CrossMark

ADVANCES IN TOXICITY ASSESSMENT

Accumulation of copper in the cell compartments of charophyte *Nitellopsis obtusa* after its exposure to copper oxide nanoparticle suspension

Levonas Manusadžianas[1] · Brigita Gylytė[1] · Reda Grigutytė[1] · Rolandas Karitonas[1] ·
Kazys Sadauskas[1] · Rimantas Vitkus[1] · Laurynas Šiliauskas[2] · Jūratė Vaičiūnienė[3]

Abstract Cu accumulation in the internodal cell of charophyte *Nitellopsis obtusa* or its compartments was investigated after 3-h-exposure to lethal effective concentrations (8-day LC_{50}) of CuO nanoparticle (nCuO) suspension or $CuSO_4$ solution, i.e. 100 mg/L nCuO or 3.18 mg Cu/L as $CuSO_4$. In both cases, the major part of Cu accumulated in the cell walls. The presence of CuO NPs in the cell wall and within the cell was visualized by scanning electron microscope images as well as confirmed by energy dispersive X-ray spectrum data. Although a threefold higher intracellular concentration of Cu was found after treatment with nCuO suspension, 3.18 mg Cu/L as $CuSO_4$ induced fast and substantial depolarization of cell membrane potential contrary to that of 100 mg/L nCuO. A delayed effect of nCuO on the survival of the cells was also observed. This suggests that internally accumulated Cu was far less active and further supports the hypothesis of delayed toxicity of internalized nCuO NPs to charophyte cells.

Responsible editor: Elena Maestri

✉ Levonas Manusadžianas
  levonas.manusadzianas@botanika.lt

1   Nature Research Centre, Institute of Botany, Žaliųjų Ežerų Str. 49, LT-08406 Vilnius, Lithuania

2   Nature Research Centre, Institute of Geology and Geography, Akademijos Str. 2, LT-08412 Vilnius, Lithuania

3   Centre for Physical Sciences and Technology, Institute of Chemistry, A. Goštauto Str. 9, LT-01108 Vilnius, Lithuania

Introduction

In recent years, numerous toxicity studies on the effects of copper oxide nanoparticles (nCuO) in various organisms have been undertaken. As metal nanoparticles (NPs) may interact with the cell directly or induce toxicity through the release of toxic metal ions, it is complicated to distinguish these effect-pathways (Perreault et al. 2014a). Several studies on microalgae (Aruoja et al. 2009), protozoa (Mortimer et al. 2010), bacteria (Bondarenko et al. 2012), or crustacean (Jo et al. 2012; Adam et al. 2014) have found that toxicity of nCuO is mediated by the release of Cu ions from the nanoparticles. However, other findings have indicated that not all observed effects could be explained by dissolved Cu from nanoparticles (Griffitt et al. 2008; Saison et al. 2010; Manusadžianas et al. 2012; Perreault et al. 2012). Furthermore, nCuO-induced toxicity may be a consequence of both NP reactivity in terms of interaction with various cellular compartments and Cu ions dissolution in lysosomes, as it has been reported in the study on human cell lines (Moschini et al. 2013). It has also been suggested that exposure medium determines whether or not free Cu ions or CuO NPs are the main mediators of toxicity in microalgae (Moos et al. 2015).

In plant and bacterial cells, prior to be internalized, the NPs have to pass semipermeable cell wall and plasma membrane barrier. Negatively charged algal cell surface is favourable for the sorption of CuO nanoparticles that bear positive charge in media of acidic to neutral pHs (Miao et al. 2015). It has been previously shown that effective diameter of cell wall pores of macrophytic algae does not exceed 4.6 nm (Proseus and Boyer 2005), while in cultured cells of various higher plants, the limiting diameter of wall pores for unrestricted pass of the molecules is up to 8.6 nm (Baron-Epel et al. 1988). It has also been suggested that the size of the pores can increase during the cell wall metabolism (Carpita and Gibeaut 1993) or interactions between the cells and nanoparticles (Navarro et al. 2008). Another factor

Springer

stemming from the cell wall, which conditions the state of nanoparticles or their aggregates attached to the cell wall surface is its slight acidity. It is known that pH equals 5.0–5.5 in the acidic zones of charophyte cell walls (Lucas and Smith 1973; Métraux et al. 1980). Studer et al. (2010) have demonstrated that 95.2% of nCuO solubilized within 3 days in non-complexing buffer solutions at pH 5.5. Similar tendency, however, in lesser extent (up to 14% at pH 6.1), has been reported for Cu^{2+} releases from CuO nanoparticles in aqueous media similar in chemistry to natural fresh waters (Odzak et al. 2014).

The uptake of NPs, once they passed the cell wall, may occur through various routes such as endocytosis (Moore 2006), carrier proteins or ion channels (Navarro et al. 2008). The internalization of nCuO particles has been visualized by means of transmission electron microscopy in blue-green algae *Mycrocystis aeruginosa* (Wang et al. 2011) and unicellular green algae *Clamydomonas reinhardtii* (Perreault et al. 2012; Melegari et al. 2013); however, the data on NPs accumulation and distribution within algal cell seem not to be sufficient. In a prior study (Gylytė et al. 2015), fast (within minutes) accumulation of Cu in *Nitellopsis obtusa* cells treated by 100 mg/L CuO nanosuspension or 3.2 mg Cu/L as $CuSO_4$ solution was found. These concentrations are equivalent, since they induced 50% cell mortality within 8 days. Besides, four to five times higher concentrations of Cu were measured in algal cells treated for up to 24 h by nCuO than $CuSO_4$. Scanning electron images confirmed the presence of nanoparticles or their agglomerates on the surface of charophyte cell (Gylytė et al. 2015); however, it was not evident whether or not CuO nanoparticles reach cell interior and if so, in what proportions it distributes between cell wall and charophyte cell interior. The main objective of our report was to explore the uptake of CuO nanoparticles into the charophyte cell as well as the Cu distribution among cell compartments. This is possible due to well-known properties of internodal cells of characean green alga such as big size, cylindrical shape and their morphology (Foissner and Wasteneys 2014), which enable mechanical separation of the compartments, namely cell wall, cytoplasm and vacuole.

