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Gene transcription patterns and energy reserves in *Daphnia magna* show no nanoparticle specific toxicity when exposed to ZnO and CuO nanoparticles.

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Abstract

There is still a lot of contradiction on whether metal ions are solely responsible for the observed toxicity of ZnO and CuO nanoparticles to aquatic species. While most experiments have studied nanoparticle effects at organismal levels (e.g. mortality, reproduction), effects at lower levels of biological organization may clarify the role of metal ions, nanoparticles and nanoparticle aggregates. In this study, the effect of ZnO and CuO nanoparticles was tested at two lower levels: energy reserves and gene transcription and compared with zinc and copper salts. *Daphnia magna* was exposed during 96 hours to 10 % immobilization concentrations of all chemicals, after which daphnids were sampled for determination of glycogen, lipid and protein concentration and for a differential gene transcription analysis using microarray. The dissolved, nanoparticle and aggregated fraction in the medium was characterized. The results showed that ZnO nanoparticles had largely dissolved directly after addition to the test medium. The CuO nanoparticles mostly formed aggregates, while only a small fraction dissolved. The exposure to zinc (both nano and metal salt) had no effect on the available energy reserves. However, in the copper exposure, the glycogen, lipid and protein concentration in the exposed daphnids was lower than in the unexposed ones. When comparing the nanoparticle (ZnO or CuO) exposed daphnids to the metal salt (zinc or copper salt) exposed daphnids, the microarray results showed no significantly differentially transcribed gene fragments. The results indicate that under the current exposure conditions the toxicity of ZnO and CuO nanoparticles to *D. magna* is solely caused by toxic metal ions.

Keywords: microarray; differential gene transcription; caloric content; aquatic; metal

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1. Introduction

Metal oxide nanoparticles are metal oxides with sizes between 1 and 100 nm. These small sizes render nanoparticles a large surface area to volume ratio with associated specific physical and chemical properties (Farre et al., 2009). As a result, metal oxide nanoparticles such as ZnO and CuO are increasingly being used by many industries for the production of consumer products and industrial applications (Chowdhuri et al., 2004; Hernández Battez et al., 2010; Ma et al., 2013). However, this increasing production of nanoparticle containing products will inevitably result in a nanoparticle runoff to the aquatic environment, where their specific properties may cause adverse effects.

Upon entering the aquatic environment, nanoparticles can behave very dynamically. At low exposure concentrations (< 0.15 mg Zn/l), ZnO nanoparticles have been shown to largely dissolve and initially form aggregates, which rapidly dissolve (Adam et al., 2014b). At higher exposure concentrations (> 1.5 mg Zn/l), the dissolution is lower and a higher aggregation of the ZnO nanoparticles is observed (Li and Wang, 2013). Most of the CuO nanoparticles aggregate heavily under acute and chronic exposure scenarios, while only a small fraction of the particles dissolves (Fan et al., 2012; Heinlaan et al., 2011; Pradhan et al., 2012). The dynamic behavior of nanoparticles is not only determined by nanoparticle characteristics (including size and surface charge) (Bagwe et al., 2006; David et al., 2012) but also by different environmental factors (including pH, ionic strength, presence of natural organic material) (Domingos et al., 2009; Keller et al., 2010; Rathnayake et al., 2014; Zhou and Keller, 2010). This dynamic nanoparticle behavior may play a role in their toxicity to different aquatic organisms.

Different studies have shown that the toxicity of CuO and ZnO nanoparticles is mainly caused by the metal ions after dissolution of the nanoparticles. As such, the acute toxicity of ZnO nanoparticles to bacteria (Blinova et al., 2010; Heinlaan et al., 2008), algae (Franklin et al., 2007), protozoa (Mortimer et al., 2010), nematoda (Ma et al., 2009) and crustacea (Heinlaan et al., 2008) and chronic toxicity of these nanoparticles to crustacea (Adam et al., 2014b) was shown to be caused by Zn²⁺ ions. Copper ions have been shown to be the main contributors in the acute toxicity of CuO nanoparticles to bacteria (Blinova et al., 2010), algae (Aruoja et al., 2009), protozoa (Mortimer et al., 2010), crustacea (Jo et al., 2012) and chronic toxicity to crustacea (Adam et al., 2015). Notter et al. (2014) showed that in 81% (for ZnO nanoparticles) and 100 % (for CuO nanoparticles) of the studies the nanoparticle form was less toxic than the dissolved metal form. However, this does not exclude the occurrence of nanoparticle specific effects under certain exposure scenarios. Additionally, some studies indicate that the formed nanoparticle aggregates also play a role in the observed nanoparticle toxicity. As such, Zhang et al. (2007) showed that smaller ZnO nanoparticle aggregates (230 nm) are more toxic to bacteria (*Escherichia coli*) than larger aggregates (2417 nm). However, in this study, the dissolved fraction from both nanoparticle types was not measured.

In most (above mentioned) papers, the effects of ZnO and CuO nanoparticles were studied at the organismal level (e.g. effects on mortality, growth, reproduction,...). However, these observed effects usually occur in a later stage of toxicity than effects at lower levels of biological organization. The effects caused at lower levels of biological organization (to which we will refer

as suborganismal levels) may provide additional insights into the toxic modes of action and may serve as an early warning system.

When an organism is exposed to a stressor, responses occurring at one of the lowest levels of biological organization involves changes in the gene transcription patterns. Many studies use microarrays to study these gene transcription patterns (Vandeghechuchte et al., 2010; Vandebrouck et al., 2009). Microarrays have the benefit to monitor tens of thousands of gene transcripts simultaneously (Karakach et al., 2010). It has been shown that metals produce specific gene transcriptional profiles in *D. magna* (Poynton et al., 2006). In the case of metal oxide nanoparticle toxicity, microarrays may be used to differentiate between the modes of action caused by nanoparticles (ZnO and CuO) and metal salts (zinc and copper salt). As such, if the formed nanoparticle aggregates have any effect on the daphnids in addition to the effects caused by the free metal ions, this would be reflected in the gene transcription patterns. So far, only one study (Poynton et al., 2011) showed distinct gene transcription patterns caused by ZnO nanoparticles and zinc salt in *D. magna*. However, in contrast with most acute and chronic *D. magna* toxicity studies (Adam et al., 2014b; Heinlaan et al., 2008), in this study different toxicity values were found for the nanoparticles and metal salt, indicating that not all ZnO nanoparticle types cause the same toxicity to this aquatic species and additional testing of different types of ZnO nanoparticles is necessary. It remains unknown whether ZnO nanoparticles showing the same toxicity as zinc salts at the organismal level are also able to cause distinct gene transcription patterns. The modes of action of CuO nanoparticles in *D. magna* are still unknown.

