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Effects of pesticide formulations containing cypermethrin and tebuconazole, individually and in mixture, on the earthworm *Eisenia fetida*

Pedro Alexandre da Rocha Costa

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The study presented below does not include all the initially planned work due to the COVID-19 pandemic, the second part of the study referring to the chronic bioassay with *Daphnia magna* could not be carried out to its end.

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Abstract

In recent years, the use of agrochemical substances has increased considerably in order to maximize crop yields. This has been increasing the presence of various types of toxic compounds in ecosystems, with potential adverse impacts on wildlife and ecosystem services. These substances often interact with one another, leading to unpredictable effects. The objective of this study was to evaluate potential effects induced by chronic exposure to a mixture of pesticide formulations extensively used in agriculture, containing cypermethrin (insecticide) and tebuconazole (fungicide). The test organism was *Eisenia fetida*, a species of high importance in edaphic ecosystems and commonly used in ecotoxicology. Tested concentrations were equivalent to double and quadruple of the recommended field application doses, which was similar to levels previously detected in the environment. As effect criteria several parameters were evaluated, namely mortality, reproduction and growth, and the activity of the enzymes glutathione *S*-transferases (GST) and acetylcholinesterase. No significant effects on the life-cycle parameters of *E. fetida* or acetylcholinesterase activity were observed. The only significant effect was an increase in GST activity in certain treatments. This may lead to higher energy demand necessary to maintain increased GST activity for detoxification of the tested compounds.

Resumo

No passado recente, o uso de agroquímicos tem vindo a aumentar consideravelmente para maximizar o rendimento da produção agrícola. Isto tem levado a um aumento da presença de vários compostos tóxicos simultaneamente em ecossistemas com potenciais impactos negativos na vida selvagem e nos serviços prestados pelos ecossistemas. Estas substâncias frequentemente interagem entre si, provocando efeitos imprevisíveis. O objetivo do presente trabalho foi avaliar potenciais efeitos induzidos pela exposição crónica a misturas de formulações de pesticidas de grande utilização agrícola contendo cipermetrina (inseticida) e tebuconazole (fungicida). O organismo teste foi *Eisenia fetida*, espécie que tem uma função muito importante em ecossistemas edáficos e é muito utilizada em Ecotoxicologia. As concentrações testadas foram equivalentes ao dobro e ao quádruplo da dose recomendada para aplicação no campo, que são semelhantes aos níveis encontrados anteriormente no ambiente. Como critérios de efeito foram estudados diversos parâmetros, nomeadamente mortalidade, reprodução e crescimento, e a atividade das enzimas glutationa *S*-transferases (GST) e acetilcolinesterase. Não foram observados efeitos significativos no ciclo de vida de *E. fetida* ou na atividade da acetilcolinesterase. O único efeito significativo foi um aumento da atividade das GST em certos tratamentos. A manutenção de atividade das GST induzidas para aumentar a eficácia da destoxificação das substâncias testadas pode requerer energia adicional.

1. Introduction

With human population growing, the need for more food has been increasing. Nowadays the methods used to improve food production involve mostly chemicals, from veterinary drugs for animal production to pesticides for plant protection and pest control (Fojtová *et al*., 2019). According to Neuwirthová and colleagues (2019), the annual pesticide use reached ca. 2 700 000 tons and is still increasing. The increasing use of pesticides in agriculture has raised concerns about their potential negative effects on non-target organisms (Claver *et al*., 2003; Hartnik *et al.*, 2008; Karise *et al.*, 2007; Subrero *et al*., 2019).

The extensive use of plant protection products in agriculture results in the increase of the concentrations of pesticides, their degradation products and metabolites in farm soils (Lushchak, 2011). Hence, soil ecosystems are considered to be the most threatened by pesticides, especially by lipophilic pesticides that have limited mobility due to high affinity towards soil organic matter (Čadková *et al*., 2013; Maund *et al*., 2002). Nevertheless, these compounds may also reach water bodies through the runoff caused by irrigation or rain, or even through wastewaters (Bereswill *et al*., 2012; Weston *et al*., 2009). Estimates indicate that 1-2% of the total amount of pesticides in agriculture enter into streams through runoff (Berenzen *et al*., 2005). Pollution sources linked to agriculture can be considered non-point sources (Weber *et al*., 2018). Water treatment plants are considered point sources of pesticides, since in many cases water treatment plants do not remove pesticides from water efficiently (Guo *et al*., 2019; Pimentão *et al*., 2019; Weber *et al*., 2018).

As stated by many authors (*e.g.*, De Gerónimo *et al.*, 2014; Komárek *et al*., 2010), pesticides behavior, persistence and mobility in soil depend on a few processes, such as sorption-desorption, volatilization, chemical and biological degradation, uptake by biota (fauna and flora) and leaching. All these depend mainly on a few physicochemical characteristics of pesticides:

- Octanol-water partition coefficient (k_{ow}), which reflects the tendency of a chemical to dissolve in organic or inorganic solvents. Generally, an increase in the octanol-water partition coefficient leads to a stronger binding to the soil organic matter the chemical is and, therefore, is less mobile in soil.
- Adsorption-desorption distribution coefficient (k_d), which depends not only on the chemical but also on the type of soil, reflects the tendency of the compound

to concentrate in soil or in water. An increase in the value of K_d leads to a stronger sorption of a chemical to the soil matrix.

- Organic carbon partition coefficient (k_{oc}) is calculated with k_d, which implies that this parameter also depends on the type of soil. It reflects the tendency of a pesticide to bind to organic matter in a specific type of soil. The higher the value of k_{oc} the stronger the binding to organic matter.

Still, even knowing all these characteristics, it is difficult to predict the behavior and fate of a soil contaminant in the environment, since it also depends on the environment characteristics, climate, frequency of application, application rates, among others (Cuevas *et al*., 2018; De Gerónimo *et al*., 2014; Komárek *et al*., 2010; Maund *et al*., 2002; Potter *et al*., 2014). For instance, fungicides have been shown to reach the target of application in lower quantities when compared to other pesticides, due to both frequency and timing of application (Potter *et al*., 2014), and the methods used in their application *(Berenzen et al*., 2005), since they are mostly applied by spraying on plant leaves, leaving a high percentage able to drift through the air, reaching distances as far as 20 meters from the site of application (Druart *et al*., 2011).

The main concerns about pesticide contamination of natural environments regard three possible outcomes: acute toxicity, leading to decreased natural populations of organisms by direct mortality (Inglesfield, 1984;Cui *et al.*, 2018); chronic or sublethal toxicity, that may lead to decreased natural population as well, but through non-lethal consequences, such as decreased fecundity, depletion of detoxifying enzymes, or disruption of the life-cycle (Rico, Sabater and Castillo, 2016; Castro *et al.*, 2018; Poulsen *et al.*, 2015); and the development of pesticide-resistant organisms (Zhou *et al*., 2011).

To avoid the development of pest resistance, pesticides are often applied in mixtures of active ingredients (Zhou *et al*., 2011), what raises another problem, since the pesticides in mixtures may cause additive or synergistic effects on no-target organisms (Kretschmann *et al.*, 2015; Nørgaard and Cedergreen, 2010). In fact, with most studies focusing on the outcome of single pesticide exposures (Das Gupta *et al*., 2011; Hartnik *et al*., 2008; Hartnik and Styrishave, 2008; Saxena *et al*., 2014), some authors have pointed out that these studies do not reflect the reality of the pesticide use consequences, since in the environment pesticides are often found in mixtures rather than as isolated substances (Chen *et al*., 2018).

A considerable part of the pesticides sold worldwide are fungicides and insecticides. According to Eurostat (Eurostat, 2020), fungicides and bactericides represented 45% of

pesticide sales in 2018 in the European Union (EU), while insecticides and acaricides represented only 11%. Insecticides and fungicides are normally applied together in agriculture and in higher frequency than herbicides (Potter, Bosch, and Strickland, 2014).

Some insecticides and fungicides that were widely used in the past have been banned in most developed countries due to their dangerous effects on the environment and human health, but some of them are still being commercialized in developing countries. Among the banned insecticides are several organochlorines, such as dichlorodiphenyltrichloroethane (DDT), aldrin and heptachlor, and organophosphates, such as parathion (Prevention and disposal of obsolete pesticides, 2019).

Among fungicides, attention should be given to the following:

- Cell membrane components inhibitors – This group of fungicides is responsible for the inhibition of the synthesis of cell membrane components such as lipids and sterols. This group includes triazoles and dicarboximides (Yang *et al.*, 2011).
- Cell division inhibitors – Fungicides like phenylureas, carbamates and benzamidazoles, disrupt the mitotic process. Carbamates and benzamidazoles both act by inhibiting the assembly of microtubules, just as the aforementioned pyridine group of herbicides (Yang *et al*., 2011).
- Protein synthesis inhibitors – This group is mainly composed by acylalanines, which block the synthesis of ribosomal ribonucleic acid (RNA), oxazolidinones, which instead act upon the transfer ribonucleic acid (t-RNA) synthesis, and tetracycline antibiotics, which bind to the ribosomes. The end-result is inhibition of protein synthesis (Yang *et al*., 2011).
- Respiration inhibitors – This group includes pyrithiones, dinitroanilines and carboximides. These compounds are responsible for the disruption of the electron transport chain and oxidative phosporilation uncouplement, leading to a decrease in adenosine triphosphate (ATP) production for the cell (Yang *et al*., 2011).

Triazoles have fungicidal activity due to their inhibitory effect upon the production of ergosterol (Cui *et al*., 2018), a main component of fungi and protozoan cell wall only. They have been proven, however, to have negative chronic effects also on other organisms, since they act by inhibiting a demethylation catalyzed by enzymes associated to the cytochrome P_{450} (CYP_{450}), an enzymatic complex crucial in detoxification of xenobiotics

and sterol synthesis, present in all kinds of organisms (Poulsen *et al*., 2015; Scott-Fordsmand and Weeks, 2000).

Among the triazole fungicides, tebuconazole was found to prevent *Daphnia magna* infection by a fungal parasite *Metschnikowia bicuspidata* (Cuco *et al*., 2017). This interference with parasitical infections may have a long-term negative outcome for the parasite population but can also remove the biotic pressure from the host species.