Materials and methods

Charophyte algae

Freshwater charophyte *N. obtusa* (Desv.) J. Groves, was harvested from Lake Obelija (54° 29′ N, 23° 83′ E), south-east Lithuania (Kostkevičienė and Sinkevičienė 2008). After separation from thalloma, single internodal cells were kept at room temperature (18–24 °C) in glass aquaria filled with equal parts of non-chlorinated tap water and artificial pond water (APW) containing 0.1 mM KH_2PO_4, 1.0 mM $NaHCO_3$, 0.4 mM $CaCl_2$, 0.1 mM $Mg(NO_3)_2$ and 0.1 mM $MgSO_4$ (unbuffered, pH 7.0–7.8) (Multi 350i, WTW, Weilheim, Germany). All chemicals were from Merck (Darmstadt, Germany). APW was also used as control medium.

Before usage of the cells for experimentation, single internodal cells (each 4–15 cm in length) were placed on Petri dishes (FGH plus, Anumbra, Šumperk, Czech) (10–15 per dish) with APW and then kept in thermostat (TC 135 S, AQUALYTIC, Dortmund, Germany) at 15–18 °C in the dark for 1 to 2 days. The acclimatization procedure before the test allowed discarding of the dead cells that had been injured during the transfer to Petri dishes. Survival of the cells was checked by gently picking up each cell with a spatula. A cell was judged to be dead if a disappearance of turgor pressure occurred when it was picked up, a state in which a cell bends on the spatula.

Preparation of the stock nCuO suspension

Powder of ultrafine CuO nanoparticles, an average particle size of less than 50 nm (mean 30 nm), was purchased from Sigma-Aldrich. Stability characteristics of nCuO suspension prepared in APW were analysed previously (Manusadžianas et al. 2012). A stock of 10 g/L CuO nanoparticles was prepared by dispersing the nanoparticles in deionized H_2O with sonication for 15 min in a bath-type sonicator at 150 W and 38 kHz (Kraintek, K-5LM, Podhájska, Slovak Republic).

Experimental design

To prepare fresh suspensions, stock suspension was sonicated for 15 min and then 20, 50, 150 or 500-μL aliquots of the stock were transferred to individual flasks (BDL, Turnov, Czech) with 50-mL APW to final concentrations of 4, 10, 30 or 100 mg/L nCuO. Each flask was sonicated for 15 min in bath sonicator with ice to prevent heating of the suspension and maintain the temperature of 15–18 °C. After sonication, the suspension was poured off the flask on the algae cells in Petri dishes as fast as within 2 s. The rapidity and the maintenance of the same manipulation manner were undertaken to have a similar portion of non-agglomerated nanoparticles and/or smaller aggregates. It has been previously shown that particle size distribution in suspension of nCuO in APW shifts toward aggregate formation after sonication within 5 s (Manusadžianas et al. 2012).

The cells in each Petri dish were rinsed twice with the APW after exposure to 100 mg/L nCuO or 3.18 Cu mg/L $CuSO_4$ (Merck, Darmstadt, Germany), both prepared in APW, for 3 h, and then left in APW for survival observation, measurement of Cu concentration in the cell and its fractions or scanning electron microscopy.

Cell lethality examination

After the exposure to nCuO or CuSO$_4$ and rinsing in APW, the cells of *N. obtusa* were kept in thermostat at 15–18 °C in the dark. Three to six replicates with 10 cells per Petri dish were used for each treatment and control (untreated cells). The survival of the cells was examined for 48 days. APW were replaced weekly.

Electrophysiological measurements

Cell resting potential of up to 20 living internodal cells was measured simultaneously according to K$^+$-anaesthesia method (Shimmen et al. 1976) modified for multichannel recording with extracellular chlorinated silver wire electrodes (Evans and Leal 1980). The details of computer-assisted experimental setup have been published previously (Manusadžianas et al. 1995, 1999, 2010).

Cell fractionation

Cells were washed with APW twice at the end of 3-h-exposure to 100 mg/L of nCuO or 3.18 mg/L Cu of CuSO$_4$. Then, a single cell was placed on a filter paper, air-dried for ~1 min until the surface was opaque. After the cell was placed on the glass plate, one end of the internodal cell was cut by sharp scalpel at approximately 4 mm distance from the node. One or two drops were obtained from the cell, when it was cautiously held by the health node in vertical position above the microtube (Orange Scientific, Graignette, Belgium). The collected fraction represents the vacuole. Visually, it looks transparent and colourless, the pH value should be at about 5.5 (Gyanes et al. 1978). The rest of cell intracellular content was gently squeezed by fingers along the cell surface up to the cut end by leaving ~1 cm unsqueezed not to contaminate with the nCuO from the cell surface. This fraction comprises the rest of vacuole and cytoplasm with the organelles. After centrifugation at 10,000 rpm for 7 min (Labnet Prism™ R Refrigerated Microcentrifuge, Edison, NJ, USA), supernatant represents vacuole-cytoplasm mixture and residuals–chloroplasts (Whitehouse and Moore 1993).

Cell wall fraction consists of what was left after separation of intracellular content. Overall, the following fractions could be isolated: intracellular content (without cell wall), cell wall, vacuole, vacuole+cytoplasm and chloroplasts.

Measurement of Cu concentration in *N. obtusa* cell

To prepare the whole cell or cell wall samples for atomic absorption spectroscopy (AAS) (Campbell and Plank 1998), algal material were dried at 25–30 °C for 4–7 days to constant dry weight, weighted (AEJ 220-4M, Kern, Balingen, Germany) and placed in a ceramic crucible (Haldenwanger,

Waldkraiburg, Germany) to destroy the combustible (organic) portion of the sample by thermal decomposition in a muffle furnace (SNOL-1,6.2,5, Borispil, Ukraine) at 450–550 °C for 2–3 h. The sample was heated with 0.5-mL nitric acid (Roth, Karlsruhe, Germany) until the acid evaporates up to a half volume and then diluted to 5 mL with deionized water. Freshly obtained cell fractions excepting the cell wall were diluted up to 4 mL, prior to AAS analysis.

Content of Cu was determined by Perkin Elmer Optima 7000 Dual View ICP Optical Emission Spectrometer (Waltham, MA, USA). Cu concentration measurements were performed at 327.393 nm. Standard Cu ion solutions of 0.100, 1.00 and 10.0 mg/L (Perkin Elmer, USA) were used for the device calibration. Cu concentration in the whole cell and cell wall was expressed as milligram per gram of cell dry weight (DW).

