VILNIUS UNIVERSITY

NATURE RESEARCH CENTRE, INSTITUTE OF BOTANY

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BIOLOGICAL EFFECTS INDUCED BY THE SUSPENSIONS OF COPPER OXIDE NANOPARTICLES IN THE CELLS OF *NITELLOPSIS OBTUSA* (DESV.) J. GROVES

Summary of doctoral dissertation

Biomedical sciences, botany (04 B)

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The research was carried out at the Nature Research Centre, Institute of Botany in 2010–2014.

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VILNIAUS UNIVERSITETAS
GAMTOS TYRIMŲ CENTRO BOTANIKOS INSTITUTAS

Brigita Gylytė

VARIO OKSIDO NANODALELIŲ SUSPENSIJŲ SUKELIAMI BIOLOGINIAI EFEKTAI *Nitellopsis obtusa* (DESV.) J. GROVES LĄSTELĖMS

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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); 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 (NPs), 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 NPs 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 (MANUSADZIANAS et al., 2012), aquatic plants (PERREAULT 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 Cu^{2+} from NPs. 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 NPs (GRIFFITT et al., 2008; MANUSADŽIANAS et al., 2012; PERREAULT et al., 2012). JIANG et al. (2009) showed that the toxicity of nano-scaled metallic 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. The role of algal cell walls as a mediator of nanoparticle toxicity is emphasized (MELEGARI et al., 2013). VAN HOECKE et al. (2008) demonstrated the adsorption of SiO_2 NPs on the cell wall of *Pseudokirchneriella 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 NPs that were observed

agglomerated inside the plant cells, but not from copper ions. Recent studies on human cell lines report that nCuO-induced toxicity is a consequence of both NPs reactivity in terms of interaction with various compartments and copper ions dissolution in lysosomes (MOSCHINI et al., 2013). However, strict differentiation between the main mechanisms of nanoparticle toxicity seems to be hardly achieved under current state of knowledge.

The aim of the study was to investigate toxicity effects of CuO nanoparticle suspensions on the cell of freshwater algae *Nitellopsis obtusa* and compare the effects with those induced by $CuSO_4$ solution.

Main tasks:

1. to evaluate the stability of CuO nanoparticle (nCuO) suspensions in artificial pond water (control solution);
2. to investigate alterations induced by nCuO suspensions and $CuSO_4$ solutions on membrane resting potential of intact *N. obtusa* cells and on K^+, Mg^{2+}-ATPase activity, electrochemical transmembrane proton potential and potassium diffusive potential in the microsomal fraction isolated from the same cells;
3. to investigate the influence of short-term exposure in nCuO suspensions and copper salt solutions on the kinetics of charophyte cell lethality;
4. to assess accumulation of copper in charophyte cell compartments – cell wall, cytoplasm and vacuole – under the influence of various copper forms.

Statements to be defended:

- ♣ Charophyte cell of *Nitellopsis obtusa* exposed for several seconds in the suspension of CuO nanoparticles accumulates sufficient amount of copper in the wall, which induces cell lethality within several days or months.
- ♣ If similar concentrations accumulate in the cells of *N. obtusa* during their exposure to copper salt solution or CuO nanosuspension, the rapid depolarization (within several minutes) of cell membrane occurs in case of copper solution only; however, both impacts lead to cell lethality.
- ♣ Within three hours of exposure in CuO nanosuspension, more than 60% of copper associated with *N. obtusa* cell accumulates in the cell wall, while major part of intracellular Cu locates in cytoplasm.

Scientific novelty of the research:

1. The response of charophyte cells of *N. obtusa* to the impact of CuO nanoparticle suspension prepared in artificial pond water was investigated for the first time at various levels of biological organization, i.e. electrophysiological reaction of the intact cell, H^+-ATPase activity of microsomal fraction isolated from the same algae and the whole cell survival.
2. For the first time, the distribution of Cu in the cell compartments – cell wall, cytoplasm, chloroplasts, cytoplasm and vacuole was investigated in charophyte cell of *N. obtusa* treated by CuO nanoparticle suspensions.
3. Extremely low exposure duration, i.e. from several seconds to several minutes, in copper salt solutions or CuO nanoparticle suspensions was found sufficient to induce cell lethality within several days or even months.

Practical importance of the research results

The effects of engineered CuO nanoparticles on the cells of charophyte *N. obtusa* that represents primary producers in freshwater ecosystems were explored. This technologically important compound, although of very low solubility in water, accumulates in aquatic organisms and may cause detrimental impacts. The data acquired in the study reveal a potential hazardousness of CuO nanoparticles, when they access aquatic environment, thus copper oxide in its nanoformulation becomes an item of environmental regulation.

Approbation of the results

The results of the study were presented at five international and one regional conferences. The material of this dissertation was published in two peer-reviewed scientific journals: one referred in the database of *ISI Web of Science* and having citation index, another referred in the database of *ISI Master List* and having no citation index.

Volume and structure of the dissertation

The dissertation includes eight chapters: Introduction, Materials and Methods, Results, Discussion, Conclusions and References (127 literature sources). Volume of the dissertation is 84 pages, and it is illustrated with 2 tables and 26 figures. The dissertation is written in Lithuanian with the summary in English.

MATERIALS AND METHODS

Plant material

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 until experimentation 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) (VOROBIEV & MANUSADŽIANAS, 1983).

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 $g \cdot L^{-1}$ 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 1, 3, 10, 30 and 100 $mg \cdot L^{-1}$ were prepared from the stock in APW and sonicated for 15 min immediately before the experimentation.

Particle characterization

The particle size was examined by the laser diffraction technique (Helos, Sympatec GmbH), which is able to measure particle sizes between 0.1 and 875 μm; measuring range from 0.2 to 90 μm was selected. The stock solution of nCuO was prepared at 10 $g \cdot L^{-1}$ in milli-Q water and was sonicated for 30 min with 3 mm ultrasonic horn (Bioblock Scientific, Vibracell) and stored at 4°C for three days in the darkness. The suspensions of 3, 10, 30 and 100 $mg \cdot L^{-1}$ were prepared in APW either 24 hours or just before measurements. For the 24-hour-sonicated batch, the suspensions in glass dishes were sonicated for 30 min with a bath-type sonicator (Bandelin Electronic Sonorex RK 510, 160-320 W and 35 kHz).

The 0-hour batch (sonicated or not) was prepared directly in the laser granulometer (which had an integrated bath-type sonicator). The samples were continuously pumped by the device and the nanoparticles were added after each measured concentration for the

non-sonicated batch. The device was being emptied after each measured concentration of sonicated batch (not to sonicate twice the same particles).

For the 24-hour samples of 100 $mg \cdot L^{-1}$ (non-sonicated or sonicated) the device did not arrive to focus the laser, and this made the measurements impossible. However, if these samples were sonicated for 60 s in the device, the measurement could be done, nevertheless, it was again impossible two minutes later. This could happen either due to high concentration or aggregation. Two consecutive measurements of 10 seconds each spaced by 5 seconds were executed.

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 $mg \cdot L^{-1}$ 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 ultrafiltration 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.

Sonication, exposure and cell manipulation

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 $mg \cdot L^{-1}$ 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.

Cell fractionation

Cells were washed with APW twice at the end of 3-h-exposure in 100 $mg \cdot L^{-1}$ of nCuO. Single cell is placed on a filter paper, air-dried for ~1 min until surface looks opaque. After the cell is placed on the glass plate, one end of the internodal cell is cut by sharp scalpel at approximately 4 mm distance from the node. One or two drops are obtained from the cell, when it is cautiously held at the health node in vertical position above the Eppendorf tube. The collected fraction represents the vacuole. Visually, it looks transparent and colourless and the pH value should be at about 5.5 (Gyanes et al., 1978).

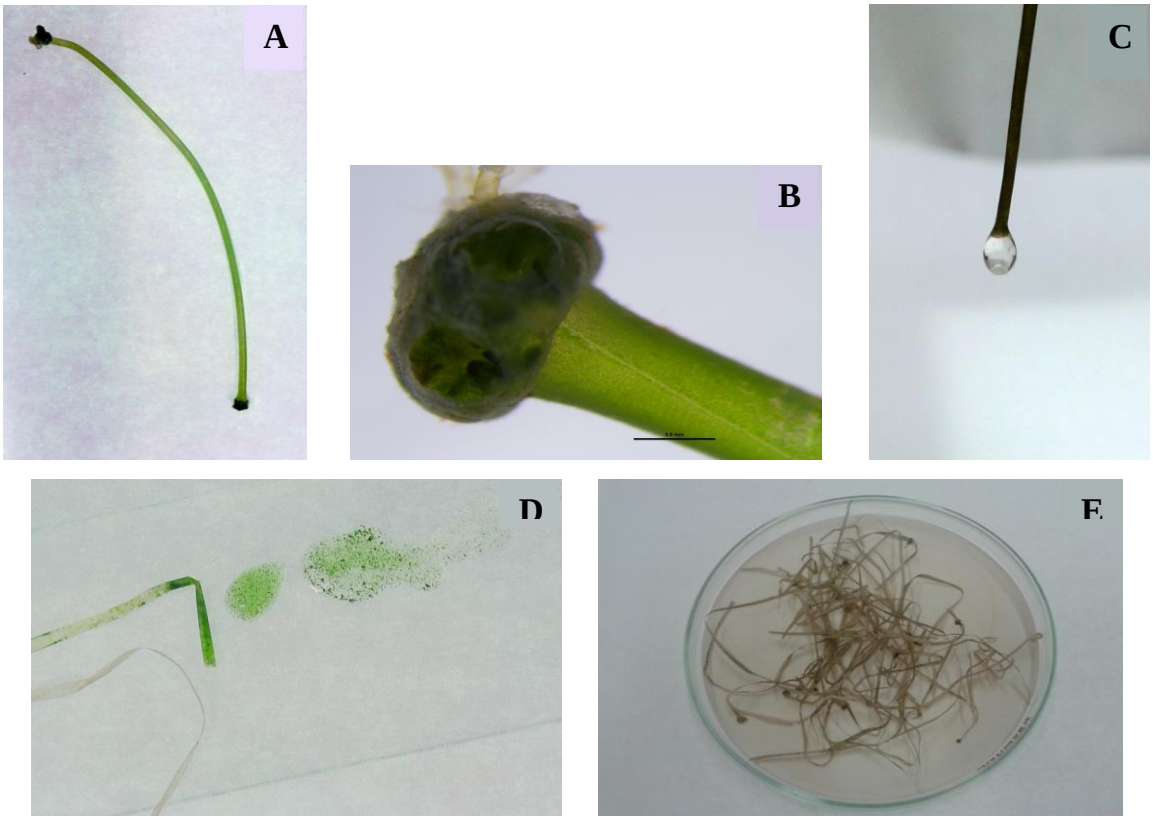

Fig. 1. Fractionation of *N. obtusa* cell. A – the whole cell; B – the node of the cell; C – drop of the vacuole; D – intracellular content; E – cell wall; Bar equals 0.5 mm.

The rest of cell intracellular content is gently squeezed by fingers along the cell surface up to the cut end yet leaving ~1 cm unsqueezed not to contaminate with the CuO from the cell surface. This fraction comprises the rest of vacuole and cytoplasm with the organelles. After centrifugation at 10000 $r \cdot min^{-1}$ for 7 min, supernatant represents vacuole-cytoplasm mixture and residuals – chloroplasts (Whitehouse & Moore, 1993).

Each of the fractions (in three replicates) is diluted with deionised water up to 4 mL and is analysed for Cu content.

Cell wall fraction consists of what is left after separation of intracellular content. It is prepared for Cu content evaluation as in case of the whole cell (see below).

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 $mg \cdot L^{-1}$ (Perkin Elmer, USA) were used for the device calibration. Copper concentration was expressed as $mg \cdot g^{-1}$ of cell dry mass (DM).

Electrophysiological experiments

Bioelectrical activity of up to 32 living internodal cells was measured simultaneously according to K^+-anaesthesia method (SHIMMEN et al., 1976), modified for multichannel recording with extra cellular chlorinated silver wire electrodes. The discrete values of membrane (plasmalemma) potential difference from distinct cells were taken every second. After amplification, the output signals were channelled into a PC by means of interface system. The data on kinetics of cell transmembrane resting potential (RP) of all 32 cells were plotted on a graphic display for visual control and stored for further analysis. The details of computer-assisted experimental setup have been published previously (MANUSADŽIANAS et al., 1999). To ameliorate physiological state of charophyte cells throughout the prolonged measurement of their bioelectrical activity (up to 30 h), the full content of control medium such as APW was used.

