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Title: Neem oil based nanopesticide as an environmentally-friendly formulation for applications in sustainable agriculture: an ecotoxicological perspective

Article Type: Research Paper

Keywords: Zein nanoparticle, nanopesticide, botanical pesticide, azadirachtin, safer by design.

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Abstract: Sustainable agriculture encourages practices that present low risks to the environment and human health. To this end, zein (corn protein) can be used to develop nanocarrier systems capable of improving the physicochemical properties of biopesticides, reducing their possible toxicity. Neem oil extracted from the Azadirachta indica tree contains many active ingredients including azadirachtin, which is the active ingredient in multiple commercially available biopesticides. In this study, we describe the preparation and characterization of neem oil-loaded zein nanoparticles, together with evaluation of their toxicity towards nontarget organisms, using Allium cepa, soil nitrogen cycle microbiota, and Caenorhabditis elegans aiming to achieve the safer by design strategy. The spherical nanoparticles showed an average diameter of 278 ± 61.5 nm and a good stability during the experiments. In the toxicity assays with A. cepa, the neem oil-loaded zein nanoparticles mitigated the increase in the DNA relative damage index caused by the neem oil. Molecular genetic analysis of the soil nitrogen cycle microbiota revealed that neem oil-loaded zein nanoparticles did not change the number of genes which encode nitrogen-fixing enzymes and denitrifying enzymes. In C. elegans, the neem oil-loaded zein nanoparticles had no toxic effect, while neem oil interfered with pharyngeal pumping and GST-4 protein expression. This neem oil-loaded zein nanoparticles showed promising results in the toxicity studies, opening perspectives for its use in crop protection in organic agriculture.

Response to Reviewers: Sorocaba, April 10th 2019.

Dear Prof. Yolanda Pico
Associate Editor
Science of the Total Environment

The authors are very thankful to the Editor and Reviewers for their valuable comments and remarks regarding thus manuscript. We have addressed all comments and suggestion adequately. The requested alterations/corrections have been inserted directly into the manuscript (significant changes are highlighted in blue) and are described below.

Yours sincerely,

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Reviewer #1:
Reviewer: This paper deals with the effects of neem-oil loaded zein particles on non-target organisms, comprising: i) plants (Allium), ii) soil nitrogen cycle, and iii) nematode. The work appear to be well performed and data well presented. The English is generally OK. My main criticisms is mainly on presentation, which needs improvements with respect to "pedagocy" and placing the data in a broader context. E.g. in the abstract the authors use the term "zein". I am not sure that most readers of STOTEN knows what this means. Also, what are the implications of the findings made. We get information that the neem oil sorbed in the zein particles increase the N2O reductase and nitrate reductase…. but what can we use this information for? The topic is interesting - how to use naturally produced pesticides and how to evaluate such compounds. So I would recommend the paper to be published, but the authors need to work with the presentation to making is more reader friendly and to address a broader audience. Some more details below.
Answer: thank you very much for your careful evaluation and suggestions for the improvement of our manuscript. We modified it in order to make it more enlightening for STOTEN readers. We answer each comment individually. Regarding to effects under soil microbiota it is important in order to understand the possible toxic effects to these organisms. In this way, thinking in agriculture, it is extremely important to monitor the nitrogen cycle species and if we have effects along these organisms this could modify the fixing nitrogen into the soil and in this context, affecting the soil quality.

Abstract
Reviewer: What is "zein"?
Answer: Thank you for your comment. In order to clarify, we have inserted in the abstract that this is a corn protein, (p. 2, l. 24).

Reviewer: What is a botanical pesticide"? Isn't this a biopesticide - I think this is the general term now.
Answer: Thank you for your comment. We agree and the term biopesticide is more appropriated. We have changed this word along the whole manuscript.

Reviewer: What is neem-oil? Sounds exoctic, but what are the main toxic ingredients, and why? Context is here lost and also why is this relevant.
Answer: Thank you for your comment. We have clarified what is neem-oil and in this way, we have inserted the following sentence: “Neem oil extracted from the Azadirachta indica tree contains many active ingredients including azadirachtin, which is the active ingredient in multiple commercially available biopesticides”, (p. 2, l.26).

Reviewer: Why testing against these three types of organisms/cycles? What is the rationale?
Answer: Thank you for your comment. We had justified in the Introduction section: "Given this background, the innovation of this study was to develop neem oil-loaded zein nanoparticles. In addition to preparation and characterization of the nanocarriers, using the novel safe by design strategy, their potential toxicity was evaluated by investigating their effects on non-target organisms (Allium cepa, nitrogen cycle bacteria, and Caenorhabditis elegans). The choice was because they are model organisms, all are used in the research of toxicity of materials making possible a broad investigation of the possible action of zein nanoparticles loaded with neem oil, since they are in different classes of organisms (plant, nematodes and microorganisms) that can come into contact with this new biopesticide in the crops", (p. 6, l. 116X).

Reviewer: What is polydispersity index?
Answer: Thank you for your comment. As requested we have inserted the following sentence: "The nanoparticle mean size distribution and polydispersity index (an indicator of the homo/heterogeneity of the size distribution calculated by the square of the standard deviation divided by the square of the mean size) were determined by the dynamic light scattering technique (DLS).", (p. 8, l. 163). Especially we removed this information from the abstract because it is very specific.

Reviewer: How can this conclusion be based on the statements above. Seems to be negative for the nitrogen cycle? And only a few number of organisms have been tested, so how can we conclude at this stage?
Answer: Thank you for your comment. We have changed the sentence, please verify the revised version of the manuscript.

Introduction
Reviewer: I don't agree that biopesticides are mainly oils. There are at least 20,000 toxic secondary metabolites and most of these are hydrophilic and not oils. If the authors want to write a paper on natural (and toxic) essential oils, then this is OK, but should be made clear from the start. Volatile oils (incl. many terpenes) has climate effects, so authors should be careful with their statements.
Answer: Thank you for your comments. We agree with the reviewer point of view and we have changed the definition of biopesticides. "Biopesticides include essential oils which are complex mixtures of substances typically containing more than sixty volatile and lipophilic compounds derived from secondary metabolites in plants, involving terpenoids such as monoterpenes, sesquiterpenes, and phenols (Campos et al., 2018; Chellappandian et al., 2018)", (p. 3, l. 48). Please verify the revised version of the manuscript.

Reviewer: I don't think this is correct. That they are non-toxic to non-target organisms.
Answer: Thank you for your comment. We have corrected the sentence: "They might be less toxic to nontarget organisms, such as humans, and have low impacts in the environment" (p. 3, l. 57).

Reviewer: it would be useful with a figure/scheme - either in the paper of in SI - with the molecular structures of the most important toxins in the neem oil, including their phys-chem properties.
Answer: Thank you very much for your suggestion. We have been inserted a new figure in the manuscript (Figure 1), (p. 4, l. 75).

Materials and Methods
Reviewer: Need more info on the composition of the neem oil (percentages of main ingredients) so that we have some basis for understanding the results
Answer: Thank you for your comment. We have inserted the azadirachtin concentration (12g/mL) in the revised version of the manuscript, (p. 6, l. 128).

Reviewer: Antisolvent?
Answer: Thank you for your comment. Just in order to explain, the antisolvent method is a principle where the active compound is dissolved in a solvent; the solution is then injected with an antisolvent solution (in which the compound is insoluble). The compound precipitates as a consequence of the change of supersaturation caused by mixing the solution and the antisolvent solution.. We have inserted this information in the revised version of the manuscript, (p. 7, l. 139).

Reviewer: What is "Pluronic F-68"? Is this sustainable? Show structures/molecular properties somewhere. What is its function?
Answer: Thank you for your comment. We have added in the revised version (p.7, l. 149) of the manuscript the following sentence: "An aqueous solution of Pluronic F-68 (a block-copolymer of ethylene oxide and propylene oxide (C3H6O.C2H4O)x) extensively used as surfactant, wetting agents and emulsifiers) (2% v/v) was prepared and was adjusted to pH 4. The presence of Pluronic F-68 decreases the surface tension of the nanoparticles and maintain the stability of the nanoparticles in suspension.

Reviewer: Reference of this statement.
Answer: Thank you for your comment. We have been added the following references, (p. 8, l. 175):
Grillo, R., dos Santos, N.Z.P., Maruyama, C.R., Rosa, A.H., de Lima, R., Fraceto, L.F., 2012. Poly(ε-caprolactone)nanocapsules as carrier systems for herbicides: Physico-chemical characterization and genotoxicity evaluation. Journal of Hazardous Materials 231–232, 1–9. https://doi.org/10.1016/j.jhazmat.2012.06.019
Grillo, R., Pereira, A.E.S., Nishisaka, C.S., de Lima, R., Oehlke, K., Greiner, R., Fraceto, L.F., 2014. Chitosan/tripolyphosphate nanoparticles loaded with paraquat herbicide: An environmentally safer alternative for weed control. Journal of Hazardous Materials 278, 163–171. https://doi.org/10.1016/j.jhazmat.2014.05.079

Reviewer: How is the polydispersity index defined and quantified?
Answer: Thank you for your comment. The polydispersivity index refers to an indicator of the homo/heterogeneity of the size distribution of particles calculated by the square of the standard deviation divided by the square of the meantime size distribution. This information was added in the revised version of the manuscript, (p. 8, l. 163).

Reviewer: What is "Span value"?
Answer: Thank you for your comment. The Span value is an additional parameter to show the width of the size distribution calculated as Span = (D90 – D10)/D50 being that D10, D50 and D90 refer, respectively, to the diameters where 10%, 50% and 90% of the particle population. This definition was added in the revised version of the manuscript (p. 8, l. 170).

Reviewer: Citation format appears a bit strange (used throughout): "Lima et al., 2010". Shouldn't this be "Lima et al. (2010)".
Answer: Thank you for your comment. We have corrected the references format.

Reviewer: Which soil (soil type, classification) - top soil? When sampled? Never dried or dried out? The organic matter content is very, very high, so this is not a normal arable soil? Particle size distribution, N content?
Answer: Thank you for your comment. We have added all requested information, (p. 10, l. 120) "Before use, the fertilized commercial soil, (14% organic matter, pH 6.80) was sieved using a 0.2 micrometer sieve, dried and separated into vessels with surface area of 0.025 m2 each, and kept moist in a heated cabinet at 25 °C for 15 days". Please verify at the revised version of the manuscript.

Results and Discussion
Reviewer: What is NTA analysis?
Answer: Thank you for your comment. The NTA (nanoparticle tracking analysis) definition was inserted in the revised version of the manuscript (p. 8, l. 173).

Reviewer: Reference for this statement
Answer: Thank you for your comment. We have added the following reference, (p. 17, l. 358):
Chuacharoen, T., Sabliov, C.M., 2016. Stability and controlled release of lutein loaded in zein nanoparticles with and without lecithin and pluronic F127 surfactants. Colloids and Surfaces A: Physicochemical and Engineering Aspects 503, 11–18.
https://doi.org/10.1016/j.colsurfa.2016.04.038

Reviewer: How can we know that?
Answer: Thank you for your comment. We have changed the sentence in order to clarify it, (p. 17, l. 359): "As determined by microelectrophoresis negative zeta potential values have been reported previously for zein nanoparticles loaded with 5-fluorouracil (-45 ± 0.3 mV) (Lai and Guo, 2011), zein nanoparticles loaded with thymol (from -34 to -40 mV) (Li et al., 2013), and zein nanoparticles stabilized with carrageenan (from -40 to -50 mV) (Cheng and Jones, 2017)"

Reviewer: Fig. 1: What does the error bars represent?
Answer: Thank you for your comment. The error bars represent the standard deviations of the measurements and this information was stated in figure 2 caption.

Reviewer: So over time the particles will aggregate and flooculate?
Answer: Thank you for your comment. As we showed in the manuscript, the particles are stable, including over 120 minutes in saline solution. The stability was determined by size distribution measurements (by DLS and NTA) and in this way we did not observe evidences of aggregate or flocculate formation.

Reviewer: What is "mitotic" index? Why do you look at mitosis?
Answer: Thank you for your comment. The mitotic index is the number of dividing cells divided by the total number of cells. This type of analysis investigates the meristematic region of the root (region of growth), rich in cellular divisions. In general, this phase is more sensitive to the exposure of the material (e.g. chemicals, nanoparticles,

etc.), allowing better observation of the changes that occur as a consequence of the toxicity of the material, such as changes in chromosomes, loss of genetic material or changes in the different phases of division, these changes being related to the genotoxic potential of the material tested.

Reviewer: What is "c-metaphases"?
Answer: Thank you for your comment. C-metaphase is a kind of chromosome alteration. We have clarified this definition in the revised version of the manuscript, (p. 19, l. 390).

Reviewer: So colchicine and azadirachtin are similar chemical structures?
Answer: Thank you for your comment. Although both of them are extracted from plants and rich in carbon and oxygen, the azadrochtin is a triterpenoid compound (C35H44O16) while colchicine is an alkaloid (C22H25NO6).

Reviewer: Fig. 3: Do not use "," as decimal comma.
Answer: Thank you for your comment. We have made the correction.

Reviewer: The decrease - to which extent?
Answer: Thank you for your comment. We have completed the information: "A similar result was reported by Kwankua et al., (2010) who found that neem oil caused a 400% increase in chromosomal aberrations in Allium cepa, that solidifies our findings that indicate the zein nanoparticles are promising carriers for neem oil, since they are able to decrease over the genotoxicity towards nontarget organisms", (p. 19, l. 403). Please verify at the revised version of the manuscript.

Reviewer: Fig. 4: What is 2-delta-delta-ct on the y-axis of subfig. A? You need a much better explanation of both the genes and the enzymes in the bacteria active in N cycling in the methods part. Why is Cu-containing nitrite reductase of interest, for instance? The conclusion from the genetic tests is not clear to me.
Answer: Thank you for your comment. We agree with the reviewer's analysis. Indeed, the way the results were presented made it difficult to analyze the data. The figure was arranged allowing a better evaluation and possible visualization of the non-occurrence of differences between the different samples used, since these are not significant. In order to provide further clarification, the soil analysis should be evaluated on the basis of the control sample that exists for each period evaluated. When using the 2-ΔΔct calculation, the soil collected at the beginning of the test, called zero soil, is used as the basis, which is used to calculate the relative quantification, so that all the results are based on differences found in relation to the initial soil that is based on the value of 1 for each gene analyzed. It is also interesting to remember that the observed changes in the quantification of the bacteria responsible for the nitrogen cycle are constant even in control soil. For the analysis of the data it is necessary that there is a constant comparison with the control sample, otherwise the analysis of the results may not be accurate, thus invalidating the analysis of the study. The 2-ΔΔCT method was used to calculate gene levels with the Ct values determined from qPCR experiments. The data were normalized considering a sample control (soil zero) and a control gene (16sRNA). To calculation of 2-ΔΔCT is based on ΔΔCT (ΔCT_sample - ΔCT_control) and ΔCT (ΔCT_alvo - ΔCT_reference) (Yuan et al., 2008; Rao et al., 2013).

Yuan JS, Wang D, Stewart CN Jr. Statistical methods for efficiency adjusted real-time PCR quantification. Biotechnol J. 2008 Jan;3(1):112-23.
Rao X, Huang X, Zhou Z, Lin X. An improvement of the 2ˆ(-delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. Biostat Bioinforma Biomath. 2013 Aug;3(3):71-85.

Reviewer: it says that the neem-oil loaded zein particles could cause release of more N2O. So this is negative, but how do we get to a conclusion on all this?
Answer: Thank you for your comment. Please disregard this statement.

Reviewer: Fig. 5: What does the error bars represent?
Answer: Thank you for your comment. The error bars represent the standard deviations (now Figure 6). We have inserted this information in the figure caption.

Reviewer: What is GST-4? Enzyme?
Answer: We apologize for the missing information. We have inserted this information in the revised version of the manuscript. GST is glutathione-S-transferase, (p.13, l. 275).

Conclusions:
Reviewer: the manus may show that the neem-zein biopesticide is not that toxic to critical functions in soil, but the paper does not provide information that the neem oil is an efficient pesticide, so this sentence is not possible based on the results presented in the paper.
Answer: Thank you for your comment. We have changed the conclusions: “In this way, more studies must be carried out to guarantee the effects of this nanopesticide before its application in agriculture. It is therefore extremely important to recognize its mechanisms of action (for both, nanopesticides and neem), as well as their possible effects at the cellular level, their efficacy and their toxicity to target organisms”. Please verify at the revised version of the manuscript, (p. 30, l. 587).

Reviewer: In conclusion there are interesting data presented, but the authors need to spend much more efforts bringing the results into a context and to reach conclusions, and to explain why the do like they do. There is too much nerdy terms and unexplained relationships which the STOTEN readers would not be aware of. For the Results and Discussion part I would prefer to have the results presented before discussion in order not to mix up things.
Answer: Based on all your comments we have changed the whole manuscript in order to better present to STOTEN readers. The manuscript quality has improved a lot. We are glad and thankful for your valuable contribution to our manuscript.

Reviewer #2:
Reviewer: The article is interesting, given the growing number of studies that are analyzing the effects of biopesticides (many of them of plant origin) on non-target organisms. Biopesticides can become a good alternative to synthetic pesticides, so any available information about their ecotoxicity or how to reduce their impacts, it is relevant. The article raises the possibility that the application of the pesticide of the Neem plant can be done with nanoparticles, which would allow to reduce the dose of application, increase its solubility and predictably this could suppose a decrease of the toxicity for the environment. For

this purpose, in this study, neem oil-loaded zein nanoparticles are synthesized and its effect is studied on three non-target organisms comparing it with the effect of neem oil (and in some cases to the zein nanoparticles). In the case of Allium cepa, its effect on the mitosis of the seeds is studied; in the case of microorganisms of a soil, the effect on enzymes associated with the nitrogen cycle. Finally, survival, reproduction, body size and pharyngeal pumping were studied in C. elegans. The approach of the article, its justification and objectives are well formulated. However, I detect two main problems:

1. The first, affects Material and Methods and Results. Some sections of Material and Methods are confusing and some of the assays cannot be well understood because there is a lack of important information on aspects such as the number of replicates or number of subjects per samples in each case or specific details about how each one was carried out. I think it is more due to the lack of explanation than to a bad design of the experiments but the reality is that essential information is not provided and neither the results reflect it. The results do not specify if they are values of a sample or means of replicates. Therefore, the ecotoxicity results must demonstrate their consistency with information relative to the comparison between replicates or between repeated experiments through standard deviation analysis or confidence limits.
2. The second point, affects the Discussion of results. The Discussion has been interspersed with the results but is poorly developed. It explains well other cases that support the results, but it misses a somewhat deeper interpretation of the results of the authors more than simply a comparison with previous results. Sometimes, previous studies are explained in more detail than their own. It is also necessary a global reflection on the implication and relevance of the set of own results with respect to the objective that was raised, as well as the future implications of these results.

Therefore, I recommend that a major revision is warranted, since I believe that the results may be of interest but the article requires a thorough review of the way in which material and methods and results are shown and to develop the discussion of the results obtained in this work. I explain my concerns in more detail below:

Answer: Thank you for your comments. We are happy for your valuable comments, which have greatly improved the quality of the manuscript. We have checked and review all points commented here, as well as the comments from reviewer # 1 and we expect the manuscript to have met the STOTEN.

2.1. Major comments:

1. Materials and Methods. Allium cepa assay.

Reviewer: The details of how A. cepa seeds were exposed to the nanoformulations are not explained. Where? How was the exhibition made? How many seeds per trial? How many replicates?

Answer: Thank you for your comment. We have inserted the requested information (p. 9, l. 194). Please look at revised version of the manuscript.

Reviewer: "This assay was performed 3 times (n = 3)". What essay: the preparation of the slides or the exposition of the seeds to the nanoparticles? "3", means that the same test was repeated three times or that each test had 3 replicates? And if so, why are not typical (or similar) deviation values shown in the results? Is there a negative control?

Answer: Thank you for your comments. We have clarified the text in order to better explain the replicates, standard deviation and negative control. Please verify at revised version of the manuscript, (p. 9, l. 199).

Reviewer: The origin of the seeds of A. cepa must also be indicated.
Answer: Thank you for your comment. We have inserted the requested information "Allium cepa seeds were purchased from Isla seeds (Brazilian company)" in materials section, (p. 6, l. 129). Please look at the revised version of the manuscript.

2. Material and Methods. Soil microbiota assay. Lines 189-196. All this paragraph is confusing and lacks much necessary information:
Reviewer: Please, indicate the origin of the soil
Answer: Thank you for your comment. The paragraph was review and rewritten: "Before use, the fertilized commercial soil, (14% organic matter, pH 6.80) was sieved using a 0.2 micrometer sieve, dried and separated into vessels with surface area of 0.025 m2 each, and kept moist in a heated cabinet at 25 °C for 15 days. Two untreated soil samples were used as the negative control. Each treatment (zein nanoparticles, neem oil-loaded zein nanoparticles, and neem oil) were tested in duplicates (two vessels containing soil for each treatment). The applications of the formulations (using sprays) were based on the dosage and number of applications of neem oil employed in the field (three applications were performed on the same sample at 7-day intervals, using a 5 mg/mL solution at a dosage of 100 L/ha)", (p. 10, l. 220). Regarding soil information, it was obtained from a commercial fertilizer obtained from a local agricultural supplier, (p. 7, l. 130).

Reviewer: How many vessels are used? Each one had an area of 0.025 m2 or was this the final sum?
Answer: Thank you for your comment. We have added the requested information (p. 10, l. 222). Please look at the revised version of the manuscript.

Reviewer: The negative control was unique or there were replicates? How many?
Answer: Thank you for your comment. We have inserted the information about the control. Please look at the revised version of the manuscript, (p. 10, l. 223).

Reviewer: Another sample was exposed to the treatments: How many replicates?
Answer: Thank you for your comment. We have inserted the requested information. Please look at the revised version of the manuscript, (p. 10, l. 224).

Reviewer: Although later (in the result section) it can be seen clearer, it is necessary to specify here that the three applications are made with intervals of 7 days on the same sample.
Answer: Thank you for your comment. We have added the requested information. Please look at the revised version of the manuscript, (p. 11, l. 228).

3. Material and Methods. C. elegans assay. Again more information is needed.
Reviewer: * Line 220. It should be clarified why two different strains are used and for what purpose

Answer: Thank you for your comment. We have clarified the requested information, (p. 12, l. 253): "C. elegans trains N2 (wild type, established as valuable experimental model due to the high level of genetic homology with humans, fast life cycle, easy maintenance and handling) and CL2166 (genetically equal to wildtype and tagged to green fluorescent protein, GFP, fused to the promoter of the detoxifying enzyme glutathione- S- transferase-4) were maintained in plates containing NGM (nematode growth media) enriched with salts and seeded with the bacterium E. coli OP50, at 20 °C". Please verify at the revised version of the manuscript.

Reviewer: It is not clear how many replicates were used for each concentration, what was the negative control, how many C. elegans there were per replicate and how many times the assay was repeated?
Answer: Thank you for your comments. We have inserted the requested information about the C. elegans amount, replicate and assay repetitions: "…using 1500 worms per replicate (per microtube), (…) Concentrations were tested in duplicates, in every experiment using C. elegans, a procedure that was repeated in three independent experiments (in different days and different batch of worms)". Please look at the revised version of the manuscript, (p. 12, l. 267).

Reviewer: In the survival test, how many C. elegans were studied? Replicates were used?
Answer: Thank you for your comment. Indeed this information is missing. We treated 1,500 worms per group, in each microtube, and we always use duplicates per independent experiments. Triplicates are used for brood size due to the loss of worms. We have inserted the requested information in the manuscript.

Reviewer: Reproduction was determined in triplicate: in plates? How many C. elegans?
Answer: Thank you for your question, this information was not clear in the manuscript. We have used triplicates per independent experiments for this specific assay, as we can lose worms. We have now clarified that in the manuscript, (p. 13, l. 282): "After scoring survival, reproduction was determined by counting the hatched larvae daily from three individual worms from each treatment transferred to NGM plate covered with E. coli OP50, during 4 reproductive days".

Reviewer: What were the different treatments?
Answer: Thank you for your comment. We have completed the information, (p. 13, l. 291). Please verify at the revised version of the manuscript.

Reviewer: 4. Material and Methods. Here it is said that the experiments were done in duplicate, but triplicates are previously mentioned. You must clarify when we refer to replicates and when the experiment is repeated more than once. It is also necessary that this be indicated for each experiment so that the results are clear enough and can be understood.
Answer: Thank you for your comment. We have corrected these mislead information, (p. 14, l. 299): "The molecular analysis of the effects of the nanoparticles on soil microbiota and C. elegans assays were performed in duplicate, and all other experiments were performed in triplicate, however, these replicates are considered one independent experiment and were repeated at least three times". Please revise at the revised version of the manuscript.

Reviewer: 5. Results / Discussion. Only reference is made to previous studies, there is no discussion of the obtained results.
Answer: Thank you for your comment. We have inserted a discussion about the relationship between the work cited and our results. Please look at the revised version of the manuscript.

Reviewer: 6. Figure 3. Lines 363-365 are results, they should not be included in the caption. However, it should be explained here if the bars are average values of replicates, the number of replicates and the standard deviation or confidence limits.
Answer: Thank you for your comment. We have corrected the figure captions as requested.

Reviewer: 7. Results / Discussion. Only previous results are discussed. A discussion of the results obtained is missing.
Answer: Thank you for your comment. We have inserted a discussion about the relationship between the work cited and our results. Please look at the revised version of the manuscript.

Reviewer: 8. Results / Discussion. The same as before (point 7)
Answer: Thank you for your comment. We have inserted the requested improvement. Please look at the revised version of the manuscript.

Reviewer: 9. Figure 4. Lines 415-416 should go to results, not in the figure caption. As before, it should be indicated if the values of the bars are means of replicates, how many, confidence limits…
Answer: Thank you for your comment. We have changed the figure caption as requested.

Reviewer: 10. Results / Discussion. Lines 432-434. I do no see in Figure 4 the assertion that the soils treated with zein nanoparticles and loaded zein nanoparticles presented higher proportions of the two enzymes. Neither the assertion of Lines 435-437. Numerical values should be indicated for the proportions.
Answer: Thank you for your comment. We have inserted the data in percentage as requested. Please look at the revised version of the manuscript.

