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LATENT CELL MORTALITY AFTER SHORT-TERM EXPOSURE OF *NITELLOPSIS OBTUSA* CELLS TO COPPER OXIDE NANOPARTICLES

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Abstract

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 [Latentinė *Nitellopsis obtusa* ląstelių žūtis jas trumpą laiką paveikus vario oksido nanodalelių suspensijomis]. – Bot. Lith., 21(2): 89–98.

The effects of 5 s–24 h exposure to CuO nanosuspension (nCuO) and $CuSO_4$ on mortality of *Nitellopsis obtusa* cells within 48 days were investigated. Based on LED_{50} kinetics, a toxicological endpoint of lethal exposure duration that induces 50% cell mortality, it was shown that mortality response of 10-min treated cells (by 3.2 and 24–79.9 mg Cu/l, respectively $CuSO_4$ and nCuO) did not differ after 12th day rewash in control medium. Larger amount of total Cu (in mg/g cell DM) was measured in charophyte cells exposed to nCuO (79.9 mg Cu/l) than to ionic Cu (3.2 mg Cu/l). The lower nCuO-induced mortality than that of Cu^{2+} during earlier rewash period could be related to the sorption of nanoparticles on the cell wall, which was confirmed by scanning electron microscope images and energy dispersive X-ray spectrum data. Effect threshold, the shortest exposure duration that induces cell mortality significantly different from the control during a post-exposure period, comprised 30 s in 24 mg Cu/l of nCuO, however, it could not be identified and was less than 5 s in 79.9 mg Cu/l of nCuO.
Keywords: charophyte cell, mortality, nCuO.

INTRODUCTION

Nanosized copper oxide particles (nCuO) are nowadays produced industrially and available commercially. The nCuO are increasingly used in technological applications, e.g. metallic coatings and antifouling paints (Turner, 2010), batteries (Yang et al., 2012), gas sensors (Zhang et al., 2006); in medicine, e.g. as an alternative to antibiotic treatment (Schrand et al., 2010); in agriculture, e.g. as pesticides or antimicrobial agents (Nair et al., 2010), etc. Alongside with other engineered nanoparticles, nCuO are emitted as particulates in smelters, metal foundries, and as torn particles from asphalt and rubber tires (Midander et al., 2007). Nanomaterials may be released to the environment with wastewater and activated sludge (Blaise et al., 2008; Singh & Agrawal, 2008). The occurrence of these small size and specific properties possessing nanoparticles in the aquatic media may cause adverse effects to different organisms.

It has already been demonstrated that nCuO are acutely toxic to many organisms including crustaceans (Heinlaan et al., 2008; Rossetto et al., 2014), microalgae (Aruoja et al., 2009), macroalgae (Manusadžianas et al., 2012), aquatic plants (Per-

reault et al., 2014) and fish (Isani et al., 2013), nevertheless, ionic copper is more toxic to most of these organisms. Several studies have indicated that the acute toxicity of nCuO observed in bacteria (Bondarenko et al., 2012), microalgae (Aruoja et al., 2009), protozoa (Mortimer et al., 2010) and crustacean (Jo et al., 2012) is due to the release of copper ions from the nanoparticles. These observations are in line with a Trojan horse-type mechanism. Other studies indicate that not all the observed effects could be explained by dissolved copper from nanoparticles (Griffitt et al., 2008; Manusadžianas et al., 2012; Perreault et al., 2012). Jiang et al. (2009) showed that the toxicity of nano-scaled aluminium, silicon, titanium and zinc oxides to bacteria were not only from the dissolved metal ions, but also from their ability to attach to the cell walls rather than to form aggregates. Van Hoecke et al. (2008) demonstrated the adsorption of SiO2 nanoparticles on the cell wall of *P. subcapitata* and thus concluded that toxicity occurs through the surface interaction. Lee et al. (2008) reported that toxicity effects on the growth inhibition of *Phaseolus radiatus* and *Triticum aestivum* seedlings clearly resulted from Cu nanoparticles that were observed agglomerated inside the plant cells, but not from copper ions. However, strict differentiation between the two toxicity mechanisms seems cannot be achieved under current experimental results.

