Ecotoxicology
DOI 10.1007/s10646-010-0542-8

Physiological and behavioural effects of imidacloprid on two ecologically relevant earthworm species (*Lumbricus terrestris* and *Aporrectodea caliginosa*)

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Accepted: 25 August 2010

Abstract Earthworms play key roles in soils and sub-lethal effects of environmental toxicants on these organisms should be taken seriously, since they might have detrimental effects on higher ecological levels. In laboratory experiments we have assessed sub-lethal effects (body mass change and cast production) of imidacloprid on two earthworm species commonly found in different agricultural soils (*Lumbricus terrestris* and *Aporrectodea caliginosa*). After 7 days of exposure in contaminated soil, a significant loss of body mass was found in both species exposed to imidacloprid concentrations as low as 0.66 mg kg^{-1} dry soil. These losses ranged from 18.3 to 39% for *A. caliginosa* and from 7.4 to 32.4% for *L. terrestris*, respectively. Changes in cast production, a new biomarker previously validated using *L. terrestris*, was assessed by soil sieving using the recommended mesh size (5.6 mm) for *L. terrestris* and three different mesh sizes for *A. caliginosa* (5.6, 4 and 3.15 mm). The 4 mm mesh size proved to be the most suitable sieve size for *A. caliginosa*. Cast production increased by 26.2% in *A. caliginosa* and by 28.1% in *L. terrestris* at the lowest imidacloprid concentration tested (0.2 mg kg^{-1} dry soil), but significantly decreased at higher concentrations (equal to and above 0.66 mg kg^{-1} dry soil) in both earthworm species after the 7 days exposure experiment. These decreases in cast production ranged from 44.5 to 96.9% in *A. caliginosa* and from 42.4 to 95.7% in *L. terrestris.* The EC_{50} for cast production were 0.84 (*L. terrestris*) and 0.76 mg kg^{-1} dry soil (*A. caliginosa*), respectively. The detected sub-lethal effects were found close to the predicted environmental concentration (PEC) of imidacloprid, which is in the range of 0.33–0.66 mg kg^{-1} dry soil. The biomarkers used in the present study, body mass change and changes in cast production, may be of ecological relevance and have shown high sensitivity for imidacloprid exposure of earthworms. The measurement of changes in cast production should be considered for inclusion in current standard tests.

Keywords Earthworms · Imidacloprid · Cast production · Body mass change

Introduction

Imidacloprid is a relatively new neonicotinoid insecticide which is commonly used worldwide in agriculture against sucking insects. It shows selective toxicity for insects (Matsuda et al. 2001), but there is evidence of effects on non-target and ecologically important organisms (Mostert et al. 2002; Iwasa et al. 2004; Kreutzweiser et al. 2009).

Earthworms are of crucial importance for soil functioning, they make important contributions to the breakdown of organic matter, soil fertility, and to the formation of soils (Edwards and Bohlen 1992, 1996). Because of their ecological importance, their high biomass in soil and their frequently observed sensitivity to relatively low concentrations of environmental toxicants, they are ideal test organisms for soil risk assessment (Bouché 1992; E.E.C. 2003).

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Published online: 05 September 2010

The few existing standard tests for earthworms are carried out mainly with *Eisenia fetida* and *Eisenia andrei* and focus on mortality, reproduction and avoidance behaviour (OECD 1984, 2004; E.E.C. 2003; ISO 2008). However, *E. fetida* and *E. andrei* are epigeic species ecologically not relevant for pesticide testing since these earthworms are absent from most agricultural soil and often claimed to be less sensitive to environmental toxicants than other earthworm species (Edwards and Coulson 1992; Spurgeon and Weeks 1998). According to our opinion, the relevance of a toxicity test is based on the possibility to link the response to indispensable soil functions (E.E.C. 2003). In this context, some behavioural endpoints are important and efforts should be made to develop, optimise and increase their use in earthworm toxicity testing. However, care must be taken, since behavioural responses might vary greatly from species to species (Gilman and Vardanis 1974).

