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The soil-dwelling earthworm *Allolobophora chlorotica* modifies its burrowing behaviour in response to carbendazim applications

Sian R Ellis[ab*], Mark E. Hodson[a], Philip Wege[c]

[a] Department of Soil Science, School of Human and Environmental Sciences, University of Reading, Whiteknights, Reading, Berkshire RG6 6DW, United Kingdom

[b] Current address: sian.ellis@wca-environment.com, WCA Environment Ltd., Brunel House, Volunteer Way, Faringdon, Oxfordshire, SN7 7YR

[c] Crop Protection Biology & Logistics, Syngenta, Jealott's Hill International Research Centre, Bracknell, Berkshire,
RG42 6ET

* corresponding author

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Abstract

Carbendazim-amended soil was placed above or below unamended soil. Control tests comprised two layers of unamended soil. *Allolobophora chlorotica* earthworms were added to either the upper or the unamended soil. After 72 hours vertical distributions of earthworms were compared between control and carbendazim-amended experiments. Earthworm distributions in the carbendazim-amended test containers differed significantly to the ‘normal’ distribution observed in the control tests. In the majority of the experiments earthworms significantly altered their burrowing behaviour to avoid carbendazim. However, when earthworms were added to an upper layer of carbendazim-amended soil they remained in this layer. This non-avoidance is attributed to 1) the earthworms’ inability to sense the lower layer of unamended soil and 2) the toxic effect of carbendazim inhibiting burrowing. Earthworms modified their burrowing behaviour in response to carbendazim in the soil. This may explain anomalous results observed in pesticide field trials when carbendazim is used as a control substance.

Keywords: earthworm, *Allolobophora chlorotica*, burrowing, avoidance behaviour, carbendazim, pesticide, field trial

1. Introduction

The fungicide carbendazim is known to be highly toxic to earthworms and is recommended for use as the reference substance in standardised guidelines for testing the effects of pesticides on earthworms in field situations (ISO, 1999). However, results using carbendazim in field trials have been highly variable (Römbke et al., 2004; Ellis, 2008). This paper reports a study into the behavioural response of *Allolobophora chlorotica* to carbendazim as part of a wider investigation into this variability.

Carbendazim has limited movement in the soil profile and studies have recorded up to 97 % of the applied total to remain in the upper 5 cm of the soil profile (Ellis, 2008; Jones et al., 2004; Holmstrup, 2000). The exposure of earthworms to carbendazim in the field will therefore, in part, be determined by their vertical distribution and their ability to detect the chemical and modify their vertical burrowing behaviour as a consequence of this. A field study (Römbke et al., 2004) showed the vulnerability of earthworms to the toxic effects of carbendazim to differ between species. This difference was attributed to the different feeding preferences of the species and their distribution in the soil profile. Species which typically feed on vegetation at the surface of the soil where carbendazim concentration was highest, including *Lumbricus terrestris* and *Lumbricus rubellus* had higher mortality than geophageous species including *Apporectodea caliginosa* which were not dependent on the surface for food and subsequently had lower exposure to the chemical (Römbke et al., 2004). While certain species may be more vulnerable due to their feeding behaviour, earthworms can occupy a range of depths in the soil profile and can adjust their burrowing depth behaviour based on soil conditions (Edwards and Bohlen, 1996). The geophageous species *A. chlorotica* for example is typically found above a depth of 8 cm when soil conditions are favourable but will burrow to below 8 cm to avoid extremes of temperature or dry soil at the surface (Gerard, 1967). In earthworm avoidance studies, in which earthworms are given a choice between horizontally adjacent soils, (usually a control, contaminant free soil and a contaminant bearing soil, e.g. Yeardley *et al.*, 1996; Natal da Luz et al., 2004; Environment Canada, 2007; ISO, 2008) the earthworm species *Eisenia andrei* (Loureiro et al., 2005) and *Eisenia fetida* (Garcia et al., 2008), have been shown to significantly avoid carbendazim and benomyl at concentrations ≥ 1 mg kg^{-1}. However, for chemicals such as carbendazim which have limited mobility through the soil profile, the most significant concentration gradient encountered in the field will be in the vertical plane and a key question is whether or not earthworms are able to modify their behaviour to avoid such chemicals. Horizontal avoidance studies provide useful information on the ability of earthworms to detect and respond to adverse concentrations of chemicals but they do not provide information on whether this avoidance driver is sufficient for earthworms to modify their normal behaviour to avoid such chemicals.