Isolation of plant cell microsomal fractions

The method of microsomal fraction isolation from *Nitellopsis obtusa* cell was performed according to Maksimov et al., 2000; Manusadžianas et al., 2002. Algal thalluses were washed with distilled water and carefully dried on filter paper. Each 10 g portion of algae was homogenised for 2 min in a porcelain potter by adding 4 mL of ice cold medium containing 875 mM sucrose, 1050 mM Tris-HCl, pH 8.0, 35 mM EDTA and 14 mM DTT. Cell debris was removed by centrifugation of the filtrate for 5 min at 4500 × *g* and then 20 min at 18000 × *g*. The obtained supernatant was centrifuged again at 91000 g for 1 h at 4 °C (Sorwall WX Ultraseries centrifuge, Thermo Scientific) to sediment the microsomal fraction. The pellet was collected, resuspended in 5 mM Tris-MES buffer (pH 7.8) and homogenised in glass potter. The resulting solution was diluted to the appropriate protein concentration with 5 mM Tris-MES buffer (pH 7.8).

H^+-ATPase activity measurement

To determine H^+-ATPase (K^+, Mg^{2+}-ATPase) activity, a medium with the following composition was used: 3 mM ATP, 3 mM $MgSO_4$, 50 mM KCl and 30 mM Tris-MES (pH 7.8 for *N. obtusa*). The reaction was initiated by adding 150 μL of membrane suspension (10–40 μg protein) to the medium and incubating at 37 °C for 30 min (Maksimov et al., 2002). The protein concentration was selected experimentally to achieve a consistent rate of ATP hydrolysis in each sample. ATP hydrolysis was not limited by substrate deficiency, reaction cofactor availability or the reaction products for 30 minutes. The reaction was stopped by the addition of chilled trichloroacetic acid to a final concentration of 3%. The principle of H^+-ATPase activity determination in these preparations is the detection of inorganic phosphate (P_{in}) that accumulates as a result of ATP hydrolysis. Inorganic phosphate colour reaction with ammonium molybdate and stannous chloride was used. H^+-ATPase activity was measured with a spectrophotometer (λ=750 nm) (Specord 210 plus, Analytic Jena) and expressed in μM of $P_{in}\cdot(\text{mg protein})^{-1}\cdot h^{-1}$.

Determination of transmembrane electrochemical potential

The transmembrane electrochemical potential of isolated microsomes was determined by a spectrofluorometer (SFR-1, Experimental Factory of the Russian Academy of Sciences, Moscow), using a potential-sensitive positively charged dye dis-C_3-(5) (λ_{excit} = 570 nm; λ_{fluor} = 670 nm; $3.3\cdot10^{-7}$M) (Tichaja and Maksimov, 1986. Potassium diffusive potential (KDP) was induced by valinomycin (8.3 nM); $\Delta\mu H^+$ was induced by 3 mM ATP and 3 mM $MgSO_4$. KDP was used as 100% for calculations of $\Delta\mu H^+$. Carbonylcyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) 0.1 μM was applied to prove the H^+-ATPase nature of ATP-induced $\Delta\mu H^+$. FCCP (0.1 μM) was added to prepared vesicle fraction as agent dissipating transmembrane proton gradient.

Protein concentration determination

Protein concentration was determined according to Bradford (1976). BSA (Sigma) was used as a standard.

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 and OriginPro 8.

RESULTS

Characterization of CuO suspensions

The data on the size of nanoparticles or their aggregates in CuO suspensions at 3–100 $mg \cdot L^{-1}$ are presented in Table 1. The smallest particles that were measured at 0-h and 24-h had the size of 200 nm or smaller; however, they are not prevalent in the suspensions with the exception of 3 $mg \cdot L^{-1}$ (sonicated and non-sonicated suspensions) and 10 $mg \cdot L^{-1}$ (sonicated suspension) in 0-h batch. For these excepting suspensions, the mode of distribution equals 500 nm. Overall, the aggregate size ranged from 0.2 μm to 80 μm. The differences between sonicated and non-sonicated suspensions were not considerable after 24 hours, since the modes of respective distributions were similar, in contrast to for freshly prepared suspensions barring two suspensions at 3 $mg \cdot L^{-1}$. Larger than 85 μm aggregates were formed in the 100 $mg \cdot L^{-1}$, both sonicated and non-sonicated suspensions, in 24-h batch, thus, in order to perform size analysis, it was necessary to sonicate them repeatedly for 60 s. The analysis yielded 4.5–5 μm size aggregates to be present. Thus, sonication has quite a short-term concentration-dependent effect on agglomerate disruption in CuO suspensions, at least under conditions of control medium used in the study. The dynamics of reagglomeration can be obviously seen from two

consecutive measurements with 5 s delay: the difference between those measurements is the highest immediately after sonication (Fig. 2, A_1 and A_2); the mode augmented from approximately 700 nm to 1200 nm. It becomes lower 3 min later (Fig. 2, B_1 and B_2) and turns to be negligible in 5 min (Fig. 2, C_1 and C_2). The drift of the modes shows that the process of reagglomeration ends within 3–5 min in 30 mg·L^{-1} nCuO suspension.

Table 1. Parameters of nanoparticle aggregate size distributions (in nm) in non-sonicated (ns) and sonicated (s) suspensions of CuO nanoparticles

Parameter of analysis	Distribution parameter, 0-h batch			Distribution parameter, 24-h batch		
	Minimum value	Mode	Maximum value	Minimum value	Mode	Maximum value
3 mg·L^{-1} (ns)	200	500	2000	200	5000	10000
3 mg·L^{-1} (s)	200	500	5000	200	5000	15000
3 mg·L^{-1} (s, 3 min)	200	500	5000	–	–	–
10 mg·L^{-1} (ns)	200	3000	20000	200	4000	15000
10 mg·L^{-1} (s)	<200	500	2000	200	4000	15000
30 mg·L^{-1} (ns)	200	5000	40000	200	5000	30000
30 mg·L^{-1} (s)	<200	1000	25000	200	4500	15000
30 mg·L^{-1} (s, 3 min)	200	3500	30000	–	–	–
30 mg·L^{-1} (s, 5 min)	200	5000	30000	–	–	–
100 mg·L^{-1} (ns)	200	6000	40000	200*	5000*	15000*
100 mg·L^{-1} (s)	200	4000	40000	200*	4500*	15000*
100 mg·L^{-1} (s, 3 min)	200	5000	80000	–	–	–

* measurement was accomplished after additional sonication.

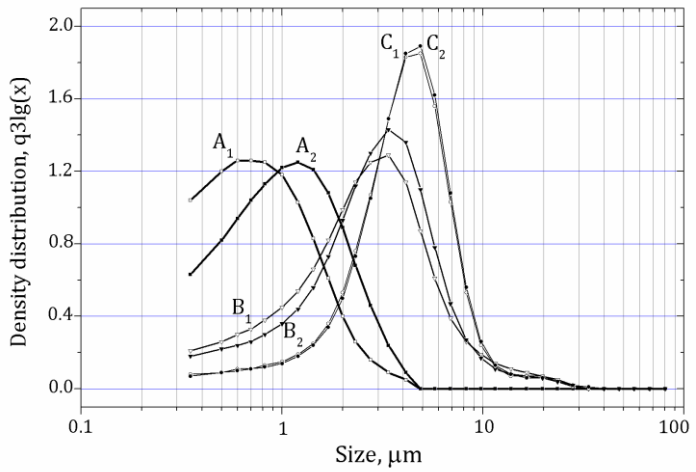

Fig. 2. Particle size distributions measured in 30 mg·L^{-1} nCuO suspension at 0-min (A_1 and A_2), 3-min (B_1 and B_2) and 5-min (C_1 and C_2) after sonication at 0-h. Measurement at respective time is represented by two curves obtained successively. Each measurement lasted 10 s with 5 s gap.

Fast sedimentation of nCuO nanoparticles (nominal nCuO suspension concentration immediately after sonication consisted of 100 mg·L^{-1} or 79.9 mg Cu·L^{-1}) was observed at the surface layer of 0–0.2 cm of the suspension (h = 1.0–1.2 cm) in Petri dishes (h = 1.5 cm, d = 11 cm). After three hours, 2% of the initial Cu concentration measured by ICP-OES remained (Fig. 3, line 1) in the dishes, where lethality observations were done. Substantial sedimentation was also observed in the 50 mL tubes in which the Cu measurements were performed. After three hours, 58% of initial Cu concentration was measured at 0.5 cm above the bottom of the tube (Fig. 3, line 2b). The concentration decreased by 42.8% at the surface layer in Petri dishes and by 10.4% at the bottom of the tubes (Fig. 3, lines 1 and 2a). Sedimentation rate in Petri dishes was constant within 1–180 min and equalled 0.24 ± 0.04 mg Cu min^{-1} (Fig. 3, line 1), while the rate in the tubes equalled 0.28 mg Cu min^{-1} within 1–60 min and 0.016 ± 0.002 mg Cu min^{-1} within 60–1440 min (Fig. 3, lines 2a and 2b, respectively).

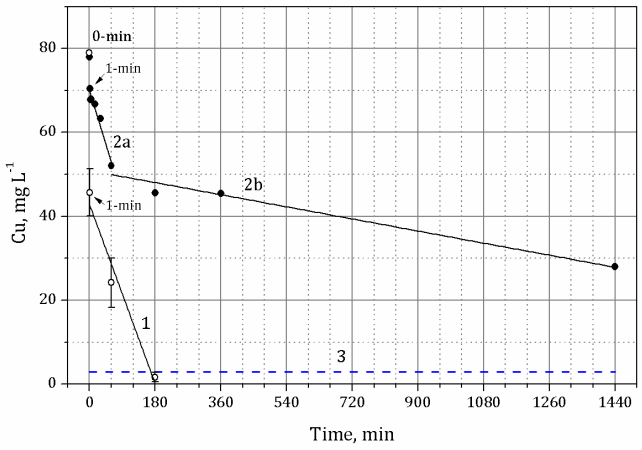

Fig. 3. Kinetics of CuO nanoparticle deposition. Cu concentration was measured at the surface layer of the suspension prepared on APW and placed in Petri dish (1) and near the bottom of measuring tube of ICP-OES spectrophotometer (2). 3 – Cu concentration measured in 3.18 mg Cu·L^{-1} of $CuSO_4$.

Electrophysiological response of *N. obtusa* to nCuO and $CuSO_4$

In order to check functional state of charophyte cells, the reference tests with $CuSO_4$ were periodically accomplished in 2006–2015. The 90-min IC_{50} endpoint, i.e. concentration that induces 50% depolarization of average resting potential within 90-min exposure, was used. According to the endpoint values generated by the reference test, the

influence of vegetation season or the storage under laboratory conditions can be evaluated (VITKUS et al., 1998). The 90-min IC_{50} for $CuSO_4$ (median value ± SD; n) equals 4.3 ± 0.46 mg·L^{-1} (n = 12). Kinetics of bioelectrical response induced by 3.18 mg Cu^{2+}·L^{-1} added as $CuSO_4$ is shown in Fig. 4. This concentration of the salt was used to compare the effects of $CuSO_4$ and nCuO. Copper salt induced depolarization starts immediately after the treatment. The cells of *N. obtusa* depolarize by ~7, ~20 and ~40% from the control level of RP, respectively within 15, 30 and 90 min (Fig. 4). This concentration is toxic to the cells, since even after short exposures depolarization does not recover in APW and proceeds further.

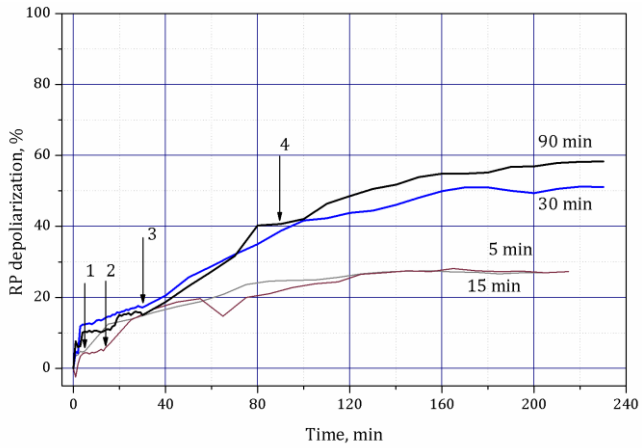

Fig. 4. The kinetics of resting potential (RP) of *N. obtusa* cells treated with 3.18 mg Cu^{2+}·L^{-1} ($CuSO_4$). Arrows show the start of rewash in APW after 5 min (1), 15 min (2), 30 min (3) and 90 min (4) treatment. Each curve represents average of 14–16 cells.

