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Assessment of the Effects of the Pesticide Imidacloprid on the Behaviour of the Aquatic Oligochaete *Lumbriculus variegatus*

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Abstract Contaminants, such as pesticides, can cause direct toxic effects when released into aquatic environments. Suitably sensitive species can help us understand and predict the impacts of such pollutants. Automated sediment toxicity testing and biomonitoring has grown rapidly, and biomonitoring instruments have proven appropriate for studying the effects of pollutants. A new approach in online biomonitoring, using the multispecies freshwater biomonitor was developed in the present study, using whole-sediment toxicity tests and behavioural responses of the freshwater oligochaete *Lumbriculus variegatus*. Endpoints, such as mortality and growth, were used to study the effects of the pesticide imidacloprid and to achieve a gradient of responses; exposures to contaminated sediments were performed over 10 days' duration (short-term tests). High mortality was observed in the three highest concentrations of imidacloprid, and inhibition of behaviour was monitored along a gradient of pesticide concentration. Exposure to imidacloprid-contaminated sediments affected growth, behaviour, and avoidance in *L. variegatus*.

Mortality, bioaccumulation, growth, and reproduction have been the most common endpoints used in the majority of studies in environmental toxicology (Leppänen and Kukkonen 1998). Data are lacking on sublethal toxicologic endpoints, such as effects on morphology or behaviour. Without this kind of information, complex biologic actions cannot be fully understood, and reliable predictions of ecologic impacts of environmental toxicants cannot be made (Rogge and Drewes 1993). Since the 1980s, there has been increasing interest in investigating sublethal endpoints (Aisemberg et al. 2005). Behaviour may be an important endpoint to elucidate mechanisms of toxicity (Macedo-Sousa et al. 2007). Once quantified, a behaviour has the potential to be used as a biomarker in the assessment of stress (Beitinger 1990). Biomonitoring offers a useful tool for the assessment of metal pollution in aquatic ecosystems (Zhou et al. 2008), and should rely on sublethal endpoint rather than on mortality alone (Macedo-Sousa et al. 2007).

Construction and functioning of the multispecies freshwater biomonitor (MFB) has been described elsewhere. Briefly, it measures online the different behaviours of aquatic species and is based on the registration of changes in a high-frequency alternating current caused by movements of organisms in their test chambers (Macedo-Sousa et al. 2008). The individual test organism is placed in a test chamber with two pairs of stainless steel-plate electrodes. Different types of behaviour (movements) generate characteristic electrical signals (Macedo-Sousa et al. 2008) that can be characterized by their amplitude and frequency. For *L. variegatus*, two different movements can be measured: peristaltic movements (0.5–1 Hz) and locomotion (1–3 Hz). The electrical signals are processed by a discrete fast Fourier transformation and generate a histogram of the occurrence of all signal frequencies in percentages (summarized in intervals of 0.5 Hz from 0 to 10 Hz), yielding a "fingerprint" of the behavioural pattern of the organism. This transformation gives the percentage of occurrence of each single frequency during a period of 4 min. The unit for measurement is the test chamber, which can have different sizes, forms, materials, and arrangements of electrodes. This method has been shown to be a valuable

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biomonitoring and toxicity testing tool using epibenthic crustaceans, insects, and planktonic and pelagic species of fish and tadpoles (Gerhardt 2000).