Insecticides are used against insects that are considered to be harmful in several contexts, including in agriculture. As there is no insecticide that exclusively acts upon one single insect species, it is expectable that insecticides can also cause stress in natural populations of beneficial insects and other fauna. Indeed, many cases have been reported along the years, with particular concern turned on the contribution of neonicotinoids and several other types of insecticides to the decline of natural populations of bees and other pollinators.

Within insecticides one can highlight the following groups:

- Acetylcholine (ACh) receptor agonists – A group constituted by nicotine and nicotinoid-based insecticides, such as imidacloprid and acetamiprid, that bind to acetylcholine (ACh) receptors thus blocking the nervous impulse transmission (Ghosal, 2020).
- Gamma-aminobutyric acid (GABA)-gated channels modulators – These insecticides act upon the nervous system as well, by allowing a higher entry of chloride ions into the neuron, which results in a higher release of the neurotransmitter, and consequently convulsions and death. This group includes lindane and cyclodiene pesticides, such as aldrin and endosulfan (Ghosal, 2020).
- Cholinesterase inhibitors – The toxicity of these pesticides, mainly used as insecticides, is mainly due to the inhibition of acetylcholinesterase (AChE), which is a key enzyme in the nervous system. It degrades the neurotransmitter acetylcholine in cholinergic synapses. AChE inhibition results in continuous stimulation of post-synaptic receptors potentially leading to paralysis and death. Anticholinesterase insecticides include organophosphates and carbamates that are both strong inhibitors of AChE and other cholinesterases (ChE) (Ghosal, 2020).
- Sodium-gated channels disruptors – As the name implies, these pesticides exert their toxicity by interfering with the sodium channels in neurons, therefore

interfering with the nervous impulse transmission, leading to convulsions and death. This group includes DDT, an organochlorine, which has been approved by the World Health Organization (WHO) in some tropical countries to eradicate malaria despite being banned in most developed countries for decades because it causes several chronic effects in humans and wildlife, including reduced fertility or increased probability of spontaneous abortions. Besides DDT, the group includes several other organochlorine insecticides. Most pyrethroids that are another class of insecticides widely used also act by this way (Ghosal, 2020).

Insecticides are among the most dangerous pesticides to wildlife and humans, since they act on the nervous system, which is present in invertebrates and vertebrates (Scott-Fordsmand and Weeks, 2000). Indeed, acute toxicity of these substances have been found for many species, such as *Eisenia fetida, Eisenia andreii, Metaphire posthuman, Daphnia magna* and *Oncorhynchus mykiss* (Hartnik *et al*., 2008; Saxena *et al*., 2014), among several others. Their sub-lethal effects may just be of as much importance, as exemplified by Claver *et al.* (2003), in which the predatory spider *Acanthaspis pedestris* was chronically affected by exposure to cypermethrin. However, this type of information is known only for a relatively small number of species.

1.1 Effects of pesticides on non-target species

Various consequences to non-target species may arise from the use of pesticides. Among them population-level consequences, inter-species consequences and ecosystem consequences deserve special attention.

The most known type of consequences is probably high mortality caused by a pesticide, leading to a fast and direct decrease in abundance of the affected populations. However, effects of pesticides can be also manifested in different forms of sublethal toxicity, like reduced life-expectancy or reproduction of a species, which eventually also lead to negative effects at the population level (Köhler and Triebskorn, 2013). Often reported cases are reduced fecundity (Claver *et al*., 2003; Yasmin and D'Souza, 2007) and/or growth rate (Campero *et al*., 2007), impaired foraging ability, endocrine disruption (Poulsen *et al*., 2015; Sancho *et al*., 2010) or hyperactivity (Saha and Kaviraj, 2008). These ultimately lead to a decrease of the population, either by mortality caused by other natural stressors or by decreased population renewal. Eventual consequences of all these sublethal effects depend also on inter-species relationships (Mills and Semlitsch, 2003).

Complex sublethal effects that may occur in result of exposure to pesticides are well shown by the study of Hayes and colleagues (2003) in which the exposure to atrazine of African clawed frogs (*Xenopus laevis*) resulted in a full conversion of 10% males into fertile females. This has been confirmed through crossing between the remaining males and the converted males, resulting in an only male descendance. The example suggests that long-term exposure to atrazine may lead to a decline in the number of females, which ultimately leads to the population decline.

More problems arise when the targeted species acquires resistance to the pesticide, which will lead to an increased use of pesticides without producing the desired effect. This may in turn result in a higher input and therefore in higher contamination of the environment. Besides this first impact, the loss of genetic diversity of the species, by elimination of non-resistant genotypes, can also be a serious consequence, as it may imply the loss of valuable genes for biotechnological applications and it may lower the chances of survival of a species against environmental changes. By acquiring resistance to pesticides, the organism may need to sacrifice growth- and/or reproduction-allocated energy, which in turn may be disadvantageous in a non-polluted environment (Walker *et al.*, 2012).

Among the consequences at the level of inter-species relationships, those that particularly should by highlighted are alterations on the competition, predation, symbiosis, and parasitism.

Competition refers to two or more species that compete for common resources, such as food/prey, water, or shelter. In this subject, one species may be more sensitive to a pesticide contaminant than its competitors and end up losing resources. This can change the dominance structure of the affected community, which, in turn, may lead to alterations in the ecosystem functioning. Also, the interspecific competition may delay the recovery of a species exposed to a toxicant. A good example was the study conducted by Knillmann and colleagues (2012) in which two species of *Daphnia*, one less sensitive than the other, were exposed to esfenvalerate. The results showed partial mortality in exposed groups and the development of negative interactions between the used species. In contrast, no negative interactions were observed in the control groups. Another example is the effect of malathion in *Culex pipiens* larvae. Malathion was reported to mitigate the increase in mortality caused by population density in high nutrient conditions (Muturi *et al*., 2010). This may have negative consequences to the human population, since adult mosquitos may be vectors of diseases (Muturi *et al*., 2010).

Predation is a relationship that involves one species feeding on another. The impacts of pesticides on this relationship may lead to two different outcomes. On the one hand, the prey may be affected and be easier to spot and capture, which would bring a higher impact on the demography of the population caused by predation. However, if the toxicant bioaccumulates in the prey, as many have been shown by past studies, it may turn out to be prejudicial to the predator who ingests a contaminated prey (Fojtová *et al*., 2019). On the other hand, if the predator is affected, it may not be able to capture the preys efficiently, leading to a decrease of the predator population (Claver *et al*., 2003; Roger *et al*., 1994; Shaw *et al*., 2006). The suppression of predators in agricultural areas may lead to increased dependence on pesticides to control pests, what would not only increase the economic cost of the agricultural activity, but also increase the frequency of pesticide use and therefore increase the input of pesticides into the environment (Baudrot *et al*., 2020). Mills and Semlitsch (2003) reported an experiment in which tadpoles of *Rana sphenocephala* were exposed to carbaryl, a carbamate insecticide, along with a competitor (*Pseudacris crucifer*) and two predators (*Notophthalmus viridescens* and *Anax spp.* larvae). In this study, effects on both competition and predation were reported. Carbaryl affected the competition between phytoplankton, the main prey of *R. sphenocephala*, and periphyton by eliminating the latter, thus allowing for a greater availability of food. In the presence of *R. sphenocephala* and its competitor only, carbaryl affected *P. crucifer* individuals, causing increased mortality, therefore favoring *R. sphenocephala*. In the joint exposure of *P. crucifer* and *R. sphenocephala* to their predators (*N. viridescens* and *Anax spp.* larvae), it was observed that *Anax* were more sensitive than all others, suffering from high mortality, and therefore leading to a reduction of the predation pressure. The decrease of the predation pressure caused an increase of the competition for the food resources between *P. crucifer* and *R. sphenocephala*. This example shows well how a pesticide can affect interactions between species and how complex these interactions can be.

Parasitism is often considered a type of predation. It involves a species that needs a host to survive and which harms the host, although only some cases of parasitism lead to direct death of the host, which is the case of parasitoids. In many cases, parasites are larval phases of organisms, whose adult phases are free-living and have a role to play in the ecosystem functioning. Parasites are often a stress factor to their hosts and may contribute to control host populations. If parasites are host specific and their host population is facing a decline due to pesticide exposure, they also face extinction. This is different from predators as these often can adapt to another diet. It is worth stressing that some parasites and parasitoids are useful as pest-control agents. As presented by Idris

and Grafius (1993), the Lepidoptera parasitoid *Diadegma insulare* is of great help in controlling the pest *Plutella xylostella*. Some pesticides may, however, pose a threat to that function as the authors observed a significant increase in mortality of the parasitoid adults when exposed to permethrin but no increase in the mortality of the larvae of *P. xylostella*.

Another type of relationship that is worth highlighting is symbiosis. Symbiosis involves two species that depend on one another to survive. In this case, if one of the symbiont species is affected, the other will be as well. Some symbionts may be pivotal to establish new ecosystems or to recover certain ecosystems, such as lichens and corals. Corals are associations between dinoflagellate algae of the genus *Symbiodinium* and an anthozoan, which gives protection to the algae, while the algae produce organic products from photosynthesis. They are known for extracting large amounts of calcium carbonate from seawater to form their exoskeletons, shaping the environment as well (Jones, 2005). Corals are known to be very sensitive organisms and have been declining in recent years. Besides global warming and changes in salinity or other abiotic factors that potentiate their decline, herbicides that act by inhibiting the photosystem II, such as diuron, have been considered a new threat. Such herbicides can penetrate the host body and while they do not have a direct negative effect on the host, they can substantially reduce the quantum yield of the algae symbionts (Jones, 2005). Another example of an important symbiotic relationship is the relationship between rhizobia bacteria and crops. As exemplified by Fox *et al.* (2007), N-fixing rhizobia bacteria are beneficial to plant productivity, as they are responsible for nitrogen fixation, an essential step to ensure soil fertility, and therefore the establishment of the symbiotic relationship is of farmer's best interest. However, pentachlorophenol, an organochlorine pesticide, prevented the establishment of that relationship.

