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29 **Interaction of Copper Based Nanoparticles to Soil, Terrestrial and Aquatic Systems:**

30 *Critical Review of the State of the Science and Future Perspectives*

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**61 Abstract**

62 In the past two decades, increased production and usage of metallic nanoparticles (NPs) has inevitably increased  
63 their discharge into the different compartments of the environment, which ultimately paved the way for their  
64 uptake and accumulation in various trophic levels of the food chain. Due to these issues, several questions have  
65 been raised on the usage of NPs in everyday life and has become a matter of public health concern. Among the  
66 metallic NPs, Cu-based NPs have gained popularity due to their cost-effectiveness and multifarious promising  
67 uses. Several studies in the past represented the phytotoxicity of Cu-based NPs on plants. However,  
68 comprehensive knowledge is still lacking. Additionally, the impact of Cu-based NPs on soil organisms such as  
69 agriculturally important microbes, fungi, mycorrhiza, nematode, and earthworms are poorly studied. This review  
70 article critically analyses the literature data to achieve a more comprehensive knowledge on the toxicological  
71 profile of Cu-based NPs and increase our understanding of the effects of Cu-based NPs on aquatic and terrestrial  
72 plants as well as on soil microbial communities. The underlying mechanism of biotransformation of Cu-based  
73 NPs and the process of their penetration into plants has also been discussed herein. Overall, this review could  
74 provide valuable information to design rules and regulations for the safe disposal of Cu-based NPs into a  
75 sustainable environment.

76

77 **Keywords:** Bioaccumulation; Bioavailability; Biotransformation; Cellular; Copper; Cytotoxic; Effects;  
78 Emissions; Exposure; Fate; Freshwater; Genotoxicity; Nanoparticles; Nanotechnology; Microorganism;  
79 Permissible levels; Phytotoxicity; Sediments; Soil; Sources; Sub-cellular; Techniques; Toxicity mechanism;  
80 Trophic transfer; Ultrastructure

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## 125 **Introduction**

126

127 In recent years, potential effects of engineered nanoparticles (ENPs), and more so of metallic and metal-oxide  
128 NPs, on aquatic and terrestrial systems have received increased attention due to their wide applications and  
129 consequential release into the environment. Metallic NPs possess unique properties for potential use in the  
130 rapidly growing nanotechnology industry (Ali et al. 2015; Arruda et al. 2015; Saleem et al. 2017). Various  
131 products containing NPs are currently in the marketplace, and many are still being added to the list (Ahmed et al.  
132 2018b; Rajput et al. 2018c; Vance et al. 2015). The Global Market for Metal Oxide Nanoparticles indicates that  
133 the metal oxide NPs production could increase from 0.27 million tons (2012) to 1.663 million tons by 2020 (The  
134 Global Market for Metal Oxide Nanoparticles to 2020). Among them, Cu-based NPs have wide applications in  
135 the field of metallurgy, electronics, automotive, fuel, transportation, machinery etc. The annual production of Cu  
136 was approximately 18.7 million metric tons in 2015 (Keller et al. 2017), out of which a small fraction of  
137 approximately 200 tons was comprised of Cu-based NPs (Keller and Lazareva 2013). Since then, the use of Cu-  
138 based NPs has been rapidly escalating into applications such as solar cells, sensor development, catalysts,  
139 hydrogen production, drug delivery, catalysts for typical C-N cross-coupling reactions and light emitting diodes  
140 (Keller et al. 2017; Rajput et al. 2017b). Due to their antimicrobial and antifungal properties, Cu-based NPs are  
141 suitable for biomedical applications and are also used in water treatment (Ben-Sasson et al. 2016), textile  
142 industries (Sedighi and Montazer 2016), food preservation, and agricultural practices (Montes et al. 2016;  
143 Ponmurugan et al. 2016; Ray et al. 2015). The rapid production and multifarious applications of Cu-based NPs  
144 in various industries have necessitated the assessment of their impacts on the environment (Ahmed et al. 2018b,  
145 c).

146 Copper (Cu) is a naturally occurring ubiquitous element present in the environment with a concentration  
147 around 60 g per ton in the Earth's crust (Ojha et al. 2017) and essential micronutrient for plant growth at certain  
148 concentrations and is known to play important roles in mitochondrial respiration, hormone signalling, cell wall  
149 metabolism, iron mobilization, and electron transport (Yruela 2009). However, at higher concentrations, Cu is  
150 generally toxic to plants and other organisms including algae, mussels, crustaceans, and fish (Aruoja et al. 2009;  
151 Braz-Mota et al. 2018; Katsumiti et al. 2018; Ruiz et al. 2015). While there is no data available on the  
152 concentration of CuO-NPs in the soil total Cu could range from 2-100 mg kg<sup>-1</sup> in unpolluted soils (Nagajyoti et  
153 al. 2010). Soil receives Cu-based NPs from direct application of agricultural nano-products and industrial wastes  
154 (Adeleye et al. 2016; Rajput et al. 2017b, 2018b). The toxic action of pesticides specifically Cu-based NPs and  
155 Cu-based nano pesticides (e.g., Kocide 3000) makes them appropriate to be used for the control of plant  
156 pathogens and pests (Anjum et al. 2015; Shahid and Khan 2017). Cu-based fungicides have been used for more

157 than a century contributing to soil contamination based on their  $\text{Cu}^{2+}$  content, allowing them to function as a  
158 reducing or oxidizing agent in biochemical reactions. Terrestrial species can have more interactions with NPs  
159 because up to 28% of the total NPs production ends into soils (Keller and Lazareva 2013). Substantially  
160 increased production of Cu-based NPs in the last decade emphasizes the need of thorough and systematic  
161 investigation of nano-Cu release, environmental fate, bioavailability, dissolution of  $\text{Cu}^+/\text{Cu}^{2+}$  ions from Cu-based  
162 NPs, exposure routes, and their toxic impacts on non-target organisms (Keller et al. 2017).

163         Plants are one of the most important entities and provide a very large surface area for NPs exposure via  
164 roots and above ground parts (Dietz and Herth 2011). For instance, the air-dispersed NPs may penetrate and  
165 transport via the stomatal openings (Pullagurala et al. 2018; Raliya et al. 2016). Different plants exhibit specific  
166 behaviours towards excess metal present in the growth medium. In particular, metal-tolerant plants could limit  
167 the uptake of NPs into photosynthetic tissues by restricting the transport of metals across the root endodermis  
168 and storing them in the root cortex; hyperaccumulating plants could compile excess NPs in the harvestable  
169 tissues (Manceau et al. 2008). The exact mechanism of plant defence towards NPs toxicity is not fully  
170 understood.

171         At present, inadequate information is available on how Cu-based NPs affect the soil organisms, for  
172 instance, agriculturally important microbes, fungi, nematodes and earthworms. The NPs may affect soil flora  
173 directly by inducing changes in the bioavailability of other toxins and nutrients or indirectly via interactions with  
174 natural organic compounds possible interactions with toxic organic compounds which may increase or decrease  
175 the toxicity of NPs (Haris and Ahmad 2017).

176         In order to get more in-depth knowledge of Cu-based NPs, this review critically assessed the literature  
177 data present over effects of Cu-based NPs on terrestrial and aquatic ecosystems, the interaction of soil microbial  
178 communities with Cu-based NPs, the bioaccumulation of Cu-based NPs in plants and their toxicity mechanism,  
179 and their biotransformation in soil (Figure1).

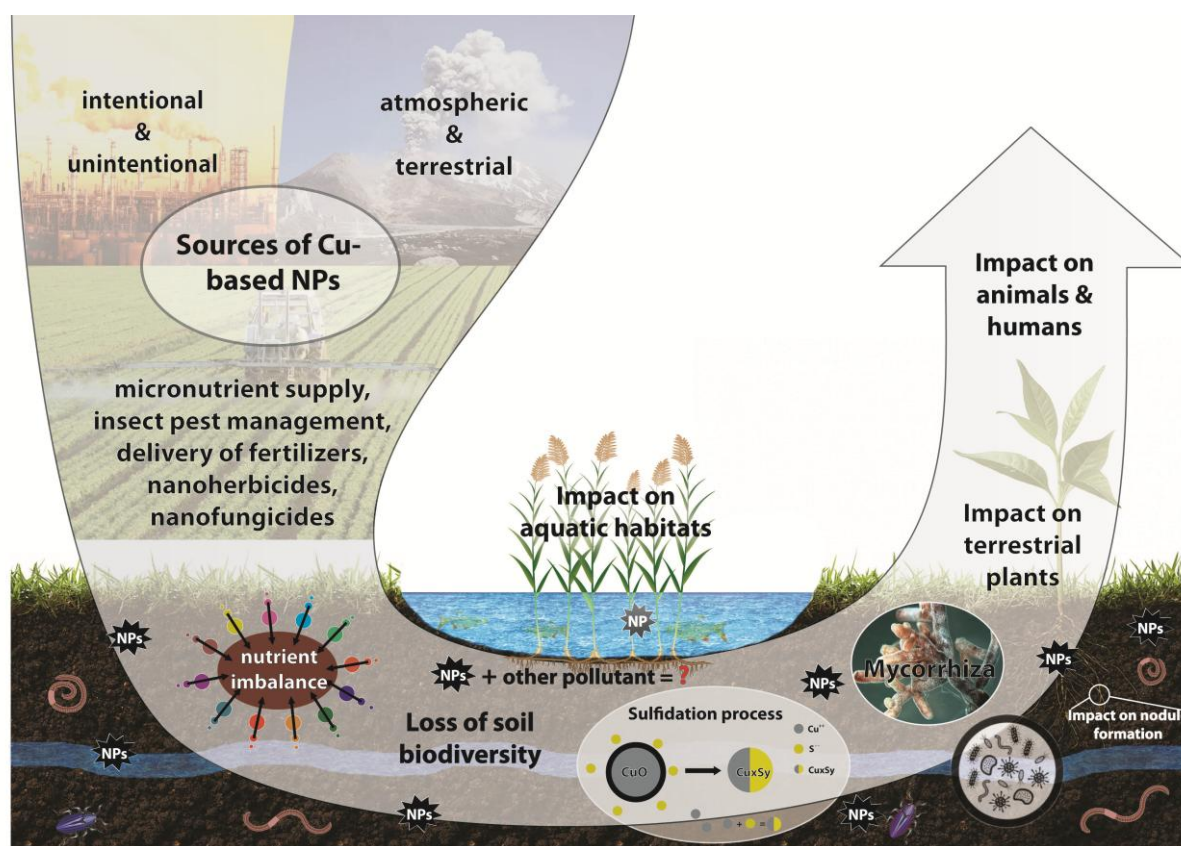
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## 181 **2 Sources, variants and fate of Cu-based NPs in the environment**

182

183 Owing to diverse applications of Cu-based NPs in the nanotechnology industry, the release of nanoscale-Cu in a  
184 different sphere of the environment is expected (Qiu and Smolders 2017). Sources of NPs include both the point  
185 and non-point sources. Point sources are comprised of production and storage units, research laboratories,  
186 disposal of nanomaterial-containing consumer products and wastewater treatment plants etc., whereas Cu  
187 discharge through non-point sources occurs through wear and tear of Cu-based NPs containing paints, cosmetic  
188 products, and cleaning agents (Rajput et al. 2018b). The Cu-NPs have potential to enter water, soil, and

189 sediments during and at the end of their life cycle (Keller et al. 2013; Slotte and Zevenhoven 2017). Soil can  
 190 receive NPs through various channels, for example, agricultural amendments of sewage sludge, atmospheric  
 191 deposition, landfills, or accidental spills during industrial production (Simonin and Richaume 2015). The Cu-  
 192 based NPs are available with various morphologies like Cu, CuO, Cu<sub>2</sub>O, Cu<sub>3</sub>N exhibiting various oxidation  
 193 states, for instance, Cu<sup>0</sup>, Cu<sup>I</sup>, Cu<sup>II</sup>, and Cu<sup>III</sup>, Cu<sup>+</sup> (Cu<sub>2</sub>O) or Cu<sup>2+</sup> (CuO) (Ojha et al. 2017). In soil, nanoscale-Cu  
 194 might be present in various forms like complexes with soil organic matters such as natural organic matter, humic  
 195 acid, fulvic acid etc., Cu-NPs containing pesticides including Kocide 3000 [nCu(OH)<sub>2</sub>], as complex with other  
 196 metal components/plant exudates etc. (Conway et al. 2015; Gao et al. 2018; Peng et al. 2017; Servin et al.  
 197 2017a).



