

1           **Detection of immune system activation in hemolymph of *Drosophila* larvae**  
2                           **exposed to chitosan-coated magnetite nanoparticles**

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## 1 **Abstract**

2 *Drosophila melanogaster* hemolymph cells are confirmed as a model to study the activation  
3 of immune system due to foreign stimuli like iron nanoparticles. The toxicity of nanoparticles  
4 is a cause for concern due to their effect on human health and the environment. The aim of  
5 this study was to detect the activation of cellular immune response in *Drosophila* larvae  
6 through the observation of hemolymph composition, DNA damage and larval viability, after  
7 the exposure to 500 ppm and 1000 ppm chitosan-coated magnetite nanoparticles for 24 hours.  
8 Our results showed activation of cellular immune response after exposure to the nanoparticles  
9 owing to the increment of hemocytes, the emergence of lamellocytes and the presence of  
10 apoptotic hemocytes. In addition, chitosan-coated magnetite nanoparticles produce DNA  
11 damage detected by comet assay as well as low viability of larvae. No DNA damage is  
12 showed at 500 ppm. The cellular toxicity is directly associated with 1000 ppm.

13 Keywords: hemolymph, apoptosis, comet assay, chitosan, magnetite nanoparticles

14

## 15 **Introduction**

16 *Drosophila melanogaster* has proved to be a suitable organism to test toxic effects of  
17 different chemical elements due to its short life cycle and abundant offspring. In *Drosophila*  
18 there are two main components of the innate immune response: the humoral and cellular  
19 systems, both of which are activated upon immune challenge. The cellular response refers to  
20 processes such as phagocytosis, encapsulation, and clotting that are directly mediated by  
21 hemocytes [1–4]. The hemolymph of *Drosophila* is composed of three types of hemocytes:  
22 plasmatocytes (95%) (macrophages) have the capacity to remove foreign material by  
23 phagocytosis; crystal cells (5%) are involved in melanin synthesis during pathogen

24 encapsulation [5] and lamellocyte, which are large flattened cells whose differentiation is  
25 induced in response to the immune system activation, i.e. the presence of foreign particles  
26 in the hemocoel.

27

28 The hemocytes of *Drosophila* are widely regarded as an excellent model for deciphering  
29 general innate immune mechanisms and DNA damage in animals [2,6–8]. *In vitro* and *in*  
30 *vivo* studies, no obvious toxicity of magnetic nanoparticles has been detected, but potential  
31 toxicity has been observed in blood and also activation of the immune systems [9].

32

33 Magnetite nanoparticles ( $\text{Fe}_3\text{O}_4\text{NPs}$ ) are a common magnetic iron oxide that have an inverse  
34 spinel structure and the electrons can hop between 2+ and 3+ oxidation states of ions in the  
35 octahedral sites at room temperature, rendering magnetite an important class of half-metallic  
36 materials. With proper surface coating, these magnetic nanoparticles can be dispersed into  
37 suitable solvents, forming homogeneous suspensions called ferrofluids [10].

38 Due to the physicochemical properties of magnetite nanoparticles ( $\text{Fe}_3\text{O}_4\text{NPs}$ ) and their  
39 application fields, biosafety information is insufficient and contradictory toxicity results have  
40 been reported due to different experimental conditions that would alter the effect of  
41 nanoparticles [11].

42 Chitosan (Ch) is the most abundant natural polysaccharide after cellulose and hemicelluloses.  
43 It is a non-toxic, biodegradable and biocompatible polysaccharide obtained from the  
44 deacetylation of chitin [12]. Chitosan provides nanoparticles with free amino and hydroxyl  
45 groups that enable the possibility to bind to a diversity of chemical groups and ions, leading  
46 to a number of applications such as protein and metal adsorption, guided drug and gene

47 delivery, magnetic resonance imaging, tissue engineering and enzyme immobilization.  
48 Furthermore, this type of nanoparticle could be used in hyperthermia treatment for destroying  
49 malignant cells [13].

50 Chitosan in *Drosophila* has been well studied. *Drosophila* has been utilized for both  
51 production of chitosan [14] and as an *in vivo* model to investigate the transport and uptake of  
52 nanoparticles covered with chitosan in the larval digestive tract after oral administration [15].

53 In this study, we have observed *in vivo*, the cellular immune system activation in the  
54 hemolymph of *Drosophila* larvae by the effect of Ch-Fe<sub>3</sub>O<sub>4</sub>NPs exposure. To achieve these  
55 objectives, third instar larvae were exposed to two concentrations of Ch-Fe<sub>3</sub>O<sub>4</sub>NPs (500 and  
56 1000 ppm) for 24 hours. Immune system activation was evaluated through hemolymph in  
57 terms of total number of hemocytes, apoptotic plasmatocytes, lamellocytes and DNA damage  
58 (comet assay). Additionally, the viability of larvae after the exposure to Ch-Fe<sub>3</sub>O<sub>4</sub>NPs was  
59 estimated.

60

## 61 **Materials and methods**

### 62 **Synthesis and characterization of Ch-Fe<sub>3</sub>O<sub>4</sub>NPs**

63 Ch-Fe<sub>3</sub>O<sub>4</sub>NPs were prepared by the protocol suggested by Gregorio *et al.* [16] with slight  
64 modifications. Transmission Electron Microscopy (TEM) micrographs were obtained using  
65 a FEI Tecnai G2 Spirit Twin at 80kV (Holland). Dynamic Light scattering (DLS) was  
66 conducted on diluted solutions previously filtered with a 220 nm PVDF filter membrane  
67 (Whatman, China), using the HORIBA LB-550 analyzer. The elemental analysis was

68 obtained by EDX which was performed in the SEM chamber (Tescan Mira3) using a Bruker  
69 X-Flash 6|30 detector with a 123 eV resolution at Mn K $\alpha$ . A sample was fixed in a stub  
70 previously covered with two layers of double coated carbon conductive tape and covering it  
71 with 20 nm of a conductive gold layer (99.99% purity) using a sputtering evaporator Quorum  
72 Q150R ES. The XRD measurement was carried out using an Empyrean diffractometer from  
73 PANalytical operating in a  $\theta$ -2 $\theta$  configuration (Bragg-Brentano geometry) and equipped with  
74 a Cu X-ray tube (K $\alpha$  radiation  $\lambda = 1.54056 \text{ \AA}$ ) operating at 40 kV and 40 mV.

75

## 76 **Exposure of *D. melanogaster* larvae to Ch-Fe<sub>3</sub>O<sub>4</sub>NPs**

77 Third instar larvae of *Drosophila melanogaster* (Oregon R+ strain), were exposed to three  
78 treatments for 24 hours: 500 and 1000 ppm Ch-Fe<sub>3</sub>O<sub>4</sub>NPs, and a control without  
79 nanoparticles. Ch-Fe<sub>3</sub>O<sub>4</sub>NPs were supplied orally through the culture media. Fly cultures and  
80 larvae exposition took place in a 22°C incubator on a 12:12 light:dark cycle. The hemolymph  
81 of exposed larvae was extracted and analyzed to detect cell immune system activation  
82 through the total number of hemocytes, apoptotic plasmatocytes and lamelocytes as well as  
83 DNA damage (comet assay).

## 84 **Hemocytes counts**

85 After the exposure, the hemolymph of thirty larvae was extracted and the hemocytes were  
86 stained with trypan blue 0,4 % (Santa Cruz Biotechnology). Three repetitions for each  
87 treatment were developed. Based on the morphology and color, normal hemocytes  
88 (transparent cells), apoptotic plasmatocytes (blue cells) and lamelocytes (large flat cells)

89 were identified. The hemocytes were counted using a Neubauer chamber in a microscope  
90 ZEISS Imager A2 (40x/0.75).

91 The number of hemocytes (normal hemocytes, apoptotic plasmatocytes and lamellocytes) in  
92 larvae exposed to 500 ppm, 1000 ppm Ch-Fe<sub>3</sub>O<sub>4</sub>NPs and non-exposed was established.

93 Statistical differences between treatments were analyzed through a one way analysis of  
94 variance (ANOVA) in the SPSS software 23.0v (Windows Version 23.0. NY: IBM Corp.  
95 <https://www-01.ibm.com/support/docview.wss?uid=swg21476197>). The Bonferroni pos hoc test  
96 was developed to compare differences between nanoparticle treatments vs. the control test  
97 for each type of hemocytes. A probability less than 0.05 ( $p < 0.05$ ) was considered  
98 statistically significant.

