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Assessment of short and long-term effects of imidacloprid on the burrowing behaviour of two earthworm species (*Aporrectodea caliginosa* and *Lumbricus terrestris*) by using 2D and 3D post-exposure techniques

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ABSTRACT

Adverse effects of agrochemicals on earthworms' burrowing behaviour can have crucial impacts on the entire ecosystem. In the present study, we have therefore assessed short- and long-term effects on burrowing behaviour in the earthworm species *Aporrectodea caliginosa* and *Lumbricus terrestris* after exposure to a range of imidacloprid concentrations (0.2–4 mg kg^{-1} dry weight (DW)) for different exposure times (1, 7, 14 d). 2D-terraria were used for the examination of post-exposure short-term effects (24–96 h), while post-exposure long-term effects were assessed by means of X-ray burrow reconstruction in three dimensional soil cores (6 weeks). For the latter each core was incubated with two specimens of *L. terrestris* and four of *A. calignosa*. Short-term effects on the burrowing behaviour (2D) of *A. caliginosa* were already detected at the lowest test concentration (0.2 mg kg^{-1} DW), whereas such effects in *L. terrestris* were not observed until exposure to concentrations 10 times higher (2 mg kg^{-1} DW). For both species tested in the 2D-terraria, "total burrow length after 24 h" and "maximal burrow depth after 24 h" were the most sensitive endpoints. 3D reconstructions of the burrow systems made by both earthworm species in the repacked soil cores revealed a significant linear decrease in burrow volume with increasing imidacloprid concentration.

Since many of the observed effects occurred at imidacloprid concentrations relevant to natural conditions and since reduced activities of earthworms in soils can have crucial impacts on the ecosystem level, our results are of environmental concern.

1. Introduction

The neonicotinoid insecticide imidacloprid is extensively used in agriculture worldwide. Currently in Germany around 1000 tons of imidacloprid are produced per year (www.bvl.de). Soil concentrations after field application can be as high as 0.66 mg kg^{-1} dry weight (DW) (Oi, 1999), which is in the range of effect concentrations for soil organisms (e.g. Luo et al., 1999; Zang et al., 2000; Lal et al., 2001; Mostert et al., 2002; Capowiez et al., 2010; Dittbrenner et al., 2010). The beneficial role of earthworms in soils, influencing a range of chemical, physical and biological processes, is beyond dispute (Scheu, 1987; Edwards and Bohlen, 1992; McCredie and Parker, 1992; Curry and Baker, 1998). Since they are, in addition, easy to culture and to handle for experiments, earthworms have become standard test organisms in ecotoxicology (OECD, 1984, 2004; EEC, 2003). During the last decades the use of behavioural endpoints to evaluate toxicity of pollutants in the environment has been generally accepted by ecotoxicologists (Little, 1990; Doving, 1991; Scherrer, 1992). Changes in behaviour of earthworms can be of crucial importance for soils, and thus can result in adverse effects on soil functions (Capowiez et al., 2006). The most frequently used behavioural test for earthworms is the standardised avoidance test (ISO, 2008), which is based on a 48 h exposure. It has proven to be very sensitive in many studies (Yeardley et al., 1996; Slimak, 1997; Natal Da Luz et al., 2004; Hund-Rinke et al., 2005; Pereira et al., 2010). However, in some cases non-avoidance of different toxicants (diazinon; chlorpyrifos; imidacloprid; ivermectin) or even significant attraction has been observed (Hodge et al., 2000; Capowiez and Bérard, 2006; Dittbrenner et al., submitted for publication a; Torkhani et al., 2011). Thus, the avoidance test is rather considered to be a measure of repellence than of toxicity (Capowiez and Bérard, 2006). Since the beneficial role of earthworms is highly dependent on their burrowing activity, examining whether and how toxicants hamper the earthworms burrowing behaviour has high ecological relevance. Both

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approaches (i.e. tests based on avoidance or burrowing activity) appear however to be complementary since modification of the burrowing behaviour is likely to occur when pollutants have not been previously avoided and earthworms consequently were exposed. Burrowing behaviour can be assessed by using 2D-terraria (Evans, 1947) or X-ray tomography in repacked soil cores (3D) (Capowiez et al., 2003a). Adverse effects on burrowing behaviour were shown several times in previous studies by means of the 2D-terraria (Hans and Beg 1992; Capowiez et al., 2003b; Capowiez and Bérard, 2006; Olvera-Velona et al., 2008). This method has the advantage of being easy, quick and inexpensive. However, it can only be used for short-term exposure periods, since space for burrow systems is very limited. In contrast, the 3D-approach opens the possibility to study long-term effects and is adapted to the study of modifications of soil functions (Capowiez et al., 2006), and thus, represents a more realistic exposure scenario. However, it is more expensive and time-consuming to carry out. Measuring chronic effects of toxicants on the environment is highly important for risk assessment, but such effects are difficult to predict from short-term experiments. With respect to imidacloprid, the majority of behavioural studies on earthworm behaviour have solely focussed on short-term effects (Capowiez et al., 2003b; Capowiez and Bérard, 2006; Capowiez et al., 2010; Dittbrenner et al., 2010) and little is known about the predictability of these results in the long run and thus about their ecological relevance.

