.01 g) was then leached with 10 ml of a 20% HNO_3 solution on a hot plate at 55°C for 30 minutes. Once cooled, the samples were filtered through a medium filter-speed filter paper (Whatman® grade 2) into 20 ml flasks, made up to 20 ml with distilled water and placed into labelled plastic tubes.

This sample preparation offered several advantages. Because arsenic analysis was required, any option involving a vigorous reaction or heating over 60°C had to be avoided. This meant that fusion or full HF acid digestions could not be performed. Samples also contained a large amount of organic matter (e.g., roots) which a simple acid leach could cope with. Finally, a mild acid leach is more representative of the bioavailable fraction of the soil matrix which was of interest in this study. However, acid leaches suffer poorer precision and repeatability/reproducibility compared to full digestions or fusions (Potts, 1989). To assess detection limits, a blank was associated with each batch (one per course) and sample preparation duplicates made in order to assess the precision of this step. Certified reference materials were not analysed as their composition is determined after full digestion only. Therefore bias errors could not be assessed.

Samples were analysed by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES, Perkin Elmer Optima 3300R2 ICP-AES unit, Perkin Elmer AS-91 autosampler and Winlab 32 Software) for lead, cadmium and copper, and by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, Perkin Elmer SCIEX Elan 5000 ICP-MS unit and Winlab 32 Software) for arsenic. Drift was monitored during ICP-AES by running a standard composed of most major and trace elements found in soils, for every tenth sample that was analysed.

The samples obtained from initial sample preparation and used for ICP-AES analysis were diluted a further 50 fold and analysed by ICP-MS. Drift was monitored by running a standard solution containing 10 ppb arsenic. A classical standard calibration approach was used for both techniques, and some samples were analysed twice to assess instrumental precision.

ORGANIC ANALYSES

The remaining sub samples were stored at –20°C prior to analysis for the organic fungicides chlorothalonil, iprodione and fenarimol. Re-distilled ethyl acetate was used as the extracting solvent, following Fucci, Ciaravolo & Mazza (1995). The frozen soil samples were placed on trays and left to defrost and dry at room temperature, until their mass remained unchanged. Ten g of soil from each were tipped into a flask and extracted twice with 8 ml of ethyl acetate (5 minutes in an ultrasonic bath). The extracts were combined after filtration through a medium grade filter paper (Whatman grade 2). The solvent was then evaporated until dryness under a stream of nitrogen, and 1 ml ethyl acetate was added back into the flask. After agitation, the extract was ready to be analysed.

Recoveries were assessed by spiking each sample with a mixture of 0.1 mg ml^{-1} chlorothalonil, iprodione and fenarimol. These were analysed and the fungicide peak integrations (minus those for unspiked samples) were matched against the peak integrations for the 0.1mg ml^{-1} standards. Results within ± 10% of the mean recovery for one site were accepted, while others were discarded and replaced by spiking and analysing another similar sub-sample.

One mg ml^{-1} stock solutions were prepared by weighing 50 mg each of pure fungicides and diluting to volume in 50 ml volumetric flasks with ethyl acetate (purity > 99%). New stock solutions were prepared every three weeks. Solutions containing 0.1 mg ml^{-1} were regularly made up from the stock solutions by dilution with ethyl acetate. The latter were analysed by GC-MSD (SCAN mode). All the compounds were identified on the chromatograms.

A Hewlett Packard 5890 Gas Chromatograph (GC) coupled with Hewlett Packard 5970 MSD was used for analysis and the GC column was of medium polarity (SE-54, 30 m, 0.25 mm internal diameter). The GC temperature programme (initial temperature: 60°C for 5 min, gradient of 10 °C min^{-1}, final temperature of 300 °C for 5 min) remained the same over the whole period of analysis. Injection of 2 µl was manual (split-less for 1-5 min). Under SCAN mode, the range of mass detection was scanned from 50 to 350 atomic mass units (amu).