Contrary to copper salt solutions, the effect of nCuO suspensions on the intact cell membrane could not be evaluated quantitatively by the 90-min IC_{50} value, since the membrane depolarizes less than 10% within 90-min exposure even at substantially increased concentrations. The stronger effect on cell resting potential became evident during prolonged exposure period and increased nCuO concentrations up to 500 mg·L^{-1} (Fig. 5). Then it was possible to calculate endpoint values from the concentration-response curves (Fig. 6). The 24-h IC_{50} value obtained for sonicated suspension equalled 36.7 (24.8–53.3) mg·L^{-1} and was lower than that for non-sonicated suspension, i.e. 122 (58.2–) mg·L^{-1} (statistically significant difference).

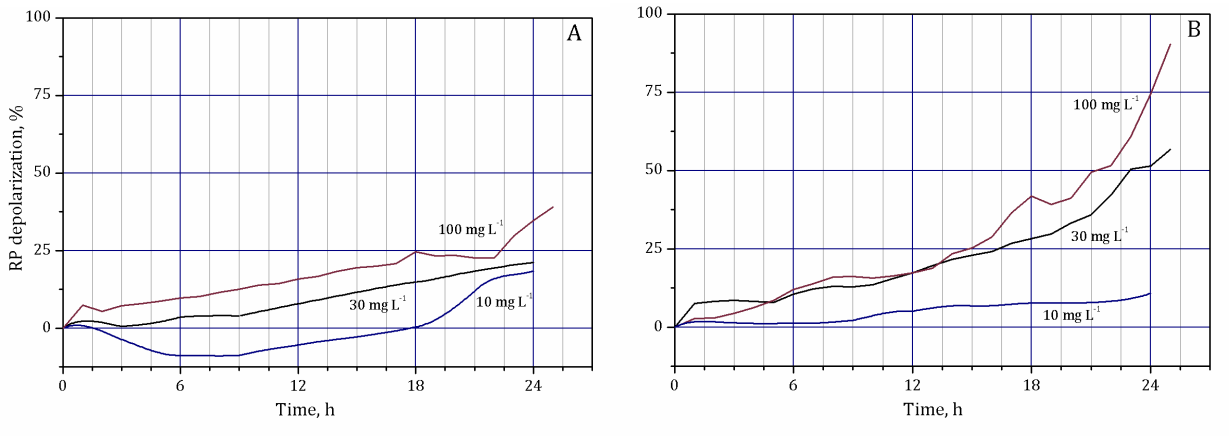

Fig. 5. Kinetics of the average resting potential (RP) of *N. obtusa* cell treated with 10–100 mg·L^{-1} non-sonicated (A) and sonicated (B) nCuO suspensions. Each curve represents average of 14–16 cell responses.

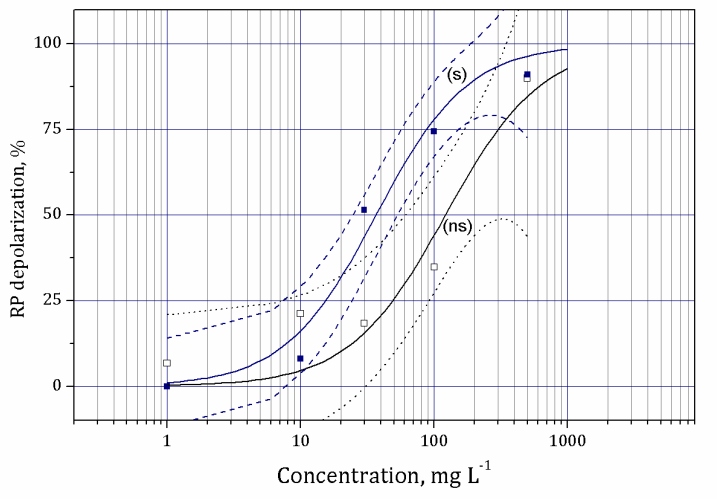

Fig. 6. Relationship between average resting potential (RP) and concentrations of sonicated (s) and non-sonicated (ns) nCuO suspensions. *N. obtusa* cells were treated for 24 hours. Each curve represents average of 14–16 cell responses. Dashed and dotted curves show respective 95% CIs.

The effect of nCuO on membrane functional activity measured in microsomal fraction isolated from *N. obtusa*

The effect of nCuO suspension on the K^+, Mg^{2+}-H^+-ATPase activity and cation transport was explored in the microsomal fraction of *N. obtusa* cells isolated immediately after exposure for 15, 60, 180 and 360 min in 100 mg·L^{-1} nCuO suspension. The inhibition of H^+-ATPase hydrolytic activity increased with the increase of exposure duration (Fig. 7 A). The longest 360 min-treatment inhibited H^+-ATPase activity by 72%, as compared to the second control at 360 minutes (K_{360}), i.e. H^+-ATPase activity in

the microsomal fraction isolated from untreated cell at the end of exposure. During six hour experiment, H^+-ATPase hydrolysing activity in control decreased by ~ 25%.

Contrary to susceptibility of H^+-ATPase activity to nCuO, no significant alterations were observed for ATP-dependent transmembrane electrochemical potential of H^+ ($\Delta\mu H^+$) (Fig. 7 B) or potassium diffusion potential (KDP) generated via valinomicin in microsomal vesicules from *N. obtusa* cells (Fig. 7 C). This shows that although K^+, Mg^{2+}-H^+-ATPase is suppressed under the influence of nCuO suspension, the active membrane transport of H^+ and the K^+ permeability remains unchanged.

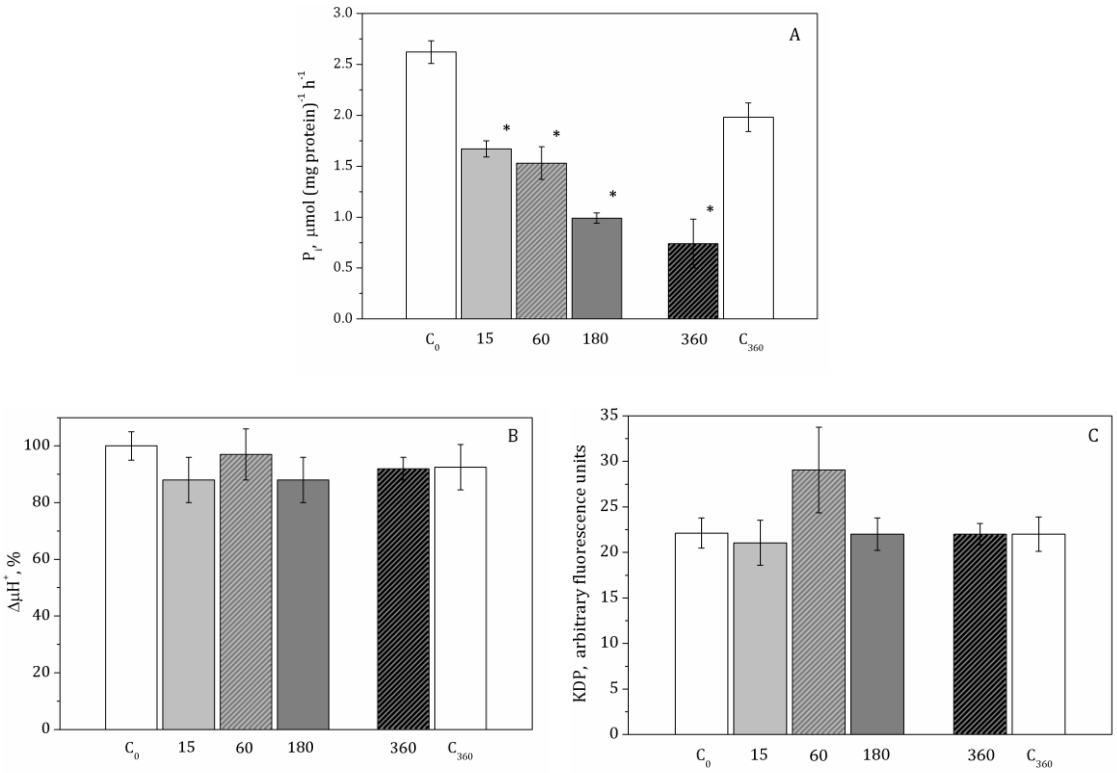

Fig. 7. The effect of exposure duration (in minutes) on H^+-ATPase activity (A), transmembrane ATP-dependent electrochemical potential ($\Delta\mu H^+$) (B) and potassium diffusion potential (KDP) (C) measured in the microsomal fraction of *N. obtusa* cells treated with 100 mg·L^{-1} of nCuO. An average ± SD (n = 3) is represented. Asterisk shows statistically significant differences from controls ($p < 0.05$). K_0 and K_{360} represent measurements done with control algae at the beginning and at the end of exposure.

Charophyte cell lethality induced by nCuO

The influence of sonication on nanosuspension toxicity

Standard duration lethality testing data for sonicated and non-sonicated nCuO suspensions are given in Table 2. The highest (10-fold) difference between the two

curves representing non-sonicated and sonicated suspensions falls at approximately 40 hours of exposure and significant difference (2-fold) remains until approximately 80 hours (Fig. 8).

Table 2. Lethality data ($mg \cdot L^{-1}$, median value ± SD) of *N. obtusa* cells treated with sonicated and non-sonicated nCuO suspensions

nCuO	96-h LC_{50}	192-h LC_{50}
Non-sonicated	4.36 ± 0.35	0.9 ± 0.10
Sonicated	2.81 ± 0.24	0.59 ± 0.23

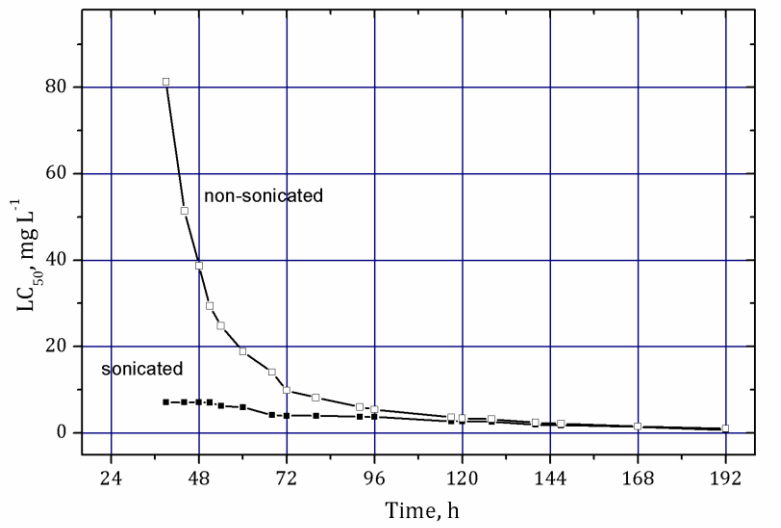

Fig. 8. Kinetics of LC_{50} values calculated for *N. obtusa* cells treated with non-sonicated and sonicated nCuO suspensions.

The influence of exposure duration on the lethality of N. obtusa cells

The influence of short-term exposure on the survival of *N. obtusa* cells was investigated by treating them with 3.18 mg Cu^{2+} L^{-1} added as $CuSO_4$ and 3.2, 7.99, 23.97 and 79.9 mg Cu L^{-1} added as 4, 10, 30 and 100 mg L^{-1} nCuO suspensions, respectively. In this study, 8-d LC_{50} value for $CuSO_4$ equalled 0.024 ± 0.007 $mg \cdot L^{-1}$ (median value ± SE). Corresponding 4-d LC_{50} value reported earlier equalled 0.128 ± 0.027 $mg \cdot L^{-1}$ (MANUSADŽIANAS et al., 2002).

The nCuO suspension (79.9 mg $Cu \cdot L^{-1}$) induced fast (within two days) and higher than 50% cell lethality if the cells were treated for one hour or longer exposures (Fig. 9 A). After four days, 50% mortality exceeded 30-min exposure duration, while 15-min exposure duration evoked 50% mortality only at the 10^{th} day of post-exposure in

APW. At the same day, lethality of the cells exposed for 3–5 minutes exceeded that in control batch significantly ($p < 0.05$). Shorter 5 s–1 min exposures induced lower mortality rates that started to differ significantly from that of control cells after one month.

