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Comparative toxicity studies on soil biota of conventional lambda-cyhalothrin formulation and lambda-cyhalothrin-loaded nanocapsulates

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Abstract

Pesticides are used in agriculture as an effective and efficient tool for controlling pests that have been enemies of crops. But during the application process, pesticides are deposited in the soil causing damages on soil organisms. To reduce the environmental impact of pesticides and increase their solubility and bioavailability; the encapsulation takes place, because this process increases the solubility and chemical stability of the pesticides, giving less contact with the soil, reducing crop damages, less losses by evaporation and prevention of the degradation of active ingredients by sunlight. The objective of this study was to compare the toxicity of a conventional lambda-cyhalothrin (LC) formulation (CF) and of a more recent lambda-cyhalothrin-loaded nanocapsulates (nano-ECF) on soil biota. The products used for the tests were: Karate® with 2.5 % of lambda-cyhalothrin and the Karate® with Zeon technology encapsulation (nanocapsules suspension, and 9.5 % p/p of lambda-cyhalothrin). Twelve concentrations (5.8-68.0 $mgLCkg^{-1}soil_{dw}$) of CF and eleven concentrations (9.1-85 $mgLCkg^{-1}soil_{dw}$) of nano-ECF, both with a dilution factor of 1.25, were tested. All ecotoxicity tests were carried out with the artificial OECD soil consisting in 70 % of fine quartz sand, 20 % of kaolin clay powder and 10 % of *Sphagnum* peat (pH=6.0 ± 0.5). The activity of arylsulphatase, dehydrogenase enzymes and changes in the nitrogen mineralization were measured in the soil samples spiked with the different CF and nano-ECF concentrations. In the soil invertebrate trial, collembolans reproduction was greatly inhibited by nano-ECF, and there was no inhibition on reproduction with the CF. The nano-ECF was also about 2X more toxic than the CF to earthworms reproduction. As for the higher plants, germination of dicotyledonous species was inhibited only by the nano-ECF. The germination of monocotyledonous was not sensitive to both formulations. The plants did not suffer any inhibition in their growth when exposed to both formulations as well. As for soil enzymatic activity (arylsulfatase, and dehydrogenase) and nitrogen mineralization; only arylsulfatase was affected by the nano-ECF. In opposition to what was expected, the nano-ECF of lambda-cyhalothrin was more toxic than the CF and frequently the difference in toxicity is remarkable.

Keywords: Nanotechnology, lambda-cyhalothrin, ecotoxicological tests

Resumo

Os pesticidas são usados na agricultura como uma ferramenta eficaz e eficiente para controlar pragas que são inimigas das culturas. Porém, durante o processo de aplicação, os pesticidas são depositados no solo, causando danos na biota do solo. Com o objetivo de reduzir o impacto ambiental dos agrotóxicos e aumentar sua solubilidade e biodisponibilidade, o processo de encapsulamento tem vindo a ganhar espaço, pois este aumenta a solubilidade e a estabilidade química dos agrotóxicos, proporcionando menos contacto com o solo, reduzindo os danos nas culturas, menores perdas por evaporação e prevenção da fotodegradação dos ingredientes ativos. Neste contexto, este estudo teve como objetivo comparar a toxicidade do ingrediente ativo lambda-cialotrina (LC) na sua formulação convencional (CF) e na mais recente formulação nanoencapsulada (nano-ECF), para a biota do solo. Os produtos utilizados para os testes foram: Karate® aglomerado contendo 2,5 % de lambda-cialotrina e o Karate Zeon® com suspensão de nanocápsulas e 9,5 % p/p de lambda-cialotrina. Foram utilizadas doze concentrações (5,8-68,0 $mgLCkg^{-1}$ de solo) de CF e onze concentrações (9,1-85,0 $mgLCkg^{-1}$ de solo) de nano-ECF, respetivamente, ambas com fator de diluição de 1,25. Todos os testes de ecotoxicidade foram realizados com o solo artificial da OCDE composto por 70 % de areia de quartzo fina, 20 % de pó de caulino e 10 % de turfa. A atividade das enzimas arilsulfatase, desidrogenase e a mineralização de azoto foram medidas nas amostras de solo com as diferentes concentrações de LC. No ensaio com invertebrados do solo, a reprodução de colêmbolos foi bastante inibida pela formulação nanoencapsulada, e não houve inibição na reprodução com a formulação convencional. A formulação nano-ECF foi cerca de 2 vezes mais tóxica que a CF na reprodução de minhocas. Quanto às plantas superiores, a germinação de espécies dicotiledóneas foi inibida apenas pela formulação nano-ECF. A germinação de monocotiledóneas não foi sensível a ambas formulações. As plantas também não sofreram nenhuma inibição no seu crescimento quando expostas a ambas as formulações. Quanto à atividade enzimática do solo (arilsulfatase e desidrogenase) e mineralização de azoto; apenas a arilsulfatase foi afetada pela formulação nano-ECF. Ao contrário do esperado, a formulação nano-ECF da lambda-cialotrina foi mais tóxico do que a CF e frequentemente a diferença na toxicidade é notável.

Palavras-chave: Nanotecnologia, lambda-cialotrina, testes ecotoxicológicos

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List of abbreviations

OCDE/OECD - Organization for Economic Cooperation and Development

FCUP – Faculty of Science of the University of Porto

NP´s - Nanoparticles

LC – Lambda-cyhalothrin

N - Nitrogen

P - Phosphorous

K - Potassium

KCL - hydrochloric acid

EEC – European Economic Community

EC – European Commission

SD – Standard deviation

EC_x - Effective concentration that causes an effect of X%

NOEC - No observed effect concentration

LOEC - Lowest observed effect concentration

WHC – Water holding capacity

1. Introduction

The new world agriculture paradigm aims to improve the decrease of the demand of land, energy and fresh water but the use of phytopharmaceutical products still favors the environmental pollution (Zhu et al. 2010). Agriculture refers to the art of cultivating fields, also representing the work and techniques used to obtain agricultural products. In addition to producing food and fiber, agriculture, also produces a wide range of goods and services, shapes the environment, affects social environmental systems and contributes to economic growth (Van Huylenbroeck et al. 2007). The presence of toxic residues in food, environmental contamination and degradation, intoxication and rampant death of living beings and the change in behavior, outbreak and selection of pests are some of the consequences of continued indiscriminate use of chemicals (De Deus and Bakonyi 2012). Sustainable agriculture should be discussed critically by science, since much of the contemporary environmental and socioeconomic problems also stem from the way science has guided the production of knowledge and the generation of agricultural technologies (Paulus and Sclindwein 2001). Since the technological advance in agriculture began, several problems arising from the widespread use of agrochemicals have been found, such as: contamination of soil, water bodies and air (during the various stages of the hydrological cycle); decreased biodiversity of aquatic and terrestrial ecosystems and bioaccumulation of pesticides in the food chains (Khandelwal et al. 2016). Within other agriculture impacts, an unacceptable degradation of soils results from the intensive use of chemical fertilizers and pesticides, the use of heavy machinery, combined with inadequate management of watersheds (Patel, Pander, and Roy 2004).

The technological development of agriculture, especially from the 21st century, incorporated a set of "advanced" or "modern" technologies aimed at increasing the production and productivity of agricultural activities. The incorporation of these technologies often occurred inappropriately to the reality of the environment, either by the way in which it was implemented, or by the nature of the technologies introduced, with social consequences and highly negative impacts on the environment (Paulus and Sclindwein 2001). However, there are three major challenges facing global agriculture in this 21st century: feeding the world population, rural poverty reduction and management of natural resources (Patel et al. 2004). However, to feed the world population, it is necessary to increase agricultural productivity without damaging the environment. This can only be attained by creating a set of inclusive policies, technologies and incentives for all types of farmers in order to motivate their responsibility for the preservation of natural resources (Hamdy 2001). So the food production challenge ahead is not small or easy. It requires increasing the productivity of complex, low yielding farming systems in ways that do not damage natural resources or the environment.

Pesticides are used in agriculture as an effective and efficient tool for controlling pests that have been enemies of crops. However, during the pesticide application process a significant amount of sprayed pesticide penetrates the soil and groundwater leading to pollution of the environment (Barry et al. 2009). Nanotechnology appeared as a promising opportunity for increasing agricultural accuracy, especially with the development of nanomaterials for the smart release of agrochemicals (Nowack and Bucheli 2007). Nanopesticides are defined as any pesticide formulation that intentionally includes entities in the nanometer size range with a wide variety of products (Kah et al. 2013). The nanopesticides as plant protection products have chemical components in their composition with at least one size dimension in the range of 1-100nm (Kookana et al. 2014). Nanoformulations can thus combine various surfactants, metals, polymers and nanomaterials (Kah and Hofmann 2014). Research on nanopesticides should be a priority for the preservation of soil quality, food security, environment and human health. The term nanopesticide covers a wide variety of products and categories (Iavicoli et al. 2017).