Reviewer: 11. Results / Discussion. The same as before (point 7)
Answer: Thank you for your comment. We have modified the discussion in order to explain our findings. Please verify at the revised version of the manuscript.

Reviewer: 12. Results / Discussion. The same as before (point 7)
Answer: Thank you for your comment. We have improved the discussion. Please look at the revised version of the manuscript.

Reviewer: 13. Figure 5. This graph shows deviation lines, but it is not indicated if they are SD or similar. Nor if the bars are average values of replicates.
Answer: Thank you for your comment. We have added this information, in figure caption 5: “Data are expressed as average of three independent experiments (n=3) normalized to % and the error bars represent the standard error”. Please verify at the revised version of the manuscript.

2.2. Minor comments:
Reviewer: 14. The pages must be numbered
Answer: Thank you for your comment. We added the page numbers.

Reviewer: 15. Highlights.
* The first highlight should indicate for whom is less genotoxic
* The last highlight is not a result of the work.
* It would be appropriate to incorporate a new highlight that would talk about the synthesis of Neem oil-loaded zein nanoparticles. This is a result of this study and is also proposed in lines 109 and 110 of the introduction as an objective.
Answer: Thank you for your comment. We have changed the highlights as requested:
- Zein nanoparticles have great potential to encapsulate neem oil
- Neem oil-loaded zein nanoparticles is less genotoxic to A. cepa than neem oil
- Biopesticide based on neem and zein nanoparticles did not change soil bacterias
- Nanoencapsulation of neem nullified the toxicity in Caenorhabditis elegans model

Reviewer: 16. Abstract. I think it's confusing to talk about "Zein nanoparticles" when in Line 28 only "neem oil-loaded zein nanoparticles" are mentioned. It should be noted that the tests are made with the two types of nanoparticles in addition to neem oil. On the other hand, I think that what should be highlighted in the abstract is the effect of the nanoparticles with neem oil that I understand are the object of study and that also showed a relative damage index lower Neem oil. This is what stands out, also in the first highlight.
Answer: Thank you for your comment. We agree with you. We have corrected the sentence. Please look at the revised version of the manuscript.

Reviewer: 17. Introduction. "The relative damage index" is a parameter that must be explained (in the field of genotoxicity).
Answer: Thank you for your comment. We have explained the term in the materials and methods section, (p. 9, l. 202): "Calculations were made of the mitotic index (MI), the damage index (DI), and the relative index (RI) which are indicators of the presence of cytotoxic, mutagenic or carcinogenic potential agents in the environment". Please look at the revised version of the manuscript.

Reviewer: 18. Introduction. This statement is not true. There are already numerous publications that show that biopesticides of plant origin (including essential oils) can be toxic to non-target organisms (Govindarajan and Benelli 2016, Kohler and Triebskorn 2013, Pino-Otin, et al. 2019, Shao and Zhang 2017).
Answer: Thank you for your comment. We have modified the sentence as requested (p. 3, l. 57): "They might be less toxic to nontarget organisms, such as humans, and have low impacts in the environment". Please look at the revised version of the manuscript.

Reviewer: 19. Introduction.. After "... and acts as a repellent." A reference is needed. Answer: Thank you for your comment. We have inserted a reference:
Campos, E.V.R., de Oliveira, J.L., Pascoli, M., de Lima, R., Fraceto, L.F., 2016. Neem Oil and Crop Protection: From Now to the Future. Frontiers in Plant Science 7. https://doi.org/10.3389/fpls.2016.01494

Reviewer: 20. Introduction. After "... Regulators, among others." A reference is needed

Answer: Thank you for your comment. We have inserted a reference as requested:
Pascoli, M., Lopes-Oliveira, P.J., Fraceto, L.F., Seabra, A.B., Oliveira, H.C., 2018b. State of the art of polymeric nanoparticles as carrier systems with agricultural applications: a minireview. Energy, Ecology and Environment 3, 137–148. https://doi.org/10.1007/s40974-018-0090-2

Reviewer: 21. Introduction. The reason why these bioindicators are selected to test the potential toxicity of the neem oil nanoparticles base of zein and not others, must be specified. What do they have in common? What information will they provide?
Answer: Thank you for your comment. We have explained the importance of the bioindicators in the introduction section (p. 6, l. 116): "The choice was because they are model organisms, all are used in the research of toxicity of materials making possible a broad investigation of the possible action of zein nanoparticles loaded with neem oil, since they are in different classes of organisms (plant, nematodes and microorganisms) that can come into contact with this new biopesticide in the crops". Please look at the revised version of the manuscript.

Reviewer: 22. Introduction. In the introduction the novelty and originality of the objectives that arise, should be commented. Have these nanoparticles been synthesized in other studies? Is it the first time that its toxicity is studied? Is it the first time that is done with these bioindicators ...? And if it has been done previously, indicate what novel aspects is what the study contributes. All this, with the necessary references and / or explaining those mentioned in lines 107 and 108.
Answer: Thank you for your comment. We have inserted the requested information in the revised version of this manuscript in order to clarify the novelty of our manuscript, (p. 6, l. 112): "Given this background, the innovation of this study was to develop neem oil-loaded zein nanoparticles. In addition to preparation and characterization of the nanocarriers, using the novel safe by design strategy their potential toxicity was evaluated by investigating their effects on nontarget organisms (Allium cepa, nitrogen cycle bacteria, and Caenorhabditis elegans)".

Reviewer: 23. Material and Methods. It is stated that the concentration used in agriculture for neem oil is 5 mg / mL. A reference or justification of this data, is needed.
Answer: Thank you for your comment. We have inserted the requested information, (p. 8, l. 160): "This concentration was chosen since in agriculture, neem oil is used at concentrations of between 4 and 6 mg/mL as recommended by the manufacturer UPL Brazil". Please look at the revised version of the manuscript.

Reviewer: 24. Material and Methods. A single concentration of nanoparticles (5mg/mL) was used in the study. However, for an adequate characterization of the toxicity of a product it is convenient to make a dose-response curve with several concentrations above and below it. This allows us to detect the concentrations in which effects begin to be seen. It cannot be ruled out, for example, that there may be an accumulation of this substance if it is applied periodically on the ground. And if this is not done, at least it should be discussed in the discussion as possible future studies.
Answer: Thank you for your comments. We have clarified this choice the text. Basically in this assay, we subjected the roots of Allium cepa to

contact by submersion in our formulation without dilution (which shows the concentration of active compound neem oil at 5mg/mL). Thus, we evaluated the maximum contact scenario that the organism could get with the nanopesticide. This model organism does not allow an assay to be carried out with the 3 applications of the compound at interval of 7 days between them due to the rapid growth of the root.

Reviewer: 25. Material and Methods. It should be clarified what is the final effect that wants to be measured with these different indices.
Answer: Thank you for your comment. We have explained what these indices indicate, (p. 10, l. 203): Calculations were made of the mitotic index (MI), the damage index (DI), and the relative index (RI) which are indicators of the presence of cytotoxic, mutagenic or carcinogenic potential agents in the environment. MI was calculated by dividing the number of cells in division by the total number of cells. DI was calculated by dividing the number of cells showing DNA alterations during the mitosis by the total number of cells in division. RI was calculated by dividing the values obtained for the treatments by the values for the negative control". Please look at the revised version of the manuscript.

Reviewer: 26. Material and Methods. The software version is advisable to indicate it.
Answer: Thank you for your comment. We have inserted the software version. Please look at the revised version of the manuscript, (p. 12, l. 250).

Reviewer: 27. Conclusions. Lines 550-560 are part of the justification and presentation of this study. They should be located in the introduction.
Answer: Thank you for your comment. As requested by you and reviewer #1, we have changed the conclusions in order to fit better with this study, (p. 30, l. 580). Please look at the revised version of the manuscript.

Reviewer: 28. The conclusions should suggest the investigations that are necessary from now on to continue characterizing the ecotoxicity of these nanoparticles in the environment. Answer: Thank you for your comment. We rewrote the conclusion as requested (p. 30, l. 587). Please look at the revised version of the manuscript.

Research Data Related to this Submission

There are no linked research data sets for this submission. The following reason is given:
Data will be made available on request

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Instituto de Ciência e Tecnologia
Câmpus de Sorocaba

Sorocaba, January 21st, 2019

Dear Dr. Damià Barceló and Dr. Jay Gan
Co-Editors in Chief
Science of the Total Environment

Please find enclosed our manuscript entitled "**Neem oil based nanopesticide as an environmentally-friendly formulation for applications in sustainable agriculture: an ecotoxicological perspective**" from Pascoli et al. to be considered for publication as article in Science of the Total Environment Journal. In this manuscript, we had developed neem oil-loaded zein nanoparticles based on an eco-friendly preparation method of encapsulation of botanical compounds aiming sustainable agriculture applications. Also, as the strategy safer by design, we use the principles of green chemistry and investigate the toxic effects on nontarget organisms (and model organisms) in order to correlate the potential toxicity of this system with the chemical composition of the nanoparticles. The results showed that this new carrier systems do not provoke toxic effects to nontarget organisms being able to decrease the toxicity caused by neem oil. The formulations presented an attractive potential for use in crop protection in sustainable agriculture contributing to the goal of sustainability. So, in this context, due the extensive toxicity studies and in special with soil organisms models we believe that this manuscript is from interest of Science of the Total Environment readers.

Sincerely yours,

Dr. Leonardo Fernandes Fraceto
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Alto da Boa Vista, Sorocaba, São Paulo,
18087-180, Brazil - e-mail: leonardo.fraceto@unesp.br

*Title Page

NEEM OIL BASED NANOPESTICIDE AS AN ENVIRONMENTALLY-FRIENDLY FORMULATION FOR APPLICATIONS IN SUSTAINABLE AGRICULTURE: AN ECOTOXICOLOGICAL PERSPECTIVE

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Sorocaba, April 10th 2019.

Dear Prof. Yolanda Pico
Associate Editor
Science of the Total Environment

The authors are very thankful to the Editor and Reviewers for their valuable comments and remarks regarding thus manuscript. We have addressed all comments and suggestion adequately. The requested alterations/corrections have been inserted directly into the manuscript (significant changes are highlighted in blue) and are described below.

Yours sincerely,

Leonardo Fernandes Fraceto
Corresponding author
E-mail: leonardo.fraceto@unesp.br

Reviewer #1:

Reviewer: This paper deals with the effects of neem-oil loaded zein particles on non-target organisms, comprising: i) plants (Allium), ii) soil nitrogen cycle, and iii) nematode. The work appear to be well performed and data well presented. The English is generally OK. My main criticisms is mainly on presentation, which needs improvements with respect to "pedagocy" and placing the data in a broader context. E.g. in the abstract the authors use the term "zein". I am not sure that most readers of STOTEN knows what this means. Also, what are the implications of the findings made. We get information that the neem oil sorbed in the zein particles increase the N2O reductase and nitrate reductase…. but what can we use this information for? The topic is interesting - how to use naturally produced pesticides and how to evaluate such compounds. So I would recommend the paper to be published, but the authors need to work with the presentation to making is more reader friendly and to address a broader audience. Some more details below.

Answer: ***thank you very much for your careful evaluation and suggestions for the improvement of our manuscript. We modified it in order to make it more enlightening for STOTEN readers. We answer each comment individually. Regarding to effects under soil microbiota it is important in order to understand the possible toxic effects to these organisms. In this way, thinking in agriculture, it is extremely important to monitor the nitrogen cycle species and if we have effects along these organisms this could modify the fixing nitrogen into the soil and in this context, affecting the soil quality.***

Abstract

Reviewer: What is "zein"?

Answer: ***Thank you for your comment. In order to clarify, we have inserted in the abstract that this is a corn protein, (p. 2, l. 24).***

Reviewer: What is a botanical pesticide"? Isn't this a biopesticide - I think this is the general term now.

Answer: ***Thank you for your comment. We agree and the term biopesticide is more appropriated. We have changed this word along the whole manuscript.***

Reviewer: What is neem-oil? Sounds exoctic, but what are the main toxic ingredients, and why? Context is here lost and also why is this relevant.

Answer: ***Thank you for your comment. We have clarified what is neem-oil and in this way, we have inserted the following sentence: "Neem oil extracted from the***

Azadirachta indica tree contains many active ingredients including azadirachtin, which is the active ingredient in multiple commercially available biopesticides", (p. 2, l.26).

Reviewer: Why testing against these three types of organisms/cycles? What is the rationale?

Answer: Thank you for your comment. We had justified in the Introduction section: "Given this background, the innovation of this study was to develop neem oil-loaded zein nanoparticles. In addition to preparation and characterization of the nanocarriers, using the novel safe by design strategy, their potential toxicity was evaluated by investigating their effects on non-target organisms (Allium cepa, nitrogen cycle bacteria, and Caenorhabditis elegans). The choice was because they are model organisms, all are used in the research of toxicity of materials making possible a broad investigation of the possible action of zein nanoparticles loaded with neem oil, since they are in different classes of organisms (plant, nematodes and microorganisms) that can come into contact with this new biopesticide in the crops", (p. 6, l. 116X).

Reviewer: What is polydispersity index?

Answer: Thank you for your comment. As requested we have inserted the following sentence: "The nanoparticle mean size distribution and polydispersity index (an indicator of the homo/heterogeneity of the size distribution calculated by the square of the standard deviation divided by the square of the mean size) were determined by the dynamic light scattering technique (DLS).", (p. 8, l. 163). Especially we removed this information from the abstract because it is very specific.

Reviewer: How can this conclusion be based on the statements above. Seems to be negative for the nitrogen cycle? And only a few number of organisms have been tested, so how can we conclude at this stage?

Answer: Thank you for your comment. We have changed the sentence, please verify the revised version of the manuscript.

Introduction

Reviewer: I don't agree that biopesticides are mainly oils. There are at least 20,000 toxic secondary metabolites and most of these are hydrophilic and not oils. If the authors want to write a paper on natural (and toxic) essential oils, then this is OK, but should be made

clear from the start. Volatile oils (incl. many terpenes) has climate effects, so authors should be careful with their statements.

Answer: ***Thank you for your comments. We agree with the reviewer point of view and we have changed the definition of biopesticides. "Biopesticides include essential oils which are complex mixtures of substances typically containing more than sixty volatile and lipophilic compounds derived from secondary metabolites in plants, involving terpenoids such as monoterpenes, sesquiterpenes, and phenols (Campos et al., 2018; Chellappandian et al., 2018)", (p. 3, l. 48). Please verify the revised version of the manuscript.***

Reviewer: I don't think this is correct. That they are non-toxic to non-target organisms.

Answer: ***Thank you for your comment. We have corrected the sentence: "They might be less toxic to nontarget organisms, such as humans, and have low impacts in the environment" (p. 3, l. 57).***

Reviewer: it would be useful with a figure/scheme - either in the paper of in SI - with the molecular structures of the most important toxins in the neem oil, including their phys-chem properties.

Answer: ***Thank you very much for your suggestion. We have been inserted a new figure in the manuscript (Figure 1), (p. 4, l. 75).***

Materials and Methods

Reviewer: Need more info on the composition of the neem oil (percentages of main ingredients) so that we have some basis for understanding the results

Answer: ***Thank you for your comment. We have inserted the azadirachtin concentration (12g/mL) in the revised version of the manuscript, (p. 6, l. 128).***

Reviewer: Antisolvent?

Answer: ***Thank you for your comment. Just in order to explain, the antisolvent method is a principle where the active compound is dissolved in a solvent; the solution is then injected with an antisolvent solution (in which the compound is insoluble). The compound precipitates as a consequence of the change of supersaturation caused by mixing the solution and the antisolvent solution.. We have inserted this information in the revised version of the manuscript, (p. 7, l. 139).***

Reviewer: What is "Pluronic F-68"? Is this sustainable? Show structures/molecular properties somewhere. What is its function?

Answer: ***Thank you for your comment. We have added in the revised version (p.7, l. 149) of the manuscript the following sentence: "An aqueous solution of Pluronic F-68 (a block-copolymer of ethylene oxide and propylene oxide ($C_3H_6O.C_2H_4O$)x) extensively used as surfactant, wetting agents and emulsifiers) (2% v/v) was prepared and was adjusted to pH 4. The presence of Pluronic F-68 decreases the surface tension of the nanoparticles and maintain the stability of the nanoparticles in suspension.***

Reviewer: Reference of this statement.

Answer: ***Thank you for your comment. We have been added the following references, (p. 8, l. 175):***

Grillo, R., dos Santos, N.Z.P., Maruyama, C.R., Rosa, A.H., de Lima, R., Fraceto, L.F., 2012. Poly(ε-caprolactone)nanocapsules as carrier systems for herbicides: Physico-chemical characterization and genotoxicity evaluation. Journal of Hazardous Materials 231–232, 1–9. https://doi.org/10.1016/j.jhazmat.2012.06.019

Grillo, R., Pereira, A.E.S., Nishisaka, C.S., de Lima, R., Oehlke, K., Greiner, R., Fraceto, L.F., 2014. Chitosan/tripolyphosphate nanoparticles loaded with paraquat herbicide: An environmentally safer alternative for weed control. Journal of Hazardous Materials 278, 163–171. https://doi.org/10.1016/j.jhazmat.2014.05.079

Reviewer: How is the polydispersity index defined and quantified?

Answer: ***Thank you for your comment. The polydispersivity index refers to an indicator of the homo/heterogeneity of the size distribution of particles calculated by the square of the standard deviation divided by the square of the meantime size distribution. This information was added in the revised version of the manuscript, (p. 8, l. 163).***

Reviewer: What is "Span value"?

Answer: ***Thank you for your comment. The Span value is an additional parameter to show the width of the size distribution calculated as Span = (D_{90} – D_{10})/D_{50} being that D_{10}, D_{50} and D_{90} refer, respectively, to the diameters where 10%, 50% and 90% of the particle population. This definition was added in the revised version of the manuscript (p. 8, l. 170).***

Reviewer: Citation format appears a bit strange (used throughout): "Lima et al., 2010". Shouldn't this be "Lima et al. (2010)".

Answer: ***Thank you for your comment. We have corrected the references format.***

Reviewer: Which soil (soil type, classification) - top soil? When sampled? Never dried or dried out? The organic matter content is very, very high, so this is not a normal arable soil? Particle size distribution, N content?

Answer: ***Thank you for your comment. We have added all requested information, (p. 10, l. 120) "Before use, the fertilized commercial soil, (14% organic matter, pH 6.80) was sieved using a 0.2 micrometer sieve, dried and separated into vessels with surface area of 0.025 m² each, and kept moist in a heated cabinet at 25 °C for 15 days". Please verify at the revised version of the manuscript.***

Results and Discussion

Reviewer: What is NTA analysis?

Answer: ***Thank you for your comment. The NTA (nanoparticle tracking analysis) definition was inserted in the revised version of the manuscript (p. 8, l. 173).***

Reviewer: Reference for this statement

Answer: ***Thank you for your comment. We have added the following reference, (p. 17, l. 358):***

Chuacharoen, T., Sabliov, C.M., 2016. Stability and controlled release of lutein loaded in zein nanoparticles with and without lecithin and pluronic F127 surfactants. Colloids and Surfaces A: Physicochemical and Engineering Aspects 503, 11–18. https://doi.org/10.1016/j.colsurfa.2016.04.038

Reviewer: How can we know that?

Answer: ***Thank you for your comment. We have changed the sentence in order to clarify it, (p. 17, l. 359): "As determined by microelectrophoresis negative zeta potential values have been reported previously for zein nanoparticles loaded with 5-fluorouracil (-45 ± 0.3 mV) (Lai and Guo, 2011), zein nanoparticles loaded with thymol (from -34 to -40 mV) (Li et al., 2013), and zein nanoparticles stabilized with carrageenan (from -40 to -50 mV) (Cheng and Jones, 2017)"***

Reviewer: Fig. 1: What does the error bars represent?

Answer: ***Thank you for your comment. The error bars represent the standard deviations of the measurements and this information was stated in figure 2 caption.***

Reviewer: So over time the particles will aggregate and flooculate?

Answer: ***Thank you for your comment. As we showed in the manuscript, the particles are stable, including over 120 minutes in saline solution. The stability was determined by size distribution measurements (by DLS and NTA) and in this way we did not observe evidences of aggregate or flocculate formation.***

Reviewer: What is "mitotic" index? Why do you look at mitosis?

Answer: ***Thank you for your comment. The mitotic index is the number of dividing cells divided by the total number of cells. This type of analysis investigates the meristematic region of the root (region of growth), rich in cellular divisions. In general, this phase is more sensitive to the exposure of the material (e.g. chemicals, nanoparticles, etc.), allowing better observation of the changes that occur as a consequence of the toxicity of the material, such as changes in chromosomes, loss of genetic material or changes in the different phases of division, these changes being related to the genotoxic potential of the material tested.***

Reviewer: What is "c-metaphases"?

Answer: ***Thank you for your comment. C-metaphase is a kind of chromosome alteration. We have clarified this definition in the revised version of the manuscript, (p. 19, l. 390).***

Reviewer: So colchicine and azadirachtin are similar chemical structures?

Answer: Thank you for your comment. Although both of them are extracted from plants and rich in carbon and oxygen, the azadrochtin is a triterpenoid compound ($C_{35}H_{44}O_{16}$) while colchicine is an alkaloid ($C_{22}H_{25}NO_6$).

Reviewer: Fig. 3: Do not use "," as decimal comma.

Answer: ***Thank you for your comment. We have made the correction.***

Reviewer: The decrease - to which extent?

Answer: ***Thank you for your comment. We have completed the information: "A similar result was reported by Kwankua et al., (2010) who found that neem oil caused a 400% increase in chromosomal aberrations in Allium cepa, that solidifies our findings that***

indicate the zein nanoparticles are promising carriers for neem oil, since they are able to decrease over the genotoxicity towards nontarget organisms", (p. 19, l. 403). Please verify at the revised version of the manuscript.

Reviewer: Fig. 4: What is 2-delta-delta-ct on the y-axis of subfig. A? You need a much better explanation of both the genes and the enzymes in the bacteria active in N cycling in the methods part. Why is Cu-containing nitrite reductase of interest, for instance? The conclusion from the genetic tests is not clear to me.

Answer: ***Thank you for your comment. We agree with the reviewer's analysis. Indeed, the way the results were presented made it difficult to analyze the data. The figure was arranged allowing a better evaluation and possible visualization of the non-occurrence of differences between the different samples used, since these are not significant. In order to provide further clarification, the soil analysis should be evaluated on the basis of the control sample that exists for each period evaluated. When using the $2^{-\Delta\Delta ct}$ calculation, the soil collected at the beginning of the test, called zero soil, is used as the basis, which is used to calculate the relative quantification, so that all the results are based on differences found in relation to the initial soil that is based on the value of 1 for each gene analyzed. It is also interesting to remember that the observed changes in the quantification of the bacteria responsible for the nitrogen cycle are constant even in control soil. For the analysis of the data it is necessary that there is a constant comparison with the control sample, otherwise the analysis of the results may not be accurate, thus invalidating the analysis of the study. The $2^{-\Delta\Delta CT}$ method was used to calculate gene levels with the Ct values determined from qPCR experiments. The data were normalized considering a sample control (soil zero) and a control gene (16sRNA). To calculation of $2^{-\Delta\Delta CT}$ is based on $\Delta\Delta CT$ ($\Delta_{CT\_sample} - \Delta_{CT\_control}$) and Δ_{CT} ($\Delta_{CT\_alvo} - \Delta_{CT\_reference}$) (Yuan et al., 2008; Rao et al., 2013).***

Yuan JS, Wang D, Stewart CN Jr. Statistical methods for efficiency adjusted real-time PCR quantification. Biotechnol J. 2008 Jan;3(1):112-23.

Rao X, Huang X, Zhou Z, Lin X. An improvement of the 2ˆ(-delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. Biostat Bioinforma Biomath. 2013 Aug;3(3):71-85.

Reviewer: it says that the neem-oil loaded zein particles could cause release of more N2O. So this is negative, but how do we get to a conclusion on all this?

Answer: ***Thank you for your comment. Please disregard this statement.***

Reviewer: Fig. 5: What does the error bars represent?

Answer: ***Thank you for your comment. The error bars represent the standard deviations (now Figure 6). We have inserted this information in the figure caption.***

Reviewer: What is GST-4? Enzyme?

Answer: ***We apologize for the missing information. We have inserted this information in the revised version of the manuscript. GST is glutathione-S-transferase, (p.13, l. 275).***

Conclusions:

Reviewer: the manus may show that the neem-zein biopesticide is not that toxic to critical functions in soil, but the paper does not provide information that the neem oil is an efficient pesticide, so this sentence is not possible based on the results presented in the paper.

Answer: ***Thank you for your comment. We have changed the conclusions: "In this way, more studies must be carried out to guarantee the effects of this nanopesticide before its application in agriculture. It is therefore extremely important to recognize its mechanisms of action (for both, nanopesticides and neem), as well as their possible effects at the cellular level, their efficacy and their toxicity to target organisms". Please verify at the revised version of the manuscript, (p. 30, l. 587).***

Reviewer: In conclusion there are interesting data presented, but the authors need to spend much more efforts bringing the results into a context and to reach conclusions, and to explain why the do like they do. There is too much nerdy terms and unexplained relationships which the STOTEN readers would not be aware of. For the Results and Discussion part I would prefer to have the results presented before discussion in order not to mix up things.

Answer: ***Based on all your comments we have changed the whole manuscript in order to better present to STOTEN readers. The manuscript quality has improved a lot. We are glad and thankful for your valuable contribution to our manuscript.***

Reviewer #2:

Reviewer: The article is interesting, given the growing number of studies that are analyzing the effects of biopesticides (many of them of plant origin) on non-target organisms. Biopesticides can become a good alternative to synthetic pesticides, so any available information about their ecotoxicity or how to reduce their impacts, it is relevant. The article raises the possibility that the application of the pesticide of the Neem plant can be done with nanoparticles, which would allow to reduce the dose of application, increase its solubility and predictably this could suppose a decrease of the toxicity for the environment. For this purpose, in this study, neem oil-loaded zein nanoparticles are synthesized and its effect is studied on three non-target organisms comparing it with the effect of neem oil (and in some cases to the zein nanoparticles). In the case of *Allium cepa*, its effect on the mitosis of the seeds is studied; in the case of microorganisms of a soil, the effect on enzymes associated with the nitrogen cycle. Finally, survival, reproduction, body size and pharyngeal pumping were studied in *C. elegans*. The approach of the article, its justification and objectives are well formulated. However, I detect two main problems:

1. The first, affects Material and Methods and Results. Some sections of Material and Methods are confusing and some of the assays cannot be well understood because there is a lack of important information on aspects such as the number of replicates or number of subjects per samples in each case or specific details about how each one was carried out. I think it is more due to the lack of explanation than to a bad design of the experiments but the reality is that essential information is not provided and neither the results reflect it. The results do not specify if they are values of a sample or means of replicates. Therefore, the ecotoxicity results must demonstrate their consistency with information relative to the comparison between replicates or between repeated experiments through standard deviation analysis or confidence limits.