Once the chromatogram peaks of chlorothalonil, iprodione and fenarimol and their related mass spectra were clearly defined, a SIM program was edited by choosing three specific amu values for each compounds. This technique dramatically improves detection and, when dealing with real samples, the signal is much less likely to suffer interferences from other compounds (from the matrix or contamination). Table 1 shows the retention times as well as the selected amu values for each compound analysed.

A classical calibration was performed by analysing 100, 10, 1 and 0.1 μg ml^{-1} chlorothalonil, iprodione and fenarimol and mixed solutions in the GC-MSD. A linear regression between peak areas and concentrations (both log-transformed) gave the equation of the calibration curve, which was accepted when the regression coefficient was higher than 0.985. The calibration curve was regularly checked by running a 10 μg ml^{-1} standard mix solution.

ARBUSCULAR MYCORRHIZAL COLONIZATION

To obtain AM colonization data, all 18 greens from all four courses were sampled on four occasions (March, June, September and December) in 1999 and 2000. On each sampling occasion, three cores, measuring 2.5 cm diameter x 5 cm deep were removed from random positions on each green. Roots of the dominant grass *P. annua* were removed from each and washed free of soil. Slide preparations of roots were examined at × 200 using a Zeiss Axiophott epifluorescence microscope fitted with a UV lamp and filters giving a transmission of 455-490 nm blue, to reveal arbuscules (Gange *et al*., 1999a) which were recorded with the cross hair eye piece method of McGonigle *et al*. (1990). At least 200 intersections were recorded per slide, to give a measure of percent root length colonized (% RLC). Mean mycorrhizal levels per green were calculated as the average of all 24 (three cores x four dates x two years) values obtained. Such a calculation takes into account inter- and intra-seasonal variation and is as accurate a measure of AM occurrence in each green as is possible.

MANIPULATIVE EXPERIMENT

A randomised block experiment, consisting of 40 x 1 m^2 plots, each separated by 2 m, was laid out on a practice putting green at course C. The sward comprised 83% *P. annua*, 14% *Agrostis stolonifera* L. and 3% *Festuca* spp. Plots were allocated at random within blocks to one of four treatments: (i) control (no fungicide applied); (ii) application of chlorothalonil; (iii) application of iprodione and (iv) application of fenarimol. Each fungicide was diluted with an appropriate volume of water to produce application rates identical to those

recommended for sports turf. These were: chlorothalonil (15,000 g a.i. ha^{-1}), iprodione (5,000 g a.i. ha^{-1}) and fenarimol (780 g a.i. ha^{-1}). Each plot received 50 ml of diluted product, equivalent to the application rate of 500 L ha^{-1} recommended for each. The first application took place on 2 July 1999 with a second application on 12 August. The green had not received any fungicide in the six months prior to the experiment and no other compounds were applied during the time of study. Levels of the three compounds used were below detectable limits in the soil.

Immediately before the first application, three 2.5 cm diameter x 5 cm deep soil cores were taken from each plot. The hole left behind was filled with sterilized top dressing (a mixture of 90% sand and 10 % loam soil). Roots of *P. annua* were extracted from each core, washed free of soil, and arbuscules within them revealed by autofluorescence microscopy (Gange *et al*. 1999a). Percent root length colonized (% RLC) by arbuscules was again recorded with the cross hair eye piece method of McGonigle *et al*. (1990), on at least 200 intersections per slide. Root samples were taken again on 12 September, 15 November and 10 January 2000.

STATISTICAL ANALYSIS

Differences between courses in metal and organic fungicide concentrations were examined with one factor ANOVA, after checking for normality and homogeneity of variances. Relations between AM fungal colonization of *P. annua* and each chemical were examined with linear regression. In all cases, mean values per green were used as replicates. The effect of fungicides on AM colonization in the manipulative experiment were examined with a repeated measures ANOVA, employing block, fungicides and dates as main effects. In this case, data were subject to the angular transformation to meet the assumptions of normality (Zar 1996).