Another response of organisms to stress, at a low level of biological organization, may be reflected by an alteration in the concentration of the available energy reserves (Canli, 2005; Vandembrouck et al., 2009). In order for aquatic species like *D. magna* to maintain the basal metabolism to survive or to maintain growth or reproduction, a higher energy expenditure may be required, by a reduction of the energy reserves, as a response to stress induced by a chemical (Sancho et al., 2009). As such, it is possible that exposing *D. magna* to ZnO or CuO nanoparticles alters their concentration of glycogen, lipids or proteins. To our knowledge, currently no studies have focused on *D. magna* energy reserves when exposed to these metal oxide nanoparticles.

In this study, the effects of ZnO and CuO nanoparticles were studied in *D. magna* at lower levels of biological organization by measuring gene transcription levels and energy reserves. Since there is still a lot of discussion on whether the toxicity of these metal oxide nanoparticles is solely caused by the toxic metal ions dissolved from the nanoparticles or whether nanoparticle specific effects occur, a comparison was made with the metal salts ZnCl₂ and CuCl₂·2H₂O.

2. Materials and Methods

2.1. Tested nanoparticles and metal salts

ZnO nanopowder (NanoSun, 30 nm) was purchased from Micronisers PTY (Australia), while the corresponding metal salt ZnCl₂ (≥ 98%) was obtained from Sigma-Aldrich (Belgium). The CuO nanopowder (< 50 nm) was purchased from Sigma-Aldrich (Belgium) and compared with CuCl₂·2H₂O (ICN Biomedicals, Belgium). The size and shape of the nanoparticles were

characterized with transmission electron microscopy (TEM FEI Philips CM30 equipped with Gatan imaging filter).

2.2. Exposure of *Daphnia magna* to ZnO and CuO nanoparticles and their corresponding metal salts

Concentration finding experiments were performed according to altered OECD guidelines (OECD 202, 2004). *D. magna* (<24 hours, 10 daphnids in 50 ml in 3 replicates) were exposed during 96 hours to a concentration range of the ZnO nanopowder and ZnCl₂ (0.04, 0.08, 0.16, 0.32, 0.64, 1.29, 2.57 mg Zn/l), CuO nanopowder (0.18, 0.39, 0.85, 1.88, 4.13, 9.08, 19.97 mg Cu/l) and CuCl₂·2H₂O (0.010, 0.015, 0.02, 0.03, 0.05, 0.07, 0.11 mg Cu/l). The test conditions (e.g. medium composition and refreshment, amount of medium and feeding regime per daphnid) were similar to the ones in the actual exposure experiment (see below). The EC₁₀ (concentration at which 10 % of the daphnids is immobilized) was calculated (using GraphPad Prism 6) after 96 hours of exposure and used as exposure concentrations in the actual experiments. Here, neonates (aged less than 24 hours) were exposed in OECD recommended ISO test medium (CaCl₂·2H₂O: 0.294 g/l, MgSO₄·7H₂O: 0.123 g/l, NaHCO₃: 0.065 g/l, KCl: 0.006 g/l, (OECD, 2004)) during 96 hours to the nanoparticles and the metal salts. Freshly prepared nanoparticle stock solutions (40 mg ZnO or CuO/l in ISO test medium) were made and sonicated for 30 min in a sonication bath (Branson 2510E-MT, 100 W, 42 KHZ). The metal salt stock solutions (40 mg ZnCl₂ or CuCl₂·2H₂O/l) were not sonicated. Small volumes of these stocks were added to ISO medium to obtain 600 ml exposure solutions of EC₁₀ immobilization concentrations: 0.52 mg Zn/l ZnO nanopowder, 0.54 mg Zn/l ZnCl₂, 1.97 mg Cu/l CuO nanoparticles, 0.063 mg Cu/l CuCl₂·2H₂O in

four replicates with four corresponding unexposed controls for both the Zn and Cu exposures. To each replicate (of the exposures and controls), 120 daphnids (< 24 hours) were added (5 ml/daphnia) and exposed during 96 hours. The medium was refreshed after 48 hours of exposure and the daphnids were exposed to a freshly prepared solution of nanoparticles or metal salts. The daphnids were fed every 48 hours with 4×10^5 cells/ml of the algae species *Raphidocelis subcapitata* and *Chlamydomonas reinhardtii* (in a 3:1 ratio). After 96 h of exposure, *Daphnia* samples (30 for lipids, 30 for proteins and glycogen, 40 for gene transcription; only surviving daphnids were sampled) were taken from the different replicates and rinsed with pure water for a few seconds to wash of surrounding exposure medium. The daphnids were transferred to bullet tubes (energy samples were stored dry; gene transcription samples were stored in 200 μ l Trizol from Invitrogen) and flash frozen in N₂. All samples were stored at -80°C until further analysis.

After 0 hours (1 – 2 hours after spiking of stock solution) and 96 hours of exposing the daphnids, water samples were taken from the medium to distinguish between the aggregated, nanoparticle and dissolved fraction. Unfiltered (containing aggregated, nanoparticle and dissolved fraction), 450 nm syringe filtered (containing aggregates smaller than 450 nm, nanoparticle and dissolved fraction; Acrodisc PP, Pall life sciences), 100 nm syringe filtered (containing nanoparticle and dissolved fraction; Puradisc PTFE, Whatman) and 3 kDa ultrafiltered (containing dissolved fraction; Microsep centrifuge filters Pall Life Sciences, using a 1 h centrifugation at 7500 g (Beckman Avanti J25)) samples were taken from the water column and acidified to 1% HNO₃. As a result, nanoparticle aggregates precipitated to the bottom of the

vessel were not included. The zinc and copper concentrations were measured by ICP-MS (inductively coupled plasma mass spectrometry; Thermo Scientific Element 2 XR) or ICP-OES (inductively coupled plasma optical emission spectrometry; Thermo scientific 6000 series). The differences in zinc or copper concentrations obtained by the different filtration procedures were compared in a one-way ANOVA, with Tukey's post tests. The physicochemical parameters (pH, temperature and oxygen concentration) were determined during the exposure (Hach HQ30d-flexi).

The *D. magna* immobilization (with standard deviation) in the actual experiment was 1.7 ± 1.2 % (control), 5.8 ± 2.5 % (ZnO), 4.8 ± 1.3 % (ZnCl₂) in the zinc exposure and 1.5 ± 1.8 % (control), 2.7 ± 1.6 % (CuO), 2.5 ± 0.7 % (CuCl₂·2H₂O) in the copper exposure.