Aquatic species are important contributors to the functioning of lotic foodwebs (Benke and Jacobi 1994; Thorp and Delong 2002), which is why they were chosen as test species for the present study. Oligochaete worms are key macroinvertebrate constituents of terrestrial and freshwater ecosystems (Edwards and Lofty 1977; Brinkhurst and Gelder 1991). The locomotion and other behavioural activities of these organisms are significant determinants of the physical, chemical, and biologic properties of soils and sediments. In addition, locomotor functions are the cornerstone of such vital functions as foraging, sexual reproduction, predator avoidance, dispersal, and general orientation to environmental cues (Drewes 1997). Aquatic oligochaetes have an extremely long history of use in pollution assessment (Chapman 2001). *Lumbriculus variegatus* (Müller 1774) is recommended for use in toxicity tests with sediments based on its ease of culture and handling, known chemical exposure history, adequate tissue mass for chemical analysis, tolerance to a wide range of sediments' physicochemical characteristics, low sensitivity to contaminants associated with sediment, and amenability to long-term exposure without feeding (Ingersoll et al. 2003). Judging by the number of internationally published articles, the most common oligochaete species used in evaluations of freshwater toxicity has been *L. variegatus* (Leppänen 1999; Aisemberg et al. 2005). This species was proposed by the American Society of Testing and Materials (ASTM 1995) as a standard organism for tests of sediment bioaccumulation and is listed by the Organization for Economic Co-operation and Development (1992) as a good organism for bioaccumulation studies. *L. variegatus* is a freshwater oligochaete known to have remarkable powers of segmental regeneration (Hyman 1916). Reproduction under laboratory conditions is always by asexual fragmentation, during which a worm spontaneously divides into two or more body fragments. Each surviving fragment then undergoes rapid regeneration of body segments to form a new head, tail, or both (Lesiuk and Drewes 1999).

All pesticides on the market have been evaluated by sets of standardized protocols (the so-called a priori evaluation). One of the aims of standardized tests is to evaluate the negative effects of pesticides on terrestrial and aquatic ecosystems. Because the direct impact on ecosystems is difficult to study, the tests are based on the detrimental effects observed in a set of model organisms that play key roles in ecosystem structure and function. However, although the European Commission (EC) encouraged the development of tests to determine sublethal effects on model organisms, most of these protocols focused on mortality (Capowiez et al. 2005). The EC recognized the importance of sublethal tests, for earthworms in particular, when the active substance is potentially persistent or applied more than once (EC 2003).

Imidacloprid [1-(6-chloro-3-pyridylmethyl)-2nitroimino-imidazolidine] is a relatively new systemic insecticide (product names Admire, Confidor, Gaucho, and Provado, manufactured by Bayer Cropscience). It was the first member of a new family, the neonicotinoids, and is chemically related to the nicotinic acetylcholine receptor (nAChR) agonists nicotine and epibatidine (Matsuda et al. 2001). It acts as an agonist of acetylcholine (Bai et al. 1991) and is therefore effective against many insects currently resistant to carbamates, organophosphates, and pyrethroids. It was first introduced to the United Kingdom in 1998 and is now marketed in >120 countries to protect >140 crops (Simms et al. 2006). It is widely used in agriculture for controlling sucking insects, as a seed dressing, for soil treatment, and as a foliar treatment for a variety of crops, including orchards. It is also used for controlling cockroaches and termites and is found in many products used for domestic pets and in gardens (Cox 2001). Some studies have shown that imidacloprid can induce behaviour modifications in parasitoid hymenoptera (Stapel et al. 2000) and termites (Thorne and Breisch 2001) (foraging and burrowing activities, respectively). The effects of imidacloprid on earthworms have been studied to a certain extent. Luo et al. (1999) and Zang et al. (2000) found sperm deformities in *Eisenia fetida* at imidacloprid concentrations as low as 0.5 mg/kg in dry soil. More recently, Mostert et al. (2000, 2002) showed that the LC_{50} for worms of the Pheretima group was 3 mg/kg in dry soil and that no effect was observed on earthworm weight at 0.66 mg/kg in dry soil. Finally, Lal et al. (2001) observed a decrease in the production of earthworm casts during a period of 120 days in field conditions. Capowiez et al. (2003) found that the behaviour of earthworms was significantly altered, noting decreases in burrow length, rate of burrow reuse, and distance covered, at concentrations of imidacloprid between 0.5 and 1 mg/kg in dry soil.