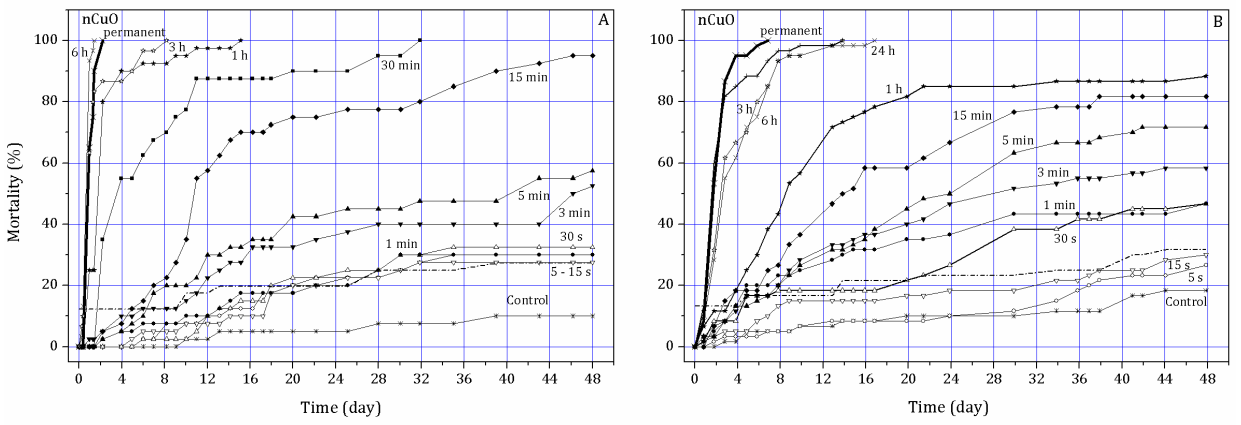

Fig. 9. Mortality kinetics of charophyte algae cells of *Nitellopsis obtusa* in control medium after exposure for 5 s–24 h to 79.9 (A) or 24 mg Cu/l (B) in nCuO suspensions. Thickened lines represent permanent exposure in corresponding medium. Dash dot lines represent statistically significant difference from controls at $\alpha = 0.05$; n = 40 and 60 cells/treatment, respectively (A) and (B).

Permanent exposure of *N. obtusa* cells in ~ three times lower nCuO concentration (23.97 mg $Cu \cdot L^{-1}$) induced 100% mortality within seven days (Fig. 9 B). The 50% lethality of the cells exposed for 3–6 h was exceeded after two days in APW. Cell mortality significantly higher than that in controls could be observed at 4th day for the cells exposed for 1 h; at 6th day for the cells exposed for 1–5 min and at 20th day for the cells exposed for 30 s. The shortest exposures of 5–15 s were not sufficient to induce mortality significantly higher than in controls during the observation period (48 days), thus 30-s-exposure duration in 23.97 mg $Cu \cdot L^{-1}$ added as nCuO suspension can be considered as a duration threshold for *N. obtusa* cells. Duration threshold is the shortest exposure duration during which the lethality in the group of treated cells is statistically significantly higher than that in control group.

The effect of lower nCuO concentrations such as 4 and 10 mg $\cdot L^{-1}$ (respectively 3.18 and 7.99 mg $Cu \cdot L^{-1}$) was similar under the condition of permanent exposure in nanosuspensions, inducing 95% mortality within 24–26 days. This mortality rate was

observed when cells were treated for 24–48 hours at 7.99 mg $Cu \cdot L^{-1}$ concentration, however, when cells were treated for 24 hours in the suspension of 3.18 mg $Cu \cdot L^{-1}$ this rate of mortality was reached after 36 days. At the latter concentration of the suspension, the 50% mortality of the cells exposed for 15 min–6 h was registered after the end of 36-day-exposure. The effect duration threshold for *N. obtusa* cells at 4 $mg \cdot L^{-1}$ nCuO suspension equals 3 min (the mortality probability statistically significantly differs from controls after 48 days). The 50% mortality of the cells exposed for 1 min–1 h at 7.99 mg $Cu \cdot L^{-1}$ nanosuspension was observed after 48 days rewash in APW. Since the mortality at exposure durations of 5 s–30 s did not differ significantly from that in controls, the effect duration threshold for 10 $mg \cdot L^{-1}$ nCuO equalled 1 minute (as for 48 observation period).

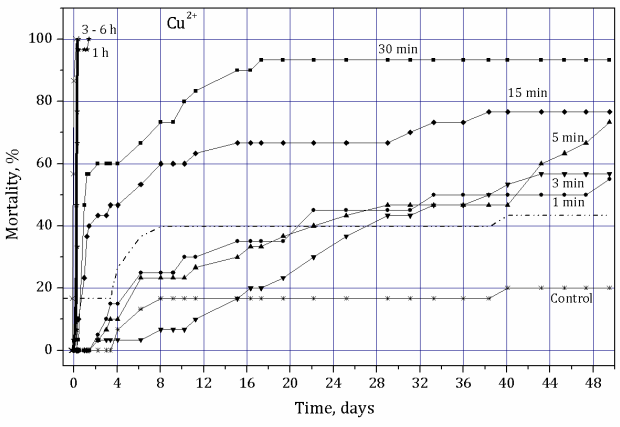

Fig. 10. Mortality kinetics of charophyte algae cells of *Nitellopsis obtusa* in control medium after exposure for 1 min–6 h to 3.18 mg/l of Cu^{2+}, added as $CuSO_4$. Thickened line represents permanent exposure in copper salt solution. Dash dot line represents statistically significant difference from controls at $\alpha = 0.05$; n = 30 cells/treatment.

When charophyte cells were treated with 3.18 mg $Cu^{2+} \cdot L^{-1}$ $CuSO_4$ solution for 15 min–6 h exposure duration, a significant from the controls mortality was observed already at the first day (Fig. 10). Within this observation period, the lethality effect equals 100% at above concentration for the cells treated permanently as well as exposed for 3–6 hours. During the second day of the cells residence in APW, 100% mortality was reached for the cells exposed for one hour. 15-min exposure induces 45% mortality effect after four days, and 60% – after eight days. Since the mortality at exposure

durations of 1 min differed significantly from that in controls, the effect duration threshold for 3.18 mg·L^{-1} nCuO equalled 1 min (as for 27 observation period).

Comparison of the effects of $CuSO_4$ (Fig. 10) and nCuO suspensions (Fig. 9) at the exposure durations of 1–6 h showed that the latter ones induce cell mortality later, i.e. can be characterized by the latent toxicity effect. Shorter exposure durations create similar cell lethality during 48-day observation periods for both treatments.

The 50% lethal effect duration values (LED_{50}) were calculated from the data obtained for the cells exposed in four concentrations of nCuO suspensions and three concentrations of $CuSO_4$. The effects induced by greater concentrations (30 and 100 mg·L^{-1} of nCuO suspensions) differed only during the first 12 days (Fig. 11), and did not significantly differ later on up to the end of experiment. Since smaller nCuO concentrations did not cause higher than 50% cell mortality within initial two weeks at the range of tested exposure durations, the LED_{50} values could not be calculated. The LED_{50} values calculated for 10 and 100 mg·L^{-1} nCuO suspensions at 35th day of post-exposure did not differ significantly, respectively 6.4 ± 2.5 and 2.7 ± 1.4 min. At the end of experiment on 62nd day, LED_{50} value (3.2 ± 0.7 min) calculated for 4 mg·L^{-1} nCuO differed from LED_{50} value (1.14 ± 0.5 min) calculated for 100 mg·L^{-1} just approximately three times.

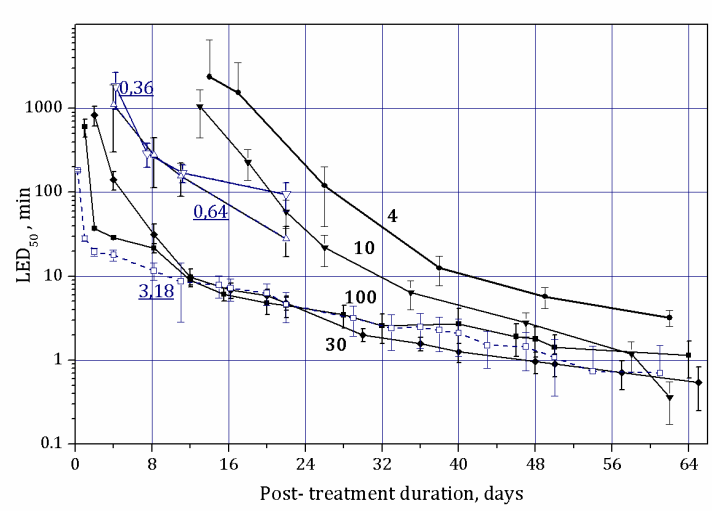

Fig. 11. Kinetics of 50% lethal exposure duration ($LED_{50,}$ median value ± SE) calculated for 0.36, 0.64 and 3.18 mg Cu·L^{-1} added as $CuSO_4$ solutions, and for 24 or 79.9 mg Cu·L^{-1} added as nCuO suspension.

LED_{50} values calculated for various copper salt concentrations are as follows: 94.5 ± 35 min (0.36 mg·L^{-1}), 27.9 ± 11 min (0.64 mg·L^{-1}) and 4.6 ± 1.8 min calculated for maximal $CuSO_4$ concentration did not differ from those for 30 and 100 mg Cu·L^{-1} nCuO concentrations.

Copper accumulation in *N. obtusa* cell and in its compartments

Cu accumulation in the cell

The dynamics of Cu accumulation in algal cells exposed to nCuO suspensions at 4, 30 and 100 mg·L^{-1} (respectively at 3.18, 24.0 and 79.9 mg Cu·L^{-1}) or $5 \cdot 10^{-5}$ M of $CuSO_4$ solution (3.18 mg Cu^{2+} L^{-1}) are presented in Fig. 12. A tight relationship between the Cu content per cell DM unit and the concentration of nCuO suspension was found in entire inspected concentration range (Fig. 12 A–C). Near-exponential augmentation of Cu content in the cells treated with 4 and 30 mg·L^{-1} of nCuO suspensions was observed within 15 min–6 h exposure durations; then, after the longest 24-h-duration, Cu content in the cells increased slightly, respectively by 13.5 and 24.3% compared to the Cu content measured in the cells after six hours of exposure (Fig. 12 A and B). At the highest 100 mg·L^{-1} nCuO concentration, the phase of exponential increase in Cu content started within 3–5 min durations and finished within 3–6 h durations. Similar to lower concentrations, Cu content in the cells increased slightly after the exposure of 24-h-duration compared to that after 6-h-duration, i.e. by 20.4% (Fig. 12 C). The Cu content per unit of DM accumulated within 5–15 s did not change up to one minute at every concentration tested. The 5-s exposure in 30 or 100 mg·L^{-1} nanosuspensions was sufficient to accumulate Cu concentrations significantly different from the control (Fig. 12 B and C). The Cu concentration measured in the cells after six hours of exposure in 4, 30 and 100 mg·L^{-1} nanosuspensions equalled, respectively (average ± SD, n): 0.31 ± 0.001 (2); 1.68 ± 0.24 (2) and 3.11 ± 2.53 mg Cu g^{-1} DM (3).

Near-exponential augmentation of Cu content in the cells treated with $CuSO_4$ at 3.18 mg Cu^{2+} L^{-1} was observed during longer exposure durations from 1 to 24 hours (Fig. 12 D). Comparison of the kinetics of Cu accumulation in nCuO suspension of 4 mg·L^{-1} (or 3.18 mg Cu·L^{-1}) and in $CuSO_4$ shows that the contents of Cu accumulated at the same exposure durations are quite similar, e.g. Cu contents at 30 min, 1 h and 6 h do

not differ (t-test, α = 0.05), however, the cells accumulate more Cu within 24 hours when treated with Cu salt than with CuO nanosuspension (Fig. 12 A and D). If one would like to accomplish similar comparison based not on the nominal Cu concentrations, but on the equal toxic effects, then the comparison has to be done between the effective concentrations. In our case, the two LC_{50} concentrations were 100 mg·L^{-1} of nCuO and 3.18 mg Cu^{2+} L^{-1} of $CuSO_4$ (note that each of these exceeds respective 8-day LC_{50} values by approximately 150 times). Substantially bigger Cu concentrations were accumulated in the treatments with nCuO suspension (Fig. 12 C and D). The ratio of Cu_{nano}/Cu_{salt} equalled at least five for all esposure durations with the exception for 24-h exposure. The maximum ratios were 19 and nine, respectively within one and three hours of exposures, i.e. during the phase of exponential increase in Cu accumulation.