Results

FUNGICIDE APPLICATION PATTERNS

The proportion of all fungicides used at each course represented by chlorothalonil, iprodione and fenarimol varied between courses (Fig. 1a). At course C, these three compounds comprised 95% of all applications, while at course D, this figure was 71%. Furthermore, the proportion of each chemical used varied, with the most dramatic difference seen with chlorothalonil. On course A, 42% of all applications were of this chemical, while the comparable figure for course D was only 4.5%. Carbendazim (mainly used for earthworm

control) was the most common constituent of the 'others' category, but was never more than 8% of total usage at any course.

There were significant differences between courses in the mean number of applications per annum of chlorothalonil and iprodione (Fig. 1b). For the former compound, course D applied significantly less than both courses A and B ($F_{3,25} = 6.7$, $P < 0.01$), while for the latter, there was a significant difference between courses A and C ($F_{3,25} = 3.1$, $P < 0.05$). No significant differences were found in the frequency of application of fenarimol between courses (Fig. 1b).

ELEMENTAL AND ORGANIC FUNGICIDE ANALYSIS

The detection limits (limits of determination) for the four elements in this study were 40.5 µg Cu kg^{-1} soil, 4.11 µg Cd kg^{-1}, 65.5 µg Pb kg^{-1} and 67 µg As kg^{-1}. There were significant differences between courses in the levels of copper ($F_{3,68} = 26.6$, $P < 0.001$), cadmium ($F_{3,68} = 33.9$, $P < 0.001$), lead ($F_{3,68} = 12.1$, $P < 0.001$) and arsenic ($F_{3,68} = 7.2$, $P < 0.001$) (Fig. 2). For the latter three elements, the newly constructed course D had much lower levels than the other three courses. However, this did not occur with copper, where levels in course D were similar to those in the older course A (Fig. 2a).

In the majority of greens, the recorded levels of all elements were very low. However, in courses A, B and C, the maximum level of lead recorded (29.9 mg kg^{-1}, 25.9 mg kg^{-1} and 70.3 mg kg^{-1}) exceeded the UK mean of 20 mg kg^{-1}. Course C had particularly high levels of lead in some greens, leading to the highest mean value overall (Fig. 2c), although this figure was not significantly different to the mean of course B.

There were highly significant positive correlations between levels of lead and arsenic (all $P < 0.001$) in courses A, B and C, suggesting that the source of these elements was lead arsenate ($PbHAsO_4$). In courses A and B there were also weak, but significant correlations ($P < 0.05$) between copper and arsenic levels, suggesting that application of Paris Green (copper acetoarsenite) was responsible for the levels of these elements.

The detection limits for the three fungicides were 1.88 µg chlorothalonil kg^{-1} soil, 242.81 µg iprodione kg^{-1} and 27.43 µg fenarimol kg^{-1}. Highly significant differences were found in chlorothalonil ($F_{3,68} = 4.2$, $P < 0.01$), iprodione ($F_{3,68} = 5.2$, $P < 0.01$) and fenarimol ($F_{3,68} = 64.6$, $P < 0.001$) levels between courses (Fig. 3). Course B had the highest levels of chlorothalonil and iprodione (Fig. 3a,b), while course D had much higher levels of fenarimol than did the other courses (Fig. 3c). These data were only a weak reflection of fungicide applications (Fig. 1b), for course B only differed from D in the application of chlorothalonil, while there were no differences in applications of fenarimol.

The mean levels of % RLC (± one s.e.) of AM colonization were: course A: 7.8 ± 1.1; course B: 11.4 ± 1.2; course C: 13.5 ± 0.8 and course D: 18.2 ± 2.2. A summary of the relations between AM fungal colonization and elemental concentrations is given in Table 2. A feature of this is that only one of the 16 regressions was significant, where a negative relation was found between cadmium and AM fungi in course C. Meanwhile, a similar picture was evident in the relations between organic fungicide levels per green and AM colonization (Table 2). Only two significant negative relations were found, for chlorothalonil in course C and fenarimol in course A. Therefore, there was little evidence that AM levels were lower in greens where elemental or organic fungicide levels were relatively high.