The aim of this study was therefore to determine whether the presence of carbendazim led to a modification of the burrowing behaviour of the earthworm *A. chlorotica.*

2. Method

2.1. Earthworm species

Allolobophora chlorotica is a widely abundant species in the UK. It was selected as a suitable species for the study as it occupies a range of depths in the soil profile, is geophageous, so is not dependent on the soil surface for feeding (Edwards and Bohlen, 1996) and is known to adjust its burrowing depth in response to unfavourable conditions (Gerard, 1967). Earthworms were collected by manual digging and hand sorting soil from a pasture field at the University of Reading farm at Sonning, Berkshire UK and kept in a 3:1 mix of sandy loam soil and sphagnum peat moss at a temperature of 15 °C until the test.

2.2. Test substance

Delsene 50 Flo, obtained from Nufarm UK Ltd. Belvedere, Kent, UK, was selected as a suitable test substance for the study as it is a commercially available water-based suspension concentrate containing carbendazim at a concentration of 500 g l^{-1}. The Delsene 50 flo was diluted using deionised water to a concentration of 46 mg l^{-1}.

2.3. Test soil

Kettering loam, a commercially available sandy loam soil obtained from Broughton Loam, Kettering, UK (Table 1 for soil properties) was used in the avoidance studies. The soil was air dried and sieved to < 2 mm prior to use. A carbendazim concentration of 8 mg kg^{-1} was used as significant avoidance behaviour was observed in previous studies using similar concentrations (Loureiro et al., 2005; Garcia et al., 2008). Using the relationship of Jänsch et al. (2006) which assumes a soil density of 1

500 kg m^{-3} this concentration is approximately twice that in soil after the typical application rate of 4 kg ha^{-1} used in field trials (ISO, 1999). The diluted carbendazim suspension was mixed thoroughly with the soil using a house-hold mixer (Kenwood A907D), to give a soil moisture content of 60 % of the soil water holding capacity. For the control soil, Kettering loam was mixed with deionised water only. The moisture contents of the carbendazim-treated and control soil were the same.

2.4. Experimental procedure

2.4.1. Arrangement of soils

The test containers comprised two sections, one section containing the carbendazim-amended soil and the other the clean unamended soil. The two sections were stacked vertically and earthworms were able to move freely between the two soils. The behavioural response of *A. chlorotica* to carbendazim was tested with the soils in two arrangements (Figure 1). The first arrangement (*Field arrangement*) reflected carbendazim application in the field with the carbendazim-amended soil at the top and the unamended soil below. In the second arrangement (*Alternative arrangement*) the carbendazim-amended soil formed the bottom section. Control tests (with unamended soil in both sections) were used to determine the natural distribution of earthworms without the influence of carbendazim. The test containers were designed to account for the typical burrowing behaviour of *A. chlorotica. Allolobophora chlorotica* usually form temporary horizontal burrows in the upper 8 cm of the soil profile (Edwards and Bohlen, 1996). The test containers comprised two open-ended, translucent PVC cylinders wrapped in black adhesive tape to exclude light, 8 cm high and with a diameter of 7.5 cm. Four hundred grams (dry weight equivalent) of soil were added to each container which were placed on top of each other. The top of the upper container was covered with mesh (1 mm size) to prevent individuals escaping and to allow light onto the surface of the soil. The bottom of the lower container was closed to prevent earthworm escape. The test containers were kept in a temperature controlled room at 18 °C with a photo period of 12:12 hours (light:dark).