198

199 Fig 1. Schematic of CuO NPs sources to environment and their effects on different ecosystems

200

201 Due to their high density, Cu-NPs tend to settle rapidly from nano to micro scale. The Cu-NPs, both in  
 202 the presence and absence of organisms may undergo micro scale aggregation with high polydispersity in water  
 203 and simple salt solutions (Adeleye et al. 2014; Conway et al. 2015; Griffitt et al. 2007). In a study by Adeleye et  
 204 al. (2014), only 20% Cu-NPs was detected after 6 h at pH 7.0 in NaCl (10mM) which suggested rapid  
 205 aggregation of Cu-NPs leading to sedimentation. On the other hand, natural organic matter released in the  
 206 environment may reduce the Cu-NPs sedimentation; for instance, approximately 40% of Cu-NPs remained

207 stabilized by organic matter released by fish even after 48 h (Griffitt et al. 2007). Indeed, the dissolution of CuO-  
208 NPs in aqueous medium is too slow; so much so that a within concentration range of 0.01-10 mg L<sup>-1</sup>, CuO-NPs  
209 showed as little as ≤1% dissolution after weeks in freshwater and after a month in seawater (Adeleye et al. 2014;  
210 Atha et al. 2012; Buffet et al. 2013; Conway et al. 2015; Hanna et al. 2013). A month after soil contaminated by  
211 CuO-NPs, an increase in labile fraction of the Cu was noted, which had negative effects on the *T. aestivum*  
212 growth (Gao et al. 2018).

213 Thus, once entered into the environment, nanoscale-Cu is expected to undergo a series of  
214 transformations and partitioning that ultimately decides its fate and bioavailability to organisms.

215

### 216 **3 Biotransformation of Cu-based NPs in soil**

217

218 Being a less dynamic component of the biosphere, the soil system has a relatively high potential for pollutants  
219 accumulation in comparison to the atmosphere and hydrosphere. Soil not only acts as a depot for pollutants but  
220 also serves as a source of contaminant input into food chains. Additionally, the soil matrix is considered  
221 abundant in natural occurring NPs which exist in both forms; as primary particles and as  
222 agglomerates/aggregates. The natural organic matter of soil influences the bioavailability of NPs through a  
223 variety of mechanisms like electrostatic interactions, ligand-exchange, hydrophobic effect, hydrogen-bonding  
224 and complexation (Philippe and Schaumann 2014). The various soil processes such as homo/hetero-aggregation,  
225 oxidation, dissolution, sulfidation, sedimentation may impact NPs toxicity (Adeleye et al. 2016; Conway et al.  
226 2015; Garner and Keller 2014; Lowry et al. 2012; Miao et al. 2015; Torres-Duarte et al. 2016). Aggregation and  
227 dissolution of NPs are generally influenced by a range of environmental factors such as pH, organic matter, ionic  
228 species and colloids. A passivation process frequently occurring under various environmental conditions is the  
229 sulfidation of CuO-NPs (Gogos et al. 2017; Ma et al. 2014). This process is expected to alter the speciation and  
230 properties of CuO-NPs significantly and might increase its apparent solubility resulting in increased  
231 bioavailability and thus eco-toxicity attributed to toxic Cu<sup>2+</sup> (Ma et al. 2014).

232 Additionally, colloidal stability of particle is one of the critical factors controlling their fate and effects  
233 (Lowry et al. 2012). The toxicity and bioavailability of Cu changes according to the Cu speciation including  
234 ionic-Cu, Cu-NPs, complexed-Cu, bulk-Cu, oxidation states and environmental factors such as pH, soil, water,  
235 sedimentation, organic matter, redox potential, plant species, and growth phase (Cornelis et al. 2014; Garner and  
236 Keller 2014; Zhang et al. 2018)

237 In soil, NPs either interact with each other forming homoaggregates or interact with different NPs and  
238 natural colloids forming heteroaggregates (Cornelis et al. 2014; del Real et al. 2018). The process of NPs

239 aggregation mainly impacts their colloidal stability which is among the key factors controlling NPs fate and  
240 impact (Bundschuh et al. 2018). The extent of aggregation correlates well with the ionic strength of the medium  
241 but not with the sedimentation rate (Conway et al. 2015). The major controlling factor for Cu-based NPs  
242 sedimentation includes phosphate and carbonate content in the matrix and the oxidation state of Cu. The  
243 dissolution of Cu-based NPs is majorly hindered by sulfidation which is often regarded as passivation process  
244 for Cu/CuO-NPs. It increases the solubility of Cu/CuO-NPs resulting in enhanced bioavailability and toxicity  
245 (Ma et al. 2014). The transformation of Cu-based NPs is further influenced by geochemical properties of soil. In  
246 line with this, low translocation of Cu-NPs was observed in organic-rich soil, whereas high translocation was  
247 noticed in sandy clay soil. The highest rate for transformation to Cu ions and adsorption complexes was detected  
248 in acidic soils (Shah et al. 2016). Under slightly acidic conditions, CuO-NPs may combine with the hydrogen  
249 ions of soil and release  $\text{Cu}^{2+}$  or  $\text{Cu}(\text{OH})^+$ . Under long-term exposure, CuO-NPs and Cu in combination with  
250 humic acid get transformed to  $\text{Cu}_2\text{S}$ , and Cu goethite complex (Peng et al. 2017).

251 Moreover, Wang et al. (2013) investigated the transformations of CuO-NPs in biological and  
252 environmental media and their effect over Cu-bioavailability, redox activity, and toxicity. The authors revealed  
253 that CuO-NPs underwent sulfidation process via sequential dissolution and re-precipitation mechanism to  
254 generate complex secondary aggregates of copper sulfide ( $\text{CuS}$ ) NPs which are considered as active catalysts for  
255 bisulfide oxidation. Although the sulfidation is considered as a natural detoxification mechanism for heavy  
256 metals, the authors suggested that it may not permanently detoxify copper as  $\text{CuS}$ -NPs but also show redox  
257 activity through the release of Cu(I) or Cu(II) by  $\text{H}_2\text{O}_2$  oxidation. In another study, wheat crop was exposed to  
258 CuO-NPs in a sand growth matrix and similar transformation of CuO to Cu (I)-sulphur complexes was noticed  
259 (Dimkpa et al. 2012). Significant reduction of CuO-NPs to  $\text{Cu}_2\text{S}$  and  $\text{Cu}_2\text{O}$  was also shown in maize during root-  
260 shoot-root translocation of CuO-NPs (Wang et al. 2012). The reason behind the transformation of Cu(II) to Cu(I)  
261 in plants may be ascribed to the presence of reducing sugars which get transported from leaf cells to roots  
262 (Huang et al. 2017; Servin et al. 2017a).

263 The leaching and mobilization of nano-Cu ions from the source material followed by their complexation  
264 with humic acids or organic acids when secreted by fungi and contained in the plant root exudates influence the  
265 biotransformation. Although CuO-NPs are often considered as insoluble materials, the presence of organic acids  
266 such as citric and oxalic acid in the environment enhances the dissolution of Cu and CuO-NPs which in turn  
267 increases their mobility and bioavailability to plants and animals. In addition, the nature of the organic acids also  
268 affects NPs dissolution significantly (Mudunkotuwa et al. 2012). Other factors affecting NPs dissolution includes  
269 pH, dissolved organic matter, biomolecular ligands, ionic strength etc. (Yu et al. 2018). All these factors  
270 determine the toxicity of Cu-based NPs by influencing the total dissolved concentration of Cu in the concerned

271 media. Among these factors, the pH has an inverse relationship with dissolution. The CuO-NPs have good  
272 solubility at lower pH which is turn down as the pH increases. However, the presence of ligands including those  
273 with amine functional groups, induce solubility of CuO-NPs at neutral pH (Wang et al. 2013). Recently,  
274 Kovacec et al. (2017) investigated potential efficacy of two phytopathogenic fungi namely *Botrytis cinerea* and  
275 *Alternaria alternata* for biotransformation of  $\text{Cu}^{2+}$  ions, micro and nanoparticulate forms of Cu and CuO. The  
276 study revealed that *B. cinerea* could transform micro and nanoparticulate forms of Cu and CuO into Cu-oxalate  
277 complex.

278 Furthermore, the waterlogged conditions as in the case of paddy fields, may influence NPs dissolution,  
279 mobility, bioavailability, accumulation, translocation and transformation. Peng et al. (2017) studied  
280 bioavailability and speciation of CuO-NPs in the paddy soil and transformation of CuO-NPs in the soil-rice  
281 system. Experimental findings showed that CuO-NPs significantly reduce the redox potential of the soil and  
282 alleviate the electrical conductivity at the maturation stage of paddy. The bioavailability of CuO-NPs showed a  
283 declining trend with rice growth, but an increase was noticed after drying-wetting cycles. Most of the Cu present  
284 in the root, shoot and leaves of the plant was found in the form of Cu-citrate. Nearly  $1/3^{\text{rd}}$  of the Cu(II) was  
285 transformed to Cu(I)-cysteine while 15.7% was present as  $\text{Cu}_2\text{O}$  in roots and 19% as Cu(I)-acetate in shoot  
286 section. In chaff, about 30% of Cu was found as Cu-citrate and Cu(I)-acetate but no CuO was reported to  
287 reached polished rice. In another study, a higher content of Cu in the form of Cu(I) in rice grain was found in the  
288 presence of sulphur (Sun et al. 2017). It was suggested that sulphur fertilization decreases the Cu content in the  
289 root, leaf, and husk of the plant yielding higher biomass but showed higher amounts of Cu in rice grains in the  
290 form of Cu(I)-cysteine and Cu(I)-acetate.

291 Therefore, the mechanism of biotransformation of Cu-based NPs includes series of chemical and  
292 biochemical reactions with soil components and living organisms.

293

#### 294 **4 Interaction of Cu-based NPs with soil organisms**

295

296 Deliberate administration of NPs into soils might have a significant impact on the living entities, as they are  
297 extremely resistant to degradation and have the potential to accumulate in the soil. The effect of NPs may also vary  
298 with varying concentration, soil properties, and enzymatic activity. Soil properties, such as pH, texture, structure,  
299 and organic matter content influence the structure of soil microbial community and the ability of pollutants to exert  
300 toxic effects on microorganisms (Simonin and Richaume 2015). As NPs have the ability to mobilize soil pollutants,  
301 comparison of the toxicity of the NPs in various soil types is much required. In order to understand the influence of

302 soil physicochemical properties on Cu-based NPs toxicity, a number of predictive models have been developed;  
303 however, these models are not always effective for other region soils (Duan et al. 2016).

