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## Toxicity evaluation of neonicotinoids to earthworm (*Eisenia fetida*) behaviors by a novel locomotion tracking assay<sup>☆</sup>

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## ABSTRACT

Pesticides are substances used for controlling, preventing, and repelling pests in agriculture. Among them, neonicotinoids have become the fastest-growing class of insecticides because of their efficiency in targeting pests. They work by strongly binding to nicotinic acetylcholine receptors (nAChRs) in the central nervous system of insects, leading to receptor blockage, paralysis, and death. Despite their selectivity for insects, these substances may be hazardous to non-target creatures, including earthworms. Although earthworms may be invasive in some regions like north America, they contribute to the development of soil structure, water management, nutrient cycling, pollution remediation, and cultural services, positively impacting the environment, particularly in the soil ecosystem. Thus, this study aimed to develop a novel earthworm behavior assay since behavior is a sensitive marker for toxicity assay, and demonstrated its application in evaluating the toxicity of various neonicotinoids. Here, we exposed *Eisenia fetida* to 1 and 10 ppb of eight neonicotinoids (acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, pestanal, thiacloprid, thiametoxam, and sulfoxaflor) for 3 days to observe their behavior toxicities. Overall, all of the neonicotinoids decreased their locomotion, showed by a reduction of average speed by 24.94–68.63% and increment in freezing time movement ratio by 1.51–4.25 times, and altered their movement orientation and complexity, indicated by the decrement in the fractal dimension value by 24–70%. Moreover, some of the neonicotinoids, which were acetamiprid, dinotefuran, imidacloprid, nitenpyram, and sulfoxaflor, could even alter their exploratory behaviors, which was shown by the increment in the time spent in the center area value by 6.94–12.99 times. Furthermore, based on the PCA and heatmap clustering results, thiametoxam was found as the neonicotinoid that possessed the least pronounced behavior toxicity effects among the tested pesticides since these neonicotinoid-treated groups in both concentrations were grouped in the same major cluster with the control group. Finally, molecular docking was also conducted to examine neonicotinoids' possible binding mechanism to Acetylcholine Binding Protein (AChBP), which is responsible for neurotransmission. The molecular docking result confirmed that each of the neonicotinoids has a relatively high binding energy with AChBP, with the lowest binding energy was possessed by thiametoxam, which consistent with its relatively low behavior toxicities. Thus, these molecular docking results might hint at the possible mechanism behind the observed behavior alterations. To sum up, the present study demonstrated that all of the neonicotinoids altered the earthworm behaviors which might be due to their ability to bind with some specific neurotransmitters and the current findings give insights into the toxicities of neonicotinoids to the environment, especially animals in a soil ecosystem.

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## 1. Introduction

Earthworms are frequently referred to as ecosystem engineers because of their roles in developing soil structure, water management, nutrient cycling, pollution remediation, and cultural functions (Blouin et al., 2013). Since they are the largest animal groups in the soil, earthworms represent the most significant component of the animal groups in the environment (Lavelle and Spain, 2002). Earthworms are beneficial in agriculture to help overcome the major issues of conventional intensive farming. For example, they help the compaction issue in humid areas by reducing the structural degradation of the soil. Besides that, earthworms can boost organic matter mineralization to improve nutrient availability, enhancing the efficiency of nutrient use by plants and reducing the risks of nitrate leaching (Bertrand et al., 2015). *Eisenia fetida* (*E. fetida*) is a species of earthworm recommended as a test organism by The Organization for Economic Cooperation and Development (OECD) for standardized acute toxicity assessment. Although earlier investigations showed that *E. fetida* is less sensitive to chemicals than other earthworm species (Fitzgerald et al., 1996; Kula, 1995; Ma and Bodt, 1994), their characteristics such as widely available, culturable, and can be easily bred in a short period on a variety of organic waste make them a practical consideration and a suitable subject for analyses of soil toxicity (Chemicals, 2005; Li et al., 2015; OECD, 1984; Yasmin and D'Souza, 2007).

The OECD has provided several appropriate chemical toxicity tests in earthworms, such as acute toxicity and reproduction tests (Wilhelm and Maibach, 2012). However, these toxicity tests have drawbacks, including being labor- and time-intensive. Additionally, specific effects of sub-lethal doses might be overlooked by these methods (Lukkari et al., 2005). Aside from the mortality and reproduction tests, behavioral tests are one of the tests with a high potential to be used in evaluating the toxicity of various pollutants. An earlier study established an alternative method as an early screening tool for evaluating soil stress due to the effects of contaminants called the "earthworm avoidance test" (Yeardley Jr et al., 1996). Since previous research on earthworms demonstrated that this soil organism would probably avoid slightly contaminated soils that impair their functions in the ecosystem (Hund-Rinke et al., 2003; Hund-Rinke and Wiechering, 2001; Salminen and Sulkava, 1996), this behavior test is one of the most reliable behavior tests for them since it exploits this earthworms behavior response (Yeardley Jr et al., 1996). Compared to acute toxicity and reproduction tests, the earthworm avoidance test offers an advantage regarding sensitivity, brief test periods, and ease of experimental design (Yeardley Jr et al., 1996). Meanwhile, burrowing behavior, which employs soil and examines the movement of the worms within the soil, is another approach to studying earthworm behavior (Djerdj et al., 2020) due to a prior study that showed that earthworms exposed to toxic concentrations of metal contaminants would be less able to perform their essential functions in soils (Edwards and Bohlen, 1996). However, burrowing behavior poses a significant issue, as it is difficult to study their native behavior since they are concealed inside the soil, thus, earthworm's locomotion will be invisible by video recording (Yeardley Jr et al., 1996). Furthermore, variability in the coloration of different body parts also makes it challenging to locate their precise location using basic image analysis (Capowiez et al., 2010). Given the significance of earthworms in the soil ecosystem, it is necessary to develop a novel and improved method as an alternative way to evaluate earthworm behavior performance. Therefore, in this study, we aimed to develop a novel behavior assay for earthworms.

Pesticides are substances used for controlling, preventing, destroying, and repelling a pest and are widely used to protect the crop from any pest attack in the agricultural area (Blacquiére et al., 2012; Borsuah et al., 2020; Elbert et al., 1998; Goulson, 2013; Hladik et al., 2014). Neonicotinoid pesticides have emerged as one of the most popular types of pesticides since they have a broad application range (Bass and Field, 2018; Borsuah et al., 2020; Goulson, 2013; Jeschke and Nauen, 2007;

Jeschke et al., 2011). These benefits have made neonicotinoids a valuable pesticide for economies, as seen by the higher yield in agriculture production brought about by their use (Aktar et al., 2009). However, despite their advantages in increasing production, a wealth of evidence showed their potentially harmful effects on non-target insects, such as bees since a previous study linked insecticides to the disappearance of honeybees from the ecosystem and other organisms, including aquatic invertebrates (Pisa et al., 2015; Spivak et al., 2011). Therefore, although their benefits to agriculture are substantial, it is also important to consider any negative impacts they may have on non-targeted organisms in the environment.

Neonicotinoids work by strongly binding to nicotinic acetylcholine receptors (nAChRs) in the central nervous system. Neonicotinoids may stimulate nerves at low concentrations, but at greater concentrations, they may block receptors, cause paralysis, or even cause death (Siregar et al., 2021; Tomizawa and Casida, 2005). It is interesting to note that neonicotinoids were discovered to be preferentially more harmful to insects due to their stronger binding to the nAChRs of insects than to vertebrates (Tomizawa and Casida, 2005). However, the possibility that neonicotinoids may also affect species other than insects should be given attention. Based on our previous study, we demonstrated that freshwater shrimp displayed a markedly decreased locomotor activity, and reduced the heartbeat and gill ventilation movement of shrimps after exposure to imidacloprid. Furthermore, imidacloprid was found to have a strong binding affinity for the freshwater shrimp acetylcholine receptor from the molecular docking results, which might be the reason for the alterations in their locomotion activity, heartbeat, and gill ventilation (Siregar et al., 2021). Previously, neonicotinoid toxicity has been tested on earthworms. Long-term exposure to imidacloprid, thiacloprid, and clothianidin affected the life cycle of *Eisenia andrei*. These neonicotinoids also significantly affected earthworm growth and maturation (van Loon et al., 2022). Five neonicotinoids toxicity also have been tested on two soil species, *Folsomia candida* and *Eisenia andrei*. The results showed that these neonicotinoids have different toxicities to each species, with imidacloprid and acetamiprid being more toxic to survival and reproduction (De Lima e Silva et al., 2020). Clothianidin also showed chronic toxicity to other earthworm species like *Lumbricus terrestris*. Chronic exposure to clothianidin showed a significant but temporary effect on food consumption and a significant effect on mortality of *L. terrestris* (Basley and Goulson, 2017). Another neonicotinoid, nitenpyram, was also discovered to have toxicity to *E. fetida*. High-concentration of nitenpyram caused DNA damage and significant oxidative stress on earthworms (Zhang et al., 2021). Moreover, prior studies in earthworms also demonstrated that neonicotinoids significantly affect the oxidative stress of earthworms by altering the reactive oxidative stress (ROS) and malondialdehyde (MDA) content, also damage the growth and DNA of earthworms (Yan et al., 2021). Bioaccumulation of neonicotinoids also has been tested before on survival, DNA damage, and reproduction of *E. Andrei*. The results prove that bioaccumulation could lead to a significant increase in DNA damage and significant effects on reproduction when earthworms were exposed to an environmental concentration of neonicotinoid (Chevillat et al., 2017). Another study also proved that low-dose imidacloprid was potent to induce cytotoxicity in *E. fetida* neurons with upregulated lipid peroxidation and ROS in the earthworm treated with low-dose imidacloprid. Low-dose imidacloprid also decreased neuron-specific enolase (NSE) expression in a dose-dependent manner, which indicated disturbances in *E. fetida* neuron cells (Huslysty et al., 2021). Furthermore, another study of six neonicotinoids on earthworms was carried out by testing the avoidance and reproduction and observed adverse effects on the avoidance behavior and reproduction at low concentrations (Ge et al., 2018). Prior studies on five neonicotinoids (imidacloprid, acetamiprid, nitenpyram, clothianidin, and thiacloprid) also demonstrated the high acute toxicity to *E. fetida* with significant adverse effects on the reproduction of *E. fetida* and the inhibition of cellulase activity (Wang et al., 2015a). Furthermore, one study used concentration addition (CA) to examine the systematic

deviations of imidacloprid and thiacloprid on earthworms. They found that there were joint effects of imidacloprid and thiacloprid in reproduction and feeding rate (Gomez-Eyles et al., 2009). A combination of several neonicotinoids like imidacloprid and acetamiprid was also found to affect by significantly decreasing the acetylcholinesterase (AChE) and cellulose activities (Teng et al., 2022). Beside their combination effect with other neonicotinoids, they also have a synergistic effect with other chemicals as demonstrated by a prior study. All thiacloprid treatments caused a high level of mortality rates on earthworms (*L. terrestris*) when combined with nitrogen, phosphorus, and potassium (NPK) fertilizer (Holmes, 2022). Taken together, these earlier findings suggested that it is crucial to look into the potential adverse effect of neonicotinoids to multiple non-target organisms. In addition, it was also intriguing to observe which neonicotinoids have the least or most harmful effect on earthworm behavior. We believed that the current study's finding would give insight on how neonicotinoid structure could affect non-target organism and help with the risk management of neonicotinoid.