2. The second point, affects the Discussion of results. The Discussion has been interspersed with the results but is poorly developed. It explains well other cases that support the results, but it misses a somewhat deeper interpretation of the results of the authors more than simply a comparison with previous results. Sometimes, previous studies are explained in more detail than their own. It is also necessary a global reflection on the implication and relevance of the set of own results with respect to the objective that was raised, as well as the future implications of these results.

Therefore, I recommend that a major revision is warranted, since I believe that the results may be of interest but the article requires a thorough review of the way in which

material and methods and results are shown and to develop the discussion of the results obtained in this work. I explain my concerns in more detail below:

Answer: ***Thank you for your comments. We are happy for your valuable comments, which have greatly improved the quality of the manuscript. We have checked and review all points commented here, as well as the comments from reviewer # 1 and we expect the manuscript to have met the STOTEN.***

2.1. Major comments:

1. Materials and Methods. *Allium cepa* assay.

Reviewer: The details of how *A. cepa* seeds were exposed to the nanoformulations are not explained. Where? How was the exhibition made? How many seeds per trial? How many replicates?

Answer: ***Thank you for your comment. We have inserted the requested information (p. 9, l. 194). Please look at revised version of the manuscript.***

Reviewer: "This assay was performed 3 times (n = 3)". What essay: the preparation of the slides or the exposition of the seeds to the nanoparticles? "3", means that the same test was repeated three times or that each test had 3 replicates? And if so, why are not typical (or similar) deviation values shown in the results? Is there a negative control?

Answer: ***Thank you for your comments. We have clarified the text in order to better explain the replicates, standard deviation and negative control. Please verify at revised version of the manuscript, (p. 9, l. 199).***

Reviewer: The origin of the seeds of *A. cepa* must also be indicated.

Answer: ***Thank you for your comment. We have inserted the requested information "Allium cepa seeds were purchased from Isla seeds (Brazilian company)" in materials section, (p. 6, l. 129). Please look at the revised version of the manuscript.***

2. Material and Methods. Soil microbiota assay. Lines 189-196. All this paragraph is confusing and lacks much necessary information:

Reviewer: Please, indicate the origin of the soil

Answer: ***Thank you for your comment. The paragraph was review and rewritten: "Before use, the fertilized commercial soil, (14% organic matter, pH 6.80) was sieved using a 0.2 micrometer sieve, dried and separated into vessels with surface area of 0.025 m² each, and kept moist in a heated cabinet at 25 °C for 15 days. Two untreated soil samples were used as the negative control. Each treatment (zein nanoparticles,***

neem oil-loaded zein nanoparticles, and neem oil) were tested in duplicates (two vessels containing soil for each treatment). The applications of the formulations (using sprays) were based on the dosage and number of applications of neem oil employed in the field (three applications were performed on the same sample at 7-day intervals, using a 5 mg/mL solution at a dosage of 100 L/ha)", (p. 10, l. 220). Regarding soil information, it was obtained from a commercial fertilizer obtained from a local agricultural supplier, (p. 7, l. 130).

Reviewer: How many vessels are used? Each one had an area of 0.025 m2 or was this the final sum?

Answer: Thank you for your comment. We have added the requested information (p. 10, l. 222). Please look at the revised version of the manuscript.

Reviewer: The negative control was unique or there were replicates? How many?

Answer: Thank you for your comment. We have inserted the information about the control. Please look at the revised version of the manuscript, (p. 10, l. 223).

Reviewer: Another sample was exposed to the treatments: How many replicates?

Answer: Thank you for your comment. We have inserted the requested information. Please look at the revised version of the manuscript, (p. 10, l. 224).

Reviewer: Although later (in the result section) it can be seen clearer, it is necessary to specify here that the three applications are made with intervals of 7 days on the same sample.

Answer: Thank you for your comment. We have added the requested information. Please look at the revised version of the manuscript, (p. 11, l. 228).

3. Material and Methods. *C. elegans* assay. Again more information is needed.

Reviewer: * Line 220. It should be clarified why two different strains are used and for what purpose

Answer: Thank you for your comment. We have clarified the requested information, (p. 12, l. 253): "C. elegans trains N2 (wild type, established as valuable experimental model due to the high level of genetic homology with humans, fast life cycle, easy maintenance and handling) and CL2166 (genetically equal to wildtype and tagged to green fluorescent protein, GFP, fused to the promoter of the detoxifying enzyme glutathione- S- transferase-4) were maintained in plates containing NGM (nematode

growth media) enriched with salts and seeded with the bacterium E. coli OP50, at 20 °C". Please verify at the revised version of the manuscript.

Reviewer: It is not clear how many replicates were used for each concentration, what was the negative control, how many *C. elegans* there were per replicate and how many times the assay was repeated?

Answer: Thank you for your comments. We have inserted the requested information about the C. elegans amount, replicate and assay repetitions: "…using 1500 worms per replicate (per microtube), (…) Concentrations were tested in duplicates, in every experiment using C. elegans, a procedure that was repeated in three independent experiments (in different days and different batch of worms)". Please look at the revised version of the manuscript, (p. 12, l. 267).

Reviewer: In the survival test, how many *C. elegans* were studied? Replicates were used?

Answer: Thank you for your comment. Indeed this information is missing. We treated 1,500 worms per group, in each microtube, and we always use duplicates per independent experiments. Triplicates are used for brood size due to the loss of worms. We have inserted the requested information in the manuscript.

Reviewer: Reproduction was determined in triplicate: in plates? How many *C. elegans*?

Answer: Thank you for your question, this information was not clear in the manuscript. We have used triplicates per independent experiments for this specific assay, as we can lose worms. We have now clarified that in the manuscript, (p. 13, l. 282): "After scoring survival, reproduction was determined by counting the hatched larvae daily from three individual worms from each treatment transferred to NGM plate covered with E. coli OP50, during 4 reproductive days".

Reviewer: What were the different treatments?

Answer: Thank you for your comment. We have completed the information, (p. 13, l. 291). Please verify at the revised version of the manuscript.

Reviewer: 4. Material and Methods. Here it is said that the experiments were done in duplicate, but triplicates are previously mentioned. You must clarify when we refer to replicates and when the experiment is repeated more than once. It is also necessary that this be indicated for each experiment so that the results are clear enough and can be understood.

Answer: ***Thank you for your comment. We have corrected these mislead information, (p. 14, l. 299): "The molecular analysis of the effects of the nanoparticles on soil microbiota and C. elegans assays were performed in duplicate, and all other experiments were performed in triplicate, however, these replicates are considered one independent experiment and were repeated at least three times". Please revise at the revised version of the manuscript.***

Reviewer: 5. Results / Discussion. Only reference is made to previous studies, there is no discussion of the obtained results.

Answer: ***Thank you for your comment. We have inserted a discussion about the relationship between the work cited and our results. Please look at the revised version of the manuscript.***

Reviewer: 6. Figure 3. Lines 363-365 are results, they should not be included in the caption. However, it should be explained here if the bars are average values of replicates, the number of replicates and the standard deviation or confidence limits.

Answer: ***Thank you for your comment. We have corrected the figure captions as requested.***

Reviewer: 7. Results / Discussion. Only previous results are discussed. A discussion of the results obtained is missing.

Answer: ***Thank you for your comment. We have inserted a discussion about the relationship between the work cited and our results. Please look at the revised version of the manuscript.***

Reviewer: 8. Results / Discussion. The same as before (point 7)

Answer: ***Thank you for your comment. We have inserted the requested improvement. Please look at the revised version of the manuscript.***

Reviewer: 9. Figure 4. Lines 415-416 should go to results, not in the figure caption. As before, it should be indicated if the values of the bars are means of replicates, how many, confidence limits…

Answer: ***Thank you for your comment. We have changed the figure caption as requested.***

Reviewer: 10. Results / Discussion. Lines 432-434. I do no see in Figure 4 the assertion

that the soils treated with zein nanoparticles and loaded zein nanoparticles presented higher proportions of the two enzymes. Neither the assertion of Lines 435-437. Numerical values should be indicated for the proportions.

Answer: ***Thank you for your comment. We have inserted the data in percentage as requested. Please look at the revised version of the manuscript.***

Reviewer: 11. Results / Discussion. The same as before (point 7)

Answer: ***Thank you for your comment. We have modified the discussion in order to explain our findings. Please verify at the revised version of the manuscript.***

Reviewer: 12. Results / Discussion. The same as before (point 7)

Answer: ***Thank you for your comment. We have improved the discussion. Please look at the revised version of the manuscript.***

Reviewer: 13. Figure 5. This graph shows deviation lines, but it is not indicated if they are SD or similar. Nor if the bars are average values of replicates.

Answer: ***Thank you for your comment. We have added this information, in figure caption 5: "Data are expressed as average of three independent experiments (n=3) normalized to % and the error bars represent the standard error". Please verify at the revised version of the manuscript.***

2.2. Minor comments:

Reviewer: 14. The pages must be numbered

Answer: ***Thank you for your comment. We added the page numbers.***

Reviewer: 15. Highlights.

* The first highlight should indicate for whom is less genotoxic

* The last highlight is not a result of the work.

* It would be appropriate to incorporate a new highlight that would talk about the synthesis of Neem oil-loaded zein nanoparticles. This is a result of this study and is also proposed in lines 109 and 110 of the introduction as an objective.

Answer: ***Thank you for your comment. We have changed the highlights as requested:***

- ***Zein nanoparticles have great potential to encapsulate neem oil***
- ***Neem oil-loaded zein nanoparticles is less genotoxic to A. cepa than neem oil***
- ***Biopesticide based on neem and zein nanoparticles did not change soil bacterias***

- ***Nanoencapsulation of neem nullified the toxicity in Caenorhabditis elegans model***

Reviewer: 16. Abstract. I think it's confusing to talk about "Zein nanoparticles" when in Line 28 only "neem oil-loaded zein nanoparticles" are mentioned. It should be noted that the tests are made with the two types of nanoparticles in addition to neem oil. On the other hand, I think that what should be highlighted in the abstract is the effect of the nanoparticles with neem oil that I understand are the object of study and that also showed a relative damage index lower Neem oil. This is what stands out, also in the first highlight.

Answer: ***Thank you for your comment. We agree with you. We have corrected the sentence. Please look at the revised version of the manuscript.***

Reviewer: 17. Introduction. "The relative damage index" is a parameter that must be explained (in the field of genotoxicity).

Answer: ***Thank you for your comment. We have explained the term in the materials and methods section, (p. 9, l. 202): "Calculations were made of the mitotic index (MI), the damage index (DI), and the relative index (RI) which are indicators of the presence of cytotoxic, mutagenic or carcinogenic potential agents in the environment". Please look at the revised version of the manuscript.***

Reviewer: 18. Introduction. This statement is not true. There are already numerous publications that show that biopesticides of plant origin (including essential oils) can be toxic to non-target organisms (Govindarajan and Benelli 2016, Kohler and Triebskorn 2013, Pino-Otin, et al. 2019, Shao and Zhang 2017).

Answer: ***Thank you for your comment. We have modified the sentence as requested (p. 3, l. 57): "They might be less toxic to nontarget organisms, such as humans, and have low impacts in the environment". Please look at the revised version of the manuscript.***

Reviewer: 19. Introduction.. After "... and acts as a repellent." A reference is needed.

Answer: Thank you for your comment. We have inserted a reference:

Campos, E.V.R., de Oliveira, J.L., Pascoli, M., de Lima, R., Fraceto, L.F., 2016. Neem Oil and Crop Protection: From Now to the Future. Frontiers in Plant Science 7. https://doi.org/10.3389/fpls.2016.01494

Reviewer: 20. Introduction. After "... Regulators, among others." A reference is needed

Answer: ***Thank you for your comment. We have inserted a reference as requested:***

Pascoli, M., Lopes-Oliveira, P.J., Fraceto, L.F., Seabra, A.B., Oliveira, H.C., 2018b. State of the art of polymeric nanoparticles as carrier systems with agricultural applications: a minireview. Energy, Ecology and Environment 3, 137–148. https://doi.org/10.1007/s40974-018-0090-2

Reviewer: 21. Introduction. The reason why these bioindicators are selected to test the potential toxicity of the neem oil nanoparticles base of zein and not others, must be specified. What do they have in common? What information will they provide?

Answer: ***Thank you for your comment. We have explained the importance of the bioindicators in the introduction section (p. 6, l. 116): "The choice was because they are model organisms, all are used in the research of toxicity of materials making possible a broad investigation of the possible action of zein nanoparticles loaded with neem oil, since they are in different classes of organisms (plant, nematodes and microorganisms) that can come into contact with this new biopesticide in the crops". Please look at the revised version of the manuscript.***

Reviewer: 22. Introduction. In the introduction the novelty and originality of the objectives that arise, should be commented. Have these nanoparticles been synthesized in other studies? Is it the first time that its toxicity is studied? Is it the first time that is done with these bioindicators ...? And if it has been done previously, indicate what novel aspects is what the study contributes. All this, with the necessary references and / or explaining those mentioned in lines 107 and 108.

Answer: ***Thank you for your comment. We have inserted the requested information in the revised version of this manuscript in order to clarify the novelty of our manuscript, (p. 6, l. 112): "Given this background, the innovation of this study was to develop neem oil-loaded zein nanoparticles. In addition to preparation and characterization of the nanocarriers, using the novel safe by design strategy their potential toxicity was evaluated by investigating their effects on nontarget organisms (Allium cepa, nitrogen cycle bacteria, and Caenorhabditis elegans)".***

Reviewer: 23. Material and Methods. It is stated that the concentration used in agriculture for neem oil is 5 mg / mL. A reference or justification of this data, is needed.

Answer: ***Thank you for your comment. We have inserted the requested information, (p. 8, l. 160): "This concentration was chosen since in agriculture, neem oil is used at concentrations of between 4 and 6 mg/mL as recommended by the manufacturer UPL Brazil". Please look at the revised version of the manuscript.***

Reviewer: 24. Material and Methods. A single concentration of nanoparticles (5mg/mL) was used in the study. However, for an adequate characterization of the toxicity of a product it is convenient to make a dose-response curve with several concentrations above and below it. This allows us to detect the concentrations in which effects begin to be seen. It cannot be ruled out, for example, that there may be an accumulation of this substance if it is applied periodically on the ground. And if this is not done, at least it should be discussed in the discussion as possible future studies.

Answer: ***Thank you for your comments. We have clarified this choice the text. Basically in this assay, we subjected the roots of Allium cepa to contact by submersion in our formulation without dilution (which shows the concentration of active compound neem oil at 5mg/mL). Thus, we evaluated the maximum contact scenario that the organism could get with the nanopesticide. This model organism does not allow an assay to be carried out with the 3 applications of the compound at interval of 7 days between them due to the rapid growth of the root.***

Reviewer: 25. Material and Methods. It should be clarified what is the final effect that wants to be measured with these different indices.

Answer: ***Thank you for your comment. We have explained what these indices indicate, (p. 10, l. 203): Calculations were made of the mitotic index (MI), the damage index (DI), and the relative index (RI) which are indicators of the presence of cytotoxic, mutagenic or carcinogenic potential agents in the environment. MI was calculated by dividing the number of cells in division by the total number of cells. DI was calculated by dividing the number of cells showing DNA alterations during the mitosis by the total number of cells in division. RI was calculated by dividing the values obtained for the treatments by the values for the negative control". Please look at the revised version of the manuscript.***

Reviewer: 26. Material and Methods. The software version is advisable to indicate it.

Answer: ***Thank you for your comment. We have inserted the software version. Please look at the revised version of the manuscript, (p. 12, l. 250).***

Reviewer: 27. Conclusions. Lines 550-560 are part of the justification and presentation of this study. They should be located in the introduction.

Answer: ***Thank you for your comment. As requested by you and reviewer #1, we have***

changed the conclusions in order to fit better with this study, (p. 30, l. 580). Please look at the revised version of the manuscript.

Reviewer: 28. The conclusions should suggest the investigations that are necessary from now on to continue characterizing the ecotoxicity of these nanoparticles in the environment. ***Answer:*** ***Thank you for your comment. We rewrote the conclusion as requested (p. 30, l. 587). Please look at the revised version of the manuscript.***

NEEM OIL BASED NANOPESTICIDE AS AN ENVIRONMENTALLY-FRIENDLY FORMULATION FOR APPLICATIONS IN SUSTAINABLE AGRICULTURE: AN ECOTOXICOLOGICAL PERSPECTIVE

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Abstract

Sustainable agriculture encourages practices that present low risks to the environment and human health. To this end, zein (corn protein) can be used to develop nanocarrier systems capable of improving the physicochemical properties of biopesticides, reducing their possible toxicity. Neem oil extracted from the *Azadirachta indica* tree contains many active ingredients including azadirachtin, which is the active ingredient in multiple commercially available biopesticides. In this study, we describe the preparation and characterization of neem oil-loaded zein nanoparticles, together with evaluation of their toxicity towards nontarget organisms, using *Allium cepa*, soil nitrogen cycle microbiota, and *Caenorhabditis elegans* aiming to achieve the safer by design strategy. The spherical nanoparticles showed an average diameter of 278 ± 61.5 nm and a good stability during the experiments. In the toxicity assays with *A. cepa*, the neem oil-loaded zein nanoparticles mitigated the increase in the DNA relative damage index caused by the neem oil. Molecular genetic analysis of the soil nitrogen cycle microbiota revealed that neem oil-loaded zein nanoparticles did not change the number of genes which encode nitrogen-fixing enzymes and denitrifying enzymes. In *C. elegans*, the neem oil-loaded zein nanoparticles had no toxic effect, while neem oil interfered with pharyngeal pumping and GST-4 protein expression. This neem oil-loaded zein nanoparticles showed promising results in the toxicity studies, opening perspectives for its use in crop protection in organic agriculture.

Keyworks: Zein nanoparticle, nanopesticide, biopesticide, azadirachtin, safer by design.

1 Introduction

Biopesticides include essential oils which are complex mixtures of substances typically containing more than sixty volatile and lipophilic compounds derived from secondary metabolites in plants, involving terpenoids such as monoterpenes, sesquiterpenes, and phenols (Campos et al., 2018; Chellappandian et al., 2018). Essential oils can be extracted from the whole plant or from isolated parts in order to obtain higher concentrations of a specific compound. Since antiquity, essential oils have been used due to their repellent, insecticidal, fungicidal, nematicidal, and bactericidal activities. They are considered safer than synthetic pesticides, having been used for human consumption and as medicines for thousands of years. They might be less toxic to nontarget organisms, such as humans, and have low impacts in the environment. Therefore, essential oils are a promising option for substituting the synthetic pesticides used in agriculture (Benelli and Pavela, 2018; de Oliveira et al., 2018; Ponsankar et al., 2016). Neem oil, which is extracted from the Indian neem tree (*Azadirachta indica Juss*.), is valued worldwide for use in the areas of human health and pest control (Lokanadhan et al., n.d.). Neem oil contains more than 300 biologically active compounds, with the major constituents being triterpenes known as limonoids (Figure 1), the most important of which is azadirachtin (Chandramohan et al., 2016; Gupta et al., 2017; Nicoletti et al., 2012). Neem oil is effective against a wide range of pests, exhibiting a broad spectrum of action due to its systemic and transmembrane activities. It inhibits feeding, reduces ecdysone, motion, and flight activity, deregulates development, suppresses fertility and reproduction, and acts as a repellent (Campos et al., 2016). In addition, neem oil can act as a fertilizer, improving the

quality of soil for crop production, hence contributing to sustainable organic agriculture. However, its use in the field is limited by its short persistence in the environment (Kumar et al., 2018; Shah et al., 2017).

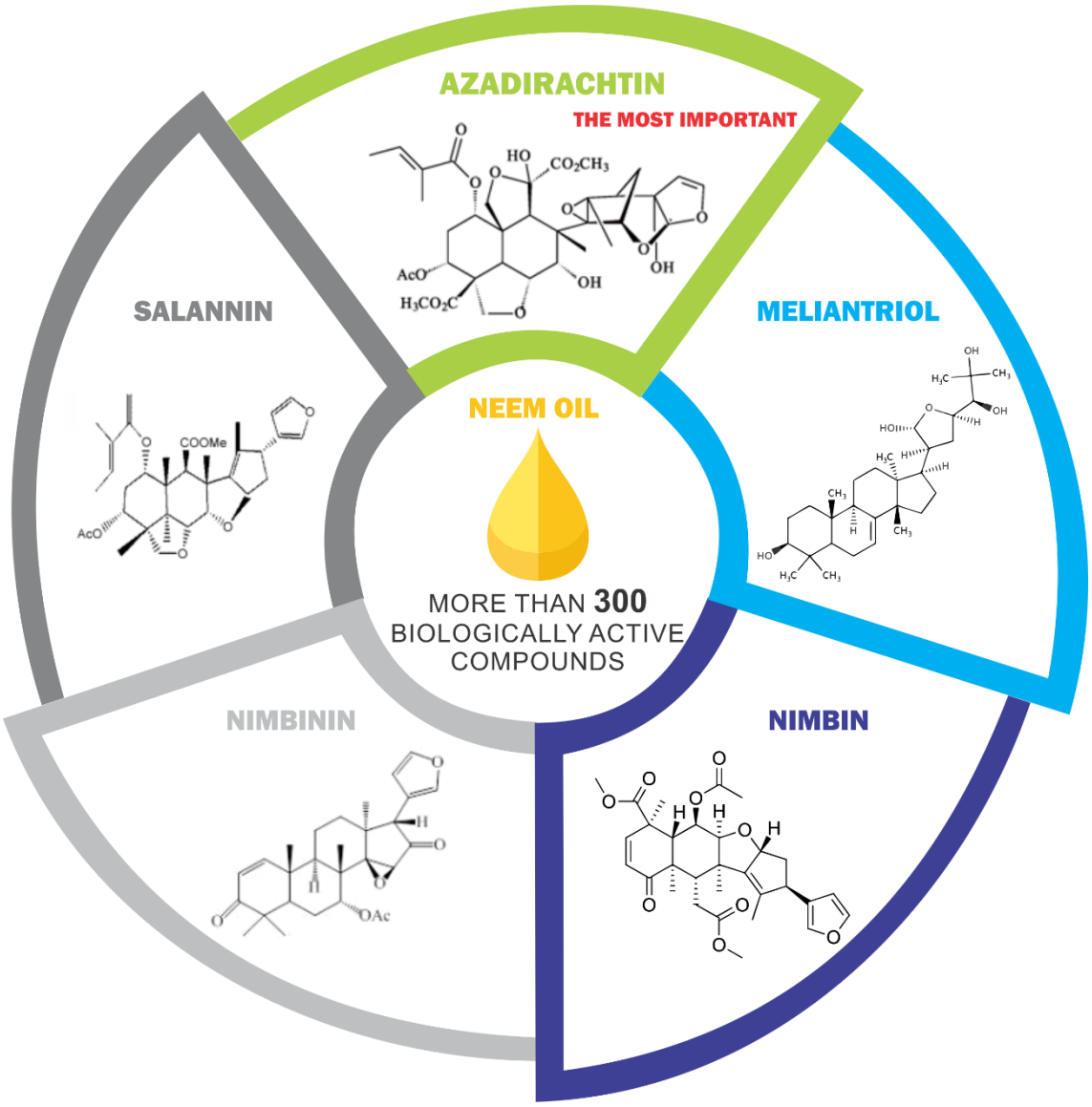


Figure 1. Chemical structures of the main active compounds of neem oil.

The application of nanotechnology in agriculture emphasizes the goal of the development of clean, safe, and environmentally friendly nanomaterials, using biocompatible and nontoxic solvents, biodegradable and biocompatible natural matrices, and energy-efficient and sustainable processes (Ashoka et al.,

2017; Bai et al., 2018; Saratale et al., 2018). Nanocarriers are capable of increasing the solubility of active compounds, while protecting them from volatilization and from degradation. The improvements in efficiency can generate better results, using lower doses and numbers of applications, hence contributing to the reduction of both environmental contamination and risks to human health (Campos et al., 2018; Choudhary et al., 2017; Oliveira et al., 2018). Different types of nanoparticle formulations are used in agriculture as herbicides, insecticides, fungicides, acaricides, fertilizers, and growth regulators, among others (Pascoli et al., 2018b). The use of polymeric nanoparticles as sustained release systems in agriculture has shown excellent results, due to their biocompatibility, biodegradability, and low toxicity (Campos et al., 2018; de Oliveira et al., 2018; de Oliveira et al., 2018b; Oliveira et al., 2018). Several studies have demonstrated the potential of formulations of biopesticides associated with polymeric nanoparticles (Campos et al., 2018; de Oliveira et al., 2018; de Oliveira et al., 2018b; Maruyama et al., 2016; Oliveira et al., 2018c; Pascoli et al., 2018). Zein nanoparticles meet the requirements of environmentally friendly nanotechnology, since zein is a naturally product that is biodegradable and biocompatible. It represents the main protein content of corn, is composed of lipophilic amino acid residues, and is not used for direct human consumption, due to its negative nitrogen balance and low water solubility (Paliwal and Palakurthi, 2014). Due to its high coating capacity, zein is used in the production of nanocarrier systems, employing a low toxicity solvent, such as ethanol, which is evaporated during the synthesis, hence causing no harm to the environment when the formulation is used in the field. Nanoparticles are capable of modifying the properties of the active substances that

encapsulated, so, it is necessary to re-screen the material in order to ensure its safe use. This involves assays using target and nontarget organisms, as well as evaluation of the behaviors of new formulations in the environment, aiming at regulation of the use of biopesticides associated with nanomaterials in crop protection (Campos et al., 2018; Dere et al., 2015; Fraceto et al., 2016; Pascoli et al., 2018; Sola et al., 2014).