In the present study, we have compared short and long-term effects of imidacloprid on the burrowing behaviour of two earthworm species (*Aporrectodea caliginosa* and *Lumbricus terrestris*) by using 2D-terraria and repacked soil cores (3D). Both methods have been conducted by firstly carrying out pre-exposure experiments of earthworms individually in Petri dishes and subsequently measuring burrowing behaviour in uncontaminated 2D-terraria or 3D-soil cores. By doing so, bias like e.g. avoidance behaviour due to the presence of a toxicant is excluded. In our experiments the measured burrowing behaviour therefore indicates effective burrowing capacities of the earthworms after toxicant exposure.

2. Material and methods

2.1. Test soil and substance

The test soil (23.4% clay, 57% silt, 19.6% sand, 2.83% organic matter, pH = 8.3 (in water), CEC = 8.2 cmol kg^{-1}) was collected from an untreated orchard located close to Avignon (France) which was abandoned for more than 10 years. The water holding capacity (WHC) of the test soil was 0.247 g g^{-1}. The soil characteristics were measured in the "Laboratoire d'Analyse des Sols" (INRA Arras) using normalised protocols (www.lille.inra.fr/las).The earthworms were acclimatised in the test soil for 7 d in a dark climate chamber (12 °C) prior to the exposure experiments. The insecticide imidacloprid was purchased from FLUKA (No.37894) and was dissolved in distilled water to different test concentrations.

2.2. Test organisms

Specimens of the species *Lumbricus terrestris* (3.35 ± 0.85 g (mean body mass ± SD)) were purchased from a fishing store in Avignon (France), while specimens of the species *A. caliginosa* (0.62 ± 0.12 g (mean body mass ± SD)) were sampled from an untreated INRA experimental orchard (no pesticide application for 5 years). For all experiments only adult earthworms were used. For the handling of the test organisms the general recommendations of Fründ et al. (2010) were followed.

2.3. Experimental design

All earthworms were pre-exposed individually to imidacloprid-treated (various concentrations) or control soil in Petri dishes before they were used for the short-term (2D) and long-term (3D) measurements of burrowing behaviour in uncontaminated soil.

2.4. Pre-exposure experiments

Prior to the exposure experiments, the soil was sieved to 3 mm and the soil water content was adjusted to 20% (i.e. 81% of the WHC) by adding distilled water. Subsequently, soil spiking was conducted according to the protocol of Capowiez et al. (2005). Therefore 40 mL of water or imidacloprid solution were added to the test soil, reaching a final soil water content of 25% (i.e. 101% of the WHC). Since the highest value for the predicted environmental concentration (PEC) of imidacloprid was found to be 0.66 mg kg^{-1} DW (Oi, 1999), we have defined this concentration to be the normal application rate (termed 1X). The earthworms were exposed to the following imidacloprid concentrations: 0 mg kg^{-1} (control); 0.2 mg kg^{-1} (0.3X); 0.66 mg kg^{-1} (1X); 2 mg kg^{-1} (3X) and 4 mg kg^{-1} DW (6X). For the measurements in the 2D-terraria the lowest concentration (0.2 mg kg^{-1} DW) was not tested in *L. terrestris* and the highest concentration (4 mg kg^{-1} DW) was not tested in *A. caliginosa*. The latter concentration proved to be highly lethal for *A. caliginosa* in preliminary range-finding tests (7 d exposure time).

The exposure experiments were carried out individually in Petri dishes (diameter = 10 cm) filled with 100 g of uncontaminated (control group with distilled water) or contaminated soil. Before and after the exposure experiments each specimen was rinsed in tap water, gently dried on filter paper and weighed. The Petri dishes were placed in dark climate chambers (12 °C) during earthworm exposure.