The ecosystem alterations resulting from pesticide contamination may have a wide range of magnitude: from small changes in species interactions to the surging of a new pest or even desertification or eutrophication. These consequences mostly derive from negative effects on the populations that play crucial roles in the ecosystem function, such as many primary producers, decomposers, and ecosystem engineering species.

As mentioned before, a new pest may rise from developing resistance to pesticides and becoming a dominant species, without much population control pressure. Such pests may have negative economic impact, especially if they end up causing drastic changes to an ecosystem. For example, the absence of a keystone species that functions as an ecosystem engineer or has a high impact on the lower levels of the ecosystem structure,

could have drastic negative consequences. One example would be earthworms, since they are responsible for cycling of organic matter from the soil, aeration of the soil, and contributing to soil fertility (Mosleh *et al*., 2003; Wang *et al*., 2019). Eradication of earthworms from an ecosystem would, thus, reduce the fertility of the soil, what may lead to a complete shift of the plant species capable of colonizing the area, and may ultimately force a change in agriculture of cultured plant species.

Another case would be the removal or a shift in the population structure of aquatic fungi from freshwater ecosystems. Since fungi play a fundamental role in the cycling and breakdown of leaf litter (Talk *et al*., 2016), a lack of these organisms could lead to an accumulation of organic matter in these freshwater ecosystems, which in a lake could lead to eutrophication. Also, bacteria and fungi are known to adapt quickly to the presence of pesticides and are able to utilize them as a source of energy, removing contaminants from the environment (Lew *et al*., 2013). Therefore, a decline or a shift in key bacteria or fungi species can make the ecosystem more sensitive to contaminants. Some studies did not found impacts of pesticides on microbial biomass in soils but they found shifts in the abundance of microbial species (Cui *et al*., 2018; El Azhari *et al*., 2018). It is still hard to predict if these shifts can have a significant impact on the ecosystem.

In conclusion, pesticide contamination should be a primary concern in ecotoxicology, as these substances have various mechanisms of action and can cause harm to different types of organisms. The understanding of the biochemical pathways through which each substance exerts toxicity is essential to grasp the full negative potential of the environmental contamination by pesticides. The current acute toxicity studies have been often shown to underestimate the damage caused by pesticides and often they reflect unrealistic scenarios. Therefore, in order to increase the knowledge in this matter, more chronic toxicity tests in more environmentally realistic scenarios, including ecological relevant concentrations of the tested substances are needed.

1.2. Cypermethrin and tebuconazole

Cypermethrin is a type II pyrethroid insecticide that acts on the sodium channels of neurons, disrupting the nervous system (Shaw *et al*., 2006). Pyrethroids have gained popularity over other insecticides since they have relatively low toxicity to mammals. However, it has been shown over the last decades that they can have highly negative effects on fish and non-target invertebrates (Hartnik and Styrishave, 2008; Saha and Kaviraj, 2008; Willis and Ling, 2004). Cypermethrin is used in aquaculture at sea and in agriculture (Shaw *et al*., 2006; Willis and Ling, 2004). Similarly, other pyrethroids closely

related to cypermethrin, such as α-cypermethrin, have been shown to be toxic to non-target insect species, like honeybees and fish (Yao *et al*., 2015). Cypermethrin is considered a potential human carcinogen by EPA (Environmental Protection Agency). Its disappearance time (DT_{50}) in soil is highly variable, ranging from <14 days to 199 days (European commission, 2005), its degradation originates 3-phenoxybenzoic acid (3-PBA) and (3-(2,2-dichlorovinyl)-2,2-dimethyl-1-cyclopropane) carboxylic acid (DCCA). The 3-PBA is thought to be an endocrine disruptor and has higher mobility than the parent compound (Yao *et al*., 2015), potentially posing a bigger threat to the soil fauna than cypermethrin itself. Since cypermethrin is a type II pyrethroid, its toxicity is expected to increase with an increase in temperature (Ghosal, 2020).

Although for some organisms acute toxicity caused by cypermethrin is the main concern, for others sublethal toxicity may lead to negative changes in their life-cycle and ecology, for example by reducing mobility, feeding capacity and the ability to fight or hide from predators (Shaw *et al*., 2006). For instance, Karise and colleagues (2007) noticed that alpha-cypermethrin decreased the foraging capability of honeybees. Hence, even though it may not have a high immediate impact on the number of bees in a short period of time, in long-term exposures this can lead to population decline due to food shortage or reduced reproduction. Besides, cypermethrin was shown to have negative effects on locomotion, mating behavior and feeding capability of spiders in laboratory studies (Claver *et al*., 2003; Shaw *et al*., 2006).

Tebuconazole is a fungicide from the azole family. These compounds block the synthesis of fungal sterols, including ergosterol, which is a main component of fungi cell membrane, thus stopping fungi development and preventing renewal of cell membranes, leading to the death of the fungi (Atmaca *et al.*, 2018; Wang *et al*., 2019; Zhu *et al*., 2014). The effect results from the inhibition of cytochrome P_{450} activity involved in sterol biosynthesis (Atmaca *et al*., 2018; Zhu *et al*., 2014). This inhibition is accomplished by binding of the azole fungicides to the *heme* group of cytochrome associated enzymes and inhibition of lanosterol 14α-demethylase (CYP51) that participates in the biosynthesis of ergosterol (Poulsen *et al*., 2015, Sancho *et al*., 2010). Since monooxygenases from CYP_{450} share the *heme* group targeted by triazoles, they can also be affected, and therefore triazoles may interfere with other enzymes belonging to the CYP superfamily (Poulsen *et al*., 2015). By affecting CYP_{450}, one of the most important enzymatic complexes in detoxification of chemical compounds (Scott-Fordsmand and Weeks, 2000), triazoles may induce synergistic toxicological interactions with other toxic compounds in exposed organisms or

cause endocrine disruption, since these enzymes are also involved in steroid hormones biosynthesis (Kretschmann *et al*., 2015; Poulsen *et al*., 2015).

Besides the mechanism of action of the compound, there are other worrying characteristics, such as the long half-life in soil and water. Tebuconazole is considered a persistent pesticide, since it takes a considerable time to dissipate in the soil. Its DT_{50} may be of 25-365 days (Fojtová *et al*., 2019). This environmental persistence along with an intensive and repetitive use may lead to relative high concentrations in soils for considerable periods of time.

Tebuconazole has been indeed detected both in soil and water of several areas. Hvězdová and colleagues (2018) found various pesticides in farm soils in Czech Republic with tebuconazole reaching concentrations up to 0.028 mg/kg of soil. In Spain, Rial-Otero and colleagues (2004) reported tebuconazole concentrations reaching 0.012 mg/kg of soil. In rivers, tebuconazole has been reported to be present at concentrations up to 3.2 µg/L in the water column (Herrero-Hernández *et al*., 2013), and 0.6 mg/kg in sediments (Smalling *et al*., 2013). In rivers near vineyards in Germany, tebuconazole concentration reached up to 9.1 µg/L in stream water (Berenzen *et al.*, 2005), exceeding the European Union (EU) safety limit. In recent studies, Poulsen *et al*., (2015) and Sancho *et al*. (2010) have reported endocrine disruption caused by tebuconazole in *Xenopus laevis* and *Danio rerio*, respectively, with observed effects in *X. laevis* at 0.1 µg/L. As tebuconazole was found to accumulate in *X. laevis* brain, it may be able to pass through the hemato-encephalic barrier and may have an impact on the neurological system (Poulsen *et al*., 2015). Tebuconazole was also observed to reduce invertebrate shredding activityprobably due to the reduction of microbial leaf decomposition (Rasmussen *et al*., 2012). As a fungicide, tebuconazole is indeed expected to have a negative impact on the natural fungi populations, which are crucial to the degradation of organic matter in freshwater and soil ecosystems (Herrero-Hernández *et al.*, 2013).

Therefore, the main concerns regarding this fungicide are the possible negative impacts on the production of hormones, which may lead to a decrease in the reproduction of organisms and a possible decrease of the capability of organisms to detoxify other xenobiotics, potentiating increasing mortality or sublethal effects of pollutants.

1.3. The problem of toxicant mixtures

A considerable part of ecotoxicological studies focus on a single toxicant that is tested in a specific species under a specific set of abiotic conditions (pH, temperature, salinity,

photoperiod, among others) that do not variate. Thus, such studies do not take into consideration interspecies dynamics, abiotic variation, and the simultaneous exposure to mixtures. Thus, more studies regarding these aspects, particularly on mixture toxicity, are needed to reinforce the basis for pesticide risk assessment (Brodeur *et al.*, 2017).

In recent years, some studies have focused on the effects of two or more stress factors on model organisms (Campero *et al*., 2007; Van Dievel *et al*., 2019), such as *Daphnia spp.* (Friberg-Jensen *et al.*, 2010; Kim *et al*., 2008), earthworms (Das Gupta *et al*., 2011; Yu *et al.*, 2012; Zhou *et al.*, 2011) or honey bees (Chen *et al*., 2019; Fine *et al.*, 2017; Wang *et al*., 2020), among others. Indeed, it is hard to find an ecosystem in which only one toxic chemical is present, especially when considering pesticides, which are often applied in mixtures by farmers (Wang *et al*., 2020). Some ecosystems are particularly susceptible to accumulate various toxic substances, like river deltas for example.

The problem with mixtures of toxicants is that their effects are often unpredictable, making the environmental risk assessment for combined effects difficult. In exposed organisms, mixtures can cause toxicological interactions that are often divided in the following types:

- Addition
- Antagonism
- Synergism
- Potentiation

Addition occurs when the effects of the mixture are approximately equal to the sum of the toxicities caused by each mixture component when tested independently. It is commonly observed when the compounds present in a mixture share a common mechanism of action or/and site of action. For example, if a given compound A at concentration X causes 20% mortality in a given species, and another compound B at a concentration Y also causes 20% mortality, then a mixture of A and B would lead to 40% mortality. These situations are often observed, however, there are exceptions, and these exceptions can have very relevant effects in the environment that should be considered in environmental risk assessments. For example, as reported by Coors and De Meester (2008), carbaryl and biotic stressors (predation threat and parasite-induced castration) exhibited additive effects on offspring body size, size at maturity and age at maturity of *Daphnia magna* but different types of interactions were observed on other evaluated endpoints.