Earthworm behavioural biomarkers range from avoidance tests (Slimak 1997; Hodge et al. 2000; Schaefer 2003) to studies on burrowing behaviour using 2D and 3D techniques (Hans and Beg 1992; Capowiez et al. 2003, 2006). Avoidance behaviour can be measured easily, but results must be interpreted carefully, since some toxicants might attract organisms, whereas others might repel them (Yeardley et al. 1996). Studying changes in earthworm behaviour via 2D and 3D techniques is very sensitive and promising, but time consuming and therefore not worth taking into account as potential standard tests (Eijsackers et al. 2001; Capowiez et al. 2006). Edwards and Lofty (1972) claimed that cast production is an important indication of earthworm activity and some studies have shown reduced cast production of earthworms (Cook et al. 1980; Lal et al. 2001) or reduced ingestion rates of earthworms after pesticide treatment (Gomez-Eyles et al. 2009). Capowiez et al. (2010) have developed a new and standardisable protocol for toxicity testing, based on changes in earthworm cast production as a proxy of changes in activity. It was successfully applied to an anecic species (*Lumbricus terrestris*) using six pesticides, but the suitability of this test for endogeic species need to be tested. In their study, these authors have found a significant decrease of cast production after exposure to imidacloprid at concentrations as low as the normal application rate (70 g ha^{-1}), but no effect for a concentration ten times lower.

On a physiological level, body mass change has already been successfully used as a biomarker in earthworms and is well established since it is an integral part of the acute and chronic standard tests. It can be interpreted as indication of general health (Leland et al. 2001; Zwahlen et al. 2003; Olvera-Velona et al. 2008).

The aim of the present study was then to assess sublethal effects of imidacloprid on two species ecologically relevant for agricultural soil (*L. terrestris* and *Aporrectodea caliginosa*) by using the earthworm cast production test and the measurement of body mass changes. Therefore the suitability of cast production test for *A. caliginosa* had to be evaluated and the test had to be adapted to this endogeic species. With the present study we also wanted to contribute to the development of the cast production test as a potential standard test. Moreover we wanted to determine if imidacloprid concentrations between the normal application rate and one-third of it would still have significant effects in both earthworm species.

Materials and methods

Test organisms, soil and pesticide

Adult earthworms of the species *A. caliginosa* were collected from an INRA experimental orchard near Avignon (France), where no pesticide was applied for 5 years. Adult and subadult earthworms of the species *L. terrestris* (raised in Canadian farms) were purchased from a fishery store in Avignon. The soil (23.4% clay, 57% silt, 19.6% sand, 28.3 g kg^{-1} organic matter, pH = 8.3 (in water), CEC = 8.2 cmol kg^{-1}, WHC = 0.247 g g^{-1}) was collected from another orchard (abandoned for about 10 years) close to Avignon (France). Before the experiments, the earthworms were acclimatized for 7 days in the orchard soil in a climate chamber (12°C) under conditions of complete darkness. Handling of our test organisms prior to the experiments was based on the general recommendations established by Fründ et al. (2010).

The insecticide imidacloprid (255.66 g/mol; purity: 99.9%) was purchased from FLUKA (No. 37894) and dissolved in distilled water to different concentrations.

Experimental design

Prior to the exposure experiments, the soil was sieved to 3 mm. Soil water content was measured and adjusted to 20% (80% of the WHC) by adding distilled water thoroughly mixed and sieved again to 3 mm. Then soil spiking (for both, pesticide exposure groups and control groups) was conducted according to the protocol of Capowiez et al. (2005) by adding 40 ml of water or solution containing the adapted pesticide concentration, reaching a final soil water content of 25% (of dry soil weight). The predicted environmental concentration (PEC) of imidacloprid was found to be in the range of 0.33–0.66 mg kg^{-1} dry soil depending on the country and crop under consideration (Oi 1999; Mostert et al. 2000). We have defined the normal application rate (1×) as to be 0.66 mg kg^{-1} dry soil corresponding to a field application rate of 244 g ha^{-1}. The soil

concentration value refers to a single application with a homogeneous distribution in the upper 5 cm of soil with a density of 1.5 kg l^{-1} and no crop interception. For both the cast production test and body mass change measurements, 25 individuals of each species were exposed to each of the following concentrations: 0 (control), 0.2 (0.3×), 0.66 (1×), 2 (3×) and 4 (6×) mg kg^{-1} dry soil (the latter concentration was only used for experiments conducted with *L. terrestris,* since it was lethal for *A. caliginosa*). Additionally the same concentrations were used to pollute the soil without earthworms as controls for the cast production test ($n = 10$ for each concentration tested).