2.5. Short-term measurements of burrowing behaviour (2D-terraria)

Burrowing behaviour was assessed by means of 2D-terraria (Evans, 1947) after 1, 7 and 14 d of exposure time using 7 replicates for each treatment ($n = 7$). A detailed description of the 2D-terraria can be found in Capowiez (2000). The 2D-terraria consisted of two glass sheets (30 cm × 42 cm) and were adapted to the according species by fixing the sheets 3 mm (*A. caliginosa*) or 5 mm (*L. terrestris*) apart. The terraria were filled with sieved soil (2 mm) and the soil water content was adjusted to 101% of the WHC. After the exposure experiments in Petri dishes, the earthworms were put individually in the 2D terraria for 4 d and were kept in dark climate chambers (at 12 °C). The burrows were marked after 24 and 96 h on transparent sheets and were then digitised. Then the following four endpoints were computed for each individual: total length of burrows after 24 and 96 h as well as maximal burrow depth after 24 and 96 h in the terraria.

2.6. Long-term measurements of burrowing behaviour (3D soil cores)

Since several detrimental effects in burrowing behaviour (2D) (e.g. reduced maximal burrow depth; reduced total burrow length) were observed in both species only after 7 d of exposure time, we have chosen the latter period as the pre-exposure duration for the 3D long-term experiments. Repacked soil cores were prepared using PVC cylinders (35 cm in length and 16 cm in diameter) lined with a mixture of sealing varnish and sharp fine sand to prevent the earthworms from crawling along the PVC walls. A hydraulic press was used to compact five cores simultaneously. Cores were compacted by applying a pressure of 270 kPa for 5 min on sieved soil (3 mm) at 23% soil water content (i.e. 93% of the WHC)

(gravimetric). This treatment resulted in a soil dry bulk density of 1.1 g cm^{-3}. To minimise variations in soil bulk density between the top and bottom of the cores, the soil was compacted stepwise in 12 layers. Each layer comprised 600 g of soil. The final thickness of each layer was approximately 2.5 cm. Before adding a new soil layer, the surface of the previous layer was gently scratched using a small rake to increase cohesion between layers and the soil water content was adjusted to 101% of the WHC by adding distilled water to the soil surface. The bottom of each core was sealed and the top was closed using a perforated lid to prevent significant water losses. 20 cores were created and 6 earthworms (2 *L. terrestris* and 4 *A. caliginosa*) were put in each core according to the different pre-exposure treatments (5 concentrations including the control) using 4 replicates (cores). One further core was incubated with only 2 *L. terrestris* (unexposed) and another one with only 4 *A. caliginosa* (unexposed). Three further cores were left without earthworms (control cores without earthworms).

The cores were incubated in a dark climate chamber at 12 °C. Water (5 mL per core) was supplied weekly. Food (5 g of dried grass) was added at the top of each core. After 6 weeks, chloroform (10 mL) was applied to each core (using a fume hood) to kill the earthworms and prevent them from further burrowing. At the end of the experiment, cores were imaged using a medical X-ray tomograph (General Electrics; brightspeed exel) at the INRA Nancy centre to obtain a set of images 1.25 mm thick every 1.25 mm. The settings at which the X-ray beam was operated were 50 mA and 120 kV.

The burrow system in each core was reconstructed following the method proposed by Pierret et al. (2002). In brief, macropores were traced starting from the darkest voxels by studying local variation in mean grey level when the current voxel was included in the current macropore. Macropores that were too small (less than 100 voxels, i.e. about 0.5 cm^3) were discarded. At this stage, the volume of macroporosity, and the number of burrows (a burrow is a set of connected voxels) were computed.

Since the tested earthworm species differed greatly in size (thus in body diameter), we studied the distribution of macropore area for each species (results not shown) in order to finally be able to define which burrows were made by which species. This was done by analysing the 2D images of cores incubated with only *L. terrestris* or *A. caliginosa*. We found that the best approximation for the allocation of macropores was that macropores larger than 25 mm^2 were most likely made by *L. terrestris* (41.55 ± 21.12 mm^2 (mean ± SD)), while smaller ones were most likely created by *A. caliginosa* (15.28 ± 6.41 mm^2 (mean ± SD)) (assuming that the interspecific interactions between earthworms would not result in variations in mean burrow diameter). In addition to the assessment of overall effects on both species in combination, the latter approach enabled us to isolate and study behavioural effects on every single species.

2.7. Statistical analysis

Data were tested for normality as well as for homogeneity and not normally distributed data sets were log-transformed. The effects of imidacloprid contamination on the burrow length of *L. terrestris* and A. *caliginosa* in the 2D-terraria were examined by using one-way ANOVA and by conducting post hoc comparisons using Tukey–Kramer HSD. For the analysis of effects on maximal burrow depth in the 2D-terraria, Kruskal–Wallis tests followed by Holm-Bonferroni corrections were carried out.