304 The toxic effect of Cu-based NPs has been shown for beneficial soil microbes such as nitrifying bacteria,  
305 nitrogen-fixing bacteria, *Arbuscular mycorrhiza* and other *Rhizobacteria*; however, it also influences other  
306 microorganisms. You et al. (2017) suggested that the soil types could play an important role in determining NPs  
307 toxicity over soil bacterial community composition and size. Recent studies showed that NPs might affect enzymatic  
308 and metabolic activities, nitrification potential, colony count and abundance of soil bacterial diversity (Colman et al.  
309 2013; Ge et al. 2011; He et al. 2016).

310 Copper ions released from the Cu-NPs can be toxic to both the pathogenic and beneficial bacteria (Lofts et al.  
311 2013). The study conducted on CuO-NPs toxicity to *Saccharomyces cerevisiae* showed increased toxicity over time  
312 due to increased dissolution of Cu ions from CuO (Kasemets et al. 2009). Furthermore, Concha-Guerrero et al.  
313 (2014) have shown that CuO-NPs were very toxic for native soil bacteria, as the formation of cavities, holes,  
314 membrane degradation, blebs, cellular collapse, and lysis in the cells of soil bacterial isolates were observed.  
315 Pradhan et al. (2011) investigated the effect of CuO-NPs on leaf microbial decomposition and found a decrease in  
316 leaf decomposition rate. The bacteria from *Sphingomonas* genus and Rhizobiales known for their importance in  
317 remediation and symbiotic nitrogen fixation appeared susceptible to Cu-NPs (Shah et al. 2016). The NPs also have  
318 significant effects on enzymatic activities (invertase, urease, catalase, and phosphatase, dehydrogenase), microbial  
319 community structure, bacterial diversity nutrient cycling, changes in humic substances, and biological nitrogen  
320 fixation. The CuO-NPs at 30-60 mg L<sup>-1</sup> affected the microbial enzymatic activity of activated sludge (Wang et al.  
321 2017). Several other studies also report Cu-NPs effects on soil microbial community, enzymatic activities and  
322 reduced C and N biomass (Ben-Moshe et al. 2013; Kumar et al. 2012; Xu et al. 2015). However, the effect of Cu-  
323 based NPs on the soil microbial community has rarely been explored. While Cu-based NPs are known to exhibit  
324 antimicrobial properties (Ingle et al. 2014), it is necessary to observe their impact on symbiotic microorganisms. It  
325 can be assumed that NPs, besides influencing plant and microbes, could affect plants-microbe associations either  
326 directly or indirectly. In this context, one of the classical examples is mycorrhizal symbiosis, which promotes plant  
327 growth enhancing the plant nutrient acquisition through uptake of mineral nutrients. The formation of Cu-NPs at the  
328 soil-root interface with the assistance of endomycorrhizal fungi was shown in *Phragmites australis*, and *Iris*  
329 *pseudoacoru* and this mechanism helped to alleviate metal stress (Manceau et al. 2008). On the other hand, metallic  
330 NPs were shown to inhibit mycorrhizal plant growth (Feng et al. 2013).

331 Furthermore, the CuO-NPs induced morphological and genetic alterations in leaf litter decomposing fungus  
332 which could impact organic matter decomposition rate (Pradhan et al. 2011). A significant negative impact on  
333 bacterial hydrolytic activity, oxidative potential, community composition and population size was also observed in

334 Bet-Dagan soil (Frenk et al. 2013). Cu-based NPs have also been reported to affect the growth and functionality of  
335 green algae, cyanobacteria, and diatoms (Anyoogu et al. 2008). The most recent findings on Cu-based NPs action on  
336 bacteria are summarized in Table 1.

337 The findings of recent studies dealing with the NPs action on bacteria are often controversial (Table 1).  
338 Though, most studies show the increased toxicity of Cu-based NPs in comparison to ionic copper at similar dose  
339 rates (VandeVoort and Arai 2018). Interesting results were also obtained when NPs interaction with pesticides was  
340 studied. Parada et al. (2019) reported no major shift in microbial species composition; however, the degradation of  
341 the pesticide was reduced. The possible explanation for this was given by Parra et al. (2019), wherein they showed a  
342 decrease in spreading of pesticide-degradation genes bearing plasmids among the bacterial community. Therefore,  
343 the current scenario demands the exploration of NPs toxicity mechanism on the soil microorganisms.

344 In addition, some studies report that Cu-based NPs can also have adverse effects on multicellular soil  
345 organisms. For instance, the CuO-NPs affected growth and neuron morphology of a transgenic *Caenorhabditis*  
346 *elegans* (Mashock et al. 2016), and disturbed immunity and reduced population density of a common earthworm  
347 *Metaphire posthuma*, which is mostly distributed across the Indian subcontinent (Gautam et al. 2018).

348 Considering the presence of Cu-based NPs in the soil, it is imperative to study their influence on soil  
349 biodiversity. The reviewed information indicates that NPs affected soil microbial community by decreasing their  
350 abundance, enzymatic activities and soil microbial biomass. Therefore, the decrease in soil microbial biomass could  
351 be a sensitive indicator for microbial changes in soils.

352

## 353 **5 Uptake and bioaccumulation of Cu-based NPs in plants**

354

355 The NPs are taken up by plant roots and transported to the aboveground plant tissues through the vascular  
356 system, depending on the composition, shape, size of NPs, and anatomy of the plants (Rico et al. 2011). On the  
357 other hand, some NPs remain adhered to the plant roots. It is well understood that NPs enter plant tissues either  
358 via root tissues (root tips, rhizodermis, and lateral root junctions) or the aboveground organs and tissues  
359 (cuticles, trichomes, stomata, stigma, and hydathodes) as well as through wounds and root junctions.  
360 Interestingly, in the event of NPs-plant interaction, some metal-tolerant plants could limit the uptake of NPs into  
361 the photosynthetic tissues by restricting the transport of metals across the root endodermis and storing them on  
362 the root cortex, whereas, hyper-accumulating plants can take up excess amounts of NPs in the harvestable tissues  
363 of plants (Manceau et al. 2008). It has been suggested that the plants can accumulate NPs in their original form  
364 or as metal ions (Cota-Ruiz et al. 2018). However, the uptake and bioaccumulation vary with varying  
365 physicochemical features of NPs (Ahmed et al. 2018b; Peng et al. 2015; Rico et al. 2011, 2015).

366 In a study, the translocation and biotransformation of CuO-NPs in rice plants were explored. It was  
367 revealed that CuO-NPs get accumulated in epidermis and exodermis regions of the plants and get precipitated  
368 with citrate or phosphate ligands or get bound to amino acids forming Cu-cysteine, Cu-citrate, and  $\text{Cu}_3(\text{PO}_4)_2$   
369 kind of products or get reduced to Cu(I) (Peng et al. 2015). Cu(I) is a highly redox active species capable of  
370 producing hydroxyl radicals by Fenton-like reactions, and so its presence in even smaller quantities has  
371 significant biological importance. Servin et al. (2017a) compared bioaccumulation of un-weathered and  
372 weathered CuO-NPs, bulk and ions in lettuce plants after 70 days. In the case of CuO-bulk, weathered material  
373 was found to decrease Cu accumulation in plant roots, whereas, weathering had a positive impact on  
374 bioaccumulation of NPs. The authors further unearthed that in roots exposed to weathered NPs, the major  
375 fraction of Cu, i.e., 94.2% was present in oxidized form as CuO, while the rest of the fraction i.e., 5.7% could  
376 bind to sulfur in reduced form as  $\text{Cu}_2\text{S}$ . In contrast, roots exposed to un-weathered NPs showed negligible  
377 biotransformation. As the ageing/weathering have a profound effect on the particle-size, particle-size  
378 distribution, surface properties, composition, reactivity etc., it is an important aspect which needs to be  
379 considered while assessing the environmental implication of Cu-based NPs. Similarly, the translocation and  
380 biotransformation of NPs is a plant-specific phenomenon which requires adequate attention.

381 The nano-phytotoxicity studies on accumulation and uptake of NPs have generated important data for  
382 understanding the fate of Cu-based NPs in plants (Ingle et al. 2014; Ma et al. 2010). Once NPs infiltrate the plant  
383 system, they may traverse to different organs (leaves, stem, and fruits) or may get compartmentalized at different  
384 locations *viz.* vacuoles, walls, stellar system, cytoplasmic matrix, lipid envelopes, and nucleus (Ahmed et al.  
385 2018b; Rajput et al. 2017b, 2018a; Rastogi et al. 2017). The translocation efficiency varies greatly in different  
386 plant species, for instance, alfalfa translocates 3-5% of Cu from root to shoot on exposure to  $0\text{-}20\text{ mg L}^{-1}$  Cu-  
387 NPs, whereas only 0.5-0.6% translocation was observed in lettuce (Hong et al. 2016). Before the plant uptake,  
388 the dissolution of Cu-NPs increases the likelihood that Cu is internalized as  $\text{Cu}^{2+}$  ions or in the form of organic  
389 complexes (Keller et al. 2017). A recent study revealed the adsorption and accumulation of Cu-based NPs in  
390 tomato plants leads to the adsorption of nano-CuO on the roots (Ahmed et al. 2018b). Similarly, maize roots  
391 showed 3.6 fold greater Cu content under CuO-NPs treatments (Wang et al. 2012). Also, the Cu content was 7  
392 times higher in shoots of maize treated with  $100\text{ mg L}^{-1}$  CuO-NPs. In this context, Zuverza-Mena et al. (2015)  
393 also reported the translocation of Cu-based NPs in cilantro and their significant accumulation in shoots.  
394 Differential accumulation profile of CuO-NPs has been reported in ryegrass and radish (Atha et al. 2012). Wheat  
395 and bean seedlings grown on dual agar media have been adequately discussed pertaining to the bioavailability of  
396 Cu-NPs and their relationship between accumulation and uptake (Woo-Mi et al. 2008). Cu-NPs were toxic to  
397 both plants and also bioavailable. A Cu ion released from Cu-NPs has negligible effects in the studied

398 concentration range, and the apparent toxicity is clearly due to Cu-NPs. Bioaccumulation increased with  
399 increasing concentration of Cu-NPs and agglomeration of particles was observed in the plant cells by using  
400 transmission-electron microscopy-energy-dispersive spectroscopy (TEM-EDX). In shoots of wheat grown in the  
401 sand matrix, the bioaccumulated Cu was detected as Cu(I)S complex and CuO (Dimkpa et al. 2012). The level of  
402 Cu accumulation in wheat shoots under CuO-NPs exposure was almost equal to the concentrations quantitated in  
403 bulk (Dimkpa et al. 2012).

404 In a very recent study, Keller and co-workers exposed leaf tissues of lettuce, collard green, and kale to  
405 nano-CuO and detected CuO-NPs in leaf surfaces by use of single particle inductively coupled plasmon mass  
406 spectroscopy (sp-ICP-MS) (Keller et al. 2018). Among all three vegetables, lettuce retained the highest amount  
407 of CuO-NPs on leaf surface even after washing. For this retention, the varying degrees of leaf surface roughness  
408 and hydrophilicity among the tested vegetables have been suggested to play an important role in holding CuO-  
409 NPs (Keller et al. 2018). Overall the data from these studies indicate that certain fractions of CuO-NPs are taken  
410 up by plants which may result in undesirable accumulation in edible plant tissues ultimately exposing humans  
411 via the food chain.