## 99 **Comet Assay**

100 The comet assay in the alkaline version was developed in the hemocytes of larvae exposed  
101 to 500 ppm and 1000 ppm Ch-Fe<sub>3</sub>O<sub>4</sub>NPs and the control larvae according to the protocol  
102 described in Alaraby *et al.* [17]. The comets were visualized through a fluorescence  
103 microscopy Olympus DP72 using a 100x/0.17 lens.

104 A hundred hemocyte comets were observed for each treatment. Image captures and comet  
105 tail length were measured using the ImageJ software version 1.50e  
106 (<https://imagej.net/Citing>). The parameters used to estimate the DNA damage were: a) the  
107 percentage (%) of DNA in the comet tail and b) the tail length ( $\mu\text{m}$ ).

108 The data was compared with an analysis of variance (ANOVA) test with the SPSS  
109 statistical software 23.0v. A probability of less than 0.05 ( $p < 0.05$ ) was considered

110 statistically significant. The Bonferroni post-hoc test was performed to compare the control  
111 versus the treatments exposed to Ch-Fe<sub>3</sub>O<sub>4</sub>NPs.

## 112 **Viability**

113 A hundred of third instar larvae were exposed to each treatment (500 ppm and 1000 ppm,  
114 and control without Ch-Fe<sub>3</sub>O<sub>4</sub>NPs until adult eclosion. The progeny eclosed from each  
115 treatment was counted. Non-eclosion after 8 days was counted as mortality. Three repetitions  
116 were performed for each treatment. The percentage of eclosed flies in each treatment was  
117 compared using the SPSS statistical software 23.0v.

## 118 **Results**

### 119 **Characterization of Ch-Fe<sub>3</sub>O<sub>4</sub>NPs**

120 Figure 1 shows the TEM micrograph of Ch-Fe<sub>3</sub>O<sub>4</sub>NPs (1A) and the shape frequency  
121 histogram (1B). The average size of 155 measured nanoparticles by TEM was  $11.0 \pm 4.7$  nm.  
122 This is compatible with the obtained DLS measurements:  $9.2 \pm 0.3$  nm.

123

124 **Fig 1. Micrographs of Ch-Fe<sub>3</sub>O<sub>4</sub>NPs.** A) TEM and B) nanoparticles frequency histogram  
125 size.

126

127 The FEG-SEM micrograph (Fig 2) shows the chitosan recovering on Fe<sub>3</sub>O<sub>4</sub>NPs. The EDS  
128 measurements have been performed by considering C, N, O, Mg, S, Cl and Fe. In order to

129 avoid biased determinations of the chemical compositions of the samples due to their  
130 inhomogeneity, we have averaged the spectra obtained from 25 points grid was averaged.  
131 The normalized weight average of each element and the standard deviation obtained by EDS  
132 analysis are listed in Table 1. We found the organic elements that comes from chitosan, C,  
133 N, and O. Chlorine comes from the inorganic salt precursors. Fe element comes from the  
134 magnetite nanoparticles. Traces of Mg and S come from the extraction process.

135

136 **Fig 2.** FEG-SEM micrograph of Ch-Fe<sub>3</sub>O<sub>4</sub>NPs.

137

138 **Table 1.** FEG-SEM EDX measurements of Ch-Fe<sub>3</sub>O<sub>4</sub>NPs in normalized wt.%

<b>Spectrum</b>	<b>C</b>	<b>N</b>	<b>O</b>	<b>Mg</b>	<b>S</b>	<b>Cl</b>	<b>Fe</b>
<b>Mean value</b>	21.5	9.9	30.3	0.4	0.7	14.1	23.1
<b>Sigma mean</b>	2.6	1.1	2.4	0.1	0.2	2.5	3.4

139

140 Samples of XRD were dried on a microscope slide at 40°C to avoid any organic degradation.  
141 Analysis XRD of the obtained average is the result of 6 different measurements from 5° to 90° ( $\theta$ -  
142 2 $\theta$ ) angle. The Fe<sub>3</sub>O<sub>4</sub>NPs crystalline nature is confirmed from the XRD analysis (Fig 3). It is  
143 found that Bragg Reflection peaks at 36.06° which coincides with the cubic phase of Fe<sub>3</sub>O<sub>4</sub>  
144 (ICSD: 96012). The lattice parameter and highest intensity plane (113) is well matched and  
145 agrees with other reported patterns [16]. Further peaks are observed around 15° and 20°. To  
146 our knowledge they correspond to impurities of the chitosan extract and its mix with the



147 chemical compounds. Hematite or metal hydroxides were not identified, which confirms the  
148 complete formation of  $\text{Fe}_3\text{O}_4$ . The Debye Scherrer's equation at the highest reflection peak  
149 (FWHM=0.168°) gives a 50 nm approximated size value for the  $\text{Fe}_3\text{O}_4$  nanoparticles. This  
150 calculated value is higher than the TEM and DLS values likely due to the agglomeration of  
151 the organic extract.

152

153 **Fig 3.** XRD pattern of the Ch- $\text{Fe}_3\text{O}_4$ NPs.

154

## 155 **Hemocytes count**

156 The changes in the total number of hemocytes and the presence of apoptotic or specialized  
157 cells were examined in order to detect the activation of immune system in the hemolymph of  
158 larvae exposed to Ch- $\text{Fe}_3\text{O}_4$ NPs. Apoptotic hemocytes were identified by the blue coloration  
159 produced by entrance of trypan blue through the cell membrane. Lamellocytes were observed  
160 as large and irregular cells (Fig 4).

161

162 **Fig 4. Hemolymph cells observed after nanoparticles exposure.** A) normal plasmatocyte,  
163 B) normal lamellocyte, C) apoptotic plasmatocyte, D) apoptotic lamellocyte (40x/0.75).

164

165 The total number hemocytes increased in larvae exposed to 1000 ppm (mean: 411.33) but  
166 decreased in the larvae exposed to 500 ppm (mean: 201.67) compared with the control group

167 (235.67). In the case of apoptotic plasmatocytes, the larvae exposed to 1000 ppm also showed  
168 an increase in the number of apoptotic plasmatocytes (mean: 54.33) compared with the 500  
169 ppm (mean: 8.6) and control group (0.33). Lamellocytes were not present in the control  
170 larvae, but this type of cell was observed in the larvae exposed to 500 ppm (1.3) and 1000  
171 ppm (13.3) (Fig 5).

172

173 **Fig 5. Hemocytes observed after nanoparticles exposure.** Total number of hemocytes,  
174 apoptotic plasmatocytes and lamellocytes counted in each treatment.

175

176 For all counted cells (total number of hemocytes, apoptotic plasmatocytes and lamellocytes)  
177 (Table 2), statistical analysis shows little difference ( $p < 0.05$ ) between the larvae exposed to  
178 500 ppm and the control test, but shows significant difference between the 1000 ppm  
179 treatment and the control test.

180 **Table 2. Hemocytes counts per treatment**

Treatment	Total hemocytes	Apoptotic plasmatocytes	Lamellocytes
Control	707	1	0
500 ppm	605	26	4
1000 ppm	1234*	163*	40*

\*Statistically significant  $p < 0.05$

181

182 **Comet Assay**

183 The comet assay was used to observe potential DNA damage in the hemocytes of larvae  
184 exposed to Ch-Fe<sub>3</sub>O<sub>4</sub>NPs. The DNA damage was detected by the presence of a comet tail in  
185 the cell nucleus. Comets without DNA damage and comets with high level of DNA damage  
186 were identified (Fig 6).

187

188 **Fig 6. Nucleus observed in the comet assay.** A) Hemocyte without comet tail, B) Hemocyte  
189 with comet tail and DNA damage. 400X (Bar = 25 μm)

190

191 DNA damage produced by exposure to each treatment in the hemocytes was estimated in  
192 function of the percentage of DNA (% of DNA) in the comet tail and the length of the comet  
193 tail. A direct association between Ch-Fe<sub>3</sub>O<sub>4</sub>NPs concentration and DNA damage was  
194 observed.

