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1 **Cutting-edge spectroscopy techniques highlight toxicity mechanisms of copper**
2 **oxide nanoparticles in the aquatic plant *Myriophyllum spicatum***

3
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16
17 **ABSTRACT**

18 Copper oxide nanoparticles (CuO-NPs) have been increasingly released in aquatic ecosystems over the
19 past decades as they are used in many applications. Cu toxicity to different organisms has already been
20 highlighted in the literature, however toxicity mechanisms of the nanoparticulate form remain unclear.
21 Here, we investigated the effect, transfer and localization of CuO-NPs compared to Cu salt on the
22 aquatic plant *Myriophyllum spicatum*, an ecotoxicological model species with a pivotal role in
23 freshwater ecosystems, to establish a clear mode of action. Plants were exposed to 0.5 mg/L Cu salt, 5
24 and 70 mg/L CuO-NPs during 96 hours and 10 days. Several morphological and physiological
25 endpoints were measured. Cu salt was found more toxic than CuO-NPs to plants based on all the

26 measured endpoints despite a similar internal Cu concentration demonstrated *via* Cu mapping by micro
27 particle-induced X-ray emission (μ PIXE) coupled to Rutherford backscattering spectroscopy (RBS).
28 Biomacromolecule composition investigated by FTIR converged between 70 mg/L CuO-NPs and Cu
29 salt treatments after 10 days. This demonstrates that the difference of toxicity comes from a sudden
30 massive Cu^{2+} addition from Cu salt similar to an acute exposure, versus a progressive leaching of Cu^{2+}
31 from CuO-NPs representing a chronic exposure. Understanding NP toxicity mechanisms can help in
32 the future conception of safer by design NPs and thus diminishing their impact on both the
33 environment and humans.

34 **Keywords:** copper, distribution, macrophyte, nanoparticle, toxicity

35

36 1. Introduction

37 The last decades have seen an exponential increase in the use of engineered nanoparticles (NPs), *i.e.*
38 particles with at least one dimension below 100 nm [1]. Their small size confers them properties which
39 are highly valuable for both domestic and industrial purposes, such as in electronics, cosmetics, drug
40 engineering or agriculture [2]. They can be found in many products of our daily life, such as paints,
41 sunscreens, toothpaste and clothing, making them omnipresent in our environment. Products
42 containing NPs increased by 91-fold between 2005 and 2020 [3]. As an example, the global annual
43 production of copper oxide NPs (CuO-NPs) was approximately 570 tons/year in 2014 and is predicted
44 to be 1600 tons/year by 2025 [4]. CuO-NPs are mainly used as biocides [5], [6], and they are included
45 in several applications in agriculture, such as fungicides and herbicides, but also as growth regulators
46 and fertilizers [7]. Such extensive use has led to a direct contamination of many ecosystems whose
47 extent started to be acknowledged by the end of the 2000's in both aquatic and terrestrial environments
48 [8]–[11]. The aquatic environment is known to be especially at risk as it acts as a sink for pollutants

49 [12]. CuO-NPs are released into aquatic ecosystems through indirect pathways, such as runoffs and
50 leaching from industrial and agricultural sites, and also through direct pathways, with the use of
51 antifouling paints [13], [14], raising a global concern for ecological impacts of such contamination
52 [15].

53 Negative impacts have been reported on several organisms, although the toxicity range varies
54 depending on the species and environmental factors. Indeed, water quality, organic matter and pH will
55 influence the colloidal stability of metal-based NPs, and thus influence their potential toxicity [16]. It
56 is therefore challenging to assess their effects on aquatic ecosystems. Several studies have been
57 performed on aquatic species to assess the mode of action and toxicity of CuO-NPs and if it stems
58 from ionic leaching or particle-specific toxicity. No consensus has been reached so far. Especially, the
59 mechanisms behind toxicity in aquatic plants remain unclear. Few studies found that aquatic plant
60 species were more sensitive to the nanoparticulate form than the ionic one [17]–[21], while another
61 study on duckweed attributed toxicity to ionic leaching from CuO-NPs [22]. Finally, one study
62 performed on a submerged rooted aquatic plant species, *Elodea nuttallii*, showed similar effects
63 between Cu salt and CuO-NP exposure on growth and down-regulation of a Cu transporter gene,
64 COPT1, after 24h of exposure [23], suggesting that toxicity was due to ionic leaching. In order to
65 understand better the toxicity mechanisms, we need to assess Cu spatial distribution which is not
66 investigated in most studies. For this purpose, biophysical techniques represent a great asset even
67 though they are not often used in environmental sciences. Indeed, spectroscopic techniques have
68 proven to be effective tools to assess metal uptake and distribution in organisms at high spatial
69 resolution [24]. The combination of micro particle-induced X-ray emission (μ PIXE) with Rutherford
70 backscattering spectroscopy (RBS) provides unique *in situ* information on elemental mapping in plant
71 tissues and is highly relevant to assess uptake of metal based NPs [25].

72 In this study, we aimed at making use of cutting-edge spectroscopic techniques to bring new and
73 original data to answer the question of the mechanisms of action implied in CuO-NP toxicity in aquatic
74 plants in comparison with Cu salt. *Myriophyllum spicatum* (L.), a submerged OECD model species
75 (OECD TG 238, 239), was used to link dissolution, adsorption, absorption, and Cu localization to
76 CuO-NP mode of action. Cu distribution was analysed using μ PIXE coupled to RBS to avoid mixing
77 the Cu signal coming from Cu adsorbed at the surface of leaves with Cu really absorbed inside the leaf
78 tissues, allowing to map Cu distribution in leaf cross-sections. Additionally, Cu toxicity was assessed
79 through “traditional” biomarkers (such as growth, dry matter content and photosystem efficiency) and
80 by evaluating its impact on plant biomacromolecule composition via Fourier-transformed infrared
81 spectroscopy (FTIR) analysis.