Imidacloprid has been found in streams and rivers and is likely to be bioavailable to aquatic organisms. Because few studies on the toxicity of imidacloprid have been relevant to lotic species (Alexander et al. 2007), we investigated the impact of imidacloprid on the behaviour of the aquatic oligochaete *L. variegatus*. In this study, a new automated sediment toxicity test using *L. variegatus* was developed to assess the effects of short-term exposures to different concentrations of the pesticide. In addition, this study was an attempt to investigate the use of the behaviour of *L. variegatus* as a tool to assess the sublethal effects of a toxic substance. Our hypothesis was that the exposure to the pesticide would cause behavioural early warning responses, particularly locomotion and peristaltic movements.

Materials and Methods

Culture

Laboratory cultures of *L. variegatus*, used throughout these tests, originated from the University of Joensuu, Finland. Animals were reared in polyethylene aquariums (8.5 × 17.5 × 12 cm), covered with lids, that contained ASTM (1980) medium (pH 7.6 ± 0.3; 20°C) in a temperature-controlled room (16:8-h light-to-dark cycle and 50% humidity). A commercially available sand-pebble mixture (grain sizes 0–8 mm) was acid washed (pH 2), ashified (for 4 h at 450°C), and used as sediment. The aquaria contained a 2-cm layer of sediment with continuous and moderated aeration. The worms were fed with Tetraphyll, applied two or three times a week (approximately 5 mg/30 worms).

Spiking

Whole sediment (sediments and associated pore water that have had minimal manipulation (United States Environmental Protection Agency [USEPA] 2000) used in the experiments had the following characteristics: 4.9% sand, 74.4% clay, and 20.7% silt; pH 6.77; ammonia 3.04 mg/kg; and total carbon content 0.54%. Solutions were prepared by dissolving the appropriate amount of imidacloprid ($C_9H_{10}ClN_5O_2$; supplied by Sigma-Aldrich) in distilled water. These solutions were immediately added to the sediment, which was then capped and rapidly shaken for 1 min. The spiked sediments were equilibrated in the dark (due to the light sensitivity of the pesticide) for a minimum of 48 h to allow the pesticide to adsorb to the sediment particles. During this time, the sediments were shaken every day for approximately 2 min. The contaminated water was replaced by ASTM water before adding the worms. The nominal imidacloprid concentrations in the sediment were 0 (uncontaminated sediment control), 0.05, 0.5, 1.0, 2.5, and 5.0 mg/kg. The sediment was sampled for imidacloprid analysis at the start and end of each test, and the overlying medium (ASTM) was sampled only at the end of the tests.

Exposure Design

Short-term (10-day) tests were performed using contaminated sediment and clean water. The exposures were conducted at 20°C in a temperature-controlled room (16:8-h light-to-dark cycle and 50% humidity) in 100-ml plastic beakers containing 35 g whole sediment and 20 ml ASTM medium under static conditions. Seven replicates per concentration were used, each with six young worms (approximately 1.5 cm; totalling 42 organisms per concentration) that were carefully introduced into the beakers with the help of a plastic Pasteur pipette. Mortality, growth (size class 1 = worm <2 cm; size class 2 = worm >2 cm but <2.5 cm; size class 3 = worm >2.5 cm), colour, and presence in sediment or water were monitored every 48 h. For monitoring, the worms were removed from the test beakers and carefully observed and measured. The sediments were replaced by newly spiked ones at day 5, and no food was added during the test. The surviving worms were collected, dried at 40°C for approximately 24 h, and then weighed. Before drying, the worms were rinsed rapidly in distilled water and gently dried with filter article. There was no attempt to remove the sediment from the intestines of *L. variegatus* by allowing a depuration period (putting the worms in water for 24 h) because an increase in worm water content could decrease or erase the negative effects on weight (Dalby et al. 1996; Capowiez et al. 2005). Imidacloprid levels in water, sediment, and whole-body samples were analysed after extraction with acetonitrile by ultraviolet high pressure liquid chromatography at 270 nm (limit of quantitation [LOQ] 0.1 µg/l for water, 0.001 mg/kg for sediment, and 0.01 mg/kg for whole body). Before analysis, the sediment, water, and whole-body samples were kept in the dark. Biomass, i.e., the dry weight of surviving organisms divided by the initial number of organisms (United States Environmental Protection Agency [USEPA]) 2000), was also calculated. Bioaccumulation factors (BAFs) were calculated according to the following formula (Barron 1995; Eq. 1):

$$\mathrm{BAF} = \text{contaminant concentration (mg/kg dry wt) in tissue/contaminant concentration (mg/kg dry wt) in sediment.} \quad (1)$$