Given this background, the innovation of this study was to develop neem oil-loaded zein nanoparticles. In addition to preparation and characterization of the nanocarriers, using the novel safe by design strategy their potential toxicity was evaluated by investigating their effects on nontarget organisms (*Allium cepa*, nitrogen cycle bacteria, and *Caenorhabditis elegans*). The choice was because they are model organisms, all are used in the research of toxicity of materials making possible a broad investigation of the possible action of zein nanoparticles loaded with neem oil, since they are in different classes of organisms (plant, nematodes and microorganisms) that can come into contact with this new biopesticide in the crops. The work opens perspectives for the use of nanobiopesticides based on neem oil in crop protection, contributing to sustainable organic agriculture as well as improved food safety.

2 Materials and Methods

2.2 Materials

Zein and Pluronic F-68 were purchased from Sigma-Aldrich. Neem oil (Azamax) containing 12g/mL of azadirachtin was acquired from UPL Brazil. Ethanol was obtained from Labsynth. *Allium cepa* seeds were purchased from

Isla seeds (Brazilian company). The soil used was obtained from a local agricultural supplier. *C. elegans* N2 (wild type) and CL2166 (dvIs19 [(gst-4p::gfp::nls] III) strains were purchased from the Caenorhabitis Genetics Center, Minnesota, USA. Other chemicals and solvents used were analytical grade and were purchased from local suppliers.

2.3 Preparation of neem oil-loaded zein nanoparticles

Zein nanoparticles were prepared by the environmentally-friendly antisolvent precipitation method (Hu and McClements, 2014) with some modifications (Pascoli et al., 2018a). The antisolvent method is a principle where the active compound is dissolved in a solvent; the solution is then injected with an antisolvent solution (in which the compound is insoluble). The compound precipitates as a consequence of the change of supersaturation caused by mixing the solution and the antisolvent solution. In this way, zein powder (2% w/v) was added to an aqueous solution of ethanol (85% v/v) and kept under magnetic stirring overnight. The zein solution was adjusted to pH 5.8, heat-treated at 75 °C for 15 min for protein densification, centrifuged, and filtered through a 0.45 µm membrane (Millipore) to remove insoluble particles. A 100 mg quantity of neem oil (in the form of an emulsifiable concentrate containing 12 g/L of azadirachtin) was added to the zein solution. An aqueous solution of Pluronic F-68 (a block-copolymer of ethylene oxide and propylene oxide ($C_3H_6O.C_2H_4O)_x$) extensively used as surfactant, wetting agents and emulsifiers) (2% v/v) was prepared and was adjusted to pH 4. The presence of Pluronic F-68 decreased the surface tension of the nanoparticles and maintain

the stability of the nanoparticles in suspension. Using a syringe, the zein solution was rapidly injected into the Pluronic solution, under stirring. The resulting colloidal dispersion was stirred for 12 h, at room temperature, in order to evaporate the ethanol, and water (pH 4.0) was then added to make up the original volume. The final concentration of neem oil in the nanoformulation was 5 mg/mL. This concentration was chosen since in agriculture, neem oil is used at concentrations of between 4 and 6 mg/mL as recommended by the manufacturer UPL Brazil.

2.4 Nanoparticle physicochemical characterization

The nanoparticle mean size distribution and polydispersity index (an indicator of the homo/heterogeneity of the size distribution of particles calculated by the square of the standard deviation divided by the square of the mean size) were determined by the dynamic light scattering technique (DLS). The zeta potential was measured by the microelectrophoresis method. These analyses were performed using a ZetaSizer Nano ZS90 system (Malvern Instruments, UK) at a fixed angle of 90° and 25 °C. The nanoparticle concentrations, size distributions, and Span values (an additional parameter to show the width of the size distribution calculated as Span = $(D_{90} - D_{10})/D_{50}$ being that D_{10}, D_{50} and D_{90} refer, respectively, to the diameters where 10%, 50% and 90% of the particle population) were also measured by nanoparticle tracking analysis (NTA), using a NanoSight LM 10 cell (green laser with wavelength of 532 nm) and a sCMOS camera, controlled by NanoSight v. 3.2 software (Grillo et al., 2012; 2014).

For these analyses, the samples were diluted 1000x in ultrapure water and in liquid medium (0.5% saline solution), at the highest concentration used in the *C. elegans* assay. Stability analyses were performed using sample aliquots removed after 0, 10, 20, 30, 40, 50, 60, 90, and 120 min of incubation in the saline solution. Each result was expressed as the average of three determinations.

Aliquots of zein nanoparticles and neem oil-loaded zein nanoparticles were collected and diluted in ultrapure water. Samples were dripped onto a silicon plate AFM sampler and kept in a desiccator for complete drying. The samples were analyzed using an Easy Scan 2 Basic BT02217 atomic force microscope (Nanosurf, Switzerland) operated in noncontact mode with TapAl-G cantilevers (BudgetSensors, Bulgaria) and tip voltage of 90 Hz. The acquired images were analyzed using Gwyddion software.

2.5 Toxicity studies

2.5.1 *Allium cepa* assay

Based on the procedure described by de Lima et al., (2010) germinated *A. cepa* seeds were exposed to the nanoformulations (zein nanoparticles, neem oil-loaded zein nanoparticles), neem oil (at a concentration of 5 mg/mL), Pluronic F-68 surfactant, and ultrapure water (negative control) in 10 mL glass beaker, in dark conditions for periods of 24 h. 10 roots were exposed to each treatment. The roots were fixed in Carnoy's reagent (methanol:acetic acid, 3:1 v/v), followed by acid hydrolysis with 1 mol/L HCl at 60 °C during 9 min. The roots were stained with Schiff reagent for 2 h. For preparation of the slides, the

meristematic region was crushed in one drop of 2% acetic-carmine, using a cover slip. Three roots exposed for each treatment were used to prepared the slides and all the cells were analyzed. This assay was repeated three independent times in different days. Calculations were made of the mitotic index (MI), the damage index (DI), and the relative index (RI) which are indicators of the presence of cytotoxic, mutagenic or carcinogenic potential agents in the environment. MI was calculated by dividing the number of cells in division by the total number of cells. DI was calculated by dividing the number of cells showing DNA alterations during the mitosis by the total number of cells in division. RI was calculated by dividing the values obtained for the treatments by the values for the negative control.

2.5.2 Molecular analysis of the effects of the nanoparticles on soil microbiota

We investigated the changes in all genes from the N cycle due the importance of this cycle for the nitrogen fixation in soil making the soil fertile by converting nitrogen into bioavailable forms that can be assimilated by living beings for production of organic molecules such as amino acids proteins and and nucleic acids. Therefore, the quantification of these functional genes involved in N transformation performed in this work improves our understanding of N-cycling soil microbiota responses to environmental impact (Hirsch and Mauchline, 2015; Fang et al., 2019).

Before use, the fertilized commercial soil, (14% organic matter, pH 6.80) was sieved using a 0.2 micrometer sieve, dried and separated into vessels with

surface area of 0.025 m^2 each, and kept moist in a heated cabinet at 25 °C for 15 days. Two untreated soil samples were used as the negative control. Each treatment (zein nanoparticles, neem oil-loaded zein nanoparticles, and neem oil) were tested in duplicates (two vessels containing soil for each treatment). The applications of the formulations (using sprays) were based on the dosage and number of applications of neem oil employed in the field (three applications were performed on the same sample at 7-day intervals, using a 5 mg/mL solution at a dosage of 100 L/ha).

The extraction of DNA from soil microorganisms was performed 7, 14, 21, and 30 days after the first application of the treatments, using a Power Soil DNA Isolation Kit (MoBio Laboratories). Quantification of the genetic material was performed by fluorescence, using a Qubit 3.0 fluorometer with the Qubit dsDNA BR Assay Kit (Invitrogen). All the samples were diluted to final concentrations of 1000 ng/mL.

Real-time polymerase chain reactions (qPCR) were performed for specific genes from nitrogen cycle bacteria: nifH (nitrogen fixation), nirK, nirS, narG, cnorB, and nosZ (denitrification). The bacterial 16S RNA gene was used as a reference. The reactions were performed using 1 µL of DNA sample, 12.5 µL of Planium SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen), 1 µL of each primer (sense and antisense), and sufficient ultrapure water to complete the final volume to 25 µL. The amplifications were conducted according to a procedure adapted from Jung et al., (2011) using a StepOne thermocycler (Applied Biosystems), with an initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 45 s, 60 °C for 45 s, and 72 °C for 45 s. The SYBR Green fluorescence emitted was measured at the end of each incubation at 72 °C.

The results were analyzed using relative quantification, with calculation of ΔΔCt ($2^{-\Delta\Delta Ct}$), employing 16S rRNA as the reference gene and the initial soil as the reference sample (Yuan et al., 2008). The calculations were performed using the StepOne Plus v2. 3 software of the equipment.

2.5.3 *Caenorhabditis elegans* assays

C. elegans trains N2 (wild type, established as valuable experimental model due to the high level of genetic homology with humans, fast life cycle, easy maintenance and handling) and CL2166 (genetically equal to wildtype and tagged to green fluorescent protein, GFP, fused to the promoter of the detoxifying enzyme glutathione- S- transferase-4) were maintained in plates containing NGM (nematode growth media) enriched with salts and seeded with the bacterium *E. coli* OP50, at 20 °C. The fertilized nematodes were synchronized by lysing them with a bleaching mixture (1% NaOCl, 0.25 M NaOH). The eggs obtained were washed with M9 buffer (0.02 M KH_2PO_4, 0.04 M Na_2HPO_4, 0.08 M NaCl, and 0.001 M $MgSO_4$) and were kept in plates containing M9 without bacteria, during 14 h, until the larvae hatched in stage L1.

Chronic exposure of the L1 worms to the negative control (0.5% NaCl) and the different formulations (zein nanoparticles, neem oil-loaded zein nanoparticles, and neem oil) was performed for 30 min with 0.05, 0.25, 0.5, and 0.75 mg/mL of the test material, using 1500 worms per replicate (per microtube), in a liquid medium (0.5% NaCl), with stirring to ensure contact of the nematodes with the treatments. Concentrations were tested in duplicates, in every experiment using *C. elegans*, a procedure that was repeated in three

independent experiments (in different days and different batch of worms). After exposure, the worms were placed with the treatment on NGM plates with *E. coli* OP50, and were kept at 20 °C for 48 h.

The wild type strain nematodes were evaluated in terms of their survival, reproduction, body size, and pharyngeal pumping. For GST-4 enzymatic expression, CL2166 strain that has GST-4 tagged to a GFP was used and the labeled xenobiotic detoxification protein was determined according to its fluorescence (Rathor et al., 2017).

For survival evaluation, 48 h after exposure a transparent grid was placed beneath the NGM plate and 18 quadrants were analyzed under a dissection microscope, obtaining a score according to the number of living animals. After scoring survival, reproduction was determined by counting the hatched larvae daily from three individual worms from each treatment transferred to NGM plate covered with *E. coli* OP50, during 4 reproductive days. Body size was evaluated by images acquired 48 h after the exposures, using an inverted microscope (MEDILUX MDL-INV-1) connected to a digital camera (SAMSUNG ST64). ImageJ software was used to measure the body lengths of 10 worms per group, in each experiment. Pharyngeal pumping was counted for 1 min using 10 worms submitted to each treatment, in order to assess the intake of the treatments. Individuals of the CL2166 transgenic strain were exposed to the different treatments (0.5% NaCl as negative control, zein nanoparticles, neem oil-loaded zein nanoparticles and neem oil) and were then transferred to microscope slides containing levamisole (1 mM) as an anesthetic. Images were acquired using an epifluorescence microscope (Nikon Eclipse 50i)

with a GFP filter (with excitation at 365 nm and emission at 420 nm), and the fluorescence was measured using ImageJ software.

2.6 Statistical analysis

The molecular analysis of the effects of the nanoparticles on soil microbiota and *C. elegans* assays were performed in duplicate, and all other experiments were performed in triplicate, however, these replicates are considered one independent experiment and were repeated at least three times, and the data were expressed as average of three independent experiments ± standard deviations, represented by error bars. Statistical analyses were performed with GraphPad Prism v. 6 software, using two-way ANOVA followed by the Tukey post-hoc test, at a significance level of $p < 0.05$.

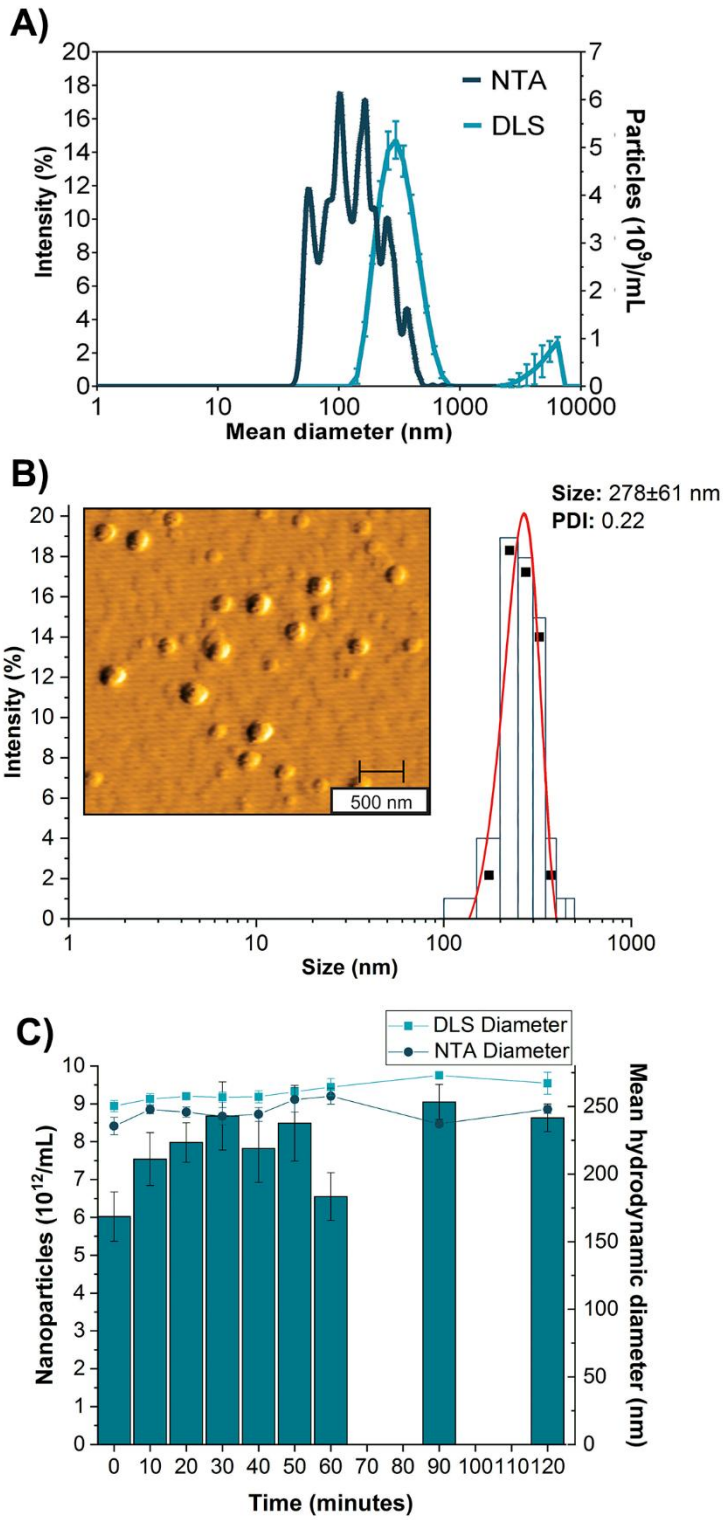
3 Results and Discussion

3.1 Physicochemical characterization of the neem oil-loaded zein nanoparticles

In order to characterize the neem oil-loaded zein nanoparticles we have measured the mean hydrodynamic diameters of the nanoparticles dispersed in water using DLS and NTA. The results obtained by DLS and NTA were 288 ± 6 and 198 ± 16 nm, respectively (Figure 2A). These results indicated that during the zein nanoparticles formation in presence the surfactant showed a range of size as described by other authors. Wu et al., (2012), using zein nanoparticles

containing thymol and carvacrol showed the mean size distribution by DLS in a range of 52 to 328 nm. In pursuance of size distribution, using atomic force microscopy (Figure 2B), the results showed that the neem oil-loaded zein nanoparticles were spherical, with a mean diameter of 278 ± 61 nm (with a concentration of nanoparticles/mL of 1.13 x 10^{12}). Using AFM, Chen et al., (2013) observed that zein nanoparticles were spherical, with sizes of around 100-200 nm. Cheng et al., (2019) reported the same size for spherical zein nanoparticles containing lutein. Oliveira et al., (2018) showed that zein nanoparticles containing geraniol and citronellal were spherical, with smooth surfaces and mean size of 90-250 nm.

However, Figure 2A and Figure 2B showed a broad size distribution curves, indicating that the particles were not monodisperse. This information was confirmed by the measurement of the polydispersity index. The value obtained for neem oil-loaded zein nanoparticles was 0.313 ± 0.005. Also, determined by NTA, the Span value calculated as described by Bender et al., (2012) was 1.3 ± 0.005. Based on both parameters a formulation is defined as monodisperse when the polydispersity index and span presented values lower than 0.2 and 1 respectively. However, in literature was described that nanoparticles prepared with matrices of natural origin (such as zein) was not monodisperse (Chuacharoen and Sabliov, 2016; Oliveira et al., 2018).



341

342 **Figure 2.** Characterization and stability of the neem oil-loaded zein
343 nanoparticles: A) Mean hydrodynamic size distribution curves obtained using
344 the DLS and NTA techniques applied to a suspension of the nanoparticles in
345 water; B) Micrograph and size distribution obtained using the AFM technique in

noncontact mode with TapAl-G cantilevers and tip voltage of 90 Hz. The image obtained was treated using Gwyddion software; C) Mean hydrodynamic size (lines) and concentration (bars) of the nanoparticles in saline medium (0.5% NaCl), as a function of time. The spherical nanoparticles showed an average diameter of 278 ± 61.5 nm with no aggregates of 288 ± 6 nm. The nanoparticles were stable over 120 minutes, under the experimental conditions. Data are expressed as average of three independent experiments (n=3) and the error bars represent the standard deviations. A significance level of $p < 0.05$ was adopted.

Also, in order to investigate the stability, we have been used the microelectrophoresis technique to measure the zeta potential of neem oil-loaded zein nanoparticles. The results showed that the zeta potential of this system was -36 ± 1 mV, which was close to the values characteristic of a stable formulation (+/-30 mV). Furthermore, in the case of this zein nanoparticles, during the preparation process we used Pluronic F-68 that provided steric hindrance, which was another factor that contributing to the stability of the zein nanoparticles in solution (Chuacharoen and Sabliov, 2016). Just in order to compare, negative zeta potential values (determined by microelectrophoresis) have been reported previously for zein nanoparticles loaded with 5-fluorouracil (-45 ± 0.3 mV) (Lai and Guo, 2011), zein nanoparticles loaded with thymol (from -34 to -40 mV) (Li et al., 2013), and zein nanoparticles stabilized with carrageenan (from -40 to -50 mV) (Cheng and Jones, 2017).

Moreover as we investigated the effect of the toxicity of these particles in models (such as *C. elegans*) that used saline medium (0.5% NaCl), the Figure 2C showed that they maintained the same mean hydrodynamic size, polydispersity, and concentration during 120 min (exposition time in *C. elegans*), while the zeta potential decreased significantly. The zeta potential was significantly lower in the saline environment, reaching -7.4 mV. This decreasing in zeta potential value in the presence of saline medium was reported in literature (de Oliveira et al., 2015; Grillo et al., 2014, 2012; Jacques et al., 2017) and explained due the greater ionic strength of the saline medium altered the ionic balance, leading to changes in the nanoparticle surface charge. It is important to pointed out that even with the low values of zeta potential (-7.4 mV) the particles kept stable in solution, showing in this way, the importance of the steric hindrance of Pluronic F-68 in neem oil-loaded zein nanoparticles.

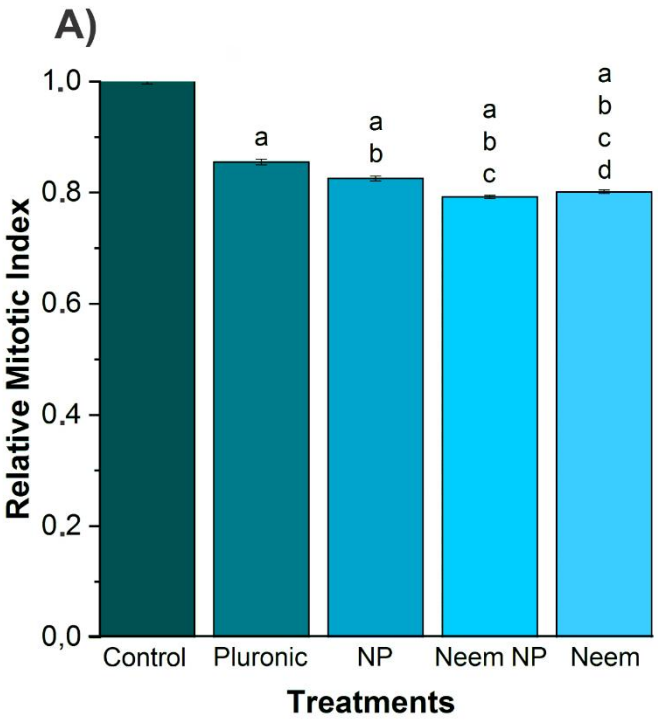
3.2 Toxicity studies

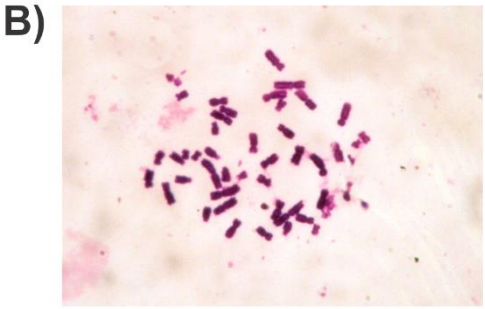
3.2.1 *Allium cepa* chromosome aberration assay

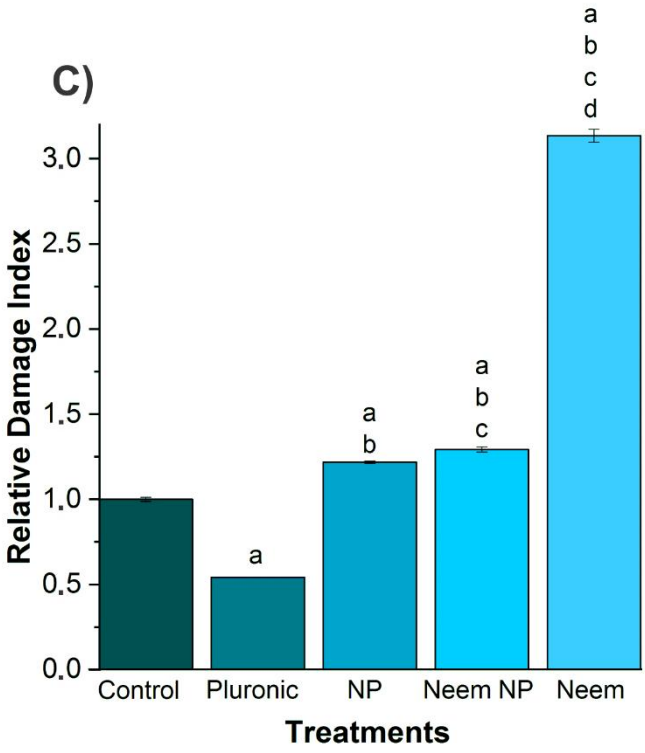
The results obtained in the *A. cepa* assay (Figure 3) showed significant differences between the control and all treatments, for both parameters evaluated (mitotic index and relative damage index). The treatments with Pluronic, zein nanoparticles, neem oil-loaded zein nanoparticles, and neem oil decreased the relative mitotic index (Figure 3A). Use of the neem oil-loaded zein nanoparticles resulted in a greater decrease in the mitotic index, compared to all other treatments. Then, our results confirm the ability of the treatments to interfer in cell mitosis, as reported by Kwankua et al., (2010) and Pasquoto-

393 Stigliani et al., (2017), that showed that neem oil extract caused a significant
394 decrease in the mitotic index of *Allium cepa* roots. The decreases in the mitotic
395 index caused by neem oil, together with the presence of chromosome
396 alteration, c-metaphase (Figure 3B), could be attributed to the azadirachtin
397 ability to interfere in mitosis usually a consequence of changes in the spindles,
398 similar to that seen with colchicine treatment, which prevents the formation of
399 spindle fibers, impairing the cell cycle progress (Soliman, 2001).

400 The results obtained for the relative damage index (Figure 3C) showed
401 that the surfactant used in production of the zein nanocarriers caused fewer
402 chromosomal changes, compared to the control. For the other treatments (zein
403 nanoparticles, neem oil-loaded zein nanoparticles, and neem oil), the
404 chromosomal changes were significantly increased. The zein nanoparticles and
405 the neem oil-loaded zein nanoparticles caused increases of 25% in the damage
406 index. However, it should be noted that neem oil alone (in the absence of
407 nanoparticles) increased the number of chromosomal aberrations by 200%. A
408 similar result was reported by Kwankua et al., (2010) who found that neem oil
409 caused a 400% increase in chromosomal aberrations in *Allium cepa*, that
410 solidifies our findings that indicate the zein nanoparticles are promising carriers
411 for neem oil, since they are able to decrease over the genotoxicity towards
412 nontarget organisms.


A)
Relative Mitotic Index
1.0
0.8
0.6
0.4
0.2
0,0
a
a
b
a
b
c
a
b
c
d
Control
Pluronic
NP
Neem NP
Neem
Treatments


B)


C)
Relative Damage Index
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
b
a
b
c
a
b
c
d
Control
Pluronic
NP
Neem NP
Neem
Treatments

413

Figure 3. Results of the *Allium cepa* aberration assay: A) Relative mitotic index values for the different treatments; B) Presence of c-metaphases in the neem oil treatment; C) Relative damage index of roots submitted to treatments for 24 h with Pluronic F-68 surfactant (280 mg/mL), zein nanoparticles (NP), neem oil-loaded zein nanoparticles (Neem NP), and neem oil (Neem), using neem oil concentrations of 5 mg/mL. Data are expressed as average of three independent experiments (n=3) and the error bars represent the standard deviations. Letters a, b, c, and d indicate a significant difference relative to the control, Pluronic, NP, and Neem NP, respectively. The significance level adopted was $p < 0.05$.