2.4.2. Earthworm addition

Earthworms were added to the containers in one of 2 ways. In both methods the earthworms were added 24 hours after the soil had been mixed and added to the containers. Five replicates were used per soil arrangement with ten individuals used per replicate. Five replicates were also used for each control. The tests were run for 72 hours to ensure that earthworms had sufficient time to burrow into the soil. After 72 hours the sections were separated using a card divider and the number of individuals in each section determined by hand sorting.

Method 1 (Fig. 1): Earthworms were added to the soil surface at the top of the test container. Thus when the carbendazim-amended soil was in the upper container earthworms were added to the upper surface of the 8 cm thick carbendazim-amended soil. This method allowed us to assess the response of the earthworms when they experienced direct dermal contact with carbendazim-amended soil.

Method 2 (Fig. 2): This was intended to be more representative of a field scenario where carbendazim would be sprayed onto the soil surface. Earthworms were initially added to unamended soil and allowed to acclimatise for 24 hours before the carbendazim-amended soil was added, either above or below the unamended soil. This method allowed us to assess whether *A. chlorotica* would modify its burrowing behaviour in response to either an over-lying or under-lying layer of carbendazim-amended soil. In this method *A. chlorotica* began the test in two different positions in the test container (either the top or bottom section), dual controls were used for both arrangements. For each arrangement, 5 replicates plus 5 dual controls were used.

2.5. Statistical analysis

The Fisher exact test in Minitab version 15 was used to determine if earthworms were significantly avoiding the carbendazim-amended soil. This test allows the distribution in the avoidance test to be compared with the normal distribution of earthworms in the controls (Natal da Luz, 2004).

3. Results

In each arrangement earthworms were observed to burrow rapidly into the soil to which they had been added. For both Method 1 (Fig. 3) and Method 2 (Figs. 4 and 5) in the control experiments there was an uneven distribution of *A. chlorotica* between the two sections. The greatest proportion of individuals had burrowed to the bottom section, below a depth of 8 cm. Therefore when analysing results from the carbendazim-amended experiments the relative proportion of earthworms in the bottom section was compared to the proportion in the bottom section in the controls. Results indicate that *A. chlorotica* does indeed modify its natural burrowing behaviour to avoid carbendazim and that exposure to carbendazim inhibits earthworm burrowing.

Method 1: Compared to the control earthworms appeared to have modified their burrowing behaviour in response to carbendazim in both the *Field* and *Alternative arrangements.* In the *Field arrangement* with the carbendazim-amended soil at the top, the majority of individuals were found in the carbendazim-amended soil (0.84 ± s.e 0.05, n = 5) and had not burrowed into the unamended soil below (Fig. 3). The proportion in the bottom soil was significantly lower than the control ($P < 0.05$). In two of the replicates, one earthworm was found dead at the surface of the test soil. In the *Alternative arrangement*, with the carbendazim-amended soil at the bottom, a significantly lower proportion of *A. chlorotica* were found in the bottom soil compared to the control (0.42 ± s.e 0.05, n = 5) ($P < 0.05$) and had not burrowed into the carbendazim-amended soil below (Fig. 3).

Method 2 In the *Field arrangement* (carbendazim-amended soil at the top) a significantly higher proportion of individuals were found in the bottom section compared to the control ($P < 0.05$). As this distribution differed significantly from the control, burrowing behaviour appears to have been modified in response to the presence of carbendazim (Figure 4). This was also apparent in the *Alternative arrangement* in which the carbendazim-amended soil formed the lower section. The majority of individuals were not found in the bottom section but instead remained in the unamended soil in the top section (0.78, s.e. ± 0.07, n = 5) (Figure 5). The proportion in the bottom soil was significantly lower than in the control ($P < 0.05$).

