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Abstract
Earthworms are important and useful soil organisms, but in agricultural soils, they are potentially exposed to a wide variety of pesticides. Insecticides represent the highest threat to earthworms and many are neurotoxic. There is a need for a reliable, relevant, simple biomarker to assess the sub-lethal effects of neurotoxic insecticides on earthworms under laboratory or field conditions. The *Aporrectodea caliginosa* earthworms were exposed to 0 (control), 0.5×, 1× (normal field application rate), and 5× concentrations of a carbamate (Pirimor®) and an organophosphate (Lorsban®) insecticides. The nerve conduction velocity (NCV) of the medial giant fibers of *A. caliginosa* earthworm was recorded on days 0, 1, 2, 3, 4, and 7 to quantify sub-lethal neurotoxic effects. Acetylcholinesterase (AChE) enzyme activity of *A. caliginosa* homogenates was measured at the conclusion of the experiment. Pirimor® but not Lorsban® induced a significant decrease in NCV on days 3, 4, and 7 at 1× and 5× doses. A significant dose-dependent decrease was observed on AChE activity to Pirimor® at the doses used but not Lorsban®. A clear relationship is observed between AChE activity and NCV in the case of Pirimor®. This study showed that NCV is a sensitive biomarker that correlates well with classical biomarker measurements such as AChE enzyme activity. This technique could be used to study the impact of insecticides on earthworms and also their recovery.

Keywords Earthworms · Ecotoxicology · Insecticides · Neurotoxicity · *Aporrectodea caliginosa* · Nerve conduction velocity

Introduction

In traditional agricultural farming systems, pesticide application can cause deleterious effects on earthworm communities (Paoletti et al. 1998; Pelosi et al. 2013). Considering their limited dispersal abilities, exposure to pesticides can negatively impact earthworm survival, biomass, physiology, and behavior (Pelosi et al. 2014). In this context, earthworms are considered as one of the best bioindicators for monitoring the terrestrial ecosystem health (Hirano and Tamae 2011). Earthworms play an important role in terrestrial ecotoxicology considered as non-target species and therefore have been used extensively to assess environmental risk and chemical toxicity of pesticides in both laboratory and fields experiments (Scott-Fordsmand and Weeks 2000; Sanchez-Hernandez 2006; Pelosi et al. 2014; Capowiez et al. 2006).

A variety of biomarkers have been used in earthworms from the molecule to the whole organism level using genomics, proteomics, metabolomics, and biochemical and physiological screening depending on the research question and the organization level of complexity (Scott-Fordsmand and Weeks 2000; Sanchez-Hernandez 2006; Pelosi et al. 2014). Environmental biomarker responses can be used to assess the impact of chemicals including pesticides which can then be used as early and sensitive signals of harm to organisms. This is a complementary approach to standard toxicity tests to evaluate sub-lethal effects of contaminants on earthworms. Biomarkers provide more information about the organism's stress response and the toxic mode of action of the substance may also be evaluated (Kammenga et al. 2000; Sanchez-Hernandez 2006; Pelosi et al. 2014).

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Among the biomarkers, physiological responses of the nervous system of earthworms were reported as early as 1980 (Drewes and McFall 1980; Drewes et al. 1980). Surprisingly, the nervous conduction velocity (NCV) biomarker has been used only in a relatively few ecotoxicology studies with invertebrates (Drewes et al. 1984; Drewes and Lingamneni 1992; Gooneratne et al. 2011; Subaraja and Vanisree 2016) but widely used with vertebrates such rats (Areti et al. 2017). Earthworms have a well-developed nervous system comprising medial (MGF) and lateral giant fibers (LGF) that run ventrally along the entire length of the body with specialization of the MGF at the anterior end along with the presence of cerebroïd ganglia, equivalent to the brain and spinal cord in higher animals. The anatomical localization of this ventral nerve cord and the relatively thin body wall in earthworms allow monitoring of the nerve action potentials that can be used to quantify the NCV using an oscilloscope. The measurement of NCV in earthworms does not require anesthesia or immobilization and presents the added advantage of allowing repeated testing of the same worm before, during, and after exposure.