For the 3D results, the number of replicates ($n = 4$) was too low and the variability of the data, as often observed for behavioural studies, was too high to use classical inference tests. The overall effect of imidacloprid concentrations on the 3D burrowing behaviour of both earthworm species was then analysed using a log-linear regression. The data for the estimation of macropores made by each species were not statistically tested since we did not find a method to accurately allocate all burrows to a definite species.

3. Results

3.1. Post-exposure modifications of burrowing behaviour in 2D

3.1.1. A. caliginosa

A. caliginosa maximal burrow depth (measured after 24 h and 96 h in the terraria) decreased with increasing imidacloprid concentration and exposure time (Table 1). The following treatments differed significantly from the respective control groups ($p < 0.05$): 1 d/3X, 7 d/0.3X, 7 d/1X, 7 d/3X, 14 d/0.3X, 14 d/1X and 14 d/3X (after 24 h in the terraria) as well as for 7 d/3X, 14 d/0.3X, 14 d/1X and 14 d/3X (after 96 h in the terraria).

The total length of burrows made by *A. caliginosa* (measured after 24 h and 96 h in the terraria) also decreased with increasing imidacloprid concentration and exposure time (Table 1). Significant differences to the control groups were observed for the following treatments ($p < 0.05$): 1 d/3X, 7 d/0.3X, 7 d/1X, 7 d/3X, 14 d/1X and 14 d/3X (after 24 h in the terraria) as well as for 1 d/1X, 7 d/3X and 14 d/3X (after 96 h in the terraria).

3.1.2. L. terrestris

In *L. terrestris* maximal burrow depth (measured after 24 h and 96 h in the terrarium) decreased with increasing imidacloprid concentration and exposure time except for the 1 d/6X treatment (Table 2). However, these decreases were only significant when maximal burrow depth was measured after 24 h in the terrarium and when the earthworms were exposed to 2 (3X) and 4 mg kg^{-1} DW (6X) for 7 d as well as to 2 (3X) mg kg^{-1} DW ($p < 0.05$).

The total length of burrows made by *L. terrestris* (measured after 24 h and 96 h in the terrarium) also decreased with increasing exposure concentration and time except for the 1 d/6X and 14 d/3X treatments (Table 2). However, significant differences in comparison to the control groups were only found after 7 d exposure to imidacloprid concentrations of 2 (3X) and 4 mg kg^{-1} DW (6X) (measured after 24 h in the terrarium) ($p < 0.05$).

3.2. Post-exposure modifications of burrowing behaviour in 3D

The 3D burrow systems made by the two uncontaminated earthworm species clearly differed visually: Burrows made by *L. terrestris* had a greater diameter and were more continuous than the ones made by *A. caliginosa* (Fig. 1). For both species, we observed a higher density of burrows in the upper part of the cores. For the combined controls (“both species”), we could visually distinguish between the burrows made by each species due to the diameter differences and no modification in burrowing patterns due to inter-specific disturbances were observed. In addition, no visual difference for the burrow systems of the pre-exposed earthworms (1X, 3X, 6X) compared to the burrow systems of control earthworms (“both species”) became obvious. In addition, no visual difference was observed between burrow systems of the pre-exposed earthworms and those of the control group.

However, quantifications of the burrow volumes enabled us to detect a significant decrease with increasing imidacloprid concentration (Fig. 2). The log-linear regression was significant ($p < 0.01$), but R^2 was low ($R^2 = 0.326$) due to the high variability. The approximated macropore area threshold used to separate burrows made by each species in 2D images (25 mm^2) was not ideal, since about 15% of the macropores were misclassified in the two cores incubated with only one species. This prevented us from applying a statistical analysis to the results. Moreover, we were not able to show significant differences in the amount of macropores created by

Table 1
Characteristics of the burrows systems made by *Aporrectodea caliginosa* (Mean ± SD) in the 2D-terraria dependent on exposure time (1, 7, 14 d) and imidacloprid concentration (0, 0.2, 0.66 and 2 mg kg^{-1} DW). Values for the contaminated groups are expressed in percentage of the respective controls. Asterisks indicate significant differences compared to the control ($p < 0.05$).