Behavioural Responses

Behaviour was measured using the MFB. Seven worms per concentration were used, and behaviour was record for 2 h (plus a 30-min acclimation period). The lower half of each test chamber was filled with sediment; the upper half contained ASTM medium (ASTM 1980). One worm was added to each test chamber, and three chambers without worms were used as controls.

Statistical Analysis

Regression analyses were carried out using Excel software (Microsoft). For each tested concentration, selected behavioural signal frequencies (ranges 0–1 and 1–3 Hz) were plotted over time. Normality and homoscedasticity were tested using SigmaStat for Windows (version 3.5) software. Original behavioural MFB data were arcsin

transformed, and the overall effect of imidacloprid concentrations on population behaviour was investigated using Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks ($p < 0.001$) followed by *posthoc* Dunn's test ($p < 0.05$) to test for significant differences (Zar 1996).

Results

The normal reddish-brown colour of the worms did not change after exposure to imidacloprid. Avoidance was measured by counting the number of worms that were not in the sediment. As seen in Fig. 1, *L. variegatus* clearly avoided contaminated sediments, and avoidance was greater (100%) for sediments contaminated with higher concentrations of the pesticide. Furthermore, the controls never avoided the sediment and were not found in the water. avoidance increased during the test period for worms exposed to 0.05 and 0.5 mg/kg.

Mortality (Fig. 2) in the controls was low (2%), demonstrating that the holding facilities and handling techniques were acceptable for conducting such tests, as required in the standard protocol, in which mean survival for controls should be 90% (ASTM 1990). After 10 days of exposure, high mortalities were observed in worms exposed to 1.0, 2.0, and 5.0 mg/kg. There was a clear positive relation between mortality and imidacloprid concentrations: Higher mortalities were observed in worms exposed to higher pesticide concentrations. A power trendline ($R^2 = 0.84$) showed that mortality increased at a specific rate.