2.3. Energy reserves in *Daphnia magna*

The pooled *Daphnia* samples (30 for glycogen and proteins, 30 for lipids) used for determination of the energy reserves were homogenized. The glycogen and protein content were extracted from the same body homogenates using perchloric acid. The glycogen content was determined using Anthrone reagent (Roe and Dailey, 1966) and the absorption was measured at 630 nm using a spectrophotometer (ELx808, Biotek instruments). A calibration curve of glycogen (Sigma-Aldrich, Belgium) was used to calculate the total glycogen concentration. The protein content was determined according to the Bradford method (Bradford, 1976). The absorption was measured at 600 nm and the total protein concentration was calculated based on a bovine serum albumin (Sigma-Aldrich, Belgium) calibration curve. The lipids were extracted based on

the chloroform-methanol extraction protocol according to Bligh and Dyer (1959) and the absorption was measured at 400 nm. Simultaneously, a calibration curve of tripalmitine (Acros Organics, Belgium) was made for calculation of the total lipid concentration in the exposed and unexposed daphnids. The different available energy fractions (glycogen, proteins, lipids expressed as μg energy reserve/daphnia) were converted to their caloric content (kJ/daphnia) using 39.5kJ/g lipid, 24 kJ/g protein, 17.5 kJ/g glycogen (De Coen and Janssen, 2003). Two-way ANOVA tests (using GraphPad Prism 6) were used to test for significant effects of the exposure to the nanoparticles and metal salts on the available energy reserves, differences between the glycogen, proteins, lipids concentrations and the interaction between both. Sidak's post tests were used to test for significant differences between the controls and exposures for the different energy reserves.

2.4. Gene transcription levels in *Daphnia magna*

For each replicate, the RNA of pooled samples (40 daphnids) was extracted using the Trizol RNA extraction method (Invitrogen), followed by a DNase treatment (Fermentas) and phenol/chloroform extractions. The quantity (absorbance at 260 nm) and purity (absorbance at 260 nm/280 nm (> 1.7) and 260 nm/230 nm (> 1.7)) of the RNA was checked with a Nanodrop spectrophotometer (ND-1000). The integrity of the RNA was checked using the Qiaxcel system (Quality control kit v2.0). The labeling of the RNA samples, hybridization on 15 K *Daphnia magna* microarrays (Agilent-023710, GEO database Platform GPL16579), washing and scanning of the arrays and statistical analysis of the array data is indicated in Appendix A. Genes (contrast of ZnO nanoparticles against their controls, ZnCl_2 against their controls, ZnO nanoparticles

against ZnCl₂) were considered significantly differentially transcribed if false discovery rate (FDR) < 0.1 and $|\log_2FC| > 0.585$ (log₂ fold change, corresponding to a 1.5 fold change). The same analysis was carried out for the Cu exposures. Raw microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo>) and are accessible through the GEO series accession number GSE54739. Sequence information of the differentially transcribed genes was obtained from the NCBI database. These sequences were blasted against the NCBI database, mapped and annotated using Blast2GO (Conesa et al., 2005) to retrieve the gene ontology information such as the biological processes and functions that are affected (up- or down-regulated) upon exposure to the nanoparticles and metal salts. The number of up- and down-regulated (both annotated and unannotated) gene transcripts was determined and pie charts were used to visualize the main GO-classes of the annotated genes. Based on the obtained results (see results 3.3), four conditions (ZnO/BI, ZnCl₂/BI, CuO/BI or CuCl₂/BI) were compared in the further analyses. Log FC values of gene fragments that were significantly differentially transcribed in at least one of these conditions were used and (significant or not significant) log FC values corresponding these gene fragments were added for the other conditions. A hierarchical clustering analysis (HCL Euclidean distance; with a bootstrap of 100 to test the significance of the tree nodes) was performed on this dataset using MultiExperiment Viewer (MEV 4.5.1). SAM (significance analysis for microarray; part of MultiExperiment Viewer) was performed on this dataset to identify the 40 key genes that indicate the main differences between the zinc and copper exposure and to visualize the transcription of these key genes in a heat map.

3. Results

3.1. Zinc and copper concentrations and exposure conditions

The daphnids were exposed to the nanoparticles (Fig. 1) and metal salts for 96 hours, with a fresh exposure solution after 0 and 48 hours of exposure. The zinc salt (Fig. 2a; 0.502 ± 0.004 mg Zn/l) dissolved (0.457 ± 0.058 mg Zn/l measured after 3 kDa filtration) immediately upon addition to the exposure medium. Most of the ZnO nanoparticles (0.498 ± 0.009 mg Zn/l) had dissolved (on average 72.6 % with min: 69.1 – max: 78.9 %; 0.362 ± 0.027 mg Zn/l measured after 3 kDa filtration) immediately after exposure of the daphnids (0 hours) (Fig. 2b). A small fraction of these nanoparticles formed aggregates larger than 450 nm (significant zinc concentration difference between 450 nm filtered and unfiltered sample). However, these aggregates had completely dissolved within 48 hours of exposure. The daphnids were exposed to a freshly prepared solution of metal salt and nanoparticles after 48 hours. The same nanoparticle dynamics occurred as during the first 48 hours: initial high dissolution and some aggregation, but total dissolution of these aggregates within 48 hours after refreshment, with no significant differences after 96 hours of total exposure.

The copper salt (Fig. 2c; 0.065 ± 0.002 mg Cu/l) dissolved directly in the ISO test medium. Only a small fraction of the CuO nanoparticles (Fig. 2d; 2.008 ± 0.017 mg Cu/l) had dissolved (0 hours after addition of the daphnids, on average 0.8 % with min: 0.7 – max: 0.8 %, 0.016 ± 0.001 mg Cu/l measured after 3 kDa filtration) directly after addition to the medium. Most of these nanoparticles formed aggregates of sizes larger than 450 nm. Visible aggregation of the nanoparticles was observed throughout the exposure. The daphnids were still exposed to highly

aggregated particles and to a small dissolved fraction (on average 0.7 % with min: 0.7 – max: 0.9 %) after 96 hours of exposure. At this time, the total measured copper concentration was lower due to nanoparticle precipitation on the bottom of the vessels (which was not sampled). High concentrations of the CuO nanoparticles (2.01 mg Cu/l) caused the same effects on *D. magna* as low copper salt concentrations (0.07 mg Cu/l). However, the dissolved concentration of both chemicals, obtained after filtration over a 3 kDa filter, was comparable (< 0.05 mg Cu/l).

The physicochemical parameters remained constant throughout the exposure. For the zinc exposure, an average pH value of 7.79 ± 0.09 and temperature of 19.7 ± 0.4 °C was measured. The oxygen concentrations had average values of 8.56 ± 0.11 mg/l (95.0 ± 0.8 %). For the copper exposure, average pH, temperature and oxygen concentration values of 7.84 ± 0.04 , 19.2 ± 0.1 °C, 8.87 ± 0.20 mg/l (96.2 ± 2.2 %) were measured.

3.2. Energy reserves in *Daphnia magna*

The energy reserves expressed as total caloric content of *D. magna* after a 96 hour exposure to the ZnO nanopowder and ZnCl₂ are shown in Fig. 3a. These exposures did not have an effect on the glycogen, protein and lipid concentrations (two-way ANOVA tests showed no significant difference of the energy reserves in the controls and exposures). Contrarily, the exposures to copper (Fig. 3b) had effects on the available energy reserves. Two-way ANOVA tests showed significant effects of the exposures (to CuO nanoparticles and copper salt) on the available total caloric content. The Sidak's post test showed no significant differences in glycogen concentration when comparing the unexposed (control) daphnids with the daphnids exposed to

CuO nanoparticles and copper salt. For the lipid content, significant differences were observed between the control and CuO nanoparticle exposed daphnids but not between the control and copper salt exposed daphnids. The protein concentrations were lower in both the nanoparticle and copper salt exposed daphnids than in the unexposed daphnids. No significant differences in glycogen, protein nor lipid concentration were observed between both copper exposures (nano and metal salt).