195 The level of DNA damage produced for each treatment was estimated in function of the %  
196 of DNA in the comet tail (Fig 7A) and the comet tail length (μm) (Fig 7B). The highest level  
197 of DNA damage was observed in the larvae exposed to 1000 ppm, followed by the larvae  
198 exposed to 500 ppm and finally the control larvae. However non-statistical differences were  
199 observed between the larvae exposed to 500 ppm and 1000 ppm (p<0.05). Therefore, both  
200 Ch-Fe<sub>3</sub>O<sub>4</sub>NPs concentrations are able to produce DNA damage in contrast with the control  
201 test (Table 3).

202 **Table 3. Statistical analysis of comet assay results**

Treatments	% DNA in tail	Tail length ( $\mu\text{m}$ )
Control vs. 500 ppm	**	**
Control vs. 1000 ppm	**	**
500 ppm vs. 1000 ppm	0.083	0.084

203

204

205 **Fig 7. DNA damage observed by the comet test assay.** Two parameters were used to

206 estimate the DNA damage: A) % of DNA in the comet tail and B) comet tail length ( $\mu\text{m}$ )

207

## 208 **Viability of larvae**

209 The viability of larvae was interpreted as the capacity of larvae to continue with the

210 metamorphosis until the adult eclosion took place after the exposure to Ch-Fe<sub>3</sub>O<sub>4</sub>NPs. The

211 parameter used to estimate the viability of larvae was the percentage of adults eclosed

212 after the exposure of the larvae to the Ch-Fe<sub>3</sub>O<sub>4</sub>NPs treatments. The lower percentage of

213 viability was observed in the larvae exposed to 1000 ppm Ch-Fe<sub>3</sub>O<sub>4</sub>NPs (51.3%). A higher

214 percentage was observed in the larvae exposed to 500 ppm (61.0%) and the highest

215 percentage of viability was observed in the control test (84.0%). It is evident that mortality

216 is directly associated to Ch-Fe<sub>3</sub>O<sub>4</sub>NPs concentration, therefore, exposure to high-dose

217 concentration of Ch-Fe<sub>3</sub>O<sub>4</sub>NPs produce high mortality of larvae (Fig 8).

218 Significant statistical differences were observed in the viability between 1000 ppm and

219 control treatment.

220

221 **Fig 8. Viability of exposed larvae.** Adults eclosed after exposure to each treatment.

222

223 Accumulation of Ch-Fe<sub>3</sub>O<sub>4</sub>NPs was observed in the midgut of the larvae after the exposure  
224 to the nanoparticles. This accumulation of Ch-Fe<sub>3</sub>O<sub>4</sub>NPs remains in the midgut of the  
225 eclosed adults (Fig 9).

226

227 **Fig 9. Accumulation of Ch-Fe<sub>3</sub>O<sub>4</sub>NPs in the midgut of third instar larvae as an adult.**

228 A) larva not exposed (control), B) larvae exposed to 1000 ppm, C) adult exposed to 1000  
229 ppm Ch-Fe<sub>3</sub>O<sub>4</sub>NPs (red arrows show accumulation of Ch-Fe<sub>3</sub>O<sub>4</sub>NPs).

230

## 231 **Discussion**

232 In *Drosophila* the cellular immune response starts in the hemolymph through the hemocytes,  
233 and the circulating immune surveillance cells play a central role in the immune response. In  
234 this response each type of hemocyte has a function. When an invading organism or particle  
235 is recognized as foreign, circulating plasmatocytes may remove it by phagocytosis and  
236 lamellocytes can complete the process by encapsulation when the particles are too large to  
237 undergo phagocytosis [18].

238

239 In this study changes were observed in the density of cell hemolymph composition in  
240 *Drosophila* larvae exposed for 24 hours to Ch-Fe<sub>3</sub>O<sub>4</sub>NPs including the increment of the  
241 number of plasmatocytes, the emergence of lamellocytes and the presence of apoptotic  
242 plasmatocytes. Additionally, DNA damage and high mortality of larvae exposed to Ch-  
243 Fe<sub>3</sub>O<sub>4</sub>NPs were observed.

244

245 The increment of plasmatocytes density and the emergence of lamellocytes were observed in  
246 larvae exposed to 500 ppm and 1000 ppm. However the effect of 1000 ppm concentration is  
247 toxic, while the exposure to 500 ppm is less nocive for the hemolymph cells. This observation  
248 could be explained due to the presence of *Drosophila* hemocytes: circulating (in hemolymph)  
249 and sessile hemocytes (in the body wall) which have different functions during the immune  
250 response. The circulating plasmatocytes in the larvae are originated through prohemocytes  
251 (embrionic macrophages); this differentiation occurs during the normal development of the  
252 larvae. However, when an event like the presence of pathogens, parasites or foreign particles  
253 are detected, the cellular immune response is activated [19], and the sessile hemocytes detach  
254 from the epithelium and enter the circulating hemolymph triggering the differentiation of  
255 plasmatocytes or lamellocytes, increasing the number of circulating hemocytes  
256 (plasmatocytes and lamellocytes). The emergence of lamellocytes could be originated by  
257 both prohemocytes differentiation and also by plasmatocytes differentiation. The  
258 plasmatocyte differentiation to originated lamellocytes is triggered by immune induction  
259 [18,20,21]. Therefore, the increase of the number of circulating plasmatocytes and the  
260 emergence of lamellocytes are evident signals of cellular immune response activation.

261

262 Larvae exposed to 1000 ppm of Ch-Fe<sub>3</sub>O<sub>4</sub>NPs showed the highest number of emergent  
263 lamellocytes (163 cells), the highest number of apoptotic hemocytes (40 cells) and the highest  
264 level of DNA damage. This suggests that high concentrations of nanoparticles could produce  
265 toxic effects in the hemocytes. This dose-concentration effect of Ch-Fe<sub>3</sub>O<sub>4</sub>NPs is supported  
266 by the statistical analysis that shows no difference between the 500 ppm treatment and the  
267 control but high significant difference between the 1000 ppm treatment and the control. The  
268 toxic effect of Ch-Fe<sub>3</sub>O<sub>4</sub>NPs at 1000 ppm concentration was demonstrated in this study. The  
269 dose concentration and the exposure time are important factors that influence the level of  
270 toxicity in hemocytes [22]. Also, the type of hemocyte is related to the toxic effect due to  
271 different structural characteristic and function of each hemolymph cells [23].

272

273 Another signal of immune system activation is the presence of apoptotic cells which is a  
274 signal of hemocyte damage and hemocyte death due to cell membrane damage, and plays a  
275 key role in immune response by eliminating cells subjected to various stress factors [24]. In  
276 this study, the high number of apoptotic cells were observed in larvae exposed to 1000 ppm  
277 Ch-Fe<sub>3</sub>O<sub>4</sub>NPs. This damage was observed by the increase of blue stained hematocytes in  
278 larvae exposed to Ch-Fe<sub>3</sub>O<sub>4</sub>NPs.

279

280 Also, the toxic effect of Ch-Fe<sub>3</sub>O<sub>4</sub>NPs on the DNA was demonstrated through the comet  
281 assay which allows for associating the percentage of DNA in the tail and the length of comet  
282 tail with the level of DNA damage. Exposure to 1000 ppm Ch-Fe<sub>3</sub>O<sub>4</sub>NPs produced the  
283 highest percentage of DNA in the comet and the highest length of comet tail. Comet assay  
284 provides a sensitive way to detect the effects of Ch-Fe<sub>3</sub>O<sub>4</sub>NPs by means of measuring DNA

285 strand breaks [25,26]; this will allow identification of the possible mode of action of  
286 nanoparticles at the molecular level.

287 In addition, the effect of Ch-Fe<sub>3</sub>O<sub>4</sub>NPs on the viability of larvae was evidently toxic,  
288 producing up to 50% of mortality in larvae exposed to 1000 ppm Ch-Fe<sub>3</sub>O<sub>4</sub>NPs, while the  
289 non-exposed larvae presented only up to 16% of mortality. The low viability is associated to  
290 the Ch-Fe<sub>3</sub>O<sub>4</sub>NPs exposure; however, the physiological mechanisms should be analyzed in  
291 future research.