412 The bioaccumulated nano-Cu or CuO is also subject to transportation and transformation in plants  
413 (Ahmed et al. 2018c). For instance, the treatment of hydroponically cultured lettuce plant with CuO/Cu-based  
414 NPs caused a greater accumulation of Cu than cupric ions (Trujillo-Reyes et al. 2014). Additionally, the xylem  
415 and phloem based transport system to shoots and back to roots were proposed for CuO-NPs accumulation in root  
416 cells, cytoplasm, intracellular space, and nuclei of xylem and cortical cells. However, the CuO-NPs was reduced  
417 from Cu (II) → Cu (I) in due course of translocation (Wang et al. 2012). A similar transformation of CuO-NPs  
418 has been reported with an elevation in the degree of saturation of fatty acids (Yuan et al. 2016). In another study,  
419 when *Zea mays* were exposed to CuO-NPs, ionic, and bulk CuO, the Cu content in root and shoot of the plant  
420 was found enhanced under CuO-NPs (Wang et al. 2012). A micro X-ray fluorescence ( $\mu$ XRF) study revealed  
421 that Cu-NPs may get accumulated in outer parts of the root (Servin et al. 2017a). The translocation of Cu-NPs  
422 also varies depending upon the growth media. For instance, alfalfa, lettuce and cilantro exposed to CuO, Cu and  
423 Cu(OH)<sub>2</sub> NPs based pesticide in soil showed >87-99% Cu accumulation mostly in roots with very little  
424 transportation to shoots and negligible in leaves (Hong et al. 2015; Zuverza-Mena et al. 2015). In some recent  
425 studies, Cu-NPs were also detected in leaves, stems, and fruits of cucumber and tomato when grown in soil  
426 system (Zhao et al. 2016a). The uptake of CuO-NPs in tomato, alfalfa, cucumber, and radish seedlings was also  
427 noticed in the range of 4-1748  $\mu\text{g g}^{-1}$  dry biomass when grown on semi-solid agar media (Ahmed et al. 2019). In  
428 a comparative study between soil and hydroponically grown tomato plants, the organ wise distribution of CuO-  
429 NPs in soil culture was found lesser than in hydroponic (Ahmed et al. 2018b). The Cu in soil grown root and

shoot of tomato plants was found lesser by 20% and 33% than in hydroponically grown plants (Ahmed et al. 2018b). This difference could be attributed to the NPs cluster formation due to the homo/hetero aggregation processes of the soil system. Besides root exposure, the atmospheric presence of Cu-based NPs also triggers their bio-uptake. For instance, during the foliar applications of Cu-NPs, most of the Cu remained in fruits or leaves with a little transport via phloem to roots. For example, *Lactuca sativa* exposed to Cu-based nano-pesticide accumulated 1350-2010 mg Cu kg<sup>-1</sup> dry biomass after 30 days (Zhao et al. 2016a, b). A small fraction (17-56 mg kg<sup>-1</sup>) of Cu was also found in roots via phloem transport (Zhao et al. 2016a). In a study, the microscopic analysis showed the presence of dense material in root cells of *O. sativum* L. treated with CuO-NPs and confirmed the presence of Cu by bulk-X-ray absorption near edge structure (XANES), and interestingly the most dominant form of dense material was CuO (Peng et al. 2015).

Being very small in size, NPs have the potential to enter, translocate, and penetrate physiological barriers to travel within the plant tissues, and microscopic studies showed the accumulation of NPs in various parts of the plant (Ahmed et al. 2018a; Rajput et al. 2018a, d).

## 6 Toxicity of Cu-based-NPs in plant system

The long-term effects of Cu-based NPs accumulation in plant systems are still scarcely known. It has been suggested that the Cu-based NPs may cause morphological, physiological, genetic, and epigenetic changes which may alter plant growth and nutritional status. Plants as primary producers are very critical for the sustainability of an ecosystem and functions as an indispensable link for perpetual food supply and human nutrition. In the environment, plant roots make close associations with soil particles and virtually everything that enters in the soil system (Ahmed et al. 2017; Anjum et al. 2013). Variants of Cu-based NPs once released in the environment may eventually enter either intentionally or accidentally into the soil-plant system. Plants in soil environment can be the non-target organisms of Cu-based NPs. The critical toxicity level of Cu in many crop species varies between 20-30 µg g<sup>-1</sup> leaf dry biomass (Anjum et al. 2015; Yruela 2009). Thus, the potential toxicity assessment of Cu-based NPs to plants is relevant to a large extent. Several studies have reported the impact of different species of Cu-NPs in various culture media such as agar, hydroponic nutrient solution, sand, filter paper, soil, and soil-sand mixtures (Dimkpa et al. 2013; Kim et al. 2013; Moon et al. 2014; Musante and White 2012) (Table 2). The exact mechanism of plant defence under NPs toxicity is not fully understood. Generally, the phytotoxicity of NPs expressed in two steps: (1) chemical toxicity based on chemical composition, and (2) stress stimuli caused by the surface, size, or shape of the NPs. The antioxidant defence machinery of plants becomes activated against external/internal NPs stress stimuli. Underexposure with NPs

462 having enough physicochemical features to exert toxicity, plants trigger their antioxidant defence mechanism to  
 463 prevent oxidative damage, as well as enhance their resistance towards NPs toxicity. For instance, cucumber  
 464 plants grown hydroponically in the presence of CuO-NPs (50 nm) were found with augmented anti-oxidative  
 465 enzymes *viz.* catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) (Kim et al. 2012). However,  
 466 *C. sativus* when grown hydroponically in the presence of Cu NPs (10-30 nm) experienced significant phytotoxic  
 467 effects which were not ameliorated by antioxidant enzymes adequately (Mosa et al. 2018). The NPs arbitrated  
 468 phytotoxicity is predominantly related to their physicochemical properties. The Cu-based NPs cause  
 469 phytotoxicity via the dissolution and release of higher concentration of ions such as  $\text{Cu}^{2+}$  or the production of  
 470 excess reactive oxygen species (ROS) (Ahmed et al. 2019; Letelier et al. 2010). ROS can affect mitochondrial  
 471 respiration, apoptosis, lipid peroxidation in the cell membrane, and induce a range of antioxidant responses  
 472 (Dimkpa et al. 2012; Shaw and Hossain 2013). Recent studies of CuO-NPs phytotoxicity showed negative  
 473 impacts on seed germination and overall plant growth of various crops such as *Lactuca sativa* (100-300 mg L<sup>-1</sup>),  
 474 *Medicago sativa* (0-20 mg L<sup>-1</sup>), *Triticum aestivum* (200 mg L<sup>-1</sup>), *Vigna radiate* (500 mg L<sup>-1</sup>), *Zea mays* (2-100  
 475 mg L<sup>-1</sup>), *Cucumis sativus* (100-600 mg L<sup>-1</sup>), *Oryza sativa* (0-1000 mg L<sup>-1</sup>), *Brassica juncea* (0-1500 mg L<sup>-1</sup>), and  
 476 *Glycine max* (50-500 mg L<sup>-1</sup>) (Rajput et al. 2017b).

477 The studies pertaining to toxicity assessment of Cu and Cu-based NPs, and understanding of its  
 478 molecular mechanism warrant more systematic and in-depth investigations. The available data on the toxicity,  
 479 chemistry, and Cu-NPs plant interactions suggesting adverse outcomes on plant growth are presented in Table 2.

480

### 481 **6.1 Effects on seed germination, morphometry and plant growth**

482

483 Seed germination commences a plant's physiological process, and therefore it is an important attribute when toxicity  
 484 of a xenobiotic is examined. The Cu-based NPs have been found to inhibit seed germination in various crops (Table  
 485 2). For instance, *Coriandrum sativum* cultivated in soil mixed with 20 and 80 mg kg<sup>-1</sup> of each Cu, CuO, and  
 486  $\text{Cu}(\text{OH})_2$  NPs (Kocide and CuPRO) exhibited significant ( $p \leq 0.05$ ) reduction in seed germination (Zuverza-Mena et  
 487 al. 2015). In another study, the seed germination by CuO-NPs was reduced to almost 50%. Similarly, treatment with  
 488 Cu-NPs at 80 mg kg<sup>-1</sup> reduces the shoot elongation by 11% (Zuverza-Mena et al. 2015). The CuO-NPs (~18.4 nm)  
 489 at 0.02-2 mg ml<sup>-1</sup> also causes severe toxicity in tomato plants (Ahmed et al. 2018b). Furthermore, *Solanum*  
 490 *lycopersicon* plants are grown in both soil and hydroponic media showed significant internalization of Cu in  
 491 different plant organs with oxidative burst and reduction in plant height and weight (Ahmed et al. 2018b). Moreover,  
 492 the Cu, CuO and core-shell Cu/CuO-NPs at different concentrations caused severe reduction in root length of  
 493 *Hordeum vulgare* L. (Shaw et al. 2014), *H. sativum* distichum (Rajput et al. 2018a), *H. vulgare* (Qiu and Smolders

494 2017), *Z. mays*, *C. sativus* (Kim et al. 2013), *T. aestivum* (Gao et al. 2018; Woo-Mi et al. 2008), and *L. sativa* (Liu et  
495 al. 2016; Trujillo-Reyes et al. 2014). The CuO-NPs (~ 40 nm) at 500 mg kg<sup>-1</sup> soil as fresh and after 28 days of  
496 mixing of CuO-NPs with soil caused a significant decrease in maximal root length (Gao et al. 2018). In the same  
497 study, it has been suggested that the exudates secreted from wheat roots in CuO-NPs amended soil enhanced the  
498 dissolution of Cu ions in pore water, which played an important role in enhanced phytotoxicity (Gao et al. 2018).  
499 Similarly, in a study by Qiu and Smolders (2017), CuO-NPs (~ 34 nm) at various concentrations ranging from 50-  
500 1000 mg kg<sup>-1</sup> at two different pH (4.8 and 5.8) increases the toxicity of CuO-NPs affecting root elongation. The  
501 CuO-NPs inhibited *C. sativus* seed germination when administered at 600 mg L<sup>-1</sup>. At this rate, only 23.3%  
502 germination was recorded over untreated of control (Moon et al. 2014). Some earlier studies also reported that CuO-  
503 NPs reduced *C. pepo* biomass by 90% (Stampoulis et al. 2009), seedling growth of *Phaseolus radiatus* and *T.*  
504 *aestivum* (Woo-Mi et al. 2008), shortened primary and lateral roots of the *B. juncea* L (Nair and Chung 2015a),  
505 affected agronomical/physiological parameters in *Origanum vulgare* (Du et al. 2018), and decreased root growth in  
506 *M. sativa* grown in hydroponic culture (Hong et al. 2015). In *Allium cepa*, 80 mg CuO-NPs L<sup>-1</sup> damaged the root cap  
507 and meristematic zone and reduced the growth of the root tip (Deng et al. 2016).

508 Morphometric observations indicated a decline in root and shoot growth for Cu-based NPs treated plants.  
509 Also, Cu-based NPs pose deleterious effects on plant germination (Deng et al. 2016; Moon et al. 2014; Nair and  
510 Chung 2015a; Rajput et al. 2018a, b). The reduction in root and shoot growth could limit the surface area for water  
511 uptake and photosynthesis respectively and consequently affects the plant performance.

512

## 513 **6.2 Effects on cellular ultrastructure**

514

515 Several studies on the ultrastructure of plants cells after Cu-based NPs exposure showed remarkable changes in  
516 plant roots and leaves. In roots, violations of the integrity of the cell wall of the epidermis and endoderm,  
517 vacuolization and disorganization of fragments in the endoplasmic reticulum, swelling of the mitochondria, and  
518 destruction of the mitochondrial cristae have been observed with rare leucoplasts with disorganized and partially  
519 destroyed thylakoid. In the chloroplasts of the leaf parenchyma, the size of starch grains and plastoglobules  
520 increased significantly; the area of the thylakoids decreased, and inter-thylakoid space expanded (Rajput et al.  
521 2018d). These changes can be indicative of lowering the photosynthetic processes with relation to CuO-NPs toxicity  
522 (Rajput et al. 2015).