To date, the first study on chronic toxicity of nCuO to *D. magna* reproductive characteristics observed throughout 21-day has been accomplished (Adam et al., 2015). The authors found that nanoparticles were a lot less toxic than copper salts if one considered nominal concentrations; however, the dissolved fraction of the nanoparticles in exposure medium fell within the same concentration range as the dissolved fraction of copper ions. It was also shown that copper concentration accumulated in daphnids appeared much higher when exposed to nanoparticles than to $CuCl_2$. Another chronic toxicity study showed that the growth of aquatic plant *Ceratophyllum demersum* reduced when concentration of Cu ions in external medium exceeded 0.6 µg/l (Thomas et al., 2013). The exceeding of this concentration lead to the increase of copper concentration inside the plant from 20 µg/g DW that was optimal up to 400 µg/g DW, within six weeks. In our previous study (Manusadžianas et al., 2012), we found that exposure to 100 mg/l nCuO suspension for five minutes caused cell death in algae *Nitellopsis obtusa* after eight days rewash in controls. Since 4-d LC_{50} for Cu^{2+} equalled 0.13 mg/l and Cu^{2+} concentration in the medium was less than 0.01 mg/l as measured by AAS, it was concluded that the observed adverse effects on *N. obtusa* cells might be attributed to nanoparticles. To further explore the mode of nCuO toxic action on this macrophytic alga, we extended exposure duration and thus aimed to compare the dynamics of (1) delayed cell mortality response after short-term exposure to various chemical forms of copper and (2) Cu accumulation in the charophyte cell.

MATERIALS AND METHODS

Charophyte algae

Freshwater charophyte *Nitellopsis obtusa* (Desv.) J. Groves was harvested from Lake Obelija, south-east Lithuania (Kostkevičienė & Sinkevičienė, 2008). After separation from thalloma, single internodal cells were kept at room temperature (18–24°C) in glass aquariums filled with equal parts of non-chlorinated tap water and artificial pond water (APW) containing (mM): 0.1 KH_2PO_4, 1.0 $NaHCO_3$, 0.4 $CaCl_2$, 0.1 $Mg(NO_3)_2$ and 0.1 $MgSO_4$ (unbuffered, pH 7.0–7.8).

Preparation of CuO nanosuspensions

Powder of ultrafine copper oxide nanoparticles, with an average particle size of less than 50 nm (mean 30 nm), was purchased from Sigma-Aldrich. A stock of 10 g/l CuO nanoparticles was prepared by dispersing the nanoparticles in deionised H_2O with sonication for 15 min in a bath-type sonicator (Intersonic, IS-2, 300 W, 35 kHz). The final working concentrations of 30 and 100 mg/l were prepared from the stock in APW and sonicated for 15 min immediately before the experimentation.

Determination of soluble Cu fraction

The concentrations of Cu ions in APW were evaluated by Perkin Elmer Optima 7000 Dual View ICP Optical Emission Spectrometer (USA) after ultrafiltration of 100 mg/l nCuO suspension in Microsep™ Advance Centrifugal Devices (Pall Corporation, USA), containing polyethersulfone membranes with a cut-off of 1 kDa, at 5000 g for 1 h. The ultrafiltra-

tion was performed on freshly prepared, sonicated for 15 min and aged for 3 h suspension.

Observation of cell lethality

Lethality of the cells of *N. obtusa* was investigated up to 72 days. The APW was used as the control. Single internodal cells (each 4–15 cm in length) were placed on Petri dishes (10 cells per dish, 4–6 replicates), preadapted for 1 to 2 d in APW, and then kept in thermostat at 15–18ºC in the dark. The preadaptation in APW before the test allowed occasionally discarding dead cells that had been injured during the transfer to the 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. The solutions of Cu $(NO_3)_2$ (Aldrich) or $CuSO_4$ (Merck) were prepared in APW and were replaced weekly during the exposure.

Exposure patterns and lethality endpoints

Two different exposure patterns and their respective toxicity endpoints were employed. Besides the routine exposure pattern for the assessment of LC_{50} (a median concentration yielding 50% cell death within certain exposure duration), the median lethal exposure duration (LED_{50}), which determines the time for 50% of the exposure group to die at a certain concentration, was used. The latter endpoint definition is similar to the 50% lethal time endpoint definition (Bliss and Stevens, 1937), but employs a different exposure pattern. To reveal the differences between these two endpoints, a convenient approach would be to distinguish exposure duration (ED, the interval from the beginning of the test, during which cells are treated with a toxicant) from the endpoint duration (*t*, time from the beginning of the test, after which mortality data are evaluated). Endpoints of LC_{50} and 50% lethal time are calculated when cells are treated permanently, that is, ED = *t*. In the rewash lethality test, the exposure pattern in LED_{50} finding protocol includes periods of exposure and successive rewash in APW; thus, the endpoint duration is a sum of both periods. The durations of exposure to Cu salt were 1 min, 3 min, 5 min, 15 min, 30 min, 1 h, 6 h and to nCuO suspension were 5 s, 15 s, 30 s, 1 min, 3 min, 5 min, 15 min, 30 min, 1 h, 6 h, 12 h, 24 h.