Fig. 4 depicts the levels of arbuscular colonization of *P. annua* in the manipulative experiment. Natural levels of colonization were about 8% of the root system colonized in early July. In control plots, there was an increase in this figure during autumn, rising to about 12%. Levels then decreased during the autumn and into winter. No significant effects of any fungicide could be found over the course of this experiment ($F_{3,36} = 2.27$, $P > 0.05$). Root biomass of *P. annua* and *A. stolonifera* showed a very similar pattern to that of AM colonization (data not shown) and there was no effect of fungicide on root production in either grass species.

Discussion

Arbuscular mycorrhizal fungi have great potential in aiding the establishment and growth of perennial grasses in golf putting greens (Gemma *et al*. 1997). Furthermore, evidence suggests that the abundance of these fungi in fine turf is negatively related to that of *P. annua* (Gange 1998) and that addition of AM inoculum to an established green may result in a decrease in the abundance of this weed (Gange *et al*. 1999b). If this biological strategy is to be successful, it is important that AM fungi form part of an integrated programme of turf management in which their presence is compatible with an array of chemicals currently in use. Studies have shown that AM colonization levels of grasses in putting greens are considerably lower than in more natural situations (Koske *et al*. 1997a; Gange *et al*. 1999b), although a surprising number of species have been found in turf (Koske, Gemma & Jackson 1997b). The results from the current study indicate that fungicidal chemicals do not appear to have an adverse effect on the levels of AM colonization in fine turf.

In the past, lead arsenate and copper acetoarsenite (Paris Green) were widely used as insecticides on turf, while cadmium chloride and various salts of mercury and copper were accepted fungicides (Greenfield 1962). Lead arsenate was probably used on turf until the late 1960's and the immobility of lead in the soil, coupled with the generally undisturbed nature of putting green soils led us to hypothesise that levels of lead and arsenic would be relatively high in the older putting greens. Although there were positive correlations between lead and arsenic and between copper and arsenic levels in the older courses, indicating the source of these elements, we found that levels of As, Cd, Cu and Pb were only a little above ambient levels reported elsewhere for UK soils (Lepp 1981). Certainly, none of the levels of any of the four elements in any green are likely to result in phytotoxicity (Carbonell *et al*. 1998; Das, Samantaray & Rout 1998; Aksoy, Hale & Dixon 1999).

There are few reports of the effects of heavy metals on AM fungi and these either show negative effects on colonization (Gildon & Tinker 1983; del Val, Barea & Azcón-Aguilar 1999) or no effect (e.g. Weissenhorn *et al*. 1995). A consistent feature of these studies is that different AM fungal ecotypes exhibit varying degrees of tolerance to heavy metals (Haselwandter, Leyval & Sanders 1994) and that indigenous fungi from polluted sites show high degrees of tolerance (Weissenhorn, Leyval & Berthelin 1993). An excellent example of this was found with *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe, in which metal tolerant isolates exist (Joner, Briones & Leyval 2000) as well as isolates that are strongly inhibited by heavy metals (del Val *et al.* 1999). *G. mosseae* was the dominant species in spore counts from similar golf courses (Gange *et al*. 1999b) and has been found in other turfgrass situations (Koske *et al*. 1997b). It is therefore possible that putting green populations of AM fungi have become more tolerant of historic heavy metal concentrations, although it must be appreciated that the current mean concentrations of Cd, Cu and Pb in our study equate with the 'non-contaminated' control levels of del Val *et al*. (1999). It is quite possible that metal concentrations have become 'diluted' over the years, with the cessation of application and regular application of top dressing, which contributes to the soil profile (Perris & Evans 1996). If putting green AM fungi are relatively tolerant, this would explain the lack of significant relations found between elemental levels and AM abundance, as found by Weissenhorn *et al*. (1995).