## 2. Materials and methods

### 2.1. Overview of experimental design

Multiple steps were taken to achieve the aims of the current study. First, we established the video recording and tracking of earthworm behavior as a crucial component of the first stage. The video recording was conducted by using a ZebraBox viewpoint machine with a light cycle to produce active movement in earthworms. Before the recording, the earthworm was placed in a self-made plastic plate with four wells and put into the ZebraBox (Fig. 1, left panel). Each well contains one earthworm, meaning four animals were recorded in a single attempt. Video results from ZebraBox were tracked using several methods to find the best tracker for earthworm behavior. Several trackers such as idTracker, UMATracker, ToxTrack, and ImageJ were tested in this experiment. Although Toxtrack and idTracker have the same principle as UMATracker, they showed some errors during the tracking process, which were observed from the trajectory results that indicated that it could not recognize and follow the earthworm movement correctly by

reading the background, instead of the earthworm. This issue caused the movement of the earthworms to become excessive and led to the over-estimation of the earthworm movement. Among the methods tested, UMATracker gave the best result by consistently showing the movement of the earthworm's centroid with the most negligible error. After finding the best method for recording and tracking earthworm behavior, the next step was to apply the method to evaluate the effects of neonicotinoid pesticides on earthworm behavior. This experiment tested eight neonicotinoids on earthworms using the direct exposure method (Fig. 1, middle panel). Briefly, the filter paper was soaked entirely in neonicotinoid test solutions. Then, earthworms were placed in a plate lined with moistened filter paper to expose earthworm skin directly to the neonicotinoid. The exposure was done for three days, and the earthworm behavior was monitored daily. Video recording was done by using a ZebraBox machine and tracked using UMATracker. Data from UMATracker in the form of X and Y coordinates were output as a.csv file and processed into behavioral endpoints using Microsoft Excel. These behavior endpoints were visualized in a graph. Afterward, the data from all endpoints were used for phenomics analysis by conducting some clustering analyses, which were heatmap and Principal Component Analysis (PCA). In addition, an *in silico* approach was conducted to explore the potential mechanism of neonicotinoids on earthworms (Fig. 1, right panel). The binding energy between neonicotinoid and earthworm acetylcholine binding protein was investigated using molecular docking. The crystal structure of earthworm acetylcholine binding protein was used to examine their binding energy to neonicotinoids to perform molecular docking. The binding energy between neonicotinoid and *Lymnaea stagnalis* acetylcholine receptors binding energy was investigated using AutoDock Vina.

### 2.2. Earthworm maintenance and genotyping

The earthworms (*Eisenia fetida*) were obtained from ZgeneBio Inc. in Taipei, Taiwan (<https://www.zgenebio.com.tw/english.html> (accessed on April 29, 2024)). In rearing earthworms, temperature and moisture are among the most important environmental factors to determine the success of earthworm culture (Lowe and Butt, 2005). According to

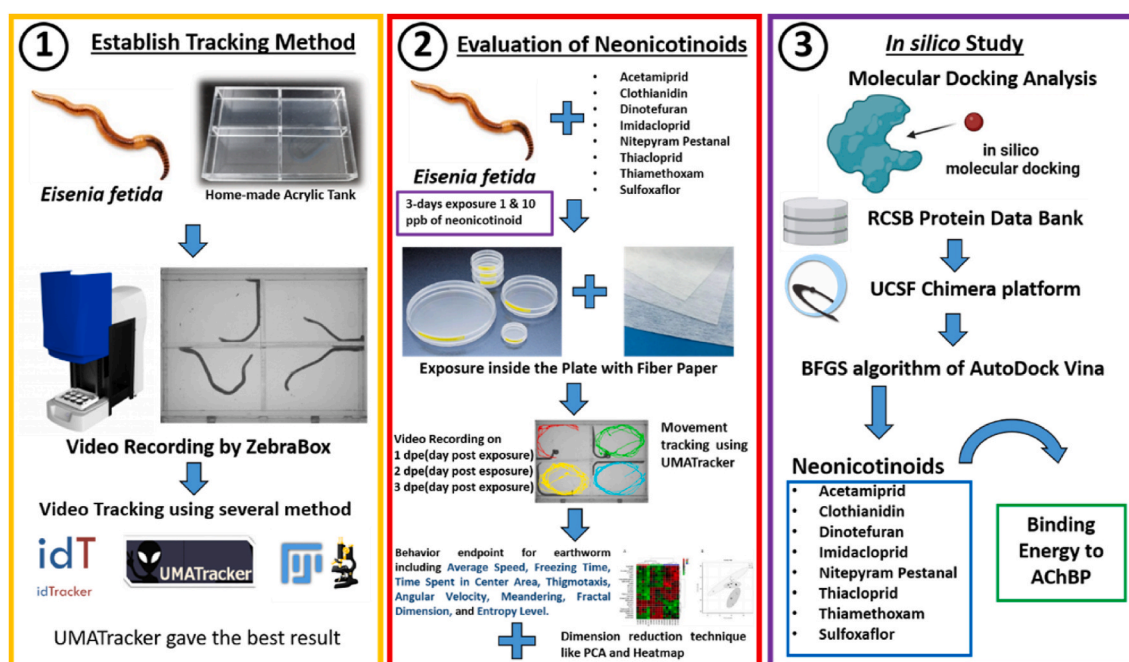


Fig. 1. Overview of the entire experimental design and workflow. In the first step, several tracking tools were tested for earthworm locomotion tracking to find the best tool. For the second step, the best performance tool for earthworm locomotion tracking was applied to evaluate the potential toxic effects of eight neonicotinoid compounds. For the final step, molecular docking was conducted for neonicotinoids and AChBP to provide the *in silico* interaction evidence.

literature studies, earthworms (*Eisenia fetida*) were kept in the soil at 20–26 °C since this temperature range is the best condition to accommodate earthworm maintenance (Hankard et al., 2005). Meanwhile, replacement water (double-distilled water (ddH<sub>2</sub>O)) was applied to the soil surface every two days to keep the soil from being dry and prevent moisture loss since based on the prior study, the optimal range of gravimetric moisture content (w%) for *E. fetida* is around 70–90% and here, the moisture content of the soil was monitored by measuring the ratio of the mass of the moisture present in the soil sample to the dried mass of the same as described in a previous study (Edwards and Arancón, 2005; Nuutinen and Butt, 1997; Rasheed et al., 2022; SU et al., 2014). Prior to the video recording, the used earthworms were selected according to their weight. Only earthworms weighing around 200–600 mg were used in the behavior assay. Furthermore, the used *E. fetida* were clitellate, which were characterized by the clitellum, as the indicator of their sexual maturation, and secreting of a protective cocoon (Martinson and Erséus, 2021). In addition, to get the specific identity of earthworms, we conducted DNA barcoding with primers targeted to mitochondrial COX-1 genes. Primer sequences used to conduct DNA barcoding were COX1-F:5'GGTCAACAAATCATAAAGATATTGG-3' and COX1-R:5'-TATACTTCTGGGTGTCGGAAGAATCA-3'. The size of amplicons is around 660 bp, and they are subjected to Sanger sequencing for species identification (Sun et al., 2017).

### 2.3. Chemical preparation

This experiment used eight insecticides from the neonicotinoid family to examine their effects on earthworms. Imidacloprid, with >98.0% purity, was purchased from Shyuanye (Shanghai Yuanye Bio-Technology Co., Shanghai, China) in a powder form with catalog number 138261-41-3. In a 100 mg powder form, Acetamiprid was purchased from Macklin (Shanghai Macklin Biochemical Co., Shanghai, China) with the catalog number 135410-20-7. A powdered form of clothianidin with catalog number 210880-91-5 was purchased from Aladdin (Aladdin Industrial Corporation, Fengxian, Shanghai). A powdered form of dinotefuran was purchased from Shyuanye (Shanghai Yuanye Bio-Technology Co., Shanghai, China) with catalog number 165252-70-0. Nitenpyram pestanal was purchased from Macklin (Shanghai Macklin Biochemical Co., Shanghai, China) in a powder form with catalog number 150824-47-8. Sulfoxaflor was purchased from Ehrenstorfer (Augsburg, Germany) in 10 mg powder form with the catalog number 946578-00-3. Thiacloprid, with a catalog number 111988-49-9, was purchased in a 100 mg powder form from Aladdin (Aladdin Industrial Corporation, Fengxian, Shanghai). Thiametoxam was purchased from Shyuanye (Shanghai Yuanye Biotechnology Co., Shanghai, China) in powder form, with a catalog number 153719-23-4. All the neonicotinoid compounds were dissolved in ddH<sub>2</sub>O and prepared into a liquid form with 1 (low) and 10 (high) ppb concentrations.

### 2.4. Neonicotinoid exposure

Previously, a research group developed a test to examine the acute toxicity of chemicals to earthworms called the contact filter paper test and the artificial soil test (Edwards, 1983), which was later adopted by the OECD (Spurgeon and Hopkin, 1995). Here, exposure of earthworms to neonicotinoids was conducted by following the guidelines of the OECD with minor modifications (OECD, 1984). In the present experiment, a Petri dish with a diameter of 15 cm with Whatman grade 1 qualitative filter paper inside as a container to hold the earthworm during the exposure period. The principle for the exposure is similar to the contact filter paper test, in which the earthworm will come directly into contact with the filter paper moistened with the chemical tested. A contact filter paper test was chosen, as it is easier to do for an early screening experiment. For the treatment groups, 15 ml of neonicotinoids at concentrations of either 1 or 10 ppb was added to the filter paper inside the Petri dish. These doses were based on the neonicotinoid

environmentally relevant concentrations mentioned in a prior study, which are around 0.2–130 ppb (Alsafran et al., 2022). According to the OECD guidelines, the total amount of pesticides was calculated in mg/cm<sup>2</sup>. Therefore, the total neonicotinoids given for exposure in each group were 1 ppb equal to  $9.7 \times 10^{-8}$  mg/cm<sup>2</sup> (low concentration) and 10 ppb equal to  $9.7 \times 10^{-7}$  mg/cm<sup>2</sup> (high concentration) while ddH<sub>2</sub>O was used for the control group. After the solution was fully absorbed into the filter paper, the earthworm was moved into the Petri dish using the wide part of a spatula to prevent any discomfort to the earthworms. This experiment was done in two replications with *n* number of four earthworms in each replication.