Experimental design

Stock suspension was sonicated for 15 min and then 150 or 500 µl aliquots of stock suspension were transferred to individual flasks with 50 ml APW to have final concentrations of 30 or 100 mg/l nCuO, i.e. 24 and 79.9 mg Cu/l, respectively. Each flask was sonicated for 15 min in bath sonicator with ice to prevent heating of the suspension and maintain the temperature at 15–18°C. After sonication, suspension was poured off the flask on the 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 larger portion of non-agglomerated nanoparticles and/or smaller agglomerates. Cells in each Petri dish were rinsed twice with the APW after exposure for 5 s–24 h, and left in APW for survival observation or measurement of Cu concentration.

Measurement of Cu concentration in the cell

The samples were washed with APW after the exposure. Algal cells were dried at 25–30°C for 4–7 days to constant dry weight. Air-dried algal material was weighted and placed in a ceramic crucible to destroy the combustible (organic) portion of the sample by thermal decomposition in a muffle furnace at 450–550°C for 2–3 h. The sample was heated with 0.5 ml nitric acid until the acid evaporates up to a half volume and then diluted to 5 ml with deionised water.

Content of Cu was determined by Perkin Elmer Optima 7000 Dual View ICP Optical Emission Spectrometer (USA). Copper concentration measurements were performed at 327.393 nm. Standard Cu ion solutions of 0.100, 1.00, 10.0 mg/l (Perkin Elmer, USA) were used for the device calibration.

Copper concentration was expressed as mg/g of cell dry mass.

Scanning electron microscopy

To prepare cell wall samples for the observation on scanning electron microscope (FEI Quanta 250, The Netherlands), charophyte cells were exposed 3 h to 100 mg/L nCuO, rinsed with control medium and air-dried on filter paper for several seconds until the surface become opaque. Then one end of the cell was cut off by razor, the interior of the cell squeezed out and left to dry. The fragments of the cell wall were used to take backscattered electron (BSE) images.

Prior to elemental analysis by energy dispersive X-ray (EDX) system, the sample was coated by a carbon layer (15–25 nm of thickness; Emitech, CA7625 attachment for carbon coating). The diameter of analyzing spot was 5 μm.

Statistical analysis

To calculate LC_{50} and LED_{50} values, the probit analysis was used. Two median effective concentrations were considered different when their confidence intervals didn't overlap, otherwise the Litchfield-Wilcoxon method was applied to confirm significance of difference for LC_{50}s. The exact Fisher test was used for comparison of quantal (mortality probabilities) effects at a certain time. In all comparisons significance level was 0.05. The statistical analysis was carried out using the software SPSS 13.0.

RESULTS

Dynamics of cell mortality

Eight-day LC_{50} values for *Nitellopsis obtusa* cells treated by copper sulphate equalled 0.024 ± 0.007 and nCuO – 0.538 ± 0.064 mg Cu/l (median value ± se). To fulfil sensitivity requirements for long-term mortality observations and chemical measurements of accumulated Cu in the cells, roughly 150 times higher concentrations than those of LC_{50}s were chosen for the comparison of mortality kinetics observed in rewash (control) medium after exposure to 3.2 mg Cu/l of Cu^{2+} and 79.9 mg Cu/l of nCuO. In the case of copper salt (Fig. 1), a significantly higher mortality over controls was observed for the cells exposed for 15 min–6 h at the first day of the rewash, reaching 45 and 60% mortality of 15 min-exposed cells at days four and eight, respectively. In the case of nCuO (Fig. 2A), a significantly higher mortality over controls was observed for the cells exposed for 1–6 h at the first day of the rewash, reaching lesser, 8 and 22%, mortality of 15 min-exposed cells at days four and eight, respectively. Corresponding differences in cell mortality observed at the fourth and eighth day after 15 min-exposure for various copper forms were significant. Later on, however, no significant differences between mortality percentages of the cells treated by Cu^{2+} or nCuO for 15 min were found during the rewash at 11th to 48th day (Fig. 1 and 2A). Similarly, mortality kinetics did not differ for the cells exposed for 30 min already starting from the 2nd and up to 48th day of rewash (Fig. 1 and 2A).