82

83 **2. Material and methods**

84 **2.1. Plant growth and Cu exposure**

85 *M. spicatum* L. (Haloragaceae) was chosen as a model species of aquatic freshwater species (see
86 supporting information for more details about plant growth). In total, two concentrations of CuO-NPs
87 were used: 5 and 70 mg/L; along with one control concentration (0 mg/L) and one CuSO₄
88 concentration containing 0.5 mg/L Cu²⁺, with $n = 10$ per concentration (see supporting information for
89 more details about exposure protocol) for either 96h or 10 days of exposure. CuO-NP concentrations
90 were chosen based on literature and preliminary experiments allowing proper Cu visualization inside
91 leaf tissues by μ PIXE/RBS. Although no information on environmental CuO-NP concentration can be
92 found, studies have highlighted Cu²⁺ concentrations up to 100 mg/kg in European topsoils [26] and Cu
93 concentration of 40 mg/kg in freshwater sediments are considered environmentally-relevant [27], [28].

94 **2.2. Nanoparticle characterization**

95 The nominal diameter of NPs was determined using Transmission Electron Microscopy (TEM, Jem-
96 1400, Jeol, USA), and imageJ software for image analysis ($n = 45$). Nanoparticle hydrodynamic
97 diameter in suspension was assessed by dynamic light scattering (DLS; Zetasizer Nano ZS90, Malvern
98 Panalytical, UK) and the software Zetasizer ($n = 3$). The zeta potential in plant culture medium was
99 measured with 12 runs for both suspensions (Zetasizer Nano ZS90, Malvern Panalytical, UK).
100 Nanoparticle dissolution at the end of exposure in plant exposure medium was assessed by ICP-OES
101 (see supplementary materials for more details).

102

103 **2.3. Copper leaching and concentration in plant samples**

104 Copper concentrations in the media were measured by sampling water at the beginning and at the end
105 of exposure from experimental units of both Cu exposure times (96 h and 10 days) in order to assess
106 effective concentrations. Copper concentration in plants was measured at the end of Cu exposure after
107 acid digestion of dry plant material. For more details about sample preparation see supporting
108 information.

109 Copper and other elemental (Ca, K, P, Fe, S, Mg, Mo, Mn, Zn) concentrations in both plants and
110 media were measured using ICP-OES (Iris Intrepid II XLD, Thermo Electron, MA, USA) with a
111 detection limit of 0.0012 mg/kg for water samples and 0.00169 mg/kg for plant samples. Different
112 controls were analysed to ensure the quality of the measurements.

113

114 **2.4. Exposure endpoints**

115 **2.4.1. Growth-related endpoints**

116 Fresh mass was measured at the beginning and at the end of exposure after having gently dried the
117 plants with blotting paper, to calculate the relative growth rate based on biomass production (RGR).
118 Samples were oven-dried at 70 °C during 72 h before weighting again to measure their dry matter
119 content (DMC).

120 RGR was calculated for each experimental unit as follows:

121
$$RGR_{i-j} = (\ln(N_j) - \ln(N_i)) / t$$

122 where RGR_{i-j} is the relative growth rate from time i to j, N_i and N_j is the endpoint (fresh mass) in
123 the test or control vessel at time i and j, respectively, and t is the time period in days from i to j.

124 DMC in % was calculated as:

125
$$\%DMC = \left(\frac{100 \times DM}{FM} \right)$$

126 where FM is the fresh mass of a plant sample, DM is its corresponding dry mass.

127

128 **2.4.2. Physiological endpoints**

129 Oxidative stress was evaluated through lipid peroxidation measurements, using the production of
130 malondialdehyde (MDA) in the samples to assess membrane integrity according to Parveen et al.
131 (2017) [29] (more details in supporting information).

132 Quantum efficiency of photosystem II was estimated at the end of exposure for each experimental unit
133 using the Fv/Fm ratio, which is the ratio of the variable (Fv) to the maximum chlorophyll fluorescence
134 (Fm), *i.e.* the maximal ability of the plant to harvest light [30]. Measurements were conducted using a

135 Diving-PAM fluorometer (Heinz Walz GmbH, Germany) in a dark chamber, 30 min after dark
136 acclimatization of the plant to ensure that all reaction centers were opened for new photons. The basic
137 settings of the Diving-PAM, namely intensity of measuring light (50: MEAS-INT) and amplification
138 factor (49: GAIN) were set to 8 and 2, respectively.

139

140 **2.4.3. Biomacromolecule composition**

141 Biomacromolecule composition was determined by Fourier transformed infrared spectroscopy (FTIR).
142 Plant samples were dried for 48 h at 105°C, then ground in thin powder (> 20 mg). Samples were
143 analysed using a FTIR microscope in attenuated total reflection (ATR) mode (Thermo Nicolet NEXUS
144 470 ESR, ThermoFisher™, Massachusetts, USA) over the frequency range of 4000 – 400 cm⁻¹ with a
145 spectral resolution of 4 cm⁻¹. One spectrum was an average of 64 scans per sample. Each powdered
146 plant was placed on the sample plate and three independent technical replicates for each sample (10
147 biological replicates per treatment) were acquired. OMNIC software was used to export experimental
148 spectra after ATR correction (OMNIC™ FTIR Software, ThermoFisher™, Massachusetts, USA).
149 FTIR data treatment was performed using Orange software [31]. Briefly, data were pre-processed
150 which implies selection of the region of interest (including most of the variance among samples),
151 vector normalization and smoothing by Savitzky-Golay filter. Using the second derivative, a principal
152 component analysis (PCA) was carried out for the different exposure times (n=30 per time of exposure
153 × condition with technical replicates). The components permitting to explain at least 70% of the
154 variance were used to perform a subsequent linear discriminant analysis (LDA). This approach
155 permitted to plot the samples and detect differences among groups of samples. When a difference was
156 detected, a logistic regression was applied to the pre-processed data to identify wavenumbers
157 contributing to the difference detected among groups by the PCLDA.

158 **2.4.4. Spatial distribution and semi-quantification of Cu**

159 Absorption and adsorption of Cu in leaves were mapped and measured using a nuclear microprobe. A
160 combination of micro-particle induced X-ray emission (μ PIXE) and Rutherford backscattered
161 spectroscopy (RBS) was used for elemental mapping and semi-quantification. Leaves were thoroughly
162 washed three times with deionized water to take off Cu lightly bound to the surface and immersed in a
163 droplet of resin (Tissue Teck Sakura[®]) to be immediately cryo-fixed by plunging the sample in
164 isopentane cooled with liquid nitrogen. Samples were then cut in thin cross-sections (40 μ m) using a
165 cryo-microtome (Leica, Germany) and finally freeze-dried (48 h, -52°C, 0.01 mbar). Freeze-dried
166 sections were analyzed at the nuclear microprobe available at the Atomic Energy Commission (CEA)
167 Center of Saclay (France) with a proton source of 3 MeV, a beam focused to 2.5 μ m and a current
168 intensity of 500 pA. Data processing was performed using Rismin software [32] to define regions of
169 interest and extract spectra, and SIMNRA [33] and GUPIX [34] codes to fit RBS and PIXE data,
170 respectively. The Cu/(K+Ca) ratio was used as a Cu enrichment indicator as K and Ca are the most
171 abundant endogenous elements.