Antagonism, as the name implies, is an interaction in which one toxicant reduces the toxicity of another. In other words, the toxicity of the mixture is lower than the sum of the

toxicities of each component, when tested separately. A typical example would be if chemicals A and B, when tested individually, would cause 20% mortality each, but the mixture of both compounds at the same concentrations would yield 15% mortality rather than 40% as expected by the additive model. Liu and colleagues (2013) observed different effects of a mixture containing dodine and bromacil, or dodine and simetryn on two test organisms, *Vibrio qinghaiensis* and *Chlorella pyrenoidosa.* In *V. qinghaiensis* the mixtures exhibited synergism (see below), but the same mixtures exhibited antagonism to *C. pyrenoidosa*. This example shows that different organisms can react differently to the same mixture of toxicants.

Synergy is observed when the toxicity of a mixture is greater than the sum of the toxicities of mixture components, when tested independently – for example, if compounds A and B are present in a mixture at concentrations causing 10% mortality each when used individually, but the actual mortality of organisms exposed to this mixture is 50% rather than 20% as predicted by the additive model. This was the case in a study conducted by Wang and colleagues (2020) in which the toxicity of binary mixtures containing chlorpyrifos, tetraconazole, acephate and various pyrethroid insecticides to honeybees (*Apis mellifera*) was tested. In this study synergistic relationships were reported for most tested mixtures.

Potentiation also implies that a mixture has higher than toxicity than the sum of mixture components individually, but only one of the components of the mixture is toxic while the other does not induce toxicity in the system at the considered concentration when used alone. This is a debated definition in the scientific community, but in my opinion, the separation of synergy and potentiation should be considered since they are substantially different and, in the specific case of potentiation, the unexpected results may be exceptionally severe to the environment. For example, let us imagine that a compound A is in a concentration known to cause 15% mortality to a species but with the addition of a compound B, which, when tested individually, caused no mortality, the mixture caused 30% mortality. Potentiation may be observed due to two mechanisms: inhibition of detoxification of one compound by another, or activation of one compound by another compound (Walker *et al.* 2012). For example, as mentioned in Walker *et al.* (2012), ergosterol biosynthesis inhibitors (EBI), such as triazoles, often exert their effect on different forms of CYP_{450} monooxygenases. These enzymes are often involved in the detoxification of xenobiotics and therefore, their inhibition may lead to a decreased detoxification of certain toxic compounds. Such effects have been observed in bees exposed to EBI fungicides and cyhalothrin. On the other hand, although CYP_{450} functions

as a detoxification enzymatic complex, it has been observed that the action of monooxygenases leads to the activation of organophosphate insecticides, increasing their toxicity (Walker *et al.*, 2012).

Usually, the substances that increase the toxicity of other substances possess similar action sites or mechanisms or affect the metabolism of a compound by the organism (Walker *et al*., 2012). For example, synergism between triazoles and other pesticides have been proved by Kretschmann *et al*. (2015), Raby *et al*. (2019) Walker and Johnston (1993). The triazole group is a good example since they act upon the CYP_{450}, an enzymatic complex with many synthetic and detoxifying functions (Walker and Johnston, 1993). These compounds can make an organism more sensitive to other substances that would normally be detoxified by the CYP_{450}, such as pyrethroid pesticides (Zare *et al*., 2020). Another example is esfenvalerate and prochloraz, a pyrethroid insecticide and imidazole fungicide, respectively, which interact resulting in a higher toxicity to *Daphnia magna* when in mixture. This mixture toxicity increases with the increase of prochloraz concentration and with the decrease of available food (Shahid *et al.*, 2019). Interacting chlorpyrifos (organophosphate) and carbofuran (carbamate) cause neurotoxicity by inhibiting cell proliferation and producing reactive oxygen species (Fu *et al*., 2018).

Although toxicological interactions are known to occur, it is virtually impossible to know all the possible combinations of pesticides and of pesticides with other environmental contaminants. Another problem is that the occurrence of toxicological interactions may vary with different abiotic factors, such as concentration of each component, pH, salinity and temperature (Delnat *et al.*, 2019; Jones, 2005; Laetz *et al*., 2014). This, coupled with the variety of other components used in commercial formulations, whose influence in the toxicity of a formulation are often overlooked (Liu, 2004; Ma *et al*., 2005; Mesnage and Antoniou, 2018), leaves a lot of possibilities open for further research.

The active substances present in pesticide formulations are normally mixed with adjuvants in these formulations, and these can sometimes also alter the toxicity of the active ingredient of a formulation (Liu, 2004; Ma *et al.*, 2005; Mesnage and Antoniou, 2018).

Solvents and other adjuvants are mixed with the active substances of pesticides in commercial formulations in order to facilitate the dilution in water and to confer a greater stability in the environment, to improve the pesticide efficacy (Liu, 2004). Some formulations may consist in up to 75% of solvent mixtures (Maliszewska and Tęgowska, 2018). The problem here is that not all of these substances are harmless to organisms,

and they can directly or indirectly affect the toxicity of a given pesticide, potentiating its environmental impact (Schmuck *et al*., 1994).

Firstly, the solvent or adjuvant itself may be toxic to a given organism. A good example was reported by Maliszewska and Tęgowska (2018) in a study with the arthropod *Blaberus giganteus* exposed to xylene, an often-used pesticide solvent. In this study an increase in lipid peroxidation and hemolymph sugar content was observed. Another good example of toxic effects caused by pesticide adjuvants comes from a study by Ma and colleagues (2005), in which three different cyanobacteria species (*Anabaena flos-aquae, Microcystis flos-aquae* and *Microcystis aeruginosa*) were exposed to various adjuvants. It was observed that the tested species had different sensitivities to the tested adjuvants, but generally they were more resistant than algae tested in earlier studies. In long term contaminations, this could lead to a shift in dominance from green algae to cyanobacteria.

Secondly, solvents and adjuvants may potentiate the environmental impact of a pesticide by delaying its degradation and increasing, thus, the probability of affecting more organisms. Not all adjuvants are intended to extend the lifetime of a pesticide, some may increase their bioavailability in order to increase the efficacy to fight a pest organism. It has to remembered, however, that this may backfire as increasing bioavailability will not only affect target species but also the non-target species, many of which are natural pest-control agents or other ecosystem service providers (Mesnage and Antoniou, 2018).

A good example is a study conducted by Chen and colleagues (2019), in which three pesticide adjuvants were tested separately on the honeybee *Apis mellifera* resulting in very low toxicity of each substance when tested individually. However, when Silwet-77 was applied in formulations with acetamiprid, it resulted in higher toxicity to *A. mellifera* when compared to the active ingredient alone (up to 4.5 times lower median lethal dose (LD_{50}) compared to the LD_{50} of acetamiprid). The exact mechanism by which the active ingredient's toxicity is increased in unknown, but this example shows that adjuvants cannot be ignored when estimating the environmental impact of pesticides and delimiting their application. Fine and colleagues (2017) reported, in turn, that organosilicones that are surfactants used as pesticide adjuvants facilitating the spread and penetration of the active ingredient into target organisms, caused an increase in mortality of *A. mellifera* larvae by somehow favoring viral infections. Exposure to organosilicone and viruses individually would result in a 4.09% and 21.56% mortality, respectively. However, when exposed to both stressors simultaneously, mortality of *A. mellifera* larvae raised to 44.45%. This suggests that pesticide adjuvants may potentiate the effects of biotic stressors as well as of chemical stressors.

Among the alternative agricultural methods, one can find biological agriculture, which utilizes biological pesticides. This is a very popular suggestion, although it is widely recognized that these techniques still require improvements in order to produce enough food to meet the market demand, and are usually very expensive due to the non-massive production of natural pesticides, among other factors (Isman, 2006). It is important to highlight here that some natural pesticides can be as hazardous, and sometimes even more toxic than synthetic ones, and the fact that they are not synthetic does not ensure no consequences to personal or ecosystem health (Isman, 2006).

Bioremediation techniques, although are not optimized, are needed for minimizing the consequences of pesticide use. This could be achieved through the use of pesticide degrading microorganisms. However, since many synthetic pesticides often disrupt biological activity, it is possible that biodegradation would be insufficient. Therefore, the suggestion already given by many authors is that the combination of chemical degradation and biodegradation is made, reducing the costs of chemical degradation and facilitating biological degradation by transformation of the active principles of the pesticides, which might compromise the efficacy of the process (Oller *et al*., 2011). With the use of these two solutions, impacts of pesticide usage would be reduced and damaged soils could be rehabilitated.

Soils are indeed the primary sink for pesticides but they are not necessarily the most affected systems, since, as evidenced by many studies, pesticides often reach streams and creeks from agricultural fields (Bereswill *et al*., 2012; Silva *et al*., 2015). They can be also transported (Kuivila and Foe, 1995) to lakes, higher order rivers, and river deltas and estuaries (Rodrigues *et al*., 2019) that often act as reservoirs for many pesticide residues found along their effluents and leaching from their margins. Since the pesticides used by farmers are often not the same, these ecosystems commonly accumulate a potentially harmful “pesticide cocktail”, which may compromise ecosystem functions (Oller *et al*., 2011; Walker *et al*., 2012).

The solutions for reducing the impact of pesticides on aquatic environments could be the creation of barriers for reducing pesticide runoff and improving pesticide removal from water in treatment plants. Pesticide runoff barriers may be applied in agricultural fields, with the intent of reducing the input of used pesticides into surface waters, resorting to riparian vegetation or other barrier structures (Bereswill *et al*., 2012). The use of riparian vegetation would also provide a form of protection from floods, improving soil structure and fertility (Power, 2010).

For the improvement of pesticide removal in water treatment plants more research and optimization methods are needed but it may be a viable solution and should be a prioritized subject in research and technology development. The bioremediation techniques are mostly identical to the previously mentioned strategies, and consist in the use of pesticide-accumulating plants in combination with microorganisms capable of degrading pesticides and chemical oxidation, if the latter is possible to apply (Oller *et al*., 2011).