The terms nanopesticide and nanofertilizer describe a very wide range of products regarding size, nature, level of development and even relevance for agricultural practices (Kah et al. 2013). In the scientific literature, the prefix "nano" has been associated until now with the notion of novelty and implicitly suggests superior properties relative to non-nano counterparts (Kah 2015). The mode of action of nanopesticides are generally common to other pesticides, but environmental fate is still poorly understood and researched. That's why is better understanding of the effect of nanopesticides on non-target organisms and of their fate after application is needed (Hupffer and Feevale 2019), as their persistence in soil may contribute for their bioaccumulation in biota and food affecting individuals along the trophic chains (Couch et al. 2016). To reduce the environmental impact of pesticides and increase their solubility and bioavailability, the encapsulation takes place as this process increases the solubility and chemical stability of the pesticides, giving less contact with the soil, reducing crop damages, less losse by evaporation and prevention of the degradation of active ingredients by sunlight (Del Valle 2004). The manipulation in the outer shell of the nanocapsule is used in the process of nano-encapsulation of the pesticide for controlled and slow release of the active ingredient, this reduces the excess flow of undesired pesticides by releasing low doses over an extended period of time (Agrawal and Rathore 2014). Although this contributes for increasing the efficiency on target species, at lower doses of application, it may also increase their dispersion in the environment and their bioavailability to non-target species.

In what regards crops fertilization, the use of nanofertilizers can help to reduce nitrogen losses in soils (Hong, Peralta-Videa, and Gardea-Torresdey 2013). Nutrients, such as N, P, and K can be encapsulated in nanotubes or nanoporous materials, coated by thin protective polymer films (Perumal and Hashim 2014). Despite that, nanofertilizers, like traditional fertilizers, also can have some environmental problems, such as eutrophication. Further, and due to the slow release of the nutrients; nanofertilizers contamination may take longer time to be identified and controlled (Hong et al. 2013). Thus, the increasing trend of the use of nanopesticides mainly in the agricultural sector raises questions about their risks and the need of performing environmental risk assessments of these materials for regulatory purposes (Kookana et al. 2014) as probably they will require a revision of existing rules and practices.

Despite the concerns described above have not yet been addressed, figure-1 illustrates potential applications of nanomaterials in agriculture, some of them already being provided by the market. Within some examples seeds treated with nanotechnology can germinate faster, giving rise to strong seedlings, with an increasing resilience to environmental stress (Adhikari, Kundu, and Rao 2016)**.** For example, urea, diammonium phosphate and simple superphosphate are some of the most used fertilizers in agricultural production to increase the scarcity of macronutrients (N and P) in soil. But due to their volatility and the flow process are generally lost in a proportion of 40-70% nitrogen and 80-90% phosphorus (Duhan et al. 2017). The use of nano-coated urea or other chemical fertilizers are pointed out as current alternatives for agricultural soil fertilization due to the ability of the nano coating to reduce the rate of fertilizer dissolution and its slow release to allow the roots of plants absorb efficiently (Ombodi and Saigusa 2000). These nanofertilizers improve the performance of conventional fertilizers by providing more nutrients to plants and releasing less to the environment (Iavicoli et al. 2017). The application of nanofertilizer significantly decreased the number of days in plant growth (crop cycle). And the supply of nanofertilizer improves source synchronization and can lead to increased fruit yield (Janmohammadi et al. 2016).

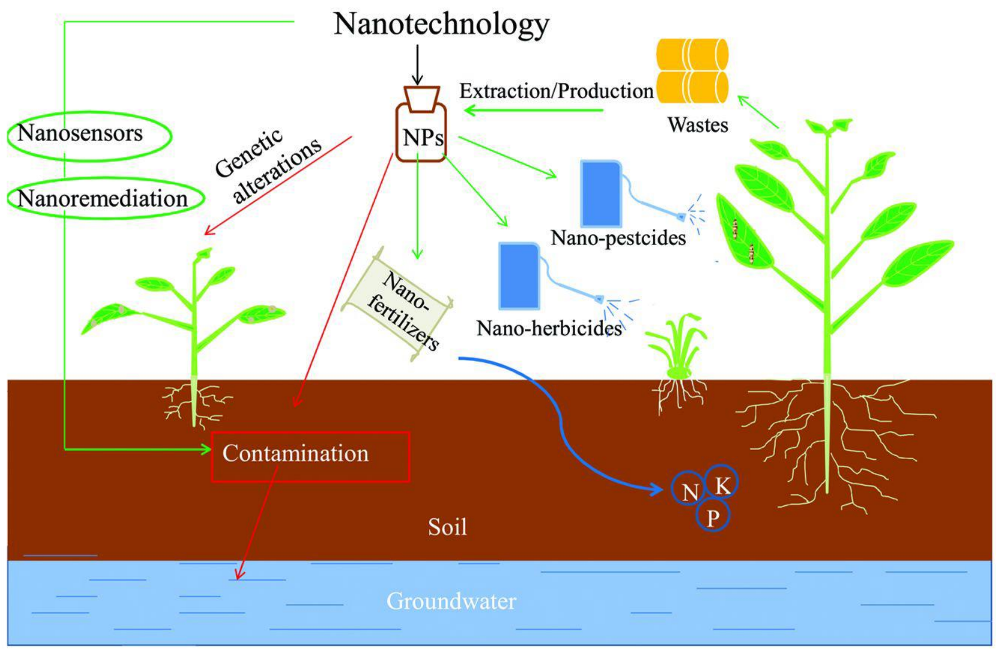

Figure-1: Potential applications of nanomaterials in agriculture (Hong et al. 2013).

1.1. Lambda-Cyhalothrin

Lambda-cyhalothrin (LC) is a synthetic insecticide that belongs to the pyrethroid chemical group. A pyrethroid pesticide mimics the naturally-occurring insecticide pyrethrins which are widely produced in the flowers of *Chrysanthemum cinerariaefolium* (Gu et al. 2007). There are two groups of pyrethroid insecticides, which are: type I and II pyrethroids. Type II pyrethoids are generally more potent than type I (Venturini 2014). Lambda-cyhalothrin is a broad-spectrum type II pyrethroid insecticide used for the control of a wide variety of arthropods (Santos et al. 2012). The mechanisms of action of pyrethroids are based on their ability to influence the sodium channels of the nervous cell membranes which are involved in the generation and conduction of the nerve impulses leading to paralysis and eventual death of the organisms after the exposure by ingestion or contact (Wang et al. 2007).

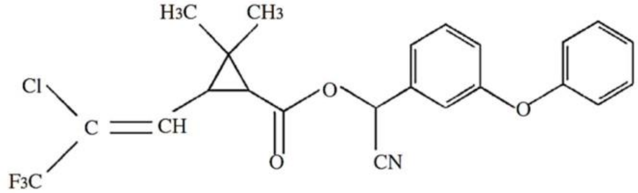

Figure-2: Structure of LC (Anadón, Martínez-Larrañaga, and Martínez 2009).

Among the various groups of pyrethroid insecticides approved for use in the European Union, there are older and newer formulations, such as Karate® and Karate Zeon®. Lambda cyhalothrin, is the active ingredient of both formulations. Karate® contains lambda- cyhalothrin in the form of water soluble granules 2.5% (w/w) and it is effective in combating various pests but is very toxic to aquatic organisms and may cause long-term adverse effects in the aquatic environment (Syngenta 2006). Karate Zeon® is a new formulation of the same active ingredient, encapsulated and in suspension containing 100 g/l or 9.5% (w/w) of lambda-cyhalothrin. It also Contains 1.2-benzisothiazole-3 (2H) –one. Karate Zeon® belongs to the new generation of photostable pyrethroids. It has good wash resistance after drying the product and has a persistence about 1 week on soil.

1.2. Risk assessment and pesticide regulation

The OECD Declaration of the Agricultural Ministers Committee defines multifunctionality of agriculture as follows: “Beyond its primary function of producing food and fibre, agricultural activity can also shape the landscape, provide environmental benefits such as land conservation, the sustainable management of renewable natural resources and the preservation of biodiversity, and contribute to the socio-economic viability of many rural areas. Agriculture is multifunctional when it has one or several functions in addition to its primary role of producing food and fibre” (Van Huylenbroeck et al. 2007).