Our data show that there is no evidence to suggest that levels of heavy metals in putting green soils, from past pesticide applications, are likely to be a significant explanatory factor for the low abundance of AM fungi. However, if metal tolerant fungi are a feature of golf greens, then future work involving the inoculation of greens with AM fungi must use isolates

that have been obtained from turfgrass. Indeed, this is the only ecologically realistic approach to AM manipulation (Read 2002) and may explain why a previous experiment, in which non-turfgrass isolates were used, was only a partial success (Gange *et al*. 1999b).

The three most-commonly applied fungicides in this study were fenarimol, chlorothalonil, and iprodione. Amongst the 'others' category, carbendazim (for earthworm control) was the most prevalent, but was only 8% of use at maximum. This chemical can reduce AM root colonization (Venedikian *et al*. 1999), but there are also studies where it has been shown to be ineffective (Schweiger, Spliid & Jakobsen 2001). We consider that in no case was application of carbendazim or other compounds frequent enough to have been a likely confounding factor in the relationships we sought between AM levels and fungicides. Chlorothalonil and iprodione have been used in a number of ecological experiments, where they have been found to be effective in reducing the abundance of AM fungi (Gange *et al*. 1990; Aziz, Habte & Yuen 1991; Wan *et al*. 1998). We found very little evidence that AM fungal abundance was related to the levels of any pesticide in the soil and, overall, levels of the three fungicides were very low. This may be because the root zone of a putting green is generally free-draining and thus chemicals are rapidly leached through it. However, previous studies indicate that this is unlikely to be a common occurrence (Cohen *et al*. 1999). Instead, it is more likely that the chemicals are degraded by microbial action (Mercadier, Vega & Bastide 1997; Armbrust 2001) and the most probable site where this occurs is the thatch layer in a golf green (Sigler *et al*. 2000). This layer of undecomposed plant material occurs at the soil surface and in some cases can be several cm deep. A moderate thatch layer is required, to improve ball bounce and putting quality (Perris & Evans 1996), but too much is regarded as problematic, as it prevents the ingress of fertilizers or even water to the root zone, and encourages disease outbreaks.

Generally, there was little indication that frequency of pesticide application was related to the amount of chemicals in the root zone, providing further evidence that chemicals may be entrapped and degraded within the thatch layer before they reach the roots. These data show the difference in pesticide usage by course managers, and it was encouraging to see that all four courses used a variety of chemicals, in an attempt to lessen the chances of resistance to any one chemical occurring. Concentrations of fenarimol were highest in the newest course, in which the rootzone was composed predominantly of sand rather than soil. It is known that retention of fenarimol in a soil is positively related to the cation exchange capacity (CEC) (Wehtje, Walker & Shaw 2000) and this may be why levels were higher in this course, as inorganic products designed to increase the CEC had been added to the rootzone.

The manipulative experiment also failed to show any effects of chlorothalonil, iprodione or fenarimol on AM colonization levels. The experiment was conducted over a six month period, which should have been ample time for any detrimental effect of iprodione or chlorothalonil on root colonisation levels to become apparent (Gange *et al*. 1990; Aziz *et al*. 1991). Given the efficacy of these chemicals in reducing AM levels in other experiments (above), this is perhaps a surprising result. It may be possible that AM fungi in putting greens are resistant to fungicides, though to our knowledge, such a phenomenon has never been recorded. Indeed, it seems unlikely for fenarimol, which is a relatively recent addition to the market. However, this is not the first time that fungicides have failed to reduce AM abundance, which Sukarno, Smith & Scott (1993) have attributed to the way in which colonization levels have been assessed. For example, they have shown that some fungicides affect root production, thus biasing results, while in other cases the amount of living fungal structures may be reduced, a fact not revealed by conventional staining methods. In our study we could detect no effect of fungicides on root production of either *P. annua* or *A. stolonifera* and chose to record arbuscules only. As these are relatively transient structures, we believe that if the fungicide had a detrimental effect on the symbiosis, it would have been revealed by this method over the course of a six month period. Instead, we suggest that the degradation of chemicals within the thatch layer meant that insufficient pesticide reached the roots and thus had no effect on AM colonization (Negre *et al*. 1997).