172

173 **2.4.5. Statistical analyses**

174 Results were analyzed using the R studio software (R Core Team (2016) V 3.3.1) and analyses were
175 performed within each exposure time (96 h and 10 days). Homoscedasticity was tested using Bartlett
176 test. Data normality was assessed with Shapiro-Wilk test on ANOVA residuals, with log-
177 transformation when normality assumption was not met. Two-way ANOVAs were performed on
178 results showing normal distribution, with or without log transformation, to assess the interactive
179 effects of Cu concentrations and time of exposure. Tukey HSD post-hoc tests were used to identify

180 significant differences among Cu concentrations and exposure times. Kruskal-Wallis tests were used in
181 dataset when no normality was found despite log-transformation. The differences in plant inorganic
182 composition resulting from exposure were assessed using a linear discriminant analysis (LDA, $n = 10$,
183 ade4 package [35]). The significance of the discriminant analyses was assessed using Monte-Carlo
184 tests with 1000 repetitions.

185 **3. Results**

186 **3.1. CuO-NP characterization, Cu²⁺ concentration and leaching**

187 Nominal diameter of CuO-NPs was on average 64.9 ± 8.5 nm according to TEM images ($n = 45$, **Fig.**
188 **1A**). Sedimentation was visually observed with NP deposition on the leaves of *M. spicatum* after the
189 first 2 hours of exposure, forming a thin black layer (**Fig. S1**). Hydrodynamic diameter measured at
190 different times showed agglomeration of NPs from the beginning of exposure with no strong evolution
191 over time, except after 10 days at the highest concentration (**Fig. 1B**). Hydrodynamic diameter based
192 on Z-average was 331 nm after 96 h of exposure for both concentrations, and 59 nm and 321 nm after
193 10 days of exposure at 5 mg/L and 70 mg/L CuO-NPs, respectively. Finally, the zeta potential of CuO-
194 NPs in Smart & Barko was -21.7 ± 3.5 mV.

195 Total Cu in the medium for all concentrations at the beginning of exposure were 0, 0.48 ± 0.01 mg/L
196 for CuSO₄, 4.44 ± 0.61 mg/L and 70.22 ± 4.73 mg/L for CuO-NPs. Cu²⁺ concentration in CuSO₄
197 treatment was 0.14 ± 0.05 mg/L after 96 h and 0.17 ± 0.03 mg/L after 10 days of exposure. Regarding
198 CuO-NP treatments, a small proportion of Cu²⁺ leached over time from the NPs, with 11.5 % and 0.8
199 % of Cu²⁺ from 5 and 70 mg/L CuO-NP suspensions measured in the water column after 96 h,
200 respectively (2-way ANOVA, $F_{1,20} = 1178.2$, $P < 0.0001$, **Fig. 1C**). After 96h, the leached Cu²⁺
201 concentrations from NPs in the water column corresponded to the Cu salt treatment with final Cu²⁺

202 concentration in the medium of 0.5 mg/L. After 10 days of exposure, the Cu²⁺ concentration in the
203 medium decreased by 86% and 79% for 5 mg/L and 70 mg/L CuO-NP suspensions, respectively,
204 likely due to NP sedimentation on sediment (**Fig. S1**) and to plant adsorption/absorption of Cu²⁺.

205

206 **3.2. Copper concentration and distribution in plants**

207 Significant differences in bulk Cu concentrations in plants were observed among treatments (2-way
208 ANOVA, $F_{3,72} = 3784.903$, $P < 0.0001$) but not between times of exposure. On average at both
209 exposure times, Cu concentrations in plants were 4.7 ± 0.5 , 8.1 ± 1.1 and 41.7 ± 4.7 mg/g dry weight
210 (DW) of plants exposed to 0.5 mg/L Cu salt, 5 and 70 mg/L CuO-NPs, respectively (**Fig. 2A**).

211 To go further into Cu internalization and localization in plant leaf, spatial distribution analysis was
212 performed on plants exposed to 0, 0.5 mg/L Cu salt and 70 mg/L CuO-NPs for 10 days. Homeostasis
213 level of Cu was found in control plants for basal metabolism (**Fig. 2B, C**), whereas significantly higher
214 accumulation of Cu was found at 0.5 mg/L Cu salt (**Fig. 2D**) and 70 mg/L CuO-NPs (**Fig. 2E**, 2-way
215 ANOVA, $F_{2,38} = 340.64$, $P < 0.0001$). The highest Cu accumulation was detected on leaf epidermis for
216 both treatments, with more than 3 times Cu level in plant sections exposed to 70 mg/L CuO-NPs
217 compared to plants exposed to 0.5 mg/L Cu salt (**Fig. 2E**). Cu accumulation decreased in parenchyma
218 and vascular cylinder and was similar for both treatments. Our results showed that Cu from both
219 treatments was internalized by the plants in parenchyma and vascular tissues.

220

221 **3.3. Copper toxicity to plants**

222 **3.3.1. Copper toxicity based on “traditional” endpoints**

223 Relative growth rate was only significantly impacted by Cu salt exposure, inhibiting growth by 57%
224 after 96 h and by 80% after 10 days of exposure (2-way ANOVA, $F_{3,72} = 12.64$, $P < 0.0001$, **Fig. 3A**).
225 CuO-NP exposure inhibited growth by 30% at 70 mg/L after 10 days of exposure, however the
226 variation among replicates was too high to highlight a significant difference.

227 Dry matter content significantly increased after 10 days of exposure at 0.5 mg/L Cu salt, with 14% of
228 DMC in exposed plants against 8% for others (2-way ANOVA, $F_{3,71} = 6.423$ $P = 0.0149$, **Fig. 3B**).

229 Lipid peroxidation levels were significantly increased at both exposure times for 0.5 mg/L Cu salt, and
230 after 10 days of exposure to 70 mg/L CuO-NPs (2-way ANOVA, $F_{3,63} = 21.559$, $P < 0.001$, **Fig. 3C**).