To better protect the multifunctionality of agro-ecosystems, the proper management of phytopharmaceutical products is of utmost importance, and there are several legislation in force for this purpose. The European Parliament and the Council of the European Union laid down in Regulation (EC) No 1107/2009 of 21 October 2009 rules for the placing of plant protection products on the market and repealing the Directives 79/117/EEC and 1/414/Council EEC, where it was regulated that, the substances should only be included in plant protection products if it has been demonstrated that they present a clear benefit for plant production and they are not expected to have any harmful effect on human or animal health or any unacceptable effects on the environment, so Karate® and Karate Zeon® are part of the various pesticides authorized by the European Union. In addition to this Regulation and Directive 2009/128/EC, a thematic strategy on the sustainable use of pesticides was adopted. In order to achieve coherence between these instruments, the user should know from the product label where, when and under what circumstances a plant protection product may be used. The directive 2009/128/EC aims to achieve a sustainable use of pesticides in the EU by reducing the risks and impacts of pesticide use on human health and the environment and promoting the use of Integrated Pest Management and of alternative approaches or techniques, such as non-chemical alternatives to pesticides. Directive 2009/128/EC on the sustainable use of pesticides and Regulation (EC) 1185/2009 on the collection of pesticide usage data.

1.3. General and specific objectives

The nanopesticides are being placed in the market and gaining relevance in terms of sales volume, without the existence of sufficient data for an effective evaluation of their risks to the environment, as their authorization still relies only on ecotoxicological data for active ingredient (Kookana et al. 2014). However, previous studies have demonstrated that the toxicity of active ingredients is completely different from that commercial formulations (Pereira et al. 2014), and this is expected to become even more meaningful for engineered nanopesticides and corresponding formulations, which were particularly tuned to improve their properties. Therefore it is of particular relevance to compare the toxicity of the same active ingredients under previous and more recent formulations in order to have a better perception of the contribution of nano-formulations to reduce the risks to non-target species. So, the general objective of this study was: To compare the toxicity of a conventional lambda-cyhalothrin formulation (CF) and of a more recent lambda-cyhalothrin-loaded nanocapsulates (nano-ECF) on soil biota. And, to make this objective possible, three specific objectives were proposed:

1. To evaluate the effect of CF and nano-ECF on the reproduction of *Folsomia candida* and *Eisenia fetida* soil invertebrates;
2. To assess the effect of CF and nano-ECF on the activity of soil microbial communities by measuring the activity of soil dehydrogenase and arylsulfatase activity as well as nitrogen mineralization;
3. To evaluate the effect of CF and nano-ECF on seed germination and on the growth of terrestrial plants;

2. Material and Methods

2.1. Insecticide formulations

In the present study, two commercial formulations containing the insecticide lambda-cyhalothrin were selected to evaluate their effect on the terrestrial species. The CF (Karate® with 2.5% of lambda-cyhalothrin) and the nano-ECF (Karate® with Zeon technology encapsulation, nanocapsules suspension, and 9.5% p/p of lambda-cyhalothrin) were obtained from Syngenta. According to the manufacturer, Zeon technology encapsulation protects the active ingredient from photo degradation, as well as, it allows high adherence of nanocapsules to the target sites with excellent control of a broad spectrum of insects (Nowack and Bucheli 2007). No available information about morphology.

2.2. Ecotoxicological test

2.2.1. Test substrate

All ecotoxicity tests were carried out with an artificial soil consisting in 70% of fine quartz sand, 20% of kaolin clay powder and 10% of *Sphagnum* peat (OECD, 2009) and (OECD, 2015). Prior to homogenizing all the soil constituents, the sand was dried at 65ºC and the *Sphagnum* peat was defaunated (through two freeze–thawing, 48h at -20 ºC) and sieved to 4 mm. The pH of OECD soil was measured in a suspension (1:5 m/v) in KCL 1 M (ISO, 2005). The pH of the soil was then adjusted to 6.0 ± 0.5 with calcium carbonated (2.5g of calcium carbonated per each 10 kg soil). The maximum water holding capacity (WHC) of soil was measured following the methodology described in OECD 232 protocol (OECD, 2009). For all ecotoxicological tests, twelve concentrations (5.8-68.0 mg (LC) kg^{-1}soil and eleven concentrations (9.1-85 mg (LC) kg^{-1}soil, both with a dilution factor of 1.25), plus the controls were tested for CF and nano-ECF, respectively. All the concentrations were obtained by dilution with deionized water. These concentrations were selected according to toxicity data previously published in the literature for the active ingredient and supported by preliminary tests (Gu et al. 2007).

2.2.2. Cultures of test organisms

For the ecotoxicological tests two species of soil invertebrates were selected, namely, oligochaetes (*E. fetida* Savigny) and collembolans (*F. candida* Willem). These organisms were chosen because of their importance in soil dynamics and functions, as well as because of their recurrent use in standardized protocols of soil testing, namely the protocols 222 (OECD, 2015) and 232 (OECD, 2009), respectively. Both organisms were obtained from laboratorial cultures (LabRisk, at the Faculty of Sciences of the University of Porto) maintained under controlled environmental conditions (20 ± 2ºC and a photoperiod of 16^L: $8h^D$). The earthworms are cultured in plastic boxes in a substrate of defaunated horse manure and *Sphagnum* peat (50:50 w/w), moisturized with deionized water. The earthworms are fed with oat and horse manure once a week. *F. candida* species are maintained in small plastic containers with a substrate composed by plaster of Paris, activated charcoal (absorb residual gases and excretions) and water (OECD, 2009). The cultures of collembolans were fed with baker's yeast and moisturized with deionized water twice a week.

2.2.3. Reproduction test with *F. candida*

The reproduction test with *F. candida* species was performed according to the standard protocol (OECD, 2015). The test was carried out in small plastic containers containing 30 g of OECD $soil_{dw}$ spiked with the insecticide formulations at the concentrations described above and the WHC of the soils adjusted to 45% of WHC_{max}. After that, ten individuals (10-12 days old) were placed in the plastic containers with the spiked soil. Each concentration of both formulations was tested in triplicate, while the control group was tested in quintuplicate. During the 28 days of test, the springtails were fed with half a teaspoon baker´s yeast twice a week and the content of water was adjusted. After 28 days of exposure, under the same conditions described for the cultures, the plastic containers were filled with water, some drops of ink were added and carefully homogenized with a glass stirring. The content of each replicate was then transferred to larger pots. Therefore, the floating individuals on the surface were photographed and the number of the adults and juveniles present in each vessel was counted using the ImageJ 1.52a Software. The validity criteria of the tests was checked and the average number of juveniles and the corresponding standard deviation is reported.

2.2.4. Reproduction Test with *E. fetida*

The reproduction test with *E. fetida* followed the standard protocol (OECD, 2015). Prior to the test, clitellated adult earthworms (300-600 mg) were acclimated in OECD soil during 24h at the same conditions of culture maintenance. The test was performed in plastic pots with 500 g of OECD $soil_{dw}$ spiked with water (control group) or with the insecticide suspensions at the pre-established concentrations. The WHC of the soil was adjusted to 45% of its maximum value. Ten clitellated earthworms were added to each pot and maintained for 28 days under controlled conditions. Each concentration was tested in triplicate, while the control groups were tested in quintuplicate. Once a week, the earthworms were fed with defaunated horse manure (5 g) and the soil humidity was adjusted with deionized water whenever necessary**.** At the end of the exposure period the adults were removed, counted and weighted and the test was prolonged for more 28 days in order to left the juveniles to hatch and grow. At the end of the test the juveniles were extracted from the soil with heat and the number of juveniles per replica was counted.

2.3. **The activity of soil microbial community**

In this study a Portuguese natural reference soil (PTRS1) was used. The soil was collected from the top 20 cm's of a field in Ervas Tenras (Pinhel, Guarda, center of Portugal: 40º44'4.27''N and 7º10'54.3''W) in a non-impacted oak forest area and without historical land-uses. The soil was validated and characterized by (Caetano et al. 2012). The collected soil was sieved through a 2 mm mesh size and stored up in polyethylene bags at -20 ºC. Three replicates per test concentration were prepared for each enzyme test. For the control, five replicates. The replicates (50 g of soil per bottle) were incubated for 30 days, at 20±2°C; photoperiod: $16h^{L}$: $8h^{D}$. During the incubation period, the soil moisture was continuously monitored and adjusted to 80% of its WHC_{max}. After the incubation period, 1 g of soil per replicate was weighted to three falcon tubes, and then frozen to -20 ºC, until analysis. The stock solution of CF and nano-ECF were diluted in the amount of water required to adjust the WHC to 80 % of its maximum value. Thereby, a total of 9 sub-replicates were made for each concentration. Before analysis, the soil was thawed at 4 °C per 24 h. The absorbance was measured spectrophotometrically and the activity in the extracts calculated based on a standard curve. The activity of arylsulphatase (ARYL), dehydrogenase (DHA) enzymes and the nitrogen mineralization (NMIN) were measured in the soil samples spiked with the different LC concentrations. These parameters were chosen because among the various soil microbial parameters, they stand out for their susceptibility to environmental changes (Crecchio et al. 2004).