523 Plastoglobules are subcompartments of thylakoids that play an important role in lipid metabolic pathways  
524 (Austin et al. 2006), the chloroplast to chromoplast transition and the formation of coloured carotenoid fibrils  
525 (Vishnevetsky et al. 1999). Previous studies showed an increased number of plastoglobules due to biotic, abiotic and

526 CuO-NPs induced stress in *Landoltia punctata* (Lalau et al. 2015). The excess concentration of CuO-NPs severely  
527 affected starch content, stomatal aperture, epidermis, endodermis, cell wall, mitochondria, nuclei and vascular  
528 bundles of *H. sativum* (Rajput et al. 2018a).

529 The identified changes in the root and leaf cell ultrastructure, especially in the photosynthetic apparatus are  
530 associated with altered plant growth and performance.

531

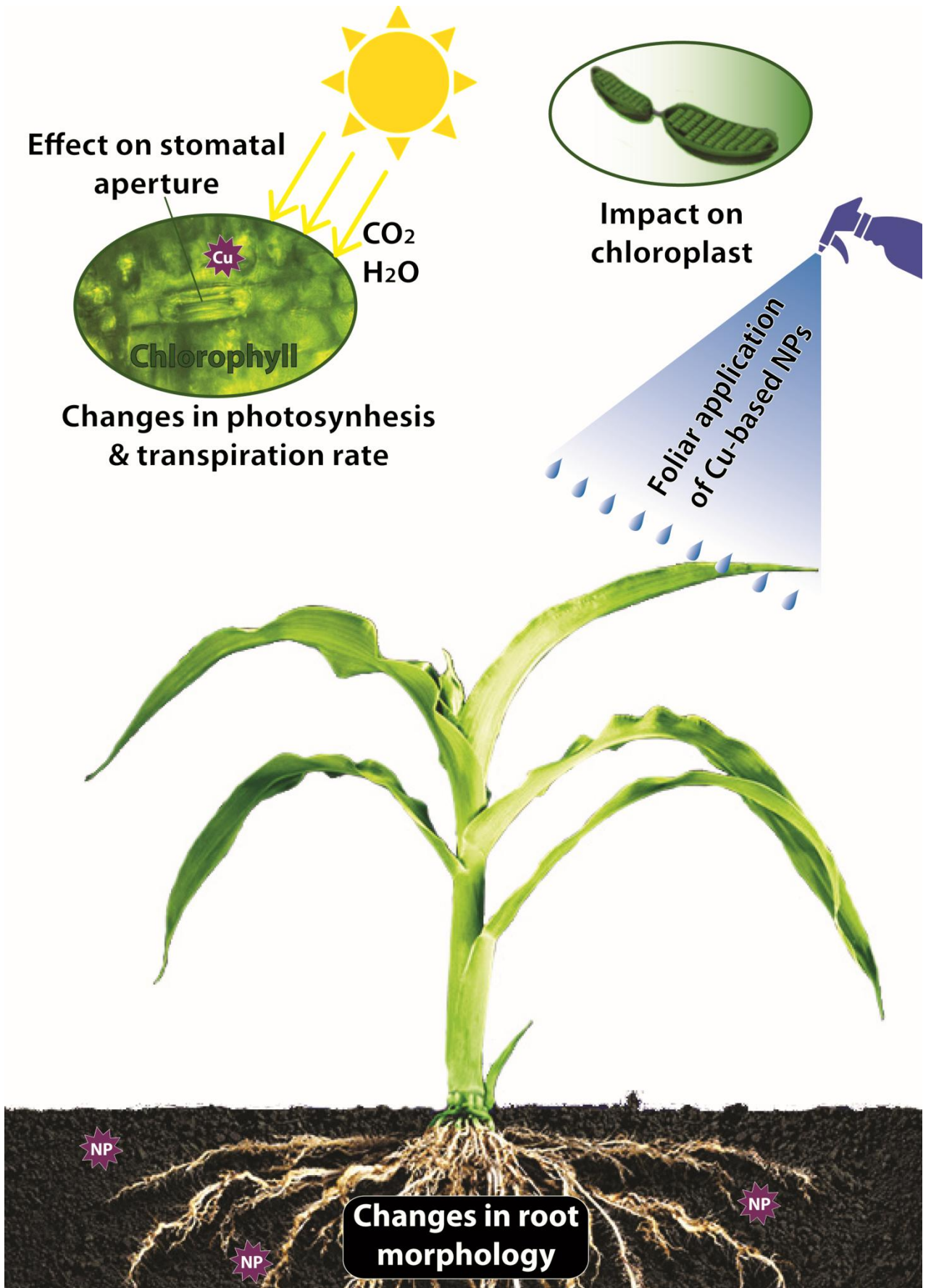
### 532 **6.3 Effects on plant physiology and photosynthetic systems**

533

534 Photosynthesis is a key process for the conversion of light energy into chemical energy, which is performed by  
535 chloroplast, and other components of the photosynthetic machinery embedded in a highly dynamic matrix and  
536 thylakoid membranes (Rottet et al. 2015). Cu-based NPs may also affect photosynthesis, and cause a decrease in  
537 electron transport, thylakoid number per granum, photosynthetic rate, transpiration rate and stomatal conductance  
538 (Da Costa and Sharma 2015; Perreault et al. 2014). Musante and White (2012) observed that both bulk Cu and Cu-  
539 NPs reduced the transpiration rate by 60-70% in *C. pepo* relative to untreated controls. For the successful  
540 photochemical phenomena, chloroplast ultrastructure, thylakoid, grana formation, and physiological activities of  
541 photosynthetic machinery are important (Miller et al. 2017; Tighe-Neira et al. 2018). Thus, any structural and  
542 ultrastructural alteration in chloroplast apparatus and functionality associated subcellular organelles such  
543 plastoglobules starch grains may adversely impact the overall photosynthesis (Figure 2). Toxic effects of CuO-NPs  
544 were further shown in experiments with *O. sativa*. The CuO-NPs decreased Fv/Fm up to a complete loss of  
545 photosystem (PS) II photochemical quenching at a concentration of 1 mg L<sup>-1</sup> and declined the photosynthetic  
546 pigment contents (Da Costa and Sharma 2015). It has further been reported that the CuO-NPs had a detrimental  
547 impact on the structure and function of the photosynthetic apparatus especially on photosynthetic pigments,  
548 chlorophyll, and grana (Tighe-Neira et al. 2018). Spring barley grown in hydroponic system showed accumulation  
549 of CuO-NPs in leaf cells and disorganized chloroplast structure and thylakoid in the mesophyll cells (Rajput et al.  
550 2018a).

551 Thus, the declining photosynthetic efficiency can be a good forecaster of NPs toxic effects on plants.

552



553

554 Fig 2. Schematic presentation of effects of Cu-based NPs on photosynthesis

#### 555 **6.4 Effects on plant metabolism and nutrient content**

556

557 Several studies have demonstrated that Cu-based NPs also significantly affect the metabolism and nutrient content  
558 of plants. For example, foliar application of Cu(OH)<sub>2</sub> nano pesticide (50-1000 nm) at 1050-2100 mg L<sup>-1</sup> alters  
559 metabolite level of *L. sativa* leaves (Zhao et al. 2016b). Gas Chromatography-Time-of-Flight Mass Spectrometry  
560 (GCTOF-MS) based analysis combined with Partial Least Squares-Discriminant Analysis (PLS-DA) multivariate  
561 analysis shows disturbance in tricarboxylic acid (TCA) cycle and amino acid related pathways (Zhao et al. 2016b;  
562 2017b). An increased level of potassium, putrescine, and spermidine in Cu(OH)<sub>2</sub> nano-pesticide treated plants has  
563 been suggested to reduce the oxidative stress and enhance the tolerance (Zhao et al. 2016b). Similarly, in cucumber  
564 grown with Cu-NPs (40 nm) in soil (200-800 mg kg<sup>-1</sup>) and hydroponics (10 and 20 mg L<sup>-1</sup>) exhibited perturbation in  
565 iron, sodium, phosphorus, zinc, sulphur, and molybdenum uptake and alterations in cucumber fruit metabolite  
566 profile (Zhao et al. 2016a). Additionally, TCA cycle and galactose metabolism also get compromised (Zhao et al.  
567 2016b). CuO and Cu(OH)<sub>2</sub> nano pesticides also decrease the level of shoot phosphorus and iron in lettuce (Hong et  
568 al. 2015). Moreover, CuO- NPs (<50 nm) at 500 mg kg<sup>-1</sup> soil has shown to reduce iron, manganese, zinc, and  
569 calcium in common bean (Dimkpa et al. 2015). Moreover, micro- and macronutrients elemental composition in  
570 cilantro has been found to be suppressed when grown with CuO-NPs (10<sup>1</sup>-10<sup>2</sup> nm) and Cu-NPs (10<sup>2</sup>-10<sup>3</sup> nm) at 0-80  
571 mg kg<sup>-1</sup> soil (Zuverza-Mena et al. 2015). The Cu-based NPs have also been documented to bring down the  
572 agronomically important characteristics of plants. The CuO-NPs (<50 nm) reduces carotenoids level in rice at 1 and  
573 1.5 mM (Shaw and Hossain 2013). Similarly, the decrease in the firmness of cucumber fruits has been reported upon  
574 treatment with CuO-NPs of <50 nm (Hong et al. 2016). Also, the grain yield of rice has been shown to reduce  
575 significantly by CuO-NPs (~ 43 nm) at 500 and 1000 mg kg<sup>-1</sup> (Peng et al. 2017).

576 Summarizing these results, it can be concluded that Cu-based NPs at a certain concentration negatively  
577 affected plant metabolism and nutrient content.

578

#### 579 **6.5 Genotoxic and cytotoxic effects**

580

581 Genotoxicity is one of the most devastating effects exerted by NPs on plants. A variety of toxic effects have been  
582 reported for NPs which may interact with biological systems via five main modes: (i) chemical effects as metal ions  
583 in solution upon dissolution; (ii) mechanical effects owing to hard spheres and defined interfaces; (iii) catalytic  
584 effects on surfaces; (iv) surface effects owing to binding of proteins to the surface, either by non-covalent or  
585 covalent mechanisms or oxidative effects; and (v) changes in the chemical environment (pH). Metal and metal oxide  
586 NPs have been shown to act as mediators of DNA damage in mammalian cells, organisms, and even in bacteria, but