	1 d				7 d				14 d			
	Control	0.2 mg kg^{-1} DW (%)	0.66 mg kg^{-1} DW (%)	2 mg kg^{-1} DW (%)	Control	0.2 mg kg^{-1} DW (%)	0.66 mg kg^{-1} DW (%)	2 mg kg^{-1} DW (%)	Control	0.2 mg kg^{-1} DW (%)	0.66 mg kg^{-1} DW (%)	2 mg kg^{-1} DW (%)
Maximal burrow depth after 24 h (cm)	23.37 (10.38)	92.38 (43.84)	69.55 (43.13)	25.14* (10.93)	20.47 (11.51)	42.74* (26.62)	40.54* (20.98)	11.92* (9.9)	21.07 (9.76)	43.29* (23.04)	22.24* (13.31)	13.08* (14.59)
Maximal burrow depth after 96 h (cm)	32.84 (11.03)	96.01 (31.83)	74.25 (37.84)	63.19 (43.59)	29.43 (12.26)	81.90 (40.9)	70.62 (48.83)	39.53* (34.79)	35.2 (8.54)	55.75* (33.34)	48.30* (32.20)	26.72* (35.69)
Total burrow length after 24 h (cm)	35.05 (10.51)	100.3 (10.53)	77.43 (20.32)	63.47* (24.7)	29.64 (9.65)	65.18* (22)	58.62* (18.03)	15.70* (12.21)	26.83 (8.35)	74.1 (32.46)	42.22* (9.45)	15.7* (15.65)
Total burrow length after 96 h (cm)	99.41 (23.07)	95.65 (20.78)	75.97* (21.64)	85.5 (31)	68.91 (20.35)	93.85 (47.37)	90.32 (17.17)	38.55* (27.24)	69.59 (21.41)	71.74 (31.42)	73.26 (18.82)	32.86* (30.34)

Table 2
Characteristics of the burrows systems made by *Lumbricus terrestris* (Mean ± SD) in the 2D-terraria dependent on exposure time (1, 7, 14 d) and imidacloprid concentration (0, 0.66, 2 and 4 mg kg^{-1} DW). Values for the contaminated groups are expressed in percentage of the respective controls. Asterisks indicate significant differences compared to the control ($p < 0.05$).

	1 d				7 d				14 d			
	Control	0.66 mg kg^{-1} DW (%)	2 mg kg^{-1} DW (%)	4 mg kg^{-1} DW (%)	Control	0.66 mg kg^{-1} DW (%)	2 mg kg^{-1} DW (%)	4 mg kg^{-1} DW (%)	Control	0.66 mg kg^{-1} DW (%)	2 mg kg^{-1} DW (%)	4 mg kg^{-1} DW (%)
Maximal burrow depth after 24 h (cm)	16.91 (10.42)	68.81 (54.54)	41.66 (50.59)	39.9 (34.04)	12.74 (7.16)	84.4 (41.05)	35.01* (37.63)	30.64* (47.79)	20.72 (7.76)	69.35 (53.53)	51.93* (55.95)	61.97 (53.99)
Maximal burrow depth after 96 h (cm)	33.02 (11.25)	92.84 (42.88)	76.6 (55.62)	105.84 (36.68)	32.82 (12.32)	77.03 (47.26)	55.41 (45.36)	90.66 (37.85)	36.69 (5.82)	93.74 (41.36)	83.13 (43.59)	97.21 (17.18)
Total burrow length after 24 h (cm)	19.48 (12)	75.88 (56.5)	51.18 (43.26)	74.76 (64.71)	20.64 (12.35)	90.16 (38.03)	31.54* (38.9)	18.93* (30.18)	25.49 (8.8)	85.92 (39.3)	86.28 (45.43)	57.83 (48.16)
Total burrow length after 96 h (cm)	58.93 (24.35)	74.17 (34.68)	69.7 (50.36)	108.52 (37.49)	72.42 (33.52)	91.72 (51.26)	45.43 (39.43)	62.34 (40.01)	78.5 (30.04)	90.53 (43.6)	107.68 (53.06)	66.01 (19.55)