### 2.5. Video recording using ZebraBox viewpoint machine

The exposure of the neonicotinoid was done for three days and the earthworm behavior was recorded daily to monitor their behavior changes. The video recording was done using a ZebraBox viewpoint machine (ViewPoint Life Sciences, Lyon, France) for 30 min after ~5 min of acclimation process to let the earthworms familiar with the environment, with the light intensity set at the maximum level. Before recording, the treated earthworms were moved from the Petri dish into a self-made acrylic plate of 13 × 8 cm as seen in Fig. 1. This acrylic plate consists of four areas and during the recording, each area has one earthworm to achieve a sample size of four in a single recording attempt. After the behavior recording process, output videos were converted into avi format with 1024 × 768 pixels of resolution and used in the following experiments.

### 2.6. The earthworm locomotion tracking using UMATracker

To evaluate the earthworm locomotion, the videos were loaded into UMATracker, developed by Yamanaka and colleagues (Yamanaka and Takeuchi, 2018). UMATracker had been utilized by prior studies as a tracking system to evaluate color preference in freshwater crayfish, locomotion activity of freshwater shrimp, and zebrafish locomotion (Boopathi et al., 2024; Feng et al., 2024; Haridevamuthu et al., 2022; Siregar et al., 2021; Suryanto et al., 2023). In this study, we used UMATracker and utilized their two features, which were FilterGenerator and Tracking. FilterGenerator (Preprocessing step) is used to design parameters for tracking on the editor panel using the visual programming environment Blockly. There are several necessary blocks such as ROI selection to exclude obstacles, morphology algorithms for noise reduction, and thresholding image algorithms. Subsequently, the images of the objects were converted to grayscale and binarized by adjusting the threshold value to obtain the output result of the converted video frame. Meanwhile, FilterGenerator was used to distinguish the object from the experimental background. The next step was using the Tracking feature for the tracking process which had several important parameters to be set, which were the number of individuals, threshold intensity, and minimum and maximum sizes of the individual. UMATracker does not track the front part of the object, instead, it tracks the center part of the object and follows the movement of the targeted object. After the tracking process was finished, the X and Y coordinates position of the earthworm in every frame was outputted in.csv file format. These X and Y coordinates position of earthworms are the primary data translated into earthworm behavior endpoints. The detailed operation protocol for earthworm tracking and endpoint calculation was provided in the supplementary file.

### 2.7. Behavioral endpoints

Following the protocol condition for recording earthworm locomotion, the next focus was extracting information from earthworm behavior. We extracted eight behavioral endpoints, including average speed, average angular velocity, meandering, thigmotaxis, time spent in the central area, fractal dimension (FD), entropy, and freezing time

movement ratio, which is a sign of anxiety in certain animal models, such as mice or zebrafish (Blanchard et al., 2001; Maximino et al., 2010), by following the previous work on zebrafish behavior tracking assay (Audira et al., 2018a, b; Audira et al., 2022). Afterward, we modified and adapted these behavior endpoints for earthworm locomotor activity evaluation. In this study, the percentage of time when the earthworms were motionless or moving at less than 0.05 cm/s was defined as freezing time. This behavior is important for animals in nature since when an animal's natural capacity to freeze turns into a defensive strategy, it reduces the possibility that a predator will notice it (Fanselow and Lester, 2013). This locomotion assessment was necessary when evaluating the effects of neonicotinoids on non-target species like earthworms since neonicotinoids may interfere with nerve transmission via the nicotinic acetylcholine receptor (nAChR) (Tomizawa and Casida, 2005). Furthermore, to test their exploratory behavior, the length of time spent in the center and thigmotaxis of earthworms were also measured. Time spent in the center behavior endpoint is defined as the total percentage of time when the position of the earthworm is in the center area of the plate. Before the calculation, the center area of the plate was determined as a circle area with a diameter of 3 cm in the middle of the well with a size of  $2 \times 3$  cm. Meanwhile, thigmotaxis refers to a specific behavior of animals where they will stay close to the walls when exploring an open space (Walz et al., 2016). In this study, we measured the thigmotaxis by calculating the average distance of the earthworm position from the central area (Supplementary Table A1). Next, in determining their movement orientation, the average angular velocity and meandering were also measured. In physics, angular velocity is a rotation rate vector that refers to how fast an object rotates or revolves relative to another point (Boyle, 2017; Essén, 1993; Gunner et al., 2020). Here, we calculated the vector by evaluating the amount of change in movement direction as computed between two successive time samples based on other behavior researchers that used zebrafish as an animal model (Audira et al., 2022; Qin et al., 2014). Meanwhile, the definition of meandering is the proportion of the total turning angle and distances of animals. Generally, a high level of meandering indicates that the animals moved in a zigzag-like pattern. Finally, correlation dimension was used in this study in the form of Fractal Dimension (FD) and entropy. Fractal dimension analysis was used to investigate the earthworm's movement pattern and complexity within the time-space structured property of the earthworm's movement while entropy measured the level of predictability and directionality of individual interactions (Eguiraun and Martinez, 2023). The formula and mathematic calculation for both FD and entropy were based on a prior study in zebrafish (Audira et al., 2022). The details about the calculated behavior endpoints can be found in the supplementary file.

## 2.8. Statistical analysis

All statistical analyses and graph plotting were conducted with GraphPad Prism (GraphPad Software version 8 Inc., La Jolla, CA, USA). The behavioral endpoints' values between the control and neonicotinoid-treated groups were compared. Initially, normality tests were conducted before the statistical analyses to evaluate the data distribution normality. Later, a One-Way Analysis of Variance (ANOVA) test continued with Dunnett's multiple comparisons test as the follow-up test was used to compare the control and treated groups, and all of the data are expressed as mean with standard error of the mean (SEM). The p-value was indicated by \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; or \*\*\*\*  $p < 0.0001$  for each significance level. Meanwhile, to categorize detailed phenotypic profiling to reduce the data complexity after all behavior endpoints had been calculated, principal component analysis (PCA) and hierarchical clustering analysis were carried out (Tecott and Nestler, 2004). As one of the most often used projection-based techniques for reducing the dimension of data sets, PCA transforms high dimensional data by projecting it onto a set of orthogonal axes and frequently visualizes two scatterplot components (Duchene and Leclercq, 1988;

Metsalu and Vilo, 2015a). PCA and hierarchical clustering analysis are two important tools that can reduce data complexity. We used a web-based ClustVis tool to create the PCA and heatmap (Metsalu and Vilo, 2015b). The mean of each behavioral endpoint value from every group (control and treatment) was summarized and pooled into a single Excel file and converted into a comma-delimited type file (.csv). This.csv data file was uploaded to the ClustVis website (<https://biit.cs.ut.ee/clustvis/>) (accessed on April 4, 2024) to get better-visualized information on PCA and heatmap.

## 2.9. Molecular docking of neonicotinoid with nAChR

Nicotinic acetylcholine receptors, belonging to a family of transmembrane neurotransmitter receptors, are ligand-gated ion channels mediating fast-acting excitatory cholinergic neurotransmission (Hendrickson et al., 2013). Structurally, nAChRs are an assembly of five subunits wherein the interfaces between subunits at the extracellular region serve as ligand-binding domains for acetylcholine (Dani, 2015). All *in silico* molecular docking experiments were performed on the UCSF Chimera platform, which was similar to the previous study on freshwater shrimp (Siregar et al., 2021). Unfortunately, since the crystal structure of *E. fetida* was not available on the protein data bank, the crystal structure of soft-bodied type snails (*L. stagnalis*) was used for the molecular docking experiment in the current study. This snail was chosen since they share a distant common ancestor with the annelid worms (Amorim et al., 2019). Moreover, both of them also possess similar characteristics like soft-bodied, slimy, squirmy, and live in the soil (Amorim et al., 2019; Zhu et al., 1994). The crystal structure of *Lymnaea stagnalis* acetylcholine binding protein (AChBP, PDB ID: 2ZJU) was retrieved from RCSB Protein Data Bank (<https://www.rcsb.org/>) and rendered to the docking platform in PDB format. The three-dimensional protein structure was processed by removing co-crystallized ligands and water molecules. Chains A and B served as the representative of the ligand-binding subunit; thus, all other protein chains were then removed. Structural refinement was done through energy minimization via the steepest descent method (100 steps; step size 0.02 Å) and conjugate gradient method (10 steps; step size 0.02 Å). Meanwhile, the ligands were prepared by optimizing their geometries using an open-source molecular builder Avogadro (version 1.2.0), and added to the docking platform as a SYBYL mol2 file (Siregar et al., 2021). Dock-prepping of ligand and protein structures was done by adding the missing hydrogen atoms and appropriate charges to the structures employing the Gasteiger charge method computed using Amber's Antechamber module (Siregar et al., 2021). The docking procedure employed a 'flexible ligand into a flexible active site' protocol, allowing the ligand to be flexible and torsion within a grid box encompassing the ligand-binding cavity of the protein. A grid box was set around the co-crystallized ligand of the protein. With all docking parameters maintained at default values, molecular docking was done following the Broyden-Fletcher-Goldfarb-Shanno (BFGS) algorithm of AutoDock Vina (version 1.5.6) (Siregar et al., 2021). The docking protocol was validated via a redocking experiment of the co-crystallized ligands. Finally, the conformation of the protein-ligand complex was visualized and analyzed using Biovia Discovery Studios (version 4.1).

## 3. Results

### 3.1. Earthworm average speed after acute exposure to the neonicotinoids

This experiment examined the locomotor activity of earthworms by checking their average speed. Here, all of the data from 1, 2, and 3 days post-exposure from each group were merged into a single dataset since based on the preliminary data, there was no statistical difference found in all measured behavior endpoints between each time interval (Supplementary Fig. 3). In addition, this data merger was done to increase the technical replicates of the data to reduce the intra-individual noise,

thus, reducing group variances and animal usage. From the results, we found that not only imidacloprid but also all tested neonicotinoids reduced earthworm locomotor activity, as shown by the significantly low average speed (Fig. 2). From all of the tested neonicotinoids, acetamiprid, dinotefuran, imidacloprid, sulfoxaflor, and thiacloprid caused a severe effect on earthworm locomotor activity. These compounds could affect the earthworm's locomotor activity even at the lowest concentration. Imidacloprid-treated earthworms' average speed was found to be the lowest, with a mean with SEM value of  $0.2721 \pm 0.14$  cm/s and  $0.1846 \pm 0.13$  cm/s for 1 ppb (low dose) and 10 ppb (high dose) respectively, followed by acetamiprid and dinotefuran, which were significantly lower than the control group with an average speed of  $0.5786 \pm 0.34$  cm/s. Meanwhile, lower concentrations of clothianidin, thiametoxam, and nitenpyram did not significantly affect earthworm's locomotor activity. However, even though these three compounds did not affect the locomotor activity of earthworms at the low dose, they still significantly reduced the average speed at the high dose (Fig. 2). Taken together, this result demonstrated that neonicotinoids could significantly lower the average speed of earthworm locomotor activity, especially when administered at a high dose (Fig. 2).