2.3.1. Soil arylsulfatase activity (ARYL)

The activity of soil ARYL was determined according to the method described by (Tabatabai and Bremner 1970) and (Schinner et al, 1996), three subsamples (1 g fresh weight) of each soil replicate were mixed with acetate buffer (0.5 M, pH 5.8) and 1 ml of substrate solution (potassium-p-nitrophenylsulfate); shaking and incubated for 1 h, at 37 ° C in 15 ml falcon tubes. After this period of incubation, 5 ml of distilled water were added, the tube were shaked and centrifuged at 3000 rpm for 4 minutes. Then 2 ml of supernatant were transferred to new tubes and 4 ml of distilled water and Sodium Hydroxide Solution (0.5 M) were added. Nitrophenol released by ARYL activity was extracted and stained with sodium hydroxide and determined spectrophotometrically at 420 nm. To perform controls, the same procedure described for tests tubes was followed but in the control tubes (3 per soil replicate) the addition of 1 ml of p-nitrophenyl sulfate solution was made after the additions of 0.5 M $CaCl_2$ and 0.5 M NaOH. The ARYL activity was expressed as µg *p*-nitrophenol (µg pNP.g^{-1}dm.h^{-1}).

2.3.2. Nitrogen mineralization (NMIN)

The NMIN was measured according to Schinner et al., (1996). Three subsamples (1 g fresh weight) were incubated for 7 days, at 40 °C. During this period, the organic forms of nitrogen led to nitrogen in inorganic form (mainly ammonium ion, $NH4^+$), which was determined by a modification of the Berthelot reaction after extraction with potassium chloride. After this period of incubation, the reaction of ammonia with sodium salicylate (NH3) in the presence of sodium dichloroisocyanurate formed a green colored complex and it was measured at 690 nm. The NMIN was expressed as µg of nitrogen per gram of soil dry weight matter and incubation time (µg pN.g^{-1}dm.d^{-1}) (Stemmer et al. 1999).

2.3.3. Dehydrogenase activity (DHA)

The DHA activity was assayed according to the method proposed by (Ohlinger 1993), so the samples were suspended in a solution of trifeniltetrazol chloride (TTC) and incubated at 40 °C for 24 hours. The triphenyl formazan (TPF) produced was extracted with acetone and measured spectrophotometrically at 546 nm and the results were expressed as µg pN.g^{-1}dm.h^{-1}. The intracellular dehydrogenase enzymes belong to the oxidoreductases and catalyse the oxidation of organic compounds by separating two-H atoms, so, the soil dehydrogenase activity provides correlative information on the biological activity and microbial populations in soil (Kumar, Chaudhuri, and Maiti 2013).

2.4. Seed germination and growth tests with terrestrial plants

The effect of both insecticide formulations on terrestrial plants was tested for, four species of terrestrial plants: two monocotyledonous species (*Zea mays* and *Triticum aestivum*) and two dicotyledonous species (*Solanum lycopersicum* and *Lactuca sativa*). The seed germination and growth tests were performed according to the standard protocol (OECD, 2006). All the seeds were purchased from a local agriculture supplier and carefully selected. The tests were carried out in plastic vessels which were filled with 200 g $soil_{dw}$ of OECD soil spiked with the different concentrations of the insecticide formulations. Twenty seeds were added to each vessel. At the beginning of the test, the first addition of water was diluted with a commercial fertilizer (NPK: 6-3-6; nitrogen (N): 6 %; phosphate (P_2O_5): 3 %; potassium (K_2O): 6 %) according to the dose recommended by the supplier. After that, the test was maintained at 20 ± 2 ºC and a photoperiod of ($16h^L$:$8h^D$) and luminosity (9.000 lux). Only five emerged seeds were maintained in the vessels after germination and the remaining counted and discarded after germination. The test was validated and initiated after the emergence of 50 % of the seeds in the control group, until complete 14 days of exposure. The parameters measured were seed germination rate, fresh weigh and dry weigh of the biomass above the soil, after drying at 60 ºC until constant weigh.

2.5. Data treatment

For all experiments, the data for each parameter is reported as mean ± standard deviation (SD). The normality of data was checked by Shapiro-Wilk. Statistical significances were calculated by univariate analysis of variance (ANOVA) with subsequent Dunnett´s post hoc test to determine specifically the difference between the control group and treatments with a 95% confidence interval, using Graph Pad Prism software 7.0a. Determination of no-observed effect concentration (NOEC) and low observed effect concentration (LOEC) was performed from ANOVA followed by Dunnett´s test ($p < 0.05$). The non-linear least squares regression analysis was used to estimate EC_{10}, EC_{20} and EC_{50} values (i.e., the concentration of LC that causes an effect of 10, 20 and 50 %, respectively in the parameter evaluation) by Statistic 7.0 software (Stat Soft, Inc., Tulsa, USA).

3. Results and Discussion

3.1. Reproduction tests with invertebrates

Table 1 describes the effective concentrations (EC_x) of both insecticide formulations and corresponding 95 % confidence intervals for the reproduction parameter measured in earthworms and collembolans exposed to different test concentrations. Figure 3 displays the average number of juveniles and corresponding standard deviation recorded as well for both species, at each treatment.

Table-1: Effective Concentration (EC_x) of CF and nano-ECF on *F. candida* and *E. fetida* species, and respective 95 % confidence intervals (95 % CI), as well as the R^2 of the non-linear models adjusted to data.

		EC_x[$mg_{(LC)}kg^{-1}{}_{(dw)}$]	**CI 95%**	**R^2**
	Nano-ECF	EC_{10}: 2.2	1.1 - 3.2	0.96
		EC_{20}: 3.2	2.1 - 4.4	0.96
F. candida		**EC_{50}: 6.4**	5.3 - 7.6	0.96
	CF	*n.d*	*n.d*	*n.d*
		n.d	*n.d*	*n.d*
		n.d	*n.d*	*n.d*
	Nano-ECF	EC_{10}: 2.2	1.2 - 3.3	0.95
		EC_{20}: 3.4	2.2 – 4.6	0.95
E. fetida		**EC_{50}: 6.9**	5.6 – 8.1	0.95
	CF	EC_{10}: 2.9	1.4 – 4.5	0.93
		EC_{20}: 5.0	3.0 – 7.0	0.93
		EC_{50}: 12.6	9.7 - 15.4	0.93

Summary of the estimated EC_{10}, EC_{20} and EC_{50} values for the soil invertebrate species tested. n.d: not determined.

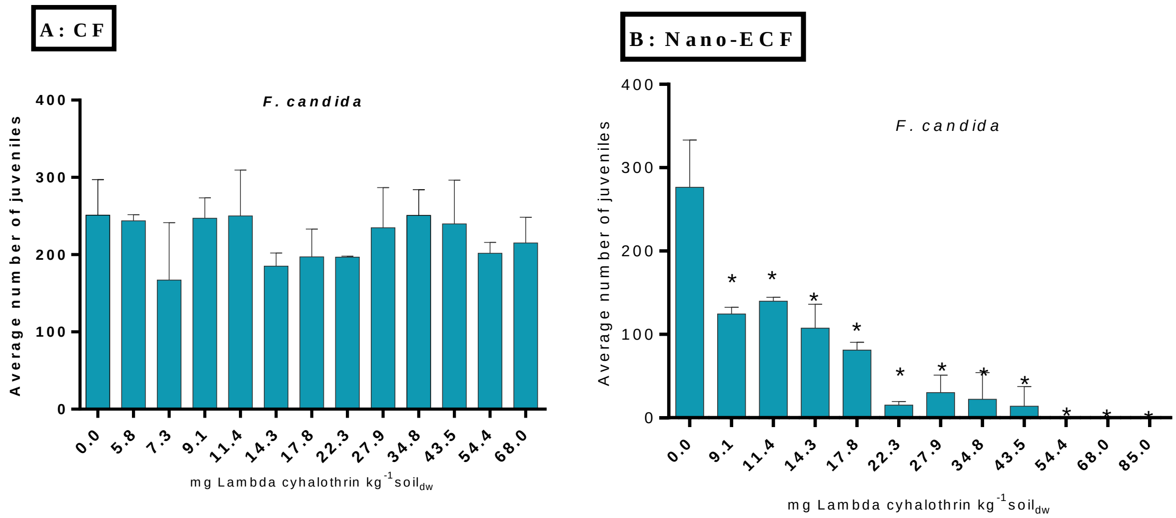

Figure-3: Average number of juveniles of *F. candida* recorded at each test concentration of the CF and of the nano-ECF, recorded after 28 days of exposure. Error bars represent standard deviation and asterisks highlight statistical differences ($p<0.05$) from the control group (0.0 mgLCkg^{-1}soil$_{dw}$), according to the Dunnet test.