587 the molecular mechanisms through which this occurs are poorly understood. For the first time, it was reported that  
588 CuO-NPs induce DNA damage in crops and grassland plants (Atha et al. 2012). The Cu-NPs, up to 20  $\mu\text{g ml}^{-1}$   
589 increased the mitotic index of actively dividing cells in *A. cepa* with a gradual decline in the mitotic index as the  
590 concentration increased (Nagaonkar et al. 2015). Smaller sized NPs, increasing concentrations, and exposure  
591 duration of NPs have been related to greater genotoxic responses, leading to mito-depressive effects in the cell  
592 cycle. Micronuclei formation, disturbed chromosomes, chromosome fragments, stickiness, bridge, laggards'  
593 chromosomes and decrease in mitotic index are the most obvious anomalies in plants exposure to silver, copper,  
594 titanium dioxide, zinc, zinc oxide, selenium oxide, multi-wall carbon nanotube, tetramethylammonium hydroxide  
595 and Bismuth (III) oxide NPs. The severity of abnormalities depending on the concentration, duration time and  
596 particle size are different. Finally, if the DNA repair mechanisms are not enough to restore these alterations, it can  
597 lead to loss of genetic material and mutation in DNA (Karami and Lima 2016). The plant DNA is also affected by  
598 cellular oxidative stress generated by Cu-based NPs. Atha et al. (2012) reported oxidative-stress induced DNA  
599 lesions in *R. sativus*, *Lolium perenne*, and *L. rigidum* by CuO-NPs (10-1000  $\text{mg L}^{-1}$ ) that include 2,6-diamino-4-  
600 hydroxy-5-formamidopyrimidine, 8-OH-dG, the 2'-deoxynucleoside form of 8-OH-G, and 4,6-diamino-5-  
601 formamidopyrimidine (Atha et al. 2012). Cu-based NPs exposure has been attributed to induce genotoxic effects and  
602 affect the normal cell cycle. Chromosomal aberrations such as sticky and disturbed chromosomes in  
603 metaphase/anaphase, c-metaphase, bridges, laggard chromosomes, disturbed telophase, and vacuolated nucleus  
604 resulted after exposure to Cu/CuO-NPs in onion and black cumin (Deng et al. 2016; Kumbhakar et al. 2016;  
605 Nagaonkar et al. 2015). These aberrations are very similar to those induced by ethyl methanesulphonate (EMS) and  
606 gamma radiation. With the use of random amplified polymorphic DNA (RAPD), the genotoxicity of CuO-NPs (~50  
607 nm) has been demonstrated in buckwheat (Lee et al. 2013). The authors demonstrated changes in DNA bands in  
608 RAPAD profiles of buckwheat exposed by 2,000 and 4,000 of CuO NPs  $\text{mg L}^{-1}$  (Lee et al. 2013). The changes in the  
609 genetic pattern induced by Cu-NPs toxicity could be attributed to changes in genomic DNA template stability due to  
610 mutations homologous recombination, deletion of large DNA segments and might be due to the strong binding of  
611 NPs with plant DNA (Ahmed et al. 2018b; Lee et al. 2013). The DNA isolated from young tomato leaves upon  
612 interaction with various concentrations of CuO-NPs exhibited concentration-dependent fluorescence quenching of  
613 acridine orange-DNA complex and ethidium bromide-DNA complex (Ahmed et al. 2018b). The CuO-NPs are able  
614 to interact with plant DNA in both intercalative and non-intercalative mode with perceptible changes in other  
615 macromolecules like amide I and II of proteins and carbohydrates (Ahmed et al. 2018b). The transfer of CuO-NPs to  
616 progeny (harvested seeds) of *Arabidopsis thaliana* has been studied by XANES in the form of CuO (88.8%),  
617 moreover, Cu in seeds has been detected as Cu-acetic acid (3.2%),  $\text{Cu}_2(\text{OH})\text{PO}_4$  (2%), and  $\text{Cu}_2\text{O}$  (6%) (Wang et al.  
618 2016). Recently, the change in the gene expression pattern of plants exposed to CuO-NPs has been reported. Wang

619 et al. (2016) documented differential expression of gene Fe-SOD and gene Aux/IAA in the regulation of *A. thaliana*  
620 root growth when exposed to 20 and 50 mg L<sup>-1</sup> CuO-NPs. Similarly, altered gene expression has been observed by  
621 surface-enhanced laser desorption/ionization-time of flight (SELDI-TOF) in cucumber seeds after treatment with  
622 nano-CuO at 600 mg L<sup>-1</sup> (Moon et al. 2014). In this study, among 34 differentially expressed proteins about 9  
623 differed from those exposed to control and bulk-CuO treated plants. A protein (5977-m/z) has been found as the  
624 most distinguished biomarker for the determination of CuO-NPs induced phytotoxicity (Moon et al. 2014).

625 Interaction of Cu-NPs with plant root exudates also influences the fate of Cu-NPs and magnitude of  
626 toxicity. Huang et al. (2017) determined the thermodynamic parameters for the interaction of Cu-NPs (40 nm) with  
627 a mixture of synthetic root exudates (SRE) and its components such as sugars, amino acids, organic acids, and  
628 phenolic acids by nano isothermal titration calorimetry. The data revealed a strong binding constant ( $K_d = 5.645 \times$   
629  $10^3 \text{ M}^{-1}$ ) for Cu-NPs SRE interaction, however, the binding of Cu<sup>2+</sup> was found stronger but varied for individual  
630 SRE components (Huang et al. 2017).

631 The DNA damage and chromosomal aberrations raise the concern about the safety associated with  
632 applications of the NPs. However, the studies on the phytotoxicity of NPs are scarce, especially with regard to its  
633 mechanisms, and on its potential uptake and subsequent fate within the food chain.

634

### 635 **6.6 Effects on plants ROS and anti-oxidative activities**

636

637 One of the widely reported toxicity mechanisms is the generation of NPs-induced ROS and consequent stimulation  
638 of cellular antioxidant defence mechanisms in plants. The NPs could enhance ROS generation in plants, and cause  
639 oxidative stress, protein oxidation, lipid peroxidation, DNA damage and finally cell death (Ahmed et al. 2018b;  
640 Mosa et al. 2018). To avoid oxidative stress, plants activate a defence mechanism involving the anti-oxidative  
641 enzymes (Rajput et al. 2015).

642 The ROS generation reportedly induces damage to cellular membranes resulting in respiratory loss and  
643 lipid peroxidation leading to disruption of vital cellular functions (Gueraud et al. 2010; Maness et al. 1999). In the  
644 presence of high concentrations, Cu can promote the generation of ROS by Fenton reaction ( $\text{Cu}^+ + \text{H}_2\text{O}_2 \rightarrow \text{Cu}^{2+} +$   
645  $\text{OH}^\bullet + \text{OH}^-$ ) due to its high redox-active nature (Halliwell and Gutteridge 1985). ROS interaction with protein  
646 sulfhydryl (-SH) groups may cause enzyme inactivation which in all likelihood may lead to necrosis, chlorosis, and  
647 growth inhibition (Das and Roychoudhury 2014; Xiong and Wang 2005; Yruela 2009). Among ROS, hydroxyl  
648 radicals formed via Haber-Weiss reaction ( $\text{H}_2\text{O}_2 + \text{O}_2^{\bullet-} \rightarrow \text{OH}^\bullet + \text{OH}^- + \text{O}_2$ ) are considered to be more toxic  
649 (Letelier et al. 2010). To mitigate the ROS stress induced by Cu-NPs, plants elevate the activity of antioxidant  
650 enzymes such as superoxide dismutase (SOD) (Wang et al. 2016), ascorbate peroxidase (APX) (Hong et al. 2015;

651 Shaw et al. 2014), glutathione reductase (GR) (Shaw et al. 2014), catalase (CAT) (Ahmed et al. 2018a,b; Trujillo-  
 652 Reyes et al. 2014), and peroxidase (POD) (Nair and Chung 2014). In addition to this, Cu-NPs arbitrated oxidative  
 653 stress can also be measured in terms of antioxidant levels and proline (Shaw and Hossain 2013; Zhao et al. 2016b).  
 654 The CuO-NPs exposure also increased the lipid peroxidation and triggered an imbalance in oxidative enzymes *viz.*  
 655 GSH, CAT and POD (Dimkpa et al. 2012). The enhanced lipid peroxidation also accompanies low GSH and  
 656 GSH/GSSG ratio (Shaw et al. 2014; Shaw and Hossain 2013) and high SOD activity that converts superoxide  
 657 radicals into hydrogen peroxide ( $O_2^{\bullet} \rightarrow H_2O_2$ ) (Kim et al. 2012; Nekrasova et al. 2011). Besides, antioxidant  
 658 enzymes enhanced malondialdehyde (MDA) content also serves as an oxidative stress marker for Cu-based NPs. For  
 659 instance, the highest levels of MDA were observed in *C. sativus* shoots and roots treated with 100 and 200, and 50  
 660 and 100 mg L<sup>-1</sup> Cu-NPs grown in a hydroponic system, respectively. An increase in MDA levels is directly  
 661 proportional to the concentration of the Cu-NPs used for the treatment (Mosa et al. 2018). Similarly, the CuO-NPs  
 662 increased lipid peroxidation and ROS in *Pisum sativum* (Nair and Chung 2015b).

663 To better understand the toxic nature of Cu-based NPs and their targeted applications, the endpoints of  
 664 toxicity should be carefully scrutinized.

665

## 666 7. Toxicity on aquatic systems

667

668 The impact of Cu-based NPs on aquatic environment is an important issue due to extensive utilization of Cu-NPs,  
 669 releasing metal ions in aqueous solution, making them bioavailable and toxic (Bondarenko et al. 2013; Chang et al.  
 670 2012; Mukherjee and Acharya 2018). The probabilistic model predicts environmental concentrations of Cu-NPs  
 671 0.06 mg L<sup>-1</sup> in major Taiwanese rivers with 95% confidence interval (CI): 0.01–0.92) (Chio et al. 2012). This model  
 672 raised concern on Cu-based NPs adverse effects on aquatic organisms. In addition, several studies highlighted  
 673 toxicity of Cu-based NPs on aquatic organisms including gill injury and acute lethality in zebrafish and toxicity to  
 674 algal species (Aruoja et al. 2009; Griffitt et al. 2007; Griffitt et al. 2009), induction of oxidative stress in the liver,  
 675 gills and muscles of juvenile *Epinephelus coioides* (Wang et al. 2014) and in mussels (Gomes et al., 2014), damage  
 676 to gill filaments and gill pavement cells of freshwater fish (Song et al. 2015b), disruption of secondary lamellae of  
 677 gills, damage in the liver showing pyknotic nuclei (Gupta et al. 2016), affected proliferation, cell cycle progression  
 678 and cell death of amphibians (Thit et al. 2013). The summarized review on NPs toxicity on aquatic habitats suggests  
 679 lethal effects on *Pseudokirchneriella Subcapitata*, *Desmodesmus subspicatus*, *Xenopus laevis*, *Rana catesbeiana*,  
 680 *Mytilus edulis*, *Mytilus galloprovincialis*, *Crassostrea virginica*, *Daphnia magna*, *Thamnocephalus platyurus*, *Danio*  
 681 *rerio*, *Lytechinus pictus*, *Oncorhynchus mykiss* and *Cyprinus carpio* (Mukherjee and Acharya 2018). Pradhan et al.  
 682 (2015) found that CuO-NPs induce oxidative stress, damage to DNA and plasma membrane of aquatic fungi.

683 Similarly, Giannetto et al. (2018) found that CuO-NPs affected oxidative stress-related genes of *Arbacia lixula*  
684 embryos. A short-term study on diatom showed that Cu-NPs inhibited the growth, photosynthesis and induced  
685 oxidative stress on *Phacodactylum tricoratum* (Zhu et al. 2017). Three different Lemnaceae species (*Spirodela*  
686 *polyrhiza*, *Lemna minor* and *Wolffia arrhiza*) commonly found in freshwater lakes exposed to Cu-NPs expressed  
687 different sensitivities (Song et al. 2015a).

688 These data suggest that the toxicity of Cu-based NPs can be influenced by the species, exposure duration,  
689 and dose.