231 An interactive effect was found between treatments and exposure time (2-way ANOVA, $F_{3,63} = 5.333$,
232 $P = 0.002$), with a significant effect of CuO-NPs on lipid peroxidation only after 10d of exposure
233 compared to Cu salt.

234 Finally, no significant effect of CuO-NPs was observed on Fv/Fm at any concentration despite the
235 deposition of a thin black layer on plant leaves, whereas Cu salt significantly decreased Fv/Fm by 12%
236 after 96 h of exposure (2-way ANOVA, $F_{3,72} = 12.956$, $P < 0.001$, **Fig. S1 & S2**).

237

238 **3.3.2. Copper toxicity based on biomacromolecule composition**

239 Biomacromolecule composition significantly changed among treatments at both exposure times (**Fig.**
240 **4**). The significant differences and the peak interpretations are listed in **Table 1**. After 96 h of
241 exposure, plants at 0.5 mg/L Cu salt segregated from other treatments based on the first and third
242 dimensions of the PCA toward the up-right corner, and plants exposed to 70 mg/L CuO-NPs
243 segregated along the first dimension on the right side (**Fig. 4A, B**). Plants exposed to Cu segregated

244 from the control based on the first dimension, with an overlap between 5 and 70 mg/L CuO-NPs
245 treatments. These differences in composition were observed mostly in proteins, phenolic compounds,
246 carbohydrates and cellulosic compounds (**Table 1**). After 10 days of exposure, plants exposed to 5
247 mg/L CuO-NPs remained the closest to control plants in terms of composition and remained on the left
248 side of the first dimension, whereas composition from plants exposed at 0.5 mg/L Cu salt and 70 mg/L
249 CuO-NPs converged on the right side of the same dimension (**Fig. 4C, D**). The plants exposed to these
250 two treatments exhibited higher absorbances for the peaks representing polysaccharides, carbohydrates
251 and proteins compared to control plants (**Table 1**).

252

253 **3.3.3 Effect of Cu exposure on plant ionome**

254 Plant exposure to ionic Cu and CuO-NPs significantly influenced their inorganic composition after 96
255 h and 10 days, as revealed by the linear discriminant analyses (LDA) based on ICP-OES
256 measurements realized on the whole plants (Monte-Carlo test, $P = 0.001$, 22.47 % and 22.62% of
257 inertia explained, respectively, **Fig S3**). A significant increase of Ca concentrations was observed at
258 both times of exposures for plants exposed to ionic Cu (2-way ANOVA, $F_{3,72} = 40.41$, $P < 0.001$),
259 whereas plants exposed to 70 mg/L CuO-NPs showed a significantly higher Ca concentration only
260 after 96 h (**Fig. S4A**). Magnesium concentration significantly increased in plants exposed to ionic Cu
261 after 96 h, and increased both in ionic Cu and 70 mg/L CuO-NPs treatments after 10 days (Kruskal-
262 Wallis, $df = 3$, $P < 0.001$, **Fig. S4B**). Sulfur concentration in plants significantly decreased after 10 d of
263 exposure to ionic Cu (2-way ANOVA, $F_{2,50} = 5.61$, $P < 0.001$, **Fig. S4C**) whereas Zn concentration
264 was significantly higher for plants exposed to 70 mg/L CuO-NPs at both times of exposure (Kruskal-
265 wallis, $df = 3$, $P < 0.005$, **Fig. S4D**).

266 These results were similar to those found by μ PIXE/RBS in plant leaves after 10 days (**Fig. S5**).
267 Calcium concentrations were significantly higher for plants exposed to ionic Cu, and found rather
268 accumulated in parenchyma and epidermis tissues (**Fig. S5A**). Sulfur was found significantly more
269 abundant in plants exposed to CuO-NPs with no difference in distribution among tissues due to
270 variation among cross-sections (**Fig. S5B**) and Zn was found at higher concentrations in plants
271 exposed to CuO-NPs at 70 mg/L, with no difference in distribution among plant tissues (**Fig. S5C**).

272 **4. DISCUSSION**

273 Our results, based on an original combination of spectroscopic techniques (FTIR and μ PIXE/RBS) and
274 a thorough characterization of NP dynamics in suspension, suggest that Cu ion leaching is the main
275 driver of CuO-NP toxicity even though a specific nano form effect cannot be excluded but would
276 remain minor. These conclusions confirmed some data available in the literature on different
277 organisms. Indeed, few studies suggest that the most prominent mode of action of CuO-NPs on
278 organisms is the leaching of ionic Cu^{2+} from NPs, inducing reactive oxygen species (ROS) production
279 and subsequent stress in organisms [15], [36]. This is shared by most heavy-metal based NPs which
280 are prone to dissolution, such as Ag-NPs or ZnO-NPs [5], [37]–[39], although some NPs, such as TiO_2
281 and CeO_2 , behave differently [40], [41]. This mode of action was shown from single-cell organisms
282 [42]–[44], to more complex organisms such as zooplankton, fish and aquatic plant species [45]–[47].
283 A big difference in toxicity can be found from one study to another in the literature even within a same
284 species, which may be related to environmental conditions [15], [18].

285 Indeed, the behavior of NPs and the subsequent ionic leaching is directly linked to water physico-
286 chemical parameters [47]. In our exposure conditions, CuO-NPs were prone to quickly form
287 agglomerates as a result of low repulsive forces, high surface energy and high ionic strength of the
288 medium leading to a high sedimentation rate, as demonstrated by the NP deposition on leaves. The

289 same kind of results was obtained in the literature for other heavy-metal based NPs [48]–[50]. The
290 presence of CaCl_2 in the Smart & Barko medium likely increased the formation of agglomerates and
291 sedimentation speed, as Ca^{2+} is known to form bridges in solution [51]. The black deposition on the
292 plant leaves visually observed suggests that CuO-NPs were tightly adsorbed at the plant surface as it
293 was not eliminated with several washing steps. Shi *et al.* (2013) noticed a similar black deposition on
294 the roots of *Elsholtzia splendens*, a terrestrial plant, after hydroponic exposure which resulted in very
295 high concentrations at the surface of plant roots [49].