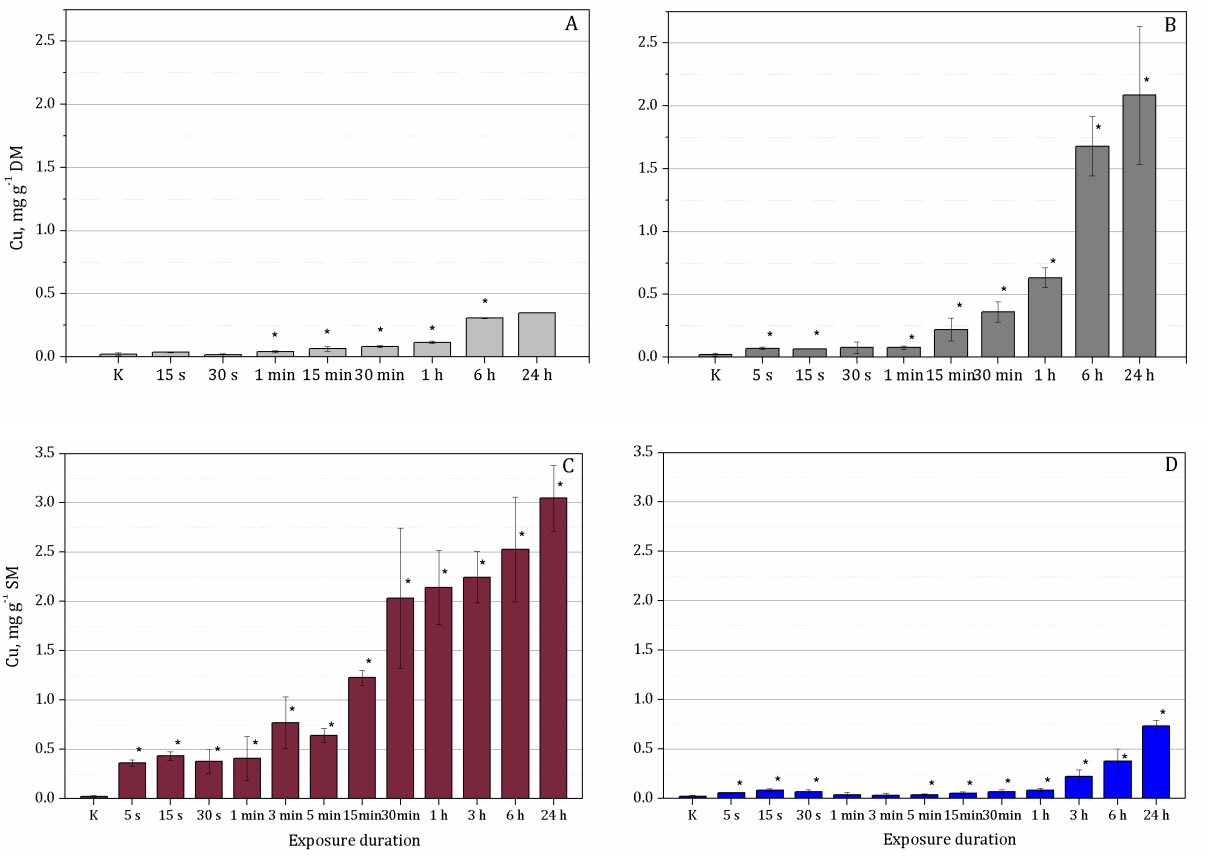

Fig. 12. Copper concentration (mean ± SD, n = 3) measured by ICP-OES in the charophyte algae cells of *Nitellopsis obtusa* exposed for various exposure durations to nCuO at 4 (A), 30 (B) and 100 mgCu·L^{-1} (C) or to $CuSO_4$ at 3.18 mg Cu·L^{-1}(D). Asterisks show statistically significant differences from controls (α = 0.05).

Cu accumulation in the cell compartments

Cu accumulation in particular compartment was investigated after 3-h-exposure period at similar lethal effective concentrations, i.e. 100 $mg \cdot L^{-1}$ nCuO suspension and 3.18 mg $Cu \cdot L^{-1}$ $CuSO_4$. As it is illustrated in the image obtained by light microscopy (Fig. 13), nCuO aggregates are located on the cell surface. The smaller or bigger aggregates covering the surface unevenly can also be seen in the images obtained by scanning electron microscopy (bright granules in Fig. 14). The concentration of Cu in distinct areas can reach up to 70% (spectrum No. 1). Very low amount of Cu or none are found in the areas enriched by insoluble $CaCO_3$ deposits (spectrum No. 2) or uncovered by the deposits cell wall areas (dark zones, spectrum No. 3).

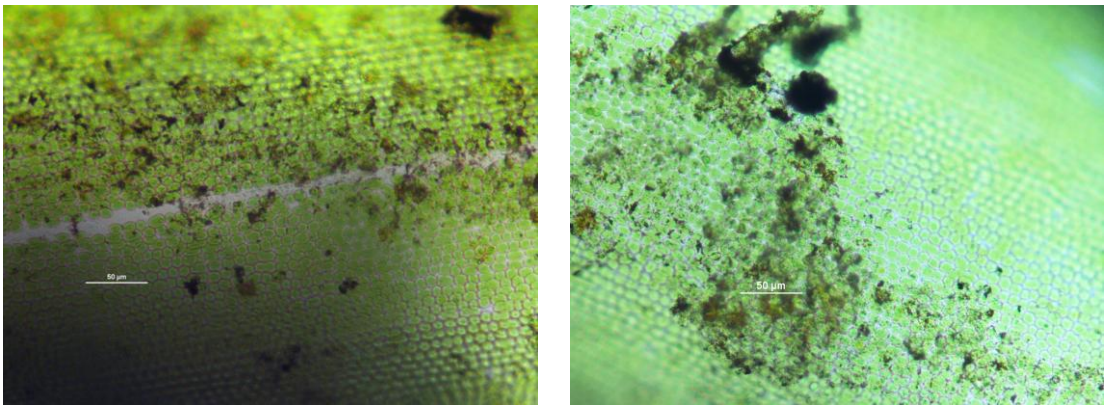

Fig. 13. The nCuO aggregates on the surface of *N. obtusa* cell. The white by chloroplasts uncovered line delimiting two cytoplasm streams that move in opposite directions can be seen on the left image. White bar equals 50 μm.

The cell surface areas covered by nCuO aggregates as well as areas with insoluble $CaCO_3$ deposits due to basic zones in the cell wall of charophyte cells (HOMBLÉ et al., 1990) can be clearly seen in Fig. 15. In this SEM image, one may distinguish uncovered areas of the cell wall as dark smooth zones that are covered by nCuO particles.

The major part of copper accumulates in the wall of the cells treated with the nCuO suspension as well as with the solution of $CuSO_4$, respectively 1.52 ± 0.29 (3) and 0.22 ± 0.012 (3) mg Cu g^{-1} DM. The amounts comprise 62.1% and 73.6% of the Cu measured in the whole cell (Fig. 15). The differences in Cu concentrations between the whole cell and the wall were significant for both copper chemical forms ($\alpha = 0.05$)

(Fig. 15). This shows that part of the copper access cell interior. Also there was found that approximately three times higher concentration occurred in the cells (without the cell walls) treated with nCuO suspension than that with $CuSO_4$ solution, respectively 0.54 ± 0.19 (3) and 0.16 ± 0.038 (3) $mg \cdot L^{-1}$ (Fig. 18).

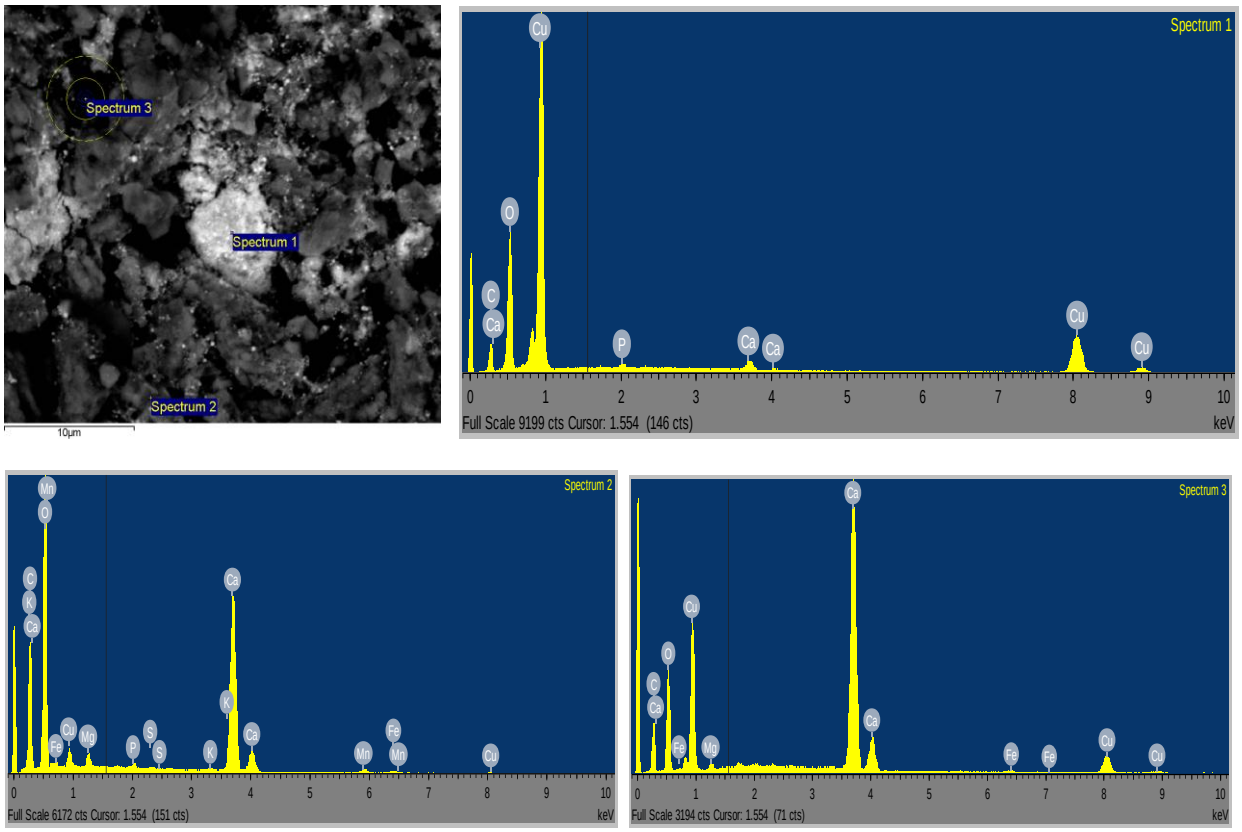

Fig. 14. SEM-BSE (scanning electron microscope-backscattered electrons) images of the cell wall of charophyte cell exposed to 100 mg/l nCuO for 3 h. Blue strips show spots where elemental analysis, an EDX (energy dispersive X-ray system), coupled to the SEM was accomplished (analysed spot comprises area with the diameter of 5 µm). The bar at the bottom of SEM image equals 10 µm.

Copper accumulated in the cells distributes as follows: 26% in chloroplasts, 12% in vacuole and approximately 60% in vacuole-cytoplasm mixture. Assuming that 90–95% of the cell volume occupies the vacuole, it was calculated that the concentration of Cu in cytoplasm together with the chloroplasts equals (average ± SD, n) 0.488 ± 0.02 $mg \cdot L^{-1}$ (3), which comprises 90% of the total copper accumulated intracellularly.