3.3. Gene transcription levels in *Daphnia magna*

The results showed that after 96 hours of exposure to EC₁₀ concentrations of the ZnO nanoparticles and ZnCl₂, significant differences in gene transcription were observed between the exposures and the control (Fig. 4). When comparing the ZnO nanoparticle exposures against the control, 29 genes, which were all down-regulated, were found to be differentially transcribed. GO information could be retrieved for 7 (24 %) of these transcripts. Of the 19 gene transcripts (18 down-regulated, 1 up-regulated) that were differentially transcribed in the daphnids exposed to ZnCl₂, compared to the control, 5 (26 %) could be annotated. However, a large overlap in these down-regulated genes was observed between the ZnO nanoparticle and ZnCl₂ exposure when compared to the control (16 identical genes, of which 4 could be annotated). Both annotated and unannotated genes were up- or down-regulated parallel in the ZnO/BI and ZnCl₂/BI exposure. Additionally, no genes were significantly differentially transcribed in the daphnids exposed to the nanoparticles, compared to the ones exposed to the zinc salt (Fig. 4).

In the copper exposure, significant differences in gene transcription were observed between the exposures (EC_{10} concentrations of the CuO nanoparticles and copper salt) and their corresponding control (Fig. 4). When comparing the CuO nanoparticle exposure against the control, 771 gene transcripts, of which 276 were down-regulated and 495 were up-regulated, were found to be differentially transcribed. GO information could be retrieved for 219 (28 %) of these transcripts. In the copper salt exposure, of the 1144 gene transcripts (501 up-regulated and 643 down-regulated transcripts) that were significantly differentially transcribed from the control, 342 (30 %) could be annotated. Large similarities were found between the CuO nanoparticle and copper salt exposure. First, a large overlap in the differentially transcribed genes (683 identical genes, of which 205 could be annotated) was seen between the CuO nanoparticle and $CuCl_2 \cdot 2H_2O$ exposure when compared to the control. Second, the overlapping annotated and unannotated gene transcripts, were either up- or down-regulated parallel in both exposures. Third, no genes were significantly differentially transcribed in the daphnids exposed to the nanoparticles, compared to the ones exposed to the copper salt (Fig. 4). However, some differences were found between the nanoparticle and metal salt exposure. First, the response to the copper salt was stronger than the response to the CuO nanoparticles in most cases (in 94 cases the $|\log FC \text{ value}|$ in the metal salt exposure was higher than in the nanoparticle exposure, while the reverse was only the case in 24 cases). Second, more genes were found to be solely significantly differentially transcribed in the metal salt exposure than in the nanoparticle exposure.

A clustering analysis (figure not shown) was performed on 1173 gene fragments (both annotated and unannotated) that were differentially transcribed in one of the exposure conditions (ZnO/BI, ZnCl₂/BI, CuO/BI, CuCl₂/BI). The results showed that when performing a hierarchical clustering (with a bootstrap value of 100), in all cases (100 %), the zinc exposures on the one hand and copper exposures on the other hand clustered together. When comparing the copper to the zinc exposure, it is clear that the response to zinc was smaller than the response to copper, as evidenced by the limited number of differentially transcribed genes in the zinc exposures and large number in the copper exposures.

The 40 key genes that can be used to indicate the main differences between the zinc and copper exposure as identified with SAM are indicated in Fig. 5. For this analysis, all annotated gene fragments that were significantly differentially transcribed in at least one of the exposure conditions (ZnO/BI, ZnCl₂/BI, CuO/BI, CuCl₂/BI) were included. Almost all key genes were up-regulated in the copper exposures, while in the zinc exposure the general trend was down-regulation. However, most of these genes were only significant in the copper exposure (significant gene transcripts are indicated in Appendix B). Exceptions occurred for GJ12967 and mfs, which were significant in both the copper and zinc exposure and p-element somatic isoform c and beta3 proteasome subunit, which were solely significant in the zinc exposure.

Most of the annotated key gene fragments (Fig. 5) were identified to be involved in metal ion binding, oxidative stress and exoskeleton. For the zinc exposure, the significantly differentially transcribed gene fragments that were identified as key genes were suggested to be involved in

reproduction, signal transduction and transmembrane transport (included in others category of Fig. 5). The other significantly differentially expressed gene fragments that were not identified as key genes were involved in the same processes as identified with the key genes (oxidative stress, transcription and translation and transport). For the copper exposure, the key genes were suggested to be mainly involved in metal ion binding, oxidative stress and exoskeleton and for a lesser extent in growth and reproduction, energy metabolism, protein phosphorylation, transcription and translation and signal transduction. The processes coding for these key genes give a good indication of the processes occurring in the whole dataset. However, some gene fragments that were significantly differentially transcribed but not identified as key genes were also involved in general stress response (e.g. heat shock proteins), which was not limited to oxidative stress and lipid metabolism in addition to the carbohydrate and protein metabolism as identified by the key genes.

4. Discussion

4.1. Effects at lower levels of biological organization in *D. magna* when exposed to ZnO nanoparticles

D. magna was exposed, during 96 hours, to a freshly prepared solution of ZnO nanoparticles or zinc salt (measured concentration of 0.5 mg Zn/l) after 0 and 48 hours of exposure. As expected, the zinc salt had from the onset completely dissolved in the test medium. During the exposure to the ZnO nanoparticles, the dynamics of the nanoparticles observed in the first (0 hours) and second (48 hours) exposure solution were similar. From the onset (0 hours of exposure), a high dissolution (on average 72.6 %) and some aggregation was observed. Dynamic light scattering

results from a previous study (Adam et al., 2014b) with similar exposure conditions and lower exposure concentrations (0.024 – 0.16 mg Zn/l ZnO nanoparticles) also showed initial nanoparticle aggregation with average sizes in the range of 244 – 373 nm. The initially formed aggregates in the current study had completely dissolved within 48 hours of exposure, similar as shown by Adam et al. (2014b) and Adam et al. (2014a).

Only a limited number of gene fragments (< 30 gene fragments) were significantly differentially transcribed in the daphnids exposed to zinc, compared to the unexposed daphnids. The expression of these gene transcripts was largely similar when comparing the exposures (nano and metal salt) with the control. Additionally, when the nanoparticle exposure was contrasted with the metal salt exposure, no genes were found to be significantly differentially transcribed. These results suggest that under the current exposure conditions, the initially formed ZnO aggregates did not cause any additional effects and that the nanoparticle toxicity is completely caused by the free zinc. These findings are in agreement with the effects observed at the organismal level under acute (Heinlaan et al., 2008; Wiench et al., 2009) and chronic (Adam et al., 2014b) exposure scenarios. The free zinc ions dissolved from the nanoparticles and the metal salt were able to cause differential gene transcription in the daphnids. However, due to the limited number of significantly differentially transcribed gene fragments in the exposed daphnids, compared to the unexposed ones, the effects of free zinc ions were very low under these exposure scenarios and should be put into perspective.