296 The Cu^{2+} concentration **leached** from CuO-NPs after 96 h was similar between the two CuO-NP
297 treatments and equivalent to the Cu salt concentration in solution (*i.e.* 0.5 mg/L). After 10 days of
298 exposure, Cu^{2+} leached from CuO-NPs was still similar between the two concentrations, but strongly
299 decreased compared to the concentration found at 96 h. This could be explained by the continuous
300 uptake of Cu^{2+} by plants, leading to Cu decrease in the water column. Furthermore, underwater
301 photosynthesis changes the pH over time through the release of HCO_3^- , which can influence colloidal
302 stability and increase the formation of agglomerates, decreasing ionic leaching [52], [53]. This,
303 combined with the formation of hetero-agglomerates with organic matter produced by *M. spicatum*,
304 can decrease further the colloidal stability and ionic leaching, as it is proportional to the surface area to
305 volume ratio [49], [51], [53]–[55].

306 In our study, CuO-NPs were less toxic to *M. spicatum* than Cu salt, especially after 96 h where no
307 effect of NPs was observed, whereas Cu^{2+} concentration in the medium was similar between Cu salt
308 and NPs. An increase in lipid peroxidation was observed after 10 days of exposure to 70 mg/L CuO-
309 NPs and was the only significant sign of toxicity when copper was provided under a NP form. On the
310 other hand, Cu salt strongly decreased growth and increased lipid peroxidation at both exposure times,
311 and increased DMC after 10 days as a result of stress [56], [57]. This result could be surprising as Cu

312 bulk concentration was higher (by a factor of 9) in plants exposed to 70 mg/L CuO-NPs compared to
313 plants exposed to Cu salts.

314 However, Cu bulk concentration does not give any information as to Cu internalization and
315 localization, especially as CuO-NP accumulation was visible on plant surface. The data provided by
316 the μ PIXE/RBS analysis confirmed that Cu mostly accumulated on the plant surface (epidermis) with
317 a factor of 3 between Cu salts and CuO-NPs 70 mg/L, even if this difference was not significant due to
318 high variation among replicates. Furthermore, when focusing on the internalized Cu, a similar
319 concentration was found in plants exposed to Cu salt and to 70 mg/L CuO-NPs. This is also in line
320 with the fact that equivalent ionic Cu concentrations were measured in the medium suggesting an
321 internalization which is mainly occurring under ionic form. Previous work has shown that ionic
322 internalization is detected through an homogenous distribution with μ PIXE, whereas a dot-like
323 distribution is linked to nanoparticulate form [24]. In our study, internalization of NPs themselves
324 cannot be excluded as some highly concentrated sub-micrometric to micrometric spots were detected
325 by μ PIXE inside leaf parenchyma, compared to the more homogeneous Cu distribution in the vascular
326 tissues. This phenomenon has also been demonstrated in other studies on both terrestrial and aquatic
327 species [18], [21], [58], [59], but further investigations using X-ray absorption spectroscopy would be
328 needed for speciation confirmation. Indeed, some studies demonstrated that aquatic species, such as
329 *Chlorella pyrenoidosa* and *Eichhornia crassipes*, were able to transform CuO-NPs into other Cu
330 species, such as Cu₂S or Cu₂O-NPs, highlighting the need to go deeper into the speciation of CuO-NP
331 once it enters biological barriers as speciation can influence both its translocation and toxicity [18],
332 [60].

333 These findings explain why such a high Cu concentration in plants exposed to CuO-NPs was found by
334 ICP, but do not explain why Cu salt was more toxic to the plants despite a similar internal

335 concentration found by μ PIXE/RBS. This difference in toxicity between Cu salt and CuO-NP
336 treatments can be explained by the sudden addition of soluble Cu salt compared to the progressive
337 leaching of ionic Cu from CuO-NPs [61]. The results for biomacromolecule composition support these
338 findings as a gradual convergence between plants exposed to 70 mg/L CuO-NPs and Cu salt was
339 observed during exposure. A strong effect of Cu salt was observed on plant composition after 96 h
340 compared to CuO-NP treatments (5 and 70 mg/L) whereas the overall ionic Cu concentration in the
341 medium was similar. This lower impact of NPs despite similar ionic concentration as Cu salt suggests
342 that the leaching was progressive, and the subsequent stress was of lower amplitude and mitigated over
343 the duration of our exposure. A change in phenolic compounds, proteins and cellulosic compounds
344 was observed in plants exposed to Cu salts, likely as the result of a stress, and possibly corresponding
345 to an antioxidant response [45], [62], [63], as well as a mechanism to maintain membrane integrity
346 [59], [64]. Additionally, ICP-OES and μ PIXE/RBS analyses showed changes in concentrations of S or
347 Zn mostly impacted by ionic Cu and by CuO-NPs to a smaller extent. It could be linked to shifts in the
348 antioxidant balance, as these elements act as co-factors for several detoxification enzymes and Cu
349 regulation pathways [65]–[67]. An interesting response was the increased Ca concentration and its
350 distribution primarily in epidermis tissues. Studies have shown that Ca was an important part of the
351 signaling pathway and stress response in plants, for instance with the calmodulin pathways for
352 signaling or the formation of egg box structures for heavy metal regulation in cell walls [66], [68].
353 More specific assays would be necessary to assess the extent of the stress response triggered by
354 exposure, and its specific pathways, by targeting mechanisms such as enzymatic activities,
355 transcription, and production of antioxidant compounds.

356 Several studies on different aquatic micro and macro-organisms have found similar results in which Cu
357 internal concentration resulting from CuO-NP exposure was not correlated to the observed toxicity,

358 and the ionic counterpart was much more toxic for a similar or lower internal concentration, supporting
359 our finding [17], [23], [42], [61], [69]. For instance Wu *et al.* (2020) demonstrated no correlation
360 between toxicity and Cu concentration in three aquatic organisms exposed to CuO-NPs, compared to
361 Cu salt [15]. Adam *et al.* (2015) found a higher toxicity in *Daphnia magna* exposed to Cu salt whereas
362 Cu concentration was higher in organisms exposed to CuO-NPs. The toxicity of CuO-NPs was
363 attributed to the Cu ions formed during NP dissolution [70]. Similarly, the marine bacteria *Vibrio*
364 *anguillarum* showed a lower sensitivity to CuO-NPs than to Cu salt, and toxic effects were attributed
365 to progressive Cu ions leaching from NPs [43].