4. Discussion

Although we did not analyse the carbendazim-amended soil used in the experiments, subsamples of the same well-mixed carbendazim-amended soil were used in all the experiments so we can be confident that concentrations of carbendazim were the same in all experiments. The aim of the investigation was to determine whether the presence of carbendazim led to a modification of burrowing behaviour and the lack of precise concentration data does not prevent this. In the current experiments no flow of water occurred through the soil (which had the same moisture content in both the carbendazim-free and carbendazim-amended parts) so it is highly unlikely that the carbendazim would have been redistributed within the soil due to movement of soil solution. Additionally studies by Ellis et al. (In press), Jones et al. (2004) and Holmstrup (2000) indicate that carbendazim is immobile in soils due to very strong partitioning onto the solid phase relative to the solution phase. Thus we can assume that any difference in earthworm behaviour between experiments is due to either exposure to the carbendazim-amended soil (Method 1, *Field arrangement*) or the detection and consequent avoidance of the carbendazim-amended soil (Method 1, *Alternative arrangement* and Method 2 *Field* and *Alternative arrangements*).

We propose two alternate explanations for the modified burrowing behaviour observed in the *Field arrangement* (the majority of individuals remaining in the carebendazim-amended soil held in the top half of the containers compared to the control in which earthworms added to the upper surface burrowed down into the soil in the bottom half of the containers, Fig. 3). The first possible explanation is that earthworms remained in the carbendazim-amended soil because there was no gradient “leading” them to the unamended soil below, i.e. the earthworms were unaware of the less challenging conditions in the bottom half of the test containers. However, as the earthworms in the control experiment clearly showed a preference for burrowing into the bottom half of the test containers this explanation can not be the complete story. Thus it seems more likely that exposure to the carbendazim disrupted the burrowing ability of the earthworms when the earthworms were placed on the upper surface of the carbendazim-amended soil. Carbendazim has been shown to disrupt conduction in the giant nerve fibre of earthworms, which is linked with earthworm mobility (Drewes et al., 1987), thus it may be possible that carbendazim reduced the ability of the earthworms to burrow. Unfortunately it is not possible to convert the concentrations used in the filter paper tests by Drewes et al. to equivalent soil concentrations. However, the concentration of carbendazim used in this study (8 mg kg^{-1}) is similar to concentrations at which both acute and chronic toxic effects have been observed in other studies. Van Gestel et al. (1992) reported an LC50 of 4.7 – 6.9 mg kg^{-1} and sublethal effects on growth at 6.0 mg kg^{-1} and reproduction at 1.92 mg kg-1 for *E. andrei*. Ellis et al (2007) reported LC50s in the range 2.47 – 16.00 mg kg^{-1} for *E. fetida*. Ellis et al. (In press) reported a reduction in surface activity of *L. terrestris* at surface carbendazim concentrations of c. 2.5 mg kg^{-1}. A third explanation (which we reject as it is contradicted by the avoidance of the carbendazim-amended soil by earthworms in the *Alternative arrangement*) is that the earthworms remained in the carbendazim-amended soil because conditions were preferable to those in the unamended soil.

By adding the earthworms to the unamended soil rather than the amended soil (Method 2), field conditions were more closely represented with the earthworms initially in carbendazim-free soil. The results of Method 2 confirm that the earthworms in the *Alternative arrangement* of Method 1 modified their burrowing behaviour to avoid the carbendazim-amended soil. In the *Field arrangement* of Method 2 (carbendazim-amended soil in the top half of the containers) significantly more earthworms were found in the bottom half of the containers relative to the control. In the *Alternative arrangement* (carbendazim-amended soil in the bottom half of the containers) significantly fewer earthworms were found in the bottom half of the containers relative to the control. This indicates that the presence of carbendazim in the soil led to the earthworms altering their burrowing behaviour to avoid burrowing into the carbendazim-amended soil. This finding is consistent with those of Loureiro et al. (2005) and Garcia et al. (2008) who observed avoidance of carbendazim at concentrations $\geq$ 1 mg kg^{-1} for *E. andrei* and *E.* fetida respectively in horizontal avoidance tests. The avoidance behaviour by earthworms of potentially toxic chemicals is well documented (e.g. Environment Canada, 2007 and references therein) and is most likely triggered by the detection of chemical substances that render the soil inhospitable by chemoreceptors located on the prosomium or buccal epithelium (Edwards and Bohlen, 1996). Thus earthworms would be able to detect the boundary between the carbendazim-free / carbendazim-amended soils and avoid entering the treated soil. Similar responses resulting in earthworms not burrowing in soils of unsuitable pH have been reported in the literature (e.g. Laverack, 1961). Thus avoidance can occur before an earthworm is in an inhospitable soil and experiments like the ones carried out here are a valid measure of earthworm avoidance behaviour despite, unlike current standardised tests (e.g. Environment Canada, 2007; ISO, 2008) all the earthworms being in the same portion of the test chambers at the start of the experiment.