1.4. *Eisenia fetida* as a test organism

Earthworms are ecologically and economically important species, since they can represent up to 80% of the soil fauna biomass, and they have important ecological functions such as soil and its microbial community structuration. They are responsible for nutrient immobilization, soil aeration, litter composting, water infiltration and plant development promotion (Das Gupta *et al*., 2011; Diao *et al*., 2011; Hartnik *et al*., 2008; Yvan *et al*., 2012). Therefore, they are precious organisms to the agricultural industry. Besides, they can be used as environmental pollution indicators for soil since they inhabit the soil, and therefore are exposed to soil contaminants throughout their life cycle. Since large quantities of soil pass through their digestive system and their body is protected by a permeable cuticula, they are susceptible to toxic chemicals present in the soil (Diao *et al.*, 2011). They are food for many amphibians, reptiles, birds, and mammals, so there is also a potential for bioaccumulation and biomagnification of toxic chemicals through the food chain (Das Gupta *et al*., 2011).

Because of their importance, earthworms have been long used in toxicity testing. Following the recommendations of internationally accepted standard protocols for chemical toxicity testing (OECD, 1984; OECD, 2016), two of the most used species in ecotoxicology are *Eisenia fetida* and *E.*. In previous laboratory studies on *E. fetida*, pesticides such as tebuconazole and cypermethrin have been shown to accumulate in its tissues (Diao *et al*., 2011; Hartnik and Styrishave, 2008; Yu *et al*., 2012).

E. fetida has been particularly extensively used in ecotoxicological studies in the past, and therefore has a well-known life-cycle, and wild populations can be found all around the globe, even though originally they were endemic to Europe (Beyer *et al*., 1985). Besides their use in ecotoxicology, they are often used in vermiculture in order to produce proteins, and in processing waste material (Venter and Reinecke, 1988). In optimal conditions, *E. fetida* can reach sexual maturity in 70-80 days, which is manifested by a well-developed clitellum (Venter and Reinecke, 1988). The cocoons are laid 4 days after

mating and, in the peak of the cocoon production (between 80 and 90 days after hatching), individuals may lay an average of 7 cocoons per worm daily (Venter and Reinecke, 1988). The incubation period of the cocoons vary from 14 to 44 days (Venter and Reinecke, 1988).

The toxicity of cypermethrin to *E. fetida* was studied Zhou *et al*. (2011) indicated an acute median lethal concentration (LC_{50}) of 86.04 mg/kg of soil, although some other studies indicated lower or higher values (Das Gupta *et al*., 2011; Inglesfield, 1984). Actual concentrations of cypermethrin found in soil are usually lower than 0.04 mg/kg, hence, mortality of *E. fetida* is not expected to occur in real-case scenarios (Kumari *et al*., 2008; Vig *et al*., 2001). Cypermethrin has been reported to have negative impacts on cocoon viability at high concentrations, such as 50 mg/kg (Zhou *et al*., 2011). Other earthworm species exposed to cypermethrin have been reported to have decreased protein levels, increased glucose levels and increased transaminases and phosphatases activity, suggesting a decrease of the worms feeding activity at a concentration of 41.14 mg/kg of cypermethrin (Mosleh *et al*., 2003). Although these effects have been observed at such high concentrations, other possible subtle effects may have been overlooked, as well as the possible chronic effects of the interaction between cypermethrin and other compounds.

Tebuconazole also did not exhibit high acute toxicity to *E. fetida* but may cause chronic negative effects. Rico *et al*. (2016) found that it increased lactate dehydrogenase (LDH) activity in *E. fetida* at concentrations of 142 mg/kg dry weight, while higher concentrations lead to inhibition of LDH. This suggests that sub-lethal concentrations may decrease organism's fitness. Mortality of earthworms due to exposure to tebuconazole is reported only at very high concentrations. For example, estimates of 7-day LC_{50} and 14-day LC_{50} were 746.3 mg/kg and 287.9 mg/kg , respectively (Chen *et al*., 2018). In another study, the 14-day LC_{50} was 180 mg/kg (Rico *et al*., 2016).

1.5. Objectives of the thesis

The goal of this study is to evaluate the chronic effects of two pesticide formulations, one containing cypermethrin and the other containing tebuconazole, alone and in mixture, on *E. fetida* using mortality, body weight, reproduction, respiration rate, and the activity of AChE and glutathione *S*-transferase (GST) enzymes as effect criteria.

2. Methodology

2.1 Earthworm cultures

Specimens of *E. fetida* were bought from Lucyna i Sławomir Siudzińscy (Kępa Oborska 20, 05-520 Konstancin – Jeziorna, Poland). They were separated by developmental stage into three categories: younglings, juveniles, and adults.

Earthworms were kept in one set of plastic containers of 78 cm x 55 cm and 47 cm high and another set of 55 cm x 36 cm and 27 cm high that allowed gaseous exchange through holes, with a plastic net to prevent the escape of earthworms, on the top of the box. They were kept in a mixture of gardening soil (made by Hollas Sp. Z o.o., ul. 3 Maja 30, 14-400 Pasłęk, Poland) and deacidified peat (pH 5.5-6.5) at 1:1 ratio, covering the bottom with approximately 5 cm of the artificial soil. They were maintained in a climatized room at 20ºC, 70% humidity and 16h light : 8h dark photoperiod. They were fed with horse manure once per week, and the moisture of the soil was also monitored once per week, by weighing the containers and adding the quantity of water needed to match the initial weight of the container.

2.2 Preparation of the test containers

In total nine different treatments were used in a full factorial design. Based on literature and preliminary studies, the low concentration represented 2x the recommended field application dose (FD) (0.6 L/ha of Sherpa 100 EC (Shp) and 1.5 L/ha of Spekfree 430 SC (Skf)) and the high concentration referred to 4x the recommended field application dose (1.2 L/ha of Sherpa 100 EC and 3.0 L/ha of Spekfree 430 SC). This corresponded to the following concentrations in the solutions: 0.10 and 0.20 mg of cypermethrin /Kg of soil and 1.07 and 2.14 mg of tebuconazole /Kg of soil. These concentrations were considered to be ecologically relevant since the values are similar to reported values for both active principles found in the environment (Kumari *et al.,* 2008; Vig *et al.,* 2001). The solutions were prepared using deionized water. Three replicates per treatment were used, consisting in separate glass jars, each containing 500g of soil..

The soil used for the experiment was LUFA 2.2, bought from LUFA Speyer (Characteristics in Table 1 in annex). The soil was dried and weighed into a glass *goblet* and then mixed with the test solutions using an electric blender (Domotec, model MS-3345) for 30 seconds. Once the test solutions were mixed into the soil, the soil was

transferred to the test containers and food was added (5 g of dry horse manure, moistened with 15 ml of water, per container).

2.3 Earthworm chronic toxicity assay

The adults were isolated from the stock culture into Petri dishes, two adults per dish, containing filter paper moistened with tap water, for 48 h in order to void the gut content. All adults were weighed after gut cleaning, before putting them into the experimental containers. Such prepared adults were randomly distributed by the test containers, 10 individuals per each, with 5-6 g of soil per individual, as advised by the OECD “Earthworm Reproduction Test” (OECD, 2016). During the entire assay, the test containers were kept in a climatized room at the same conditions as the cultures (20 ± 2ºC, 70% humidity, 16 h light and 8 h dark photoperiod). The moisture of the test soil was kept at 50-60% of the total water holding capacity, using tap water.

For the first four weeks the earthworms were fed once a week with 5 g of dried horse manure per worm. After the first four-week period, the adults were removed from the containers and the mortality was accounted. Afterwards individual’s guts were voided for 7 h. Then two individuals per replicate were tested for respiration rate, as described in section 2.4, and weighed as before the experiment. The individuals that were not tested for respiration had the same period of gut voiding and were equally weighed. Finally, the adults were put into cryotubes and frozen at -80ºC for further analysis. The cocoons produced during the first four weeks were counted manually and returned to the test container, and food was provided in the same quantity as before.

After the second four-week period, the younglings were counted, weighed and frozen at -80ºC for further analysis. Finally, the unhatched cocoons were counted and discarded at the end of the 8-week exposure period.

2.4 Earthworm respiration rate measurement

To measure the respiration rate, a 30-channel Micro-Oxymax respirometer (Columbus Instruments, USA) was used. Two worms per replicate were selected, to have approximately the same size. Before the start of the test, each individual was put into a test chamber, inside plastic tubes with a stripe of humidified filter paper and a net in the top of each, in order to avoid that the individuals would escape and enter the air tubes of the respirometer. Two series of respiration rate measurements were conducted, using 27 individuals per series.

The experimental setup was set with 27 test chambers, air sampling interval of 3 h, for 12 intervals (36 h), of which only 8 were used (making up to 24 h of measurements) at 20ºC, with the same photoperiod as used in the chronic toxicity assay (16 h light and 8 h dark).

2.5 Earthworm biomarker determinations

For the biomarker determinations all the individuals were individually homogenized in 2.5 ml plastic tubes containing phosphate buffer (PB), utilizing a tissue homogenizer (Gen-Bio PRO200). The homogenization process was executed with the tubes dipped in ice in order to prevent overheating of the samples, which could compromise protein integrity. After homogenization 50 µl of the homogenized sample was stored at -80ºC as a backup and the remaining part of the sample was centrifuged for 10 minutes at 15 000 g and 4ºC. The supernatant was then collected and stored at -80ºC for biomarker analyses, and the pellet discarded.

The chosen biomarkers were AChE, which plays a crucial role in the removal of neurotransmitters from synaptic clefts and may indicate subtle chronic effects (Ghosal *et al*., 2018; Walker *et al.,* 2012), and GST, which are important detoxification enzymes involved in the removal of many xenobiotics, including some pesticides (Scott-Fordsmand and Weeks, 2000).

2.5.1 Earthworm protein quantification

The protein quantification was done according to the Bradford method (Bradford, 1976). A calibration curve per 96-well plate was created using bovine serum albumin (BSA) standard solutions (concentrations 0, 0.25, 0.5, 1 and 1.4 mg/ml), prepared from the protein standard (200 mg/ml BSA) from Sigma-Aldrich®. In each well 5 µl of sample and 250 µl of Bradford reagent were applied, in 3 replicates per sample. After the addition of the Bradford reagent the plate was shaken for 30 seconds and incubated at 25ºC for 15 minutes before reading the absorbance at 595 nm wavelength, using a Synergy HTX multi-mode reader (Biotek®).