366 Overall, exposure to Cu salt can be considered as an acute exposure to ionic Cu, triggering a rapid and
367 strong change in plant physiology, whereas exposure to CuO-NPs corresponds to a chronic exposure to
368 ionic Cu, inducing progressive physiological adjustments of lower amplitude. The antioxidant balance
369 can mitigate a chronic exposure over time by inducing progressive physiological changes [62], [64],
370 whereas an acute exposure could lead to a tipping point where the stress can no longer be copped with
371 [23], [71].

372

373 **5. CONCLUSION**

374 This study assessed the toxicity of CuO-NPs compared to Cu ions from CuSO₄ salt on *M. spicatum*, a
375 model aquatic plant species. Based on our observations, the toxicity of CuO-NPs appeared driven by a
376 progressive ionic leaching from NPs and was found less toxic than Cu salt, as the plant was able to
377 adapt and mitigate stress over time through physiological changes. Cutting-edge techniques showed
378 that most of the Cu leached from NPs was adsorbed at the plant leaf surface rather than absorbed,
379 which would have not been possible with other bulk analyses such as ICP-OES. μ PIXE/RBS provided

380 a new perception of the links between toxicity and accumulation regarding heavy metal-based NPs.
381 Thanks to high-throughput FTIR technique, we were able to visualize global biomacromolecule
382 composition shifts resulting from exposure. In future experiments, we will investigate more specific
383 response patterns such as phenolic compound production and cell walls components. These findings do
384 not exclude a nanospecific toxicity mechanism, as NP internalization was suggested by μ PIXE, but
385 further studies on speciation within organisms remain to be done.

386

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394

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396 Formal analysis, Writing - Original draft preparation, **AE:** Writing – original draft preparation,
397 Funding Acquisition, Supervision, **CA:** Data acquisition, **SS:** Resources, Data acquisition, Data
398 analysis, **HCM:** Resources, Data acquisition, **CL:** Conceptualization, Data acquisition, Data analysis,
399 Writing-Original draft preparation, Supervision.

400

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- 644

645 **Table 1.** Spectral assignment and significant differences in biomacromolecule composition of
 646 *M. spicatum* exposed to Cu salt at 0.5 mg/L and CuO-NPs at 5 and 70 mg/L during 96 hours and
 647 10 days. Peak numbers refer to Figure 4, the significant differences are either between (-)
 648 concentrations, or one treatment differs from all others (alone). Significant differences were set
 649 at p-value < 0.05 and were calculated with ANOVAs on Orange software for each wavenumber.

Peaks	Significant differences	Wavenumber cm ⁻¹	Definition of the spectral assignment
96h	1	0.5	1630-1610 C=O stretching carbonyl, C=C aromatic ring vibration [72] related to phenolic compounds [73]
	2	0 - 0.5, 70	1580-1570 C=N and N-H stretching from proteins [74]
	3	0 - 0.5, 70	1482-1470 C-H bending, structural carbohydrate [75]
	4	0 - 0.5, 70	1390-1380 C-H bending vibrations [76]
	5	0.5	1210-1180 C-O stretching from alcohol, esters, amide III from proteins, from polysaccharides in cellulosic compounds [77]
	6	0 - 5, 70	1065 S=O stretching from sulfoxides [72], C-O stretching from polysaccharides [74]
	7	0 - 0.5, 70	1017 C-O stretch from carbohydrates [75], [78]
	8	0 - 70	1000-985 C=C bending from alkene, C-O stretching from polysaccharides [79]
	9	0.5	940-900 C=O, C=C bending from alkene [80]
10d	1	0.5	C=O stretching, esters from lipids, polysaccharides from cellulose [74], [81] and phenolic compounds [73]
	2	0	1574 C=N and N-H stretching from proteins [74]
	3	5 - 0	1502 C=C aromatic stretching bond [76]
	4	0, 5 - 0.5, 70	1430-1370 C-H bending vibrations [76]
	5	0	1320 C-O, C-H and C-N stretching vibration in polysaccharides, aromatic amines and cellulosic compounds [75], [82]
	6	0	1200-1175 C-O stretching from polysaccharides in cellulosic compounds [75], [77]
	7	0, 5, 70 - 0, 5	1140-1125 C-O stretching from carbohydrates [75]
	8	0 - 0.5	1098 C-C and C-O stretches in carbohydrate [73]
	9	0 - 70	1037-1004 OH and C-OH stretching from cell wall polysaccharides [74]

650

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Figure captions

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653

654 **Figure 1.** (A) Nominal diameter of CuO-NPs through Transmission Electron Microscope, (B) NP
655 hydrodynamic diameter in Smart and Barko medium (C) Ionic Cu²⁺ leached from CuO-NP
656 suspensions of 5 and 70 mg/L after 96 hours and 10 days. Different lowercase letters represent
657 significant differences among experimental conditions (HSD Tukey test after 2-way ANOVA), $n = 6$.

658

659 **Figure 2.** (A) Cu concentrations in *Myriophyllum spicatum* plants measured with ICP-OES $n = 10$ and
660 (B, C, D, E) distribution of Cu analysed by micro-particle induced X-ray emission coupled to
661 Rutherford backscattered spectroscopy in leaf cross-section. (B) displays semi quantitative information
662 (Cu/(K+Ca)) in the different tissues of the cross-section (all: data for the full section, ep: epidermis,
663 par: parenchyma, vc: vascular cylinder). Lowercase letters indicate significant differences among
664 conditions according to HSD-Tukey test following 2-way ANOVA ($p < 0.05$) \pm SE, $n = 4$. Maps show
665 without (C) or with Cu contamination: 0.5 mg/L Cu salt (D) or 70 mg/L CuO nanoparticles (E) for 10
666 days. Scale bar: 20 μ m. Color scale in the third map of CuO-NPs condition has been set to the same
667 level than the Cu map of the Cu salt condition for easier comparison.