5. Conclusion

Carbendazim is used as a reference substance in standardised guidelines for testing the effects of pesticides on earthworms in field situations. If carbendazim application fails to reduce field populations of earthworms to between 40 and 80 % of those in control plots the trial is declared invalid (ISO, 1999). Our results indicate that earthworms may be able to avoid the effects of carbendazim by modifying their burrowing behaviour. This should be borne in mind when determining earthworm population size after application of test chemicals. It is possible that a failure to recover an acceptable number of earthworms from trial plots, which would be interpreted as excess mortality may simply be due to avoidance of treated soil by earthworms. Therefore in field trials when sampling after application of pesticides and control substances care should be taken to sample both outside the treated plot and to sufficient depths so that earthworms exhibiting such behaviour are included in counts of earthworm numbers.

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Figure captions

Figure 1. Diagrammatic representation of method 1 for assessing vertical avoidance behaviour of earthworms in which earthworms are added to the upper surface of the upper soil.

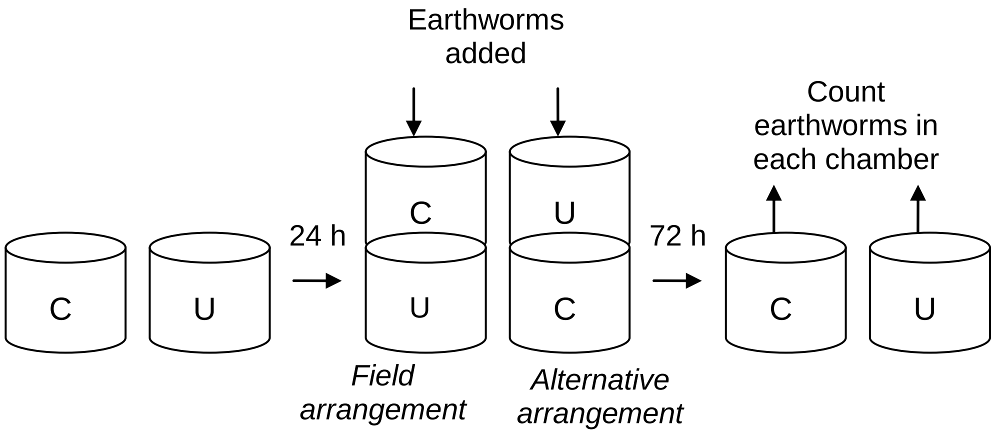
Figure 2. Diagrammatic representation of method 2 for assessing vertical avoidance behaviour of earthworms in which earthworms are added to the upper surface of the unamended soil.

Figure 3. Mean proportional distribution of *Allolobophora chlorotica* in test containers in the upper and lower soils in the *Field* (carbendazim-amended soil in the upper section) and *Alternative* (carbendazim-amended soil in the bottom section) arrangements with *A. chlorotica* being added to the upper soil upper surface (Method 1). Error bars = standard deviation, n = 5. * = significantly different from the Control.

Figure 4. Mean proportional distribution of *Allolobophora chlorotica* in test containers in the upper and lower soils in the *Field arrangement* (carbendazim-amended soil in the upper section) with *A. chlorotica* being added to the unamended soil (Method 2). Error bars = standard deviation, n = 5. * = significantly different from the control.