690

### 691 **7.1 Toxicity on aquatic plants**

692

693 There are potentially many sources of NPs in the aquatic ecosystem such as geogenic sources, industrial sources  
694 including medical and pharmaceutical, runoff from household's farms, leaching from landfills etc. Xenobiotic  
695 substances could have a great impact on aquatic biota as well as constitute a serious danger for the aquatic  
696 ecosystem (Moore 2006). One of the anthropogenic sources of Cu-based NPs in the aquatic system is polymer-  
697 coating found in marine paints or fabric with antimicrobial and biocidal properties. This kind of material is used  
698 for antifouling of boats and immersed structures, and CuO-NPs are frequently one of the ingredients (Almeida et  
699 al. 2007). A study showed that CuO-NPs alone ( $0.004 \text{ g L}^{-1}$ ) is less toxic to green alga *Chlamydomonas*  
700 *reinhardtii* than CuO-NPs coated with the polymer after 6 h of exposition (Melegari et al. 2013). Nonetheless,  
701 CuO-NPs still decreased the activity of PS II and were found responsible for the generation of ROS. There were  
702 observations for significantly higher intracellular Cu accumulation in the form of aggregate as compared to Cu-  
703 free samples (Perreault et al. 2012). Similar results were observed in the plant *Lemna gibba* such as  
704 morphological changes like abscission of the fronds from the colonies, decrease in frond size and whitening of  
705 the fronds (Perreault et al. 2014). Both observations indicate that surface modification of NPs in order to enhance  
706 their stabilization changes their mechanism of toxicity which seems to be an important issue for expanding  
707 applications of Cu-based NPs in future. Aruoja et al. (2009) performed tests on the bioavailability of Cu-based  
708 pollutants. The authors confirmed that Cu from CuO-NPs was 141-fold more bioavailable to aquatic flora in  
709 comparison to that from bulk CuO. The greater toxicity of CuO-NPs was seen in algae *Pseudokirchneriella*  
710 (Aruoja et al. 2009) and plant *Lemna minor* (Song et al. 2015a). That is consistent with the previous statement  
711 that the Cu bioavailability rather than the total concentration is the primary toxicity (Campbell 1995). However,  
712 Perreault et al. (2012) pose a hypothesis that during CuO-NPs solubilisation, a soluble form of copper, mostly  
713  $\text{Cu}^{2+}$  ions are released which can spread into the medium and become the main factor for CuO-NPs toxicity that  
714 is similar to the danger posed by  $\text{CuSO}_4$ . The *P. stratiotes* plants grown in the presence of Cu-NPs ( $1000 \text{ mg L}^{-1}$

715 <sup>1</sup>) for 14 days exhibited discolouration along with the visible signs of turgor loss in mesophilic cells.  
716 Morphological changes in the root system were more prominent. In comparison to the control plant, blackening  
717 of roots together with inhibition of new growth roots, and a decrease in plant weight, amino acids, and the  
718 content of ascorbic acid reduced by 63% was observed in exposed plants (Olkhovych et al. 2016). The  
719 morphological changes were also observed for plant *L. gibba* in the form of leaf reduction and detachment of  
720 fronds from the plant. The symptoms were detected after 24 h CuO-NPs exposure with 1.0 mg L<sup>-1</sup> (Perreault et  
721 al. 2014). The growth inhibition was observed at 6.4 mg L<sup>-1</sup> microalgae culture and for *L. minor* at 10 mg L<sup>-1</sup> in  
722 comparison to Cu-free samples (Melegari et al. 2013, Song et al. 2016). The Cu-based NPs exposure on aquatic  
723 flora is mostly reflected in photosystem dysfunction. The chlorophyll content of *L. minor* decreased with the  
724 increase in concentration at 100 mg L<sup>-1</sup> CuO-NPs (Song et al. 2016). In the algal culture of *C. reinhardtii*, the  
725 decrease of total chlorophyll and carotenoids was observed at 1000 mg L<sup>-1</sup> when exposure lasted for 72 h  
726 (Aruoja et al. 2009). For microalgae, *Pseudokirchneriella* 6.4 mg L<sup>-1</sup> was sufficient to evoke abnormality in  
727 photosynthetic system performance (Melegari et al. 2013). In the study of Perreault et al. (2014), lower  
728 photosynthetic electron transport rate for *L. gibba* was observed. The Cu-NPs at a concentration higher than 1 mg  
729 L<sup>-1</sup> clearly suppresses photosynthesis on *Elodea densa* (waterweed) while low concentration (<0.25 mg L<sup>-1</sup>) has a  
730 positive impact on photosynthesis effectiveness (Nekrasova et al. 2011). The main feature of Cu-based NPs is  
731 that they have the ability to cross the plasma membrane that results in alteration of subcellular organelles. This  
732 condition substantially may cause oxidative stress which is connected to increased enzymatic activity (i.e., POD,  
733 CAT, and SOD) (Melegari et al. 2013). The production of ROS may be the result of conditions when plants are  
734 subjected to harmful stress conditions. The chloroplasts and mitochondria of plant cells are important in  
735 intracellular generators of ROS. Internal O<sub>2</sub> concentration is high during photosynthesis, and chloroplasts are  
736 particularly prone to generate ROS; therefore, these cytotoxic ROS can remarkably disrupt normal metabolism  
737 through oxidative damage of lipids, nucleic acids, and proteins.

738 In general Cu-based pollutants induce various responses within the photosynthetic organism. The  
739 changes seem to be the most prominent for the CuO-NPs and Cu-NPs following by CuSO<sub>4</sub> and bulk CuO. The  
740 Cu-NPs toxicity heavily depends on dosage and further surface modification.

741

## 742 ***7.2 Toxicity on aquatic animals***

743

744 There is currently a significant gap in our knowledge about CuO-NPs toxicity to aquatic animals. In general, the  
745 Cu(O) NPs toxicity may be a potential environmental concern for crustaceans, as LC50 values are within an order of  
746 magnitude of predicted wastewater concentrations, while chronic and developmental toxicity are a more relevant

747 concern for fishes (Braz-Mota et al. 2018). A few studies have noted bioactivity in these animals at high  
748 concentrations ( $20 \mu\text{g L}^{-1}$ ). The release of manufactured Cu-based NPs into the aquatic environment is rather rarely  
749 known (Moore 2006). Nevertheless, it was proven that NPs association with naturally occurring colloids may affect  
750 their bioavailability and uptake into cells and organisms. Uptake by endocytic routes was previously identified as  
751 probable major mechanisms of entry into cells; potentially leading to various types of toxic cell injury (Moore  
752 2006). Griffitt et al. (2009) demonstrated that the effects of Cu-NPs were not solely due to the release of soluble  
753 metals into the water column. These studies highlight the need for further studies focused on understanding the  
754 mechanisms of NPs toxicity to aquatic organisms as dissolution and the presence of a generic NPs response are not  
755 sufficient to explain the observed effects.

756 Sedimentation following hetero-aggregation with organic matter and free anions poses a threat due to  
757 benthic, sediment-dwelling and filter feeding organisms. In marine systems, NPs can be absorbed by  
758 microorganisms and transferred to the next trophic levels by consumption. Filter feeders, especially bivalves,  
759 accumulate CuO-NPs through trapping them in mucus prior to ingestion. Benthic fauna may directly ingest sediment  
760 CuO-NPs. In fish, uptake is principally via the gut following drinking, whilst CuO-NPs caught in gill mucus may  
761 affect respiratory processes and ion transport. Currently, environmentally realistic CuO-NPs concentrations are  
762 unlikely to cause significant adverse acute health problems, however, sub-lethal effects e.g. oxidative stress have  
763 been noted in many organisms, often deriving from the dissolution of  $\text{Cu}^{2+}$ , and this could result in chronic health  
764 impacts (Baker et al. 2014).

765 The effect of waterborne Cu-NPs and copper sulphate on rainbow trout (*Oncorhynchus mykiss*) in the  
766 context of physiology and accumulation was also evaluated by Shaw et al. (2012). Overall, these data showed that  
767 Cu-NPs have similar types of toxic effects to  $\text{CuSO}_4$ , which can occur at lower tissue Cu concentrations than  
768 expected for the dissolved metal. It was also proved that CuO-NPs can induce toxicity to the freshwater shredder  
769 (*Allogamus ligonifer*) (Pradhan et al. 2012).

770 Abdel-Khalek et al. (2015) compared the toxicity of CuO-NPs to Nile Tilapia (*Oreochromis niloticus*) with  
771 its bulk counterpart and reported that the  $\text{LC}_{50/96 \text{ h}}$  of CuO bulk particles (BPs) was higher than that of NPs  
772 indicating that CuO-NPs are more toxic. The CuO-NPs could exert more toxic effects despite the fact that they are  
773 smaller in size than the CuO-BPs, and they can form aggregates in suspensions. The authors demonstrated CuO  
774 (BPs & NPs) induced biochemical alterations and oxidative stress in *O. niloticus*, which suggest ecological  
775 implications of CuO-NPs released in aquatic ecosystems. The study conducted by Braz-Mota et al. (2018) aimed to  
776 understand the effects of CuO-NPs and Cu on two ornamental Amazon fish species: dwarf cichlid (*Apistogramma*  
777 *agassizii*) and cardinal tetra (*Paracheirodon axelrodi*). For fish exposed to 50% of the  $\text{LC}_{50}$  for CuO-NPs, aerobic  
778 metabolic rate ( $\text{MO}_2$ ), gill osmoregulatory physiology and mitochondrial function, oxidative stress markers, and

779 morphological damage were evaluated. The results revealed species specificity in metabolic stress responses. An  
780 increase of  $MO_2$  was noted in cardinal tetra exposed to Cu, but not CuO-NPs, whereas  $MO_2$  in dwarf cichlid showed  
781 little change with either treatment. In contrast, mitochondria from dwarf cichlid exhibited increased proton leak and  
782 a resulting decrease in respiratory control ratios in response to CuO-NPs and Cu exposure. This uncoupling was  
783 directly related to an increase in ROS levels. The authors revealed different metabolic responses between these two  
784 species in response to CuO-NPs and Cu, which are probably caused by the differences between species natural  
785 histories, indicating that different mechanisms of toxic action of the contaminants are associated to differential  
786 osmoregulatory strategies among species.

787 Gupta et al. (2016) described the effect of Cu-NPs exposure in the physiology of the common carp  
788 (*Cyprinus carpio*) using biochemical, histological and proteomic approaches. The results indicated that the activity  
789 of oxidative stress enzymes catalase, superoxide dismutase, and glutathione-S-transferase were significantly  
790 increased in the kidney, liver and gills of the treated groups when compared to control. Histological analysis  
791 revealed that after exposure, disruption of the secondary lamellae of gills, liver damage with pyknotic nuclei and  
792 structural disarray of the kidney occurred. Proteomic analysis of the liver showed down-regulation of several  
793 proteins including the ferritin heavy chain, Rho guanine nucleotide exchange factor 17-like, cytoglobin-1, regulation  
794 of diphosphomevalonate decarboxylase and selenide & water dikinase-1.

795 The effect of Cu-NPs on the development of zebrafish embryos was depicted by Sun et al. (2016). The  
796 exposure to CuO-NPs at concentrations of  $12.5 \text{ mg L}^{-1}$  or higher leads to abnormal phenotypes and induces an  
797 inflammatory response in a dose-dependent pattern. Moreover, exposure to CuO-NPs at high doses results in an  
798 underdeveloped liver and a delay in retinal neurodifferentiation accompanied by reduced locomotor ability. The  
799 authors demonstrated that short-term exposure to CuO-NPs at high doses shows hepatotoxicity and neurotoxicity.  
800 On the other hand, cellular and molecular responses of adult zebrafish after exposure to CuO-NPs or ionic Cu were  
801 tested by Vicario-Pares et al. (2018). Another study performed by Bai et al. (2010) was undertaken to test the  
802 toxicity of nano-Cu suspension to zebrafish embryos. It was found that nano-Cu retarded the hatching of zebrafish  
803 embryos and caused morphological malformation of the larvae. The authors claimed that high concentrations ( $>0.1$   
804  $\text{mg L}^{-1}$ ) of nano-Cu can kill the gastrula-stage zebrafish embryos. Denluck et al. (2018) investigated the role of the  
805 chorion in nanomaterial toxicity. The authors found that the presence of the chorion inhibited Cu-NPs toxicity:  
806 while dechorionated embryonic zebrafish exposed to Cu-NPs had an  $LC_{50}$  of  $2.5 \pm 0.3 \text{ mg L}^{-1}$ , a chorion-intact had  
807  $LC_{50}$  of  $13.7 \pm 0.8 \text{ mg L}^{-1}$ . In summary, embryo sensitivity increased by at least one order of magnitude when  
808 chorions were removed.