Petri dishes (diameter = 10 cm) were filled with 100 g of uncontaminated (control group with distilled water) or contaminated moist soil. For the exposure experiments each earthworm was rinsed in tap water, gently dried on filter paper, weighed and randomly put in individual Petri dishes. Afterwards the dishes were placed in a dark chamber at 12°C. After 7 days of exposure, the earthworms were taken out of the dishes with soil, rinsed, gently dried on a filter paper and weighed again and the soil was kept apart for the cast production test. The weighing procedures before and after the experiment were done without voiding the guts of the earthworms, so body mass change was rather an indication for feeding activity than for growth (the latter is limited for adult earthworms during an exposure time of 7 days).

The cast production test was conducted according to the protocol of Capowiez et al. (2010). Therefore, the soil in each dish was taken out carefully and sieved (diameter = 15 cm) manually by consistently shaking the sieve for 10 s. To determine the cast production of *L. terrestris*, we used a sieve with a mesh size of 5.6 mm since Capowiez et al. (2010) showed that values found for smaller mesh sizes (4 and 3.15 mm) were either very low or zero. For *A. caliginosa* the soil was sieved using a set of sieves (mesh sizes = 5.6, 4 and 3.15 mm). The additional 3.15 and 4 mm sieves were chosen in order to determine the most suitable mesh size for this species since cast produced by *A. caliginosa* were relatively smaller.

The remaining soil in each sieve was weighed and cast production was computed by subtracting the mass of the remaining soil in the control sieves (control without earthworms) from the corresponding mass in the sieves of the test samples. Since a linear correlation was found for *L. terrestris* between weight of produced casts and earthworm biomass (Capowiez et al. 2010), every single value for cast weight in each Petri dish was divided by the body mass of the corresponding earthworm. This resulted in cast production values expressed as cast fresh weight per earthworm fresh body mass per exposure day (g g^{-1} day^{-1}). Because we could not find a significant correlation between cast production and earthworm body mass in *A. caliginosa*, expressing cast production rate dependant on earthworm body mass became dispensable. But for the sake of a better comparison, we have used cast production rate (in g g^{-1} fresh body mass day^{-1}) in both species as a unit for expressing the amount of produced casts.

Statistical analysis

Data (body mass change or cast production) were tested for normality and homoscedasticity and were log-transformed when necessary. For both species, the effect of imidacloprid concentration either on body mass change or cast production was analysed by a one-way ANOVA and post-hoc comparisons were done using Tukey-HSD. The relationship between cast production and earthworm body mass was investigated using linear regression.

Median effective concentrations (EC_{50}) for cast production were computed using the linear interpolation (ICp) method.

Results

No mortality was observed except that one individual died at one time in the 0.3× and 1× exposure group of *L. terrestris* and in the 3× exposure group of *A. caliginosa*.

Body mass change

For *A. caliginosa* significant losses in body mass were observed ($p < 0.0001$; $F = 115.2$; df = 3). The 1× and 3× exposure groups were significantly different from the control group at $p < 0.05$ (Table 1). For *L. terrestris* body mass change differed significantly as well ($p < 0.0001$; $F = 50.8$; df = 4). The 1×, 3× and 6× exposure groups were significantly different (compared to the control group) at $p < 0.05$ (Table 2).

Cast production

First of all the results obtained for *A. caliginosa* using three different sieve sizes (3.15, 4 and 5.6 mm) were compared. Four and 5.6 mm mesh size resulted in the same significant differences, but higher cast weights were obtained when using the 4 mm mesh size (Table 3). For 3.15 mm mesh size no significant difference between control group and 1× exposure group was observed (Table 3).