### 3.2. Earthworm freezing time movement ratio after acute exposure to the neonicotinoids

The freezing time movement ratio, another endpoint associated with locomotion, was also calculated in the current study. The results revealed an increased freezing time movement ratio in treated earthworms, notably at high concentrations (Fig. 3). These findings are consistent with Fig. 2, which shows that neonicotinoid treatment reduced earthworm speed. Compared to the control, which had a mean with SEM of freezing time movement ratio of approximately  $13.86 \pm 12\%$ , 10 ppb of sulfoxaflor had the most significant detrimental effects, with a mean with SEM of freezing time movement ratio around  $59.02 \pm 30\%$  (Fig. 3). Sulfoxaflor was followed in terms of the severity of effects on the freezing time movement ratio by thiacloprid, clothianidin, and imidacloprid. In contrast to other neonicotinoid pesticides, thiametoxam treatment at high concentrations caused a less pronounced shift in the freezing movement, while at low concentrations, it had no noticeable effects. In summary, it has been demonstrated that neonicotinoids increase the frequency of freezing movements in earthworms, which in turn affects their locomotor activity.

### 3.3. Earthworm time spent in the center area after acute exposure to the neonicotinoids

To examine the exploratory behaviors of earthworms, the amount of time earthworms stayed in the center area was measured to investigate their movement across the plate. Interestingly, neonicotinoids were also found to have an impact on the exploratory behaviors of the earthworm, as seen by the longer time earthworms spent in the center area. These findings indicated that earthworms treated with neonicotinoids remained in the center of the well longer than the earthworms of the control group, which moved and explored the well's periphery and center. For instance, compared to the control group, which spent just 4.4% of the observation period in the middle of the tank, the treatment group that was administered with a high dose of sulfoxaflor spent a statistically higher percentage of time spent in the center area of the tank ( $57.2 \pm 39\%$ ) (Fig. 4). Following treatment with sulfoxaflor, acetamiprid, dinotefuran, imidacloprid, and nitenpyram, the amount of time spent at the center of the tank was longer than it was in the control group. Nevertheless, thiametoxam, clothianidin, and thiacloprid did not appear to affect the earthworm's normal tendency to stay in the center. Both low and high doses of these three neonicotinoids did not affect time spent in the center area of the treated earthworm at all. While, sulfoxaflor showed the highest time spent in the center area, thiametoxam low dose became the lowest with 3.79 % time spent in the center area (Fig. 4). The result is intriguing because it raises the possibility that neonicotinoids could also affect the exploratory behavior of earthworms.

### 3.4. Earthworm thigmotaxis after acute exposure to the neonicotinoids

In order to further examine the effect of neonicotinoids on the exploratory behavior of earthworms, thigmotaxis was calculated. Results showed that several neonicotinoids could significantly affect the thigmotaxis levels by reducing the thigmotaxis compared to the control group (Fig. 5). A higher thigmotaxis value indicated that the earthworm stayed close to the wall when exploring the plate. The control group had a mean with SEM of thigmotaxis value around  $2.358 \pm 0.3$  cm, indicating that the normal earthworm behavior would stay close to the wall when they move across the acrylic plate (Fig. 5). Meanwhile, acetamiprid, dinotefuran, imidacloprid, nitenpyram, and sulfoxaflor altered the thigmotaxis of earthworms when applied in high concentrations. Among these compounds, sulfoxaflor showed the lowest mean with SEM of thigmotaxis results at  $1.5523 \pm 0.6$  cm. The results also showed that

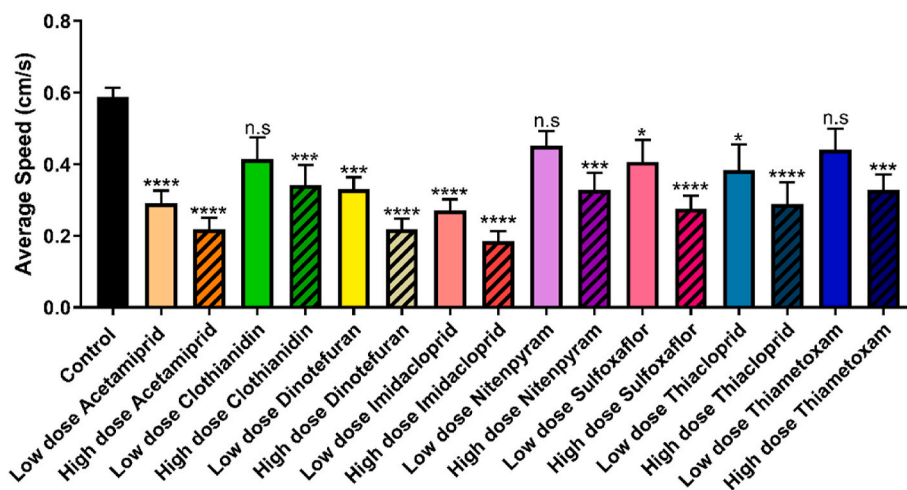


Fig. 2. The average speed of earthworms after 1, 2, and 3 days of exposure to neonicotinoids (acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, sulfoxaflor, thiacloprid, and thiametoxam) in 0 (control), 1 (low dose) and 10 ppb (high dose). Data are presented as mean with SEM and were analyzed by using One Way ANOVA followed by Dunnet's multiple comparison test ( $n = 8$  for each group; \* $p < 0.05$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ).

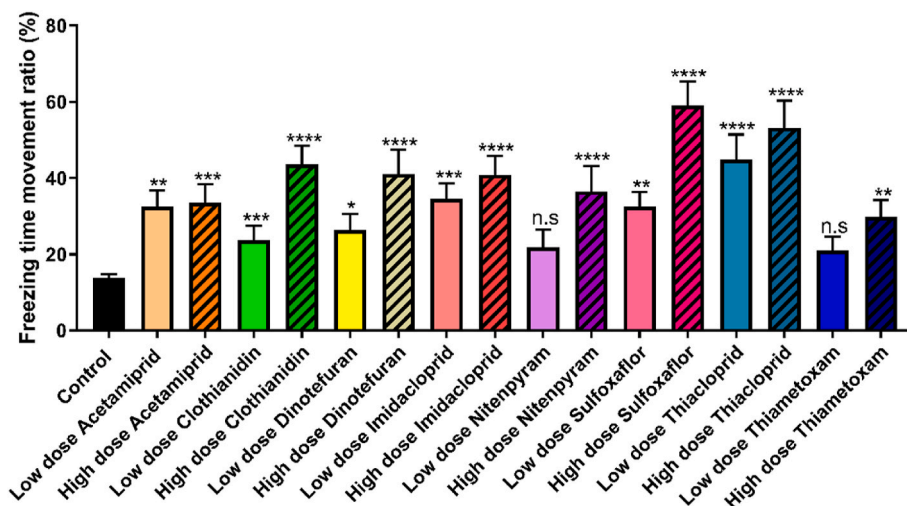


Fig. 3. Freezing time of earthworms after 1, 2, and 3 days of exposure to neonicotinoids (acetamidrid, clothianidin, dinotefuran, imidacloprid, nitenpyram, sulfoxaflor, thiachloprid, and thiametoxam) in 0 (control), 1 (low dose) and 10 ppb (high dose). Data are presented as mean with SEM and were analyzed by using One Way ANOVA followed by Dunnet’s multiple comparison test (n = 8 for each group; \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; \*\*\*\*p < 0.0001).

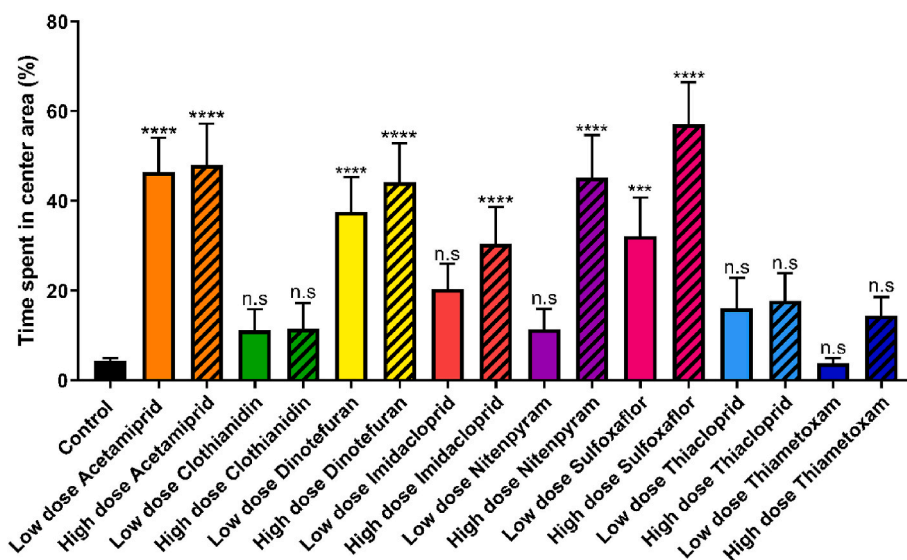


Fig. 4. Time spent in the center area of earthworms after 1, 2, and 3 days of exposure to neonicotinoids (acetamidrid, clothianidin, dinotefuran, imidacloprid, nitenpyram, sulfoxaflor, thiachloprid, and thiametoxam) in 0 (control), 1 (low dose) and 10 ppb (high dose). Data are presented as mean with SEM and were analyzed by using One Way ANOVA followed by Dunnet’s multiple comparison test (n = 8 for each group; \*\*\*p < 0.001; \*\*\*\*p < 0.0001).

the sulfoxaflor-treated earthworm did not stay close to the walls or stayed close to the center area. On the other hand, the thigmotaxis values of groups exposed to thiametoxam, clothianidin, and thiachloprid were comparable to the thigmotaxis value of the control group, suggesting that these treated earthworms still showed similar behavior where they stayed close to the walls. Reduced thigmotaxis values complemented the longer time spent in the center area, supporting the theory that pesticide exposure could affect earthworm exploratory behavior.