The nano-ECF significantly affected collembolans reproduction at all concentrations tested [figure-3B: F (11. 26) = 39.21; $p<0.0001$]. And the estimated EC_{50} value was 6.4 mg/kg soil$_{dw}$. In opposition collembolans reproduction was not significantly affected by the CF of the insecticide at any of the concentrations tested [figure-3B: F (12. 28) = 1.545; $p<0.1666$]. A NOEC value > 68 mg/kg soil$_{dw}$ and < 9.1 mg/kg soil$_{dw}$ can be obtained for CF and nano-ECF, respectively, clearly reflecting the different toxicity of both formulations. Similar results were obtained by K. Achazi (2002) when comparing the effects of lambda-cyhalothrin and cypermethrin pyrethroids in conventional formulations and found no toxicity to *F. candida* even at the highest concentrations tested. The nanoformulation of plant protection products have a physical form with at least one size dimension in the range of 1-100 nm (Kookana et al. 2014). The small size of nanoparticles can easily increase their availability in the soil, which can easely come in contact with organisms affecting their physiological conditions. The toxicity observed after the exposure of *F. candida* to nano-ECF may be explained precisely by these aspects.

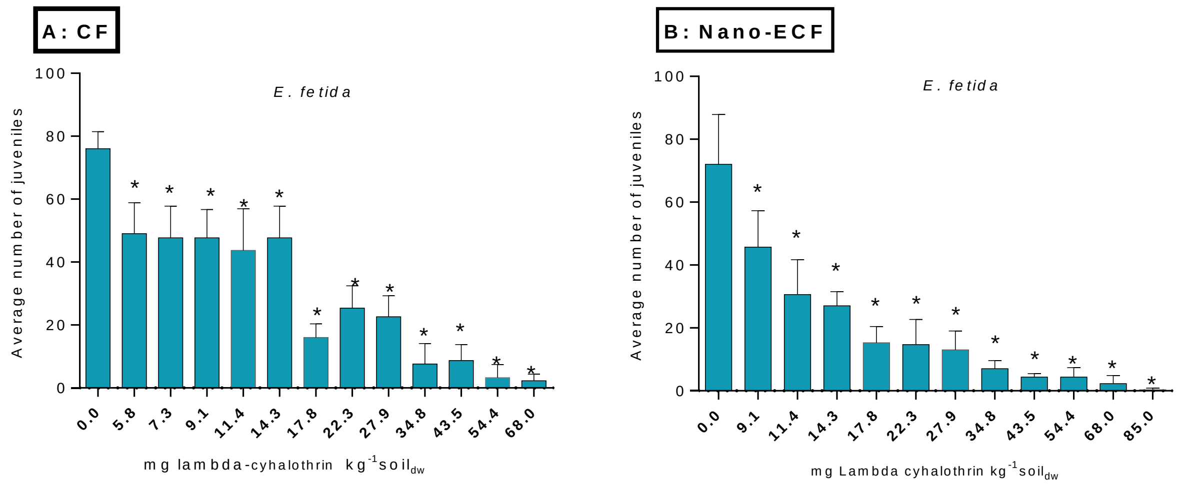

Figure-4: Average number of juveniles of *E. fetida* recorded at each test concentration of the CF and of the nano-ECF, recorded after 56 days of exposure. Error bars represent standard deviation and asterisks highlight statistical differences ($p<0.05$) from the control group (0.0 mgLCkg^{-1}soil$_{dw}$), according to the Dunnet test.

The nano-ECF significantly affected earthworms (*E.fetida*) reproduction at all concentrations tested [figure-4B: F (11. 26) = 25.50; $p<0.0001$], the estimated EC_{50} value was 6.9 mg/kg soil$_{dw}$, (table1). Also the CF of the insecticide significantly affected earthworms (*E.fetida*) reproduction at all concentrations tested [figure-4A: F (12. 28) = 32.37; $p<0.0001$]. The estimated EC_{50} value for CF was 12.6 mg/kg soil$_{dw}$. Earthworms were more sensitive than collembolans, at least to the CF. Although without a remarkable difference in their reproductive output, earthworms were more sensitive to the nano-ECF. According Nowack et al (2007), nanoformulations of insecticides have good pest control effectiveness, but they may also have several disadvantages such as no specificity, and uncertain environmental behavior. Ram (2014), also agrees that nanotechnology can have unknown consequences on soil, health, biodiversity and the environment. As there is no standardization for nanotechnology use and testing, products incorporating nanomaterials are being produced without an appropriate hazardous assessment. Pyrethroids can easily cross membranes because they are lipophilic compounds, thus having a high affinity for biological tissues (He et al. 2008). Wang (2012), found that pyrethroids are very toxic to *E. fetida* in contact toxicity tests, which indicate they are absorbed through the skin. The level of nanotoxicity to aquatic and terrestrial organisms depends on the chemical composition, size and concentration of the nanocapsules (Dubey and Mailapalli 2013). In this study the results showed the nano-ECF is more toxic than conventional insecticide, probably because of its composition, size and concentration that can easily be absorbed. Earthworms growth and reproduction have been used as important endpoints used in environmental ecotoxicity (Wu et al. 2011). Lambda-cyhalothrin is slightly to highly toxic to terrestrial and aquatic organisms (He et al. 2008), and in our study we also concluded that earthworms were more sensitive to both formulations when compared to collembolans

3.2. The activity of soil microbial enzymes and nitrogen mineralization

Figure-5 describes the effect of the concentrations of both lambda-cyhalothrin formulations in the activity of arylsulfatase enzyme in soils.

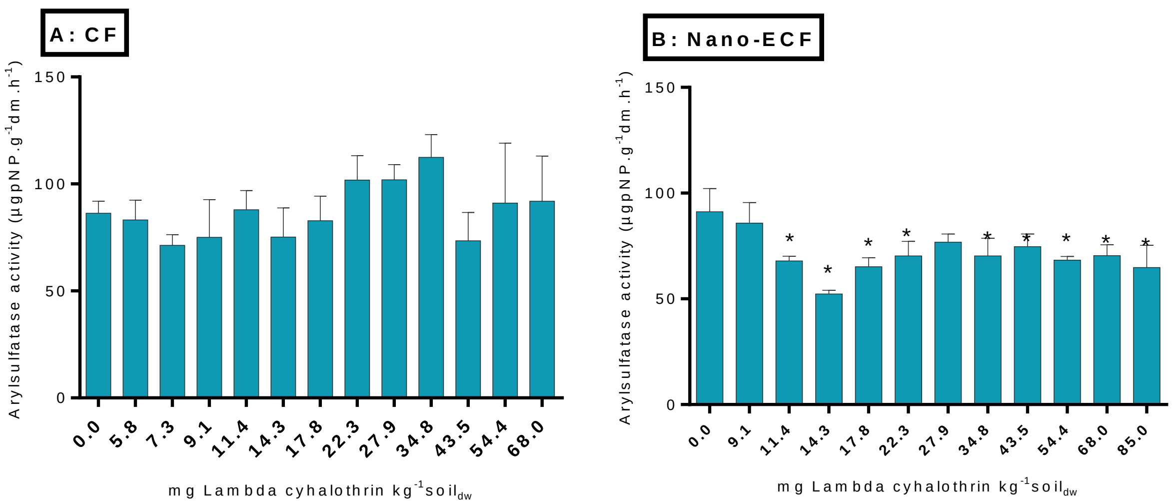

Figure-5: Effect of different concentrations of CF and nano-ECF in the activity of the soil arylsulfatase. Bars represent average values, error bars account for standard deviation. The asterisks highlight statistical differences ($p<0.05$) from the control group (0.0 $mgLCkg^{-1}soil_{dw}$).

Arylsulfatase activity was significantly influenced by the application of nano-ECF to the soil [figure-5B: $F_{(11.\ 25)} = 6.875$; $p<0.0001$], but there was no influence of CF on the activity of this enzyme [figure-5A: $F_{(12.\ 27)} = 2.466$; $p=0.0251$]. The enzymatic activities in the soil are disturbed by pesticides that hit the soil during pest control, therefore, long-term application of pesticides should be well studied in order not to disturb the biochemical balance and reduce soil fertility and productivity (Hussain et al. 2009). Generally, pesticides do not affect arylsulfatase activity in soil (Niemi et al. 2009). In contrast, from the results reported in this study arylsulfatase activity was affected. Pesticides active ingredients may directly interact with soil enzymes by binding with the active groups of the proteins and consequently affect their catalytic activities

(Klose and Tabatabai 1999; Tabatabai and Bremner 1970). Commercial formulations with non-pesticide additives may have different effects on microbial functions than pure active ingredients (Floch et al. 2011).

Figure-6 describes the effect of the concentrations of both lambda-cyhalothrin formulations in the nitrogen mineralization in soils.