292

293 Accumulation of Ch-Fe<sub>3</sub>O<sub>4</sub>NPs in the midgut of exposed larvae was observed; this  
294 accumulation was transferred to the adult during the metamorphosis. This event could be  
295 associated to the immune response through cellular events such as phagocytosis and humoral  
296 events that include lysis and melanization [27]. Another explanation could be related to the  
297 low capacity of larvae to excrete the nanoparticles. The effect of this accumulation has not  
298 been analyzed in this study. However new studies should be conducted to establish if  
299 agglomeration of Ch-Fe<sub>3</sub>O<sub>4</sub>NPs in the digestive tract are produced by plasmatocytes through  
300 phagocytosis, and additionally accumulation of nanoparticles should be estimated.

301

## 302 **Conclusion**

303

304 Activation of cellular immune response was observed in the hemolymph of *Drosophila*  
305 larvae through the increment of hemocytes density, the emergence of lamellocytes and the  
306 presence of apoptotic hemocytes after the exposure to Ch-Fe<sub>3</sub>O<sub>4</sub>NPs. In addition, DNA  
307 damage detected in hemocytes by the comet assay, and the low viability of larvae is directly



308 associated to the dose concentration Ch-Fe<sub>3</sub>O<sub>4</sub>NPs. The toxic effect of nanoparticles is higher  
309 in larvae exposed to 1000 ppm concentration, while 500 ppm could have toxic risks but have  
310 not been detected in this study.