3.2.2 Effects of the nanoparticles on soil bacteria involved in the nitrogen cycle

Soil microbiota are considered soil quality parameters once they are responsible for regulating several important soil processes such as organic matter decomposition, degradation of organic pollutants and transformation of nutrients (Fang et al., 2019). The Nitrogen (N) cycle consists of several N transformation processes which are performed by bacteria that have specific genes to encode enzymes involved in each stage of the cycle including *nifH* (encoding nitrogenase reductase, nitrogen-fixing enzyme: reduction of nitrogen gas in ammonia), *amoA* (encoding ammonia monooxygenase, nitrification enzyme: conversion of ammonia to hydroxylamine), *haO* (encoding hydroxylamine oxidase, nitrification enzyme: oxidation of hydroxylamine to

nitrite) *narG* (encoding nitrate reductase, first two denitrification steps: reduction of nitrate to nitrite) *nirK* and *nirS* (encoding Cu-containing nitrite reductase and nitrite reductase, respectively, first two denitrification steps: catalyze the reduction of nitrite to nitric oxide), *cnorB* (encoding nitric oxide reductase, second two denitrification steps: reduces nitric oxide to nitrous oxide) and *nosZ* (encoding nitrous oxide reductase; second two denitrification steps: reduction of nitrous oxide to molecular nitrogen) (Hirsch and Mauchline, 2015; Ouyang et al., 2018).

In this context, soil analysis should be evaluated based on control sample that exists for each period evaluated. The percentages of nitrogen cycle genes (Figure 4B) show that after 7 days there is a small amount of bacteria that present the cnorB gene, but this also presents small amount in the control, indicating a homogeneity between the samples and the non-alteration of the genes compared to the control (possible observation in 5A and 5B, referring to 7 days after exposure). The results in time of 14 days after exposure it is possible to observe the presence of bacteria that have the *cnorB* gene, being the proportions similar to those found in control soil. The concentration of bacteria (time 14 days) presents a greater variation in relation to the control, but the existing proportion of each type of bacteria responsible for the maintenance of the nitrogen cycle is similar between the treatments and the control. It is also possible to observe an increase the *nifH* gene, responsible for the nitrogen fixation, especially in control sample, and in other treatments this still remains with a low relative quantification. After 21 days the quantification shows that in relation to the number of genes that participate in the cycle the treatments are matched in a smaller quantity to control, it is possible to observe a decrease in

the quantification of cnorB and a slight increase in the amount of *nirS* gene, responsible for the second step of denitrification. In the end experiment (after 30 days of exposure) the increase *nosZ* and *cnorB* genes indicates an increase in final steps of the nitrogen cycle, being observed in all the samples evaluated, including in control. It was possible to observe greater homogeneity between the samples in relation to both the quantification and the distribution have great similarity indicating that the soil, in relation to the bacteria responsible for the nitrogen cycle do not seem to suffer changes in the presence of the evaluated compounds.

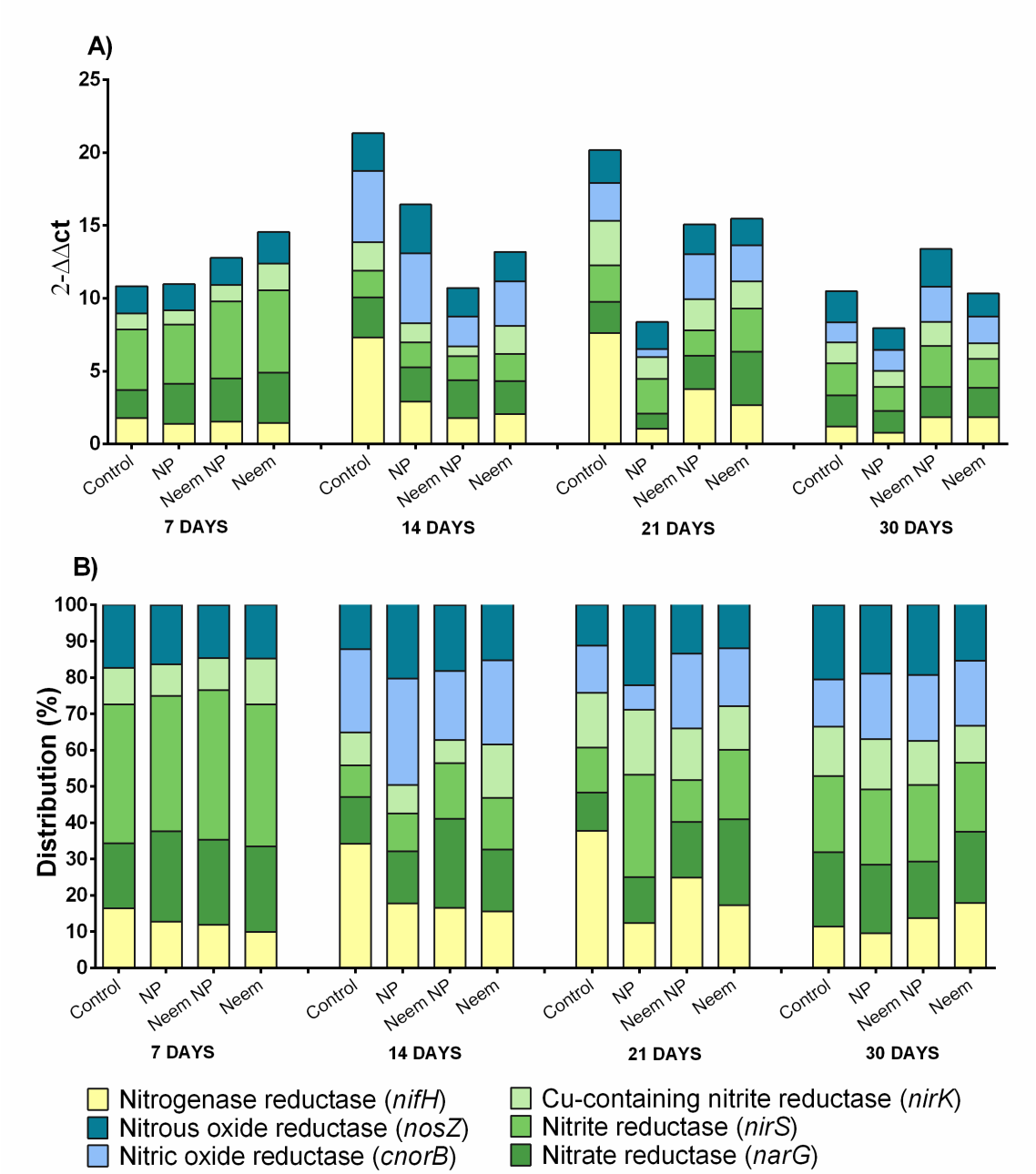


Figure 4. Molecular analysis of the genes of bacteria associated with the nitrogen cycle (*nifH, nosZ, cnorB, nirK, narG, and nirS*). A) Relative quantification of genes by qPCR and B) proportions of genes in the control soil and soils exposed to the zein nanoparticles (NP), neem oil-loaded zein nanoparticles (Neem NP), and neem oil (Neem), at 7, 14, 21, and 30 days after the initial treatment. Data are expressed as average of three independent experiments (n=3).

In according to our results, Pasquoto-Stigliani et al., (2017) investigated the behavior of bacteria involved in the nitrogen cycle when exposed to poly(ε-caprolactone) nanocapsules loaded with neem and showed that the differences in the proportions of these bacteria, compared to the control, varied during the experiment, with no significant difference after 300 days. Maruyama et al., (2016) evaluated atrazine and imazethapyr nanocapsules, showed lower effects on the bacterial profile associated with the nitrogen cycle, in the soil displayed in comparison with control. Yang et al., (2013) and Guilger et al., (2017) analyzed the effects of silver nanoparticles on nitrogen-fixing, nitrifying, and denitrifying bacteria, and found that the nitrifying bacteria were significantly affected, while the nitrogen-fixing and denitrifying organisms were not.

The use of molecular analysis of the genes of soil microbiota involved in the nitrogen cycle to investigate the possible toxicity of new materials, especially nanoparticles, is still recent and the literature is very limited. Further detailed studies are needed and are essential to ensure the safe use of newly emerging technologies.

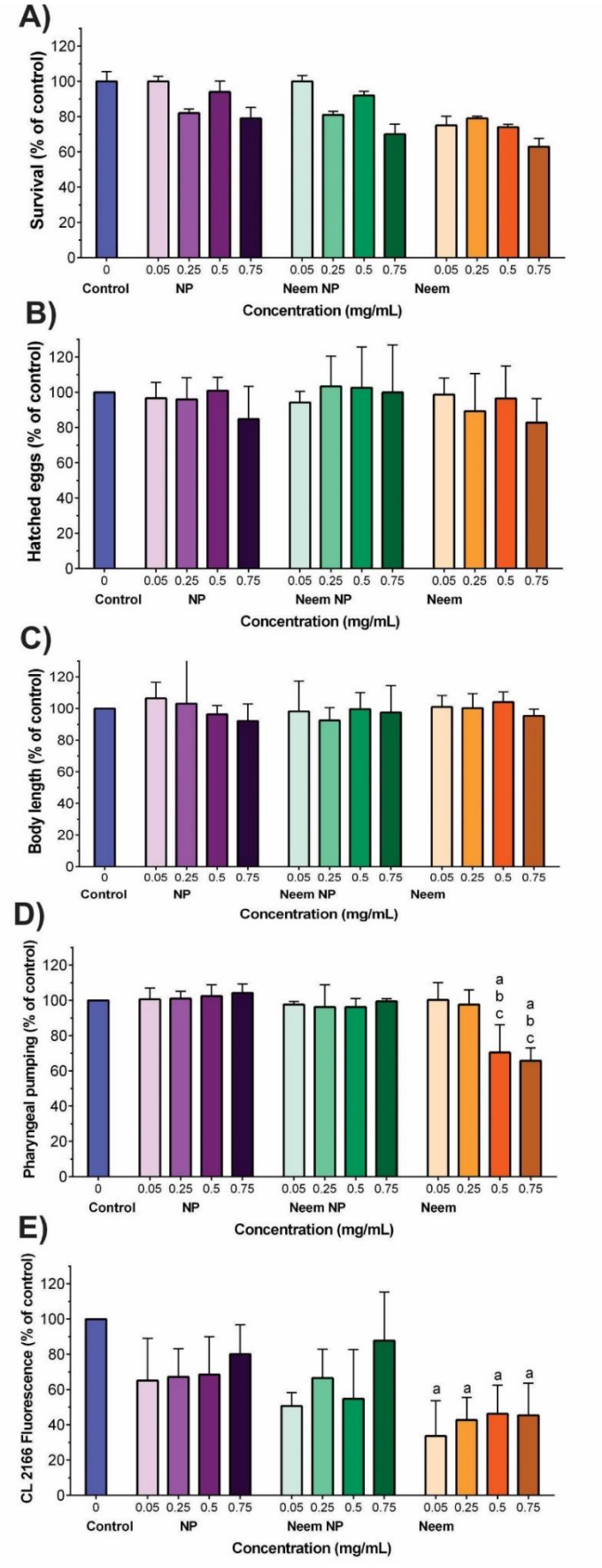
3.2.3 Effects of the formulations on the nematode *C. elegans*

The results of toxicity assays performed with *C. elegans* (Figure 5) showed that the survival, reproduction, and body length of the worms did not present significant differences after exposure to the zein nanoparticles, neem oil-loaded zein nanoparticles, and neem oil (using neem oil concentrations of 0.05, 0.25, 0.5, and 0.75 mg/mL). This lack of toxicity for a nontarget organism

504 is very promising for the advance of neem oil-loaded zein nanoparticle
505 research. These endpoints have been validated as the basic triad for safety
506 assessment. Even if mortality rate does not increase following exposure to a
507 toxicant, the reproductive system and the development of the worms are very
508 sensitive and may show tenuous signs of cellular damage (Tejeda and Olivero,
509 2016). That because during the larval stages, mitosis and meiosis are in fast
510 speed and it has been demonstrated that toxicants, pesticides included, can
511 disrupt cell cycle, elevate DNA double-strand break formation, activate
512 apoptosis and increase embryonic lethality (Shin et al., 2019). Of note, another
513 study evaluating the toxicity of zein nanoparticles loaded with the antidiabetic
514 drug glibenclamide (with an average size of 190 nm and a surface charge of
515 −37 mV) and showed that the formulation exerted significant hypolipidemic
516 activity in *C. elegans*, without causing any toxic effect (Lucio et al., 2017). In
517 contrast, nanoparticles toxicity can be detected in this animal model. Jacques et
518 al. (2017) have shown that different NPs interfered in the survival and vital
519 parameters of *C. elegans*. Solid lipid nanoparticles with or without atrazine and
520 simazine (sizes of 293 ± 3 and 288 ± 6 nm, respectively) and polymeric
521 nanoparticles with or without atrazine (sizes of 367 ± 13 and 305 ± 12 nm,
522 respectively) depicted dose-dependent increases of lethality and decreases of
523 *C. elegans* body length. Chitosan/tripolyphosphate nanoparticles, produced
524 using a natural biopolymer, with or without paraquat (sizes of 262 ± 14 and 246
525 ± 7 nm, respectively), caused increased mortality, but did not alter reproduction
526 or worm length in the surviving animals, therefore providing evidences that
527 natural biopolymers can be more compatible to nontarget organisms (Jacques
528 et al., 2017). Using another nontarget organism Deng et al., (2013) and Wang

et al., (2013) investigated the subacute, acute, and subchronic toxicity of neem oil towards mice and the only significant result was after 90 days, when the mice treated with neem oil at a dose of 1600 mg/kg/day presented several degrees of lesions in the testes, liver, and kidneys. However, the lesions were decreased or eliminated after a 30-day recovery period not demonstrating critical toxicity to the organism studied, in the same way that it happened in our research. (Wang et al., 2013).

In the present work, the pharyngeal pumping of the worms (Figure 5D) decreased significantly in the treatments with neem oil at concentrations of 0.5 and 0.75 mg/mL, compared to the control group, while the neem oil-loaded zein nanoparticles caused no significant alterations in the worms. Pharyngeal pumping is an indicator of a healthy worm and is mainly controlled by cholinergic and glutamatergic innervation, as well as by dopamine and serotonin (Raizen, 2012). Reduced pharyngeal pumping can lead to dietary restriction (Powolny et al., 2011). The results suggested that the zein nanoparticle formulation was able to decrease the toxicity of neem oil in this organism. These findings were in agreement with the work of Sanches Moraes et al., (2016) who reported the ability of polymeric nanocapsules to decrease the toxic effects of clozapine in *C. elegans*.


A)
Survival (% of control)
B)
Hatched eggs (% of control)
C)
Body length (% of control)
D)
Pharyngeal pumping (% of control)
E)
CL 2166 Fluorescence (% of control)
0
0.05 0.25 0.5 0.75
Control
NP
Neem NP
Neem
Concentration (mg/mL)

549

Figure 5. Toxicity assay using *C. elegans* exposed for 48 h to 0.05, 0.25, 0.5, and 0.75 mg/mL of zein nanoparticles (NP), neem oil-loaded zein nanoparticles (Neem NP), and neem oil (Neem). The wild type strain was evaluated for A) survival rate, B) brood size, C) body length, and D) pharyngeal pumping. The transgenic CL2166 strain was evaluated for E) fluorescence intensity, which indicates levels of GST-4 expression. The neem oil caused decreases in pharyngeal pumping and GST-4 expression. Data are expressed as average of three independent experiments (n=3) normalized to % and the error bars represent the standard deviation. Letters a, b, and c indicate a significant difference relative to the control, NP, and Neem NP, respectively. A significance level of $p < 0.05$ was considered.

As shown in Figure 5E, the treatments with neem oil at all concentrations caused significant decreases in fluorescence intensity, indicating reduced GST-4 expression, compared to the untreated animals. The zein nanoparticles and neem oil-loaded zein nanoparticles did not affect the GST-4 enzyme levels. GST-4 is involved in cellular detoxification and cell defense, so the reduction induced by neem oil could lead to oxidative stress and cell death (Lindblom and Dodd, 2006). The results showed that the neem oil decreased GST-4 levels in *C. elegans* by up to 66%, compared to the control, representing a threat since this protein is regulated by protective transcription factors, promoting longevity and resistance to stress (Rathor et al., 2015). It should be highlighted that the neem oil-loaded zein nanoparticles did not affect this parameter, providing further evidence that the new zein nanoparticle system was capable of reducing toxicity towards nontarget organisms (Figure 6). In previous studies, it has been

found that treatments using extracts of *Lavandula latifolia*, *Melissa officinalis*, *Origanum vulgare* (Gayoso et al., 2018), *Ginkgo biloba* (Kampkotter et al., 2007) and antioxidant compounds such as quercetin (Büchter et al., 2015) led to reduced GST-4 expression. It should be noted that the antioxidant capacity of pure neem oil has been demonstrated in several previous studies (Mattos et al., 2017; Rinaldi et al., 2017; Sithisarn et al., 2005).

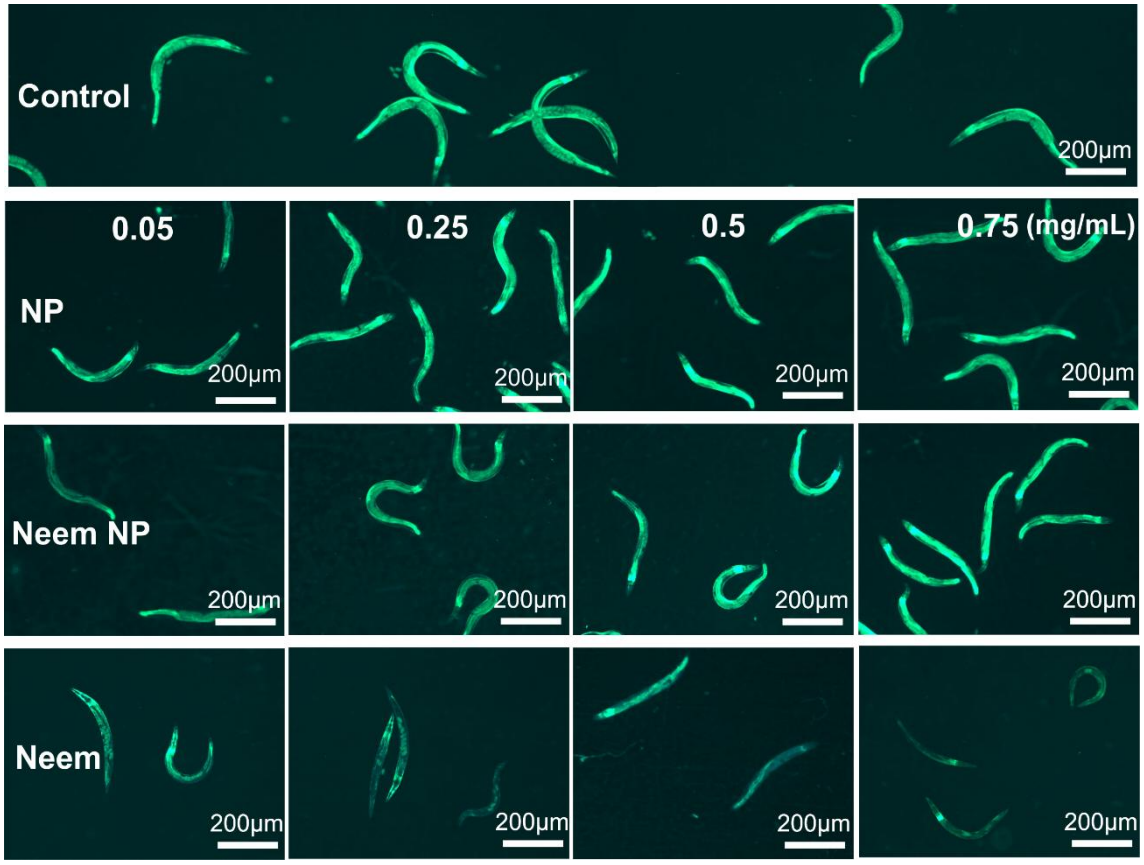


Figure 6. Images of the CL2166 transgenic strain exposed to the zein nanoparticles, neem oil-loaded zein nanoparticles, and neem oil (using neem oil concentrations of 0.05, 0.25, 0.5, and 0.75 mg/mL) for 48 hours acquired using an epifluorescence microscope (Nikon Eclipse 50i) with a GFP filter. It is possible to observe the decrease in the intensity of the fluorescence emitted by worms treated with neem oil at all concentrations used, indicating a decrease in GST-4 expression.

4 Conclusions

The neem oil-loaded zein nanoparticles developed in this work presented good colloidal characteristics and stability in different media. In the *A. cepa* analysis, the use of zein nanoparticles decreased the relative damage index caused by neem oil. In relation to the microbiota of the soil nitrogen cycle, the response to the neem oil-loaded zein nanoparticles was similar to that observed for control. In tests using C. elegans, the organism was susceptible to the effects of neem oil, while the nanoparticles did not show potential toxicity. In this way, more studies must be carried out to guarantee the effects of this nanopesticide before its application in agriculture. It is therefore extremely important to recognize its mechanisms of action (for both, nanopesticides and neem), as well as their possible effects at the cellular level, their efficacy and their toxicity to target organisms. A final consideration is that the definition of the risks associated with nanobiopesticides requires a multidisciplinary approach and that, in order to be sustainable and safe, it is crucial to ensure the awareness and use of correct management practices between farmers and the wider population.

Abbreviations

GST-4, glutathione S-transferase 4; CL2166, transgenic fluorescence *C. elegans* type; AFM, atomic force microscopy; qPCR, real-time polymerase chain reactions; L1, first *C. elegans* larval stage; GFP, green fluorescent protein; DLS, dynamic light scattering; NTA, nanoparticle tracking analysis; NP,

612 zein nanoparticles; Neem NP, neem oil-loaded zein nanoparticles; Neem, neem
613 oil.

614

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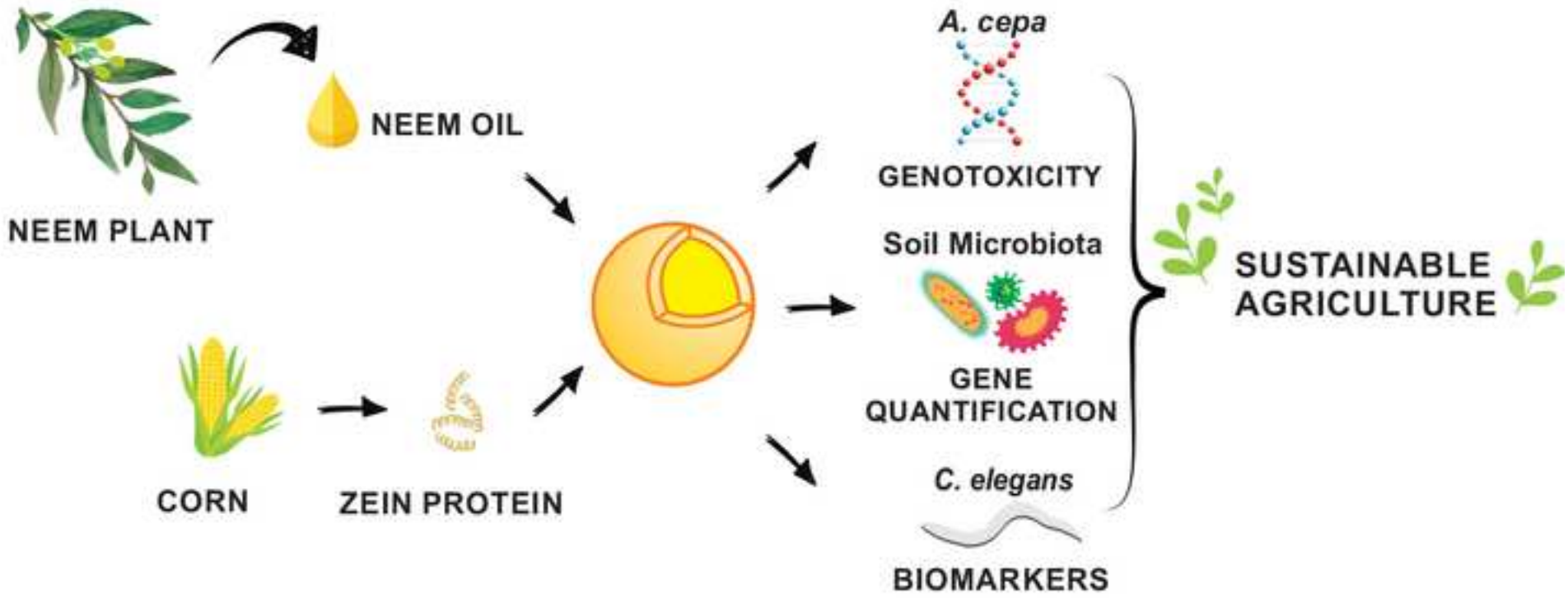
*Graphical Abstract



***Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)**

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Instituto de Ciência e Tecnologia
Câmpus de Sorocaba

Sorocaba, April 10th, 2019

Highlights

- Zein nanoparticles have great potential to encapsulate neem oil
- Neem oil-loaded zein nanoparticles is less genotoxic to *A. cepa* than neem oil
- Biopesticide based on neem and zein nanoparticles did not change soil bacterias
- Nanoencapsulation of neem nullified the toxicity in Caenorhabditis elegans model

NEEM OIL BASED NANOPESTICIDE AS AN ENVIRONMENTALLY-FRIENDLY FORMULATION FOR APPLICATIONS IN SUSTAINABLE AGRICULTURE: AN ECOTOXICOLOGICAL PERSPECTIVE

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Abstract

Sustainable agriculture encourages practices that present low risks to the environment and human health. To this end, zein (corn protein) can be used to develop nanocarrier systems capable of improving the physicochemical properties of biopesticides, reducing their possible toxicity. Neem oil extracted from the *Azadirachta indica* tree contains many active ingredients including azadirachtin, which is the active ingredient in multiple commercially available biopesticides. In this study, we describe the preparation and characterization of neem oil-loaded zein nanoparticles, together with evaluation of their toxicity towards nontarget organisms, using *Allium cepa*, soil nitrogen cycle microbiota, and *Caenorhabditis elegans* aiming to achieve the safer by design strategy. The spherical nanoparticles showed an average diameter of 278 ± 61.5 nm and a good stability during the experiments. In the toxicity assays with *A. cepa*, the neem oil-loaded zein nanoparticles mitigated the increase in the DNA relative damage index caused by the neem oil. Molecular genetic analysis of the soil nitrogen cycle microbiota revealed that neem oil-loaded zein nanoparticles did not change the number of genes which encode nitrogen-fixing enzymes and denitrifying enzymes. In *C. elegans*, the neem oil-loaded zein nanoparticles had no toxic effect, while neem oil interfered with pharyngeal pumping and GST-4 protein expression. This neem oil-loaded zein nanoparticles showed promising results in the toxicity studies, opening perspectives for its use in crop protection in organic agriculture.