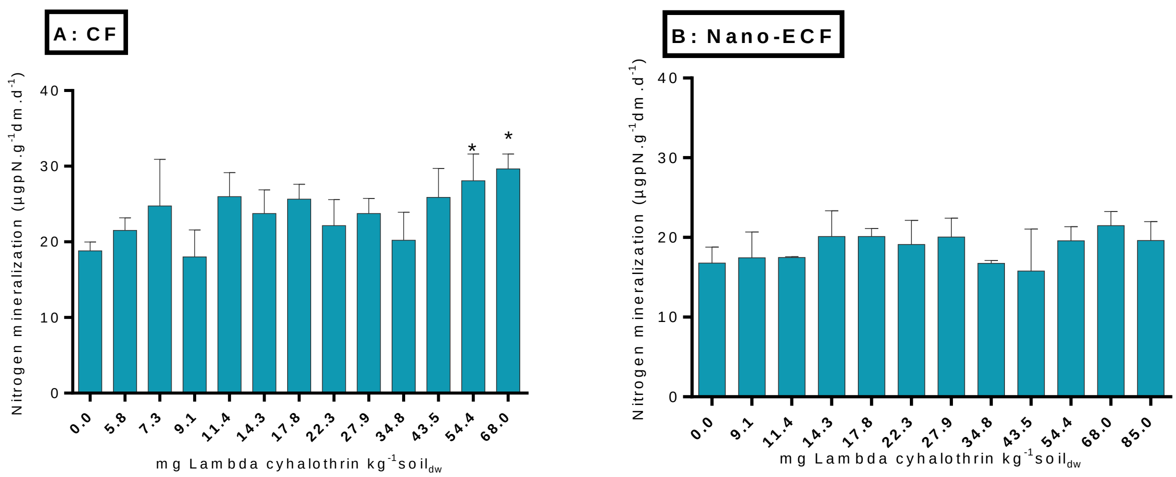

Figure-6: Effect of different concentrations of CF and nano-ECF in the nitrogen mineralization. Bars represent average values, error bars account for standard deviation. The asterisks highlight statistical differences ($p<0.05$) from the control group (0.0 $mgLCkg^{-1}soil_{dw}$).

As for nitrogen mineralization it can be noted that the highest concentrations (54.4 and 68.0 $mgLCkg^{-1}soil_{dw}$), of the CF, had significantly stimulated the nitrogen mineralization [figure-6A: F (12. 27) = 3.675; p=0.0024]. But nano-ECF had no significant effect on this soil microbial parameter [figure-6B: F (11. 25) = 1.470; p=0.2047]. Probably the conventional insecticide stimulated the activities of ammonifying and nitrifying bacteria that are responsible for the mineralization of organic nitrogen (Hussain et al. 2009). Pyrethroid insecticides, as λ-cyhalothrin, stimulate N mineralization and nitrification because some ingredients of pesticide formulation could be used as substrates to soil microbial community (Kaczyn and Kozdro 2006).

Figure-7 describes the effect of the concentrations of both lambda-cyhalothrin formulations in the activity of dehydrogenase enzyme in soils.

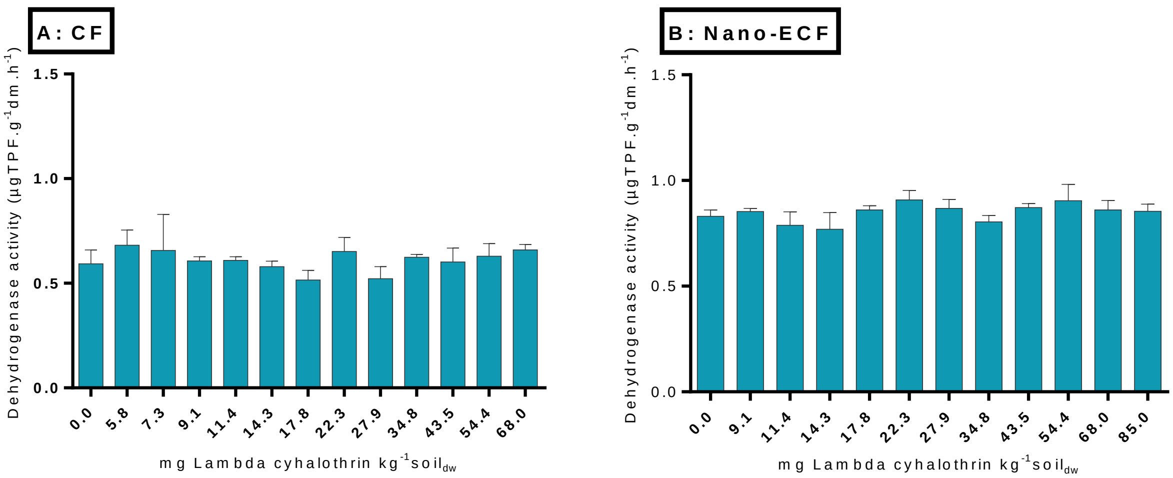

Figure-7: Effect of different concentrations of CF and nano-ECF in the activity of the soil dehydrogenase. Bars represent average values, error bars account for standard deviation. There was no significant differences ($p>0.05$) from the control group (0.0 mgLCkg^{-1}soil$_{dw}$).

The activity of soil dehydrogenases is a good indicator of general soil microbial activity, as it occurs only in viable cells (Muñoz-Leoz et al. 2013). Dehydrogenase activity was not significantly affected by both the CF [figure-7A: F (12. 27) = 1.664; $p=0.1323$] and by the nano-ECF [figure-7B: F (11. 25) = 2.585; $p=0.0339$], at all the concentrations tested.

3.3. Seed germination and terrestrial plants growth tests

Figure-8: Average number of emerged seeds in *Z. mays*, *T. aestivum*, *L. sativa* and *S. lycopersicum* after exposure in the soil OECD to CF (A, A1, A2 and A3) and nano-ECF (B, B1, B2 and B3), respectively. Error bars represent standard deviation. The asterisks highlight statistical differences ($p<0.05$) from control group (0.0 $mgLCkg^{-1}soil_{dw}$).

As regards higher plant tests, it was found that in the test with dicotyledonous (*S. lycopersicum* and *L. sativa*), the nano-ECF significantly inhibited seeds germination [figure-8B1: F (11. 26) = 7.094; p<0.0001] and [figure-8B2: F (11. 26) = 4.092; p=0.0015]. However, no statistically significant differences were recorded on average seeds germination number of the fours species at the different concentrations of the CF of the insecticide. Because of its high reactivity nanomaterials usually show greater toxicity than the same material of larger size (Oberdürster 2000). Castillo-Michael et al (2010), found that nanomaterials may decrease seed germination rate of soybean (*Glycine max*).