### 3.5. Earthworm average angular velocity after acute exposure to the neonicotinoids

The next behavioral endpoint is the average angular velocity, a behavior endpoint that is related to the animals’ movement orientation. From the results, the earthworms revealed a significantly increased angular velocity after 72 h of neonicotinoid treatment, indicating that the majority of them had an aberrant movement direction (Fig. 6). All of

the tested neonicotinoids at high concentrations significantly increased the number of earthworms rotating across the plate, as evidenced by the higher angular velocity with clothianidin, which had the highest mean with SEM value of the angular velocity at  $108 \pm 14.9^\circ/s$  that was significantly higher than the control group with a mean with SEM of average angular velocity around  $58.57 \pm 20.1^\circ/s$ . Other neonicotinoids, including acetamidrid, thiachloprid, and imidacloprid also showed a significantly higher average angular velocity even at low doses. Meanwhile, the lowest average angular velocity was demonstrated by  $54.82 \pm 19.9^\circ/s$ , which was quite similar to the control. At low concentrations, however, several neonicotinoids including dinotefuran, nitenpyram, sulfoxaflor, and thiametoxam had little to no effect on the orientation of the earthworms’ movement and less of an effect on the angular velocity.

### 3.6. Earthworm meandering after acute exposure to the neonicotinoids

This study also measured meandering, another behavior endpoint that is also related to an animal movement orientation. Similar to the

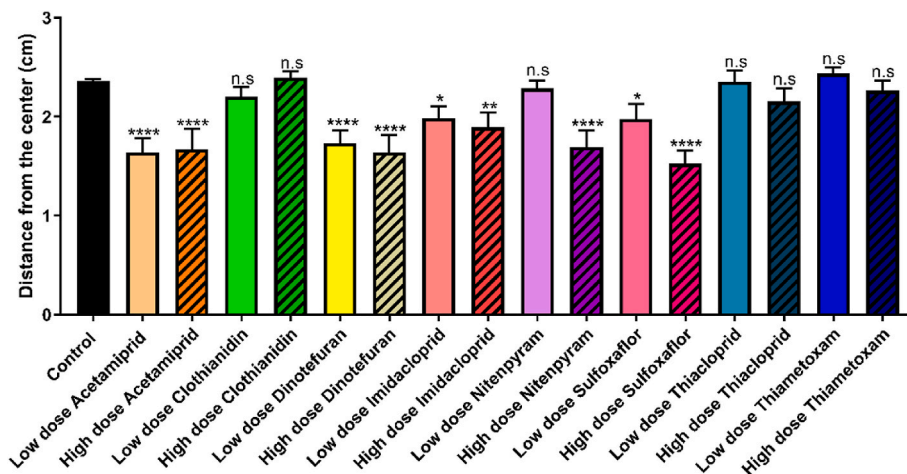


Fig. 5. Thigmotaxis of earthworms after 1, 2, and 3 days of exposure to neonicotinoids (acetamidrid, clothianidin, dinotefuran, imidacloprid, nitenpyram, sulfoxaflor, thiacloprid, and thiametoxam) in 0 (control), 1 (low dose) and 10 ppb (high dose). Data are presented as mean with SEM and were analyzed by using One Way ANOVA followed by Dunnet’s multiple comparison test ( $n = 8$  for each group; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.0001$ ).

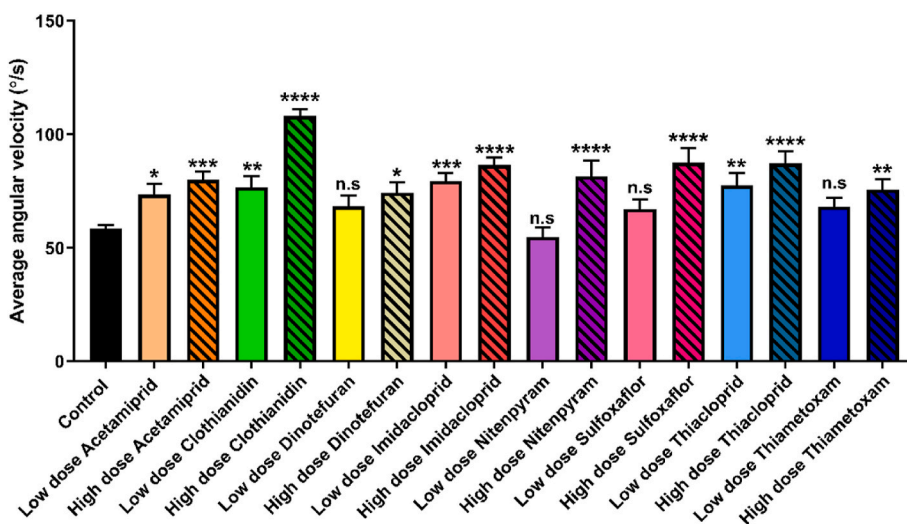


Fig. 6. Average angular velocity of earthworms after 1, 2, and 3 days of exposure to neonicotinoids (acetamidrid, clothianidin, dinotefuran, imidacloprid, nitenpyram, sulfoxaflor, thiacloprid, and thiametoxam) in 0 (control), 1 (low dose) and 10 ppb (high dose). Data are presented as mean with SEM and were analyzed by using One Way ANOVA followed by Dunnet’s multiple comparison test ( $n = 8$  for each group; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ).

previously mentioned angular velocity data, the results showed that neonicotinoid treatments could affect the meandering of earthworms. After exposure to acetamidrid, clothianidin, dinotefuran, imidacloprid, sulfoxaflor, and thiacloprid, earthworms displayed meandering values higher than the control, indicating that they were more often to move in a zigzag-like pattern (Fig. 7). The meandering value was highest in the thiacloprid-treated group, registering at the mean of  $234229^\circ/\text{m}$  at the high concentration. Imidacloprid and thiacloprid at low concentrations also significantly increased the meandering of earthworms. On the other hand, earthworm meandering values were unaffected by nitenpyram and thiametoxam exposure compared to the control group (Fig. 7).

### 3.7. Earthworm fractal dimension after acute exposure to the neonicotinoids

Since it can be difficult and complex to interpret earthworm behavior data based on the behavior endpoints collected from their motions and due to the need, fractal dimension (FD) was calculated. Results showed that FD was statistically lower in all the neonicotinoid-treated groups than in the control group (Fig. 8). These results revealed that the

movement of the treated earthworms was less complex than that of the untreated ones. The control group, in contrast, showed a relatively high mean with SEM of FD ( $0.7367 \pm 0.29$ ), which suggests greater complexity and more rapid movement. The FD was statistically lower in the groups treated with sulfoxaflor, imidacloprid, acetamidrid, and thiacloprid than in the control group (Fig. 8). For low and high doses, the thiacloprid-treated group showed the lowest mean with SEM of FD values of  $0.3362 \pm 0.3\%$  and  $0.2636 \pm 0.2$  respectively. On the other hand, thiametoxam, clothianidin, and nitenpyram did not significantly change the FD value of earthworms when used at low doses. These findings support the results for average speed and freezing time (Figs. 2 and 3), indicating that faster motility would probably result in a high FD value. In conclusion, neonicotinoid-treated earthworms displayed less complexity in their spatial movement than untreated earthworms and became less active.

### 3.8. Earthworm entropy after acute exposure to the neonicotinoids

Entropy, another nonlinear measurement, was also calculated in the present study. Here, the earthworms treated with neonicotinoids had

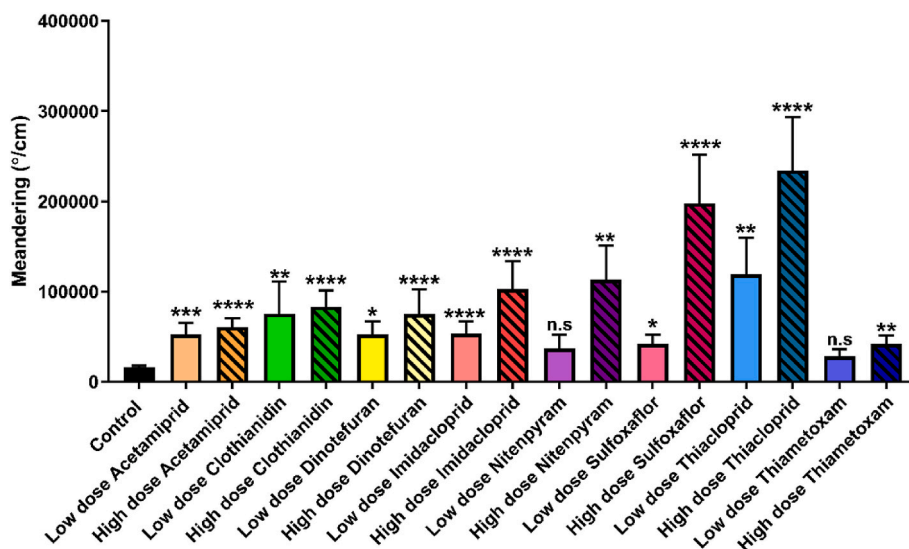


Fig. 7. Meandering of earthworms after 1, 2, and 3 days of exposure to neonicotinoids (acetamidiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, sulfoxaflor, thiacloprid, and thiametoxam) in 0 (control), 1 (low dose) and 10 ppb (high dose). Data are presented as mean with SEM and were analyzed by using One Way ANOVA followed by Dunnet’s multiple comparison test ( $n = 8$  for each group; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ).

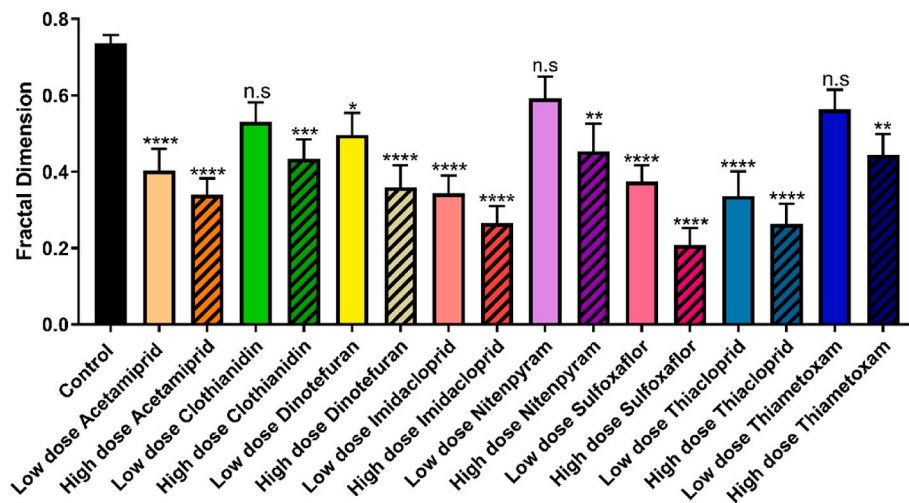


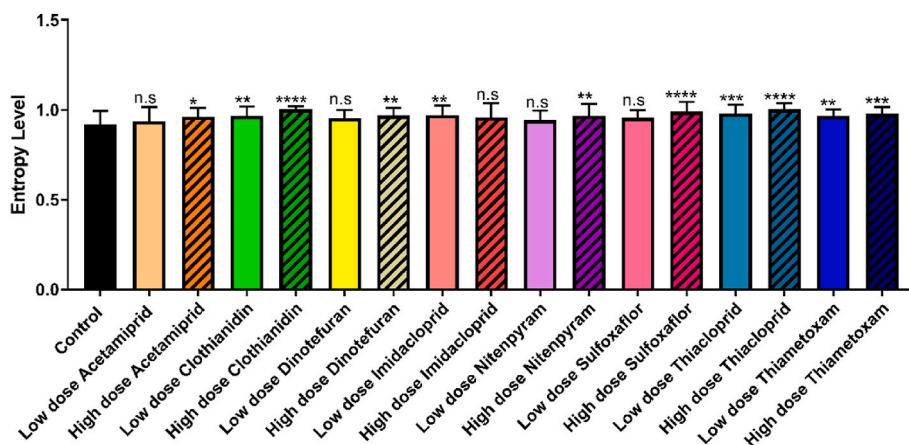
Fig. 8. Fractal dimension of earthworm after 1, 2, and 3 days of exposure to neonicotinoid insecticide (acetamidiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, sulfoxaflor, thiacloprid, and thiametoxam) in 0 (control), low and high dose. Data were based on the trajectories calculated by UMATracker. Data are presented as mean with SEM. For statistical differences, data were analyzed by using One Way ANOVA followed by Dunnet’s multiple comparison test ( $n = 8$  for each group; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ).