Keyworks: Zein nanoparticle, nanopesticide, biopesticide, azadirachtin, safer by design.

1 Introduction

Biopesticides include essential oils which are complex mixtures of substances typically containing more than sixty volatile and lipophilic compounds derived from secondary metabolites in plants, involving terpenoids such as monoterpenes, sesquiterpenes, and phenols (Campos et al., 2018; Chellappandian et al., 2018). Essential oils can be extracted from the whole plant or from isolated parts in order to obtain higher concentrations of a specific compound. Since antiquity, essential oils have been used due to their repellent, insecticidal, fungicidal, nematicidal, and bactericidal activities. They are considered safer than synthetic pesticides, having been used for human consumption and as medicines for thousands of years. They might be less toxic to nontarget organisms, such as humans, and have low impacts in the environment. Therefore, essential oils are a promising option for substituting the synthetic pesticides used in agriculture (Benelli and Pavela, 2018; de Oliveira et al., 2018; Ponsankar et al., 2016). Neem oil, which is extracted from the Indian neem tree (*Azadirachta indica Juss*.), is valued worldwide for use in the areas of human health and pest control (Lokanadhan et al., n.d.). Neem oil contains more than 300 biologically active compounds, with the major constituents being triterpenes known as limonoids (Figure 1), the most important of which is azadirachtin (Chandramohan et al., 2016; Gupta et al., 2017; Nicoletti et al., 2012). Neem oil is effective against a wide range of pests, exhibiting a broad spectrum of action due to its systemic and transmembrane activities. It inhibits feeding, reduces ecdysone, motion, and flight activity, deregulates development, suppresses fertility and reproduction, and acts as a repellent (Campos et al., 2016). In addition, neem oil can act as a fertilizer, improving the

quality of soil for crop production, hence contributing to sustainable organic agriculture. However, its use in the field is limited by its short persistence in the environment (Kumar et al., 2018; Shah et al., 2017).

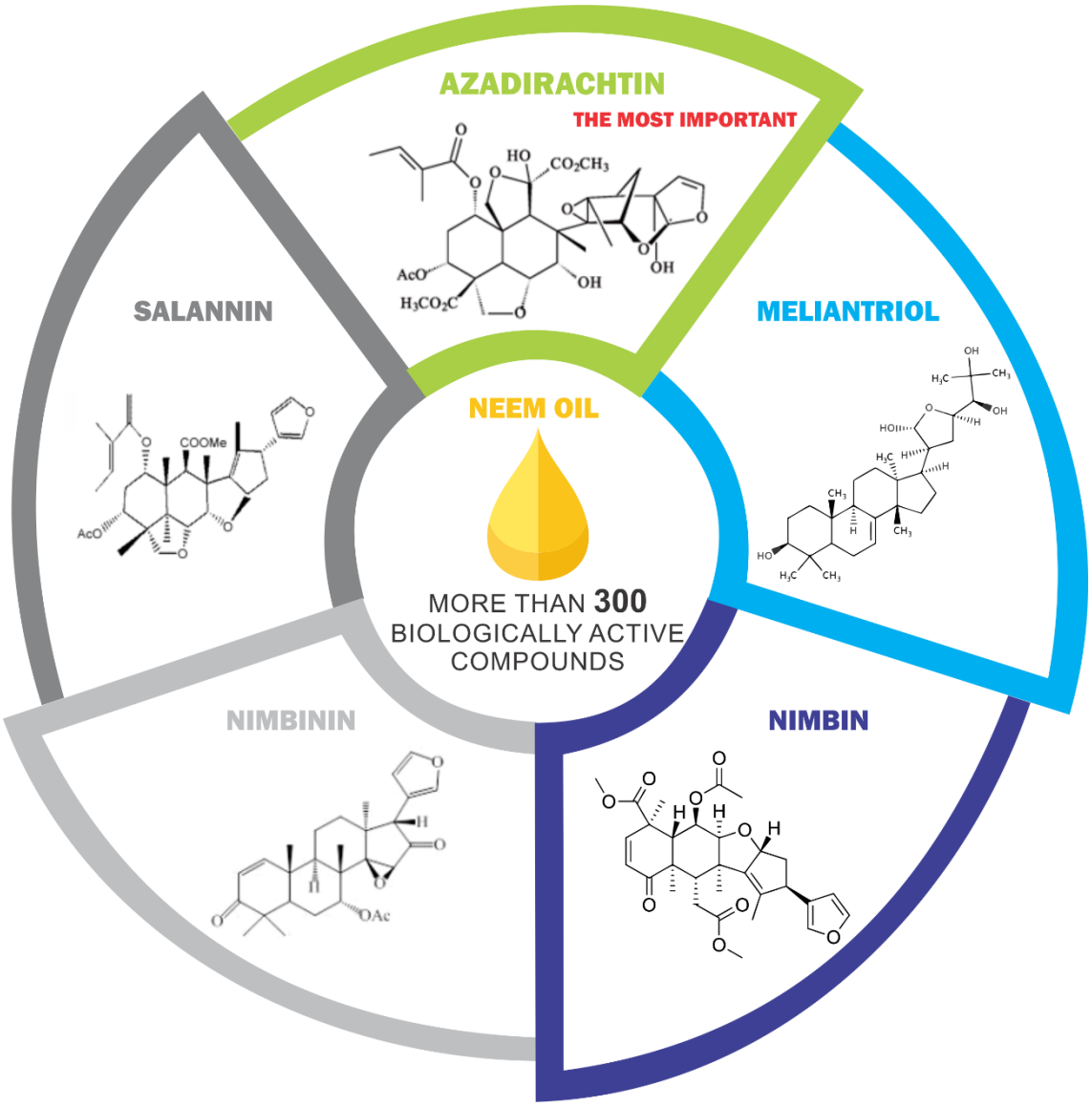


Figure 1. Chemical structures of the main active compounds of neem oil.

The application of nanotechnology in agriculture emphasizes the goal of the development of clean, safe, and environmentally friendly nanomaterials, using biocompatible and nontoxic solvents, biodegradable and biocompatible natural matrices, and energy-efficient and sustainable processes (Ashoka et al.,

2017; Bai et al., 2018; Saratale et al., 2018). Nanocarriers are capable of increasing the solubility of active compounds, while protecting them from volatilization and from degradation. The improvements in efficiency can generate better results, using lower doses and numbers of applications, hence contributing to the reduction of both environmental contamination and risks to human health (Campos et al., 2018; Choudhary et al., 2017; Oliveira et al., 2018). Different types of nanoparticle formulations are used in agriculture as herbicides, insecticides, fungicides, acaricides, fertilizers, and growth regulators, among others (Pascoli et al., 2018b). The use of polymeric nanoparticles as sustained release systems in agriculture has shown excellent results, due to their biocompatibility, biodegradability, and low toxicity (Campos et al., 2018; de Oliveira et al., 2018; de Oliveira et al., 2018b; Oliveira et al., 2018). Several studies have demonstrated the potential of formulations of biopesticides associated with polymeric nanoparticles (Campos et al., 2018; de Oliveira et al., 2018; de Oliveira et al., 2018b; Maruyama et al., 2016; Oliveira et al., 2018c; Pascoli et al., 2018). Zein nanoparticles meet the requirements of environmentally friendly nanotechnology, since zein is a naturally product that is biodegradable and biocompatible. It represents the main protein content of corn, is composed of lipophilic amino acid residues, and is not used for direct human consumption, due to its negative nitrogen balance and low water solubility (Paliwal and Palakurthi, 2014). Due to its high coating capacity, zein is used in the production of nanocarrier systems, employing a low toxicity solvent, such as ethanol, which is evaporated during the synthesis, hence causing no harm to the environment when the formulation is used in the field. Nanoparticles are capable of modifying the properties of the active substances that

encapsulated, so, it is necessary to re-screen the material in order to ensure its safe use. This involves assays using target and nontarget organisms, as well as evaluation of the behaviors of new formulations in the environment, aiming at regulation of the use of biopesticides associated with nanomaterials in crop protection (Campos et al., 2018; Dere et al., 2015; Fraceto et al., 2016; Pascoli et al., 2018; Sola et al., 2014).

Given this background, the innovation of this study was to develop neem oil-loaded zein nanoparticles. In addition to preparation and characterization of the nanocarriers, using the novel safe by design strategy their potential toxicity was evaluated by investigating their effects on nontarget organisms (*Allium cepa*, nitrogen cycle bacteria, and *Caenorhabditis elegans*). The choice was because they are model organisms, all are used in the research of toxicity of materials making possible a broad investigation of the possible action of zein nanoparticles loaded with neem oil, since they are in different classes of organisms (plant, nematodes and microorganisms) that can come into contact with this new biopesticide in the crops. The work opens perspectives for the use of nanobiopesticides based on neem oil in crop protection, contributing to sustainable organic agriculture as well as improved food safety.

2 Materials and Methods

2.2 Materials

Zein and Pluronic F-68 were purchased from Sigma-Aldrich. Neem oil (Azamax) containing 12g/mL of azadirachtin was acquired from UPL Brazil. Ethanol was obtained from Labsynth. *Allium cepa* seeds were purchased from

Isla seeds (Brazilian company). The soil used was obtained from a local agricultural supplier. *C. elegans* N2 (wild type) and CL2166 (dvIs19 [(gst-4p::gfp::nls] III) strains were purchased from the Caenorhabitis Genetics Center, Minnesota, USA. Other chemicals and solvents used were analytical grade and were purchased from local suppliers.

2.3 Preparation of neem oil-loaded zein nanoparticles

Zein nanoparticles were prepared by the environmentally-friendly antisolvent precipitation method (Hu and McClements, 2014) with some modifications (Pascoli et al., 2018a). The antisolvent method is a principle where the active compound is dissolved in a solvent; the solution is then injected with an antisolvent solution (in which the compound is insoluble). The compound precipitates as a consequence of the change of supersaturation caused by mixing the solution and the antisolvent solution. In this way, zein powder (2% w/v) was added to an aqueous solution of ethanol (85% v/v) and kept under magnetic stirring overnight. The zein solution was adjusted to pH 5.8, heat-treated at 75 °C for 15 min for protein densification, centrifuged, and filtered through a 0.45 µm membrane (Millipore) to remove insoluble particles. A 100 mg quantity of neem oil (in the form of an emulsifiable concentrate containing 12 g/L of azadirachtin) was added to the zein solution. An aqueous solution of Pluronic F-68 (a block-copolymer of ethylene oxide and propylene oxide ($C_3H_6O.C_2H_4O)_x$) extensively used as surfactant, wetting agents and emulsifiers) (2% v/v) was prepared and was adjusted to pH 4. The presence of Pluronic F-68 decreased the surface tension of the nanoparticles and maintain

the stability of the nanoparticles in suspension. Using a syringe, the zein solution was rapidly injected into the Pluronic solution, under stirring. The resulting colloidal dispersion was stirred for 12 h, at room temperature, in order to evaporate the ethanol, and water (pH 4.0) was then added to make up the original volume. The final concentration of neem oil in the nanoformulation was 5 mg/mL. This concentration was chosen since in agriculture, neem oil is used at concentrations of between 4 and 6 mg/mL as recommended by the manufacturer UPL Brazil.

2.4 Nanoparticle physicochemical characterization

The nanoparticle mean size distribution and polydispersity index (an indicator of the homo/heterogeneity of the size distribution of particles calculated by the square of the standard deviation divided by the square of the mean size) were determined by the dynamic light scattering technique (DLS). The zeta potential was measured by the microelectrophoresis method. These analyses were performed using a ZetaSizer Nano ZS90 system (Malvern Instruments, UK) at a fixed angle of 90° and 25 °C. The nanoparticle concentrations, size distributions, and Span values (an additional parameter to show the width of the size distribution calculated as Span = $(D_{90} - D_{10})/D_{50}$ being that D_{10}, D_{50} and D_{90} refer, respectively, to the diameters where 10%, 50% and 90% of the particle population) were also measured by nanoparticle tracking analysis (NTA), using a NanoSight LM 10 cell (green laser with wavelength of 532 nm) and a sCMOS camera, controlled by NanoSight v. 3.2 software (Grillo et al., 2012; 2014).

For these analyses, the samples were diluted 1000x in ultrapure water and in liquid medium (0.5% saline solution), at the highest concentration used in the *C. elegans* assay. Stability analyses were performed using sample aliquots removed after 0, 10, 20, 30, 40, 50, 60, 90, and 120 min of incubation in the saline solution. Each result was expressed as the average of three determinations.

Aliquots of zein nanoparticles and neem oil-loaded zein nanoparticles were collected and diluted in ultrapure water. Samples were dripped onto a silicon plate AFM sampler and kept in a desiccator for complete drying. The samples were analyzed using an Easy Scan 2 Basic BT02217 atomic force microscope (Nanosurf, Switzerland) operated in noncontact mode with TapAl-G cantilevers (BudgetSensors, Bulgaria) and tip voltage of 90 Hz. The acquired images were analyzed using Gwyddion software.

2.5 Toxicity studies

2.5.1 *Allium cepa* assay

Based on the procedure described by de Lima et al., (2010) germinated *A. cepa* seeds were exposed to the nanoformulations (zein nanoparticles, neem oil-loaded zein nanoparticles), neem oil (at a concentration of 5 mg/mL), Pluronic F-68 surfactant, and ultrapure water (negative control) in 10 mL glass beaker, in dark conditions for periods of 24 h. 10 roots were exposed to each treatment. The roots were fixed in Carnoy's reagent (methanol:acetic acid, 3:1 v/v), followed by acid hydrolysis with 1 mol/L HCl at 60 °C during 9 min. The roots were stained with Schiff reagent for 2 h. For preparation of the slides, the

meristematic region was crushed in one drop of 2% acetic-carmine, using a cover slip. Three roots exposed for each treatment were used to prepared the slides and all the cells were analyzed. This assay was repeated three independent times in different days. Calculations were made of the mitotic index (MI), the damage index (DI), and the relative index (RI) which are indicators of the presence of cytotoxic, mutagenic or carcinogenic potential agents in the environment. MI was calculated by dividing the number of cells in division by the total number of cells. DI was calculated by dividing the number of cells showing DNA alterations during the mitosis by the total number of cells in division. RI was calculated by dividing the values obtained for the treatments by the values for the negative control.

2.5.2 Molecular analysis of the effects of the nanoparticles on soil microbiota

We investigated the changes in all genes from the N cycle due the importance of this cycle for the nitrogen fixation in soil making the soil fertile by converting nitrogen into bioavailable forms that can be assimilated by living beings for production of organic molecules such as amino acids proteins and and nucleic acids. Therefore, the quantification of these functional genes involved in N transformation performed in this work improves our understanding of N-cycling soil microbiota responses to environmental impact (Hirsch and Mauchline, 2015; Fang et al., 2019).

Before use, the fertilized commercial soil, (14% organic matter, pH 6.80) was sieved using a 0.2 micrometer sieve, dried and separated into vessels with

surface area of 0.025 m^2 each, and kept moist in a heated cabinet at 25 °C for 15 days. Two untreated soil samples were used as the negative control. Each treatment (zein nanoparticles, neem oil-loaded zein nanoparticles, and neem oil) were tested in duplicates (two vessels containing soil for each treatment). The applications of the formulations (using sprays) were based on the dosage and number of applications of neem oil employed in the field (three applications were performed on the same sample at 7-day intervals, using a 5 mg/mL solution at a dosage of 100 L/ha).

The extraction of DNA from soil microorganisms was performed 7, 14, 21, and 30 days after the first application of the treatments, using a Power Soil DNA Isolation Kit (MoBio Laboratories). Quantification of the genetic material was performed by fluorescence, using a Qubit 3.0 fluorometer with the Qubit dsDNA BR Assay Kit (Invitrogen). All the samples were diluted to final concentrations of 1000 ng/mL.

Real-time polymerase chain reactions (qPCR) were performed for specific genes from nitrogen cycle bacteria: nifH (nitrogen fixation), nirK, nirS, narG, cnorB, and nosZ (denitrification). The bacterial 16S RNA gene was used as a reference. The reactions were performed using 1 µL of DNA sample, 12.5 µL of Planium SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen), 1 µL of each primer (sense and antisense), and sufficient ultrapure water to complete the final volume to 25 µL. The amplifications were conducted according to a procedure adapted from Jung et al., (2011) using a StepOne thermocycler (Applied Biosystems), with an initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 45 s, 60 °C for 45 s, and 72 °C for 45 s. The SYBR Green fluorescence emitted was measured at the end of each incubation at 72 °C.

The results were analyzed using relative quantification, with calculation of ΔΔCt ($2^{-\Delta\Delta Ct}$), employing 16S rRNA as the reference gene and the initial soil as the reference sample (Yuan et al., 2008). The calculations were performed using the StepOne Plus v2. 3 software of the equipment.

2.5.3 *Caenorhabditis elegans* assays

C. elegans trains N2 (wild type, established as valuable experimental model due to the high level of genetic homology with humans, fast life cycle, easy maintenance and handling) and CL2166 (genetically equal to wildtype and tagged to green fluorescent protein, GFP, fused to the promoter of the detoxifying enzyme glutathione- S- transferase-4) were maintained in plates containing NGM (nematode growth media) enriched with salts and seeded with the bacterium *E. coli* OP50, at 20 °C. The fertilized nematodes were synchronized by lysing them with a bleaching mixture (1% NaOCl, 0.25 M NaOH). The eggs obtained were washed with M9 buffer (0.02 M KH_2PO_4, 0.04 M Na_2HPO_4, 0.08 M NaCl, and 0.001 M $MgSO_4$) and were kept in plates containing M9 without bacteria, during 14 h, until the larvae hatched in stage L1.

Chronic exposure of the L1 worms to the negative control (0.5% NaCl) and the different formulations (zein nanoparticles, neem oil-loaded zein nanoparticles, and neem oil) was performed for 30 min with 0.05, 0.25, 0.5, and 0.75 mg/mL of the test material, using 1500 worms per replicate (per microtube), in a liquid medium (0.5% NaCl), with stirring to ensure contact of the nematodes with the treatments. Concentrations were tested in duplicates, in every experiment using *C. elegans*, a procedure that was repeated in three

independent experiments (in different days and different batch of worms). After exposure, the worms were placed with the treatment on NGM plates with *E. coli* OP50, and were kept at 20 °C for 48 h.

The wild type strain nematodes were evaluated in terms of their survival, reproduction, body size, and pharyngeal pumping. For GST-4 enzymatic expression, CL2166 strain that has GST-4 tagged to a GFP was used and the labeled xenobiotic detoxification protein was determined according to its fluorescence (Rathor et al., 2017).

For survival evaluation, 48 h after exposure a transparent grid was placed beneath the NGM plate and 18 quadrants were analyzed under a dissection microscope, obtaining a score according to the number of living animals. After scoring survival, reproduction was determined by counting the hatched larvae daily from three individual worms from each treatment transferred to NGM plate covered with *E. coli* OP50, during 4 reproductive days. Body size was evaluated by images acquired 48 h after the exposures, using an inverted microscope (MEDILUX MDL-INV-1) connected to a digital camera (SAMSUNG ST64). ImageJ software was used to measure the body lengths of 10 worms per group, in each experiment. Pharyngeal pumping was counted for 1 min using 10 worms submitted to each treatment, in order to assess the intake of the treatments. Individuals of the CL2166 transgenic strain were exposed to the different treatments (0.5% NaCl as negative control, zein nanoparticles, neem oil-loaded zein nanoparticles and neem oil) and were then transferred to microscope slides containing levamisole (1 mM) as an anesthetic. Images were acquired using an epifluorescence microscope (Nikon Eclipse 50i)

with a GFP filter (with excitation at 365 nm and emission at 420 nm), and the fluorescence was measured using ImageJ software.

2.6 Statistical analysis

The molecular analysis of the effects of the nanoparticles on soil microbiota and *C. elegans* assays were performed in duplicate, and all other experiments were performed in triplicate, however, these replicates are considered one independent experiment and were repeated at least three times, and the data were expressed as average of three independent experiments ± standard deviations, represented by error bars. Statistical analyses were performed with GraphPad Prism v. 6 software, using two-way ANOVA followed by the Tukey post-hoc test, at a significance level of $p < 0.05$.

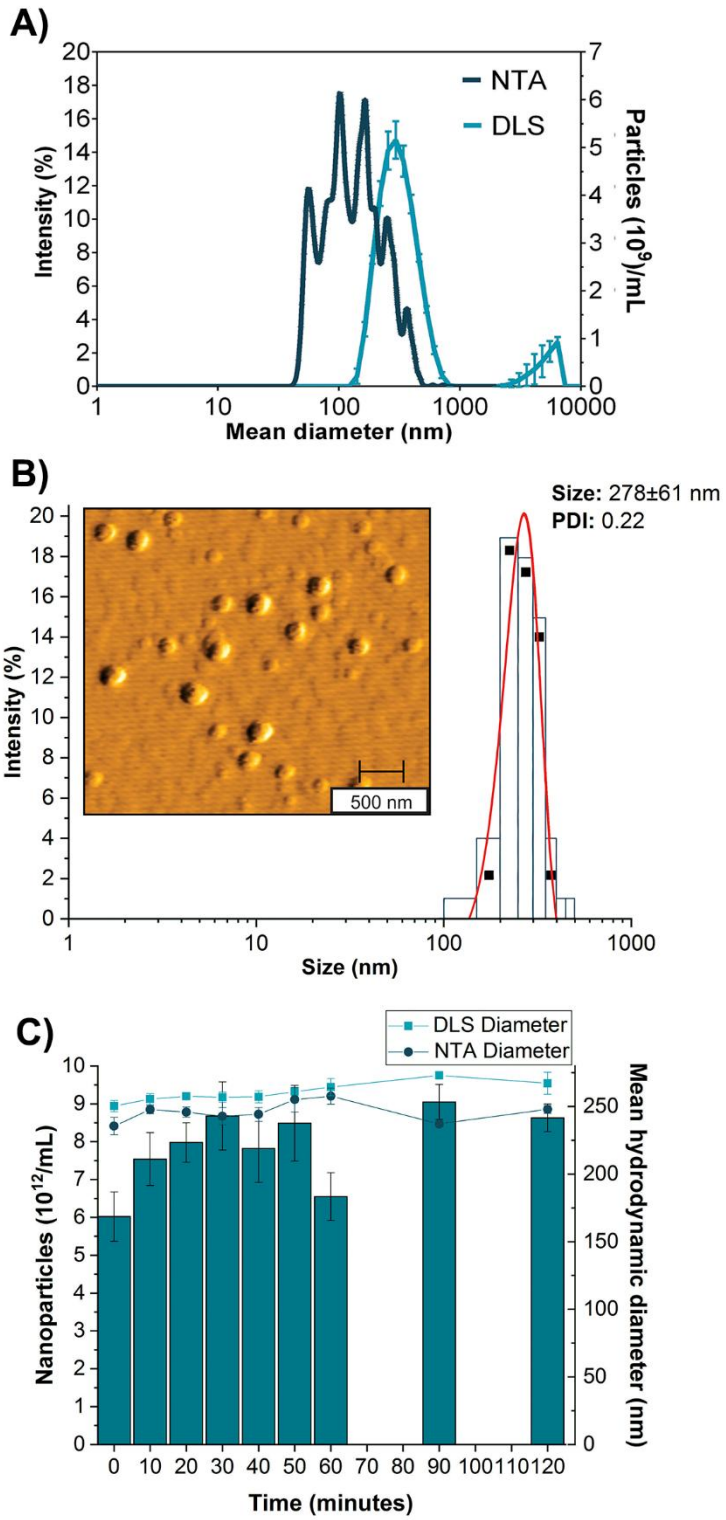
3 Results and Discussion

3.1 Physicochemical characterization of the neem oil-loaded zein nanoparticles

In order to characterize the neem oil-loaded zein nanoparticles we have measured the mean hydrodynamic diameters of the nanoparticles dispersed in water using DLS and NTA. The results obtained by DLS and NTA were 288 ± 6 and 198 ± 16 nm, respectively (Figure 2A). These results indicated that during the zein nanoparticles formation in presence the surfactant showed a range of size as described by other authors. Wu et al., (2012), using zein nanoparticles

containing thymol and carvacrol showed the mean size distribution by DLS in a range of 52 to 328 nm. In pursuance of size distribution, using atomic force microscopy (Figure 2B), the results showed that the neem oil-loaded zein nanoparticles were spherical, with a mean diameter of 278 ± 61 nm (with a concentration of nanoparticles/mL of 1.13 x 10^{12}). Using AFM, Chen et al., (2013) observed that zein nanoparticles were spherical, with sizes of around 100-200 nm. Cheng et al., (2019) reported the same size for spherical zein nanoparticles containing lutein. Oliveira et al., (2018) showed that zein nanoparticles containing geraniol and citronellal were spherical, with smooth surfaces and mean size of 90-250 nm.

However, Figure 2A and Figure 2B showed a broad size distribution curves, indicating that the particles were not monodisperse. This information was confirmed by the measurement of the polydispersity index. The value obtained for neem oil-loaded zein nanoparticles was 0.313 ± 0.005. Also, determined by NTA, the Span value calculated as described by Bender et al., (2012) was 1.3 ± 0.005. Based on both parameters a formulation is defined as monodisperse when the polydispersity index and span presented values lower than 0.2 and 1 respectively. However, in literature was described that nanoparticles prepared with matrices of natural origin (such as zein) was not monodisperse (Chuacharoen and Sabliov, 2016; Oliveira et al., 2018).