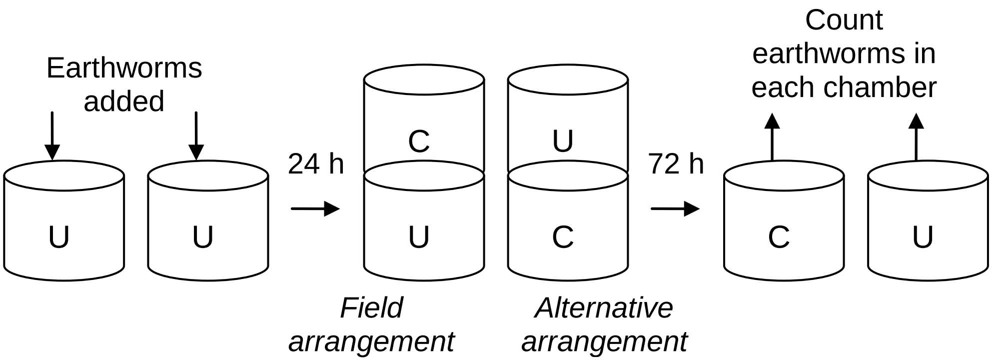
Figure 5. Mean proportional distribution of *Allolobophora chlorotica* in test containers in the upper and lower soils in the *Alternative arrangement* (carbendazim-amended soil in the bottom section) with *A. chlorotica* being added to the unamended soil (Method 2). Error bars = standard deviation, n = 5. * = significantly different from the Control.

Figure 1.



C = carbendazim-amended soil U = unamended soil

Figure 2.