statistically higher entropy levels than the control group (Fig. 9). These findings suggest that the administration of neonicotinoids to earthworms may influence the degree of predictability of their movement; in this case, a greater entropy value denotes an increase in their unpredictable movement. As entropy increased, earthworm movement treated with neonicotinoids became more erratic. The entropy level of the earthworms was unaffected by low concentrations of acetamidiprid, dinotefuran, nitenpyram, and sulfoxaflor.

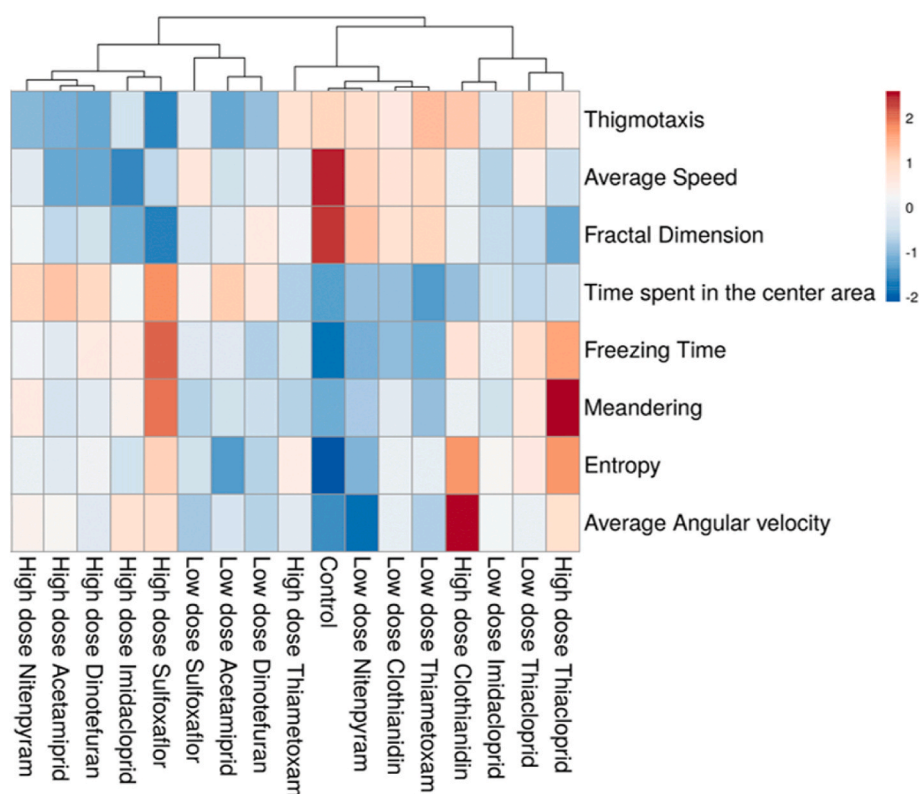
### 3.9. Heatmap and PCA of earthworm behavior

Next, the complexity of the earthworm behavior data was reduced and grouped into clusters using the PCA analysis and heatmap. The heatmap analysis shows fingerprint-like data where each compound exhibits its unique patterns and the accuracy of these data improves as the number of endpoint data increases. Here, we analyzed eight neonicotinoid pesticides by using eight different behavior endpoints.

According to the heatmap and PCA result, all experimental groups were divided into two main clusters. The control and low-concentration groups of nitenpyram, thiametoxam, clothianidin, imidacloprid, and thiacloprid were clustered together in a single cluster (right side of the heatmap). Conversely, another cluster (left side of the heatmap) was mostly comprised of neonicotinoids at the high dose (Fig. 10). After integrating the data with the heatmap, the PCA revealed that at the low concentration, several neonicotinoid-treated groups, such as thiametoxam, clothianidin, and nitenpyram were comparable to the control (Fig. 11). These results are consistent with the behavior data, indicating that the earthworm behaviors were not significantly affected by the lower concentration of these neonicotinoids while their behaviors would be more significantly affected by higher concentrations of these neonicotinoids.



**Fig. 9.** The entropy of earthworms after 1, 2, and 3 days of exposure to neonicotinoid insecticide (acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, sulfoxaflor, thiacloprid, and thiametoxam) in 0 (control), low and high dose. Data were based on the trajectories calculated by UMATracker. Data are presented as mean with SEM. For statistical differences, data were analyzed by using One Way ANOVA followed by Dunnet's multiple comparison test ( $n = 8$  for each group; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ ).

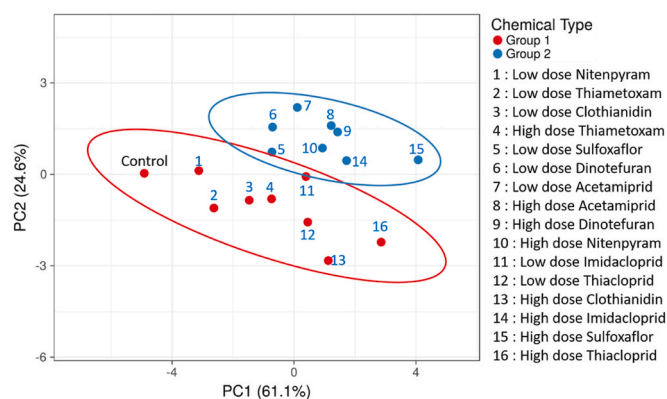


**Fig. 10.** Hierarchical clustering analysis of multiple behavior endpoints in earthworms after exposure to eight different neonicotinoids. Color in the heatmap represents the changes in earthworm behavior compared to control. The red color indicates a higher value while the blue color indicates a lower value of the behavior endpoints. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

### 3.10. Molecular docking of neonicotinoids to nAChBP

Molecular docking studies were carried out to assess and visualize the binding behavior of neonicotinoids toward nicotinic acetylcholine receptors (nAChRs). As mentioned above, since there was limited information on the crystal structure of earthworm nAChR, the acetylcholine binding protein (AChBP) from the snail *Lymanea stagnalis* was used for the *in-silico* evaluation of binding mechanisms of the neonicotinoids. The ligand-binding region (LBR) for AChBPs is highly conserved. Thus, considered valuable ligand-binding domain surrogates for nAChRs, especially for molecular docking experiments (Ihara et al.,

2008). Three nicotinoids, namely acetamiprid, nitenpyram, and dinotefuran, exhibited the highest binding affinity toward the ligand-binding domain of AChBP. Each compound gave a binding energy (BE) of  $-6.1$  kcal/mol, comparable to the reference compound, imidacloprid (Table 1, Fig. 12). One of the primary reasons for the relatively strong attachment of acetamiprid onto the enzyme's binding domain is its chloropyridine moiety being densely strengthened by LBR residues. The pyridine ring interacted with Cys187 of subunit A and Met114 of subunit B through intermolecular pi-sulfur bonding and with Tyr185A through stacked pi-pi forces. The chloro substituent, on the other hand, is held via pi-alkyl/alkyl interactions with Trp53B, Tyr164B, and Tyr185A. Also,



**Fig. 11.** Principal component analysis (PCA) of multiple behavior endpoints in earthworms after exposure to eight different neonicotinoids. Two major clusters from hierarchical clustering are marked with red (cluster 1) and blue (cluster 2) colors. Unit variance scaling is applied to rows; singular value decomposition (SVD) with imputation is used to calculate principal components. X and Y-axis show principal component 1 with 61.1% and principal component 2 with 24.6%, which explains the total variance. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the cyano group is stabilized by intermolecular carbon-hydrogen bonding (4.56 Å) with Tyr113B and hydrogen bonding (4.43 Å) with Met114B. Similar to acetamiprid, nitenpyram's strong binding is attributed to its chloropyridine moiety, where the aromatic ring participated in pi-sulfur bonding with Cys187A, Cys188A, and Met114B. Pi-pi stacking can also be observed between the aromatic core and Tyr185A. The chloro substituent is affixed by pi-alkyl interplay against Trp53B, Tyr164B, and Tyr185A. As for its hydrophilic side chain, the secondary methylamine is held by H-bonding (4.66 Å) with Trp143A and C-H bonding (3.68 Å) with Met114B, while the nitro group is held by H-bonding (3.73 Å) with Met114B and C-H bonding with Tyr113B and Thr144A. In the case of dinotefuran, its tetrahydrofuran ring is nestled to the binding region through pi-alkyl/alkyl interactions against Trp53B, Trp143A, Tyr185A, and Met114B. Two relatively strong H-bonding are observed between one of the nitrogen molecules of the guanidine moiety (4.37 Å) against Trp143A and between the nitro group (4.63 Å) against Arg104B. Thiacloprid is another notable compound with a relatively good binding affinity to the enzyme (BE = -6.0

**Table 1**

Summary of molecular docking scores and non-van der Waals interacting residues of the bound neonicotinoids against *L. stagnalis* AChBP.