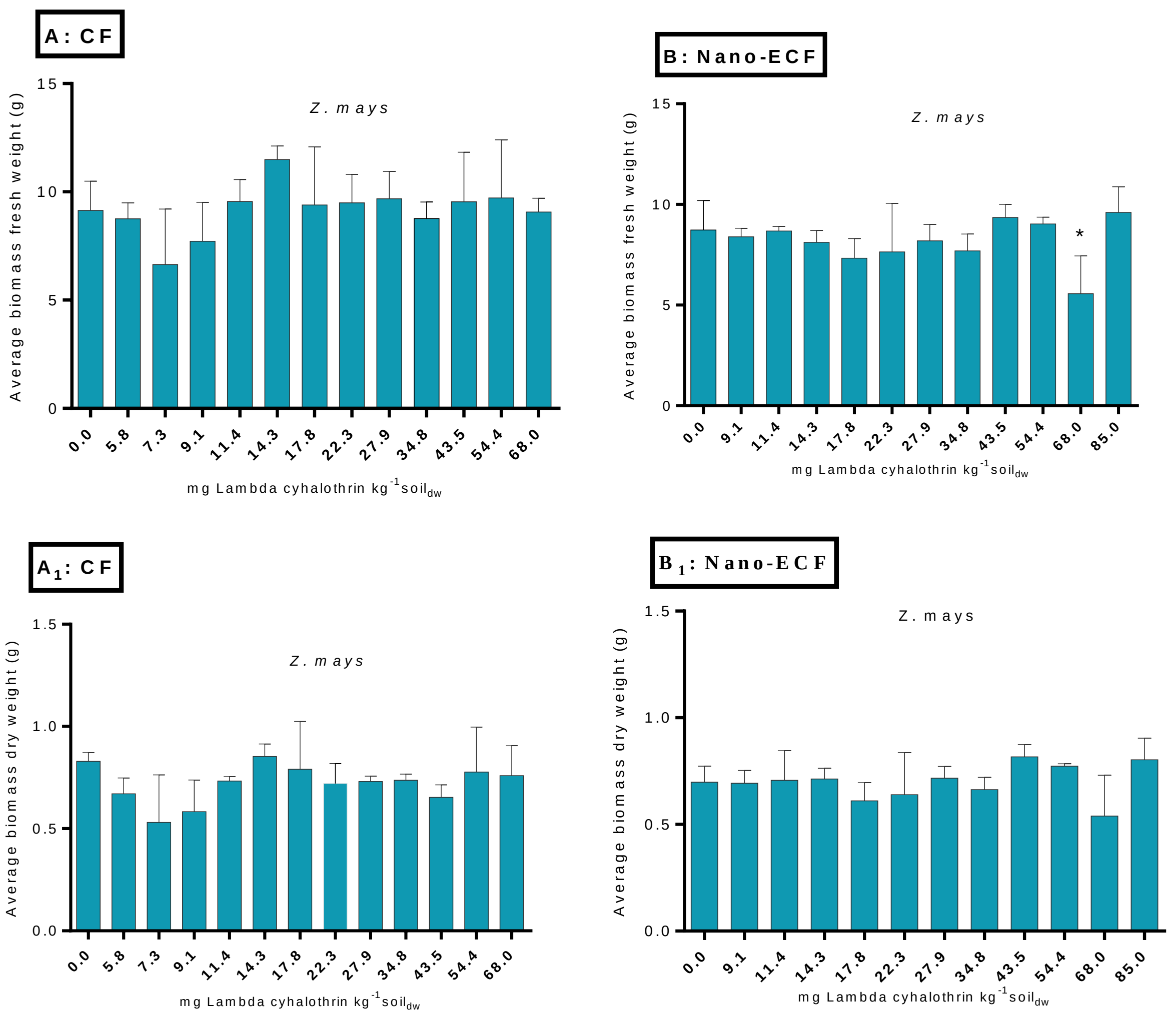

Figure-9: Average biomass fresh weight (A and B) and average biomass dry weight (A1 and B1) in *Z. mays*, after exposure in the soil OECD to CF (A and A1) and nano-ECF (B and B1), respectively. Error bars represent standard deviation. The asterisks highlight statistical differences (p<0.05) from control group (0.0 mgLCkg^{-1}soil$_{dw}$).

According to the data obtained for fresh and dry biomass, both formulations of lambda-cyhalothrin tested showed a no significant effect on *Z. mays* [figure-9A: F (11. 26) = 2.467, p=0.0286]; [figure-9B: F (12. 28) = 1.356, p=0.2441]; [figure-9A1: F (11. 26) = 1.759, p=0.11152] and [figure-9B1: F (12. 28) = 1.654, p=0.1328].

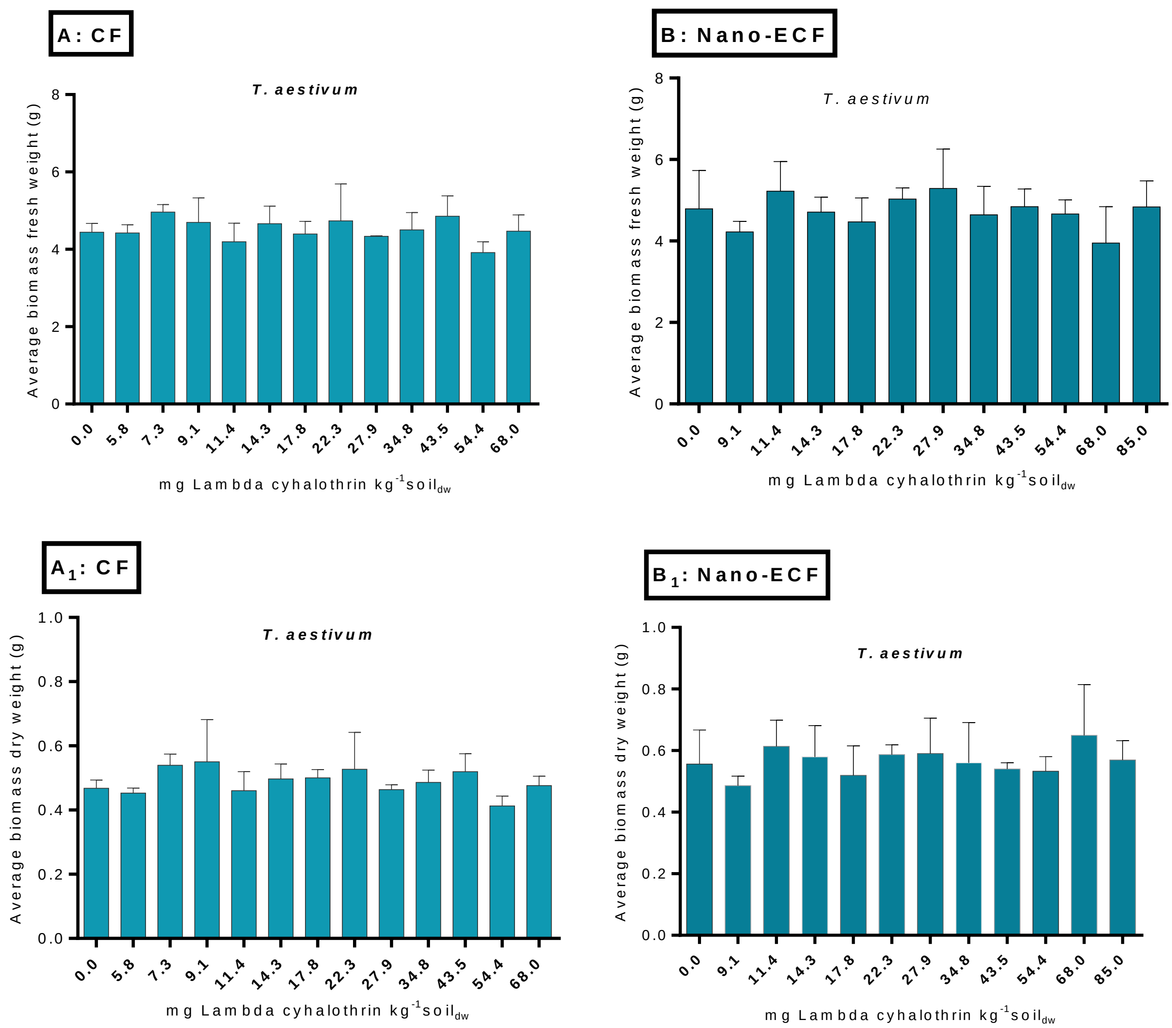

Figure-10: Average biomass fresh weight (A and B) and average biomass dry weight (A1 and B1) in *T. aestivum*, after exposure in the soil OECD to CF (A and A1) and nano-ECF (B and B1), respectively. Error bars represent standard deviation. There was no significant differences ($p>0.05$) from the control group (0.0 mgLCkg^{-1}soil$_{dw}$).

The same trend was recorded for *T. aestivum* (monocotyledonous), as no significant effects were caused by the exposure to both, CF and nano-ECF formulations [figure-10A: F (11. 26) = 0.9788, p=0.4889]; [figure-10B: F (12. 28) = 1.199, p=0.3311]; [figure-10A1: F (11. 26) = 0.6304, p=0.7867] and [figure-10B1: F (12. 28) = 1.369, p=0.2381].