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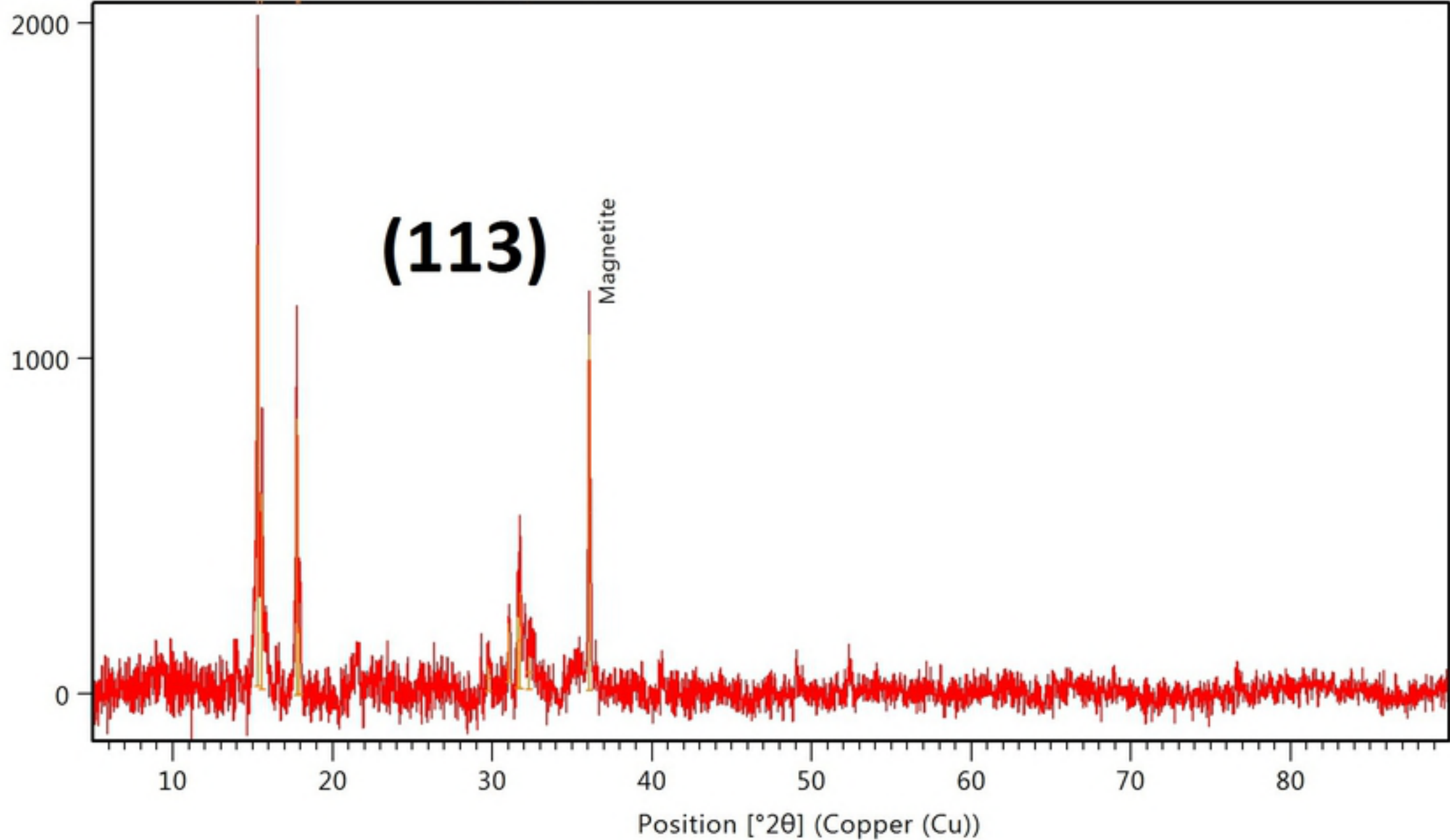
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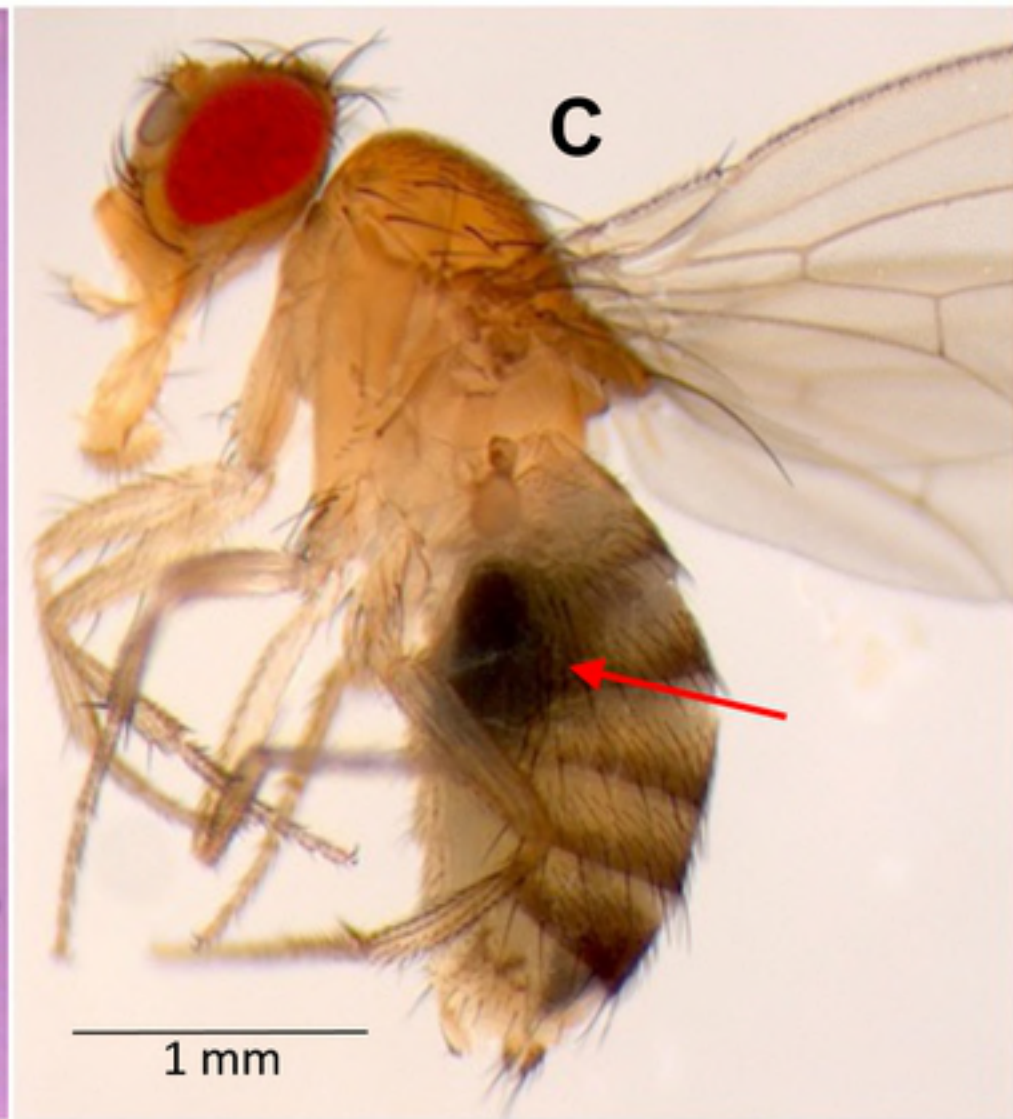
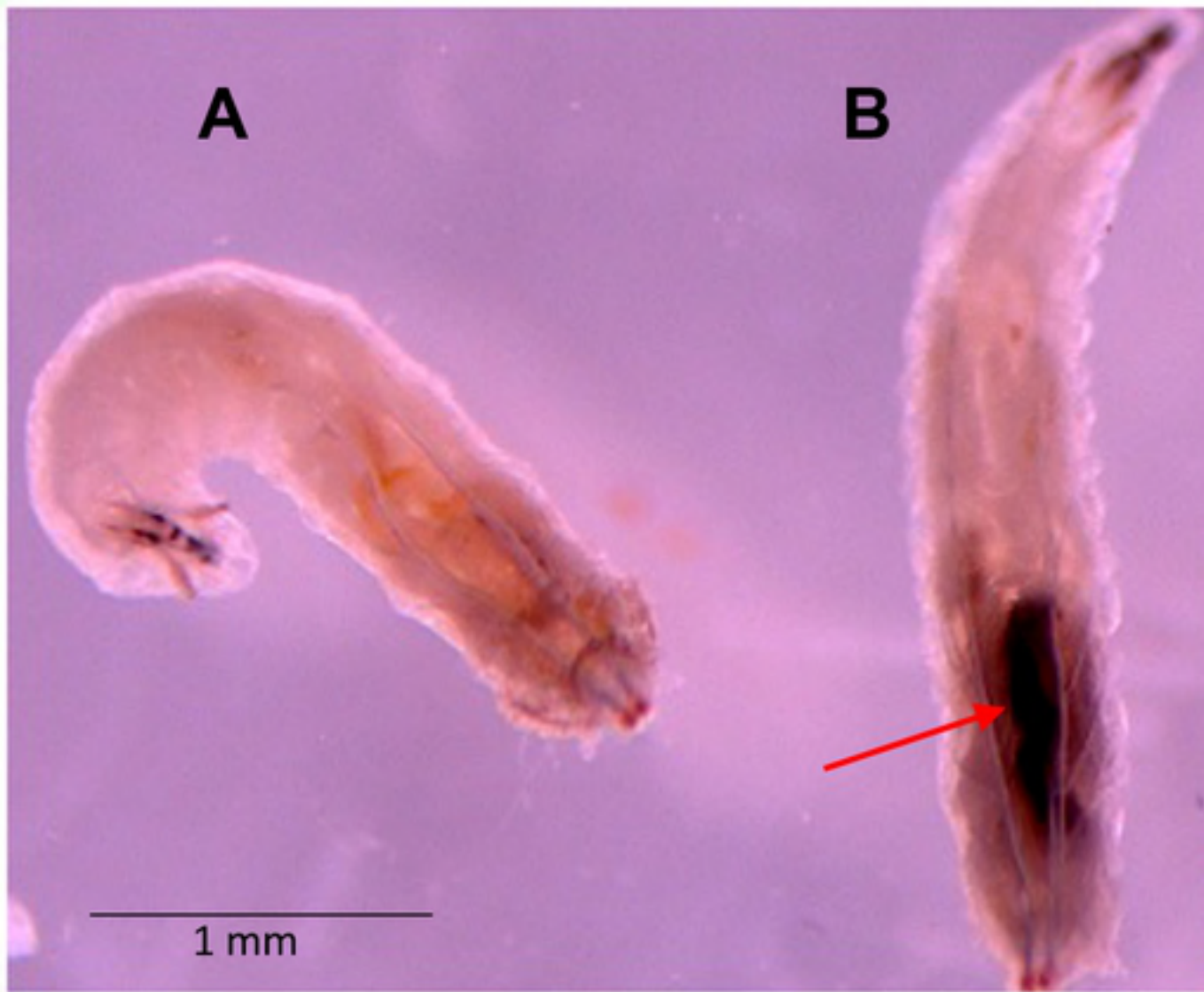
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