C = carbendazim-amended soil U = unamended soil

Figure 3

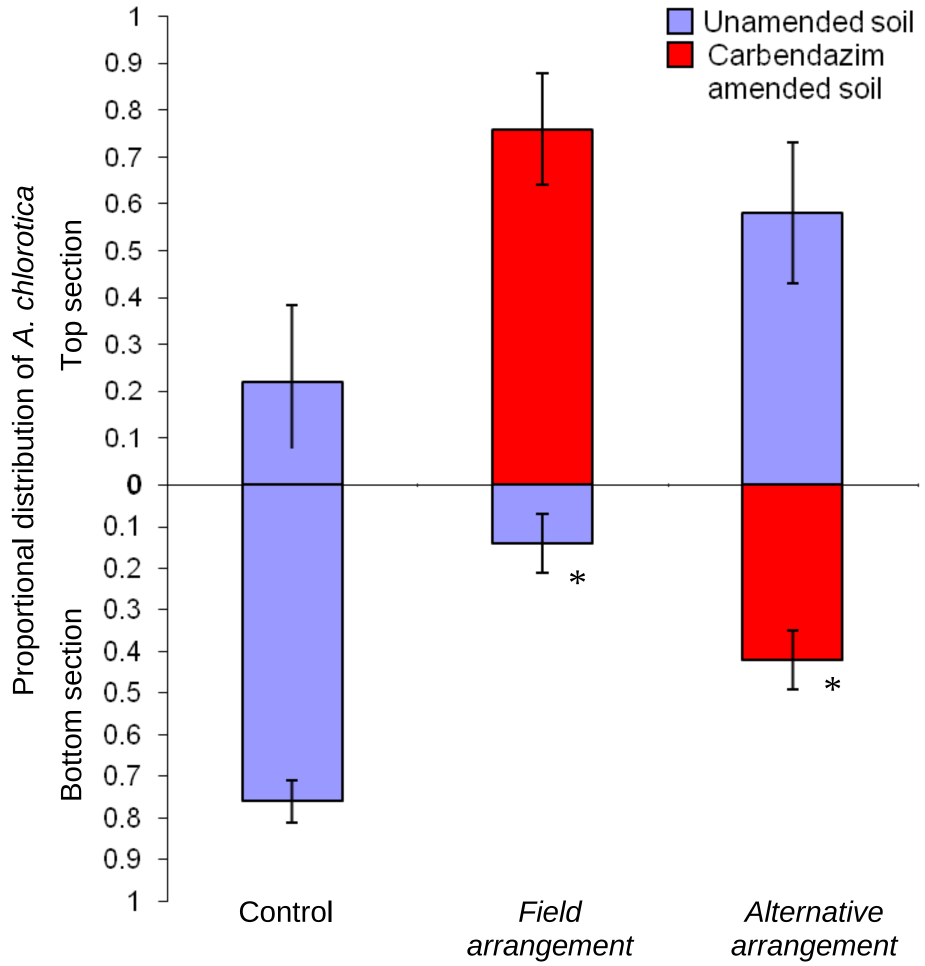

Unamended soil
Carbendazim amended soil
Proportional distribution of A. chlorotica
Top section
Bottom section
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Control
Field arrangement
Alternative arrangement