The aim of this study was to assess the usefulness of the NCV in earthworms as a biomarker and to compare this physiological response with a biochemical response following insecticide exposure. We chose two insecticides currently used in agrosystems: a carbamate (Pirimor®) and an organophosphate (Lorsban®). Pirimor® (pirimicarb, 2-(dimethylamino)-5,6-dimethylpyrimidin-4-yl-dimethylcarbamate) is a commonly used insecticide, belonging to the carbamate family. Formulations containing this compound are used to control aphids in a wide range of agricultural and horticultural crops including cereals, potatoes, and other vegetables and fruit by inhibiting the acetylcholinesterase (AChE) enzyme at the nerve endings (Ecobichon 2001). Lorsban® (chlorpyrifos-ethyl) is an OP insecticide widely used in the world to control pests in agricultural crops. We monitored the impact of these two insecticides on the NCV of *Aporrectodea caliginosa* over a 1-week exposure period. At the end of the experiment, the NCV responses were correlated to AChE enzyme activity, the molecular target of the chosen insecticides.

Material and methods

Earthworms were originally collected from an abandoned organic orchard farm (for 10 years) near Lincoln University and genotypically typed by Bate (2015) at the Lincoln University toxicology laboratory. The progeny from this colony were used in the study. Species identification was by amplification and sequencing of the 16s RNA and *A. caliginosa* cytochrome oxidase subunit 1 genes (Bate 2015). Degenerate primers specifically designed for annelid speciation experiments were used for this analysis. The *A. caliginosa* earthworms were placed in plastic boxes containing soil from the same orchard.

Soil spiking and experimental procedures

For the exposure studies, we used two commercially available solutions: Pirimor® 50 (500 g kg^{-1} active ingredient (a.i.), Syngenta) as a carbamate insecticide and Lorsban® (500 g L^{-1} a.i., Dow Agrosciences NZ Ltd.) as an organophosphorus insecticide. The insecticide concentrations were chosen based on the recommended field application rate in New Zealand and calculation of the predicted environmental concentration (PEC). Briefly, it is based on a single application at normal application rate, with a homogeneous distribution in the first 5 cm of soil with no crop interception, and a soil density of 1.5 kg L^{-1}. The normal application rate (referred to 1×) corresponds to 0.66 a.i. mg kg^{-1} soil for pirimicarb (250 g ha^{-1}) and to 0.53 a.i. mg kg^{-1} soil for Lorsban (200 g ha^{-1}). For each insecticide, three different doses were tested 0.5×, 1×, and 5×. Prior to the exposure, the soil was sieved at 2 mm and its water content was adjusted to 21% (approximately to 81% of the water holding capacity of soil) by adding distilled water. Then, 100 g of soil was spiked with 4 mL of the different insecticide solutions (or 4 mL of distilled water for the control) leading to final soil water content of 25% (gravimetric) and thoroughly mixed, as currently done using normalized tests.

Earthworms ($n = 128$) were placed individually in plastic container filled with 100 g of soil each (either control or polluted soil) to limit inter-specific interactions and to prevent cascade death (Sheppard and Evenden 1992). The experiment was conducted at ~ 17 °C in a dark room for 7 days. Day 0 corresponds to the beginning of the experiment when earthworms were placed in control and test soils. For each pesticide, eight earthworms were used per dose. The whole experiment was repeated twice. Earthworm weights (without gut voiding) were measured on days 0 and 7. Nerve conduction velocity measurements were performed at days 0, 1, 2, 3, 4, and 7. At day 7, the earthworms were frozen and used for biochemical measurements (protein and acetylcholinesterase activities).

Conduction velocity measurement

Immediately prior to the NCV measurement, each earthworm was rinsed in de-ionized water and placed on a printed circuit board with parallel metallic grid lines (electrodes) etched onto it. MGF activity was evoked by a light tactile stimulus to the anterior end of the earthworm with a small rubber band. Each stimulation induces action potentials recorded and visualized on the oscilloscope as a global potential. The electrical signal generated by the MGF and detected by two parallel electrode

pairs was fed into differential recording amplifiers, filtered, and displayed as multiple traces on a storage oscilloscope (Tektronix TDS model 250) as described by Gooneratne et al. (2011). The impulse amplitude ranged from 20 to 50 mV, with signal-to-noise ratio varying from about 5:1 to 10:1. MGF peak-to-peak time intervals were measured and converted to velocity by dividing the conduction distance between the electrode points (10 mm) by the conduction time as displayed on the oscilloscope. Measurements are expressed in meter per second. The mean of three readings for each earthworm at each time point was recorded.