While a significant but low positive relationship between cast production and earthworm body mass was found for the control group of *L. terrestris* ($p = 0.001$ and $r^2 = 0.2$), cast production (for all mesh sizes) of the control group of *A. caliginosa* did not correlate with earthworm body mass.

Significant differences in cast production were observed in the earthworm species *A. caliginosa* ($p < 0.0001$;

Table 1 Mean initial body mass (+SD), mean body mass after 7 days (+SD) and mean body mass after 7 days relative to initial body mass (+SD) (expressed in percentage) of *Aporrectodea caliginosa* after imidacloprid exposure for 7 days ($n = 25$)

	0 mg kg^{-1} dry soil	0.2 mg kg^{-1} dry soil	0.66 mg kg^{-1} dry soil	2 mg kg^{-1} dry soil
Initial body mass (g)	0.61 (0.15)	0.66 (0.19)	0.60 (0.16)	0.65 (0.20)
Body mass after 7 days (g)	0.67 (0.18)	0.68 (0.18)	0.54 (0.14)	0.46 (0.13)
Relative body mass after 7 days (%)	109.3 (10.6)	104.5 (7.5)	**91** (**9.3**)	**70.3** (**5.9**)

Values in bold are significantly different from the control values ($p < 0.05$)

Table 2 Mean initial body mass (+SD), mean body mass after 7 days (+SD) and mean body mass after 7 days relative to initial body mass (+SD) (expressed in percentage) of *Lumbricus terrestris* after imidacloprid exposure for 7 days ($n = 25$)

	0 mg kg^{-1} dry soil	0.2 mg kg^{-1} dry soil	0.66 mg kg^{-1} dry soil	2 mg kg^{-1} dry soil	4 mg kg^{-1} dry soil
Initial body mass (g)	4.10 (1.09)	3.91 (1.03)	3.95 (0.68)	4 (1.58)	4.12 (1)
Body mass after 7 days (g)	4.74 (1.35)	4.56 (1.29)	4.28 (0.8)	3.82 (1.69)	3.43 (1.26)
Relative body mass after 7 days (%)	115.6 (7.9)	116.6 (9.4)	**108.4** (**12.1**)	**95.5** (**8.3**)	**83.3** (**4.7**)

Values in bold are significantly different from the control values ($p < 0.05$)

Table 3 Effect of mesh size on the estimation of mean cast production (+SD) (in g cast weight g^{-1} earthworm body mass day^{-1}) of *Aporrectodea caliginosa* after exposure to different concentrations of imidacloprid for 7 days ($n = 25$)

Mesh size (mm)	0 mg kg^{-1} dry soil	0.2 mg kg^{-1} dry soil	0.66 mg kg^{-1} dry soil	2 mg kg^{-1} dry soil
3.15	2.63 (1.27)	3.38 (1.75)	1.8 (1.7)	**−0.07** (**1.32**)
4	2.56 (1.27)	3.23 (1.57)	**1.42** (**1.34**)	**0.08** (**1.23**)
5.6	1.89 (1.06)	2.44 (1.18)	**0.93** (**0.9**)	**−0.01** (**0.83**)

Values in bold are significantly different from the control values ($p < 0.05$)

$F = 35.8$; df = 3). In comparison to the control group, mean cast production significantly decreased for the 1× and 3× exposure groups (−44.5 and −96.9% respectively) at $p < 0.05$ (Table 3).

Compared to the control group, mean cast production in the species *L. terrestris* was found to be significantly different in all exposure groups ($p < 0.0001$; $F = 139.1$; df = 4). For the 0.3× group cast production increased (+28.1%) and considerably decreased in the 1×, 3× and 6× exposure groups (−42.4, −57.2 and −95.7%, respectively) at $p < 0.05$ (Fig. 1). The EC_{50} for changes in cast production were 0.84 (*L. terrestris*) and 0.76 mg kg^{-1} dry soil (*A. caliginosa*), respectively. They were calculated by also including the induction of cast production in the 0.3× exposure groups in both species.