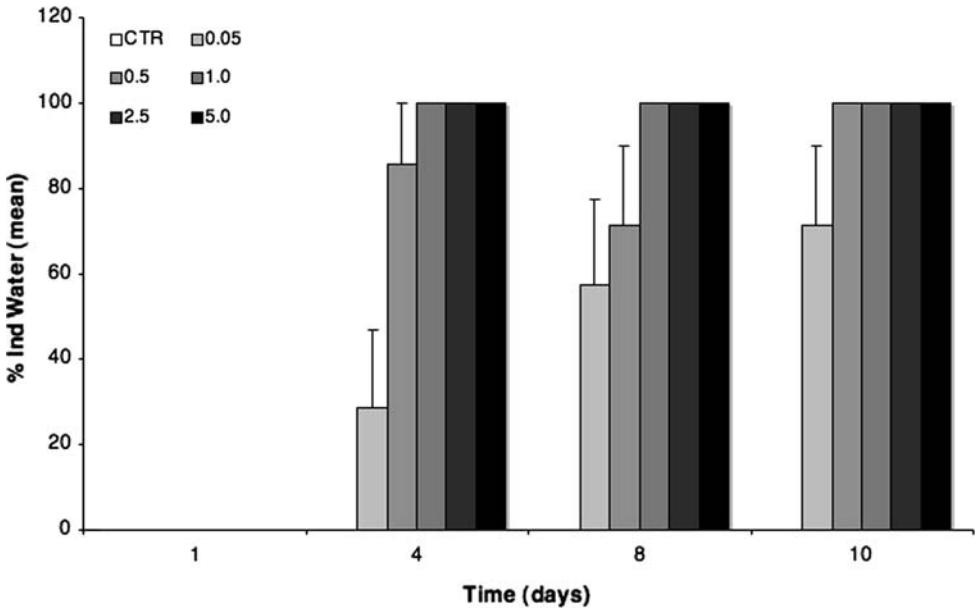

Fig. 1 Percentage of individuals in water (mean ± STDEV) throughout the test

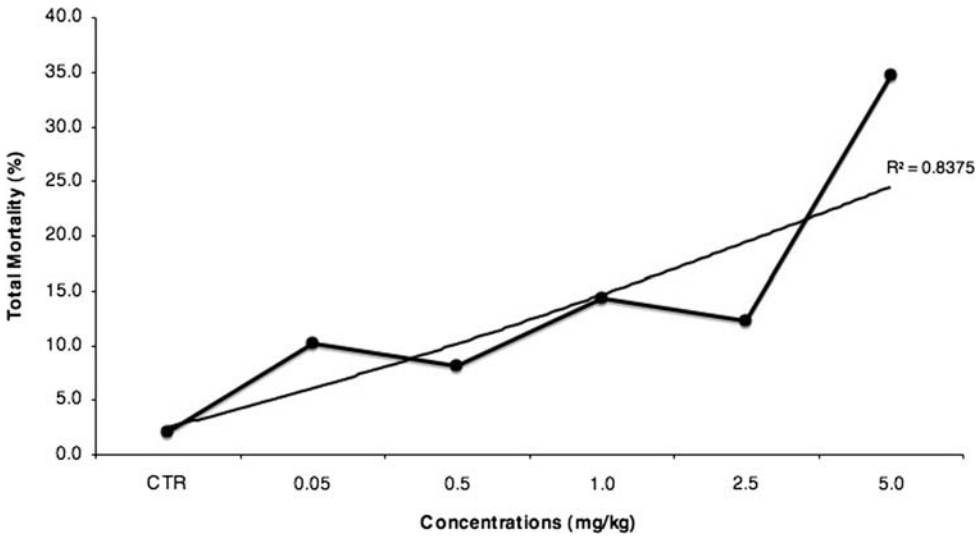

Fig. 2 Total mortality (%) of *L. variegatus* at the end of the short-term (10-day) test

After the short-term test, it was clear that the growth of the worms was inhibited by their exposure to sediments contaminated with imidacloprid (Fig. 3). All tested concentrations induced a growth inhibition; in fact, worms exposed to higher concentrations (2.5 and 5.0 mg/kg) did not grow at all. Biomass, according to the USEPA (2000), is the dry weight of surviving organisms divided by the initial number of organisms. Biomass data (Table 1) corroborated the results of mortality and growth. As expected, biomass decreased with increasing imidacloprid concentrations because mortality was higher and growth was lower. Biomass values (Fig. 3) decreased in a concentration-dependent fashion.

Behavioural tests showed that exposure to imidacloprid strongly inhibited both locomotion and peristaltic movements (Fig. 4). The average frequency of peristaltic movements on day 1 (Fig. 4a) was high for all worms, with no significant differences between sediments ($p > 0.05$). However, after 10 days of exposure to collected sediments (Fig. 4b), significant differences ($p < 0.05$) were found among the worms. Activity decreased with increased pesticide concentrations. The variances were not homogeneously distributed, but the results of one-way ANOVA on ranks indicated a significant concentration effect ($p < 0.05$) for both locomotion and peristaltic movement.