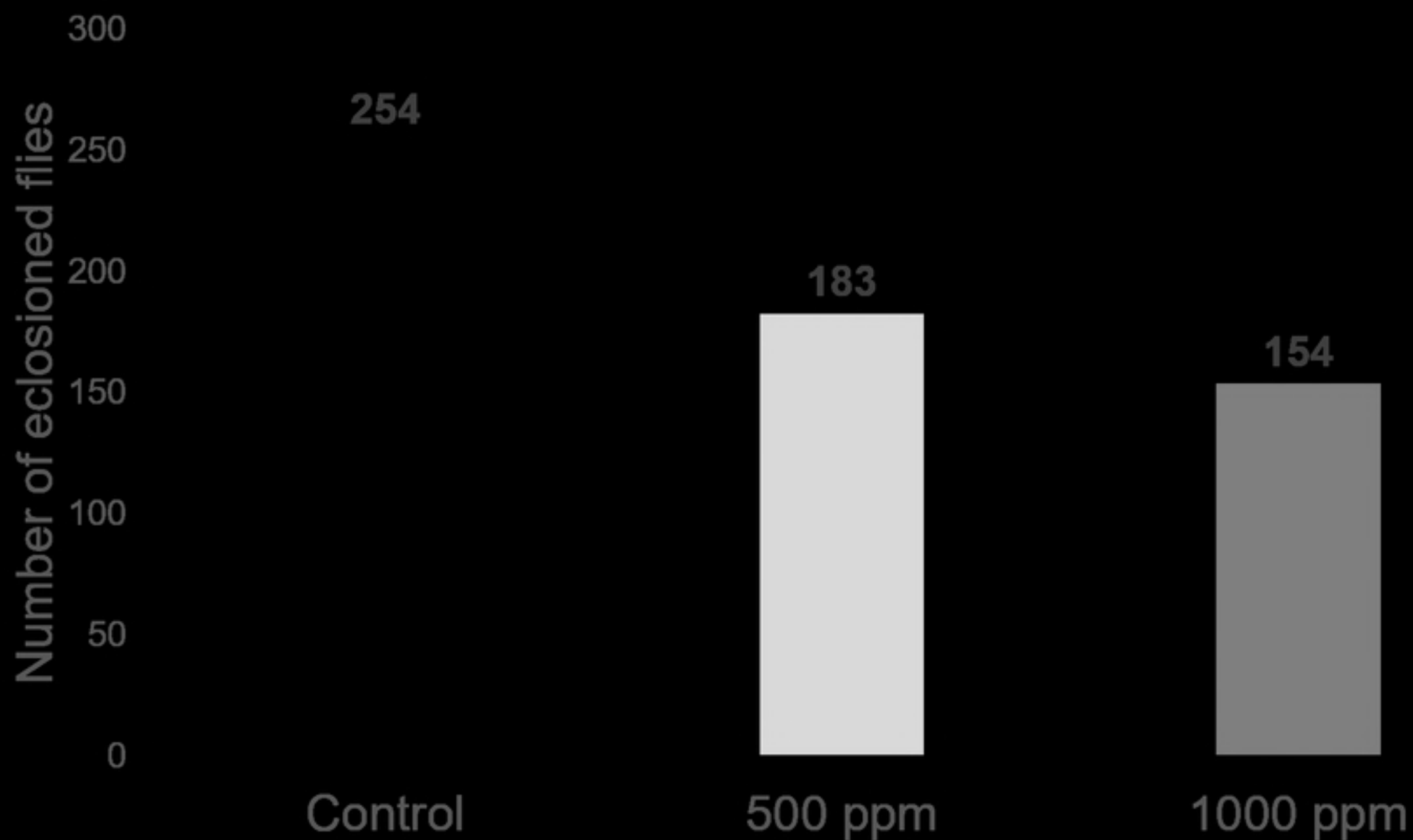
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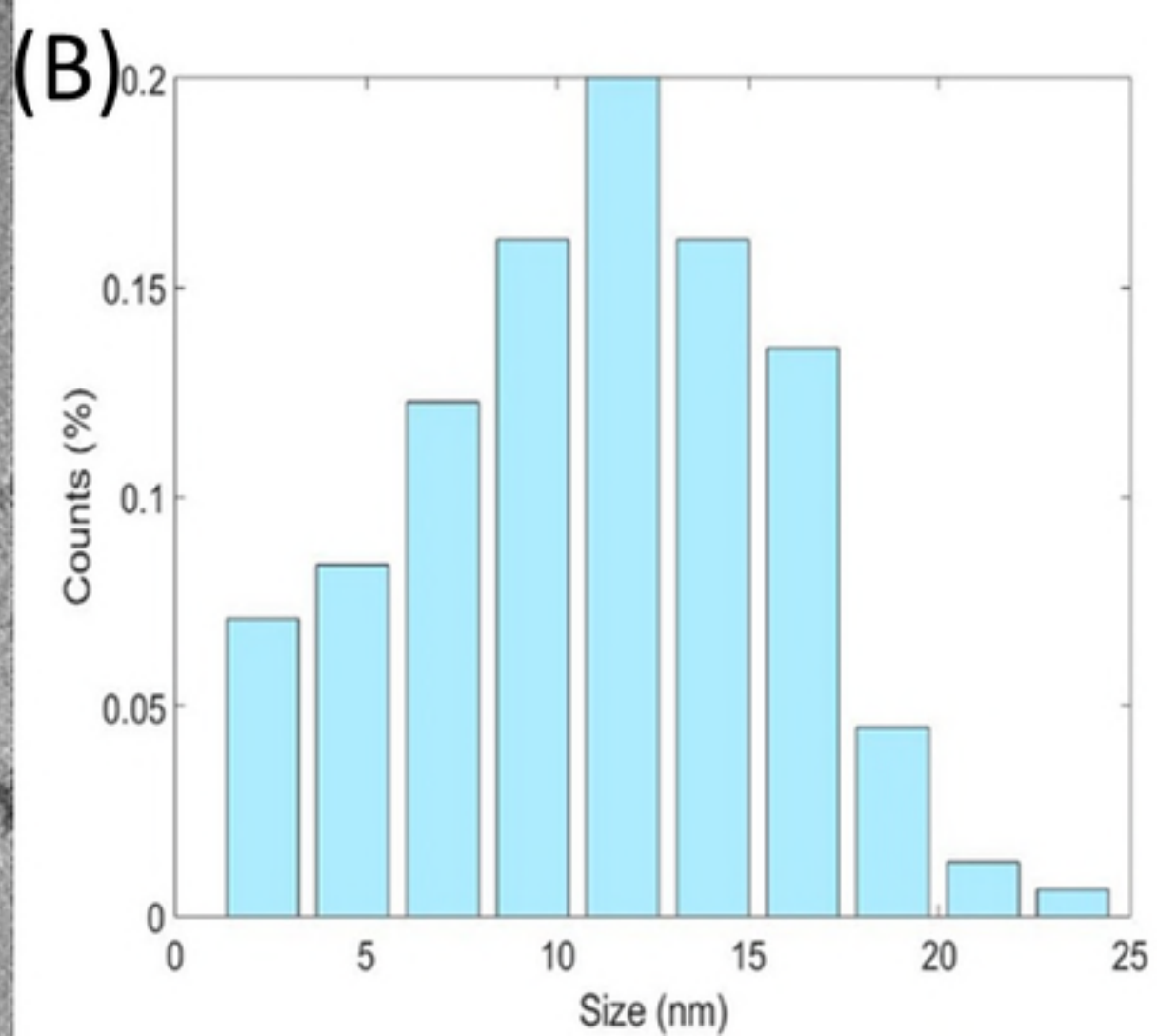
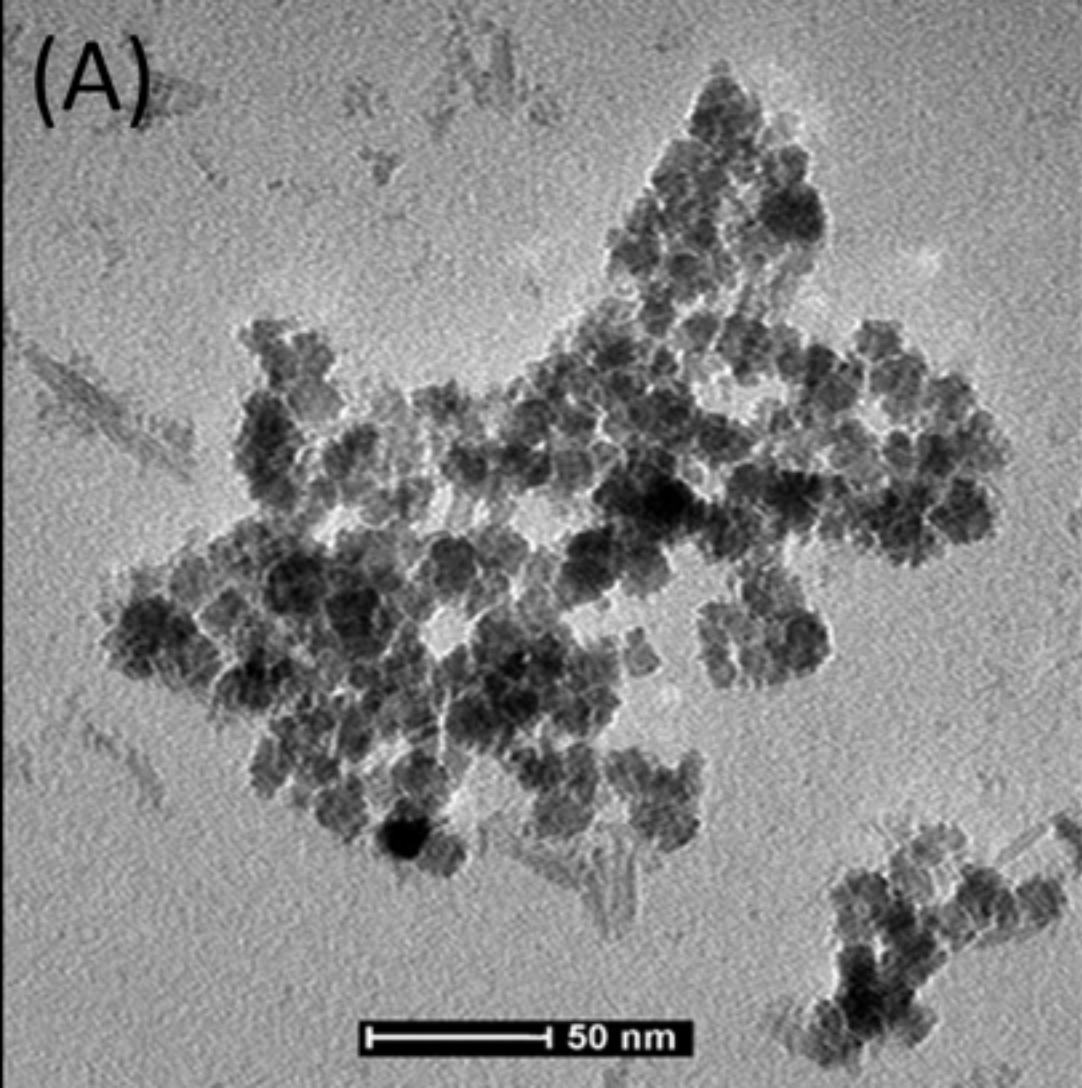
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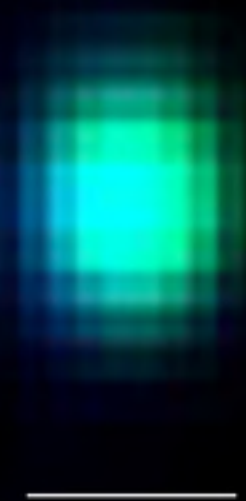