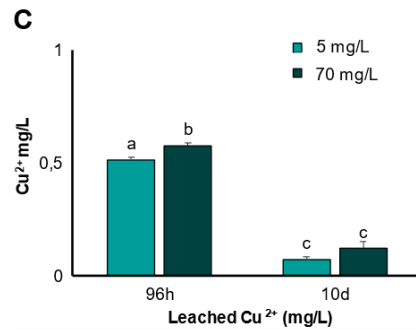
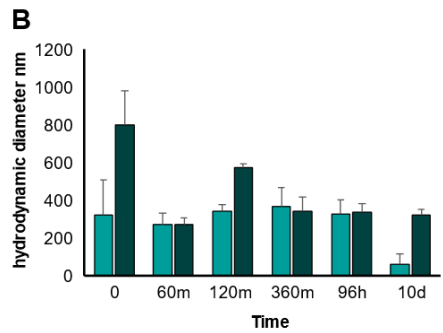
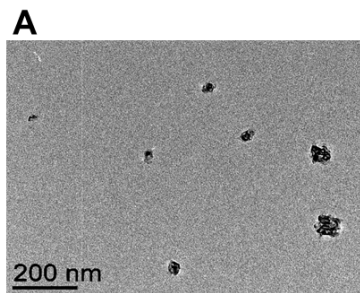
668

669 **Figure 3.** (A) Relative growth rates with Cu²⁺ leached from CuO-NPs in mg/L displayed in italic, (B)
670 Dry matter content in % and (C) Malondialdehyde in nmol/g fresh weight of *M. spicatum* exposed to
671 0, 0.5 mg/L Cu²⁺ from CuSO₄, 5 and 70 mg/L CuO-NPs for 96 hours or 10 days. 2-way ANOVA P-
672 values for Cu effects are provided; similar lowercase letters indicate conditions that did not
673 significantly differ (HSD Tukey test), \pm SE, $n = 10$ except for MDA where $n = 6$.

674 **Figure 4.** Biomacromolecule composition of *M. spicatum* analyzed by FTIR exposed to Cu salt at 0.5
675 mg/L and CuO-NPs at 5 and 70 mg/L during (A) 96 hours and (C) 10 days with significant differences
676 among treatments highlighted by a logistic regression marked with black arrows (n=120 per exposure
677 time), and PCLDA analyses of the FTIR spectra for exposures during (B) 96 hours and (D) 10 days.
678 Numbers in A, C refers to the peaks listed in Table 1.