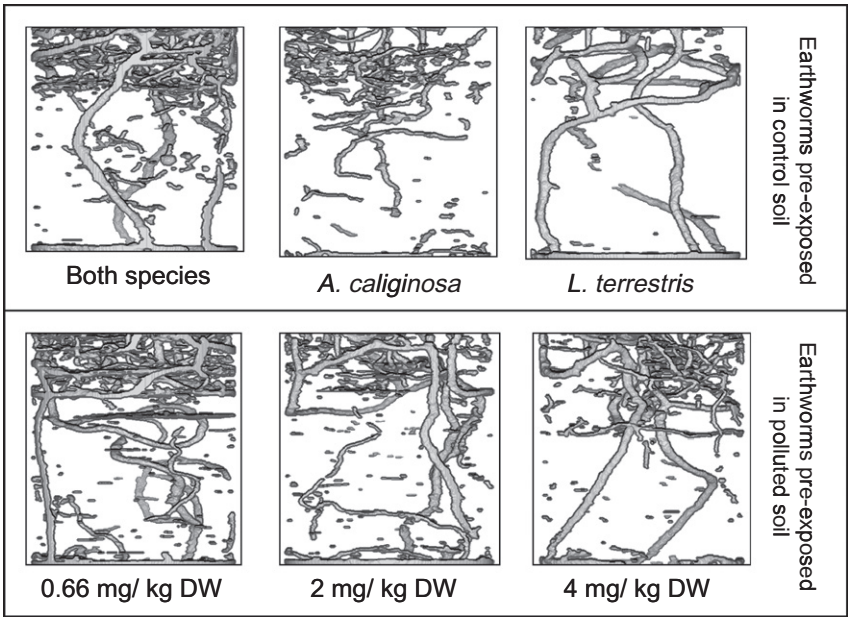

Fig. 1. Examples for 3D reconstructions of the burrow systems made by *L. terrestris* and *A. caliginosa* in the repacked soil cores during 6 weeks of incubation. Colours range from soft to dark grey according to the distance from the point of observation. Burrow systems shown in Fig. 1 on top were observed after exposure to control soil of either both species (4 *A. caliginosa* and 2 *L. terrestris*), or each species separately (4 *A. caliginosa* or 2 *L. terrestris*). The 3 burrow systems shown at the bottom – made by both species (4 *A. caliginosa* and 2 *L. terrestris*) – were observed after pre-exposure to different imidacloprid concentrations (0.66, 2 and 4 mg kg^{-1} DW).

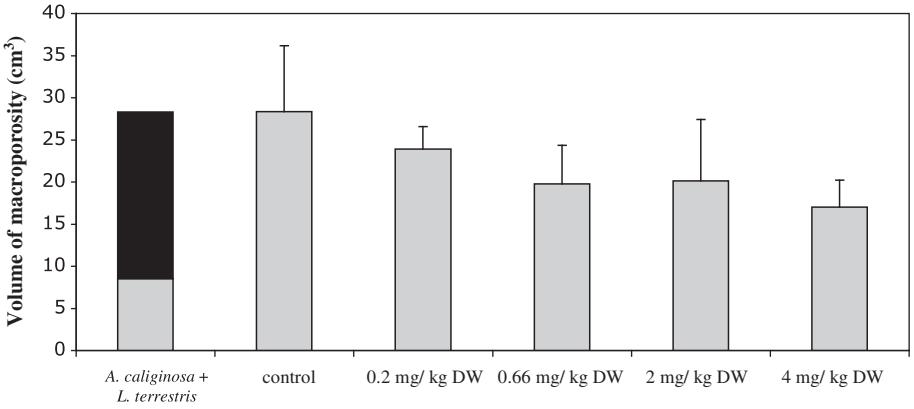

Fig. 2. Mean volume of macroporosity (+SD) measured in uncontaminated soil cores each incubated with 2 *L. terrestris* and 4 *A. caliginosa* for 6 weeks after a 7 d pre-exposure to different imidacloprid soil concentrations (0.2, 0.66, 2 and 4 mg kg^{-1} DW). The first bar is the sum of the macroporosity found in the mono-specific cores with either 2 *L. terrestris* (grey) or 4. *A. caliginosa* (black).

each species, even if a trend of fewer macropores for *A. caliginosa* at the highest imidacloprid concentration was observed in comparison to the control.

4. Discussion

In the present study both short- and long-term effects on behaviour were detected for a similar range of concentrations (0.2–4 mg kg^{-1} DW), for which previous ecotoxicological studies have observed different sub-lethal effects on earthworms after imidacloprid exposure ranging from changes in burrowing behaviour, sperm deformities, body mass losses to reduced cast production rates (e.g. Luo et al., 1999; Lal et al., 2001; Mostert et al., 2002; Capowiez et al., 2003b; 2010; Dittbrenner et al., 2010). Since behavioural changes can have detrimental effects on higher levels of biological organisation (e.g. changes in reproductive behaviour might affect population size) (Little, 1990; Doving, 1991; Scherrer, 1992) and since imidacloprid is frequently used in agriculture and was shown in the present study to have adverse effects on the earthworms' burrowing behaviour, already at concentrations relevant to natural conditions, (predicted environmental concentration (PEC) = 0.33–0.66 mg kg^{-1} DW) (Oi, 1999; Mostert et al., 2000), our results might be of great environmental concern.