Determination of soluble Cu

The concentrations of dissolved Cu in 100 mg/L nCuO suspension at pH 7.7 and 5.5 (pH was adjusted by the addition of 0.1 M HCl) as well as intracellular medium, vacuole and cytoplasm+vacuole fractions were evaluated spectrophotometrically after their ultrafiltration in Microsep™ Advance Centrifugal Devices (Pall Corporation, Ann Arbor, MI, USA) containing polyethersulfone membranes with a cut-off of 1 kDa, at 5000 g (5430R, Eppendorf, Hamburg, Germany) for 1 h (Adam et al. 2014). For Cu measurements in ultrafiltrates, freshly prepared unbuffered suspensions sonicated for 15 min and aged for 3 h were used.

Scanning electron microscopy

For the analysis by scanning electron microscope (FEI Quanta 250, the Netherlands), the samples of cell wall and cell interior (vacuole or vacuole+cytoplasm) were obtained as described in previous section. Then, small fragments of the cell wall cut with a razor or droplets of respective cell interior fraction were placed on a sample stub and left to dry at room temperature and were used to obtain backscattered electron images. Prior to elemental analysis by energy dispersive X-ray (EDX) spectroscopy, the sample was coated by a carbon layer (15–25 nm of thickness; Emitech, CA7625 (Quorum technologies, Laughton, UK) attachment for carbon coating). The diameter of analysed spot was 5 μm.

Statistical analysis

Comparison between intracellular Cu concentrations before and after ultrafiltration was done by using one-way ANOVA and two planned contrasts for treatments with nCuO suspension and CuSO$_4$ solution at a significance level 0.05. There were three independent replicates per treatment. The statistical

analysis was carried out using the software PASW Statistics 18.0 (Predictive Analytics Software, IBM).

Results

Cu distribution in the cell compartments

Cu accumulation in the whole cell or particular compartment was investigated after 3-h-exposure at similar lethal effective concentrations (8-day LC_{50}) multiplied by approximately 150, i.e. 100 mg/L nCuO suspension and 3.18 mg Cu/L as $CuSO_4$. In both cases, the major part of Cu accumulated in the cell walls, respectively, 1.21 ± 0.23 mg Cu/g DW and 0.17 ± 0.010 mg Cu/g DW (Table 1). The overall cellular Cu concentration after treatment with corresponding concentrations of nCuO and $CuSO_4$ was found to be 1.94 ± 0.17 and 0.24 ± 0.027 mg/g DW (Table 1). As it is illustrated in the SEM image of the cell wall (Fig. 1B), metallic aggregates of approximately 0.2 μm or larger in size (bright spots) were located on the nCuO-treated cell surface unevenly. Elemental analysis of such areas (spectrum 1, Fig. 1) by energy dispersive X-ray spectroscopy confirmed high content of Cu (up to 70%). Relatively low amount of Cu or none was found in the areas enriched by insoluble $CaCO_3$ deposits (spectrum 2, Fig. 1). Similarly, the SEM image showed the presence of nCuO aggregates (bright spots) associated with intracellular content of charophyte cell after exposure to 100 mg/L nCuO suspension for 3 h (Fig. 2). The NP aggregates associated with the vacuolar material were tracked during the inspection of vacuole fraction that was air-dried before analysis by the SEM.

The levels of total Cu concentration found in untreated *N. obtusa* cells and in their cell walls equalled 24 ± 1.4 and 20 ± 1.3 μg/g DW, respectively (Table 1). For comparison, 20 μg/g DW is necessary for optimal functioning of aquatic plant *Ceratophyllum demersum* (Thomas et al. 2013). Approximately three times higher Cu concentration was measured in cell interior content (without cell walls) treated with nCuO suspension than in those with $CuSO_4$ solution, respectively, 0.54 ± 0.19 and 0.16 ± 0.037 mg/L (Fig. 3). However, overall concentration of Cu when evaluated on the basis of the measurements in distinctive parts of nCuO-treated cells, i.e. chloroplasts, cytoplasm-vacuole mixture and vacuole, was lower and equalled 0.25 ± 0.072 mg/L ($n = 3$, data not shown). Further, assuming that charophyte cell vacuole occupies 90% of the cell volume (Saltman and Christensen 1961), it was calculated that concentration of Cu in cytoplasm (including chloroplasts) equals 1.44 ± 0.55 mg/L (Table 1). It was impossible to measure Cu concentrations in the cell fractions obtained after the exposure to $CuSO_4$.

Intracellular concentrations of soluble Cu

Ionic Cu concentration measured in ultrafiltrates of intracellular content of nanosuspension-treated cells was found to be 0.061 ± 0.038 mg Cu^{2+}/L ($n = 3$), i.e. approximately 10% of the total intracellular Cu concentration 0.54 ± 0.19 mg Cu/L ($n = 3$) (Fig. 3). In the case of $CuSO_4$, the concentration of ionic Cu equalled 0.094 ± 0.050 mg Cu^{2+}/L ($n = 3$), i.e. approximately 60% of the total intracellular Cu concentration 0.160 ± 0.037 mg Cu/L ($n = 3$) (Fig. 3).

Ionic Cu concentration measured in the charophyte cell compartments after treatment with nCuO consisted of 0.013 ± 0.023 mg Cu^{2+}/L ($n = 3$) in the vacuole and 0.14 ± 0.069 mg Cu^{2+}/L ($n = 3$) in the cytoplasm-vacuole mixture (without chloroplast fraction).

The influence of pH on ionic Cu concentration in the nCuO suspension

The concentration of Cu^{2+} measured in the ultrafiltrate of the nominal 100 mg/L nCuO in APW at pH 7.7 and aged for 3 h equalled 0.025 ± 0.006 mg/L ($n = 3$). This comprised 0.033% of the total Cu concentration of 76.4 ± 0.51 mg/L ($n = 3$) measured in the initial suspension at zero time. The concentration of ionic Cu measured in the suspension ultrafiltrate after 3 h at pH 5.5 was higher by 42.5% in relation to that of pH 7.7.