Based on the key gene analysis, the main processes that were influenced were involved in signal transduction and reproduction, while metabolic effects were not observed. Names of gene fragments that were found to be differentially transcribed in our study are indicated in *italics*.

4.1.1. Signal transduction

The plasma membranes of all cells contain specific receptors. When extracellular molecules bind to these receptors a biochemical chain of reactions, better known as signal transduction, can occur (Horton et al., 2006). It has been shown that metal ions can trigger signal transduction systems which can regulate the expression of genes to evoke responses to changes in the metal ion concentration in bacteria (Singh et al., 2014). Also in our study, effects of zinc on signal transduction were observed. The signal transduction gene, *GJ12967*, plays a role in the Rab GTPase activity, which has been suggested to be involved in endocytosis (Somsel Rodman and Wandinger-Ness, 2000). Since endocytosis has been suggested as one of the uptake mechanisms of zinc in the cell (Mouat et al., 2003), the down-regulation of a gene coding for *GJ12967* (in both exposures) may indicate that the intracellular zinc concentration of *D. magna* is decreased by decreased uptake.

4.1.2. Energy metabolism

When exposed under the current conditions, no effects on energy metabolism were observed. The gene transcription results showed no differential gene regulation involved in energy metabolism (Fig. 4). Also the direct measurements (Fig. 3) showed no effects of the ZnO nanoparticles nor zinc salt on the available energy reserves. The absence of effects at the

exposed concentrations (0.5 mg Zn/l), suggest that exposure to zinc triggers a limited number of genes involved in regulation mechanisms, which could be important to maintain the homeostasis. This is in agreement with a study by Muysen and Janssen (2002), indicating that the zinc concentration in *D. magna* can be actively regulated at concentrations up to 0.6 mg Zn/l.

4.1.3. Reproduction

One way for *D. magna* to maintain its basal metabolism involves a reduced reproduction when exposed to a stressor (Villarroel et al., 2009). Muysen and Janssen (2002) have shown that the regulation of zinc in *D. magna* involves eliminating the excessive amount of zinc through their exoskeleton during molting, causing effects on reproduction. The down-regulation of a gene coding for *p-element somatic isoform c* in our study may suggest that as a response to increased zinc concentrations, the basal metabolism of *D. magna* may be maintained through reduced reproduction. Under the current exposure scenarios (0.5 mg Zn/l), effects on reproduction are expected to occur in the further development of *D. magna* since a chronic toxicity assay (Adam et al., 2014b) already showed a reduction of reproduction by 50 % at lower concentrations of 0.112 (with 95 % confidence interval: 0.074 – 0.170) mg Zn/l for ZnO nanoparticles and 0.082 (with 95 % confidence interval: 0.050 – 0.133) mg Zn/l for zinc salt. When *D. magna* was exposed during 24 hours to similar concentrations of zinc salt (0.5-1 mg Zn/l) as in the current exposure, a down-regulation of some genes coding for reproduction was also observed by Poynton et al. (2008).

4.2. Effects at lower levels of biological organization in *D. magna* when exposed to CuO nanoparticles

D. magna was exposed, for 96 hours, to a freshly prepared solution of CuO nanoparticles and CuCl₂·2H₂O after 0 and 48 hours of exposure. The copper salt (measured concentration of 0.065 mg Cu/l after 0 hours) dissolved completely upon addition to the test medium. During the exposure to the CuO nanoparticles (measured concentration of 2.008 mg Cu/l after 0 hours), the dynamics observed in the first (0 hours) and second (48 hours) solution were similar. From the onset (0 hours of exposure), a low dissolution (on average 0.8 %) and high aggregation was observed. In a similar study (under the same exposure conditions and at concentrations of 0.230 – 1.438 mg Cu/l CuO nanoparticles) a dynamic light scattering test showed that during the initial exposure (0 hours) the average nanoparticle aggregate sizes in the medium were between 312 and 364 nm (Adam et al., 2015). Similar aggregate sizes are expected in the current study. After 96 hours of exposure, the dissolved fraction remained constant in the current study, while the CuO nanoparticles formed large aggregates. It is remarkable that throughout the exposure the dissolved concentration of the CuO nanoparticles corresponded with the dissolved concentration of the copper salt. Similar nanoparticle dynamics were observed by Adam et al. (2014a) and Adam et al. (2015).

In the copper exposures, much more gene fragments (> 700 gene fragments) were significantly differentially transcribed in the exposed daphnids than in the unexposed control daphnids. Similar as for the zinc exposure, a largely similar transcription (large overlap in differentially transcribed genes which were up- or down-regulated parallel) was seen when comparing both

exposures (CuO nanoparticle and copper salt) with the control. Additionally, no significantly differentially transcribed genes were seen when comparing the nanoparticle to the metal salt exposure. However, in most cases, the response of the commonly transcribed genes was stronger (higher $|\log FC|$ values) in the copper salt exposure than in the nanoparticle exposure. Additionally, a lot of genes were only significantly differentially transcribed in the copper salt exposure (compared with the control). These additional observed effects may be a result of small differences in dissolved copper concentration. After 0 and 96 hours of exposure, the measured dissolved concentration of the copper salt is slightly higher than the dissolved concentration of the CuO nanoparticles. As a result, in the copper salt exposure, the daphnids were (instantly) exposed to somewhat higher copper ion concentrations, which may have resulted in the observed stronger response. Since these dissolved concentration differences were very small, they did not cause measurable effects at the organismal level. Additionally, there is a large overlap (see below) in the annotated processes and functions and their regulation when comparing genes that were transcribed in both exposures with genes that were solely transcribed in the copper salt exposure. These results suggest that, under the current exposure conditions, despite the high aggregation and low dissolution of CuO nanoparticles in the test medium, the observed effects are caused by the free copper ions dissolved from the nanoparticles. At the organismal level, similar effects were shown under acute (Jo et al., 2012) and chronic (Adam et al., 2015) exposure scenarios. The copper ions were able to cause differential gene transcription in *D. magna* at different levels.

Based on the key gene analysis, the main processes that were influenced were involved in metal ion binding, oxidative stress and exoskeleton, while the metabolism and growth were influenced to a lesser extent. Names of gene fragments that were found to be differentially transcribed in our study are indicated in *italics*.