The results obtained for burrowing behaviour by means of 2D-terraria showed that significant adverse effects in *A. caliginosa* occurred at very low imidacloprid concentrations (at 0.2 mg kg^{-1} DW after 7 and 14 d as well as at 0.66 mg kg^{-1} DW after 1 d), while significant negative effects for *L. terrestris* were only detected after exposure to ten times higher concentrations (2 and 4 mg kg^{-1} DW after 7 d of exposure). In a previous study on effects of imidacloprid on the burrowing behaviour of two other earthworm species (*Aporrectodea nocturna* and *Allolobophora icterica*) also using 2D-terraria, significantly reduced burrow lengths, burrow reuse rates and covered distances were observed for both species starting at soil concentrations of 0.5 mg kg^{-1} DW (Capowiez et al., 2003b). However, in the latter approach – unlike the experimental

design of the present study – the earthworms were not pre-exposed in Petri dishes, but were directly put into 2D-terraria filled with contaminated soil. Therefore changes on the effective burrow capacities of the earthworms could not be measured and thus the study could not reveal whether the detected effects were only due to physiological damage caused by the toxicant or whether the effects were influenced by bias (e.g. avoidance behaviour). In our study, however, we were able to exclude such bias and to demonstrate that the effects on burrowing behaviour were due to physiological damage in the earthworms.

Nevertheless, a lower sensitivity of *L. terrestris* compared with *A. caliginosa* towards imidacloprid has also been observed in previous studies in terms of heat shock protein (hsp70) induction, body mass change and cast production rate (Dittbrenner et al., 2010, submitted for publication a, submitted for publication b) and might generally be due to species-specific differences in sensitivity, higher ingestion rates of *A. caliginosa* and/or to the relatively low surface-volume ratio of *L. terrestris*. With respect to behavioural measurements by means of 2D-terraria, this method was only used with small earthworm species in the past (*A. nocturna*, *A. icterica*, *A. caliginosa*) (Capowiez et al., 2003b; Olvera-Velona et al., 2008). Even if the 2D-terraria were adapted to *L. terrestris* in the present study, they still might have been too small in order to offer sufficient space to detect effects more precisely. For example the maximal burrow depth of *L. terrestris* could easily exceed 40 cm (Shipitalo and Butt, 1999). In addition the burrow systems of *L. terrestris* are less complex than the ones of endogeic species (*A. caliginosa*) (Jégou et al., 1998; Bastardie et al., 2003a) meaning that behavioural effects in *A. caliginosa* might become more obvious at an earlier stage. Moreover anecic species like *L. terrestris* are known to reuse their burrows more often and to show less overall burrowing activity than endogeic species such as *A. caliginosa* (Bastardie et al., 2003a; Capowiez et al., 2003a). All of these facts might add up and lead to a hindered detection of detrimental effects on burrowing behaviour in *L. terrestris* compared with *A. caliginosa*, when using the present approach. However, one of our previous studies on sub-lethal effects of imidacloprid on *L. terrestris* showed significant cellular alterations as well as significant body mass losses occurring in this species already at lower imidacloprid concentrations (e.g. 0.66 mg kg^{-1} DW after 7 d) (Dittbrenner et al., submitted for publication b). This might support that the present 2D measurements were rather limited to sensitively detect sublethal effects in *L. terrestris*.

In general, total burrow length and maximal burrow depth after 24 h incubation in the 2D-terraria proved to be the most sensitive endpoints in the present study. Total burrow length represents a very important behavioural aspect, since it indicates general burrowing activity. Changes in maximal burrow depth should also be of high ecological importance, since reduced depths of earthworm burrow systems under natural conditions might have detrimental effects on gas/water transfer properties of soils and as a consequence might affect the whole ecosystem (Bastardie et al., 2003b; Capowiez et al., 2006).

While for *L. terrestris* significant effects on burrowing behaviour (2D) were found only after 7 d exposure time, significant detrimental effects for *A. calignosa* were found for all exposure periods (1, 7 and 14 d), and the number of significant endpoints grew with increasing exposure time. It seems very likely that *A. caliginosa* strongly suffered from the imidacloprid exposure towards the highest concentrations (0.66 and 2 mg kg^{-1} DW) and – unlike *L. terrestris* – was not able to adapt to the presence of the insecticide in our experiments. However, when looking at the effects measured during incubation in the 2D-terraria, one can clearly recognise that for both total burrow length as well as maximal burrow depth, the number of significant effects diminished with increasing incubation time (24 h vs 96 h). This makes evident, that without the presence of the insecticide after the exposure the earthworms were capable of recovering from adverse effects.