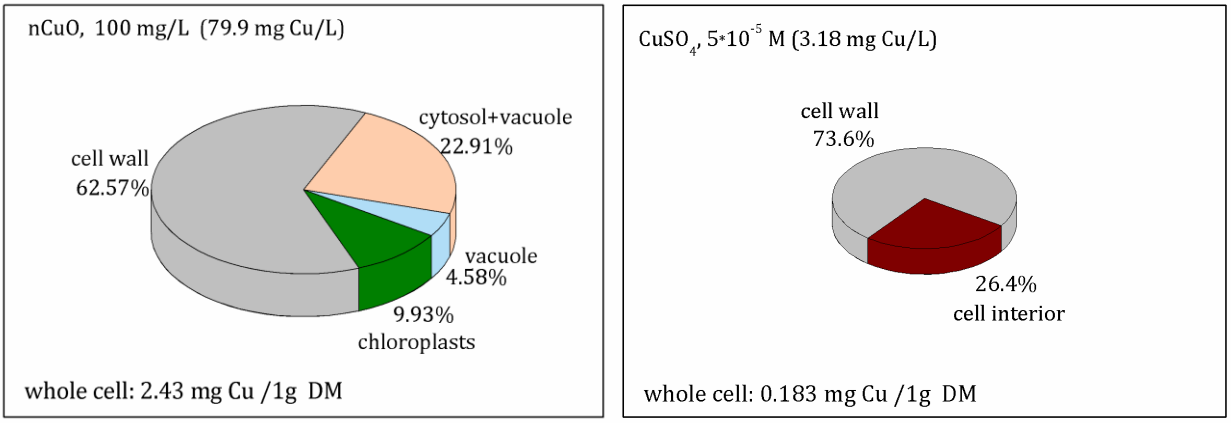

Fig. 15. Copper distribution within compartments of charophyte cell of *N. obtusa* after 3-h exposure in 100 mg·L^{-1} of nCuO suspension and in 3.18 mg Cu·L^{-1} of $CuSO_4$ solution.

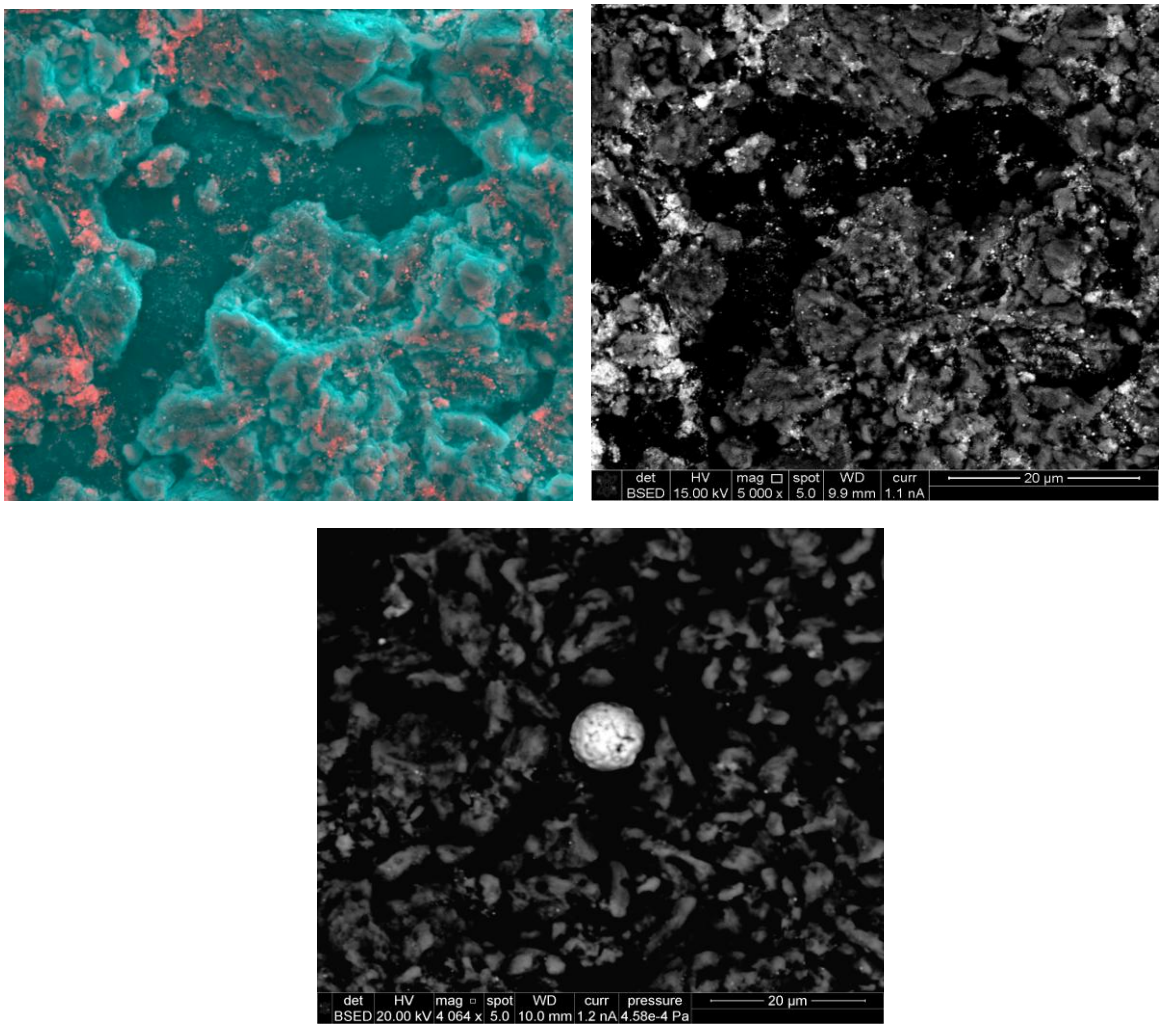

Fig. 16. SEM images of charophyte cell of *N. obtusa* cells. The nCuO aggregates are marked in red. The image of aggregate with diameter of 10 µm is shown at the bottom picture; the smaller particle aggregates are 0.8–1.2 µm. The bar at the bottom in the left equals 20 µm.

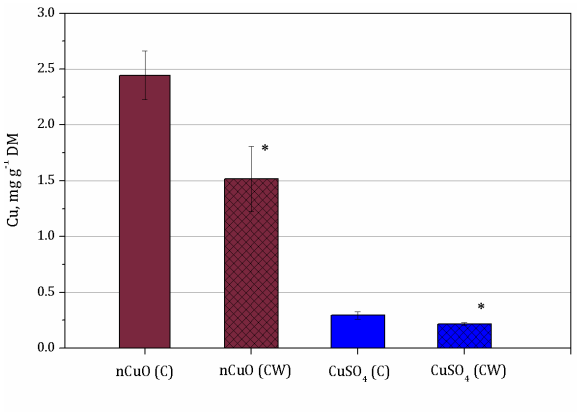

Fig. 17. Cu accumulation in the whole *N. obtusa* cell (C) and in the cell wall (CW) (mg g-^{1} DM) after exposure for 3 h in nCuO suspension (100 mg·L^{-1}) or $CuSO_4$ solution (3.18 mg Cu L^{-1}). An average ± SD (n = 3) is represented.

The soluble copper concentration in intracellular medium was assessed by using ultrafiltration through 1 kDa filter. The concentration of Cu^{2+} in suspension-treated cells was found to be 0.061 ± 0.037 mg Cu^{2+} L^{-1} (n = 3), i.e. one tenth of the total copper concentration 0.539 ± 0.187 mg Cu·L^{-1} (n = 3) (Fig. 17). In the case of $CuSO_4$, the concentration of Cu^{2+} equalled 0.094 ± 0.050 mg Cu^{2+} L^{-1} (n = 3), i.e. approximately 60% of the total copper concentration 0.160 ± 0.038 mg Cu·L^{-1} (n = 3) (Fig. 17).

Intracellular Cu concentration in the charophyte cell compartments consisted (average ± SD, n; mg·L^{-1}): 0.039 ± 0.006 (n = 3) in the vacuole, 0.20 ± 0.10 (3) in the vacuole-cytoplasm mixture and 0.072 ± 0.037 (6) in the chloroplasts; soluble copper (Cu^{2+}) concentration equalled 0.013 ± 0.023 (n = 3) in the ultrafiltrates of vacuole and 0.139 ± 0.069 (3) in the vacuole-cytoplasm mixture. Considering that vacuole occupies (90–95%) of the total charophyte cell volume and that vacuole is mixed with the cytoplasm, Cu^{2+} distribution among the compartments was evaluated. It was found that vacuole contains 50% and mixed vacuole-cytoplasm fraction – 27% of the whole intracellular soluble copper. Chloroplast fractions were not ultrafiltered due to their structure. Although these Cu^{2+} concentrations did not differ significantly from those measured for Cu in corresponding compartments, the possibility that nCuO particles entered the cell interior medium cannot be rejected. Indeed, the nanoparticle aggregates attached to chloroplasts (light microscopy, Fig. 19) and to vacuole or vacuole-cytoplasm

fragments are seen in SEM images (Fig. 20). SEM analysis on elemental content revealed that spectra contain the peaks of Cu (up to 20% of the total content).

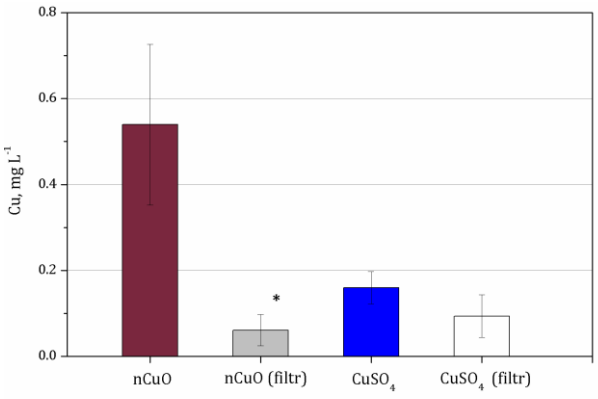

Fig. 18. Intracellular Cu concentration in *N. obtusa* cell and in the filtrates (filtr) expressed inmg·L^{-1}. Cells were exposed for 3 h in nCuO suspension (100 mg·L^{-1}) and $CuSO_4$ solution (3.18 mg Cu·L^{-1}). An average ± SD (n=3) is represented.

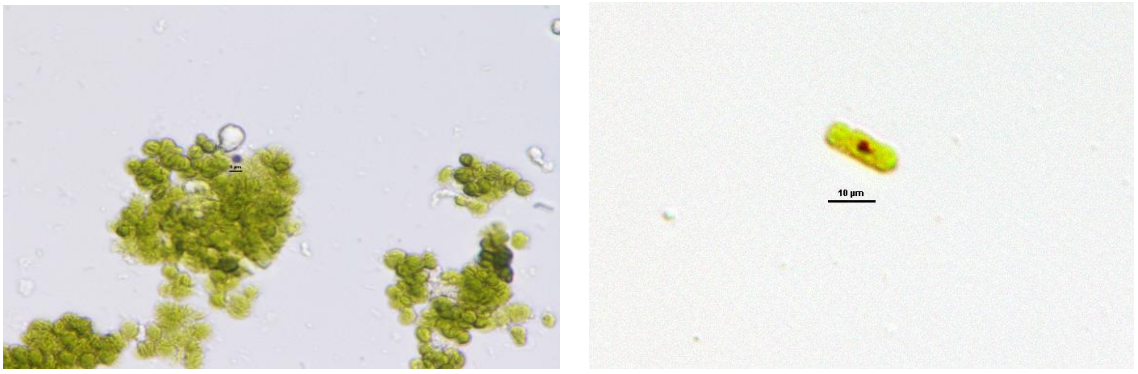

Fig. 19. The nCuo aggregates in the suspension of chloroplasts (A) and associated with the distinct chloroplast (B). The bars equal 5 µm (A) and 10 µm (B) distances.

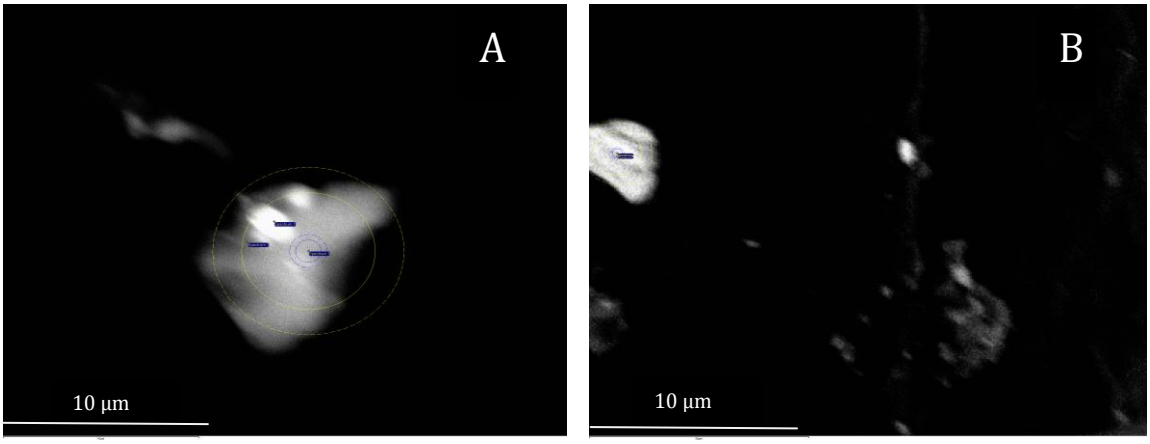

Fig. 20. The nCuO aggregates in the vacuole (A) and mixed cytoplasm-vacuole fraction (B) of *N. obtusa*. Dark blue bars label the spots of elemental analysis performed. Bars equal 10 µm.