4.2.1. Metal ion binding

All key genes involved in metal binding were up-regulated in both copper exposures. The up-regulation of metal binding proteins suggests that increasing exposure to copper evokes a defense mechanism, causing specific proteins to bind to copper ions and thereby protect the exposed daphnids. Examples of these up-regulated key gene transcripts are *nicotianamine synthase* and *cytochrome b5*. Increased expression of nicotianamine synthase results in increased biosynthesis of nicotianamine. Nicotianamine is a known metal chelator in higher plants (Takahashi et al., 2003). As a result, if the function of nicotianamine in *D. magna* is the same as in higher plants, the increased transcription of a gene fragment coding for increased biosynthesis of nicotianamine is expected to directly enhance the tolerance of the exposed species to high levels of copper by chelating copper (Kim et al., 2005). Cytochrome b5, a heme protein which serves as an electron donor in a number of biochemical reactions including fatty acid desaturation and hydroxylation (Smith et al., 1998), has also been shown to bind to copper ions (Reid et al., 1987). Comparable with our study, Poynton et al. (2008) found an up-regulation of several transcripts coding for metal binding proteins when exposing *D. magna* during 24 hours to copper salt concentrations of 0.006 and 0.030 mg Cu/l.

4.2.2. Oxidative stress

The generation of reactive oxygen species (ROS) makes part of normal cell metabolism and defense. Under normal cell conditions, ROS are counteracted by antioxidant defense mechanisms. However, exposure to metals can increase ROS levels, causing an imbalance between ROS and antioxidants, which may lead to oxidative stress (Leonard et al., 2004). In the current study (Fig. 5), the up-regulation of all key genes involved in oxidative stress when exposed to CuO nanoparticle and copper salt indicates that copper ions increased oxidative stress levels in *D. magna*. Oxidative stress has also been identified in other studies as one of the toxicity mechanisms of CuO nanoparticles (Mwaanga et al., 2014) and copper ions (Barata et al., 2005) in *D. magna*. We will discuss a few examples of affected transcripts that are involved in oxidative stress in the current study.

Serine-type endopeptidases cleave peptide bonds of proteins and are known to be involved in several physiological functions including digestion, blood coagulation and immune response (Hedstrom, 2002). The up-regulation of gene fragments coding for serine-type endopeptidases (e.g. *GH22866* and *chymotrypsin-like proteinase 5a precursor*) suggests increasing levels of ROS (Aoshiba et al., 2001) as part of the immune response. Serine-type endopeptidase inhibitors (e.g. *kazal-type serine protease inhibitor 4 precursor* and *serine protease serpin*) have been shown to inhibit these serine-type endopeptidases which can thereby lower ROS concentrations (Zhang and Ma, 2008).

A gene fragment coding for *T23o_droer* was up-regulated in both copper exposures. This heme enzyme, also known as indoleamine 2,3-dioxygenase, catalyzes the catabolism of tryptophan and its main physiological role is modulating the immune activation in the digestive tract (Soliman et al., 2010). Linked to this immune response, *T23o_droer* has been shown to protect organisms from oxidative stress by scavenging the superoxide anion (Daley-Yates et al., 1988).

In both copper exposures, an up-regulation of a gene fragments coding for *glutathione-S-transferase* was observed. The metabolic role of glutathione-S-transferase is detoxification of reactive electrophiles by catalyzing their reactions with glutathione, which reduces the interactions between the reactive electrophiles and essential proteins and nucleic acids (Josephy, 2010). As a result, glutathione-S-transferase may play a direct role in lowering the oxidative stress levels by interaction with reactive oxygen species (Sadler and von Elert, 2014). Neonate *D. magna* exposed during 48 hours to CuSO_4 concentrations of 0.013 mg/l also showed up-regulated expression of glutathione-S-transferase confirming the occurrence of oxidative stress caused by copper ions (Watanabe et al., 2007).

The primary role of *formaldehyde dehydrogenase* (up-regulated gene in the copper exposures) is the metabolism of formaldehyde. This enzyme may be indirectly involved in oxidative stress since alternative substrates for formaldehyde dehydrogenase can be toxic aldehydic fatty acid derivatives that are formed during the degradation of lipid membranes in a situation of oxidative stress (Díaz et al., 2003; Singh et al., 2013).

Some genes coding for metal ion binding, including *GK19701* (also known as zinc finger C2H2) (Kielbowicz-Matuk, 2012) and *CDK-activating kinase assembly factor MAT 1* (Strocchi et al., 2003) have also been identified to be linked to increasing oxidative stress levels. These genes may play a secondary role in oxidative stress, since the primary function of zinc finger C2H2 is DNA-binding involved in biological processes such as development, differentiation, tumor suppression (Duan et al., 2008) and the assembly factor MAT 1 is part of the CDK-activating kinase which primary function is to control the transition between the different phases of the cell cycle (Kaldis et al., 1996).

4.2.3. Energy metabolism

Other effects of the exposure to copper could be seen at the metabolic level. In order to survive, all organisms require energy that can be achieved by oxidation of glucose, fatty acids or amino acids. These are stored in the organism as carbohydrates (e.g. glycogen), lipids and proteins (Lehninger et al., 2005). However, when the organism is exposed to a stressor, additional energy may be required to resist this stressor. As a result, the organism will need to rely more on its energy reserves than under unexposed circumstances, in order to maintain its basal metabolism (Arzate-Cárdenas and Martínez-Jerónimo, 2012). This may cause a net decrease in energy reserves when the energy supplied by food intake is not sufficient for compensation. It is also possible that when exposed to a stressor, energy reserves are lower due to lower food intake as a result of decreased filtration and ingestion (Ferrando and Andreu, 1993). Our results showed that exposure to copper had direct effects on the available energy reserves of *D. magna* since the total caloric content was lower in the exposed organisms than in the unexposed ones. Some

differentially expressed key genes explain the lowering of the carbohydrate and protein content.

An up-regulation of a gene fragment coding for *endo- α -polygalactosaminidase* precursor was observed. Endo- α -polygalactosaminidase is a very rare enzyme that belongs to the family of glycoside hydrolases (Naumov and Stepushchenko, 2011). Although the primary function of this enzyme is not known, it is possible that this glycoside hydrolase enzyme is involved in the breakdown of the glycosidic bonds of carbohydrates, as identified by the GO-analysis.

Sarcosine is a natural amino acid that plays a significant role in various physiological processes as the main metabolic source of glutathione, serine, creatine and purines of living cells (Amaral et al., 2013). Sarcosine is a known by-product in the synthesis and degradation of the amino acid glycine (Stipanuk and Caudill, 2013). The up-regulation of a gene fragment coding for *mitochondrial sarcosine* suggests the breakdown of glycine (identified GO-process) in the copper exposure. It may be possible that this breakdown of glycine is indirectly involved in the production of glutathione (Martinov et al., 2010), which is known to be involved in stress response. These results suggest that *D. magna* needs to rely on its energy reserves to resist the internal copper. When exposed to lower concentrations of copper salt (0.006 mg Cu/l) than in the current study, a microarray study already showed a down-regulation of several gene transcripts coding for digestion and nutrient absorption in adult *D. magna* (16-18 day old) exposed during 24 hours, which can be linked to lower energy reserves (Poynton et al., 2006).