Charophyte cell responses to $CuSO_4$ solutions and nCuO suspensions

Kinetics of cell bioelectrical response induced by 0.64–3.18 mg Cu/L as $CuSO_4$ and 100 mg/L nCuO are shown in Fig. 4. Depolarization of cell resting potential (RP) starts immediately when cells are treated with $CuSO_4$ and decreases by 40% from its initial level within 3 h at 0.64 mg Cu^{2+}/L, and within one and a half hour at 3.18 mg Cu^{2+}/L. In the latter case, the depolarization proceeds further even after the replacement of $CuSO_4$ solution into the control medium (APW). On the contrary, depolarization induced by nCuO suspension was slow, not exceeding 12% within 3 h, reaching only less than 20% within 6 h (Fig. 4).

The effect of 3.18 mg Cu/L added as $CuSO_4$, or 3.20, 7.99, 23.97 and 79.89 mg Cu/L added as 4, 10, 30 and 100 mg/L nCuO suspensions, respectively, on the survival of characean cells was investigated by treating them for 3 h and then keeping in the control medium. A substantial mortality up to 90% was observed already within 7–8 h from the beginning of the treatment with $CuSO_4$ solution (Fig. 5). Similarly, both 30 and 100 mg/L nCuO suspensions induced similar mortality; however, within 3–8 days. The cells treated for 3 h at lower nCuO concentrations, 4 and 10 mg/L, evoked substantial lethality

Table 1 Concentration of Cu in the compartments of *N. obtusa* cells treated by nCuO (100 mg/L) and CuSO$_4$ (3.18 mg Cu/L) for 3 h. Data represent mean ± standard deviation (number of independent replicates).

Compartment	Control (untreated)	nCuO treatment	CuSO$_4$ treatment
Whole cell	0.024 ± 0.014 (mg/g DW) (3)	1.94 ± 0.17 (mg/g DW) (3)	0.24 ± 0.027 (mg/g DW) (3)
Cell wall	0.020 ± 0.013 (mg/g DW) (4)	1.21 ± 0.23 (mg/g DW) (3)	0.17 ± 0.010 (mg/g DW) (3)
Vacuole		0.12 ± 0.036 (mg/L) (3)	nd
Cytoplasm-vacuole		0.22 ± 0.077 (mg/L) (3)	nd
Chloroplasts		1.24 ± 0.74 (mg/L) (3)	nd
Cytoplasm[a]		1.44 ± 0.55 (mg/L) (3)	

nd values below the detection limit

[a] The numbers were calculated from Cu concentration values measured in the fractions of vacuole, cytoplasm-vacuole mixture and chloroplasts as well as presuming that the volume of vacuole comprises 90% of the cell volume

later, for example, reaching 50% within approximately 24 days (Fig. 5).

Discussion

Recent data on CuO NPs suggest two main mechanisms of toxic action to the cell, i.e. through their solubilization by releasing Cu ions and through direct interaction of NPs with the cellular systems (Navarro et al. 2008; Misra et al. 2012; Wang et al. 2016). The first one relates to the questions, where and in what circumstances ions are released, e.g. in the suspension media or in the cell and the second one—where NPs are localized, e.g. associated on the cell surface or internalized within the cell. The presence of CuO NPs or their aggregaates attached to the cell wall of *N. obtusa* cell have been confirmed (Fig. 1) by SEM images and elemental analysis (this paper and Gylytė et al. 2015). Various NPs localized on and in the walls of plant cells have been visualized in the roots of *Phaseolus radiatus* and *Triticum aestivum* (Cu, Lee et al. 2008), *Schoenoplectus tabernaemontani* (CuO, Hua et al. 2014), and in unicellular green alga *Pseudokirchneriella subcapitata*