Figure 4

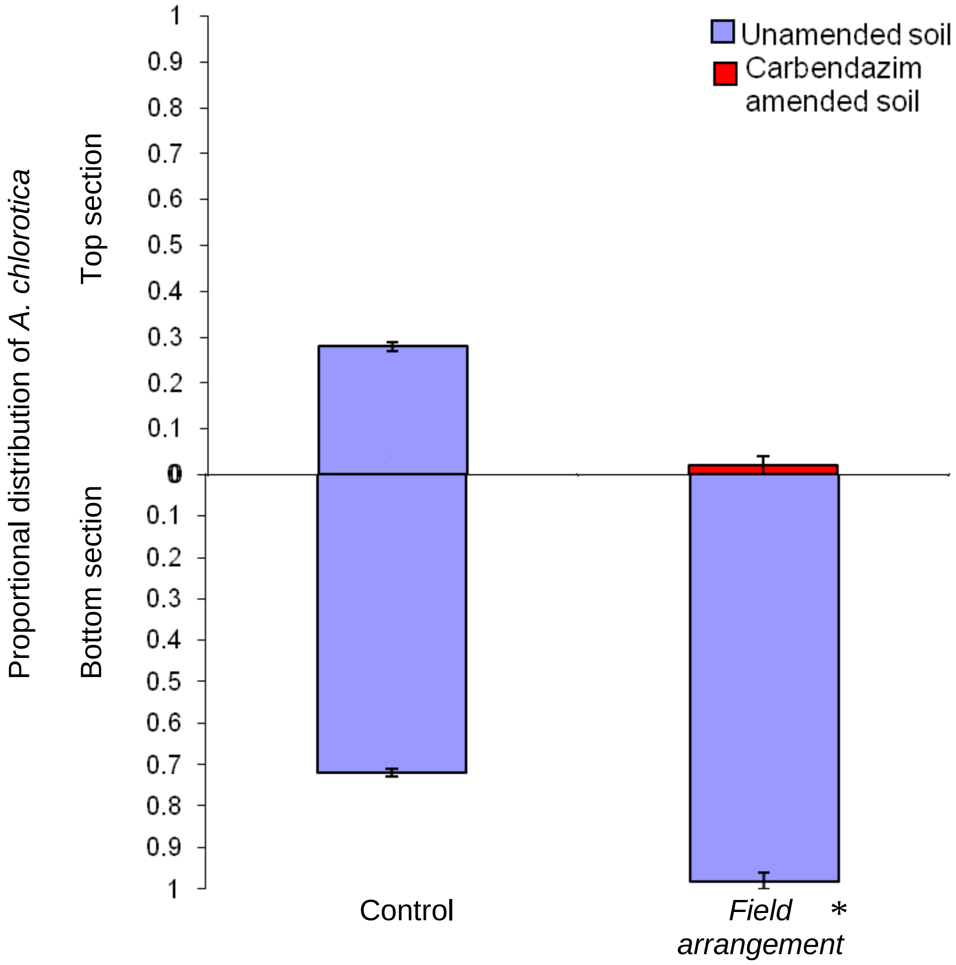
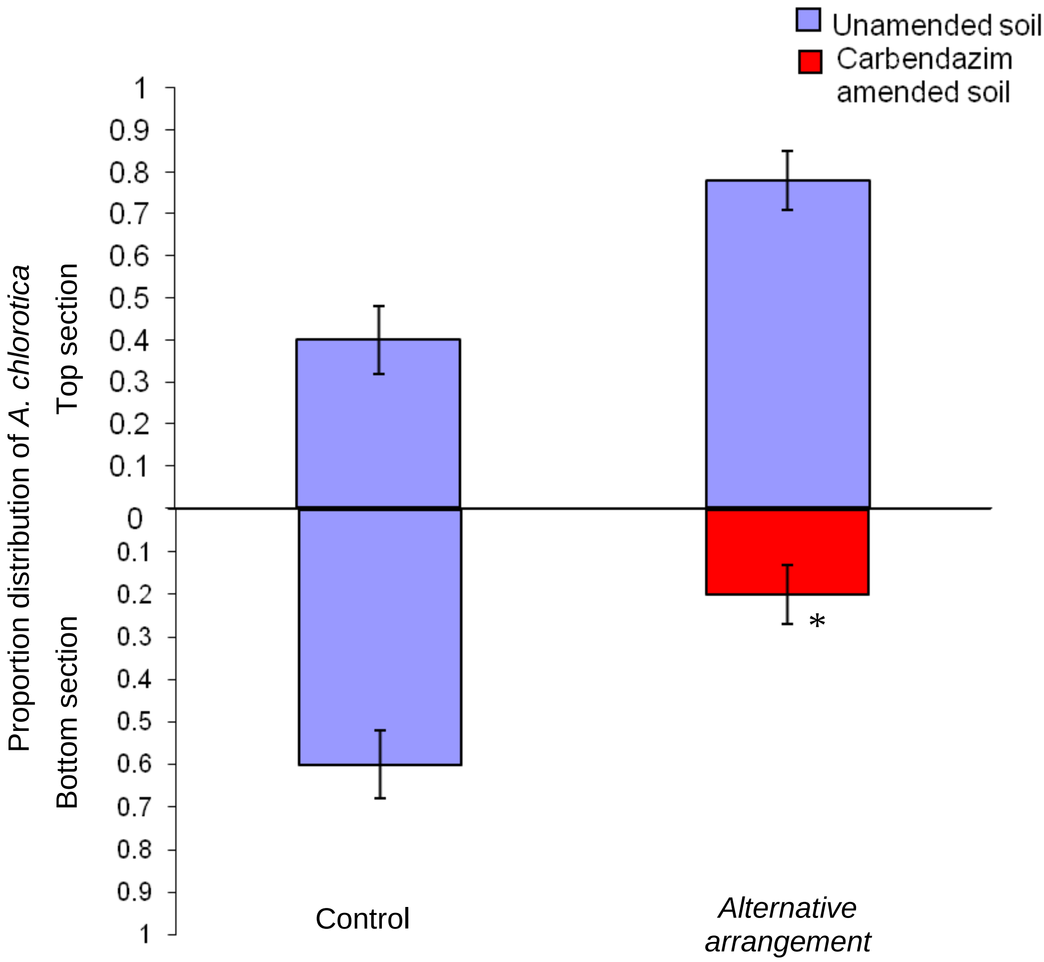

Unamended soil
Carbendazim amended soil
Proportional distribution of A. chlorotica
Top section
Bottom section
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Control
Field arrangement
*

Figure 5



Unamended soil
Carbendazim amended soil
Proportion distribution of *A. chlorotica*
Top section
Bottom section
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
*
Control
Alternative arrangement

Table 1. Selected mean chemical and physical properties of the Kettering loam test soil (n = 3 $\pm$ standard error).

Soil property	
pH	6.2 $\pm$ 0.2
Organic matter content / %	7.06 $\pm$ 0.09
Texture	11.8 $\pm$ 1.3 % clay
	21.7 $\pm$ 0.3 % silt
	66.9 $\pm$ 1.0 % sand
Water holding capacity / %	29 $\pm$ 4