Protein measurements and acetylcholinesterase activity assays

Each earthworm was homogenized in ice (Janke and Kunkel IKA-WERK Ultra-Turrex Homogenier) for 20 s in 20% (*w*/*v*) 10 mM Tris-HCl, pH 7.3, 10 mM NaCl supplemented with protease inhibitors (aprotinin, leupeptin and pepstatin = 5 μg mL^{-1}, antipain = 1 μg mL^{-1}, trypsin inhibitor = 1 mg mL^{-1}). The homogenates were centrifuged at 3000*g* for 10 min at 4 °C and the supernatants were stored in 10% glycerol as an enzyme-stabilizing agent at − 20 °C until analysis.

All biochemical measurements were conducted spectrophotometrically in quadruplicate using a microplate reader (synergy HT, Bio-Tek). AChE activity was monitored at 412-nm wavelength and 25 °C according to Ellman et al. (1961) in a reaction medium (0.2 mL final volume) containing 4 μL of earthworm homogenate extract, 0.375 mM DTNB (5, 5′ di thiobis 2-nitro-benoic acid), and 3 mM of substrate ACSCh (acetylthiocholine iodide) in 0.1 M sodium phosphate buffer pH 7 (Rault et al. 2008). The total protein content was determined using the Lowry method according to Markwell et al. (1978), using bovine serum albumin as the standard.

The enzyme assays were expressed as units per milligram of total protein (U mg^{-1} protein). One unit of enzyme activity is defined as 1 μmol of substrate hydrolyzed per minute under the experimental conditions described above.

Statistic analysis

Results are expressed as means + standard deviations. Normality and homoscedasticity of the data were tested or visually assessed using qplots. For each pesticide, we used two one-way ANOVA to analyze the effects of pesticide concentration on earthworm weight loss and AChE activity. For each pesticide, we applied ANOVA with repeated measures in time to analyze the effects of pesticide concentration on NCV since measurements were applied to the same individuals at different dates. Post hoc test (Tukey HSD) was used to compare the means. In all cases, the significant level was set at $p < 0.05$.

Results

No morphological or physiological abnormalities were observed in any of the earthworms during the exposure period to the two pesticides. The weight of earthworms decreased slightly during the experiment and for all the groups (around 5–6%) but no significant difference was observed between the control and exposed earthworms (data not shown).

Nerve conduction velocity

The mean NCV of earthworms placed in control soil was 12.83 ± 0.53 m/s on day 0 and did not significantly change during the experiment (Fig. 1a, b). The exposure of earthworms to soil contaminated with 1× and 5× concentrations of Pirimor® significantly decreased the NCV (Fig. 1a). Concerning the soil polluted with 5×, a loss of about 25% was observed on NCV after 3-day exposure (~ 10 ± 0.094 vs 13.26 ± 0.42 m/s). This decrease was maintained at days 4 and 7. The NCV was also significantly decreased in earthworms in soil polluted with the dose 1× but only on days 4 and 7. No significant difference was observed between 1× and 5×. After Lorsban® exposure, no significant differences were observed

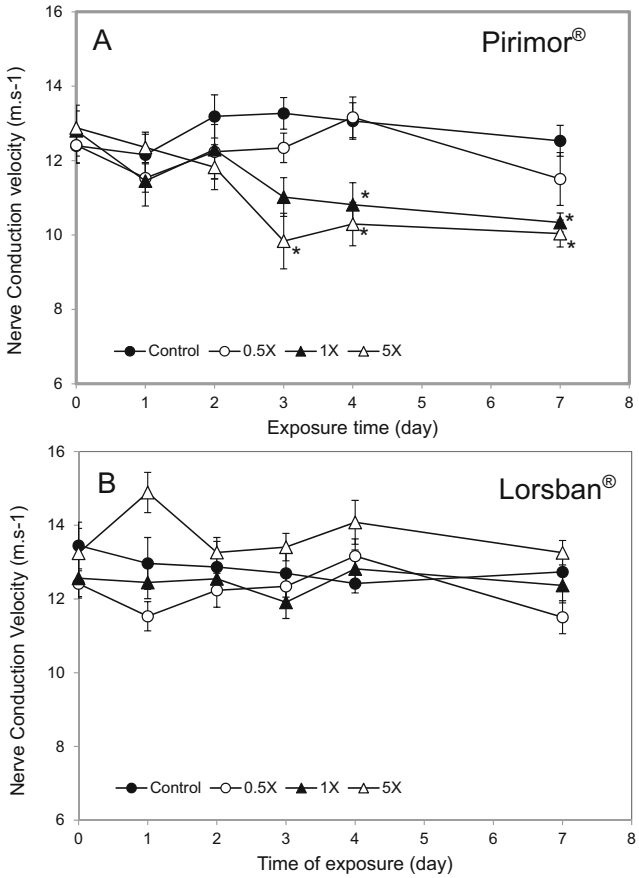

Fig. 1 Nerve conduction velocity (mean + SD) in *A. caliginosa* earthworms exposed to 0 (control), 0.5×, 1×, and 5× concentrations of Pirimor® (**a**) and Lorsban® (**b**) for 7 days

at any dose or any stage of the experiment on the NCV of earthworms (Fig. 1b).