At higher exposure durations such as 1–6 h or permanent (thick lines in Fig. 1 and 2A), the toxicity of nCuO suspensions were lower than those of Cu^{2+}. For example, although all cells exposed for 3 h to respective copper forms survived, the mortality observed after 10 h of rewash in control medium was 100 and 10%, respectively for cells exposed to 3.2 mg Cu/l as Cu^{2+} and 79.9 mg Cu/l as nCuO (Fig. 1 and 2A). Moreover, 15–20% of the cells exposed to nCuO for 1–3 h survived for 2–4 days. Obviously, the curves of cell mortality kinetics for 6 h or permanent exposure were steeper for Cu^{2+} than those for nCuO (Fig. 1 and 2A).

At lower, 3–5 min, exposure durations to 3.2 mg Cu/l of Cu^{2+} and 79.9 mg Cu/l of nCuO, the mortality curves did not differ significantly during the whole rewash period either between respective exposures or between the copper forms (Fig. 1 and 2A). Cell mortality induced by 1 min exposure to nCuO differed from 5 min at rewash durations longer than 12 days and differed also from that of Cu^{2+} at rewash durations longer than four days. No significant mortality differences among short exposures of 5 s, 15 s, 30 s and 1 min could be found (Fig. 2A).

To further compare the kinetics of cell mortality induced by various copper forms, the effect thresholds were investigated. The effect threshold repre-

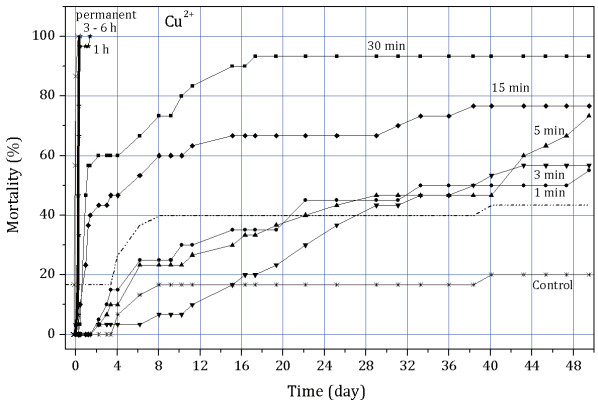

Fig. 1. Mortality kinetics of charophyte algae cells of *Nitellopsis obtusa* in control medium after exposure for 1 min–6 h to 3.18 mg/l of Cu^{2+}, added as $CuSO_4$. Thickened line represents permanent exposure in copper salt solution. Dash dot line represents statistically significant difference from controls at α = 0.05; n = 30 cells/treatment

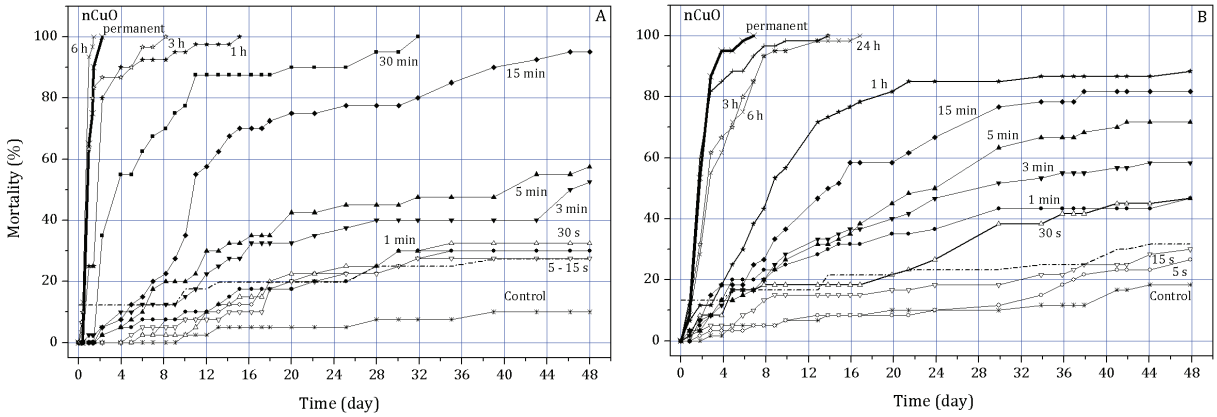

Fig. 2. Mortality kinetics of charophyte algae cells of *Nitellopsis obtusa* in control medium after exposure for 5 s–24 h to 79.9 (A) or 24 mg Cu/l (B) in nCuO suspensions. Thickened lines represent permanent exposure in corresponding medium. Dash dot lines represent statistically significant difference from controls at $\alpha = 0.05$; n = 40 and 60 cells/treatment, respectively (A) and (B)

sents the shortest exposure duration that induces cell mortality significantly different from the control, during a post-exposure period (i.e. rewash) in control medium (mortality curves coincide or are above the dashed line in Figs. 1 and 2).