Neonicotinoid	Binding energy, kcal/mol	Non-van der Waals interacting residue	
		Chain A	Chain B
acetamiprid	-6.1	Cys187, Tyr185	Trp53, Tyr113, Met114, Tyr164
clothianidin	-5.7	Ser142, Trp143, Tyr185, Tyr192	Trp53, Arg104, Leu112, Met114
dinotefuran	-6.1	Trp143, Tyr185	Trp53, Arg104, Met114
nitenpyram	-6.1	Trp143, Thr144, Cys187, Cys188, Tyr185	Trp53, Tyr113, Met114, Tyr164
sulfoxaflor	-5.9	Trp143, Cys187, Cys188, Glu190, Tyr192	Gln73, Arg104, Leu112
thiacloprid	-6.0	Trp143, Tyr185, Cys187	Trp53, Arg104, Leu112, Met114, Tyr164
thiametoxam	-5.4	Trp143, Thr144, Cys188, Tyr192	Arg104, Met114
imidacloprid (reference)	-6.1	Trp143, Cys187, Tyr192	Arg104, Leu112, Met114

kcal/mol). The pyridine core of thiacloprid is stabilized by stacked pi-pi with Tyr185A and pi-sulfur with Cys187A and Met114B. The chlorine substituent of pyrimidine showed pi-alkyl/alkyl interplay against Trp53B, Trp164B, Tyr185A, and Met114B. A similar pi-alkyl interplay aided the stabilization of the thiazolyl moiety with Arg104B and Leu112B. Also, a conventional H-bonding is noted between the nitrogen of the cyanamide group and Met114B. Finally, sulfoxaflor, clothianidin, and thiametoxam were shown to exhibit slightly lower binding affinities (BE of -5.9 kcal/mol, -5.7 kcal/mol, and -5.4 kcal/mol, respectively) compared to the reference compound imidacloprid (Fig. 12 and Table 1).

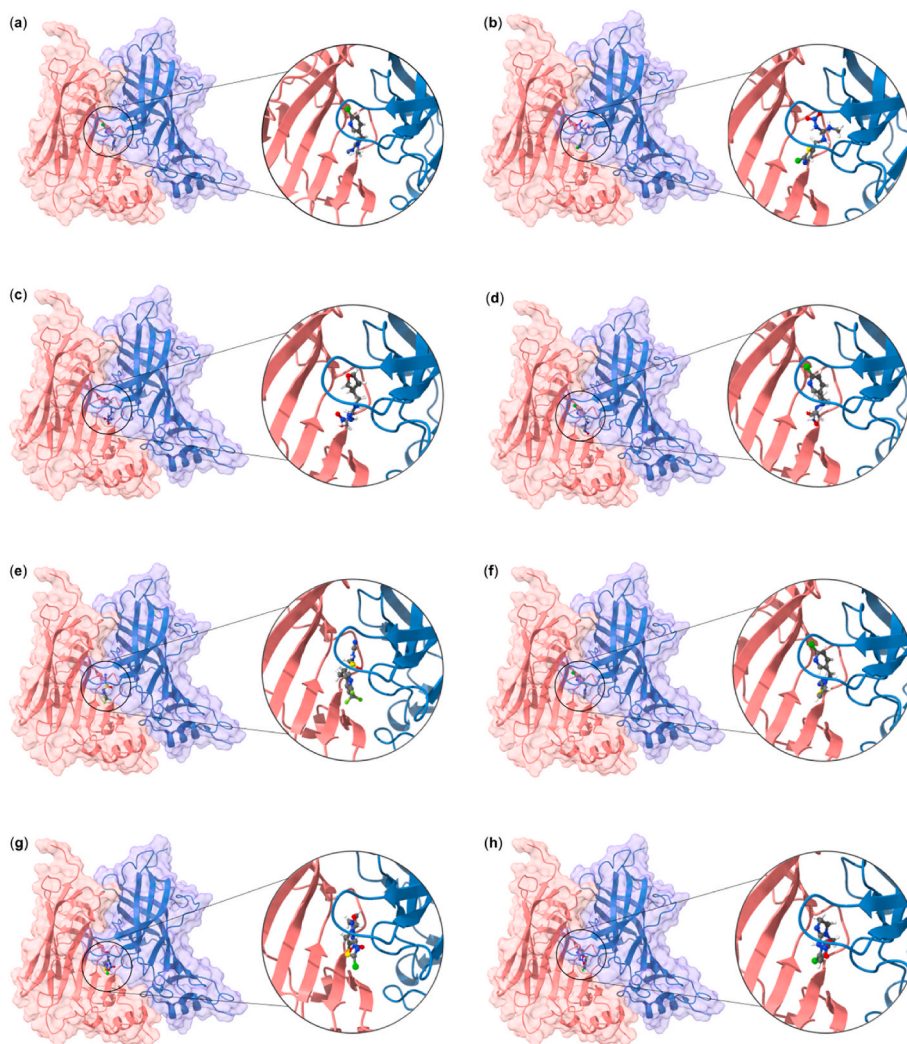
## 4. Discussion

### 4.1. A novel method for assessing earthworms behavior

Earthworms are a useful animal model to represent the soil environment, and studying their behavior can provide an alternative perspective on ecotoxicity investigations. Here, we conducted an in-depth experiment to evaluate earthworm behavior to determine its potential for determining the environmental toxicity of neonicotinoids by creating a novel and straightforward method to analyze earthworm locomotor behavior in a high-throughput manner. The technique described in this paper can be applied as a benchmarking tool for evaluating earthworm locomotor behavior since up to now there have yet to be any assay that could directly observe earthworm behavior. Since earthworms are buried in the soil, it was challenging to monitor their natural behavior, which is a problem in earlier research on earthworm behavior (Yeardley Jr et al., 1996). Our approach addressed this issue and enabled the tracking of earthworm motion and the extraction of behavioral alterations following exposure to pollutants. Therefore, we focused on the most critical aspects to evaluate earthworm behavior, including (i) behavior observation (recording) and (ii) behavior tracking, which were translated to (iii) behavior endpoints. A self-made transparent acrylic plate with four separate areas was employed as part of the setup for the behavior observation to allow for direct movement observation, making it possible to observe four individual earthworms in each recording to observe earthworm movement using ZebraBox (Fig. 1, Supplementary Fig. 1A).

The tracking technique used to examine earthworm activity is the first step that became the priority of the present study. As mentioned above, UMATracker provided better tracking outcomes than the other tracking systems, such as IdTracker and ToxTrac, since it provided more reliable and consistent results (Supplementary Fig. 2).

Similar to idTracker and ToxTrac, UMATracker marks and tracks the center of mass, a position defined relative to an object or system of objects, of the organism (Pérez-Escudero et al., 2014). For rigid objects, this center of mass will be easier to define and usually located at the centroid (Minetti et al., 2011). Meanwhile, due to their lack of a bony structure and a constant body shape, the earthworms were more challenging to monitor using ToxTrack and idTracker since these trackers could mark outside of the earthworm body, which could lead to overestimation or underestimation (Edwards and Bohlen, 1996). However, even though UMATracker produced tracking results that were more dependable and consistent (Supplementary Figs. 1 and 2), this tracker still faces limitations including biased tracking by overestimating or underestimating the direction of the worms' heads and tails as their center of mass makes them identifiable (Supplementary Fig. 1B). Therefore, the use of AI might be beneficial to monitor earthworm activities in the future. Actually, previous research has created a Deep Learning-based AI system to monitor earthworm-digging activity by using two cameras to record the earthworms' burrowing motion while artificial intelligence (AI) assisted in locating them (Djerdj et al., 2020). Nevertheless, the current developed earthworm tracking test successfully demonstrated in this research showed that it is possible to watch the behavior of earthworms directly. Even though the experiment was



**Fig. 12.** Top docking poses of (a) acetamidiprid, (b) clothianidin, (c) dinotefuran, (d) nitenpyram, (e) sulfoxaflor, (f) thiacloprid, (g) thiamethoxam, and (h) reference compound imidacloprid within the ligand-binding region of *L. stagnalis* AChBP at the interface of chain A (purple) and chain B (pink). A wide-angle view of the electron density map of the bound complex (left) and a focused view (right) are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

conducted in a setting other than the earthworm's natural habitat, it can still be regarded as the first screening effort to determine how neonicotinoids affect earthworm behavior.

The behavior endpoints that were extrapolated from the movement of the earthworms were another area of emphasis for the current study. After comparing their average speed between 10, 20, and 30 min of observation time, we chose the 30 min of observation time as the most suitable duration for this experiment due to several reasons. First, while the average speed of earthworms was exceptionally rare to be studied, there is one particular study that measured the average speed of *Lumbricus* (Quillin, 1999). Based on the equation from their research article, a zoologist constructed a graph of a relationship between the speed of the earthworms and their size (Campbell, 2000). Based on this graph, we could predict that the earthworms with a weight ranging from 0.2 to 0.6 g should possess an average speed that ranges from 0.5 to 0.8 cm/s, which was relatively comparable with the results from 30 min of observation (Supplementary Fig. 1C). Second, the 30 min observation time was chosen to reduce the time required to conduct this test, enhancing the efficiency of this method. In addition, light was also provided during the test since based on our preliminary test, the earthworms exhibited a higher activity in the presence of light compared to the dark environment (Supplementary Fig. 1D). This is typical

behavior of earthworms as shown in a previous study that demonstrated earthworms reaction to strong and intense light by avoiding it and turning away from the stimulus (Harper, 1905; Mishra et al., 2019). When their entire body is exposed to light, earthworms will retreat into their burrow if the anterior end is stimulated and move forward if the posterior end is triggered (Harper, 1905; Mishra et al., 2019). Therefore, by applying the optimized condition to record earthworm locomotion behavior for continuous 30 min in light conditions for three consecutive days, we could maximize earthworm locomotion activity and obtain better dynamic changes of locomotion impairment before and after the chemical treatment.

#### 4.2. Application of the developed method in investigating the behavior toxicities of neonicotinoids on earthworms

Neonicotinoid works by disturbing the nervous system, targeting the nicotinic acetylcholine receptor (nAChR) that is responsible for nerve signaling (Bass and Field, 2018). Excessive use of this pesticide leads to the contamination of aquatic and soil ecosystems. There has been documentation of neonicotinoid pesticides in several investigations (Bonmatin et al., 2015; Sánchez-Bayo et al., 2016). Based on these investigations, we utilized the newly developed method to investigate the

neonicotinoid toxicity on earthworm behavior. A prior study in earthworms revealed a harmful and toxic effect of neonicotinoids on *E. fetida*. The earthworm's epidermal and midgut cells were significantly harmed by the neonicotinoids imidacloprid, acetamiprid, nitenpyram, clothianidin, and thiacloprid, which also impaired their capacity to reproduce (Wang et al., 2015a). Results in this study showed a consistent result where neonicotinoids in 1 and 10 ppb concentrations, were able to alter locomotor activity, exploratory behavior, and movement orientation of earthworms that were similar to the findings from a prior study on freshwater shrimp treated with imidacloprid. When given imidacloprid at ppb levels, freshwater shrimp (*Neocaridina denticulata*) had considerably reduced locomotor activity, a decreased heartbeat, and altered gill ventilation movement (Siregar et al., 2021). In addition to behavior and heart rate, a previous study also inspected imidacloprid's molecular docking to the nAChRs of freshwater shrimp and discovered that imidacloprid had a high affinity binding to the nAChRs of *N. denticulata* (Siregar et al., 2021). This demonstrates that imidacloprid, whose primary target is insects, may also affect other creatures such as shrimp (Siregar et al., 2021) and earthworms as demonstrated in this study. Furthermore, the current finding was also consistent with a previous study on brown shrimp (*Farfantepenaeus aztecus*), which found that exposure to imidacloprid immobilized this species of brown shrimp, reduced their locomotion, produced involuntary leg movement, and eventually caused death (Al-Badran et al., 2019). Earlier investigations on zebrafish larvae and *Daphnia* (Hussain et al., 2020) were also in line with the current study, where pesticide exposure caused an alteration in the locomotion of both organisms. In addition, recent studies in earthworms showed that there was a significant decrease in body weight of earthworm and significant changes in enzyme activities such as SOD, CAT, GST, and POD after exposure to clothianidin (Chowdhary et al., 2023). Another recent study in *Eisenia fetida* also demonstrated similar results, in which imidacloprid induced oxidative stress and led to DNA damage of *E. fetida*. Furthermore, it showed a synergistic effect with microplastic and caused a significantly lower level of SOD, CAT, and ANN (Baihetiyaer et al., 2023). These prior studies in earthworms also supported current findings, which displayed the toxic effects of neonicotinoids by markedly altering their behavior. Combined with the current findings, these results highlight the neonicotinoids potential environmental risks, especially to earthworms.