2.5.2 Earthworm AChE activity measurement

The AChE activity was measured according to Ellman *et al.* (1961). Briefly, this was achieved by applying 5 µl of the sample, 180 µl of 0.5 M PB, 10 µl of 0.01 M 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) solution (prepared in 0.1 M Tris-HCl) and 5 µl of 0.1 M acetylthiocholine (ACTC) to each well of the 96-well microplate. After the addition of the

substrate (ACTC) the microplate was shaken for 6 seconds and then the absorbance was measured at 405 nm wavelength, using a Synergy HTX multi-mode reader (Biotek®), for 4 minutes and 22 seconds, with readings each 42 seconds.

2.5.3 Earthworm GST activity measurement

For the measurement of the GST activity, the GST Assay Kit (Sigma-Aldrich®) was used. Briefly, into each well 4 µl of sample was applied, followed by the addition of 196 µl of the master mix (98% Dulbecco's Phosphate Buffered Saline, 1% L-glutathione reduced and 1% CDNB). The absorbance was measured at 340 nm wavelength, using a Synergy HTX multi-mode reader (Biotek®), for 7 min and 10 seconds, with readings each 42 seconds.

2.6 Statistical analysis

Data from all evaluated parameters were analyzed with two-way ANOVA in SPSS®. Before using ANOVA, all measured parameters were checked for homogeneity of variances, through Brown-Forsythe test, and for normal distribution, through the Kolmogorov Smirnov normality test.

The Body Mass Change Index (BMCI) was calculated using the average weight of each batch of individuals (of each treatment) by the following formula:

$$BMCI = \frac{Final\ weight - Initial\ weight}{Initial\ weight}$$

The cocoon hatchability was estimated by the following formula:

$$Cocoon\ hatchability = \frac{Number\ of\ juveniles}{Number\ of\ cocoons}$$

3. Results

After four weeks of exposure none of the tested treatments showed any mortality, neither in adults nor juveniles. The results of the statistical analysis of all effect criteria are presented in Table 1. The BMCI showed no significant differences among treatments (Figure 1).

Table 1 - Results of the Analysis of variance for different effect criteria. N = 27; BMCI - body mass change index.

Effect criteria	Factor	Level	N	Mean ± SD	F and p
BMCI (body mass change index)	Shp	0	9	0.122 ± 0.028	F_{Shp} =0.290, p =0.817
		2	9	0.092 ± 0.028	
		4	9	0.104 ± 0.028	
	Skf	0	9	0.055 ± 0.028	F_{Skf} =2.477, p =0.810
		2	9	0.126 ± 0.028	
		4	9	0.137 ± 0.028	
	Interaction				$F_{Interaction}$ =1.438, p =0.459
Rate of consumption of O_2 (µl of O_2/ h/ g of body mass)	Shp	0	9	83.549 ± 3.221	F_{Shp} =0.443, p =0.645
		2	9	86.293 ± 3.221	
		4	9	84.891 ± 3.221	
	Skf	0	9	90.722 ± 3.221	F_{Skf} =2.483, p =0.095
		2	9	76.804 ± 3.221	
		4	9	87.206 ± 3.221	
	Interaction				$F_{Interaction}$ =1.750, p =0.156
AChE activity (U/min/mg of protein)	Shp	0	9	6.006 ± 0.396	F_{Shp} =0.202, p =0.817
		2	9	5.914 ± 0.396	
		4	9	5.350 ± 0.396	
	Skf	0	9	5.486 ± 0.396	F_{Skf} =0.211, p =0.810
		2	9	6.000 ± 0.396	
		4	9	5.784 ± 0.396	
	Interaction				$F_{Interaction}$ =0.913, p =0.459
GST activity (µmol/ml/min)	Shp	0	9	0.505 ± 0.051	F_{Shp} =1.808, p =0.171
		2	9	0.581 ± 0.051	
		4	9	0.642 ± 0.051	
	Skf	0	9	0.452 ± 0.051	F_{Skf} =5.118, p =0.008
		2	9	0.596 ± 0.051	
		4	9	0.681 ± 0.051	
	Interaction				$F_{Interaction}$ =1.321, p =0.270

Number of juveniles (U)	Shp	0 2 4	9 9 9	83.333 ± 7.894 76.778 ± 7.894 83.667 ± 7.894	F_{Shp} =0.242, p =0.787
	Skf	0 2 4	9 9 9	92.000 ± 7.894 69.667 ± 7.894 82.111 ± 7.894	F_{Skf} =2.010, p =0.163
	Interaction				$F_{Interaction}$ =2.844, p =0.055
Number of cocoons (U)	Shp	0 2 4	9 9 9	22.000 ± 1.814 18.444 ± 1.814 16.444 ± 1.814	F_{Shp} =2.405, p =0.119
	Skf	0 2 4	9 9 9	20.889 ± 1.814 15.111 ± 1.814 20.889 ± 1.814	F_{Skf} =3.380, p =0.057
	Interaction				$F_{Interaction}$ =1.509, p =0.242
Cocoon hatchability	Shp	0 2 4	9 9 9	3.922 ± 0.741 4.067 ± 0.741 6.022 ± 0.741	F_{Shp} =2.506, p =0.110
	Skf	0 2 4	9 9 9	4.833 ± 0.741 4.467 ± 0.741 4.711 ± 0.741	F_{Skf} =0.063, p =0.939
	Interaction				$F_{Interaction}$ =0.182, p =0.945

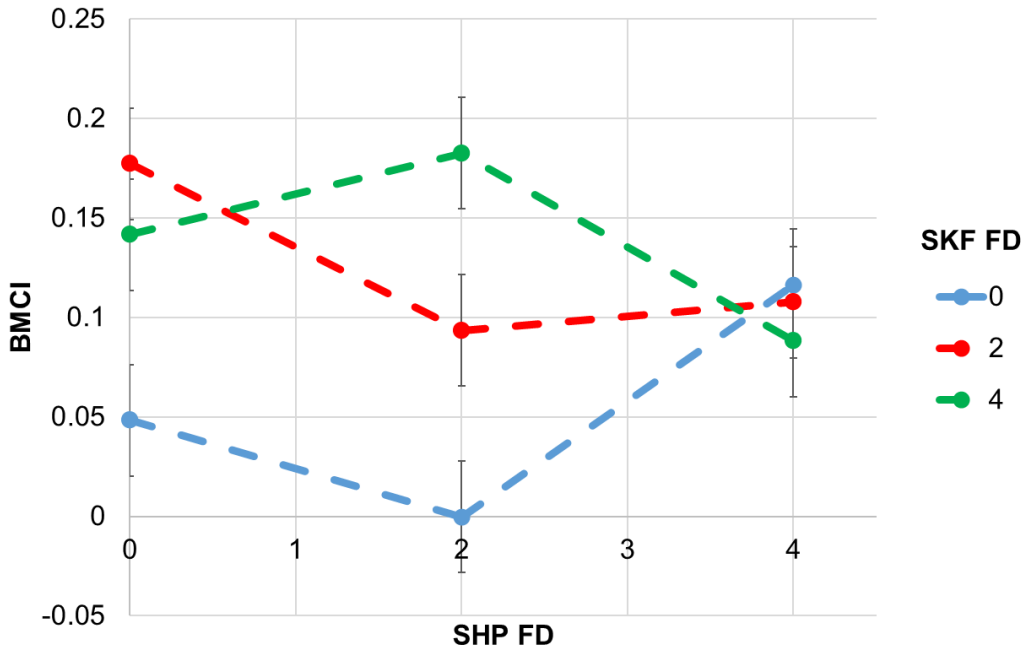

Figure 1 - Mean adult Body Mass Change Index (BMCI) after a 4-week period of exposure to double and quadruple recommended application doses (FD) of Sherpa 100 EC (Shp) and Spekfree 430 SC (Skf). No significant effects were observed at $p \leq 0.05$.

The treatments did not show any significant effect on O_2 consumption rate and no significant interaction between the pesticides was found (Table 1, Figure 2).

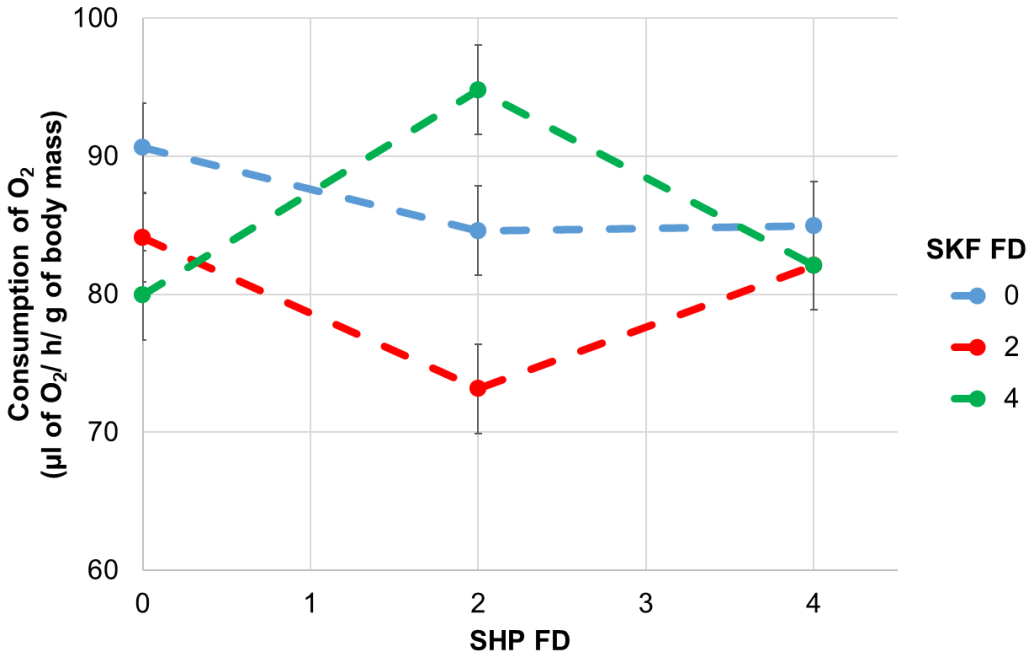

Figure 2 - Mean respiration rate (rate of O_2 consumption (µl of O_2/ h/ g of body mass)) of *Eisenia fetida* at different Shp and Skf doses expressed in relation to the FD; no significant effect of the pesticides or their interaction was found at $p \leq 0.05$.