Acetylcholinesterase enzyme activity and correlation with the NCV

A significant decrease in the AChE activity was observed in earthworms at doses 1× and 5× following Pirimor® exposure (Fig. 2) but not after exposure to Lorsban®. No significant variations were observed for proteins. Additionally, a clear relationship was observed between the decrease of both NCV and AChE activity (Fig. 3, $R^2 = 0.91369$).

Discussion

Insecticides can affect the physiology or behavior of individuals that in turn can have impacts on populations or ecosystem functions in a variety of ways. The nervous system plays a major role in the physiology of all species, responsible for the control of almost all body functions. In the earthworms, the nervous system is implicated in locomotion, rapid escape behavior, and digestive and vascular functions (Wu 1939a, b; Ladd Prosser and Zimmerman 1943; Millott 1943; O'Gara et al. 1982). In the present study, we recorded the NCV of *A. caliginosa* during a 7-day exposure period to two neurotoxic insecticides, Pirimor® and Lorsban®.

The basal values of the NCV in *A. caliginosa* were similar to those reported in a previous study using the same species (Gooneratne et al. 2011). Pirimicarb induced a significant reduction of the NCV from day 3 at the highest dose and 1 day later at exposure concentrations corresponding to the normal application rate in New Zealand agricultural systems. This decrease in NCV remained at similar levels until the end of the experiment on day 7 and correlated well with the significant reduction in AChE enzyme activity in the earthworms.

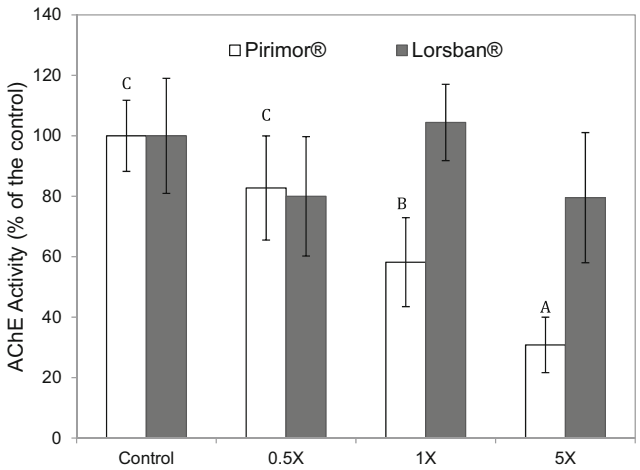

Fig. 2 Response of acetylcholinesterase activity in *A. caliginosa* after 7-day exposure to 0 (control), 0.5×, 1×, and 5× of Pirimor® and Lorsban®

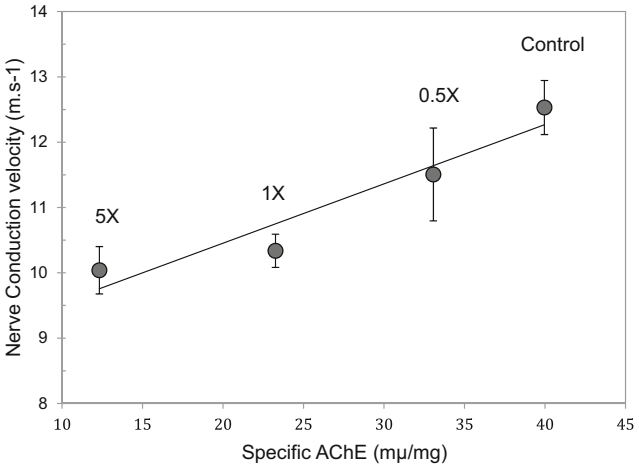

Fig. 3 Correlation between nerve conduction velocity (m s^{-1}) and specific acetylcholinesterase activity (mμ mg^{-1}) after 7 days of exposure with Pirimor®