One of the most important characteristics for measuring the complexity of the system is the FD, which measures the complexity of fractal patterns as a ratio of the change in detail to the change proportionally (Eguiraun et al., 2014). In behavioral assays, unhealthy animals in pain typically exhibit lower FD values than the normal groups (Audira et al., 2022; Nimkerdphol and Nakagawa, 2008). Previous research has demonstrated that lower FD values are linked to unfavorable well-being situations defined by less complex and more repetitive movement. On the other hand, a more complex movement pattern is related to a greater FD value (Deakin et al., 2019). The results of the current study, which indicated that neonicotinoid treatment decreased the FD value in treated earthworms (Fig. 8), were supported by these previously published data. The present data imply that treated earthworms have poor health and move slowly and repetitively. This result is consistent with locomotor activity data, which showed that treated earthworms had longer freezing periods and moved more slowly on average speed. The decreased level of FD value was responsible for the earthworm's reduced movement and exploratory behavior. We discovered that neonicotinoid exposure can drastically lower earthworm FD values, which was consistent with other studies. The two neonicotinoids that significantly reduced FD were imidacloprid and sulfoxaflor. This finding was in line with a study on earthworms that evaluated the toxicity of sulfoxaflor. In their study, sulfoxaflor was shown to be an extremely hazardous contaminant to earthworms. This substance may cause oxidative harm to earthworms at sub-lethal levels (Fang et al., 2018). To sum up, the present study successfully demonstrated that earthworms exposed to neonicotinoids displayed reduced locomotor activity, altered movement

orientation, and diminished exploratory behavior. The findings of the fractal dimension study offered convincing proof that neonicotinoids might have unfavorable effects on non-target creatures like earthworms. In addition, the results also showed the high potency of the current method as a screening tool for the preliminary assessment of pesticides and their mixture in the soils.

#### 4.3. Phenomics analysis and *in silico* result on neonicotinoid effect on earthworms behavior

The significance of phenomics analysis is in its potential to provide a distinct viewpoint that is comprehensible to all readers (Chitayat and Rudan, 2016). According to previous studies, phenomics has been used to investigate behavior in mice and zebrafish since PCA and heatmap clustering analysis could help reduce the complexity of the behavioral data (Audira et al., 2020; Maroteaux et al., 2012). According to the PCA result, the tested neonicotinoids were divided into two different groups. Sulfoxaflor in both low and high doses, clustered far away from the control group, indicating that sulfoxaflor-treated earthworms exhibited a distinct behavior than the control groups. According to a prior study, sulfoxaflor was proven as a toxic pollutant to earthworms (Zhang et al., 2020). Sulfoxaflor can cause oxidative damage to earthworms, in which SOD, CAT, and GST were significantly inhibited and MDA content was accumulated. Furthermore, metabolomics metabolism was also altered, causing DNA damage (Fang et al., 2018). These prior studies of the sulfoxaflor effect in earthworms were in line with the present study. Sulfoxaflor, among the other tested neonicotinoids, showed severe effects on earthworm behavior on several endpoints. This translated correctly to the phenomics analysis and was indeed consistent with previous studies in earthworms (Fang et al., 2018; Zhang et al., 2020). Other interesting neonicotinoids that showed interesting results were imidacloprid, acetamiprid, and thiacloprid since they were also grouped in a different cluster from the control. Imidacloprid has been proven to cause detrimental effects on various animals other than insects (Iwasa et al., 2004; Sawasdee and Köhler, 2009; Siregar et al., 2021; Wang et al., 2015b). Imidacloprid itself proves to be highly toxic to earthworms by causing a higher mortality rate after exposure (De Lima e Silva et al., 2017). A study in earthworms also proves that imidacloprid affected the hatchability, growth, acetylcholinesterase (AChE) activity, and DNA damage (Wang et al., 2015b). All these data supported our result of the imidacloprid effect on earthworm behavior. Finally, other compounds like acetamiprid and thiacloprid also showed a severe effect in the present study. This result was somewhat similar to a prior study showing that both acetamiprid and thiacloprid are toxic to soil invertebrates (Renaud et al., 2018) as they could affect their reproductive, behavioral, immune, and antioxidant systems (Feng et al., 2015; Saggiolo et al., 2019b). Taken together, the phenomics approach in this study provides a promising alternative approach to evaluate the effects of neonicotinoids on earthworm behavior.

*In silico* testing was also carried out to investigate the mechanism of action behind neonicotinoids on earthworms. This work used molecular docking to look at the acetylcholine receptor binding affinity of selected neonicotinoids (Fig. 12). To test the cholinergic receptor, we used snails' acetylcholine receptors, which are mollusks with soft bodies comparable to earthworms, and both burrow in the soil. The molecular docking results demonstrated that all neonicotinoids have binding affinity and compatibility with the cholinergic receptors, as the binding energy is nearly identical. Interestingly, thiametoxam has the lowest binding energy of the neonicotinoids tested at 5.4 kcal/mol. The result was in line with behavioral results, where thiametoxam displayed the least harmful effect in behavioral tests among all the neonicotinoids examined in this experiment, which may be due to its low binding energy (Figs. 2–9). The current finding is also consistent with a prior study done on *Eisenia andrei*, where it was discovered that thiametoxam had less harmful effects than imidacloprid. Thiametoxam, which has a greater EC<sub>50</sub> than imidacloprid, also caused negligible mortality and body weight loss in

that study (Alves et al., 2013). Similar outcomes to our experiment were also seen in earlier research on the soil invertebrate species *Folsomia candida* and *Eisenia andrei*. Thiametoxam had no harmful effects on *F. candida*, whereas the other neonicotinoids led to weight loss in *F. candida* and *E. andrei*. Thiametoxam was also the least harmful in tests for survival and reproduction in *E. andrei* (De Lima e Silva et al., 2020). Alves et al. previously noted that up to 1000 mg/kg of a commercial thiametoxam formulation, earthworm survival was unaffected (Alves et al., 2013). Thiametoxam was also shown in other articles to not affect the survival rates of *E. andrei* but negatively impact its capacity for reproduction (Ritchie et al., 2019; Robinson, 2001). All of these studies support the findings of our *in silico* and behavior results. In fact, these results are also in line with the phenomics analysis result, where thiametoxam was in the same groups as control, indicating that thiametoxam showed a rather similar result with control.

Next, the outcomes of the *in silico* analysis also revealed that among the neonicotinoids examined, imidacloprid and acetamiprid have the highest binding energies (Table 1). The strong binding affinities of these two neonicotinoids may have contributed to the observed harmful effects of imidacloprid and acetamiprid on earthworm behavior, as shown by the much lower average speed level and greater freezing time ratio, indicating impairments in their locomotion activity (Figs. 2 and 3). According to earlier investigations in earthworms, acetamiprid was ten times more toxic to *E. Andrei* (De Lima e Silva et al., 2020). Furthermore, research in *E. fetida* showed that acetamiprid could cause a toxic effect at biochemical and genotypic levels by inducing ROS accumulation and changing antioxidant enzyme activities, leading to oxidative stress (Li et al., 2018). Acetamiprid has also been proven to affect the reproductive, behavioral, immune, and antioxidant systems of *E. andrei* (Saggiaro et al., 2019a). Meanwhile, a prior study also found that imidacloprid significantly affected the reproduction rate in earthworms, reduced the body weight of *E. andrei*, and caused high mortality in the worms (Alves et al., 2013) while another experiment showed that imidacloprid was able to impact the behavior of two species of earthworms (*Aporrectodea nocturna* and *Allolobophora icterica*). Furthermore, imidacloprid exposure also decreased burrowing activity in *A. nocturna* and *A. icterica* after 24 h (Capowiez and Bérard, 2006) and had been proven to modify the behavior of earthworms, reduce survival, and negatively impact reproduction. It has also been demonstrated to damage DNA and increase oxidative stress in *E. fetida* (Wang et al., 2016). Taken together, *in silico* results along with behavioral and phenomics analysis in the present study supported each other and according to the results of behavior and molecular docking data, there was a tendency for more severe effects on behavior when the binding affinity was high. In conclusion, the present study offers evidence of neonicotinoid toxicity to earthworms based on *in vivo* assessments of their behavioral endpoints and *in silico* data.

## 5. Conclusion

In summary, the present study successfully developed a novel assay for assessing earthworms' behavior and applied this method to assess the behavior toxicity of neonicotinoids on earthworms, considering neonicotinoids as one of the most widely used pesticides in the world, and uncontrolled use of these substances might pollute the environment. Even at relatively low concentrations, neonicotinoids were demonstrated to affect the behavior of earthworms in different magnitudes and these behavioral results were supported by *in silico* and phenomic approach results. Thus, it is crucial to pay attention to neonicotinoid use and any potential negative consequences on the environment. Despite the information provided in this study, more research is required to determine the precise mechanism by which the neonicotinoid influences earthworm behavior. More improvement is warranted to increase the value of the earthworm behavior assay developed in this study for ecotoxicity study. Nevertheless, our findings indicated that behaviors of *E. fetida* could be a potential alternative endpoint to evaluate the toxicities of compounds in soil organisms since behavior is more sensitive

than other markers, such as mortality or growth tests.

## CRedit authorship contribution statement

**Petrus Siregar:** Writing – original draft, Data curation, Conceptualization. **Yu-Chen Hsieh:** Methodology, Data curation. **Gilbert Audira:** Methodology, Data curation. **Michael Edbert Suryanto:** Methodology, Formal analysis. **Allan Patrick Macabeo:** Software. **Ross D. Vasquez:** Software. **Chung-Der Hsiao:** Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2024.124111>.

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