DISCUSSION

The extensive investigation of toxicological properties of engineered CuO nanoparticles has been fostered since documented confirmation of their appearance in hydrosphere (BLAISE et al., 2008; TURNER, 2010) as well as the fact that these nanoparticles are not stable. Copper ions, although they are important microelements, released from CuO nanoparticles may be poisonous to aquatic organisms. The toxicity data on the responses of various organisms belonging to different trophic chains such as alga, invertebrates, rotifers, bacteria, protozoa, fish, etc. were summarized and it was concluded that aquatic biota is more susceptible to soluble copper than to its nanoform (ADAM et al., 2015). Lethality data obtained in our study with charophyte cells of *Nitellopsis obtusa* confirmed above generalization, since the ratio of 8-d LC_{50} values for nCuO and $CuSO_4$ (respectively 0.69 and 0.024 mg $Cu \cdot L^{-1}$) equals 23. Similar ratio (35), has been found for unicellular algae *Pseudokirchneriella subcapitata* based on 3-d growth inhibition data (ARUOJA et al., 2009).

A fast electrophysiological reaction represented by alterations of cell membrane (plasma membrane) resting potential (RP) is observed when charophyte cell is affected by deleterious chemical or physical factors. Toxicity is related with the cell RP depolarization, i.e. decrease of its absolute value. The 50% resting potential depolarization during 45–90 min (IC_{50}) is used as an endpoint in the standardized electrophysiological algal biotest (Charatox) (MANUSADŽIANAS et al., 1999, 2010; BARTUSEVIČIENĖ & MANUSADŽIANAS, 2003). Relatively fast changes in electro-physiological reaction induced by testing solution are considered as a symptom of acute effect on *N. obtusa*. 90-min IC_{50} values generated by this test are usually greater than 4-d LC_{50} values obtained for the same alga. In the case of heavy metal ions, IC_{50} values are greater than those of LC_{50} values up to three orders of magnitude (MANUSADŽIANAS et al., 2002). For organic chemicals, the ratio of 90-min IC_{50} and 4-d LC_{50} from several orders to one order of magnitude, and for complex solutions such as wastewater it is approximately the same (MANUSADŽIANAS et al., 2007). In the case of $CuSO_4$, the ratio of 90-min IC_{50} and 8-d LC_{50} values equals approximately 180. In the case of nCuO suspension, 8-d. LC_{50} = 0.69 mg/L (the average value found for non-sonicated and

sonicated suspensions, Table 2), therefore it could be expected that substantial depolarization of the cells treated with ~ 120 mg·L^{-1} nCuO concentration should be observed. However, during 90-min exposure period, there was not induced RP depolarization that could be linked to the toxic action, even if concentration was increased up to 1000 mg·L^{-1} (MANUSADŽIANAS et al., 2012), although depolarization occurs immediately at, for example, 3.18 mg Cu^{2+}·L^{-1} added as $CuSO_4$ (Fig. 4). The RP depolarization was reached at 30–100 mg·L^{-1} of nCuO, when treatment duration prolonged up to 24 hours (Fig. 5). One of the reasons of very weak effect of nCuO suspension on *N. obtusa* cells during the 90-min treatment duration could be an insufficient amount of relatively small and likely bioactive nanoparticles due to their fast aggregation.

Bioavailability of nanoparticles dispersed in aquatic medium can be increased with the minimum influence to the chemical properties of the medium by ultrasonic homogenisation of the suspension (HANDY et al., 2012). The sonication duration most often used lasts up to one hour. Nanoparticle dispersion is not thermodynamically stable, since two processes of opposite directions take place, i.e. particle aggregation and their separation. The nanoparticle dispersions can be relatively stable under certain conditions from several hours to several days, however, stability of the suspension composed of smaller particles than 100 nm is low – from several minutes to several hours (HANDY et al., 2008). Moreover, the state of the suspension depends on chemical and physical properties of the medium (SMITH et al., 2007). In our study, the characterization of nCuO suspension (prepared on APW) was conducted by laser diffraction technique and focused rather on the size distribution of NP aggregates than primary NPs. It was found that aggregates formed in nCuO suspensions of 3–100 mg·L^{-1} can be disrupted by sonication; however, aggregates set back within 15 s after the sonication stops (Fig. 2). Thus, while reagglomeration depended on suspension concentration, the effect of sonication could be characterized as short-term, since the relationship between the modes of particle size distribution and concentration could not be established (Table 1). It is worth mentioning that nanosuspensions used in our study consist of particle aggregates (> 200 nm) rather than the particles of nominal size (~ 50 nm); nevertheless, the presence of minor part of small particles could not be neglected. Taking into account the fast reagglomeration

during the post-sonication period, it is important to treat the cells with the suspension immediately after sonication as well follow strictly the procedures of experimentation. This was essential when the effect threshold for particular suspension of particular concentration was established (it will be discussed later).

Due to high energy impact during the sonication, certain part of CuO or Cu nanoparticles can be ionized, thus toxic concentration of copper ions may appear. The amount of soluble copper depends on sonication duration, NP concentration as well as on chemical composition of the media. It has been reported that 1 $g \cdot L^{-1}$ nCu suspension made on deionized water releases 0.3 $mg \cdot L^{-1}$ (0.03%) of Cu^{2+} within one hour (LEE et al., 2008). Another study informed the release of 0.06 $mg \cdot L^{-1}$ (0.12%) of Cu^{2+} from 50 $mg \cdot L^{-1}$ nCuO suspension made on 25% of Hoagland medium within one hour of sonication (ZHANG et al., 2014). Our data show that 30-min sonication of 100 $mg \cdot L^{-1}$ nCuO suspension prepared on the medium as twice lesser in nutrient content than that of the latter study, yields 0.033 $mg \cdot L^{-1}$ (0.033%) of Cu^{2+} within 3-h sonication. Such concentration is not lethal for *N. obtusa* cells (at least for four days) and does not induce cell membrane depolarization.

The comparison of the effects induced by non-sonicated and sonicated nCuO suspensions to charophyte cell electrophysiological reaction and lethality shows that non-sonicated suspensions were less toxic than sonicated ones at all concentrations tested. Nevertheless, the suspensions even not affected by sonication were toxic to charophyte cells (8-d $LC_{50} = 0.79 \pm 0.10$ $mg \cdot L^{-1}$), and this presumes the noxious influence of engineered NPs under nature conditions. Earlier it was reported that TiO_2 nanoparticles used in formulations of paints for house façades wash out into surface waters at concentrations as big as 0.6 $mg \cdot L^{-1}$ (KAEGI et al., 2008), thus it seems credible that nCuO that is widely used in technological processes and specifically as antifouling paints as well, could not enter the environment at effective concentrations.

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 of chemical analysis of accumulated Cu in the cells, i.e.

3.18 mg·L^{-1} of Cu^{2+} and 79.9 mg Cu·L^{-1} of nCuO (Figs. 9A and 10). It was revealed that $CuSO_4$ solution was more toxic than nCuO to the cells treated for 15 min–6 h at above concentrations within first two days during the post-treatment period; however, lethality did not significantly differ starting from the 12^{th} day. This is well illustrated by the curves of LED_{50} kinetics obtained for 3.18 mg·L^{-1} of Cu^{2+} and 30–100 mg·L^{-1} of nCuO concentrations (Fig. 11). It means that 10-min treatment with 3.18 mg·L^{-1} of Cu^{2+} (as $CuSO_4$) and 79.9 mg Cu·L^{-1} (as nCuO) induce similar toxic effect on charophyte cell survival on the 12^{th} day and later on. 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 three 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. 12 B and C). The accumulated amount of Cu depended on exposure concentration in the range of 4–100 $mg \cdot L^{-1}$ nCuO (Fig. 12 A and C).

To answer the question of nanoparticle bioavailability, several alternatives could be analysed. First, NPs access intracellular medium as particles and dissociate there, or second, they are bound in the cell wall, dissociate gradually and then copper ions enter the cell through conventional ion transport pathways.

As it was mentioned before, significant cell depolarization does not occur up to 6^{th} hour of treatment even at high nCuO concentrations, while depolarization of *N. obtusa* cell membrane initiates after five minutes of treatment with 3.18 $mg \cdot L^{-1}$ $CuSO_4$ and continues after the change to rewash in APW (Fig. 4). 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.12D). It has been recently 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). As it was shown by the laser diffraction analysis of CuO nanoparticle sizes in the suspension, the aggregates set back within shortly after the

sonication stops; therefore, keeping in mind the size of pores in the cell wall, minor part of NPs might be able to pass cell wall and plasma membrane barriers. 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. We found that Cu^{2+} concentration comprises only one tenth of the total Cu measured intracellularly (Fig. 18).

The internalization of nanoparticles can be proved by electron microscopy. The images with nCuO-treated green alga *C. reinhardtii* can be found in recent scientific publications (MELEGARI et al., 2013; PERREAULT et al., 2012), however, it is complicated to decide unquestionably the location of aggregates due to uneven distribution of organelles in this unicellular alga (MELEGARI et al., 2013). In this respect, charophyte cell represents unique object for the investigation of internalized substances due to its size and clearly separated main inner compartments, in particular the huge vacuole occupying up to 95% of cell volume, which is separated from the cytoplasm by the inner membrane (tonoplast). Thus, it was found that major part of associated with the cell Cu accumulates in the cell wall (60–70%) irrespective whether cells treated either with nCuO or $CuSO_4$ (Fig. 17). The adhesion of SiO_2 NPs on the cell wall surface of unicellular green alga *P. subcapitata* has been reported (VAN HOECKE et al., 2008); however, the authors concluded that no particles are seen on the images obtained by transmission electron microscopy.

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.

CONCLUSIONS

1. The CuO nanoparticle (nCuO) aggregates (up to 85 μm) are formed in suspensions of 3–100 $mg \cdot L^{-1}$ prepared on artificial pond water (APW). After sonication aggregates are disintegrated, however, their restoration occurs within 3–5 minutes, immediately after the end of sonication. The aggregates settle fast in nCuO suspensions prepared on APW, contrary to those prepared on deionized water. The speed of aggregate deposit equals 0.24 ± 0.04 mg $Cu \cdot min^{-1}$ at the surface layer of suspension. This generic instability of nCuO suspensions prepared on APW should be taken into account while performing biological experiments or envisaging the impact to aquatic biota.

2. Short-term (5 s) exposure of charophyte cells of *N. obtusa* in 100 $mg \cdot L^{-1}$ nCuO suspension is sufficient to induce cell lethality significantly different from that in control after one month; therefore, the toxicity response is caused by the nanoparticles associated with the algal cell.

3. nCuO suspensions at concentrations 1–100 $mg \cdot L^{-1}$ do not induce rapid depolarization of *N. obtusa* cell membrane, which is observed in the case of copper salt solutions, instead a significant membrane depolarization occurs after 6–12 h lag period; moreover, 3-h-exposure in the media that evoke similar lethality of the cells (respectively, 100 $mg \cdot L^{-1}$ nCuO and 3.18 mg $Cu \cdot L^{-1}$ $CuSO_4$) leads to a higher level of accumulated intracellular Cu concentration in the case of nCuO than that of $CuSO_4$ (respectively, 0.54 $mg \cdot L^{-1}$ and 0.16 $mg \cdot L^{-1}$), hence, intracellular Cu concentration measured in the cells treated with nCuO cannot be explained by the dissociation of nCuO particles, since then Cu^{2+} (at 54 $mg \cdot L^{-1}$) had to induce substantial cell depolarization.

4. CuO nanosuspensions inhibit enzymatic ATPase activity in the microsomal fraction isolated from *N. obtusa* cells, a key factor that regulates membrane transport, while transmembrane proton electrochemical potential and potassium diffusion potential remain stable. This and latent electrophysiological response of the intact cell show that at least during the initial 6-h-exposure period, the algal cell maintains homeostasis due to likely inactive intracellular copper form.