Sediment, Water, and Whole-Body Analysis

Concentrations of pesticide present in the initial and final sediment samples were as expected (Table 2), thus confirming the adequacy of the protocol designed for spiking the sediments with imidacloprid. Differences were found between the initial and final values of imidacloprid in the sediment: after 10 days, despite frequent sediment change, concentrations of the pesticide were much lower. Water samples collected at the end of the test showed some imidacloprid present due to pesticide degradation. As expected, whole-body tissues presented a higher level of imidacloprid when exposed to higher concentrations of the pesticide (Table 1). Worms exposed to higher levels of imidacloprid (2.5 and 5.0 mg/kg) had higher concentrations of the pesticide in whole-body tissues, which confirms a great ability to absorb and store this chemical.

Table 1 Bioaccumulation and biomass data[a]

Concentration (mg/kg)	Bioaccumulation (mg/kg)	Biomass (mg)
CTR	<LOQ	502.7
0.05	4.06	456.9
0.5	4.02	452.3
1.0	6.16	443.3
2.5	9.73	308.0
5.0	27.8	219.0

[a] LOQ 0.001 mg/kg

Discussion

Growth of *L. variegatus* was particularly affected by exposure to contaminated sediments. Even the lowest concentration of imidacloprid (0.05 mg/kg) was observed to inhibit growth. Hence, growth was inhibited by exposure to imidacloprid-contaminated sediments. Acute (short-term) tests are useful for identifying highly toxic chemicals, but they do not test key life events, during which