Similarly, no significant effect of the treatments on AChE activity was found (Table 1, Figure 3. Although, significant differences in GST activity among treatments were observed: worms exposed to the 4xSkf FD and Shp 4xFD + Skf 2xFD treatments had significantly higher GST activity than control individuals, with no evidence of interaction between the compounds (Table 1, Figure4).

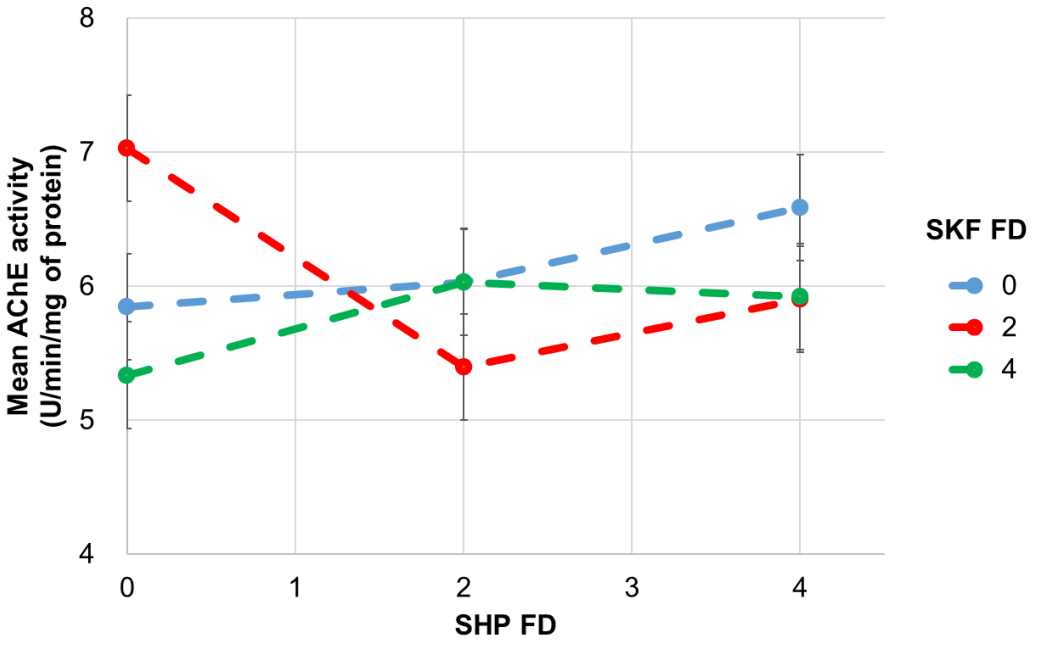

Figure 3 - Mean AChE activity (in U/min/mg of protein) of *Eisenia fetida* at different Shp and Skf doses expressed in relation to the FD; no significant effect of the pesticides or their interaction was found at $p \leq 0.05$.

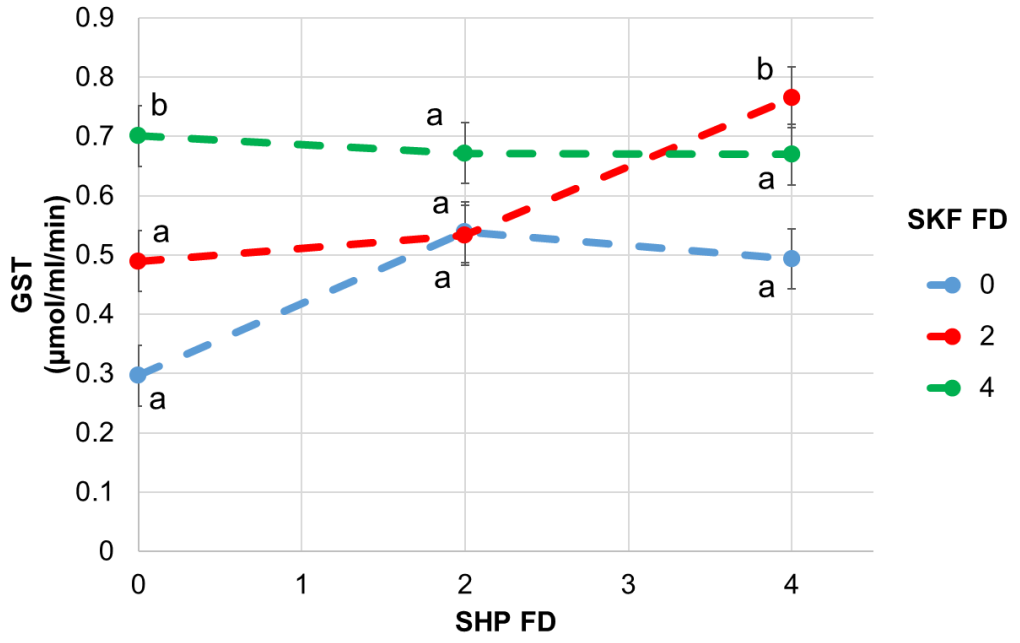

Figure 4 - Mean GST activity (in µmol/ml/min) of *Eisenia fetida* at different Shp and Skf doses expressed in relation to the FD; treatments marked with different letters are significantly different at $p \leq 0.05$.

The mean number of cocoons and juveniles produced per treatment are shown in figure 5 and 6, respectively. No significant effect of the treatments was found for both endpoints (Table 1). The cocoon hatchability per treatment is shown in figure 7. None of these parameters show any significant treatment effect (Table 1).

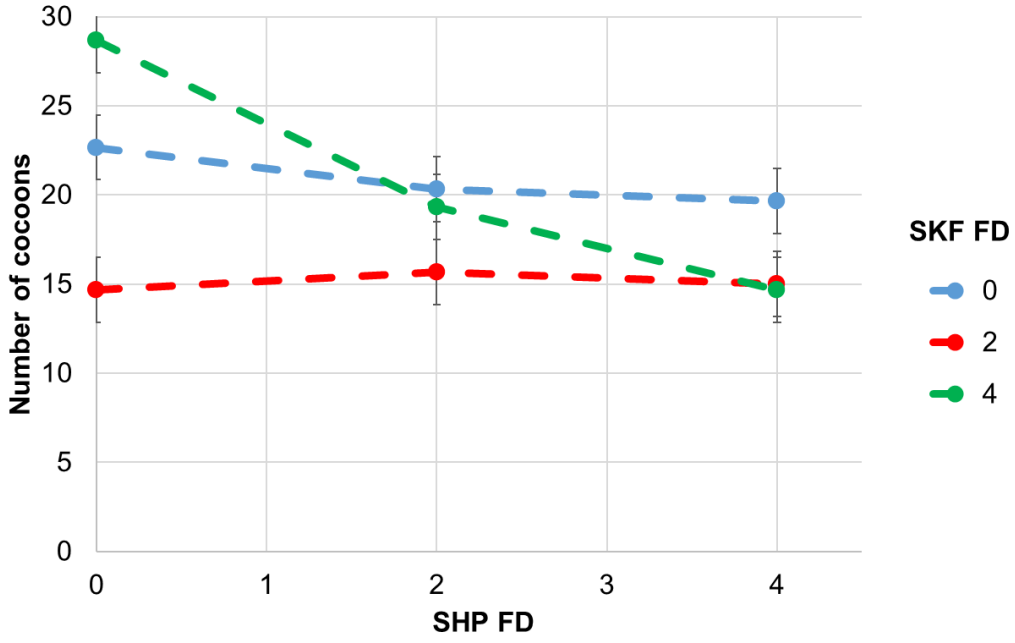

Figure 5 - Mean number of cocoons of *Eisenia fetida* at different Shp and Skf doses expressed in relation to the FD; no significant effect of the pesticides or their interaction was found at $p \leq 0.05$.

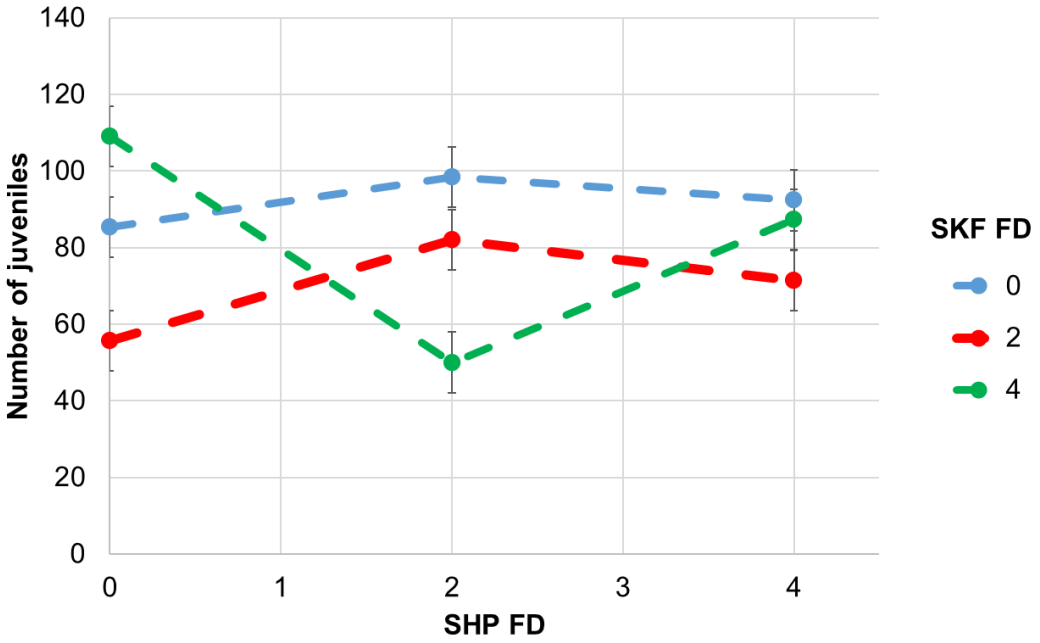

Figure 6 - Mean number of juveniles of *Eisenia fetida* at different Shp and Skf doses expressed in relation to the FD; no significant effect of the pesticides or their interaction was found at $p \leq 0.05$.