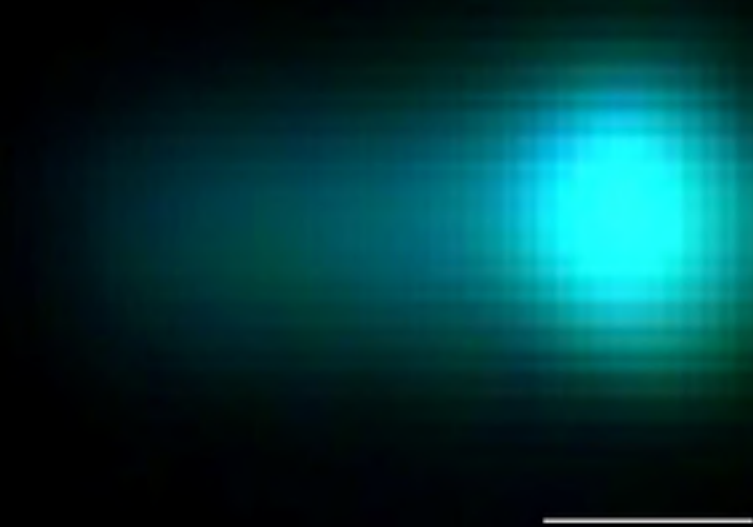




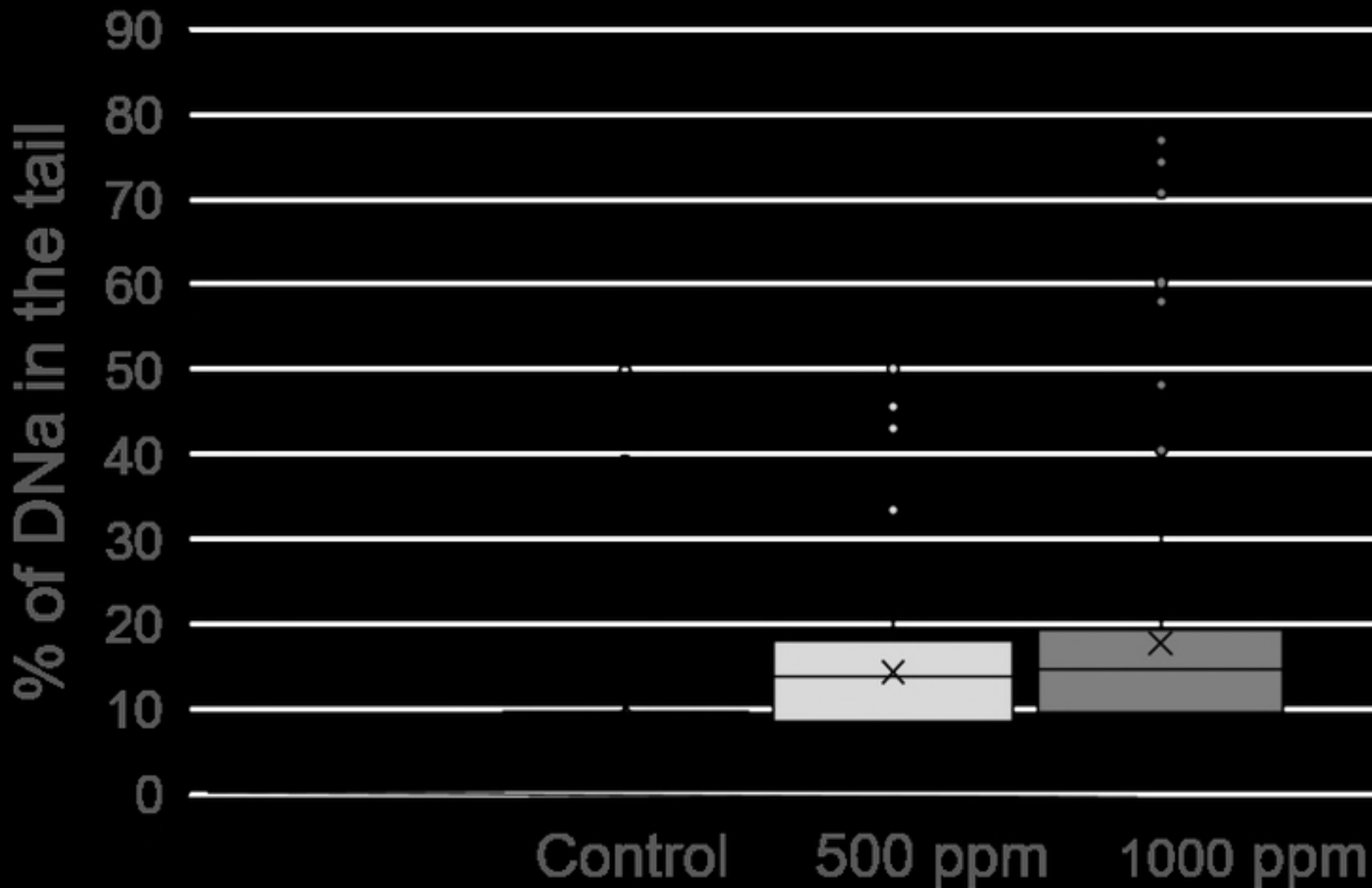
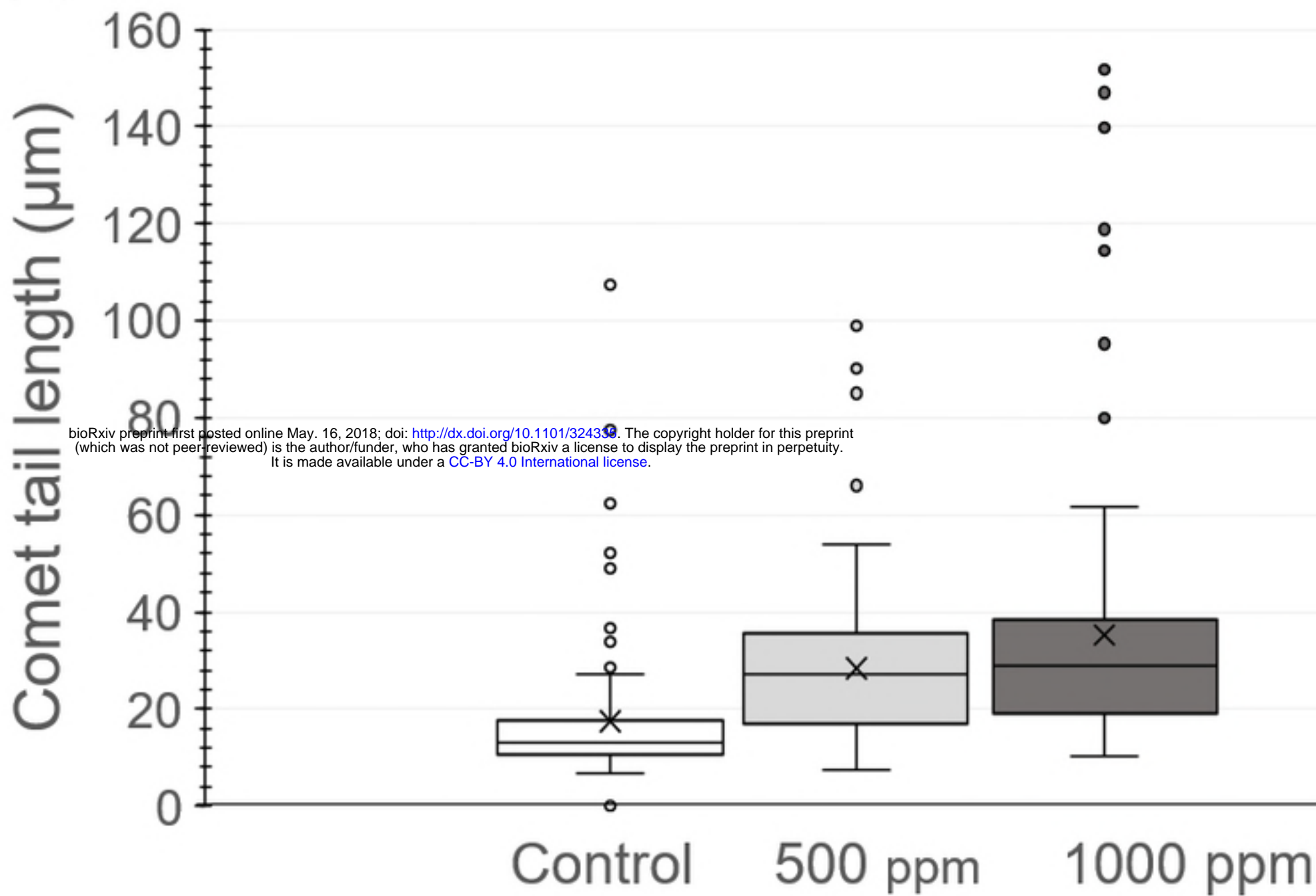