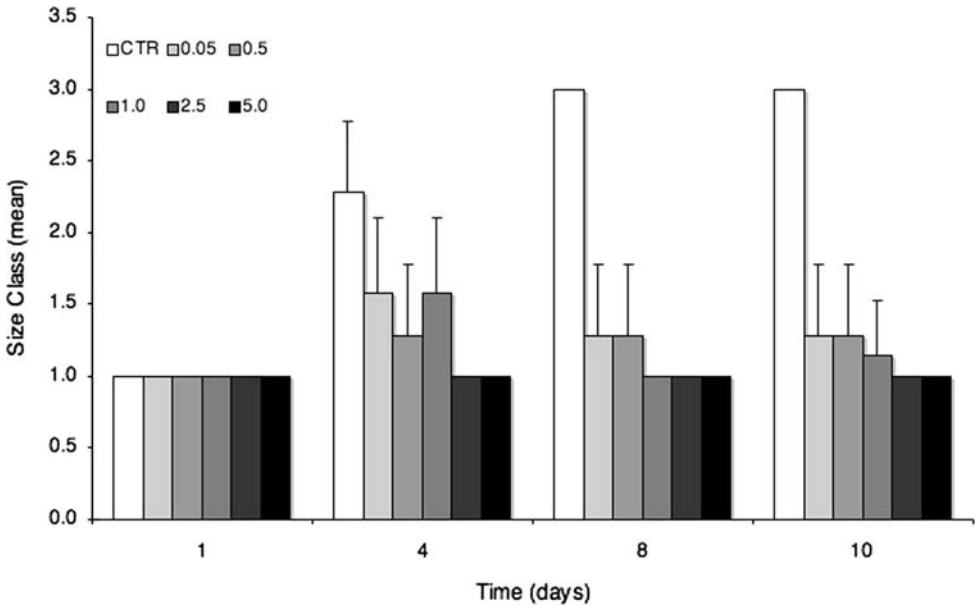

Fig. 3 Growth (size class) of worms (mean ± STDEV) throughout the test

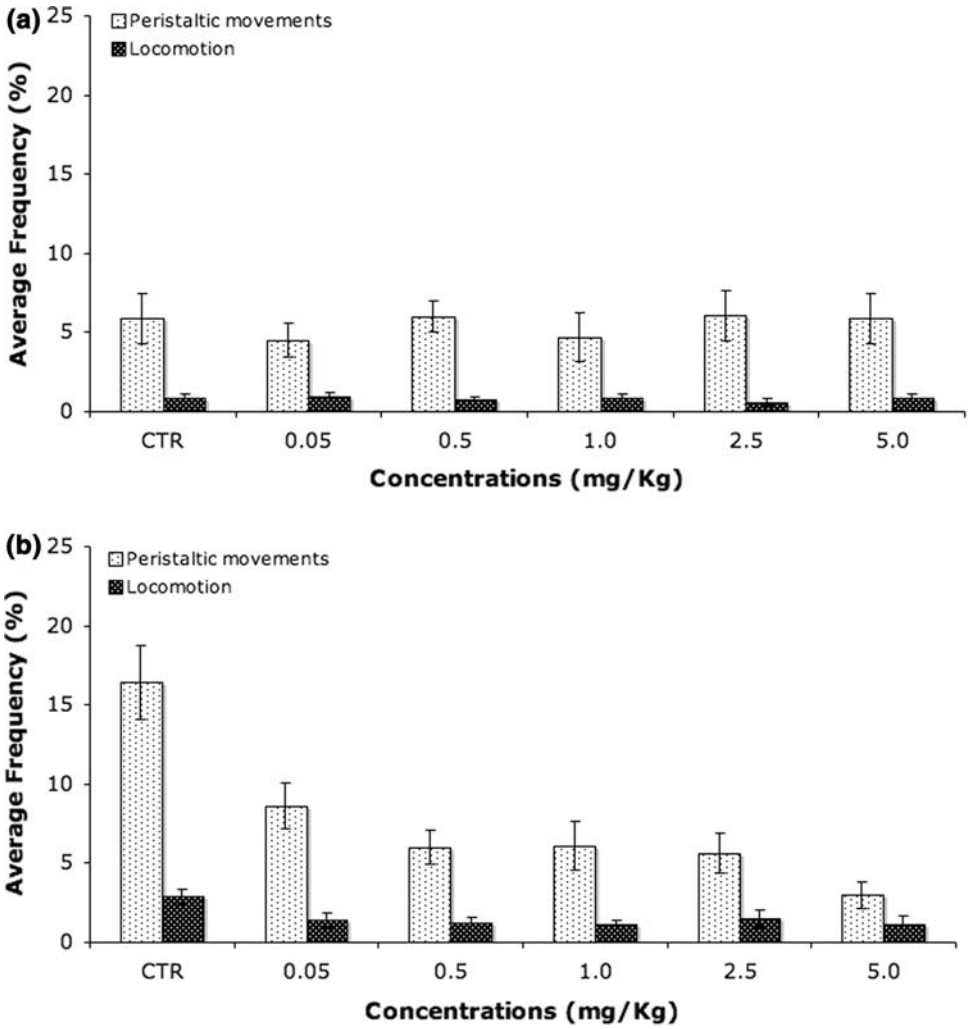

Fig. 4 Average frequency (%) (±SE) of locomotion and peristaltic movements throughout the test (A = day 1; B = day 10)

Table 2 Imidacloprid concentration in sediment and water samples (mean ± STDEV)[a]

Concentration (mg/kg)	Sediment in initial samples (mg/kg)	Sediment in final samples (mg/kg)	Water in final samples (mg/L)
CTR	<LOQ	<LOQ	<LOQ
0.05	0.05 ± 0.032	0.05 ± 0.051	0.39 ± 0.0004
0.5	0.58 ± 0.030	0.14 ± 0.009	0.55 ± 0.0006
1.0	0.80 ± 0.211	0.20 ± 0.021	0.86 ± 0.0009
2.5	2.27 ± 0.030	0.30 ± 0.016	1.38 ± 0.0014
5.0	4.58 ± 0.428	0.64 ± 0.103	2.99 ± 0.0030

[a] LOQ 0.001 mg/kg

sensitivity to toxicants may be increased (Scarlett et al. 2007). Because *L. variegatus* does not have a chitinous exoskeleton, it can only accumulate toxicants in soft tissues, simplifying the interpretation of the relation between survival and body concentrations of toxic materials (Meyer et al. 2002). Considerable quantities of imidacloprid were found in the worms' whole-body tissues. The chloragogen cells of lumbricid worms (such as *L. variegatus*), which surround the gut and the large blood vessels, contain numerous granules and chloragosomes, which are capable of binding toxic cations and organic xenobiotcs, thus enabling the worms to survive mild poisoning (Fischer 1977). Contaminants may accumulate from ingested sediment particles by desorption followed by absorption across the gut wall in the presence of digestive fluids (Weston et al. 2000), which can explain the high values of imidacloprid present in the whole-body tissues.