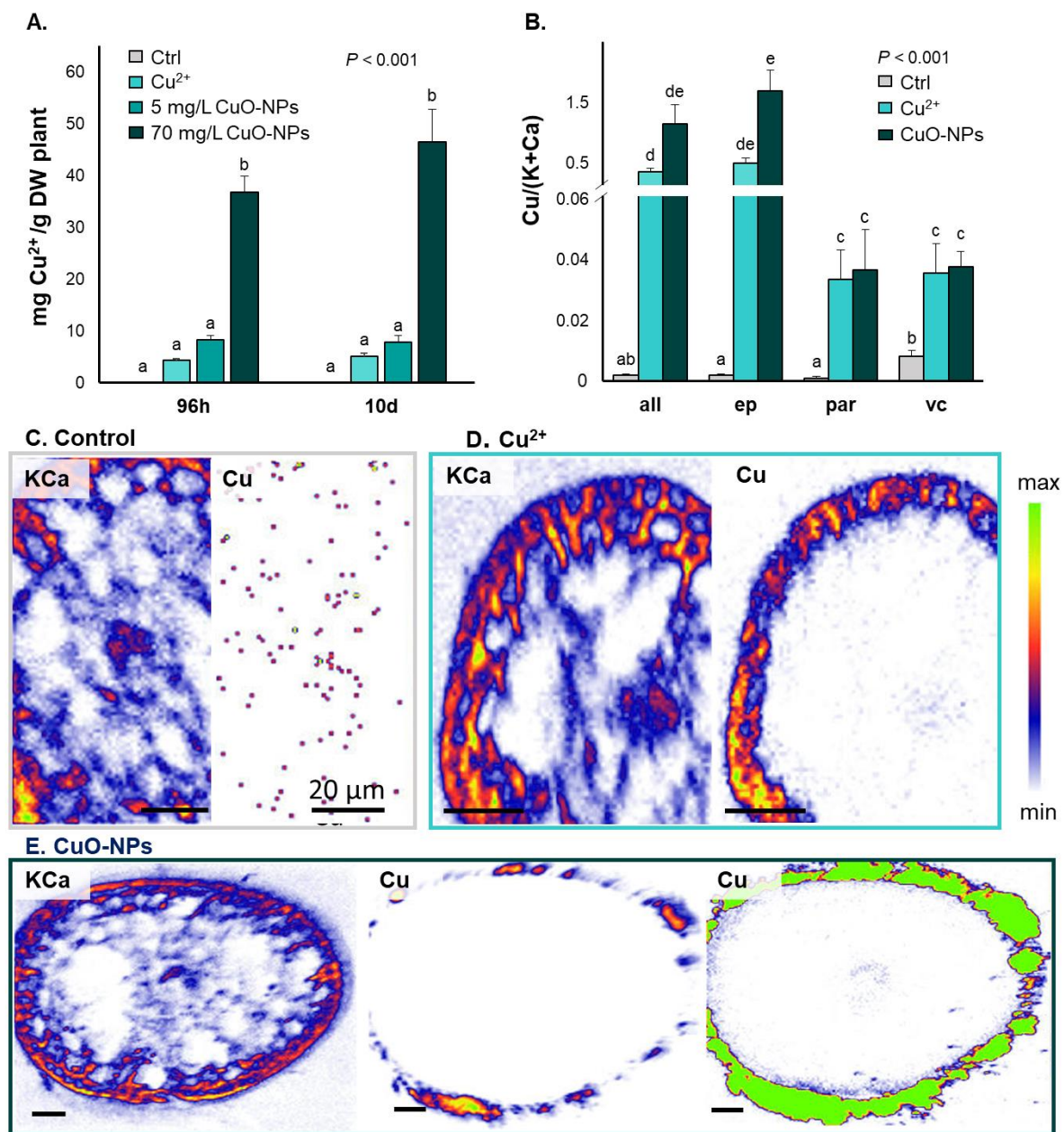
679

680 **Figure 1**



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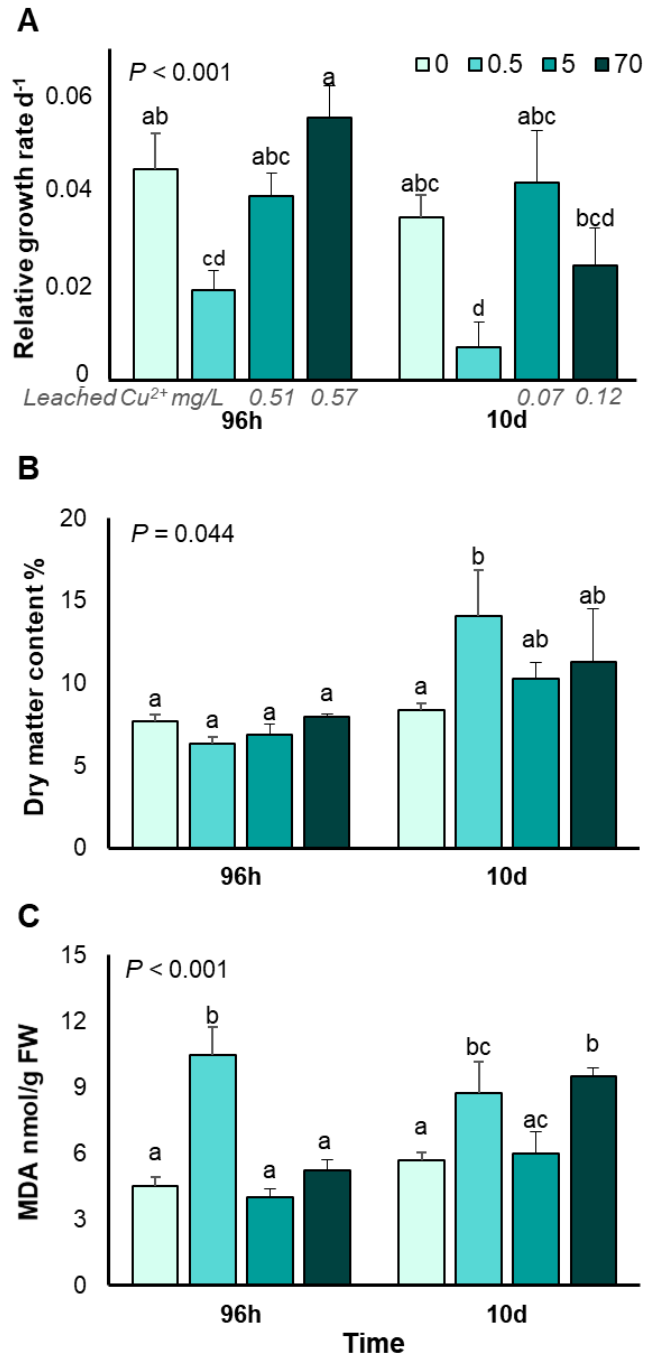
682 **Figure 2**



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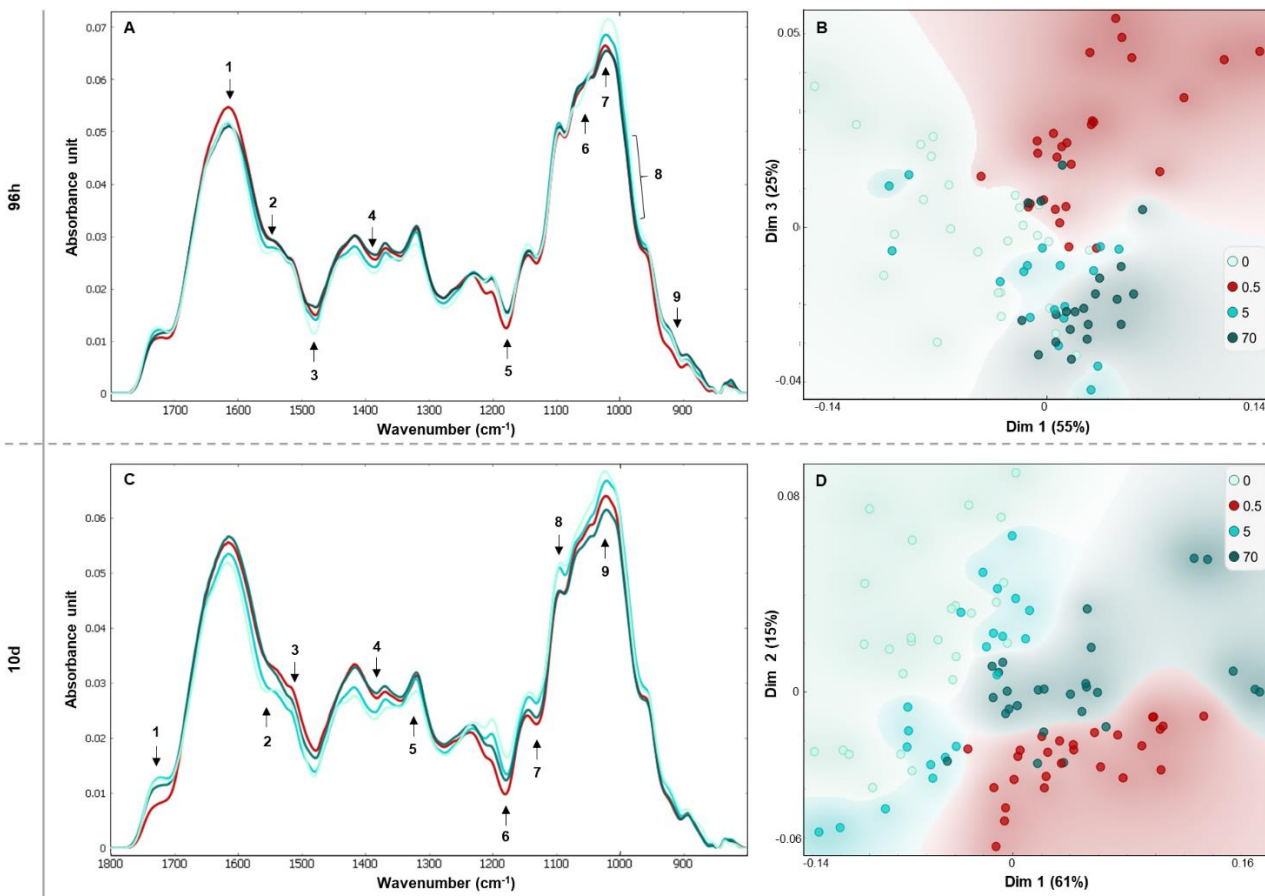
685 **Figure 3**



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687

688 **Figure 4**



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