341

342 **Figure 2.** Characterization and stability of the neem oil-loaded zein
343 nanoparticles: A) Mean hydrodynamic size distribution curves obtained using
344 the DLS and NTA techniques applied to a suspension of the nanoparticles in
345 water; B) Micrograph and size distribution obtained using the AFM technique in

noncontact mode with TapAl-G cantilevers and tip voltage of 90 Hz. The image obtained was treated using Gwyddion software; C) Mean hydrodynamic size (lines) and concentration (bars) of the nanoparticles in saline medium (0.5% NaCl), as a function of time. The spherical nanoparticles showed an average diameter of 278 ± 61.5 nm with no aggregates of 288 ± 6 nm. The nanoparticles were stable over 120 minutes, under the experimental conditions. Data are expressed as average of three independent experiments (n=3) and the error bars represent the standard deviations. A significance level of $p < 0.05$ was adopted.

Also, in order to investigate the stability, we have been used the microelectrophoresis technique to measure the zeta potential of neem oil-loaded zein nanoparticles. The results showed that the zeta potential of this system was -36 ± 1 mV, which was close to the values characteristic of a stable formulation (+/-30 mV). Furthermore, in the case of this zein nanoparticles, during the preparation process we used Pluronic F-68 that provided steric hindrance, which was another factor that contributing to the stability of the zein nanoparticles in solution (Chuacharoen and Sabliov, 2016). Just in order to compare, negative zeta potential values (determined by microelectrophoresis) have been reported previously for zein nanoparticles loaded with 5-fluorouracil (-45 ± 0.3 mV) (Lai and Guo, 2011), zein nanoparticles loaded with thymol (from -34 to -40 mV) (Li et al., 2013), and zein nanoparticles stabilized with carrageenan (from -40 to -50 mV) (Cheng and Jones, 2017).

Moreover as we investigated the effect of the toxicity of these particles in models (such as *C. elegans*) that used saline medium (0.5% NaCl), the Figure 2C showed that they maintained the same mean hydrodynamic size, polydispersity, and concentration during 120 min (exposition time in *C. elegans*), while the zeta potential decreased significantly. The zeta potential was significantly lower in the saline environment, reaching -7.4 mV. This decreasing in zeta potential value in the presence of saline medium was reported in literature (de Oliveira et al., 2015; Grillo et al., 2014, 2012; Jacques et al., 2017) and explained due the greater ionic strength of the saline medium altered the ionic balance, leading to changes in the nanoparticle surface charge. It is important to pointed out that even with the low values of zeta potential (-7.4 mV) the particles kept stable in solution, showing in this way, the importance of the steric hindrance of Pluronic F-68 in neem oil-loaded zein nanoparticles.

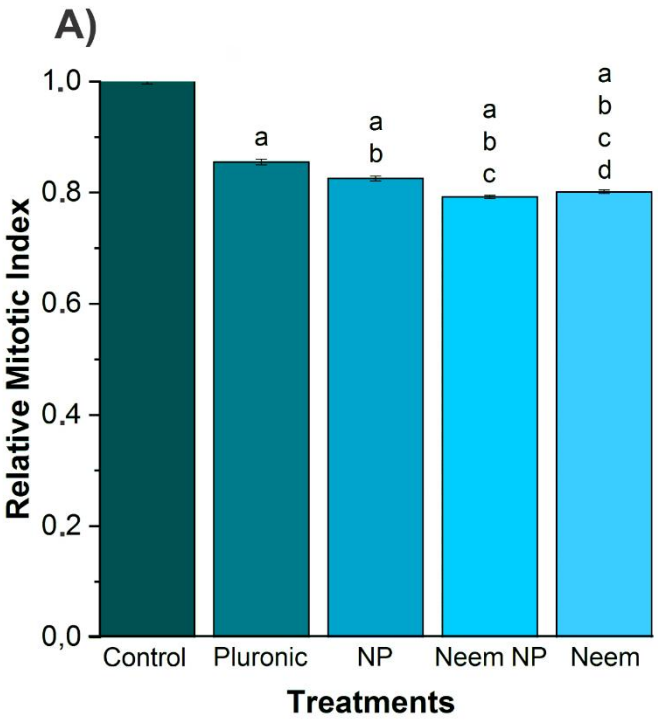
3.2 Toxicity studies

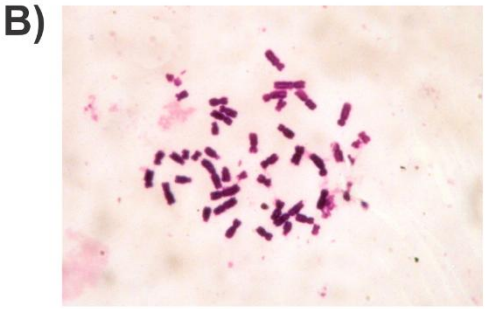
3.2.1 *Allium cepa* chromosome aberration assay

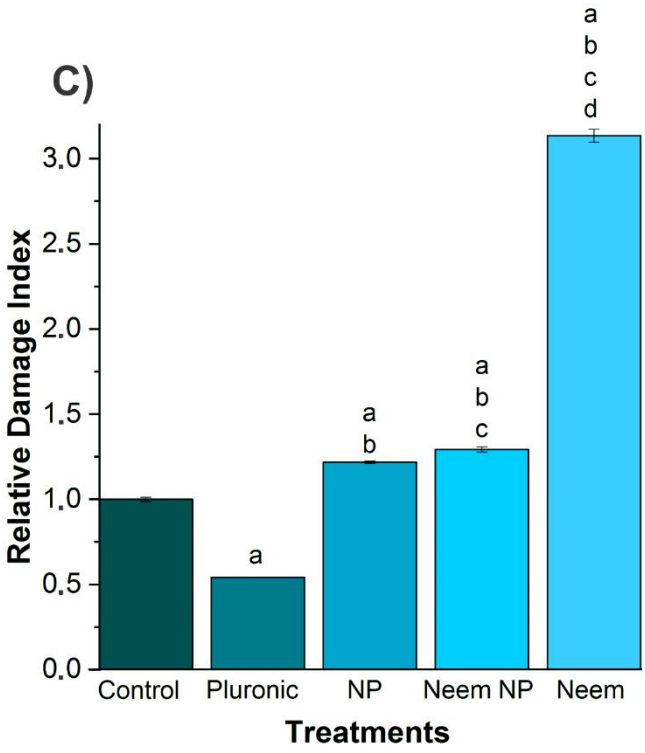
The results obtained in the *A. cepa* assay (Figure 3) showed significant differences between the control and all treatments, for both parameters evaluated (mitotic index and relative damage index). The treatments with Pluronic, zein nanoparticles, neem oil-loaded zein nanoparticles, and neem oil decreased the relative mitotic index (Figure 3A). Use of the neem oil-loaded zein nanoparticles resulted in a greater decrease in the mitotic index, compared to all other treatments. Then, our results confirm the ability of the treatments to interfer in cell mitosis, as reported by Kwankua et al., (2010) and Pasquoto-

393 Stigliani et al., (2017), that showed that neem oil extract caused a significant
394 decrease in the mitotic index of *Allium cepa* roots. The decreases in the mitotic
395 index caused by neem oil, together with the presence of chromosome
396 alteration, c-metaphase (Figure 3B), could be attributed to the azadirachtin
397 ability to interfere in mitosis usually a consequence of changes in the spindles,
398 similar to that seen with colchicine treatment, which prevents the formation of
399 spindle fibers, impairing the cell cycle progress (Soliman, 2001).

400 The results obtained for the relative damage index (Figure 3C) showed
401 that the surfactant used in production of the zein nanocarriers caused fewer
402 chromosomal changes, compared to the control. For the other treatments (zein
403 nanoparticles, neem oil-loaded zein nanoparticles, and neem oil), the
404 chromosomal changes were significantly increased. The zein nanoparticles and
405 the neem oil-loaded zein nanoparticles caused increases of 25% in the damage
406 index. However, it should be noted that neem oil alone (in the absence of
407 nanoparticles) increased the number of chromosomal aberrations by 200%. A
408 similar result was reported by Kwankua et al., (2010) who found that neem oil
409 caused a 400% increase in chromosomal aberrations in *Allium cepa*, that
410 solidifies our findings that indicate the zein nanoparticles are promising carriers
411 for neem oil, since they are able to decrease over the genotoxicity towards
412 nontarget organisms.


A)
Relative Mitotic Index
1.0
0.8
0.6
0.4
0.2
0,0
a
a
b
a
b
c
a
b
c
d
Control
Pluronic
NP
Neem NP
Neem
Treatments


B)


C)
Relative Damage Index
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
b
a
b
c
a
b
c
d
Control
Pluronic
NP
Neem NP
Neem
Treatments

413

Figure 3. Results of the *Allium cepa* aberration assay: A) Relative mitotic index values for the different treatments; B) Presence of c-metaphases in the neem oil treatment; C) Relative damage index of roots submitted to treatments for 24 h with Pluronic F-68 surfactant (280 mg/mL), zein nanoparticles (NP), neem oil-loaded zein nanoparticles (Neem NP), and neem oil (Neem), using neem oil concentrations of 5 mg/mL. Data are expressed as average of three independent experiments (n=3) and the error bars represent the standard deviations. Letters a, b, c, and d indicate a significant difference relative to the control, Pluronic, NP, and Neem NP, respectively. The significance level adopted was $p < 0.05$.

3.2.2 Effects of the nanoparticles on soil bacteria involved in the nitrogen cycle

Soil microbiota are considered soil quality parameters once they are responsible for regulating several important soil processes such as organic matter decomposition, degradation of organic pollutants and transformation of nutrients (Fang et al., 2019). The Nitrogen (N) cycle consists of several N transformation processes which are performed by bacteria that have specific genes to encode enzymes involved in each stage of the cycle including *nifH* (encoding nitrogenase reductase, nitrogen-fixing enzyme: reduction of nitrogen gas in ammonia), *amoA* (encoding ammonia monooxygenase, nitrification enzyme: conversion of ammonia to hydroxylamine), *haO* (encoding hydroxylamine oxidase, nitrification enzyme: oxidation of hydroxylamine to

438 nitrite) *narG* (encoding nitrate reductase, first two denitrification steps: reduction
439 of nitrate to nitrite) *nirK* and *nirS* (encoding Cu-containing nitrite reductase and
440 nitrite reductase, respectively, first two denitrification steps: catalyze the
441 reduction of nitrite to nitric oxide), *cnorB* (encoding nitric oxide reductase,
442 second two denitrification steps: reduces nitric oxide to nitrous oxide) and *nosZ*
443 (encoding nitrous oxide reductase; second two denitrification steps: reduction of
444 nitrous oxide to molecular nitrogen) (Hirsch and Mauchline, 2015; Ouyang et al.,
445 2018).

446 In this context, soil analysis should be evaluated based on control
447 sample that exists for each period evaluated. The percentages of nitrogen cycle
448 genes (Figure 4B) show that after 7 days there is a small amount of bacteria
449 that present the cnorB gene, but this also presents small amount in the control,
450 indicating a homogeneity between the samples and the non-alteration of the
451 genes compared to the control (possible observation in 5A and 5B, referring to
452 7 days after exposure). The results in time of 14 days after exposure it is
453 possible to observe the presence of bacteria that have the *cnorB* gene, being
454 the proportions similar to those found in control soil. The concentration of
455 bacteria (time 14 days) presents a greater variation in relation to the control, but
456 the existing proportion of each type of bacteria responsible for the maintenance
457 of the nitrogen cycle is similar between the treatments and the control. It is also
458 possible to observe an increase the *nifH* gene, responsible for the nitrogen
459 fixation, especially in control sample, and in other treatments this still remains
460 with a low relative quantification. After 21 days the quantification shows that in
461 relation to the number of genes that participate in the cycle the treatments are
462 matched in a smaller quantity to control, it is possible to observe a decrease in

the quantification of cnorB and a slight increase in the amount of *nirS* gene, responsible for the second step of denitrification. In the end experiment (after 30 days of exposure) the increase *nosZ* and *cnorB* genes indicates an increase in final steps of the nitrogen cycle, being observed in all the samples evaluated, including in control. It was possible to observe greater homogeneity between the samples in relation to both the quantification and the distribution have great similarity indicating that the soil, in relation to the bacteria responsible for the nitrogen cycle do not seem to suffer changes in the presence of the evaluated compounds.

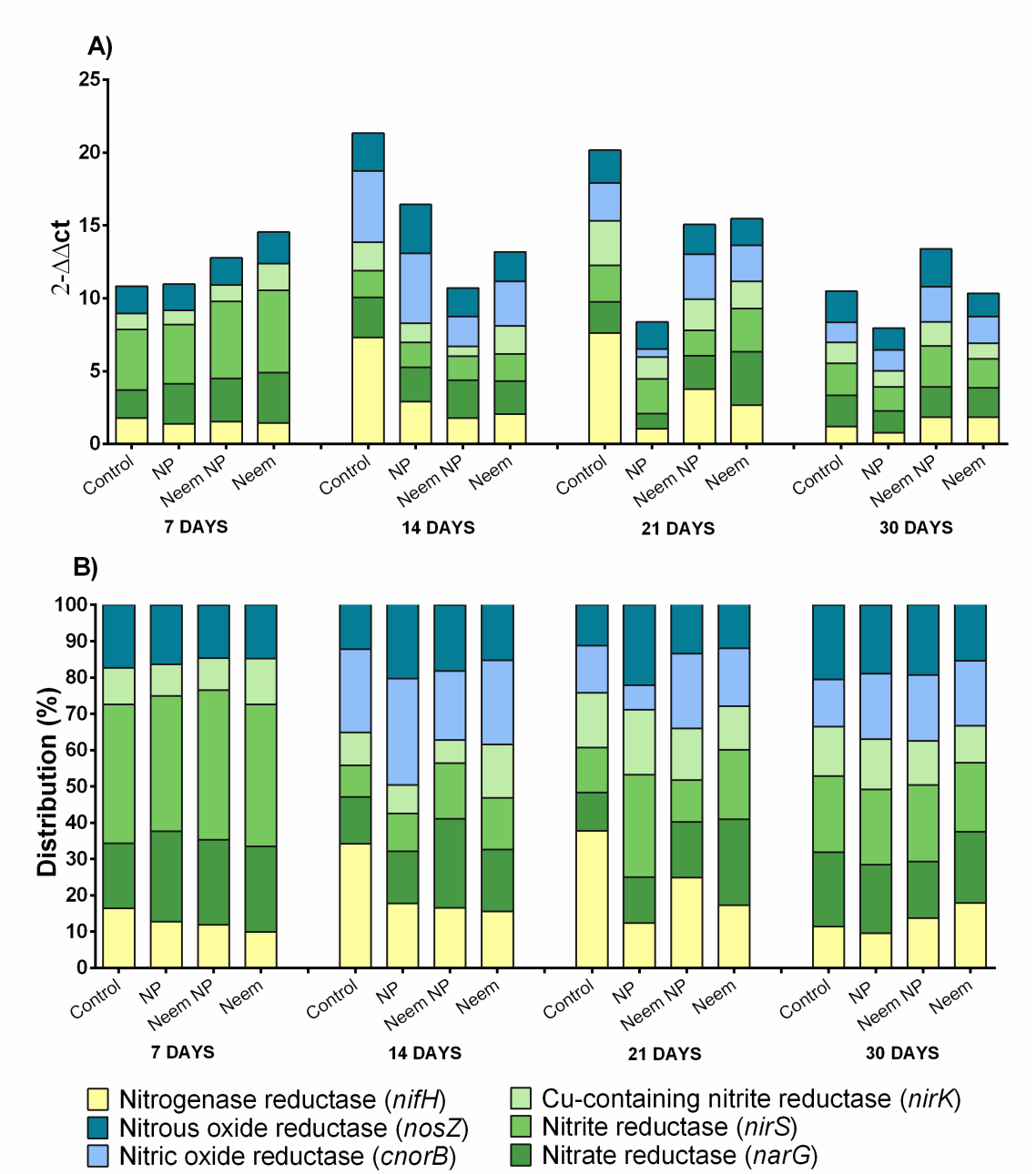


Figure 4. Molecular analysis of the genes of bacteria associated with the nitrogen cycle (*nifH, nosZ, cnorB, nirK, narG, and nirS*). A) Relative quantification of genes by qPCR and B) proportions of genes in the control soil and soils exposed to the zein nanoparticles (NP), neem oil-loaded zein nanoparticles (Neem NP), and neem oil (Neem), at 7, 14, 21, and 30 days after the initial treatment. Data are expressed as average of three independent experiments (n=3).

In according to our results, Pasquoto-Stigliani et al., (2017) investigated the behavior of bacteria involved in the nitrogen cycle when exposed to poly(ε-caprolactone) nanocapsules loaded with neem and showed that the differences in the proportions of these bacteria, compared to the control, varied during the experiment, with no significant difference after 300 days. Maruyama et al., (2016) evaluated atrazine and imazethapyr nanocapsules, showed lower effects on the bacterial profile associated with the nitrogen cycle, in the soil displayed in comparison with control. Yang et al., (2013) and Guilger et al., (2017) analyzed the effects of silver nanoparticles on nitrogen-fixing, nitrifying, and denitrifying bacteria, and found that the nitrifying bacteria were significantly affected, while the nitrogen-fixing and denitrifying organisms were not.

The use of molecular analysis of the genes of soil microbiota involved in the nitrogen cycle to investigate the possible toxicity of new materials, especially nanoparticles, is still recent and the literature is very limited. Further detailed studies are needed and are essential to ensure the safe use of newly emerging technologies.

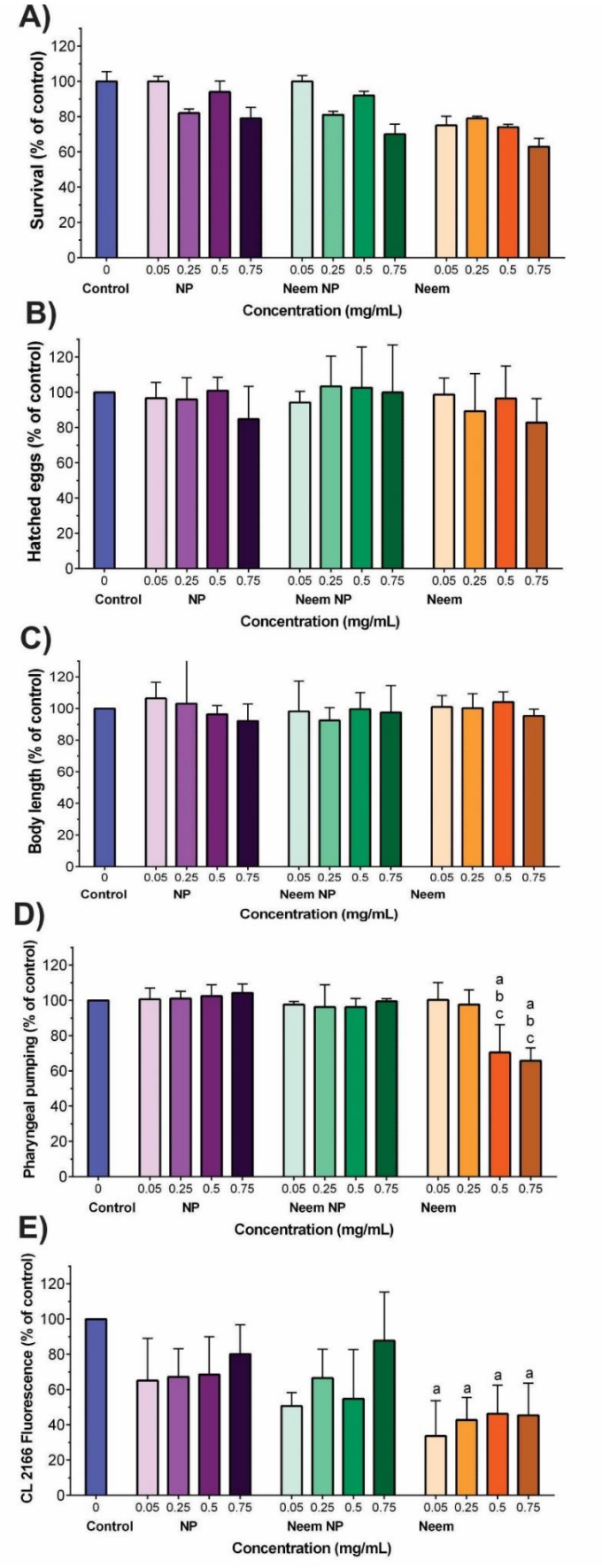
3.2.3 Effects of the formulations on the nematode *C. elegans*

The results of toxicity assays performed with *C. elegans* (Figure 5) showed that the survival, reproduction, and body length of the worms did not present significant differences after exposure to the zein nanoparticles, neem oil-loaded zein nanoparticles, and neem oil (using neem oil concentrations of 0.05, 0.25, 0.5, and 0.75 mg/mL). This lack of toxicity for a nontarget organism

504 is very promising for the advance of neem oil-loaded zein nanoparticle
505 research. These endpoints have been validated as the basic triad for safety
506 assessment. Even if mortality rate does not increase following exposure to a
507 toxicant, the reproductive system and the development of the worms are very
508 sensitive and may show tenuous signs of cellular damage (Tejeda and Olivero,
509 2016). That because during the larval stages, mitosis and meiosis are in fast
510 speed and it has been demonstrated that toxicants, pesticides included, can
511 disrupt cell cycle, elevate DNA double-strand break formation, activate
512 apoptosis and increase embryonic lethality (Shin et al., 2019). Of note, another
513 study evaluating the toxicity of zein nanoparticles loaded with the antidiabetic
514 drug glibenclamide (with an average size of 190 nm and a surface charge of
515 −37 mV) and showed that the formulation exerted significant hypolipidemic
516 activity in *C. elegans*, without causing any toxic effect (Lucio et al., 2017). In
517 contrast, nanoparticles toxicity can be detected in this animal model. Jacques et
518 al. (2017) have shown that different NPs interfered in the survival and vital
519 parameters of *C. elegans*. Solid lipid nanoparticles with or without atrazine and
520 simazine (sizes of 293 ± 3 and 288 ± 6 nm, respectively) and polymeric
521 nanoparticles with or without atrazine (sizes of 367 ± 13 and 305 ± 12 nm,
522 respectively) depicted dose-dependent increases of lethality and decreases of
523 *C. elegans* body length. Chitosan/tripolyphosphate nanoparticles, produced
524 using a natural biopolymer, with or without paraquat (sizes of 262 ± 14 and 246
525 ± 7 nm, respectively), caused increased mortality, but did not alter reproduction
526 or worm length in the surviving animals, therefore providing evidences that
527 natural biopolymers can be more compatible to nontarget organisms (Jacques
528 et al., 2017). Using another nontarget organism Deng et al., (2013) and Wang

et al., (2013) investigated the subacute, acute, and subchronic toxicity of neem oil towards mice and the only significant result was after 90 days, when the mice treated with neem oil at a dose of 1600 mg/kg/day presented several degrees of lesions in the testes, liver, and kidneys. However, the lesions were decreased or eliminated after a 30-day recovery period not demonstrating critical toxicity to the organism studied, in the same way that it happened in our research. (Wang et al., 2013).

In the present work, the pharyngeal pumping of the worms (Figure 5D) decreased significantly in the treatments with neem oil at concentrations of 0.5 and 0.75 mg/mL, compared to the control group, while the neem oil-loaded zein nanoparticles caused no significant alterations in the worms. Pharyngeal pumping is an indicator of a healthy worm and is mainly controlled by cholinergic and glutamatergic innervation, as well as by dopamine and serotonin (Raizen, 2012). Reduced pharyngeal pumping can lead to dietary restriction (Powolny et al., 2011). The results suggested that the zein nanoparticle formulation was able to decrease the toxicity of neem oil in this organism. These findings were in agreement with the work of Sanches Moraes et al., (2016) who reported the ability of polymeric nanocapsules to decrease the toxic effects of clozapine in *C. elegans*.


A)
Survival (% of control)
B)
Hatched eggs (% of control)
C)
Body length (% of control)
D)
Pharyngeal pumping (% of control)
E)
CL 2166 Fluorescence (% of control)
0
0.05 0.25 0.5 0.75
Control
NP
Neem NP
Neem
Concentration (mg/mL)

549

Figure 5. Toxicity assay using *C. elegans* exposed for 48 h to 0.05, 0.25, 0.5, and 0.75 mg/mL of zein nanoparticles (NP), neem oil-loaded zein nanoparticles (Neem NP), and neem oil (Neem). The wild type strain was evaluated for A) survival rate, B) brood size, C) body length, and D) pharyngeal pumping. The transgenic CL2166 strain was evaluated for E) fluorescence intensity, which indicates levels of GST-4 expression. The neem oil caused decreases in pharyngeal pumping and GST-4 expression. Data are expressed as average of three independent experiments (n=3) normalized to % and the error bars represent the standard deviation. Letters a, b, and c indicate a significant difference relative to the control, NP, and Neem NP, respectively. A significance level of $p < 0.05$ was considered.

As shown in Figure 5E, the treatments with neem oil at all concentrations caused significant decreases in fluorescence intensity, indicating reduced GST-4 expression, compared to the untreated animals. The zein nanoparticles and neem oil-loaded zein nanoparticles did not affect the GST-4 enzyme levels. GST-4 is involved in cellular detoxification and cell defense, so the reduction induced by neem oil could lead to oxidative stress and cell death (Lindblom and Dodd, 2006). The results showed that the neem oil decreased GST-4 levels in *C. elegans* by up to 66%, compared to the control, representing a threat since this protein is regulated by protective transcription factors, promoting longevity and resistance to stress (Rathor et al., 2015). It should be highlighted that the neem oil-loaded zein nanoparticles did not affect this parameter, providing further evidence that the new zein nanoparticle system was capable of reducing toxicity towards nontarget organisms (Figure 6). In previous studies, it has been

found that treatments using extracts of *Lavandula latifolia*, *Melissa officinalis*, *Origanum vulgare* (Gayoso et al., 2018), *Ginkgo biloba* (Kampkotter et al., 2007) and antioxidant compounds such as quercetin (Büchter et al., 2015) led to reduced GST-4 expression. It should be noted that the antioxidant capacity of pure neem oil has been demonstrated in several previous studies (Mattos et al., 2017; Rinaldi et al., 2017; Sithisarn et al., 2005).