809           The toxicity of Cu-based NPs in aquatic environment appears to be one of the most important issues for  
810 assessing whole ecosystem safety. With no doubts, zebrafish embryos are excellent models for the study of  
811 nanomaterial-biological interactions and toxicity.

812

### 813 **8 Techniques used to detect the presence of Cu in plant tissues treated with Cu-based NPs**

814

815 It has already been mentioned that new developments in nanotechnology industry increase the amount of such  
816 engineered nanomaterials in the environment, particularly in soils and aquatic ecosystems. This could lead to  
817 unpredicted consequences in the nearest future as plants play a vital role in the ecosystem and worldwide food  
818 supply. That is why NPs detection in environmental samples is of importance (Chaudhry et al. 2008; Mukherjee et  
819 al. 2016). However, not all methods are applicable to this problem due to low concentrations of NPs in  
820 environmental samples and experimental complications in sample preparation. Still, there are several modern  
821 techniques which are being widely applied to detect the presence, visualise the distribution and analyse chemical  
822 properties of NPs in plant tissues or in the soil. The available detection methods could be classified into three broad  
823 sections: spectroscopy, diffraction and imaging. However, the most comprehensive results could be obtained using  
824 the combination of all three methods. Besides, one of the most sensitive techniques is Atomic Absorption  
825 Spectroscopy (AAS). However, this method is destructive and requires special sample preparation procedures.

826           Different types and combinations of electron microscopy techniques offer environmental scientists a wide  
827 range of capabilities. Scanning Electron Microscopy (SEM) gives a possibility to find and locate metal NPs which  
828 usually have higher electron density. SEM microscopes are often equipped with EDX that extend analytical  
829 capabilities to qualitative determination of elements present in the sample and quantitative determination of element  
830 concentration, thus opening a possibility to study the chemical composition of NPs. High-Resolution TEM reveal  
831 the shape and morphology of tiny NPs of several nanometers in diameter. Selected Area Electron Diffraction  
832 (SAED) and images acquired in bright and dark-field modes could be used to study NPs phase composition and  
833 distribution in the samples. Microscopes equipped with Electron Energy Loss Spectra (EELS) cameras are capable  
834 of revealing the oxidation state of 3d transition metals at nanoscale resolution (Tan et al. 2012). Moreover, these  
835 electron-based methods could be combined in one microscope that provides a great possibility to study the presence,  
836 distribution, chemical composition, morphology, shape and size distribution of NPs in soils and plants. However, the  
837 shortcomings of the method are the limitations on the size of the sample, special sample preparation procedures and  
838 the requirement of ultra-high vacuum.

839           Furthermore, X-Ray Fluorescence (XRF) is one of the powerful tools to estimate the relative quantity of  
840 elements present in the sample semi-quantitatively (mass %). Often laboratory equipment has a focused X-ray beam

841 up to 20-50 micrometres ( $\mu$ -XRF) that gives a possibility to obtain element concentration maps of the samples with  
842 appropriate resolution. The latter could be used to detect and locate NPs aggregation in plants. There is also a  
843 particular interest in portable XRF devices (pXRF) (McLaren et al. 2012) for agronomic and environmental science  
844 applications as it opens possibilities to conduct in field studies. Such equipment could be used to relate plant  
845 conditions to elemental nutrient deficiencies in the soil (Towett et al. 2016). However, such devices are limited to  
846 spectroscopic data and low sensitivity. On the contrary, sub-micron resolution and high sensitivity of synchrotron-  
847 based micro- and nano- X-ray techniques open new possibilities to investigate the interactions between plants and  
848 engineered nanomaterials. Synchrotron-based techniques require minimal sample preparation, are non-destructive,  
849 offer the best balance between sensitivity, chemical specificity, and spatial resolution (Castillo-Michel et al. 2017).  
850 These techniques are particularly adapted to investigate localization and speciation of NPs in plants:  $\mu$ -XRF and  
851 synchrotron X-ray fluorescence mapping (SR-XFM) offers multi-elemental detection with resolution down to the  
852 tens of nm, in combination with spatially resolved X-ray absorption spectroscopy ( $\mu$ -XAS or  $\mu$ -XANES) speciation.  
853 Moreover, such synchrotron-based techniques could be combined with  $\mu$ -XRD (micro X-Ray Diffraction) and  $\mu$ -  
854 FTIR (micro Fourier-Transform Infrared Spectroscopy) techniques in one beamline (Cotte et al. 2017).

855         One of the most promising methods to detect the presence of NPs at environmentally relevant concentration  
856 is sp-ICP-MS (Laborda et al. 2014; Laborda et al. 2013). It gives a possibility to obtain qualitative information about  
857 the presence of particulate and/or dissolved forms, quantitative information as particle number as well as mass  
858 concentrations, and characterization information about the mass of element/s per particle and particle size (Laborda  
859 et al. 2016).

860         TEM remains one of the main tools to analyse Cu-based NPs distribution (Lee et al. 2008; Nhan Le et al.  
861 2016) and composition in plants (Trujillo-Reyes et al. 2014; Wang et al. 2011). The XRF technique was applied to  
862 reveal the elemental composition of *C. sativus* shoot and root samples treated with Cu-NPs (Mosa et al. 2018). The  
863 microscopic analysis showed the presence of dense material in root cells of *O. sativum* L. treated with CuO-NPs and  
864 confirmed the presence of Cu by bulk-XANES, and the most dominant form of Cu was from CuO-NPs (Peng et al.  
865 2015). A combination of  $\mu$ -XRF and  $\mu$ -XANES was used to study bioaccumulation un-weathered (U) and weathered  
866 (W) CuO-NPs, bulk and ionic form by lettuce (Servin et al. 2017b). The  $\mu$ -XRF analysis of W-NP-exposed roots  
867 showed a homogenous distribution of Cu in the tissues, while  $\mu$ -XANES analysis of W-NP-exposed roots showed  
868 near complete transformation of CuO to Cu (I)-sulfur and oxide complexes in the tissues. Duran et al. (2017)  
869 showed that CuO-NPs did not affect seed germination of *Phaseolus vulgaris* L., but seedling weight gain was  
870 promoted by 100 mg Cu L<sup>-1</sup> and inhibited by 1000 mg Cu L<sup>-1</sup> of 25 nm CuO and CuSO<sub>4</sub>. The  $\mu$ -XRF analysis  
871 showed that most of the Cu taken up remained in the seed coat with Cu hotspots in the hilum. Moreover,  $\mu$ -XANES  
872 unravelled that most of Cu remained in its pristine form. Zhao et al. (2017a) showed significant growth inhibition on

873 both roots and shoots of *E. crassipes* after 8-day exposure of CuO-NPs (50 mg L<sup>-1</sup>) which was much higher than that  
874 of the bulk CuO particles and dissolved Cu<sup>2+</sup> ions of the same Cu concentration. The XANES was used to reveal the  
875 presence of CuO-NPs as well as Cu<sub>2</sub>S and other Cu species in roots, submerged leaves, and emerged leaves of plants  
876 providing solid evidence of the transformation of CuO-NPs. Electron microscopy remains one of the most widely  
877 used tools to study distribution, morphology and composition of metal NPs in plants. The possibilities that such  
878 synchrotron radiation techniques as  $\mu$ -XRF and  $\mu$ -XANES open to environmental scientists could significantly  
879 change the situation in the sense of revealing precise information on its structure. Moreover, an sp-ICP-MS becomes  
880 one of the most promising technique to obtain the presence and size distribution of NPs at environmentally relevant  
881 concentrations.

882

### 883 **9 Conclusion and future outlook**

884

885 The literature unequivocally suggests that the higher concentrations of Cu-based NPs are detrimental to beneficial  
886 soil microorganisms, food crops, aquatic animals and plants. The toxicity of Cu-based NPs is influenced by their  
887 composition, capping/coating material, size, and interactions with environmental components such as abiotic factors  
888 (e.g. pH) and microbial/plant secretions, and naturally occurring organic matter etc. Furthermore, the phytotoxicity  
889 may vary with the varying physiology/anatomy of plant species. Cu-based NPs are either taken up by organisms  
890 (internal efficiency) or adsorbed on external structures (external efficiency). The adherence and bioaccumulation  
891 may also be changed by physicochemical properties of Cu-based NPs, plant genotypes, and  
892 physical/chemical/biological transformation. The available studies considered in this review showed the inadequate  
893 characterization of Cu-based NPs, which could be the major obstacle in properly assessing its toxicity. Moreover,  
894 the disposal/discharge of Cu-based NPs into the environment is not regulated appropriately. After reviewing those  
895 studies, many questions still persist unanswered when the behaviour and fate of Cu-based NPs in biological systems  
896 are taken into consideration. For instance, most of the studies on Cu-based NPs and plants interactions were  
897 performed on agar or in hydroponic media which do not reflect the actual interaction in the more realistic  
898 environment such as the soil system. The fate of Cu-based NPs, their toxicity and accumulation in the soil can vary  
899 significantly in different soil types due to the difference in pH, organic matter content and composition, etc.  
900 Therefore, understanding the connection between association and dissociation/dissolution of adequately  
901 characterized Cu-based NPs in a range of environmental media and the physiology/anatomy of affected organisms is  
902 most urgently needed to further our knowledge regarding the potential toxicity exerted by Cu-based NPs. After all,  
903 we conclude that Cu-based NPs comprised of Cu-NPs, CuO-NPs, and nano-Cu based products used in agricultural  
904 practices have a great potential to negatively impact soil and aquatic micro/macro biota. The current scenario also

905 emphasizes the regulated and safe dumping of waste containing Cu-based NPs into agro-ecosystems. In the future,  
906 the concentration of Cu-based NPs in edible parts of food crops must be measured carefully before supplying the  
907 products to consumers.

908 It is also crucial to develop a unified methodology for testing the NPs toxicity in natural environments.  
909 With the help of this methodology, joint research should be conducted to determine the toxicity of the same NPs  
910 under different climatic conditions and soil types. Such international research could help to develop the permissible  
911 levels of Cu-based NPs application and determine the threshold levels of their contents in different soils. The  
912 kinetics of NPs dissolution and migration to the groundwater should be specifically considered to avoid their  
913 accumulation above the safe levels. Sustainable use of Cu-based NPs could help to utilize the beneficial effects of  
914 their application (i.e. in the form of nanopesticides) without posing a threat to the living organisms.

915 The increased application of Cu-based NPs clearly indicates their negative impact on ecosystems. It is,  
916 therefore, imperative to explore Cu-based NPs toxicity and behaviour in water, living organisms (biota), soil and  
917 sediments individually, and their toxicity in a combination of other metallic NPs. Past and future research must  
918 be placed in the context of current risk assessments associated with Cu-based NPs, their use, distribution, and  
919 release in the environment.

920

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925

#### 926 **Conflict of interest**

927 The authors declare that they have no conflict of interest.

928

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