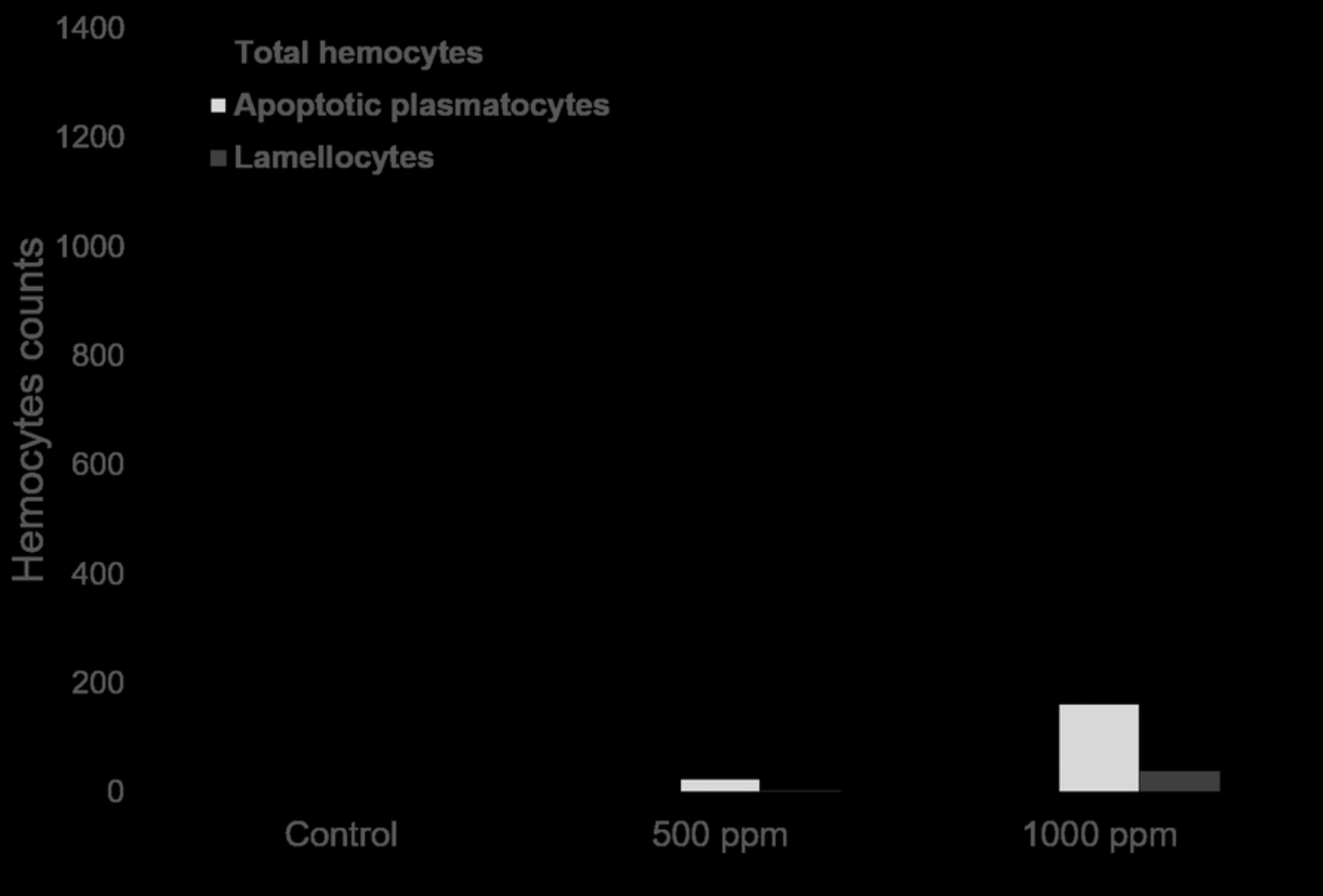


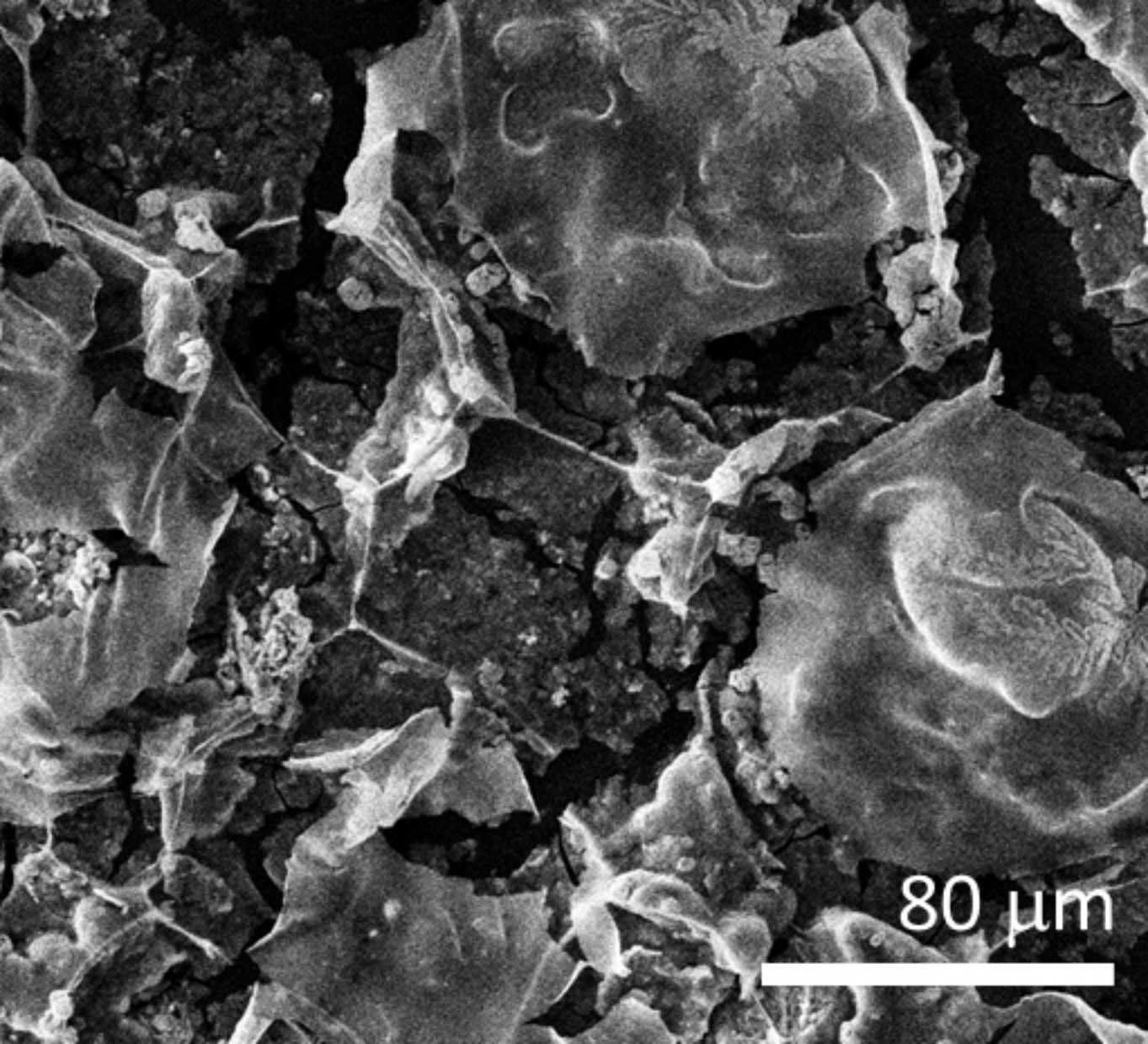
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