5. Major part of associated with the charophyte cell of *N. obtusa* (60–70%) is accumulated in cell wall, when algal cells are treated by the concentrations that induce similar lethality (respectively, 79.9 mg $Cu \cdot L^{-1}$ nCuO and 3.18 mg $Cu \cdot L^{-1}$ $CuSO_4$). In the case of nanosuspension, 90% of intracellular Cu is located in cytoplasm and chloroplasts; while a half of intracellular soluble copper (Cu^{2+}) is located in vacuole.

LIST OF PUBLICATIONS

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VARIO OKSIDO NANODALELIŲ SUSPENSIJŲ SUKELIAMI BIOLOGINIAI EFEKTAI *Nitellopsis obtusa* (DESV.) J. GROVES LĄSTELĖMS

SANTRAUKA

Bendru sutarimu nanomedžiagoms priskiriamos cheminės struktūros, kurių bent vienas matmuo yra nuo 1 iki 100 nm (HANDY ET AL., 2008). Su nanotechnologijomis susijusios ūkio sritys – elektronika ir chemijos pramonė (pvz., kompleksinės dažų dangos automobilių pramonėje); biomedicina ir farmacija, kai dėl palankaus paviršiaus ploto ir masės santykio nanodalelės panaudojamos kaip katalizatoriai ar dėl savo ypatingos erdvinės atomų konglomeratų struktūros – kaip vaistų nešikliai; aplinką tausojančios technologijos (saulės baterijos) ir, pagaliau, plataus vartojimo sfera – apsauginių kremų gamyba ir kosmetika. Taigi, tiek dėl nekontroliuojamų priežasčių, tiek gamyboje dabar ir ateityje numatomo nanomedžiagų pramonės augimo esama pavojaus, kad nemažai nanodalelių papildomai toms, kurios susidaro gamtoje degant miškams ar po vulkanų išsiveržimų, pateks į hidrosferą, taip pat ir per pramonines bei municipalines nuotekas. Duomenys apie nanojunginių poveikį žmogaus organizmui sistemingai kaupiami kelis dešimtmečius (GEISER ET AL., 2005; OBERDOERSTER ET AL., 2005), tuo tarpu duomenų apie vandens biotos nanotoksikologiją esama santykinai nedaug (BLAISE ET AL., 2008).

Pastaruoju metu, sprendžiant iš publikacijų, atkreiptas dėmesys į metalinių ar metalo oksido nanodalelių sukeliamus toksinius efektus įvairiems vandens organizmams. Tarp jų didesnė dalis tyrinėjimų tenka gyvūniniams objektams, vėžiagyviams ir žuvims, ir tik nedidelė – fitotoksiškumo tyrimams. Metalinių nanojunginių sukelti toksikologiniai efektai grindžiami dviem veikimo mechanizmais – pirmas, kai visi stebimi poveikiai yra siejami su pačių nanodalelių, pvz., CuO, tirpumu tam tikroje terpėje paskleidžiant Cu jonus (pvz., HEINLAAN et al., 2008; ARUOJA et al., 2009), ir antras, kai ekotoksiškumo efektai siejami tik arba bent tam tikra dalimi su pačių nanodalelių veikimu (GRIFFITT et al., 2008; MANUSADŽIANAS et al., 2012; PERREAULT et al., 2012). Tiriant dumblių ląstelių atsaką yra koncentruojamasi į ląstelės sienelės, kaip nanodalelių kaupiklio ir toksiškumo mediatoriaus vaidmenį. VAN HOECKE et al. (2008) pabrėžia žaliadumblių *Pseudokirchneriella subcapitata* ląstelės sienelės svarbą ląstelei sąveikaujant su nanodalelėms. Kita vertus, skelbiama, kad ne vario jonai, o būtent viduląstelinėje terpėje susikaupę vario nanodalelės ar jų agregatai yra atsakingi už toksinį poveikį aukštesniesiems augalams *Phaseolus radiatus* and *Triticum aestivum* (LEE et al., 2008). Pagaliau, remiantis žmogaus ląstelių linijos tyrimais, teigiama, kad jose nCuO sukeliamo toksiškumo priežastis yra abu minėti poveikio mechanizmai – tiek nanodalelių *per se*, kurios

aptiktos įvairiuose ląstelių kompartmentuose, tiek ir dėl vario jonų atsiradimo nanodalelėms irstant lizosomose (MOSCHINI ET AL., 2013). Taigi, šiuo metu nCuO poveikio mechanizmo aspektai nėra pakankamai atskleisti.

Šiuo darbu siekiama ištirti CuO nanodalelių suspensijų toksikologinio poveikio ypatybes gėlavandenių dumblių *Nitellopsis obtusa* ląstelėms, registruojant jų atsakus įvairiuose biologinio organizuotumo lygmenyse ir palyginant su vario druskų tirpalų poveikiu. Taip pat, – išnaudojant menturdumblio ląsteles, kaip unikalų tyrimo objektą su aiškia pagrindinių kompartmentų lokalizacija, ištirti vario pasiskirstymą ląstelėje veikiant CuO nanodalelių suspensijoms.

Tyrimų hipotezė: Vario oksido nanodalelių suspensijos toksinis poveikis *Nitellopsis obtusa* ląstelei pobūdis negali būti paaiškinamas vien terpėje esančių ir su ląstelės sienele susijusių CuO nanodalelių disociacija.

Darbo tikslas: Ištirti CuO nanodalelių suspensijų toksikologinio poveikio ypatybes gėlavandenio dumblio *Nitellopsis obtusa* ląstelėms ir palyginti jas su $CuSO_4$ tirpalo poveikiu.

Tyrimo uždaviniai: (1) Ištirti nCuO suspensijų, pagamintų menturdumblių kontroliniame tirpale, stabilumą; (2) Ištirti nCuO suspensijomis paveiktų *N. obtusa* ląstelių membranos ramybės potencialo ir iš tų pačių ląstelių išskirtos mikrosomų frakcijos K^+, Mg^{2+}-ATPazinio aktyvumo, nuo ATP priklausomo protonų elektrocheminio potencialo bei kalio difuzinio potencialo pokyčius palyginant juos su vario druskų tirpalų poveikiu; (3) Ištirti ir palyginti trumpalaikės ekspozicijos vario druskų tirpaluose arba vario oksido nanosuspensijose įtaką menturdumblių ląstelių žuvimo kinetikai; (4) Ištirti vario kaupimąsi menturdumblių ląstelėje ir atskirose jos dalyse –sienelėje, citoplazmoje ir vakuolėje, veikiant skirtingoms vario cheminėms formoms.

Ginamieji teiginiai: (1) Ląstelės sienelė per trumpą, kelių sekundžių trukmės, ekspoziciją vario oksido nanosuspensijoje gali sukaupti vario kiekį, pakankamą sukelti ląstelių mirtingumą, kuris pasireiškia po kelių dienų ar mėnesių; (2) Jei eksponuojant menturdumblių ląsteles CuO nanosuspensijoje arba vario druskos tirpale ląstelėse susikaupia panašios toksinės vario koncentracijos, greita (po keliolikos minučių) ląstelės membranos depoliarizacija pasireikš tik vario druskų tirpalo poveikio atveju, tačiau vėliau mirtingumą sukels tiek vienas, tiek kitas poveikis; (3) Per 3-ų valandų *N. obtusa* ląstelių ekspoziciją vario oksido nanodalelių suspensijoje daugiau nei 60% asocijuoto su ląstele Cu susikaupia sienelėje, o didžioji viduląstelinio Cu dalis (apie 80%) aptinkama citoplazmoje.

Darbo mokslinis naujumas. Pirmą sykį tirtas menturdumblio *N. obtusa* ląstelių atsakas įvairiuose biologinio organizuotumo lygmenyse, ląstelės membranos elektrofiziologinės

reakcijos ir mikrosomų frakcijos H^+-ATPazinio aktyvumo bei ląstelės išgyvenamumo, veikiant CuO nanodalelių suspensijoms terpėje, chemiškai artimoje gamtiniams vandenims. Pirmą kartą tirtas sukauptų CuO nanodalelių pasiskirstymas *N. obtusa* ląstelės kompartmentuose – sienelėje, chloroplastuose, citoplazmoje ir vakuolėje. Nustatyta itin trumpa, nuo kelių sekundžių iki kelių minučių trukmės, ekspozicija vario druskų tirpale arba vario oksido nanosuspensijoje, sukelianti ląstelių žūtį po kelių dienų ar net mėnesių.

Darbo praktinė reikšmė. Ištirtas technologiškai svarbaus cheminio junginio, vario oksido nanodalelių paveikumas žvaigždėtojo maurašakio, kuris yra reikšmingas gėlavandenių producentų atstovas, ląstelėms. Šis junginys, nors mažai vandenyje tirpus, gali susikaupti vandens organizmuose ir sukelti toksikologines pasekmes. Gauti rezultatai įrodo vandens taršos šiomis nanodalelėmis potencialų pavojingumą, todėl į juos turi būti atsižvelgta aplinkos kontrolės reikalavimuose.

Išvados:

1. Vario oksido nanodalelių 3–100 $mg \cdot L^{-1}$ koncentracijų suspensijose (nCuO), pagamintose artimoje gamtiniams vandenims terpėje (kontrolinė tirpalas), susiformuoja agregatai (iki 85 μm), kurie išardomi apdorojant suspensijas ultragarsu, tačiau sonifikaciją nutraukus, per 3–5 minutes jie atsistato. CuO nanosuspensijose, pagamintose kontroliniame tirpale, priešingai nei dejonizuotame vandenyje, vyksta spartus agreguotų nanodalelių nusėdimas – nusėdimo greitis paviršiniame sluoksnyje $0{,}24 \pm 0{,}04$ mg $Cu \cdot min^{-1}$. Tai parodo nCuO suspensijų nepastovumą, į kurį būtina atsižvelgti atliekant biologinius eksperimentus bei prognozuojant poveikį vandens organizmams;

2. Trumpalaikės (5 sek.) menturdumblio *N. obtusa* ląstelių ekspozicijos 100 $mg \cdot L^{-1}$ koncentracijos nCuO suspensijoje pakanka, kad po mėnesio mirtingumas reikšmingai skirtųsi nuo kontrolės, todėl galima teigti, kad toksinį atsaką sukelia su ląstele asocijuotos CuO nanodalelės.

3. Vario oksido nanosuspensijos (1–100 $mg \cdot L^{-1}$) nesukelia greitos, būdingos vario druskų tirpalų poveikiui, *N. obtusa* ląstelių membranos depoliarizacijos (ji pasireiškia žymiai vėliau, po 6–12 val.), be to, po 3 val. ekspozicijos vienodą letalumą sukeliančiose terpėse (100 $mg \cdot L^{-1}$ nCuO ir 3,18 mg $Cu \cdot L^{-1}$ $CuSO_4$) viduląstelinėje terpėje sukaupiama daugiau bendrojo vario paveikus nCuO negu $CuSO_4$ (atitinkamai, 0,54 $mg \cdot L^{-1}$ ir 0,16 $mg \cdot L^{-1}$), todėl atsiradusios dėl CuO nanosuspensijos poveikio viduląstelinės vario koncentracijos negalima paaiškinti vien vario oksido nanodalelių disociacija, nes tokia (0,54 $mg \cdot L^{-1}$) Cu^{2+} koncentracija turėtų sukelti stiprią ląstelės depoliarizaciją.

4. Tiriant iš *N. obtusa* ląstelių išskirtą mikrosomų frakciją, nustatyta, kad per pirmąsias 6 val. CuO nanosuspensijos slopina medžiagų pernašą reguliuojančių ATPazių aktyvumą, tačiau neturi įtakos mikrosomų vezikulėse sukuriamam protonų elektrocheminių potencialų skirtumui ir kalio difuziniam potencialui. Šis ir latentiškas intaktinės ląstelės elektrofiziologinis atsakas rodo, kad dumblių gyvybinės funkcijos nėra greitai sutrikdomos dėl, tikėtina, neaktyvios viduląstelinės vario formos buvimo.

5. Paveikus *N. obtusa* ląsteles vienodą letalumą sukeliančiais $CuSO_4$ (3,18 mg $Cu \cdot L^{-1}$) tirpalu arba CuO (100 mg$\cdot L^{-1}$) nanosuspensija, didžioji asocijuoto su menturdumblio ląstele vario dalis (60–70%) yra sukaupiama sienelėje. Veikiant nanosuspensijai, apie 90% viso viduląstelinio Cu lokalizuojasi citoplazmoje ir chloroplastuose, o pusė viduląstelinio tirpiojo vario (Cu^{2+}) – vakuolėje.