Fig. 1 SEM images of the cell wall of charophyte cell exposed to 100 mg/L nCuO for 3 h. **a** *Dark regions* represent "clean" cell wall surface (*arrow 1*), *grey regions* represent cell surface covered by calcium carbonate deposits (*arrow 2*). *Plus (+)* indicates points where elemental EDX-analysis, an energy dispersive X-ray spectroscopy coupled to the SEM was accomplished (analysed spot comprised the area of 5 μm in a diameter), *rectangle* indicates enlarged area shown on the B. **b** CuO nanoparticle aggregates are shown in turquoise colour. Spectrum 1 (s1) and spectrum 2 (s2) represent the areas with high Cu and high Ca contents, respectively.
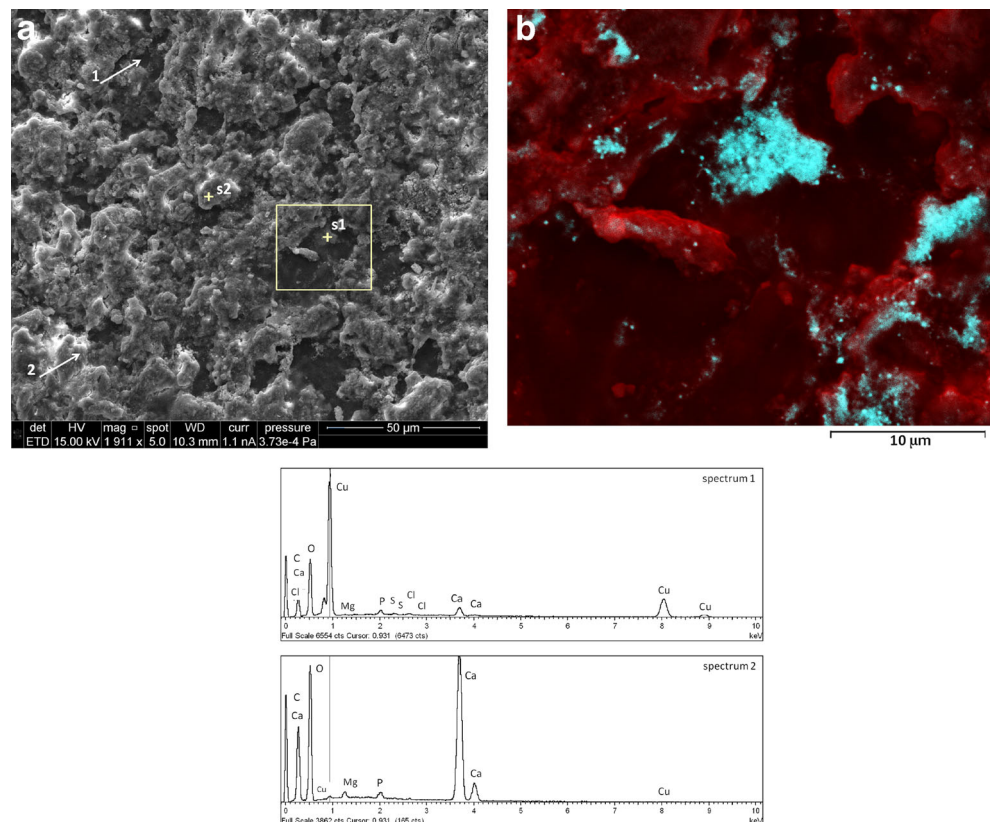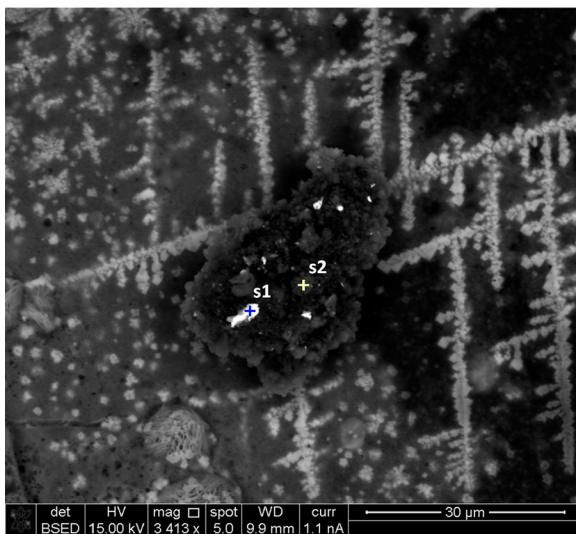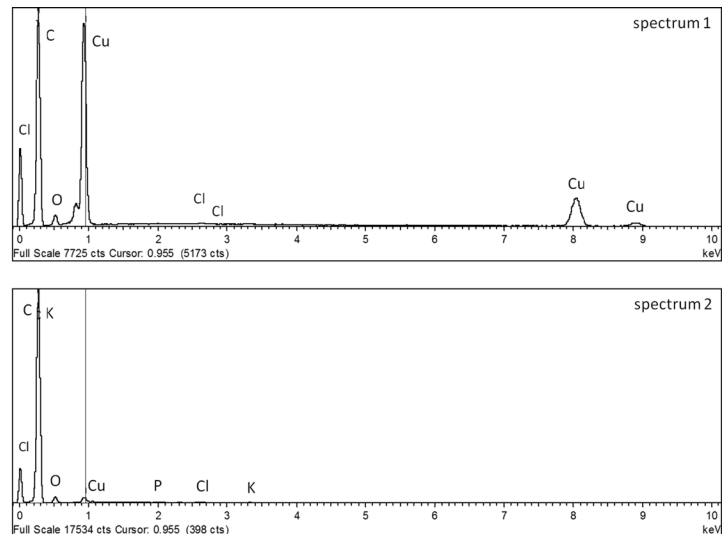
Fig. 2 SEM images of CuO aggregates (*bright spots*) associated with intracellular content found in the vacuole of *N. obtusa* cell exposed to 100 mg/L nCuO for 3 h. *Plus* (+) indicates points where elemental EDX-

analysis was accomplished (analysed spot comprised the area of 5 μm in a diameter). Spectrum 1 (s1) and spectrum 2 (s2) represent the areas with high Cu and low Cu contents, respectively

(SiO$_2$, Van Hoecke et al. 2008). The presence of CuO NPs was also shown in the cell interior of characean cell (Fig. 2), the most veracious in the vacuole compartment. In most characean cells, the central vacuole occupies about 95% of the cell volume (Beilby and Casanova 2014) and can be collected after the node(s) of internodal cell was(were) cut. Minor contamination of the drops of freely seeping vacuole sap can also be due to disintegrated vacuolar membrane (tonoplast) and/or cytoplasm. By using similar fractionation method into vacuole and cytoplasm (including cell wall) from *Chara australis*, Oikawa et al. (2011) have justified high purity of the compartments, respectively, 99.9 and 93.1%, as followed from the results with two compartment-specific marker enzymes. For photosynthetic organisms, CuO NPs have been

observed internalized in blue-green alga *M. aeruginosa* (Wang et al. 2011) and unicellular green alga *C. reinhardtii* (Perreault et al. 2012, Melegari et al. 2013). Uptake and bioaccumulation of CuO NPs have also been suggested in aquatic macrophytes *Landoltia punctata* (Shi et al. 2011) and *Lemna gibba* (Perreault et al. 2014b), while Lalau et al. (2015) could not observe NPs in tissues and cells of *L. punctata*.

Most of Cu accumulated in the charophyte cell was measured in the cell wall fraction, irrespective whether cells were exposed for 3 h to 100 mg/L of nCuO suspension or 3.2 mg/L ionic Cu as CuSO$_4$, corresponding to 63 and 74%. It has to be mentioned that the concentration of Cu determined in the walls of nanosuspension-exposed cells represents the least
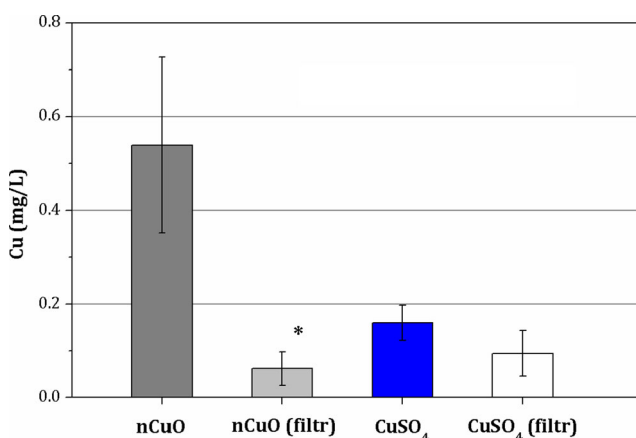