As hypothesised, it was possible to detect early warning signals in exposed worms, proving that behavioural parameters may be included in risk-assessment protocols.

These experiments showed that imidacloprid significantly changed the behaviour of *L. variegatus* in terms of both locomotion and peristaltic movements, confirming the findings of Alexander et al. (2007). The decreased oligochaete movement could increase predation risk by limiting the ability to avoid capture (Drewes 1997). Previous experiments using earthworms showed that imidacloprid can also change burrowing behaviour at concentrations between 0.5 and 1 mg/kg (Capowiez et al. 2003). In another experiment, Capowiez and Bérard (2006) observed that several aspects of earthworm behaviour (e.g., distance travelled, oscillations) or of the resulting burrow systems (e.g., area, topology, sinuosity, and depth) were affected by imidacloprid concentrations. Imidacloprid is a potential contaminant of surface and ground waters because of its persistence in soil (half-life of 48–190 days), high solubility (514 mg/L at 20°C), and low octanol water partition coefficient (log kow = 0.57) (Fossen 2006). It is not easy to spike sediments homogeneously with a contaminant, but, bearing this in mind, the effectiveness of our spiking protocol appears satisfactory.

Decrease in biomass seems to be a sensitive endpoint because it was possible to detect decreases in biomass even at low concentrations of imidacloprid. Capowiez et al. (2005) observed that weight loss in earthworms was a sensitive biomarker for exposure to imidacloprid even at low concentrations (0.5 mg/kg dry soil). To explain weight decrease, these investigators proposed different but not exclusive factors: (1) inactivity, as a direct response to the insecticide or as a mechanism of avoidance or (2) physiologic causes, such as less efficient assimilation or development of a costly mechanism of detoxification. We believe that these two factors may also be responsible for the biomass decrease observed in the present study.

An interesting observation was that all surviving worms exposed to concentrations >0.05 mg/kg were found together in a single aggregate. This also seems to be a result of exposure to imidacloprid and raises many questions. Is it a defense mechanism? Does it help the worms survive longer? Further investigation is needed to address these questions.

Oligochaetes feed on subsurface sediments and egest onto the sediment's surface, hence recycling deposited material. At high worm densities, reworking can considerably modify the structure of sediments (Krezoski and Robbins 1985; McCall and Fisher 1980). Thus, when the presence of imidacloprid affects the survival, growth, and behaviour of *L. variegatus*, it also affects the balance of the ecosystem. Because it is likely that behaviour inhibition could have a severe effect on oligochaete performance in the environment, this endpoint is important to evaluate the impacts of pollutants and pesticides on sediment ecosystems. Although behaviour cannot replace standard toxicity endpoints, we suggest that it should be introduced as an additional parameter. It is a rapid approach (faster than mortality and growth) and thus important in early warning systems. Behaviour integrates many cellular processes and is essential to the viability of the organism, its population, and its community. Using behaviour as a parameter, results can also be obtained at ecologically relevant concentrations (lower than lethal concentrations), which does not always happen with mortality and growth. Therefore, observations of behaviour provide a unique toxicologic perspective, linking the biochemical and ecologic consequences of environmental contamination (Little and Finger 1990). Because behaviour is important in activities such as predator avoidance, sexual interactions, and feeding, an impact on individual behaviour leads to an impact on population dynamics.

Conclusion

The pesticide imidacloprid proved to affect the population of *L. variegates* by decreasing survival, inhibiting behaviour, interfering with the growth process, and shortening life span. Growth and avoidance proved to be sensitive sublethal endpoints for imidacloprid contamination. Behaviour tests, in addition to classical sediment tests, should be conducted.

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