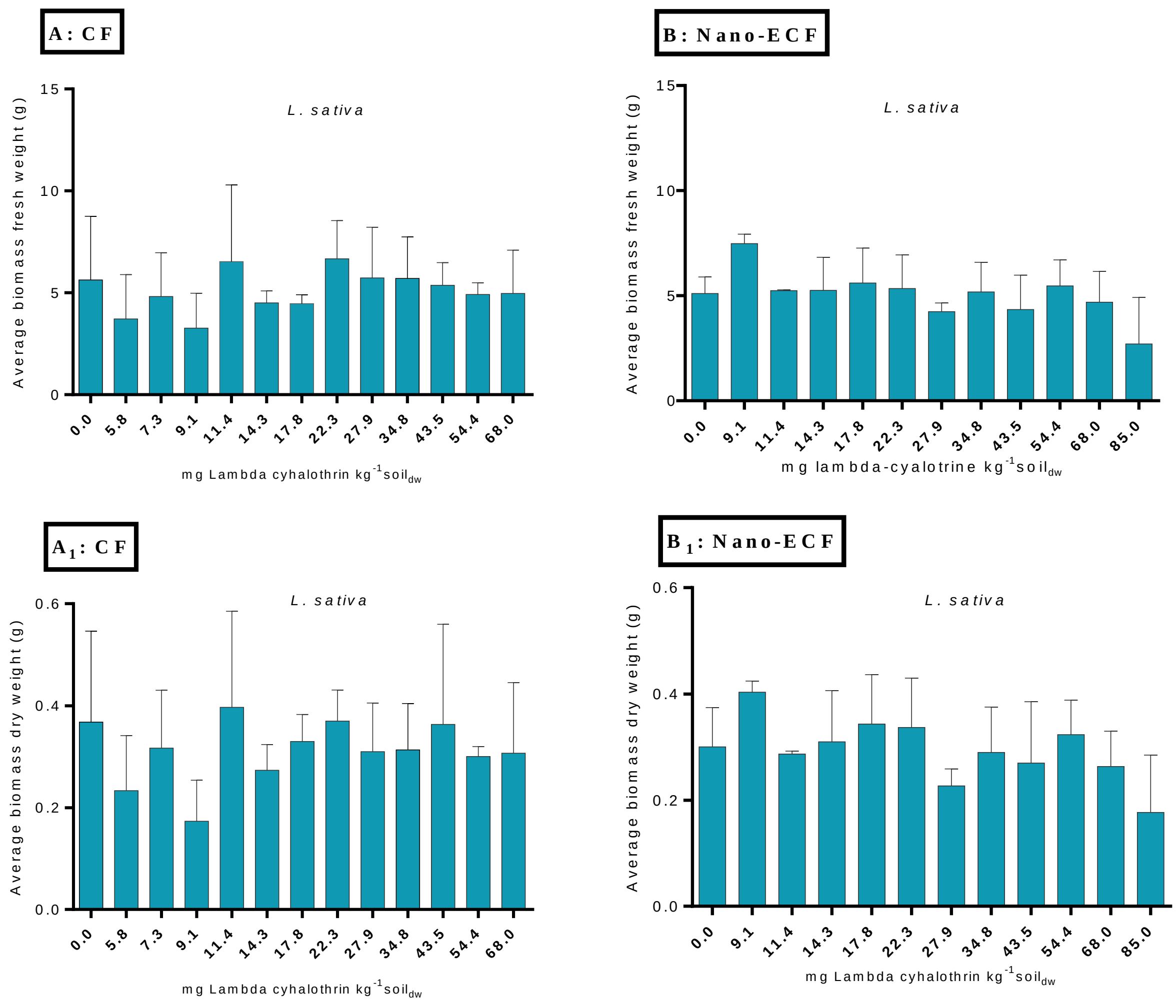

Figure-11: Average biomass fresh weight (A and B) and average biomass dry weight (A1 and B1) in *L. sativa*, after exposure in the soil OECD to CF (A and A1) and nano-ECF (B and B1), respectively. Error bars represent standard deviation. There was no significant differences (p>0.05) from the control group (0.0 mgLCkg^{-1}soil$_{dw}$).

According to the data obtained for fresh and dry biomass weight, both insecticide formulations showed no significant effects in growth parameters of *L. sativa* (dicotyledonous), [figure-11A: F (11. 26) = 2.087, p=0.0602]; [figure-11B: F (12. 28) = 0.6292; p=0.7996]; [figure-11A1: F (11. 26) = 1.652, p=0.1420] and [figure-11B1: F (12. 28) = 0.7452, p=0.6974].

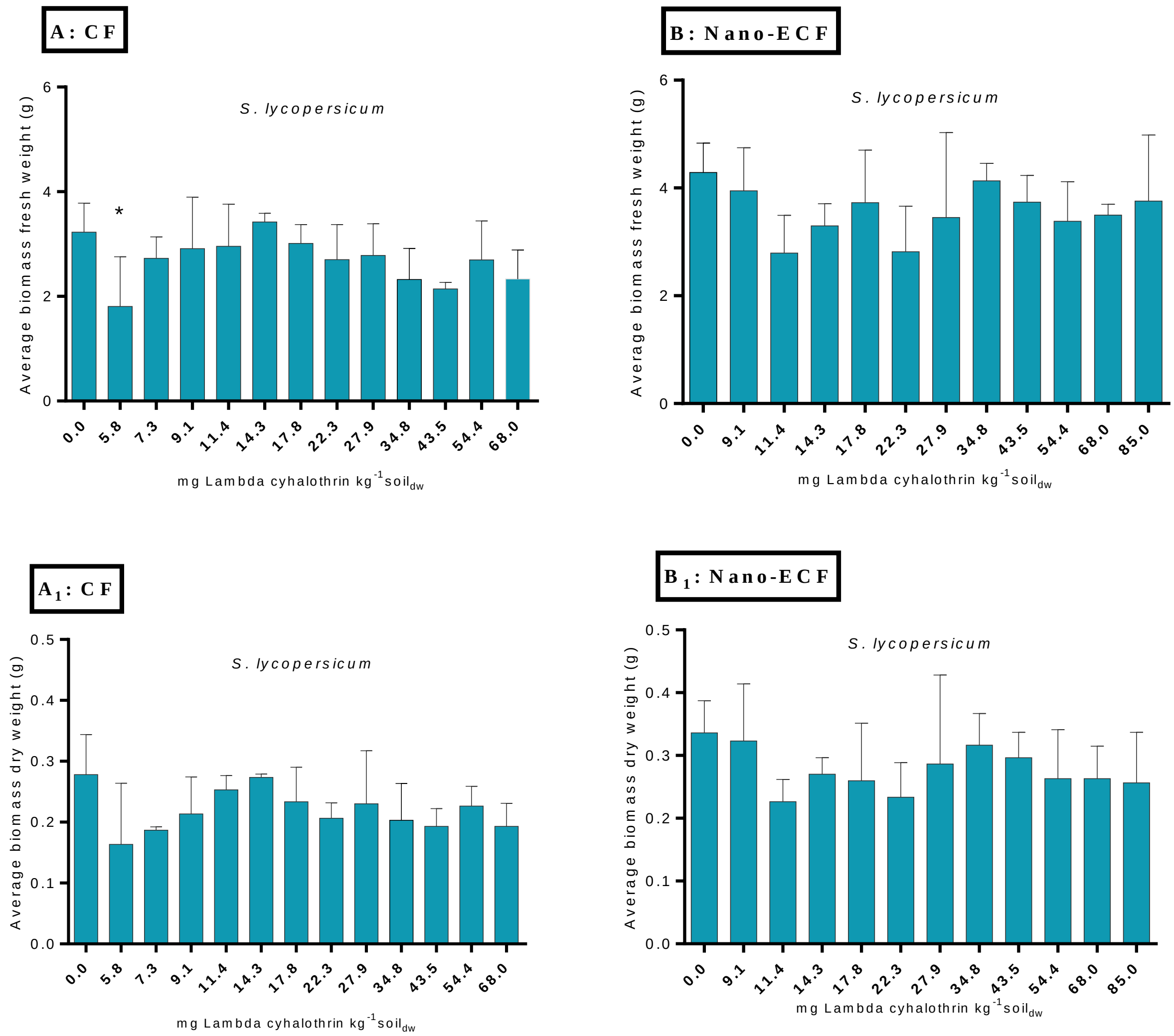

Figure-12: Average biomass fresh weight (A and B) and average biomass dry weight (A1 and B1) in *S. lycopersicum*, after exposure in the soil OECD to CF (A and A1) and nano-ECF (B and B1), respectively. Error bars represent standard deviation. The asterisks highlight statistical differences ($p<0.05$) from control group (0.0 $mgLCkg^{-1}soil_{dw}$).

The growth of *S. lycopersicum* (dicotyledonous) [figure-12A: F (11. 26) = 1.123, p=0.03836]; [figure-12B: F (12. 28) = 1.633, p=0.7996]; [figure-12A1: F (11. 26) = 0.8245, p=0.6177] and [figure-12B1: F (12. 28) = 1.350, p=0.2471] was also not affected by both insecticide formulations of the insecticide tested. In the study by (Moore and Kröger 2010), no significant differences were observed in the growth of rice seedling coleoptile exposed in lambda-cyhalothrin as well. Agreeing with Yang (2017), the main factors influencing the effects on plants are the characteristics of the nanomaterials (concentration/size/category/stability), the plant seed (size/species), the plant growth medium, the plant growth stage and the NP coating material.

4. Conclusion

Regarding the toxicity of conventional lambda-cyhalothrin formulation (CF) and lambda-cyhalothrin-loaded nanocapsulates (nano-ECF) on soil biota, it can be concluded that: in the soil invertebrate trial, collembolans (*F. candida*) reproduction was greatly inhibited by nano-ECF, and there was no inhibition on reproduction with the CF. The nano-ECF was also about 2X more toxic than the CF to earthworms (*E. fetida*) reproduction. As for the higher plants, germination of dicotyledonous species was inhibited only by the nano-ECF. The germination of monocotyledonous was not sensitive to both formulations. The plants did not suffer any inhibition in their growth when exposed to both formulations as well. As for soil enzymatic activity (arylsulfatase, and dehydrogenase) and nitrogen mineralization; only arylsulfatase was affected by the nano-ECF. In a general, and in opposition to what was expected, the nano-ECF of clorpyrifos was more toxic than the conventional formulation and frequently the difference in toxicity is remarkable. Thus, also it is announced that nanopesticides can be applied at lower doses, given their highest efficiency, it still unclear if they are safer considering that such difference in toxicity may not be compensated by lower application doses.

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