In general, mean cast production rate was about four times higher in *A. caliginosa* (2.56 g g^{-1} day^{-1}) than in *L. terrestris* (0.65 g g^{-1} day^{-1}) when comparing the control groups (mesh size = 4 and 5.6 mm respectively).

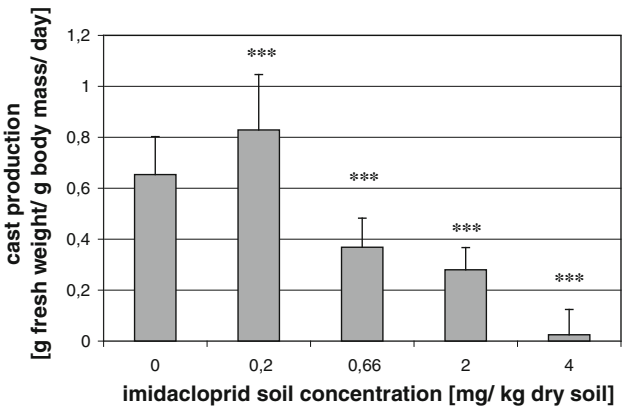

Fig. 1 Mean cast production (+SD) (in g casts g^{-1} earthworm body mass day^{-1}) of *Lumbricus terrestris* after exposure to different concentrations of imidacloprid for 7 days ($n = 25$). A sieve with a mesh size of 5.6 mm was used. *Asterisks* indicate significant differences from the control ($p < 0.001$)

Discussion

Imidacloprid is known to cause different sub-lethal effects in earthworms as e.g. at behavioural, physiological as well as on molecular levels (Luo et al. 1999; Schaefer 2003; Capowiez et al. 2006; Olvera-Velona et al. 2008; Gomez-Eyles et al. 2009). The LC_{50} value of imidacloprid in different earthworm species (*E. fetida*, *L. terrestris*, *Aporrectodea nocturna*, *Allolobophora icterica, and Pheretima* group) is between 2.3 and 4 mg kg^{-1} dry soil (Luo et al. 1999; Mostert et al. 2002; Capowiez et al. 2006; Gomez-Eyles et al. 2009).

Changes in earthworm body mass (as a biomarker of effect) are thought to be of ecological relevance. It is often assumed that high losses in body mass may lead to negative effects on reproduction and survival (Capowiez et al. 2005; Olvera-Velona et al. 2008). We found body mass change to be significantly different from the control groups starting for concentrations of imidacloprid as low as 0.66 mg kg^{-1} dry soil for both species. Our results proved to be in the range of those found in previous studies, in which significant effects of imidacloprid on body mass change in different earthworm species (*E. fetida*, *A. nocturna*, *A. icterica*, and *L. terrestris*)—exposed in soils with different properties—were found at concentrations between 0.5 and 1.91 mg kg^{-1} dry soil (Mostert et al. 2002; Capowiez et al. 2005; Gomez-Eyles et al. 2009). Nevertheless, one has to bear in mind that in our study body mass change was rather a measure of earthworm activity (filling of the gut) than of growth, since the exposure time was relatively short and since *L. terrestris* and *A. caliginosa* are slower growing species than the common test species *E. fetida* or *E. andrei*. However, the entire biological implications (for the ecosystems functions) of these losses in body mass due to reduced earthworm activity are hard to predict from such short-term exposures.

The measurement of changes in cast production is a new and promising behavioural biomarker in ecotoxicology. It is of ecological relevance, since reduced cast production demonstrates a reduced (feeding) activity and in consequence might have indirect impacts on soils (Capowiez et al. 2010). Indeed cast production is an important function of earthworm activities i.e. organic matter mixing with soil aggregates and thus organic matter protection and hot spots for micro-organisms (Binet and Le Bayon 1999).