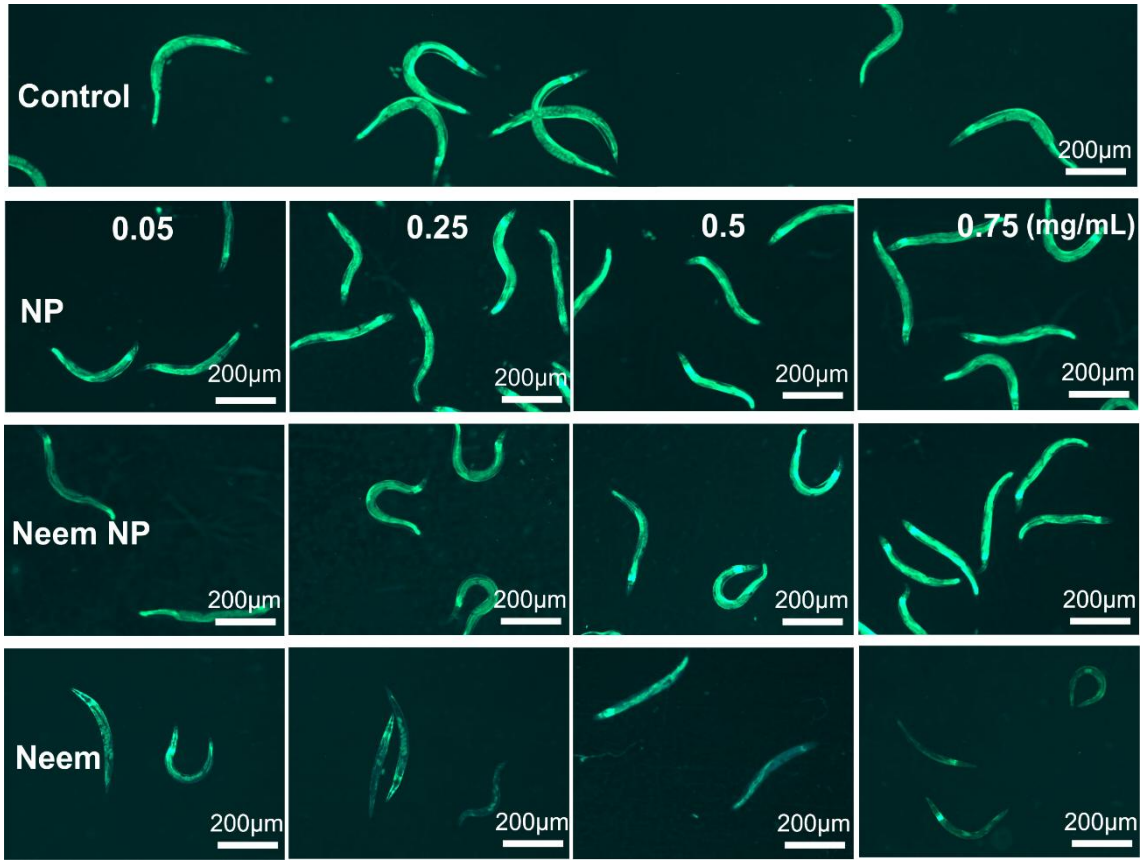


Figure 6. Images of the CL2166 transgenic strain exposed to the zein nanoparticles, neem oil-loaded zein nanoparticles, and neem oil (using neem oil concentrations of 0.05, 0.25, 0.5, and 0.75 mg/mL) for 48 hours acquired using an epifluorescence microscope (Nikon Eclipse 50i) with a GFP filter. It is possible to observe the decrease in the intensity of the fluorescence emitted by worms treated with neem oil at all concentrations used, indicating a decrease in GST-4 expression.

4 Conclusions

The neem oil-loaded zein nanoparticles developed in this work presented good colloidal characteristics and stability in different media. In the *A. cepa* analysis, the use of zein nanoparticles decreased the relative damage index caused by neem oil. In relation to the microbiota of the soil nitrogen cycle, the response to the neem oil-loaded zein nanoparticles was similar to that observed for control. In tests using C. elegans, the organism was susceptible to the effects of neem oil, while the nanoparticles did not show potential toxicity. In this way, more studies must be carried out to guarantee the effects of this nanopesticide before its application in agriculture. It is therefore extremely important to recognize its mechanisms of action (for both, nanopesticides and neem), as well as their possible effects at the cellular level, their efficacy and their toxicity to target organisms. A final consideration is that the definition of the risks associated with nanobiopesticides requires a multidisciplinary approach and that, in order to be sustainable and safe, it is crucial to ensure the awareness and use of correct management practices between farmers and the wider population.

Abbreviations

GST-4, glutathione S-transferase 4; CL2166, transgenic fluorescence *C. elegans* type; AFM, atomic force microscopy; qPCR, real-time polymerase chain reactions; L1, first *C. elegans* larval stage; GFP, green fluorescent protein; DLS, dynamic light scattering; NTA, nanoparticle tracking analysis; NP,

612 zein nanoparticles; Neem NP, neem oil-loaded zein nanoparticles; Neem, neem
613 oil.

614

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Figure 1

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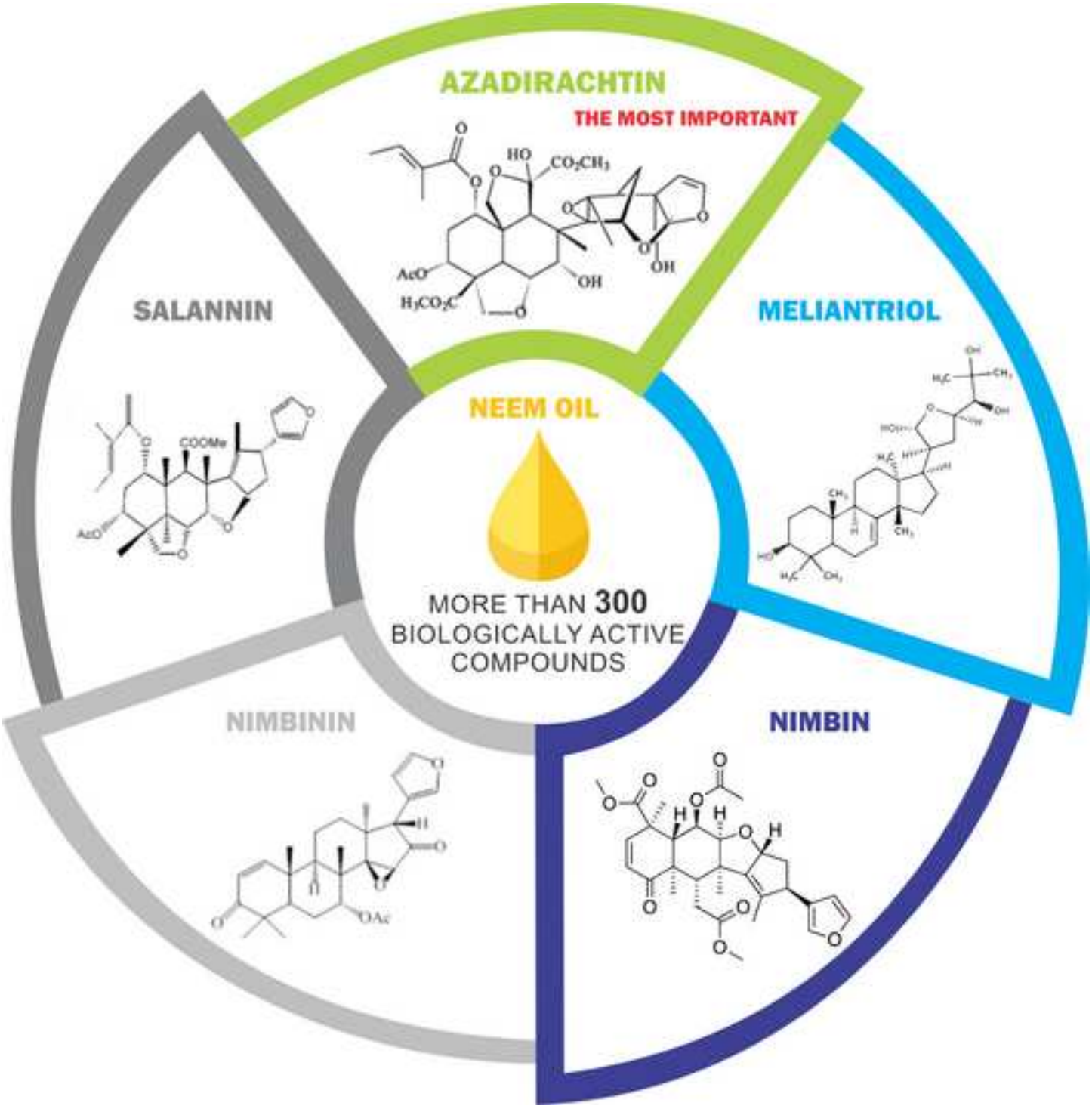

Figure 2

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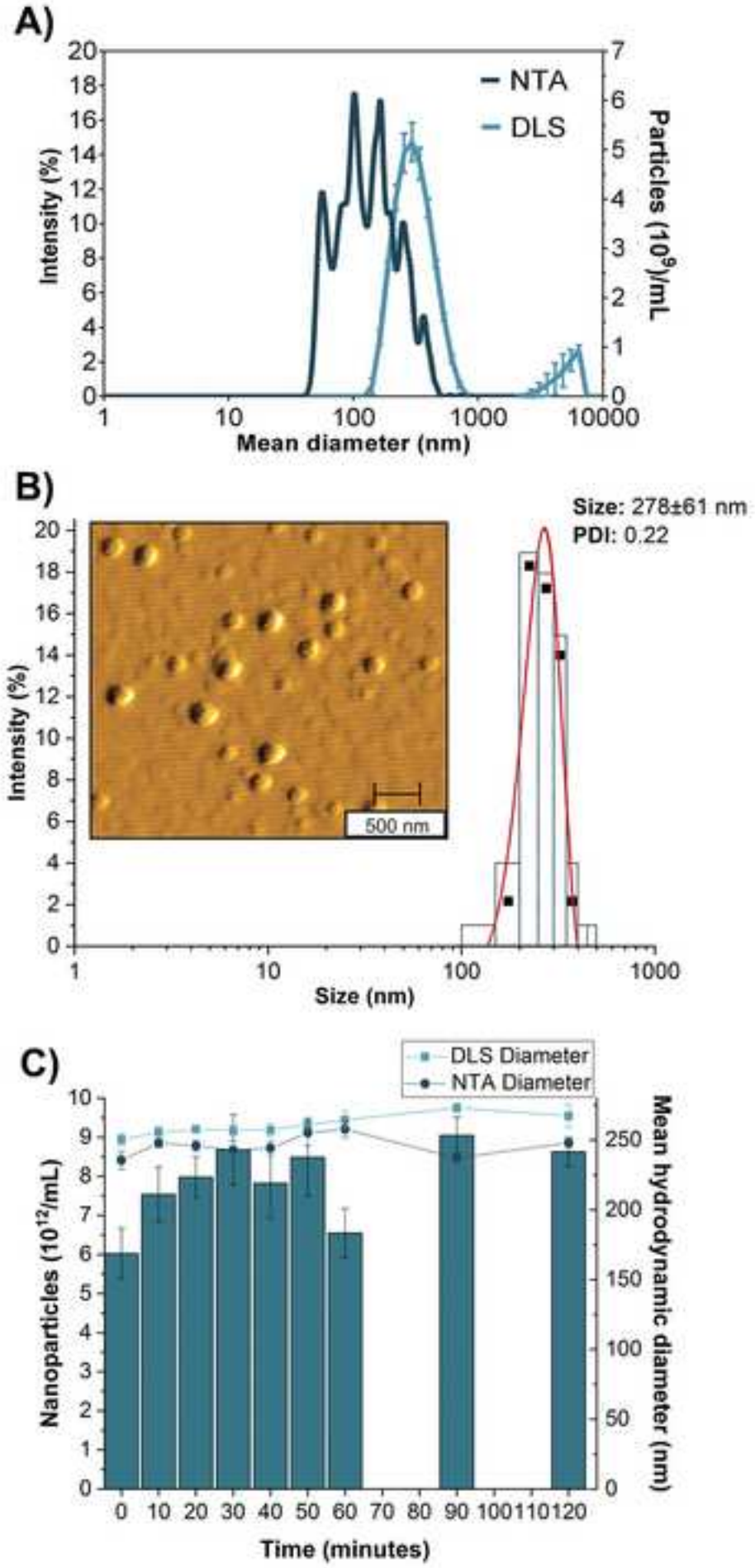

Figure 3

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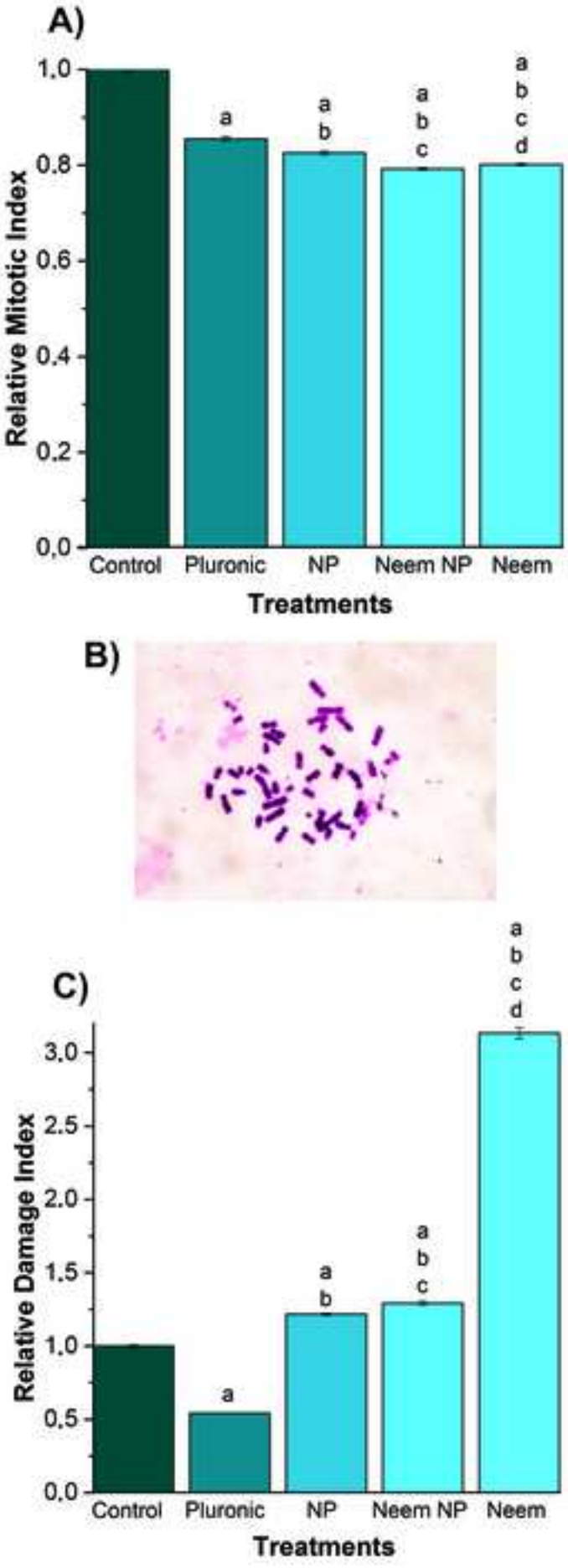

Figure 4

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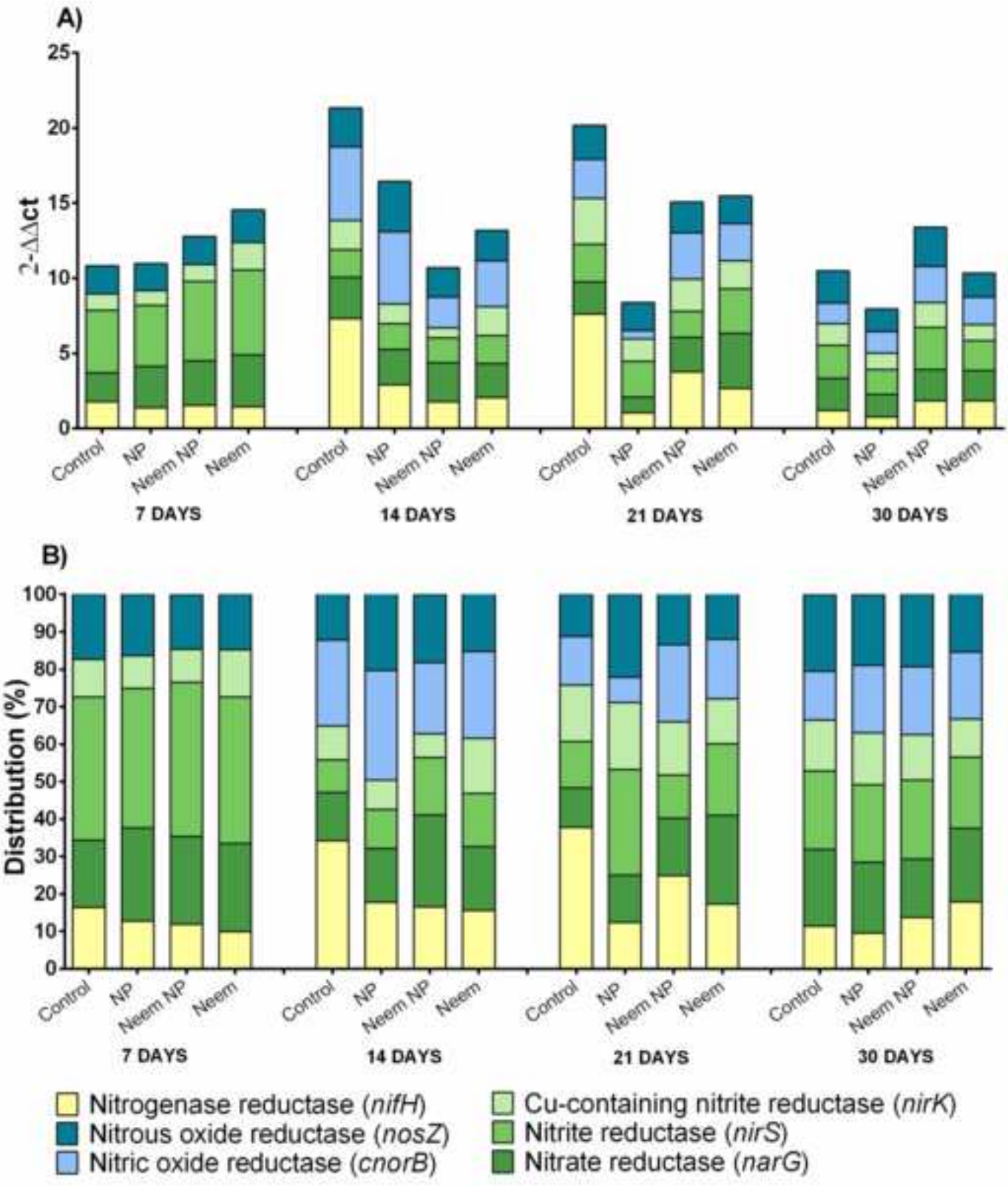

Figure 5

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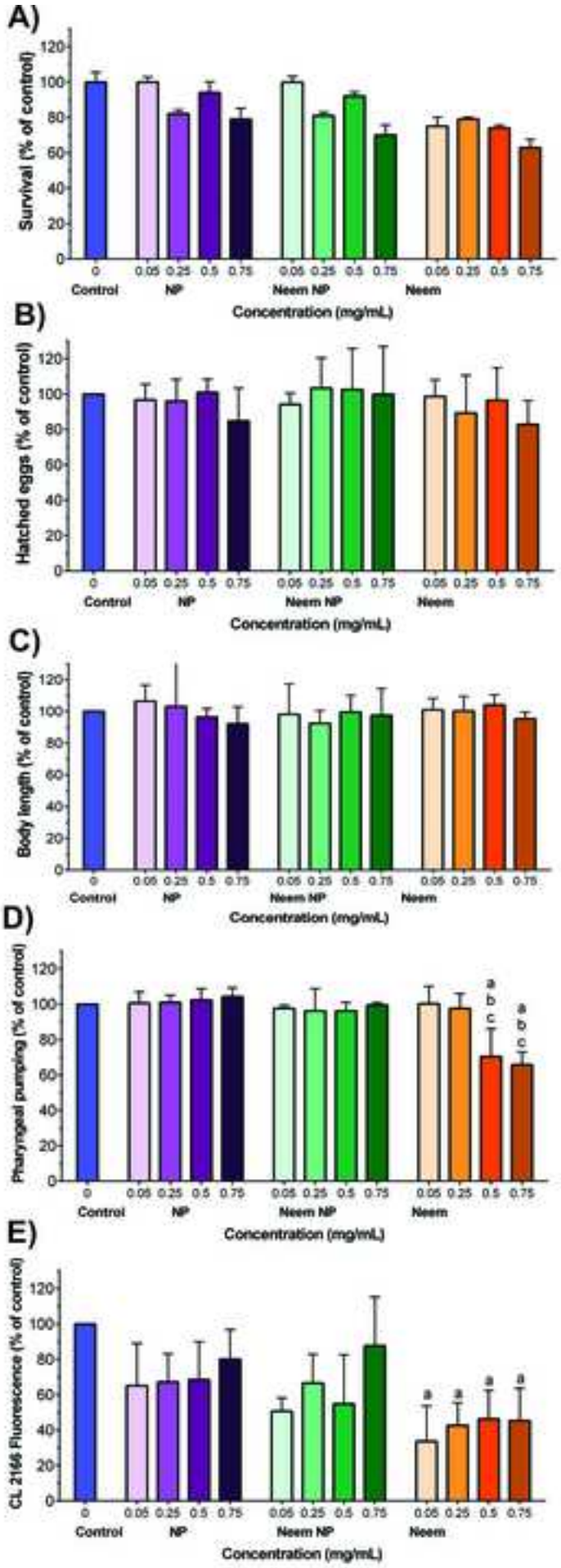

Figure 6

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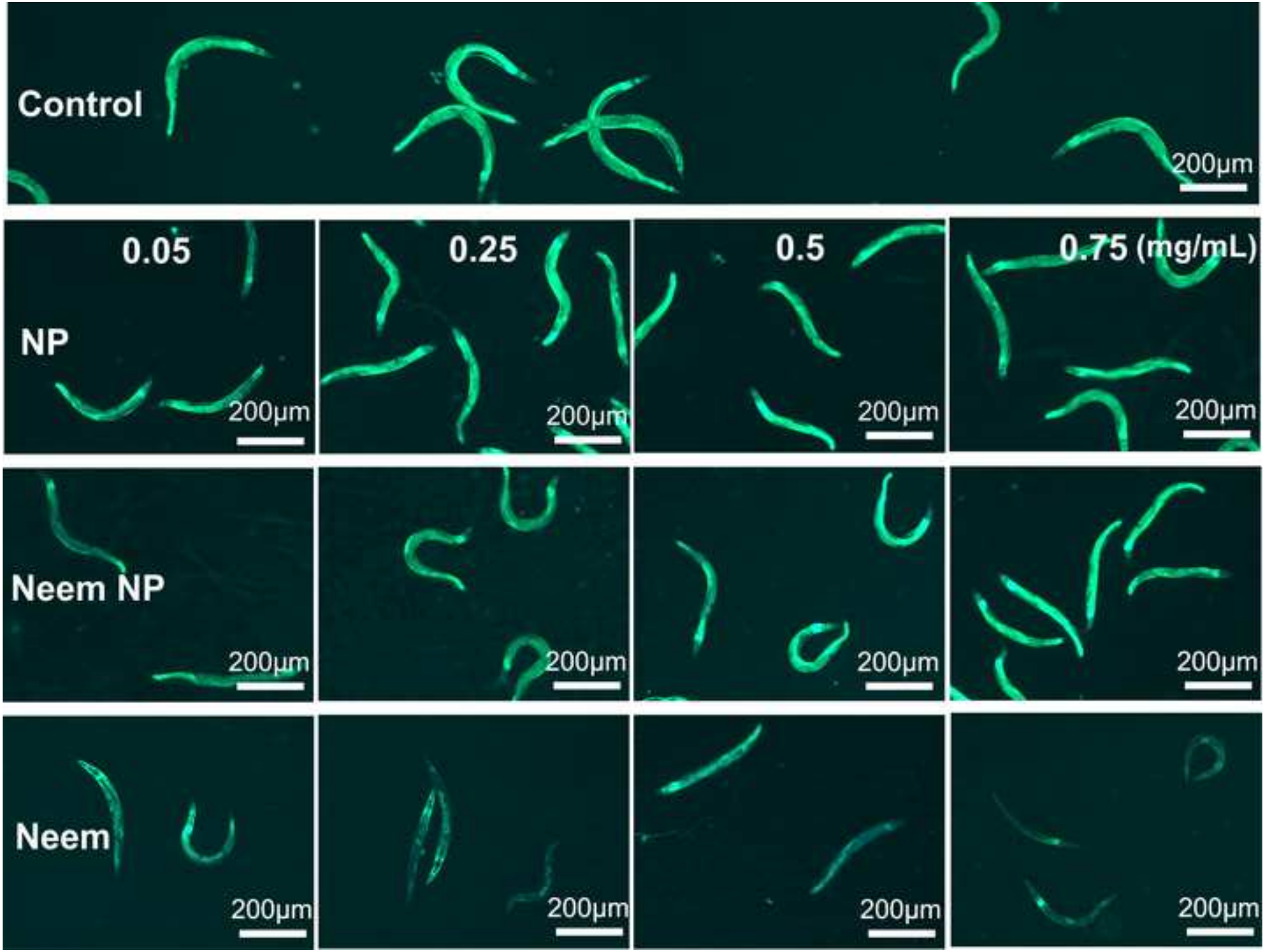

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Mônica Pascoli: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Data Curation, Project Administration, Writing – Original Draft, Review & Editing, Funding Acquisition. **Mauricio T. Jacques and Danielle A. Agarrayua:** Methodology, Validation, Formal Analysis. **Daiana S. Avila and Renata Lima:** Project Administration, Writing – Review & Editing, Supervision. **Leonardo F. Fraceto:** Conceptualization, Supervision, Resources, Project Administration, Writing – Review & Editing, Funding Acquisition.