The data reported here do not provide any evidence that toxic chemicals are the reason for the relatively low levels of AM fungi seen in putting green soils and we suggest that two alternative explanations are worth investigating. The first concerns soil P levels, which can be high in some putting greens (Baker, Binns & Cook 1997). Indeed, it is well known that AM fungi tend to become less abundant and less functional at high soil P (Demiranda & Harris 1994). However, if this is so, it still does not explain why such a relatively high diversity of AM species has been recorded in turfgrass soils (Koske *et al*. 1997b). There has been no study of the occurrence of AM fungi in fine turf in relation to soil P status and we suggest that this is an important area that needs to be addressed.

The second feature of fine turf is the intensity of mowing throughout the year, which in a golf green results in a height of about 4.5 mm for most of the growing season (Perris & Evans 1996). Although in ecological studies occasional mowing may have little effect on AM colonization (Eom *et al*. 1999; Smilauer 2001) such an intensity of foliage removal is certain to affect the capacity of the plant to direct carbon to the mycorrhiza (Jakobsen, Smith &

Smith 2002). Therefore, in any future manipulation of AM populations in turf, factors that affect the carbon economy of the plant will need to be considered too.

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Figure legends

Fig. 1. (a) The extent of use of chlorothalonil, iprodione and fenarimol at four golf courses, expressed as a proportion of all fungicides used. (b) The mean number of applications per annum, over an 8 year period, of chlorothalonil, iprodione and fenarimol.

Fig. 2. Mean concentration of copper (a), cadmium (b), lead (c) and arsenic (d) in putting green soils at four golf courses. Values are means of all 18 greens per course, vertical lines represent one standard error. Letters above bars indicate significant differences between means (Tukey HSD test).

Fig. 3. Mean concentration of chlorothalonil (a), iprodione (b) and fenarimol (c) in putting green soils at four golf courses. Values are means of all 18 greens per course, vertical lines represent one standard error. Letters above bars indicate significant differences between means (Tukey HSD test).

Fig. 4. Changes in AM colonization of *P. annua* roots in control and fungicide treatments in the manipulative experiment (n = 10 for each treatment). Vertical lines represent one standard error.

Table 1. Organic fungicides retention times and selected atomic mass units (amu) for analysis by GC-MS.

Fungicide	Retention time (min)	amu 1	amu 2	amu 3
Chlorothalonil	20.7	108.8	123.9	263.78
Iprodione	26.7	186.8	243.9	270.9
Fenarimol	28.4	106.9	138.9	218.9

Table 2. Summary of results from linear regression analyses, testing for relationships between elemental and organic fungicide levels and arbuscular mycorrhizal colonization in putting greens at four golf courses. In all cases, n = 18.

	Course A			Course B			Course C			Course D		
Fungicide	*F*	P	R^2, %	*F*	P	R^2, %	*F*	P	R^2, %	*F*	P	R^2, %
Copper	0.01	NS	0.007	0.47	NS	2.9	2.59	NS	13.9	0.08	NS	0.05
Cadmium	1.36	NS	7.8	0.02	NS	0.14	4.74	< 0.05	22.8	0.32	NS	1.9
Lead	0.66	NS	3.9	0.48	NS	2.49	0.90	NS	5.3	0.03	NS	1.9
Arsenic	0.03	NS	0.002	1.35	NS	7.8	1.41	NS	8.1	1.68	NS	9.5
Chlorothalonil	0.88	NS	5.2	0.09	NS	0.06	6.84	< 0.05	29.9	1.9	NS	10.6
Iprodione	2.63	NS	14.1	2.63	NS	14.1	1.37	NS	7.9	0.11	NS	0.07
Fenarimol	8.43	< 0.05	34.5	0.77	NS	4.6	1.66	NS	9.4	0.06	NS	0.04