Fig. 3 Cu concentration in intracellular (cell wall-free) medium of *N. obtusa* cells exposed for 3 h to nCuO suspension (100 mg/L) and CuSO$_4$ solution (3.18 mg Cu/L) before and after ultrafiltration (filtr). Data represent the mean ± standard deviation (*n* = 3 independent replicates). *Asterisk* is for significant difference between Cu concentrations in the samples before and after ultrafiltration at $\alpha = 0.05$
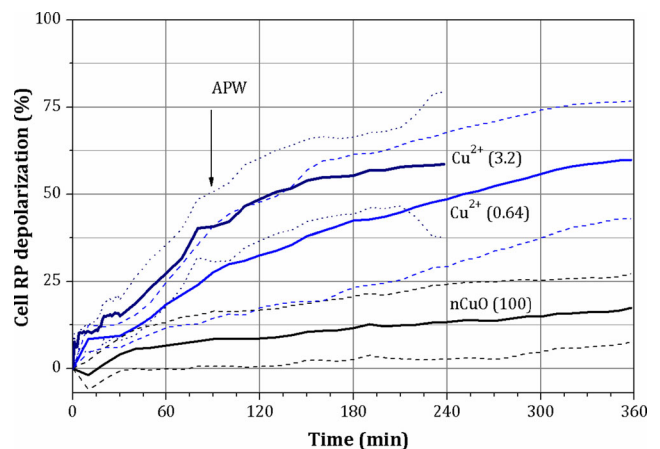

Fig. 4 Kinetics of resting potential (RP) depolarization of *N. obtusa* cells treated continuously with 0.64 mg Cu/L as CuSO$_4$ or 100 mg/L of nCuO. The treatment with 3.18 mg Cu/L as CuSO$_4$ was stopped at 90th minute by replacing with APW. *Bold curves* show average cell RP (*n* = 9 and 12 cells, respectively, for CuSO$_4$ of 0.64 and 3.18 mg Cu/L, and *n* = 20 for nCuO) and the *dashed* or *dotted curves* show corresponding standard deviations
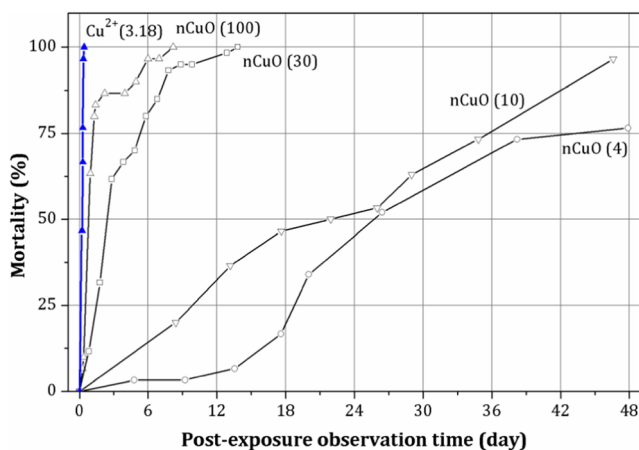
Fig. 5 Mortality kinetics of charophyte algae cells of *N. obtusa* in control medium after exposure for 3 h to 4, 10, 30 and 100 mg/L of nCuO suspensions or 3.18 mg Cu/L as CuSO₄. Each *curve* represents 30–60 cells/treatment. Mortality of untreated cells within 36-day observation period did not exceed 10%

concentration, since some part of nCuO NPs attached loosely to cell surface could be lost during mechanical squeeze out of the internal cell content. Post-exposure wash with ethylenediamintetraacetic acid, in order to remove loosely bound metal from the cell wall of marine diatom *Thalassiosira weissflogii* exposed to 5 mg/L of nCuO or Cu added as $CuCl_2$, yielded 2–5% of the total accumulated Cu (Bielmyer-Fraser et al. 2014). Interestingly, the percentage of Cu concentration in the walls of this diatomic algae cell comprised almost 85% from the total accumulated Cu for nCuO-treated cells, i.e. *major* part as it was obtained for *N. obtusa* in our study. Whereas lower concentration (28%) was obtained for the cells treated by ionic Cu, showing that the proportion of intracellular versus cell wall concentrations in diatoms for soluble Cu is opposite to that in characean cells.

Of the total intracellular Cu concentration measured in *N. obtusa* cells after exposure to nanosuspension, roughly one tenth was found to be soluble, i.e. ionic, Cu. The part of intracellular Cu^{2+} could be generated by the NPs attached to the cell wall with its milieu at pH 5.0–5.5 as it was assessed for characeans (Lucas and Smith 1973; Métraux et al. 1980). We found that solubility of 100 mg/L of nCuO in APW increases by more than 40% at pH 5.5 compared to negligible 0.033% at pH 7.7. Similar relationship between the release of Cu ions from nCuO and acidity in various media has been reported (Studer et al. 2010, Odzak et al. 2014). Another source for the appearance of intracellular Cu ions might be vacuole compartment with pH 5.25–5.5 in *N. obtusa* (Gyanes et al. 1978; Mimura and Kirino 1984).

To compare the effects of nCuO and ionic Cu on charophyte cell, isobolic concentrations were chosen, i.e. concentrations equivalent to those inducing similar 8-day-lethal effect (Gylytė et al. 2015). In our study, a threefold larger intracellular concentration of Cu measured after 3-h-

treatment with nCuO suspension suggested that internally accumulated Cu was far less active than that found after the same exposure duration to CuSO₄ solution. Indeed, external 3.18 mg/L of ionic Cu concentration in the medium lead to intracellular Cu concentration equal to 0.16 mg/L, which induced fast (within 90-min), substantial and irreversible cell membrane depolarization, whereas 100 mg/L nCuO conditioned augmentation of intracellular Cu concentration up to 0.54 mg/L, however, yielded negligible depolarization (Fig. 4), and this depolarization has been shown to be insignificant even at elevated up to 1 g/L of nCuO (Manusadžianas et al. 2012). Thus, the lack of immediate cell depolarization in the case of exposure to nCuO serves as evidence that intracellular Cu accumulated in charophyte cells after exposure to nCuO have to be, in major part, in nanoparticle form.

In the present study, an early attempt to evaluate accumulation of Cu in the main parts of charophyte cell exposed to nCuO suspension or Cu salt solution was done. Mechanical separation of charophyte cell into cell wall, protoplasm and vacuole compartments applied in the study has also been used earlier to explore the distribution of metals among cell compartments by means of radioactive labelling (Hampson 1967) or localization and dynamics of metabolites in the vacuole and cytoplasm by applying metabolomic approach (Oikawa et al. 2011). The author of the first reference mentioned that some contamination of protoplasm with vacuolar sap could take place, while the authors of the second study emphasized high purity of vacuole fraction identified by using enzymatic marker after its separation from the rest of the cell. Since we found that both treatments lead to the exhibition of only minor part of Cu in the cell interior relatively to the amount accumulated in the cell wall, an accurate characterization and quantification of isolated cell fractions in terms of their purity are necessary to better understand the localization of CuO NPs and/or generated ionic Cu concentrations within the charophyte cell.