In the case of Cu^{2+} (Fig. 1), the effect threshold locates within exposure durations lesser than 1 min. For this exposure, the mortality significantly exceeds that of controls at day 27. In the case of nCuO, even the shortest exposures of 5–15 s to 79.9 mg Cu/l (Fig. 2A) yielded mortality rates bigger than those of controls at day 30 and later. These exposures were not sufficient to exceed mortality effect threshold during the whole observation period of 48 days. At lower 24 mg Cu/l of nCuO, the lowest exposure exceeding the threshold was identified as 30 s, since shortest exposures of 5–15 s didn't yield cell mortality different to that of control (Fig. 2B).

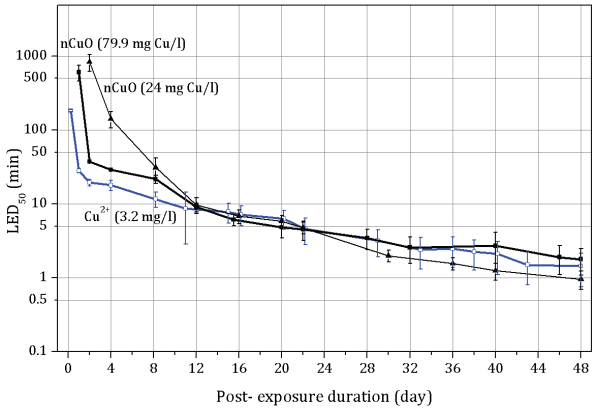

Fig. 3. Kinetics of 50% lethal exposure duration (LED_{50}, median value ± se) calculated for 3.2 mg/l of Cu^{2+} added as $CuSO_4$ solution and 24 or 79.9 mg Cu/l added as nCuO suspension

The courses of LED_{50} median values calculated for two nCuO suspensions and $CuSO_4$ solution against post-exposure duration can be divided into two parts at day 12 (Fig. 3). Although all three curves indicate growing lethality to *N. obtusa* cells over post-exposure duration, the Cu^{2+} shows the highest, nCuO of 79.9 mg Cu/l lesser and nCuO of 24 mg Cu/l the least toxicity during the initial 12-day post-exposure period. For example, comparison of LED_{50} values for 79.9 mg Cu/l as nCuO and 3.2 mg Cu/l as Cu^{2+} at the first day during the rewash in control medium gives a factor of approximately 20. No differences in 50% lethal effect durations could be seen at day 12 and later up to day 48 neither between various concentrations of nCuO, nor among ionic and nano forms of copper.

Copper oxide nanoparticle solubility

The concentration of Cu^{2+} measured in the ultrafiltrate of aged for three hours the nominal 100 mg/l nCuO in APW, equalled 0.025 ± 0.006 mg/l. This comprised 0.033% of measured total Cu concentration of 76.40 ± 0.51 mg/l measured in the initial suspension at zero time.

Dynamics of Cu accumulation

The data on accumulation of Cu in charophyte cells exposed for 5 s–24 h to 3.2 mg Cu/l supplemented as $CuSO_4$ or 79.9 mg Cu/l as 100 mg/l nCuO are presented in Fig. 4. Significantly higher concentrations of Cu were measured by ICP-OES in the

cells treated by nCuO than $CuSO_4$. At all exposure durations except 24 h, the ratio of Cu_{nano}/Cu_{salt} was at least 5, reaching 19 and 9 at 1 and 3 h, respectively.

Scanning electron microscopy images of the cell wall are shown at different magnifications in Fig. 5. The cells were exposed to 100 mg/l nCuO for 3 h. Cell surface is covered by insoluble calcium carbonate (arrows on the right picture) depositing under natural conditions in the lakes at pH 7–8. Relatively lesser part of surface area seen as dark regions represents an uncovered cell wall area. The relation of bright spots to nCuO aggregates was confirmed by elemental analysis. At the spots of analysis, 1 and 2 (Fig. 5, right picture), Cu comprised, respectively, 67.2 and 61.1% of the total measured elements including oxygen, while only 0.36% was found at the spot 3.