Thus, no recovery was observed for these short-term exposure times, which is in agreement with the results obtained by Rault et al. (2008) regarding AChE inhibition in this species. A previous study showed the impact of carbofuran, another carbamate pesticide, on *Lumbricus terrestris* nervous activity (Drewes and Lingamneni 1992) within 1 h of contact on paper filter. A marked decrease in NCV, after exposure to several other insecticides by dermal contact or by immersion, was recorded using the same technique (Drewes et al. 1988). A 2-min immersion in diazinon, an OP insecticide, induced an inhibition of the NCV of *L. terrestris*. However, in the present study, using more environmentally relevant conditions (polluted soil), we did not observe an effect of Lorsban® which is also an OP insecticide. Based on the electrophysiology results, we can hypothesize that the mode of exposure, the doses used, and/or the earthworm species considered could influence the NCV. *A. caliginosa* is the most common earthworm found in agricultural soils in the world, and it has been shown that this species is very sensitive to insecticides (Rault et al. 2008; Jouni et al. 2018). To our knowledge, this is the first time that effect on NCV was observed after pesticide exposure in soil following recommended doses. So far, impact on *A. caliginosa* NCV has been reported only after 7 days of exposure to soils contaminated with acid mine drainage (Gooneratne et al. 2011).

To our knowledge, direct ecotoxicity of pirimicarb on *A. caliginosa* AChE has not been reported yet, although it is well established that carbamate insecticides decrease this enzyme activity in other earthworm species (Scott-Fordsmand and Weeks 2000) or non-target organisms as, for example, bees (Belzunces et al. 1992). In the present study, we have shown that Lorsban® did not affect AChE activity in *A. caliginosa* earthworms following exposure to 7 days. Previous results have reported that either higher doses or longer exposure time (18 days) is needed to induce significant inhibition of AChE

with Dursban (chlorpyrifos) on the earthworm *L. terrestris* (Collange et al. 2010). Then, chlorpyrifos seems to be less toxic on earthworms than other OP insecticides for which AChE inhibition following exposure was reported (Scott-Fordsmand and Weeks 2000; Rault et al. 2008; Olvera-Velona et al. 2008; Martinez-Morcillo et al. 2013, Jouni et al. 2018). Such a discrepancy between insecticides could be due to the different OPs, length of exposure, or doses used. The correlation between AChE activity and NCV after 7 days showed a clear relationship. In future experiments, it will be interesting to study the changes in these two biomarkers in the first days of exposure in order to know if this correlation starts early. This electrophysiological technique is less invasive and then could be used as a warning tool in ecotoxicological assessment without sacrifice.

During the experiment, significant variations were not observed in the earthworm body weight. Only a slight weight loss between 5 and 6% was recorded. These weight losses were lower than the recommended threshold of 15–20% (Bembridge 1998). This observation led us to conclude that weight loss is a marker more suited to monitor the effects of long-term exposure. It should be noted, for example, that *A. caliginosa* did not show significant weight loss after 8 days of exposure to Oleobladan (parathion ethyl), an OP insecticide (Rault et al. 2008).

The NCV records in earthworms could be viewed as a missing link between biochemical and behavioral markers. It is often claimed that ecotoxicological studies require multi-approaches with different biomarkers acting at different biological levels of organization from the molecular level to behavioral studies. In earthworms, several studies have attempted to evaluate the potential link between biochemical parameters such as AChE activity and behavioral responses (Gupta and Sundararaman 1991; Olvera-Velona et al. 2008; Pereira et al. 2010; Jordaan et al. 2012; Martinez-Morcillo et al. 2013, Jouni et al. 2018). Despite the importance of biochemical biomarkers as early warning tools, the significance of these studies based on such biomarkers has been questioned mainly because the changes induced at sub-individual level do not necessarily have a negative impact at higher levels of biological organization. In this context, considering that some insecticides such as OP and carbamate act primarily on the nervous system, it will be interesting to integrate this physiological biomarker, NCV in environmental assessment studies in order to better understand the chain of events and the mechanism(s) implied in the disturbance of these environmentally relevant organisms. Moreover, this technique is not intrusive and allows fine temporally analysis of effects using repeated measures on the same individuals, thus decreasing the variability between individuals. In addition, it is cheap and the NCV records could represent an ideal link between cellular and integrated functions of earthworms exposed to a range of pesticides (Subaraja and Vanisree 2016) and xenobiotics (Gooneratne et al. 2011). We can also consider this biomarker to understand potential mechanisms implicated in functional earthworm behaviors and their dysfunctions, such as a decrease in burrowing activity following exposure to imidacloprid (Capowiez et al. 2006) or a decrease in cast production following parathion exposure (Jouni et al. 2018).

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