With respect to imidacloprid exposure of *L. terrestris* and *A. caliginosa*, measuring behavioural effects by means of 2D-terraria was more sensitive than by means of the avoidance test (ISO 17512-1 (2008)) – the most frequently used test to investigate impacts on behaviour in earthworms (Dittbrenner et al., submitted for publication a). In the latter study, the test showed no significant avoidance behaviour in *L. terrestris* and *A. caliginosa* for imidacloprid soil concentrations up to 2 mg kg^{-1} DW. However, a recently developed behavioural bioassay – the earthworm cast production test (Capowiez et al., 2010) – proved to be of a similar sensitivity (in the case of *L. terrestris* even of higher sensitivity) to the post-exposure 2D-burrowing behaviour assessment, when effects of imidacloprid on the earthworm species *L. terrestris* and *A. caliginosa* were examined. Significantly reduced cast production rates were observed for both species after 7 d of exposure to imidacloprid concentrations as low as 0.66 mg kg^{-1} DW (Dittbrenner et al., 2010).

The 3D burrow reconstructions of the control treatments are in agreement with the typical burrowing patterns known for each species (Jégou et al., 1998; Langmaack et al., 1999), indicating adequate conditions for both species in the repacked soil cores. The overall burrowing activity – measured by means of macropore volume in the soil cores – decreased significantly and log-linearly with increasing imidacloprid concentration (from 0.2 to 4 mg kg^{-1} DW). The mean burrow volume in the cores with earthworms pre-exposed to the highest imidacloprid concentration (4 mg kg^{-1} DW) showed a decrease of about 40% relative to the earthworms pre-exposed in control soil. Although we did not find a perfect way to classify macropores according to the earthworm species in the present study, our approximation by means of macropore area gave satisfying results. By using this approximation and then separating the burrow systems of both species, a trend for greater sensitivity of *A. caliginosa* compared to *L. terrestris* could be elucidated at the highest imidacloprid concentration, which, however, could not be confirmed statistically. In comparison to the results obtained by the 2D measurements, the 3D measurements were less sensitive, but proved to be a mere confirmation of the effects observed for burrowing activity in the 2D-terraria. Significant long-term effects of imidacloprid on the burrowing behaviour of *L. terrestris* and *A. caliginosa* were measured in the soil cores, but due to a relatively low replicate number only a linear regression could be used to analyse the present data.

However, it was not very surprising to discover that the detected long-term effects (3D) were less obvious than the measured short-term effects (2D), given that the earthworms spent 6 weeks in uncontaminated soil cores compared to 96 h in uncontaminated 2D-terraria enabling them to recover from the sub-lethal effects. Nevertheless, the results obtained by means of 3D burrow reconstruction should raise great concern about the frequent use of imidacloprid in agriculture, since a reduced burrowing activity of earthworms over a longer period of time might have crucial effects on soils. Moreover, under natural conditions effects are likely to be more severe than the ones measured in the soil cores, since (1) the half-life of imidacloprid in soils can be greater than 1 year (Sabbagh et al., 2002) resulting in a long-term exposure of earthworms, and (2) because a number of different pesticides are often used in one plot favouring mixture toxicity. Comparing the present post-exposure method for assessing long-term effects on burrowing behaviour (3D) with the direct-exposure method developed by Capowiez et al. (2003a) in terms of adequacy, the first one seems to be better suited for testing of rapidly degraded toxicants, while the latter might be preferred when testing rather persistent pollutants (e.g. copper). This is due to big differences in half-life of different pollutants.

In conclusion, we have found significant adverse effects of imidacloprid on the burrowing behaviour of two earthworm species (*A. caliginosa* and *L. terrestris*) in environmentally relevant concentrations in both short- and long-term experiments in the laboratory. With respect to the measurements of burrowing activity, the results obtained from the 3D reconstruction of burrows proved to be a mere confirmation of the results observed by means of 2D-terraria, but were less sensitive. Due to the frequent use of imidacloprid in agriculture and its relatively long half-life in soils, our results are of environmental concern. From a methodological point of view, 2D terraria appeared to be a convenient tool (high number of replicates, responses after only 24 or 96 h) to analyse ecologically meaningful modifications of earthworm behaviour but for short term effects. 3D reconstructions provided a relevant tool to determine modifications of earthworm behaviour and some of their effects on the soil functioning (e.g. gas transfer properties, water infiltration). However the cost and the complexity of this method generally results in a low number of replicates and thus prevents a powerful statistical analysis.

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