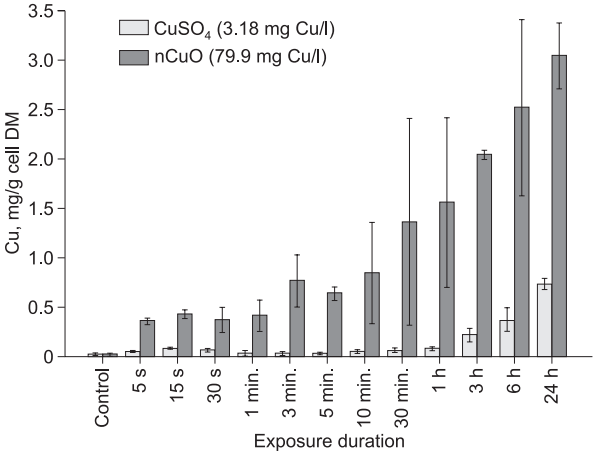

Fig. 4. Copper concentration (mean ± sd, n = 3) measured by ICP-OES in the charophyte algae cells of *Nitellopsis obtusa* exposed to Cu^{2+} (3.18 mg Cu/l as $CuSO_4$) or Cu (79.9 mg Cu/l as 100 mg/l of nCuO) for various exposure durations. Both concentrations induce 50% cell mortality within eight days. Control represents unexposed cells. All values significantly differ from controls at $\alpha = 0.05$ barring 1 and 3 minutes exposures in $CuSO_4$

Cu comprised, respectively, 67.2, 61.1 and 0.36% of the total measured elements including oxygen at the spots of analysis, 1, 2 and 3 (Fig. 5, right picture).

DISCUSSION

The results of the present study demonstrate that nanoparticulate copper was less toxic to charophyte cells than its soluble form, when mass added is considered. The ratio for respective 8-day LC_{50}s equalled 27, which was close to 23 found for the same cells within 4-day exposure (Manusadžianas et al., 2012) and to 35 found for 72-h EC_{50}s of growth inhibition in *Pseudokirchneriella subcapitata* (Aruoja et al., 2009). As for the algae, the higher toxicity of copper salts was affirmed by the comparison of species

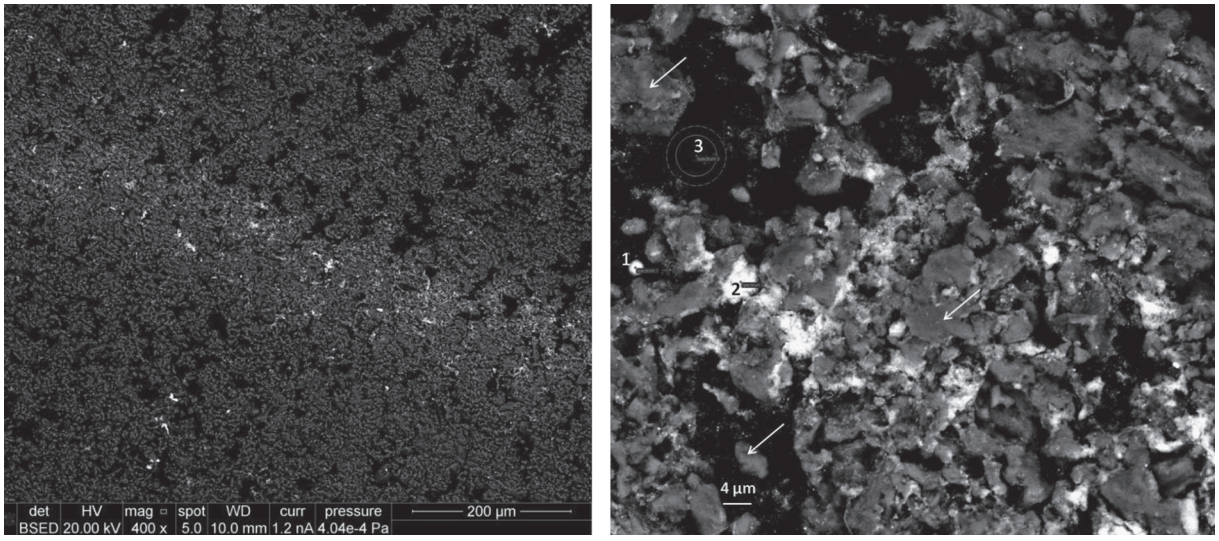

Fig. 5. SEM-BSE (scanning electron microscope-backscattered electrons) images of the cell wall of charophyte cell exposed to 100 mg/l nCuO for 3 h. Dark regions represent "clean" cell wall surface not covered by calcium carbonate deposits. The bright spots are CuO nanoparticle aggregates. On the right picture, the numbers show spots where elemental analysis, an EDX (energy dispersive X-ray system), coupled to the SEM was accomplished. Arrows show calcium carbonate deposits

sensitivity distributions (Adam et al., 2015). In this review, responses of various species including algae, moss, fungi, crustacea, protozoa, mollusca, fish, etc., to Cu^{2+} and nCuO were analysed. The overall difference in sensitivities precludes different toxicity mechanisms or, at least, temporal characteristics of the action of the two copper chemical forms.