In general, the data of our study show that more than three times higher Cu concentration accumulated intracellularly when internodal cells of *N. obtusa* were treated for 3 h by nCuO than by CuSO₄ at used concentrations. Nevertheless, contrary to CuSO₄, the toxic effects of nCuO were delayed. In our previous study (Gylytė et al. 2015), it has been shown that even very short, up to 1 min, exposure to 100 mg/L nCuO causes cell death eventually, after several weeks. The mechanism of adverse action in charophyte cells has to include the occurrence of Cu ions due to slow dissolution of nCuO particles or its aggregates associated with the cell wall and/or within the cell. However, the details of this process need to be explored. In particular, although the intracellular ionic Cu can be measured by ultrafiltration, it is difficult or even impossible to separate from the total Cu concentration measured the part of Cu ions that dissolved from the nCuO and were immediately bound on various intracellular sites. We found that approximately one tenth of the total intracellular Cu

comprised Cu ions when *N. obtusa* was exposed to nCuO; however, no visible depolarization in comparison to that of CuSO$_4$-induced could be observed. To decide whether or not ionic Cu release from nCuO comprises a primary mechanism of CuO NPs toxic action in charophyte cell, further investigation on accumulation of Cu in the cells exposed to various nCuO or Cu salt concentrations and treatment durations is needed to relate intracellular soluble Cu concentrations with bioelectrical reaction of the cell membrane.

References

Adam N, Leroux F, Knapen D, Bals S, Blust R (2014) The uptake of ZnO and CuO nanoparticles in the water-flea *Daphnia magna* under acute exposure scenarios. Environ Pollut 194:130–137

Aruoja V, Dubourguier HC, Kasemets K, Kahru A (2009) Toxicity of nanoparticles of CuO, ZnO and TiO$_2$ to microalgae *Pseudokirchneriella subcapitata*. Sci Total Environ 407:1461–1468

Baron-Epel O, Gharyal KP, Schindler M (1988) Pectins as mediators of wall porosity in soybean cells. Planta 175:389–395

Beilby MJ, Casanova MT (2014) The physiology of *Characean* cells. The physiology of characean cells. Springer-Verlag, Berlin, Heidelberg

Bielmyer-Fraser GK, Jarvis TA, Lenihan HS, Miller RJ (2014) Cellular partitioning of nanoparticulate versus dissolved metals in marine phytoplankton. Environ Sci Technol 48:13443–13450

Bondarenko O, Ivask A, Käkinen A, Kahru A (2012) Sub-toxic effects of CuO nanoparticles on bacteria: kinetics, role of Cu ions and possible mechanisms of action. Environ Pollut 169:81–89

Campbell C, Plank C (1998) Preparation of plant tissue for laboratory analysis. In: Kalra Y (ed) Handbook of methods for plant analysis. CRC Pres, Boca Raton, pp. 37–51

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J 3:1–30

Evans TD, Leal JR (1980) Silver:silver halide electrode construction and preparation. Lab Pract 8:846–847

Foissner I, Wasteneys GO (2014) *Characean* internodal cells as a model system for the study of cell organization. Int Rev Cell Mol Biol 311:307–364

Griffitt RJ, Luo J, Gao J, Bonzongo J-C, Barber DS (2008) Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. Environ Toxicol Chem 27:1972–1978

Gyanes M, Andrianov VK, Bulychev AA, Kurella GA (1978) Light-induced H$^+$ accumulation in the vacuole of *Nitellopsis obtusa*. J Exp Bot 29:1185–1195

Gylytė B, Manusadžianas L, Sadauskas K, Vitkus R, Jurkonienė S, Karitonas R, Petrošius R, Skridlaitė G, Vaičiūnienė J (2015) Latent cell mortality after short-term exposure of *Nitellopsis obtusa* cells to copper oxide nanoparticles. Bot Lith 21:89–98

Hampson M (1967) Uptake of radioactivity by aquatic plants and location in the cells: II. Uptake of cerium-144 by the freshwater plant *Nitella opaca*. J Exp Bot 18:34–53

Jo HJ, Choi JW, Lee SH, Hong SW (2012) Acute toxicity of Ag and CuO nanoparticle suspensions against *Daphnia magna*: the importance of their dissolved fraction varying with preparation methods. J Hazard Mater 227-228:301–308

Kostkevičienė J, Sinkevičienė Z (2008) A preliminary checklist of Lithuanian macroalgae. Bot Lith 14:11–27

Lalau CM, de Mohedano RA, Schmidt ÉC, Bouzon ZL, Ouriques LC, dos Santos RW, da Costa CH, Vicentini DS, Matias WG (2015) Toxicological effects of copper oxide nanoparticles on the growth rate, photosynthetic pigment content, and cell morphology of the duckweed *Landoltia punctata*. Protoplasma 252:221–229

Lee W-M, An Y-J, Yoon H, Kweon H-S (2008) Toxicity and bioavailability of copper nanoparticles to the terrestrial plants mung bean (*Phaseolus radiatus*) and wheat (*Triticum aestivum*): plant agar test for water-insoluble nanoparticles. Environ Toxicol Chem 27:1915–1921

Lucas WJ, Smith FA (1973) The formation of alkaline and acid regions at the surface of *Chara corallina* cells. J Exp Bot 24:1–14

Manusadžianas L, Caillet C, Fachetti L, Gylytė B, Grigutytė R, Jurkonienė S, Karitonas R, Sadauskas K, Thomas F, Vitkus R, Férard J-F (2012) Toxicity of copper oxide nanoparticle suspensions to aquatic biota. Environ Toxicol Chem 31:108–114

Manusadžianas L, Sadauskas K, Vitkus R (2010) Comparative study of indices used in toxicity evaluation of effluents. Desalination 250:383–389

Manusadžianas L, Vitkus R, Pörtner R, Märkl H (1999) Phytotoxicities of selected chemicals and industrial effluents to *Nitellopsis obtusa* cells, assessed by using a rapid electrophysiological charophyte test. ATLA Altern to Lab Anim 27:379–386

Manusadžianas L, Vitkus R, Sakalauskas V (1995) Wastewater toxicity assessment using electrophysiological response of charophyte *Nitellopsis obtusa*. Environ Toxicol Water Quality 10:49–56