4.2.4. Exoskeleton and growth

Under the current exposure conditions, evidence was observed that the basal mechanisms, including growth during early development, were maintained. During the *Daphnia* life cycle, the exoskeleton is shed of by molting and replaced by a new cuticle, growing underneath the old one. Under the current conditions no clear regulation of gene transcripts coding for exoskeleton was observed. When exposed to both the CuO nanoparticles and copper salt, an up-regulation of gene transcripts coding for chitinases (*brain chitinase and chia, chitinase 1 precursor*; required for molting) and down-regulation of a gene transcript coding for structural constituents of the exoskeleton (*GK15738*) was observed. Differentially transcribed genes involved in molting or exoskeleton may be artifacts occurring due to delayed molting. These genes are known to vary during the molting cycle and hence slight variations in molting (i.e. delay molting due to reduced growth) may affect these genes. Poynton et al. (2008) also observed no clear regulation but both up- and down-regulation of gene transcripts coding for exoskeleton when *D. magna* was exposed during 24 hours to different concentrations of copper (0.002, 0.006, 0.030 mg Zn/l). The up-regulated gene transcripts may suggest that under the exposed conditions in the current study, the molting of the daphnids is promoted. Increased molting may stimulate the daphnia growth (Ebert, 2005). An up-regulation of a gene transcript coding for growth (*xnr5-2 protein*) confirms these results. Arzate-Cárdenas and Martínez-Jerónimo (2012) indicate that in the early stage of *Daphnia* development (neonates and juveniles), energy reserves are used to maintain growth when exposed to a toxicant. Our results suggest that in the daphnids exposed to the nanoparticles and metal salts the daphnids had to

rely more on its energy reserves than under unexposed circumstances in order to maintain the basal metabolism and growth.

5. Conclusions

In this study, the effects of ZnO nanoparticles and CuO nanoparticles were tested at low levels of biological organization (energy reserves and gene transcription) in *D. magna*. Our results showed that the exposure to ZnO nanoparticles and zinc salt did not lower the energy reserves in *D. magna*. Contrarily, the exposure to CuO nanoparticles and copper salt, lowered the *Daphnia* total caloric content. In the zinc exposures only a small number of significantly differentially transcribed genes was observed between the exposures and control, while many genes were significantly differentially transcribed in the copper exposed daphnids, compared to the control. For both exposures (zinc and copper), a largely similar transcription and overlap in GO-processes was observed when comparing the nanoparticles and metal salts with their control. Additionally, no significantly differentially transcribed genes were observed between the nanoparticle and corresponding metal salt exposure. These results suggests that under the current exposure conditions the ZnO and CuO nanoparticle toxicity to *D. magna* is solely caused by metal ions dissolved from the nanoparticles. Under different exposure conditions (e.g. different nanoparticles, environmental conditions) the occurrence of nanoparticle specific suborganismal effects should not be excluded but this will require additional research.

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Figures

Fig. 1. Transmission electron microscopic images of ZnO (a) and CuO (b) nanoparticles.

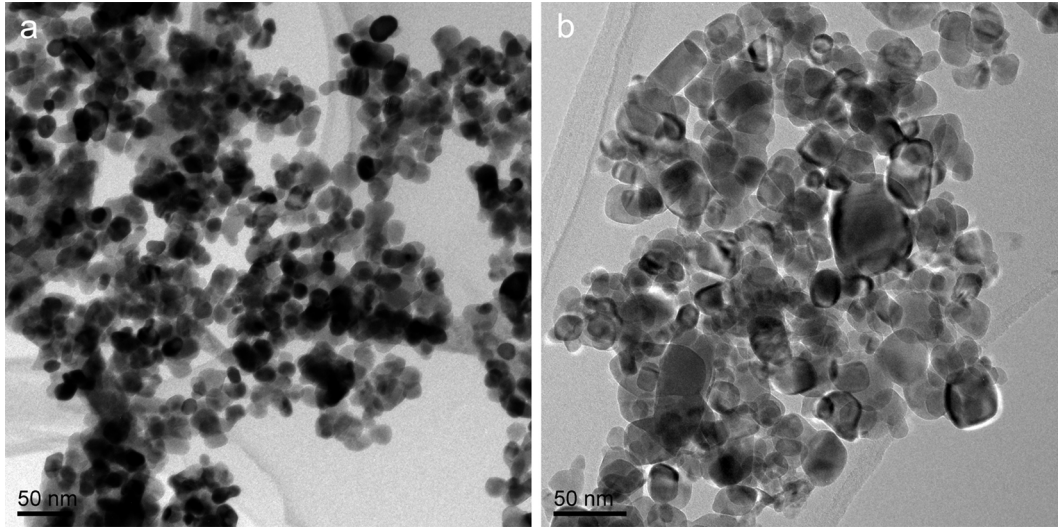


Fig. 2. Measured zinc or copper concentration (with standard deviations for three replicates) in the 3 kDa, 100 nm, 450 nm filtered and unfiltered samples after 0 and 96 hours of exposure to nominal concentrations of 0.54 mg Zn/l of the ZnCl₂ (a), 0.52 mg Zn/l of the ZnO nanopowder (b), 0.063 mg Cu/l of the CuCl₂.2H₂O (c), 1.97 mg Cu/l of the CuO nanopowder (d). One-way ANOVA with Tukey's post test (different letters on graph indicate significant differences) are indicated. The controls (not indicated on graph) in the unfiltered samples, corresponding to the zinc exposure, had average concentrations of 0.014 ± 0.006 mg Zn/l, while the controls, corresponding to the copper exposure, had average concentrations of 0.0037 ± 0.0004 mg Cu/l.