To explore initial stages of toxicological impact, the cells were treated with the respective copper formulation for a short time and then placed in control solution for survival check. The background for choosing salt and nano-suspension concentrations was respective 8-day LC_{50} values that were multiplied by approximately 150 to fulfil sensitivity requirements. The comparison of mortality curves obtained after the 15 min–6 h exposure duration (Fig. 1 and 2A) revealed obviously stronger effect of copper ions than of nanoparticle suspension during the first two days of post-exposure in control medium, however, the toxic effects become undistinguishable later, i.e. starting from 12th day, as it is clearly seen from the LED_{50} kinetics (Fig. 3, bold lines). It means that either 10-min exposure to 3.2 mg Cu/l as Cu^{2+} or 24–80 mg Cu/l as nCuO causes similar toxic effect to charophyte cell after 12 days and later. One of the possible reasons of the delayed toxic action of NPs might be the sorption of nCuO particles on the cell wall, where from they, under the influence of excreted H^+ through the cell membrane, could release Cu^{2+} gradually. The presence of nCuO aggregates on the cell surface of *N. obtusa* was confirmed by SEM and elemental analysis (Fig. 5). The ability of nanoparticles to attach to algal cell walls has been earlier demonstrated in several studies, e.g. SiO_2 for *P. subcapitata* (Van Hoecke et al., 2008). The attachment of nanoparticles to the cell wall and slightly acidic phase of algal cell walls compose favourable conditions for degrading CuO NPs. From the direct measurements with pH-microelectrodes on the surface of charophyte cell walls it is known that, in acidic zones, pH equals 5.0 for *Chara corallina* (Lucas & Smith, 1973) and 5.5 for *Nitella* (Métraux et al., 1980). The solubility of CuO nanoparticles was investigated in non-complexing buffer solutions at pH 5.5 and 7.4 (Studer et al., 2010). Since 95.2% and < 0.1% of nCuO dissolved respectively at these pHs within 3 days, authors concluded that pH effects play the main role in Cu^{2+} release from nCuO. Similar tendency, however, in lesser extent, were reported for Cu^{2+} releases from 45 nm CuO nanoparticles in aqueous media similar in chemistry to natural fresh waters, i.e. 5–14 and 0.3–1%, respectively at pH 6.1 and 7.7 (Odzak et al., 2014). We measured negligible amount of Cu^{2+} in freshly prepared and aged for 3 h nCuO suspension, i.e. 0.033% at pH 7.7. Although it is difficult to evaluate actual dissolution of nCuO under conditions of algal cell walls, certain release of Cu^{2+} into premembrane phase from copper nanoparticles adsorbed on the surface of *N. obtusa* cells could happen.

The amount of Cu associated with charophyte cell, under our experimental conditions, exceeded that of control already after 5 s of exposure in nCuO suspension and it increased with the exposure duration (Fig. 4). From the chemical analysis undertaken in this study it is not possible to distinguish copper chemical forms, however, indirect data allow to preclude on bioactivity/bioavailability of nCuO. Earlier obtained electrophysiological data revealed the absence of cell membrane depolarization within 6 h at concentrations up to 79.9 mg Cu/l added as nCuO (Manusadžianas et al., 2012), while depolarization started immediately and augmented irrespective of the exposure was continuous or exchanged to control medium just after five minutes of treatment with 3.2 mg Cu^{2+} (current study, data not shown). This indicates that copper ions, in relation to CuO nanoparticles, are highly bioactive to charophyte cell and exceed the tolerable to aquatic plants Cu concentrations very fast, reaching 250 ppm of DM within 3 h (Fig. 4). It has been found that intracellular copper above 20 ppm of DM accumulated in *Ceratophyllum demersum* impaired plant growth during exposure in nutrient solution supplemented with $CuSO_4$ concentrations higher than 10 nM (Thomas et al., 2013).