Melegari SP, Perreault F, Costa RHR, Popovic R, Matias WG (2013) Evaluation of toxicity and oxidative stress induced by copper oxide nanoparticles in the green alga *Chlamydomonas reinhardtii*. Aquat Toxicol 142-143:431–440

Métraux J-P, Richmond PA, Taiz L (1980) Control of cell elongation in *Nitella* by endogenous cell wall pH gradients: multiaxial extensibility and growth studies. Plant Physiol 65:204–210

Miao L, Wang C, Hou J, Wang P, Ao Y, Dai S, Lv B (2015) Effects of pH and natural organic matter (NOM) on the adsorptive removal of CuO nanoparticles by periphyton. Environ Sci Pollut Res 22:7696–7704

Mimura T, Kirino Y (1984) Changes in cytoplasmic pH measured by 31P-NMR in cells of *Nitellopsis obtusa*. Plant Cell Physiol 25:813–821

Misra SK, Dybowska A, Berhanu D, Luoma SN, Valsami-Jones E (2012) The complexity of nanoparticle dissolution and its importance in nanotoxicological studies. Sci Total Environ 438:225–232

Moore MN (2006) Do nanoparticles present ecotoxicological risks for the health of the aquatic environment? Environ Int 32:967–976

Moos N, Maillard L, Slaveykova VI (2015) Dynamics of sub-lethal effects of nano-CuO on the microalga *Chlamydomonas reinhardtii* during short-term exposure. Aquat Toxicol 161:267–275

Mortimer M, Kasemets K, Kahru A (2010) Toxicity of ZnO and CuO nanoparticles to ciliated protozoa *Tetrahymena thermophila*. Toxicology 269:182–189

Moschini E, Gualtieri M, Colombo M, Fascio U, Camatini M, Mantecca P (2013) The modality of cell-particle interactions drives the toxicity of nanosized CuO and TiO$_2$ in human alveolar epithelial cells. Toxicol Lett 222:102–116

Navarro E, Baun A, Behra R, Hartmann NB, Filser J, Miao AJ, Quigg A, Santschi PH, Sigg L (2008) Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. Ecotoxicology 17:372–386

Odzak N, Kistler D, Behra R, Sigg L (2014) Dissolution of metal and metal oxide nanoparticles in aqueous media. Environ Pollut 191: 132–138

Oikawa A, Matsuda F, Kikuyama M, Mimura T, Saito K (2011) Metabolomics of a single vacuole reveals metabolic dynamism in an alga *Chara australis*. Plant Physiol 157:544–551

Perreault F, Popovic R, Dewez D (2014a) Different toxicity mechanisms between bare and polymer-coated copper oxide nanoparticles in *Lemna gibba*. Environ Pollut 185:219–227

Perreault F, Samadani M, Dewez D (2014b) Effect of soluble copper released from copper oxide nanoparticles solubilisation on growth and photosynthetic processes of *Lemna gibba* L. Nanotoxicology 8: 374–382

Perreault F, Oukarroum A, Melegari SP, Matias WG, Popovic R (2012) Polymer coating of copper oxide nanoparticles increases nanoparticles uptake and toxicity in the green alga *Chlamydomonas reinhardtii*. Chemosphere 87:1388–1394

Proseus TE, Boyer JS (2005) Turgor pressure moves polysaccharides into growing cell walls of *Chara corallina*. Ann Bot 95:967–979

Saison C, Perreault F, Daigle JC, Fortin C, Claverie J, Morin M, Popovic R (2010) Effect of core-shell copper oxide nanoparticles on cell culture morphology and photosynthesis (photosystem II energy distribution) in the green alga, *Chlamydomonas reinhardtii*. Aquat Toxicol 96:109–114

Saltman P, Christensen T (1961) Relative volumes of cytoplasm and vacuole in *Nitella*. Nature 191:825–826

Shi J, Abid AD, Kennedy IM, Hristova KR, Silk WK (2011) To duck-weeds (*Landoltia punctata*), nanoparticulate copper oxide is more inhibitory than the soluble copper in the bulk solution. Environ Pollut 159:1277–1282

Shimmen T, Kikuyama M, Tazawa M (1976) Demonstration of two stable potential state of plasmalemma of *Chara* without tonoplast. J Membr Biol 30:249–270

Studer AM, Limbach LK, Van Duc L, Krumeich F, Athanassiou EK, Gerber LC, Moch H, Stark WJ (2010) Nanoparticle cytotoxicity depends on intracellular solubility: comparison of stabilized copper metal and degradable copper oxide nanoparticles. Toxicol Lett 197:169–174

Thomas G, Stärk HJ, Wellenreuther G, Dickinson BC, Küpper H (2013) Effects of nanomolar copper on water plants—comparison of biochemical and biophysical mechanisms of deficiency and sublethal toxicity under environmentally relevant conditions. Aquat Toxicol 140-141:27–36

Van Hoecke K, De Schamphelaere KAC, Van der Meeren P, Lucas S, Janssen CR (2008) Ecotoxicity of silica nanoparticles to the green alga *Pseudokirchneriella subcapitata*: importance of surface area. Environ Toxicol Chem 27:1948–1957

Wang D, Lin Z, Wang T, Yao Z, Qin M, Zheng S, Lu W (2016) Where does the toxicity of metal oxide nanoparticles come from: the nanoparticles, the ions, or a combination of both? J Hazard Mater 308: 328–334

Wang Z, Li J, Zhao J, Xing B (2011) Toxicity and internalization of CuO nanoparticles to prokaryotic alga *Microcystis aeruginosa* as affected by dissolved organic matter. Environ Sci Technol 45:6032–6040

Whitehouse DG, Moore AL (1993) Isolation and purification of functionally intact chloroplasts from leaf tissue and leaf tissue protoplasts. In: Graham JM, Higgins JA (eds) Biomembrane Protocols. Methods in Molecular Biology. Humana Press, Totowa, pp. 123–131

Zhang D, Hua T, Xiao F, Chen C, Gersberg RM, Liu Y, Ng WJ, Tan SK (2014) Uptake and accumulation of CuO nanoparticles and CdS/ZnS quantum dot nanoparticles by *Schoenoplectus tabernaemontani* in hydroponic mesocosms. Ecol Eng 70:114–123