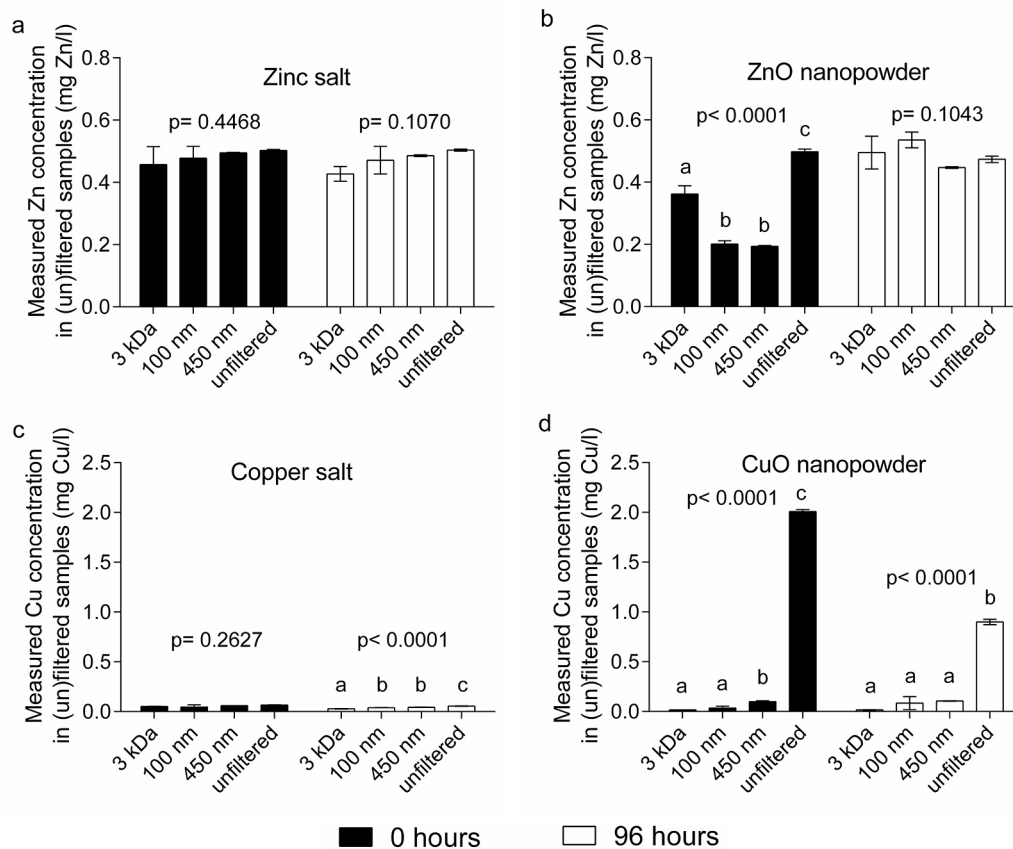


Fig. 3. Total caloric content (glycogen, lipid and protein concentration with standard deviation) in *D. magna* after 96 hours of exposure to the ZnO nanopowder, ZnCl₂ and its corresponding control (a) and after 96 hours of exposure to CuO nanopowder, CuCl₂·2H₂O and its corresponding control (b). Significance levels of two-way ANOVA tests in which the effect of the exposure to nanoparticles and metal salts (indicated on graph; p=0.3977 for zinc, p< 0.0001 for zinc and copper), differences in energy reserves (p< 0.0001 for copper) and the interaction between both (p= 0.6106 for zinc, p< 0.0001 for copper) are tested. Sidak's post test, testing for differences between the controls and exposures for glycogen, lipid and protein concentration, are indicated with different letters.

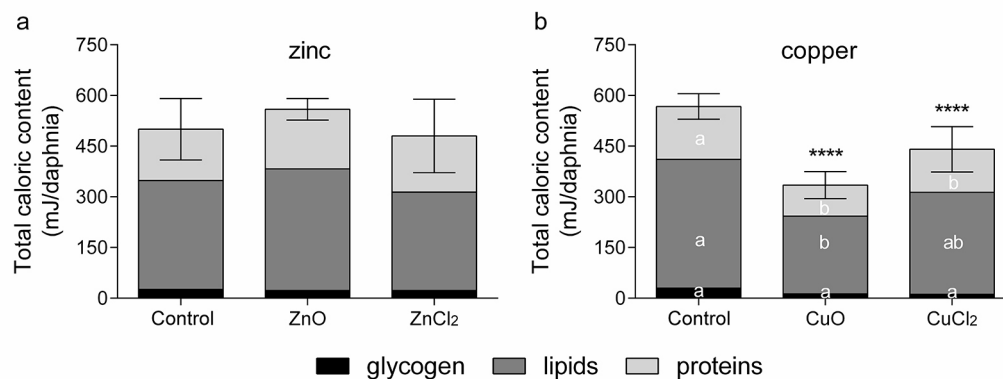


Fig. 4. The number of significantly differentially transcribed annotated and unannotated genes (up- or down-regulated) in the zinc exposure (when comparing ZnO with control; ZnCl₂ with control; ZnO with ZnCl₂) and the copper exposure (when comparing CuO with control; CuCl₂.2H₂O with control; CuO with CuCl₂.2H₂O). The venn diagrams indicate the number of significantly differentially transcribed genes that occurred solely in the ZnO or CuO nanoparticle exposure, solely in the ZnCl₂ or CuCl₂ exposure and in both nanoparticle and metal salt exposure (all compared with their control). The pie charts indicate the proportion of GO-classes of the annotated gene transcripts (hypothetical proteins and undefined isoforms were not included) that occurred for each condition. Since no genes were differentially transcribed when comparing ZnO with ZnCl₂ and CuO with CuCl₂.2H₂O, no pie charts are available here.

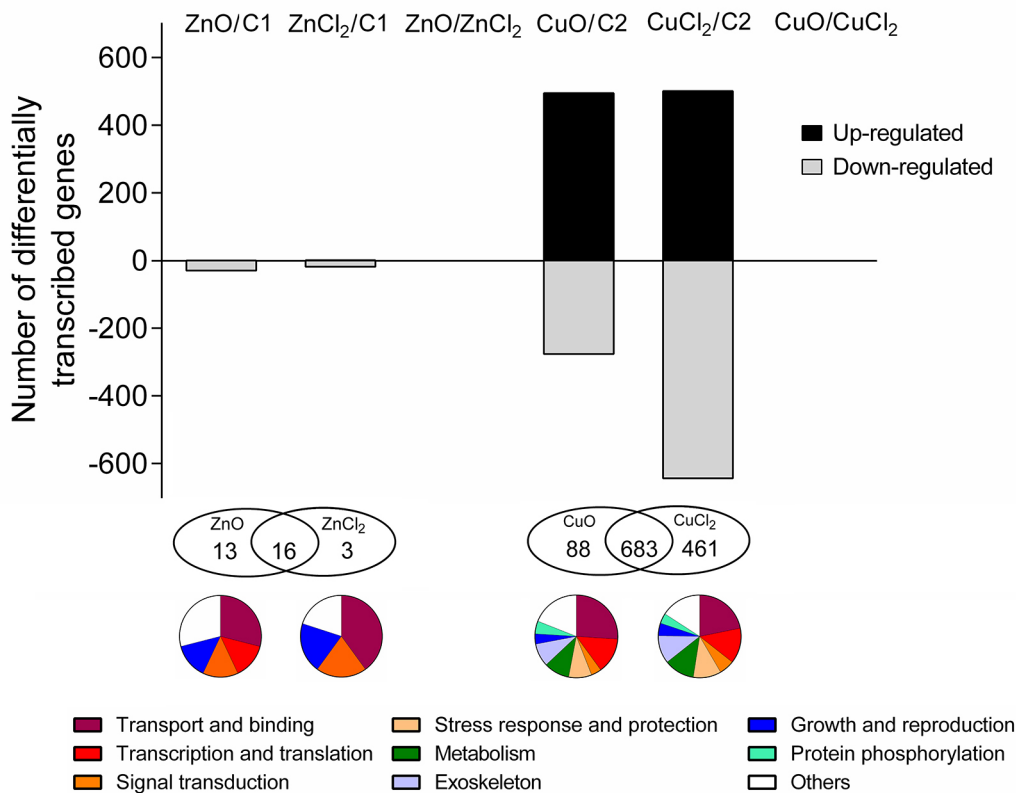


Fig. 5. Heat map with clustering analysis of the 40 key gene fragments (with sequence description and GO-process or function) identified with SAM. Green color indicates down-regulation of genes, while red color indicates the up-regulation of genes.