Whether or not CuO nanoparticles may pass a mechanical barrier of thick and rigid cell wall of charophyte cell and later plasma membrane? The effective diameter of cell wall pores of macrophytic alga *C. corallina* was evaluated not to exceed 2.1 nm (Berestovsky et al., 2001). Another study analysing polysaccharide entry into cell walls of the same algae concluded the interstices in the wall matrix had a diameter of 4.6 nm (Proseus & Boyer, 2005). In cultured cells of various higher plants, the limiting diameter of wall pores for unrestricted pass

of the molecules was determined to be 3.5–5.2 nm (Carpita et al., 1979) or 6.6–8.6 nm (Baron-Epel et al., 1988). Moreover, it was suggested that the size of the pores can increase during the cell wall metabolism (Carpita & Gibeaut, 1993) or interactions between the cells and nanoparticles (Navarro et al., 2008), thus facilitating the nCuO internalization (Melegari et al., 2013). The nCuO suspension used in the present study has been characterized by laser diffraction technique earlier (Manusadžianas et al., 2012). Because reagglomeration of nanoparticles occurs fast, within up to five minutes after sonication, only the smallest of the minor part of copper oxide particles could directly pass the barrier of cell wall and plasma membrane. As for the latter, it is known that one of the possible internalization pathways proceeds through endocytosis, e.g. in cyanobacteria *Microcystis aeruginosa* (Wang et al., 2011) and in unicellular green alga *Chlamydomonas reinhardtii* (Melegari et al., 2013). In this alga, the intracellular Cu content of nCuO-treated algal cells for 6 h was twice the amount accumulated for only the soluble fraction (Perreault et al., 2012). A similar Cu accumulation tendency was obtained in our study with *N. obtusa* cells treated for the similar exposure duration, yet at bigger nominal concentration of nCuO; however, in what proportions it distributes between cell wall and charophyte cell interior remains to be explored.

To conclude, the fast electrophysiological reaction induced by copper ions contrary to nCuO as well as similar mortality response to both copper chemical forms within later stages of post-exposure, suggest that, initially, the major part of Cu measured in the cell presents as nanoparticles or their agglomerates adsorbed on the surface of the cell and only fraction of the small nanoparticles might penetrate charophyte cell wall and then the membrane. Later on, toxicity to charophyte cell is likely caused by the Cu^{2+} released from adsorbed nanoparticles and/or internalized small nanoparticles. To further identify whether or not nanoparticles accumulate intracellularly, experiments on various compartments of charophyte cell are needed. This would be possible due to well-known properties of internodal charophyte cells such as cylindrical shape, big size and clear separation of the compartments, namely cell wall, cytoplasm and vacuole.

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LATENTINĖ *NITELLOPSIS OBTUSA* LĄSTELIŲ ŽŪTIS JAS TRUMPĄ LAIKĄ PAVEIKUS VARIO OKSIDO NANODALELIŲ SUSPENSIJOMIS

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Santrauka

Buvo ištirtas *Nitellopsis obtusa* ląstelių, paveiktų 5 sek.–24 val. CuO nanosuspensija (nCuO) ir $CuSO_4$ tirpalu, mirtingumas per 48 dienas nuo eksperimento pradžios. Iš mirtingumo kinetikos duomenų buvo įvertintas toksikologinis rodiklis LET_{50}, reiškiantis letaliosios ekspozicijos trukmę, sukeliančią 50 % ląstelių žūtį. Gauta, kad po 10 min. ekspozicijos $CuSO_4$ (3,2 mg Cu/l) arba nCuO (24–79,9 mg Cu/l) žuvusių ląstelių skaičius nebesiskiria po 12-os atplovimo kontrolinėje terpėje dienos. Didesnis bendrojo Cu kiekis (mg/g ląstelių SM) buvo išmatuotas menturdumblių ląstelėse, paveiktose nCuO (79.9 mg Cu/l) nei $CuSO_4$ (3.2 mg Cu/l). Mažesnis CuO nanodalelių nei Cu^{2+} sukeltas mirtingumas per pradinį atplovimo laikotarpį sietinas su nanodalelių sorbcija ląstelės sienelėje – tai buvo patvirtinta vizualinės ir elementų analizės pagalba, panaudojant skenuojantį elektroninį mikroskopą. Nustatyta trumpiausia poveikio trukmė, sukelianti postekspoziciniu laikotarpiu ląstelių žūtį, statistiškai reikšmingai besiskiriančią nuo kontrolės – 30 sek., jei ląstelės buvo paveiktos 24 mg Cu/l nCuO suspensija, ir mažiau nei 5 sek., jei – 79,9 mg Cu/l nCuO.