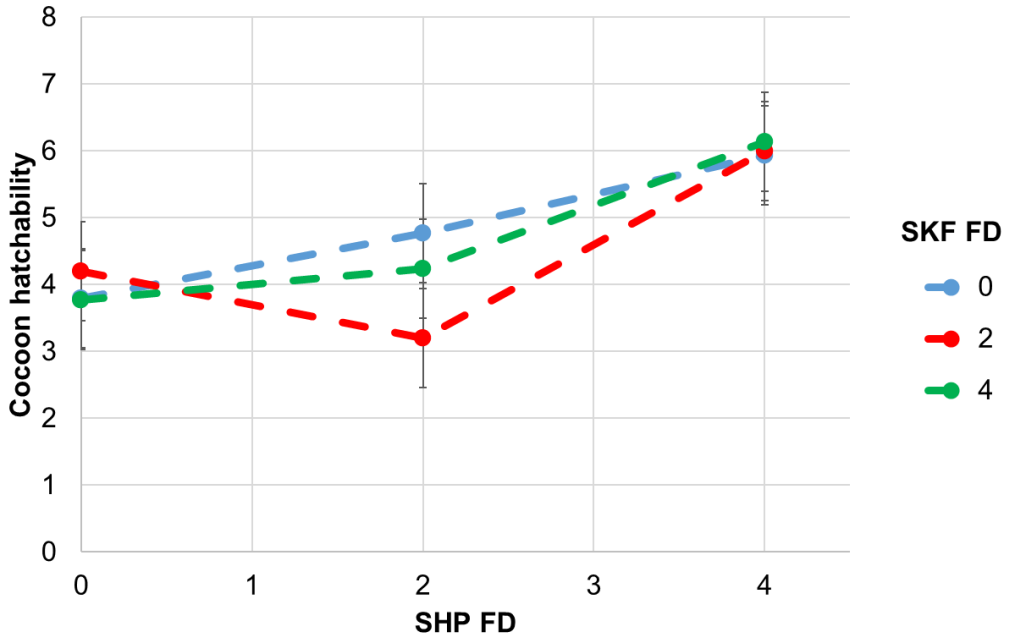

Figure 7 – Cocoon hatchability of *Eisenia fetida* at different Shp and Skf doses expressed in relation to the FD; no significant effect of the pesticides or their interaction was found at $p \leq 0.05$.

4. Discussion

The presented results suggest that the formulations tested in this study, either alone or in the mixture, do not impact significantly *E. fetida* life cycle up to the quadruple of the recommended application doses. Among all the endpoints measured in *E. fetida* only the GST activity was significantly affected by the exposure to Spekfree pesticide, applied separately and in mixture with Sherpa. Sherpa alone did not affect GST activity so it can be assumed that the increased GST activity in 4xShp FD+2xSkf FD mixture was due to the Spekfree effect, especially that GST activity in this treatment was almost identical to that found in organisms exposed to 4xSkf FD alone, and no significant interaction between the two formulations was found. The induction of GST activity suggests that this enzyme is involved in the detoxification of Spekfree, although the increase was only significant in organisms exposed to 4xSkf and 4xShp+2xSkf FD treatments. Because only two doses of each pesticide were used, it is not possible to assess the type of dose-response relationship, but the highest doses used in this experiment were already four times higher than the recommended application doses for each tested pesticide. Hence, it can be safely concluded that the results of the experiment cover environmentally realistic scenarios, since these values are close to the previously found concentrations in agricultural fields (Kumari *et al.,* 2008, Vig *et al.,* 2001).

The absence of mortality in both single-pesticide exposures is in agreement with LC_{50}s reported in the reviewed literature (Chen *et al*., 2018; Inglesfield, 1984; Saxena *et al*., 2014), since these were above the concentrations used in this study. Also, no impact was observed on reproduction at the concentrations of cypermethrin and tebuconazole used in this particular bioassay, as expected taking into account the concentrations indicated in the literature (Zhou *et al.*, 2008).

It is hard to compare the results to earlier experiments, since most of the studies on these substances focused exclusively on acute toxicity, and no study was found in the published literature regarding the exposure of *E. fetida* to the mixture of cypermethrin and tebuconazole. It also has to be kept in mind that this assay was performed with commercial formulations, actually used by farmers, rather than on the active ingredients as in previous studies. The formulations always contain other substances besides active ingredients, which may alter toxic responses of organisms.

Although the results of the study suggest that both Sherpa and Spekfree pesticides are safe to earthworms at recommended field application rates, the significant increase in GST activity at 4xSkf FD suggests that the worms actively detoxify at least the fungicide formulation (Skf), and that they needed to induce GST activity. The maintenance of high GST activity for long periods may need additional energy (Oliveira *et al*., 2012). Therefore, organisms may need to allocate energy from growth and reproduction to detoxification processes (Sibly and Calow, 1989). According to the works of Sibly and Calow (1989), individuals spend their energy on both growth and reproduction when the conditions are favourable, but any response to a stress factor will require some energy to be diverted from these processes to let the organism to cope with the stress factor. In this case, GST seems to play a role in the detoxification of at least one of the tested pesticides in the earthworms. This could represent an additional energy cost to the long-term survival of an individual. This observation may suggest that a 4-week period for mortality assessment and 8-week period for fecundity assessment may not be enough to detect more subtle yet potentially significant effects of pesticides on earthworms.

Also, there is the possibility of the existence of negative effects that were not assessed in this assay. One of these endpoints could be teratogenic effects and the effects on the growth and development of juveniles that were born in the contaminated soil that might be interesting to assess in further studies. For a successful assay evaluating the impact on more than one generation, a 25-week test period would be required, since the juveniles of the second generation (direct descendants of the first batch of *E. fetida* that are put into the test containers at the beginning of the assay) would take between 40 to 60 days to attain sexual maturity (Venter and Reinecke, 1988).

It has to be noted that the results of fecundity measurements may be prone to error due to the design of the OECD test itself. The OECD chronic test with *E. fetida* indicates that after a 4-week period of cocoon incubation in the soil the juveniles should be removed and accounted for. But cocoons are reported to hatch even 44 days after deposition into the soil (Venter and Reinecke, 1988), and since the substances tested could delay the incubation period of the cocoons, there is the possibility of some of the cocoons still being viable and unhatched. Therefore, juveniles to be born from those cocoons were not accounted for at the end of the assay. This could be avoided with an extension of the assay time by, perhaps, additional 4 weeks, providing fresh food each week. The additional benefit of such a modified test which would be that the juveniles could grow to reach sizes big enough to allow their weighing. This could not be done in the current setup

since the juveniles were too small at the end of the assay to be safely washed before weighing.

Another interesting endpoint to be measured would be the effect on regeneration capability, especially that both these substances have been reported to have endocrine disrupting properties. The regeneration mechanism of earthworms is not fully understood but it is believed to be based on dedifferentiation and posterior differentiation of tissues by the action of an endocrine system. Therefore, this would be a pertinent endpoint to evaluate. Also, the abovementioned diversion of part of energy resources to detoxification could handicap the regeneration capabilities which is a very energy demanding process. This was not assessed in this study due to specific OECD test design and time limitations.

Finally, substances like tebuconazole may not directly affect the tested organism but it may affect its diet since, according to Edwards (2004), fungi are a key part of earthworm diet and these may be affected by fungicides like tebuconazole and be absent or their populations structures may be altered. In natural environments food availability may be a limiting factor for earthworms and they may have to consume larger volumes of soil than in controlled laboratory experiments with food supplied *ad libitum*. Taking this into account, this assay may not represent all the naturally occurring consequences to *E. fetida* populations since these organisms were in good nutritional state and were not exposed to any predators or parasites.

5. Conclusions

This study showed that the formulations Sherpa 100 EC and Spekfree 430 SC, containing the pesticides cypermethrin and tebuconazole, respectively, do not have significant impacts on the survival, growth, and reproduction of adult individuals of *E. fetida* at double or quadruple of the recommended field application doses. However, sublethal effects cannot be excluded in natural conditions, where food is limited, and temperature and soil moisture are not stable. The study highlighted the need of more research, such as to investigate effects on further generations, and on regeneration capability of the exposed individuals.

For a future in-depth amelioration of this assay three different temperatures should be preferably used (corresponding to winter, summer, and mid-season temperatures) and the bioassay conditions should be directed to the type of ecosystems where the model organism naturally occurs and where the toxic substances are found. This requires more resources and more time expenditure, but it could shed light on possible chronic sublethal

impacts that might be missed by the current model of bioassays. Besides, the addition of a regeneration capability test as an endpoint could be easily added to the protocols involving earthworms as model organisms. This addition would be considering a very relevant aspect of the earthworm biology and a possible serious impact on the natural population’s survival.

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7. Annex

Table 2- Detailed composition of LUFA 2.2 soil, provided by LUFA Speyer (version 1 D12-18, 15.03.2018).

(Mean values of different batch analysis ± standard deviation. All values refer to dry matter)	
Organic carbon (% C)	1.71 ± 0.30
Nitrogen (% N)	0.18 ± 0.03
pH value (0.01 M $CaCl_2$)	5.6 ± 0.4
Cation exchange capacity (meq/100g)	9.2 ± 1.4
Particle size distribution (mm) according to German DIN (%):	
<0.002	8.3 ± 0.9
0.002 - 0.006	2.8 ± 0.9
0.006 - 0.02	4.8 ± 0.4
0.02 – 0.063	6.9 ± 0.7
0.063 – 0.2	35.3 ± 2.0
0.2 – 0.63	41.0 ± 1.5
0.63 – 2.0	0.8 ± 0.2
Soil type	Loamy sand (IS)
Particle size distribution (mm) according to USDA (%):	
<0.002	8.0 ± 1.5
0.002 – 0.05	13.7 ± 1.0
0.05 – 2.0	78.3 ± 1.0
Soil type	Sandy loam
Maximum water holding capacity (g/100g)	44.8 ± 2.9
Weight per volume (g/1000ml)	1205 ± 41