In this study, cast production increased in both species after exposure to the lowest concentration of imidacloprid (0.2 mg kg^{-1} dry soil), but only significantly for *L. terrestris*, while the exposures to higher concentrations (0.66–4 mg kg^{-1} dry soil) caused significant decreases for both species. Other studies have observed reduced total or surface cast production after imidacloprid exposure for *L. terrestris* at concentrations starting from 0.5 mg kg^{-1} dry soil using the same test soil as was used in this study (Capowiez et al. 2006, 2010). In former studies reduced total or surface cast production was also described after exposure to a range of other different pesticides and species in laboratory as well as in field experiments (Lal et al. 2001; Capowiez et al. 2010). In our study, the increase of cast production after exposure to the lowest concentration might be explained by higher earthworm activity due to escaping or avoidance behaviour and/or an increased metabolic rate possibly caused by detoxification processes. Avoidance behaviour may not harm earthworms directly, but could still have negative impacts on soils, like consequences related to reduced leaf-litter breakdown (Kreutzweiser et al. 2009). The drastic decrease in cast production at higher concentrations found in this study might be due to the neurotoxic effect of imidacloprid inhibiting earthworms in their normal feeding behaviour. In general, such biphasic dose responses (low-dose stimulation and high-dose inhibitory effect) are referred to as the phenomenon of hormesis and can often be observed in earthworms after exposure to environmental agents (Spurgeon et al. 2004; Hackenberger et al. 2008; Zhang et al. 2009).

Cast production rate in the control groups was about four times higher in *A. caliginosa* compared to *L. terrestris*. This could possibly be explained by higher ingestion/egestion rates of endogeic species (Lavelle et al. 1989), but also by allometric differences of metabolic rates due to body mass differences. The cast production rate found for the control group of *L. terrestris* (0.65 g g^{-1} fresh body body mass day^{-1}) is close to the one described by Capowiez et al. (2010) for the same species (between 0.8 and 0.9 g g^{-1} fresh body mass day^{-1}), indicating a good reproducibility. In previous studies on cast production of *A. caliginosa* (Scheu 1987; Curry and Baker 1998), casts were dried before weighing and therefore direct comparisons with our results are difficult.

However, the measurement of changes in cast production proved to be a sensitive biomarker for sub-lethal effects in both tested earthworm species. This was unclear prior to our study, since the mean body mass of *A. caliginosa* was about one-sixth/seventh of the one of *L. terrestris* [which was used as a model organism in the protocol of Capowiez et al. (2010)] and therefore their casts could have been too small to separate from the soil using the sieving method. Compared to the results of two biggest mesh sizes (5.6 mm and 4 mm), the use of the smallest mesh size (3.15 mm) did not result in significant differences between control and 1× exposure group and therefore 3.15 mm seemed to be the least suitable mesh size for the sieving test with *A. caliginosa* under our experimental conditions. The two biggest mesh sizes used to assess the cast production of *A. caliginosa* led to the same significant differences, but the sieve with the 4 mm mesh size proved to be more suitable, because it led to higher cast weights (Table 3). In general, using the sieving method always has to be adapted to the chosen earthworm species.

Even if this biomarker seems not to be suited for epigeic earthworm species (Capowiez et al. 2010), it seems to be very likely that the method functions well in anecic and endogeic earthworm species.

In conclusion, sub-lethal effects of imidacloprid on two earthworm species were found in the range of the predicted environmental concentrations (PEC) (0.33–0.66 mg kg^{-1}

dry soil) of this substance. Considering the extensive use of imidacloprid in agriculture, the effects could be of high importance, since they might be related to constraints of earthworms in their role as "soil engineers" and consequently may have crucial impacts on soils.

Both parameters tested, body mass change and cast production, proved to be sensitive biomarkers, easy and rapid to handle. The recently developed cast production test seems promising for future earthworm ecotoxicity testing and might be considered for inclusion in current standard tests or even as potential standard test itself.

Acknowledgments The authors are grateful to Kate Stevens for carefully reading the manuscript. Odile Mascle is thanked for her help. Evangelisches Studienwerk and FAZIT-Stiftung are highly acknowledged for financial support. The authors also thank the anonymous reviewers for their suggestions and comments.

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